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Genetic variation of longtail tuna *Thunnus tonggol* landed in four fish markets in Indonesia based on mitochondrial DNA

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Abstract. *Astarini IA, Ningsih EY, Simanungkalit D, Ardiana SA, Al Malik MD, Yusmalinda NLA, Sembiring A, Pertiwi NPD, Cahyani NKD, Collins A. 2021. Genetic variation of longtail tuna* Thunnus tonggol *landed in four fish markets in Indonesia based on mitochondrial DNA. Biodiversitas 22: 1644-1651.* Longtail tuna (*Thunnus tonggol*, Family: Scombridae) is an economically valuable neritic species found in tropical and subtropical waters in the Indo-Pacific region. High catch numbers, which have been decreasing, could negatively impact this tuna's population level. Little research has been conducted on the longtail tuna population in Indonesia. This study aims to determine the genetic diversity and potential population structure of longtail tuna landed in four fish markets in Indonesia (representing three sampling locations because two markets are relatively close to each other) based on sequences of a region of mitochondrial control region (d-loop). A total of 101 samples, out of 110, were identified and confirmed as *T. tonggol* species by amplifying and sequencing a fragment of d-loop (amplicons ranging from 482 - 523 bp). Neighbor-joining analysis resulted in a topology with all samples grouped into one clade with an average genetic distance of 0.020. Meanwhile, haplotype diversity (Hd) and nucleotide diversity (π) values of the longtail tuna samples were 0.9939 and 0.0192, respectively. The fixation index (Fst) value was -0.00507, with $p > 0.05$, which indicates that there is no significant population structure among the longtail tuna collected from four fish markets representing three sampling locations. The results of this analysis can be used as basic data in planning sustainable fisheries management efforts.

Keywords: Control region, genetic conservation, genetic distance, haplotype

INTRODUCTION

Tuna is one of the three largest fisheries commodities in Indonesia, after shrimp and demersal fish (Habibi et al. 2011). Tuna makes up a significant part of the global seafood market, worth more than $42 billion USD value per year, and are vulnerable to overfishing (Tidd et al. 2018). Tuna fishing globally has reached 7.7 million tons/year, with total Indonesian catch reaching 16% of total global take (Alfajri 2017). Indonesia is the largest tuna exporting country in Southeast Asia with tuna export volume of 209,410 tons and production value reaching 768.4 million USD in 2013 (Alfajri 2017). This relatively high catch number is feared to cause a decrease in tuna populations, especially the longtail tuna (*Thunnus tonggol*).

Longtail tuna (*T. tonggol*) is a neritic species found in tropical and subtropical waters (Restiangsih and Hidayat 2018; Collette and Graves 2019). General characteristics of longtail tuna are a maximum body length of 142 cm and maximum weight of 35.9 kg (Griffiths et al. 2010; Collette and Graves 2019). The fish possesses an upper body that is bluish-black, a long tail base, silvery-white belly with oval spots arranged horizontally, a finlet that is yellow with gray edges, 2 dorsal fins, and a yellow anal fin (White et al. 2013; Collette and Graves 2019).

The longtail tuna has a coastal distribution in the Indo-Pacific region (Kumar and Kocour 2015) and is being exploited by commercial fisheries in several countries throughout the Indo-Pacific. Throughout the Indian Ocean region, the highest contributions to longtail tuna catches were from Iran (34%) and Indonesia (31%), followed by Taiwan, Thailand, Oman, Pakistan, Malaysia, India, and Australia (Abdussamad et al. 2012).

Decreasing numbers of the longtail tuna catch raise concerns that genetic variation of longtail tuna is being decreased as a result of exploitation (Riccioni et al. 2010; Siriraksophon 2017). Also, catch statistics may be inaccurate due to misidentification (Mohri et al. 2013). Misidentification of catch estimates of certain tuna species may be caused by the similarity of morphological

characters of several tuna species, i.e., yellowfin tuna (*T. albacares*) and longtail tuna (*T. tonggol*) (Mohri et al. 2013). Genetic conservation strategies that incorporate molecular identification methods to determine species identity will more accurately reveal the status and health of fish populations. It is essential to understand the genetic information of longtail tuna as a basis for guiding sustainable fisheries resource management policies.

