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Spathebothriidea: survey of species, scolex and egg morphology, and interrelationships of a non-segmented, relictual tapeworm group (Platyhelminthes: Cestoda)*

Roman Kuchta[1], **Rebecca Pearson**[2], **Tomáš Scholz**[1,3], **Oleg Ditrich**[3] and **Peter D. Olson**[2]

[1] Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic;

[2] Department of Life Sciences, Natural History Museum, London, United Kingdom;

[3] Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

* This paper is dedicated to Michael 'Mick' David Brunskill Burt (1938–2014) whose recent passing represents a great loss to cestodology.

Abstract: Tapeworms of the order Spathebothriidea Wardle et McLeod, 1952 (Cestoda) are reviewed. Molecular data made it possible to assess, for the first time, the phylogenetic relationships of all genera and to confirm the validity of *Bothrimonus* Duvernoy, 1842, *Diplocotyle* Krabbe, 1874 and *Didymobothrium* Nybelin, 1922. A survey of all species considered to be valid is provided together with new data on egg and scolex morphology and surface ultrastructure (i.e. microtriches). The peculiar morphology of the members of this group, which is today represented by five effectively monotypic genera whose host associations and geographical distribution show little commonality, indicate that it is a relictual group that was once diverse and widespread. The order potentially represents the earliest branch of true tapeworms (i.e. Eucestoda) among extant forms.

Keywords: Eucestoda, taxonomy, scanning electron microscopy, 28S rDNA, 18S rDNA, ITS2, phylogenetic relationships, distribution

The order Spathebothriidea Wardle et McLeod, 1952 exhibits all of the hallmarks of a relictual group that was once diverse and widespread, but is today represented by a small number of refugial species whose host associations and geographical distribution show little commonality. Multiple lines of evidence suggest that the group is indeed ancient and potentially represents the earliest branch of true tapeworms (i.e. Eucestoda) among extant forms. Morphologically, they share features of both the eucestodes (including a hexacanth embryonic stage and the serial repetition of the reproductive organs) and the 'cestodarian' sister group Amphilinidea (in their utilisation of amphipod intermediate hosts, lack of external segmentation and a scolex lacking either bothrial or acetabulate holdfast structures).

Molecular phylogenetic analyses have also provided independent support for a basal position of the group (Olson and Caira 1999, Kodedová et al. 2000, Olson et al. 2001, 2008, Waeschenbach et al. 2007, 2012). Progenesis is common in the group (Sandeman and Burt 1972, Okaka 2000) and has been used as evidence of their 'primitive' position (Beveridge 2001), albeit this argument pre-supposes the original definitive (or only) host of the tapeworm ancestor to be an invertebrate. Geographically, they have a disjunctive distribution across the northern hemisphere (i.e. a circumboreal distribution), with each of the five described genera parasitising disparate groups of freshwater, euryhaline or marine fishes.

A total of 15 nominal species within five genera have been proposed, with the first species described as *Taenia truncata* by Pallas (1781) (see details in Caira et al. 2014). The taxonomy and systematics of the group has been reviewed by Nybelin (1922), Burt and Sandeman (1969), Gibson (1994) and Protasova and Roitman (1995). In this paper, we provide a survey of all species together with new data on scolex, egg and microthrix morphology. Variable regions of rDNA were characterised from newly collected material in order to test for intraspecific variation and to examine the interrelationships of the group. Sequence data were also characterised from formalin-fixed material of the sturgeon-hosted species *Bothrimonus fallax* Lühe, 1900, allowing us to test its taxonomic validity and phylogenetic affinities using data independent of morphology for the first time.

Address for correspondence: R. Kuchta, Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic. Phone +420 38 777 5488; Fax: +420 38 531 0388; E-mail: krtek@paru.cas.cz

MATERIALS AND METHODS

Specimens examined

The present study was based on morphological and molecular evaluations of specimens recently collected by the authors and their collaborators, as well as on historical collections deposited in the following museums: Göteborgs Naturhistoriska Museum, Göteborg, Sweden (GNM); Helminthological collection of the Institute of Parasitology, Czech Academy of Sciences, České Budějovice (IPCAS); Muséum National d'Histoire Naturelle, Paris, France (MNHN); Natural History Museum, London, UK (BMNH); and United States National Parasite Collection, Beltsville, MD, USA (USNPC).

The following newly collected material was studied:

(i) *Cyathocephalus truncatus* (Pallas, 1781): 10 specimens from *Salmo trutta* Linnaeus (Salmoniformes: Salmonidae), River Brenta, Italy (45°07'N; 12°02'E; collected by B. Dezfuli 27 July 1997 and February 2007; IPCAS C-13/1); 2 specimens from *Coregonus lavaretus* (Linnaeus) (Salmoniformes: Salmonidae), Lake Segozevo, Karelia, Russia (63°30'N; 33°75'E; L.G. Poddubnaya, 17 July 2004; IPCAS TS-04/136, and on June 2005; IPCAS TS-06/9); pieces of both specimens were used for molecular study.

(ii) *Diplocotyle olrikii* Krabbe, 1874: 30 specimens from *Myoxocephalus scorpius* (Linnaeus) (Scorpaeniformes: Cottidae), Petunia Bay, Isfjorden, Svalbard Archipelago, Arctic Ocean (78°68'N; 16°45'E) (R. Kuchta and O. Ditrich, see details in Table 2; IPCAS C-333/4); 2 specimens sequenced, and *Gymnocanthus tricuspis* (Reinhardt) (Scorpaeniformes: Cottidae), from the same locality (R. Kuchta and O. Ditrich IPCAS C-333/5; two progenetic specimens from the body cavity of *Marinogammarus* sp. (Amphipoda: Gammaridae), off St. Andrews, Scotland, UK (56°21'N; 2°48'W; P.D. Olson, R. Kuchta and J. Brabec, 5 April 2004, 1 infected out of 1 673 examined amphipods; IPCAS C-645/1).

(iii) *Spathebothrium simplex* Linton, 1922: 2 specimens from *Liparis fabricii* Krøyer (Scorpaeniformes: Liparidae), Petunia Bay, Isfjorden, Svalbard Archipelago, Arctic Ocean (78°68'N; 16°45'E), (R. Kuchta and O. Ditrich 1 August 2008; IPCAS C-568); pieces of both specimens used for DNA sequencing; 1 specimen from *Liparis atlanticus* (Jordan et Evermann), off Rye Beach, New Hampshire, USA (42°58'7"N; 70°45'49"W; R. Kuchta, 10 April 2009).

The following museum material was studied:

(i) *Bothrimonus fallax*: 10 specimens from *Acipenser nudiventris* Lovetsky (Salmoniformes: Salmonidae), Caspian Sea, Russia (leg. Pilarcki; IPCAS C-382/1); 1 specimen Kulai (= Aygedzor, Tavush Province), Armenia (leg. E. Lönnberg; 22 May 1899; GNM 2524); 10 specimens from *Acipenser stellatus* Pallas, Agrakhanski Bay, Caspian Sea, Russia (leg. B. Kuperman; 29 April 1966; IPCAS C-382/2); 2 specimens from *A. stellatus*, off Dagestan, Caspian Sea, Russia (IPCAS C-382/2); 1 specimen from *Huso huso* (Linnaeus), Kizil-Aga, Turkmenistan (leg. B. Kuperman; 2 June 1962; IPCAS C-382/3) (all hosts Acipenseriformes: Acipenseridae).

(ii) *Cyathocephalus truncatus*: 2 specimens from *Coregonus lavaretus*, Lake Baikal, Russia (leg. O. Rusinek; 20 July 1997; IPCAS C-13/3); 10 specimens from *Salmo trutta*, River Tirino, Italy (23 March 1977; IPCAS C-13/1); 1 specimen from *Gammarus italicus* Goedmakers et Pinkster (Amphipoda: Gammaridae), Tirino River, Italy (27 April 1977; IPCAS C-13/1).

(iii) *Diplocotyle olrikii*: 2 specimens from *Oncorhynchus gorbuscha* (Walbaum) (Salmoniformes: Salmonidae), Pacific Ocean, Canada (leg. M. Burt; BMNH 1982.10.1.24–26); 1 specimen from *Salvelinus* sp. (Salmoniformes: Salmonidae), Kamchatka Bay, Kamchatka, Russia (leg. B. Kuperman; 19 April 1966; IPCAS C-333/2); 10 specimens from *Salvelinus alpinus* (Linnaeus), Ottostrand, East Greenland (leg. H. Fleisher; 19 August 1947; BMNH 1961.2.22.17–20 and 1963.10.7.7–50); 15 specimens from *Salmo salar* Linnaeus, Newfoundland, Canada (leg. M. Burt; BMNH 1982.9.20.144–169); 10 specimens from *Salmo trutta*, Osnes, R. Selfjort, Iceland (leg. R.E. Wauch; 5 July 1980; BMNH 1982.3.3.1–25); 1 specimen from *Thymallus arcticus* (Pallas) (Salmoniformes: Salmonidae), Chukotka, Far East, Russia (leg. B. Kuperman; 1962; IPCAS C-333/3); 2 specimens from *Monoporeia affinis* (Lindström) (Amphipoda: Pontoporeiidae) Tvarminne, Baltic Sea, Finland (leg. E.T. Valtonen; 5 August 1985; BMNH 1988.3.17.2–6).

