HAYATI
Journal of Biosciences

Vol. 33 No. 2, March 2026 345-359
DOI:10.4308/hjb.33.2.345-359
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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Molecular Characterization of *Anisakis* spp.: Potential Source of Fish-borne Zoonosis in Coastal Living Environment in Semarang, Indonesia

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ARTICLE INFO

Article history:
Received April 21, 2025
Received in revised form August 8, 2025
Accepted August 17, 2025
Available Online November 10, 2025

KEYWORDS:
Anisakiasis,
Anisakis spp.,
cox2 gene,
genetic diversity

ABSTRACT

Anisakis spp. are marine nematodes known to cause anisakiasis, a zoonotic disease transmitted through fish consumption with diverse clinical manifestations. In Indonesia, a country with extensive marine biodiversity and high fish consumption, data on anisakiasis remain limited. This study aimed to characterize the *Anisakis* spp. from the most significant commercial fish market in Semarang, Indonesia. A total of 17 *Rastrelliger* sp. were sampled. PCR amplification targeting the mitochondrial *COX2* gene was used to identify the species of *Anisakis* spp. molecularly. Phylogenetic relationships, nucleotide diversity, and neutrality indices were assessed. The haplotype structures were visualized using the Haplotype Network. Subsequently, 7 of 17 (41.17%) *Rastrelliger* sp. samples were infected with *A. typica*. Molecular analysis revealed two species, *A. typica* and *H. amoyense*, with high haplotype diversity (1.00±0.016, diversity±SD). Phylogenetic analysis revealed two major clades: *A. typica* (Semarang and Southern Makassar) and *H. amoyense* (Semarang, China, and Bangladesh), with both species indicating high genetic connectivity. Neutrality indices suggested purifying selection and population expansion for both species (dN-dS = -5.017). These findings highlight the genetic variability and zoonotic potential of *Anisakis* spp. in the commercial fish market, emphasizing the need for surveillance of fish-borne parasitic infections in Indonesia.

1. Introduction

Anisakis is well-known for its ability to cause ichthyozoonosis disease (Ozuni *et al*. 2021). The genus *Anisakis* is a vital parasite due to its prevalence in seafood and its adverse impact on human health. Its zoonotic significance has also been globally considered as an emerging zoonotic parasite (Shamsi *et al*. 2017). *Anisakis* is classified in the phylum Nemathelminthes, class Secernentea, order Ascarida, suborder Ascaridina, superfamily Ascaridoidea, family Anisakidae, and subfamily Anisakinae (Aibinu *et al*. 2019). Among the nine species in the genus *Anisakis*,

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only *Anisakis simplex* (s.s.) and *Anisakis pegreffii* were identified as zoonotic pathogens (confirmed by molecular markers) (Mattiucci *et al*. 2018). There were 0.32/100,000 zoonotic cases worldwide in 2021 that are linked to the *Anisakis* species (Orphanet 2023). Anisakiasis, a fish-borne zoonosis, is becoming more widely acknowledged as an emerging human disease. Anisakiasis is a fish-borne parasitic zoonosis caused by the third-stage larval nematodes of the genus *Anisakis* (Mattiucci *et al*. 2013). Abdominal pain, diarrhea, nausea, and vomiting are frequently linked to anisakiasis. However, the clinical presentation of this infection varies, ranging from asymptomatic cases to severe conditions necessitating emergency medical intervention (Nieuwenhuizen and Lopata 2013).

Clinical manifestations of anisakiasis are diverse and often depend on the location and extent of larval invasion. The most commonly reported symptoms include acute abdominal pain, nausea, vomiting, and diarrhea, typically occurring within hours after ingestion of contaminated raw or undercooked seafood (Pontone *et al*. 2012; Kim *et al*. 2018; Patiño & Olivera 2019; Quiñones-Silva *et al*. 2019). In gastric anisakiasis, patients often experience sudden epigastric pain, nausea, and vomiting due to larval penetration of the stomach lining (Pontone *et al*. 2012; Lalchandani *et al*. 2018). In contrast, intestinal anisakiasis may present with more delayed and nonspecific symptoms such as lower abdominal pain, bloating, or signs mimicking appendicitis, Crohn's disease, or intestinal obstruction, sometimes progressing to complications like intussusception or perforation (López-González *et al*. 2010; Baeza-Trinidad *et al*. 2015; Kim *et al*. 2018; Choi *et al*. 2019; Cózar-Bernal *et al*. 2023).

In addition to gastrointestinal involvement, anisakiasis may also present through allergic symptoms such as urticaria, generalized rash, angioedema, and, in severe cases, anaphylaxis, particularly in sensitized individuals (González Quijada *et al*. 2005; Pontone *et al*. 2012; Quiñones-Silva *et al*. 2019). These allergic reactions can occur independently or concurrently with digestive symptoms, a condition known as gastroallergic anisakiasis (Pontone *et al*. 2012; Patiño & Olivera 2019; D'Amelio *et al*. 2023). In rare cases, larvae may migrate to extra-gastrointestinal sites, including the pancreas, liver, or lungs, resulting in atypical presentations and posing diagnostic challenges (Ugenti *et al*. 2004; Dinas *et al*. 2024).

