Parasitology International 62 (2013) 309–319

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

# Molecular identification and larval morphological description of *Contracaecum pelagicum* (Nematoda: Anisakidae) from the anchovy *Engraulis anchoita* (Engraulidae) and fish-eating birds from the Argentine North Patagonian Sea

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ARTICLE INFO

*Article history:*
Received 6 October 2011
Received in revised form 18 February 2013
Accepted 1 March 2013
Available online 14 March 2013

*Keywords:*
*Phalacrocorax atriceps*
*Contracaecum pelagicum*
Larval transmission
mtDNA cox2
rrnS
ITS-1
ITS-2

ABSTRACT

Anisakids use invertebrates as paratenic and/or intermediate hosts as a basic feature of larval transmission. The third-stage larva usually develops in invertebrates which are prey items of finfish paratenic hosts. *Contracaecum* larvae molt twice inside the egg and hatch as free third-stage larvae ensheathed in the second-stage larval cuticle. Copepods act as paratenic or obligatory hosts, usually ingesting these free L3 larvae, and fish act as intermediate/paratenic or metaparatenic hosts preying on infected copepods. Fish-eating birds acquire L3 larvae by ingesting infected fish where they develop into the fourth-stage larvae and adults. Objectives of this work were to establish the specific correspondence between *Contracaecum pelagicum* L3 larvae parasitizing the anchovy *Engraulis anchoita*, and the adults parasitizing the Magellanic penguin *Spheniscus magellanicus* and the Imperial shag *Phalacrocorax atriceps* through the use of molecular markers; and, to evaluate the anisakid L3 larval recruitment and infection caused by ingestion of anchovy by *S. magellanicus*. Sixteen specimens of *Contracaecum* L3 larvae were analyzed from *E. anchoita* from Bahía Engaño, Chubut, eight adult nematodes from *S. magellanicus* and six adult specimens from *P. atriceps* both from the Valdés Peninsula, Chubut. All nematodes were sequenced for three genes: mitochondrial cytochrome oxidase 2 (mtDNA *cox2*), mitochondrial ribosomal RNA (*rrnS*), and the internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA region. Phylogenetic analyses were performed by using Maximum Parsimony (MP) analysis by PAUP. In addition, studies under SEM and LM were carried out on L3 larvae. All L3 individuals from *E. anchoita*, adults from *S. magellanicus*, and *P. atriceps* clustered in the same clade, well supported in the MP tree inferred from the mtDNA *cox2*, and *rrnS* gene sequences analyses. Further, the sequence alignments of L3 larvae and adults of *C. pelagicum* here obtained at the ITS-1 and ITS-2 regions of the rDNA matched the sequences of *C. pelagicum* previously deposited by us in GenBank. Nematode recruitment ($R_o$) was equal to 33.07 (7.20–91.14) L3 larvae for *C. pelagicum* in each penguin's meal of anchovy. The MP tree topologies obtained from mtDNA *cox2* and *rrnS* genes demonstrated that specimens of *Contracaecum* L3 larvae from *E. anchoita* and *C. pelagicum* from *S. magellanicus* as well as from *P. atriceps* constitute a unique clade, well-distinct and supported from all the others formed by the *Contracaecum* spp. sequenced so far for these genes. Molecular markers are considered to be an effective tool to elucidate larval transmission. The *Contracaecum* L3 larval recruitment value showed that many worms fail to establish in the bird digestive tract, probably because they are below a critical size. Further work is needed to elucidate other factors (e.g., physiological, immunological) that control nematode populations in the penguin digestive tract.



## 1. Introduction

In most anisakid species, the use of invertebrates as paratenic and/or intermediate hosts is a basic feature of larval transmission. The infective stage (third-stage larva L3) usually develops in invertebrates which are eaten by vertebrate (mainly fish) intermediate/paratenic hosts. The larva generally remains as L3 in the new host but, occasionally, development to the fourth-stage has been observed. The L3 can also increase in size and show genital primordium development — "precocial phenomenon" [1,2,5,6]. This process can occur also in the invertebrate intermediate host when teleost fish are definitive hosts.

*Contracaecum pelagicum* Johnston and Mawson, 1942, probably has a cycle with invertebrate paratenic hosts such as copepods, euphausids and amphipods, and vertebrate intermediate/paratenic hosts such as teleost fish (e.g., *Engraulis anchoita* and *Merluccius hubbsi*). These are

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http://dx.doi.org/10.1016/j.parint.2013.03.001

prey items of the Magellanic penguin *Spheniscus magellanicus* Foster, and the Imperial shag *Phalacrocorax atriceps* King — definitive hosts of *C. pelagicum* (Fig. 1) [3,4].

The *C. pelagicum* life cycle develops as suggested by Moravec [7] who described the transmission of *Contracaecum rudolphii* (=*spiculigerum*) (Hartwich, 1964) through several fish species and the Great Cormorant *Phalacrocorax carbo sinensis* (Blumenbach). Thus, embryonated eggs are expelled with fish-eating bird feces, falling into the sea and developing into the first (L1), second (L2) and third-stage (L3) larvae. The latter hatches ensheathed in the L2 cuticle and attaches itself to the seabed (Fig. 1) [7, Garbin et al., unpublished results]. Unknown copepods would act as invertebrate paratenic hosts and ingest the L3 which escapes from the L2 cuticle within the new host. The L3 does not grow within the invertebrate [7, Garbin et al., unpublished results].

The present work starts with the hypothesis that *E. anchoita* would be the vertebrate intermediate/paratenic (metaparatenic) host acquiring L3 larvae by ingesting these planktonic copepods. The L3 larva increases in size inside the fish which would be the link for piscivorous birds to acquire and develop L4 larvae and adults of this species (Fig. 1) [7, Garbin et al., unpublished results].

The complex life cycles of anisakids make larval transmission experiments difficult. Such work requires handling intermediate and paratenic hosts and the identification of third stage larval species that lack characteristic features such as caudal papillae, lips and spicules. Thus, there are few studies on larval transmission based on experimental infections [8–12]. Mature eggs from *C. rudolphii* parasitizing *Phalacrocorax* spp. were incubated under different laboratory conditions and hatched L2 larvae were used to infect copepods which were then offered as food to several fish species. Then, developed L3 larvae were found encapsulated in host mesenteries [8]. Semenova [10] studied the life cycle of *Contracaecum micropapillatum* (Stossich, 1890) and was able to infect experimentally several copepod species with L3 larvae which then were used to infect frogs and fish. Third stage-larvae migrated to the body cavity of these hosts, encapsulated and showed to be infective to pelicans, in which developed the adult stage. Due to the logistics required to reproduce the entire anisakid life cycle, in addition to the difficulty of identifying larval species, molecular genetic studies could facilitate studies of larval transmission. For example, Shamsi et al. [13] described and characterized genetically different morphotypes of *Contracaecum* larvae using sequence data of internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA, and assigned them to specified *Contracaecum* adults in definitive hosts.

A further question is to ascertain how many anisakid larvae can be acquired by fish-eating birds during feeding. We only know that Magellanic penguin stomach contents average 430 g with a range from 80 to 1183 g. [14]. Penguins can also eat several anchovy individuals at each feeding. But, the number of ingested L3 larvae remains uncertain. As yet, no studies have been done on this.

Therefore, the main aims of this work were: i) to establish larval transmission and the specific correspondence – identification of specimens as the same species – between L3 larvae parasitizing anchovy, and the fourth-stage larvae and adults parasitizing *S. magellanicus* and *P. atriceps*, and ii) to evaluate anisakid L3 larval recruitment in *S. magellanicus* by quantifying the infection caused by a typical meal of anchovy.

