



Molecular identification and pathology of *Anisakis pegreffii* (Nematoda: Anisakidae) infection in the Mediterranean loggerhead sea turtle (*Caretta caretta*)

Mario Santoro^{a,*}, Simonetta Mattiucci^a, Michela Paoletti^{a,b}, Annalisa Liotta^c, Barbara Degli Uberti^d, Giorgio Galiero^d, Giuseppe Nascetti^b

^a Department of Public Health and Infectious Diseases – Section of Parasitology, “Sapienza” University of Rome, P.le Aldo Moro, 00185 Rome, Italy

^b Department of Ecology and Sustainable Economic Development, Tuscia University, Viale dell'Università, 01100 Viterbo, Italy

^c Tartanet, Centro di Recupero Tartarughe Marine, Brancalione, Reggio Calabria, Italy

^d Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute n 9, Portici, Naples, Italy

ARTICLE INFO

Article history:

Received 12 March 2010

Received in revised form 4 August 2010

Accepted 16 August 2010

Keywords:

Anisakis pegreffii

Caretta caretta

Loggerhead sea turtle

Pathological changes

Mediterranean Sea

ABSTRACT

Nematodes of genus *Anisakis* spp. parasitize a wide range of marine hosts with marine mammals (mainly cetaceans) serving as definitive hosts, while fish, squid and other invertebrates serve as paratenic or intermediate hosts. Sea turtles can act as accidental or paratenic hosts for *Anisakis* spp. larvae, harbouring third-stage larvae unable to complete their life cycle in an ectothermic vertebrate. Post-mortem examination of 96 loggerhead sea turtles (*Caretta caretta*) stranded along the Italian coast of the Mediterranean Sea showed infection by *Anisakis* larvae Type I from 4 of 6 locations that were identified as belonging to *Anisakis pegreffii* by sequence analyses of the mtDNA *cox2*. Thirteen turtles (11 males and 2 females) were infected with *A. pegreffii*. Larvae were detected through gross necroscopy from 7 turtles, while in other 6 positive loggerhead sea turtles *A. pegreffii* larvae were revealed by histopathology. Pathological changes associated with *A. pegreffii* larvae in the stomach and intestine included necrosis and granulomatous response compatible with larvae migration. The role of the loggerhead sea turtle as an accidental host in the life cycle of this nematode is also discussed. This study is the first description of pathological changes associated with *A. pegreffii* in a sea turtle.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Nematodes of genus *Anisakis* spp. parasitize a wide range of marine hosts with marine mammals, especially cetaceans, serving as definitive hosts, while fish, squid and other invertebrates serve as paratenic or intermediate hosts (Mattiucci and Nascetti, 2008). Eggs of *Anisakis simplex* are shed by marine mammals into the marine environment where they then embryonate to second-stage larvae and are ingested by small crustaceans, such as mem-

bers of the *Euphausia* and *Thysanoessa* genera (Smith, 1971; Klimpel et al., 2004). When infected crustacea are ingested by fish or squids, the third-stage larvae of the parasite encapsulates on the visceral tissues, mainly in the mesenteries and liver. *A. simplex sensu lato* third-stage larvae may also migrate into the muscle tissue of the fish paratenic host (Skov et al., 2009). Fourth-stage larvae and adult parasites are found in the alimentary tract of marine mammals, which acquire them via ingestion of infected fish (Smith, 1971; Klimpel et al., 2004). Sea turtles can act as accidental or paratenic hosts for *Anisakis* spp. larvae, harbouring third-stage larvae unable to complete their life cycle in an ectothermic vertebrate (Glazebrook and Campbell, 1990; Santoro and Mattiucci, 2008).

* Corresponding author. Tel.: +39 06 49914894; fax: +39 06 49914894.
E-mail address: marisant@libero.it (M. Santoro).

Larval forms of *A. simplex* s.l. have been reported from a variety of captive and wild sea turtles around the world, but the larvae collected have been identified in those hosts as *A. simplex* or *Anisakis* spp. using only light microscopy (Burke and Rodgers, 1982; Glazebrook and Campbell, 1990; Piccolo and Manfredi, 2003; Orós et al., 2005; Badillo, 2007; Innis et al., 2009; Valente et al., 2009). Larval identification of *Anisakis* spp. to the specific level is not possible by using morphological characters; two larval morphotypes indicated as Types I and II (*sensu* Berland, 1961) have been described. On the other hand, molecular-genetic methodologies, widely applied to the species of the genus *Anisakis* has demonstrated that larval *Anisakis* Type I is shared so far by 6 distinct species, including the species of the *A. simplex* complex (i.e. *A. simplex sensu stricto*, *A. simplex* C and *A. pegreffii*), *A. typica*, *A. ziphidarum* (see Mattiucci and Nascetti, 2008), and *A. nascettii* Mattiucci et al., 2009. Whereas, the larval *Anisakis* Type II is shared so far by three different species: *A. physeteris*, *A. brevispiculata* (see Mattiucci et al., 2001), and *A. paggiae* Mattiucci et al., 2005. These anisakid species are characterized by different geographical distribution and definitive host preference (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009). Among the genetic molecular markers to be used for the larval identification of *Anisakis* spp. (reviewed in Mattiucci and Nascetti, 2008), the sequence analysis of the mtDNA *cox2* gene locus has been used in this paper for the identification to the species level of the larvae of *Anisakis* recovered in loggerhead sea turtle (*Caretta caretta*). Indeed this gene has been proved to be a useful genetic marker to detect cryptic species of nematodes of the genus *Anisakis* (Mattiucci et al., 2009) as well as to study the haplotype polymorphisms and nucleotide diversity of *Anisakis* spp. (in preparation).

