Contracaecum bioccai n. sp. from the brown pelican *Pelecanus occidentalis* (L.) in Colombia (Nematoda: Anisakidae): morphology, molecular evidence and its genetic relationship with congeners from fish-eating birds

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Abstract *Contracaecum bioccai* n. sp. is described from the brown pelican *Pelecanus occidentalis* (L.) in northern Colombia (Totumo Marsh) based on 20 enzyme loci studied using multilocus allozyme electrophoresis. Moreover, genetic relationships between the new taxon and related congeners are presented based on allozyme data-sets and sequence analyses (519 bp) of the mtDNA-*cox2* gene. Fixed allele differences were found at some of the allozyme loci analysed in comparison with other *Contracaecum* spp. from pelicans and cormorants [i.e. the sibling species of the *C. rudolphii* Hartwich, 1964 complex, *C. septentrionale* Kreis, 1955, *C. micropapillatum* (Stossich, 1890), *C. microcephalum* (Rudolphi, 1809) and *C. pelagicum* Johnston & Mawson, 1942]. The genetic distance, at the allozyme level, between *C. bioccai* n. sp. and its congeners ranged from D_{Nei} = 0.80 versus *C. septentrionale* to D_{Nei} = 1.40 versus *C. micropapillatum*. The genetic distance at the mtDNA *cox-2* level ranged, on average, from K-2P = 0.12 versus the *C. rudolphii* species complex to K-2P = 0.15 versus *C. micropapillatum*. An overall concordant tree topology, obtained from UPGMA and NJ tree analyses inferred from allozyme data, as well as from MP, UPGMA and NJ inferred from *mtDNA-cox2* sequence analysis, showed *C. bioccai* n. sp. as a separated lineage to the other *Contracaecum* spp. A concordant result was also obtained by PCA analysis based on both the allozyme and mtDNA *cox-2* data-sets. All of the tree topologies, derived from the phylogenetic analysis inferred from both allozymes and mtDNA data-sets, were in substantial agreement and depicted *C. bioccai* as closely related to the sibling species of the *C. rudolphii* complex (*C. rudolphii* A and *C. rudolphii* B) and *C. septentrionale*. Morphological analysis and a differential diagnosis based on male specimens of *C. bioccai*, which had been genetically characterised by both allozyme markers and mtDNA sequences analysis with respect to morphologically related congeners, enabled the detection of differences in a numbers of characters, including spicule length, the morphology of the distal end of the spicule and the distribution patterns of the distal caudal papillae.

Introduction

Adult nematodes of the genus *Contracaecum* Railliet & Henry, 1912 have been reported, at the adult stage, in fish-eating birds (and seals) throughout the world. They are common parasites of the gut and proventriculus of waterbirds, often causing pathological lesions in their hosts (Huizinga, 1971; Fagerholm et al., 1996; Abollo et al., 2001; Dezfuli et al., 2002). Among the species of *Contracaecum* considered as valid by Hartwich (1964), *C. rudolphii* Hartwich, 1964 (*sensu lato*) (see Hartwich, 1964), *C. multipapillatum* (von Drasche, 1882) (see Lucker, 1941), *C. micropapillatum* (Stossich, 1890) and *C. microcephalum* (Rudolphi, 1809) have been recorded as parasites of various fish-eating birds from the Western Hemisphere (Huizinga, 1971; Courtney & Forrester, 1974; Grimes et al., 1989; Humphrey et al., 1978; Deardoff & Overstreet, 1980; Dyer et al., 2002; Navone et al., 2000; Dronen et al., 2003; Torres et al., 2005). In addition, *C. mexicanum* Flores-Barroeta, 1957 has been described from *Pelecanus occidentalis* (L.) near Acapulco, Mexico (Flores-Barroeta, 1957) and later reported from the same host in Central America by Dyer et al. (2002). Morphological characters have generally been used to differentiate the species of *Contracaecum* from fish-eating birds (Hartwich, 1964; Fagerholm, 1989, 1991; Fagerholm et al., 1996).

Previous studies, using multilocus allozyme electrophoresis (Bullini et al., 1986; Nascetti et al., 1990, 2000; D'Amelio et al., 1990; Mattiucci et al., 2002) have detected the presence of a high genetic heterogeneity within the species *C. rudolphii* collected from *Phalacrocorax carbo sinensis* (Blumenbach), the Eurasian subspecies of the great cormorant. Allozyme markers have indeed detected the presence of two sibling species within *C. rudolphii*, which have been named *C. rudolphii* A and *C. rudolphii* B (Bullini et al., 1986; Nascetti et al., 1990; D'Amelio et al., 1990); these species often occur sympatrically in the same individual hosts in various geographical areas of the European Boreal region (Mattiucci et al., 2002). Recent studies based on the SSCP analysis of the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of ribosomal DNA (rDNA) have also detected two sibling species within *C. rudolphii* (*sensu lato*) recovered from the Eurasian subspecies of the cormorant which likely correspond to *C. rudolphii* A and B; these studies have also established the validity of *C. septentrionale* Kreis, 1955 (see Li et al., 2005). Moreover, other genetic/molecular markers applied to the systematics of *Contracaecum* have enabled the study of genetic relationships between some *Contracaecum* spp. from fish-eating birds and those which are parasites of seals (Nascetti et al., 1990; Nadler et al., 2000, 2005).

The aim of this study is to analyse the genetic variation, inferred from nuclear (20 allozyme-loci) and mitochondrial (mtDNA *cox-2* sequences) markers, in specimens of *Contracaecum* collected from the brown pelican *Pelecanus occidentalis* in the Totumo Marsh, northern Colombia, which correspond morphologically with *C. rudolphii* (*s. l.*) in order to: (a) quantify the genetic divergence, inferred at the nuclear and mitochondrial level, between the *Contracaecum* material from Colombia with respect to the sibling species of the *C. rudolphii* complex (i.e. *C. rudolphii* A and *C. rudolphii* B) and to other *Contracaecum* spp. from waterbirds (i.e. *C. septentrionale, C. microcephalum*, *C. micropapillatum* and *C. pelagicum*); (b) establish the genetic relationship between this taxon and congeners which are available for genetic studies; and (c) morphologically characterise this taxon.

Materials and methods

Parasite material

A total of 40 adult specimens of a species of *Contracaecum*, collected from the stomach of the brown pelican *Pelecanus occidentalis* in the Totumo Marsh (northern Colombia), were genetically tested. In addition, samples of the two sibling species, *C. rudolphii* A and *C. rudolphii* B (see Bullini et al., 1986; Nascetti et al., 1990; Mattiucci et al., 2002), collected from *Phalacrocorax carbo sinensis*, the 'Eurasian subspecies' of the great cormorant, were included in this study for genetic comparison (Table 1). Furthermore, specimens of the following *Contracaecum* spp. from fish-eating birds were also considered: *C. septentrionale* from *P. aristotelis* (L.), *C. microcephalum* from *P. pygmaeus* (Pallas), *C.micropapillatum* from the white pelican *Pelecanus onocrotalus* (L.) at Aswan (Egypt), and finally, *C. pelagicum* from *Spheniscus*

Table 1 *Contracaecum* spp. which have been studied genetically

Parasite	Host	Nh	N *allozyme*	N *mtDNA* and specimen code	*Sampling locality*	*Collector*
Contracaecum sp.	*Pelecanus occidentalis* (Pelecanidae)	3	40	10 (CSP1-CSP2-CSP3-CSP4-CSP5-CSP6-CSP7-CSP8-CSP9-CSP10)	Totumo marsh (Northern Colombia)	J. Olivero-Verbel
C. rudolphii A	*Phalacrocorax carbo sinensis* (Phalacrocoracidae)	1	39	2 (CRA9-CRA10)	Gdansk Bay (Poland)	P. Myjak
C. rudolphii B	*Phalacrocorax carbo sinensis* (Phalacrocoracidae)	2	20	3 (CRB3-CRB4-CRB5)	Tarquinia salt marshes (Latium region, Italy)	G. Nascetti
C. rudolphii B	*Phalacrocorax carbo sinensis* (Phalacrocoracidae)	3	54	3 (CRB9-CRB10-CRB11)	Matsury Lakes (Poland)	P. Myjak
C. rudolphii (*s. l*)	*Phalacrocorax carbo sinensis* (Phalacrocoracidae)	3	140	3 (CRB1-CRB2-CRB12)	Oristano Lagoon, Sardinia (Italy)	S. Mattiucci
C. rudolphii (*s. l*)	*Phalacrocorax carbo sinensis* (Phalacrocoracidae)	2	120	8 (CRA1-CRA2-CRA3-CRA4-CRA5-CRA6-CRA7-CRA8); 3 (CRB6-CRB7-CRB8)	Venice lagoon, (North of Italy)	S. Mattiucci
C. septentrionale	*Phalacrocorax carbo carbo* (Phalacrocoracidae)	1	20	2 (CS3-CS5)	Hüsavik (Iceland)	B. Berland
	Phalacrocorax aristotelis (Phalacrocoracidae)	1	11	3 (CS1-CS2-CS4)	Karmoy (Norway)	B. Berland
C. microcephalum	*Phalacrocorax pygmaeus* (Phalacrocoracidae)	2	30	4 (CMP1-CMP2-CMP3-CMP4)	Scutari Lake (Montenegro region)	G. Nascetti
C. micropapillatum	*Pelecanus onocrotalus* (Pelecanidae)	1	40	5 (CMI1-CMI2-CMI3-CMI4-CMI5)	Assuan (Egypt)	G. Nascetti
C. pelagicum	*Spheniscus magellanicus* (Spheniscidae)	1	20	3 (CP1-CP2-CP3)	Peninsula Valdés (Argentina)	L. Garbin

Nh, number of hosts; *N* allozyme, number of specimens studied at 20 enzyme loci; *N* mtDNA, number of specimens sequenced at the mtDNA *cox2* gene

magellanicus (Forster) on the Argentine coast. The number of specimens tested, collecting sites and hosts of the *Contracaecum* spp. considered in this study are given in Table 1. The birds were found dead in the areas of collection. Nematodes collected from the proventriculus and stomach of their hosts were repeatedly washed in saline solution, then kept frozen at –70°C until analysed.

