Morphological and molecular characterization of tetraphyllidean merocercoids (Platyhelminthes: Cestoda) of striped dolphins (*Stenella coeruleoalba*) from the Western Mediterranean

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SUMMARY

Two types of tetraphyllidean merocercoids, *Phyllobothrium delphini* and *Monorygma grimaldii*, are well known from most cetaceans world-wide. The role of cetaceans in the life-cycle of these merocercoids is unclear because their specific identity is as yet unknown. The problem is compounded by poor descriptions of both merocercoids. We used light and scanning electron microscopy, and histological techniques to provide a thorough description of merocercoids collected from 11 striped dolphins, *Stenella coeruleoalba*, from the Spanish Mediterranean. We also described, for the first time, specimens of *P. delphini* with immature proglottides. Our merocercoids were morphologically similar to those described previously, except in the structure of the apical organ. Intra- and inter-sample variability in the morphology of the apical organ suggested that it degenerates during larval development. A subsample of 16 specimens of *P. delphini* and *M. grimaldii* was characterized for the D2 variable region of the large subunit ribosomal RNA gene (LSU) and compared with published tetraphyllidean cestode LSU sequences. *P. delphini* showed 2 unique signatures that differed from one another by a single base, whereas all sequences of *M. grimaldii* were identical. This suggests that each type may represent a single species, contrary to previous speculations based on morphological data. All merocercoid specimens formed a clade together with *Clistobothrium montaukensis*. Based on the low degree of divergence, all specimens of this clade are predicted to be congeneric.

Key words: Tetraphyllidea, merocercoid, *Phyllobothrium delphini*, *Monorygma grimaldii*, *Clistobothrium montaukensis*, striped dolphin, molecular diagnostics.

INTRODUCTION

Tetraphyllidean merocercoids (terminology of larval cestodes follows Chervy, 2002) have been reported frequently from most cetacean species and some pinnipeds world-wide (Delyamure, 1955; Dailey & Brownell, 1972; Dailey, 1985; Bester, 1989; Raga, 1994, and references therein). Generally, 2 types have been recognized, i.e. *Phyllobothrium delphini* (Bosc, 1802) van Beneden, 1868, encysted in the subcutaneous blubber, usually in the abdominal area, and *Monorygma grimaldii* (Moniez, 1889) Baylis, 1919, encysted mainly in the peritoneum of the abdominal cavity. Both types of larvae have a scolex bearing an apical sucker and 4 monolocular bothridia with accessory suckers, but the scolex of *P. delphini* is large, has folded bothridia and is connected to a bladder through a short, thick filament, whereas the scolex of *M. grimaldii* is small, has bothridia with simple margins and is connected to the bladder through a very long and thin filament (Southwell & Walker, 1936; Skrjabin, 1970). Occasionally, other larval cestode types with bothridia lacking accessory suckers have been recorded, encysted in the subcutaneous blubber of some cetaceans (Markowski, 1955; Skrjabin, 1964; Siquier & Le Bas, 2003).

Even though *P. delphini* and *M. grimaldii* have been reported frequently from marine mammals in the last two centuries, there are surprisingly few accurate descriptions, especially of the scolex. This could be explained, at least in part, by the difficulty to recover the invaginated scolex intact, especially in the case of *M. grimaldii*, whose small scolex is invaginated at the end of a very thin and fragile filament. To date, only Mendonça (1984) has published

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histological sections of the scolex of *P. delphini* in which the gross morphology can be distinguished. Siquier & Le Bas (2003) were apparently the first to use scanning electron microscopy (SEM) to describe the scolex of *P. delphini*. Neither method has been employed in the case of *M. grimaldii*.

Most authors believe that *P. delphini* and *M. grimaldii* use marine mammals, especially cetaceans, as a means of infecting predatory or scavenging elasmobranchs (e.g. Southwell & Walker, 1936; Johnston & Mawson, 1939; Dollfus, 1964; Testa & Dailey, 1977; Walker, 2001). However, the specific identity of these larvae is not known. In the case of *P. delphini*, some authors (Guiart, 1935; Delyamure, 1955; Testa & Dailey, 1977) have recognized different morphotypes. Dailey (1985) suggested that these morphotypes may represent distinct species, whereas morphological uniformity in *M. grimaldii* suggests that these larvae represent a single species. There is, however, the possibility that observed 'morphotypes' represent different phases of development of the same species (Siquier & Le Bas, 2003). The use of molecular systematic techniques is necessary to help clarify these taxonomic issues.

During the parasitological examination of striped dolphins, *Stenella coeruleoalba* (Meyen, 1833), stranded on the Mediterranean coasts of Spain, a number of specimens of *P. delphini* and *M. grimaldii* were obtained. In this study we provide detailed morphological and morphometric descriptions of both types of larvae by using light microscopy, SEM, and fine histology. We also found specimens of *P. delphini* with immature proglottides and provide, for the first time, a description of this material, which may be useful for shedding light on the ontogenetic changes that occur in these larvae. Finally, we provide a molecular characterization of both larval types in order to further elucidate the identities and phylogenetic affinities of *P. delphini* and *M. grimaldii*.

MATERIALS AND METHODS

Morphological description

Eleven striped dolphins, *Stenella coeruleoalba*, stranded during 1998–2001 along the Western Mediterranean coast of Spain (between 40°13′N, 0°17′E and 37°52′N, 0°45′W) were necropsied for parasites. The sample consisted of 3 males (range of total length 155–193 cm) and 8 females (range of total length 154–211 cm). The subcutaneous blubber, peritoneum and mesenteries of the abdominal cavity were inspected immediately and a sample of the cestodes encysted removed while living. All striped dolphins were infected with both larval types. About 25 specimens of each type were collected from each dolphin (the total number of merocercoids per dolphin was not counted). All merocercoids were washed in saline (9‰), examined in a Petri dish with saline and

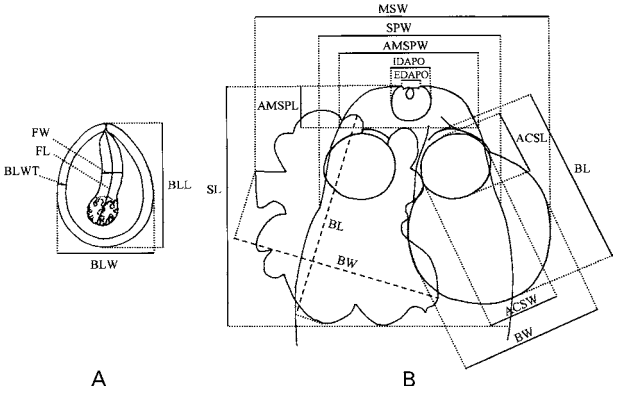

Fig. 1. Schematic drawings of a tetraphyllidean merocercoid showing the measurements taken in this study. (A) Specimen *in toto*. (B) Scolex (bothridium on the left – *Phyllobothrium delphini*; bothridium on the right – *Monorygma grimaldii*). For each specimen, measurements of bothridial structures were obtained from 2 bothridia and averaged. BLL, bladder length; BLW, bladder width; BLWT, bladder wall thickness; FL, filament length; FW, filament width; SL, scolex length; MSW, maximum scolex width; SPW, scolex proper width (measured at the level of the scolex mid-length); AMSPL, apical modification of scolex proper length; AMSPW, apical modification of scolex proper width; EDAPO; external diameter of the apical organ; IDAPO, internal diameter of the apical organ; BL, bothridium length; BW, bothridium width (equivalent to bothridium loculus width); ACSL, bothridial accessory sucker length; ACSW, bothridial accessory sucker width.

described under a stereomicroscope. The entire filament and scolex were subsequently removed from the cyst. The filament was cut near to the scolex and left in saline until the scolex was fully evaginated.