Furthermore, chronic anisakiasis may develop when larval remnants persist in host tissues, inducing prolonged inflammatory responses such as eosinophilic granuloma formation, which can mimic neoplastic or autoimmune gastrointestinal disorders (Bridet *et al*. 2016; D'Amelio *et al*. 2023; Caramello *et al*. 2003). The broad spectrum of these clinical features highlights the importance of considering anisakiasis in the differential diagnosis of acute and chronic gastrointestinal symptoms, particularly in patients with a history of consuming raw or undercooked seafood (Cavallero *et al*. 2018)

Human hosts can be infected by eating raw, smoked, marinated, salted, or undercooked seafood that larval stages have contaminated (Macchioni *et al*. 2021). Consumption of locally caught wild fish and homemade seafood preparations is considered a risk factor for the spread of the zoonotic species (Shamsi 2019). If locally sourced wild fish are not carefully examined, the risk of contamination increases, potentially resulting in the transmission of severe zoonotic disease (Bao *et al*. 2017). Preferred fish species differ amongst nations and communities, and may be consumed raw, partially cooked, fermented, or dried. A few of these preparations have been linked to incidences of human anisakiasis in several nations across the world and may pose a health concern to people (Mattiucci *et al*. 2018). In Europe, particularly in Spain, the incidence is notably high, with approximately 8,000 annual cases of anisakiasis linked to traditional raw fish dishes like marinated anchovies (Fuentes *et al*. 2022). Similarly, in Japan, over 20,000 annual cases of anisakiasis are linked to high raw fish consumption (Takeda *et al*. 2020). Despite these significant numbers, the actual burden of human anisakiasis remains uncertain due to insufficient epidemiological data (Guardone *et al*. 2018).

In Indonesia, specific data on anisakiasis cases remain underreported. However, as the largest archipelagic country with vast marine biodiversity, Indonesia has the potential for a high prevalence of fish parasites like *Anisakis* spp. The high level of fish consumption in Indonesia further increases the risk of anisakiasis, especially as fish is often traditionally or partially cooked in local culinary practices. The study of marine fish parasitology remains understudied in Indonesia, and only a few preliminary findings have been published internationally (Palm *et al*. 2017). Although several studies have identified the presence of *Anisakis* spp. in Indonesian marine fish, most of them have focused primarily on morphological identification or basic molecular detection using

single-gene barcoding techniques (e.g., COI or ITS markers). Comprehensive molecular investigations, such as analyses of genetic diversity, phylogenetic relationships, and neutrality tests (e.g., Tajima's D or Fu's Fs), remain largely unexplored. Molecular characterization plays a crucial role in parasitological studies, particularly for *Anisakis* spp., as it enables accurate species-level identification, which is essential for assessing the pathogenic potential of each species. Some species, such as *A. simplex* and *A. pegreffii*, are known to cause severe anisakiasis in humans, while others have uncertain or lower zoonotic potential. Moreover, analyzing genetic diversity through molecular markers allows for the detection of local or novel strains, which may exhibit different biological behaviors or pathogenicity. Neutrality tests, such as Tajima's D and Fu and Li's statistics, further help to identify signatures of mutation or selection pressure within parasite populations. When these molecular data are integrated, they provide a more comprehensive picture of zoonotic risk, supporting both epidemiological surveillance and food safety management in regions where seafood consumption is high. Thus, this study aims to identify the molecular characterization and infection level of *Anisakis* spp. in commercial fish of coastal areas near Semarang, Indonesia.

2. Materials and Methods

2.1. Sampling Site

The fish samples were obtained from the most significant commercial fish market, called Kobong market, which is located in the center of Semarang City, Central Java Province, Indonesia (Figure 1). This market is known as the biggest supplier of fish around Semarang City. The fish come from the surrounding coastal area in Java. This market is strategically located near the major fishing zones, particularly in the Java Sea, which is known for its abundance of fish species, making the local population potentially exposed to marine-borne parasites, such as *Anisakis* spp.

2.2. Parasites Collection and Processing

A total of 17 fresh marine fish of *Rastrelliger* sp. were obtained from Kobong Market (Figure 2). All samples were briefly weighed using a digital scale, and height was measured with a ruler. The abdomens of fish were dissected horizontally to find the parasites. Once observed, the nematodes were directly isolated and washed in saline solution, pH 7.0, to be examined microscopically and identified based on the morphological characteristics. The selection of *Rastrelliger* sp. (commonly known as Indian mackerel) as the target fish species was based on its high consumption and availability in local markets, making it a relevant source for assessing potential zoonotic risks in the population. Moreover, a previous study has reported that *Rastrelliger* sp. are among the shared hosts of *Anisakis* larvae in tropical and subtropical waters, with a relatively high prevalence observed in several regions, including Indonesia and surrounding marine zones (Setyobudi *et al*. 2019). Prevalence was calculated as the number of infected hosts divided by the total number of fish examined, and intensity as the total number of *Anisakis* larvae. As for the sample's coding criteria, each sample is labeled with "SMG" to indicate its origin from Semarang, followed by a three-digit number representing the collection order (e.g., "008" for the 8th sample). If Anisakidae larvae are found, the letter "A" is added to the code (e.g., SMG-A-008). This system simplifies tracking and analysis of the samples.

2.3. DNA Extraction, Amplification, and Sequencing

Genomic DNA extraction was performed on nematodes isolated from *Rastrelliger* sp. specimens using InstaGene™ Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Nematodes were manually disrupted using pestles and gently mixed with 50 µL of InstaGene™ Matrix. The mixture was incubated at 56°C for 30 minutes and vortexed consecutively every 15 minutes. The mixture was subsequently boiled for 8 minutes and centrifuged at 12.000 rpm for 3 minutes before storage at -20°C. The PCR was used to amplify the mitochondrial *COX2* gene. The *cox2* gene was amplified from the genomic DNA using Promega GoTaq® Green Master mix (Madison, WI, USA) with primers 211F 5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3' and 210R 5'-CAC CAA CTC TTA AAA TTA TC-3'(Mattiucci *et al*. 2011). The amplicon products (± 600 bp and ± 300 bp) were sent for Sanger Sequencing.

