

## GENETIC RELATIONSHIPS AMONG *ANISAKIS* SPECIES (NEMATODA: ANISAKIDAE) INFERRED FROM MITOCHONDRIAL *COX2* SEQUENCES, AND COMPARISON WITH ALLOZYME DATA

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**ABSTRACT:** The genetic relationships among 9 taxa of *Anisakis* Dujardin, 1845 (*A. simplex* (sensu stricto), *A. pegreffii*, *A. simplex* C., *A. typica*, *A. ziphidarum*, *A. physeteris*, *A. brevispiculata*, *A. paggiae*, and *Anisakis* sp.) were inferred from sequence analysis (629 bp) of the mitochondrial *cox2* gene. Genetic divergence among the considered taxa, estimated by *p*-distance, ranged from  $p = 0.055$ , between sibling species of the *A. simplex* complex, to  $p = 0.12$ , between morphologically differentiated species, i.e., *A. ziphidarum* and *A. typica*. The highest level was detected when comparing *A. physeteris*, *A. brevispiculata*, and *A. paggiae* versus *A. simplex* complex (on average  $p = 0.13$ ) or versus *A. typica* (on average  $p = 0.14$ ). Sequence data from the newly identified *Anisakis* sp. poorly aligned with other *Anisakis* species but was most similar to *A. ziphidarum* ( $p = 0.08$ ). Phylogenetic analyses based upon Parsimony and Bayesian Inference, as well as phenetic analysis based upon Neighbor-Joining *p*-distance values, generated similar tree topologies, each well supported at major nodes. All analyses delineated two main clades, the first encompassing *A. physeteris*, *A. brevispiculata*, and *A. paggiae* as a sister group to all the remaining species, and the second comprising the species of the *A. simplex* complex (*A. simplex* (s.s.), *A. pegreffii* and *A. simplex* C.), *A. typica*, *A. ziphidarum*, and *Anisakis* sp. In general, mtDNA-based tree topologies showed high congruence with those generated from nuclear data sets (19 enzyme-loci) and with morphological data delineating adult and larval stages of the *Anisakis* spp.; however, precise positioning of *A. typica* and *A. ziphidarum* remain poorly resolved, though they consistently clustered in the same clade as *Anisakis* sp. and the *A. simplex* complex. Comparison of anisakid data with those currently available for their cetacean-definitive hosts suggests parallelism between host and parasite phylogenetic tree topologies.

The genus *Anisakis* Dujardin, 1845, previously considered to include only 3 valid species (Davey, 1971), has been substantially redefined in the past 2 decades using nuclear markers based on multilocus allozyme electrophoresis (Mattiucci et al., 2005, and ref. therein). Genetic variation among and within populations has also demonstrated the existence of reproductively isolated individuals, i.e., "biological species," within single populations and the single morphospecies recognized as valid by Davey (1971). Thus, among other things, allozymes data have advanced (1) the detection of sibling species within the previously defined *A. simplex* s.l. (Nascetti et al., 1986; Mattiucci et al., 1997); (2) validation of the unique taxonomic status of *A. brevispiculata*, based upon reproductive isolation from the sympatric species, *A. physeteris* (Mattiucci et al., 2001); (3) genetic characterization of *A. physeteris* (Mattiucci et al., 1986), and *A. typica* (Mattiucci et al., 2002); (4) the discovery and description of *A. ziphidarum*, a parasite of beaked whales (Paggi et al., 1998), and *A. paggiae*, a parasite of the pigmy and dwarf sperm whales (Mattiucci et al., 2005); and (5) the characterization of anisakid larval stages, disclosing that *A. simplex* (s.s.), *A. pegreffii*, *A. simplex* C., *A. typica*, and *A. ziphidarum* are Type I morphotypes (sensu Berland, 1961), whereas *A. physeteris*, *A. brevispiculata*, and *A. paggiae* are Type II morphotypes (Berland, 1961) (Mattiucci et al., 2002, 2004, 2005).

Progress in molecular systematics has also expanded the use of other molecular markers to recognize species of *Anisakis*, i.e., as internal transcribed spacer (ITS)-RFLP (D'Amelio et al.,

2000). Nevertheless, taxonomic aspects of parasites belonging to the genus still require resolution. In this regard, genetic divergence inferred from cytoplasmic genes and/or other nuclear genes, can assist in confirming phylogenetic relationships among anisakid parasites and in testing hypotheses for host-parasite coevolutionary events.

Considering the high genetic heterogeneity evidenced by nuclear markers (Mattiucci et al., 2001; Mattiucci et al., 2005), the objective of the present study was to use mitochondrial (mt) DNA data to further clarify genetic relationships among *Anisakis* taxa. The cytochrome oxidase 2 (*cox2*) gene was chosen as the target sequence because mtDNA is well known to evolve at faster rate than nuclear DNA and may potentially provide useful information for phylogenetic reconstruction of closely related species of nematodes, beyond the confamilial genera (Blouin et al., 1998). Herein, we evaluated sequence data (629 bp) from the mtDNA-derived *cox2* gene of several specimens belonging to 9 *Anisakis* spp. to better estimate genetic divergence among all currently recognized species including 1 taxon thus far undescribed. In addition, the mtDNA-based tree topologies were compared with those previously inferred from the nuclear genes (allozyme data sets).

### MATERIALS AND METHODS

#### Parasite material

A total of 45 specimens belonging to 9 *Anisakis* species recognized so far by allozymes markers (i.e., *A. simplex* (s.s.), *A. pegreffii*, *A. simplex* C., *A. typica*, *A. ziphidarum*, *Anisakis* sp., *A. physeteris*, *A. brevispiculata*, and *A. paggiae*) were studied. All collection data are summarized in Table I. All nematodes were adults; 39 were previously tested and assigned to species level by allozyme markers according to the methods reported elsewhere (Mattiucci et al., 1997, 2001, 2002, 2005) (Table I). In some cases ( $n = 6$ ), specimens were preserved in alcohol and/or formalin rather than frozen (Table I). All the cetacean hosts listed in Table I were stranded animals. They included 7 species of dolphins (Delphinidae and Phocoenidae) and 4 species of beaked

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TABLE I. *Anisakis* spp. from definitive hosts sequenced at the mtDNA *cox2* gene. N = number of adult specimens studied.

