Molecular and morphological characterization of *Bolbosoma balaenae* (Acanthocephala: Polymorphidae), a neglected intestinal parasite of the fin whale *Balaenoptera physalus*

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Abstract

Post-mortem examination of a fin whale *Balaenoptera physalus* stranded in the Mediterranean Sea led to the finding of *Bolbosoma balaenae* for the first time in this basin. In this work, we describe new structural characteristics of this parasite using light microscopy and scanning electron microscopy approaches. Moreover, the molecular and phylogenetic data as inferred from both ribosomal RNA 18S-28S and the mitochondrial DNA cytochrome oxidase *c* subunit 1 (*cox*1) for adult specimens of *B. balaenae* are also reported for the first time. Details of the surface topography such as proboscis's hooks, trunked trunk spines of the pre-bulbar foretrunk, ultrastructure of proboscis's hooks and micropores of the tegument are shown. The 18S + 28S rRNA Bayesian tree (BI) as inferred from the phylogenetic analysis showed poorly resolved relationships among the species of *Bolbosoma*. In contrast, the combined 18S + 28S + mtDNA *cox*1 BI tree topology showed that the present sequences clustered with the species of *Bolbosoma* in a well-supported clade. The comparison of *cox*1 and 18S sequences revealed that the present specimens are conspecific with the cystacanths of *B. balaenae* previously collected in the euphausiid *Nyctiphanes couchii* from the North Eastern Atlantic Ocean. This study provided taxonomic, molecular and phylogenetic data that allow for a better characterization of this poor known parasite.

Introduction

Polymorphid acanthocephalans belonging to the genus *Bolbosoma* Porta, 1908 comprise 12 valid species (Amin, 2013). Of these, at least 10 (all described at the adult stage) have been reported in the intestinal tract of a range of oceanic whales and dolphins (Amin, 2013; Felix, 2013). The life cycle of *Bolbosoma* species has been not yet completely elucidated. However, it has been suggested that pelagic crustaceans (euphausiids and copepods) and fishes serve as intermediate and paratenic hosts, respectively (Measures, 1992; Hoberg *et al.*, 1993; Dailey *et al.*, 2000; Gregori *et al.*, 2012). Marine mammals serve as definitive hosts; they become infected by ingestion of infected preys. In marine mammals, the species of *Bolbosoma* may cause different degrees of enteritis due to their ability to perforate mucosal surface for anchoring to the muscular layer (Parona, 1893; Porta, 1906; Dailey *et al.*, 2000; Arizono *et al.*, 2012; Kaito *et al.*, 2019).

Bolbosoma balaenae (Gmelin, 1790) Porta, 1908 type species, has been described as *Sipunculus lendix* Phipps, 1774 in a sei whale *Balenoptera borealis* Lesson, 1828 from the Arctic waters. After its original description, *B. balaenae* was reported as sporadic finding in four oceanic odontocetes [i.e. the northern bottlenose whale *Hyperoodon ampullatus* Lacépède, 1804, spinner dolphins *Stenella longirostris* Gray, 1828, spotted dolphins *S. attenuata* Gray, 1846, and the pygmy sperm whale *Kogia breviceps* Golvan, 1961 (Gregori *et al.*, 2012; Felix, 2013)] and, at least, in other five mysticetes species as regular hosts: the common minke whale *B. acutorostrata* Lacépéde, 1804, the fin whale *B. physalus* Linneus, 1758, the blue whale *B. musculus* Linneaus, 1758, the humpback whale *Megaptera novaeangliae* Borowski, 1781, and the grey whale *Eschrichtius robustus* Lilljeborg, 1861 (Golvan, 1961; Zdzitowiecki, 1991; Dailey *et al.*, 2000; Felix, 2013). Regarding its geographical distribution, *B. balaenae* is known from Antarctic and Arctic waters, Southwest Atlantic Ocean, Tasman Sea and northern California coast to date (Zdzitowiecki, 1991; Dailey *et al.*, 2000; Gregori *et al.*, 2012; Felix, 2013).

The identification of *Bolbosoma* species is hardly based on the morphological characters alone, because of its similarities with congeneric species, and/or the old poor original description and redescriptions (Phipps, 1774; Van Cleave, 1953). Moreover, the presence of a wide variability of morphological characters of the anterior extremity in *Bolbosoma* spp. has been

reported (Porta, 1906; Meyer, 1933; Van Cleave, 1953; Petrochenko, 1956; Zdzitowiecki, 1991). Likely, due to the old, opportunistic and scattered findings around its geographical range, *B. balaenae* remains a little known parasite: no microscopic images and molecular data exist for adult specimens of *B. balaenae*. Moreover, interest in *Bolbosoma* species increased recently by reason of their potential zoonotic role. At least, eight cases of human infection with *Bolbosoma* sp. and a case for *B. capitatum* causing clinical signs and intestinal lesions have been reported from Japan and related to the consumption of uncooked fish flesh (Arizono *et al.*, 2012; Kaito *et al.*, 2019).

Aims of the present study were to: (1) report the first occurrence of *B. balaenae* from a fin whale in the Mediterranean Sea; (2) describe new morphological characters of the species by using traditional microscopy and scanning electron microscopy (SEM); (3) carry out the molecular characterization of the species and to study its phylogenetic relationships with congener species and other polymorphid species maturing in marine hosts.

Materials and methods

Parasitological study

An immature female fin whale measuring 14.4 m in total length was found stranded in a cove of Capri Island (Tyrrhenian Sea) in southern Italy on 8 November 2020. At necropsy, approximately 1 m of duodenum showing the occurrence of acanthocephalans embedded into the intestinal wall or free in the lumen was cut and moved to the laboratory, where parasites were counted, rinsed in saline solution and preserved in ethanol 70% or frozen (−20°C) for morphological and molecular analyses, respectively. Morphological measurements were obtained from 20 relaxed adult specimens (10 females and 10 males) using a compound microscope and a stereomicroscope equipped with ZEN 3.1 imaging system (Zeiss). To study the proboscis and the pattern of hook spination, the bulb of acanthocephalans was dissected using scissors and tweezers under the stereomicroscope, and proboscis and neck were displayed and clarified in Amman's lactophenol. To study the testes and cement glands, the male specimens were dissected and organs were displayed and measured under the stereomicroscope. Acanthocephalans were morphologically classified following the identification keys proposed by Meyer (1933), Van Cleave (1953) and Petrochenko (1956). Copromicroscopic examination was performed on a sample of feces obtained from the rectum and a standard flotation method with Sheather's sucrose solution (specific gravity 1.27) was used to detect and measure parasite eggs.

