Advancing understanding of the taxonomy and diversity of the genus Contracaecum in the great white pelican (Pelecanus onocrotalus)

Advancing understanding of the taxonomy and diversity of the genus *Contracaecum* in the great white pelican (*Pelecanus onocrotalus*)

Monica Caffara[1*#], Perla Tedesco[1#], Nadav Davidovich[2], Sean A. Locke[3], Andrea Gustinelli[1], Roni King[4], Michelle Nuytten[1], Marialuisa Nuzzo[1], Maria Letizia Fioravanti[1]

[1] Department of Veterinary Medical Sciences, Alma Mater Studiorum University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy

[2] Israeli Veterinary Services, Bet Dagan 5025001, Israel

[3] Department of Biology, University of Puerto Rico, Box 9000, Mayagüez 00681-9000, Puerto Rico

[4] Israel Nature and Parks Authority, 3 Am Ve'Olamo St., Jerusalem, 95463, Israel

*Corresponding author: monica.caffara@unibo.it

# these authors contributed equally

Abstract

Despite the wide distribution and health importance of anisakids of the genus *Contracaecum*, epidemiological data on their occurrence in definitive bird hosts are scarce, particularly from certain parts of the world that represent important wintering sites or migration stopovers for different bird species. In the present study, *Contracaecum* spp. infecting six great white pelicans (*Pelecanus onocrotalus*) in Israel were identified using light and scanning electron microscopy and phylogenetic analyses of nuclear internal transcribed spacer (ITS) and mitochondrial cytochrome *c* oxidase II (*cox*2). A PCR-RFLP method was also developed and applied to screen large numbers of *Contracaecum* parasites. Most (415/455) worms recovered were *C. micropapillatum*, followed by *C. gibsoni* (31/455), *C. quadripapillatum* (8/455) and *C. multipapillatum* E (1/455). *Contracaecum micropapillatum* from Israel and *C. bancrofti* from Australia are distinguishable by *cox*2 but less

23 well resolved with ITS sequences, and could not be distinguished morphologically. Worms with
24 *cox*2 matching *C. gibsoni* had ITS matching specimens identified as *C. multipapillatum* A. To the
25 authors' knowledge, this represents the first of such studies in Israel, and provides useful data on
26 the ecology and distribution of different *Contracaecum* species of health and economic interest.

27

28 Key words: *Contracaecum*; taxonomy; great white pelican; Israel.

29

30

31

32

Introduction

Anisakids of the genus *Contracaecum* Railliet and Henry 1912 are widely distributed in aquatic ecosystems (freshwater, brackish and marine) where they undergo a heteroxenous life cycle, involving a wide range of paratenic hosts (crustaceans, planktivorous and piscivorous fish; Anderson 2000; Valles-Vega et al. 2017) and definitive hosts, including marine mammals (pinnipeds, cetaceans) and piscivorous birds (Mattiucci and Nascetti 2008).

The genus *Contracaecum* comprises over 60 species, the majority of which have been described from fish-eating birds but also in marine mammals (Yamaguti 1935; Hartwich 1964; Baruš et al. 1978; Ángeles-Hernández et al. 2020). In birds, massive infections may occur as a result of the continuous ingestion of paratenic hosts; third-stage larvae undergo further development and moult, becoming fourth stage and eventually adults in the proventriculus and stomach (Fagerholm and Overstreet 2008), where they cause hemorrhages, ulcerations and necrosis, leading in some instances to a fatal outcome (Rokicki et al. 2011; Kumar et al. 2019).

The taxonomic status of several *Contracaecum* spp. is uncertain due to their morphological similarity and needs to be investigated with both morphological and molecular analysis. One pair of species needing such attention is *Contracaecum bancrofti* and *C. micropapillatum*. Johnston and Mawson (1941) described *Contracaecum bancrofti* from the Australian pelican *Pelecanus conspicillatus* sampled throughout Eastern Australia. The adults of *C. bancrofti* were distinguished from an older, similar species described in Croatia, *C. micropapillatum* (Stossich 1890), on the basis of the position of vulva and size of eggs in females, and on the length of spicules in males (Johnston and Mawson 1941). However, Hartwich (1964) considered *C. bancrofti* a synonym of *C. micropapillatum*. Based on a variety of morphometric data, Shamsi et al (2009) considered *C. bancrofti* to be valid, and provided the first ITS rDNA sequences from this species. Although aware

of the work of Shamsi et al (2009), Li et al (2016) nonetheless considered the two species as synonyms and reported *C. micropapillatum* in North America, Africa, Europe, China, and Australia, albeit without providing any support for this decision.

Adults of *C. bancrofti* infecting *Pelecanus conspicillatus* have been reported from several parts of Australia, including Peron Island in the Northern Territory, Thompson River and Burnett River in Queensland, Sydney Zoological Gardens in New South Wales, Geelong and Healesville in Victoria and Morgan in South Australia (Johnston and Mawson 1941; Shamsi et al. 2009). Although Shamsi et al. (2009) considered *C. bancrofti* endemic in Australia, this species has also been reported in the American white pelican *P. erythrorhynchos* along the coasts of Texas (McDaniel and Patterson 1966) and in Mexico (Yamaguti 1961). To our knowledge, neither the occurrence of *C. bancrofti* outside Australia nor its genetic differentiation from *C. micropapillatum* have been assessed with molecular data. This is partly because different genetic markers have been sequenced in these two species, namely ITS rDNA in *C. bancrofti* (Shamsi et al. 2009), 28S rDNA and *cox*2 mtDNA in *C. micropapillatum* (Nadler et al. 2000; Mattiucci et al. 2008, 2010).

The ITS rDNA sequences from adult *C. bancrofti* (Shamsi et al. 2009) allowed identification of larval stages in freshwater fishes, including *Cyprinus carpio* (Shamsi et al. 2018a), *Carassius auratus*, *Gambusia holbrooki*, *Hypseleotris* sp., *Melanotaenia fluviatilis*, *Misgurnus anguillicaudatus*, *Nematalosa erebi* and *Retropinna semoni* in eastern Australia (Shamsi et al. 2018b). Possibly due to their small size, location deeply embedded in fish host tissues, and paucity of morphological features useful for species identification, the larvae of *C. bancrofti* have not been reported elsewhere. Nevertheless, the diversity of infected hosts (eight fish species from eight different families), suggests that *C. bancrofti* may also occur in a variety of other host species.

The present study is part of a larger project focused on the parasitic fauna of piscivorous birds collected in Israel. Sampled birds included Pelecanidae belonging to the species *P. onocrotalus*, which were found infected with parasitic nematodes of the genus *Contracaecum*. The aim of this work was to characterize these parasites to the species level with morphometrical (by both light microscopy and Scanning Electron Microscopy - SEM) and molecular methods based on two markers (i.e. ITS rDNA and *cox*2 mtDNA) to confirm their taxonomic position. To date, no such studies have been carried out in Israel, and therefore our work was also intended to elucidate the ecology and distribution of *Contracaecum* species in scarcely investigated hosts and geographical areas.

Materials and Methods

Contracaecum sampling

Four hundred and fifty-five adults of *Contracaecum* spp. were collected from the gastric mucosa of six great white pelican (*Pelecanus onocrotalus*) collected and processed fresh, from five localities under permits 2020/42659 and 2021/42855 from the Israel Nature and Parks Authority (Fig. 1). The nematodes were washed in saline and preserved in 70% ethanol for morphological and molecular analysis. For some adults, the anterior and posterior portions were preserved in 10% neutral buffered formalin for SEM. Moreover, two additional specimens of *Contracaecum bancrofti* were obtained from *Pelecanus conspicillatus* sampled in Australia.

