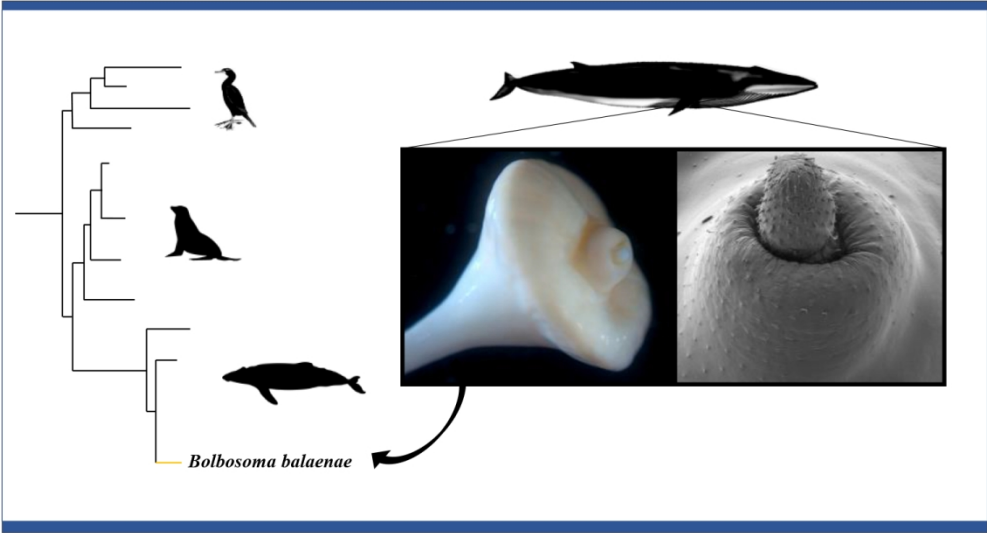


Molecular and morphological characterization of *Bolbosoma balaenae* (Acanthocephala: Polymorphidae), a neglected intestinal parasite of the fin whale *Balaenoptera physalus*

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| Abstract: | <p>Post-mortem examination of a fin whale <i>Balaenoptera physalus</i> stranded in the Mediterranean Sea led to the finding of <i>Bolbosoma balaenae</i> for first time in this basin. In this work, we describe new structural characteristics of this parasite using light microscopy and scanning electron microscopy (SEM) approaches. Moreover, the molecular and phylogenetic data as inferred from both ribosomal RNA 18S-28S and the mitochondrial DNA cytochrome oxidase c subunit 1 (cox1) for adult specimens of <i>B. balaenae</i> are also reported for the first time. Details of the surface topography such as proboscis's hooks, trunked trunk spines of the prebulbar 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 <i>Bolbosoma</i>. In contrast, the combined 18S+28S+mtDNA cox1 BI tree topology showed that the present sequences clustered with species of <i>Bolbosoma</i> in a well-supported clade with a high probability value. The comparison of cox1 and 18S sequences revealed that the present specimens are conspecific with the cystacanths of <i>B. balaenae</i> previously collected in the euphausiid <i>Nyctiphanes couchii</i> from the North Eastern Atlantic Ocean. This study provided taxonomic, molecular and phylogenetic data that allow for a better characterisation of this poor known parasite.</p> |

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

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26 Abstract

27 Post-mortem examination of a fin whale *Balaenoptera physalus* stranded in the
28 Mediterranean Sea led to the finding of *Bolbosoma balaenae* for first time in this basin. In
29 this work, we describe new structural characteristics of this parasite using light microscopy
30 and scanning electron microscopy (SEM) approaches. Moreover, the molecular and
31 phylogenetic data as inferred from both ribosomal RNA 18S-28S and the mitochondrial DNA
32 cytochrome oxidase *c* subunit 1 (*cox1*) for adult specimens of *B. balaenae* are also reported
33 for the first time. Details of the surface topography such as proboscis's hooks, trunked trunk
34 spines of the prebulbar foretrunk, ultrastructure of proboscis's hooks and micropores of the
35 tegument are shown. The 18S+28S rRNA Bayesian tree (BI) as inferred from the
36 phylogenetic analysis showed poorly resolved relationships among the species of *Bolbosoma*.
37 In contrast, the combined 18S+28S+mtDNA *cox1* BI tree topology showed that the present
38 sequences clustered with species of *Bolbosoma* in a well-supported clade with a high
39 probability value. The comparison of *cox1* and 18S sequences revealed that the present
40 specimens are conspecific with the cystacanths of *B. balaenae* previously collected in the
41 euphausiid *Nyctiphanes couchii* from the North Eastern Atlantic Ocean. This study provided
42 taxonomic, molecular and phylogenetic data that allow for a better characterisation of this
43 poor known parasite.

44

45 **Key words:** *Balaenoptera physalus*, fin whale, *Bolbosoma balaenae*, ribosomal RNA 18S-
46 28S, mitochondrial DNA *cox1*, phylogenetic analysis, Mediterranean Sea

48 Key Findings

- 49 • First report of *Bolbosoma balaenae* in a cetacean from the Mediterranean Sea
- 50 • First integrative taxonomy of adults of *B. balaenae* by morphology and multilocus
- 51 analysis
- 52 • New morphological features of *B. balaenae* are shown
- 53 • *B. balaenae* represents a distinct phylogenetic lineage from other polymorphid
- 54 acanthocephalans
- 55 • ~~Present specimens of *B. balaenae* are conspecific with the cystacanths previously~~
- 56 ~~sequenced from Atlantic cuphausiid *Nyctiphanes couchii*~~

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73 Introduction

74 Polymorphid acanthocephalans belonging to the genus *Bolbosoma* Porta, 1908 comprise 12
75 valid species (Amin, 2013). Of these, at least 10 (all described at the adult stage) have been
76 reported in the intestinal tract of a range of oceanic whales and dolphins (Amin, 2013; Felix,
77 2013). The life cycle of *Bolbosoma* species has been not yet completely elucidated. However,
78 it has been suggested that pelagic crustaceans (euphausiids and copepods) and fishes serve as
79 intermediate and paratenic hosts, respectively (Measures, 1992; Hoberg *et al.*, 1993; Dailey *et*
80 *al.*, 2000; Gregori *et al.*, 2012). Marine mammals serve as definitive hosts; they become
81 infected by ingestion of infected preys. In marine mammals, the species of *Bolbosoma* may
82 cause different degrees of enteritis due to their ability to perforate mucosal surface for
83 anchoring to the muscular layer (Parona, 1893; Porta, 1906; Dailey *et al.*, 2000; Arizono *et*
84 *al.*, 2012; Kaito *et al.*, 2019).