For SEM, the anterior portion of five acanthocephalan specimens was also fixed overnight in 2.5% glutaraldehyde, then transferred to 40% ethanol (10 min), rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO_4 for 2 h, and dehydrated in ethanol series, critical point dried and sputter-coated with platinum. Observations were made using a JEOL JSM 6700F scanning electron microscope operating at 5.0 kV (JEOL, Basiglio, Italy).

Molecular and phylogenetic analyses

Caudal portion of 10 specimens of *B. balaenae* (comprising three specimens studied for SEM) was used for molecular analyses. Total genomic DNA from ∼2 mg of each specimen was isolated using the Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the standard manufacturer-recommended protocol.

Two regions (18S and 28S) of the nuclear ribosomal RNA (rRNA) and a fragment of the mitochondrial DNA (mtDNA *cox*1) were amplified. The near-complete small subunit (ssrDNA, 18S) (∼1800 bp) was amplified using the forward

5′-AGATTAAGCCATGCATGCGT-3′ and reverse 5′-GCAGGTT CACCTACGGAAA-3′ primers (Garey *et al.*, 1996; García-Varela *et al.*, 2002, 2013). The near-complete large subunit (lsrDNA, 28S) (∼2900 bp) was amplified using two overlapping PCR fragments of 1400–1500 bp. Primers for the amplicon 1 were forward 5′-CAAGTACCGTGAGGGAAAGTTGC-3′ and reverse 5′-CTT CTCCAAC(T/G)TCAGTCTTCAA-3′; primers for the amplicon 2 were forward 5′-CTAAGGAGTGTGTAACAACTCACC and reverse 5′-CTTCGCAATGATAGGAAGAGCC-3′ (García-Varela and Nadler, 2005). A partial (∼700 bp) sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) was amplified using the primers LCO1490 (5-GGTCAACAAATCATAAAGATATTGG-3) and HCO2198 (5-TAAACTTCAGGGTGACCAAAAAATCA-3) (Folmer *et al.*, 1994). Polymerase chain reactions (PCRs) were performed in a 25 µL volume containing 0.6 µL of each primer 10 mM, 2 µL of $MgCl_2$ 25 mM (Promega), 5 µL of 5× buffer (Promega), 0.6 µL of dNTPs 10 mM (Promega), 0.2 µL of Go-Taq Polymerase (5 U µL^{-1}) (Promega) and 2 µL of total DNA. PCR temperature conditions for rRNA amplifications were the following: 95°C for 3 min (initial denaturation), followed by 40 cycles at 94°C for 1 min (denaturation), 52–56°C (optimized for the 18S and 28S amplification, respectively) for 1 min (annealing), 72°C for 1 min (extension) and followed by post-amplification at 72°C for 7 min. PCR cycling parameters for the mtDNA *cox*1 amplifications were the following: 95°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 1 min (denaturation), 45°C for 1 min (annealing), 72°C for 1 min (extension) and followed by post-amplification at 72°C for 7 min.

PCR amplicons were purified using the AMPure XP kit (Beckman Coulter) following the standard manufacturer-recommended protocol and Sanger sequenced from both strands, using the same primers, through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, Carlsbad), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Contiguous sequences were assembled and edited using MEGAX v. 11 (Kumar *et al.*, 2018). Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis *et al.*, 2008).

The 18S, 28S and *cox*1 datasets were aligned with all the sequences of species of genera *Andracantha*, *Bolbosoma* and *Corynosoma* (Polymorphidae) available in GenBank, using ClustalX v. 2.1 (Larkin *et al.*, 2007), as described in García-Varela *et al.* (2013) (see Table 1).

Sequences were combined (18S + 28S and 18S + 28S + *cox*1), using SequenceMatrix (Vaidya *et al.*, 2011), while the best partition schemes and best-fit models of substitution were identified using Partition Finder (Lanfear *et al.*, 2012) with the Akaike information criterion (AIC; Akaike, 1973). Sequences obtained in the present study were deposited in GenBank under the accession numbers MZ047218-MZ047227 (18S), MZ047231-MZ047240 (28S) and MZ047272-MZ047281 (*cox*1).

Phylogenetic trees of the 18S + 28S and 18S + 28S + *cox*1 gene loci were constructed using the Bayesian inference (BI) with MrBayes, v. 3.2.7 (Ronquist and Huelsenbeck, 2003). The Bayesian posterior probability analysis was performed using the MCMC algorithm, with four chains, 0.2 as the temperature of heated chains, 5 000 000 generations, with a subsampling frequency of 500 and a burn-in fraction of 0.25. Posterior probabilities were estimated and used to assess support for each branch. Values with a 0.90 posterior probability were considered well-supported. Trees were drawn using FigTree v. 1.3.1 (Rambaut, 2009). The phylogenetic trees were rooted using *Hexaglandula corynosoma* (Travassos, 1915) Petrochenko, 1958 and *Polymorphus brevis* (Van Cleave, 1916) Travassos, 1926 as outgroups, according to García-Varela *et al.* (2021). Genetic distances were computed using the Kimura 2-Parameters (K2P) model (Kimura, 1980)

Table 1. Species, stage (L: larva; A: adult), host, locality and accession numbers of sequences of *cox*1, 28S and 18S of genera *Andracantha*, *Corynosoma* and *Bolbosoma* included in the Bayesian inference shown in Figs 3 and 4