Three samples of merocercoids were used. The first sample was used to make observations under compound and stereomicroscopes, and to take morphometric measurements. The bladder and filament were drawn in 20 specimens of each morphotype. The bladder was drawn from live specimens that had been in the refrigerator for at least 1 h, allowing the worms to relax. The bladder of relaxed specimens acquired an oblong, flattened shape in all specimens, thus minimizing the potential deformation of the bladder in active animals. The filament and the width of the bladder wall were drawn in 70% (v/v) ethanol-fixed material. The scoleces of 20 live specimens of each morphotype from each of 2 dolphins were fixed in hot 70% (v/v) ethanol by shaking them vigorously in a circular movement. They were then stained with eosin, washed in tap water, dehydrated in 70% (v/v) ethanol and cleared with lactophenol. Temporary mounts were made on cavity slides to avoid deforming the scoleces. Drawings were made with the aid of a drawing tube connected to a compound or stereo light microscope. The internal diameter of the apical organ was visible only in 13 individuals of *P. delphini*. We measured 16 homologous metrics (Fig. 1) from drawings of each selected specimen.

Three specimens of *P. delphini* showing initial proglottization were fixed in 70% (v/v) ethanol and drawn. Characters described in Fig. 1 were measured from drawings of proglottized specimens, except for the internal diameter of the apical organ, which was not visible, and the bladder size because the bladder of these specimens was not collected intact. Seven proglottides of *P. delphini* were fixed in 70% (v/v) ethanol, stained with haematoxylin, dehydrated in an ethanol series, cleared in xylene and mounted in Canada balsam. Three additional proglottides were used to obtain transverse sections. Proglottides were processed as indicated above, and thick transverse sections were obtained by cutting the proglottides with a razor blade after they were hardened in xylene. All measurements are in micrometres unless otherwise stated.

A second sample of 5 specimens of *P. delphini* and 5 specimens of *M. grimaldii* was processed for observation with a vacuum scanning electron microscope (SEM) or an environmental scanning electron microscope (ESEM). Specimens were killed and fixed in a hot solution of 10% (v/v) formalin in Sörensen's phosphate buffer (0·1 M, pH 7·2). For SEM, merocercoids were dehydrated in an ethanol series, critical-point dried in liquid CO_2, mounted on specimen stubs using conductive carbon paint, sputter coated with gold-palladium to a thickness of 25–30 nm in a Bio-Rad Sc 500 coating unit and examined in a S-4100 SEM at 5 kV. Specimens for ESEM were washed in saline solution and observed directly with a Philips ESEM XL-30 with a gaseous secondary electron detector.

A third set of specimens was processed for histology. Seven scoleces of *M. grimaldii* and 10 of *P. delphini* were fixed in hot buffered 10% (v/v) formalin, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections (7–10 μm) were stained with haematoxylin and eosin, mounted in Entellan (Merck) and observed under a light microscope. Two proglottides of *P. delphini* fixed in 70% (v/v) ethanol were similarly processed.

Voucher specimens have been deposited in the helminth collection of the Natural History Museum, London (NHM; accession nos.: *Phyllobothrium delphini*, BMNH 2003.10.29.1-10; *Monorygma grimaldii*, BMNH 2003.10.29.11-20).

Molecular analysis

In addition to the collections described above, 8 specimens of *P. delphini* and 8 specimens of *M. grimaldii* from 3 host individuals were preserved in 95% ethanol for molecular diagnostic analysis. Specimens of *P. delphini* showing proglottization (see above) were excluded due to their poor condition of preservation, and scoleces were retained for vouchers prior to genomic DNA (gDNA) extraction and deposited in the helminth collection of the NHM (Accession nos. BMNH 2004.8.18.6-21). gDNA was extracted from the specimens using a Qiagen DNeasy™ tissue kit and used for PCR as described by Olson *et al.* (2003). A fragment (~1400 bp) of the nuclear large subunit ribosomal RNA gene (LSU; spanning domains D1-D3) was amplified using primers LSU5 (5′-TAG GTC GAC CCG CTG AAY T TA AGC-3′) and 1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′) and the middle portion spanning the variable D2 region (~650 bp) sequenced bidirectionally using internal primers 300F (5′-CA A GTA CCG TGA GGG AAA GTT-3′) and ECD2 (5′-CTT GGT CCG TGT TTC AAG ACGGG-3′). This region of the LSU has proven informative for both diagnostic and phylogenetic work in tetraphyllidean and related taxa (e.g. Brickle *et al.* 2001; Reyda & Olson, 2003). Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp., ver. 4), and leading and trailing regions of the sequences without overlap were removed prior to analysis. Sequences are available from GenBank under Accession nos. AY741591-1606.

Sequences were screened using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their orthology with the LSU genes of cestodes, and aligned by eye using MacClade ver. 4.06 (Maddison & Maddison, 2000) together with available tetraphyllidean LSU sequences (21 taxa). Phylogenetic affinities of the merocercoid sequences with previously characterized adult tetraphyllidean taxa ($n=19$) were estimated by Bayesian analysis using MrBayes ver. 3.b4 (Huelsenbeck & Ronquist, 2001). Based on the results of MrModeltest ver. 1.1b (Nylander, 2002; a simplified version of ModelTest by Posada & Crandall, 1998), a general time reversible model of nucleotide substitution incorporating among-site rate variation was specified, and the analysis run over 1 million generations, sampling topologies every 100th generation. Other program parameters were as specified in Olson *et al.* (2003). A consensus tree was constructed using the 'sumt' command with a 'burnin' value of 250 and the 'contype=allcompat' option. Trees were rooted using *Echeneibothrium maculatum* Woodland, 1927 based on prior analysis of tetraphyllidean and related LSU sequences (see Reyda & Olson, 2003). Comparisons of uncorrected genetic distances (shown parenthetically as the percentage difference; i.e. no. of substitutions/no. of sites compared*100) were calculated using PAUP* ver. 4.0b10 (Swofford, 2001) based on a re-alignment of taxa from the clade (marked with an asterisk in Fig. 5) including only the unique merocercoid sequences together with *Clistobothrium montaukensis* Ruhnke, 1993. The high similarity of these sequences necessitated only a single 1 bp insertion in the alignment.

Table 1. Measurements of *Phyllobothrium delphini* from the present study and the literature

(Scolex measurements have been taken from evaginated scoleces, except for those of Testa & Dailey (1977). Range (mean ± S.D.) [coefficient of variation (%)]. Measurements in micrometres unless otherwise stated.)

