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3 ORIGINAL PAPER

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5 **Morphological and molecular observations on the status of *Crassicauda***
6 ***magna*, a parasite of the subcutaneous tissues of the pygmy sperm whale**
7 **with a re-evaluation of the systematic relationships of the genus**
8 ***Crassicauda***

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14 Nucleotide sequence data reported in this paper are available in the GenBank database

15 under the accession number KM233410

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Abstract Members of the genus *Crassicauda* (Nematoda: Spirurida) are parasites of the body tissues of whales and dolphins. Owing to the large size of worms and difficulties in the recovery of entire nematodes from the tissues of hosts, limited information is available on morphological descriptions of both male and female worms. Furthermore, there are currently no available sequence data for this genus to assist with such identifications. This paper describes for the first time features of the anterior extremity and the male tail of *Crassicauda magna*, suggesting that *Crassicauda duguyi* may be a synonym of this species. In addition, molecular data are presented for the genus for the first time suggesting that the genus belongs within the superfamily Acuarioidea rather than within the Habronematoidea, in which it is currently placed.

Keywords *Crassicauda magna* . description . molecular phylogeny . Cetacea . Nematoda

38 **Introduction**

39 Members of the spirurid nematode genus *Crassicauda* are parasites of the body tissues of
40 whales and dolphins (Cetacea) (Delyamure 1955). Most species described to date occur
41 in the urogenital and renal systems of their hosts, although *C. magna* occurs primarily,
42 but not exclusively in the neck musculature of the pygmy sperm whale *Kogia breviceps*.

43 *Crassicauda magna* was first described from the subcutaneous tissues of the neck
44 region of *K. breviceps*, by Johnston and Mawson (1939) based on specimens collected in
45 South Australia and from Moreton Bay in Queensland, Australia. While Johnston and
46 Mawson (1939) reported that the largest fragment they examined was 3.7 m long, the
47 entire length of the nematode remains undetermined. The description they were able to
48 provide was meagre, with only the features of the cephalic extremity being used as the
49 basis for their description. The species has subsequently been reported from the same
50 host species in Australia and New Zealand (Duignan 2000) as well as off the coast of
51 Spain (Abollo et al. 1998) and the Atlantic coast of the USA (Bateman et al. 2014),
52 although none of these publications provide data on museum specimens deposited to
53 substantiate the identifications made. A related species, *C. duguyi*, was reported from the
54 neck muscles of *K. breviceps* collected on the western coast of France (Dollfus 1966) and
55 the species has also been reported from the Caribbean (Mignucci-Giannoni et al. 1998).
56 Dollfus (1966) considered it impossible to distinguish his new species from *C. magna*,
57 because, in spite of its wide geographical distribution, its anatomy was poorly known
58 with only the anterior end having been described by Johnston and Mawson (1939). Due
59 to the large size of these nematodes, they have frequently been described from fragments,
60 with either only the anterior (Johnston and Mawson, 1939: *C. magna*) or posterior end

(Dollfus 1966: *C. duguyi*) being described, leading to uncertainties in their identification. As a specific example, *Crassicauda pacifica* described from the kidney of a fin whale, *Balaenoptera physalis*, by Margolis and Pike (1955) based only on the anterior end of the nematode, was subsequently shown to be a synonym of *C. boopis*, once entire nematodes were collected (Lambertsen 1985).

Difficulties of this type could be readily overcome by the use of molecular methods for which the dissection of entire nematodes from the tissues of whales would not be necessary. However, there are currently no available sequence data for this genus to assist with such identifications.

The collection of new specimens of *C. magna* from a pigmy sperm whale beached in Moreton Bay, Queensland, Australia, resulted in the acquisition of fragments including three male tails as well as material preserved in ethanol, and therefore suitable for obtaining sequence data. As cardinal morphological features of the male tail have not been reported previously, they are described in the current paper together with other collections of the parasite from the Australian region including a description of the anterior end, and a comparison with *C. duguyi*, described from the same host species from the Mediterranean (Dollfus 1966).

In this paper, we provide novel morphological data for *C. magna* as well as the first set of sequence data as a basis for the future molecular identification of species of *Crassicauda* from whales. In addition, the molecular data presented here suggests an alternative taxonomic allocation of *Crassicauda* and therefore, potentially, the Crassicaudinae.

