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TAXONOMY OF THE HAPLOPORINAE NICOLL, 1914 AND BUNOCOTYLINAE DOLLFUS, 1950 (DIGENEA) FROM MEDITERRANEAN MULLETS (TELEOSTEI): MORPHOLOGICAL AND MOLECULAR APPROACHES

MARÍA ISABEL BLASCO COSTA

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Valencia, Enero 2009

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TESIS DOCTORAL

POR

MARÍA ISABEL BLASCO COSTA

DIRECTORES

JUAN ANTONIO BALBUENA DÍAZ-PINÉS

ANETA KOSTADINOVA

VALENCIA, ENERO DE 2009

B

D. JUAN ANTONIO BALBUENA DÍAZ-PINÉS, Profesor Titular del Departament de Zoologia de la Facultad de Ciencias Biológicas de la Universitat de València y

Dª ANETA KOSTADINOVA, Investigadora del Instituto de Parasitología de la Academia de Ciencias de la República Checa y del Laboratorio Central de Ecología General de la Academia de Ciencias de Bulgaria

CERTIFICAN que Dª María Isabel Blasco Costa ha realizado bajo nuestra dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título: "Taxonomy of the Haploporinae Nicoll, 1914 and Bunocotylinae Dollfus, 1950 (Digenea) from Mediterranean mullets (Teleostei): morphological and molecular approaches", para optar al grado de Doctora en Ciencias Biológicas.

Y para que así conste, en cumplimiento de la legislación vigente, expedimos el presente certificado en Paterna a 26 de Enero de 2009

Firmado: Juan Antonio Balbuena Díaz-Pinés Firmado: Aneta Kostadinova

A mi familia y
a mi Carlitos

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SUMMARY

Mullets (Mugilidae) offer an excellent system in which to study the geographical, ecological and evolutionary aspects of host-parasite associations. Existing data indicate that two trematode subfamilies specific to mullets, the Bunocotylinae of the Hemiuridae, and the Haploporinae of the Haploporidae, are generally most abundant and most widely distributed in different mugilid species worldwide. However, despite numerous previous records, their morphological variability and taxonomic diversity has not been addressed in detail.

The thesis is aimed at developing a modern taxonomic framework, combining morphological and molecular data, based on the examination of large samples (676 fish) of *Mugil cephalus*, *Liza aurata*, *L. ramado* and *L. saliens* collected at two localities (Ebro Delta and off Santa Pola), on the Spanish Mediterranean coasts, as well as comparative material from the Black Sea and the Sea of Japan. This study provides new taxonomic knowledge of two major groups of mullet digeneans, the subfamily Bunocotylinae of the Hemiuridae and the subfamily Haploporinae of the Haploporidae. In the case of the latter, the study examined the taxonomic consistency of digenean identification based on a combination of morphological, morphometric and molecular-sequence data from abundant newly collected material and used a molecular phylogenetic approach to test hypotheses for the relationships of genera within the Haploporidae and the position of the group within the phylogeny of the Digenea.

The following objectives were targeted in the study:

(i) A taxonomic revision of the genus *Saturnius* (Hemiuridae: Bunocotylinae);

(ii) A taxonomic revision of the Mediterranean haploporid genera *Haploporus*, *Dicrogaster*, *Forticulcita*, *Lecithobotrys* and *Saccocoelium*;

(iii) A phylogenetic analysis of Mediterranean haploporids using sequence data for the ribosomal DNA gene fragments to evaluate the degree of species differentiation and the validity of the haploporine genera, and to assess interspecific and intergeneric relationships within the taxonomic framework of the Haploporinae based on morphology;

(iv) Assessment of the systematic position and phylogenetic relationships of the families Haploporidae and Haplosplanchnidae within the phylogeny of the Digenea as inferred from the ribosomal RNA gene sequences.

As a synthesis of the study the following main conclusions were drawn:

The species diversity of *Saturnius* is higher than previously known, as evidenced by the description of three new species: *S. minutus* Blasco-Costa et al., 2006, *S. dimitrovi* Blasco-Costa et al., 2006. and *S. overstreeti* Blasco-Costa et al., 2008. The distinct species status of

the new taxa was validated by means of discriminant morphometric analysis. The revision of the allocation of the nominal species resulted in a refined diagnosis of *Saturnius* and a key to the species; four species are considered *species inquirendae*.

Haploporus is considered a monotypic Mediterranean haploporine genus. *H. benedeni* is redescribed and *H. lateralis* is considered to be its junior synonym. Five species parasiting *Valamugil* spp. from the Indo-West Pacific region, *H. indicus*, *H. spinosus, H. magnisaccus, H. mugilis* and *H. muscolosaccus*, are considered *incertae sedis* with respect to their generic affiliation. *H. pacificus, H. pseudoindicus* and *H. musculosaccus* are believed to be *species inquirendae* and *H. lossii* is considered to be a *nomen nudum*. A new generic diagnosis is provided.

The status of the nominal species of *Dicrogaster* is re-assessed. *D. perpusilla* and *D. contracta* are redescribed on the basis of new material from *Liza* spp. The latter two species and *D. fastigata* are considered valid. *D. fragilis* is considered a junior synonym of *D. fastigata* and *D. maryutensis* is considered to be *nomen nudum.* The two Mediterranean forms, *D. perpusilla* and *D. contracta* were further distinguished by multivariate morphometric analyses. A refined diagnosis of *Dicrogaster* and a key to its species is given.

A new species belonging to *Forticulcita*, *F. gibsoni* Blasco-Costa et al. in press, is described and distinguished from the other two species in the genus by its significantly smaller body size and most of its metrical data. *F. gibsoni* was also distinguished by means of a multivariate morphometric analysis from the two Mediterranean *Dicrogaster* spp. to which it exhibits superficial similarity. A refined diagnosis of *Forticulcita* and a key to its species is presented.

Lecithobotrys is considered a monotypic Mediterranean haploporine genus. *Lecithobotrys putrescens* is redescribed. *L. aegyptiacus* is considered to be a synonym of *Saccocoelium tensum* and *L. brisbanensis* and *L. vitellosus* are regarded as *species inquirendae.* A new generic diagnosis is provided.

Saccocoelium is revised and a refined diagnosis and a key to its recognised species is presented. *S. obesum* and *S. tensum* are redescribed and three new species, *Saccocoelium cephali* Blasco-Costa et al. in press, *S. brayi* n. sp. and *S. currani* Blasco-Costa et al. in press, are described. The five Mediterranean species of *Saccocoelium* were distinguished by multivariate morphometric analyses. *Lecithobotrys helmymohamedi*, *S. portsaidensis*, *S. saoudi* and *Neosaccocoelium aegyptiacus* are considered to be synonyms of *S. tensum* and *Neosaccocoelium* a synonym of *Saccocoelium*. *Lecithobotrys mugilis* is transferred to

Unisaccus and *Lecithobotrys sprenti* is transferred to *Unisaccus S. megasacculum* is transferred to *Elliptobursa*. *S. tripathi* is considered to be a *species inquirenda*.

Three new haploporine genera are established for parasites of mullets. *Ragaia* n. g. was erected for a new species, *R. lizae* Blasco-Costa et al. in press, from *Liza ramada* in the Ebro Delta on the Mediterranean Coast of Spain. Two genera, *Pseudolecithobotrys* Blasco-Costa et al. in press and *Pseudodicrogaster* Blasco-Costa et al. in press, were erected to accommodate species from the North Pacific previously placed in other genera, *Lecithobotrys stomachicola* and *Dicrogaster japonica*, respectively. A key to the ten recognised genera of the Haploporinae is presented.

A more refined estimation of the amount of genetic and morphological differentiation for closely related species of *Saccocoelium* from sympatric mullets in the Mediterranean was achieved. The molecular data corroborated the taxonomic decisions based on morphology with respect to the distinct status of the species. However, the results based on ITS2 sequences did not rule out the possibility for even higher species diversity within *Saccocoelium*. The observed patterns of species and genetic diversity at the limited geographical scale of the study indicated that factors linked to features of the haploporid life-cycle modify the effect of the enhanced host encounter due to the similar feeding ecology of the mullet hosts. Consequently, the possibility for sympatric speciation in the system studied, and in Haploporidae in general, might be higher. Two non-exclusive hypotheses are discussed with respect to the speciation patterns.

Multivariate statistical analyses provided important means for assessment of intra- and interspecific morphological variation and for testing the hypothesis of a morphometric separation between specimens of different species/populations in the studied subfamilies both comprising genera composed of morphologically similar species. The results of both approaches, PCA and LDA, were concordant when applied simultaneously; the latter demonstrating consistently the morphometric variables that best distinguish species groups. Both applications are, therefore suggested as valuable tools in the recognition of cryptic species and species delimitation in the studied taxa as well as in constructing species identification keys.

The first application of molecular analysis to evaluate the taxonomic framework of the Haploporidae based on morphology and to assess the relationships at the generic level revealed (i) strong support for the monophyly of the Haploporinae, *Dicrogaster* and *Saccocoelium*, and the position of *Ragaia* within the Haploporinae; (ii) evidence for rejection of the Dicrogasterinae and the synonymy of *Saccocoelioides* and *Lecithobotrys*; and (iii)

support for the distinct status of *Saccocoelium* in relation to *Haploporus*. Forticulcitinae n. subf. is erected for *Forticulcita* based on the presence of a well-delimited eversible intromittent copulatory organ, a feature unique in the Haploporidae, and the hypothesis of the Haploporinae based on molecular data. This action resolved *Saccocoelioides* and, by extension the Chalcinotrematinae, as sister group to the Haploporinae.

The phylogenetic affinities of the Haploporidae within the Digenea were found to clearly lie with other members of the Gorgoderoidea in the recently circumscribed suborder Xiphidiata. The monophyletic Haploporidae was resolved as sister to the Atractotrematidae on most occasions; however recognition of an independent superfamily was unsupported. The previously presumed relationship of the Haplosplanchnidae with Haploporidae was refuted and the former was confirmed to be a monophyletic distinct lineage in a basal position within the 'higher plagiorchiidans' supporting its currently elevated taxonomic status, i.e. suborder Haplosplanchnata.

Different gene fragments and analyses resulted in different phylogenetic hypotheses for the haploporids within the Xiphidiata. Despite the existence of few if any biological features that suggested a common origin, a sister relationship between the haploporids and atractotrematids and the paragonimids and troglotrematids was found in almost all phylogenies estimated. Therefore, the Haploporidae + Atractotrematidae were considered to represent a lineage that exhibits a secondarily simplification of the life-cycle and a host switch from tetrapods to fish. The morphological and developmental characters of these two families do not, therefore, reflect those of their most recent common ancestor and such evidence used for inferring phylogenetic affinities will be misleading.

RESUMEN

1. INTRODUCCIÓN GENERAL

Los mugílidos ofrecen un sistema excelente para estudiar las relaciones parásito-hospedador en sus aspectos geográficos, ecológicos y evolutivos. La información disponible sugiere que este sistema parásito-hospedador está caracterizado por (i) un alto grado de intercambio de parásitos en áreas tales como los mares Mediterráneo, Rojo o Negro, donde los mugílidos son muy diversos y las especies viven en simpatría; y (ii) por la presencia de cierto número de especies parásitas congenéricas estrictamente específicas a nivel local, que son próximas a aquellas de áreas adyacentes (Esch y Fernández, 1993). Sin embargo, estas conclusiones están basadas en decisiones taxonómicas según el criterio de los autores de estudios puntuales y fragmentarios, mientras que, hasta ahora, no se han realizado grandes esfuerzos por formular unas bases taxonómicas consistentes que permitan establecer la especificidad al hospedador.

La bibliografía existente indica que dos grupos de trematodos específicos de mugílidos, la subfamilia **Bunocotylinae** Dollfus, 1950 de la familia Hemiuridae Looss, 1899 y la familia **Haploporidae** Nicoll, 1914, son generalmente los más abundantes y ampliamente distribuidos en mugílidos de todo el mundo (Gibson, 2002; Overstreet y Curran, 2005). Sin embargo, a pesar de las numerosas citas, su variabilidad morfológica y diversidad taxonómica no se han estudiado en detalle. Por ello, uno de los requerimientos básicos para delimitar con exactitud las comunidades parásitas de los mugílidos, como es una taxonomía bien establecida para una rápida y fiable identificación de los parásitos, es prácticamente inexistente. Esta situación puede llevar a la interpretación errónea de los patrones de diversidad parasitaria en comunidades de hospedadores mugílidos simpátricos, retrasando, por tanto, el progreso de los estudios ecológicos.

La familia Hemiuridae es grande y está formada por numerosas subfamilias cuyas species habitan mayoritariamente en el estómago de peces marinos. La característica diagnóstica más relevante de esta familia es el ecsoma, una parte posterior del cuerpo que es protusible, la cual parece facilitar la supervivencia en las regiones ácidas del estómago (Gibson y Bray, 1979). En cambio, los miembros de cuatro de las subfamilias habitan en otras partes del estómago o en el intestino, y éstas (incluyendo Bunocotylinae) están desprovistas de ecsoma o se presenta de manera vestigial (Gibson, 2002). Los hemiúridos están pobremente representados en mugílidos, con solamente 19 especies nominales registradas hasta ahora en todo el mundo: 1 especie de *Hemiurus* Rudolphi, 1809, 1 de *Parahemiurus* Vaz y Pereira, 1930, 1 de *Lecithocladium* Lühe, 1901, 3 de *Lecithochirium* Lühe, 1901, 6 de *Saturnius* Manter, 1969, 2 de *Bunocotyle* Odhner, 1928, 4 de *Aphanurus* Looss, 1907 y 1 de

Opisthadena Linton, 1910 (Pankov *et al*., 2006). El pequeño género de hemiúridos específicos de mugílidos, ***Saturnius***, representado actualmente por 6 especies, parece ser el más diverso de los hemiúridos en mugílidos. De esas seis especies, sólo *S. papernai* Overstreet, 1977, ha sido encontrada en diferentes hospedadores en el Mediterráneo, pero se sabe poco sobre su variabilidad morfológica. Además, algunas especies de *Saturnius* fueron descritas en base a muy pocos especímenes y algunas de las características que han demostrado ser útiles para distinguir especies no han sido caracterizadas suficientemente en las descripciones originales (p.ej., Yamaguti, 1970; Fischthal, 1977; Rekharani y Madhavi, 1985; Domnich y Sarabeev, 1999; Peng *et al*., 2004).

La familia Haploporidae comprende un grupo de parásitos del tracto digestivo de peces marinos, estuarinos y dulceacuícolas. Según la revisión taxonómica más reciente de la familia, se reconocen 4 subfamilias: Haploporinae Nicoll, 1914; Chalcinotrematinae Overstreet y Curran, 2005; Megasoleninae Manter, 1935 y Warentrematinae Srivastava, 1937 (ver Overstreet y Curran, 2005). **Haploporinae** Nicoll, 1914 es un grupo relativamente pequeño de digeneos y bastante desconocido que, con una sola excepción [*Haploporus pacificus* (Manter, 1963)], parasitan mugílidos de agua marina o salobre, mayoritariamente en el Mediterráneo. Los miembros de esta subfamilia se caracterizan por poseer un vitelario compuesto por uno o más grupos de folículos fusionados o compactos, o dos grupos de unos pocos folículos distinguibles, y un útero que ocupa la mayoría de la parte posterior del cuerpo (*hindbody*) y se extiende hasta la altura del saco hermafrodítico (Overstreet y Curran, 2005). Muchas de las especies fueron sucintamente descritas en el Mediterráneo por Looss (1902), dando lugar a la erección de la mayoría de géneros en este área: *Haploporus* Looss, 1902, *Saccocoelium* Looss, 1902; *Lecithobotrys* Looss, 1902 and *Dicrogaster* Looss, 1902. A estos le siguieron tres más (*Unisaccus* Martin, 1973, *Forticulcita* Overstreet, 1982 and *Rondotrema* Thatcher, 1999), por lo que actualmente la subfamilia comprende siete géneros (ver Overstreet y Curran, 2005).

Aunque se han realizado estudios sobre algunos aspectos de la morfología y sistemática de grupos específicos dentro de la familia Haploporidae, y Haploporinae en particular, todavía falta información consolidada para establecer una taxonomía sólida. Uno de los principales problemas radica en la dificultad de preparación de los ejemplares de estos grupos para su examen morfológico. Los haplopóridos se caracterizan por poseer un tegumento extremadamente delicado, por lo que especímenes preparados inadecuadamente dan lugar a descripciones incompletas y engañosas (Overstreet y Curran, 2005). Como

resultado, la definición de taxones puede resultar errónea, pudiendo no estar basada en apomorfías que faciliten la clasificación del grupo en un contexto filogenético. En consecuencia, el estatus de muchas especies ha sido reconsiderado. La revisión taxonómica de Overstreet y Curran (2005) ha esclarecido de manera notable la situación a nivel genérico y supragenérico de los Haploporidae y, por ello, este estudio se ha ceñido a la reordenación taxonómica propuesta por estos autores.

Sin embargo, aunque Overstreet y Curran (2005) ya propusieron algunas nuevas combinaciones, la taxonomía a nivel de especie todavía necesita atención. Debido probablemente a las limitaciones de la publicación del libro *Key to the Trematoda* (Jones *et al*., 2005), el rango de especies considerado para establecer las diagnosis de los géneros no fue indicado con claridad. Además, el estado actual de la taxonomía de los haploporinos todavía presenta ciertas cuestiones sin resolver. De particular importancia para este estudio es el escaso o prácticamente inexistente conocimiento sobre la variabilidad morfológica y morfométrica a nivel de especies para la mayoría de géneros de la subfamilia. Esto es quizás la causa de que se haya sugerido la existencia de una sola especie polimórfica en tres de los cinco géneros de Haploporinae en el Mediterráneo, lo cual contrasta fuertemente con la situación de otros grupos de digeneos en peces simpátricos en el Mediterráneo, los espáridos, en los que se ha encontrado una diversidad de especies mucho mayor de lo que se conocía previamente como resultado de la combinación de estudios morfológicos, morfométricos y moleculares (*e.g*. Jousson *et al.*, 2000; Jousson y Bartoli, 2002). Un estudio reciente en mugílidos del Mediterráneo aplicando este enfoque, dio como resultado el descubrimiento de un nuevo género de Bunocotylinae y permitió corroborar la clasificación taxonómica actual de la subfamilia Bunocotylinae y explorar las relaciones de la superfamilia Hemiuroidea (Pankov *et al*., 2006).

El uso de **técnicas moleculares** ha contribuido sustancialmente a la comprensión de la diversidad de especies y ciclos vitales, así como de las relaciones filogenéticas dentro de los Digenea, el mayor grupo de metazoos endoparásitos (Cribb *et al*., 2001). Los estudios de **sistemática molecular** se pueden realizar a diferentes niveles usando la molécula con el grado de variabilidad adecuado y un grupo de taxones para comparar, representando la escala de tiempo evolutiva apropiada (Hillis *et al.*, 1996). Los métodos moleculares pueden distinguir especies crípticas (Avise, 2004) y han sido ampliamente utilizados para identificar bacterias y protozoos que poseen muy pocas características morfológicas apreciables (*e.g.* Perkins, 2000; Jenga *et al*., 2001). La identificación a nivel de especie mediante técnicas

moleculares requiere el uso de marcadores muy variables. Por ejemplo, los digeneos son un grupo extraordinariamente diverso caracterizado por la presencia de un gran número de **especies morfológicamente similares y/o crípticas**, en los que el empleo de los dos espaciadores internos transcritos del gen ribosómico (**ITS1 y ITS2**) ha sido de especial relevancia (*e.g.* Anderson y Barker, 1993; Despres *et at.,* 1995; Jousson *et al*, 1998a,b, 2000; Tkach *et al*., 2000; Snyder y Tkach, 2001; Jousson y Bartoli, 2001, 2002; Nolan y Cribb, 2004, 2006; Miller y Cribb, 2007).

Se considera que los estudios de sistemática molecular que obtienen los resultados más concluyentes son los que pretenden inferir hipótesis filogenéticas para esclarecer las relaciones entre géneros y familias, dado que en estudios a niveles taxonómicos más altos existe mayor nivel de homoplasia (Olson y Tkach, 2005). Los estudios que se han llevado a cabo por el momento en los digeneos utilizando como marcadores las regiones **18S y/o 28S** del gen ribosómico han permitido mejorar nuestra comprensión de las relaciones entre linajes **supraespecíficos** (géneros, subfamilias y entre familias cercanas) (ver Blair, 1993; Blair y Barker, 1993; Lumb *et al*., 1993; Blair *et al*. 1998b; Fernández *et al*., 1998 a, b, 2000; Tkach *et al.,* 1999, 2001a,b, 2002, 2003; Snyder y Loker, 2000; Lockyer *et al*., 2003; Bray *et al*., 2005; Chambers y Cribb, 2006; Pankov *et al*., 2006; Choudhury *et al.,* 2007; Curran *et al*., 2006, 2007). Sin embargo, hasta ahora no ha habido ningún intento de esclarecer las relaciones dentro de la familia Haploporidae.

Recientemente, se han desarrollado estudios sobre la filogenia de los digeneos a nivel **suprafamiliar**, siendo los análisis más completos el de Cribb *et al*. (2001) y, posteriormente, el de Olson *et al*. (2003). Sus estudios partieron de trabajos previos, ya que las regiones 18S y 28S han sido comúnmente utilizadas para investigar filogenias de los trematodos, dando lugar a la mayor base de datos de caracteres moleculares en este grupo (Olson y Tkach, 2005).

A pesar de que el estudio de Olson *et al*. (2003) incluyese un amplio rango de familias de digeneos (hasta 77), otras familias con escaso número de representantes y con afinidades filogenéticas desconocidas o no muy claras quedaron desatendidas. En el caso de estos grupos, se requiere un mayor esfuerzo de muestreo y secuenciación para representar su diversidad (Olson y Tkach, 2005). Estudios posteriores se han centrado en clados concretos dentro de la filogenia de los digeneos (*e.g.* Bray *et al*., 2005; Pankov *et al*., 2006; Choudhury *et al*., 2007). La representación de Haploporidae en estudios filogenéticos ha sido bastante limitada: sólo un taxón fue incluido en Cribb *et al*. (2001) (*Pseudomegasolena ishigakiense* Machida y Kamiya, 1976 la cual fue transferida a Atractotrematidae por Overstreet y Curran,

(2005)) y uno más fue incluido por Olson *et al*. (2003) (*Hapladena nasonis* Yamaguti, 1970). Estos autores encontraron la posición de la familia Haploporidae entre las más variables, pero según el análisis combinado, Haploporidae y Atractotrematidae formaron un clado que era grupo hermano del formado por Paragonimidae Dollfus, 1939 y Troglotrematidae (Odhner, 1914). Los cuatro taxones estaban incluidos dentro de la superfamilia Gorgoderoidea Looss, 1901, en el suborden Xiphidiata Olson, Cribb, Tkach, Bray y Littlewood, 2003. Sin embargo, el único carácter morfológico que une a las superfamilias en Xiphidiata no está presente en Haploporidae, lo cual podría indicar una posición errónea en la filogenia, tal y como sugirieron Olson *et al*. (2003). Además, Haploporidae resultó ser parafilética, ya que uno de sus representantes, *P. ishigakiense*, apareció incluido junto a los representantes de Atractotrematidae, por lo que estos autores sugirieron que se disolviese esta última familia. Sin embargo, *P. ishigakiense* se transfirió posteriormente a Atractotrematidae (Overstreet y Curran, 2005).

Posteriormente, miembros de Haploporidae han sido incidentalmente incluidos en unos pocos estudios (ver Bray *et al.*, 2005; Curran *et al.*, 2006, 2007; Choudhury *et al.*, 2007) pero, hasta el momento, sólo un taxón más ha sido secuenciado, *Saccocoelioides* sp. (ver Curran *et al*., 2006). A pesar de la compleja y controvertida historia taxonómica de esta familia, sus afinidades continúan sin resolverse.

Esta Tesis Doctoral se llevó a cabo en el contexto de un proyecto internacional titulado: '*Evaluación del efecto de una especie invasora en comunidades locales de mugílidos en el Mediterráneo: aproximación a las comunidades parásitas'*. Ello permitió disponer de una gran cantidad de muestras de cuatro especies de mugílidos del Mar Mediterráneo occidental para su análisis, *Mugil cephalus* L, *Liza aurata* (Risso), *L. ramado* (Risso) and *L. saliens* (Risso), muestreados en dos localidades de la costa española, frente al Delta del Ebro y Santa Pola. Igualmente, se dispuso de material para comparar procedente de la costa búlgara del Mar Negro y de la costa rusa del Mar del Japón. La gran cantidad de material parasitario recolectado permitió plantear un estudio comparativo de su variabilidad morfológica y genética para responder a las siguientes preguntas:

- ¿Cuál es la 'verdadera' diversidad de especies de Bunocotilinae y Haploporinae?
- ¿Cuales son los niveles de variabilidad intraespecífica con respecto a la morfología y morfometría en ambas subfamilias?

- ¿Respaldan los datos moleculares el marco taxonómico de Haploporinae basado en la morfología?
- ¿Cuáles son las relaciones filogenéticas a nivel supragenérico y los grupos más próximos a la familia Haploporidae dentro de los Digenea?

2. JUSTIFICACIÓN Y OBJETIVOS

El presente trabajo pretende el avanzar en el conocimiento taxonómico de dos grupos mayoritarios de digénidos de mugílidos, la subfamilia Bunocotylinae, de la familia Hemiuridae y la subfamilia Haploporinae, de la familia Haploporidae. En el caso de esta última, se examinará la consistencia taxonómica en la identificación de estos grupos de digeneos mediante en una combinación de datos morfológicos, morfométricos y moleculares del abundante material recolectado y se aplicarán métodos filogenéticos moleculares para comprobar las hipótesis sobre las relaciones entre los géneros dentro de Haploporinae y la posición de los mismos en la filogenia de los Digenea.

Los objetivos específicos que se plantean en este estudio son:

1. Realizar una revisión taxonómica del género *Saturnius* (Hemiuridae: Bunocotylinae) basada en un detallado estudio morfológico y morfométrico de las muestras recolectadas y de material procedente de museos, y la construcción de claves de identificación a nivel de especie y un listado de hospedadores y distribución para cada una de ellas.
2. Llevar a cabo una revisión taxonómica de los haplopóridos del Mediterráneo, concretamente de los géneros *Haploporus*, *Dicrogaster*, *Forticulcita*, *Lecithobotrys* y *Saccocoelium*, mediante un estudio detallado de las nuevas muestras recolectadas y de material de museos, y una evaluación crítica de la bibliografía. Asímismo, se elaborarán claves de identificación de géneros y especies y un listado de hospedadores y distribución para cada especie.
3. Efectuar un análisis filogenético de los haplopóridos mediterráneos utilizando las secuencias genéticas de la subunidad grande y del segundo espaciador interno transcrito del ARN ribosómico, para evaluar el grado de diferenciación entre especies y la validez de los géneros de haploporinos y averiguar las relaciones interespecíficas

y intergenéricas en el contexto taxonómico de la familia Haploporidae basado en la morfología.

4. Investigar la posición sistemática y las relaciones filogenéticas de las familias Haploporidae y Haplosplanchnidae en la filogenia de los digeneos, inferida mediante las secuencias genéticas de las subunidades grande y mediana del ARN ribosómico.

3. MATERIAL Y MÉTODOS GENERALES

3.1. Muestreo de los hospedadores y recolección de la fauna parásita

Se tomaron muestras de mugílidos en dos localidades de la costa Mediterránea española, frente al Delta del Ebro (40º30'– 40º50'N, 0º30'– 1º10'E) y en Santa Pola; en ésta última se tomaron muestras del mar (38º00' – 38º20'N, 0º10'– 0º40'W) y de una laguna salobre (38º10'N, 0º39'E). Dado que esta Tesis Doctoral se desarrolló como parte de un proyecto internacional, se examinó también material procedente de mugílidos del Mar Negro y del Mar del Japón. Los peces del Mar Negro se muestrearon en Sozopol, Bulgaria (42º26'–42º19'N, 27º40'–28º05'E) y las muestras del Mar del Japón se tomaron del Río Kievka, Rusia (las coordenadas exactas se desconocen). Adicionalmente, se tomaron muestras en las localidades españolas durante 2007-2008 para conseguir material parasitológico de algunas especies para completar el estudio molecular.

Se muestrearon y analizaron un total de 698 mugílidos con el fin de recolectar parásitos. Se analizaron en fresco submuestras de 5-10 peces de cada especie y de cada muestreo para conseguir material vivo para el estudio morfológico y molecular. Esto era crucial ya que los haplopóridos son muy frágiles y se degradan muy rápido después de morir. El resto de peces se congelaron a -20ºC para su examen posterior. Se recogieron, identificaron y contaron todos los parásitos metazoos. Para la recolección de los parásitos se siguió un protocolo estandarizado (Kostadinova *et al*., 2004), disponible en http://cetus.uv.es/mullpardb/index.html.

3.2. Procesamiento de los especimenes para el estudio morfológico

Los especímenes vivos se sacrificaron en solución salina muy caliente (temperatura próxima a la ebullición), se fijaron en alcohol 70% y se tiñeron con acetocarmín férrico (Georgiev *et*

al., 1986), se deshidrataron mediante un tren de alcoholes y se transparentaron con dimetil-ftalato y se montaron en preparaciones permanentes con bálsamo de Canadá. El material tipo de las nuevas especies y material de referencia (*vouchers*) de otras estudiadas se depositaron en la Colección del Museo Británico, en el Museo de Historia Natural de Londres (BMNH).

Las medidas fueron tomadas a partir de las ilustraciones realizadas mediante la cámara clara en el microscopio óptico. Se examinaron también especímenes del material tipo y de referencia de diferentes colecciones de muesos de varias especies de Bunocotylinae y de Haploporinae (ver Tabla 3.4 y Tabla 3.5), con la finalidad de compararlos con los de nuestras muestras. Se realizó a su vez estudio bibliográfico exhaustivo, así como, una búsqueda en la base de datos 'Host-Parasite Database' mantenida en el Museo de Historia Natural de Londres (Gibson *et al.*, 2005) para recopilar todos los datos de hospedadores, parásitos y distribución.

3.3. Análisis estadísticos morfométricos

En este estudio se emplearon dos técnicas de análisis multivariante: análisis de componentes principales (PCA) y análisis discriminante lineal (LDA). El primer análisis es una técnica para reducir la mayoría de la variación de un conjunto de datos con múltiples variables en una o pocas dimensiones (p.ej. Flury y Riedwyl, 1988; Tabachnick y Fidel, 2007). Mediante esta técnica se evaluó la relación multivariante entre los especímenes, sin tener en cuenta su identidad.

El segundo análisis (LDA con procedimiento paso a paso) se utiliza para determinar cuáles son las variables que mejor discriminan entre dos o más grupos existentes, pero en este caso, la existencia de esos grupos se conoce *a priori*. Asimismo, esta técnica también se utiliza para asignar entidades a los grupos (p.ej. McLachlan, 1992; Tabachnick y Fidel, 2007). Aquí se aplicó el LDA a especímenes asignados *a priori* a varios grupos definidos por la identificación de especies en base a la morfología (y también en base a la especie de hospedador y/o la localidad de origen en el caso de *Saturnius* spp.) con la finalidad de evaluar las diferencias morfométricas entre los morfotipos y seleccionar las variables que permiten una separación óptima entre las especies o grupos.

3.4. Procesado del material para el estudio molecular

Los especímenes se fijaron vivos en alcohol 100% y se guardaron a -20ºC para ser transferidos posteriormente a un tampón TNES urea 300 µl [10 mM Tris-HCl (pH 8), 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea]. El ADN genómico (ADNg) fue extraído utilizando un sólo espécimen cada vez mediante el protocolo de fenol-cloroformo descrito en Holzer *et al*. (2004). Alternativamente, se usó el tampón 1M Tris-EDTA (pH 8) para reemplazar el etanol del tejido fijado de los especímenes y el ADNg se extrajo utilizando el kit comercial Qiagen® DNeasy™ tissue kit siguiendo las instrucciones del fabricante.

Se amplificaron las secuencias de las siguientes regiones ribosómicas: 18S (casi completa); 28S (parcial; dominios D1-D3; ~1400-1600 pb) e ITS2 (completa). Para ello se usaron los cebadores indicados en la Tabla 3.4. Mediante la reacción en cadena de la polimerasa (PCR) se amplificó cada región en un volumen total de 30 µl que contenía ~1,5 unidades de la enzima polimerasa Thermoprime Plus DNA polymerase (ABgene, Epsom, UK) y tampón a 10× con 1,5 mM $MgCl_2$, 0,2 mM de cada dNTP, 0,5 mM de cada cebador para la PCR y unos 20-70 ng de ADNg. En otros casos, para la PCR de amplificación se utilizó el kit Ready-To-Go™ (Amersham Pharmacia Biotech) PCR *Beads*.

Los perfiles del termociclador utilizados para la PCR según la combinación de cebadores para cada región amplificada se detallan en la Fig. 3.1. El producto de PCR fue cortado del gel o purificado directamente utilizando el kit Qiagen QIAquick™ PCR Purification y se secuenciaron ambas cadenas. Las secuencias contiguas fueron ensambladas y editadas con los programas Bioedit 7.0.5. (©1997-2005, Hall, 1999) o Sequencher™ 3.1.1 (GeneCodes Corp.,) y alineadas automáticamente con Clustal X (Thompson *et al*., 1997).

3.5. Análisis filogenéticos

Los alineamientos se especifican en los Capítulos 6-8. En los alineamientos que incluían dos regiones, éstas se analizaron individualmente y combinadas, utilizando métodos de Inferencia Bayesiana (BI) y de máxima parsimonia (MP). Los análisis de MP se realizaron con PAUP* 4.0b10 (Swofford, 2002) mediante búsqueda heurística, con el número de réplicas de la búsqueda detallado en cada capítulo, con adición de taxones aleatoria y '*tree-bisection-reconnection branch-swapping*', con los caracteres desordenados y con el mismo peso para todos los caracteres, y considerando los huecos como datos desconocidos. El soporte para

cada nodo fue estimado mediante muestreo con reemplazamiento (bootstrap). Los análisis BI se realizaron con el programa MrBayes versión 3.1.2 (Ronquist y Huelsenbeck, 2003). Previamente al análisis, se estimó el mejor modelo de sustitución nucleotídica utilizando el programa ModelTest version 3.06 (Posada y Crandall, 1998), independientemente para cada alineamiento o partición. Los modelos seleccionados en cada caso se especifican en la sección de materiales y métodos de cada capítulo (Capítulos 6-8). Los análisis se realizaron para un millón de generaciones con una frecuencia de muestreo de 100. Se construyeron árboles consenso con la media de la longitud de las ramas, utilizando los árboles después de alcanzar los valores del logaritmo de la verosimilitud (*log-likelihood*) y la asíntota de la curva de sustitución de parámetros. El soporte para los nodos se estimó como las probabilidades posteriores (Huelsenbeck *et al*., 2001).

4. REVISIÓN TAXONÓMICA DE *SATURNIUS* (HEMIURIDAE: BUNOCOTYLINAE)

En 1977, Overstreet sugirió que "Si este artículo estimula a examinar mugílidos de diferentes localidades, algunos de los especímenes que encontrarán serán, probablemente, especies nuevas y formas diferentes". A pesar de la gran cantidad de información bibliográfica sobre estudios parasitológicos en diferentes especies de mugílidos del Mediterráneo, la única especie que se cita del género *Saturnius* es *S. papernai*. Sin embargo, la mayoría de esas citas no está documentada, por lo que no es sorprendente que al realizar un amplio trabajo como el presente sobre la variabilidad morfológica de los bunocotílinos que parasitan mugílidos, se haya descubierto una mayor diversidad del género. De hecho, hemos identificado y descrito tres especies nuevas, *S. minutus* Blasco-Costa et al. (2006) y *S. dimitrovi* Blasco-Costa et al. (2006) en *M. cephalus* de la cuenca mediterránea y *S. overstreeti* Blasco-Costa et al. (2008) en *M. cephalus* y *M. soiuy* (Basilewsky) del Mar del Japón. Mediante análisis univariantes y multivariantes se demostró que las tres especies presentes en el Mediterráneo eran distinguibles utilizando morfometría. En particular, debido a que la diferencia más evidente entre las tres especies era el tamaño, se decidió aplicar además un LDA con una corrección para el tamaño (Darroch y Mosimann, 1985) que redujese su efecto sobre las demás variables. Así, las diferencias que se observaron entre las especies fueron atribuidas mayoritariamente a la forma y no al tamaño (Junger *et al.*, 1995). El LDA también permitió identificar seis variables que diferenciaban claramente las especies. Posteriormente, se

añadieron al conjunto de datos previo los especímenes de *S. papernai* encontrados en *L. aurata* que presentaban diferencias superficiales respecto a la forma 'típica' en *M. cephalus*. El LDA diferenció las tres especies, mostrando una dispersión mayor de los especímenes de *S. papernai* a lo largo del primer eje, pero no se observó hiato entre los especímenes que parasitaban diferentes hospedadores.

Uno de los descubrimientos importantes del presente estudio fue la detectión de un ciclocele (*cyclocoel:* unión de los ciegos en la parte distal) en las tres especies descritas a pesar de la dificultad para su observación, ya que esa región suele encontrarse enmascarada por el útero y los huevos, siendo más fácil de apreciar en individuos juveniles. La presencia (en el género *Bunocotyle*) y ausencia (en *Saturnius*) del ciclocele había sido utilizada hasta el momento como una de las características importantes para diferenciar los dos género dentro de Bunocotylinae (p.ej. Gibson, 2002).

El examen de material tipo de *S. papernai* y *S. segmentatus* Manter, 1969, así como de los especímenes recolectados de esas especies en este trabajo, aportó nuevos datos sobre la variabilidad morfológica, especialmente en el caso de *S. papernai*, así como la detección de de ciertas estructuras (pseudoseptos y/o expansiones musculares laterales, detalles de la parte final de la genitalia) que no se habían descrito con claridad anteriormente. El estudio del material tipo de *S. maurepasi* Overstreet, 1977 añadió algunos detalles a la descripción de la especie, mientras que en el caso de *S. belizensis* Fischthal, 1977 y *S. mugilis* (Yamaguti, 1970) no se pudo obtener información adicional relevante, especialmente sobre la parte terminal de la genitalia, por lo que se sugirió que se tomasen nuevas muestras en el futuro que permitan describir mejor estas estructuras. La revisión del material tipo de *Bunocotyle constrictus* Domnich y Sarabeev, 1999 y *Saturnius valamugilis* Rekharani y Madhavi, 1985 y las descripciones de *B. mugilis* por Solonchenko (1976) y *S. mugilis* por Dmitrieva y Gaevskaya (2001) nos llevó a sugerir que se considerasen material de dudosa identificación y especies *inquirendae* en el caso de *B. constrictus* y *S. valamugilis*. Como resultado de la reevaluación de las especies de *Saturnius* se identificaron los caracteres que mejor permiten diferenciar las especies y éstos fueron utilizados para la elaboración de unas claves de identificación de las especies y para la nueva diagnosis del género propuesta en este estudio.

5. REVISIÓN TAXONÓMICA DE LA SUBFAMILIA HAPLOPORINAE

5.1. Género *Haploporus*

Haploporus es el género tipo de la familia Haploporidae y de la subfamilia Haploporinae. Algunos autores sugirieron que la especie descrita por Looss (1902), *H. lateralis* Looss, 1902, y la especie tipo para la que él mismo erigió el género *Haploporus, H. benedeni* Looss, 1902, podían ser sinónimas (Dawes, 1947; Fares y Maillard, 1974), en especial si se considera que Looss se basó en uno o muy pocos especímenes para describirlas (en el caso de *H. benedeni* la redescribió a partir del material tipo). Asimismo, sus descripciones son muy breves y algunas de las diferencias que se identifican podían explicarse por la contracción de unos especímenes (como se indica en la propia descripción) o por el grado de desarrollo de los individuos (miracidios sin mancha ocular y menor tamaño de los especímenes). Por estas razones y algunas más detalladas en el capítulo 5 de esta Tesis, *H. lateralis* se consideró sinónimo de *H. benedeni*. Ésta fue redescrita con nuevo material recolectado para el presente estudio procedente de *L. ramada* del Mediterráneo occidental, y de material depositado en la Colección del Museo de Historia Natural de Londres, procedente de *C. labrosus* del Atlántico noreste.

El estudio del material tipo de *H. spinosus* Machida, 1996 y *H. magnisaccus* Machida, 1996 confirmó la morfología general de las descripciones originales y aportó información esencial sobre la parte terminal de la genitalia que se encuentra dentro del saco hermafrodítico en los haplopóridos. En el caso de *H. pseudoindicus* Rekharani y Madhavi, 1985, que fue descrito a partir de un solo espécimen, se requiere nuevo material en buenas condiciones para poder establecer su estatus. Se observó, además, que *H. pseudoindicus* podría estar estrechamente relacionada con las especies de *Haploporus* descritas en *Valamugil* spp., en particular con *H. mugilis.* Las especies de *Haploporus* que parasitan *Valamugil* spp. difieren bastante del concepto original de Looss (1902) sobre la morfología del género *Haploporus* y no parecen formar un grupo natural (por diferencias respecto a varios caracteres morfológicos). Sin embargo, este estudio comparativo reveló que dichas especies presentan 16 características comunes, que no son compartidas por *H. benedeni*, especie tipo y, a nuestro entender, única representante del género *Haploporus*. En consecuencia, consideramos las especies de *Haploporus* de *Valamugil* spp. *incertae sedis* con respecto al género. Además, se precisa material adicional de las especies *H. pacificus* (Manter, 1963) (sin. *Neohaploporus pacificus* Manter, 1963), *H. pseudoindicus* y *H. musculosaccus* Machida, 2003 para poder

esclarecer su estatus, por lo que en este estudio fueron consideradas especies *inquirendae* y *H. lossii* Al-Bassel, 1990, que fue descrita únicamente en una Tesis Doctoral (Al-Bassel, 1990), se consideró un *nomen nudum*.

5.2. Género *Dicrogaster*

El material recolectado para este estudio de las dos especies del género *Dicrogaster*, *D. perpusilla* Looss, 1902 (especie *tipo*) y *D. contracta* Looss, 1902 procedente de *Liza* spp. en el Mediterráneo, hizo posible reevaluar su estatus, las cuales se habían considerado sinónimas por algunos autores (Dawes, 1947 y Sarabeev y Balbuena, 2003). Además de las redescripciones morfológicas de estas especies, que han aportado información completamente nueva sobre su variabilidad morfológica, se diferenciaron también las dos formas mediterráneas de *Dicrogaster* mediante análisis estadísticos univariantes y multivariantes (PCA y LDA) utilizando variables morfométricas. El LDA se realizó una primera vez con todas las variables y una segunda sólo con las ratios, y en ambos casos se pudieron diferenciar los dos grupos. Las siguientes cinco variables fueron seleccionadas como las mejores para diferenciar las especies: (i) longitud de la vesícula seminal externa, (ii) longitud del testículo, (iii) longitud del vitelario, (iv) la anchura de la ventosa ventral respecto al cuerpo y (v) el índice de elongación.

Tras la revisión bibliográfica del resto de especies de *Dicrogaster*, se sugirió que *D. fragilis* Fernández Bargiela, 1987 es un sinónimo de *D. fastigata* Thatcher y Sparks, 1958, puesto que las diferencias entre ambas especies podrían atribuirse al maceramiento de los especímenes de *D. fragilis* (falta de espinas, tegumento muy frágil y ventosa ventral ligeramente más grande), mientras que la mayoría de rangos de las variables morfológicas se solapaban. *D. maryutensis* Al-Bassel, 1990 se consideró *nomen nudum* dado que fue descrita sólo en una Tesis Doctoral no publicada (Al-Bassel, 1990). En este trabajo elaboramos una clave de identificación a nivel de especie y añadimos ciertos detalles a la diagnosis del género.

5.3. Género *Forticulcita* Overstreet, 1982

En este estudio se describió una especie nueva, *F. gibsoni* Blasco-Costa et al., en prensa (a) partir de ejemplares procedentes de *M. cephalus* en el Mediterráneo español. Esta especie se distingue de las otras dos única especies del género, *F. glabra* Overstreet, 1982 (especie tipo) y *F. mugilis* Hassanine, 2007 por su cuerpo considerablemente más pequeño y por diferencias

en la mayoría de variables morfométricas. Además, *F. gibsoni* se caracteriza por poseer una región estrecha en la parte anterior al poro genital (a modo de 'cuello') y por una estructura similar a una 'cola' en la parte más distal de la región posterior del cuerpo (*hindbody*) desprovista de órganos, de tal manera que el útero queda restringido a la parte más anterior de la región posterior del cuerpo y el testículo aparece en una posición más anterior. *F. gibsoni* se diferenció de *F. glabra* por presentar la parte anterior del cuerpo hasta el margen anterior de la ventosa ventral (*forebody*) más larga, una menor ratio de anchura de la ventosa ventral en relación a la oral y huevos más grandes. Del mismo modo, se distinguió de *F. mugilis* por una menor ratio de anchura de la ventosa ventral en relación a la oral, el saco hermafrodítico mucho más largo que la ventosa ventral y los huevos más pequeños. En base a las características de las tres especies se describió una nueva diagnosis del género y una clave de identificación de las especies.

Los haplopóridos de tamaño más pequeño en el Mediterráneo, *D. perpusilla, D. contracta* y *F. gibsoni*, podrían ser confundidos a primera vista debido a su tamaño, por lo que mediante un análisis discriminante se evaluaron las variables morfométricas que mejor diferencian las tres especies, y estas son las siguientes: (i) anchura del cuerpo, (ii) tamaño de la vesícula seminal externa, (iii) anchura del vitelario, (iv) longitud del saco hermafrodítico en relación a la longitud de la ventosa ventral, (v) anchura de la ventosa ventral en relación a la anchura del cuerpo y (vi) índice de elongación.

5.4. Género *Lecithobotrys* Looss, 1902

A partir del material recolectado de *Liza* spp. en el presente estudio, se redescribió por primera vez en detalle la especie tipo, *L. putrescens* Looss, 1902, la cual fue brevemente descrita en 1902 a partir de un sólo individuo. Overstreet y Curran (2005) sugirieron que *L. putrescens* podría considerarse congenérica con *Haploporus*, pero nosotros mostramos que *Lecithobotrys* y *Haploporus* pueden diferenciarse en (i) la distribución del pigmento ocular, (ii) la forma y tamaño de las vesículas seminales, (iii) el atrio genital y (iv) la estructura del vitelario.

Las revisión de la literatura existente sobre el resto de especies de *Lecithobotrys* nos llevó a considerar *L. aegyptiacus* Hassan, El-Aziz, Khidr y Abu Samak, 1990 como sinónimo de *S. tensum*, en especial por (i) la presencia de estructuras esclerotizadas en el conducto hermafrodítico, característica diagnóstica del género *Saccocoelium*, y (ii) el solapamiento entre la práctica totalidad de las variables morfométricas. Se consideró *L. brisbanensis*

(Martin, 1974) (sin. *Paralecithobotrys brisbanensis* Martin, 1974) y *L. vitellosus* Sharma y Gupta, 1970 como especies *inquirenda* ya que la primera no presentaba las características diagnosticas del género, a excepción de la estructura del vitelario, y la descripción de la segunda se basó en un único ejemplar, que se encontraba deteriorado.

5.5. Género *Saccocoelium* Looss, 1902

Se obtuvo abundante material de *Saccocoelium* en todas las especies de hospedadores analizadas. Los ejemplares de este género presentaron un alto grado de variabilidad morfológica, lo que promovió el estudio amplio y minucioso que hemos realizado sobre este género. La especie tipo, *S. obesum* Looss, 1902, y *S. tensum* Looss, 1902 fueron redescritas con material procedente de *Liza* spp. de la costa mediterránea española. Además, en el caso de *S. obesum*, se dispuso de material adicional procedente de la costa búlgara del Mar Negro. Se identificaron tres morfotipos, dos grandes, uno en cada localidad, y uno pequeño en el Mediterráneo, por lo que se consideró que *S. obesum* podía representar un complejo de especies. Ello nos llevó a considerar la necesidad de llevar a cabo estudios moleculares con este material, los cuales se iniciaron y desarrollaron de manera paralela a los morfológicos. La información proporcionada por los datos morfológicos, morfométricos y moleculares reveló que la forma pequeña de *S. obesum* (*sensu lato*) representaba una nueva especie, *S. brayi* n. sp., y se diferenció de *S. obesum* (*sensu stricto*) y de las demás especies del género. El examen del material identificado como *S. tensum* aportó gran cantidad de información novedosa sobre la variabilidad morfológica y morfométrica de la especie, mostrando el presente material descrito un rango de variabilidad más estrecho en comparación con los datos encontrados en la bibliografía. Los especímenes de *Saccocoelium* procedentes de *M. cephalus* se asignaron a 2 nuevas especies, *S. currani* Blasco-Costa et al., en prensa (c) y *S. cephali* Blasco-Costa et al., en prensa (c), gracias al empleo de datos morfológicos, morfométricos y moleculares (estos últimos sólo en el caso de *S. cephali*). El amplio rango de variabilidad atribuido a *S. tensum* en algunos estudios previos de diferentes hospedadores podría deberse, bien a la variabilidad como consecuencia de la parasitación sobre diferentes especies hospedadoras o bien, en el caso concreto de estudios que utilizan material de *M. cephalus*, al hecho de incluir, por error, diferentes especies en la muestra descrita. *S. obesum*, *S. tensum* y las tres nuevas especies del Mediterráneo fueron, asimismo, comparadas y diferenciadas mediante métodos univariantes y multivariantes que permitieron identificar las variables morfométricas que mejor distinguen los grupos/poblaciones estudiados.

Además de las cinco especies anteriores, reconocimos la validez de *S. gohari* Ramadan, Saoud, Ashour y Mansour, 1989 mientras que *Lecithobotrys helmymohamedi* Ramadan, Saoud, Ashour y Mansour, 1988, *S. portsaidensis* El-Shahawi, El-Gindy, Imam y Al-Bassel, 1992, *S. saoudi* El-Shahawi, El-Gindy, Imam y Al-Bassel, 1992, y *Neosaccocoelium aegyptiacus* El-Shahawi, El-Gindy, Imam y Al-Bassel, 1992 las consideramos como sinónimos de *S. tensum*; y *Neosaccocoelium* El-Shahawi, El-Gindy, Imam y Al-Bassel, 1992 por tanto, sinónimo de *Saccocoelium*. Overstreet y Curran, 2005 revisaron las especies *Lecithobotrys mugilis* Rekharani y Madhavi, 1985, de la cual sugirieron su transferencia a *Saccocoelium* pero no la formalizaron, y *L. sprenti* Martin, 1973 que transfirieron a *Saccocoelium* [= *Saccocoelium sprenti* (Martin, 1973)]. La revisión que hemos llevado a cabo reveló que estas especies presentan considerables diferencias respecto a la diagnosis del género *Saccocoelium*, a la vez que cumplen las características diagnósticas de otro género, *Unisaccus*, en el que se han realojado como *U. mugilis* (Rekharani y Madhavi, 1985) Blasco-Costa et al., en prensa y *U. sprenti* (Martin, 1973) Blasco-Costa et al., en prensa, respectivamente. A su vez, *S. megasacculum* Liu, Wang, Peng, Yu y Yang, 2004 fue transferida al género *Elliptobursa* Wu, Lu y Zhu, 1996 como *E. megasacculum* (Liu, Wang, Peng, Yu y Yang, 2004) Blasco-Costa et al., en prensa. El género *Elliptobursa*, a pesar de pertenecer a la familia Monorchiidae Odhner, 1911, en la reciente revisión de su familia es considerado como un género perteneciente a Haploporidae (Madhavi, 2008). En el caso de *S. tripathi* Dutta, 1995 (= *Saccocoelium tripathi* Datta y Manna, 1998) fue considerada especie *inquirenda* en el presente estudio dado que la descripción original fue muy somera y no se identificó ningún carácter morfológico que permita reconocerla.

La reevaluación de estos taxones permitió identificar nuevas características morfológicas que parecen estar conservadas en todas las especies del género consideradas como válidas y han demostrado su utilidad para diferenciar *Saccocoelium* de los otros géneros de haploporinos. Por ello, dichas características han sido incluidas en la nueva diagnosis del género y han permitido la elaboración de una clave de identificación de las especies.

5.6. Tres nuevos géneros y una clave para los géneros en Haploporinae

En el transcurso de este estudio se erigieron tres nuevos géneros de haploporinos, uno de ellos procedente del material nuevo estudiado y los otros dos como resultado de la revisión taxonómica de los géneros de haplopóridos previamente mencionados. El género *Ragaia* Blasco-Costa et al., en prensa se erigió para la nueva especie *R. lizae* Blasco-Costa et al., en

prensa, en *Liza ramada* en el Delta del Ebro, en la costa mediterránea española. *Ragaia* se diferenció de los demás géneros por poseer una combinación única de los siguientes caracteres: (i) una ventosa ventral muy musculosa, dos veces más grande que la ventosa oral, (ii) un saco hermafrodítico grande y musculoso de tamaño similar al de la ventosa ventral, (iii) y el ovario y vitelario situados próximos a la parte más posterior del cuerpo. Dos géneros, *Pseudolecithobotrys* Blasco-Costa et al., en prensa (d) y *Pseudodicrogaster* Blasco-Costa et al., en prensa (b), se erigieron para acomodar especies del Pacífico norte que estaban alojadas previamente en otros géneros, *Lecithobotrys stomachicola* Machida, 1996 y *Dicrogaster japonica* Machida, 1996, respectivamente. *Pseudodicrogaster* se puede reconocer por presentar (i) la vesícula seminal interna y externa tubulares, siendo la externa mucho más corta, (ii) la ratio de las dimensiones de las ventosas, (iii) el saco hermafrodita piriforme y grande, (iv) la posición del testículo y (v) la presencia de dos manchas oculares en los miracidios desarrollados. *P. stomachicola* Machida, 1996 se diferenció de las demás especies de *Lecithobotrys* por poseer (i) ventosas del mismo tamaño y más musculosas, (ii) el testículo subcilíndrico, (iii) la vesícula seminal externa tubular y serpenteante, (iv) un saco hermafrodita estrecho y arqueado, (v) la vesícula seminal marcadamente alargada, casi subcilíndrica, (vi) un conducto hermafrodítico largo, (vii) metratermo largo y musculoso, (viii) cáscara de los huevos gruesa, (ix) vitelario formado por dos grupos de masas de vitelógenas grandes y compactas, y (x) el sitio de infección.

Puesto que en este trabajo se erigieron estos tres nuevos géneros, que se suman a los siete ya presentes en la subfamilia Haploporinae, elaboramos una clave de identificación de los 10 géneros actuales.

6. EVIDENCIA MORFOLÓGICA Y MOLECULAR DE ESPECIACIÓN SIMPÁTRICA EN *SACCOCOELIUM* EN MUGÍLIDOS MEDITERÁNEOS

Los haplopóridos parásitos de mugílidos representan un sistema excelente para estudiar el papel de los filtros de encuentro y de compatibilidad en la especiación simpátrica debido a su modo de transmisión pasiva y la ecología trófica similar de sus hospedadores definitivos. Para resolver el estatus taxonómico de las especies de *Saccocoelium* aplicamos una combinación de técnicas moleculares y morfológicas. Se secuenciaron las regiones ribosomales 28S e ITS2 de múltiples réplicas de los ocho morfotipos de *Saccocoelium* spp. que fueron identificados mediante un análisis multivariante de datos morfométricos. Los análisis moleculares

mostraron únicamente cuatro genotipos que corroboraron la identidad de las especies *S. obesum*, *S. tensum* y *S. cephali*, y sustentaron sólidamente, junto con las diferencias morfométricas, la identidad de la nueva especie *S. brayi* n. sp. Por el contrario, dos morfotipos presentes en *M. cephalus* compartieron el mismo genotipo y cuatro formas de *S. tensum* aparecieron también genéticamente idénticas, incluso con el marcador más variable (ITS2), confirmando por tanto, la hipótesis de la existencia de una sola especie polimórfica en ambos casos.

La división en dos linajes de las especies de *Saccocoelium* en la filogenia sugirió una diferenciación genética y morfológica asociada al hospedador intermediario, un gasterópodo, en vez de al hospedador definitivo como consecuencia de coespeciación. La elevada diversidad de especies sugiere la existencia de factores relacionados con las características del ciclo de vida de los haplopóridos que modificasen el efecto del filtro de encuentro y, por tanto, se plantearon dos hipótesis no excluyentes. En primer lugar, es obvio pensar que la existencia de un mayor número de especies que el previamente conocido refleje la adaptación a diferentes especies de hospedador intermediario, ya que se sabe que los digeneos presentan una gran especificidad por el primer hospedador intermediario (Pearson, 1972; Adamson y Caira, 1994), lo cual apoyaba esta hipótesis. Como hipótesis alternativa, el patrón de especies y diversidad genética a una escala tan reducida como la de este estudio (aprox. 500 km, en muchos casos estando presentes varias especies en las mismas localidades) puede ser el resultado de la adaptación local debido a la dispersión de las larvas, tanto de los gasterópodos como de los digeneos. En particular, el desarrollo directo del primer hospedador de *S. tensum*, *Hydrobia ventrosa* y, por tanto, el alto grado de diferenciación entre poblaciones y el poco flujo genético entre ellas (Foltz, 2003; Wilke y Davis, 2000), además de la heterogeneidad de los hábitats en el Mediterráneo (Bartoli y Gibson, 2007), podrían propiciar diferente susceptibilidad de los hospedadores a infecciones por *Saccocoelium*, y haplopóridos en general. Por otro lado, la ausencia del hospedador secundario en los haplopóridos, implicaría un menor grado de mezcla de clones (ver Criscione *et al*., 2005; Criscione y Blouin, 2006), como ha sido observado en otros grupos con ciclos de vida de dos hospedadores (Theron et al., 2004; Mulvey et al. 1991). En consecuencia, concluimos que la baja dispersión de la fase infectiva de los haplopóridos debe favorecer el aislamiento de los clones, con una homogeneización genética a través del amplio filtro de encuentro. Sin embargo, la dispersión limitada y agregada del primer hospedador, junto con la multiplicación por 'clonación' y el patrón de enquistamiento de la fase infectiva del parásito darán lugar a pocas oportunidades

de reproducción cruzada y la diferenciación de las poblaciones a pequeña escala. Por todo esto, la posibilidad de especiar en simpatría debido a la estructura espacial de las poblaciones del primer hospedador parece elevada en *Saccocoelium*, y en Haploporidae en general.

7. RELACIONES FILOGENÉTICAS DE LOS HAPLOPÓRIDOS DEL MEDITERRÁNEO INFERIDAS A PARTIR DE LAS SECUENCIAS DEL 28S E ITS2 rADN

En esta Tesis Doctoral se evaluó por primera vez la taxonomía tradicional, basada en la morfología, de la familia Haploporidae mediante datos moleculares que permitieron establecer las relaciones a nivel genérico dentro de la subfamilia Haploporinae. Se obtuvo la secuencia parcial de la subunidad grande (28S) del ARN ribosómico y la completa del segundo espaciador interno (ITS2) de representantes de seis de los diez géneros presentes en Haploporinae. Estas secuencias se alinearon con las disponibles de otras dos subfamilias y con las existentes de la posible familia sinónima Atractotrematidae. Los análisis moleculares mostraron: (i) una relación muy próxima entre la familia Atractotrematidae Yamaguti, 1939 y Haploporidae; (ii) una base sólida a favor de la monofilia de Haploporinae, *Dicrogaster* y *Saccocoelium*, y la posición de *Ragaia* dentro de Haploporinae; (iii) evidencia para rechazar la subfamilia Dicrogasterinae (ver Yamaguti, 1958) y la sinonimia entre *Saccocoelioides* y *Lecithobotrys* (Yamaguti, 1958 y Nasir y Gómez, 1976); y (iv) soporte para reconocer el distinto estatus de *Saccocoelium* en relación con *Haploporus* (género tipo) (Overstreet y Curran, 2005).

Lecithobotrys y *Haploporus* aparecieron estrechamente relacionados, especialmente según los análisis del 28S, y presentaron la menor divergencia genética entre géneros, incluso mostrando divergencias similares a las encontradas entre especies del mismo género (p.ej. en *Saccocoelium* y *Dicrogaster*) lo cual tiende a apoyar la posible sinonimia sugerida por Overstreet y Curran (2005). No obstante, concluimos que sería deseable disponer de más datos moleculares de otras especies de ambos géneros antes de proceder a un cambio nomenclatural. El amplio muestreo de especies de *Dicrogaster* y *Saccocoelium* confirmó la validez de estas especies y, rechazó, por tanto, las sinonimias propuestas previamente entre *D. perpusilla* y *D. contracta*, y entre *S. tensum* y *S. obesum*.

El género *Saccocoelioides* Szidat, 1954, que fue recientemente transferido a la subfamilia Chalcinotrematinae Overstreet y Curran, 2005, apareció dentro del clado de

Haploporinae, pero esa posición esta relacionada con la posición del género *Forticulcita,* que apareció en la posición más basal de la subfamilia. La presencia en *Forticulcita* de un órgano copulador intromitente (terminología según Overstreet, 1982) bien diferenciado es, además, una característica única dentro de la familia, así como la presencia de una sola masa de vitelario (sólo presente en *D. fastigata* Thatcher y Sparks, 1958). Por lo tanto, esta importante apomorfía, que no se había considerado nunca hasta ahora, junto con la hipótesis aquí planteada según los datos moleculares, sugieren la posibilidad de que *Forticulcita* merezca un rango taxonómico más elevado, por lo que proponemos una nueva subfamilia, Forticulcitinae n. subf. para las especies del género *Forticulcita*. Como resultado de esta decisión, *Saccocoelioides* y, por extensión la subfamilia Chalcinotrematinae, se resolvió como grupo hermano de Haploporinae. Sin embargo, la posición de la subfamilia Megasoleninae Manter, 1935, representada por una especie del género *Hapladena* Linton, 1910, permaneció sin resolver, quizás debido a que la secuencia pertenece a una especie aberrante dentro del grupo. Sin duda, un muestreo más amplio de miembros de Atractotrematidae y de las subfamilias de Haploporidae mejorará el conocimiento sobre las relaciones, de forma que se pueda establecer una clasificación en base a grupos naturales.

8. DIFERENTES GENES - DIFERENTES SOLUCIONES: RELACIONES FILOGENÉTICAS DE DOS FAMILIAS CONTROVERTIDAS DENTRO DE LOS DIGENEA INFERIDAS MEDIATE 18S Y 28S rADN.

La posición sistemática y las afinidades de las familias Haploporidae y Haplosplanchnidae Poche, 1926 fueron investigadas mediante nuevas secuencias de cinco y tres taxones, respectivamente. Se realizaron dos alineamientos con las secuencias del 18S y 28S conjuntamente; el primero incluyó un mayor número de taxones (alineamiento de las secuencias empleadas en Olson *et al*., 2003, junto con las nuevas secuencias obtenidas). Mientras que el segundo se ciñó a los taxones más próximos a Haploporidae, es decir, aquellos que fueron encontrados en el estudio previamente mencionado. Los análisis se aplicaron considerando, por un lado, las regiones secuenciadas combinadas y, por otro, cada región de manera independiente (sólo en el análisis reducido). La hipótesis filogenética obtenidas mediante BI de secuencias del 18S y 28S combinadas en el contexto de los Digenea mostró una clara afinidad entre Haploporidae y los miembros de la superfamilia Gorgoderoidea Looss, 1901, en el recientemente establecido suborden Xiphidiata Olson Cribb, Tkach, Bray y Littlewood, 2003. La familia Haploporidae resultó ser monofilética y

grupo hermano de Atractotrematidae en la mayoría de ocasiones, siendo la posición de *Hapladena* la que convirtió a Haploporidae y Atractotrematidae en parafiléticas en algunas ocasiones, pero sin apoyo significativo. La erección de una superfamilia aparte para Atractotrematidae y Haploporidae como se había sugerido previamente (Jones, 2003; Overstreet y Curran, 2003; Curran *et al*, 2006) no fue apoyada por las hipótesis obtenidas. La presunta relación entre las familia Haplosplanchnidae y Haploporidae quedó rotundamente rechazada dada la posición de Haplosplanchnidae como linaje monofilético independiente y siendo menos derivado dentro de los plagiórquidos superiores, lo que confirma la elevación de su rango taxonómico a suborden (Haplosplanchnata Olson Cribb, Tkach, Bray y Littlewood, 2003) por Olson *et al*. (2003).

Las diferentes regiones del gen ribosómico utilizadas y los diferentes análisis dieron lugar a diferentes hipótesis filogenéticas para la posición los haplopóridos en los Xiphidiata. A pesar de la existencia de muy pocas o ninguna característica biológica que sugiera un origen común, encontramos una relación estrecha entre los clados de Haploporidae y Atractotrematidae y Paragonimidae y Troglotrematidae en prácticamente todas las filogenias inferidas. Por tanto, Haploporidae + Atractotrematidae parece ser que representan un linaje con una simplificación secundaria del ciclo de vida y una captura de hospedador de tetrápodos a peces. Los caracteres morfológicos y del desarrollo de Haploporidae no representan las de su ancestro más cercano y si se utilizaran como evidencias para inferir relaciones filogenéticas darían resultados espurios.

CONCLUSIONES

El estudio morfológico comparativo de las subfamilias Bunocotylinae y Haploporidae se llevó a cabo considerando el conocimiento sobre los caracteres taxonómicos relevantes y teniendo como propósito: (i) evaluar la importancia de nuevos caracteres morfológicos; (ii) redescribir las especies del Mediterráneo con el nuevo material recolectado; y (iii) realizar una revisión crítica de las especies nominales de cada género. Además, la variabilidad intra e interespecífica de los caracteres morfológicos se estudió por medio de análisis estadísticos multivariantes basados en varias muestras poblacionales. Como resultado, obtuvimos información sobre la adscripción de las especies a determinados géneros y diagnosis más detalladas de nueve géneros y una estimación más exacta de la riqueza de especies, de la morfología y de la posición sistemática de los parásitos más diversos de mugílidos.

Finalmente, mediante una aproximación molecular se pudo comprobar, de manera independiente, la situación taxonómica de la subfamilia Haploporinae establecida mediante la comparación morfológica, y permitió evaluar la diversidad taxonómica y las relaciones filogenéticas a diferentes escalas taxonómicas. Como síntesis del estudio, éstas son las principales conclusiones:

1. La diversidad de especies del género de Bunocotylinae *Saturnius* es mayor de lo que se creía hasta el momento, como se evidencia por la descripción de tres nuevas especies: *S. minutus* en *Mugil cephalus* en la costa mediterránea de España; *S. dimitrovi* en *M. cephalus* de la costa búlgara del Mar Negro y de la costa mediterránea española; y *S. overstreeti* en *M. soiuy* y *M. cephalus* de la costa rusa del Mar del Japón. El estatus de los nuevos taxones se ha validado mediante los análisis discriminantes que permitieron identificar cinco variables que contribuían en un 100% a la correcta identificación de los grupos. La revisión de las especies nominales emplazadas en *Saturnius* ha dado lugar a una refinada diagnosis del género y a la elaboración de unas claves de identificación a nivel de especie. Dos especies, *Bunocotyle constrictus* y *Saturnius valamugilis*, han sido consideradas especies *inquirendae* en el presente trabajo, así como *B. mugilis* de Solonchenko (1976), , y *S. mugilis* de Dmitrieva y Gaevskaya (2001) se han considerado citas dudosas..
2. *Haploporus* se considera un género de haploporinos mediterráneos monotípico. Se ha redescrito *H. benedeni* (especie tipo) y se ha considerado *H. lateralis* como su sinónimo. Cinco especies parásitas de *Valamugil* spp. en la región del Índico y del oeste del Pacífico (*H. indicus*, *H. spinosus, H. magnisaccus, H. mugilis* y *H. muscolosaccus)*, han sido consideradas *incertae sedis* en cuanto al género. Consideramos *H. pacificus* (sin. *Neohaploporus pacificus* Manter, 1963)*, H. pseudoindicus* y *H. musculosaccus* especies *inquirendae* y *H. lossii* Al-Bassel, 1990 un *nomen nudum*. Se ha presentado una nueva diagnosis, tratando de evitar la situación anterior que daba cabida a cualquier especie, y conservando el concepto original de Looss (1902).
3. El estatus de las especies nominales de *Dicrogaster* Looss, 1902 se ha reconsiderado mediante el estudio morfológico comparativo del nuevo material recolectado en la costa española y la evaluación crítica de los datos publicados. *D.*

perpusilla (especie tipo) y *D. contracta* se han redescrito utilizando material nuevo procedente de varias especies de *Liza*. Estas dos especies junto con *D. fastigata* se han considerado especies válidas, mientras que *D. fragilis* se ha considerado sinónimo de *D. fastigata*. *D. maryutensis* se considera como *nomen nudum*. Las dos formas mediterráneas de *Dicrogaster, D. perpusilla* y *D. contracta*, se diferenciaron también mediante análisis estadísticos multivariantes. Se ha presentado una diagnosis más detallada de este género *Dicrogaster* y una clave de identificación de las especies consideradas válidas.

4. Se ha descrito una nueva especie perteneciente al género *Forticulcita*, *F. gibsoni*, parásita de *M. cephalus* en el Mediterráneo occidental. Ésta se ha diferenciado de las otras dos especies del género por tener un cuerpo sustancialmente más pequeño, y por la diferencia en prácticamente todas las demás las características morfométricas. *F. gibsoni* también se ha distinguido mediante análisis estadísticos multivariantes con datos morfométricos de las dos formas mediterráneas de *Dicrogaster*, puesto que se parecían superficialmente. Se ha redefinido la diagnosis del género y se ha preparado una clave de identificación de las especies.
5. *Lecithobotrys* Looss, 1902 se considera como género monotípico de haploporinos Mediterráneos. Se redescribe *L. putrescens* Looss, 1902 con el nuevo material recolectado de especies de *Liza*. *L. aegyptiacus* se ha considerado sinónimo de *Saccocoelium tensum* y por otro lado, *L. brisbanensis* (sin. *Paralecithobotrys brisbanensis* Martin, 1974) y *L. vitellosus* se consideran especies *inquirendae.* Hemos presentado una nueva diagnosis para el género.
6. Se ha revisado el género *Saccocoelium* Looss, 1902, presentándose una nueva diagnosis y clave de identificación para las especies reconocidas en él. *S. obesum* Looss, 1902 (especie tipo) y *S. tensum* Looss, 1902 han sido redescritas y se han descrito tres especies nuevas, *Saccocoelium cephali* n. sp., *S. brayi* n. sp. y *S. currani* n. sp. Las cinco especies presentes en el Mediterráneo se han diferenciado también mediante análisis multivariantes. *Lecithobotrys helmymohamedi*, *S. portsaidensis*, *S. saoudi* y *Neosaccocoelium aegyptiacus* se han considerado sinónimos de *S. tensum* y *Neosaccocoelium* sinónimo de *Saccocoelium*. *Lecithobotrys mugilis* ha sido reubicado en *Unisaccus* como *U. mugilis* (Rekharani y Madhavi, 1985), así como *Lecithobotrys sprenti* Martin, 1973 [= *Saccocoelium sprenti* (Martin, 1973) Overstreet y Curran, 2005] ha sido transferido a *Unisaccus*

como *U. sprenti* (Martin, 1973). *S. megasacculum* ha sido transferido al género *Elliptobursa* como *E. megasacculum* (Liu, Wang, Peng, Yu y Yang, 2004). *S. tripathi* Dutta, 1995 (sin. *Saccocoelium tripathi* Datta y Manna, 1998) ha sido considerada especie *inquirenda*.

7. Se establecen tres nuevos géneros de haploporinos en este estudio. *Ragaia* se ha erigido para una nueva especie, *R. lizae*, en *Liza ramada* en el Delta del Ebro, en la costa mediterránea española. Dos géneros, *Pseudolecithobotrys* y *Pseudodicrogaster*, han sido erigidos para acomodar especies del Pacífico norte que pertenecían a otros géneros, *Lecithobotrys stomachicola* y *Dicrogaster japonica*, respectivamente. Se ha elaborado una clave de identificación de los 10 géneros existentes en Haploporinae.
8. Este estudio ha sido el primero en caracterizar especies congenéricas de la familia Haploporidae mediante una aproximación morfológica y molecular. Ello ha permitido una valoración de las diferencias genéticas y morfológicas entre ellas. Estas diferencias son típicas entre especies próximas del género *Saccocoelium* que parasitan especies simpátricas de hospedadores mugílidos en el Mediterráneo. Los datos moleculares han corroborado las decisiones taxonómicas basadas en la morfología con respecto a la validez de diferentes especies en *Saccocoelium,* como es el caso de *S. obesum* (*sensu stricto*) y *S. tensum*, y ha apoyado el reconocimiento de las dos especies nuevas mencionadas anteriormente, *S. brayi* n. sp. y *S. cephali*, por lo que se ha rechazado la hipótesis de una sola especie de *Saccocoelium* en el Mediterráneo. Sin embargo, los resultados obtenidos mediante la secuenciación del fragmento ITS2 no refutan la posibilidad de que exista una diversidad de especies de *Saccocoelium* aún mayor. A su vez, se ha encontrado que los patrones de especies y diversidad genética observados a la escala reducida de este estudio indican que hay factores ligados a las características del ciclo de vida de los haplopóridos que modifican el efecto del filtro abierto de encuentro, debido a la similitud en la alimentación de los hospedadores. En consecuencia, la posibilidad de especiación simpátrica y de mayor diversidad de especies en este sistema estudiado, y en Haploporidae en general, debe ser mayor, proponiéndose dos hipótesis no excluyentes.
9. Hemos demostrado la utilidad de los análisis multivariantes para averiguar la variabilidad intra e interespecífica así como para comprobar las hipótesis de

diferenciación morfométrica entre los especímenes de distintas especies/poblaciones en los grupos estudiados, géneros que poseen especies morfológicamente muy similares. Los resultados de ambos análisis estadísticos, PCA y LDA, han sido concordantes cuando fueron aplicados simultáneamente. El LDA mostró de manera consistente las variables morfométricas que mejor separaban los grupos de especies. Se ha sugerido, por tanto, que ambos métodos estadísticos son herramientas valiosas para reconocer especies crípticas y delimitar especies de los taxones aquí estudiados, así como a la hora de desarrollar claves de identificación de especies.

10. La clasificación de la familia Haploporidae basada en la morfología se evaluó por primera vez mediante datos moleculares en esta tesis doctoral, a la vez que se esclarecieron las relaciones dentro de la subfamilia Haploporinae a nivel de género. Los análisis moleculares han revelado: (i) una relación muy próxima entre las familias Atractotrematidae y Haploporidae; (ii) un apoyo significativo de la monofilia de la subfamilia Haploporinae, *Dicrogaster* y *Saccocoelium*, y la posición de *Ragaia* dentro de Haploporinae; (iii) evidencias para rechazar la subfamilia Dicrogasterinae y la sinonimia entre *Saccocoelioides* y *Lecithobotrys*; y (iv) evidencia significativa para reconocer el distinto estatus de *Saccocoelium* en relación con *Haploporus*. El amplio muestreo de especies de *Dicrogaster* y *Saccocoelium* ha confirmado el reconocimiento del distinto estatus de estas especies y rechazando, por tanto, las sinonimias previas. *Saccocoelioides*, recientemente incluido en la subfamilia Chalcinotrematinae, ha aparecido dentro del clado de Haploporinae, lo cual estaba íntimamente relacionado con la posición del género *Forticulcita,* que ha aparecido como el género más basal de la subfamilia. También se ha detectado que *Forticulcita* posee un órgano copulador intromitente bien diferenciado, característica única dentro de la familia. Esta importante apomorfía, junto con la hipótesis molecular, han respaldado la decisión de proponer una nueva subfamilia, Forticulcitinae n. subf. para *Forticulcita*. Este hecho hace que *Saccocoelioides* y, por extensión la subfamilia Chalcinotrematinae, sea grupo hermano de Haploporinae. La posición de la subfamilia Megasoleninae, representada por una especie del género *Hapladena,* permanece sin resolver.
11. Dentro de los Digenea, los Haploporidae muestran claras afinidades filogenéticas con los miembros de la familia Gorgoderoidea Looss, 190, estando incluidos en el

suborden Xiphidiata Olson Cribb, Tkach, Bray y Littlewood, 2003, recientemente establecido. Haploporidae aparece como un clado monofilético y grupo hermano de la familia Atractotrematidae en la mayoría de ocasiones. Sin embargo, las diferentes soluciones han sugerido que no se reconoce la erección de una superfamilia aparte para Atractotrematidae y Haploporidae, como se había sugerido. La presunta relación entre las familia Haplosplanchnidae y Haploporidae es rechazada y se confirma la posición de Haplosplanchnidae como linaje monofilético independiente y menos derivado en los 'Plagiorchiida superiores', apoyando la elevación de su rango taxonómico a suborden (Haplosplanchnata Olson Cribb, Tkach, Bray y Littlewood, 2003).

12. Las diferentes regiones del gen ribosómico utilizadas y los diferentes análisis han dado lugar a diferentes hipótesis filogenéticas para los haplopóridos en los Xiphidiata. A pesar de la existencia de muy pocas o ninguna característica biológica que sugiera un origen común, se encontró en prácticamente todas las filogenias estimadas una relación cercana entre los haplopóridos y los paragonímidos y los troglotremátidos. Por tanto, se ha considerado que Haploporidae + Atractotrematidae pueden representar un linaje con una simplificación secundaria en el ciclo de vida y una captura de hospedador de tetrápodos a peces. Se ha visto que los caracteres morfológicos y del desarrollo de estas dos familias no representan las de su ancestro más cercano y si se utilizasen como evidencias para inferir relaciones filogenéticas obtendríamos posiblemente resultados erróneos.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Host group studied

Grey mullets (Pisces: Mugilidae) (referred to as mullets thereafter in the thesis) represent a family of teleost fish with a worldwide distribution. Thomson (1997) recognised 14 genera for a total of 62 valid species. Most of these are included in the genera *Mugil* L. and *Liza* Jordan & Swain, which have 12 and 23 species, respectively. Six sympatric species occur commonly in the Mediterranean, namely *Chelon labrosus* (Risso), *Liza aurata* (Risso), *L. ramado* (Risso), *L. saliens* (Risso), *Mugil cephalus* L. and *Oedalechilus labeo* (Cuvier) (Matallanas, 1990). Two additional species, *Liza carinata* (Valenciennes) and *Mugil soiuy* (Basilewsky) have recently spread to the eastern Mediterranean from the Red Sea and the Black Sea, respectively (Thomson, 1997; Kaya *et al*., 1998).

Mullets tolerate a large salinity and oxygen concentration gradient and this enables them to dwell in lagoons, estuaries and the sea (Thomson, 1966). They exhibit a seasonal habitat shift due to offshore migration during the spawning season but shifts are also observed in immature fish in some seasons (Cardona, 2000). Mullets undergo a diet change from juveniles to adults (Cardona & Castelló, 1994; Cardona *et al*., 2001) and there is a trophic overlap among species, generally during the summer (Cardona, 2001). Of particular relevance to the present study is the fact that mullets are detritivorous (Cardona, 1994) and are also shown to behave as efficient planktivorous pump-filters that forage simultaneously on plankton and benthos (see Cardona *et al*., 2001 and references therein). This feeding behaviour would ensure completion in this host group of parasite life-cycles which involve encystment on the substrate (*e.g.* haploporid and haplosplanchnid digeneans) and within planktonic crustaceans (*e.g.* hemiuroidean digeneans, anisakid nematodes, tetraphyllidean cestodes).

Mullets offer an excellent system to study the geographical, ecological and evolutionary aspects of host-parasite associations. Current evidence suggests that this host-parasite system is characterised by (i) a high degree of exchange of mullet parasites in areas, such as the Mediterranean, Red Sea and Black Sea, where mullets are diverse and sympatric, and (ii) the presence of a number of local, strictly specific congeneric parasite species that are closely related to those in adjacent areas (Esch & Fernández, 1993). These conclusions, however, are based on the original authors' taxonomy of largely scattered literature sources and no effort has been made, to date, towards a taxonomically consistent assessment of host specificity.

Mullets harbour a generally diverse parasite fauna, which includes representatives of all major parasitic groups. Thus, considering protozoans, helminths and crustaceans, 39 species have been reported from mullets in the eastern Mediterranean, at least 29 species from the northern Red Sea and at least 43 species from the Black Sea where studies are continuing (see Gaevskaya *et al*., 1975; Paperna & Overstreet, 1981; Gaevskaya & Korniychuk, 2003 for reviews). A number of new monogenean species have been described recently in the Mediterranean (*e.g.* Sarabeev & Balbuena, 2004a; Sarabeev *et al.*, 2005; Rubtsova *et al.*, 2006). However, existing data on parasites of mullets in general, and in the Mediterranean region in particular, are largely scattered both in space and time and, with a few exceptions (Paperna, 1975; Skinner, 1975; Fernández-Bargiela, 1987; Solonchenko & Tkachuk (1985); Merella & Garippa, 1998, 2000, 2001; Domnich & Sarabeev, 2000a, b; Sarabeev & Domnich, 2000; Valles-Ríos *et al.*, 2000) no focused faunistic studies have been carried out worldwide. Studies which contribute to the knowledge of the parasite fauna of mullets represent mostly isolated descriptions (typically the original descriptions) of individual species, whereas the prevailing part of host-parasite records in this system is a by-product of regional faunistic studies on a wide range of fish species, mullets being typically characterised by a low study effort (*e.g.* Vlassenko, 1931; Fischthal & Kuntz, 1963; Paperna, 1964; Yamaguti, 1970; Solonchenko, 1976, 1982; Orecchia & Paggi, 1978; Paggi *et al*., 1979, 1988; Romero & Galeano, 1981; Orecchia *et al*., 1988; Lester & Sewell, 1989; Radujković & Raibaut, 1989; Radujković *et al*., 1989; Saoud *et al*., 1990; Luque & Oliva, 1993; D'Amelio *et al*., 1995; Di Cave *et al*., 1997; Knoff *et al*., 1997).

Nevertheless, existing data indicate that two trematode groups specific of mullets, the genus *Saturnius* Manter, 1969 of the subfamily Bunocotylinae Dollfus, 1950 of the Hemiuridae Looss, 1899 and the family Haploporidae Nicoll, 1914, are generally most abundant and broadly distributed in different mugilid species worldwide (see also Gibson, 2002; Overstreet & Curran, 2005). However, despite the numerous records, their morphological variability and taxonomic diversity has not been addressed in detail. Thus, the basic requirement for accurate delineation of the parasite communities, *i.e.* a well-developed taxonomy for a fast and reliable identification of the parasites, is still needed. This situation can lead to misinterpretation of parasite biodiversity patterns in communities of sympatric mullet hosts, thus delaying the development of ecological studies; these indeed to date are few (*e.g.* Knoff *et al.*, 1997; Dzikowski *et al.*, 2003).

1.2. *Saturnius* Manter, 1969 (Hemiuridae: Bunocotylinae)

The family Hemiuridae is a large group with numerous subfamilies that occur mostly in the stomach of marine teleosts. The major diagnostic feature of the family is the ecsoma, a protusible, posterior region of the body, which appears to enable the worm to survive in the acid regions of the stomach (Gibson & Bray, 1979). However, members of four subfamilies occur in other parts of the stomach or in the intestine; in these (including Bunocotylinae) the ecsoma is lost or vestigial (Gibson, 2002). The Hemiuridae is poorly represented in mullets with only 19 nominal species so far recorded worldwide (*i.e*. *Hemiurus* Rudolphi, 1809 – 1 species, *Parahemiurus* Vaz & Pereira, 1930 – 1, *Lecithocladium* Lühe, 1901 – 1, *Lecithochirium* Lühe, 1901 – 3, *Saturnius* Manter, 1969 – 6, *Bunocotyle* Odhner, 1928 – 2, *Aphanurus* Looss, 1907 – 4, and *Opisthadena* Linton, 1910 – 1) (Pankov *et al.*, 2006).

The small hemiurid genus of stomach parasites specific to mullets, *Saturnius* Manter, 1969 until recently represented by six species, appears the most diverse hemiurid group in mullets. Of these, only *S. papernai* Overstreet, 1977, has been recorded in different hosts from the Mediterranean. However, there is a lack of studies characterising morphological variability of the species in this genus especially in the Mediterranean where mullet parasite faunas are relatively well-studied (see references above). Furthermore, some species of *Saturnius* were described on the basis of merely a few specimens and features that have proved useful for species discrimination were not sufficiently characterised in the original descriptions (*e.g*. Yamaguti, 1970; Fischthal, 1977; Rekharani & Madhavi, 1985; Domnich & Sarabeev, 1999; Peng *et al*., 2004), which prompted the revision of the genus presented herein (Chapter 4).

1.3. Subfamily Haploporinae Nicoll, 1914 (Digenea: Haploporidae)

The Haploporidae is a cosmopolitan group of parasites from the alimentary canal of marine, estuarine and freshwater fishes. According to the latest taxonomic revision, the family is formed by four subfamilies: Haploporinae Nicoll, 1914; Chalcinotrematinae Overstreet & Curran, 2005; Megasoleninae Manter, 1935 and Waretrematinae Srivastava, 1937 (see Overstreet & Curran, 2005). The Haploporinae is a relatively small group of poorly known digeneans which, with a single exception [*i.e. Haploporus pacificus* (Manter, 1963)], parasitise marine or brackishwater mugilid fishes, mostly in the Mediterranean. Haploporines are characterised by the possession of a vitellarium composed of one or two coalesced or

compact groups or two groups of a few distinct follicles, and a uterus that occupies much of the hindbody and reaches anteriorly to the level of the hermaphroditic sac (Overstreet & Curran, 2005). Many species from the Mediterranean were briefly described by Looss (1902), and this resulted in the erection of most of the genera from this area, *i.e. Haploporus* Looss, 1902, *Saccocoelium* Looss, 1902; *Lecithobotrys* Looss, 1902, and *Dicrogaster* Looss, 1902. Following the addition of three genera (*Unisaccus* Martin, 1973, *Forticulcita* Overstreet, 1982 and *Rondotrema* Thatcher, 1999), the subfamily is now considered to comprise seven genera (see Overstreet & Curran, 2005).

Although studies exist on some aspects of the morphology and systematics of specific groups within the Haploporidae and Haploporinae in particular, a thorough and well-grounded concept of their taxonomy and classification is still lacking, mostly because these worms are especially difficult to prepare for morphological examination. They possess an extremely delicate tegument and inadequately prepared specimens can result in incomplete and misleading descriptions (Overstreet & Curran, 2005). This, in turn, leads to defining taxa erratically rather than on soundly based apomorphs which prevents both identification and attempts to a phylogenetic classification concept for the group. Consequently, the status of many species has been reconsidered. The recent taxonomic revision of Overstreet & Curran (2005) has greatly clarified the situation at the generic/suprageneric level; their taxonomic framework is followed in the present study.

However, although some new combinations were made by Overstreet & Curran (2005), species-level taxonomy is still unsettled; due to volume limitations in the *Keys to the Trematoda* the range of species considered for the development of the generic diagnoses has not been clearly indicated. Further, the current status of the taxonomy of the haploporines still comprises a number of unsolved problems. Of particular importance to the present study is the poor/or virtual lack of knowledge on the morphological and morphometric variation at the species level throughout most genera in the subfamily. This perhaps has led to the fact that a hypothesis of a single polymorphic species has been suggested in the case of Mediterranean representatives of three out of the five genera of the Haploporinae recorded in the region (see Chapters 5, 6 for details) and is in sharp contrast with the situation with another group of digeneans in sympatric Mediterranean fish, sparids, for which a much higher than previously known species diversity was revealed as a result of combined morphological, morphometric and molecular studies (*e.g.* Jousson *et al.*, 2000; Jousson & Bartoli, 2002). A recent study on Mediterranean mullets, applying this approach, resulted in the discovery of a new

bunocotyline genus and allowed the current taxonomic classification of the Bunocotylinae to be tested and relationships within the Hemiuroidea to be explored (Pankov *et al*., 2006).

1.4. Advances in the application of molecular approaches to trematode systematics

'All levels of systematics are enjoying a renaissance' (Hillis *et al.*, 1996). This quote reflecting the impact of the advent and the new developments of molecular biological techniques (reviewed by Avise, 2004; Hillis *et al*., 1996), notably the polymerase chain reaction, is fully justified with regard to parasitic platyhelminths as evidenced by the explosive development of studies using molecular data in the last decade.