	Sample‡											
Variable†	I *n* = 20/40§	II *n* = 3	III *n* = ?/1#	IV *n* = 3	V *n* = 15	VI *N* = 10	VII *n* = 2	VIII *n* = ?	IX *n* = ?	X *n* = 2	XI *n* = 5	XII *n* = ?
BLL (mm)	5–15·1 (10·3 ± 2·5) [24·7]	—	10–12	5–8·5 (6·7)	4·5–10·2 (6·8)	4·9–11·2 (7·8)	5–7·7*	4–13·5	9–15	6–6·5	12–18	14–22·3 (16·7)
BLW (mm)	2·3–9·3 (5·9 ± 1·9) [31·4]	—	3–4	5–6 (5·5)	4–5 (4·8)	4–6·1 (5·3)	3·4–6·1*	3–7·5	5–8	4·5	6–10	5–9 (6·9)
BLWT	173–833 (479 ± 172) [35·8]	—	—	612–918 (734)	748–1400	—	—	—	—	500–800	—	120–260 (200)
FL (mm)	1·5–12·9 (7·4 ± 2·7) [36·4]	20·7–29·3 (24·2 ± 4·6)	—	6·3–9·6 (8·4)	2·4–4·7 (3·3)	2·7–4·1	—	c.8·5–20·5	0·4–1·4	2·5	12–14	5–12 (8·8)
FW	774–2564 (1634 ± 505) [30·9]	710–1002 (874 ± 149)	—	816–1305 (1074)	1050–2110 (1660)	—	—	—	—	—	2000–4000	1200–2800 (1780)
SL	1220–2300 (1640 ± 228) [13·9]	1571–2413 (1954 ± 426)	1400	1122–1428 (1265)	1050–1250 (1140)	2400–2700	1167–1500*	c.2400*	—	1200*	2000 2000*	1280–1650 (1435) 1143*
MSW	1600–3200 (2163 ± 361) [16·7]	1898–3175 (2609 ± 651)	1200	979–1305 (1189)	1120–1670 (1320)	1800–2100 2182*	1967–2000*	c.3200*	c.1730*	1356*	3000 3000*	1540–2000 (1760) 1643*
SPW	860–2227 (1385 ± 273) [19·7]	1255–1556 (1450 ± 169)	—	—	—	—	—	—	—	—	—	—
AMSPL	40–420 (197 ± 101) [51·3]	71–127 (93 ± 30)	—	—	—	—	167–267*	c.500*	—	133*	120 333*	250*
AMSPW	400–1130 (769 ± 157) [20·5]	694–825 (738 ± 76)	—	—	370–500 (430)	771	1033*	c.900*	—	467*	1000*	321*
EDAPO	40–130 (74 ± 20) [27·1]	65–159 (111 ± 47)	—	—	—	101*	67*	70 95*	56*	67*	c.130*	64*
IDAPO	50–170 (91 ± 30) [32·6]	—	—	71–97 (84)	78–112 (94)	103–123	167–200*	—	122*	—	120–150	240–400 (314) 143*
BL	1185–2090 (1471 ± 212) [14·4]	1434–2032 (1791 ± 316)	—	—	c.1240*	c.1310*	1083–1100*	1150 2000*	678–700 c.890*	1033*	1567*	884*

BW	1025–1880 (1303 ± 210) [16·2]	1102–1651 (1438 ± 294)	—	—	c.1100*	c.1140*	1017–1117*	500 1200*	867–886 c.940*	700*	1583*	955*
ACSL	220–325 (274 ± 29) [10·7]	273–349 (313 ± 38)	—	—	—	—	c.270–333*	276*	200*	244*	133–200*	277*
ACSW	230–360 (288 ± 33) [11·5]	333–382 (351 ± 27)	190	203–267 (239)	120–170 (144) c.210*	102–180 (138)	—	160–200 250*	207–226 206*	280 278*	200–350 242–250*	360–600 (442) 321*

† Abbreviations as in Fig. 1.
‡ Host and source of the sample: I. *Stenella coeruleoalba*, present study; II. *Stenella coeruleoalba*, present study (proglottized specimens); III. *Delphinus delphis*, Baer (1932); IV. *Delphinus delphis* and *Lagenorhynchus obscurus*, type 9 of Testa & Dailey (1977); V. *Delphinus delphis*, *Stenella coeruleoalba* and *Globicephala melas*, Raga (1985); VI. *Globicephala melas*, Balbuena (1991); VII. whales (host not specified), Skrjabin (1972); VIII. *Kogia breviceps*, Johnston & Mawson (1939); IX. *Physeter catodon*, type 6 of Delyamure (1955); X. *Physeter catodon*, Skrjabin (1964); XI. *Arctocephalus australis*, Southwell & Walker (1936); XII. *Arctocephalus pusillus*, Mendonça (1984).
* Measured from published drawing Raga (1985): measurements from Fig. 43 Balbuena (1991): measurements from Fig. 3.1.11 Skrjabin (1972): scolex measurements from Figs 4 and 5, bladder measurements from Figs 7 and 8 Johnston & Mawson (1939): measurements from Figs 13 and 14 Delyamure (1955): measurements from Fig. 129 Skrjabin (1964): measurements from Fig. 2 Southwell & Walker (1936): measurements from Figs 2 and 3 Mendonça (1984): measurements from Fig. 2.
§ Measurements of BLL, BLW, BLWT, FL and FW are based on 20 specimens; the remaining measurements are based on 40 specimens.
# Measurements of SL, MSW and ACSW are based on 1 specimen; the number of specimens used to measure BLL and BLW is not reported.

Terminology

Some of the morphological terms have been inconsistently and ambiguously used to describe *M. grimaldii* and *P. delphini*. For instance, many authors have used the term 'myzorhynchus' to refer to the apical region of the scolex of these larvae (Baer, 1932; Southwell & Walker, 1936; Dollfus, 1964; Testa & Dailey, 1977; Mendonça, 1984; Raga, 1985; Balbuena, 1991; Soulier, 1993; Siquier & Le Bas, 2003), but the use of this term has often been questioned (Wardle & McLeod, 1952; Euzet, 1959; Caira, Jensen & Healy, 1999). To avoid further confusion, in this study we have adopted the workable definitions provided by Caira *et al*. (1999) for some conflicting characters of the scolex. We used 'apical modification of scolex proper' for the apical region of *P. delphini* and *M. grimaldii* because this region is externally modified as a mobile, dome-shaped structure, and its tissue is internally continuous with that of the scolex proper (see Results). Likewise, we used 'apical organ' for the structure we observed on the tip of the scolex because there was a discrete, histological boundary between this structure and the surrounding tissue (Caira *et al*. 1999).

