Species Identification of *Anisakis* Parasites in Mae Klong Mackerel from Thailand Using DNA Barcoding

Tanawat Chaiphongpachara[1,*] and Sedthapong Laojun[2]

[1] Department of Public Health and Health Promotion, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram, 75000, Thailand, E-mail: tanawat.ch@ssru.ac.th

[2] Department of Public Health and Health Promotion, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram, 75000, Thailand, E-mail: sedthapong.la@ssru.ac.th

*Corresponding author

Abstract

Anisakis parasites significantly impact public health. Unlike most other fish-borne parasites, which are typically associated with freshwater seafood, Anisakids are primarily linked to marine fish. This parasite has also been detected in Mae Klong mackerel. However, the morphological similarities among members of the Anisakis genus make species-level identification through morphological methods challenging. Consequently, DNA barcoding was employed to confirm the species level of the Anisakis parasite found in Mae Klong mackerel. Our study determined that the Anisakis parasites from Maeklong mackerel were identified as A. typica, based on comparisons with published sequences in the GenBank database. The results of the phylogenetic tree analysis corroborated this identification, demonstrating a clear clustering of our samples with A. typica sequences from the database. Thus, we underscore the importance of safe consumption practices to mitigate the risk of Anisakis infections and emphasizes the need for public education on the dangers of consuming raw fish, advocating adherence to food safety guidelines, such as thorough cooking or appropriate freezing.

Keywords: parasites, *Anisakis* species, mackerel, species identification, DNA Barcoding

1. Introduction

Anisakids are roundworms found in the gastrointestinal tract of fish and are the causative agent of anisakiasis in humans (Baird et al., 2014). Anisakids, which are nematodes or roundworms, belong to the phylum Nemathelmintes, class Nematoda, order Ascarida, suborder Ascaridina, superfamily Ascaridoidea, and family Anisakidae. According to current taxonomic data, the family Anisakidae comprises several genera such as *Anisakis*, *Pseudoterranova*, *Contracaecum*, and Hysterothylacium (Choi et al., 2011; Nieuwenhuizen & Lopata, 2013). Among these, three species from the genus *Anisakis*—*A. simplex* sensu stricto, *A. pegreffii*, and *A. physeteris*—are identified as the primary agents causing infections in humans. Briefly, the life cycle of *Anisakis* species involves several hosts, including cetaceans as definitive hosts, zooplankton as intermediate hosts, and fish and cephalopods as either intermediate or paratenic hosts. Additionally, humans can become accidental hosts in this cycle; infection typically occurs through the consumption of raw or undercooked fish containing live third-stage *Anisakis* larvae (Baird et al., 2014; Nuchjangreed et al., 2006). The severity of the resulting condition, anisakiasis, can vary from mild to severe, manifesting symptoms in the stomach, intestines, and even causing uterine complications and allergic reactions (Nieuwenhuizen & Lopata, 2013). The

disease was first reported in the 1960s in the Netherlands. To date, there have been approximately 20,000 reported cases of anisakiasis worldwide, with the majority of cases occurring primarily in Japan, Europe, and the United States (Fitzsimmons et al., 2014; Sugiyama et al., 2022).

In Thailand, the first documented case of intestinal anisakiasis occurred in 1993 when a patient with acute abdominal obstruction was diagnosed through the examination of a parasite in a biopsy from the small intestine (Hemsrichart, 1993). Previous surveillance efforts have investigated parasites in various marine fish species, revealing *Anisakis* infections in several fish. More recently, this parasite has also been detected in Maeklong mackerel (Chaiphongpachara et al., 2022). However, due to the significant morphological similarities among members of the *Anisakis* genus, species-level identification using solely morphological methods has proven challenging (Lim et al., 2015). Consequently, molecular biology techniques are essential to ensure accurate species confirmation.

DNA barcoding is a method of specimen identification that utilizes short, standardized DNA segments to identify organisms at the species level (Ratnasingham & Hebert, 2007). Although various genetic loci have been proposed for this purpose, the mitochondrial genes cytochrome *c* oxidase I and II (*COI* and *COII*) are most commonly employed as markers. The process begins by amplifying the target DNA region using Polymerase Chain Reaction (PCR), followed by verifying the success of the PCR. Subsequently, the DNA barcode is sequenced, and the organism is identified by comparing the reference species sequences with the database.

To confirm the *Anisakis* species found in Maeklong mackerel, DNA barcoding based on *COII* gene was used for species identification. The results of this study will assist in disease surveillance and inform strategies for safer mackerel consumption.

1.1 Research Objective

This study aims to identify the species of the *Anisakis* parasite found in Maeklong mackerel through the use of DNA barcoding.

