Parasitology International 64 (2015) 222–228

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Molecular identification of *Hysterothylacium aduncum* specimens isolated from commercially important fish species of Eastern Mediterranean Sea using mtDNA *cox1* and ITS rDNA gene sequences

Emre Keskin [a,*], Cafer Erkin Koyuncu [b], Ercument Genc [a]

[a] Department of Fisheries and Aquaculture, Agricultural Faculty, 06110 Diskapi, Ankara, Turkey
[b] Fish Diseases Lab., Department of Aquaculture, Fisheries Faculty, Mersin University, 33169 Yenisehir, Mersin, Turkey

ARTICLE INFO

Article history:
Received 23 May 2014
Received in revised form 7 December 2014
Accepted 21 December 2014
Available online 24 December 2014

Keywords:
Hysterothylacium aduncum
mtDNA *cox1*
ITS rDNA
Sparus aurata
Diplodus vulgaris
Solea solea

ABSTRACT

The presence of a Raphidascarid parasitic nematode *Hysterothylacium aduncum* (Rudolphi, 1802) in two sparid fish (*Sparus aurata* and *Diplodus vulgaris*) and one soleid fish (*Solea solea*) was investigated in this study. A total of 868 individuals; 385 *S. aurata*, 437 *D. vulgaris* and 46 *S. solea* were collected from the Mersin Bay between February 2013 and January 2014 and examined. Variations in the prevalence, mean intensity, and mean abundance of the parasite were 14.55%, 2.05, and 0.30 for *S. aurata*, 4.12%, 2.44, and 0.10 for *D. vulgaris*, and 15.22%, 3.29, and 0.50 for *S. sole* respectively.

Nucleotide sequences of 1398 base pair long fragment of 18S rRNA-ITS1-5.8S rRNA-ITS2-28S rRNA region and 641 base pair long fragment of mtDNA cytochrome c oxidase I (*cox1*) gene were used in molecular identification of isolated parasites at species level. All the parasite samples were identified as *H. aduncum* based on nucleotide sequence comparisons. Both ITS rDNA and mtDNA *cox1* sequences revealed a genetic variation among *H. aduncum* specimens isolated from different fish species, while only mtDNA *cox1* sequences were indicating a mean genetic distance of 0.010 among *H. aduncum* specimens of the same host species.

1. Introduction

The phylogenetic studies based on molecular markers divide nematodes into two classes: Enoplea and Rhabditea. The genus *Capillaria* and *Pseudocapillaria* are known as marine fish parasitic species and found in the class of Enoplea, subclass of Dorylaimia. The heteroxenous marine ascaridoid nematodes (use intermediate hosts) such as *Anisakis*, *Hysterothylacium* and *Pseudoterranova* are classified under the Rhabditia subclass [1,2].

Hysterothylacium is one of the common genera in the family Raphidascarididae. *Hysterothylacium* has been found to be a valid genus which now includes some species previously considered as members of the junior synonym *Thynnascaris*. These species were considered members of *Contracaecum* in the past [3].

Hysterothylacium is usually found in benthic and pelagic fishes [4]. The adult stages are normally found in the alimentary canal of marine fishes and also reported from the freshwater fishes [5,6]. Third stage larvae have been found encapsulated in the mesentery and viscera. Fourth-stage larvae were found in the intestinal canal and also free in the abdominal space. Infected fish act as transport hosts [2,7].

In the literature, *Hysterothylacium aduncum* has been reported from the Northeastern Atlantic [8,9], the Mediterranean Sea [10–18], the Black Sea [19], the Adriatic Sea [20], the Pacific and Atlantic waters of North America [21,22], Northwest Pacific, [23] and the Pacific Ocean costs of Japan [24].

On the subject of molecular characterization of Raphidascarid *Hysterothylacium*, Martín-Sánchez et al. [25] provided valuable results at the taxonomic level. Klimpel et al. [1], Amor et al. [26], and Farjallah et al. [11] determined population-specific patterns with the different localities off the Tunisian coasts. Vardić Smrzlić et al. [17] provided some data on the molecular characterization of Anisakidae larvae from Adriatic Sea and recently Guo et al. [27] used molecular methods by sequencing and analyzing the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA). The improvements in molecular parasitology have been reviewed by Mattiucci and Nascetti [28] but there are still very limited molecular studies that confirm the molecular identification of the *H. aduncum* at species level, other than data based on ITS rDNA sequences.

Therefore, this study was designed to investigate the *H. aduncum* infections and to describe the molecular identification of *H. aduncum* by sequencing the region spanning the ITS1, the 5.8S and the ITS2 of nuclear DNA and cytochrome c oxidase I region, known as the DNA barcode, in two sparid fish species, *Sparus aurata* (gilthead sea bream) and *Diplodus vulgaris* (two banded bream), and one soleid fish species,

* Corresponding author. Tel./fax: +90 3125961722.
E-mail address: keskin@ankara.edu.tr (E. Keskin).

http://dx.doi.org/10.1016/j.parint.2014.12.008
1383-5769/

Solea (common sole) from the Mersin Bay, the southern coast of Turkey (Northeast Mediterranean Sea). This is the first molecular study using mtDNA *cox1* gene sequences in identification of *H. aduncum* at species level and in investigation of genetic variability of specimens isolated from different hosts, comparing nucleotide sequence data of ITS rDNA and mtDNA *cox1* genes.

2. Materials and methods

2.1. Sampling

Gilthead seabream *S. aurata* Linnaeus, 1758 and common two-banded seabream *D. vulgaris* (Geoffroy Saint-Hilaire, 1817) were sampled monthly from commercial fishing area of Mersin Bay, Eastern Mediterranean Sea (36°10′58.08″N–33°58′37.43″E and 36°32′00.70″N–35°19′32.54″E) during February 2013 to January 2014 and common sole, *S. solea* (Linnaeus, 1758) were sampled from the same catching area between February 2013 and April 2013.

