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Hysterothylacium amoyense (Nematoda: Raphidascarididae) infecting *Trichiurus lepturus* (Scombriformes: Trichiuridae) from Demak, Central Java, Indonesia

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Abstract. *Utami AMR, Murwantoko, Istiqomah I, Triyanto, Setyobudi E. 2022.* Hysterothylacium amoyense *(Nematoda: Raphidascarididae) infecting* Trichiurus lepturus *(Scombriformes: Trichiuridae) from Demak, Central Java, Indonesia. Biodiversitas 23: 1030-1037.* This study aims to determine parasitic infection and identify the larvae of anisakid nematode found on the hairtail *Trichiurus lepturus* caught on the northern coast of Demak District, Central Java Province, Indonesia. One hundred seventy-eight hairtail samples were collected from fishermen who caught the fish at the Java Sea north coast of Demak. Fish specimens were measured (length) and weighed, then examined for anisakid larvae examination in the abdominal cavity, internal organs, and muscle. Data analysis included prevalence, mean intensity, and distribution of anisakid larvae on target organs. Selected samples of collected anisakid larvae were identified molecularly using PCR direct sequencing method. The results showed that the hairtail at the northern coast of Demak District was susceptible to anisakid larvae infection, on the category "frequently infected" with the prevalence of 56% and low mean intensity of infection of 2.43 larvae/host. Anisakid larvae were mainly found in the abdominal cavity (67%); followed by the digestive tract (19%), liver (13%), and gonads (1%). There were no anisakid larvae infecting the muscle tissue. Molecular analyses identified the anisakid infecting *Trichiurus lepturus* as *Hysterothylacium amoyense*.

Keywords: Anisakid, *Hysterothylacium*, intensity, nematode, prevalence

INTRODUCTION

Anisakid nematodes are frequently found as parasites of various marine organisms. Anisakid worms have a complicated life cycle that involves crustaceans, squid, fish, fish-eating birds, and marine mammals that act as intermediate and final hosts (Smith and Wooten 1978). The transmission of anisakid nematodes occurs throughout the food web at different trophic levels (Angeles-Hernandez et al. 2020). Anisakid nematodes consist of representatives of the Anisakidae and Raphidascarididae (Fagerholm 1991), with several important genera related to their presence as fish parasites, such as the genera *Anisakis*, *Pseudoterranova*, *Contracaecum*, and *Hysterothylacium*. The genus *Anisakis* is the most commonly studied; however, other genera might also have equal impact and importance. For example, currently, around 101 species of *Hysterothylacium* have been described (Bezerra et al. 2020) and reported to parasitize marine fish (Shamsi et al. 2011; Li et al. 2013; Guo et al. 2014; Cavallero et al. 2015; El-Asely et al. 2015; Kong et al. 2015; Gazzonis et al. 2017; Barcala et al. 2018; Chen et al. 2018; Khammassi et al. 2020).

Anisakid is one of the parasite groups that draws attention to its presence in fisheries products due to its zoonotic potential (EFSA-BIOHAZ 2010). Humans become accidental hosts by consuming raw or undercooked seafood infected by larval anisakid nematodes. Although these nematodes cannot complete their life cycle in the human body, their presence can cause disease problems or initiate immune hypersensitivity or allergic reaction (Klimpel and Palm 2011). Among anisakid nematodes, the genus *Anisakis*, *Pseudoterranova* and *Contracaecum* get much more attention due to their pathogenicity and impact on human health (Mehrdana and Buchmann 2017). Nevertheless, *Hysterothylacium aduncum* was reported infecting a 55-year-old man in Japan after consuming raw cod (*Gadus macrocephalus*) with complaints of chronic abdominal pain and diarrhea (Yagi et al. 1996). Besides their responsibility to health impact, i.e., a food-borne zoonosis and allergic reactions, the presence of anisakid nematodes causes unattractiveness and consumer distrust of fisheries products, as well as loss of economic profits in the fisheries industry (Bao et al. 2019). On the other hand, anisakid nematodes have been applied as biological indicators for some ecological studies. Farjallah et al. (2008) investigated the presence of *Anisakis* spp. and its possibility for stock discrimination of several marine species at the North African coasts of the Mediterranean Sea. Mattiucci et al. (2018) studied the Atlantic herring (*Clupea harengus*) stock from North-East Atlantic fishing grounds based on the population genetic structure of *Anisakis* spp. The variation on anisakid infection has also been used to describe the stock of common squid (*Todarodes pacificus*) along the Korean Peninsula (Setyobudi et al. 2013).