2. Methodology

1. *Anisakis* samples

Anisakis samples used in this study were obtained from mackerel (*Rastrelliger kanagurta*) purchased in November 2016 from a fish market in Samut Songkhram Province, Thailand, as part of a previous study. Each third instar larva of *Anisakis* parasites was separated and stored in a sterile 1.5 ml micro-centrifuge tube containing 70% ethanol, and kept at -20°C in the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Campus, Thailand.

2. DNA extraction, polymerase chain reaction, and DNA sequencing

Anisakis samples stored at -20°C were subjected to DNA extraction using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan), following the manufacturer's protocols. The mitochondrial cytochrome oxidase II (*COII*) gene was then amplified via PCR with two universal primers: forward (5′-TTT TCT AGT TAT ATA GAT TGR TTT YAT-3′) and reverse (5′-CAC CAA CTC TTA AAA TTA TC-3′). PCR mixtures were prepared in 50 µl volumes containing 5 µl of genomic DNA, 1x reaction buffer, 2.5 mM $MgCl_2$,

0.4 mM dNTPs, 0.3 µM of each primer, 5 U of Taq polymerase, and distilled water. The PCR cycling conditions included an initial denaturation at 94°C for 3 min, followed by 34 cycles of 30 s at 94°C, 60 s at 46°C, and 90 s at 72°C, concluding with a final extension at 72°C for 10 min. Successful amplification of the *COII* gene was indicated by the presence of a band at approximately 591 bp on a 1.5% agarose gel. The resulting PCR products were subsequently purified and bidirectionally sequenced by SolGent, Inc. (Daejeon, Korea).

3. Species identification via Genetic Database Comparison

After *COII* gene sequencing, the forward and reverse sequences were initially trimmed and subsequently aligned to assemble into a consensus sequence using BioEdit software version 7.2.5 (Hall, 1999). The consensus sequences of *Anisakis* samples were then compared with previously published sequences in the GenBank database via the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the percentage identity matches were calculated.

4. Phylogenetic analysis

Phylogenetic analysis was carried out to examine the relationships among Anisakis species. This analysis employed the neighbor-joining (NJ) method using MEGA 11 software (Tamura et al., 2021). Branch support for the NJ tree was assessed through bootstrapping with 1000 replicates to ensure reliability.

3. Results

1. DNA Barcoding

Four *Anisakis* samples, obtained from mackerel at a fish market in Samut Songkhram Province, Thailand, were assessed for species identification via BLAST by comparing their DNA sequences with reference sequences in the GenBank database, as detailed in Table 1. Four samples matched with the reference *A. typica* sequences in the database. The percentage identity ranged from 93.57% to 100%. These high levels of sequence identity strongly support the accurate identification of the samples as *A. typica*.

Table 1. BLAST results of our Anisakis samples found in Mae Klong Mackerel from Thailand.

Anisakis species	ID in this study	Species match	% Identity (*n*)
Unknown	001	*Anisakis typica*	94.25%–100% (34)
Unknown	002	*Anisakis typica*	94.92%–100% (34)
Unknown	003	*Anisakis typica*	93.57%–100% (34)
Unknown	004	*Anisakis typica*	93.57%–100% (34)

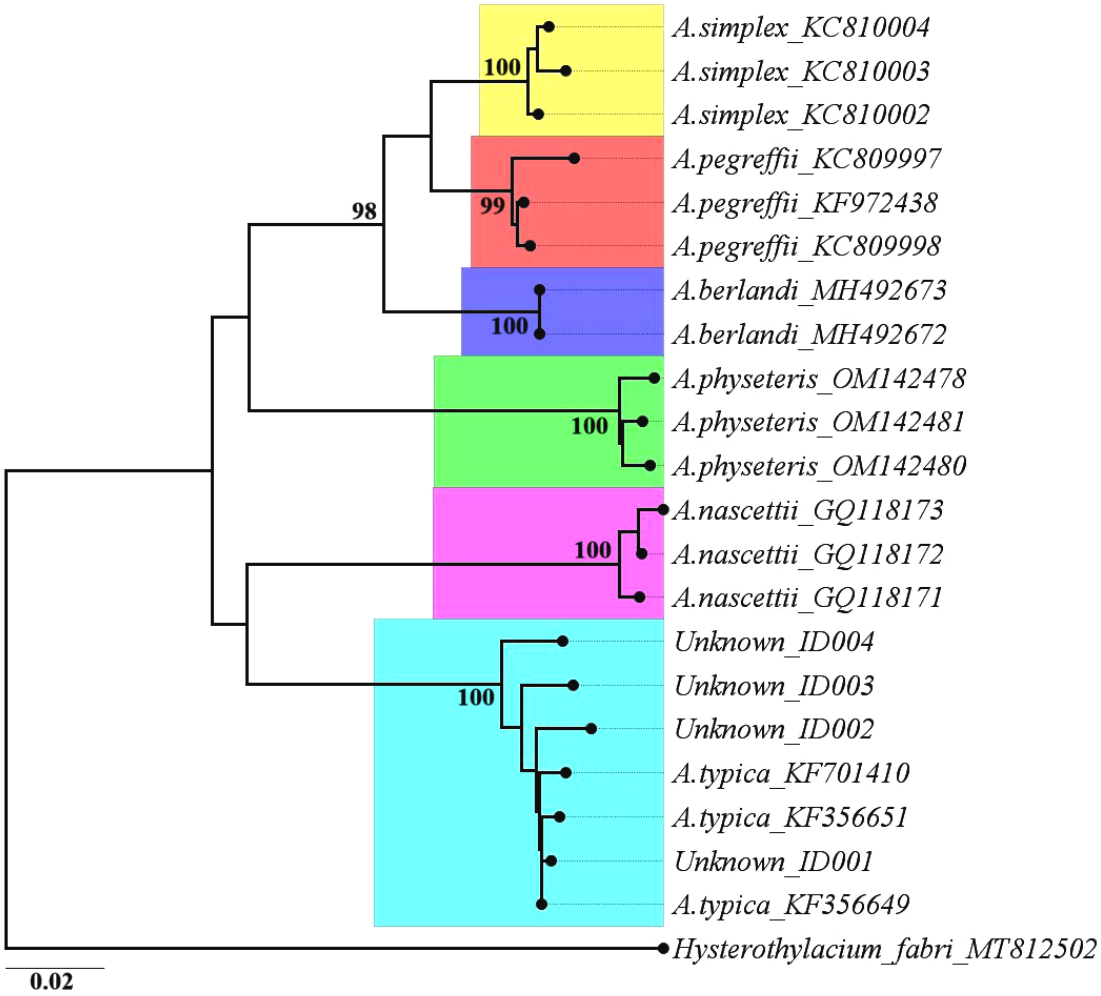
DNA reference sequences from the GenBank database that had 100% query coverage were used for comparison with our sequences.

