Genetic characterization of members of the genus *Contracaecum* (Nematoda: Anisakidae) from fish-eating birds from west-central Florida, USA, with evidence of new species

S. D'AMELIO[1]*, N. B. BARROS[2], S. INGROSSO[1], D. A. FAUQUIER[2], R. RUSSO[3] *and* L. PAGGI[1]

[1] *Department of Public Health Science, University of Rome "La Sapienza", P. le Aldo Moro, 5, 00185 Rome, Italy*
[2] *Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, FL34236, USA*
[3] *Department of Pathology, University of California San Diego, La Jolla, CA 92093, USA*

(*Received 24 October 2006; revised 20 December 2006 and 15 January 2007; accepted 15 January 2007; first published online 13 March 2007*)

SUMMARY

Specimens of *Contracaecum* spp. from *Phalacrocorax auritus* and *Pelecanus occidentalis* from Florida were characterized by sequencing of the small subunit of the mitochondrial ribosomal RNA gene (*rrnS*) and by PCR-based RFLP analysis of the same gene and of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. Analyses of the *rrnS* sequence data using the MP and UPGMA approaches yielded trees with similar topologies, delineating 3 main clusters. Specimens from *Ph. auritus*, morphologically assigned to *C. rudolphii* (s.l.), were part of the cluster comprising also the other 2 species of the *C. rudolphii* complex (A and B), but representing a genetically distinct group, potentially corresponding to a distinct lineage within the complex, provisionally named as *C. rudolphii* C. The second cluster comprised 5 individuals from *P. occidentalis*, which formed a genetically relatively homogeneous group. The *rrnS* data indicate that these specimens (indicated as *Contracaecum* sp. 1) are clearly genetically different from the morphologically most closely related species, i.e. *C. rudolphii* (s.l.) and *C. microcephalum*, and could represent a new species. The third cluster comprised 7 specimens from *P. occidentalis* morphologically assigned to *C. multipapillatum* (s.l.). These were shown to be genetically homogeneous and related to but quite distinct from *C. multipapillatum* from Greece, although additional studies are needed to assess their status. PCR-RFLP based markers for the quick identification of these taxa are provided.

Key words: *Contracaecum*, *Phalacrocorax auritus*, *Pelecanus occidentalis*, nuclear and mitochondrial ribosomal DNA, Florida, USA.

INTRODUCTION

Contracaecum Railliet and Henry, 1912 is one of the largest ascaridoid genera comprising approximately 50 species. Most of these are (at adult-stage) parasites of fish-eating birds, whereas some are parasites of seals and dolphins. According to a recent molecular phylogenetic study (Nadler *et al*. 2000), the latter are more closely related to species of the genus *Phocascaris* Høst, 1932, from marine mammals than to *Contracaecum* from birds.

Avian *Contracaecum* are commonly reported in all regions of the world and occur in a very large number of host species (Mozgovoi, 1953; Hartwich, 1964; Barus *et al*. 1978). Previous multilocus enzyme electrophoretic studies of *Contracaecum rudolphii* Hartwich, 1964 (D'Amelio *et al*. 1990; Cianchi *et al*. 1992; Mattiucci *et al*. 2002) have revealed a marked genetic heterogeneity in several genetic loci for specimens collected from *Phalacrocorax carbo sinensis* (the Eurasian subspecies of the great cormorant) from different geographical localities. These results suggested the co-existence of multiple genetically distinct, reproductively isolated operational taxonomic units (sibling species), namely *C. rudolphii* A and *C. rudolphii* B. *C. rudolphii* A predominantly infects cormorants dwelling in brackish waters of coastal lagoons in Europe, whereas *C. rudolphii* B is harboured by cormorant colonies living in freshwater lagoons in central Europe (Mattiucci *et al*. 2002). The evaluation of the first (ITS-1) and/or second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) has yielded genetic markers for the specific identification of a range of anisakids from various host groups, including birds (D'Amelio *et al*. 2000; Zhu *et al*. 2000, 2001, 2002; Hu *et al*. 2001; Abollo *et al*. 2003). More recently, a study employing markers in the ITS (Li *et al*. 2005) has provided additional support for the hypothesis that

* Corresponding author: Department of Public Health Science, University of Rome "La Sapienza", P. le Aldo Moro, 5, 00185, Rome, Italy. Tel: +39 0649914671. Fax: +39 0649914644. E-mail: stefano.damelio@uniroma1.it

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doi:10.1017/S003118200700251X Printed in the United Kingdom

https://doi.org/10.1017/S003118200700251X Published online by Cambridge University Press

C. *rudolphii* represents a complex of at least 2 sibling species and that *Contracaecum septentrionale* Kreis, 1955, from *Phalacrocorax aristotelis* (shag), is a valid, separate species. Extending previous studies, the present investigation focused on the genotypic identification of individual specimens of *Contracaecum* from the double-crested cormorant (*Phalacrocorax auritus*) and the brown pelican (*Pelecanus occidentalis*) from west-central Florida, USA, and compared them with members of the same genus characterized previously using morphological and molecular approaches.