In this paper, for the first time, we report on the molecular identification of *A. pegreffii* larvae from loggerhead sea turtle and we describe their pathological significance in an accidental host.

2. Materials and methods

2.1. Sample collection

Post-mortem examination was performed on 96 loggerhead sea turtles stranded between July 2006 and April 2009 in 6 locations from the Central Mediterranean of southern Italy including the Tyrrhenian coast of Campania region ($n=32$; 12 females, 13 males and 7 of unidentified sex), the Tyrrhenian ($n=18$; 9 females, 4 males and 5 of unidentified sex) and Ionian ($n=23$; 10 females, 8 males and 5 of unidentified sex) coasts of Sicily, the Tyrrhenian ($n=4$; 2 females and 2 males) and Ionian coast of Calabria region ($n=16$; 9 females and 7 males), and the Ionian coast of Apulia region ($n=3$; 1 female and 2 males) (Fig. 1). Loggerhead sea turtles examined were from 4 sea turtles recovery centres including Anton Dohrn Zoological Station in Naples (Campania); Tartanet, Brancalone (Reggio Calabria) and Isola Capo Rizzuto (Crotone) recovery centres (Calabria); and Centro Regionale Recupero Fauna Selvatica e Tartarughe Marine in Comiso, Sicily. Most (>90%) stranded turtles died from interactions with fishing activities. All turtles were frozen until necropsy. The curved carapace length notch-

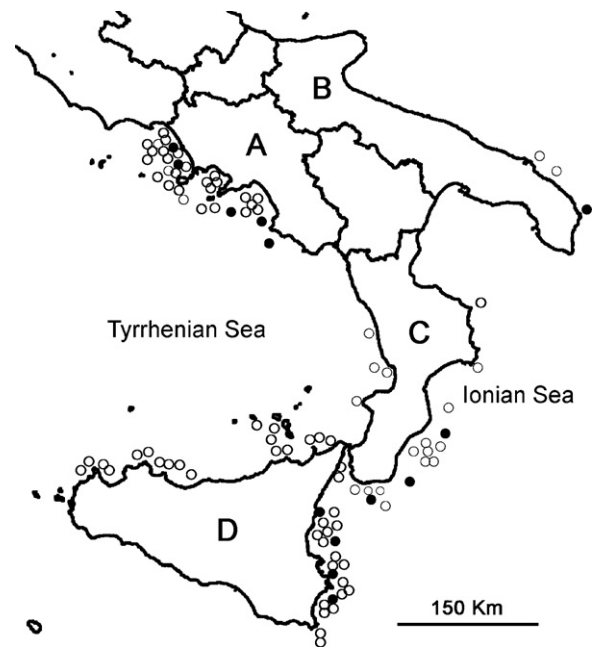


Fig. 1. Sites of stranding of loggerhead sea turtles, *Caretta caretta* on the Central Mediterranean (southern Italy coast) between 2006 and 2009, including Campania (A), Apulia (B), Calabria (C) and Sicily (D) coasts. (●) infected; (○) uninfected by *Anisakis pegreffii*.

to tip (CCL) was measured to the nearest cm, and each turtle was categorized by age classes using Bolten (1999) criteria: early juveniles (CCL ≤ 40 cm); late juveniles or sub-adults (CCL between 40 and 69 cm) and adults (CCL ≥ 70 cm).

Oesophagus, stomach, small and large intestines, spleen, liver, and coelomic cavity were carefully examined for macroscopic lesions, and *Anisakis* specimens were collected, counted and stored at -20°C for molecular analysis. Tissue samples from the organs with gross lesions were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at $6\text{ }\mu\text{m}$, and stained with haematoxylin and eosin. Sections from selected cases were also stained with Brown and Brenn Gram's stain and Ziehl–Neelsen methods to detect bacteria and acid-fast organisms, respectively.