DNA barcoding techniques provide fast and accurate means for identifying cryptic organisms (Rahayu and Nugroho 2015). One locus that is often used in species delimitation and population analysis is the non-coding gene known as d-loop or control region. This mtDNA locus is a region involved in the control of mtDNA replication and transcription processes and has relatively high mutation and polymorphism rates, thus rendering nucleotide sequences that vary greatly between individuals (Rahayu and Nugroho 2015). D-loop contains the most varied DNA sequences of all animal mtDNA genomes (Savira 2012). Therefore, it can be informative when studying variation, diversity, and genetic structure of animal populations (Astuti and Kurniati 2010). In the case of tunas, genetic analysis of d-loop has been widely employed, for example in *Thunnus* spp. (Joka 2013), *Thunnus albacares* (Akbar et al. 2014), *Thunnus tonggol* (Willette et al. 2016), and *Thunnus obesus* (Pertiwi et al. 2017).

This study aims to complement understanding of the genetic diversity of *T. tonggol* by determining the genetic diversity and potential population structure of landed longtail tuna obtained from four fish markets in Indonesia, including Kedonganan-Bali, Muncar-Banyuwangi, Pabean-Surabaya, and Sagulung-Batam. The d-loop mitochondria DNA marker is used to investigate the population structure of longtail tuna across the study areas. Understanding longtail tuna populations will help identify genetic stocks, which would be an important source of information to help policymakers in managing sustainable fisheries of this species in Indonesia, especially in these areas of study.

MATERIALS AND METHODS

Sample collections

This research is based on collections conducted from December 2018 to June 2019. Fin clips of longtail tuna were collected from four fish markets in Indonesia including Bali (n = 41), Banyuwangi (n=20), Surabaya (n = 28) and Batam (21), for a total of 110 samples (Figure 1). Samples were preserved in 96% ethanol before being transported to the laboratory for DNA extraction. Interviews with local fishermen were conducted to confirm the catch location of the longtail tuna samples, which were within 50 miles offshore of each port.

