GENETIC RELATIONSHIPS AMONG SPECIES OF *CONTRACAECUM* RAILLIET & HENRY, 1912 AND *PHOCASCARIS* HÖST, 1932 (NEMATODA: ANISAKIDAE) FROM PINNIPEDS INFERRED FROM MITOCHONDRIAL *COX2* SEQUENCES, AND CONGRUENCE WITH ALLOZYME DATA

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Summary:

The genetic relationships among 11 taxa, belonging to the genus *Contracaecum* (*C. osculatum* A, *C. osculatum* B, *C. osculatum (s.s.)*, *C. osculatum* D, *C. osculatum* E, *C. osculatum baicalensis*, *C. mirounga*, *C. radiatum*, *C. ogmorhini (s.s.)*, *C. margolisi*) and *Phocascaris (Phocascaris cystophorae*), parasites as adults of seals, were inferred from sequence analysis (519 bp) of the mitochondrial cytochrome c oxidase subunit II (mtDNA *cox2*) gene. Phylogenetic analyses obtained from Parsimony (MP) and Neighbour-Joining (NJ) K2P distance values generated similar topologies, each well supported at major nodes. All analyses delineated two main clades: the first encompassing the parasites of the phocid seals, *i.e.* the *C. osculatum* species complex, *C. osculatum baicalensis*, *C. mirounga* and *C. radiatum*, with the latter two species forming a separate subclade; the second including the parasites of otarids, *i.e. C. ogmorhini (s.s.)* and *C. margolisi*. An overall high congruence between mtDNA inferred tree topologies and those produced from nuclear data sets (20 allozyme loci) was observed. Comparison of the phylogenetic hypothesis here produced for *Contracaecum* spp. plus *Phocascaris* with those currently available for their definitive hosts (pinnipeds) suggests parallelism between hosts and parasite phylogenetic tree topologies.

KEY WORDS : *Contracaecum*, *Phocascaris*, seals, mtDNA *cox2*, allozymes, phylogeny, host-parasite cophylogenetic aspects.

Anisakid nematodes of the genera *Contracaecum* Railliet & Henry, 1912 and *Phocascaris* Höst, 1932 have an aquatic life cycle and as adults are parasitic in homeothermic hosts. Adults of the genus *Contracaecum* are commonly reported in several seals and fish-eating birds from all over the world. Species of the genus *Phocascaris* use seals, only, as final hosts.

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Specific morphological features in the cephalic part of the interlabia, present in the species of genus *Contracaecum* from birds and seals are considered as diagnostic with respect to species of genus *Phocascaris* in which interlabia are reduced or absent (Höst, 1932). Despite this morphological character, Berland (1964) suggested that all the species of *Contracaecum* from seals and those of *Phocascaris* should belong to the same genus, because of their similar life cycles and definitive hosts (seals). First allozyme studies, carried out on this group of anisakids, have demonstrated that the species of *Phocascaris* are genetically close to members of *Contracaecum* that are parasitic as adults in seals (Orecchia *et al.*, 1986; Nascetti *et al.*, 1990). Conversely, *Contracaecum* spp. maturing in pinnipeds are genetically very distant, sharing no alleles with those *Contracaecum* spp. maturing in fish-eating birds, even if they exhibit similar morphological features (presence of "interlabia") (Nascetti *et al.*, 1990; Orecchia *et al.*, 1986). Indeed, allozyme studies have suggested that the genus *Contracaecum* is highly genetically heterogeneous and polyphyletic. Recent studies (Nadler *et al.*, 2000) based on the LSU rDNA sequence data carried out on several taxa of *Contracaecum* (including species of *Contracaecum* from seals and from waterbirds previously identified by allozyme markers) supported the hypothesis that species of genus *Phocascaris* are nested within the clade of the species of *Contracaecum* hosted in phocids, thus supporting the monophyly of *Phocascaris* spp. with those *Contracaecum* species from phocids.

In addition, previous studies using allozyme markers have demonstrated the reproductive isolation and absence of gene flow among sympatric and allopatric populations of *Contracaecum* spp. hosted by pinnipeds from Arctic and Antarctic regions. They have proved the existence, within some nominal species of the genus *Contracaecum* maturing in seals, of several biological species, often morphological very similar, and, at times, identical (sibling species). This is the case in *C. osculatum sensu lato*, considered previously as a cosmopolitan species and parasitic in various definitive seal hosts, which actually comprises at least six sibling species. They are: the arctic members *C. osculatum* A, *C. osculatum* B, *C. osculatum (s.s.)* (Nascetti *et al.*, 1993;

Mattiucci *et al.*, 1998) plus *C. osculatum baicalensis* (D'Amelio *et al.*, 1995), and the two Antarctic members (*C. osculatum* D and *C. osculatum* E) (Orecchia *et al.*, 1994). Single-strand conformation polymorphism (SSCP) based identification of members of the *Contracaecum osculatum* complex using genetic markers in the internal transcribed spacers of ribosomal DNA (Zhu *et al.*, 2000) and in the three mitochondrial DNA regions cytochrome-c oxidase subunit I (COI), small and large subunits of rRNA (ssrRNA and lsrRNA, respectively) (Hu *et al.*, 2001) allowed the unequivocal differentiation of all the taxa previously disclosed by allozyme markers, except of the two Antarctic taxa, *C. osculatum* D and *C. osculatum* E (Zhu *et al.*, 2000; Hu *et al.*, 2001).

Allozymes and partial sequence analysis of the mitochondrial cytochrome oxidase b (mtDNA *cytb*) have also demonstrated that the morphospecies *C. ogmorhini* Johnston & Mawson, 1941, which is sometimes also considered a synonym of *C. osculatum (s.l.)* (Johnston & Mawson, 1945; Hartwich, 1964) includes two sibling species (*C. ogmorhini (s.s.)* and *C. margolisi*) (Mattiucci *et al.*, 2003; Timi *et al.*, 2003). The existence of two species within *C. ogmorhini (s.l.)* was also suggested by using molecular markers in the internal transcribed spacers of ribosomal DNA (Zhu *et al.*, 2001). Moreover, allozyme markers have also demonstrated that these anisakid species are ecologically differentiated by their host preference, ecological niche, and life cycle pathway (Nascetti, 1992; Bullini *et al.*, 1994, 1997; Mattiucci & Nascetti, 2007, 2008).

