

The plerocercus of *Ditrachybothridium macrocephalum* Rees, 1959 from two deep-sea elasmobranchs, with a molecular analysis of its position within the order Diphyllidea and a checklist of the hosts of larval diphyllideans

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Abstract

New collections were made of *Ditrachybothridium macrocephalum* Rees, 1959 from the deep-sea elasmobranchs *Apristurus laurussonii* (Saemundsson) and cf. *Rajella bigelowi* (Stehmann) in the northern Atlantic Ocean, allowing studies of the morphology of its plerocercus. The material has also allowed an estimate of its position within the Diphyllidea inferred from molecular data for the first time. The plerocercus is withdrawn, but not invaginated, within a cyst with a thick vacuolated wall. The encysted strobila is distinct, with several putative segments and advanced genital anlagen, and the scolex is fully developed with a distinct apical pad. 28S rDNA (D1-D3 regions) sequences were generated from both encysted and excysted *D. macrocephalum*, as well as from other diphyllidean and pseudodiphyllidean (outgroup) taxa for use in phylogenetic analyses. Maximum parsimony and Bayesian inference analyses both showed that the diphyllidean genus *Echinobothrium* is paraphyletic without inclusion of the only two other diphyllidean genera, *Ditrachybothridium* Rees, 1959 and *Macrobothridium* Khalil & Abdul-Salam, 1989. A list of published records of larval diphyllideans, their hosts and localities is included.

Introduction

The diphyllidean genus *Ditrachybothridium* was established by Rees (1959) for *D. macrocephalum* Rees, 1959 and elevated to family status (Ditrachybothridiidae) by Schmidt (1970). As far as we are aware, no publication has reported more recent collections of this unusual diphyllidean genus (the report by Williams, 1960, is based on the same material that was described by Rees), which remained monotypic until Faliex et al. (2000) described *D. piliformis* Faliex, Tyler & Euzet, 2000 from the southern Pacific Ocean. The collection of new material of *D. macrocephalum* has allowed further examination of the morphology of its plerocercus and represents the first opportunity to examine its phylogenetic position within the Diphyllidea using molecular data.

In the recent review of the 'larval stages or metacestodes' of cestodes, Chervy (2002) stated that the larval stage of members of the order Diphyllidea should be considered a 'plerocercus'. Beveridge

(2001) could find no record of a complete diphyllidean life-cycle having been published, but he did point out that observations have been made on the larval stages of species of *Echinobothrium* van Beneden, 1849 (see Table 1), and it is clear that they occur in various organs of marine crustaceans and molluscs. The structure is most clearly illustrated by Ruzskowski (1928) and Ramadevi & Rao (1974), but other useful morphological observations are found in the figures and/or descriptions published by Lespés (1857), Leuckart & Pagenstecher (1858), Anantaraman (1963), Dollfus (1964), Shimazu (1975), Cake (1976), Shimazu (1982) and Jones & Beveridge (2001). It has been clear since the description of Lespés (1857) that the scolex is not invaginated, but retracted in the cyst, and since Leuckart & Pagenstecher (1858) that a distinct strobila with evidence of segmentation may be present. Observations presented here make it clear that the general arrangement in *Ditrachybothridium* is similar to that in *Echinobothrium*, and this similarity is reinforced by new molecular data.

Materials and methods

Collections and microscopy

New collections were made aboard the RRS *Discovery* in October, 2002. Fishes were caught with a semi-balloon otter trawl and were dead on arrival on deck. They were dissected under a dissecting microscope and fixed and preserved in 100% molecular grade ethanol or fixed in Berland's fluid and preserved in 80% ethanol. Tissue samples of the hosts were also preserved in 100% molecular grade ethanol and digital photographs of the hosts taken. Specimens were stained with Mayer's paracarmine, cleared in beechwood creosote and mounted in Canada balsam. Measurements were made through a drawing tube on an Olympus BH-2 microscope, using a Digicad Plus digitising tablet and Zeiss KS100 software adapted by Imaging Associates, and are quoted in micrometres as the range followed by the mean in parentheses. Bright field, polarised light, and differential interference contrast images were captured using a Leica DM 5000B microscope and 480 digital camera. The following abbreviation is used: BMNH, the British Museum (Natural History) Collection at The Natural History Museum, London, UK.