The Northern Coast of Java (Fisheries Management Area 712) is one of the areas with a relatively high level of fish exploitation, especially of demersal fish resources.

Ministerial Decree of Maritime and Fisheries Number 50 of 2017 recorded that the potential source of demersal fish in the Java Sea is 657,525 tons/year. It has been exploited by 526,020 tons/year or 83% of the Total Allowable Catch (TAC). Demak is one of the regencies with enormous fisheries resources regarding both demersal and pelagic fish. The fisheries resource commodities in the northern coast of Demak District are very diverse, consisting of hairtail, pomfret, mackerel, tuna, shrimp, anchovies, mackerel, ponyfish, snapper, bigeye, threadfin, and squid (Demak District Central Statistics Agency 2019).

Hairtail (*Trichiurus* spp.) is an economically important demersal fish that is an export commodity globally (FAO 2020). Anisakid larval infections on hairtail have been widely reported (Borges et al. 2012; Kong et al. 2015; Kim et al. 2016; Youssir et al. 2017; Sonko et al. 2020), with the different species of anisakid infecting each region. Borges et al. (2012) reported *Hysterothylacium* sp. and *Anisakis typica*, infecting hairtail from Rio de Janeiro coast, Brazil. Kim et al. (2016) noted *Anisakis pegreffii* was the most dominant (98.7%) infecting the large head hairtail in Korea, besides another anisakid such as *Hysterothylacium* sp. and a hybrid genotype (*A. simplex* × A. *pegreffii*). Observation on hairtail collected from several locations on the East China Sea and the Pacific coast of central Japan showed the differences in the prevalence and species of anisakid infecting those fish. The presence of *Anisakis simplex* s.s., *A. pegreffii*, *A. typica*, *Hysterothylacium* sp., *H. aduncum*, *H. amoyense*, *H. fabri*, and the recombinant genotype of *Anisakis simplex* s.s. and *A. pegreffii* have been recorded (Kong et al. 2015).

Anisakid larvae have also been reported to infect hairtail in Indonesian waters (Setyobudi et al. 2007; Setyobudi et al. 2011; Palm et al. 2017; A'yun et al. 2021). Such studies were primarily conducted on the southern coast of Java and found only anisakid from the genus *Anisakis*. Similar studies in the northern coast of Java have not been widely carried out. Therefore, this study aims to determine the infection and molecular identification of anisakid on hairtails (*T. lepturus*) from the northern coast of Demak District Central Java. In this study, we reported for the first-time infection of anisakid from the genus *Hysterothylacium* on hairtails (*T. lepturus*) from the studied location.

MATERIAL AND METHOD

Study area

Fish sampling and parasite collection

In total, 178 samples of hairtail were obtained from fishermen. The fish were caught on the northern coast of Demak District, Central Java Province, Indonesia (Figure 1).

The fish samples were weighed using an analytical balance and the length was measured using a ruler. Then, the fish were dissected and screened for parasites in the abdomen, internal organs, and muscle. The presence of anisakid was observed in the abdominal cavity, digestive tract, liver and gonad. The anisakid larvae were observed for their external appearance and morphology, namely color and size. Anisakid larvae are characterized by cylindrical body shape, pointed at the tip of the body, white or yellowish in color, coiled in the fish's body cavity, and 15-25 mm in length (Rahma et al. 2016). Anisakid nematode larvae were washed up with physiological saline and preserved with absolute ethanol for further molecular identification.