2. Phylogenetic tree

The NJ tree based on *COII* sequences was constructed to assess the relationships among *Anisakis* species, with *Hysterothylacium fabri* serving as the outgroup, as shown in Figure 1. This analysis revealed that our four *Anisakis* samples formed a cluster within the *A. typica* group, effectively differentiating them from other species. Positioned at the bottom of the tree,

H. fabri served as the outgroup, distinctly separate from all *Anisakis* species, consistent with its taxonomic classification outside the *Anisakis* genus.

Figure 1. Neighbor-Joining tree based on COII gene sequences of Anisakis species from GenBank, alongside four unidentified species in Mae Klong Mackerel from Thailand. Hysterothylacium fabri was used as an outgroup. Bootstrap support values of 90% or higher are indicated for significant branches



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4. Discussion

Our study determined that the *Anisakis* parasites found from Mae Klong mackerel were identified as *A. typica*, based on comparisons with published sequences in the GenBank database. The results of the phylogenetic tree analysis further supported this identification, showing a clear clustering of our samples with *A. typica* sequences from the database. This finding aligns with previous research indicating the presence of this parasite in mackerel in Thailand (Chaiphongpachara, 2019; Chaiphongpachara et al., 2022). Furthermore, previous studies have indicated that *A. typica* is the dominant species in neighboring countries such as Malaysia, Indonesia, and the Philippines, with *A. pegreffii* being the second most prevalent (See

et al., 2022). Although this parasite is not considered a major human pathogen, precautionary measures are recommended.

The results demonstrated that DNA barcoding can effectively distinguish among species within the *Anisakis* genus. However, the BLAST analysis revealed a significant interspecific variation in *A. typica*, with sequence identities ranging from 93.57% to 100%. This variation suggests that *A. typica* may represent a species complex or exhibit regional differences. The previous research supports this, having identified two genetic groups of *A. typica* between South America and Asian countries, based on the *COII* gene (Chaiphongpachara et al., 2022). However, almost no variation was observed in the internal transcribed spacer (ITS) region of ribosomal DNA, indicating genetic variation rather than a complex species group. Our study underscores the risk of mackerel being infected with *Anisakis* parasites, emphasizing the need for careful consumption practices (Chai et al., 2005). Local communities should be informed about the risks of consuming raw fish, which may be contaminated with parasites and pose health risks if not properly handled (Pravettoni et al., 2012; Chaiphongpachara, 2019; Debenedetti et al., 2019). Additionally, infections can be prevented by cooking or, for those who consume raw fish, by freezing it at -35°C or colder for 15 hours, or at -20°C or colder for seven days (Chaiphongpachara, 2019).

5. Conclusions

This study confirmed the species of *Anisakis* parasites in Mae Klong mackerel as *A. typica* through DNA barcoding. The research underscores the importance of safe consumption practices to mitigate the risk of *Anisakis* infections. It emphasizes the need for public education about the dangers of consuming raw fish and the necessity of adhering to food safety guidelines, such as thorough cooking or appropriate freezing.

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