Molecular analyses

Separate genomic DNA (gDNA) extractions of *D. macrocephalum* were made from a portion of one excysted worm from cf. *Rajella bigelowi* (BMNH 2004.1.6.6-11) and from an entire encysted worm (manually removed from its cyst) from *Apristurus laurussonii* (BMNH 2004.1.6.1-5). In addition, gDNA was extracted from three diphyllidean species: *Echinobothrium* cf. *bonasum* Williams & Campbell, 1980 (BMNH 2004.2.18.44; 2004.3.18.1-4) collected from *Rhinoptera bonasus* from the Gulf of California, Baja, Mexico; *Echinobothrium* sp. (2 individuals; BMNH 2003.3.6.23-28; 31-34) collected from *Raja* spp., north Atlantic Ocean; and *Macrobothridium rhynchobati* Khalil & Abdul-Salam, 1989 (BMNH 2004.18.3.101) from *Rhinobatos typus* collected from Yorkey's Knob., Queensland, Australia. For outgroup comparison, gDNA was extracted from a non-diphyllid pseudophyllidean, *Marsipometra hastata* (Linton, 1897) Cooper, 1917 (BMNH 2004.4.13.1) from *Polyodon spathula* collected in Mississippi, USA. Extractions were used to amplify the D1-D3 regions (c.1,400 nucleotides) of the nuclear large subunit ribosomal DNA (28S rDNA) which has been shown to be informative in cestodes for

both diagnostic (Brickle et al., 2001; Reyda & Olson, 2003) and phylogenetic (Olson et al., 2001, 2003) purposes. PCR and direct cycle-sequencing of PCR products were performed as described by Olson et al. (2003). New sequences were assembled and edited using Sequencher™ ver. 4 (GeneCodes Corp.), submitted to GenBank (nos AY584861-67) and aligned by eye using MacClade ver. 4 (Maddison & Maddison, 2002). To these were added three previously published diphyllidean sequences (*Echinobothrium harfordi* McVicar, 1976, AF286921; *E. chisholmae* Jones & Beveridge, 2001, AF286922; and *Macrobothridium rhynchobati*, AF286923) and five published pseudophyllidean sequences (*Abothrium gadi* van Beneden, 1871, AF286945; *Anantrum tortum* (Linton, 1905) Overstreet, 1968, AF286941; *Anchistrocephalus microcephalus* (Rudolphi, 1819) Monticelli, 1890, AF286946; *Bothriocephalus scorpii* (Müller, 1776), AF286942; and *Eubothrium crassum* (Bloch, 1779) Nybelin, 1922, AF286947). See Olson et al. (2001) for the host and collection localities of previously published sequences. Maximum parsimony and Bayesian inference analyses were conducted using PAUP* ver. 4.0b10 (Swofford, 2001) and MrBayes ver. 3.0b4 (Huelsenbeck & Ronquist, 2001), respectively, as described in Olson et al. (2003). Pseudophyllidean species were restricted to the outgroup for parsimony analysis, whereas only *Anchistrocephalus microcephalus* was used in Bayesian inference due to the single taxon outgroup constraint of MrBayes. Previous molecular phylogenetic studies using this region of the 28S rDNA have shown the Diphyllidea to be most closely related to the non-diphyllid pseudophyllideans (Olson et al., 2001), although their phylogenetic position has yet to be resolved satisfactorily, and is thus subject to change in the light of new data.

Family Ditrachybothridiidae Schmidt, 1970

Genus *Ditrachybothridium* Rees, 1959

***Ditrachybothridium macrocephalum* Rees, 1959**

Material studied

Ex *Apristurus laurussonii* (Saemundsson), Scyliorhinidae, spiral valve. Goban Spur, northeastern Atlantic (49°47' N, 11°58' W, depth 1,240-1,360 m, 19.x.2002). BMNH 2004.1.6.1-5.

Ex deep-water ray, possibly *Rajella bigelowi* (Stehmann), Rajidae, spiral valve. Porcupine Seabight,

Table 1. Records of larval stages of the Diphyllidea.