MATERIALS AND METHODS

Parasites

A total of 117 nematodes, comprising 95 from 20 double-crested cormorants (*Ph. auritus*) and 22 from 4 brown pelicans (*P. occidentalis*) from Sarasota Bay, on the Gulf coast of Florida (Sarasota and Manatee Counties), were used in the present study (see Table 1). The birds included immatures and adults found dead or which were euthanized in 2004 and 2005 during periods of prolonged and intense 'red tide' caused by the dinoflagellate *Karenia brevis*, which also impacted on marine mammals (*cf.* Flewelling *et al*. 2005). Nematodes collected from the stomach of these fish-eating birds at necropsy were placed in 70% ethanol. The anterior and posterior ends of individual specimens were removed, cleared in lactophenol for morphological studies, and the remaining part of each worm was used for molecular analysis. For morphological identification, characters considered of diagnostic value for anisakid nematodes (Fagerholm, 1991) and those proposed for *Contracaecum* from birds (Barus *et al*. 1978) were considered (e.g., morphology of lips and interlabial tips, length of spicule and morphology of the spicule tip, and patterns of the male caudal papillae). The specimens from double-crested cormorants were identified as *Contracaecum rudolphii* (s.l.). Fifteen specimens from the brown pelican were identified as *Contracaecum multipapillatum* (Von Drasche, 1882) Baylis, 1920, whereas 7 specimens did not correspond to any of the described species of *Contracaecum*, displaying some characters consistent with *C. rudolphii* or of *Contracaecum microcephalum* (Rudolphi, 1809).

Twenty-eight specimens used for comparative purposes were identified by microscopy and by using genetic markers defined previously in the internal transcribed spacers of nuclear ribosomal DNA (Li *et al*. 2005), and belonged to *C. rudolphii* complex (A and B) from the great cormorant (*Phalacrocorax carbo sinensis*) from northeastern Italy, *C. multipapillatum* from the Dalmatian pelican (*Pelecanus crispus*) from Greece and *C. microcephalum* from the small cormorant (*Phalacrocorax pygmaeus*) from Montenegro (see Table 1).

Isolation of genomic DNA

DNA was isolated using the Wizard® Genomic DNA purification kit (Promega), according to the manufacturer's protocol. In brief, body portions from individual nematodes were each placed in 600 μl of a mixture containing 0·5 M ethylene diamine tetraacetic acid (EDTA) plus Nuclei Lysis solution and then crushed employing a sterile pestle. An aliquot of 17·5 μl of proteinase K (20 mg/ml; Promega) was added to each tube, which was incubated at 55 °C for 3 h. An aliquot of 3 μl of RNase solution (4 mg/ml) was added, and the tubes were incubated at 37 °C for 30 min. Subsequently, 200 μl of protein precipitation solution were added, the tubes vortexed and chilled on ice for 5 min, and the DNA precipitated with ethanol. Each DNA pellet was air-dried for 20 min and dissolved in 100 μl of DNA rehydration solution.

PCR amplification

The ITS (plus intervening 5·8S rRNA gene) was amplified by PCR using 4·0 μl of template DNA (20–40 ng), 10 mM Tris-HCl (pH 8·3), 50 mM KCl (Applied Biosystems), 3 mM $MgCl_2$ (Applied Biosystems), 1 mM of dNTPs (Promega), 50 pM of each the forward primer NC5 (5′-GTAGGTGAACCT-GCGGAAGGATCATT-3′) and the reverse primer NC2 (5′-TTAGTTTCTTCCTCCGCT-3′) (Zhu *et al*. 2000) and 2·5 U of AmpliTaq Gold™ (Applied Biosystems) in a final volume of 50 μl. The PCR was performed in a GeneAmp PCR System 2400 (Applied Biosystems) under the following conditions: 10 min at 95 °C (initial denaturation), 30 cycles of 30 sec at 95 °C (denaturation), 40 sec at 52 °C (annealing) and 75 sec at 72 °C (extension), and a final elongation step of 7 min at 72 °C. The amplification of the *rrnS* was performed using 4·0 μl of template DNA (20–40 ng), 10 mM Tris-HCl (pH 8·3), 50 mM KCl (Applied Biosystems), 3 mM $MgCl_2$ (Applied Biosystems), 1 mM of dNTPs (Promega), 50 pM of the forward primer MH3, 5′-TTGTTCCAGA-ATAATCGGCTAGACTT, 50 pM of the reverse primer MH4.5, 5′-TCTACTTTACTACAACT-TACTCC) and 0·5 μl of AmpliTaq Gold™ (Promega) in a 50 μl final volume of reaction. The conditions of PCR 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) and 30 sec at 72 °C (extension), and a final elongation step of 7 min at 72 °C.

A negative control (i.e., without genomic DNA) was included in each amplification. Aliquots (5 μl) of individual PCR products were detected on agarose gels (1%), stained with ethidium bromide (10 mg/ml) and detected upon ultraviolet transillumination. Gel images were captured electronically and analysed using the program MULTI-ANALYST (v.1.1, Bio-Rad).