Molecular approaches may be particularly effective in situations (*e.g.* closely related species) where morphological variation is limited. Furthermore, molecular systematics offers comparative and reproducible evolutionary data in addition to the mainstay of parasite systematics, *i.e.* the morphological examination. This explains why the bulk of studies on parasitic worms using a molecular approach occupy two extremes, *i.e.* either circumscribing major lineages and estimating their relationships, or delineating species and strains, the latter typically focused on those of medical or economic importance (Olson & Tkach, 2005).

The adoption of molecular techniques has substantially advanced our understanding of species diversity and life histories, and phylogenetic interrelationsips within the Digenea, the largest group of internal metazoan parasites of animals (Cribb *et al*., 2001). Molecular systematics studies at different taxonomic levels can be driven by using a molecule with the appropriate level of variability and a set of systematic comparisons representing the appropriate evolutionary timescale (Hillis *et al.*, 1996). The ribosomal RNA gene is ideally suited to address systematic questions at different taxonomic levels because it contains regions which exhibit different rates of evolution *i.e.* highly conserved (18S, 5.8S and 28S) and highly variable (ITS) fragments (Hillis & Dixon, 1991). Sequence data of these regions have been widely applied to molecular systematics of digeneans (reviewed in Nolan & Cribb, 2005; Olson & Tkach, 2005). The 18S region is among the slowest evolving sequences found in living organisms and is used to infer deep phylogenetic relationships among ancient lineages (Hillis & Davis, 1986). Unlike in cestodes, 18S sequences of disparately related digeneans have shown far less variability (Cribb *et al*., 2001) resulting in short internodes and low nodal support (Olson & Tkach, 2005). However, the 28S region is larger and evolves faster than 18S but possesses regions with levels of gene conservation similar to those of 18S (Hillis & Dixon, 1991) that make it suitable for studying relationships among genera as well

as related families. The 5.8S fragment, in contrast, is too short and of little use for inferring phylogenies across large time scales (Hillis & Dixon, 1991; Hershkovitz & Lewis, 1996; Coleman, 2003). However, its conserved nature offers the opportunity to develop primers for PCR amplification and sequencing of the two internal transcribed ribosomal spacers. The latter exhibit the fastest evolutionary rate in the ribosomal gene but, at the same time, they are thought to be relatively conserved within a species or genus. Consequently, these regions have been commonly exploited for the exploration of species boundaries in digeneans (see Nolan & Cribb, 2005 for an extensive review) and assessment of phylogenetic relationships at several nested taxonomic scales (briefly summarised below).

1.4.1. Lowest scale: Species distinction

Species distinction using solely morphological features has proved to be difficult and problematic in many digenean families. A number of factors can lead to both under- and overstimates of parasite diversity (reviewed in Nolan & Cribb, 2005). These include the small size of the adult stages in combination with the paucity of taxonomic features and the uncertainty over their validity (*e.g.* Luton *et al.*, 1992, Leon-Regagnon *et al*., 1999); high-morphological similarities between closely related species (*i.e*. cryptic species) (Tkach *et al*., 2000b); time-lag between primary genetic speciation and morphological differentiation (Jousson *et al*., 2000); phenotypic plasticity (especially age and host-induced variation; Galazzo *et al*., 2002); lack of conserved and hard structures (Jousson & Bartoli, 2001) and the fact that many digenean life-cycle stages such as the cercariae and metacercariae lack distinctive morphological characters, making their association with adults by morphological characters alone impossible (Jousson *et al.,* 1998b, 1999; Bartoli & Jousson, 2003).

Molecular genetic methods can distinguish otherwise cryptic species (Avise, 2004) and have been widely applied to identify species of bacteria and protozoans lacking major distinguishing morphologies (*e.g.* Perkins, 2000; Jenga *et al*., 2001). Identification at the species level using a molecular approach requires the use of a highly variable marker. This is associated with the wide application to digeneans, a group of extraordinary diversity characterised by the presence of large numbers of morphologically similar and cryptic species, of the two ITS rRNA gene regions (ITS1 and ITS2).

This has helped to uncover a large number of cryptic/sibling and morphologically similar species of at least 19 digenean families (see Nolan & Cribb, 2005 for an extensive review) (*e.g.* Anderson & Barker, 1993; Despres *et at.,* 1995; Jousson *et al.*, 1998a,b, 2000;

Tkach *et al*., 2000b; Snyder & Tkach, 2001; Jousson & Bartoli, 2001, 2002; Nolan & Cribb, 2004, 2006; Miller & Cribb, 2007) as well as to elucidate life-cycle stages (Jousson *et al.,* 1999; Bartoli *et al*., 2000; Jousson & Bartoli, 2000; Bartoli & Jousson, 2003; Picard & Jousson, 2001; Dvorak *et al*., 2002). Of the 63 studies reviewed in Nolan & Cribb (2005), 14 analysed the entire ITS region, 44 used the entire ITS2 region and 24 analysed partial or complete ITS1 (incl. 6 using entire ITS).

In general, smaller differences were found in ITS2 (larger number of studies with interspecific variation <1.0%) than in the ITS1 region, in which the greatest divergences of the ITS occur. Nevertheless, the ITS2 region was found to successfully discriminate digenean species from many digenean families and can be expected to continue to do so (Nolan & Cribb, 2005). As mentioned above, the difficulties in preparing haploporid material and the unclear morphological boundaries of some species, offer an interesting case study for the application of a molecular approach.

1.4.2. Systematics and phylogenetic relationships of supraspecific groups

Molecular systematic studies aimed at assessment of phylogenetic hypotheses in order to elucidate interrelationships of genera and families were considered to produce the most conclusive results due to the higher levels of homoplasy in studies at higher taxonomic levels (Olson & Tkach, 2005). Examples have accumulated using three different regions of the ribosomal RNA gene, to mention a few:

- **18S:** Hemiuroidea (see Blair *et al*., 1998b); Fasciolidae and Paragonimidae (see Blair, 1993); Gyliauchenidae (see Blair & Barker, 1993); Lepocreadiidae and Fellodistomidae (see Lumb *et al*., 1993; Hall *et al*., 1999); Brachycladidae and Nasitrematidae (associated with an analysis of mtDNA sequences, see Fernández *et al*., 1998 a, b, 2000).
- **ITS:** Didymozoidae (see Anderson & Barker, 1998); Echinostomatidae (see Grabda-Kazubska *et al*., 1998; associated with an analysis of *nad1* mt gene sequences, Kostadinova *et al*., 2003); Paragonimidae (associated with *cox1* mt gene sequence analysis, Blair *et al*., 1999); Mesometridae (see Jousson *et al*., 1998b).
- **Partial 28S:** various genera and families assigned to the suborder Plagiorchiata (see Tkach *et al.,* 1999, 2001a,c, 2002, 2003; Curran *et al*., 2006, 2007); Schistosomatidae (see Snyder & Loker, 2000); Quadrifoliovariinae of the Lecithasteridae (associated with analysis of ITS2 sequences, Chambers & Cribb, 2006).

- **Combined 18S and 28S:** Schistosomatidae (studies reviewed in Rollinson *et al.*, 1997, *e.g.* Snyder, 2004; associated with *cox 1* mt gene sequence analysis, Lockyer *et al*., 2003); Acanthocolpidae (see Bray *et al*., 2005); Bunocotylinae and Hemiuroidea (see Pankov *et al*., 2006); Allocreadiidae (see Choudhury *et al.*, 2007).

The studies carried to date have improved our understanding of the relationships between a number of supraspecific lineages (genera, subfamilies and closely related families). However, no attempt has been made to date to assess the interrelationships within the family Haploporidae.

1.4.3. Phylogenetic relationships at the suprafamilial level within the Digenea

Comprehensive studies of digenean phylogeny at the suprafamilial taxonomic levels have developed recently (resulting from the accumulation of molecular data in the last decade, see Olson & Tkach, 2005 for a review) the first being the analysis of Cribb *et al*. (2001). These authors used morphological and molecular data (complete 18S sequences) for 75 digenean taxa, representing 55 families to examine the basic patterns of digenean phylogeny. Olson *et al*. (2003) conducted the broadest molecular phylogenetic analysis of the Digenea based on a combination of complete 18S and partial 28S rDNA sequences for 163 species of 77 families, which represented the major lineages of the Digenea. Their analysis built upon earlier studies since the two target ribosomal regions, 18S and 28S, have typically been used in trematode phylogenetic investigations and this has resulted in the largest database of molecular characters for this group (Olson & Tkach, 2005).

Further studies have analysed specific clades within the previously assessed phylogenies (*e.g.* Bray *et al*., 2005; Pankov *et al*., 2006; Choudhury *et al*., 2007) since in spite of the wide coverage of the study of Olson *et al*. (2003), there were unattended groups (some small families) with unknown or unclear phylogenetic affinities for which a higher effort was needed to provide greater representation of their range of diversity (Olson & Tkach, 2005). The representation of the Haploporidae in phylogenetic analyses at this level has been rather limited: a single taxon, *Pseudomegasolena ishigakiense* Machida & Kamiya, 1976 (transferred to Atractotrematidae by Overstreet & Curran, 2005) in the study of Cribb *et al*. (2001); the latter species plus *Hapladena nasonis* Yamaguti, 1970 were included in the analysis of Olson *et al.* (2003). These authors found the family Haploporidae among the most labile in its placement, but in the analysis of the combined dataset it was found together with

the Atractotrematidae clustering to the Paragonimidae Dollfus, 1939 and Troglotrematidae (Odhner, 1914) within the Gorgoderoidea Looss, 1901 in the suborder Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003. However the Haploporidae lacks the unique character that unites all superfamilies in the Xiphidiata, the stylet in the cercariae, which could point to a misplacement of this family. Moreover, the Haploporidae + Atractotrematidae were found to be paraphyletic, suggesting sinking the latter family. A few further studies included incidentally members of the Haploporidae (see Bray *et al.*, 2005; Curran *et al.*, 2006, 2007; Choudhury *et al.*, 2007) but to date only one new sequence (*Saccocoelioides* sp.) has been added (Curran *et al*., 2006). Despite the complex and controversial taxonomic history of this family (see details in Chapter 8), the affinities of the haploporids remain unresolved.

In summary, the review of the recent developments in trematode taxonomy supports the idea that studies that incorporate both, morphological and molecular data, will provide better descriptions and interpretations of biological diversity than those that focus on just one approach (Hillis *et al.*, 1996; Nolan & Cribb, 2005). It might be expected, therefore, that increased sampling for rDNA sequences of the species of Haploporidae could be instrumental to clarification of the position of this family in the phylogeny of the Digenea and to the assessment of the relationships within the family as well as its suprageneric content. At the species level, the presence of molecular data would improve our understanding of both genetic and morphological variability of haploporids.

1.5. This study

This study has been carried out within the framework of an international project 'Evaluating the effect of an invasive species on local mullet communities in the Mediterranean: A parasite community approach' financed by INTAS (03-51-5998). The study thus profited from the examination of large samples of four mullet species in the Western Mediterranean, *Mugil cephalus*, *Liza aurata*, *L. ramado* and *L. saliens*, collected at two localities (Ebro Delta and off Santa Pola) on the Spanish coasts as well as from the availability of comparative material collected off the Bulgarian Black Sea coast and off the Russian coasts of the Sea of Japan.

The large specimen collection gained in the course of the study enabled a comparative assessment of the morphological and genetic variation by addressing the following questions:

- What is the actual species diversity of the Bunocotilinae and Haploporinae?
- What are the levels of intraspecific morphological/morphometric variability within the two subfamilies?
- Do molecular data support the taxonomic framework of the Haploporinae based on morphology?
- What are the phylogenetic relationships at suprageneric level and the affinities of the Haploporidae within the Digenea?

CHAPTER 2

AIM AND OBJECTIVES

Aim

The study aims to facilitate the advancement of taxonomic knowledge of two major groups of mullet digeneans, the subfamily Bunocotylinae of the Hemiuridae and the subfamily Haploporinae of the Haploporidae. In the case of the latter, the study examines the taxonomic consistency of digenean identification based on a combination of morphological, morphometrical and molecular sequence data from abundant newly collected material and uses the application of a molecular phylogenetic approach to test hypotheses for the relationships of genera within the Haploporidae and the position of this group within the phylogeny of the Digenea.

Objectives

- To carry out a taxonomic revision of the genus *Saturnius* (Hemiuridae: Bunocotylinae) based on a detailed morphological and morphometrical study of both newly collected and museum material, and to construct identification keys to the species level and a host-parasite-distribution list for each species.

- To undertake a taxonomic revision of five Mediterranean haploporid genera, *Haploporus*, *Dicrogaster*, *Forticulcita*, *Lecithobotrys* and *Saccocoelium,* based on a detailed morphological study of newly collected and museum material and a critical evaluation of the literature, and to construct identification keys to the generic and species level plus host-parasite-distribution lists for each species.

- To attempt a phylogenetic analysis of Mediterranean haploporids using sequence data for the large subunit and the second internal transcribed spacer of the ribosomal RNA gene in order to evaluate the degree of species differentiation and the validity of the haploporine genera, and to assess interspecific and intergeneric relationships within the taxonomic framework of the Haploporinae based on morphology.

- To assess the systematic position and phylogenetic relationships of the families Haploporidae and Haplosplanchnidae within the phylogeny of the Digenea as inferred from the small and large subunits of the ribosomal RNA gene.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. Fish sampling and parasite collection

Mugilids were sampled at two localities on the Spanish western Mediterranean coast, the Ebro Delta (40º30'– 40º50'N, 0º30'– 1º10'E) and off Santa Pola. Fish was sampled from two sites at the latter locality, off the seashore (38º00' – 38º20'N, 0º10'– 0º40'W) and in a brackishwater lagoon near the sea (38º10'N, 0º39'E). Given that the development of the present thesis was part of an international project, additional mullet specimens from the Black Sea and the Sea of Japan were examined for parasites. Specimens from the Black Sea were collected off Sozopol, Bulgaria (42º26'–42º19'N, 27º40'–28º05'E) and those from the Sea of Japan were sampled in the Kievka River, Russia (exact coordinates unknown) (see Fig 3.1). Selective additional sampling was carried out in the Spanish localities during 2007-2008 to collect more specimens of selected species for sequencing.

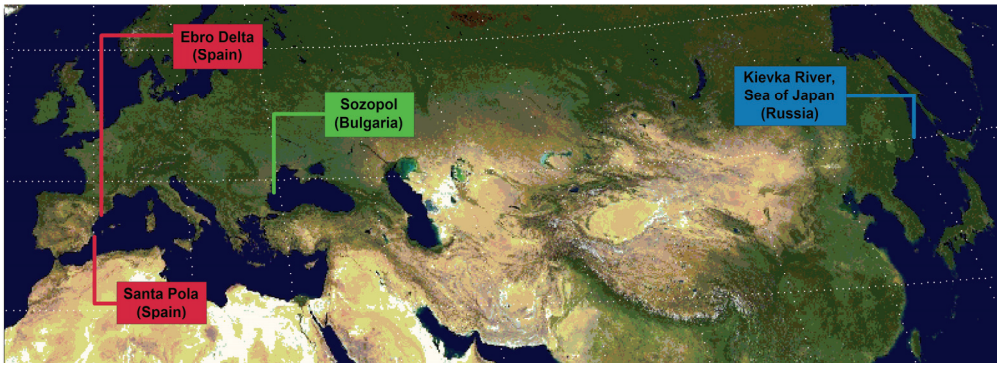

Fig. 3.1. Origin localities of the material studied throughout the thesis.

A total of 698 mullets (*M. cephalus, L. aurata, L. ramado* and *L. saliens*, see Fig 3.2 below) from the Western Mediterranean was collected and examined for parasites (see Table 3.1). A sub-sample (5-10 fish) of each host species per sample was examined fresh in order to collect live material for the morphological and molecular study. This is very important given that the trematodes and especially haploporids are fragile and degrade very rapidly after death. Thus, the study of fresh material allows appropriate fixation for subsequent morphological study and DNA isolation. The remaining fish were frozen at -20ºC and examined at a later stage when all parasites were collected, identified and counted. Parasites were collected according to a standardised protocol (Kostadinova *et al*., 2004) available as text and video at http://cetus.uv.es/mullpardb/index.html. In order to collect bunocotyline digeneans, the stomach of the fish was dissected longitudinally in a separate petri dish. The internal

Fig 3.2. Host species analysed.

stomach lining was removed and gently scraped in saline solution. The muscular walls of the stomach were scraped as well, to separate worms, if attached, and washed with saline solution. The washings were examined under a high magnification stereomicroscope (×6-40 or ×8-80). To collect haploporine digeneans, the intestinal pyloric caeca and intestine were removed and analysed separately. Each caecum was dissected longitudinally, the content scraped in saline solution and examined. The intestine was opened longitudinally and the usually abundant content was scraped into a large volume of saline solution, left to sediment in a tall conical glass vessel (0.4 L), decanted at least twice before examination and the parasites collected from the sediment. The walls of the caeca and the intestine were then examined under high magnification.

Table 3.1. Number of fishes analysed for this study.

2004-2005	Santa Pola (Sea)	Santa Pola (lagoon)	Ebro Delta
Liza aurata	102	-	101
Liza saliens	-	-	86
Liza ramado	48	10	48
Mugil cephalus	60	122	121

3.2. Processing of materials for morphological study

Digeneans were killed in near-boiling saline solution, fixed in 70% alcohol, stained with iron acetocarmine (Georgiev *et al*., 1986), dehydrated through a graded alcohol series, cleared in dimethyl phthalate and examined as permanent mounts in Canada balsam.

Type and voucher specimens are deposited in the British Museum (Natural History) Collection at the Natural History Museum, London (BMNH). The number of specimens used for descriptions and morphometric analyses (with BMNH collection numbers) are provided together with species descriptions in Chapters 4 and 5. Measurements were taken from illustrations, which were made using a drawing tube at high magnification (×400). All measurements are in micrometres. Metrical data when incorporated in the descriptions are given as ranges followed by the mean ± standard deviation (where applicable) in parentheses. Relative proportions were also calculated on the basis of published illustrations of the species included for comparison. Abbreviations for the metrical features used in the text and tables are given in Tables 3.2 (*Saturnius*) and Table 3.3 (Haploporinae). Additional specimens from museum collections examined for comparative purposes included type- and voucher material of a number of species of *Saturnuis* and the Haploporinae. Details for this material are provided on Table 3.4 and Table 3.5.

Table 3.2. Abbreviations of the metrical features used in the text and tables: *Saturnius*.

Abbreviation	Feature
BW/BL	Body width as a % of body length
FO/BL	Forebody as a % of body length
LSL/BL	Posteriormost pseudosegment length as a % of body length
LSW/LSL	Posteriormost pseudosegment width as a % of its length
MFL/VSL	Ventral sucker muscular flange length as a % of ventral sucker length
LSW/BW	Posteriormost pseudosegment width as a % of body width
VSW/BWVS	Ventral sucker width as a % of body width at the level of the anterior margin of the ventral sucker
MFW/BWVS	Ventral sucker flange cone width as a % of body width at the anterior border of the ventral sucker
MFW/VSW	Ventral sucker flange cone width as a % of the width of the ventral sucker
MFW/MFL	Ventral sucker flange cone width as a % of length
VL/BL	Vitellarium length as a % of body length
OSL/VSL	Sucker length ratio
OSW/VSW	Sucker width ratio

An exhaustive search of the literature and the Host-Parasite Database maintained at the Natural History Museum, London (Gibson *et al.*, 2005) was carried out in order to compile host-parasite-distribution records. The distribution lists follow the FAO's 'Major Fishing Areas' (http://www.fao.org/figis/servlet/) expressed in numerical form.

3.3. Morphometric statistical analyses

Two multivariate statistical methods were applied to morphometric data. Principal Components Analysis (PCA) was used to examine the multivariate relation among the specimens irrespective of their identity. PCA is a technique for summarizing most of the variation in a multivariate data set in fewer dimensions (*e.g.* Flury & Riedwyl, 1988; Tabachnick & Fidel, 2007). The first component is the linear combination that accounts for the maximum variance. Geometrically, it corresponds to the direction of the longest axis through the scatter of data points. Subsequent principal components take up maximal variance, subject to being orthogonal to all preceding component axis (Klingenberg, 1996).

Table 3.3. Abbreviations of the metrical features used in the text and tables: Haploporinae.

Abbreviation	Feature
Measurements	
BL	Body length
BW	Maximum body width
OSL	Oral sucker length
OSW	Oral sucker width
PL	Prepharynx length
PHL	Pharynx length
PHW	Pharynx width
OL	Oesophagus length
VSL	Ventral sucker length
VSW	Ventral sucker width
HSL	Hermaphroditic sac length
HSW	Hermaphroditic sac maximum width
GAL	Genital atrium length
GAW	Genital atrium maximum width
ISVL	Internal seminal vesicle length
ISVW	Internal seminal vesicle maximum width
ML	Metraterm length
MW	Metraterm width
ESVL	External seminal vesicle length
ESVW	External seminal vesicle maximum width
TL	Testis length
TW	Testis width
OVL	Ovary length
OVW	Ovary width
VL	Vitelline masses length
VW	Vitelline masses width
EL	Egg length
EW	Egg width
Distances	
FO	Forebody length
UEND	Post-uterine field length
CEND	Post-caecal field length
TEND	Post-testicular field length
Ratios	
BW/BL	Maximum body width as a % of body length
FO/BL	Length of the forebody as a % of body length
OSL/VSL	Sucker length ratio
OSW/VSW	Sucker width ratio
VSW/BW	Width of ventral sucker as a % of maximum body width
VL/PHL	Vitelline masses length as a % of pharynx length
HSL/VSL	Hermaphroditic sac length as a % of ventral sucker length
TEND/BL	Post-testicular field length as a % of body length
CEND/BL	Post-caecal field length as a % of body length.

Table 3.4. Comparative material of the Bunocotylinae studied from museum collections. *Abbrevations:* BMNH: British Museum (Natural History) Collection at the Natural History Museum, London UK; HWML: Harold W. Manter Laboratory, Lincoln, Nebraska, USA and USNPC: United States National Parasite Collection, Beltsville, Maryland, USA.

Species	Accession Number
Saturnius papernai Overstreet, 1977	BMNH 1975.9.29.4-5 (holotype and paratype)
	HWML 20248 (2 paratypes)
	USNPC 73271.00 (1 paratype)
Saturnius papernai of Dimitrov et al. (1998)	BMNH 1997.6.23.1-2 (10 vouchers)
Saturnius segmentatus Manter, 1969	USNPC 071219.00 (holotype)
	HWML 634-635 (6 paratypes)
Saturnius maurepasi Overstreet, 1977	BMNH1975.9.29.1-3 (holotype and 2 paratypes)
	HWML 20247 (1 paratype)
	USNPC 073270.00 (4 paratypes)
Saturnius mugilis (Yamaguti, 1970)	USNPC 063747.00 (holotype and paratype)
Saturnius belizensis Fischthal, 1977	USNPC 074167.00 (paratype)
Saturnius valamugilis Rekharani & Madhavi, 1985	BMNH 1984.6.28.16 (holotype)
Bunocotyle constrictus Domnich & Sarabeev, 1999	Schmal'gausen Institute of Zoology, Ukrainian Academy of Sciences No. 122-69 (paratype I.I.) labelled "*Bunocotyle constrictus* sp. n. det. V. Sarabeev (2 specimens mounted on the slide: 1 paratype and 1 distorted juvenile haplopoporid). Two other slides provided by V. Sarabeev: (i) Labelled "*M. soiuy* 56-15. 26.vii.1997. Molochnyi Liman. Stomach. Coll. Sarabeev. *Bunocotyle* sp. new. 3 specimens" (3 juvenile specimens); (ii) Labelled "*M. soiuy* 174-59. 16.vii.1998. Molochnyi Liman. Stomach. Coll. Sarabeev. *Bunocotyle constrictus*. 22.vii.1998" (5 specimens).

Table 3.5. Comparative material of the species of Haploporinae studied from museum collections. *Abbrevations:* BMNH, British Museum (Natural History) Collection at the Natural History Museum, London, UK; NSMT, National Museum of Nature and Science, Tokyo, Japan.

Species	Accession Number
Dicrogaster japonica Machida, 1996	NSMT-Pl 967 (1 holotype and 26 paratypes)
Dicrogaster perpusilla Looss, 1902	BMNH 1986.5.20.155-156 (neotype)
Haploporus benedeni (Stossich, 1887)	BMNH 1986.5.20.160-163 (1 voucher)
Haploporus magnisaccus Machida, 1996	NSMT-Pl 4317 (3 paratypes)
Haploporus mugilis Liu & Yang, 2002	BMNH 2001.8.6.1-2. (1 paratype)
Haploporus pseudoindicus Rekharani & Madhavi, 1985	BMNH 1984.6.28.19 (holotype)
Haploporus spinosus Machida, 1996	NSMT-Pl 4365, 4709 (17+16 paratypes)
Lecithobotrys mugilis Rekharani & Madhavi, 1985	BMNH 1984.6.28.17 (holotype and 7 paratypes)
Lecithobotrys putrescens Looss, 1902	BMNH 1991.2.4.7-9 (2 vouchers)
Lecithobotrys stomachicola Machida, 1996	NSMT-Pl 4318, 4345 (8 paratypes)

Linear Discriminant Analysis (LDA with backward stepwise procedure) is a method used to determine which variables discriminate between two or more naturally occurring groups, where the existence of the groups is known *a priori*, and to allocate entities to groups (*e.g*. McLachlan, 1992; Tabachnick & Fidel, 2007). In the present study it was applied to the specimens, which were assigned to several *a priori* groups defined by their species identification based on morphology (plus species and locality of origin in the case of Mediterranean *Saturnius* spp.), in order to evaluate the morphometric differences between the species and select the variables yielding optimal separation between the species/groups.

Prior to the analyses, metrical data were ln-transformed and subjected to univariate (ANOVA) statistical tests to assess the overall variation. Square-root transformation was applied to the relative proportions/ratios which were analysed separately. The univariate and multivariate statistical analyses were carried out using either SPSS ®12.0 (SPSS Inc.; Norušis, 2002), PAST 1.42 (Hammer *et al.*, 2001) or Statistica ® 6.0 (StatSoft Inc.).

3.4. Processing of materials for molecular study

Specimens fixed live in 100% EtOH and stored at -20ºC were subsequently transferred into 300 µl TNES urea [10 mM Tris-HCl (pH 8), 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M

urea]. Genomic DNA (gDNA) was extracted from single specimens using a phenol-chloroform protocol as described in Holzer *et al*. (2004). Alternatively, 1M Tris-EDTA (pH 8) buffer was used to replace ethanol from the tissue of some specimens and gDNA was extracted using Qiagen® DNeasy™ tissue kit following the manufacturer's protocol, except for the proteinase-K incubation period, which was extended overnight, and the gDNA was further concentrated to a volume of ~30 µl using Millipore Microcon® columns.

Table 3.6. Primers used for gene fragment amplification (PCR) and/or sequencing (Seq).

Gene fragment / Primer	Sequence (5'-3')	Direction	Application	Source
18S rDNA				
ERIB1	ACCTGGTTGATCCTGCCAG	Forward	PCR + Seq	Barta et al. (1997)
ERIB10	CTTCCGCAGTTCACCTACGG	Reverse	PCR + Seq	Barta et al. (1997)
WormA	GCGAATGGCTCATTAAATCAG	Forward	PCR + Seq	Littlewood & Olson (2001)
WormB	CTTGTTACGACTTTTACTTCC	Reverse	PCR + Seq	Littlewood & Olson (2001)
LIN3	GCGGTAATTCCAGCTCCA	Forward	Seq	Lin et al. (1999)
LIN10	CACTCCACGAACTAAGAA	Reverse	Seq	Lin et al. (1999)
300F	AGGGTTCGATTCCGGAG	Forward	Seq	Littlewood & Olson (2001)
600R	ACCGCGGCKGCTGGCACC	Reverse	Seq	Littlewood et al. (2000)
1270R	CCGTCAATTCCTTTAAGT	Reverse	Seq	Littlewood & Olson (2001)
930F	GCATGGAATAATGGAATAGG	Forward	Seq	Littlewood & Olson (2001)
1270F	ACTTAAAGGAATTGACGG	Forward	Seq	Littlewood et al. (2000)
1630R	TAAGGGCATCACAGACCTG	Reverse	Seq	Littlewood et al. (2000)
28S rDNA				
U178	GCACCCGCTGAAYTTAAG	Forward	PCR + Seq	Lockyer et al. (2003)
L1642	CCAGCGCCATCCATTTTCA	Reverse	PCR + Seq	Lockyer et al. (2003)
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	Forward	PCR + Seq	Littlewood et al. (2000)
1500R	GCTATCCTGAGGGAAACTTCG	Reverse	PCR + Seq	Tkach et al. (1999)
L300F	CAAGTACCGTGAGGGAAAGTTG	Forward	Seq	Littlewood et al. (2000)
ECD2	CTTGGTCCGTGTTTCAAGACGGG	Reverse	Seq	Littlewood et al. (2000)
LSU1200R	GCATAGTTCACCATCTTTCGG	Reverse	Seq	Littlewood et al. (2000)
ITS2 rDNA				
3S	GTACCGGTGGATCACGTGGCTAGTG	Forward	PCR + Seq	Anderson & Barker (1993)
ITS2.2	CCTGGTTAGTTTCTTTTCCTCCGC	Reverse	PCR + Seq	Anderson & Barker (1993)

Sequences of the near-complete 18S, partial (domains D1-D3; ~1400-1600 bps) 28S and complete ITS2 rDNA regions were amplified using primers listed in Table 3.6. Polymerase chain reaction (PCR) amplifications were performed in a total volume of 30 µl containing ~1.5 units of Thermoprime Plus DNA polymerase (ABgene, Epsom, UK) and 10× buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of each PCR primer and about 20 - 70 ng of template DNA. Alternatively, PCR amplifications were carried out using Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads (each containing ~1.5 units *Taq* DNA polymerase, 10 mM Tris-HCl at pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP and stabilisers, including BSA), 20-70 ng of template DNA and 10 mM of each PCR primer. The thermocycling profiles applied using different primer combinations for gene fragment amplifications are shown in Fig 3.3. PCR amplicons were either gel-excised from a 1% agarose gel in sodium acetate buffer (Brody & Kern, 2004) or purified directly using

Qiagen QIAquick™ PCR Purification Kit, and cycle-sequenced from both strands using ABI BigDye™ Terminator v3.1 Ready Sequencing Kit, alcohol-precipitated, and run on an ABI 3730 automated sequencer. Primers used for cycle sequencing of each rRNA gene fragment are listed in Table 3.6. Contiguous sequences were assembled and edited using either Bioedit v7.0.5. (©1997-2005, Hall, 1999) or Sequencher™ (GeneCodes Corp., ver. 3.1.1) and submitted to GenBank (see Table 3.7). Alignments and data sets used in the specific analyses are described in Chapters 6-8.

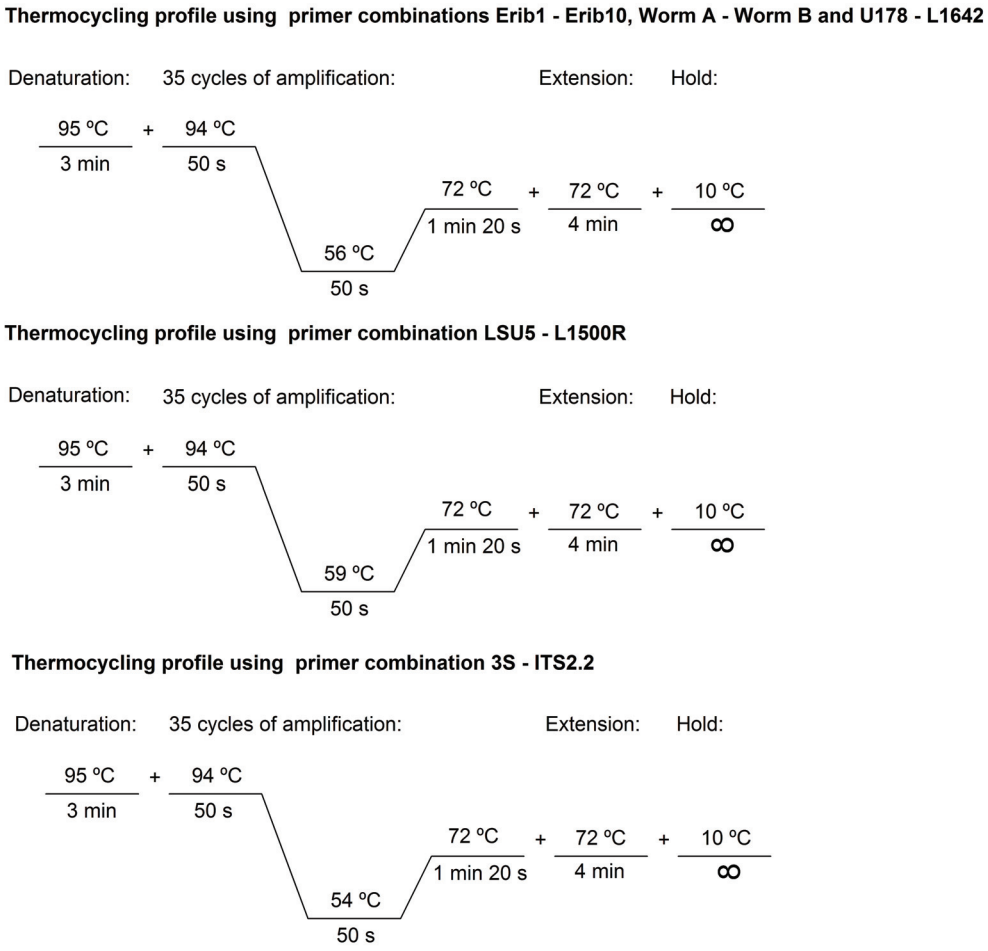

Fig. 3.3. Thermocycling profiles applied for each primer combination.

Table 3.7. Newly sequenced species, their hosts, number of sequence replicates for the three rDNA regions and EMBL/GenBank and BMNH accession numbers for sequences and morphological types/vouchers, respectively. *Abbrevations:* MNHN: Muséum National d'Histoire Naturelle, Paris.

Family/Species	Host	No. of replicates	EMBL/GenBank accession numbers			BMNH accession numbers
Haploporidae			**18S**	**28S**	**ITS2**	
Dicrogaster perpusilla	*L. ramado*	5	FJ211230	FJ211238	FJ211248	2008.10.7.6-11
Dicrogaster contracta	*L. ramado*/*L. aurata*	4/3	FJ211256/5	FJ211262/1	FJ211268/7	2008.10.7.14-16 /2008.10.7.12-13
Forticulcita gibsoni	*M. cephalus*	1	FJ211226	FJ211239	FJ211249	2008.10.7.61-76
Haploporus benedeni	*L. ramado*	4	FJ211228	FJ211237	FJ211247	2008.10.7.52-55
Lecithobotrys putrescens	*L. saliens*	2	FJ211229	FJ211236	FJ211246	2008.10.7.56-60
Saccocoelium brayi	*L. saliens*	1	FJ211227	FJ211234	FJ211244	2008.10.7.82-83
Saccocoelium cephali	*M. cephalus*	1	FJ211232	FJ211233	FJ211243	2008.10.7.23-25
Saccocoelium obesum	*L. ramado* /*L. aurata*	3/1	FJ211253/4	FJ211259/60	FJ211265/6	2008.10.7.38-39
Saccocoelium tensum	*L. ramado* /*L. aurata*	1/6	FJ211251/2	FJ211257/8	FJ211263/4	2008.10.7.44/2008. 10.7.41-43
Ragaia lizae	*L. aurata*	1	FJ211231	FJ211235	FJ211245	2008.10.7.19
Saccocoelium sp.	*M. cephalus*	8	-	FJ211233	FJ211243	-
Haplosplanchnidae						
Haplosplanchnus pachysomus	*L. ramado*	3	FJ211224	FJ211241	-	-
Haplosplanchnus purii	*M. cephalus*	1	FJ211225	FJ211242	-	MNHN JNC2180
Schikhobalotrema sparisomae	*L. aurata*	2	FJ211223	FJ211240	-	-

3.5. Phylogenetic analyses

The sequence datasets were analysed individually and/or combined using Bayesian inference (BI) and maximum parsimony (MP) methods. MP analyses were performed with PAUP* 4.0b10 (Swofford, 2002) using a heuristic search strategy with the number of search replicates provided in the materials and methods sections of the respective chapters, random-addition taxa sampling, tree-bisection-reconnection branch-swapping, with all characters run unordered with equal weights and with gaps treated as missing data. Nodal support was estimated by bootstrap analysis. BI analyses were conducted using Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Prior to analyses the best model of nucleotide substitution was estimated using ModelTest 3.06 (Posada & Crandall, 1998) independently for each dataset/partition. The models selected are specified in the materials and methods sections of the respective chapters. The analyses were run over 1 million generations with a sampling frequency of 100. Consensus trees with mean branch lengths were constructed based on trees saved after 'burn-in', that was estimated by plotting log-likelihoods against generation and determining when these values and substitution parameters had plateaued. Nodal support was estimated as posterior probabilities (Huelsenbeck *et al*., 2001).

CHAPTER 4

TAXONOMIC REVISION OF THE GENUS *SATURNIUS* (HEMIURIDAE: BUNOCOTYLINAE)

4.1. Background

Saturnius Manter, 1969 is a small hemiurid genus (subfamily Bunocotylinae Dollfus, 1950) of stomach parasites specific to mullets (Mugilidae), a family with a wide world distribution in marine and brackish water in temperate and tropical regions. Until the present study, this genus included six nominal species: *S. segmentatus* Manter, 1969, *S. mugilis* (Yamaguti, 1970), *S. maurepasi* Overstreet, 1977, *S. belizensis* Fischthal, 1977, *S. papernai* Overstreet, 1977 and *S. valamugilis* Rekharani & Madhavi, 1985.

Although all mullet host species in the Mediterranean basin have been subjected to intensive studies, the existing records indicate the presence of only two species, *S. papernai* and *S. mugilis*. *S. papernai* has been reported in *Mugil cephalus* (see Dimitrov *et al.*, 1998; Merella & Garippa, 1998, 2001; Domnich & Sarabeev, 2000a; Dmitrieva & Gaevskaya, 2001; Zhdamirov & Mal'tsev, 2001); *M. soiuy* (see Sarabeev, 2000; Domnich & Sarabeev, 2000a,b,c,d; Sarabeev & Domnich, 2000; Dmitrieva & Gaevskaya, 2001; Zhdamirov & Mal'tsev, 2001); *Liza aurata* (see Domnich & Sarabeev, 2000a; Dmitrieva & Gaevskaya, 2001; Pronkina, 2001; Gaevskaya & Korniychuk, 2003); and *L. ramado* (see Di Cave *et al.*, 1997). Records of *S. mugilis* include reports from *M. cephalus* (see Gaevskaya & Korniychuk, 2003); *L. aurata* (see Dmitrieva & Gaevskaya, 2001; Zhdamirov & Mal'tsev, 2001; Gaevskaya & Korniychuk, 2003); and *L. saliens* (see Solonchenko & Tkachuk, 1985; Dmitrieva & Gaevskaya, 2001; Gaevskaya & Korniychuk, 2003). In addition, material unidentified to species level belonging to *Saturnius* has been reported in *Chelon labrosus*, *L. aurata*, *L. ramado* and *L. saliens* in the Western Mediterranean (see D'Amelio *et al.*, 1995; Merella & Garippa, 1998, 2000, 2001).

However, although the number of *Saturnius* spp. records from the Mediterranean and the Black Sea may appear high, most are not original (e.g. Dmitrieva & Gaevskaya, 2001; Gaevskaya & Korniychuk, 2003), whereas the remaining appear to reiterate results of single host population samples; virtually none of these provide supportive evidence for the species identification [only Dimitrov *et al.* (1998) and Zhdamirov & Mal'tsev (2001), the latter provide some measurements in an abstract].

A survey of the parasites of mullets off the Mediterranean coast of Spain and examination of additional material from the Black Sea coast of Bulgaria and off the Russian coast of the Sea of Japan was carried out in the course of the present study. Examination of various population samples with the application of multivariate morphometric approach (PCA, LDA) revealed higher species diversity within this genus. This prompted a re-

examination of the type-materials of *Saturnius* spp. because some species were described only on the basis of a few specimens, and features that have proved useful for species discrimination were not sufficiently characterised in the original descriptions. This chapter provides a revision of the genus *Saturnius*, paying particular attention to a re-examination of the type materials of the nominal species, critical assessment of published descriptions (when type-material unavailable) and host-parasite data, and detailed study of the species diversity and morphometric variation (see sections 4.4. & 4.5.) of *Saturnius* spp. in mullets in the Mediterranean basin. All results of the study have been published (see Blasco-Costa *et al.*, 2006, 2008).

4.2. Generic diagnosis

Body elongate, cylindrical, with maximum width at level of ventral sucker flange. Tegument unarmed, with fine longitudinal and transverse striations. Three circular muscular flanges present around body. Anteriormost flange surrounds equator of oral sucker, forms muscular papillae in type-species only. Second flange at level of ventral sucker, forms 2 lateral sub-conical protuberances provided with concentric muscles. Third flange located in posterior third of last pseudosegment, forms tegumental ridge dorsally. Body with 5-7 pseudosegments separated by 4-6 transverse fibrous septa. Oral sucker muscular, subterminal. Ventral sucker strongly muscular, subspherical, anterior to mid-body. Prepharynx absent; pharynx subspherical. Oesophagus short. 'Drüsenmagen' present. Caeca with constrictions at levels of septa; cyclocoel present in at least some species. Testes 2, smooth, in tandem or slightly oblique, in middle pseudosegments of hindbody. Seminal vesicle saccular or wide-tubular, antero-dorsal to ventral sucker, similar in size to or much larger than sinus-sac. Pars prostatica external, just posterior or dorsal to sinus-sac, vesicular, lined with anuclear blebs and surrounded by prostatic cells; enters sinus-sac at its base or connects dorsally to mid-level of sinus-sac. Sinus-sac elongate-oval, contains short muscular hermaphroditic duct, lined by intensely stained cells. Genital pore at level of anteriormost septum, median. Ovary oval to transverse-oval, in anterior part of last pseudosegment, ventral to caeca, contiguous with and sometimes partly overlapping vitellarium. Mehlis' gland and uterine seminal receptacle present. Laurer's canal not observed. Vitellarium compact, smooth, elongate-oval, larger than ovary. Uterus thin-walled, extends posterior to vitellarium. Metraterm short. Eggs numerous. Excretory pore wide, terminal or subterminal; vesicle Y-shaped; stem saccular distally; arms unite at level of pharynx. In mullets (Mugilidae). Type-species *S. segmentatus* Manter, 1969

4.3. Review of species

Saturnius segmentatus Manter, 1969

Material studied

Type-material: Ex *Mugil cephalus* L. Stomach. Moreton Bay, Queensland, Australia. Holotype USNPC 071219.00; paratypes HWML 634-635 (6 specimens).

New material: Ex *Mugil cephalus* L. Stomach. Kievka River, Russia (southeastern coast of the Sea of Japan) (23.vi.2005), area 61. BMNH 2007.1.24.28-37 (10 specimens).

Records

References: 1. Manter (1969); 2. Rekharani & Madhavi (1985); 3. Present study.

Descriptions: 1; 2 (figure); 3.

Definitive hosts: *Mugil cephalus* L. (1, 2, 3).

Distribution: Area 71 (Moreton Bay, Queensland, Australia) (1); area 57 (India) (2); area 61 (Kievka River, Russia, coast of Sea of Japan) (3).

Description (Fig. 4.1; Table 4.1)

Body robust, elongate, cylindrical, with maximum width at level of ventral sucker flange. Tegument unarmed, with fine longitudinal and transverse striations.

Three circular muscular flanges occur around body: (i) anterior (oral sucker) flange well developed, bears muscular papillae (9-12 in types; 9-11 in new material) (Fig. 4.1B,C) surrounding equator of oral sucker; (ii) second (ventral sucker) flange strongly muscular, forms 2 lateral sub-conical protuberances provided with concentric muscles, with cone length (anterior-posterior) 51-72 (types) and 48-72 (new material) and width (lateral) 24-36 (types) and 18-38 (new material) (21-27% and 19-37% of body width at level of ventral sucker, respectively); tegumental ridge caused by musculature of second flange present on both ventral and dorsal surfaces just posterior to ventral sucker posterior margin; and (iii) third flange located in posterior third of last pseudosegment, well developed, forms tegumental ridge dorsally.

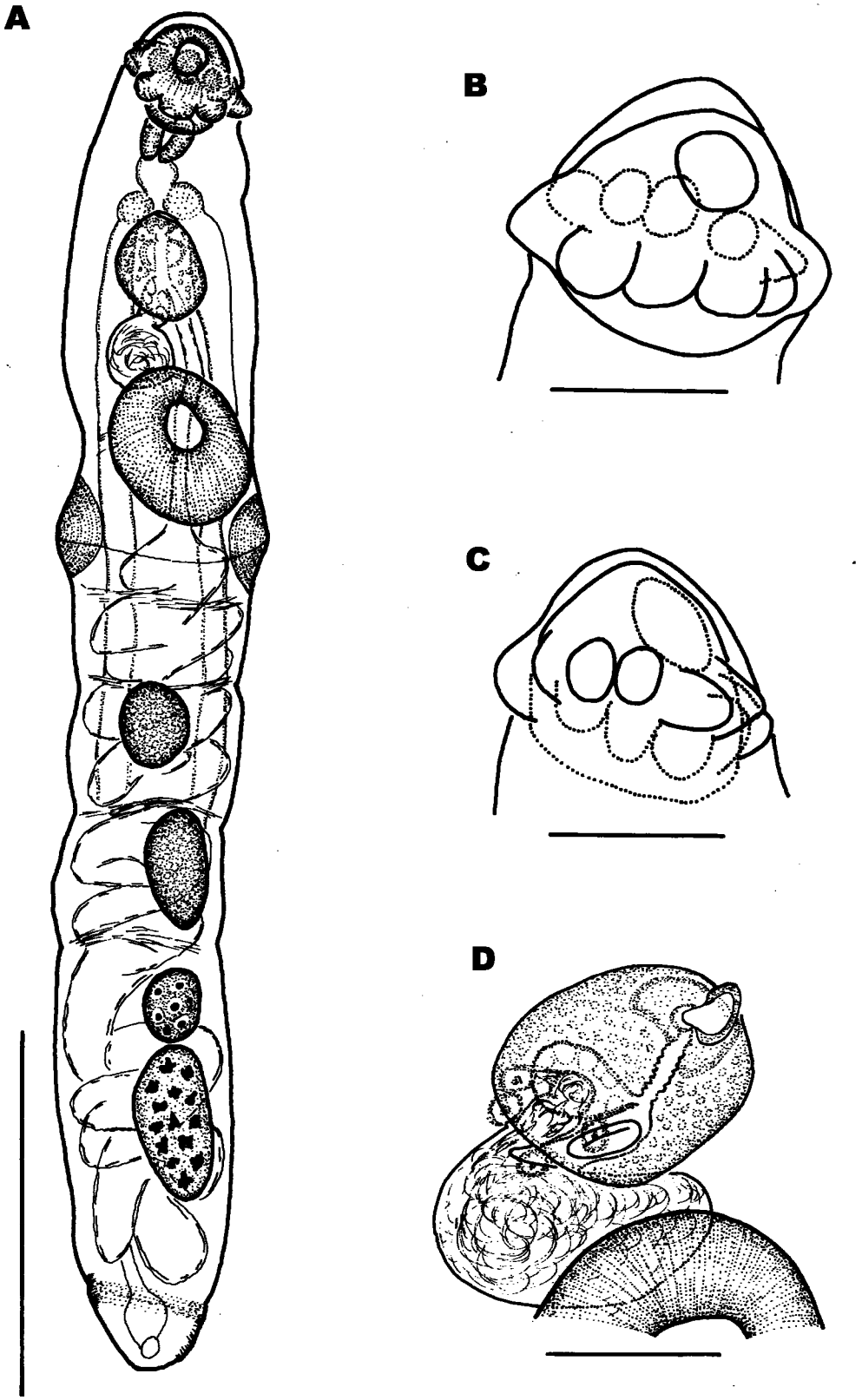

Fig. 4.1. *Saturnius segmentatus* ex *Mugil cephalus* from the Sea of Japan. **A.** General morphology, ventral view with uterus in outline. **B-C.** Ventro-lateral and dorso-lateral view of anterior extremity with muscular papillae. **D.** Terminal genitalia. *Scale-bars*: **A**, 200 μm; **B-D,** 50 μm.

Body with 5-6 pseudosegments (5 in 29% and 90% of types and new material, respectively) separated by 4-5 transverse fibrous septa. Anteriormost septum in forebody, very faint or absent (in 35% and 65% of all specimens, respectively). Additional median group of few faint muscle fibres with no connection to lateral body margins present at mid-level of ventral sucker in 65% of all specimens (Fig. 4.1A). Hindbody always has 4 thick septa forming 4 pseudosegments of different sizes; anterior smallest, middle pair similar in size and posteriormost largest, 35-38% (types) and 22-32% (new material) of body length.

Pre-oral lobe well developed. Oral sucker muscular, with subterminal aperture. Ventral sucker strongly muscular, subspherical, larger than oral sucker, anterior to mid-body. Prepharynx absent; pharynx muscular, subspherical. Oesophagus shorter than pharynx. Caeca wide, thick-walled, with constrictions at levels of septa, form 'Drüsenmagen' just anterior to first septum; most posterior regions masked by ovary, vitellarium and uterus, but appear united at level of third muscular flange in 2 specimens of type-series.

Testes 2, subspherical to triangular, smooth, in tandem or slightly oblique, in middle pseudosegments of hindbody. Seminal vesicle thin-walled, saccular, with attenuated anterior portion recurved or bent once in some specimens, antero-dorsal to ventral sucker, similar in size to or larger than sinus-sac. Pars prostatica external, dorsal to sinus-sac, small, vesicular, lined with anuclear blebs and surrounded by prostatic cells, opens into sinus-sac dorsally at mid-level (Fig. 4). Sinus-sac elongate-oval, contains short muscular hermaphroditic duct lined by intensely stained cells. Genital pore at level of anteriormost septum, median, round, supported by characteristic faintly-muscular 'collar' (Fig. 4.1D).

Ovary oval, in anterior part of last pseudosegment, ventral to caeca, contiguous with and sometimes partly overlapping vitellarium ventrally. Small Mehlis' gland observed in 1 paratype. Uterine seminal receptacle distinct (in holotype and 2 paratypes) postero-dorsal to vitellarium; Laurer's canal not observed. Vitellarium compact, smooth, elongate-oval, larger than ovary, length 10-16% (types) and 9-13% (new material) of body length. Uterus thin-walled, extends posterior to vitellarium. Metraterm joins sinus-sac at about its mid-length. Eggs very numerous.

Excretory pore wide, terminal (types) to ventro-subterminal (new material); vesicle Y-shaped; stem saccular distally; bifurcation not observed; arms unite at level of pharynx. Six papillae present around excretory pore.

Remarks

This species was originally described in some detail (Manter, 1969). Overstreet (1977) further clarified a number of morphological features in the type-material and added substantially to the description. The present study provides additional data on the morphometric variation which was detected mainly in the differences between the means (Table 4.1); apart from the somewhat smaller body size of the newly collected worms, all metrical features exhibit overlapping ranges for both the new and the type-material.

Manter (1969) figured at least four lateral papillae (see his fig. 1) but did not describe them. Overstreet (1977) described 10 papillae in the types whereas a variable number of papillae (9-12) was observed in this study. In the original description of *S. segmentatus,* Manter (1969) mentioned only '2 flanges or ridges encircling body'. The re-examination of both the type- and newly collected material confirmed the presence of a strongly developed third flange located close to the posterior extremity and also forming a detectable tegumental ridge, which is clearly seen in the latter material.

Manter (1969) also described the body of *S. segmentatus* with four septa, all located in the hindbody. Overstreet (1977) provided information on additional septa, describing a 'major' septum in the forebody at the level of the genital pore and two less prominent septa: (i) a few fibres extending from the ventral sucker ('oral sucker' [?sic]) to the margins of the forebody; and (ii) transverse fibres at the posterior end forming a septum which prevents the uterus from reaching the posterior extremity. The present study revealed that the anteriormost septum in the forebody is very indistinct or absent; this results in a variable number of apparent septa (four or five). However, the few muscular fibres [see (i) above] cannot be considered as a 'less prominent septum' and the septum close to posterior extremity [see (ii) above] in fact represents the tegumental ridge formed by the strongly developed third flange (figured in Overstreet, 1977). Therefore, the body of *S. segmentatus* is comprised of five or six pseudosegments.

Unfortunately, the type-material does not help clarify the structure of the terminal genitalia (e.g. the location of the pars prostatica and its connection with the seminal vesicle and the sinus-sac). The original observations of Manter (i.e. 'sinus-sac pyriform, with small spherical basal sac containing sperm cells, and thick-walled genital sinus about half length of sac') was corrected by Overstreet (1977), who considered that an external prostatic vesicle exists but is not what Yamaguti (1971) (and Manter, see above) interpreted as a spherical basal sac inside the sinus-sac. Re-examination of the type-material confirmed that this

Table 4.1. Morphometric data for *Saturnius segmentatus* Manter, 1969 ex *Mugil cephalus*.

Locality	Moreton Bay, Queensland, Australia	Moreton Bay, Queensland, Australia		Kievka River, coast of Sea of Japan	
Source	Manter (1969)	Present study (type-material) (n=6)		Present study (new material) (n=10)	
	Range	Range	Mean	Range	Mean
Measurements					
Body length	661-866	761-877	835	656-764	717
Body width at ventral sucker flange	148-159	138-191	168	96-159	128
Body width at ventral sucker	-	112-151	129	89-124	107
Forebody length	171-205	193-234	212	161-224	193
Oral sucker	- × 62-65	56-62 × 52-67	59 × 61	50-69 × 49-65	58 × 57
Ventral sucker	- × 93-101	92-99 × 86-105	96 × 95	82-107 × 69-103	94 × 85
Flange at ventral sucker	-	51-72 × 24-36	62 × 31	48-72 × 18-38	55 × 30
Pharynx	31-36 × 33	32-34 × 34-36	33 × 35	25-37 × 26-35	30 × 30
Sinus-sac	-	32-58 × 22-28	49 × 25	59-82 × 40-63	69 × 53
Pars prostatica	-	28-37 × 17-22	32 × 17	22-37 × 16-25	29 × 20
Seminal vesicle	-	60-110 × 37-49	87 × 43	41-90 × 32-44	62 × 39
Anterior testis	-	67-82 × 45-99	73 × 77	27-80 × 26-57	51 × 42
Posterior testis	-	49-80 × 65-92	67 × 77	36-66 × 30-58	54 × 41
Ovary	-	60-84 × 65-103	68 × 83	27-54 × 22-54	38 × 41
Vitellarium	-	86-119 × 79-97	100 × 89	62-89 × 34-59	75 × 44
Last pseudosegment	250-319	271-322 × 135-165	302 × 153	146-235 × 88-126	193 × 108
Eggs	26-28 × 12-13	22-27 × 9-13	24 × 11	18-26 × 9-13	21 × 11
Ratios					
Sucker length ratio	-	1:1.6-1.7	1:1.6	1:1.6-1.8	1:1.6
Sucker width ratio	1:1.4-1.6	1:1.4-1.7	1:1.6	1:1.2-1.7	1:1.5
BW/BL (%)	23*	16-24	20	15-21	18
VSW/BWVS (%)	-	70-88	75	71-89	79
FO/BL (%)	c. 25-33	24-28	25	25-34	28
VL/BL (%)	-	10-16	12	9-13	10
MFL/VSL (%)	-	55-75	67	50-67	58
MFW/VSW (%)	-	27-36	32	26-49	35
MFW/BWVS (%)	-	21-27	24	19-38	28
MFW/MFL (%)	-	33-71	51	38-79	54
LSL/BL (%)	> 33	35-38	36	22-32	27
LSW/BW (%)	-	83-109	92	73-105	85
LSW/LSL (%)	-	44-56	51	40-86	57

* Measured from the original drawing.

Table 4.2. Morphometric data for the type-specimens of *Saturnius mugilis* and *S. belizensis.*

Species	*S. mugilis* (n=2)	*S. belizensis* (n=1)
Measurements		
Body length	709-785	604
Body width at ventral sucker flange	138-151	84
Body width at ventral sucker	110-137	64
Forebody length	187-208	189
Oral sucker	49-56 × 52-65	28 × 30
Ventral sucker	80-107 × 84-84	45 × 41
Flange at ventral sucker	64-65 × 27-31	35 × 13
Pharynx	26-32 × 33-37	22 × 24
Sinus-sac	43-43 × 30-30	23 × 17
Pars prostatica	15-32 × 17-26	-
Seminal vesicle	77-90 × 36-37	65 × 39
Anterior testis	47-54 × 43-45	41 × 45
Posterior testis	45-50 × 41-62	45 × 49
Ovary	64-77 × 58-73	37 × 49
Vitellarium	75-75 × 65-71	54 × 52
Last pseudosegment	239-262 × 94-112	187 × 103
Eggs	20-22 × 10-13	21-22 × 9-11
Ratios		
Sucker length ratio	1:1.6-1.9	1:1.6
Sucker width ratio	1:1.6	1:1.4
BW/BL (%)	19	14
VSW/BWVS (%)	76	64
FO/BL (%)	26	31
VL/BL (%)	10-11	9
MFL/VSL (%)	61-80	78
MFW/VSW (%)	37	32
MFW/BWVS (%)	20-28	20
MFW/MFL (%)	42-48	37
LSL/BL (%)	33-34	31
LSW/BW (%)	68-74	123
LSW/LSL (%)	39-43	55

structure is difficult to observe in most cases because it overlaps the sinus-sac dorsally. However, the newly collected material exhibits a vesicular pars prostatica lying dorsally to the sinus-sac and opening into the hermaphroditic duct at about its mid-level. As a result, the hermaphroditic duct in *S. segmentatus* appears shorter (in relation to the sinus-sac) than in the other *Saturnius* spp. (with the exception of *S. mugilis* and *S. overstreeti* described below; Blasco-Costa et al., 2008) due to the junction of the pars prostatica being at the base of the sinus-sac in the latter species. Manter (1969) also illustrated the metraterm as entering the sinus-sac at its mid-level and this feature was confirmed in the new material. The 'precaecal sacs' of Manter (1969) and Overstreet (1977) exhibit the structure of 'Drüsenmagen'.

Peng *et al*. (2004) recently described material from *Liza carinata* (Valenciennes) from the Taiwan Strait which they identified as *S. segmentatus*. However, this material apparently lacks the most characteristic feature of *S. segmentatus*, i.e. muscular papillae surrounding the oral sucker, since the authors have described and illustrated only two lateral protuberances at level of oral and ventral sucker and stated that these were clearly observed only in the ventral plane. The material described by Peng *et al.* (2004) keys down (see below) to, and appears most similar to, *S. maurepasi* due to the following characters: (i) flange at level of ventral sucker not prominent, mound-shaped; (ii) body with seven pseudosegments separated by six transverse fibrous septa; (iii) small pars prostatica; (iv) sucker ratio; and (v) shape and size of the seminal vesicle. The third flange located in the posterior third of the last pseudosegment is rather weakly developed in S. *maurepasi* and has probably been overlooked by Peng *et al.* (2004). However, the size of the eggs in the description of Peng *et al.* (2004) is outside the range of S. *maurepasi* [24-29 × 14-15 (mean 27 × 14) *vs* 21-24 × 9-13 μm]. Although the material from Taiwan clearly represents a misidentification, further work and material are needed to establish the status of this form.

***Saturnius belizensis* Fischthal, 1977**

Material studied

Type-material: Ex *Mugil curema* Valenciennes. Stomach. Offshore at Belize City, Belize. Paratype USNPC 074167.00.

Record

Reference: 1. Fischthal (1977).

Description: 1.

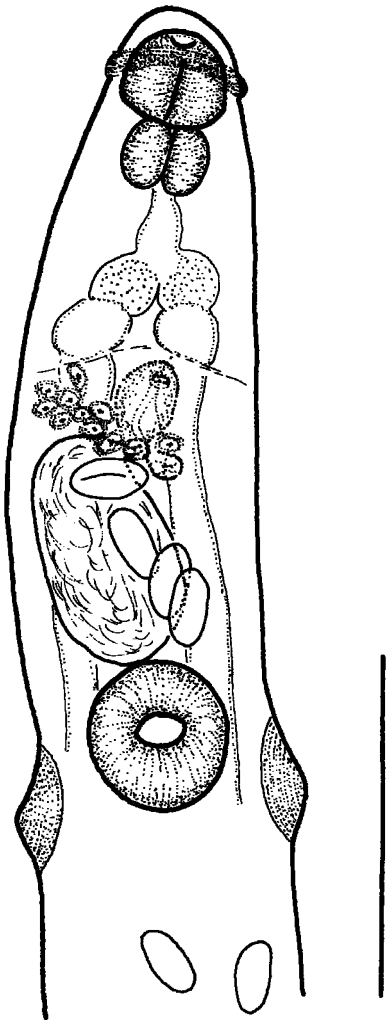

Fig. 4.2. *Saturnius belizensis* ex *Mugil curema* from offshore at Belize City, Belize. Forebody of the paratype, ventral view. *Scale-bar*: 100 μm.

Definitive host: *Mugil curema* (1).

Distribution: Area 31 (off Belize City, Caribbean Sea) (1).

Description (Fig. 4.2; Table 4.2)

Body with 5 pseudosegments separated by 4 fibrous septa. First fibrous septum at level of genital pore in forebody, consists of indistinct muscle fibres. Oesophagus short; caeca form 'Drüsenmagen' anterior to first septum, not visible posterior to ovary. Seminal vesicle elongate-saccular. Prostatic vesicle very small (*c.* 1/3 of size of sinus-sac), obscured by several layers of large prostatic cells, enters sinus-sac at its base. Sinus-sac elongate-oval; hermaphroditic duct not seen.

Remarks

Fischthal (1977) originally described *S. belizensis* with three transverse septa located in the hindbody, blind caeca and a sinus-sac 'containing sperm-filled prostatic vesicle posteriorly and thick-walled hermaphroditic duct'. Re-examination of the paratype revealed a faint septum at the level of the genital pore and, although it was not possible to trace the hermaphroditic duct, the structure that appears to be a small pars prostatica is external and presumably enters the sinus-sac at its base. Newly collected material from the type-host and locality is needed to elucidate the structure of the terminal genitalia.

***Saturnius dimitrovi* Blasco-Costa, Pankov, Gibson, Balbuena, Raga, Sarabeev & Kostadinova, 2006**

Syn. *S. papernai* Overstreet, 1977 of Dimitrov *et al*. (1998)

Type-host: Mugil cephalus L.

Other hosts: Liza aurata (Risso)*, Liza ramada* (Risso)*, Chelon labrosus* (Cuvier).

Type-locality: Off the Black Sea coast of Bulgaria (at Sozopol).

Other locality: Ebro Delta and Off Santa Pola (Spanish Mediterranean coast).
Site: Embedded between the grinding stomach lining and the layer of glandular cells.
Type-material: Holotype BMNH 2005.11.10.20; paratypes BMNH 2005.11.10.21-24
Voucher material: BMNH 2005.11.10.25-29.
Etymology: The species is named for Dr Georgi Dimitrov, formerly of the Central Laboratory of Ecology, Bulgarian Academy of Sciences, who collected the material.

Additional material
Ex *Liza aurata* (Risso). Stomach. Ebro Delta, Spain (22.ix.2005; 05.x.2005).
Ex *M. cephalus* L. Stomach. Ebro Delta (02.vi.2004; 22.vi.2004; 14.x.2004; 16.xi.2004; 20.v.2005; 31.v.2005; 22.ix.2005; 05.x.2005) and off Santa Pola, Spain (28.v.2004; 18.vi.2004; 04.x.2004; 09.xi.2004; 07.vi.2005; 15.vi.2005; 03.x.2005; 16.xi.2005; 21.xi.2005).
Ex *L. ramado* (Risso). Stomach. Ebro Delta, Spain (26.v.2004; 02.vi.2004; 22.vi.2004).
Ex *Chelon labrosus* (Cuvier). Stomach. Off Santa Pola, Spain (02.xi.2005).

Records
References: 1. Dimitrov *et al.* (1998); 2. Blasco-Costa *et al.* (2006); 3. Present study.
Descriptions: 1; 2.
Definitive hosts: *Mugil cephalus* L. (1, 2, 3); *Liza aurata* (Risso) (3); *L. ramado* (Risso) (3); *Chelon labrosus* (Cuvier) (3).
Distribution: Area 37, subarea 1 (Western Mediterranean) (2, 3); area 37, subarea 4 (Black Sea) (1, 2).

Description (Fig. 4.3)
[Based on 30 whole-mounted adult specimens; metrical data in Tables 4.3, 4.4] Body elongate, cylindrical, with rounded posterior extremity and maximum width at level of ventral sucker flange; width at mid-level of ventral sucker 94-128 (106 ± 9). Tegument unarmed, with fine longitudinal and transverse striations.

Three circular muscular flanges present around body: (i) anterior (oral sucker) flange well developed, located at mid-level of oral sucker; (ii) second (ventral sucker) flange strongly muscular, forming 2 lateral conical protuberances provided with concentric muscles, with cone length (anterior-posterior) 48-70 (60 ± 6) and width (lateral) 21-35 (29 ± 3) [20-34% (27 ± 4%) of body width at mid-level of ventral sucker], with anterior margin located at

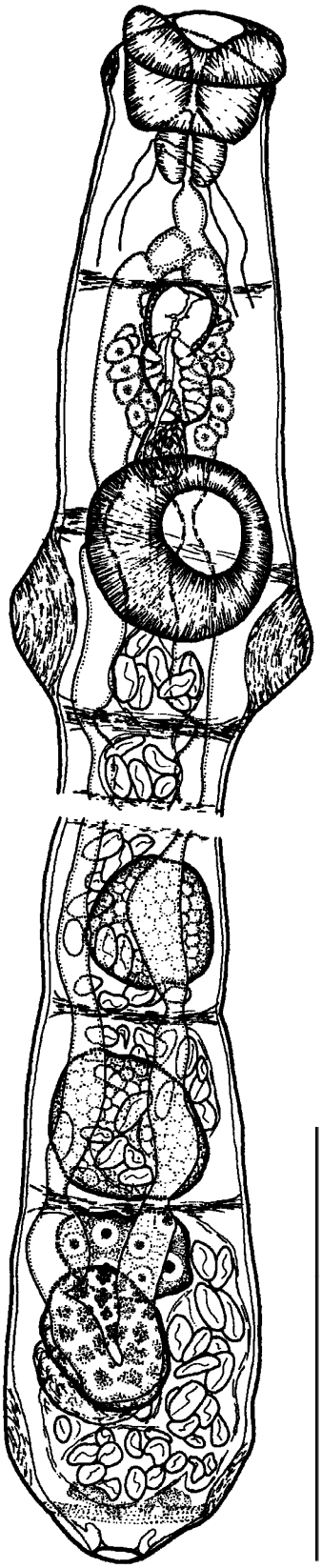

Fig. 4.3. *Saturnius dimitrovi* Blasco-Costa et al., 2006 ex *Mugil cephalus*. Holotype in 2 pieces. Ventral view of forebody and dorsal view of hindbody. *Scale-bar*: 200 μm.

level of posterior half of ventral sucker; tegumental ridge present on ventral surface posterior to margin of ventral sucker; (iii) third flange located in posterior third of last pseudosegment, well developed.

Body with 7 pseudosegments separated by 6 transverse thick fibrous septa. Anteriormost septum at level of genital pore; second septum at anterior level of ventral sucker flange; 4 other septa form 4 pseudosegments of different sizes located entirely in hindbody; anterior smallest, middle pair similar in size and posteriormost largest, 22-28% (24 ± 2%) of body length.

Oral sucker thick-walled, sub-oval, with terminal opening. Ventral sucker strongly muscular, subspherical, larger than oral sucker, anterior to mid-body. Prepharynx absent; pharynx muscular, subspherical. Oesophagus short. Intestinal bifurcation near first septum. Caeca relatively wide, with constrictions at septa; most posterior region difficult to observe in adult worms due to the overlap of ovary, vitellarium and uterus; caeca observed to unite posterior to vitellarium to form cyclocoel in 5 specimens (see e.g. holotype, Fig. 4.3).

Testes 2, subspherical to elongate-oval, smooth, in tandem, occupy most of respective pseudosegments in middle of hindbody, contiguous or slightly separated. Seminal vesicle thick-walled, elongate-oval, antero-dorsal to ventral sucker, usually larger than sinus-sac. Pars prostatica external, elongate-oval, vesicular, similar in size to sinus-sac, lined with anuclear blebs, surrounded by large prostatic cells which overlap sinus-sac and anterior half of seminal vesicle. Sinus-sac thin-walled, round to elongate-oval, contains eversible hermaphroditic duct lined by small intensely stained cells which forms temporary sinus-organ. Genital pore a wide transverse slit, median, at level of first septum.

Ovary oval to subtriangular, in anterior part of last pseudosegment, overlaps vitellarium somewhat dorsally. Uterine seminal receptacle large, dorsal to posterior part of vitellarium; Laurer's canal not observed. Vitellarium compact, suboval to elongate-oval, larger than ovary, length *c*.11% of body length. Uterus thin-walled, fills free space in pseudosegments of hindbody; metraterm not observed. Eggs operculate, numerous.

Excretory pore wide, terminal to dorso-subterminal; vesicle elongate-tubular, divides in anterior hindbody; arms unite at level of pharynx.

Remarks

Dimitrov *et al.* (1998) redescribed what they believed to be *S. papernai* from *Mugil cephalus* off the Bulgarian Black Sea coast. However, the newly collected material of *S. papernai* from the Mediterranean showed a number of differences when compared to the description of these authors. Thus, the size of the ventral sucker in *S. papernai* is smaller, the seminal vesicle is longer and the posteriormost pseudosegment is larger in relation to body length (see Dimitrov *et al.*, 1998). After re-examination of the voucher material, a conclusion was reached that this material can be distinguished from both the original description *S. papernai* by Overstreet (1977) and the new collection of *S. papernai* from the Western Mediterranean in the above characteristics.

S. dimitrovi resembles *S. maurepasi*, *S. valamugilis* and *S. papernai* in the number and distribution of the muscular septa. However, the pars prostatica in *S. maurepasi* is small in relation to the sinus-sac and seminal vesicle which are longer; and the posteriormost pseudosegment is longer in relation to the body length and the suckers are smaller, although the upper range for body size is higher. S. *valamugilis* was described from specimens with a similar size of body, which, however, possess smaller suckers, pharynx, testes and ovary; the different host and distant geographical area also tend to support the distinct status of the two species.

S. dimitrovi appears most similar to the sympatric (both forms occur in mullets from the Ebro Delta and the Black Sea) *S. papernai* (*sensu stricto*), which was originally described from *M. cephalus* on the Sinai coast of the Eastern Mediterranean (Overstreet, 1977). Similarities include: the presence of three clearly distinguishable muscular flanges; the number of septa, which are strongly muscular; the somewhat asymmetrical appearance of the anterior margin of the oral sucker; and the overlap in the lower range of most metrical features. However, the posteriormost pseudosegment in *S. papernai* is wider and distinctly more elongate in relation to the body; the ventral sucker muscular flange length is similar to

Table 4.3. Morphometric data for *Saturnius* spp.

Species	*S. minutus*	*S. dimitrovi*		*S. papernai* Overstreet, 1977	*S. papernai* Overstreet, 1977	*S. segmentatus* Manter, 1969	*S. mugilis* (Yamaguti, 1970)	*S. maurepasi* Overstreet, 1977	*S. belizensis* Fischthal, 1977	*S. valamugilis* Rekharani & Madhavi, 1984
Host	*M. cephalus*	*M. cephalus*	*M. cephalus*	*M. cephalus*	*M. cephalus*	*M. cephalus*	*M. cephalus*	*M. cephalus*	*M. curema*	*V. cunnesius*
Source	Present study	Present study (Ebro Delta)	Present study (Off Sozopol)	Present study	Overstreet (1977)	Manter (1969)	Yamaguti (1970)	Overstreet (1977)	Fischthal (1977)	Rekharani & Madhavi (1984)
Body length	364-542	692-840	599-750	687-1183	635-1021	661-866	400-750	497-1085	415-594	672-720
Body width at ventral sucker flange	73-115	94-111	95-128	107-165	93-190	148-159	80-150	77-139	65	80-128
Forebody length	133-184	210-259	170-231	240-412	-	171-205	-	-	155-185	-
Oral sucker	31-54 × 39-70	57-74 × 54-67	48-61 × 57-82	55-91 × 57-91	38-64 × 51-87	- × 62-65	35-50 × 42-70	29-42 × 33-48	28-29 × 31	31-39 × 39-40
Ventral sucker	56-74 × 56-74	84-94 × 82-94	74-103 × 80-116	47-79 × 57-84	48-78 × 51-90	- × 93-101	80-90 × 80-90	46-75 × 49-77	40-44 × 44	39-47 × 54
Pharynx	20-32 × 19-31	27-40 × 30-37	32-38 × 29-36	27-65 × 27-48	30-48 × 29-42	31-36 × 33	23-35 × 21-37	22-32 × 19-33	18-21 × 18	19-39 × 19-23
Oesophagus length	7-29	-	-	25-59	-	-	23-35	-	25-30	-
Sinus-sac	23-41 × 15-26	40-54 × 22-35	32-48 × 29-38	37-74 × 27-45	-	-	-	-	22-44 × >15	-
Pars prostatica	18-38 × 12-24	40-59 × 25-37	29-48 × 23-36	40-77 × 23-54	-	-	-	-	-	-
Seminal vesicle	12-47 × 11-37	42-91 × 35-54	36-63 × 24-50	52-109 × 32-81	-	-	65-95 × 28-42	-	-	70-85 × 39-46
Anterior testis	22-47 × 22-47	44-91 × 42-54	50-74 × 44-80	34-113 × 44-94	41-90 × 44-99	-	35-70 × 46-58	28-55 × 30-62	35-48 × 38	- × 27-39
Posterior testis	22-45 × 20-57	57-84 × 44-59	53-76 × 46-82	38-105 × 42-105	52-99 × 45-116	-	as above	29-58 × 38-71	35-48 × 45	- × 31-42
Ovary	22-37 × 21-46	37-62 × 49-82	34-69 × 42-76	42-101 × 36-120	46-87 × 44-107	-	35-58 × 46-70	26-70 × 39-91	30-40 × 37	42-58 × 39
Vitellarium	29-64 × 22-51	79-104 × 49-77	44-95 × 42-84	45-124 × 50-103	67-145 × 58-138	-	70-116 × 46-86	57-106 × 44-94	44-59 × 37	81-85 × 58-62
Last pseudosegment length	65-150	178-220	137-181	176-358	173-273	250-319	-	173-373	120-195	210-240
Eggs	19-24 × 9-12	22-24 × 10-13	22-24 × 10-13	21-26 × 9-14	20-32 × 10-17	26-28 × 12-13	21-25 × 11-15	17-26 × 10-16	22-24 × 11-15	-
Body ratio (BW/BL %)	19-25	13-17	17-27	12-19	15*	23*	19*	11-22	15*	10*
Sucker length ratio (VSL/OSL)	1:1.21-2.25	1:1.14-1.52	1:1.35-1.94	1:0.65-1.21	-	-	-	-	1:1.38-1.57	1:1.21-1.26**
Sucker width ratio (VSW/OSW)	1:0.90-1.82	1:1.31-1.56	1:1.08-1.67	1:0.73-1.18	1:0.8-1.3	1:1.44-1.6	-	1:1.3-1.8	1:1.42	1:1.35-1.39**
FO/BL %	28-39	27-32	27-32	30-37	28-35	c. 25-33	25*	25-34	37*	27-29
LSL/BL %	17-29	23-26	22-28	25-35	23-31	>33	-	30-37	29-33	26-34

*Measured from original drawing; **Calculated

the length of ventral sucker (means for MFL/VSL, 99 ± 13 *vs* 69 ± 8%); and the latter is less prominent in *S. papernai*. Furthermore, although almost all measurements of *S. dimitrovi* exhibit an overlap with the lower range of *S. papernai* (see Table 4.3), the univariate analyses of variance revealed that the two species are distinguishable with respect to 35 of the 41 metrical features used (see section 4.4., this chapter). Finally, *S. dimitrovi* and *S. papernai* can be easily distinguished, even under low magnification, considering the relative size of ventral sucker only (means for VS/BWVS, 83 ± 5 *vs* 61 ± 5%, see Table 4.4).

The small form from the Western Mediterranean described here as *S. minutus* (see below) resembles *S. dimitrovi* in the degree of development of the ventral sucker and the muscular flanges (in relation to body width) and in its generally larger sucker-ratios than those of *S. papernai* (i.e. the ventral sucker is much larger in comparison to the oral sucker in these two forms). However, *S. minutus* is smaller, with smaller eggs and ventral sucker, and narrower pars prostatica and posteriormost pseudosegment; the body width at the level of the anterior border of the ventral sucker is also smaller. Although there is some overlap between the upper and the lower ranges of variation of many metrical features in *S. minutus* and *S. dimitrovi*, respectively, univariate analyses clearly discriminated the two species (section 4.4., this chapter). Finally, the muscular septa are very poorly developed in *S. minutus* and form six pseudosegments, whereas those in *S. dimitrovi* are rather thick and form seven. All these comparisons support the distinct species status of *S. dimitrovi*. Although *S. dimitrovi* was originally described based on material from *M. cephalus* in the Black Sea (Blasco-Costa *et al.*, 2006) the completion of the identification of additional abundant Mediterranean material (three localities, two seasons, 676 fish) revealed three new host records: *Liza aurata, L. ramado* and *Chelon labrosus*. The prevalence patterns of infection by the three sympatric *Saturnius* spp. observed in the newly collected Mediterranean material suggest that all previous records of *S. papernai* from the Mediterranean should be treated with caution. The prevalence ranges in Table 4.5, although showing geographical and habitat variation, clearly indicate that *S. dimitrovi* and *S. minutus* attain their highest prevalence in *M. cephalus* (samples from the Ebro Delta and Santa Pola Lagoon, respectively), whereas *S. papernai* reaches a maximum prevalence in *L. aurata*. It is apparent that the three species coexist in the two areas of the western Mediterranean sampled, where they also infect multiple hosts. Therefore, all non-documented records of *S. papernai* (*sensu lato*) need confirmation.

Table 4.4. Means for the variables for *Saturnius* spp. from mullet subjected to univariate statistical analyses.

Species	*S. minutus*	*S. dimitrovi*	*S. papernai*
Locality	Off Santa Pola	Off Sozopol & Ebro Delta	Off Sozopol & Ebro Delta
	Mean ± SD	Mean ± SD	Mean ± SD
Body length	431 ± 42	691 ± 74	994 ± 149
Body width (at ventral sucker flange)	92 ± 11	130 ± 14	141 ± 16
Body width (at anterior margin of ventral sucker)	74 ± 6	106 ± 9	119 ± 12
Forebody length	155 ± 16	205 ± 23	326 ± 45
Oral sucker	41 ± 6 × 56 ± 7	58 ± 7 × 67 ± 7	71 ± 9 × 78 ± 10
Ventral sucker	67 ± 4 × 66 ± 4	87 ± 6 × 88 ± 7	66 ± 8 × 73 ± 7
Pharynx	25 ± 2 × 25 ± 3	35 ± 3 × 33 ± 2	44 ± 8 × 40 ± 5
Oesophagus length	19 ± 5	28 ± 9	37 ± 8
Sinus-sac	32 ± 4 × 21 ± 4	40 ± 6 × 32 ± 3	54 ± 11 × 37 ± 5
Pars prostatica	27 ± 5 × 17 ± 3	42 ± 7 × 29 ± 4	62 ± 9 × 38 ± 7
Seminal vesicle	33 ± 9 × 22 ± 4	54 ± 14 × 39 ± 9	82 ± 17 × 57 ± 14
Anterior testis	33 ± 8 × 33 ± 6	65 ± 10 × 55 ± 9	72 ± 19 × 67 ± 12
Posterior testis	34 ± 5 × 35 ± 8	65 ± 8 × 61 ± 10	73 ± 17 × 70 ± 17
Ovary	30 ± 5 × 33 ± 7	46 ± 8 × 60 ± 10	62 ± 13 × 71 ± 20
Vitellarium	42 ± 9 × 37 ± 7	76 ± 16 × 62 ± 10	87 ± 22 × 76 ± 14
Last pseudosegment	86 ± 14 × 63 ± 8	169 ± 23 × 109 ± 8	278 ± 45 × 141 ± 24
Muscular flange cone length (anterior-posterior)	46 ± 5	60 ± 6	65 ± 11
Muscular flange cone width (lateral)	23 ± 3	29 ± 3	28 ± 5
Eggs	21 ± 1 × 10 ± 1	23 ± 1 × 11 ± 1	24 ± 2 × 12 ± 1
Body ratio (BW/BL) %	21 ± 2	19 ± 3	14 ± 2
Sucker length ratio (VSL/OSL)	1:1.66 ± 0.25	1:1.51 ± 0.18	1:0.93 ± 0.11
Sucker width ratio (VSW/OSW)	1:1.19 ± 0.13	1:1.32 ± 1.26	1:0.95 ± 0.12
FO/BL %	36 ± 1	30 ± 1	33 ± 2
LSL/BL %	20 ± 3	24 ± 2	28 ± 2
LSW/LSL %	73 ± 11	66 ± 9	51 ± 9
MFL/VSL %	69 ± 8	69 ± 8	99 ± 13
LSW/BW %	68 ± 6	84 ± 6	101 ± 13
VSW/BWVS %	89 ± 7	83 ± 5	61 ± 5
MFW/BWVS %	31 ± 4	27 ± 4	23 ± 4

Table 4.5. Ranges for prevalence (%) of *Saturnius* spp. in mullets (30 samples, 676 fish) sampled in Spring and Autumn (2004-2005) at two localities off the western Mediterranean coast of Spain.

	S. papernai	*S. dimitrovi*	*S. minutus*
Ebro Delta			
Mugil cephalus	6.7-36.7	22.6-86.7	13.3-36.7
Liza aurata	33.0-76.7	0-6.7	0-10.0
L. saliens	0-12.9	-	0-3.3
L. ramado	0-9.1	0-6.1	-
Chelon labrosus	+	-	-
Santa Pola (sea)			
M. cephalus	5.0-20.0	10.0-30.0	0-3.3
L. aurata	10.0-23.3	-	-
L. ramado	16.7-26.7	-	-
Santa Pola (brackishwater lagoon)			
M. cephalus	-	2.0-27.6	21.0-90.0
C. labrosus	+	+	-

+ Present but prevalence not calculated (sample size < 15 fish).

Saturnius maurepasi **Overstreet, 1977**

Material studied

Type-material: Ex *Mugil cephalus* L. Stomach. Off Deer Island in Davis Bayou, Mississippi Sound, USA. Holotype BMNH1975.9.29.1; paratypes BMNH1975.9.29.2-3, USNPC 073270.00, HWML 20247 (5 specimens).

Records

References: 1. Overstreet (1977); 2. Romero & Galeano (1981); 3. Fernandes & Goulart (1992); 4. Knoff *et al.* (1997); 5. Present study.

Descriptions: 1; 3.

Definitive hosts: *Mugil cephalus* L. (1, 2); *M. liza* Valenciennes (3); *M. platanus* Günther (4).

Distribution: Area 31 (Mississippi Sound, USA) (1); area 31 (off Santa Marta, Colombia) (2); area 41 (Brazilian coast of SW Atlantic) (3, 4).

Description (Table 4.6)

Flange at level of ventral sucker not prominent, mound-shaped. Third flange rather weakly developed, located in posterior region of posteriormost pseudosegment. Second transverse septum dorsal to ventral sucker, rather wide (*c.*1/2 of ventral sucker length) in holotype and 1

Table 4.6. Comparative morphometric data for *Saturnius overstreeti* and *S. maurepasi* (type-material).

Species	*S. overstreeti* (n=5)	*S. maurepasi* (n=5)
Measurements		
Body length	905-1,000	782-1,098
Body width at ventral sucker flange	99-142	120-135
Body width at ventral sucker	96-124	101-122
Forebody length	242-277	237-316
Oral sucker	50-61 × 51-61	36-41 × 38-48
Ventral sucker	76-90 × 72-90	62-69 × 67-77
Flange at ventral sucker	37-48 × 16-28	30-51 × 10-16
Pharynx	28-36 × 28-42	28-34 × 26-32
Sinus-sac	51-80 × 34-46	28-39 × 11-24
Pars prostatica	29-38 × 14-22	15-15 × 11-11
Seminal vesicle	235-339 × 32-46	82-146 × 32-50
Anterior testis	66-70 × 54-72	43-64 × 50-84
Posterior testis	66-83 × 77-99	47-64 × 41-65
Ovary	48-70 × 50-69	49-71 × 69-88
Vitellarium	64-104 × 69-101	80-97 × 80-94
Last pseudosegment	258-304 × 130-163	262-376 × 150-165
Eggs	21-24 × 11-12	21-24 × 9-13
Ratios		
Sucker length ratio	1:1.5-1.6	1:1.6-1.9
Sucker width ratio	1:1.4-1.6	1:1.5 -1.8
BW/BL (%)	11-14	12-16
VSW/BWVS (%)	73-77	63-68
FO/BL %	25-28	29-32
VL/BL (%)	6-11	7-12
MFL/VSL (%)	47-53	43-76
MFW/VSW (%)	20-31	20-23
MFW/BWVS (%)	15-23	8-16
MFW/MFL (%)	43-58	31-38
LSL/BL (%)	28-32	31-34
LSW/BW (%)	101-131	113-134
LSW/LSL (%)	46-58	41-63

paratype. 'Drüsenmagen' present. Caecal termination not traceable (masked by uterine coils packed with eggs). Pars prostatica vesicular, small (*c.*1/2 of sinus-sac), difficult to distinguish, short, in most cases covered by several layers of prostatic cells, enters sinus-sac at its base. Seminal vesicle large, wide-tubular, much longer than sinus-sac. Uterine seminal receptacle seen in 3 specimens.

Remarks

Because this species was originally described in a great detail (Overstreet, 1977), only a few details are included here. *S. maurepasi* is distinguished by its: body with seven pseudosegments separated by five fibrous septa; mound-shaped flange at the level of the ventral sucker; spherical suckers; small pars prostatica; and wide-tubular seminal vesicle, which is much longer than the sinus-sac. The 'minor septum at the posterior end of the body' in the original description appears to be the tegumental ridge of the weakly developed third muscular flange.

***Saturnius minutus* Blasco-Costa, Pankov, Gibson, Balbuena, Raga, Sarabeev & Kostadinova, 2006**

Syn. *Saturnius* n. sp. of Pankov *et al.* (2006)

Type-host: Mugil cephalus L.

Type-locality: Off the Mediterranean coast of Spain (at Santa Pola).

Site: Embedded between the grinding stomach lining and the layer of glandular cells.

Type-material: Holotype BMNH 2005.11.10.1; paratypes BMNH 2005.11.10.2-5.

Voucher material: BMNH 2005.11.10.6-9.

Additional material

Ex *Liza aurata* (Risso). Stomach. Ebro Delta, Spain (22.ix.2005; 05.x.2005).

Ex *L. saliens* (Risso). Stomach. Off Santa Pola, Spain (22.vi.2004).

Records

References: 1. Blasco-Costa *et al.* (2006); 2. Present study.

Description: 1.

Definitive hosts: *Mugil cephalus* L. (1), *Liza aurata* (Risso) (2), *L. saliens* (Risso) (2).

Distribution: Area 37, subarea 1 (Western Mediterranean) (1, 2).

