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GENETIC CHARACTERIZATION OF LONGTAIL TUNA *(Thunnus tonggol* BLEEKER, 1851) BASED ON PARTIAL SEQUENCE OF *16S* rRNA MITOCHONDRIAL GENE

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ABSTRACT

Although the Longtail tuna *(Thunnus tonggol)* is an important fish in Indonesia, the population structure has not been investigated. In this study, the genetic differences in geographic scale are analyzed to provide a clear picture of the structure of *T. tonggol* populations along a transect stretching from Pemangkat (western Kalimantan) to Pekalongan in the Java Sea. We also analyzed SNPs in the mitochondrial *16S* rRNA gene of *T. tonggol* as potential molecular marker for the identification of the origin within species. In total, three polymorphic sites (all represent singleton dimensions) were identified in the sequence analysis of the 570-bp fragment among a total of 97 *T. tonggol* individuals from Pekalongan and Pemangkat. Based on these polymorphic sites, four haplotypes were identified. The Pemangkat samples had higher amount of haplotype and nucleotide diversity (h = 0.1556 ± 0.0680 and Π = 0.000277 ± 0.000432), meanwhile samples Pekalongan showed lower levels of diversity (h = 0.0400 ± 0.0380 and Π = 0.000070 ± 0.000209). The study revealed a single, intermixing population of *T. tonggol* across the sampled location. No significant structuring was observed between other pairwise comparisons, indicating gene flow between geographically adjacent locations.

Keywords: Longtail tuna; 16S rRNA; genetic characteristic

INTRODUCTION

Longtail tuna, *Thunnus tonggol* (Bleeker 1851), is the second smallest of eight *Thunnus* species and grows to a maximum size of 142 cm total length (TL) and 35.9 kg (Froese & Pauly, 2010). It is an economically important pelagic species inhabiting tropical and subtropical provinces of the Indo-Pacific region. From northern Australia, Papua New Guinea, and Indonesia, and northwest through Malaysia and Thailand, the species has a continuous distribution to the northeast to southern Japan and to the northwest to Iran and the Red Sea (Froese & Pauly, 2010). Their distribution is unique compared to other *Thunnus* species in that they nearly exclusively occupy neritic areas close to landmasses and are rarely found offshore (Yesaki, 1993).

Global catches of longtail tuna increased substantially to around 100,000 t yr-1 in 1985 and continued to increase to 248,000 t in 2007. Annual reported catches have been variable for most countries, although over the past decade four countries such as: Thailand, Indonesia, Malaysia, and Iran, have contributed most to the global catch (Griffiths *et al.*, 2010). Good resource management is vital if continued economic progress and poverty alleviation is to be achieved. In many parts of Southeast Asia, fisheries resources have become depleted due to inadequate management, but in the case of tonggol, there exists a current opportunity to ensure sustainable use (Willette *et al.,* 2016). Decreased natural resources necessitate the development of molecular markers that can be used for stock assessment and fishery management, population dynamics, and phylogenetic relationship analyses. Neritic tuna species that exist in shallower coastal waters of Southeast Asia have received less attention than their pelagic counterparts in molecular genetic studies. Further, population patterns may be distinct from pelagic tuna species due to the influence of availability of shelf habitats and localized ocean currents (Willette *et al.,* 2016).

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Microsatellites are widely used as molecular markers in population genetics. However, with the development of high-throughput screening strategies, single nucleotide polymorphisms (SNPs) have increasingly been used as molecular markers due to several advantages, including processing efficiency, ease in both scoring and standardizing genotypes across laboratories, and the high density in which they are observed across most genomes (Vignal *et al*., 2002; Anderson & Garza, 2006).