85 *Bolbosoma balaenae* (Gmelin, 1790) Porta, 1908 type species, has been described as
86 *Sipunculus lendix* Phipps, 1774 in a sei whale *Balenoptera borealis* Lesson, 1828 from the
87 Arctic waters. After its original description, *B. balaenae* was reported as sporadic finding in
88 four oceanic odontocetes (i.e., the northern bottlenose whale *Hyperoodon ampullatus*
89 Lacépède, 1804, spinner dolphins *Stenella longirostris* Gray, 1828, spotted dolphins *S.*
90 *attenuata* Gray, 1846, and the pygmy sperm whale *Kogia breviceps* Golvan, 1961 (Gregori *et*
91 *al.*, 2012; Felix, 2013) and, at least, in other five mysticetes species as regular hosts: the
92 common minke whale *B. acutorostrata* Lacépède, 1804, the fin whale *B. physalus* Linneus,
93 1758, the blue whale *B. musculus* Linneaus, 1758, the humpback whale *Megaptera*
94 *novaeangliae* Borowski, 1781, and the grey whale *Eschrichtius robustus* Lilljeborg, 1861
95 (Golvan, 1961; Zdzitowiecki, 1991; Dailey *et al.*, 2000; Felix, 2013). Regarding its
96 geographical distribution, *B. balaenae* is known from Antarctic and Arctic waters, Southwest

97 Atlantic Ocean, Tasman Sea, and northern California coast to date (Zdzitowiecki, 1991;
98 Dailey *et al.*, 2000; Gregori *et al.*, 2012; Felix, 2013).

99 The identification of *Bolbosoma* species is hardly based on the morphological
100 characters alone, because of its similarities with congeneric species, and/or the old poor
101 original description and redescriptions (Phipps, 1774; Van Cleave, 1953). Moreover, the
102 presence of a wide variability of morphological characters of the anterior extremity in
103 *Bolbosoma* spp. has been reported (Porta, 1906; Meyer, 1933; Van Cleave, 1953;
104 Petrochenko, 1956; Zdzitowiecki, 1991). Likely, due to the old, opportunistic, and scattered
105 findings around its geographical range, *B. balaenae* remains a little known parasite: no
106 microscopic images and molecular data exist for adult specimens of *B. balaenae*. Moreover,
107 interest in *Bolbosoma* species increased recently by reason of their potential zoonotic role. At
108 least, 8 cases of human infection with *Bolbosoma* sp. and a case for *B. capitatum* causing
109 clinical signs and intestinal lesions, have been reported from Japan and related to
110 consumption of uncooked fish flesh (Arizono *et al.*, 2012; Kaito *et al.*, 2019).

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

116

117 **Materials and methods**

118 *Parasitological study*

119 An immature female fin whale measuring 14.4 meters in total length was found stranded in a
120 cove of Capri Island (Tyrrhenian Sea) in southern Italy on November 8, 2020. At necropsy,
121 approximately 1 meter of duodenum showing the occurrence of acanthocephalans embedded

122 into the intestinal wall or free in the lumen was cut and moved to the laboratory, where
123 parasites were counted, rinsed in saline solution, and preserved in ethanol 70% or frozen (-
124 20°C) for morphological and molecular analyses, respectively. Morphological measurements
125 were obtained from 20 relaxed adult specimens (10 females and 10 males) using a compound
126 microscope and a stereomicroscope equipped with ZEN 3.1 imaging system (Zeiss). To study
127 the proboscis and the pattern of hook spination, the bulb of acanthocephalans was dissected
128 using scissors and tweezers under the stereomicroscope, and proboscis and neck were
129 displayed and clarified in Amman's lactophenol. To study the testes and cement glands, the
130 male specimens were dissected and organs were displayed and measured under the
131 stereomicroscope. Acanthocephalans were morphologically classified following the
132 identification keys proposed by Meyer (1933), Van Cleave (1953) and Petrochenko (1956).
133 Copromicroscopic examination was performed on a sample of faeces obtained from the
134 rectum and a standard flotation method with Sheather's sucrose solution (specific gravity
135 1.27) was used to detect and measure parasite eggs.

136 For SEM, the anterior portion of five acanthocephalan specimens was also fixed
137 overnight in 2.5% glutaraldehyde, then transferred to 40% ethanol (10 min), rinsed in 0.1 M
138 cacodylate buffer, postfixed in 1% OsO₄ for 2 h, and dehydrated in ethanol series, critical
139 point dried and sputter-coated with platinum. Observations were made using a JEOL JSM
140 6700F scanning electron microscope operating at 5.0 kV (JEOL, Basiglio, Italy).