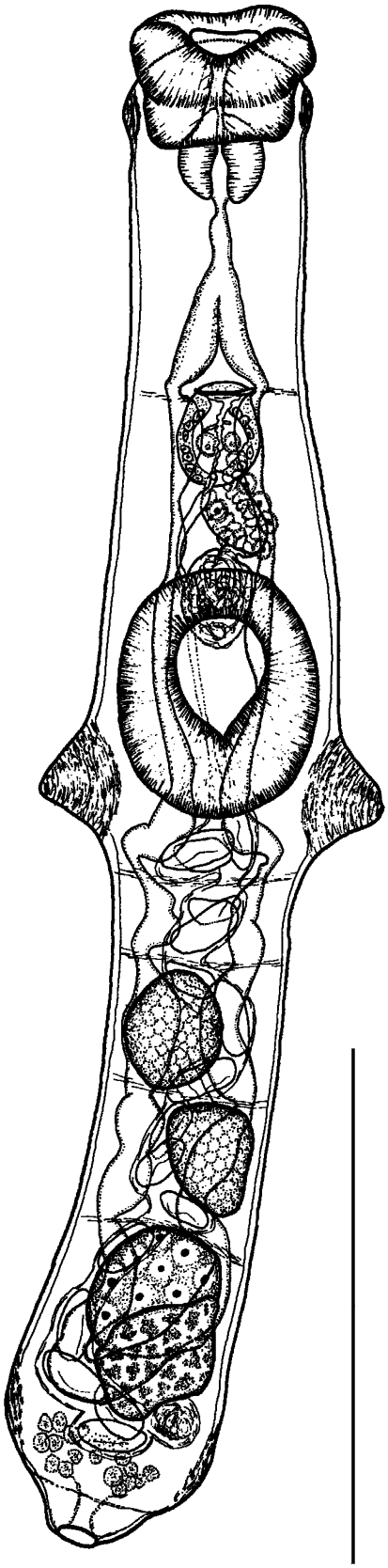

Fig. 4.4. *Saturnius minutus* Blasco-Costa et al., 2006 ex *Mugil cephalus*. Holotype, ventral view. *Scale-bar*: 150 μm.

Description (Fig. 4.4, 4.5)

[Based on 19 whole-mounted adult specimens; metrical data for 15 adult worms in Tables 4.3, 4.4] Body minute, elongate, cylindrical, with rounded posterior extremity and maximum width at level of ventral sucker flange; width at mid-level of ventral sucker 65-91 (74 ± 5). Tegument unarmed, with fine longitudinal and transverse striations.

Three circular muscular flanges present around body: (i) anterior (oral sucker) flange weakly developed, located at base of oral sucker, seen as faint thickening in lateral view; (ii) second (ventral sucker) flange strongly muscular, forming 2 lateral sub-conical protuberances provided with concentric muscles, with cone length (anterior-posterior) 34-59 (47 ± 6) and width (lateral) 17-34 (24 ± 4) [24-47% (32 ± 5%) of body width at mid-level of ventral sucker], with anterior margin located at level of posterior third of ventral sucker; tegumental ridge also distinguishable on ventral surface of whole-mounts posterior to posterior margin of ventral sucker; (iii) third flange located in posterior third of last pseudosegment, in some specimens relatively poorly developed.

Body typically with 6 pseudosegments separated by very faint transverse fibrous septa, difficult to see in many cases. Anteriormost septum at level of genital pore, not observed in 6 specimens (32%); 4 other septa (first located just posterior to ventral sucker flange) form 4 pseudosegments of different sizes in hindbody; anterior smallest, middle pair similar in size and posteriormost largest, 17-29% (22 ± 3%) of body length.

Oral sucker simple, transverse-oval, with terminal opening. Ventral sucker strongly muscular, subspherical, larger than oral sucker, at mid-body. Prepharynx absent; pharynx muscular, subspherical. Oesophagus very short, in some specimens surrounded by minute gland-cells. Intestinal bifurcation at about middle of first pseudosegment. Caeca relatively wide, with several constrictions; most posterior region impossible to observe in adult specimens since it is masked by ovary, vitellarium and uterus; in juvenile specimens, however, caeca can be seen to unite posterior to vitellarium to form cyclocoel (Fig. 4.5)

Testes 2, subspherical, smooth, in tandem or slightly oblique, occupy most of respective pseudosegments, in middle of hindbody, contiguous. Seminal vesicle thin-walled, elongate-oval, antero-dorsal to ventral sucker, similar in size to sinus-sac. Pars prostatica external, elongate-oval, vesicular, similar in size to sinus-sac and seminal vesicle, lined with anuclear blebs, surrounded by large prostatic cells which also partly overlap sinus-sac and anterior part of seminal vesicle. Sinus-sac thin-walled, elongate-oval, contains eversible hermaphroditic duct lined by small intensely stained cells and which forms temporary sinus-organ. Genital pore a transverse slit, median, at level of first septum (when visible).

Ovary oval to subtriangular, in anterior part of last pseudosegment, partly overlaps vitellarium dorsally. Uterine seminal receptacle present; Laurer's canal not observed. Vitellarium compact,

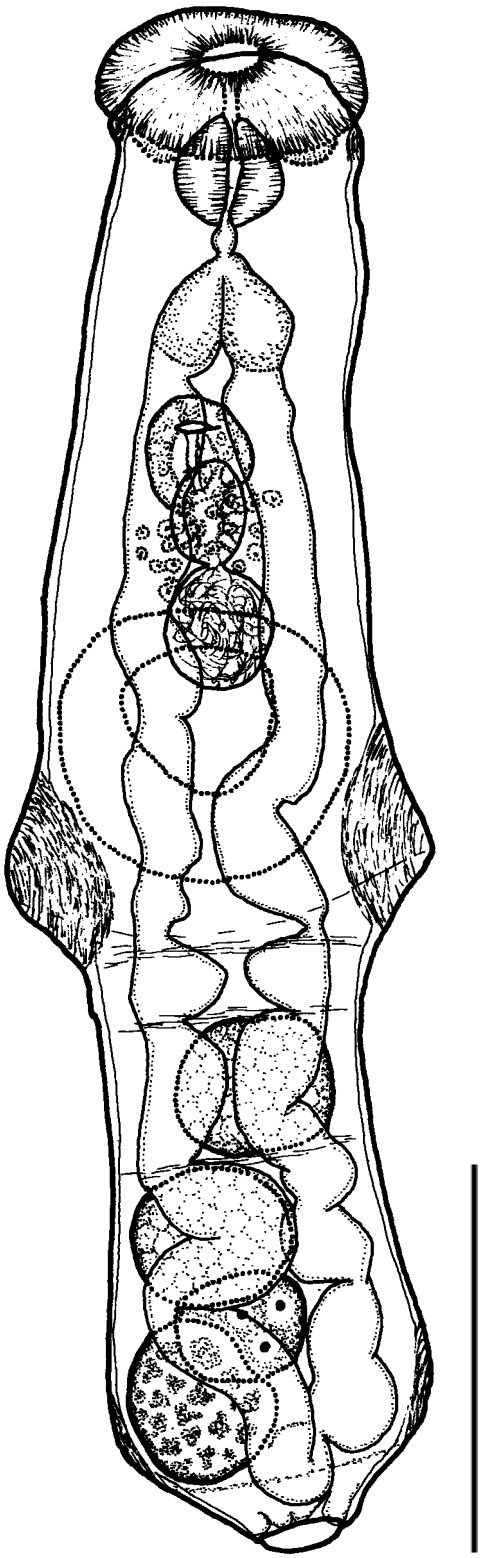

Figure 4.5. Juvenile *Saturnius minutus* Blasco-Costa et al., 2006. *Scale-bar*: 100 μm.

round to elongate-oval, larger than ovary, length *c*.10% of body length. Uterus thin-walled, slightly coiled between ventral sucker and posterior extremity; metraterm not seen. Eggs operculate, not numerous, large in relation to size of body.

Excretory system not visible; pore wide, terminal to dorso-subterminal.

Remarks

This small form from *M. cephalus* from off Santa Pola exhibits the diagnostic characteristics of *Saturnius*. However, unlike other *Saturnius* spp., all the septa are very poorly developed such that only five are visible; as a result, the body appears to be divided into six pseudosegments. *S. minutus* can be distinguished from all previously known species of *Saturnius* by its: (i) more posterior position of the ventral sucker flange so that its lateral sub-conical tips are equidistant from both anterior and posterior extremity; (ii) substantially shorter last pseudosegment; and (iii) generally smaller LSL/BL ratio.

Three species, *S. segmentatus*, *S. papernai* and *S. valamugilis*, are much larger forms (see Manter, 1969; Overstreet,1977; Rekharani & Madhavi, 1985) which, in addition to the features listed above, can also be distinguished from *S. minutus* as follows:

- *S. segmentatus* and *S. papernai* have a larger ventral sucker, pharynx, testes, ovary and vitellarium. In addition, the eggs and sucker-ratio in *S. segmentatus* are also larger. Although the latter is the only species possessing the same number (five) and similar location of the muscular septa, it differs clearly from *S. minutus* in the presence of 10 muscular papillae around the oral sucker (see Overstreet, 1977); the latter author also noted the presence of additional septa. On the other hand, *S. papernai* is clearly distinguishable from *S. minutus* by the presence of six strongly muscular septa, the ventral sucker is smaller in relation to the body width (VSL/BWVS = 52-71 *vs* 81-97%) and the ventral sucker muscular flange is less prominent.
- *S. valamugilis* possesses smaller suckers and a larger posteriormost pseudosegment, seminal vesicle, ovary and vitellarium; this species was also described with a body divided internally into seven to eight pseudosegments. Finally, it was described from a different host [*Valamugil cunnesius* (Valenciennes)] in the Bay of Bengal.

The three other *Saturnius* spp., i.e. *S. mugilis*, *S. maurepasi* and *S. belizensis*, which show overlapping lower ranges for body length (see Yamaguti, 1970; Overstreet, 1977; Fischthal, 1977), differ from *S. minutus* as follows:

- *S. mugilis* has a larger ventral sucker, seminal vesicle, ovary and vitellarium. This species also lacks a septum at the level of the genital pore.

- *S. maurepasi* possesses a pars prostatica which is distinctly smaller in relation to the seminal vesicle, which occupies a large proportion of the second pseudosegment, and a larger vitellarium, the length of which represents a greater proportion of the body length.
- *S. belizensis* is a much thinner form, with smaller suckers and pharynx. Furthermore, this species was described from *Mugil curema* from the Carribean Sea off Belize and possesses only three septa located in the hindbody.

The above comparisons justify the erection of *S. minutus*. Final identification of the newly collected Mediterranean material revealed two new host records for *S. minutus*: *L. aurata* and *L. saliens*. Pankov *et al.* (2006) described *Robinia aurata* Pankov, Webster, Blasco-Costa, Gibson, Littlewood, Balbuena & Kostadinova, 2006 from *Liza aurata* (Mugilidae) from the Spanish Mediterranean. These authors developed a phylogenetic hypothesis for the Bunocotylinae from sequence data analyses based on partial lsrDNA and complete ssrDNA combined (22 species) and V4 domain of the ssrRNA gene (37 species). These analyses supported the erection of the new genus and the concept of Gibson (2002), who recognised two genera (*Bunocotyle* and *Saturnius*) in the subfamily Bunocotylinae. In these analyses a new sequence was obtained for *S. minutus* (referred to as *Saturnius* n. sp. to avoid nomenclatural problems due to uncertainty concerning the first publication of the name), which was described in Blasco-Costa *et al.* (2006). *Saturnius* n. sp. of Pankov *et al.* (2006) is therefore listed as a synonym of *S. minutus*.

***Saturnius mugilis* (Yamaguti, 1970) Overstreet, 1977**

Syn. *Bunocotyle mugilis* Yamaguti, 1970

Material studied

Type-material: Ex *Mugil cephalus* L. Stomach. Off Hawaii. Holotype and one paratype USNPC 063747.00.

Records

References: 1. Yamaguti (1970); 2. Overstreet (1977).

Descriptions: 1; 2.

Definitive host: *Mugil cephalus* L. (1, 2).

Distribution: Area 77 (Hawaii) (1, 2).

Description (Fig. 4.6; Table 4.2)

This species was originally well described by Yamaguti (1970) and adequately redescribed by Overstreet (1977), who transferred *Bunocotyle mugilis* Yamaguti, 1970 to *Saturnius*. A few additional observations follow (measurements in Table 4.2).

No transverse fibrous septum present in forebody of both specimens. Two groups of 3-4 weak, muscular fibres seen on right side of paratype at level of ventral sucker (Fig. 4.6); these do not reach lateral margin of body and are absent in holotype. Third muscular flange located close to posterior extremity present but weakly developed. Intestinal caeca not traceable posterior to vitellarium. Small, vesicular pars prostatica distinguished in both specimens, dorsal to and entering sinus-sac anterior to its base; few prostatic cells visible at base of pars prostatica. Metraterm enters sinus-sac just anterior to pars prostatica (holotype) or more anteriorly (i.e. closer to genital pore in paratype, Fig. 4.6).

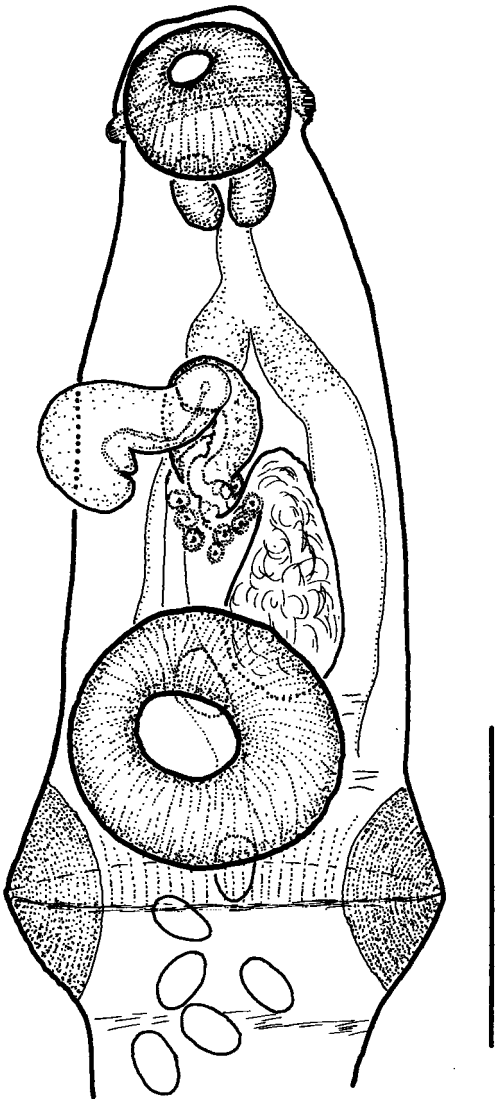

Fig. 4.6 *Saturnius mugilis* ex *Mugil cephalus* from Hawaii. Forebody of the paratype, ventral view. *Scale-bar*: 100 µm.

Remarks

Characteristic features of this species include: body with five pseudosegments, separated by four fibrous septa, entirely located in the hindbody; a strongly developed muscular flange at the level of ventral sucker; subspherical suckers; an elongate-saccular seminal vesicle almost twice as long as the sinus-sac; and a relatively small, vesicular pars prostatica (*c*.1/2 of sinus-sac).

The two groups of muscular fibres observed at the mid-level of the ventral sucker in the paratype are not well enough developed to assume the presence of a septum. These observations, plus the fact that the first septum was not observed at the level of the genital pore in both specimens have lead to the conclusion that septa in *S. mugilis* number four and are located entirely in the hindbody. Overstreet (1977) suggested that 'the caeca seem to end blindly rather than unite behind the vitellarium'. Re-examination of the type-material did not lead to observation of either caeca 'apparently united behind the vitelline gland' (Yamaguti, 1970) or blind caeca, due to the

presence of uterine loops tightly packed with eggs in the postvitelline region. The 'ejaculatory bulb' of Yamaguti (1970) apparently represents a small pars prostatica (as indicated by Overstreet, 1977), with a few small prostatic cells associated with it. The present observations on the entrance of the metraterm into the sinus-sac anterior to pars prostatica (as described by Yamaguti, 1970) deviate from the description of Overstreet (1977, i.e. at the base of sinus-sac). Additional and preferably well-fixed material is definitely needed in order to obtain a more detailed redescription of *S. mugilis*. Unfortunately, most of the reports of this species from a rather distant region (the Black Sea) are not documented and the specimens are unavailable for re-examination; their identification is therefore considered here somewhat dubious [see also 'Remarks' on *Bunocotyle mugilis* of Solonchenko (1976) below].

***Saturnius overstreeti* Blasco-Costa, Montero, Gibson, Balbuena, Raga, Shvetsova & Kostadinova, 2008**

Type-host: Mugil soiuy Basilewsky.
Other host: Mugil cephalus L.
Type-locality: Razdol'naya River, Russia (flowing into the northern Amur Bay, Sea of Japan) (12.x.2004), area 61.
Other locality: Kievka River, Russia (southeastern coast of the Sea of Japan) (17.vi.2005), area 61.
Site: Embedded between the grinding stomach lining and the layer of glandular cells.
Type-material: Holotype BMNH 2007.1.24.46; paratypes BMNH 2007.1.24.47-48.
Etymology: The species is named for Professor Robin Overstreet, Gulf Coast Research Laboratory, University of Southern Mississippi, in recognition to his contribution to the knowledge of the genus that has inspired this study.

Description (Fig. 4.7; Table 4.6)
Body robust, elongate, cylindrical, with wide-rounded posterior extremity and maximum width of 152-166 at mid-level of posteriormost pseudosegment. Tegument unarmed, with distinct transverse and fine longitudinal striations.

Three strongly developed circular muscular flanges present around body: (i) anterior (oral sucker) flange well developed, located at mid-level of oral sucker; (ii) second (ventral sucker) flange muscular, forms 2 lateral conical protuberances provided with concentric

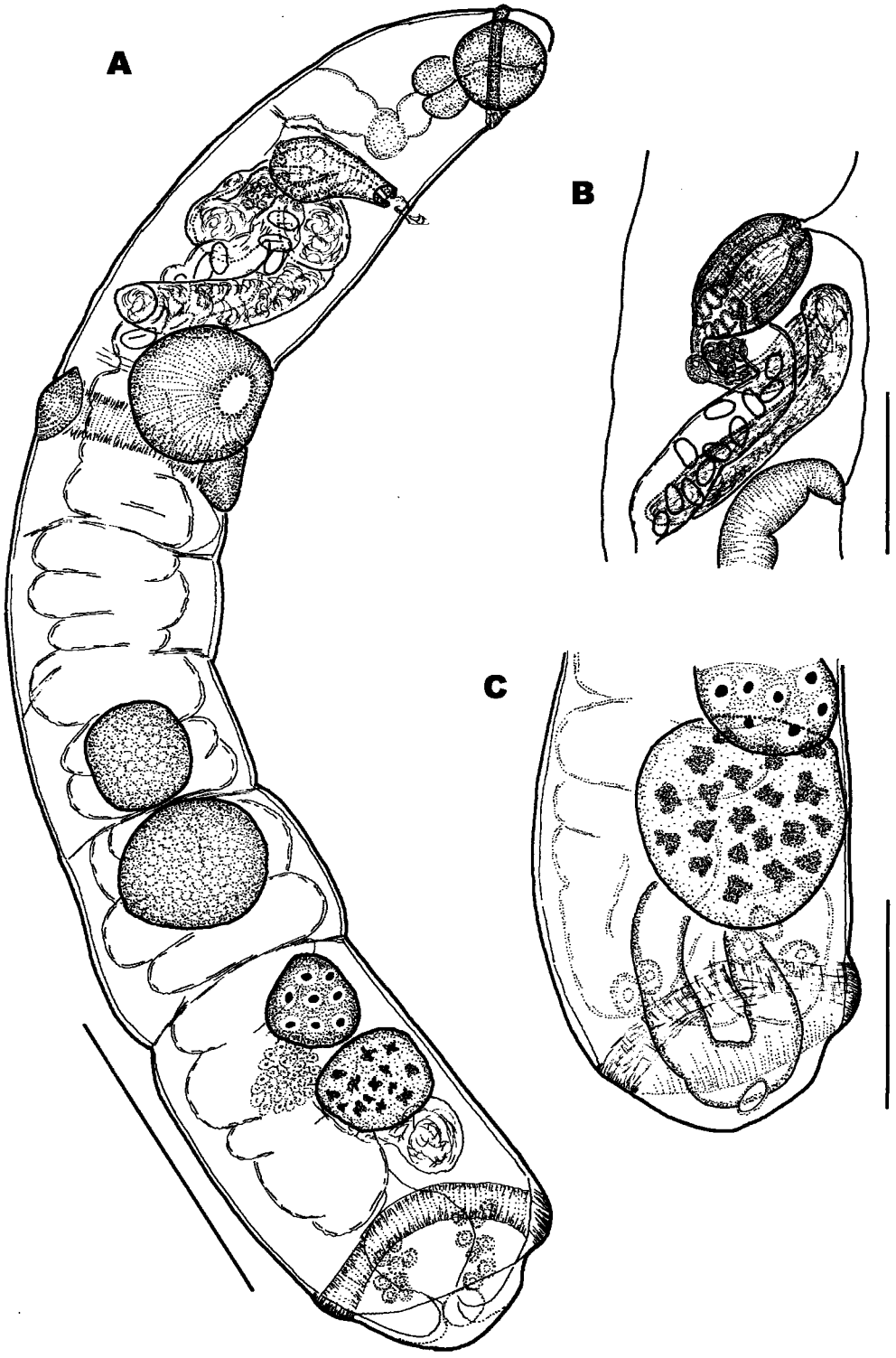

Fig. 4.7 *Saturnius overstreeti* Blasco-Costa et al., 2008. **A.** Holotype ex *Mugil soiuy* from the Sea of Japan: general morphology, ventro-lateral view with uterus in outline. **B.** Paratype ex *M. cephalus*: terminal genitalia, lateral view. **C.** Paratype ex *M. cephalus*: posterior extremity. *Scale-bars*: **A.**, 200 μm; **B.**, **C.**, 100 μm.

muscles, with cone length (anterior-posterior) 37-48 and width (lateral) 16-28 (15-23% of body width at level of ventral sucker), with anterior margin located at level of posterior quarter of ventral sucker and tegumental ridge present on both ventral and dorsal surface; and (iii) third flange located in posterior third of last pseudosegment, strongly muscular.

Body with 6 pseudosegments separated by 5 transverse relatively thick fibrous septa. Anteriormost septum at level of genital pore; 4 other septa form 4 pseudosegments of different sizes located entirely in hindbody; anterior smallest, middle pair similar in size and posteriormost largest, 28-32% of body length. One additional median group of muscle fibres with no connection to lateral body margins present at mid-level of ventral sucker.

Pre-oral lobe distinct. Oral sucker muscular, spherical, subterminal. Ventral sucker strongly muscular, spherical, larger than oral sucker, anterior to mid-body. Prepharynx absent; pharynx muscular, subspherical. Oesophagus short. Caeca wide, thick-walled, form 'Drüsenmagen' just anterior to first septum, unite close to posterior extremity to form cyclocoel (Fig. 4.7).

Testes 2, subspherical, smooth, in tandem, occupy most of fourth and fifth pseudosegments in middle of hindbody, contiguous or separated. Seminal vesicle thin-walled, wide-tubular, coiled, extends dorsally to the mid-level of ventral sucker, longer than forebody. Pars prostatica external, elongate-oval, vesicular, much smaller than sinus-sac, lined with anuclear blebs, surrounded by relatively small prostatic cells, passes into sinus-sac mid-dorsally. Sinus-sac with muscular walls, elongate-oval, contains eversible hermaphroditic duct lined by small intensely stained cells; duct forms temporary sinus-organ. Genital pore median, at level of first septum.

Ovary oval to subtriangular, in anterior part of last pseudosegment. Mehlis' gland similar in size to ovary, contiguous with ovary and vitellarium. Uterine seminal receptacle large, postero-dorsal to vitellarium; Laurer's canal not observed. Vitellarium compact, suboval to elongate-oval, larger than ovary, with length 6-11% of body length. Uterus thin-walled, fills free space in pseudosegments of hindbody; metraterm enters hermaphroditic duct at mid-level of sinus-sac. Eggs numerous.

Excretory pore wide, ventro-subterminal; vesicle stem saccular; arms unite at level of pharynx.

Remarks

The form described from *Mugil soiuy* and *M. cephalus* from the coast of the Sea of Japan exhibits the diagnostic characteristics of *Saturnius*. It is characterised by its large robust body

which only overlaps the upper size limits of *S. papernai* and *S. maurepasi* (see Overstreet, 1977). It can be distinguished from these two species as follows:

S. papernai is clearly distinguishable from *S. overstreeti* by the presence of six stout, transverse, muscular septa forming seven pseudosegments (*vs* five septa and six pseudosegments), the saccular seminal vesicle (*vs* wide-tubular), the flared anterior margin of the cup-shaped oral sucker and the fact that the first septum in the hindbody is located just posterior to the ventral sucker flange. Furthermore, the ventral sucker in *S. papernai* is transverse-oval and smaller in relation to the width of body (VSW/BWVS = 52-71 *vs* 73-77%) and the ventral sucker muscular flange is longer in relation to ventral sucker (MFL/VSL= 76-118 *vs* 47-53%).

S. overstreeti appears most similar to *S. maurepasi*, which was originally described from *M. cephalus* in the Mississippi Sound, USA (Overstreet, 1977). Similarities include the shape of the oral and ventral suckers; the longer posteriormost pseudosegment (in relation to body length, LSL/BL=28-32 *vs* 31-34%); the overlap of some metrical features (e.g. size of pharynx, testes, ovary, vitellarium and eggs, see Table 4.6); and the large elongate-tubular seminal vesicle. However, the suckers in *S. overstreeti* are larger; the ventral sucker is larger in relation to the body width (VSW/BWVS = 73-77 *vs* 63-68%), whereas the sucker ratio is smaller (length ratio 1:1.48-1.56 *vs* 1:1.59-1.92; width ratio 1:1.41-1.61 *vs* 1:1.53-1.76); and the ventral sucker muscular flange is more prominent (width 16-28 *vs* 10-16 μm; MFW/BWVS = 15-23 *vs* 8-16%; MFW/MFL = 43-58 *vs* 31-38%). Finally, the sinus-sac and pars prostatica in *S. overstreeti* n. sp. are much larger (51-80 × 34-46 *vs* 28-39 × 11-24 μm and 29-38 × 14-22 *vs* 15 × 11 μm); the pars prostatica enters the sinus-sac at its mid-level (*vs* at its base); and the seminal vesicle is substantially longer (235-339 × 32-46 *vs* 82-146 × 32-50 μm).

The above comparisons and the substantial geographical separation of the two forms justify the erection of *S. overstreeti*.

Saturnius papernai **Overstreet, 1977**

Material studied

Type-material: Ex *Mugil cephalus* L. Bardawil Lagoon, NW Sinai, Egypt (UAR). Holotype BMNH 1975.9.29.4; paratypes BMNH 1975.9.29.5; USNPC 073271.00; HWML 20248 (5 specimens).

New material: Ex *M. cephalus* L. Stomach. Ebro Delta, Spain. Voucher material: BMNH 2005.11.10.10-19.

Ex *Liza aurata* (Risso). Stomach. Ebro Delta (26.v.2004; 02.vi.2004, 22.vi.2004; 16.xi.2004; 20.v.2005; 31.v.2005; 22.vi.2005; 22.ix.2005; 05.x.2005) and off Santa Pola, Spain (14.v.2004; 19.v.2004; 07.vi.2005; 29.vi.2005; 04.vii.2005; 03.x.2005; 08.xi.2005). BMNH 2007.1.24.38-45.

Ex *L. ramado* (Risso). Stomach. Ebro Delta (26.v.2004; 02.vi.2004; 22.vi.2004) and off Santa Pola, Spain (04.x.2004; 09.xi.2004; 07.vi.2005; 03.x.2005; 02.xi.2005).

Ex *Chelon labrosus* (Cuvier). Stomach. The Ebro Delta (20.v.2005; 01.vi.2005) and off Santa Pola, Spain (02.xi.2005).

Ex *L. aurata* (Risso) and *L. saliens* (Risso). Stomach. Off Sozopol, Bulgarian Black Sea coast. 32 specimens. Voucher material: BMNH 2005.11.10.10-19.

Records

References: 1. Overstreet (1977); 2. Di Cave *et al.* (1997); 3. Merella & Garippa (1998); 4. Domnich & Sarabeev (2000a) 5. Merella & Garippa (2001); 6. Dmitrieva & Gaevskaya (2001); 7. Pronkina (2001); 8. Gaevskaya & Korniychuk (2003); 9. Blasco-Costa *et al.* (2006); 10. Present study.

Descriptions: 1; 9; 10.

Definitive hosts: *Mugil cephalus* L. (1, 3, 4, 5, 6, 9, 10); *M. soiuy* Basilewsky (6, 8); *Liza aurata* (Risso) (4, 6, 7, 8, 9, 10); *L. ramado* (Risso) (2, 10); *L. saliens* (Risso) (9, 10); *Chelon labrosus* (Cuvier) (10).

Distribution: Area 37, subarea 3 (Eastern Mediterranean) (1); area 37, subarea 1 (Western Mediterranean) (2, 3, 5, 9, 10); area 37, subarea 4 (Black Sea) (6, 7, 8, 9) and Azov Sea (4, 6).

Description (Fig. 4.8; Table 4.7)

[Based on 27 whole-mounted adult specimens; metrical data in Tables 4.3, 4.4]. Body large, elongate, cylindrical, with obtuse posterior extremity and maximum width at levels of ventral sucker flange and posterior flange. Width at mid-level of ventral sucker 95-136 (119 ± 12). Tegument unarmed, with fine longitudinal and transverse striations.

Three circular muscular flanges present; (i) anterior (oral) flange well developed, muscular, located approximately at mid-level of oral sucker; (ii) second (ventral sucker) flange strongly muscular, forming 2 lateral circular protuberances, provided with concentric muscles, with cone length (anterior-posterior) 46-91% (65 ± 11%) and width (lateral) 19-36%

(28 ± 5%) [17-32% (23 ± 4%) of body width at mid-level of ventral sucker], located mostly posterior to ventral sucker; tegumental fold caused by musculature of second flange at some distance from posterior margin of ventral sucker; (iii) third flange located in posterior third of last pseudosegment, well developed.

Body with 7 clearly distinguishable pseudosegments separated by thick transverse fibrous septa. Anteriormost septum at level of genital pore; second septum at anterior level of ventral sucker flange; 4 other septa form 4 pseudosegments of different sizes located entirely in hindbody: anterior smallest, middle pair similar in size and posteriormost largest, 25-35% (28 ± 2%) of body length.

Oral sucker thick-walled, cup-shaped, with terminal aperture; anterior portion of sucker protrusible, with flared anterior rim. Ventral sucker muscular, oval to transversely oval, smaller than oral sucker. Prepharynx absent; pharynx muscular, elongate-oval. Oesophagus short; intestine bifurcates at about middle of first septum. Caeca wide, with constrictions at levels of septa; most posterior portions masked by the ovary, vitellarium and uterus; cyclocoel observed in 1 juvenile specimen (Fig. 4.8B).

Testes 2, spherical or with slightly irregular shape, smooth, slightly oblique, in fifth and sixth pseudosegments. Seminal vesicle thick-walled, elongate-oval, curves dorsally to anterior portion of ventral sucker, distinctly larger than sinus-sac. Pars prostatica external, large, elongate-oval, vesicular, lined with large anuclear blebs and surrounded by large prostatic cells which partly overlap sinus-sac and anterior part of seminal vesicle. Sinus-sac thick-walled, elongate-oval, contains eversible hermaphroditic duct lined by intensely stained cells. Genital pore a transverse slit, median, at level of first septum.

Ovary oval to subtriangular, in anterior part of last pseudosegment, ventral to caeca and partly overlapping vitellarium dorsally. Uterine seminal receptacle large, postero-dorsal to vitellarium; Laurer's canal not observed. Vitellarium compact, smooth, elongate-oval, larger than ovary, length *c*.9% of body length. Uterus thin-walled, extends posterior to vitellarium. Eggs very numerous.

Excretory pore wide, terminal to dorso-subterminal; arms of excretory vesicle unite at level of pharynx.

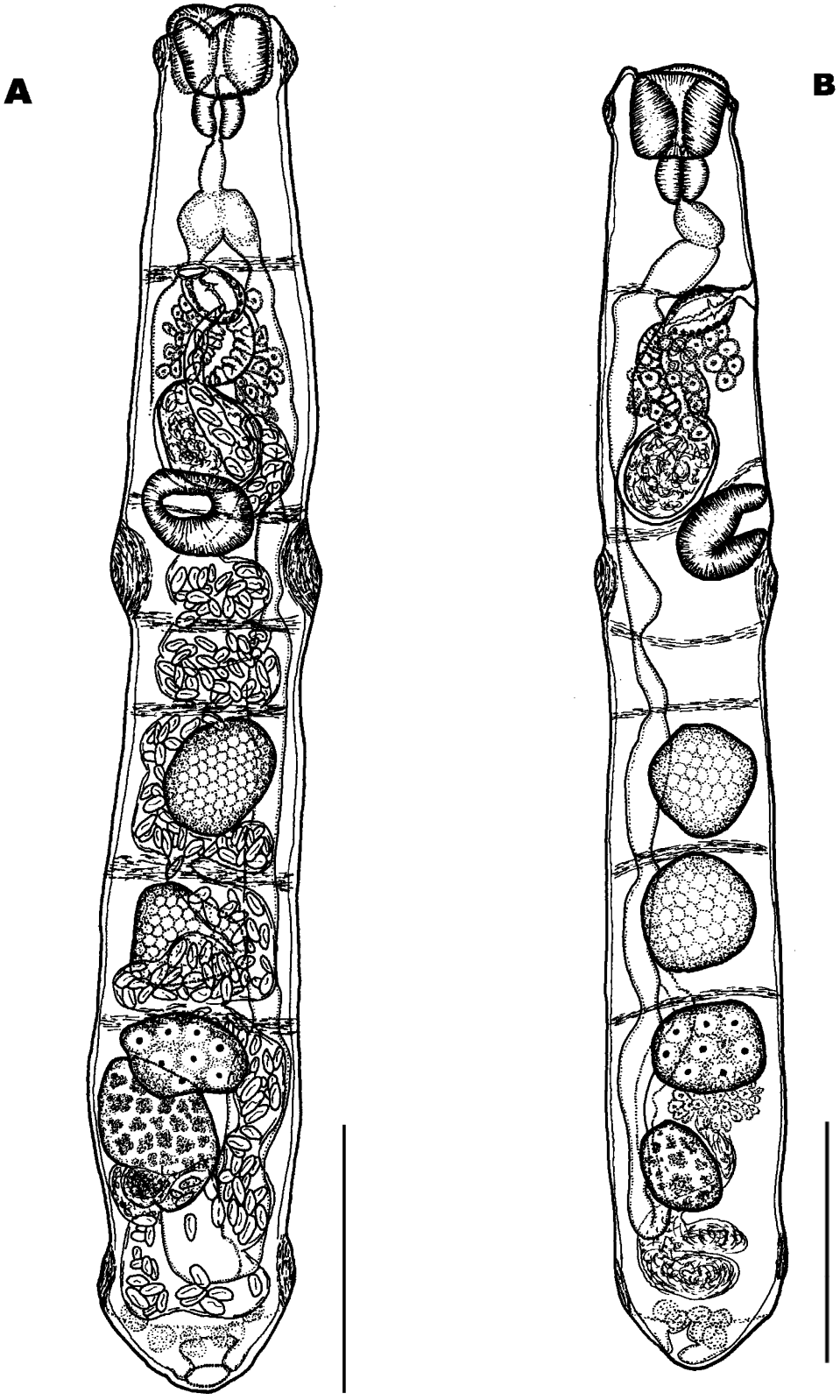

Fig. 4.8. A. *Saturnius papernai* ex *Mugil cephalus*. Ventral view. **B.** Juvenile *S. papernai*. *Scale-bars*: 200 μm.

Small morph in *L. aurata* (Fig. 4.9): The material from *L. aurata* exhibits a higher variation of the metrical features (see Table 4.7 for a comparison of the metrical data from both fresh and frozen material from *L. aurata* with the previous descriptions of *S. papernai*). Thus the forebody is somewhat longer (in relation to body length, FO/BL=34-56 *vs* 28-37%), the last pseudosegment is relatively wider (LW/LL= 57-82 *vs* 31-72%), and the size of body and most of the other metrical features (correlated with body size) was found to vary below or within the lower ranges reported for *S. papernai* (Overstreet, 1977; Blasco-Costa *et al.*, 2006; Table 4.7). However, the size of the eggs and the ratios (with the exception of those mentioned above) are within the known range of *S. papernai*. Furthermore, both 'small' and 'typical' forms of *S. papernai* were found to occur together in the same populations and in the same individuals of *L. aurata*. There were no significant morphometric differences between the fresh and frozen material (Kolmogorov-Smirnov test, $p > 0.05$) with the exception of the width of the seminal vesicle ($p = 0.017$) and VSW/BWVS ($p = 0.030$).

Remarks

This species has been adequately described (Overstreet, 1977) and recently redescribed on the basis of abundant material from the Western Mediterranean and the Black Sea (see above and Blasco-Costa *et al.*, 2006). The re-examination of the types revealed the presence of a uterine seminal receptacle in 4 specimens. However, it was impossible to observe caecal endings (masked by the uterine coils filled with eggs) in any of the type-specimens.

Of the six species of *Saturnius,* only *S. mugilis* and *S. papernai* have previously been reported from the Mediterranean basin. However, only two documented records of the latter species exist (see Overstreet, 1977; Dimitrov *et al.*, 1998). As shown above, the latter record represents a species new to science, i.e. *S. dimitrovi*. On the other hand, the newly collected *S. papernai* from off the Ebro Delta described here from *M. cephalus* agrees very well with the original description of the species given by Overstreet (1977) (see Table 4.7 for metrical data). It also exhibits the main differential characters which distinguish *S. papernai* from its congeners: a thick-walled, cup-shaped oral sucker with a flared anterior rim; muscular, oval to transverse-oval ventral sucker, smaller than oral sucker; ventral sucker flange mound-shaped, not well developed; large pars prostatica comparable in size to sinus-sac and seminal vesicle; saccular seminal vesicle; and relatively short posteriormost pseudosegment (Overstreet, 1977; Blasco-Costa *et al.*, 2006).

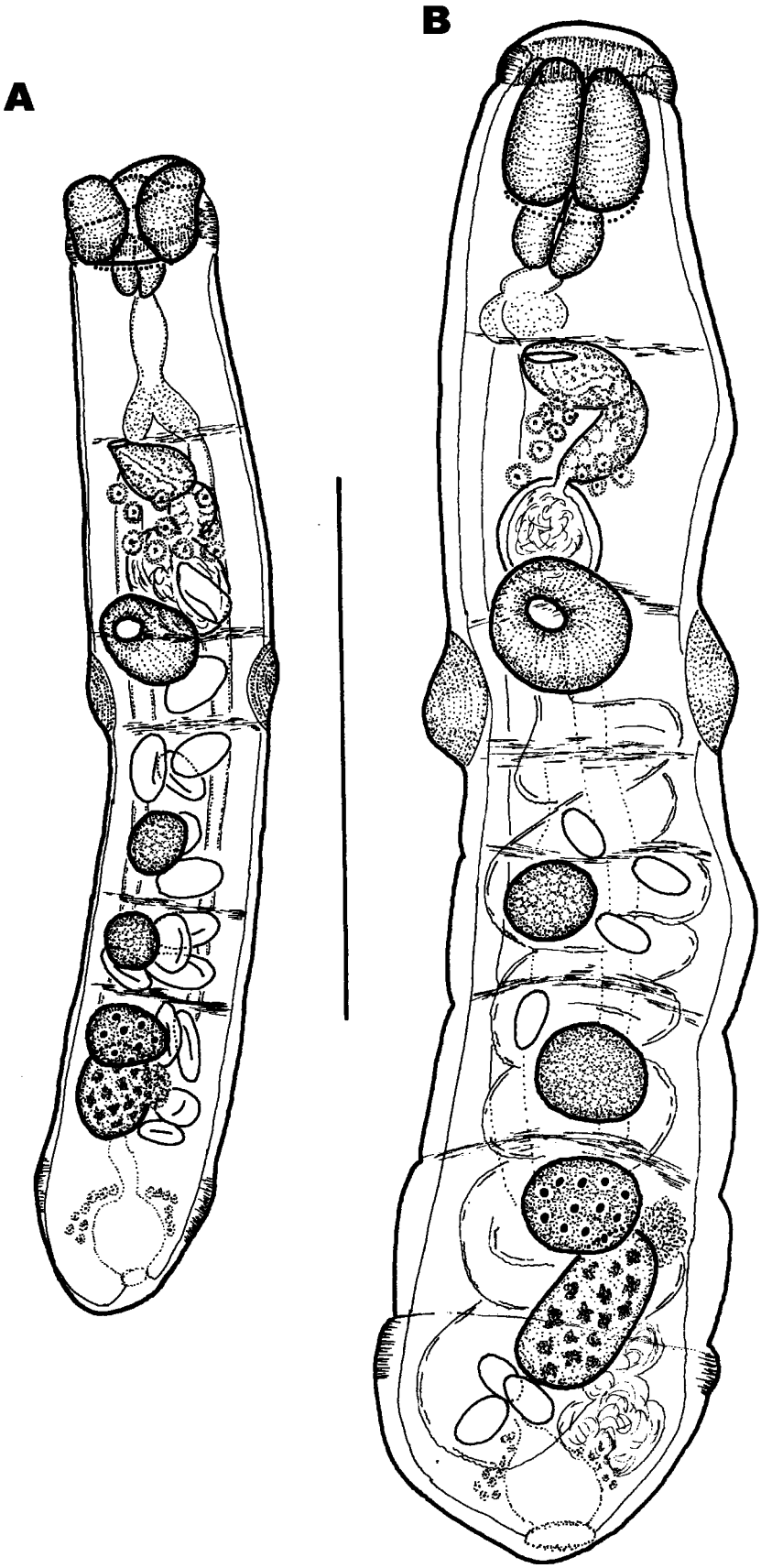

Fig. 4.9. *Saturnius papernai* ex *Liza aurata* from the Ebro Delta, Spain. **A.** Neogravid specimen. **B.** Fully-gravid specimen with uterus in outline. *Scale-bar*: 200 μm.

Table 4.7. Morphometric data for *Saturnius papernai* Overstreet, 1977.

Host	*Mugil cephalus*	*M. cephalus*	*Liza aurata*	*L. aurata*
Locality	Bardawil lagoon (Egypt)	Ebro Delta	Ebro Delta & off Santa Pola	Ebro Delta & off Santa Pola
Source	Overstreet (1977) (n=5)	Blasco-Costa et al. (2006) (n=26)	Present study (fresh material, n=8)	Present study (frozen material, n=28)
Measurements				
Body length	635-1021	687-1183	427-576	454-593
Body width at ventral sucker flange	93-190	107-165	70-117	81-104
Body width at ventral sucker	-	95-136	69-101	72-93
Forebody length	-	240-412	161-288	160-233
Oral sucker	38-64 × 51-87	55-91 × 57-91	38-59 × 47-54	38-57 × 38-56
Ventral sucker	48-78 × 51-90	47-79 × 57-84	34-50 × 39-57	38-47 × 44-62
Flange at ventral sucker	-	46-91 × 19-36	31-54 × 10-26	35-54 × 12-21
Pharynx	30-48 × 29-42	27-65 × 27-48	25-34 × 21-34	25-37 × 23-33
Sinus-sac	-	37-74 × 27-45	34-51 × 21-29	29-39 × 19-31
Pars prostatica	-	40-77 × 23-54	20-52 × 18-31	37-49 × 16-28
Seminal vesicle	-	52-109 × 32-81	40-68 × 30-45	33-67 × 23-45
Anterior testis	41-90 × 44-99	34-113 × 44-94	22-51 × 19-52	23-53 × 19-52
Posterior testis	52-99 × 45-116	38-105 × 42-105	22-56 × 18-57	24-59 × 24-58
Ovary	46-87 × 44-107	42-101 × 36-120	24-46 × 29-58	28-44 × 32-48
Vitellarium	67-145 × 58-138	45-124 × 50-103	35-67 × 29-54	37-61 × 26-56
Last pseudosegment	173-273	176-358 × 80-173	114-163 × 66-128	120-160 × 81-105
Eggs	20-32 × 10-17	21-26 × 9-14	23-25 × 11-12	20-26 × 10-15
Ratios				
Sucker length ratio	-	1:0.7-1.2	1:0.7-0.9	1:0.7-1.1
Sucker width ratio	1:0.8-1.3	1:0.7-1.2	1:0.8-1.2	1:0.9-1.3
BW/BL (%)	-	12-19	16-21	15-19
VSW/BWVS (%)	-	52-71	45-66	55-68
FO/BL %	28-35	30-37	34-56	34-42
VL/BL (%)	-	4-12	8-12	7-11
MFL/VSL (%)	-	76-118	74-129	77-123
MFW/VSW (%)	-	28-49	26-53	21-39
MFW/BWVS (%)	-	17-32	14-29	13-26
MFW/MFL (%)	-	33-56	31-54	28-51
LSL/BL (%)	-	25-35	22-29	23-30
LSW/BW (%)	-	51-120	93-110	94-114
LSW/LSL (%)	-	31-72	57-82	60-78

The present study, therefore, represents the second documented record of *S. papernai* in the Mediterranean (see Blasco-Costa et al., 2006, 2008). *L. saliens* is a new host record and *L. aurata* is the first documented record for *S. papernai* in the Mediterranean basin.

Although a higher variation in most of the metrical features of *S. papernai* from *L. aurata* was detected (extending the known range for this species and perhaps reflecting the colonisation of a different host), the results of the multivariate analyses (see section 4.4. below) verified its identification. The variation in the metrical features within the *S. papernai* set of specimens was always less than between the three species (i.e. *S. papernai*, *S. dimitrovi* and *S. minutus*). *L. ramado* and *C. labrosus* are new host records for *S. papernai* (*s. str.*) (see 'Remarks' on *S. dimitrovi* above).

Species inquirendae

***Saturnius valamugilis* Rekharani & Madhavi, 1985**

Material studied

Type-material: *Valamugil cunnesius* (Richardson). Stomach. Off Visakhapatnam, Waltair Coast, India. Holotype BMNH 1984.6.28.16.

Record

Reference, Description: Rekharani & Madhavi (1985).
Definitive host: *Valamugil cunnesius* (Richardson).
Distribution: Area 57 (Off Waltair Coast, India).

Remarks

This species was described on the basis of two specimens in poor condition (probably dead when fixed and overstained). Some major discrepancies between the original description and the types exist. Thus, a mound-shaped muscular flange just posterior to the ventral sucker (*vs* 'ventral sucker without any muscular thickening') was observed. The body has seven (*vs* seven or eight) pseudosegments separated by six transverse septa. The caeca were described and figured as blind, but this is impossible to observe due the presence of uterine coils filled with eggs between the vitellarium and the posterior extremity. The pars prostatica was described as a 'short male duct and its surrounding prostatic cells' enclosed in the sinus-sac. Although poorly figured for the holotype (fig. 14 of Rekharani & Madhavi, 1985), none of

these are discernible in the types. Mehlis'gland cannot be seen and the uterus extends fairly close to the posterior extremity, including what is described as a 'minor posterior segment' (*vs* 'extending posterior to vitellarium but not into minor posterior segment'). Finally, apart from the subventral (and not terminal) excretory pore, no other details of the excretory system described by Rekharani & Madhavi (1985) are visible, and the 'minor posterior segment' appears problematical (a misinterpretation?). However, a faint muscular flange was observed in the posterior quarter of the last pseudosegment. Additional well-preserved material from the type-host and locality is needed in order to establish the taxonomic status of *S. valamugilis*.

Bunocotyle constrictus Domnich & Sarabeev, 1999
Syn. *Saturnius papernai* of Domnich & Sarabeev (2000)

Material studied
Type-material: Ex *Mugil soiuy* Basilewsky. Stomach. Molochnyi Liman (11.x.1997), Sea of Azov. Paratype I.I. Schmal'gausen Institute of Zoology, Ukrainian Academy of Sciences No. 122-69 labelled "*Bunocotyle constrictus* sp. n. det. V. Sarabeev (2 specimens mounted on the slide: 1 paratype and 1 distorted juvenile haplopoporid). Two other slides were provided by V. Sarabeev: (i) Labelled "*M. soiuy* 56-15. 26.vii.1997. Molochnyi Liman. Stomach. Coll. Sarabeev. *Bunocotyle* sp. new. 3 specimens" (3 juvenile specimens); (ii) Labelled "*M. soiuy* 174-59. 16.vii.1998. Molochnyi Liman. Stomach. Coll. Sarabeev. *Bunocotyle constrictus*. 22.vii.1998" (5 specimens).

Records
References: 1. Domnich & Sarabeev (1999); 2. Domnich & Sarabeev (2000a); 3. Domnich & Sarabeev (2000b); 4. Domnich & Sarabeev (2000c); 5. Domnich & Sarabeev (2000d); 6. Sarabeev (2000); 7. Sarabeev & Domnich (2000).
Description: 1.
Definitive host: *Mugil soiuy* Basilewsky.
Distribution: Area 37, subarea 4 (Azov Sea) (1, 2, 3, 4, 5, 6, 7).

Remarks
Domnich & Sarabeev (1999) described *Bunocotyle constrictus* Domnich & Sarabeev, 1999 from *M. soiuy* in the Azov Sea, which they later considered a misidentification of *S. papernai*

(see Domnich & Sarabeev, 2000 a,b,c,d; Sarabeev, 2000; Sarabeev & Domnich, 2000). However, the original description is based on much smaller worms than *S. papernai* (360-620 × 79-122 *vs* 635-1021 × 93-190 μm, see Overstreet, 1977), with a 'tail appendage', a ventral sucker 'somewhat larger than oral', three pseudosepta all located in the hindbody (first anterior to the anterior testis, second between the testes and third between the posterior testis and the ovary) and caeca united to form a cyclocoel. The terminal genitalia of *B. constrictus* were said to be 'characteristic for the genus' (i.e. *Bunocotyle*). Neither the sinus-sac nor the pars prostatica were detected. The species was distinguished from *Bunocotyle mugilis* [as described by Solonchenko (1982), see below] in by its 'striated cuticle, bindings in posterior part of body, larger gastral suckers, and location in the intestine of hosts'. In addition to their smaller size, the presence of only three septa and the different structure of the terminal genitalia, the testes, ovary and vitellarium in the worms from *M. soiuy* exhibit much lower ranges when compared to the original description of *S. papernai*.

The re-examination of the paratype and the voucher material of *B. constrictus* provided by one of the authors revealed that all specimens were in poor condition. Three of the eight voucher specimens were juvenile. Most morphological features could not be observed in the specimens, and of the terminal genitalia in particular. The presence of some fibrous septa could only be detected in the paratypes, which suggests that the material belongs to *Saturnius*. The use of juvenile specimens can explain the substantially small dimensions provided by Domnich & Sarabeev (1999) for the lower range of all metrical characters. However, the egg-size measurements provided by these authors are apparently erroneous (i.e. 9-28 × 6-19 μm), since all eggs in the paratype are collapsed; among the five voucher specimens only five eggs were not collapsed and relatively good for taking measurements; the latter measured 22-26 × 11-13 μm. In the light of these discrepancies, further material is needed to establish the identity of the form in *M. soiuy* from the Sea of Azov.

Questionable records

Bunocotyle mugilis of Solonchenko (1976)

Records

References: 1. Solonchenko (1976); 2. Solonchenko & Tkachuk (1985).

Description: 1.

Definitive hosts: 'Kefali' (mugilid) (1); *Liza saliens* (Risso) (2).

Distribution: Area 37, subarea 4 (Azov Sea) (1, 2).

Remarks
Solonchenko (1976) made the first report of *Bunocotyle mugili*s Yamaguti, 1970 in the Azov Sea from the stomach of 'kefali' (a vernacular used for several mugilids). This record was reiterated by Solonchenko (1982) and by Solonchenko & Tkachuk (1985) with the host name *L. saliens*. However, the brief description and the figure of Solonchenko (1976) illustrate a species of *Bunocotyle* (see Gibson, 2002 and detailed comments of Dimitrov *et al.*, 1998) rather than *Saturnius* (i.e. absence of transverse fibrous septa and absence of sinus-sac; ventral and oral sucker similar in size; presence of subventral and lateral protuberances on the oral the sucker; opening of metraterm into the genital atrium).

***Saturnius mugilis* of Dmitrieva & Gaevskaya (2001)**

Records
References: 1. Dmitrieva & Gaevskaya (2001); 2. Gaevskaya & Korniychuk (2003).
Definitive hosts: *Liza aurata* (Risso) (1, 2); *L. saliens* (Risso) (1, 2).
Distribution: Area 37, subarea 4 (Azov Sea, Black Sea) (1, 2); Azov Sea (1).

Remarks
Both of the above references represent non-documented records, the second reiterating the first and also perhaps the records by Solonchenko (1976, 1982) and Solonchenko & Tkachuk (1985), and should, therefore, be treated with caution. New material is needed to confirm the presence of *S. mugilis* (Yamaguti, 1970) in the Black and Azov Seas.

***Saturnius* spp. innom.**

Records
References: 1. Lester & Sewell (1989); 2. D'Amelio *et al.* (1995); 3. Merella & Garippa (1998); 4. Merella & Garippa (2000); 5. Merella & Garippa (2001).
Definitive hosts: *Mugil cephalus* L. (1); *Liza aurata* (Risso) (3, 4, 5); *L. saliens* (Risso) (4, 5); *Liza ramado* (Risso) (2, 5); *Chelon labrosus* (Cuvier) (5).
Distribution: Area 71 (Great Barrier Reef, W Pacific) (1); area 37, subarea 1 (Western Mediterranean) (3, 4, 5) (?2 'Italy').

4.4. Morphometric variability of the three Mediterranean species of *Saturnius*

Measurements were recorded from a set of 85 specimens (23 *S. minutus*, 30 *S. dimitrovi* and 32 *S. papernai*); these include eight *S. minutus* additional to the type-series, and the type-material of *S. papernai* (5 specimens). Metrical data were ln-transformed and initially subjected to univariate (ANOVA, a posteriori contrasts) statistical tests. Univariate analyses of variance revealed that *S. papernai, S. dimitrovi* and *S. minutus* are morphometrically distinguishable with respect to the most metrical features used (all $p<0.0001$, see Table 4.4 for means). Moreover, although a range overlap was detected in egg-size (see Table 4.3), the three species differed significantly with respect to both the length ($F_{1, 174}=114.6$, $p<0.0001$; multiple comparisons $p<0.001$) and width ($F_{1, 174}=101.6$, $p<0.0001$; multiple comparisons $p<0.0001$) of the eggs (see also Table 4.3 and Fig. 4.10).

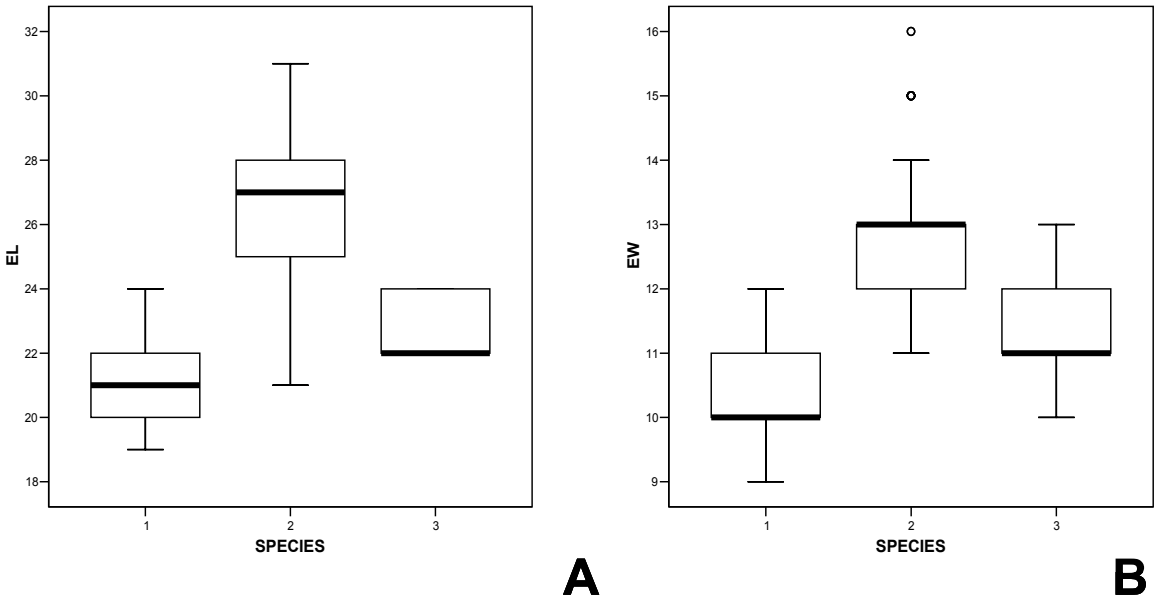

Fig. 4.10. Box-plots for egg length (**A**, EL) and width (**B**, EW) measurements in *Saturnius* spp. Species codes: 1, *Saturnius minutus*; 2, *S. papernai*; 3, *S. dimitrovi*.

Secondly, two LDA were applied to a set of 69 specimens for which data for all variables measured were available (body length, egg-size and ratios were excluded from these analyses). These specimens were distributed in six *a priori* groups defined by their species identification and geographical origin, namely *S. minutus* from off the Mediterranean coast of Spain (n=12), *S. papernai* from off the Black Sea coast of Bulgaria (n=7), *S. papernai* from off the Mediterranean coast of Spain (n=19), *S. dimitrovi* from off the Black Sea coast of Bulgaria (n=20) and *S. dimitrovi* from off the Mediterranean coast of Spain (n=6), plus the type-material of *S. papernai* (n=5) from the Eastern Mediterranean.

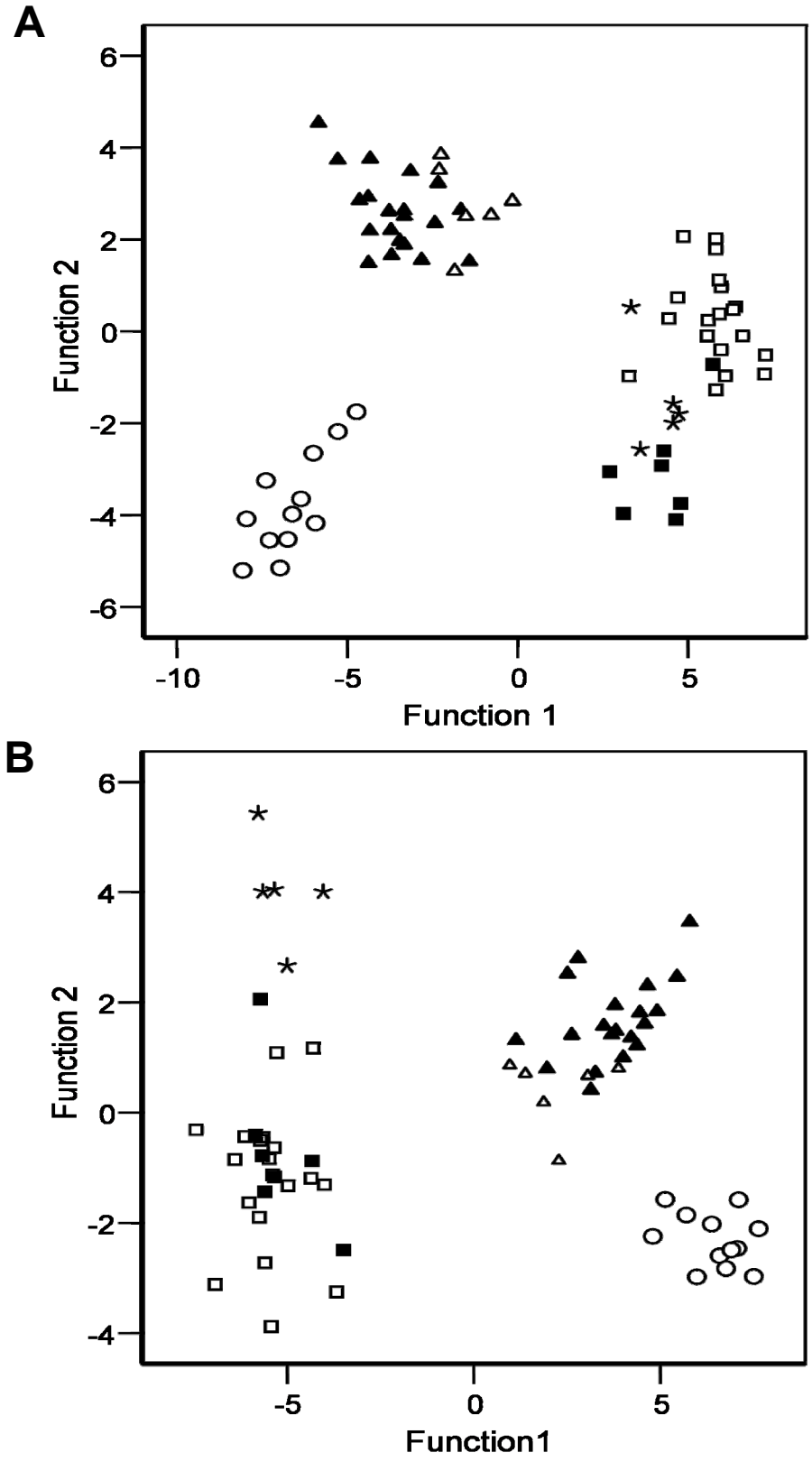

Fig. 4.11. Plot of the 69 specimens of *Saturnius* spp. against the first and second discriminant functions resulting from stepwise LDAs with ln-transformed data (**A**), and size-adjusted data (**B**). Legend: ○ *S. minutus* from Spain, □ *S. papernai* from Spain, ■ *S. papernai* from Bulgaria, ⁎ *S. papernai* type-material, Δ *S. dimitrovi* from Spain, ▲ *S. dimitrovi* from Bulgaria.

In the first analysis (Fig. 4.11A), ln-transformed metrical data were subjected to a stepwise LDA to select those variables yielding optimal separation between the groups. Since the three *Saturnius* spp. differed substantially in body size, a second analysis (Fig. 4.11B) was carried out in order to test whether discrimination between the groups still occurred after controlling for this factor. A stepwise LDA was run using a size-adjusting technique consisting in substitution of the original metric variables by the ratios formed by dividing each variable by the geometric mean of all variables (Darroch & Mosimann, 1985). It has been shown that this approach satisfactorily minimises the effect of body size in multivariate analyses (Jungers *et al.*, 1995).

Both the conventional LDA with ln-transformed data (Analysis A) and its size-adjusted counterpart (Analysis B) were able to correctly allocate all specimens to their species designations based on morphology (i.e. 100% successful classification rate, see Figure 6 for a plot of the specimens against the first two discriminant functions). Furthermore, both models generated by the stepwise procedures selected the same set of six variables for optimal separation between samples: the length of the forebody, ventral sucker and posterior testis, the length and width of the posteriormost pseudosegment, and the width of the muscular flange at ventral sucker. Almost all specimens were also assigned to the correct population (locality) with the exception of three (Analysis A, misclassification rate 4.4%) and 11 specimens (Analysis B, misclassification rate 15.9%) (see Table 4.8 for details).

Finally, a PCA was performed -to a set of 105 specimens belonging to the three Mediterranean species of *Saturnius* after adding the ‘small’ and ‘typical’ forms of *S. papernai* from *L. aurata* to the set of specimens examined above. The set therefore comprised *S. minutus* (n=12); *S. dimitrovi* (n=26); *S. papernai* ex *M. cephalus* (n=31); and *S. papernai* ex *L. aurata* (n=36). This analysis based on 28 metrical variables aimed to test whether (i) the metrical variability observed in the material from *L. aurata* confirms its identification as *S. papernai* and (ii) the entire set of specimens of *S. papernai* can still be distinguished from *S. minutus* and *S. dimitrovi*.

The first two principal components explained more than 70% of the variation in the data set. A plot of the specimens in the first plane of the PCA (Fig. 4.12) shows three well-separated groups that correspond to the original species designations. There was a higher dispersion in the group representing *S. papernai* along the first principal component, perhaps due to the noticeably increased number of the specimens. Still, the plot indicates the absence of a hiatus in the two-dimensional plane between the material originating from *L. aurata* (filled squares) and *M. cephalus* (open squares and stars).

Table 4.8. Classification results of two stepwise LDAs with (A) ln-transformed data and (B) size-adjusted data applied to six samples of 69 specimens of *Saturnius* defined by species and geographical origin.

Analysis		Actual group membership						
		S. minutus (S)	*S. papernai* (S)	*S. papernai* (B)	*S. dimitrovi* (B)	*S. dimitrovi* (S)	*S. papernai* (T)	
A	*S. minutus* (S)	12	0	0	0	0	0	12
	S. papernai (S)	0	19	0	0	0	0	19
	S. papernai (B)	0	1	6	0	0	0	7
	S. dimitrovi (B)	0	0	0	18	2	0	20
	S. dimitrovi (S)	0	0	0	0	6	0	6
	S. papernai (T)	0	0	0	0	0	5	5
B	*S. minutus* (S)	12	0	0	0	0	0	12
	S. papernai (S)	0	14	5	0	0	0	19
	S. papernai (B)	0	3	4	0	0	0	7
	S. dimitrovi (B)	0	0	0	18	2	0	20
	S. dimitrovi (S)	0	0	0	1	5	0	6
	S. papernai (T)	0	0	0	0	0	5	5

S, Spanish Mediterranean coast; B, Bulgarian Black Sea coast; T, type-material of *S. papernai*.

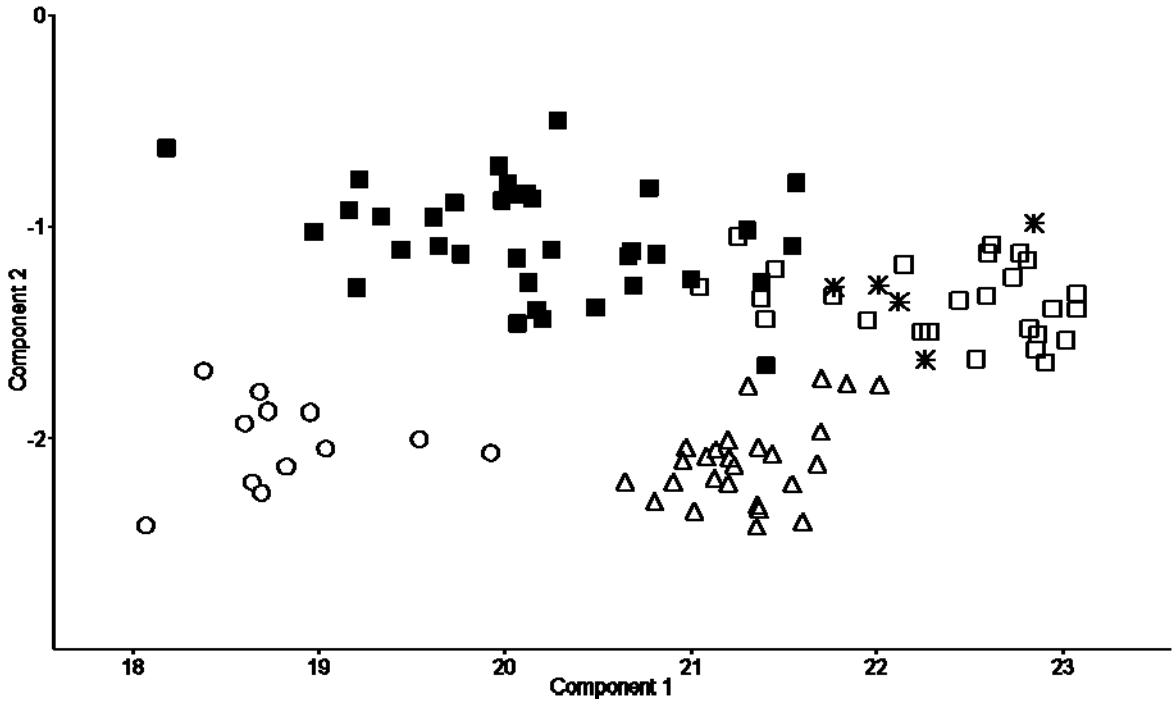

Fig. 4.12. Plot of the 105 specimens of *Saturnius* spp., including 'small' and 'typical' forms of *S. papernai* from *Liza aurata* in the first plane of the PCA. *Legend*: ○ *S. minutus*, Δ *S. dimitrovi*, □ *S. papernai* ex *M. cephalus*, ■ *S. papernai* ex *L. aurata*, ∗ *S. papernai* (type-material ex *M. cephalus*).

4.5. Concluding remark

In his revision of *Saturnius,* Overstreet (1977) suggested that "If this report stimulates examination of mullet from different localities, some encountered worms probably will be new species and different forms". As shown in section 4.1. above, the large body of records from all mullet species in the Mediterranean were related mainly with *S. papernai*. However, most of them are non-documented. It is therefore not surprising that this first large-scale morphological study on bunocotyline mullet parasites, has increased the diversity of the genus by adding three new species (see Blasco-Costa *et al.* 2006, 2008).

In concert, univariate and multivariate analyses have demonstrated that the Mediterranean species, *S. papernai, S. dimitrovi* and *S. minutus*, are morphometrically distinguishable. These results also verified that using the methods of LDA classification, the discrimination of the three closely related and possibly sympatric (*S. minutus* has not yet been recovered from Black Sea mullet populations) species of *Saturnius* may be achieved using a limited number of metrical variables. In particular, by applying the size-adjusted LDA, the large difference in size between the three forms is controlled for and, thus, the differentiation observed should be mostly attributable to shape differences between the species (Junger *et al.*, 1995). The LDAs were also efficient at assigning the specimens of each species to the correct population (locality). Notably, the present analyses suggest that interpopulational distances are smaller than interspecific, thus reinforcing the notion of three distinct morphometric forms of *Saturnius* spp. (as shown in Fig. 4.11 and Table 4.8). Although the intraspecific differences might point to the existence of geographical morphometric variants (some of which might be clonal siblings), they might well reflect pseudoreplication derived from samples coming from related hosts or fixed/processed differently (*e.g*. in the case of the type-material of *S. papernai* and *S. dimitrovi* collected by Dimitrov *et al.*, 1998). Future efforts, focusing on a detailed morphological study of large sets of specimens from *Liza* spp. and *M. soiuy*, may help testing the prediction of Overstreet (1977), thus revealing the actual diversity of *Saturnius* in the Mediterranean.

One important finding of the present study is the presence of a cyclocoel in all three species described. It should be stressed that the most posterior region of the intestine was very difficult to observe in adult specimens due to its dorsal location; it was obscured by the ovary, vitellarium and uterus which typically overlap it ventrally. Observation was most complicated in the specimens of *S. papernai* due to the large number of eggs (75-602), a significant

proportion of which was located in the last body pseudosegment. Examination of the type-material of *S. papernai* revealed a similar situation, where it was impossible to clearly observe blind caeca. However, caeca can be seen to unite posterior to the vitellarium to form a cyclocoel in juvenile specimens of *S. papernai* and *S. minutus*, as well as in the holotype of *S. dimitrovi* (which was mounted dorsally and also had fewer eggs in the posteriormost pseudosegment). The presence (in *Bunocotyle* Odhner, 1928) and absence (in *Saturnius*) of cyclocoel has so far been used as an important feature in distinguishing the two genera within the Bunocotylinae (e.g. Gibson, 2002). Although at present it appears that the presence of cyclocoel is a common feature for most *Saturnius* spp., it was not reinforced in the generic diagnosis due to difficulties in observation.

4.6. Key to the recognised species of *Saturnius*

1a. Muscular papillae surrounding oral sucker present..... *S. segmentatus*
1b. Muscular papillae surrounding oral sucker absent 2

2a. Muscular flange at level of ventral sucker prominent, nipple-shaped 3
2b. Flange at ventral sucker level not well developed, mound-shaped 5

3a. Anteriormost transverse fibrous septum at level of genital pore always present, strongly developed. Second septum at level of ventral sucker present, thick .... *S. dimitrovi*
3b. Anteriormost transverse fibrous septum at level of genital pore absent or very faint. Second septum at level of ventral sucker absent 4

4a. Body length < 550 μm. Pars prostatica distinct, as large as sinus-sac, enters sinus-sac at its base. Seminal vesicle small, comparable in size to sinus-sac. Last pseudosegment > 1/3 of body length (LSL/BL= 17-29%) ..... *S. minutus*
4b. Body length > 700 μm. Pars prostatica relatively small, *c.* 1/2 of sinus-sac, enters sinus-sac at about its mid-level. Seminal vesicle *c.* twice as large as sinus-sac. Last pseudosegment > 1/3 of body length (LSL/BL= 33%) ..... *S. mugilis*

5a. Body with 7 pseudosegments separated by 6 transverse fibrous septa. Second septum at level of ventral sucker present, thick 6

5b. Body with 5-6 pseudosegments separated by 4-5 transverse fibrous septa. Second septum at level of ventral sucker absent 7

6a. Oral and ventral suckers spherical; sucker length ratio 1:1.6-1.9, width ratio 1:1.5-1.8. Pars prostatica small, *c*.1/2 of sinus-sac. Seminal vesicle wide-tubular, much longer than sinus-sac ..... *S. maurepasi*

6b. Oral sucker cup-shaped with flared anterior margins; ventral sucker transverse-oval; sucker length ratio 1:0.7-1.5, width ratio 1:0.7-1.3. Pars prostatica similar to or larger than sinus-sac. Seminal vesicle saccular, as large as sinus-sac ..... *S. papernai*

7a. Body length < 650 μm, with 5 pseudosegments. Ventral sucker flange long in relation to ventral sucker length (MFL/VSL = 78%). Muscular flange close to posterior extremity absent. Seminal vesicle saccular. Pars prostatica enters sinus-sac at its base ..... *S. belizensis*

7b. Body length > 900 μm, with 6 pseudosegments. Ventral sucker flange short in relation to ventral sucker length (MFL/VSL = 47-53%). Muscular flange close to posterior extremity present, strongly muscular. Seminal vesicle wide-tubular. Pars prostatica enters sinus-sac near its mid-level ..... *S. overstreeti*

CHAPTER 5

TAXONOMIC REVISION OF THE SUBFAMILY HAPLOPORINAE

5.1. Genus *Haploporus* Looss, 1902

Syn. *Neohaploporus* Manter, 1963

5.1.1. Background

Haploporus, the type-genus of the family Haploporidae, comprised originally two species: *H. benedeni* (Stossich, 1887) (type-species) and *H. lateralis* Looss, 1902. Further studies described and assigned eight nominal species to this genus: *H. longicollum* Vlassenko, 1931; *H. indicus* Rekharani & Madhavi, 1985; *H. pseudoindicus* Rekharani & Madhavi, 1985; *H. lossii* Al-Bassel, 1990; *H. magnisaccus* Machida, 1996; *H. spinosus* Machida, 1996; *H. mugilis* Liu & Yang, 2002 and *H. musculosaccus* Machida, 2003 (see Vlassenko, 1931; Rekharani & Madhavi, 1985; Al-Bassel, 1990; Machida, 1996; Liu & Yang, 2002; Machida, 2003). Dawes (1947) considered *H. lateralis* Looss, 1902 a synonym of *H. benedeni* Looss, 1902. Skrjabin (1956) erected *Wlassenkotrema* Skrjabin, 1956 for *H. longicollum*. Yamaguti (1958, 1971) treated *Wlassenkotrema* as a junior synonym of *Haploporus*, whereas Overstreet & Curran (2005) considered *Wlassenkotrema longicollum* a synonym of *Saccocoelium obesum* Looss, 1902 and listed *Wlassenkotrema* as a synonym of *Saccocoelium* in their recent revision of the Haploporidae. These authors also transferred *Neohaploporus pacificus* Manter, 1963 to *Haploporus* regarding *Neohaploporus* a synonym of the latter.

5.1.2. Generic diagnosis

Body elongate-oval, with maximum width at level of testis. Tegument armed. Eye-spot pigment dispersed between pharynx and anterior border of oral sucker. Oral sucker subterminal, spherical. Ventral sucker spherical, smaller to equal in size to oral sucker, in middle third of body. Forebody about third of body length. Prepharynx relatively short. Pharynx subglobular. Oesophagus about twice length of pharynx. Intestinal bifurcation dorsal to ventral sucker. Caeca two, relatively narrow, end blindly at mid-body. Testis single, subspherical, sinistral to median, adjacent or just posterior to ventral sucker (distance from posterior margin of ventral sucker less than half length of ventral sucker). External seminal vesicle contiguous with hermaphroditic sac, saccular, subglobular, similar in size to internal seminal vesicle. Hermaphroditic sac subglobular to slightly elongate-oval, antero-dorsal to and not extending posterior to ventral sucker, with length up to twice that of ventral sucker. Internal seminal vesicle thin-walled, subglobular saccular, occupies more than half of hermaphroditic sac. Pars prostatica indistinct, tubular; prostatic cells large. Hermaphroditic

duct unarmed, faintly muscular, less than third length of hermaphroditic sac. Genital atrium apparently absent. Genital pore median, between pharynx and ventral sucker. Ovary median to sinistral, spherical, dorsal to or just posterior to ventral sucker, anterior and adjacent to or overlapping testis dorsally. Uterine seminal receptacle present; blind seminal receptacle absent. Uterus extensive, occupies almost entire hindbody. Metraterm indistinct, short. Eggs numerous, operculate; developed miracidia with two fused eye-spots. Vitellarium two symmetrical, separated, compact, smooth or slightly irregular masses, smaller than pharynx, at level of ovary. Excretory vesicle Y-shaped, pore terminal. In mullets (Mugilidae). Type-species: *H. benedeni* Looss, 1902.

5.1.3. Review of species

***Haploporus benedeni* (Stossich, 1887) Looss, 1902**

Syns (?)*Distomum viviparum* van Beneden, 1870; *Distomum benedeni* Stossich, 1887; *Haploporus lateralis* Looss, 1902

Material studied

Ex *Liza ramado* (Risso). Intestine. Off Santa Pola, Spain (24.viii.2007). BMNH 2008.10.27.52-55.

Ex *Chelon labrosus*. Intestine. West Thurrock, UK, Collected by I. Galder (13.ii. 1985). BMNH 1986.5.20.160-163.

Records

References[*]: 1. (?) van Beneden (1870, as *Distomum viviparum*); 2. Stossich (1887, as *Distomum benedeni*); 3. Looss (1902, also as *Haploporus lateralis*); 4. Nicoll (1914); 5. Dawes (1947); 6. Ergens (1960, as *H. lateralis*); 7. Paperna (1964, also as *H. lateralis*); 8. Fares & Maillard (1974); 9. Orecchia & Paggi (1978); 10. Paggi *et al.* (1979); 11. Solonchenko & Tkachuk (1985, as *H. lateralis*); 12. Orecchia *et al.* (1988); 13. Paggi *et al.* (1988); 14. Radujković & Raibaut (1989); 15. Radujković *et al.* (1989); 16. D'Amelio *et al.* (1995); 17. Di Cave *et al.* (1997); 18. Merella & Garippa (1998); 19. Domnich & Sarabeev (2000b, as *H. lateralis*); 20. Domnich & Sarabeev (2000a, as *H. lateralis*); 21. Domnich & Sarabeev (2000c, as *H. lateralis*); 22. Maltsev & Zhdamirov (2000, as *H. lateralis*); 23. Sarabeev (2000, as *H. lateralis*); 24. Sarabeev & Domnich (2000, as *H. lateralis*); 25.

[*] A series of ultrastructure studies on a trematode identified as *H. benedenii* ex *C. labrosus* (see Sampour & Mas-Coma, 2004; Sampour, 2005, 2006) and *H. lateralis* ex *L. aurata* (see Sampour, 2008) lack locality data and supportive evidence for the identification of the parasites; these are therefore not included in the list of records.

Dmitrieva & Gaevskaya (2001, as *H. lateralis*); 26. Merella & Garippa (2001); 27. Nizova *et al.* (2001, as *H. lateralis*); 28. Al-Bassel (2003, also as *H. lateralis*); 29. Dzikowski *et al.* (2003, also as *H. lateralis*); 30. Öztürk & Aydogdu (2003); 31. Overstreet & Curran (2005); 32. Ragias *et al.* (2005); 33. Present study.

Descriptions: 1? (incomplete figure only); 2; 3; 6; 8; 15; 31(figure only); 33.

Definitive hosts: *Chelon labrosus* (Risso) (1?, 2, 3, 4, 5, 8, 10, 12, 15, 26, 32, 33) (type-host); *Mugil cephalus* L. (3?, 7, 8, 9, 10, 11, 13, 18, 30); *L. aurata* (Risso) (3, 7, 8, 11, 13, 20, 25, 29, 32); *L. saliens* (Risso) (7, 8, 11, 26, 31); *L. ramado* (Risso) (6, 7, 8, 10, 12, 13, 14, 15, 16, 17, 26, 28, 29, 32, 33); *Mugil soiuy* Basilewsky (19, 20, 21, 22, 23, 24, 25, 27).

Distribution: Area 37, subarea 2 (Central Mediterranean) (type-locality: off Trieste, Adriatic Sea) (2, 3, 6, 12, 14, 15, 16); area 37, subarea 1 (Western Mediterranean) (1?, 8, 9, 10,18, 26, 31, 33) (13?,17?Italy); area 37, subarea 3 (Eastern Mediterranean) (7, 16, 28 , 29, 32); area 37, subarea 4 (Black Sea): 4.2. Black Sea (25, 27, 30), 4.3. Azov Sea (11, 19, 20, 21, 22, 23, 24, 25); area 27, (Northeast Atlantic) (4, 5, 33).

Description (Fig. 5.1; Table 5.1)

[Based on 6 whole-mounted adult specimens.] Body elongate-oval, with maximum width at mid-hindbody, at level of testis; width 32-44% of body length. Tegument thick, armed with large sharp spines (up to 11). Eye-spot pigment abundant, dispersed between pharynx and anterior border of oral sucker. Oral sucker spherical, with subterminal aperture. Ventral sucker spherical, similar in size to oral sucker to slightly smaller (sucker length ratio 1:0.88-1.21; width ratio 1:0.83-1.01), in second third of body. Forebody 30-37% of body length.

Prepharynx very short to almost similar in length to pharynx (PL/PHL=0.05-0.86); pharynx spherical, large in relation to oral sucker. Oesophagus about twice length of pharynx; intestinal bifurcation dorsal to ventral sucker; caeca 2, relatively narrow, end blindly at about mid-body, masked by uterus filled with eggs.

Testis single, sinistral to median, sub-spherical, smooth, adjacent or just posterior to ventral sucker (distance from posterior margin of ventral sucker less than half length of ventral sucker); post-testicular space 36-44% of body length. External seminal vesicle contiguous with hermaphroditic sac, saccular, subglobular, similar in size to internal seminal vesicle. Hermaphroditic sac mostly in forebody, subglobular to slightly elongate-oval, antero-dorsal to and not extending posterior to ventral sucker, similar in length up to twice length of ventral sucker (HSL/VSL=104-183%), contains internal seminal vesicle, many large prostatic cells, and indistinct metraterm and hermaphroditic duct. Internal seminal vesicle thin-walled,

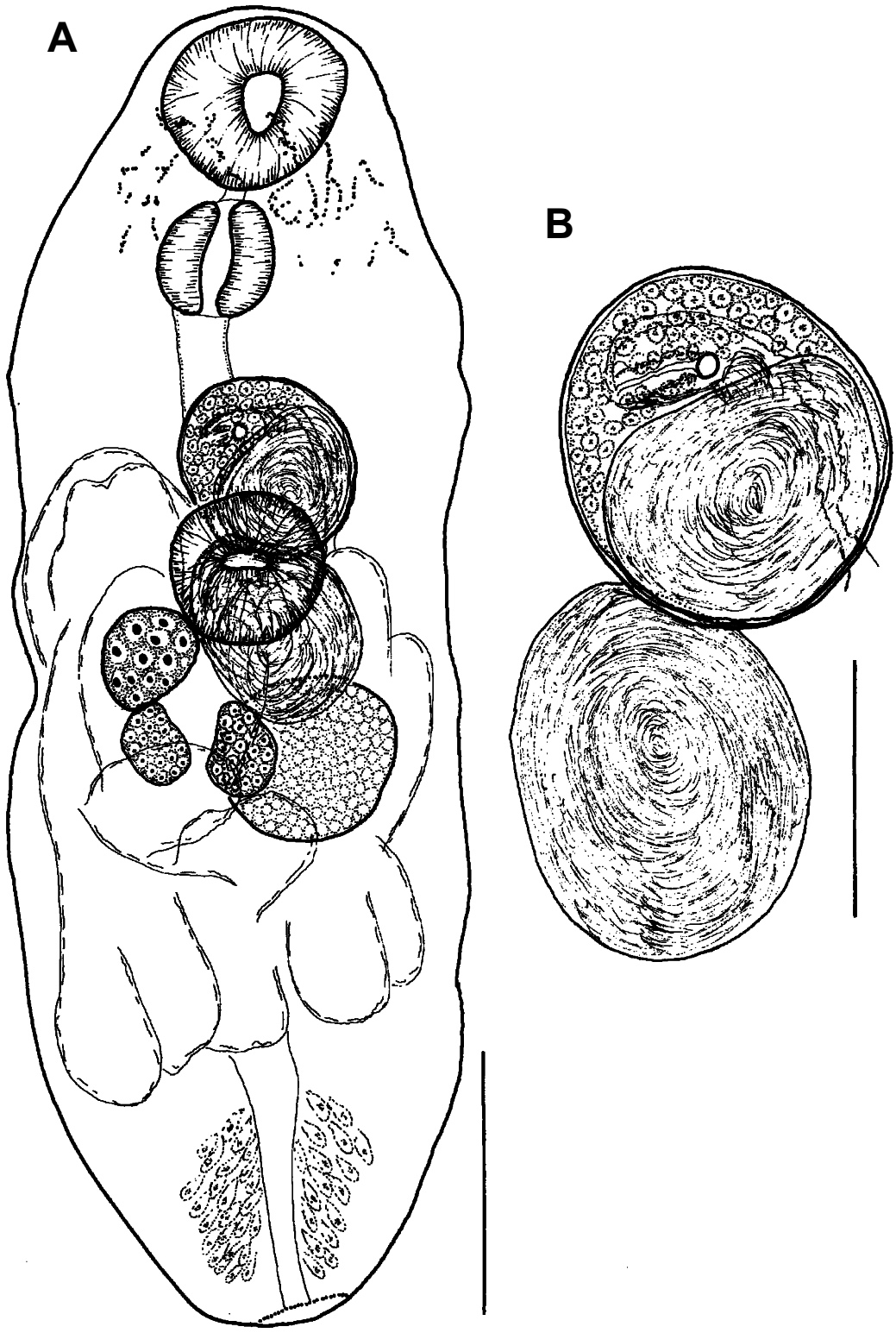

Fig. 5.1. *Haploporus benedeni* ex *Liza ramado*. **A.** Ventral view with uterus in outline. **B.** Terminal genitalia. *Scale-bars*: **A**, 200 μm; **B**, 100 μm.

subglobular saccular, occupies more than half of hermaphroditic sac. Hermaphroditic duct unarmed, faintly-muscular, thin-walled, length less than third length of hermaphroditic sac. Genital atrium apparently absent. Genital pore wide, round, median, between pharynx and ventral sucker.

Ovary spherical, median to sinistral, dorsal to or just posterior to ventral sucker, anterior and adjacent to or overlapping testis dorsally. Uterine seminal receptacle present; blind seminal receptacle absent. Laurer's canal and Mehlis' gland not observed. Uterus thin-walled, extensive, occupies almost entire hindbody. Metraterm indistinct, short, joins hermaphroditic duct close to seminal vesicle. Eggs numerous, operculate; developed miracidia with 2 fused eye-spots. Vitellarium 2 symmetrical, separated, smooth or slightly irregular, compact masses of small coalesced follicles, at level of ovary.

Stem of excretory vesicle wide tubular, surrounded by strongly stained large cells; bifurcation and excretory arms obscured by uterus; pore terminal, wide.

