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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Accepted for publication 14th April, 2004

**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 The plerocercus is withdrawn, but not invaginated, within a cyst with a thick vacuolated wall.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 pseudophyllidean (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 metaccestodes’ ofcestodes, 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 Ruszkowski (1928) and Ramadevi & Rao (1974), but other useful morphological observations are found in the figures and/or descriptions published by Lepsés (1857), Leuckart & Pagenstecher (1858), Anantaraman (1963), Doll-fus (1964), Shimazu (1975), Cake (1976), Shimazu (1982) and Jones & Beveridge (2001). It has been clear since the description of Lepsés (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 2004.18.3.101), 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-diphyllobothrid pseudophyllidean, *Marsipometra hastata* (Linton, 1897) Cooper, 1917 (BMNH 2004.4.1.3.1) 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; *Bothrioccephalus 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-diphyllobothrid 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,