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

Prasetyarti Utami*, Niken Satuti Nur Handayani, and Rony Marsyal Kunda
1Study Program of Biology, Faculty of Mathematics and Natural Science, Open University, Jakarta, Indonesia
2Department of Genetics, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia
3Study Program of Veterinary Science, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia
*Corresponding author: prasetyarti@ut.ac.id

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

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 zoooparasite 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. peggiae, and A. nascettii, whereas type II L3 Anisakis consists of A. peggiae, A. physeteris, and A. brevispiculata (Mattuici and Nascetti, 2008; Mattiucci et al., 2009). Mattiucci et al. (2011); Arizon et al. (2012), noted there were only A. simplex s.s, A. pegreffii, and A. physeteris cause anisakidiosis in human. Furthermore, Koinari et al. (2013) stated that the most Anisakis sp. that can cause anisakidiosis 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. Sakania and McKerrow (1989), Audicana and Kennedy (2008), and Koinari et al. (2013) described anisakidiosis in human was marked by several symptoms, such as sudden abdominal pain, nausea, vomiting, 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 anisakidiosis 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;
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 rosei, 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.

**Morphology 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 RNase 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 gel colored by bioatlas fluorescence (Genaid). 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 sequencings

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. ziphidaram (access number DQ116430), A. physeteris (access number DQ116432), A. brevispiculata (access number DQ116433), A. pagiae (access number DQ116434), and A. nascettii (access number DQ116431).

RESULTS AND DISCUSSION

The observational results of 65 mackerel tuna fishes (Euthynnus affinis) found Anisakid larvae as shown on Table 1.

| Target | F/R | Base order | Base numbers | Tm (°C) |
|--------|-----|------------|--------------|--------|
| COII (530 bp) | F | TGTAGTTTGCTTTTCGGTGTTCT | 23 | 55.0 |
| R | GCCCCACAAATCTCTGAACAA | 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).

Mattucci *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](image1.png)

**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

| #Anisakis_physeteris | ATG TTC AAC CTA CGA AAA ACC CAC CCA CTC ATA AAA ATC CTC AAC | [45] |
|---------------------|-------------------------------------------------------------|------|
| #Anisakis_1          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |
| #Anisakis_2          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |
| #Anisakis_3          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |
| #Anisakis_4          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |
| #Anisakis_5          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |
| #Anisakis_6          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |
| #Anisakis_7          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [45] |

| #Anisakis_physeteris | AAA GCC TTC ATT GTA CCA ACA CCA TCC AAT ATC TCT GCC TGA | [90] |
|---------------------|---------------------------------------------------------|------|
| #Anisakis_1          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |
| #Anisakis_2          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |
| #Anisakis_3          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |
| #Anisakis_4          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |
| #Anisakis_5          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |
| #Anisakis_6          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |
| #Anisakis_7          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [90] |

| #Anisakis_physeteris | ACA GCC TTC TCA TCA GTC GCC CAC TGC CGA GAC GTA AAC TAT | [225] |
|---------------------|---------------------------------------------------------|------|
| #Anisakis_1          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |
| #Anisakis_2          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |
| #Anisakis_3          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |
| #Anisakis_4          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |
| #Anisakis_5          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |
| #Anisakis_6          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |
| #Anisakis_7          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [225] |

| #Anisakis_physeteris | TCC TAC CTT TAC AAA GAA ACA TGA AAC GGA GTA ATC CTA | [360] |
|---------------------|---------------------------------------------------------|------|
| #Anisakis_1          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |
| #Anisakis_2          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |
| #Anisakis_3          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |
| #Anisakis_4          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |
| #Anisakis_5          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |
| #Anisakis_6          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |
| #Anisakis_7          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [360] |

