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Genetic and Morphological Evidences for the Existence of a New Species of Contracaecum (Nematoda: Anisakidae) Parasite of Phalacrocorax brasilianus (Gmelin) From Chile and Its Genetic Relationships with Congeners From Fish-Eating Birds

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GENETIC AND MORPHOLOGICAL EVIDENCES FOR THE EXISTENCE OF A NEW SPECIES OF *CONTRACAECUM* (NEMATODA: ANISAKIDAE) PARASITE OF *PHALACROCORAX BRASILIANUS* (GMELIN) FROM CHILE AND ITS GENETIC RELATIONSHIPS WITH CONGENERS FROM FISH-EATING BIRDS

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ABSTRACT: *Contracaecum australe* n. sp. is described from the Neotropic cormorant *Phalacrocorax brasilianus* in Chile based on morphology and the sequence analyses of multiple loci, i.e., mitochondrial cytochrome oxidase 2, mtDNA *cox-2*, the small subunit of the mitochondrial ribosomal RNA gene, *rrnS*, and the ITS-1 and ITS-2 regions of nuclear ribosomal DNA. Moreover, sequence analysis of the same genes was carried out on the morphospecies *Contracaecum chubutensis* Garbin et al. (2008) from *Phalacrocorax atriceps*. Further, genetic relationships are presented between *C. australe* n. sp. and *C. chubutensis* with respect to the related congeners from fish-eating birds previously characterized genetically on the same genetic markers, i.e., *Contracaecum rudolphii* A, B, C, D, and E, *Contracaecum septentrionale*, *Contracaecum microcephalum*, *Contracaecum bioccai*, *Contracaecum pelagicum*, *Contracaecum micropapillatum*, *Contracaecum gibsoni*, and *Contracaecum overstreeti*. Several phylogenetic analyses (MP, NJ, and BI) inferred from mitochondrial genes (*cox-2*, *rrnS*) were congruent in depicting *C. australe* n. sp. and *C. chubutensis* as forming distinct clades, highly supported, from the remainder of the *Contracaecum* taxa considered; thus, it validates their specific status. Further, analyses of the ITS-1 and ITS-2 sequence data of *C. australe* n. sp. and *C. chubutensis* supported their distinction with respect to the 2 sibling species, *C. rudolphii* D and *C. rudolphii* E, previously detected from Phalacrocoracidae of Australia. Morphological analysis and the differential diagnosis of male specimens of *C. australe* n. sp. enabled the detection of differences in a number of characters, including spicule length, peculiar shape of male tail, paracloacal papillae disposition, and shape and bifurcation depth of interlabia. According to the genetic and morphological results obtained, the erection of a new taxon from fish-eating birds of the Austral region is given and its formal description is presented. Phylogenetic trees support both *C. australe* n. sp. and *C. chubutensis* as being included in the same clade with the previously detected species from cormorants, i.e., *C. rudolphii* A, B, C, and *C. septentrionale*. The finding of *C. australe* n. sp. and *C. chubutensis* parasites of *Ph. brasilianus* and *Ph. atriceps*, respectively, appears to support a host–parasite association between the *C. rudolphii* A, B, and C, *C. septentrionale*, *C. chubutensis*, and *C. australe* n. sp. and different species of cormorants belonging to *Phalacrocorax*.

Species of *Contracaecum* Railliet and Henry, 1912 are parasites of aquatic organisms in freshwater, brackish, and marine ecosystems. Definitive hosts are usually piscivorous birds and pinnipeds (Anderson, 2000; Mattiucci et al., 2008; Mattiucci and Nascetti, 2008). Among the fish-eating birds, various species of cormorants (Phalacrocoracidae) from all over the world have been reported as definitive hosts of these nematodes (Anderson, 2000; Mattiucci et al., 2008). The Neotropical cormorant, *Phalacrocorax brasilianus* (Gmelin, 1789) (Pelecaniformes: Phalacrocoracidae) lives in both freshwater and marine environments (Harrison, 1985) and is widely distributed from southern South America, i.e., Argentina and Chile, to Texas, North America (Morrison et al., 1979; Araya and Millie, 1991; Telfair and Morrison, 1995). Cormorant chicks (*Phalacrocorax* spp.) may be seriously affected by diseases of parasitic origin, mainly due to the habit of food regurgitation from parents to their chicks (Kuiken et al., 1999).

There are few records of *Contracaecum* spp. parasitizing cormorants in South America. *Contracaecum travassosi* Gutiérrez, 1943, was originally described as a parasite of *Phalacrocorax atriceps albiventer* Lesson from the Península Valdés, Argentinean Sea coast (Gutiérrez, 1943), and later it was found in the proventriculus of *Ph. brasilianus* off the Uruguayan coast (Lent and Freitas, 1948). Malacalza et al. (1998) reported *Contracaecum* sp. in regurgitated pellets of *Ph. a. albiventer* from the Chubut coast, Argentina. Garbin et al. (2008) described *Contracaecum chubutensis* parasitizing *Ph. atriceps* King in the same area. In addition, *Contracaecum pelagicum* was found in 2 marine birds, *Spheniscus magellanicus* Forster and *Thalassarche melanophris* Temminck (Diomedeidae) (Garbin et al., 2007). Recently, *C. pelagicum* was recorded in *Ph. atriceps* on the Punta León coast, Chubut, Argentina (Garbin, 2009).

The genetic characterization of the latter species has been recently provided in comparison with other species of the genus' parasites of aquatic birds (Mattiucci et al., 2008). The species of *Contracaecum* reported in *Ph. brasilianus* to date include *Contracaecum caballeroi* Bravo Hollis, 1939, described in *Ph. brasilianus* from off the Uruguayan sea coast (Lent and Freitas, 1948). Vicente et al. (1996) provided a concise description of 4 specimens of *Contracaecum spiculigerum* (= *C. rudolphii*) collected from *Ph. brasilianus* and *Anhinga anhinga* (Linnaeus, 1758) from Mato Grosso and Rio de Janeiro, Brazil. Specimens of *Contracaecum rudolphii* (s. l.) and larval stages of *Anisakis* and *Pseudoterranova* species were found in the proventriculus of *Ph. brasilianus*, with species of *Anisakis* being the most abundant (Torres et al., 2000, 2005). Amato et al. (2006) redescribed *C. rudolphii* from *Ph. brasilianus* occurring in Rio Grande do Sul, southern Brazil.

Genetic data inferred from allozymes (Bullini et al., 1986; Mattiucci et al., 2002, 2008, 2010), the direct sequencing of mtDNA *cox-2* gene (Mattiucci et al., 2008, 2010), the SSCP analysis of the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS of the ribosomal DNA (rDNA) (Li et al., 2005), and the PCR-based RFLP analysis of the same gene

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(D'Amelio et al., 2007; Zhu et al., 2007) were used to identify 2 sibling species of the *C. rudolphii* s. l. complex, referred to as *C. rudolphii* A and B. Genetic evidence based on the small subunit of the mitochondrial ribosomal RNA gene (*rrnS*), by PCR-based RFLP analysis of the same gene and of the internal transcribed spacers (ITS) of nuclear ribosomal DNA (D'Amelio et al., 2007), permitted the detection of a further sibling species of the *C. rudolphii* complex, which was designated as *C. rudolphii* C from *Phalacrocorax auritus* Lesson, in Florida. More recently, another 2 siblings, *C. rudolphii* D and *C. rudolphii* E from *Phalacrocorax carbo* and *Phalacrocorax various* (Gmelin) in Australia (Shamsi et al., 2009a, 2009b) were genetically characterized using sequence analysis of the ITS-1 and ITS-2 regions of rDNA; their morphological descriptions were also provided (Shamsi et al., 2009a, 2009b).

Combining different genetic–molecular and morphological evidence, it was also possible to discover and describe new taxa of *Contracaecum* as parasites of aquatic birds, i.e., *Contracaecum bioccai* Mattiucci, Paoletti, Olivero-Verbel, Baldiris, Arroyo-Salgado, Garbin, Navone, and Nascetti, 2008 from *Pelecanus occidentalis* (L.) in Colombia and *Contracaecum pyripapillatum* Shamsi, Gasser, Beveridge, and Shabani, 2008 from *Pelecanus conspicillatus* (Temminck) in Australia. *Contracaecum gibsoni* Mattiucci, Paoletti, Consuegra-Solorzano, and Nascetti, 2010 and *Contracaecum overstreeti* Mattiucci, Paoletti, Consuegra-Solorzano, and Nascetti, 2010 co-infected *Pelecanus crispus* (L.) in Greece (see Mattiucci et al., 2010).

In the present paper, we analyzed morphological and molecular data inferred from the sequence analysis of the mitochondrial cytochrome oxidase 2 gene (mtDNA *cox-2*), the mitochondrial ribosomal RNA gene (*rrnS*), and the internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2 regions). The effort was designed to: (1) demonstrate the presence–absence of a new *Contracaecum* taxon in the Neotropical cormorant *Ph. brasilianus* (Gmelin) off the Chile coast; (2) genetically characterize the *C. chubutensis* thus far only morphologically described (Garbin et al., 2008); and (3) compare the genetic relationships of several *Contracaecum* species parasitizing cormorants and other fish-eating birds from different regions of the world.

MATERIALS AND METHODS

Parasite material

Four dead *Ph. brasilianus* from a breeding colony of the Santa Elena lagoon, VIII Region, Chile (37°15′S, 72°28′W) were necropsied during 2006–2008. Specimens of *C. chubutensis* were collected from *Ph. atriceps* at Bahia Bustamante, Chubut Province, Argentina (45°11′S, 66°30′W). During necropsy, their entire digestive tracts were removed and frozen at −20 C until they could be examined. After thawing, their contents were washed with water on a sieve with a mesh of 0.25 mm and the sediment was placed in Petri dishes. Isolated nematodes were fixed and stored in 70% ethanol until morphological and genetic analyses could be undertaken.

Morphological study

Thirty adult nematodes (20 males and 10 females) of *Contracaecum* spp. collected from *Ph. brasilianus* were examined morphologically. For each adult specimen, the overall body length was measured directly. The middle part of the body was then separated from the rest of the body and used to genetically characterize the individual specimens by sequencing of the mtDNA *cox2* gene. The anterior and posterior parts were then cleared and mounted in lactophenol (1:1) for morphological studies. Specimens were studied using a compound microscope (×100–400) and a drawing apparatus. Measurements are presented in mm, except where indicated. Several characters considered diagnostic for anisakid nematodes (Fagerholm, 1989, 1991; Paggi et al., 2000) were analyzed, including interlabial structure, the pattern of distribution of male caudal papillae, spicule length and tip shape, and the size and pattern of the caudal papillae, all of which were labelled according to the nomenclature proposed by Fagerholm (1989). To consider allometric variation, spicule length measurements were related to either total body length or to tail length. In addition, a cecum to appendix ratio was obtained.

Some specimens were dried by the critical point method, then observed and photographed using an SEM (Jeol® JSV 6063 LV, Jeol Ltd., Akishma City, Tokyo, Japan). Holotype, allotype, and paratype specimens were stored in 70% ethanol and deposited in the Helminthological Collection of Museo de La Plata (CHMLP).

