Morphological and molecular characterization of adults and larvae of *Crassicauda* spp. (Nematoda: Spirurida) from Mediterranean fin whales *Balaenoptera physalus* (Linnaeus, 1758)

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**A B S T R A C T**

*Crassicauda* boopis is known to infect the kidneys and vascular system of mysticetes included *Balaenoptera physalus* and has been recently reported in Mediterranean waters. Identification at the species level relies on the observation of morphological features of the adult parasites, but field conditions during necropsy and the massive reaction of the host's immune system often prevent optimal conservation of the extremities. Moreover, larval stages of *Crassicauda* have never been described and no sequences are available in public databases to help such identification. Adult and larvae of *Crassicauda* were isolated from four specimens of *B. physalus* and studied with morphological and molecular techniques. Specimens of *C. anthonyi*, *C. grampicola* and *Crassicauda* sp. isolated from *Ziphius cavirostris*, *Grampus griseus*, *Stenella coeruleoalba* and *Tursiops truncatus* respectively were studied as well. Sequences of nuclear markers 18S and ITS-2 and of mitochondrial gene cox1 were obtained and phylogenetic relationships within the genus *Crassicauda* were analysed. Analysis of the ITS2 grouped the different species in accordance with morphological identification, as already evidenced in literature for other Spirurida. A higher intra-specific variability was observed for the cox1 gene, for which two species (*C. grampicola* and *C. anthonyi*) did not appear as monophyletic in the tree. Well-developed non-attached larval specimens in the intestinal lumen of a whale calf were molecularly identified as *C. boopis*, allowing new insights on the life cycle of this species. This work broadens the genetic database on cetaceans parasites, allowing species identification even in challenging field conditions or in poor conservation of the samples; moreover, the first morphological description of *C. boopis* larvae is provided.

1. Introduction

Parasites of the order Spirurida are a diverse group of large nematodes affecting terrestrial and aquatic vertebrates. The genus *Crassicauda* Leiper and Atkinson, 1914 (Spirurida: Tetrameridae) infect different species of cetaceans, both toothed and baleen whales. Different organ tropism and consequent pathogenic impact are described among the members of this genus, whose localization spans over subcutaneous tissues, cranial sinuses and the urogenital system (Lambertsen, 1986; Geraci and St. Aubin, 1987; Jabbar et al., 2015). *Crassicauda boopis* Baylis, 1920, with its large size and localization in the renal and circulatory systems, is considered one of the most pathogenic species in whales, similarly to *Crassicauda anthonyi* Chabaud, 1962 in Cuvier’s beaked whales (Díaz-Delgado et al., 2016). Infections by *C. boopis* have been reported in fin whales (*Balaenoptera physalus*, Linnaeus, 1758), humpback (*Megaptera novaeangliae*, Borowski 1781) and blue whales (*Balaenoptera musculus*, Linnaeus, 1758) (Baylis, 1920; Cockrill, 1960; Lambertsen, 1992; Lempereur et al., 2017). Reports of this species have been provided from the North and South Pacific Ocean (Baylis, 1920; Rees, 1953; Margolis and Pike, 1955; Delyamure, 1955; Cockrill, 1960) and the North Atlantic Ocean (Hamilton, 1914, 1915; Lambertsen, 1985, 1986; Lempereur et al., 2017). Recently, *C. boopis* has been recorded in fin whales stranded along the coast of the Mediterranean Sea (Marcer et al., 2019) and nematodes tentatively classified as the same species have been observed in a fin whale stranded along Atlantic Spanish coasts, close to the Gibraltar strait (Fernández-Maldonado, 2018). Thrombosis of the renal veins and of the vena cava, abscission and fibrosis of the kidneys are lesions commonly associated with the presence of cephalic portions of female nematodes within the lumen of the vena cava and with the presence of the body of the parasites within renal parenchyma. The impairment of blood supply to the kidney can finally result in congestive renal failure (Lambertsen,
Few hypotheses on the life cycle of *Crassicauda* spp. have been formulated (Lambertsen, 1992; Díaz-Delgado et al., 2016). The presence of an intermediate or paratenic host is a reliable hypothesis, as the presence of an intermediate or paratenic host is a reliable hypothesis, as evidenced by observations on *Crassicauda* spp. in Cuvier’s beaked whale (Ziphus cavirostris, G. Cuvier, 1823), and from the pterygoid sinuses of a Risso’s dolphin (*Grampus griseus*, G. Cuvier, 1823), were morphologically identified as *C. anthonyi* (4 cephalic portions, 1 male tail and 1 female tail) and *C. grampicola*, Johnston and Mawson, 1941 (2 cephalic portions, 2 male tail and 2 female tail), respectively. One cephalic portion, one male tail and one female tail of both species were deposited at the Natural History Museum of London (NHMUK) (*C. anthonyi*: 2018.4.25.3–4; *C. grampicola*: 2018.4.25.1–2). Finally, fragments of *Crassicauda* sp. isolated from subcutaneous tissues of two bottlenose dolphins (*Tursiops truncatus*, Montagu, 1821) and of one striped dolphin (*Stenella coeruleoalba*, Meyen, 1833) were included in the study. Due to the lack of the cephalic and caudal extremities, these nematodes could not be identified to species level. Data of the hosts and nematodes analyzed in this study are listed in Supplementary Table 1.