2.4. Data Analysis

Evolutionary relationships among the isolates were systematically analyzed and visualized through phylogenetic reconstruction using MEGA 11 software (https://www.megasoftware.net). The phylogenetic analysis employed the Maximum Likelihood method, and the statistical robustness of the tree topology

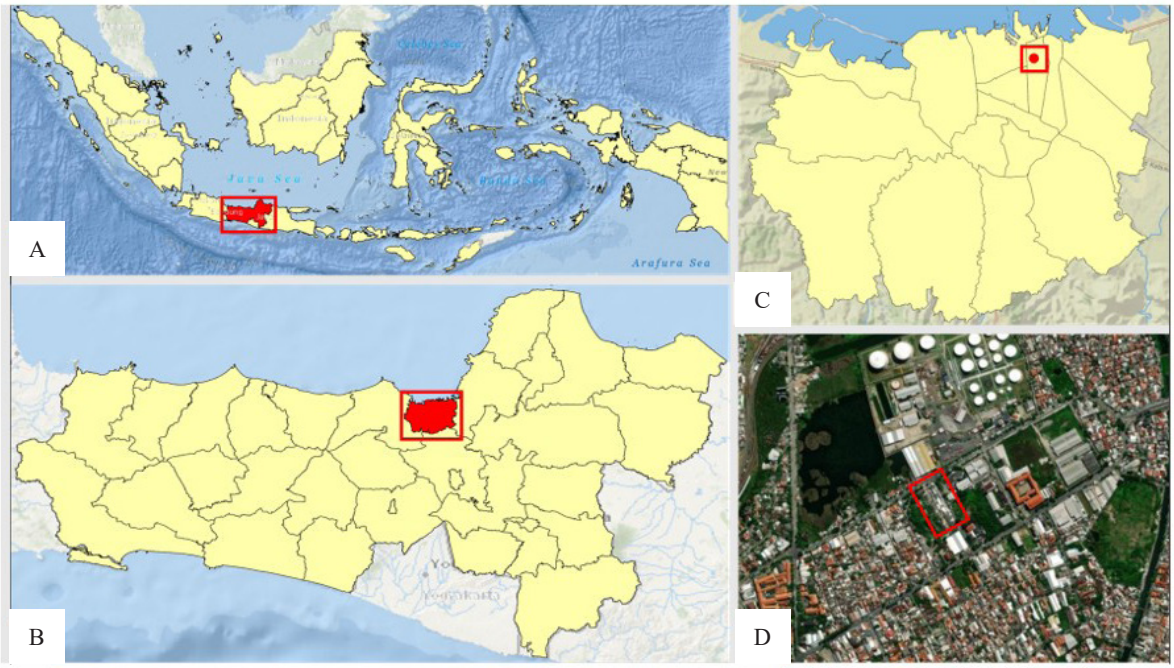

Figure 1. Sampling site location in Semarang, Central Java Province, Indonesia. Sequential geographic panels showing: (A) location within the Indonesian archipelago; (B) Semarang City (red) in Central Java Province; (C) Semarang City administrative boundaries with red circles indicating Kobong Market location; (D) satellite imagery of Kobong Market, the primary commercial fish market serving as the regional distribution hub for marine fishery products from Java Sea fishing zones, where fish samples were collected for this study

Figure 2. Representative specimens of the examined fish species of *Rastrelliger* sp.

was evaluated through bootstrap analysis with 1,000 replicates. Furthermore, nucleotide diversity (π) was defined using DnaSP software version 5 (Librado and Rozas 2009). The McDonald and Kreitman (MK test) and Tajima's D test were run by DnaSP to statistically measure the adaptive evolution and neutral mutation hypothesis within the samples. Haplotype network analysis was performed to elucidate evolutionary relationships and mutational pathways among linked allelic variants using Network software version 10.2.0.0 (Fluxus Technology Ltd, Suffolk, UK).

3. Results

3.1. Prevalence of Ascaridoid Nematode Larvae in Examined Samples

A total of 17 species of *Rastrelliger* sp. were examined, with an average body length of 20.9 cm and an average weight of 111.8 g. Detailed morphometric data for each specimen are presented in Table 1.

The association of fish length and fish weight with the presence of ascaridoid nematode larvae is determined using a non-parametric comparative test, the Mann-Whitney U-test (CI=95%; α=0.05), as the total sample (n) < 30. The test indicates a significant difference in fish length and weight between infected and non-infected fish (p values = 0.005 and 0.002, respectively).

The overall prevalence of infection was nine positive ascaridoid nematode infections of 17 all collected fish samples, 52.94% (9/17), and larvae were mainly found in the fatty tissues of the infected collected fish samples. Furthermore, 7 of 17 (41.17%) *Rastrelliger* sp. samples were infected with *Anisakis typica*, and 4 of 17 (23,52 %) samples were contaminated with *Hysterothylacium amoyense*. The morphology of the larvae of ascaridoid nematodes, examined using a stereomicroscope, showed that the bodies and ventriculi of the larvae were long and cylindrical, attenuated at both ends. These larvae also displayed short, rounded tails with a characteristic cylindrical bentel protruding mucron (Figure 3).

3.2. Molecular Identification of L3 Ascaridoid Nematode Larvae

The identification of 6 larval samples using PCR targeting the mtDNA *COX2* region showed the presence of 5 bands at 600 to 650 bp (SMG-001, SMG-002, SMG-008, SMG-012, SMG-016) and one band at 300 to 400 bp (SMG-007) (Figure 4).