Species	Stage	Host	Locality	cox1	28S	18S	References
Andracantha gravida	A	*Phalacrocorax auritus*	Yucatan, Mexico	EU267822	EU267814	EU267802	García-Varela *et al.* (2009)
Andracantha leucocarboi	A	*Leucocarbo chalconotus*	New Zealand	MF527025	MF401623	–	Presswell *et al.* (2018)
Andracantha sigma	A	*Eudyptula minor*	New Zealand	MF527034	MF401624	–	Presswell *et al.* (2018)
Andracantha phalacrocoracis	A	*Phalacrocorax pelagicus*	Hokkaido, Japan	LC465396	LC461973	–	Sasaki *et al.* (2019)
Corynosoma australe	A	*Phocarctos hookeri*	New Zealand	JX442191	JX442180	JX442168	García-Varela *et al.* (2013)
Corynosoma hannae	L	*Peltorhamphus novaezeelandiae*	New Zealand	KX957726	–	–	Hernandez-Orts *et al.* (2016)
Corynosoma validum	A	*Callorhinus ursinus*	St. Paul Island, Alaska	JX442193	JX442182	JX442170	García-Varela *et al.* (2013)
Corynosoma villosum	L	*Pleurogrammus azonus*	Hokkaido, Japan	LC465336	LC461969	–	Sasaki *et al.* (2019)
Corynosoma obtuscens	A	*Callorhinus ursinus*	St. Paul Island, Alaska	JX442192	JX442181	JX442169	García-Varela *et al.* (2013)
Corynosoma enhydri	A	*Enhydra lutris*	Monterey Bay, California	DQ089719	AY829107	AF001837	García-Varela and Nadler (2006)
Corynosoma magdaleni	A	*Phoca hispida saimensis*	Lake Saimaa, Finland	EF467872	EU267815	EU267803	García-Varela *et al.* (2009)
Corynosoma semerme	L	*Osmerus dentex*	Hokkaido, Japan	LC465392	LC461963	–	Sasaki *et al.* (2019)
Corynosoma strumosum	A	*Phoca vitulina*	Monterey Bay, California	EF467870	EU267816	EU267804	García-Varela *et al.* (2009)
*Bolbosoma balaenae**	L A	*Nyctiphanes couchii* *Balaenoptera physalus*	Spain Capri Island, Italy	JQ061132 MZ047272–MZ047281	– MZ047231–MZ047240	JQ040306 MZ047218–MZ047227	Gregori *et al.* (2012) Present study
Bolbosoma caenoforme	A	*Salvelinus malma*	Tauj Bay, Russia	KF156891	–	KF156879	Malyarchuk *et al.* (2014)
Bolbosoma sp.	L	*Callorhinus ursinus*	St. Paul Island, Alaska	JX442190	JX442179	JX442167	García-Varela *et al.* (2013)
Bolbosoma turbinella	A	*Eschrichtius robustus*	Monterey Bay, California	JX442189	JX442178	JX442166	García-Varela *et al.* (2013)
Bolbosoma vasculosum	–	*Lepturacanthus savala*	Indonesia	–	–	JX014225	Verweyen *et al.* (2011)
Hexaglandula corynosoma	A	*Nyctanassa violacea*	La Tovara, Mexico	EU189488	EU267817	EU267808	Guillén-Hernández *et al.* (2008), García-Varela *et al.* (2009)
Polymorphus brevis	A	*Nycticorax nycticorax*	Michoacan, Mexico	DQ089717	AY829105	JX442171	García-Varela and Nadler (2006), García-Varela *et al.* (2013)

The *cox*1 sequence of *Bolbosoma balaenae* of Gregori *et al.* (2012) was erroneously deposited in GenBank under the name *Rhadinorhynchus pristis*.
–, data not reported.

with 1000 bootstrap re-samplings, using MEGA Software, version 7.0 (Kumar *et al.*, 2018).

Results

Parasitological study

A total of 142 specimens of acanthocephalans yellowish in colour were collected from the examined tract of the duodenum. Most specimens were firmly embedded with their proboscis and cephalic bulb within the muscular layer of the intestinal wall, having perforated the mucosal and submucosal surfaces, and few specimens were found free in the intestinal lumen. Gross changes consisted of oedematous thickening of the duodenal wall with the occurrence of 5–10 mm large, green-dark multifocal nodular lesions scattered throughout the muscular layer.