RESULTS

Morphological description

Phyllobothrium delphini (Bosc, 1802) van Beneden, 1868

Description of merocercoid (Table 1, Figs 2 and 3)

Bladder sub-spherical to ovoid, with scolex invaginated at end of short thick filament connected to bladder. Filament with 2 osmoregulatory canals running longitudinally along each side, forming zigzag pattern. Scolex with 4 monolocular bothridia with folded margins and round anterior accessory sucker, occupying 14·8–22·4% (18·8 ± 1·8%, $n = 40$) of bothridium length (Fig. 2A and B). Loop of osmoregulatory canal enters each bothridium and runs parallel to bothridium margin, at distance of about quarter of bothridial diameter. Each bothridium connected to scolex proper by short stout stalk (BS in Fig. 2C and D). Posterior half of bothridial loculus free. Anterior part of bothridium with free rims. Margins of bothridial loculus unite with upper part of accessory sucker (Fig. 2A and B).

Apical modification of scolex proper dome-shaped in frontal plane, conical in sagittal plane, and cruciform in apical view, with bothridia located between cross-arms. Apical modification of scolex proper neither invaginable nor retractable, but continuously moving and deforming in live specimens. Histological sections of this region reveal muscular bundles and some basophilic cells (Fig. 2E). No histological differences between apical region and scolex proper (Fig. 2C and E).

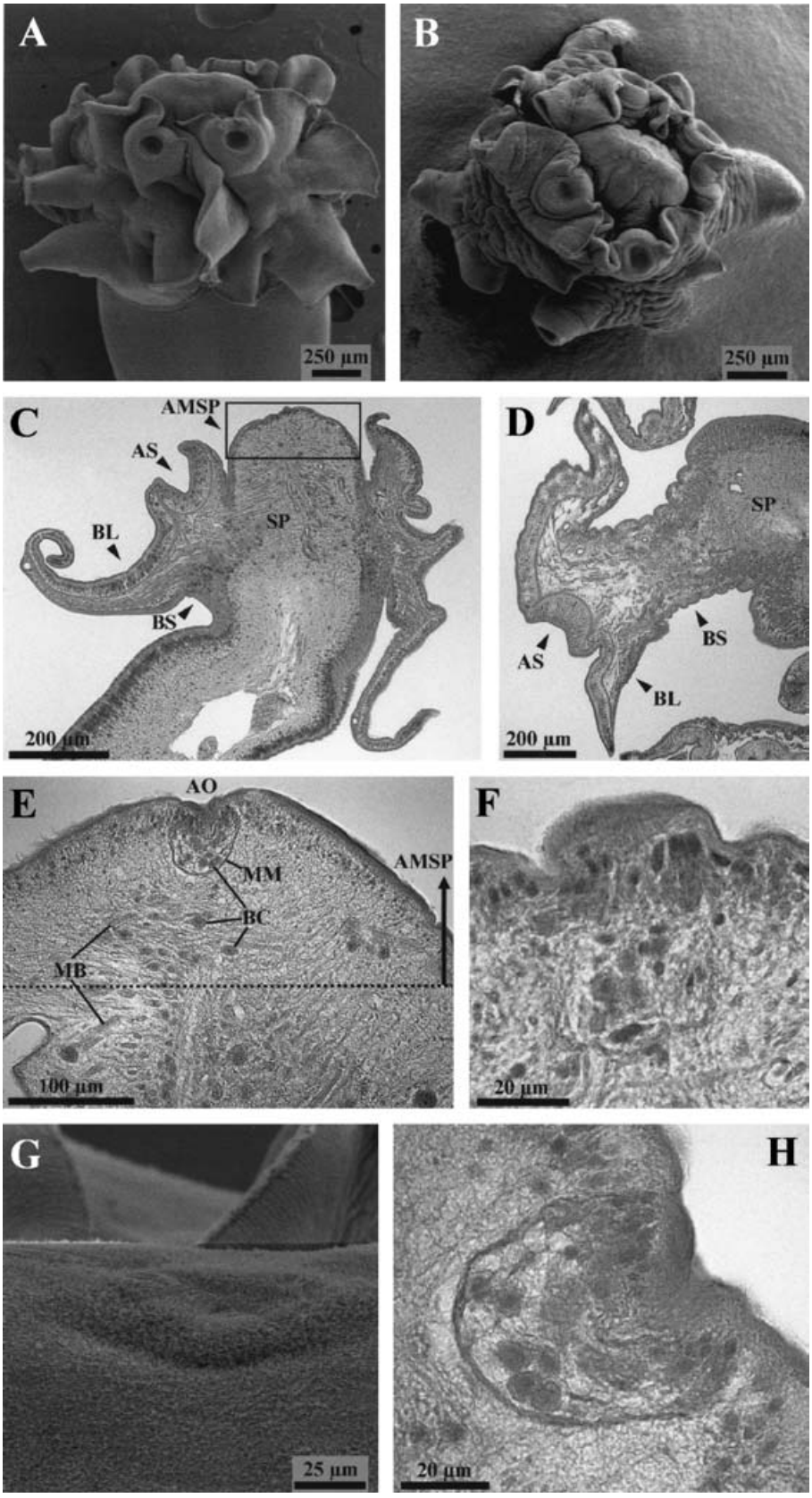

Fig. 2. Scanning electron micrographs and histological sections of the scolex of *Phyllobothrium delphini* collected from Mediterranean striped dolphins. (A) Lateral view of scolex; (B) apical view of scolex; (C) longitudinal section of the scolex; (D) transversal section of the scolex; (E) longitudinal section of the apical modification of scolex proper and the apical organ; (F) longitudinal section of the apical organ protruded; (G) apical organ (SEM); (H) longitudinal section of the apical organ resembling a collapsed cup. AO, apical organ; AMSP, apical modification of scolex proper; AS, accessory sucker; BL, bothridial loculus; BC, basophilic cells; BS, bothridial stalk; MB, muscular bundles; MM, muscular membrane; SP, scolex proper.

