

Research Article

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# The molecular phylogeny of the digenean family Opecoelidae Ozaki, 1925 and the value of morphological characters, with the erection of a new subfamily

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**Abstract:** Large and small rDNA sequences of 41 species of the family Opecoelidae are utilised to produce phylogenetic inference trees, using brachycladioids and lepecreadioids as outgroups. Sequences were newly generated for 13 species. The resulting Bayesian trees show a monophyletic Opecoelidae. The earliest divergent group is the Stenakrinae, based on two species which are not of the type-genus. The next well-supported clade to diverge is constituted of three species of *Helicometra* Odhner, 1902. Based on this tree and the characters of the egg and uterus, a new subfamily, the Helicometrinae, is erected and defined to include the genera *Helicometra*, *Helicometrina* Linton, 1910 and *Neohelicometra* Siddiqi et Cable, 1960. The subfamily Opecoelinae is found to be monophyletic, but the Plagioporinae is paraphyletic. The single representative of the Opecoelininae (not of the type genus) is nested within a group of deep-sea ‘plagioporines’. The two representatives of the Opistholebetidae are embedded within a group of shallow-water ‘plagioporine’ species. The Opistholebetidae is reduced to subfamily status *pro tem* as its morphological and biological characteristics are distinctive. This implies that as opecoelid systematics develops with more molecular evidence, several further subfamilies will be recognised. Many of the morphological characters were found to be homoplasious, but the characters defining the Helicometrinae and Opecoelinae, such as filamented eggs, reduced cirrus-sac and uterine seminal receptacle, are closely correlated with the inferred phylogeny.

**Keywords:** Digenea, Opecoelinae, Plagioporinae, Stenakrinae, Opecoelininae, rDNA sequence, Helicometrinae

This article contains supporting information (Figs. S1, S2) online at <http://folia.paru.cas.cz/suppl/2016-63-013.pdf>

The family Opecoelidae Ozaki, 1925 is the largest digenean family with over 90 genera and nearly 900 species, almost solely found in marine and freshwater teleost fishes. It is now considered to belong in the superfamily Opecoeloidea Ozaki, 1925 (see Littlewood et al. 2015) or the Brachycladioidea Odhner, 1905 (see Curran et al. 2006). Molecular studies have begun to elucidate the complexity of this large family, but as yet no clear pattern is emerging. There are several genera that are quite large, i.e. *Plagioporus* Stafford, 1904 (with 55 species), *Podocotyle* Dujardin, 1845 (55), *Macvicaria* Gibson et Bray, 1982 (51), *Coitocaecum* Nicoll, 1915 (50), *Opecoelus* Ozaki, 1925 (43), *Opegaster* Ozaki, 1928 (37), *Pseudopecoelus* Von Wicklen, 1946 (37) and *Neolebouria* Gibson, 1976 (25). The distinguishing characters for these genera are weak and rife with homoplasy, and as a result their demarcation and validity is constantly under discussion and disagreement.

For example, Cribb (2005a), in his key to the genera of the family, recognised the genus *Opegaster* as separate from *Opecoelus* on a “character of equivocal value”, but

that was “entrenched in the literature”. On the other hand, Aken’Ova (2007) reviewed the controversy relating to these genera and synonymised them, forming many new combinations. Manter (1940) said “Actually some species seem close to the border line between the 2 genera”. Crowcroft (1947) stated in relation to this problem that “The preferable course would seem to be the grouping of such similar species into one genus until such time as sufficiently clear sub-groups appear to warrant the setting up of several genera”. Bray and Justine (2013) described a new species of *Opegaster*, discussed the generic distinction and stated “any worker who has struggled with opecoelid systematics will know, separation of taxa in this family is largely arbitrary and is a prime candidate for molecular solutions”.

Cribb (2005a) reckoned that the “subfamily level classification within the Opecoelidae is complex and remains unsatisfactory”. He recognised four subfamilies, the Opecoelinae Ozaki, 1925, Plagioporinae Manter, 1937, Stenakrinae Yamaguti, 1970 and Opecoelininae Gibson et Bray, 1984. These taxa are distinguished by the charac-

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ters of the male terminal genitalia and the female proximal genitalia. The Opecoelinae and Opecoelininae are characterised by a reduced or absent cirrus-sac, and are distinguished by the canalicular seminal receptacle being found only in the latter subfamily. The Plagioporinae and Stenakrinae share well-developed and muscular cirrus-sacs, and are differentiated by the presence of a canalicular seminal receptacle in the former. The family Opistholebetidae Fukui, 1929 has been considered close to the Opecoelidae and has been thought likely to be embedded within the Opecoelidae (Cribb 2005b).

This study is an attempt to use new and existing molecular data to assess the value of characters in the family and to present a preliminary phylogenetic estimate of the family.

## MATERIALS AND METHODS

Fourteen partial large subunit nuclear ribosomal RNA gene (*lsrDNA* = 28S rDNA) sequences (representing 13 species) and 13 almost complete small subunit nuclear ribosomal RNA gene (*ssrDNA* = 18S rDNA) sequences were newly generated (see Table 1) following the methodology outlined in Bray et al. (2012). These were aligned together with 29 published *lsrDNA* sequences (representing 28 species) and seven published *ssrDNA* sequences (representing seven species) using MAFFT v.6.611b (Kato et al. 2005) with 1000 cycles of iterative refinement and the *genafpair* algorithm. The alignment included four outgroup species representing the superfamilies Brachycladioidea (*Zalophotrema hepaticum*, *Stephanostomum pristis* [Deslongchamps, 1824]) and Lepocreadioidea Odhner, 1905 (*Preptetos caballeri* Pritchard, 1960, *Enenterum aureum* Linton, 1910). Outgroup choice was informed by trees based on published *lsrDNA* data from 556 species, representing 24 superfamilies and 97 families (see Littlewood et al. 2015). Alignments were examined by eye using Mesquite v.3.03 (Maddison and Maddison 2015). Ambiguously aligned positions were excluded manually. Alignments with indicated exclusion sets are available from the NHM Data Portal at <http://dx.doi.org/10.5519/0009364>. GenBank accession numbers for newly generated sequences are given in Table 1. Uncorrected p-distances were calculated using PAUP\* v4.0b10 (Swofford 2003).