DNA amplification and sequencing

The 519-bp fragment of the mitochondrial cytochrome oxidase 2 gene (mtDNA *cox-2*) was analyzed from 6 specimens of *C. chubutensis* n. sp. and from 12 of the new species. A 470-bp fragment of the small subunit of the mitochondrial ribosomal RNA gene (*rrnS*) was analyzed in 6 specimens of *C. chubutensis* and in 6 of the new species. A 451-bp fragment of the ITS-1 and 284 bp of the ITS-2 regions were analyzed in 3 specimens of *C. chubutensis* and 3 of the new species. 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 (Valentini et al., 2006). The *cox-2* gene from each specimen of *Contracaecum* was amplified according to the procedures as reported in Mattiucci et al. (2010) with the primers 211F 5′-TTTTCTAGTTATATAGATTGRTTYAT-3′ and 210R 5′-CACCAA-CTCTTAAAATTATC-3′ from Nadler and Hudspeth (2000) spanning the mtDNA nucleotide position 10,639–11,248 as defined in *Ascaris suum* (GenBank X54253).

Polymerase chain reaction (PCR)

Amplification was carried out in a volume of 50 µl containing 30 pmol of each primer, $MgCl_2$ 2.5 mM (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey), PCR buffer 1× (Amersham), DMSO 0.08 mM, dNTPs 0.4 mM (Sigma-Aldrich, St. Louis, Missouri), 5 U of *Taq* Polymerase (Amersham), 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 sec, 46 C for 1 min and 72 C for 1.5 min, followed by post-amplification at 72 C for 10 min. The PCR product was purified using PEG precipitation and automated DNA sequencing was performed by Macrogen Inc. (Seoul, Korea) using primers 210 and 211.

The amplification of the small subunit of the mitochondrial ribosomal gene, *rrnS*, was performed according to the procedures reported in D'Amelio et al. (2007) with the primers MH3 (forward; 5′-TTGTTCCA-GAATAATCGGCTAGACTT-3′) and MH4.5 (reverse; 5′-TCTACTT-TACTACAACTTACTCC-3′). The PCR conditions were as follows: 10 min at 95 C (initial denaturation), 35 cycles of 30 sec at 95 C (denaturation), 30 sec at 55 C (annealing), 30 sec 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 according to the procedure reported in Shamsi et al. (2009a, 2009b) with the primers sets SS1 (forward; 5′-GTTTCCGTAGGTGAACCTGCG-3′) and NC13R (reverse; 5′-GCTGCGTTCTTCATCGAT-3′) and the ITS-2 region with the primers sets SS2 (forward; 5′-TTGCAGACACATTGAGCACT-3′) and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′). The PCR 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, and 1.5 U Taq polymerase (Promega) in a thermocycler using the following conditions: 4 C for 5 min (initial denaturation), followed by 30 cycles at 94 C for 30 sec (denaturation), at 55 C for 30 sec (annealing), at 72 C for 30 sec (extension), and a final extension at 72 C for 5 min. The PCR products were examined on a 1% agarose gel, stained with 1.5 µl of Gel-Red (Biotium Inc., Hayward, California), and analyzed using a gel documentation system. 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.

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The sequences of the specimens of *Contracaecum* spp. from *Ph. brasilianus* and *C. chubutensis* in *Phalacrocorax atriceps* were compared to those already obtained in our previous studies on mtDNA *cox2* deposited in GenBank. Sequences examined were from the 2 sibling species of *C. rudolphii* (s. l.) complex, i.e., *C. rudolphii* A and *C. rudolphii* B of Bullini et al. (1986) from the Eurasian subspecies of the great cormorant *Phalacrocorax carbo sinensis* (Blumenbach) from Italian coastal lagoons; *C. bioccai* Mattiucci, Paoletti, Olivero-Verbel, Baldiris, Arroyo-Salgado, Garbin, Navone, and Nascetti, 2008, from the brown pelican *P. occidentalis* (L.) in Colombia; *Contracaecum septentrionale* Kreis, 1955 from *Phalacrocorax aristotelis* (L.) off Norway; *Contracaecum microcephalum* (Rudolphi, 1809) from *Phalacrocorax pygmaeus* (L.) off Montenegro; *Contracaecum micropapillatum* (Stossich, 1890) sampled in the white pelican *Pelecanus onocrotalus* (L.) in Egyptian waters; *C. pelagicum* Johnston and Mawson, 1942 from *S. magellanicus* (Forster) off Argentina; and, finally, *C. gibsoni* Mattiucci, Paoletti, Consuegra-Solorzano, and Nascetti, 2010, and *C. overstreeti* Mattiucci, Paoletti, Consuegra-Solorzano, and Nascetti, 2010 from the Dalmatian pelican, *P. crispus*, off the coast of Greece.

The sequences of the mitochondrial *rrnS* region of the ribosomal DNA obtained for the specimens of *Contracaecum* from *Ph. brasilianus* and *C. chubutensis* from *Ph. atriceps* were compared to those already obtained for C. *rudolphii* C from *Ph. auritus* and deposited in GenBank. Sequences obtained in the present study for *Contracaecum* spp. included, for comparative purposes: *C. rudolphii* A, *C. rudolphii* B, *C. bioccai*, *C. septentrionale*, *C. microcephalum*, *C. micropapillatum*, *C. pelagicum*, *C. gibsoni*, and *C. overstreeti.*

Finally, the sequences of ITS-1 and ITS-2 regions of the rDNA obtained for *Contracaecum* from *Ph. brasilianus* and *C. chubutensis* were also compared with those previously obtained for the same gene from *C. rudolphii* D and *C. rudolphii* E isolated from *Ph. carbo* and *Ph. various*, respectively.

Sequence analysis

The *cox-2* and *rrnS* sequences obtained were aligned using Clustal X (Larkin et al., 2007). Phylogenetic scrutiny was performed using maximum parsimony (MP) and neighbor-joining (NJ) analyses, based on *p-distance* values, by PAUP* (Swofford, 2003) for mtDNA *cox-2* and *rrnS* datasets. The optimal evolution schematic for the datasets was the GTR+I+G model, as determined by using Akaike Information Criterion (AIC) (Posada and Buckley, 2004), and implemented in the software Modeltest 3.6 (Posada and Crandall, 1998) among 56 possible alternative models. The parameters for the model inferred from the mtDNA *cox2* sequences data were the proportion of invariable sites (I) = 0.6020, distribution shape parameter (α) = 0.8138, and nucleotide frequencies A = 0.19, C = 0.07, G = 0.27, T = 0.47. The parameters for the model inferred from *rrnS* rDNA were the proportion of invariable sites (I) = 0.5937, distribution shape parameter (α) = 0.7905, and nucleotide frequencies A = 0.20, C = 0.06, G = 0.27, T = 0.47. The reliabilities of the phylogenetic relationships were evaluated using nonparametric bootstrap analysis (Felsenstein, 1985) for the MP and NJ trees. Bootstrap values $\geq$70 were considered well supported (Hillis and Bull, 1993; Morrison, 2006).

Bayesian inference (BI) analysis (Larget and Simon, 1999) was performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) on full consensus sequences. The optimal evolution model of our dataset for the Bayesian analysis was determined using Akaike Information Criterion (AIC) (Posada and Buckley, 2004), as implemented in the software Model test 3.7 (Posada and Crandall, 1998) associated with PAUP* (Swofford, 2003). This analysis supported GTR+I+G as the best-fit substitution model for the data. The parameters for the model inferred were the proportion of invariable sites (I) = 0.6020, distribution shape parameter (α) = 0.8524, and nucleotide frequencies A = 0.19, C = 0.07, G = 0.27, T = 0.45. For Bayesian analysis, 4 incrementally heated Markov chains (using default heating values) were run for 1,000,000 generations, sampling the Markov chains at intervals of 100 generations. Of 20,002 samples summarized from 2 runs, 19,502 were included in the analysis. Posterior probabilities were estimated and used to assess support for each branch in inferred phylogenies; probabilities where $P < 0.05$ were indicative of significant support (Reeder, 2003).

The sequences of *Contracaecum* from *Ph. brasilianus* and *C. chubutensis* from *Ph. atriceps* from Argentina were compared to those already obtained for mtDNA *cox-2* for *Contracaecum* spp. from waterbirds in our previous studies (Mattiucci et al., 2008, 2010) and deposited in GenBank with the following accession numbers: EF513501, EF513502, EF513503, EF513505, EF558891, EF122202, EF535570 (*C. rudolphii* sp. A); EF558894, EF558896, EF513506, EF513507, EF513509, EU852349 (*C. rudolphii* sp. B); EF122205, EF513512, EF513513 (*C. septentrionale*); EF122208, EF5135017, EF5135018, EF513519 (*C. microcephalum*); EF122206, EF122207, EF513514, EF513515, EF513516, EU852350 (*C. micropapillatum*); EF513494, EF513495, EF558899, EF513497, EF513498, EF513499 (*C. bioccai*); EF122210, EF535568, EF535569 (*C. pelagicum*); EU852337–EU852342 (*C. gibsoni*); and EU852343–EU852348 (*C. overstreeti*).

Further, for a genetic comparison with other *Contracaecum* spp. 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 (EF014283) deposited in GenBank. 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, retrievable from GenBank under the accession numbers: FM210251, FM210252, FM210253, FM210262, FM210263, FM210264, FM210258, E-FM210259, FM210260, FM210270, FM210271, and FM210272. *Sulcascaris sulcata* from *Caretta caretta* of the Mediterranean Sea was included as outgroup to root the phylogenetic trees (GenBank HQ328505).

DESCRIPTION

Contracaecum australe n. sp.

(Fig. 1; Tables I, II)

General morphology (20 adult specimens: 10 males and 10 females from Santa Elena lagoon, VIII Región, Chile): Body entirely transversally striated (Fig. 1a, b, e, g). Conspicuous cephalic collar with V-shaped lateral region without striations (Fig. 1a, b). Three bifurcated interlabia (Fig. 1a–c). Lips longer than interlabia with 1 shallow apical notch (Fig. 1a, c). Lips with 2 conspicuous and lobed auricles, each with 2 prominent sensory pits at external end (Fig. 1a–c). Lip papillae present, 2 on the dorsal lip and 1 on each ventrolateral lip (Fig. 1a, c). Ventriculus with solid posterior appendix, intestinal cecum well developed, longer than ventricular appendix.

Male (holotype): Body length 27.28. Maximum body width 0.78. Distance from anterior end to nerve ring and deirids 0.61 and 0.63, respectively. Esophagus length 3.76; intestinal cecum length 2.48; ventriculus length 0.23; ventricular appendix length 1.25, cecum to appendix ratio 1.98. Spicules of equal length reaching almost half of body length. Spicule length 13.20; body to spicule length ratio 2.07. Tail length 0.24. Caudal extremity conical, bearing 27 to 32 precloacal papillae pairs. Pts-zone (= first 25 precloacal transverse striae) including 2 pairs precloacal papillae (Fig. 1e). Six pairs postcloacal papillae: 2 large subventral paracloacal pairs side by side, 2 subventral pairs, 2 sublateral pairs. One pair of phasmids between both sublateral papillae pairs (Fig. 1e, g). Cuticular constrictions on caudal extremity between precloacal papillae (Fig. 1e, g). Marked distal tail constriction between postparacloacal and subventral papillae (Fig. 1f, arrow). Median plaque (median papilla) very conspicuous lying on anterior cloaca rim (Fig. 1g, arrow). Spicule distal tip extended and rounded; length of free distal end shorter than spicule width (0.02 vs. 0.03) (Fig. 1d). Spicule wings slope distally toward shaft and insert at different points (Fig. 1d) (male paratypes, see Table I.).

Female (allotype): Body length 37.31. Maximum body width 1.03. Distance from anterior end to nerve ring and deirids 0.62 and 0.71, respectively. Esophagus length 3.63; intestinal cecum length 2.57; ventriculus length 0.27; ventricular appendix length 0.78. Vulva in anterior half of body. Distance from anterior end to vulva 9.58. Tail length 0.49. One pair of distal phasmids. Embryonated egg diameter 0.07. (Female paratypes, see Table II).