2.2. Parasitological examination

Fish were obtained from commercial fishermen and fish samples were measured for total length (TL, cm) and weight (W, g) individually before the parasitological examination. All the nematodes studied were counted. After that nematodes were measured with the micrometric ocular. Standard parasitic procedures were used. In the cranial and post-cranial dissection, parasites were found only from internal lumen of digestive tract. Alimentary canals of fish specimens were longitudinally opened and parasites were removed. Isolated parasites were washed in physiological saline solution, fixed in 70% ethanol for the molecular investigation and also stored in 4% buffered formalin for the routine study. Fixed nematodes within the formalin, cleared in glycerin for light microscope (phase-contrast Nikon H550L) examination and for the identification. The parasite specimens were morphologically identified as L4 stage larvae of *Hysterothylacium* sp. [3,7,8,27,29–32].

Isolated parasites were counted, then prevalence (*P*%), mean intensity (m*I*) and mean abundance (m*A*) of *Hysterothylacium* sp. were calculated regarding the procedures reported in Bush et al. [33].

2.3. DNA analyses

Genomic DNA was isolated from *Hysterothylacium* sp. L4 stage larvae (total 157 out of 182 specimens; *S. aurata*: 100/115, *D. vulgaris*: 39/44 and *S. solea*: 18/23. Remaining specimens kept in fixatives) using the Promega Wizard® Genomic DNA Purification Kit according to the manufacturers' protocol. Quality and quantity of the DNA were determined using NanoDrop™ ND-1000 spectrophotometer and were visualized on a 1% agarose gel electrophoresis. DNA samples were all set to a final concentration of 50 ng/μl. Eluted DNA samples were used either immediately in polymerase chain reaction (PCR) or stored at −20 °C.

Two regions were selected to amplify with PCR. The first region includes a small fragment from 18S rRNA, complete ITS1-5.8S rRNA-ITS2 region and a small fragment of 28S rRNA. The second region includes a fragment of mtDNA *cox1* gene, known as the DNA barcode region for the eukaryotic species. Both protein coding and non-coding regions were amplified using species specific primers designed for this study. Primer sequences, fragment lengths and PCR conditions are presented in Table 2. PCR yields were subjected to a bidirectional direct sequencing using ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA) platform. Sequencing was performed using the same primers designed for the PCR.

2.4. Data analyses

Alignment of the forward and reverse nucleotide sequences was conducted using ClustalW 2.1 and MEGA 6.0.5 software [34] with the default gap and extension penalties. Basic Local Alignment Search Tool (BLAST) program was used in comparison with previously published reference sequences in order to make species level molecular identification. BOLD Systems Identification database was also used for mtDNA *cox1* sequences. Nucleotide sequences of *Hysterothylacium* sp. specimens were deposited as haplotypes into GenBank database (Accession numbers KJ748530–KJ748537).

Nucleotide composition was calculated using MEGA 6.0.5 software. jModelTest [35] was used to choose the best fitting model for our dataset according to the corrected Akaike information criterion. Maximum likelihood phylogenies were reconstructed with Jukes Cantor model with a discrete Gamma distribution for ITS rDNA and with Tamura-Nei model with a discrete Gamma distribution for mtDNA *cox1* datasets using PAUP 4.0b10 software [36] with transition/transversion ratio calculated in the JModelTest. Bootstrap test with 1000 replicates was used to check confidence in the topologies. Fst values according to host species were also calculated [36].

Genetic distance (pairwise) among the haplotypes and previously submitted sequences gathered from GenBank database were calculated using Jukes Cantor and Tamura-Nei model for ITS rDNA and mtDNA *cox1*, respectively. Genetic distance matrix of both datasets including relative standard errors was given in. Evolutionary relationship among the haplotypes was analyzed using Neighbor Joining (NJ) trees. Phylogenetic trees were constructed using Jukes Cantor, Tamura Nei, and Kimura 2-parameter models (according to datasets) with bootstrap tests of 1000 replicates. Building phylogenetic trees from intraspecific datasets is complicated because of high number of samples and small genetic distances among the specimens. Network analysis helps to express the alternative evolutionary path possibilities in the form of cycles [37]. Consequently, we also used Median Joining (MJ) network to evaluate the relationship among *Hysterothylacium* sp. haplotypes, using Network 4.612 software [37].

3. Results

3.1. Parasitological indices

A total of 868 fish were examined in this study (from February 2013 to January/April 2014 from the Mersin Bay, Northeast Mediterranean Sea) by means of presence of nematodes. Fifty-six of 385 *S. aurata*, 18 of 473 *D. vulgaris* and 7 of 39 *S. solea* specimens were found to be parasitized. Common characteristics of the L4 stage larvae were found as distinct 3 lips, short interlabia and tail shapes typically spinous were

Table 1
Data of the fish hosts examined, number of specimens examined, and levels of infection.

Examined fish	N_{upf}	W_{upf}	TL_{upf}	N_{pf}	W_{pf}	TL_{pf}	N_p	*P* (%)	*mI*	*mA*
Sparus aurata	329	78.83 ± 19.02	16.59 ± 1.55	56	85.64 ± 22.20	17.11 ± 2.07	115	14.55	2.05	0.30
Diplodus vulgaris	419	104.68 ± 40.52	17.11 ± 2.4	18	92.02 ± 23.91	16.68 ± 3.02	44	4.12	2.44	0.10
Solea solea	39	61.17 ± 20.49	19.53 ± 2.22	7	61.01 ± 14.66	19.96 ± 1.72	23	15.22	3.29	0.50

N_{upf}: the number of un-parasitized fish, W_{upf}: live weight of un-parasitized fish, TL_{upf}: total length of un-parasitized fish, N_{pf}: the number of parasitized fish, W_{pf}: live weight of parasitized fish, TL_{pf}: total length of parasitized fish, N_p: the number of parasites, *P*: prevalence, mI: mean intensity, mA: mean abundance.

Table 2
Primer sequences designed for ITS rDNA and mtDNA *cox1* regions, PCR contents and thermal cycler conditions.