MrModeltest v.2.3 (Nylander 2004) was used to select appropriate models of nucleotide substitution using the Akaike information criterion. Phylogenetic trees for partitions *lsrDNA*, *ssrDNA* and *lsr + ssrDNA* were constructed using Bayesian inference (BI) with MrBayes v.3.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Likelihood settings were set to nst = 6, rates = invgamma, ngammat = 4 (equivalent to the GTR + I +  $\Gamma$  model of nucleotide evolution). In the concatenated *lsr + ssrDNA* analyses model parameters were estimated separately for each partition. Four chains (temp = 0.2) were sampled every 1000 generations and run for 10000000 generations. 6000000 generations were discarded as 'burn-in', at which point the average standard deviation of split frequencies were <0.01. Nodes with <0.95 posterior probabilities (pp) were collapsed in the *lsr + ssrDNA* tree (Fig. 1). Trees in nexus format are available from the NHM Data Portal at <http://dx.doi.org/10.5519/0009364>.

## RESULTS

Of the aligned 1857 nucleotide positions in the *ssrDNA* alignment, 58 were excluded. Of the aligned 1364 nucleotide positions in the *lsrDNA* alignment, 137 were excluded. Thus the concatenated *lsr+ssrDNA* dataset consisted of 3221 positions, of which 195 were excluded (see NHM Data Portal at <http://dx.doi.org/10.5519/0009364> for alignments indicating exclusion sets). Because none of the strongly supported nodes in the individual gene partition trees (Figs. S1, S2) conflicted with the topology obtained from the concatenated *lsr+ssrDNA* dataset (Fig. 1), description of the results will be based on the latter.

Under our current taxon sampling, the monophyly of the Opecoelidae was well supported (1.00 pp). The earliest diverging clade was composed of two species of *Biospeodotrema* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014 from a deep sea (hydrothermal vent) teleost; these are recognised here as probable representatives of the subfamily Stenakrinae Yamaguti, 1970. The clade forming the sister group to the remaining opecoelids was composed of the genus *Helicometra* Odhner, 1902, represented here by three species.

The remainder of the tree was split into two well-supported clades, one composed of members of the subfamily Opecoelinae, and a group of deep-sea and freshwater 'plagioporines' (Plagioporinae in Fig. 1) in which the opecoeline *Buticulotrema thermichthysi* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014 nested. The other was composed of taxa mostly representing the subfamily Plagioporinae, consisting of three lineages whose relationships were unresolved. *Pseudopycnadena tendu* Bray et Justine, 2007 grouped separately from all other plagioporines as part of a polytomy including Clades A and B. Clade A consisted mostly of species of *Macvicaria*, which did not form a monophyletic group. However, sister-group relationships could be established for *M. mormyri* (Stossich, 1885) + *M. crassigula* (Linton, 1910) and *M. obovata* (Molin, 1859) + *M. maamouriae* Antar, Georgieva, Gargouri et Kostadinova, 2015. The recently described species *M. bartolii* Antar, Georgieva, Gargouri et Kostadinova, 2015, a replacement name for Mediterranean worms previously considered *M. crassigula*, took an unresolved position within Clade A. *Macvicaria dubia* (Stossich, 1905) also took an unresolved position in a subclade of Clade A, which amongst other *Macvicaria* representatives also included a clade composed of the opistholebetids, *Maculifer* sp. and *Opistholebes amplicoelus* Nicoll, 1915 and a well-supported but unresolved clade composed of *Gaevskajatrema perezi* (Mathias, 1926), *Propycnadenoides philippinensis* Fischthal et Kuntz, 1964 and *Peracreadium idoneum* (Nicoll, 1909).

Clade B was composed of a non-monophyletic *Allopodocotyle* Pritchard, 1966, where *Allopodocotyle* sp. A ex *Scolopsis bilineatus* (Bloch) formed the earliest diverging lineage and where *Allopodocotyle epinepheli* (Yamaguti, 1942) + *Allopodocotyle* sp. B ex *Epinephelus coioides* (Hamilton) were sister taxa grouping in an unresolved as-