All the collected nematodes (larvae and adults) were preserved in 70% ethanol. Adult parasites (cephalic and caudal ends) and the whole body of larvae, selected for morphological identification, were clarified in Amman’s lactophenol solution and in 10% glycerol and 70% alcohol solution respectively. All samples were subsequently observed and measured with a light microscope (Olympus, ACH 40X-2) by NIS-Elements D software (Nikon).

2.2. Molecular analyses

DNA was isolated from the following specimens: 7 adult nematodes from fin whales; 4 larvae from mesenteric arteries and intestinal nodules and 3 larvae from the intestinal lumen of fin whales; 2 specimens of *C. anthonyi*; 3 specimens of *C. grampicola*; 6 fragments of *Crassicauda* sp. (Supplementary Table 1 and Table 1). Extraction was performed using Nucleospin® Tissue Kit (Macherey-Nagel, Germany), according to the manufacturer’s instructions.

The small subunit (SSU; 18S) was amplified by polymerase chain reaction, using the primers G18S4–F (5′-GCTTGTTCTCAAAGATTAA GCC-3′) and reverse 136-R (5′-TATCCTTCTGGATGTTCACCTAC-3′) (Nadler et al., 2007). The PCR for 18S region was performed in a 30 μl reaction volume, comprising 3 μl DNA, 2 mM MgCl₂, 0.25 mM dNTPs (MBI Fermentas, Germany), 1X PCR buffer, 0.5 μM of each forward and reverse primer, 1U Platinum Taq DNA Polymerase (Invitrogen). Molecular biology grade water was added up to the final volume. Cycling conditions comprised an initial activation step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58.7 °C for 30 s, 72 °C for 80 s, with a final extension step of 72 °C for 7 min. The PCR products were resolved through electrophoresis runs in 2% agarose gel with SYBR® Safe DNA gel stain (Invitrogen™, USA). The amplicons of PCR (fragments of expected size 1700 bp) were directly sequenced at Macrogen (Macrogen Europe, the Netherlands) using PCR primers. Additionally, a couple of internal primers were designed specifically with software Primer3 to facilitate sequencing the 18S fragment, i.e. forward primer 437F (5′-AATCTGGAAGGCATGCAC-3′) and reverse 1279R (CTCTGGGCA TGAGGAGGTAC-3′) (length of internal sequence: 842 bp).

The chromatograms quality was evaluated using the software ChromasPro version 2.4.3 (Technelysium Pty Ltd, Australia). The consensus sequences were assembled with the program SeqMan available in the DNASTar package. The consensus sequences were compared with the non-redundant data base available in the GeneBank® database using

(F. Marcer, et al., 1992).
Further the samples were characterized through the amplification of the ITS-2 fragment of the rDNA and of the mt-cox1 gene region. Primers D (5′-GAGTGCAGAAGACGACATG-3′) and reverse B1 (5′-TCTTGTTAGTTTCTTTTCCT-3′) were used for the ITS-2 region (Traversa et al., 2004) and forward primer JB3 (5′-TTTTTGGGCATCCTGAGGTGTAT-3′) and reverse JB4.5 (5′-TAAAGGAAAGACAATATGA AAATG-3′) were used for cox1 (Bowles et al., 1992).

The PCR for ITS-2 region was performed in a 30 μl reaction, comprising 3 μl DNA, 2.5 mM MgCl₂, 0.25 mM dNTPs (MBI Fermentas, Germany), 1X PCR buffer, 0.5 μM of each forward and reverse primer, 1U Platinum Taq DNA Polymerase (Invitrogen). Molecular biology grade water was added up to the final volume. Cycling conditions comprised an initial activation step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, with a final extension step of 72 °C for 5 min.

