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Microscopic ossicle analyses and the complete mitochondrial genome sequence of Holothuria (Roweothuria) polii (Echinodermata; Holothuroidea) provide new information to support the phylogenetic positioning of this sea cucumber species

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18 January 2025

1 **Microscopic ossicle analyses and the complete mitochondrial genome sequence of *Holothuria***
2 ***(Roweothuria) polii* (Echinodermata; Holothuroidea) provide new information to support the**
3 **phylogenetic positioning of this sea cucumber species**

4

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13

14 **Short title:**
15 Ossicles and mtDNA information from *Holothuria polii*

16 **Abstract**

17 Sea cucumbers (Holothuroidea) are ecologically important organisms for their bioturbation and
18 alkalinization activities of the seabed. These species are extensively fished as they are considered
19 luxury food. Sea cucumbers are also relevant for biomedical studies and the production of bioactive
20 compounds. A few initiatives are recently evaluating sea cucumbers as novel aquaculture species.
21 The aim of this study was to provide morphological and genetic information useful for the
22 identification of *Holothuria polii*, the white spot sea cucumber (a common species of the
23 Mediterranean Sea). We generated the complete sequence of the mitochondrial DNA (mtDNA)
24 genome of this species and combined it with a detailed ossicle characterization of the sequenced
25 specimen by scanning electron microscopic analysis. Ossicles (known also as sclerites) are
26 anatomical features that can discriminate Holothuroidea species, including the closely related ones of
27 the genus *Holothuria*. The complete mitochondrial genome was assembled, functionally annotated
28 and then used to evaluate the phylogenetic relationship of *H. polii* against the other few Holothuroidea
29 species for which the whole mtDNA was available. The 15,907 bp *H. polii* mtDNA sequence has the
30 same gene order already reported for *H. scabra*, *H. forskali* and other species of the same class. *Cox1*
31 and 16S gene sequences were informative for species identification across the genus and could be
32 used for the authentication of commercialized *Holothuria* spp. The mitochondrial genome sequence
33 presented here provides the basis to a future analysis of the variability of *H. polii* populations in the
34 Mediterranean region.

35

36 **Keywords:** mtDNA; phylogeny; sandfish; sclerite; species identification; white spot sea cucumber.

37 **1. Introduction**

38 Within the phylum Echinodermata (Leuckart, 1854), which comprises five classes of marine 39 invertebrates, the class Holothuroidea (known as sandfishes or sea cucumbers) includes about 1400 40 species (Pawson, 2007). This class represents 90% of the deep-sea floor biomass, therefore its species 41 are considered amongst the most dominant organisms in the world (e.g.: Pawson and Pawson, 2008; 42 De Leo et al., 2010).

43 Holothuroid phylogeny is subject to controversies and ambiguities and demands close 44 inspection and perhaps re-evaluations. Kerr and Kim (2001) used 47 morphological traits and 45 performed a cladistic analysis to assess the relationship among the orders belonging to the class 46 Holothuroidea initially established by Bronn (1860). Recent molecular data obtained from both 47 mitochondrial and nuclear markers identified seven orders (Miller et al., 2017).

48 Within this class, to date the nuclear genome of only three species has been preliminarily 49 assembled and genome scaffolds are available for *Apostichopus parvimensis*, *A. japonicus* and 50 *Australostichopus mollis* (Kudtarkar and Cameron, 2017). A total of 10 complete mitochondrial 51 genomes has been obtained for species of this class (only nine of which are available in GenBank; 52 Long et al., 2016; Kudtarkar and Cameron, 2017; Wang et al., 2019).

53 In several regions of the world, holothurian fishing and rearing are practiced to supply specific 54 food markets, mainly driven by Asian countries (Han et al., 2016). Therefore, several efforts have 55 been made to study and catalogue sea cucumber exploitation hotspots all over the world. Over-56 exploitation caused genetic flow loss among populations and in some places their complete extinction 57 (Friedman et al., 2011; Soliman et al., 2016). One of the main reasons of this excessive harvest is the 58 rising demand of the Asian luxury food and traditional medicine markets (Purcell et al., 2014). 59 Moreover, sandfishes consumption, as traditional and valuable food, has raised interests on the 60 nutritional properties of these animals, highlighting the presence of antioxidant molecules together 61 with a high protein content and a low fat level, also in the dried form, known as "bêche-de-mer" or 62 *trepang* (Wen et al., 2010; Roggatz et al., 2016). Other reasons of interests on sea cucumbers derive

63 by their use as models for tissue and organ regeneration, by their peculiar adaptations and by their
64 relevance for bioactive compounds production, such as holothurins (García-Arrarás and Dolmatov,
65 2010; Jaeckle and Strathmann, 2013; Zhang et al., 2017).

66 *Holothuria (Roweothuria) polii* (Delle Chiaje, 1823), also known as white spot cucumber, is a
67 neritic marine organism belonging to the Holothuriidae family (Aspidochirotida). This species lives
68 in a depth between 0 to 250 m along the coasts of Mediterranean and Black Seas, Suez Gulf and in
69 some spots of the Atlantic Ocean coasts (Coll et al., 2010). *Holothuria polii*, as other holothurians,
70 plays an important ecological role as a detritivorous in benthic communities doing both a bioturbation
71 of the marine sediments and a buffer activity counteracting the effects of water acidification and
72 showing physiological plasticity (Vergara-Chen et al., 2010; Yuan et al., 2018). *Holothuria polii* is
73 also one of the most exploited species in terms of commercial trade for food and pharmaceutical
74 purposes in the Mediterranean area. Turkey has the main sea cucumber commercial fishery where
75 about 80% of the harvested holothurians belongs to *H. polii* species (González-Wangüemert et al.,
76 2014). The high market demand, mainly from Asian countries, is increasing the risk to deplete wild
77 stocks of this species, with a reduction of biodiversity and benthic biomass, which might break the
78 ecological and chemical marine balances (Purcell et al., 2016; Pawson and Pawson 2008). Therefore,
79 efforts are necessary to preserve wild sea cucumber populations and to establish new efficient rearing
80 methods and aquaculture production systems for this species (Bell et al., 2007; Purcell et al., 2013;
81 Ren et al., 2014; Beltran-Gutierrez et al., 2016).