| #Anisakis_physeteris | CTA ACA GTA ATA GCA GCC GCT TTC GTT GCC TAC TCT CAG CTA TCG TGA | [405] |
|---------------------|---------------------------------------------------------|------|
| #Anisakis_1          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |
| #Anisakis_2          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |
| #Anisakis_3          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |
| #Anisakis_4          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |
| #Anisakis_5          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |
| #Anisakis_6          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |
| #Anisakis_7          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [405] |

| #Anisakis_physeteris | GGA CAG ATG TCA TCA GTC GCC CTA CCA TCA GTA CTC ACA | [450] |
|---------------------|---------------------------------------------------------|------|
| #Anisakis_1          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [450] |
| #Anisakis_2          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [450] |
| #Anisakis_3          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [450] |
| #Anisakis_4          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [450] |
| #Anisakis_5          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [450] |
| #Anisakis_6          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [450] |
| #Anisakis_7          | T...... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... | [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 (GenBank AB241057.1). 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

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|----|----|----|----|
| Anisakis physeteris | 11 | | | | | | | | | | | |
| Anisakis 1 | 11 | | | | | | | | | | | |
| Anisakis 2 | 10 | 0 | | | | | | | | | | |
| Anisakis 3 | 10 | 0 | 0 | | | | | | | | | |
| Anisakis 4 | 11 | 0 | 0 | 0 | | | | | | | | |
| Anisakis 5 | 11 | 0 | 0 | 0 | 0 | | | | | | | |
| Anisakis 6 | 11 | 0 | 0 | 0 | 0 | 0 | | | | | | |
| Anisakis 7 | 11 | 0 | 0 | 1 | 0 | 0 | 0 | | | | | |
| Anisakis simplex | 21 | 20 | 20 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |
| Anisakis typica | 32 | 32 | 34 | 33 | 34 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| Anisakis brevispiculata | 54 | 56 | 56 | 53 | 54 | 53 | 53 | 53 | 52 | 52 | 52 | 53 |
| Anisakis paggiae | 61 | 62 | 60 | 60 | 60 | 61 | 62 | 62 | 61 | 62 | 62 | 62 |
| Anisakis nascettii | 68 | 68 | 68 | 68 | 68 | 69 | 70 | 70 | 68 | 68 | 68 | 68 |

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

122
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, Sphyraena 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-planetivore-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).

ACKNOWLEDGEMENTS

The author would like to thank LPPM-UT through Hibah Penelitian Lanjut Bidang Ilmu Tahun 2015, 08 April 2015, for funding this research.

REFERENCES

Abe, N., K. Tominaga, and I. Kimata. 2006. Usefulness of PCR-restriction fragment length polymorphism analysis of the internal transcribed spacer region of rDNA for identification of Anisakis simplex complex. Jpn. J. Infect. Dis. 59:60-62.

Arizono, N., M. Yamada, T. Tegoshi, and M. Yoshikawa. 2012. Anisakis simplex sensu stricto and Anisakis pegreffii: Biological characteristics and pathogenic potential in human anisakiasis. Foodborne Pathog. Dis. 9:517-521.

Audiciana, T.M., I.J. Ansotegui, L.F. De Corres, and M.W. Kennedy. 2002. Anisakis simplex: Dangerous-dead and alive? Trends Parasitol. 18:20-25.

Audiciana, M.T. and M.W. Kennedy. 2008. Anisakis simplex: From obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21:360-379.

Berland, B. 1961. Nematodes from some Norwegian marine fishes. Sarsia. 2:1-50.

Cavallero, S., S.A. Nadler, L. Paggi, N.B. Barros, and S. D’Amelio. 2011. Molecular characterization and phylogeny of anisakid nematodes from cetaceans from south-eastern Atlantic coasts of USA, Gulf of Mexico, and Caribbean Sea. Parasitol. Res. 108:781-792.

Cavallero, S., Lugas, A., Bruschi, F., Smelio, S.D. 2012. Molecular identification of Anisakis spp. from fishes collected in the Tyrrhenian Sea (NW Mediterranean). Vet. Parasitol. 187:563-566.