141

142 *Molecular and phylogenetic analyses*

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

147 Two regions (18S and 28S) of the nuclear ribosomal RNA (rRNA) and a fragment of
148 the mitochondrial DNA (mtDNA *cox1*) were amplified. The near-complete small subunit
149 (ssrDNA, 18S) (~1800 bp) was amplified using the forward 5'-
150 AGATTAAGCCATGCATGCGT-3' and reverse 5'-GCAGGTTACCTACGGAAA-3'
151 primers (Garey *et al.*, 1996; Garcia-Varela *et al.*, 2002, 2013). The near-complete large
152 subunit (lsrDNA, 28S) (~2900 bp) was amplified using 2 overlapping PCR fragments of
153 1400-1500 bp. Primers for the amplicon 1 were forward 5'-
154 CAAGTACCGTGAGGGAAAGTTGC-3' and reverse 5'-CTTCTCCAA
155 C(T/G)TCAGTCTTCAA-3'; primers for the amplicon 2 were forward 5'-
156 CTAAGGAGTGTGTAACAACCTCACC and reverse 5'-
157 CTTCGCAATGATAGGAAGAGCC-3' (García-Varela and Nadler, 2005). A partial (~700
158 bp) sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) was amplified
159 using the primers LCO1490 (5-GGTCAACAAATCATAAAGATATTGG-3) and HCO2198
160 (5-TAAACTTCAGGGTGACCAAAAAATCA-3) (Folmer *et al.*, 1994). Polymerase chain
161 reactions (PCRs) were performed in a 25 µL volume containing 0.6 µL of each primer 10
162 mM, 2 µL of MgCl₂ 25 mM (Promega), 5 µL of 5× buffer (Promega), 0.6 µL of dNTPs 10
163 mM (Promega), 0.2 µL of Go-Taq Polymerase (5U/µL) (Promega) and 2 µL of total DNA.
164 PCR temperature conditions for rDNA amplifications were the following: 95°C for 3 min
165 (initial denaturation), followed by 40 cycles at 94°C for 1 min (denaturation), 52-56°C
166 (optimized for the 18S and 28S amplification, respectively) for 1 min (annealing), 72°C for 1
167 min (extension) and followed by post-amplification at 72°C for 7 min. PCR cycling
168 parameters for the mtDNA *cox1* amplifications were the following: 95°C for 5 min (initial
169 denaturation), followed by 40 cycles at 95°C for 1 min (denaturation), 45°C for 1 min
170 (annealing), 72°C for 1 min (extension) and followed by post-amplification at 72°C for 7
171 min.

172 PCR amplicons were purified using the AMPure XP kit (Beckman coulter) following
173 the standard manufacturer-recommended protocol and Sanger sequenced from both strands,
174 using the same primers, through an Automated Capillary Electrophoresis Sequencer 3730
175 DNA Analyzer (Applied Biosystems), using the BigDye® Terminator v3.1 Cycle Sequencing
176 Kit (Life Technologies). Contiguous sequences were assembled and edited using MEGAX v.
177 11 (Kumar *et al.*, 2018). Sequence identity was checked using the Nucleotide Basic Local
178 Alignment Search Tool (BLASTn) (Morgulis *et al.*, 2008).

179 The 18S, 28S and *cox1* data sets were aligned with all the sequences of species of
180 genera *Andracantha*, *Bolbosoma* and *Corynosoma* (Polymorphidae) available in GenBank,
181 using ClustalX v. 2.1 (Larkin *et al.*, 2007), as described in García-Varela *et al.* (2013) (see
182 Table 1).

183 Sequences were combined (18S+28S and 18S+28S+*cox1*), using SequenceMatrix
184 (Vaidya *et al.*, 2011), while the best partition schemes and best-fit models of substitution
185 were identified using Partition Finder (Lanfear *et al.*, 2012) with the Akaike information
186 criterion (AIC; Akaike, 1973). Sequences obtained in the present study were deposited in
187 GenBank under the accession numbers MZ047218-MZ047227 (18S), MZ047231-MZ047240
188 (28S) and MZ047272-MZ047281 (*cox1*).

189 Phylogenetic trees of the 18S+28S and 18S+28S+*cox1* gene loci were constructed
190 using the Bayesian inference (BI) with MrBayes, v. 3.2.7 (Ronquist and Huelsenbeck, 2003).
191 The Bayesian posterior probability analysis was performed using the MCMC algorithm, with
192 four chains, 0.2 as the temperature of heated chains, 5,000,000 generations, with a
193 subsampling frequency of 500 and a burn-in fraction of 0.25. Posterior probabilities were
194 estimated and used to assess support for each branch. Values with a 0.90 posterior probability
195 were considered well-supported. Trees were drawn using FigTree v. 1.3.1 (Rambaut, 2009).
196 The phylogenetic trees were rooted using *Hexaglandula corynosoma* (Travassos, 1915)

197 Petrochenko, 1958 and *Polymorphus brevis* (Van Cleave, 1916) Travassos, 1926 as
198 outgroups, according to García-Varela *et al.* (2021). Genetic distances were computed using
199 the Kimura 2-Parameters (K2P) model (Kimura, 1980) with 1000 bootstrap re-samplings,
200 using MEGA Software, version 7.0 (Kumar *et al.*, 2018).

201

202 **Results**

203 *Parasitological study*

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

211 Based on the morphological characters, all the acanthocephalans were identified as *B.*
212 *balaenae* (Figs. 1 and 2). Specimens of *B. balaenae* differ from all other species of
213 *Bolbosoma* having unarmed bulb and proboscis armed with 24 rows of hooks with 7-8 hooks
214 per row. Proboscis was cylindrical showing hooks of different sizes and morphology, first 5
215 with roots and last 2-3 with rootless (Fig. 1C, 1D; Table 2). A field of trunked trunk spines
216 restricted to the prebulbar foretrunk variable in number (from 5 to 9 irregular circles) was
217 distinguished by SEM study alone (Fig. 2). Observation of the detailed surface morphology
218 allowed also to highlight the features and unique ultrastructure of proboscis's hooks showing
219 shallow longitudinal grooves, as well as the micropores of about 100-120 nanometres on the
220 tegument of the foretrunk (Fig. 2). Most important diagnostic morphological measurements
221 of *B. balaenae* and their mature eggs observed at the copromicroscopic analysis (Fig. 1H) are

222 listed in table 2. Voucher specimens have been deposited at the Zoological Collection of the
223 Stazione Zoologica Anton Dohrn in Naples (Italy) with the following accession number:
224 SZN-ACA0001.