| Parasite                        | N | Preservation method | Host  | Collection locality                             | Specimen code       |
|---------------------------------|---|---------------------|---|---|---------------------|
| <i>A. simplex</i> s.s.          | 4 | frozen -80 C        | <i>Pseudorca crassidens</i> (Delphinidae)           | Northeast Pacific Ocean (Canadian coast)        | AS11-AS12-AS13-AS14 |
| <i>A. simplex</i> s.s.          | 4 | frozen -80 C        | <i>Globicephala melas</i> (Delphinidae)             | Northeast Atlantic Ocean (Spanish coast)        | AS01-AS02-AS03-AS04 |
| <i>A. simplex</i> s.s.          | 2 | frozen -80 C        | <i>Phocoena phocoena</i> (Phocoenidae)              | Northeast Pacific Ocean (Vancouver Island)      | AS09-AS10           |
| <i>A. simplex</i> s.s.          | 1 | frozen -80 C        | <i>Balaenoptera acutorostrata</i> (Balaenopteridae) | Northeast Atlantic (Norwegian coast)            | AS06                |
| <i>A. pegreffii</i>             | 2 | frozen -80 C        | <i>Delphinus delphis</i> (Delphinidae)              | Northeast Atlantic Ocean (Spanish coast)        | AE01-AE02           |
| <i>A. pegreffii</i>             | 2 | frozen -80 C        | <i>Tursiops truncatus</i> (Delphinidae)             | Southeast Atlantic Ocean (South African coast)  | AE03-AE04           |
| <i>A. simplex</i> C             | 2 | frozen -80 C        | <i>Pseudorca crassidens</i> (Delphinidae)           | Northeast Pacific coast (Vancouver Island)      | AC02-AC07           |
| <i>A. simplex</i> C             | 1 | frozen -80 C        | <i>Lissodelphis borealis</i> (Delphinidae)          | Northeast Pacific Ocean (Californian coast)     | AC10                |
| <i>A. typica</i>                | 2 | frozen -80 C        | <i>Sotalia fluviatilis</i> (Delphinidae)            | Western North Atlantic Ocean (Brazilian coast)  | AT01-AT02           |
| <i>A. typica</i>                | 1 | frozen -80 C        | <i>Stenella attenuata</i> (Delphinidae)             | Western North Atlantic Ocean (Florida coast)    | AT03                |
| <i>A. typica</i>                | 2 | formalin 10%        | <i>Steno bredanensis</i> (Delphinidae)              | Caribbean Sea                                   | AT07-AT10           |
| <i>A. typica</i>                | 2 | formalin 10%        | <i>Tursiops truncatus</i> (Delphinidae)             | West Atlantic Ocean (Caribbean Sea)             | AT11-AT12           |
| <i>A. ziphidarum</i>            | 2 | frozen -80 C        | <i>Mesoplodon layardii</i> (Ziphiidae)              | Southeast Atlantic Ocean (South African coast)  | AZ01-AZ02           |
| <i>A. ziphidarum</i>            | 2 | frozen -80 C        | <i>Ziphius cavirostris</i> (Ziphiidae)              | Southeast Atlantic Ocean (South African coast)  | AZ03-AZ04           |
| <i>Anisakis</i> sp.             | 3 | frozen -80 C        | <i>Mesoplodon mirus</i> (Ziphiidae)                 | Southeast Atlantic Ocean (South African coast)  | AN01-AN02-AN03      |
| <i>Anisakis</i> sp.             | 2 | ethanol 70%         | <i>Mesoplodon grayi</i> (Ziphiidae)                 | Western South Pacific Ocean (New Zealand coast) | AN04-AN05           |
| <i>A. physeteris</i>            | 4 | frozen -80 C        | <i>Physeter macrocephalus</i> (Physeteridae)        | Mediterranean Sea                               | AP01-AP02-AP03-AP04 |
| <i>A. brevispiculata</i>        | 2 | frozen -80 C        | <i>Kogia breviceps</i> (Kogiidae)                   | Southeast Atlantic Ocean (South African coast)  | AB02-AB03           |
| <i>A. brevispiculata</i>        | 2 | frozen -80 C        | <i>Kogia breviceps</i> (Kogiidae)                   | West Atlantic Ocean (Florida coast)             | AB04-AB05           |
| <i>A. paggiae</i>               | 2 | frozen -80 C        | <i>Kogia breviceps</i> (Kogiidae)                   | West Atlantic Ocean (Florida coast)             | AK04-AK05           |
| <i>Pseudoterranova ceticola</i> | 1 | frozen -80 C        | <i>Kogia sima</i> (Kogiidae)                        | Caribbean Sea                                   | PC01                |

whales (Ziphiidae) and the sperm whale (Physeteridae), 2 species of Kogiidae, and 1 species of orqual (Balaenopteridae) (Table I).

### DNA amplification and sequencing

A 629-bp portion of the *cox2* gene was analyzed for all the specimens of *Anisakis* spp. listed in Table I. Their GenBank accession numbers are *A. simplex* s.s. (DQ116426), *A. pegreffii* (DQ116428), *A. simplex* C. (DQ116429), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *Anisakis* sp. (DQ116431), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), and *A. paggiae* (DQ116434). Each sequence number represents the consensus sequences from all the specimens of each *Anisakis* species (Table I). Reference specimens and isolated DNA samples were stored at the Section of Parasitology, the Department of Public Health Sciences, University of Rome "La Sapienza" in Mattiucci's collection of anisakid nematodes.

Total DNA was extracted from 2 mg of individual nematode tissue, using the Wizard Genomic DNA Purification Kit, (Promega, Madison, Wisconsin). Total DNA from some specimens preserved in formalin (Table I) were extracted using cetyltriethylammonium bromide as described by Yang et al., (1997). The *cox2* gene from each species of *Anisakis* was amplified using the primers 210 5' CACCAACTCT-TAAAATTATC and 211 5' TTTTCTAGTTATATAGATTGRTTYAT from Nadler and Hudspeth (2000) spanning mtDNA nucleotide position 10,639–11,248 as defined in *Ascaris suum* (Accession X54253). PCR amplification was carried out in a volume of 50  $\mu$ l containing 30 pmol of each primer, MgCl<sub>2</sub> 2.5 mM (Amersham Pharmacia Biotech. Inc., Piscataway, New Jersey), PCR buffer 1 $\times$  (Amersham), DMSO 0.08 mM, dNTPs 0.4 mM (Sigma-Aldrich, St. Louis, Missouri) 5 U of *Taq* Polymerase (Amersham), and 10 ng of total DNA. The mixture was denatured at 94 C for 3 min, followed by 34 cycles at 94 C for 30 sec, 46 C for 1 min, 72 C for 1 min and 30 sec, followed by postamplification at 72 C for 10 min. The PCR product was purified using PEG precipitation, and automated DNA sequencing was performed by Macrogen Inc. (Seoul, Korea) using primers 210 and 211.

### Sequence analysis

The *cox2* sequences were aligned using ClustalW (Thompson et al., 1994) as implemented in BioEdit 7.0.1 (Hall, 1999), using default parameters. Phylogenetic analyses by maximum parsimony (MP) were performed using PAUP\* 4.0b10 (Swofford, 2003). Two MP analyses were performed; 1 using all sequence data, and the other using only the first and second positions of the amino acid codon. For both analyses, heuristic search with TBR branch swapping and random addition of sequence was used. All characters were treated as unordered, and nucleotide substitutions in each gene segment were equally weighted. Neighbor-Joining (NJ) analysis, based on *p*-distance was performed using MEGA 2.1 program (Kumar et al., 2001).