3.3. Phylogenetic Analysis

The first major clade in the tree comprises sequences identified as *A. typica*, which includes both Semarang samples and reference sequences from Southern Makassar, Indonesia, obtained from GenBank NCBI. Several

Table 1. Morphometric characteristics (length and weight), infection status, intensity, and prevalence of ascaridoid nematode larvae in *Rastrelliger* sp. fish samples from Semarang, Indonesia

Sample codes	Length (cm)	Weight (g)	Infection status of ascaridoid nematode larvae	Intensity (larvae per fish)
SMG-001	20.5	98	+	2
SMG-002	22.5	135	+	1
SMG-003	21.5	100	-	0
SMG-004	19.5	82	-	0
SMG-005	23.5	142	+	1
SMG-006	23.5	143	+	1
SMG-007	23.0	162	+	2
SMG-008	21.0	118	+	2
SMG-009	22.0	143	+	7
SMG-010	21.5	124	-	0
SMG-011	20.0	93	-	0
SMG-012	22.0	126	+	1
SMG-013	19.5	86	-	0
SMG-014	22.0	118	-	0
SMG-015	19.0	77	-	0
SMG-016	22.5	136	+	1
SMG-017	12.5	18	-	0
Mean	20.9	111.8	Prevalence: 52.94% (9/17)	
Std.Dev	2.5	34.6		

SMG : Semarang isolates

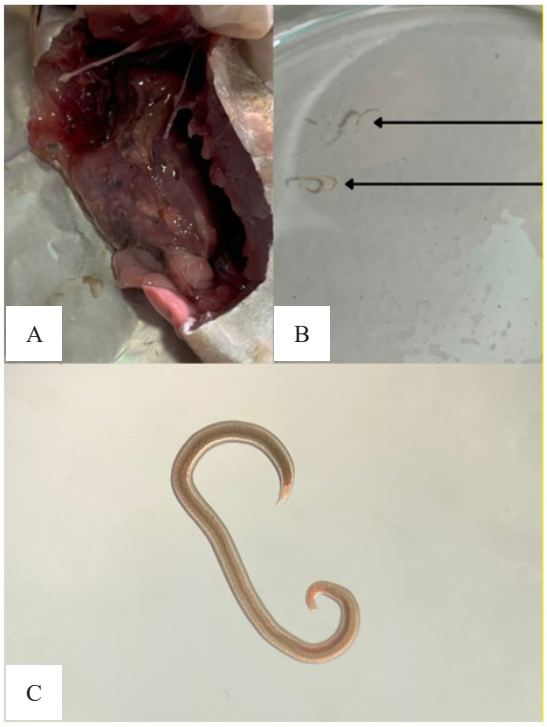

Figure 3. Dissected fish showing internal organs: (A) Larvae observed in the fish's body cavity; (B) Microscopic image of the third-stage; (C) Larvae parasite found in this study

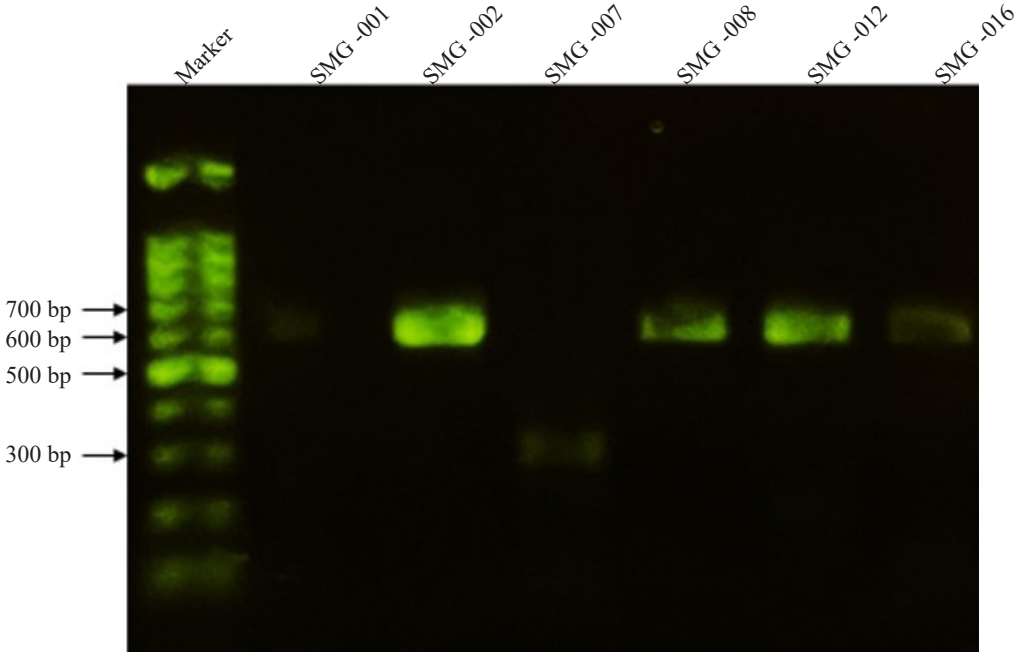

Figure 4. PCR profiles obtained by amplification of mtDNA *COX2* using the primers 211F and 210R showed that samples SMG-001, SMG-002, SMG-008, SMG-012, and SMG-016 (*Anisakis typica*) had a DNA band measuring approximately 600 bp. In contrast, SMG-007 (*Hysterothylacium amoyense*) showed a DNA band measuring approximately 300 bp

Semarang samples, including SMG A10, SMG A9, SMG A15, SMG A32, and SMG A45, cluster closely with reference sequences KC928266, KC928265, and KC928264, which are identified as *A. typica* from Southern Makassar. Other Semarang samples, which are SMG A22, SMG A37, SMG A38, SMG A4, and SMG A5, form a distinct subclade within *A. typica*. This group is genetically differentiated but still closely related to the Southern Makassar population (Figure 5). The second major clade comprises sequences identified as *H. amoyense*, including both Semarang samples and reference sequences from China and Bangladesh, which show closer genetic relationships, as evidenced by high bootstrap values (up to 99). SMG A48 aligns closely with Chinese reference sequences (MF120248), while SMG A23 clusters with sequences from Bangladesh (ON109766, ON109758) (Figure 5).

