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

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

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12 **Abstract**

13 Despite the wide distribution and health importance of anisakids of the genus *Contracaecum*,
14 epidemiological data on their occurrence in definitive bird hosts are scarce, particularly from
15 certain parts of the world that represent important wintering sites or migration stopovers for
16 different bird species. In the present study, *Contracaecum* spp. infecting six great white pelicans
17 (*Pelecanus onocrotalus*) in Israel were identified using light and scanning electron microscopy and
18 phylogenetic analyses of nuclear internal transcribed spacer (ITS) and mitochondrial cytochrome c
19 oxidase II (*cox2*). A PCR-RFLP method was also developed and applied to screen large numbers of
20 *Contracaecum* parasites. Most (415/455) worms recovered were *C. micropapillatum*, followed by
21 *C. gibsoni* (31/455), *C. quadripapillatum* (8/455) and *C. multipapillatum* E (1/455). *Contracaecum*
22 *micropapillatum* from Israel and *C. bancrofti* from Australia are distinguishable by *cox2* but less

23 well resolved with ITS sequences, and could not be distinguished morphologically. Worms with
24 *cox2* 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.

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33 **Introduction**

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

39 The genus *Contracaecum* comprises over 60 species, the majority of which have been described
40 from fish-eating birds but also in marine mammals (Yamaguti 1935; Hartwich 1964; Baruš et al.
41 1978; Ángeles-Hernández et al. 2020). In birds, massive infections may occur as a result of the
42 continuous ingestion of paratenic hosts; third-stage larvae undergo further development and
43 moult, becoming fourth stage and eventually adults in the proventriculus and stomach (Fagerholm
44 and Overstreet 2008), where they cause hemorrhages, ulcerations and necrosis, leading in some
45 instances to a fatal outcome (Rokicki et al. 2011; Kumar et al. 2019).

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

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

59 Adults of *C. bancrofti* infecting *Pelecanus conspicillatus* have been reported from several parts of
60 Australia, including Peron Island in the Northern Territory, Thompson River and Burnett River in
61 Queensland, Sydney Zoological Gardens in New South Wales, Geelong and Healesville in Victoria
62 and Morgan in South Australia (Johnston and Mawson 1941; Shamsi et al. 2009). Although Shamsi
63 et al. (2009) considered *C. bancrofti* endemic in Australia, this species has also been reported in
64 the American white pelican *P. erythrorhynchos* along the coasts of Texas (McDaniel and Patterson
65 1966) and in Mexico (Yamaguti 1961). To our knowledge, neither the occurrence of *C. bancrofti*
66 outside Australia nor its genetic differentiation from *C. micropapillatum* have been assessed with
67 molecular data. This is partly because different genetic markers have been sequenced in these two
68 species, namely ITS rDNA in *C. bancrofti* (Shamsi et al. 2009), 28S rDNA and *cox2* mtDNA in *C.*
69 *micropapillatum* (Nadler et al. 2000; Mattiucci et al. 2008, 2010).

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

78 The present study is part of a larger project focused on the parasitic fauna of piscivorous birds
79 collected in Israel. Sampled birds included Pelecanidae belonging to the species *P. onocrotalus*,
80 which were found infected with parasitic nematodes of the genus *Contracaecum*. The aim of this
81 work was to characterize these parasites to the species level with morphometrical (by both light
82 microscopy and Scanning Electron Microscopy - SEM) and molecular methods based on two
83 markers (i.e. ITS rDNA and *cox2* mtDNA) to confirm their taxonomic position. To date, no such
84 studies have been carried out in Israel, and therefore our work was also intended to elucidate the
85 ecology and distribution of *Contracaecum* species in scarcely investigated hosts and geographical
86 areas.

87 **Materials and Methods**

88 ***Contracaecum* sampling**

89 Four hundred and fifty-five adults of *Contracaecum* spp. were collected from the gastric mucosa of
90 six great white pelican (*Pelecanus onocrotalus*) collected and processed fresh, from five localities
91 under permits 2020/42659 and 2021/42855 from the Israel Nature and Parks Authority (Fig. 1).

92 The nematodes were washed in saline and preserved in 70% ethanol for morphological and
93 molecular analysis. For some adults, the anterior and posterior portions were preserved in 10%
94 neutral buffered formalin for SEM. Moreover, two additional specimens of *Contracaecum*
95 *bancrofti* were obtained from *Pelecanus conspicillatus* sampled in Australia.

96 **Morphological study**

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

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

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

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

122 **Molecular study**

123 For molecular analysis, genomic DNA was initially extracted from 51 adults using a PureLink®
124 Genomic DNA Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions.
125 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 *cox2* 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 *cox2* 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 *cox2*, 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 *cox2* 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.