Chai, J.Y., K.D. Murrell, A.J. Lymbery. 2005. Fish-borne parasitic zoonoses: Status and issues. Int. J. Parasitol. 35:1233-1254.

Daluy, 2003. Prevalensi Anisakis pada Delapan Jenis Ikan Laut TPI Daerah Istimewa Yogyakarta. Thesis. Pasca Sarjana Universitas Gadjah Mada. Yogyakarta.

D’Amelio, S., K.D. Mathiopoulos, C.P. Santos, O.N. Pugachev, S.C. Webb, M. Picano, and L. Paggi. 2000. Genetic markers in ribosomal DNA for the identification of members of the genus Anisakis (Nematoda: Ascaridoidea) defined by polymerase-chain-reaction-based restriction fragment length polymorphism. Int. J. Parasitol. 30:223-226.

D’Amelio, S., S. Cavallero, N.O. Dronen, N.B. Barros, and L. Paggi. 2011. Two new species of Contracaecum Railliet & Henry, 1912 (Nematoda: Anisakidae), C. fagerholmi n. sp. and C. rudolphfi from the brown pelican Pelecanus occidentalis in the Northern Gulf of Mexico. Syst. Parasitol. 81:1-16.
Fossette, S., M.H. Jorgensen, M.V. Jensen, J. Kiszka, M. Berube, N. Bertrand, and M. Vely. 2014. Humpback whale (Megaptera novaeangliae) post breeding dispersal and southward migration in the western Indian ocean. *J. Exp. Marine Biol. Eco.* 450:6-14.

Gutiérrez-Galindo, J.F., A.C. Osanz-Mur, and M.T. Mora-Ventura. 2010. Occurrence and infection dynamics of anisakid larvae in *Scomber scombrus*, *Trachurus trachurus*, *Sarda pilchardus*, and *Engraulis encrasicolus* from Tarragona (NE Spain). *Food Control* 21:1550-1555.

Hutomo, M., Burhanuddin, and P. Hadidjaja. 1978. Observations on the incidence and intensity of infection of nematode larvae (families Anisakidae) in certain marine fishes of waters around Panggang Island, Seribu Islands. *Mar. Res. Indonesia.* 21:49-60.

Indaryanto, F.R., Y. Wardiatno, and R. Tauria. 2015. Inventarisasi cacing parasitik pada ikan kembung di perairan Teluk Banten dan Pelabuhan Ratu. *J. Vet.* 16(2):196-203.

Kiewska, A., J. Dzido, O. Shukhaltar, and J. Rokicki. 2009. Anisakid parasites of fishes caught on the African shelf. *J. Parasitol.* 95:639-645.

Koinan, M., S. Karl, A. Elliot, and A.J. Lynbery. 2013. Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea. *Vet. Parasitol.* 193:126-133.

Kumar, S., K. Tamura, J. Dudley, and M. Nei. 2001. Molecular evolutionary genetics analysis (MEGA) software version 6.0. *Mol. Biol. Evol.* 24:1596-1609.

Mattiucci, S. and G. Nascetti. 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. *Adv. Parasitol.* 66:47-148.

Mattiucci, S., M. Paoletti, and S.C. Webb. 2009. *Anisakis nascetti* sp. (Nematoda: Anisakidae) from beaked whales of the southern hemisphere: Morphological description, genetic relationships between congeners and ecological data. *Syst. Parasitol.* 74:199-217.

Mattiucci, S., M. Paoletti, F. Bornini, M. Palumbo, R.P. Macarone, V. Gomes, A. Casati, and G. Nascetti. 2011. First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. *BMC Inf. Disease.* Doi:10.1186/1471-2334-11-82.

Nadler, S.A., S. D’Amelio, M.D. Dailey, L. Paggi, S. Siu, and J.A. Saknanai. 2005. Molecular phylogenetics and diagnosis of Anisakis, Pseudoterranova, and Contracaecum from northern Pacific marine mammals. *J. Parasitol.* 91:1413-1429.

