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A polyphasic approach with molecular phylogeny for the characterization of *Anisakis pegreffii* (Anisakidae: Nematoda) in fishes from Adriatic Sea

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Summary

In this study we investigated the morphometric and molecular characterization of a liver encapsulated third-stage larval population of *Anisakis* spp. infecting *Merluccius merluccius* and *Lophius piscatorius* caught in the Adriatic Sea waters (southern Italy). A polyphasic approach based on PCR-RFLP profiles of the ITS region, mitochondrial COI (cytochrome c oxidase subunit 1), sequencing and molecular phylogeny of ITS and mitochondrial COI was used to identify *Anisakis* larvae collected from fish samples. PCR-RFLP analysis showed three banding pattern corresponding to the peculiar pattern of *A. pegreffii*. Sequence data from ribosomal ITS and mitochondrial COI were analysed by Neighbour Joining, Minimum Evolution and Maximum Parsimony methods to evaluate the phylogenetic relationships among *A. simplex sensu lato*. The phylogenetic trees obtained for both ITS and COI revealed the existence of three distinct clades for *A. simplex sensu sricto*, *A. simplex* C and *A. pegreffii* and the sequences obtained in this study clearly clustered together with *A. pegreffii* sequences present in the database.
Histopathological observations of anisakid nematode specimens detected on the liver surface of *M. merluccius* are illustrated. Encapsulated specimens of the L3 stage of the nematode were similar in size and morphometry to those found into the peritoneal cavity. *Anisakis* larvae encapsulated on the liver surface within dense and pearl coloured envelops caused host hepatic tissue necrosis, large cavities and oedematous liver spots to the host.

Keywords: *Anisakis pegreffii*; Adriatic Sea; mitochondrial COI; ITS; PCR-RFLP

Introduction

Anisakis Dujardin 1845 (Nematoda: Anisakidae) is a genus of parasitic nematodes, which possess a complex-life cycle involving marine organisms worldwide. The occurrence of anisakid nematodes is of great concern for human health both as potential causative agents of anisakiasis and as food-borne allergens. The morphospecies *A. simplex sensu lato* (*s.l.*) exists as a complex of cryptic species, morphologically similar but with different genetic traits. *Anisakis simplex s.l.* consists of three sibling species, namely *A. simplex sensu stricto* (*s.s.*), *A. pegreffii* and *A. simplex* C differing in their genetic structure and in ecological traits, such as geographic distribution and host preference (Mattiucci *et al*., 1997). *Anisakis simplex s.s.* is mainly distributed in the northern Atlantic and Pacific Oceans, *A. simplex* C in the Pacific coasts and in the Southern hemisphere and *A. pegreffii* in the Mediterranean Sea and in the Southern hemisphere (Abollo *et al*., 2003). The precise identification of *Anisakis* larvae up to species level is very difficult because no obvious morphological differences have been reported especially in differentiating *A. simplex s.s*. from *A. pegreffii*. The PCR-based approaches have been recently used for accurate diagnosis and the study of the systematic evolution of anisakid nematodes (Umehara *et al*., 2006; Mattiucci & Nascetti, 2008; Cavallero *et al*., 2011; 2012). PCR-RFLP analysis and sequencing of the internal transcribed spacers of the ribosomal DNA have demonstrated that the ITS containing region is a specific and suitable marker for the identification of *Anisakis* members.
In the Adriatic and Ionian Sea waters the occurrence of *Anisakis simplex* (Roudolphi 1809, det. Krabbe 1878) in the peritoneal cavity of *Merluccius merluccius* L. 1758 and *Sardina pilchardus* Walb. was already reported by Larizza & Vovlas (1995). On May-June 2011, a large increase of anisakid populations was detected in *M. merluccius* and *Lophius piscatorius* L. 1758 specimens from the Adriatic Sea waters and an abundance of liver encapsulated larvae of *Anisakis* were detected.
In the present study, we report the PCR-RFLP analysis of the ITS and the sequencing of the ITS, the D3 domain of

the 28S rDNA and a portion of the *mt*DNA COI (cytochrome c oxidase subunit 1) of *Anisakis* L3 larvae extracted from *M. merluccius* and *L. piscatorius*. In addition, the pathogenic effects (histopathology) of *Anisakis* larvae in the *Merluccius* liver, together with a morphological and morphometrical comparison of peritoneal and liver encapsulated specimens are reported.