82 In parallel to conservation actions, it is important to develop new methods and tools to simplify
83 the identification of *H. polii*. This is needed for species authentication of luxury holothurian food
84 products to identify frauds and illegal trades of this species, recently studied as a new candidate for
85 aquaculture (Conand et al., 2018; Rakaj et al., 2019). Morphological identification of species within
86 the *Holothuria* genus is mainly based on the shape, size and fine details of endodermal ossicles (or
87 sclerites) which are calcified structures that are part of the echinoderm endoskeleton (Koehler, 1924;
88 Tortonese, 1965; Aydin and Erkan, 2015). However, the morphological analysis has often led to

89 wrong species assignment of holothurians because ossicles, within species, can change shape,
90 typologies and location in different body regions (Cutress, 1996; Massin et al., 2000). In addition, a
91 detailed and complete characterization of ossicles is not available for most species. Hybridization
92 events between sympatric species and subspecies, that can lead to animals with mixed morphological
93 features might add confounding factors (Uthicke et al., 2005; Yoshida et al., 2012; Kim et al., 2013).
94 Thus, molecular information is therefore necessary in order to complete and clarify the inter and
95 intraspecific diversity of sea cucumbers (Aydin and Erkan, 2015; Dettaï et al., 2011; Valente et al.,
96 2015). At present, molecular phylogeny of the Holothuriidae family, based only on *cox1,* 16S
97 mitochondrial DNA (mtDNA) sequences and 18S nuclear sequences, is still unresolved for some
98 subgenera resulting in paraphyletic groups including *Roweothuria*, which in turn includes *H. polii*
99 (Kerr et al., 2005; Honey-Escandon et al., 2011; Borrero-Pérez et al., 2010).

100 In this study, as a first step for a detailed description of the genetic variability in *H. polii*
101 populations, we sequenced the complete mitochondrial genome of this species and compared this
102 mitogenome with the available mtDNA sequences of other holothurian species. In addition,
103 mitochondrial genome information of *H. polii* was evaluated in a comparative analysis with ossicle
104 morphology and distribution obtained using detailed microscopy ispection. The produced molecular
105 and morphological results filled a gap in the phylogenetic analysis of *H. polii* and provided important
106 classification and identification tools, also useful for the authentication of this species in food and
107 drug preparations.

108

109 **2. Materials and Methods**

110 **2.1. Specimen and morphological characterization**

111 A sea cucumber specimen was collected in the western coast of Sardinia (Oristano province).
112 The identification of the species was conducted following dichotomous keys (Tortonese, 1965;
113 Koehler, 1924) and using the criteria reported by Aydin and Erkan (2015) who suggested the use of
114 complementary information on bathymetry and body coloration and shape.

115

116 ## 2.2. Microscopic analyses of the ossicles

117 Ossicles were analysed by using optical microscopy and scanning electron microscopy (SEM).
118 Microscopy samples were prepared starting from 1 g of different tissues using sodium hypochlorite
119 to eliminate non-calcified material. Preparations were from the internal tegument portion of the
120 *bivium* (dorsal part of the animal) including papillae, tentacles, anal tegument or wall and the
121 tegument around the calcareous ring. After the digestion of the organic matrix, specimens were
122 carefully washed with bi-distilled water, taking care to preserve the ossicles. Optical microscopy
123 analysis of the dorsal body wall was performed to check the cleanliness of the digested organic matrix.
124 The visualization and the image acquisition were performed using an optical microscope Laborlux
125 12 (Leitz, Wetzlar, Germany), resuspending purified ossicles in a solution of bi-distilled water. The
126 ossicles were visualized with a 40X magnification and acquired by Infinity 1-5C camera software
127 (Teledyne Lumenera, Lumenera Corp. 7 Capella Crt. Ottawa, Ontario, Canada K2E 8A7). For the
128 SEM analyses, purified ossicle samples were mounted on a glass slide stuck on an aluminum stub
129 using Silver conductive glue (Silver Print, Provac AG). Specimens were sputtered with 2 nm gold
130 particles using K500 instrument (Emitec, Lohmar, Germany) at 30 mA for 2 min. Ossicles were
131 observed using a SEM 515 microscope (Philips, Electronic Instruments, Eindhoven, The
132 Netherlands) at 10 kV with a spot size of 20 nm. Images acquisition was performed with a K-5 camera
133 (Pentax, Tokyo, Japan).

134

135 ## 2.3. DNA extraction, library preparation and next generation sequencing

136 DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega
137 Corporation, Madison, Wisconsin, USA) following the manufacturer's instructions. Genomic DNA
138 was quality checked performing an electrophoresis on a 0.8% agarose gel and quantified using the
139 Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

140 Next generation sequencing included the following procedures for the library preparation.
141 Genomic DNA was sheared through sonication. Next, after fragment end repair, sequencing adapters
142 were ligated to both ends and DNA was amplified in an indexing PCR. Then library size distribution
143 was confirmed using Bioanalyzer instrument (Agilent, Santa Clara, CA, USA) and size selection was
144 performed using the BluePippin System (Sage Science, Beverly, Massachusetts, USA) with a 2%
145 agarose gel cassette. Finally, the library was sequenced on an Illumina (San Diego, CA, USA) HiSeq
146 2500 with 2 × 150 PE rapid run chemistry and 100 bp reads were obtained with an insert size of 450
147 bp and an inner distance of 250 bp.
148
149 **2.4. Next generation sequencing reads, mtDNA assembly and annotation**
150 Quality of the reads was evaluated using FastQC v.0.1.1.7
151 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) that highlighted very high-quality
152 reads. No other filtering procedures were adopted. Reads were assembled via the iterative approach
153 implemented in MITObim 1.9.1 (Hahn et al., 2013) using the cytochrome-oxidase subunit 1 (*cox1*)
154 sequence of *H. polii* (GenBank: KJ493895.1) as reference. To evaluate the quality and the reliability
155 of the assembled genome, reads were subsequently mapped on it with BWA tool 0.7.17 (Li and
156 Durbin, 2009) by computing with SAMtool v1.7 (Li et al., 2009) the following parameters: i) breadth
157 and depth of coverage and ii) the length of the insert size. The annotation of the constructed mtDNA
158 genome was obtained by using MITOS WebServer (http://mitos.bioinf.uni-leipzig.de) and by
159 manually curating gene boundaries using the NCBI tool ORFfinder
160 (https://www.ncbi.nlm.nih.gov/orffinder/). RNA genes were validated with MFold
161 (http://unafold.rna.albany.edu) and ARWEN (http://mbioserv2.mbioekol.lu.se/ARWEN/) software
162 tools (Zuker, 2003; Bernt et al., 2013; Laslett and Canbäck, 2007). Then, the mitochondrial genome
163 map was prepared using GenomeVx (http://wolfe.gen.tcd.ie/GenomeVx/), setting the *cox1* gene as
164 the starting point of the mtDNA (Conant and Wolfe, 2008). The complete annotated mitochondrial