**Table 1.** List of species and details of provenance and GenBank Numbers. Taxa are listed according to Fig. 1.

Superfamily Family	Parasite species	Host	Locality	28S Gen-Bank No.	18S Gen-Bank No.	References
Brachycladioidea						
Brachycladiidae	<i>Zalophotrema hepaticum</i> Stark et Alvey, 1929	<i>Zalophus californianus</i>	California, USA	AY222255	AJ224884	Cribb et al. (2001), Olson et al. (2003)
Acanthocolpidae	<i>Stephanostomum pristin</i> (Deslongchamps, 1824)	<i>Phycis phycis</i>	Scandola, Corsica	DQ248222	DQ248209	Bray et al. (2005)
Lepocreadioidea						
Lepocreadiidae	<i>Preptetos caballeri</i> Pritchard, 1960	<i>Naso vlamingii</i>	Heron Island	AY222236	AJ287563	Cribb et al. (2001), Olson et al. (2003)
Enenteridae	<i>Enenterum aureum</i> Linton, 1910	<i>Kyphosus vaigiensis</i>	Moorea, French Polynesia	AY222232	AY222124	Olson et al. (2003)
Opecoeloidea						
Opecoelidae	<i>Biospeedotrema jolliveti</i> Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014	<i>Ventichthys biospeedoi</i>	South East Pacific Rise	KF733988	KF733985	Bray et al. (2014)
Opecoelidae	<i>Biospeedotrema biospeedoi</i> Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014	<i>Thermichthys hollisi</i>	South East Pacific Rise	KF733986		Bray et al. (2014)
Opecoelidae	<i>Helicometra boseli</i> Nagaty, 1956	<i>Sargocentron spiniferum</i>	New Caledonia	KU320600	KU320587	Present study
Opecoelidae	<i>Helicometra epinepheli</i> Yamaguti, 1934	<i>Epinephelus fasciatus</i>	New Caledonia	KU320597	KU320584	Present study
Opecoelidae	<i>Helicometra manteri</i> Andres, Ray, Pulis, Curran et Overstreet, 2014	<i>Prionotus alatus</i>	Gulf of Mexico	KJ701238		Andres et al. (2014a)
Opecoelidae	<i>Helicometra manteri</i>	<i>Bellator egretta</i>	Gulf of Mexico	KJ701239		Andres et al. (2014a)
Opecoelidae	<i>Dimerosaccus oncorhynchi</i> (Eguchi, 1931)	<i>Salvelinus curilus</i>	Kedrovaya River, Russia	FR870262		Shedko et al. (2015)
Opecoelidae	<i>Anomalotrema koiae</i> Gibson et Bray, 1984	<i>Sebastes viviparus</i>	Shetland Islands	KU320595	KU320582	Present study
Opecoelidae	<i>Pseudopecoeloides tenuis</i> Yamaguti, 1940	<i>Priacanthus hamrur</i>	New Caledonia	KU320605	KU320592	Present study
Opecoelidae	<i>Opecoeloides furcatus</i> (Bremser in Rudolphi, 1819)	<i>Mullus surmuletus</i>	Corsica	AF151937		Tkach et al. (2001)
Opecoelidae	<i>Opecoeloides fimbriatus</i> (Linton, 1934)	<i>Micropogonias undulatus</i>	North Western Gulf of Mexico	KJ001211		Andres et al. (2014b)
Opecoelidae	<i>Bathycreadium brayi</i> Pérez-del-Olmo, Dallarés, Carrasón et Kostadinova, 2014*	<i>Trachyrincus scabrurus</i>	Mediterranean Spain	JN085948		Constenla et al. (2011)
Opecoelidae	<i>Plagiocirrus loboides</i> Curran, Overstreet et Tkach, 2007	<i>Fundulus nottii</i>	Mississippi, USA	EF523477		Curran et al. (2007)
Opecoelidae	<i>Podocotyloides brevis</i> Andres et Overstreet, 2013	<i>Conger esculentus</i>	off western Puerto Rico	KJ001212		Andres et al. (2014b)
Opecoelidae	<i>Neolebouria lanceolata</i> (Price, 1934)	<i>Polymixia lowei</i>	Eastern Gulf of Mexico	KJ001210		Andres et al. (2014b)
Opecoelidae	<i>Allopodocotyle margolisi</i> Gibson, 1995	<i>Coryphaenoides mediterraneus</i>	Rockall Trough	KU320596	KU320583	Present study
Opecoelidae	<i>Gaevskajatrema halosauropsi</i> Bray et Campbell, 1996	<i>Halosauropsis macrochir</i>	Goban Spur	AY222207	AJ287514	Cribb et al. (2001), Olson et al. (2003)
Opecoelidae	<i>Buticulotrema thermichthysi</i> Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014	<i>Thermichthys hollisi</i>	South East Pacific Rise	KF733987	KF733984	Bray et al. (2014)
Opecoelidae	<i>Pseudopycnadena tendu</i> Bray et Justine, 2007	<i>Pseudobalistes fuscus</i>	New Caledonia	FJ788506		Bray et al. (2009)
Opecoelidae	<i>Macvicaria bartolii</i> Antar, Georgieva, Gargouri et Kostadinova, 2015	<i>Diplodus annularis</i>	Bay of Bizerte, Tunisia	KR149465		Antar et al. (2015)
Opecoelidae	<i>Macvicaria mormyri</i> (Stossich, 1885)	<i>Lithognathus mormyrus</i>	Scandola, Corsica	AF184256		Tkach et al. (2001)
Opecoelidae	<i>Macvicaria crassigula</i> (Linton, 1910)	<i>Calamus bajonado</i>	Gulf of Mexico	KJ701237		Andres et al. (2014a)
Opecoelidae	<i>Macvicaria dubia</i> (Stossich, 1905)	<i>Oblada melanura</i>	Bay of Bizerte, Tunisia	KR149470		Antar et al. (2015)
Opistholebetidae	<i>Maculifer</i> sp.	<i>Diodon hystrix</i>	Heron Island	AY222211	AY222109	Olson et al. (2003)
Opistholebetidae	<i>Opistholebes amplicoeilus</i> Nicoll, 1915	<i>Tetractenos hamiltoni</i>	Stradbroke Island	AY222210	AJ287550	Cribb et al. (2001), Olson et al. (2003)
Opecoelidae	<i>Macvicaria obovata</i> (Molin, 1859)	<i>Gibbula adansonii</i>	Ebro Delta, Spain	JQ694146		Born-Torrijos et al. (2012)
Opecoelidae	<i>Macvicaria maamouriae</i> Antar, Georgieva, Gargouri et Kostadinova, 2015	<i>Lithognathus mormyrus</i>	Bizerte Lagoon, Tunisia	KR149468		Antar et al. (2015)

(continued.)

Table 1. Continued.

Superfamily Family	Parasite species	Host	Locality	28S Gen-Bank No.	18S Gen-Bank No.	References
Opecoeloidea						
Opecoelidae	<i>Gaevskajatrema perezii</i> (Mathias, 1926)	? <i>Symphodus</i>	Scandola, Corsica	AF184255		Tkach et al. (2001)
Opecoelidae	<i>Propycaenoides philippinensis</i> Fischthal et Kuntz, 1964	<i>Gymnocranius grandoculis</i>	New Caledonia	KU320604	KU320591	Present study
Opecoelidae	<i>Peracreadium idoneum</i> (Nicoll, 1909)	<i>Anarhichas lupus</i>	North Sea	AY222209	AJ287558	Cribb et al. (2001), Olson et al. (2003)
Opecoelidae	<i>Allopodocotyle</i> sp. A	<i>Scolopsis bilineatus</i>	New Caledonia	KU320599	KU320586	Present study
Opecoelidae	<i>Allopodocotyle epinepheli</i> (Yamaguti, 1942)	<i>Epinephelus cyanopodus</i>	New Caledonia	KU320598	KU320585	Present study
Opecoelidae	<i>Allopodocotyle</i> sp. B	<i>Epinephelus coioides</i>	Bali	KU320607	KU320606	Present study
Opecoelidae	<i>Pacificreadium serrani</i> (Nagaty et Abdel-Aal, 1962)	<i>Plectropomus leopardus</i>	New Caledonia	KU320602	KU320589	Present study
Opecoelidae	<i>Cainocreadium labracis</i> (Dujardin, 1845)	<i>Gibbula adansonii</i>	Ebro Delta, Spain	JQ694144		Born-Torrijos et al. (2012)
Opecoelidae	<i>Cainocreadium lintoni</i> (Siddiqi et Cable, 1960)	<i>Epinephelus morio</i>	Off northern Virgin Islands	KJ001208		Andres et al. (2014b)
Opecoelidae	<i>Bentholebouria blatta</i> (Bray et Justine, 2009)	<i>Pristipomoides argyrogrammicus</i>	New Caledonia	KU320608		Present study
Opecoelidae	<i>Bentholebouria blatta</i>	<i>Pristipomoides argyrogrammicus</i>	New Caledonia	KU320606	KU320593	Present study
Opecoelidae	<i>Bentholebouria colubrosa</i> Andres, Pulis et Overstreet, 2014	<i>Pristipomoides aquilonaris</i>	Eastern Gulf of Mexico	KJ001207		Andres et al. (2014b)
Opecoelidae	<i>Macvicaria macassarensis</i> (Yamaguti, 1952)	<i>Lethrinus miniatus</i>	Heron Island	AY222208	AJ287533	Cribb et al. (2001), Olson et al. (2003)
Opecoelidae	<i>Hamacreadium 'mutabile'</i>	<i>Lutjanus fulviflamma</i>	New Caledonia	KU320601	KU320588	Present study
Opecoelidae	<i>Hamacreadium</i> sp.	<i>Lethrinus miniatus</i>	New Caledonia	KU320603	KU320590	Present study
Opecoelidae	<i>Hamacreadium mutabile</i> Linton, 1910	<i>Lutjanus griseus</i>	Eastern Gulf of Mexico	KJ001209		Andres et al. (2014b)