Morphological study

Fifty males and 50 females were randomly selected from the 6 Israeli birds and observed under a dissection microscope to first evaluate gross morphology and to record total length (TL), then under a light microscope (Leica Microsystems, Wetzlar, Germany) with the aid of a digital Nikon DS-Fi1 camera and image-acquisition software (Nikon Nis-Elements D3.0). A section of each worm was then removed for DNA extraction (central 5 mm, where taxonomically informative features

are absent). Anterior and posterior portions of the parasite body then were clarified in Amman's lactophenol to measure internal structures by light microscope. Morphometric analysis was conducted following Yamaguti (1935), Hartwich (1964), and Baruš et al. (1978). Measurements are given in millimeters unless otherwise indicated. The two specimens of *C. bancrofti* from Australia were subject to the same treatment.

For SEM, anterior and posterior portions of the nematodes were dehydrated through a graded ethanol series, subjected to critical point drying, sputter-coated with gold palladium, and observed using a Phenom XL G2 Desktop SEM (Thermo Fisher Scientific, Eindhoven, The Netherlands) operating at 5 kV.

Measurements of specimens of *C. micropapillatum* and *C. bancrofti* were compared using data from the present and other studies using non-metric multidimensional scaling and ANOSIM (999 permutations, crossed design of species × worm sex) in PRIMER-E (Auckland, NZ). Morphometric distances were based on normalized measurements of eight features (lengths of whole body, esophagus, intestinal cecum, ventricular appendix, tail, left and right spicules, and distance of vulva to anterior end, transformed by subtracting the mean from the observed value and dividing the result by the standard deviation, for each variable). As only two specimens of *C. bancrofti* were available, and individual-specimen-level data from *C. micropapillatum* are not available from prior publications, six additional data points (equivalent to artificial specimens) were extracted from other studies based on reported minimum, maximum and mean (if given) or range midpoint, for each sex, for each of the eight aforementioned measurements.

Molecular study

For molecular analysis, genomic DNA was initially extracted from 51 adults using a PureLink® Genomic DNA Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The ITS rDNA was amplified with primers NC5_f (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and

126 NC2_r (5'-TTAGTTTCTTCCTCCGCT-3') (Zhu et al. 1998). A fragment of *cox*2 mtDNA was amplified
127 from 33 adults (among the 51) with primers 211_f (5'-TTTTCTAGTTATATAGATTGRTTTYAT-3') and
128 210_r (5'-CACCAACTCTTAAAATTATC-3') of Mattiucci et al (2008) following the same protocol. The
129 PCR products were electrophoresed on a 1% agarose gel stained with SYBR Safe DNA Gel Stain
130 (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5X TBE. For sequencing, the amplicons were
131 excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel, Düren, Germany),
132 and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany). The DNA trace files
133 were assembled with Contig Express (VectorNTI Advance 11 software, Invitrogen, Carlsbad, CA,
134 USA), and the consensus sequences of the ITS rDNA after separating the two regions (ITS1 and
135 ITS2) and *cox*2 mtDNA were compared with published data by BLAST tools
136 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using
137 BioEdit 7.2.5 (Hall 1999), p-distance and maximum-likelihood (ML) tree (T92+G+I substitution
138 model for ITS and KHY+G+I for *cox*2, bootstrap of 1,000 replicates for both genes) were obtained
139 using MEGA 7 (Kumar et al. 2016). The ITS1 and ITS2 rDNA sequences were concatenated and
140 used to build a ML tree together with the sequences of *Contracaecum* spp. reported by Mattiucci
141 et al (2020). The *cox*2 gene was also aligned with the sequences reported by Mattiucci et al (2020),
142 with *Pseudoterranova ceticola* (DQ116435) and *Anisakis pegreffii* (MT912471) as outgroups. The
143 sequences generated in this study have been deposited in GenBank under accession numbers
144 ON714944-88 (cox2 mtDNA) and ON736806-38 (ITS rDNA).
145 The initial sequencing of *Contracaecum* from Israel indicated mixed species infections in individual
146 birds, molecular work was conducted on all remaining adult worms. The middle portions of 404
147 adult males and females were subjected to a fast DNA extraction method using Chelex®100
148 (Sigma-Aldrich, Darmstadt, Germany). Briefly, 300 µl of 5% Chelex®100 solution in sterile DNA/RNA
149 free molecular grade water was added to the central piece of body and heated at 95 °C for 10 min.

and then centrifuged at full speed for 5 min. The supernatant was removed and diluted 1:10 for downstream molecular analyses. The ITS rDNA of the extracts was amplified as reported above and then 10 µl were subjected to PCR-RFLP with the restriction enzymes *Msp*I (Zhu et al. 2007) and *Ssp*I to distinguish among species of *Contracaecum*. The second enzyme was selected after running an *in-silico* digestion of the whole ITS rDNA of some sequenced specimens, with the software NEBcutter 3.0 (https://nc3.neb.com/NEBcutter/). After digestion (37 °C for 90 min) the specimens were electrophoresed on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5X TBE for 90 min. In each digestion reaction, previously Sanger-sequenced specimens were included as positive controls.

Results

Molecular analyses

Overall, of 455 adult worms from six Israeli pelicans examined with a combination of morphometric and molecular analyses, 415 were identified as *C. micropapillatum*, 31 as *C. gibsoni*, 8 as *C. quadripapillatum* and 1 as *C. multipapillatum* E (Table 1). As described below, most of these identifications were based on PCR-RFLP, and the entire ITS rDNA array was sequenced in 51 worms from Israel, with partial *cox*2 sequenced in 33 of the same specimens. Both ITS rDNA and *cox*2 mtDNA were also sequenced in two specimens of *C. bancrofti* from *P. conspicillatus* in Australia, and these data supported the distinct status of *C. bancrofti*.

Thirty-four worms from Israel with identical ITS rDNA sequences were 99.5% similar to adults of *C. bancrofti* from Australia, comprising data from Shamsi et al. 2009 (EU839568-EU839566) and from two specimens of *C. bancrofti* from Australia newly sequenced in the present study. In phylogenetic analysis, all the aforementioned ITS sequences fell in a well-supported clade

containing two subclades of sequences from Israel and Australia with moderate support (95%, 79%) (Fig. 2). Sequences of *cox*2 from 25 of these 34 adult worms from Israel matched (97-99.2% similarity) those of *C. micropapillatum* (EU852350, Mattiucci et al. 2010, EF513514-16 and EF122206-07, Mattiucci et al. 2008) and were 92.6% similar to the *cox*2 from two specimens of *C. bancrofti* from Australia, newly sequenced herein. In phylogenetic analysis of *cox*2, specimens from Israel and Australia were resolved into two clades with strong (≥98%) bootstrap support (Fig. 3). Taken together, these analyses support the separation of *C. micropapillatum* and *C. bancrofti*. Further evidence of the validity of *C. bancrofti* occurred in the form of gaps in the ITS rDNA alignment. A 12-bp gap unique to *C. bancrofti* began at position 120 in ITS1, which corresponded to an insertion of "TTGCTAAATTAA" in *C. multipapillatum* sequences and "TTGCTTATTTAG" in *C. quadripapillatum*. At the 3' end of ITS1, an insertion of 7 bp (position 420-426 bp, "TATTTAG") occurred in *C. bancrofti* only. In ITS2, we observed 3 insertions in the *C. bancrofti* sequences (position 449-456 bp "GAATATCT", position 495-507 bp "AAAGACGAGAAAA" and position 555-569 bp "TCCTTGCTTAGTTTG") corresponding to deletions in the other two species. The ITS sequences from the adult specimens of *C. micropapillatum* from Israel were also 99.7% similar to ITS from larvae of an unidentified species of *Contracaecum* from *Tilapia zillii* from Kenya (KF990496, Otachi et al. 2014), indicating a possible transmission path of this *C. micropapillatum*. The 34 ITS sequences of *C. micropapillatum* from Israeli pelicans differed by 2.5% from *C. multipapillatum* and 2.4-2.5% from *C. quadripapillatum*.