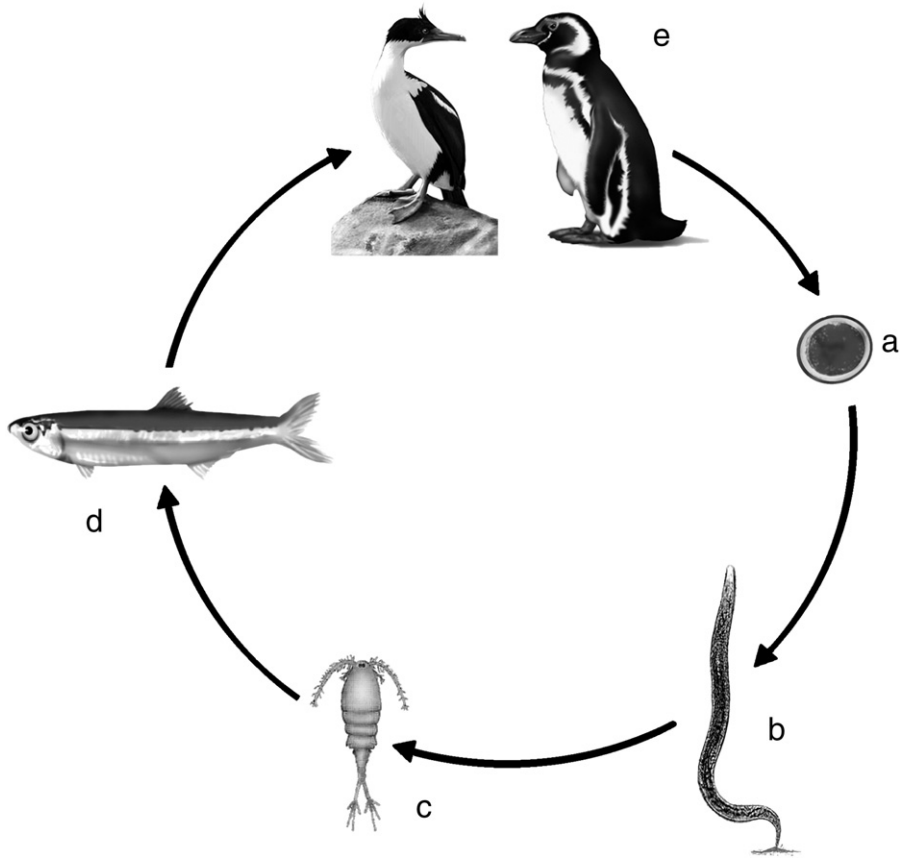


**Fig. 1.** Hypothetical life cycle of *Contracaecum pelagicum*. a) Developing egg(s) containing first, second and third-stage larvae; b) third-stage larvae attached to the substratum and ensheathed in the L2 cuticle; c) crustacean (copepod), first invertebrate paratenic (or obligatory?) host, ingesting later L3 larvae; d) *Engraulis anchoita*, second vertebrate intermediate/paratenic (metaparatenic) host ingesting infected crustacean; e) *Spheniscus magellanicus* and *Phalacrocorax atriceps*, definitive hosts acquiring L3 larvae by eating anchovy.

**Table 1**
*Contracaecum* spp. genetically studied from definitive hosts. N and specimen code = number of specimens sequenced at the mtDNA *cox2* and *rrnS* genes. * = Specimens from D'Amelio et al. (2007).

| Parasite | Host | N and specimen code<br>*cox-2 rrnS* | | Gene Bank accession number | Collection locality |
|---|---|---|---|---|---|
| *C. pelagicum* | *Spheniscus magellanicus* (Spheniscidae) | 4 (CPE1-CPE2-CPE3-CPE4) | 4 (CPE1-CPE2-CPE3-CPE4) | EF122210, EF535568, EF535569 | Peninsula Valdés, Chubut (Argentina) |
| | *Engraulis anchoita* (Engraulidae) | 2 (CPE11-CPE12) | 2 (CPE11-CPE12) | | Bahía Engaño, Chubut waters (Argentina) |
| | *Phalacrocorax atriceps* (Phalacrocoracidae) | 2 (CPE14-CPE15) | 2 (CPE14-CPE15) | | Peninsula Valdés, Chubut (Argentina) |
| *C. australe* | *Phalacrocorax brasilianus* (Phalacrocoracidae) | 4 (CAU1-CAU2-CAU3-CAU4) | 4 (CAU6-CAU7-CAU8-CAU9) | GQ847532, GQ847533, GQ847534, GQ847535, GQ847536, GQ847537, GQ847538; GQ847539, GQ847540, GQ84741, GQ84742, GQ84743, GQ84744 | Laguna Santa Elena, Chile |
| *C. rudolphii* A | *Phalacrocorax carbo sinensis* (Phalacrocoracidae) | 5 (CRA1-CRA2-CRA3-CRA4-CRA5) | 4 (CRA2-CRA4-CRA8-ACOR31*) | EF513501, EF513502, EF513503, EF513505, EF558891, EF122202, EF535570 | Venice lagoon, (north of Italy) |
| *C. rudolphii* B | *Phalacrocorax carbo sinensis* (Phalacrocoracidae) | 2 (CRB1-CRB2) | | EF558894, EF558896, EF513506, EF513507, EF513509, EU852349 | Oristano lagoon, Sardinia Island (Italy) |
| | *Phalacrocorax carbo sinensis* (Phalacrocoracidae) | 1 (CRB4) | 1 (CRB3) | | Tarquinia salt marshes (Latium region, Italy) |
| | *Phalacrocorax carbo sinensis* (Phalacrocoracidae) | 1 (CRB6) | 2 (CRB5-BCOR41*) | | Venice lagoon, (north of Italy) |
| | *Phalacrocorax carbo sinensis* (Phalacrocoracidae) | 1 (CRB10) | 2 (CRB10-CRB11) | | Matsury Lake (Poland) |
| *C. rudolphii* C | *Phalacrocorax auritus* (Phalacrocoracidae) | – | 1 (CCOR52*) | EF014283 | Sarasota Bay, west-central Florida (USA) |
| *C. septentrionale* | *Phalacrocorax carbo carbo* (Phalacrocoracidae) | 2 (CSE3-CSE7) | | EF122205, EF513512, EF513513 | Hϋsavik (Iceland) |
| | *Phalacrocorax aristotelis* (Phalacrocoracidae) | 3 (CSE1-CSE2-CSE6) | 4 (CSE1-CSE2-CSE4-CSE5) | | Karmoy (Norway) |
| *C. chubutensis* | *Phalacrocorax atriceps* (Phalacrocoracidae) | 4 (CCH1-CCH2-CCH3-CCH4) | 4 (CCH1-CCH2-CCH3-CCH4) | HQ328504 | Bahía Bustamante, Chubut (Argentina) |
| *C. microcephalum* | *Phalacrocorax pygmaeus* (Phalacrocoracidae) | 4 (CMP1-CMP2-CMP3-CMP4) | 2 (CMP2-CMP3) | EF122208, EF5135017, EF5135018, EF513519 | Scutari Lake (Montenegro region) |
| *C. micropapillatum* | *Pelecanus onocrotalus* (Pelecanidae) | 6 | (CMI1-CMI2-CMI3-CMI4-CMI5-CMI7) | 3 | |
| (CMI1-CMI2-CMI5) | EF122206, EF122207, EF513514, EF513515, EF513516, EU852350 | Assuan (Egypt) | | | |
| *C. bioccai* | *Pelecanus occidentalis* (Pelecanidae) | 6 (CBO2-CBO3-CBO5-CBO7-CBO8-CBO9) | 3 (CBO2-CBO8-CBO10) | EF513494, EF513495, EF558899, EF513497, EF513498, EF513499 | Totumo marsh (north of Colombia) |
| *C. gibsoni* | *Pelecanus crispus* (Pelecanidae) | 4 (CMG1-CMG2-CMG3-CMG4) | 5 (CMG1-CMG2-CMG3-CMG6-PC1*) | EU852337-EU852342 | Psatatopi (Greece) |
| *C. overstreeti* | *Pelecanus crispus* (Pelecanidae) | 4 (CMO1-CMO2-CMO3-CMO4) | 3 (CMO2-CMO3-CMO5) | EU852343-EU852348 GQ847532, GQ847533 | Psatatopi (Greece) |

