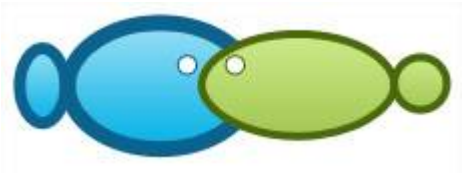

A brief assessment of NADH dehydrogenase 2 (ND2) gene in tuna fish for species determination through DNA barcoding

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Abstract. DNA barcoding has been used to identify fish species, especially for authenticating fishery products. In the authentication process for processed tuna products, DNA barcoding is required due to its accuracy and the small number of tissue samples needed for the process. As a standard gene marker, the COI gene has shortcomings in differentiating several fish species. This study aimed to examine the ability of the mitochondrial NADH dehydrogenase 2 (ND2) gene marker in DNA barcoding for the determination of tuna species. Variation within 1,042 bp of ND2 gene in 13 species of three tuna groups (bluefin, yellowfin, and other tuna groups) showed better performance than marker genes in previous studies (COI gene, CYB gene, and 16S rRNA gene) to be used in DNA barcoding. There were 296 observed points of interspecific variation, of which 49 points were able to distinguish members of the genus *Thunnus* from other tuna genera. There are no identical sequences from all the compared species. The final results provided prospects for the use of the ND2 gene species identification of tuna through DNA barcoding and the development of practical methods (e.g. PCR-RFLP) for the authentication of tuna products.

Key Words: bluefin, DNA barcoding, ND2 gene, tuna, yellowfin.

Introduction. The use of DNA barcoding has shown an important role in the authentication of fish species and fishery products (Rasmussen & Morrissey 2008). Many processed fish products are labeled with a label that does not match the fish ingredients used (Xiong et al 2019). In general, morphological features are used to identify various species of tuna but this requires highly skilled human resources. Nowadays, this method is difficult to be used in identifying products that are already in the form of filets and canned fish (Bottero et al 2007). On the other hand, consumers have the right to be informed of the identity of the goods purchased, both raw and processed tuna, so that proper identification methods are important to continue (Aranishi et al 2005). The prospect of using DNA barcoding technology has opened up opportunities for precise species identification, even if only from a small number of tissue specimens (Dudu et al 2016). Specifically for tuna, sequencing of several genes from mitochondrial DNA is still used because it can reliably differentiate many of these fish species (Wulansari et al 2015).

DNA barcoding research has been carried out to identify tuna species in Indonesia, but only based on the Cytochrome B (CYB/CytB) gene (Wulansari et al 2015; Nurilmala et al 2016). Since 2003, it has been proposed that the cytochrome-c-Oxidase I gene (often abbreviated as the COI or cox1 gene) from mitochondria is the standard DNA barcoding gene for most animals (Hebert et al 2003). Nonetheless, the COI gene has limitations in distinguishing several fish species (Imtiaz et al 2017). In other animals, as reported by Lv et al (2014), the COI gene may not necessarily provide reliable results compared to other genes when used for DNA barcoding. As an alternative to COI, the mitochondrial NADH dehydrogenase 2 (ND2) gene is also used due to better genetic distance among closely related species, such as cichlid fish (Kocher et al 1995), birds (Luttrell et al 2020), and dolphins (Caballero et al 2015). Luttrell et al (2020) even

declared that ND2 gene tree was more statistically robust, has a minimum of 1.5 times greater genetic distance between sister clades, and also resolves paraphyly in two clades when compared to COI gene.

As a standard gene, the COI gene has been developed to study fish biodiversity. However, in the prior study regarding tuna fish, this gene is still considered unable to distinguish all tuna species (Kolondam 2020a). Previous studies have also explored the capabilities of other genes, for example, the CYB gene (Kolondam 2020b) and 16S rRNA (Kolondam 2022), in an effort to find better markers. Considering the capability of ND2 gene, this research was conducted with the aim to assess the ability of the mitochondrial ND2 gene in DNA barcoding for the determination of tuna species.

Material and Method

Mitochondrial DNA of tuna fish. This research was conducted entirely by in silico analysis from January to March 2023. The research retrieved sequence information from NCBI (National Center for Biotechnology Information) GenBank. The ND2 gene sequence from the tuna specimen was taken directly from the mitochondrial genome (mitogenome). Detailed information on all sequences for analysis is listed in Table 1. This table contained 13 specimens from different tuna species, complete with its unique GenBank accession numbers. These tuna species were divided into three groups, namely bluefin tuna, yellowfin tuna, and other types of tuna that are closely related.