Material and methods

*Nematode preparation for microscopic observation*s
Nematodes for microscopic observations extracted from encapsulated structures were fixed in 4 % formaldehyde solution + 1 % propionic acid and mounted permanently in dehydrated glycerine following Seinhorst's (1959) method. Morphometrics of fish larvae from the body cavity and from liver encapsulated structures are listed in Table 1.
Under LM observations *Anisakis* larvae were characterized by measurements of body length, oesophagus extension, excretory pore position, tail and final mucron length. Specimens for scanning electron microscopy (SEM) were processed according to Eisenback's (1985) method but mainly glycerine-infiltrated specimens were used for this study.

Histopathology
Liver tissue portions with encapsulated nematodes were excised and placed in appropriate fixative (formaldehyde + acetic acid solution). After 48 h of fixation, tissues were dehydrated by treatment in a gradient of tertiary butyl alcohol series (50, 70, 85 and 100 %), infiltrated and embedded in 58 % melting point paraffin blocks. Embedded material was sectioned in 10 – 12 µm thick sections by rotary microtome, mounted on glass slides, stained with safranin and fast green and mounted permanently in dammar xylene (Johansen, 1940).

DNA extraction, amplification and sequencing
DNA was extracted from individual L3 larvae. Specimens were handpicked and singly placed on a glass-slide in 3 µl of the lysis buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 15 mM $MgCl_2$, 0.1 % Triton X100, 0.01 % gelatine with 90 µg/ml proteinase K) and then cut into small pieces by using a sterilized syringe needle under a dissecting microscope. The samples were incubated at 65 ºC for 1 h and then at 95 ºC for 15 min to deactivate the proteinase K. The crude DNA of each specimen was amplified by using the following sets of primers (*i*) D3A-D3B expansion segment of 28S rRNA gene using the primers D3A (5'-GACCCGTCTTGAAACACGGA-3') and reverse D3B (5'-TCGGAAGGAACCAGCTACTA-3'); (*ii*) ITS1-5.8-ITS2-rRNA using 18S ext (5'-TTGATTACGTCCCTGCC CTTT-3') and 28S ext (5'-TTTCACTCGCCGTTACTAA GG-3'); (iii) and a portion of the *mt*DNA COI using the primers: COI-F1 (5'-CCTACTATGATTGGTGGTTTTGG TAATTG-3') and COI-R2 (5'-GTA GCAGCAGTAAAAT AAGCACG-3'). The size of ampli-fication products was determined by comparison with the molecular marker Ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 µl on a 1.5 % agarose gel.

PCR-RFLP
The PCR products, containing the ITS region, from three individual nematodes both of hake (*M. merluccius*) and chilled monkfish (*L. piscatorius*) were digested with the following restriction enzymes: *Alu* I (Roche), *Dde* I (Roche), *Hae* III (Roche), *Hinf* I (Roche), *Hpa* II (Roche) and *Rsa* I (Roche) (10 U of enzyme for each digestion) at 37 ºC overnight. The digested DNA fragments were loaded onto 2.5 % agarose gel and visualized by ethidium bromide staining gel. All gel images were stored digitally.