Based on the morphological characters, all the acanthocephalans were identified as *B. balaenae* (Figs 1 and 2). Specimens of *B. balaenae* differ from all other species of *Bolbosoma* having unarmed bulb and proboscis armed with 24 rows of hooks with
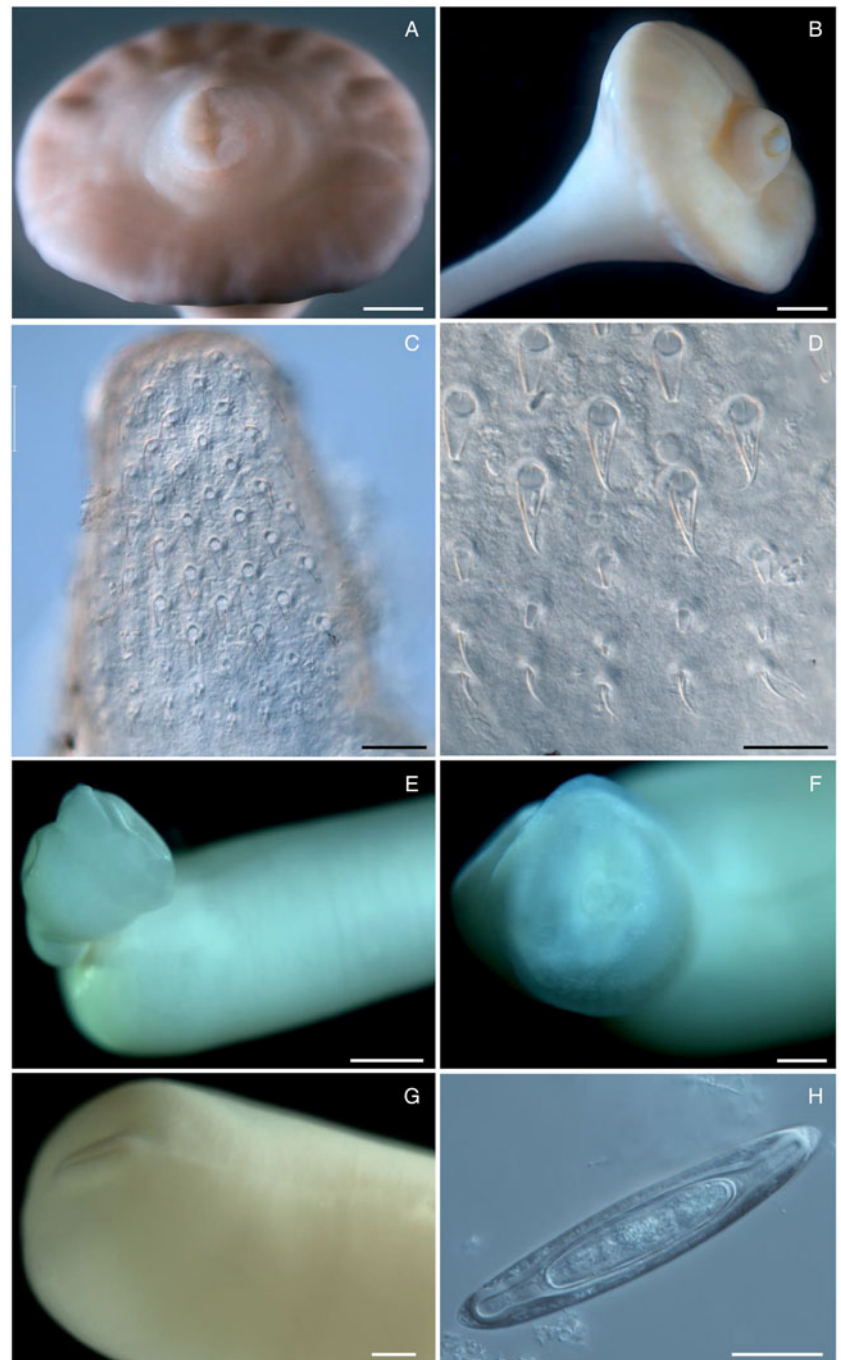
Fig. 1. Microscopic features of *Bolbosoma balaenae* from the intestine of the fin whale from the southern Italy. Anterior extremity frontal (A, female) and lateral (B, male) views (scale bar: 1000 μm). Proboscis (C, scale bar: 50 μm) and particular of proboscis basal hooks (D, scale bar: 100 μm). Bursa lateral (E, scale bar: 1000 μm) and ventral (F, scale bar: 500 μm) views. Genital pore of female in lateral view (G, scale bar: 500 μm). Mature egg (H, scale bar: 20 μm).

7–8 hooks per row. Proboscis was cylindrical showing hooks of different sizes and morphology, first 5 with roots and last 2–3 with rootless (Fig. 1C and D; Table 2). A field of trunked trunk spines restricted to the prebulbar foretrunk variable in number (from 5 to 9 irregular circles) was distinguished by SEM study alone (Fig. 2). Observation of the detailed surface morphology allowed also to highlight the features and unique ultrastructure of proboscis's hooks showing shallow longitudinal grooves, as well as the micropores on the tegument of the foretrunk (Fig. 2). Most important diagnostic morphological measurements of *B. balaenae* and their mature eggs observed at the copromicroscopic analysis (Fig. 1H) are listed in Table 2. Voucher specimens have been deposited at the Zoological Collection of the Stazione Zoologica Anton Dohrn in Naples (Italy) with the following accession number: SZN-ACA0001.

Molecular and phylogenetic analyses

The BLASTn analysis of the 18S sequences retrieved a similarity between 99.70 and 100% with sequences from GenBank belonging to *B. balaenae* (JQ040306), *Bolbosoma* sp. (JX442167) and *B. turbinella* (JX442166). The BLASTn analysis of 28S sequences produced a percentage of similarity of 99.60% with *Bolbosoma* sp. (JX442179) from the northern fur seal *Callorhinus ursinus* Linnaeus, 1758 available in GenBank. The mtDNA *cox*1 sequences shared a similarity of ~99% with *B. balaenae* (JQ061132) from the euphausiid *Nyctiphanes couchii* (Bell, 1853), erroneously deposited in GenBank under the name *Rhadinorhynchus pristis* by Gregori *et al.* (2012).