## 2. Material and methods

### 2.1. Parasite materials

A total of 202 anchovies were obtained from fishing boats at Rawson's Port, Chubut province, Argentina, in January 2007. Anchovies were caught on the coastal zone of Bahía Engaño (43°32′S, 64°25′W), Chubut, a foraging point for Magellanic penguins. Fish were analyzed for nematodes and 40 of them (20%) were weighed and measured. Some *Contracaecum chubutensis* individuals parasitizing *P. atriceps* from Bahía Bustamante, Chubut province, were also sampled.

### 2.2. Morphometric analyses

A subset of 10 *Contracaecum* third-stage larvae (L3) parasitizing *E. anchoita* from Bahía Engaño were cleared in lactophenol, examined by light microscope and identified to genus level. Another 3 specimens were dried by the critical point method, then observed and photographed using a SEM (Jeol® JSV 6063 LV). Unless otherwise stated measurements are given in millimeters and expressed by the mean followed by the range in parentheses. Morphometric means for *C. pelagicum* L3 larvae of *E. anchoita* from Bahía Engaño were compared with those of *C. pelagicum* fourth-stage larvae (L4) of *S. magellanicus* from Mar del Plata, and *C. pelagicum* of *S. magellanicus* from the Valdés Peninsula already surveyed morphometrically by Garbin et al. [3]. Morphometric means were analyzed with a 1-way ANOVA with significant difference values of $P < 0.05$.

### 2.3. Calculation for recruitment of anisakid larvae

Prevalence (P), mean intensity (MI), and mean abundance (MA) were calculated for all anisakid L3 larvae genera parasitizing the anchovy [15].

The recruitment index (R) for third-stage larvae was proposed:

$$\mathbf{R_o} = \mathbf{MA}\cdot\mathbf{Fi}.$$

where:

MA mean abundance of a nematode genus found in fish
#Fi number of fish ingested by a host

### 2.4. DNA amplification and sequencing

A subset of two *Contracaecum* L3 larvae collected from the fish (*E. anchoita)* from Bahía Engaño (codes: CPE11, CPE12) were first examined under light microscope in ethanol 70%. The 519 bp fragment of the mitochondrial cytochrome oxidase II (mtDNA *cox-2*) gene was analyzed for these two *Contracaecum* L3 larvae, two *C. pelagicum* adult specimens from *P. atriceps* (codes: CPE14, CPE15) and four *C. pelagicum* adult specimens from *S. magellanicus* (codes: CPE1, CPE2, CPE3 and CPE4). Further, a 470 bp fragment of the small subunit of the mitochondrial ribosomal RNA gene (*rrnS*) was analyzed in the same specimens of *Contracaecum* L3 larvae from the fish, four *C. pelagicum* adult specimens from *S. magellanicus* and two *C. pelagicum* adult specimens from *P. atriceps*. Moreover, the larval stages of *Contracaecum* and two *C. pelagicum* adult specimens from *P. atriceps* were sequenced for a 451 bp fragment of the ITS1 and 284 bp of the ITS2 regions.

The total DNA was extracted from 2 mg of tissue from a single nematode using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) or cetyltrithylammonium-bromide protocol (CTAB method) [16].

The mtDNA*cox2* gene from each specimen of *Contracaecum* was amplified according to the procedures as reported by Mattiucci et al. [17] with the primers 211F (5′-TTT TCT AGT TAT ATA GAT TGR TTY AT-3′) and 210R (5′-CAC CAA CTC TTA AAA TTA TC-3′) from Nadler and Hudspeth [18] spanning the mtDNA nucleotide position 10,639–11,248

**Table 2**
Morphometrical data of *Contracaecum* sp. third-stage larvae (L3) parasitizing *E. anchoita* from Mar del Plata, *Contracaecum pelagicum* larvae L3 on *E. anchoita* from Bahía Engaño, and *C. pelagicum* fourth-stage larvae (L4) on *S. magellanicus* from Península Valdés and Mar del Plata.

References: L = length; W = width; DAE = distance from anterior end; (*) Significant difference by 1-way ANOVA between *C. pelagicum* L3 from *E. anchoita* and L4 from *S. magellanicus*.

| Species | *Contracaecum pelagicum* | *Contracaecum* sp. | *Contracaecum* type I | *Contracaecum* type II | *Contracaecum* type III | *Contracaecum pelagicum* | *Contracaecum pelagicum* |
|---|---|---|---|---|---|---|---|
| Larval stage | L3 | L3 | L3 | L3 | L3 | L4 | L4 |
| Type host | *E. anchoita* | *E. anchoita* | *Mugil cephalus* | *Scomber australasicus; Seriola lalandi* | *Platycephalus laevigatus* | *S. magellanicus* | *S. magellanicus* |
| Locality | Bahía Engaño, Chubut sea waters | Mar del Plata, Buenos Aires sea waters | Victoria and Western Australian sea waters | Victoria, South Australian sea waters | Victoria, South Australian sea waters | Península Valdés, Chubut coast | Mar del Plata, Buenos Aires coast |
| References | Present study | Timi et al., 2001 | Shamsi et al., 2011 | Shamsi et al., 2011 | Shamsi et al., 2011 | Garbin et al., 2007 | Garbin et al., 2007 |
| Body L | 4,78 (3,89–5,47) | 5,01 (4,06–5,82) | 27.2 (23.1–34.3) | 4.38 (3.63–5.30) | 2.46 (1.40–3.38) | 6.40 (4.52–7.84) | 5,23 (3,77–6,68) |
| Maximum body W | 0,18 (0,15–0,22) | 0,19 (0,16–0,24) | 1.11 (0.89–1.27) | 0.25 (0.20–0.32) | 0.13 (0.06–0.20) | 0.20 (0.16–0.24) | 0,20 (0,12–0,27) |
| Nerve ring (DAE) | 0,19 (0,13–0,22) | 0,20 (0,16–0,24) | 0.41 (0.36–0.46) | 0.29 (0.10–0.78) | 0.16 (0.09–0.20) | 0.24 (0.08–0.33) | 0,24 (0,17–0,28) |
| Deirids (DAE) | 0,26 (0,22–0,30) | 0,29 (0,24–0,34) | – | – | – | 0.29 (0.21–0.38) | 0,33 (0,22–0,43) |
| Esophagus L | 0,47 (0,38–0,59) | 0,50 (0,40–0,63) | 3.42 (3.09–4.06) | 0.87 (0.46–1.45) | 0.46 (0.24–0.67) | 0.99 (0.74–1.26)* | 1,08 (0,76–1,38)* |
| Intestinal caecum L | 0,18 (0,12–0,30) | 0,26 (0,21–0,34) | 2.83 (2.36–3.49) | 0.43 (0.18–0.75) | 0.25 (0.07e0.41) | 0.56 (0.41–0.70)* | 0,74 (0,50–0,94)* |
| Ventricular appendix L | 0,24 (0,17–0,36) | – | 1.03 (0.89–1.19) | 0.83 (0.60–1.22) | 0.37 (0.23–0.49) | 0.41 (0.30–0.67) | 0,34 (0,22–0,43) |
| Ventriculus + Ventricular appendix L | 0,32 (0,23–0,41) | 0,36 (0,30–0,43) | – | – | – | 0.43 (0.36–0.64) | 0,42 (0,28–0,51) |
| Excretory gland (DAE) | 0,75 (0,63–0,98) | 0,69 (0,39–0,96) | – | – | – | 2.06 (1,40–2,37)* | 1,59 (0,97–2,08)* |
| Tail L | 0,12 (0,10–0,16) | 0,10 (0,09–0,13) | 0.19 (0.13–0.24) | 0.15 (0.10–0.31) | 0.09 (0.07–0.13) | 0.14 (0.12–0.16) | 0,13 (0,10–0,16) |