Cloning, sequencing and phylogenetic analysis
The D3 amplified product was purified from gel and directly sequenced. PCR products of the ITS region from two individual nematodes for each fish host were purified for cloning and sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified ITS fragments were cloned in TA cloning vector (Invitrogen) and four clones from *M. merluccius* and two from *L. piscatorius* were sequenced by MWG Eurofins (Germany). Purified COI fragments from both *M. merluccius* and *L. piscatorius* were also cloned and sequenced.
Both ITS and COI sequences obtained in this study were aligned with the corresponding ITS and COI sequences of *A. pegreffii, A. simplex s.s.* and *A. simplex* C deposited in the database. Alignments were performed using ClustalW (Thompson *et al*., 1994). Sequence alignments were manually edited using BioEdit in order to improve the default parameters of the multialignment.
Phylogenetic trees, obtained for both ITS and *cox*1 datasets, were performed with Neighbour-Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods using the MEGA version 4 software (Tamura *et al*., 2007). The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method.
New sequences have been submitted to the GenBank database under the accession numbers: HE997163-HE997164 for COI and HE997158-HE997162 for the ITS.

Results

Hosts: The study was carried out on hake specimens (*M. merluccius*) caught on May – June 2011 and chilled monkfish (*L. piscatorius*) caught on May 2012 in the Adriatic Sea.

Morphology of encapsulated specimens: The L3 *Anisakis* larvae obtained from fish organs (body cavity and liver), are morphologically and morphometrically identical (Fig. 1; Table 1). The cuticle of encapsulated specimens was 13 – 16 µm thick, usually with distinct transverse striations along entire body (Fig. 1F – G). In *face* view the triangular oral opening visible, and the V-shaped boring

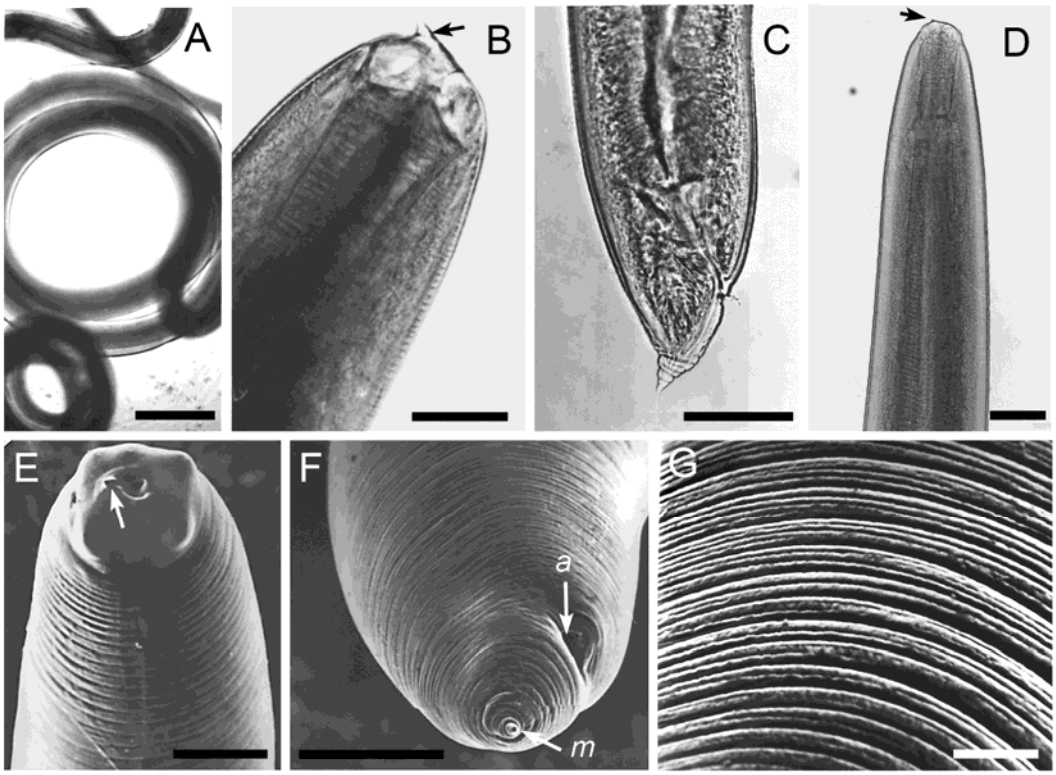

Fig. 1. *Anisakis pegreffii* larvae (L3). Light (A – D) and SEM (E – G) microscopy illustrations:
A: A coiled specimen extracted from a liver encapsulated structure. B, D & E: Anterior nematode body extremity in lateral and face view. *(note the arrowed distinct boring tooth in these figures).* C & F: Posterior extremity *(arrowed in these figures a = anus; and tail terminus spine m = mucron),* G: Fine body annulation along entire body
(Scale bars: A = 1.0 mm; B – F = 150 µm; G = 50 µm)