225

226 *Molecular and phylogenetic analyses*

227 The BLASTn analysis of the 18S sequences retrieved a similarity between 99.70% and 100%
228 with sequences from GenBank belonging to *B. balaenae* (JQ040306), *Bolbosoma* sp.
229 (JX442167), and *B. turbinella* (JX442166). The BLASTn analysis of 28S sequences
230 produced a percentage of similarity of 99.60% with *Bolbosoma* sp. (JX442179) from the
231 northern fur seal *Callorhinus ursinus* Linnaeus, 1758 available in GenBank. The mtDNA
232 *cox1* sequences shared a similarity of ~99% with *B. balaenae* (JQ061132) from the
233 euphausiid *Nyctiphanes couchii* (Bell, 1853), erroneously deposited in GenBank under the
234 name *Rhadinorhynchus pristis* by Gregori *et al.* (2012).

235 The combined 18S+28S phylogenetic Bayesian tree, including sequences of species within
236 the three genera (*Andracantha*, *Bolbosoma* and *Corynosoma*) of the family Polymorphidae,
237 showed poorly resolved relationships, especially within the genus *Bolbosoma* (Fig. 3). In
238 contrast, the concatenated BI tree topology of the three gene loci 18S+28S+*cox1* showed that
239 the obtained sequences from *Bolbosoma* here analyzed clustered in a highly supported clade
240 (100% of probability value) (Fig. 4). This clade including also the sequences available in
241 GenBank of the polymorphid cystacanth obtained from *N. couchii* (JQ061132, JQ040306),
242 resulted to be clearly distinct from the other species of the genus *Bolbosoma*, whose
243 sequences at those analyzed gene loci, were available in GenBank (Fig. 4). The distance
244 values between the present sequences of *B. balaenae* and the sequences from *N. couchii*
245 were: K2P= 0.017±0.005 at the mtDNA *cox1* and K2P= 0.004±0.002 at the 18S rRNA
246 (present sequences *versus* JQ040304-JQ040306). While, at the interspecific level, the

247 mtDNA *cox1* sequences of *B. balaenae* showed a higher value of differentiation (K2P=
248 0.165±0.020) with respect to the closest sequence of *B. caeniforme* (KF156891). No
249 sequences of *B. caeniforme* were available in GenBank for the 28S gene locus.

250

251 Discussion

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

257 These uncommon records suggest that *Bolbosoma* spp. are only occasional in the
258 Mediterranean basin, likely transported from migrating individuals from the Atlantic Ocean.
259 Helminth parasites have been extensively used as biological tags of marine vertebrates in host
260 population structure studies. Recently, we used anisakid nematodes of the dwarf sperm whale
261 *Kogia sima* Owen 1866 and trypanorhynch cestodes of the sunfish *Mola mola* Linnaeus, 1758
262 to suggest the possible existence of a resident population or migration routes of their hosts,
263 respectively (Santoro *et al.*, 2018; 2020). The fin whale is the most abundant mysticete in the
264 Mediterranean Sea (Panigada and Notarbartolo di Sciara, 2012) with the occurrence of both
265 resident and migrating populations confirmed by genetic studies (Bérubé *et al.*, 1998). For the
266 migrating fin whale populations, a general movement trend towards the northeast North
267 Atlantic in spring-summer and towards the Mediterranean during fall-winter has been
268 suggested (Geijer *et al.*, 2016). The present finding of a parasite known from geographical
269 areas far from the Mediterranean basin seems to suggest that the present fin whale would be a
270 migrating and not a resident individual.

271 Regarding the source of infection, *Bolbosoma* cystacanths have been found in fish
272 (Scombridae, Scorpaenidae, Carangidae, Trichiuridae, Gempylidae, Salmonidae, Berycidae,
273 Lophotidae, Gadidae and Belonidae (www.nhm.ac.uk/research-curation/research/projects/
274 host-parasites/index.html) and crustaceans (euphausiids and copepods) (Measures, 1992;
275 Hoberg *et al.*, 1993; Dailey *et al.*, 2000; Gregori *et al.*, 2012). Recently, Gregori *et al.* (2012)
276 found cystacanths identified as *B. balaenae* in 0.04% of the euphausiid *N. couchii* specimens
277 examined from the Atlantic Galician waters (Spain). The source of the infection of the
278 present fin whale with *B. balaenae* remains unknown; it could be plausible that the fin whale
279 acquired the infection by ingestion of infected crustaceans and/or fish during the migration
280 from the Atlantic to the Mediterranean Sea waters.

281 Most of the morphological characters of adult specimens of *B. balaenae* were not
282 detailed by earlier authors so that comparisons with the present material are limited. For
283 instance, males/females combined total length were 80-160 mm in the original description
284 (reported in Porta, 1906) and 190 to 205 mm in Van Cleave (1953), while data on the
285 measurements of the hook proboscis are missed as well as the measurements of most
286 characters listed in table 2. Regarding the number of rows of hooks and the number of hooks
287 per longitudinal row of the proboscis, the present data correspond to previous data (Meyer,
288 1933; Van Cleave, 1953; Zdzitowiecki, 1991). In contrast, previous descriptions of prebulbar
289 foretrunk of *B. balaenae* using optical microscopy alone reported apparently contrasting data
290 on the presence/absence and numbers of circles of spines: 6 circles in Meyer (1933), 0 in Van
291 Cleave (1953), and up to 10 circles of spines in Zdzitowiecki (1991). Moreover, in
292 cystacanths morphologically identified as *B. balaenae* found encapsulated in the
293 cephalothorax of *N. couchii*, Gregori *et al.* (2012) described a single field of trunk spines
294 restricted to the foretrunk and composed of 4 to 6 irregular circles of small spines adjacent to
295 the neck. Finally, Bennett *et al.* (2021) found 7 circles of spines in an immature individual

296 identified as *B. balaenae* in a blue penguin *Eudyptula novaehollandiae* Stephens 1826 from
297 New Zealand. The present observation regarding the occurrence of trunked trunk spines on
298 the prebulbar foretrunk of adult individuals of *B. balaenae* differentiated by SEM alone
299 supports the hypothesis of Van Cleave (1953), according to which the trunk spines show
300 wide variability in number, and these may be lost along the parasite life span.