as defined in *Ascaris suum* (GenBank X54253). For the *cox-2* gene, amplification was carried out in a volume of 50 μl containing 30 pmol of each primer, MgCl2 2.5 mM (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), PCR]buffer 1× (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), DMSO 0.08 mM, NTPs 0.4 mM (Sigma-Aldrich, St. Louis, MO), 5 U of Taq polymerase (Amersham Pharmacia Biotech. Inc.,

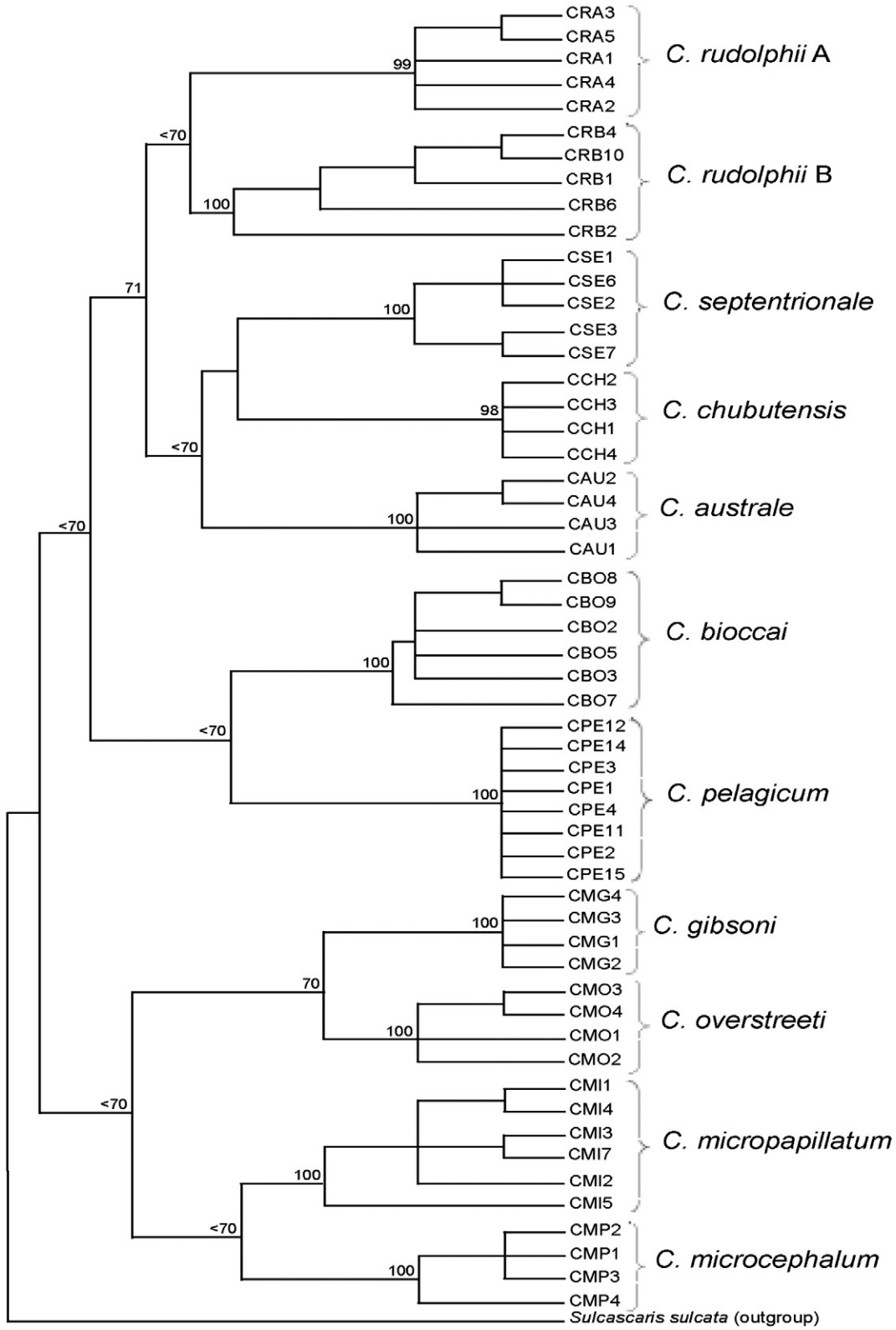


**Fig. 2.** Maximum Parsimony (MP) bootstrap consensus tree performed by PAUP* (Swofford, 2003), on 1000 replicates inferred from the mtDNA *cox-2* (519 bp) sequence analysis of larvae and adults of *C. pelagicum* with respect to sequences of other *Contracaecum* spp. previously characterized genetically on the basis of the same gene and deposited in GenBank (Garbin et al., 2011). 172 polymorphic sites and 157 parsimony informative sites. Bootstrap values are reported at the nodes (values ≥70 were considered well supported). *Sulcascaris sulcata* was used as outgroup.