tooth (8 – 10 µm long) is located ventrally to the mouth. The alimentary canal was circular, with triangular lumen (Figs 2D, F). The short tail (c value = 0.8 – 1.8) ends with a distinct and not reflexed mucro (19.0 – 33.5 µm long).

Table 1. Measurements of *Anisakis pegreffii* larvae from different organs (intestine and liver) of *Merluccius merluccius*

Characters	Body cavity population	Liver encapsulated specimens
n	20	20
Body length (mm)	16.5 – 28.0	17.0 – 29.6
Body width (mm)	0.3 – 0.5	0.3 – 0.5
Oesophagus length (mm)	2.1 – 3.1	2.1 – 3.1
Ventriculus length (mm)	0.6 – 0.9	1.0 – 1.3
Ventriculus width (mm)	0.1 – 0.3	0.2 – 0.4
Tail (mm)	0.1	0.1
Anal body width (µm)	69.3 – 150.6	70.3 – 155.5
Tail mucron (µm)	17.3 – 32.0	19.0 – 33.5
Boring tooth (µm)	5.5 – 14.5	5.5 – 14.0
a	42.8 – 59.4	44.8 – 66.5
b	6.8 – 11.1	7.4 – 11.1
c	133.3 – 288.9	166.4 – 233.0
c'	0.7 – 1.8	0.8 – 1.8

Liver histology: The liver histological examination revealed *Anisakis* larvae encapsulated on the liver surface within dense and pearl coloured envelops causing host hepatic tissue necrosis, large cavities and oedematous liver spots (Fig. 2). The parasitic specimen for each capsule was coiled 4 – 5 times in a spiral position. A distinct compact capsule structure (35 – 45 µm thick) contained single nematode specimens.

PCR-RFLP analysis

Amplification of the ITS region in hake and chilled monkfish produced a fragment of approximately 1.2 kb in length for all specimens. Digestion with *Hinf*I enzyme produced six fragments of approximately 331, 285, 242, 106, 100 and 67 bp matching the peculiar restriction pattern of *A. pegreffii* both in *M. merluccius* and *L. piscatorius* (Fig. 3). Some larvae showed with *Hinf* I an extra band of about 600 bp. With *Alu* I enzyme, a restriction profile was obtained of 422, 395, 163, 85 and 84 bp. *Hae* III enzyme provided a pattern of three fragments of 627, 343 and 179. *Eco* RI enzyme produced two fragments of 468 and 682. *Rsa* I enzyme produced four fragments of 564, 305, 259 and 22 bp. *Hpa* II enzyme produced four bands of 608, 400, 100 and 42 bp.