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Table 1. Parasite specimens examined in the present study (C-cor and Pel) and those used for comparative purposes (Pc, A-cor, B-cor, Php), including information regarding their hosts and geographical origin (Cor = cormorant, Pel = pelican, Pc = *Pelecanus crispus*, Php = *Phalacrocorax pygmaeus*)

(Representative sequences for each taxon have been submitted to GenBank and have the following Accession numbers: EF014283 (C-cor 52), EF030717 (Pel28), EF030716 (Pel3), EF014280 (Pc1), EF014281 (A-cor31), EF014279 (B-cor41), EF014282 (Php1).)

Parasite species	Host species	Number of hosts	Number of specimens analysed by PCR-RFLP	Codes of specimens sequenced for *rrnS*	Geographical origin
Contracaecum rudolphii C	*Phalacrocorax auritus*	20	95	C-cor5, C-cor81, C-cor21, C-cor68, C-cor35, C-cor2, C-cor36, C-cor52, C-cor1, C-cor54, C-cor4, C-cor47	Sarasota Bay, west-central Florida, USA
Contracaecum multipapillatum (s.l.)	*Pelecanus occidentalis*	4	15	Pel12, Pel6, Pel7, Pel1, Pel28, Pel4, Pel18	Sarasota Bay, west-central Florida, USA
Contracaecum sp. 1	*Pelecanus occidentalis*	4	7	Pel20, Pel2, Pel9, Pel3, Pel10	Sarasota Bay, west-central Florida, USA
Contracaecum multipapillatum (s.l.)	*Pelecanus crispus*	1	1	Pc1	Psatatopi, Greece
Contracaecum rudolphii A	*Phalacrocorax carbo sinensis*	8	14	A-cor21, A-cor48, A-cor7, A-cor53, A-corM1, A-cor39, A-cor6, A-cor24, A-cor37, A-cor1, A-corD6, A-cor60, A-cor31, A-cor23	Venice Lagoon, Italy
Contracaecum rudolphii B	*Phalacrocorax carbo sinensis*	9	12	B-cor35, B-cor52, B-cor50, B-corD10, B-cor40, B-corD9, B-cor42, B-corT1, B-cor41, B-corT2, B-corF, B-cor611	Venice Lagoon, Italy
Contracaecum microcephalum	*Phalacrocorax pygmeus*	1	1	Php1	Scutari Lake, Montenegro

Sequencing and analysis

Twenty-four *rrnS* amplicons representing the 117 nematode specimens from Florida (indicated as C-cor and Pel in Table 1) were purified by SureClean Product Insert (Bioline), following the manufacturer's instructions. The pellets were re-suspended in 30 μl of H_2O and subjected to automated sequencing using BigDye ver. 3.1 Terminator chemistry in a 3730xl DNA sequencer (Applied Biosystem). Nucleotide sequences were aligned using the program ClustalX (Thompson *et al*. 1997) and adjusted manually after careful checking for misalignments. Analyses were conducted using MEGA (version 3.1). A UPGMA tree was constructed based on Kimura's 2-parameter model (Gamma) (Kimura, 1980). A maximum parsimony (MP) analysis was conducted using an heuristic search (Close-Neighbor-Interchange), and a 70% majority-rule consensus tree was obtained. The robustness of clades of UPGMA and MP trees was assessed using 1000 bootstrap replications. *Contracaecum osculatum* A, a species reported from pinnipeds, was chosen as an outgroup. The monophyly of *Contracaecum* species from pinnipeds and their differentiation from avian *Contracaecum* has been demonstrated previously (Nadler *et al*. 2000).

PCR-RFLP analysis

For each DNA locus, amplicons (95 from specimens from *Ph. auritus* and 22 from those from *P. occidentalis*) were digested with the restriction endonuclease *Tsp509*I (for the ITS), or *Rsa*I or *Dde*I (for *rrnS*). Digests were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide (10 mg/ml), detected upon transillumination and the sizes of fragments determined by comparison with a 100 bp DNA ladder as size marker (Promega).

RESULTS

The *rrnS* sequences from 24 nematodes from double-crested cormorants and brown pelicans from west-central Florida were aligned with those from the 2 sibling species of the *C. rudolphii* complex (i.e., *C. rudolphii* A and *C. rudolphii* B) from great cormorants (*Ph. carbo sinensis*) from northeastern Italy, from *C. multipapillatum* from Dalmatian pelicans (*P. crispus*) from Greece and from *C. microcephalum* from small cormorants (*P. pygmaeus*) from Montenegro. The sequences had an A+T content of 70·0% in average, consistent with percentages obtained previously for mitochondrial genes in