Taxonomic summary

Hosts: Phalacrocorax brasilianus (Gmelin, 1789) (Phalacrocoracidae).
Localities: Santa Elena lagoon, VIII Región, Chile.
Infection sites: Stomach.
Prevalence: Four of 4 infected (100%).
Mean intensity and range: 21.3 (4–87).

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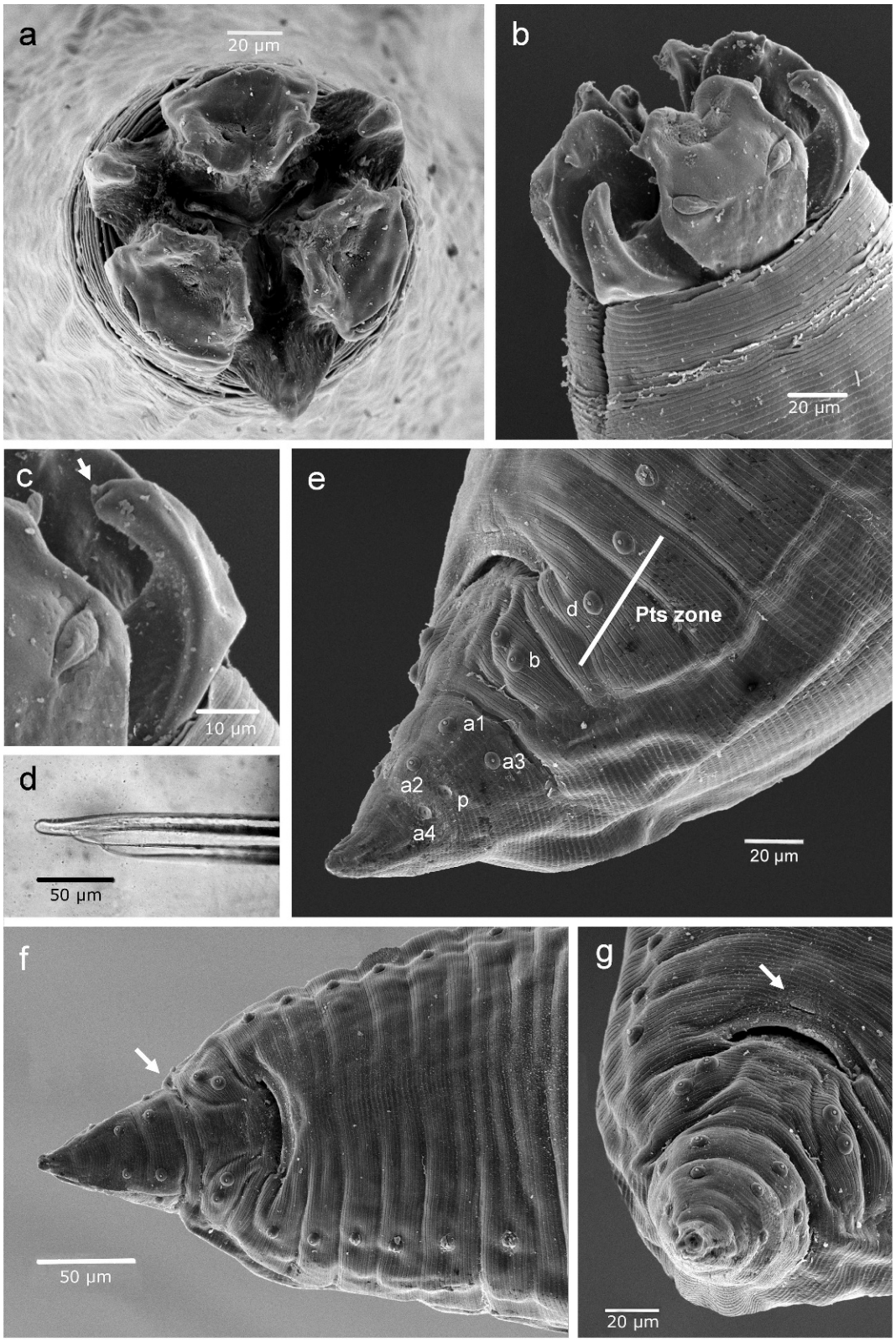

FIGURE 1. *Contracaecum australe* n. sp. from *Ph. brasilianus* from Santa Elena lagoon. (**a**) Anterior end apical view. (**b**) Anterior end, laterodorsal view, cephalic collar, dorsal lip, interlabia, cephalic lip papillae, lip auricles, auricle tips, and V-shaped lateral region without striations. (**c**) Bifid interlabia, showing bifurcation (arrow), and lip auricle tip. (**d**) Distal spicule end, lateral view. (**e**) Posterior male end, precloacal and postcloacal papillae distribution: a1–a2 = distal subventral papillae, a3–a4 = distal sublateral papillae, b = paracloacal papilla pair, d = proximal precloacal papillae, p = phasmid. (**f**) Posterior male end, precloacal papillae, postcloacal papillae, distal tail constriction (arrow). (**g**) Posterior male end, precloacal papillae, medial plaque (arrow).

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TABLE I. Morphometrical data of *Contracaecum australe* n. sp. male specimens from *Phalacrocorax brasilianus* from Santa Elena lagoon, VIII Región, Chile.

	Species						
	Contracaecum australe n. sp.	*Contracaecum chubutensis* Garbin et al., 2008	*Contracaecum caballeroi* Bravo Hollis, 1939	*Contracaecum rudolphii* Hartwich, 1964	*C. rudolphii* Hartwich, 1964	*Contracaecum travassosi* Gutiérrez, 1943	*C. travassosi* Gutiérrez, 1943
References*	Present paper	Garbin et al., 2008	Lent and Freitas, 1948	Hartwich, 1964	Amato et al, 2006	Gutiérrez, 1943	Morgan et al., 1949
Type host	*Ph. brasilianus*	*Ph. atriceps*	*Anhinga anhinga*	*Ph. carbo*		*Ph. albiventer* (=*Ph. atriceps*)	*Pandion haliaetus carolinensis*
Other hosts	—	—	*Ph. brasilianus*	*Phalacrocorax* spp. Phalacrocoracidae Charadriiformes Ciconiiformes	*Phalacrocorax* spp. Phalacrocoracidae Charadriiformes Ciconiiformes	*Ph. brasilianus*	*Ph. brasilianus*
Localities	VIII Región, Chile	Bahía Bustamante Chubut, Argentina	México Uruguay	Various	Various	Chubut coast, Argentina	United States
Males (n)	10	10	3	39	30	No data	5
Body L	23.24 (13.90–28.40)	25.06 (14.32–38.58)	24.29–26.97	12.10–33.90	25 (18–31)	16.10–25.40	42 (34–58)
Maximum body W	0.75 (0.64–0.93)	0.77 (0.43–0.98)	0.53–0.64	0.24–0.95	0.48 (0.31–0.60)	0.70–1.10	0.74 (0.58–0.85)
Nerve ring (DAE)	0.63 (0.58–0.68)	0.52 (0.36–0.60)	0.43–0.45	—	—	0.45–0.64	0.61 (0.49–0.70)
Deirids (DAE)	0.65 (0.58–0.79)	0.64 (0.46–0.84)	0.44–0.48	—	—	—	—
Esophagus L	3.62 (2.62–4.60)	3.39 (2.32–4.50)	3.18–3.48	2.03–4.26	3.10 (2.40–3.80)	2.80–4.10	4.6 (3.5–7.0)
Intestinal cecum L	2.41 (1.56–3.24)	2.25 (1.50–2.76)	2.71–3.01	1.53–3.68	2.40 (2.10–2.90)	1.90–3.20	3.1 (2.1–4.4)
Ventriculus L	0.28 (0.2–0.38)	0.23 (0.13–0.30)	0.10–0.10	—	—	—	—
Ventricular appendix L	1.17 (0.87–1.41)	0.67 (0.46–0.80)	0.51–0.61	—	1.00 (0.80–1.20)	0.74–1.30	—
Spicule L	11.97 (9.60–15.88)	9.95 (6.40–12.60)	0.90–1.09	R: 4.46–9.19 L: 4.05–9.98	R: 6.20 (4.50–7.50) L: 7.10 (5.90–8.20)	7.70–11.10	R: 8.9 (7.2–11.4) L: 9.4 (7.5–12.9)
Tail L	0.22 (0.18–0.24)	0.20 (0.17–0.26)	0.13–0.15	0.14–0.24	0.21 (0.14–0.24)	0.19–0.26	0.30 (0.26–0.38)
PrPP	27–32	35–43	40	27–43	30	26–30	30
BL:MBW	28.31–39.12 (34.12)	33.19 (27.68–39.32)	42.14–45.83†	52.3 (29.4–98.1)	52.9 (47.9–68.6)	23.00–23.09†	58.62–68.23†
BL:EL	6.03–8.87 (7.14)	7.33 (6.15–9.27)	7.64–7.75†	8.01 (5.25–10.78)	7.1–10.9 (8.1)	5.75–6.19†	8.28–9.71†
BL:TL	97.92–138.89 (117.42)	124 (83.74–214.32)	179.80–186.84†	131.8 (74.1–197.2)	91.3–145.5 (122.7)	84.74–97.69†	130.76–152.63†
EL:ICL	1.37–1.68 (1.52)	1.52 (1.44–1.63)	1.16–1.17†	1.3 (1.11–1.54)	0.9–1.6 (1.3)	1.28–1.49†	1.59–1.67†
EL:VAL	2.25–3.99 (3.13)	4.90 (3.49–6.02)	5.70–6.23†	3.25 (1.82–4.25)	2.8–3.6 (3.2)	3.15–3.78†	—
BL:SL	1.41–2.77 (1.90)	2.58 (2.18–3.14)	24.74–26.99†	3.86 (2.06–5.69)	3.1–5.4 (3.8)	2.09–2.28†	4.49–4.72†

* L = length; W = width; BL:MBW = body length to maximum body width ratio; BL:EL = body length to esophagus length ratio; BL:TL = body length to tail length ratio; EL:ICL = esophagus length to intestinal cecum length ratio; EL:VAL = esophagus length to ventricular appendix length ratio; BL:SL = body length to spicule length ratio; PrPP = precloacal papillae pairs; DAE = distance from anterior end.
† Ratios calculated with maximum and minimum values.

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TABLE II. Morphometrical data of *Contracaecum australe* n. sp. female specimens from *Phalacrocorax brasilianus* from Santa Elena lagoon, VIII Región, Chile.

