2005

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J. Parasitol., 91(6), 2005, pp. 1413–1429

MOLECULAR PHYLOGENETICS AND DIAGNOSIS OF *ANISAKIS*, *PSEUDOTERRANOVA*, AND *CONTRACAECUM* FROM NORTHERN PACIFIC MARINE MAMMALS

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ABSTRACT: Individual specimens of *Anisakis*, *Pseudoterranova*, and *Contracaecum* collected from marine mammals inhabiting northern Pacific waters were used for comparative diagnostic and molecular phylogenetic analyses. Forty-eight new sequences were obtained for this study of 14 *Anisakis* taxa, 8 *Pseudoterranova* taxa, 4 *Contracaecum* taxa, and 4 outgroup species. Partial 28S (LSU) and complete internal transcribed spacer (ITS-1, 5.8S, ITS-2) ribosomal DNA was amplified by the polymerase chain reaction and sequenced. Sequences of ITS indicated that *Pseudoterranova* specimens from *Zalophus californianus* (California sea lion), *Mirounga angustirostris* (northern elephant seal), *Phoca vitulina* (harbor seal), *Enhydra lutris* (sea otter), and *Eumetopias jubatus* (Steller's sea lion) exactly matched *P. decipiens* s. str., extending the host and geographic range of this species. *Anisakis* from northern Pacific marine mammals were most closely related to members of the *A. simplex* species complex. Comparison of *Anisakis* ITS sequences diagnosed the presence of *A. simplex* C in 2 *M. angustirostris* hosts, which is a new host record. *Anisakis* specimens from *Phocoena phocoena* (harbor porpoise), *Lissodelphis borealis* (Pacific rightwhale porpoise), and *E. jubatus* included 3 ITS sequences that did not match any known species. *Contracaecum* adults obtained from *Z. californianus* were most closely related to *C. ogmorhini* s.l. and *C. rudolphii*, but ITS sequences of these *Contracaecum* specimens did not match *C. ogmorhini* s. str. or *C. margolisi*. These novel *Anisakis* and *Contracaecum* ITS sequences may represent previously uncharacterized species. Phylogenetic analysis of LSU sequences revealed strong support for the monophyly of Anisakinae, *Contracaecum* plus *Phocascaris*, *Pseudoterranova*, and *Anisakis*. Phylogenetic trees inferred from ITS sequences yielded robustly supported relationships for *Pseudoterranova* and *Anisakis* species that are primarily consistent with previously published phenograms based on multilocus electrophoretic data.

Anisakid nematodes are common parasites of marine mammals, and have a worldwide distribution. Larvae of these nematodes are a major problem for commercial fishing industries (Rohlwing et al., 1998), and are potential human health hazards, both as causative agents of anisakiasis (Sakanari and McKerrow, 1989), and as potential food-borne allergens (Moneo et al., 2000; Baeza et al., 2001). Numerous studies employing data from multilocus enzyme electrophoresis have revealed that morphospecies of *Anisakis*, *Contracaecum*, and *Pseudoterranova* consist of genetically differentiated sibling species with different geographic and host distributions (Mattiucci et al., 1986; Nascetti et al., 1986; Orecchia et al., 1986; Paggi et al., 1991; Nascetti et al., 1993; Mattiucci et al., 1997, 1998; Paggi, Mattiucci et al., 1998; Paggi et al., 2000; Mattiucci et al., 2003). These population-level allozyme studies have been instrumental in detecting evidence of genetic heterogeneity (noninterbreeding individuals within populations) among large samples of ascaridoids collected from paratenic or definitive hosts in nature (Paggi and Bullini, 1994; Bullini et al., 1997), and have led to the discovery and description of several new species. Such studies have facilitated the development of other molecular diagnostic tools for these species, in particular, those based on the polymerase chain reaction (PCR) including PCR-restriction fragment length polymorphism (PCR-RFLP) (Zhu, Gasser, Podolska, and Chilton, 1998; D'Amelio et al., 2000; Kijewska et al., 2002; Shih, 2004), single-strand conformational polymorphism (SSCP) of PCR products (Zhu, Gasser, Podolska, and Chilton, 1998; Zhu et al., 2000; Hu et al., 2001; Zhu et al., 2001, 2002), and direct sequencing of PCR-amplified DNA (Zhu, Gasser, Podolska, and Chilton, 1998; Nadler et al., 2000; Zhu et al., 2000; Hu et al., 2001; Zhu et al., 2001, 2002; Mattiucci et al., 2003). These DNA-based diagnostic techniques are advantageous because individual alcohol-preserved adults and larvae can be identified; in contrast, allozyme techniques require enzymatic activities that are only preserved in frozen tissues. Diagnostic DNA markers from a single genetic locus can be quite useful for nematode identification (Gasser et al., 1996; Zhu, Gasser, Podolska, and Chilton, 1998; D'Amelio et al., 2000; Nadler et al., 2003). However, such single-locus studies are of considerably less utility for finding and delimiting new species in nature (Nadler, 2002, 2005). Nevertheless, unexpected nucleotide sequence variation at even 1 locus provides valuable information that can lead to testing hypotheses of species using multilocus datasets (allozymes or DNA based approaches) with reference to explicit species concepts (Adams, 1998; Nadler, 2002).

The most detailed studies of North American marine mammal ascaridoids have focused on hosts from Canadian Atlantic waters (Brattey and Ni, 1992; Brattey and Stenson, 1993; Brattey and Davidson, 1996). These investigations have characterized ascaridoids in *Halichoerus grypus* (gray seal, GS), *Phoca vitulina* (harbor seal, HS), *Phoca hispida* (ringed seal, RS), *Cystophora cristata* (hooded seal, HDS), *Phoca groenlandica* (harp seal, HRS), and *Erignathus barbatus* (bearded seal, BS), and have used molecular methods to identify species of *Anisakis*, *Pseudoterranova*, *Contracaecum*, *Phocascaris*, when assessing parasite abundance and host distribution. Studies using molecular tools to diagnose ascaridoids in North American Pacific waters have been very limited in scope. For *Anisakis*, investigators have used allozyme and PCR-RFLP techniques to document adults of *A. simplex* C (Mattiucci et al., 1997; D'Amelio et al., 2000) from *Pseudorca crassidens* (false killer whale) and *A. simplex* s. str. from *Phocoena phocoena* (harbor porpoise, HP) and *P. crassidens* (Mattiucci et al., 1997; D'Amelio et al., 2000) occurring in Canadian Pacific waters. *Contracaecum ogmorhini* s.l. was reported from *Zalophus californianus* (Cali-

Received 19 November 2004; revised 21 March 2005; accepted 21 March 2005.
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fornia sea lion, CSL) in Mexican and Californian Pacific waters (Fagerholm and Gibson, 1987), and these authors predicted this parasite would be found in additional host species from the Pacific, including *Callorhinus ursinus* (northern fur seal, NFS) and *Eumetopias jubatus* (Steller's sea lion, SSL). More recent genetic studies show that *C. ogmorhini* s.l. consists of 2 sibling species, *C. ogmorhini* s. str. and *Contracaecum margolisi* (Mattiucci et al., 2003), with the latter species diagnosed genetically (Zhu et al., 2001; Mattiucci et al., 2003) from 1 locality (Vancouver Island, Canada) in *Z. californianus* (CSL). The third common genus of marine mammal ascaridoid, *Pseudoterranova*, is also a complex of at least 5 species (Paggi et al., 1991; Mattiucci et al., 1998; George-Nascimento and Urrutia, 2000; Paggi et al., 2000; Zhu et al., 2002) that can be diagnosed by allozyme markers (Paggi et al., 2000), nucleotide sequences (Zhu et al., 2002), and, for adult males, morphometric differences (Di Deco et al., 1994; Mattiucci et al., 1998; George-Nascimento and Urrutia, 2000; Paggi et al., 2000). Reports of *Pseudoterranova decipiens* from North American Pacific waters predate the common use of molecular markers to identify species, thus, *Pseudoterranova* taxa from these hosts have not yet been investigated relative to recently described species.

In the present study, specimens of *Anisakis*, *Pseudoterranova*, and *Contracaecum* collected from marine mammals inhabiting North American Pacific waters were sequenced for ITS ribosomal DNA (rDNA), compared with diagnostic sequences for known species, and used to infer phylogenies. Some of these specimens were also sequenced for a region of LSU rDNA and analyzed in a comparative phylogenetic context to assess relationships among taxonomic groups of aquatic ascaridoids.

MATERIALS AND METHODS

Taxa for molecular systematics

Nematodes (Table I) were obtained from stranded marine mammals by staff at the Marine Mammal Center, Sausalito, California. Ascaridoids were collected at necropsy, preserved in 95% ethanol, and stored at −20 C. Anterior and posterior ends of specimens were removed, cleared in lactophenol, and diagnosed by microscopy. Morphologically, the northern Pacific specimens corresponded to *A. simplex* s.l., *P. decipiens* s.l., or *C. ogmorhini* s.l. Four of the other *Anisakis* specimens used for comparative study were diagnosed to species based on morphology (*A. typica*, *A. ziphidarum*, *A. physeteris*, and *A. brevispiculata*), and all such reference species were confirmed by using diagnostic allozyme markers. Voucher specimens of northern Pacific specimens have been retained in the University of California-Davis frozen tissue collection. *Contracaecum* and *Pseudoterranova* reference ITS sequences were obtained from GenBank as were 34 of 56 partial LSU sequences used for phylogenetic analysis (Table I). Additional ascaridoids sequenced for comparative analysis of LSU rDNA included *Parascaris equorum*, *Ascaris suum*, *Baylisascaris procyonis*, *Hysterothylacium auctum*, and *Raphidascaris acus* (Table I).

DNA amplification and sequencing

DNA was extracted from pieces of individual nematodes using commercial kits (DNAzol, Molecular Research Center Inc., Cincinnati, Ohio; MasterPure™, Epicentre Technologies, Madison, Wisconsin). A region of the 5′ end of the nuclear large subunit ribosomal RNA gene (LSU rDNA) containing the D2 and D3 divergent domains was amplified using a forward PCR primer (#391, 5′-AGCGGAGGAAAAGAA ACTAA, or #538, 5′-AGCATATCATTTAGCGGAGG) in combination with a reverse primer (#390, 5′-ATCCGTGTTTCAAGACGGG, or #501, 5′-TCGGAAGGAACCAGCTACTA). A region of nuclear rDNA including the internal transcribed spacers (ITS-1, ITS-2) and 5.8S subunit was amplified using primers that anneal to the 3′ end of the 18S rDNA (#93, 5′-TTGAACCGGGTAAAAGTCG) and the 5′ end of the 28S rDNA (#94, 5′-TTAGTTTCTTTTCCTCCGCT). PCR reactions (25 μl) consisted of 0.5 μM each primer, 200 μM deoxynucleoside triphosphates, and $MgCl_2$, ranging from 1.5 to 3 mM as required for specific amplification. Proofreading polymerase (0.5 unit, Finnzymes DNAzyme EXT, MJ Research, Waltham, Massachusetts) was used for PCR, and cycling parameters included denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 30 sec, 48–57 C for 30 sec, and 72 C for 1 min, followed by a postamplification extension at 72 C for 7 min. PCR products were prepared for direct nucleotide sequencing by using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product presequencing kit, USB Corporation, Cleveland, Ohio). PCR products were cloned when they could not be successfully sequenced directly. For cloning, PCR products were washed 3 times with TE buffer (pH 7.0) by spin filtration (Millipore Ultrafree-MC 30,000 NMWL, Millipore Corporation, Bedford, Massachusetts), ligated into pGEM-T vector (Promega, Madison, Wisconsin), and cloned into JM109 *Escherichia coli*. Plasmid DNA was obtained using Qiaprep spin miniprep kits (Qiagen, Valencia, California). Sequencing reactions were performed using dye-terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer (PE Applied Biosystems, Boston, Massachusetts). All sequences were completely double stranded for verification using reactions primed from the PCR or vector primers and 2 or more internal sequencing primers. Site polymorphisms in directly sequenced PCR products were recorded only when both alternative nucleotide peaks were present in all sequence reactions representing both DNA strands. If the heights of the alternative nucleotide peaks at polymorphic sites were not equal, the height of the minor peak was required to significantly exceed background terminations and comprise at least 25% of the major peak to be scored as a polymorphism (Nadler et al., 2003). For cloned rDNA, conflicts between clones were recorded as polymorphisms. Phred base-calling was used before contig assembly with CodonCode Aligner (Version 1.2.2).

