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Anisakis sp. and *Hysterothylacium* sp. larvae in anchovies (*Engraulis encrasicolus*) and chub mackerel (*Scomber colias*) in the Mediterranean Sea: molecular identification and risk factors.

Alessia Libera **Gazzonis**[a*§], Serena **Cavallero**[b*], Sergio Aurelio **Zanzani**[a], Emanuela **Olivieri**[a,c], Renato **Malandra**[d], Valerio **Ranghieri**[e], Stefano **D'Amelio**[b], Maria Teresa **Manfredi**[a]

[a] Department of Veterinary Medicine, Università degli Studi di Milano, via Celoria 10, 20133 Milan (Italy)

[b] Department of Public Health and Infectious Diseases, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Roma (Italy)

[c] Department of Veterinary Medicine, University of Perugia, Via S.Costanzo 4, 06126 Perugia, Italy (Italy)

[d] Veterinary Department, ATS Milano Metropolitana (Italy)

[e] Veterinary practitioner, Milan (Italy)

[*] **These two Authors have contributed equally to the work.**

[§] **corresponding Author**

Abstract

Larval ascaridoids in fish destined to human consumption represent an important public health issue, besides to be an economical problem. Indeed, marine ascaridoids are the etiological agents of the fish-borne zoonosis anisakidosis. Due to an increase of new cases reported worldwide, a continuous monitoring of infection in fish is mandatory. The study was aimed to evaluate the risk of infection by larval ascaridoids in fishes from Mediterranean Sea. Two species of fishes among those representing a major potential threat for human health were selected. Epidemiological and molecular study was carried out. At Milan Fish Market, Italy, 179 anchovies (*Engraulis encrasicolus*) and 84 chub mackerels (*Scomber colias*) caught in different fishing areas in the Mediterranean Sea were sampled and inspected for the presence of larvae. For each fish, larvae were counted and morphologically identified. Predictors of infections were investigated through general linear models. A subsample of 100 larvae was molecular characterized with PCR–RFLP targeting the nuclear ribosomal internal transcribed spacer (ITS) region. Moreover, 26 *Hysterothylacium* spp. larvae were analyzed by sequencing of both nuclear ITS and mitochondrial ribosomal *rrn*S regions.

Overall, 1080 anisakids larvae were collected from 103 anchovies (P=57.5%) and 53 chub mackerels (P=63.09%). Larvae were morphologically identified as *Anisakis* Type I larvae (P=6.14% in anchovies and P=55.95% in chub mackerels) and as *Hysterothylacium* spp. (P=54.18% in anchovies and P= 13.09% in chub mackerels). Fishing area and fish weight resulted predictors of both *Anisakis* Type I land *Hysterothylacium* spp. infections in anchovies; in chub mackerels, only fishing areas resulted to be associated to both infections. Molecular analysis on ITS region identified *Anisakis pegreffii*, heterozygote genotype between *A. pegreffii* and *A. simplex* sensu stricto, and *Hysterothylacium aduncum*. Sequences analysis on *Hysterothylacium* specimens revealed a great homogeneity in *rrn*S marker, with eight variable nucleotides and an average evolutionary divergence over all sequence of 0.3%.

Keywords

Anisakis; *Hysterothylacium*; *Engraulis encrasicolus*; *Scomber colias*; risk of infection

1. Introduction

The presence of larval parasitic nematodes in fish or fish products intended for human consumption causes economic and medical problems: alongside with the loss of marketability of fish, larval nematodes belonging to Anisakidae family may cause a fish-borne zoonosis known as anisakidosis, while nematodes of Raphidascarididae family are commonly considered not zoonotic or of negligible concern (Klimpel and Palm, 2011).

These nematodes comprise a parasitic group widely distributed at geographical level, with a complex life cycle depending on aquatic ecosystem and various intermediate, paratenic and definitive hosts at different levels in the food-web (Anderson, 1992; Koie, 2001).

Humans may become accidental hosts acquiring the infection by consuming raw or lightly cooked fish and cephalopods, paratenic hosts for anisakids, infected with third-stage larvae. Considering the zoonotic potential, the relevant genera of the family Anisakidae are *Anisakis* and *Pseudoterranova*, in particular the *Anisakis simplex* and *Pseudoterranova decipiens* complexes of species, although larvae of *Contracaecum* have been rarely associated with the disease in humans (Hochberg and Hamer, 2010; Shamsi and Butcher, 2011). Larvae of *Hysterothylacium* spp. (Raphidascarididae) are not considered pathogenic for human, although members of this genus may be involved in allergic reactions due the ingestion of infected fish (Valero et al., 2003).

In Mediterranean countries, *Anisakis pegreffii* is the main etiological agent of anisakiasis, due to the widespread presence of this species in paratenic and definitive hosts of Mediterranean waters (Mattiucci and D'Amelio, 2014). Among traditional fish dishes considered to be of high risk for human disease, Spanish boquerones and Italian marinated anchovies are mentioned. In recent years, new cases of anisakiasis have been increasingly reported worldwide and it is now considered an emerging disease (Carrera et al., 2016; Mladineo et al., 2016).

Anisakidosis is considered an emerging disease in Europe, with an increasing of notified cases also in countries where the disease was sporadically reported, due to the consumption of traditional dishes and to the increasing consumption of exotic food products with raw fish (i.e. sushi, sashimi, etc.). In Italy, particularly, few cases have been reported mainly from the southern regions and associated to the consumption of raw fish (Fumarola et al., 2009; Maggi et al., 2000; Mattiucci et al., 2011; Pampiglione et al., 2002).

Therefore, a continuous monitoring of anisakid infections in fish destined to human consumption appears needed, particularly regarding certain species.

Panel of experts from the European Food Safety Authority (EFSA) released a scientific opinion on zoosanitary parasite control of fishery products for human consumption. They indicated protection and prevention as priorities and recommended a continuous research in parasites of public health

importance in fishery products, regarding prevalence, intensity, anatomical location, as well as geographical and seasonal distribution (EFSA, 2010; Pico-Duran et al., 2016).

Following the EFSA guidelines (2010), the study was aimed to evaluate the risk of infection by larval ascaridoids in fishes from Mediterranean Sea. For the present survey, fish originating from different areas of Mediterranean Sea were collected at Milan Fish Market, thus depicting an example of fish consumed in Northern Italy. Among fishes representing a major potential threat for human health, two species were selected: anchovies and chub mackerels. In particular, anchovies are often consumed raw in Italian regions. Further, these are among the most commonly consumed fish in Italy, representing 23% of the national fishery production (data of Ministry of Agricultural Food and Forestry Policies); in some Italian regions anchovies are often prepared cured or marinated and their consumption is assumed as the major cause of anisakiasis in Italy (Mattiucci and D'Amelio, 2014). Chub mackerel is a pelagic-neritic fish and it is recognized as one of the species more at risk of infection by anisakids, being at the top of the trophic chain in Mediterranean Sea (Piras et al., 2014). In the present study, epidemiological study and molecular identification were carried out in order to analyze the risk factors that may influence the infection in fish and infer the human risk for anisakiasis posed by the consumption of the surveyed fishes.