Knowledge of genetic population is important in planning and implementing appropriate management strategies for the *T. tonggol.* Furthermore, efforts regarding to reduce pressures on natural populations through domestication and increase production through selective breeding, the basic information related to genetic conditions of this species is necessary. Here, the genetic differences in widely geographic area were analyzed to provide a clear picture of the structure of *T. tonggol* populations along a transect stretching from Pemangkat (western Kalimantan) to Pekalongan in the Java Sea. In this study, we also analyzed SNPs in the mitochondrial *16S* rRNA gene of *T. tonggol* as potential molecular marker for the identification of the origin within species. The results can provide important biological data as basic information to properly plan policy and management of *T. tonggol*.

MATERIALS AND METHODS

Area Study, Sample Collection and Preservation

Sampling conducted at two locations. The first location is 6°51'S, 109°41'E (Pekalongan) and the second one is 1°10'N, 108°58'E (Pemangkat) (Figure 1). Tissue samples of *T. tonggol* were collected from whole specimens landed at fish port. Tissue samples were taken from the flank of the fish and preserved in 95% ethanol until molecular analysis. Total 97 individuals that were used for the partial mitochondrial *16S* rRNA gene analysis were identified at the sampling site and then transported to the laboratory for DNA extraction.

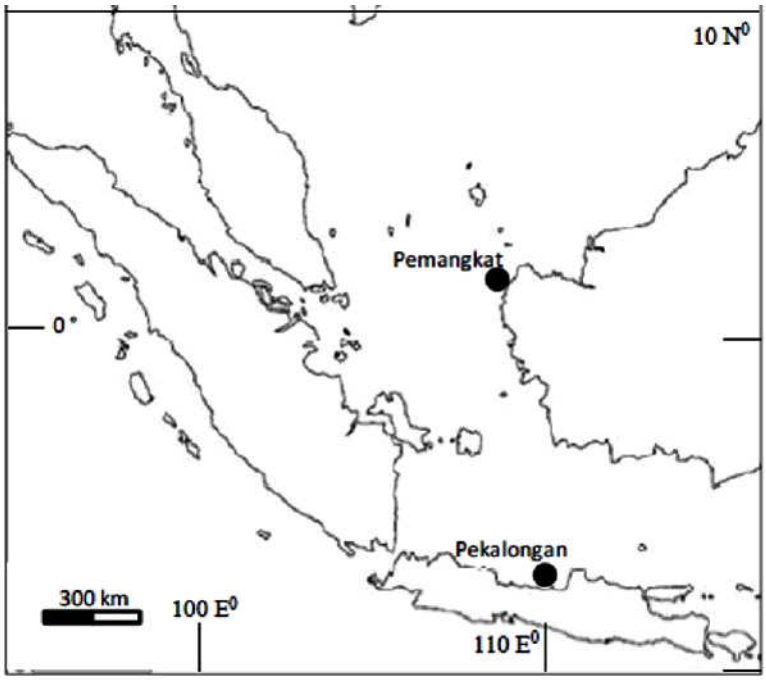

Figure 1. Sampling locations of *T. tonggol* in Pekalongan and Pemangkat.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from muscle tissue of each specimen using genomic extraction kit GENEAID. DNA was purified, and then dissolved in 100 μL elution buffer and stored in -20 °C. Partial sequences (570 bp) of the mitochondrial *16S* rRNA gene were analyzed from *T. tonggol* individuals from Pekalongan (n = 50) and Pemangkat (n = 47). The targeted DNA region was amplified using the *16S* rRNA universal primers: 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') and 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') (Palumbi, 1996). Polymerase chain reaction (PCR) amplification of *16S* rRNA fragment was carried out in a 25 µL of reaction volume consisting of 2.5 µL of 10x PCR Buffer (1x PCR buffer contained 100 mM Tris-HCL, 500 mM KCL) and dNTPs (8mM), 2 µL of $MgCl_2$, 1.25 µL of both primers, 0.125 µL of 2.5 U *Taq* DNA polymerase, and 1 µL of DNA template. PCR cycling parameters included an

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initial denaturing phase of 2 min at 94 °C followed by 35 cycles of 30 sec at 94 °C, 40 sec at 52 °C and min at 72 °C and ended with a final extension for 10 min at 72 °C. All PCR products were checked for positive amplification by visualizing them in a 1% agarose gel stained with ethidium bromide. Successfully amplified PCR product was purified and sequenced by commercial sequencing facilities (Macrogen, South Korea). Sequencing of all samples were done in both directions (forward and reverse), so there were 194 single-pass DNA sequences fitted for 97 individuals.