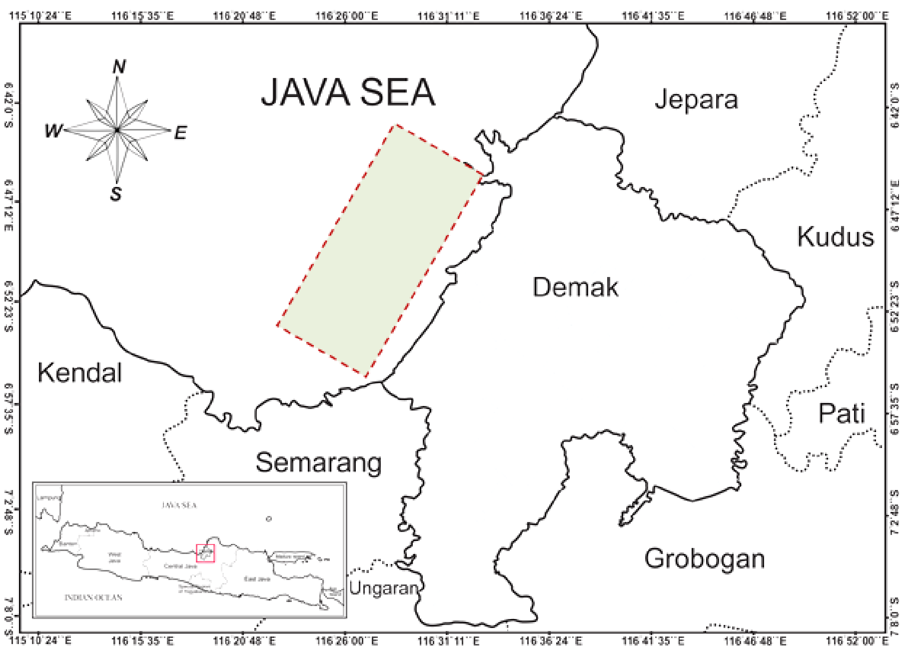

Figure 1. Predicted fishing ground of *Trichiurus lepturus* capture from northern coast of Demak District, Central Java, Indonesia

Molecular identification

Molecular identification was conducted by the PCR Direct Sequencing method. DNA genome of Anisakid larvae was extracted using DNA Mini Kit Tissue Protocol. The ITS rDNA region (ITS1-5.8S-ITS2) region was amplified using PCR with primer A (forward) (5'- GTC GAA GGT GAA TTC GTA CCT GAA GCG GGA TCA - 3') and primer B (reverse) (5'- GCC GGA TCC TCC GAA TGG TTA TCT TTT GTT CCT-3') (D'Amelio et al. 2000). Amplification of DNA was carried out in the following conditions: pre-denaturation at 95°C for 10 minutes, following 30 cycles: denaturation at 95°C for 40 seconds, annealing at 55°C for 40 seconds, extension at 72°C for 75 seconds, and final extension at 72°C for 7 minutes. The amplification products were then electrophoresed to determine the presence of target amplified DNA fragments. DNA amplification products were sequenced in Singapore's 1st base laboratory through PT. Genetika Science Indonesia. The nucleotide sequences were processed with Bioedit and Mega X software. BLAST analysis was performed to determine the anisakid species and determine similarities to other species previously reported in GenBank on the ncbi.nlm.nih.gov website. The phylogenetic tree of the sequences with several other anisakid DNA sequences was constructed using Mega X software (Kumar et al. 2018).

Data analysis

Data analysis consisted of the prevalence, mean intensity, and target organ of infection. The prevalence (P) was calculated based on the number of fish infected with parasites divided by the total of fish examined (%), whereas the mean intensity (MI) was calculated as an infection average of parasites on the infected fish (larvae/individual) (Bush et al. 1997). The relationship between host length and intensity of infection was determined by correlation analysis using Microsoft Excel.