	Species						
	Contracaecum australe n. sp.	*Contracaecum chubutensis* Garbin et al., 2008	*Contracaecum caballeroi* Bravo Hollis, 1939	*Contracaecum rudolphii* Hartwich, 1964	*C. rudolphii* Hartwich, 1964	*Contracaecum travassosi* Gutiérrez, 1943	*C. travassosi* Gutiérrez, 1943
References*	Present paper	Garbin et al., 2008	Lent and Freitas, 1948	Hartwich, 1964	Amato et al, 2006	Gutiérrez, 1943	Morgan et al., 1949
Type host	*Ph. brasilianus*	*Ph. atriceps*	*Anhinga anhinga*	*Ph. carbo*		*Ph. albiventer* (=*Ph. atriceps*)	*Pandion haliaetus carolinensis*
Other hosts	—	—	*Ph. brasilianus*	*Phalacrocorax* spp. Phalacrocoracidae Charadriiformes Ciconiiformes	*Phalacrocorax* spp. Phalacrocoracidae Charadriiformes Ciconiiformes	*Ph. brasilianus*	*Ph. brasilianus*
Localities	VIII Región, Chile	Bahía Bustamante Chubut, Argentina	México Uruguay	Various	Various	Chubut, Argentina	United States
Females (n)	10	10	No data	36	30	No data	2
Body L	31.60 (25.44–41.23)	29.60 (21.98–35.33)	—	10.10–57.60	41.8 (23–52	22.7–31.5	53–55
Maximum body W	0.94 (0.66–1.16)	0.90 (0.61–1.27)	—	0.29–1.51	0.8 (0.5–1.1)	1.10–1.50	1.1–1.3
Nerve ring (DAE)	0.58 (0.50–0.68)	0.56 (0.48–0.62)	—	—	—	0.59–0.73	0.75–0.76
Deirids (DAE)	0.68 (0.57–0.82)	0.69 (0.58–0.80)	—	—	—	—	—
Esophagus L	3.24 (1.52–3.95)	3.05 (1.19–4.28)	—	1.62–5.48	4.2 (2.4–5.4)	3.50–4.80	5.0–5.1
Intestinal cecum L	2.13 (1.30–2.86)	1.91 (1.08–2.93)	—	1.28–4.12	2.9 (1.6–3.6)	2.70–4.20	4.0–4.3
Ventriculus L	0.25 (0.14–0.28)	0.24 (0.16–0.26)	—	—	—	0.89–1.60	—
Ventricular appendix L	0.70 (0.57–0.91)	0.74 (0.66–0.94)	—	—	1.2 (0.6–1.5)	—	—
Vulva (DAE)	9.26 (8.25–10.87)	9.57 (8.32–11.56)	—	5.12–17.7	15.2 (9.7–21.3)	18.60–21.00	24
Tail L	0.39 (0.28–0.58)	0.41 (0.30–0.65)	—	0.19–0.63	0.4 (0.2–0.6)	0.43–0.54	0–43–0.45
Embrionated egg	0.068 (0.063–0.071)	0.070 (0.06–0.07)	—	0.059–0.073	0.105 (0.099–0.106)	0.068	0.062

* L = length; W = width; DAE = distance from anterior end.

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TABLE III. GenBank accession numbers of the specimens of *Contracaecum australe* n. sp. and *Contracaecum chubutensis* sequenced at *cox-2*, *rrnS*, ITS-1, and ITS-2 loci. They are reported with their codes appearing in the text and figures.

Species	*Cox-2*	*rrnS*	ITS-1	ITS-2
Contracaecum australe n. sp.	CAU1 = GQ847532; CAU2 = GQ847533 CAU3 = GQ847534; CAU4 = GQ847535 CAU5 = GQ847536; CAU6 = GQ847537 CAU7 = GQ847538; CAU8 = GQ847539 CAU9 = GQ847540; CAU10 = GQ84741 CAU11 = GQ84742; CAU12 = GQ84743 CAU13 = GQ84744	CAU8 = HQ333520	CAU8 = HQ389545	CAU8 = HQ389547
Contracaecum chubutensis	CCH1 = HQ328504	CCH1 = HQ333521	CCH1 = HQ389546	CCH1 = HQ389548

Voucher specimens deposited: Holotype (male) (6208 CHMLP), allotype (female) (6209 CHMLP), and 18 paratypes (6210 CHMLP); Helminthological Collection of Museo de La Plata, La Plata, Buenos Aires, Argentina. Holotype accession number in GenBank is GQ847539.

Etymology: Contracaecum australe is named because of its occurrence as a parasite of the Neotropic cormorant from the Austral Hemisphere.

Remarks

According to the morphological characters considered as diagnostic for species of *Contracaecum*, i.e., the length of the spicules, the morphology of the distal end of the spicule, and the bifurcation of the interlabial tip (sensu Hartwich, 1964), our specimens collected from the *Ph. brasilianus* would be assigned to *C. rudolphii* Hartwich 1964 sensu lato (see Hartwich, 1964). However, according to the morphological comparison of the new specimens, the material has been assigned to *C. australe* n. sp.

The new species possesses a distal tail constriction which seems to be absent in the original description of *C. rudolphii* (Hartwich, 1964), even though it is present in other descriptions, i.e., that of Abollo et al. (2001) (Fig. 1). The median plaque (Fig. 1g) observed in *C. australe* also seems to be absent in *C. rudolphii* (s. l.), or perhaps it was not observed by other authors (Hartwich, 1964; Abollo et al., 2001; Amato et al., 2006). Moreover, *C. australe* appears shorter and thicker, as is suggested by the body length to maximum body width ratio: 23.00–23.09 versus 29.4–98.1 (Table I). In addition, male spicules of *C. australe* are longer than those of *C. rudolphii* (9.60–15.88 mm vs. 4.05–9.98 mm), with a larger BL:SL, 1.41–2.77 versus 2.06–5.69. However, according to Hartwich (1964) and Barus et al. (2000), *C. rudolphii* (s. l.) has a great variability in the size of spicules (Table I). It has been also demonstrated that *C. rudolphii* (sensu Hartwich, 1964) (s. l.) is a complex of sibling species. *Contracaecum australe* differs from both *C. rudolphii* sp. A and *C. rudolphii* sp. B (of Bullini et al., 1986). The former species has longer spicules compared to those observed for the 2 sibling species infecting the great cormorant *Phalacrocorax carbo sinensis*. Spicules from *C. rudolphii* sp. A are 6.8–7.2 mm and 8.6–9.5 mm in *C. rudolphii* B (Mattiucci et al., 2008). Moreover, the geographical distribution and host of the 2 siblings are different. Finally, the sequence analysis of the mtDNA *cox-2*, *rrnS* and of the ITS-1 and ITS-2 regions presented here demonstrates that *C. australe* is genetically distinct from *C. rudolphii* A, *C. rudolphii* B, *C. rudolphii* C, *C. rudolphii* D, and *C. rudolphii* E (Figs. 5–7). C*ontracaecum rudolphii* C (see D'Amelio et al., 2007) from *Ph. auritus* has not been morphologically described. As for *C. rudolphii* D and *C. rudolphii* E from Australian cormorants (see Shamsi et al., 2009b), the new species differs from *C. rudolphii* D for the spicule length (3.90–6.60 mm in *C. rudolphii* D vs. 9.60–15.88 mm in *C. australe* and from *C. rudolphii* E, in which the spicule lengths range from 5.53–6.13 mm). Moreover, the host and geographical distributions are different.

Among those *Contracaecum* spp. reported from cormorants belonging to Phalacrocoracidae, *C. caballeroi* is also reported as a parasite of *Ph. brasilianus*; it possesses significantly shorter spicules (0.90–1.09 mm vs. 9.60–15.88 mm) and, therefore, has a much higher body to spicule length ratio: 24.74–26.98 versus 1.41–2.77 (Lent and Freitas, 1948) (Table I). *Contracaecum australe* also differs morphologically from *C. chubutensis* Garbin Diaz, Cremonte, and Navone, 2008; the new species has a well-marked constriction of the tail tip, an oblique position of the paracloacal papillae, lips with no notches, entire or barely bifurcated interlabia, longer spicules (9.60–15.88 mm vs. 5.34–12.60 mm), and a smaller body to spicule length ratio: 1.41–2.77 versus 2.18–3.14.

Contracaecum septentrionale Kreis, 1955 greatly resembles *C. australe*; however, the precloacal papillae number is smaller, the paracloacal papillae seem to have an inverse oblique disposition, the tail looks more curved, and the spicule end appears to be more blunt in *C. septentrionale* (Kreis, 1955). Further, the host and geographical distribution of *C. septentrionale* is different; all the genetic data presented here also support the distinction of *C. australe* from *C. septentrionale*.

Contracaecum travassosi is also similar to *C. australe*, although spicules in the former species are shorter (7.70–11.10 vs. 9.60–15.88). Therefore, the BL:SL ratio is less variable (2.09–2.28 vs. 1.41–2.77), the bifurcation of interlabia is more marked, the body width is greater and, consequently, so is the body to maximum body width ratio: 23.00–23.09 versus 28.31–39.12. Further, according to the original description given by Gutierrez (1943), the paracloacal papillae are double, even if in the original figure they appear to be as 2 separate papillae, but very close to each other. Specimens of *C. travassosi* described from osprey, *Pandion haliaetus* (L.) (Accipitridae), in North America (Morgan et al., 1949) are significantly thinner (Table I) and the tail length is longer based on the body to tail length ratio: 84.74–97.69 versus 97.92–138.89. A morphological re-examination of a male paratype of *C. travassosi* provided clear evidence that the paracloacal papillae are double.

Contracaecum australe is also morphologically distinct from other *Contracaecum* species that parasitize waterbirds, i.e., it differs from *Contracaecum variegatum* Rudolphi, 1809 from red-throated loon, *Gavia stellata* (Pontoppidan) (Gaviidae), because the latter 2 species possess almost double the number of precloacal papillae and shorter spicules (4.40–4.86 mm vs. 9.60–15.88 mm), and, therefore, a larger body to spicule length ratio (BL:SL 4.00–6.50 vs. 1.41–2.77) (Fagerholm et al., 1996). Moreover, *C. variegatum* is genetically distinct from the new species based on mtDNA *cox-2* analysis (data not shown).

Contracaecum magnipapillatum (= *Contracaecum magnicollare*) Johnston and Mawson, 1941 differs from *C. australe* of black noddy *Anous minutus* Chapin (Laridae) because it lacks bifurcated interlabia (Fagerholm et al., 1996) and its spicules are smaller (2.62–3.70 vs. 9.60–15.88 mm) and, therefore, has a higher BL:SL ratio (4.20–6.00 vs. 1.41–2.77). *Contracaecum plagiaticum* has 8 postcloacal papillae pairs (1 more subventral papillae pair) and shorter spicules (2.32–3.49 mm vs. 9.60–15.88 mm), BL:SL ratio 4.80–5.40 versus 1.41–2.77 (Lent and Freitas, 1948). *Contracaecum pelagicum* Johnston and Mawson, 1942 from several hosts (Portes-Santos, 1984; Silva et al., 2005; Garbin et al., 2007; Garbin, 2009) can be differentiated from *C. australe*, mainly for its bifurcation on interlabia and shorter spicules (3.07–5.07 mm vs. 9.60–15.88 mm); moreover, *C. pelagicum* is also genetically distinct from *C. australe* (Figs. 3, 4). *Contracaecum multipapillatum* (von Drasche, 1882) from great egret *Ardea alba* greatly differentiates from *C. australe* in terms of the number of papillae and the pattern of postcloacal papillae; further, *C. multipapillatum* s. l. has no bifurcated interlabia (Navone et al., 2000). *Contracaecum australe* differs also from *C. gibsoni* and *C. overstreeti* described from *P. crispus*; the latter species does not have a bifurcated interlabia, and they also have shorter spicules and a different pattern of distribution of proximal papillae (see Mattiucci et al., 2010). *Contracaecum bioccai* Mattiucci et al., 2008 from *P. occidentalis* has shorter and subequal spicules (right 5.80–6.20 mm, left 6.00–6.50 mm vs. 9.60–15.88 mm), conspicuous bifurcated interlabia, and the a4 sublateral

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Cox-2

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                                10        20        30        40        50        60        70        80        90       100
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C. australe n.sp.       GTTTTTATTTCAAGAGTAAAAAGATTGAATATCAATTTGGTGAGCTTTTATGTAGTGTTTTCCCGACTTTAATTTTGGTTATGCAGATGATCCCTTCTTT
C. chubutensis          ..........T..A.............G.............A................T..T.....G.....A.....A................
C. rudolphii A          ..........T..A.....G........G.....G.....G........G.............A.....G...........A..A.............
C. rudolphii B          ..........T..A.....G........G...........A..A.....G.............A.....G...........A..A.............
C. septentrionale       ..........T................G.............A.....G...........T..T.....G.....A.....A..A..A...........
C. bioccai              ..........T..A.....G........G.....G.........................T..A.....G...........A..A...G.A........
C. pelagicum            ..........T........G.......G.....G........A................T..A.....G...........A......G.G........
C. microcephalum        ..........T................G.....G.....G...T.G..G...........T..T.....G................A..T........
C. micropapillatum      ....C.....T.....G................G.....G..A................T..T.....G................G.T........
C. gibsoni              ..........T..A..A..G..A.....G.....G.....G.............G.....T..T...C.T.....A..G...................
C. overstreeti          ..........T.....G..G.....C..G.....G.....A.............G.....T..T.....G.....A.....A.....A..T........