2. Material and methods

2.1 Fish sampling and visual inspection

A total of 179 anchovies (*Engraulis encrasicolus*) and 84 chub mackerels (*Scomber colias*) were sampled at Milan Fish Market, between April and December 2014. Fish originated from Adriatic and Tyrrhenian Seas: specifically, anchovies were caught in Tyrrhenian Sea (FAO zone 37.1.3) and Adriatic Sea (FAO zone 37.2.1, FAO zone 37.2.2), whereas mackerels came from the Adriatic Sea (FAO zone 37.2.2).

Each fish was measured, weighted and submitted to inspective analysis for the presence of nematodes larvae. Third stage larvae of nematode ascaridoids were isolated from the visceral surface and body cavity of the fresh fish; larvae encysted in fillets were carefully removed. The visceral organs were separated and then carefully observed with a stereomicroscope. Collected larvae were washed with saline solution and stored in 70% ethanol until further examination. For each specimen and irrespectively of the localization in the fish body, larvae were counted and identified according to their morphological features by a light microscope at 100 or 400× magnification (Hurst, 1984; Petter, 1969).

2.2 Statistical analysis

Epidemiological parameters including prevalence, intensity, abundance and the parameter k of the negative binomial distribution were calculated for *Anisakis* spp. and *Hysterothylacium* spp. larvae recorded in both anchovies and chub mackerels (Bush et al., 1997; Wilson et al., 2001). Pearson's chi-square was used to test for the difference between prevalence values for both larval genera. General linear models (GLMs) with binomial negative distribution and logarithmic link were performed separately for anchovies and chub mackerels to investigate on predictors of *Anisakis* and *Hysterothylacium* infections, using the number of larvae as independent variable. For chub mackerels, a second GLM was run to verify the influence of considered variables on the number of *Anisakis* larvae found encysted in the fillet.

The following explanatory variables were inserted in each full model: fish body length (continuous variable, measured in centimetres), fish weight (variable, measured in grams), and fishing area; in addition, the interactions between length/weight and fishing area were considered. Final models were developed by backward elimination. Statistical analysis was performed with SPSS 22.0 software (IBM, Chicago, IL).

2.3 Molecular identification of species

2.3.1 PCR-RFLP

A selected subsample of 100 third stage larvae of ascaridoids (80 from anchovies and 20 from chub mackerels), randomly selected, were characterized at genetic level using a molecular approach based on PCR–RFLP of the nuclear ribosomal internal transcribed spacer (ITS) region, since it is informative for taxonomic/diagnostic purposes (Abollo et al., 2003; D'Amelio et al., 2000; De Liberato et al., 2013; Pontes et al., 2005). Genomic DNA was isolated from entire larvae using the Wizard Genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer's protocol.

The entire ITS region (ITS-1, 5.8S, ITS-2), of around 1000 base pairs, was amplified using 20ng of template DNA, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2 (Bioline), 40 mM of nucleotide mix (Promega), 50 pmol/µl of NC5 primer forward (5-GTAGGTGAACCTGCGGAAGGATCAT-3) and NC2 reverse primer (5-TTAGTTTCTTCCTCCGCT-3) (Zhu et al., 2000), and 1.0 U of BIOTAQ DNA Polymerase (Bioline) in a final volume of 50µl. PCR was carried out using the following parameters: 10 min at 95°C, thirty cycles of 30 s at 95°C, 40 s at 52°C and 75 s at 72°C, with a final extension of 7 min at 72°C. A negative control was included in each amplification. Aliquots of individual PCR products were separated by electrophoresis using agarose gels (1%), stained with GelRed (25 µg/ml) and detected by the use of ultraviolet transillumination. Gel images were captured electronically and analyzed using Bio-Rad's Image Lab software.

The two endonucleases *Hinf*I and *Hha*I were used to digest positive amplicons in order to identify larval nematodes at species level. Digestions were performed with incubations of three and half hours at 37°C. The fragments obtained were separated by 2% agarose gel electrophoresis, visualized as above and the sizes were determined by comparison with a 100 bp DNA ladder marker (Promega).

2.3.2 Sequences analyses

Twenty-six third stage larvae belonging to *Hysterothylacium* genus were analyzed by sequencing of both nuclear and mitochondrial ribosomal regions ITS and *rrn*S, respectively. The *rrnS* mitochondrial gene were amplified using the primers MH3 (forward: 5'-TTGTTCCAGAATAATCGGCTAGACTT-3') and MH4 (reverse: 5'-TCTACTTTACTACAACTTACTCC-3') (Abollo et al., 2003). The amplification was performed using the same protocol condition mentioned before and the following thermal profile: 10 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, and a final elongation step of 7 min at 72°C.

Positive amplicons of *Hysterothylacium* spp. were purified for sequence analyses using SureClean (Bioline), following the manufacturer's instructions. The pellets were sequenced by MWG Eurofins DNA external service.

Nuclear ribosomal sequences ITS belonging to *Hysterothylacium* genus retrieved from Genbank were selected for phylogenetic comparisons to sequences obtained in the present survey. Accession numbers and specimens codes are available in Table 1. *Anisakis simplex* s.l. was selected as outgroup for phylogenetic analysis with ITS (KM273046). Electropherograms were manually checked using Trace implemented in MEGA6 (Tamura et al., 2011), software used also to align mitochondrial sequences obtained for *rrn*S region. Web-PRANK tool (Loytynoja and Goldman, 2005) was used to align nuclear ribosomal ITS region and three distinct datasets were generated in order to better decipher polymorphisms: DATASET1 with specimens from the present study and all retrievable *Hysterothylacium* spp GenBank sequences; in the DATASET2 an outgroup was added; DATASET3 included only *Hysterothylacium* previously reported from the Mediterranean basin together with sequences here obtained. Distance-based phylogenetic tree were generated using the Neighbor-Joining method with 1000 bootstrap pseudoreplications to infer node support at branches. Lastly, representative sequences of partial ITS were used to run the BLAST search tool, in order to confirm species identity.

Mitochondrial ribosomal marker *rrn*S was investigated at intraspecific level, due to the lack of retrievable sequences from other *Hysterothylacium* species for comparison.

3. Results

3.1 Parasitic infection

Anchovies had an average length of 11.4 cm±0.9 standard deviation (SD) and weighted 15.3 gr (±5.1 SD). Chub mackerels' mean size was 20.7 cm (±3.6 SD) and weighted 129.7gr (±84.7 SD).

