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# Genetic identification and distribution of the parasitic larvae of *Anisakis pegreffii* and *Anisakis simplex* (s. s.) in European hake *Merluccius merluccius* from the Tyrrhenian Sea and Spanish Atlantic coast: Implications for food safety

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ABSTRACT

The consumption of the hake *Merluccius merluccius* is widespread in European countries, where this fish has a high commercial value. To date, different larval species of *Anisakis* have been identified as parasites in *M. merluccius* from European waters, *Anisakis pegreffii* and *Anisakis simplex* (s. s.) being the two most common. The aim of the study is to present data on the occurrence of *Anisakis* spp. larvae in the viscera and flesh of *M. merluccius*. Consequently, the distribution and infection rates of different species of *Anisakis* in different sites (viscera, and dorsal and ventral fillets) were investigated in hake caught in the central Tyrrhenian Sea (FAO 37.1.3) and the NE Atlantic Ocean (FAO 27 IXa). A sample of N = 65 fish individuals (length > 26 cm) was examined parasitologically from each fishing ground. The fillets were examined using the pepsin digestion method. A large number (1310) of *Anisakis* specimens were identified by multilocus allozyme electrophoresis (MAE) and mtDNA *cox2* sequence analysis; among these, 814 larvae corresponded to *A. simplex* (s. s.) and 476 to *A. pegreffii*. They were found to infect both the flesh and the viscera. The two species co-infected the same individual fish (both in the viscera and in the flesh) from the FAO 27 area, whereas only *A. pegreffii* was found in hake from the Tyrrhenian Sea. The average parasite burden of *A. pegreffii* in hake from the Tyrrhenian Sea was significantly lower to that observed from hake off the Atlantic coast of Spain, both in prevalence and in abundance. In addition, whereas no significant difference in overall prevalence values was recorded between the two *Anisakis* species in the viscera of the FAO 27 sample, significant differences were found in the abundance levels observed between these species in the flesh, with *A. simplex* (s. s.) exhibiting significantly higher levels than that observed for *A. pegreffii* ($p < 0.001$). Given that the pathogenic role in relation to man is known for these two species of *Anisakis*, both the flesh inspection and the infection rates of the different anisakid species assume particular importance in terms of assessing the risk they pose to humans.



## 1. Introduction

The European hake *Merluccius merluccius* (L.) is one of the most important and widely distributed fish species, occurring in both Western European (Casey and Pereiro, 1995) and Mediterranean Sea fisheries (Ardizzone and Corsi, 1997; Oliver and Massutí, 1995). The existence of different intra-specific stocks in European waters has been suggested on the basis of a multi-methodological approach. In fact, North-East Atlantic and Mediterranean Sea populations of *M. merluccius* can be considered as separate stocks (Abaunza et al., 2001; Alheit and Pitcher, 1995; Cimmaruta et al., 2005; Inada, 1981; Lundy et al., 1999; Mattiucci et al., 2014a; Milano et al., 2014; Roldan et al., 1998). These Atlantic and Mediterranean hake populations exhibit remarkable differences in demographic and life history traits, such as growth rate, size at maturity, recruitment patterns and spawning season (Froese and Pauly, 2013, and references therein). The species is caught commercially throughout its range, and it is the most important demersal species caught off Western Europe. It commands a high price in Europe, and is mostly sold fresh in both Italian and Spanish markets.

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The nematode genus *Anisakis* contains species which are parasites of marine organisms, with crustaceans as first intermediate hosts, fishes and squid as second intermediate or paratenic hosts, and mainly cetaceans as definitive hosts (Mattiucci and Nascetti, 2006, 2008). Larval stages of *Anisakis* spp. commonly infect the viscera and musculature of many teleost species (Mattiucci and Nascetti, 2008). Nine species of *Anisakis* have been detected and characterised genetically (Mattiucci et al., 2009, 2014b). Their third stage larvae, recovered from fish hosts, exhibit morphotypes indicated as *Anisakis* Type I or Type II (*sensu* Berland, 1961), but they cannot be identified to species based on traditional morphological analysis. Among the genetic methodologies used for the species identification of *Anisakis* larvae, allozyme markers, based on several diagnostic loci, can be used to recognise the species at any of their life-history stages. In addition, among the other molecular markers used for the species detection of *Anisakis* (Mattiucci et al., 2014b), the high substitution rate of the mtDNA *cox2* sequences so far found in *Anisakis* spp. also enables identification.

Different species of *Anisakis* occur commonly in *M. merluccius*. The two most common in hake from Mediterranean and NE Atlantic waters are *Anisakis pegreffii* and *Anisakis simplex* (s. s.), respectively (Mattiucci et al., 2004; Valero et al., 2006). Statistically significant differences observed in the distribution patterns of genetically identified *Anisakis* spp. larvae have also been used as biomarkers in the stock characterisation of fish species in European waters (Mattiucci et al., 2004, 2014a). However, no detailed information has been available regarding the infection levels by different species of *Anisakis* in the viscera and flesh of hake in Mediterranean and NE Atlantic waters.

In addition to ecological aspects related to the differential distribution of *Anisakis* spp. larvae detected in this host, data on the distribution of larval *Anisakis* spp. in the edible parts of the fish, as compared with the viscera, are important, since larval *Anisakis* are etiological agents of human anisakiasis (Van Thiel et al., 1960). Over the last 30 years, there has been an increase in reported cases of human anisakiasis throughout the world. This is probably attributable to: *i*) the application of diagnostic techniques, including the molecular identification of parasites removed by gastroendoscopy (D'Amelio et al., 1999; Fumarola et al., 2009; Mattiucci et al., 2013; Umehara et al., 2007) and in surgically removed granulomas (Mattiucci et al., 2011); *ii*) the increasing global demand for seafood; and *iii*) a growing preference for raw or lightly cooked food, especially in many western countries, with the increased risk of exposure to live parasites (EFSA, 2010). So far, among the nine species of *Anisakis* detected genetically (Mattiucci et al., 2009), *A. simplex* (s. s.) and *A. pegreffii* are recognised as zoonotic species causing human anisakiasis (D'Amelio et al., 1999; Umehara et al., 2007; Mattiucci et al., 2011, 2013).