Reliabilities of phylogenetic relationships were evaluated using non-parametric bootstrap analysis (Felsenstein, 1985) with 1,000 replicates for MP and NJ trees. Bootstrap values exceeding 70 were considered well supported (Hills and Bull, 1993).

Bayesian Inference (BI) analyses (Larget and Simon, 1999) were performed using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), on full consensus sequences. Four incrementally heated Markov Chains (using default heating values), were run for 1,000,000 generations, sampling the Markov Chains at intervals of 100 generations, where 10,000 samples were discarded as "burn-in." The optimal model of sequence evolution for Bayesian analyses was assessed using hierarchical-likelihood ratio test (hLRT) as implemented in the software Modeltest 3.6 (Posada and Crandall, 1998) associated with PAUP\*. This analysis supported the HKY + I +  $\Gamma$  model (Hasegawa et al., 1985) as the best fit substitution model for the data. The parameters for the model inferred were proportion of invariable sites = 0.6048, shape parameter ( $\alpha$ ) = 0.7389, nucleotide frequencies (A = 0.20, C = 0.08, G = 0.24, T = 0.48) and transition:transversion ratio = 6.246. Posterior probabilities were estimated and used to assess support for each branch in inferred phylogeny with probabilities where *P*  $\geq$  95% being indicative of significant support (Reeder, 2003).

Two *cox2* sequences of *Anisakis*, (Nadler and Hudspeth, 2000) with the accession numbers AF179905 (*Anisakis* sp. NH clone 1) and AF179906 (*Anisakis* sp. NH clone 4), and here indicated with code AC1 and AC4 respectively, were also included in the phylogenetic analysis.

Moreover, specimens of *Pseudoterranova ceticola* (Deardoff and Overstreet, 1981) from *Kogia breviceps* of Florida, previously identified at species level by morphological analysis and by allozyme markers, were also sequenced at the *cox2* gene (GenBank DQ116435, this study) and considered as outgroup to root the *Anisakis* phylogenetic trees, based on the sister-group relationship of *Anisakis* and *Pseudoterranova* previously evidenced in the ribosomal and mitochondrial DNA analyses (Nadler and Hudspeth, 2000).

## RESULTS

### *Cox2* mt-DNA sequence differentiation

A 629-bp portion of the *cox2* gene was sequenced for 45 specimens of *Anisakis* that have been genetically characterized thus far. The 2 *Anisakis* specimens collected from *Steno bredanensis* and those from *Tursiops truncatus* stranded along the Caribbean Sea (preserved in formalin) (Table I), and those from *Stenella attenuata* from Florida coast as well, were found to match the sequences of the species identified by allozyme markers, as *A. typica* from *Sotalia fluviatilis* from the Brazilian coast (Table I).

The 2 sequences previously deposited in GenBank and defined as *Anisakis* sp. NH clone 1 (AF179905), and as *Anisakis* sp. NH clone 4 (AF179906) (Nadler and Hudspeth 2000) were matched with the sequences of those specimens characterized by allozyme markers, as *A. simplex* C collected from *Pseudorca crassidens* from Northeast Pacific waters (Table I).

*Cox2* sequences of specimens collected from *Mesoplodon mirus* (Table I) didn't match the alignment with *cox2* sequences from other genetically described taxa of *Anisakis*. Moreover, these specimens, tested by allozymes, have showed alleles at some enzymatic loci not previously observed in any of the previously described taxa of *Anisakis* (data not shown).

The *cox2* fragment in all the *Anisakis* spp. analyzed was found to be A + T rich (60.7%, 64.9%, and 74.7%, at the first, second, and third codon positions, respectively) (Table II; Fig. 1).

Genetic divergence based on *p*-distance values was evaluated. The lowest level of interspecific genetic distance was found between sibling species of the *A. simplex* complex (on average, *p* = 0.055, range 0.045–0.061). Values ranging from 0.107 to 0.126 were observed when comparing the *A. simplex* complex with the *A. typica* and/or *A. ziphidarum*. Similar values (on average, *p* = 0.116) were obtained when *A. physeteris*, *A. brevispiculata*, or *A. paggiae* were compared with *A. ziphidarum*. The highest values, ranging from 0.128 to 0.158, were observed when comparing the same group of species and the sibling species of the *A. simplex* complex (on average, *p* = 0.133) or *A. typica* (on average, *p* = 0.142).

At the amino acid level, a total of 31 variable positions were identified for the *Anisakis* species, with the average variation ranging from 1.4% between *A. ziphidarum* versus *Anisakis* sp. and 2.4% among the sibling species of *A. simplex* complex to as high as 7.7% between morphologically distinct species (i.e., *A. physeteris*, *A. brevispiculata*, and *A. paggiae* versus the *A. simplex* complex).

### Phylogenetic relationships among *Anisakis* spp.

Parsimony analysis using all codon positions in the analysis generated a tree (Fig. 2) showing 2 main clades with 1 cluster, highly supported, consisting of the sibling species of the *A. simplex* complex, *A. typica*, *A. ziphidarum*, and *Anisakis* sp.,

TABLE II. Composition of the *cox2* gene of each *Anisakis* species organized by codon position.

| Parasite species         | First position |      |      |      | Second position |      |      |      | Third position |      |      |      | All positions |      |      |      |
|--------------------------|----------------|------|------|------|-----------------|------|------|------|----------------|------|------|------|---------------|------|------|------|
|                          | T              | A    | C    | G    | T               | A    | C    | G    | T              | A    | C    | G    | T             | A    | C    | G    |
| <i>A. simplex</i> (s.s)  | 34.3           | 26.7 | 13.8 | 25.2 | 38.1            | 26.7 | 15.2 | 20.0 | 66.0           | 8.6  | 2.9  | 22.5 | 46.1          | 20.7 | 10.7 | 22.6 |
| <i>A. pegreffii</i>      | 33.8           | 26.7 | 14.3 | 25.2 | 38.1            | 26.7 | 15.2 | 20.0 | 64.1           | 8.6  | 4.3  | 23.0 | 45.3          | 20.7 | 11.3 | 22.7 |
| <i>A. simplex</i> C      | 33.3           | 26.7 | 14.8 | 25.2 | 38.1            | 26.7 | 15.2 | 20.0 | 64.6           | 10.5 | 3.3  | 21.5 | 45.3          | 21.3 | 11.1 | 22.3 |
| <i>A. typica</i>         | 33.8           | 27.1 | 14.8 | 24.3 | 38.1            | 26.2 | 15.7 | 20.0 | 56.9           | 9.6  | 8.1  | 25.4 | 42.9          | 21.0 | 12.9 | 23.2 |
| <i>A. ziphidarum</i>     | 35.2           | 27.1 | 13.3 | 24.3 | 38.6            | 26.2 | 15.2 | 20.0 | 62.2           | 9.1  | 3.8  | 24.9 | 45.3          | 20.8 | 10.8 | 23.1 |
| <i>Anisakis</i> sp.      | 36.7           | 11.9 | 27.1 | 24.3 | 38.6            | 15.2 | 26.2 | 20.0 | 59.3           | 6.2  | 12.0 | 22.5 | 44.8          | 11.1 | 21.8 | 22.3 |
| <i>A. physeteris</i>     | 32.9           | 27.1 | 14.8 | 25.2 | 38.6            | 26.7 | 15.2 | 19.5 | 65.1           | 14.8 | 0.5  | 19.6 | 45.5          | 22.9 | 10.2 | 21.5 |
| <i>A. brevispiculata</i> | 32.4           | 26.7 | 15.7 | 25.2 | 38.6            | 26.7 | 15.2 | 19.5 | 60.3           | 16.3 | 3.3  | 20.1 | 43.7          | 23.2 | 11.4 | 21.6 |
| <i>A. paggiae</i>        | 34.3           | 27.1 | 14.3 | 24.3 | 39.0            | 26.2 | 14.8 | 20.0 | 63.6           | 17.2 | 3.8  | 15.3 | 45.6          | 23.5 | 11.0 | 19.9 |
| Avg.                     | 34.1           | 25.2 | 15.9 | 24.8 | 38.4            | 25.3 | 16.4 | 19.9 | 62.5           | 11.2 | 4.7  | 21.6 | 44.9          | 20.6 | 12.4 | 22.1 |
| Percentage of A + T      | 59.3           |      |      |      | 63.7            |      |      |      | 73.7           |      |      |      | 65.5          |      |      |      |