The combined 18S + 28S phylogenetic Bayesian tree, including sequences of species within the three genera (*Andracantha*, *Bolbosoma* and *Corynosoma*) of the family Polymorphidae, showed poorly resolved relationships, especially within the
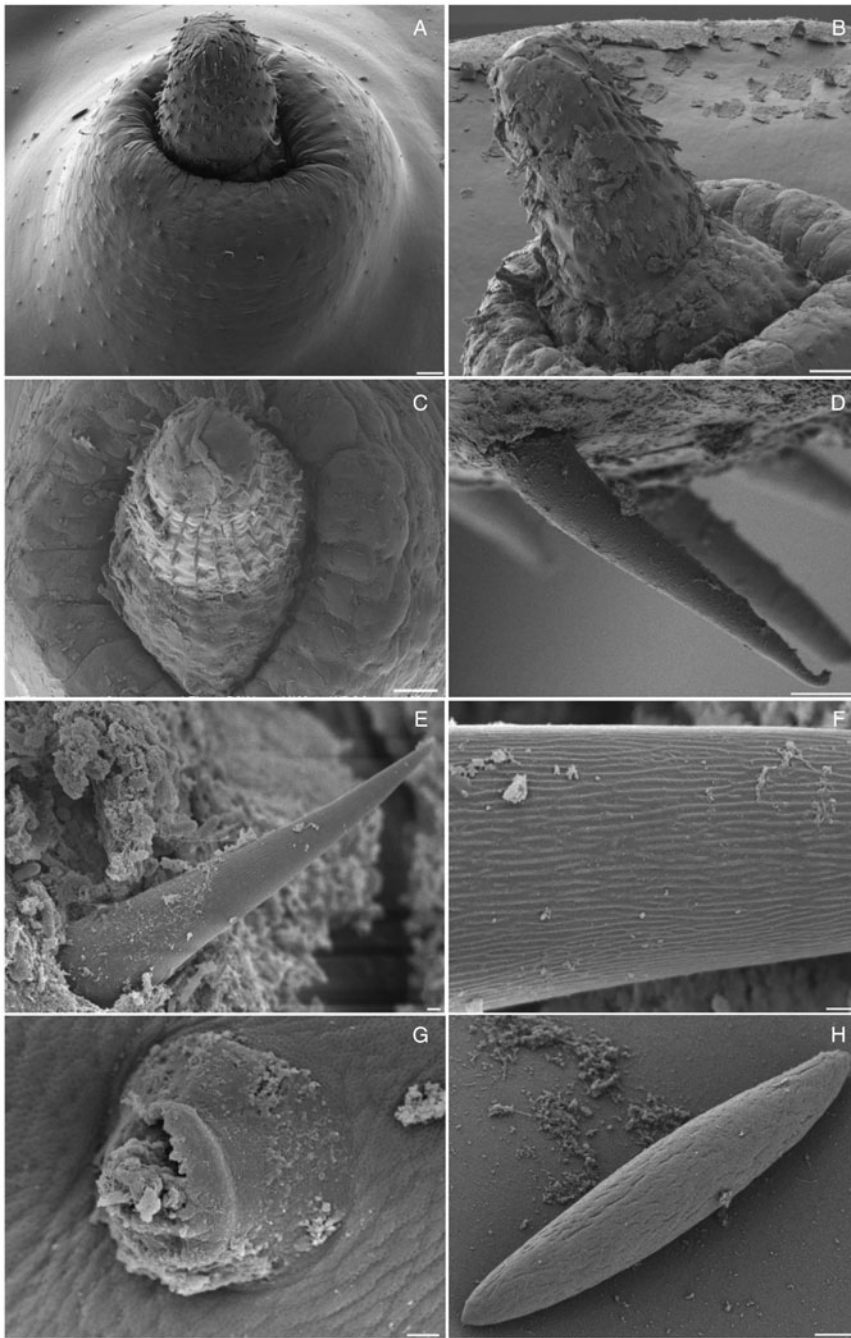
Fig. 2. Scanning electron micrographs of *Bolbosoma balaenae* from the intestine of the fin whale from the southern Italy. General view of prebulb and proboscis of a female (A, scale bar: 100 μm). Note the circles of trunked trunk spines on the prebulb. Lateral (B) and apical (C) views of proboscis and neck (scale bar: 100 μm) of a male. High magnification of an apical (D, scale bar: 10 μm) and a basal (E, scale bar: 1 μm) proboscis hook. High magnification of an apical proboscis hook surface (F, scale bar: 1 μm) showing longitudinal grooves. A high magnification of a truncated trunk spine (G, scale bar: 1 μm). Note the body wall micropores on the tegument of the prebulb. Mature egg (H, scale bar: 10 μm).

genus *Bolbosoma* (Fig. 3). In contrast, the concatenated BI tree topology of the three gene loci 18S + 28S + *cox*1 showed that the obtained sequences from *Bolbosoma* here analysed clustered in a highly supported clade (100% of probability value) (Fig. 4). This clade, including also the sequences available in GenBank of the polymorphid cystacanths obtained from *N. couchii* (JQ061132, JQ040306), resulted to be clearly distinct from the other species of the genus *Bolbosoma*, whose sequences at those analysed gene loci were available in GenBank (Fig. 4). The distance values between the present sequences of *B. balaenae* and the sequences of cystacanths from *N. couchii* were: K2P = 0.017 ± 0.005 at the mtDNA *cox*1 and K2P = 0.004 ± 0.002 at the 18S rRNA (present sequences *vs* JQ040304-JQ040306). While, at the interspecific level, the mtDNA *cox*1 sequences of *B. balaenae* showed a higher value of differentiation (K2P = 0.165 ± 0.020) with respect to the closest sequence of *B. caenoforme* (KF156891). No sequences of *B. caenoforme* were available in GenBank for the 28S gene locus.

Discussion

Previous reports of *Bolbosoma* species from the marine mammals in the Mediterranean Sea are limited to *B. capitatum* (Parona, 1893; Porta, 1906) in a long-finned pilot whale *Globicephala melas* Traill, 1809, and *B. vasculosum* (only immature specimens in a common dolphin *Delphinus delphis*; Van Cleave, 1953). Recently, a single specimen of *Bolbosoma* sp. later identified as *B. capitatum* was collected from 1 of 7 fin whales (Marcer *et al.*, 2019).

These uncommon records suggest that *Bolbosoma* spp. are only occasional in the Mediterranean basin, likely transported from migrating individuals from the Atlantic Ocean. Helminth parasites have been extensively used as biological tags of marine vertebrates in host population structure studies. Recently, we used anisakid nematodes of the dwarf sperm whale *Kogia sima* Owen, 1866 and trypanorhynch cestodes of the sunfish *Mola mola* Linnaeus, 1758 to suggest the possible existence of a resident

Table 2. Measurements (mean value ± standard deviation with range in parenthesis) of main diagnostic characters in *Bolbosoma balaenae* found in a fin whale from southern Italy