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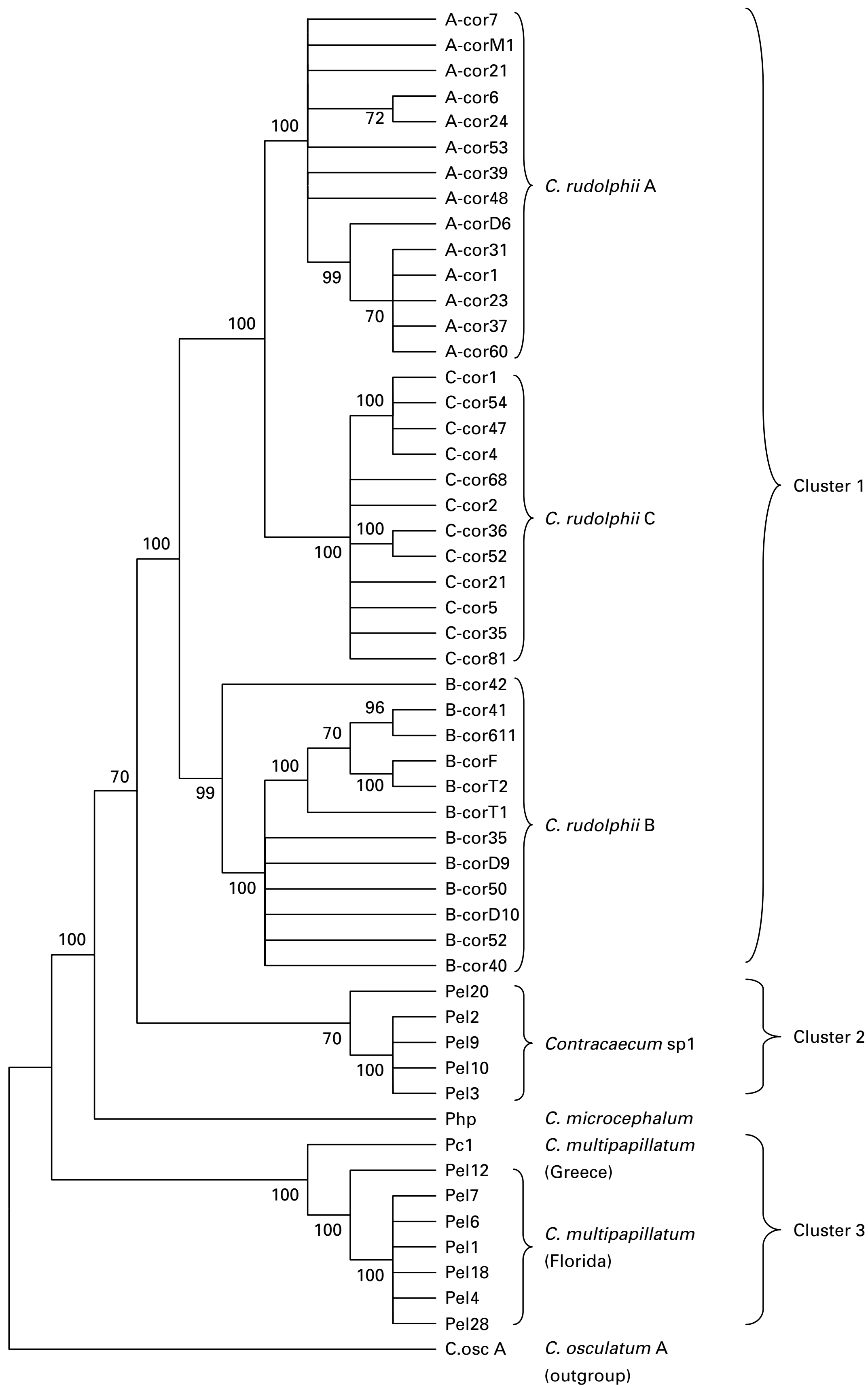

Fig. 1. Maximum Parsimony 70% majority-rule bootstrap consensus tree based on *rrnS* gene sequences. Bootstrap percentages of clades (based on 1000 iterations) are shown at internal nodes.