The present paper aims to: *i)* review the genetic structure of eleven taxa from pinnipeds belonging to the genus *Contracaecum* and *Phocascaris*, based on allozyme markers; *ii)* infer a phylogenetic hypothesis for these species, based on the mitochondrial cytochrome-c oxidase 2 (mtDNA *cox2*) sequence analysis; *iii)* compare their genetic relationship based on mtDNA *cox2* with that provided by allozyme data; and *iv)* gather preliminary data on host-parasite co-phylogenetic aspects.

MATERIAL AND METHODS

PARASITE MATERIAL

Anisakid specimens belonging to 11 *Contracaecum* taxa so far recognized genetically by allozyme markers (*i.e. C. osculatum* A, *C. osculatum* B, *C. osculatum (sensu stricto), C. osculatum* D, *C. osculatum* E, *C. osculatum baicalensis*, *C. radiatum*, *C. mirounga*, *C. ogmorhini (sensu stricto), C. margolisi*, plus *Phocascaris cystophorae*) were considered in the present paper. The anisakid specimens were first identified to species level by allozymes; they were then sequenced separately from individual nematodes at the mitochondrial DNA region of the *cox2*. All collection data and specimens analyzed are summarized in Table I. The pinniped hosts, reported in Table I, were stranded animals. They include: seven species of "true seals" (Family Phocidae) belonging to the subfamily Phocinae

Parasite	Host	*N* allozyme	*N* mtDNA *and specimen code*	Collection locality
C. osculatum A	*Erignathus barbatus* (Phocidae)	20	3 (COA1-COA2-COA3)	Newfoundland (Canada)
C. osculatum B	*Pagophilus groenlandicus* (Phocidae)	10	10 (COB1-COB2-COB3-COB4-COB5-COB6-COB7-COB8-COB9-COB10)	Newfoundland (Canada)
C. osculatum (s.s.)	*Halichoerus grypus* (Phocidae)	10	2 (COC1-COC2)	Bothnian Bay (Baltic Sea)
C. osculatum D	*Leptonychotes weddellii* (Phocidae)	10	2 (COD1-COD2)	Ross Sea (Antarctica)
C. osculatum E	*Leptonychotes weddellii* (Phocidae)	10	5 (COE1-COE2-COE3-COE4-COE5)	Ross Sea (Antarctica)
C. osculatum baicalensis	*Phoca sibirica* (Phocidae)	5	4 (CBA1-CBA2-CBA3-CBA4)	Lake Baikal
P. cystophorae	*Cystophora cristata* (Phocidae)	3	2 (PCYS1-PCYS2)	Newfoundland (Canada)
C. radiatum	*Leptonychotes weddellii* (Phocidae)	10	3 (CRA1-CRA2-CRA3)	Ross Sea (Antarctica)
C. mirounga	*Mirounga leonina* (Phocidae)	10	2 (CMI8-CMI9)	Peninsula Valdes (Argentine coast)
C. mirounga	*Mirounga leonina* (Phocidae)	20	6 (CMI1-CMI2-CMI4-CMI5-CMI6-CMI7)	Antarctica
C. ogmorhini (s.s.)	*Arctocephalus australis* (Otariidae)	10	2 (COGM1-COGM2)	Argentine coast
C. margolisi	*Zalophus californianus* (Otariidae)	4	4 (CMAR1-CMAR2-CMAR3-CMAR4)	California coast

Table I. – Taxa of *Contracaecum* and *Phocascaris* from pinnipeds analyzed genetically. *N* allozyme = number of specimens studied at 20 enzyme loci; *N* mtDNA = number of specimens sequenced at the mtDNA *cox2* gene.

and Monachinae, and two species of fur seals (Family Otariidae) (*i.e. Zalophus californianus* and *Arctocephalus australis*) (Table I).

Multilocus allozyme electrophoresis

All of the anisakids were adults. They were tested by multilocus allozyme electrophoresis. 20 enzyme loci shared by all the *Contracaecum* species here considered, were analysed in the present study. The following enzymes were studied: idditol dehydrogenase (IDDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (ICDH), 6-phosphogluconate dehydrogenase (6PGDH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), superoxide dismutase (SOD), nucleoside phosphorylase (NP), aspartate aminotransferase (AAT), adenylate kinase (AK), colorimetric esterase (cEST), peptidase (LEU-ALA) (PEP C), mannose phosphate isomerase (MPI), glucose phosphate isomerase (GPI), and phosphoglucomutase (PGM). Details on the enzyme-loci genetically analyzed and electrophoretic procedures used are given in previous papers (Nascetti *et al.*, 1993; Mattiucci *et al.*, 2003). The nematode specimens were identified to species level by diagnostic markers according to those reported elsewhere (Nascetti *et al.*, 1993; Orecchia *et al.*, 1994; Mattiucci *et al.*, 2003).

DNA amplification and sequencing

45 *Contracaecum* specimens previously genetically characterized at allozyme level, were sequenced using mtDNA *cox2*. A 519 bp fragment of the cytochrome oxidase 2 (*cox2*) gene was analysed for all the specimens of *Contracaecum* spp. listed in Table I. Their GenBank accession numbers are: EU477203 (*Contracaecum osculatum* A), EU477204 (*Contracaecum osculatum* B), EU477206 (*Contracaecum osculatum* (s.s.)), EU477205 (*Contracaecum osculatum* D), EU477207 (*Contracaecum osculatum* E), EU477208 (*Contracaecum osculatum baicalensis*), EU477209 (*Phocascaris cystophorae*), EU477210 (*Contracaecum radiatum*), EU477213 (*Contracaecum mirounga*), EU477211 (*Contracaecum ogmorhini* (s.s.)), EU477212 (*Contracaecum margolisi*). Total DNA was extracted from 2 mg of tissue from a single nematode using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) or cetyltriethylammonium bromide (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 µl containing 30 pmol of each primer, $MgCl_2$ 2.5 mM (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), PCR buffer 1 × (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), DMSO 0.08mM, 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 210R and 211F. Reference specimens and isolated DNA samples were stored at the Section of Parasitology of the DSSP, "Sapienza" – University of Rome.