84

85 **Materials and methods**

86 Morphological identification

87 An adult-sized female pygmy sperm whale was found dead in January 2014, beached
88 near Burpengary Creek in Moreton Bay, Queensland (27° 10' S, 153° 2' E). During the
89 recovery of whale parts for deposition at the Queensland Museum (registered as QM
90 JM20051) subcutaneous tissue samples containing nematode fragments were also
91 collected (Fig. 1). Specimens of *Crassicauda* were retrieved, including the tails of three
92 male specimens (registered as QM G233693) and were fixed in 70% ethanol. For
93 morphological examination, these nematode fragments were cleared in lactophenol and
94 line drawings were made using an Olympus BH-2 microscope with a drawing tube.
95 Measurements were made with an ocular micrometer and are presented in millimetres.

96 Additional Australian specimens held in the South Australian Museum (SAM)
97 were also examined. These included: 'Syntypes', Port Victoria, South Australia (34° 29'
98 S, 137° 28' E), collected April, 1937 (SAM 42833) and Sandgate, Moreton Bay,
99 Queensland (27° 19' S 153° 04' E) collected June, 1935 (SAM 1599, 1607); one
100 specimen (239 cm long, tail missing) collected from neck region, Maslin's Beach, South
101 Australia (35° 13' S, 138° 28' E), February 1991 (SAM 19592); one specimen (190 cm
102 long, tail missing) from abdominal and diaphragmatic regions from Point Sinclair, South
103 Australia (32° 06' S, 132° 58' E), 9.x.2003 (SAM 45361); one specimen (277 cm long,
104 tail missing) from neck region from Point Ricardo, Victoria (37° 48' S, 148° 38' E),
105 19.viii.1975 (SAM 18425); one specimen, fragments from axillary region from Brooklyn,
106 Hawkesbury River, New South Wales (33° 33' S, 151° 14' E), 2002 (SAM 32826). The

anterior end of one specimen (SAM 19592) was also cleared in lactophenol and the cephalic features examined.

Additional comparative material studied:

Additional specimens of *C. magna*, *C. duguyi* from *K. breviceps* were examined in the Natural History Museum, London (BMNH) and the Muséum national d'Histoire naturelle, Paris (MNHN) as follows:

1 specimen identified as *C. magna* (anterior end), Gower Peninsula, Wales (BMNH 1980.2421).

1 male tail, identified as *C. ? duguyi*, South Africa (BMNH 1982.2052).

2 male tails, identified as *C. duguyi*, King's Beach, Port Elizabeth, South Africa (BMNH 1982. 767-768).

Holotype of *C. duguyi*, Charente-Maritime, France (MNHN DB7:36).

Molecular characterisation

Genomic DNA was isolated from one worm from Moreton Bay, Queensland, by suspending a small piece in a 200 µl of 20 mM Tris-HCl (pH 8.0), 100 mM EDTA, and 1% sodium dodecyl-sulphate containing 10 mg/ml proteinase K (Amresco Inc., Solon, Ohio, USA) and incubating at 37 °C for 16 h. Genomic DNA was column-purified from the homogenised suspension with the Wizard[®] DNA Clean-Up Kit (Promega, Madison, WI, USA), following the manufacturer's recommendations. The small subunit (SSU;

18S) nuclear ribosomal DNA was amplified from 20 ng of genomic DNA with the polymerase chain reaction (PCR) using G18S4-F (5'-GCTTGTCTCAAAGATTAAGCC - 3') and reverse 136-R (5'- TGATCCTTCTGCAGGTTACCTAC - 3') primers (Nadler et al. 2007). PCRs were conducted in 50 µl volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each primer, and 1 U of *GoTaq* polymerase (Promega). PCR cycling conditions were 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 80 sec, followed by 72 °C for 7 min. Following the examination on a 1.5 % agarose gel, PCR amplicons were treated with shrimp alkaline phosphatase and exonuclease I (Werle et al. 1994), and subjected to automated DNA sequencing (BigDye ® Terminator v.3.1 chemistry, Applied Biosystems, Foster City, CA, USA) using PCR primers (G18S4 and 136) in separate reactions. The quality of each nucleotide sequence was appraised using the program Geneious Pro 6.5 (Biomatters Ltd., Auckland, New Zealand) (Drummond et al. 2011).

The consensus SSU sequence determined was then subjected to BLASTn analysis (<http://blast.ncbi.nlm.nih.gov>) to establish the 'top hits' to all nucleotide sequences available in current databases and identities (in %) calculated by pairwise comparisons. Subsequently, the consensus sequence was aligned with the selected subset of related sequences belonging to the members of order Spirurida (Blaxter et al. 1998; Almeyda-Artigas et al. 2000; Smythe et al. 2006; Wijová et al. 2006; Moravec 2007; Nadler et al. 2007; Honisch and Krone 2008; Laetsch et al. 2012). The analysis of these sequence data was conducted using Bayesian inference (BI), employing the Monte Carlo Markov Chain (MCMC) method of MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and

Huelsenbeck 2003). The likelihood parameters were set based on the Akaike Information Criteria (AIC) test in jModelTest 2.1.5 (Darriba et al. 2012). AIC revealed the general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites (GTR+ Γ +I) as the ‘best’ model. Posterior probabilities (pp) were calculated for 2,000,000 generations, utilizing four simultaneous tree-building chains, with every 100th tree being saved.