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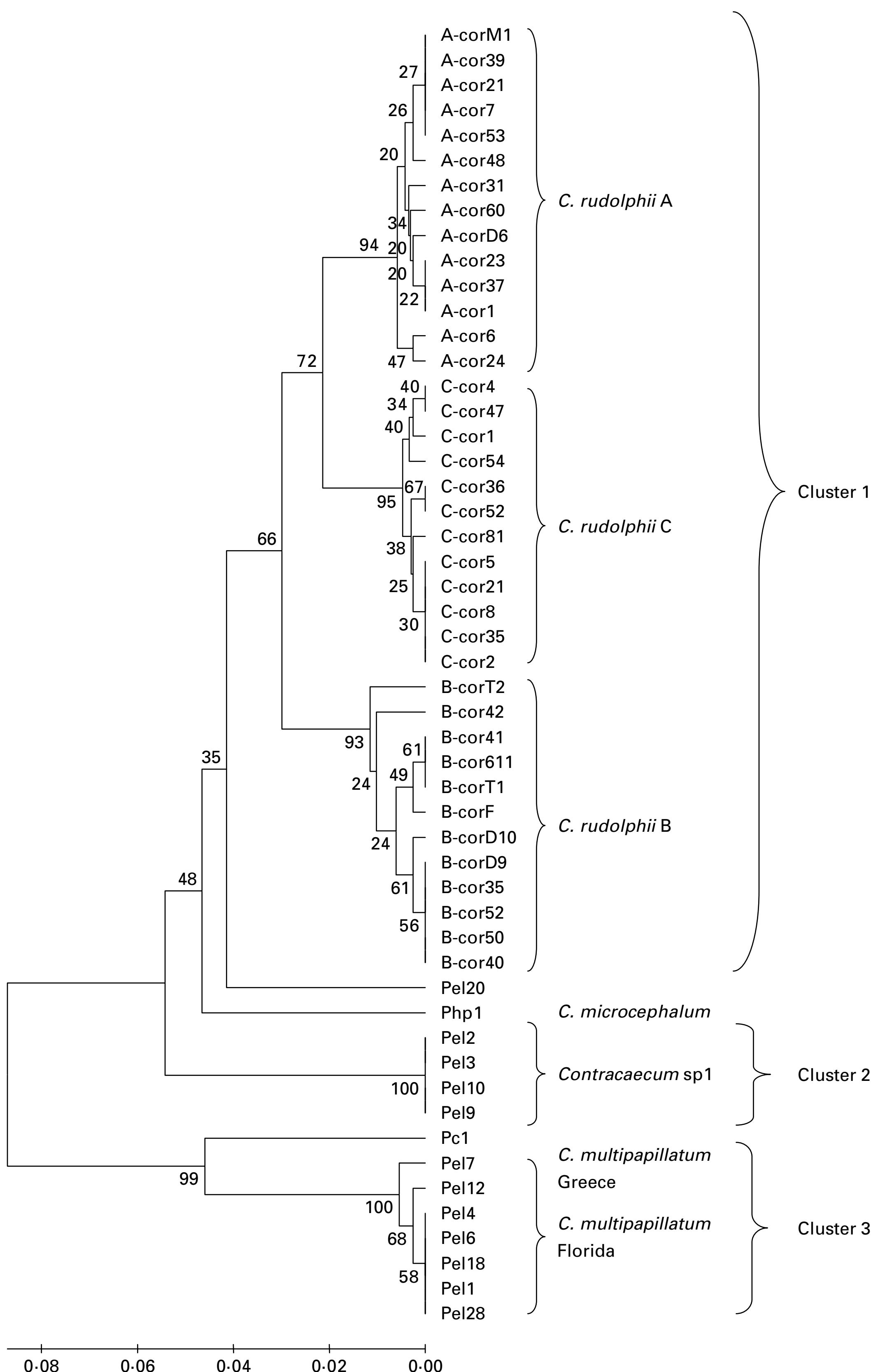

Fig. 2. UPGMA tree constructed on a genetic distance matrix based on Kimura's 2-parameter model (Gamma), based on *rrnS* gene sequences. Bootstrap percentages of clades (based on 1000 iterations) are shown at internal nodes.

ascaridoid nematodes (Hu *et al*. 2001). Analyses of the mitochondrial *rrnS* sequence data using the MP (70% majority-rule bootstrap consensus) (Fig. 1) and UPGMA (Fig. 2) approaches yielded trees with similar topologies, delineating 3 main clusters.

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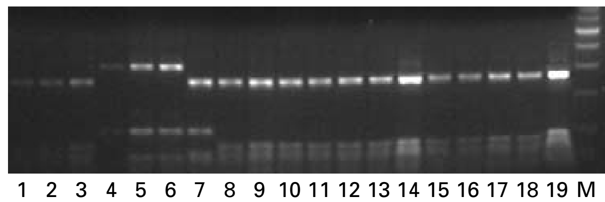

Fig. 3. A representative gel displaying the RFLP profiles following the digestion of *rrnS* amplicons with restriction endonuclease *Rsa*I. 1–3 *C. rudolphii* B; 4–6 *C. rudolphii* A; 7–19 *C. rudolphii* C; M 100 bp DNA ladder.

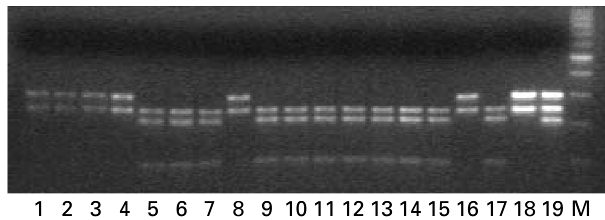

Fig. 4. A representative gel displaying the RFLP profiles following the digestion of *rrnS* amplicons with restriction endonuclease *Dde*I. 1–2 *C. rudolphii* B; 3–4 *C. rudolphii* A; 5–7, 9–15, 17 *C. rudolphii* C; 8, 16, 18 *C. multipapillatum* (Florida), 19 *C. microcephalum*; M 100 bp DNA ladder.

Cluster 1

The first cluster (*cf.* Figs 1 and 2), comprises specimens assigned to the morphospecies *C. rudolphii* (s.l.) and contains 3 subclusters. A bootstrap value of 100 confirmed the robustness of this clustering. Two of these subclusters comprised specimens of *C. rudolphii* A and *C. rudolphii* B from cormorants from northeastern Italy, previously characterized by sequencing of the ITS-1 and ITS-2 (*cf.* Li *et al.* 2005). All of the individuals from the double-crested cormorants from Florida formed a single cluster (referred to henceforth as *C. rudolphii* C), which was distinct from those containing *C. rudolphii* A and *C. rudolphii* B.