3.4. Genetic Diversity

A total of 13 *A. typica* (At) and 7 *H. amoyense* (Ha) were sequenced. *A. typica* had 99 variable sites and 102 SNPs, whereas *H. amoyense* had just 62 variable sites and 62 SNPs. The two species had 148 variables and 180 SNPs (Table 2). The two species, *A. typica* and *H. amoyense*, have high haplotype diversity (Hd = 1.000). *A. typica* has a higher nucleotide diversity (π) of 0.046 than *H. amoyense*, which is 0.04339. It indicates the species *A. typica* type has higher nucleotide variation than *H. amoyense* (Table 2). Tajima's D values are negative in *A. typica* (-1.646) and *H. amoyense* (-1.326), indicating population expansion or purifying selection in both species, albeit with varying intensities. Total numbers that are less than zero (0.503) suggest that there is no significant growth in the population. Fu and Li's (D*/F*) neutrality index with negative results indicates the existence of purifying

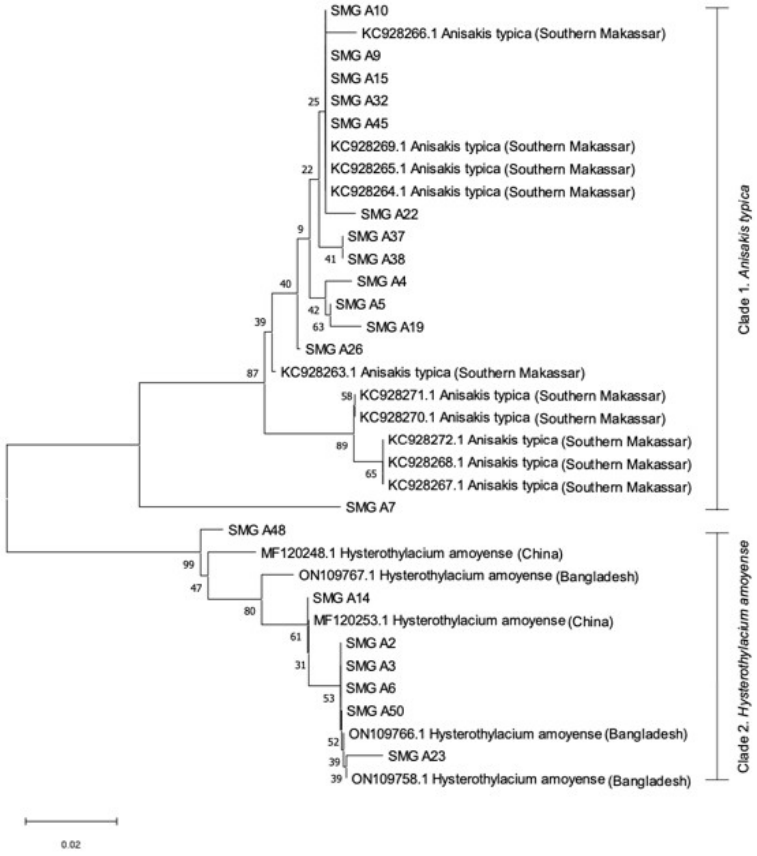

Figure 5. Phylogenetic analysis of the mtDNA *COX2* gene of *Anisakis typica* and *Hysterothylacium amoyense*. To assess the phylogenetic tree reliability, bootstraps were tested at 1000 replicates

selection or population expansion in both species, but with varying intensities. Positive total numbers indicate slow population growth. In general, *A. typica* has higher genetic diversity than *H. amoyense*, as evidenced by the greater variety of variables, SNPs, and π values (Table 2).

3.5. McDonald-Kreitman (MK) Test

Polymorphic changes in SMG-At type synonymous (n = 83) were more than non-synonymous (n=15), and in SMG-Ha type synonymous (n=58) are more than non-synonymous (n=3), indicating a predominance of neutral mutations. In contrast, detrimental non-synonymous mutations tend to be eliminated through purifying selection. The dominance of synonymous (n=32) differences suggests that natural selection eliminates most of the harmful non-synonymous mutations, supporting purifying selection. Neutrality index <1 indicates that positive selection may be at work, but with a value of (p=0.604), this result is not statistically significant. Negative values on dN-dS (-5.017) indicate robust purifying selection. This means that detrimental non-synonymous mutations are not allowed to persist in the population, maintaining the stability of protein function (Table 3).

3.6. Haplotype Relationships

The Haplotype network distinctly segregates the two species into separate clusters; grey circles represent *A. typica*, while green circles denote *H. amoyense* (Figure 6). The clustering of SMG isolates closely within the network, particularly in haplotypes H03, H04, H07, H08, H10, H11, H12, H14, H15, H16, H17, H18, H24, H27, H28, and H29, suggests a localized genetic structure indicative of shared ancestry or limited gene flow with other geographic populations. In contrast, GenBank isolates, including haplotypes H21, H22, H23, H25, H26, and H30, exhibit broader dispersal across the network, reflecting greater genetic divergence potentially driven by geographic or ecological factors (Figure 6).

4. Discussion

4.1. Prevalence of Ascaridoid Nematode Larvae in Examined Samples

The parasitic nematode fauna of marine fish species in Semarang, Indonesia, is poorly known. We present a report of molecularly identified ascaridoid nematodes of the genera *A. typica* and *H. amoyense* found in several types of fish sold in Kobong Market, Semarang. *Anisakis* spp. is a heteroxenous life cycle in the aquatic environment, where marine mammals, especially cetaceans, act as definitive hosts, small crustaceans act as secondary hosts, and fish and squid act as intermediate hosts or third parasites. This highly complex life cycle is crucial for their distribution and existence (Aibinu *et al*. 2019). Based on current information, this is the first molecular report of an *Anisakis* species in Semarang City.