Primer sequence	Target region	Amplified fragment length	Tm	Reaction content (25 μl)	PCR condition
HaITS-F: 5′-GAAGACTTCTTAGAGGGACA-3′ HaITS-R: 5′-CAGCGGGTAGTCACGA-3′	ITS rDNA	1398 bp	53 °C	10 mM Tris–HCl (pH 8.3) 50 mM KCl	95 °C (2 min) 30 cycles of:
HaCOI-F: 5′-TTTTTATTTGGTTTGTGGTCTGGTA-3′ HaCOI-R: 5′-ATGCTGAATAATTAATGGGTTACCA-3′	mtDNA *cox1*	639 bp	59 °C	2,5 mM $MgCl_2$ 0.3 mM dNTP mix 1 mM of each primer 0.5 U DNA Polymerase 5 μl DNA	94 °C (30 s) 53/59 °C (45 s) 72 °C (1 min) and 72 °C (10 min)

detected. The larvae lengths (mm) and nerve ring widths (mm) were measured as;

Host 1: *Sparus aurata*, 18.29 ± 4.31 (11.4–24.7, n = 23) and 0.29 ± 0.07 (0.18–0.37, $n = 11$)

Host 2: *Solea solea*, 16.72 ± 4.02 (10.8–24.6, $n = 14$) and 0.28 ± 0.08 (0.17–0.36, $n = 12$)

Host 3: *Diplodus vulgaris*, 18.29 ± 4.31 (10.9–23.2, $n = 15$) and 0.25 ± 0.06 (0.16–0.33, $n = 8$) respectively.Total lengths and weights of un-parasitized and parasitized fish samples are shown in Table 1.

The raphidascarid parasitic nematodes were found only in the stomach and intestine during the endo-parasitic examination of the infected fish. The identified nematodes were classified as L4 form larvae of *Hysterothylacium* sp. Numbers of *Hysterothylacium* sp. worms per gilthead sea bream, common two banded seabream and common sole were 1-4, 1-5 and 2-5, respectively.

Prevalence (*P*) values for *Hysterothylacium* sp. for parasitized gilthead sea bream, common two banded seabream, and common sole were calculated as 14.55%, 4.12%, and 15.22%, respectively. Also mean intensity (*mI*) values were found to be 2.05, 2.44, and 3.29, and the mean abundance (m*A*) values were 0.30, 0.10, and 0.50, respectively (Table 1).

3.2. Molecular analyses

The comparison of the obtained sequences with those available in GenBank resulted with a match for the species *H. aduncum* with an identification ratio of >99%, using both the mtDNA cox1 and ITS rDNA genes.

3.2.1. ITS rDNA dataset

ITS rDNA gene, including a small fragment from 18S rRNA, complete ITS1-5.8S-ITS2 region and a small fragment of 28S rRNA was successfully amplified using the species specific primer pair HaITS-F and HaITS-R. The final length of the aligned nucleotide sequence was 1398 base pairs. No insertion or deletion was detected among aligned sequences. Nucleotide sequences were composed of 431 base pairs long partial 18S rRNA sequence, 431 base pairs long complete ITS1 sequence, 157 base pairs long complete 5.8S rRNA sequence, 347 base pairs long complete ITS2 sequence, and 32 base pairs long partial 28S rRNA sequence. Nucleotide composition, in terms of G + C, for each region was between 51.2% and 51.5%. Nucleotide composition of 28S rRNA region (G + C = 62.5%) was not used in calculations as only a 32 base pairs-long fragment of it was amplified. Nucleotide composition was 22.2% adenine, 23.0% cytosine, 28.4% guanine, and 26.4% thymine. Number of sequences, number of haplotypes, nucleotide diversity and number of polymorphic sites were given in Table 3.

Pairwise genetic distance matrix indicated a genetic divergence among *H. aduncum* specimens isolated from different hosts in our study and among different *H. aduncum* populations such as Black Sea, Baltic Sea, Adriatic Sea, North Sea, Pacific, and Atlantic from GenBank. All the *H. aduncum* specimens isolated from the same host species belonged to the same haplotype. However, mean pairwise genetic distance among *H. aduncum* specimens isolated from different host species were in the range of 0.002–0.003 (±0.001), indicating a genetic variation based on host species. Pairwise genetic distance among *H. aduncum* specimens from this study and previously submitted sequences was 0.001–0.012 (±0.001). None of the *H. aduncum* haplotypes found in this study showed absolute match with any of the previously submitted sequences. Specimens from Black Sea, North Sea, Portugal, and Tunisia showed no difference based on sequenced fragments of the ITS rDNA gene. Although there is no insertion or deletion detected among sequences from this study, one deletion at sequences from South Korea (702733) and Tunisia (HQ270427), one insertion at sequence from Portugal (KF923931), two insertions at same positions from sequences of Japan (AB277826), China (KF736937) and South Korea (HQ702733) were detected among the compared sequences. The Fst value of the dataset according to host species was calculated as 0.286.

Neighbor-joining tree clearly indicated four distinct clusters among specimens from different populations (Fig. 1). *H. aduncum* specimens from this study were grouped under three clusters according to their hosts, while sequences from Japan, South Korea and China were clustered together close to another group of sequences from Portugal, Denmark, Croatia, Turkey and Tunisia. The sequence from Poland was separated from the rest with a single branch. Bootstrap supports were all found to be greater than 73%. The clustering pattern correlated with geographical distribution of the specimens. Median joining network confirms the divergence of populations under four distinct clusters separated with three mutational vectors (Fig. 2).

Table 3
Number of individuals, total number of sequences, haplotype numbers, nucleotide diversity and parsimony informative polymorphic sites according to datasets.

Host	*H. aduncum* Individuals	Total sequences	Number of haplotypes		Nucleotide diversity		Polymorphic sites[a]	
			mtDNA *cox1* dataset	ITS rDNA dataset	mtDNA *cox1* dataset	ITS rDNA dataset	mtDNA *cox1* dataset	ITS rDNA dataset
Diplodus vulgaris	39	157	2 (18/21)	1	0.010 (SE: 0.003)	0.004 (SE: 0.001)	13/641	10/1398
Solea solea	18		2 (12/6)	1				
Sparus aurata	100		1 (100)	1				

[a] All the polymorphic sites were found parsimony informative.