Results

Morphological description (based on Australian specimens)

Anterior end bluntly rounded; oral opening bilobed, laterally compressed in apical view; surrounded by cuticular plate (ptilina); large lateral rounded papillae (pseudolabia) projecting beyond anterior margins of head, merging posteriorly into cuticular plate, possibly bearing three papillae (2 cephalic papillae and amphid); lateral margins of cuticular plate with irregular projections, larger projections lateral, bearing prominent but fine transverse striations. Buccal capsule 200 μ m long, 90 μ m wide; walls parallel, sclerotised, 10 μ m thick. Deirids 0.40 mm from anterior end; excretory pore 0.55 mm from anterior end; nerve ring 0.50 mm from anterior end; muscular oesophagus 2.25 mm long.

Male tail tightly coiled in 2 complete coils, not possible to unroll in fixed specimens; tail terminating bluntly, cloaca subterminal, with phasmids posterior to cloaca; 2 lateral expansions of cuticle present anterior to cloaca with irregularly arranged superficial striations; single median pre-cloacal papilla present with paired neural terminations; 5 pairs of sessile lateral papillae, extending anteriorly from median pre-cloacal papilla,

each with single neural termination; cuticle between columns of lateral papillae with parallel transverse striations. Left spicule robust, 2.65 long, heavily sclerotised; anterior extremity enlarged; distal extremity blunt, distinctly narrowed, with transparent flange visible only in ventral views. Right spicule shorter, 1.15 long, simple, heavily sclerotised with blunt tip and rib on right side extending from expanded proximal region of spicule four fifths of distance to distal extremity.

Molecular analysis

A PCR amplicon of about 1,700 bp was detected on agarose gels. DNA sequencing revealed that the SSU sequence of *C. magna* determined herein (GenBank accession number KM233410) was 1,641 bp long with 50.15% G+C content. BLAST analysis revealed this consensus SSU sequence to be most similar to those of *Stegophorus macronectes*, *Synhimantus laticeps*, *Synhimantus hamatus* and *Echinuria borealis* (acuariid nematodes) in public databases. Pairwise comparisons revealed that this SSU sequence had the maximum identity (97.1 to 99.3%) to *Stegophorus macronectes*, *Synhimantus laticeps*, *Synhimantus hamatus* and *Echinuria borealis*. In order to provide statistical support for the relationships of the SSU sequence determined here with sequence data for these spiruroids and other spiruroids were aligned (over a consensus length of 1,921 nucleotide positions) and subjected to phylogenetic analysis using BI method. This analysis showed that the consensus SSU sequence representing *C. magna* from the pygmy sperm whale grouped, with strong statistical support (pp = 0.99), with four sequences from *St macronectes*, *Sy laticeps*, *Sy hamatus* and *E. borealis* to the exclusion of all 44 other sequences of Spirurida (see Fig. 10).

Discussion

The current specimens were identified as *C. magna* based on their occurrence in the subcutaneous tissues of the thoracic region of the type host, *K. breviceps*, as well as the fact that the original description of the species by Johnston and Mawson (1939) included specimens collected from this host species from Moreton Bay, Queensland. As the species is incompletely described, the opportunity was taken here to describe the features of the male tail as well as those of the anterior end.

Dollfus (1966) recognised 11 species within the genus: *C. anthonyi*, *C. bennetti*, *C. boopis*, *C. crassicauda*, *C. fuelleborni*, *C. giliakiana*, *C. grampicola*, *C. magna*, *C. pacifica* and *C. tortilis*. Subsequently, Skrjabin (1966, 1969) added *C. delamureana* from the sei whale, *Balaenoptera borealis* and *C. costata* from the kidney of the southern right whale *Eubalaena australis*. Lambertsen (1985) has shown that *C. pacifica* is a synonym of *C. boopis*, while an additional species, *C. carbonelli* has been added, from *Globicephala melas* from the Mediterranean (Raga and Balbuena 1990).

Of these species, only *C. anthonyi*, *C. crassicauda*, *C. duguyi* and *C. giliakiana* possess spicules (Dollfus 1966), while in *C. costata* the spicules are vestigial (Skrjabin 1969). However apart from *C. duguyi*, the longest spicules, those of *C. crassicauda* (600 μm), are much shorter than those of *C. magna* described here (1.15, 2.65 mm).