Remarks

Stossich (1887) briefly described *Distomum benedeni* Stossich, 1887 which he believed to be identical with *Distomum viviparum* van Beneden, 1870 for which van Beneden (1870) provided only an incomplete drawing. Looss (1902) described briefly (including metrical data limited to the size of the body, suckers, pharynx and eggs) the three type-specimens collected by Stossich (1887) and erected *Haploporus* Looss, 1902 to accommodate *H. benedeni* (Stossich, 1887) and *H. lateralis* Looss, 1902, which he described from *L. aurata* and *C. labrosus* in the Adriatic Sea. Looss (1902) did not provide differential diagnoses for the two *Haploporus* spp. but indicated that in *H. benedeni*: (i) the oral sucker is larger than the ventral sucker (*vs* slightly but notably smaller in *H. lateralis*); (ii) the hermaphroditic sac ('pseudocirrus sac') is smaller than ventral sucker and does not reach its posterior margin (*vs* larger than the ventral sucker and reaching close to its posterior margin in *H. lateralis*); and (iii) the eggs are somewhat larger and contain miracidia with X-shaped eye-spots (*vs* miracidia with no eye-spots in *H. lateralis*).

However, both descriptions of Looss (1902) were based on few specimens (apparently a single specimen in the case of the metrical data for *H. lateralis*) and, as he stated, the specimens described as *H. benedeni* were contracted, which might explain the more rounded posterior extremity and the ventrally curved forebody. Furthermore, the oral sucker appears smaller than the ventral in the associated drawings (see figs 5-8 in Looss, 1902) of the

Table 5.1. Comparative metrical data for *Haploporus*.

Source	Present study		Present study	Looss (1902)	Looss (1902) (as *H. lateralis*)	Ergens (1960) (as *H. lateralis*)	Fares & Maillard (1974)		Radujković et al. (1989)
Host	*L. ramado*		*C. labrosus*	*C. labrosus*	*C. labrosus, L. aurata*	*L. ramado*	*M. cephalus*, *L. aurata*, *L. ramado*, *L. saliens*, *C. labrosus*		*C. labrosus, L. ramado*
	Range	Mean	Range	Range	Range	Range	Range	Mean	Range
Measurements									
BL	1,007-1,298	1,157	1,045	1,250	800-950	1,260-1,494	640-2,090	1,380	1,330-2,200
BW	344-516	415	456	600	380	450-486	230-650	460	500-620
OSL	94-145	124	167	-	-	108-126	90-220	-	170-210
OSW	119-147	135	152	250	112	162	90-220	150	160-180
PL	5-81	35	8	-	-	-	-	40	90-120
PHL	83-102	94	80	120	68	72-90	50-110	90	80-120
PHW	82-122	101	72	120	68	72-81	-	-	90-110
OL	178	-	-	-	-	126-198	-	-	-
VSL	114-132	125	148	-	-	108	80-190	-	140-170
VSW	111-137	128	148	190	120	126	80-190	130	130-160
HSL	119-241	161	160	-	-	-	100-270	180	180-220
HSW	86-140	123	133	-	-	-	-	-	150-180
ISVL	78-178	123	137	-	-	-	-	-	-
ISVW	53-119	90	80	-	-	-	-	-	-
ESVL	109-152	133	95	-	-	-	-	60	-
ESVW	53-116	98	53	-	-	-	-	-	-
TL	124-231	173	163	-	-	162-234	100-340	240	220-230
TW	132-224	167	144	-	-	126-216	-	-	130-160
OVL	56-102	76	80	-	-	144	70-230	160	100-200
OVW	68-99	84	72	-	-	72-90	-	-	80-110
VL	43-70	61	97	-	-	72-81	-	-	-
VW	46-59	51	86	-	-	54-72	-	-	-
EL	38-43	40	40-43 (mean 41)	45-53	42-45	43-50	40-70	55	35-40
EW	24-27	26	23-25 (mean 24)	30-34	23-26	23-28	20-35	27	20-22

Table.5.1. Continued .

Source	Present study		Present study	Looss (1902)	Looss (1902) (as *H. lateralis*)	Ergens (1960) (as *H. lateralis*)	Fares & Maillard (1974)		Radujković et al. (1989)
Host	*L. ramado*		*C. labrosus*	*C. labrosus*	*C. labrosus, L. aurata*	*L. ramado*	*M. cephalus*, *L. aurata*, *L. ramado*, *L. saliens*, *C. labrosus*		*C. labrosus, L. ramado*
	Range	Mean	Range	Range	Range	Range	Range	Mean	Range
Distances									
FO	320-447	386	323	-	-	-	-	-	-
CEND	c. 452	-	-	-	-	-	-	-	-
TEND	367-508	470	418	-	-	-	-	-	-
UEND	180-292	224	61	-	-	-	-	-	-
Ratios									
BW/BL (%)	32-40	36	44	28-39*	41*	-	39*	-	29*
FO/BL (%)	30-37	33	31	29-33*	28-33*	-	28*	-	29*
OSL/VSL	1:0.88-1.21	1:1.02	1:0.89	-	-	-	1:0.86-0.88	0.87	1:1.00*
OSW/VSW	1:0.83-1.01	1:0.95	1:0.97	1:0.86-0.93*	1:1.08*	-	-	-	1:0.78*
HSL/VSL (%)	104-183	128	108	136-146*	127-158*	-	150*	-	200*
CEND/BL (%)	-	-	-	44*	48-49*	-	50*	-	55*
TEND/BL (%)	36-44	41	40	27-31*	44-46*	-	44*	-	49*
UEND/BL (%)	16-24	19	6	5-12*	19*	-	24*	-	40*

* Estimated from the published drawing.

specimens of both species (sucker width ratio 1:0.86-0.93 and 1:1.08, respectively), and the hermaphroditic sac is larger than ventral sucker (HSL/VSL=136-146% and 127-158%, respectively) and does not reach its posterior margin in both species (figs 5-8). Finally, whereas the small difference in egg-size may be due to the paucity of specimens studied by Looss (1902), the absence of eye-spots in the miracidia clearly reflects the degree of their development (Fares & Maillard, 1974; Overstreet & Curran, 2005); the smaller size of the specimen of *H. lateralis* described by him supports this notion (see Table 5.1). These data support the opinion of Dawes (1947), who considered the two species synonymous; a possible synonymy has also been suggested by Fares & Maillard (1974), who also provided a detailed description of *H. benedeni* and its life-cycle in the western Mediterranean.

Despite the high number of records of both *H. benedeni* and *H. lateralis* in most mugilid species from the Mediterranean basin, there are only three studies which provide descriptions of the material (Ergens, 1960; Fares & Maillard, 1974; Radujković *et al.*, 1989). The present data, therefore, expand the range of variation of the metrical characters of *H. benedeni* (Table 5.1). The data of Ergens (1960) and Radujković *et al.* (1989) show an overall agreement with the original description of *H. benedeni* and/or the present study. However, it appears that Fares & Maillard (1974) incorporated metrical data from juvenile specimens in their description of *H. benedeni* based on material from the western Mediterranean. This may explain why the lower limits for the size of the body (and all organs) provided by these authors fall well outside the minimum values previously reported for either species (see Table 5.1 for details). Fares & Maillard (1974) obtained experimentally juvenile worms 10 days post-infection (dpi) which measured 630 × 350 μm (this size is very close to the minima provided in their description, *i.e.* 640 × 230) μm; at 24 dpi they observed first small eggs in worms measuring 950 × 350 μm. Fares & Maillard (1974) recovered at 30 dpi adult worms (measuring 1,130 × 350 μm) with eggs, but still not containing fully-developed miracidia. Therefore, the lower limits for the measurements provided by Fares & Maillard (1974) should be excluded in further comparisons based on metrical data. Another departure of the description of the material of Fares & Maillard (1974) from both the original description and those by Ergens (1960) and Radujković *et al.* (1989), and the present study, is the statement that the hermaphroditic duct ‘est longée latéralement par deux rangées d’épines en croissant’. The present redescription, on the contrary, suggest that the hermaphroditic duct is unarmed; this character along with the lack of a muscular genital atrium clearly discriminates between *Haploporus* and *Saccocoelium* (see also section 5.5, this chapter; Blasco-

Costa *et al.*, 2009c). Other distinguishing features of *Haploporus* resulting from the present comparative study are summarised in the generic diagnosis (above).

Other nominal species

***Haploporus lossii* Al-Bassel, 1990 nom. nud.**

Record, Reference: Al-Bassel (2003).
Definitive host: *Liza ramado* (Risso).
Distribution: Area 37, subarea 3 (Eastern Mediterranean) (Egypt).

Remark
This species, presumably described in a PhD thesis and only reported as a record by the same author, is a *nomen nudum*.

***Haploporus pacificus* (Manter, 1963) Overstreet & Curran, 2005 sp. inq.**
Syn. *Neohaploporus pacificus* Manter, 1963

Record
References: 1. Manter (1963); 2. Overstreet & Curran (2005).
Descriptions: 1, 2 (figure of the holotype only).
Definitive host: '*Scatophagus argus* (Bloch) (?)' (type-host).
Distribution: Area 71: Western Central Pacific (type-locality: Fiji).

Remarks
Manter (1963) erected *Neohaploporus* Manter, 1963 to accommodate *Haploporus pacificus* Manter, 1963 from a marine scatophagid off Fiji. He distinguished the new genus from the haploporid genera possessing paired vitelline masses, *i.e. Haploporus*, *Saccocoelium* and *Wlassenkotrema* Skrjabin, 1956, in possessing lymphatic vessels in forebody, a feature which Manter (1963) listed as present in several genera of the Haploporidae but not in genera with a reduced vitellarium, such as *Haploporus* and *Saccocoelium*. Overstreet & Curran (2005) interpreted the structures described as lymphatic vessels as gland-cells with associated ducts leading to the oral sucker (also observed by these authors at the posterior extremity of the holotype). They considered the latter (irrespective of interpretation) as a feature of specific

significance only and transferred *N. pacificus* to *Haploporus,* making *Neohaploporus* a junior synonym of *Haploporus*. Although this action is tentatively accepted here, it should be noted that *H. pacificus* exhibits differences from the type-species from Mediterranean mullets whose significance at the generic level needs to be further explored: (i) long prepharynx and oesophagus which bifurcates posterior to the ventral sucker; (ii) long caeca terminating posterior to the mid-hindbody; (iii) testis located at a greater distance from the ventral sucker; (iv) hermaphroditic sac located mostly in the forebody; and (v) a distinct, long ?pars prostatica, described by Manter (1963) as 'a narrow, sinuous tube leading backward from anterior end of seminal vesicle to join metraterm near middle of hermaphroditic sac' [this structure appears to be tubular and even longer in the figure of the holotype given by Overstreet & Curran (2005)]. Furthermore, the differences in the structure and the shape of the vitellarium between the original description [vitelline masses defined as 'non-follicular, one on each side of ovary, each a shortly-branched or bulbed tube' in the generic diagnosis of *Neohaploporus* given by Manter (1963)] and the illustration of the holotype presented by Overstreet & Curran (2005) require additional material to be studied in order to fully assess the morphological variability of *H. pacificus,* especially with regard to the fact that this is the only species assigned to *Haploporus* which infects a non-mugilid fish [although the questionable identification of the type-host (see Manter, 1963) may cause additional problems].

***Haploporus pseudoindicus* Rekharani & Madhavi, 1985 sp. inq.**

Material studied

Type-material: Ex *Liza macrolepis* (Smith, 1846). Intestine. Off Visakhapatnam, Waltair coast, India. BMNH 1984.6.28.19 (holotype).

Record

Reference, Description: Rekharani & Madhavi (1985).

Definitive host: *Liza macrolepis* (Smith, 1846) (type-host).

Distribution: Area 57: Eastern Indian Ocean (type-locality: Off Visakhapatnam (brackish waters), Bay of Bengal, India).

Additional morphological data (Fig. 5.2)

Hermaphroditic sac large, elongate-oval, somewhat displaced by fixation so it appears anterior to genital pore, with thin walls. Internal seminal vesicle elongate-oval, occupies about third of hermaphroditic sac, connects to hermaphroditic duct via large vesicular pars prostatica; prostatic cells large, in two distinct groups on both sides of pars prostatica. Hermaphroditic duct wide, about half length of sac, thin-walled, armed with minute spines. Genital atrium distinct; anterior half narrow, thin-walled; posterior half strongly muscular, armed with circle of spines (*c*.20) at base. Metraterm thick-walled, glandular, with length about third length of hermaphroditic sac.

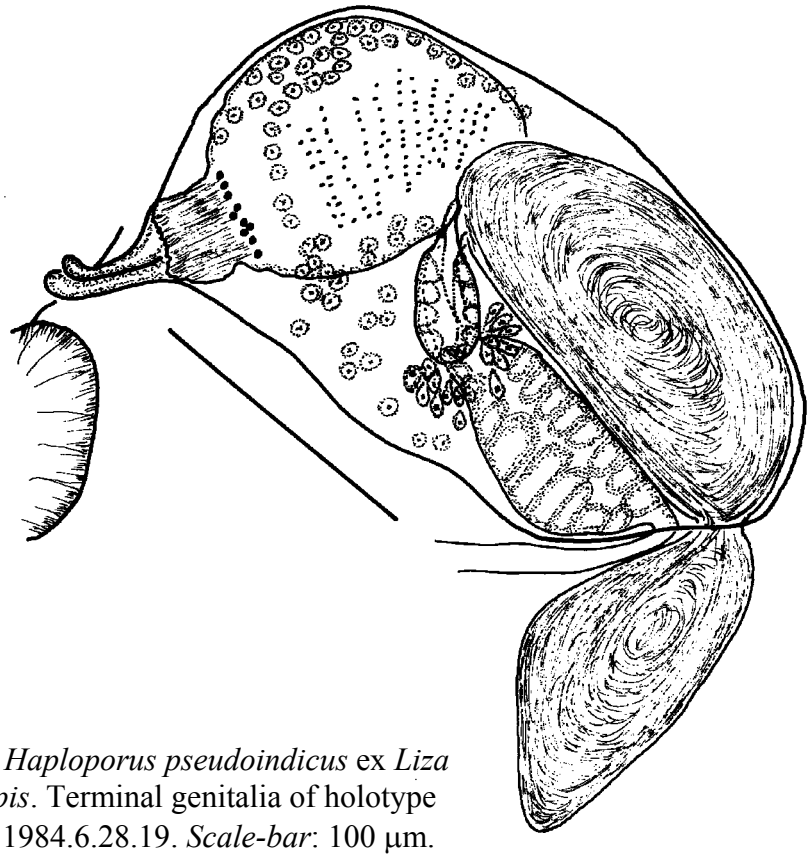

Fig. 5.2. *Haploporus pseudoindicus* ex *Liza macrolepis*. Terminal genitalia of holotype (BMNH 1984.6.28.19. *Scale-bar*: 100 μm.

Remarks

H. pseudoindicus was described on the basis of a single specimen with malformed eggs. Rekharani & Madhavi (1985) described the pars prostatica as short; it appears to be illustrated as anterior to the seminal vesicle in their figure 3. However, a vesicular pars prostatica was distinguished in the course of reexamination of the type-material, with two groups of

associated cells just anterior to the metraterm (illustrated originally as an enlargement of the metraterm, see their fig. 3). The latter was described as thick-walled; a lining of large cells was observed. Finally, the presence of fine spination of the hermaphroditic duct and distal part of the muscular region of the genital atrium (illustrated as sinuous in their fig. 3) was observed in the type. The different structure of the terminal genitalia revealed by the re-examination of the holotype indicates a possible close relationship of *H. pseudoindicus* with the species complex described from *Valamugil* spp. in the Indo-West Pacific (see below) and with *H. mugilis* Liu & Yang, 2002 in particular (Liu & Yang, 2002**)**. However, the state of the Indian specimen does not permit a decision to be made. Additional material is needed to establish the status of this form.

Species in *Valamugil* spp. from the Indo-West Pacific region

The brief generic diagnoses given by Looss (1902) for the haploporine genera erected based on Mediterranean material appear to have been reflected in subsequent misleading identifications and generic assignations. Thus Overstreet & Curran (2005) noted that the placement of a few species of *Haploporus* and *Saccocoelium* is difficult. These authors stated: 'in addition to two sympatric species reported by Looss (1902b), there is one new combination indicated below and three new Indo-West Pacific species described from mugilids by Rekharani & Madhavi (1985) and Machida (1996)'. Although two new species were described in the latter papers each (*i.e.* four Indo-West Pacific forms), the species considered as members of *Haploporus* by Overstreet & Curran (2005) are supposed to be *H. benedeni*, *H. lateralis*, *H. indicus*, *H. pseudoindicus*, *H. spinosus*, *H. magnisaccus* and *H. pacificus* (the latter transferred from *Neohaploporus*, see Overstreet & Curran, 2005). The generic diagnosis given by these authors appears to encompass the wide morphological variation occurring between the above-listed species and thus supports this assumption. Two further species, *H. mugilis* Liu & Yang, 2002 and *H. musculosaccus* Machida, 2003, have recently been described and assigned to *Haploporus*. Therefore, considering the synonymy suggested above, *Haploporus* contains at present the type-species, *H. benedeni*, parasitising several species of mullet in the Mediterranean; one species, *H. pacificus*, recovered in a scatophagid of a doubtful identification in the Western Central Pacific; and a group of a further five species described from *Valamugil* spp. (*V. cunnesius* and *V. engeli*) in the Indo-West Pacific. In addition to the departures in *H. pacificus* from the morphology of the type-species of *Haploporus* discussed above, comparative morphological data indicate that the

species complex from *Valamugil* spp. may not belong to *Haploporus* (both with respect to its original definition and the present diagnosis). Although a comparison of the original descriptions does not clearly indicate that these species form a natural grouping, 16 common, but striking, deviations from both the original and subsequent descriptions of the type-species of *Haploporus* were detected:

- Body elongate-fusiform, with maximum width at level of ventral sucker *vs* elongate-oval, with maximum width at level of testis;
- Eye-spot pigment not reported or figured (except in *H. indicus*) *vs* dispersed between pharynx and anterior border of oral sucker;
- Oral sucker terminal, funnel-shaped, transversely elongate *vs* subterminal, spherical;
- Ventral sucker located in first third of body *vs* in middle third;
- Oesophagus very long (3-7× length of pharynx) *vs* 2× length of pharynx;
- Caeca usually long and slender, ending blindly at mid-hindbody or more posterior (except in *H. musculosaccus*) *vs* relatively narrow, ending blindly at mid-body;
- Testis highly elongate, elliptical, located well posterior to ventral sucker (distance from posterior margin of the latter 2.5-9× length of ventral sucker) *vs* subspherical, adjacent or just posterior to ventral sucker (distance from posterior margin of the latter less than 1/2 of ventral sucker length);
- External seminal vesicle elongate, subcylindrical *vs* saccular, subglobular;
- Hermaphroditic sac elongate (length>3× length of ventral sucker), typically extending posterior to ventral sucker (up to 2 lengths of ventral sucker; except *H. musculosaccus*) *vs* subglobular to slightly elongate-oval (length up to twice length of ventral sucker), antero-dorsal to and not extending posterior to ventral sucker;
- Internal seminal vesicle elongate-oval to tubular, occupying less than half of hermaphroditic sac *vs* subglobular/saccular, occupying more than half of hermaphroditic sac;
- Pars prostatica distinct, vesicular *vs* indistinct, tubular;
- Hermaphroditic duct long (1/3-1/2 length of hermaphroditic sac), muscular, may be armed with spines *vs* unarmed, faintly muscular, relatively short (less than 1/3 length of hermaphroditic sac);
- Genital atrium shallow to large, with muscular or glandular walls, usually armed with spines or spine-like structures *vs* absent;

- Ovary located well apart from ventral sucker *vs* dorsal to or just posterior to ventral sucker;
- Metraterm thick-walled, glandular, long (1/3-1/2 length of hermaphroditic sac) *vs* indistinct, very short;
- Vitellarium a single compact dumbbell-shaped mass, larger than pharynx *vs* two symmetrical, separated, compact masses, smaller than pharynx.

Since the Indo-West Pacific species of *Haploporus* described from *Valamugil* spp. apparently do not fit the historical or current (derived from the features characteristic for the type-species, *H. benedeni*) generic diagnosis of *Haploporus,* it can be concluded that they have been assigned to a wrong genus. However, because of the features listed above, they do not appear to correspond to any recognised haploporine generic diagnosis (see above and also Overstreet & Curran, 2005; Blasco-Costa *et al*., 2009b). These species are therefore retained here in the Haploporinae but as *incertae sedis* with respect to their generic affiliation. A possible accommodation could be sought within the West Pacific genus *Elliptobursa* Wu, Lu & Zhu, 1996, which was originally allocated to the Monorchiidae Odhner, 1911 (see Wu, Lu & Zhu, 1996), but it is considered a haploporid genus 'as evidenced by the presence of a single testis, a long external seminal vesicle, a well-developed prostatic complex, and a long hermaphroditic duct wrongly interpreted as a cirrus' in a new revision of this family by Madhavi (2008). The forms from *Valamugil* spp. in the Indo-West Pacific resemble *Elliptobursa* spp. in: (i) the structure of the vitellarium (*i.e.* a single compact dumbbell-shaped mass); (ii) the large and distinctly elongate hermaphroditic sac, extending far posterior to the ventral sucker; (iv) the strongly elongate testis, located at a significant distance from the ventral sucker; (v) the distinctly anterior position of the ventral sucker; (iii) the long caeca, reaching well posterior in the hindbody; and (v) the presence of a vesicular pars prostatica and well-developed genital atrium. However, there is an apparent lack of agreement on the placement of *Elliptobursa* within the Haploporinae (compare the concepts of Overstreet & Curran, 2005 and Madhavi, 2008). Moreover, a possible transfer of this genus to the Haploporinae requires a revision of its species to clarify/verify the structure of the terminal genitalia (male and female ducts have been described as separate in *Elliptobursa* spp. but this feature was questioned by Madhavi, 2008). A detailed comparative morphology study of the forms described from Indo-West Pacific mugilids, preferably combined with a molecular test for their phylogenetic affinities, is clearly necessary. A lack of data at present prevents more

definitive suggestions regarding the generic affiliation of the Indo-West Pacific species assigned to *Haploporus*; these are listed below with comments on their morphology.

Haploporus indicus **Rekharani & Madhavi, 1985**

Record

Reference, Description: Rekharani & Madhavi (1985).

Definitive host: *Valamugil cunnesius* (Valenciennes, 1836) (type-host).

Distribution: Area 57: Eastern Indian Ocean (type-locality: Off Visakhapatnam (brackish waters), Bay of Bengal, India).

Remarks

This species was distinguished from *H. benedeni* and *H. lateralis* by having the testis in the middle of hindbody and caecal bifurcation anterior to ventral sucker, the claviform shape of the hermaphroditic sac which extends far posterior to ventral sucker, and much smaller eggs (Rekharani & Madhavi, 1985). Additional features which associate *H. indicus* with the forms from the Indo-West Pacific *Valamugil* spp. include a fusiform body, with its maximum width at the level of the ventral sucker; a terminal, funnel-shaped oral sucker; an oesophagus about 3× the length of the pharynx; long, slender caeca terminating at mid-hindbody; an elliptical testis, located at a distance from the ventral sucker of *c*.4× the length of the latter; long (length about 3× the length of the ventral sucker) hermaphroditic sac located mostly in the hindbody and extending posterior to the ventral sucker of up to twice the length of the latter; the presence of a vesicular pars prostatica; an ovary located far posterior to the ventral sucker; and a thick-walled metraterm of about half the length of the hermaphroditic sac.

Haploporus magnisaccus **Machida, 1996**

Material studied

Type-material: Ex '*Mugil cephalus*?'. Intestine. Off Ambon, Indonesia. 23.i.1993. Paratypes NSMT-Pl 4317 (3 specimens). One specimen mounted together with the paratypes of *H. spinosus* (NSMT-Pl 4365) ex *C. crenilabis*. 14.v.1993.

Record

Reference, Description: 1. Machida (1996).

Definitive host: *Crenimugil crenilabis* (Forsskål) [corrected to *Moolgarda seheli* (Forsskål) (syn. of *Valamugil seheli* (Forsskål)) by Machida (2003)] (type-host), '*Mugil cephalus*(?)'.

Distribution: Area 61: Northwest Pacific (type-locality: Off Nago, Okinawa Prefecture, Japan); area 71: Northwest Pacific (Off Ambon, Indonesia).

Additional morphological data (Fig.5.3; Table 5.2)

Hermaphroditic sac large, elongate-oval, with muscular walls, antero-dorsal to and extending posterior to ventral sucker (up to length of the latter; length about 3× length of ventral sucker). Internal seminal vesicle tubular, occupies more than half length of hermaphroditic sac, connects to hermaphroditic duct via relatively large vesicular pars prostatica. Prostatic cells large, in 2 distinct groups on both sides of pars prostatica. Hermaphroditic duct long (more than half length of sac), widening anteriorly, with thick strongly muscular walls. Genital atrium large, 144 × 108 internally in ventral view; 93 × 80 in apical view), with thick double-layered walls; inner layer strongly muscular up to 29 thick; outer layer less muscular, up to 32 thick; latter armed with row of small spines (7-9 × 2-4). Circle of spines associated with strongly stained muscular bundles present internally and more anterior (bases of some of spines visible apically, see Fig. 5.3A). Genital pore small, oval (25 × 11). Metraterm thick-walled, glandular, length *c*.1/4 of length of hermaphroditic sac. External seminal vesicle lined with large cells posteriorly. [One of 3 paratypes had apical region of terminal genitalia everted (Fig. 5.3B); this revealed median double line of small spines internally (which represents internal lateral armament of genital atrium) and ventral part of what appears to be ring of larger spines seen apically in other specimen (see Fig. 5.3A,B for comparison)].

Remarks

H. magnisaccus appears to have been placed in *Haploporus* due to its resemblance to other *Hapoloporus* species described in the same study by Machida (1996) (*i.e.* intestinal bifurcation posterior to ventral sucker) and was not differentiated from any other species of the genus. The structure of the terminal genitalia described from the type-material above largely departs from the description of Machida (1996). Thus, he described the hermaphroditic duct as surrounded by tall, thin-walled glandular cells and

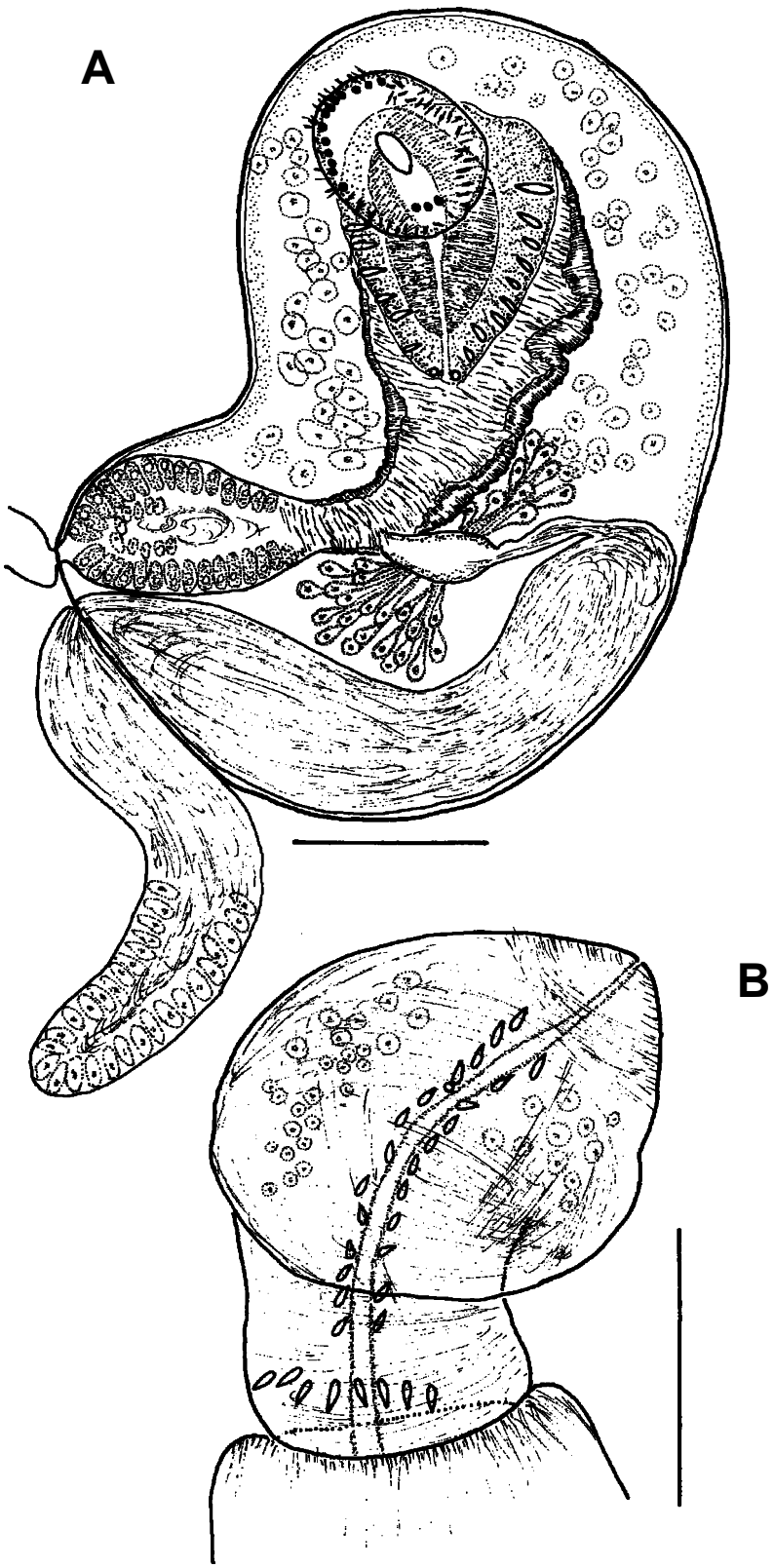

Fig. 5.3. *Haploporus magnisaccus* ex '*Mugil cephalus*?'. Terminal genitalia of paratypes (NSMT-Pl 4317). Spine bases of apical spines in black. *Scale-bars*: 100 μm.

about 100 short, thick-walled diverticula, all enclosed by longitudinal muscle bundles, whereas in fact the muscular hermaphroditic duct is distingushable from the genital atrium, which is clearly armed with spines (and not diverticula, although associated with muscular fibres). On the other hand, present observations suggest that the circle of 'nearly 40 diverticula' around the genital pore actually comprises a smaller number (*c*.25) of slightly larger (than those present posterolaterally) spines which appear attached to the anterior rim of the genital atrium; the strongly-staining muscular bundles might have been included in the count of 'diverticula' given by Machida (1996). With the exception of the armament of the hermaphroditic duct in the original description, *H. magnisaccus* exhibits a striking similarity to *H. mugilis,* described recently from *Valamugil engeli* in China (Liu & Yang, 2002), both forms possessing: (i) gonads located at a substantial distance from the ventral sucker; (ii) a dumbbell-shaped vitellarium; (iii) an elongate testis; (iv) a large hermaphroditic sac which extends well posterior to posterior margin of ventral sucker; (v) tubular internal and external seminal vesicles; (vi) a muscular genital atrium; and (vii) a strongly-developed, thick-walled metraterm.

***Haploporus mugilis* Liu & Yang, 2002**

Material studied

Type-material: Ex *Mugil engeli* (Bleeker). Intestine. Off Xiamen, China. Paratype BMNH 2001.8.6.1-2.

Record

Reference, Description: Liu & Yang (2002).

Definitive host: *Valamugil engeli* (Bleeker) (type-host).

Distribution: Area 61: Northwest Pacific (type-locality: Off Xiamen, Fujian Province, China).

Remarks

Liu & Yang (2002) provided a detailed description of *H. mugilis,* which they distinguished from *H. magnisaccus* based on the interpretation of spines as diverticula by Machida (1996) despite the above-cited similarities in the general morphology and the overlapping ranges for some metrical characters (Table 5.2). However, *H. mugilis* has a much narrower body, shorter hermaphroditic sac length, and smaller ventral sucker and testis width. Although these metrical differences may represent geographical variation or reflect the parasitism of different

Table 5.2. Comparative metrical data for *Haploporus magnisaccus* and *H. mugilis*.

Species	*H. magnisaccus*	*H. mugilis*
Host	*Valamugil seheli*, *Mugil cephalus*(?)	*Valamugil engeli*
Source	Machida (1996)	Liu & Yang (2002)
	Range	Range
BL	1,900-2,500	1,320-2,320
BW	580-680	290-520
OSL	80-110	66-104
OSW	130-180	108-170
PL	up to 60	10-46
PHL	80-100	62-90
PHW	70-100	40-90
OL	up to 900	320-744
VSL	140-150	88-134
VSW	140-170	108-136
HSL	400-480	224-336
HSW	240-290	144-272
ISVL	190-370	124-270
ISVW	-	50-86
ESVL	130-280	96-204
ESVW	-	50-76
HDL	150-250	138-180
HDW	75*	80-110
ML	175*	86-140
MW	75*	44-88
TL	340-640	288-536
TW	200-270	100-188
OVL	110-150	78-116
OVW	140-200	80-120
EL	34-42	39-44
EW	18-26	19-22
FO/BL (%)	22-35	22*
Sucker ratio	1:0.90-1.20	1:0.88-1.21

* Estimated from the published drawing.

hosts, the reexamination of the paratype confirmed the very different structure of the genital atrium (as originally described), the armament of the hermaphroditic duct with very small spines (absent in *H. magnisaccus,* albeit this may be due to the fixation of the latter material in AFA) and the smaller genital atrium (67 *vs* 93×80 μm). These differences tend to support the distinct status of *H. mugilis*.

***Haploporus musculosaccus* Machida, 2003 sp. inq.**

Record

Reference, Description: Machida (2003).

Definitive host: *Valamugil seheli* (Forsskål, 1775) (as *Moolgarda seheli*) (type-host).

Distribution: Area 61: Northwest Pacific (type-locality: Off Nago, Okinawa Prefecture, Japan).

Remarks

This bizarre form described from *Moolgarda seheli* in the same collection of hosts from off Nago (Japan) clearly exhibits some resemblance to the two species previously described by Machida (1996) in the shape of the body, seminal vesicles and testis, the position of the gonads, and the structure of the vitellarium. However, the location of some uterine loops in the forebody in combination with the terminal, funnel-shaped oral sucker with sensory papillae around the orifice, bear no resemblance to any of the currently recognised haploporine genera (see Overstreet & Curran, 2005; Blasco-Costa *et al.*, 2009b). In fact, *H. musculosaccus* departs morphologically from the complex of species described from *Valamugil* spp. discussed above due to its: (i) bipartite (‘retort-shaped’) hermaphroditic sac, located entirely anterior to the ventral sucker; (ii) slender ‘membranous’ hermaphroditic duct; (iii) more anterior intestinal bifurcation; and (iv) saccular caeca which terminate at a shorter distance posterior to ventral sucker.

Previous experience, *i.e.* assuming that the specimen in best condition was selected as the holotype by Machida (2003), and the fact that one of the 10 type-specimens was described as ‘anomalous’ (lacking the muscular portion of the hermaphroditic sac), deterred a re-examiniation the type-material. The illustration of the holotype (fig. 1 in Machida, 2003), however, indicates that the specimens were apparently poorly fixed; this might have lead to organ displacement. Clearly, further work and additional well-preserved material from the type-host and locality are needed to establish the status of this form.

***Haploporus spinosus* Machida, 1996**

Material studied

Type-material: Ex *Crenimugil crenilabis*. Intestine. 14.v.1993, 30.ix.1994. Paratypes NSMT –Pl 4365, 4709 (17+16 specimens; one specimen of *H. magnisaccus* present on the former slide, see above).

Record

Reference, Description: Machida (1996).

Definitive host: Crenimugil crenilabis (Forsskål) [corrected to *Moolgarda seheli* (Forsskål) (syn. of *Valamugil seheli* (Forsskål)) by Machida (2003)] (type-host).

Distribution: Area 61: Northwest Pacific (type-locality: Off Nago, Okinawa Prefecture, Japan).

Additional morphological data

(Fig. 5.4)

Examination of the paratypes helped clarify the structure of the terminal genitalia as follows:

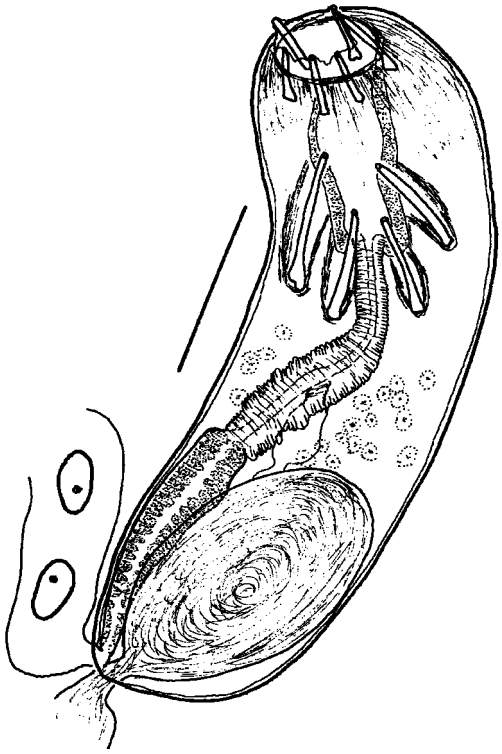

Fig. 5.4. *Haploporus spinosus* ex *Valamugil seheli*. Terminal genitalia of holotype (NSMT-Pl 4709). *Scale-bar*: 100 μm.

Hermaphroditic sac large, elongate-oval, subcylindrical, with thin walls (up to 4 thick), located mostly in hindbody. Internal seminal vesicle elongate-oval, occupies about third of hermaphroditic sac, connects to hermaphroditic duct via relatively large vesicular pars prostatica, with large prostatic cells sparsely distributed laterally. Hermaphroditic duct long, more than third length of sac, strongly muscular, thick-walled; pars prostatica connects *via* short muscular canal. Genital atrium deep (114 × 49), with thick (9-10) non-muscular (?glandular) walls and 2 pairs of long spines (60-89) located in parenchyma on both sides of its posterior half and further

posterior (1 pair enter basal to atrium on either side of muscular hermaphroditic duct and 1 pair laterally further anterior, at about mid-length of atrium). Spines with somewhat enlarged bases with 2 knobs (width at base 11-17, see Fig. 5.4), connected to wall of genital atrium by 2 strongly-developed muscular bundles each, and with tips which appear broken (in studied paratypes we found only 1 spine 106 long which was entire). Genital pore wide, oval (61 × 39), armed with 6 shorter spines (30-43) with similarly enlarged knobbed bases (width at base 4-9) and broken tips; latter are connected to thin festooned membrane when partly everted (Fig. 5.4). Metraterm thick-walled, glandular with muscular terminal portion, with length about third length of hermaphroditic sac. In many specimens caeca reach far beyond level of ovary close to posterior extremity.

Remarks

This species was described in some detail (Machida, 1996) from a host which appears to have been *Valamugil seheli* [host corrected by Machida (2003) to *Moolgarda seheli*, which is a synonym of *V. seheli*]. Machida (1996) did not provide explicit argumentation for the generic affiliation of *H. spinosus* and differentiated the new species from existing *Haploporus* spp. by the bifurcation of the caeca posterior to the ventral sucker and the hermaphroditic duct being armed with four long and six short spines.

The re-examination of the paratypes confirmed the observations of Machida (1996) with respect to the general morphology of the material fixed in AFA, which, although abundant, does not provide adequate specimens for a detailed observation of the terminal genitalia. Nevertheless, the structure of the terminal genitalia was sufficiently clarified (Fig. 5.4) so that the part described by Machida (1996) as 'armed' cannot be considered to represent the hermaphroditic duct. As shown in Fig. 5.4, the hermaphroditic duct can be clearly distinguished from what appears to be a genital atrium ensuing from the strongly muscular nature of its walls (*vs* glandular). The size of the spines that were good for measuring in the paratypes deviates somewhat from the data given by Machida (1996). Thus, whereas the length range of the small spines approaches the single measurement in the original description (30-43 *vs* 50 µm), the length range for the long spines observed is about half the size of that measured by this author (60-89 *vs* 120-160 µm); this might be due to the fact that unbroken spines are present in the holotype (figs. 4-5 in the original description), which was unavailable for reexamination. All large spines in the paratypes, except one, were lacking tips; the latter was however also somewhat shorter.

5.2. Genus *Dicrogaster* Looss, 1902

5.2.1. Background

Looss (1902) erected *Dicrogaster* Looss, 1902 to accommodate *D. perpusilla* Looss, 1902 (type-species) and *D. contracta* Looss, 1902 from *C. labrosus* in the Adriatic Sea off Trieste (Looss, 1902). Three additional species of haploporid digeneans have since been described and assigned to this genus: *D. fastigata* Thatcher & Sparks, 1958; *D. fragilis* Fernández Bargiela, 1987; and *D. japonica* Machida, 1996. Dawes (1947) and Sarabeev & Balbuena (2003) considered the Mediterranean forms, *i.e. Dicrogaster contracta* Looss, 1902 and *D. perpusilla* Looss, 1902, conspecific. However, with the exception of Sarabeev & Balbuena (2003), no attempt has been made at a critical evaluation of the features distinguishing the species; this is perhaps due to their disparate distribution and the difficulties with specimen preparation (Overstreet & Curran, 2005).

5.2.2. Diagnosis

Body small, oval to fusiform, tapered or rounded posteriorly. Tegument armed. Eye-spot pigment concentrated on either side of pharynx. Oral sucker subterminal spherical. Ventral sucker spherical, muscular, equal to or larger than oral sucker, in middle third of body or more posterior. Forebody at about third of body length. Prepharynx absent or very short. Pharynx muscular, subspherical. Oesophagus short to relatively long. Intestinal bifurcation dorsal to ventral sucker or slightly posterior. Caeca two, sac-like, short. Testis single, subspherical to elongate, median, smooth. External seminal vesicle contiguous with hermaphroditic sac, saccular to elongate. Hermaphroditic sac subglobular to elongate-oval, antero-dorsal to and not extending posterior to ventral sucker. Internal seminal vesicle thin-walled, saccular, elongate-oval. Pars prostatica indistinct to conspicuos (*D. fastigata*), prostatic cells numerous. Hermaphroditic duct armed, at least half length of hermaphroditic sac. Genital atrium apparently absent or shallow, non-muscular. Genital pore round, median, between pharynx and ventral sucker. Ovary submedian, oval, dorsal to ventral sucker to somewhat posterior, pretesticular or at level of testis. Uterine seminal receptacle present; blind seminal receptacle absent. Uterus thin-walled, occupies entire hindbody. Metraterm short. Eggs numerous, length half to up to twice length of pharynx, operculate. Developed miracidia with single or two fused eye-spots. Vitellarium two (one in *D. fastigata*) adjacent elongate-oval compact masses

of small follicles, contiguous with testis and ovary. Excretory vesicle Y-shaped, stem tubular, with small sphincter in some species; pore terminal. In mullets (Mugilidae). Type-species: *D. perpusilla* Looss, 1902.

5.2.3. Review of species

Dicrogaster perpusilla Looss, 1902

Material studied

Ex *Liza saliens* (Risso). Intestine. Ebro Delta, Spain (40º30'–40º50'N, 0º30'–1º10'E; 22.vi.2004; 31.v.2005). BMNH 2008.10.7.1-5.

Ex *L. ramado* (Risso). Intestine. Lagoon near Santa Pola, Spain (38º10'N, 0º39'E; 15.vi.2005). BMNH 2008.10.7.6-11.

Comparative material

D. perpusilla neotype ex *Chelon labrosus*. Off West Thurrock, UK (BMNH 1986.5.20.155-156).

Records

References: 1. Looss (1902); 2. Oguz & Bray (2006); 3. Present study.

Descriptions: 1; 3.

Definitive hosts: *Chelon labrosus* (Risso) (type-host) (1); *Liza saliens* (Risso) (2, 3); *L. ramado* (Risso) (2, 3); *L. aurata* (Risso) (2, 3); *Mugil cephalus* L. (3).

Distribution: Area 37, subarea 2 (Central Mediterranean) (type-locality: Adriatic Sea off Trieste) (1); area 37, subarea 3 (Eastern Mediterranean) (2); area 37, subarea 1 (Western Mediterranean) (3).

Description (Fig. 5.5; Table 5.3)

[Based on 29 whole-mounted adult specimens.] Body minute; posterior quarter tapered, 'tail-like', retractile; shape elongate-fusiform (17 worms; see Fig. 5.5B) to rounded posteriorly when 'tail' withdrawn (12 worms; see Fig. 5.5B); maximum width at mid-level of ventral sucker, 32-56 (43)% of body length. Tegument thin, armed with fine spines, *c.*3 long, striated in posterior quarter when 'tail' everted. Eye-spot pigment concentrated on either side of pharynx (at its mid-level); dispersed granules also present anteriorly and posteriorly. Oral

sucker subspherical, with subterminal aperture. Ventral sucker spherical, strongly muscular, distinctly larger than oral sucker [sucker length ratio 1:1.24-2.36 (1:1.80); width ratio 1:1.17-1.97 (1:1.50)], in middle third of body or more posterior (8 worms). Forebody long, 26-57 (38)% of body length.

Prepharynx apparently absent to similar in length to pharynx; pharynx muscular, subspherical. Oesophagus up to twice length of pharynx; intestinal bifurcation at level of anterior margin of ventral sucker or slightly posterior; caeca 2, sac-like, short, end blindly anterior to posterior margin of ventral sucker at 34-49 (41%) from posterior extremity.

Testis single, median, subspherical, smooth, located posterior to ventral sucker in worms with fully-extended 'tail' and dorsal to ventral sucker in specimens with invaginated or contracted 'tail'; post-testicular space 15-49 (27)% of body length. External seminal vesicle contiguous with hermaphroditic sac, saccular, small, globular, somewhat smaller than internal seminal vesicle. Hermaphroditic sac prominent, thin-walled (<2 thick), subglobular to elongate-oval, antero-dorsal to ventral sucker (entirely anterior in single worm with everted, narrow 'tail'), reaches posteriorly as far as its mid-level, usually slightly shorter than ventral sucker [HSL/VSL=62-122 (87)%], contains internal seminal vesicle, numerous small prostatic cells, short metraterm and hermaphroditic duct. Internal seminal vesicle thin-walled, saccular, elongate-oval, occupying up to half of hermaphroditic sac (typically one third). Hermaphroditic duct more than half length of hermaphroditic sac, wide, slightly muscular; its distal half is eversible and lined by small tubercles. Genital atrium very shallow, non-muscular. Genital pore round, median, about half-way between pharynx and ventral sucker or more anterior.

Ovary oval, submedian, dorsal to ventral sucker, pretesticular. Uterine seminal receptacle observed in single worm; Mehlis' gland and Laurer's canal not seen. Uterus thin-walled, occupies entire hindbody. Metraterm short and wide (30 × 32; *c*.40% of length of hermaphroditic sac), joins hermaphroditic duct close to seminal vesicle. Eggs numerous (up to 36), length up to twice length of pharynx, operculate; developed miracidia with single eye-spot. Vitellarium 2 adjacent compact masses of small follicles, smooth, elongate-oval, contiguous with testis and/or ovary, usually smaller than pharynx [VL/PHL=49-141 (90)%].

Stem of excretory vesicle tubular, with small sphincter, 13-18 × 8-13, located at some distance (*c*.1/5 of body length) from posterior extremity; bifurcation and excretory arms obscured by eggs; pore terminal.

Remarks

The material described here exhibits the diagnostic characteristics of *Dicrogaster* and actually represents the smallest form observed in a large collection of fully mature *Dicrogaster* spp. from the western Mediterranean based on the examination of 698 mugilid fishes (three species of *Liza* and *Mugil cephalus*). In addition to the metrical differences of almost all of the internal organs, which fell outside the known lower range of variation for both *D. contracta* and *D. fastigata* (Tables 5.3, 5.4), other distinctive characters of *D. perpusilla* include: (i) a ventral sucker which is large in relation to the body and distinctly larger than the oral sucker; (ii) a thin-walled hermaphroditic sac antero-dorsal to the ventral sucker; (iii) a short, wide metraterm with thin walls; (iv) a vitellarium comprising two adjacent compact masses of follicles; and (v) eggs which are up to twice the length of the pharynx.

D. perpusilla resembles *D. contracta* in the ability of the posterior extremity to retract (Fig. 5.5B) and in having a ventral sucker distinctly larger than the oral. In addition to the smaller size of the body and organs, and the features listed above, *D. perpusilla* can also be distinguished from *D. contracta* by the following: (i) a hermaphroditic sac reaching to the mid-level of the ventral sucker (*vs* restricted to the forebody); (ii) a small internal seminal vesicle occupying less than half of the hermaphroditic sac (*vs* more than half of the hermaphroditic sac); (iii) a short, wide metraterm (less than half *vs* about two-thirds the length of the hermaphroditic sac); and (iv) larger eggs in relation to pharynx (length up twice *vs* half the length of tpharynx). Although *D. perpusilla* was found in all four host species studied, a high prevalence of infection was observed only in *L. saliens* (20.0% *vs* 1.0-4.4% in the other three hosts; Table 5.5).

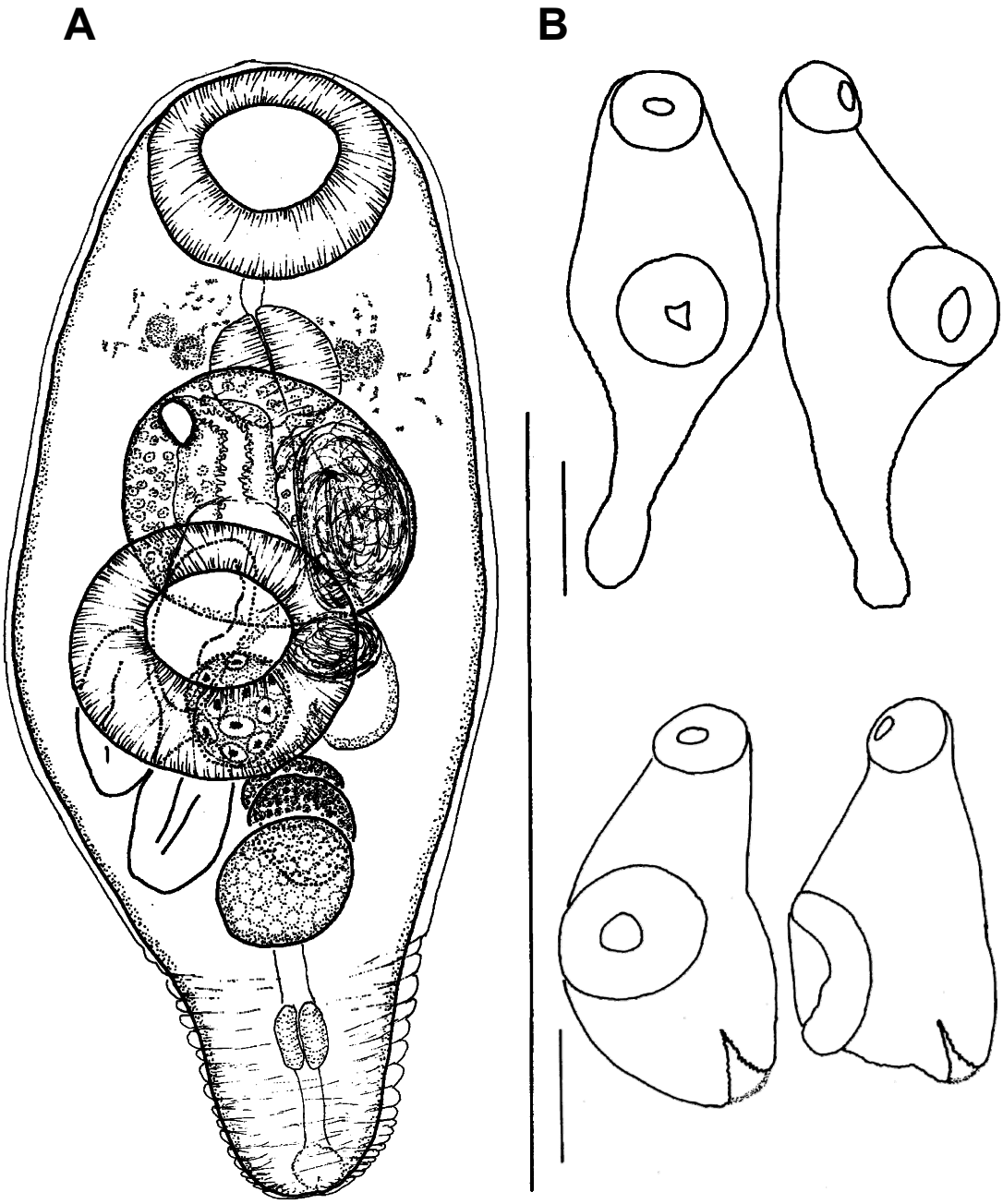

Fig. 5.5. *Dicrogaster perpusilla* Looss, 1902 ex *Liza ramado*. **A.** Ventral view. **B.** Outlines (ventral and lateral views) showing the shape of specimens when the 'tail' is everted and withdrawn. *Scale-bars*: **A**, 200 µm; **B**, 100 µm.

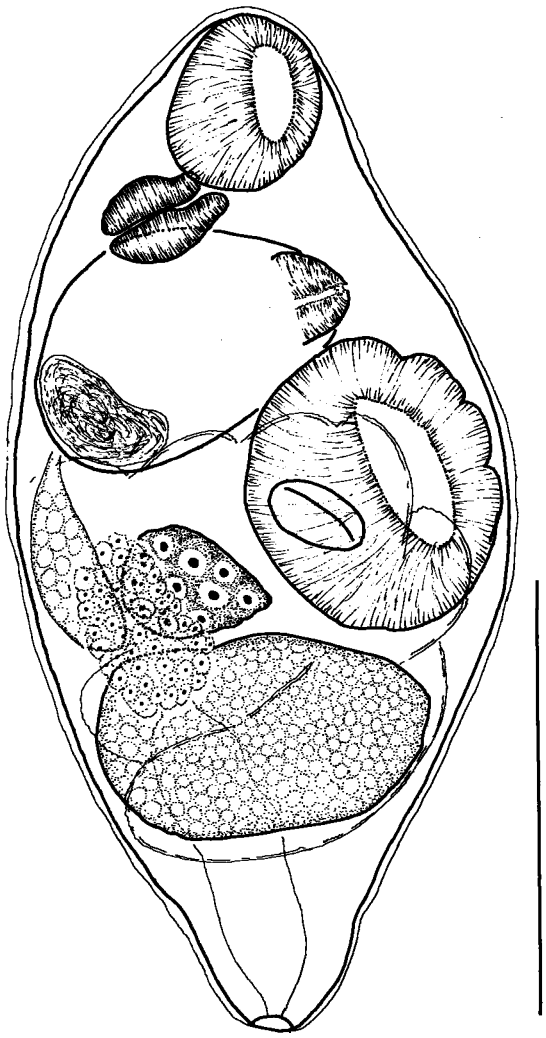

Fig. 5.6. Neotype of *Dicrogaster perpusilla* Looss, 1902 ex *Chelon labrosus*. Off West Thurrock, UK (BMNH 1986.5.20.155-156). Dorsolateral view with uterus in outline. *Scale-bar*: 200 µm.

Both original descriptions of the Mediterranean species of *Dicrogaster* are brief (including metrical data limited to the size of the body, suckers, pharynx and eggs) and based only on a few worms (Looss, 1902). All subsequent records of *Dicrogaster* in mugilids from the Mediterranean, Black, Azov and Caspian Seas (plus a fish farm in Egypt) refer to *D. contracta.* Unfortunately, most of these are non-documented. Sarabeev & Balbuena (2003) examined three samples of *D. contracta* from the NE Atlantic, Azov Sea and Spanish Mediterranean and compared the metrical data with the descriptions of this species given by other authors, as well as with the original descriptions of *D. contracta* and *D. perpusilla* of Looss (1902). They considered the two species synonymous, a decision not supported here, and selected a neotype for *D. perpusilla*. Unfortunately, the neotype represents a neogravid (*i.e.* bearing 16 eggs, all collapsed) dorso-laterally mounted specimen and is unrecognisable (all visible structures outlined in Fig. 5.6). In view of this, it is pragmatic to treat this neotype designation as one would any unrecognisable type-material and to continue to use the original conception of the type-species.

***Dicrogaster contracta* Looss, 1902**

Material studied

Ex *Liza ramado* (Risso). Intestine. Ebro Delta, Spain (40º30'–40º50'N, 0º30'–1º10'E; 26.v.2004). BMNH 2008.10.7.12-13.

Ex *L. aurata* (Risso). Intestine. Ebro Delta, Spain (26.v.2004) BMNH 2008.10.7.14-16.

Records

References: 1. Looss (1902); 2. Fares & Maillard (1974); 3. Orecchia & Paggi (1978)*; 4. Paggi *et al.* (1979)*; 5. Solonchenko & Tkachuk (1985)*; 6. Ibragimov (1988)*; 7. Orecchia *et al.* (1988)*; 8. Paggi *et al.* (1988)*; 9. Radujković *et al.* (1989); 10. D'Amelio *et al.* (1995)*; 11. Oguz (1995)*; 12. Di Cave *et al.* (1997)*; 13. Domnich & Sarabeev (2000a)*; 14. Domnich & Sarabeev (2000b)*; 15. Domnich & Sarabeev (2000c)*; 16. Sarabeev (2000)*; 17. Sarabeev & Domnich (2000)*; 18. Dmitrieva & Gaevskaya (2001)*; 19. Merella & Garippa (2001)*; 20. Al-Bassel (2003)*; 21. Ragias *et al.* (2005)*; 22. Present study.

Descriptions: 1; 2; 9; 22.

Definitive hosts: *Chelon labrosus* (Risso) (type-host) (1, 2, 4, 7, 9, 19, 21); *Mugil cephalus* L. (2, 3, 4, 8, 21); *M. soiuy* Basilewsky (13, 14, 15, 16, 17, 18); *Liza aurata* (Risso) (5, 6, 8, 15, 18, 19, 21, 22); *L. ramado* (Risso) (2, 4, 6, 8, 10, 11, 12, 19, 20, 21, 22); *L. saliens* (Risso) (2, *5, 19).

Distribution: Area 37, subarea 2 (Central Mediterranean) (type-locality: Adriatic Sea off Trieste) (1, 7, 9, 10); area 37, subarea 1 (Western Mediterranean) (2, 3, 4, 19, 22) (?8, 12 'Italy'); area 37, subarea 3 (Eastern Mediterranean) [10, 20 (Fayoum fish farm, Egypt), 21]; area 37, subarea 4 (Black Sea): 4.1 Sea of Marmara (11), 4.2 Black Sea (18), 4.3 Azov Sea (5, 13, 14, 15, 16, 17, 18); Caspian Sea (6).

Description (Fig. 5.7; Table 5.3)

[Based on 9 whole-mounted adult specimens.] Body small, plump, oval, with tail-like narrowing of posterior quarter but rounded posterior extremity in specimens with retracted 'tail' (6 worms); maximum width at level of ventral sucker, 44-67 (58)% of body length. Tegument thick, armed with strong spines *c.*3-6 long, with striated appearance in posterior fifth of body. Eye-spot pigment in 2 groups on either side of pharynx (at its mid-level). Oral sucker subspherical, muscular, subterminal. Ventral sucker subspherical, strongly muscular, distinctly larger than oral sucker [sucker length ratio 1:1.38-1.62 (1:1.49); width ratio 1:1.11-1.75 (1:1.38)], in middle third of body or more posterior (1 worm). Forebody long, 29-66 (42)% of body length.

Prepharynx absent to short (up to approx. half length of pharynx); pharynx large, muscular, subspherical. Oesophagus short, as long as pharynx to slightly longer. Intestinal

* Dubious records without a description or figure – see 'Remarks'.

bifurcation in forebody; caeca 2, sac-like, short, end blindly usually anterior to posterior margin of ventral sucker at 39-51 (43)% from posterior extremity.

Testis single, median, elongate-oval, smooth, located in last third of body, more anterior and closer to ventral sucker in specimens with withdrawn 'tail'; post-testicular space 21-33 (27)% of body length. External seminal vesicle dorsal to ventral sucker, adjacent to hermaphroditic sac, saccular, large, elongate-oval, similar in size to internal seminal vesicle. Hermaphroditic sac massive, thick-walled (4-8 thick), elongate-oval, anterior to ventral sucker, comparable in size to ventral sucker [HSL/VSL=76-122 (96)%], contains internal seminal vesicle, numerous large prostatic cells, short metraterm and hermaphroditic duct. Internal seminal vesicle thin-walled, saccular, elongate-oval, usually occupying more than half of hermaphroditic sac. Hermaphroditic duct approx. half length of hermaphoditic sac, wide, slightly muscular; its distal half is eversible and lined by distinct tubercles. Genital atrium very shallow. Genital pore round, median, at level of pharynx or just posterior.

Ovary oval, submedian, dorsal to ventral sucker to somewhat posterior (anterior to ventral sucker in 1 specimen with inverted 'tail'), pretesticular. Uterine seminal receptacle present in all worms. Mehlis' gland observed in 1 worm from *L. aurata*, 85 × 59. Laurer's canal not seen. Uterus thin-walled, occupies entire hindbody and expands dorsally to level of pharynx into forebody. Metraterm relatively long (about two-thirds length of hermaphroditic sac), narrow, with thick glandular walls, joins hermaphroditic duct close to seminal vesicle. Eggs abundant, usually approx. half length of pharynx, operculate; developed miracidia with single eye-spot. Vitellarium 2 adjacent large compact masses of small follicles, smooth, elongate-oval, contiguous with ovary or somewhat posterior; usually as long as pharynx [VL/PHL=75-130 (100)%].

Stem of excretory vesicle tubular, with relatively thick (*c*.3) epithelium and large muscular sphincter, 29-48 × 32-51 (38 × 42), located close to posterior extremity; bifurcation and excretory arms obscured by uterus; pore terminal.

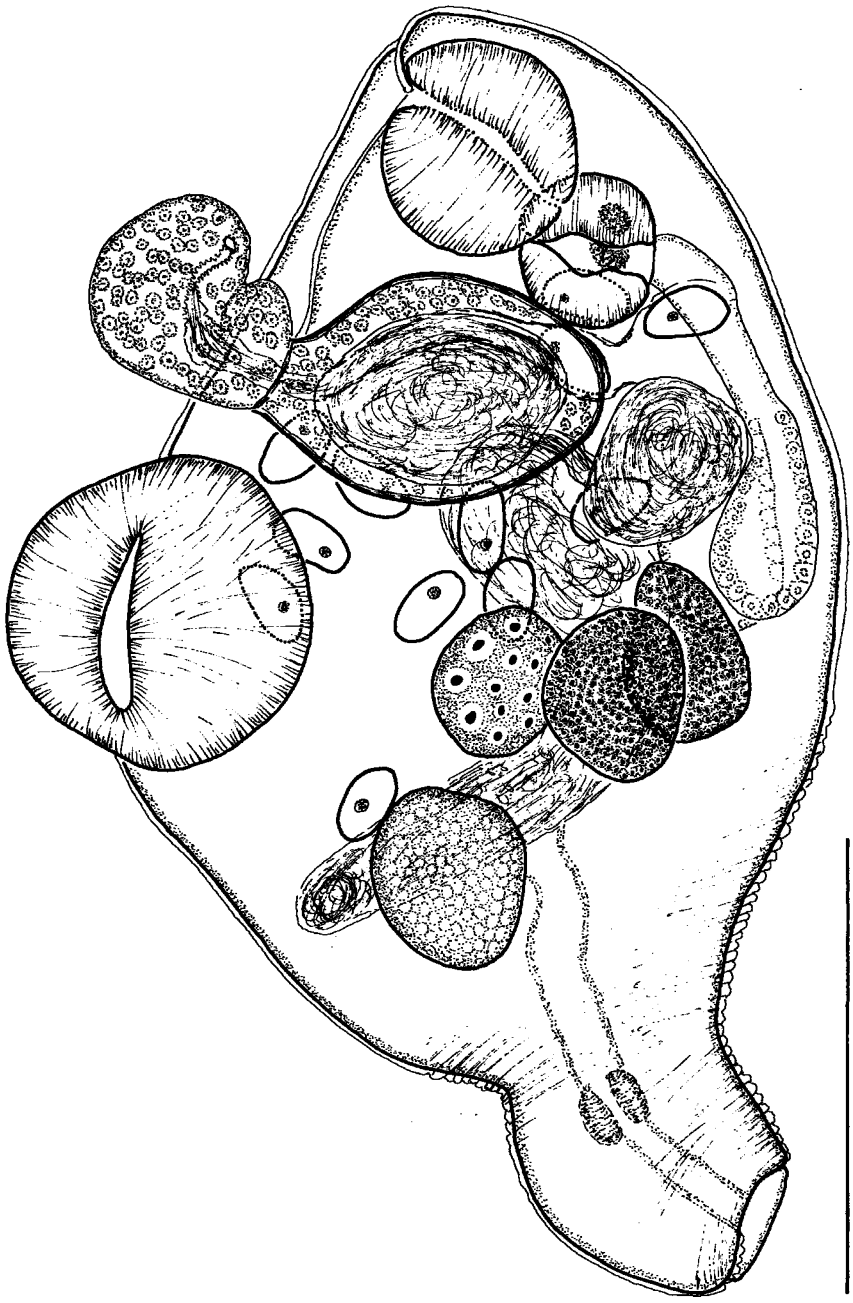

Fig. 5.7. *Dicrogaster contracta* Looss, 1902 ex *Liza ramado*. Lateral view. *Scale-bar*: 200 µm.

Table 5.3. Comparative data for *Dicrogaster* spp. recorded from the Mediterranean.

Species	*D. perpusilla*			*D. contracta*		*D. contracta*	*D. contracta*	
Locality	Ebro Delta & lagoon near Santa Pola			Ebro Delta		Kotor Bay (Adriatic Sea)	lagoons of Languedoc-Rousillon	
Source	Present study			Present study		Radujković et al. (1989)	Fares & Maillard (1974)	
	Range	Mean ± SD	CV (%)	Range	Mean	Range (n=1)	Range	Mean
Measurements								
BL	203-467 (300-330)*	327 ± 55	16.7	590-904 (450)*	744	860	460-1490	830
BW	96-168	143 ± 18	12.8	312-525	425	390	270-650	390
OSL	37-84	55 ± 10	18.7	94-139	120	140	-	-
OSW	38-102 (66)*	66 ± 11	16.4	96-181 (100)*	141	130	90-160	130
PL	0-37	13 ± 11	88.3	0-48	13	short	20	-
PHL	16-45 (18)*	30 ± 7	22.3	61-88 (47)*	74	60	40-80	60
PHW	19-46	33 ± 6	17.4	69-98 (34)*	80	60	-	-
OL	32-83	55 ± 17	31.7	61-144	102	2× PHL	-	-
VSL	67-118	97 ± 14	14.4	140-197	177	180	-	-
VSW	69-142 (100)*	98 ± 17	17.3	134-229 (125)*	190	180	100-260	170
HSL	52-114	84 ± 16	18.5	144-224	180	190	140-290	190
HSW	40-75	53 ± 7	14.0	102-184	139	120	-	-
ISVL	24-53	38 ± 8	22.4	83-155	112	-	-	-
ISVW	14-42	27 ± 7	24.7	61-136	89	-	60-100	-
ESVL	21-35	26 ± 5	19.3	58-131	100	-	-	--
ESVW	16-24	21 ± 3	14.3	37-117	75	-	-	-
HDL	35-85	53 ± 18	33.8	61-112	80	-	100	-
HDW	14-32	24 ± 8	31.3	40-56	47	-	-	-
TL	29-46	37 ± 5	14.4	75-133	108	180	110-230	170
TW	29-43	33 ± 4	12.5	69-107	87	190	-	-
OVL	19-48	31 ± 9	30.4	43-150	80	100	70-180	110
OVW	21-40	28 ± 6	21.7	56-122	85	100	-	-
VL	17-41	27 ± 5	19.7	58-98	73	-	-	-

Table 5.3. Continued.

Species	*D. perpusilla*			*D. contracta*		*D. contracta*	*D. contracta*	
Locality	Ebro Delta & lagoon near Santa Pola			Ebro Delta		Kotor Bay (Adriatic Sea)	lagoons of Languedoc-Rousillon	
Source	Present study			Present study		Radujković et al. (1989)	Fares & Maillard (1974)	
	Range	Mean ± SD	CV (%)	Range	Mean	Range (n=1)	Range	Mean
VW	11-26	17 ± 4	21.8	40-67	51	-	-	-
EL	41-52 (53)*	45 ± 2	5.6	32-43 (35-40)*	39 ± 3 (CV=6.8%)	45-56	35-70	50
EW	22-26 (25)*	24 ± 2	6.6	17-26 (23)*	22 ± 2 (CV=10.4%)	25-35	20-50	30
Distances								
FO	69-174 (180)*	125 ± 25	19.8	210-476 (240)*	306	-	-	-
CEND	101-222	142 ± 40	28.3	238-464	318	-	-	-
TEND	40-165	88 ± 41	46.6	158-301	197	-	-	-
UEND	22-99	70 ± 24	34.2	80-168	112	-	-	-
Ratios								
Elongation index	1.8-2.9 (1.6)**	2.4 ± 0.3	12.9	1.5-2.3 (1.8)**	1.8	1.4**	2.0**	-
FO/BL (%)	26-57 (39)**	38 ± 7	17.4	29-66 (35)**	42	34**	36**	-
OSL/VSL	1:1.24-2.36 (1.50)**	1:1.80 ± 0.29	15.9	1:1.38-1.62 (1:1.40)**	1:1.49	-	-	-
OSW/VSW	1:1.17-1.97 (1.57)**	1:1.50 ± 0.24	15.8	1:1.11-1.75 (1:1.36)**	1:1.38	-	1:1.11-1.67	1:1.32
VSW/BW (%)	56-97	68 ± 11	17.0	38-51	45	39**	46**	-
HSL/VSL (%)	62-122	87 ± 16	18.9	76-122	96	-	-	-
TEND/BL (%)	15-49 (17)**	27 ± 10	35.7	21-33 (18)**	27	17**	14**	-
VL/PHL (%)	49-141	90 ± 23	25.9	75-130	100	-	-	-
CEND/BL (%)	34-49 (44)**	41 ± 6	15.0	39-51	43	52**	51**	-

* Data from Looss (1902) in parentheses; ** Estimated from the original drawing.

Remarks

Generally, the new material of *D. contracta* from *L. ramado* and *L. aurata* agrees morphologically with that described by Looss (1902), especially in terms of the range of egg-size, despite the fact that his description is based on only a single specimen from a different mullet host, *Chelon labrosus.* Characteristic features of *D. contracta,* which may serve to distinguish it from the other species of the genus, include: (i) the posterior quarter of body narrows to form a retractable 'tail-like' region; (ii) the ventral sucker, although distinctly larger than the oral sucker, is small in relation to the body; (iii) the hermaphroditic sac is muscular, comparable in length to the ventral sucker and located in the forebody; (iv) the metraterm is relatively long, with thick glandular walls; and (v) the eggs are small in relation to the size of the pharynx. It appears that infections with *D. contracta* are restricted to *L. ramado* and *L. aurata,* at least along the Spanish coast of the western Mediterranean (Ebro Delta and off Santa Pola); infection levels are also low (Table 5.5). The rarity of *D. contracta* in the present study (over two seasons in each of two years), unfortunately, casts some doubt on the correct identification of the numerous non-documented records (see above). Therefore, all records (marked with an asterisk above) where only the species name is listed (and the material neither described nor figured) are considered dubious.

The morphometric data from a single specimen described by Radujković *et al.* (1989) from *L. ramado* in Kotor Bay (Adriatic Sea) generally fall within the range observed in the present material (Table 5.3), with the exception of the larger and more posteriorly located testis and smaller elongation index, although these differences might well have been caused by the flattening of the specimen during mounting.

The description of *D. contracta* by Fares & Maillard (1974), and particularly their fig. 15, agree with the present redescription of *D. contracta* in the following key characteristics: (i) a ventral sucker much larger than the oral sucker; (ii) an intestinal bifurcation located in the forebody; (iii) a relatively long, narrow metraterm; and (iv) eggs which are small in relation to size of the body. However, the limited metrical data provided by these authors exhibit notable variations from the material described above, especially with regard to the lower and upper limits for the size of the body and the width of the ventral sucker (all below and above the range observed in the present material, respectively; Table 5.3). Furthermore, the upper limits of the length of hermaphroditic sac, testis, ovary and egg-size are outside the range observed here, whereas the means of the width of the body and suckers and the length of pharynx are smaller. A possible explanation for these differences in the metrical data may

be related to the fact that Fares & Maillard (1974) combined together samples from various mugilid species (*Liza ramado*, *L. saliens*, *M. cephalus* and *C. labrosus*) studied in the lagoons of Languedoc-Rousillon, France. However, during the course of the present extensive sampling, *D. contracta* appears, despite the overall high annual variations in parasite prevalence, to be a rather rare parasite of *L. ramado* (overall prevalence 2.0%) and *L. aurata* (overall prevalence 0.5%), with maxima of 6.3 and 3.4%, respectively (both from fish samples, collected in the Ebro Delta). None of the 303 *M. cephalus* examined harboured *D. contracta*, but another form, somewhat similar to *Dicrogaster* spp. in appearance, was recovered solely from this host (*Forticulcita gibsoni*, see below). It seems likely, therefore, that the metrical data of Fares & Maillard (1974) are based on composite material of more than one haploporid species. However, this cannot explain the extremely high upper limits (and means) for egg-size observed by these authors, since these are well above the known range for *Dicrogaster* spp. (Table 5.3). Apart from the serial sections of three specimens, no evidence can be found in their paper suggesting that the specimens measured by Fares & Maillard (1974) were mounted in Canada balsam (see Fares & Maillard, 1974, p. 37). The numbers in the 'material studied' section are also rather confusing. Thus, 20 specimens were 'étudiés in vivo' and 10 specimens were 'fixés in toto', whereas the means of the measurements are based on 15 specimens. Consequently, it must be concluded that the measurements provided by these authors are based on a mixture of live and fixed material and cannot, therefore, be compared to the descriptions based on material mounted in Canada balsam.

***Dicrogaster fastigata* Thatcher & Sparks, 1958**

Syn. *Dicrogaster fragilis* Fernández Bargiela, 1987 (new synonym)

Records

References: 1. Thatcher & Sparks (1958); 2. Overstreet (1971); 3. Rawson (1973); 4. Skinner (1975); 5. Fernández Bargiela (1987) (also as *D. fragilis*); 6. Luque & Oliva (1993) (also as *D. fragilis*); 7. Thatcher (1993); 8. Knoff *et al.* (1997).

Descriptions: 1; 2; 5.

Definitive hosts: *Mugil cephalus* L. (1, 2, 3, 4, 5, 6, 7); *M. platanus* Günther (8).

Distribution: Area 31 (Western Central Atlantic) (type-locality: Grand Isle, Louisiana, USA) (1, 2, 3, 4, 7); area 41, subarea 2 (Central Southwest Atlantic) (8); area 87, subarea 2 (Central Southeast Pacific) (5, 6).

Table 5.4. Metrical data reported for *Dicrogaster fastigata*.

Species	*D. fastigata*	*D. fastigata*	*D. fastigata*	*D. fastigata*	*D. fastigata*	*D. fragilis*
Locality		Grand Isle, Louisiana (USA)	Louisiana & Mississippi (USA)	Sapelo Island, Georgia (USA)	Arica & Concepción (Chile)	Concepción (Chile)
Source	Global range	Thatcher & Sparks (1958)	Overstreet (1971)	Overstreet (1971)	Fernández Bargiela (1987)	Fernández Bargiela (1987)
	Range	Range	Range	Range	Range	Range
Measurements						
BL	274-1,094	274-860	477-818	582-1,094	630-910	580-1,110
BW	140-449	140-330	159-356	226-449	250-390	210-390
OSL	37-93	42-83	37-72	61-93	-	65-125
OSW	40-107	42-83	40-93	77-107	77-95	65-125
PL	-	-	Up to ½ PHL	Less than ½ PHL	24-60	10-55
PHL	28-60	28-52	30-49	42-58	41-60	42-95
PHW	28-63	28-55	33-51	35-63	36-60	31-95
OL	67-217	-	67-217 (1- 6 × PHL)	≤ 7 × PHL	108-190	75-320
VSL	62-121	62-94	65-121	70-107	-	-
VSW	62-126	62-94	63-126	79-109	67-103	65-115
HSL	149-310	-	-	-	149-310	170-265
HSW	95-130	-	-	-	95-130	65-95
ISVL	-	-	-	-	-	-
ISVW	-	-	-	-	-	-
ESVL	-	-	-	-	-	-
ESVW	-	-	-	-	-	-
HDL	-	-	-	-	-	-
HDW	-	-	-	-	-	-
TL	72-226	73-162	79-226	72-203	140-150	67-96
TW	44-140	49-97	63-140	44-128	85-110	38-84
OVL	35-127	35-87	49-93	63-116	77-127	60-72
OVW	35-91	35-87	40-84	44-91	36-55	57-65
VL	31-128	31-83	42-100	65-128	60-100	42-115
VW	31-119	31-83	33-84	51-119	60-100	42-115
EL	35-56	42-52	36-56	35-51	46-53	36-60
EW	15-28	15-21	18-28	19-26	20-26	19-24
Distances						
FO	-	-	-	-	-	-
CEND	-	-	38-55	-	-	-
TEND	-	-	-	-	-	-
UEND	-	-	-	-	-	-
Ratios						
Elongation index	2.5-2.6	2.5*	2.6*	-	2.5*	2.4*
FO/BL (%)	15-30	19*	16-30	15-25	23*	-
OSL/VSL	-	-	-	-	-	-
OSW/VSW	1:0.9-1.6	1:0.92*	1:0.9-1.6	1:0.9-1.1	1:1.0*	1:1.0*
HSL/VSL (%)	-	-	-	-	-	-
TEND/BL (%)	10-51	36*	10-46	21-51	30*	39*
VL/PHL (%)	-	-	-	-	-	-
CEND/BL (%)	38-55	51*	38-55	38-55	49*	48*

*, Estimated from the published drawing

Remarks

This species was incompletely described by Thatcher & Sparks (1958) based on material from *M. cephalus* at Grand Isle, Louisiana, USA, and distinguished from *D. perpusilla* and *D. contracta* by its vitellarium comprising a single compact mass of follicles and its relatively smaller suckers. Overstreet (1971) redescribed *D. fastigata* based on abundant material from the Gulf of Mexico region (Louisiana, Mississippi and Sapelo Island, Georgia), thus adding substantial detail to the morphology (notably of the structure of the terminal genitalia) and the range of intraspecific morphometric variation.

The only further documented record is that of Fernández Bargiela (1987) from *M. cephalus* in two Chilean localities (Arica and Concepción). In the latter locality, she also described a new species of *Dicrogaster* with a single vitelline mass, *D. fragilis* Fernández Bargiela, 1987, which appears to be poorly distinguished from *D. fastigata* (*i.e.* by having a fragile tegument devoid of spines; slightly larger suckers and pharynx; and a smaller ovary, testis and hermaphroditic sac). However, the ranges for the size of the ventral sucker, gonads and hermaphroditic sac fit the known range for *D. fastigata* and the data for the size of oral sucker and pharynx, although exhibiting somewhat higher upper limits, overlap with the known ranges (Table 5.4). Therefore, *D. fragilis* is considered here a synonym of *D. fastigata* due to the lack of reliable characters justifying its recognition.

D. fastigata can be distinguished from the other *Dicrogaster* species by the small size, in relation to the body, of the ventral sucker, the short forebody, long hermaphroditic sac and the vitellarium consisting of a single compact mass of follicles which is distinctly larger than the pharynx.

Table 5.5. Overall prevalence (%) of *Dicrogaster* spp. in mullets (30 samples, 673 fish) sampled at two localities: Ebro Delta and Santa Pola.

	D. perpusilla	*D. contracta*
Mugil cephalus	1.3	-
Liza aurata	4.4	0.5
L. saliens	20.0	-
L. ramado	1.0	2.0

***Dicrogaster maryutensis* Al-Bassel, 1990 nom. nud.**

Records

References: 1. El-Shahawi & Al-Bassel (1992); 2. Al-Bassel (2003).

Definitive host: *Liza ramado* (Risso) (1, 2).

Distribution: Area 37, subarea 3 (Eastern Mediterranean) (Egypt) (1, 2).

Remark

This species, presumably described in a PhD thesis and only reported as a record by the same author, is a *nomen nudum*.

***Dicrogaster* spp. innom.**

Records

References: 1. Saoud *et al.* (1990); 2. Tantalean *et al.* (1992); 3. Carnevia & Speranza (2003).

Definitive hosts: *Mugil cephalus* L. (1, 2); *M. platanus* Günther. (3) *Liza ramado* (Risso) (1).

Distribution: Egypt (1); River Moche (Peru) (2); River Plate (Uruguay) (3).

5.2.4. Morphometric statistical comparisons

Measurements recorded from 55 specimens (29 *Dicrogaster perpusilla* and 9 *D. contracta*) were ln-transformed and subjected to univariate (ANOVA) statistical tests in order to assess the degree of overlap with respect to the metrical variables. The univariate analyses of variance of 26 metrical features and nine relative proportions/ratios indicated that *D. perpusilla* and *D. contracta* are morphometrically distinguishable with respect to all variables used except for the length of the prepharynx (ANOVA, all $p<0.0001$). Furthermore, despite the small range overlap in egg-size (Table 5.3), the two species differed significantly with respect to both the length ($F_{(1, 52)}=54.15$, $p<0.0001$) and width ($F_{(1, 52)}=5.08$, $p=0.028$) of the eggs. Finally, three of the nine ratios also proved useful in differentiation of the two Mediterranean *Dicrogaster* spp.: the elongation index and VSW/BW (ANOVA, both $p<0.0001$); and OSL/VSL ($p=0.002$). Specimens of *D. perpusilla* exhibited significantly higher values for the elongation index, sucker length ratio and relative width of the ventral sucker (see also Table 5.3).

A PCA was performed to examine the multivariate relation between the specimens of *Dicrogaster* irrespective of their identity. The first two principal components run on the correlation matrix between 26 metrical variables of the 38 specimens of *Dicrogaster* explained 87% of the variation in the dataset. A plot of the specimens in the first plane of the PCA (Fig. 5.8) showed two well-separated groups that correspond to *D. perpusilla* and *D. contracta.* The width of body, hermaphroditic sac and testis had the highest coefficients on the first component, which explained 81.8% of the total variance, while the length of prepharynx, oral sucker and oesophagus had important contributions to the second principal component. A LDA was applied to all specimens assigned to *a priori* groups defined by their species identification based on morphology in order to evaluate the morphometric differences between the two species. First, ln-transformed metrical data for 26 variables were subjected to a backward stepwise procedure of the LDA to select the variables yielding optimal separation between the species. The latter resulted in a 100% correct classification with no overlap in the ranges for canonical scores of the specimens (ranges 1.5 to 6.0 for *D. perpusilla* and -9.5 to -14.5 for *D. contracta*).

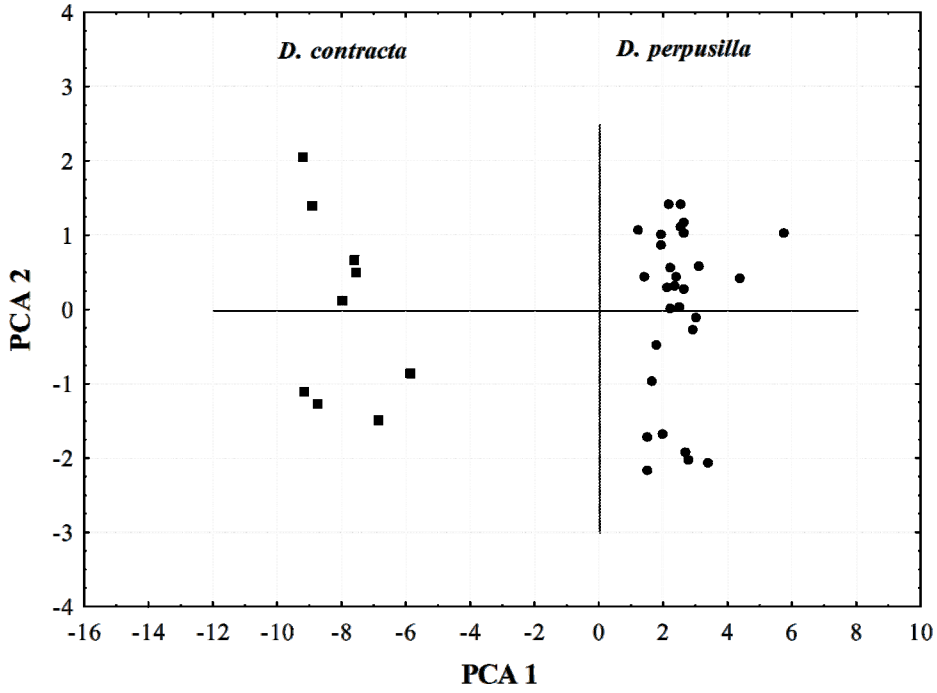

Fig. 5.8. Plot of the 38 specimens of *Dicrogaster* spp. in the first plane of the PCA

A second backward stepwise LDA was carried out using ratio data only (10 variables, after a square-root transformation). In this analysis one *D. contracta* was misclassified (accuracy of 97.4%; range for canonical scores 1.0 to -4.0 and 1.0 to 4.5 for *D. perpusilla* and *D. contracta*, respectively). The following variables were the most important for the discrimination of the two Mediterranean *Dicrogaster* spp.: (i) the length of the external seminal vesicle; (ii) the length of the testis; (iii) the length of the vitellarium; (iv) the relative width of the ventral sucker (VSW/BW); and (v) the elongation index.

5.2.5. Key to the recognised species of *Dicrogaster*

1a. Ventral sucker small in relation to body; width <1/3 of body width, located in first third of body (*i.e.* forebody relatively short). Hermaphroditic sac length 2-3× length of ventral sucker (HSL/VSL=220-300%). Vitellarium a single large compact mass of follicles distinctly larger than pharynx ..... *D. fastigata*

1b. Ventral sucker large in relation to body (VSW/BW = 38-97%), located in middle third of body or more posteriorly (*i.e.* forebody relatively long). Hermaphroditic sac comparable to ventral sucker in length (HSL/VSL=62-154%). Vitellarium two compact adjacent or overlapped masses of follicles; each mass smaller or similar in size to pharynx 2

2a. Body minute (BL=203-467 μm). Ventral sucker large in relation to body (VSW/BW=56-97%). Intestinal bifurcation at level of anterior margin of ventral sucker or slightly posterior. Hermaphroditic sac thin-walled, antero-dorsal to ventral sucker. Internal seminal vesicle occupies < 1/2 of hermaphroditic sac. Metraterm short and wide (< 1/2 of hermaphroditic sac length), with thin walls. Eggs 41-53×22-26 μm; length up to twice length of pharynx ..... *D. perpusilla*

2b. Body distinctly larger (BL=590-904 μm). Ventral sucker small in relation to body (VSW/BW=38-51%). Intestinal bifurcation in forebody. Hermaphroditic sac muscular, anterior to ventral sucker. Internal seminal vesicle occupies > 1/2 of hermaphroditic sac. Metraterm relatively long (*c.*2/3 of hermaphroditic sac length), narrow, with thick glandular walls. Eggs 32-43×17-26 μm; length av. *c.*1/2 length of pharynx .... *D. contracta*

5.3. Genus *Forticulcita* Overstreet, 1982

5.3.1. Background

Overstreet (1982) erected *Forticulcita* Overstreet, 1982 for *F. glabra* Overstreet, 1982 (type-species) from *Valamugil seheli* (Forsskål) in the Red Sea. This species was later reported, only once, form the Mediterranean by D'Amelio *et al.* (1995). A single species, *F. mugilis* Hassanine, 2007, was added to the genus based on material from *Crenimugil crenilabis* (Forsskål) in the northern Red Sea (off Sharm El-Sheikh, Egypt, see Hassanine, 2007).