A total of 103 anchovies (57.5%) and 53 chub mackerels (63.09%) resulted infected by third stage larvae of nematode ascaridoids and an overall of 1080 larval nematodes was collected. Larvae were identified as *Anisakis* Type I larvae (15 in anchovies and 697 in chub mackerels) and as *Hysterothylacium* spp. (326 in anchovies and 42 in chub mackerels). *Anisakis* prevalence resulted to be higher in chub mackerels (55.95%) than in anchovies (6.14%) (Pearson's chi-square, *p*-value=0.0001); on the contrary, the prevalence of *Hysterothylacium* sp. was higher in anchovies (54.18%) than in chub mackerels (13.09%) (*p*-value=0.0001). A small number of fish resulted to be infected by both *Anisakis* Type I and *Hysterothylacium* spp. larvae (P=2.79% and P=5.95% in anchovies and mackerels, respectively). In 13 infected chub mackerels caught in Southern Adriatic Sea, *Anisakis* Type I larvae were also found encysted in the fillets. These fish were heavier (mean weight= 283.3 gr) and longer (mean length= 26.5 cm) than the overall of sampled chub mackerels. On the contrary, no anchovies showed migrated larvae in the fillet (Table 2 and 3).

Different epidemiological values were registered according to the fishing area: higher prevalence of both *Anisakis* Type I larvae and *Hysterothylacium* spp. infection were registered in anchovies from Adriatic Sea in comparison to Tyrrhenian Sea. Chub mackerels from Southern Adriatic Sea showed higher prevalence by *Anisakis* Type I than fish from Middle Adriatic Sea, whereas higher abundance of *Hysterothylacium* spp. infection was recorded in fish from Middle Adriatic Sea if compared to fish from Southern Adriatic Sea. For both anchovies and mackerels, k parameter was calculated, describing a binomial negative distribution of larvae for both *Anisakis* Type I larvae and *Hysterothylacium* spp. (Table 2 and 3). However, the k values observed were different according to parasite and host species, with a very low value observed for *Anisakis* Type I from chub mackerel, probably due to the high number of *Anisakis* larvae detected in this host.

Data resulting from risk factors analysis obtained in the final models are shown in Table 4 (anchovies) and Table 5 (chub mackerels). Concerning anchovies, the fishing area was predictor of infection for both *Anisakis* Type I and *Hysterothylacium* sp. Fish from Adriatic Sea resulted to be more at risk of infections than those from Tyrrhenian Sea. The risk of both *Anisakis* Type I and *Hysterothylacium* sp. infections increased with fish weight.

As regards chub mackerels, only the variable "fishing area" was retained in the final model; it is interesting to notice that fish caught in Southern Adriatic Sea resulted to be at higher risk of

Anisakis Type I infection and at lower risk of *Hysterothylacium* spp. infection in comparison to those caught in Middle Adriatic Sea. The model run only on data concerning *Anisakis* larvae in the fillet, confirmed that the risk of infection was associated to the increase of fish weight.

3.2 Molecular identification of species

3.2.1 PCR-RFLP

PCR amplification of the ITS in *Anisakis* spp. produced a fragment of about 960 bp while in the raphidascaridid *Hysterothylacium* sp. produced a fragment of about 1100bp. Among the 100 specimens processed, 55 gave successful amplification. Amplicons were subsequently submitted to RFLP and three taxonomic units were identified: *Anisakis pegreffii* (17 specimens from eight anchovies and nine specimens from nine chub mackerels), hybrid genotype between *A. pegreffii* and *A. simplex* sensu stricto (two specimens from two anchovies and one specimen from a chub mackerel), and *Hysterothylacium aduncum* (22 specimens from 18 anchovies and four specimens from two chub mackerels).

3.2.2 Hysterothylacium spp. *sequences analyses*

About the 26 specimens sequenced for ITS, nine isolates gave usable results for comparisons. Lengths of sequences alignments used in the datasets were 528bp for DATASET1, 1625bp for DATASET2 and 780bp for DATASET3. Alignments are available as supplementary material (S1, S2, S3). Samples here analyzed showed a low level of polymorphism, with only one variable site of an isolates showing heterozygote residue Y (C/T) in comparison to the other isolates all showing T.

The representative partial ITS sequence was compared to GenBank using BLAST showing 99% coverage and 100% of identity with several *H. aduncum* sequences (KP670310, KU306720, KT852549, KP979763, KR349114, KM272443).

The NJ tree obtained describes a well supported cluster (100% bootstrap value) including all sequences here analyzed, *H. aduncum* and *H. auctum*; and *H. fabri* as sister branch (Figure 1). *H. auctum* and *H. aduncum* sequences cluster together in the same very well supported node (95) while the differentiation between these two species is still under debate.

PCR amplification of the *rrnS* produced a fragment of about 550 bp; 16 on 26 specimens analyzed gave usable electropherograms, and entire alignment of 450bp and partial dataset of 252bp are available as supplementary material (S4). Representative *rrnS* haplotypes were deposited in GenBank under the following accession numbers: MF000685 to MF000691. The sequences analysis indicated homogeneity also in this mitochondrial marker, revealing the presence of eight variable nucleotides and an average evolutionary divergence over all sequence pairs of 0.3%.

4. Discussion

The results of the present survey confirmed the presence of anisakids in both fish species, with differences associated to the biology of the species and to the fishing area.

Particularly, in anchovies a prevalence of *Anisakis* type I of 6.14% was recorded, with low values of abundance (0.08) and intensity (1.36).

Previously published data on *Anisakis* spp. in anchovies from Mediterranean Sea reported prevalence values varying considerably among different fishing areas. Cavallero et al. (2015) found 0.5% of infected anchovies in Northern Adriatic Sea, similarly to values reported on *Anisakis* infection of 1% in Tyrrhenian Sea (De Liberato et al., 2013). In other fishing areas, on the contrary, higher prevalence values were recorded: 65% of infected anchovies, with a mean intensity of 2.8 in body cavity and 2 in muscle, were reported in Sardinia (Piras et al., 2014), and a prevalence of 81.7% was found in anchovies caught in Northern Adriatic, with high values of abundance and intensity (6.89 and 8.44, respectively) (Mladineo and Poljak, 2014). More recently, Casti et al. (2017) reported a prevalence of 25.9% in anchovies from the Gulf of Asinara, in Sardinia.

Differences in epidemiological parameters reported in literature could be attributed to different variables including different fishing areas or differences in fishing seasons and, as a consequence, differences in fish body size. Indeed, in the present study, the increasing of fish body size expressed as weight, resulted to be associated to the infection, as previously described (Mladineo and Poljak, 2014; Mladineo et al., 2012; Rello et al., 2009). Besides to differences in sampled population, different techniques used for larvae detection could also affect sensitivity, especially in the case of a low burden of infection.

Low prevalence values and especially the low burden of infection in terms of intensity and abundance may result in an underestimation of the infection. Indeed, during sanitary controls fish are randomly selected for inspection and fish infected by one or few larvae may be unnoticed.