Phylogenetic analyses

Sequences determined in this study (indicated in Table I), plus those obtained from GenBank, were aligned using ProAlign Version 0.5 (Löytynoja and Milinkovitch, 2003). For each alignment, a ProAlign guide tree was constructed using corrected (for multiple hits) pairwise distances; this guide tree was used to estimate the hidden Markov model parameters (δ and ϵ) for progressive multiple alignment. Program (Java) memory and band widths were increased, as required, to complete the alignment. The minimum posterior probability of sites was used as the criterion for detecting and removing unreliably aligned sequence. To reduce the likelihood of excluding correctly aligned sites, the filter threshold was set to 60% minimum posterior probability (Löytynoja and Milinkovitch, 2003). Pairwise sequence differences (absolute distances) were determined from multiple alignments using PAUP* 4.0b10 (Swofford, 1998). Phylogenetic trees were inferred using unweighted maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP* executed on a dual-processor Linux computer. Gaps were treated as missing data in parsimony analyses. Modeltest Version 3.06 (Posada and Crandall, 1998) was used to compare the fit of nucleotide substitution models for datasets using the Akaike information criterion. The best-fit ML model for each dataset was used for likelihood analysis.

For the 56-taxa LSU dataset, heuristic MP searches were conducted using 500 replicates of random taxon addition with tree-bisection-reconnection (TBR) branch swapping. Bootstrap MP searches were conducted using 1,000 pseudoreplicates, with random taxon addition and a search time limit of 30 sec per pseudoreplicate. Maximum likelihood trees were inferred using a neighbor-joining (NJ) starting tree, with heuristic searching of tree space by TBR branch swapping. The bootstrap ML tree was produced using 100 pseudoreplicates of heuristic searches as indicated for individual ML trees, except each replicate was limited to 800 sec. Maximum parsimony and ML trees were rooted using *Heterocheilus tunicatus* (Ascaridoidea, Heterocheilidae), a choice supported by previous analyses of molecular datasets (Nadler and Hudspeth, 1998, 2000).

For the *Anisakis* and *Pseudoterranova* ITS datasets, MP searches were conducted using the branch-and-bound method; bootstrap MP trees were inferred using 1,000 pseudoreplicates of branch-and-bound. Maximum likelihood and bootstrap ITS ML trees were inferred using

the same methods as for the LSU dataset. The sister-group relationship of *Anisakis* and *Pseudoterranova* recovered in analysis of ribosomal DNA and mitochondrial sequences (Nadler and Hudspeth, 1998, 2000) and for regions of LSU sequence (this study) was the basis for using *Anisakis* to root the *Pseudoterranova* ITS tree, and in a separate analysis, *Pseudoterranova* to root the *Anisakis* ITS tree. Thus, these ITS analyses cannot be used to test the monophyly of *Pseudoterranova* or Anisakis. Phylogenetic analyses that would permit more comprehensive comparisons and tests of monophyly with ITS sequences through inclusion of more ''basal'' ascaridoid taxa were not undertaken because of substantial regions of positional homology uncertainty in ITS alignments that included more taxonomically diverse ascaridoids.

RESULTS

28S rDNA sequence data and analysis

Sequences of LSU rDNA from 9 *Pseudoterranova* specimens (8 *P. decipiens* s.l. and 1 *P. decipiens* s. str.) were identical. These included individual nematodes obtained from 2 *P. vitulina* (HS), 3 *Z. californianus* (CSL), 1 *Mirounga angustirostris* (northern elephant seal, NES), 1 *Enhydra lutris* (sea otter, SO), and 1 *E. jubatus* (SSL). These sequences also matched the LSU sequence of *P. decipiens* s. str. (Nadler and Hudspeth, 1998). Likewise, LSU sequences of 4 *Contracaecum* specimens obtained from 4 *Z. californianus* (CSL) were identical and matched the sequence of *C. ogmorhini* s. str. (Nadler et al., 2000) obtained from *Arctocephalus pusillus pusillus* (Cape fur seal, CFS). Pairwise comparisons of Anisakis LSU sequences showed absolute nucleotide distances ranging from 0 to 5 differences. All 3 Anisakis individuals from *M. angustirostris* (NES) hosts were identical in sequence, as were specimens from 1 *E. jubatus* (SSL) and 1 *Lissodelphis borealis* (Pacific rightwhale porpoise, PRP).

For phylogenetic analysis of LSU sequences, using ProAlign to detect and remove unreliably aligned sites by their posterior probabilities excluded 52 of 712 alignment sites. This dataset of 660 characters included 206 parsimony informative sites. Maximum parsimony analysis of the LSU dataset yielded 4 most parsimonious trees of 774 steps (C.I. = 0.53). The strict consensus of these trees (Fig. 1) depicted *Anisakis* plus (*Pseudoterranova, Terranova*) as monophyletic, with 100% bootstrap support. Likewise, representatives of *Pseudoterranova* and *Anisakis* were each monophyletic, with 100% and 93% bootstrap support, respectively. The strict consensus of MP trees also revealed a monophyletic *Contracaecum* plus *Phocascaris* clade, with 100% bootstrap support. Anisakidae (sampled genera included *Anisakis*, *Pseudoterranova*, *Contracaecum*, *Phocascaris*) was not a clade in the strict consensus of MP trees or in the bootstrap majority-rule MP consensus tree. Genera in the Raphidascarididae (*Hysterothylacium, Goezia, Iheringascaris*, and *Raphidascaris*) were monophyletic in the MP trees, but this clade received low bootstrap support (69%). Taxa from the Ascaridinae (*Parascaris*, *Ascaris*, *Toxascaris*, *Baylisascaris*) were strongly supported as monophyletic (99% bootstrap), but Ascarididae was not monophyletic because of the exclusion of *Toxocara*. In general, relationships among these clades, e.g., Raphidascaridae, Ascaridinae, and Anisakinae, representing the deepest nodes in the phylogenetic hypothesis, were not reliably supported as assessed by MP bootstrap resampling (Fig. 1).

Maximum likelihood analysis of the 660-character LSU dataset yielded a single tree (Fig. 2). *Anisakis* plus (*Pseudoterranova, Terranova*) was monophyletic in the ML tree with 100% bootstrap support. Like for MP trees, *Pseudoterranova*, *Anisakis*, and *Contracaecum* + *Phocascaris* were each monophyletic, with ≥99% support in ML bootstrap trees. Unlike the MP result, the ML tree recovered a monophyletic Anisakidae, but this clade was not found in the ML bootstrap majority-rule consensus tree. The Raphidascarididae received moderate (89%) support in the ML bootstrap tree. Genera representing Ascaridinae were strongly supported as monophyletic (100% bootstrap), but representatives of the Toxocarinae (*Toxocara*, *Porrocaecum*) were more closely related to anisakids (*Toxocara*) or the clade that included anisakids plus raphidascarids in the ML tree. As found for bootstrap MP analysis, the deepest nodes in the phylogenetic tree were not reliably supported as assessed by bootstrap resampling with ML inference (Fig. 2).

ITS rDNA sequence data and analysis

Sequences of ITS rDNA from 7 of the 8 *Pseudoterranova* specimens from Pacific waters (obtained from 5 different host species, Table I), were identical and matched the sequence of *P. decipiens* s. str. (Zhu et al., 2002). One *Pseudoterranova* sequence from a *Z. californianus* (CSL) host (CSL, 4994) was polymorphic (A/G) at 1 ITS-2 site (position 50 in the alignment of Zhu et al., 2002) that was an adenine in the other CSL *Pseudoterranova* specimens. This polymorphic nucleotide occurred at the single site that distinguishes the ITS sequences of *P. decipiens* s. str. (adenine at this site) from *Pseudoterranova azarasi* (guanine at this site).

Sequences of ITS rDNA from the 4 *Contracaecum* specimens obtained from 4 *Z. californianus* (CSL) hosts were identical except for 1 ITS-1 polymorphism in 1 individual (CSL, 4836). These sequences were not identical with any of the *C. ogmorhini* s.l. published sequences (Zhu et al., 2001), which are highly conserved, with only 1 ITS-1 site and 2 ITS-2 site differences (Fig. 3) between austral (*C. ogmorhini* s. str.) and boreal (*C. margolisi*) species. Three of 4 *Contracaecum* individuals sequenced from *Z. californianus* hosts in this study matched the sequence of *C. margolisi* (from a Pacific Canada *Z. californianus*) at all 3 sites (1 ITS-1, 2 ITS-2) that distinguish *C. ogmorhini* s. str. from *C. margolisi*, including the diagnostic ITS-2 *Bst*N1 restriction site (Zhu et al., 2001). The polymorphic (C/T) ITS-1 site found in 1 individual (Fig. 1, position 254) occurred at a site that distinguishes *C. ogmorhini* s. str. (cytosine at this site) from *C. margolisi* (thymine at this site). All 4 of the *Contracaecum* sequences from *Z. californianus* hosts had 1 ITS-1 difference (position 407, a transition) that distinguished them from *C. ogmorhini* s. str. and *C. margolisi*. Three additional ITS-1 sequence sites were different for published *C. ogmorhini* s.l. sequences (Zhu et al., 2001) and the 4 *Contracaecum* sequences from *Z. californianus* hosts obtained from California. The characteristics of these differences are consistent with potential errors in the previously published sequences. For example, GA dinucleotides at positions 91–92 are AG in the newly obtained sequences (Fig. 3) and also in 6 *Contracaecum osculatum* sequences (Zhu et al., 2000). Similarly, there are 2 C nucleotides at positions 240–241 (trailing C also in 6 *C. osculatum* sequences) and 2 T nucleotides at positions 409–410 rather than the single nucleotide indicated in each case for previously published sequences (Zhu et al., 2001).

Pairwise comparisons of 14 *Anisakis* ITS sequences showed

TABLE I. Specimen information and GenBank accession numbers for taxa used for comparative analyses.

