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Research Article

Morphometric analysis and genetic identification of *Hysterothylacium* larvae (Nematoda) in marine fish in the Aegean Sea

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ABSTRACT

The classification of *Hysterothylacium* larval types in the Aegen Sea (FAO fishing area 37.3.1) is incomplete and still requires further investigation. This study aimed to offer morphological identification, supported by entire ITS sequence data, of *Hysterothylacium* larval types infecting six out of sixteen commonly consumed marine fish species from the Aegean Sea: *Boops boops, Trachurus mediterraneus, Spicara maena, Upeneus moluccensis, Engraulis encrasicolus,* and *Sardina pilchardus*. This study describes three morphotypes IV, V, and VIII in the Aegean Sea for the first time using both morphological and genetic data. We also report *Spicara maena* as new hosts for larval morphotype V. Herein, the presence of potential zoonotic larval type VIII has been found in *B. boops*, *E. encrasicolus*, and *S. pilchardus* in the Aegean Sea, and may pose a public health risk.

Keywords: Aegean sea, *Hysterothylacium* morphotypes, ITS, Sequencing

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Introduction

Belonging to the Raphidascarididae family, *Hysterothylacium* species are marine ascaridoid nematodes. These nematodes are recognized to infect many marine fish and are thought to be potentially transmissible to humans (8, 32, 33). Up to now, researchers have described 18 unique larval morphotypes of *Hysterothylacium* (2, 7, 12, 16, 19, 24, 25, 26, 28, 29). Analysis of rDNA ITS–1 and ITS–2 sequences, along with molecular data from adult parasites, has been effective in determining the species of several *Hysterothylacium* larval morphotypes (6, 7, 10-12, 14, 16, 17, 21, 22, 24, 25, 26, 28, 29).

Within the Aegean Sea, researchers have identified *H. fabri* larvae and *Hysterothylacium* spp. larvae in marine fish based on morphology but have not specified the detailed larval morphotypes (4, 18, 30), and only larval specimens of *H. aduncum*, *H. fabri*, and *H. reliquens* have been genetically characterized (18, 30). However, comprehensive morphological and molecular information regarding *Hysterothylacium* morphotypes present from Aegean Sea remains insufficient. The specific species and molecular characteristics of these morphotypes within the Aegean Sea are not yet fully understood. Hence, present investigation aimed to use a taxonomic approach combining detailed morphological characterization with complete ITS sequence data to examine the *Hysterothylacium* larva morphotype infecting marine fish selected from the Aegean Sea.

Materials and Methods

***Sampling and Parasitological Examinations*:** Sixteen frequently consumed fish species, including garfish, *Belone belone* (L.) (n=8), bogue, *Boops boops* (L.) (n=14), European anchovy, *Engraulis encrasicolus* (L.) (n=20), little tunny, *Euthynnus alletteratus* (R.) (n=1), sand steenbras, *Lithognathus mormyrus* (L.) (n=5), mullet, *Mugil* spp. (n=1), red mullet, *Mullus barbatus* (L.) (n=10), striped red mullet, *M. surmuletus* (L.) (n=15), European pilchard, *Sardina pilchardus* (W.) (n=10), salema, *Sarpa*

salpa (L.) (n=5), brushtooth lizardfish, *Saurida undosquamis* (R.) (n=4), scorpionfish, *Scorpaena* spp. (n=3), blotched picarel, *Spicara maena* (L.) (n=10), Mediterranean horse mackerel, *Trachurus mediterraneus* (S.) (n=10), piper gurnard, *Trigla lyra* (L.) (n=1), and goldband goatfish, *Upeneus moluccensis* (B.) (n=47) were freshly obtained from the local fish markets. All marine fish species were caught in the Turkish Aegean Sea coasts (FAO fishing area 37.3.1) between 2022 and 2023. Fish were transported to the laboratory in iced containers. Fish were dissected under a stereomicroscope and examined for nematode presence using the incubation method (27). All nematodes found were counted and preserved in 70% ethanol for subsequent morphological and molecular analysis. Each collected larva was divided into three segments. The anterior and posterior segments were cleared using Amman's lactophenol and then morphologically identified under a light microscope, following established identification keys (6, 14, 21, 24). DNA extraction was performed on the middle segments. Randomly chosen representatives of morphotypes were measured for their morphological features and then DNA sequenced to determine their species (6, 14, 21, 24).