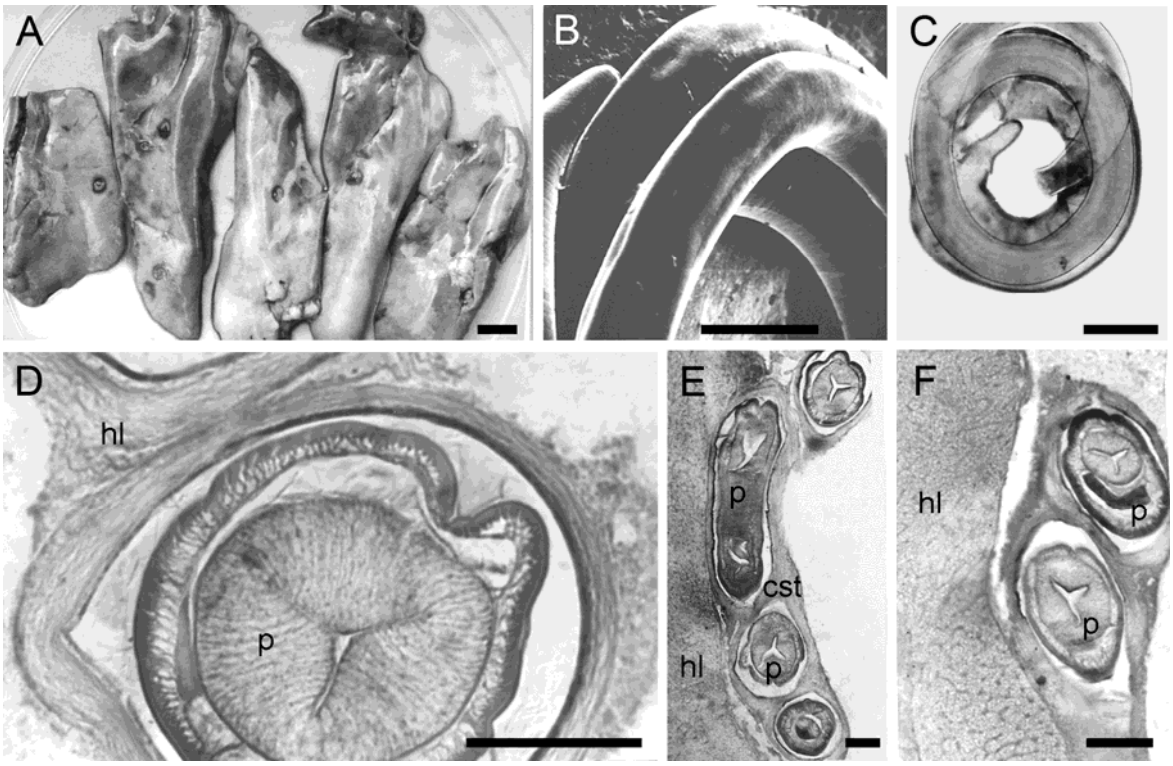

Fig. 2. Host relationship of encysted *Anisakis pegreffii* larvae (L3).
A: Hepatic lobules heavily infected. B: Coiled nematode specimen extracted from a liver encapsulated structure. C: The empty pearl envelop encasement of the nematode from infected liver. D – F: Histological sections showing the encysted parasite specimens (p) in the host liver (hl) tissues. E: Distinct layer of capsule structure (cst) into the hepatic lobules
(Scale bars: A = 10 mm; B = 0.5 mm; C = 1.0 mm; D – F = 150 µm)

Sequence of the ribosomal regions

The sequences of the entire ITS were determined for 3 larvae of hake and 2 larvae of chilled monkfish. No intra-specific differences were found in the sequences of these specimens. The ITS sequences from hake were identical to those from chilled monkfish. BLAST search at NCBI revealed that the ITS sequences obtained were identical to the deposited sequences of *A. pegreffii* and *A. simplex s.s*. But the ITS sequences from hake and chilled monkfish showed a C at the two variable sites that specifically identified *A. pegreffii*. No recombinant genotypes were identified.

The direct sequencing of the D3 expansion domain of the 28S rRNA gene produced a sequence of 320 bp. BLAST search at NCBI revealed that the D3 expansion domain showed 100 % similarity with the corresponding sequences of *Anisakis simplex s.l.*

