

IDENTIFICATION OF GENETIC DIVERSITY CYTOCHROME OXIDASE SUBUNIT II (COII) MITOCHONDRIAL GENE AS GENETIC MARKER FOR ANISAKIS SPECIES IN *Euthynnus affinis*

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ABSTRACT

This study aimed to get specific genetic marker for *Anisakis* sp. identification on mackerel tuna using gene sequence cytochrome oxidase subunit II (COII) mitochondrial deoxyribonucleic acid (mtDNA) and to identify taxonomic affiliation between *Anisakis* sp. from Indonesia and others *Anisakis* sp. from GenBank database. This study started with sample collections at three fish auctions in Cilacap (Central Java), morphology classification, DNA isolation, and molecular based identification using polymerase chain reaction (PCR) and sequencing methods. Molecular based identification of *Anisakis* used gene amplification COII mtDNA as a cell target prior to sequence. Morphology characteristic results showed that *Anisakis* nematodes which infected mackerel tuna classified as type II L3 larvae. Molecular based identification showed significant result, which found 530 bp COII DNA gene fragment similar to target cell. Gene sequencing alignment results of COII *Anisakis* gene compared with GenBank showed 11 different nucleotide sites that can be used as genetic barcode for Indonesian *Anisakis* sp. This study showed that *Anisakis* sp. infected mackerel tuna in Java Sea is *Anisakis physeteris* and considered as zoonosis.

Key words: *Anisakis*, COII gene, genetic diversity, genetic marker, zoonosis

ABSTRAK

Penelitian ini bertujuan mendapatkan penanda genetik spesifik untuk identifikasi spesies *Anisakis* pada ikan tongkol menggunakan urutan gen cytochrome oxidase subunit II (COII) mitochondrial deoxyribonucleic acid (mtDNA) serta mengetahui afiliasi taksonomi spesies *Anisakis* asal Indonesia dengan spesies *Anisakis* lainnya dari database GenBank. Penelitian ini diawali dengan koleksi sampel di tiga Tempat Pelelangan Ikan (TPI) di kabupaten Cilacap Jawa Tengah, karakterisasi morfologi, isolasi DNA, serta dilanjutkan dengan identifikasi berbasis molekuler dengan metode polymerase chain reaction (PCR) dan pengurutan. Identifikasi *Anisakis* berbasis molekuler dilakukan dengan amplifikasi gen COII mtDNA sebagai gen target, kemudian diurutkan. Hasil karakterisasi morfologi menunjukkan bahwa nematoda *Anisakis* yang menginfeksi ikan tongkol termasuk larva L3 tipe II. Identifikasi berbasis molekuler memberikan hasil yang baik, dengan ditemukan fragmen DNA gen COII berukuran 530 bp sesuai gen target. Hasil penjejakan urutan gen COII *Anisakis* dengan pembandingan dari GenBank memperlihatkan adanya 11 situs nukleotida pembeda yang dapat dijadikan penanda genetik (barcode) untuk spesies *Anisakis* asal Indonesia. Hasil penelitian ini menunjukkan bahwa spesies *Anisakis* yang menginfeksi ikan tongkol di perairan laut Jawa merupakan spesies *Anisakis physeteris* dan bersifat zoonosis.

Kata kunci: *Anisakis*, gen COII, keragaman genetik, penanda genetik, zoonosis

INTRODUCTION

Health and disease monitoring in specific fish species are important things to do because the disease, particularly due to parasite, holds a key role in aquatic biology (Indaryanto *et al.*, 2015). According to Indaryanto *et al.* (2015) and Cavallero *et al.* (2012), parasitic worms were one of large group of parasites that can infect fish and created biological and economic loss. Nematodes from *Anisakis* genus are one of parasite that can live inside fish and squid during larvae stage and sea mammal during adult stage (Yman, 2003; Chai *et al.*, 2005; Cavallero *et al.*, 2012). Histopathology and parasitology examination also found that *Anisakis* sp. is the most zooparasite found in sea fish and mammals (Siagian *et al.*, 2010; Cavallero *et al.*, 2011; D'Amelio *et al.*, 2011; Setyobudi *et al.*, 2011).