The ITS sequences of eight adults from Israel were 99.7% similar to *C. quadripapillatum* (OK138879-80, Hamoud and Younis 2022) from *Heterobranchus bidorsalis* from Lake Nasser (Egypt), identical to *Contracaecum* sp. 2 (MT477131) from an unknown fish species sampled in Ethiopia, 99.6% similar to *Contracaecum* sp. (MZ727197, Abdallah and Thabit 2021, unpublished) from *Lates niloticus* both from Egypt, and 99.3% similar to *Contracaecum* sp. 1-8 (FM210434,

Shamsi and Aghazadeh-Meshgi 2011) from barboid fish in Iran. These ITS sequences formed a distinct and well-supported lineage in phylogenetic analysis (Fig. 2). The *cox*2 sequences of two the eight specimens in this clade were most similar (87.6%) to *C. osculatum* A sensu Nascetti et al (JN786334). As described below, these worms were identified as *C. quadripapillatum*.

Another eight specimens yielded ITS sequences identical to *C. multipapillatum* (MH400190, Pronkina and Spiridonov 2018) from *Chelon auratus* from the Black Sea and with 98.7% similarity to *C. multipapillatum* D (AM940056, Shamsi et al. 2008) from Australian *P*. *conspicillatus*. In five of these eight adults with these ITS rDNA matching *C. multipapillatum*, *cox*2 showed 99.6-100% similarity with *C. gibsoni* (EU852337, syn *C. multipapillatum* A, Mattiucci et al. 2010) from *P. crispus* from Greece (intraspecific p-distance 0%-0.1%).

In one adult specimen from Israel, both ITS (ON723788) and *cox2* (ON736838) were identical to *C. multipapillatum* E (OL830790, OL809970, Davidovich et al. 2022) from hybrid tilapia farmed in Israel.

Through PCR-RFLP analysis, 413 adults were identified as *C. micropapillatum*, 31 as *C. gibsoni*, 8 as *C. quadripapillatum* and one as *C. multipapillatum* E. These results were based on *Msp*I banding patterns of 330-315-230 bp for *C. micropapillatum*, 560-360 bp for *C. gibsoni* and 600-360 bp for *C. quadripapillatum*, while *C. multipapillatum* E was not digested. The enzyme *Ssp*I was able to distinguish better between *C. gibsoni* and *C. quadripapillatum*, producing 550-300 bp fragments for the former and no cut for the latter (undigested band of 1000 bp) (Fig. 4).

Morphological descriptions

Contracaecum micropapillatum Johnston & Mawson, 1941

Synonym: *Ascaris micropapillata* Stossich, 1890

Site in host: stomach.

Representative DNA sequences: ON714944-77 (ITS rDNA), ON736808-32 (*cox*2 mtDNA)

Adult stage: Body stout. Cuticle transversally striated. Lips longer than wide, one dorsal and two sub-ventral (Figs. 5a-b; 6a). Two oval cephalic papillae on dorsal lip and a single papilla on each subventral lip. Interlabium reaching approximately ¾ of lip length, narrow, wider at base, with distinctly bifurcated tip (Fig. 5c). Excretory pore at base of lips. Collar area with fine cuticular annulations interrupted laterally. Deirids at approximately same level as nerve ring. Oesophagus muscular, ending in round ventriculus with short ventricular appendix. Intestinal caecum two to three times longer than ventricular appendix.

Males (*n* = 50): Total length 10-34.2 (17.2 ± 4.9). Nerve ring 0.17-0.67 (0.48 ± 0.1) from anterior end. Oesophagus 2.01-4.56 (3.27 ± 0.59) long, 12.3–25.5% (19.7%) of body length. Ventricular appendix 0.47-1.15 (0.85 ± 0.14) long, 17.8–42.1% (26.7%) of esophageal length. Intestinal cecum 1.47-3.79 (2.5 ± 0.5) long, 55.7–86.3% (75.8%) of esophageal length. Three pairs of double post-cloacal papillae (Fig. 5d). Phasmids approximately at level of third pair of post-cloacal papillae. Precloacal papillae simple, arranged in two longitudinal rows (Fig. 5e). Spicules similar, subequal, 8.6-19% (14%) of body length. Right spicule 1.02–3.37 (2.45 ± 0.56) long; left spicule 1.05–3.48 (2.4 ± 0.56) long, with rounded tip and folded longitudinal alae (Fig. 5f-g).

Females (*n* = 50): Total length 10-40.7 (18.8 ± 9.4). Nerve ring 0.25-0.92 (0.45 ± 0.13) from anterior end. Oesophagus 1.67-5.69 (3.29 ± 1.02) long, 12.7–22.3% (18.7%) of body length. Ventricular appendix 0.44-1.67 (0.81 ± 0.27) long and 14.2–40.2% (25.2%) of esophageal length. Intestinal cecum 1.34-4.87 (2.48 ± 0.84) long, 62.7–89.1% (75.6%) of esophageal length. Vulva in anterior

241 half of body, 1.76-14.11 (6.68 ± 3.7) from anterior end (Fig. 6b); Tail conical, 0.17-0.54 (0.33 ± 0.09)
242 long, with pointed tip (Fig. 6c-d).

243 **Remarks**

244 Overall, the validity of *C. bancrofti* was well supported by molecular data. While divergence levels
245 and phylogenetic analysis of ITS did not present overwhelming support for considering Australian
246 *C. bancrofti* separate from *C. micropapillatum* (the older species), the indels in the ITS alignment
247 and all aspects of *cox*2 analysis provide strong support for both species. These results are not
248 surprising, as ITS may vary little or not at all between recently separated species (Zhu et al. 2000;
249 Blouin 2002).

250 Morphological analysis of genetically characterized specimens revealed no characters that clearly
251 distinguish *C. bancrofti* and *C. micropapillatum*. As characterized by Johnston and Mawson
252 (1941b), *C. bancrofti* was reported having interlabia with bifid tips and the male tail with three
253 pairs of double papillae, and was differentiated from the morphologically similar *C.*
254 *micropapillatum* based on the length of the spicules, size of the eggs and position of the vulva.

255 Hartwich (1964), who considered *C. bancrofti* a synonym of *C. micropapillatum*, reported spicule
256 lengths (1.21–3.53 mm) for *C. micropapillatum*, which closely overlap lengths we observed in 50
257 genetically identical specimens of this species (1.02–3.48 mm) collected in Israel. In contrast,
258 spicules 2.2–2.8 mm long were measured by Johnston and Mawson (1941) in an unknown number
259 of specimens of *C. micropapillatum*. In comparison, spicule lengths of 2.23–3.17 mm long were
260 recorded in 18 specimens of *C. bancrofti* measured by Shamsi et al (2009), and suggested that this
261 narrower range of spicule lengths supported the validity of *C. bancrofti*, separate from *C.*
262 *micropapillatum*, and raised the possibility that Hartwich's material included multiple species. Our

263 results support the validity of *C. bancrofti*, as per Shamsi et al (2009), but also indicate that spicule
264 length variation may not be a useful character for distinguishing the two species.

265 The caudal papillae in the post-cloacal region also appear to be inconclusive for resolving *C.*
266 *bancrofti* and *C. micropapillatum*, as these show the same pattern in both species, i.e. three pairs
267 of double papillae (Stossich 1890; Cram 1927; Hartwich, 1964; Shamsi et al. 2009). The SEM
268 micrographs of *C. micropapillatum* genetically characterized in the present study show the first
269 pair of post-cloacal papillae are generally fused (Fig. 5d), while the second and third pairs may be
270 single but adjacent, or shifted backwards (Fig. 5h), possibly due to differences in the
271 developmental stage, or to intraspecific variability.