In comparison to anchovies, chub mackerels showed a higher prevalence of *Anisakis* Type I (55%), as previously reported by Abattouy et al. (2011), that found 57% of infected chub mackerels caught in Mediterranean coast of Morocco. The prevalence values observed are lower than those reported by Piras et al. (2014), ranging from 96% to 100%. However, the average size of fish here analyzed is smaller with respect to those of the above studies. Although our data on weight or body length did not show a significant association to the infection, in chub mackerels the body size expressed as body length or weight demonstrated to be a risk factor associated to the *Anisakis* infection (Abattouy et al., 2011). Fish body weight resulted only to be associated to the presence of *Anisakis* larvae recorded in the fillet. Indeed, 15.5% of chub mackerels hosting *Anisakis* Type I larvae in

fillets had a higher average size in comparison to fish in which larvae were only found in coelomic cavity. In terms of weight and length, chub mackerels with larvae encysted in fillets were similar to fish sampled by Piras et al. (2014) that found 20.7% of chub mackerels with larvae in muscle. On the contrary, in anchovies, larvae were only found in body cavity, as previously reported for this species (Cavallero et al., 2015).

Further, the origin of chub mackerels resulted to be statistically associated to *Anisakis* Type I larvae infection, with fish caught in Southern Adriatic showing the highest prevalence, abundance and intensity (P=66.04%, A=12.45, I=18.86) when compared to fish caught in middle Adriatic (P=38.71%, A=1.19, I=3.08).

In the present survey, another parasitic nematode was found, with different patterns of infection in the investigated host species.

Although not considered pathogenic for humans, the presence of *Hysterothylacium* spp. larvae may cause depreciation of fish because of to the aesthetic problem, causing repulse from consumers (Abollo et al., 2001); a high overall prevalence (54.18%) was registered in anchovies, with a peak in fish caught in Adriatic Sea where prevalence values reaches 83.3%. Cavallero et al. (2015) reported in Northern Adriatic Sea a lower prevalence of *Hysterothylacium* spp. in anchovies (27%), combined with lower abundance and intensity values. Considering Tyrrhenian Sea, a lower prevalence (23.72%) was registered if compared to Adriatic Sea; however, the values here reported resulted higher when compared to previous data 0.7% from the same locality (De Liberato et al., 2013).

In chub mackerels, prevalence values of *Hysterothylacium* spp. infection resulted lower than in anchovies, with 13.01% of infected fish, with low values of abundance and intensity. Similar results were already reported (Madrid et al., 2016), recording a prevalence of *Hysterothylacium* sp. of 4.8% with an abundance value of 0.1 in mackerels (*Scomber scombrus*) caught in Mediterranean Sea.

It is worth noting that the fishing area of both anchovies and chub mackerels resulted associated to fish infection also in the case of *Hysterothylacium* sp.; however, differently from *Anisakis* that showed the highest burden of infection in Southern Adriatic Sea, the highest prevalence of *Hysterothylacium* sp. infection was registered in Northern and Middle Adriatic Sea.

In the present survey, different patterns of *Anisakis* and *Hysterothylacium* spp. infections were therefore registered in anchovies and chub mackerels, stating the importance of monitoring of fish species potentially representing a hazard for public health.

Furthermore, molecular analysis allowed the identification of *Anisakis* larvae at species level. PCR-RFLP identified *Anisakis* type I larvae as *A. pegreffii* and the hybrid genotype between *A. pegreffii* and *A. simplex* sensu stricto, both recorded in anchovies and chub mackerels. *A. pegreffii* was

demonstrated as a species able to cause human anisakiasis (Mattiucci et al., 2013), and even hybrids genotype between the *Anisakis simplex* sensu stricto and *A. pegreffii* have been recently discussed in terms of pathogenic potential in comparison to parental species (Arcos et al., 2014; del Carmen Romero et al., 2013).

Concerning *Hysterothylacium* spp., the sequencing of ITS region revealed homology with sequences belonging to *H. aduncum* species. Phylogenetic reconstruction showed similarity among the specimens analyzed with retrieved sequences of *H. aduncum* and *H. auctum*. The presence of *H. aduncum* in the same cluster indicated that further investigations using additional genomic regions should be performed to solve evolutionary branching pattern. Identity of the Baltic species *H. auctum* is not well resolved in the tree and it is still under debate. Moreover, sequences from congeneric species as *H. incurvum* and *H. corrugatum* and species previously reported in the same area as *H. petteri* (Mattiucci et al., 2014) were not available in Genbank and further investigations are needed in order to better understand the phylogenetic relationships and species boundaries among *Hysterothylacium* spp. Finally, analysis on *rrnS* mitochondrial marker were performed for the first time on this species adding information on its genetic background and on molecular markers potentially used for diagnostic purposes, due to the high homology revealed among specimens within species, with an average evolutionary divergence over all sequence pairs of 0.3%.

Conclusions

The spread of *Anisakis* larval infection was confirmed in fishes destined to human consumption: the chub mackerel resulted strongly infected and hosting zoonotic species. On the contrary, a lower risk of *Anisakis* infection was registered in anchovies, with no larvae found in the fillets and with differences also associated to fishing areas. Nevertheless, the frequent consumption of raw, marinated anchovies highlights that the risk for humans should not be underestimated, supporting the need of continuous survey on such fish species, combining morphologic and molecular analysis. However, a comprehensive analysis of risk factors of human anisakiasis associated to the occurrence of anisakid nematodes in fish species should be integrated by systematic data on larval migration to fillets, preservation methods as well as studies on the trend of spreading habits of raw fish consumption.

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References

Abattouy, N., Valero, A., Benajiba, M.H., Lozano, J., & Martin-Sanchez, J. (2011). *Anisakis simplex* s.l. parasitization in mackerel (*Scomber japonicus*) caught in the North of Morocco - Prevalence and analysis of risk factors. *International Journal of Food Microbiology, 150*, 136-139.

Abollo, E., Gestal, C., & Pascual, S. (2001). Anisakis infestation in marine fish and cephalopods from Galician waters: an updated perspective. *Parasitology Research, 87*, 492-499.

Abollo, E., Paggi, L., Pascual, S., & D'Amelio, S. (2003). Occurrence of recombinant genotypes of *Anisakis simplex* s.s. and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. *Infection Genetics and Evolution, 3*, 175-181.

Anderson, R.C. (1992). The Superfamily Ascaridoidea. In: Anderson, R.C. (Ed.), Nematode parasites of vertebrates: their development and transmission, pp. 253-256.

Arcos, S.C., Ciordia, S., Roberston, L., Zapico, I., Jimenez-Ruiz, Y., Gonzalez-Munoz, M., Moneo, I., Carballeda-Sangiao, N., Rodriguez-Mahillo, A., Albar, J.P., & Navas, A. (2014). Proteomic profiling and characterization of differential allergens in the nematodes *Anisakis simplex* sensu stricto and A. pegreffii. *Proteomics, 14*, 1547-1568.

Bush, A.O., Lafferty, K.D., Lotz, J.M., & Shostak, A.W. (1997). Parasitology meets ecology on its own terms: Margolis et al revisited. *Journal of Parasitology, 83*, 575-583.