and a second that includes *A. physeteris*, *A. brevispiculata*, and *A. paggiae*. The second clade received moderate support (>50%) at MP bootstrap analysis. In the first clade, the relationship of *A. typica* with respect to the other *Anisakis* taxa included within the clade was not well supported (50%) (Fig. 2). Upon removing the third codon positions, the tree topology again depicted 2 main clusters: the first was well supported and included *A. simplex* (s.s.), *A. pegreffii*, and *A. simplex* C; and the second was very poorly supported (<<50% bootstrap), and consisted of 2 subclades, not supported as well; 1 comprised of *A. physeteris*, *A. brevispiculata*, and *A. paggiae* and the second consisting of *A. typica*, *A. ziphidarum*, and *Anisakis* sp. (data not shown).

A congruent tree topology to MP, based on all codon positions, was generated by NJ inferred from *p-distance* values (Fig. 3). The same 2 main clusters were produced: as with previous tree topology, the 3 species of the *A. simplex* complex, *A. typica*, *A. ziphidarum*, and *Anisakis* sp. form a well separate and supported cluster. *Anisakis typica* clustered within the clade of *A. ziphidarum* and *Anisakis* sp., but it received a very low bootstrap value. On the other hand, both MP and NJ trees, based on *p-distance* analyses showed *A. ziphidarum* and *Anisakis* sp. to be monophyletic (Figs. 2 and 3).

The BI analysis (Fig. 4) produced a tree topology similar to that obtained by MP, and by NJ albeit with less support (<50%), for the subclade including *A. typica* and *Anisakis* sp.; *A. ziphidarum* was partitioned into a separate clade basal to the most related species within the *A. simplex* complex.

In MP, NJ, and BI tree topologies (Figs. 2–4), a close association, highly supported by all the analyses, was observed between *A. brevispiculata* and *A. physeteris* whereas *A. paggiae* appeared as a sister species to this group; however, BI and NJ tree topology showed stronger support for the placement of *A. brevispiculata* and *A. physeteris* than that generated by MP analysis.

Finally, strong support was received, in all the phylogenetic elaborations (MP, NJ, and BI), for the clade formed by the sibling species of the *A. simplex* complex with a close relationships between *A. simplex* s.s. and *A. pegreffii*.

## DISCUSSION

### *cox2*-derived phylogenetic relationships among *Anisakis* spp. and a comparison with the allozyme data

Allozymes have been used to demonstrate reproductively isolated populations and to provide genetic markers for several

anisakid taxa now recognized as species. In the present study, sequence data generated from the *cox2* gene support previous allozyme studies regarding high genetic heterogeneity within the genus *Anisakis* (Mattiucci et al., 2002, and Mattiucci et al., 2005). A phylogenetic hypothesis for 9 taxa is supported by *cox2* gene data. The strong A + T bias observed herein is consistent with that found elsewhere for nematode mtDNA (Thomas and Wilson, 1991; Okimoto et al., 1992; Anderson et al., 1998; Blouin et al., 1998; Blouin, 2002) and can limit the phylogenetic value of mtDNA beyond the level of related species (Blouin et al., 1998; Blouin, 2002; Nadler and Hudspeth, 2000). The genetic divergence of mtDNA among the *Anisakis* taxa evaluated herein is of the same order found among other related nematode species (Blouin et al., 1998; Zarlenga et al., 1998; La Rosa et al., 2001).

All the tree topologies derived from the phylogenetic analyses were in substantial agreement where each depicted *A. physeteris*, *A. brevispiculata*, and *A. paggiae* as a sister group to the remaining anisakids analyzed (*A. simplex* (s.s.), *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphidarum*, and *Anisakis* sp.). The clade formed by the former species received moderate levels of support (>50 and <70% bootstrap in the MP and NJ;  $P > 80\%$  and <95% in the BI), whereas the remaining species formed a monophyletic grouping highly supported when analyzed by MP, BI, or NJ based on *p-distance* analysis.

The new taxon, *Anisakis* sp., showed a close relationship with *A. ziphidarum*, which supports findings generated from allozyme data (data not shown). Indeed, allozymes were used to detect this reproductively isolated taxon in sympatry with *A. ziphidarum* within the same definitive host, the beaked whale *Mesoplodon mirus* from South African waters. The estimation, at allozyme level, of *Anisakis* sp. genetic divergence from *A. ziphidarum* was  $D_{Nei} = 0.69$  (data not shown). Unfortunately, only pre-adult specimens corresponding to this taxon have been identified thus far, thereby, limiting the morphological description and proper naming of this species. Type I larval stages (sensu Berland, 1961) corresponding to this species were also identified from *Aphanopus carbo* from Madeira and from *Trachurus trachurus* from the North Atlantic (Mattiucci et al., in press). Other genetic studies and expanded sampling from other definitive hosts are required to fully characterize this novel taxon.