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

159

160 **Results**

161 **Molecular analyses**

162 Overall, of 455 adult worms from six Israeli pelicans examined with a combination of
163 morphometric and molecular analyses, 415 were identified as *C. micropapillatum*, 31 as *C. gibsoni*,
164 8 as *C. quadripapillatum* and 1 as *C. multipapillatum* E (Table 1). As described below, most of these
165 identifications were based on PCR-RFLP, and the entire ITS rDNA array was sequenced in 51 worms
166 from Israel, with partial *cox2* sequenced in 33 of the same specimens. Both ITS rDNA and *cox2*
167 mtDNA were also sequenced in two specimens of *C. bancrofti* from *P. conspicillatus* in Australia,
168 and these data supported the distinct status of *C. bancrofti*.

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

173 containing two subclades of sequences from Israel and Australia with moderate support (95%,
174 79%) (Fig. 2). Sequences of *cox2* from 25 of these 34 adult worms from Israel matched (97-99.2%
175 similarity) those of *C. micropapillatum* (EU852350, Mattiucci et al. 2010, EF513514-16 and
176 EF122206-07, Mattiucci et al. 2008) and were 92.6% similar to the *cox2* from two specimens of *C.*
177 *bancrofti* from Australia, newly sequenced herein. In phylogenetic analysis of *cox2*, specimens
178 from Israel and Australia were resolved into two clades with strong ($\geq 98\%$) bootstrap support (Fig.
179 3). Taken together, these analyses support the separation of *C. micropapillatum* and *C. bancrofti*.
180 Further evidence of the validity of *C. bancrofti* occurred in the form of gaps in the ITS rDNA
181 alignment. A 12-bp gap unique to *C. bancrofti* began at position 120 in ITS1, which corresponded
182 to an insertion of "TTGCTAAATTAA" in *C. multipapillatum* sequences and "TTGCTTATTTAG" in *C.*
183 *quadripapillatum*. At the 3' end of ITS1, an insertion of 7 bp (position 420-426 bp, "TATTTAG")
184 occurred in *C. bancrofti* only. In ITS2, we observed 3 insertions in the *C. bancrofti* sequences
185 (position 449-456 bp "GAATATCT", position 495-507 bp "AAAGACGAGAAAA" and position 555-569
186 bp "TCCTTGCTTAGTTTG") corresponding to deletions in the other two species. The ITS sequences
187 from the adult specimens of *C. micropapillatum* from Israel were also 99.7% similar to ITS from
188 larvae of an unidentified species of *Contraecaecum* from *Tilapia zillii* from Kenya (KF990496, Otachi
189 et al. 2014), indicating a possible transmission path of this *C. micropapillatum*. The 34 ITS
190 sequences of *C. micropapillatum* from Israeli pelicans differed by 2.5% from *C. multipapillatum*
191 and 2.4-2.5% from *C. quadripapillatum*.

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

197 Shamsi and Aghazadeh-Meshgi 2011) from barboid fish in Iran. These ITS sequences formed a
198 distinct and well-supported lineage in phylogenetic analysis (Fig. 2). The *cox2* sequences of two
199 the eight specimens in this clade were most similar (87.6%) to *C. osculatum* A sensu Nascetti et al
200 (JN786334). As described below, these worms were identified as *C. quadripapillatum*.

201 Another eight specimens yielded ITS sequences identical to *C. multipapillatum* (MH400190,
202 Pronkina and Spiridonov 2018) from *Chelon auratus* from the Black Sea and with 98.7% similarity
203 to *C. multipapillatum* D (AM940056, Shamsi et al. 2008) from Australian *P. conspicillatus*. In five of
204 these eight adults with these ITS rDNA matching *C. multipapillatum*, *cox2* showed 99.6-100%
205 similarity with *C. gibsoni* (EU852337, syn *C. multipapillatum* A, Mattiucci et al. 2010) from *P.*
206 *crispus* from Greece (intraspecific p-distance 0%-0.1%).

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

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

216

217 **Morphological descriptions**

218 *Contracaecum micropapillatum* Johnston & Mawson, 1941

219 *Synonym: Ascaris micropapillata* Stossich, 1890

220 *Site in host:* stomach.

221 *Representative DNA sequences:* ON714944-77 (ITS rDNA), ON736808-32 (*cox2* mtDNA)

222 *Adult stage:* Body stout. Cuticle transversally striated. Lips longer than wide, one dorsal and two
223 sub-ventral (Figs. 5a-b; 6a). Two oval cephalic papillae on dorsal lip and a single papilla on each
224 subventral lip. Interlabium reaching approximately $\frac{3}{4}$ of lip length, narrow, wider at base, with
225 distinctly bifurcated tip (Fig. 5c). Excretory pore at base of lips. Collar area with fine cuticular
226 annulations interrupted laterally. Deirids at approximately same level as nerve ring. Oesophagus
227 muscular, ending in round ventriculus with short ventricular appendix. Intestinal caecum two to
228 three times longer than ventricular appendix.