Multilocus allozyme electrophoresis (MAE)

The genetic variation of *Contracaecum* spp. samples was investigated by standard horizontal starch gel electrophoresis at 20 enzyme loci. These are: idditol dehydrogenase (*Iddh*, EC 1.1.1.14), malate dehydrogenase *(Mdh-1*, *Mdh-2*, *Mdh-3*, *Mdh-4*, EC 1.1.1.37), isocitrate dehydrogenase (*Icdh*, EC 1.1.1.42), 6–phosphogluconate dehydrogenase (*6Pgdh*, EC 1.1.1.43), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, EC 1.2.1.12), superoxide dismutase (*Sod-1*, *Sod-2*, EC 1.15.1.1), nucleoside phosphorylase (*Np*, EC 2.4.2.1), aspartate amino transferase (*Aat-2*, EC 2.6.1.1), adenylate kinase (*Adk-2*, EC 2.7.4.3), colorimetric esterase (*cEst-1*, EC 3.1.1), leucine-alanin peptidase (*PepC-1, PepC-2*, EC 3.4.11), mannose phosphate isomerase (*Mpi*, EC 5.3.1.8), glucose phosphate isomerase (*Gpi*, EC 5.3.1.9) and phosphoglucomutase (*Pgm-1, Pgm-2*, EC 5.4.2.2). Their staining procedures are reported, in detail, in Nascetti et al. (1993) and Mattiucci et al. (1997). Isozymes were numbered in order of decreasing mobility from the most anodal one. Allozymes were named using numbers indicating their mobility (in mm, standardised conditions) relative to the most common allele, designated as *100*, found in the reference population, a *Contracaecum rudolphii* A sample in *Phalacrocorax carbo sinensis* from brackish coastal lagoons at Oristano, Sardinia, Italy. The statistical significance of departures from the Hardy-Weinberg equilibrium was estimated using the $\chi 2$ test. The genetic divergence of populations and species was estimated using the following indices: standard genetic distance – D_{Nei} (Nei, 1972) and chord-distance – *Dc* (Cavalli-Sforza & Edwards, 1967). Population genetic analysis was performed using BIOSYS-2 software (Swofford & Selander, 1989). Genetic relationships between the species of the *C. rudolphii* complex and the other *Contracaecum* species considered here, were evaluated by UPGMA and Neighbour Joining (NJ) cluster analysis using PHYLIP version 3.57 (Felsenstein, 1995). Boostrap consensus analysis (500 replicates) was carried out to verify the robustness of the topologies obtained by PHYLIP (Felsenstein, 1995). PCA analysis was performed usingy SYN-TAX 2000 (Podani, 2005).

DNA amplification and sequencing

Some of the *Contracaecum* specimens genetically characterised at the allozyme level were also sequenced using mtDNA *cox-2*. A 519 bp fragment of the cytochrome oxidase 2 (*cox2*) gene was analysed for all the specimens of *Contracaecum* spp. listed in Table 1. Total DNA was extracted from 2 mg of tissue from a single nematode using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) and/or cetyltrithylammonium bromide (Murray et al., 1980; Valentini et al., 2006). The *cox2* gene from each species of *Contracaecum* was amplified using 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 & Hudspeth (2000) spanning mtDNA nucleotide position 10,639-11,248 as defined in *Ascaris suum* (Genbank X54253). PCR (polymerase chain reaction) amplification was carried out in a volume of 50 (l containing 30 pmol of each primer, $MgCl_2$ 2.5 mM (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), PCR buffer 1 X (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), DMSO 0.08 mM, dNTPs 0.4 mM (Sigma-Aldrich, St. Louis, MO), 5 U of *Taq* Polymerase (Amersham Pharmacia Biotech. Inc., 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 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. Reference specimens and isolated DNA samples were stored at the Section of Parasitology of the DSSP, "Sapienza" University of Rome.

Sequence analysis

The *cox2* sequences were aligned using ClustalW (Thompson et al., 1994), and a square matrix based on *p*-distance and K-2P was performed using

MEGA3.1 (Kumar et al., 2001). Phylogenetic analyses at the interspecific level were performed using "maximum parsimony" (MP) in PAUP* version 4.0 (Swofford, 2003). UPGMA and Neighbour-Joining (NJ) analyses, based on K-2P values, were performed using the MEGA3.1 program (Kumar et al., 2001). The reliabilities of the phylogenetic relationships were evaluated using nonparametric bootstrap analysis (Felsenstein, 1985) for the MP and NJ trees. PCA analysis on mtDNA sequences was performed using SYN-TAX 2000 (Podani, 2005). Sequences were deposited in GenBank with the following accession numbers: *C. bioccai* n. sp: EF122209, EF513494–513500 and EF558899–558900; *C. rudolphii* A: EF122201–122202, EF35570, EF513500–513501, EF558891–558892; *C. rudolphii* B: EF122203–122204, EF513506–513511 and EF558892–558896; *C. septentrionale*: EF122205, EF5135012–513513 and EF558897–558898; *C. pelagicum*: EF122210 and EF535568–535569; *C. micropapillatum*: EF122 206–122207 and EF5135014–EF513516; *C. microcephalum*: EF122208 and EF5135017–EF513519. The reliabilities of phylogenetic relationships were evaluated using nonparametric bootstrap analysis (Felsenstein, 1985) for NJ and MP trees. Bootstrap values ≥70 were considered well supported (Hills & Bull, 1993). Sequences at the mtDNA *cox-2* of *Pseudoterranova ceticola* Deardoff & Overstreet, 1982 from *Kogia breviceps* (Genbank DQ116435) and *Contracaecum osculatum* (Rud., 1802) (*sensu stricto*) (Nascetti et al., 1993) from *Halichoerus grypus* (Genbank EF122211) were included as outgroup to root the *Contracaecum* phylogenetic trees, based on the relationships of *Contracaecum* (*s. s.*). and *Pseudoterranova* spp., previously demonstrated in ribosomal and mitochondrial DNA analyses (Nadler et al., 2000, 2005).

Morphological study

For each adult specimen (n = 11), the overall body length was first measured directly. The middle part of the body was then cut and used to identify the individual specimen by both allozyme markers and by sequencing of the mtDNA *cox-2* gene. The anterior and posterior parts were cleared and mounted in lactophenol (1:1) for morphological studies. Specimens were studied using a compound microscope (×100–400) with a drawing apparatus. Measurements are presented in millimetres, except where indicated. Several characters considered of diagnostic use for anisakid nematodes (Fagerholm 1989, 1991; Paggi et al., 2000) have been analysed; they include interlabial structure, the pattern of distribution of male caudal papillae, spicule length and tip shape, and the size and pattern of the caudal papillae, which were labelled according to the nomenclature proposed by Fagerholm (1989). In order to consider allometric variation, caudal measurements of each male specimen were related to either total body length or to tail length; these were: spicule length/body length (*spi/len*); diameter of distal papilla 1/tail (*dd1/tail*); diameter distal papilla 2/tail (*dd2/tail*); diameter distal papilla 3/tail (*dd3/tail*); diameter distal papilla 4/tail (*dd4/tail*); distance between distal papilla 1 and d2/tail (*d1-d2/tail*), d1 and d3/tail (*d1-d3/tail*), d2 and d4/tail (*d2-d4/tail*), d3 and d4/tail (*d3-d4/tail*), d1 and d4/tail (*d1-d4/tail*), and paracloacal papilla and d3 (*pc-d3/tail*). Paratypes of *Contracaecum travassosi* Gutierrez, 1943 (CHIOC no 14426), and *C. plagiaticum* Lent & Freitas, 1948 (CHIOC no. 16630), from the Helminthological Collection of the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, were borrowed for examination.