                                110       120       130       140       150       160       170       180       190       200
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C. australe n.sp.       GAGTTTACTTTATTACTATGGTTTAATAAACCTGGATAGTAGTTTAACTGTGAAGGTTACTGGCCATCAGTGGTATTGAAGTTATGAATTTAGGGATATC
C. chubutensis          ...............T.............T..T...........................T...........C..................T......
C. rudolphii A          A..............T........G........T...........G...............T.......................G............
C. rudolphii B          ....C.G........T................T...........................T.....A................G.....A......
C. septentrionale       A.....G........T........G.....T..T...........G.....A...........T.....A...............G...........T
C. bioccai              ...............T.............T........A.....G...........C.....T........A.....G..A.....G.....T......
C. pelagicum            A..............T......C.......TT....C......C.T..G...........G..T.....A..A........A..C..............
C. microcephalum        ......GT.G.....T.....G..G.....TT....C..G.....G.....T...........G.......................G............
C. micropapillatum      A......T.G.....T........G..G..TT....C..G.....G.....T........C..G..C..A..................G...........T
C. gibsoni              ......GT.G.....T........G......T............G...A.T..A..C.....G..C...........G..C.....G...........T
C. overstreeti          ....C.GT.G.....T...........G..T.............G..G..T...........T.......................G...........T

                                210       220       230       240       250       260       270       280       290       300
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C. australe n.sp.       CCTGGCTTAGAGTTTGACTCTTATATGAAGTCTCTGGATCAATTGGAATTGGGGGAGCCTCGTCTTTTAGAGGTTGATAACCGTTGTGTTGTACCTTGTG
C. chubutensis          .....T...........T...............T.......G.....G.....T..............G.........................G....
C. rudolphii A          .....T..G........T...............T.A.....G.........................G...........T...........G.......
C. rudolphii B          ..C..T..G........T...............T.A..C..G.....G...........C........G...........T...........T.......
C. septentrionale       .....T..G.....C..T........A......T.......G.....G.....C.......................................G....
C. bioccai              ..G..GC.T........T..............GT....C..G...........C..A...........G.....G.................G.......
C. pelagicum            ......C.G.....................A...T..........A..T.....T..A..G........G.....G.....T...................
C. microcephalum        ..G..G..G........T...............T....C..G..A..GG.T..A..............G.....G.....T........AA.T.......
C. micropapillatum      .....T..G........T...............T.............GC..............T.A..G...........T...........T.......
C. gibsoni              .....TC.G..A.....T...........A...T..........A..G.......................A........T...........T.......
C. overstreeti          .....T..G........T...............T.............G...............C...C.G..A..A.....T.........A.T.......

                                310       320       330       340       350       360       370       380       390       400
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C. australe n.sp.       ATACTAATATTCGTTTTTGTATTACTTCTGGGGATGTTATTCATTCTTGGGCTTTGCCTAGGCTTTCTATTAAGTTGGATGCTATGAGGGGCATTCTTAC
C. chubutensis          ..........C........C........C....................A...........T...........A.................G.......G
C. rudolphii A          ..........C...................G.......................................T..T.....A.G
C. rudolphii B          ......................C.....G..T........C..C.....A........C..............AC.............T..T.......G
C. septentrionale       ..................................................A.....T...........A...........A..T..A.......G
C. bioccai              .C..................G..........T......................A.....A...........A..............T..T...T.G.G
C. pelagicum            ....C......................G..T.....G..C........A.....................................G.......G
C. microcephalum        ..........C..G...........G..G........G................A.....TT.G........AC.T...........T..G...T.A..
C. micropapillatum      ............................G........G...............C.A.....T..............A.....A.....T..T...T.G..
C. gibsoni              ............................G...........C...........G.....C..AT.G...........A........A..T..T...T.G.G
C. overstreeti          ............................C............................C..T...........A...........A..T..T...T.G.G

                                410       420       430       440       450       460       470       480       490       500
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C. australe n.sp.       AACTTTGTCCTATAGTTTCCCTATTGTGGGTGTTTTTTATGGTCAGTGTTCAGAGATTTGTGGTGCCAACCATAGTTTTATACCTATTGCTTTGGAGGTG
C. chubutensis          G........T........T...C.......G.............A.....G..............T..T...........G..............A...
C. rudolphii A          G........T........T...........G.............A........A...........T..............G..................
C. rudolphii B          G.....A..T..C.....T........A..A.............A.....G..A...........T...............................A
C. septentrionale       G.................T..CC.......G.................G.............C..T..T..........G...................A
C. bioccai              T...C.T..T........T...G....A...........C...........T..A...........T..T...........G..C........A.....T
C. pelagicum            T........T........T...........A....................T..A...........T..............G..A...........A..A
C. microcephalum        T.....A..T........T...G....T..............G..A.....T..............A..T...................T.........T
C. micropapillatum      .........T........T...G.G.....G.............A.....T...........G..T..............G..C..............T
C. gibsoni              T........T.....G..T...G....T..C...........G..A.....T..............T.........................A..A..T
C. overstreeti          T..C..A..T............G.G..T..............G..A.....T..............T..............G................T