Genetic data analysis

Allozyme data obtained here was combined with that gathered in previous allozyme studies performed by the authors. Genetic divergence at interspecific level was estimated using Nei's indices of standard genetic distance (D_{Nei}, (Nei, 1972) and chord distance (*Dc*, Cavalli-Sforza & Edwards, 1967)). Population genetic analyses were performed using BIOSYS-2 software (Swofford & Selander, 1989). Genetic relationships between the considered species of *Contracaecum* and *Phocascaris* were evaluated by UPGMA and Neighbour-Joining (NJ) cluster analysis using PHYLIP version 3.57 (Felsestein, 1995). Bootstrap consensus analysis (500 replicates) was carried out to verify the robustness of the topologies obtained by PHYLIP (Felsestein, 1995).
The *cox2* sequences were aligned using Clustal W (Thompson *et al.*, 1994), and a square matrix based on *p*-distance and K2P was performed using MEGA 3.1 (Kumar *et al.*, 2001). Phylogenetic analyses at the interspecific level were performed using "maximum parsimony" (MP) by PAUP* version 4.0 (Swofford, 2003). UPGMA and Neighbour-Joining (NJ) analyses, based on K2P values, were performed using the MEGA 3.1 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. Bootstrap values ≥ 70 were considered well supported (Hills & Bull, 1993). Sequences at the mtDNA *cox2* of *Pseudoterranova ceticola* Deardoff & Overstreet, 1982 from *Kogia breviceps* (Genbank DQ116435) was 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 & Hudspeth, 2000).

RESULTS

Allozyme differentiation

At allozyme level, the genetic divergence observed between the considered taxa of *Contracaecum* and *Phocascaris* are reported in Table II. The

Species	*COSA*	*COSB*	*COSS*	*COSD*	*COSE*	*CBAI*	*PCYS*	*CRAD*	*CMIR*	*COGM*	*CMAR*
C. osculatum A *(COSA)*	–	0.53	0.59	0.40	0.36	0.44	0.47	0.63	0.68	0.84	0.83
C. osculatum B *(COSB)*	0.41	–	0.66	0.52	0.50	0.51	0.66	0.65	0.70	0.86	0.85
C. osculatum (s.s.) (COSS)	0.57	0.80	–	0.60	0.56	0.65	0.55	0.75	0.74	0.90	0.89
C. osculatum D *(COSD)*	0.23	0.38	0.65	–	0.40	0.52	0.63	0.67	0.73	0.85	0.83
C. osculatum E *(COSE)*	0.20	0.36	0.53	0.25	–	0.53	0.57	0.62	0.68	0.85	0.84
C. osculatum baicalensis (CBAI)	0.30	0.27	0.41	0.60	0.50	–	0.60	0.72	0.75	0.87	0.85
P. cystophorae (PCYS)	0.45	0.83	0.46	0.68	0.51	0.64	–	0.68	0.73	0.90	0.90
C. radiatum (CRAD)	0.72	0.76	1.30	0.78	0.67	0.79	0.96	–	0.70	0.87	0.84
C. mirounga (CMIR)	1.00	1.06	1.13	1.15	0.86	1.20	1.07	0.95	–	0.88	0.87
C. ogmorhini (s.s.) (COGM)	1.30	1.35	1.37	1.40	1.48	1.70	1.90	1.60	1.81	–	0.40
C. margolisi (CMAR)	1.35	1.40	1.42	1.39	1.45	1.70	1.89	1.75	1.80	0.30	–

Table II. – Matrix of average values of genetic distance calculated by Cavalli-Sforza and Edwards (D_c above the diagonal) and by Nei (D_{Nei}, below the diagonal) between species of *Contracaecum* and *Phocascaris* from pinnipeds, so far characterized genetically.

Species	*COSA*	*COSB*	*COSS*	*COSD*	*COSE*	*CBAI*	*PCYS*	*CRAD*	*CMIR*	*COGM*	*CMAR*
C. osculatum A *(COSA)*	–	0.09	0.10	0.09	0.09	0.06	0.09	0.13	0.14	0.16	0.15
C. osculatum B *(COSB)*	0.08	–	0.09	0.09	0.10	0.08	0.10	0.14	0.13	0.15	0.15
C. osculatum (s.s.) (COSS)	009	0.08	–	0.11	0.10	0.10	0.07	0.16	0.15	0.14	0.15
C. osculatum D *(COSD)*	0.08	0.09	0.10	–	0.05	0.09	0.11	0.13	0.13	0.16	0.16
C. osculatum E *(COSE)*	0.08	0.09	0.10	0.05	–	0.09	0.11	0.14	0.13	0.14	0.15
C. osculatum baicalensis (CBAI)	0.05	0.07	0.09	0.08	0.09	–	0.09	0.15	0.12	0.15	0.16
P. cystophorae (PCYS)	0.08	0.08	0.06	0.09	0.10	0.08	–	0.16	0.15	0.16	0.16
C. radiatum (CRAD)	0.12	0.13	0.14	0.12	0.12	0.13	0.13	–	0.09	0.14	0.15
C. mirounga (CMIR)	0.12	0.12	0.13	0.12	0.12	0.11	0.13	0.09	–	0.14	0.15
C. ogmorhini (s.s.) (COGM)	0.14	0.13	0.13	0.14	0.13	0.14	0.14	0.13	0.13	–	0.05
C. margolisi (CMAR)	0.13	0.13	0.13	0.14	0.13	0.14	0.14	0.14	0.14	0.05	–