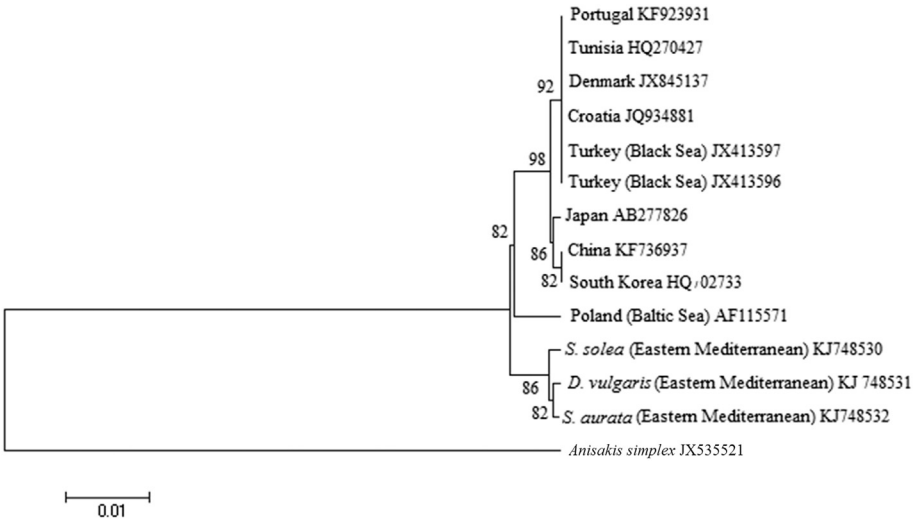

Fig. 1. Neighbor joining tree based on ITS rDNA sequences. Phylogenetic relationship was inferred using neighbor joining method. Bootstrap confidence percentage is shown next to branches. Genetic distances were calculated using the Jukes Cantor + G method. *H. fabri* was used as out-group.

3.2.2. The mtDNA cox1 dataset

The mtDNA *cox1* region amplified in this study consists of 641 base pairs long fragment of mitochondrial cytochrome c oxidase I region. The mtDNA *cox1* region was successfully amplified using the species-specific primer pair of HaCOI-F and HaCOI-R. No insertion or deletion was detected in the aligned dataset. Translated protein sequences resulted with no stop codons confirming the functional mitochondrial gene and lack of numts. The mtDNA *cox1* data generated two haplotypes for *H. aduncum* specimens isolated from *S. solea* and *D. vulgaris*, and one haplotype for specimens isolated from *S. aurata*. A total of 5 haplotypes were detected using mtDNA *cox1* dataset. Mean G + C composition of amplified region was calculated as 35.2%. Nucleotide composition was 21.8% adenine, 16.1% cytosine, 19.1% guanine, and 43.0% thymine. Number of sequences, number of haplotypes, nucleotide diversity and number of polymorphic sites were given in Table 3.

Genetic distance matrix was constructed using only haplotypes of this study as no other published mtDNA *cox1* sequences were found in BOLD and GenBank databases. Genetic distance among *H. aduncum* specimens isolated from same host species was calculated as 0.005 (±0.003) for two haplotypes of *D. vulgaris* and 0.003 (±0.002) for two haplotypes of *S. solea*. No genetic distance was calculated for specimens isolated from *S. aurata* as they were clustered under same haplotype. On the other hand, genetic distance range among *H. aduncum* specimens isolated from different host species was calculated between 0.010 and 0.014 (±0.004). The overall genetic distance of the mtDNA *cox1* dataset was found as 0.010 (±0.003).

Neighbor joining tree indicates that three distinct clusters were correlated with host species in which *H. aduncum* specimens were isolated from *D. vulgaris* seems to be the most divergent among haplotypes. Although the specimens isolated from host species *S. solea* and *S. aurata* were clustered under the same main branch, their sub branches were separated according to the host species they are sampled from (Fig. 3). Median joining network confirms the divergence of populations into four haplotypes separated with four mutational vectors (Fig. 4).

4. Discussion

According to Deardorff and Overstreet [3] the excretory pore of *Hysterothylacium* sp. is located at or near the level of the nerve ring. Additional distinctive specifications of the L4 stage larvae were

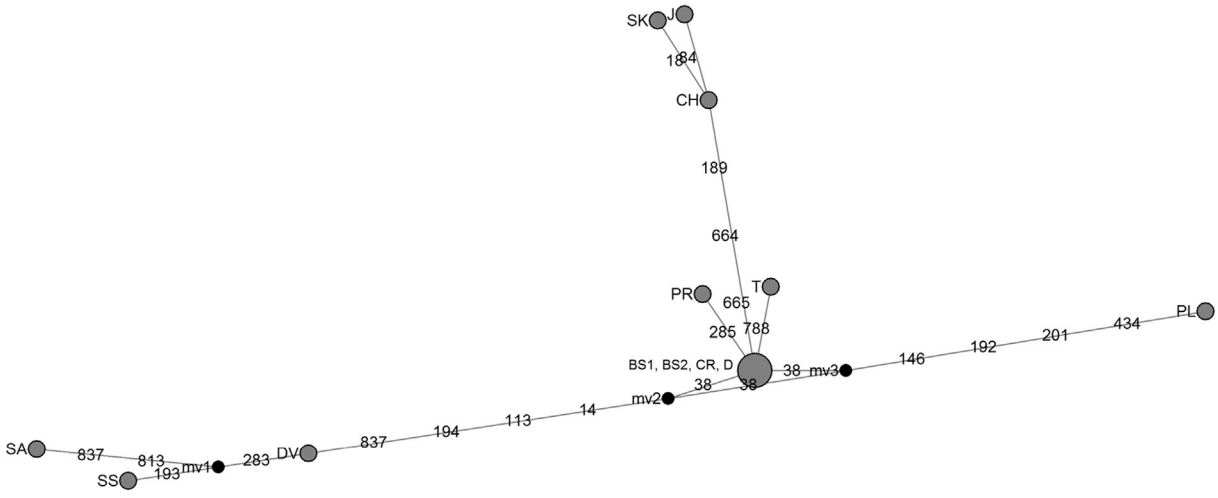

Fig. 2. MJ network based on ITS rDNA sequences of examined *H. aduncum* specimens. Median vectors (mv) were shown with black squares and population nodes were drawn as gray, proportional to frequencies. Numbers indicate mutated base positions. Haplotypes: BS1: JX413596 Black Sea Turkey, BS2: JX413597 Black Sea Turkey, CH: KF736937 Yellow Sea China, CR: JQ934881 Adriatic Sea Croatia, D: JX845137 North Sea Denmark, DV: Eastern Mediterranean (*Diplodus vulgaris*), J: AB277826 Japan, PL: AF115571 Baltic Sea Poland, PR: KF923931 Portugal, SA: Eastern Mediterranean (*Sparus aurata*), SK: HQ702733 South Korea, SS: Eastern Mediterranean (*Solea solea*), T: HQ270427 Tunisia.