165 sequence has been deposited in ENA within the project number PRJEB31737 and the accession
166 number NC_045029.
167
168 **2.5 Molecular species assignment and phylogenetic analyses**
169 Molecular analyses were performed using the most representative sequences of the species of
170 holothurians present in NCBI nucleotide database. A portion of 412 bp of all holothurian *cox1* gene
171 sequences including other sequences from *H. polii* and including the *de novo* assembled *H. polii*
172 portion of *cox1* (from positions 312 to 723 of the mtDNA genome) was used to confirm the obtained
173 species assignment using BLASTn analysis (Altschul et al., 1990). Similarly, a portion of 443 bp the
174 16S rRNA sequence (from position 15,206 to 15,648 of the mtDNA genome) was compared with the
175 most representative 16S holothurian sequences in order to confirm the *cox1* outputs.
176 The MEGA X software suite (Kumar et al., 2018) was used to compute codon usage, nucleotide
177 composition statistics and to carry out the phylogenetic analyses. Three phylogenetic trees were
178 computed. The first one was based on the complete aminoacidic sequence of *cox1* gene of the
179 holothurians for which the complete mtDNA was available in NCBI (https://www.ncbi.nlm.nih.gov/)
180 while the second one was based on the complete 16S rRNA sequences of the same species of
181 holothurians. A total of ten species plus the outgroup *Strongylocentrotus purpuratus* (accession
182 NC_001453) were considered (Table S2). Initially, BLASTn analysis was used to compare *cox1* and
183 16S rRNA gene sequences and confirm species identification. *Cox 1* nucleotide sequences were
184 translated using the Echinoderm mitochondrial genetic code and the multiple sequence alignment
185 (MSA) was obtained using the CLUSTALW algorithm (Thompson et al., 1994). A *maximum*
186 *likelihood* (ML) phylogenetic tree was obtained using default settings (molecular evolutionary model:
187 Jones – Tailor – Thorton) with 1,000 bootstrap replicates (Jones et al., 1992). The second
188 phylogenetic tree was computed starting from the MSA of 16S rRNA sequences obtained using the
189 Q-INS-i algorithm implemented in the online version of MAFFT v7.427 (Katoh et al., 2019) in which
190 secondary structure of RNA were considered. Then, the evolutionary history was inferred by using

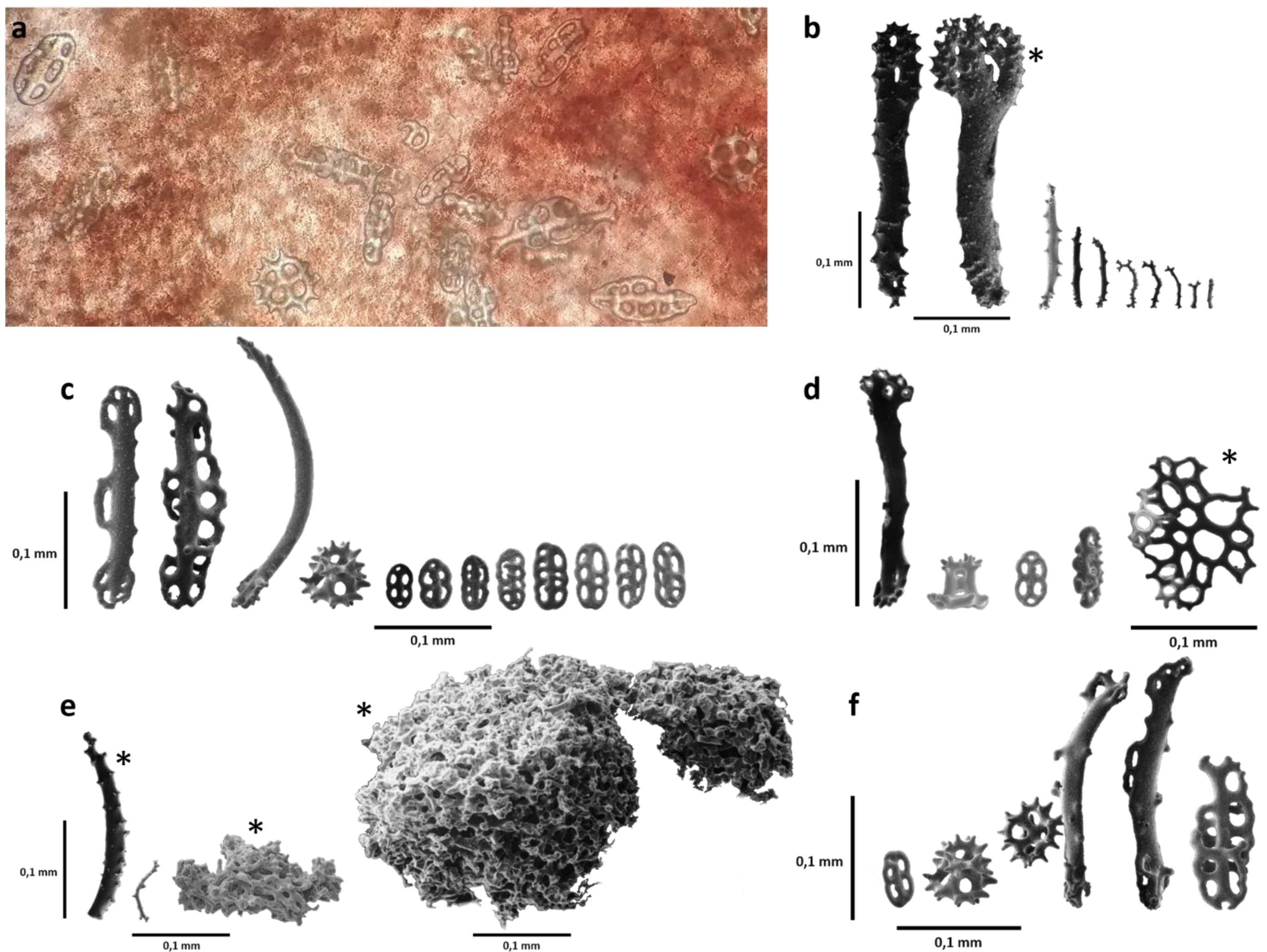
191 the *maximum likelihood* (ML) method and General Time Reversible model with 1,000 bootstrap
192 replicates (Nei and Kumar, 2000).
193 The third phylogenetic tree was built by considering the complete mitochondrial DNA sequence
194 of nine holothurian species plus the outgroup *Strongylocentrotus purpuratus* (accession
195 NC_001453.1) excluding the *Peniagone sp.* mtDNA because of the lack of *trnC* gene in the
196 annotation. Genomes were aligned using MAFFT v7.427 with default settings (Katoh and Standley,
197 2013). The MSA was manually curated and a Neighbour Joining (NJ) phylogenetic tree was obtained
198 by computing evolutionary distances using the Maximum Composite Likelihood method (Tamura et
199 al., 2004). The rate variation among sites was modelled with a gamma distribution (shape parameter
200 = 1).
201 Finally, the gene order of the *H. polii* mtDNA genome was compared with those of the other
202 holothurians mitochondrial genomes in order to detect putative rearrangements within the
203 Holothuroidea class.
204