Species	Host	Host ID*	Stage	Collection locality	GenBank accession no.†	
					LSU	ITS
Anisakis brevispiculata	*Kogia breviceps*		Adult	Coast of Florida, USA	—	AY826719
Anisakis pegreffi	*Micromesistius poutassou*		Larva	Tyrrhenian Sea, Italy	—	AY826720
Anisakis physeteris	*Physeter catodon*		Adult	Tyrrhenian Sea, Italy	—	AY826721
Anisakis simplex C	*Pseudorca crassidens*		Adult	Nanaimo, Canada	—	AY826722
Anisakis simplex s. str.	*Trachurus trachurus*		Larva	Cantabrian Sea, Spain	—	AY826723
Anisakis sp.	*Mirounga angustirostris*	NES 1940	Adult	San Francisco, Baker Beach, California, USA	AY821754	AY821739
Anisakis sp.	*Mirounga angustirostris*	NES 4	Adult	Ana Nuevo Island, California, USA	AY821755	AY821736
Anisakis sp.	*Mirounga angustirostris*	NES 2013	Adult	Half Moon Bay, California, USA	AY821757	AY821746
Anisakis sp.	*Phocoena phocoena*	HP C141	Adult	San Francisco, Ocean Beach, California, USA	AY821759	AY821749
Anisakis sp.	*Lissodelphis borealis*	PRP C140	Adult	Drakes Beach, California, USA	—	AY821740
Anisakis sp.	*Lissodelphis borealis*	PRP C140	Adult	Drakes Beach, California, USA	AY821758	AY821745
Anisakis sp.	*Eumetopias jubatus*	SSL 17	Adult	Seward, Alaska, USA	AY821756	AY821738
Anisakis sp. Clone 2	*Sebastes sp.*		Larva	Northern California Coast, USA	U94749	—
Anisakis sp. Clone 3	*Sebastes sp.*		Larva	Northern California Coast, USA	U94750	—
Anisakis typica	*Stenella longirostris*		Adult	Coast of Brazil	—	AY826724
Anisakis ziphidarum	*Ziphius cavirostris*		Adult	South Africa	—	AY826725
Ascaris suum	*Sus scrofa*		Adult	Cassopolis, Michigan, USA	AY826773	—
Baylisascaris procyonis	*Procyon lotor*	FDL 7	Adult	Cheshire, Connecticut, USA	AY826774	—
Baylisascaris transfuga	*Ursus americana*	DJR 410	Adult	Pocahontus County, West Virginia, USA	U94754	—
Contracaecum o. baicalensis	*Phoca sibirica*		Adult	Lake Baikal, Russia	AF226589	—
Contracaecum eudyptulae	*Eudyptula minor*		Adult	Philip Island, Victoria, Australia	AF226586	—
Contracaecum margolisi	*Zalophus californianus*		Adult	Vancouver Island, Canada	—	AJ291470, AJ291471
Contracaecum microcephalum	*Phalacrocorax pygmaeus*		Adult	Scutari Lake, Yugoslavia	AF226573	—
Contracaecum micropapillatum	*Pelecanus onocrotalus*		Adult	Assuan, Egypt	AF226587	—
*Contracaecum mirounga*e	*Mirounga leonina*		Adult	King George Island, Antarctica	AF226581	—
Contracaecum multipapillatum	*Pelecanus crispus*		Adult	Psatatopi, Greece	AF226574	—
Contracaecum multipapillatum cl 1	*Mugil curema*		Larva	Grand Lagoon, Horn Island, Mississippi	U94755	—
Contracaecum multipapillatum cl 3	*Mugil curema*		Larva	Grand Lagoon, Horn Island, Mississippi	U94756	—
Contracaecum ogmorhini	*Arctocephalus pusillus pusillus*		Adult	South Africa	AF226582	—
Contracaecum osculatum A	*Erignathus barbatus*		Adult	St. Anthony, Newfoundland, Canada	AF226583	—
Contracaecum osculatum B	*Phoca groenlandica*		Adult	Front, Newfoundland, Canada	AF226580	—
Contracaecum osculatum s. str	*Myoxocephalus quadricomis*		Larva	Geta, Åland, Finland	AF226576	—
Contracaecum radiatum	*Leptonychotes weddelli*		Adult	Weddell Sea, Antarctica	AF226577	—
Contracaecum rudolphii A	*Phalacrocorax carbo*		Adult	Policoro, Italy	AF226585	—
Contracaecum rudolphii B	*Phalacrocorax carbo*		Adult	Policoro, Italy	AF226579	—
Contracaecum septentrionale	*Phalacrocorax carbo*		Adult	Husavik, Iceland	AF226588	—
Contracaecum sp.	*Zalophus californianus*	CSL 4881	Adult	Monterey, California, USA	AY821771	AY821753
Contracaecum sp.	*Zalophus californianus*	CSL 4966	Adult	Morro Bay, California, USA	AY821768	AY821752
Contracaecum sp.	*Zalophus californianus*	CSL 4836	Adult	Santa Cruz, California, USA	AY821769	AY821750
Contracaecum sp.	*Zalophus californianus*	CSL 5034	Adult	San Francisco, Crissy Field, California, USA	AY821770	AY821751
Goezia pelagia	*Chaetodipterus faber*		Adult	East Ship Island, Mississippi Gulf Coast, USA	U94758	—
Heterocheilus tunicatus	*Trichechus manatus*		Adult	Citrus County, Florida, USA	AF226592	—
Hysterothylacium auctum	*Zoarces viviparus*		Adult	Geta, Åland, Finland	AF226591	—
Hysterothylacium fortalezae	*Lutjanus campechanus*		Adult	25 mi S Horn Island, Mississippi Gulf Coast, USA	U94760	—

TABLE I. Continued.

Species	Host	Host ID*	Stage	Collection locality	GenBank accession no.† LSU	ITS
Hysterothylacium pelagicum	*Coryphaena hippurus*		Adult	Gulf Coast of Mississippi, USA	AF226590	—
Hysterothylacium reliquens	*Micropogonias undulatus*		Larva	Davis Bayou, Ocean Springs, Mississippi, USA	U94762	—
Iheringascaris inquies	*Rachycentron canadum*		Adult	Petit Bois oil rig, Mississippi Gulf, USA	U94763	—
Parascaris equorum	*Equus caballus*		Adult	Baton Rouge, Louisiana, USA	AY821775	—
Phocascaris cystophorae	*Cystophora cristata*		Adult	Front, Newfoundland, Canada	AF226578	—
Phocascaris phocae	*Phoca groenlandica*		Adult	Sotra, Norway	AF226584	—
Phocascaris sp.	*Phoca groenlandica*		Adult	Gulf of St. Lawrence, Newfoundland, Canada	AF226575	—
Porrocaecum depressum	*Strix varia*		Adult	Baton Rouge, Louisiana, USA	U94765	—
Pseudoterranaova decipiens s. str.	*Myoxocephalus scorpius*		Larva	Dantzic Point, Burin Peninsula, Newfoundland, Canada	U94766	—
Pseudoterranaova sp.	*Zalophus californianus*	CSL 4934	Adult	Muir Beach, Marin County, California, USA	AY821766	AY821748
Pseudoterranaova sp.	*Zalophus californianus*	CSL 4994	Adult	Monterey, California, USA	AY821764	AY821747
Pseudoterranaova sp.	*Zalophus californianus*	CSL 5024	Adult	Monterey, California, USA	AY821762	AY821743
Pseudoterranaova sp.	*Phoca vitulina*	HS 1421	Adult	Pacifica, Rockaway Beach, California, USA	AY821763	AY821744
Pseudoterranaova sp.	*Phoca vitulina*	HS 2	Adult	Richardson Bay, California, USA	AY821765	AY825253
Pseudoterranaova sp.	*Eumetopias jubatus*	SSL	Adult	Seward, Alaska, USA	AY821767	AY821737
Pseudoterranaova sp.	*Mirounga angustirostris*	NES 5	Adult	Santa Cruz, California, USA	AY821760	AY821741
Pseudoterranaova sp.	*Enhydra lutris*	SO	Adult	Monterey, California, USA	AY821761	AY821742
Pseudoterranova azarasi Pa1	*Eumetopias jubatus*		Adult	Iwani, Japan	—	AJ413973, AJ413974
Pseudoterranova bulbosa Pb1	*Erignathus barbatus*		Adult	Newfoundland, Canada	—	AJ413969, AJ413971
Pseudoterranova bulbosa Pb5	*Erignathus barbatus*		Adult	Newfoundland, Canada	—	AJ413970, AJ413972
Pseudoterranova cattani Pc1	*Otaria byronia*		Adult	Concepcion, Chile	—	AJ413981, AJ413983
Pseudoterranova cattani Pc6	*Otaria byronia*		Adult	Concepcion, Chile	—	AJ413982, AJ413984
Pseudoterranova decipiens s.l. PdCA1	*Chaenocephalus aceratus*		Larva	South Shetland Islands, Antarctica	—	AJ413979, AJ413980
Pseudoterranova decipiens s. str. PdOe1	*Osmerus eperalanus*		Larva	Elbe estuary, Germany	—	AJ413975, AJ413978
Pseudoterranova decipiens s. str. PdOe5	*Osmerus eperlanus*		Larva	Elbe estuary, Germany	—	AJ413976, AJ413977
Pseudoterranova decipiens s. str. Pd1	*Phoca vitulina*		Adult	Newfoundland, Canada	—	AJ413967, AJ413968
Pseudoterranova krabbei Pk1	*Halichoerus grypus*		Adult	Froya Island, Norway	—	AJ413965, AJ413966
Raphidascaris acus	*Esox lucius*		Adult	Geta, Åland, Finland	AY821772	—
Terranova caballeroi	*Nerodia cyclopion*		Adult	Hammond, Louisiana, USA	U94767	—
Toxascaris leonina	*Vulpes vulpes*		Adult	Brookings, South Dakota	U94769	—
Toxocara canis	*Canis familiaris*		Adult	DeKalb, Illinois, USA	U94768	—

* Host identification (ID) designators were not assigned by all collectors.
† Underlined GenBank numbers are new submissions sequenced herein.