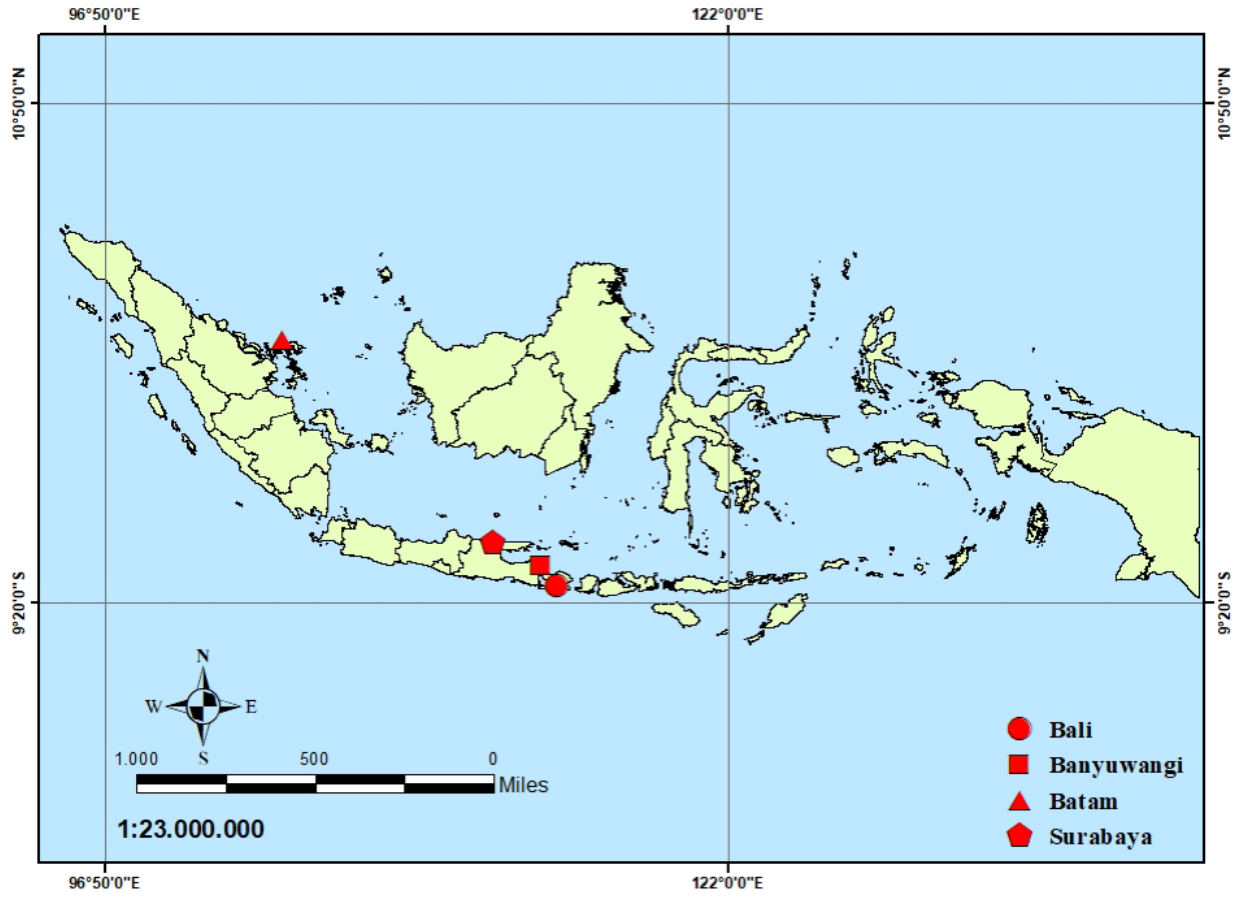

Figure 1. Sampling locations at four fish markets in Indonesia

Molecular analysis

Molecular analysis was carried out at the Yayasan Biodiversitas Indonesia (Bionesia) Laboratory, Bali. DNA extracts were isolated using 10% chelex (Walsh et al. 1991). Extracted DNA was then used to amplify, using Polymerase Chain Reaction (PCR) methods, a fragment of the mitochondrial DNA (mtDNA) control region (d-loop) locus. PCR methods were carried out following the methods in Allen et al. (2017) using the forward primer (CRK: 5 '- AGC TCA GCG CCA GAG CGC CGG TCT TGT AAA - 3') and reverse primer (CRE: 5– 'CCT GAA GTA GGA ACC AGA TG - 3') (Lee et al. 1995).

Each PCR reaction was 22 µl in volume, consisting of reagent solution containing 12.5 µl ddH_2O, 2.5 µl 10x PCR buffer (PE-II), 2.5 µl dNTP, 2.0 µl $MgCl_2$ and 1.25 µl primary CRK - CRE and 0.125 µl of PE Amplitaq, and 3 ul of DNA template. Each microtube was vortexed for 30 seconds to homogenize the samples. PCR reaction and thermocycling profile were modified from the methods in Allen et al. (2017), with an initial denaturation of 94°C for 10 s, 38 cycles of 94°C for 15 s, 50°C for 30 s, 72°C for 45 s, with final extension of 72°C for 5 min. PCR products were visualized using 1% gel agarose stained by Biotium® *gel red stain*. Successfully amplified products were then sent to a DNA sequencing facility and sequenced using Big Dye Chain Termination.