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

311 Observation of the detailed surface morphology of the present material using SEM
312 allowed also to highlight the features and unique ultrastructure of proboscis's hooks, showing
313 shallow longitudinal grooves, as well as the micropores of the tegument of foretrunk
314 supposed to be a specialized system implicated in absorptive function (Heckmann *et al.*,
315 2013). According to Heckmann *et al.* (2013) micropores on the tegument showing different
316 sizes and shapes have been described in at least 16 acanthocephalan species. The different
317 ultrastructural pattern of proboscis's hooks has been studied as a potential diagnostic feature
318 to differentiate among species of *Centrorhynchus* and species of related genera, but no
319 conclusive results were obtained (Amin *et al.*, 2015; 2018). No mention is done on both
320 ultrastructure of proboscis's hooks and epidermal micropores from previously published

321 papers reporting SEM observation of *B. capitatum*, *B. vasculosum*, and *B. turbinella* (Amin
322 and Margolis, 1998; Costa *et al.*, 2000; da Fonseca *et al.*, 2019). Future studies comparing the
323 ultrastructure features among *Bolbosoma* species could reveal if the present findings might
324 yield important information to help identify this species.

325 The combination of morphological and molecular studies is considered a very useful
326 approach to resolve taxonomic ambiguities within the genera of Polymorphidae (García-
327 Varela *et al.*, 2013). Unfortunately, out of the 12 species of *Bolbosoma* considered as valid,
328 DNA sequences for only six of those are available in GenBank. Moreover, from the current
329 23 *Bolbosoma* sequences available, only four are from adult parasites obtained from their
330 definitive hosts: three of them are belonging to *B. turbinella* (18S, 28S and *cox1*) from the
331 grey whale, and one to *B. nipponicum* (ITS1/ITS2 region) from the common minke whale.
332 Before the present study, four sequences of *B. balaenae* (including three of 18S and one of
333 *cox1*) were available in GenBank, all from same cystacanths (Gregori *et al.*, 2012). However,
334 the sequence of *cox1* (JQ040303) deposited in GenBank as *B. balaenae* belongs to *R. pristis*
335 (an acanthocephalan of Rhadinorhynchidae family), while the sequence deposited as *R.*
336 *pristis* (JQ061132.1) belongs to *B. balaenae*. Likely an error occurred by Gregori *et al.*
337 (2012) at moment of sequence submission and the names of sequences used in the mentioned
338 study were inverted.

339 In the present study, the BI phylogenetic analysis based on the combined (18S+28S)
340 rRNA data produced poorly resolved clades among species of *Andracantha*, *Corynosoma* and
341 *Bolbosoma*. Moreover, from the obtained results it is clear that the gene locus 18S is not
342 diagnostic for the genetic identification of *Bolbosoma* species. While, the phylogenetic tree
343 herein inferred from combining the sequences obtained at the three gene loci
344 (18S+28S+*cox1*) from adult individuals of *B. balaenae* and those sequences at the same gene
345 loci available in GenBank, has shown that the species of *Andracantha*, *Bolbosoma*, and

346 *Corynosoma* are comprising, respectively, in three distinct and well-supported major clades
347 (Fig. 4). These findings are in agreement with previous phylogenetic elaborations provided
348 by García-Varela *et al.* (2013) and Presswell *et al.* (2018). In addition, the combined BI
349 inferred from 18S+28S+cox1 gene sequences supports, with high probability values, that the
350 so far genetically characterized species of *Bolbosoma*, including *B. balaenae*, represent distinct
351 phylogenetic lineages.

352 The phylogenetic pattern obtained is congruent with the life cycles of members of
353 these three genera (i.e., *Andracantha*, *Bolbosoma* and *Corynosoma*), which involve teleost
354 marine fish as paratenic hosts. It has been suggested that the shared ecological feeding
355 behaviour among different definitive hosts could have provided many opportunities for co-
356 speciation and host-switching events and could have accompanied the evolutionary pathways
357 of these polymorphid species (Dailey *et al.*, 2000; Aznar *et al.*, 2006; García-Varela *et al.*,
358 2013, 2021; Presswell *et al.*, 2018).

359 Finally, the only report of pathological changes associated with Acanthocephala of the
360 genus *Bolbosoma* in a Mediterranean cetacean was reported by Parona (1893) who described
361 a severe intestinal parasitosis caused by *B. capitatum* in a long-finned pilot whale. Parona
362 (1893) reported the occurrence of at least 25305 individual parasites strictly embedded in the
363 muscular layer along the first 12 meters of the intestine. Dailey *et al.* (2000) described gross
364 multifocal transmural abscesses encapsulating proboscis of *B. balaenae* along the first 7.5 m
365 of the ileum in a juvenile gray whale. The present results agree with the gross pathological
366 changes described by Parona (1893) and Dailey *et al.* (2000) and confirm that *B. balaenae*
367 may cause enteritis also in the fin whale.

368

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377

378 **Conflicts of Interest**

379 The authors declare there are no conflicts of interest.

380

381 **Ethical Standards**

382 Not applicable.

383

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563 **Figure legends**

564 **Fig. 1.** Microscopic features of *Bolbosoma balaenae* from the intestine of the fin whale from
565 the southern Italy. Anterior extremity frontal (A, female) and lateral (B, male) views (scale
566 bar: 1000 μm). Proboscis (C, scale bar: 50 μm) and particular of proboscis basal hooks (D,
567 scale bar: 100 μm). Bursa lateral (E, scale bar: 1000 μm) and ventral (F, scale bar: 500 μm)
568 views. Genital pore of female in lateral view (G, scale bar: 500 μm). Mature egg (H, scale
569 bar: 20 μm).