2.2. Molecular identification of *Anisakis* larvae

All the *Anisakis* larvae Type I ($n=41$), at third-stage, recovered from the loggerhead sea turtles were identified by using molecular markers. A 629 bp fragment of the cytochrome oxidase 2 (*cox2*) gene was analysed. Total DNA was extracted from 2 mg of tissue from a single nematode using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and/or cetyltrimethylammonium bromide (Mattiucci et al., 2009). The *cox2* gene from *Anisakis* spp. was amplified using the primers 211F 5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3' and 210R 5'-CAC CAA CTC TTA AAA TTA TC-3' from Nadler and Hudspeth (2000) spanning the mtDNA nucleotide position 10,639–11,248 as defined in *Ascaris suum* (Genbank X54253). PCR (polymerase chain reaction) amplification was carried out in a volume of $50\text{ }\mu\text{l}$ containing 30 pmol of

each primer, MgCl_2 2.5 mM (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), PCR buffer 1× (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), DMSO 0.08 mM, dNTPs 0.4 mM (Sigma–Aldrich, St. Louis, MO), 5 U of *Taq* Polymerase (Amersham Pharmacia Biotech. Inc., Piscataway, NJ) and 10 ng of total DNA. The mixture was denatured at 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1.5 min, followed by post-amplification at 72 °C for 10 min. The PCR product was purified using PEG precipitation and automated DNA sequencing was performed by MacroGen Inc. (Seoul, Korea) using primers 210 and 211. The sequences obtained were compared to those already obtained for the same gene for all the *Anisakis* spp. above reported and previously deposited by us in GenBank with their accession numbers. They are the following: *A. simplex* s.s. (DQ116426), *A. pegreffii* (DQ116428), *A. simplex* C (DQ116429), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), *A. paggiae* (DQ116434) and *A. nascettii* (DQ116431), as reported in Mattiucci et al. (2009). Additionally, the specimens from loggerhead sea turtles were also compared with other *A. pegreffii* specimens collected from anchovies (*Engraulis encrasicolus*) from the Mediterranean Sea (Mattiucci et al., 2009).

The *cox2* sequences were aligned using Clustal W (Thompson et al., 1994). Phylogenetic analysis was performed by MEGA 4.0 (Tamura et al., 2007), using Maximum Parsimony (MP) and Neighbour-joining (NJ) based on *p-distance* values. The reliabilities of the phylogenetic relationships were evaluated using nonparametric bootstrap analysis (Felsenstein, 1985) for the MP and NJ trees. Bootstrap values ≥ 70 were considered well supported (Hillis and Bull, 1993). The sequences obtained for the specimens of *A. pegreffii* here analysed were compared to those already obtained for the same gene for *Anisakis* spp., in our previous studies (Valentini et al., 2006). Sequence at the mtDNA *cox2* of *Pseudoterranova decipiens* (s.s.) from *Phoca vitulina* was included as outgroup to root the *Anisakis* phylogenetic tree. Nucleotide diversity (π) at the mtDNA *cox2* level was estimated by DnaSPver.5 software (Librado and Rozsa, 2009).

3. Results

3.1. Molecular identification of *Anisakis* larvae

All *Anisakis* larvae Type I from loggerhead sea turtles, sequenced at the *cox2* gene (629 bp) clustered in the same well supported clade, in both the Maximum Parsimony (MP) and Neighbour-joining (NJ) analysis (Fig. 2) with specimens of the species *A. pegreffii*, previously sequenced (Valentini et al., 2006; Mattiucci et al., 2009) and deposited in GenBank. The mtDNA *cox2* sequences of *Anisakis* larvae obtained from loggerhead sea turtles matched the sequence of *A. pegreffii* previously deposited by us in GenBank with 99% of similarity. Further, the larval *A. pegreffii* from loggerhead sea turtles were clustering in the same well supported clade with the *A. pegreffii* larvae collected from anchovies of the Mediterranean Sea in both MP and NJ analysis (Fig. 2). At the intraspecific level, nucleotide diversity found in the sample of *A. pegreffii* from logger-

head sea turtles was $\pi = 0.004$. Sequence of *A. pegreffii* from loggerhead sea turtle is deposited in GenBank under the Accession number FJ940755. A very low level of genetic differentiation at the mtDNA *cox2* level (*p-distance* was on average = 0.008), has been found between the sample of *A. pegreffii* from loggerhead sea turtles and the sample of *A. pegreffii* so far sequenced from the Mediterranean Sea.