(iv) *Didymobothrium rudolphi* (Monticelli, 1890): 1 specimen from *Pegusa cadenati* Chabanaud (Pleuronectiformes: Soelidae), off Cape Verde, Atlantic Ocean (leg. P. Chabanaud, 10 January 1952; MNHN bD 52); 4 specimens from *Pegusa lascaris* (Risso), off Banyuls-sur-Mer, Mediterranean Sea, France (leg. O. Nybelin; 11 October 1959; GNM 481); 4 specimens, off Plymouth, English Channel, UK (D. Gibson; May 1972; BMNH 1989.1.31.38–41); 10 specimens, off northern Portugal, Atlantic Ocean (J.M. Marques; 2003; BMNH 2006.10.4.9–20); 2 spec. from *Solea solea* (Linnaeus) (Pleuronectiformes: Soelidae), off Turkey, Mediterranean Sea (M.C. Oğuz; BMNH 1993.1.21.10; 1997.9.30.1).

Morphological studies

Tapeworms collected by the present authors were washed in saline and those for morphological studies, including scanning electron microscopical (SEM) observations and histology, were fixed with hot 4% formaldehyde solution. Additional specimens or parts of specimens were preserved in 96% molecular-grade ethanol for molecular study. Whole-mounted specimens were stained with Schuberg's hydrochloric carmine and mounted in Canada balsam. Cross-sections of the strobila (thickness 15 µm) were stained with haematoxylin-eosin, using standard histological techniques. Several scoleces and segments were prepared for SEM following the procedure outlined by Kuchta and Caira (2010). The microthrix terminology follows Chervy (2009). The development of the eggs liberated from the uterus of unstained specimens of *D. olrikii* and *S. simplex* using fine needles were placed in distilled water and observed under light microscopy. In a survey of the species, illustrations (line drawings) of individual taxa are not provided because they were presented in several taxonomic accounts, such as those of Nybelin (1922), Burt and Sandeman (1974), Gibson (1994), and Protasova and Roitman (1995). For comparison eggs of *Amphilina foliacea* (Rudolphi, 1819) from *Acipenser ruthenus*, Danube River, Slovakia (M. Oros; 20 April 2011; IPCAS C-44/1), were also studied using light microscopy (LM) and SEM.

Molecular characterisation from fresh material

Molecular ribosomal data were characterised from newly collected specimens of *Cyathocephalus truncatus* (2 spec. sequenced), *Diplocotyle olrikii* (n = 8) and *Spathebothrium sim-*

Table 1. New sequences of spathebothriideans used in phylogenetic analyses.

Access. No. 28S rDNA/ITS2	Coll. No.	Parasite	Host	Locality
*	TS 04/10	*Bothrimonus fallax*	*Acipenser nudiventris*	Caspian Sea, Russia
KJ400369/ KJ400374	TS 04/136	*Cyathocephalus truncatus*	*Coregonus lavaretus*	Lake Segozevo, Russia
KJ400370/ KJ400375	TS 06/9	*Cyathocephalus truncatus*	*Coregonus lavaretus*	Lake Segozevo, Russia
KJ400366/ KJ400379	TS 09/271	*Diplocotyle olrikii*	*Gymnocanthus tricuspis*	Svalbard, Arctic Ocean
KJ400363	TS 09/275	*Diplocotyle olrikii*	*Myoxocephalus scorpius*	Svalbard, Arctic Ocean
KJ400367/ KJ400378	TS 09/279	*Diplocotyle olrikii*	*Myoxocephalus scorpius*	Svalbard, Arctic Ocean
KJ400368	TS 09/280	*Diplocotyle olrikii*	*Gymnocanthus tricuspius*	Svalbard, Arctic Ocean
KJ400361	TS 09/282	*Diplocotyle olrikii*	*Myoxocephalus scorpius*	Svalbard, Arctic Ocean
KJ400364	TS 09/283	*Diplocotyle olrikii*	*Myoxocephalus scorpius*	Svalbard, Arctic Ocean
KJ400362/ KJ400377	TS 09/284	*Diplocotyle olrikii*	*Myoxocephalus scorpius*	Svalbard, Arctic Ocean
KJ400365/ KJ400376	AR3	*Diplocotyle olrikii*	*Myoxocephalus scorpius*	Svalbard, Arctic Ocean
KJ400372/ KJ400373	AR23	*Spathebothrium simplex*	*Liparis fabricii*	Svalbard, Arctic Ocean

* partial 18S rDNA (see sequence on Fig. 25).

plex (n = 2) (see Table 1). Following Marques et al. (2007), the D2 region of the 28S rDNA gene (~660 bps) was characterised for all specimens (n = 12) and the ITS-2 region (~600 bps) for each of the specimens, except for replicates of *D. olrikii* that proved identical based on comparison of 28S sequences (n = 8) (Table 1). These data were combined with published 28S and ITS2 sequences of these three taxa as well as *Didymobothrium rudolphii* from the work of Marques et al. (2007). Attempts were also made to characterise the cytochrome *c* oxidase subunit I gene, used for 'species barcoding' in many groups (especially arthropods and vertebrates), using a variety of primer combinations from Littlewood et al. (2008) and Moszczynska et al. (2009), but without success.

Ethanol in the samples was replaced by Tris-EDTA buffer through two rinses and genomic DNA extracted with a DNeasy kit (Qiagen, Venlo, Neatherlands) and a hand-held homogeniser fitted with sterile plastic pestles. PCR was performed using PuRe Taq Ready-To-Go beads (GE Healthcare, Little Chalfont, UK). The D1–D3 region of the 28S was amplified using primers LSU5 and 1200R (Olson et al. 2001) and the ITS2 using primers ITS2.3S (GGT ACC GGT GGA TCA CGT GGC TAG TG) and ITS2.2 (CCT GGT TAG TTT CTT TTC CTC CGC). Cycling parameters were as follows: denaturation (95 °C/5 min), amplification (40 cycles 95 °C/30 s, 52 °C/30 s, 72 °C/90 s), extension (72 °C/1 min). Products were visualised on agarose gels, purified using Qiagen spin columns and sequenced directly by Sanger sequencing. The 28S products were sequenced using internal primers 300F and ECD2 (Olson et al. 2001), whereas the ITS2 products were sequenced using the PCR primer combinations.

Molecular characterisation of *Bothrimonus fallax* from formalin-fixed material

Only formalin-fixed museum specimens of *Bothrimonus fallax* were available for study despite prolonged efforts to obtain fresh material, especially from Iran (see Discussion). Attempts to extract gDNA were made from a range of samples from museum collections, all with unknown type of fixation, but presumed to have had prolonged exposure to formalin and possibly additional harmful chemicals such as industrial methylated ethanol. These samples were homogenised in 1 ml of water using a Precellys 24 lysis and homogenisation machine (Bertin Technologies, Paris, France) with ceramic beads at 6 500 rpm for 23 s. Tissues were separated from the water and gDNA extracted using a QIAmp DNA FFPE tissue kit (Qiagen) specifically designed for formalin-fixed and paraffin-embedded samples.

A nested PCR approach was used in an attempt to amplify products from both the 28S and 18S rDNA genes. For both genes a large combination of primers was tried, targeting small (~350 bp) fragments from larger (~1 000 bp) initial PCR products (even when the initial products were not visible). However, this approach either failed to produce results or resulted in sequences amplified via nested PCR that proved to be contaminants (e.g. fungi). We therefore tried to design spathebothriidean-specific primers using the 18S and 28S alignments of Olson et al. (2008) to find priming regions that were putatively unique to spathebothriidean taxa (to avoid amplification of other organisms, including other tapeworms) and that would amplify very short (~100–200 bp) fragments (to accommodate highly fragmented gDNA samples) of highly variable regions of the 18S and 28S genes (e.g. 18S – V4, V7, V9).

Initial PCR amplification was done as above with PCR beads and 0.1–1 µl of the PCR products was used directly for secondary, nested amplifications using a combination of spathebothridean-specific primers. The cycling profile for nested PCR was as above except that only 25 cycles were run and the extension time was reduced to 30 s. Gradient PCR was also used to test a range of annealing temperatures between 55 °C and 60 °C. Because of the small fragment size, products were visualised on 3.5% agarose gels and cleaned using a Microcon YM30 centrifugal filter device prior to Sanger sequencing as described above.