Data Analysis

Sequence chromatograms were displayed and edited manually using BIOEDIT (Applied Biosystems, Foster City, CA, USA). After the editing process, multiple alignment was carried out using MAFFT (Katoh & Standley, 2013). Additional sequences were obtained from BLAST searches of the public domain database GenBank (*T. tonggol* [accession numbers HQ425780.1], *T. alalunga* [accession numbers LN908909.] and *T. orientalis* [accession number KF906721.1]). Gene diversity (Nei & Tajima, 1981), nucleotide diversity *(*Π*)* (Nei, 1987), Analysis of Molecular Variance (AMOVA) (Excoffier *et al.,* 1992), and fixation index (*Fst)* with the permutation test were calculated using the ARLEQUIN 3:01 (Excoffier *et al.,* 2005). Neighbor-Joining *(NJ)* method is used to reconstruct phylogenetic relationships among haplotype with MEGA version 4 (Tamura *et al*., 2007). The partial mitochondrial *16S* rRNA gene sequences of every analyzed specimen were submitted to GenBank database.

RESULTS AND DISCUSSION

Results

Sequence Variation and Haplotype Distribution

Nucleotide sequences were obtained from all 97 fish individuals. PCR method amplified 570 bp DNA fragment of the mitochondrial *16S* rRNA gene that spans sequence position 2031 – 2600 bp of *T. tonggol* taken from GenBank with accession number HQ425780. In total, 3 polymorphic sites (all represent singleton dimensions) were identified in the sequence analysis of the 570 bp fragment among a total of 97 *T. tonggol* individuals from Pekalongan and Pemangkat (Figure 2). Based on these polymorphic sites, four haplotypes were identified.

CCTCTTGCAAAAACAAAGAATAAGAGGTCCAGCCTGCCCTGTGACTATATGTTTAACGGCCG
CGGTATTTTAACCGTGCGAAGGTAGCGCAATCACTTGTCTTTTAAATGGAGACCTGTATGAAT
GGCATTACGAGGGCTTAACTGTCTCCTTTTTCTAGTCAGTGAAATTGATCTCCCCGTGCAGA
AGCGGGGAT**G**TAACCATAAGACGAGAAGACCCTATGGAGCTTTAGACACCAAGGCATATCA
TGTCAAACACCCCTAAACAAAGGACTAAACCAAATGAATCATGCCCCCATGTCTTTGGTTGG
GGCGACCGCGGGGAAATAAAAAACCCCCACGTGGAATGGGAGTACTACCTCCTACAACCAA
GAGCT**G**CAGCTCTAAAAAACAGAATATCTGACCAATAAGATCCGGCAACGCCGATCAACGG
ACCGAGTTACCCTAGGGATAACAGCGCAATC**C**CCTTTTAGAGCCCATATCGACAAGGGGGT
TTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTGTTAAGGGTTCGTTTG
TTCAACGATTAAAGT

Figure 2. Nucleotide sequence of a partial DNA fragment (570 bp) of *T. tonggol 16S* rRNA gene. Single nucleotide polymorphism locations are designated in bold and underline.

Haplotypes composition and the position of bp variation of *T. tonggol* based on *16S* rRNA gene are presented in Table 1 and Table 2. Among four haplotypes, haplotype 1 is found in all sample locations, while the others (haplotype 2, haplotype 3 and haplotype 4) are unique Pekalongan samples have 1 site specific haplotype (haplotype 3), whereas Pemangkat samples have 2 site specific haplotypes (haplotype 2 and haplotype 3). Haplotype 1 is the most frequent (96%) and the frequencies of the remaining haplotypes are very low and in a low nucleotide diversity.