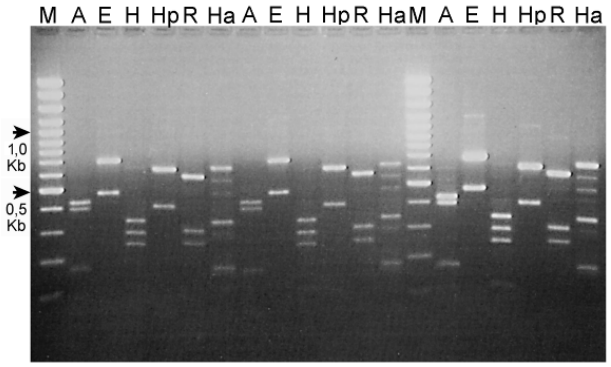

Fig. 3. PCR-RFLP profiles of *Anisakis pegreffii* obtained by digestion of ITS region with the restriction enzymes *Alu* I, *Eco* RI, *Hinf* I, *Hpa* II, *Rsa* I and *Hae* III

mtDNA COI analysis

The amplification of the *mt*DNA COI produced a fragment of 710 bp in length. The nucleotide sequences of *mt*DNA COI gene obtained in this study showed 94 – 95 % similarities with other published sequences of *A. simplex s.s*. In addition, the translated amino acid sequence of our *mt*DNA COI gene showed 95 – 96 % similarities with the corresponding sequences of *A. simplex s.s.* present in the database confirming the species level of our sequences. Furthermore, 11 variable sites were found between *A. pegreffii* and *A. simplex* CO I protein.

Phylogenetic relationships

Phylogenetic analyses were carried out on the ITS and mitochondrial COI datasets. Phylogenetic trees, obtained for both ITS and COI datasets, were performed with Neighbour-Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods by using MEGA version 4 software (Tamura *et al*., 2007). The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method.