RESULTS AND DISCUSSION

Results

A total of 178 hairtail samples with lengths ranging from 35-64 cm and weight between 22.3-248.3 grams were used in this study. A total of 243 anisakid nematode larvae were collected, with red-brown and white-brown color and length ranging from ±1-3 cm. The larva has a conical tail and is relatively short in size at the posterior part. The hairtail *T. lepturus* in the northern coast of Demak District is susceptible to infection with anisakid nematodes with a moderate prevalence of 56% and mean intensity of 2.43 larvae/individual.

Figure 2 shows that most of the *T. lepturus* fish were infected with anisakid larvae with a low intensity (≤5 larvae/individual), which was 91%. As much as 8% of *T. lepturus* fish had an infection intensity of 5-10 larvae/individual, and only 1% were infected with high-intensity anisakid (>10 larvae/individual).

The distribution of anisakid infections with the highest percentage to the lowest was the abdominal cavity (67%), digestive tract (19%), liver (13%), and gonads (1%) (Figure 3).

The correlation between the length of hairtail and the intensity of anisakid infection following the equation y = 0.0161x + 1.6942 and with a coefficient of determination R^2: 0.0017, indicates that the number of parasites that infect hairtails are influenced by fish length as much as 0.17%, while the rest is influenced by factors other (Figure 4).

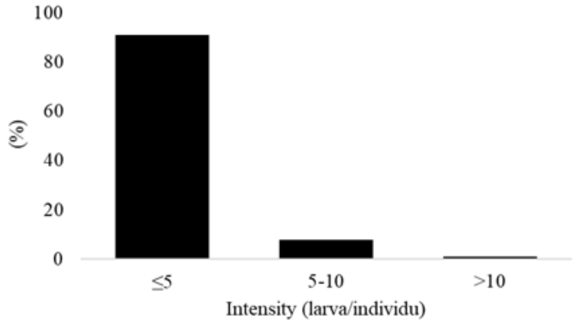

Figure 2. Distribution intensity of anisakid nematode on *Trichiurus lepturus* from the northern coast of Demak District, Central Java, Indonesia

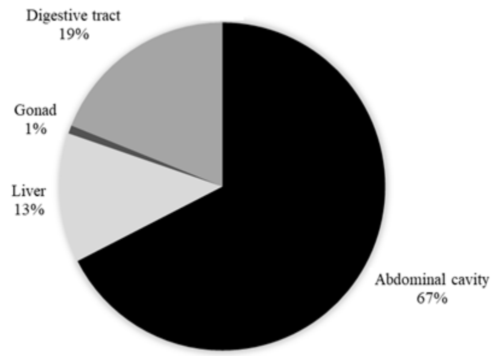

Figure 3. Distribution of anisakid larvae infection location on *Trichiurus lepturus* from the northern coast of Demak District, Central Java, Indonesia

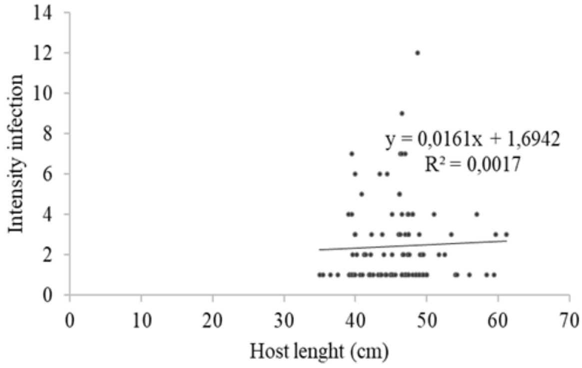

Figure 4. The relationship of fish host length and intensity of anisakid larvae infection

The results of anisakid nucleotide sequencing with the target area of ITS region had a nucleotide sequence length of about 900 bp (974 bp, 919 bp, and 920 bp) with nucleotide differences in 8 sites (Figure 5). The nucleotide sequences from this study were submitted to Genbank under the accession numbers: OL376800, OL376801, and OL376802 (Figure 6).