In addition to the consumer health implications, anisakid nematodes also impact fish quality, in terms of aesthetics due to their repellent appearance, should they occur in fish intended for human consumption (Karl and Levsen, 2011).

The aims of the present work were to obtain data regarding the prevalence and distribution of *Anisakis* species in the flesh of European hake caught in the Mediterranean and NE Atlantic waters and to provide epidemiological data for a risk assessment for this zoonotic parasite in sea food.

## 2. Materials and methods

### 2.1. Fish sampling and parasitological survey

A total of 130 specimens of European hake *M. merluccius* were sampled between November 2012 and May 2013 (Table 1). In particular, 65 were caught by commercial fishing net in the area FAO 27 (western Iberian Sea, ICES division IXa, 42°6′N, 9°28′W) of the NE Atlantic Ocean, while 65 specimens were caught in the area FAO 37.1.3 (Tyrrhenian Sea, 39°45′N, 14°42′E) of the Mediterranean Sea (Table 1). Fish, randomly obtained from fishing vessels at their landings from the two fishing areas, were kept in a refrigerated box until their arrival at the laboratory for parasitological examination. The temperature was monitored during the transport using a data logger, and it did not exceed 3 °C. Fish were weighed to the nearest 0.1 g and measured (total length) to the nearest 0.1 cm. The mean weight and mean length of the hake were, respectively, 329.98 ± 90.98 g and 35.95 ± 3.75 cm for the 65 specimens from area FAO 27, and 286.06 ± 110.25 g and 33.86 ± 4.07 cm for the 65 fish of FAO 37.1.2 area (Table 1).

A traditional parasitological survey was carried out for the detection of larval *Anisakis* spp. The visceral cavity, digestive tract, liver, gonads and mesenteries of each individual fresh fish were first examined under a dissecting microscope using standard parasitological procedures. Afterward, all the viscera were digested in a pepsin solution, according to the procedures reported by Llarena-Reino et al. (2013) in order to recover undetected parasites. The fish flesh was filleted and separated into the hypaxial (ventral) and epaxial (dorsal) regions, following the horizontal septum; afterwards, each part was digested separately in a pepsin solution, according to Llarena-Reino et al. (2013). Digestion was carried out in an acid solution (pH = 1.5) with HCl at 0.063 M. The assay uses liquid pepsin at a concentration of 0.5%, and a ratio of 1:10 sample weight/solution volume was used. The digestion was carried out at 37 °C, for 30 min. After digestion, the solution obtained was poured carefully through a sieve into a beaker; any *Anisakis* spp. larvae were easily detected on the sieve and collected.

All the nematodes obtained from the parasitological inspection and pepsin digestion were washed in physiological saline, counted and stored at minus 50 °C until genetic/molecular identification.

### 2.2. Multilocus allozyme electrophoresis (MAE)

Genetic identification of the larval specimens of *Anisakis* spp. collected during the parasitological survey (Table 1) was first undertaken using multilocus allozyme electrophoresis (MAE) on the frozen samples. A total of 1310 specimens were genetically identified using MAE. Each single specimen was crushed in distilled water, and a small amount of homogenized larva was separated to be further sequenced at the mtDNA *cox2* gene. Standard horizontal starch gel electrophoresis was performed at those enzyme loci, which have been proven to be

**Table 1**
Number of larval specimens of *Anisakis simplex* (s. s.), *Anisakis pegreffii*, and of F1 (first generation) hybrids between the two species, identified by allozyme loci in *Merluccius merluccius* from the two sampling areas, according to the site of infection in the host.

| | Viscera | | | Ventral flesh | | | Dorsal flesh | | |
|---|---|---|---|---|---|---|---|---|---|
| | *A. simplex* (s. s.) | *A. pegreffii* | F1 | *A. simplex* (s. s.) | *A. pegreffii* | F1 | *A. simplex* (s. s.) | *A. pegreffii* | F1 |
| FAO 27 Atlantic coast of Spain | 452 | 265 | 16 | 311 | 48 | 3 | 51 | 9 | 1 |
| FAO 37.1.2 Tyrrhenian Sea | 0 | 140 | 0 | 0 | 9 | 0 | 0 | 5 | 0 |

diagnostic for the three sibling species (Mattiucci et al., 1997, 2009, 2014a, 2014b; Mattiucci and Nascetti, 2006). These are: adenylate kinase (*Adk-2*, EC 2.7.4.3), leucine–alanine peptidase (*PepC-1*, *PepC-2*, EC 3.4.11), superoxide dismutase (*Sod-1*, EC 1.15.1.1), leucine-amino peptidase (*Lap-2*, EC 3.4.11.1) and leucine–leucine peptidase (*Pep B*, EC 3.4.11). The staining procedures used have been reported in detail by Mattiucci et al. (1997). Isozymes were numbered in order of decreasing mobility from the most anodal one. Allozymes were identified by numbers indicating their mobility (in mm, standardized conditions) relative to the most common allele, designated as *100*, found in the reference population (*i.e. A. pegreffii* from the Mediterranean Sea). The statistical significance of departures from the Hardy–Weinberg equilibrium was estimated using the $\chi^2$ test. Genetic analysis was performed using BIOSYS-2 software (Swofford et al., 1997).