229 *Males* ($n = 50$): Total length 10-34.2 (17.2 ± 4.9). Nerve ring 0.17-0.67 (0.48 ± 0.1) from anterior
230 end. Oesophagus 2.01-4.56 (3.27 ± 0.59) long, 12.3–25.5% (19.7%) of body length. Ventricular
231 appendix 0.47-1.15 (0.85 ± 0.14) long, 17.8–42.1% (26.7%) of esophageal length. Intestinal cecum
232 1.47-3.79 (2.5 ± 0.5) long, 55.7–86.3% (75.8%) of esophageal length. Three pairs of double post-
233 cloacal papillae (Fig. 5d). Phasmids approximately at level of third pair of post-cloacal papillae.
234 Precloacal papillae simple, arranged in two longitudinal rows (Fig. 5e). Spicules similar, subequal,
235 8.6-19% (14%) of body length. Right spicule 1.02–3.37 (2.45 ± 0.56) long; left spicule 1.05–3.48
236 (2.4 ± 0.56) long, with rounded tip and folded longitudinal alae (Fig. 5f-g).

237 *Females* ($n = 50$): Total length 10-40.7 (18.8 ± 9.4). Nerve ring 0.25-0.92 (0.45 ± 0.13) from anterior
238 end. Oesophagus 1.67-5.69 (3.29 ± 1.02) long, 12.7–22.3% (18.7%) of body length. Ventricular
239 appendix 0.44-1.67 (0.81 ± 0.27) long and 14.2–40.2% (25.2%) of esophageal length. Intestinal
240 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 *cox2* 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

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

287 In multivariate analysis of eight measurements of *C. micropapillatum* and *C. bancrofti* in the
288 present and other studies, the two species were poorly separated (Table 2, Fig. 7). In an ANOSIM
289 test, morphometric variation between sexes and species was statistically significant, although
290 modest in magnitude between sexes within the two species ($R=0.24$, $P=0.019$), and negligible
291 between same-sex individuals of the two species ($R=0.025$, $P=0.017$). These results were
292 essentially the same if data from Hartwich (1964), who included data from non-Australian sources
293 of *C. "bancrofti"*, were excluded from MDS (not shown) or ANOSIM (sex $R=0.253$, $P=0.017$, species
294 $R=0.034$, $P=0.01$).

295
296 *Contracaecum gibsoni* Mattiucci, Paoletti, Solorzano and Nascetti, 2010

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

298 *Site in host:* Ventriculus.

299 *Representative DNA sequences:* ON723780-87 (ITS rDNA), ON736833-37 (*cox2* mtDNA);

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

306 *Males* ($n = 4$): Total length 15.0–44.0 (27 ± 12.3). Nerve ring 0.35–0.41 (0.39 ± 0.035) from anterior
307 end. Oesophagus 2.95–6.65 (4.89) long. Ventricular appendix 0.53–0.70 (0.70) long. Intestinal
308 caecum 2.10–2.28 (2.50) long; intestinal caecum/ventricular appendix length ratio 2.5–4.5 (3.8).
309 Spicules slightly subequal; right spicule 1.82–2.50 (2.11 ± 0.29) long; left spicule 1.75–2.18 ($1.99 \pm$
310 0.19) long; spicule tip pointed. Ratio spicules length/total length (spi/len) 0.04–0.12 (0.07).
311 Precloacal papillae simple, forming 2 subventral lines (Fig. 8d). Five pairs of proximal papillae
312 posterior to cloaca, lateral to paracloacal papillae, four of which pyriform in shape (Fig. 8e-f).
313 Single pair of double paracloacal papillae; 4 pairs of distal papillae. Single pair of small papilla-like
314 phasmids lateral to the distal pair (d4) of post-cloacal papillae (Fig. 8e). Tail 0.21 – 0.24 ($0.23 \pm$
315 0.017) long.

316 *Females* ($n = 16$) Total length 13.0–63.0 (36.9 ± 14.5). Nerve ring 0.32–0.60 (0.49 ± 0.083) from
317 anterior end. Oesophagus 3.20 – 6.79 (5.10 ± 1.22) long. Ventricular appendix 0.85–1.42 ($1.10 \pm$
318 0.16) long. Intestinal caecum 2.38 – 6.36 (4.31 ± 1.24) long, intestinal caecum/ventricular appendix
319 length ratio 2.7 – 4.4 (3.8). Vulva in first third of body, 6.92 – 17.33 (12.90 ± 2.95) from anterior
320 end. Tail conical, 0.21 – 0.60 (0.48 ± 0.09) long (Fig. 9c-d).

