Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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

1 **Advancing understanding of the taxonomy and diversity of the genus *Contracaecum* in the great**

2 **white pelican (*Pelecanus onocrotalus*)**

3 Monica Caffara[1]*[#], Perla Tedesco[1#], Nadav Davidovich[2], Sean A. Locke[3], Andrea Gustinelli[1], Roni

4 King[4], Michelle Nuytten[1], Marialuisa Nuzzo[1], Maria Letizia Fioravanti[1]

5 [1] Department of Veterinary Medical Sciences, Alma Mater Studiorum University of Bologna, Via Tolara di

6 Sopra 50, 40064 Ozzano Emilia (BO), Italy

7 [2] Israeli Veterinary Services, Bet Dagan 5025001, Israel

8 [3] Department of Biology, University of Puerto Rico, Box 9000, Mayagüez 00681-9000, Puerto Rico

9 [4] Israel Nature and Parks Authority, 3 Am Ve'Olamo St., Jerusalem, 95463, Israel

10 *Corresponding author: monica.caffara@unibo.it

11 # these authors contributed equally

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 (*cox*2). 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 *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

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 *cox*2 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 *cox*2 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

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

6

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.

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 *Msp*I (Zhu et al. 2007)

153   and *Ssp*I 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 *cox*2 sequenced in 33 of the same specimens. Both ITS rDNA and *cox*2

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

8

173 containing two subclades of sequences from Israel and Australia with moderate support (95%,

174 79%) (Fig. 2). Sequences of *cox*2 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 *cox*2 from two specimens of *C.*

177 *bancrofti* from Australia, newly sequenced herein. In phylogenetic analysis of *cox*2, specimens

178 from Israel and Australia were resolved into two clades with strong (≥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 *Contracaecum* 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 *Contracaecum* sp. 2 (MT477131) from an unknown fish species sampled in

195 Ethiopia, 99.6% similar to *Contracaecum* sp. (MZ727197, Abdallah and Thabit 2021, unpublished)

196 from *Lates niloticus* both from Egypt, and 99.3% similar to *Contracaecum* 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 *cox*2 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*, *cox*2 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 *Msp*I 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 *Ssp*I 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 (*cox*2 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 ¾ 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 *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

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 (*cox*2 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.

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

19

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

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"

22

References

496   **References**

497   Anderson RC (2000) Nematode parasites of vertebrates: their development and transmission,

498   second edition. CABI, Wallingford.

499   Ángeles-Hernández JC, Gómez-de Anda FR, Reyes-Rodríguez NE, Vega-Sánchez V, García-Reyna PB,

500   Campos-Montiel RG, Calderón-Apodaca NL, Salgado-Miranda C, Zepeda-Velázquez AP (2020)

501   Genera and species of the Anisakidae family and their geographical distribution. Animals, 10:

502   2374. https://doi.org/10.3390/ani10122374

503   Baruš V, Sergeeva TP, Sonin MD, Ryzhikov KM (1978) Helminths of fish-eating birds of the

504   Palaearctic Region: Nematoda. In: Rysavy B, Ryzhikov KM (Eds) Helminths of Fish-Eating

505   Birds. Springer, Praha, p 318

506   Blouin MS (2002) Molecular prospecting for cryptic species of nematodes: mitochondrial DNA

507   versus internal transcribed spacer. Int J Parasitol 32:527-531. doi: 10.1016/s0020-

508   7519(01)00357-5.

509   Cram EB (1927) Bird parasites of the nematode suborders Strongylata, Ascaridata, and Spirurata.

510   Bull US Natl Mus. p 492. https://doi.org/10.5479/si.03629236.140.1

511   Crivelli AJ, Leshem Y, Mitchev T, Jerrentrup H (1991) Where do palearctic great white pelicans

512   (*Pelecanus onocrotalus*) presently overwinter? Rev Ecol-Terre Vie 46:145-171

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

514   D'Amelio S, Lombardo F, Pizzarelli A, Bellini I, Cavallero S (2020) Advances in omic studies drive

515   discoveries in the biology of *Anisakid* nematodes. Genes 11:801.

516   https://doi.org/10.3390/genes11070801

517   Davidovich N, Tedesco P, Caffara M, Yasur-Landau D, Gustinelli A, Drabkin V, Minkova E, Aflalo O,

518   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

565 congruence with allozyme data. Parasite 15:408–419.

566 https://doi.org/10.1051/parasite/2008153408

567 Mattiucci S, Sbaraglia GL, Palomba M, Filippi S, Paoletti M, Cipriani P, Nascetti G (2020) Genetic

568 identification and insights into the ecology of *Contracaecum rudolphii* A and *C. rudolphii* B

569 (Nematoda: Anisakidae) from cormorants and fish of aquatic ecosystems of Central Italy.

570 Parasitol Res 119:1243–1257. https://doi.org/10.1007/s00436-020-06658-8

571 McDaniel B, Patterson I (1966) Nematode infestation of a white pelican found along the gulf coast

572 of Texas. Southwest Nat 11:312-312

573 Nadler SA, Hudspeth DSS (2000) Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on

574 three genes and morphology: hypotheses of structural and sequence evolution. J Parasitol

575 86:380-393. https://doi.org/10.1645/0022-3395(2000)086[0380:POTANA]2.0.CO;2

576 Nascetti G, Bullini L, Cianchi R, Paggi L, Orecchia P, Mattiucci S, D'Amelio S, Berland B (1990).

577 Genetic relationships among anisakid species belonging to the genera *Contracaecum* and

578 *Phocascaris*. Bull Soc Fr Parasitol 8:261

579 Otachi EO, Szostakowska B, Jirsa F, Fellner-Frank C (2014) Parasite communities of the elongate

580 tigerfish *Hydrocynus forskahlii* (Cuvier 1819) and redbelly tilapia *Tilapia zillii* (Gervais 1848)

581 from Lake Turkana, Kenya: influence of host sex and size. Acta Parasitol 60:9–20.

582 https://doi.org/10.1515/ap-2015-0002

583 Poulin R (2007) Are there general laws in parasite ecology? Parasitology 134:763–776.

584 https://doi.org/10.1017/S0031182006002150

585 Pronkina NV, Spiridonov SE (2018) Morphological and molecular characterization of anisakid

586 juveniles from the golden grey mullet of the Black Sea. Russ J Nematol 26:87-92.

587 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

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

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

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

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

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

Fig. 7 Non-metric multidimensional scaling of eight morphometric distances in the present and other studies of *C. micropapillatum* (Stossich 1890) and *C. bancrofti* Johnston and Mawson 1941. Points are shape- and color-coded by worm sex and species (see inset legend) and labelled by source, with unlabelled points representing individual worms in the present study. Other studies 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

700