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Molecular Identification of Larvae of a Tetraphyllidean Tapeworm (Platyhelminthes: Eucestoda) in a Razor Clam as an Alternative Intermediate Host in the Life Cycle of *Acanthobothrium brevissime*

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ABSTRACT: Dwarf razor clams (*Ensis minor*) in the Gulf of Mexico are known to be infected with plerocercoid larvae of a tetraphyllidean tapeworm. Here, we show that these larvae live unencysted in the intestinal lumen of the clam. Morphologically, the larvae are similar to (although significantly larger than) tapeworm larvae previously described living in the gut of amphioxus (*Branchiostoma floridae*) from the same habitat. Sequence data from the D2 region of the 28S rDNA from clam-infecting larvae were identical to the sequence of *Acanthobothrium brevissime* isolated as larvae from amphioxus and as adults from a stingray (*Dasyatis say*). The sequence data leave little doubt that the dwarf razor clam and the amphioxus are alternative intermediate hosts in the life cycle of *A. brevissime*.

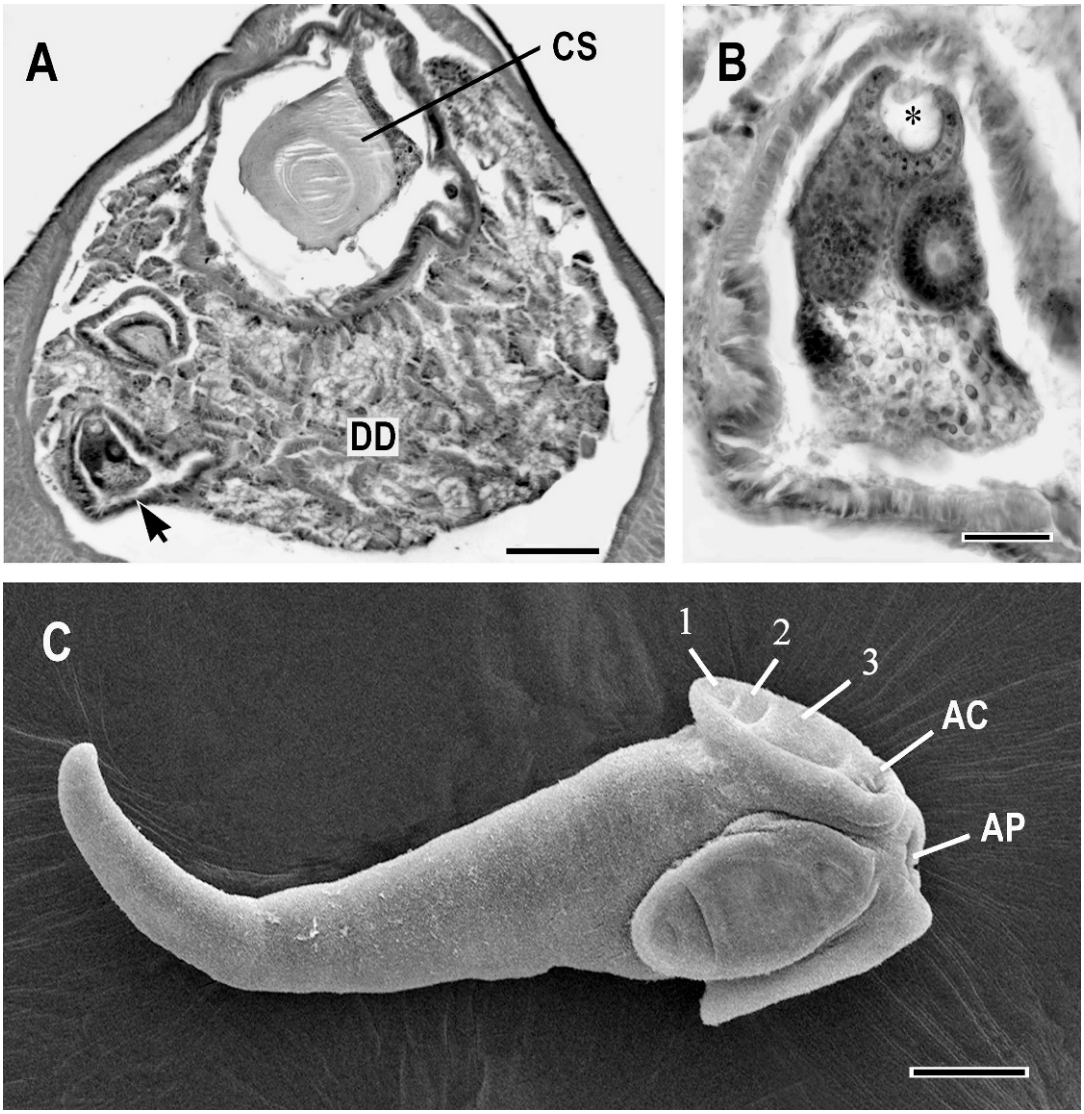