Parasite	Host	Site	Locality	Reference	Comment
Mollusca: Gastropoda					
<i>Echinobothrium levicolle</i> Lespés, 1857	Nassariidae: <i>Nassa reticulata</i>	Hepatopancreas	?	Lespés (1857)	Description and figures
<i>E. lateriporum</i> Subhadratha, 1948	Nassariidae: <i>Bullia melanoides</i> Muricidae: <i>Murex tropa</i>	Liver mass Liver mass	Madras	Anantaraman (1963)	Description and figures
<i>Echinobothrium</i> sp.	Buccinidae: <i>Cantharus cancellarius</i> Nassariidae: <i>Nassarius vibex</i>	Digestive gland	Northern Gulf of Mexico	Cake (1976)	Not figured Figured
<i>E. lateriporum</i> <i>nigracanthum</i> Reimer, 1975	Nassariidae: <i>Bullia melanoides</i>	?	Off Madras	Reimer (1975)	Described, not figured
Mollusca: Bivalvia					
<i>Echinobothrium</i> sp.	Solenidae: <i>Solen vagina</i>	Foot	?	Kunstler (1888)	No description or figures
Crustacea: Amphipoda					
<i>E. pigmentatum</i> Ostrowski de Núñez, 1971	Unidentified amphipod	?	Off Argentina	Ostrowski de Núñez (1971)	Figured and described
<i>E. typus</i> van Beneden, 1858	Gammaridae: <i>Gammarus locusta</i> Oedicerotidae: <i>Perioculoides longimanus</i>	Haemocoel Haemocoel	Belgian coast Wimereux	van Beneden (1858) Monticelli (1890)	No description or figures No description or figures
Crustacea: Decapoda					
<i>E. typus</i>	Crangonidae: <i>Crangon</i> sp. Paguridae: <i>Pagurus</i> sp.	Found amongst crustacean remains in stomach of <i>Raja clavata</i>	Heligoland	Leuckart & Pagenstecher (1858)	Figured

Table 1. continued

Parasite	Host	Site	Locality	Reference	Comment
<i>E. benedeni</i> Ruszkowski, 1928	Hippolytidae: <i>Hippolyte varians</i>	Haemocoel	Roscoff	Ruszkowski (1928)	Description and figures
<i>E. lateriporum</i> Subhappradha, 1948	Matutidae: <i>Matuta victor</i>	Free in gut	Madras	Anantaraman (1963)	Description and figures
<i>E. affine</i> Diesing, 1863	Portunidae: <i>Carcinus maenus</i>	Haemocoel	Roscoff	Dollfus (1964)	Description and figures
	Dorippidae: <i>Ethusa mascarone</i>	Body-cavity	Western Mediterranean	Vivares (1971)	Description, no figure
	Dorippidae: <i>Ethusa mascarone</i>	Body-cavity	Golfe du Lion	Vivares (1973)	No description or figure
<i>E. reesae</i> Ramadevi, 1969	Pasiphaeidae: <i>Leptocheila aculeicaudata</i>	Haemocoel	Waltair coast	Ramadevi & Rao (1974)	Description and figures
<i>Echinobothrium</i> sp.	Pasiphaeidae: <i>Leptocheila</i> sp.	Cephalothoracic haemocoel	South China Sea	Shimazu (1982).	Description and figures
<i>E. chisholmae</i> Jones & Beveridge, 2001	Penaeidae: <i>Penaeus longistylus</i>	Body-cavity	Heron Island	Jones & Beveridge (2001)	Described, not figured
Crustacea: Euphausiacea					
<i>Echinobothrium</i> sp.	Euphausiidae: <i>Euphausia similis</i>	?	Suruga Bay	Shimazu (1975)	Description and figure
Elasmobranchia					
<i>E. typus</i>	Rajidae: <i>Raja clavata</i>	Gut	Belgian coast	van Beneden (1858)	No description or figures
<i>E. reesae</i>	Dasyatidae: <i>Himantura walga</i>	stomach	Waltair coast	Ramadevi & Rao (1974)	Description and figures
<i>Ditrachybothridium macrocephalum</i> Rees, 1959	<i>Himantura uarnak</i> Scyliorhinidae: <i>Apristurus laurussonii</i> Rajidae: ? <i>Rajella bigelowi</i>	Spiral valve	Goban Spur	Present study	

northeastern Atlantic (51°09' N, 11°55' W, depth 1,200 m, 3.x.2002). BMNH 2004.1.6.6-11.