***Molecular and Phylogenetic Analysis*:** A total of six nematodes were used for DNA extraction. DNA was individually isolated from middle segments of nematodes with the GeneJET Genomic DNA Purification Kit, ThermoFisher Scientific, Vilnius, Lithuania according to the manufacturer's instructions. The concentration and purity of the isolated DNA were determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). The entire ITS region (ITS–1, 5.8S subunit, ITS–2) was amplified using the primer sets NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (34), and PCR cycling conditions described by Pekmezci et al. (17, 18). PCR products were electrophoresed in 2% agarose gel (Prona) in a TBE buffer (Invitrogen), stained with ethidium bromide (Sigma-Aldrich) and visualized by UV illumination. The amplification of ITS products was purified with a PCR purification kit (PureLink™ PCR Purification Kit, Invitrogen) and sequenced in both directions with the same primers using the Big Dye Terminator kit (Applied Biosystems) on an ABI PRISM 310 genetic analyzer (Applied Biosystems). The quality of the sequences was checked using Geneious Prime (13). Sequences were verified by forward and reverse comparisons, assembled, and edited with Geneious Prime. The consensus sequences were compared with all *Hysterothylacium* species sequences using the BLASTn via GenBank (1). Subsequently, the validated sequences were selected from GenBank. Geneious Prime was used to concatenate ITS–1 and ITS–2 sequences (12, 21, 28, 29). The ITS–1 and ITS–2 sequences were aligned using ClustalW in MEGA X (31). Gblocks program was used to remove poorly aligned or highly variable regions from the alignments (3). These alignments were subsequently used in phylogenetic analysis. A Neighbor–Joining (NJ) tree was generated in MEGA X, employing the Kimura-2-parameter model as the best-fitting model of sequence evolution for the ITS–1 and ITS–2 regions using the complete deletion option (15). The robustness of the NJ tree was assessed using bootstrap analysis with 10 000 replicates (5). Bootstrap values equal to or greater than 70% were displayed at the internal nodes to indicate statistical support (9).

***Epidemiological Data*:** Prevalence (P) and mean intensity (MI) were determined according to Rózsa et al. (23) by using Quantitative Parasitology (20).

Results

In the current study, three distinct larval morphotypes were identified as types IV, V, and VIII. These larvae were found to be in their third and fourth stages of development. A comprehensive description of the morphotypes, along with their genetic characterization, is presented in the following sections.

Hysterothylacium Larval Type IV

***Morphological Description*:** Ten fourth–stage larvae were examined. The body length averaged 10.5 mm (range: 9.5–12 mm) with a width of 125 µm (range: 98–196 µm). Developed labia were present, but a tooth was absent. The nerve ring and excretory pore were located 302 µm (range: 265–335 µm) and 382 µm (range: 332–405 µm), respectively, from the anterior part. The oesophagus, measuring 930 µm (range: 770–1070 µm), comprised 8.8% of the body length. The ventriculus was nearly sub-spherical, with a length of 85 µm (range: 70–152 µm). The ventricular appendix (650 µm; range: 462–859 µm) was significantly longer than the intestinal caecum (75 µm; range: 51–151 µm), representing 69.8% and 8.06% of the oesophageal length, respectively. The ratio of the intestinal caecum to the ventricular appendix was 1:8.6. The conical tail, 112 µm in length (range: 83–183 µm) and 1.06% of the body length, was covered in many or several rows of spines (Figure 1).