RFLP analysis of representative *rrnS* amplicons using *Rsa*I (Fig. 3) produced 4 fragments of 271, 81, 64 and 37 bp for *C. rudolphii* C, a pattern similar to that of *C. rudolphii* B (270, 82, 64 and 49 bp) but distinct from *C. rudolphii* A, which had fragments of 330, 110 and 49 bp. To distinguish *C. rudolphii* B from *C. rudolphii* C, amplicons were digested with *Dde*I. Analysis of amplicons from specimens from Florida displayed fragments of 199, 175 and 84 bp, whereas *C. rudolphii* A and B revealed fragments of 294 and 183 bp (Fig. 4). Hence, the use of the endonucleases *Rsa*I and *Dde*I provided a reliable means of delineating the 3 taxa within *C. rudolphii* (s.l.) by PCR-RFLP. In order to provide independent support for the existence of *C. rudolphii* C within the complex, PCR-RFLP analysis of ITS amplicons with *Tsp509*I was conducted, yielding fragments of 480, 220, 170 and 80 bp, compared

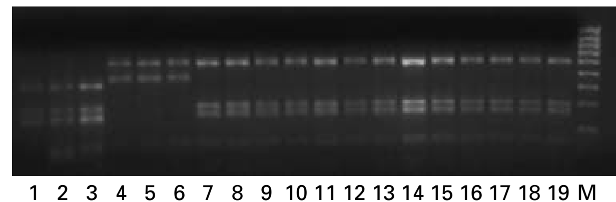

Fig. 5. A representative gel displaying the RFLP profiles following the digestion of ITS amplicons with restriction endonuclease *Tsp509*I. 1–3 *C. rudolphii* A; 4–6 *C. rudolphii* B; 7–19 *C. rudolphii* C; M 100 bp DNA ladder.

with fragments of 330, 220, 170 and 80 bp for *C. rudolphii* A and fragments of 480, 360 and 80 bp for *C. rudolphii* B (Fig. 5). Using the PCR-RFLP analyses of *rrnS* and ITS (employing the 3 enzymes), all 95 specimens from *Ph. auritus* from Florida were identified as *C. rudolphii* C.

Cluster 2

A second cluster, comprising 5 individual specimens from pelicans from Florida (Pel2, Pel3, Pel9, Pel10 and Pel20), was shown to represent a genetically unique entity and thus designated as *Contracaecum* sp. 1, as distinct from all 3 currently recognized members of the *C. rudolphii* complex and from *C. microcephalum* (code Php1) from a small cormorant (*Ph. pygmaeus*) from the Scutari Lake (Montenegro). There was 70–100% support for *Contracaecum* sp. 1 being a distinct group (Figs 1 and 2). The position of specimen Pel20 was uncertain, grouping within or external to cluster 2 (Figs 1 and 2).

RFLP analysis of representative *rrnS* amplicons using *Dde*I yielded distinct patterns between *C. microcephalum* and specimens Pel2, Pel3, Pel9 and Pel10. However, a single mutation at nucleotide position 278 for specimen Pel20 revealed the absence of a site for *Dde*I, such that the profile for the latter specimen was identical to that of *C. microcephalum*. PCR-RFLP analysis of ITS amplicons with *Tsp509*I produced fragments of 550 and 380 bp for *Contracaecum* sp. 1 (Fig. 6, lane 3), as distinct from the patterns produced for each of the 3 recognized members of the *C. rudolphii* complex (Fig. 6, lanes 4, 5 and 6) and from that of *C. microcephalum* (Fig. 6, lane 2).

Cluster 3

A third cluster, well defined by UPGMA tree analysis, comprised the sequence of *C. multipapillatum* from *P. crispus* from Greece (specimen's code Pc1) and the remaining individuals from *P. occidentalis* from Florida (Pel1, Pel4, Pel6, Pel7, Pel12, Pel18 and Pel28). In the MP tree (Fig. 1), specimen Pc1 was related to, but not a sister to, specimens from *P. occidentalis*. These specimens,

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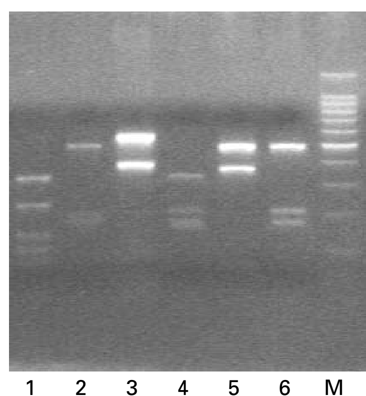

Fig. 6. A representative gel displaying the RFLP profiles following the digestion of the ITS amplicons with restriction endonuclease *Tsp509*I. 1 *C. multipapillatum* (Florida); 2 *C. microcephalum*; 3 *Contracaecum* sp. 1; 4 *C. rudolphii* A; 5 *C. rudolphii* B; 6 *C. rudolphii* C; M 100 bp DNA ladder.

morphologically identified as *C. multipapillatum*, were genetically 'homogeneous' but distinct from *C. multipapillatum* from Greece. Henceforth, they are referred to as *C. multipapillatum* (Florida). PCR-RFLP analysis of ITS amplicons with *Tsp509*I produced fragments of 330, 240, 150 and 110 bp for *C. multipapillatum* (Florida). The same analysis identified 15 specimens from *P. occidentalis* from Florida as *C. multipapillatum* (Florida) and 7 specimens as *Contracaecum* sp. 1.