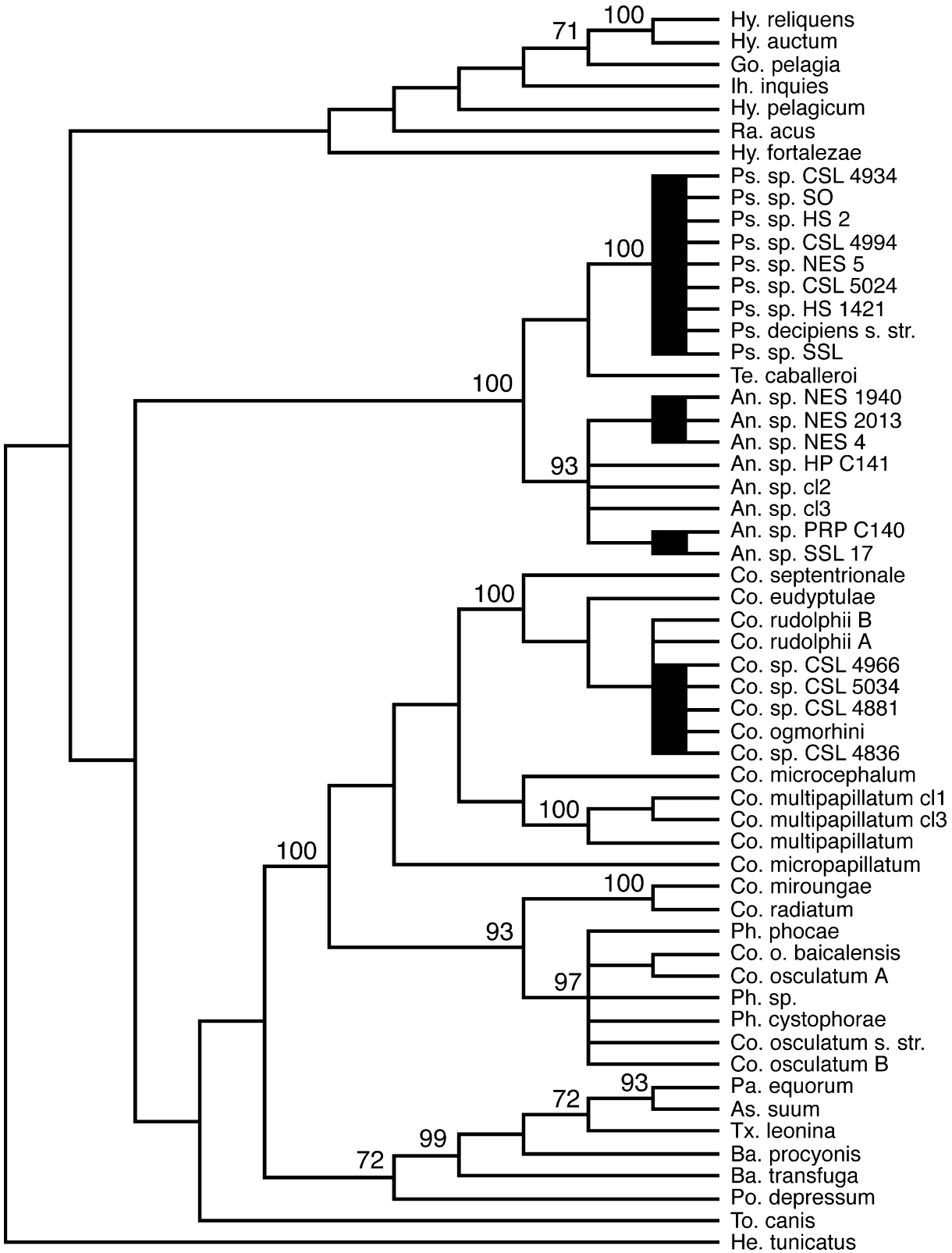

FIGURE 1. Strict consensus of 4 equally parsimonious trees inferred from LSU rDNA sequences. Trees inferred using unweighted MP from a ProAlign alignment after excluding sites with minimum posterior probabilities of 60% or less (52 of 712 sites). These 4 MP trees required 774 steps and had a C.I. of 0.53. Bootstrap percentages of clades (≥70%) as inferred by MP are shown above internal nodes. Vertical black bars mark *Anisakis*, *Contracaecum*, and *Pseudoterranova* taxa with identical LSU sequences as inferred from pairwise comparison of the alignment without exclusion of sites. Tree rooted by *Heterocheilus tunicatus* outgroup. Abbreviations for genera: An (*Anisakis*), As (*Ascaris*), Ba (*Baylisascaris*), Co (*Contracaecum*), Go (*Goezia*), Hy (*Hysterothylacium*), Ih (*Iheringascaris*), Pa (*Parascaris*), Ph (*Phocascaris*), Po (*Porrocaecum*), Ps (*Pseudoterranova*), Ra (*Raphidascaris*), Te (*Terranova*), To (*Toxocara*), Tx (*Toxascaris*). Abbreviations for host species and individual host identifier follow selected parasite species: CSL (California sea lion), HP (harbor porpoise), HS (harbor seal), NES (northern elephant seal), PRP (Pacific rightwhale porpoise), SO (sea otter), SSL (Steller's sea lion).

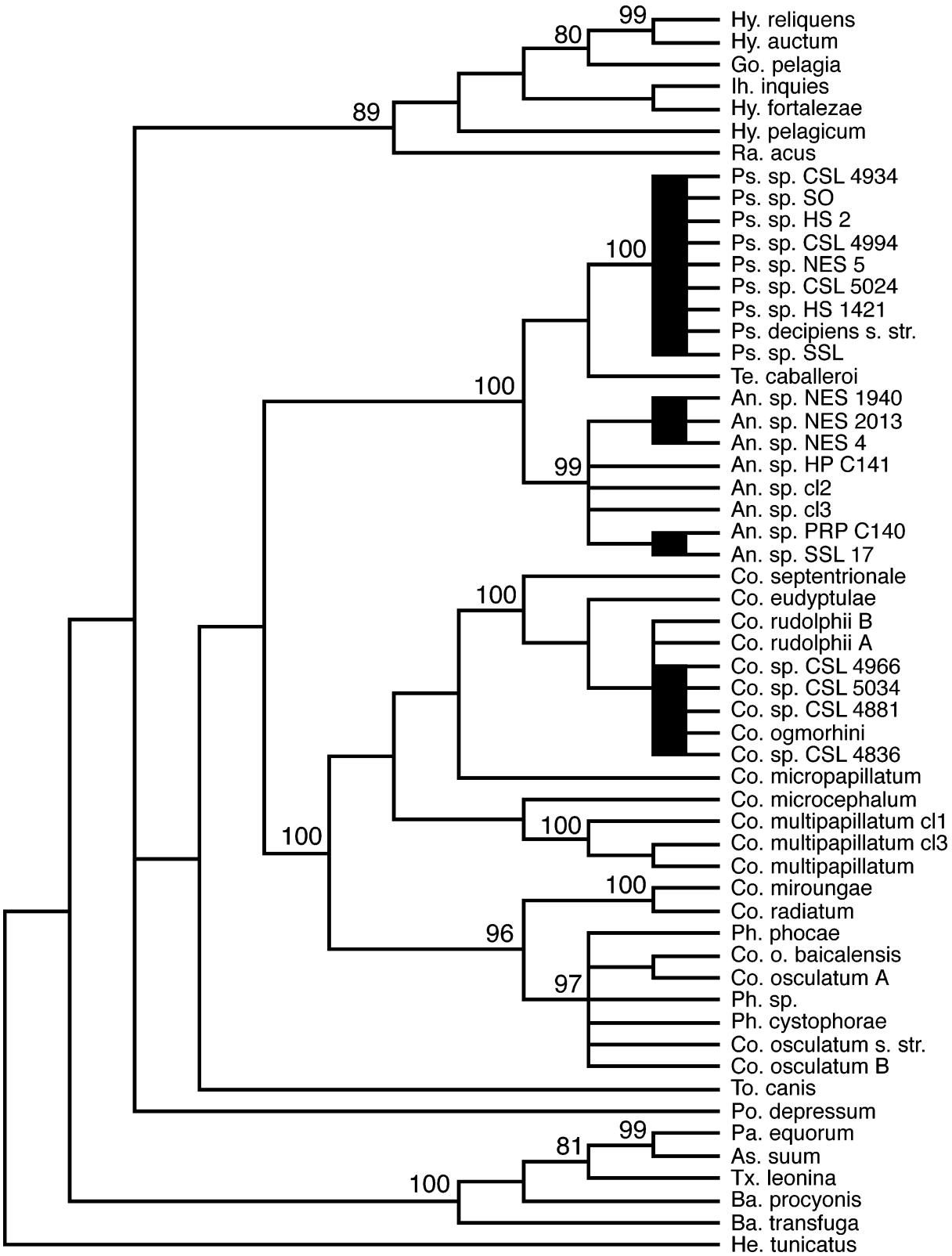

FIGURE 2. Maximum likelihood tree inferred from LSU rDNA sequences. Tree inferred from a ProAlign alignment after excluding sites with minimum posterior probabilities of 60% or less (52 of 712 sites). Vertical black bars mark *Anisakis*, *Contracaecum*, and *Pseudoterranova* taxa with identical LSU sequences as inferred from pairwise comparison of the alignment without exclusion of sites. Bootstrap percentages of clades (≥70%) as inferred by ML are shown above internal nodes. Likelihood search conducted using TVM+I+G model with gamma shape = 0.7169 and Pinvar = 0.3443 as selected by ModelTest. Tree rooted by *Heterocheilus tunicatus* outgroup. Abbreviations for genera: An (*Anisakis*), As (*Ascaris*), Ba (*Baylisascaris*), Co (*Contracaecum*), Go (*Goezia*), Hy (*Hysterothylacium*), Ih (*Iheringascaris*), Pa (*Parascaris*), Ph (*Phocascaris*), Po (*Porrocaecum*), Ps (*Pseudoterranova*), Ra (*Raphidascaris*), Te (*Terranova*), To (*Toxocara*), Tx (*Toxascaris*). Abbreviations for host species and individual host identifier follow selected parasite species: CSL (California sea lion), HP (harbor porpoise), HS (harbor seal), NES (northern elephant seal), PRP (Pacific rightwhale porpoise), SO (sea otter), SSL (Steller's sea lion).

```
                                        10         20         30         40         50         60
C. ogmorhini s. str. APD      ATCGAGCTAA ACCAAAAAGT CTCCTTACGT GCATAAATTC CATTTGCGCG TAATCGTGAG
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        70         80         90         100        110        120
C. ogmorhini s. str. APD      CCATGCAGCG AGTCATACAC ATGTGGTGGC GACCGTCGGC TGTTTTTCRT TTGGCTGACA
C. ogmorhini s. str. APP      .......... .......... .......... .......... ........A. ..........
C. margolisi                  .......... .......... .......... .......... ........A. ..........
Contracaecum CSL 4966         .......... .......... .......... AG........ ........A. ..........
Contracaecum sp. CSL 4836     .......... .......... .......... AG........ ........A. ..........

                                        130        140        150        160        170        180
C. ogmorhini s. str. APD      ATGGCTTATG GCTTGCTGTG TGTTGAGGGG AAGTGAGTGA TCCGATATGC TAGAAAGGCG
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        190        200        210        220        230        240
C. ogmorhini s. str. APD      GATCGATGGC GCTCATTTCC TCGTTATTCT CAACAACGGT GTCCACTTTG GCGTCTACGC
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        250        260        270        280        290        300
C. ogmorhini s. str. APD      -TCACCTAGC TATCGCCCGG ACCGTCGGTA GCGATGAAAG GTGGGGAGAA AGTTCCTCTC
C. ogmorhini s. str. APP      -.......... .......... .......... .......... .......... ..........
C. margolisi                  -.......... ...T...... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     C.......... ...T...... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     C.......... ...Y...... .......... .......... .......... ..........

                                        310        320        330        340        350        360
C. ogmorhini s. str. APD      TATGAGTTGA GCAGACTTAA TGAGCCCTGC GTGAGGGCCG CCAAAACCCA AAACACAACC
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        370        380        390        400        410        420
C. ogmorhini s. str. APD      GTTTCTTTTC ATTTCCGAAG TTGACCGATG AGTCGAGGCG TCCCGCTGT- CCATTCTTGG
C. ogmorhini s. str. APP      .......... .......... .......... .......... ..........- ..........
C. margolisi                  .......... .......... .......... .......... ..........- ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... ......C..T ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... ......C..T ..........

                                        430        440        450        460        470        480
C. ogmorhini s. str. APD      ATATGCGGGC GTGTTGATGA GTCGTTAACT AATATTCAAT ACTATCCGCA CAATGCTTCA
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        490        500        510        520        530        540
C. ogmorhini s. str. APD      GACGGTTCGT GTGAAGCGTG TGGTGCATTC GACAAGCAGT GTCCCTTTGG GGCGCTCCTT
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        550        560        570        580        590        600
C. ogmorhini s. str. APD      GCCTGGTTTG AACGGCAAAT TATTGCAAAG GTTTACTCGG TAAGCAGCAA TAATGGCCGT
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .T........ .......... ......R... T......... .......... ..........
Contracaecum sp. CSL 4966     .T........ .......... .......... T......... .......... ..........
Contracaecum sp. CSL 4836     .T........ .......... .......... T......... .......... ..........

                                        610        620        630        640        650        660
C. ogmorhini s. str. APD      AAGTGTGAGA TTGATTGTGT ACGTCCCTCG ATGCGGCCCC CAGTATTTGT TGACTGCCTC
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                        670        680        690        700        710        720
C. ogmorhini s. str. APD      TGGTGGTGAC TGGGGGTTAA GTATCGGATT ATCGAAAGAA TGTGACATGT CTTATACGGT
C. ogmorhini s. str. APP      .......... .......... .......... .......... .......... ..........
C. margolisi                  .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4966     .......... .......... .......... .......... .......... ..........
Contracaecum sp. CSL 4836     .......... .......... .......... .......... .......... ..........

                                      728
C. ogmorhini s. str. APD      TATGTGCT
C. ogmorhini s. str. APP      ........
C. margolisi                  ........
Contracaecum sp. CSL 4966     ........
Contracaecum sp. CSL 4836     ........
```

FIGURE 3. Alignment of ITS-1 (positions 1–451) and ITS-2 (452–728) sequences representing *C. ogmorhini* s.l. taxa. Dots indicate identity with the first sequence and dashes are inferred insertion-deletion events; standard IUB ambiguity codes are used, i.e., Y = C/T, R = A/G. *Contracaecum* CSL 4966 and 4836 include the diversity of sequences found in 4 specimens from *Zalophus californianus* obtained from California waters. Sequences of *Contracaecum ogmorhini* s. str. from austral localities are represented by specimens from *Arctocephalus pusillus pusillus* (APP) and *Arctocephalus pusillus doriferus* (APD), whereas *C. margolisi* is from a *Z. californianus* from Pacific Canada.