Phylogenetic analyses

All bioinformatic and phylogenetic analyses were conducted using Geneious ver. 6.1.6 (Biomatters, Auckland, New Zealand; available from http://www.geneious.com). The closely related 'cestodarian' (Amphilinidea and Gyrocotylidea) and eucestode groups (Caryophyllidea) were considered as outgroups (Olson et al. 2001, 2008), but representative sequences showed little homology for the highly variable regions of 28S, 18S and ITS-2 sequenced. One exception was the 28S D1–D3 region of *Amphilina foliacea* that was similar in length and composition to the spathebothriidean sequences and was thus used as an outgroup in these analyses. Based on these results, *Spathebothrium simplex* was used as a functional outgroup for analyses of the ITS-2, whereas results of the 18S analyses based on only

100 bps of data were left unrooted. A nucleotide substitution model was chosen using MrModelTest (ver. 2.; Nylander 2008) and applied to both maximum likelihood and Bayesian analyses, and parsimony analyses were run with all changes given equal weight.

RESULTS

Taxonomic account

Several revisions of spathebothriidean tapeworms have been carried out (Gibson 1994, Protasova and Roitman 1995), but only one study (Burt and Sandeman 1969) synonymised the genera *Diplocotyle* Krabbe, 1874 and *Didymobothrium* Nybelin, 1922 with *Bothrimonus* Duvernoy, 1842. The validity of these genera was tested by morphological and molecular approach. The following species of the Spathebothriidea are considered to be valid based on a critical revision of published data combined with new data obtained in this study (all nominal taxa are available in the Global Cestode Database; Caira et al. 2014):

Family **Acrobothriidae** Olsson, 1872

Bothrimonus Duvernoy, 1842

Bothrimonus sturionis Duvernoy, 1842 – type species

Synonym: *Disymphytobothrium paradoxum* Diesing, 1854.

Type and only host: *Acipenser oxyrhynchus* Mitchill (Acipenseriformes: Acipenseridae).

Type locality: Wabasch River (near the mouth to the Ohio River), Mississippi basin, Ohio, USA.

Distribution: USA.

Morphological description: Duvernoy (1842), Protasova and Roitman (1995).

Remarks. *Bothrimonus sturionis*, the type species of the genus, was described from a single specimen collected from *Acipenser oxyrhynchus* in the USA by M. Lesueur in 1835. No other record of the species from sturgeons in North America exists and thus Markevich (1951) questioned the validity of the species, whereas Skryabina (1974) considered it to be a valid taxon. We concur with Protasova and Roitman (1995) that the misidentification of *Diplocotyle olrikii* from an atypical host is the most probable explanation for the record, but this assumption cannot be confirmed because the type material of *B. sturionis* is not known to be extant (J.-L. Justine, MNHN, Paris, France – pers. comm.). Therefore, the species is tentatively retained as valid in the present account.

Bothrimonus fallax Lühe, 1900 Figs. 1, 3, 11, 19

Synonyms: *Bothrimonus pachycephalus* Linstow, 1904; *Bothrimonus caspicus* Cholodkovsky, 1914.

Type host: *Acipenser ruthenus* Linnaeus (Acipenseriformes: Acipenseridae).

Additional hosts: *Acipenser gueldenstaedtii* Brandt et Ratzeburg, *A. nudiventris* Lovetsky, *A. persicus* Borodin, *A. stellatus* Pallas, *A. sturio* Linnaeus, *Huso huso* (Linnaeus).

First intermediate host: Freshwater gammarid *Dikerogammarus haematobaphes* (Eichwald) (Amphipoda: Gammaroidea) (Sudarikov and Kurochkin 1964).

Type locality: Black Sea off Romania.

Distribution: Caspian Sea (off coasts of Azerbaijan, Iran, Kazakhstan, Russia and Turkmenistan), River Kura (Azerbaijan) and River Volga (Russia), also sporadically reported from the Black Sea.

Morphological descriptions: Nybelin (1922), Protasova and Roitman (1995).

Selected references: Lühe (1900), Cholodkovsky (1914), Nybelin (1922), Dogiel and Bychowsky (1938), Izyumova (1977), Sattari (2002), Mamedova (2005), Sattari and Mokhayer (2005), Noei (2010, 2011).

Remarks. *Bothrimonus fallax* was described by Lühe (1900) from specimens collected by Volz (1899) off the Black Sea coast off Romania, but all other records are from the Caspian Sea (Nybelin 1922, Protasova and Roitman 1995, present study), especially from its southern parts off Azerbaijan and Iran (Mamedova 2005, Sattari and Mokhayer 2005, Noei 2010, 2011). The prevalence of *B. fallax* in sturgeons is usually low, only about 5% (Sattari and Mokhayer 2005, Noei 2011), with the highest prevalence up to 14% in summer (Sattari 2002).

Nybelin (1922) and Protasova and Roitman (1995) provided the most detailed morphological descriptions of the species, which is characterised by the presence of an incomplete septum dividing the lumen of the funnel-like scolex (Fig. 11) and genital pores that alternate, being located either on the dorsal or on the ventral side of the body. The life cycle has not been fully elucidated, but the freshwater gammarid *Dikerogammarus haemobaphes* has been identified as an intermediate host by Sudarikov and Kurochkin (1964).

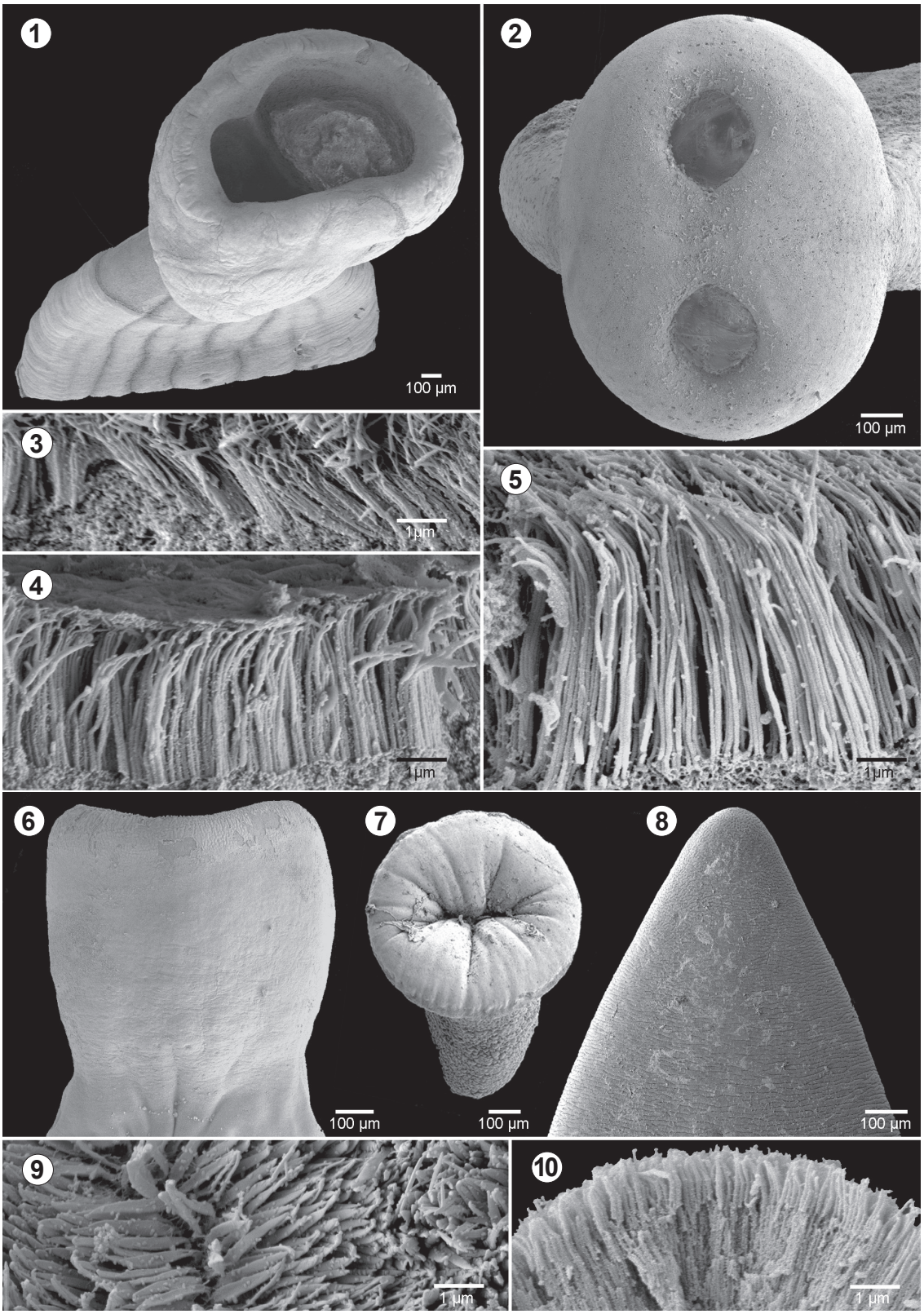
Bothrimonus fallax is mostly found in sturgeons during their marine life phase, but it has been found twice in sturgeons migrating upstream as far as 450 km from the coast (Mikailov 1963, Ivanov 1968). Some authors (e.g. Markov et al. 1967, Bauer et al. 2002) have reported younger fish to be more heavily infected than older ones, whereas others found young sturgeons to be entirely uninfected (e.g. Skryabina 1974, Sattari 2002).