Description (Figures 1, 2)

[Based on 4 specimens from *Apristurus laurussonii* and 1 from cf. *Rajella bigelowi* (measurements from the latter are in square brackets).] Cyst elongate-oval or pyriform; 1,998-3,548 × 788-1,388 (2,849 × 1,042) [4,048 × 1,134] (Figures 1, 2A). Narrow channel leading from pore at one extremity of cyst to cavity 380-748 (540) [351] long, with glandular sheath (Figure 2D). Strobila originates at base of cavity, long, coiled, with evidence of segmentation and median deeply-stained anlagen; 3,669-5,787 × 271-427 (4,813 × 333) [7,223 × 325]. Scolex sagittiform, with 2 bothria covered in spines, birefringent in polarised light (i.e. not microtriches) (Figure 2C), 1,074-1,491 × 307-549 (1,320 × 430) [1,623 × 504]; scolex oriented in various directions; apical organ small, peg-like (Figure 2F). Small concavity surrounded by gland-cells at antiporal end of cyst (Figure 2G). Cyst-wall of varying thickness, with large vacuoles (Figure 2B).

Sequence analysis

The full 28S rDNA alignment was 1,296 characters, of which 276 were excluded due to alignment ambiguity. Of the remaining 1,020 characters, 639 were invariant, 85 were unique to single taxa (autapomorphic) and 296 were parsimony informative. Sequences from both the encysted and excysted specimens of *D. macrocephalum* were identical, as were those of *Echinobothrium* sp. from *Raja* spp. Similarly, the sequence of *Macrobothridium rhynchobati* collected from Yorkey's Knob, Queensland, was the same as that collected from Darwin, Northern Territory, Australia (AF286923). Both maximum parsimony (Figure 3A) and Bayesian inference (Figure 3B) produced identical trees for the Diphyllidea, with *Ditrachybothridium* and *Macrobothridium* Khalil & Abdul-Salam, 1989 embedded among species of *Echinobothrium*. *E. bonasum* from the cownose ray was found to be the most basal member of the diphyllidean species analysed.

Discussion

The morphological arrangement we describe and illustrate here is, in essence, identical to that described

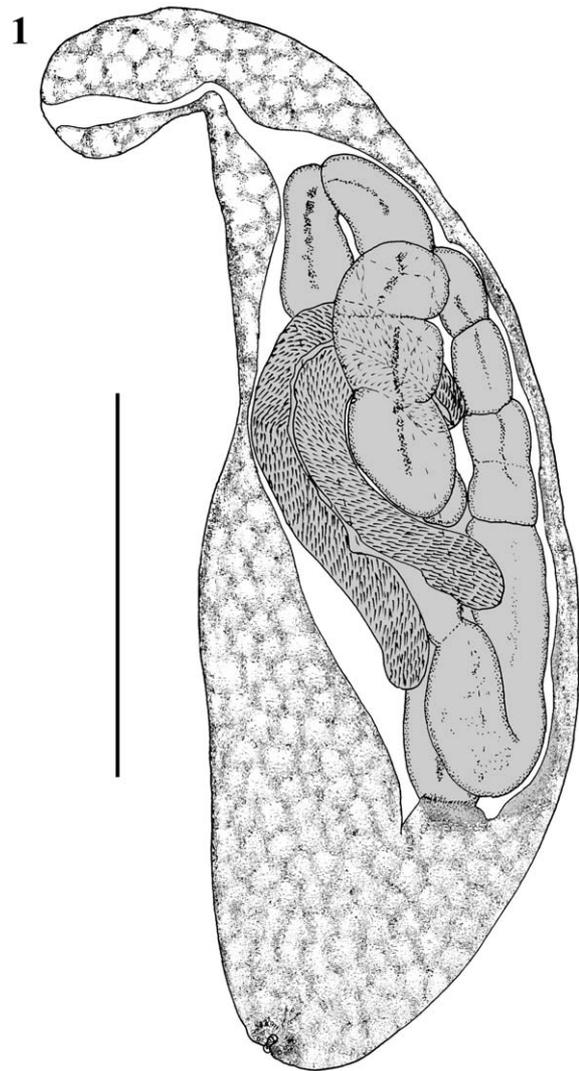


Figure 1. *Ditrachybothridium macrocephalum* Rees, 1959 from *Apristurus laurussonii*. Drawing of complete encysted larval worm. Scale-bar: 1 mm.

earlier for *Echinobothrium* species. Ruzskowski's (1928) figures (particularly 3 and 10) of *E. benedeni* Ruzskowski, 1928 illustrate this point, showing all basic features, even a distinct strobila, although this is markedly shorter than in *D. macrocephalum*. His Figure 9, a section through the cyst, shows similar large vacuoles as are found in the cyst wall of *D. macrocephalum*. Ramadevi & Rao (1974) described a similar elongate strobila in *E. reesae* Ramadevi, 1969, with clear segmentation and well-developed genital anlagen. The strobila of *Echinobothrium* sp., as described by Shimazu (1982), is also elongate, with segmentation and advanced gonads. None of our