570

571 **Fig. 2.** Scanning electron micrographs of *Bolbosoma balaenae* from the intestine of the fin
572 whale from the southern Italy. General view of prebulb and proboscis of a female (A, scale
573 bar: 100 μm). Note the circles of trunked trunk spines on the prebulb. Lateral (B) and apical
574 (C) views of proboscis and neck (scale bar: 100 μm) of a male. High magnification of an
575 apical (D, scale bar: 10 μm) and a basal (E, scale bar: 1 μm) proboscis hook. High
576 magnification of an apical proboscis hook' surface (F, scale bar: 1 μm) showing longitudinal
577 grooves. A high magnification of a truncated trunk spine (G, scale bar: 1 μm). Note the body
578 wall micropores on the tegument of the prebulb. Mature egg (H, scale bar: 10 μm).

579

580 **Fig. 3.** Phylogenetic concatenated tree from Bayesian inference based on 18S and 28S
581 sequences of *B. balaenae* obtained in the present study, with respect to the sequences of
582 species of genera *Andracantha*, *Bolbosoma* and *Corynosoma*, at the same gene loci available
583 in GenBank. The analysis was performed by MrBayes, v. 3.2.7, using the GTR + G
584 substitution model. *Hexaglandula corynosoma* and *Polymorphus brevis* were used as
585 outgroup. The sequences obtained in this study are in bold.

586

587 **Fig. 4.** Phylogenetic concatenated tree from Bayesian inference based on 18S+28S+cox1
588 sequences of *B. balaenae* obtained in the present study, with respect to the sequences of
589 species of genera *Andracantha*, *Bolbosoma* and *Corynosoma*, at the same gene loci available
590 in GenBank. The analysis was performed by MrBayes, v. 3.2.7, using the GTR + G
591 substitution model. *Hexaglandula corynosoma* and *Polymorphus brevis* were used as
592 outgroup. The sequences obtained in this study are in bold.

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Table 1. Species, stage (L: larva; A: adult), host, locality, and accession numbers of sequences of *cox1*, 28S and 18S of genera *Andracantha*, *Corynosoma* and *Bolbosoma* included in the Bayesian inference shown in the figures 3 and 4. -: data not reported.

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

The *cox1* sequence of *Bolbosoma balaenae* of Gregori *et al.* (2012) was erroneously deposited in GenBank under the name *Rhadinorhynchus pristis*.

Table 2. Measurements (mean value \pm standard deviation with range in parenthesis) of main diagnostic characters in *Bolbosoma balaenae* found in a fin whale from southern Italy. Measurements are in micrometres except when stated. Ten elements for each characters were measured **except for the bursa for which the measurements were obtained from four specimens with everted bursa.**

| Characters | Male (n = 10) | Females (n = 10) |
|----------------------------------|---|---|
| Total length (cm) | 11.3 \pm 0.91 (10.1-12.8) | 13.6 \pm 0.75 (12.8-14.5) |
| Width at middle of body (mm) | 2.4 \pm 0.06 (2.4-2.5) | 4 \pm 0.94 (3-5.1) |
| Bulb length (mm) | 5.2 \pm 0.30 (5.1-5.6) | 6.1 \pm 0.82 (5-7.1) |
| Bulb width (mm) | 5.1 \pm 0.23 (4.9-5.4) | 5.9 \pm 0.61 (4.9-6.6) |
| Prebulb length (mm) | 1 \pm 0.18 (0.8-1.2) | 1 \pm 0.21 (0.8-1.3) |
| Prebulb width at base (mm) | 1.4 \pm 0.26 (1.1-1.5) | 1.8 \pm 0.25 (1.6-2.1) |
| Proboscis length | 564.6 \pm 10.44 (598.4-613.1) | 611 \pm 38.05 (561-648.7) |
| Proboscis width at basal hook | 499.9 \pm 3.51 (496.1-503.1) | 483.9 \pm 67.65 (425.5-572.9) |
| Neck length | 573 \pm 48.44 (518.4-610.8) | 517.8 \pm 61.04 (454-595.4) |
| Lemnisci length | 4005.4\pm219.02 (3828.4-4250.7) | 3157.2\pm873.27 (2211.7-4369.8) |
| Proboscis hook 1 length | 66.8 \pm 15.58 (54.5-88.6) | 51.8 \pm 3.36 (46.2-55.1) |
| Proboscis hook 1 width | 10.5 \pm 1.72 (8.2-12.1) | 11.7 \pm 3.48 (5.7-16.6) |
| Proboscis hook 2 length | 61.8 \pm 7.33 (56.4-74.4) | 61.6 \pm 9.46 (79.2-53.6) |
| Proboscis hook 2 width | 13.3 \pm 2.40 (10.2-16.8) | 14.4 \pm 1.95 (11.4-18.2) |
| Proboscis hook 3 length | 63.2 \pm 4.40 (59.4-69.1) | 57.72 \pm 10.68 (40.01-87.99) |
| Proboscis hook 3 width | 14.55 \pm 0.96 (13.19-15.49) | 13.7 \pm 3.13 (11.5-20.9) |
| Proboscis hook 4 length | 60.6 \pm 3.53 (56.8-65.7) | 51.1 \pm 7.65 (39.2-67.1) |
| Proboscis hook 4 width | 16.1 \pm 2.05 (14.2-18.7) | 14.5 \pm 3.39 (11.3-20.9) |
| Proboscis hook 5 length | 67 \pm 4.35 (61.8-73.9) | 51.3 \pm 8.97 (40.2-70.8) |
| Proboscis hook 5 width | 19.2 \pm 1.42 (16.7-20.9) | 14.9 \pm 3.35 (9.5-20.7) |
| Proboscis hook 6 length | 58.3 \pm 11.71 (44.4-70.1) | 57.7 \pm 12.41 (40.3-81.7) |
| Proboscis hook 6 width | 15.5 \pm 4.22 (11.2-19.8) | 16.7 \pm 3.78 (13.9-24.7) |
| Proboscis hook 7 length | 37.6 \pm 1.12 (36.8-39.5) | 35.3 \pm 7.65 (21.4-55.5) |
| Proboscis hook 7 width | 8.2 \pm 0.97 (7.1-9) | 9.4 \pm 2.56 (5.7-11.5) |
| Proboscis hook 8 length | 28.5 \pm 3.96 (24.7-35.3) | 34.1 \pm 7.41 (22.8-46.8) |
| Proboscis hook 8 width | 4.8 \pm 1.37 (3.8-7.5) | 7.1 \pm 1.48 (4.9-11.2) |
| Anterior testis length | 3621.5\pm350.1 (3044.5-3949.4) | - |
| Anterior testis width | 1308.5\pm143.13 (1065.3-1406.6) | - |
| Posterior testis length | 3479.5\pm348.46 (2866.5-3712.6) | - |
| Posterior testis width | 1431.6\pm105.53 (1275-1441) | - |
| Cement glands length (cm) | 5\pm0.97 (4-6.5) | - |
| Bursa diameter | 2260.2\pm0.18 (2020.1-2480.3) | - |
| Egg length | - | 140.6 \pm 6.74 (132.1-149.5) |