Cyathocephalus Kessler, 1868

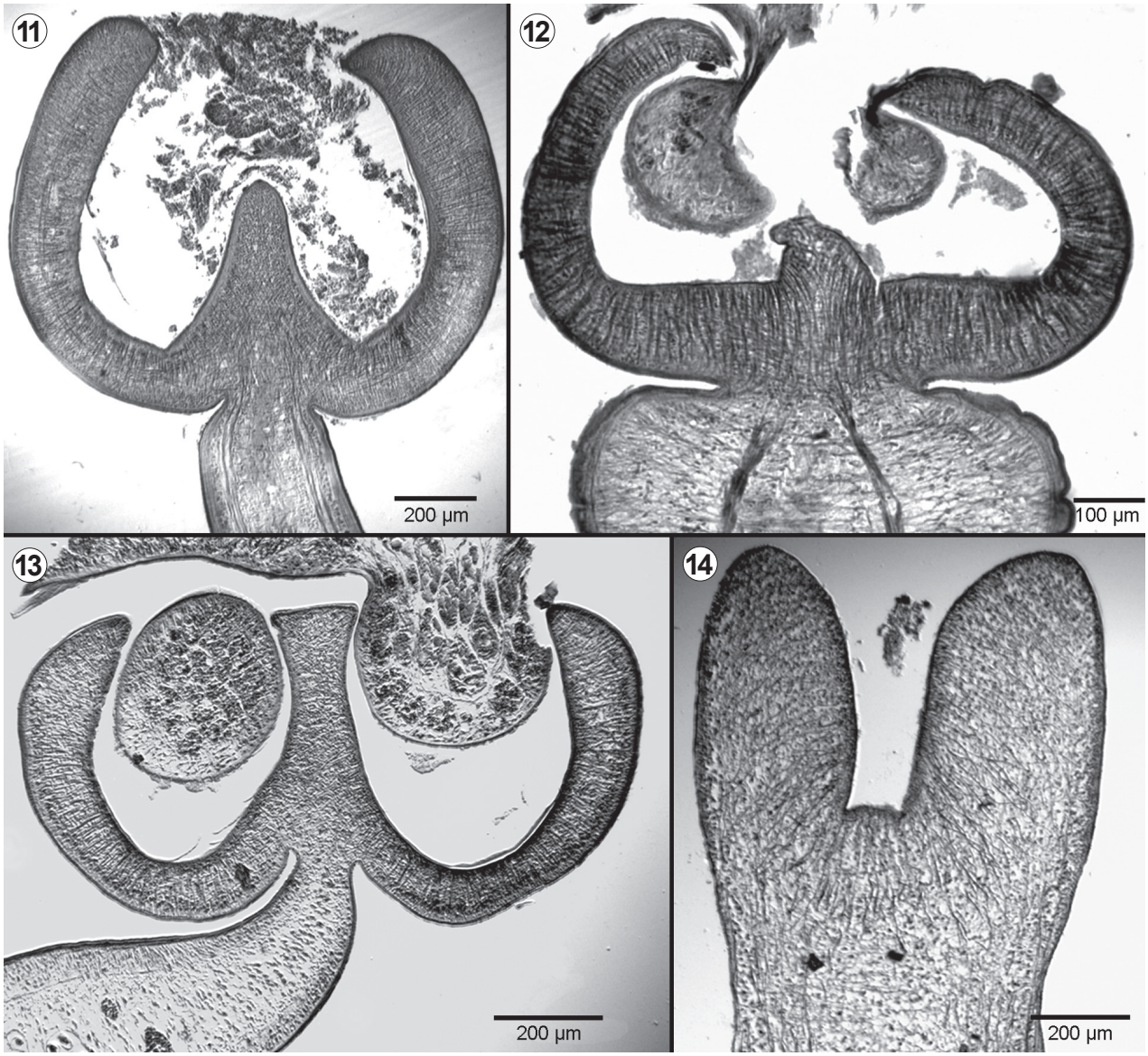
Cyathocephalus truncatus (Pallas, 1781) Kessler, 1868 – type and only species Figs. 6, 7, 9, 14, 20

Synonyms: *Taenia truncata* Pallas, 1781; *Vermis esocis lucii* Rudolphi, 1810; *Dubium esocis lucii* Rudolphi, 1819; *Cephalocotylum esocis lucii* Diesing, 1850; *Cyathocephalus tuba* (von Siebold, 1837) Bairol, 1853; *Acrobothrium typicum* Olsson, 1872; *Cyathocephalus truncata* Southwell, 1913; *Cyathocephalus americanus* Cooper, 1917.

Type host: *Esox lucius* Linnaeus (Esociformes: Esocidae).



Figs. 1–10. Spathebothriidean cestodes; scanning electron micrographs. **Figs. 1**, **3.** *Bothrimonus fallax* from *Acipenser nudiventris*, Caspian Sea, Russia. **Figs. 2**, **5.** *Diplocotyle olrikii* from *Myoxocephalus scorpius*, Petunia Bay, Svalbard, Arctic Ocean. **Fig. 4.** *Diplocotyle olrikii* from *Marinogammarus* sp., off St. Andrews, Scotland, UK. **Figs. 6**, **7**, **9.** *Cyathocephalus truncatus* from *Salmo trutta*, Brenta River, Italy. **Figs. 8**, **10.** *Spathebothrium simplex* from *Liparis fabricii*, Petunia Bay, Svalbard, Arctic Ocean. Scolex in apical view (1, 2, 7) and dorsoventral (6, 8). Surface of the scolex covered with capilliform filitriches, magnification 10 000× (3–5, 9, 10).



Figs. 11–14. Spathebothriidean cestodes; photomicrographs of histological sections through the scolex. **Fig. 11.** *Bothrimonus fallax* from *Acipenser nudiventris*, Caspian Sea, Russia. **Fig. 12.** *Didymobothrium rudolphii* from *Pegusa lascaris*, off Banyuls-sur-Mer, Mediterranean Sea, France. **Fig. 13.** *Diplocotyle olrikii* from *Myoxocephalus scorpius*, Petunia Bay, Svalbard, Arctic Ocean. **Fig. 14.** *Cyathocephalus truncatus* from *Salmo trutta*, River Brenta, Italy.

Additional hosts: Mostly salmonids and coregonids, such as species of *Salmo* Linnaeus, *Salvelinus* Richardson and *Coregonus* Linnaeus (see Protasova and Roitman 1995 for a comprehensive list of hosts).

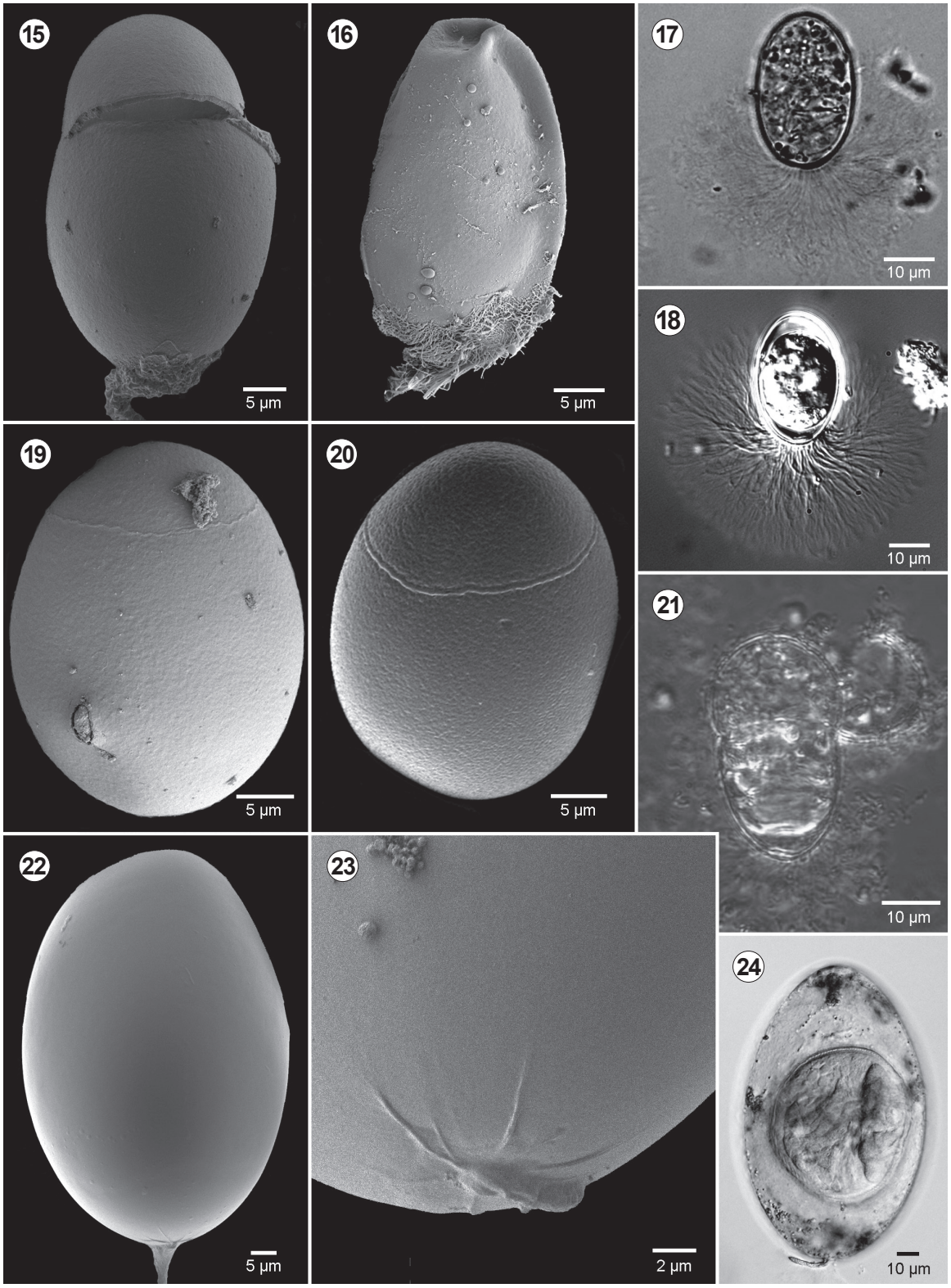
First intermediate hosts: Freshwater gammarids (Amphipoda: Gammaroidea), e.g. species of *Echinogammarus* Stebbing, *Eulimnogammarus* Bazikalova, *Gammarus* Linnaeus, *Gmelinoides* Bazikalova, *Pallasea* Sars, *Poekilogammarus* Stebbing, *Pontogammarus* Sowinsky, and *Pontoporeia* Krøyer. Amin (1978) reported a plerocercoid of *C. truncatus* from *Mysis relicta* Lovén (Amphipoda: Mysidae) (see Protasova and Roitman 1995 for a more exhaustive list).