                                510
                        ....|....|....|....
C. australe n.sp.       ACTTTACTTGATAATTTTA
C. chubutensis          .....G.............
C. rudolphii A          .....GT.G..........
C. rudolphii B          .....GT.G..........
C. septentrionale       .....GT.G..........
C. bioccai              ......T.G..........
C. pelagicum            .....GT.A..........
C. microcephalum        ..C..GT.G..........
C. micropapillatum      .....GT.G..........
C. gibsoni              .....GA.A..........
C. overstreeti          .....GA.G..........
```

FIGURE 2. Alignment of mtDNA *cox-2* (519 bp) sequences of *C. australe* n. sp. and *C. chubutensis* with other *Contracaecum* spp. previously sequenced (Mattiucci et al., 2010) and deposited in GenBank. The alignment was performed using BioEdit (Hall, 1999). One representative of each unique sequence was included for the comparison. Dot indicates identity.

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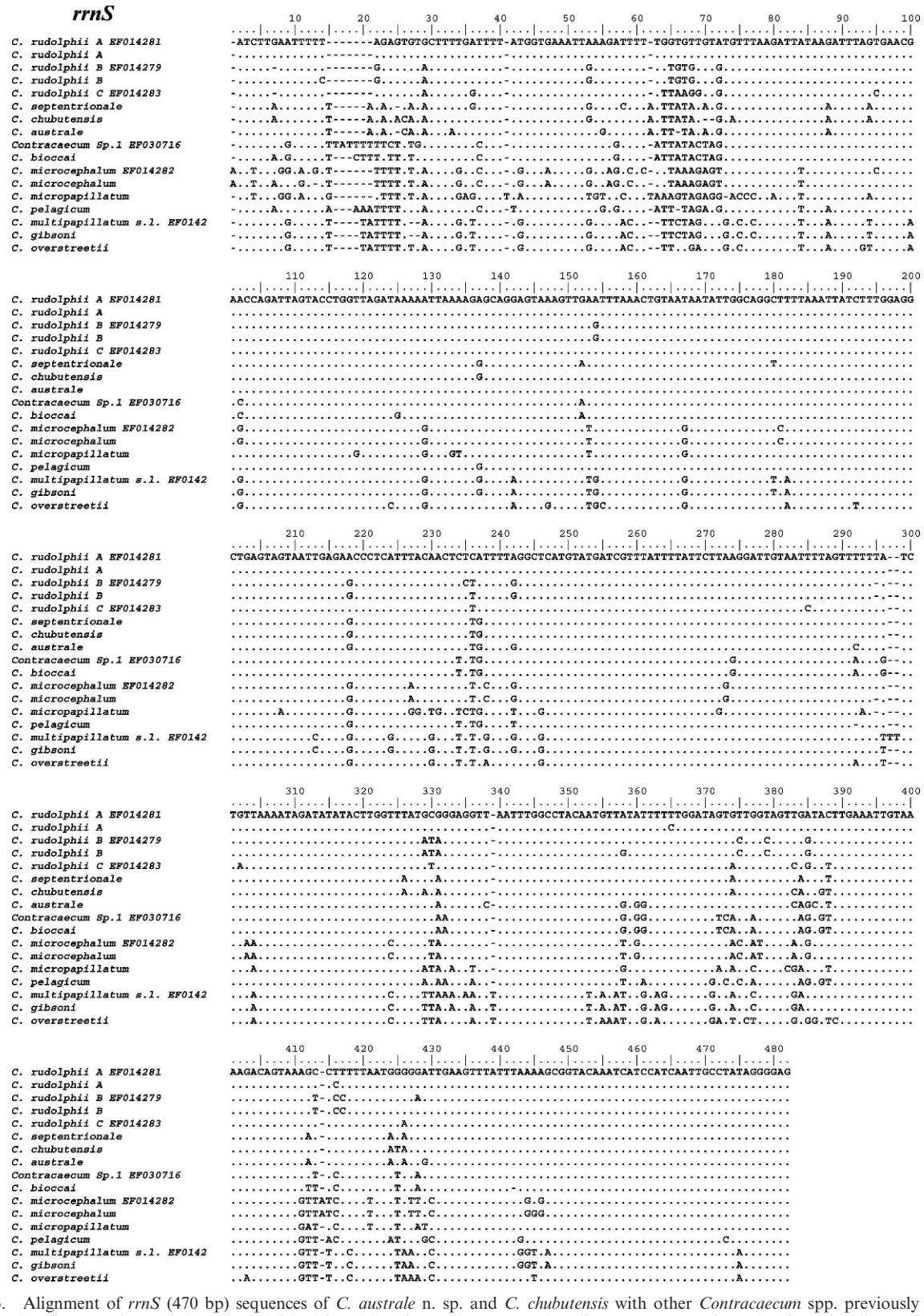

FIGURE 3. Alignment of *rrnS* (470 bp) sequences of *C. australe* n. sp. and *C. chubutensis* with other *Contracaecum* spp. previously deposited in GenBank under the accession numbers as published in D'Amelio et al. (2007). The alignment was performed using BioEdit (Hall, 1999). One representative of each unique sequence was included for the comparison. Dot indicates identity and dash indicates gap.

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papillae pair unites with the a2 subventral pair, forming a double, 3-paired subventral row. *Contracaecum microcephalum* (Rudolphii, 1809) has very short spicules (1.40–3.65 mm vs. 9.60–15.88 mm), different in the BL:SL ratio (5.06–15.05 vs. 1.41–2.77).

Genetic differentiation between *C. australe* n. sp. and *C. chubutensis* with respect to other congeners from waterbirds

To provide support for the existence and the validity of *C. australe* as a new species and of *C. chubutensis*, morphologically described in our previous studies, the same 13 specimens of the first taxon and 3 of the second were sequenced at the mtDNA *cox-2* locus. Further, some specimens among those sequenced at the mtDNA *cox-2* locus were also sequenced at the *rrnS* locus and at the ITS-1 and ITS-2 regions of the nuclear rDNA (Table III). The sequences obtained for the specimens of *C. australe* n. sp. and *C. chubutensis* are deposited in GenBank under the accession numbers indicated in Table III.

The specimens of *Contracaecum* from *Ph. brasilianus* indicated that *C. australe* did not match any of the previously reported sequences for the 3 genes examined here or any of those previously deposited in GenBank. Similarly, the specimens of *C. chubutensis* did not match any of the congener species previously scrutinized or deposited in GenBank. The sequence alignments of *C. australe* n. sp. and *C. chubutensis*, in comparison with others that have been investigated, are shown in Figures 2, 3, 4a, b.

The individuals corresponding to *C. australe* n. sp. all clustered in the same clade, well supported in the MP tree (Fig. 5) as well as in the BI (Fig. 6) inferred from the mtDNA *cox-2* sequence analysis. In these trees, the clade formed by the specimens of *C. australe* was quite distinct from all the previously genetically characterized species of *Contracaecum.* Similarly, *C. chubutensis* forms, at both MP and BI (Figs. 5, 6) inferred from the same mtDNA *cox2* sequences analysis, a distinct clade from all the *Contracaecum* spp. Moreover, both *C. australe* n. sp. and *C. chubutensis* form 2 quite-distinct clades (Fig. 6). Nonetheless, *C. australe* and *C. chubutensis* are closely related to the other *Contracaecum* parasites of cormorants, i.e., *C. rudolphii* A, *C. rudolphii* B, *C. rudolphii* C, and *C. septentrionale*. Indeed, a congruent tree topology inferred from mtDNA *cox-2* and *rrnS* sequence analyses (Figs. 5–7) was generated. The same sub-clade was produced with the tree topology inferred from MP and BI from mtDNA *cox-2* (Figs. 5, 6), as well as from MP and NJ of the *rrnS* (Fig. 7), including *C. australe*, the species in the *C. rudolphii* complex (*C. rudolphii* sp. A, *C. rudolphii* sp. B, and *C. rudolphii* C) plus *C. septentrionale* and *C. chubutensis*. Moreover, this sub-clade (Fig. 5, 7) was distinct from all other *Contracaecum* species considered in the comparison, although it did not receive a high bootstrap value (<70) in all the analyses inferred from different genes.

Pairwise comparisons of the *p-distance* values inferred from mtDNA *cox-2* (Table IV) sequences range from 0.08 to 0.10 between *C. australe* and *C. chubutensis* with respect to *C. rudolphii* A and *C. rudolphii* B. On the other hand, *C. australe* versus *C. chubutensis* shows a value of *p-distance* = 0.09. Moreover, *C. chubutensis* was found to be genetically more related to *C. septentrionale*, from which it has been demonstrated to show, however, a *p-distance* value of 0.06. With respect to *C. septentrionale*, *C. australe* exhibits a *p-distance* value of 0.10, whereas *C. australe* shows much larger values, i.e., 0.13 to 0.14 with respect to other morphologically distinct species such as *C. micropapillatum* or *C. gibsoni* (Table III).

Similarly, the *p-distance* estimated at the *rrnS* DNA (Table IV) exhibits a value of 0.03 between *C. australe* and *C. chubutensis*, whereas a value of *p-distance* = 0.04 was observed between *C. australe* and *C. rudolphii* C. Similar numbers were observed between *C. australe* and *C. chubutensis* with respect to *C. rudolphii* A, *C. rudolphii* B, and *C. septentrionale* as well (Table IV).

Finally, at the ITS-1 and ITS-2 regions of the rDNA, *C. australe* exhibits *p-distance* values (Table V) ranging from 0.02 to 0.03 with respect to *C. chubutensis* and other species of the *C. rudolphii* complex, i.e., *C. rudolphii* D and *C. rudolphii* E. Sequence polymorphisms at the ITS-1 and ITS-2 were detected in *C. australe* at alignment positions 303, 333, and 418, and 74, 83, 88, 159, 160, 273, and 280, respectively (Fig. 4a, b). Sequence polymorphism was detected at alignment position 116 of the ITS-1 and at position 31 of the ITS-2 in *C. chubutensis* (Fig. 4a, b).

DISCUSSION

A congruent topology was obtained in all the phylogenetic analyses inferred from mtDNA *cox-2* and *rrnS* DNA sequences (Figs. 2–4). The MP, NJ, and BI tree topologies show that all *C. australe* specimens sequenced form a well-defined clade and separated clearly from *C. rudolphii* A, *C. rudolphii* B, *C. rudolphii* C, and *C. septentrionale*. High support was received in all the phylogenetic elaborations for the clade formed by *C. australe* as a new species. Similarly, the MP, NJ, and BI tree topologies obtained from the sequences analyses of the mtDNA *cox-2* and *rrnS* DNA demonstrated that the specimens of *C. chubutensis* form a well-distinct clade from *C. australe*, as well as from the other *Contracaecum* species sequenced for these genes.