Results

Genetic evidence for a new species of *Contracaecum* and genetic differentiation between it and its congeners

Allozyme data

Genetic variation between the specimens reported in Table 1 was examined at 20 enzyme loci. According to the diagnostic loci existing between *C. rudolphii* A and *C. rudolphii* B, detected in previous allozyme studies (Mattiucci et al., 2002), the sample of *C. rudolphii* (*s. l.*) collected from Venetian coastal lagoons (northern Italy) (Table 1) was found to include 95 specimens of *C. rudolphii* A, with the alleles *Mdh-4*^{100}, *Sod-1*^{100} and *PepC-1*^{100}, while 25 specimens were found to correspond to *C. rudolphii* B, having the alleles *Mdh-4*^{103}, *Sod-1*^{87} and *PepC-1*^{105}. On the basis of the same diagnostic enzyme loci, of the 140 specimens collected from *Phalacrocorax carbo sinensis* at the Oristano Lagoon, Sardinia, it was

possible to attribute 102 specimens to *C. rudolphii* A and 38 to *C. rudolphii* B. All of the specimens recovered from the same host, *P. carbo sinensis*, in north-eastern Poland (Mazury Lakes) (Table 1) were genetically identified as *C. rudolphii* B.

On the other hand, the sample of 40 adult specimens of *Contracaecum* sp., collected from the brown pelican *Pelecanus occidentalis* in the Totumo Marsh, northern Colombia, exhibited (Table 1) at some enzyme loci (*Mdh-4, Icdh, Adk-2, PepC-1*) distinct and unique alleles, which were not observed in other studied *Contracaecum* species from fish-eating birds (Table 2) or in specimens morphologically identified as *C. multipapillatum* (*s. l.*) and occurring concurrently in the same host individuals (Mattiucci et al., unpubl. data). Consequently, they correspond to a new gene pool and are considered to represent a new species of *Contracaecum*. At the allozyme level, the new taxon was found to be genetically more closely related to the sibling species *C. rudolphii* A and *C. rudolphii* B (av. D_{Nei} = 0.90) and to *C. septentrionale* (D_{Nei} = 0.80) rather than to *C. microcephalum* (D_{Nei} = 1.30), *C. pelagicum* (D_{Nei} = 1.19) and *C. micropapillatum* (D_{Nei} = 1.40) (Table 3).

mtDNA cox-2 data

The alignments of the mtDNA *cox-2* sequences (519 bp) for the new taxon and for genetically characterised species of *Contracaecum* are reported in Fig. 1.

The *cox2* fragment in all of the *Contracaecum* spp. analysed was found to be AT rich (64.2%, 70.1% and 60.7%, respectively at the first, second and third codon positions). Moreover, 155 (30%) variable sites were identified for the 519 bp analysed. At the amino acid level, a total of 28 variable positions were found in the *Contracaecum* species studied.

Some of the *Contracaecum* specimens collected from *Phalacocorax carbo sinsensis* in the Venetian Lagoon (Table 1) matched the sequences of the species identified at the allozyme level as corresponding to *C. rudolphii* A or to *C. rudolphii* B. Similarly, some individuals sequenced for the mtDNA *cox-2* gene, recovered from *P. carbo sinsensis* at Oristano and previously characterised at the allozyme level as corresponding to the sibling species *C. rudolphii* B, matched the sequences observed for this sibling species identified by allozymes in cormorants from the Mazury Lakes in Poland (Table 1).

On the other hand, the specimens of the new species recovered from the brown pelican in Colombia and genetically characterised at the allozyme level did not match any of the sequences of the species studied genetically (e.g. *C. rudolphii* A and B, *C. septentrionale, C. microcephalum, C. micropapillatum* and *C. pelagicum*).

In Table 4 the values of genetic differentiation at the *mtDNA cox-2* gene level, inferred from *p-distance* and Kimura-2-parameter (K-2P) distance values, are reported. The lowest interspecific values of differentiation were found in the comparison of the new gene pool of *Contracaecum* with respect to the two sibling species of the *C. rudolphii* complex (av. K-2P = 0.12) and to *C. septentrionale* (K-2P = 0.12) (Table 4). Higher values of *p-distance* and K-2P were found when comparing the new species with *C. pelagicum* (K-2P = 0.13), *C. microcephalum* (K-2P = 0.14) and a morphologically well-differentiated species, *C. micropapillatum* (K-2P = 0.15) (Table 4).

Morphological data

Adult specimens of the new taxon indicated above were also studied morphologically. As a consequence of the genetic (at both allozyme and mitochondrial levels) and the morphological evidence (see below), it was considered as new to science and named *Contracaecum bioccai* n. sp.

***Contracaecum bioccai* n. sp.**

Type-host: Pelecanus occidentalis (L.).
Type-locality: Totumo Marsh, northern Colombia.
Site: Stomach.
Type-material: Holotype (anterior and posterior regions of a male); collector Jesus Olivero-Verbel, Universidad de Cartagena; Natural History Museum, London, BMNH 2007.6.15-1. Paratypes: 3 males and 1 female (anterior and posterior regions) from the same host; Collection of the Section of Parasitology, Department of Public Health Sciences, "Sapienza" – University of Rome; 1 male and 1 female, Natural History Museum, London, BMNH 2007.6.15.2-3.
Other material examined: Three males and 3 females from the same host and locality.

Table 2 Allele frequencies at 20 enzyme loci in *Contracaecum bioccai* n. sp. in comparison to those observed in other species of the genus from fish-eating birds

Locus	*C. bioccai* n. sp.	*C. rudolphii* A	*C. rudolphii* B	*C. septentrionale*	*C. micropapillatum*	*C. microcephalum*	*C. pelagicum*
Iddh							
80	–	0.03	0.11	–	1.00	–	–
90	–	0.08	0.85	0.08	–	–	–
95	–	0.02	–	–	–	–	–
100	0.98	0.82	0.02	0.92	–	0.98	1.00
109	0.02	0.03	0.02	–	–	0.02	–
115	–	0.02	–	–	–	–	–
Mdh-1							
72	–	–	–	0.05	–	–	–
84	–	0.01	0.02	0.02	1.00	–	–
90	–	–	–	0.93	–	–	–
96	–	–	–	–	–	1.00	–
100	1.00	0.80	0.98	–	–	–	–
108	–	0.19	–	–	–	–	1.00
Mdh-2							
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-3							
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-4							
100	–	1.00	–	–	–	–	–
103	–	–	1.00	1.00	–	–	–
104	1.00	–	–	–	–	–	–
105	–	–	–	–	–	1.00	–
107	–	–	–	–	–	–	1.00
113	–	–	–	–	1.00	–	–
Icdh							
90	–	0.02	–	–	–	–	–
97	–	–	–	–	–	–	1.00
100	1.00	0.51	1.00	0.99	–	1.00	–
107	1.00	–	–	–	–	–	–
112	–	0.47	–	0.10	1.00	–	–
6Pgdh							
80	–	0.05	0.02	–	–	–	–
85	–	0.03	–	0.03	–	–	–
92	–	–	–	–	–	0.02	–
95	–	–	–	–	1.00	–	–
98	0.02	–	–	0.94	–	0.98	–
100	–	0.87	0.98	–	–	–	1.00
114	0.98	0.05	–	0.03	–	–	–
Gapdh							
100	1.00	1.00	1.00	–	1.00	1.00	–
107	–	–	–	1.00	–	–	–
110	–	–	–	–	–	–	1.00

Table 2 continued

Locus	*C. bioccai* n. sp.	*C. rudolphii* A	*C. rudolphii* B	*C. septentrionale*	*C. micropapillatum*	*C. microcephalum*	*C. pelagicum*
Sod-1							
87	–	–	1.00	–	–	–	–
97	–	1.00	–	1.00	–	–	1.00
100	1.00	–	–	–	1.00	1.00	–
Sod-2							
100	–	1.00	1.00	–	1.00	1.00	–
105	1.00	–	–	1.00	–	–	–
110	–	–	–	–	–	–	1.00
Np							
80	1.00	–	–	0.03	–	–	–
88	–	0.29	0.01	0.95	–	–	–
92	–	–	–	–	–	–	1.00
100	–	0.71	0.99	0.02	–	–	–
136	–	–	–	–	1.00	–	–
138	–	–	–	–	–	1.00	–
Aat-2							
87	–	–	–	0.03	–	–	–
90	–	–	–	–	1.00	–	–
92	1.00	–	–	–	–	1.00	–
100	–	1.00	1.00	0.97	–	–	–
105	–	–	–	–	–	–	1.00
Adk-2							
90	–	–	–	1.00	–	–	–
100	–	1.00	1.00	–	–	1.00	1.00
102	–	–	–	–	1.00	–	–
105	1.00	–	–	–	–	–	–
cEst-1							
90	1.00	–	0.02	–	–	–	1.00
94	–	–	0.01	–	–	–	–
98	–	0.07	0.07	0.06	–	–	–
100	–	0.90	0.90	0.94	–	–	–
104	–	0.03	–	–	–	1.00	–
106	–	–	–	–	1.00	–	–
PepC-1							
100	–	1.00	–	1.00	–	1.00	–
105	–	–	1.00	–	1.00	–	–
108	–	–	–	–	–	–	1.00
110	1.00	–	–	–	–	–	–
PepC-2							
80	–	–	–	–	1.00	–	–
100	1.00	0.94	0.99	–	–	1.00	–
104	–	0.06	0.01	–	–	–	–
108	–	–	–	–	–	–	1.00
110	–	–	–	1.00	–	–	–