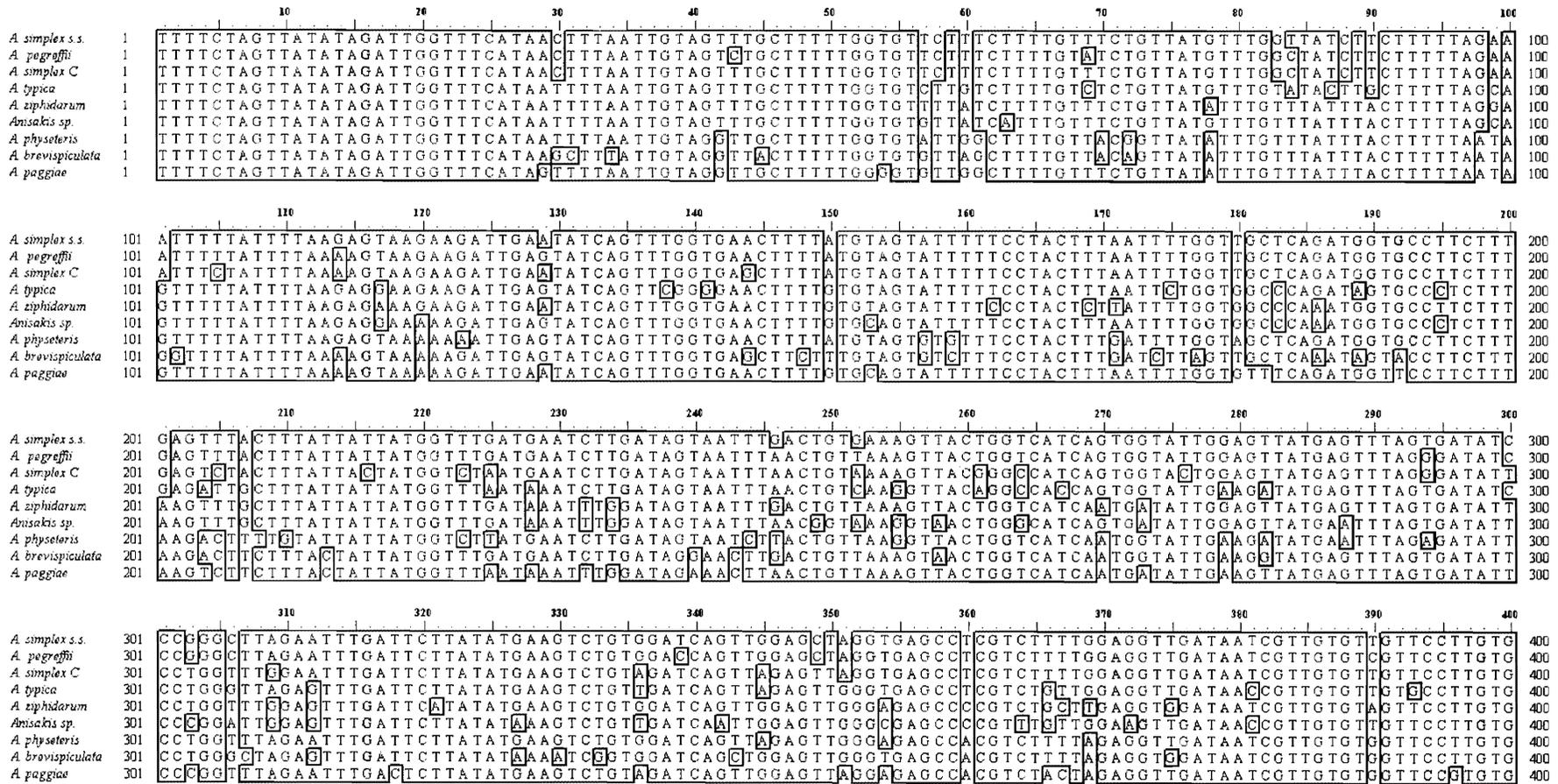


FIGURE 1. Alignment of mtDNA *cox2* sequences (629 bp) for all currently recognized *Anisakis* species. Each sequence represents the consensus sequences from the specimens of each species presented in Table I.

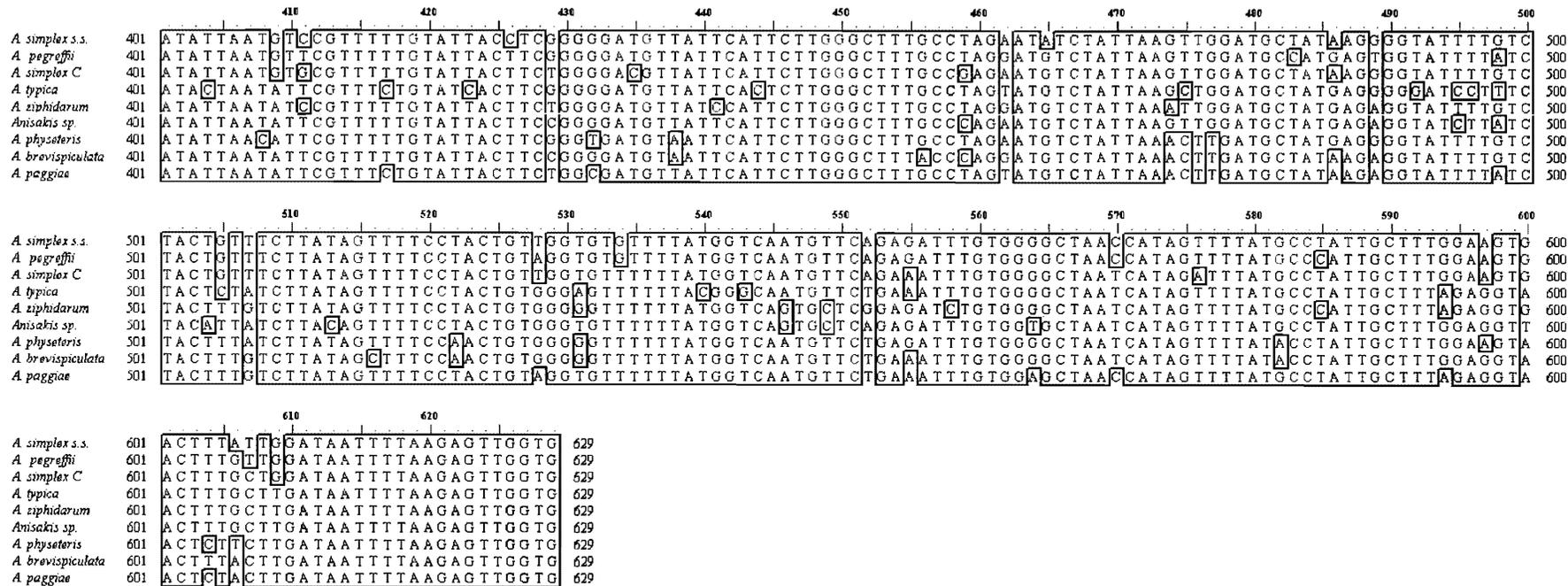


FIGURE 1. Continued.