5.3.2. Diagnosis

Body fusiform, with maximum width at level of ventral sucker. Tegument armed. Eye-spot pigment dispersed between oral sucker and hermaphroditic sac level. Oral sucker subterminal. Ventral sucker about size of oral sucker or larger. Forebody up to a third of body length. Prepharynx short. Pharynx large, subspherical. Oesophagus 2-6 times length of pharynx. Intestinal bifurcation posterior to ventral sucker. Caeca two, sac-like, end blindly at about mid-body or more posterior. Testis single, dextral to submedian. External seminal vesicle tubular, distinctly longer than internal seminal vesicle. Hermaphroditic sac elongate, subcylindrical, arcuate. Internal seminal vesicle tubular to elongate-oval. Hermaphroditic duct narrow, unarmed. Ejaculatory organ distinctly muscular, cylindrical. Genital atrium shallow. Genital pore median, just anterior to ventral sucker. Ovary pretesticular, contiguous with or overlapping testis. Uterine seminal receptacle present. Metraterm long. Eggs numerous, operculate; developed miracidia with single or two fused eye-spots. Vitellarium a single large spherical to subtriangular compact mass of small follicles, at level of or posterior to gonads. Excretory system Y-shaped, pore terminal, wide. In mullets (Mugilidae). Type-species: *F. glabra* Overstreet, 1982.

5.3.3. Review of species

Forticulcita glabra Overstreet, 1982

Records

References: 1. Overstreet (1982); 2. D'Amelio *et al.* (1995).

Description: 1.

Definitive hosts: *Valamugil seheli* (Forsskål) (1); *Liza ramado* (Risso) (2).

Distribution: Area 51, subarea 1 (Red Sea) (type-locality: Gulf of Aqaba) (1); area 37, subarea 3 (Eastern Mediterranean) (2).

Remark

This is the type-species of the genus, which was described in sufficient detail from a mullet species in the Red Sea (Overstreet, 1982) and subsequently recorded in the Mediterranean (D'Amelio *et al*., 1995). Characteristic features of *F. glabra* include a short forebody, conspicuous caeca and small eggs.

Forticulcita gibsoni Blasco-Costa, Montero, Balbuena, Raga & Kostadinova, in press

Type-host: *Mugil cephalus* (L.).

Type-locality: Off Santa Pola, Spain (38º00'–38º20'N, 0º10'–0º40'E; 04.x.2004). Area 37, subarea 1 (Western Mediterranean).

Other locality: Ebro Delta, Spain (40º30'– 40º50'N, 0º30'–1º10'E). Area 37, subarea 1 (Western Mediterranean).

Site: Intestine.

Type-material: Holotype BMNH 2008.10.7.61; paratypes BMNH 2008.10.7.62-76.

Etymology: The species is named for Dr David Gibson, Natural History Museum, London, UK in recognition of his immense contribution to the development of studies on taxonomy of marine fish digeneans at the University of Valencia.

Description (Fig. 5.9; Table 5.6)

[Based on 17 whole-mounted adult specimens.] Body long, fusiform, narrowing to 'neck' region anterior to genital pore and long (approx. third to almost half of body length) 'tail' region in hindbody. 'Tail' typically devoid of organs (except stem of excretory vesicle; single

uterine loop reaches its middle in 5 specimens). Maximum body width at level of ventral sucker or more posterior; width 22-36 (29)% of body length. Tegument thin, armed with minute spines to level of testis; 'tail' region with fine striations. Eye-spot pigment dispersed between oral sucker and hermaphroditic sac. Oral sucker small, slightly transversely oval, with subterminal aperture. Ventral sucker spherical, small, about size of oral sucker [sucker length ratio 1:0.91-1.51 (1:1.20); sucker width ratio 1:0.87-1.21 (1:1.05)], in second quarter of body. Forebody short, 22-31 (25)% of body length.

Prepharynx apparently absent to short (up to about half of pharynx length); pharynx large, muscular, subspherical. Oesophagus 2-6 times length of pharynx; intestinal bifurcation typically well posterior to ventral sucker (dorsal to its posterior third in 6 worms). Caeca 2, sac-like, length about twice width, with thick epithelium, end blindly far posterior to ventral sucker in about middle of body, 45-61 (52)% from posterior extremity.

Testis single, dextral, elongate-oval, smooth, in second quarter of body, posterior to ventral sucker (postero-dorsal to it in 2 worms); post-testicular space 45-57 (52)% of body length. External seminal vesicle just posterior and contiguous with hermaphroditic sac, wide-tubular to elongate-oval when filled with sperm, long, distinctly longer (about twice) than internal seminal vesicle, approaches hermaphroditic sac in length. Hermaphroditic sac thin-walled, very long, narrow (average length 3 × width), antero-dorsal to ventral sucker, reaches posteriorly to distance equivalent to ventral sucker length (dorsal to posterior margin of ventral sucker in 3 worms), at least twice as long as ventral sucker [HSL/VSL=191-293 (242)%], contains internal seminal vesicle, numerous large prostatic cells (vesicular pars prostatica not observed), long metraterm, short hermaphroditic duct and eversible ejaculatory organ (terminology of Overstreet, 1982). Internal seminal vesicle thin-walled, saccular, elongate-oval, occupies about third of hermaphroditic sac. Hermaphroditic duct *c*.20% of hermaphroditic sac length, narrow. Ejaculatory organ muscular, cylindrical, 127-152 × 32-40 when evaginated. Genital atrium shallow, 10-14, but distinct. Genital pore round, median, just anterior to ventral sucker.

Ovary subglobular, in second quarter of body, adjacent or close to posterior margin of hermaphroditic sac, occasionally more posterior (dorsal to testis in 1 worm) or anterior (dorsal to ventral sucker in 1 worm), contiguous with or overlaps testis ventrally, or at level of testis but separated by uterine loop (5 worms). Uterine seminal receptacle, Mehlis' gland and Laurer's canal not observed. Uterus thin-walled, restricted to anterior half of hindbody. Metraterm long (*c*.50-65% of hermaphroditic sac length), with thick glandular walls. Eggs numerous, small in relation to body (average egg-length 80% of pharynx length); developed

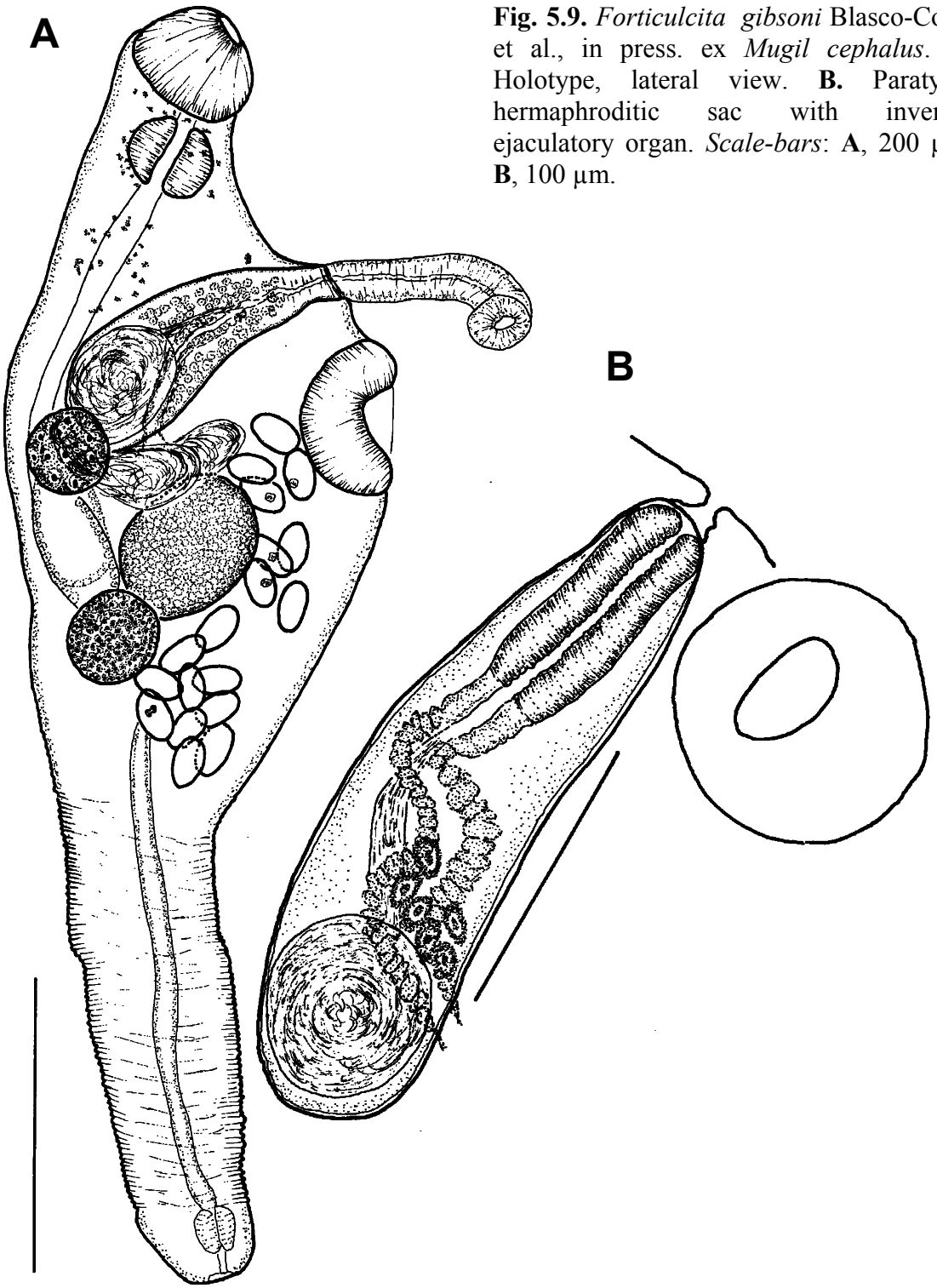

Fig. 5.9. *Forticulcita gibsoni* Blasco-Costa et al., in press. ex *Mugil cephalus*. **A.** Holotype, lateral view. **B.** Paratype, hermaphroditic sac with inverted ejaculatory organ. *Scale-bars*: **A**, 200 μm; **B**, 100 μm.

miracidia with single or 2 fused eye-spots. Vitellarium a single large compact mass of small follicles, smooth, spherical, posterior to gonads (at level of testis in 2 worms), at least as large as pharynx [VL/PHL=100-154% (122)%].

Stem of excretory vesicle tubular, long, with relatively thick (*c*.3) epithelium and large muscular sphincter, 16-35 × 14-32 (25 × 23), located close to posterior extremity; bifurcation at mid-hindbody; anterior limits of excretory arms obscured by uterus; pore terminal, wide.

Remarks

This strongly elongate haploporine form, which was preliminary identified as a 'strange *Dicrogaster*', was unexpectedly discovered in the samples from off Santa Pola. The material keys down to and appears most similar to *Forticulcita glabra* Overstreet, 1982 described from *V. seheli* in the Gulf of Aqaba (Northern Red Sea) due to the following characters: (i) the alimentary tract includes two distinct caeca; (ii) the vitellarium is a single mass of compact follicles; (iii) the caecal length is less than half of the body length; (iv) the external seminal vesicle is very elongate; (v) the hermaphroditic sac is very elongate; (vi) there is a long metraterm; (vii) the hermaphroditic duct is unarmed; and (viii) the ejaculatory (intromittent) organ is muscular, cylindrical, eversible and unarmed (see Overstreet, 1982; Overstreet & Curran, 2005). However, *F. glabra* is a much larger and robust form, with a somewhat shorter forebody in relation to body-length, a strongly muscular ventral sucker which is distinctly larger than the oral sucker, and a more posteriorly located testis. Furthermore, although the elongation index for *F. glabra,* as estimated from fig. 1 of Overstreet (1982), appears to be very similar to that of the present material, the latter posses a narrow 'neck' region anterior to the genital pore and long striated 'tail' region in the hindbody which is devoid of organs. Finally, the bulk of the uterus is restricted to the anterior half of the hindbody in *F. gibsoni*, and almost all of the metrical data are outside the lower range observed in *F. glabra* (see Table 5.6 above), except for the eggs which are larger (34-44 × 18-24 *vs* 25-34 × 14-17 μm).

F. gibsoni differs from the recently described material from the mugilid *Crenimugil crenilabis* (Forsskål) in the Red Sea, *F. mugilis* Hassanine, 2007, in most of the metrical features, due to the fact that the latter species is much larger than *F. glabra* (see Hassanine, 2007; Table 5.6). In addition, the egg-size of *F. gibsoni* is much smaller (34-44 × 18-24 *vs* 45-54 × 30-36; mean 39 × 21 *vs* 49 × 33 μm); the ventral sucker is smaller in relation to oral

sucker (sucker width ratio 1: 0.87-1.21 *vs* 1:1.55-1.79; mean 1:1.05 *vs* 1:1.67); the testis is more anterior (TEND/BL = 45-57 *vs* 30%); the hermaphroditic sac is long in relation to the ventral sucker (*c.*2-3 times longer *vs* nearly as long as the ventral sucker); and the external seminal vesicle is wider (mean 44 *vs* 25 μm) (Table 5.6). The morphological differences listed above, in combination with the substantial geographical and host separation of the three forms, justify the distinct status of *F. gibsoni*.

A comparison with the only form of *Dicrogaster* which possesses a single vitellarium, *D. fastigata*, is pertinent in view of the initial misidentification. The two species show a number of other similarities: (i) body shape (the original description of *D. fastigata* only); (ii) a ventral sucker similar in size to the oral sucker and located in the second quarter of the body, *i.e.* forebody short; (iii) a long oesophagus and caeca terminating far posterior to the ventral sucker; (iv) a long hermaphroditic sac; and (v) generally overlapping ranges of metrical data (see Tables 5.4, 5.6). However, the intestinal bifurcation in *F. gibsoni* is located well posterior to the ventral sucker, the hermaphroditic duct is very short (*vs* more than half the length of the hermaphroditic sac) and unarmed, the genital pore is more posterior, the uterus is confined to the anterior half of the body, the metraterm is much longer, and the vitellarium is spherical and posterior to the gonads. Furthermore, none of the descriptions of *D. fastigata* indicate the presence of an eversible hermaphroditic duct (as observed, for example, in *D. contracta*, see above), whereas many specimens of *F. gibsoni* exhibited an everted muscular ejaculatory organ (the latter was also distinguishable when withdrawn). Finally, although a range overlap exists in the metrical data [*D. fastigata* showing a much wider range of variation for characters which exhibited low values of the coefficient of variation (CV<10%; Table 5.6) in the specimen set of *F. gibsoni* described herein]; *D. fastigata* also appears distinctly less elongate (elongation index 2.5-2.6 *vs* 2.8-4.6) and possesses a more posteriorly located testis.

Forticulcita mugilis **Hassanine, 2007**

Record

Reference, Description: Hassanine (2007).

Definitive host: *Crenimugil crenilabis* (Forsskål).

Distribution: Area 51, subarea 1 (Red Sea) (type-locality: Sharm El-Sheikh, South Sinai, Egypt).

Remark

This species was recently described in detail (Hassanine, 2007). *F. mugilis* is distinguished from the other species of the genus by its largest size, largest oral and ventral suckers, longest forebody and largest eggs.

5.3.4. Multivariate morphometric analyses

Given the preliminary misidentification of the specimens of *F. gibsoni* (as commented above), the metrical data of the Mediterranean species of *Dicrogaster* were combined with those for *F. gibsoni* and subjected to LDA in order to asses which metrical variables better discriminate the three small Mediteranean forms of Haploporinae. The measurements recorded from 55 specimens (29 *Dicrogaster perpusilla*, 9 *D. contracta* and 17 *F. gibsoni*) were ln-transformed and a LDA was applied to all specimens assigned to *a priori* groups defined by their species identification based on morphology. First, ln-transformed metrical data for 26 variables were subjected to a backward stepwise procedure of the LDA to select the variables yielding optimal separation between the species. A second backward stepwise LDA was carried out using proportion/ratio data only (10 variables, after a square-root transformation). The two backward stepwise LDA procedures clearly separated the specimens of the three species, *D. perpusilla*, *D. contracta* and *F. gibsoni*, described in the present study from mugilid fishes of the western Mediterranean (accuracy of 100%; Wilks' Lambda=0.00148; $F_{(8,98)}$=306.47, p<0.0001 and 0.019; $F_{(6,100)}$=103.65. p<0.0001) (Fig. 5.10). The following variables were the most important in the discrimination of the three species: (i) the width of the body; (ii) the size of the external seminal vesicle; (iii) the width of the vitellarium; (iv) the relative length of the hermaphroditic sac (HSL/VSL); (v) the relative width of the ventral sucker (VSW/BW); and (vi) the elongation index.

Table 5.6. Comparative metrical data for *Forticulcita* spp.

Species	*F. gibsoni*			*F. glabra*	*F. mugilis*
Locality	Off Santa Pola & Ebro Delta (Western Mediterranean)			Gulf of Aqaba (Red Sea)	Off Sharm El-Sheikh (Red Sea)
Source	Present study			Overstreet (1982)	Hassanine (2007)
	Range	Mean±SD	CV (%)	Range	Range (mean)
Measurements					
BL	777-1,024	893 ± 79	8.9	1,185-1,767	1,814-2,761 (2,287)
BW	201-299	253 ± 26	10.1	348-516	530-810 (670)
OSL	69-93	79 ± 6	8.0	103-123	200-245 (222)
OSW	80-107	95 ± 8	8.9	118-167	200-245 (222)
PL	0-35	16 ± 10	60.0	short	66-93 (79)
PHL	45-62	50 ± 5	9.7	66-86	130-176 (153)
PHW	48-70	63 ± 5	8.1	83-96	138-186 (162)
OL	116-296	200 ± 50	24.9	-	571-825 (698)
VSL	82-109	95 ± 9	9.3	183-230	-
VSW	85-115	100 ± 8	8.2	189-222	310-439 (374)
HSL	183-261	231 ± 20	8.6	-	344-490 (417)
HSW	54-96	75 ± 11	15.0	-	118-160 (139)
ISVL	53-104	72 ± 15	21.3	-	-
ISVW	35-77	58 ± 13	22.7	-	-
ESVL	123-197	160 ± 29	18.0	-	308-435 (371)
ESVW	34-62	44 ± 9	21.3	-	23-28 (25)
HDL	48-122	78 ± 39	49.9	short	-
HDW	21	-	-	-	-
TL	75-127	100 ± 17	16.9	218-388	125-168 (146)
TW	62-90	75 ± 10	13.1	124-208	142-187 (164)
OVL	51-137	69 ± 20	29.4	42-147	-
OVW	47-81	60 ± 10	15.9	44-96	118-170 (144)
VL	53-71	61 ± 6	9.6	77-208	93-130 (111)
VW	46-64	56 ± 5	9.5	71-166	115-132 (120)
EL	34-44	39 ± 3	7.1	25-34	45-54 (49)
EW	18-24	21 ± 1	5.0	14-17	30-36 (33)
Distances					
FO	184-291	225 ± 29	13.0	-	543-930 (736)
CEND	397-622	474 ± 60	12.6	-	-
TEND	395-538	464 ± 43	9.3	-	436-625 (530)
UEND	147-442	288 ± 86	29.8	-	-
Ratios					
Elongation index	2.76-4.60	3.57 ± 0.52	14.5	3.5*	3.4*
FO/BL (%)	22-31	25 ± 2	9.9	17-22	30-33
OSL/VSL	1:0.91-1.51	1:1.20 ± 0.16	13.0	-	-
OSW/VSW	1:0.87-1.21	1:1.05 ± 0.08	7.7	1:1.2-1.7 (1:1.4-1.5)**	1:1.55-1.79 (1:1.67)
HSL/VSL (%)	191-293	242 ± 27	11.1	-	106*
TEND/BL (%)	45-57	52 ± 4	7.2	28-41	30*
VL/PHL (%)	100-154	122 ± 15	12.5	-	-
CEND/BL (%)	45-61	52 ± 5	10.2	37-54	32*

*, Estimated from the published drawing; **, When suckers not distorted, see Overstreet (1982).

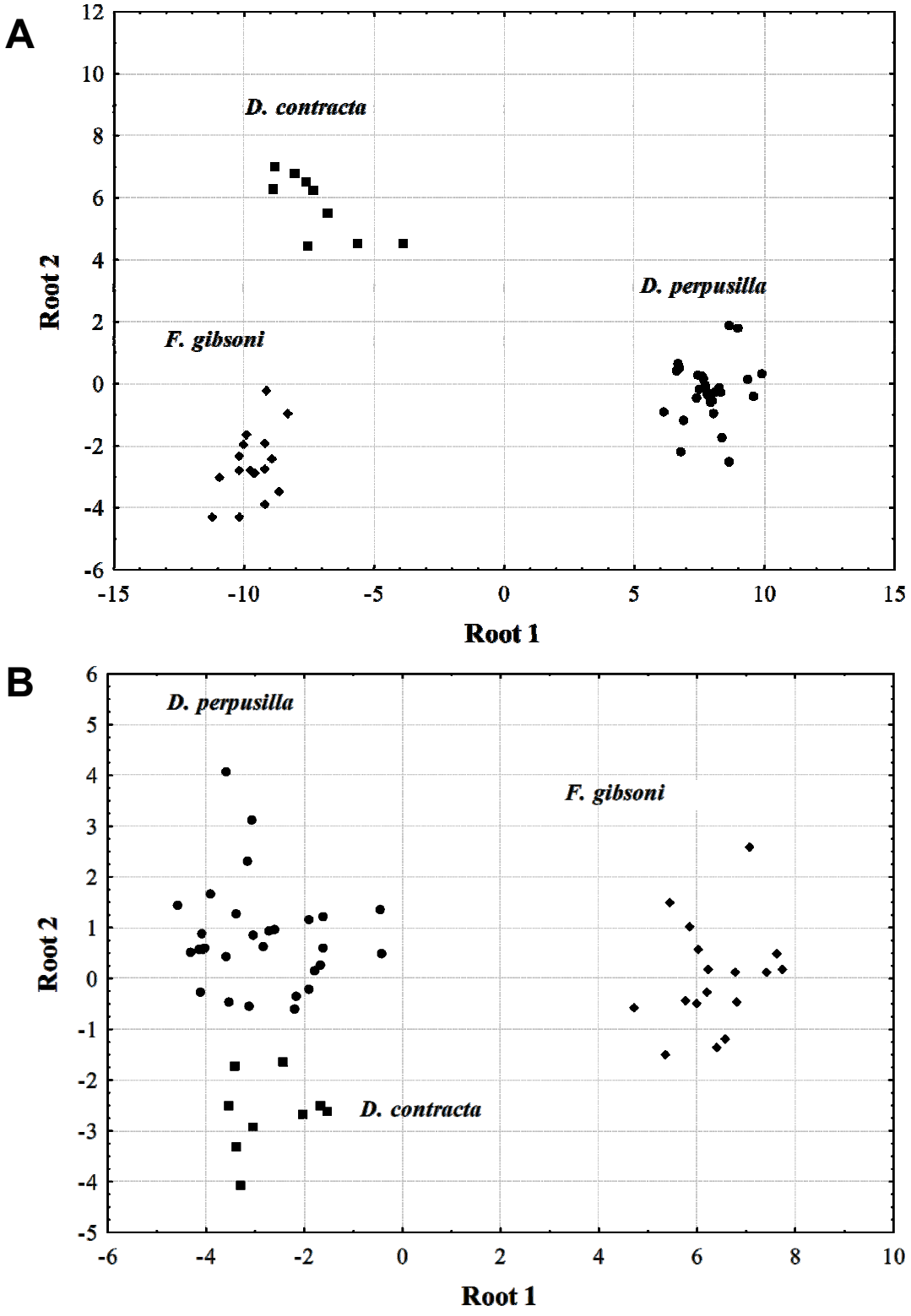

Fig. 5.10. Plots of the 55 haploporine specimens (*D. perpusilla*, *D. contracta* and *F. gibsoni*) against the first and second canonical discriminant functions resulting from backward stepwise LDA procedures. **A.** All three sympatric species, variant with 26 metrical variables. **B.** All three sympatric species, variant with 9 relative proportions/ratios.

5.3.5. Key to the species of *Forticulcita*

1a. Body small (BL<1,050 μm; BW<300 μm). Suckers small (OSW<110 μm; VSW<120 μm); ventral sucker smaller in relation to oral sucker (width ratio<1:1.21). Testis more anteriorly located (TEND/BL=45-57%). Eggs 34-44 × 18-24 μm ……... ..... *F. gibsoni*

1b. Body larger (BL>1,100 μm; BW>300 μm). Suckers larger (OSW>110 μm; VSW>150 μm); ventral sucker larger in relation to oral sucker (width ratio>1:1.21). Testis more posterior (TEND/BL=28-41%) 2

2a. Body large (BL>1,800 μm; BW>500 μm). Forebody relatively long (FO/BL=30-33%). Suckers large, muscular (OSW>200 μm; VSW>300 μm). Eggs 45-54 × 30-36 μm ..... *F. mugilis*

2b. Body smaller (BL<1,800 μm; BW<520 μm). Forebody short (FO/BL=17-22%). Suckers smaller (OSW<180 μm; VSW<250 μm). Eggs 25-34 × 14-17 μm .... *F. glabra*

5.4. Genus *Lecithobotrys* Looss, 1902

5.4.1. Background

After the erection of *Lecithobotrys* Looss, 1902 for *L. putrescens* Looss, 1902 from *L. aurata* in the Adriatic Sea (Looss, 1902), six nominal species have been assigned to this genus: *L. vitellosus* Sharma & Gupta, 1970; *L. sprenti* Martin, 1973; *L. mugilis* Rekharani & Madhavi, 1985; *L. helmymohamedi* Ramadan, Saoud, Ashour & Mansour, 1989; *L. aegyptiacus* Hassan, El-Aziz, Khidr & Abu Samak, 1990 and *L. stomachicolum* Machida, 1996 (see Sharma & Gupta, 1970; Martin, 1973c; Rekharani & Madhavi, 1985; Ramadan *et al.*, 1989b; Hassan *et al.*, 1990a; Machida, 1996). Overstreet & Curran (2005) transferred *L. sprenti* to *Saccocoelium*, and *Paralecithobotrys brisbanensis* Martin, 1974 to *Lecithobotrys,* synonymised *L. helmymohamedi* with *Saccocoelium tensum* Looss, 1902 and considered the status of *L. mugilis* uncertain because of the extremely large eggs and short caeca. While the present study shows agreement with some of these actions, *Unisaccus* Martin, 1973 is considered to be a better placement for *L. sprenti* and *L. mugilis* (see section 5.5. this chapter, and Blasco-Costa *et al.*, 2009c).

Species of *Saccocoelioides* Szidat, 1954 have at least temporarily been included within *Lecithobotrys*. Thus Martin (1973c) transferred *Saccocoelioides magniovatus* Szidat, 1954 to this genus and suggested that the same action should be taken with respect to *S. magnus* Szidat, 1954. Rekharani & Madhavi (1985) indicated that *S. octavus* Szidat, 1970 may also belong to *Lecithobotrys* and Nasir & Gómez (1976) considered *Saccocoelioides* Szidat, 1954 to be a junior synonym of the latter genus. In their revision of the Haploporidae, Overstreet & Curran (2005) temporarily accepted *Lecithobotrys* as a valid genus and reorganised and transferred *Saccocoelioides* (*sensu stricto*) to the new subfamily Chalcinotrematinae Overstreet & Curran, 2005.

5.4.2. Generic diagnosis

Body fusiform to pyriform, with maximum width posterior to testis. Tegument unarmed. Eye-spot pigment abundant, spread thoughout body, with concentrations between pharynx and mid-level of oral sucker. Oral sucker subterminal, transversely oval. Ventral sucker spherical, smaller than oral sucker, in second quarter of body. Forebody short, up to third of body length. Prepharynx distinct, much shorter than to similar in length to pharynx. Pharynx

elongate oval. Oesophagus up to three times length of pharynx. Intestinal bifurcation at level of posterior margin of hermaphroditic sac or just posterior. Caeca two, relatively narrow, end blindly at mid-hindbody. Testis single, subspherical, median, at some distance posterior to ventral sucker (distance from posterior margin of ventral sucker at least length of ventral sucker). External seminal vesicle contiguous with hermaphroditic sac, elongate-oval, curved, distinctly larger than internal seminal vesicle. Hermaphroditic sac elongate-oval, antero-dorsal to ventral sucker but may extend posterior to it, similar in length to twice length of ventral sucker. Internal seminal vesicle thin-walled, saccular, elongate-oval, occupies up to third of hermaphroditic sac. Pars prostatica vesicular, prostatic cells small. Hermaphroditic duct unarmed, faintly-muscular, less than third length of hermaphroditic sac. Genital atrium with muscular walls. Genital pore median, just anterior to ventral sucker. Ovary submedian, spherical, just anterior or dorsal to anterior region of testis. Uterine seminal receptacle not observed; blind seminal receptacle absent. Uterus occupies most of hindbody. Metraterm indistinct, short. Eggs numerous, operculate; developed miracidia with two fused eye-spots. Vitellarium two symmetrical separated lateral clusters of 7-10 distinct subglobular groups of small coalesced follicles, at anterior level of ovary and testis or more anterior. Excretory vesicle large, Y-shaped; stem wide tubular, arms thick-walled, with ramified appearance; pore terminal, narrow. In mullets (Mugilidae). Type-species: *L. putrescens* Looss, 1902.

5.4.3. Review of species

Lecithobotrys putrescens Looss, 1902

Material studied

Ex *Liza saliens* (Risso). Intestine. Ebro Delta, Spain. (22.vi.2004). BMNH 2008.10.27.56-60.

Ex *L.aurata* (Risso). Intestine. Fogliano, Italy. Collected by Paggi *et al.* BMNH 1991.2.4.7-9.

Records

References: 1. Looss (1902); 2. Paperna (1964); 3. Paperna & Overstreet (1981); 4. Merella & Garippa (1998); 5. Merella & Garippa (2001); 6. Al-Bassel (2003); 7. Ragias *et al*. (2005); 8. Present study.

Descriptions: 1; 8.

Definitive hosts: *Liza aurata* (Risso) (type-host) (1, 7, 8); *L. ramado* (Risso) (2, 3, 5, 6, 7); *L. saliens* (Risso) (4, 8); *Mugil cephalus* L. (2, 3).

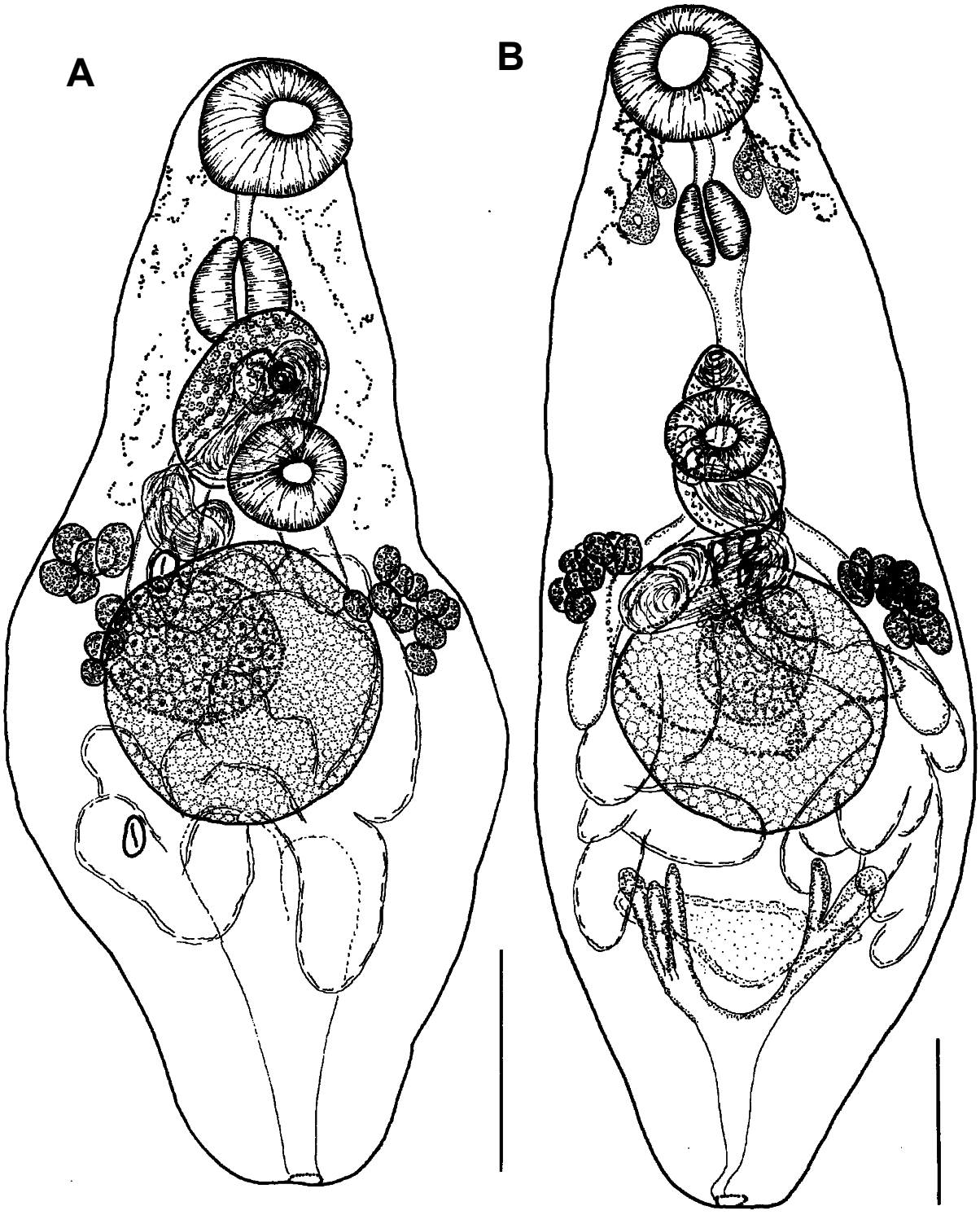

Fig. 5.11. *Lecithobotrys putrescens* ex *Liza saliens*. Ventral views with uterus in outline. *Scale-bars*: 200 μm.

Distribution: Area 37, subarea 2 (Central Mediterranean) (type-locality: off Trieste, Adriatic Sea) (1); area 37, subarea 3 (Eastern Mediterranean) (2, 3, 6, 7); area 37, subarea 1 (Western Mediterranean) (4, 5, 8).

Description (Figs. 5.11-5.13; Table 5.7)

[Based on 11 whole-mounted adult specimens.] Body fusiform to pyriform, with maximum width in last third of body, posterior to testis; width 23-39% of body length. Tegument thin, armed with small spines. Eye-spot pigment abundant, spread throughout body (notably dense in juvenile worms), with concentrations between pharynx and mid-level of oral sucker. Two groups of 2 gland-cells each present on either side of pharynx/prepharynx in some specimens. Oral sucker subterminal, transversely oval. Ventral sucker spherical, smaller than oral sucker (sucker length ratio 1: 0.56-0.81; width ratio 1:0.63-0.73), in second quarter of body. Forebody 25-37% of body length.

Prepharynx distinct, much shorter to similar in length to pharynx (PL/PHL=0.3-1.1); pharynx elongate oval. Oesophagus up to three times length of pharynx; intestinal bifurcation at level of posterior margin of hermaphroditic sac or just posterior; caeca 2, relatively narrow, end blindly at mid-hindbody (36-53% of body length from posterior extremity).

Testis single, large, median, sub-spherical, smooth, at some distance posterior to ventral sucker (distance from posterior margin of ventral sucker at least length of ventral sucker); post-testicular space 29-44% of body length. External seminal vesicle contiguous with hermaphroditic sac, saccular, elongate-oval, curved, distinctly larger than internal seminal vesicle. Hermaphroditic sac small, thin-walled, elongate-oval, antero-dorsal to and/or extending posterior to ventral sucker, similar in length to twice length of ventral sucker (HSL/VSL=109-224%), contains internal seminal vesicle, vesicular pars prostatica (26-68 × 21-48), numerous small prostatic cells, indistinct metraterm and short hermaphroditic duct. Internal seminal vesicle thin-walled, saccular, elongate-oval, occupies up to third of hermaphroditic sac. Hermaphroditic duct unarmed, faintly muscular, less than third length of hermaphroditic sac. Genital atrium with muscular walls. Genital pore round, median, just anterior to ventral sucker.

Ovary spherical, submedian, dorsal to anterior part of testis. Uterine seminal receptacle not observed; blind seminal receptacle absent. Laurer's canal not observed. Mehlis' gland visible in two specimens, 89 × 83-109. Uterus thin-walled, occupies most of hindbody. Metraterm indistinct, joins hermaphroditic duct dorsal to seminal vesicle. Eggs numerous, operculate; developed miracidia with 2 fused eye-spots. Vitellarium 2 symmetrical separated

lateral clusters of 7-10 distinct subglobular groups of small coalesced follicles, at anterior level of ovary and testis or more anterior.

Excretory vesicle large, Y-shaped; stem wide tubular; arms thick-walled, with ramified appearance in larger specimens; pore terminal, narrow.

Remarks

The original description of *Lecithobotrys putrescens* by Looss (1902) was brief (including metrical data limited to the size of the body, suckers, pharynx and eggs) and based on a single worm. None of the subsequent records documents the morphology of the species. Although the specimens from *L. aurata* were slightly larger than those from *L. saliens,* they are smaller than the type-specimen of *Lecithobotrys putrescens* (Table 5.7). Despite these differences, the illustration of Looss (fig. 14 in Looss, 1902) clearly represents the species described herein. The new morphological observations enabled a refinement of the generic diagnosis of *Lecithobotrys* (see above). Overstreet & Curran (2005) suggested that the latter may be congeneric with *Haploporus*. However, *Lecithobotrys* can be distinguished from *Haploporus* based on: (i) the distribution of eye-spot pigment (spread throughout entire body *vs* dispersed between levels of the

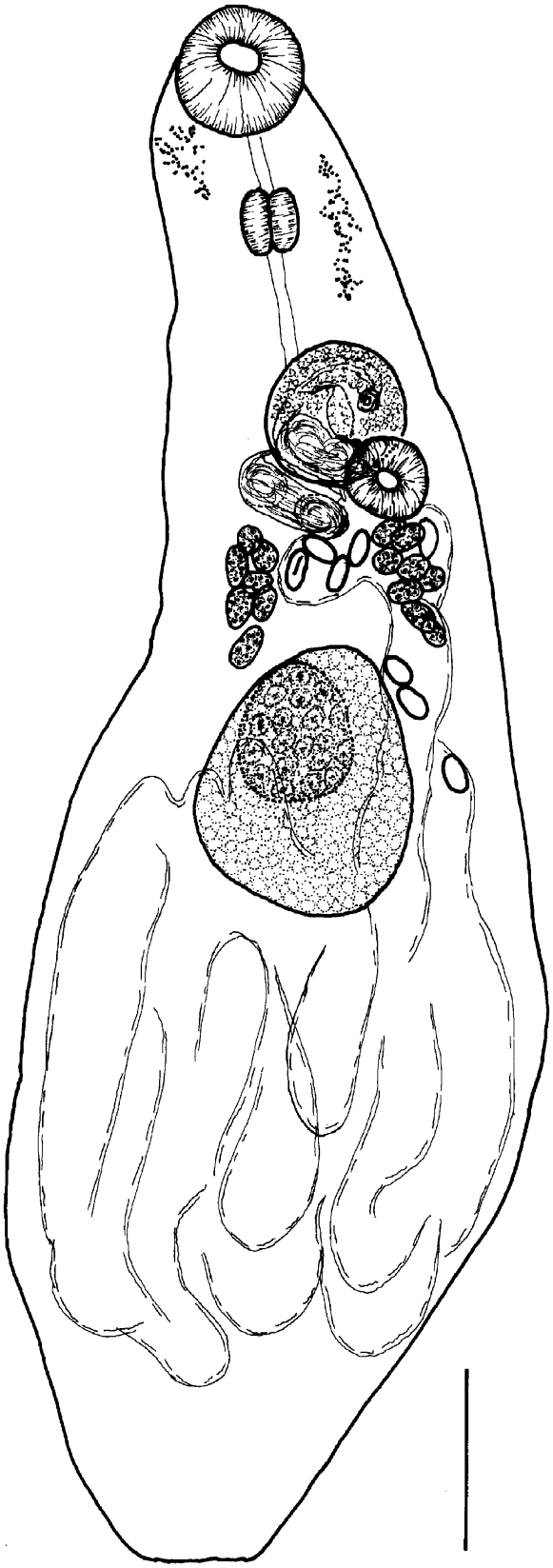

Fig. 5.12. *Lecithobotrys putrescens* ex *Liza aurata* (BMNH 1991.2.4.7-9). Ventral view with uterus in outline. *Scale-bar*: 200 μm.

Table 5.7. Comparative metrical data for *Lecithobotrys putrescens.*

Source	Looss (1902)	Present study		Present study
Host	*L. aurata*	*L. saliens*		*L. aurata*
	Range	Range	Mean	Range
Measurements				
BL	2, 300	1,035-1,528	1,300	1,732-1,818
BW	750	304-536	425	604-714
OSL	-	109-161	134	128-140
OSW	210	121-181	150	146-171
PL	-	25-89	59	55-61
PHL	-	81-106	93	73-104
PHW	100	71-94	85	61-85
OL	-	190-304	254	287
VSL	-	67-121	93	92-104
VSW	150	78-129	103	98-116
HSL	-	114-258	170	163-169
HSW	-	72-134	103	150-154
GAL		51-63	55	-
GAW		23-61	44	25
ISVL	-	61-176	92	93-146
ISVW	-	33-66	53	59-76
ESVL	-	56-357	183	196-249
ESVW	-	40-91	60	53-84
TL	-	170-329	231	305-323
TW	-	139-330	222	256-311
OVL	-	63-176	117	134-159
OVW	-	65-164	123	134-195
EL	44-47	37-46	41	40-44
EW	26-28	21-26	22	19-21
No. of follicles in each lateral cluster	7	7-10		7-9
Distances				
FO	-	299-486	423	482-488
CEND	-	468-792	564	732
TEND	-	349-655	467	732-805
UEND	-	68-383	203	192-195
Ratios				
BW/BL (%)	30	23-43	33	35-39
FO/BL (%)	34	25-37	32	27-28
OSL/VSL	-	1:0.56-0.86	0.70	1:0.66-0.81
OSW/VSW	1:0.71	1:0.63-0.80	0.70	1:0.67-0.68
HSL/VSL (%)	154	110-224	165	141-172
TEND/BL (%)	40	29-43	36	42-44
CEND/BL (%)	40	36-53	43	40
UEND/BL (%)	17	6-25	15	11

* Estimated from the published drawing.

pharynx and oral sucker); (ii) the shape and size of the seminal vesicles (both elongate-oval and external distinctly larger than internal *vs* subglobular and similar in size); (iii) the genital atrium (distinct, with muscular walls *vs* absent); and (iv) the structure of the vitellarium (in two separated lateral clusters of distinct subglobular groups of small coalesced follicles *vs* two separated compact masses).

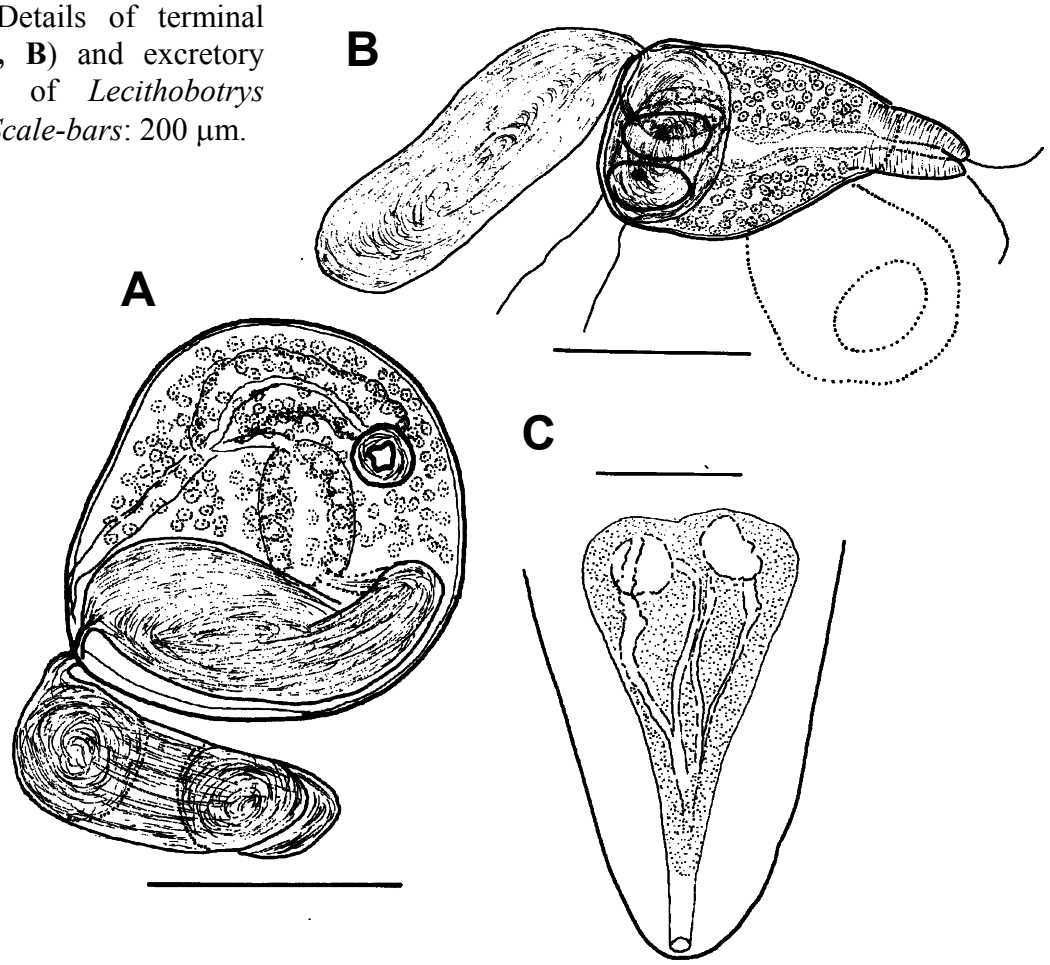

Fig. 5.13. Details of terminal genitalia (**A, B**) and excretory vesicle (**C**) of *Lecithobotrys putrescens*. *Scale-bars*: 200 μm.

Species transferred to other genera

***Saccocoelium* Looss, 1902**

***Saccocoelium tensum* Looss, 1902**

Syn. *Lecithobotrys aegyptiacus* Hassan, El-Aziz, Khidr & Abu Samak, 1990 (new synonym)

Record

Reference, Description: Hassan *et al*. (1990a).

Definitive hosts: *Liza ramado* (Risso) (type-host).
Distribution: Area 37, subarea 3 (Eastern Mediterranean) (type-locality: off Ras El-Bar, Egypt).

Remarks
This species was described as having saccular caeca, well-developed muscular genital atrium and hermaphroditic duct lined with crescentic sclerotised structures (see fig. 1A in Hassan *et al*., 1990a), all features characteristic of *Saccocoelium* Looss, 1902 (see Looss, 1902; Fares & Maillard, 1974; section 5.5. this chapter; Blasco-Costa *et al*., 2009c). All metrical data for *L. aegyptiacus* vary within the range of *S. tensum* (see below) with the exception of the somewhat larger ventral sucker (180-230 *vs* 95-170 μm), which might be due to combining measurements taken from both live and fixed material (see Hassan *et al*., 1990a). In view of this, *L. aegyptiacus* is regarded as a synonym of *S. tensum.*

Species inquirendae

***Lecithobotrys brisbanensis* (Martin, 1974) Overstreet & Curran, 2005**
Syn. *Paralecithobotrys brisbanensis* Martin*,* 1974

Records
References: 1. Martin (1974); Overstreet & Curran (2005).
Descriptions: 1, 2 (figure only).
Definitive host: *Mugil cephalus* L. (type-host).
Distribution: Area 71: Australia (1, 2) (type-locality: Brisbane River, Queensland).

Remarks
Overstreet & Curran (2005) based on the original description and examination of additional specimens transferred *Paralecithobotrys brisbanensis* Martin, 1974 to *Lecithobotrys*, a decision not accepted here. Thus, although *P. brisbanensis* bears some resemblance to *Lecithobotrys* in the structure of the vitellarium (in two lateral clusters of 7-8 follicles each) and sucker ratio, this distinctly more elongate (cylindrical) form lacks a muscular genital atrium and possesses an armed hermaphroditic duct lined with tiny spines or tubercles, features which are considered important at the generic level here (see above). *P. brisbanensis* also has a poorly developed hermaphroditic sac which is smaller in relation to the ventral

sucker [HSL/VSL=63-92%, estimated from published drawings (fig. 1 in Martin, 1974, and fig. 12.9 in Overstreet & Curran, 2005)]. Since the type-material is unavailable (see Overstreet & Curran, 2005), the description of new material from the type-species and locality, especially with regard to the morphology of the terminal genitalia, is needed to assess the generic affiliation of *P. brisbanensis*.

***Lecithobotrys vitellosus* Sharma & Gupta, 1970**

Record
Reference, Description: Sharma & Gupta (1970).
Definitive hosts: *Liza parsia* (Hamilton) (type-host).
Distribution: Area 57: India (type-locality: Madras (now Chennai), India.

Remark
The description of *L. vitellosus* is based on a single flattened (resulting in a dispersion of the vitelline follicles) specimen with abnormal eggs (measuring 22 × 15 µm). Further data are needed to assess the validity of *L. vitellosus* and its generic affiliation.

***Lecithobotrys* spp. innom.**

Record
Reference: Saoud *et al*. (1990).
Definitive hosts: *Liza ramado* (Risso); *Mugil cephalus* L.; *Chelon labrosus* (Risso).
Distribution: Egypt.

5.5. Genus *Saccocoelium* Looss, 1902

Syn. *Neosaccocoelium* El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 (new synonym); *Wlassenkotrema* Skrjabin, 1956

5.5.1. Background

Looss (1902) erected *Saccocoelium* Looss, 1902 for *S. obesum* Looss, 1902 (type-species) and *S. tensum* Looss, 1902, parasites of mullets (*M. cephalus*, *L. aurata* and *C. labrosus*) in the Adriatic Sea (off Trieste, Italy). Further studies have described and assigned six nominal species to this genus: *S. beauforti* Hunter & Thomas, 1961; *S. gohari* Ramadan, Saoud, Ashour & Mansour, 1989; *S. saoudi* El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992; *S. portsaidensis* [sic] El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992; *S. tripathi* Dutta, 1995; and *S. megasacculum* Liu, Wang, Peng, Yu & Yang, 2004 (Hunter & Thomas, 1961; Ramadan *et al*., 1989a; El-Shahawi *et al*., 1992; Dutta, 1995; Liu *et al*., 2004). *S. beauforti* was considered a member of *Skrjabinolecithum* Belous, 1954 by Manter (1963), tentatively transferred to *Saccocoelioides* Szidat, 1954 by Overstreet (1971), and finally assigned by Overstreet & Curran (2005) to *Culuwiya* Overstreet & Curran, 2005. As mentioned above, the latter authors also transferred *Lecithobotrys sprenti* Martin, 1973 and *L. helmymohamedi* Ramadan, Saoud, Ashour & Mansour, 1989 to *Saccocoelium*, the latter species being considered a synonym of *S. tensum*. Overstreet & Curran (2005) stated that the status of *Lecithobotrys mugilis* Rekharani & Madhavi, 1985 is uncertain and suggested a probable affiliation with *Saccocoelium* if the vitelline follicles were dispersed due to the fixation of the specimens under pressure. The extent of intra- and interspecific variation in *Saccocoelium* spp. is virtually unknown, since most species within the genus are known only from their original descriptions. Although the two Mediterranean forms, *S. tensum* and *S. obesum*, both described by Looss (1902), are the most widely reported species, there are few documented reports providing data on their morphology and some authors (*e.g*. Mikailov, 1958; Fischthal & Kunz, 1963; Ferreti & Paggi, 1965; Moravec & Libosvárský, 1975) considered them conspecific.

5.5.2. Generic diagnosis

Body elongate-oval, elongate-fusiform to subcylindrical, with tapered or rounded posterior extremity with bell-shaped concavity (type). Tegument armed. Eye-spot pigment dispersed

mostly on either side of pharynx. Oral sucker subterminal, spherical to transversely oval, muscular. Ventral sucker subspherical, muscular, smaller or larger than oral sucker. Forebody short to long. Prepharynx absent to long. Pharynx strongly muscular, large, elongate-oval to subcylindrical. Oesophagus short to long. Intestinal bifurcation dorsal to posterior to ventral sucker, typically dorsal to posterior third of hermaphroditic sac. Caeca two, sac-like, with thick lining of cells, end blindly at mid-body to mid-hindbody. Testis single, subspherical to elongate, in middle or last third of body. External seminal vesicle just posterior or slightly dorsal to hermaphroditic sac, saccular, globular to elongate-oval, similar in size to or smaller than internal seminal vesicle. Hermaphroditic sac thick-walled, muscular, elongate-oval, antero-dorsal to ventral sucker to reaching back well posterior to it, much longer than ventral sucker. Internal seminal vesicle thin-walled or lined by a layer of cells, saccular, elongate-oval, occupies up to half of hermaphroditic sac. Pars prostatica vesicular; prostatic cells numerous. Hermaphroditic duct wide, thick-walled; walls lined with crescentic, sclerotised structures. Genital atrium prominent, with well developed muscular walls. Genital pore median, between pharynx and ventral sucker. Ovary round, pretesticular, dorsal to ventral sucker or contiguous with testis. Uterine seminal receptacle distinct; blind seminal receptacle absent. Mehlis' gland small or diffuse. Uterus thin-walled, occupies almost entire hindbody (restricted to third quarter of body in *S. currani*). Metraterm distinct, joins hermaphroditic duct close to internal seminal vesicle. Eggs numerous, operculate; developed miracidia with single or two fused eye-spots. Vitellarium two symmetrical separated masses of loosely coalesced follicles, at level of ovary or more posterior. Excretory vesicle Y-shaped; stem tubular to wide-tubular, with small sphincter close to excretory pore in some species; pore terminal or subterminal. In mullets (Mugilidae). Type-species: *S. obesum* Looss, 1902.

5.5.3. Review of species

***Saccocoelium obesum* Looss, 1902**

Syns *Haploporus longicollum* Vlassenko, 1931; *Wlassenkotrema longicollum* (Vlassenko, 1931) Skrjabin, 1956

Material studied

Ex *Liza aurata* (Risso). Intestine. Ebro Delta, Spain (40º30'–40º50'N, 0º30'–1º10'E; 02.vi.2004). BMNH 2008.10.7.38-39.

Ex *L. aurata* (Risso). Intestine. Sozopol, Bulgaria (42º26'–42º19'N, 27º40'–28º05'E; 11.ix.2004). BMNH 2008.10.7.40.

Records

References: 1. Looss (1902); 2. Nicoll (1914); 3. Vlassenko (1931, as *Haploporus longicollum*); 4. Butskaya (1952, as *H. longicollum*); 5. Reshetnikova (1955, as *H. longicollum*); 6. Mikailov (1958, also as *H. longicollum*)*; 7. Fischthal & Kunz (1963)*; 8. Ferretti & Paggi (1965) *; 9. Fares & Maillard (1974); 10. Moravec & Libosvárský (1975) *; 11. Orecchia & Paggi (1978); 12. Ibragimov (1988); 13. Orecchia *et al*. (1988); 14. Paggi *et al*. (1988); 15. Radujković & Raibaut (1989); 16. Radujković *et al*. (1989); 17. Gaevskaya & Dmitrieva (1993); 18. D'Amelio *et al*. (1995); 19. Oguz (1995); 20. Di Cave *et al*. (1997); 21. Merella & Garippa (1998); 22. Domnich & Sarabeev (2000a); 23. Domnich & Sarabeev (2000b); 24. Domnich & Sarabeev (2000c); 25. Sarabeev (2000); 26. Sarabeev & Domnich (2000); 27. Dmitrieva & Gaevskaya (2001, also as *Wlassenkotrema longicollum*); 28. Merella & Garippa (2001); 29. Pronkina (2001); 30. Al-Bassel (2003); 31. Gaevskaya & Korniychuk (2003, as *W. longicollum*); 32. Ragias *et al*. (2005); 33. Present study.

Descriptions: 1; 3; 9; 16; 17; 29; 33.*

Definitive hosts: *Chelon labrosus* (Risso) (type-host) (1, 2, 9, 13, 16, 28); *Mugil cephalus* L. (1, 3, 4, 5, 7, 8, 9, 11, 17, 27); *M. soiuy* Basilewsky (22, 23, 24, 25, 26, 27); *Liza aurata* (Risso) (1, 5, 9, 12, 13, 14, 15, 16, 17, 21, 24, 27, 28, 29, 31, 32, 33); *L. ramado* (Risso) (4, 7, 9, 10, 13, 15, 16, 18, 19, 20, 27, 28, 30, 32); *L. saliens* (Risso) (5, 6, 9, 12, 17, 21, 27, 28, 31).

Distribution: Area 37, subarea 2 (Central Mediterranean) (type-locality: off Trieste, Adriatic Sea) (1, 8, 13, 15, 16); area 37, subarea 1 (Western Mediterranean) (9, 11, 21, 28, 33); area 37, subarea 3 (Eastern Mediterranean) (7, 10, 30, 32); area 37, subarea 4 (Black Sea): 4.1. Sea of Marmara (19), 4.2. Black Sea (4, 5, 17, 27, 29, 31, 33), 4.3. Azov Sea (22, 23, 24, 25, 26, 27); area 27 (Northeast Atlantic) (2); Caspian Sea (6, 12).

Description (Figs. 5.14, 5.15; Table 5.8)

[Based on 12 whole-mounted adult specimens (large morphs 1 and 2 in Table 5.8).] Body elongate, cylindrical, with bell-shaped concavity at posterior extremity, with bluntly rounded posterior extremity and maximum width at level of ventral sucker; width 25-34% of body length. Tegument thick (8-13), armed with large (8-13 in length) sharp spines reaching to

* Authors marked with an asterisk considered *S. obesum* and *S. tensum* synonymous

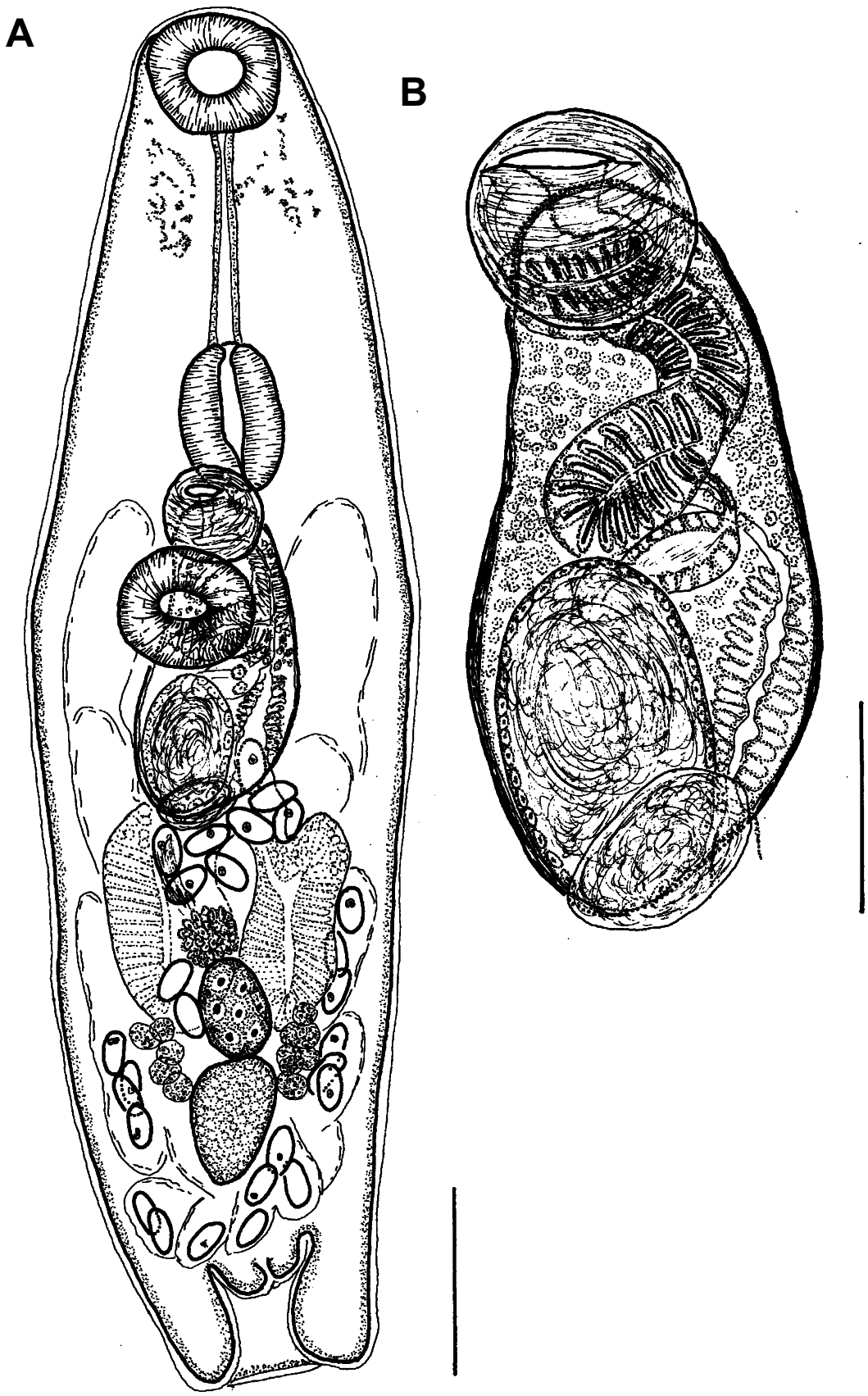

Fig. 5.14. *Saccocoelium obesum* Looss, 1902 ex *Liza aurata*. Large morph 1 from the Ebro Delta (Spain). **A.** Ventral view with uterus in outline. **B.** Hermaphroditic sac. *Scale-bars*: **A**, 200 µm; **B**, 100 µm.

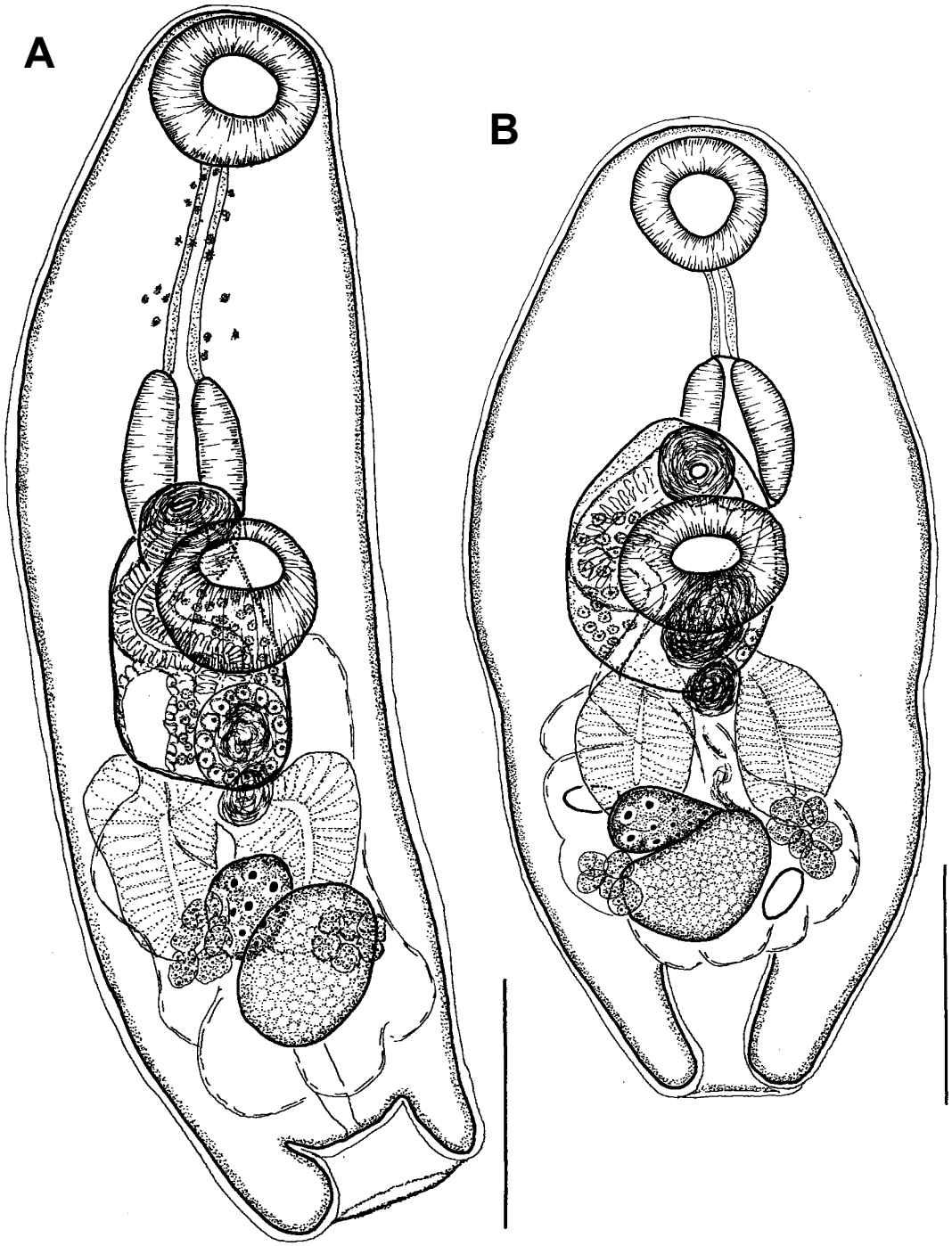

Fig. 5.15. *Saccocoelium obesum* Looss, 1902 ex *Liza aurata*. **A**. Large morph 2 from off Sozopol (Bulgarian Black Sea coast), ventral view with uterus in outline. **B**. *S. brayi* n. sp. (small morph from the Ebro Delta, Spain), ventral view of paratype with uterus in outline. *Scale-bars*: 200 μm.

posterior extremity. Eye-spot pigment abundant, dispersed on either side of anterior half of prepharynx. Oral sucker spherical, muscular, with ventrally subterminal aperture. Ventral sucker spherical, muscular, similar in size to oral sucker (sucker length ratio 1:0.99-1.17; width ratio 1:0.88-1.07), in second quarter of body. Forebody long, 37-49% of body length.

Prepharynx very long (PL/PHL=0.8-2.0); pharynx strongly muscular, large, elongate. Oesophagus up to twice length of pharynx. Intestinal bifurcation posterior to or at level of posterior margin of hermaphroditic sac; caeca 2 sac-like, large, with thick lining of elongate cells, end blindly in about middle of hindbody at 22-29% from posterior extremity.

Testis single, median, subspherical to elongate-oval, smooth, in last quarter of body; post-testicular field 10-23% of body length. External seminal vesicle saccular, globular to elongate-oval, thin-walled (<2), much smaller than internal seminal vesicle. Hermaphroditic sac large, thick-walled (5-8), muscular, elongate-oval, reaches back well posterior to posterior margin of ventral sucker, much longer than ventral sucker (HSL/VSL=159-261%), contains internal seminal vesicle, vesicular pars prostatica (56 × 46), numerous small prostatic cells, metraterm and hermaphroditic duct. Hermaphroditic duct wide (51-76), faintly-muscular, thick-walled; walls lined with crescentic, sclerotised structures (Fig. 5.14B). Internal seminal vesicle thick-walled (8-15), lined by layer of large cells, saccular, elongate-oval, occupies up to third of hermaphroditic sac. Genital atrium prominent, with strongly developed muscular walls (Figs. 5.14, 5.15). Genital pore median, at posterior level of pharynx.

Ovary round to elongate-oval, median to sinistral, well posterior to hermaphroditic sac, contiguous with or slightly separated from testis. Uterine seminal receptacle distinct; blind seminal receptacle absent. Mehlis' gland diffuse, 68-83 × 71-76. Laurer's canal not observed. Uterus thin-walled, occupies almost entire hindbody. Metraterm distinct, joins hermaphroditic duct close to seminal vesicle. 'Uterine vesicle' (*sensu* Fares & Maillard, 1974) not observed, but metraterm dilate at base of hermaproditic sac in some specimens. Eggs numerous, operculate; developed miracidia with single eye-spot (in some cases with appearance of 2 fused eye-spots). Vitellarium 2 separated elongate-oval, lobulate masses of coalesced follicles, lateral at level of ovary or more posterior.

Excretory vesicle tubular; bifurcation and anterior limits of excretory arms obscured by uterus; pore terminal at base of bell-shaped depression in posterior margin.

Table 5.8. Comparative metrical data for *Saccocoelium obesum* and *S. brayi* n. sp.

Species	*S. obesum*		*S. obesum*		*S. brayi* n. sp.		*S. obesum*		*S. obesum*	*S. obesum*	
Source	Present study (large morph 1)		Present study (large morph 2)		Present study (small morph)		Fares & Maillard (1974)		Radujkovic et al. (1989)	Gaevskaya & Dmitrieva (1993) (immat. worms)	
Locality	Ebro Delta		Off Sozopol, Black Sea coast (Bulgaria)		Ebro Delta		Languedoc-Roussillon lagoons (Mediterranean coast of France)		Kotor Bay (Adriatic Sea)	Black Sea (Ukraine)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Range	Mean
Measurements											
BL	1,384-1,574	1,477	949-1,184	1,058	810-997	879	1,200-1,930	1,470	1,570-2,500	900-1,130	1,020
BW	397-483	440	286-354	314	387-488	428	300-730	510	500-800	390-480	430
OSL	124-144	134	110-127	118	101-142	118	-	-	150-190	100-125	110
OSW	137-187	151	119-149	133	99-157	130	120-230	180	130-240	116-125	130
PL	185-304	241	116-185	158	0-76	56	40-250	150	250-430	113-219	180
PHL	142-177	160	137-159	146	132-187	150	-	-	110-200	100-125	114
PHW	104-134	115	78-111	94	85-159	123	100-180	120	110-180	50-100	77
OL	185-316	269	168-268	206	89-271	167	-	-	-	-	148
VSL	132-154	140	124-132	129	99-132	116	-	-	170-230	125-153	136
VSW	127-177	149	121-154	135	116-170	147	130-210	170	130-220	122-144	136
HSL	245-350	295	210-228	218	187-299	241	280-550	380	530-690	338-448	388
HSW	149-185	167	127-152	138	144-177	164	-	-	210-310	163-200	173
GAL	66-101	82	53-101	74	40-83	67	-	-	-	-	-
GAW	96-114	106	73-91	81	58-96	75	-	-	-	-	-
ISVL	127-187	155	86-121	110	99-158	125	-	-	-	-	-
ISVW	53-119	86	63-81	70	63-94	80	-	-	-	-	-
ESVL	62-109	86	53-71	-	46-114	65	-	-	-	-	-
ESVW	56-73	67	35-37	-	43-53	50	-	-	-	-	-
TL	86-154	125	101-137	126	114-210	149	120-260	190	160-280	163-188	177
TW	80-127	107	89-119	102	94-172	123	-	-	100-260	175-213	188
OVL	78-114	101	76-89	83	53-99	76	90-190	130	100-150	63-100	72
OVW	71-94	80	63-94	74	81-109	93	-	-	90-120	63-88	68
VL	88-125	102	72-121	87	71-123	97	-	-	-	-	-
VW	46-100	72	34-61	48	54-68	59	-	-	-	-	-
EL	50-58	53 ± 2	46-55	51 ± 3	43-55	50 ± 3	40-90	60	45-60	-	-
EW	26-31	29 ± 1	26-32	28 ± 2	26-29	27 ± 1	20-50	30	25-35	-	-

Table 5.8. Continued.

Species	*S. obesum*		*S. obesum*		*S. brayi* n. sp.		*S. obesum*		*S. obesum*	*S. obesum*	
Source	Present study (large morph 1)		Present study (large morph 2)		Present study (small morph)		Fares & Maillard (1974)		Radujkovic et al. (1989)	Gaevskaya & Dmitrieva (1993) (immat. worms)	
Locality	Ebro Delta		Off Sozopol, Black Sea coast (Bulgaria)		Ebro Delta		Languedoc-Roussillon lagoons (Mediterranean coast of France)		Kotor Bay (Adriatic Sea)	Black Sea (Ukraine)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Range	Mean
Distances											
FO	511-756	607	359-486	430	314-374	341	-	-	-	-	-
UEND	127-202	153	40-101	82	76-157	117	-	-	-	-	-
CEND	349-448	386	210-304	253	230-326	267	-	-	-	-	-
TEND	147-319	264	121-205	156	73-225	161	-	-	-	-	-
Ratios											
BW/BL (%)	25-34	30	27-32	30	43-59	48	32*	-	35*	46*	-
FO/BL (%)	37-49	41	38-43	41	35-41	38	38*	-	44*	37*	-
OSL/VSL	1:0.99-1.08	1:1.04	1:1.04-1.17	1:1.10	1:0.87-1.09	1:0.99	-	-	-	-	-
OSW/VSW	1:0.88-1.05	1:0.99	1:0.91-1.07	1:1.02	1:1.00-1.28	1:1.17	1:0.86-1.09	1:0.90	-	-	-
HSL/VSL (%)	159-261	212	163-177	169	189-227	202	230*	-	277*	282*	-
TEND/BL (%)	10-23	18	11-17	15	9-23	18	15*	-	11*	13*	-
CEND/BL (%)	24-29	26	22-26	24	26-33	30	29*	-	27*	31*	-

* Estimated from the published drawing.

Remarks

Dawes (1947) listed *S. tensum* as a synonym of *S. obesum*, and this was followed by Mikailov, 1958; Fischthal & Kunz, 1963; Ferreti & Paggi, 1965; and Moravec & Libosvárský, 1975). Fischthal & Kuntz (1963) apparently presented pooled metrical data for three specimens from *M. cephalus* in Egyptian waters, which are consequently unsuitable for comparison with the present data. Fares & Maillard (1974) studied the life-cycles of *S. tensum* and *S. obesum* and provided detailed descriptions of all of the life-cycle stages. The morphological and life-history data of these authors substantiate the distinct species status of these two Mediterranean species of *Saccocoelium*. Skrjabin (1956) erected *Wlassenkotrema* Skrjabin, 1956 for *Haploporus longicollum* Vlassenko, 1931, originally described by Vlassenko (1931) from the Black Sea. This species was treated as junior subjective synonym *S. obesum* by Overstreet & Curran (2005), an action which is accepted here. Consequently, all records of *H. longicollum* and *W. longicollum* are listed above as *S. obesum*.

The material described herein exhibited the diagnostic characteristics of *S. obesum*: (i) elongate, cylindrical body with bell-shaped concavity at bluntly rounded posterior extremity of body; (ii) long forebody; (iii) long prepharynx; and (iv) strongly-developed muscular walls of the genital atrium (Looss, 1902; Fares & Maillard, 1974). Three morphs of *S. obesum* were distinguished in the present material (Table 5.8, Figs. 5.14A, 5.15). The two larger exhibited only slight metrical differences, perhaps due to the geographical distance between populations originating from the Ebro Delta (Spanish Mediterranean) and Sozopol (Black Sea), respectively. However, the third form collected in the same Spanish locality had a smaller, less elongate body and a larger sucker width ratio (Fig. 5.15B; Table 5.8).

Despite this variability, all three morphs were correctly allocated to *S. obesum* in the global multivariate analysis (see section 5.4.4. below). Although showing lower upper limits and/or means for some metrical features (BL, BW, OSW, HSL, TL, OVL, EL and EW), the large morph from the Ebro Delta appears most similar morphologically to the material described by Fares & Maillard (1974) from the Western Mediterranean. The upper limits of egg-size provided by these authors, however, fall well outside the maximum values previously reported for this species (*i.e.* 90 × 50 *vs* 60 × 35 μm; see Radujković *et al*., 1989 and Table 5.8 for the ranges). It is possible that the measurements of Fares & Maillard (1974) are erroneous, since the largest egg illustrated close to the testis appears to measure only 33 × 22 μm (estimated from their fig. 5). Overall, the recognition of three morphs of *S. obesum* indicated the presence of a species complex which is clearly differentiated morphologically

from the remaining *Saccocoelium* spp., a notion that warranted a molecular test for consistent genetic variation. Examination and sequencing (ITS2 and 28S rDNA, see Chapter 6) of additional material from *L. aurata* and *L. saliens* from the Ebro Delta confirmed the distinctness of the 'small morph' for which the name *S. brayi* n. sp. is proposed.

***Saccocoelium brayi* n. sp.**

Type-host: *Liza aurata* (Risso).
Other host: *Liza saliens* (Risso).
Type-locality: Ebro Delta, Spain (40º30'– 40º50'N, 0º30'–1º10'E).
Site: Pyloric caeca of the intestine.
Type-material: Holotype BMNH 2008.10.07.77; paratypes BMNH 2008. 10.07.78-83.
Etymology: The species is named for Dr Rodney A. Bray of the Natural History Museum, London, in recognition of his contributions to platyhelminth taxonomy, systematics and evolution.

Description (Fig. 5.16; Table 5.8)
[Based on 10 whole-mounted adult specimens; measurements in Table 5.8.] Body elongate-oval, plump, with bell-shaped concavity at posterior extremity, with bluntly rounded posterior extremity and maximum width at posterior level of ventral sucker; width 43-59% of body length. Pre-oral lobe strongly developed 25-51. Tegument thick (5-8), armed with large sharp spines (8-10) reaching to posterior extremity. Eye-spot pigment abundant, dispersed on either side of anterior part of pharynx and oral sucker. Oral sucker spherical, with ventral aperture. Ventral sucker cup-shaped, similar in size to or slightly larger than oral sucker (sucker length ratio 1:0.87-1.09; width ratio 1:1.00-1.28), in second quarter of body. Forebody relatively long, 35-41% of body length. Prepharynx absent or short (PL/PHL=0-0.6); pharynx strongly muscular, elongate-oval, larger than oral sucker. Oesophagus similar in length to pharynx. Intestinal bifurcation at level of posterior margin of ventral sucker; caeca two, sac-like, large, with thick lining of cells, end blindly in about middle of hindbody at 26-33% from posterior extremity.

Testis single, median to dextral, sub-globular, smooth, in last quarter of body; post-testicular field 9-23% of body length. External seminal vesicle saccular, sub-globular, thin-walled (<2), smaller than internal seminal vesicle. Hermaphroditic sac massive, thick-walled (5-6), muscular, elongate-oval, reaches up to length of ventral sucker distance posteriorly in

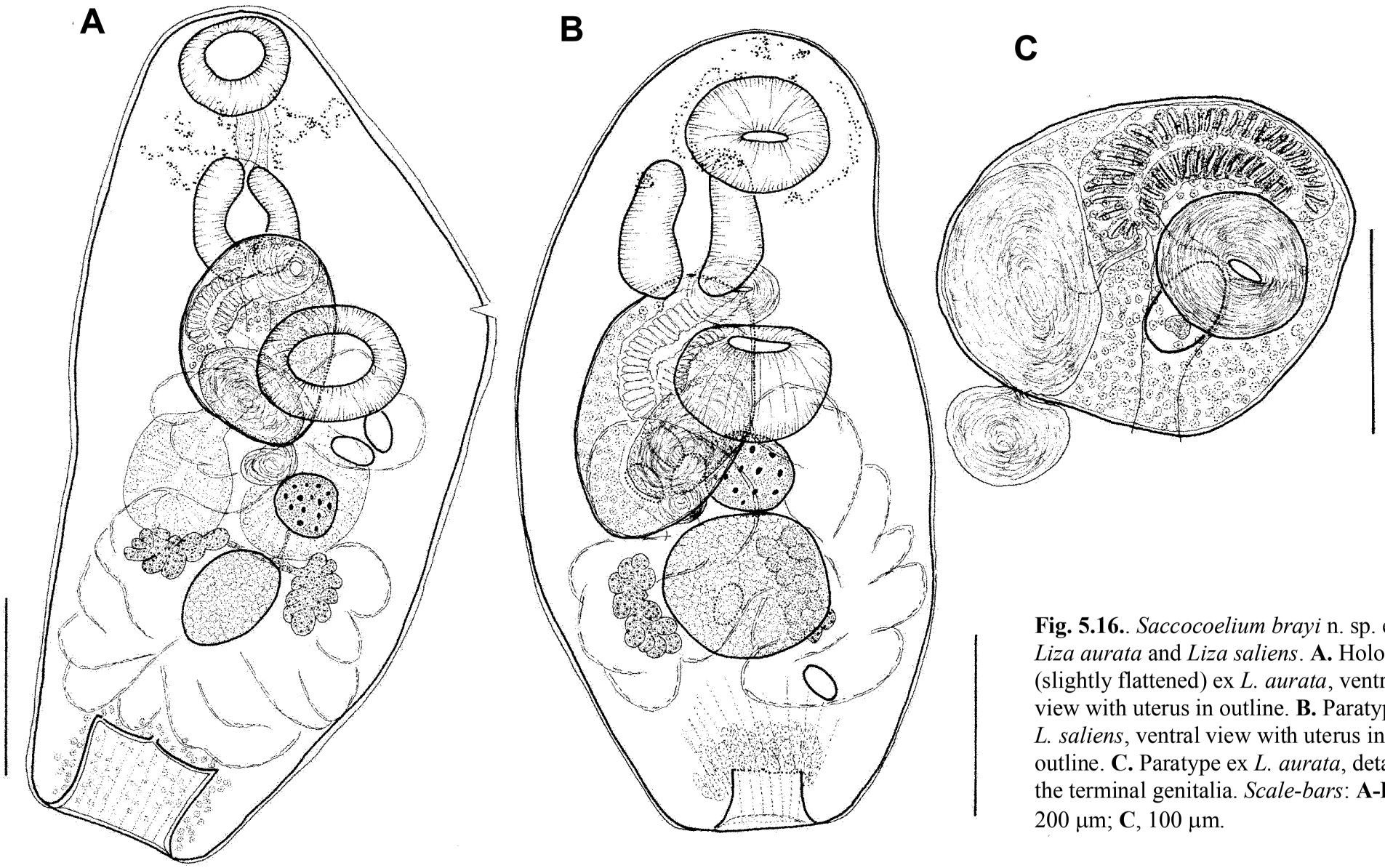

Fig. 5.16.. *Saccocoelium brayi* n. sp. ex *Liza aurata* and *Liza saliens*. **A.** Holotype (slightly flattened) ex *L. aurata*, ventral view with uterus in outline. **B.** Paratype ex *L. saliens*, ventral view with uterus in outline. **C.** Paratype ex *L. aurata*, detail of the terminal genitalia. *Scale-bars*: **A-B**, 200 μm; **C**, 100 μm.

hindbody, much longer than ventral sucker (HSL/VSL=189-227%), contains internal seminal vesicle, numerous small prostatic cells, metraterm and hermaphroditic duct. Hermaphroditic duct wide (c.51-89), faintly-muscular, thick-walled; walls lined with crescentic, sclerotised structures. Internal seminal vesicle lined by a layer of cells, saccular, elongate-oval, occupies up to half of hermaphroditic sac. Genital atrium with strongly developed muscular walls. Genital pore median, at posterior level of pharynx.

Ovary round to transverse-oval, median to dextral, contiguous with hermaphroditic sac, posterior margin of ventral sucker and testis, overlapping the latter dorsally in some specimens. Uterine seminal receptacle distinct; blind seminal receptacle absent. Mehlis' gland and Laurer's canal not observed. Uterus thin-walled, occupies almost entire hindbody; metraterm distinct, joins hermaphroditic duct close to seminal vesicle. Eggs numerous, operculate; developed miracidia with single eye-spot. Vitellarium 2 separated elongate-oval clusters of large follicles, lateral at level of testis. Excretory pore terminal; details of excretory vesicle not observed (masked by uterus).

Remarks

The present material exhibits some of the diagnostic characteristics of and appears most close morphologically to *S. obesum*, *i.e.* bell-shaped concavity at posterior extremity, bluntly rounded posterior extremity of body, long forebody, large pharynx and strongly-developed muscular walls of the genital atrium (Looss, 1902; Fares & Maillard, 1974; Blasco-Costa *et al*., 2009c; see above). However, *S. obesum* is characterised by a much larger elongate cylindrical body [BW/BL=25-34 *vs* 43-59% (mean 30 *vs* 48 %)], distinctly longer prepharynx (185-304 *vs* 0-76; PL/PHL=0.8-2.0 *vs* 0-0.6) and a somewhat smaller sucker width ratio (mean 1:0.99 *vs* 1:1.17) due to the ventral sucker being wider than long in *S. brayi* n. sp. Although the measurements of the testis and eggs exhibit overlapping ranges, the upper limits in *S. brayi* are higher for the former (mean 149 × 123 *vs* 125 × 107 in *S. obesum*) and lower for the latter (mean 50 × 27 *vs* 53 × 29, see Table 5.8). Furthermore, the genital atrium appears more prominent in *S. obesum* whereas it is less muscular in *S. brayi* (mean 82 × 106 *vs* 67 × 75) and vesicular pars prostatica is apparently absent in the latter species. The above comparisons coupled with the consistent multivariate morphometric differentiation and observed genetic divergence (see Chapter 6) support the distinct species status of *S. brayi* n. sp.

***Saccocoelium cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga & Kostadinova, in press**

Type-host: *Mugil cephalus* L.
Type-locality: Ebro Delta, Spain (40º30'–40º50'N, 0º30'–1º10'E; 14.x.2004).
Site: Intestine.
Type-material: Holotype BMNH 2008.10.07.23; paratypes BMNH 2008.10.07.24-25.
Etymology: The species name indicates the type-host.

Description (Fig. 5.17; Table 5.9)
[Based on 10 whole-mounted adult specimens; measurements of 7 adults with means in parentheses.] Body elongate-fusiform, small, with tapered posterior extremity and maximum width at junction of first and second body thirds (at mid-body in 2 specimens); width 26-42 (36)% of body length. Tegument 2-3 thick, armed with large, sharp spines (3-5 in length) reaching close to posterior extremity. Eye-spot pigment dispersed mainly on either side of pharynx; pigment granules also present dorsal to oral sucker. Oral sucker spherical to slightly transversely oval, muscular, with ventrally subterminal aperture. Ventral sucker subspherical, muscular, smaller than oral sucker [sucker length ratio 1:0.64-0.82 (0.71); sucker width ratio 1:0.63-0.84 (1:0.74)], in second quarter of body. Forebody 28-37 (32)% of body length.

Prepharynx apparently absent (6 specimens) or short; pharynx strongly muscular, large, elongate-oval. Oesophagus 2-3 times length of pharynx. Intestinal bifurcation dorsal to posterior third of hermaphroditic sac (dorsal to ventral sucker in 1 specimen); caeca 2, sac-like, large, ending blindly in middle of hindbody, at 20-44 (33)% from posterior extremity.