FIGURE 1. (**A**) Cross section through the viscera of a dwarf razor clam (*Ensis minor*) showing a tapeworm larva (arrowed) in the lumen of the intestine. (**B**) Enlargement of the larva in A; the apical sucker is indicated by an asterisk. (**C**) Scanning electron micrograph of a tapeworm larva after removal from the clam's intestine; 1, 2, 3, and AC indicate, respectively, the 3 loculi and the accessory sucker on 1 of the 4 bothridia. AP, apical sucker; CS, crystalline style; DD, digestive diverticula. Scale bars = 200 μm (A), 50 μm (B, C).

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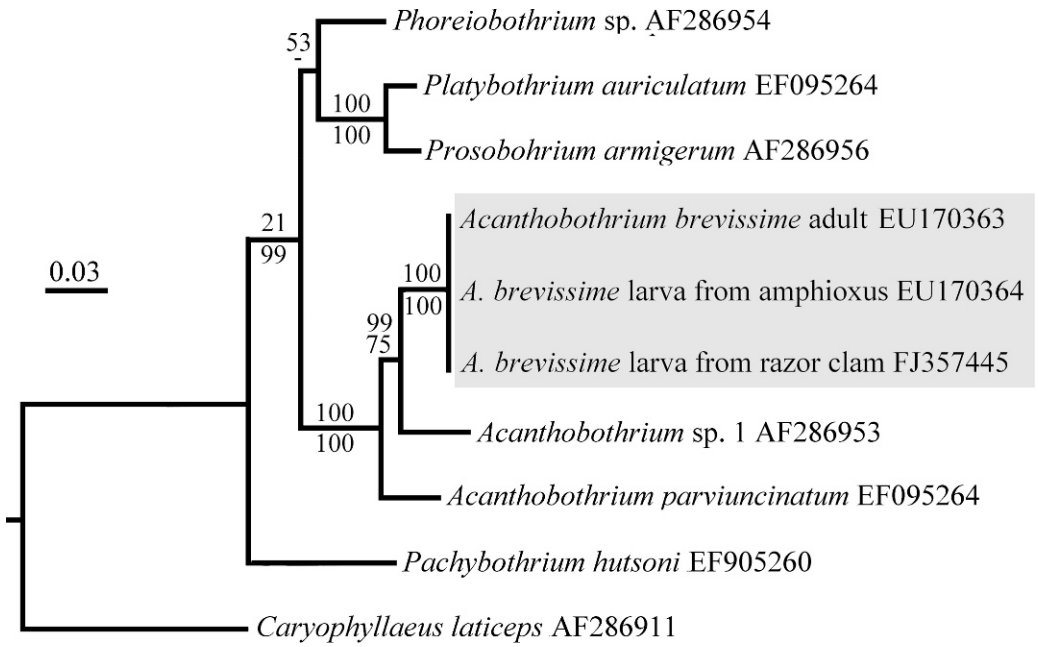

FIGURE 2. Most-likely tree based on the D2 region of 28S rDNA from a basal cestode, 4 relatively close relatives of *Acanthobothrium*, and all available sequences for *Acanthobothrium*. The GenBank accession number is given after each species name. The numbers above and below the nodes are, relatively, maximum likelihood bootstrap values and maximum parsimony jackknife values. The scale line indicates the number of substitutions per site. The shaded box emphasizes that the sequence from tapeworm larvae parasitizing the dwarf razor clam is identical to the sequence of adult and larval *Acanthobothrium brevissime* parasitizing, respectively, an elasmobranch and an amphioxus.