Characters	Male (*n* = 10)	Females (*n* = 10)
Total length (cm)	11.3 ± 0.91 (10.1–12.8)	13.6 ± 0.75 (12.8–14.5)
Width at middle of body (mm)	2.4 ± 0.06 (2.4–2.5)	4 ± 0.94 (3–5.1)
Bulb length (mm)	5.2 ± 0.30 (5.1–5.6)	6.1 ± 0.82 (5–7.1)
Bulb width (mm)	5.1 ± 0.23 (4.9–5.4)	5.9 ± 0.61 (4.9–6.6)
Prebulb length (mm)	1 ± 0.18 (0.8–1.2)	1 ± 0.21 (0.8–1.3)
Prebulb width at base (mm)	1.4 ± 0.26 (1.1–1.5)	1.8 ± 0.25 (1.6–2.1)
Proboscis length	564.6 ± 10.44 (598.4–613.1)	611 ± 38.05 (561–648.7)
Proboscis width at basal hook	499.9 ± 3.51 (496.1–503.1)	483.9 ± 67.65 (425.5–572.9)
Neck length	573 ± 48.44 (518.4–610.8)	517.8 ± 61.04 (454–595.4)
Lemnisci length	4005.4 ± 219.02 (3828.4–4250.7)	3157.2 ± 873.27 (2211.7–4369.8)
Proboscis hook 1 length	66.8 ± 15.58 (54.5–88.6)	51.8 ± 3.36 (46.2–55.1)
Proboscis hook 1 width	10.5 ± 1.72 (8.2–12.1)	11.7 ± 3.48 (5.7–16.6)
Proboscis hook 2 length	61.8 ± 7.33 (56.4–74.4)	61.6 ± 9.46 (79.2–53.6)
Proboscis hook 2 width	13.3 ± 2.40 (10.2–16.8)	14.4 ± 1.95 (11.4–18.2)
Proboscis hook 3 length	63.2 ± 4.40 (59.4–69.1)	57.72 ± 10.68 (40.01–87.99)
Proboscis hook 3 width	14.55 ± 0.96 (13.19–15.49)	13.7 ± 3.13 (11.5–20.9)
Proboscis hook 4 length	60.6 ± 3.53 (56.8–65.7)	51.1 ± 7.65 (39.2–67.1)
Proboscis hook 4 width	16.1 ± 2.05 (14.2–18.7)	14.5 ± 3.39 (11.3–20.9)
Proboscis hook 5 length	67 ± 4.35 (61.8–73.9)	51.3 ± 8.97 (40.2–70.8)
Proboscis hook 5 width	19.2 ± 1.42 (16.7–20.9)	14.9 ± 3.35 (9.5–20.7)
Proboscis hook 6 length	58.3 ± 11.71 (44.4–70.1)	57.7 ± 12.41 (40.3–81.7)
Proboscis hook 6 width	15.5 ± 4.22 (11.2–19.8)	16.7 ± 3.78 (13.9–24.7)
Proboscis hook 7 length	37.6 ± 1.12 (36.8–39.5)	35.3 ± 7.65 (21.4–55.5)
Proboscis hook 7 width	8.2 ± 0.97 (7.1–9)	9.4 ± 2.56 (5.7–11.5)
Proboscis hook 8 length	28.5 ± 3.96 (24.7–35.3)	34.1 ± 7.41 (22.8–46.8)
Proboscis hook 8 width	4.8 ± 1.37 (3.8–7.5)	7.1 ± 1.48 (4.9–11.2)
Anterior testis length	3621.5 ± 350.1 (3044.5–3949.4)	–
Anterior testis width	1308.5 ± 143.13 (1065.3–1406.6)	–
Posterior testis length	3479.5 ± 348.46 (2866.5–3712.6)	–
Posterior testis width	1431.6 ± 105.53 (1275–1441)	–
Cement glands length (cm)	5 ± 0.97 (4–6.5)	–
Bursa diameter	2260.2 ± 0.18 (2020.1–2480.3)	–
Egg length	–	140.6 ± 6.74 (132.1–149.5)
Egg width	–	31.2 ± 1.78 (27.8–33.9)

Measurements are in micrometres except when stated. Ten elements for each character were measured except for the bursa for which the measurements were obtained from four specimens with everted bursa.

population or migration routes of their hosts, respectively (Santoro *et al.*, 2018, 2020). The fin whale is the most abundant mysticete in the Mediterranean Sea (Panigada and Notarbartolo di Sciara, 2012) with the occurrence of both resident and migrating populations confirmed by genetic studies (Bérubé *et al.*, 1998). For the migrating fin whale populations, a general movement trend towards the northeast North Atlantic in spring-summer and towards the Mediterranean during fall-winter has been suggested (Geijer *et al.*, 2016). The present finding of a parasite known from geographical areas far from the Mediterranean basin seems to suggest that the present fin whale would be a migrating and not a resident individual.

Regarding the source of infection, *Bolbosoma* cystacanths have been found in fish [Scombridae, Scorpaenidae, Carangidae, Trichiuridae, Gempylidae, Salmonidae, Berycidae, Lophotidae, Gadidae and Belonidae (www.nhm.ac.uk/research-curation/research/projects/host-parasites/index.Html)] and crustaceans (euphausiids and copepods) (Measures, 1992; Hoberg *et al.*, 1993; Dailey *et al.*, 2000; Gregori *et al.*, 2012). Recently, Gregori *et al.* (2012) found cystacanths identified as *B. balaenae* in 0.04% of the euphausiid *N. couchii* specimens examined from the Atlantic Galician waters (Spain). The source of the infection of the present fin whale with *B. balaenae* remains unknown; it could be plausible that the fin whale acquired the infection by
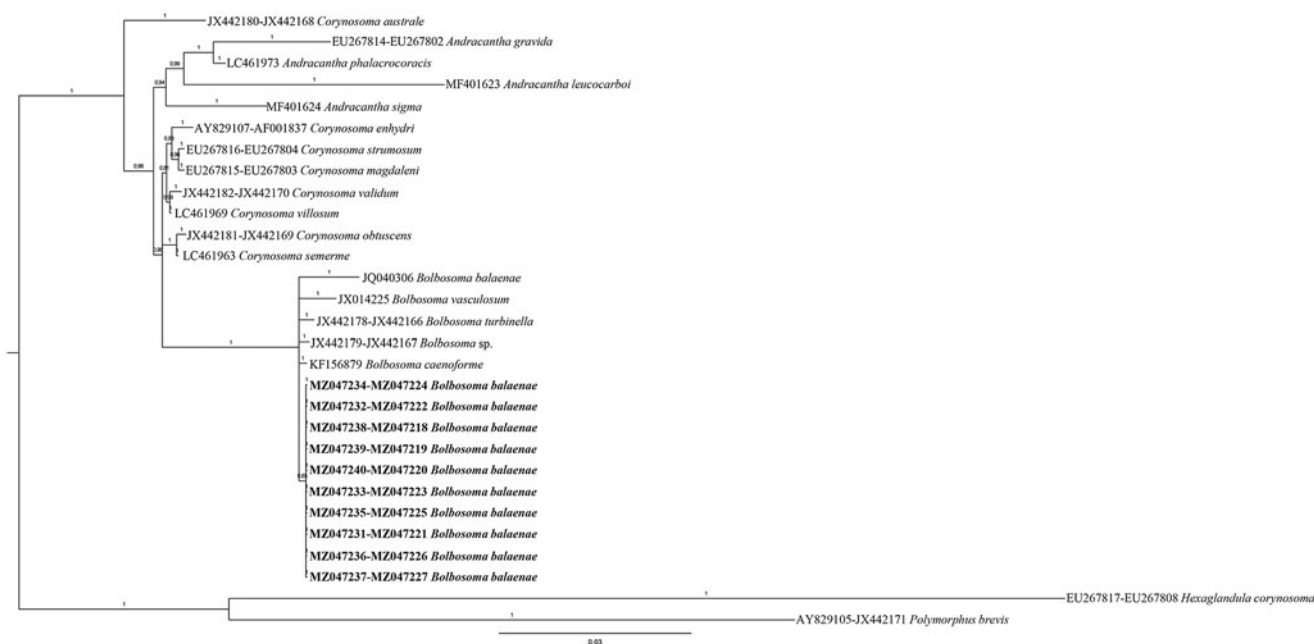
Fig. 3. Phylogenetic concatenated tree from Bayesian inference based on 18S and 28S sequences of *B. balaenae* obtained in the present study, with respect to the sequences of species of genera *Andracantha*, *Bolbosoma* and *Corynosoma*, at the same gene loci available in GenBank. The analysis was performed by MrBayes, v. 3.2.7, using the GTR + G substitution model. *Hexaglandula corynosoma* and *Polymorphus brevis* were used as outgroup. The sequences obtained in this study are in bold.