Host: *Boops boops*, *Trachurus mediterraneus*, *Spicara maena*, *Upeneus moluccensis*

Site of Infection: On the internal organs and abdominal cavity

Prevalence and Intensity: P: 7.14% (1/14), MI: 4, a number of larvae: 4 in *B. boops*; P: 100% (10/10), MI: 8, total number of larvae: 80 in *T. mediterraneus*; P: 10% (1/10), MI: 3, a number of larvae: 3 in *S. maena*; P: 2.1% (1/47), MI: 3, a number of larvae: 3 in *U. moluccensis*

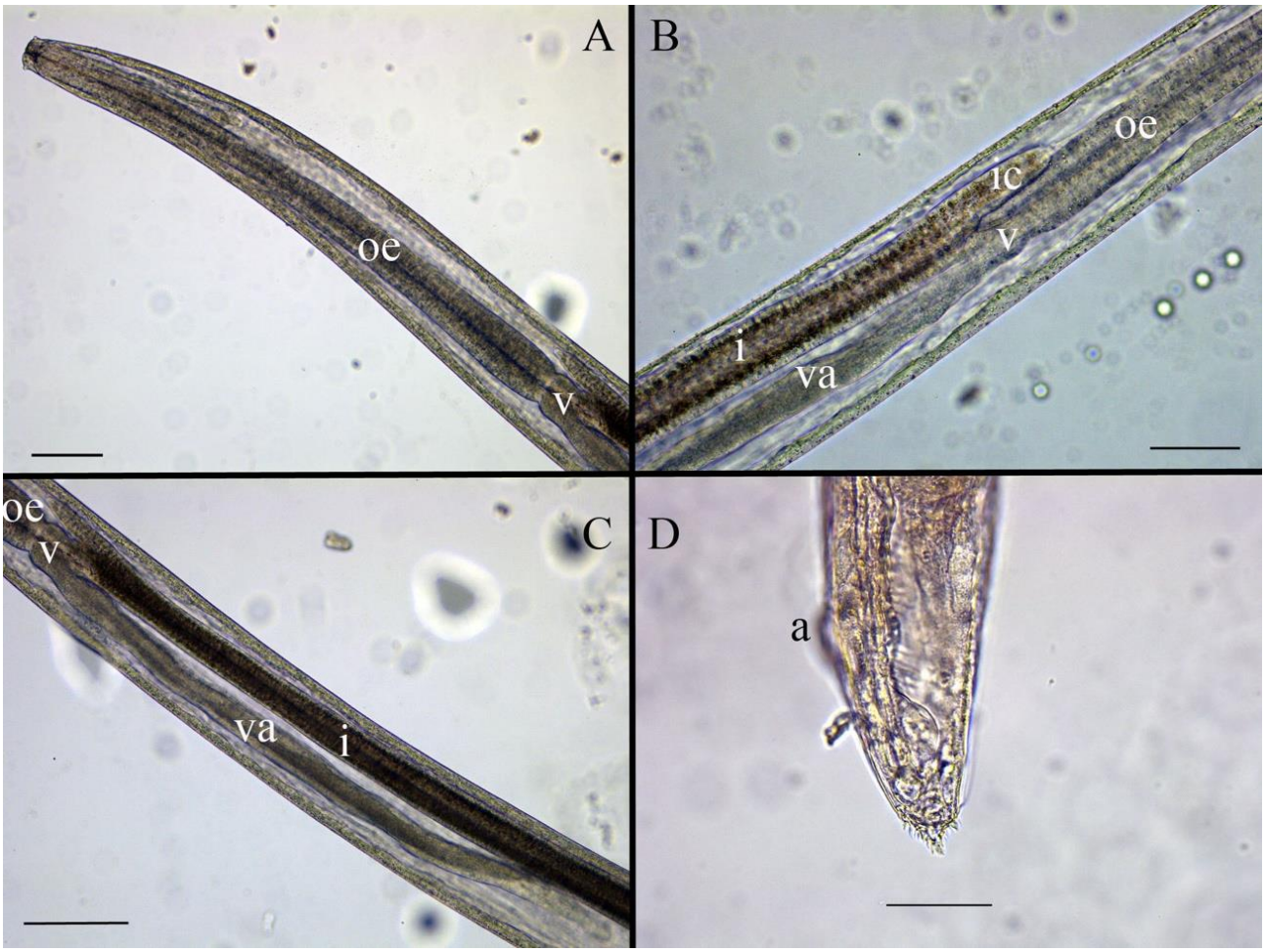

Figure 1. Microphotographs showing main morphological features of *Hysterothylacium* larval type IV from the Aegean Sea. A. Anterior end of *Hysterothylacium* larval type IV (scale: 100 µm), B–C. Oesophagus–ventriculus–intestine junction of *Hysterothylacium* larval type IV (scales B and C: 50 µm and 100 µm, respectively), D. Posterior end of *Hysterothylacium* larval type IV (scale: 50 µm). a: Anus, i: Intestine, ic: Intestinal caecum, oe: Oesophagus, v: Ventriculus and va: Ventricular appendix.

Molecular Data: Three specimens (OZ–1 from *T. mediterraneus*, OZ–2 from *B. boops*, OZ–3 from *S. maena*) underwent entire ITS region sequencing. The 924 bp sequences showed no intraspecific variation. Larval morphotype OZ–1 was deposited in GenBank (OQ971706). ITS–1 and ITS–2 sequences from this study exhibited 100% identity with *Hysterothylacium* larval type IV sequences (ITS–1: MF693071; ITS–2: MF693087) previously reported from the Spanish Mediterranean (21). The entire ITS region of OZ–1 also matched type IV from the Turkish Black Sea (6). Phylogenetic analysis (NJ ITS tree) clustered the OZ–1 larval morphotype with *H. fabri* (L4) (KU948632, KX083575) and *Hysterothylacium* larval types III (MF693070–MF693086, MZ313182) and IV (MF693071–MF693087, MZ313187), supported by a strong bootstrap value (6, 18, 21) (Figure 4).

Hysterothylacium Larval Type V