Although larvae of marine tapeworms are difficult to identify to species on the basis of morphology, they can be linked to taxonomically known adult stages by comparing their rDNA sequences (Brickle et al., 2001; Dezfuli et al., 2002; Agustí et al., 2005; Aznar et al., 2007; Holland et al., 2009). For another aspect of tapeworm life cycles, i.e. the utilization of alternative intermediate hosts, species determination of larvae by such molecular methods has only rarely been accomplished (Ito et al., 2002). The present study concerns a marine tapeworm, *Acanthobothrium brevissime* Linton, 1909, known to infect the bluntnose stingray *Dasyatis say* (Lesueur) as a definitive host and the Florida amphioxus *Branchiostoma floridae* Hubbs as an intermediate host (Holland et al., 2009). The question of whether the life cycle of this tapeworm species might include alternative intermediate hosts was raised by some incidental observations of Wardle (1974) and Cake (1976), who found tapeworm larvae in molluscs and very tentatively suggested the parasite might be *A. brevissime*. Here, molecular sequence data from the D2 region of the 28S rDNA from the cestodes leave little doubt that the tapeworm larvae from a razor clam are indeed *A. brevissime*.

Specimens of the dwarf razor clam, *Ensis minor* Dall, approximately 4 cm long, were collected by shovel and sieve at a depth of 1 m at 2 Tampa Bay locations: 20 at Apollo Beach on 13 July 2008 and 10 at Saint Petersburg on 14 July 2008. Live clams were dissected in a dish of seawater to isolate the tapeworm larvae, which were conspicuous due to their incessant cycle of extension and retraction. For 14 of the 27 larvae found, lengths at maximum extension were recorded with an ocular micrometer before they were preserved either for DNA sequencing in 80% ethanol or for morphological observation in freshly prepared 4% paraformaldehyde in 0.1 M MOPS buffer (pH 7.5) containing 1 mM EGTA, 2 mM $MgSO_4$ and 0.5 M NaCl (referred to below simply as PFA). For scanning electron microscopy, PFA-fixed larvae were rinsed briefly in distilled water, dehydrated in a graded ethanol series, critical point dried from CO_2, attached to stubs with double-sided adhesive tape, sputter coated with a 15-nm layer of gold and palladium, and viewed in a Cambridge Instruments 360 scanning electron microscope (Cambridge Instruments, Cambridge, U.K.). Ten additional clams were fixed in PFA, embedded in paraffin, cut as a complete series of 25-μm thick serial sections, and stained with Mayer's hematoxylin and eosin.

DNA was extracted from an ethanol-fixed larva with a DNeasy blood and tissue kit (Qiagen, Valencia, California), and a region of rDNA including the 725-base pair D2 loop was amplified with the 300F and ECD2 primers of Littlewood et al. (2000). This sequence was gel purified and bi-directionally sequenced on an ABI automated sequencer using Big Dye Readymix reaction kit (ABI–Perkin Elmer, Foster City, California). Forward and reverse sequences were reconciled and edited using Sequencher v.4.7 (Gene Codes Corp., Ann Arbor, Michigan). Voucher specimens of 6 larvae were fixed in 80% ethanol, a fixative suitable for both molecular and morphological work (Holland et al., 2009), and have been deposited in the Benthic Invertebrates Collection at Scripps Institution of Oceanography (SIO-BIC-Pt55).