Table 1

Mitochondrial DNA of tuna fish used in this study

Group	*Species*	*Accession number*	*References*
Bluefin tuna (*Thunnus*)	*Thunnus alalunga*	AB101291.1	Manchado et al (2016)
	Thunnus maccoyii	KF925362.1	Li et al (2016)
	Thunnus obesus	GU256525.1	Martinez-Ibarra et al (2016a)
	Thunnus orientalis	KF906721.1	Chen et al (2014)
	Thunnus thynnus	AP006034.1	Satoh et al (2016)
Yellowfin tuna (*Neothunnus*)	*Thunnus albacares*	NC_014061.1	Martinez-Ibarra et al (2010)
	Thunnus atlanticus	NC_025519.1	Márquez et al (2016)
	Thunnus tonggol	NC_020673.1	Martinez-Ibarra et al (2013)
Other tuna species	*Auxis rochei*	KM651784.1	Li et al (2014a)
	Auxis thazard	AB105447.1	Catanese et al (2008)
	Euthynnus affinis	NC_025934.1	Li et al (2014b)
	Euthynnus alletteratus	NC_004530.1	Infante et al (2006)
	Katsuwonus pelamis	JN086155.1	Martinez-Ibarra et al (2016b)

Mitochondrial genomic DNA obtained from GenBank was 16.5 kbp each. From this one specimen, the ND2 gene was separated from the other genes and the non-coding region. The location of the gene can be found from the description of the name of the gene, the region of the gene (in the form of a number), along with the coding sequence of the amino acid sequence encoded by the gene. The 1,047 bp long ND2 gene of each specimen was extracted for analysis.

Multiple sequence alignment. Sequence alignment was performed using the MUSCLE algorithm (Edgar 2004) which is integrated with Geneious v5.6 software (Kearse et al 2012). The differences in each nucleotide point are shown by comparing the nucleotides of all specimens in the same position. The percentage similarity prepared as part of the Geneious software was calculated based on sequence alignment. Consensus is also shown using the nucleotide notation of the nucleotide variation at the point where there is a difference.

Results and Discussion

Polymorphism of ND2 gene sequence in tuna fish. The ability of the ND2 gene as a DNA barcode for the determination of tuna species is considered excellent. Based on Figure 1, it can be seen that there are 296 sites of difference in nucleotides between the 13 tuna specimens. The nucleotide variation found at these points can confirm the sequence differences between the genus *Thunnus* (bluefin and yellowfin groups) in general and other types of tuna groups (genera of *Auxis*, *Euthynnus*, and *Katsuwonus*). Variation started at nucleotide number 30 to nucleotide number 1,042. None of the compared species has an identical ND2 sequence.

Figure 1. Sequence variation in ND2 gene sequence among 13 species of tuna fish.

The genus *Thunnus* (bluefin and yellowfin) mostly shared the same nucleotides and can be distinguished from the other tuna genera at 49 sites on the ND2 gene sequence (Figure 1). The variation points were at position number 54, 78, 84, 87, 96, 102, 120, 132, 147, 165, 171, 186, 213, 225, 243, 246, 277, 300, 306, 372, 390, 414, 454, 464, 465, 471, 474, 594, 609, 624, 625, 627, 645, 651, 721, 723, 727, 774, 792, 856, 915, 926, 937, 957, 969, 987, 1,008, and 1,026. According to Dooley et al (2005), this variation has the potential to be developed as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for rapid identification of species without the need for DNA sequencing.

At certain positions, there are also variations that can distinguish the bluefin group, the yellowfin group, and other tuna groups. At the nucleotide position number 345, there is a thymine (T) for the bluefin tuna group and a cytosine (C) for the yellowfin tuna group. For the other tuna genera, it is not uniform in this position. For *A. thazard*, there was a guanine (G) at that position. *A. rochei*, although in the same genus as *A. thazard*, has adenine (A) in that position as members of the other two distinct genera. For other types of tuna, there are uniformities at 22 different nucleotide sites with the bluefin and yellowfin groups. These sites are nucleotide number 54, 78, 120, 132, 147, 186, 231, 246, 363, 414, 464, 465, 471, 624, 625, 627, 721, 727, 774, 926, 937, and 1,014.