272 In genetically identified female specimens of *C. micropapillatum*, the distance of the vulva from
273 anterior end varied from 1.8-14.1 mm, which overlaps data reported by Shamsi et al (2019) and
274 Hartwich (1964). Therefore, female morphology and morphometry is not of value in distinguishing
275 *C. bancrofti* from *C. micropapillatum*, and in fact, has seldom been used in specific diagnosis of
276 *Contracaecum* and other anisakids.

277 Other morphometric features of the adult stage (total body length, length of esophagus, intestinal
278 cecum, ventricular appendix) of *C. micropapillatum* were on average higher in Stossich (1896) and
279 Cram (1927) compared to data reported by Hartwich (1964). Our values include also
280 measurements of smaller specimens and are more similar to the ranges reported by Hartwich
281 (1964).

282 Additionally, both *C. micropapillatum* (Stossich, 1890, Hartwich, 1964 as reported in Baruš et al.,
283 1978) and *C. bancrofti* (Johnston and Mawson, 1941; Shamsi et al., 2009) were described as having
284 bifid interlabia. In the current study, the bifid appearance of interlabia (Fig. 5c) was confirmed in

light microscopy as well as in several SEM micrographs that clearly show this feature in both lateral and apical views of prepared specimens.

In multivariate analysis of eight measurements of *C. micropapillatum* and *C. bancrofti* in the present and other studies, the two species were poorly separated (Table 2, Fig. 7). In an ANOSIM test, morphometric variation between sexes and species was statistically significant, although modest in magnitude between sexes within the two species (R=0.24, P=0.019), and negligible between same-sex individuals of the two species (R=0.025, P=0.017). These results were essentially the same if data from Hartwich (1964), who included data from non-Australian sources of *C*. "*bancrofti*", were excluded from MDS (not shown) or ANOSIM (sex R=0.253, P=0.017, species R=0.034, P=0.01).

Contracaecum gibsoni Mattiucci, Paoletti, Solorzano and Nascetti, 2010

Synonyms: *Contracaecum multipapillatum* sp. A of Nascetti et al. (1990)

Site in host: Ventriculus.

Representative DNA sequences: ON723780-87 (ITS rDNA), ON736833-37 (*cox*2 mtDNA);

Adult stage: Body stout. Cuticle transversally striated. Dorsal and ventro-lateral lips with slight medial depression on upper margin (Figs. 8a-c, 9a-b); dorsal lip with 2 double papillae; each ventro-lateral lip with 1 double papilla, 1 single papilla and 1 amphid. Interlabia triangular, wider at base, with rounded non-bifurcate tip (Figs. 8b, 9a). Excretory pore at base of lips. Oesophagus with globular ventriculus. Ventriculus with posterior appendix. Intestinal caecum three to four times longer than ventricular appendix.

Males (*n* = 4): Total length 15.0–44.0 (27 ± 12.3). Nerve ring 0.35–0.41 (0.39 ± 0.035) from anterior end. Oesophagus 2.95–6.65 (4.89) long. Ventricular appendix 0.53–0.70 (0.70) long. Intestinal caecum 2.10–2.28 (2.50) long; intestinal caecum/ventricular appendix length ratio 2.5–4.5 (3.8). Spicules slightly subequal; right spicule 1.82–2.50 (2.11 ± 0.29) long; left spicule 1.75–2.18 (1.99 ± 0.19) long; spicule tip pointed. Ratio spicules length/total length (spi/len) 0.04–0.12 (0.07). Precloacal papillae simple, forming 2 subventral lines (Fig. 8d). Five pairs of proximal papillae posterior to cloaca, lateral to paracloacal papillae, four of which pyriform in shape (Fig. 8e-f). Single pair of double paracloacal papillae; 4 pairs of distal papillae. Single pair of small papilla-like phasmids lateral to the distal pair (d4) of post-cloacal papillae (Fig. 8e). Tail 0.21 – 0.24 (0.23 ± 0.017) long.

Females (*n* = 16) Total length 13.0–63.0 (36.9 ± 14.5). Nerve ring 0.32–0.60 (0.49 ± 0.083) from anterior end. Oesophagus 3.20 – 6.79 (5.10 ± 1.22) long. Ventricular appendix 0.85–1.42 (1.10 ± 0.16) long. Intestinal caecum 2.38 – 6.36 (4.31 ± 1.24) long, intestinal caecum/ventricular appendix length ratio 2.7 – 4.4 (3.8). Vulva in first third of body, 6.92 – 17.33 (12.90 ± 2.95) from anterior end. Tail conical, 0.21 – 0.60 (0.48 ± 0.09) long (Fig. 9c-d).

Remarks

Overall, measurements of our male and female specimens overlap data reported by Mattiucci et al (2010), although our samples include also smaller and larger specimens. With respect to male features, the average ratio spi/len, spicule shape and pattern of caudal papillae are the same as those reported in the original description of *C. gibsoni* (Mattiucci et al. 2010); such features have been considered as useful taxonomic criteria for distinguishing genetically detected sibling species of *Contracaecum* (Mattiucci et al. 2010). Particularly, as suggested by Shamsi and colleagues (2008), the arrangement and shape of the caudal papillae of males could be useful to differentiate

cryptic species of the *C. multipapillatum* complex. In addition to its original description, the present work adds further morphological material providing the first SEM images of *C. gibsoni* male and female adults. Particularly, SEM micrographs of the caudal region of male specimens, showed the presence of five proximal pairs of single papillae of which four pyriform in shape (Fig. 8e), a feature here reported for the first time in *C. gibsoni*.

Contracaecum quadripapillatum Saad, Younis & Rabei, 2018

Site in host: Stomach and oesophagus.

Representative DNA sequences: ON714979-86 (ITS rDNA), ON736806-07 (*cox*2 mtDNA)

Adult stage: Body stout. Cuticle transversally striated. Dorsal and ventro-lateral lips with central depression on upper margin (Figs. 10a, 11a); dorsal lip with 2 ovate papillae; each ventro-lateral lip with 1 ovate papilla. Interlabia triangular, wider at base, with rounded non-bifurcate tip (Fig. 10b). Short cuticular collar, interrupted laterally at the base of lips. Intestinal caecum three to five times longer than ventricular appendix.

Males: (*n* = 3) Total length 21.0–42.4 (20.8 ± 21.2). Nerve ring 0.45–0.63 (0.54 ± 0.09) from anterior end. Oesophagus 3.60–4.99 (4.29 ± 0.069) long. Ventricular appendix 0.60–0.83 (0.74 ± 0.12) long. Intestinal caecum 2.06–4.39 (3.22 ± 1.16) long; intestinal caecum/ventricular appendix length ratio 2.5–5.6 (4.5). Spicules subequal, with rounded, spoon-like, flattened tip (Fig. 10c-d). Right spicule 2.27 -2.52 (2.39 ± 0.13) long, left spicule 1.87 -2.72 (2.32 ± 0.42) long 11.2-11.5% of body length. Post-cloacal papillae consisting in two pairs of single papillae, followed by one pair of double papillae; remaining post-cloacal papillae arranged in three rows, first and second rows containing four papillae on each side forming quadrilateral shape, third row with one papilla on each side (Fig. 10e-f).