### 2.3. DNA extraction, amplification and sequencing of the mtDNA cox2 gene

A certain number of the *Anisakis* spp. larvae first identified by allozymes (*i.e.* 23 specimens from the Tyrrhenian Sea and 45 from the Atlantic coast of Spain) (Table 1) were sequenced at the mtDNA *cox2* gene. The total DNA was extracted, using the cetyltrimethylammonium bromide method (CTAB), from 2 mg of homogenized tissue from each single nematode (Valentini et al., 2006). The mitochondrial cytochrome *c* oxidase subunit II (*cox2*) gene 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′) (Nadler and Hudspeth, 2000) spanning the mtDNA nucleotide position 10,639–11,248, as defined in *Ascaris suum* [GenBank X54253]. The PCR (polymerase chain reaction) was carried out using the following conditions: 94 °C for 3 min (initial denaturation), followed by 34 cycles at 94 °C for 30 s (denaturation), 46 °C for 60 s (annealing) and 72 °C for 90 s (extension), followed by post amplification at 72 °C for 10 min (Valentini et al., 2006).

The sequences obtained at the mtDNA *cox2* for those larval specimens analysed in the present study were compared with those already obtained for the same gene in the species *A. pegreffii* and *A. simplex* (s. s.) and with respect to the other species of *Anisakis*. Therefore, the following mtDNA *cox2* sequences of *Anisakis* spp., retrievable from GenBank, were used for the identification of those larval specimens previously identified by allozymes: *A. simplex* (s. s.) (DQ116426), *A. pegreffii* (DQ116428), *Anisakis berlandi* (KC809999), *Anisakis typica* (DQ116427), *Anisakis ziphidarum* (DQ116430), *Anisakis nascettii* (FJ685642), *Anisakis physeteris* (DQ116432), *Anisakis brevispiculata* (DQ116433) and *Anisakis paggiae* (DQ116434).

Phylogenetic trees were rooted using the ascaridids *A. suum* and *Toxocara canis* as outgroups, as reported in Mattiucci et al. (2014b).

### 2.4. Statistical analysis of the epidemiological data

Infection levels of *Anisakis* spp. larvae in hake were presented as prevalence (P, %) with 95% CI (determined with Sterne's method) and abundance (A), following Bush et al. (1997), Rozsa et al. (2000) and Reiczigel (2003), using the Software Quantitative Parasitology QPweb implemented for the web (Reiczigel and Rozsa, 2005). The statistical significance of the differences observed in the prevalence and abundance values of the infestation by larvae of the two different species were assessed by the Fisher's exact test and bootstrap t-test, respectively, using the Software Quantitative Parasitology QPweb (Reiczigel and Rozsa, 2005). Differences were considered significant when $p< 0.05$.

Differences in the average abundance of *A. pegreffii* between localities and between *A. simplex* (s. s.) and *A. pegreffii* in the different tissues (*i.e.* viscera and flesh) for each locality (*i.e.* Tyrrhenian Sea *versus* Atlantic coast of Spain) were evaluated by means of a Kruskal–Wallis one-way ANOVA.

Finally, the relationship between the observed abundance values by *Anisakis* spp. larvae infecting in the viscera and flesh of the hake sampled in the Tyrrhenian Sea *versus* those from off the Atlantic coast of Spain was measured by means of a linear regression analysis.

An overall MANOVA test of multivariate regression was performed to evaluate the possible dependence between the size and weight of the hake and its parasitic burden in the form of *Anisakis* spp. larvae recovered from the viscera and flesh, respectively. Raw data were fourth-root transformed and 'zero-adjusted' by adding 1 as a 'dummy variable' to all cells (Clarke and Warwick, 2001), due to the great number of uninfected individuals. The significance of the regression was given by Rao's F statistics computed from the Wilks lambda with one-tailed F-test.

The phylogenetic analysis of the sequence datasets obtained from the *Anisakis* spp. larval specimens examined was carried out by Maximum Parsimony (MP) by using PAUP* (Swofford, 2003). MP analysis was performed using the heuristic search with tree-bisection-reconnection (TBR) branch-swapping algorithm; the reliabilities of the phylogenetic relationships were evaluated using nonparametric bootstrap analysis on 1000 pseudoreplicates (Felsenstein, 1985). Bootstrap values ≥70 were considered well supported (Hillis and Bull, 1993).

## 3. Results

### 3.1. Genetic identification of Anisakis spp. larvae using MAE and sequences analysis of mtDNA cox2 gene

A total of 2990 *A. simplex* (s. l.) larvae were collected from *M. merluccius* captured in the two fishing grounds (FAO 27 IXa and FAO 37.1.2) (Table 2). A large number (1310) of the *Anisakis* spp. larvae collected were identified by allozyme electrophoresis (MAE) (Table 2). According to the alleles observed at the diagnostic loci, *i.e.* $Sod\text{-}1^{100}$, $Adk\text{-}2^{100}$, $PepB^{100}$, $PepC\text{-}1^{100}$ and $PepC2^{100}$, 476 specimens were assigned to the species *A. pegreffii*; whereas, according to the diagnostic alleles $Sod\text{-}1^{105}$, $Adk\text{-}2^{105}$, $PepB^{70}$, $PepC\text{-}1^{90}$ and $PepC2^{96}$, as indicated in Mattiucci et al. (1997, 2014a, 2014b), 814 larvae corresponded to the species *A. simplex* (s. s.) (Table 2). More specifically, 154 larvae were identified as *A. pegreffii* in hake samples from the Tyrrhenian Sea and 322 from those caught off the Atlantic coast of Spain. Furthermore, *A. simplex* (s. s.) was identified ($n = 814$) only in the hake from off the Atlantic coast of Spain. Twenty larvae collected from hake in the FAO 27 area exhibited a heterozygote genotype between *A. pegreffii* and *A. simplex* (s. s.) at all of the diagnostic allozyme loci, likely representing F1 hybrid genotypes. No hybrid genotypes were observed in larval *Anisakis* collected from hake in the Tyrrhenian Sea.