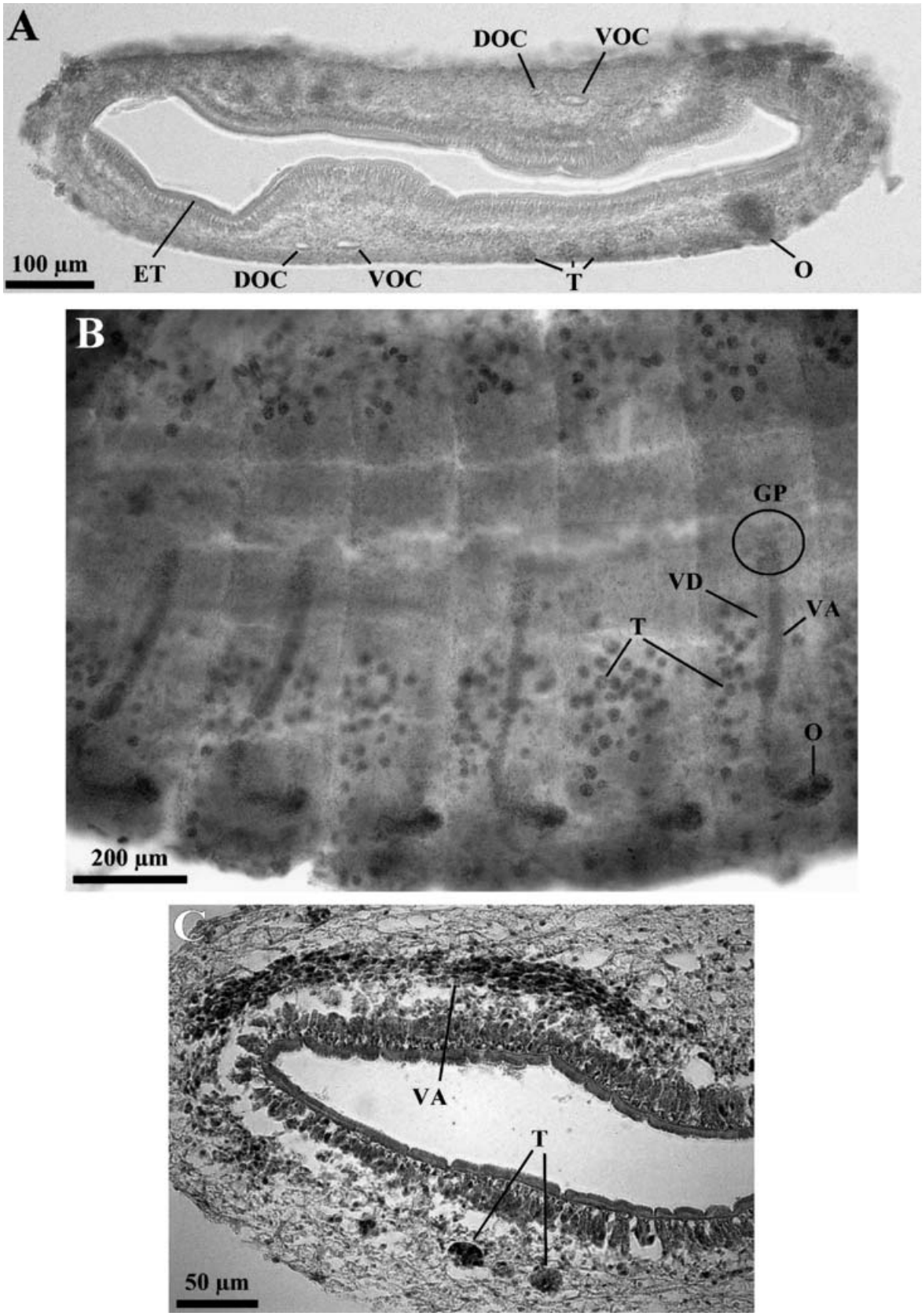

Fig. 3. Immature proglottides in a filament of *Phyllobothrium delphini*. The filament of this specimen is invaginated (i.e. the external tegument is internal), and laterally flattened (osmoregulatory canals appear running longitudinally along its central axis). (A) Transverse thick section of a proglottis; (B) whole mount; (C) transverse histological section of a proglottis. ET, external tegument; DOC, dorsal osmoregulatory canal; GP, approximate location of the genital pore; O, primordium of ovary; VD, primordium of vas deferens; T, testes; VA, primordium of vagina; VOC, ventral osmoregulatory canal.

Apical organ located at tip of apical modification of scolex proper. Some shape variability was found: in some individuals, apical organ resembled collapsed cup (Fig. 2E, G and H), whereas in other individuals it was protruded and more rounded (Fig. 2F). Internally, apical organ with sac-like structure delimited by thin muscular membrane, containing basophilic cells but not radial muscles (Fig. 2F and H).

Nine specimens of *P. delphini* collected from 1 striped dolphin showed initial proglottization along filament. In some individuals, only anterior part of filament proglottized, whereas in others the entire filament was proglottized. Proglottized individuals with filaments much longer than non-proglottized individuals (Table 1). Scolex of proglottized worms either evaginated or invaginated within filament, and morphologically very similar to that of non-proglottized individuals from same dolphin. Accessory suckers of bothridia occupying 16·6–19·0% ($17{\cdot}6 \pm 1{\cdot}3\%$, $n=3$) of bothridium length. Apical organ dome-shaped in all individuals. Scolex structures larger than in non-proglottized specimens (Table 1). Proglottis length increases further from scolex. Neck measured in 2 specimens 274 and 295 in length. Proglottides with genital primordia of male and female systems in most developed specimen are shown in Fig. 3. Round testes clearly distinguishable in 7 proglottides (Fig. 3A, B and C), numbering 90–134 (107 ± 18) per proglottis and measuring 20–31 (26 ± 3, $n=42$). Large group of cells (probably primordium of ovary) observed in middle of proglottides (Fig. 3A and B). Thick cord of cells (probably primordium of vagina) arises from ovary and runs transversally to lateral margin (Fig. 3B and C). Thin cord of cells (probably primordium of vas deferens) runs parallel to thick cord (Fig. 3B). According to arrangement of cell cords, genital pores appeared irregularly alternating (Fig. 3B).

Site: encysted in the subcutaneous blubber, especially in the posterior body half of striped dolphins.

Monorygma grimaldii (Moniez, 1889) Baylis, 1919
Description of merocercoid (Table 2, Fig. 4)
Bladder ovoid to almond-shaped, connected to internal long slender filament ending with invaginated scolex. In live specimens, filament tangled in fluid filled bladder; when larva fixed in 70% (v/v) ethanol, fluid precipitates around filament, such that it appears to be surrounded by amorphous porous parenchyma. Filament bears 2 osmoregulatory canals running longitudinally along each edge, tracing close zigzag pattern. Scolex bears 4 sessile monolocular bothridia with simple edges and anterior accessory sucker; accessory sucker occupies 25·6–41·1% ($31{\cdot}7 \pm 3{\cdot}3\%$, $n=40$) of bothridial length (Fig. 4A and B). Bothridia slightly tapered anteriorly, attached to scolex proper only by anterior part of bothridial loculus. Margins of bothridial loculus unite laterally with accessory sucker (Fig. 4A).

Apical modification of scolex proper large, subspherical, dome-shaped, neither invaginable nor retractable, but continuously moving and deforming in live specimens. Histological sections of this region showed muscular bundles and basophilic cells (Fig. 4C and D). No histological differences between apical region and scolex proper (Fig. 4C and D).

Apical organ located at tip of apical modification of scolex proper, cup-shaped in some individuals (Fig. 4E and F) and conical or knob-shaped in others (Fig. 4G and H). Histological sections revealed that apical organ has sac-like structure, is slightly muscular and with some basophilic cells but lacks radial muscle fibres, and is delimited from surrounding tissue by thin muscular membrane (Fig. 4F and H).

Site: Most worms encysted in the peritoneum of the abdominal cavity of striped dolphins; some of them also in the peritoneum of testes and the mesentery of the rectum and uterus.