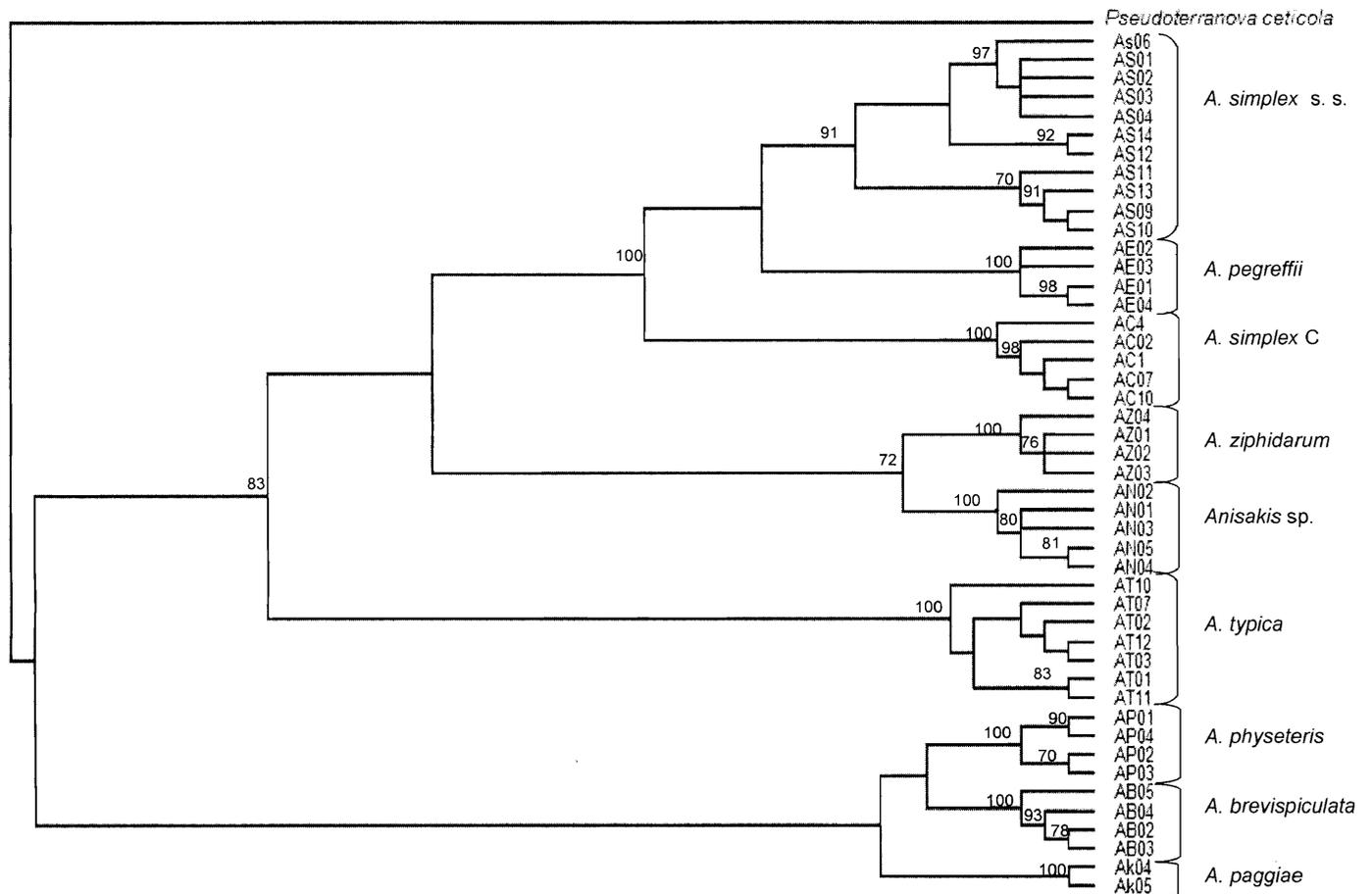


FIGURE 2. *Cox2*-derived Maximum Parsimony (MP) tree for the 46 anisakid DNA representing all currently recognized taxa. The tree topology depicts the strict consensus MP tree, over the 71 most parsimonious trees (CI = 0.5725, HI = 0.4275, RI = 0.8842, RC = 0.5062) (187 parsimony-informative characters). Specimen codes are referenced in Table I. Bootstrap values were calculated over 1,000 replicates; percentages  $\geq 70\%$  are shown at the internal nodes.

Discordance among the phylogenetic analyses surfaced in the unresolved placement of *A. typica* within either a subclade with *A. ziphidarum* and *Anisakis* sp., or within a clade containing the *A. simplex* complex. However, low bootstrap values appeared in all the trees suggesting that further analyses on *A. typica* collected from more individuals, other geographic areas, and additional hosts are needed to clarify its phylogenetic position with respect to the species of *A. simplex* complex and *A. ziphidarum*. It should be noted, however, that its placement within the clade as 1 of the *A. simplex* complex is supported by MP, BI, and NJ analyses (Figs. 2–4) with varying levels of support.

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 (allozyme data) generated previously (Mattiucci et al., 2001, 2002, 2005). Allozyme clustering (Fig. 5) depicted the species *A. physeteris*, *A. brevispiculata*, and *A. paggiae* as a sister group, highly supported, with respect to the other *Anisakis* taxa (Fig. 5), and this was evidenced by the *cox2* data as well. In addition, *A. paggiae* appears to share a common ancestor with *A. brevispiculata* (Fig. 5), and this is well supported by the NJ allozyme data. Finally, allozyme tree topology clearly demonstrated that *Anisakis* sp. formed a monophyletic group with *A. ziphi-*

*darum*. Although the position occupied by *A. typica* remains an enigma, consistent tree topologies were observed between nuclear gene products and mitochondrial genes, indicating close genetic relationships among the species of the *A. simplex* complex (*A. simplex* (s.s.), *A. pegreffii*, and *A. simplex* C) (Figs. 2–5). Data also support the group of species formed by *A. physeteris*, *A. brevispiculata*, and *A. paggiae* as basal sister taxa in all the phylogenetic elaborations from 2 different data sets.

### Morphology and host–parasite relationships

The high genetic heterogeneity of the *Anisakis* spp. studied here is supported by morphology of the species belonging to this genus as well, where 2 major clades can be delineated as follows: (1) the ventriculus, at adult stage, is short, never sigmoid, and broader than it is long in the species *A. physeteris*, *A. brevispiculata*, and *A. paggiae* (Mattiucci et al., 2005), and longer than it is broad, and often sigmoidal in shape, in the other clade; (2) male spicules that are short, stout, and of similar length can be observed in *A. physeteris*, *A. brevispiculata*, and *A. paggiae* (Mattiucci et al., 2005), and which are thin, long, and often unequal (equal in *A. ziphidarum*; see Paggi et al., 1998) in the other clade; and (3) Type II larval morphology (sensu Berland, 1961) is characteristic of *A. physeteris*, *A. brev-*