In addition, some of the specimens of *A. pegreffii* and *A. simplex* (s. s.) previously identified by allozymes (MAE) were sequenced at the mitochondrial *cox2* gene (mtDNA *cox2*). Twenty-three larvae of *Anisakis* spp. collected from hake in the Tyrrhenian and 31 from the hake of the Atlantic coast of Spain were a 99% or 100% match with the sequence at the mtDNA *cox2* gene of *A. pegreffii* previously deposited in GenBank (Mattiucci et al., 2014b). Similarly, 14 specimens of *Anisakis* spp. from hake caught of the Atlantic coast of Spain were a 99% or 100% match with the sequence deposited in GenBank for the species *A. simplex* (s. s.) at the gene mtDNA *cox2* (Mattiucci et al., 2014b). The strict consensus of the Maximum Parsimony (MP) tree (Fig. 1), inferred from the mtDNA *cox2* sequence datasets depicted all the specimens of *A. pegreffii* ($n = 54$) as forming a unique phylogenetic lineage with the sequence deposited in GenBank, and well distinct from the other phylogenetic lineages formed by different species of the *A. simplex* (s. l.) complex (Fig. 1). On the other hand, the specimens of *A. simplex* (s. s.) from Atlantic hake ($n = 14$) clustered in the same clade in the MP analysis, representing a distinct phylogenetic lineage from the other species of *Anisakis* considered (Fig. 1).

**Table 2**
Total number (*n*) of *Merluccius merluccius* examined from the two different fishing grounds, with values of mean length (expressed in millimetres) and mean weight (expressed in grams), reported with the range of larvae collected per fish ($N_{range}$), the number (*N*) of larvae of *Anisakis* collected, number ($N_{MAE}$) of larvae identified by MAE, and number (*N* mtDNA *cox2*) of larvae sequenced at mtDNA *cox2* gene.

| | *n* | Mean length (Range) | Mean weight (Range) | $N_{range}$ | *N* | $N_{MAE}$ (% of tot) | *N* mtDNA *cox2* |
|---|---|---|---|---|---|---|---|
| FAO 27 IXa Atlantic coast of Spain | 65 | 35.9 (29.0–45.0) | 329.2 (160.8–497.0) | 0–353 | 2836 | 1244 (44%) | 45 |
| FAO 37.1.2 Tyrrhenian Sea | 65 | 33.9 (26.3–47.0) | 286.0 (120.1–631.0) | 0–17 | 154 | 154 (100%) | 23 |

This finding confirmed the results achieved using allozymes with regard to the species identification of the larvae from hake, *i.e.* these specimens belong to *A. simplex* (s. s.) and *A. pegreffii* (Table 2).

### 3.2. Parasitic infection levels of A. pegreffii and A. simplex (s. s.)

Only 2 of a total of 111 fish that were infected by specimens of *Anisakis* (1.8%) presented nematodes in the flesh when the viscera were negative.

Regarding the fish sampled from the Atlantic waters (area FAO 27), only 1 of the 65 hake examined was free of *Anisakis* spp. larvae; 2 hake were parasitized by *A. simplex* (s. s.) only and 1 specimen was infected by *A. pegreffii* larvae only. The remaining 61 fish reported a mixed infection by both *A. simplex* (s. s.) and *A. pegreffii*. Furthermore, 53 fish had *Anisakis* spp. in the fillets, 35 of which had a mixed infection of both *Anisakis* species.

With regard to hake in the Tyrrhenian Sea, 47 were infected by *A. pegreffii* only and 8 of these exhibited an infection in the flesh.

Prevalence (P) with 95% CI (determined with Sterne's method) and abundance (A) estimates of infections by larvae of *A. simplex* (s. s.) and *A. pegreffii* in hake taken from the two fishing grounds are reported in Table 3. Due to the fact that *A. simplex* (s. s.) was not found in fish from the Tyrrhenian Sea, the statistical analysis within and between locations refers exclusively to the parasite burden of *A. pegreffii*.

In the hake caught off the Atlantic coast of Spain (FAO 27), *A. simplex* (s. s.) and *A. pegreffii* co-infected the same individual fish host, both in the viscera and in the flesh (Table 3). However, while no significant difference ($p = 0.36$) in the prevalence values was recorded between the two *Anisakis* species found in the viscera of the host (P = 98.5% for *A. simplex* (s. s.) and P = 93.8% for *A. pegreffii*), the abundance of *A. simplex* (s. s.) was significantly higher than that observed for *A. pegreffii* (A = 20.51 and A = 11.46, respectively, $p = 0.045$). In particular, in relation to the infestation by both *Anisakis* species in the flesh of the hake, the prevalence (P) of *A. simplex* (s. s.) was significantly higher than that of *A. pegreffii* in both ventral and dorsal fillets, being respectively P = 78.5% *vs* P = 53.8% ($p = 0.0051$) and P = 38.5% *vs* P = 10.8% ($p = 0.0004$). *A. simplex* (s. s.) was significantly more abundant in the hypaxial ventral fillet (with a value of A = 8.17 for *A. simplex* (s. s.) and A = 1.41 for *A. pegreffii*, $p = 0.0026$) (Table 3, and Fig. 1). No statistical difference ($p = 0.411$) was recorded in the abundance values for the two species in the dorsal fillet (Table 3).