Fig. 4. Phylogenetic concatenated tree from Bayesian inference based on 18S + 28S + *cox*1 sequences of *B. balaenae* obtained in the present study, with respect to the sequences of species of genera *Andracantha*, *Bolbosoma* and *Corynosoma*, at the same gene loci available in GenBank. The analysis was performed by MrBayes, v. 3.2.7, using the GTR + G substitution model. *Hexaglandula corynosoma* and *Polymorphus brevis* were used as outgroup. The sequences obtained in this study are in bold.
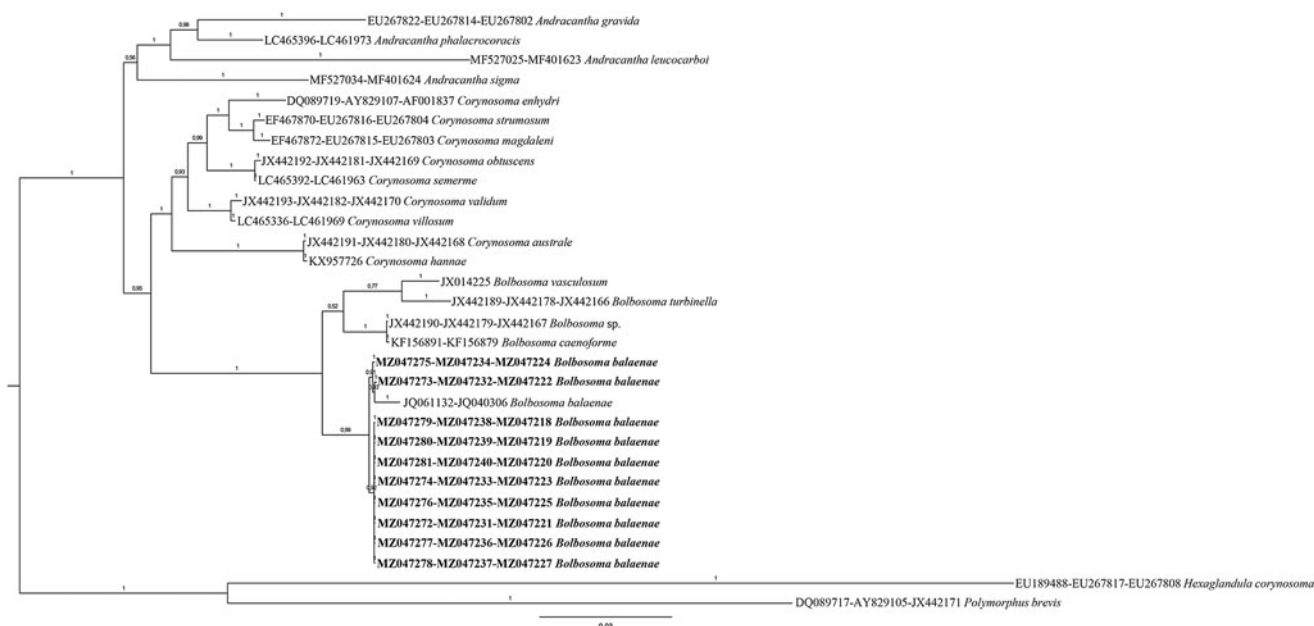
ingestion of infected crustaceans and/or fish during the migration from the Atlantic to the Mediterranean Sea waters.