\* this species was identified as *Bathycreadium elongatum* by Constenla et al. (2011), but Pérez-del-Olmo et al. (2014) re-identified the material.

semblage together with (*Pacificreadium serrani* (Nagaty et Abdel-Aal, 1962) (*Cainocreadium labracis* (Dujardin, 1845), *Cainocreadium lintoni* (Siddiqi et Cable, 1960))), (*Bentholebouria blatta* (Bray et Justine, 2009), *Bentholebouria colubrosa* Andres, Pulis et Overstreet, 2014) and (*Macvicaria macassarensis* (Yamaguti, 1952) (*Hamacreadium 'mutabile'*, *Hamacreadium mutabile* Linton, 1910, *Hamacreadium* sp.)).

## DISCUSSION

### Monophyly of the Opecoelidae

The monophyly of the family is convincingly demonstrated, with good support. The variety of types of terminal genitalia and proximal female genitalia discussed below all appear to be derived within the family.

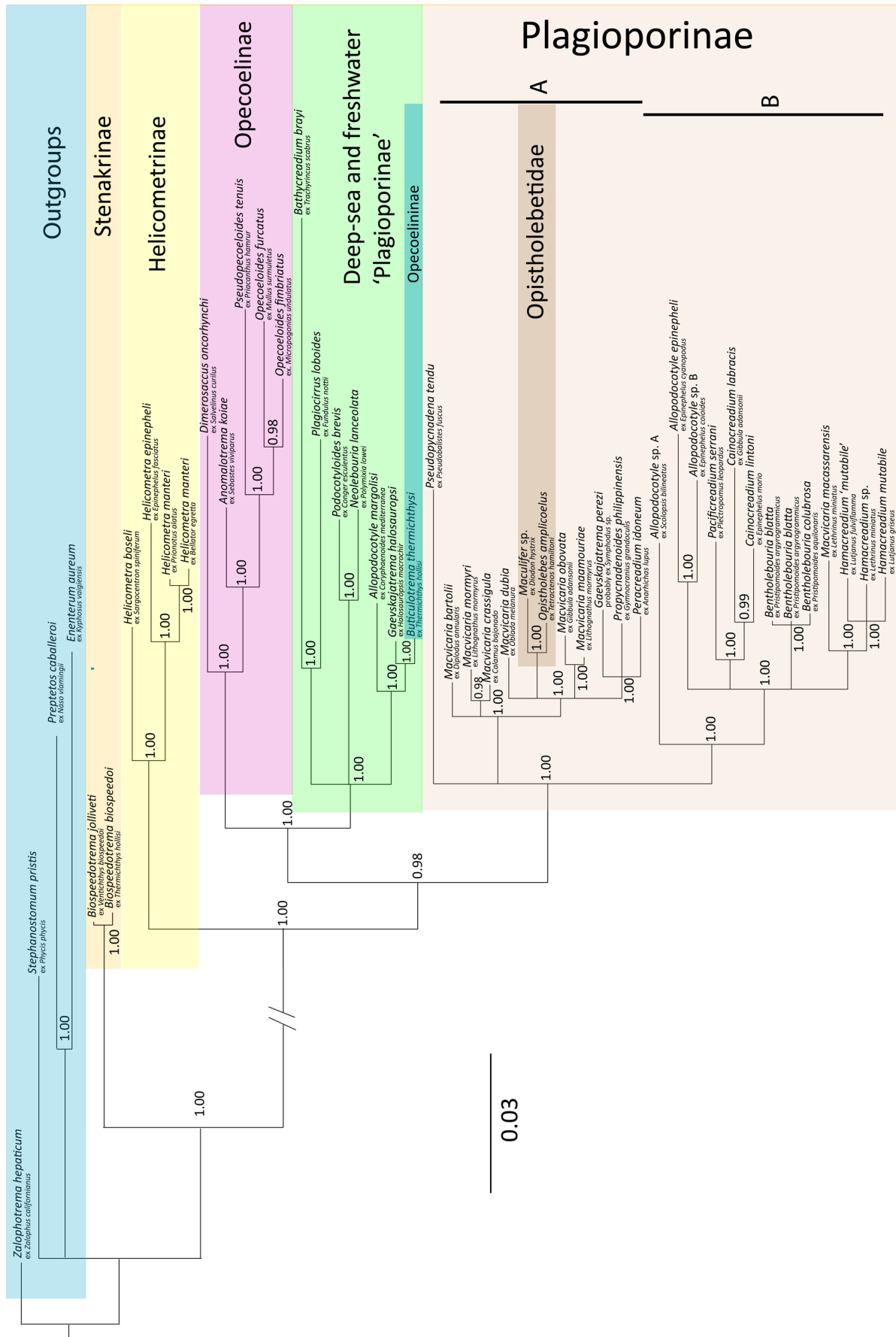
### Subfamily Stenakrinae

Gibson and Bray (1982) defined the subfamily as “forms possessing both a distinct cirrus-sac, containing an internal seminal vesicle, and a uterine seminal receptacle, but lacking both a naked seminal vesicle and a distinct canalicular seminal receptacle” and then later (Gibson and Bray 1984) they suggested that these characteristics “would appear to be the most primitive” in the family. The well-supported position of the putative stenakrines, *Biospeedotrema* spp., as the earliest diverging lineage adds some weight to this view. The long branch separating this subfamily from the remainder of the Opecoelidae may indicate that this taxon

should be at the family rank, but unfortunately, no molecular data of species of *Stenakron* Stafford, 1904. are yet available. Whilst the position of *Biospeedotrema* is well supported in the analyses of *ssrDNA* (Fig. S1) and the concatenated dataset of *lsr+ssrDNA* (Fig. 1), in the *lsrDNA* tree the support is low (0.67 pp; Fig. S2). Other evidence, such as the tree produced by Shedko et al. (2015), lends support to the possibility that *Biospeedotrema* is not closely related to the Opecoelidae.