*A. pegreffii* was present in hake in both the sampling areas. The mean parasite burden, in terms of abundance (F = 3.885, $p = 0.001$) differed significantly between the Atlantic and Tyrrhenian Sea samples.

Prevalence and abundance values were significantly higher in the Atlantic fish than in those from the Tyrrhenian Sea (Table 3). *A. pegreffii* larvae were significantly more common in the viscera of Atlantic hake (P = 93.8, A = 11.46) than from those in the Tyrrhenian Sea (P = 70.8, A = 2.15) (prevalence $p = 0.001$ and abundance $p = 0.002$) (Table 2). The host's ventral fillet had significantly higher levels of *A. pegreffii* in terms of both prevalence and abundance in the Atlantic as compared with the Tyrrhenian Sea (respectively, P = 53.8% *vs* P = 9.2% $p = 0.0001$; A = 1.41 *vs* A = 0.14 $p = 0.06$) (Table 3, and Fig. 1). The dorsal fillet of Tyrrhenian Sea hake showed no significant difference in the level of infection by *A. pegreffii* from that observed in the same part of the body in fish taken in the Atlantic.

Hake sampled from the Atlantic showed significant differences in abundance between the different infection sites for both *A. simplex* (s. s.) and *A. pegreffii* (one-way ANOVA, $F_{2,192} > 40$ and $p < 0.0001$ in both infections). The same trend was also observed in the average abundance of the infection by *A. pegreffii* between the different host tissues in the fish collected in the Tyrrhenian Sea (one-way ANOVA, $F_{2,192} = 53.56$, $p < 0.0001$). Fig. 3 shows the spatial distribution and relative frequencies of the two larval *Anisakis* species detected in the flesh. In the area of sympatry, the hypaxial (ventral) fillet of the hake hosted the majority of both the larvae (531) of *A. simplex* (s. s.) and (92) *A. pegreffii*, compared with the epaxial (dorsal) fillet (109 of *A. simplex* (s. s.), 19 of *A. pegreffii*).

Concerning the relationship between the parasite burden of *Anisakis* spp. and fish size, the overall MANOVA test showed a significantly positive correlation between the body length of the hake and the level of infection ($p = 1.71E-06$), whereas no correlation was found between infection level and the weight of the fish in either locality. Multivariate regression analysis showed that the relative dependence between the size of the hake and the parasite burden of *Anisakis* spp. (overall MANOVA, F = 4.912, $p < 0.0001$) was mainly related to host length (Wilks' lambda = 0.64, $p < 0.0001$). The abundance of *Anisakis* (*A. simplex* (s. s.) plus *A. pegreffii*) in the viscera of the Atlantic hosts reached a higher degree of correlation with respect to host size (cumulative $r^2 = 0.977$), in comparison with the abundance values observed in the flesh (cumulative $r^2 = 0.692$); the latter was mostly due to infection by *A. simplex* (s. s.) ($r^2 = 0.522$, F = 33.88, $p < 0.001$). A relationship between the burden of *A. simplex* (s. s.) in the viscera and flesh of Atlantic hake was found ($r = 0.912$) and a similar, but with a lower level of significance ($r = 0.586$), was found for *A. pegreffii*. Conversely, the same relationship did not occur for *A. pegreffii* in the different host tissues of fishes sampled in the Tyrrhenian Sea ($r = 0.018$).

## 4. Discussion

In the present survey, two species of *Anisakis* were identified as larvae in the European hake from two different fishing grounds: they are *A. simplex* (s. s.) and *A. pegreffii*. They occurred in sympatry and sintopy in fish caught in the Atlantic FAO 27 IXa area, whereas only *A. pegreffii* was detected in fish collected from the Tyrrhenian Sea. The occurrence of F1 hybrid genotypes in this sympatric area of the two species was also documented on the basis of the same genetic markers (allozymes) in this host (Mattiucci et al., 2004). Both *Anisakis* species occurred in the viscera and flesh of the fish. However, significant differences were found in the infection levels in terms of both the geographical origin of the fish (fishing grounds) and the infection site of the two *Anisakis* species. For instance, the overall parasite burden of *A. pegreffii* reported in area FAO 37.1.2 (Tyrrhenian Sea) was significantly lower than that of the same parasite species identified in *M. merluccius* from area FAO 27 IXa in the Atlantic (Table 3, Fig. 2). *A. simplex* (s. s.) infection exhibited significantly higher values than *A. pegreffii* in both the viscera and the flesh of hake (Tables 2 and 3 and Fig. 1). The higher infection levels shown by *A. simplex* (s. s.) with respect to *A. pegreffii* in the present