Type locality: St. Petersburg, Russia.

Distribution: Europe (Bosna and Herzegovina, Estonia, Finland, France, Germany, Italy, Lithuania, Macedonia, Norway, Poland, Serbia, Slovakia, Sweden, Switzerland, UK, Ukraine), Asia (Mongolia, Russia – Far East, Karelia, Kola Peninsula, Krasnodar Region, Lakes Ladoga and Onega, Rybinsk Reservoir, Siberia) and North America (Canada – Alberta, British Columbia, Manitoba, Newfoundland, Northwest Territories, Ontario, Quebec; Greenland; USA – Alaska, Michigan, Montana, Oregon, Wisconsin).

Morphological descriptions: Nybelin (1922), Protasova and Roitman (1995).

Selected references: Cooper (1918), Nybelin (1922), Protasova and Roitman (1995).



Figs. 15–24. The eggs of spathebothriidean and amphilinidean cestodes; scanning electron micrographs and photomicrographs. **Fig. 15.** *Diplocotyle olrikii* from *Myoxocephalus scorpius*, Petunia Bay, Svalbard, Arctic Ocean. **Fig. 16.** *Spathebothrium simplex* from *Liparis fabricii*, Petunia Bay, Svalbard, Arctic Ocean. **Fig. 17.** *Diplocotyle olrikii* from *Marinogammarus* sp., off St. Andrews, Scotland, UK. **Figs. 18**, **21.** *Spathebothrium simplex* from *Liparis atlanticus*, off Rye Beach, New Hampshire, USA. Hatching of coracidium (21). **Fig. 19.** *Bothrimonus fallax* from *Acipenser nudiventris*, Caspian Sea, Russia. **Fig. 20.** *Cyathocephalus truncatus* from *Coregonus lavaretus*, Lake Segozero, Karelia, Russia; **Figs. 22–24.** *Amphilina foliacea* (Amphilinidea) from *Acipenser ruthenus*, River Danube, Slovakia.

Remarks. *Cyathocephalus truncatus* is the most common spathebothriidean, with prevalence up to 80–90% and intensity up to 80 parasites per fish (Dechtiar and Loftus 1965). It infects a wide spectrum of freshwater fish, especially salmonids and coregonids, but has also been reported from unrelated fish such as sturgeons (*Acipenser baerii* Brandt, *A. ruthenus* Linnaeus and *A. sturio*) in Europe and Asia (Skryabina 1974). The species is unique among spathebothriideans in its possession of a funnel-shaped apical organ without a septum.

Plerocercoids of *C. truncatus* may reach maturity in the intermediate host (gammarid amphipods). Progenetic, i.e. egg-bearing, plerocercoids were found in the body cavity of *Gammarus pulex* after 24 days of development at 25 °C (Okaka 2000). The tapeworm has been reported as a pathogen of fish, which may retard the growth of its host (Wiśniewski 1932, Vik 1958) or even cause the mass mortality of trout (Huitfeldt-Kaas 1927). It has been found that gammarids infected with *C. truncatus* were more susceptible to predation by alpine charr, *Salvelinus alpinus* (Linnaeus), than non-infected gammarids, probably because of parasite-induced alterations in behaviour or visibility (Knudsen et al. 2001).

Didymobothrium Nybelin, 1922

Didymobothrium rudolphii (Monticelli, 1890) Nybelin, 1922 – type and only species Fig. 12

Synonyms: *Cestoideum paradoxum* Rudolphi, 1819; *Cephalocotyleum soleae* Zschokke, 1887; *Cyathocephalus catinatus* Riggenbach, 1898.

Type host: Not explicitly mentioned in the original description, but *Solea solea* (Linnaeus) (Pleuronectiformes: Soleidae) was mentioned first in the original description.

Additional hosts: *Pegusa cadenati* Chabanaud (new host), *P. impar* (Bennett), *P. lascaris* (Risso), *Solea senegalensis* Kaup (all Pleuronectiformes: Soleidae).

Type locality: Mediterranean Sea, off Naples, Italy.

Distribution: Off Atlantic coasts of Europe and Africa (14–50°N, with the northernmost record from off Playmouth, UK and the southernmost record from off Cape Verde) and Mediterranean Sea – off coasts of Europe (France, Greece, Italy, Portugal, Spain, Turkey), off coast of Africa (Algeria).

Selected references: Orecchia et al. (1985), Renaud and Gabrion (1988), Álvarez et al. (2002), Marques and Cabral (2007), Marques et al. (2007), Oguz and Bray (2008).

Remarks. This species is a typical parasite of flatfishes of the family Soleidae. Its distribution is limited to the Atlantic and Mediterranean coasts of Europe and Africa (no reliable record from northern Europe or North America is available). Papoutsoglou (1976) and Sanmartín Durán et al. (1989) reported *D. rudolphii* from flatfishes of the family Scophthalmidae, namely *Lepidorhombus boscii* (Risso) and *L. whiffiagonis* (Walbaum), but these records are doubtful and need verification.

Marques et al. (2007) provided new morphological and molecular data for *D. rudolphii* from the sand sole *P. lascaris* collected off the Atlantic coast of Portugal. Based on analyses of 28S and ITS2, these authors detected two cryptic species, differing in their gene sequences and the seasonal pattern of occurrence north to south along the Portuguese coast. Renaud and Gabrion (1988) also reported the occurrence of cryptic species of *D. rudolphii* in the Mediterranean (which they referred to as *Bothrimonus nylandicus*) based on allozyme data. However, multivariate analysis of 20 morphological characters could only show one form to be more slender and elongate than the other with no discrete or consistent character differences found that could reliably distinguish individuals (Marques et al. 2007). Thus, neither Renaud and Gabrion (1988) nor Marques et al. (2007) chose to formally describe and name a new species, and the genus remains monotypic.

Recently, several ultrastructural studies on *D. rudolphii* have been published, with the main focus being on the spermiogenesis, vitellogenesis and the fine morphology of the ovary and eggs (Poddubnaya et al. 2007, Bruňanská and Poddubnaya 2010, Swiderski et al. 2010). The ultrastructural features support a view of the close relationships between the Spathebothriidea and the Diphyllobothriidea and the basal position of the Spathebothriidea within the Eucestoda. Two parallel rows of cortical microtubules were reported from the proximal part of the two-axoneme region of the spermatozoon for the first time in a eucestode (Bruňanská and Poddubnaya 2010). The comparative data demonstrate that vitelline material has unique features that may differ in species of three spathebothriidean genera and may be used for the recognition of separate taxa (Poddubnaya et al. 2006, 2007, Bruňanská and Poddubnaya 2010, Swiderski et al. 2010).

Diplocotyle Krabbe, 1874

Diplocotyle olrikii Krabbe, 1874 – type and only species Figs. 2, 4, 5, 13, 15, 17

Synonyms: *Bothrimonus nylandicus* Schneider, 1902; *Diplocotyle cohaerens* Linstow, 1903; *Bothrimonus intermedius* Cooper, 1917.

Type host: *Salvelinus alpinus* (as *Salmo carpio* Linnaeus – corrected by Zhukov 1963) (Salmoniformes: Salmonidae).