The PCR for cox1 region was performed in a 30 μl reaction volume, comprising 3 μl DNA, 2.5 mM MgCl₂, 0.5 mM dNTPs (MBI Fermentas, Germany), 1X PCR buffer, 1.25 μM of each forward and reverse primer, 1U Platinum Taq DNA Polymerase (Invitrogen). Molecular biology grade water was added up to the final volume. Cycling conditions comprised an initial activation step at 95 °C for 2 min, followed by 35 cycles of 94 °C for 40 s, 50 °C for 30 s, 72 °C for 30 s, with a final extension step of 72 °C for 5 min.

The PCR products were resolved through electrophoresis runs in 2% agarose gel with SYBR® Safe. The PCR products (fragments of expected size 510 bp and 710 bp for ITS-2 and cox1 respectively) were sequenced at Macrogen (Macrogen Europe, the Netherland) using the same PCR primer pairs and chromatograms processed as previously described.

### 2.3. Phylogenetic analysis

Independent data set including the 18S, cox1 and ITS2 sequences obtained in the present study as well as orthologous counterparts belonging to Crassicauda genus available in GenBank (C. magna KX354835 and KM233410; C. boopis: KY263809 and C. gilbikiana: LC057236-37; LC057239-41; LC057243-46), were created in accordance to in accordance to (see results). When available, sequences obtained from members of the same superfamily were included as outgroup. The multiple alignments were performed with the program Muscle (Edgar, 2004) implemented in MEGA7 software (Kumar et al., 2016). When coding regions were considered (i.e. cox1), the alignment was performed at codon level. To guarantee phylogenetic trees reliability, distantly related outgroup sequences were maintained only when alignment quality was high and no evidences of substitution saturation were detected. Phylogenetic trees were computed with the MEGA7 software (Kumar et al., 2016) according to the Maximum likelihood method by applying the best fitting evolutionary model selected by the program itself (i.e. HKY + 1 + G and HKY + G for the cox1 and ITS2 genes, respectively). To assess the statistical support to the tree topologies, 10,000 bootstrap replicates were calculated.

### 3. Results

#### 3.1. Morphological description of nematodes collected from B. physalus

##### 3.1.1. Adult nematodes

Specimens of C. boopis (1 head, 2 male tails and 2 female tails) were deposited at the NHMUK (Crassicauda boopis 2015.10.11.1-4).

Morphometric data of adult nematodes isolated from the kidneys of fin whales were in accordance to the description of C. boopis in literature (Baylis, 1920; Delyamure, 1955; Lambertsen, 1985).

**Female.** Elongated body with maximum length of cephalic fragments of 60.5 cm. The anterior end (measured specimens: n = 2) lying free in the lumen of vena cava, narrowing towards the head, with an evident constriction at 53.5 ± 2.12 μm (range 52–55 μm) from the buccal opening, with a diameter of 90.5 ± 10.5 μm (83–98 μm). The buccal cavity appeared laterally compressed (21.5 ± 108 μm); two lips in lateral position, triangular in shape (74 μm wide at the base and 30 μm high), each with a small apical labial papilla. Two lateral and four submedial cephalic papillae were visible on the anterior end (Fig. 1a, lower inset; Fig. 3i). The esophagus was encircled by a nerve ring at 275 ± 0.7 μm (274.0–275.0 μm) from anterior extremity (Fig. 1a) (esophagus length 1200 μm). Paired ovaries and uteri were visible.

The posterior end (measured specimens n = 3), found in the ureters, had a genital constriction at 5600 ± 503 μm (range 5000–6000 μm) from the tip of the tail, where the vulva opened ventrally (Fig. 1b). The pinéal appendage contained last part of intestine that ended at 417 ± 38.6 μm (range 375–451) from the terminal end.

**Male.** A cephalic extremity (measured specimens: n = 1) was strongly fixed in the renal parenchyma and the fragment was 8.5 cm long. In the anterior part of the parasite a constriction was observed at about 50 μm from the top, giving the extremity a triangular shape. The
mouth was characterized by a slit with two evident triangular lips (88 μm wide at the base and 33 μm high) with two small labial papillae and two well visible lateral cephalic papillae. The buccal cavity appeared laterally compressed (20 × 110 μm).