Mean genetic distance

A matrix of mean genetic distances based on the *rrnS* sequences (Kimura 2-parameters index), among members grouped according to the topology of the *rrnS* tree, is given in Table 2. The values of genetic distances between taxa of cluster 1, corresponding to the members of the *C. rudolphii* complex, range between 0·043 and 0·063, and are the lowest values observed in this study, consistent with their present status as sibling species. Higher values were calculated within and among members of the other clusters, ranging from 0·126 between *C. microcephalum* and *Contracaecum* sp. 1, to 0·192 between *C. multipapillatum* (s.l.) from Greece and *Contracaecum* sp. 1.

Preliminary morphological examination of C. rudolphii *C and* Contracaecum *sp. 1*

Morphological examination of the 2 taxa supports their genetic identification. Specifically, *C. rudolphii* C differs significantly from both *C. rudolphii* A and *C. rudolphii* B in the length of the spicules, which shows non-overlapping values (A: 6·50–7·90, B: 8·11–10·00, C: 5·20–5·96, in mm). *Contracaecum* sp. 1 differs from all congeners other than *C. microcephalum* and *C. rudolphii* (s.l.). *Contracaecum* sp. 1 can be distinguished from *C. microcephalum* by (i) the length of spicules (in mm) being longer (4·82–5·83) than the latter (1·43–3·65) according to the descriptions by Hartwich (1964) and Barus *et al.* (1978) and by (ii) the shape of the distal tip of the spicule having a longer free distal tip (distance of most distal insertion of alae to rounded distal point of spicules). This new species can be distinguished from *C. rudolphii* sensu Hartwich, 1964 by (i) interlabial tips rounded and not bifurcated, (ii) lips slightly longer than wide, and (iii) dorsal lip with a slight deepening on upper margin rather than a deep depression. A formal description of the 2 species and a detailed analysis of the morphological differentiation of *C. rudolphii* C from the other members of the *C. rudolphii* complex and of *Contracaecum* sp. 1 from congeners will be the subject of separate articles.

DISCUSSION

There are few records of *Contracaecum* species from the double-crested cormorant from the south-eastern USA. The following species have been recorded: *C. multipapillatum* from Louisiana (Deardorff and Overstreet, 1980), *Contracaecum* specimens identified as *C. microcephalum* from Mississippi, Louisiana and Florida (Deardorff and Overstreet, 1980) but with a spicule length and a tip morphology in accordance with *C. rudolphii*.

Several studies of members of the genus *Contraceacum* from the brown pelican from the Gulf of Mexico and Caribbean Sea have been carried out, and the following records exist: *C. mexicanum*, a species described from Acapulco, Mexico (Flores–Barroeta, 1957), from Venezuela (Diaz-Ungria, 1978, 1979) and Puerto Rico (Dyer *et al.* 2002); *C. multipapillatum* from the Gulf of Mexico (Courtney and Forrester, 1974; Courtney *et al.* 1977; Deardoff and Overstreet, 1980; Grimes *et al.* 1989), and Puerto Rico (Dyer *et al.* 2002); *C. rudolphii* (sometimes reported as *C. spiculigerum*) from the Gulf of Mexico (Hutton, 1964; Huizinga, 1966, 1971; Courtney and Forrester, 1974; Deardoff and Overstreet, 1980) and Puerto Rico (Bunkley-Williams and Williams, 1994); *Contracaecum* sp. or spp. from the Gulf of Mexico (Courtney *et al.* 1977; Humphrey *et al.* 1978; Deardorff and Overstreet, 1980; Greve *et al.* 1986; Dronen *et al.* 2003).

In the present study, the genetic data obtained support the existence of 2 new species, and possibly a third. The first new taxon comprises nematodes collected from the stomach of double-crested cormorants from Florida, morphologically assigned to *C. rudolphii* (s.l.). These specimens were found to be part of the cluster comprising the other 2 species of the complex (*C. rudolphii* A and *C. rudolphii* B), but representing a genetically distinct group, potentially corresponding to a distinct lineage within the

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Table 2. Matrix of genetic distances, using Kimura 2-parameters index, between taxa based on groups identified by UPGMA and Maximum Parsimony analysis

	C. rudolphii A	*C. rudolphii* B	*C. rudolphii* C	*Contracaecum* sp. 1	*C. microcephalum*	*C. multipapillatum* s.l. (Greece)
C. rudolphii A						
C. rudolphii B	0·057					
C. rudolphii C	0·043	0·063				
Contracaecum sp. 1	0·104	0·108	0·098			
C. microcephalum	0·105	0·078	0·094	0·126		
C. multipapillatum s.l. (Greece)	0·169	0·171	0·176	0·192	0·182	
C. multipapillatum s.l. (Florida)	0·176	0·165	0·174	0·181	0·161	0·092