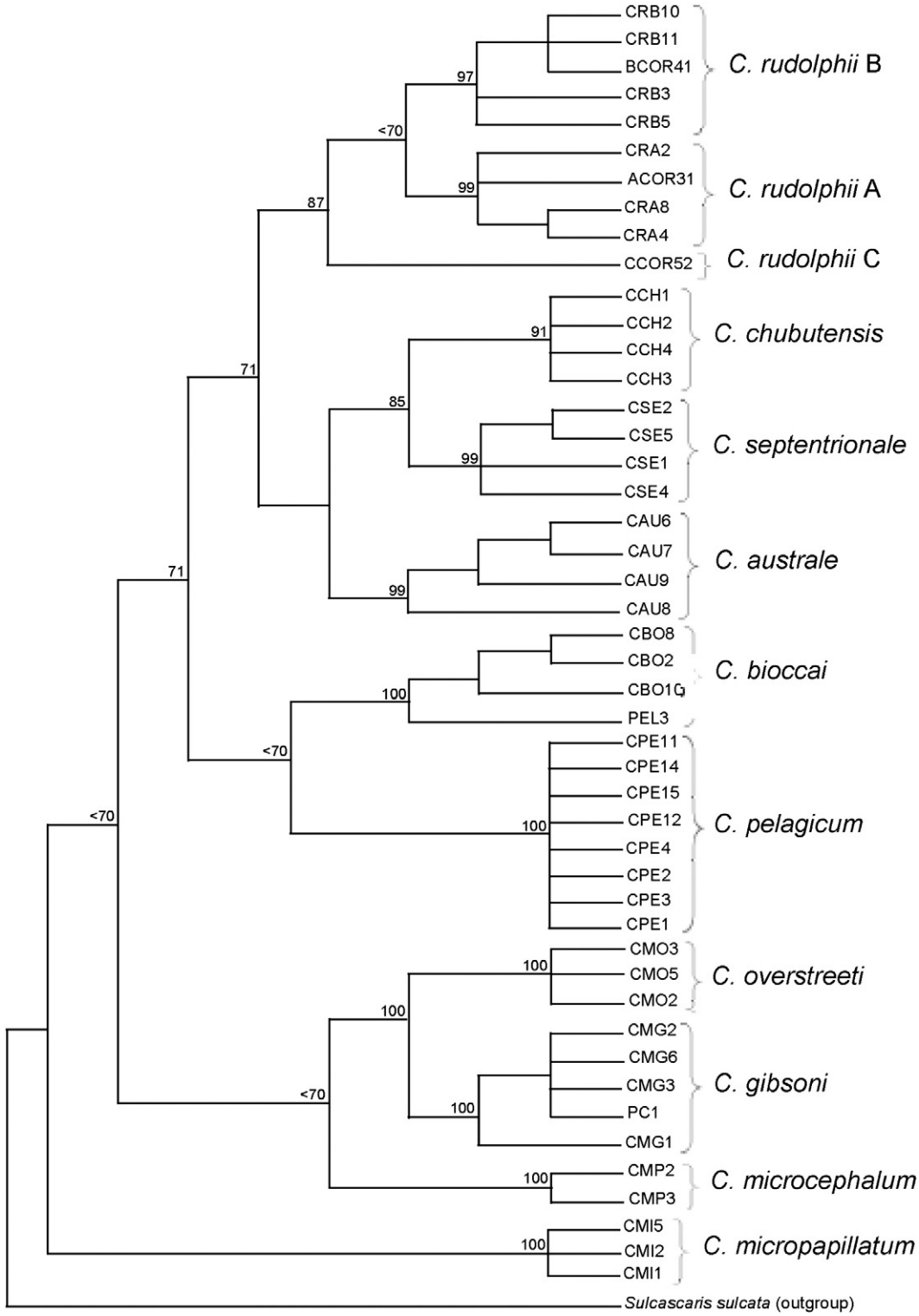


**Fig. 3.** Condensed Maximum Parsimony (MP) consensus tree performed by PAUP* (Swofford, 2003), inferred from *rrnS* sequences analysis (470 bp) of larvae and adults of *C. pelagicum* with respect to sequences of other *Contracaecum* spp. previously characterized genetically on the basis of the same gene and deposited in GenBank (Garbin et al., 2011). 148 polymorphic sites and 139 parsimony informative sites. Bootstrap values are reported at the nodes (values ≥ 70 were considered well supported). *Sulcascaris sulcata* was used as the outgroup.

Piscataway, NJ) and 10 ng of total DNA. The mixture was denatured at 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1.5 min, followed by post-amplification at 72 °C for 10 min.

The amplification of the small subunit of the mitochondrial ribosomal gene, *rrnS*, was performed according to the procedures reported in D'Amelio et al. [19] with the primers MH3 (5′-TTGTTCCAGAATAA TCGGCTAGACTT-3′) and MH4.5 (5′-TCTACTTTACTACAACTTACTCC-3′). The *rrnS* amplification was performed using 50 μl reaction mixture containing 0.5 μl AmpliTaq Gold (AmpliTaq Gold, Applied Biosystems, USA), 5 μl of 10 × PCR buffer II (Applied Biosystems), 5 μl of MgCl2 (3 mM, Applied Biosystems), 4 μl of dNTPs (dCTP, dGTP, dATP, dTTP) (Promega), 0.5 μl each primer (50 pmol/μl) and 20 ng of template DNA.

The PCR conditions were as follows: 10 min at 95 °C (initial denaturation), 35 cycles of 30 s at 95 °C (denaturation) 30 s at 55 °C (annealing), 30 s at 72 °C (extension), and a final elongation step of 7 min at 72 °C.

The amplification of the ITS-1 region was carried out with the primers SS1 (5′-GTTTCCGTAGGTGAACCTGCG-3′) and NC13R (5′-GCTGCGTTCT TCATCGAT-3′) and the ITS-2 region with the primers SS2 (5′-TTGC AGACACATTGAGCACT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′), according to the procedure reported in Shamsi et al. [20,21]. The PCR (in 50 μl of final volume) was performed in 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM MgCl2, 250 μM each of dNTP, 50 pmol of each primer, 1 U Taq polymerase (Promega) and 10 ng of template DNA, using the following conditions: 94 °C for 5 min (initial denaturation), 30 cycles at 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 30 s (extension), and a final extension at 72 °C for 5 min.

All the PCR products were examined on a 1% agarose gel, stained with 1.5 μl of Gel-Red (Biotium), analyzed using a gel documentation system (KODAK Gel Logic 100 Imaging System) and purified using Wizard® SV Gel and PCR Clean-Up System (Promega). DNA sequencing was performed by Macrogen Inc. (Seoul, Korea) using the couple of primers for each gene analyzed.

Reference specimens and isolated DNA samples are stored at the Department of Public Health and Infectious Diseases, of the "Sapienza — University of Rome", Rome, Italy. The sequences of the mtDNA *cox2* (519 bp) obtained from the specimens of *Contracaecum* from *E. anchoita*, *S. magellanicus* and *P. atriceps* were compared with those already obtained in our previous studies (Table 1) [17,22]. Number, code, and sampling site of *Contracaecum* spp. genetically studied (mtDNA *cox2* and *rrnS* genes) from definitive hosts are reported in Table 1.

The sequences (470 bp) of the mitochondrial *rrnS* region of the ribosomal DNA obtained for the specimens of *Contracaecum* L3 larvae from *E. anchoita* and adults from *S. magellanicus* and *P. atriceps* were compared with those already obtained for other *Contracaecum* spp. here considered and with respect to *C. rudolphii* C from *Phalacrocorax auritus* and *Contracaecum septentrionale* from *Phalacrocorax carbo carbo* [19] deposited in GenBank (Table 1).

Finally, the sequences obtained in the same larval and adult specimens of *C. pelagicum* at the ITS-1 (451 bp) and ITS-2 (284 bp) regions of the rDNA were compared with those previously obtained for the same gene for *C. rudolphii* D and *C. rudolphii* E from *P. carbo* and *Phalacrocorax varius* respectively, from the austral hemisphere by Shamsi et al. [21] and deposited in GenBank.