However, evidence for *C. australe* and *C. chubutensis* as 2 distinct species was also supported by analyses of ITS-1 and ITS-2 sequence data. Indeed, alignment of the ITS-1 and ITS-2 showed differences in both regions of the 2 taxa with respect to sibling species of the *C. rudolphii* complex, including *C. rudolphii* D and *C. rudolphii* E. Genetic characterization of these 2 species revealed a distance value for *C. rudolphii* D and *C. rudolphii* E at the same level as that between *C. rudolphii* A and *C. rudolphii* B (Table V). Currently, there are no ITS-1 and ITS-2 sequences available and deposited in GenBank for *C. rudolphii* C. However, the clear distinctiveness of *C. australe* and *C. chubutensis* from *C. rudolphii* C was clearly supported by the sequence analysis of the mitochondrial *rrnS* ribosomal DNA region (Fig. 7).

Our sequence data also allowed for the identification of a specimen belonging to the taxon previously identified as *Contracaecum* sp.1 in D'Amelio et al. (2007) as corresponding to *C. bioccai* of Mattiucci et al. (2008), as well as to a specimen of *C. multipapillatum* s. l. in D'Amelio et al. (2007), and also corresponding to the species *C. overstreeti* of Mattiucci et al., 2010 (Fig. 7).

Tree topologies obtained are also congruent in showing the existence of a well-separated sub-clade formed by all the *Contracaecum* species so far characterized in Phalacrocoracidae, i.e., *C. rudolphii* A, *C. rudolphii* B, *C. rudolphii* C, *C. septentrionale*, *C. chubutensis*, and the new taxon, *C. australe*, albeit this clade did not receive very high bootstrap values inferred from either of the analyses performed (Figs. 5–7).

All the tree topologies derived from the phylogenetic analyses were in substantial agreement with each other in depicting *C. chubutensis* as forming a sub-clade, albeit not always well supported, with *C. septentrionale*, parasite of a cormorant species, i.e., *Ph. aristotelis*, of the boreal hemisphere.

To date, only 3 *Contracaecum* species have been found to parasitize the Neotropic cormorant, *Ph. brasilianus* (Lent and Freitas, 1948; Torres et al., 2000; Amato et al., 2006), and none matches morphologically with the new species *C. australe*. All results obtained previously indicate that the new species is consistently separated from *Contracaecum* spp. that parasitize cormorants, not only genetically but also morphologically. The peculiar distal tail constriction, and the other constrictions between proximal precloacal papillae observed in male specimens of *C. australe*, seems to be a common feature on the *Contracaecum* spp. that infect Phalacrocoracidae, even more so in the well-differentiated clade formed by the 4 species (Kreis, 1955; Abollo et al., 2001). Another shared feature by *Contracaecum* spp. from cormorants is the lip shape, which has

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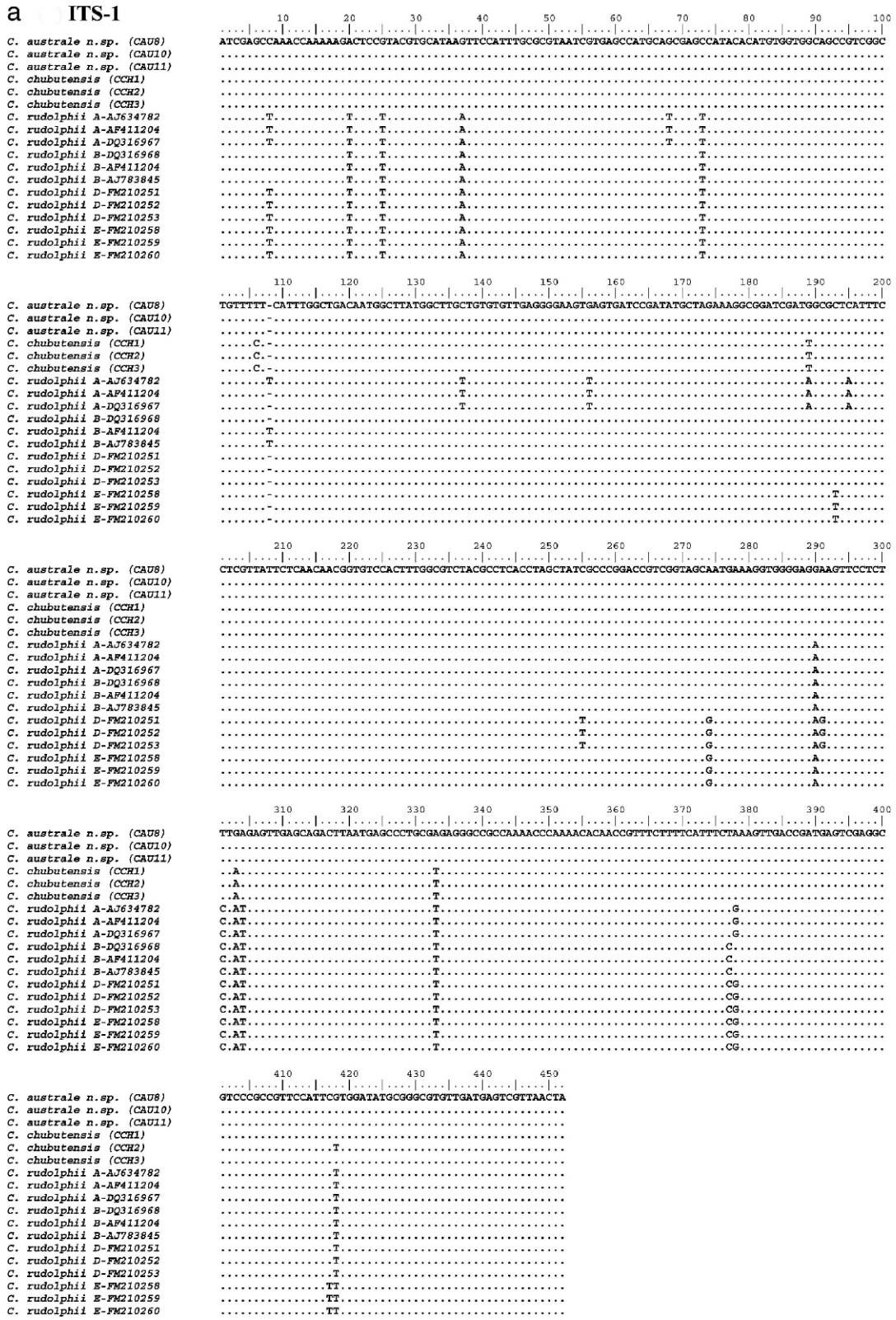

FIGURE 4. Alignment of sequences of nuclear ribosomal DNA ITS-1 (**a**) (451 bp) and ITS-2 (**b**) (284 bp) regions of *C. australe* n. sp. and *C. chubutensis* with respect to the siblings of the *C. rudolphii* complex sequenced so far at those loci and deposited in GenBank under the accession numbers as given in Shamsi et al. (2009). The alignment was performed using BioEdit (Hall, 1999). Three representative sequences for each *C. rudolphii* A, *C. rudolphii* B, *C. rudolphii* D, and *C. rudolphii* E were included for the comparison. Dot indicates identity and dash indicates gap.

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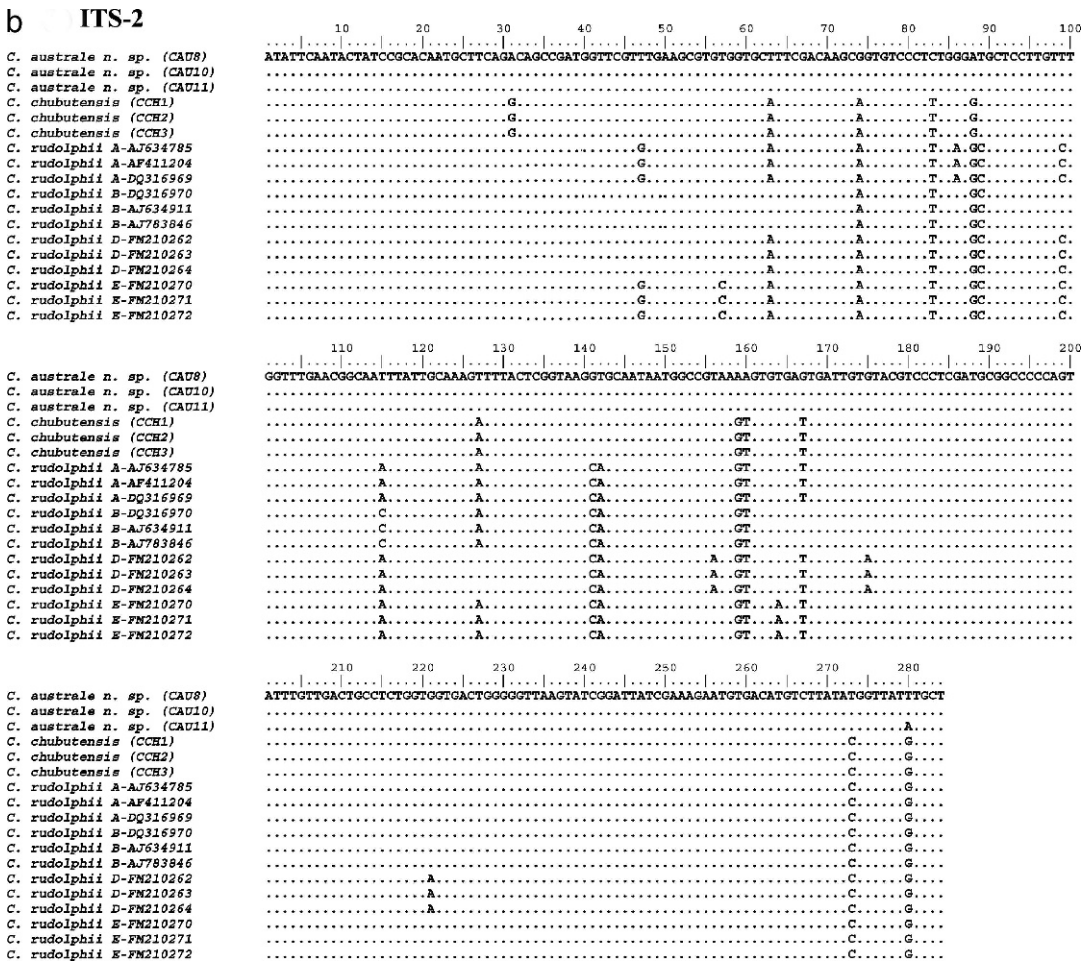

FIGURE 4. Continued.

rudimentary notches forming only 1 fissure and conspicuous auricles with conspicuous tips. The interlabium bifurcation is not as marked as that observed in *C. pelagicum* parasitizing the Magellanic penguin, and *C. chubutensis* in the imperial cormorant, *Ph. atriceps* (see Garbin et al., 2007, 2008). Paracloacal papillae appear to be smaller and their disposition is in an oblique angle with respect to the body axis. Finally, longer spicules seem to be common on the *Contracaecum* spp. parasitizing Phalacrocoracidae from both hemispheres. The only exception would be *C. chubutensis*, which does not show a well-marked distal constriction, lips with 3 obvious notches with smaller auricles, larger paracloacal papillae placed at a right angle with respect to the body axis, and shorter spicules (Garbin et al., 2008). In addition, these features of *C. chubutensis* resemble those also observed on the other closely related *Contracaecum* spp., i.e., *C. pelagicum* and *C. bioccai*, forming another sub-clade in the phylogenetic tree (Figs. 5–7), even though they are parasites in 3 different marine bird orders (Pelecaniformes, Sphenisciformes, and Procellariformes) from South America (Garbin et al., 2007; Mattiucci et al., 2008).

Morphological analysis and the differential diagnosis of genetically identified male specimens of *C. australe* have revealed differences in a number of features. These include absolute measurements of spicule length, the peculiar distal tail constriction observed in male specimens, the angle disposition of paracloacal papillae, and the interlabium shape and its bifurcation depth. Similar characters have been shown in previous studies to be useful diagnostic characters for anisakid nematodes (Fagerholm, 1989, 1991; Mattiucci et al., 2008, 2009, 2010).

The present study further indicates that molecular markers, such as those provided by different genes as used here, i.e., mtDNA *cox-2*, *rrnS*, and the ITS-1 and ITS-2 regions, are useful for distinguishing cryptic species of *Contracaecum* spp. among waterbirds (Mattiucci et al., 2008, 2010). In the present case, the DNA sequence analysis at multiple loci corroborated the evidence for *C. australe* and *C. chubutensis* as separate species. Detecting DNA barcodes in these genes may be helpful in the future for discriminating taxa where species overlapping and co-infection of the same definitive host may occur, especially when morphological differences are often difficult to discern.

The present study supports the evidence that the combining of morphology and molecular tools in delimiting and diagnosing of sibling species represents a valuable and efficient approach in the systematic studies of parasites, as recently underlined by Perez-Ponce de Leon and Nadler (2010). Indeed, this methodological approach has been successful recently in the discovery and description of siblings and new taxa of anisakid nematodes (Shamsi et al., 2008, 2009a, 2009b; Mattiucci et al., 2009, 2010).