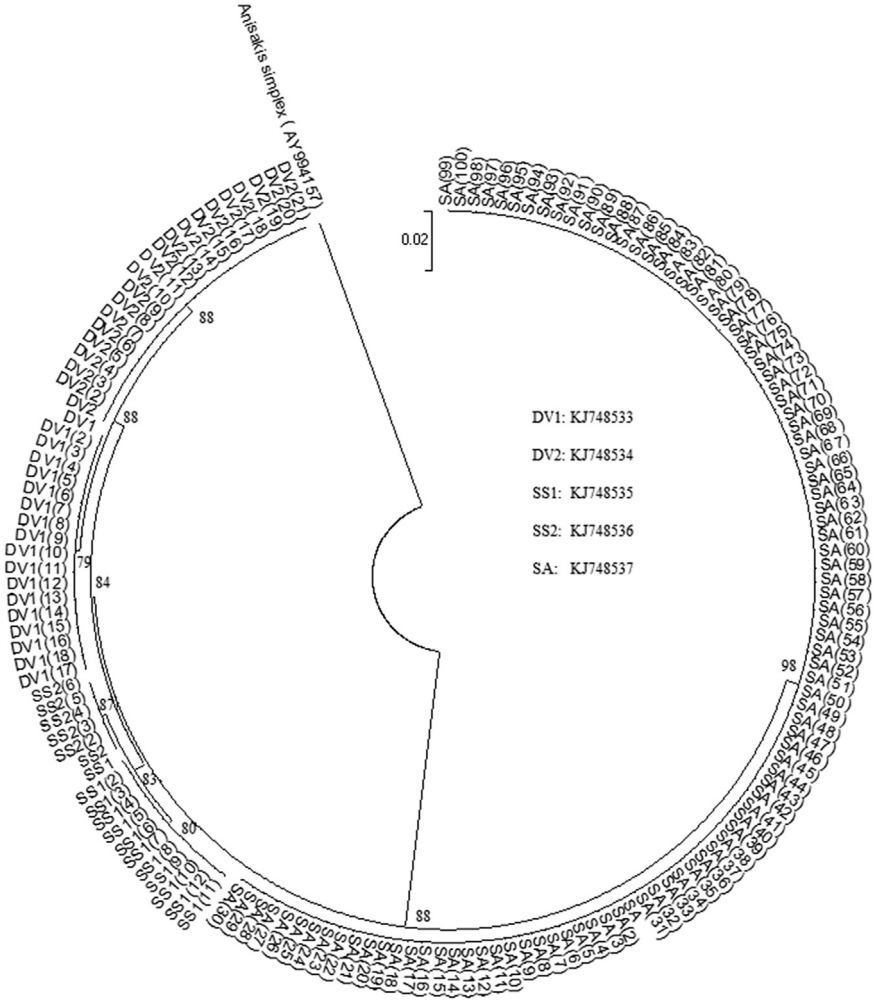

Fig. 3. Circular neighbor joining tree based on mtDNA *cox1* sequences. Phylogenetic relationship was inferred using neighbor joining method. The sum of branch length was calculated as 0.21772246. Bootstrap confidence percentage is shown next to branches. Genetic distances were calculated using both Tamura Nei/Kimura 2-parameter method as both has given the same topology.

characterized by the typical spinous shape of tail and also arrangement of the digestive organs, especially the excretory pore position (just behind the nerve ring). Therefore all of the examined nematodes removed from the fish hosts in the study are considered to belong to the species *H. aduncum* (Rudolphi 1802).

Anisakids are typically found in carnivorous fishes and their natural transmission takes place in specific habitats where the hosts consume parasitized prey, other intermediate or paratenic host [15,31]. According to Genc [12], prevalence levels of *Hysterothylacium* sp. in *S. aurata* ($N = 100$), *D. vulgaris* ($N = 117$) and *S. solea* ($N = 72$) from the Iskenderun Bay (close to the Mersin Bay) from June 2000 to May 2001 were found as 1%, 0.85%, and 0%, respectively.

In May 2003 and April 2004, Kalay et al. [15] isolated *H. aduncum* specimens from two sparids. The prevalence and the mean intensity values were noted as 6.25%, 1.92 for *S. aurata* ($N = 208$), and 6.08%, 2 for *D. vulgaris* ($N = 263$). Moreover, *H. aduncum* was reported from common guitarfish, *Rhinobatos rhinobatos*, an elasmobranch fish species sampled ($N = 244$) in Northeastern Mediterranean Sea, Iskenderun Bay. Genc et al. [13] stated that the prevalence value of *H. aduncum* (in L4 stage) was varied from 7.69% to 78.57% (March 2003–February 2005).