### 2.5. Sequence analysis

The ITS-1 and ITS-2 sequences obtained were aligned using Clustal X [24] and BioEdit [25] software program. Phylogenetic scrutiny was performed using Maximum Parsimony (MP) analysis, by PAUP [26] for mtDNA *cox2* and *rrnS* datasets. The reliabilities of the phylogenetic relationships and specific correspondence between *Contracaecum* larvae and adults were evaluated using nonparametric bootstrap analysis [27,28] for the MP tree. Bootstrap values ≥70 were considered well supported [29,30]. The sequences of *Contracaecum* L3 larvae from *E. anchoita*

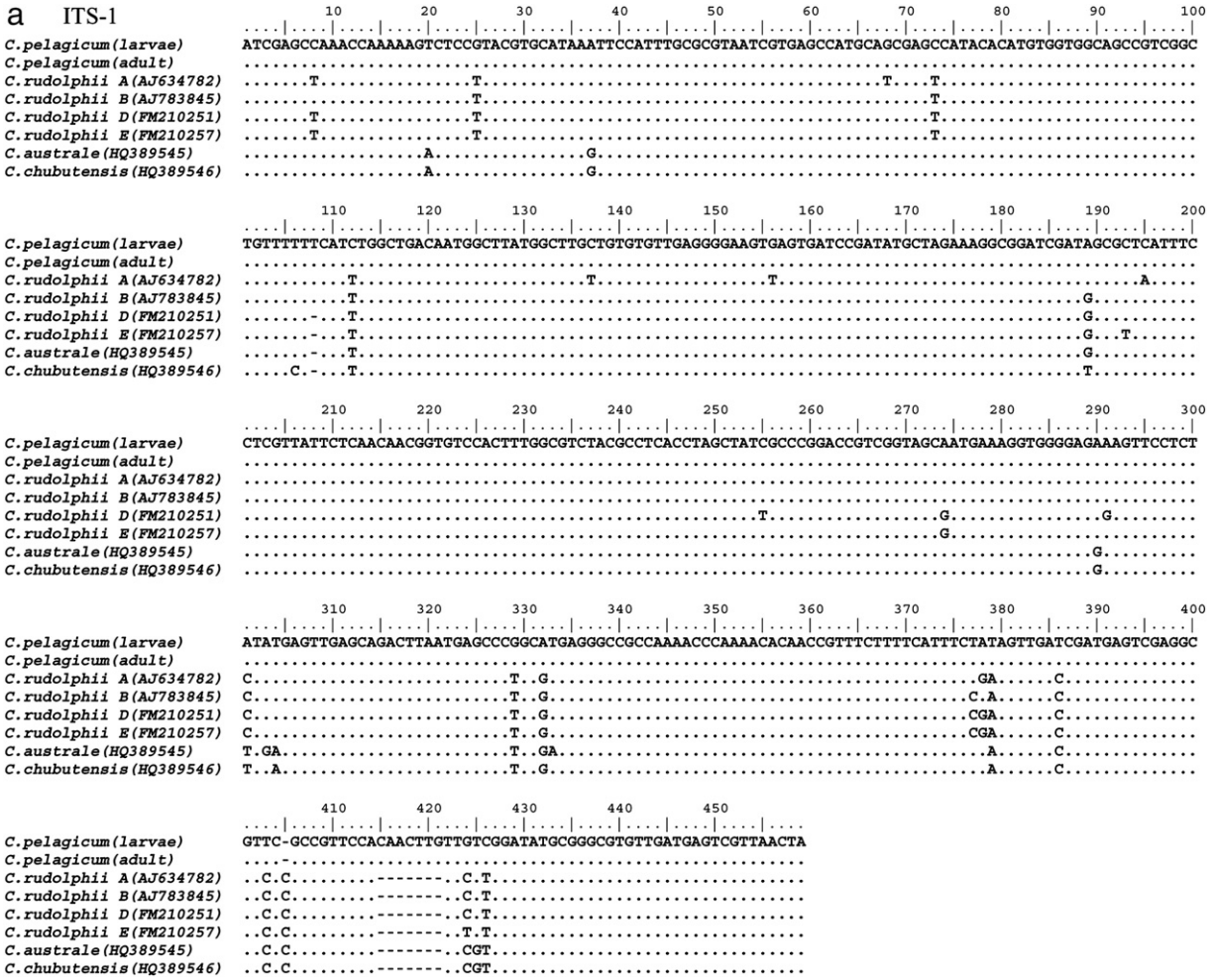


**Fig. 4.** Alignment sequences of nuclear ribosomal DNA. a) ITS-1 (451 bp), and b) ITS-2 (284 bp) regions of larvae and adults of *C. pelagicum* sequences respectively from *A. anchoita* and *P. atriceps* respectively, in comparison with *C. rudolphii* A, B, D and E as reported in Shamsi et al. (2009). The alignment was performed using BioEdit (Hall, 1999). Dots indicate identities and dashes indicate gaps.

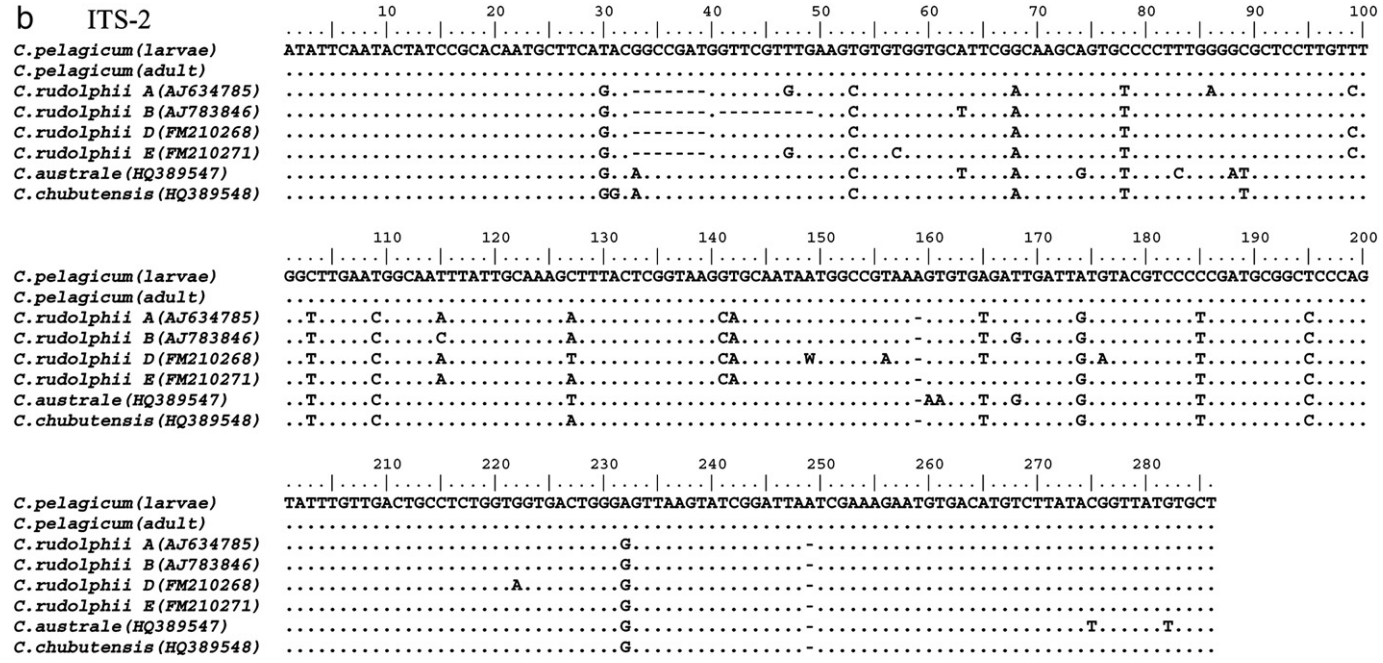


**Fig. 4b.** (*continued*).

and adult specimens from *S. magellanicus* and *P. atriceps* from Argentina were compared with those already obtained for mtDNA *cox2* for *Contracaecum* spp. from water birds in our previous studies [17,31] and deposited in GenBank (Table 1). Further, for a genetic comparison with other *Contracaecum* so far described from other fish-eating species of *Phalacrocorax*, the sequences obtained for the mitochondrial gene *rrnS* in the present study were compared with those available for 1 specimen of *C. rudolphii* C deposited in GenBank (Table 1). Finally, the sequences obtained for the ITS-1 and ITS-2 regions of the rDNA were compared with those of *C. rudolphii* D and *C. rudolphii* E (GenBank, accession numbers: FM210262 and FM210270). In addition, the specimens characterized for ITS-1 and ITS-2 from *C. rudolphii* A and *C. rudolphii* B [22] were included in the comparison. Both for mtDNA *cox-2* and *rrnS* genes, *Sulcascaris sulcata* from *Caretta caretta* from the Mediterranean Sea was included as outgroup to root the phylogenetic trees (GenBank accession numbers: HQ328505, for mtDNA *cox2* and EF180080 for *rrnS*).