205 3. Results

206 3.1. Species identification based on ossicle analysis

207 The application of dichotomous keys (Tortonese, 1965; Koehler, 1924) and the use of
208 information on the bathymetry and body coloration and shape (Aydin and Erkan, 2015) indicated that
209 the analysed specimen was from *H. polii*. To confirm this assignment, ossicles were microscopically
210 analysed.
211 SEM analyses of the ossicles revealed smooth surfaces, perforated buttons and tables as
212 common features of the ossicle morphology; some examples are shown in Figure 1. The different
213 analysed parts of the body showed various types of ossicles, some of which having different shapes,
214 as already reported on the dichotomous keys (Tortonese, 1965; Koehler, 1924). In particular, the
215 *papillae* tegument contained spiny rods, coral-like rods and some large concretions not described in
216 the literature yet. The internal tegument covering the calcareous ring showed the presence of both

217 regular and irregular smooth buttons, knobbed buttons and smooth tables. In addition, rods with
218 smooth surface and enlarged perforated ends were present. Tentacles showed only rods of different
219 size and shape. The external anal tegument contained smooth tables, buttons and rods, the latter with
220 irregular shape. A few other ossicles had a rod-like convoluted shape, with large and numerous holes.
221 Finally, the dorsal tegument (*bivium*) showed smooth tables, regular and irregular buttons, curved
222 rods perforated at the end and rod-like convoluted ossicles (Figure 1). Most of these ossicles were
223 already described by Moussa and Wirawati (2018) in *H. polii* and led to the identification of this
224 species. However, thus far several shapes described here have not been reported in this species. Figure
225 1 describes the novel ossicle types.



226

227 **Figure 1.** a. Example of sclerites included in the organic matrix of *Holothuria polii* seen with optical microscope (40X).

228 b. c. d. e. f. Sclerites seen with SEM with scalebars near and below for each body portion analysed. b: Tentacles. c: *bivium*.
229 d: Internal tegument of calcareous ring. e: Dorsal *papilla*. f: Anal tegument.
230 * indicates novel sclerites not yet described in this species.
231