Data analysis

Sequences were edited and aligned using the CLUSTAL W algorithm in MEGA X (Kumar et al. 2018). A distance-based topology was obtained using the Neighbor-Joining method (Saitou and Nei 1987) with 1000 bootstrap replications. Sequences of *Thunnus obesus* (JN572738.1), *Thunnus albacares* (JN572794.1), *Thunnus orientalis* (JN631250.1), and *Euthynnus affinis* (JN655119.1) from Genbank (http://ncbi.nlm.nih.gov) were used as an outgroup. In addition to the outgroup, one sequence of *T. tonggol* (KC313300.1) was added to the analysis to confirm the samples as *T. tonggol*. For population genetic analysis, Banyuwangi and Bali samples were treated as one population site, with Surabaya and Batam as different population sites. Genetic diversity analysis to measure haplotype diversity (Hd) (Nei 1987) and nucleotide diversity (π) was done in DNAsp 5.10 (Rozas et al. 2017). The Analysis of Molecular of Variance (AMOVA) in Arlequin Ver.3.5 (Excoffier and Lischer 2010) was used to assess population genetic structure.

RESULTS AND DISCUSSION

Result

From the total of 110 samples from four fish markets, nine samples were not identified as *T. tonggol*. Among those nine samples, four were identified as *T. albacares* and five as *Euthynnus affinis*. This left 101 samples confirmed as *T. tonggol*, represented by amplicons 411 base pair (bp) long (Figure 2). All sequences have been deposited in Genbank with accession numbers (MW658015-MW658124). The neighbor-joining topology showed that all the longtail tuna samples from the four fish markets grouped into one clade. The resulting topology also revealed that the Genbank sequence of *T. tonggol* (KC313300.1) nested with the collected samples rather than the outgroup species (*T. obesus*, *T. albacares*, *T. orientalis*, *E. affinis*), confirming that the samples collected were longtail tuna (*T. tonggol*). Genetic distance within the *T. tonggol* specimens is 0.02, whereas the genetic distance between *T. tonggol* and its outgroup is 0.199.

For the population genetic analysis, samples from Banyuwangi and Bali were treated as one sampling location (Bali* population) because of their close proximity. Genetic diversity analysis shows that among the 101 samples collected, 82 different haplotypes were found, with a haplotype diversity value of 0.9939, and a nucleotide diversity value of 0.0192 (Table 1). The haplotype distribution (Figure 3) showed nine (9) haplotypes to be shared in different sampling locations; eight (8) found to be shared by two sampling locations, and only one (1) haplotype shared among all the sampling locations. 73 non-singleton haplotypes were found, with 34, 17, and 22 unique haplotypes acquired within the sampling locations of Bali*, Batam, and Surabaya, respectively.

Population genetic analysis using Analysis of Molecular Variance (AMOVA) showed that the structures of the longtail tuna were not significantly different within Bali* (Bali and Banyuwangi), Surabaya, and Batam (FST-value = -0.00507; *p-value* >0.05) (Table 3). This result indicates a mixing population of longtail tuna from the four fish markets.

Discussion

Longtail tuna (*Thunnus tonggol*) is a neritic species found in Indo-Pacific shallow, coastal, tropical and subtropical waters (Restiangsih and Hidayat 2018). This tuna species has a wide distribution across Indonesia, including Aceh (Rahmah et al. 2019), Java Sea (Widodo et al. 2011; Fitriani et al. 2020; Hidayat et al. 2020), Bali (Mahmud et al. 2019), Lombok (Setyadji and Nugraha 2015), East Kalimantan (Alfian et al. 2020), Sulu and Sulawesi Sea (Wanchana et al. 2015). Indonesia is listed as one of countries with the highest contributions to longtail tuna catches (31%) in Asia (Kumar and Kocour 2015; Abdussamad et al. 2012). However, this species' similar morphological characters with those of other tuna compromise accurate identification of the fish, leading to mislabeling and uncertainty in published catch estimates (Pauly and Froese 2012). Thus, molecular identification methods can help improve the determination of species identity and the accurate assessment of the status of populations. This study aims to determine the genetic diversity and potential population structure among longtail tuna obtained from four fish markets in Indonesia, including Kedonganan-Bali, Muncar-Banyuwangi, Pabean-Surabaya, and Sagulung-Batam, using the d-loop mitochondrial DNA marker.