## 3. Results

Only *Contracaecum* sp. third stage larvae (L3) were found in *E. anchoita* specimens: no *Contracaecum* sp. fourth stage larvae (L4) were seen. These L3 Larvae were studied for molecular characterization and described morphologically.

### 3.1. Description

**Contracaecum pelagicum** Johnston & Mawson, 1942

*Third stage larvae* (L3)

*Description (10 specimens from *E. anchoita* from Bahía Engaño, Argentinean Sea, Table 2, Fig. 5 a–g).

Body cuticle transversally striated (Fig. 5a–d), vestigial cephalic collar (Fig. 5a, b, d). Non-developed lips with tiny vestigial papillae. Conspicuous boring tooth (Fig. 5a, b). Non-visible amphids and interlabia. Ventral excretory pore (Fig. 5a, b). Inconspicuous deirids (Fig. 5d). Small ventricle. Intestinal caecum placed dorsally to esophagus, shorter than ventricular appendix (Fig. 5e, f). Conical tail without mucron. Non-visible phasmids (Fig. 5c, g), and genital primordia (Fig. 5e).

### 3.2. Recruitment of anisakid L3 larvae

Ecological parameters for *C. pelagicum* L3 parasitizing *E. anchoita* were: P = 52.5%, MI = 2.81 and MA = 1.47. Mean anchovy weight was 19.08 g (12.89–29.45 g). A penguin might ingest an average of 22.5 anchovies with a minimum of 4.9 and a maximum of 62 individuals daily. In this way, nematode recruitment ($R_o$) was equal to 33.07 (7.20–91.14) L3 larvae for *C. pelagicum* in each meal of anchovy.

### 3.3. Molecular/genetic identification of larval and adult stages of C. pelagicum

The specimens of *Contracaecum* L3 larvae from *E. anchoita* matched 99% the previously reported gene sequences for the mtDNA*cox2* in *C. pelagicum* from *S. magellanicus* examined previously and deposited in GenBank (codes EF122210, EF535568, EF535569) [20], also reported in the present paper. All the larval individuals from the fish *E. anchoita* (CPE14 and CPE15), the adults from *S. magellanicus* (CPE1-CPE4), and *P. atriceps* (CPE11 and CPE12) clustered in the same clade, very well supported in the MP tree (Fig. 2) inferred from the mtDNA *cox-2* sequences analysis. The sequences of the mtDNA *cox2* of larval *C. pelagicum* are deposited in GenBank under the accession number NJ580992 and NJ580993.

Similarly, the larval specimens of *Contracaecum*, the adults from *S. magellanicus*, and those from *P. atriceps* clustered in the same clade, very well supported in the MP tree inferred from the *rrnS* sequence analysis (Fig. 3). The sequences of the *rrnS* gene in larval *C. pelagicum* were deposited in GenBank under the accession number NJ580990 and NJ580991.

On the other hand, in these two trees the clade formed by the adults and larval specimens of *C. pelagicum* respectively from *S. magellanicus* and *P. atriceps* and *E. anchoita* was quite distinct from clades of all so far genetically characterized species of *Contracaecum* maturing in fish-eating birds (Figs. 2 and 3).

Further, the sequence alignments (Fig. 4a and b) of larval and adults of *C. pelagicum* here obtained at the ITS-1 and ITS-2 regions of the rDNA, were found to be distinct from those so far obtained in other *Contracaecum* spp. genetically characterized on the basis of the same locus [20,22].

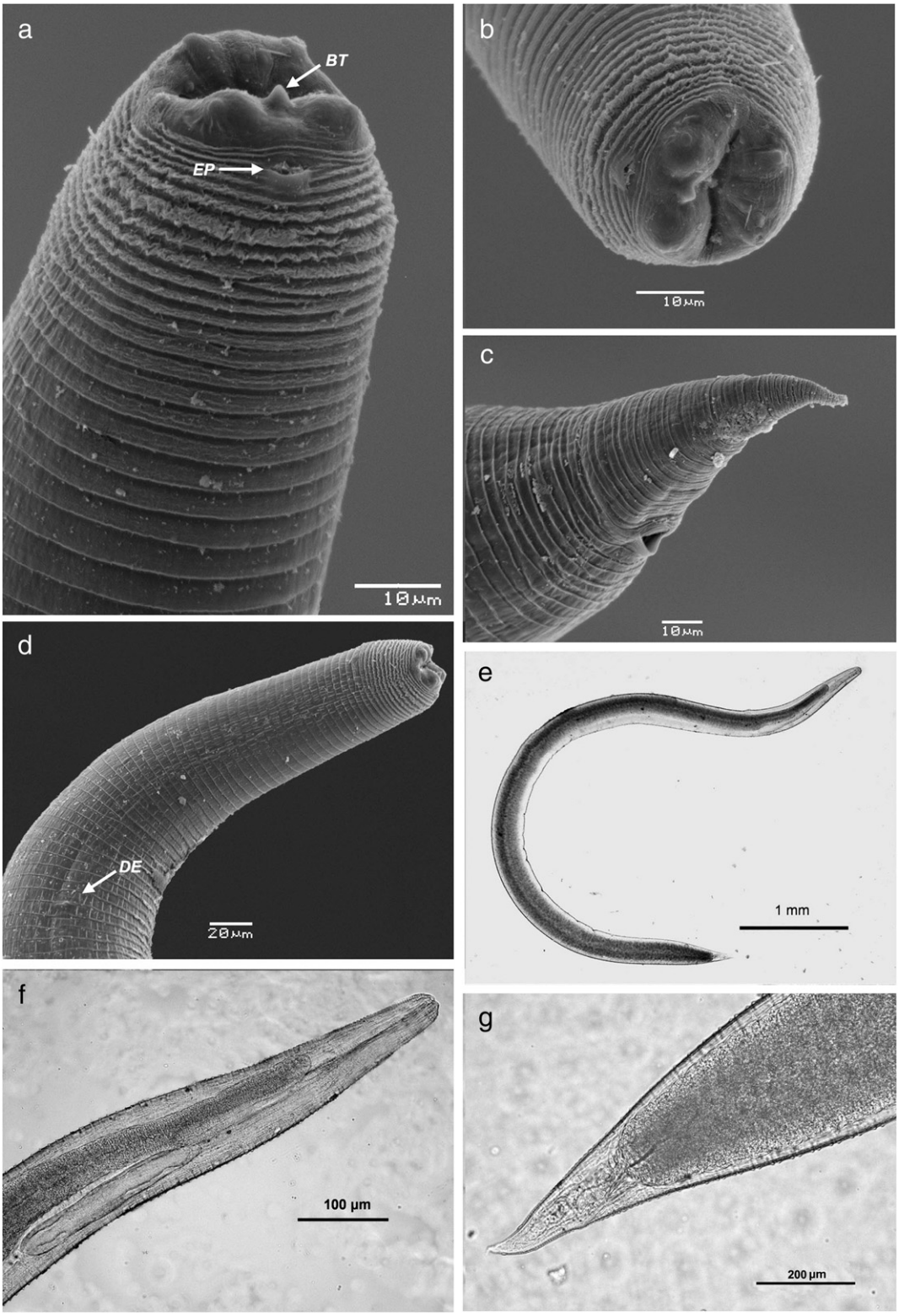


**Fig. 5.** *Contracaecum pelagicum* third-stage larvae (L3) under scanning electron microscope (SEM) and light microscope (LM). a) Anterior end lateral view showing the boring tooth (BT) and excretory pore (EP); b) anterior end sagittal view; c) Posterior end lateral view showing the cloaca; d) anterior end lateral view showing a deirid (DE); e) entire larva; f) anterior end showing esophagus, intestinal caecum, ventricle, and ventricular appendix; g) posterior end.