Genetic Diversity and Population Genetic Differences

Haplotypes of *16S* rRNA gene sequences between two sites are summarized in Table 3. Samples from Pemangkat show higher amount of haplotype and nucleotide diversity (h = 0.1556 ± 0.0680 and Π = 0.000277 ± 0.000432), while samples from Pekalongan show lower levels of diversity (h = 0.0400 ± 0.0380 and Π = 0.000070 ± 0.000209).

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Table 1. Haplotype composition of *T. tonggol* based on *16S* rRNA gene

	Haplotype			
	1	**2**	**3**	**4**
Pekalongan	49	0	0	1
Pemangkat	45	1	1	0

Table 2. Position of bp variation of *T. tonggol* based on *16S* rRNA gene

Haplotype	**Polymorphic site**		
	1286	**1466**	**1553**
T. tonggol (HQ.425780.1)	G	G	C
haplotype 1	G	G	C
haplotype 2	A	G	C
haplotype 3	G	A	C
haplotype 4	G	G	T

Table 3. Location, sample size and genetic data of *T. tonggol* based on *16S* rRNA gene

No	**Location**	**S. size**	**Site polymorpism**	**Trans**	**Transv**	**Indel**	**Nucleotides composition (%)**
1	Pekalongan	50	1	1	0	0	T = 22.8 C = 24.2 A = 30.0 G = 23.0
2	Pemangkat	47	2	2	0	0	T = 22.8 C = 24.2 A = 30.0 G = 23.0

AMOVA analysis indicates that most of genetic variation is found within samples (99.95%) and only (0.05%) the variations is found among samples (Table 4). Furthermore there is a difference between two samples location although the magnitude is very small.

Table 4. Analysis of molecular variance of *T. tonggol* base on *16S rRNA* gene

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among populations	1	0.032	0.00001 Va	0.05
Within populations	95	2.937	0.03092 Vb	99.95
Total	96	2.969	0.03094	100
Fixation Index F_{ST} : 0.00047				

Fixation index (*Fst)* and mean genetic distances (*d)* between paired samples inform that there is no significant genetic difference (based on 1,023 permutation test) between samples from Pekalongan and Pemangkat (*P*-value = 0.49071 ± 0.01695; d = 0.0001091).

Phylogeny reconstruction

Neighbor-Joining tree using kimura 2 parameters (Figure 3) was reconstructed from 7 haplotypes (4 haplotypes *T. tonggol* samples obtained by this study/ MH124639-MH124735 and 3 haplotypes samples, as

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comparison, generated from public domain database/ HQ425780, KF906721 and AB101291). Based on phylogeny tree, common haplotypes of Pekalongan and Pemangkat samples (haplotype 1 to 4) are monophyletic with *T. tonggol.* Sequence analysis of PCR products shows over 99% sequence identity to*16S* rRNA gene of *T. tonggol*/MH124639-MH124735.