352 *Females*: (*n* = 4) Total length 31.0–64.0 (40.5 ± 15.7). Nerve ring 0.33–0.56 (0.46 ± 0.10) from
353 anterior end. Oesophagus 4.73–5.65 (5.08 ± 0.44) long. Ventricular appendix 0.85–1.09 (0.95 ±
354 0.1) long. Intestinal caecum 3.95–4.46 (4.28 ± 0.32) long. Intestinal caecum/ventricular appendix
355 length ratio 3.8–4.9 (4.5). Vulva in first third of body, 7.73 – 15.38 (10.48 ± 3.36) from anterior end
356 (Fig. 11b). Tail conical, 0.38 – 0.47 (0.42 ± 50.5) long (Figs. 11c-d).

357 **Remarks**

358 Overall, measurements of male and female specimens of *C. quadripapillatum* reported by Saad
359 and colleagues (2018) fall within a narrower range than our observations, possibly because Saad et
360 al (2018) measured experimentally obtained specimens belonging to exactly the same
361 developmental stage, while specimens analyzed in the present study were recovered from
362 naturally infected birds who likely acquired parasites in multiple feeding events. With respect to
363 male morphological features of taxonomic value, the average ratio spi/len was slightly higher in
364 our specimens (11% as compared to 8% reported by Saad et al. 2018), while spicule shape and
365 pattern of caudal papillae are similar to those described by the latter authors. While the pyriform-
366 shaped precloacal papillae in some cases (Fig. 10f) resemble *C. pyripapillatum*, the post-cloacal papillae of
367 *C. quadripapillatum* form a square (Fig. 10e) and the tip of male's spicules is completely different (fig. 10d)
368 from *C. pyripapillatum*. In addition, the ITS rDNA of *C. pyripapillatum* is distant from *C. quadripapillatum*
369 (Fig 2).

370

371 **Discussion**

372 Prior to this study, in several valid species of *Contracaecum*, only either ITS rDNA or *cox*2 mtDNA
373 were available, making comparison of sequences impossible. For example, only ITS was available
374 in Australian *C. bancrofti*, while in *C. micropapillatum*, only *cox*2 and 28S rDNA had been

sequenced. We resolved this problem by obtaining data from both markers in both species and avoided potential confusion from single-marker results (particularly ITS). While ITS sequences from Israeli and Australian isolates in the *C. bancrofti* + *C. micropapillatum* clade formed reciprocally monophyletic subclades, they had only moderate statistical support and were nearly identical (99.5% similarity). However, the *cox*2 of the specimens formed well-supported clades with unambiguous levels of sequence divergence, and gaps unique to *C. bancrofti* in the ITS alignment were not reflected in divergence calculations, or in phylogenetic analysis. We attempted to make further distinctions in sequenced specimens of these two species with both light microscopy and SEM, with particular attention to characters useful in distinguishing anisakids, such as interlabial structure, distribution pattern of male caudal papillae, spicule length and tip shape, size and pattern of caudal papillae (Fagerholm 1989; Mattiucci et al. 2010), as well as quantitative visualization of morphometric variation in *C. micropapillatum* and *C. bancrofti* in the present and past studies. Ultimately, while molecular evidence supports the validity of *C. bancrofti* as described by Shamsi et al (2009), no morphological distinctions were observed that reliably separate this species from *C. micropapillatum*. Morphological variation in Table 2 and Fig 7 may be inflated by variation in preservation methods among studies, or mixed-species infections undetected in non-molecular studies (i.e., those other than Shamsi et al. 2009 and the present work), but even stable characters such as spicule length are notably uninformative. In the future, sequences of *cox*2 from regional isolates of *C. micropapillatum* are needed to verify the wide geographic distribution and diverse host range that ITS-based and morphological records imply for this species, which has been reported from all the continents (China, Australia, Croatia, England, Congo, Mexico, USA), as well as in birds from several different families (Li et al. 2016), namely: Anseriformes (Anatidae): *Bucephala clangula* (L.); *Mergus squamatus* Gould; *Spatula clypeata* L.; Charadriiformes (Stercorariidae): *Stercorarius pomarinus* (Temminck); Pelecaniformes (Ardeidae):

399 *Ardea alba* L.; *A. purpurea* L.; *Butorides striata* L., *B. striata atricapilla* (Afzelius); *Nyctanassa*
400 *violacea* (L.); Pelecaniformes (Pelecanidae): *Pelecanus crispus* Bruch; *P. conspicillatus* Temminck; *P.*
401 *erythrorhynchos* Gmelin; *P. onocrotalus* L.; *P. rufescens* Gmelin; *Pelecanus* sp.; Pelecaniformes
402 (Phalacrocoracidae): *Microcarbo africanus* (Gmelin); *M. pygmaeus* (Pallas); *Phalacrocorax*
403 *brasilianus* (Gmelin); *P. africanus* (Gmelin); *P. carbo* (L.); *Phalacrocorax* sp.

404 Joint analysis of both nuclear and mitochondrial markers was also necessary to resolve conflicting
405 ITS and *cox2* results from *C. gibsoni*. In five adults from Israel collected in the present study, *cox2*
406 matched *C. gibsoni* (syn *C. multipapillatum* A of Nascetti et al. 1990) described from *P. crispus* in
407 Greece (p-distance 0-0.1% to data from Mattiucci et al. 2010). Had we obtained only ITS
408 sequences, however, these specimens of *C. gibsoni* could have been mis-identified within the
409 already-complicated *C. multipapillatum* complex, because the ITS matched data from specimens
410 identified as *C. multipapillatum* (syn *C. multipapillatum* A of Nascetti et al. 1990). In addition to the
411 *cox2* results, morphological features of these specimens overlapped with *C. gibsoni* as described
412 by Mattiucci et al (2010), and were supported with SEM micrographs of adult male and female
413 structures, to better characterize features poorly visible by light microscopy.

414 *Contracaecum gibsoni* was described from the Dalmatian pelican *P. crispus* L. (Pelecaniformes:
415 Pelecanidae) from Greece, and is now reported for the first time in *P. onocrotalus* L. from Israel.
416 According to Mattiucci et al (2010), species in the *C. multipapillatum* complex are restricted to the
417 families Pelicanidae and Ardeidae from Central and South America, but in the Mediterranean
418 areas they have been found only in pelicans, as demonstrated for *C. gibsoni* and *C. overstreeti*.

419 The adults of *C. quadripapillatum* were first described by Saad et al (2018) after experimental
420 infection of *P. erythrorhynchos* fed *Clarias lazera* from Lake Nasser, South Egypt naturally infected
421 with L3s. These authors also obtained ITS rDNA sequences from *C. quadripapillatum* to which our

422 specimens are identical (p-distance 0%). We here provide a new PCR-RFLP (*Ssp*I) assay to
423 distinguish *C. quadripapillatum* from *C. gibsoni*, which are not resolved by the *Msp*I enzyme of Zhu
424 et al (2007), as well as *cox*2 mtDNA from both species, and new records in *P. onocrotalus* sampled
425 in Israel.

426 The status and distributions of species of *Contracaecum* encountered here should be considered
427 alongside the habits and movements of their definitive host, the great white pelican *P.*
428 *onocrotalus*, one of the largest members of the family Pelecanidae. Populations of this gregarious
429 bird are distributed in eastern Europe, Asia and Africa. However, great white pelicans are not
430 found in Australia, which is inhabited by *P. conspicillatus*. The disjunct distributions of the
431 definitive hosts (*P. onocrotalus*, *P. conspicillatus*) are of interest given the apparent sister
432 relationship and lack of morphological distinctions between their parasites, *C. bancrofti* and *C.*
433 *micropapillatum*. Interestingly, phylogenetic analysis indicates *P. onocrotalus* is a basal, sister
434 lineage to an Old-World clade of pelicans that includes *P. conspicillatus* (Kennedy et al. 2013).