Recently, there are nine species from *Anisakis* genus, divided into two types. Type I L3 *Anisakis* consists of *Anisakis simplex sensu stricto* (s.s.), *A. pegreffii*, *A. simplex C*, *A. typical*, *A. ziphridarum*, and *A. nascettii*, whereas type II L3 *Anisakis* consists of *A. paggiae*, *A. physeteris*, and *A. brevispiculata* (Mattiucci

and Nascetti, 2008; Mattiucci *et al.*, 2009). Mattiucci *et al.* (2011); Arizono *et al.* (2012), noted there were only *A. simplex* s.s., *A. pegreffii*, and *A. physeteris* cause anisakidosis in human. Furthermore, Koinari *et al.* (2013) stated that the most *Anisakis* sp. that can cause anisakidosis were *Anisakis* Dujardin genus, 1845.

Anisakis infection in human reported first time more than 50 years ago by Van Thiel (Audicana and Kennedy, 2008) which described as 'a very unusual finding' that is the discovery of sea nematodes in the middle of intestinal eosinophilic mass taken from one patient's abdomen with abdominal pain. Sakanari and McKerrow (1989), Audicana and Kennedy (2008), and Koinari *et al.* (2013) described anisakidosis in human was marked by several symptoms, such as sudden abdominal pain, nausea, vomitus, diarrhea, allergic reaction, and in serious case can cause shock in patient. Mackerel fish is intermediate, paratenic, and definitive host for *Anisakis* nematode that can cause anisakiasis or anisakidosis in human (Umehara *et al.*, 2006; Siagian *et al.*, 2010; Cavallero *et al.*, 2012).

There are approximately 20.000 cases recently reported in various places in the world, more than 90% cases happened in Japan (Audicana *et al.*, 2002;

Cavallero *et al.*, 2011). *Anisakis* larvae study in Indonesia usually used phenotype character. Hutomo *et al.* (1978) used three fish species from Thousand Islands sea, namely *Rastrelliger kanagurta*, *Decapterus rosselli*, and *Sardinella sirm* which identified as type I L3 *Anisakis* and type B Terranova. Daulay (2003) analyzed *Anisakis* larvae infection and its causal factors in sea fish at south coast fish auction, Yogyakarta. Setyobudi *et al.* (2007) analyzed prevalence, intensity, and distribution of *Anisakis* larvae in layur fish (*Trichiurus* sp.) at south coast Purworejo Regency. Utami (2013) identified *Anisakis* sp. in mackerel, selar, and tengiri swanggi fish at Cilacap fish auction according to its morphology characteristic. The results demonstrated successfully discovered and identified *Anisakis* sp. larvae are stage III larvae with most number of *Anisakis* sp. were found in selar fish. Suadi *et al.* (2007) studied layur fish (*Trichiurus* sp.) population which landed at Cilacap fish harbor using *Anisakis* sp. parasite as biological marker. Indaryanto *et al.* (2015) inventoried parasitic worms in mackerel (*Rastrelliger* sp.) at sea of Banten Bay and Pelabuhan Ratu. Study results concluded finding of *A. typica* in mackerel fish without support information.

Berland (1961) and Koinari *et al.* (2013) stated that *Anisakis* nematodes could be identified according to its morphology and molecular data. Before establishment of *polymerase chain reaction* (PCR) and sequencing technique based molecular, ventriculus and digestive tract were used as marker to differentiate species in *Anisakis* genus (Berland, 1961), and only type I and II *Anisakis* could be differentiated from its ventriculus length and presence of tail spine (*mucron*) (Berland, 1961; Mattiucci *et al.*, 2011; Arizono *et al.*, 2012; Sohn *et al.*, 2014).

Recently, PCR is the most popular method used to characterize *Anisakis* sp. form its different locus, such as *ribosomal internal transcribed spacer* (ITS) region (Zhu *et al.*, 1998; D'Amelio *et al.*, 2000; Nadler *et al.*, 2005; Pontes *et al.*, 2005; Abe *et al.*, 2006; Umehara *et al.*, 2006; Zhu *et al.*, 2007; Kijewska *et al.*, 2009), and

cytochrome C oxidase subunit II (COII) mitochondria region (Valentini *et al.*, 2006; Mattiucci *et al.*, 2009, 2011; Cavallero *et al.*, 2011; D'Amelio *et al.*, 2011; Setyobudi *et al.*, 2011).

In Indonesia, there is no reported study about genetic biodiversity of *Anisakis* sp. in mackerel fish with COII gene as a genetic barcode, hence we conducted this study. In this study, COII gene was amplified using PCR method. Amplicon then purified with chromatography column then sequenced to determine nucleotide orders. Potency of COII gene sequence as genetic barcode of *Anisakis* sp. species was proven with analyzed genetic biodiversity in each species using MEGA 6.0 version program (Kumar, 2001).