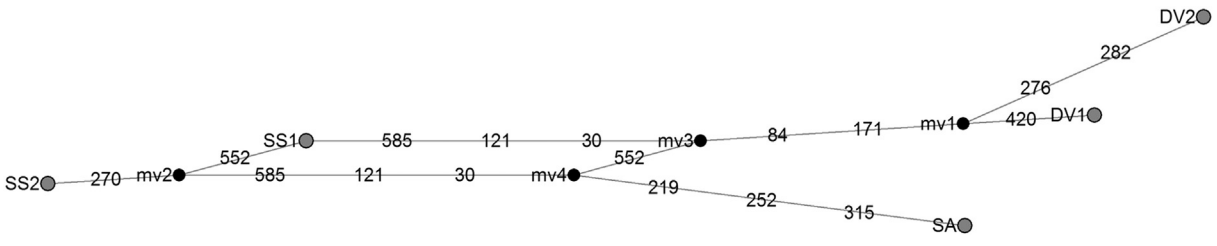

Fig. 4. MJ network based on mtDNA *cox1* sequences of examined *H. aduncum* specimens. Median vectors (mv) were shown with black squares and population nodes were drawn as gray, proportional to frequencies. Numbers are indicating mutated base positions. A total of 5 haplotypes found: DV1: Eastern Mediterranean (*Diplodus vulgaris*) Haplotype-1, 18 individuals (46.15%); DV2: Eastern Mediterranean (*Diplodus vulgaris*) Haplotype-2, 21 individuals (53.85%); SA: Eastern Mediterranean (*Sparus aurata*), 100 individuals; SS1: Eastern Mediterranean (*Solea solea*) Haplotype-1, 12 individuals (66.67%); SS2: Eastern Mediterranean (*Solea solea*) Haplotype-2, 6 individuals (33.33%).

In the present study, *H. aduncum* was the only endoparasite detected in the examined *S. aurata*, *D. vulgaris* and *S. solea* from the Mersin Bay. Our parasitological indices including *P*, m*I* and also m*A* were found to be slightly higher than the previously published studies mentioned above [12,13,15].

According to Marques and Cabral [38], estimation of the parasitological indices (their variance and bias) depends on sample size of fish (in the Monte Carlo simulation procedures). Estimates of prevalence were not significantly affected by sample size whereas mean abundance and mean intensity were affected by even a single sample. Thus, underestimation of values was more perceptible in small (<40) sample sizes.

In the current study fish sample sizes were recorded as 385 for gilthead seabream, 437 for common two banded seabream and 46 for common sole, respectively. This makes our sparid sample size more reliable than the soleid sample size in terms of confidence interval. Although sample size of soleid fish is just over acceptable limit of 40, results of molecular data showed a significant divergence among the *H. aduncum* specimens, even at intragenic levels, related to their host and sampling populations.

Previous studies based on nucleotide sequences of ITS rDNA gene clearly demonstrated the effectiveness of this genetic marker in molecular identification of ascaridoid species [1,19,26,39–44]. Nucleotide sequences of L4 stage larvae of *H. aduncum* specimens isolated from three commercially important fish species caught off the Eastern Mediterranean cost are also shown to be effective in molecular identification at species level, using both ITS rDNA and mtDNA *cox1* regions. Results indicated that the ITS rDNA sequences from our study are highly similar to those already submitted to GenBank in previous studies [1,19,26,42,45–47]. We were not able to compare mtDNA *cox1* sequences, as this is the first molecular study using mtDNA *cox1* sequences in identification of *H. aduncum*.

Comparisons of ITS rDNA sequences from this study to sequences from GenBank were indicating two main clades. First clade includes *H. aduncum* specimens isolated in this study, in which three haplotypes were found according to host fish species. Second clade three distinct groups, separating Asian coast samples (Japan, China and South Korea) under one branch, European coast samples (Turkey, Croatia, Denmark, Portugal and Tunisia) in another and leaving the sample from Baltic Sea (Poland) at another. European cost samples from Turkey were isolated from the Black Sea [19] and were clearly distinct from the specimens in this study that were isolated from Eastern Mediterranean Sea. Both the neighbor-joining tree and the median-joining network show that the haplotypes were correlated with the geographical variation.

Moreover, mtDNA *cox1* data pointed out an intragenic variation among *H. aduncum* specimens isolated from the same host species. Two haplotypes were detected for *H. aduncum* specimens isolated both from *S. solea* and *D. vulgaris*. On the other hand, specimens isolated from *S. aurata* showed a single haplotype.

The low intraspecific genetic distance among *H. aduncum* specimens has already been reported by Klimpel et al. [1]. Their result based on ITS rDNA data was indicating a genetic variation of only three indels at ITS2 sequences, and exactly the same sequences at ITS1 and 5.8S rRNA genes from geographically distant populations. Klimpel et al. [1] suggested that studies with higher sample sizes should be used to confirm the phylogenetic pattern and using more polymorphic markers will increase the chance to detect intraspecific variation. Results based on mtDNA *cox1* dataset in our study confirm these suggestions as the amplified fragment of mtDNA *cox1* gene used in this study revealed multiple haplotypes within *H. aduncum* specimens isolated from same host species.

4.1. Conclusions

As a result, this study provides new data on the occurrence of *H. aduncum* in three commercially important marine fish species caught off the Eastern Mediterranean coast.

Studies on molecular identification of nematodes typically use the ribosomal DNA small subunit (SSU) [48], the ribosomal DNA large subunit (LSU) [49,50] or the internal transcribed spacer (ITS) region [49,51] for the advantage of the availability of universal nematode primers and genus level phylogenetic resolution. The mtDNA *cox1* has been distinguished lately as a potential marker with an amplification success across a wide taxonomic range, lack of insertions and deletions making the alignment easy and variability pattern making species level identifications possible. Besides advantages of being a mitochondrial gene, mtDNA *cox1* is translated into an evolutionary conserved protein, making it a better molecular identification tool over SSU, LSU, and ITS rDNA [52].

In this study, mtDNA *cox1* sequences were used for the first time to identify *H. aduncum* at species level. Along with mtDNA *cox1* sequences, also ITS rDNA which is a widely used genetic marker in previous studies on molecular identification was also used to confirm the results. Both datasets resulted with an intraspecific variation among *H. aduncum* specimens sampled from different host species. Intraspecific genetic variation was also found between ITS rDNA sequences of *H. aduncum* specimens from this study and previously submitted sequences downloaded from GenBank. Also different haplotypes were detected from the *H. aduncum* specimens isolated from the same host species and the same location (Eastern Mediterranean) using the mtDNA *cox1* nucleotide sequences. Calculated Fst value of 0.286 between specimens of different host species also confirms the pattern. Fst values range from 0 to 1. Zero means a total panmixis, which can be explained by a free interbreeding among populations. Contrary to zero, one points out a population structure with no genetic relationship. Fst values <0.05 indicate a population structure with no genetic differentiation, while Fst value >0.25 points out a large genetic differentitation [53]. Fst value of 0.286 calculated in our study stands for a high rate of genetic differentitation among the populations of *H. aduncum* specimens from different fish hosts.