Table III. – Matrix on average values of genetic differentiation calculated by Kimura-2-parameter (K2P, above the diagonal) and by P-distance (D_p, below the diagonal) between taxa of *Contracaecum* and *Phocascaris* from pinnipeds, so far studied genetically.

lowest genetic distance values were those reported among the *C. osculatum* species complex and between the members of *C. ogmorhini*, ranging from on average, D_{Nei} = 0.20 between *C. osculatum* A and *C. osculatum* E to D_{Nei} = 0.80 between *C. osculatum (s.s.)* and *C. osculatum* B. Higher genetic distance values were those found between the members of the *C. osculatum* complex with respect to *C. mirounga* ($D_{Nei} \approx$ 1.00) or *C. radiatum* ($D_{Nei} \approx$ 0.80). Finally, the highest values were those estimated between the species of the *C. osculatum* complex in comparison with the species of the *C. ogmorhini* complex $D_{Nei} \approx$ 1.50 (Table II).

COX2 MTDNA SEQUENCE DIFFERENTIATION

A 519-bp portion of the *cox2* gene was sequenced for 42 specimens of *Contracaecum* that have been genetically characterized by allozymes. Alignment of mtDNA *cox2* sequences for all currently recognized species of *Contracaecum* and *Phocascaris*, obtained by BioEdit (Hall, 1999) is reported in Figure 1.

Genetic divergence among the 11 *Contracaecum* taxa was estimated by K2P and *p-distance* methods (Table III). The lowest genetic divergence was that found among the sibling species of *C. osculatum* complex and *C. ogmorhini* species complex (on average, K2P ≈ 0.09 and K2P = 0.06, respectively). Higher values were observed in the comparison between the members of the *C. osculatum* complex and *C. mirounga* (on average, K2P ≈ 0.13), or with respect to *C. radiatum* (on average, K2P ≈ 0.14). The highest values were those observed in the comparison of the sibling species of *C. osculatum* complex with respect to *C. ogmorhini* species complex (on average K2P ≈ 0.15) (Table III).

GENETIC RELATIONSHIPS AMONG *CONTRACAECUM* SPP.

Parsimony analysis (MP) based on mtDNA *cox2* sequence data, using all the codon positions, generated a tree (Fig. 2) showing two main clusters. The first consists of the sibling species of *C. osculatum* complex, *C. mirounga* and *C. radiatum*, while the second cluster includes the species of the *C. ogmorhini* complex, always well supported. Interestingly, in the first clade, the species *P. cystophorae* is clustering within the same clade formed by the *C. osculatum* species complex. In addition, a subclade is produced by *C. radiatum* and *C. mirounga*, highly supported (Fig. 2). A congruent tree topology to MP was generated by NJ inferred from the K2P distance analysis (Fig. 3). The same two main clusters were produced: as with the previous analysis, the species of the *C. osculatum* complex plus *P. cystophorae* and *C. oscu-*