complex. Although allopatric with respect to the existing species of the *C. rudolphii* complex, described from central and southern Europe and, recently, from Qinghai Lake, China (Zhu *et al.* 2007), this third clade seems to represent a new species within the *C. rudolphii* complex (here provisionally named as *C. rudolphii* C). In anisakid nematodes, speciation processes, and associated levels of genetic variation, are not always followed by morphological differentiation. Speciation processes have led to a number of sibling species within this group of nematodes, including 3 species within *Anisakis simplex* (s.l.) (Mattiucci *et al.* 1997), 5 species within *Pseudoterranova decipiens* (s.l.) (Paggi *et al.* 1991, 2000; Mattiucci *et al.* 1998; Zhu *et al.* 2002), 5 species within *C. osculatum* (s.l.) (Nascetti *et al.* 1993; Orecchia *et al.* 1994; Zhu *et al.* 2000; Hu *et al.* 2001). Regarding anisakids from fish-eating birds, sibling species (*C. rudolphii* A and *C. rudolphii* B) were proposed within the *C. rudolphii* complex using allozyme markers (D'Amelio *et al.* 1990; Mattiucci *et al.* 2002) and later confirmed by rDNA sequence data (Li *et al.* 2005). The assessment at species level classification based on haploid data has been discussed recently by Padgett *et al.* (2005), who stated that "*genetically defined clades, corresponding to distinct evolutionary lineages are consistent with their recognition as separate species*". Also, the genetic differentiation of the *C. rudolphii* C clade from both *C. rudolphii* A and *C. rudolphii* B based on nuclear (diploid) ITS data clearly supports its recognition as a distinct species within the complex.

C. rudolphii C is a sister taxon to *C. rudolphii* A. From a geographical perspective, one could expect that species A and B should be more related to each other, as both species are found in sympatry in Italy. However, species A and B have a broad geographical distribution. For instance, *C. rudolphii* A has been detected in the Atlantic coast of Spain (Abollo *et al.* 2001), whereas *C. rudolphii* B has been recorded in inland waters in central Europe and China (Zhu *et al.* 2007). From an ecological viewpoint, Mattiucci *et al.* (2002) considered *C. rudolphii* A as a species occurring in brackish waters, as is *C. rudolphii* C, in contrast to *C. rudolphii* B which occurs mostly in freshwater habitats. Therefore, it is possible that the tree topology representing the 3 sibling species reported in this study may reflect ecological niches rather than their geographical distributions.

Five individuals from *P. occidentalis* from Florida formed a genetically relatively homogeneous group (*Contracaecum* sp. 1) compared with members of the *C. rudolphii* complex as well as *C. microcephalum*, indicating the existence of a distinct cluster. The *rrnS* sequence data indicate that these specimens are clearly genetically different both from all current members of the *C. rudolphii* complex and from *C. microcephalum*, and may represent an additional species. Morphologically, such specimens have no bifurcated interlabia as is typical for *C. microcephalum*, but their spicule length and spicule tip shape is consistent with these features for *C. rudolphii*. Specimens showing the same morphological characters were described by Deardorff and Overstreet (1980) from *Ph. auritus* and *P. occidentalis* from the northern Gulf of Mexico, suggesting that they could represent a new species. The possibility of a new species of *Contracaecum* from white and brown pelicans from Galveston Bay, Texas, has also been proposed by Dronen *et al.* (2003).

Seven specimens from *P. occidentalis* from Florida were shown to be genetically homogeneous and related to but quite distinct from *C. multipapillatum* from Greece. According to their morphology, these specimens have shown to be related to *C. multipapillatum* (s.l.), a species that may represent a complex of species, according to preliminary data presented by Nascetti *et al.* (2000). A species similar morphologically to *C. multipapillatum* and named *C. mexicanum* was described by Flores–Barroeta (1957). However, the original description of *C. mexicanum* reports spicule lengths ranging from 2·6 to 2·8 mm, whereas the individuals studied herein possessed spicule lengths ranging from 1·62 to 2·10 mm, consistent with *C. multipapillatum* (s.l.). The 7 specimens examined in the present study may

https://doi.org/10.1017/S003118200700251X Published online by Cambridge University Press

be members of a distinct taxon within a *C. multipapillatum* complex, although further investigation is needed to test this hypothesis.

The 2 host species (the double-crested cormorant and the brown pelican) investigated herein were shown to harbour multiple genetically distinct groups/species of *Contracaecum*, *i.e.*, *C. rudolphii* C in the double-crested cormorants and *Contracaecum* sp. 1 and *C. multipapillatum* (s.l.) in the brown pelicans. These bird species are sympatric in Sarasota Bay and other areas of the Gulf of Mexico and have largely overlapping dietary habits (Hatch and Weseloh, 1999; Shield, 2002), suggesting that these differences could be the product of adaptation of adult forms to their definitive hosts. Therefore, host specificity seems to play a more important role than trophic ecology.

The present study provides evidence for at least 2 new species in the genus *Contracaecum*. A detailed analysis of the morphological differentiation of *C. rudolphii* C from the other members of the *C. rudolphii* complex and of *Contracaecum* sp. 1 from congeners will permit their formal description and nomenclatural designation.

We wish to thank very much the editor (Professor Robin B. Gasser) and three anonymous referees, whose comments and suggestions have significantly improved the manuscript. This research was partially supported by grants from the Ministero delle Politiche Agricole, Alimentari e Forestali – Sesto Piano Triennale della Pesca e dell'Acquacoltura.

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