Additional hosts: This species has been reported from almost 50 species from 14 families (see Protasova and Roitman 1995), the most common being species of salmonids (Salmonidae), righteye flounders (Pleuronectidae) and scorpionfish (Scorpaenidae).

First intermediate hosts: Marine and estuarine gammarid amphipods (Amphipoda: Gammaroidea), e.g. species of *Anonyx* Krøyer, *Dogielinotus* Gurjanova, *Eogammarus* Birstein, *Gammarus* Fabricius, *Marinogammarus* Sexton et Spooner, *Monoporeia* Bousfield, *Psammonyx* Bousfield and *Spinulogammarus* Tzvetkova (listed alphabetically; see

Table 2. Prevalence of *Diplocotyle olrikii* Krabbe, 1874 in fishes in Petunia Bay, Svalbard.

Sampling dates	2008 20. 7.–10. 8.	2009 25. 7.–5. 8	2010 10. 8.–10. 9	2011 10. 7.–10. 8.	2012 1. 7.–15. 7.	2013 7. 8.–25. 8.	Total
Fish host				*Myoxocephalus scorpius*			
No. examined	10	96	145	92	116	66	525
Prevalence (%)	30	7	6	14	12	3	6.1
Fish host				*Gymnocanthus tricuspis*			
No. examined	0	31	73	20	20	21	165
Prevalence (%)	0	3	14	15	5	0	9.9

Protasova and Roitman 1995 for species list).

Type locality: Greenland (probably inland waters, but not specified in the original description).

Distribution: Circumboreal, i.e. arctic and subarctic brackish coastal water north of 40°N: Atlantic Ocean, off Europe (Finland, France, Germany, Island, Lithuania, Norway, Russia, Sweden, UK), Pacific Ocean, off Asia (Russia) and off North America (Canada, USA – Alaska).

Selected references: Burt and Sandeman (1969), Sandeman and Burt (1972), Protasova and Roitman (1995), Desdevises et al. (1998).

Remarks. This species was described from *Salvelinus alpinus* from Greenland (Krabbe 1874). It is a relatively common parasite of a wide spectrum of teleost fish, with salmonid and scorpaenid fish probably being the most suitable definitive hosts. Records from some fish hosts, such as eels [*Anguilla rostrata* (Lesueur) – Anguilliformes] and capelin [*Mallotus villosus* (Müller) – Osmeriformes], need verification and may represent accidental infections, as suggested by their very low prevalence (Protasova and Roitman 1995).

The species has a circumboreal distribution and is always distributed close to the coast in brackish water; no reliable records from fish taken far from the coast are known (Sandeman and Burt 1972). Several authors observed seasonality in the occurrence of adults in fish, with the highest prevalence being in summer, i.e. from June to August, with almost no infection being recorded in winter (Sandeman and Burt 1972, Haldorson 1984, Bouillon and Dempson 1989, present study). Examination of large numbers of two fish species, *Myoxocephalus scorpius* (Linnaeus) (525 specimens examined) and *Gymnocanthus tricuspis* (Reinhardt) (165 specimens; both Scorpaeniformes), from off Svalbard during six consecutive summer seasons (2008–2013) showed conspicuous fluctuations in the prevalence values between individual years, from 0 to 15% in *G. tricuspis* and 3 to 30% in *M. scorpius*, and a tendency for prevalence to decline from July to September was also observed (Table 2).

Plerocercoids were reported in gammarids between February and April in Scotland by Sandeman and Burt (1972) and present data, but Protasova et al. (2010) reported infections in amphipods from Sea of Okhotsk all year round. The prevalence of infection in gammarids is usually as low as about 0.1% (Sandeman and Burt 1972, present study), but Protasova et al. (2010) reported as much as 14% of amphipods infected.

Spathebothriidae Yamaguti, 1934

Spathebothrium Linton, 1922

Spathebothrium simplex Linton, 1922 – type and only species Figs. 8, 10, 16, 18, 21

Type host: *Liparis liparis* (Linnaeus) (Scorpaeniformes: Liparidae).

Additional hosts: *Careproctus roseofuscus* Gilbert et Burke, *Crystallias matsushimae* Jordan et Snyder, *Liparis atlanticus* (Jordan et Evermann), *L. callyodon* (Pallas), *L. coheni* Able, *L. fabricii* Krøyer (new host record), *L. fucensis* Gilbert, *L. gibbus* Bean (Scorpaeniformes: Liparidae).

First intermediate hosts: Marine gammarids *Locustogammarus locustoides* (Brandt) and *Megamoera dentate* Bate (Amphipoda: Gammaroidea) – (Protasova et al. 2010).

Type locality: Woods Hole, Massachusetts, USA, Atlantic Ocean (collected in 1904–1905).

Distribution: Arctic coastal marine waters, north of 35°N: North Atlantic Ocean (off Canada, USA), Arctic Sea (off Norway – Svalbard [new geographical record], Russia) and North Pacific Ocean (off Japan, USA – California and Washington, Russia – Chukotka, Sea of Okhotsk).

References: Linton (1922, 1941), Yamaguti (1934), Hart and Guberlet (1936), Polyanskii (1955), Zhukov (1963), Munson (1974), Linkletter (1977), Nahhas and Krupin (1977), Muzzall (1980), Appy and Burt (1982), Machida and Araki (1992, 1994), McDonald and Margolis (1995), Hansson (1998), Protasova et al. (2010).

Remarks. *Spathebothrium simplex* is a specific parasite of liparid fishes and have a circumboreal distribution. Detailed morphological descriptions were provided by Linton (1922) and Hart and Guberlet (1936). The parasite has also been reported, probably accidentally, from gadids (Gadidae) and eelpouts (Zoarcidae) (Linton 1941, Appy and Burt 1982, Machida and Araki 1992).

Plerocercoids of *S. simplex* have been found recently by Protasova et al. (2010) in two species of amphipods from the Sea of Okhotsk with a high prevalence (25%). The prevalence of infection in fish hosts may reach up to 75–100%, whereas the intensity of infection is usually only about two worms per fish (Munson 1974, Nahhas and Krupin 1977, Muzzall 1980, present study).

Table 3. Morphology of the eggs of spathebothriidean cestodes.

Species	Habitat	Filaments	Formation of oncosphere	Reference
Bothrimonus fallax	freshwater	absent	unknown	present study
Cyathocephalus truncatus	freshwater	absent	30 days	Okaka (1989)
Didymobothrium rudolphii	marine	present	unknown	Marques et al. (2007)
Diplocotyle olrikii	marine	present	5 days	Sanderman and Burt (1972), present study
Spathebothrium simplex	marine	present	8 days	present study

Morphology of attachment organs

The present study, based on the evaluation of new and museum material, made it possible to provide new data on the scolex morphology of five species of the order, including information from scanning electron micrographs and histological sections. Four scolex conditions can be recognised in individual genera:

(1) Anterior end of the body not differentiated – *Spathebothrium simplex* (Fig. 8).

(2) Single funnel-shaped organ – *Cyathocephalus truncatus* (Figs. 6, 7, 14).

(3) Forwardly directed sucker-like attachment organ with lumina completely separated internally by median septum – *Didymobothrium rudolphii* (Fig. 12; fig. 3A–B in Marques et al. 2007) and *Diplocotyle olrikii* (Figs. 2, 13).

(4) Forwardly directed sucker-like attachment organ with lumina completely fused and with only rudimentary septum at base – *Bothrimonus fallax* (Figs. 1, 11).

The scolex of spathebothriideans is uniformly covered by filitriches. Capilliform filitriches were observed using SEM for the first time on both *Bothimonus fallax* (although only material of poor-quality was available; length of filitriches ~2 μm) and *Spathebothrium simplex* (length up to 6 μm), and also on both *Diplocotyle olrikii* (length up to 10 μm) and *Cyathocephalus truncatus* (Figs. 3–5, 9, 10).

Egg morphology

Light and scanning electron microscopical observation of eggs liberated from the uterus of freshly collected *Diplocotyle olrikii* and *Spathebothrium simplex* and museum specimens of *Bothrimonus fallax*, *Cyathocephalus truncatus* and *Didymobothrium rudolphii* enabled us to present new data on egg morphology; these are summarised in Table 3. All spathebothriidean eggs have a large operculum and the surface is smooth or slightly roughened (Figs. 15–21). In addition, the eggs of *Amphilina foliacea* from *Acipenser ruthenus* were observed using SEM for comparison. Only a single polar filament was observed and an operculum is absent (Figs. 22–24).

Phylogenetic analyses

No differences in the sequences of any gene was found among individuals of the same species (see Discussion), and molecular data strongly supported the monophyly of each genus. Interrelationships of the genera (except for *Bothrimonus*) based on 28S rDNA (Fig. 25A) supported *Spathebothrium* as the most basal taxon, followed by *Diplocotyle*, and a sister relationship between *Cyathocephalus* and the two genotypes of *Didymobothrium* (see Marques et al. 2007). ITS-2 analyses (Fig. 25B), which used *Spathebothrium* as a functional outgroup, supported the same arrangement of (*Diplocotyle* (*Cyathocephalus* + *Didymobothrium*)). However, neither data partition strongly resolved the interrelationships between these genera, and comparison with outgroup sequences highlighted little homology between the tapeworm orders within these highly variable regions of rDNA.