Carrera, M., Gallardo, J.M., Pascual, S., Gonzalez, A.F., & Medina, I. (2016). Protein biomarker discovery and fast monitoring for the identification and detection of Anisakids by parallel reaction monitoring (PRM) mass spectrometry. *Journal of Proteomics, 142*, 130-137.

Casti, D., Scarano, C., Piras, M.C., Merella, P., Muglia, S., Piras, F., Garippa, G., Spanu, C., & De Santis, E.P. (2017). Occurrence of Nematodes of the Genus *Anisakis* in Mediterranean and Atlantic Fish Marketed in Sardinia. *Italian Journal of Food Safety, 6*, 6185.

Cavallero, S., Magnabosco, C., Civettini, M., Boffo, L., Mingarelli, G., Buratti, P., Giovanardi, O., Fortuna, C.M., & Arcangeli, G. (2015). Survey of *Anisakis* sp and *Hysterothylacium* sp in sardines and anchovies from the North Adriatic Sea. *International Journal of Food Microbiology, 200*, 18-21.

D'Amelio, S., Mathiopoulos, K.D., Santos, C.P., Pugachev, O.N., Webb, S.C., Picanco, M., & Paggi, L. (2000). Genetic markers in ribosomal DNA for the identification of members of the

391 genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based
392 restriction fragment length polymorphism. *International Journal for Parasitology, 30*, 223-226.
393 De Liberato, C., Bossu, T., Scaramozzino, P., Nicolini, G., Ceddia, P., Mallozzi, S., Cavallero, S.,
394 & D'Amelio, S. (2013). Presence of Anisakid Larvae in the European Anchovy, *Engraulis*
395 *encrasicolus*, Fished Off the Tyrrhenian Coast of Central Italy. *Journal of Food Protection, 76*,
396 1643-1648.
397 del Carmen Romero, M., Valero, A., Navarro-Moll, M.C., & Martín-Sánchez, J. (2013).
398 Experimental comparison of pathogenic potential of two sibling species *Anisakis simplex* s.s.
399 and *Anisakis pegreffii* in Wistar rat. *Tropical Medicine & International Health, 18*, 979-984.
400 EFSA, 2010. EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on risk assessment
401 of parasites in fishery products. Scientific Opinion on risk assessment of parasites in fishery
402 products. *EFSA Journal, 8*, 9.
403 Fumarola, L., Monno, R., Ierardi, E., Rizzo, G., Giannelli, G., Lalle, M., & Pozio, E. (2009).
404 *Anisakis pegreffi* Etiological Agent of Gastric Infections in Two Italian Women. *Foodborne*
405 *Pathogens and Disease, 6*, 1157-1159.
406 Guo, Y.-N., Xu, Z., Zhang, L.-P., Hu, Y.-H., & Li, L. (2014). Occurrence of *Hysterothylacium* and
407 *Anisakis* nematodes (Ascaridida: Ascaridoidea) in the tanaka's snailfish *Liparis tanakae*
408 (Gilbert & Burke) (Scorpaeniformes: Liparidae). *Parasitology Research, 113*, 1289-1300.
409 Haarder, S., Kania, P.W., & Buchmann, K. (2013). Comparative infectivity of three larval
410 nematode species in three different salmonids. *Parasitology Research, 112*, 2997-3004.
411 Hochberg, N.S., & Hamer, D.H. (2010). Anisakidosis: Perils of the Deep. *Clinical Infectious*
412 *Diseases, 51*, 806-812.
413 Hurst, R.J. (1984). Identification and description of larval *Anisakis simplex* and *Pseudoterranova*
414 *decipiens* (Anisakidae: Nematoda) from New Zealand waters. *New Zealand Journal of Marine*
415 *and Freshwater Research, 18*, 177-186.
416 Klimpel, S., & Palm, H.W. (2011). Anisakid Nematode (Ascaridoidea) Life Cycles and
417 Distribution: Increasing Zoonotic Potential in the Time of Climate Change? *Progress in*
418 *Parasitology, 2*, 201-222.
419 Knoff, M., Felizardo, N.N., Iniguez, A.M., Maldonado, A., Jr., Torres, E.J.L., Pinto, R.M., &
420 Gomes, D.C. (2012). Genetic and morphological characterisation of a new species of the genus
421 *Hysterothylacium* (Nematoda) from *Paralichthys isosceles* Jordan, 1890 (Pisces: Teleostei) of
422 the Neotropical Region, state of Rio de Janeiro, Brazil. *Memorias Do Instituto Oswaldo Cruz,*
423 *107*, 186-193.

Koie, M. (2001). Experimental infections of copepods and sticklebacks *Gasterosteus aculeatus* with small ensheathed and large third-stage larvae of *Anisakis simplex* (Nematoda, Ascaridoidea, Anisakidae). *Parasitology Research, 87*, 32-36.

Li, L., Liu, Y.Y., & Zhang, L.P. (2012). Morphological and genetic characterization of *Hysterothylacium zhoushanensis* sp nov (Ascaridida: Anisakidae) from the flatfish *Pseudorhombus oligodon* (Bleeker) (Pleuronectiformes: Paralichthyidae) in the East China Sea. *Parasitology Research, 111*, 2393-2401.

Liu, Y.Y., Xu, Z., Zhang, L-P., & Li, L. (2013). Redescription and genetic characterization of *Hysterothylacium thalassini* Bruce, 1990 (Nematoda: Anisakidae) from marine fishes in the South China Sea. *Journal of Parasitology, 99*, 655-661.

Loytynoja, A., & Goldman, N. (2005). An algorithm for progressive multiple alignment of sequences with insertions. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 10557-10562.

Madrid, E., Gil, F., Garcia, M., Debenedetti, A.L., Trelis, M., & Fuentes, M.V. (2016). Potential risk analysis of human anisakiasis through the consumption of mackerel, *Scomber scombrus*, sold at Spanish supermarkets. *Food Control, 66*, 300-305.

Maggi, P., Caputi-Iambrenghi, O., Scardigno, A., Scoppetta, L., Saracino, A., Valente, M., Pastore, G., & Angarano, G. (2000). Gastrointestinal infection due to *Anisakis simplex* in southern Italy. *European Journal of Epidemiology, 16*, 75-78.

Mattiucci, S., & D'Amelio, S. (2014). Anisakiasis. In: Bruschi, F. (Ed.), Helminth Infections and their Impact on Global Public Health. Springer-Verlag Wien.

Mattiucci, S., Fazii, P., De Rosa, A., Paoletti, M., Megna, A.S., Glielmo, A., De Angelis, M., Costa, A., Meucci, C., Calvaruso, V., Sorrentini, I., Palma, G., Bruschi, F., & Nascetti, G. (2013). Anisakiasis and gastroallergic reactions associated with *Anisakis pegreffii* infection, Italy. *Emerging Infectious Diseases, 19*, 496-499.

Mattiucci, S., Garcia, A., Cipriani, P., Santos, M.N., Nascetti, G., & Cimmaruta, R. (2014). Metazoan parasite infection in the swordfish, *Xiphias gladius*, from the Mediterranean Sea and comparison with Atlantic populations: implications for its stock characterization. *Parasite 21*.