Table 3 Average of standard genetic distance of Nei (1972) (D_{Nei}, below the diagonal) and chord distance of Cavalli-Sforza & Edwards (1967) (Dc, above the diagonal), inferred from 20 enzyme-loci, between the *Contracaecum* spp. from fish-eating birds

Species	*C. bioccai* n. sp.	*C. rudolphii* A	*C. rudolphii* B	*C. septentrionale*	*C. microcephalum*	*C. micropapillatum*	*C. pelagicum*
C. bioccai n. sp.	–	0.70	0.66	0.60	0.78	0.79	0.75
C. rudolphii A	0.90	–	0.44	0.61	0.60	0.73	0.71
C. rudolphii B	0.91	0.35	–	0.68	0.68	0.76	0.78
C. septentrionale	0.80	0.70	0.88	–	0.72	0.80	0.76
C. microcephalum	1.20	0.58	0.98	0.84	–	0.65	0.75
C. micropapillatum	1.40	1.03	1.34	1.70	0.70	–	0.75
C. pelagicum	1.19	0.96	1.32	1.25	1.19	1.20	–

dd4 = 10 (10) µm; distal papillae d1, d2 and d4 disposed in longitudinal row, with d3 lateral to d1; distance between d1 and d2 (*d1-d2*) = 30 (30) µm, between d2 and d4 (*d2-d4*) = 30 (30) µm, between d1 and d4 (*d1-d4*) = 60 (60) µm, between d3 and d4 (*d3-d4*) = 50–60 (50) µm, between d1 and d3 (*d1-d3*) = 30–40 (40) µm; distance between pc and d3 (*pc-d3*) = 60 (60) µm. One pair of very small papilla-like phasmids situated lateral to d4. Ratios between absolute caudal measurements related to body/tail length are: *spi/len*: 0.25–0.28 (0.25), *dd1/tail*: 0.04 (0.04), *dd2/tail*: 0.04 (0.04), *dd3/tail:* 0.04 (0.04), *dd4/tail*: 0.04 (0.04), *d1-d2/tail*: 0.11–0.12 (0.12), *d1-d3/tail:* 0.11–012 (0.12), *d2-d4/tail* 0.11–0.12 (0.12), *d3-d4/tail*: 0.22–0.25 (0.25), *d1-d4/tail*: 0.21–0.25 (0.25), *pc-d3/tail*: 0.22–0.24 (0.22).

Female

Total length 26–34. Oesophagus 4.40–4.60 in length, 0.40–0.50 in width. Ventricular appendix 0.60–0.90 in length. Intestinal caecum 3.20–3.60 in length. Vulva in first third of body. Eggs subspherical, 40–50 µm.

Differential diagnosis

According to the morphological characters considered as of diagnostic value for species of *Contracaecum* from fish-eating birds (i.e. the length of the spicules, the morphology of the distal end of the spicule and the bifurcation of the interlabial tip – see Hartwich, 1964), our specimens collected from *Pelecanus occidentalis* in Colombia would be assigned to the species *C. rudolphii* (*s. l.*). However, the genetic/molecular markers from multilocus allozyme electrophoresis applied here have demonstrated that the specimens belong to a taxon clearly distinct from the previously recognised sibling species *C. rudolphii* A and *C. rudolphii* B. Previous findings of *C. rudolphii* (*s. l.*) from the central and southern fish-eating birds of Central and South America include: *C. rudolphii* (*s. l.*) recorded in the Neotropic cormorant *Phalacrocorax brasilianus* (Gmelin) from southern Chile (Torres et al., 2005), in *P. auritus* (Lesson) from the coast of Texas, USA (Fedynich et al., 1997), in *P. albiventer* (Lesson) from Punta Leon, Argentine coast (Malacalza et al., 1998), in *Pelecanus occidentalis* from Florida (Courtney & Forrester, 1974) and in *P. occidentalis* from Puerto Rico (Dyer et al., 2002). Moreover, other species of

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C. micropapillatum	1.40	1.03	1.34	1.70	0.70	–	0.75
C. pelagicum	1.19	0.96	1.32	1.25	1.19	1.20	–

dd4 = 10 (10) µm; distal papillae d1, d2 and d4 disposed in longitudinal row, with d3 lateral to d1; distance between d1 and d2 (*d1-d2*) = 30 (30) µm, between d2 and d4 (*d2-d4*) = 30 (30) µm, between d1 and d4 (*d1-d4*) = 60 (60) µm, between d3 and d4 (*d3-d4*) = 50–60 (50) µm, between d1 and d3 (*d1-d3*) = 30–40 (40) µm; distance between pc and d3 (*pc-d3*) = 60 (60) µm. One pair of very small papilla-like phasmids situated lateral to d4. Ratios between absolute caudal measurements related to body/tail length are: *spi/len*: 0.25–0.28 (0.25), *dd1/tail*: 0.04 (0.04), *dd2/tail*: 0.04 (0.04), *dd3/tail:* 0.04 (0.04), *dd4/tail*: 0.04 (0.04), *d1-d2/tail*: 0.11–0.12 (0.12), *d1-d3/tail:* 0.11–012 (0.12), *d2-d4/tail* 0.11–0.12 (0.12), *d3-d4/tail*: 0.22–0.25 (0.25), *d1-d4/tail*: 0.21–0.25 (0.25), *pc-d3/tail*: 0.22–0.24 (0.22).

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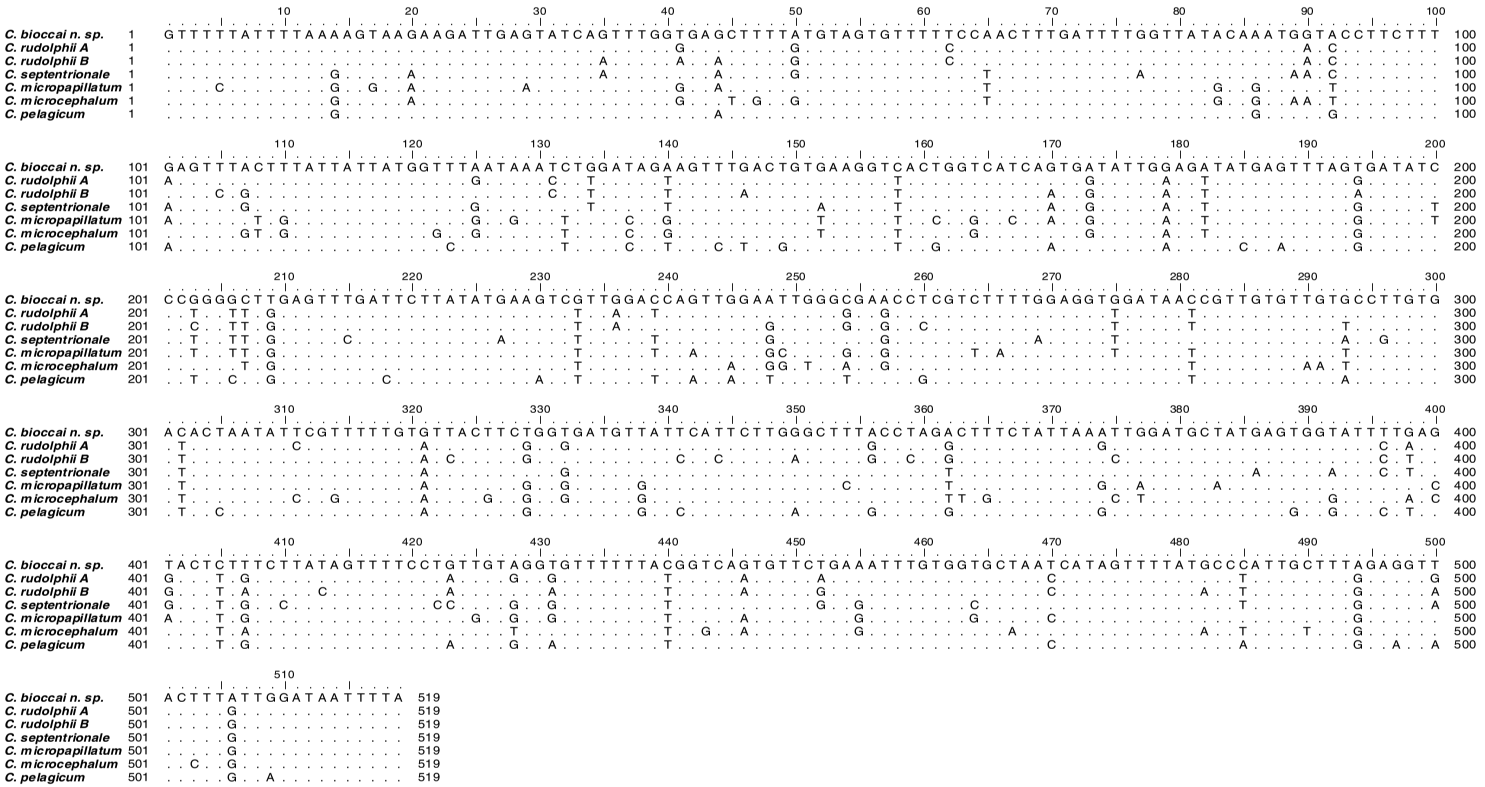