***Morphological Description*:** A single third–stage larva was examined. The body was measured 9.2 mm in length and 220 µm in width. Three poorly developed lips were present, but a boring tooth was absent. The nerve ring and excretory pore were located 288 µm and 384 µm from the anterior end, respectively. The oesophagus, 621 µm in length, comprised 6.7% of the body length. The ventriculus was sub-spherical, measuring 55 µm in length and 70 µm in width. The ventricular appendix (547 µm) was significantly longer than the intestinal caecum (112 µm), which represented 18.03% of the oesophageal length. The ratio of intestinal caecum to ventricular appendix was 1:4.88. The elongated tail, measured 104 µm in length and 1.1% of the body length, possessed a single prominent terminal spine (mucron) (Figure 2).

Figure 2. Microphotographs showing main morphological features of *Hysterothylacium* larval type V from the Aegean Sea. A. Anterior end of *Hysterothylacium* larval type V (scale: 200 µm), B. Anterior end detail of *Hysterothylacium* larval type V (scale: 50 µm), C. Oesophagus–ventriculus–intestine junction of *Hysterothylacium* larval type V (scale: 100 µm), D. Posterior end of *Hysterothylacium* larval type V (scale: 50 µm). E) Detail of the tail showing the mucron of *Hysterothylacium* type V (scale: 50 µm). a: Anus, i: Intestine, ic: Intestinal caecum, oe: Oesophagus, v: Ventriculus, va: Ventricular appendix and m: Mucron.

Host*: *Spicara maena

***Site of Infection*:** Abdominal cavity

***Prevalence and Intensity*:** P: 10% (1/10), MI: 1, a number of larvae: 1

***Molecular Data*:** A single specimen (OZ–4) underwent sequencing of the complete ITS region, yielding a sequence of 820 bp. The OZ–4 was deposited in GenBank with accession number OQ971708. The entire ITS region showed 100% identity with *Hysterothylacium* larval type V sequences (KX815378, KU594481, KU594482) previously reported from the Tunisian Mediterranean (14) and the Brazilian Atlantic coasts (16). ITS tree grouped the larval morphotype V (OZ–4) with *H. deardorffoverstreetorum* larvae (JF730199, MK039161) and *Hysterothylacium* larval type V (KX815378, KU594481), supported by a strong bootstrap value (14, 16) (Figure 4).