## 4. Discussion

The sequence analysis of multiple loci (i.e. mtDNA cox2, *rrnS* genes, and rDNA ITS-1 and ITS-2 regions) corroborate the evidence that L3 larvae parasitizing the anchovy *E. anchoita* belong to the species *C. pelagicum*, a parasite at the adult stage of the Magellanic penguin *S. magellanicus* and the Imperial shag *P. atriceps*.

The MP tree topologies obtained from the sequence analyses of the mtDNA *cox2* and *rrnS* demonstrated that the specimens of *Contracaecum* L3 larvae from *E. anchoita* and *C. pelagicum* from *S. magellanicus* as well as

from *P. atriceps* constitute a unique clade well-supported and distinct from all the others formed by *Contracaecum* species sequenced so far for these genes. Tree topologies obtained are also congruent in showing that *C. pelagicum* is closely related to the species *Contracaecum bioccai*.

Few morphological differences were observed between the *Contracaecum* sp. L3 larvae in *E. anchoita* from the marine littoral of the Valdés Peninsula compared with those *Contracaecum* sp. L3 described by Timi et al. [32] from the coast of Mar del Plata. However, L3 from both sampling sites showed differences with respect to *C. pelagicum* L4 found in *S. magellanicus* from the Valdés Peninsula and Mar del Plata [3]. The esophagus is twice as large, the intestinal caecum is longer, and the excretory gland lies further back in L4 larvae specimens in penguins from sampling sites. These differences would correspond to metamorphic changes that L3 larvae suffer when molting to L4 larvae in the definitive host. Several authors, who studied the life cycle of other *Contracaecum* species, concur [8,11,33]. Since it has a wide geographic range, *Contracaecum* sp. L3 larvae found in anchovies from Mar del Plata sea waters by Timi et al. [32] probably also belong to the species *C. pelagicum*.

Some morphological resemblances and differences in these larvae were found with respect to those L3 larvae types described by Cannon [34] and Shamsi [13]. Present study L3 specimens (PSL3E) are at least five times shorter than those *Contracaecum* type I L3 larvae found in *Mugil cephalus* by Cannon, and *Mugil dussumieri*, *Mugil strongylocephalus* and *M. cephalus* in Australian sea waters found by Shamsi. Specimens from the present study also are twice as big as *Contracaecum* type III L3 larvae found by Shamsi [11] in *Platycephalus laevigatus*, even though, PSL3E seem to be similar according with *Contracaecum* type III illustrations [13]. On the other hand, PES keep a great similarity with those *Contracaecum* type II L3 larvae found by Cannon in *Apogon fasciata*, *Platycephalus arenarius*, *Pseudorhombus arsius*, and *Pseudorhombus jenynsii* in Australian waters. However, the ventricular appendix and intestinal caecum are twice as big in the latter specimens, and the intestinal caecum/ventricular appendix ratio (IC/VA) is 0.5 whereas in the PSL3E it is 0.75 [34]. It is the same with those *Contracaecum* type II L3 larvae found by Shamsi [13] in *Scomber australasicus* and *Seriola lalandi*, the IC/VA is 0.48. In addition, *Contracaecum* type II was identified genetically as *Contracaecum ogmorhini sensu stricto* according to the internal transcribed spacers ITS-1 and ITS-2 [13]. So that, these latter fishes might be prey items of pinnipeds from Australian waters since *C. ogmorhini* is a regular parasite of sea mammals.

Although the specific correspondence established here between *C. pelagicum* L3 larvae and its adults represents an important advance in terms of parasite transmission in the Anisakidae, other intermediate/paratenic hosts should also be studied. Several surveys of nematode transmission identify copepods as the main transmitters of *Contracaecum* L3 larvae, with euphausids and amphipods playing a lesser role [2,7,8,11]; all these invertebrates are prey items of *E. anchoita* [35]. However, this hypothesis might be corroborated by further work.

On the other hand, the hake *Merluccius hubbsi* might be a second intermediate/paratenic host of *C. pelagicum* L3 larvae since some authors have reported *Contracaecum* sp. L3 larvae in this fish [35–37]. The hake feeds on the anchovy and hake is in the diet of the Magellanic penguin and the Imperial shag in the same marine coastal area [31,38]. This could be corroborated by using molecular markers.

In addition, *P. atriceps* is found to be parasitized by *C. chubutensis* in more southerly nesting colonies from Bahia Bustamante, Chubut, Argentina [38,39]. For this reason, new collections of *Contracaecum* nematodes in the shag's prey items from this site and analysis with molecular markers should be carried out to identify which prey item might be transmitting *C. chubutensis* L3 larvae to the Imperial shag.

Results of *Contracaecum* L3 larval recruitment showed that only a few worms ingested with the fish become established in the bird digestive tract contrasting that only an average of 33 larvae per day is ingested with a meal of anchovy and the ecological parameters on *C. pelagicum* L4 larvae (P = 100%, MI = 283, and MA = 283), and adults (P = 40%, MI = 149 and MA = 60) in *S. magellanicus* penguins from the Valdés Peninsula (n = 20) [3]. Therefore, some factors could limit nematode numbers counted in the bird such as fish allocation to nestlings, inmunological barriers, sampling season and time, and other physiological ones. Some authors assert that L3 anisakid larvae must attain a certain body size to be established in the host digestive tract. If shorter, they pass out into the intestine and are digested [40,41]. Studies are required to reveal what other factors control worm burdens in the penguin digestive tract.

The present study demonstrates the value of combining morphology and molecular tools in the identification of larval stages of anisakid nematodes, especially those occurring in fish and maturing in fish-eating birds. Application of these methods will contribute to the elucidation of anisakid life cycles. Previous studies have used molecular tools to identity larval nematodes of the genus *Contracaecum* maturing in waterbirds [42,21]. In addition, this approach has been successfully used recently to discover and describe siblings and new taxa of *Contracaecum* maturing in fish-eating birds as well as to identify their larval stages [18,21–23,32,42,43]. Therefore, the application of genetic/molecular tools is of particular importance in acquiring information about the life cycle of these nematodes.

## Conflict of interest statement

There is no conflict of interest.

## Acknowledgments

This research was funded by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 309), the Universidad Nacional de La Plata (N628), and partially by the Italian Ministry of Education, MIUR-PRIN 2008. We would like to thank Lic. Sonia Laurenti for her help with anchovy sampling. Authors are very grateful to Dr. Steve Webb for reading the manuscript and correcting the English language.

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