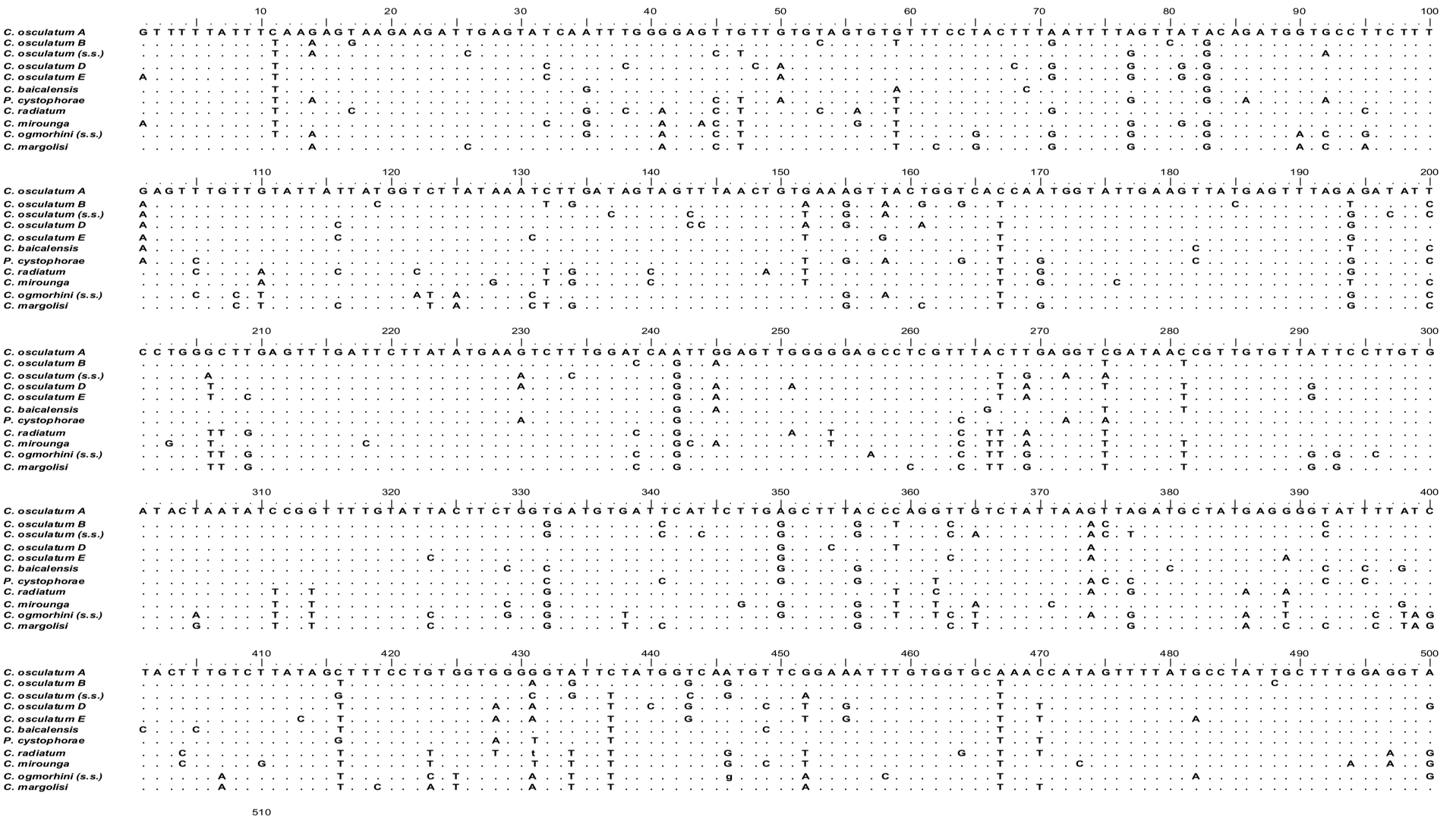

Fig. 1 – Alignment of mtDNA *cox2* sequences (519 bp) for all currently recognized species of *Contracaecum* and *Phocascaris*, obtained by BioEdit (Hall, 1999). One representative of each unique sequence for each taxon was included in the comparison. Dots indicate identity.

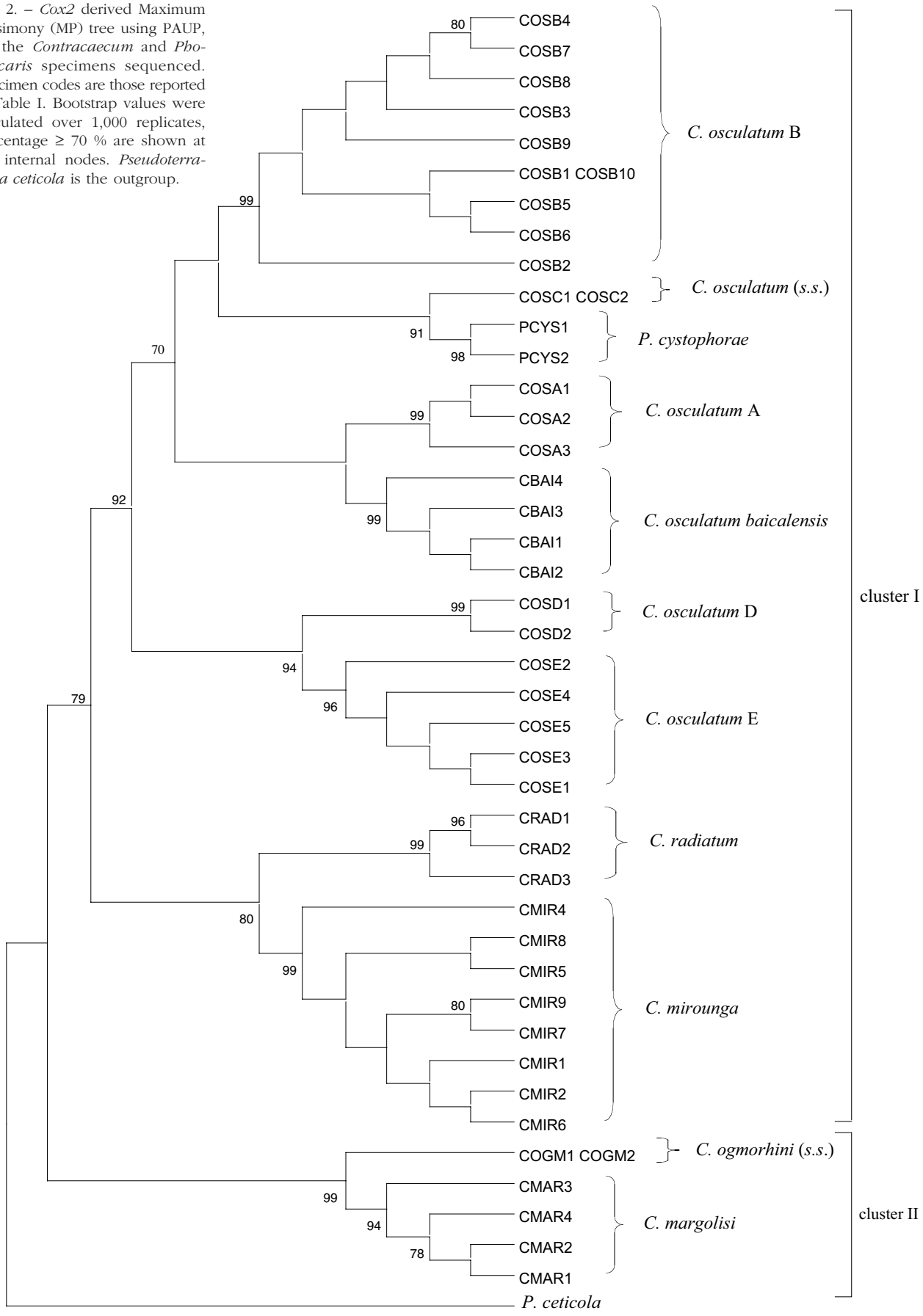

Fig. 2. – *Cox2* derived Maximum Parsimony (MP) tree using PAUP, for the *Contracaecum* and *Phocascaris* specimens sequenced. Specimen codes are those reported in Table I. Bootstrap values were calculated over 1,000 replicates, percentage ≥ 70 % are shown at the internal nodes. *Pseudoterranova ceticola* is the outgroup.