Fig. 1 Alignment of mtDNA *cox-2* (519 bp) sequences of *Contracaecum* species, using BioEdit (Hall, 1999). One representative of each unique sequence was included for the comparison. Dots indicate identity

Table 4 Genetic distance values, calculated using the *Kimura-2-parameter* K2P (above the diagonal) and *p-distance* (below the diagonal), between *Contracaecum* spp. from fish-eating birds, inferred from 519 bp of mtDNA *cox-2*

Species	*C. bioccai* n. sp.	*C. rudolphii* A	*C. rudolphii* B	*C. septentrionale*	*C. microcephalum*	*C. micropapillatum*	*C. pelagicum*
C. bioccai n. sp	–	0.12	0.13	0.12	0.14	0.15	0.13
C. rudolphii A	0.11	–	0.09	0.09	0.14	0.13	0.12
C. rudolphii B	0.12	0.08	–	0.11	0.15	0.16	0.14
C. septentrionale	0.11	0.08	0.10	–	0.13	0.12	0.14
C. microcephalum	0.12	0.13	0.14	0.12	–	0.12	0.17
C. micropapillatum	0.14	0.12	0.14	0.11	0.11	–	0.14
C. pelagicum	0.11	0.11	0.12	0.12	0.15	0.13	–

Contracaecum reported as parasitising *P. occidentalis* include *C. mexicanum* Flores-Barroeta, 1957 and *C. multipapillatum* (see Dyer et al., 2002), from which *C. bioccai* n. sp. can be easily distinguished by not having a double paracloacal papilla (pc), the distribution pattern of the proximal papillae and the length of the spicules. Moreover, *C. bioccai* is genetically very distinct, at the allozyme level, with respect to *C. multipapillatum* (*s. l.*) collected in the same host and locality (Mattiucci et al., unpubl.). Deardoff & Overstreet (1980) reported, in the brown pelican *P. occidentalis* and the American white pelican *P. erythrorhynchus* (Gmelin) from the Gulf of Mexico, concurrent infections of *C. microcephalum* with *C. multipapillatum* (*s. l.*) and *C. rudolphii* (*s. l.*). However, according to the length of the spicules and the distinctly bifurcate tips of the interlabia, *C. bioccai* cannot be assigned to *C. microcephalum* (*sensu* Hartwich, 1964 and Barus et al., 1978), from which it is genetically well differentiated at both the allozyme and the mtDNA levels. According to the above-mentioned morphological features, the new taxon is also morphologically differentiated from *C. variegatum* (Rudolphi, 1809) (*sensu* Hartwich, 1964 and Barus et al., 1978) by the shape of the spicules, which are sharply pointed distally in *C. variegatum* (see Hartwich, 1964). The new taxon is also genetically distinct in both allozyme and mtDNA *cox-2* analyses from *C. variegatum* (*s. l.*) (Mattiucci et al., unpubl.). Finally, it also differs morphologically from *C. oschmarini* Mosgovoy, 1950, described from *Uria lomvia* in Eastern Europe and later synonymised by Hartwich (1964) with *C. variegatum*, in terms of shorter spicules (4.6–4.9 versus 5.8–6.2 mm) with a different shape; the host and geographical distribution so far reported for that species are also different. *C. bioccai* also differs morphologically from *C. septentrionale* by its shorter spicules (5.8–6.5 versus 12.57–15.16 mm). Furthermore, it has been shown, in the present paper, that it is also genetically well differentiated, at both allozyme and mtDNA levels, from *C. septentrionale*.

Among those species retained as synonyms by Hartwich (1964) and later reconsidered by Barus et al. (1978), there is a species which our specimens do resemble, i.e. *C. travassosi* Gutierrez, 1943, originally described from *Phalacrocorax albiventer* (King) in Argentina. This resemblance is based on the

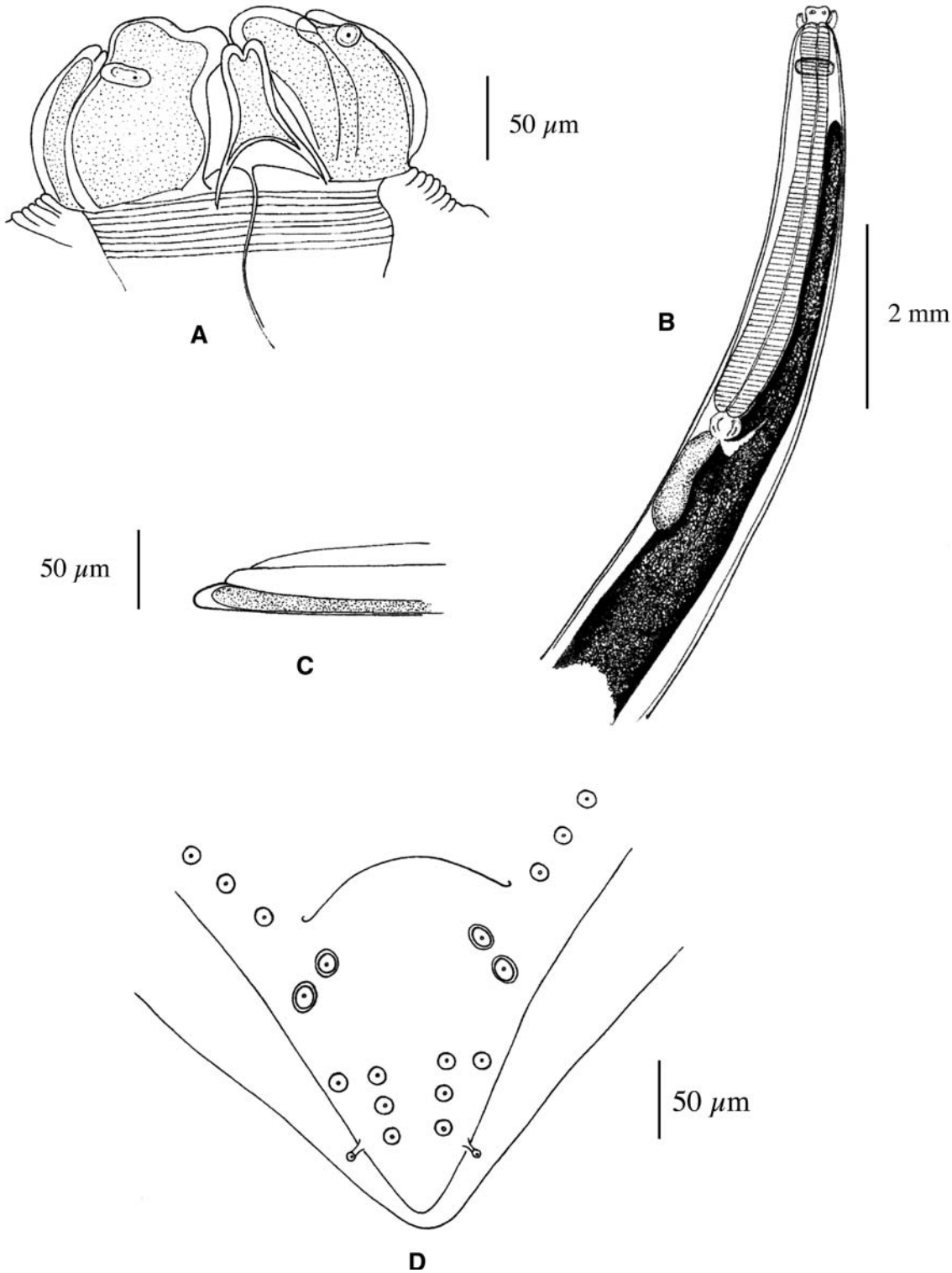

Fig. 2 *Contracaecum bioccai* n. sp. from *Pelecanus occidentalis*. A. Anterior end (subdorsal view); B. Anterior end (dorsal view); C. Distal end of spicule; D. Posterior end of male (ventral view)