Molecular analysis

A total of 586 characters was included in the analysis of the D2 variable region of the LSU gene, of which 250 were parsimony informative. Three unique LSU signatures were present among the merocercoid sequences, and a consensus tree resulting from Bayesian analysis (Fig. 5) showed that these formed a clade together with *C. montaukensis* and a previously published sequence of a metacestode collected from squid (*Loligo gahi*; see Brickle *et al.* 2001). Sequences of *P. delphini* were identical except for 1 which differed by a single G/A transition (0·16%), whereas all sequences of *M. grimaldii* were identical and differed from the LSU signature of *P. delphini* by only 3 C/T transitions (0·48%), the most common substitution class due to the regular formation of G-T, as well as G-C, pair bonds in the secondary structure of rDNA (see comparison of substitution classes in Olson *et al.* 2001). The genetic distance between the signature of *P. delphini* with that of *C. montaukensis* was 1·7% and between *M. grimaldii* and *C. montaukensis* was 1·1%, whereas between the metacestode from *Loligo gahi* and *C. montaukensis* was 0·32%.

DISCUSSION

The morphology of the tetraphyllidean merocercoids described in this study agrees well with the available descriptions of *P. delphini* and *M. grimaldii*, except for the structure of the apical organ. In some descriptions of both types of larvae, the apical organ was not described (Linton, 1905; Baer, 1932; Guiart, 1935; Garippa, Scala, & Pais, 1991). However, most authors described or illustrated the apical organ as an apical sucker (Southwell & Walker, 1936; Delyamure, 1955; Dollfus, 1964; Skrjabin, 1964;

Table 2. Measurements of *Monorygma grimaldii* from the present study and the literature

(Scolex measurements have been taken from invaginated scoleces, except for data of the present study and those of Skrjabin (1970). Range (mean ± S.D.) [coefficient of variation (%)]. Measurements in micrometres unless otherwise stated.)

	Sample‡					
Variable†	I $n = 20/40$§	II $n = 1$	III $n = 1$	IV $n = 15$	V $n = 5$	VI $n = ?$
BLL (mm)	5·7–27·3 (13·7 ± 5·4) [39·6]	—	25	18–25 (21)	18·5–26	9·8–25
BLW (mm)	3·9–11·5 (7·7 ± 2·3) [30·0]	—	—	15–18 (16)	13·2–20·5	6·6–21
BLWT	385–1312 (843 ± 241) [28·7]	—	—	890–1070	—	400–1000
FL (mm)	3–415·7 (151·8 ± 122·9) [81·0]	—	—	240–423 (286)	185–280	81–226
FW	178–390 (270 ± 64) [23·6]	—	246	520–630 (540)	350–820	500–1400
SL	498–869 (680 ± 76) [11·1]	517*	—	c.680*	250–442 c.660*	260–540 581*
MSW	414–877 (602 ± 100) [16·6]	c.320*	520	c.390*	530–670 c.370*	500–550 572*
SPW	172–549 (318 ± 76) [23·9]	—	—	—	—	c.400*
AMSPL	104–262 (183 ± 35) [19·1]	117*	—	167*	133*	188*
AMSPW	204–475 (334 ± 69) [20·7]	208*	—	226–288 (274) 207*	208–223 183*	309*
EDAPO	26–96 (57 ± 15) [27·0]	—	—	—	—	47*
IDAPO	53–131 (84 ± 20) [24·1]	—	97–102	100–125 (118)	96–110	68–74 75*
BL	375–599 (469 ± 60) [12·8]	367*	—	500–550 (530) c.450*	500–550 c.430*	250–390 356*
BW	220–480 (308 ± 55) [17·7]	c.120*	—	310–350 (340)	310–330	150–230 239*
ACSL	107–196 (148 ± 22) [15·0]	100*	—	67*	83*	76–114 127*
ACSW	133–248 (172 ± 25) [14·8]	100*	85–102	130–150 (139)	120–150	95–137 155*

† Abbreviations as in Fig. 1.

‡ Host and source of the sample: I. *Stenella coeruleoalba*, present study; II. *Lagenorhynchus acutus*, drawing of Baylis (1919) reproduced in Dollfus (1964); III. *Tursiops truncatus*, Dollfus (1964); IV. *Delphinus delphis*, *Stenella coeruleoalba*, *Tursiops truncatus* and *Globicephala melas*, Raga (1985); V. *Globicephala melas*, Balbuena (1991); VI. *Physeter catodon* and *Balaenoptera physalus*, Skrjabin (1970).

* Measured from published drawing: Dollfus (1964): measurements from Fig. 7 (Baylis, 1919); Raga (1985): measurements from Fig. 42; Balbuena (1991): measurements from Fig. 3.1.10; Skrjabin (1970): measurements from Fig. 1.

§ Measurements of BLL, BLW, BLWT, FL and FW are based on 20 specimens; the remaining measurements are based on 40 specimens.