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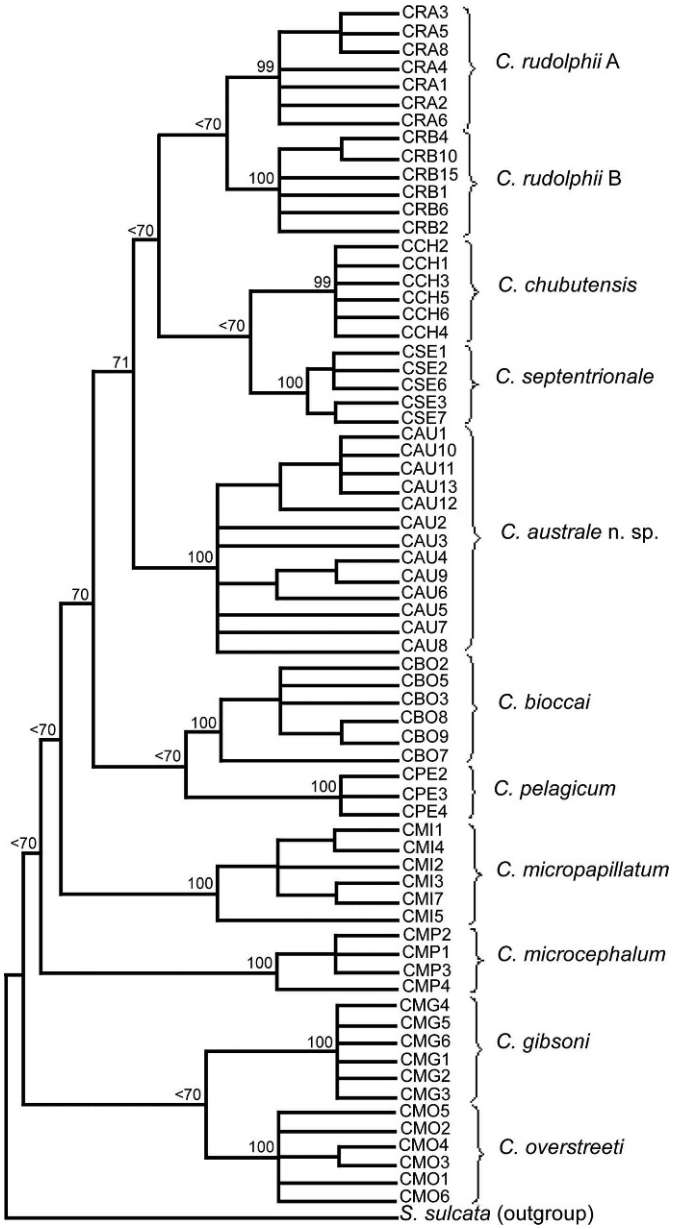

FIGURE 5. Maximum parsimony (MP) bootstrap consensus tree performed by PAUP* (Swofford, 2003) on 1,000 replicates, using the GTR+I+G evolution model (by Modeltest 3.1; Posada and Crandall, 1998) showing the genetic relationship of *C. australe* n. sp. and *C. chubutensis* with respect to *Contracaecum* spp. previously sequenced at the mtDNA *cox-2*. Bootstrap values are reported at the nodes (MP values: above; neighbor-joining values: below). *Sulcascaris sulcata* was used as outgroup.

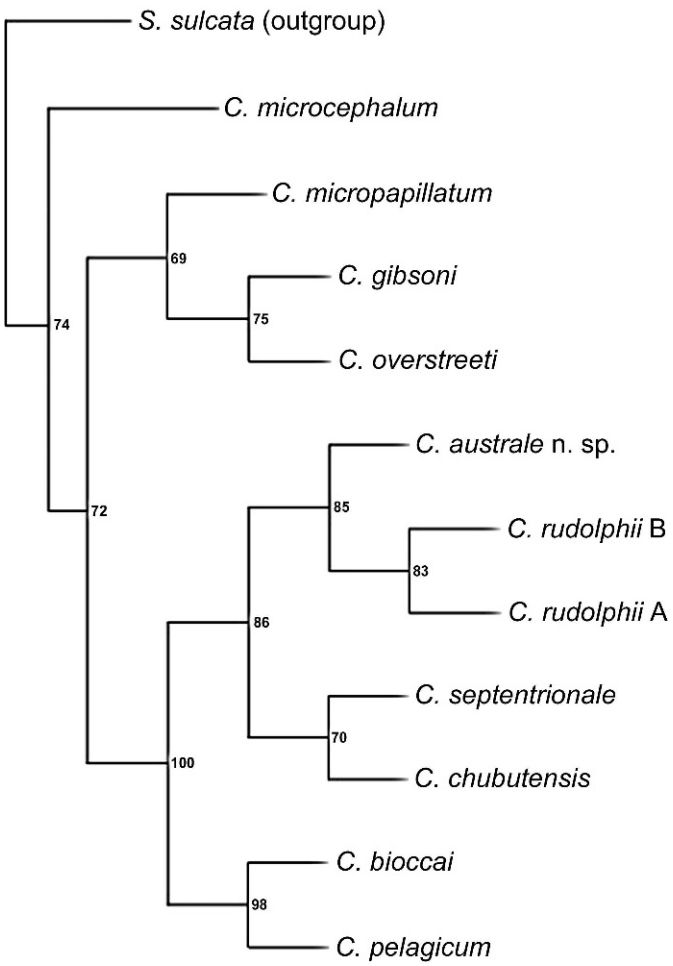

FIGURE 6. Consensus tree inferred by Bayesian analysis (BI) carried out using MrBayes3.1 software program (Ronquist and Huelsenbeck, 2003), inferred from mtDNA *cox-2* sequences analysis of *Contracaecum* spp. studied, on 1,000,000 generations. The BI was performed using the GTR+I+G model as the best-fit substitution model for the data. The parameters for the model inferred were the proportion of invariable sites (I) = 0.6020, distribution shape parameter (α) = 0.8524, and nucleotide frequencies A = 0.19, C = 0.07, G = 0.27, T = 0.45. Numbers at the nodes are posterior probabilities recovered by the Bayesian analysis with a significant support of $P \geq 95\%$. *Sulcascaris sulcata* was used as outgroup.

The application of molecular tools is of particular importance for identification of adults to the species level but also for larval stages occurring in fish. However, no data are so far available on the occurrence of the larval stage of *C. australe*. Studies on the Neotropic cormorant diet have not been conducted as extensively on the Atlantic coast as on the Pacific coast (Kalmbach et al., 2001; Gil de Weir et al., 2003; Barquete et al. 2008). In central Chile, Kalmbach et al. (2001) noted that the most frequent prey items were toad fish *Aphos porosus* (Batrachoididae) and tilefish *Prontilus luplaris* (Pinguipedidae) and, to a lesser extent, anchovy *Engraulis ringens* (Engraulidae). However, the role of these species in the *C. australe* life cycle has yet to be determined. Garbin et al. (2007) hypothesized that *Engraulis anchoita* may be an intermediate–paratenic host for *C. pelagicum* in the Magellan penguin *S. magellanicus* from Península Valdés coast, Chubut, Argentina, based on bird feeding behavior and preliminary molecular identification of the larval stages (data not shown).

The phylogenetic data here presented seem to confirm a general rule that all the *Contracaecum* species genetically characterized to date, i.e., *C. rudolphii* A, *C. rudolphii* B, *C. rudolphii* C, *C. septentrionale*, *C. chubutensis*, and *C. australe* form a well supported clade. Moreover, their main definitive hosts also form a supported clade (Hughes and Page, 2007), suggesting the existence of a possible parallelism between the phylogeny of the anisakid *Contracaecum* parasites of fish-eating birds and that

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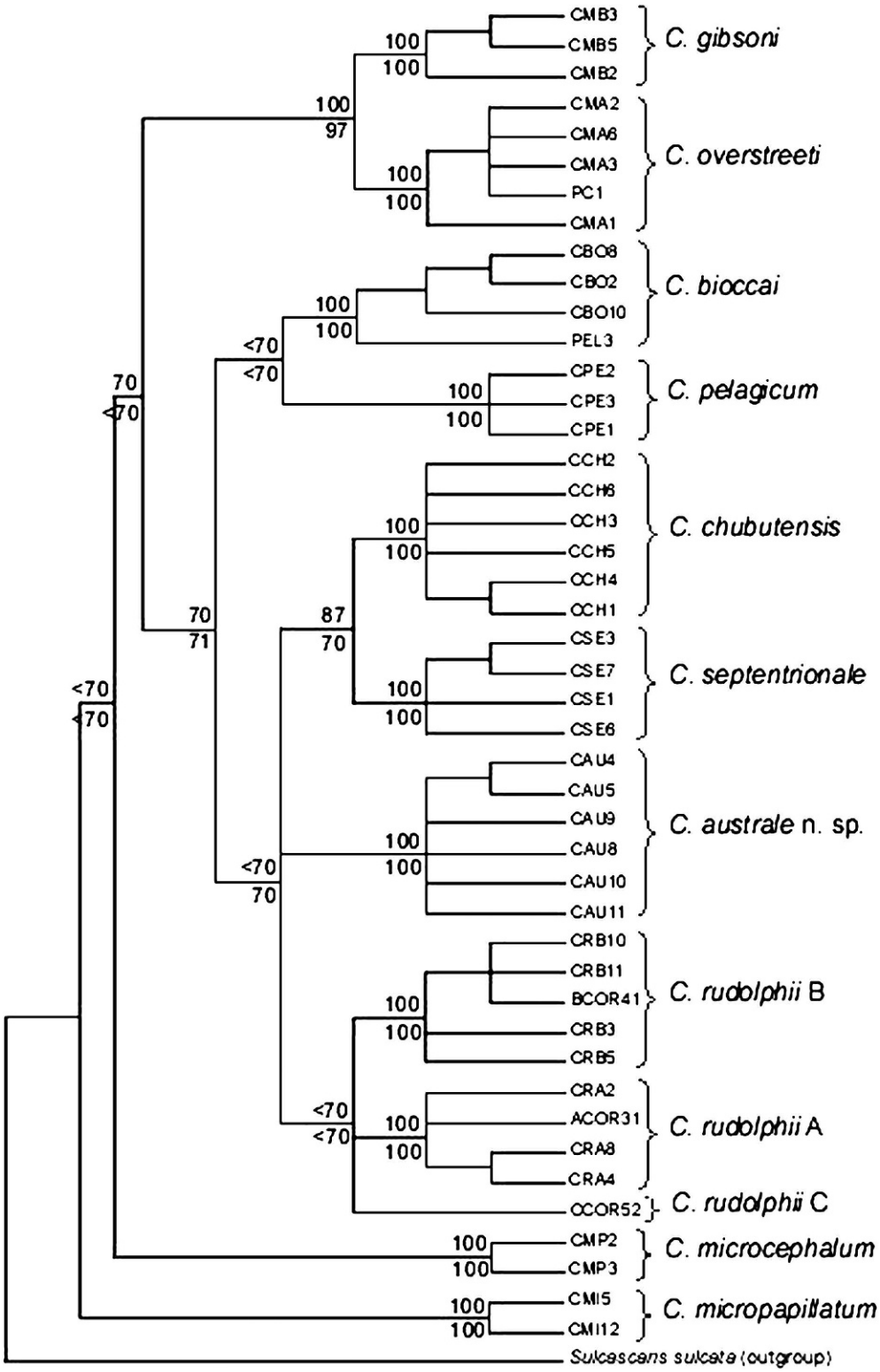

FIGURE 7. Condensed MP and neighbor-joining (NJ) bootstrap consensus trees by PAUP* (Swofford, 2003) on 1,000 replicates, based on *rrn*S sequences analysis of specimens of *Contracaecum* spp. sequenced in the present study in comparison with those sequenced by D'Amelio et al. (2007) and reported in the analysis with the following GenBank accession numbers: EF014281 (A-cor31), EF014279 (B-cor41), EF014283 (C-cor52), EF030716 (Pel3), EF014282 (Php1), EF014280 (Pc1). MP tree was obtained by bootstrap method on 1,000 replicates. The MP was performed using the GTR+I+G evolution model (by Modeltest 3.1; Posada and Crandall, 1998). Proportion of invariable sites (I) = 0.5937; gamma distribution shape parameter (G) = 0.7905; 166 polymorphic sites; 154 parsimony-informative sites; base frequencies A = 19%, C = 7%, G = 27%, T = 47%. Bootstrap values are shown at the nodes: MP and NJ values are shown above and below the nodes, respectively. *Sulcascaris sulcata* was used as outgroup.

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TABLE IV. Pairwise *p-distance* values inferred from mtDNA *cox*-2 (above the diagonal) and *rrnS* (below the diagonal) sequences analysis between *Contracaecum australe* n. sp. and *Contracaecum chubutensis* and versus other *Contracaecum* spp. so far sequenced at the same loci. Accession number for *rrnS* of *Contracaecum rudolphii* C deposited in GenBank is reported.

Species	CAU	CCH	CRA	CRB	CRC	CSE	CBO	CPE	CMP	CMI	CMG	CMO
Contracaecum australe n. sp. (CAU)	—	0.09	0.08	0.10	*	0.10	0.12	0.12	0.14	0.13	0.14	0.12
Contracaecum chubutensis (CCH)	0.03	—	0.09	0.10	*	0.06	0.12	0.11	0.13	0.13	0.14	0.11
Contracaecum rudolphii A (CRA)	0.04	0.03	—	0.08	*	0.08	0.11	0.11	0.13	0.12	0.12	0.12
C. rudolphii B (CRB)	0.04	0.03	0.03	—	*	0.10	0.12	0.13	0.14	0.14	0.12	0.11
C. rudolphii C (CRC) (EF014283)	0.04	0.04	0.03	0.03	—	*	*	*	*	*	*	*
Contracaecum septentrionale (CSE)	0.03	0.01	0.04	0.03	0.04	—	0.11	0.12	0.12	0.11	0.13	0.11
Contracaecum bioccai (CBO)	0.03	0.04	0.04	0.05	0.05	0.04	—	0.11	0.12	0.14	0.14	0.12
Contracaecum pelagicum (CPE)	0.04	0.04	0.06	0.05	0.05	0.04	0.05	—	0.15	0.13	0.15	0.14
Contracaecum microcephalum (CMP)	0.06	0.07	0.07	0.06	0.06	0.06	0.06	0.07	—	0.11	0.13	0.11
Contracaecum micropapillatum (CMI)	0.06	0.05	0.06	0.05	0.06	0.05	0.07	0.07	0.08	—	0.12	0.11
Contracaecum gibsoni (CMG)	0.07	0.06	0.07	0.07	0.08	0.05	0.08	0.06	0.07	0.06	—	0.10
Contracaecum overstreeti (CMO)	0.08	0.07	0.08	0.07	0.08	0.07	0.09	0.08	0.07	0.08	0.05	—

* The values were not calculated because sequences data of mtDNA cox2 in *C. rudolphii* C are missing.

TABLE V. Pairwise *p-distance* values inferred from the ITS-1 (above the diagonal) and ITS-2 (below the diagonal) sequences analysis between *Contracaecum australe* n. sp. and *Contracaecum chubutensis* and versus members of the *Contracaecum rudolphii* complex. Sequences and their accession numbers of the species *C. rudolphii* D and *C. rudolphii* E are those reported in Table III.

Species	CAU	CCH	CRA	CRB	CRD	CRE
Contracaecum australe n. sp. (CAU)	—	0.01	0.02	0.01	0.02	0.02
Contracaecum chubutensis (CCH)	0.02	—	0.02	0.01	0.02	0.02
Contracaecum rudolphii A (CRA)	0.03	0.02	—	0.01	0.01	0.01
C. rudolphii B (CRB)	0.02	0.01	0.01	—	0.01	0.01
C. rudolphii D (CRD)	0.03	0.02	0.01	0.01	—	0.01
C. rudolphii E (CRE)	0.03	0.02	0.01	0.01	0.01	—

proposed so far for their phalacrocoracid definitive hosts. Similar host–parasite associations between anisakid nematodes and their cetacean hosts have been demonstrated (Mattiucci and Nascetti, 2008).

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