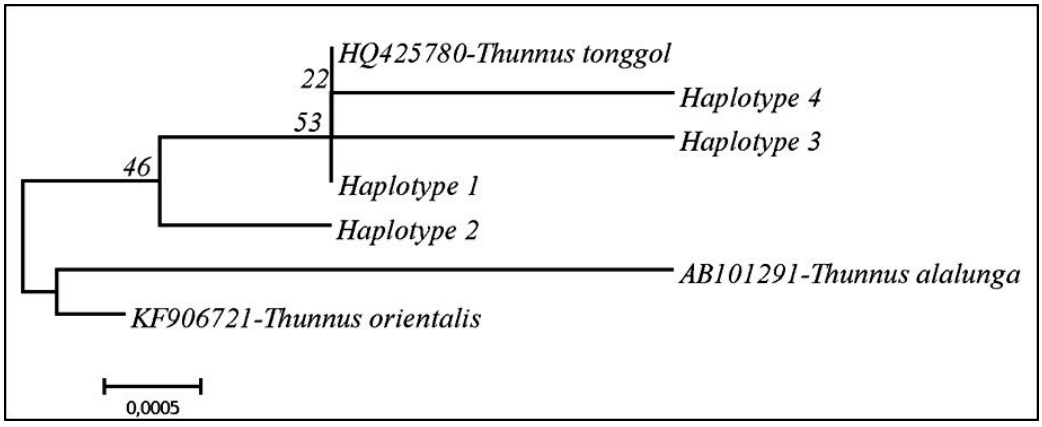

Figure 3. Neighbour-Joining phylogeny using Kimura 2-parameter haplotype of *T. tonggol* based on *16S* rRNA mitochondrial gene.

Discussion

Mitochondrial DNA is a popular molecular marker because it is uniparental and has a high evolutionary rate, lack of introns, large copy number in every cell, and limited recombination (Galtier *et al*., 2009). Although the *cytochrome c oxidase subunit I* (*COI*) gene is the most well-known molecular marker for the analysis of intraspecific and interspecific relationships in many marine fish and shellfish, the *16S* rRNA mitochondrial gene has also been proven to be a good marker to differentiate marine species (Craig *et al*., 2001).

Results from this study revealed a single, intermixing population of *T. tonggol* across the sampled locations. Sample haplotypes did not differ between Pekalongan and Pemangkat, which are separated by 500 km. No significant structuring was observed between other pairwise comparisons, tindicating gene flow between geographically adjacent locations. Long tail tuna *(T. tonggol)* is considered one species of neritic tuna, inhabiting the shallow waters of Southeast Asia and is well known for its extensive expansion in the geographic range. For example, the eastern little tunas (*Euthynnus affinis*), which share the same habitat and are often caught incidentally by long tail tuna, studied in the Philippines (5 sites) and Malaysia (1 site) inferred a panmictic stock and suggested this was due to the migratory nature of the species, larval dispersal, and the contiguous nature of the continental shelf between Malaysia and the Philippines (Santos *et al*., 2010). Tag and recapture studies in the waters of Australia revealed that movements of tagged long tail tuna could reach as far as 600 km away (Griffiths *et al*., 2010).

These results have direct management implications in recommending that *T. tonggol* from Pekalongan and Pemangkat should be managed as a single stock. Understanding the distribution of fish stocks is an important component for understanding stock status and the design and implementation of management regimes. The importance and the existing pressures on *T. tonggol* resources and the need for a more solid basis for understanding stock distribution have all been highlighted by previous authors. These justify studies like the present one for unraveling population structure of target fisheries via molecular genetic tools (Willette *et al.,* 2016). Therefore, this study is an important first step in providing insight for stock management of this neritic species.

Another interesting finding of this study is SNPs data. SNPs are now widely used for both linkage analyses and biodiversity studies (Moorhead *et al*., 2003; Pearson *et al*., 2004). Although further analysis of samples from both Pekalongan and Pemangkat is necessary, the information described here could prevent false labeling of imported *T. tonggol* and contribute to food safety. Also, these results show that the *16S* rRNA gene, which is generally used for the identification of species (Ivanova *et al*., 2007), can be used for the identification of the origin within species.

CONCLUSION

Long tail tuna *(T. tonggol)* is considered one species of neritic tuna, inhabiting the shallow waters of Southeast Asia and is well known for its extensive expansion in the geographic range. There is

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intermixing population of *T. tonggol* between Pekalongan and Pemangkat, which are separated by 500 km. No significant structuring was observed between other pairwise comparisons, indicating gene flow between geographically adjacent locations. Thus, *T. tonggol* from Pekalongan and Pemangkat should be managed as a single stock and SNPs method can be used for this type of study.

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