TABLE II. Pairwise nucleotide distances (absolute differences) for ITS-1, 5.8S, and ITS-2 sequences between *Anisakis* taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *A. typica*													
2. *A.* sp. SSL 17	149												
3. *A. pegreffii*	151	2											
4. *A.* sp. PRP C140	149	0	2										
5. *A.* sp. PRP C140	149	0	2	0									
6. *A.* sp. HP C141	149	3	5	3	3								
7. *A. simplex* s. str.	148	2	4	2	2	1							
8. *Anisakis* NES 1940	147	4	6	4	4	5	4						
9. *A. simplex* C.	147	4	6	4	4	5	4	0					
10. *A.* sp. NES 4	146	4	6	4	4	5	4	0	0				
11. *A.* sp. NES 2013	147	6	8	6	6	7	6	2	2	1			
12. *A. ziphidarum*	128	43	45	43	43	44	44	44	44	43	45		
13. *A. physeteris*	153	129	131	129	129	129	130	132	132	131	132	128	
14. *A. brevispiculata*	144	126	128	126	126	124	125	124	124	123	124	123	37

a large range of absolute distances (Table II), with many comparisons showing 2 or more differences. Comparisons involving *Anisakis typica*, *Anisakis ziphidarum*, *Anisakis physeteris*, or *Anisakis brevispiculata* yielded large pairwise distances. Two of 3 *Anisakis* specimens from *M. angustirostris* (NES) were identical with *A. simplex* C, and these were the only matches between *Anisakis* reference ITS sequences and the 7 *Anisakis* from hosts inhabiting northern Pacific waters (Fig. 4). Identical sequences were found between 2 specimens from *L. borealis* (PRP) and 1 specimen from *E. jubatus* (SSL). This sample of 7 *Anisakis* individuals included 3 unique sequences from 5 individual nematodes (hosts SSL 17, PRP C140, HP C141, NES 2013) that did not match ITS sequences of *A. brevispiculata*, *Anisakis simplex* s. str., *Anisakis simplex* C., *Anisakis pegreffii*, *A. physeteris*, *A. typica*, and *A. ziphidarum*.

For the phylogenetic analysis of *Anisakis* taxa, using ProAlign to detect and remove unreliably aligned sites by their posterior probabilities excluded 198 of 922 ITS sites. This dataset of 724 characters included 96 phylogenetically informative characters. Maximum parsimony analysis of this ITS dataset yielded 3 most parsimonious trees of 240 steps (C.I. = 0.88); ML analysis yielded 3 trees of equal likelihood. The strict consensus of the MP trees (Fig. 5) depicted *A. physeteris* plus *A. brevispiculata* as the sister group to the remaining *Anisakis* taxa. This clade was reliably supported in the MP bootstrap tree (92%) but was not recovered in the ML bootstrap tree. The remaining 12 ingroup *Anisakis* were monophyletic in ML and MP consensus trees, with 100% bootstrap support. *Anisakis* taxa from hosts collected in northern Pacific waters were nested within the strongly supported clade that included *A. pegreffii*, *A. simplex* s. str., and *A. simplex* C. A clade including *A. simplex* s. str., *A. pegreffii*, and specimens from *P. phocoena* (HP), *L. borealis* (PRP), and *E. jubatus* (SSL) was recovered in the strict consensus of MP and ML trees and received moderate bootstrap support.

For the phylogenetic analysis of *Pseudoterranova* taxa, using ProAlign to detect and remove unreliably aligned sites excluded 83 of 681 ITS characters. This yielded a dataset of 598 characters that included 23 phylogenetically informative sites. Maximum parsimony analysis yielded 2 equally parsimonious trees of 114 steps (C.I. = 0.93); ML analysis recovered 2 trees of equal likelihood. A strict consensus of MP trees (Fig. 6) and 1 for ML trees depicted *P. decipiens* s.l. from *Chaenocephalus aceratus* (Blackfin icefish) as sister to the remaining taxa, which were supported as monophyletic with moderate support in MP and ML bootstrap analyses. *Pseudoterranova* specimens from hosts inhabiting northern Pacific waters were part of a clade in MP and ML consensus trees that included *P. decipiens* s. str. and *P. azarasi*.

DISCUSSION

Molecular approaches to delimiting and identifying anisakid nematodes have markedly influenced our understanding of their systematics and biodiversity (Paggi and Bullini, 1994; Bullini et al., 1997). For example, in a detailed morphologically based revision of *Anisakis*, Davey (1971) recognized 3 valid species (*A. simplex*, *A. typica*, and *A. physeteris*) and retained 4 others as species inquirendae (*Anisakis dussumierii*, *Anisakis insignis*, *Anisakis schupakovi*, and *Anisakis alexandri*). Multilocus allozyme methods, which have a long history of application to investigations of *Anisakis* diversity (Mattiucci et al., 1986; Nascetti et al., 1986; Orecchia et al., 1986; Paggi and Bullini, 1994; Bullini et al., 1997), have independently supported the validity of *A. simplex* s. str., *A. typica*, *A. physeteris*, and *A. schupakovi*, plus other species not recognized as valid by Davey (1971), such as *A. brevispiculata* (Mattiucci et al., 2001). These methods have also proved to be powerful tools for revealing cryptic *Anisakis* species, such as members of the *A. simplex* complex, including *A. simplex* C and *A. pegreffii*.

Multilocus protein electrophoresis with population-level sampling of *Anisakis*, *Contracaecum*, and *Pseudoterranova* has been used to detect evidence of distinct biological species in natural populations and develop allozyme keys for their identification (Mattiucci et al., 1997, 1998; Paggi et al., 2000). Reference individuals initially characterized by allozymes have also been used to develop DNA-based approaches for species identification such as PCR-RFLP and direct sequencing of ITS rDNA or mitochondrial DNA. For molecular systematics, multilocus approaches offer significant theoretical advantages for

```
                                310        320        330        340        350        360
A. simplex s. str.      GACTTAATGA GCCACG--CT AGGTGGCCGC CAAAACCCAA AACACAACCG GTCTATTTGA
A. simplex C            .......... ......--.. .......... .......... .......... ..........
A. pegreffii            .......... ......--.. .......... .......... .......... ..........
A. ziphidarum           .......... ......--.. .......... .......... .......... ..........
A. physeteris           .......... ......--.. T......... .......... .........A ..........
A. brevispiculata       .......... ...G..--.. T..C...... .......... .........A ..........
A. typica               .......... ......CT.. .......... ..G....... ......C.AA T.G---...T
Anisakis sp. PRP C140   .......... ......--.. .......... .......... .......... ..........
Anisakis sp. HP C141    .......... ......--.. .......... .......... .......... ..........
Anisakis sp. NES 2013   .......... ......--.. .......... .......... .......... ..........

                                370        380        390        400        410        420
A. simplex s. str.      CATTGTTA-- TTTCATTGTA TGTGTTGAAA ATGTATTACG GTGAACTGTC TTCACG-GTT
A. simplex C            ........-- .......... .......... .......... .......... ......-..C
A. pegreffii            ........-- .......... .......... .......... .......... ......-...
A. ziphidarum           ........-- C..---.... .......... .......... .......... ...G..-...
A. physeteris           .......CAG .A.GCG.TG. CA.TAC.TT. ........T. .CA....A.. ...GT.----
A. brevispiculata       .......CAG .A.GCG.TG. CA.TA..TT. ........T. .CA....A.. ...G..----
A. typica               ....TGAC-- ----.....T GA..A...TT .......GT. C.AG.GCA.. ..TG.AATCA
Anisakis sp. PRP C140   ........-- .......... .......... .......... .......... ......-...
Anisakis sp. HP C141    ........-- .......... .......... .......... .......... ......-...
Anisakis sp. NES 2013   ........-- .......... .......... .......... .......... .......-..C

                                430        440        450        460        470        480
A. simplex s. str.      TTTCT----- ----GGACTG TGAAGCATTC GGCAAGCAAT TGCTGTTGTG TTGTTGGTGA
A. simplex C            .....----- ----...... .......... .......... .......... ..........
A. pegreffii            .....----- ----...... .......... .......... .......... ..........
A. ziphidarum           ...AG----- ----T..... .......... ...G...... ..T..C.... ......A...
A. physeteris           -GCTC----- ----C.G.C. ....A..... ........G. .---..C... ........--.T
A. brevispiculata       -G.T.----- ----C.G... .......... ........G. .---.C.... ........--.T
A. typica               C....CTCAG ATTGT..T.. .......... ...G...G.. ..T...C... ......C.T.
Anisakis sp. PRP C140   .....----- ----...... .......... .......... .......... ..........
Anisakis sp. HP C141    ...T.----- ----...... .......... .......... .......... ..........
Anisakis sp. NES 2013   .....----- ----...... .......... .......... .......... ..........

                                490        500        510        520        530        540
A. simplex s. str.      TTCTATCATG G--------- --ACAATATG ACGAGCGGTT CCTTGCTTAG TG--ATGAC-
A. simplex C            .......... .--------- --........ ...C...... .......... ..--....T-
A. pegreffii            .......... .--------- --........ .......... .......... ..--......-
A. ziphidarum           ..GAG..--- .--------- --........ G.ACA..... .......... .T--....A-
A. physeteris           .GG.------ ---------- ---.GG.C.T TGA.T....C GA...GCGGC ----TC...-
A. brevispiculata       CGG.------ ---------- ---.GG.C.C GG--T....C GA...ACAGC ----....T-
A. typica               AGG.GA.GAT TGAATCGGCA CCG.GCG.CA CGACA..... .......... .TTG....AC
Anisakis sp. PRP C140   .......... .--------- --........ .......... .......... ..--......-
Anisakis HP C141        .......... .--------- --........ .......... .......... ..--......-
Anisakis sp. NES 2013   .......... .--------- --........ ...C...... .......... ..--....T-

                                550        560        570        580        590        600
A. simplex s. str.      -AAAAGAAGA CGTCAACACC GAATCTACTA TA------CT ACTAATACTA GTATATAGGT
A. simplex C            -.........  .......... .......... ..------.. .......... ..........
A. pegreffii            -.........  .......... .......... ..------.. .......... ..........
A. ziphidarum           G.G.......  .......... ...C...... ..------.. ....G..... ..G.......
A. physeteris           -----.GCTC .T.GCTT.GT TGT.G.GTG. GG------AG ..GTCA..AC C-GA.C--.A
A. brevispiculata       -----.GCTC .T.GCTT.GT TGT.G.GTG. AG------AG ..GTTA..AC C-GA.C--.G
A. typica               A......C.T .CCGC..... C..CG.CTGC ..AACACTAG ....GAG..G ..G.C...AG
Anisakis sp. PRP C140   -.........  .......... .......... ..------.. .......... ..........
Anisakis sp. HP C141    -.........  .......... .......... ..------.. .......... ..........
Anisakis sp. NES 2013   A.........  .......... .....C.... ..------.. .......... ..G.......
```