435 Crivelli and Schreiber (1984) distinguished two geographically separate populations of the great
436 white pelican: one in Africa and the other in eastern Europe and Asia. The African population is
437 sedentary, living under tropical climatic conditions and the Eurasian population is migratory,
438 visiting the Palearctic in spring and summer, during the breeding season (Crivelli et al. 1991). A few
439 hundred of the migratory *P. onocrotalus* regularly winter in Israel, where they arrive from July to
440 September, while thousands of individuals continue their migration to winter either in Sudan or in
441 eastern central Africa (e.g. Ethiopia, Kenya, Uganda, Tanzania or Zaïre). Several wintering grounds
442 are also known in western and southern Asia, in Russia, Iran, Iraq, Pakistan and India (Scott and
443 Carp 1982; Van der Ven 1987, 1988; Crivelli et al. 1991). The same wetlands are used as stopovers,
444 both in spring and autumn. Crivelli and colleagues (1991) reported that Great White Pelicans do
445 not feed systematically at each stopover, either because the wetlands visited do not provide

446 favorable feeding conditions or because the fish density is too low, or because the costs in time
447 and energy would be too great. The fish ponds situated in northern Israel provide a favorable
448 feeding environment being extremely rich in fish (Sarig 1990) and are subject to intensive foraging
449 by migrating pelicans (Crivelli et al. 1991). In contrast, the Mediterranean Sea is rarely used as a
450 feeding site by Great White Pelicans, which is consistent with the lack of recovered *Contracaecum*
451 species that are distributed in brackish or saltwater environments (e.g. *Contracaecum rudolphii* A).
452 In addition, the numerical dominance of *C. micropapillatum* compared to the other species
453 encountered, as revealed by RFLP analysis, is consistent with its putatively wide geographic
454 distribution (Poulin 2007), which extends beyond the range of *P. onocrotalus* to the Americas. The
455 relative abundance of *C. micropapillatum* is also consistent with the general frequency with which
456 this species is reported (Shamsi et al. 2009; 2019).

457 Taken together, our results provide further illustration that more than one marker (preferably
458 independent, e.g., one nuclear, one mitochondrial) provide better support for distinguishing
459 helminths characterized by genetic variability and lacking clear morphological differences, such as
460 those within the *Anisakis simplex*, *Pseudoterranova decipiens* and *Contracaecum multipapillatum*
461 complexes (Nadler and Hudspeth, 2000; Paggi et al., 2000; Mattiucci et al., 2005) and other
462 anisakids (Valentini et al., 2006; Mattiucci et al., 2008; 2010; 2020; D'Amelio et al. 2020).

463 Such data should be generated in any study focused on the genetic diversity of this group of
464 parasites, to help clarifying not only their taxonomy but also possible cospeciation patterns
465 between *Contracaecum* spp. and different families of their definitive hosts, as already suggested
466 for other anisakid taxa (Mattiucci and Nascetti 2006; 2008).

467

468 **Funding**

469 This study was supported by the Israeli Veterinary Services and by the University of Bologna, Italy.
470 Scanning Electron Microscopy acquired thanks to the Department of Excellence Project 2018-2022 funded
471 by the Italian Ministry of Education, Universities and Research. SAL was supported by the National Science
472 Foundation (DEB award 1845021).

473 **Conflict of interest**

474 The authors declare no competing interests.

475 **Availability of data and material**

476 The DNA sequences generated in this study have been deposited on the public database GenBank.

477 **Author contributions**

478 Monica Caffara and Perla Tedesco wrote the main manuscript text, carried out the analyses and
479 prepared all the figures. Michelle Nuytten and Marialuisa Nuzzo provided the technical support for
480 SEM and molecular analyses. Nadav Davidovich and Roni King carried out the sampling and
481 necropsies. Sean Locke carried out the multivariate analysis and revised the manuscript. Andrea
482 Gustinelli and Maria Letizia Fioravanti revised the manuscript. All authors reviewed the manuscript
483 and approved the final manuscript.

484 **Ethical approval**

485 “Not applicable”

486 **Consent to participate**

487 “Not applicable”

488 **Consent for publication**

489 “Not applicable”

Acknowledgements

The authors are grateful for the constructive comments of anonymous reviewers on early versions of this manuscript, and express particular thanks to the editor dr. Shokoofeh Shamsi, who kindly provided specimens of *C. bancrofti* from Australia.

References

Anderson RC (2000) Nematode parasites of vertebrates: their development and transmission, second edition. CABI, Wallingford.

Ángeles-Hernández JC, Gómez-de Anda FR, Reyes-Rodríguez NE, Vega-Sánchez V, García-Reyna PB, Campos-Montiel RG, Calderón-Apodaca NL, Salgado-Miranda C, Zepeda-Velázquez AP (2020) Genera and species of the Anisakidae family and their geographical distribution. Animals, 10: 2374. https://doi.org/10.3390/ani10122374

Baruš V, Sergeeva TP, Sonin MD, Ryzhikov KM (1978) Helminths of fish-eating birds of the Palaearctic Region: Nematoda. In: Rysavy B, Ryzhikov KM (Eds) Helminths of Fish-Eating Birds. Springer, Praha, p 318

Blouin MS (2002) Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int J Parasitol 32:527-531. doi: 10.1016/s0020-7519(01)00357-5.

Cram EB (1927) Bird parasites of the nematode suborders Strongylata, Ascaridata, and Spirurata. Bull US Natl Mus. p 492. https://doi.org/10.5479/si.03629236.140.1

Crivelli AJ, Leshem Y, Mitchev T, Jerrentrup H (1991) Where do palearctic great white pelicans (*Pelecanus onocrotalus*) presently overwinter? Rev Ecol-Terre Vie 46:145-171

Crivelli AJ, Schreiber RW (1984) Status of Pelecanidae. Biol Conserv 30:147-156

D'Amelio S, Lombardo F, Pizzarelli A, Bellini I, Cavallero S (2020) Advances in omic studies drive discoveries in the biology of *Anisakid* nematodes. Genes 11:801. https://doi.org/10.3390/genes11070801

Davidovich N, Tedesco P, Caffara M, Yasur-Landau D, Gustinelli A, Drabkin V, Minkova E, Aflalo O, Morick D, Fioravanti ML (2022) Morphological description and molecular characterization of

519 *Contracaecum* larvae (Nematoda: Anisakidae) parasitizing market-size hybrid tilapia
520 (*Oreochromis aureus* x *Oreochromis niloticus*) and red drum (*Sciaenops ocellatus*) farmed in
521 Israel. Food Waterborne Parasitol 26:e00147. https://doi.org/10.1016/j.fawpar.2022.e00147
522 Fagerholm HP (1989) Intra-specific variability of the morphology in a single population of the seal
523 parasite *Contracaecum osculatum* (Rudolphi) (Nematoda: Ascaridoidea), with a redescription
524 of the species. Zool Scr 18:33–41. https://doi.org/10.1111/j.1463-6409.1989.tb00121.x
525 Fagerholm HP, Overstreet RM (2008) Ascaridoid Nematodes: *Contracaecum*, *Porrocaecum*, and
526 *Baylisascaris*. In: Atkinson CT, Thomas NJ, Hunter DB (eds) Parasitic Diseases of Wild Birds.
527 Wiley-Blackwell, Iowa, USA, pp 413–433
528 Hall T.A. (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program
529 for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95-98
530 Hamouda AH, Younis AE (2022) Molecular characterization of zoonotic anisakid *Contracaecum*
531 spp. larvae in some fish species from Lake Nasser, Egypt. Aquac Res 53:2548-2561.
532 https://doi.org/10.1111/are.15774
533 Hartwich G (1964) Revision der vogelparasitischen nematoden Mitteleuropas II. Die gattung
534 *Contracaecum* Railliet and Henry, 1912 (Ascaridoidea). Mitt Zool Mus Berl 40:15‑53
535 Israeli Veterinary Services, 2017. Pre-marketing control of locally grown edible fish: procedure.
536 https://www.gov.il/he/departments/policies/moag-pro-082 (in Hebrew)
537 Johnston TH, Mawson PM (1941) Ascaroid nematodes from Australian birds. Trans R Soc S Aust
538 65:110-5
539 Kennedy M, Taylor SA, Nádvorník P, Spencer HG (2013) The phylogenetic relationships of the
540 extant pelicans inferred from DNA sequence data. Mol Phyl Evol 66:215-222.
541 https://doi.org/10.1016/j.ympev.2012.09.034.