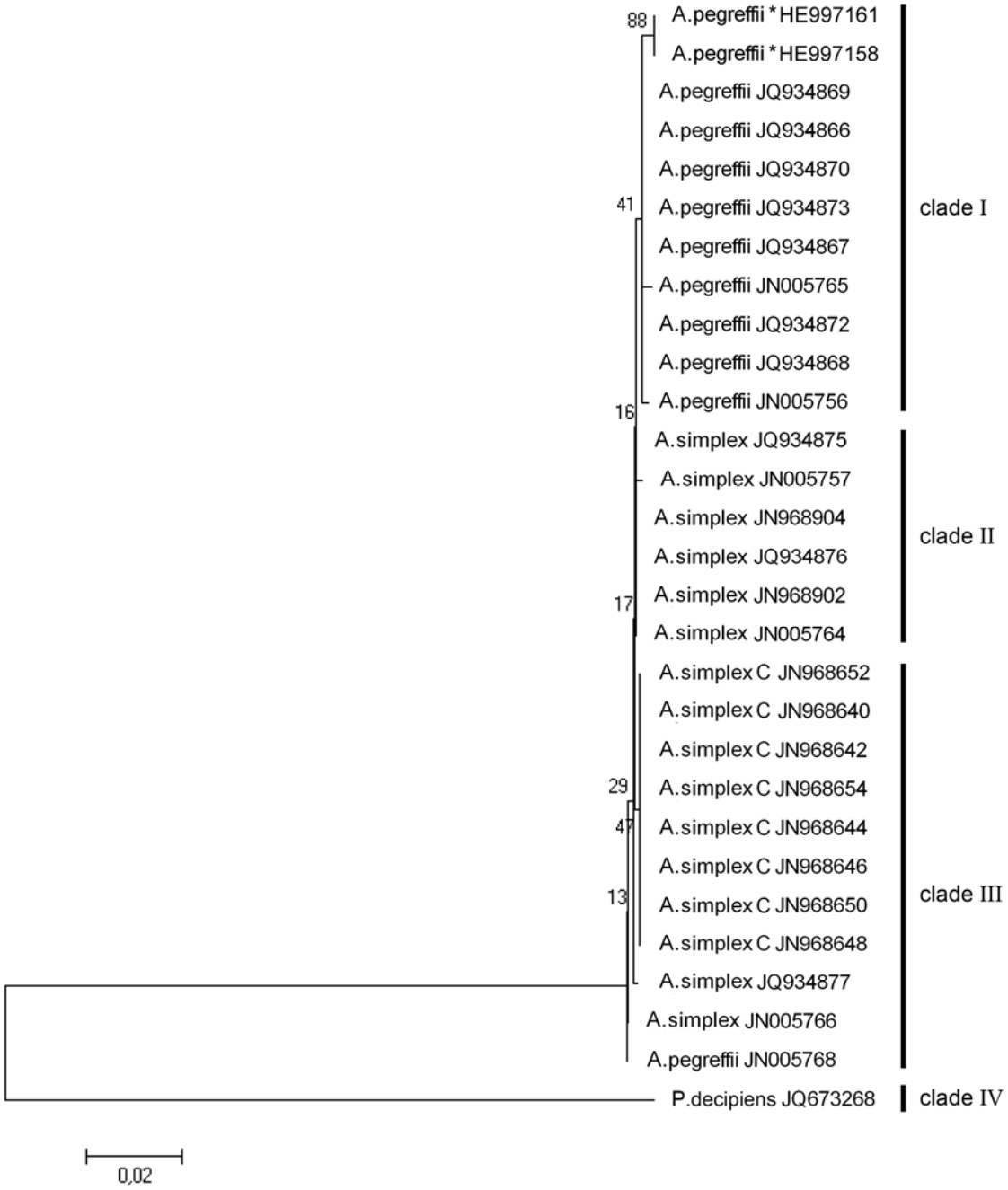

Fig. 4. Phylogenetic tree based on the ITS sequences of *Anisakis* species including the present ITS sequences. The tree was constructed using the maximum parsimony criteria by MEGA, version 4 (Tamura *et al*., 2007). * indicated the sequences obtained in this study

For phylogenetic analysis of the ITS region the 8 most representative sequences of *A. simplex* C, 8 sequences of *A. simplex s.s.* and 9 sequences of *A. pregreffii* were aligned along with the *A. pegreffii* sequences from hake and chilled monkfish obtained in our study. *Pseudoterranova decipiens* ITS sequence was used as outgroup. Phylogenetic trees generated by the Neighbour-Joining, Maximum Parsimony and Minimum Evolution methods showed no significant conflict in branching order and support level, so only NJ tree is shown (Fig. 4). In this phylogenetic tree four clades were evident with *P. decipiens* in basal position. Clade I grouped all sequences of *A. pegreffii*. Clade II contained *A. simplex s.s.* sequences. Clade III consisted of *A. simplex C*.

For phylogenetic analysis of the COI sequences, 12 sequences of *A. simplex* were aligned along with the two COI sequences from hake and chilled monkfish. The corresponding COI sequence of *A. physeteris* was used as outgroup. In the MP tree, *A. physeteris* was at the basal position, instead *A. pegreffii* COI sequences formed a well supported group interspersed with the sequences of *A. simplex*. In the NJ and ME trees *A. pegreffii* was at the basal position of the trees, instead *A. physeteris* grouped with *A. simplex* sequences.