Mattiucci, S., Paoletti, M., Borrini, F., Palumbo, M., Palmieri, R.M., Gomes, V., Casati, A., & Nascetti, G. (2011). First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. *Bmc Infectious Diseases 11*.

Mehrdana, F., Bahlool, Q.Z.M., Skov, J., Marana, M.H., Sindberg, D., Mundeling, M., Overgaard, B.C., Korbut, R., Strom, S.B., Kania, P.W., & Buchmann, K. (2014). Occurrence of zoonotic

458 nematodes *Pseudoterranova decipiens, Contracaecum osculatum* and *Anisakis simplex* in cod
459 (*Gadus morhua*) from the Baltic Sea. *Veterinary Parasitology, 205*, 581-587.
460 Mladineo, I., & Poljak, V. (2014). Ecology and Genetic Structure of Zoonotic *Anisakis* spp. from
461 Adriatic Commercial Fish Species. *Applied and Environmental Microbiology, 80*, 1281-1290.
462 Mladineo, I., Popovic, M., Drmic-Hofman, I., & Poljak, V. (2016). A case report of *Anisakis*
463 *pegreffii* (Nematoda, Anisakidae) identified from archival paraffin sections of a Croatian
464 patient. *Bmc Infectious Diseases 16*.
465 Mladineo, I., Simat, V., Miletic, J., Beck, R., & Poljak, V. (2012). Molecular identification and
466 population dynamic of *Anisakis pegreffii* (Nematoda: Anisakidae Dujardin, 1845) isolated from
467 the European anchovy (*Engraulis encrasicolus* L.) in the Adriatic Sea. *International Journal of*
468 *Food Microbiology, 157*, 224-229.
469 Pampiglione, S., Rivasi, F., Criscuolo, M., De Benedittis, A., Gentile, A., Russo, S., Testini, M., &
470 Villani, M. (2002). Human anisakiasis in Italy: A report of eleven new cases. *Pathology*
471 *Research and Practice, 198*, 429-434.
472 Pekmezci, G.Z., Yardimci, B., Onuk, E.E., & Umur, S. (2014). Molecular characterization of
473 *Hysterothylacium fabri* (Nematoda: Anisakidae) from *Zeus faber* (Pisces: Zeidae) caught off
474 the Mediterranean coasts of Turkey based on nuclear ribosomal and mitochondrial DNA
475 sequences. *Parasitology International, 63*, 127-131.
476 Petter, A.J. (1969). Survey on nematodes of fishes in the Nantes area. Identification of ascaris
477 larvae parasiting sardines (in relation with eosinophilic granuloma observed in man in the area.
478 *Annales de parasitologie humaine et comparee, 44*, 559-579.
479 Pico-Duran, G., Pulleiro-Potel, L., Abollo, E., Pascual, S., & Munoz, P. (2016). Molecular
480 identification of *Anisakis* and *Hysterothylacium* larvae in commercial cephalopods from the
481 Spanish Mediterranean coast. *Veterinary Parasitology, 220*, 47-53.
482 Piras, M.C., Tedde, T., Garippa, G., Virgilio, S., Sanna, D., Farjallah, S., & Merella, P. (2014).
483 Molecular and epidemiological data on *Anisakis* spp. (Nematoda: Anisakidae) in commercial
484 fish caught off northern Sardinia (western Mediterranean Sea). *Veterinary Parasitology, 203*,
485 237-249.
486 Pontes, T., D'Amelio, S., Costa, G., & Paggi, L. (2005). Molecular characterization of larval
487 anisakid nematodes from marine fishes of Madeira by a PCR-based approach, with evidence
488 for a new species. *Journal of Parasitology, 91*, 1430-1434.
489 Rello, F.J., Adroher, F.J., Benitez, R., & Valero, A. (2009). The fishing area as a possible indicator
490 of the infection by anisakids in anchovies (*Engraulis encrasicolus*) from southwestern Europe.
491 *International Journal of Food Microbiology, 129*, 277-281.

Shamsi, S., & Butcher, A.R. (2011). First report of human anisakidosis in Australia. *Medical Journal of Australia, 194*, 199-200.

Szostakowska, B., Myjak, P., Kur, J., & Sywula, T. (2001). Molecular evaluation of *Hysterothylacium auctum* (Nematoda, Ascaridida, Raphidascarididae) taxonomy from fish of the southern Baltic. *Acta Parasitologica, 46*, 194-201.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution, 28*, 2731-2739.

Valero, A., Terrados, S., Diaz, V., Reguera, V., & Lozano, J. (2003). Determination of IgE in the serum of patients with allergic reactions to four species of fish parasite anisakids. *Journal of Investigational Allergology and Clinical Immunology, 13*, 94-98.

Wilson, K., Bjørnstad, O.N., Dobson, A.P., Merler, S., Poglayen, G., Randolph, S.E., Read, A.F., & Skorping, A. (2001). Heterogeneities in macroparasite infections - patterns and processes. In: Hudson, P.J., Rizzoli, A., Grenfell, B.T., Heesterbeek, H., Dobson, A.P. (Eds.), The Ecology of Wildlife Diseases. OUP, pp. 6–44.

Zhu, X.Q., D'Amelio, S., Paggi, L., & Gasser, R.B. (2000). Assessing sequence variation in the internal transcribed spacers of ribosomal DNA within and among members of the *Contracaecum osculatum* complex (Nematoda : Ascaridoidea : Anisakidae). *Parasitology Research, 86*, 677-683.

513 **Figure 1**. NJ tree inferred from ITS sequences analyzed in the present paper (indicated as "ALG")
514 together with retrieved GenBank sequences from related *Hysterothylacium* species and one
515 outgroup (DATASET2). Bootstrap support is indicated at nodes.

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517

518 **Supplementary material**

519 Table 1 - Data about *Hysterothylacium* spp. nuclear ribosomal ITS sequences retrieved from
520 GenBank and used for comparative purposes. Parasitic species, Genbank accession number,
521 geographical origin of sample, host species and references are available.