Testis single, large in relation to body (length 14-23% of body length), median, elongate-oval (subtriangular in 2 worms), smooth, located in middle or last third of body and far from ventral sucker; post-testicular field 13-27 (21%) of body length. External seminal vesicle saccular, elongate-oval, thin-walled, slightly smaller than internal seminal vesicle, between hermaphroditic sac and ovary (overlapping these dorsally in 1 specimen). Hermaphroditic sac with thick (3-5) wall, muscular, elongate-oval, reaches back well posterior to posterior margin of ventral sucker, substantially longer than ventral sucker [HSL/VSL=228-313 (273)%], contains internal seminal vesicle, numerous large prostatic cells, metraterm and hermaphroditic duct. Hermaphroditic duct 37-45 (40) in width, slightly muscular, thick-walled; walls lined with crescentic, sclerotised structures (Fig. 5.17B). Internal seminal vesicle with thick (2) wall, saccular, elongate-oval, occupies about third of

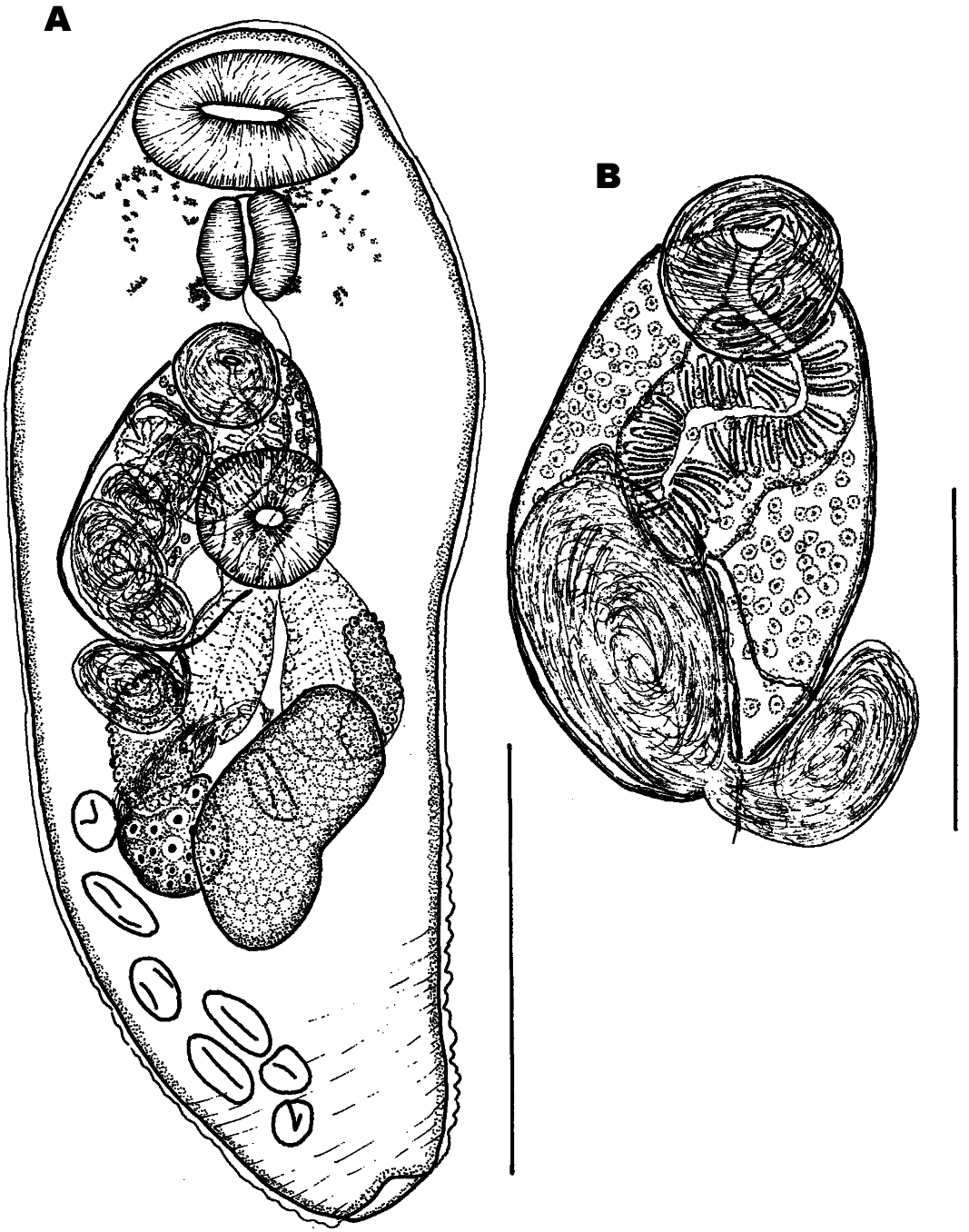

Fig. 5.17. *Saccocoelium cephali* Blasco-Costa et al., in press ex *Mugil cephalus*. **A.** Holotype, ventral view. **B.** Hermaphroditic sac of paratype. *Scale-bars*: **A**, 200 μm; **B**, 100 μm.

Table 5.9. Comparative metrical data for *Saccocoelium tensum* [ranges for the data of Looss (1902), Fares & Maillard (1974), Gaevskaya & Dmitrieva (1993) and the present study], *S. cephali* ., *S. currani* and *S. gohari.*

Species	*S. tensum*	*S. cephali*		*S. currani*		*S. gohari*		*S. gohari*	
Locality		Ebro Delta (Spain)		Ebro Delta (Spain)		Lake Qarun (Egypt)		Black Sea (Ukraine)	
Source	Combined data	Present study		Present study		Ramadan et al. (1989)		Sarabeev & Balbuena (2004)	
	Range	Range	Mean	Range	Mean ± SD	Range	Mean	Range	Mean
Measurements									
BL	600-1,400	496-664	583	1,075-1,570	1,396 ± 122	1,020-1,210	1,110	826-1,534	1,050
BW	200-550	173-230	207	352-574	429 ± 54	260-390	330	212-543	314
OSL	105-139	64-83	76	124-162	144 ± 10	70-120	90	98-157	118
OSW	76-150	78-104	88	124-195	166 ± 16	70-130	100	-	-
PL	0-63	0-14	4	0-53	24 ± 16	10-50	30	4-17	9
PHL	77-152	46-58	53	89-104	95 ± 4	40-60	50	48-76	60
PHW	51-142	42-51	47	77-106	92 ± 8	50-70	60	45-64	54
OL	149-276	77-192	143	157-374	288 ± 66	190-320	260	154-294	230
VSL	94-137	53-66	57	104-134	118 ± 10	70-80	80	56-98	76
VSW	95-170	60-69	65	104-144	122 ± 11	70-90	80	67-101	79
HSL	180-330	142-166	154	207-291	244 ± 23	160-190	180	210-274	234
HSW	111-258	94-104	100	144-202	175 ± 21	110-130	120	126-188	156
GAL	38-51	32-48	40	51-76	62 ± 13	63*	-	-	-
GAW	51-61	42-56	49	61-73	64 ± 4	40*	-	-	-
ISVL	61-202	70-110	94	78-197	140 ± 40	70-150	110	53-146	108
ISVW	35-111	32-54	43	66-152	97 ± 25	-	-	64-126	97
ESVL	44-147	48-99	69	58-228	111 ± 50	40-120	80	39-76	50
ESVW	43-81	32-37	33	46-137	68 ± 25	-	-	42-140	83
TL	83-413	77-136	107	76-259	150 ± 45	270-370	320	266-336	300
TW	67-351	61-99	80	81-147	116 ± 21	110-170	140	160-213	187
OVL	60-163	51-96	61	61-139	92 ± 20	90-140	130	59-140	91
OVW	51-138	38-61	52	56-139	90 ± 24	70-100	90	56-134	97
VL	74-131	58-93	76	56-171	116 ± 26	60-150	100-110^{+}	70-126	94-111^{+}
VW	35-68	26-56	40	32-93	60 ± 14	30-50	40-50	31-70	48
EL	37-49	42-43	42	37-51	43 ± 3	33-36	35	38-67	45
EW	21-27	22-23	22	21-29	24 ± 2	17-19	18	17-25	22

Table 5.9. Continued.

Species	*S. tensum*	*S. cephali*		*S. currani*		*S. gohari*		*S. gohari*	
Locality		Ebro Delta (Spain)		Ebro Delta (Spain)		Lake Qarun (Egypt)		Black Sea (Ukraine)	
Source	Combined data	Present study		Present study		Ramadan et al. (1989)		Sarabeev & Balbuena (2004)	
	Range	Range	Mean	Range	Mean ± SD	Range	Mean	Range	Mean
Distances									
FO	256-390	171-200	187	324-476	393 ± 43	-	-	-	-
UEND	25-250	48-91	64	190-724	491 ± 138	-	-	-	-
CEND	195-407	99-272	207	486-850	683 ± 88	-	-	-	-
TEND	134-380	66-154	124	503-929	729 ± 117	-	-	-	-
Ratios									
BW/BL (%)	33-56	26-42	36	25-39	31 ± 4	22-32	26	20-35	29
FO/BL (%)	26-36	28-37	32	23-32	28 ± 4	30*	-	32*	-
OSL/VSL	1:0.80-1.10	1:0.64-0.82	1:0.71	1:0.69-1.00	1:0.82 ± 0.10	-		-	-
OSW/VSW	1:0.75-1.13	1:0.63-0.84	1:0.74	1:0.65-0.84	1:0.73 ± 0.05	1:0.67-1.15	1:0.84	1:0.60-0.90 (1:0.64*)	1:0.67
HSL/VSL (%)	158-255	228-313	273	160-254	209 ± 25	210*	-	306*	-
TEND/BL (%)	16-36	13-27	21	44-61	52 ± 6	22*	-	36*	-
CEND/BL (%)	23-39	20-44	33	44-55	49 ± 4	55*	-	63*	-

* Estimated from the published drawing; $^{+}$, Means for each vitelline mass.

hermaphroditic sac. Genital atrium relatively deep, with weakly-developed muscular walls (Fig. 5.17). Genital pore median, just anterior to ventral sucker (5 worms) or more anterior (5 worms).

Ovary round to elongate-oval, submedian, between testis and hermaphroditic sac, contiguous with testis. Uterine seminal receptacle distinct; blind seminal receptacle absent. Mehlis' gland small; Laurer's canal not observed. Uterus thin-walled, occupies almost entire hindbody, reaches close to posterior extremity [UEND/BL= 8-16 (11)%]. Metraterm distinct, joins hermaphroditic duct close to seminal vesicle. Eggs numerous, operculate; developed miracidia with single eye-spot. Vitellarium comprises 2 separated, elongate-oval masses of large coalesced follicles, lateral at level of ovary or more posterior.

Excretory vesicle wide-tubular, with small thickening (11 wide) close to excretory pore; bifurcation and excretory arms obscured by eggs; pore terminal.

Remarks

S. cephali differs from all other species of *Saccocoelium* in its distinctly smaller dimensions (size of body and most organs, see Tables 5.8-5.10) and in possessing a large testis (in relation to its body size; TL/BL=14-23 *vs* 5-16%). In the size of testis and the genital atrium, and the ratios BW/BL, FO/BL, TEND/BL and CEND/BL, *S. cephali* resembles only *S. tensum*. However, due to the substantially smaller dimensions of the body and most of its organs (virtually half the size), univariate statistical comparisons clearly discriminated the two species ($p<0.0001$ for BL, BW, OSL, OSW, PHL, PHW, VSL, VSW, HSL, FO, CEND, TEND; $p<0.05$ for PL, OL, HSW, ISVW, OVL, OVW, VL, UEND). Furthermore, the ventral sucker in *S. cephali* is less muscular and always smaller than oral (sucker length ratio, $p=0.004$; width ratio, $p=0.0002$; see Table 5.9) and the genital atrium, although less muscular in both species compared to others, appears more prominent in *S. cephali*. Although ranges for egg length and width tend to overlap, the eggs in *S. cephali* differed significantly form those of the three Mediterranean species (see section 5.5.4. below). Finally, both multivariate approaches (PCA and LDA, section 5.5.4.) clearly indicate a separate allocation of the four sympatric Mediterranean species described here, the discrimination being achieved using a small number of metrical variables. These comparisons support the distinct species status of *S. cephali*.

***Saccocoelium currani* Blasco-Costa, Montero, Gibson, Balbuena, Raga & Kostadinova, in press**

Type-host: *Mugil cephalus* L.
Type-locality: Ebro Delta, Spain (40º30'–40º50'N, 0º30'–1º10'E; 02.vi.2004).
Site: Intestine.
Type-material: Holotype BMNH 2008.10.7.26; paratypes BMNH 2008.10.7.27-37.
Etymology: The species is named for Dr Stephen Curran, Gulf Coast Research Laboratory, University of Southern Mississippi, in recognition to his contribution to haploporid taxonomy.

Description (Fig. 5.18; Table 5.9)
[Based on 16 whole-mounted adult specimens.] Body elongate-fusiform, distinctly tapered posterior to level of testis, with maximum width just posterior to level of ventral sucker; width 25-39% of body length. Tegument thin, armed with fine spines (2-3 in length). Eye-spot pigment dispersed on either side of pharynx. Oral sucker slightly transversely oval, ventro-subterminal, muscular, larger than ventral sucker [sucker length ratio 1:0.69-1.00 (0.82); width ratio 1:0.65-0.84 (0.73)]. Ventral sucker spherical, in second quarter of body. Forebody 23-32 (28)% of body length.

Prepharynx typically short (apparently absent in 1 worm), shorter than pharynx [PL/PHL=0-0.5 (0.3)]; pharynx muscular, elongate-oval. Oesophagus 1.6–4 times length of pharynx. Intestinal bifurcation at level of posterior margin of hermaphroditic sac (at level of ventral sucker in 2 worms); caeca 2, sac-like, with thick lining of round cells, end blindly in middle of body, at 44-55 (49)% from posterior extremity.

Testis single, usually dextral (median in 7 worms), elongate-oval, smooth, in middle third of body, lateral and contiguous with or slightly separated from ovary (9 worms), post-ovarian (5 worms), pre-ovarian (1 worm), located close to ventral sucker; post-testicular field very long, 44-61 (52)% of body length. External seminal vesicle just posterior to hermaphroditic sac, saccular, globular to elongate-oval, thin-walled (<2), smaller than internal seminal vesicle (larger in 1 worm). Hermaphroditic sac large, thick-walled (wall 5-13 thick), muscular, elongate-oval, antero-dorsal to ventral sucker, much longer than ventral sucker [HSL/VSL=161-254 (209)%], contains internal seminal vesicle, numerous large prostatic cells, metraterm and hermaphroditic duct. Internal seminal vesicle thin-walled (<2), saccular, elongate-oval, occupies up to half of hermaphroditic sac. Hermaphroditic duct wide (42-63), indistinctly muscular; wall thick, lined with crescentic, sclerotised structures (Fig. 5.18B).

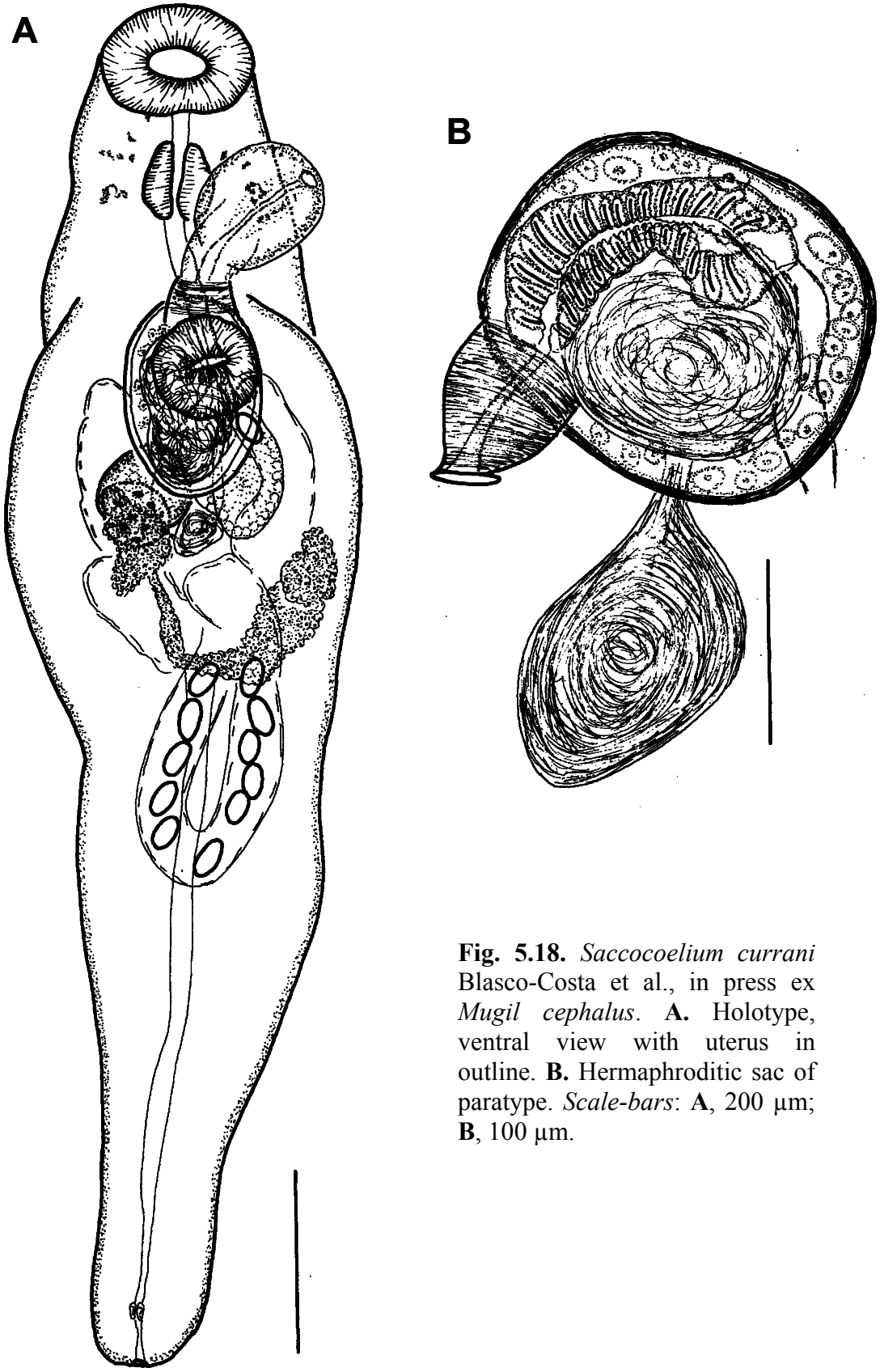

Fig. 5.18. *Saccocoelium currani* Blasco-Costa et al., in press ex *Mugil cephalus*. **A.** Holotype, ventral view with uterus in outline. **B.** Hermaphroditic sac of paratype. *Scale-bars*: **A**, 200 µm; **B**, 100 µm.

Genital atrium with well-developed muscular walls (Fig. 5.18). Genital pore median, close to anterior margin of ventral sucker (at level of posterior pharynx in 1 worm).

Ovary subspherical, overlaps posterior third of hermaphroditic sac ventrally (more anterior and dorsal to ventral sucker in 1 worm). Uterine seminal receptacle distinct; blind seminal receptacle absent. Mehlis' gland diffuse, 89 × 78. Laurer's canal not observed. Uterus thin-walled, restricted to third quarter of body [(UEND/BL=28-52 (37)%]. Metraterm joins hermaphroditic duct close to internal seminal vesicle. Uterine vesicle not observed but metraterm dilate in 2 specimens. Eggs numerous, operculate; developed miracidia with single eye-spot (in some cases with appearance of 2 fused eye-spots). Vitellarium 2 elongate-oval, lobulate masses of small coalesced follicles, at level of ovary or slightly posterior. Vitelline ducts and vitelline reservoir prominent, resulting in horseshoe-like appearance of vitelline complex in most cases.

Excretory vesicle tubular with small sphincter, 13-25 × 8-13, located at 13-99 from excretory pore; bifurcation and anterior limits of excretory arms not observed; pore ventro-subterminal.

Remarks

Characteristic features of *S. currani* include: (i) an elongate-fusiform body which is distinctly tapered posterior to the testis; (ii) the testis located close to the ventral sucker; (iii) a uterus restricted to the third quarter of the body; and (iv) a long postcaecal field. These features serve to distinguish the new species from all other species of *Saccocoelium*. Furthermore, *S. currani* differs from *S. tensum* in having: more developed muscular walls of the genital atrium; a smaller sucker ratio; and higher upper limits for a number of characters (body length; size of the oral sucker; length of oesophagus and hermaphroditic duct; size of the external seminal vesicle and vitellarium; length of forebody and post-uterine, postcaecal and post-testicular fields) (Table 5.9).

Due to its elongate-fusiform body, *S. currani* appears similar to *S. gohari*, described from mugilids in Lake Qarun (Egypt) (Ramadan *et al*., 1989a), but the latter has: a large and very elongate testis (mean size 320 × 140 *vs* 150 × 116 μm), which occupies more than half of the hindbody; a larger ovary; and smaller eggs (mean size 35 × 18 *vs* 43 × 24 μm). In addition, the new species exhibits greater values in terms of both ranges and means for almost all of the remaining morphometric features (Table 5.9). The above differences, coupled with

the results of the multivariate comparisons with the two sympatric species *S. obesum* and *S. tensum* (section 5.5.4 below) support the distinct status of *S. currani*.

***Saccocoelium gohari* Ramadan, Saoud, Ashour & Mansour, 1989**

Records

References: 1. Ramadan *et al*. (1989a); 2. Al-Bassel (2003).

Descriptions: 1.

Definitive hosts: *Mugil cephalus* L. (type-host) (1); *Liza ramado* (Risso) (1, 2); *Chelon labrosus* (Risso) (1).

Distribution: Area 37, subarea 3 (Eastern Mediterranean) (type-locality: Lake Qarun, Egypt) (1, 2).

Remarks

This species was described in sufficient detail by Ramadan *et al*. (1989a) based on material from *M. cephalus*, *L. ramado* and *C. labrosus* in Lake Qarun (Egypt) and distinguished from other *Saccocoelium* spp. by its very elongate testis, prominent uterine seminal receptacle, oval shape of the vitelline masses and sucker-ratio. While the latter three characters cannot serve as discriminating features, *S. gohari* is characterised by the smallest size of eggs and ventral sucker, the length of the pharynx and the width of the genital atrium (Table 5.9), in addition to a distinctly elongate testis. The material from *M. cephalus* in the Black Sea (near Kerch) described as *S. gohari* by Sarabeev & Balbuena (2004b) appears to be a misidentification, since the eggs are much larger [38-67 × 17-25 *vs* 33-36 × 17-19 (mean 45 × 22 *vs* 35 × 18) µm], the testis is rounded and more anteriorly located [TEND/BL=36 *vs* 22% (estimated from the published drawings)], the hermaphroditic sac is much larger both in absolute dimensions [length 210-274 (234) *vs* 160-190 (180) µm; width 126-188 (156) *vs* 110-130 (120) µm] and in relation to the ventral sucker [HSL/VSL= 306 *vs* 210% (estimated from the published drawings)] (Table 5.9).

***Saccocoelium tensum* Looss, 1902**

Syns *Lecithobotrys helmymohamedi* Ramadan, Saoud, Ashour & Mansour, 1988; *Saccocoelium helmymohamedi* (Ramadan, Saoud, Ashour & Mansour, 1988) Overstreet & Curran, 2005; *Saccocoelium portsaidensis* [sic] El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 (new synonym); *S. saoudi* El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 (new

synonym); *Neosaccocoelium aegyptiacus* [sic] El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 (new synonym)

Material studied

Ex *Liza aurata* (Risso). Intestine. Ebro Delta, Spain (40º30'–40º50'N, 0º30'–1º10'E; 02.vi.2004; 22.vi.2004); off Santa Pola, Spain (38º00'–38º20'N, 0º10'–0º40'E; 19.v.2004). BMNH 2008.10.27.41-43.

Ex *L. ramado* (Risso). Intestine. Ebro Delta, Spain (26.v.2004). BMNH 2008.10.27.44-48.

Records

References: 1. Looss (1902); 2. Vlassenko (1931); 3. Chernyshenko (1955); 4. Reshetnikova (1955); 5. Mikailov (1958)*; 6. Fischthal & Kuntz (1963)*; 7. Paperna (1964); 8. Fares & Maillard (1974); 9. Moravec & Libosvárský (1975)*; 10. Paggi *et al*. (1979); 11. Solonchenko & Tkachuk (1985); 12. Brglez & Paradiznik (1988); 13. Ibragimov (1988); 14. Orecchia *et al*. (1988); 15. Paggi *et al*. (1988); 16. Ramadan *et al*. (1989b) (as *Lecithobotrys helmymohamedi*); 17. Radujković *et al*. (1989); 18. Ramadan *et al*. (1989a); 19. Hassan *et al*. (1990a); 20. El-Shahawi *et al*. (1992) (as *Saccocoelium portsaidensis*, *S. saoudi* and *Neosaccocoelium aegiptyacus*); 21. Gaevskaya & Dmitrieva (1993); 22. Zabodash & Semenenko (1994); 23. D'Amelio *et al*. (1995); 24. Di Cave *et al*. (1997); 25. Merella & Garippa (1998); 26. Domnich & Sarabeev (2000a); 27. Domnich & Sarabeev (2000b); 28. Domnich & Sarabeev (2000c); 29. Domnich & Sarabeev (2000d); 30. Maltsev & Zhdamirov (2000); 31. Sarabeev & Domnich (2000); 32. Sarabeev (2000); 33. Dmitrieva & Gaevskaya (2001); 34. Merella & Garippa (2001); 35. Nizova *et al*. (2001); 36. Pronkina (2001); 37.[2] Al-Bassel (2003) (also as *Saccocoelium saoudi, Lecithobotryes* [sic] *helmymohamedi* and *Neosaccocoelium aegyptiacus*); 38. Gaevskaya & Korniychuk (2003); 39. Ragias *et al*. (2005); 40. Present study.

Descriptions: 1; 8; 16; 17; 18; 19; 20; 21; 36; 40.

Definitive hosts: *Chelon labrosus* (Risso) (type-host) (1, 8, 10, 18, 34, 39); *Mugil cephalus* L. (2, 4, 6, 7, 8, 10, 11, 12, 15, 18, 20, 21, 28, 33, 39); *M. soiuy* Basilewsky (22, 26, 27, 28, 29, 30, 31, 32, 33, 35); *Liza aurata* (Risso) (3, 4, 7, 8, 10, 11, 13, 15, 21, 25, 28, 33, 34, 36, 38, 39, 40); *L. ramado* (Risso) (6, 7, 8, 9, 10, 15, 16, 18, 19, 20, 23, 24, 25, 33, 34, 37, 39, 40); *L. saliens* (Risso) (4, 5, 7, 8, 11, 13, 14, 17, 21, 33, 34, 38, 39).

[2] Authors marked with an asterisk considered *S. obesum* and *S. tensum* synonymous.

Distribution: Area 37, subarea 2 (Central Mediterranean) (type-locality: Adriatic Sea, off Trieste) (1, 12, 14, 17); area 37, subarea 1 (Western Mediterranean) (8, 10, 15, 25, 34, 49) (?23, 24 'Italy'); area 37, subarea 3 (Eastern Mediterranean) [6, 7, 9, 16, 18, 19, 20, 23, 37 (Fayoum fish farm, Egypt), 39]; area 37, subarea 4 (Black Sea): 4.2 Black Sea (2, 3, 4, 21, 33, 35, 36, 38), 4.3. Azov Sea (11, 22, 26, 27, 28, 29, 30, 31, 32, 33); Caspian Sea (5, 13).

Description (Fig 5.19; Table 5.10)

[Based on 14 whole-mounted adult specimens.] Body elongate-oval, typically with rounded posterior extremity and maximum width at mid-body (at level of ovary, 8 specimens) or in posterior third of body (at level of testis, 6 specimens); width 37-56 (42)% of body length. Tegument thick (5-8), armed with large, sharp spines (length 8-10) reaching to posterior extremity. Eye-spot pigment dispersed mostly on either side of pharynx; pigment granules also present dorsal to oral sucker. Three groups of gland-cells present: (i) 5 gland-cells on either side of anterior part of hermaphroditic sac opening ventrally; (ii) 3 gland-cells on either side of oral sucker opening ventrally; and (iii) 4 (2+2) larger median cells at level of genital pore opening on dorsal lip of oral sucker (Fig. 5.19C). Oral sucker spherical, muscular, with ventrally subterminal aperture. Ventral sucker subspherical, muscular, similar in size to oral sucker [sucker length ratio 1:0.80-1.10 (1:0.94); sucker width ratio 1:0.82-1.13 (1:1.01)], in second quarter of body. Forebody 26-36 (32) % of body length.

Prepharynx apparently absent (5 specimens) or short; pharynx strongly muscular, large, elongate-oval. Oesophagus up to twice length of pharynx. Intestinal bifurcation posterior to ventral sucker, typically dorsal to posterior third of hermaphroditic sac (posterior to latter in 6 worms); caeca 2, sac-like, large, end blindly in middle of hindbody, at 23-39 (31)% from posterior extremity.

Testis single, median to somewhat sinistral, elongate-oval (subspherical in 2 worms), smooth, located in middle or last third of body far from ventral sucker; post-testicular field 16-36 (26%) of body length. External seminal vesicle just posterior or slightly dorsal to hermaphroditic sac, saccular, elongate-oval, thin-walled (< 2), similar in size to internal seminal vesicle. Hermaphroditic sac thick-walled (5-13), muscular, elongate-oval, reaches back well posterior to posterior margin of ventral sucker (antero-dorsal to ventral sucker in 4 worms), much longer than ventral sucker [HSL/VSL=158-255 (196%)], contains internal seminal vesicle, vesicular pars prostatica measuring 53-61 × 38-44 (57 × 40, n=3), numerous small prostatic cells, metraterm and hermaphroditic duct. Hermaphroditic duct wide, 76-152 ×

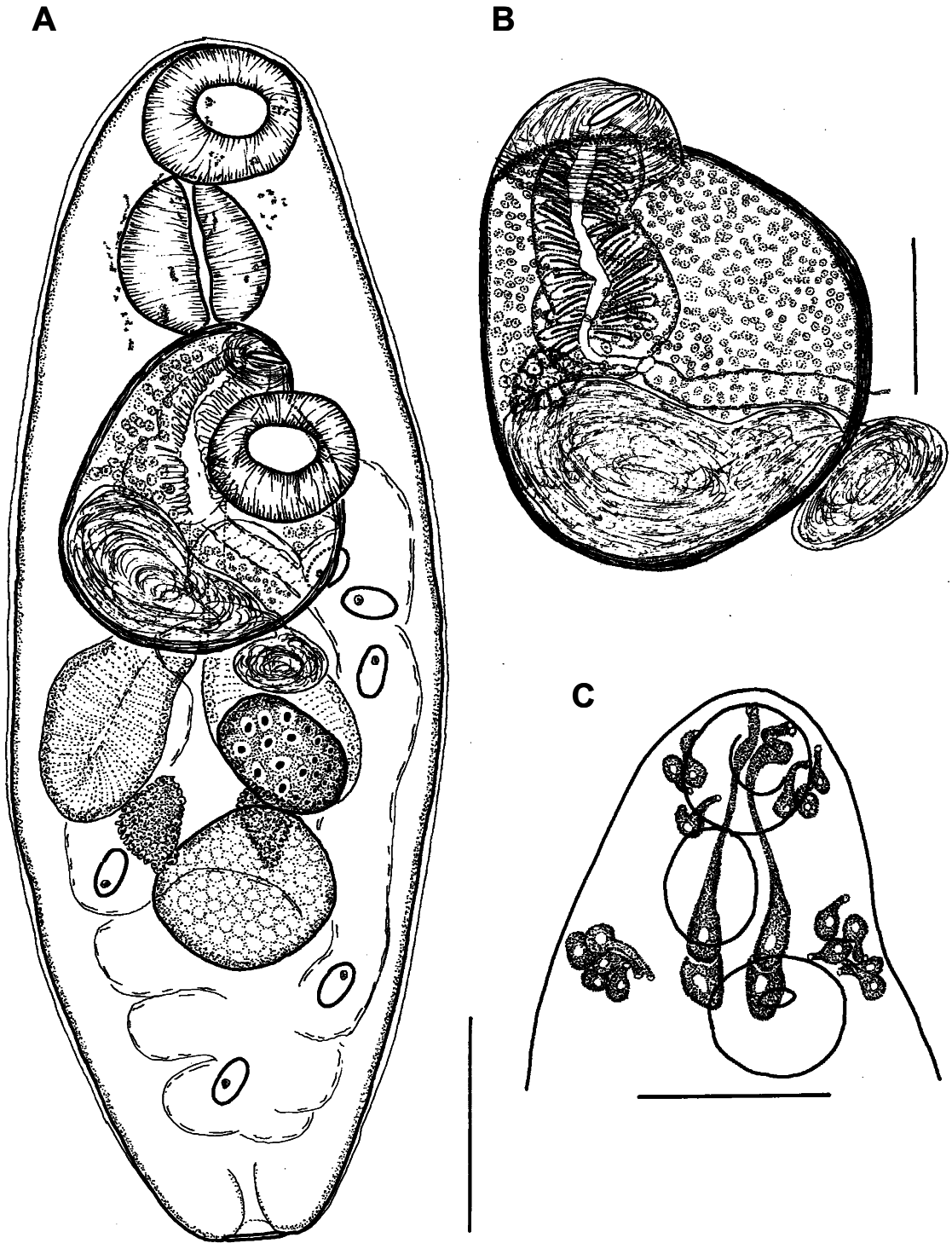

Fig.5.19. *Saccocoelium tensum* Looss, 1902 ex *Liza ramado* and *L. aurata*. **A.** Specimen ex *L. ramado*, ventral view with uterus in outline. **B.** Specimen ex *L. ramado*, hermaphroditic sac. **C.** Specimen ex *L. aurata*, ventral view, showing the distribution of gland-cells in the forebody. *Scale-bars*: **A**, 200 µm **B-C**, 100 µm.

Table 5.10. Comparative metrical data for *Saccocoelium tensum.*

Source	Present study		Fares & Maillard (1974)		Radujkovic et al. (1989)	Ramadan et al. (1989)		Hassan et al. (1990)	Gaevskaya & Dmitrieva (1993)	
Locality	Ebro Delta (Spain)		Languedoc-Roussillon Lagoons (Mediterranean coast of France)		Kotor Bay (Adriatic Sea)	Lake Qarun (Egypt)		Off Ras El-Bar (Egypt)	Off Sevastopol (Black Sea)	
	Range	Mean±SD	Range	Mean	Range	Range	Mean	Range	Range	Mean
Measurements										
BL	853-1,133	1,011 ± 70	580-1,400	980	1,300	560-820	690	510-1,120	750-1,230	880
BW	321-548	428 ± 61	190-550	440	690	210-260	240	220-450	300-460	390
OSL	105-139	125 ± 10	-	-	160	70-80	80	70-110	69-125	98
OSW	114-149	134 ± 10	80-190	120	180	80-100	90	80-120	94-150	121
PL	0-63	16 ± 19	-	-	-	10-20	20	6-19	13-63	27
PHL	109-152	121 ± 10	-	-	110	50-70	60	30-80	88-125	100
PHW	94-142	107 ± 12	40-120	80	120	60-70	70	40-80	66-85	74
OL	149-268	218 ± 38	-	-		90-220	160	79-213	200-276	205
VSL	94-137	117 ± 11	-	-	110	80-100	90	-	72-131	110
VSW	106-149	135 ± 12	60-170	120	110	80-120	100	110-150	94-150	121
HSL	180-314	230 ± 39	130-270	220	330	110-180	150	93-199	225-330	275
HSW	111-258	149 ± 36	-	-	150	100-180	140	93-186	125-200	160
GAL	38-51	42 ± 6	+	-	+	+	-	+	+	-
GAW	51-61	53 ± 4	+	-	+	+	-	+	+	-
ISVL	61-202	94 ± 37	-	-	-	30-70 (83*)	-	-	-	-
ISVW	35-111	57 ± 23	-	-	-	-	-	-	-	-
ESVL	44-147	87 ± 30	80	-	-	50-60 (80*)	-	-	-	-
ESVW	43-81	62 ± 14	-	-	-	-	-	-	-	-
TL	83-167	116 ± 26	100-220	190	240	120-140	130	67-173	150-413	272
TW	67-172	91 ± 29	-	-	360	90-110	100	53-173	200-351	264
OVL	66-116	87 ± 15	60-180	120	160	70-100	90	40-110	100-163	118
OVW	51-130	83 ± 24	-	-	120	60-90	80	40-120	75-138	96
VL	74-131	103 ± 18	-	-	-	70-90	80	-	-	-
VW	35-68	53 ± 8	-	-	-	30-50	40	-	-	-
EL	37-49	44 ± 3	30-70	50	50-55	33-35	34	22-58	38-44	41
EW	21-27	24 ± 2	20-50	30	30-35	16-18	17	19-32	19-25	21

Table 5.10. Continued.

Source	Present study		Fares & Maillard (1974)		Radujkovic et al. (1989)	Ramadan et al. (1989)		Hassan et al. (1990)	Gaevskaya & Dmitrieva (1993)	
Locality	Ebro Delta (Spain)		Languedoc-Roussillon Lagoons (Mediterranean coast of France)		Kotor Bay (Adriatic Sea)	Lake Qarun (Egypt)		Off Ras El-Bar (Egypt)	Off Sevastopol (Black Sea)	
	Range	Mean±SD	Range	Mean	Range	Range	Mean	Range	Range	Mean
Distances										
FO	256-390	325 ± 38	-	-	-	-	-	-	-	-
UEND	25-250	120 ± 63	-	-	-	-	-	-	-	-
CEND	195-407	310 ± 59	-	-	-	-	-	-	-	-
TEND	134-380	264 ± 68	-	-	-	-	-	-	-	-
Ratios										
BW/BL (%)	37-56	42 ± 6	33*	-	-	26-32	28	44*	38*	-
FO/BL (%)	26-36	32 ± 3	32*	-	-	-	-	36*	31*	-
OSL/VSL	1:0.80-1.10	1:0.94 ± 0.10	-	-	-	-	-	1:1.24*	1:0.91*	-
OSW/VSW	1:0.82-1.13	1:1.01 ± 0.08	1:0.75-1.04	1:1.0	-	1:1.25-1.43	1:1.25	1:1.35*	-	-
HSL/VSL (%)	158-255	196 ± 27	232*	-	-	180*	-	-	250*	-
TEND/BL (%)	16-36	26 ± 6	12*	-	-	15*	-	-	16*	-
CEND/BL (%)	23-39	31 ± 4	32*	-	-	34*	-	-	36*	-

* Estimated from the published drawing; +, No measurement but structure illustrated.

58-94 (n=2), slightly muscular, thick-walled; walls lined with crescentic, sclerotised structures (Fig. 5.19B). Internal seminal vesicle thin-walled (<2), elongate-oval, saccular, occupies up to third of hermaphroditic sac. Genital atrium relatively deep, with weakly-developed muscular walls (Fig. 5.19A,B). Genital pore median, usually at about halfway between pharynx and ventral sucker (11 worms), but observed at posterior pharyngeal level (2 worms) and at anterior margin of ventral sucker (1 worm).

Ovary round to elongate-oval, median to sinistral, closely posterior to hermaphroditic sac, separated from testis by uterine coils (8 worms) or anterior to and contiguous with testis (5 worms), dorsal at posterior testicular level (1 worm). Uterine seminal receptacle distinct; blind seminal receptacle absent. Mehlis' gland small, diffuse, 43 × 76 (n=1). Laurer's canal not observed. Uterus thin-walled, occupies almost entire hindbody, reaches close to posterior extremity [UEND/BL=3-24 (12)%]. Metraterm distinct, joins hermaphroditic duct close to internal seminal vesicle. Uterine vesicle not observed but metraterm dilate in single specimen. Eggs numerous, operculate; developed miracidia with single eye-spot. Vitellarium comprises 2 separated subtriangular to elongate-oval masses of coalesced follicles, lateral at level of ovary or more posterior.

Excretory vesicle tubular, slightly dilate in some specimens, with small sphincter, 25-43 × 15-48, close to excretory pore, bifurcates posterior to ovary; anterior limits of excretory arms not observed; pore terminal or ventro-subterminal.

Remarks

The brief original description of *S. tensum* (see Looss, 1902), based on a single specimen (apparently now lost) from *C. labrosus*, contains metrical data for the sizes of the body, suckers, pharynx and eggs only, and these, with the exception of the egg size, fall below the lower limits of the sample from *L. aurata* described above. Although *S. tensum* is the most widely reported species of *Saccocoelium* in the Mediterranean basin (see above), only a relatively few documented records provide information on its morphological variability.

Comparison with published data shows a narrower range of variation in the specimen sample studied (Table 5.10), which may reflect the fact that previous descriptions include combined metrical data for material from various mullet species. The lower limits of the size of body, oral sucker, pharynx and ventral sucker in descriptions by Fares & Maillard (1974), Ramadan *et al*. (1989a), Hassan *et al*. (1990b) and Gaevskaya & Dmitrieva (1993) are below the limits observed in the present material, whereas the samples described by Fares & Maillard (1974), Radujković *et al*. (1989) and Gaevskaya & Dmitrieva (1993) exhibit gonad

sizes outside the upper limits of described here (Table 5.10). However, these descriptions of *S. tensum* were probably based on composite material from more than one host species; this notably involved specimens from *M. cephalus* (except those of Hassan *et al*., 1990b) and may therefore have included *S. cephali* n. sp. described above. Characteristic features of *S. tensum* include: (i) an elongate-oval body with a rounded posterior extremity; (ii) less developed muscular walls of the genital atrium; (iii) the testis located far from the ventral sucker; (iv) the uterus reaching close to the posterior extremity; and (v) a relatively short postcaecal field.

El-Shahawi *et al*. (1992) erected *Neosaccocoelium* El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 to accommodate an apparently aberrant form based on three worms from *Mugil capito* in Lake Qarun, Egypt. They distinguished the new genus, within the Haploporinae, in 'having a coiled tubular-like vitellaria and distinguishable zygote-shaped eggs'. Although the meaning of the latter statement cannot be understood, it appears that the former discriminating feature represents a misinterpretation, since their figure 1 and the photos (plate I, photos 1, 2) clearly illustrate abnormal worms with the uterus sparsely filled with vitelline material. Otherwise *N. aegyptiacus* [sic] El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 agrees well with the morphometric features of *S. tensum* (Table 5.11), with which it is considered synonymous. Consequently, *Neosaccocoelium* becomes synonymous with *Saccocoelium*.

El-Shahawi *et al*. (1992) also described *S. saoudi* El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 based on five worms from *M. cephalus* in Lake Qarun, Egypt. The differentiating features (*i.e.* shorter oesophagus, caeca of unequal length, oval vitelline masses and small eggs) listed by these authors appear of little discriminating value when all metrical data are included to show the variation found in *S. tensum* (Table 5.11). *S. saoudi* is, therefore, considered its synonym.

These same authors (El-Shahawi *et al*., 1992) also described *S. portsaidensis* [sic] El-Shahawi, El-Gindy, Imam & Al-Bassel, 1992 on the basis of four apparently macerated specimens (body lacking spines) from *Mugil capito* at an Al-Rasswa fish farm, Egypt. It appears that maceration and improper handling has led these authors to a misinterpretation of the peculiar appearance of the oral sucker (folded ventrally, fig. 2) and the hermaphroditic duct as a 'terminal bi-lipped sac-like oral sucker' and 'globular-shaped hermaphroditic duct', respectively. Morphologically *S. portsaidensis* is indistinguishable from *S. tensum* (Table 5.11) and is here considered synonymous with the latter.

Korniychuk (2001) reported *M. soiuy* as a new host record for *S. tensum* in the Black Sea (Sevastopol Bay). However, the four juvenile specimens are poorly described and

Table 5.11. Metrical data for *Saccocoelium portsaidensis*, *S. saoudi* and *Neosaccocoelium aegyptiacus* compared with the range for *S. tensum* (see Table 5.9).

	S. tensum	*S. portsaidensis*	*S. saoudi*	*N. aegyptiacus*
Locality		El-Shahawi et al. (1992)	El-Shahawi et al. (1992)	El-Shahawi et al. (1992)
Source		Al-Rasswa fish farm (Egypt)	Lake Qarun (Egypt)	Lake Qarun (Egypt)
	Range	Range	Range	Range
Measurements				
BL	600-1,400	800-890	1,220-1,330	1,170-1,420
BW	200-550	280-350	390-500	370-460
OSL	105-139	-	100-130	100-150
OSW	76-150	-	110-120	130-160
PL	0-63	100-120	20-40	67-70
PHL	77-152	20-23	50-60	65-96
PHW	51-142	19-23	70-90	100-105
OL	149-276	160-180	150-170	360-380
VSL	94-137	-	100-140	110-140
VSW	95-170	100-120	120-140	120-150
HSL	180-330	130-150	200-230	220-270
HSW	111-258	130-140	150-180	130-170
GAL	38-51	-	-	-
GAW	51-61	-	-	-
ISVL	61-202	33-48	90-100	91-99
ISVW	35-111	-	-	48-57
ESVL	44-147	55-60	-	80-89
ESVW	43-81	-	-	43-48
HDL	76-152	85-96	79-85	100-120
HDW	58-94	-	-	-
TL	83-413	150-170	200-240	150-180
TW	67-351	160-180	170-190	130-135
OVL	60-163	84-100	120-150	96-110
OVW	51-138	70-80	90-110	75-95
VL	74-131	60-99	90-130	-
VW	35-68	30-43	50-80	-
EL	37-49	35-37	39-43	32-36
EW	21-27	21-23	20-24	20-24
Ratios				
BW/BL (%)	33-56	38*	36*	26-39 (35*)
FO/BL (%)	26-36	36*	28*	31*
OSL/VSL	1:0.80-1.10	-	-	-
OSW/VSW	1:0.75-1.13	-	1:1.0-1.05	1:0.93
HSL/VSL (%)	158-255	133*	220*	169*
TEND/BL (%)	16-36	29*	30*	45*
CEND/BL (%)	23-39	43*	58*	38*

illustrated and the range of all measurements falls well below the size range of *S. tensum*. Two eggs were observed in one specimen; these were probably immature, since their size (24-27 × 14-16 μm) is far below the range of *Saccocoelium* spp. (Tables 5.8-5.11). Therefore, this record is regarded as questionable.

Species transferred to other genera

***Elliptobursa* Wu, Lu & Zhu, 1996**

***Elliptobursa megasacculum* (Liu, Wang, Peng, Yu & Yang, 2004) Blasco-Costa et al., in press**

Syn. *Saccocoelium megasacculum* Liu, Wang, Peng, Yu & Yang, 2004

Record

Reference, Description: Liu *et al*. (2004).

Definitive hosts: *Liza carinata* (Valenciennes) (type-host).

Distribution: Area 61, (Northwest Pacific) (type-locality: off Xiamen, Fujian Province, China).

Remarks

Liu *et al*. (2004) described *Saccocoelium megasacculum* Liu, Wang, Peng, Yu & Yang, 2004 from *Liza carinata* in the Taiwan Strait. Although the authors state that 'the new species clearly falls within *Saccocoelium*', their material exhibits the following departures from the generic diagnosis of *Saccocoelium* (see Overstreet & Curran, 2005): (i) hermaphroditic duct lacking sclerotised structures; (ii) hermaphroditic sac with large vesicular pars prostatica lined with large anuclear cells (not described but figured, figs 1-2 of Liu *et al*., 2004) which occupies up to half of the hermaphroditic sac; and (iii) vitellarium composed of two round, closely connected compact masses. These features are characteristic of *Elliptobursa* Wu, Lu & Zhu, 1996, which was erected for *E. singlorchis* Wu, Lu & Zhu, 1996 described from *Liza affinis* on the Guangdong coast of China (Wu *et al*., 1996).

Elliptobursa was originally allocated to the Monorchiidae Odhner, 1911, but it is considered a haploporid genus 'as evidenced by the presence of a single testis, a long external seminal vesicle, a well-developed prostatic complex, and a long hermaphroditic duct wrongly interpreted as a cirrus' in a new revision of this family (Madhavi, 2008). Although not

described, a well-developed genital atrium is illustrated in *S. megasacculum* (figs 1-2 of Liu *et al*., 2004) and its appearance is similar to that in *E. singlorchis* (see Liu *et al*., 2004; Wu *et al*., 1996). Morphologically these two forms appear very similar, the main differences being the shorter caeca, the wider hermaphroditic sac [110-130 (120) *vs* 70-108 (89) µm] and vesicular pars prostatica [80-110 (90) *vs* 50-58 (54) µm], the somewhat smaller vitelline masses [20-30 × 20-30 (30 × 20) *vs* 33-48 × 35-48 µm] and the larger eggs [40-50 × 20-30 (50 × 20) *vs* 35-43 × 18-20 (38 × 20) µm] in *S. megasacculum*. In view of these considerations, *S. megasacculum* is transferred to *Elliptobursa* as *E. megasacculum* (Liu, Wang, Peng, Yu & Yang, 2004) n. comb.

Unisaccus Martin, 1973

Unisaccus mugilis (Rekharani & Madhavi, 1985) Blasco-Costa et al., in press

Syn. *Lecithobotrys mugilis* Rekharani & Madhavi, 1985

Material studied

Ex *M. cephalus* L. Off Visakhapatnam, India. BMNH 1984.6.28.17 (holotype and paratypes).

Record

Reference, Description: Rekharani & Madhavi (1985).

Definitive hosts: *Mugil cephalus* L.*, Liza macrolepis* (Smith); *Valamugil cunnesius* (Valenciennes) (type-host hereby designated as *M. cephalus*, the host of the holotype).

Distribution: Area 57: India (type-locality: off Visakhapatnam, brackish waters).

Remarks

This species was allocated to *Lecithobotrys* Looss, 1902 by Rekharani & Madhavi (1985), who provided no justification for the generic affiliation of their material. However, the exceptionally large eggs (larger than the testis) are not concordant with the generic diagnosis of *Lecithobotrys* (*i.e.* relatively small eggs; see Overstreet & Curran, 2005). Overstreet & Curran (2005) considered the status of *L. mugilis* Rekharani & Madhavi, 1985 uncertain and suggested that, if the vitelline follicles were slightly dispersed because of the pressure during fixation, the species could probably be allocated to *Saccocoelium*. However, the morphology of *L. mugilis* does not agree with the diagnostic features of *Saccocoelium* (*i.e.* vitellarium in two distinct groups of follicles, caeca sac-like, hermaphroditic duct containing sclerotised

structures, and uterus extensive and containing relatively small eggs). In fact, the description of *L. mugilis* complies with the generic features of *Unisaccus* Martin, 1973 [*i.e.* a combination of long prepharynx (much longer than the pharynx) and a well-developed pharynx, hermaphroditic sac about twice as long as wide, and vitellarium in form of clusters of subspherical follicles; see Overstreet & Curran, 2005)]. Two short caeca were described and figured by Rekharani & Madhavi (1985). However, the re-examination of the type-material failed to confirm this, since no distinct margins of the caeca could be observed. Although the types appear in poor condition and are all mounted laterally, the distribution of the small vitelline follicles is more similar to *Unisaccus* than *Saccocoelium*. Furthermore, *Unisaccus* is the only haploporine genus with distinctly large eggs, both in absolute dimensions and in relation to body size (Table 5.12). Therefore, *L. mugilis* is transferred to *Unisaccus* as *U. mugilis* (Rekharani & Madhavi, 1985) n. comb. This species can be differentiated from the other species of the genus, *i.e. U. brisbanensis* Martin, 1973, *U. spinosus* Martin, 1973 and *U. overstreeti* (Ahmad, 1987), by its much smaller size of both body and organs (see Martin, 1973; Ahmad, 1987). In addition, the egg-size ranges of the three *Unisaccus* spp. do not overlap with those of *U. mugilis*. The eggs in *U. overstreeti* and *U. spinosus* are distinctly larger [82-100 × 52-60 and 90-106 × 40-66 (mean 102 × 53) *vs* 78-79 × 31-37 μm], whereas those in *U. brisbanensis* are markedly smaller [46-64 × 24-29 (mean 58 × 27) μm].

***Unisaccus sprenti* (Martin, 1973) Blasco-Costa, Montero, Gibson, Balbuena, Raga & Kostadinova, in press**

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

Records

References: 1. Martin (1973c); 2. Paperna & Overstreet (1981).

Description: 1.

Definitive hosts: *Liza argentea* (Quoy & Gaimard) (type-host) (1, 2); *Mugil cephalus* L. (1, 2).

Distribution: Area 71: Australia (type-locality: Brisbane River and tributaries, Queensland) (1, 2).

Table 5.12. Comparative data for *Unisaccus* spp.

	U. overstreeti	*U. mugilis* n. comb.	*U. brisbanensis*	*U. spinosus*	*U. sprenti* n. comb.
Locality	Panjim, Goa	India	Australia	Australia	Australia
Source	Ahmad (1987)	Rekharani & Madhavi (1985)	Martin (1973)	Martin (1973)	Martin (1973)
	Range	Range	Range (Mean)	Range (Mean)	Range (Mean)
Measurements					
BL	710-940	394-407	700-840 (780)	500-774 (700)	660-755 (728)
BW	250-330	135-195	260-400 (320)	146-399 (274)	135-182 (164)
OSL	-	39-51	73-90 (75)	112-188 (159)	80-103 (86)
OSW	75-130	41-54	64-100 (90)	130-217 (192)	90-106 (103)
PL	100-150	58-89	170-280 (230)	195-290 (248)	60
PHL	75-82	27-31	22-44 (35)	72-116 (91)	60-70 (64)
PHW	65-80	39-58	30-68 (55)	130-200 (174)	60-90 (71)
OL	35-45	38-58	*c.*170-280 (230)	*c.*72-116 (91)	*c.*180
VSL	-	54-58	-	-	79-100 (87)
VSW	135-208	39-54	80-84 (82)	97-16?0 (131)	80-105 (86)
HSL	120-150	99-117	106-166 (129)	203-300 (260)	220-290 (250)
HSW	65-80	54-60	90-190 (150)	130-260 (210)	113-133 (124)
ISVL	60-75	-	-	-	-
ISVW	30-35	-	-	-	-
ESVL	100-120	-	-	-	-
ESVW	28-35	-	-	-	-
HDL	65-75	-	-	-	-
HDW	28-32	-	-	-	-
TL	-	62-78	97-147 (122)	73-206 (171)	113-246 (187)
TW	62-90	39-58	56-101 (81)	80-210 (168)	60-77 (69)
OVL	-	35-46	13-18 (16)	43-73 (61)	73-106 (90)
OVW	122-150	35-39	12-22 (17)	43-73 (61)	50-80 (72)
EL	82-100	78-79	46-64 (58)	90-106 (102)	60-77 (69)
EW	52-60	31-37	24-29 (27)	40-66 (53)	33-40 (37)
Distances					
FO	300-350	170-175	-	-	-
TEND	50-110	54-78	-	-	-
Ratios					
OSW/VSW	1:1.6-1.8	1 :1.0-1.2	-	-	-

Remarks

Martin (1973c) described a new species from *Liza argentea* and *M. cephalus,* which he assigned to *Lecithobotrys* without comment. Overstreet & Curran (2005) considered this assignation erroneous and transferred *L. sprenti* Martin, 1973 to *Saccocoelium* 'primarily because of the short saccular gut and relatively large eggs'. However, *Saccocoelium* cannot be considered the best placement for *L. sprenti* which lacks a well-developed muscular genital atrium, and possesses vitellarium composed of small compact globular follicles and eggs which are large in relation to the body. The latter two features, in combination with the long prepharynx, well-developed pharynx and the single caecum (as illustrated in a ventral view, fig. 24 of Martin, 1973c), suggest that *Unisaccus* might be a better repository for *L. sprenti*. This species resembles *U. spinosus* Martin, 1973 morphologically (described from the same host and locality), especially in the structure of the hermaphroditic sac and nature of the padded lining of the hermaphroditic duct (each pad bears two spines). However, *L. sprenti* has

smaller eggs [60-77 × 33-40 (69 × 37) *vs* 90-106 × 40-66 (102 × 53) µm], a narrower body, hermaphroditic sac and testis, a smaller oral sucker and pharynx, and a shorter prepharynx (Table 5.12). Consequently, *L. sprenti* is transferred to *Unisaccus* as *U. sprenti* (Martin, 1973) n. comb.

Species inquirenda

***Saccocoelium tripathi* Dutta, 1995**

Syn. *Saccocoelium tripathi* Datta & Manna, 1998

Records

References: 1. Dutta (1995); 2. Datta & Manna (1998).

Descriptions: 1; 2.

Definitive hosts: *Mugil* sp. (type-host) (1, 2).

Distribution: India (type-locality: Chilka Lake, Orissa) (1, 2).

Remarks

This species was originally rather poorly described and with a wrong label on the illustration of the holotype (fig. 4) by Dutta (1995). The description and illustration were reiterated with no additional information by Datta & Manna (1998). *S. tripathi* Dutta, 1995 is considered a *species inquirenda,* since it differs from the recognised species of the genus in the absence of a muscular genital atrium and sclerotised structures associated with the hermaphroditic duct, and its peculiar V-shaped asymmetrical vitellarium. Additional well-preserved material from the type-host and locality is needed in order to obtain an adequate description of *S. tripathi* and to clarify its position.

***Saccocoelium* spp. innom.**

Records

References: 1. Saoud *et al*. (1990); 2. Toman (1992).

Definitive hosts: *Mugil cephalus* L. (1); *Liza ramado* (Risso) (1); *Chelon labrosus* (Risso) (1); *C. crenilabis* (Forsskål) (2).

Distribution: Egypt (1); Off North Indian Ocean Isles (Seychelles) (2).

5.5.4. Statistical comparisons

Measurements recorded from 53 specimens (16 of *Saccocoelium obesum* complex, 14 *S. tensum*, 7 *S. cephali* and 16 *S. currani* were ln-transformed and subjected to univariate statistical tests (ANOVA) and multivariate analyses (PCA, LDA). Univariate analyses of variance of 26 metrical features and seven relative proportions/ratios indicated that *S. obesum* (the three morphs pooled), *S. tensum*, *S. cephali* and *S. currani* are morphometrically distinguishable with respect to all variables used (all $p<0.0001$). Furthermore, despite the range overlap in egg-size (Tables 5.8-5.10), the four groups differed significantly with respect to both the length ($F_{(3,178)}=144.57$, $p<0.0001$) and width ($F_{(3,172)}=115.16$, $p<0.0001$) of the eggs).

The first two principal components in a PCA run on the correlation matrix between metrical variables explained 65% of the variation in the dataset. A plot of the specimens in the first plane of the PCA (Fig. 5.20A) confirmed the identification and showed two well-separated groups that correspond to *S. cephali* and *S. currani*. It also showed a higher dispersion in the group representing *S. obesum* complex along the first principal component, with specimens belonging to the large morph 2 and *S. brayi* n. sp. appearing closer to *S. tensum*. This overlap, however, has no significance for the morphological separation of the *S. obesum* species complex from *S. tensum*, which can be achieved solely using the structure of the posterior extremity of the body. The size of body and suckers and the length of the hermaphroditic sac had the highest coefficients on the first component, which explained 53.1% of the total variance, while the length of pharynx and post-testicular, postuterine and postcaecal fields had important contributions to the second principal component.

Two backward stepwise LDA procedures run on metrical variables and relative proportions/ratios (seven variables, after a square-root transformation) separated the four species with an accuracy of 100% (Fig. 5.20B) and 92.4% (Fig. 5.20C), respectively (Wilk's Lambda=0.0006; $F_{(15,\ 124)}=111.85$, $p<0.0001$ and Wilk's Lambda=0.0259; $F_{(9,114)}=44.31$, $p<0.0001$, respectively). Using relative proportions/ratios as independent variables in the second analysis, three specimens of *S. tensum* were misclassified (two specimens as *S. obesum* (*sensu lato*) and 1 as *S. cephali*) and a single *S. brayi* n. sp. was misclassified as *S. tensum*. In the analysis based on metrical data, the first canonical function clearly discriminates between the two new species and *S. obesum* – *S. tensum* specimens, the latter being well separated by the second function (Fig. 5.20B). Although specimens of *S. currani* were well separated from the others by the first canonical function, the discrimination of the

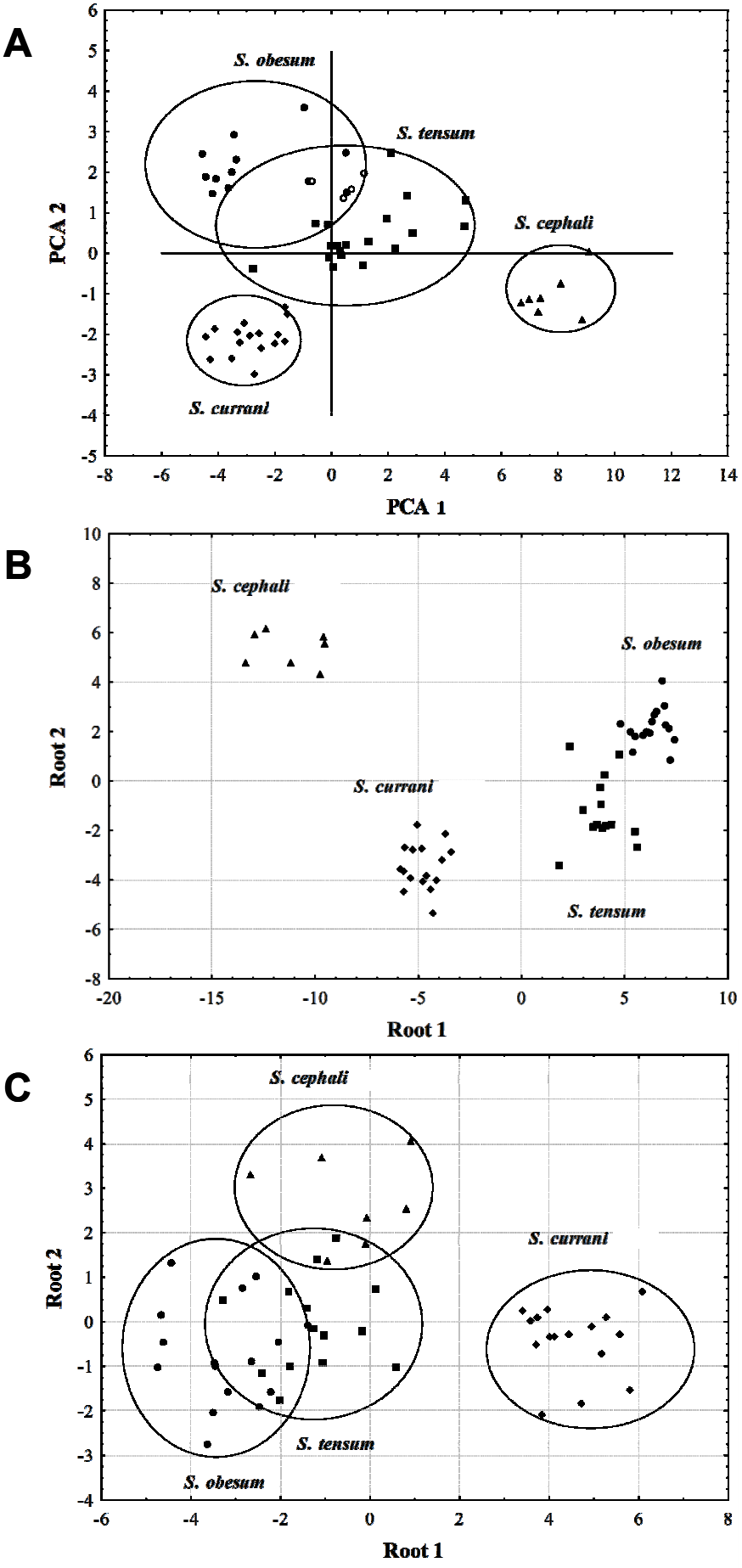

Fig. 5.20. Plots of the 51 specimens of *Saccocoelium* spp. **A.** In the first plane of the PCA. Empty circles indicate specimens of *S. brayi* n. sp. **B.** Against the first and second canonical discriminant functions (LDA, 26 metrical variables). **C.** Against the first and second canonical discriminant functions (LDA, 7 relative proportions/ratios).

four species was not as clear-cut in the analysis based on relative proportions/ratios only (Fig. 5.20C). Overall, the following variables were the most important in the speciediscrimination: (i) the size of the oral sucker; (ii) the length of the pharynx; (iii) the width of the hermaphroditic sac; (iv) the width of the genital atrium; (v) the sucker width ratio; (vi) the relative width of body; and (vii) the relative length of the post-testicular field. Three of these variables (ii, iv and vii, see above) were used subsequently in the construction of the key to the species.

5.5.5. Key to the recognised species of *Saccocoelium*

1a. Body small (BL<700 μm; BW<230 μm), elongate-fusiform. Testis large in relation to body (TL/BL=14-23%) ..... *S. cephali*

1b. Body larger (BL>850 μm; BW>320 μm), subcylindrical, elongate-fusiform to elongate-oval. Testis small in relation to body (TL/BL=5-16%) 2

2a Body with bell-shaped concavity in bluntly rounded posterior extremity. Forebody long (FO/BL=35-49%). Genital atrium muscular, strongly developed. Wall of internal seminal vesicle thick, lined by layer of large cells 3

2b Body elongate-oval to fusiform, with tapered posterior extremity. Forebody short (FO/BL<35%). Genital atrium less developed. Wall of internal seminal vesicle thin, without layer of large cells 4

3a Body elongate, subcylindrical (BW/BL=25-34%). Prepharynx long (PL/PHL=0.8-2.0) ..... *S. obesum*

3b Body plump, elongate-oval (BW/BL=43-59%). Prepharynx short (PL/PHL=0-0.6) ..... *S. brayi* n. sp.

4a Testis very elongate (length more than twice width), occupies more than half of hindbody. Ventral sucker and pharynx small (VSL≤80 μm; VSW≤90 μm; PHL≤60 μm). Eggs small (33-36 × 17-19 μm) ..... *S. gohari*

4b Testis subglobular, occupies less than third of hindbody. Ventral sucker and pharynx large (VSL>90 μm; VSW>90 μm; PHL>75 μm). Eggs large (37-51 × 21-29 μm) 5

5a Body elongate-fusiform, distinctly tapered posterior to testis. Genital atrium muscular, well developed (GAL=51-76 μm; GAW=61-73 μm). Testis located close to ventral sucker (TEND/BL=44-61%). Uterus restricted to third quarter of body (UEND/BL=28-52%). Postcaecal field long (CEND/BL=44-55%) ..... *S. currani*

5b Body elongate-oval with rounded posterior extremity. Genital atrium less developed (GAL=38-51 μm; GAW=51-61 μm). Testis located far from ventral sucker, in middle or last third of body (TEND/BL=16-36%). Uterus reaches to close to posterior extremity (UEND/BL=3-24%). Postcaecal field shorter (CEND/BL=23-39%) ..... *S. tensum*

5.6. Erection of three new genera and a key to the genera of the subfamily Haploporinae

5.6.1. Background

Until recently the subfamily Haploporinae comprised seven genera: *Haploporus*, *Saccocoelium*, *Lecithobotrys*, *Dicrogaster*, *Unisaccus*, *Forticulcita* and *Rondotrema* Thatcher, 1999 (see Overstreet & Curran, 2005). The present study resulted in the erection of three new genera. One is based on a new species parasitising *L. aurata* from the Mediterranean, and two resulted from the re-examination of the type-material of two Japanese species from mullets, originally described as *Dicrogaster japonica* Machida, 1996 and *Lecithobotrys stomachicola* Machida, 1996 (see Machida, 1996; Blasco-Costa *et al*., 2009b).

5.6.2. *Pseudodicrogaster* Blasco-Costa, Montero, Gibson, Balbuena & Kostadinova, in press

Generic diagnosis

Body inverse-pyriform, with maximum width at level of ventral sucker. Tegument thin, armed with minute spines. Oral sucker subterminal, similar in size to ventral sucker. Ventral sucker transversely elongate, fairly close to oral sucker. Forebody less than quarter of body length. Prepharynx distinct. Pharynx muscular, subspherical. Oesophagus long, two to three times length of pharynx. Intestinal bifurcation posterior to ventral sucker. Caeca two, short, saccular, end blindly at about mid-body level. Testis single, elongate-oval to sigmoid, large, occupying more than third of posterior half of body. External seminal vesicle just posterior to hermaphroditic sac, tubular, much smaller than internal seminal vesicle. Hermaphroditic sac thin-walled, pyriform, typically postero-dorsal to ventral sucker, much larger than ventral sucker. Pars prostatica small; prostatic cells few, small. Hermaphroditic duct lined with rows of unarmed pads. Internal seminal vesicle very long, tubular, coiled, occupying more than third of hermaphroditic sac. Metraterm thick-walled, strongly muscular, curved. Genital atrium apparently absent. Genital pore median, at level of anterior margin of ventral sucker. Ovary transversely elongate to sub-triangular, pre-testicular. Uterine seminal receptacle curved between hermaphroditic sac and ovary. Uterus entirely in hindbody, reaches close to posterior extremity. Eggs not numerous, operculate; developed miracidia with two distinct eye-spots. Vitellarium a single compact, transversely elongate, dumb-bell-shaped mass,

approx. twice size of ovary. Excretory vesicle small, subspherical; stem lined with small cells; pore a relatively wide dorso-subterminal slit. In mullets (Mugilidae).Type- and only species: *P. japonica* (Machida, 1996) Blasco-Costa et al., in press.

***Pseudodicrogaster japonica* (Machida, 1996) Blasco-Costa, Montero, Gibson, Balbuena & Kostadinova, in press**

Syn. *Dicrogaster japonica* Machida, 1996

Type-material: NSMT-Pl 967: 1 holotype and 26 paratypes. *Mugil cephalus* L. Intestine. 21.v.1992. Off Fukaura, Ehime Prefecture, Japan.

Description (Fig. 5.21; Table 5.13)

[Based on 27 whole-mounted adult specimens (types); measurements of holotype given in text; complete metrical data in Table 5.13.] Body relatively large, inverse pyriform, 1,015 long, with maximum width 506 (50% of body length) at level of ventral sucker (in 22 specimens) or more posterior (in 5 specimens). Tegument thin, armed with minute spines. Eye-spot pigment not observed (presumably affected by fixation). Oral sucker subterminal, oval, 109 × 121. Ventral sucker oval, 106 × 142, similar in size to oral sucker [sucker length ratio 1:0.97; width ratio 1:1.17], close to oral sucker. Forebody very short, 159 (16% of body length).

Prepharynx distinct in most specimens, 40. Pharynx muscular, subspherical, 51 × 51. Oesophagus lined with small intensely stained cells, 127 in length, *c.*2.5 times length of pharynx. Intestinal bifurcation well posterior to ventral sucker; caeca 2, sac-like, with lipoid vacuolation of gastrodermis, end blindly close to middle of body (at 45% from posterior extremity).

Testis single, sinistral to median, elongate-oval to sigmoid, large, 311 × 159, occupies more than third of posterior half of body; post-testicular field 16.2% of body length. External seminal vesicle just posterior to hermaphroditic sac, reaches vitellarium and ovary, tubular, curved, 202 × 68, much smaller than internal seminal vesicle. Hermaphroditic sac thin-walled, elongate-oval, pyriform, typically postero-dorsal to ventral sucker, 311 × 210, much larger than ventral sucker [HSL/VSL=293%)]. Internal seminal vesicle very long, tubular, coiled, 810 × 43, occupies more than third of hermaphroditic sac. Pars prostatica small, 38 × 20-25 (visible in holotype and 2 paratypes only); prostatic cells few, small. Hermaphroditic duct

lined with *c*.8 longitudinal rows of unarmed pads (Fig. 5.21B), 228 × 68, more than half length of hermaphroditic sac. Genital atrium absent. Genital pore round, median, at level of anterior margin of ventral sucker.

Ovary dextral to median, transversely elongate to sub-triangular, 91 × 137, anterior to and contiguous with testis. Mehlis' gland and Laurer's canal not observed. Uterine seminal receptacle distinct, curved between hermaphroditic sac and ovary. Uterus thin-walled, entirely in hindbody, reaches close to (*i.e.* 114 or 11.2% of body length from) posterior extremity. Metraterm thick-walled, strongly muscular, curved, 127 × 51. Eggs not numerous, 48 × 24-27, operculate; few contain developed miracidia with 2 distinct eye-spots. Vitellarium a single compact, smooth mass, transversely elongate, narrowing slightly in middle to form dumb-bell-shape, approx. twice size of ovary, anterior to or overlapping ovary dorsally, 73 × 220.

Excretory vesicle small, subspherical; stem lined with small cells; arms not observed; pore a relatively wide dorso-subterminal slit.

Remarks

Machida (1996) did not provide argumentation for the generic affiliation of *Dicrogaster japonica* and only commented the difference between the shape of the internal seminal vesicle in *D. contracta* and his new species. As part of the taxonomic revision of *Dicrogaster* the type-material of *D. japonica* was examined. This examination showed that this species does not agree with the diagnosis of *Dicrogaster* proposed by Overstreet & Curran (2005) in terms of the peculiar lining of the hermaphroditic duct (see below), having both an internal and an external seminal vesicle which are tubular, caeca more than twice the length of the ventral sucker, and eggs with two eye-spots.

The terminal genitalia, the structure of the vitellarium and the location of the main bulk of the uterus of this species, as described above, are consistent with the characteristics of the subfamily Haploporinae as defined and keyed by Overstreet & Curran (2005). Of the seven genera recognised by these authors in this subfamily (plus *Ragaia* Blasco-Costa et al., in press and *Pseudolecithobotrys* n. g., see below), only *Forticulcita* and *Pseudolecithobotrys* exhibit the tubular condition of both the internal and external seminal vesicles observed in the type-material of *D. japonica* (see also Machida, 1996; Overstreet & Curran, 2005).

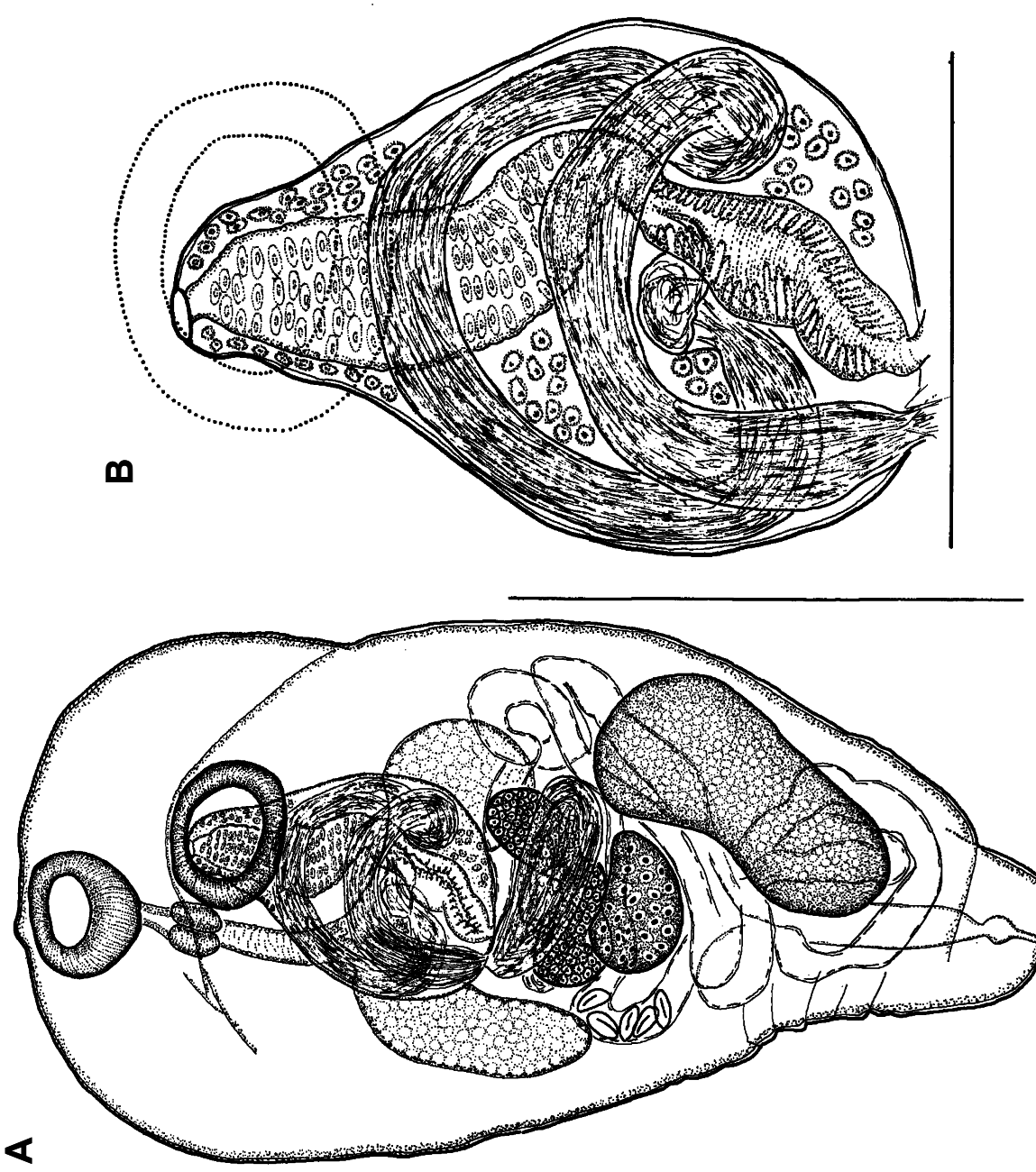

Fig. 5.21. *Pseudodicrogaster japonica* **A.** Ventral view of holotype with uterus in outline; **B.** Terminal genitalia. *Scale-bars:* **A**, 500 μm; **B**, 200 μm.

Forticulcita is known by its type-species, *F. glabra* Overstreet, 1982, a parasite of *Valamugil seheli* (Mugilidae) in the Gulf of Aqaba (Red Sea) (Overstreet, 1982), *F. mugilis* Hassanine, 2007, a parasite of *Crenimugil crenilabis* (Mugilidae) in the northern Red Sea (Hassanine, 2007), and a new species, *Forticulcita gibsoni* Blasco-Costa et al., in press from *Mugil cephalus* from western Mediterranean (described above; Blasco-Costa *et al.*, 2009a). *Forticulcita* also has a single, compact vitellarium, an unarmed hermaphroditic duct and a muscular metraterm, but differs from Machida's material in its fusiform body shape, elongate arcuate hermaphroditic sac, internal seminal vesicle much shorter than the external seminal vesicle, testis located near the middle of the body and miracidia with a single or two fused eye-spots. *Pseudolecithobotrys* n. g. erected to accommodate *Lecythobotrys stomachicola* Machida, 1996 as *P. stomachicola* (Machida, 1996) n. comb., differs from *Pseudodicrogaster* in the notably different structure of the vitellarium which represents two lateral clusters of large compact vitelline follicles (*vs* single compact dubm-bel-shaped mass). In view of these differences, *Pseudodicrogaster* is proposed and *D. japonica* is transferred to it as *P. japonica* (Machida, 1996) n. comb.

It is worth noting that, in the original description of *D. japonica,* Machida (1996) stated that the hermaphroditic duct is 'lined with fusiform cells'. A similar condition has been described in other haploporids as: (i) 'pads bearing tiny denticles' in *Skrjabinolecithum lobolecithus* (Martin, 1973); (ii) 'spirally arranged pads about 20 long, each bearing 2 spines about 7 μm long and supported by a delicate sclerotized basal lattice about 7 μm long' in *Unisaccus brisbanensis* Martin, 1973; and (iii) 'spiraling row of pads, each pad with 2 or rarely 3 spines on a reticular sclerotized base' in *U. spinosus* Martin, 1973 (see Martin, 1973a,b). Although not described, unarmed pads have been illustrated in *Pseudohapladena megaorchis* Liu & Yang, 2002 and *P. lizae* Liu & Yang, 2002 (see Liu & Yang, 2002). This condition appears to have been derived separately in several haploporid groups, notably in the Waretrematinae Srivastava, 1937 and the Haploporinae.

Table 5.13. Measurements of the type-series (n = 27) of *Pseudodicrogaster japonica*.

Character	Range	Mean ± SD
Body	681-1075 × 349-549	884 ± 104 × 445 ± 58
Forebody length	83-253	174 ± 44
Oral sucker	63-109 × 91-147	84 ± 13 × 117 ± 13
Prepharynx length	0-40	15 ± 11
Pharynx	38-73 × 38-67	51 ± 9 × 54 ± 6
Oesophagus length	91-223	151 ± 33
Ventral sucker	78-121 × 101-162	98 ± 10 × 125 ± 15
Hermaphroditic sac	167-311 × 154-291	208 ± 31 × 221 ± 35
Internal seminal vesicle	278-810 × 25-51	484 ± 146 × 36 ± 6
External seminal vesicle	81-278 × 23-71	165 ± 51 × 39 ± 12
Hermaphroditic duct	139-291 × 33-73	214 ± 35 × 53 ± 11
Metraterm	96-316 × 25-51	189 ± 64 × 35 ± 7
Testis	190-445 × 101-268	327 ± 58 × 170 ± 36
Ovary	63-114 × 68-154	82 ± 13 × 115 ± 23
Vitellarium	51-124 × 139-263	88 ± 17 × 178 ± 30
Post-caecal field	293-519	403 ± 59
Post-uterine field	23-215	103 ± 45
Post-testicular field	43-256	135 ± 57
Sucker length ratio	1:0.98-1.54	1:1.18 ± 0.13
Sucker width ratio	1:0.89-1.37	1:1.08 ± 0.12
Oesophagus/pharynx length ratio	1:2.0-5.1	1:3.0 ± 0.8
BW/BL (%)	39-64	51 ± 6
FO/BL (%)	11-28	20 ± 4
HSL/VSL (%)	165-306	214 ± 34
TEND/BL (%)	4-25	15 ± 6
CEND/BL (%)	38-52	46 ± 4
UEND/BL (%)	3-28	12 ± 6

5.6.3. *Pseudolecithobotrys* Blasco-Costa, Gibson, Balbuena, Raga & Kostadinova, in press

Diagnosis

Body elongate-fusiform, tapered posteriorly, with maximum width at level of ventral sucker. Tegument armed. Eye-spot pigment absent; gland-cells present on either side of pharynx. Oral sucker subterminal, subspherical, muscular. Ventral sucker spherical, muscular, similar in size to oral sucker, in second quarter of body. Forebody short, up to third of body length. Prepharynx distinct, wide, much shorter than pharynx. Pharynx elongate oval. Oesophagus up to twice length of pharynx. Intestinal bifurcation close posterior to ventral sucker. Caeca of variable shape (single or two), end blindly at about mid-body. Testis single, distinctly elongate, subcylindrical, in posterior half of body, well posterior to ventral sucker. External seminal vesicle contiguous with hermaphroditic sac, tubular, winding, much longer than internal seminal vesicle. Hermaphroditic sac narrow to club-shaped, arcuate, antero-dorsal to ventral sucker, may extend posterior to it; length twice length of ventral sucker. Internal seminal vesicle thin-walled, strongly elongate, subcylindrical, occupies up to third of hermaphroditic sac. Pars prostatica vesicular; prostatic cells small. Hermaphroditic duct muscular, lined with intensely-staining cells, long; length more than two-thirds length of hermaphroditic sac. Genital atrium with muscular walls. Genital pore median, just anterior to ventral sucker. Ovary dextral, globular, at mid-distance between ventral sucker and testis or more posterior. Uterine seminal receptacle present; blind seminal receptacle absent. Uterus occupies most of hindbody. Metraterm muscular, long, up to a third of hermaphroditic sac length. Eggs numerous, operculate, thick-shelled; developed miracidia with two fused eye-spots. Vitellarium two symmetrical, well separated lateral clusters of 9-10 large oval to triangular compact vitelline follicles, at level of ovary. Excretory vesicle tubular; pore terminal or subterminal. In stomach of mullets (Mugilidae). Type- and only species: *P. stomachicola* (Machida, 1996) n. comb.

***Pseudolecithobotrys stomachicola* (Machida, 1996) Blasco-Costa, Gibson, Balbuena, Raga & Kostadinova, in press**

Syn. *Lecithobotrys stomachicola* Machida, 1996

Material studied

Type-material: Ex *Crenimugil crenilabis*. Cardiac stomach. Paratypes NSMT-Pl 4291; 4353; 4377; 4748 (30 specimens). Ex '*Mugil cephalus*?'. Cardiac stomach. Off Ambon, Indonesia. 23.i.1993, 01.ii.1993. NSMT-Pl 4318, 4345 (8 specimens).

Records

Reference, Description: Machida (1996).

Definitive hosts: *Crenimugil crenilabis* (Forsskål) [corrected to *Moolgarda seheli* (Forsskål) (syn. of *Valamugil seheli* (Forsskål)) by Machida (2003)] (type-host); '*Mugil cephalus*(?)'

Distribution: Area 61: Northwest Pacific (type-locality: off Nago, Okinawa Prefecture, Japan); area 71 Northwest Pacific (off Ambon and Lombok, Indonesia).

Additional morphological data (Figs. 5.22)

Re-examination of the paratypes permitted a more detailed description of some organs, especially the terminal genitalia, as follows:

External seminal vesicle tubular, winding. Hermaphroditic sac large, arcuate, distinctly elongate (more than three times as long as wide), attenuated anteriorly, extends well posterior to ventral sucker. Internal seminal vesicle very elongate, subcylindrical, occupies more than 2/3 length of hermaphroditic sac, connects to hermaphroditic duct *via* large vesicular pars prostatica; latter with muscular sphincter at base. Hermaphroditic duct long (length more than 2/3 length of hermaphroditic sac), muscular, lined with intensely-staining cells. Genital atrium distinct, with muscular walls. Genital pore small, oval. Metraterm thick-walled, very muscular, long (length up to third length of hermaphroditic sac). Eggs 60-63 × 37-40 (only well-positioned eggs measured), possess rather thick hyaline shells (2-4 thick). Vitellarium comprises 2 clusters of large compact vitelline masses which represent individual vitelline follicles and not groups of coalesced follicles (Fig. 5.22B).

Remarks

This species was originally well described by Machida (1996). Due to the wide variation in the shape of the caeca, he provided separate descriptions for the two morphs he distinguished: (i) forms with double caeca (including specimens with 'perfectly bifurcating caeca without a stem' and 'caecum bifurcating at the middle, having a short stem and branches'; and (ii) forms with single caecum (including specimens with 'caecum not bifurcating, having two protuberances at the termination' and 'caecum completely single, saccular'). Machida (1996) pointed out the similarities with respect to the shape of caeca and distinguished the new species from species of both *Lecithobotrys* and *Pseudounicoelium* Ahmad, 1987, which appear similar with respect to the structure of the vitellarium but differ in the structure of the alimentary tract. In a recent revision of the Haploporidae, Overstreet & Curran (2005) considered *Pseudounicoelium* synonymous with *Unisaccus* Martin, 1973 and included in their key and the generic diagnosis of the latter genus two states: (i) single sac-like caecum, longer than wide, with or without bifurcations posteriorly; and (ii) relatively short caeca, never more than twice long as wide. A comparison of the morphological features of the present material with the generic diagnosis of *Unisaccus* Martin, 1973 given by Overstreet & Curran (2005) revealed a number of differences: (i) eye-spot pigment absent *vs* diffuse; (ii) oral sucker subterminal *vs* terminal; (iii) hermaphroditic sac more than three times as long as wide *vs* twice as long as wide; (iv) hermaphroditic duct unramed *vs* armed in most species; (v) metraterm muscular and long *vs* relatively short; and (vi) testis distinctly elongate and subcylindrical *vs* spherical to ellipsoidal. Some of the above characters are a matter of interpretation [(ii), (v)], dependent on the state of the material [(i), (iv)] and/or variable [(iv)]. However, the re-examination of *P. stomachicola* revealed that the latter exhibits substantial disagreement with the morphology of the species included in *Unisaccus* in terms of: the structure of the terminal genitalia (*i.e.* its more elongate arcuate hermaphroditic sac; the presence of a vesicular pars prostatica and muscular genital atrium; more muscular and distinctly longer metraterm and hermaphroditic duct; and more elongate seminal vesicles, see Fig. 5.13C); the strongly elongate subcylindrical testis; the markedly smaller thick-shelled eggs; and the unusual site of infection.

Overstreet & Curran (2005) included *L. stomachicola* in *Lecythobotrys* with a note on the variation of the shape of the caecum/caeca. *P. stomachicola* resembles *Lecithobotrys* (as defined above, this chapter) in the presence of a muscular genital atrium and a vesicular pars prostatica but differs in having: (i) notably muscular suckers of similar size *vs* ventral sucker smaller than oral; (ii) a subcylindrical testis *vs* subspherical; (iii) a tubular, winding external

seminal vesicle *vs* elongate-oval; (iv) a narrow to club-shaped, arcuate hermaphroditic sac *vs* elongate-oval; (v) a markedly elongate, subcylindrical internal seminal vesicle *vs* saccular and elongate-oval; (vi) a long (length more than two-thirds that of the hermaphroditic sac), muscular hermaphroditic duct *vs* indistinctly muscular and less than a third the length of the hermaphroditic sac; (vii) long (up to a third of the length of the hermaphroditic sac), muscular metraterm *vs* short and indistinct; (viii) thick-shelled eggs *vs* thin-shelled (this feature has not been yet observed in other species of the Haploporidae); (ix) a vitellarium in two clusters of large compact vitelline masses *vs* formed by distinct subglobular groups of small coalesced follicles; and (ix) the site of infection. In view of the differences discussed above, *Pseudolecithobotrys* n. g. is proposed and *L. stomachicola* is transferred to it as *P. stomachicola* (Machida, 1996) n. comb.