### Helicometra

Although previous phylogenetic studies based on ITS ribosomal DNA (Jousson et al. 1999, Born-Torrijos et al. 2012, Barnett et al. 2014) provided conflicting and ambiguous results with regard to the position of *Helicometra*, our topology clearly places the genus *Helicometra* as sister group to the Opecoelinae and Plagioporinae. This reinforces the view that ITS sequences are clearly more useful for species distinction than higher level phylogeny (Nolan and Cribb 2005). As far as we are aware, no one has seen fit to recognise this genus (which has hitherto been considered a plagioporine along with its close relatives *Helicometrina* Linton, 1910 and *Neohelicometra* Siddiqi et Cable, 1960), as a separate, named taxon. In addition to its distinct phylogenetic position, this group is also characterised by striking morphological characters, i.e. the filamented eggs and the helical uterus. We, therefore, feel justified in erecting a new subfamily for *Helicometra* and morphologically similar worms.



**Fig. 1.** Bayesian analysis of the concatenated *lsr* + *ssr* DNA dataset constructed using MrBayes v3.2 under the GTR + I + G model; 10 000 000 generations, 6 000 000 generations 'burn-in'. All nodes with < 0.95 posterior probability have been collapsed. The branch length scale indicates number of substitutions per site. The length of the truncated branch is 0.11.

### Subfamily Helicometrinae subfam. n.

ZooBank number for subfamily:

urn:lsid:zoobank.org:act:32FC2816-B7CE-42B5-B34A-9E54B9DBD152

**Diagnosis.** Opecoelidae. Body oval to elongate, with almost parallel margins, rounded posteriorly. Oral sucker unspecialised or distinctly funnel-shaped. Ventral sucker unspecialised, larger or smaller than oral sucker. Caeca blind, extending close to posterior end of body, or form ani. Testes two, or several (3–9), usually deeply lobed, occasionally almost entire, tandem to oblique; well separated from posterior end of body. Genital pore median or slightly submedian, bifurcal to pre-bifurcal. Ovary usually distinctly lobed, occasionally almost entire. Uterus distinctly helical, restricted to area between ovary and anterior testis and genital pore. Eggs with unipolar filaments. Vitelline follicles may enter forebody or be restricted to hindbody, extend posteriorly beyond testes to posterior end of body. Excretory vesicle extends to ovary. In many marine fish families; cosmopolitan.

Type genus: *Helicometra* Odhner, 1902 (syns. *Stenopera* Manter, 1933, *Allosthenopera* Baeva, 1968, subgenus *Metahelicometra* Yamaguti, 1971). Other genera included in this subfamily due to their morphological similarity: *Helicometrina* Linton, 1910, *Neohelicometra* Siddiqi et Cable, 1960.

*Helicometra boseli* Nagaty, 1956 is here segregated as sister to *H. epinepheli* Yamaguti, 1934 + *H. manteri* Andres, Ray, Pulis, Curran et Overstreet, 2014. It is morphologically distinct from typical *Helicometra* spp. and Bray and Justine (2014), in discussing specimens from the same batch as that sequenced, considered that it may be appropriate to resurrect *Stenopera* Manter, 1933, to accommodate this and similar worms.

Interestingly, the two specimens of *H. manteri* which occurred in different hosts, *Prionotus alatus* Goode et Bean and *Bellator egretta* (Goode et Bean), in the Gulf of Mexico, differed by 7 nucleotides across the ribosomal RNA array (2433 bp) sequenced by Andres et al. (2014a) (see also branch lengths in Fig. 1 and Fig. S2). Although this amounts to only 0.3% difference, it may indicate that *H. manteri* may either be a complex of closely related species or an unusually genetically diverse population. Andres et al. (2014a) did note that *H. manteri* from *Bellator* spp. had ‘a slightly more prominent lobed ovary than specimens recovered from *P. alatus*’ (see also Figs. 1–3 in Andres et al. 2014a), but did not consider this sufficient evidence to separate them into discrete species. More elaborate morphological and molecular study of *H. manteri* across its host range ought to shed light on this issue.

Filamented eggs also occur in members of the plagioporine genus *Diplobulbus* Yamaguti, 1934, but it is not included here in the Helicometrinae as the filaments occur on both poles of the egg, the uterus reaches to the posterior extremity of the body and is not helical, and its ITS2 sequence places it distant from *Helicometra* (see Barnett et al. 2014).

### Remaining Opecoelidae

The remaining Opecoelidae form a monophyletic clade which is subdivided into two major clades: (Opecoelinae + deep sea and freshwater ‘Plagioporinae’ + opecoelinine *Buticulotrema thermichthysi*) and (Plagioporinae with Opistholebetidae nested). Frustratingly, no morphological synapomorphies are apparent for these disparate groups.

### Opecoelinae

This is a well-supported group, both in terms of molecular and morphological evidence. The subfamily Opecoelinae is characterised by possessing both a naked seminal vesicle and a uterine seminal receptacle, but lacking both a distinct cirrus-sac containing an internal seminal vesicle (a vestige of a cirrus-sac is present in some genera including the type-genus *Opecoelus*), and a canalicular seminal receptacle (Gibson and Bray 1982). The five species studied here all show these, apparently derived, characters. The subfamily is also characterised by a variety of posterior caecal arrangements. *Dimerosaccus oncorhynchi* (Eguchi, 1931), which forms the sister taxon to the remaining opecoelines, has typical digenean blind caecal endings (Shimazu 1980, Shedko et al. 2015). *Anomalotrema koi-ae* Gibson et Bray, 1984, which forms the sister taxon to *Opecoeloides* spp. and *Pseudopecoeloides tenuis* Yamaguti, 1940, has caeca which open separately ventrally to the excretory pore (Gibson and Bray 1984). The remaining three species, *Pseudopecoeloides tenuis*, *Opecoeloides furcatus* (Bremser in Rudolphi, 1819) and *O. fimbriatus* (Linton, 1934), share the same caecal endings, i.e. an uroproct, where the posterior caecal extremities open into the posterior part of the excretory vesicle (Yamaguti 1940, Sogandares-Bernal and Hutton 1959, Jousson and Bartoli 2000). Sogandares-Bernal and Hutton (1959) reckoned that “(t)he presence of 2 ani or of a uroproct is dependent upon the contraction of the posterior end of the body in our specimens” and stated: “We believe that when the uroproct is formed very close to the posterior end of the body it does not serve as a good generic character.” However, in most well-fixed specimens, and unless occluded by vitelline follicles, it is usually possible to distinguish these features. The Opecoelinae appears to be the most robust opecoelid subfamily of those presently recognised (see also Barnett et al. 2014) and there is no need for change.