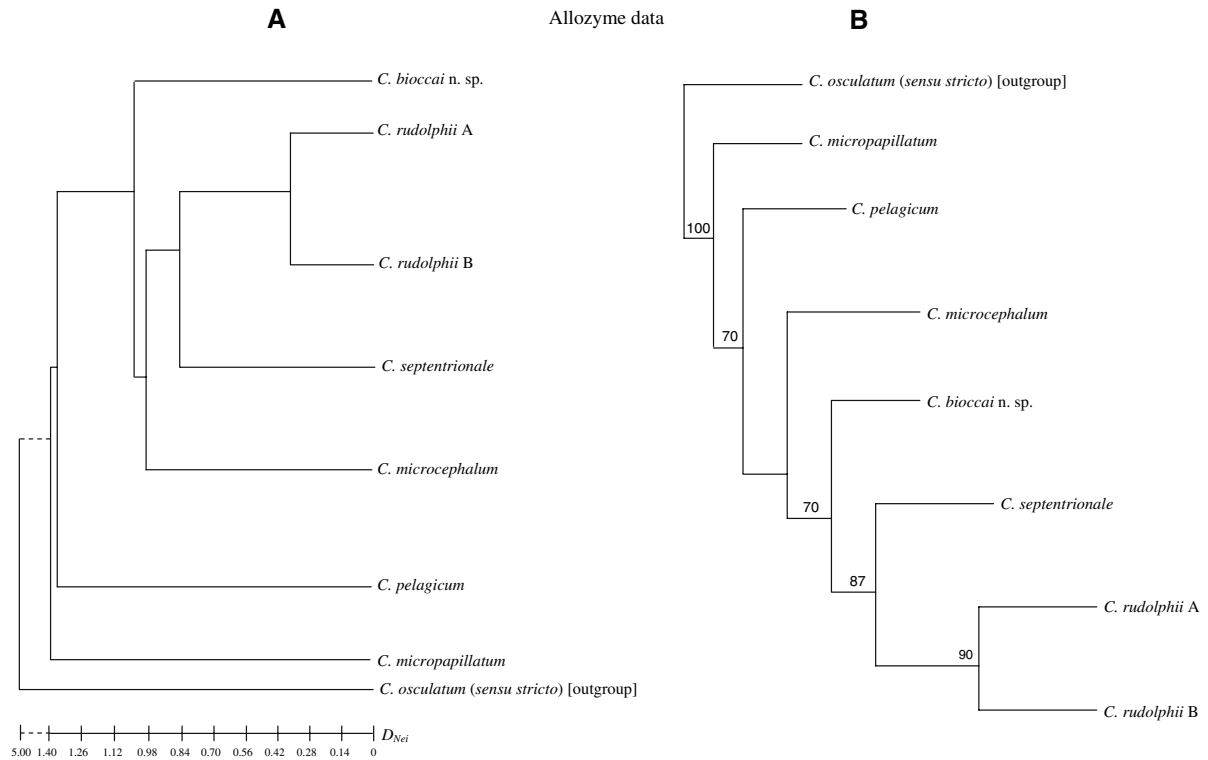

Fig. 3 UPGMA dendrogram from (A) D_{Nei} (Nei, 1972) genetic distance values and (B) NJ using the Cavalli-Sforza & Edwards (1967) chord distance, *Dc*, based on 20 enzyme loci, showing the genetic relationships between *C. bioccai* n. sp. and other *Contracaecum* spp. (bootstrap values >500 replicates; percentages ≥70% are shown at the internal nodes). *C. osculatum* (*s. s.*) is the outgroup in the NJ analysis

longitudinal arrangement of the distal (d1, d2, d3 and d4) papillae, as illustrated in the original figure and later reported by Morgan et al. (1949) in material from *Pandion haliaetus* (L.) in North America. According to the original description given by Gutierrez (1943), the paracloacal papillae are double; however, in the original figure they appear to be two separate papillae, although they are very close to each other. A morphological re-examination of one male paratype *C. travassosi* showed no clear evidence that the paracloacal papillae are separate and the length of the spicules is different from our specimens (5.8–6.2 mm versus 9.3 mm in the *C. travassosi* paratype), the ratio *spi/len* is 0.34 (versus 0.25–0.28 in *C. bioccai*), and the host and the known geographical distribution of *C. travassosi* are different.

The new taxon is also morphologically distinct from *C. plagiaticum* Lent & Freitas, 1948, described from *Nycticorax nyctycorax* (Forster) in Uruguay; indeed, a morphological re-examination of one male paratype of this species revealed that the new taxon has much longer spicules (5.8–6.2 versus 3.2 mm).

Another species which the new taxon might resemble is *C. pelagicum* Jonhston & Mawson, 1942, described from *Diomedea* spp. off Australia and later reported from the albatross off Uruguay (Lent & Freitas, 1948) and *Spheniscus magellanicus* (Forster) off Brazil (Portes-Santos, 1984), and recently described from *S. magellanicus* and *Diomedea melanophris* (Temmick) on the Argentine coast (Garbin et al., 2007). However, *C. pelagicum* has shorter spicules (4.3–4.5 versus 5.8–6.2 mm) and sharp-pointed distal ends of the spicules. Furthermore, it has been shown, in the present paper, that *C. bioccai* is also genetically distinct, at both allozyme (D_{Nei} = 1.19) and mtDNA-*cox2* (K-2P = 0.13) levels from *C. pelagicum* from *S. magellanicus* on the Argentine coast.

Finally, *C. bioccai* was found to be morphologically distinct from the sibling species *C. rudolphii* A

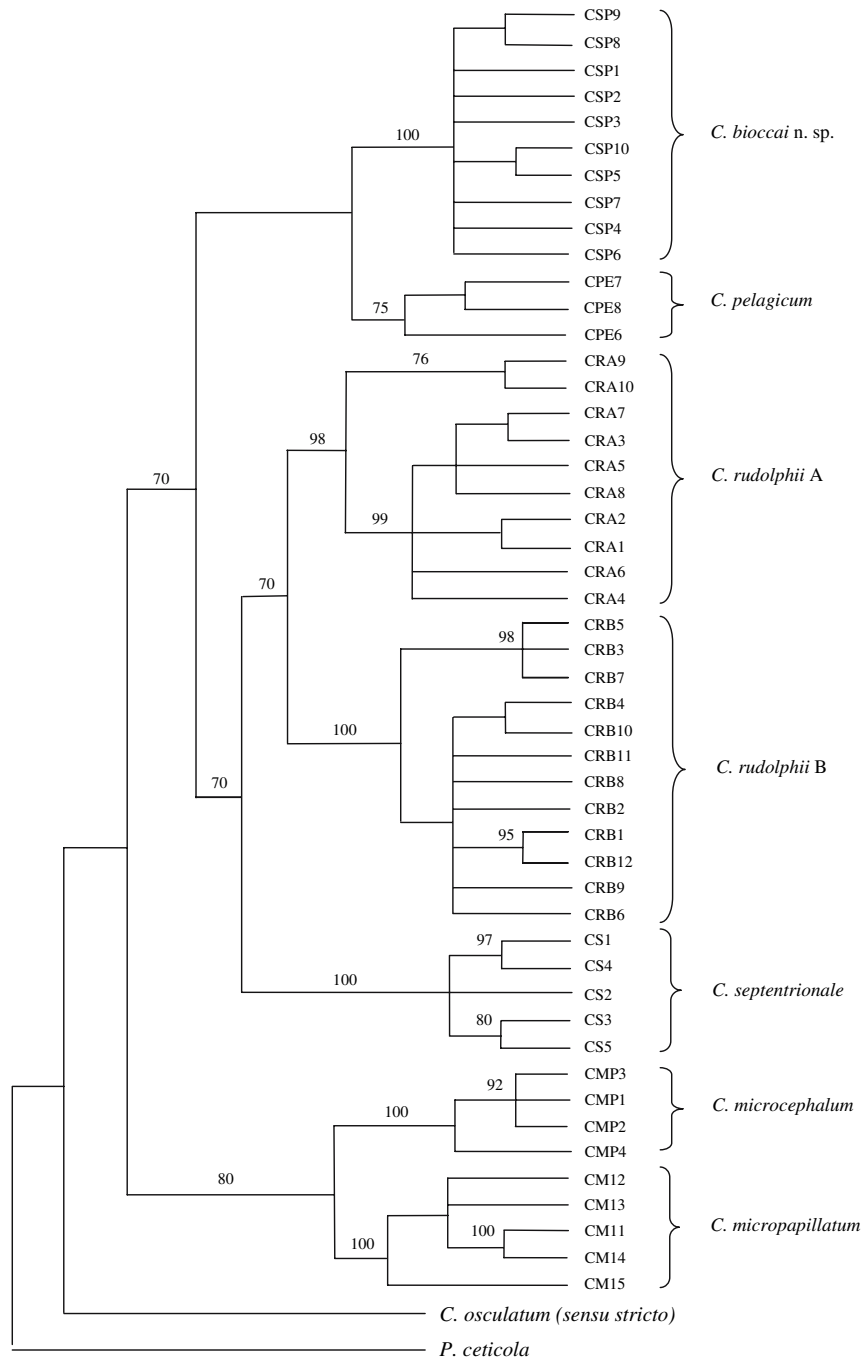

Fig. 4 *Cox-2* derived Maximum Parsimony (MP) tree using PAUP for the *Contracaecum* specimens sequenced (see Table 1 for specimen codes). The MP tree was obtained by the bootstrap method with a heuristic search on 100 replicates. There are 146 parsimony-informative characters and 22 variable parsimony uninformative characters. Bootstrap values ≥70% are shown at the internal nodes. *C. osculatum* (*s. s.*) and *Pseudoterranova ceticola* are outgroups

and *C. rudolphii* B, having shorter spicules (5.8–6.2 mm versus 6.8–7.2 for *C. rudolphii* A and 8.6–9.5 mm for *C. rudolphii* B) and a different spicule length/body length ratio (0.25–0.28 versus 0.31–0.32 in *C. rudolphii* A and 0.38–0.40 in *C. rudolphii* B). In addition, in *C. bioccai* the distal papillae d1, d2 and d4 are in a longitudinal row and d3 is lateral to d1 (Fig. 2), whereas in *C. rudolphii* A and B the distal papillae d1, d2, d3 and d4 form a rectangle, with d3 and d4 being lateral. Furthermore, in *C. rudolphii* A the size of the d2 papilla is smaller (5–6 μm) than in *C. bioccai* (10 μm).