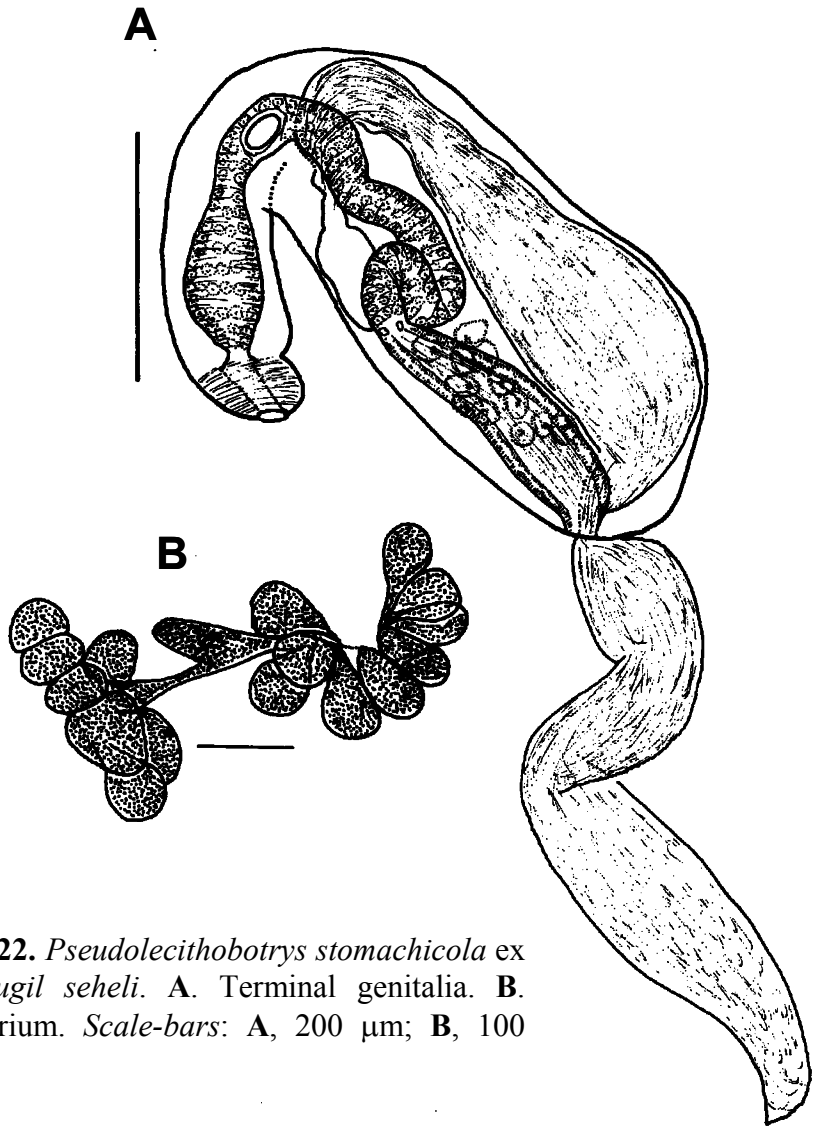

Fig. 5.22. *Pseudolecithobotrys stomachicola* ex *Valamugil seheli*. **A**. Terminal genitalia. **B**. Vitellarium. *Scale-bars*: **A**, 200 μm; **B**, 100

5.6.4. *Ragaia* Blasco-Costa, Montero, Gibson, Balbuena & Kostadinova, in press

Diagnosis

Body elongate-oval, with maximum width at mid-level of ventral sucker and bell-shaped concavity at posterior extremity. Tegument thin, armed with minute spines. Eye-spot pigment dispersed between oral sucker and mid-level of pharynx. Oral sucker subterminal, spherical, muscular. Ventral sucker strongly muscular, twice as large as oral sucker, in middle of body. Forebody more than third of body length. Prepharynx short. Pharynx strongly muscular, large. Oesophagus about three times length of pharynx. Intestinal bifurcation approximately mid-dorsal to ventral sucker. Caeca two, relatively narrow, end blindly in middle of hindbody. Testis single, subspherical, dextral, adjacent to ventral sucker. External seminal vesicle contiguous with hermaphroditic sac, saccular, thick-walled, much smaller than internal seminal vesicle. Hermaphroditic sac extends length of anterior half of body posterior to pharynx, muscular, elongate-oval, antero-dorsal and similar in length to ventral sucker. Internal seminal vesicle thick-walled, elongate-oval saccular, occupying nearly third of hermaphroditic sac. Pars prostatica small; prostatic cells numerous, very small. Hermaphroditic duct muscular, unarmed, more than half length of hermaphroditic sac. Genital atrium relatively deep, with muscular walls. Genital pore median, between pharynx and ventral sucker. Ovary sinistral, subspherical, fairly close to posterior extremity. Uterine seminal receptacle curved between ventral sucker and ovary. Mehlis' gland small, sinistral, contiguous with ovary and vitellarium. Uterus extensive, occupies almost entire hindbody, reaches to mid-level of ovary. Metraterm about third of hermaphroditic sac length. Eggs numerous, operculate; developed miracidia with two contiguous eye-spots. Vitellarium two separated, compact, smooth masses, each slightly smaller than pharynx, fairly close to posterior extremity. Excretory system not observed. In mullets (Mugilidae). Type- and only species: *R. lizae* n. sp.

Etymology: The genus is named for Professor Juan Antonio Raga in recognition to his immense effort towards the development of studies on marine fish parasites at the University of Valencia, Spain. Its gender is feminine.

***Ragaia lizae* Blasco-Costa, Montero, Gibson, Balbuena & Kostadinova, in press**

Type-host: *Liza ramado* (Risso) (Mugilidae).

Type-locality: Ebro Delta, Mediterranean coast of Spain (40º30'–40º50'N, 0º30'–1º10'E; 26.v.2004).

Site: Pyloric caeca.

Type-material: Holotype BMNH 2008.10.7.19. Paratypes*.

Description (Figs. 5.23)

[Based on 4 specimens.] Body elongate-oval, 1,050-1,178 long, with maximum width 650-689 (59-62% of body length) at mid-level of ventral sucker; bell-shaped concavity with membranous lining at posterior extremity. Tegument thin, armed with minute spines. Eye-spot pigment dispersed between oral sucker and mid-level of pharynx. Two groups of large gland-cells [2 (2+1)] on either side of pharynx. Oral sucker spherical, muscular, with ventrally subterminal aperture, 167-172 × 165-177. Ventral sucker strongly muscular, slightly transversely elongate, 345-352 × 350-377, twice as large as oral sucker [sucker length ratio 1:2.05-2.07; width ratio 1:2.12-2.13], in middle of body. Forebody 380-407 long, 35-36% of body length.

Prepharynx short, 10-15. Pharynx strongly muscular, large, subspherical, 118-128 × 120-137. Oesophagus with small dilation anteriorly, 352-364 long, *c*.3 times length of pharynx. Intestinal bifurcation approx. mid-dorsal to ventral sucker; caeca 2, elongate, relatively narrow, end blindly in middle of hindbody, at 18-19.8% from posterior extremity.

Testis single, dextral, subspherical, smooth, 235-248 × 218-225, just posterior to and slightly overlapping ventral sucker dorsally; post-testicular field 19.2-19.8% of body length. External seminal vesicle just posterior to hermaphroditic sac, saccular, elongate-oval, thick-walled (5-8 thick), much smaller than internal seminal vesicle, 95-105 × 69-73. Hermaphroditic sac muscular, thick-walled (8-13 thick), elongate-oval, somewhat constricted in middle, reaches back dorsally to mid-ventral sucker level, 340-354 × 218-228, similar in length to ventral sucker [HSL/VSL=98-101%)], contains internal seminal vesicle, numerous very small prostatic cells and relatively long muscular unarmed hermaphroditic duct of more

* The paratype specimens upon which this description was based were accidentally destroyed after acceptance of the manuscript while being prepared for deposition. Attempts will be made to collect vouchers.

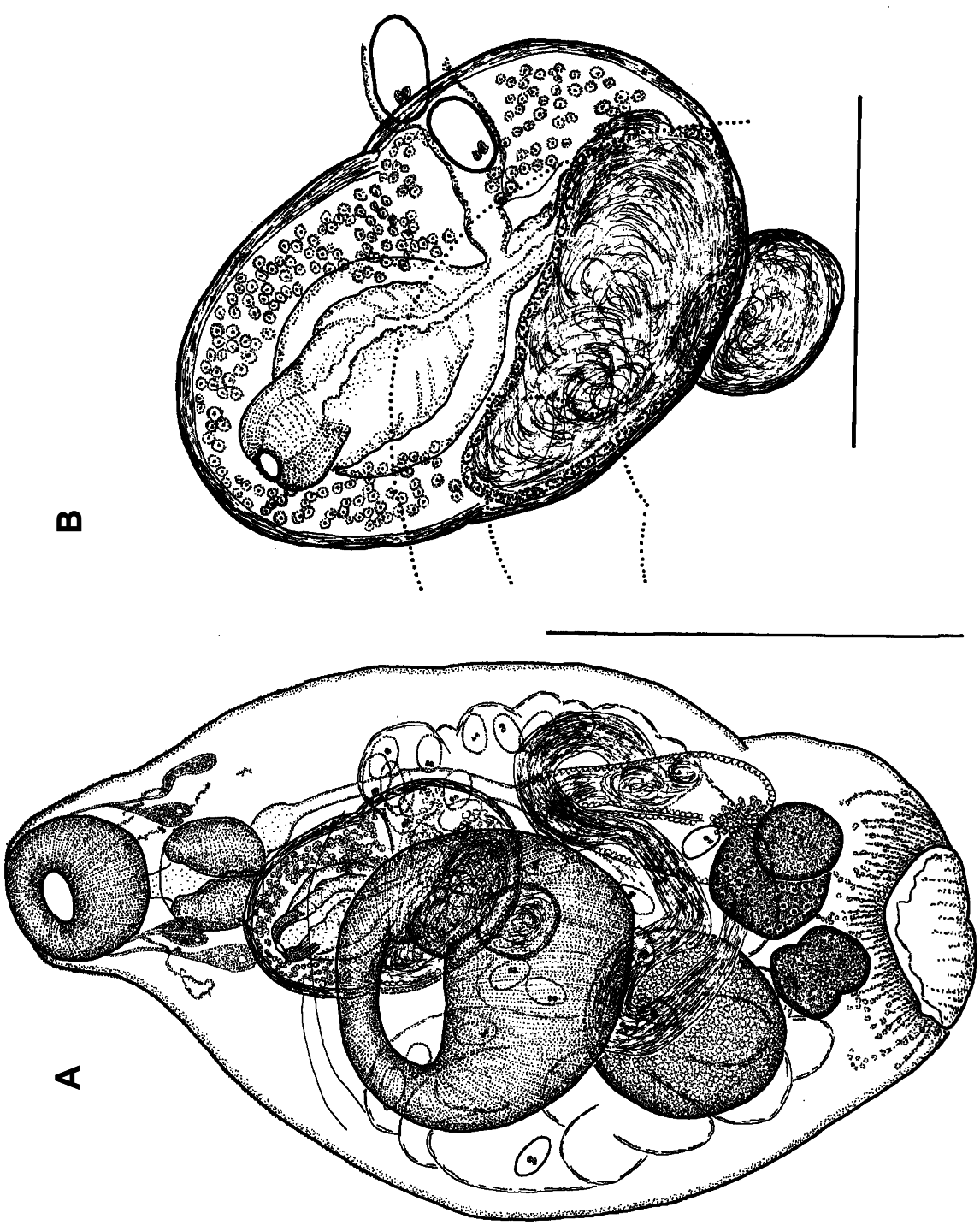

Fig. 5.23. *Ragaia lizae*. **A.** Ventral view of holotype with uterus in outline; **B.** Terminal genitalia. *Scale-bars*: **A**, 500 µm; **B**, 200 µm.

than half its length (duct 165-177 × 78-89). Internal seminal vesicle elongate-oval, saccular, 225-238 × 98-105, occupies nearly third of hermaphroditic sac, with thick (8) lining of strongly staining cells. Genital atrium relatively deep, with muscular walls. Genital pore round, median, at about half-distance between pharynx and ventral sucker.

Ovary sinistral, subspherical, 126-144 × 128-137, fairly close to posterior extremity, postero-lateral to testis. Uterine seminal receptacle distinct, rather elongate, curved between ventral sucker and ovary. Mehlis' gland small, 73 × 38, sinistral, contiguous with ovary and vitellarium. Laurer's canal not observed. Uterus thin-walled, extensive, occupies almost entire hindbody, reaching back to mid-level of ovary. Metraterm approx. third length of hermaphroditic sac. Eggs numerous, 53-61 × 29-35, with length approx. half length of pharynx, operculate, contain developed miracidia with 2 contiguous but distinct eye-spots. Vitellarium 2 separated entire, compact masses, subspherical, slightly shorter than pharynx (VL/PHL= 85.2-90.6%); posterior to testis and close to posterior extremity, 109-116 × 96-101; dextral mass constricted in middle in holotype.

Excretory system not observed.

Remarks

Ragaia lizae agrees well with the family diagnosis of the Haploporidae and exhibits the main distinguishing features of the Haploporinae (see Overstreet & Curran, 2005): (i) vitellarium comprising two compact masses of follicles lying adjacent to the ovary; and (ii) uterus occupying much of the hindbody and not confined to an area anterior to the testis.

Within the Haploporinae, which currently accommodates seven genera (see above), the new genus exhibits a unique combination of: (i) a strongly muscular ventral sucker which is twice as large as the oral sucker; (ii) a large, muscular hermaphroditic sac similar in length to the ventral sucker; (iii) a saccular, thick-walled internal seminal vesicle which is much larger than the external seminal vesicle; and (iv) the ovary and vitellarium located rather close to the posterior extremity (the former level with the posterior half of the testis, the latter just post-testicular). It is worth noting that the new genus resembles *Pholeohedra* Cribb, Pichelin & Bray, 1998 (subfamily Waretrematinae Srivastava, 1937) in the presence of a bell-shaped concavity at the posterior extremity.

Although *Ragaia* keys down to *Haploporus*/*Lecithobotrys* in the generic key of Overstreet & Curran (2005), these genera do not appear closely related to the new form. In

addition to the features listed above, *Ragaia* can be distinguished from these genera as follows:

- In *Haploporus* the hermaphroditic sac is much larger than the ventral sucker, the genital atrium is very shallow and lacks muscular walls, the caeca are longer than the ventral sucker, and the internal and external seminal vesicles are of similar size.
- In *Lecithobotrys* the vitellarium consists of two groups of large distinct follicles and the fully-developed miracidia have a single eye-spot.

The substantial morphological differences discussed above warrant the erection of a new genus.

In view of the changes to the subfamily proposed in the present study, a revised key is presented below.

5.6.5. Key to the genera of the subfamily Haploporinae

1a. Internal and external seminal vesicles tubular 2
1b. Internal and external seminal vesicles saccular 4

2a. Vitellarium two lateral clusters of large compact vitelline follicles .... *Pseudolecithobotrys*
2b. Vitellarium a single compact mass of follicles 3

3a. Hermaphroditic sac elongate-oval, pyriform. Hermaphroditic duct lined with pads. External seminal vesicle much shorter than internal .... *Pseudodicrogaster*
3b. Hermaphroditic sac elongate, subcylindrical. Hermaphroditic duct without pads. External seminal vesicle much longer than internal ..... *Forticulcita*

4a. Caecum single, sac-like, occasionally with slight bifurcation posteriorly ....… *Unisaccus*
4b. Two distinct caeca 5

5a. Caeca tubular to subcylindrical 6
5b. Caeca sac-like 7

6a. Vitellarium two clusters of distinct subspherical follicles .....*Lecithobotrys*

6b. Vitellarium two masses of coalesced follicles 8

7a. Hermaphroditic duct armed or containing sclerotised structures 9

7b. Hermaphroditic duct unarmed and without sclerotised structures ..... *Ragaia*

8a. Caeca narrow, tubular, terminate in posterior quarter of body. Forebody very short (distance between suckers less than length of pharynx)..... *Rondotrema*

8b. Caeca wide, elongate, terminate at about mid-body. Forebody longer (distance between suckers more than twice length of pharynx)..... *Haploporus*

9a. Vitellarium two (one in *Dicrogaster fastigata*) adjacent masses composed of tight conglomerate of follicles. Genital atrium shallow, non-muscular..... *Dicrogaster*

9b. Vitellarium two separated masses of loosely coalesced follicles. Genital atrium prominent, with strongly developed muscular walls..... *Saccocoelium*

CHAPTER 6

MORPHOLOGICAL AND MOLECULAR EVIDENCE OF SYMPATRIC SPECIATION IN *SACCOCOELIUM* IN MEDITERRANEAN MULLETS

6.1. Introduction

Digeneans of the family Haploporidae parasitic in mullets offer a good system to study the role of encounter and compatibility filters (*sensu* Combes, 1995; Combes & Théron, 2000) on sympatric speciation. The parasites utilise simple two-host life-cycles in which the dispersal stages (gymnocephalous cercariae) leave the intermediate snail host and encyst in the open to be ingested by typically detritivorous mullets (Cardona, 2000; Cardona *et al*., 2001) in which the adult stages develop. This passive ingestion transmission pattern combined with the similar feeding ecology of the definitive hosts as a group, suggests a relaxed encounter filter and this is supported by the notion that mullet host-parasite systems are, in general, characterised by a high degree of exchange of parasites in areas, such as the Mediterranean and the Red and Black Seas, where a number of mullet species occur in sympatry (Esch & Fernandez, 1993). Although host-parasite records tend to support the notion that host-associated factors have little influence on species diversity in the haploporids, these records probably reflect the lack of knowledge of the intra- and interspecific morphological and genetic variation within this group.

Saccocoelium Looss, 1902 was erected for two species (*S. obesum* Looss, 1902 and *S. tensum* Looss, 1902) discovered in the Adriatic Sea. Further studies described and assigned eight species to this genus (six nominal and two transferred from *Lecithobotrys*, see detailed account in section 5.5.1). Still today most species of *Saccocoelium* are only known from their original descriptions. Although *S. obesum* and *S. tensum* are the most widely reported in virtually all sympatric Mediterranean mullet species, there are few documented records providing data on their morphology and some authors have considered them to be synonymous (listed in section 5.5.1)

As part of the present study the status of the species of *Saccocoelium* was re-assessed based on examination of newly collected material and critical evaluation of published descriptions. This revision resulted in the descriptions of two new species from *M. cephalus*, *S. cephali* Blasco-Costa et al., 2009c and *S. currani* Blasco-Costa et al., 2009c; redescriptions of *S. obesum* and *S. tensum* based on material from *Liza* spp.; discrimination with the aid of multivariate analyses of the four Mediterranean species; and a key to the species (see section 5.5; Blasco-Costa *et al*., 2009c). However, the unexpected diversity of haploporids parasitising *M. cephalus* (see sections 5.3 & 5.5; Blasco-Costa *et al*., 2009 a,c) and the wide intraspecific morphological variation in the species parasitising *Liza* spp. detected in the course of the study, prompted simultaneous sequencing of the rRNA genes.

In this chapter morphological and DNA sequence data of the 28S and ITS2 rRNA genes are used to resolve the taxonomic status of *Saccocoelium* species parasitising sympatric mullets in the western Mediterranean. In particular, this study provides a test of the hypothesis for the species delimitation based on morphology of *S. obesum*, *S. tensum* and *S. cephali* and examines the phylogenetic affinities of the forms parasitising *M. cephalus* and *Liza* spp. The system selected for study is small but characteristic for the known size of the genera of the subfamily Haploporinae and thus may provide an example for the application of a combined approach to species diversity within the Haploporidae.

6.2. Materials and methods

6.2.1. Morphological data

The material studied comprises trematodes from four mullet species (*M. cephalus*, *L. aurata*, *L. ramado* and *L. saliens*) collected at Ebro Delta, off Santa Pola and in a brackishwater lagoon near Santa Pola. Trematodes were processed as described in section 3.2. Abbreviations of the metrical features examined are listed in section 3.4. Eight morphotypes of *Saccocoelium* were distinguished on morphological grounds and sequenced in the course of the study: two of *S. obesum* ex *Liza* spp.; four of *S. tensum* ex *Liza* spp.; and two ex *M. cephalus* (*S. cephali* and *Saccocoelium* sp.).

6.2.2. Statistical analyses

Multivariate statistical analyses were performed on 26 metrical variables. First, a principal components analysis (PCA) was applied to scrutinize the multivariate relationship between the 52 specimens assigned to eight *a priori* groups corresponding to the delineated morphotypes within *Saccocoelium* spp. Secondly, a linear discriminant analysis (LDA; backward stepwise procedure) was applied to 51 specimens, which were assigned to seven *a priori* groups (morphotype 4 of *S. tensum* was excluded because it was represented by a single specimen), in order to evaluate the morphometric differences between them and to identify the variables yielding optimal separation. The squared Mahalanobis distances between the group centroids obtained in the LDA were used to perform a test for association between genetic and morphological distance between recognised morphotypes by applying a method based on the permutation of distance matrices. Mantel test for matrix correlation was carried

out by regressing the Mahalanobis distances on the distances in the percentage of sequence difference matrix (ITS2 region only). The significance of the best regression model was tested with a randomisation approach (9,999 random permutations of the dependent variable matrix) (Manly, 1997) using RT 2.1 program (Western EcoSystems Technology, Inc., Cheyenne, Wyoming).

6.2.3. Molecular data

Isolation, amplification and purification of gDNA, and sequence alignment follow the protocols described in Chapter 3. A total of 26 sequence replicates for both complete ITS2 and partial (domains D1-D3; ~1400 bps) 28S rDNA regions were obtained for the eight morphotypes of *Saccocoelium*, and *F. gibsoni* and *H. benedeni* used as outgoup taxa. Sequences were submitted to GenBank (see Table 3.7 for host, replicates and accession numbers).

Newly generated sequences of *Saccocoelium* spp. were aligned together with the new sequences of the outgroup taxa and adjustments made by eye using MacClade 4.08 (Maddison and Maddison, 2005). Sequences for both gene fragments were concatenated and regions of ambiguous alignment were defined in a character exclusion set. Pairwise distances for each rDNA region were calculated from the trimmed (to match the shortest sequence) aligned sequences with the absolute pairwise character difference (gaps treated as missing data) and the percentage of pairwise character differences on a total of 451 and 1189 unambiguously aligned positions for the ITS2 and the 28S rDNA, respectively.

6.2.4. Molecular analysis

ITS2 and 28S rDNA data partitions were analysed individually and combined by the methods of maximum parsimony (MP) and Bayesian inference (BI). MP analyses were performed using a heuristic search strategy with 1,000 search replicates, random-addition taxa sampling, tree-bisection-reconnection branch-swapping, with all characters run unordered with equal weights and with gaps treated as missing data. Nodal support was estimated by bootstrap analysis (heuristic search strategy with 1,000 pseudoreplicates and 100 random sequence addition each). Prior to BI analyses, the nucleotide substitution model was estimated using ModelTest 3.06 (Posada & Crandall 1998) independently for each data partition. The model GTR+Γ (general-time-reversible model including gamma distributed among-site rate

variation) was estimated as the one fitting the data best. BI analyses were run over 1 million generations with a sampling frequency of 100. Consensus trees with mean branch lengths were constructed using trees after log-likelihood values and substitution parameters plateau at approx. generation number 12,700 and 6,600 for the ITS2 and 28S rDNA regions, respectively; and at 6,400 for the combined analysis. Nodal support was estimated as posterior probabilities (Huelsenbeck *et al.* 2001).

6.3. Results

6.3.1. Morphological data

Two morphotypes of S. obesum *ex* Liza *spp.*

Detailed description of *S. obesum* based on material of two morphotypes (labelled 'large morphs 1 and 2' from *L. aurata* in the Ebro Delta and the Black Sea, respectively) is provided in section 5.5 (see also Blasco-Costa *et al.*, 2009c). Examination and sequencing of additional material of the third morphotype (initially collected from *L. aurata* in the Ebro Delta and labelled as 'small morph' in section 5.5) from *L. saliens* from the same locality confirmed the distinctness of this morph described as *S. brayi* n. sp. (description and remarks given in section 5.5).

Four morphotypes of S. tensum *ex* Liza *spp.*

In addition to the material from *L. aurata* and *L. ramado* identified and described as *S. tensum* in Chapter 5 and Blasco-Costa *et al.* (2009c) which is labelled as morphotype 1 here, three isolates which closely resembled *S. tensum* (see Fig. 6.1) were found in *L. ramado* (labelled as morphotypes 2-4). It is worth noting that morphotype 1 was collected in fish from the Ebro Delta, morphotype 2 was the only species of *Saccocoelium* found in the brackishwater lagoon near Santa Pola, whereas morphotypes 3 and 4 were collected in hosts fished off Santa Pola.

The specimens tentatively identified as *S. tensum* generally showed a morphological homogeneity but the three morphotypes ex *L. ramado* gradually exhibited increasing upper ranges for most morphometric features (Table 6.1), the larger two forms being distinctly larger and with measurements of the ventral sucker, and the length of genital atrium, testis, vitelline and postcaecal fields varying outside the ranges for the morphotypes 1 and 2.

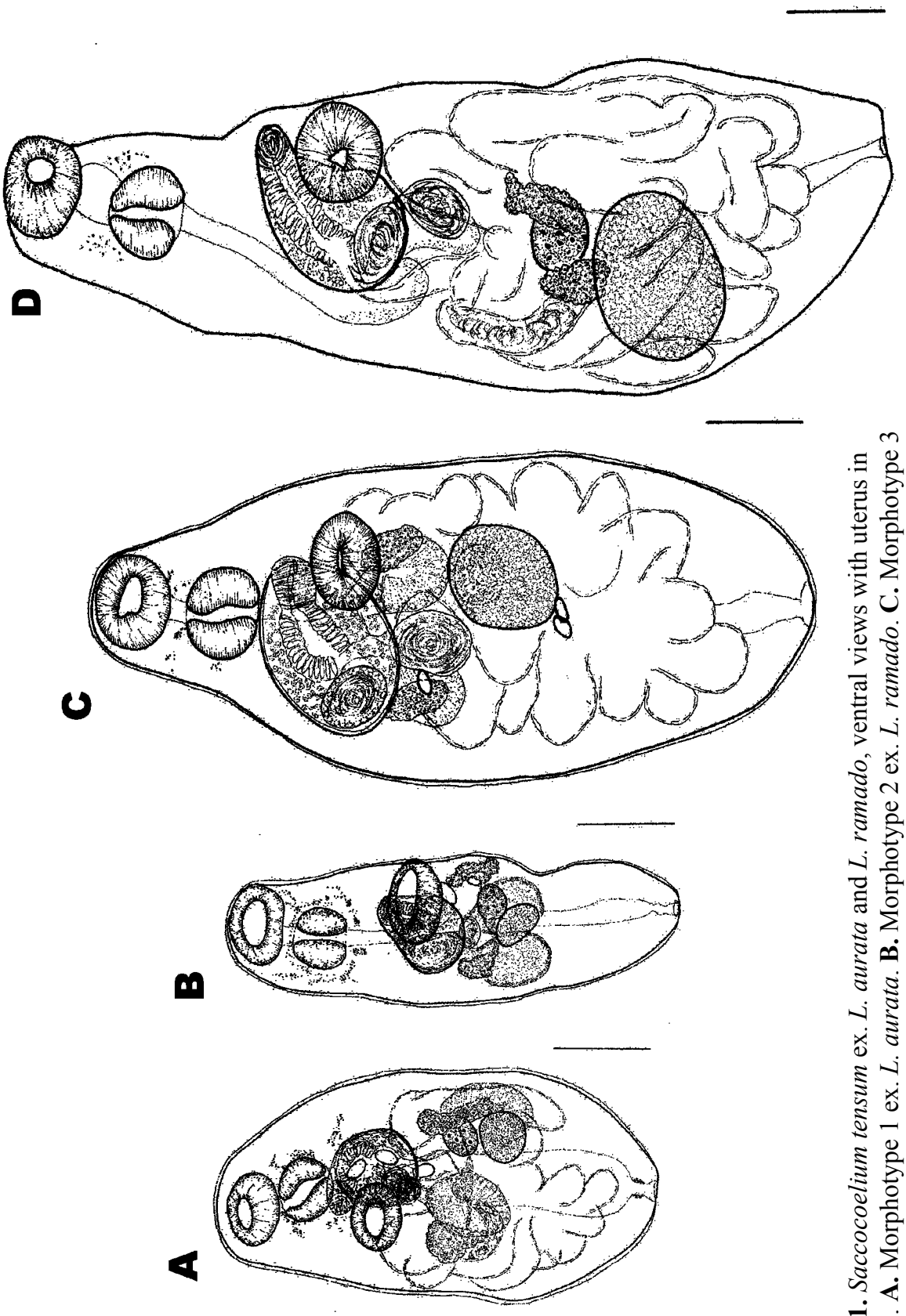

Fig. 6.1. *Saccocoelium tensum* ex. *L. aurata* and *L. ramado*, ventral views with uterus in outline. **A.** Morphotype 1 ex. *L. aurata*. **B.** Morphotype 2 ex. *L. ramado*. **C.** Morphotype 3 ex. *L. ramado*. **D.** Morphotype 4 ex. *L. ramado*. *Scale-bars:* 200µm.

Table 6.1. Comparative morphometric data for the four morphotypes of *S. tensum* ex *L. aurata* and *L. ramado*.

Morphotype	**1**		**2**		**3**	**4**
Host	*L. aurata*		*L. ramado*		*L. ramado*	*L. ramado*
	Range	Mean	Range	Mean	Range	n=1
Measurements						
BL	853-1,133	1,011	890-1,271	1,101	1,240-1,510	1,865
BW	321-548	428	278-434	356	521-584	560
OSL	105-139	125	110-148	137	101-152	154
OSW	114-149	134	139-162	149	139-167	181
PL	0-63	16	5-45	32	33-99	68
PHL	109-152	121	110-137	128	130-154	152
PHW	94-142	107	110-132	120	137-170	171
OL	149-268	218	192-308	235	220-240	309
VSL	94-137	117	114-157	131	137-147	177
VSW	106-149	135	140-185	156	139-177	180
HSL	180-314	230	131-238	211	235-349	316
HSW	111-258	149	104-168	143	197-253	233
GAL	38-51	42	42-76	59	61-114	76
GAW	51-61	53	50-76	62	43-83	73
ISVL	61-202	94	101-126	116	134-177	149
ISVW	35-111	57	45-76	60	86-114	99
ESVL	44-147	87	48-87	64	111-170	147
ESVW	43-81	62	40-70	54	73-114	94
TL	83-167	116	58-134	101	170-235	266
TW	67-172	91	57-91	73	106-197	316
OVL	66-116	87	46-106	73	81-137	-
OVW	51-130	83	53-87	66	116	-
VL	74-131	103	74-151	104	137-159	163
VW	35-68	53	35-67	48	71-73	76
EL	37-49	44	45-51	48	43-47 (45)	-
EW	21-27	24	24-28	26	25-27 (26)	-
Distances						
FO	256-390	325	325-456	393	353-458	584
UEND	25-250	120	92-227	138	86-129	175
CEND	195-407	310	240-378	320	503-663	863
TEND	134-380	264	211-336	284	245-539	385
Ratios						
BW/BL (%)	37-56	42	29-38	33	37-39	30
FO/BL (%)	26-36	32	34-40	36	27-30	31
OSL/VSL	1:0.80-1.10	1:0.94	1:0.85-1.06	1:0.96	1:0.97-1.32	1.15
OSW/VSW	1:0.82-1.13	1:1.01	1:1.03-1.14	1:1.09	1:0.83-1.06	0.99
HSL/VSL (%)	158-255	196	105-184	161	201-237	179
TEND/BL (%)	16-36	26	23-31	26	17-36	21
CEND/BL (%)	23-39	31	24-33	29	47	46

Specimens of all three morphotypes were distinctly more elongate than those of morphotype 1 as shown by the lower upper limits (30-39 *vs* 56%) and means (30-33 *vs* 42%) for the ratio BW/BL (see also Fig. 6.1). These differences reflected in the separation of the specimens of the morphotypes 3 and 4 in the multivariate analyses (below). Although the morphometric differentiation in the two-dimensional plane was not as clear for the morphotypes 1 and 2, the latter were distinguished prior to sequencing in the following characters: (i) more elongate,narrower body; (ii) long forebody; and (iii) hermaphroditic sac shorter in relation to ventral sucker which also appears large in relation to body (Fig. 6.1B). As shown in Table 6.1 the specimens of morphotype 2 also exhibit larger means for the size of pharynx, suckers, genital atrium and eggs, the length of prepharynx, oesophagus and internal seminal vesicle but possess smaller gonads. The specimens of this morphotype were all gravid adults bearing 30-89 eggs (30, 36, 44, 48, 76, 68 and 89, respectively) and the number of eggs was not correlated with body length ($p = 0.119$).

Two Saccocoelium *morphotypes ex M. cephalus*

Finding of yet another form parasitising *M. cephalus* in the Ebro Delta, an extensively sampled locality in which two new species, *S. cephali* and *S. currani* were already described (see section 5.5; Blasco-Costa *et al.*, 2009c; unfortunately, the attempts to sequence the latter species failed), was unexpected. However, a third form, *Saccocoelium* sp., was distinguished morphologically in this host, which differed from *S. cephali* (described from the voucher specimens of the present molecular study; see Chapter 5) in its distinctly larger and more elongate body (BW/BL=22-26% *vs* 26-42%; mean 24 *vs* 36%) with maximum width at level of ventral sucker (*vs* at junction of first and second body thirds), larger suckers, testes, ovary and vitelline masses and somewhat smaller eggs (see Fig. 6.2. and Table 6.2). The specimens of *Saccocoelium* sp. also possessed a longer oesophagus, more anteriorly terminating caeca (at mid-body *vs* mid-hindbody) and a more anterior location of the most posterior uterine loops (UEND/BL=22-42 *vs* 8-16%). The size differences could not be attributed to growth since some of the specimens of the larger form, *Saccocoelium* sp., were neogravid. However, lack of sufficient number of fully gravid worms prevented confident identification of the species.

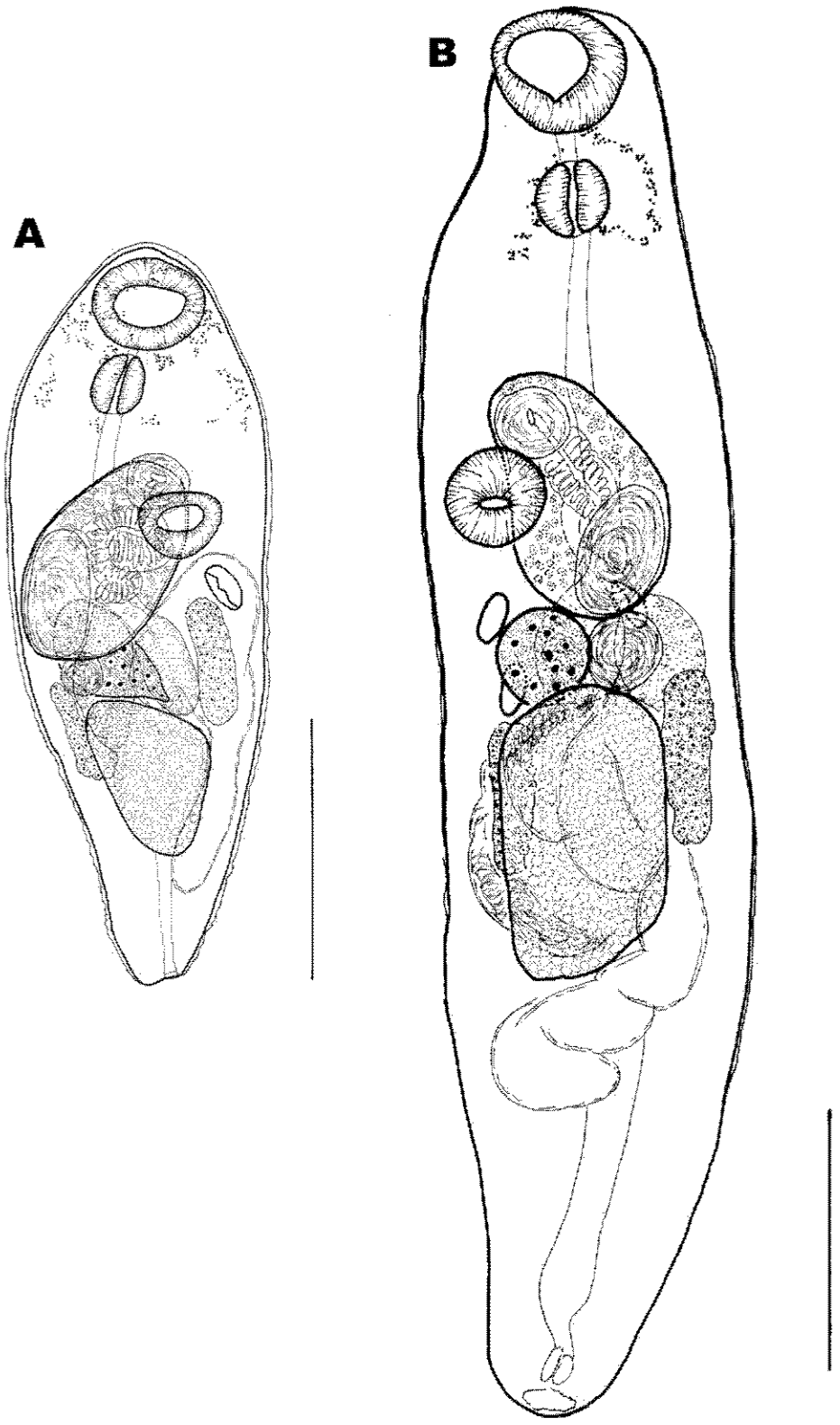

Fig. 6.2. *Saccocoelium* morphotypes ex *M. cephalus*. **A.** *Saccocoelium cephali*, ventral view of a paratype with uterus in outline. **B.** *Saccocoelium* sp., ventral view with uterus in outline. *Scale-bars*: 200 μm.

Table 6.2. Comparative morphometric data for *S. cephali* and *Saccocoelium* sp. ex *M. cephalus*.

	S. cephali		*Saccocoelium* sp.	
	Range	Mean	Range	Mean
Measurements				
BL	496-664	583	875-1,088	985
BW	173-230	207	198-270	236
OSL	64-83	76	90-96	95
OSW	78-104	88	104-126	113
PL	0-14	4	0-22	10
PHL	46-58	53	51-58	53
PHW	42-51	47	46-62	55
OL	77-192	143	216-288	255
VSL	53-66	57	64-75	72
VSW	60-69	65	64-80	74
HSL	142-166	154	155-200	175
HSW	94-104	100	106-147	118
GAL	32-48	40	37-40	39
GAW	42-56	49	38-58	47
HDL	-	-	c. 88-96	-
HDW	-	-	c. 30-50	-
ISVL	70-110	94	88-133	107
ISVW	32-54	43	64-80	73
ESVL	48-99	69	54-107	71
ESVW	32-37	33	46-85	60
TL	77-136	107	218-355	284
TW	61-99	80	128-189	156
OVL	51-96	61	70-114	92
OVW	38-61	52	72-120	93
VL	58-93	76	70-142	116
VW	26-56	40	42-55	47
EL	42-43	42	39	-
EW	22-23	22	20	-
Distances				
FO	171-200	187	298-336	317
CEND	99-272	207	378-548	475
TEND	66-154	124	163-474	311
UEND	48-91	64	235-368	313
Ratios				
BW/BL	26-42	36	22-26	24
FO/BL	28-37	32	31-36	33
VSL/OSL	1:0.64-0.82	1:0.71	1:0.71-0.78	1:0.76
VSW/OSW	1:0.63-0.84	1:0.74	1:0.60-0.77	1:0.66
HSL/VSL	228-313	273	239-267	248
TEND/BL	13-27	21	19-52	32
CEND/BL	20-44	33	47-56	51

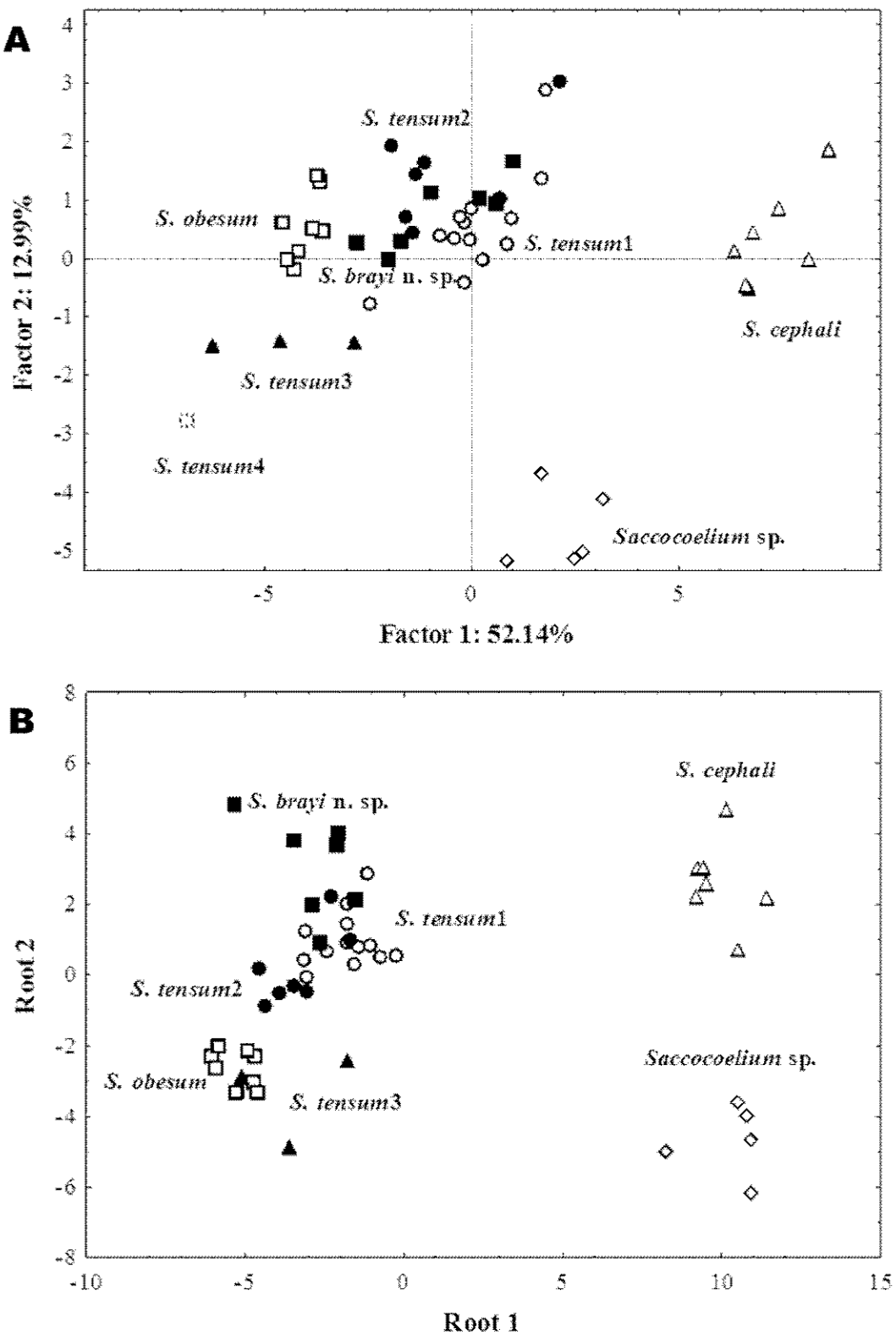

Fig. 6.3. Plots of the 52 specimens of *Saccocoelium* spp. **A.** In the first plane of the PCA. **B.** Against the first and second canonical discriminant functions (LDA).

Morphometric variation of* Saccocoelium *spp.

The considerable morphological variability encountered in the present collection required a thorough examination to determine the species status of the morphotypes. Therefore, the multivariate approach adopted in Chapters 4 and 5 was followed. First, PCA was applied to two principal components of the PCA run on the correlation matrix between 26 metrical variables of the eight morphotypes explained 65.1% of the variation in the data-set comprising 52 specimens. The size of the body, suckers, pharynx, hermaphroditic sac, and the length of the forebody had the highest coefficients on the first component, which explained 52.1% of the total variance (eigenvalue 13.6), whereas testis size and the length of post-uterine and post-caecal fields had important contributions to the second principal component which explained a further 13.0% of the variance (eigenvalue 3.4). A plot of the specimens in the first plane of the PCA (Fig. 6.3A) shows two well-separated groups along both the first and second axis that correspond to the two morphotypes from *M. cephalus*. *S. obesum* (*sensu stricto*) (*s.s.*) and *S. brayi* n. sp. specimens appeared close, but separated along the first axis. On the other hand, although the morphotypes of *S. tensum* did not show a clear clustering pattern, the specimens of morphotypes 1 and 2 were more closely located in the two-dimensional plane, whereas those of morphotypes 3 and 4 appeared somewhat separated along the second axis. The specimens of *S. brayi* n. sp. appeared closer and overlapped morphotypes 1 and 2 of *S. tensum* in this analysis based on morphometric data only. However, the new species can be readily distinguished from *S. tensum* using only the structure of the posterior extremity of the body.

Secondly, a backward stepwise LDA run on 26 metrical variables separated the seven morphotypes with 100% accuracy (Fig. 6.3B) (Wilk's Lambda=0.00023; approximate $F_{(30, 162)}$=38.30, $p<0.0001$). The first canonical function clearly discriminates the two morphotypes ex *M. cephalus* from the remaining specimens whereas the second canonical function contributes to the discrimination between *S. obesum* (*s.s.*) and *S. brayi* n. sp. and between the former and the morphotypes 1 and 2 of *S. tensum*. The discriminatory power of the model was associated with only five variables (four already identified by the PCA, see above). The size of the pharynx and the length of the muscular genital atrium exhibited strong correlation with the first axis and length of body and width of testis were associated with the discrimination along the second axis of the two-dimensional plane.

6.3.2. Molecular analysis

A total of 21 sequence replicates of both 28S and ITS2 rRNA gene regions was obtained from individuals of the eight morphotypes of *Saccocoelium* spp. Multiple replicates (Table 3.5) obtained for the two rDNA regions from *S. tensum*, *S. obesum* and *Saccocoelium* sp. specimens showed no variation within the sequences for either region. The 28S and ITS2 sequences for all morphotypes examined were aligned together with the sequences for the two outgroup taxa. The alignment of the 28S rDNA incorporated a total of 1,189 included characters (bp and gaps) and the alignment of the ITS2 represented a total of 451 characters. Comparative sequence analysis revealed four unique genotypes for each of the two rDNA regions examined: (i) *S. obesum* (*s.s.*); (ii) *S. brayi* n. sp.; (iii) *S. tensum* (all four morphotypes); and (iv) *S. cephali*/*Saccocoelium* sp.

Table 6.3. Pairwise nucleotide sequence comparisons between taxa, calculated as percentage of nucleotide differences (gaps treated as missing data) for the aligned ITS2 (above the diagonal; N=451 nt) and 28S rDNA (below the diagonal; N=1189 nt) sequences.

Taxon	*S. cephali*	*S. tensum*	*S. obesum*	*S. brayi*	*H. benedeni*	*F. gibsoni*
Saccocoelium cephali	-	3.1	7.2	7.2	6.3	16.8
Saccocoelium tensum	2.5	-	7.2	6.3	6.5	16.0
Saccocoelium obesum	4.1	3.4	-	1.6	8.8	17.5
Saccocoelium brayi n. sp.	4.3	3.7	0.8	-	9.0	17.4
Haploporus benedeni	7.7	7.1	7.7	8.0	-	15.4
Forticulcita gibsoni	9.4	9.1	9.1	9.6	9.7	-

Differences in the 28S sequence among species of *Saccocoelium* ranged from 0.8 to 4.3% (9-51 nucleotide sites) and those in the ITS2 sequence ranged from 1.6 to 7.2% (7-33 nt) (Table 6.3). The smallest differences were observed between the two morphotypes of *S. obesum* (*sensu lato*) [*i.e. S. obesum* (*s.s.*) and *S. brayi* n. sp.] both showing the highest percentage of sequence difference with the other two *Saccocoelium* genotypes. However, the two morphotypes from *M. cephalus* which appeared morphologically distinct (*S. cephali* and *Saccocoelium* sp., see above), shared the same genotype for both regions analysed and the four morphotypes of *S. tensum* (one from *L. aurata* and three from *L. ramado*) were genetically identical for the two rDNA regions. Mantel test aimed to assess the congruence between the genetic (ITS2 region) and morphometric differentiation between the seven morphotypes resulted in a high p-value (p=0.332) leading to acceptance of the null hypothesis of the lack of correlation between the genetic and morphometric distance matrices in the

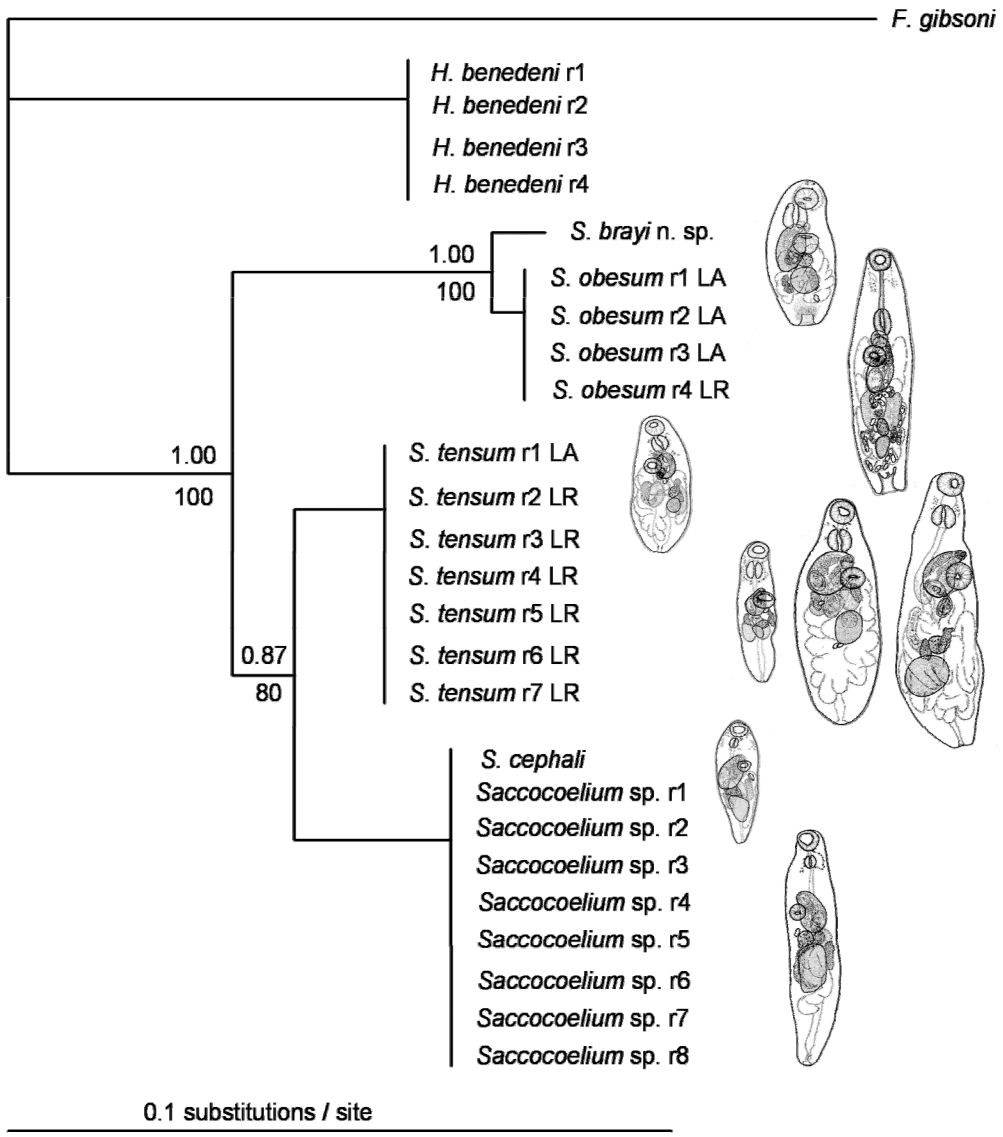

Fig. 6.4. Tree topology derived from the combined 28S and ITS2 rRNA gene sequences using Bayesian analysis with posterior probability values above and MP bootstrap values below the branches. *Abbreviations*: r followed by number, sequence replicate number; LA, *Liza aurata*; LR, *Liza ramado*.

present material. Figure 6.4 presents the tree topology generated with BI analyses of the combined dataset (28S and ITS2). MP of the combined dataset produced a single most-parsimonious tree (length 336, consistency index 0.896) with strong nodal support for all clades (not shown). The tree topologies obtained in independent analyses of the two gene regions shared the same branching patterns and showed similar levels of support. Within the ingroup, two strongly supported clades were recognised, one formed by morphotypes of *S. tensum* from *L. aurata* and *L. ramado* and morphotypes from *M. cephalus*, and the other comprising *S. obesum* (*s.s.*) and *S. brayi* n. sp., the latter subtended by a longer branch.

6.4. Discussion

The present study is the first parallel molecular and morphological attempt focused on characterisation of a group of congeneric species within the Haploporidae, a poorly known digenean family characterised by a long history of scattered poorly documented records, inadequate descriptions, poor specific diagnoses and extensive synonymy (Overstreet & Curran, 2005). The family has also been found to be highly labile in its placement in the most comprehensive molecular phylogenetic analysis of the Digenea performed to date (Olson *et al*., 2003), and this reflects the difficulty in characterizing the group at effectively all taxonomic levels. Combining sequence data analysis with a detailed morphological and multivariate morphometric study of the specimens has permitted a more refined estimation of the amount of genetic and morphological differentiation that is typical for closely related species of *Saccocoelium* from sympatric mullets in the Mediterranean.

One important result is that molecular data corroborated the decisions based on morphology with respect to the distinct status of the species of *Saccocoelium*, *i.e. S. obesum*, *S. tensum* and *S. cephali*, described in detail in section 5.5 and Blasco-Costa *et al*. (2009c), thus rejecting the hypothesis of a single species in Mediterranean mullets (*e.g*. Dawes, 1947; Mikailov, 1958; Fischthal & Kunz, 1963; Ferreti & Paggi, 1965; Moravec & Libosvárský, 1975). Furthermore, as expected from morphological data, the analysis of both gene regions confirmed the distinct species status of *S. obesum* (*s.s.*) and supported the recognition of *S. brayi* n. sp. (Fig. 5.16). Results based on sequence divergence in the ITS2 are difficult to interpret, especially in cases when low interspecific variation is detected between congeners. In such situations, one is most likely to either fail to recognise multiple sibling species or to create new ‘species’ destined for synonymy (see Nolan & Cribb, 2005 for an extensive review). The amount of genetic variation between *S. obesum* (*s.s.*) and *S. brayi* n. sp. was

lower than that observed between *S. tensum* and *S. cephali* and this would suggest their recent separation. Further, the morphological differences between the morphotypes of *S. obesum* were depicted prior to sequencing (section 5.5; Blasco-Costa *et al*., 2009c). This, coupled with the observed sequence divergence in the 28S and ITS2 regions, the former slightly above the minima (*e.g.* 0.2-0.4% in Cryptogonimidae, see Miller & Cribb, 2007a,b) and the latter higher than or closely approaching the lowest levels reported between congeneric taxa in other marine digenean systems (*e.g.* 0.5% in Didymozoidae, see Anderson & Barker, 1998; 0.3% in Sanguinicollidae, see Nollan & Cribb, 2006; 0.4-1.4% in Cryptogonimidae, see Miller & Cribb, 2007a,b), support the decision to recognize the distinct species status of *S. brayi* n. sp. Further sequencing, especially of material from the Black Sea, will be useful to test the hypothesis for higher diversity in the *S. obesum* complex (see section 5.5; Blasco-Costa *et al*., 2009c) and to reveal the divergence rates in this morphologically and genetically distinct lineage of *Saccocoelium*.

The lack of genetic differentiation among the four morphotypes of *S. tensum* and the two morphotypes from *M. cephalus* was unexpected based on *a priori* examination of morphology. This uncertainty is reflected in the larger number of replicate sequences examined in this clade (15 *vs* 5, see Table 3.5). The initial hypothesis of polymorphism in the case of *S. tensum* was based on the long list of synonyms from the preceding comparative morphological study (section 5.5; Blasco-Costa *et al*, 2009c), the scarcity of diagnostic features and the gradually overlapping ranges for morphometric data in the present material. Indeed, this results show that the 28S and ITS2 sequence data do not support the division of *S. tensum* into four distinct taxa, thus suggesting a wider phenotypic plasticity of a single species in the cluster of morphotypes of *S. tensum vs* the hypothesis of the presence of cryptic species supported by multivariate morphometric analyses. Another unexpected result was that *S. cephali* and *Saccocoelium* sp. had identical sequences, especially of the more variable ITS2 region, in spite of the apparent boundaries were indicated by comparative morphology and multivariate statistical analysis. One explanation for the observed lack of genetic differentiation in the ITS2 region within the *S. tensum* – *S. cephali* clade may be a slower rate of evolution than that found in the *S. obesum* (*s.s.*) + *S. brayi* n. sp. lineage, the latter exhibiting a considerably longer branch length. To date just a single convincing example for identical ITS2 sequences in genuinely different species exists (Blair *et al*., 1997, see Nolan & Cribb, 2005 for a detailed discussion). Therefore a more conservative approach is adopted here, considering the distinct species status only for the *Saccocoelium* isolates that are supported by both morphological and molecular evidence. However, sufficient data for their

morphological differentiation are provided and voucher material of the morphotypes studied here is deposited, in order to enable their recognition should future studies on different loci (*e.g*. ITS1 or mitochondrial genes) offer evidence validating their species distinction.

The lineage division of the *Saccocoelium* spp. may appear to reflect genetic and morphological differentiation associated with the first intermediate hosts rather than diversification as a function of co-speciation with the definitive hosts. Life-cycle data available for *S. tensum* and *S. obesum* (if extended to the other members of their respective clades) tend to support this suggestion. Cercariae of *S. tensum* develop in *Hydrobia acuta* and *H. ventrosa*, whereas those of *S. obesum* develop in *Rissoa* spp. (Fares & Maillard, 1974; these authors also reported numerous unsuccessful attempts to infect experimentally *Hydrobia* spp. with *S. obesum*). The free-living cercariae of both species encyst in the open by attaching to the substrate and are thus available for passive ingestion by the definitive pump-filtering, detritivorous mullet hosts (Cardona *et al*., 2001). Host-parasite data, although recently updated, are still wanting due to the large body of non-original (*i.e.* re-iterating results of single host/population studied), non-documented (*i.e.* with no supportive evidence for the species identification provided) records (see section 5.5; Blasco-Costa *et al*., 2009c). Nevertheless, documented records for both *S. obesum* and *S. tensum* include five of the six widespread sympatric Mediterranean mullet species (*i.e*. *C. labrosus*, *M. cephalus*, *L. aurata*, *L. ramado* and *L. saliens*) as definitive hosts (section 5.5; Blasco-Costa *et al*., 2009c).

The fact that the two presumably snail host-associated clades are separated by a larger sequence divergence in comparison with contrasts between definitive host-parasite associations (*i.e*. 6.3-7.2% *vs* 1.6-3.1%) suggests that parasite specificity at the level of the definitive host does not serve as an important force for speciation in the mullet-haploporid system in the Mediterranean. This situation is similar to the one observed in a Mediterranean sparid-digenean system comprising three host species occurring in sympatry (see Jousson *et al*., 2000). This might be due to a wide open encounter filter in the system studied as a result of the combination of habitat/trophic overlap between the mullet hosts and the distinctly 'passive' transmission of the infective metacercariae attached to the substrate and ingested non-selectively. However, mathematical modelling (Kawecki, 1998) suggests that selection favours genotypes with a strong preference for one or few host species at the expense of those which parasitise several species thus strengthening the concept of alloxenic speciation (*sensu* Euzet & Combes, 1980; see also Combes, 1995) as a unique way to restore specialisation to host species (Combes & Théron, 2000). This scenario seems plausible in terms of the elevated diversity of haploporids in *M. cephalus*, a species considered as the most divergent among the

mullets in the Mediterranean (Rossi *et al*., 2004) and that exhibits complex, age-structured habitat selection patterns (Cardona, 2000).

However, the higher than previously believed species diversity, confirmed by the present molecular data indicates the action of factors, linked to the features of the haploporid life cycle, which modify the effect of the enhanced host encounter. Two non-exclusive hypotheses can be suggested for the observed patterns of species and genetic diversity in the studied system. The first, and an obvious prediction, is that the existence of more species of *Saccocoelium* reflects adaptation to different sympatric snail hosts. The relationship with the snail intermediate hosts can be viewed as the most important component of the compatibility filter since digeneans exhibit highest specificity to their molluscan hosts (Pearson, 1972; Adamson & Caira, 1994) and this supports this suggestion.

An alternative hypothesis is that the higher parasite diversity at the limited geographical scale of the study (c. 500 km, often species co-occurring in the same locality) is a result of local adaptation governed by larval dispersal of both, snails and digeneans. Larval spatial distributions and dispersal ability have been linked to genetic differentiation among free-living marine organisms (*e.g.* Tatarenkov & Johannesson, 1998; Boisselier-Dubayle & Gofas, 1999; Riginos & Victor, 2001). Of particular relevance to the system under study is the fact that *Hydrobia ventrosa* (first intermediate host of *S. tensum* and two other haploporid species) is a species with direct development (*i.e.* crawl-away juveniles emerge after metamorphosis from egg masses deposited on the substrate) and this results in high population level differentiation and low gene flow between populations (Foltz, 2003; Wilke & Davis, 2000). The poor dispersal and the heterogeneity of habitats (see Bartoli & Gibson, 2007 for comment on lagoonal types in the western Mediterranean) may therefore provide a setting for the development of differential susceptibility in the populations of this host towards infection with *Saccocoelium* spp. and haploporids in general. On the other hand, the lack of a second intermediate host in the haploporid life cycle and encystment in the open suggest that lower degree of clonal mixing (see Criscione *et al*., 2005; Criscione & Blouin, 2006) may exist in this system, similar to that observed in digenean species utilising two host life-cycle strategies (*e.g. Schistosoma mansoni*, see Theron *et al*., 2004; *Fascioloides magna*, see Mulvey *et al*. 1991). Thus the micro-spatial dispersal of the haploporid infective stages may provide prerequisites for clonal isolation which overcomes genetic homogenisation *via* enhanced encounter. The limited dispersal and clumped distribution of intermediate hosts coupled with the clonal multiplication and the encystment pattern of the infective larvae, would lead to restricted opportunities for cross-fertilisation and population level

differentiation over small spatial scales. Thus, a possibility for sympatric speciation conferred through the spatial structure of the first intermediate host populations might be high in the system studied and in haploporid digeneans in general.

Summarizing the results of the present study, the decision adopted is that distinct species status is only valid for the *Saccocoelium* isolates that are supported by both morphological and molecular evidence. The present data suggest that circumscribing species using solely morphological criteria may be misleading; however, the results do not rule out the possibility for even higher species diversity within the studied digenean group. Recent studies suggest that increased sampling effort and the application of a combined molecular and morphological approach reveals the presence of cryptic species and higher taxa especially in poorly known digenean groups in spite of low divergences recorded in the rRNA gene regions (*e.g.* Jousson & Bartoli, 2001; Nolan & Cribb, 2004, 2006; Chambers & Cribb, 2006; Miller & Cribb, 2007a,b). By describing sequence and morphological divergence across the lowest taxonomic levels, this study provides a test case that demonstrates which genetic and morphological markers can be used for diagnostic analysis in the Haploporidae.

CHAPTER 7

PHYLOGENETIC RELATIONSHIPS OF THE MEDITERRANEAN HAPLOPORIDAE INFERRED FROM 28 S AND ITS2 RDNA SEQUENCE DATA

7.1. Introduction

The Haploporinae Nicoll, 1914, one of the four currently recognised subfamilies within the Haploporidae Nicoll, 1914 (see Overstreet & Curran, 2005), represents a group of poorly known digeneans which is restricted to marine or brackish water Mugilidae. Looss (1902) erected the majority of its genera (*i.e. Haploporus* Looss, 1902, *Dicrogaster* Looss, 1902, *Lecithobotrys* Looss, 1902 and *Saccocoelium* Looss, 1902) for a few species which he described from Mediterranean mullets (see Chapter 5). His descriptions and generic diagnoses were brief and based on a small number of specimens; this has resulted in subsequent misleading identifications and synonymies leading to an underestimation of the diversity of Mediterranean haploporines. Thus, Dawes (1947) considered *Haploporus lateralis* Looss, 1902 a synonym of *H. benedeni* Looss, 1902; Dawes (1947) supported by Mikailov (1958), Fischthal & Kuntz (1963), Ferretti & Paggi (1965) and Moravec & Libosvárský (1975) regarded *Saccocoelium obesum* Looss, 1902 and *S. tensum* Looss, 1902 synonymous; and Dawes (1947) and Sarabeev & Balbuena (2003) synonymised *Dicrogaster contracta* Looss, 1902 with *D. perpusilla* Looss, 1902.

The problems in haploporine taxonomy extend to generic recognition as well. Thus *Saccocoelioides* Szidat, 1954, originally assigned to the Haploporinae by Szidat (1954), was considered a subgenus of *Lecithobotrys* by Yamaguti (1958; later reinstated, see Yamaguti, 1971) and a junior synonym of *Lecithobotrys* by Nasir & Gómez (1976). Overstreet & Curran (2005) temporarily accepted the validity of *Lecithobotrys*, reorganised *Saccocoelioides* and transferred *Saccocoelioides* (*sensu stricto*) to the new subfamily Chalcinotrematinae Overstreet & Curran, 2005. These authors also suggested that *Lecithobotrys* may be synonymous with *Haploporus* and indicated that the placement of some species of *Haploporus* and *Saccocoelium* Looss, 1902 is difficult. Yamaguti (1958) erected the Dicrogasterinae Yamaguti, 1958 for *Dicrogaster* based on the presence of a single vitellarium (*vs* vitellarium in two symmetrical masses in his concept of the Haploporinae, see Yamaguti, 1958; 1971) but this action was not accepted by Overstreet & Curran (2005).

In spite of the large number of records of haploporine species, especially in Mediterranean mullets, there are surprisingly few documented records (*i.e.* supplied with a description or figure) or taxonomic studies contributing to the knowledge of morphological variation in this group (see detailed lists in Chapter 5; Blasco-Costa *et al*., 2009a,b).

The revision of the Mediterranean genera of the Haploporidae carried out in the present study proved the validity of six of the species (*i.e. Haploporus benedeni* (type-species), *Dicrogaster*

contracta, *D. perpusilla*, *Lecithobotrys putrescens*, *Saccocoelium obesum* and *S. tensum*) originally described by Looss (1902). Four new species (*Saccocoelium cephali*, *S. currani, S. brayi* and *Forticulcita gibsoni*) were described, and a new genus *Ragaia* for a new species, *R. lizae*, from the Ebro Delta was erected (Chapter 5; Blasco-Costa *et al.*, 2009a,b,c). Simultaneously, the second internal ribosomal spacer (ITS2) region and the partial large subunit rRNA (28S) gene of haploporine representatives of all Mediterranean haploporine genera were sequenced.

This chapter presents an evaluation of the taxonomic framework of the Haploporinae based on morphology (Overstreet & Curran, 2005; Blasco-Costa *et al.*, 2009a,b,c; Chapter 5) using ribosomal DNA sequence data generated from 10 species representing six out of the ten genera currently recognised within the subfamily. The monophyly of the Haploporinae is tested by incorporation of the only available sequence data for two non-haploporine haploporid and two atractotrematid species and the relationships at the generic level are assessed for the first time. More specifically, the molecular data allowed an independent test of the previous hypotheses for the synonymy of *Lecithobotrys* and *Saccocoelioides* as well as for the status of *Haploporus*, *Lecithobotrys* and *Saccocoelium*. In two cases the data provided an opportunity to test earlier suggested synonymies at the species level.

7.2. Materials and methods

Taxon sampling

Specimens representing all Mediterranean haploporid genera were collected from mullets (*M. cephalus*, *L. aurata*, *L. ramado* and *L. saliens*) at three localities along the Mediterranean coast of Spain: Ebro Delta, off Santa Pola and in a brackish water lagoon near Santa Pola. In total, 34 sequence replicates of both ITS2 and partial 28S rDNA regions of 10 species were obtained (see Table 3.7 for hosts and sequence/specimen accession numbers). Multiple replicates for the two gene regions of six species [*D. perpusilla* (5); *D. contracta* (7); *H. benedeni* (4); *L. putrescens* (2); *S. obesum* (4); and *S. tensum* (7)] revealed no variation within the sequences. All haploporid taxa sequenced are described morphologically in Chapter 5 (see also Blasco-Costa *et al.*, 2009a,b,c). Type and voucher material has been deposited in the British Museum (Natural History) Collection at the Natural History Museum, London (BMNH) and sequences were submitted to GenBank (see Table 3.5 for accession numbers).

Sequencing and phylogenetic analysis

Isolation, amplification and purification of gDNA, and sequence alignment followed the protocols described in Chapter 3. The new ITS2 rDNA and partial 28S rDNA sequences were aligned in two independent datasets, the latter including the chalcinotrematine haploporid *Saccocoelioides* sp., the megasolenine haploporid *Hapladena nasonis* Yamaguti, 1970*,* and two species (*Atractotrema sigani* Durio & Manter, 1969 and *Pseudomegasolena ishigakiensis* Machida & Kamiya, 1976) of the closely related Atractotrematidae Yamaguti, 1939 (see Olson *et al*., 2003) for which sequences were available for this region only (see GenBank accession number in Appendix 1). *Paragonimus westermani* (Kerbert, 1878) (Paragonimidae) (GenBank accession numbers: 28S, DQ836244; ITS2, DQ836243), *Preptetos trulla* (Linton, 1907) (28S, AY222237) (Lepocreadiidae) and *Preptetos laguncula* Bray & Cribb, 1996 (ITS2, AF392439) were chosen to root the phylogenetic trees. Sequences were aligned and adjustments made by eye using MacClade 4.08 (Maddison & Maddison, 2005) and regions of ambiguous alignment in each dataset were defined in a character exclusion set.

The two datasets were analysed individually using Bayesian inference methods (BI) and maximum parsimony (MP). Prior to BI analyses the best model of nucleotide substitution was estimated using ModelTest version 3.06 (Posada & Crandall, 1998) independently for each data set. This was the general time reversible model, with estimates of invariant sites and gamma distributed among site rate variation (GTR+I+Γ) in the case of 28S dataset and general time reversible model, with gamma distributed among site rate variation (GTR+Γ) in the case of ITS2 dataset. The analyses were run for 1 million generations with a sampling frequency of 100. Consensus trees with mean branch lengths were constructed after log-likelihood values and substitution parameters plateaued at approximately generation number 12,300 and 30,000 for the 28S and ITS2 regions, respectively. Nodal support was estimated as posterior probabilities (Huelsenbeck *et al.*, 2001). MP analyses were performed with PAUP* 4.0b10 (Swofford, 2002) using a heuristic search strategy with 1,000 search replicates, random-addition taxon sampling, tree-bisection-reconnection branch-swapping, with all characters run unordered with equal weights and with gaps treated as missing data. Nodal support was estimated by bootstrap analysis (heuristic search strategy with 1,000 pseudoreplicates and 100 random sequence addition each). The analyses were run for 1 million generations with a sampling frequency of 100. Distance matrices for each rDNA region were constructed from the trimmed (to match the shortest sequence) aligned sequences with the absolute pairwise character difference (gaps treated as missing data) and the

percentage of pairwise character differences.

7.3. Results

There was a total of 337 and 1,029 included (*i.e.* alignable) characters in the ITS2 and 28S data sets, respectively. Of these, 179 (53%) and 649 (63%) were invariant, and 84 (25%) and 232 (23%) informative under the principles of parsimony, respectively. Within the Haploporinae (*sensu* Overstreet & Curran, 2005; Blasco-Costa *et al*., 2009b; Chapter 5), the interspecific sequence variability ranged from 2.1 to 10.9% in the ITS2 and from 0.9 to 4.8% in the 28S region. Intergeneric divergence overlapped with the species-level data ranging between 6.7-21.2% and 4.6-11.4%, respectively. The upper limits of intergeneric divergence within the Haploporinae were set by *Forticulcita* whereas the pair *Lecithobotrys-Haploporus* showed the lowest percent of sequence divergence (Table 7.1). Comparisons at the suprageneric level (28S dataset only) largely overlapped with the intergeneric data for haploporine subfamilies (range of 9.1-14.5%) and were slightly higher 12.3-15.8% for family-level comparisons (see Tables 7.1 for details). The data available for two representatives of other haploporid subfamilies (*i.e.* the Megasoleninae Manter, 1935 and the Chalcinotrematinae) and two species of the Atractotrematidae, the latter considered the closest (Overstreet & Curran, 2005; Curran *et al.*, 2006) and possibly synonymous with the Haploporidae (see Olson *et al.*, 2003), allowed their inclusion in the analyses of 28S rDNA sequences. Figure 7.1 presents the tree topology generated by BI analysis of the 28S and ITS2 datasets. MP analysis depicted the same tree topology and both showed similar high support for the branch pattern. The Haploporinae formed a strongly supported monophyletic clade, with *Saccocoelioides* sp. nested within it. Within this clade, *Forticulcita* occupied a basal position, sister to *Saccocoelioides* and the rest of the haploporines which formed two clades with high support: (i) *Saccocoelium* spp. and (ii) *Dicrogaster* spp. with *Ragaia* clustered to it but with no support + *Lecithobotrys-Haploporus* clade. The placement of *Ragaia* was unsupported in the analyses of both datasets; it appeared either as sister taxon to *Dicrogaster* (28S, see Fig. 7.1A) or *Saccocoelium* (ITS2, see Fig. 7.1B). The topology depicted by the analyses of the ITS2 dataset was unsupported for most nodes, except for *Saccocoelium* spp., *S. obesum-S. brayi* clade, and the Haploporinae (however excluding *Forticulcita*) which had high posterior probabilities; the latter two also strongly supported by MP bootstrap (Fig. 7.1B). Although the species of *Saccocoelium* formed a monophyletic group in both MP and BI analyses, different relationships were depicted using ITS2 rDNA sequences. Thus, *S.*

cephali clustered with *S. tensum* in the analyses of the 28S dataset whereas in the analyses based on ITS2 it appeared basal to the remaining species all collected from *Liza* spp. (Figs 7.1A-B). Regarding the suprageneric relationships (28S dataset only), in both analyses *Hapladena* appeared basal in the Haploporidae, but poorly supported, whereas, the Atractotrematidae formed a strongly supported clade sister to the Haploporidae.

Table 7.1. Pairwise nucleotide sequence comparisons between taxa, calculated as percentage of nucleotide differences (gaps treated as missing data) for the aligned 28S and ITS2 rDNA sequences.

28S rDNA sequences (N=1029 nt)														
	Taxon	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	*Saccocoelium cephali*	-													
2	*Saccocoelium tensum*	2.6	-												
3	*Saccocoelium obesum*	4.6	3.8	-											
4	*Saccocoelium brayi*	4.8	4.2	0.9	-										
5	*Ragaia lizae*	6.3	5.8	5.8	6.4	-									
6	*Lecithobotrys putrescens*	7.5	7.2	7.6	7.9	6.4	-								
7	*Haploporus benedeni*	8.6	7.9	8.5	8.9	6.8	4.6	-							
8	*Dicrogaster perpusilla*	8.2	7.6	8.1	8.5	6.8	8.1	8.2	-						
9	*Dicrogaster contracta*	6.6	5.8	6.2	6.6	5.2	6.5	6.8	4.6	-					
10	*Forticulcita gibsoni*	10.5	10.5	10.5	11.0	10.7	10.9	10.7	11.4	10.2	-				
11	*Saccocoelioides sp.*	9.8	9.6	9.9	10.2	9.7	10.6	11.2	11.0	9.4	9.1	-			
12	*Pseudomegasolena ishigakiense*	13.1	12.4	12.9	13.3	13.2	13.9	13.6	14.6	13.5	12.7	12.3	-		
13	*Atractotrema sigani*	15.1	14.0	14.0	14.3	15.3	15.2	14.6	15.8	14.9	14.8	14.3	12.0	-	
14	*Hapladena nasonis*	13.5	13.0	12.9	13.0	13.7	14.2	13.1	14.5	13.3	13.9	13.5	13.2	15.8	-

ITS2 rDNA sequences (N=337 nt)											
	Taxon	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	*Saccocoelium cephali*	-									
2	*Saccocoelium tensum*	5.1	-								
3	*Saccocoelium obesum*	10.9	10.2	-							
4	*Saccocoelium brayi*	10.9	9.0	2.1	-						
5	*Ragaia lizae*	9.4	8.2	14.1	13.2	-					
6	*Lecithobotrys putrescens*	10.3	10.0	14.4	14.7	8.8	-				
7	*Haploporus benedeni*	9.6	9.6	13.6	13.9	8.8	6.7	-			
8	*Dicrogaster perpusilla*	11.2	10.5	13.7	13.1	11.3	11.0	10.6	-		
9	*Dicrogaster contracta*	10.2	9.6	13.3	12.4	10.7	11.0	9.4	8.7	-	
10	*Forticulcita gibsoni*	21.1	19.7	21.1	21.2	19.4	21.0	19.3	20.6	17.3	-

7.4. Discussion

The systematic position and taxonomy of the Haploporidae still offer challenges at various levels. Thus, the poor knowledge of the morphological and molecular diversity within this family (large and small subunit rDNA sequence data available for just two species, the megasolenine *Hapladena nasonis* Yamaguti, 1970 and the chalcinotrematine *Saccocoelioides* sp.) has resulted in contradictory (morphological data, see Brooks *et al.*, 1985) or unclear

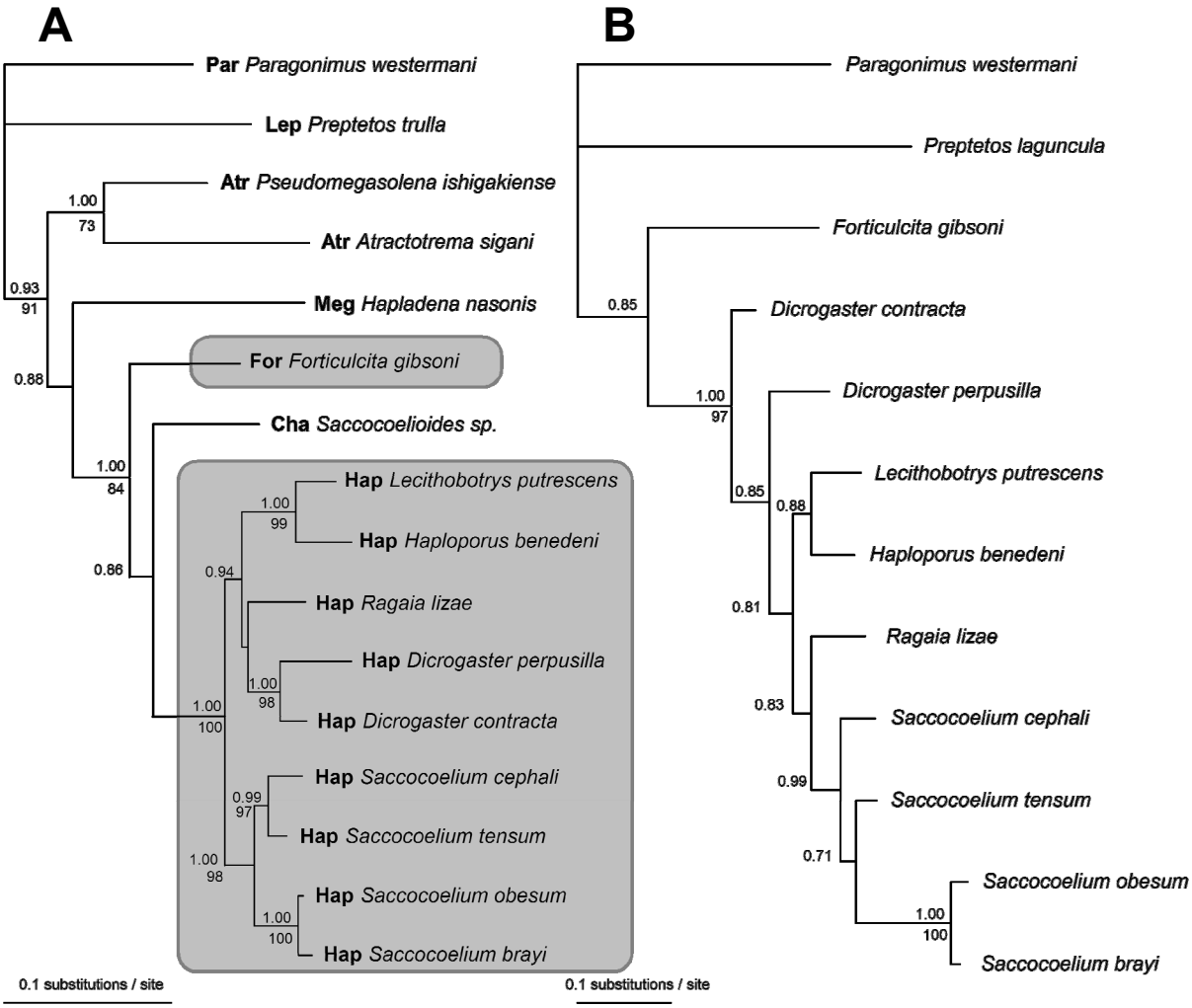

Fig. 7.1. Trees derived from BI employing a GTR+I+Γ substitution model for 28S dataset (**A**) and GTR+Γ substitution model for ITS2 dataset (**B**). Nodal support is provided by posterior probabilities (number above or only number; not shown if < 0.70) and by maximum parsimony bootstrap percentages (number below; not shown if < 70%). Shaded areas indicate the Haploporinae *sensu* Overstreet & Curran (2005). *Abbreviations*: Atr, Atractotrematidae; Cha, Chalcinotrematinae; For, Forticulcitinae n. subfam.; Hap, Haploporinae; Lep, Lepocreadiidae; Meg, Megasoleninae; Par, Paragonimidae.

(molecular data, see Olson *et al.*, 2003) concepts for the placement of the family Haploporidae in the classification scheme of the Digenea. The recent taxonomic revision of Overstreet & Curran (2005) has greatly clarified the situation at the generic/suprageneric level. As shown by Olson & Tkach (2005) molecular systematic studies aimed at these taxonomic levels have produced more conclusive results. The first attempt at assessment of interrelationships of the Haploporidae thus provides a molecular-based test of the taxonomic framework based on morphology. Moreover, the wider sampling within the family conducted here can help improve knowledge on the relationships at higher taxonomic levels.

Comparisons of genetic distance

Relatively low values of intergeneric variation of 4.6-8.6% and 6.7-14.7% for the 28S and ITS2, respectively, were observed within the Haploporinae (excluding *Forticulcita*, see below). These fall within the range observed in Cryptogonimidae (28S: 3.8-8.4%; ITS2: 6.6-12%, see Miller & Cribb, 2007a, b) and Didymozoidae (ITS2: 3.0-19.0, see Anderson & Barker, 1998) and well below the one reported in Bivesiculidae (ITS2: 16.0 to 36.0%, see Cribb *et al.*, 1998b). The intergeneric divergence appears closely associated with the interspecific sequence variation recorded in the latter three examples for which data are available at both levels, *i.e.* very low in Cryptogonimidae (0.2-0.4% in the 28S and 0.4-7.1% in the ITS2) and Didymozoidae (0.5% in the ITS2) *vs* 8.1-11.6% in the ITS2 of *Bivesicula*, (see Anderson & Barker, 1998; Cribb *et al.*, 1998b; Miller & Cribb, 2007a, b). Therefore, the lower limits of genetic differentiation observed in our study at the species level (0.9% and 2.1% in the 28S and ITS2, respectively) may not be exceptional and may have implications for species recognition within the Haploporidae. This, coupled with the lack of correlation between the morphological and genetic differentiation within *Saccocoelium* (Chapter 6) supports a prediction for discovery of sibling species within other haploporid lineages.

Subfamily-level interrelationships

The present results illustrate, with considerable resolution, the relationships among and within the genera of the Haploporidae included in the analysis. Overall, the 28S rDNA dataset produced trees with better resolution, presumably due to the higher degree of homoplasy in

the ITS2 dataset; the former strongly supported the grouping of the Mediterranean genera of the Haploporinae in a robust cluster.

Skrjabin (1956) placed *Dicrogaster* within the *Haploporinae*, whereas Yamaguti (1958) erected the Dicrogasterinae Yamaguti, 1958 for this genus. Overstreet & Curran (2005) did not accept this subfamily, an opinion well supported by our molecular analysis which places *Dicrogaster* within the Haploporinae as sister to *Haploporus* (type-genus) and *Lecithobotrys* (Fig. 7.1A). The two species of *Dicrogaster* differed considerably in terms of the two rDNA regions and this confirms their distinct species status, in agreement with the morphological study (Chapter 5; Blasco-Costa *et al.*, 2009a).

Lecithobotrys and *Haploporus,* the type-genus of the Haploporinae, were strongly associated especially in the analyses of 28S dataset. Further, the type-species of both genera exhibited the lowest percent of sequence difference, which falls within the interspecific range observed within *Saccocoelium* and *Dicrogaster*. These results tend to support the possible synonymy suggested by Overstreet & Curran (2005) but sequence data from more species of both genera is desirable to adequately circumscribe their limits before a nomenclatural change can be recommended. *Lecithobotrys* is here distinguished morphologically from *Haploporus* based on (i) the distribution of eye-spot pigment (spread throughout entire body *vs* dispersed between levels of the pharynx and oral sucker); (ii) the shape and size of the seminal vesicles (both elongate-oval and external distinctly larger than internal *vs* subglobular and similar in size); (iii) the genital atrium (distinct, with muscular walls *vs* absent); and (iv) the structure of the vitellarium (in two separated lateral clusters of distinct subglobular groups of small coalesced follicles *vs* two separated compact masses; see Chapter 5). Altogether then, there appears to be a considerable disjunction between morphology and molecules in the case of *Lecithobotrys* and *Haploporus*.

The present phylogenetic hypotheses did not resolve the closest affinities of *Ragaia*, erected recently for *R. lizae* from the Mediterranean, although its inclusion among the haploporines was well supported. It is possible that its sister genus has not yet been described since only four recognised genera are not included in the present analyses due to lack of data: *Unisaccus, Pseudodicrogaster* and *Pseudolecithobotrys* from the Indo-West Pacific and the poorly defined *Rondotrema* parasiting non-mugilid fishes in the Southwest Atlantic. The largely disparate geographical distribution of these genera, however, makes the possibility of a close relationship with *Ragaia* seem unlikely.

Saccocoelium formed a strongly supported monophyletic group. The wider sampling within this genus allowed confirmation of the distinct status of *S. tensum* and *S. obesum* (thus

rejecting previously suggested synonymy, see above) and the two recently described species, *S. cephali* and *S. brayi*. The results provide no evidence to question the distinct status of *Saccocoelium* (especially in relation to *Haploporus*) as the two genera clustered in different, well-defined clades. A characteristic feature of *Saccocoelium* that distinguishes it morphologically from *Haploporus* and all known genera of the Haploporidae is the presence of a prominent genital atrium with strongly developed muscular walls (Chapter 5; Blasco-Costa *et al.*, 2009b,c). Therefore, the problems with species affiliation to either genus (Overstreet & Curran, 2005) are rather due to poor differential diagnoses and misplacements and not to morphological similarity resulting from a close phylogenetic relationship.

Phylogenetic inference at the familial level

The present results clearly resolve the distinct status of *Saccocoelioides* (recently transferred to the subfamily Chalcinotrematinae, see Overstreet & Curran, 2005) and *Lecithobotrys* thus rejecting the synonymy suggested by Yamaguti (1958) and Nasir & Gómez (1976). The two genera were found clustering in different well-supported groups, *Saccocoelioides* being earlier divergent than *Lecithobotrys*. However, *Saccocoelioides*, which has recently been transferred to the subfamily Chalcinotrematinae (see Overstreet & Curran, 2005), was nested within the Haploporinae (*sensu* Overstreet & Curran, 2005) and this is largely associated with the position of *Forticulcita* which was resolved as the most basal haploporine genus (Fig. 7.1); *F. gibsoni* also exhibited the highest percent of sequence difference with all other species of the Haploporinae.