Most of the morphological characters of adult specimens of *B. balaenae* were not detailed by earlier authors so that comparisons with the present material are limited. For instance, males/females combined total length were 80–160 mm in the original description (reported in Porta, 1906) and 190–205 mm in Van Cleave (1953), while data on the measurements of the hook proboscis are missed as well as the measurements of most characters listed in Table 2. Regarding the number of rows of hooks and the number of hooks per longitudinal row of the proboscis, the present data correspond to previous data (Meyer, 1933; Van Cleave, 1953; Zdzitowiecki, 1991). In contrast, previous descriptions of prebulbar foretrunk of *B. balaenae* using optical microscopy alone reported apparently contrasting data on the presence/absence and numbers of circles of spines: 6 circles in Meyer (1933), 0 in Van Cleave (1953) and up to 10 circles of spines in Zdzitowiecki (1991). Moreover, in cystacanths morphologically

identified as *B. balaenae* found encapsulated in the cephalothorax of *N. couchii*, Gregori *et al.* (2012) described a single field of trunk spines restricted to the foretrunk and composed of 4–6 irregular circles of small spines adjacent to the neck. Finally, Bennett *et al.* (2021) found seven circles of spines in an immature individual identified as *B. balaenae* in a blue penguin *Eudyptula novaehollandiae* Stephens, 1826 from New Zealand. The present observation regarding the occurrence of trunked trunk spines on the prebulbar foretrunk of adult individuals of *B. balaenae* differentiated by SEM alone supports the hypothesis of Van Cleave (1953), according to which the trunk spines show wide variability in number, and these may be lost along the parasite life span.

The species of the genus *Corynosoma* (a polymorphid genus very close to *Bolbosoma*) use the flattened, spiny foretrunk as a very efficient device that assists the proboscis to adhere to the gut wall but is also able to put the ventral hindtrunk into contact with the substratum, reinforcing attachment (Aznar *et al.*, 2006, 2018). Aznar *et al.* (2016) reported that cystacanths and adults of *Corynosoma cetaceum* (a parasite of the stomach of dolphins) exhibited a wide range of fold spine reduction and variability, suggesting that they are generated before the adult stage, when spines are functional for attachment to the stomach wall of its definitive host. This assumes that the foretrunk spines should not be regarded as a diagnostic taxonomic character within the genus *Corynosoma* (Aznar *et al.*, 2016) as well as in the genus *Bolbosoma*.

Observation of the detailed surface morphology of the present material using SEM allowed also to highlight the features and unique ultrastructure of proboscis's hooks, showing shallow longitudinal grooves, as well as the micropores of the tegument of foretrunk supposed to be a specialized system implicated in absorptive function (Heckmann *et al.*, 2013). According to Heckmann *et al.* (2013) micropores on the tegument showing different sizes and shapes have been described in at least 16 acanthocephalan species. The different ultrastructural pattern of proboscis's hooks has been studied as a potential diagnostic feature to differentiate among species of *Centrorhynchus* and species of related genera, but no conclusive results were obtained (Amin *et al.*, 2015, 2018). No mention is done on both ultrastructure of proboscis's hooks and epidermal micropores from previously published papers reporting SEM observation of *B. capitatum*, *B. vasculosum* and *B. turbinella* (Amin and Margolis, 1998; Costa *et al.*, 2000; da Fonseca *et al.*, 2019). Future studies comparing the ultrastructure features among *Bolbosoma* species could reveal if the present findings might yield important information to help identify this species.

The combination of morphological and molecular studies is considered a very useful approach to resolve taxonomic ambiguities within the genera of Polymorphidae (García-Varela *et al.*, 2013). Unfortunately, out of the 12 species of *Bolbosoma* considered as valid, DNA sequences for only six of those are available in GenBank. Moreover, from the current 23 *Bolbosoma* sequences available, only four are from adult parasites obtained from their definitive hosts: three of them are belonging to *B. turbinella* (18S, 28S and *cox*1) from the grey whale, and one to *B. nipponicum* (ITS1/ITS2 region) from the common minke whale. Before the present study, four sequences of *B. balaenae* (including three of 18S and one of *cox*1) were available in GenBank, all from the same cystacanths (Gregori *et al.*, 2012). However, the sequence of *cox*1 (JQ040303) deposited in GenBank as *B. balaenae* belongs to *R. pristis* (an acanthocephalan of Rhadinorhynchidae family), while the sequence deposited as *R. pristis* (JQ061132.1) belongs to *B. balaenae*. Likely an error occurred by Gregori *et al.* (2012) at the moment of sequence submission and the names of sequences used in the mentioned study were inverted.

In the present study, the BI phylogenetic analysis based on the combined (18S + 28S) rRNA data produced poorly resolved clades among species of *Andracantha*, *Corynosoma* and *Bolbosoma*. Moreover, from the obtained results, it is clear that the gene locus 18S is not diagnostic for the genetic identification of *Bolbosoma* species. While the phylogenetic tree herein inferred from combining the sequences obtained at the three gene loci (18S + 28S + *cox*1) from adult individuals of *B. balaenae* and those sequences at the same gene loci available in GenBank has shown that the species of *Andracantha*, *Bolbosoma* and *Corynosoma* are comprising, respectively, in three distinct and well-supported major clades (Fig. 4). These findings are in agreement with previous phylogenetic elaborations provided by García-Varela *et al.* (2013) and Presswell *et al.* (2018). In addition, the combined BI inferred from 18S + 28S + *cox*1 gene sequences supports, with high probability values, that the so far genetically characterized species of *Bolbosoma*, including *B. balenae*, represent distinct phylogenetic lineages.

The phylogenetic pattern obtained is congruent with the life cycles of members of these three genera (i.e. *Andracantha*, *Bolbosoma* and *Corynosoma*), which involve teleost marine fish as paratenic hosts. It has been suggested that the shared ecological feeding behaviour among different definitive hosts could have provided many opportunities for co-speciation and host-switching events and could have accompanied the evolutionary pathways of these polymorphid species (Dailey *et al.*, 2000; Aznar *et al.*, 2006; García-Varela *et al.*, 2013, 2021; Presswell *et al.*, 2018).

Finally, the only report of pathological changes associated with Acanthocephala of the genus *Bolbosoma* in a Mediterranean cetacean was reported by Parona (1893) who described a severe intestinal parasitosis caused by *B. capitatum* in a long-finned pilot whale. Parona (1893) reported the occurrence of at least 25 305 individual parasites strictly embedded in the muscular layer along the first 12 m of the intestine. Dailey *et al.* (2000) described gross multifocal transmural abscesses encapsulating proboscis of *B. balaenae* along the first 7.5 m of the ileum in a juvenile grey whale. The present results agree with the gross pathological changes described by Parona (1893) and Dailey *et al.* (2000) and confirm that *B. balaenae* may cause enteritis also in the fin whale.

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