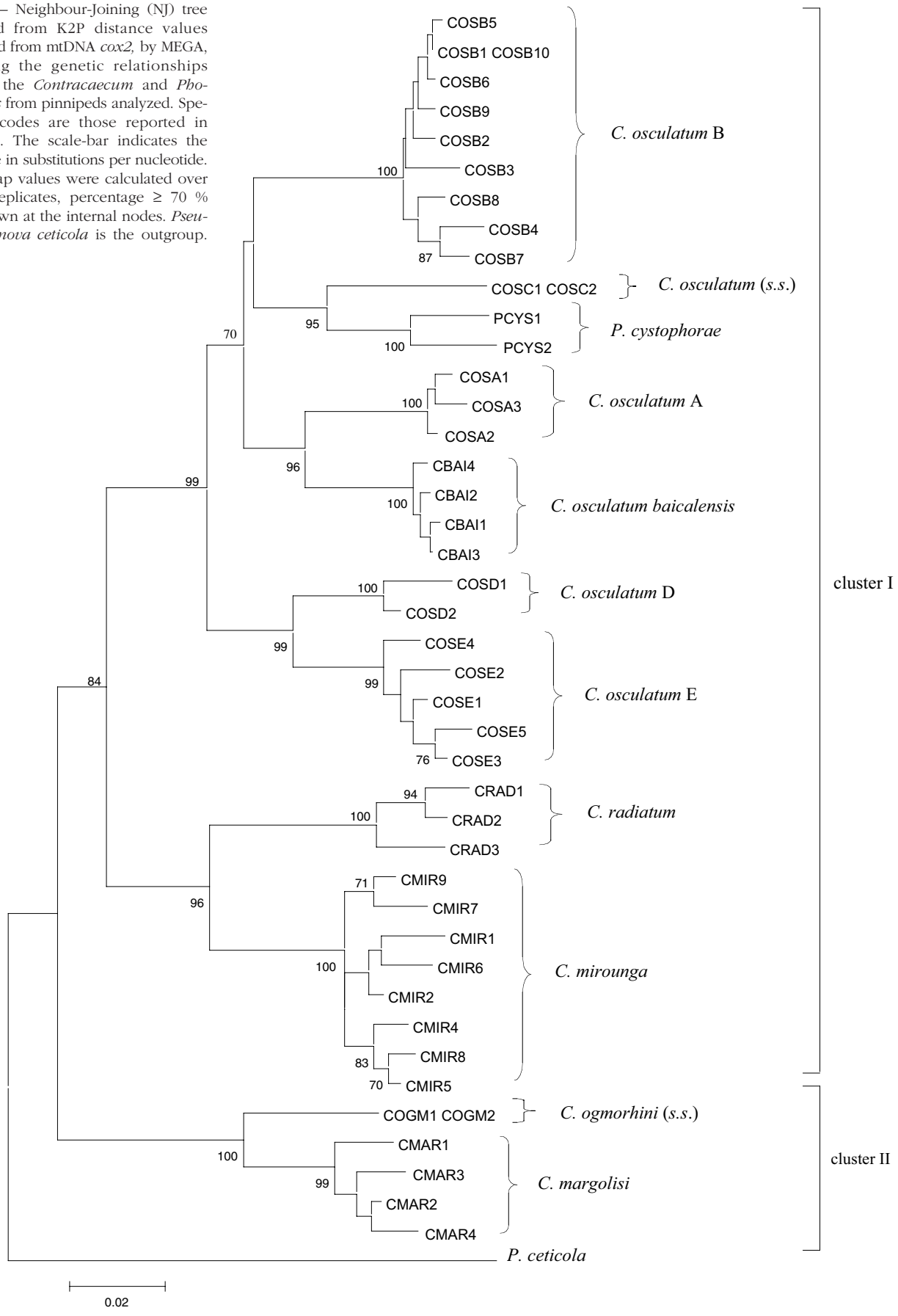

Fig. 3. – Neighbour-Joining (NJ) tree inferred from K2P distance values obtained from mtDNA *cox2,* by MEGA, showing the genetic relationships among the *Contracaecum* and *Phocascaris* from pinnipeds analyzed. Specimen codes are those reported in Table I. The scale-bar indicates the distance in substitutions per nucleotide. Bootstrap values were calculated over 1,000 replicates, percentage ≥ 70 % are shown at the internal nodes. *Pseudoterranova ceticola* is the outgroup.