The anisakid nematode infecting *T. lepturus* was molecularly identified as *Hysterothylacium amoyense*, with a similarity of 99.45-99.56% with the same species from other regions (Table 1). The genetic relationship of *H. amoyense* found in *T. lepturus* on the northern coast of Demak District with other anisakid nematodes is shown in Figure 7.

Anisakid nematodes infecting *T. lepturus* on the northern coast of Demak District were in the same cluster as *H. amoyense* from Iraq and China.

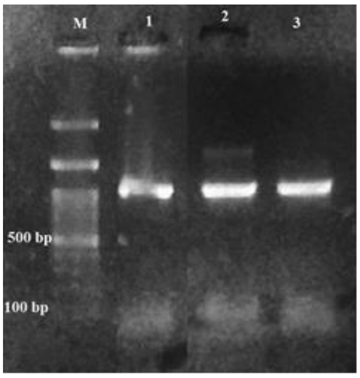

Figure 5. PCR visualization using the rDNA ITS region of anisakid larvae on *Trichiurus lepturus* from the northern coast of Demak District, Central Java, Indonesia. Note: M: Marker; 1-3: Anisakid sample from *Trichiurus Lepturus*

Table 1. BLAST results of anisakid nematodes infecting *Trichiurus lepturus* from the northern coast of Demak District, Central Java, Indonesia

Acc. no.	Spesies	Site	Query cover	Per. Ident (%)
MT269312	*Hysterothylacium amoyense*	China	100	99.46
MW411818	*Hysterothylacium amoyense*	Iraq	100	99.46
MW404622	*Hysterothylacium amoyense*	Iraq	100	99.46
MH211527	*Hysterothylacium amoyense*	Iraq	100	99.46
MZ509280	*Hysterothylacium* sp.	Iraq	100	99.56
MH211555	*Hysterothylacium zhoushanense*	China	100	98.26
KP252133	*Hysterothylacium amoyense*	China	99	99.56
MF539812	*Hysterothylacium amoyense*	China	99	99.45

Discussion

Hairtail (*T. lepturus*) caught on the northern coast of Demak District was susceptible to infection with anisakid larvae. The number of larvae collected was 243 larvae with lengths ranging from ±1-3 cm. *Hysterothylacium* larvae can reach 3-5 cm in length with a whitish color (Li et al. 2008). *Hysterothylacium* is characterized by the anterior end with 3 lips; dorsal lip slightly smaller than subventrals; a conical tail and relatively short at the posterior part (Li et. 2007), excretory pore at slightly posterior of nerve ring, presence of intestinal caecum and ventricular appendix (Guo et al. 2020). The prevalence of anisakid nematode infection found in hairtails on the Northern Coast of Demak District was 56%, and the mean intensity of infection was 2.53 larvae/individual. The prevalence of anisakid infection found in hairtails at the northern coast of Demak District was in the category of very frequent infection (56%).