Arising from the present phylogenetic solutions two hypotheses can be suggested. If the current classification of Overstreet & Curran (2005) is considered, *i.e. Forticulcita* as basal within the Haploporinae, the position of *Saccocoelioides* sp. would result in paraphyly of the Chalcinotrematinae, which was previously suggested by Overstreet & Curran (2005) when they erected the subfamily. *Saccocoelioides* was included in the latter subfamily based on vitelline follicles surrounding the testis, the presence of a short oesophagus and an uterine loop anterior to the ventral sucker, and the developed miracidia having pigmented eye-spots (Overstreet & Curran, 2005). However, the vitellarium in *Saccocoelioides* is not as well-developed as in the other genera of the Chalcinotrematinae and the vitelline follicles are arranged in two symmetrical groups rather than irregularly dispersed in lateral fields in the hindbody. Whereas the presence of eye-spots depends on the development of the miracidia (Overstreet & Curran, 2005), the structure of the vitellarium in *Saccocoelioides* suggests a

closer resemblance to Haploporinae than Chalcinotrematinae. Definitely, additional sequences of identified species (preferably the type-species) of *Saccocoelioides* and other chalcinotrematines are required to test whether they form a natural group. The present results indicate that (i) *Saccocoelioides* may belong to the Haploporinae or (ii) *Saccocoelioides* and by extension the Chalcinotrematinae is the closest group to the Haploporinae considering the range of taxa examined.

On the other hand, if *Forticulcita* exhibits features not seen again in the Haploporidae, it may be basal in the evolution of this group. In this case *Forticulcita* should be considered apart from the Haploporinae by an elevation of its taxonomic status. This will resolve *Saccocoelioides* and the Chalcinotrematinae as sister group to the Haploporinae. In fact *Forticulcita* possesses some diagnostic morphological features that appear to be unique in the Haploporidae: a single vitelline mass (present only in *Dicrogaster fastigata* Thatcher & Sparks, 1958), and an eversible ejaculatory organ (terminology of Overstreet, 1982). This muscular structure (long and cylindrical when everted, see section 5.3; Blasco-Costa *et al.*, 2009a) is present in all three species of the genus, *i.e*. *F. glabra* Overstreet, 1982, *F. gibsoni* Blasco-Costa *et al.*, in press, and *F. mugilis* Hassanine, 2007; described as eversible hermaphroditic duct for the latter (see section 5.3; Overstreet, 1982; Blasco-Costa *et al*., 2009a; Hassanine, 2007).

However, the presence of an intromittent ejaculatory organ has not been previously considered an important apomorphy; this feature although originally included in the generic diagnosis of *Forticulcita* by Overstreet (1982) is not mentioned in the generic, subfamilial or familial diagnoses in the recent revision of Haploporidae (see Overstreet & Curran, 2005). Although the ability of the hermaphroditic duct to evert has been observed in some haploporids (*e.g.* Martin, 1974; Machida, 1996; see Chapter 5; Blasco-Costa *et al*., 2009a,b,c) the presence of a well-delimited eversible intromittent copulatory organ is considered here an important discriminating feature at the subfamilial level. This, combined with the present hypothesis of the Haploporinae inferred from rDNA sequence data, suggests that taxonomic elevation of *Forticulcita* is warranted. Therefore, the subfamily Forticulcitinae is erected for the latter with the following diagnosis:

Forticulcitinae n. subfam.

Haploporidae. Body fusiform, with maximum width at level of ventral sucker. Tegument armed. Eye-spot pigment dispersed between oral sucker and hermaphroditic sac. Oral sucker

subterminal. Ventral sucker about size of oral sucker or larger. Forebody short. Prepharynx short. Pharynx large, subspherical. Oesophagus 2-6 times length of pharynx. Caeca two, sac-like, end blindly at about mid-body or more posterior. Testis single, dextral to submedian. External seminal vesicle tubular, distinctly longer than internal seminal vesicle. Hermaphroditic sac elongate, subcylindrical. Internal seminal vesicle tubular to elongate-oval. Hermaphroditic duct narrow. Ejaculatory organ muscular, cylindrical. Genital atrium shallow. Genital pore median, just anterior to ventral sucker. Ovary pretesticular, contiguous with or overlapping testis. Metraterm long. Eggs numerous, operculate; developed miracidia with single or two fused eye-spots. Vitellarium a single large spherical to subtriangular compact mass of small follicles, at level of or posterior to gonads. Excretory system Y-shaped, pore terminal, wide. Type-species: *F. glabra* Overstreet, 1982

Although *Hapladena* appeared as the most basal taxon in the Haploporidae, the relationships of the sole species of the subfamily Megasoleninae for which sequence is currently available, *H. nasonis,* remained unresolved. Its position was also found to be labile, as sister to either the Atractotrematidae or the Haploporidae, in an analysis within a much wider taxonomic framework (Chapter 8). This species was found to form a strongly supported clade with the Atractotrematidae in an analysis of the relationships of the Acanthocolpidae Lühe, 1906 (see Bray *et al.*, 2005), a sister taxon to the Haploporidae (see Olson *et al.*, 2003). In addition, *H. nasonis* grouped as a sister taxon to the newly sequenced chalcinotrematine haploporid, *Saccocoelioides* sp. in a study on a different set of taxa closely-related to the Haploporidae (Curran *et al.*, 2006). The poor support of the latter relationship was interpreted as evidence for a distant relationship between the two subfamilies. However, these authors have excluded from the analysis the sequence of the second atractotrematid species (*i.e. Atractotrema sigani*) and this might have affected the solution. Although the present results sustain the assumption of a distant relationship between the Megasoleninae and the Haploporinae, also supported by host-parasite data (all members of the former subfamily occur in marine reef fishes and none was found in a mugilid, see Overstreet & Curran, 2005), it is unfortunate that only a single taxon of the most speciose megasolenine genus has so far been used in all molecularly tested hypotheses. However, *H. nasonis* appears to be an aberrant ('atypically elongate' see Overstreet & Curran, 2005) representative of the Megasoleninae. Clearly, the relationships of this subfamily would be better understood if sequences of type-taxa were incorporated in future analyses.

Finally, the support for the close relationship between the Atractotrematidae and the

Haploporidae was strong. Wider sampling within the Atractotrematidae and the remaining subfamilies of the Haploporidae would improve the knowledge on the relationships so that natural groups within the Haploporidae are defined and the validity of the Atractotrematidae is assessed.

CHAPTER 8

DIFFERENT GENES – DIFFERENT SOLUTIONS: PHYLOGENETIC AFFINITIES OF TWO CONTROVERSIAL FAMILIES WITHIN THE DIGENEA INFERRED FROM NUCLEAR rDNA

8.1. Introduction

The phylogenetic relationships and systematic position of the digenean (Platyhelminthes: Trematoda) families Haploporidae Nicoll, 1914 and Haplosplanchnidae Poche, 1926 have long been controversial. Members of these families are mainly parasites of brackish and estuarine, and to a lesser extent marine teleost fishes throughout the world. Although studies exist on aspects of the morphology and systematics of specific groups within these families, a thorough and well-grounded concept of their classification is lacking because these worms are especially difficult to assess morphologically. Consequently both families have a complex taxonomic history and the viewpoints of taxonomists have varied greatly as to their content and phylogenetic affinities.

Since La Rue (1957) produced the first 'modern' taxonomic system for the Digenea, several authors have proposed classifications based initially on morphological characters (*e.g.* Brooks *et al*., 1985; Pearson, 1992) and recently on molecular data (*e.g.* Cribb *et al*., 2001; Olson *et al*., 2003). La Rue (1957) incorporated the Haplosplanchnidae within the order Echinostomida on the basis of life history data provided by Cable (1954). Mehra (1961) erected the superfamily Haploporoidea Nicoll, 1914 within the Echinostomida for the Haploporidae and the Waretrematidae Srivastava, 1937 relating them by 'preacetabular genital pore in the forebody and the presence of a single testis and a hermaphroditic sac containing vesicula seminalis interna, pars prostatica, ductus ejaculatorius, metraterm and ductus hermaphroditicus'. He also included without comment the Haplosplanchnidae and the Megaperidae Manter, 1934 within the Haploporoidea.

Brooks *et al.* (1985) inferred a phylogeny of the Digenea based on characters derived from morphology and life cycle data. They erected a new order, the Haploporiformes Brooks, O'Grady & Glen, 1985 for the Haploporidae (with the Atractotrematidae Yamaguti, 1939, the Megasolenidae Manter, 1935 and the Warentrematinae Srivastava, 1937 as included groups), the Haplosplanchnidae and the Megaperidae. The phylogenetic hypothesis suggested by these authors related the Haploporiformes with the Echinostomiformes La Rue, 1957 and the Hemiuriformes Travassos et al., 1969. However, Pearson (1992) critically revised the character argumentation in the database of Brooks *et al.* (1985) and found that extensive morphological homoplasy and invalid character assessment resulted in a drastic decline in resolution as compared with the analysis of Brooks *et al.* (1985). In particular, the position of the order Haploporiformes erected by Brooks *et al.* (1985) was left unresolved in the analysis (Pearson, 1992).

The affinities of the Haplosplanchnidae and the Haploporidae assessed by morphological features are considerably different from those assessed using molecular data. The first molecular study incorporating a wide range of digenean taxa was conducted by Cribb *et al.* (2001) who used complete sequences of the small subunit ribosomal RNA gene (18S rDNA) and morphological and life cycle characters for 75 digenean species. Their data set included two haplosplanchnid (*Hymenocotta mulli* Manter, 1961 and *Schikhobalotrema* sp.) and one haploporid species [*Pseudomegasolena ishigakiense* Machida & Kamiya, 1976 now considered to belong to Atractotrematidae (see Overstreet & Curran, 2005)]. Cribb *et al.* (2001) found little support for the relationship between the Haploporidae and the Haplosplanchnidae previously suggested (Mehra, 1961 and Brooks *et al*., 1985). Olson *et al.* (2003) used complete 18S rDNA and partial large subunit ribosomal RNA (28S rDNA) sequences of 163 digenean taxa in the most recent study to estimate the phylogeny of the Digenea. In their analyses, in which one species of the Haploporidae (*Hapladena nasonis* Yamaguti, 1970) was included, the two families, the Haplosplanchnidae and Haploporidae, were among the most labile in their placement. Olson *et al.* (2003) distinguished for the first time the Haplosplanchnoidea Poche, 1926 at a higher level, forming the suborder Haplosplanchnata Olson, Cribb, Tkach, Bray & Littlewood, 2003, whereas the Haploporidae and the Atractotrematidae were found to be paraphyletic (suggesting sinking the latter family) and were placed within the Gorgoderoidea Looss, 1901 as the sister clade to the Paragonimidae Dollfus, 1939 and Troglotrematidae (Odhner, 1914) in the suborder Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003.

Jones (2005) agreed with the distinct status of the Haplosplanchnoidea and its removal from the Echinostomatoidea Looss, 1899 following the results of Olson *et al.* (2003), but treated the Haploporoidea as a distinct superfamily for ease of identification and because the two included families, the Haploporidae and the Atractotrematidae were found to be so labile in the phylogenies of Olson *et al.* (2003). Overstreet and Curran (2005) related morphologically the Atractotrematidae to the Haploporidae because of the possession of a hermaphroditic sac. These authors recognized four subfamilies within the Haploporidae: the Haploporinae Nicoll, 1914, the Waretrematinae, the Megasoleninae Manter, 1935, and the Chalcinotrematinae Overstreet & Curran, 2005.

Subsequent molecular studies on a lower taxonomic scale that included species of the Haploporidae show different solutions at a familial level: (i) the Haploporidae left unresolved within the families of the Xiphidiata (see Bray *et al*., 2005; Curran *et al.,* 2007); (ii) the Haploporidae and Atractotrematideae placed as a distinct lineage either basal to, or sister

taxon to the Gorgoderoidea Looss, 1901 + Plagiorchioidea Lühe, 1901 (both members of Xiphidiata) (see Curran *et al*., 2006); (iii) the Haploporidae and Atractotrematidae placed as sister group to the Monorchioidea, completely out of the Xiphidiata (Choudhury *et al* 2007). Taken together, these studies show that the Haploporidae and Haplosplanchnidae have been poorly studied, have had a controversial taxonomic history, and prove unstable in phylogenetic analyses of the Digenea.

In this chapter, the position of the Haploporidae and Haplosplanchnidae is investigated by improving the taxon sampling on a larger data set used recently for estimating a Digenean phylogeny (Olson *et al* 2003). New molecular sequences for complete 18S and partial 28S rDNA of five species belonging to five haploporid genera and three species belonging to two genera of the Haplosplanchnidae have been added. Additionally, the affinities of the Haploporidae to the closest families have been studied in deep by analysing the 18S & partial 28S rDNA sequences in separate and combined analyses for a reduced data set. Present results are discussed in the context of previous hypotheses and morphological and life-cycle traits of the groups.

8.2. Materials and methods

Taxon sampling

A full list of the taxa used in this study is given in Appendix 1. In addition to the published sequences from Olson *et al.* (2003); Cribb *et al.* (2001); Littlewood & Olson (2001); Lockyer *et al.* (2003) and Tkach *et al.* (2000a, 2001 a, b, c, 2003), newly characterized complete 18S and partial 28S (D1-D3) rDNA sequences of representatives of all Mediterranean genera of the families Haploporidae and Haplosplanchnidae are added. These include five species belonging to five haploporid genera (*i.e. Haploporus benedeni, Lecithobotrys putrescens, Saccocoelium obesum, Forticulcita gibsoni* and *Dicrogaster perpusilla*) including the type genus of the family, *Haploporus,* and three species of the Haplosplanchnidae (*i.e. Haplosplanchnus pachysomus, H. purii* and *Schikhobalotrema sparisomae*), two of them belonging to the type genus, *Haplosplanchnus*. Seven of these species were collected from mullets off the Mediterranean coast of Spain and *Haplosplanchnus purii* Srivastava, 1937 ex *Mugil cephalus* from Anse Vata, New Caledonia was kindly provided by Dr R.A. Bray (see Appendix1 for hosts, localities and accession numbers). Voucher material has been deposited

in the Parasitic Worms Collection at the Natural History Museum, London (BMNH) and sequences were submitted to GenBank (see Table 3.7).

DNA extraction, amplification and sequencing

Isolation, amplification and purification of gDNA, and sequence alignment follow the protocols described in Chapter 3. Two gene fragments were amplified and sequenced: the nuclear 18S ribosomal RNA and 28S ribosomal RNA. Near-complete 18S rDNA sequences were amplified using the primer combinations ERIB1 + ERIB10 or Worm-A + Worm-B (Table 3.6). Partial (domains D1-D3; ~1400 bps) 28S rDNA sequences were amplified using primers LSU5 + 1500R or U178 + L1642.

Alignments

New sequences were incorporated into an existing alignment of 170 taxa of Olson *et al.* (2003). Adjustments to the alignment were made by eye using MacClade (Maddison & Maddison 2005, ver. 4.08) and sequences of the two gene fragments were concatenated and regions of ambiguous alignment redefined in a character exclusion set in MacClade. Regions containing gaps in the majority of taxa were also excluded from analyses even if these regions were alignable among the minority of taxa possessing the insertions due to the ambiguity of decisions on homology. Alignment adjustments were made independently for two data sets. The first, referred further to as total data set, included the 163 digenean taxa of Olson *et al.* (2003) plus this eight newly sequenced taxa and seven aspidogastrean taxa designated as outgroup. This data set was constructed in order to determine the placement of the two families within the phylogeny of the digeneans. Analyses of the total data set of the Digenea were conducted on the combined 18S rDNA and partial 28S rDNA data partitions. A far more restricted taxon set, only considering the closest clades relative to the Haploporidae was prepared to study the relationships of the Haploporidae with its closest digenean families. The restricted data set included taxa closely related to the Haploporidae [30 species plus one species of Lisorchiidae and two species of Monorchiidae designated as functional outgroups since the two families appeared as basal to the Haploporidae in all analyses of Olson *et al.* (2003)]. This dataset was analysed using: (i) 18S rDNA; (ii) 28S rDNA (including an additional, previously published sequence of *Saccocoelioides* sp. in the ingroup); and (iii) the combined data (see Table 8.1). These partitions were studied in order to allow comparison with previous studies (Bray *et al* 2005; Curran *et al* 2006, 2007), some of them based on single gene analysis.

Phylogenetic analyses

Two analyses were conducted for each data partition. Maximum parsimony analyses (MP) were performed using a heuristic search strategy with 1,000 search replicates, random-addition taxa sampling, tree-bisection-reconnection branch-swapping, with all characters run unordered with equal weights and with gaps treated as missing data. Bootstrap values (BV) were estimated by using a fast-heuristic search strategy, with 10,000 pseudoreplicates and 10 random sequence additions/replicate each for the total data set and a heuristic search strategy with 1,000 pseudoreplicates and 10 random sequence additions/replicate for the restricted data set. Bayesian inference analyses (BI) were conducted using MrBayes (Ronquist & Huelsenbeck, 2003, ver. 3.1.2). The GTR+I+Γ nucleotide substitution model (general-time-reversible model including estimates of invariant sites and gamma distributed among-site rate variation) was estimated independently for each data partition to provide the best fit to the data using ModelTest (Posada & Crandall, 1998, ver. 3.7 macX). Analyses of the total data set were run for 2 million generations and of the restricted data set for 1 million, with samples recorded every 100 generations. Consensus trees with mean branch lengths were constructed using the 'sumt' command in MrBayes based on trees saved after substitution parameters reached stationarity. Nodal support was estimated as posterior probabilities (PP) (Huelsenbeck *et al*., 2001).

Table 8.1. Data partitions and tree statistics.

Data partition	No. of ingroup (outgroup) taxa	No. of characters (%)			Tree statistics		
		Included	Constant	Parsimony informative sites	No. equally parsimonious trees	Length (steps)	CI
Digenea data set							
18S rDNA+28S rDNA	171 (7)	2432	1153	1024	872	11612	0.196
Clades related to the Haploporidae data set							
18S rDNA+28S rDNA	30 (3)	2729	1767	679	8	3487	0.419
18S rDNA	30 (3)	1627	1239	246	115	1149	0.470
28S rDNA	31 (3)	1102	525	435	3	2373	0.391

8.3. Results

The result of the BI analysis of the total data set is shown in Fig. 8.1; MP tree is shown in Fig. 8.2. Trees from BI and MP analyses of the combined data partition of the restricted data set are shown in Fig. 8.3; those based on the 18S rDNA and 28S rDNA data partitions individually are shown in Figs 8.4, 8.5 (solutions for 18S rDNA data partition in Fig. 8.4; those for 28S rDNA data partition in Fig. 8.5). Data partitions and tree statistics for each data set and analysis are given in Table 8.1.

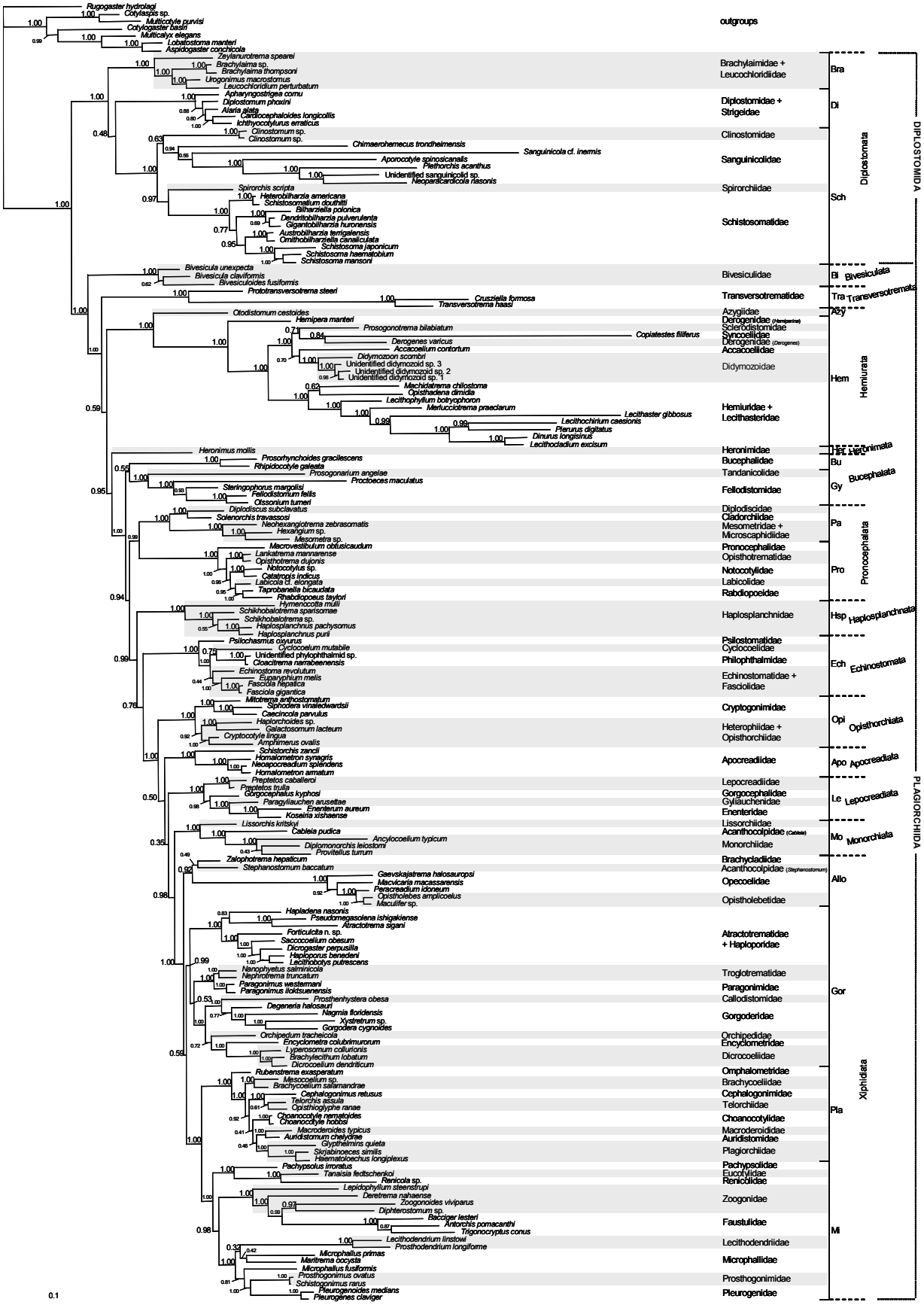

Fig. 8.1. Analysis of the total data set estimated from the combined data. Tree derived from Bayesian inference (BI) employing a GTR+I+Γ substitution model nodal support shown as posterior probabilities.

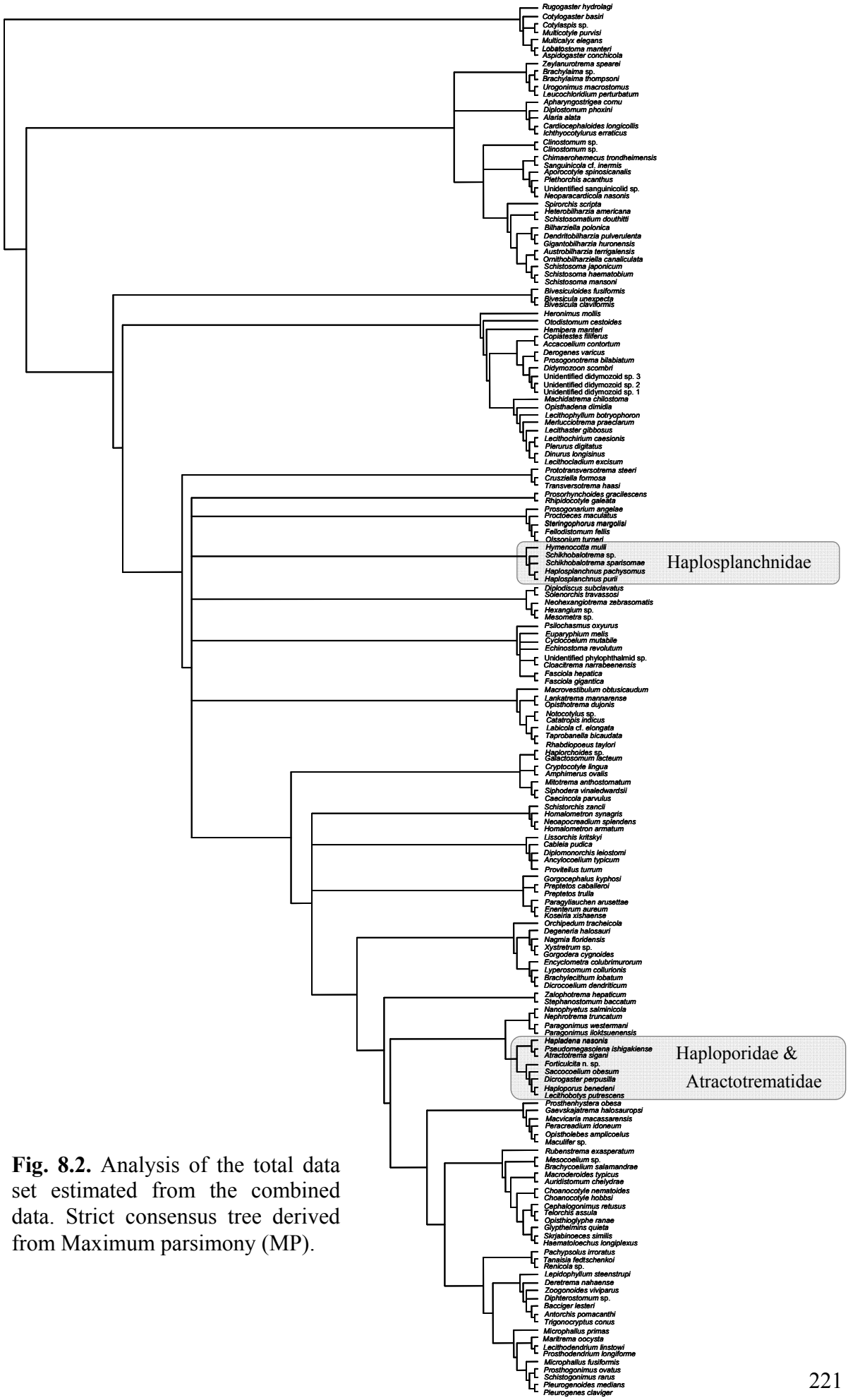

Fig. 8.2. Analysis of the total data set estimated from the combined data. Strict consensus tree derived from Maximum parsimony (MP).

Global phylogenetic analysis

The digeneans consistently formed two major lineages with strong nodal support that corresponded to the two orders of the phylogenetically-based classification of Olson *et al*. (2003): the Diplostomida Olson *et al.,* 2003 and the Plagiorchiida La Rue, 1957. However, most interrelationships of superfamilies and families within the diverse Plagiorchiida were labile. The newly sequenced taxa of the Haplosplanchnidae clustered together with the previously sequenced species in Olson *et al.* (2003) resulting in a monophyletic group. The Haplosplanchnidae formed a distinct lineage with high support in a basal position within the 'higher plagiorchiida' (*sensu* Olson *et al*., 2003) in the BI tree, diverging prior to the Echinostomata, Opisthorchiata, Apocreadiata, Lepocreadiata, Monorchiata and Xiphidiata. Support for the Xiphidiata was strong, although the internal structure of the clade differed slightly from that shown in Olson *et al*. (2003). The Haploporidae + Atractotrematidae appeared here as sister clade to the rest of the members of the Gorgoderoidea (closest) with high PP, instead of to the Paragonimidae + Troglotrematidae (Olson *et al*., 2003); and to the Plagiorchioidea + Microphalloidea (furthest) (Fig. 8.1). The Allocreadioidea, as recognized by Olson *et al.* (2003), was found basal in the Xiphidiata clade and not basal to the Plagiorchioidea + Microphalloidea. The Atractotrematidae was placed within the Haploporidae in this analysis, as in the previous study of Olson *et al.* (2003).

Relationships among the plagiorchiidans were poorly resolved by MP analysis. Equally parsimonious trees had a low consistency index (Table 8.1) thus, the strict consensus tree (Fig. 8.2) resulted in polytomies in which the Haplosplanchnidae and Haploporidae were included. In the MP consensus tree, the Haplosplanchnidae appeared as a distinct monophyletic lineage (BV = 99%) but its position within the Plagiorchiida was unresolved. The Haploporidae was similarly supported as monophyletic (albeit with low support: BV = 47%) and as a sister lineage to the Atractotrematidae (BV = 80%). In contrast to BI, the MP strict consensus tree showed the superfamily Gorgoderoidea as defined by Olson *et al.* (2003) to be paraphyletic, consisting of three separate clades.

Phylogenetic analyses of the restricted data set

Different gene fragments and analyses resulted in different phylogenetic hypotheses for the restricted data sets. Thus, BI analyses of the combined and 28S rDNA data partitions resolved the Haploporidae and the Atractotrematidae as well-supported, reciprocally monophyletic

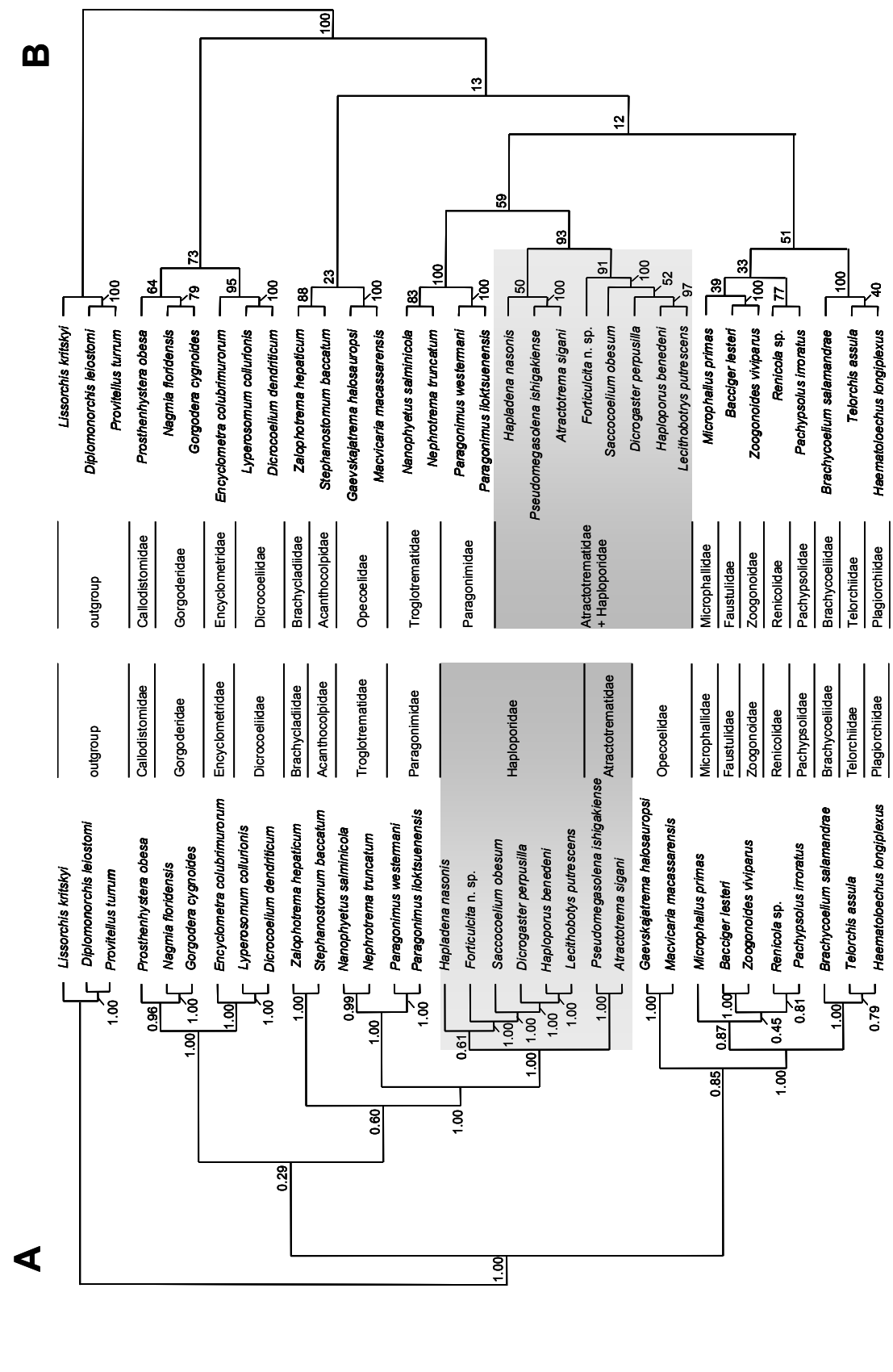

Fig. 8.3. Analysis of the restricted data set based on analysis of the combined data. **A.** Tree derived from Bayesian inference employing a GTR+I+Γ substitution model nodal support shown as posterior probabilities. **B.** Tree derived from Maximum Parsimony with nodal support based on bootstrapping.

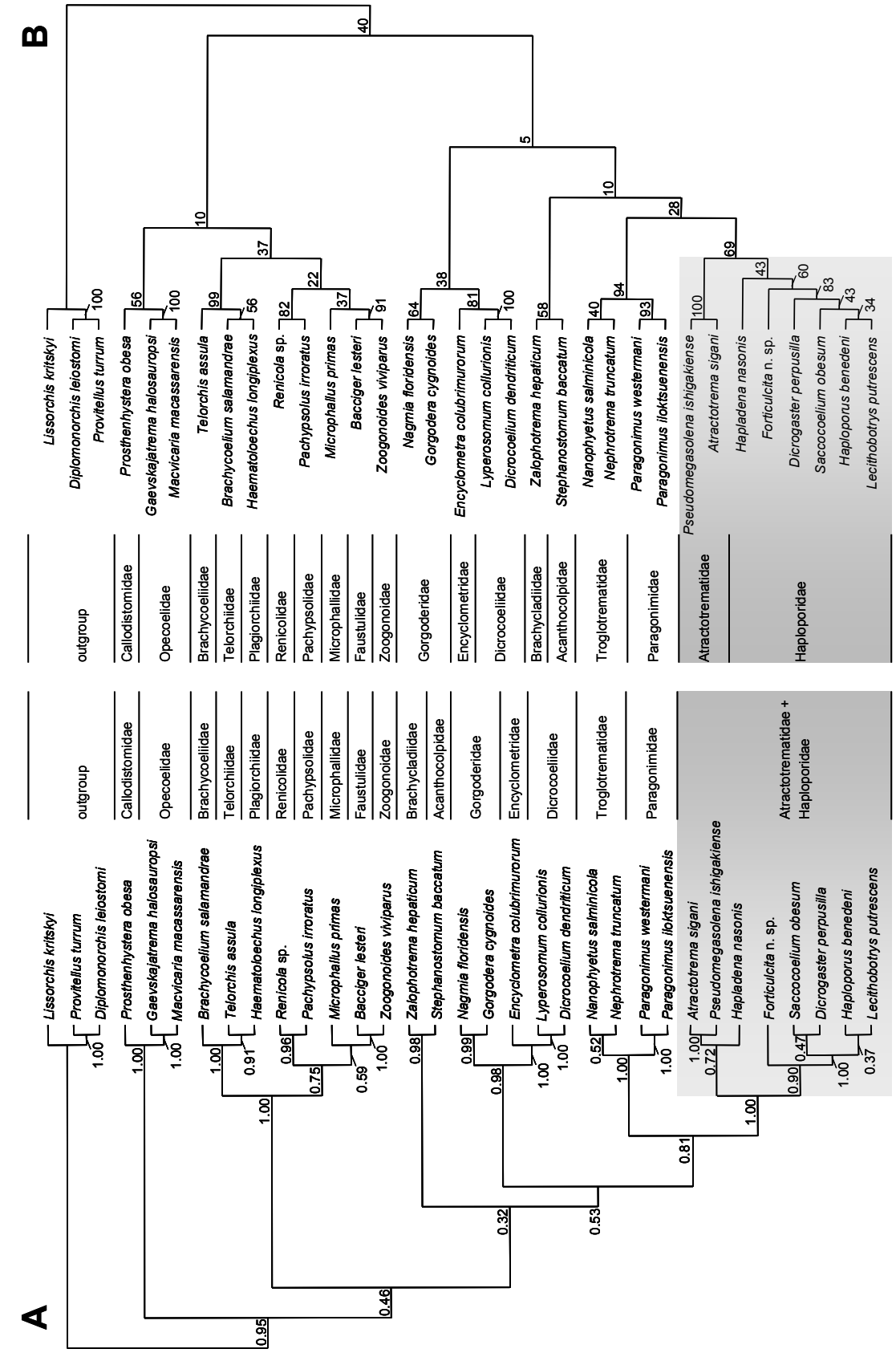

Fig. 8.4. Analysis of the restricted data set based on analysis of 18S only. **A**. Tree derived from Bayesian inference employing a GTR+I+Γ substitution model with nodal support shown as posterior probabilities. **B**. Tree derived from Maximum Parsimony with nodal support based on bootstrapping.

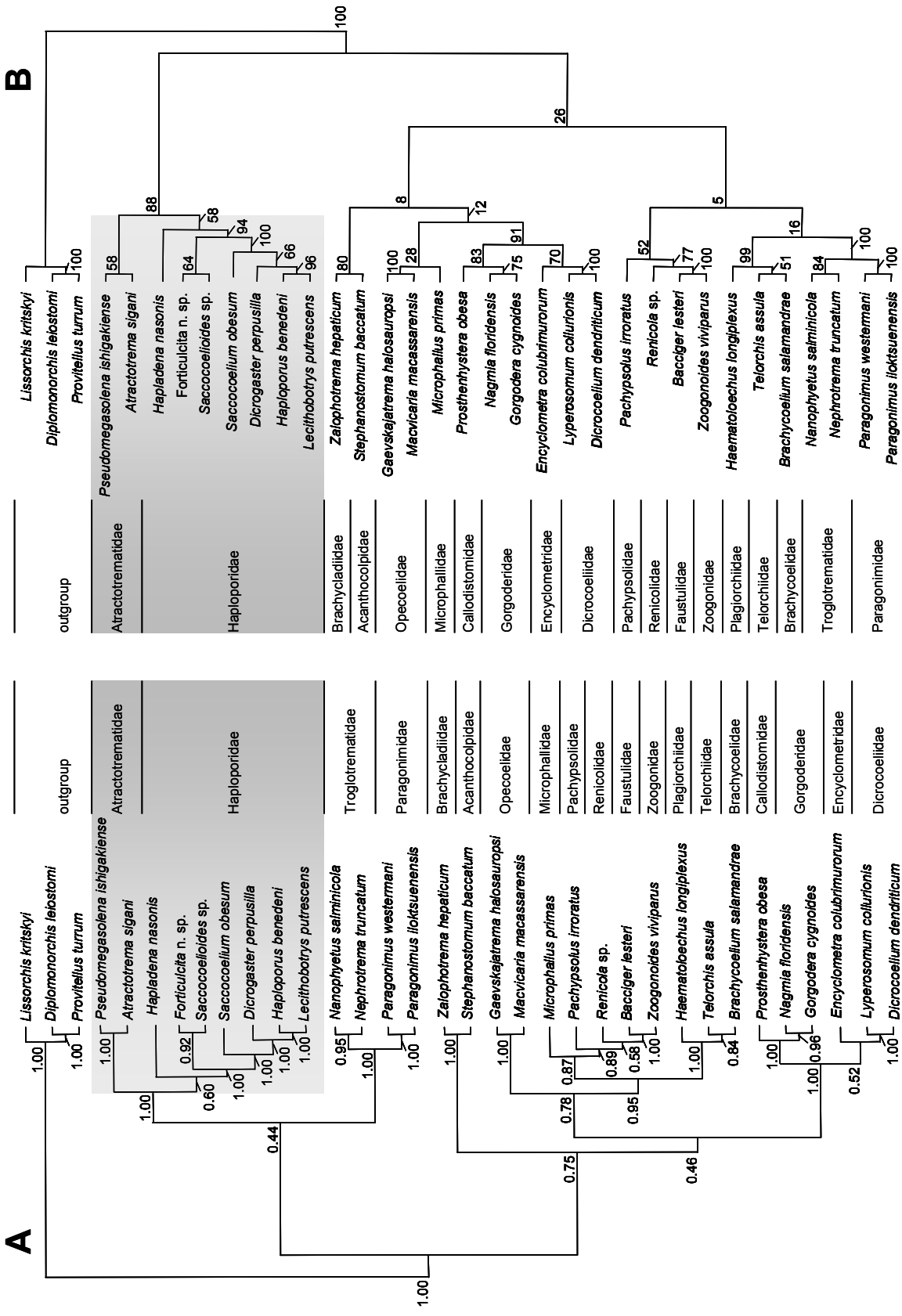

Fig. 8.5. Analysis of the restricted data set based on analysis of 28S only. **A**. Tree derived from Bayesian inference employing a GTR+I+Γ substitution model with nodal support shown as posterior probabilities. **B**. Tree derived from Maximum Parsimony with nodal support based on bootstrapping.

sister clades (Figs 8.3A, 8.5A), whereas in the BI analysis of the 18S rDNA data partition (Fig. 8.4A) the Haploporidae appeared paraphyletic due to the placement of *Hapladena nasonis* as a sister species to the members of the Atractotrematidae, albeit with low support. *Saccocoelioides* sp., for which 28S rDNA sequence was only available fell well within the Haploporidae clade (Fig. 8.5A–B). Consistently in the three BI analyses of the restricted data set, the Haploporidae + Atractotrematidae clade was resolved as the sister group to the Paragonimidae + Troglotrematidae although the PP of this affinity varied depending on the data partition (strongly supported in the combined analysis and unsupported in both individual partition solutions). Support for the monophyly of the Gorgoderoidea differed among the analyses, with 18S supporting monophyly of the group (Fig. 8.4A), 28S showing the group to be polyphyletic (Fig. 8.5A) and the combined analysis resulting in paraphyly of the group (Fig. 8.3A). The MP analyses of the restricted data set provided unresolved trees above the family level (from five to eight major clades). The difference in signal between the two genes was reflected in both the BI and MP analyses (Figs 8.3, 8.5).

8.4. Discussion

Position of the Haplosplanchnidae in the phylogeny of the Digenea

The present study, in which species of three of the four subfamilies of the Haplosplanchnidae were represented, supports the monophyly of the family with the placement of all sequenced taxa (including the type species of the type genera) within the clade. The position of Haplosplanchnidae in a well supported, distinct lineage is congruent with the hypothesis of Olson *et al*. (2003) and supports the elevation of its taxonomic level. The most characteristic features of the family are ‘the presence of a single intestinal caecum lined with prominent cells, occurrence of a single testis and the absence of a cirrus-sac’ (Madhavi, 2005). The life cycle of the haplosplanchnids includes cerithiid gastropods as first intermediate hosts and cercariae that encyst on vegetation to be transmitted to the definitive host *via* passive ingestion (Cable, 1954). Definitive hosts are herbivorous teleosts (mullets, beloniforms and kyphosids) that inhabit a great variety of environments, from freshwater and estuaries in temperate waters to coral reefs in marine tropical waters.

Monophyly of the Haploporidae

Olson *et al.* (2003) found the Haploporidae to be paraphyletic due to the position of *Pseudomegasolena ishigakiense* outside of the clade. Consequently, this species was transferred recently to the Atractotrematidae (see Overstreet & Curran, 2005), thereby making the Haploporidae in Olson *et al.* (2003) monophyletic. The high support of the clade *Pseudomegasolena* + *Atractotrema* observed by the latter authors, as well as in the analyses in Chapter 7 confirms the taxonomic decision of Overstreet & Curran (2005). In the present analyses, the Haploporidae was found monophyletic in all but three poorly supported solutions [BI for the combined gene fragments of the total data set (Fig. 8.1) and the18S rDNA data partition of the restricted data set (Fig. 8.5A); and MP for the combined data partition of the restricted data set (Fig. 8.3B)] in which *H. nasonis* clustered basal to the Atractotrematidae. This species is assigned to the Megasoleninae Manter, 1935 whereas the newly sequenced haploporid taxa (including the type species of the type genus of the family) belong to the Haploporinae. The position of *Hapladena* in the Atractotrematidae can be considered a misplacement most likely due to *H. nasonis* being a taxon relatively distant to both the Atractotrematidae and the Haploporinae. The only available sequence representing the Chalcinotrematinae was that of 28S rDNA of *Saccocoelioides* sp. (Curran *et al.,* 2006). Its clustering within the Haploporinae (Fig. 8.5; see also Chapter 7) suggests that either *Saccocoelioides* or *Forticulcita* is misplaced, or that the Chalcinotrematinae, erected to include the haploporids infecting freshwater (but not mugilid) fishes in Central and South America and Central Africa (Overstreet & Curran, 2005), does not reflect a natural grouping.

Until recently, the Atractotrematidae had been considered synonymous with the Haploporidae (Durio & Manter, 1969; Nasir & Gómez, 1976; Ahmad, 1985; Cribb *et al*., 1998b). However, Overstreet & Curran (2005) regarded the distinct status of the Atractotrematidae tentatively. The present results tend to support the validity of the Atractotrematidae as the sister clade to the Haploporidae (Figs. 8.3A, 8.4B, 8.5).

Affinities of the Haploporidae

In the present study as well as in previous molecular studies of the 'higher plagiorchiid' taxa (Tkach *et al*., 2000a; Tkach *et al.*, 2001a; Bray *et al*., 2005; Curran *et al*., 2007) the deep nodes are generally poorly supported and the relationships inferred change depending on the gene or method of analysis used (see *e.g.* Olson *et al*., 2003). Thus, assessing relationships above the family and superfamily levels is tenuous. However, a sister relationship between the Haploporidae + Atractotrematidae and the Paragonimidae + Troglotrematidae was found in almost all phylogenies that conforms with the previous study of the Digenea (figs. 2-5 in Olson *et al*., 2003). This affiliation was neither expected by morphology nor life-cycle traits. For example, the two lineages are readily distinguished morphologically by (i) the location of the genital pore relative to the ventral sucker and (ii) the presence *vs* absence of a hermaphroditic sac enclosing the male terminal genitalia (internal seminal vesicle and pars prostatica), metraterm and hermaphroditic duct. Moreover, although life cycles of members of the Atractotrematidae are still unknown, the typical haploporid life cycle involves two hosts: a gastropod of the superfamily Rissooidea as the first intermediate host and herbivorous fishes as definitive hosts (e.g. Fares & Maillard, 1974; Overstreet & Curran, 2005). In addition, cercariae lack oral stylets and encyst on vegetation. In contrast, paragonimids and troglotrematids possess a typical three-host life cycle (Yamaguti, 1975). First intermediate hosts are gastropods of at least five families (Hydrobiidae, Thiaridae, Pomatiopsidae, Viviparidae and Pleuroceridae). Cercariae bearing stylets infect the second intermediate host (freshwater crabs or fish) by penetration or ingestion. Metacercariae in the second intermediate host are then transferred *via* ingestion to a variety of mammalian definitive hosts such as cats, dogs, foxes, raccoons or even humans, causing paragonimiasis. Thus without the influence of molecular data, there are few if any biological features to suggest a common origin of these groups.

Placement of the Haploporidae within the Digenea

Analyses based on the partial 28S have shown the Haploporidae and Atractotrematidae in a basal position within the Xiphidiata (*e.g.* Curran *et al*., 2006), or as sister to it (present study, Fig. 8.5), or more basal still within the 'higher plagiorchiida' and completely outside of the Xiphidiata (see fig. 1 in Olson *et al*., 2003). These solutions are different from those inferred with 18S as well as 18S in combination with 28S, despite the fact that the majority of

phylogenetically informative characters in the combined analyses stemmed from the 28S gene. Thus, although the 18S gene shows fewer informative characters than the 28S, the signal it contains appears to be more consistent.

Based on rDNA, Olson *et al.* (2003) classified the Haploporidae + Atractotrematidae within the Gorgoderoidea. However, Jones (2005) and Overstreet & Curran (2005) both recognised and retained the Haploporoidea for ease of identification. The present analysis based on a larger extent of Haploporidae does not support the Haploporoidea [despite the distinct morphological features outlined by Overstreet & Curran (2005)] depicting that the phylogenetic affinities of the Haploporidae and Atractotrematidae clearly lie with other members of the Gorgoderoidea as defined by Olson *et al*. (2003 Choudhury *et al*. (2007) depicted the Haploporidae and the Atractotrematidae outside the Xiphidiata and as sister group to the families Lissorchiidae and Monorchiidae [including *Cableia* as suggested by Bray *et al*. (2005)] although with low support. Their solution could be considered the most plausible as the position within the Xiphidiata is itself unexpected as they lack the sole synapomorphy of the group: cercariae possessing stylets used to penetrate the second intermediate host (Cribb *et al*., 2003). However, this feature is also lacking in some members of the Acanthocolpidae, and other distinctions as described above suggest that the Haploporidae + Atractotrematidae represent a lineage that have secondarily simplified their life cycle and switched from tetrapod to fish hosts. Their morphological and developmental characters do not, therefore, reflect those of their most recent common ancestor and such evidence used for inferring phylogenetic affinities will be misleading.

The fact that the haploporids infect an ecological rather than a phylogenetic group of fishes (Overstreet and Curran, 2005) suggests that the herbivorous diet of their hosts may be secondary for fishes (derived from a carnivorous/omnivorous diet). Although additional evidence is needed to explain the ‘leap’ from a three-host life cycle it appears that a host dietary shift is responsible for the emergence of host-parasite associations with novel lineages.

CHAPTER 9

CONCLUSIONS

A detailed comparative morphological study of the Bunocotylinae Dollfus, 1950 and the Haploporinae Nicoll, 1914 was carried out taking into account the knowledge of traditional characters of taxonomic importance and aiming at (i) evaluation of the significance of new morphological characters; (ii) a redescription of the Mediterranean species on the basis of newly collected material; and (iii) a critical revision of the allocation of the nominal species. Furthermore, the intra-/interspecific variability of morphological characters was assessed by means of multivariate statistical analyses based on various population samples. This resulted in a redefinition of generic boundaries and refined diagnoses of nine genera and a more accurate estimation of the species richness, morphology and systematic position of the most diverse parasites in mullets, *Saturnius* spp. and the haploporine haploporids. Finally, the application of a molecular approach provided an independent test of the taxonomic framework of the Haploporinae established from comparative morphology, and permitted an evaluation of the taxonomic diversity and phylogenetic relationships at different taxonomic scales. As a summary of the study, the following main conclusions can be drawn:

9.1. The species diversity of the bunocotyline genus *Saturnius* Manter, 1969 is higher than previously thought, as evidenced by the description of three new species: *S. minutus* Blasco-Costa et al., 2006 in *Mugil cephalus* off the Mediterranean coast of Spain; *S. dimitrovi* Blasco-Costa et al., 2006 in *M. cephalus* off the Bulgarian Black Sea coast and the Spanish Mediterranean coast; and *S. overstreeti* Blasco-Costa et al., 2008 in *Mugil soiuy* and *M. cephalus* from the Russian coast of the Sea of Japan. The distinct species status of the new taxa is validated by means of discriminant morphometric analysis which led to the identification of five variables which solely contribute to a 100% correct allocation of specimens to species. The revision of the nominal species resulted in a refined diagnosis of *Saturnius* and a key to the species; two species, *Bunocotyle constrictus* Domnich & Sarabeev, 1999 and *S. valamugilis* Rekharani & Madhavi, 1985, are considered *species inquirendae* as well as *B. mugilis* of Solonchenko (1976) and *Saturnius mugilis* of Dmitrieva & Gaevskaya (2001) are regarded questionable records.

9.2. *Haploporus* Looss, 1902 is considered a monotypic Mediterranean haploporine genus. The type species, *H. benedeni* (Stossich, 1887), is redescribed and *H. lateralis* Looss, 1902 is considered to be its junior synonym. Five species parasitising *Valamugil* spp. from the Indo-West Pacific region, *H. indicus* Rekharani & Madhavi, 1985, *H. spinosus* Machida, 1996, *H. magnisaccus* Machida, 1996, *H. mugilis* Liu & Yang, 2002 and *H. muscolosaccus* Machida,

2003, are considered *incertae sedis* with respect to their generic affiliation. *H. pacificus* (Manter, 1963) (syn. *Neohaploporus pacificus* Manter, 1963), *H. pseudoindicus* Rekharani & Madhavi, 1985 and *H. musculosaccus* are believed to be *species inquirendae* and *H. lossii* Al-Bassel, 1990 is considered to be a *nomen nudum*. A new diagnosis, avoiding 'catching-all-species' and taking into account the original concept of Looss (1902) is provided.

9.3. The status of the nominal species of *Dicrogaster* Looss, 1902 is re-assessed by means of a comparative morphological study based on newly collected material from the western Mediterranean and a critical evaluation of the published data. *D. perpusilla* Looss, 1902 (type-species) and *D. contracta* Looss, 1902 are redescribed on the basis of new material from *Liza* spp. The latter two species and *D. fastigata* Thatcher & Sparks, 1958 are considered valid. *D. fragilis* Fernández Bargiela, 1987 is considered a junior synonym of *D. fastigata*, and *D. maryutensis* Al-Bassel, 1990 is considered to be *nomen nudum*. The two Mediterranean forms, *D. perpusilla* and *D. contracta* are further distinguished by multivariate morphometric analyses. A refined diagnosis of *Dicrogaster* and a key to its species is given.

9.4. A new species belonging to *Forticulcita* Overstreet, 1982, *F. gibsoni* Blasco-Costa et al., in press, is described from *M. cephalus* from the western Mediterranean. It is distinguished from the other two species in the genus by its significantly smaller body size and most of its metrical data. *F. gibsoni* is also distinguished by means of a multivariate morphometric analysis from the two Mediterranean species of *Dicrogaster*, to which it exhibits superficial similarity. A refined diagnosis of *Forticulcita* and a key to its species is presented.

9.5. *Lecithobotrys* Looss, 1902 is considered a monotypic Mediterranean haploporine genus. *L. putrescens* Looss, 1902 is redescribed based on newly collected material form *Liza* spp. *L. aegyptiacus* Hassan, El-Aziz, Khidr & Abu Samak, 1990 is synonymised with *Saccocoelium tensum* Looss, 1902 and *L. brisbanensis* (Martin, 1974) (syn. *Paralecithobotrys brisbanensis* Martin, 1974) and *L. vitellosus* Sharma & Gupta, 1970 are regarded as *species inquirendae*. A new generic diagnosis is provided.

9.6. *Saccocoelium* Looss, 1902 is revised and a refined diagnosis and a key to its recognised species are presented. *S. obesum* Looss, 1902 (type-species) and *S. tensum* are redescribed and three new species, *S. cephali* Blasco-Costa et al., in press, *S. brayi* n. sp. and *S. currani*

Blasco-Costa et al., in press, are described. The five Mediterranean species of *Saccocoelium* are distinguished by multivariate morphometric analyses. *Lecithobotrys helmymohamedi* Ramadan et al., 1988, *S. portsaidensis* El-Shahawi et al., 1992, *S. saoudi* El-Shahawi et al., 1992 and *Neosaccocoelium aegyptiacus* El-Shahawi et al., 1992 are considered to be synonyms of *S. tensum* and *Neosaccocoelium* El-Shahawi et al., 1992 is synonymised with *Saccocoelium*. *Lecithobotrys mugilis* Rekharani & Madhavi, 1985 is transferred to *Unisaccus* Martin, 1973 as *U. mugilis* (Rekharani & Madhavi, 1985) n. comb., and *L. sprenti* Martin, 1973 [= *Saccocoelium sprenti* (Martin, 1973) Overstreet & Curran, 2005] is transferred to *Unisaccus* as *U. sprenti* (Martin, 1973) n. comb. *S. megasacculum* Liu et al., 2004 is transferred to *Elliptobursa* Wu, Lu & Zhu, 1996 as *E. megasacculum* (Liu et al., 2004) n. comb. *S. tripathi* Dutta, 1995 (syn. *S. tripathi* Datta & Manna, 1998) is considered to be a *species inquirenda*.

9.7. Three new haploporine genera are established for parasites of mullet. *Ragaia* Blasco-Costa et al., in press is erected for a new species, *R. lizae* Blasco-Costa et al., in press, from *Liza ramada* in the Ebro Delta on the Mediterranean Coast of Spain. Two genera, *Pseudolecithobotrys* n. g. and *Pseudodicrogaster* Blasco-Costa et al., in press, are erected to accommodate species from the North Pacific previously placed in other genera, *Lecithobotrys stomachicola* Machida, 1996 and *Dicrogaster japonica* Machida, 1996, respectively. A key to the ten recognised genera of the Haploporinae is presented.

9.8. The first parallel molecular and morphological attempt to characterise a group of congeneric species within the Haploporidae Nicoll, 1914 permitted a more refined estimation of the amount of genetic and morphological differentiation, which is typical for closely related species of *Saccocoelium* from sympatric mullets in the Mediterranean. The molecular data corroborated the taxonomic decisions based on morphology with respect to the distinct status of the species of *Saccocoelium*, *i.e. S. obesum (sensu stricto)* and *S. tensum*, and supported the recognition of *S. brayi* n. sp. and *S. cephali*, thereby rejecting the hypothesis of a single species in Mediterranean mullets. However, the results based on ITS2 sequences do not rule out the possibility for even higher species diversity within *Saccocoelium*. Thus, the observed patterns of species and genetic diversity at the limited geographical scale of the study indicates that factors linked to features of the haploporid life-cycle modify the effect of the enhanced host encounter due to the similar feeding ecology of the mullet hosts.

Consequently, the possibility for sympatric speciation and even higher species diversity in the system studied, and in Haploporidae in general, might be higher.

9.9. Multivariate statistical analyses provide an important means for assessment of intra- and interspecific morphological variation and for testing the hypothesis of a morphometric separation between species/populations in the studied parasite groups both comprising genera composed of morphologically similar species. The results of both approaches, PCA and LDA, were concordant when applied simultaneously; the latter demonstrating consistently the morphometric variables that best distinguish species groups. Therefore, both techniques are suggested as valuable tools in the recognition of cryptic species and species delimitation in the studied taxa, as well as in constructing species identification keys.

9.10. For the first time, molecular data were used to evaluate the taxonomic framework of the Haploporidae based on morphology and to assess the relationships within the Haploporinae at the generic level. Molecular analysis revealed: (i) a close relationship between the Atractotrematidae Yamaguti, 1939 and the Haploporidae; (ii) strong support for the monophyly of the Haploporinae, *Dicrogaster* and *Saccocoelium*, and the position of *Ragaia* within the Haploporinae; (ii) evidence for rejection of the Dicrogasterinae Yamaguti, 1958 and the synonymy of *Saccocoelioides* Szidat, 1954 and *Lecithobotrys*; and (iii) support for the distinct status of *Saccocoelium* in relation to *Haploporus*. The wide sampling within the genera *Dicrogaster* and *Saccocoelium* confirmed the distinct status of the included species, thus rejecting previously suggested synonymies. *Saccocoelioides*, recently transferred to the Chalcinotrematinae Overstreet & Curran, 2005, was nested within the Haploporinae and this was largely associated with the position of *Forticulcita*, resolved as the most basal haploporine genus. *Forticulcita* also possesses a well-delimited eversible intromittent copulatory organ, a feature unique in the Haploporidae. This important apomorphy in association with the hypothesis of the Haploporinae based on molecular data, supported the erection of Forticulcitinae n. subf. for *Forticulcita*. This action resolved *Saccocoelioides* and, by extension the Chalcinotrematinae, as sister group to the Haploporinae. The position of the megasoleninae *Hapladena* Linton, 1910 remained unresolved.

9.11. The phylogenetic affinities of the Haploporidae within the Digenea clearly lie with other members of the Gorgoderoidea Looss, 1901 in the recently circumscribed suborder Xiphidiata Olson et al., 2003. The monophyletic Haploporidae was resolved as sister to the

Atractotrematidae on most occasions; however the different solutions found suggest that recognition of an independent superfamily for the Haploporidae and the Atractotrematidae is unsupported. The previously presumed relationship between Haplosplanchnidae Poche, 1926 and Haploporidae is refuted and the former is confirmed to be a monophyletic, distinct lineage in a basal position within the 'higher plagiorchiida' supporting its currently elevated taxonomic status, *i.e.* suborder Haplosplanchnata Olson et al., 2003.

8.12. Different gene fragments and analyses resulted in different phylogenetic hypotheses for the haploporids within the Xiphidiata. Despite the existence of few, if any, biological features that suggest a common origin, a sister relationship between the haploporids and atractotrematids and the paragonimids and troglotrematids was found in almost all phylogenies estimated. Therefore, the Haploporidae + Atractotrematidae are considered to represent a lineage that exhibits a secondary simplification of the life-cycle and a host-switch from tetrapods to fish. Therefore, the morphological and developmental characters of these two families do not reflect those of their most recent common ancestor and the use of such evidence for inferring phylogenetic affinities will be misleading.

APPENDIX 1

Appendix 1. Exemplar taxa of the families included in the analyses in Chapter 8, hosts, localities and GenBank accession numbers.

Classification of taxa	Host/Locality	18S rDNA	28S rDNA
Subclass ASPIDOGASTREA			
Order Aspidogastrida			
Family Aspidogastridae			
Aspidogaster conchicola	*Quadula postulosa,* Tennessee River, Onile, Tennessee, USA	AJ287478	AY222162
Cotylaspis sp.	*Pelodiscus sinensis*, Chilinh, HaiDuongVietnam	AY222083	AY222165
Cotylogaster basiri	*Poganias cromis,* Gulf of Mexico, Mississippi, USA	AY222082	AY222164
Lobatostoma manteri	*Trachinotus blochii,* HI, Australia	L16911	AY157177
Multicotyle purvisi	*Siebenrockiella crassicollis,* Malaya	AJ228785	AY222166
Family Multicalycidae			
Multicalyx elegans	*Callorhinchus milii,* Hobart, Tasmania, Australia	AJ287532	AY222163
Order Stichocotylidae			
Family Rugogastridae			
Rugogaster hydrolagi	*Callorhinchus milii,* Hobart, Tasmania, Australia	AJ287573	AY157176
Subclass DIGENEA			
Order Diplostomida			
Family Brachylaimidae			
Brachylaima sp.	*Mus musculus,* lab infection, Queensland, Australia	AY222084	AY222167
Brachylaima thompsoni	*Blarina brevicaudata,* Wisconsin, USA	AY222085	AF184262
Zeylanurotrema spearei	*Bufo marinus,* Daintree region, Queensland, Australia	AY222088	AY222170
Family Leucochloridiidae			
Leucochloridium perturbatum	*Turdus merula,* Záhlinice, Czech Republic	AY222087	AY222169
Urogonimus macrostomus	*Anas platyrhynchus,* lab infection, Ukraine	AY222086	AY222168
Family Diplostomidae			
Alaria alata	*Nyctereutes procyonoides,* Kherson Region, Ukraine	AY222091	AF184263
Diplostomum phoxini	*Phoxinus phoxinus,* Aberystwyth, Wales, UK	AY222090	AY222173
Family Strigeidae			
Apharyngostrigea cornu	*Ardea cinerea,* Kherson Region, Ukraine	AY222092	AF184264
Cardiocephaloides longicollis	*Larus ridibundus,* Kherson Region, Ukraine	AY222089	AY222171
Ichthyocotylurus erraticus	*Coregonus autumnalis,* Lough Neagh, Northern Ireland, UK	AJ287526	AY222172
Family Clinostomidae			
Clinostomum sp.	*Hypseleotris galii,* Moggil Creek, Queensland, Australia	AY222094	AY222175
Clinostomum sp.	*Rana catesbeiana,* Reelfoot lake, Tenneesse, USA	AY222095	AY222176
Family Sanguinicolidae			
Unidentified sanguinicolid sp.	*Arothron meleagris,* Moorea, French Polynesia	AY222107	AY222205
Aporocotyle spinosicanalis	*Merluccius merluccius,* Off Orkney Islands, NE Atlantic	AJ287477	AY222177
Chimaerohemecus trondheimensis	*Chimaera monstrosa,* Korsfjorden, Bergen, Norway	AY157213	AY157239
Neoparacardicola nasonis	*Naso unicornis,* LI, Australia	AY222097	AY222179
Plethorchis acanthus	*Mugil cephalus,* Brisbane River, Queensland, Australia	AY222096	AY222178
Sanguinicola cf. *Inermis*	*Lymnaea stagnalis,* Warminia-Mazury Region, Poland	AY222098	AY222180
Family Spirorchiidae			
Spirorchis scripta	*Trachemys scripta scripta,* Van Cleave, Mississippi, USA	AY222093	AY222174
Family Schistosomatidae			
Austrobilharzia terrigalensis	*Batillaria australis,* Iron Cove, Sydney Harbour, Australia	AY157223	AY157249
Bilharziella polonica	*Anas platyrhynchus,* Kheson Oblast, Ukraine	AY157214	AY157240
Dendritobilharzia pulverulenta	*Gallus gallus,* Bernallio County, New Mexico,USA	AY157215	AY157241
Gigantobilharzia huronensis	*Agelaius phoeniceus,* Wisconsin, USA	AY157216	AY157242
Heterobilharzia americana	*Mesocricetus auratus,* lab infection, UK	AY157220	AY157246
Ornithobilharziella canaliculata	*Larus delawarensis,* Donley County, Texas, USA	AY157222	AY157248
Schistosoma haematobium	*Mesocricetus auratus,* lab infection, UK	Z11976	AY157263
Schistosoma japonicum	*Mus musculus,* lab infection, UK	AY157226	AY157607
Schistosoma mansoni	*Mus musculus,* lab infection, UK	M62652	AY157173
Schistosomatium douthitti	*Mesocricetus auratus,* lab infection, Indiana, USA	AY157221	AY157247
Orden Plagiorchiida			
Family Bivesiculidae			
Bivesicula claviformis	*Epinephelus quoyanus,* LI, Australia	AJ287485	AY222182
Bivesicula unexpecta	*Acanthochromis polyacanthus,* HI, Australia	AY222099	AY222181
Bivesiculoides fusiformis	*Atherinomorus capricornensis,* HI, Australia	AY222100	AY222183
Family Transversotrematidae			
Crusziella formosa	*Crenimugil crenilabis,* HI, Australia	AJ287491	AY222185
Prototransversotrema steeri	*Acanthopagrus australis,* Iluka, Queensland, Australia	AY222101	AY222184
Transversotrema haasi	*Caesio cuning,* HI, Australia	AJ287583	AY222186
Family Azygiidae			
Otodistomum cestoides	*Raja montagui,* North Sea, UK	AJ287553	AY222187
Family Derogenidae			
Derogenes varicus	*Hippoglossoides platessoides,* North Sea, UK	AJ287511	AY222189
Hemiperina manteri	*Latridopsis forsteri,* Tasmania, Australia	AY222105	AY222196
Family Hemiuridae			
Dinurus longisinus	*Coryphaena hippurus,* Port Royal, Kingston, Jamaica	AJ287501	AY222202
Lecithochirium caesionis	*Caesio cuning,* HI, Australia	AJ287528	AY222200
Lecithocladium excisum	*Scomber scombrus,* North Sea, UK	AJ287529	AY222203
Machidatrema chilostoma	*Kyphosus vaigiensis,* Moorea, French Polynesia	AY222106	AY222197
Merlucciotrema praeclarum	*Cataetyx laticeps,* Goban Spur, NE Atlantic	AY222107	AY222205
Opisthadena dimidia	*Kyphosus cinerascens,* HI, Australia	AJ287549	AY222198
Plerurus digitatus	*Scomberomorus commerson,* HI, Australia	AJ287562	AY222201
Family Lecithasteridae			
Lecithaster gibbosus	*Merlangius merlangus,* North Sea, UK	AJ287527	AY222199
Lecithophyllum botryophorum	*Alepocephalus bairdii,* Goban Spur, NE Atlantic	AY222107	AY222205

Appendix 1. Continued (i).

Classification of taxa	Host/Locality	18S rDNA	28S rDNA
Family Accacoeliidae			
Accacoelium contortum	*Mola mola,* North Sea, UK	AJ287472	AY222190
Family Didymozoidae			
Unidentified didymozoid sp. 1	*Epinephelus cyanopodus,* HI, Australia	AY222103	AY222193
Unidentified didymozoid sp. 2	*Taeniura lymma,* HI, Australia	AY222102	AY222192
Unidentified didymozoid sp. 3	*Apogon cookii,* HI, Australia	AY222104	AY222194
Didymozoon scombri	*Scomber scombrus,* North Sea, UK	AJ287500	AY222195
Family Sclerodistomidae			
Prosogonotrema bilabiatum	*Caesio cuning,* HI, Australia	AJ287565	AY222191
Family Syncoeliidae			
Copiatestes filiferus	*Trachurus murphyi,* New Zeland	AJ287490	AY222188
Family Heronimidae			
Heronimus mollis	*Chelydra serpentine,* Pawnee County, Nebraska, USA	AY222118	AY116878
Family Bucephalidae			
Prosorhynchoides gracilescens	*Lophius piscatorius,* North Sea, UK	AJ228789	AY222224
Rhipidocotyle galeata	*Eutrigla gurnardus,* North Sea, UK	AY222119	AY222225
Family Fellodistomidae			
Fellodistomum fellis	*Anarhichas lupus,* North Sea, UK	Z12601	AY222282
Olssonium turneri	*Alepocephalus agassizi,* Porcupine Seabright, NE Atlantic	AJ287548	AY222283
Proctoeces maculatus	*Archosargus probatocephalus,* Gulf of Mexico, Mississippi, USA	AY222161	AY222284
Steringophorus margolisi	*Spectrunculus grandis,* Rockall Trough, NE Atlantic	AJ287578	AY222281
Family Tandanicolidae			
Prosogonarium angelae	*Euristhmus lepturus,* Moreton Bay, Queensland, Australia	AJ287564	AY222285
Family Microscaphidiidae			
Hexangium sp.	*Siganus fuscescens,* HI, Australia	AJ287522	AY222215
Neohexangiotrema zebrasomatis	*Zebrasoma scopes,* LI, Australia	AJ287544	AY222214
Family Mesometridae			
Mesometra sp.	*Sarpa salpa,* Mediterranean Sea, Perpignan (fish market), France	AJ287537	AY222216
Family Diplodiscidae			
Diplodiscus subclavatus	*Rana ridibunda,* Kokaljane, Bulgaria	AJ287502	AY222212
Family Cladorchiidae			
Solenorchis travassosi	*Dugong dugon,* Lucinda, Queensland, Australia	AY222110	AY222213
Family Pronocephalidae			
Macrovestibulum obtusicaudum	*Trachemys scripta scripta,* George County, Mississippi, USA	AY222111	AY116877
Family Opisthotrematidae			
Lankatrema mannarense	*Dugong dugon,* Townsville, Queensland, Australia	AY222116	AY222222
Opisthotrema dujonis	*Dugong dugon,* Townsville, Queensland, Australia	AY222117	AY222223
Family Notocotylidae			
Catatropis indicus	*Cairina moschata,* lab infection, Armidale, Australia	AY222114	AY222220
Notocotylus sp.	*Lymnaea palustris,* Leckford Estate, Stockbridge, UK	AJ287547	AY222219
Family Rabdiopoeidae			
Rhabdiopoeus taylori	*Dugong dugon,* Lucinda, Queensland, Australia	AY222113	AY222218
Taprobanella bicaudata	*Dugong dugon,* Townsville, Queensland, Australia	AY222112	AY222217
Family Labicolidae			
Labicola cf. *elongata*	*Dugong dugon,* Lucinda, Queensland, Australia	AY222115	AY222221
Family Haplosplanchnidae			
Haplosplanchnus pachysomus	*Liza ramada*, Santa Pola and Ebro Delta, Spain	FJ211224	FJ211241
Haplosplanchnu purii	*Mugil cephalus*, Anse Vata, New Caledonia	FJ211225	FJ211242
Schikhobalotrema sparisomae	*Liza aurata*, Ebro Delta, Spain	FJ211223	FJ211240
Schikhobalotrema spp.	*Scarus rivulatus*, HI, Australia	AJ287574	AY222238
Hymenocotta mulli	Crenimugil crenilabis, HI, Australia	AJ287524	AY222239
Family Psilostomidae			
Psilochasmus oxyurus	*Anas platyrhynchus,* Kherson Region, Ukraine	AY222135	AF151940
Family Echinostomatidae			
Echinostoma revolutum	*Mesocricetus auratus,* lab infection, UK	AY222132	AY222246
Euparyphium melis	*Nyctereutes procyonoides,* Kherson Region, Ukraine	AY222131	AF151941
Family Fasciolidae			
Fasciola gigantica	*Bos taurus,* St. Louis, Senegal	AJ011942	AY222245
Fasciola hepatica	*Capra hircus,* Saudi Arabia	AJ004969	AY222244
Family Philophthalmidae			
Cloacitrema narrabeenensis	*Batillaria australis,* Iron Cove, Sydney Harbour, Australia	AY222134	AY222248
Unidentified phylophthalmid sp.	*Batillaria australis,* Moreton Bay, Queensland, Australia	AY222133	AY222247
Family Cyclocoelidae			
Cyclocoelum mutabile	*Calidris canutus,* Fair Isle, Scotland, UK	AJ287494	AY222249
Family Heterophyidae			
Cryptocotyle lingua	*Littorina littorea,* Isle of Sylt, North Sea, Germany	AJ287492	AY222228
Galactosomum lacteum	*Phalacrocorax carbo,* Kherson Region, Ukraine	AY222120	AY222227
Haplorchoides sp.	*Arius graeffei,* Lake Wivenhoe, Queensland, Australia	AJ287521	AY222226
Family Opisthorchiidae			
Amphimerus ovalis	*Trionyx muticus,* George County, Mississippi, USA	AY222121	AY116876
Family Cryptogonimidae			
Caecincola parvulus	*Micropterus salmoides,* Pascagoula River, Mississippi, USA	AY222123	AY222231
Siphodera vinaledwardsii	*Sciaenops ocellatus,* South of Horn Island, Mississippi, USA	AY222122	AY222230
Mitotrema anthostomatum	*Cromileptes altivelis,* HI, Australia	AJ287542	AY222229

Appendix 1. Continued (ii).

Classification of taxa	Host/Locality	18S rDNA	28S rDNA
Family Apocreadiidae			
Homalometron armatum	*Lepomis microlophus,* Pascagoula River, Mississippi, USA	AY222130	AY222241
Homalometron synagris	*Scolopsis monogramma,* HI, Australia	AJ287523	AY222243
Neoapocreadium splendens	*Scolopsis monogramma,* LI, Australia	AJ287543	AY222242
Schistorchis zancli	*Zanclus cornutus,* Moorea, French Polynesia	AY222129	AY222240
Family Lepocreadiidae			
Preptetos caballeroi	*Naso vlamingi,* HI, Autralia	AJ287563	AY222236
Preptetos trulla	*Ocyurus chrysurus,* Port Royal, Kingston, Jamaica	AY222128	AY222237
Family Gorgocephalidae			
Gorgocephalus kyphosi	*Kyphosus vaigiensis,* LI, Australia	AY222126	AY222234
Family Enenteridae			
Enenterum aureum	*Kyphosus vaigiensis,* Moorea (fish market), French Polynesia	AY222124	AY222232
Koseiria xishaense	*Kyphosus vaigiensis,* HI, Australia	AY222125	AY222233
Family Gyliauchenidae			
Paragyliauchen arusettae	*Pomacanthus sexstriatus,* Ningaloo, Australia	AY222127	AY222235
Family Lissorchiidae			
Lissorchis kritskyi	*Carpiodes cyprinus,* Pascagoula River, Mississippi, USA	AY222136	AY222250
Family Acanthocolpidae			
Cableia pudica	*Cantherines pardalis,* HI, Australia	AJ287486	AY222251
Stephanostomum baccatum	*Eutrigla gurnardus,* North Sea, UK	AJ287577	AY222256
Family Monorchiidae			
Ancylocoelium typicum	*Trachurus trachurus,* North Sea, UK	AJ287474	AY222254
Diplomonorchis leiostomi	*Leiostomus xanthurus,* Ocean Springs, Mississippi, USA	AY222137	AY222252
Provitellus turrum	*Pseudocaranx dentex,* HI, Australia	AJ287566	AY222253
Family Atractotrematidae			
Atractotrema sigani	*Siganus lineatus*, LI, Australia	AJ287479	AY222267
Pseudomegasolena ishigakiense	*Scarus rivulatus,* HI, Australia	AJ287569	AY222266
Family Haploporidae			
Hapladena nasonis	*Naso unicornis*, LI, Australia	AY222146	AY222265
Saccocoelioides sp.	Host not reported, Nicaragua	-	EF032696
Haploporus benedeni	*Liza ramada,* Santa Pola, Spain	FJ211228	FJ211237
Saccocoelium obesum	*Liza aurata*, Ebro Delta, Spain	FJ211254	FJ211260
Dicrogaster perpusilla	*Liza ramada*, brackish lagoon at Santa Pola, Spain	FJ211230	FJ211238
Lecithobotrys putrescens	*Liza saliens*, Ebro Delta, Spain	FJ211229	FJ211236
Forticulcita n. sp.	*Mugil cephalus*, Santa Pola, Spain	FJ211226	FJ211239
Family Paragonimidae			
Paragonimus iloktsuenensis	*Rattus norvegicus,* Amami Island, Japan	AY222141	AY116875
Paragonimus westermani	*Canis familiaris,* Hyogo, Japan	AY222140	AY116874
Family Troglotrematidae			
Nanophyetus salminicola	*Oncorhynchus mykiss,* Benton County (hatchery), Oregon, USA	AY222138	AY116873
Nephrotrema truncatum	*Neomys anomalus,* Zakarpatska Region, Ukraine	AY222139	AF151936
Family Callodistomidae			
Prosthenhystera obesa	*Hoplias* sp., Rio Itaya, 50km from Iquitos, Peru	AY222108	AY222206
Family Gorgoderidae			
Degeneria halosauri	*Halosauropsis macrochir,* NE Atlantic Ocean	AJ287497	AY222257
Gorgodera cygnoides	*Rana ridibunda,* Kokaljane, Sofia, Bulgaria	AJ287518	AY222264
Nagmia floridensis	*Rhinoptera bonasus,* East Ship Island, Mississippi, USA	AY222145	AY222262
Xystretrum sp.	*Sufflamen chrysopterus,* LI, Australia	AJ287588	AY222263
Family Orchipedidae			
Orchipedum tracheicola	*Cygnus olor,* Drumpellier Loch, Scotland, UK	AJ287551	AY222258
Family Dicrocoeliidae			
Brachylecithum lobatum	*Corvus corone,* Záhlinice, Czech Republic	AY222144	AY222260
Dicrocoelium dendriticum	*Ovis aries,* Spain	AY11236	AY222261
Lyperosomum collurionis	*Sylvia atricapilla,* Záhlinice, Czech Republic	AY222143	AY222259
Family Encyclometridae			
Encyclometra colubrimurorum	*Natrix natrix,* Kiev Region, Ukraine	AY222142	AF184254
Family Opecoelidae			
Gaevskajatrema halosauropsi	*Halosauropsis macrochir,* Goban Spur, NE Atlantic Ocean, UK	AJ287514	AY222207
Macvicaria macassarensis	*Lethrinus miniatus,* HI, Australia	AJ287533	AY222208
Peracreadium idoneum	*Anarhichas lupus,* North Sea, UK	AJ287558	AY222209
Family Opistholebetidae			
Maculifer sp.	*Diodon hysterix,* HI, Australia		
Opistholebes amplicoelus	*Tetractenos hamiltoni,* Stradbroke Island, Queensland, Australia	AJ287550	AY222210
Family Brachycladiidae			
Zalophotrema hepaticum	*Zalophus californianus,* California, USA	AJ224884	AY222255
Family Omphalometridae			
Rubenstrema exasperatum	*Crocidura leucodon,* Bulgaria	AJ287572	AY222275
Family Brachycoeliidae			
Brachycoelium salamandrae	*Salamandra salamandra,* Zakarpatska Region, Ukraine	AY222160	AF151935
Mesocoelium sp.	*Bufo marinus,* Brisbane, Queensland, Australia	AJ287536	AY222277
Family Macroderoididae			
Macroderoides typicus	*Lepisosteus platostomus,* Reelfoot Lake, Tennessee, USA	AY222158	AF433673
Family Auridistomidae			
Auridistomum chelydrae	*Chelydra serpentine,* Jackson County, Mississippi, USA	AY222159	AY116872
Family Choanocotylidae			
Choanocotyle hobbsi	*Chelodina oblonga*, Perth, Australia	AY116868	AY116865
Choanocotyle nematoides	*Emydura* sp., New South Wales, Australia	AY116867	AY116862

Appendix 1. Continued (iii).

Classification of taxa	Host/Locality	18S rDNA	28S rDNA
Family Plagiorchiidae			
Haematoloechus longiplexus	*Rana catesbeiana,* Keith County, Nebraska, USA	AJ287520	AY222280
Glypthelmins quieta	*Rana catesbeiana,* Keith County, Nebraska, USA	AJ287517	AY222278
Skrjabinoeces similis	*Rana ridibunda,* Kokaljane, Sofia, Bulgaria	AJ287575	AY222279
Family Cephalogonimidae			
Cephalogonimus retusus	*Rana ridibunda,* Kokaljane, Sofia, Bulgaria	AJ287489	AY222276
Family Telorchiidae			
Opisthioglyphe ranae	*Rana arvalis,* Ivano-Frankivsk Region, Ukraine	AY222157	AF151929
Telorchis assula	*Natrix natrix,* Kiev Region, Ukraine	AY222156	AF151915
Family Pachypsolidae			
Pachypsolus irroratus	*Lepidochelys olivacea,* Oaxaca, Mexico	AJ287554	AY222274
Family Renicolidae			
Renicola sp.	*Numenius arquata,* Kherson Region, Ukraine	AY222155	AY116871
Family Eucotylidae			
Tanaisia fedtschenkoi	*Anas platyrhynchus,* Kherson Region, Ukraine	AY222154	AY116870
Family Zoogonidae			
Deretrema nahaense	*Thalassoma lunare,* LI, Australia	AJ287498	AY222273
Diphterostomum sp.	*Scolopsis monogramma,* HI, Australia	AY222153	AY222272
Lepidophyllum steenstrupi	*Anarhichas lupus,* North Sea, UK	AJ287530	AY157175
Zoogonoides viviparus	*Callionymus lyra,* North Sea, UK	AJ287590	AY222271
Family Faustulidae			
Antorchis pomacanthi	*Pomacanthus sexstriatus,* HI, Autralia	AJ287476	AY222268
Bacciger lesteri	*Selenotoca multifasciata,* Moreton Bay, Queensland, Australia	AJ287482	AY222269
Trigonocryptus conus	*Arothron nigropunctatus,* HI, Australia	AJ287584	AY222270
Family Lecithodendriidae			
Lecithodendrium linstowi	*Nyctalus noctula,* Sumy Region, Ukraine	AY222147	AF151919
Prosthodendrium longiforme	*Myotis daubentoni,* Kiev Region, Ukraine	AY222148	AF151921
Family Microphallidae			
Maritrema oocysta	*Hydrobia ulvae,* Belfast Lough, Northern Ireland, UK	AJ287534	AY220630
Microphallus fusiformis	*Hydrobia ulvae,* Belfast Lough, Northern Ireland, UK	AJ287531	AY220633
Microphallus primas	*Hydrobia ulvae,* Belfast Lough, Northern Ireland, UK	AJ287541	AY220627
Family Pleurogenidae			
Pleurogenes claviger	*Rana temporaria,* Kiev Region, Ukraine	AY222152	AF151925
Pleurogenoides medians	*Rana lessonae,* Kiev Region, Ukraine	AY222151	AF433670
Family Prosthogonimidae			
Prosthogonimus ovatus	*Pica pica,* Chernigiv Region, Ukraine	AY222149	AF151928
Schistogonimus rarus	*Anas querquedula,* Kherson Region, Ukraine	AY222150	AY116869

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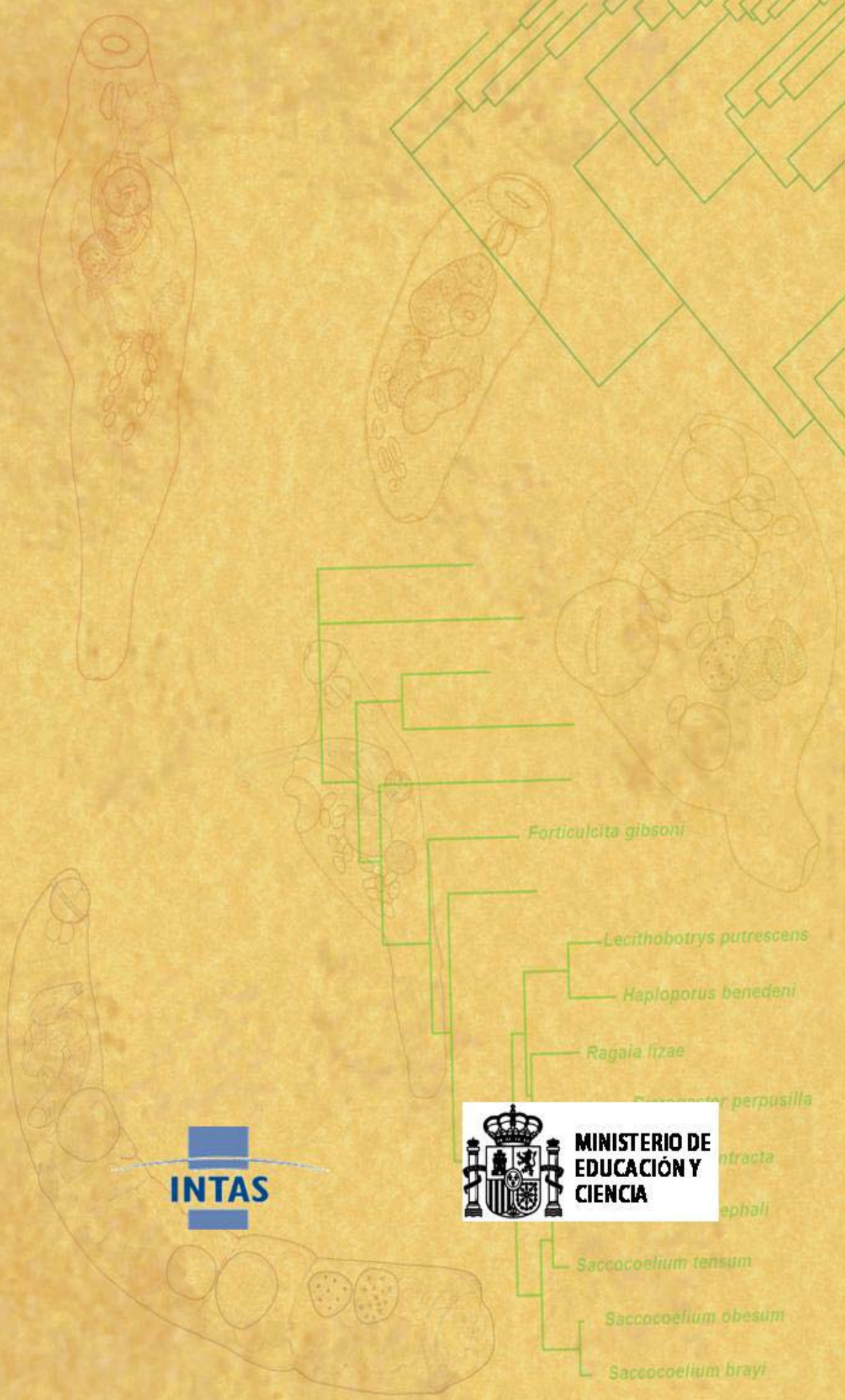
UVEG

TAXONOMY OF THE HAPLOPORINAE Nicoll, 1914 AND BUNOCOTYLINAE Dollfus, 1950 (DIGENEA) FROM MEDITERRANEAN MULLETS (TELEOSTEI): MORPHOLOGICAL AND MOLECULAR APPROACHES

María Isabel Blasco Costa



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