Molecular characterisation and phylogenetic affinities of *Bothrimonus fallax*

Extraction of usable gDNA from formalin-fixed, 'historical' museum samples of *Bothrimonus fallax* (and other spathebothriidean species) proved almost impossible, even when attempting to amplify high-copy genes such as rDNA. Only a single, ~150 bp 18S product was amplified from one sample of *B. fallax* (from *Acipenser nudiventris*, Caspian Sea, Russia) via nested PCR using putatively spathebothriidean-specific primers Spath18S_1470F (GGTGGCGTTTCAGTGAGACTG) and Spath18S_1600R (GAACCCGGAAGTAAACGCT).

The validity of the sequence was examined by BLAST comparison after the removal of the priming regions (leaving 100 bps of unique sequence) demonstrating that it was both unique among all known sequences, and most similar to the 18S sequences of other spathebothriidean tapeworms. Pairwise comparisons showed it to be 92% similar to *Cyathocephalus truncatus*, 80% to *Diplocotyle olrikii*, 76% to *Spathebothrium simplex* and 69% to *Didymobothrium rudolphii* and an unrooted phylogeny (Fig. 25C) supported its placement as the sister genus to *Cyathocephalus*, thus rejecting the synonymy of the genera *Bothrimonus*, *Didymobothrium* and *Diplocotyle* by Burt and Sandeman (1969).

DISCUSSION

Morphology of attachment organs of cestodes is one of the key morphological characteristics that readily differentiate individual orders (Wardle and McLeod 1952, Schmidt 1986, Khalil et al. 1994). The most widely distributed forms of attachment organs are:

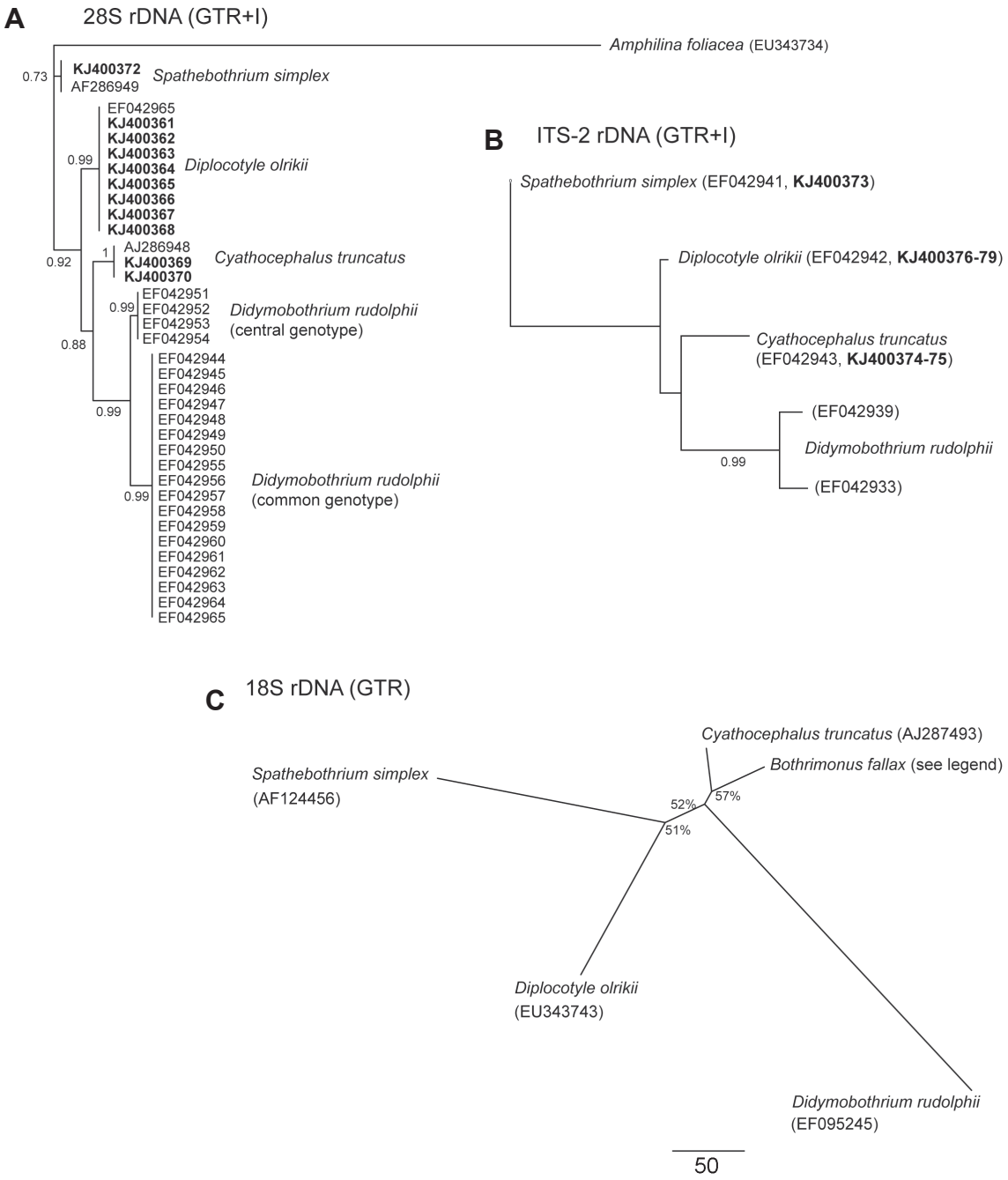


Fig. 25. Phylogenetic analyses of the Spathebothriidea. **A** – Bayesian inference tree based on partial 28S rDNA and rooted with *Amphilina foliacea* (Amphilinidea); posterior probabilities shown at nodes. **B** – Maximum likelihood topology based on ITS-2 data rooted with *Spathebothrium simplex* as a functional outgroup. **C** – Parsimony analysis of 18S rDNA (100 bps) with bootstrap support. Note the affinity of *Bothrimonus fallax* to *Cyathocephalus truncatus*, the only freshwater taxon in the group. Sequence accessions new to this study shown in bold. Partial sequence of 18S of *Bothrimonus fallax*: CCCTATTTAGCTGTCTTCCTAGTGGGTGCATTGTTGATCGGTAGCTTTGTGTTGCTGGTTGGTGGTGTATCTGTTGGTTGATGGTTGGGGTGGTTGACCT.

(i) bothrium-like attachment structures, i.e. grooves of different shape, size and depth on the ventral and dorsal sides of the scolex that do not consist of radial muscular fibres, are not membrane-bound and are thus not delimited from the surrounding parenchyma (Wardle and McLeod 1952); these are present in lower tapeworm orders, such as the Caryophyllidea, Diphyllobothriidea, Haplobothriidea, Trypanorhyncha, Diphyllidea and Bothriocephalidea; and

(ii) acetabulate structures, i.e. bothridia and suckers (in both cases, the attachment organ is membrane-bound and thus clearly delimited from surrounding tissues). The latter structures are typical of higher groups of tapeworms,