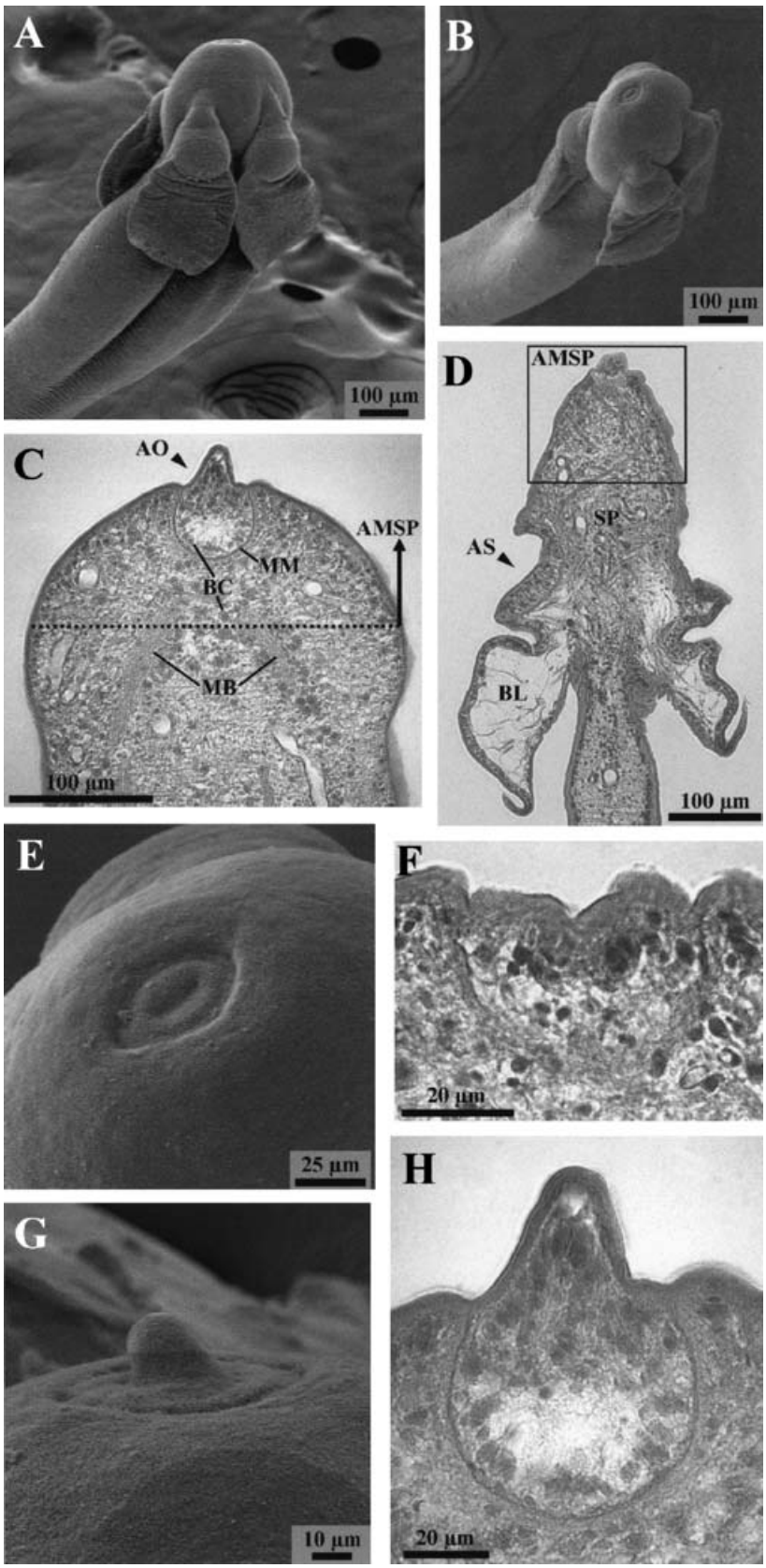

Fig. 4. Scanning electron micrographs and histological sections of the scolex of *Monorygma grimaldii* collected from Mediterranean striped dolphins. (A) Lateral view of scolex; (B) apical view of scolex; (C) longitudinal section of the apical modification of scolex proper and the apical organ; (D) longitudinal section of the scolex; (E) non-everted apical organ (SEM); (F) longitudinal section of a cup-shaped apical organ; (G) everted apical organ to form a knob-like structure (SEM); (H) longitudinal section of an everted apical organ. AO, apical organ; AMSP, apical modification of scolex proper; AS, accessory sucker; BL, bothridial loculus; BC, basophilic cells; MB, muscular bundles; MM, muscular membrane; SP, scolex proper.

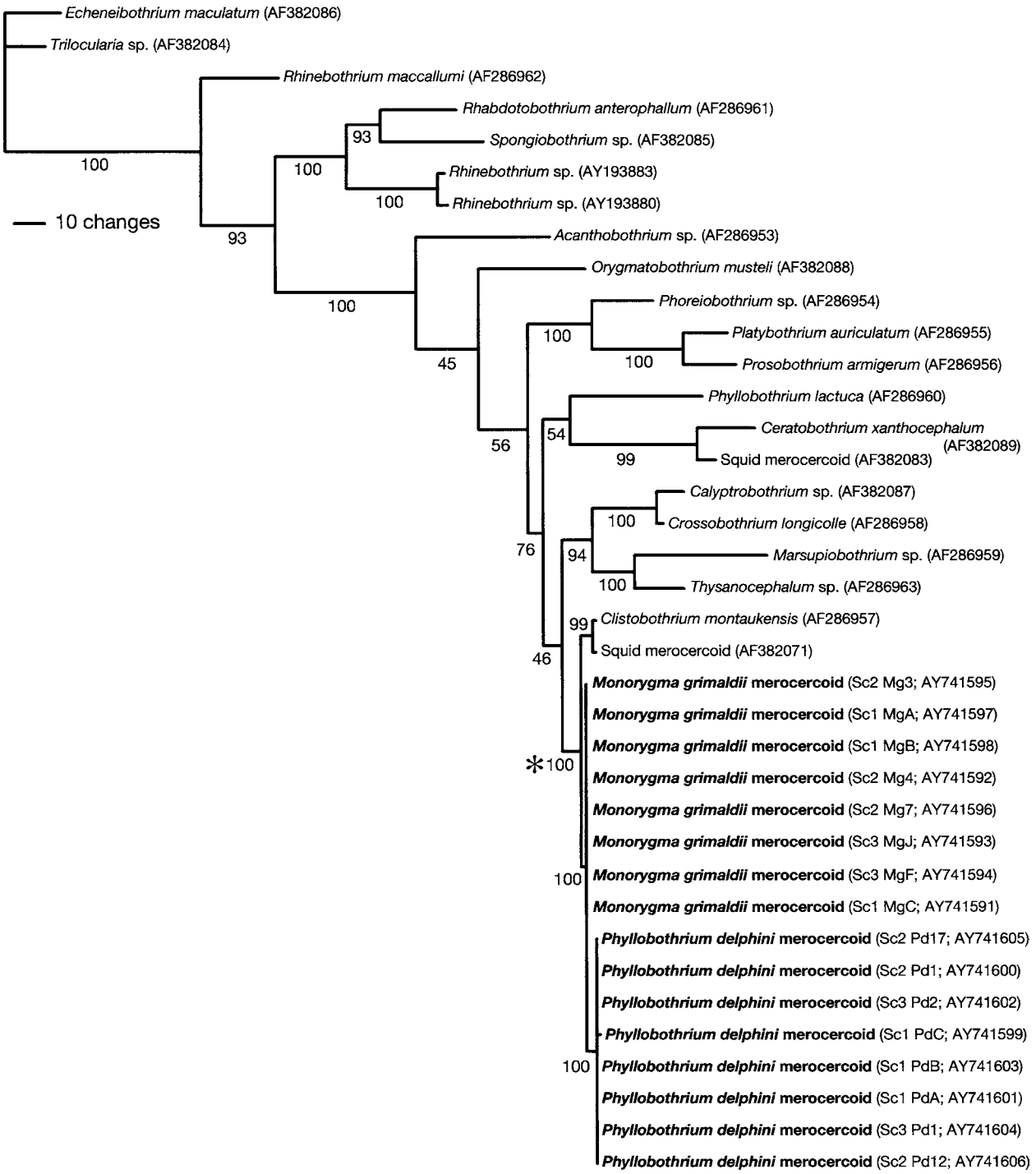

Fig. 5. Results of Bayesian analysis with nodal support shown as percentage posterior probabilities; N.B. branch lengths are proportional to observed character change, not as estimated by Bayesian analysis. Re-alignment and direct comparison of genetic distances were compared for the taxa inclusive of the clade indicated by an asterisk (see text). GenBank sequence Accession numbers shown parenthetically.

Skrjabin, 1970, 1972; Testa & Dailey, 1977; Mendonça, 1984; Raga, 1985; Balbuena, 1991; Siquier & Le Bas, 2003). In specimens of *P. delphini*, Johnston & Mawson (1939) described this organ as an apical plug. In our specimens, the apical organ always appeared as a sac-like structure delimited by a thin muscular membrane. However, we found structural variability even in specimens from the same individual dolphin; in some individuals, the apical organ resembled a collapsed cup, whereas in others it was dome-shaped. Although the causes of this variability are unclear, Hamilton & Byram (1974) described the *in vitro* development of plerocercoids of the onchobothriid *Acanthobothrium* sp. and found substantial changes in the structure of the apical sucker that are relevant for our case (see also Chambers, Cribb & Jones, 2000): in early development, a typical sucker (i.e. a cup-shaped structure with radial

musculature) was observed. Later on, the apical sucker began to degenerate, and subsequently collapsed and lost the radial muscles. Then, the degenerated sucker everted, acquiring a dome-shape appearance. Finally, the sucker detached from the scolex. The entire process was observed to occur at the larval stage since it was not accompanied by proglottization and sexual differentiation of worms (Hamilton & Byram, 1974). Accordingly, there is the possibility that the variability observed in the apical organ of *P. delphini* and *M. grimaldii* may result from a similar degenerative process (compare Figs 2E, F, H and 4C, F, H with Figs 8–12 in Hamilton & Byram, 1974). In fact, several authors (Southwell & Walker, 1936; Wardle & McLeod, 1952) suggested that the apical sucker of *P. delphini* disappears during development. It is therefore possible that the specimens described as having an apical sucker were in an early stage of development, while the specimens we have described were in intermediate phases of development.