***Hysterothylacium* Larval Type VIII**

***Morphological Description*:** Ten third-stage larvae were examined. The body length was averaged 9.8 mm (range: 9.2–11.2 mm) with an average width of 184 µm (range: 155–210 µm). Developed labia were absent. A boring tooth was present. The nerve ring and excretory pore were located 317 µm (range: 292–349 µm) and 431 µm (range: 385–438 µm), respectively, from the anterior part. The oesophagus, measuring 1038 µm (range: 810–1496 µm), comprised 10.5% of the total body length. The ventriculus was almost sub-spherical, with a length of 45 µm (range: 39–58 µm). The ventricular appendix measured 458 µm (range: 391–511 µm), representing 44.1% of the oesophageal length, while the intestinal caecum measured 392 µm (range: 332–465 µm) and represented 37.7% of oesophageal length. The ratio of intestinal caecum to ventricular appendix length was 1:0.85. The conical tail, 163 µm in length (range: 120–211

µm), represented 1.6% of the body length and terminated in a single prominent spine (mucron) (Figure 3).

***Host*:** *Boops boops, Engraulis encrasicolus, Sardina pilchardus*

***Site of Infection*:** Abdominal cavity

***Prevalence and Intensity*:** P: 7.14% (1/14), MI: 3, a number of larvae: 3 in *B. boops*; P: 50% (10/20), MI: 1.9, a number of larvae: 19 in *E. encrasicolus*; P: 20% (2/10), MI: 2, a number of larvae: 4 in *S. pilchardus*

***Molecular Data*:** Sequencing of the entire ITS region was performed on two specimens

(OZ–5 from *E. encrasicolus* and OZ–6 from *B. boops*), producing 924 bp sequences with no intraspecific nucleotide differences. The sequence for larval morphotype OZ–5 was deposited in GenBank with accession number OQ971707. We found that the ITS–1 and ITS–2 sequences from our samples shared 100% similarity with *Hysterothylacium* larval type VIII sequences (ITS–1: MF693073–MF693078; ITS–2: MF693089–MF693094) previously reported in the North-East Atlantic Ocean (21). Furthermore, our ITS–1 and ITS–2 sequences were identical to adult *H. aduncum* sequences (ITS–1: MF693081–MF693085 and ITS–2: MF693097–MF693101 (21). The complete ITS region of OZ–5 showed 100% identity to larval type VIII (MZ313180) and *H. aduncum* larvae (JX413596, JX413597) in the Turkish Black Sea coasts (6, 17). Phylogenetic analysis using an NJ tree confirmed that larval morphotype VIII (OZ–5) clustered with *H. aduncum* adults (MW131974, MF693084–MF693100), *H. aduncum* fourth–stage larvae (JX413596), and *Hysterothylacium* types VIII (MF693073–MF693089, MZ313180) and IX (MF693080–MF693096, MZ313183) (6, 17, 21) (Figure 4), suggesting a consistent genetic profile across different geographic regions.