Further studies using different host species from distinct geographical locations and sequencing other polymorphic genes could result in a more detailed analysis of the population structure of *H. aduncum*.

References

[1] Klimpel S, Kleinertz S, Hanel R, Rückert S. Genetic variability in *Hysterothylacium aduncum*, a raphidascarid nematode isolated from sprat (*Sprattus sprattus*) of different geographical areas of the northeastern Atlantic. Parasitol Res 2007;101:1425–30.

[2] Anderson RC. Nematode Parasites of Vertebrates: Their Development and Transmission. Wallingford: CAB; 2000.

[3] Deardorff TL, Overstreet RM. Larval *Hysterothylacium* (=*Thynnascaris*) (Nematoda: Anisakidae) from fishes and invertebrates in the Gulf of Mexico. Proc Helminthol Soc Wash 1981;48:113–26.

[4] Moser M, Hsieh J. Biological tags for stock separation in Pacific herring *Clupea harengus pallasi* in California. J Parasitol 1992;78:54–60.

[5] Moravec F, Nagasawa K, Urawa S. Some fish nematodes from fresh water in Hokkaido, Japan. Folia Parasitol 1985;32:305–16.

[6] Saglam N. Infection of *Hysterothylacium aduncum* (Nematoda: Anisakidae) in farmed rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). Afr J Agric Res 2013;8(47):5953–7.

[7] Køie M. Aspects of the life cycle and morphology of *Hysterothylacium aduncum* (Rudolphi, 1802) (Nematoda, Ascaridoidea, Anisakidae). Can J Zool 1993;71:1289–96.

[8] Køie M. Nematode parasites in teleosts from 0 to 1540 m depth off the Faroe Islands (the North Atlantic). Ophelia 1993;38:217–43.

[9] Klimpel S. Distribution of nematodes of the family Anisakidae in commercially important fish species from the central and northern North Sea. Z Fischk 2005;7(2):161–8.

[10] Petter AJ, Maillard C. Ascarides de poissons de Méditerranée occidentale. Bull Mus Natl d'Histoire Nat, Paris, 4e série, sec. A, 9; 1987 773–98.

[11] Farjallah S, Ben Slimane B, Blel H, Amor N, Said K. Anisakid parasites of two forkbeards (*Phycis blennoides* and *Phycis phycis*) from the eastern Mediterranean coasts in Tunisia. Parasitol Res 2006;100:11–7.

[12] Genc E. The endoparasites and histopathologies found in the some commercial teleosts in the Bay of Iskenderun, Turkey. PhD Thesis Adana, Turkey: University of Çukurova; 2002.

[13] Genc E, Yildirim YB, Basusta N, Cekic M. Seasonal variation of *Hhysterothylacium aduncum* infection in the common guitarfish, *Rhinobatos* in Iskenderun Bay (North-easthern Mediterranean Sea) Turkey. Proc of the Int Workshop on Med Cartilaginous Fish with Emphasis on South-East Med, Istanbul-Turkey; 2006.