The posterior end (measured specimens n = 3), found in ureters, was narrowing with a coiled tail (2 turns); the cloaca opened at 1040.6 ± 57.2 μm from anterior extremity (Figs. 2f and 3g). In other three specimens, a vestigial genital pore was located at 209.5 μm (999-1106 μm)-from the tip of the tail, with no spicules. Eighteen caudal papillae were visible (Fig. 1c).

3.1.2. Larvae

All measures in this section are average values; please refer to Table 2 for ranges and standard deviation values.

**Intestinal nodules.** Larvae (n = 7) contained in the intestinal mucosa of one of the adult whales showed the following morphometric features: total length of the body 8356.0 μm, width 81.5 μm. Anterior end was sharp; two lips with terminal rounded tooth, 2 lateral and 4 submedial prominent papillae were observed (Fig. 2a and Fig. 3a and b). Excretory pore was located at 209.5 μm from anterior extremity and anus at 62.0 μm from tip of tail. The posterior end, starting from the cloacal opening, terminated with a rounded tip (Figs. 2b and 3c).

**Mesenteric artery walls.** Larvae were included in the thickness of the vessels’ intimal layer (n = 4). Morphological features were generally similar to those of the larvae included in the intestinal nodules. Total length of the body was 10,613.8 μm, width 107.3 μm. A triangular shaped head, bulging at the base, was observed. Excretory pore at 171.7 μm from anterior extremity (Figs. 2c and 3d). The intestinal tube was evident in central and terminal segment of the body opening in the anus at 71.4 μm from the tip of the tail (Figs. 2d and 3e).

**Intestinal lumen.** Free larvae inside intestinal lumen (n = 9) had a length of 30,417.4 μm and width of 151.1 μm. The anterior end was sharp, with two lips and six cephalic papillae (two lateral and four submedial). The buccal cavity was compressed and measured 63.9 μm × 15.2 μm. Excretory pore was located at 269.3 μm from anterior extremity (Figs. 2e and 3f). In three specimens, the posterior portion had a terminal restriction in which a cloaca with pre and post cloacal papillae (9–10 pairs) was visible at 141.9 μm from the tip of the tail (Figs. 2f and 3g). In other three specimens, a vestigial genital pore and anus were observed at 544.1 μm and 101.7 μm from the tip of the tail respectively (Fig. 3h).

Cuticle transversely stratified all along the body length was observed in all larval stages.

**Table 2**

Mean, standard deviation and range (in brackets) of morphometric data of larval elements (μm). INL, Intestinal Nodules larvae; MAL, Mesenteric Arteries Larvae; FIL, Free Larvae in Intestinal lumen. M = immature male; F = immature female.

|                  | INL            | MAL            | FIL            |
|------------------|----------------|----------------|----------------|
| Total length     | 8356.81 ± 679.36 | 10613.79 ± 1096.75 (9838.27-11389.31) | 30417.45 ± 3377.37 (28018.41-34279.7) |
| Mid body width   | 81.53 ± 6.81 (69.54-85.04) | 107.33 ± 16.90 (91.78-130.23) | 151.08 ± 19.09 (124.19-178.00) |
| Head width       | 39.29 ± 2.46 (35.25-42.32) | 41.49 ± 8.20 (35.68-47.29) | 66.05 ± 9.27 (56.54-78.89) |
| Distance pseudolabia – head base | 25.69 ± 5.56 (18.9-38.49) | 21.73 ± 7.80 (16.21-27.25) | 36.08 ± 2.55 (32.06-39.20) |
| Distance excretory pore-cephalic end | 209.46 ± 15.02 (226.79-200.03) | 171.72 | 269.28 ± 19.22 (235.32-299.50) |
| Tooth length     | 3.24 ± 0.88 (2.51-4.01) | 2.78 ± 0.17 (2.65-2.90) | / |
| Head cuticle thickness | 2.08 ± 0.55 (1.08-2.66) | 1.44 ± 0.36 (1.19-1.70) | 4.42 ± 1.51 (2.31-6.40) |
| Width of tail at anal opening | 61.15 ± 7.82 (47.65-73.99) | 64.69 ± 4.79 (64.07-72.93) | 99.34 ± 11.84 (88.12-113.59) |
| Width of tail at anal opening | / | / | 154.01 ± 18.28 (139.61-174.58) |
| Distance anus – terminal end | 62.01 ± 6.79 (57.84-71.03) | 71.44 ± 5.33 (60.91-72.62) | 101.67 ± 8.48 (98.11-107.18) |
| Distance cloacal opening/genital pore – terminal end | / | / | 141.98 ± 0.88 (141.01-142.75) |
| Cuticle thickness at mid body | 5.63 ± 1.90 (4.28-9.74) | 5.48 ± 1.69 (3.71-7.99) | 7.82 ± 2.51 (4.46-12.56) |
| Cuticle thickness in terminal end | 4.22 ± 1.01 (3.01-5.93) | 4.43 ± 1.20 (2.36-4.55) | 4.89 ± 1.05 (3.74-5.67) |
| Length of buccal cavity | / | / | 63.91 ± 3.49 (59.4-68.61) |
| Width of buccal cavity | / | / | 15.19 ± 3.67 (10.01-22.41) |
3.2. Molecular and phylogenetic analysis