Phylogenetic analyses were by maximum likelihood and maximum parsimony. The cestodes included in the analyses comprised, in addition to the clam-infecting larvae, adults of *Caryophyllaeus laticeps* (Pallas, 1781) (the outgroup), *Pachybothrium hutsoni* (Southwell, 1911), *Phoreiobothrium* sp. Linton, 1889, *Platybothrium auriculatum* Yamaguti, 1952, *Prosobothrium armigerum* Cohn, 1902, and all available larval and adult sequences for species of *Acanthobothrium* van Beneden, 1849. The GenBank accession numbers of the species analyzed are included in Figure 2. Alignment was with Clustal X v.1.83.1 (Chenna et al., 2003), which is available through TreeBASE (www.trebase.org). Maximum likelihood was carried out in RaxML v7.0.4 (Stamatakis, 2006) after selecting the appropriate model of evolution using the AIC in Modeltest (GTR + Γ) (Posada, 2005), and node support was calculated with 350 bootstrap replicates. Maximum parsimony was conducted using a heuristic search in PAUP* v4.0b10 (Swofford, 2000) with TBR branch swapping and 1,000 random sequence addition replicates. Branch support was estimated using 1,000 jackknife replicates.

Histological examination showed that 1 of the serially sectioned clams contained 5 larval tapeworms, all located in the lumen of the intestine (Fig. 1A), close to its origin from the stomach. The larvae, which are not encysted (Fig. 1B), are plerocercoids (fluid-filled spaces lacking and anterior end everted), as defined by Chervy (2002). Scanning electron microscopy (Fig. 1C) shows that the larval body ranges from approximately cylindrical to cone-shaped, tapering posteriorly. Toward the anterior end, there are 4 attachment organs in the form of bothridia, each divided into 3 loculi and an anterior region in the form of an accessory sucker; however, no rudiments of bothridial hooks are present. There is also a single apical sucker at the anterior end of the body.

The tapeworm larvae from the clam are morphologically similar to those that Holland et al. (2009) discovered infecting amphioxus. However, when measured alive and extended, the former are significantly longer

(mean of 746 μm ± standard deviation of 193 μm; n = 14) than the latter (mean = 564 ± standard deviation of 128 μm; n = 58, data of Holland et al., 2009); an unpaired *t*-test gives a 2-tailed *P*-value ≤ 0.0001. Overnight fixation in either PFA or ethanol caused the larvae to shrink to about 70% of their live, extended length. It is not known why the tapeworm larvae from the clams are markedly larger than those from amphioxus, although the size difference might reflect a greater abundance of resources in the clam relative to amphioxus. Of the 30 clams dissected, 3 were found to be infected (prevalence of infection = percentage of hosts harboring 1 or more parasite = 10%). The 2 infected clams at Apollo Beach hosted 20 and 5 larvae, respectively, while the single infected clam at Saint Petersburg hosted 2 larvae.

Figure 2 shows the results of the phylogenetic analysis of the base sequence from the D2 region of the 28S rDNA. The sequence for *A. brevissime* diverges by about 6% from other species of *Acanthobothrium*, i.e., by 42–44 altered base pairs in the 725-base pair D2 region. In contrast, identical sequences in the D2 region leave little doubt that different cestode life cycle stages belong to the same species. In the present study, the D2 sequence of the clam-inhabiting cestode larva exactly matched that of *A. brevissime*. Therefore, this tapeworm species can utilize amphioxus (Holland et al., 2009) and the dwarf razor clam, *E. minor* (present study), as alternative intermediate hosts. It is possible that there are more alternative intermediate hosts for *A. brevissime* than the ones found so far because morphologically similar tapeworm larvae have been found in 7 other bivalve species and in 1 gastropod living in the Gulf of Mexico (Wardle, 1974; Cake, 1976). This possibility requires further testing by rDNA sequencing. A further complexity in the life cycle of many parasitic helminths is the presence of a sequence of intermediate (paratenic) hosts. Such a sequence can occur when the larvae are passed along to successive predators before reaching the definitive vertebrate host. One example might be the molluscivorous gastropod, *Neverita duplicata* (Say), which Cake (1976) found was parasitized by a tapeworm larva similar to the ones described here. It is not known whether this gastropod becomes infected directly by developmental stages of the tapeworm or acquires tapeworm larvae by eating parasitized bivalves.

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