### Deep-sea and freshwater ‘Plagioporinae’ + *Buticulotrema* (Opecoelininae)

Seven species constitute the sister clade to the Opecoelinae, five of which are deep-sea plagioporine-like worms, one is a freshwater species and one, confusingly, is a hydrothermal vent ‘opecoelinine’. This clade is strongly supported (1.00 pp), but the relationships of the three well-supported internal lineages are not resolved.

Although well supported (1.00 pp), the clade including *Bathycradium brayi* Pérez-del-Olmo, Dallarés, Carrassón et Kostadinova, 2014 and *Plagiocirrus loboides* Curran, Overstreet et Tkach, 2007 is puzzling. *Bathycradium brayi* was collected from 600–647 m depth in the Mediterranean

Sea (Pérez-del-Olmo et al. 2014), in contrast to the freshwater form *P. lobooides* from the Pascagoula River in Mississippi (Curran et al. 2007). It should be noted, however, that the branch-length to both species is long. There are no apparent synapomorphies for these two worms. It is likely that the type-genus of the subfamily Plagioporinae, i.e. *Plagioporus*, now considered a freshwater genus, will be in this clade. The clade consisting of *Podocotyloides brevis* Andres et Overstreet, 2013 from 200 m depth (Andres and Overstreet 2013) and *Neolebouria lanceolata* (Price, 1934) from 329–430 m (Andres et al. 2014b), both in the Gulf of Mexico/Puerto Rico region, is biologically reasonable, although, apart from being typical ‘plagioporines’, they share no obvious morphological synapomorphy (Price 1934).

The third clade (1.00 pp) includes three deep-sea forms: *Allopodocotyle margolisi* Gibson, 1995 from 1 745–2 220 m depth from the Rockall Trough off NW Scotland (Gibson 1995), *Gaevskajatrema halosauropsi* Bray et Campbell, 1996 at 2 570 m depth from the Goban Spur, NE Atlantic Ocean (new depth data) and *Buticulotrema thermichthysi* from 2 598 m depth from a hydrothermal vent site on the South East Pacific Rise (Bray et al. 2014). The placement of *B. thermichthysi* in this position is perplexing. Bray et al. (2014) stated that the material of the hydrothermal vent worms they described had endured suboptimal fixation, but the study of wholmounts and sections show fairly unambiguously that the cirrus-sac is missing in this species. The seminal receptacle is described as saccular, presumably canalicular, which is the arrangement found in the closest relatives in the tree.

### The main Plagioporinae clade

#### Separate position of *Pseudopycnadena*

*Pseudopycnadena tendu*, a robust, oval species from a coral reef fish off New Caledonia (Bray and Justine 2007), grouped separately from the remaining members of this clade. It is rather atypical morphologically, with its broadly oval cirrus-sac containing a massive field of large gland-cells, and the annular ridge on the ventral surface which, it is presumed, functions as an accessory attachment organ. Nevertheless, in general, the terminal genitalia and proximal female system conform to the normal plagioprine pattern.

### Clade A

#### Non-monophyly of *Macvicaria*

The species of *Macvicaria* in clade A parasitise sparid fishes (Linton 1910, Bartoli et al. 1993, Antar et al. 2015) in the western Mediterranean Sea and the Gulf of Mexico, but they do not constitute a monophyletic group. *Macvicaria obovata* and *M. maamouriae* appear as sister taxa and, although they are similar species and are both found in sparids in the Mediterranean Sea, they have no obvious synapomorphies (Bartoli et al. 1989, Antar et al. 2015). The *M. obovata* sequence is derived from parthenitae in

a snail, but the identification has been verified by ITS comparison by Born-Torrijos et al. (2012).

*Macvicaria crassigula* and *M. mormyri* also appear as sister taxa. The former species was originally described off the Dry Tortugas, Florida (Linton 1910) and the sequence is from a worm from the Gulf of Mexico and from a congener of the type-host, whereas *M. mormyri* is a Mediterranean worm (Bartoli et al. 1993). Although reported many times in both the Mediterranean and the Gulf of Mexico region, *M. crassigula* has never been described in detail. It is likely that it does not occur in the Mediterranean Sea, or at least some of the Mediterranean records are of *M. bartolii* (see Antar et al. 2015). No obvious synapomorphies unite *M. mormyri* and *M. crassigula*. *Macvicaria macassarensis*, from lethrinids and nested in clade B, is clearly only distantly related to the sparid parasites.

### More comments on Clade A

The monophyletic clade composed of *Peracreadium idoneum*, *Propycnadenoides philippinensis* and *Gaevskajatrema perezi* is not characterised by any obvious synapomorphies or distributional or host similarities. All are fairly typical plagioporines. *Peracreadium idoneum* is a north Atlantic species found in wolffishes (*Anarhichas* spp., Anarhichadidae) (Nicoll 1909, Bray 1987), *P. philippinensis* is found in large-eye bream (*Gymnocranius* spp., Lethrinidae) in the western Pacific Ocean (Bray and Cribb 1989) and *Gaevskajatrema perezi* is a poorly known species from labrids supposedly found in the north-eastern Atlantic, Mediterranean and Black Seas (Gibson and Bray 1982). Provenance data on the specimen of *G. perezi* are vague, particularly in relation to host.

The nesting of the two opistholebetids within a clade of plagioporines poses a taxonomic challenge. Both are from the coast of Queensland and bear similar morphological and biological characteristics. *Opistholebes amplicoeus* is known only from tetraodontids from this region. Cribb (2005b) considered that there were four distinguishing features for the family Opistholebetidae: the posterior position of the ventral sucker, the presence of a post-oral (muscular) ring, the presence of pigment granules and the parasitism in diodontid and tetraodontid fishes. None of these features occur in the related plagioporines. The position of the opistholebetids identified here, nested deep among typical opecoelids, makes the retention of family-level recognition for the group untenable. However, the biological and morphological distinctness of the group suggest to us that it should continue to be recognised in a supra-generic taxon, in this case the subfamily Opistholebetinae Fukui, 1929, stat. emend.