3.2. Sampling data

Ninety-one of turtles were immature (juveniles and sub-adults) and 5 were adults. Forty-three were females, 36 were males and 17 were of unknown sex. Of the 13 turtles infected with *A. pegreffii*, 11 were males and 2 were females. Infected chelonians ranged in CCL from 22 to 57 cm. Larvae of *A. pegreffii* were found in turtles from all locations except from Tyrrhenian coasts of Calabria and Sicily. Prevalence (*P*) of infection was as following: 1 of 3 from the Ionian Sea of Apulia coast, 3 of 16 (*P* = 18.7%) from Ionian Sea of Calabria coast, 4 of 23 (*P* = 17.3%) from Ionian Sea of Sicily and 5 of 32 (*P* = 15.6%) from Tyrrhenian Sea of Campania coast. *Anisakis* specimens have been deposited in the U.S. National Parasite Collection, Beltsville (registration no. USNPC 101631).

3.3. Gross and histopathological findings

A total of 41 larvae were macroscopically found in 7 turtles with an intensity of infection ranging from 1 to 21. The larvae were found free in the coelomic cavity (in a single turtle), free in the stomach (in 7 turtles) or intestine (in 3 turtles), embedded in the wall of stomach (in 4 turtles), or encapsulated on surface of liver (in 4 turtles). Six other infections were revealed by histological examination of gastrointestinal tissues.

At gross examination, haemorrhagic foci and ulcers ranging approximately from 1 to 4 cm in diameter were found on thickened gastric mucosal surface of all infected turtles. These lesions were associated with occasional worms embedded in the ulcerous areas, while others had migrated from the gastric lumen to the serosa. A single turtle had multifocal ulcerative lesions with caseous material on the mucosa of upper intestine associated with *Anisakis* larvae in the sub-mucosa.

Microscopically, the gastric mucosa was hyperplastic with focal accumulation of heterophils and rare macrophages. Larvae surrounded by a connective tissue capsule with occasional necrotic debris and multinucleated giant cells were within mucosa, sub-mucosa and muscularis (Fig. 3). Older lesions were characterized by the accumulation of necrotic material, degenerate heterophils and cellular debris occasionally mixed with bacterial colonies. Some coiled larvae were observed encapsulated on liver surface and appeared as round to ovoid white in colour measuring approximately 0.5 cm in diameter. Mechanical compression of the liver parenchyma by *Anisakis* larvae was not evident, and there were no gross pathological changes.

In cross-section, at level of the nematode intestine, the larvae had a diameter measuring approximately 500 μm $\times$ 300 μm with a thin cuticle, approximately