FIGURE 4. Alignment of ITS-1 (positions 1–395) and ITS-2 (396–765) sequences of *Anisakis* taxa. One representative of each unique sequence was included for comparison. Dots indicate identity with the first sequence and dashes are inferred insertion-deletion events.

```
                                310        320        330        340        350        360
A. simplex s. str.      GACTTAATGA GCCACG--CT AGGTGGCCGC CAAAACCCAA AACACAACCG GTCTATTTGA
A. simplex C            .......... ......--.. .......... .......... .......... ..........
A. pegreffii            .......... ......--.. .......... .......... .......... ..........
A. ziphidarum           .......... ......--.. .......... .......... .......... ..........
A. physeteris           .......... ......--.. T......... .......... .........A ..........
A. brevispiculata       .......... ...G..--.. T..C...... .......... .........A ..........
A. typica               .......... ......CT.. .......... ..G....... ......C.AA T.G---...T
Anisakis sp. PRP C140   .......... ......--.. .......... .......... .......... ..........
Anisakis sp. HP C141    .......... ......--.. .......... .......... .......... ..........
Anisakis sp. NES 2013   .......... ......--.. .......... .......... .......... ..........

                                370        380        390        400        410        420
A. simplex s. str.      CATTGTTA-- TTTCATTGTA TGTGTTGAAA ATGTATTACG GTGAACTGTC TTCACG-GTT
A. simplex C            ........-- .......... .......... .......... .......... ......-..C
A. pegreffii            ........-- .......... .......... .......... .......... ......-...
A. ziphidarum           ........-- C..---.... .......... .......... .......... ...G..-...
A. physeteris           .......CAG .A.GCG.TG. CA.TAC.TT. ........T. .CA....A.. ...GT.----
A. brevispiculata       .......CAG .A.GCG.TG. CA.TA..TT. ........T. .CA....A.. ...G..----
A. typica               ....TGAC-- ----.....T GA..A...TT .......GT. C.AG.GCA.. ..TG.AATCA
Anisakis sp. PRP C140   ........-- .......... .......... .......... .......... ......-...
Anisakis sp. HP C141    ........-- .......... .......... .......... .......... ......-...
Anisakis sp. NES 2013   ........-- .......... .......... .......... .......... .......-..C

                                430        440        450        460        470        480
A. simplex s. str.      TTTCT----- ----GGACTG TGAAGCATTC GGCAAGCAAT TGCTGTTGTG TTGTTGGTGA
A. simplex C            .....----- ----..... .......... .......... .......... ..........
A. pegreffii            .....----- ----..... .......... .......... .......... ..........
A. ziphidarum           ...AG----- ----T..... .......... ...G...... ..T..C.... ......A...
A. physeteris           -GCTC----- ----C.G.C. ....A..... ........G. .---..C... .........T
A. brevispiculata       -G.T.----- ----C.G... .......... ........G. .---.C.... .........T
A. typica               C....CTCAG ATTGT..T.. .......... ...G...G.. ..T...C... ......C.T.
Anisakis sp. PRP C140   .....----- ----..... .......... .......... .......... ..........
Anisakis sp. HP C141    ...T.----- ----..... .......... .......... .......... ..........
Anisakis sp. NES 2013   .....----- ----..... .......... .......... .......... ..........

                                490        500        510        520        530        540
A. simplex s. str.      TTCTATCATG G--------- --ACAATATG ACGAGCGGTT CCTTGCTTAG TG--ATGAC-
A. simplex C            .......... .--------- --........ ...C...... .......... ..---...T-
A. pegreffii            .......... .--------- --........ .......... .......... ..---.....-
A. ziphidarum           ..GAG..--- .--------- --........ G.ACA..... .......... .T--....A-
A. physeteris           .GG.------ ---------- ---.GG.C.T TGA.T....C GA...GCGGC ----TC...-
A. brevispiculata       CGG.------ ---------- ---.GG.C.C GG--T....C GA...ACAGC ----....T-
A. typica               AGG.GA.GAT TGAATCGGCA CCG.GCG.CA CGACA..... .......... .TTG....AC
Anisakis sp. PRP C140   .......... .--------- --........ .......... .......... ..---.....-
Anisakis HP C141        .......... .--------- --........ .......... .......... ..---.....-
Anisakis sp. NES 2013   .......... .--------- --........ ...C...... .......... ..---....T-

                                550        560        570        580        590        600
A. simplex s. str.      -AAAAGAAGA CGTCAACACC GAATCTACTA TA------CT ACTAATACTA GTATATAGGT
A. simplex C            -......... .......... .......... ..------.. .......... ..........
A. pegreffii            -......... .......... .......... ..------.. .......... ..........
A. ziphidarum           G.G....... .......... ...C...... ..------.. ....G..... ..G.......
A. physeteris           -----.GCTC .T.GCTT.GT TGT.G.GTG. GG------AG ..GTCA..AC C-GA.C--.A
A. brevispiculata       -----.GCTC .T.GCTT.GT TGT.G.GTG. AG------AG ..GTTA..AC C-GA.C--.G
A. typica               A......C.T .CCGC..... C..CG.CTGC ..AACACTAG ....GAG..G ..G.C...AG
Anisakis sp. PRP C140   -......... .......... .......... ..------.. .......... ..........
Anisakis sp. HP C141    -......... .......... .......... ..------.. .......... ..........
Anisakis sp. NES 2013   A......... .......... .....C.... ..------.. .......... ..G.......
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FIGURE 4. Continued.

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                                610        620        630        640        650        660
A. simplex s. str.     GAGGTGCTTT TGGTGGTCAC AAAAGTGACA AGTATGCCAT TTCATAGGGG CAACAACCAG
A. simplex C           .......... .......... .......... .......... .......... ..........
A. pegreffii           .......... .......... .......... .......... .......... ..........
A. ziphidarum          .......... .......... ........A. .......... .......... ..........
A. physeteris          C-....A.A. .....A..G. .....C..A. .........C .......... ..........
A. brevispiculata      C-....A.A. .....A..G. .C...C..G. ...G.....C .......... ..........
A. typica              .T.T..GG.G ..A.TT.G.T GGTCACA.A. GTGCC..... .......T.. ..........
Anisakis sp. PRP C140  .......... .......... .......... .......... .......... ..........
Anisakis sp. HP C141   .......... .......... .......... .......... .......... ..........
Anisakis sp. NES 2013  .......... .......... .......... .......... .......... ..........

                                670        680        690        700        710        720
A. simplex s. str.     CATACGT--- -------GAT AAGTTGGCTG GTTGATGAAA C----GGCAA CGGAATG---
A. simplex C           .......--- -------... .......... .......... .----..... .......---
A. pegreffii           .......--- -------... .......... .......... .----..... .......---
A. ziphidarum          .....A.--- -------... .......... .......... .----..... .......---
A. physeteris          .......--- ---------- GT........ .........C -TGTT..... ....G..---
A. brevispiculata      .......--- ---------- .T........ .........C .TGTT..... ....G..---
A. typica              .......CTA TGATACTAG. .G........ .......... .----..... .......TGC
Anisakis sp. PRP C140  .........--- -------... .......... .......... .----..... ..........---
Anisakis sp. HP C141   .........--- -------... .......... .......... .----..... ..........---
Anisakis sp. NES 2013  .........--- -------... .......... .......... .----..... ..........---

                                730        740        750        760
A. simplex s. str.     --ACGGACGT CTATGTGATC AAA-AATGAT ACTATTTGAC CTCAG
A. simplex C           --........ .....T.... ...-...... .......... .....
A. pegreffii           --........ .....T.... ...-...... .......... .....
A. ziphidarum          --.T.----- -....T.... ...-....T. TA........ .....
A. physeteris          --.....--.. G.G----... .GG-..C.T. TG........ .....
A. brevispiculata      --.....--.. G.G----... .GG-..C.T. TG........ .....
A. typica              GC.T.C.T.. GATC.A..AG CG.T....T. CG........ .....
Anisakis sp. PRP C140  --........ .....T.... ...-...... .......... .....
Anisakis sp. HP C141   --........ .....T.... ...-...... .......... .....
Anisakis sp. NES 2013  --........ .....T.... ...-...... .......... .....
```

FIGURE 4. Continued.

detecting and delimiting species when investigating natural populations (Nadler, 2002, 2005), whereas DNA diagnostic markers based on a single locus are most useful as practical tools for identification of known species from either larvae or adults. Single-locus studies based on sequencing or SSCP methods have the potential to reveal previously uncharacterized genetic variation, as in this study. However, interpreting this variation is often difficult, in particular, when relatively few individuals have been studied. Although such unique genetic variants may represent the derived states characteristic of distinct evolutionary species (and the markers of noninterbreeding populations as inferred for biological species), an alternative interpretation is that they represent intraspecific polymorphisms. When the genetic differentiation between known species and newly discovered genetic variants is large, it is tempting to hypothesize that the new variant represents an uncharacterized species, even when these data come from 1 locus. More typically, there are few differences between new variants and known species, as in this study, and multilocus studies of population samples are required to assess whether the genetic differences are fixed and the pattern consistent with separate species. Nevertheless, discovering novel sequences, as reported here for specimens of *Anisakis* and *Contracaecum*, is valuable because it reveals that previously unrecognized species may exist. Genetic data for fully testing hypotheses of new species (species delimitation) must come from additional research, such as comparisons of nucleotide sequences from multiple loci (and interpretation of multiple gene trees) or from multilocus protein electrophoresis (and population genetic analysis).

Pseudoterranova adults collected from hosts inhabiting northern Pacific waters, including *Z. californianus* (CSL), *M. angustirostris* (NES), *P. vitulina* (HS), *E. lutris* (SO), and *E. jubatus* (SSL), had LSU and ITS sequences that were identical with *P. decipiens* s. str. (formerly called *P. decipiens* B). These results represent new host records for *P. decipiens* s. str. in *M. angustirostris*, *E. lutris*, and *E. jubatus*, extending the confirmed host and geographic range of this anisakid, which is geographically widespread in pinniped hosts of the Northern Hemisphere. Surveys of marine mammals from Pacific waters using morphological approaches have reported *P. decipiens* s.l. or *Pseudoterranova* sp. from these same hosts plus *C. ursinus* (NFS), in which it has a high prevalence (Spraker et al., 2003). *Pseudoterranova decipiens* s. str. is 1 of 4 species from the *P. decipiens* complex, which also includes *Pseudoterranova bulbosa*, *Pseudoterranova krabbei*, and *P. azarasi* (Mattiucci et al., 1998; Paggi et al., 2000). The latter was redescribed based on specimens from *E. barbatus* (BS) and *E. jubatus* (SSL) collected in Japanese waters of the northern Pacific Ocean. These investigators tested 237 individual adult *P. decipiens* s.l. from these 2 host species using allozyme electrophoresis and only