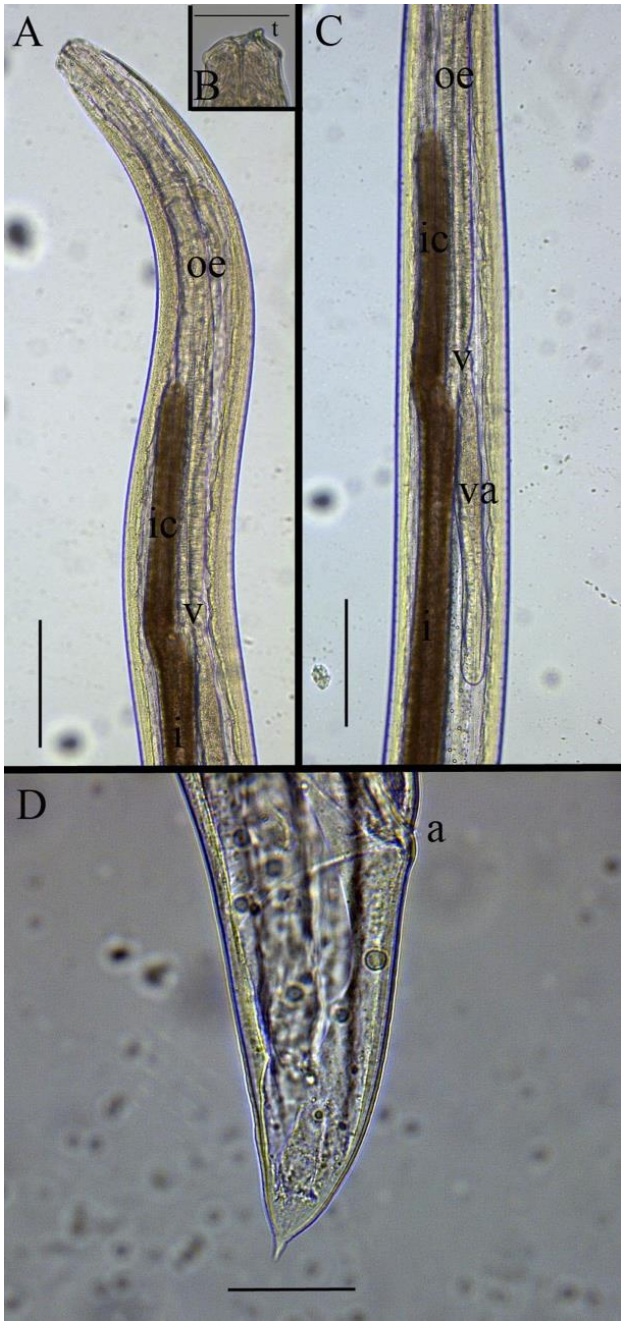

Figure 3. Microphotographs showing main morphological features of *Hysterothylacium* larval type VIII from the Aegean Sea. A. Anterior end of *Hysterothylacium* larval type VIII (scale: 200 µm), B. Anterior end detail of *Hysterothylacium* larval type VIII (scale: 50 µm), C. Oesophagus–ventriculus–intestine junction of *Hysterothylacium* larval type VIII (scale: 200 µm), D. Posterior end of *Hysterothylacium* larval type VIII (scale: 50 µm). a: Anus, i: Intestine, ic: Intestinal caecum, oe: Oesophagus, t: Boring tooth, v: Ventriculus and va: Ventricular appendix.