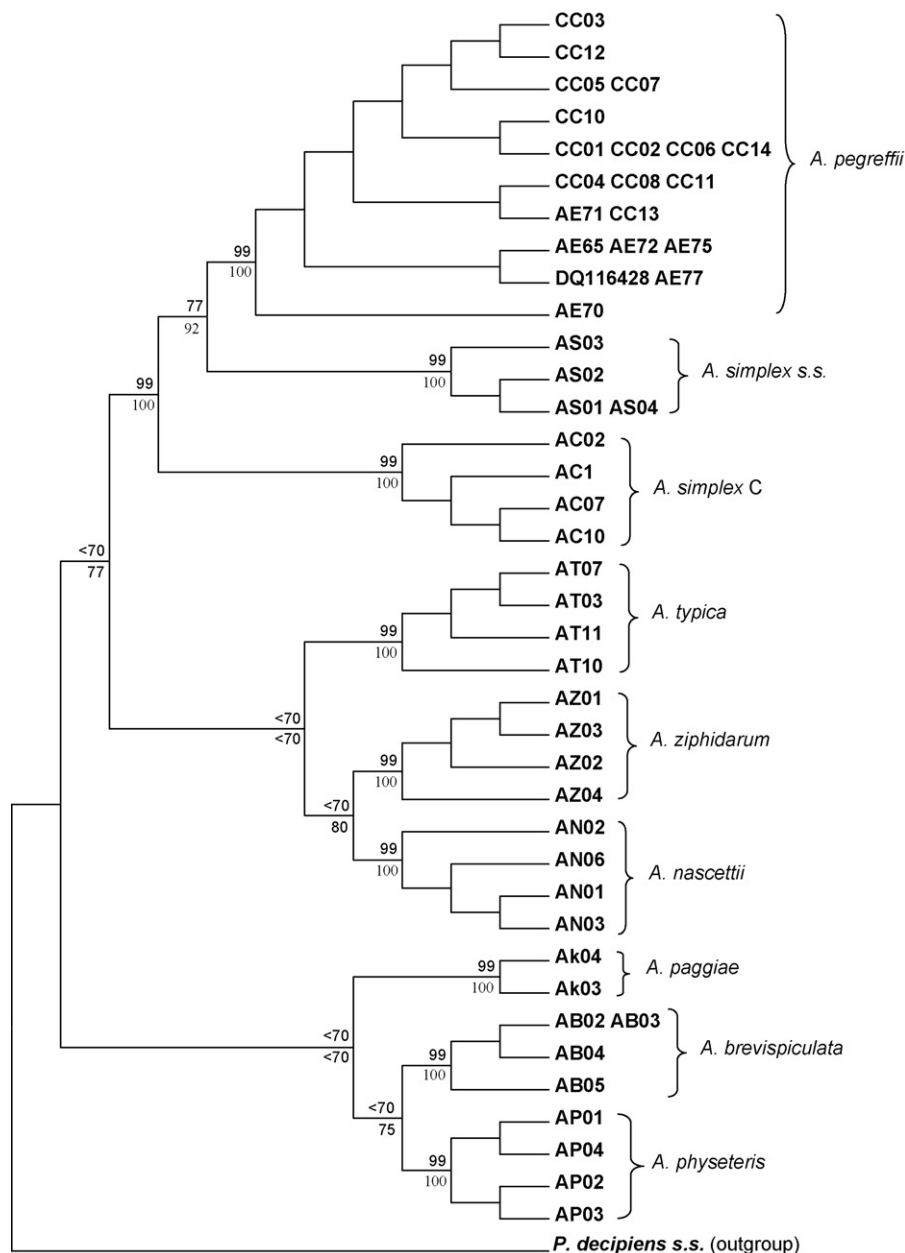


Fig. 2. Cox2 derived Maximum Parsimony (MP) inferred by MEGA 4.0 consensus tree inferred by MEGA 4 for the specimens of *Anisakis pegreffii* from loggerhead sea turtle, *Caretta caretta* (CC) sequenced for the mtDNA *cox2* (629 bp). The MP tree was obtained by the bootstrap method with heuristic search. They were 213 polymorphic sites, 178 parsimony-informative sites. Sequences of *A. pegreffii* from *Engraulis encrasicolus* are reported with the code AE71, AE65, AE72, AE70, AE75, AE77. Number of bootstrap replicates = 1000, bootstrap percentages of the clades ≥ 70 are shown at the nodes: MP and NJ values are reported above and below the nodes, respectively. Sequences from the other *Anisakis* spp. are those analysed in Mattiucci et al. (2009) and deposited in Genbank. *Pseudoterranova decipiens s.s.* was used as the outgroup.

10 μ m thick, lacking lateral alae. Polymyarian muscle cells, separated into 4 bundles by the chords, had 2 wing-like distal lobes. The intestine was circular with an amorphous lumen. The excretory cells were banana-shaped and located ventrally to intestine (Fig. 3).

Diffuse yellow granulomatous 0.2 mm lesions, were found on the capsule and parenchyma of the liver of 2 turtles. On histology, these lesions were characterized by foci of necrotic debris mixed with bacteria and surrounded by

multinucleated giant cells; bacteria were acid-fast negative. One turtle had infection with *A. pegreffii* larvae and the other did not.

4. Discussion

The identification of the larval nematodes recovered from the examined loggerhead sea turtles, as belonging to the species *A. pegreffii* was very well supported by both

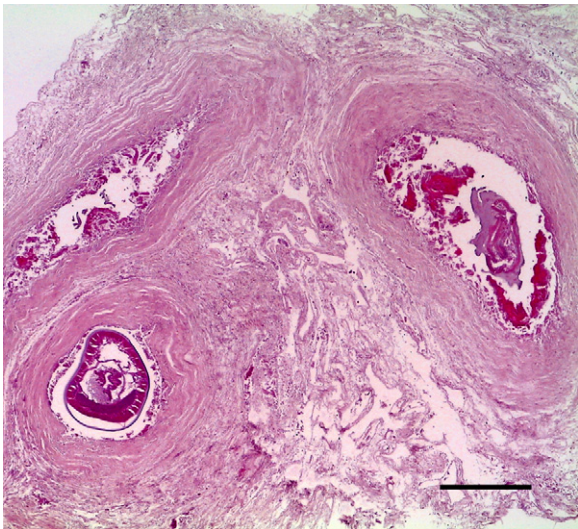


Fig. 3. Granulomatous reaction by *Anisakis pegreffii* larvae in the gastric wall of a sub-adult loggerhead sea turtle *Caretta caretta*. Transverse sections of the larvae are well visible surrounded by connective tissue capsule, necrotic debris and multinucleated giant cells. Hematoxylin and eosin. Bar = 350 μ m.

the MP and NJ analysis of the mtDNA *cox2* sequences. The tree topologies were in agreement in showing that they were included in the same clade formed by the specimens of *A. pegreffii* previously sequenced for the same gene.