Our re-descriptions of *P. delphini* and *M. grimaldii* provide the most complete account of morphometric data of these larvae to date and illustrate the range of morphological variation in specimens collected from one host species in a single locality. Minor differences of both *P. delphini* and *M. grimaldii* were found with respect to published data, but the ranges of morphometric variables generally overlapped. Moreover, part of the morphometric variability observed could be attributable to differences in the processing methods (see references in Table 1 and 2), or the degree of development of the larvae (Siquier & Le Bas, 2003).

Although diagnosing species via molecular analysis is still at an early stage, it is reasonable to expect that the low variation ($<0{\cdot}5\%$) observed within the samples of *P. delphini* may be well within the genetic variation of a single morphological species (see, e.g. Brickle *et al.* 2001), and may thus represent population-level differences. Based on previous descriptions and their own morphological data, Testa & Dailey (1977) defined 11 morphotypes of *P. delphini* that were suggested to represent different species (see also Dailey, 1985); up to 6 morphotypes were found in a single host species in the same locality. A cursory examination of samples of *P. delphini* from Mediterranean striped dolphins suggested the presence of at least 4 of the morphotypes described by Testa & Dailey (1977), yet genetic data point to the existence of only 2 genetic signatures that differ by a single base. It is likely therefore that the morphological variation reported by early authors (Guiart, 1935; Delyamure, 1955) and by Testa & Dailey (1977) may be intraspecific, at least in part, and perhaps associated with ontogenetic changes (see Siquier & Le Bas, 2003).

The names *Phyllobothrium delphini* and *Monorygma grimaldii* have been used historically as convenient labels to recognize the 2 types of merocercoids commonly found in marine mammals; however, the taxonomic validity of these names is questionable. Apparently, neither *P. delphini* nor *M. grimaldii* fulfill the current diagnosis of their putative genera (see Ruhnke, 1996 and Euzet, 1994, respectively). Obviously, the problem is that the generic concepts of tetraphyllideans are based on adults, so the use of diagnostic criteria for *P. delphini* and *M. grimaldii* can be justified only if their scolex is not modified through development to the adult stage. At present, we can state only that the scolex morphology of proglottized specimens of *P. delphini* does not differ substantially compared to that of non-proglottized specimens. Therefore, a molecular analysis is fundamental to positively match these larval forms with adult specimens of '*P. delphini*' and '*M. grimaldii*'.

Bayesian analysis indicated that both types of merocercoids cluster together with adult specimens of *C. montaukensis* collected from sharks off the Western Atlantic coast of Montauk, New York, and with some metacestodes collected from squid in the Falkland Islands (Brickle *et al.* 2001); all these taxa are separated by slight genetic distances. Bayesian analysis also indicated that *P. delphini* is far separated from *Phyllobothrium lactuca* van Beneden, 1849, the type species of the genus *Phyllobothrium* van Beneden, 1849. These results have 2 interesting taxonomic implications. First, the analysis suggests that *P. delphini* should be removed from the genus *Phyllobothrium*. Second, it is premature to determine whether the low degree of genetic divergence observed in the clade containing *P. delphini*, *M. grimaldii*, *C. montaukensis* and the squid metacestode represent population, strain or species-level differences but, based on the levels of inter-generic genetic distances as evident in Fig. 5, it might be reasonable to assume that all of these taxa are congeneric. In the case of *P. delphini*, this suggestion seems to be supported by morphological data. If we assume that the structure of the scolex of proglottized specimens of *P. delphini* does not change substantially in the definitive host (see Freeman, 1973), the morphology of the scolex would fit with the description given by Ruhnke (1993) in the generic diagnosis of *Clistobothrium* Dailey & Vogelbein, 1990: 'scolex with 2 dorsal and 2 ventral pedunculate bothridia and dome-shaped or cruciform apical region. Myzorhynchus absent. Each bothridium with single apical, muscular, round sucker. Posterior loculus foliose or folding flap of tissue'. Thus, the only difference between *P. delphini* and the adult of species of *Clistobothrium* is that the latter lacks an apical organ. However, we have suggested above that the apical organ degenerates during the larval development and may disappear at the adult stage. Our taxonomic suggestion agrees with the opinions of several authors (Southwell & Walker, 1936; Johnston & Mawson, 1939; Wardle &

Mc Leod, 1952), who speculated that *P. delphini* might actually be *Clistobothrium tumidum* (Linton, 1922) Ruhnke, 1993 (syn. *Phyllobothrium tumidum*) based on the morphological resemblance of the scolex. Our taxonomic suggestion is also compatible with ecological data. The 3 known species of *Clistobothrium* are restricted to large pelagic sharks of the family Lamnidae (mackerel sharks) that are known to feed on cetaceans and pinnipeds, as well as on cephalopods (Linton, 1922; Dailey & Vogelbein, 1990; Ruhnke, 1993). In addition, species of *Clistobothrium* have been reported in localities of the Pacific, the Atlantic and the Mediterranean basins (Linton, 1922; Euzet, 1959; Dailey & Vogelbein, 1990; Ruhnke, 1993), where *P. delphini* and *M. grimaldii* are also known to occur (see Raga, 1994, and references therein).

The generic assignation of *M. grimaldii* is much more problematic. The scolex morphology of these merocercoids is very different from that of *P. delphini* and that of *Clistobothrium* (the scolex morphology of squid metacestodes from Falkland Island has not been described, see Brickle *et al*. (2001)). We were unable to find proglottized specimens of *M. grimaldii*, so we ignore whether there might be substantial changes in scolex morphology when merocercoids begin proglottization. However, according to the slight differences observed between non-proglottized and proglottized specimens of *P. delphini*, we should not expect deep transformations in proglottized *M. grimaldii*.

In summary, our study provides, for the first time, a detailed morphological analysis of *P. delphini* and *M. grimaldii*, and a description of proglottized specimens of *P. delphini*. We also provide the first molecular analysis of the 2 larval types, which reveals that little variability exists within each type, and that both types might be congeneric with *C. montaukensis*. This suggestion is supported by morphological and ecological data in the case of *P. delphini*, but not in the case of *M. grimaldii*. The lack of congruence between molecular and morphological analyses will be solved definitively when a complete phylogenetic tree of the Tetraphyllidea, as well as molecular data from adult forms of Mediterranean species of tetraphyllideans, are available.

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