Amplification and sequencing of 18S gene resulted in 23 good quality sequences and their alignment (1600 bp long) proved them to be all identical. The Blast research in GeneBank revealed them to be highly similar (99.88%) also to the sequence of C. magna (Acc. number KM233410) and C. boopis (Acc. number KY263809) already deposited. Thus these sequences proved useless to discriminate among different Crassicauda species.

Amplification of ITS-2 fragment gave 22 sequences (alignment length 435 bp) of good quality (Acc. Numbers MK631888 - MK631909). The specimens, represented by fragments of adult parasites belonging to the species C. boopis were split in two clusters. All specimens belonging to C. grampicola exhibited identical sequences. The same evidence was reported for specimens of C. anthonyi. Specimens, represented by fragments of adult parasites, assigned to Crassicauda sp. exhibited a single sequence. Two ITS-2 sequences of the larvae found free in the intestinal lumen (FIL) appeared to be identical to that of C. boopis. On the other side, larval specimens included in the intestinal nodules (INL) and in the mesenteric artery’s wall (MAL) were part of a genetically distant cluster (p-distance ranging between 9 and 9.2%) compared to C. boopis cluster. The phylogenetic analyses of all sequenced individuals is provided in Fig. 4.

Amplification of cox1 gene gave 19 sequences (alignment length 259 bp) of good quality (Acc. Numbers MK621821 - MK621839). The phylogenetic tree based on the cox1 sequence provided a much complex pattern. The samples of adult C. boopis and free intestinal larvae were classified in a single cluster. On the other hand, specimens of C. anthonyi and C. grampicola were intermingled in two independent clusters comprising substantially identical sequences despite the species difference (Fig. 5). Samples from Crassicauda sp. formed a single monophyletic cluster. Two sequences from mesenteric (MALm) and intestinal larvae (INL7) respectively formed, similarly to what observed with the ITS2 gene, an independent group, loosely related (p-distance ranging between 8.2 and 8.5%) to the C. boopis one. The analysis of the topology of the obtained cox1 sequences reveals that, with the exception of adults Crassicauda sp. and C. boopis, other species of Crassicauda do not form monophyletic groups.

Details of all the obtained sequences are reported in Supplementary Table 1.

4. Discussion

Fourteen species have been described inside the genus Crassicauda, i.e. C. crassicauda (Creplin, 1929), C. giliaiana Skrjabin and Andreeva, 1934, C. anthonyi, C. bennetti Spaul, 1926, C. grampicola, C. boopis (syn. C. pacifica), C. magna Johnston and Mawson, 1939 (syn. C. duguyi), C. tortilis Skrjabin (1959), C. delamureana Skrjabin, 1966, C. fuelleborni Baylis, 1932, C. costata Skrjabin (1969), C. carbonelli Raga and Bal-buena, 1990. These species occur in the kidneys (C. giliaiana, C. anthonyi, C. bennetti, C. boopis, C. tortilis, C. delamureana, C. costata), reproductive system (C. crassicauda, C. carbonelli, C. fuelleborni), pterygoid sinuses (C. grampicola) and subcutaneous tissues and “gill slit” gland (C. magna) of the host (Keenan-Bateman et al., 2018). Among them, four species have been described in the kidneys of mysticetes, i.e. C. boopis, C. tortilis, C. delamureana, C. costata showing low host-specificity. The morphological identification of adult specimens is primarily based upon presence of the spicules, which are absent in C. boopis and C. tortilis and present in C. delamureana and C. costata (Skrjabin, 1969). C. boopis has been isolated in fin whale, blue whale and humpback whale, whereas C. tortilis has been described in Balae-roptera musculus (Skrjabin, 1973) (Skrjabin, 1959; Lambertsen, 1992). The distinction between the two species relies mainly upon measurements, that are rather similar. In the present study, the isolation of the
intact tails and heads of males and females of C. boopis allowed the identification of the species in three fin whales, by comparison with the description of the species made by Lambertsen (1985) and Delyamure (1955).