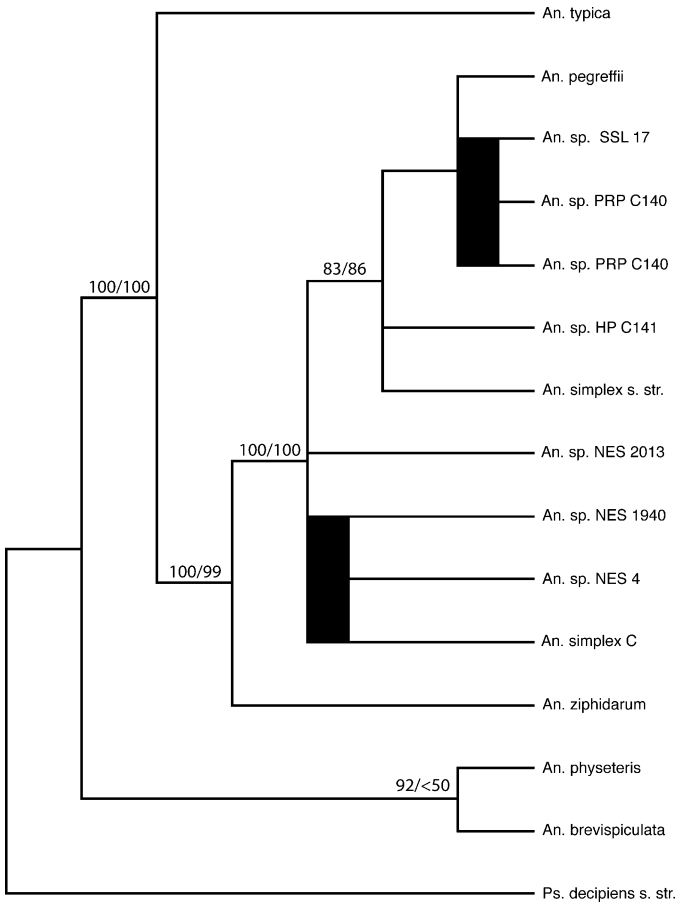

FIGURE 5. Strict consensus of 3 equally parsimonious trees inferred from *Anisakis* ITS rDNA sequences. Trees inferred using unweighted MP from a ProAlign alignment after excluding sites with minimum posterior probabilities of 60% or less (198 of 922 sites). These 3 MP trees required 240 steps and had a C.I. of 0.88. Bootstrap percentages of clades (≥70%) are shown above internal nodes, with MP values listed first, followed by ML values. Vertical black bars mark *Anisakis* taxa with identical ITS sequences as inferred from pairwise comparison of the alignment without exclusion of sites. Tree rooted by *Pseudoterranova decipiens* s. str. sequence. Abbreviations for host species and individual host identifier follow selected parasite species: HP (harbor porpoise), NES (northern elephant seal), PdCa (blackfin icefish), PdOe (european smelt), PRP (Pacific rightwhale porpoise), SSL (Steller's sea lion).

found individuals of *P. bulbosa* and *P. azarasi*. Although *P. azarasi* was not found in the small sample of nematodes examined in these North American hosts, 1 individual was polymorphic for the only ITS site that distinguishes *P. azarasi* from *P. decipiens* s. str. Additional research is needed to determine if this site is polymorphic within *P. decipiens* s. str. or *P. azarasi* (compromising the diagnostic utility of this ITS site in this case), or alternatively if this individual represents an F1 hybrid. Genetic evidence has revealed 1 instance of hybridization between *P. decipiens* s. str. and *P. krabbei* (Paggi et al., 1991), and hybridization of other anisakids has been suggested based on ITS sequences (Abollo et al., 2003).

Previous systematic analyses of *Pseudoterranova* species have included comparisons based on allozyme genetic distances (Paggi et al., 1991; Bullini et al., 1997; Paggi et al., 2000) and a phenogram of uncorrected ITS rDNA distances (Zhu et al., 2002). The allozyme studies indicated a close genetic relationship between *P. decipiens* s. str. and *P. azarasi* (Bullini et al., 1997; Paggi et al., 2000), with the topology: ((((*P. decipiens* s. str., *P. azarasi*), *P. krabbei*), *P. bulbosa*), *P. decipiens* E) (Bullini et al., 1997). Considering only taxa that were identified to species, the ITS phenogram (Zhu et al., 2002) also depicted greatest similarity between *P. decipiens* s. str. and *P. azarasi*; however, their ITS phenogram was different from the allozyme tree: (((((*P. decipiens* s. str., *P. azarasi*), *P. bulbosa*), *P. cattani*), *P. krabbei*), *P. decipiens* Ca1). Zhu et al. (2002) have hypothesized that *P. decipiens* Ca1 is most likely equivalent to *P. decipiens* E. Maximum parsimony and ML analyses of ITS sequences (Fig. 6) also depicted a clade including *P. azarasi* and *P. decipiens* s. str.; however, the remaining topology was different from the previously published ITS analysis but was the same as the allozyme phenogram (excepting the absence of *P. cattani* from the allozyme study). Differences between these 2 analyses of ITS sequences are not unexpected, given that methods of inferring phenograms and phylogenetic trees involve very different assumptions. One potentially interesting phylogenetic result is that the Southern Hemisphere *Pseudoterranova* Ca1 and *P. cattani* are not sister taxa, suggesting a more complex evolutionary history than might be explained by a simple biogeographic scenario.

Contracaecum adults collected from *Z. californianus* (CSL) hosts inhabiting northern Pacific waters were identical in LSU

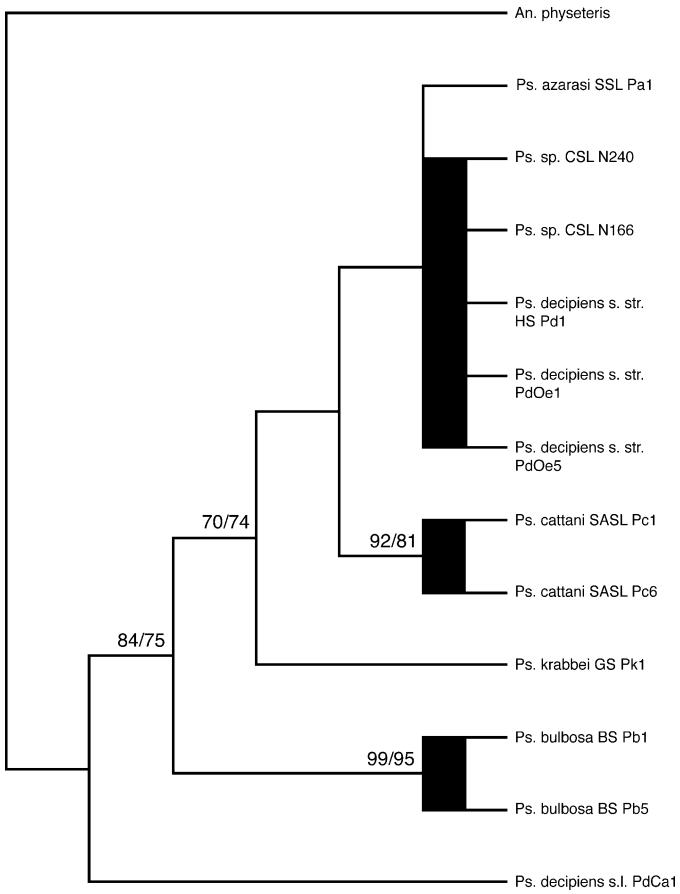

FIGURE 6. Strict consensus of 2 equally parsimonious trees inferred from *Pseudoterranova* ITS rDNA sequences. Only 2 of 8 new *Pseudoterranova* sequences representing hosts from northern Pacific waters were included; these sequences represented all the variation among these 8 sequences. Vertical black bars mark taxa with identical ITS sequences as inferred from pairwise comparison of the alignment without exclusion of sites. Trees inferred using unweighted MP from a ProAlign alignment after excluding sites with minimum posterior probabilities of 60% or less (83 of 681 sites). These MP trees required 114 steps and had a C.I. of 0.93. Bootstrap percentages of clades (≥70%) are shown above internal nodes, with MP values listed first, followed by ML values. Abbreviations for host species and individual host identifier follow selected parasite species: BS (bearded seal), CSL (California sea lion), GS (grey seal), HS (harbor seal), NES (northern elephant seal), PdCa (blackfin icefish), PdOe (european smelt), PRP (Pacific rightwhale porpoise), SASL (South American sea lion), SSL (Steller's sea lion).

sequence with *C. ogmorhini* s. str., but their ITS sequences matched neither *C. ogmorhini* s. str. nor *C. margolisi*. This apparent conflict between LSU and ITS rDNA can be explained by the more conservative substitution rate of this LSU region. All 4 *Contracaecum* individuals from *Z. californianus* differed from *C. margolisi* and *C. ogmorhini* s. str. at 1 site; 4 other ITS-1 site differences were noted, but the nature of these differences suggest they may represent errors in the previously published sequences. One individual specimen was polymorphic at the only ITS-1 site that is different between *C. ogmorhini* s. str. and *C. margolisi*. Although this individual nematode would not be confused with *C. ogmorhini* s. str. or *C. margolisi* due to the difference in ITS sequence at position 407 (Fig. 3), the presence of such site polymorphisms in the few individuals of *C. ogmorhini* s.l. examined indicates that more individuals should be sequenced to confirm that sites with differences are fixed within species. From a phylogenetic perspective, the LSU analysis establishes that the *Contracaecum* from these *Z. californianus* is most closely related to *C. ogmorhini* s.l. and *C. rudolphii*. Phylogenetic analysis of ITS sequences was not undertaken for *Contracaecum* because of the potential sequence errors previously noted and the lack of ITS variation (Fig. 3). Additional genetic studies are required to determine whether these novel sequences from California sea lion hosts represent a previously unrecognized species.

Anisakis adults collected from northern Pacific marine mammals included individuals of *A. simplex* C from 2 *M. angustirostris* (NES) hosts; this is a new host record for this species. None of the other 5 individuals had ITS sequences that matched known species. Specimens from *L. borealis* (PRP) and *E. jubatus* (SSL) were most closely related to *A. pegreffii*, as inferred from phylogenetic analysis of ITS rDNA. The unique sequence from *P. phocoena* (HP) was part of the clade that included *A. pegreffii* and *A. simplex* s. str. All the specimens from northern Pacific marine mammals were nested within the clade that included ((*A. pegreffii*, *A. simplex* s. str.) *A. simplex* C); these represent the 3 known species within the *A. simplex* species complex and are characterized (along with *A. typica* and *A. ziphidarum*) by having type I larvae sensu Berland (1961), which may represent the apomorphic (derived) state within *An-*

isakis. Thus, these 3 unique ITS sequences may represent previously unidentified species of the *A. simplex* complex, but testing this hypothesis will require additional data. Previously published assessments of *Anisakis* relationships have been based on analysis of allozyme data (Bullini et al., 1997; Mattiucci et al., 1997; Paggi, Mattiucci et al., 1998; Mattiucci et al., 2002). Allozyme studies that have included explicit trees have indicated that a close genetic relationship exists among species of the *A. simplex* complex, with phenograms depicting the topology: ((((*A. simplex* s. str., *A. simplex* C), *A. pegreffii*), *A. ziphidarum*), *A. physeteris*) (Mattiucci et al., 1997; Paggi, Nascetti et al., 1998). In contrast to these multilocus phenograms, the MP and ML analyses of ITS sequences supported a sister-group relationship for *A. pegreffii* and *A. simplex* s. str., with *A. simplex* C as sister to this clade (Fig. 5). Although the ITS phylogeny contains more species than in the published allozyme phenograms, there is good agreement between phenetic clustering of allozyme data and phylogenetic analysis of ITS sequences.