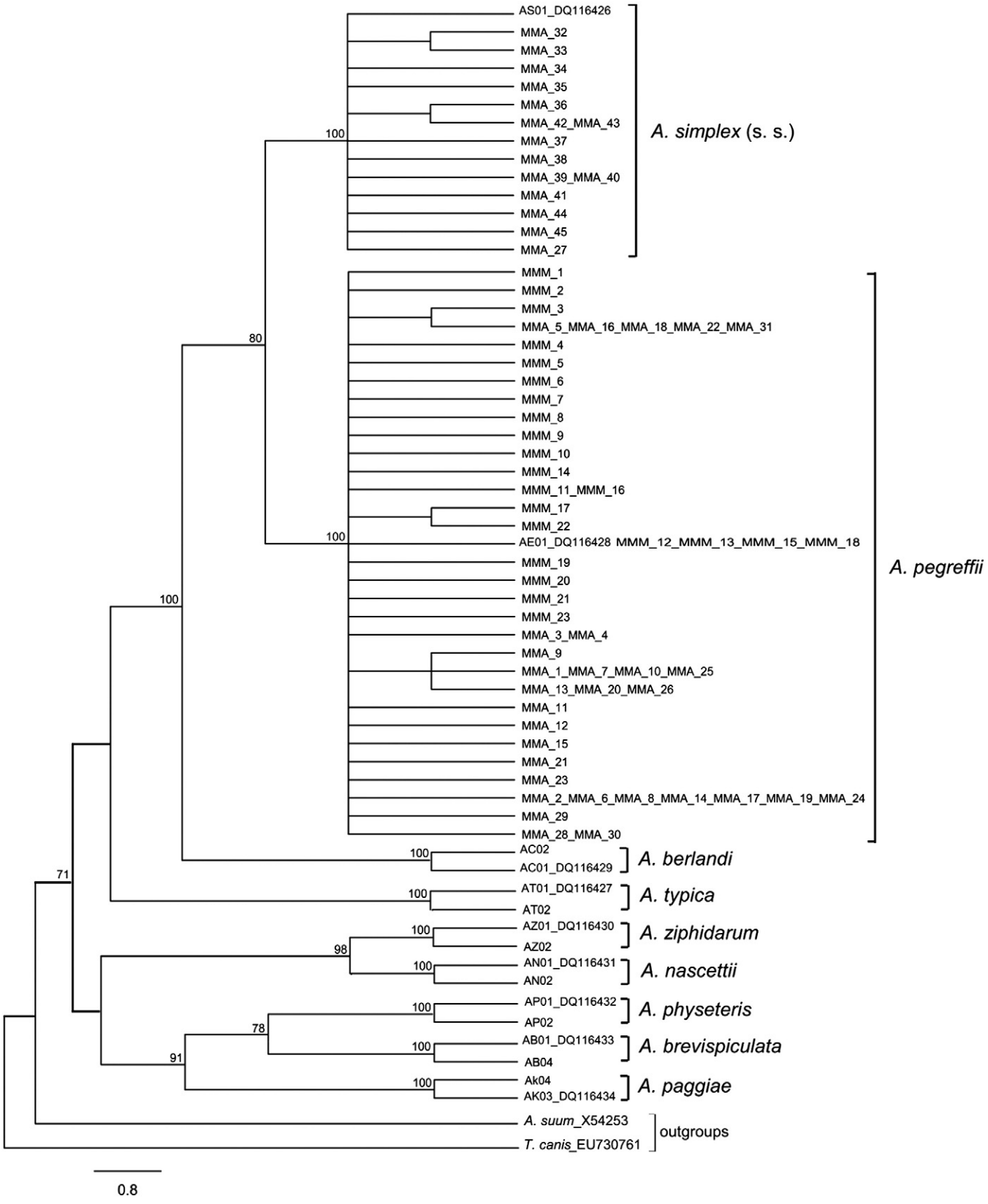


**Fig. 1.** Maximum Parsimony (MP) bootstrap consensus tree inferred by PAUP*4.0 (bootstrap method with heuristic search) (Swofford, 2003) for the *Anisakis pegreffii* and *Anisakis simplex* (s. s.) specimens collected from *Merluccius merluccius* collected in the Tyrrhenian Sea (FAO 37.1.2) and Atlantic Ocean (Spanish coast — FAO 27 IXa) sequenced at mtDNA *cox2*, with respect to the other *Anisakis* spp., previously sequenced for the same gene and deposited in GenBank. The analysis was run on 1000 pseudoreplicates; bootstrap values (≥70) are shown at the nodes. *Toxocara canis* and *Ascaris suum* were used as the outgroups.

**Table 3**
Prevalence (P) and abundance (A) infection levels of *Anisakis simplex* (s. s.) and *Anisakis pegreffii* larvae identified in *Merluccius merluccius* from the two sampling areas and according to the site of infection in the host.

| | Viscera | | | | | | Ventral flesh | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | P (%) | | $P^1$ | A | | $P^2$ | P (%) | | $P^1$ | A | | $P^2$ |
| | *A. simplex* (s. s.) | *A. pegreffii* | | *A. simplex* (s. s.) | *A. pegreffii* | | *A. simplex* (s. s.) | *A. pegreffii* | | *A. simplex* (s. s.) | *A. pegreffii* | |
| FAO 27 Atlantic coast of Spain | 98.5 (0.92–0.99) | 93.8 (0.85–0.98) | ns | 20.15 ± 29.31 | 11.46 ± 16.64 | * | 78.5 (0.67–0.87) | 53.8 (0.41–0.66) | *** | 8.17 ± 15.78 | 1.41 ± 2.42 | * |
| FAO 37.1.2 Tyrrhenian Sea | – | 70.8 (0.58–0.81) | – | – | 2.15 ± 3.04 | – | – | 9.2 (0.04–0.20) | – | – | 0.14 ± 0.50 | – |
| $P^3$ | – | *** | | – | – | | – | *** | | – | – | |
| $P^4$ | – | – | | – | * | | – | – | | – | * | |

$P^1$ = significance level (Fisher's exact test) of differences between prevalences.
$P^2$ = significance level of differences between mean abundances (bootstrap 2-sample t-test).
$P^3$ = significance level of differences (Fisher's exact test) between prevalences.
$P^4$ = significance level of differences between mean abundances (bootstrap 2-sample t-test).
ns = not significant.
*** $p < 0.001$.
* $p < 0.05$.

study generally agree with those previously reported for these two species in European hake from the same fishing grounds (Mattiucci et al., 2004), despite the fact that the latter authors did not report the infection levels in the flesh of this fish.