The parasite examination showed a higher prevalence of *A. typica* parasites (41.17%) than *H. amoyense* parasites (23.52%) in the local fish populations studied in warmer marine environments. In another research study, *H. amoyense* was identified in 5% of samples collected from marine fish in the East China Sea, while *A. Typica* showed a significant presence in a wide range of host species (Kong *et al*. 2015; Utami *et al*. 2022). Conversely, studies in Japan and Europe

Table 2. Estimates of nucleotide diversity, haplotype diversity, and neutrality indices of SMG samples

ID	No. of samples	No. of variable sites	SNPs	No. of haplotype	Diversity ± S.D.		Tajima's D	Fu and Li's	
					Haplotype (Hd)	Nucleotide (π)		D*	F*
At type	13	99	102	13	1.000 ± 0.030	0.046 ± 0.018	-1.646	-1.671	-1.906
Ha type	7	62	62	7	1.000 ± 0.076	0.043 ± 0.021	-1.326	-1.337	-1.478
Total	20	148	180	20	1.000 ± 0.016	0.126 ± 0.014	0.503	0.047	0.217

SNPs : Single nucleotide polymorphisms; At = *Anisakis typica*; Ha = *Hysterothylacium amoyense*; S.D. = standard deviation

Table 3. The McDonald-Kreitman (MK) test on SMG distinguishes between *Anisakis typica* (At) and *Hysterothylacium amoyense* (Ha) types

ID	Polymorphic changes within SMG-*At* type		Polymorphic changes within the SMG-*Ha* type		Fixed differences between the *At* and *Ha* types		Neutrality index (*p-value*)	dN-dS
	Syn	Non-syn	Syn	Non-syn	Syn	Non-syn		
SMG	83	15	58	3	32	7	0.697 ($p = 0.604$)	-5.017

SMG : Semarang isolates; Syn = synonymous; Non-syn = non-synonymous; At = *Anisakis typica*; Ha = *Hysterothylacium amoyense*

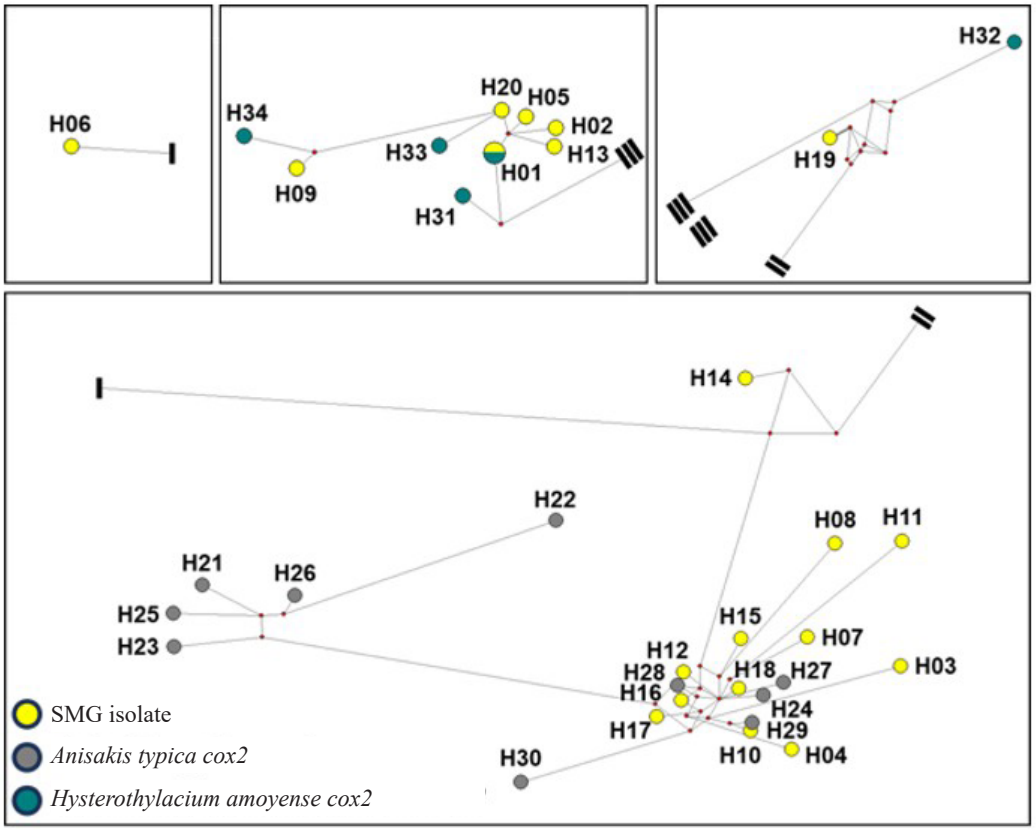

Figure 6. Haplotype network of *Anisakis typica* and *Hysterothylacium amoyense* based on mitochondrial *cox2* sequences, including isolates obtained from GenBank and Semarang (SMG) samples. Each circle represents a unique haplotype, with the size proportional to the number of isolates sharing that haplotype. Red dots along the connecting lines indicate mutational steps between haplotypes. Yellow dots were isolates from Semarang; gray and green dots were reference genes from other geographical areas

have documented varying prevalence rates, with *A. typica* being more prevalent in colder waters, while *H. Amoyense* is frequently encountered in warmer marine environments (Bao *et al*. 2022). In some studies, *A. typica* has been documented to be less prevalent than *H. amoyense*. In a study conducted in Bangladesh, *A. typica* was identified in 10% of fish viscera samples (Bao *et al*. 2022). This geographical variation suggests that environmental factors significantly influence the distribution of these nematodes.