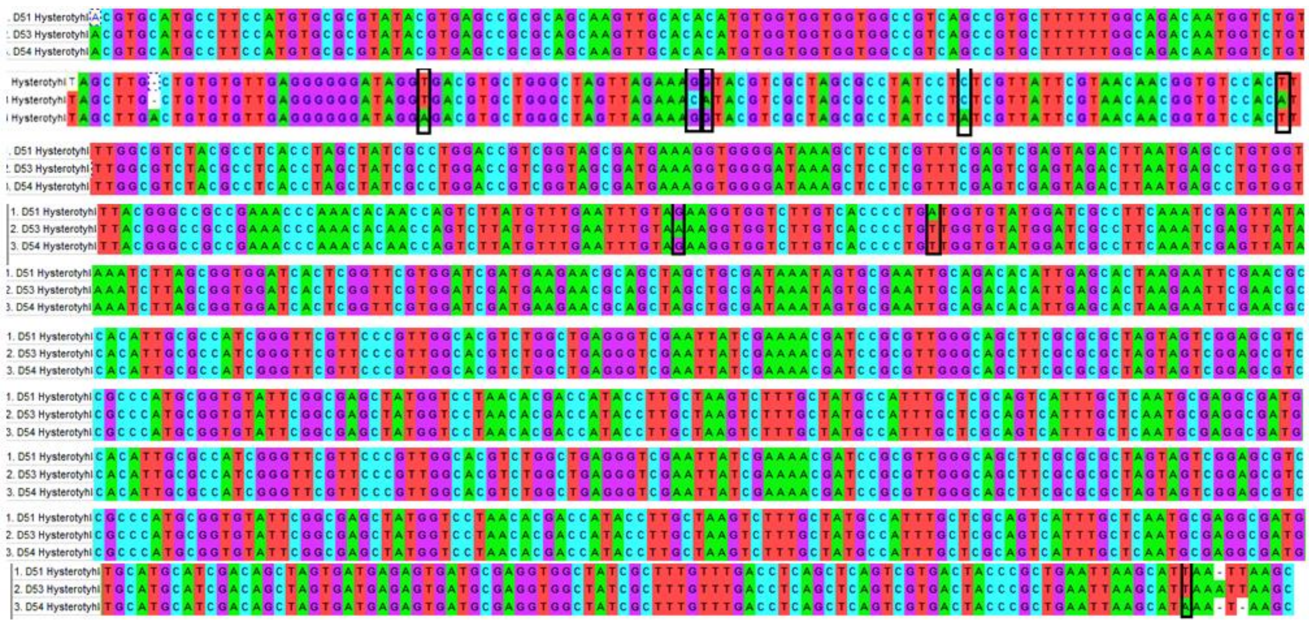

Figure 6. The result of nucleotide alignment of the three sequences of anisakid nematodes of hairtails (*Trichiurus lepturus*) from the present study