542 Kumar S, Periyasamy A, Nitin Venkatesh Ranga Rao, Sunil SS, Kumara HN, Sundararaj P,
543 Chidananda G, Sathish A (2019) Multiple infestations of gastrointestinal parasites – Probable
544 cause for high mortality of Spot-billed Pelican (*Pelecanus philippensis*) at Kokrebellur
545 Community Reserve, India. Int J Parasitol Parasites Wildl 9:68-73.
546 https://doi.org/10.1016/j.ijppaw.2019.04.001.

547 Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7
548 for bigger datasets. Mol Biol Evol 33:1870-1874. https://doi.org/10.1093/molbev/msw054

549 Li L, Gibson DI, Zhang LP (2016) An annotated catalogue of the ascaridoid nematode parasites of
550 Chinese vertebrates. Syst Parasitol 93:1-35. https://doi.org/10.1007/s11230-015-9617-5

551 Mattiucci S, Nascetti G (2006) Molecular systematics, phylogeny and ecology of anisakid
552 nematodes of the genus *Anisakis* Dujardin, 1845: an update. Parasite 13(2):99-113.
553 https://doi.org/10.1051/parasite/2006132099

554 Mattiucci S, Nascetti G (2008) Advances and trends in the molecular systematics of anisakid
555 nematodes, with implications for their evolutionary ecology and host parasite co-
556 evolutionary processes. Adv Parasitol 66:47-148. https://doi.org/10.1016/S0065-
557 308X(08)00202-9

558 Mattiucci S, Paoletti M, Solorzano AC, Nascetti G (2010) *Contracaecum gibsoni* n. sp. and *C.*
559 *overstreeti* n. sp. (Nematoda: Anisakidae) from the dalmatian pelican *Pelecanus crispus* (L.) in
560 Greek waters: genetic and morphological evidence. Syst Parasitol 75:207–224.
561 https://doi.org/10.1007/s11230-009-9220-8

562 Mattiucci S, Paoletti M, Webb SC, Sardella N, Timi JT, Berland B, Nascetti G (2008) Genetic
563 relationships among species of *Contracaecum* Railliet & Henry, 1912 and *Phocascaris* Host,
564 1932 (Nematoda: Anisakidae) from pinnipeds based on mitochondrial cox2 sequences, and

congruence with allozyme data. Parasite 15:408–419. https://doi.org/10.1051/parasite/2008153408

Mattiucci S, Sbaraglia GL, Palomba M, Filippi S, Paoletti M, Cipriani P, Nascetti G (2020) Genetic identification and insights into the ecology of *Contracaecum rudolphii* A and *C. rudolphii* B (Nematoda: Anisakidae) from cormorants and fish of aquatic ecosystems of Central Italy. Parasitol Res 119:1243–1257. https://doi.org/10.1007/s00436-020-06658-8

McDaniel B, Patterson I (1966) Nematode infestation of a white pelican found along the gulf coast of Texas. Southwest Nat 11:312-312

Nadler SA, Hudspeth DSS (2000) Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J Parasitol 86:380-393. https://doi.org/10.1645/0022-3395(2000)086[0380:POTANA]2.0.CO;2

Nascetti G, Bullini L, Cianchi R, Paggi L, Orecchia P, Mattiucci S, D'Amelio S, Berland B (1990). Genetic relationships among anisakid species belonging to the genera *Contracaecum* and *Phocascaris*. Bull Soc Fr Parasitol 8:261

Otachi EO, Szostakowska B, Jirsa F, Fellner-Frank C (2014) Parasite communities of the elongate tigerfish *Hydrocynus forskahlii* (Cuvier 1819) and redbelly tilapia *Tilapia zillii* (Gervais 1848) from Lake Turkana, Kenya: influence of host sex and size. Acta Parasitol 60:9–20. https://doi.org/10.1515/ap-2015-0002

Poulin R (2007) Are there general laws in parasite ecology? Parasitology 134:763–776. https://doi.org/10.1017/S0031182006002150

Pronkina NV, Spiridonov SE (2018) Morphological and molecular characterization of anisakid juveniles from the golden grey mullet of the Black Sea. Russ J Nematol 26:87-92. https://doi.org/10.24411/0869-6918-2018-10008

588 Rokicki J, Sołtysiak Z, Dziekońska-Rynko J, Borucińska J (2011) Pathology associated with
589 *Contracaecum rudolphii* (Nematoda: Anisakidae) infection in the great cormorant
590 *Phalacrocorax carbo* (L. 1758). Helminthol 48:28-35. https://doi.org/10.2478/s11687-011-
591 0006-6

592 Saad AI, Younis AE, Rabei JM (2018) Experimental life cycle of *Contracaecum quadripapillatum* n.
593 sp. in white pelican (*Pelecanus erythrorhynchos*) at Lake Nasser, Egypt: Morphological and
594 genetic evidences. J Egypt Soc Parasitol 48:587-598

595 Sarig S (1990) The fish culture industry in Israel in 1989. Israeli J Aquacult Bamidgeh 42:39-45

596 Scott DA, Carp E (1982) A midwinter survey of wetlands in Mesopotamia, Iraq, 1979. Sandgrouse,
597 4: 60-76.

598 Shamsi S (2019) Parasite loss or parasite gain? Story of *Contracaecum* nematodes in antipodean
599 waters. Parasite Epidemiol Contr 4: e00087. https://doi.org/10.1016/j.parepi.2019.e00087

600 Shamsi S, Aghazadeh-Meshgi M (2011) Morphological and genetic characterisation of selected
601 *Contracaecum* (Nematoda: Anisakidae) larvae in Iran. Iran J Fish Sci 10:356-361

602 Shamsi S, Gasser R, Beveridge I, Shabani AA (2008) *Contracaecum pyripapillatum* n. sp.
603 (Nematoda: Anisakidae) and a description of *C. multipapillatum* (von Drasche, 1882) from
604 the Australian pelican, *Pelecanus conspicillatus*. Parasitol Res 103:1031–1039.
605 https://doi.org/10.1007/s00436-008-1088-z

606 Shamsi S, Steller E, Chen Y (2018a). New and known zoonotic nematode larvae within selected fish
607 species from Queensland waters in Australia. Int J Food Microbiol 272:73–82.
608 https://doi.org/10.1016/j.ijfoodmicro.2018.03.007