Nematode species	**Genbank accession number**	**Geographical origin of sample**	**Host species**	**Reference**
H. liparis	KF601900	China	*Liparis tanakae*	(Guo et al., 2014)
H. zhoushanensis	JX028282	East China Sea	*Pseudorhombus oligodon*	(Li et al., 2012)
H. auctum	AF115571	South Baltic Sea	*Zoarces viviparus*	(Szostakowska et al., 2001)
H. aduncum	JX845137	Denmark: North Sea	Salmonids	(Haarder et al., 2013)
H. aduncum	KP670310	Adriatic sea	*Engraulis encrasicolus*	(Cavallero et al., 2015)
H. tetrapteri	KF601901	"chinese waters"	"marine fishes"	Li et al., unpublished
H. fabri	KC852206	Egypt	*Zeus faber*	(Pekmezci et al., 2014)
H. thalassini	JX982129	China	*Priacanthus macracanthus*	(Liu et al., 2013)
H. deardorffoverstreetorum	JF730204	Brazil	*Paralichthys isosceles*	(Knoff et al., 2012)
H. bidentatum	AY603539	-	-	Kijewska et al., unpublished
H. longilabrum	JQ520159	South China Sea	marine fishes	(Li et al., 2012)
H. rigidum	HF680324	Ireland: Porcupine Bank	*Lophius piscatorius*	Canas et al., unpublished
Anisakis simplex (outgroup)	KM273046	Baltic Sea	*Gadus morhua*	(Mehrdana et al., 2014)

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524 Table 2 - Parameters of parasitization by *Anisakis* type I and *Hysterothylacium* spp. in anchovies from different fishing areas: number of infected
525 hosts, number of larvae recovered, epizootiological parameters (prevalence, mean intensity, mean abundance, k index of aggregation).

Fishing area	**overall**		**North Tyrrhenian Sea**		**Northern Adriatic Sea**		**Southern Adriatic Sea**	
Examined fishes								
Weight: mean (SD)	15.27 (5.14)		18.71 (7.33)		14.29 (2.15)		12.88 (1.89)	
Length: mean (SD)	11.41 (0.96)		11.9 (1.33)		11.35 (0.59)		11 (0.53)	
N	179		59		60		60	
Parasite	***Anisakis* type I**							
	overall		**North Tyrrhenian Sea**		**Northern Adriatic Sea**		**Southern Adriatic Sea**	
	overall	incisted	overall	incisted	overall	incisted	overall	incisted
Infected fishes								
Weight: mean (SD)	17.03 (5.09)	-	27.1 (2.26)	-	14.45 (0.36)	-	15.08 (1.16)	-
Length: mean (SD)	11.83 (0.82)	-	13.3 (0.42)	-	11.47 (0.29)	-	11.54 (0.52)	-
N	11	0	2	0	4	0	5	0
N larvae	15	0	3	0	4	0	8	0
Prevalence (95% CI)	6.14 (3.47-10.67)	0 (0-2.1)	3.38 (0.93-11.54)	0 (0-6.11)	6.67 (2.62-15.93)	0 (0-6.02)	8.33 (3.61-18.06)	0 (0-6.02)
Mean Intensity (SD) (Range)	1.36 (0.674) (1-3)	-	1.5 (0.707) (1-2)	-	1 (0) (1-1)	-	1.6 (0.894) (1-3)	-
Mean Abundance (SD)	0.08 (0.365)	-	0.05 (0.289)	-	0.07 (0.252)	-	0.13 (0.503)	-
k parameter	0.56	-	-	-	-	-	-	-
Parasite	***Hysterothylacium* spp.**							
	overall		**North Tyrrhenian Sea**		**Northern Adriatic Sea**		**Southern Adriatic Sea**	
	overall	incisted	overall	incisted	overall	incisted	overall	incisted
Infected fishes								
Weight: mean (SD)	14.52 (3.37)	-	17.35 (6.75)	-	14.56 (2.2)	-	13.25 (1.72)	-
Length: mean (SD)	11.34 (0.71)	-	11.64 (1.22)	-	11.44 (0.6)	-	11.08 (0.48)	-
N	97	0	14	0	50	0	33	0
N larvae	326	0	16	0	199	0	111	0
Prevalence (95% CI)	54.18 (46.88-61.32)	0 (0-2.1)	23.72 (14.7-35.98)	0 (0-6.11)	83.33 (71.96-90.68)	0 (0-6.02)	55 (42.49-66.91)	0 (0-6.02)
Mean Intensity (SD) (Range)	3.36 (3.345) (1-20)	-	1.14 (0.363) (1-2)	-	3.98 (3.711) (1-20)	-	3.36 (3.111) (1-11)	-
Mean Abundance (SD)	1.82 (2.976)	-	0.27 (0.52)	-	3.32 (3.698)	-	1.85 (2.845)	-
k parameter	0.044	-	-	-	-	-	-	-

526 Table 3 - Parameters of parasitization by *Anisakis* type I and *Hysterothylacium* spp. in chub mackerels from different fishing areas: number of
527 infected hosts, number of larvae recovered, epizootiological parameters (prevalence, mean intensity, mean abundance, k index.

	overall		**Middle Adriatic Sea**		**Southern Adriatic Sea**	
Examined fishes						
Weight: mean (SD)	129.68 (84.75)		81.41 (44.94)		157.91 (89.99)	
Length: mean (SD)	20 (3.58)		18.38 (2.34)		22.04 (3.5)	
N	84		31		53	
Parasite	***Anisakis* type I**					
	overall		**Middle Adriatic Sea**		**Southern Adriatic Sea**	
	overall	encysted	overall	encysted	overall	encysted
Infected fishes						
Weight: mean (SD)	162.68 (96.23)	283.35 (82.37)	108.69 (62.45)	-	181.2 (99.43)	283.35 (82.37)
Length: mean (SD)	22.21 (3.64)	26.56 (1.95)	20.01 (2.82)	-	22.97 (3.62)	26.56 (1.95)
N	47	13	31	0	35	13
N larvae	697	166	37	0	660	166
Prevalence (95% CI)	55.95 (45.3-66.07)	15.48 (9.28-24.7)	38.71 (23.73-56.18)	0 (0-11.03)	66.04 (52.6-77.31)	24.53 (14.93-37.57)
Mean Intensity (SD)	14.83 (24.279)	12.76 (8.86)	3.08 (2.193)	-	18.86 (27.028)	12.76 (8.86)
(Range)	(1-111)	(2-35)	(1-8)		(1-111)	(2-35)
Mean Abundance (SD)	8.3±19.533	1.98±5.739	1.19±2.024	-	12.45±23.642	1.97±5.73
k parameter	0.0004	-	-	-	-	-
Parasite	***Hysterothylacium* spp.**					
	overall		**Middle Adriatic Sea**		**Southern Adriatic Sea**	
	overall	encysted	overall	encysted	overall	encysted
Infected fishes						
Weight: mean (SD)	101.55 (69.04)	-	105.12 (76.67)	-	85.5 (2.96)	-
Length: mean (SD)	19.34 (3.34)	-	19.64 (3.66)	-	18± (0.42)	-
N	11	0	9	0	2	0
N larvae	42		39		3	
Prevalence (95% CI)	13.09 (7.48-21.95)	0 (0-4.37)	29.03 (16.09-46.59)	0 (0-11.03)	3.77 (1.04-12.75)	0 (0-6.76)
Mean Intensity (SD)	3.82 (4.956)	-	4.33 (5.385)	-	1.5 (0.707)	-
(Range)	(1-18)		(1-18)		(1-2)	
Mean Abundance (SD)	0.5 (2.154)	-	1.26 (3.425)	-	0.06 (0.305)	-
k parameter	0.111	-	-	-	-	-

528 Table 4 - Risk factors analysis for *Anisakis* Type I and *Hysterothylacium* spp. infections in
529 anchovies according to multivariate analysis. Prevalence (P%), coefficients (β), standard error (S.E.)
530 of the coefficients, test statistic (the Wald statistic), degrees of freedom (d.f.), the odds ratio (OR) of
531 an event occurring with 95% confidence interval (CI), and are given for each variable.