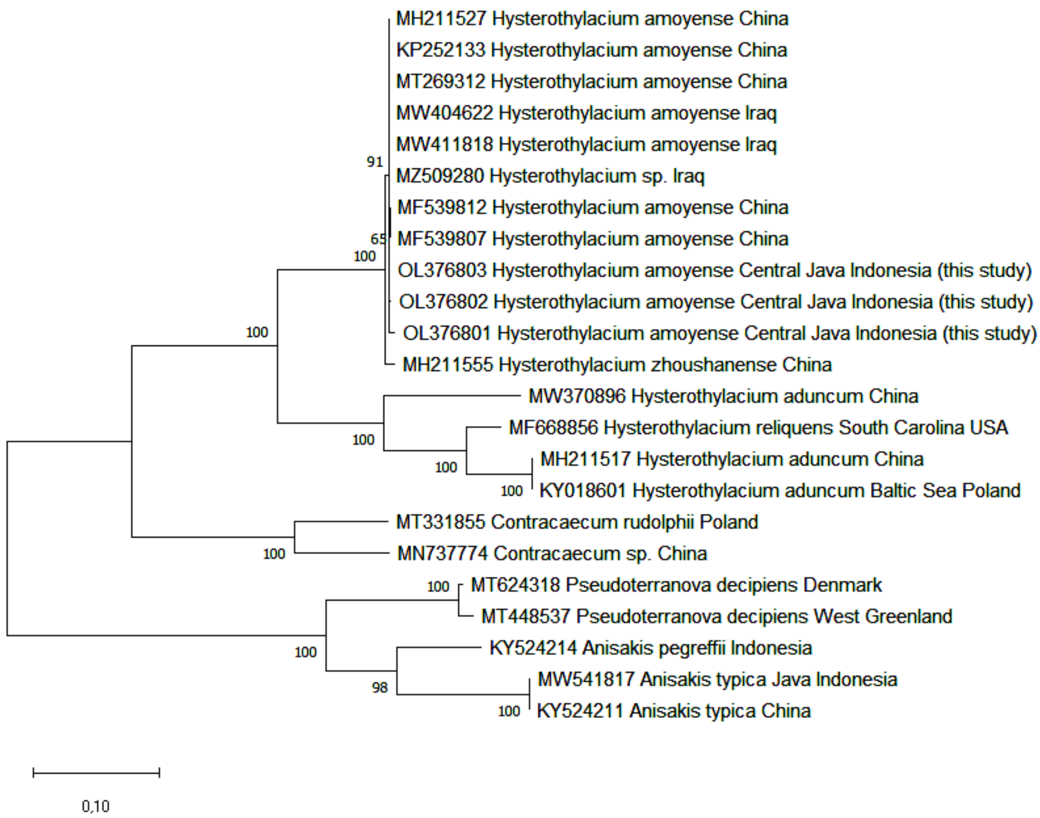

Figure 7. Molecular phylogenetic tree showing genetic relationships between samples of *Hysterothylacium amoyense* species, based on rDNA ITS region. The phylogenetic tree was created using the Maximum Likelihood method according to the Tamura-Nei model (bootstrap: 1000)

However, the mean intensity of infection was at a low level (2.53 larvae/individual). A similar level of anisakid larvae infection, moderate prevalence, and low mean intensity was also reported on *T. lepturus* originating from Pangandaran waters, West Java (P: 45.5%; MI: 4.4 larvae/individual) (A'yun et al. 2021). As a comparison, different infection levels were found on hairtails (*T. lepturus*) caught at Rio de Janeiro coast, with a prevalence of 51.56%, but with a high intensity of 55 larvae/individual (Borges et al. 2012). Differences in infection may be possible due to regional differences and anisakid host preferences (Shamsi et al. 2011).

The prevalence and mean intensity of anisakid based on body length classes showed various values. One of the main factors affecting parasitism for almost all fish species is host size (in terms of length and weight) (Debenedetti et al. 2019). A'yun et al. (2021) mentioned that anisakid infection levels in terms of prevalence and intensity increased in line with increasing the body length of *T. lepturus*. The older fishes have a longer time preying on infected small fish throughout the food web, taking a higher risk of infection and acting as accumulating hosts compared to small host fishes. Thus, the prevalence and intensity of infection will increase with the age of the fish (Abattuoy et al. 2011). Another factor affecting anisakid larvae infection is the host food and feeding habits. Rohit et al. (2015) mentioned that fishes and crustaceans are the main prey of *T. lepturus*. Small marine crustaceans play an essential role as an intermediate host in the life cycle and distribution of *Hysterothylacium* (Koie 1993). Increasing in the prevalence of anisakid can also occur due to the reproduction period of *T. lepturus*. Behavior in preying small fish and marine crustacea will increase due to the energy requirements used for the reproductive process (Borges et al. 2012).

In the present study, anisakid larvae were mostly found infecting hairtails (*T. lepturus*) in the body cavity (67%). Other target organs were the digestive tract by 19%, liver by 13%, and gonads by 1%. Anisakid nematodes enter the host's body by being swallowed by the host, survive in the digestive tract, and possibly migrate to the body cavity (A'yun et al. 2021). Other studies also showed a similar result of the target organ of anisakid larvae infection on hairtails, i.e., mostly in the body cavity (Setyobudi et al. 2011; Kong et al. 2015; A'yun et al. 2021). As additional information, the most prominent site of infection of anisakid larvae in hairtails from Korea was found in the viscera (Kim et al. 2016). Differences in abundance can be influenced by parasite species, fish age, infected fish species, and environmental conditions. The adult stage of the genus *Hysterothylacium* lives in the digestive tract of marine fish; however, the larval stage of this genus usually infects invertebrates and marine fish in the body cavity (Koie 1993).