MATERIALS AND METHODS

Sample Collections

Mackerel tuna fish samples were collected from three fish auctions in Cilacap, Central Java. A total of 65 mackerel tuna fish were collected from three fish auctions in Cilacap Regency, Central Java consist of 20 fish were collected from Lengkong Fish Auction, 25 fish from Samudera Fish Auction, and 20 fish from Rajawarit Fish Auction (Figure 1). The samples collections were done from May until June 2015. *Anisakis* samples were collected by carried out a necropsy on fish body organ especially abdomen, digestive tract, liver, and muscles. External part observation was done in gill and entire body surface, mainly fin and back. Seven nematodes successfully collected and fixated in *RNA buffer* later for morphology and molecular examination at Biochemistry Laboratory, Faculty of Veterinary Medicine, Gadjah Mada University.

Morpholgy Analysis

Lactophenol solution was drop on *Anisakis* samples for 24 hours. Then the body length measurement was performed directly, followed by documented using Olympus Bx50 camera with 100x zoom. Body parts

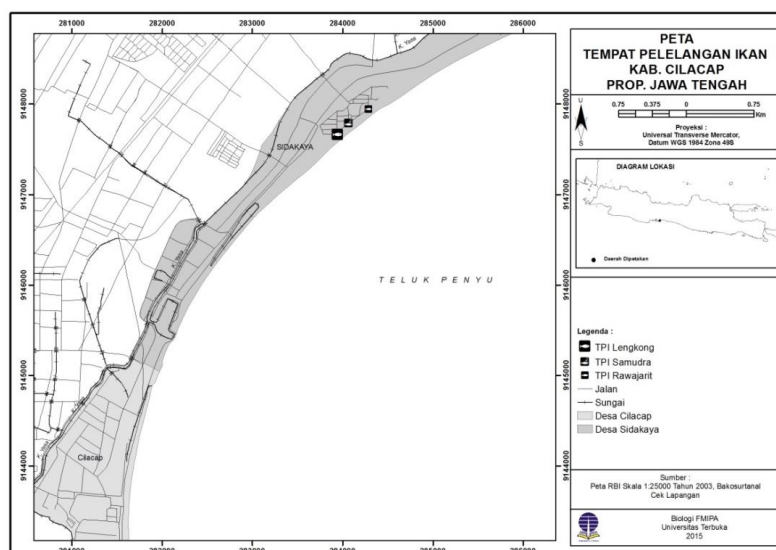


Figure 1. Map of *Anisakis* sample collections locations in Cilacap

measured were anterior part of cephalic organ and posterior part of caudal organ (Figure 2).

DNA Isolation

Total deoxyribonucleic acid (DNA) from each individual of *Anisakis* was isolated from body parts using DNA isolation kit (Qiagen) matched with protocol from its manufacture, with combination of RNAase solution treatment. The quality of isolated DNA results were observed using electrophoresis method on 1% agarose gel and buffer 1xTBE on submarine electrophoresis device (Hoefer, USA). Observation was performed using UV-transilluminator with 260 nm wave lengths after provided by gel with bioatlas buffer (Geneaid) functioned as fluorescence. The isolated DNA was then stored at -20° C degree before PCR stage. A total concentration of 50 ng/mL of isolated DNA was used in PCR process.

Primer Design

Primer designed was carried out using *primer3online* program (http://www.genome.wi.mit.edu/cgi-bin/primer3.cgi/results_from-primer3) based on mitochondria genome sequence data of *Anisakis simplex* (access number AB241057.1). Primary base order for COII gene amplification from *Anisakis* sp. samples was presented in Table 1.

COII Gene Amplification Using PCR Methods

In this study, DNA was amplified using PCR machine (Infinigen). DNA amplification was performed in conditions: initial denaturation for 5 minutes at 94° C continued with denaturation at 94° C for 30 seconds, 50° C for primary attachment (annealing), 72° C for 1 minute for elongation; amplification was done for 35 cycles, then post elongation for 5 minutes at 72° C.