Previous authors (Mattiucci et al., 2014a; Suzuki et al., 2010; Valero et al., 2006) have noted that infection levels of different species of *Anisakis* vary depending on the geographical origin of the host fish. Such differences in the infection levels of *A. pegreffii* from the two fishing grounds could be related to the fact that the Atlantic coast of Spain is an area inhabited by several cetacean species, such as 'oceanic dolphins' and baleen whales, which are suitable definitive hosts for these nematodes in Atlantic waters (Mattiucci and Nascetti, 2008). Whereas, in the Mediterranean Sea, a lower level of infection by *Anisakis* has been related to higher levels of habitat disturbance, which affects the population density of the suitable intermediate and definitive hosts directly involved in the life-cycle of this parasite (Mattiucci and Nascetti, 2006, 2008).

The differential spatial distribution and relative frequencies of the two larval *Anisakis* species detected in the flesh of hake have significance in terms of food safety. This study contains the first report differentiating species of *Anisakis* from the flesh fishes (in this case hake). Previous reports concerned with infection levels of *A. simplex* (s. s.) (*i.e.* Herreras et al., 2000, in Argentinean hake *Merluccius hubbsi*, and Valero et al., 2006) gave no genetic identification of the *Anisakis* spp. larvae collected from the flesh; thus no co-infection by different *Anisakis* species was detected.

When hake were found co-infected by the two species in our study, significant greater numbers of *A. simplex* (s. s.) were observed. These findings accord with previous data from other fish host species (Karl and Levsen, 2011; Llarena-Reino et al., 2013; Levsen and Karl, 2014; Levsen and Lunestad, 2010) and confirm that most of the *Anisakis* spp. larvae penetrating the fish flesh are located in its ventral fillet, mainly in the musculature surrounding the visceral organs. However, a statistically lower significant relative proportion was recorded in the flesh for *A. pegreffii* as compared to *A. simplex* (s. s.) (Fig. 3). Indeed, *A. simplex* (s. s.) larvae (640) outnumbered (by almost 6 to 1) the average number (111) of *A. pegreffii* in the flesh. Similar findings were previously observed in *Scomber japonicus* from the Pacific coast of Japan, co-infected by the same two species of *Anisakis*, where the average number of *A. simplex* (s. s.) per fish was 12 times higher than that of *A. pegreffii* (see Suzuki et al., 2010). Moreover, the same authors also found that the penetration rate of *A. simplex* (s. s.) in agar was higher than that of *A. pegreffii*. They concluded that human anisakiasis reported in Japan (Umehara et al., 2007) is mainly caused by *A. simplex* (s. s.), because

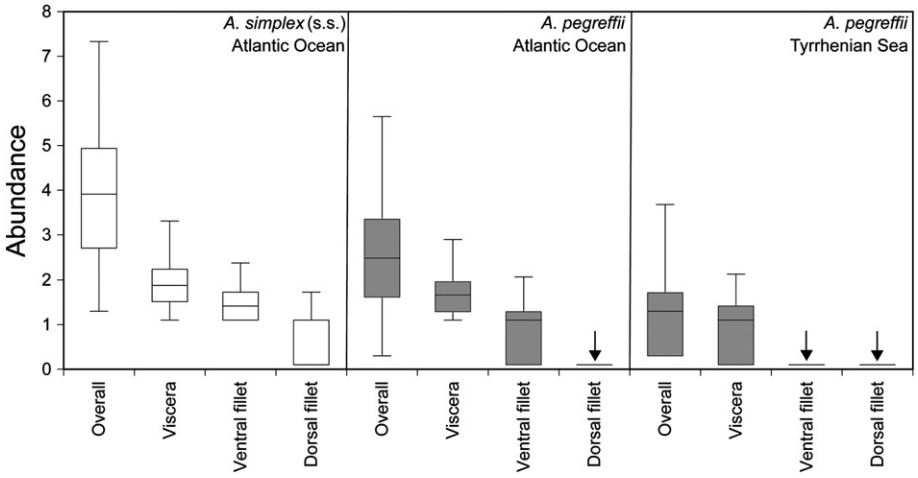


**Fig. 2.** Boxplots showing the distribution of genetically identified *Anisakis simplex* (s. s.) (white boxes) and *Anisakis pegreffii* (grey boxes) larvae (four square root transformed) in different host tissues of *Merluccius merluccius* sampled from the Atlantic area FAO 27 IXa and the Tyrrhenian Sea area FAO 37.1.3. Overall data on the "x" axis are the sum of ventral and dorsal flesh infestation; black arrows indicate extremely low values of infection. Differences for all groups were significant, with $p < 0.001$ (Kruskal–Wallis ANOVA).

| Dorsal flesh | | | | | | Total | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P (%) | | P[1] | A | | P[2] | P (%) | | P[1] | A | | P[2] |
| *A. simplex* (s. s.) | *A. pegreffii* | | *A. simplex* (s. s.) | *A. pegreffii* | | *A. simplex* (s. s.) | *A. pegreffii* | | *A. simplex* (s. s.) | *A. pegreffii* | |
| 38.5 (0.27–0.51) | 10.8 (0.05–0.21) | *** | 1.68 ± 8.08 | 0.29 ± 1.51 | ns | 98.5 (0.92–0.99) | 93.8 (0.85–0.98) | ns | 30.00 ± 44.71 | 13.17 ± 18.63 | * |
| – | 7.7 (0.03–0.17) | – | – | 0.08 ± 0.27 | – | – | 73.8 (0.62–0.83) | – | – | 2.37 ± 3.10 | – |
| – | ns | | – | – | | – | *** | | – | – | |
| – | – | | – | ns | | – | – | | – | * | |

this parasite is able to penetrate the flesh of the fish species at a greater rate than *A. pegreffii*. Similar observations have been made during experimental infection of rainbow trout and olive flounder with larvae of the same two species; indeed, larvae of *A. simplex* (s. s.) were found to have migrated to the flesh, whereas those of *A. pegreffii* remained in the body cavity (Quiazon et al., 2011). Analogously, Abattouy et al. (2011) found in the flesh only the 5.5% of the total larval *A. pegreffii* identified from *S. japonicus* caught in north Moroccan Mediterranean waters, while the remaining 94.5% infected the viscera.