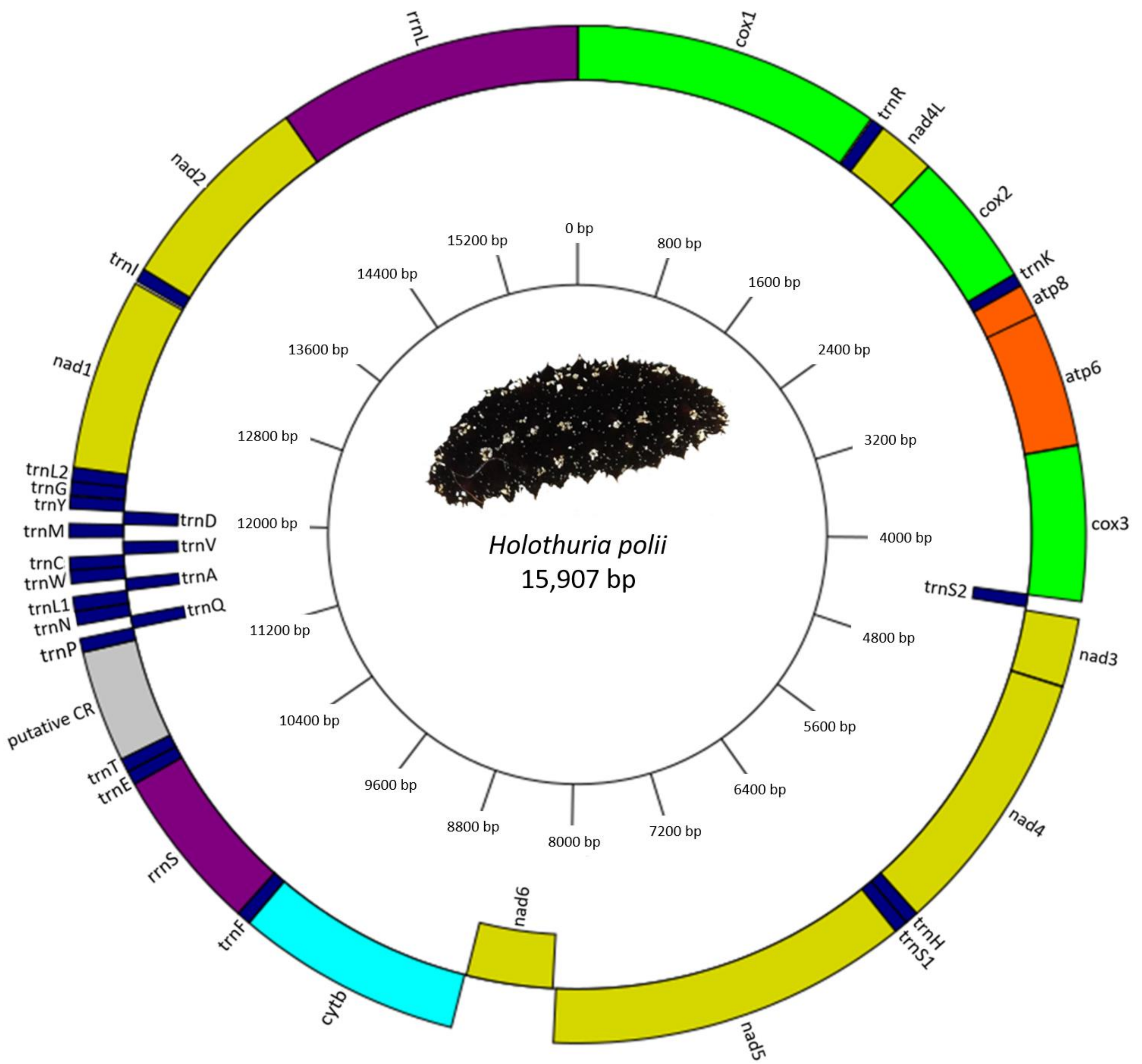
232 **3.2. Description of the complete mtDNA genome of *Holothuria polii***

233 The complete mitochondrial genome of *H. polii* was obtained from whole DNA sequencing
234 raw data (38,340 read pairs). Breadth and depth of coverage were 100% and 480X, respectively, with
235 an average inner distance of 250 bp, as expected. No polymorphisms were detected after variant
236 calling analysis, excluding the presence of heteroplasmy. Table S1 shows the complete annotation
237 and organization of the *H. polii* genome. This mitogenome consisted of 15,907 bp and included 22
238 tRNA genes, 13 protein-coding genes, 2 rRNA genes and a putative control region (D-Loop). The
239 light strand encoded 5 tRNAs and the *nad6* gene, whereas the heavy strand encodes 17 tRNAs, 12
240 protein-coding genes, the 2 rRNAs and the longest unassigned non-coding region of 566 bp (Figure
241 2). Given that the second longest unassigned region was 26 bp long, the 566 bp unassigned sequence
242 was considered the putative control region, which had an A-T content of 63.8% (the highest value
243 over the whole mtDNA sequence). This mtDNA genome had an overall A-T content equal to 58.3%.
244 The nucleotide frequencies were: A=30.9%, T=27.4, C=25.6% and G=16.1%. The coding region
245 consisted of 3,775 amino acids with a total of 3,786 codons. The most frequently used amino acids
246 were leucin (16.6%) and isoleucin (9.9%), whereas the most frequently used codon was ATA (Ile)
247 with a frequency of 5.55%, followed by CTA (Leu; 5.49%).

248 All 13 protein-coding genes started with the ATG codon (Met). Nine genes terminated with the
249 TAA codon, 2 genes terminated with TAG and 2 genes had incomplete stop codons (T--) which are
250 quite common in animal mitochondrial genes (Ojala et al., 1981). No rearrangements were found in
251 this species and the gene order was the same as that of the mtDNA genome of *Apostichopus japonicus*,
252 *Parastichopus californicus*, *P. nigripunctatus*, *P. parvimensis*, *H. scabra*, *H. forskali* and also with

253 that of the animal model *Strongylocentrotus purpuratus* (Echinoidea). The strand position of the

254 genes was shared among all these species (Figure S1).



255

256

257 **Figure 2.** Circular visualization and organization of the complete *Holothuria polii* mtDNA. External genes of the circle

258 are encoded by the positive strand (5'→3') and internal genes are encoded by the negative strand (3'→5'). The specimen

259 picture is reported inside the circle.