The description of the male tail provided here is very similar that of *C. duguyi*. Dollfus (1966) provided excellent illustrations of his species, showing the coiled tail, the pair of lateral inflations on the tail, the papillae distributed exclusively anterior to the cloaca and

spicules measuring 2.0 and 0.6 mm respectively. The right spicule is shorter than the specimen measured here, and the distribution of papillae is slightly different. Dollfus (1966) reported five papillae on each side although his illustration (Fig. 16) shows a pre-cloacal papilla, four papillae on the right side and five papillae on the left side. Dollfus (1966) did not describe the spicule morphology in detail. In one specimen of *C.?* *duguyi* (BMNH 1982.767-8), the right spicule is everted and bears a terminal filiform spur united with the body of the spicule by a transparent flange. This feature is not observable when the spicule is retracted, as in the available Australian material described here. The type specimen of *C. duguyi* was re-examined but is mounted in balsam and is extremely dark such that while the spicules are visible, the distribution of the papillae is not. In spite of these minor differences, *C. duguyi* is probably a synonym of *C. magna*.

The morphology of the anterior end is described in detail for the first time. Johnston and Mawson (1939) provided illustrations of the anterior end but not apical views. The cuticular plate described here appears to be unique within the genus and may facilitate identification of the species in the future. Specimens of the type species, *C. crassicauda* were examined (BMNH 1915.11.27.1, 1921.8.30.1-6) including an apical view of the anterior extremity, to confirm that these features were not present in that species. The cephalic and labial papillae were not clearly identifiable. On each lateral pseudolabium were three structures which may have been cephalic papillae and an amphid, one median and two sub-lateral, but it was not possible to confirm this suggestion. If and when more abundant material becomes available, it may be possible to identify these structures using scanning electron microscopy.

The molecular phylogeny presented here places *Crassicauda* within a clade comprised of members of the family Acuariidae and the superfamily Acuarioidea. All morphological classifications to date (Chabaud 1975; Bain et al. 2014), have included *Crassicauda* and the sub-family Crassicaudinae in the family Tetrameridae and the superfamily Habronematoidea. The Acuarioidea are differentiated from the Habronematoidea by the possession of cuticular ornamentation in the form of cordons, collarettes or ptilina derived from anterior cuticular structures (Bain et al. 2014). *C. magna* clearly possesses a perioral structure which might be identifiable as a ptilina, therefore justifying its allocation to this superfamily. However, other members of the genus do not possess such structures. Resolution of this anomaly will rely upon additional molecular data for the Crassicaudinae to assess the potential hypothesis that the genus *Crassicauda* is not para or poly-phyletic. Consequently, the initial genetic data presented in this paper pose rather than answer questions with respect to the phylogenetic relationships of the Crassicaudinae.

In conclusion, while the current report provides additional morphological and molecular data on the identification of *C. magna* and provides a molecular tool for the future identification of species within this genus and subfamily, the preliminary data presented here raise questions as to its familial and superfamilial allocation which can only be addressed with additional molecular studies of these nematodes.

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Captions to figures:

Fig. 1. *Crassicauda magna* in subcutaneous tissues of adult female pigmy sperm whale beached in Moreton Bay, Queensland, Australia.

Figs. 2-6. The anterior end of *Crassicauda magna*. 2. Anterior end, dorso-ventral view showing deirids and nerve ring. 3. Anterior end, lateral view showing excretory pore (left side), deirid and nerve ring. 4. Buccal capsule, dorso-ventral view. 5. Buccal capsule, lateral view. 6. Anterior end, apical view. Scale bars 0.1 mm.

Figs. 7-10. Features of the male tail of *Crassicauda magna*. 7. Male tail, lateral view showing coiling. 8. Mail tail, ventral view, showing papillae and cuticular ornamentation; only the distal region of the left spicule is shown. 9. Lateral left aspect of mail tail showing left spicule and papillae; the right spicule is shown in outline only. 10. Right spicule, view from right side. Scale bars: fig. 1, 1 cm; figs. 2-4. 0.1 mm.

Fig. 11. Genetic relationships of *Crassicauda magna* from pygmy sperm whale with Spiruroida from various hosts. The relationships were inferred based on phylogenetic analyses of the small subunit (18S) nuclear ribosomal DNA sequence data using Bayesian Inference (BI) method. Nodal support (above or below) is given as a posterior probability. Figures in parentheses indicate unique GenBank accession number of each sequence used herein. Superfamilies of nematodes included in the analysis are also

372 shown on the right side. The scale bar indicates the number of inferred substitutions per
373 nucleotide site.
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377 **Fig. 1.**

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379 **Figs. 2-6**

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381 **Figs. 7-10**

Fig. 11

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