To date, knowledge of the life cycle of Crassicauda species is incomplete, but hypotheses on transmission, migration and development of larvae have been formulated for C. boopis, by observing pathological findings in infected fin whales (Lambertsen, 1986).

In this study, larval nematodes with morphological features of progressively more advanced developmental stages were isolated from mesenteric arteries, intestinal walls and intestinal lumen of fin whales, respectively. The phylogenetic analysis performed on 18S sequences of the species made by Lambertsen (1985) and Delyamure (1955).

The ITS-2 sequences of C. magna were identical to each other, as well as highly similar to C. magnus, as already evidenced by Diaz-Delgado et al. (2016) and Lempereur et al. (2017). Analysis of the ITS-2 sequences efficiently grouped together specimens morphologically identified as the same species (i.e. C. grampicola and C. anthonyi), as reported for other nematode families (Chilton, 2004; Powers et al., 1997) included the Spirurida (Traversa et al., 2004). Interestingly, the ITS-2 sequences of the well-developed larvae isolated from intestinal lumen of the newborn calf were identical to those from adult C. boopis collected from the kidneys of adult whales, and this may open new insights on migration and development of C. boopis larvae. The hypothesis on the migration of C. boopis larvae indeed suggests the passage of the immature parasites through the gut's mucosal and submucosal layers after ingestion to reach the mesenteric arteries' walls. Still according to the lesions pattern, they finally reach the aorta and renal arteries either by migrating within vessels' walls or by getting into the blood flow (Lambertsen, 1986). In our case, larvae of C. boopis found within intestinal lumen of the calf, were found to be in an advanced developmental stage; thus, we speculate the migration through the intestinal walls could occur quite late in the developmental process of the nematode. Alternatively, different migration routes could actually exist for C. boopis in newborn calves, and aberrant migrations cannot be excluded as well. Remarkably, larvae detected in the mesenteric arteries demonstrated quite peculiar genetic features in the ITS2 (p-distance to C. boopis = 9%) (and cox1 gene as well - p-distance to C. boopis = 8.2%). If this variability can be explained by a remarkable within-species variability or if it is due to the detection and sequencing of still unclassified Crassicauda species remains to be elucidated. Unfortunately, the absence of additional data on ITS-2 sequences from other Crassicauda species prevents a further specimen characterization. We speculate that at least two other species should be molecularly characterized to perform comparison with the larvae recovered, i.e. C. crassicauda and C. tortilis. The first one is the only other species of Crassicauda described in fin whales. In the study by Lambertsen (1992), adults of C. crassicauda and C. tortilis were simultaneously present with adult C. boopis and with larvae migrans in the mesenteric arteries; no hypothesis on the life cycle of this species have been formulated. On the other side, C. tortilis is the only other species described in the kidneys of a mysticete (Balaenoptera musculus). Assuming that similar final localization could correspond to similar intrasomatic migration route - as observed for Crassicauda sp. in Cuvier’s beaked whale’s kidneys (Diaz-Delgado et al., 2016) and considering that
the genus *Crassicauda* is characterized by a relatively low host specificity inside the suborders of the odontocetes and mysticetes, a wider molecular assessment inside the genus would be essential to characterize related species and investigate the life cycle with confidence.

Sequencing and phylogenetic analysis of the mitochondrial cox1 gene revealed a less interpretable pattern, since samples morphologically classified in the same species demonstrated a heterogeneous mitochondrial genetic background and were not monophyletic in the tree. The actual causes of the observed phenomenon remain elusive and further analysis based on more extended dataset should be performed to investigate this issue. Therefore the need for collaborative efforts among research groups and the definition of shared reference genes and regions to be sequenced appears pivotal to understand the evolutionary relationship among *Crassicauda* genus members. Nevertheless, although preliminary, the obtained evidences and especially the comparison between morphological and genetic features, suggests ITS2 to be a more reliable marker for taxa identification within the *Crassicauda* genus.

Overall, the results of the present study emphasize the limits of current classification criteria and the need of a much more extensive and systematic analysis of the morphometric and genetic features of the genus *Crassicauda*, in order to establish an updated, shared and handier classification scheme. This work contributes both to enrich the genetic sequence data bank and the molecular taxonomy of this genus; it represents an important starting point for identification of ruined fragments or immature stages of *Crassicauda* spp.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.06.004.

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