Since *M. merluccius* is the most important demersal species caught off Western Europe, this study of the presence and localization of *Anisakis* (s. l.) species in this fish offers a crucial food safety device for assessing risks associated with these parasites. The fillets, as the edible part of the fish, represent the real risk to the consumer when harbouring zoonotic *Anisakis* larvae. During fish processing, the viscera and other internal organs of the body cavity of the fish are removed; however, when significant numbers of *Anisakis* larvae have migrated into the musculature, that operation alone is insufficient to reduce the potential zoonotic risk to humans, especially when fish is consumed raw or undercooked. Thus, it is important to ascertain the parasite burden of the different species of *Anisakis* in the edible parts of food fishes (*i.e.* the fillets) as compared to those not routinely consumed by humans (*i.e.* the viscera).

Data so far acquired suggest that *A. pegreffii* and *A. simplex* (s. s.) differ in their site of infection in the fish host, with *A. pegreffii* showing a lower propensity to invade the flesh. It has been also observed, in experimental infections, that at high temperatures (generally >25 °C), *Anisakis* larvae appear more likely to migrate to the flesh; however, a lower percentage of larvae migrating to the flesh was observed for *A. pegreffii* (Quiazon et al., 2011).

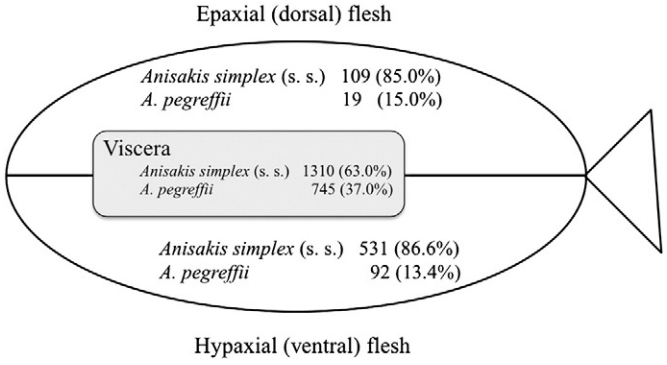


**Fig. 3.** Relative distribution of genetically identified larvae of *Anisakis simplex* (s. s.) and *Anisakis pegreffii* in the viscera and flesh of *Merluccius merluccius* caught in the Atlantic area FAO 27 IXa.

The findings of the present study should be compared with those obtained in other fish species. Indeed, it has been also suggested that the ability of *Anisakis* spp. larvae to migrate to the flesh could be related to differences in the nature of the flesh tissues in various fish species, such as the fatty acid content (Smith, 1983). The different capabilities between the two species, *A. pegreffii* and *A. simplex* (s. s.), to penetrate into the tissue of accidental hosts as humans and other vertebrates, were also observed in other studies (Arizono et al., 2012; Del Carmen Romero et al., 2013).

Our study suggests that, while *A. simplex* (s. s.) showed a significant positive correlation between the proportion of larvae in the viscera and the flesh, in the case of *A. pegreffii* this trend was not statistically significant, with always a lower percentage of larvae migrating to the flesh, despite the presence of great numbers of these larvae found in the body cavity and viscera of the hake examined. This result is consistent with similar observations reported by Karl and Levsen (2011) in grey gurnards. Indeed these authors reported in this host "*…a significantly positive relationship between both abundance and intensity of the larvae in the flesh and the viscera ($r = 0.64$ and $r = 0.59$, respectively), i.e. the number of larvae in the flesh tend to increase with increasing infection level in the viscera…*". Only 1.8% of the fish positive for *Anisakis* exhibited an infestation in the flesh and no larvae in the viscera. Karl and Levsen emphasized that fish which "*…appear to be free of nematodes in the viscera may still carry A. simplex larvae in the flesh, i.e. the larval infection level in the viscera cannot be used as reliable indicator for the approximate Anisakis burden in the flesh…*".

Thus, we highlight the importance of a larval anisakid survey which includes not only the inspection of viscera, but, more importantly, an evaluation of worm numbers in the fish flesh, as the latter represents the main threat of human infection.

In this respect, the correct genetic/molecular identification of the anisakid nematodes involved in the fish infection represents the basis for an epidemiological survey intended to identify the zoonotic species involved.

Detailed information on the distribution of different *Anisakis* species in a fish could reform inspection procedures for the fishing industry. Recent results show that current procedures, such as candling and visual inspection, are inadequate for detecting *Anisakis* larvae in the flesh of various fish species (Llarena-Reino et al., 2013; Levsen et al., 2005). Pepsin digestion does provide a valuable tool for the detection of anisakid nematodes in the fish flesh. When applied to fresh fillets, this technique does keep the parasites alive and available for identification using

genetic/molecular methodologies; it also allows the recovery of dead worms from frozen material. The only limits of pepsin digestion method are that it is time consuming and difficult to adopt for the commercial mass sampling of huge amounts of fish (European Food Safety Authority (EFSA), 2010).

The relatively low prevalence of *A. pegreffii* so far observed with respect to *A. simplex* (s. s.) in the flesh of a commercially important fish species, such as the European hake, is of public health interest. It also provides a baseline, which can be used to pursue similar studies on these anisakid parasites in other food-fish species in European waters.

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