PCR products were detected by migrated them on 1% agarosa gel using 1xTBE buffer in submarine electrophoresis device (Hoefer, USA), then observed

using UV-transilluminator with 260 nm wave lengths after gel colored by bioatlas fluorescence (Geneaid). DNA marker with the size of 100 bp was used as molecular weight marker. Good PCR result was marked with single band with the nucleotide length is 530 bp.

DNA sequencing

The PCR product was purified using GFX Column purification kit, furthermore it was used as DNA template for DNA sequencing reaction. Each samples was completed two sequencing reactions, using forward primer and reverse primer. The PCR product of COII gene was sequenced. Good sequencing results were marked with single peak spectrophotogram results and without noise.

Data analysis

Double alignments of nucleotide sequence of COII gene were analyzed using ClustalW software (Thompson *et al.*, 1994). Results analysis was done based on nucleotide sequencing of COII gen using MEGA 6.0 version program (Kumar, 2001). The genetical length was analyzed with Kimura methods using two parameters (Kumar, 2001). Phylogenetic tree was analyzed according to its nucleotide sequence using neighbor joining methods with bootstrap value at 1000x. Comparison data was took from GenBank, there were *A. simplex* (s.s.) (access number DQ116426), *A. pegreffii* (access number DQ116428), *A. simplex C* (access number DQ116429), *A. typica* (access number DQ116427), *A. ziphidarum* (access number DQ116430), *A. physeteris* (access number DQ116432), *A. brevispiculata* (access number DQ116433), *A. paggiae* (access number DQ116434), and *A. nascettii* (access number DQ116431).

RESULTS AND DISCUSSION

The observational results of 65 mackerel tuna fishes (*Euthynnus affinis*) found *Anisakis* larvae as shown on

Table 1. Primary nucleotide order for COII gene amplification of *Anisakis* sp.

Target	F/R	Base order	Base numbers	Tm (° C)
COII (530 bp)	F	TGTAGTTTGCTTTTCGGTGTCT	23	55,0
	R	GCCCCACAAATCTCTGAACA	20	55,3

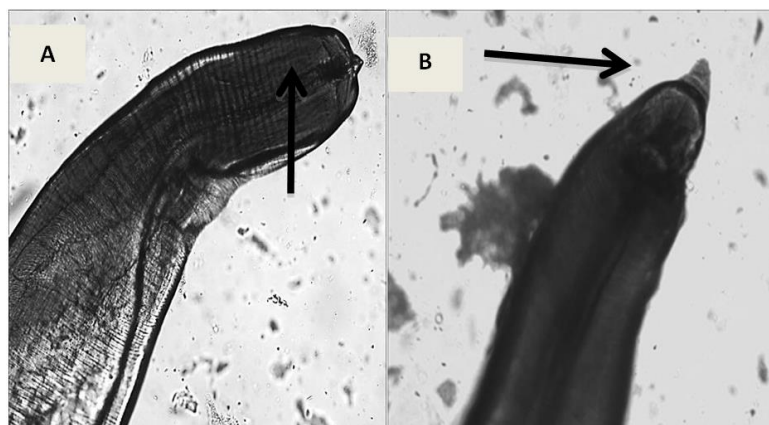


Figure 2. The morphology of *Anisakis* sp. A= Anterior tip (cephalic), arrow sign showed boring tooth; B= Posterior tip (caudal), arrow sign showed anus

Figure 2. The morphology characterization such as cephalic and caudal length showed positive results of *Anisakis* sp. (Figure 2).

Mattiucci *et al.* (2011) and Sohn *et al.* (2014) stated that stage III *Anisakis* larvae (L3) has special

feature called drilling tooth “boring tooth” in its anterior tip for perforated and hanged on intestine wall. Morphology identification results revealed that in *Anisakis* sp. found on mackerel fish shows sharpened area on its anterior and posterior part.

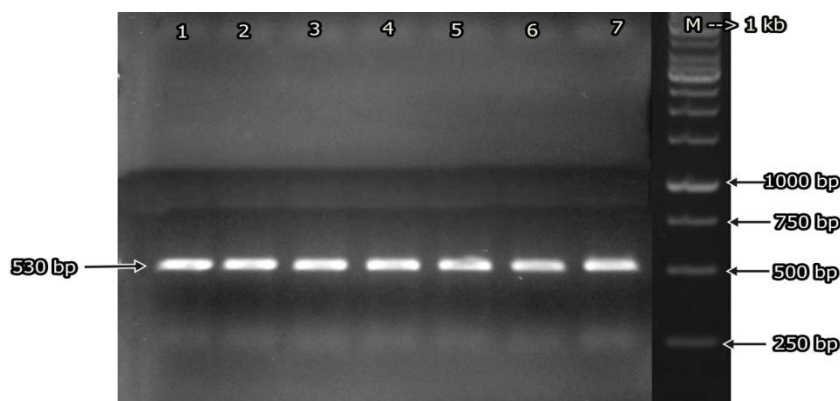


Figure 3. PCR products of *Anisakis* sp. COII gene on 1% agarose gel. 1Kb= DNA ladder 1000 bp, 1-7= PCR products of *Anisakis* samples