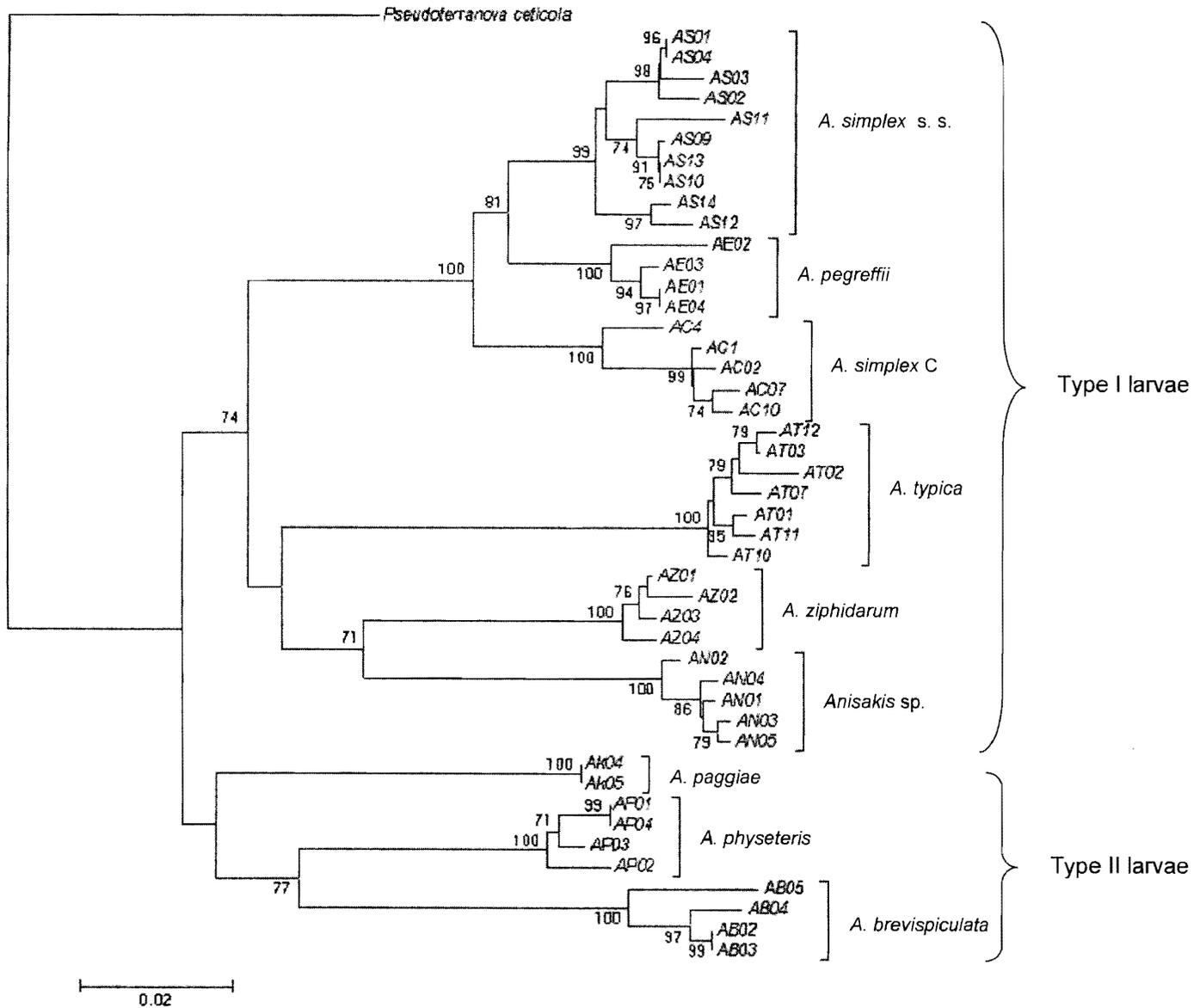


FIGURE 3. Neighbor-Joining (NJ) tree inferred from *p*-distance values, by MEGA, showing the genetic relationships among the *Anisakis* spp. analyzed. The scale bar indicates the distance in substitutions per nucleotide. Specimen codes are referenced in Table I. Bootstrap values were calculated over 1,000 replicates; percentages  $\geq 70\%$  are shown at the internal nodes.

*ispiculata*, and *A. paggiae* (Mattiucci et al., 2001, 2004, 2005), whereas Type I morphology (sensu Berland, 1961) can be found in the species of the *A. simplex* complex, *A. typica*, *A. ziphidarum*, and *Anisakis* sp.

The presence of the 2 clades is supported also by ecological data and specific host-parasite relationships. The sperm whales (i.e., *Physeteris catodon*, *Kogia breviceps*, and *K. sima*) are hosts for the *A. physeteris*, *A. brevispiculata*, and *A. paggiae* (Mattiucci et al., 2001, 2005) that are included in the first clade. Oceanic dolphins in Delphinidae, Arctic dolphins in Monodontidae, and porpoises in Phocoenidae are hosts of the species of the *A. simplex* complex and of *A. typica* (Mattiucci et al., 1997, 1998, 2002, 2005), and the beaked whales *Ziphius cavirostris*, *Mesoplodon layardii*, *M. mirus*, and *M. grayi* are hosts of *A. ziphidarum* (Paggi et al., 1998 and present data) and *Anisakis* sp., that are partitioned into the second clade.

Phylogenetic relationships proposed here and elsewhere (Mattiucci et al., 2005) for species of genus *Anisakis* seem to align with that of their cetacean hosts (Milinkovitch, 1995; Nikaido et al., 2001). The phylogeny of cetaceans proposed by Milinkovitch (1995), based on mtDNA (12S, 16S, and *cytb* partial sequences) and myoglobin sequences, and by Nikaido et al., (2001), based on retroposon analysis, indicated the branching order of the cetacean lineages where the sperm whale and the pygmy sperm whales (Physeteridae and Kogiidae) represent basal taxa, followed by the beaked whales and freshwater and marine dolphins as the most derived. In accordance with that analysis, the branching order proposed for the *Anisakis* taxa showed that nematodes from the sperm whale and the pygmy sperm whales (*A. physeteris*, *A. brevispiculata*, and *A. paggiae*) always occupy a basal lineage followed by those parasitizing the beaked whales (*A. ziphidarum* and *Anisakis* sp.). Those spe-