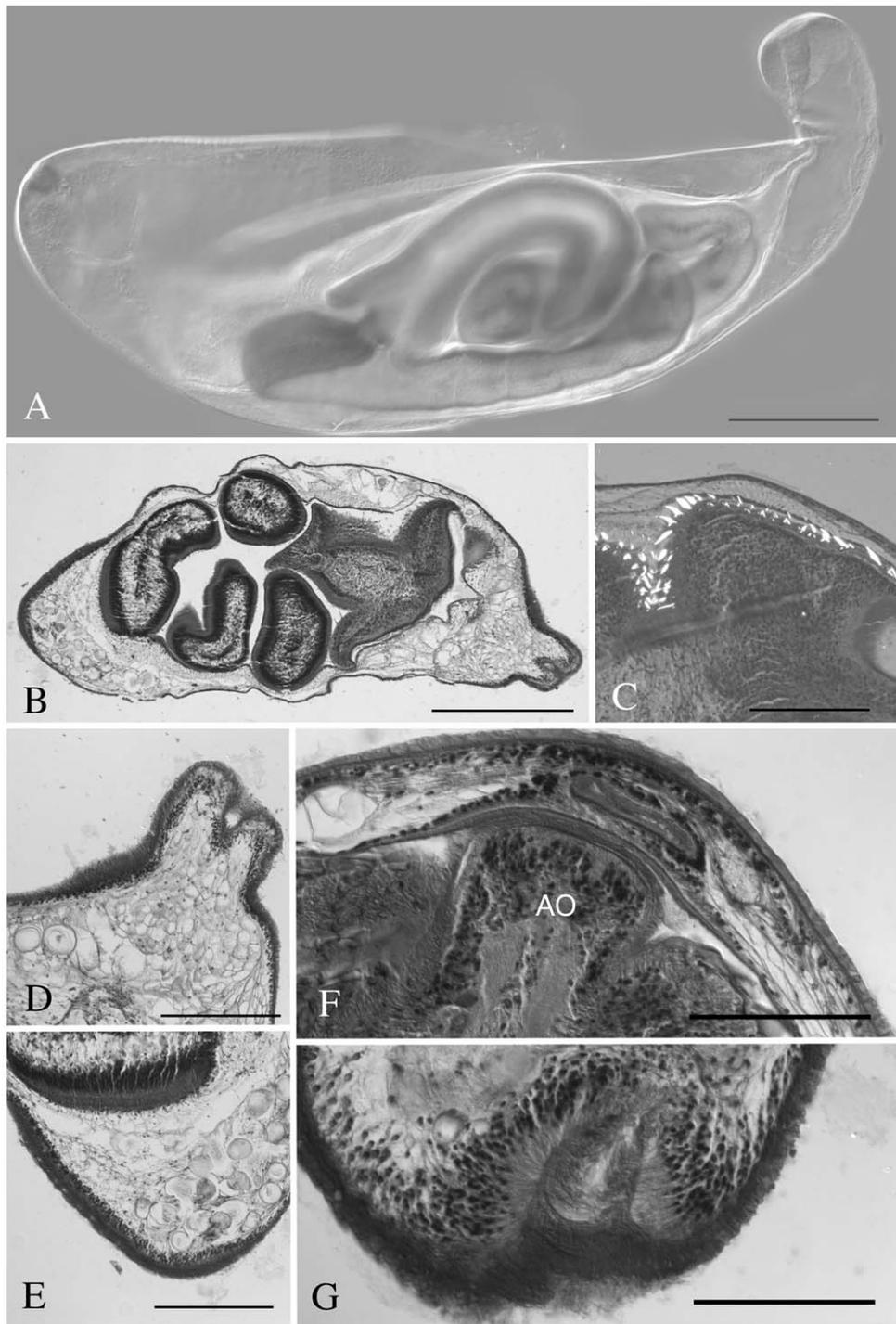


Figure 2. *Ditrachybothridium macrocephalum* Rees, 1959 from *Apristurus laurussonii*, whole-mount and sagittal sections. A. Composite image of whole-mounted larval cyst showing early segmentation of strobila (cf. Figure 1); B. Larval cyst showing vacuolate cyst wall, strobila, scolex and opening of channel connecting cyst cavity to exterior; C. Scolex showing birefringence of hooks under polarised light; D. Poral extremity showing pore of channel connecting cyst cavity to exterior; E. Aporal extremity showing vacuolate cells in cyst-wall; F. Poral extremity showing apical organ (AO), section of pore channel and hooks; G. Blind concavity at aporal extremity of cyst. Scale-bars: A-B, 500 μm ; C-E, G, 200 μm ; F, 100 μm .