### Clade B

The worms in Clade B are all typical plagioporines and in many cases are difficult to distinguish. An isolated undescribed *Allopodocotyle* sp. A from a nemipterid fish is the sister to the remaining internal clades, which form a 4-way polytomy. Two species of *Allopodocotyle* from *Epinephelus* spp. in the Indo-West Pacific Region form

a well-supported clade. These are morphologically practically indistinguishable, but clearly distinct genetically. The position of *Allopodocotyle margolisi* in the other major opecoelid clade, along with other deep-sea fish parasites, indicates that some convergence appears to have occurred. *Allopodocotyle margolisi* is the only deep-sea member of the nominal genus and is genuinely deep-sea, having been found at depth between 1700–3500 m off the west coast of Scotland and at the Mid-Atlantic Ridge (Gibson 1995, Kellermanns et al. 2009).

The two closely related species of *Bentholebouria* Andres, Pulis et Overstreet, 2014 formed a well-supported clade. *Bentholebouria blatta* was represented by two identical sequences from worms collected from the same host and locality. Both species are found in relatively deep-water lutjanids of the genus *Pristipomoides* Bleeker (see Bray and Justine 2009, Andres et al. 2014b). Intriguingly and surprisingly, the *lsrDNA* sequence of *B. colubrosa* from the Gulf of Mexico differed by only two nucleotide positions, i.e. 0.016%, from that of *B. blatta* from New Caledonia.

The final clade in B includes four morphologically similar species, three of them belonging to the genus *Hamacreadium* Linton, 1910. The sister to *Hamacreadium* spp. is *Macvicaria macassarensis*, which is from the same host, *Lethrinus miniatus* (Forster), as *Hamacreadium* sp. and is placed in *Macvicaria* based on the entire, rather than lobed ovary (Bray and Cribb 1989, Cribb 2005a). It also differs from *Hamacreadium* spp. in the excretory vesicle, which reaches to the posterior edge of the ventral sucker (Yamaguti 1952, Bray and Cribb 1989), but not into the forebody as is characteristic of *Hamacreadium*. *Hamacreadium mutabile* is a widely reported parasite mainly of lutjanid fishes, and our worms from New Caledonia have been identified as this species (Justine et al. 2012). Morphologically it is similar to the Gulf of Mexico form, but molecular evidence from *lsrDNA* suggests that *H. 'mutabile'* and *H. mutabile* are as distinct from each other, as *Hamacreadium* sp. and *H. 'mutabile'* are from each other, i.e. 0.2% difference in pairwise sequence comparisons. The Gulf of Mexico *H. mutabile* material is from the type host in the eastern Gulf of Mexico, close to the type locality of Dry Tortugas, Florida (Linton 1910, Andres et al. 2014a). The relationships amongst the species of *Hamacreadium* are not resolved.

### Type species

The following species are the type species of their respective genera: *Biospeedotrema jolliveti* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014, *Dimerosaccus oncorhynchi*, *Pseudopecoeloides tenuis*, *Opecoeloides furcatus*, *Propychnadenoides philippinensis*, *Gaevskajatrema perezii*, *Opistholebes amplicoeelus*, *Bentholebouria colubrosa*, *Pacificreadium serrani*, *Cainocreadium labracis* and *Hamacreadium mutabile*. If these are correctly identified, then they can be taken as genuine representatives of the genera in question.

### Generic polyphyly

Of the nine genera represented by more than one species, six appear monophyletic: *Biospeedotrema* (2 spe-

cies), *Helicometra* (3), *Opecoeloides* Odhner, 1928 (2), *Bentholebouria* (2), *Cainocreadium* Nicoll, 1909 (2), *Hamacreadium* (3). On the other hand, three are polyphyletic: *Allopodocotyle* (4), *Gaevskajatrema* Gibson et Bray, 1982 (2), *Macvicaria* (7). The four species of *Allopodocotyle* appear in three places in the tree and the seven *Macvicaria* species appear in five places. There are about 13 nominal species in *Allopodocotyle* and 51 in *Macvicaria*. *Macvicaria* and *Allopodocotyle* in particular are considered particularly unsatisfactory genera, with dubious, general, pleiomorphic defining characteristics, such as details of the vitelline distribution and the shape of the ovary.

### Morphological characters

The morphological differentiating characters were discussed in detail by Cribb (2005a). Here we comment on these characters in the light of our molecular tree.

**Body shape:** This is a difficult character to define as it is a continuum. Four opecoelines are elongate and as they are monophyletic it might be considered a useful character. Some of the other worms are also on the border-line of this character state, e.g. *Bathycreadium brayi* and *Podocotyloides brevis*, but it does appear to be the case that really elongate worms are most common in the Opecoelinae.

**Tegument:** Occasional spines, usually absent. None of the species studied have tegumental spines.

**Oral sucker:** All species studied have an oval subterminal oral sucker.

**Ventral sucker:** All species but one have an unelaborated ventral sucker. The ventral sucker of *Propychnadenoides philippinensis* has muscular lamellar lips.

**Ventral sucker peduncle:** This character is found in a monophyletic group of opecoelines (*Pseudopecoeloides* Yamaguti, 1940 and *Opecoeloides*). One plagioporine species, *Podocotyloides brevis*, also has this character.

**Ventral sucker position:** One species, *Opistholebes amplicoeelus*, actually has a posteriorly situated ventral sucker. It was this character that led, in part, to the previous recognition of the Opistholebetidae.

**'Accessory sucker':** This appears as an autapomorphy of the genus *Opecoeloides*.

**Gut length:** Several species scattered in the tree have relatively short caeca, i.e. not reaching significantly into the post-testicular region. The only combination of taxa for which this might be a synapomorphy is the two *Biospeedotrema* spp.

**Gut termination:** Blind caeca are by far the most common arrangement. Two ani occur in *Anomalotrema koiae*; a uroproct occurs in *Pseudopecoeloides* and *Opecoeloides*. As discussed above it appears that these states may be a progression. These appear to be a useful characters and, in our tree, occur only in the opecoelines. *Bathycreadium brayi* has a cyclocoel.

**Excretory vesicle:** A long excretory vesicle extending into the forebody is a characteristic of *Hamacreadium* species, but it also occurs in *Pacificreadium serrani*, where it becomes distinctively diverticulate. In general, excretory vesicle length appears to be an informative character, although difficult to define in some cases. A very short excre-



tory vesicle is found in *Biospeedotrema* and *Opistholebes* Nicoll, 1915 and is probably a useful generic character, but with considerable homoplasy.