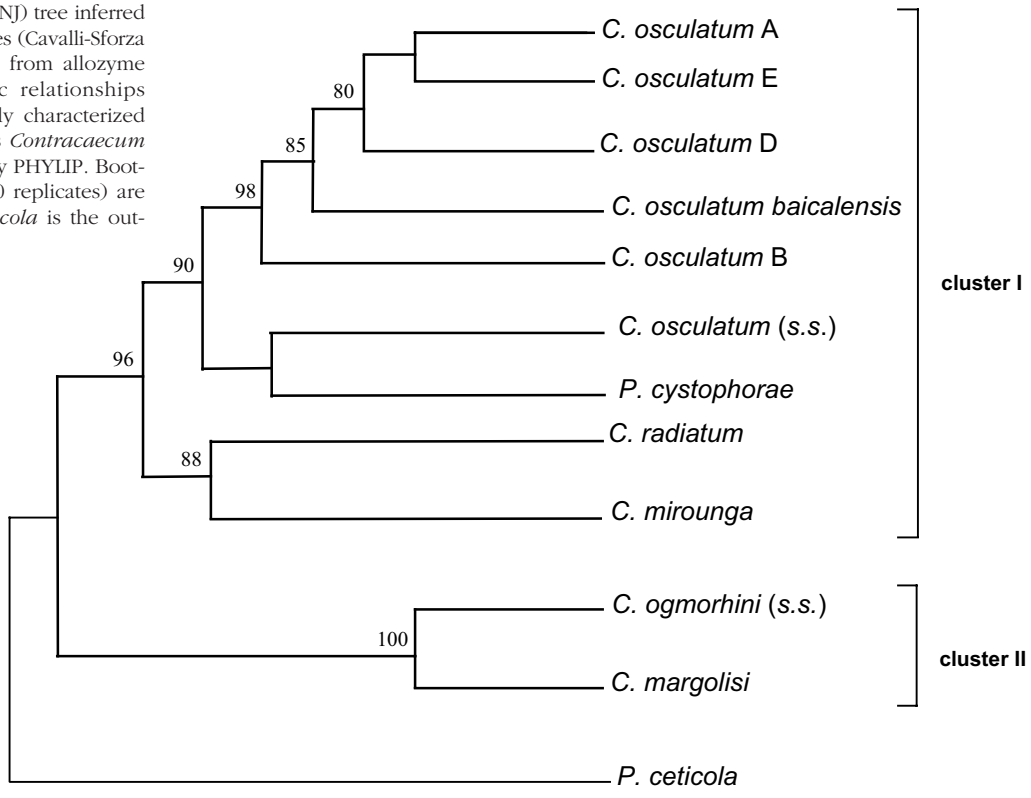

Fig. 4. – Neighbour-Joining (NJ) tree inferred from *Dc* chord distance values (Cavalli-Sforza & Edwards, 1967) obtained from allozyme data, showing the genetic relationships among the so far genetically characterized taxa belonging to the genus *Contracaecum* and *Phocascaris,* obtained by PHYLIP. Bootstrap values (more than 500 replicates) are given. *Pseudoterranova ceticola* is the outgroup.

latum baicalensis form a clade receiving a well supported bootstrap value. In all the elaborations, the species *C. ogmorhini (s.s.)* and *C. margolisi* form always a basal lineage in the tree topology, always well supported.

Cluster analysis carried out by different methods (UPGMA) (data not shown) and NJ (Fig. 4) on the considered taxa of *Contracaecum* and *Phocascaris* from seals, based on the D_{Nei} and *Dc* values, generated similar topologies. Two main clusters were found consistently supported by high bootstrap values, including respectively: cluster 1) a subclade formed by the five members of the *C. osculatum* complex (*i.e. C. osculatum* A*, C. osculatum* B*, C. osculatum (s.s.), C. osculatum* D and *C. osculatum* E), plus *C. osculatum baicalensis* and *Phocascaris cystophorae*; in this main cluster, *C. mirounga* is clustering with *C. radiatum*, forming a separate subclade; cluster 2) formed by the two members *C. ogmorhini (s.s.)* and *C. margolisi* (Fig. 4).

DISCUSSION

COX2 BASED PHYLOGENETIC RELATIONSHIPS AMONG *CONTRACAECUM* SPP. AND COMPARISON WITH ALLOZYME DATA

Allozymes have demonstrated reproductive isolation between *Contracaecum* taxa from pinnipeds and provided specific genetic markers for their recognition at any life-history stage (Nascetti *et al*., 1993; Orecchia *et al*., 1994; Mattiucci *et al.,* 2003). In the present study, sequence data generated from the mitochondrial *cox2* region support previous allozyme studies in recognizing the existence of distinct *Contracaecum* species based upon evidence of independent evolutionary lineages. The use of different genetic character states inferred from independent data sets provides strong evidence of high heterogeneity within the genus *Contracaecum*, and implies the presence of genetically distinct taxa of *Contracaecum* from seals.

A phylogenetic hypothesis for all the taxa so far recognized genetically, was here provided by allozyme data and supported by mtDNA *cox2* gene data. All the tree topologies derived from the phylogenetic analyses were in substantial agreement where each depicted *C. ogmorhini (s.s.)* and *C. margolisi* as a sister group to the remaining species of *Contracaecum* from the pinnipeds analyzed, forming a monophyletic grouping highly supported when analyzed by MP, and NJ based on K2P distance values, and also by NJ allozymes.

Phocascaris cystophorae showed close genetic relationship with *C. osculatum (s.s.)* and nested within the subclade formed by the *C. osculatum* species complex. Moreover, sequences of the mtDNA *cox2* provided unambiguous phylogenetic evidence for the two Antarctic members of the *C. osculatum* complex, *C. osculatum* D and *C. osculatum* E, as distinct species, whose

reproductive isolation was previously demonstrated in sympatry in the same definitive hosts by allozyme markers (Orecchia *et al.,* 1994).

An overall high congruence was found between the tree topologies obtained from the mitochondrial data sets studied here and the phenetic clustering gathered from nuclear data sets (allozymes) generated previously (Arduino *et al.,* 1995; Bullini *et al.*, 1997) and here reviewed also including the taxa *C. mirounga* and *C. osculatum baicalensis* (Fig. 4). Indeed, allozyme clustering, obtained from 20 allozyme loci shared by all the *Contracaecum* taxa here considered, depicted the existence of two main clusters as well: one formed by the species of *C. osculatum* complex, plus *P. cystophorae* and the species from the monachine seals, *i.e. C. radiatum* and *C. mirounga*. These latter two species form a separate subclade. Moreover, high congruence was found in showing *P. cystophorae* as nesting in the subclade of the first cluster. The discordant placement of *C. osculatum* A as closely related to the Antarctic species *C. osculatum* E in the tree generated from allozymes is in contrast to that obtained from *cox2* sequences (Fig. 4) where *C. osculatum baicalensis* shows a closer relationship.

Finally, both *cox2* phylogeny derived and clustering based on allozymes clearly demonstrated that the species of the *C. osculatum* complex plus *P. cystophorae* formed a monophyletic group well supported in all the elaborations, and that *C. ogmorhini (s.s.)* and *C. margolisi* are basal sister taxa in all the elaborations from the two different data sets. High congruence was also found in showing the close genetic relationship between *P. cystophorae* and *C. osculatum (s.s.)* as inferred from both the data analyses (Figs 2, 3 and 4).

These findings are in agreement with the phylogenetic hypothesis based on LSU rDNA sequence data sets by Nadler *et al.* (2000) inferred for seven among the eleven *Contracaecum* taxa from pinnipeds here considered, in depicting the species of the *C. osculatum* complex (except *C. osculatum* D and *C. osculatum* E, which were not included in the elaboration by the Authors) plus *Phocascaris* spp., as forming a monophyletic group. In addition, high consistence between the phylogenetic hypothesis provided by LSU rDNA sequences (Nadler *et al.*, 2000) and that furnished by mtDNA *cox2,* was also found in indicating *C. osculatum baicalensis* as the most related species to *C. osculatum* A, and showing *C. radiatum* and *C. mirounga* genetically closely related each other, forming a monophyletic group.