321 **Remarks**

322 Overall, measurements of our male and female specimens overlap data reported by Mattiucci et al
323 (2010), although our samples include also smaller and larger specimens. With respect to male
324 features, the average ratio spi/len, spicule shape and pattern of caudal papillae are the same as
325 those reported in the original description of *C. gibsoni* (Mattiucci et al. 2010); such features have
326 been considered as useful taxonomic criteria for distinguishing genetically detected sibling species
327 of *Contracaecum* (Mattiucci et al. 2010). Particularly, as suggested by Shamsi and colleagues
328 (2008), the arrangement and shape of the caudal papillae of males could be useful to differentiate

329 cryptic species of the *C. multipapillatum* complex. In addition to its original description, the
330 present work adds further morphological material providing the first SEM images of *C. gibsoni*
331 male and female adults. Particularly, SEM micrographs of the caudal region of male specimens,
332 showed the presence of five proximal pairs of single papillae of which four pyriform in shape (Fig.
333 8e), a feature here reported for the first time in *C. gibsoni*.

334
335 *Contracaecum quadripapillatum* Saad, Younis & Rabei, 2018

336 *Site in host:* Stomach and oesophagus.

337 *Representative DNA sequences:* ON714979-86 (ITS rDNA), ON736806-07 (*cox2* mtDNA)

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

343 *Males:* ($n = 3$) Total length 21.0–42.4 (20.8 ± 21.2). Nerve ring 0.45–0.63 (0.54 ± 0.09) from
344 anterior end. Oesophagus 3.60–4.99 (4.29 ± 0.069) long. Ventricular appendix 0.60–0.83 ($0.74 \pm$
345 0.12) long. Intestinal caecum 2.06–4.39 (3.22 ± 1.16) long; intestinal caecum/ventricular appendix
346 length ratio 2.5–5.6 (4.5). Spicules subequal, with rounded, spoon-like, flattened tip (Fig. 10c-d).
347 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
348 body length. Post-cloacal papillae consisting in two pairs of single papillae, followed by one pair of
349 double papillae; remaining post-cloacal papillae arranged in three rows, first and second rows
350 containing four papillae on each side forming quadrilateral shape, third row with one papilla on
351 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 ($0.95 \pm$
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 *cox2* 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 *cox2* and 28S rDNA had been

375 sequenced. We resolved this problem by obtaining data from both markers in both species and
376 avoided potential confusion from single-marker results (particularly ITS). While ITS sequences from
377 Israeli and Australian isolates in the *C. bancrofti* + *C. micropapillatum* clade formed reciprocally
378 monophyletic subclades, they had only moderate statistical support and were nearly identical
379 (99.5% similarity). However, the *cox2* of the specimens formed well-supported clades with
380 unambiguous levels of sequence divergence, and gaps unique to *C. bancrofti* in the ITS alignment
381 were not reflected in divergence calculations, or in phylogenetic analysis. We attempted to make
382 further distinctions in sequenced specimens of these two species with both light microscopy and
383 SEM, with particular attention to characters useful in distinguishing anisakids, such as interlabial
384 structure, distribution pattern of male caudal papillae, spicule length and tip shape, size and
385 pattern of caudal papillae (Fagerholm 1989; Mattiucci et al. 2010), as well as quantitative
386 visualization of morphometric variation in *C. micropapillatum* and *C. bancrofti* in the present and
387 past studies. Ultimately, while molecular evidence supports the validity of *C. bancrofti* as
388 described by Shamsi et al (2009), no morphological distinctions were observed that reliably
389 separate this species from *C. micropapillatum*. Morphological variation in Table 2 and Fig 7 may be
390 inflated by variation in preservation methods among studies, or mixed-species infections
391 undetected in non-molecular studies (i.e., those other than Shamsi et al. 2009 and the present
392 work), but even stable characters such as spicule length are notably uninformative. In the future,
393 sequences of *cox2* from regional isolates of *C. micropapillatum* are needed to verify the wide
394 geographic distribution and diverse host range that ITS-based and morphological records imply for
395 this species, which has been reported from all the continents (China, Australia, Croatia, England,
396 Congo, Mexico, USA), as well as in birds from several different families (Li et al. 2016), namely:
397 Anseriformes (Anatidae): *Bucephala clangula* (L.); *Mergus squamatus* Gould; *Spatula clypeata* L.;
398 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 (*SspI*) assay to
423 distinguish *C. quadripapillatum* from *C. gibsoni*, which are not resolved by the *MspI* enzyme of Zhu
424 et al (2007), as well as *cox2* 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 Zaire). 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

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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
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494

495

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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 *cox2* 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 *MspI* (a) and *SspI* (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

678 1941; C=Cram 1927; H=Hartwich 1964. Data point sizes for male worms are proportionate to
679 spicule lengths (see inset legend). Two-dimensional stress=0.16

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

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

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

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

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