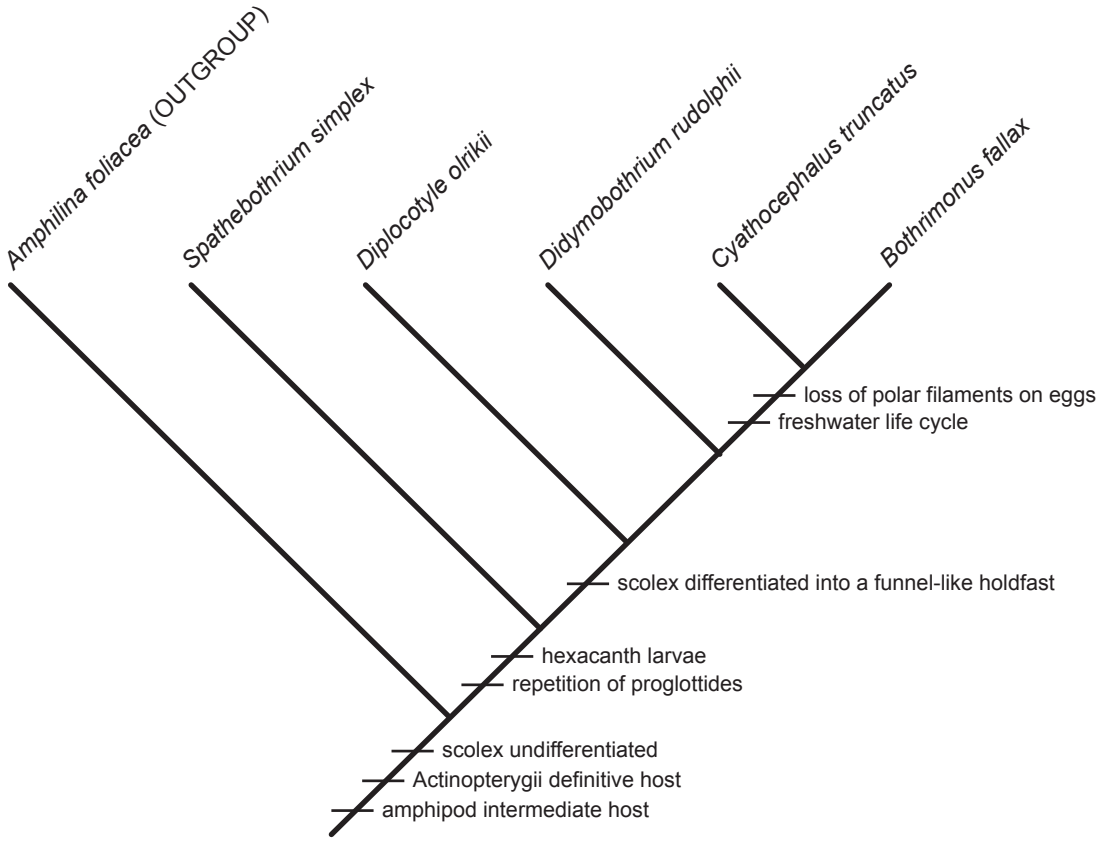


Fig. 26. Summary tree showing major synapomorphies of constituent groups of the Spathebothriidea.

such as the 'Tetraphyllidea-related' groups (including the Proteocephalidea), Tetrabothriidea and Cyclophyllidea (Olson et al. 2001).

In the Spathebothriidea, attachment organs open apically and thus differs markedly from those present in other cestode groups. These organs are paired or unpaired deep depressions that are separated completely or partly by a muscular septum. However, the absence of any differentiated structure at the anterior end of the most basal species, *Spathebothrium simplex*, indicates that a 'funnel-like' scolex evolved in the group later, and diversified rather modestly among the four other species. None of the above conditions appear to have been shared in the species of any extant cestode groups. It is thus reasonable to assume that the existing bothrium-like and acetabulate attachment organs, present in other eucestodes, except for caryophyllideans, represent evolutionary innovations that appeared after the divergence of the spathebothriideans.

The unique morphology of the attachment organs present in spathebothriideans (see Burt and Sandeman 1974 for detailed data on the scolex morphology) indicates their independent origin in this group, rather than support the results of rDNA-based studies of Olson and Caira (1999), Kodedová et al. (2000), Olson et al. (2001, 2008) and Waeschenbach et al. (2007), who found support for the Spathebothriidea as the earliest branching 'true' tapeworms (Eucestoda), implying that the monozoic body plan of the Caryophyllidea represents a secondary loss of strobilation in the adult phase of their life cycle.

However, support for the early branching pattern of tapeworms using ribosomal data has been both weak and conflicting between the small (18S) and large (28S) rDNA genes, and the Caryophyllidea has been alternatively supported as the earliest branching group by at least some of the analyses in the same studies. Most recently, Waeschenbach et al. (2012) added large regions of mitochondrial, protein-coding sequences to the rDNA data, and found that whereas rDNA data supported a basal position of the Spathebothriidea, mitochondrial data supported the Caryophyllidea as the earliest lineage, followed by the Spathebothriidea. Although these authors concluded that the weight of the evidence supports a most basal position for the Caryophyllidea (Waeschenbach et al. 2012), a robust solution for the early branching pattern continues to be problematical.

Two types of filitriches have been observed on the scolex of *Cyathocephalus truncatus* by Levron et al. (2008): small acicular filitriches up to 3 μm long and large capilliform filitriches measuring up to 10 μm in length. In *Diplocotyle olrikii*, Burt and Sandeman (1974) reported microtriches up to 4 μm long in plerocercoids from gammarids and adults from fish. In the same species, much

longer (up to 10 µm) capilliform filitriches were observed in the present study (Figs. 4, 5). The differences may have been caused by the measuring of filitriches from TEM and SEM photomicrographs. Marques et al. (2007) found microtriches of only 2 µm in length on the scolex and body of *Didymobothrium rudolphii*, whereas the surface of *S. simplex*, which was studied here for the first time, is covered with capilliform filitriches up to 6 µm long (Fig. 6). It can be concluded that the morphology of microtriches and their distribution are highly uniform in spathebothriidean cestodes, which appears to be a typical feature of more basal tapeworms (Levron et al. 2008, Chervy 2009).

The eggs of spathebothriideans are unique among cestodes in several aspects. They are mostly unembryonated (i.e. they do not contain a fully developed oncosphere with six hooks – a hexacanth) and operculate when laid, which resembles the situation in most caryophyllidean and some bothriocephalidean cestodes (Mackiewicz 1972, Kuchta et al. 2008). However, the eggs of spathebothriideans are unique in that the operculum is much larger (i.e. its diameter represents 20–40% of the total length of the egg) than those of other cestode groups (Bothriocephalidea, Caryophyllidea and Diphyllobothriidea), in which the diameter of the operculum always represents less than 10% of egg length, and usually only ~5% (Protasova and Roitman 1995; R.K. – unpubl. data).

Another unique character of spathebothriideans that has not been observed in any group of tapeworms is the presence of external filaments on the surface of the posterior pole of the eggs of the marine genera *Didymobothrium*, *Diplocotyle* and *Spathebothrium* (Burt and Sandeman 1969, Munson 1974, Marques et al. 2007, present study). The function of the filaments is most likely one of adhesion to the aquatic vegetation that is foraged by amphipods (Burt and Sandeman 1969). Interestingly, these structures have never been reported on the eggs of the freshwater species *B. fallax* and *C. truncatus* (Okaka 1989, Protasova and Roitman 1995, present study), which indicates a different mode of transmission.

Molecular data allowed us to test for intraspecific variation in *Diplocotyle* (n = 8) and *Spathebothrium* (n = 2) for the first time, using the same variable rDNA regions that readily separate cryptic species within the genus *Didymobothium* (see Marques et al. 2007). However, no variation was detected, even when comparing samples collected from opposite sides of the Atlantic. This could be a reflection of low sample sizes, as species of both *Spathebothrium* and *Diplocotyle* are exceedingly difficult to obtain in the wild, or, if taken at face value, suggests that each species is panmictic across its geographical range. Interrelationships of the genera are also difficult to assess due to a high level of divergence between the taxa, but there was at least consistency among the rDNA regions (Fig. 25). Based on analyses of 28S data, including an outgroup taxon (the cestodarian *Amphilina foliacea*), *Spathebothrium* is the most basal member of the group, whereas *Cyathocephalus* and *Didymobothrium* form the most derived clade. The 18S data, although only totalling 100 bps, allowed us to assess the interrelationships of all five genera with molecular data for the first time. This analysis suggests that *Bothrimonus*, represented by *B. fallax*, is most closely related to *Cyathocephalus*, and thus together form a freshwater clade of spathebothriideans. This affinity is also supported by their shared lack of polar filaments on the eggs (see above) (Fig. 26).

Over more than ten years, we have made numerous attempts to obtain fresh (i.e. live) specimens of *B. fallax* through contacts with parasitologists or ichthyologists in Eastern Europe and the Middle East. We have also enquired about the possibility of obtaining ethanol-preserved specimens from private or museum collections. However, no channel we pursued has been able to provide either fresh or ethanol-preserved specimens and it is highly likely that populations of *B. fallax* have been in decline for many years, together with most sturgeon populations, particularly in the Caspian Sea (Choudhury and Dick 2000, Bauer et al. 2002). For example, the most recent survey of sturgeon parasites in Iran found no *B. fallax* and reported an overall decline in parasite diversity (Sattari and Monkhayer 2005).

Consequently, this unique and interesting cestode species may well be on its way to extinction, even if its sturgeon hosts manage to survive current threats to their populations. It is therefore critical that any future collection of *B. fallax* minimally includes fixation of their genomic DNA (e.g. using ethanol), and ideally for examination of both gDNA and mRNA (e.g. using liquid nitrogen or RNALater (Qiagen)), as the information contained in these molecules represents the most promising historical record we have of this evolutionarily pivotal tapeworm lineage.

The relictual nature of the Spathebothriidea is indicated by their morphology, life history, biogeography and molecular data. We conclude that the five described genera, well separated by host ranges and disjunctive distributions, represent refugia of a previously more diverse and widespread clade of early-branching 'true' tapeworms, potentially the first to evolve proglottisation (i.e. repetition of the reproductive organs) as a strategy for increasing fecundity. Apart from the existence of two cryptic forms of *Didymobothrium rudolphii* detected by Renaud and Gabrion (1988) and Marques et al. (2007), we find no evidence to recognise multiple species in any of the genera, while providing multiple lines of data to support the distinctiveness and thus validity of the genera themselves. Having been recognised for more than 150 years, we suggest that these taxa are likely to represent the totality of extant spathebothriidean diversity, and that the sturgeon-hosted species *Bothrimonus fallax*, if still extant, may be close to extinction as a result of significant and prolonged declines in their host populations.

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