This study indicates that the sequences analysis at the mtDNA *cox2* gene are useful markers (barcodes) for distinguishing *Anisakis* spp. as well as other anisakid nematodes (Mattiucci et al., 2010). Further, at the intraspecific level, a very low level of genetic differentiation at mitochondrial level has been found between the sample of *A. pegreffii* from loggerhead sea turtles and the sample of *A. pegreffii* so far sequenced from fish and cetaceans of the Mediterranean Sea; similar values are those reported for conspecific populations of anisakid nematodes (Mattiucci and Nascetti, 2008). Indeed, higher level of differentiation, at the same mitochondrial gene level, are those observed at the inter-specific level between *Anisakis* spp. (Mattiucci and Nascetti, 2006). The mtDNA *cox2* gene revealed to be highly polymorphic in *Anisakis* spp. (Valentini et al., 2006; Mattiucci et al., 2009, unpublished data). The nucleotide diversity value so far observed, at the mitochondrial level, in the sample of *A. pegreffii* from loggerhead sea turtles is at the same level as that observed in the *A. pegreffii* populations so far sequenced from the Mediterranean Sea (on average, $\pi = 0.005$). On the other hand, nucleotide diversity value so far estimated at mtDNA *cox2* level in the Mediterranean population of *A. pegreffii* is generally lower with respect to that observed in other populations of the same species from the austral region (on average, $\pi = 0.016$) (our unpublished data, in preparation). The last finding seems to be in accordance with the genetic diversity estimates observed in the same populations of *A. pegreffii* previously based on allozyme level (Mattiucci and Nascetti, 2007).

A. pegreffii, first described from the digestive tract of a Mediterranean monk seal (*Monachus monachus*), captured in eastern Sardinia (Italy) (Campana-Rouget and Biocca, 1955), is the main sibling species within *A. simplex* complex present in fish and squids from the Mediterranean Sea (see Mattiucci et al., 1997; Mattiucci and Nascetti, 2008). In the Mediterranean Sea, its definitive hosts include dolphins, such as *Tursiops truncatus*, *Stenella coeruleoalba*, and as intermediate/paratenic hosts, 18 fish species and 1 squid species have been reported by Mattiucci and Nascetti (2008). The finding of *A. pegreffii* in the stranded individuals of loggerhead sea turtles along the Italian coast is in accordance with the geographical distribution of this anisakid species so far documented by Mattiucci and Nascetti (2008). On the other hand, the absence of other species of the genus *Anisakis*, such as, for instance, *A. simplex* s.s., which is a species mainly distributed in the North Atlantic Sea, seems so far to suggest that these individuals of loggerhead sea turtles were from a Mediterranean population. Indeed, *Anisakis* spp. have been proved to be used as a good biological tags in suggesting the possible origin and migratory routes of their intermediate/paratenic hosts (Mattiucci et al., 2007).

In sea turtles, *A. simplex* (s.l.) larvae have been previously found in captive green sea turtles (*Chelonia mydas*) in Australia (Burke and Rodgers, 1982; Glazebrook and Campbell, 1990), in free-ranging Atlantic loggerhead sea turtles in Canary Islands of Spain (Orós et al., 2005) and Portugal (Valente et al., 2009), and in free-ranging Kemp's ridley sea turtles (*Lepidochelys kempii*) from MA (USA) (Innis et al., 2009). In the Mediterranean Sea, Piccolo and Manfredi (2003) and Badillo (2007) from the Adriatic Sea and from the coast of the Valencian Community of Spain found one of 14 and one of 44 loggerhead sea turtles parasitized with 10 *Anisakis* larvae respectively. Prevalence of infection in loggerhead sea turtles ranged from 7.1% (Piccolo and Manfredi, 2003) to 35.7% (Valente et al., 2009). Common site of infection was the gastrointestinal tract, however larvae were also found in liver, spleen, lungs and coelomic cavity (Glazebrook and Campbell, 1990; Piccolo and Manfredi, 2003; Orós et al., 2005; this study).

In captive green turtles, the apparent source of infection with *Anisakis* spp. were fresh sardines (*Harengula ovalis*) and members of Atherinidae family on which chelonians were fed (Burke and Rodgers, 1982; Glazebrook and Campbell, 1990). In free-ranging turtles the source of infection is unknown. In the present study, fish belonging to the families Engraulidae (such as *E. encrasicolus*) as well as unidentified fish species and benthic invertebrates were in the stomach contents of infected chelonians. *E. encrasicolus* has been found as parasitized by *A. pegreffii* in Mediterranean basin waters (Mattiucci and Nascetti, 2008). However, loggerhead sea turtles seem unable to catch fast-swimming animals as pelagic fish, which would be consumed only if already impaired or dead (Plotkin et al., 1993; Laurent and Lescure, 1994). Possibly, if infection by *A. pegreffii* in the examined turtles was acquired by preying on infected fish, it is likely that loggerhead sea turtles became infected by feeding on fish as discarded by-catch (see Tomás et al., 2001).