609 Shamsi S, Turner A, Wassens S (2018b) Description and genetic characterization of a new
610 *Contracaecum* larval type (Nematoda: Anisakidae) from Australia. J Helminthol 92:216–222.
611 https://doi.org/10.1017/S0022149X17000360
612 Shamsi S, Norman R, Gasser R, Beveridge I (2009) Redescription and genetic characterization of
613 selected *Contracaecum* spp. (Nematoda: Anisakidae) from various hosts in Australia.
614 Parasitol Res 104:1507-1525. https://doi.org/10.1007/s00436-009-1357-5
615 Shamsi S, Stoddart A, Smales L, Wassens S (2019) Occurrence of *Contracaecum bancrofti* larvae in
616 fish in the Murray–Darling Basin. J Helminthol 93:574-579.
617 https://doi.org/10.1017/S0022149X1800055X
618 Stossich M (1890) Elminti della Croazia. Glasnik Hrvatskoga Naravoslovnoga Društva 5:129-136.
619 Stossich M (1896) Il Genere *Ascaris* Linné. Lavoro monografico. Boll Soc Adriat Sci Nat Trieste
620 17:9–114
621 Valentini A, Mattiucci S, Bondanelli P, Webb SC, Mignucci-Giannone AA, Colom-Llavina MM,
622 Nascetti G (2006) Genetic relationships among *Anisakis* species (Nematoda: Anisakidae)
623 inferred from mitochondrial *cox*2 sequences, and comparison with allozyme data. J Parasitol
624 92:156-166. https://doi.org/10.1645/GE-3504.1
625 Valles-Vega I, Molina-Fernández D, Benítez R, Hernández-Trujillo S, Adroher FJ (2017) Early
626 development and life cycle of *Contracaecum multipapillatum s.l.* from a brown pelican
627 *Pelecanus occidentalis* in the Gulf of California, Mexico. Dis Aquat Org 125:167-178.
628 https://doi.org/10.3354/dao03147
629 Van Der Ven J (1987) Asian waterfowl 1987. Midwinter bird observations in most Asian countries.
630 IWB, Slimbridge, England
631 Van Der Ven J (1988) Asian waterfowl 1988. Midwinter bird observations in most Asian countries.
632 IWRB, Slimbridge, England

633 Yamaguti S (1935) Studies on the helminth fauna of Japan. Part 12. Avian nematodes, I. J J Zool
634 6:403–431

635 Yamaguti S (1961) Systema Helminthum, vol. 3. The Nematodes of Vertebrates. Part II.
636 Interscience, New York, p 1575

637 Zhu X, Gasser RB, Podolska M, Chilton NB (1998) Characterisation of anisakid nematodes with
638 zoonotic potential by nuclear ribosomal DNA Sequences. Int J Parasitol 28:1911–1921.
639 https://doi.org/10.1016/S0020-7519(98)00150-7

640 Zhu X, D'Amelio S, Paggi L, Gasser RB (2000). Assessing sequence variation in the internal
641 transcribed spacers of ribosomal DNA within and among members of the *Contracaecum*
642 *osculatum* complex (Nematoda: Ascaridoidea: Anisakidae). Parasitol Res 86:677–683.
643 https://doi.org/10.1007/pl00008551

644 Zhu XQ, D'Amelio S, Gasser RB, Yang TB, Paggi L, He F, Lin RQ, Song HQ, Ai L, Li AX (2007) Practical
645 PCR tools for the delineation of *Contracaecum rudolphii* A and *Contracaecum rudolphii* B
646 (Ascaridoidea: Anisakidae) using genetic markers in nuclear ribosomal DNA. Mol Cell Probes
647 21:97–102. https://doi.org/10.1016/j.mcp.2006.08.004

648

649 **Captions**

650 **Fig. 1** Map of Israel with detail of the sampling locality together with the number of *Contracaecum*
651 spp. collected from each *Pelecanus onocrotalus*

652 **Fig. 2** Maximum-Likelihood tree based on the concatenated ITS1-ITS2 rDNA sequences showing
653 the relationship between *C. micropapillatum* (condensed, containing 34 sequences GB acc. n.
654 ON714944-77), *C. bancrofti*, *C. gibsoni*, *C. quadripapillatum* and *C. multipapillatum* E described in

655 the present study (in bold) and the congeneric *Contracaecum* species. The tree is drawn to scale,
656 with branch length measured in the number of substitutions per site

657 **Fig. 3** Maximum-Likelihood tree based on the *cox*2 mtDNA sequences showing the relationship
658 between *C. micropapillatum* (condensed pink clade containing 25 sequences GB acc. n. ON736808-
659 32, expanded at right), *C. bancrofti*, *C. gibsoni*, *C. quadripapillatum* and *C. multipapillatum* E
660 described in the present study (in bold) and other *Contracaecum* species. The inset at right shows
661 sequences from the present study nested with *C. micropapillatum* of Mattiucci et al (2008, 2010)
662 from *P. onocrotalus* sampled in Egypt and Greece. The tree is drawn to scale, with branch length
663 measured in the number of substitutions per site

664 **Fig. 4** PCR-RFLP pattern of *C. micropapillatum, C. gibsoni*, *C. quadripapillatum* and *C.*
665 *multipapillatum* E after digestion with *Msp*I (a) and *Ssp*I (b)

666 **Fig. 5** SEM micrographs of *C. micropapillatum* adult males: a) anterior end, showing dorsal lip (dl);
667 b) anterior end, showing subventral lip (svl); c) detail of bifid interlabium; d) ventral view of caudal
668 end, showing two rows of single pre-cloacal papillae, and two rows of double post-cloacal papillae;
669 e) detail of precloacal papillae; f) caudal end with everted spicule; g) detail of spicule tip; h)
670 specimen with unusual pattern of post-cloacal papillae

671 **Fig. 6** SEM micrographs of *C. micropapillatum* adult females: a) anterior end, showing dorsal lip
672 (dl) and subventral lip (svl); b) detail of vulva; c) lateral view of caudal end; d) detail of caudal tip

673 **Fig. 7** Non-metric multidimensional scaling of eight morphometric distances in the present and
674 other studies of *C. micropapillatum* (Stossich 1890) and *C. bancrofti* Johnston and Mawson 1941.
675 Points are shape- and color-coded by worm sex and species (see inset legend) and labelled by
676 source, with unlabelled points representing individual worms in the present study. Other studies
677 are S=Shamsi et al. 2009; N=Norman (2005) cited in Shamsi et al. 2009; J=Johnston and Mawson

1941; C=Cram 1927; H=Hartwich 1964. Data point sizes for male worms are proportionate to spicule lengths (see inset legend). Two-dimensional stress=0.16

Fig. 8 SEM micrographs of *C. gibsoni* adult males: a) subventral view of anterior end showing the shape of subventral lip and the position of the amphid (arrow); b) apical view of anterior end showing dorsal lip (dl) with two ovoid papillae, subventral lips (svl) and simple non-bifurcated interlabium (il); c) detailed ventral view of anterior end; d) ventral view of caudal end showing precloacal papillae; e) ventral view of caudal end showing the characteristic pattern of paracloacal and post-cloacal papillae; f) detail of the pyriform-shaped proximal and paracloacal papillae

Fig. 9 SEM micrographs of *C. gibsoni* adult females: a) subapical view of anterior end showing dorsal lip (dl) with two ovoid papillae, subventral lips (svl) and simple non-bifurcated interlabium (il); b) subapical view of anterior end showing the shape of subventral lips; c) caudal end; d) detail of caudal tip, with phasmid (ph)

Fig. 10 SEM micrographs of *C. quadripapillatum* adult males: a) subventral view of anterior end showing the shape of the subventral lip with single ovoid papilla and short cuticular collar; b) apical view of anterior end showing dorsal lip (dl) with two ovoid papillae, subventral lips (svl) and simple non-bifurcated interlabium (il); c) ventral view of caudal end with everted spicule; d) detail of spicule tip; e) detail of the caudal end showing the characteristic pattern of caudal papillae, particularly, the first (d1) and second (d2) pair of distal papillae forming a square; f) detail of the caudal end showing pyriform-shaped paracloacal papillae

Fig. 11 SEM micrographs of *C. quadripapillatum* adult females: a) ventral view of anterior end, showing the excretory pore (ep) at the base of ventral interlabium, between subventral lips (svl); b) detail of the vulva; c) caudal end; d) detail of caudal tip