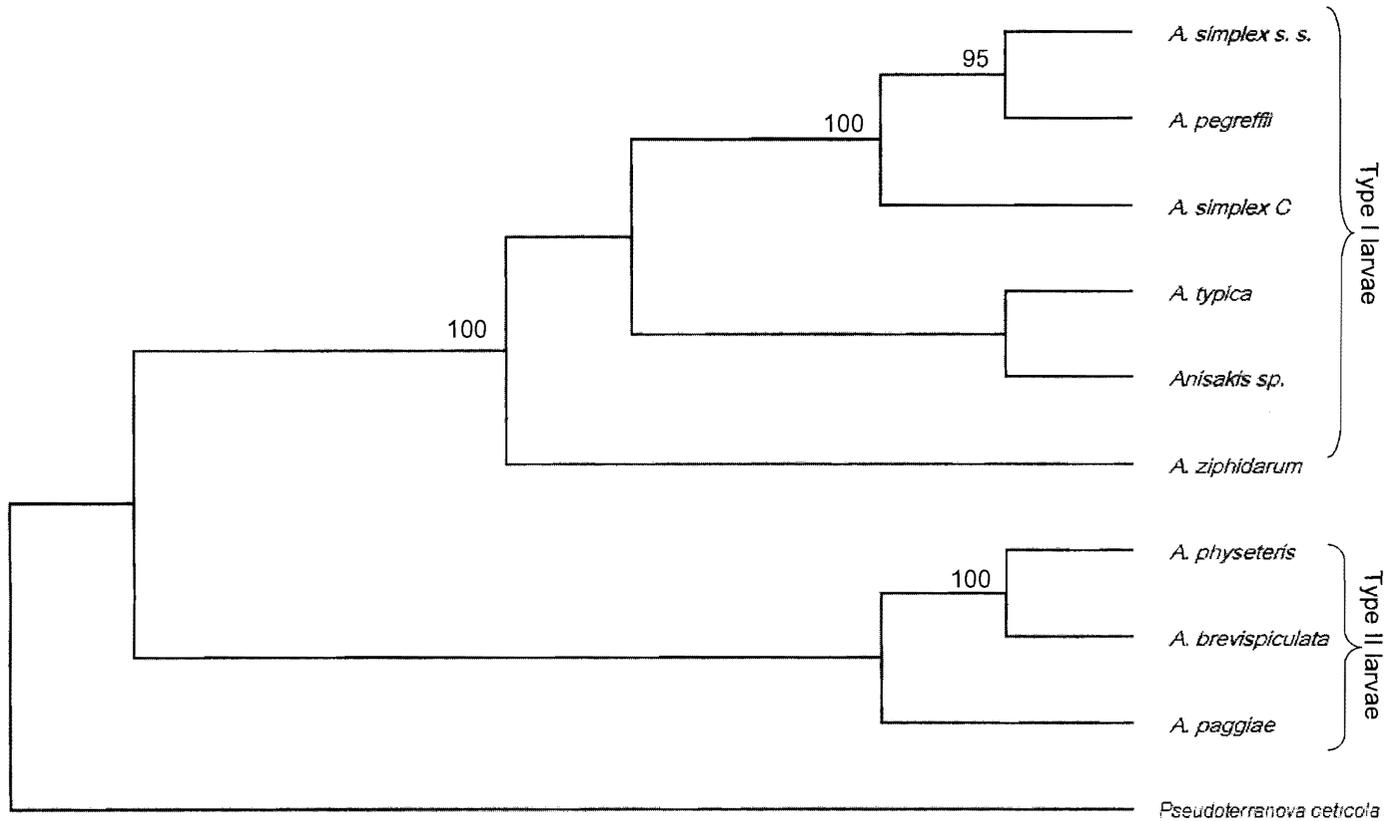


FIGURE 4. Bayesian Inference (BI) tree of the consensus sequences per species, using as substitutions model HKY + I +  $\Gamma$  model (T-Ratio = 6.2464,  $\alpha$  = 0.7389 Pinvar = 0.6048, A = 0.20, C = 0.08, G = 0.24, and T = 0.48). Four incrementally heated Markov Chains, 1,000,000 gen. and 10,000 samples were discarded as “burn-in.” Values of  $P \geq 95\%$ , indicative of significant support, are shown at the internal nodes.

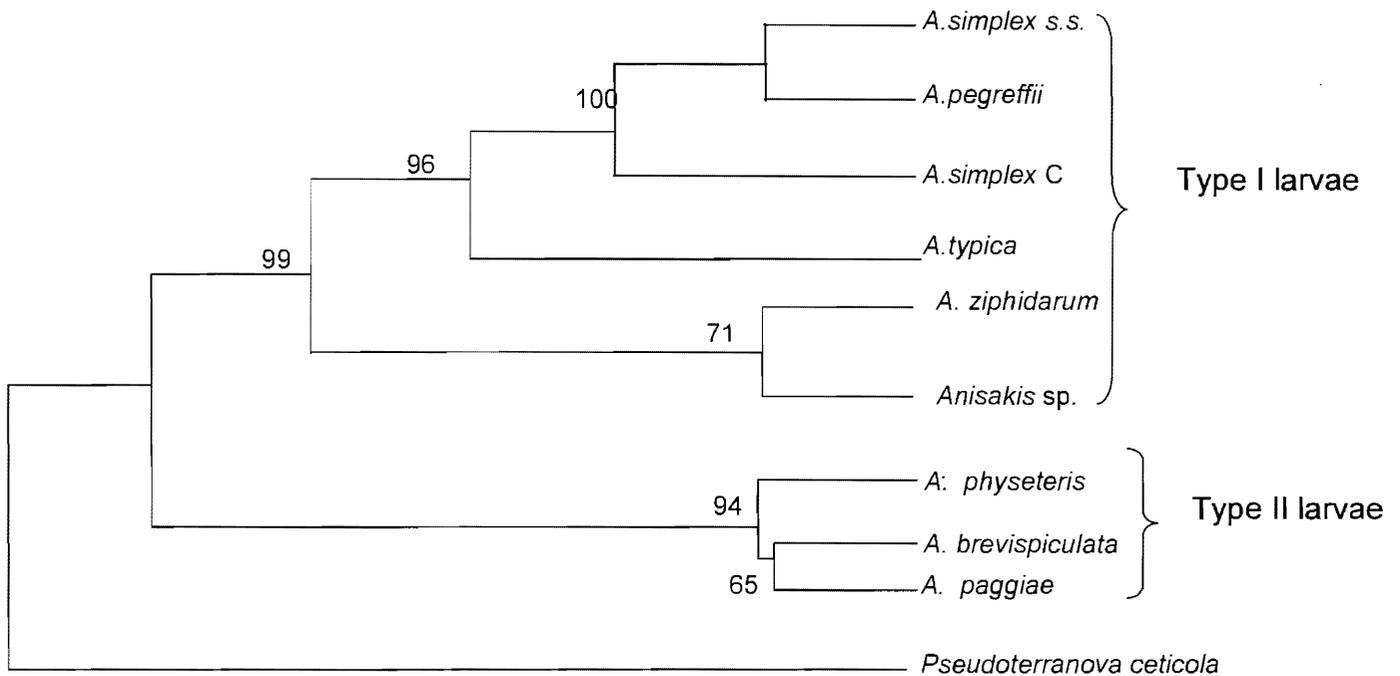


FIGURE 5. Consensus Neighbor-Joining (NJ) tree, showing the genetic relationships among *Anisakis* species from Cavalli-Sforza and Edwards (1967) chord-distance values, based on 19 enzyme loci (modified from Mattiucci et al., 2005). Bootstrap values (more than 500 replicates) are given. *Pseudoterranova ceticola* as outgroup.

cies from “oceanic dolphins” (the definitive hosts of the *A. simplex* complex) consistently appear as the most derived, suggesting some level of parallelism or coevolutionary event could have accompanied the speciation of these endoparasitic nematodes and their definitive hosts. Clearly, a broader data set is needed to confirm cospeciation and/or host-switching events. In addition, phylogenetic analysis using other molecular targets could provide supporting evidence about the evolutionary history of this group of marine ascaridoid nematodes.

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