Granulomatous hepatic serositis associated with *Anisakis* larvae was reported in 10 of 88 loggerhead sea turtles from Canary Islands (Orós et al., 2005). Hepatic lesions described by Orós et al. (2005) were similar to those here found in 2 turtles, but we could not link hepatic lesions to anisakids. In fish which represents the natural paratenic hosts for *Anisakis* spp., larvae are coiled inside a connective tissue capsule on the serosa of pyloric ceca, liver and large intestine. Tissue alterations including mechanical compression and displacement of internal organs and granulomatous reactions and necrosis of liver are evident in massive infestations of fish hosts (Smith, 1971; Hauck and May, 1977; Klimpel et al., 2004). In marine mammal definitive hosts *Anisakis* spp. have been associated with gastric ulcers and haemorrhages (Geraci and Aubin, 1987; Raga et al., 2009).

A. pegreffii may be considered as pathogenic and accidental parasite in sea turtles. Probably, when an infected host is ingested by a loggerhead sea turtle, *Anisakis* larvae “change” the turtle for a fish, then they migrate from the gastrointestinal tract to organs of the coelomic cavity. Because definitive hosts of *A. pegreffii* are cetaceans and these do not feed on sea turtles, it is plausible to affirm that the loggerhead sea turtle physiologically functions as a paratenic hosts, but ecologically represents a dead end.

Acknowledgments

We are grateful to the Centro Regionale Recupero Fauna Selvatica e Tartarughe Marine (Comiso), Tartanet (Brancaleone), Centro di Recupero di Isola Capo Rizzuto (Crotone) and Anton Dohrn Zoological Station (Naples), for providing the turtle carcasses. Part of the research was granted by Funds of MIUR-PRIN 2008. The first draft of manuscript was improved by the comments and advices from Dr. Thierry Work.