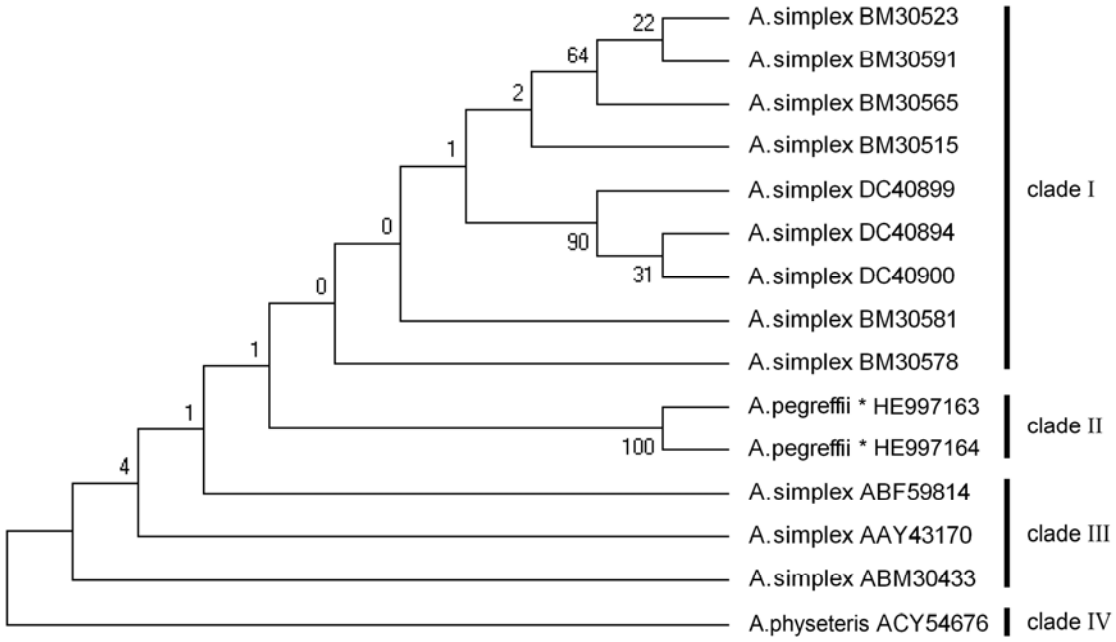

Fig. 5. Phylogenetic tree based on Cox I sequences of *Anisakis* species including the present Cox I sequences. The tree was constructed using the maximum parsimony criteria by MEGA, version 4 (Tamura *et al.*, 2007). * indicated the sequences obtained in this study

Discussion

The ability to identify species of *Anisakis* has important implications for investigating their systematics, population biology and ecology as well as for controlling anisakiasis. Classical-morphometrical species identification of *Anisakis simplex s.l.* is hindered because of similarities among species and intra-specific morphometrical variability. Using a molecular polyphasic approach such as PCR-RFLP, sequencing of nuclear and mitochondrial markers and phylogenetic analysis, we were able to specifically identify *A. pegreffii* in *M. merluccius* and *L. piscatorius* from Adriatic Sea waters.

The unequivocal identification of *A. pegreffii* by using the entire ITS was possible for the specific restriction pattern obtained with the enzyme *Hinf* I and the diagnostic nucleotides C and C at alignment positions 475 and 491 peculiar for *A. pegreffii* (Abollo *et al*., 2003; Abe *et al*., 2005; Ceballos-Mendiola *et al*., 2010). These findings clearly demonstrated that the heterozygote pattern observed with *Hinf* I enzyme is due to an incomplete digestion of the amplified DNA. In addition, the sequence analysis of the mitochondrial COI of *A. pegreffii* showed 4 – 5 % of dissimilarities with the corresponding sequences of *A. simplex s.s.* confirming that it is a reliable marker for species discrimination.

Phylogeny of the ITS sequences with ME, NJ and MP produced identical trees and allowed clear separation of *A. simplex s.s*., *A. simplex* C and *A. pegreffii*. In addition, all sequences belonging to the same *Anisakis* species clustered together (Fig. 4) revealing a high intra-specific variability. Thus, heterogeneity of ITS did not preclude species discrimination. Only one ITS sequence identified as *A. pegreffii* present in GenBank grouped with *A. simplex* C. suggesting a misidentification of nematode species.

The phylogenetic tree of COI with MP (Fig. 5) clearly revealed that *A. pegreffii* is a sister species of *A. simplex* supporting the taxonomic position of this species as found in Vardić Smrzlić *et al*. (2012).

In conclusion, the present study establishes the importance of using polyphasic identification such as the PCR-RFLP method, sequencing and phylogenetic analyses highlighting the time consuming aspect and difficulty of a correct identification at species level within the *Anisakis simplex* group. More detailed analysis of the ITS sequence alignment will allow the design of species-specific primers for unequivocal diagnostics of *A. simplex s.s.*, *A. simplex* C, *A. pegreffii* and closely related species.

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