| | | |
|-----------|---|-----------------------|
| Egg width | - | 31.2±1.78 (27.8-33.9) |
|-----------|---|-----------------------|

For Peer Review

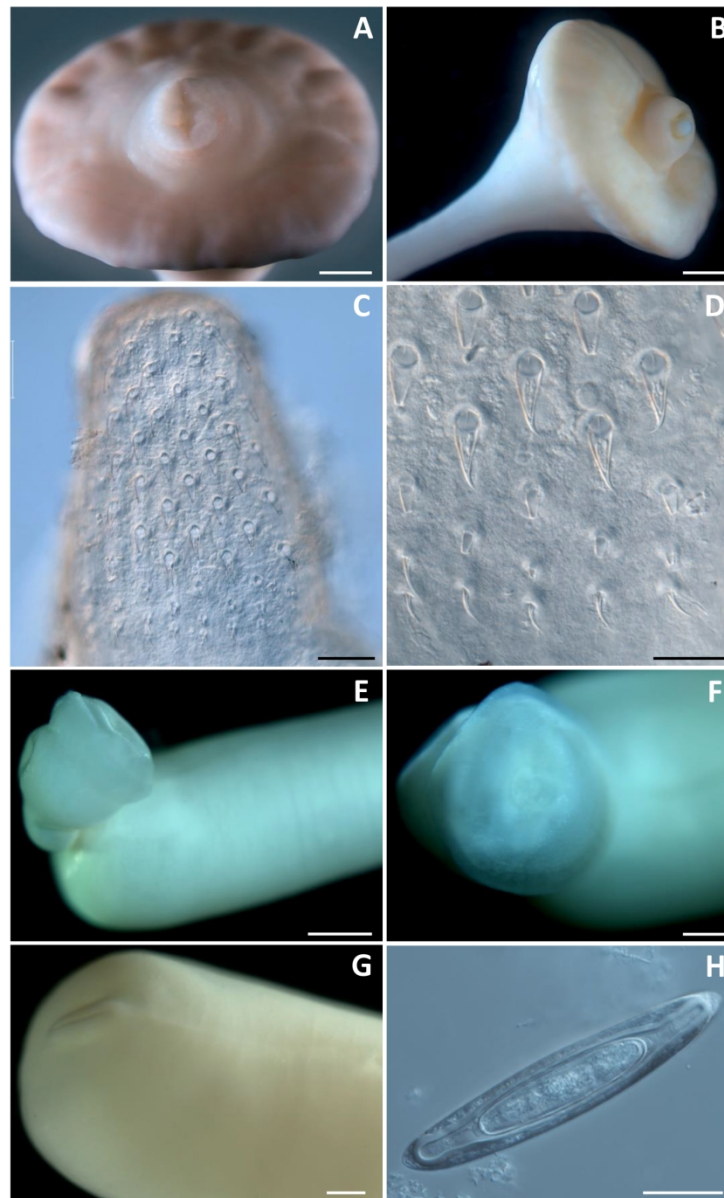


Fig. 1. Microscopic features of *Bolbosoma balaenae* from the intestine of the fin whale from the southern Italy. Anterior extremity frontal (A, female) and lateral (B, male) views (scale bar: 1000 μm). Proboscis (C, scale bar: 50 μm) and particular of proboscis basal hooks (D, scale bar: 100 μm). Bursa 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).