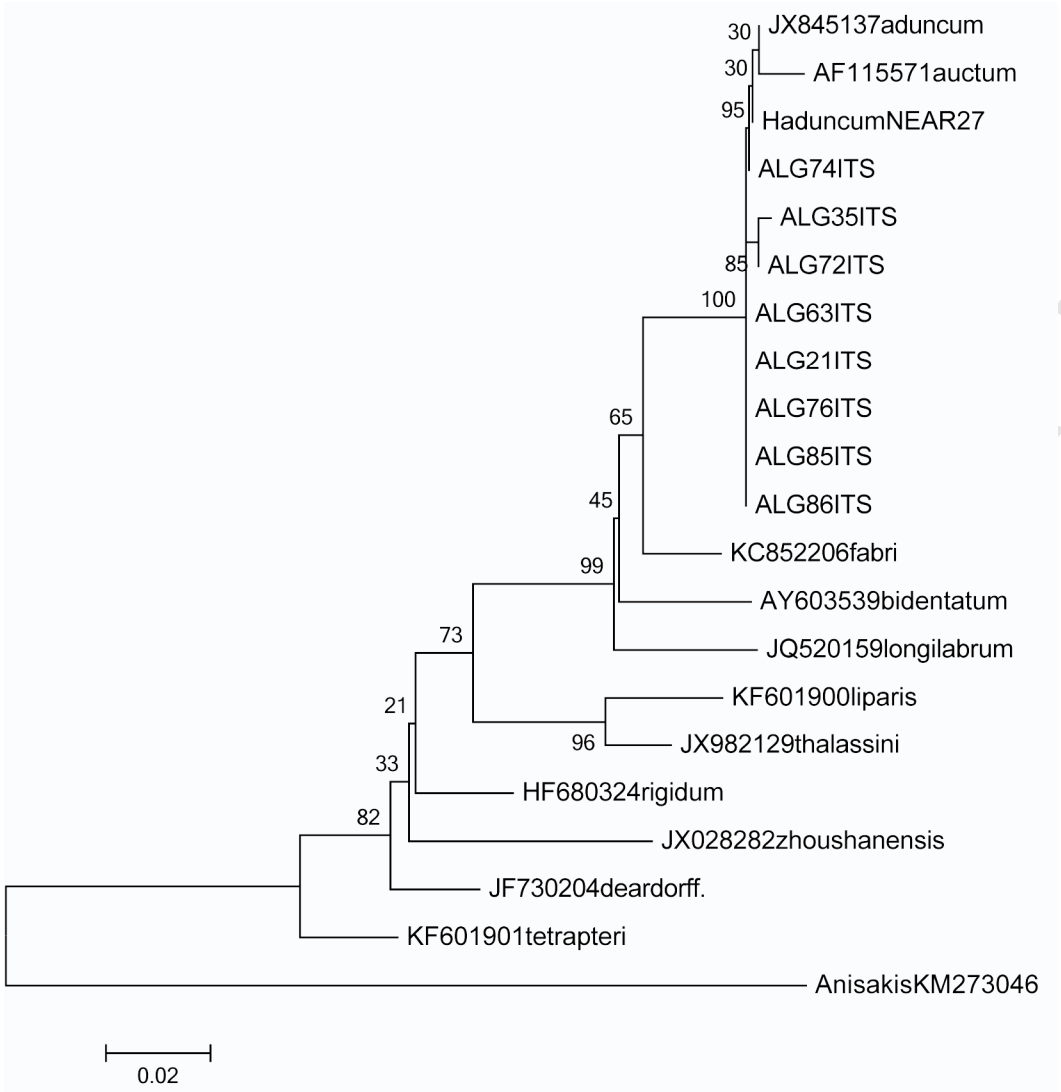
Parasite	Variable	P%	β±S.E.	Wald statistic	d.f.	OR (95% CI)	*p*-value
Anisakis	Intercept		-11.414±3.527				0.0001
	Fishing area			7.189	2		0.027
	Northern Tyrrhenian Sea (reference)	3.38	0			1	
	Northern Adriatic Sea	6.67	3.579±1.698	4.442		35.834 (1.285-999.175)	0.035
	Southern Adriatic Sea	8.33	4.719±1.8341	6.619		112.034 (3.077-4079.146)	0.01
	Fish weight		0.342±0.1266	7.301	1	1.408 (1.099-1.805)	0.007
Hysterothylacium	Intercept		-2.619±0.6997	14.009			0.0001
	Fishing area			59.741	2		0.0001
	Northern Tyrrhenian Sea (reference)	23.72	0			1	
	Northern Adriatic Sea	83.33	2.785±0.3624	59.059		16.197 (7.961-32.952)	0.0001
	Southern Adriatic Sea	55	2.311±0.3921	34.735		10.086 (4.677-21.753)	0.0001
	Fish weight		0.068±0.0315	4.684	1	1.071 (1.006-1.1389)	0.03

532
533

534 Table 5 - Risk factors analysis for *Anisakis* Type I and *Hysterothylacium* spp. infections in chub
535 mackerels according to multivariate analysis. Prevalence (P%), coefficients (β), standard error (S.E.)
536 of the coefficients, test statistic (the Wald statistic), degrees of freedom (d.f.), the odds ratio (OR) of
537 an event occurring with 95% confidence interval (CI), and are given for each variable.

Parasite	Category	P%	β±S.E.	Wald statistic	d.f.	OR (95% CI)	*p*-value
Anisakis	Intercept		2.522±0.1428				0.0001
	Fishing area			69.025	1		0.0001
	Middle Adriatic Sea	38.71	-2.345±0.2823	69.025		0.096 (0.055-0.167)	0.0001
	Southern Adriatic Sea (reference)	66.04	0			1	
Anisakis (incisted)	Intercept		-3.828±0.5035				0.0001
	Fish weight		0.021±0.0024	76.086	1	1.021 (1.016-1.026)	0.0001
Hysterothylacium	Intercept		-2.872±0.5935				0.0001
	Fishing area			23.452	1		0.0001
	Middle Adriatic Sea	29.03	3.101±0.6404	23.452		22.226 (6.335-77.976)	0.0001
	Southern Adriatic Sea (reference)	3.77	0			1	

538



Highlights

Larval ascaridoids in anchovies and chub mackerels from Mediterranean Sea were investigated

Anisakis Type I and *Hysterothylacium* spp. were identified in both fish

Molecular analysis identified *A. pegreffii*, hybrid genotype (A. pegreffi/ *A. simplex* s. s.) and *H. aduncum*

Novel information on *rrnS* mitochondrial gene of *H. aduncum* was achieved

Both fishes represented a sanitary risk for consumers.