The sequencing of anisakid nucleotide of ITS region resulted in sequences around 900 bp long (974 bp, 919 bp, and 920 bp) with a difference of 8 bp from a total of 920 bp. The study of Costa et al. (2018) showed that the length of the *Hysterothylacium* nucleotide sequence for the ITS area is approximately 900 bp. The BLAST analyses confirmed that the anisakid samples isolated from *T. lepturus* caught from the northern coast of Demak District was *H. amoyense* species. The highest similarity with those species was 99.46%, with Accession Number MT269312.1 originating from Chinese waters, MW411818.1, and MW404622.1 from Iraq waters. The phylogenetic tree based on the ITS region between nematodes forms two main clades: the first clade is an outgroup with species from the genera *Anisakis* and *Pseudoterranova*; the second clade is divided into two: one clustering together *Contracaecum* spp., and another clustering species of *Hysterothylacium*, which consists of *H. amoyense*, *H. zhoushanense*, *H. aduncum*, and *H. reliquens* (Figure 7).

Hysterothylacium is a nematode commonly reported to infect freshwater, estuarine and marine fish (Simsek et al. 2018). The adult stage *Hysterothylacium* could live in the digestive tract of fish, with fish acting as definitive hosts (Koie 1993). The genus *Hysterothylacium* includes at least 67 species with high morphological variability, both in larval and adult form. Several *Hysterothylacium* larvae have been characterized molecularly and synchronized with the adult form (Shamsi et al. 2013; Roca-Geronès et al. 2018; Hossen and Shamsi 2019). Intraspecific genetic differences may occur due to host variability and environmental changes. The species of the families Anisakidae and Raphidascarididae exhibit various intermediate hosts and low host specificity, resulting in wide geographical distribution (Simsek et al. 2018). *Hysterothylacium* has also been reported to infect Indonesian marine fishes. Theisen (2019) noted the presence of *Hysterothylacium* sp. on several economically essential fishes such as *Sardinella lemuru*, *Caesio cuning*, *Selar crumenophthalmus*, *Decapterus russelli*, *Atule mate*, *Scomberoides tol*, *Selaroides leptolepis* and *Selar boops*. The larval stage of *Hysterothylacium* sp. has also been reported infecting white-streaked grouper (*Epinephelus ongus*) from the Karimunjawa Island (Neubert et al. 2016) and Kepulauan Seribu, Java, Indonesia (Koepper et al. 2020).

In this study, we conducted identification based on the sequences of the rDNA ITS region. *Hysterothylacium amoyense* has been reported to infect several fish species, including the demersal fish's bar tail flathead, *Platycephalus indicus* (Najjari et al. 2016); white-spotted conger *Conger myriaster* (Chen et al. 2018); and Japanese threadfin bream, *Nemipterus japonicus* (Guo et al. 2020). Previous studies on anisakid nematode infection on *T. lepturus* found only the genus *Anisakis*, namely *A. typica* (Setyobudi et al. 2011; Palm et al. 2017; A'yun et al. 2021). In this study, we confirmed the presence of *H. amoyense* in hairtails (*T. lepturus*) in the Java Sea.

Anisakid nematode infection can affect the fish quality and value, resulting in economic losses for the fishing industry (Bao et al. 2019). Research on the occurrence, host species, identification, and zoogeography of anisakid nematode in Indonesian marine waters is still rare. Moreover, the life cycle for most species of *Hysterothylacium* remains unclear; the intermediate hosts, definitive hosts, and their life cycle are still unknown (Lopes et al. 2011). Therefore, more research efforts are necessary to elucidate the data and information of anisakid nematode infection and distribution, including members of the genus *Hysterothylacium*.

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