Molecular phylogenetic studies of anisakids and other ''aquatic'' ascaridoids have been relatively limited with respect to species representation (Nadler and Hudspeth, 1998; Nadler et al., 2000; Nadler and Hudspeth, 2000). Phylogenetic support for Anisakidae and Raphidascarididae has varied according to both the genes analyzed and the types of analytical methods used (Nadler and Hudspeth, 1998; Zhu, Gasser, and Chilton, 1998; Nadler and Hudspeth, 2000; Shih, 2004). For example, phylogenetic analyses of SSU or LSU sequences recovered a raphidascarid clade, but did not find evidence of a monophyletic Anisakidae, whereas combined analysis of SSU and LSU sequences yielded a monophyletic Anisakidae using ML inference but not by MP (Nadler and Hudspeth, 1998). Such problems may be addressed by adding taxa, or characters, or both (Graybeal, 1998; Mitchell et al., 2000); however, in the most comprehensive phylogenetic analysis of Ascaridoidea, which included data from 3 genes plus morphological characters, support for the anisakid clade remained weak (Nadler and Hudspeth, 2000). To provide increased taxon representation, published and new 28S sequences were combined in an analysis that used a probabilistic approach to determine which sites were ambiguous with respect to multiple alignment and warranted exclusion from the phylogenetic analyses. In these analyses, *Contracaecum* diversity was well represented, and raphidascarids included 4 of 9 genera. Anisakinae was less well represented, with only members of the *A. simplex* complex, *Terranova*, and 1 species of *Pseudoterranova*. The latter genus shows relatively little ITS diversity among known species (Zhu et al., 2002), and, therefore, additional *Pseudoterranova* 28S sequences are unlikely to substantially alter the LSU tree. In contrast, *Anisakis* taxa show substantial ITS sequence diversity (Fig. 4), and because species with the most divergent ITS sequences (*A. typica*, *A. physeteris*, *A. brevispiculata*) are absent from the LSU trees, these trees cannot be considered definitive statements about *Anisakis* monophyly. Parsimony and ML analyses indicated that the Raphidascarididae, *Contracaecum* plus *Phocascaris*, and *Anisakinae* (*Pseudoterranova*, *Anisakis*, *Terranova*) are each monophyletic, the latter 2 groups with consistently strong (MP and ML) bootstrap support. Anisakidae was recovered in the ML analysis, but without reliable bootstrap support; this clade was absent from the MP tree. In general, clade support was weak at deeper nodes in the LSU gene tree as evidenced by low bootstrap values for both MP and ML. In this case, adding taxa did not appear to increase the resolution of rDNA trees when testing the monophyly of the Anisakidae or Ascarididae. Improving phylogenetic resolution for deeper nodes in the evolutionary history of Ascaridoidea will apparently require additional gene sequences, perhaps those with relatively conservative rates of evolutionary change (Nadler, 1995).

ACKNOWLEDGMENTS

We thank Derek Hyman and Eugene Bolotin for assistance with contig assembly and database management. This study was supported in part by NSF Tree of Life grant DEB-0228692.

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                                 10         20         30         40         50         60
A. simplex s. str.      ATCCAAAACG AACGAAAAAG TCTCCCAACG TGCATACCTT CCATTTGCAT GTTGTTGTGA
A. simplex C            .......... .......... .......... .......... .......... ..........
A. pegreffii            .......... .......... .......... .......... .......... ..........
A. ziphidarum           .......... ...T...... .......... ........G. .......... ..........
A. physeteris           .......... ...----... .......... ........G. .......... ..........
A. brevispiculata       .......... ...----... .......... ........A. .......... ..........
A. typica               .......... ...T...... .......... ........GC .......... ..........
Anisakis sp. PRP C140   .......... .......... .......... .......... .......... ..........
Anisakis sp. HP C141    .......... .......... .......... .......... .......... ..........
Anisakis sp. NES 2013   .......... .......... .......... .......... .......... ..........

                                 70         80         90         100        110        120
A. simplex s. str.      GCCACATGGA AACTCGTACA CAC-----GT GGTGGCAGCC GTCTGCTGTG CTTTTTTTAG
A. simplex C            .......... .......... ...-----.. .......... .......... ..........
A. pegreffii            .......... .......... ...-----.. .......... .......... ..........
A. ziphidarum           ...G...... .......... ..T-----.. .......... .......... .......GT.
A. physeteris           ...G...... .....A.... ..T-----.. .......... .......... ....A..GT.
A. brevispiculata       ...G...... .....A.... ..T-----.. .......... .......... ....A..GT.
A. typica               ...G..C... .......... .GTTTGTG.. ....AT.... .......... .G..CG..G.
Anisakis sp. PRP C140   .......... .......... ...-----.. .......... .......... ..........
Anisakis sp. HP C141    .......... .......... ...-----.. .......... .......... ..........
Anisakis sp. NES 2013   .......... .......... ...-----.. .......... .......... ..........

                                 130        140        150        160        170        180
A. simplex s. str.      GCAGACAATG GCTTACGAGT GGCC-GTG-T GCTTGTTGAA CAACGGTGAC CAATTTGGC-
A. simplex C            .......... .......... ....-...-. .......... .......... .........-
A. pegreffii            .......... .......... ....-...-. .......... .......... .........-
A. ziphidarum           -......... .......... ...T-...-. .......... .......... .........-
A. physeteris           -......... .....T.... ....-...-. ..C....... .......... .........-
A. brevispiculata       -......... .....T.... ....-...-. ..C....... .......... .........-
A. typica               .......... .......... ....T...CC .......... .......... .........T
Anisakis sp. PRP C140   .......... .......... ....-...-. .......... .......... .........-
Anisakis sp. HP C141    .......... .......... ....-...-. .......... .......... .........-
Anisakis sp. NES 2013   .......... .......... ....-...-. .......... .......... .........-

                                 190        200        210        220        230        240
A. simplex s. str.      GTCTACGCCG TATCTAGCTT CTGCCTGGAC CGTCAGTTGC GATGAAAGAT GCGGAGAAAG
A. simplex C            .......... .......... .......... .......... .......... ..........
A. pegreffii            .......... .......... .......... .......... .......... ..........
A. ziphidarum           .......... .......... .C........ ....G..... .......... ......G...
A. physeteris           .......... .......... .......... .......A.. .......... ..........
A. brevispiculata       .......... C......... .......... ....G..A.. .......... ..........
A. typica               .......... .......... .C........ ....G..A.. .......... ......G...
Anisakis sp. PRP C140   .......... .......... .......... .......... .......... ..........
Anisakis sp. HP C141    .......... .......... .......... .......... .......... ..........
Anisakis sp. NES 2013   .......... .......... .......... .......... .......... ..........

                                 250        260        270        280        290        300
A. simplex s. str.      TTCCTTTGTT TTGGCTGCTA ATCATCATTG ATGAGCAGTA GCTTAAGGCA GAGTTGAGCA
A. simplex C            .......... .......... .......... ........C. .......... ..........
A. pegreffii            .......... .......... .......... ........C. .......... ....C.....
A. ziphidarum           .........G ......---T .......... ..C...T.-- -----.T... ....C.....
A. physeteris           .....C.... ...A.----- -....TCG.. G--GC..--- -----.CA.. .G..C.....
A. brevispiculata       .....C.... .....----- -..G.TC..C GA..C..--- -----.CT.. .G........
A. typica               .....C.--- ---------- ---------- ---------- -------T.G ..........
Anisakis sp. PRP C140   .......... .......... .......... ........C. .......... ....C.....
Anisakis sp. HP C141    .......... .......... .......... .......... .......... ..........
Anisakis sp. NES 2013   .......... .......... .......... ........C. .......... ..........
```

FIGURE 4. Alignment of ITS-1 positions 1–300 for sequences of *Anisakis* taxa. One representative of each unique sequence was included for comparison. Dots indicate identity with the first sequence and dashes are inferred insertion-deletion events. Positions 301–395 of ITS-1 and sequences for ITS-2 (positions 396–765) are correct as found in the original paper.

Published in the *Journal of Parasitology* (2007) 93(2): 444. DOI: 10.1645/0022-3395(2007)93[444:E]2.0.CO;2.

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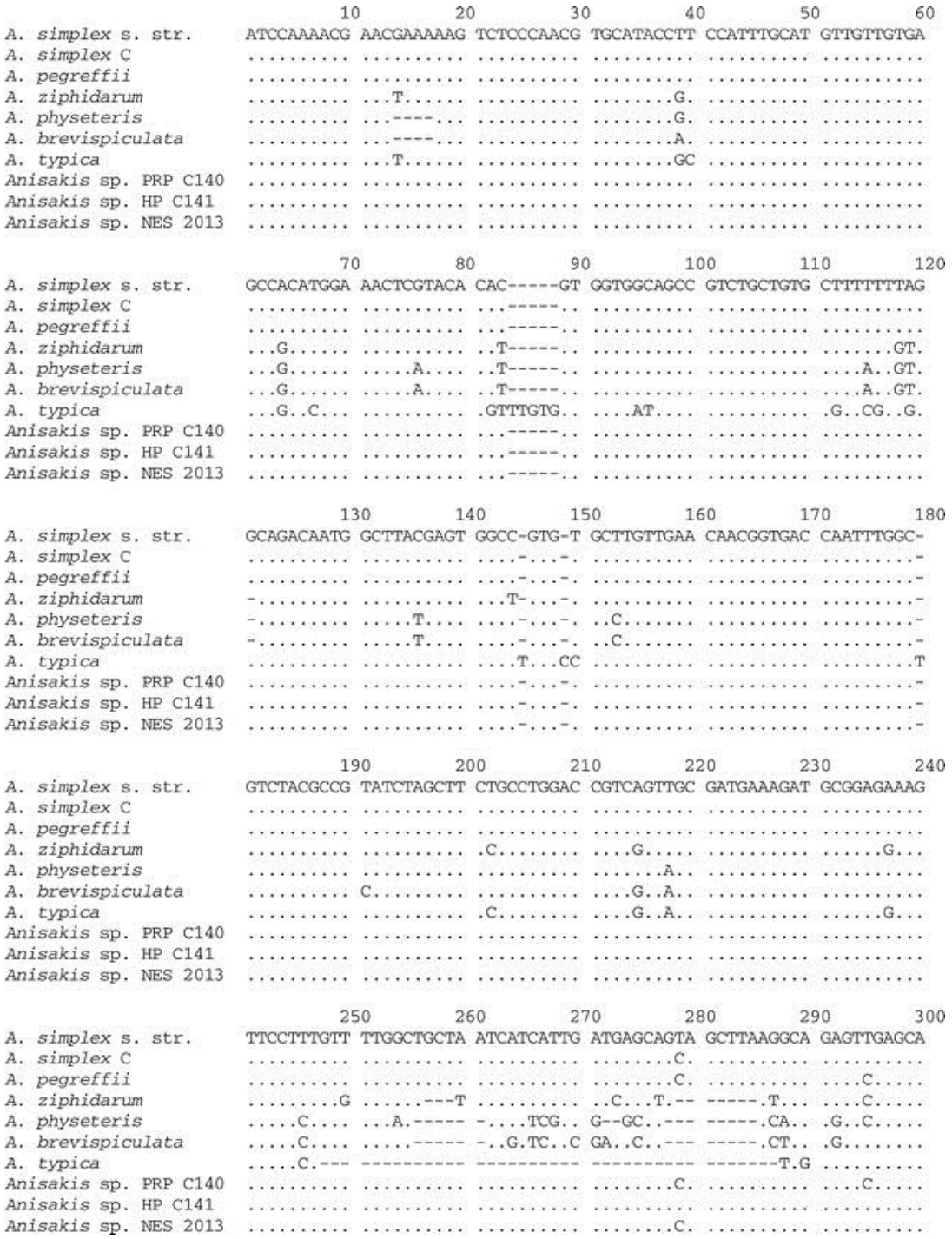

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