# <i>Anisakis_physeteris</i>	ATG TTC AAC CTA CGC AAA ACC CAC CCA CTC ATA AAA ATC CTC AAC [45]
<i>Anisakis_1</i>A.T.A. ... [45]
<i>Anisakis_2</i>A.T.A. ... [45]
<i>Anisakis_3</i>A.A. ... [45]
<i>Anisakis_4</i>A.A. ... [45]
<i>Anisakis_5</i>A.T.A. ... [45]
<i>Anisakis_6</i>A.T.A. ... [45]
<i>Anisakis_7</i>A.T.A. ... [45]
# <i>Anisakis_physeteris</i>	AAA GCC TTC ATT GAC CTA CCA ACA CCA TCC AAC ATC TCT GCC TGA [90]
<i>Anisakis_1</i>T. ... [90]
<i>Anisakis_2</i>T. ... [90]
<i>Anisakis_3</i>T. ... [90]
<i>Anisakis_4</i>T. ... [90]
<i>Anisakis_5</i>A. ..T. .C. ... [90]
<i>Anisakis_6</i>A. ..T. .C. ... [90]
<i>Anisakis_7</i>A. ..T. .C. ... [90]
# <i>Anisakis_physeteris</i>	ACA GCC TTT TCA TCA GTC GCC CAC ATC TGC CGA GAC GTA AAC TAT [225]
<i>Anisakis_1</i>T.C. [225]
<i>Anisakis_2</i>T.C. [225]
<i>Anisakis_3</i>T.C. [225]
<i>Anisakis_4</i>T.C. [225]
<i>Anisakis_5</i>T.C. [225]
<i>Anisakis_6</i>T.C. [225]
<i>Anisakis_7</i>T.C. [225]
# <i>Anisakis_physeteris</i>	TCC TAC CTT TAC AAA GAA ACA TGA AAC ACC GGA GTA ATC CTA CTA [360]
<i>Anisakis_1</i>T. ... [360]
<i>Anisakis_2</i>T. ... [360]
<i>Anisakis_3</i>T. ... [360]
<i>Anisakis_4</i>T. ... [360]
<i>Anisakis_5</i>T.G. [360]
<i>Anisakis_6</i>T.G. [360]
<i>Anisakis_7</i>T.G. [360]
# <i>Anisakis_physeteris</i>	CTA ACA GTA ATA GCA ACC GCT TTC GTT GGC TAC GTC CTC CCA TGA [405]
# <i>Anisakis_1</i>	T..C.C. ... [405]
# <i>Anisakis_2</i>	T..C.C. ... [405]
# <i>Anisakis_3</i>	T..C.C. ... [405]
# <i>Anisakis_4</i>	T..C.C. ... [405]
# <i>Anisakis_5</i>	T..C.C.A. ..T. ... [405]
# <i>Anisakis_6</i>	T..C.C.A. ..T. ... [405]
# <i>Anisakis_7</i>	T..C.C.A. ..T. ... [405]
# <i>Anisakis_physeteris</i>	GGA CAG ATG TCA TTC TGA GGC GCT ACA GTA ATT ACA AAC CTA CTA [450]
<i>Anisakis_1</i>A. ..C. ... [450]
<i>Anisakis_2</i>A. ..C. ... [450]
<i>Anisakis_3</i>A. ..C. ... [450]
<i>Anisakis_4</i>A. ..C.T. ... [450]
<i>Anisakis_5</i>A. ..C.C. ... [450]
<i>Anisakis_6</i>A. ..C.C. ... [450]
<i>Anisakis_7</i>A. ..C.C. ... [450]

Figure 4. Alignment result of *Anisakis* sp. mtDNA COII gene sequence from the research compared to *A. physeteris* from GenBank and the polymorphic sites that could be utilized as genetic barcode

Boring tooth is observed in anterior part but there is no excretory pore found, whereas in its posterior part there is no mucron but anus is observed.