Piras, M.C., T. Tedde, G. Garippa, S. Virgilio, D. Sanna, S. Farajallah, and P Merelle. 2014. Molecular and epidemiological data on *Anisakis* spp. (Nematoda: Anisakidae) in commercial fish caught off northern Sardinia (western Mediterranean Sea). *Vet. Parasitol.* 203:237-240.

Pontes, T., S. D’Amelio, G. Costa, and L. Paggi. 2005. Molecular characterization of larval anisakid nematodes from marine fishes of Madeira by a PCR-based approach, with evidence for a new species. *J. Parasitol.* 91:1430-1434.

Poizzo, G. 2013. Integrating animal health surveillance and food safety: The safety of Anisakis. *Rev. Sci. Tech. Off. Int. Epic.* 32:487-496.

Sakanari, J.A. and J.H. McKerrow. 1989. Anisakiasis. *Clin. Microbiol. Rev.* 2:278-284.

Santoro, M., S. Mattucci, M. Paoletti, A. Liotta, B.D. Uberti, G. Galiero, and G. Nascetti. 2010. Molecular identification and pathology of *Anisakis pegreffii* (Nematoda: Anisakidae) infection in the Mediterranean loggerhead sea turtle (*Caretta caretta*). *Vet. Parasitol.* 174:65-71.

Setyobudi, E., S. Helmatti, and Soeparno. 2007. Infeksi *Anisakis* sp. pada layar (*Trichiurus* sp.) di Pantai Selatan Kabupaten Purworejo. *J. Perikanan* 9(1):142-148.

Setyobudi, E., C.H. Jeon, C.H. Lee, K.B. Seong, and J.H. Kim. 2011. Occurrence and identification of *Anisakis* spp. (Nematoda: Anisakidae) isolated from chum salmon (*Oncorhynchus keta*) in Korea. *Parasitol. Res.* 108:585-592.

Siagian, F.E., G.T.Y. Siagian, and A.O. Siagian. 2010. Kelainan yang berhubungan dengan larva *Anisakis* sp. *Majalah Ked. FK. UKL.* 27(3):122-129.

Sohn, W.M., J.M Kang, and B.K. Na. 2014. Molecular Analysis of *Anisakis* type I larvae in marine fish from three different sea areas in Korea. *Korean J. Parasitol.* 4:383-389.

Suadi, S. Helmatti, and R. Widanangroem. 2007. Population and parasite parameter in hairtail (*Trichiurus* spp.) landed at PPS Cilacap. *J. Fish. Sci.* 9(2):226-232.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, Position-specific gap penalties and weight matrix choice. *Nucleic Acid Res.* 22:4673-4680.

Umehara, A., Y. Kawakami, T. Matsu, J. Araki, and A. Uchida. 2006. Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. *Parasitol. Int.* 55:267-271.

Utami, P. 2013. Identifikasi *Anisakis* sp. pada beberapa ikan laut di beberapa tempat pelelangan ikan (TPI) Cilacap. *J. Matematika Sains dan Teknologi.* 15(1):21-28.

Valentini, A., S. Mattucci, P. Bondaneli, S.C. Webb, A.A. Mignucci-Giannone, M.M. Colom-Llavina, and G. Nascetti. 2006. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial cox2 sequences, and comparison with alloyme data. *J. Parasitol.* 92:156-166.

Widjanarko, A.P. 2015. Identifikasi, Prevalensi dan Intensitas *Anisakis* spp. (Nematoda Anisakisidae) in certain marine fishes of waters around Panggang Island, Seribu Islands. *J. Perikanan* 14(1):142-149.

Yman, L. 2003. Spesifik IgE in the diagnosis of parasite-induced allergy. *Allergy.* 59:14-17.

Zhu, X., R.B. Gasser, M. Podolska, and N.B. Chilton. 1998. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *Int. J. Parasitol.* 28:1911-1921.