Host-parasite association and co-phylogenetic aspects

The presence of the two major clusters evidenced in the phylogenetic hypothesis of *Contracaecum* from pinnipeds here proposed is supported also by ecological evidences regarding specific host-parasite relationships and host preferences evidenced so far in the *Contracaecum* species from seals from Arctic and Antarctic regions (Nascetti, 1992; Nascetti *et al.,* 1993; Bullini *et al.*, 1997; Mattiucci *et al.*, 2003; Mattiucci & Nascetti, 2007).

The true seals belonging to the Family Phocidae are the main definitive hosts of the members of the *C. osculatum* complex from the Arctic region; indeed, the bearded seal *Erignathus barbatus* is the main definitive host for the species *C. osculatum* A in both the North Atlantic and Pacific Ocean waters, while the harp seal *Pagophilus groenlandicus*, and the harbour seal *Phoca vitulina* are suitable hosts for *C. osculatum* B in both Atlantic and Pacific waters; finally, *C. osculatum (s.s.)* is the only species present in the grey seal *Halichoerus grypus* from Baltic Sea (Nascetti *et al.*, 1993; Brattey & Stenson, 1993; Mattiucci *et al.*, 1998; Paggi *et al.*, 1998). In addition, the species *C. osculatum baicalensis* represents an endemic nematode anisakid species of the relict Baikal seal, *Phoca sibirica* (D'Amelio *et al.,* 1995). The taxon *C. radiatum* was so far recognized genetically in the Antarctic region only in the Weddell seal *Leptonychotes weddellii* (Arduino *et al.*, 1995), although the species was morphologically reported also in the leopard seal *Hydrurga leptonyx* (Baylis, 1937). The taxon *C. mirounga* has been so far characterized genetically as hosted by the southern elephant seal *Mirounga leonina* and, occasionally, also occurring in sympatry with *C. ogmorhini (s.s.)* in the southern fur seal *Arctocephalus australis* from the southern hemisphere (Mattiucci *et al.,* 2003).

The otarids in the southern fur seals belonging to the genus *Arctocephalus* are the definitive hosts of the species *C. ogmorhini (s.s.),* while the northern fur seal (*Zalophus californianus*) is the main definitive host so far detected for *C. margolisi*.

Phylogenetic relationships proposed here for the species of *Contracaecum* from pinnipeds seem to align that of their definitive hosts as suggested by Arnason *et al.* (1995) using the complete sequences of the mitochondrial cytochrome b gene (mtDNA *cytb*) of the Phocidae, Odobenidae and Otariidae, and recently by Démére *et al.* (2003) using a composite tree inferred from the basic topology of generic level, morphological and molecular data, and fossil taxa to propose an integrated hypothesis for pinniped evolutionary biogeography. According to these data elaborations, the Pinnipedia includes three major monophyletic clades: 1) the Otariidae (fur seals and sea lions), 2) the Odobenidae (walrus), and 3) the Phocidae (true seals), *plus* the extinct desmatophocids (Démére *et al.,* 2003). In this combined tree, the fur seals and sea lions, including the Otariinae (*Zalophus californianus*) and the Arctocephalinae (*Arctocephalus* spp.), with the *Arctocephalus* spp. from the Southern hemisphere are repre-

sented, in the host phylogenetic tree inferred from different data sets (Arnason, *et al.*, 1995; Démére *et al.*, 2003), as the basal groups. In accordance with that analysis, the branching order here proposed for the *Contracaecum* taxa depicts that nematodes from the Otariidae (*i.e. C. ogmorhini (s.s.)* from *Arctocephalus* spp. and *C. margolisi* from *Zalophus californianus*) always occupy a basal lineage of the parasite phylogenetic tree, with the species of the *C. osculatum* complex from the Phocinae (true seals) as the most derived.

CONCLUDING REMARKS

The present study demonstrated clearly the usefulness of the mitochondrial *cox2* gene for unequivocal recognition of seal-parasitizing *Contracaecum* taxa so far genetically characterized by allozymes. On the other hand, analyses of the substitution patterns for mtDNA genes of nematodes have suggested that they are very useful markers for identifying and differentiating cryptic species and for determining relationships of closely related species (Blouin *et al.,*1998; Blouin, 2002; Hu *et al.*, 2004 and references therein, Hu & Gasser, 2006). The same mtDNA region was demonstrated to be useful in distinguishing closely related anisakid taxa previously characterized by allozyme markers, belonging to the genus *Anisakis* (Valentini *et al.*, 2006), and to the genus *Contracaecum* maturing in fish-eating birds (Mattiucci *et al.*, 2008). The genetic divergence of mtDNA here estimated among *Contracaecum* from seals is of the same level as that previously found between other anisakid species (Valentini *et al.*, 2006; Mattiucci *et al.,* 2008). Bootstrap analysis has revealed an overall high congruence between the genetic relationships proposed for this group of anisakid species inferred from two different data sets (nuclear and mitochondrial). Bootstrap analysis based on both genetic markers also strongly supported the hypothesis that species of *Phocascaris* are more closely related to members of the *C. osculatum* complex; a result consistent with nuclear rDNA evidence (Nadler *et al.*, 2000). This finding supports Berland's proposal that the *Contracaecum* species that have seals as definitive hosts should be included in a same genus with *Phocascaris* species. Moreover, as *Phocascaris* was erected for those species devoid of "interlabia", the morphological character of "interlabia" has proved to be of no taxonomic value in the seal ascarids.

Finally, the parallelism between host and parasite phylogenies so far discovered between *Contracaecum* spp. (plus *Phocascaris* spp.) and seals seems to suggest that some level of co-evolution, including co-divergence and host-switching events, could have accompanied the speciation of these group of nematodes and their definitive hosts. A broader set of data is needed to confirm these findings. Evidence for such co-phylogenetic events has recently been reported between *Anisakis* taxa and their cetacean definitive hosts by Mattiucci & Nascetti (2006, 2008).

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