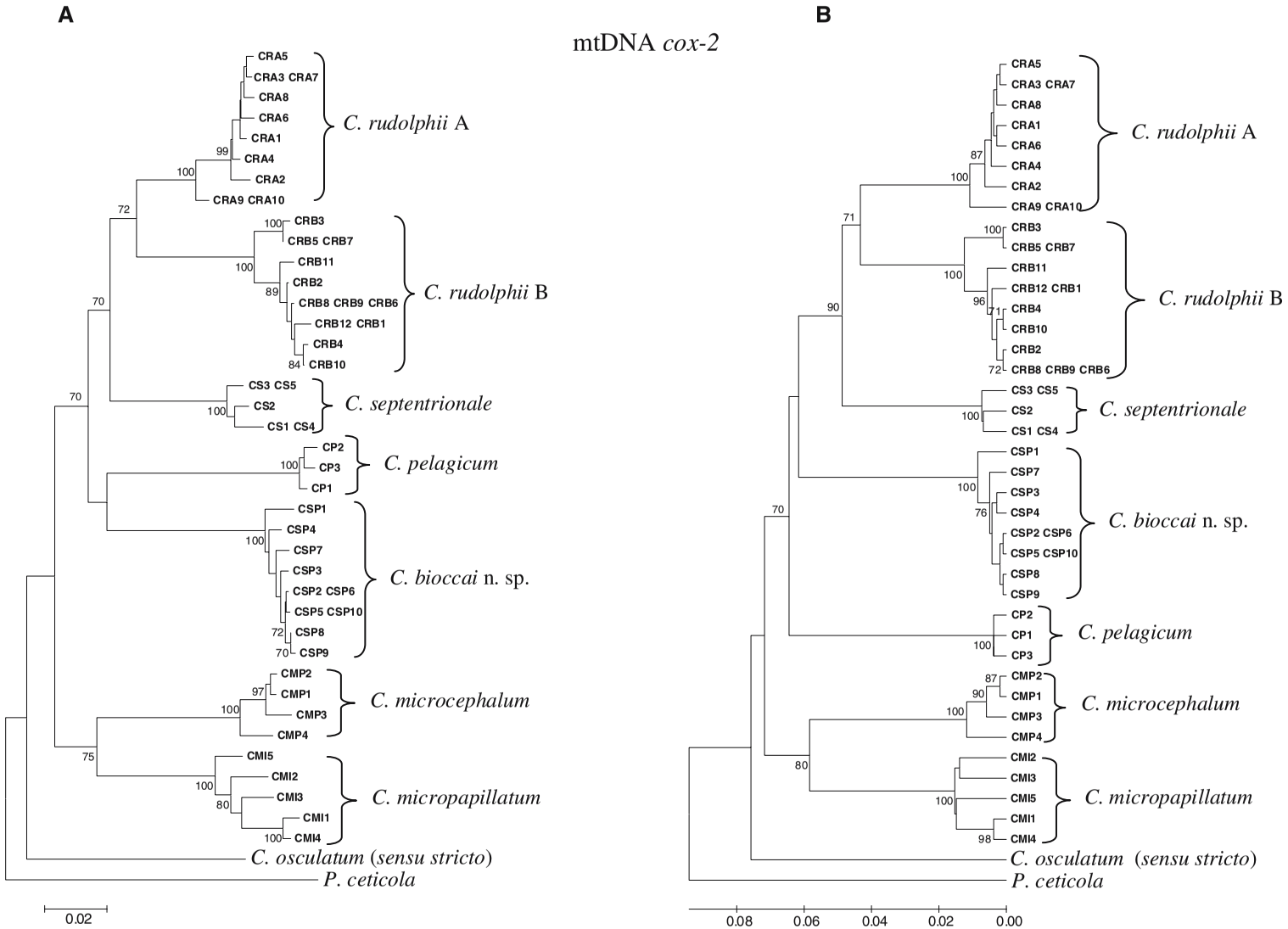

Fig. 5 UPGMA (A) and Neighbour-Joining (NJ) (B) tree inferred from K-2P distance values (using MEGA 3.1), showing the genetic relationships between the studied *Contracaecum* spp. The scale-bar indicates the distance in substitutions per nucleotide. Specimen codes are referenced in Table 1. Bootstrap values were calculated for >1,000 replicates; percentages ≥70% are shown at the internal nodes. *C. osculatum* (*s. s.*), and *Pseudoterranova ceticola* are outgroups

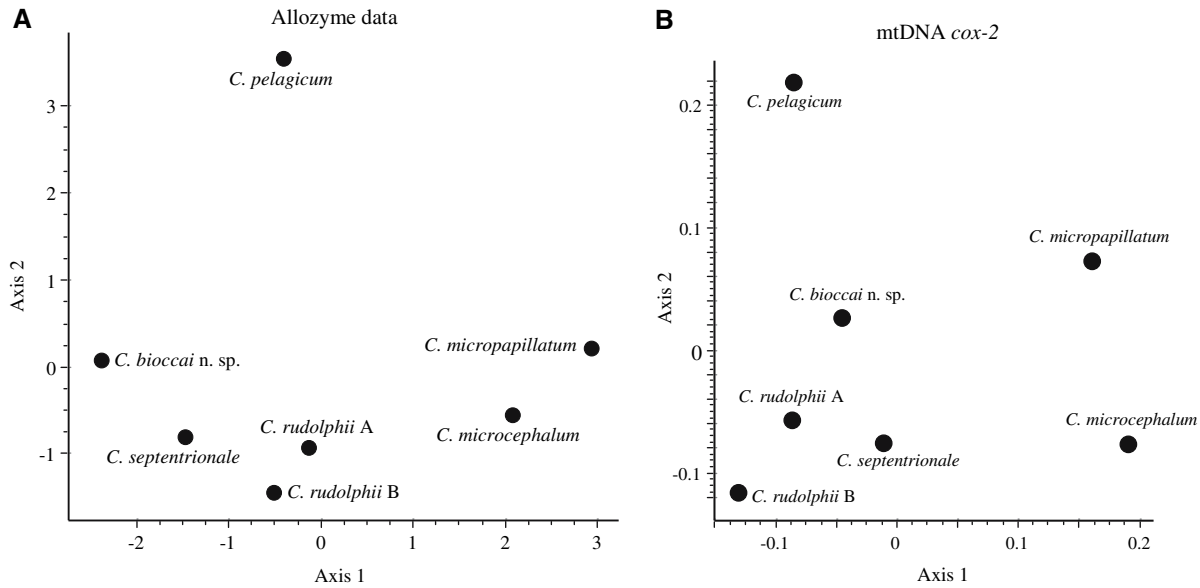

Fig. 6 Principal Component Analysis (PCA) based on (A) allele frequencies obtained at 20 enzyme loci and (B) K-2P distance values from mtDNA *cox-2* sequences, showing the genetic relationships between the studied *Contracaecum* spp. Percentage variance explained by the two components are, respectively, 40% and 28% (A), 37% and 31% (B)

Genetic relationships between species of *Contracaecum*

Cluster analysis carried out on the studied populations and species of *Contracaecum* from fish-eating birds, as inferred from allozyme data (genetic distance values, D_{Nei} and *Dc*) determined using different methods (UPGMA and NJ) (Fig. 3), generated similar topologies. *C. bioccai* n. sp. clusters with the species of the *C. rudolphii* complex, *C. septentrionale* and *C. microcephalum* using UPGMA, while *C. pelagicum* and *C. micropapillatum* form two distinct clades (Fig. 3A). The position of *C. bioccai* in relation to the species of the *C. rudolphii* complex and *C. septentrionale* is well supported in the NJ analysis by the *Dc* values (Fig. 3B).