Excretory pore: In two species, *Pseudopycnadena tendu* and *Opistholebes amplicolus*, the excretory pore is displaced onto the dorsal surface. These species are not particularly closely related. It seems likely that this character is of some value, as has been found in the Lepocreadiidae (see Bray 2005), but our sample of worms with this character is too small to be informative.

Testis number: All species studied have two testes.

Testis arrangement: Tandem and symmetrical testes arrangements are scattered amongst the species in the tree. In two cases symmetrical testes may be a synapomorphy for a clade, i.e. the two *Biospeedotrema* spp. and (*Gaevskajatrema halosauropsi* + *Buticulotrema thermichthysi*). The condition may be of value, but tends to homoplasy.

Testis shape: Species with entire or lobate testes are found throughout the tree. Lobed testes are found in all three *Helicometra* species sampled, although this state is not invariant in the genus (Sekerak and Arai 1974).

Cirrus-sac presence: Significant reduction in the cirrus-sac is a good character defining the Opecoelinae. The reduction may be in the size where, as in *Dimerosaccus* Shimazu, 1980, the cirrus-sac now encloses only a small distal portion of the male terminal genitalia. On the other hand, it may be, as in *Anomalotrema* Zhukov, 1957, reduced to a thin membrane. Ultimately, the cirrus-sac may be completely absent, as in the monophyletic group (*Pseudopecoeloides* + *Opecoeloides*). Parallel complete reduction in the cirrus-sac appears to have occurred in *Buticulotrema thermichthysi*.

Genital pore: This may be submedian or median. This seemingly slight difference, in fact, appears to be important even if prone to homoplasy. Several monophyletic groups have a median genital pore, e.g. *Biospeedotrema* spp., *Helicometra* spp., 'Opistholebetidae' and *Pacificreadium* Durio et Manter, 1968 + *Cainocreadium*. An isolated case is *Peracreadium idoneum*.

Ovary: Presence or absence of lobation of the ovary may be of some value, although to be utilised with caution. It is exhibited by the monophyletic groups *Hamacreadium* spp., *Opecoeloides* spp., *Bentholebouria* spp., *Pacificreadium* + *Cainocreadium* and *Hamacreadium* spp. Elsewhere in the tree this character appears scattered in isolated species.

Vitellarium anterior extent: In all the opecoeline species and (*Allopodocotyle epinepheli* + *Allopodocotyle* sp. B ex *Epinephelus coioides*) the vitellarium does not reach into the forebody. Elsewhere the character occurs sporadically. This character has often, but not invariably, been used as a generic character. It is not always reliable, even as a specific character (as in *Opecoelus variabilis* Cribb, 1985, see Cribb 1985).

Vitellarium posterior extent: The vitellarium does not extend posteriorly beyond the testes in the Stenakrinae and *Gaevskajatrema*. This appears to be a good character, but with some homoplasy.

Egg size: Members of the genera *Choerodonicola* Cribb, 2005 and *Diplobulbus* have relatively tiny eggs (< 32 µm long). No species with eggs of this size were sampled.

Egg filament: A unipolar filament is a good character, a synapomorphy of the Helicometrinae. Bipolar filaments, which occurs only in *Diplobulbus*, do not occur amongst the species in our sample.

There are thus remarkably few useful morphological characters available to distinguish a large number of taxa. There clearly is substantial homoplasy, and uncertainty about the nature of the basal condition, in the characters that we do have. It therefore comes as no surprise that molecular phylogenetic analysis shows our current classification to be seriously deficient.

### Concluding remarks

It seems obvious that the present subfamily structure of the Opecoelidae is quite unsatisfactory. Given the incomplete molecular data set that is presently available for analysis, it is clear that we should step warily in proposing change. Two issues arise. How important is it to recognise subfamilial taxa and what is presently plausible? For a taxon comprising so many genera and species, we consider it desirable that subfamilies are recognised if and as they become convincing and informative. Some clades recognised here are morphologically and (in some cases) biologically distinct and their recognition at the subfamily level seems useful. In this category we consider that the Opecoelinae and Stenakrinae, as presently recognised, show signs of remaining useful and robust taxa.

We argue, in addition, that the genus *Helicometra* and its relatives are now unarguably distinct from all other opecoelid clades and that it is appropriate to recognise a subfamily for them. Of the remaining taxa, we conclude that the Plagioporinae can now be considered no more than a work-in-progress which will ultimately require the recognition of multiple subfamilies. In our view, these certainly cannot yet be distinguished reliably, especially in the absence of sequence information for the type-genus *Plagioporus*. However, we do observe that the close relationship of two opistholebetid genera to taxa in one minor clade of 'plagioporines' renders it no longer defensible to recognise the Opistholebetidae as a distinct family. As a concept, this taxon is appealing given the combination of its relatively distinct morphology and narrow host range. We propose that it should now be recognised at the subfamily level.

Such recognition implies that ultimately we may need to recognise many further subfamilies of opecoelids; on the basis of the topology discussed here it could easily be as many as ten. However, for a taxon as rich and complex as the Opecoelidae this should come as no real surprise. We thus can look forward to the challenge that lies ahead of the combined recognition of the clades of opecoelids and the understanding of their biological and evolutionary basis, confused as it is by conservative morphology and rampant homoplasy.

The study has also shown that the dissatisfaction with the generic boundaries voiced by many earlier authors (see

Cribb 2005a) is justified. One third of the genera represented by more than one species are polyphyletic. Not only are they polyphyletic, but their constituents are well separated in the tree.

This study has contributed significantly to our understanding of the value of morphological characters in opecoelid systematics. The characters separating the Opecoelinae, namely the reduced or absent cirrus-sac and the uterine seminal receptacle, seem in this case to be highly informative, but these characters, separately, are found elsewhere in the tree. The reduced cirrus-sac of *Buticulotrema thermichthysi*, the only putative representative of the subfamily Opecoelininae (see Gibson and Bray 1984, Bray et al. 2014), suggests that this character is homoplasious and that this subfamily is embedded within a plagioporo-

rine-like clade. The uterine seminal receptacle found in the Stenakrinae, represented here by the two species of *Biospeedotrema*, similarly appears to be convergent. The lack of samples from the type genera of these two subfamilies is to be regretted and emphasises the need for much further work. Nevertheless, this exercise has given us many useful insights into the phylogeny and systematics of this difficult group.

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