Based on morphology analysis, *Anisakis* sp. found in mackerel fish classified as type II L3 *Anisakis* larvae since there was no mucron at its posterior tip. This result similar with Widjanarko (2015) who discovered *Anisakis* larvae in karo and mackerel tuna fish which were classified as type II L3 *Anisakis* larvae. Based on Mattiucci and Nascetti (2009), type II L3 *Anisakis* nematodes included *A. paggiae*, *A. physeteris*, and *A. brevispiculata* (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009).

A total of 7 isolated DNA samples were amplified for the COII genes by PCR technique which producing amplicons of 540 bp. Subsequently, the PCR products underwent electrophoresis in 1% agarose gel with 1 kb DNA ladder (1st base) (Figure 3). The 540 bp PCR products was obtained after the primer utilized in the PCR was aligned to mitochondrial genome sequence of *Anisakis simplex* with the access number of (AB241057.1) using BLAST software from the website http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

The alignment result of the 530 nucleotides of *Anisakis* showed 11 different sites, which function as

genetic barcode (Figure 4). The 11 nucleotide sites that could be utilized as barcode for Indonesian *Anisakis* sp. are on the 5th site (A/T), 40th site (A/C), 54th site (T/C), 210th site (T/C), 225th site (C/T), 344th site (T/C), 361st site (T/C), 381st site (C/T), 387th site (C/T), 414th site (A/G), an 417th site (C/A). Mattiucci et al., (2009); Santoro et al. (2010), described that mtDNA COII gene could be used as barcode to distinguish *Anisakis* at species level. Valentini et al. (2006); Santoro et al. (2010) stated that mtDNA COII gene nucleotides are highly polymorphic among the different *Anisakis* species. Santoro et al. (2010) reported that COII gene could be function as barcode to identify *A. pegreffii* that infected barrel-shaped turtles (*Celonia mydas*), fishes, and cetacean organisms in the Mediterranean Sea.

Table 2 presents the difference of nucleotides number between the *Anisakis* sample in the research and the species from GenBank. It showed that the difference ranged from zero (0) to 1 nucleotide (nt) among the *Anisakis* samples in the research and up to 11 when compared to *A. physeteris* from GenBank.

There was no significant difference among the COII gene sequence and the *Anisakis* sp. in the research, showing that there is no significant diversity among the samples. There is 1 nucleotide difference between

Table 2. Matrix of the difference between COII gene nucleotide sequence of *Anisakis* in the research and the *Anisakis* from GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>Anisakis physeteris</i>													
2 <i>Anisakis</i> 1						11							
3 <i>Anisakis</i> 2						10	0						
4 <i>Anisakis</i> 3						10	0	0					
5 <i>Anisakis</i> 4						11	0	0	1				
6 <i>Anisakis</i> 5						11	0	0	0	0			
7 <i>Anisakis</i> 6						11	0	0	0	0	0		
8 <i>Anisakis</i> 7						11	0	0	1	0	0	0	
9 <i>Anisakis simplex</i>						21	20	20	21	21	21	21	21
10 <i>Anisakis pegreffii</i>						32	32	34	33	34	32	32	32
11 <i>Anisakis typica</i>						42	42	41	41	42	42	43	42
12 <i>Anisakis brevispiculata</i>						54	56	56	53	54	53	53	53
13 <i>Anisakis paggiae</i>						61	62	60	60	60	61	62	62
14 <i>Anisakis nascettii</i>						68	68	68	68	69	69	70	70

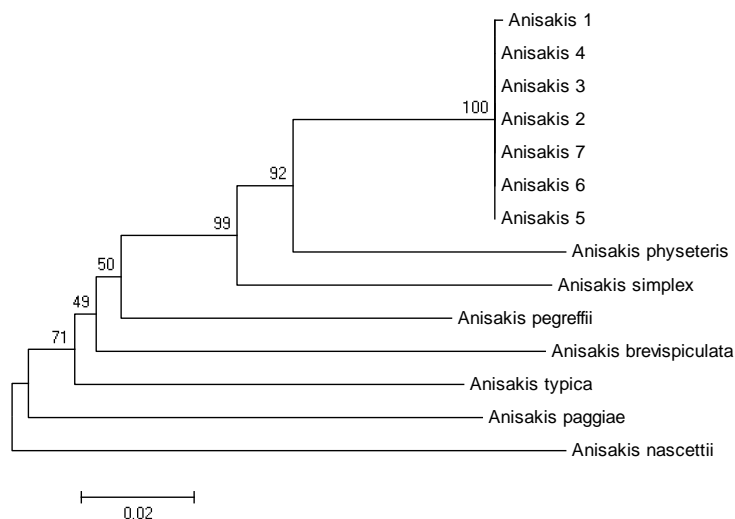


Figure 5. *Anisakis* phylogram, from the research based on COII gene nucleotide sequence (530 bp) compared to the species from GenBank