A. typica and *H. amoyense* can be differentiated morphologically, especially at the third larval stage (L3), which plays an essential role in parasite diagnosis. *A. typica* has a mucron and elongated ventriculus; it generally has a sturdy body with smooth cuticles without protrusions, and three prominent lips with well-defined and neatly arranged head papillae, but no interlabial. The tail is conical and features a small mucron, with an elongated ventriculus that merges obliquely into the gut, and the caudal papilla is rare or not apparent at all. In contrast, *H. amoyense* larvae show a more complex variety of body shapes, have alae laterales and protrusions or small spines on the cuticle, especially posteriorly. The lips are also three in number, but interlocked, separated by the interlabium, and the absence of a mucron. The tail of the larvae is usually round or pointed without a mucron and is equipped with caudal papillae that can be very numerous, depending on the species. The body size of *H. amoyense* tends to be larger than that of *A. typica* (Adroher *et al*. 2020). The morphological characteristics of L3 of various *Anisakis* species are very similar, so it is not possible to identify species by morphology alone because it is less accurate. Therefore, from a molecular perspective, these two genera can be clearly distinguished through phylogenetic analyses based on ITS markers and the mitochondrial *cox2* gene, which show that *A. typica* and *H. amoyense* are in genetically distinct clades (Da Fonseca *et al*. 2016).

A. typica is commonly found in subtropical and tropical waters, especially in Brazilian waters (Di Azevedo *et al*. 2017). Pagrus fish caught in the Brazil Current at the same latitude showed the presence of *A.*

typica in it. This suggests that the Brazil Current tends to move from high depth to shallower areas around the convergence. The presence of *A. typica* has also been found in several dolphin species, namely *Del phinidae*, *Phocoena phocoena*, and *Pontoporia blainvillei*, in subtropical and tropical waters (Lanfranchi *et al*. 2018). Most dolphins are tropical marine animals that have warm temperatures, so the distribution of *Anisakis* parasites is highly dependent on the definitive host (Mattiucci *et al*. 2002). *A. typica* was also the *Anisakis* species that dominated *Z. conchifer* infection in continental slope waters at 94–117 m depth. This suggests a greater influence of the warmer Brazilian Current than other marine waters on the prevalence of *Anisakis* spp. Thus, the influence of subtropical waters is related to the infective stage of the parasite in the intermediate host or the migration behavior of hosts from lower latitudes (Lanfranchi *et al*. 2018). On the other hand, *Hysterothylacium* spp. was the most common nematode parasite and had a higher infection rate than any other parasite species reported from the Northwest Mediterranean before 2015 (Bušelić *et al*. 2017). An increase in the number of copepods in the ocean due to global warming is one of the causes of the high parasitic infection of *Hysterothylacium* spp. in sardines, as an intermediate host, in the Northwest Mediterranean (Køie 1993). Parasite prevalence is also influenced by the difference between northern and southern waters, i.e., cold and warm waters (Frigola-Tepe *et al*. 2022)—infection with *Hysterothylacium* spp. The movement of these nematodes may also influence parasites. A higher prevalence of parasite infection occurs in fish living in warm waters because of the availability of intermediate hosts for *Hysterothylacium* spp—parasites in warm waters (Klimpel & Rückert 2005).

In fish and squid muscle or mantle, *H. amoyense* is generally considered non-zoonotic because it is found at low infection levels (Bao *et al*. 2022). Due to the limited records of human infection by *Hysterothylacium* spp., claims about its zoonotic potential are controversial. (Fernández-Caldas *et al*. 1998; Serrano *et al*. 2023).

Marine fish are intermediate hosts, and marine mammals are commonly infected by *A. typica* (Mattiuci *et al*. 2002). Based on a previous study, *A. typica* was found at high prevalence in *Decapterus macrosoma* and distributed in tropical and temperate marine waters (See *et al*. 2022). This nematode infects the fatty tissue of the fish, such as the gonads, and migrates through the fish's visceral organs. *A. typica* presence poses a potential risk if fish is consumed raw or undercooked (Kim *et al*. 2013). The parasite can cause allergic reactions due to its allergenic proteins, but these reactions are rare or unreported in human cases (See *et al*. 2022). Therefore, potential zoonotic implications of *A. typica* have definitive evidence of it causing human anisakiasis (See *et al*. 2022). Zoonotic infections are primarily linked to *A. simplex* and *A. pegreffii*, which can cause anisakiasis, such as nausea, abdominal pain, vomiting, and diarrhea (Mattiuci *et al*. 2015; Palm *et al*. 2017; Chou *et al*. 2011; Cipriani *et al*. 2018). Nevertheless, *A. typica* causes food-safety problems in the fisheries products in Indonesia (Palm *et al*. 2008; Palm *et al*. 2017).

4.2. Molecular Identification of L3 Ascaridoid Nematode Larvae

The molecular identification of *Anisakis* species using PCR targeting the mtDNA *COX2* region has been well-documented in various studies (Mattiucci *et al*. 2011; Utami *et al*. 2017). For instance, a previous study reported that *A. typica* typically produces a band size of approximately 600 bp when analyzed using this method (Mattiucci *et al*. 2011). An additional study also demonstrated that *Anisakis* species produce distinct band sizes in PCR analyses, with *A. typica* consistently yielding bands around 600 bp (Utami *et al*. 2017). This aligns with our findings for samples SMG-001, SMG-002, SMG-008, SMG-012, and SMG-016, all of which produced bands within the expected range. Furthermore, a previous study on the molecular identification of *H. amoyense* using PCR targeting the mtDNA *COX2* region reported band sizes ranging from approximately 356 to 583 bp, which also aligns with our findings for this species (SMG-007) (Bao *et al*. 2022).

4.3. Phylogenetic Analysis

A. typica has been identified in various fish species across different regions. The presence of *A. typica* in *Rastrelliger* sp., which exhibits a close genetic relationship with populations in the southern region of Makassar, has also been documented in the southern coastal area of East Java (Setyobudi *et al*. 2019). This observation stands in contrast to the northern coastal regions of Java, where worm populations exhibited genetic proximity to the Makassar, South China Sea, and Egypt regions (Setyobudi *et al*. 2023). This discrepancy is presumably attributable to variations in the fish species utilized as hosts and the distinct characteristics of the marine environment across these regions. Notably, the species *H. amoyense* was exclusively observed in the Demak region, exhibiting genetic affinities with populations in China, potentially attributable to its geographical

proximity (Bao *et al*. 2022). However, this study also documented a population related to Bangladesh, a finding that has not been previously reported.