Table 2. Records of excysted adult and subadult *Ditrachybothridium*.

Parasite	Host	Site	Locality	Reference	Comment
<i>Ditrachybothridium macrocephalum</i> Rees, 1959	Rajidae: <i>Raja fullonica</i> , <i>R. circularis</i> Scyliorhinidae: <i>Scyliorhinus canicula</i>	Spiral valve	Off NW Scotland near St Kilda	Rees (1959) Williams (1960)	Not fully mature
	Scyliorhinidae: <i>Galeus melastomus</i>	Spiral valve	NW Utsira, northern North Sea	Present study	Early ovigerous
	Scyliorhinidae: <i>Apristurus</i> sp.	Spiral valve	Porcupine Seabight	Present study	Short excysted fragment
<i>Ditrachybothridium piliformis</i> Faliex, Tyler & Euzet, 2000	Scyliorhinidae: <i>Galeus</i> sp.	Spiral valve	South Pacific Ocean near Vanuatu	Faliex et al. (2000)	Ovigerous
<i>Ditrachybothridium</i> sp.	Rajidae: <i>Raja fyllae</i>	Spiral valve	Off NW Scotland west of St Kilda	BMNH 1982.4.26.261	Scolex and fragment of strobila

specimens exhibited the tape-like tail described by Dollfus (1964) and Shimazu (1975, 1982).

Despite its unusual morphology, the rDNA data presented here support a position of *Ditrachybothridium* embedded among species of *Echinobothrium*, the closest being a unidentified species collected from *Raja* spp. in the deep waters of the north Atlantic Ocean. Similarly, *Macrobothridium* is also found embedded among species of *Echinobothrium*, appearing closest to the only other diphyllidean representative from Australia in the analysis, *E. chisholmae* Jones & Beveridge, 2001. Based on these data alone, both *Ditrachybothridium* and *Macrobothridium* should be considered unusual forms of a sole diphyllidean genus, *Echinobothrium*, although formal taxonomic revision is beyond the scope of this study. This partly contrasts with the morphological phylogenetic studies of Ivanov & Hoberg (1999) and Caira et al. (1999, 2001), all of which placed *Ditrachybothridium* as the basal lineage of the Diphyllidea. However, this position was possibly influenced by its lack of typical 'diphyllidean' characters (e.g. dorsal and ventral apical hooks), which would group the monotypic *Macrobothridium* and species of *Echinobothrium* together to the exclusion of *Ditrachybothridium*. Paraphyly of *Echinobothrium* was nevertheless also suggested by the analysis of Ivanov & Hoberg (1999) in that *Macrobothridium* was found, as herein, embedded among species of the former genus.

Faliex et al. (2000) discussed the host relationships of *Ditrachybothridium* and came to the conclusion that the 'natural' hosts of *D. macrocephalum* in the north Atlantic are deep-water scyliorhinids and, *inter*

alia, mentioned *Apristurus laurussonii*. The presence of plerocerci in this host reported here supports this view, although no excysted forms were found. An excysted form, i.e. a scolex and a short length of immature strobila was, however, previously recovered from *Apristurus* sp. (Porcupine Seabight, 49°32' N, 12°48', depth 1541 m, 16.viii.1997: BMNH 2000.1.19.5). Several excysted worms were found in the ray, but none showed much maturation of the strobila beyond that seen in the plerocercus. The prediction of Faliex et al. (2000) on the definitive hosts of *D. macrocephalum* is further reinforced by the finding of a fully mature (early gravid) specimen from the relatively deep-water scyliorhinid *Galeus melastomus* (unpublished data: NW Utsira, northern North Sea: BMNH 1973.6.11.11-13).

Diphyllidean cestode larval stages have been reported in a variety of molluscs, crustaceans and elasmobranchs (Table 1). It is likely that the elasmobranchs listed (Tables 1, 2) have acquired the parasite by consumption of infected invertebrates.