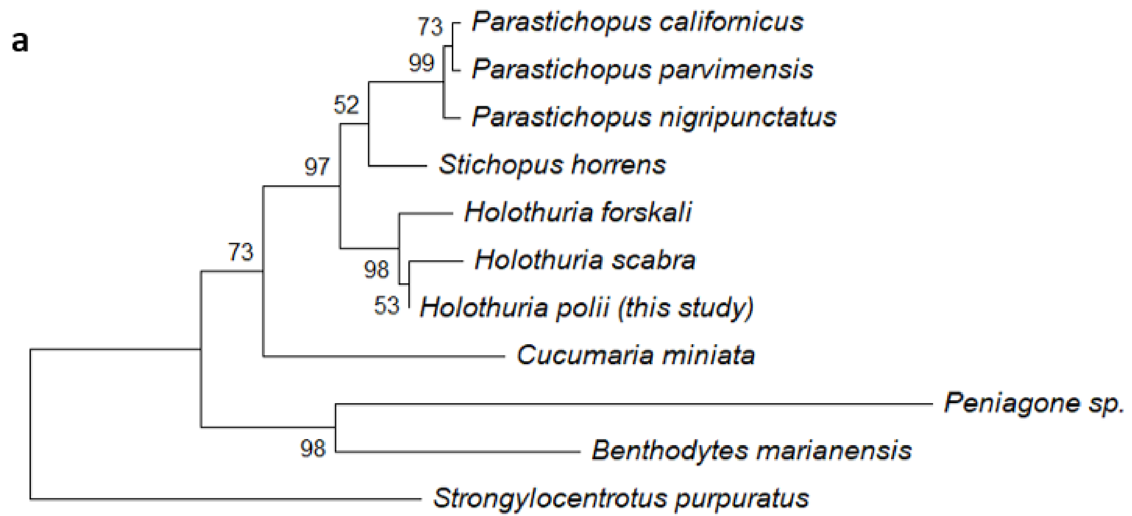
260

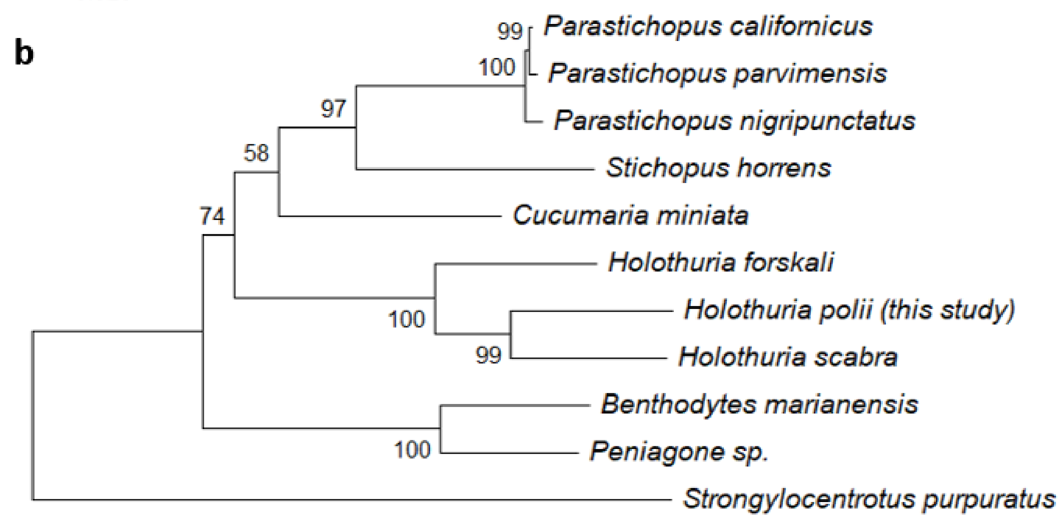
261 3.3. Molecular identification and phylogenetic analyses

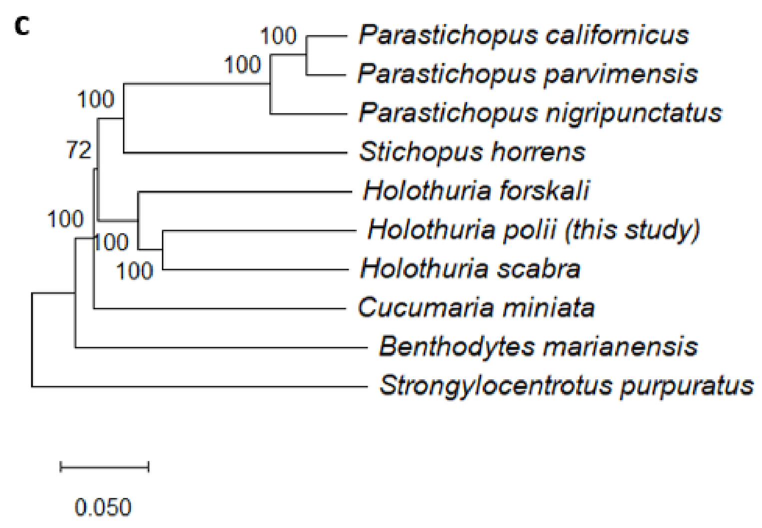
262 The molecular identification of the species based on the selected portions of *cox1* gene using
263 BLASTn analysis showed a range from 98% to 100% of identity with *cox1* of *H. polii* sequences
264 already deposited in NCBI, whereas the second higher identity (90%) was obtained for *H. tubulosa*
265 (GenBank: KJ719549.1). The BLASTn analysis with the portion of the 16S rRNA sequence obtained
266 similar results (identity ranged from 98% to 100% with *H. polii* sequences and was 94% with the
267 second closest species). These results confirmed that these two genes (or some portions of them) of
268 the *H. polii* mtDNA genome are informative in terms of species identification (Uthicke et al., 2010;
269 Kerr et al., 2005). Table S3 shows a comparison between the main ossicle types and *cox1* and 16S
270 molecular markers; in particular, the main rod, button and table morphology have been compared
271 together with the results of BLASTn analysis among our *H. polii* specimen, *H. polii* from other
272 studies, *H. tubulosa* and *H. scabra* (Moussa and Wirawati 2018; Aydin and Erkan 2015; Massin et
273 al., 2000).

274 Regarding the phylogenetic analyses, in order to obtain a robust phylogenetic evaluation, a
275 *maximum-likelihood* phylogenetic tree was constructed based on the entire aminoacidic sequence of
276 the *cox1* gene of 10 species belonging to 4 orders of the Holothuroidea class for which the whole
277 mtDNA was available in GenBank (Table S2). Parallelly, another ML tree was obtained based on the
278 sequences of 16S rRNA using the same dataset and in both cases the outgroup *Strongylocentrotus*
279 *purpuratus* was chosen. The phylogenetic trees are reported in Figure 3.

280 *H. polii cox1* and 16S rRNA sequence obtained in this study grouped with the two extant
281 mtDNA of the genus with a high bootstrap value showing a strong reliability of the node.