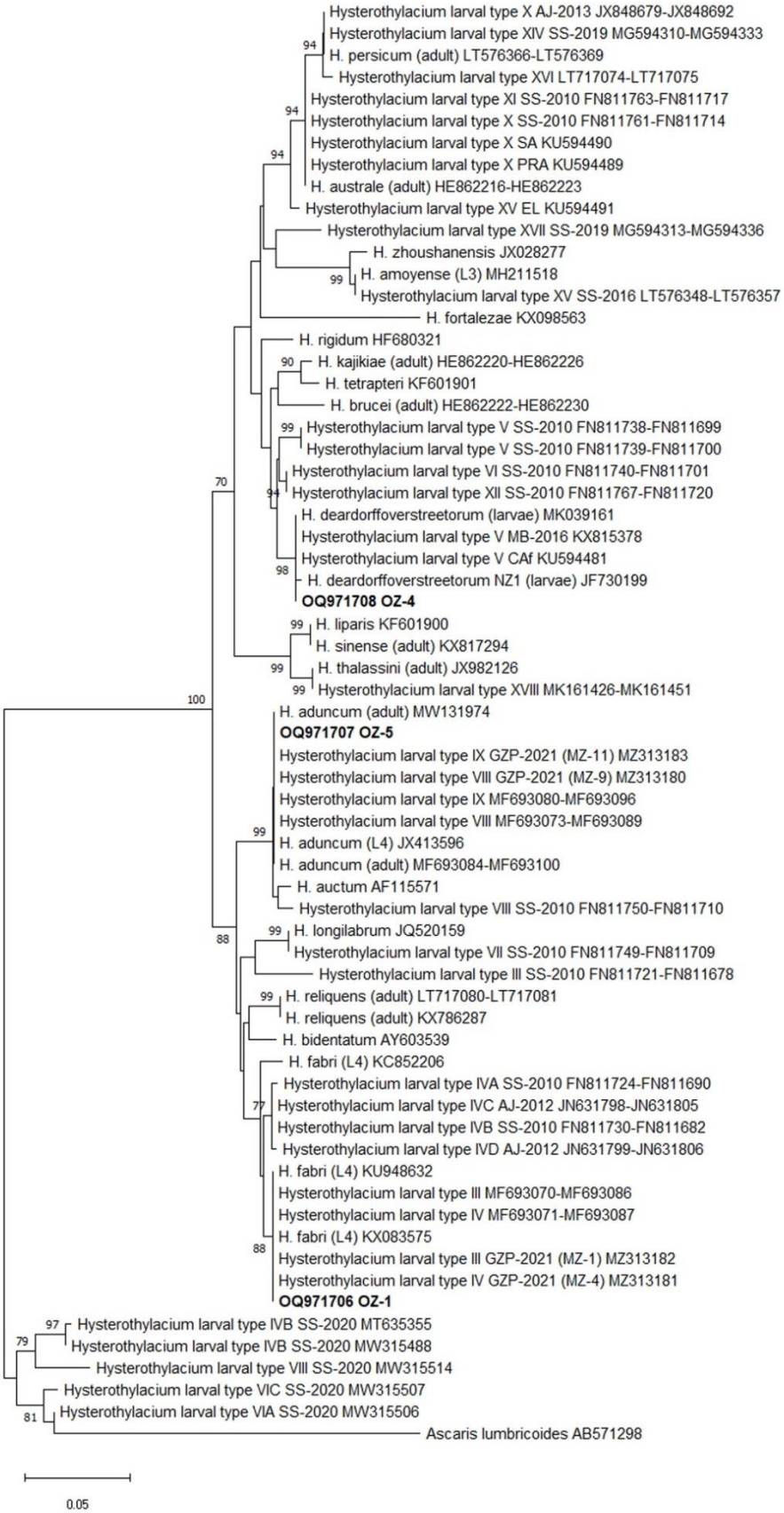

Figure 4. Phylogenetic relationships between *Hysterothylacium* larval types IV, V, and VIII from the Aegean Sea and other *Hysterothylacium* species as inferred by Neighbor-Joining tree obtained from combined ITS–1 and ITS–2. If one number or two numbers after taxa are presented, one number refers to ITS (ITS–1 and ITS–2) regions and two numbers indicate ITS1 and ITS2 sequences, respectively. The scale bar indicates the distance in substitutions per nucleotide. Bootstrap values were calculated over 10 000 replicates and percentages ≥ 70% are shown at the internal nodes.

Discussion and Conclusion

This study provides morphological and genetic descriptions of three *Hysterothylacium* morphotypes IV, V, and VIII found in four species of marine fish from the Aegean Sea (FAO fishing area 37.3.1). Currently, there is a limited understanding of the specific identities and taxonomic relationships of *Hysterothylacium* morphotypes in the Aegean Sea. Therefore, the entire ITS region of these morphotypes from the Aegean Sea was sequenced, representing their first molecular characterization. Previous studies have reported the presence of *H. fabri* larvae and *Hysterothylacium* spp. larvae in various marine fish species from the Aegean Sea (4, 18, 30); however, these studies did not provide specific identification of larval morphotypes. Our study demonstrates that *Hysterothylacium* morphotypes IV and VIII in the Aegean Sea represent the fourth and third stages larvae of *H. fabri* and *H. aduncum*, respectively. Currently, genetic analyses have established a connection between *Hysterothylacium* morphotypes VIII and IX and well-characterized *H. aduncum* adults from the North-East Atlantic Ocean (21). Furthermore, the whole ITS sequence obtained for our *Hysterothylacium* morphotype VIII (OQ971707) matched perfectly with the sequences reported for *Hysterothylacium* morphotype VIII genotype B (21). Although *Hysterothylacium* morphotypes III and IV have been morphologically described, their specific molecular identity remains unknown, as they have not yet been definitively matched to adult *Hysterothylacium* species using molecular data (10, 11, 21, 24). The only previous molecular identification of fourth-stage *H. fabri* larvae (KC852206) was achieved by sequencing the entire ITS region from a *Zeus faber* specimen collected in the Aegean Sea (18). Our analysis of *Hysterothylacium* larval type IV (OQ971706), obtained in this study, revealed 98.9% sequence similarity with the complete ITS region (KC852206) previously identified in fourth-stage *H. fabri* larvae in the Aegean Sea (18). No sequences for morphotype IV from the Aegean Sea are currently available in GenBank. Analysis of the complete ITS sequence of *Hysterothylacium* larval type IV (OQ971706) in this study revealed a 100% sequence identity with *Hysterothylacium* larval type IV sequences (ITS–1: MF693071; ITS–2: MF693087) previously characterized from the Spanish Mediterranean coast (21). *Hysterothylacium* larval morphotype IV is known to exhibit ITS sequence variations, leading to its classification into five distinct genotypes (A through E) (10, 11, 12, 21, 24). Based on our findings, the larval type IV (OQ971706) isolated from the Aegean Sea was assigned to genotype E.