These observations increase our small fund of knowledge on the morphology of diphyllidean larval stages. Beveridge (2001) coded five 'metacestode characters' in his cladistic study of cestode life-cycle characters. In the Diphyllidea, three of these were coded with a query. His character 9 is coded as '(1) scolex invaginated or retracted' and our observation agrees with this. Character 10 is queried, but our observations suggest that '(0) primary lacuna absent' is most appropriate as we have detected no evidence of it. The apical organ is clearly seen in sections and, therefore, character 11 should be coded (0). For character

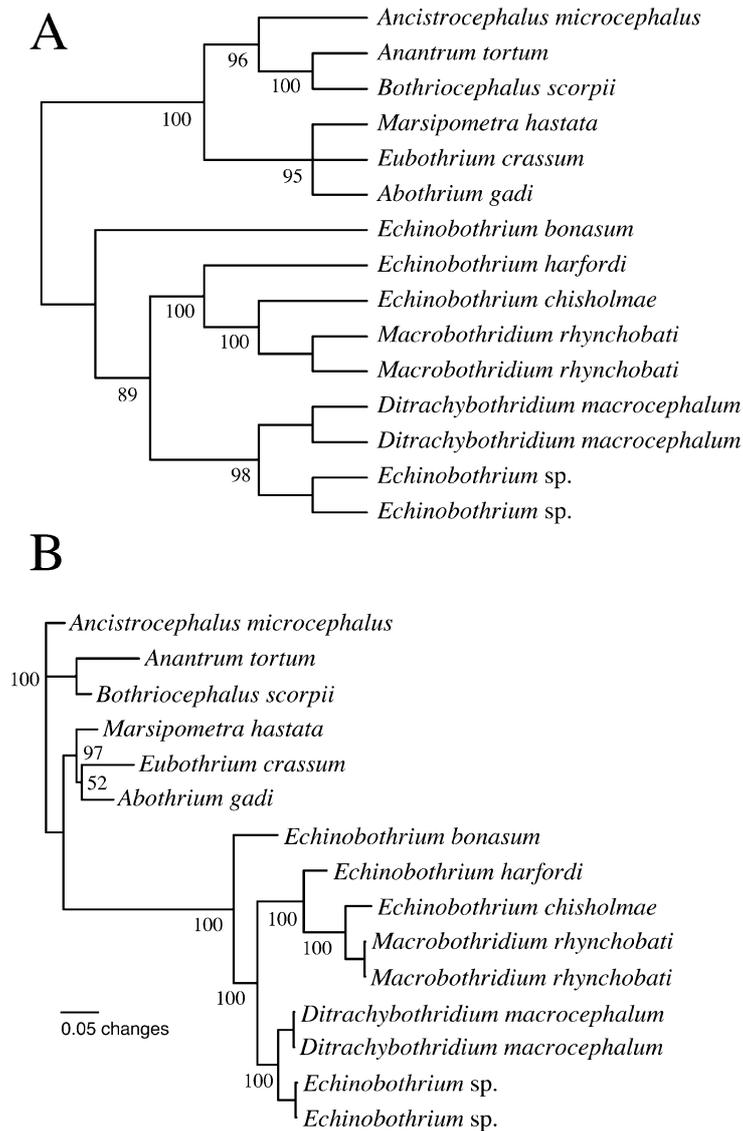


Figure 3. Phylogenetic analyses of diphyllidean species based on partial 28S rDNA (D1-D3). A. Strict consensus of two trees resulting from maximum parsimony analysis. Nodes show bootstrap percentages > 50%; B. Phylogram of consensus resulting from Bayesian inference. Nodes show Bayesian posterior probabilities.

12 the 'metacercoid' is coded as (4) a 'neoplerocercus' (a lapsus for 'plerocercus' – Beveridge, pers. comm.). No evidence of a cercomer is available in our specimens (character 13), but the tape-like tail described by Dollfus (1964) and Shimazu (1975, 1982) may represent the remnants, although no authors mention hooks on the tail as would be expected on a cercomer.

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mens of *Echinobothrium bonasum*. Stephen Curran, Ash Bullard and Robin Overstreet provided specimens of *Marsipometra hastata* and Roman Kuchta helped confirm its identity. PDO was supported by a Wellcome Trust Senior Fellowship (043965/Z/95/Z) to Tim Littlewood.

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