References

- Badillo, F.J., 2007. Epizootos y parasitos de la tortuga boba (*Caretta caretta*) en el Mediterraneo Occidental. Doctoral dissertation, Universitat de Valencia.
- Berland, B., 1961. Nematodes from some Norwegian marine fishes. *Sarsia* 2, 1–50.
- Bolten, A.B., 1999. Techniques for measuring sea turtles. In: Eckert, K.L., Bjørndal, K.A., Abreu-Grobois, F.A., Donnelly, M. (Eds.), *Research and Management Techniques for the Conservation of Sea Turtles*. IUCN/SSC Marine Turtle Specialist Group, Washington, DC, pp. 110–114.
- Burke, J.B., Rodgers, L.J., 1982. Gastric ulceration associated with larval nematodes (*Anisakis* sp. Type I) in pen reared green turtles (*Chelonia mydas*) from Torres Strait. *J. Wildl. Dis.* 18, 41–46.
- Campana-Rouget, Y., Biocca, E., 1955. Une nouvelle espèce d'*Anisakis* chez un phoque méditerranéen. *Ann. Parasitol.* 30, 477–480.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Geraci, J.R., Aubin, St.D.J., 1987. Effects of parasites on marine mammals. *Int. J. Parasitol.* 17, 407–414.
- Glazebrook, J.S., Campbell, R.S.F., 1990. A survey of diseases of marine turtles in northern Australia. I. Farmed turtles. *Dis. Aquat. Org.* 9, 83–95.
- Hauck, A.K., May, E.B., 1977. Histopathologic alterations associated with *Anisakis* larvae in Pacific herring from Oregon. *J. Wildl. Dis.* 13, 290–293.
- Hillis, D.M., Bull, J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42, 182–192.
- Innis, C., Nyaoke, A.C., Williams III, C.R., Dunnigan, B., Merigo, C., Woodward, D.L., Weber, E.S., Frasca Jr., S., 2009. Pathologic and parasitologic findings of cold-stunned Kemp's ridley sea turtles (*Lepidochelys kempii*) stranded on Cape Cod, Massachusetts, 2001–2006. *J. Wildl. Dis.* 45, 594–610.
- Klimpel, S., Palm, H.W., Ruckert, S., Piatkowski, U., 2004. The life cycle of *Anisakis simplex* in the Norwegian Deep (northern North Sea). *Parasitol. Res.* 94, 1–9.
- Laurent, L., Lescure, J., 1994. L'hivernage des tortues caouannes *Caretta caretta* (L.) dans le sud de Tunisie. *Rev. Ecol.* 49, 63–86.
- Librado, P., Rozsa, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452.
- Mattiucci, S., Abaunza, P., Damiano, S., García, A., Santos, M.N., Nascetti, G., 2007. Distribution of *Anisakis* larvae identified by genetic markers and their use for stock characterization of demersal and pelagic fish from European waters: an update. *J. Helminthol.* 81, 117–127.
- Mattiucci, S., Nascetti, G., 2006. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. *Parasitol.* 13, 99–113.
- Mattiucci, S., Nascetti, G., 2007. Genetic diversity and infection levels of anisakid nematodes parasitic in fish and marine mammals from Boreal and Austral hemispheres. *Vet. Parasitol.* 148, 43–57.
- Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of *Anisakis* nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. *Adv. Parasitol.* 66, 47–148.
- Mattiucci, S., Nascetti, G., Cianchi, R., Paggi, L., Arduino, P., Margolis, L., Bratley, J., Webb, S.C., D'Amelio, S., Orecchia, P., Bullini, L., 1997. Genetic and ecological data on the *Anisakis simplex* complex with evidence for a new species (Nematoda, Ascaridoidea Anisakidae). *J. Parasitol.* 83, 401–416.
- Mattiucci, S., Nascetti, G., Dailey, M., Webb, S.C., Barros, N., Cianchi, R., Bullini, L., 2005. Evidence for a new species of *Anisakis* Dujardin, 1845: morphological description and genetic relationships between congeners (Nematoda: Anisakidae). *Syst. Parasitol.* 61, 157–171.
- Mattiucci, S., Paggi, L., Nascetti, G., Abollo, E., Webb, S.C., Pascual, S., Cianchi, R., Bullini, L., 2001. Genetic divergence and reproductive isolation between *Anisakis brevispiculata* and *Anisakis physeteris* (Nematoda: Anisakidae). *Int. J. Parasitol.* 31, 9–14.
- Mattiucci, S., Paoletti, M., Consuegra-Solorzano, A., Nascetti, G., 2010. *Contracaecum gibsoni* n. sp. and *C. overstreeti* n. sp. (Nematoda: Anisakidae) from the Dalmatian pelican *Pelecanus crispus* (L.) in Greek waters: genetic and morphological evidence. *Syst. Parasitol.* 75, 207–224.
- Mattiucci, S., Paoletti, M., Webb, S.C., 2009. *Anisakis nascettii* n. sp. (Nematoda: Anisakidae) from beaked whales of the southern hemisphere: morphological description, genetic relationships between congeners and ecological data. *Syst. Parasitol.* 74, 199–217.
- Nadler, S.A., Hudspeth, D.S.S., 2000. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. *J. Parasitol.* 86, 380–393.
- Orós, J., Torrent, A., Calabuig, P., Déniz, S., 2005. Diseases and causes of mortality among sea turtles stranded in the Canary Islands, Spain (1998–2001). *Dis. Aquat. Org.* 63, 13–24.
- Piccolo, G., Manfredi, M.T., 2003. New reports on parasites of marine turtles stranded along the Italian coasts. In: Margaritoulis, D., Demetropoulos, A. (Eds.), *Proceedings of the First Mediterranean Conference on Marine Turtles*. Barcelona Convention–Bern Convention–Bonn Convention (CMS), Nicosia, pp. 207–211.
- Plotkin, P.T., Wicksten, M.K., Amos, A.F., 1993. Feeding ecology of the loggerhead sea turtle *Caretta caretta* in the north-western Gulf of Mexico. *Mar. Biol.* 115, 1–15.
- Raga, J.A., Fernández, M., Balbuena, J.A., Aznar, F.J., 2009. Parasites. In: Perrin, W.F., Würsig, B., Thewissen, H.G.M. (Eds.), *The Encyclopedia of Marine Mammals Parasites*. Academic Press/Elsevier, pp. 821–830.
- Santoro, M., Mattiucci, S., 2008. Sea turtle parasites. In: Wehrmann, I.S., Cortés, J. (Eds.), *Marine Biodiversity of Costa Rica, Central America*. Springer, Netherlands, pp. 507–519.
- Skov, J., Kania, P.W., Olsen, M.M., Lauridsen, J.H., Buchmann, K., 2009. Nematode infections of maricultured and wild fishes in Danish waters: a comparative study. *Aquaculture* 298, 24–28.
- Smith, J.W., 1971. *Thysanoessa inermis* and *T. longicaudata* (Euphausiidae) as first intermediate hosts of *Anisakis* sp. (Nematoda: Ascaridata) in the Northern Sea, to the north of Scotland and at Faroe. *Nature* 234, 478.

- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weigh matrix choice. *Nucleic Acid Res.* 22, 4673–4680.
- Tomás, J., Aznar, F.J., Raga, J.A., 2001. Feeding ecology of the loggerhead turtle *Caretta caretta* in the western Mediterranean. *J. Zool.* 255, 525–532.
- Valente, A.L., Delgado, C., Moreira, C., Ferreira, S., Dellinger, T., Pinheiro de Carvalho, M.A.A., Costa, G., 2009. Helminth component community of the loggerhead sea turtle, *Caretta caretta*, from Madeira Archipelago, Portugal. *J. Parasitol.* 95, 249–252.
- Valentini, A., Mattiucci, S., Bondanelli, P., Webb, S.C., Mignucci-Giannone, A., Colom-Llavina, M.M., Nascetti, G., 2006. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial *cox-2* sequences, and comparison with allozyme data. *J. Parasitol.* 92, 156–166.