282

283 **Figure 3.** a. Maximum Likelihood (lnL -2,753.67) phylogenetic tree obtained using the *cox1* aminoacidic sequence of
284 the available mtDNA of different holothurian species, rooted with the outgroup *Strongylocentrotus purpuratus*. The
285 bootstrap test values (1,000 replicates) are shown next to the branches.
286 b. Maximum Likelihood (lnL -10,936.53) phylogenetic tree obtained using the 16S rRNA sequence of the available
287 mtDNA of different holothurian species, rooted with the outgroup *Strongylocentrotus purpuratus*. The bootstrap test
288 values (1,000 replicates) are shown next to the branches.
289 c. Neighbour-Joining phylogenetic tree obtained with complete mtDNA genome sequences of holothurian species.
290 The bootstrap test values (1,000 replicates) are shown next to the branches.
291
292 Generally, with the exception of *Cucumaria miniata* position in the 16S tree, all the
293 phylogenetic trees show similar topologies. The NJ phylogenetic tree based on the complete
294 sequences of holothurians confirmed the topology obtained with the ML tree based on aminoacidic
295 *cox1* sequences: *H. polii* clustered with *H. scabra* and together they formed a clade with *H. forskali*.
296 The three *Parastichopus* species clustered together while *Benthodytes marianensis* was the closest
297 species to the outgroup, the echinoid *Strongylocentrotus purpuratus* (Figure 3).
298
299 **Discussion**
300 The main informative morphological features used to identify holothurian species are the
301 ossicles, that are calcite structures inserted into the integument, which provide the typical rigidity of
302 the body of the animal (Cutress et al., 1996). It is important to highlight that the whole intraspecific
303 diversity of the shape and of the surface of the ossicles, as well as their position on the different body
304 parts is still largely unknown for most holothurian species, including *H. polii* (Borrero-Pèrez et al.,
305 2009). *Holothuria polii* is described to have different types of ossicles (buttons, tables, rods and
306 others) with a smooth surface (Tortonese, 1965; Koehler, 1924). Despite the predominance of smooth
307 ossicles in the analysed specimen, the presence of some knobbed and rugose buttons could have
308 potentially led to an uncorrected species assignment. These types of ossicles are characteristics of *H.*
309 *tubulosa*, which is described to have only rugose ossicles, in addition to tables similar to that of *H.*

310 *polii*. It is still unclear if hybridization events could occur in sympatric species (like *H. polii* and *H.*
311 *tubulosa*), and if mixed ossicle types (and in which proportion and body positions) could be present.
312 The ossicle morphological phenotype could be misleading as already observed by other authors
313 (Moussa and Wirawati, 2018). The broad spectrum of ossicle shapes described in this study using
314 the SEM images included types that, to our knowledge, were never detected before in *H. polii*. These
315 observations might suggest that the entire variability of this morphological trait is not completely
316 known and further investigations are needed also to evaluate the potential confounding hybridization
317 events.

318 Despite these not completely clarified questions, all other morphological elements, in addition
319 to molecular evidences based on mtDNA support the assignment of the analysed specimen to *H. polii*.
320 Molecular analyses of *cox1* and 16S sequences were concordant to unambiguously assign the
321 specimen to the *H. polii* suggesting that molecular data may be more robust than morphological
322 descriptors, as already noted by others (Miller et al., 2017). Moreover, in some contexts it is not
323 possible to analyse ossicles (for instance in highly processed sea cucumber food or in environmental
324 studies).

325 The complete mitochondrial genome of *H. polii* shows an A-T content in line with the other
326 holothurian mitogenomes analysed. The comparison of the gene order showed that *H. polii* shares the
327 gene position on mtDNA with that of all other holothuroids (with the exception of *Stichopus horrens*,
328 *Cucumaria miniata*, *Benthodytes marianensis*) and also with that of sea urchin *Strongylocentrotus*
329 *purpuratus*, suggesting that this may represent the ancestral condition. Phylogenetic analyses
330 confirmed that the represented four orders are clearly supported by robust nodes corroborating the
331 phylogenetic position of *H. polii* closer to *H. scabra* in respect of *H. forskali*. In particular, these trees
332 highlighted a distinct clade of abyssal sea cucumbers *Peniagone sp.* and *Benthodytes marianensis*
333 (Elasipodida) while the Synallactida, Holothuriida and Dendrochirota orders form a cluster together
334 confirming the Pneumonophora as sister group to Elasipodida (Miller et al., 2017). The *Cucumaria*
335 *miniata* different position in the 16S rRNA tree in respect of *cox1* and complete mtDNA trees can be

336 explained with the less suitability of mitochondrial rRNA genes for species delimitation in DNA
337 barcoding and for the low gap between intra and interspecific divergences in these portions in respect
338 of coding genes (Nijman and Aliabadian, 2010; Vences et al., 2005).

339 *H. polii* plays an important ecological role for the Mediterranean marine environment. This
340 species is subject to massive illegal overfishing and extensive over utilization (Friedman et al., 2011;
341 Purcell et al., 2013; 2014). For these reasons, it is important to develop and validate methods and
342 tools that could be used for species identification and to monitor the distribution and biodiversity of
343 *H. polii* and related species. Therefore, molecular tools based on mtDNA sequences could become
344 very relevant for these purposes, in addition to the use for the authentication of food products based
345 on holothurian species. The mtDNA information we provided for *H. polii* filled an important gap in
346 this context.

347

348

349 **Conclusions**

350 A more extensive description of ossicle variability is still needed to obtain a better description
351 of holothurian species. It appeared however clear that ossicle data should be coupled with molecular
352 data to confirm species identification as we did in our study. The mtDNA sequence can be used for
353 species identification which might be relevant in particular to monitor the trades of holothurians for
354 the extensive use of these species in the food luxury market. The complete mtDNA sequence of *H.*
355 *polii* will be also useful as a starting point to monitor genetic diversity of this species and evaluate
356 the success of conservation programmes of the marine ecological communities that are highly
357 dependent by sea cucumbers.

358

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362 **Conflict of interest**

363 The authors declare that there is no conflict of interests regarding the publication of this article.

364

365 **References**

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555 ## Supplementary materials:

556

557 **Table S1**. Functional annotation of the complete mitochondrial DNA of *Holothuria (Roweothuria) polii*.