4.4. Genetic Diversity

Nucleotide diversity and haplotype diversity act as indicators to determine the level of genetic diversity present in a population. The nucleotide diversity values of *A. typica* and *H. amoyense* were lower than the haplotype diversity values of *A. typica* and *H. amoyense*, indicating a recent population expansion to a smaller effective population size after congestion (Chaiphongpachara *et al*. 2022). Higher nucleotide diversity values indicate greater genetic variation in a population, leading to increased mutation. Conversely, lower nucleotide diversity values indicate less genetic variation in a population, leading to lower mutation rates (Guo *et al*. 2019). A high haplotype diversity value indicates that genetic lineage variation in a population is also high, and vice versa (Putra *et al*. 2024). The Tajima's D values of *A. typica* and *H. amoyense* also the Fu and Li's D* and F* values for both species showed all negative values, supporting population size in Semarang. If a population is balanced between genetic drift and selective mutation, then Tajima's D value becomes zero (Chaiphongpachara *et al*. 2022). Tajima's D value can produce positive results if the population shows a stable state, while negative results occur if the population is expanding. Likewise, Fu and Li's D* and F* values reflect genetic diversity. High values of these indicators indicate that population expansion has rarely occurred recently, while low values indicate recent population growth or selection (Omori and Wu 2017). Based on the Tajima's D and the Fu and Li's D* and F* values, our results show that there is growth in the population. This genetic evidence supports the hypothesis of recent population expansion in both *A. typica* and *H. amoyense*. Although both species belong to the family Anisakidae, they are classified under different genera. Despite this taxonomic difference, the similar patterns of genetic diversity and demographic expansion observed in both species may indicate that they are responding to similar ecological or environmental pressures in the coastal waters of Semarang.

4.5. McDonald-Kreitman (MK) Test

The McDonald-Kreitman test explores the fact that mutations in coding regions are divided into two types: nonsynonymous mutations and synonymous mutations (Eyre-Walker and Keightley 2007). This study demonstrates that synonymous mutations are more prevalent than nonsynonymous mutations in the *A. typica* and *H. amoyense*, indicating a neutral evolutionary pattern. Synonymous mutations tend not to alter protein function and do not have a significant impact on individual fitness, leading to the accumulation of mutations without strong selection effects. In contrast, deleterious nonsynonymous mutations, which alter protein structure or function, are more likely to be eliminated through purifying selection. This process removes mutations that can decrease an individual's fitness (Vitti *et al*. 2013). The dominance of synonymous differences supports the theory that natural selection works to maintain the stability of protein function by minimizing the impact of deleterious mutations. This finding aligns with earlier studies, which emphasised purifying selection as a significant mechanism in preserving genetic stability, particularly in genes involved in essential functions (Brunet *et al*. 2021). This process is vital for the survival and evolutionary success of these organisms, as it maintains a balance between the necessity for genetic stability and the pressures of adapting to changing host conditions.

4.6. Haplotype Relationships

The mutational steps between haplotypes are shown through the red dots on the connecting line. The findings confirm that the complex life cycle of these nematodes does not limit gene exchange but enhances it through the high mobility of the different hosts, such as fish and marine mammals (Mattiucci and Nascetti 2008). The distinct clustering of grey and green circles representing *A. typica* and *H. amoyense*, respectively, confirms their genetic divergence and supports their classification as separate species. The concept of reproductive isolation between these clades is reinforced by mutually monophyletic clades, suggesting that they represent different evolutionary lineages without the same haplotype (Cipriani *et al*. 2022).

This clear bifurcation underscores the genetic distinctiveness between the two taxa, supporting their taxonomic classification as separate species. The absence of overlapping haplotypes between the two clusters reinforces the reproductive isolation and genetic barriers that maintain species integrity. Notably, haplotype 14 (H14) from SMG is positioned distantly from the primary cluster, indicating unique genetic variation that may be shaped by localized evolutionary pressures or genetic drift. H01 appears to be a central haplotype, serving as a key node connected to other SMG haplotypes, including H20, H05, H02, and H13. This positioning

suggests that H01 may represent a common ancestral haplotype or a highly conserved sequence within the population. In contrast, peripheral haplotypes such as H06 and H19 are linked by longer branches, indicating a greater number of mutational steps and highlighting their genetic divergence. The distinct isolation of H06 may reflect divergence driven by local selective pressures or restricted gene flow within the population.

4.7. Health Implications and Recommendations

In conclusion, the findings reveal significant genetic diversity and connectivity among *A. typica* and *H. amoyense*, underscoring their potential public health implications due to high fish consumption practices in Indonesia. This research advances the field by providing molecular insights into the prevalence and genetic variability of these parasites, which is essential for developing effective monitoring and control strategies. The risk of anisakiasis is particularly relevant in areas like Semarang, where a previous study conducted in 2020 identified the presence of *Anisakis* spp. in five mackerel fish collected from various aquaculture sites across the city (Febrina *et al*. 2020). These findings indicate that local fishery products may serve as a reservoir for zoonotic parasites, heightening the need for public awareness and food safety interventions. Future research should focus on expanding the geographical scope of sampling and investigating the epidemiological implications of anisakiasis, particularly regarding local culinary practices that may increase infection risk. Additionally, ongoing research should explore the ecological factors influencing the distribution of these parasites to inform public health policies effectively.

Acknowledgements

The authors would like to express their gratitude to the Faculty of Public Health, Universitas Diponegoro, Semarang, Indonesia, for providing the facilities and resources necessary for the completion of this study.

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