Anisakis sample no 3 and 4, as well as Anisakis sample no 4 and 7, while there was no differences (0 nt) among other samples.

It could be concluded that the Anisakis samples found in this research are from the same species because there are no significant nucleotide difference. The diversity percentage of COII gene nucleotide sequence between the Anisakis in the research compared to species from GenBank is presented as phylogenetic tree diagram using neighbor joining method with a bootstrap value of 1000 times. Figure 5 shows the Anisakis phylogenetic tree based on the nucleotide sequence of COII gene.

Figure 5 showed that all of the Anisakis samples studied creates a branch, and closely related to *A. physeteris* (100%) which have been saved in the GenBank database (Valentini *et al.*, 2006; Mattiucci *et al.*, 2009). This molecular data is consistent and support the previous study by Widjanarko (2015) on the characterization of *Anisakis* sp. along the coastal port of Sedang, Gunung Kidul, Yogyakarta, based on their morphology characteristics. This research proves that the Anisakis nematodes which infected tuna fishes included type II L3 Anisakis from *A. physeteris*.

Previously, there was no information on infection from type II L3 Anisakis in Indonesian waters. Mattiucci and Nascetti (2009) also found no type II L3 Anisakis in Asian waters but found some in the Middle East waters, southern ocean of the African continent and South Pacific. The prevalence of individual Anisakis found in this research was 7 nematodes out of 65 tuna fishes studied.

This result indicates that the distribution of type II L3 Anisakis larvae in Java Sea is low. Mattiucci and Nascetti (2008), Mattiucci *et al.* (2009), and Widjanarko (2015), stated that the low intensity and distribution of type II L3 Anisakis larvae could be influenced by the presence of end phase hosts from the ocean mammals. Gutiérrez-Galindo *et al.* (2010) reported the infection by type II L3 *Anisakis* sp. in *Micromesistius poutassou* and *Trachurus trachurus* fish in a low prevalence along the Alboran and Catalan waters. Piras *et al.* (2014) also reported that there was *A. physeteris* nematode infections in different prevalence in some fish species collected from north Sardinia (North West Mediterranean Sea), with high prevalence among *Micromesistius poutassou* and *M. merluccius*, and low prevalence among *P. blennoides*, *S. pilchardus*, *Sphyrna viridensis*, *Trachurus mediterraneus*, *T. Trachurus*, and *S. pilchardus*.

Mattiucci and Nascetti (2008) and Mattiucci *et al.* (2009) reported that the only end phase host of Type II L3 Anisakis was the sperm whale (*Physeteris macrocephalus*). In general, sperm whales migrate during mating or birth seasons (Fossette *et al.*, 2014). The presence of Anisakis nematode in marine environment is influenced by several factors such as hosts, nature condition, and the feeding habit of the infected fishes. In general, tuna fishes are sea predators that consume crustaceans, small pelagic fishes, and cephalopods. Widjanarko (2015) studied on tuna fishes

in Gunung Kidul, Yogyakarta, found many large sized crustaceans in the digestive tract of tuna. Piras *et al.* (2014) and Pozio (2013) stated that Anisakis life cycle includes small crustaceans-plantivore-piscivore-sea mammals-large crustaceans was rarely involved in Anisakis nematode life cycle. Pozio (2013) added that the low prevalence of Anisakis nematode infection in certain fish species could be affected by the number of infected crustaceans.

CONCLUSION

The *Anisakis* sp. infecting tuna fishes in the three fish markets in Cilacap District, Central Java, was type II L3 Anisakis larvae of *A. physeteris* (100%) and one of zoonotic organism. There were 32 nucleotide sites in COII mtDNA that could be utilized as genetic barcode for *A. physeteris* from Indonesia (Java Sea) compared with the *A. physeteris* from the database (GenBank).

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