Gene	Start	End	Strand	Lenght (bp)	tRNA codon	Intergenic nucleotides	Start codon	Stop codon	Aminoacids
cox1	1	1557	H	1557		0	ATG	TAG	518
trnR	1566	1632	H	67	CGA	8			
nad4L	1633	1929	H	297		0	ATG	TAA	98
cox2	1930	2617	H	688		0	ATG	T—	229
trnK	2619	2682	H	64	AAG	1			
atp8	2683	2850	H	168		0	ATG	TAA	55
atp6	2844	3527	H	684		-7	ATG	TAA	227
cox3	3530	4312	H	783		2	ATG	TAA	260
trnS2	4311	4381	L	71	TCA	-2			
nad3	4400	4744	H	345		18	ATG	TAA	114
nad4	4748	6104	H	1357		3	ATG	T—	452
trnH	6106	6172	H	67	CAC	1			
trnS1	6174	6241	H	68	AGC	1			
nad5	6242	8074	H	1833		0	ATG	TAG	610
nad6	8092	8580	L	489		17	ATG	TAA	162
cytb	8589	9731	H	1143		8	ATG	TAA	380
trnF	9733	9803	H	71	TTC	1			
rrnS	9804	10632	H	829		0			
trnE	10633	10701	H	69	GAA	0			
trnT	10703	10772	H	70	ACA	1			
Putative Control Region	10773	11338	H	566		0			
trnP	11339	11407	H	69	CCA	0			
trnQ	11404	11473	L	70	CAA	-4			
trnN	11476	11545	H	70	AAC	2			
trnL1	11547	11618	H	72	CTA	1			
trnA	11618	11684	L	67	GCA	-1			
trnW	11685	11752	H	68	TGA	0			
trnC	11753	11816	H	64	TGC	0			
trnV	11820	11889	L	70	GTA	3			
trnM	11916	11985	H	70	ATG	26			
trnD	11987	12058	L	72	GAC	1			
trnY	12059	12123	H	65	TAC	0			
trnG	12126	12195	H	69	GGA	2			
trnL2	12195	12265	H	71	TTA	-1			
nad1	12266	13237	H	972		0	ATG	TAA	323
trnI	13251	13318	H	68	ATC	13			
nad2	13319	14362	H	1044		0	ATG	TAA	347
rrnL	14363	15907	H	1545		0			

Table S2. List of the Holothuroidea used for phylogenetic analyses available in GenBank including *Holothuria polii* obtained in this study.

Species	**Accession number**	**mtDNA Lenght (bp)**
Benthodytes marianensis	MH208310	17,567
Holothuria forskali	NC_013884	15,841
Holothuria scabra	NC_027086	15,779
Holothuria polii (this study)	NC_045029	15,907
Cucumaria miniata	NC_005929	17,538
Parastichopus nigripunctatus	NC_013432	16,112
Parastichopus californicus	NC_026727	16,727
Parastichopus parvimensis	NC_029699	16,120
Peniagone sp. *	KF915304	15,507
Stichopus horrens	NC_014454	16,257

* This species has not been used for the complete mtDNA NJ tree.

Table S3. Comparison between the main morphological and molecular markers used for *Holothuria polii* species identification. The percentages represent the identity and the coverage of the first more similar result and the related accession number in BLASTn for each species. Images of the holothurians different from our specimen derive from Moussa and Wirawati 2018; Aydin and Erkan 2015; Massin et al. 2000.

	Buttons	Tables	Rods	*cox1* (% BLASTn identity; % coverage, accession number)	16S (% BLASTn identity; %coverage, accession number)
Holothuria polii this study				100% LR694133	100% LR694133
Holothuria polii other studies				99.76%; 100% JN207607	99.77%; 99% LC176660
Holothuria tubulosa				89.95%; 96% KJ719549	93.24%; 100% GU797633
Holothuria scabra				84.69%; 98% GQ920784	86.94%; 100% JQ657264

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Figure S1. Gene order comparison between the outgroup *Strongylocentrotus purpuratus* (Echinoidea) and the holothurians with the mtDNA available in NCBI. The underlined genes are encoded by the internal strand.

Strongylocentrotus purpuratus

nad1	trnI	nad2	rrnL	cox1	trnR	nad4L	cox2	trnK	atp8	atp6	cox3	trnS2	nad3	nad4	trnH	trnS1	nad5	nad6	cob	trnF	rrnS	trnE	trnT	trnP	trnQ	trnN	trnL1	trnA	trnW	trnC	trnV	trnM	trnD	trnY	trnG	trnL2

Parastichopus parvimensis, Parastichopus nigripunctatus, Parastichopus californicus, Apostichopus japonicus, Holothuria forskali, Holothuria scabra, Holothuria polii (this study)

nad1	trnI	nad2	rrnL	cox1	trnR	nad4L	cox2	trnK	atp8	atp6	cox3	trnS2	nad3	nad4	trnH	trnS1	nad5	nad6	cob	trnF	rrnS	trnE	trnT	trnP	trnQ	trnN	trnL1	trnA	trnW	trnC	trnV	trnM	trnD	trnY	trnG	trnL2

Stichopus horrens

nad1	trnI	nad2	rrnL	cox1	trnR	nad4L	cox2	trnK	atp8	atp6	cox3	trnS2	nad3	nad4	trnH	trnS1	nad5	nad6	cob	trnF	rrnS	trnE	trnT	trnP	trnQ	trnN	trnL1	trnA	trnW	trnC	trnV	trnD	trnY	trnG	trnM	trnL2

Cucumaria miniata

nad1	trnI	nad2	rrnL	cox1	trnR	trnE	trnP	trnN	trnL1	trnW	trnV	nad4L	cox2	trnK	atp8	atp6	cox3	trnS2	nad3	nad4	trnH	trnS1	nad5	nad6	cob	trnF	rrnS	trnT	trnQ	trnA	trnC	trnM	trnD	trnY	trnG	trnL2

Benthodytes marianensis

nad1	trnI	nad2	trnT	rrnL	cox1	trnR	nad4L	cox2	trnK	atp8	atp6	cox3	trnS2	nad3	nad4	trnH	trnS1	nad5	nad6	cob	trnF	rrnS	trnE	trnA	trnW	trnM	trnG	trnP	trnQ	trnN	trnL1	trnC	trnV	trnD	trnY	trnL2