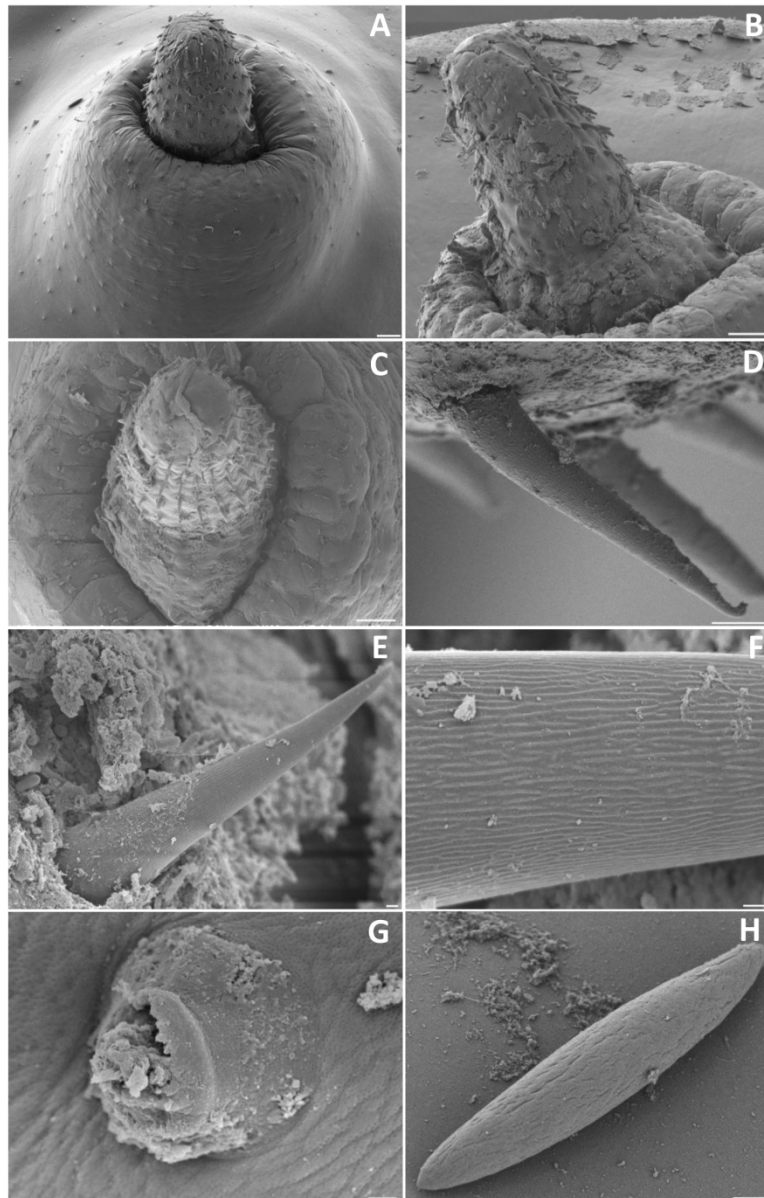


Fig. 2. Scanning electron micrographs of *Bolbosoma balaenae* from the intestine of the fin whale from the southern Italy. General view of prebulb and proboscis of a female (A, scale bar: 100 μ m). Note the circles of trunked trunk spines on the prebulb. Lateral (B) and apical (C) views of proboscis and neck (scale bar: 100 μ m) of a male. High magnification of an apical (D, scale bar: 10 μ m) and a basal (E, scale bar: 1 μ m) proboscis hook. High magnification of an apical proboscis hook' surface (F, scale bar: 1 μ m) showing longitudinal grooves. A high magnification of a truncated trunk spine (G, scale bar: 1 μ m). Note the body wall micropores on the tegument of the prebulb. Mature egg (H, scale bar: 10 μ m).

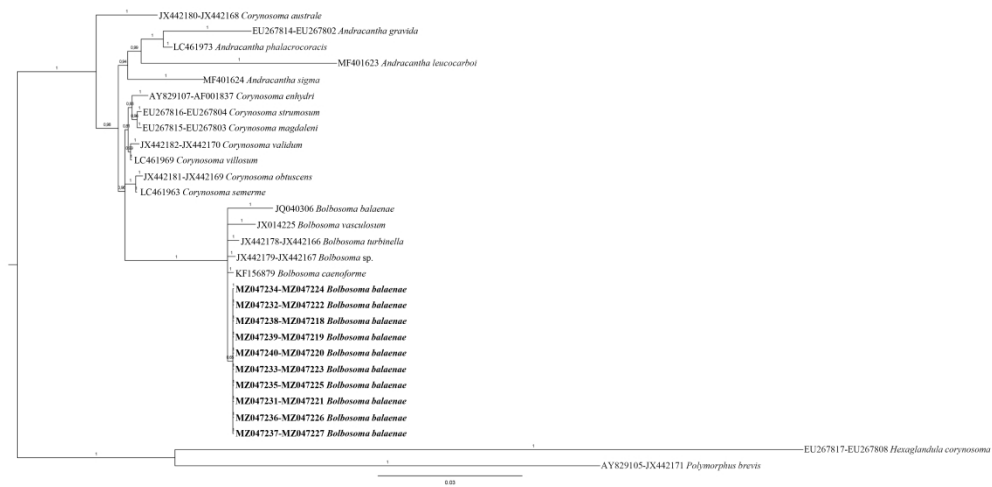


Fig. 3. Phylogenetic concatenated tree from Bayesian inference based on 18S and 28S sequences of *B. balaenae* obtained in the present study, with respect to the sequences of species of genera *Andracantha*, *Bolbosoma* and *Corynosoma*, at the same gene loci available in GenBank. The analysis was performed 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.

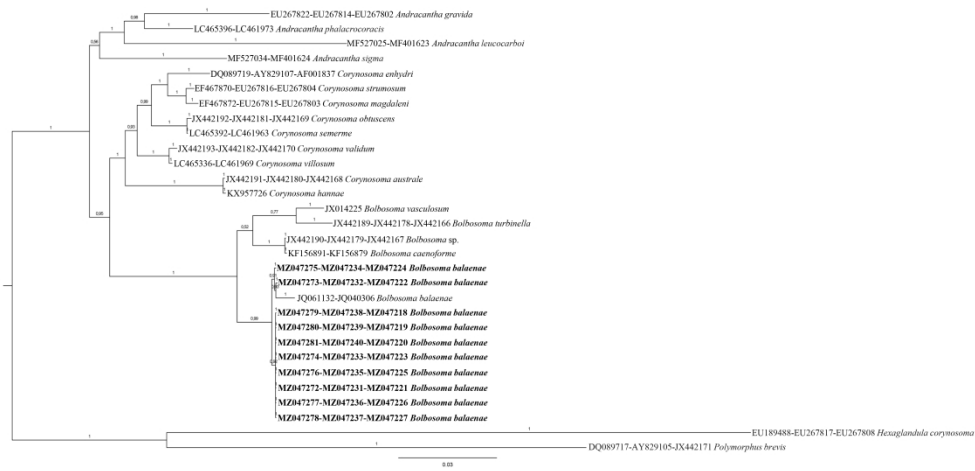


Fig. 4. Phylogenetic concatenated tree from Bayesian inference based on 18S+28S+cox1 sequences of *B. balaenae* obtained in the present study, with respect to the sequences of species of genera *Andracantha*, *Bolbosoma* and *Corynosoma*, at the same gene loci available in GenBank. The analysis was performed 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.