A congruent position for *C. bioccai*, which was included in the same main cluster with the species of *C. rudolphii* complex, *C. septentrionale* and *C. pelagicum*, was generated by MP sequence analysis (Fig. 4) and by using UPGMA and NJ inferred from the K-2P values for the mtDNA *cox-2* (Fig. 5). In both the MP and NJ analyses (Figs. 4, 5), *C. bioccai* appears genetically related to *C. pelagicum*, although the clade formed by *C. bioccai* and *C. pelagicum* is not well supported by a high level of bootstrap. In all the analyses (UPGMA and NJ) inferred from allozyme (Fig. 3) and mtDNA-*cox2* sequence data (UPGMA, NJ and MP) (Figs. 4, 5), a close association, highly supported by all of the analyses, was observed between *C. rudolphii* A, *C. rudolphii* B and *C. septentrionale*; whereas *C. bioccai* appeared to be a sister species to this group and genetically related to *C. pelagicum* based on mtDNA *cox-2* analysis, albeit with less support using both NJ and MP (<70) (Fig. 4A). On the other hand, based on UPGMA in the allozyme analysis, *C. bioccai* is not closely related to *C. pelagicum* (Fig. 3A); the same result was obtained using UPGMA analysis inferred from the mtDNA *cox-2* (Fig. 5B).

In the MP, UPGMA and NJ tree topologies, inferred from mtDNA *cox2*, *C. micropapillatum* and *C. microcephalum* form a well-separated clade, which appear to be the sister group to the other studied *Contracaecum* spp. from fish-eating birds; this is well supported in the MP, NJ and UPGMA analyses (Figs. 4, 5).

A spatial representation of the genetic relationships between *C. bioccai* and the other members of *Contracaecum* studied is also given in Fig. 6, which shows the PCA analysis based on allele frequencies (Fig. 6A) and mtDNA *cox-2* sequences

(Fig. 6B). This shows that *C. bioccai*, *C. septentrionale* and *C. rudolphii* A and B cluster far apart from both *C. pelagicum* and *C. micropapillatum.*

Discussion

In nematode species delimitation, taxa are recognised based upon evidence of independent evolutionary lineages reflected in the form of derived character states unique to the same individuals of a species (Adams, 1998). Indeed, genetically defined clades, corresponding to distinct evolutionary lineages, are consistent with their recognition as separate species (Padgett et al., 2005). Moreover, it has been repeatedly indicated that the use of different genetic character states inferred from independent data-sets provides stronger evidence for separate species than evidence from a single locus (Nadler, 2002). On the other hand, mtDNA is of particular value for testing hypotheses of lineage exclusivity for closely related nematode species (Nadler, 2002). In the present paper, nuclear markers (20 enzyme loci) and sequences of the mtDNA *cox-2* (519 bp) provided concordant evidence for *C. bioccai* n. sp. as a good species. Evidence for *C. bioccai* as a valid species was demonstrated by the presence of allozyme loci fixed for alternative and unique alleles in this taxon with respect to other *Contracaecum* spp. so far studied genetically; this finding was corroborated by the unambiguous phylogenetic evidence inferred from the mtDNA *cox-2*.

C. bioccai n. sp. was readily differentiated from the sibling species of the *C. rudolphii* complex (i.e. *C. rudolphii* A and B), at both nuclear and mtDNA levels, from some of its congeners (*C. spiculigerum, C. microcephalum* and *C. micropapillatum*) and from one which it resembles morphologically (*C. pelagicum*). The new taxon is also genetically well differentiated at both allozyme (D_{Nei} = 1.8) and mtDNA *cox-2* (K-2P = 0.15) levels from *C. multipapillatum* (*sensu lato*) recovered from the same host (*P. occidentalis*) in the same locality (Totumo Marsh) of northern Colombia (Mattiucci et al., unpubl.). *C. bioccai*, although not occurring sympatrically with species of the *C. rudolphii* complex, exhibited, at the allozyme level, several loci fixed for alternative alleles when compared with the studied species of this complex. The genetic distance values observed between *C. bioccai* and the studied species of *Contracaecum* are at the same level as those previously observed between allopatric sibling species of *Contracaecum* from pinnipeds in the boreal and austral regions (e.g. D_{Nei} = 0.48 between *C. osculatum* (*sensu stricto*) and *C. osculatum* D) (Nascetti et al., 1993; Orecchia et al., 1994; Mattiucci et al., 2003). Moreover, *C. bioccai* showed, with respect to morphologically well differentiated species (e.g. *C. micropapillatum*), the same level of genetic divergence as that obtained between other species of *Contracaecum* from pinnipeds, which are also morphologically distinct taxa [e.g. D_{Nei} = 1.35 between *C. osculatum* (*s. s.*) and *C. radiatum* (v. Linstow, 1907)] (Arduino et al., 1995).

At the mtDNA level, the strong A + T bias observed herein is consistent with that found elsewhere in other anisakid nematodes (Valentini et al., 2006) or, in general, for nematode mtDNA (Blouin et al., 1998). The genetic divergence of mtDNA among *Contracaecum* spp. which have been studied genetically is of the same order as found between other nematode species (Zarlenga et al., 1998; Hu & Gasser, 2006) and related anisakids. For instance, based on mtDNA *cox-2* sequences (629 bp), genetic divergence estimated between *Anisakis* spp. ranged from K-2P = 0.05 (between the sibling species of the *A. simplex* complex) to K-2P = 0.14 [between morphologically different taxa, such as *A. pegreffii* Campana-Rouget & Biocca, 1955 and *A. physeteris* (Baylis, 1923)] (Valentini et al., 2006; Mattiucci & Nascetti, 2006).

All the tree topologies, derived from the phylogenetic analysis inferred from both allozymes and mtDNA data-sets, were in substantial agreement where they depicted *C. bioccai* as closely related to the sibling species of the *C. rudolphii* complex and *C. septentrionale*. However, discordance between the phylogenetic analyses, inferred from the two data-sets (allozymes and mtDNA *cox-2*), surfaced in the unresolved placement of *C. pelagicum* within either a subclade with *C. bioccai*, as obtained in the MP and NJ analyses from the mtDNA *cox-2* (Figs. 4, 5A), or forming a separate clade, as indicated by allozyme data (Fig. 3) and UPGMA from the mtDNA *cox-2* (Fig. 5B). However, low bootstrap values appeared in all the trees, except for the UPGMA from the allozymes (Fig. 3B), suggesting that other molecular markers applied to *C. pelagicum*, as well as to other species of *Contracaecum* from other penguins and

albatrosses, are needed to clarify the phylogenetic position of *C. pelagicum* with respect to other *Contracaecum* spp. from fish-eating birds.

Strong support was received, from all the phylogenetic elaborations, for the clade formed by the sibling species *C. rudolphii* A, *C. rudolphii* B and *C. septentrionale* (Figs. 3–5). This finding is congruent with those previously inferred from LSU rDNA sequence data (Nadler et al., 2000, 2005). The clade formed by *C. micropapillatum* and *C. microcephalum* at the mtDNA *cox-2* level received strong support in the MP, NJ and UPGMA trees; however, this finding appears incongruent with the LSU rDNA sequences analysis (Nadler et al., 2000, 2005).

Morphological analysis and differential diagnosis of genetically identified male specimens of *C. bioccai*, compared with species of *Contracaecum* which have been studied genetically and others reported from the Western Hemisphere, highlighted differences in a numbers of male characters. These include absolute measurements of spicule length, the morphology of the spicule tip and the distribution pattern of the distal papillae of the tail. Such characters have been shown in previous studies to be useful diagnostic criteria for anisakid nematodes (Fagerholm 1989, 1991), as well as for discriminating genetically detected sibling species (Paggi et al., 2000).

Considering the definitive hosts of the studied species of *Contracaecum* from fish-eating birds, our findings appear to indicate a host-parasite association between the species of the *C. rudolphii* complex and *C. septentrionale* and birds belonging to the family Phalacrocoracidae. Indeed, the sibling species *C. rudolphii* A and *C. rudolphii* B are the main nematode parasites of the Eurasian cormorant subspecies *Phalacrocorax carbo sinensis* in both freshwater and brackish waters (Mattiucci et al., 2002). In fact, *C. rudolphii* B is the only genetically identified sibling species in cormorant colonies from freshwater localities (e.g. the Mazury Lakes, Poland), suggesting that this species has a freshwater life-cycle, involving fish, such as *Rutilus rutilus*, as an intermediate host. *Contracaecum* larvae Type 1 (*sensu* Moravec, 1994) infecting this fish species in freshwater lakes was recognised genetically as *C. rudolphii* B (unpublished data). Whereas larvae of *C. rudolphii* A have been genetically identified in *Anguilla anguilla* and *Dicentrarchus labrax* from the coastal lagoons of Italy, suggesting that this species has a life-cycle mainly involving brackish fish species (Mattiucci et al., 2002). Finally, *C. septentrionale* has only been recorded as an adult, i.e. in *Phalacrocorax aristotelis* in the Artic Boreal Region. Although the finding of *C. bioccai* in *Pelecanus occidentalis* appears not to follow this general rule, this host's distributional range is very great and extends from the sandy beaches to the lagoons of Central America, and its feeding habits include both marine and brackish water fish species. Moreover, the area of the Totumo Marsh in northern Colombia is a relatively closed body of water with a low level of salinity, and it is inhabited by several water birds from the Western Hemisphere which might represent other suitable definitive hosts for this parasite.

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