[14] Rello FJ, Adroher FJ, Valero A. *Hysterothylacium aduncum*, the only anisakid parasite of sardines (*Sardina pilchardus*) from the southern and eastern coasts of Spain. Parasitol Res 2008;104:117–21.
[15] Kalay M, Donmez AE, Koyuncu CE, Genc E, Sahin G. Seasonal variation of *Hysterothylacium aduncum* (Nematoda: Raphidascarididae) infestation in sparid fishes in the Northeast Mediterranean Sea. Turk J Vet Anim Sci 2009;33:517–23.
[16] Dural M, Genc E, Sangun MK, Güner Ö. Accumulation of some heavy metals in *Hysterothylacium aduncum* (Nematoda) and its host sea bream, *Sparus aurata* (Sparidae) from North-Eastern Mediterranean Sea (Iskenderun Bay). Environ Monit Assess 2011;174(1–4):147–55.
[17] Vardić-Smrzlić IV, Valić D, Kapetanović D, Kurtović B, Teskeredžić E. Molecular characterisation of Anisakidae larvae from fish in Adriatic Sea. Parasitol Res 2012;111: 2385–91.
[18] Morsy K, Bashtar AR, Abdel-Ghaffar F, Mostafa N. New host and locality records of two nematode parasites *Dujardinnascaris mujibii* (Heterocheilidae) and *Hysterothylacium aduncum* (Anisakidae) from the common seabream *Pagrus*: a light and scanning electron microscopic study. Parasitol Res 2013;112:807–15.
[19] Pekmezci GZ, Bolukbas CS, Gurler AT, Onuk EE. Occurrence and molecular characterization of *Hysterothylacium aduncum* (Nematoda: Anisakidae) from *Merlangius merlangus euxinus* and *Trachurus* off the Turkish coast of Black Sea. Parasitol Res 2013;112:1031–7.
[20] Petter AJ, Radujkovic J. Parasites des Poissons marins du Montenegro: nematodes. Acta Adriat 1989;30:195–236.
[21] Margolis L, Arthur JR. Synopsis of the parasites of fishes of Canada. Bull Fish Res Board Can 1979;199:269.
[22] Marcogliese DJ. Larval parasitic nematodes infecting marine crustaceans in eastern Canada. 3. *Hysterothylacium aduncum*. J Helminthol Soc Wash 1996;63:12–8.
[23] Shih HH, Jeng MS. *Hysterothylacium aduncum* (Nematoda: Anisakidae) infecting a herbivorous fish, *Siganus fuscescens*, off the Taiwanese coast of the northwest Pacific. Zool Stud 2002;41(2):208–15.
[24] Moravec F, Nagasawa K. *Ichthyofilaria japonica* sp.n. (Philometridae) and some other nematodes from Hokkaido, Japan. Vest cs Spolec Zool 1985;49:211–23.
[25] Martin-Sánchez J, Paniagua I, Valero A. Contribution to the knowledge of *Hysterothylacium aduncum* through electrophoresis of the enzymes glucose phosphate isomerase and phosphoglucomutase. Parasitol Res 1998;84:160–3.
[26] Amor N, Farjallah S, Merella P, Said K, Ben Slimane B. Molecular characterization of *Hysterothylacium aduncum* (Nematoda: Raphidascaridae) from different fish caught off the Tunisian coast based on nuclear ribosomal DNA sequences. Parasitol Res 2011;109:1429–37.
[27] Guo YN, Xu Z, Zhang LP, Hu YH, Li L. Occurrence of *Hysterothylacium* and *Anisakis* nematodes (Ascaridida: Ascaridoidea) in the tanaka's snailfish *Liparis tanakae* (Gilbert & Burke) (Scorpaeniformes: Liparidae). Parasitol Res 2014;113:1289–300.
[28] Mattiucci S, Nascetti G. Advances and trends in the molecular systematics of Anisakid nematodes, with implications for their evolutionary ecology and host–parasite co-evolutionary processes. Adv Parasitol 2008;66:47–148.
[29] Deardorff TL, Overstreet RM. Review of *Hysterothylacium* and *Iheringascaris* (both previously = *Thynnascaris*) Nematoda: Anisakidae) from the northern Gulf of Mexico. Proc Biol Soc Wash 1980;93:1035–79.
[30] Berland B. *Hysterothylacium aduncum* (Nematoda) in fish. ICES identification leaflets for diseases and parasites of fish and shellfish, Leaflet No. 44; 1991(4 pp.).
[31] Berland B. Biology of *Hysterothylacium* species. Parasitol Int 1998;47(1):26.
[32] Berland B. Musing on Nematod Parasites. Bergen: Fisgen OG Havet; 2006.
[33] Bush AO, Lafferty KD, Lotz JM, Shostak AW. Parasitology meets ecology on its own terms: Margolis et al. revisited. J Parasitol 1997;83:575–83.
[34] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.
[35] Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol 2008;25: 1253–6.
[36] Posada D. Using Modeltest and PAUP* to select a model of nucleotide substitution. In: Baxevanis AD, Davison DB, Page RDM, Petsko GA, Stein LD, Stormo GD, editors. Current Protocols in Bioinformatics. New York: John Wiley& Sons; 2003. p. 6.5.1–6.5.14.
[37] Bandelt HJ, Forster P, Rohl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 1999;1:37–48.
[38] Marques JF, Cabral HN. Effects of sample size on fish parasite prevalence, mean abundance and mean intensity estimates. J Appl Ichthyol 2007;23:158–62.
[39] Zhu XQ, D'Amelio S, Palm HW, Paggi L, George-Nascimento M, Gasser RB. SSCP-based identification of members within the *Pseudoterranova decipiens* complex (Nematoda:Ascaridoidea: Anisakidae) using genetic markers in the internal transcribed spacers of ribosomal DNA. Parasitology 2002;124:615–23.
[40] D'Amelio S, Mathiopoulos KD, Santos CP, Pugachev ON, Webb SC, Picanco M, et al. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidae) defined by polymerase chain reaction-based restriction fragment length polymorphism. Int J Parasitol 2000; 30:223–6.
[41] Zhang L, Hu M, Shamsi S, Beveridge I, Li H, Xu Z, et al. The specific identification of anisakid larvae from fishes from the Yellow Sea, China, using mutation scanning-coupled sequence analysis of nuclear ribosomal DNA. Mol Cell Probes 2007;21: 386–90.
[42] Kellermanns E, Klimpel S, Palm HW. Molecular identification of ascaridoid nematodes from the deep-sea onion-eye grenadier (*Macrourus berglax*) from the East Greenland Sea. Deep Sea Res I Oceanogr Res Pap 2007;54:2194–202.
[43] Farjallah S, Ben Slimane B, Busi M, Paggi L, Amor N, Blel H, et al. Occurrence and molecular identification of *Anisakis* spp. from the North African coasts of Mediterranean Sea. Parasitol Res 2008;102(3):371–9.
[44] Farjallah S, Merella P, Ingrosso S, Rotta A, Ben Slimane B, Garippa G, et al. Molecular evidence for the occurrence of *Contracaecum rudolphii* A (Nematoda: Anisakidae) in shag *Phalacrocorax aristotelis* (Linnaeus) (Aves: Phalacrocoracidae) from Sardinia (western Mediterranean Sea). Parasitol Int 2008;57:437–40.
[45] Szostakowska B, Myjak P, Kur J. Identification of anisakid nematodes from the Southern Baltic Sea using PCR-based methods. Mol Cell Probes 2002;16:111–8.
[46] Shih HH. Parasitic helminth fauna of the cutlass fish, *Trichiurus lepturus* L., and the differentiation of four anisakid nematode third stage larvae by nuclear ribosomal DNA sequences. Parasitol Res 2004;93:188–95.
[47] Zhu XQ, Podolska M, Liu JS, Yu HQ, Chen HH, Lin ZX, et al. Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA. Parasitol Res 2007;101: 1703–7.
[48] Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, et al. A molecular evolutionary framework for the phylum Nematoda. Nature 1998;392:71–5.
[49] De Ley P, De Ley IT, Morris K, Abebe E, Mundo-Ocampo M, et al. An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. Philos Trans R Soc Lond B 2005;360: 1945–58.
[50] Subbotin SA, Ragsdale EJ, Mullens T, Roberts PA, Mundo-Ocampo M, Baldwin JG. A phylogenetic framework for root lesion nematodes of the genus Pratylenchus (Nematoda): evidence from 18S and D2–D3 expansion segments of 28S ribosomal RNA genes and morphological characters. Mol Phylogenet Evol 2008;48: 491–505.
[51] Floyd R, Abebe E, Papert A, Blaxter M. Molecular barcodes for soil nematode identification. Mol Ecol 2002;11:839–50.
[52] Prosser SW, Velarde-Aguilar MG, León-Règagnon V, Hebert PD. Advancing nematode barcoding: a primer cocktail for the cytochrome c oxidase subunit I gene from vertebrate parasitic nematodes. Mol Ecol Resour 2013;13:1108–15.
[53] Derycke S, Backeljau T, Moens T. Dispersal and gene flow in free-living marine nematodes. Front Zool 2013;10:1–12.