Our type V represents the first documented occurrence of this morphotype in the Aegean Sea (FAO fishing area 37.3.1). The BLASTn search indicated that our type V sequence did not correspond to any available adult *Hysterothylacium* spp. sequences in GenBank. Moreover, our type V from the Aegean Sea exhibited 99.6–100% sequence identity with *H. deardorffoverstreetorum* from Brazil, as reported by Pantoja et al. (16). The species status of *H. deardorffoverstreetorum* is debated, as it was described based solely on third and fourth larval stages, and adult morphology is essential for proper species identification (16). In our opinion, the species identity of *Hysterothylacium* type V larvae cannot be definitively resolved without genetically characterizing and matching them to a reliably identified adult specimen. This larval type has previously been reported from *Caulolatilus chrysops, Gymnothorax vicinus, Lagocephalus laevigatus, Lutjanus carponotatus, Menticirrhus americanus, Merluccius hubbsi, Mullus barbatus, M. surmuletus, Pagellus erythrinus, Paralichthys isosceles, Prionotus punctatus, Zenopsis conchifer* in Australian, Brazilian, and Tunisian Mediterranean waters (14, 16, 24). This study also reports *Spicara maena* as new hosts for larval type V.

A consumer from Spain experienced epigastralgia associated with larval type VIII, identified as the third larval stage of *H. aduncum* (8). The present study found this morphotype in three edible fish species within the Aegean Sea: *Boops boops*, *Engraulis encrasicolus*, and *Sardina pilchardus*. The presence of this potential zoonotic larval type in these fish species may pose a public health risk. Except for the Aegean Sea, this larval type has been frequently reported in *E. australis, E. encrasicolus, Merlangius merlangus, Micromesistius poutassou, Mullus barbatus ponticus, Neoplatycephalus richardsoni, Platycephalus richardsoni, P. bassensis, P. fuscus, Sardinops sagax, S. neopilchardus, Scomber australasicus, Seriola hippos, S. lalandi, Sillago flindersi*, and *T. trachurus* from the North-East Atlantic, Western Mediterranean, Black Sea, and Australian coastal waters (6, 10, 11, 12, 16, 21).

In conclusion, using a comprehensive approach, this study identifies larval *Hysterothylacium* morphotypes in the Aegean Sea and presents the first report of complete ITS data for larval morphotypes IV, V, and VIII in this region. Notably, *S. maena* is identified as a new host for *Hysterothylacium* morphotype V. The combined use of morphological and molecular methods provides a more comprehensive assessment of *Hysterothylacium* taxonomy and diversity in the Aegean Sea.

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Ethical Statement

Not applicable.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

All authors have contributed equally to all aspects of this study, including the conception, methodology, data analysis, and manuscript preparation.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request. Nucleotide sequences generated in this study were deposited in the GenBank database under the accession numbers OQ971706-OQ971708.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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