Resolution of ND2 gene for DNA barcoding of tuna fish. Out of the 13 species tested in this study using the ND2 gene sequence, no species was observed to have identical sequence (Table 2). The variations in 49 sites were able to distinguish all the species. From Table 2, the degree of similarity between species in the genus *Thunnus* ranges from the lowest, 94.5% (*T. atlanticus* vs. *T. thynnus*) to the highest 99.2% (*T. thynnus* vs. *T. orientalis*). The level of similarity in the bluefin group is between 95.4% (*T. maccoyii* vs. *T. thynnus*) to 99.2% (*T. thynnus* vs. *T. orientalis*). The similarity within the Yellowfin group ranged from 98.0% (*T. albacares* vs. *T. tonggol*) to 98.9% (*T. atlanticus* vs. *T. albacares*).

Table 2

ND2 gene sequence similarity among tuna species

#	Species	Similarity (%)												
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
1	*Thunnus alalunga*	100												
2	*Thunnus maccoyii*	96.0	100											
3	*Thunnus obesus*	95.6	97.3	100										
4	*Thunnus orientalis*	99.0	95.8	95.6	100									
5	*Thunnus thynnus*	98.9	95.4	95.2	**99.2**	100								
6	*Thunnus atlanticus*	94.8	97.1	97.1	94.6	**94.5**	100							
7	*Thunnus tonggol*	95.3	97.8	98.0	95.3	94.9	98.3	100						
8	*Thunnus albacares*	95.1	97.1	97.3	95.0	94.8	98.9	98.0	100					
9	*Auxis rochei*	84.6	85.0	85.4	84.4	84.3	84.6	85.2	84.0	100				
10	*Auxis thazard*	83.4	83.2	83.7	83.7	83.5	83.6	83.3	83.1	91.5	100			
11	*Euthynnus affinis*	84.2	83.8	83.8	84.1	84.2	83.2	83.6	83.6	84.6	84.5	100		
12	*Euthynnus alletteratus*	83.4	82.7	83.0	83.5	83.2	82.5	82.8	82.6	85.2	84.5	91.5	100	
13	*Katsuwonus pelamis*	84.6	85.2	85.0	84.3	84.3	85.0	85.1	85.4	87.3	86.7	86.5	86.8	100

The COI/cox1 gene, which is a standard animal DNA barcoding gene (Pentinsaari et al 2016; Yang et al 2018; Wu et al 2019), has 248 points of difference along 1,551 bp. However, this COI gene was unable to differentiate between *T. orientalis* and *T. thynnus*, also all the species studied shared at least 91% similarity (Kolondam 2020a). For the CYB/CytB (cytochrome b) gene, which is widely used for phylogenetic studies (Brisse et al 2003; Martínez et al 2012; Liu et al 2020), also has 248 points of difference from 1,141 bp (Kolondam 2020b). The CYB gene has a higher resolution than the COI gene in previous studies which can be seen from its ability to distinguish all the species studied. The 16S rRNA gene has 99 positions of nucleotide differences from a total length of 1,695 bp (Kolondam 2022). The 16S rRNA gene is also able to distinguish all tuna species, but its resolution is considered to be lower than CYB gene when considering its similarity value to the COI gene (more than 90%).

By comparing previous studies using the same accession number mitochondrial DNA, the ND2 gene has shown better performance during in silico analysis to obtain the ideal DNA barcoding marker gene. This performance can be used as a promising prospect for designing fast species determination methods, for example using PCR-RFLP. Yao et al (2020) succeeded in designing the PCR-RFLP method to identify four species of tuna sashimi products based on the CYB gene. Lin & Hwang (2007) also succeeded in differentiating eight species of tuna from canned fish products using the CYB gene. It has been proven from this study that the ND2 gene is superior to the CYB gene by Kolondam (2020b) using an equivalent comparison (using analysis from the same mitochondrial genome and using more reference species than previous studies). This finding can lead to the development of a better PCR-RFLP method designed from ND2 gene sequences.

Conclusions. Based on the variations in 13 species of three groups of tuna fish compared, the ND2 gene has shown better performance than the marker genes in previous studies (COI gene, CYB gene, and 16S rRNA gene). There were 296 sites of interspecific variation observed, of which 49 points were able to distinguish members of the genus Thunnus from other tuna genera. There is no identical sequence of ND2 gene among 13 species studied. This study provided prospects for the use of the ND2 gene for identification through DNA barcoding, as well as for the development of a more practical way of determining tuna species using PCR-RFLP.

Conflict of interest. The authors declare that there is no conflict of interest.

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