Unlike all other *Thunnus* species, oceanodromous, longtail tuna occupy shallow waters and do not undergo diel vertical migration, limiting their niche and migration

(Griffiths 2020). Longtail tuna has been reported to occupy the coastal areas close to landmasses and is rarely found beyond continental shelf waters (Yesaki 1994). This information is in-line with our finding where the fishermen from these four markets also reported catching the longtail tuna within 50 miles offshore of each port and accord with our understanding based on surveying other fish markets around Indonesia. Longtail tuna is more commonly sold at fish markets adjacent to ocean beds with extensive shallow topography.

Table 1. Genetic diversity of longtail tuna (*Thunnus tonggol*) populations from all study locations

Sample locations	N	Hd	π	Hn
Bali and Banyuwangi	52	0.9902	0.0195	43
Batam	21	1.0000	0.0181	21
Surabaya	28	1.0000	0.0197	28
All	101	0.9939	0.0192	82

Note: N: Number of samples, Hd: Haplotype diversity, π: Nucleotide diversity, Hn: Number of Haplotype

Figure 2. Neighbor-Joining (NJ) topology generated from 411 bp of mtDNA control region sequence data from *Thunnus tonggol* species, with node support assessed using 1000 bootstrap replications

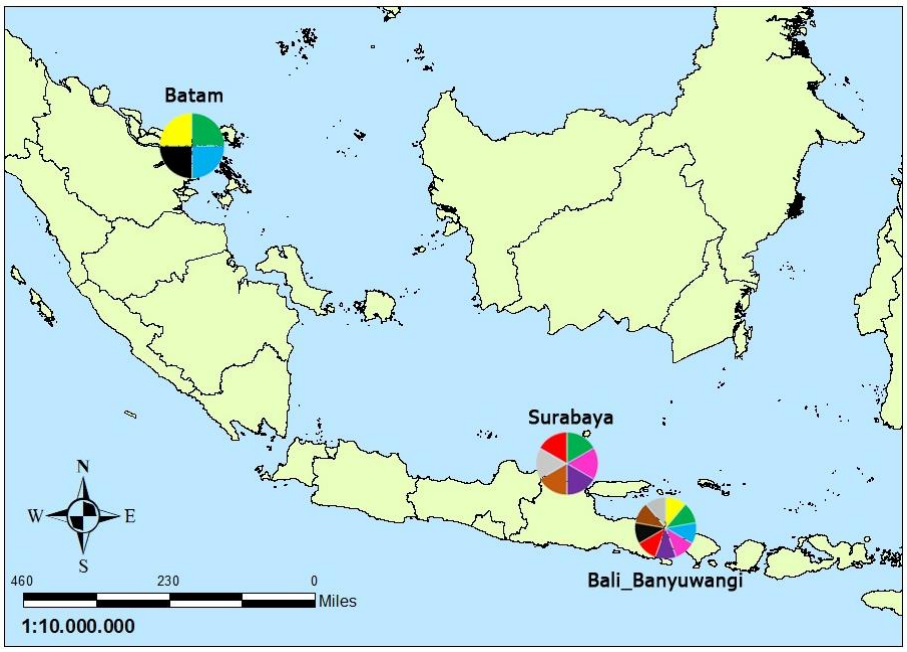

Figure 3. The distribution of shared haplotypes of longtail tuna (*Thunnus tonggol*) between study locations

Neighbor-joining analysis showed that 101 samples were confirmed as longtail tuna (*T. tonggol*). The Genbank *T. tonggol* were nested onto a similar clade with the samples collected (Figure 2). Comparison results with Genbank data using BLAST (Basic Local Alignment Search Tool) show the similarity of the samples collected with *T. tonggol* data is 97% - 100%. Thus, this study found that 101 of 110 samples collected from four fish markets were accurately identified as *Thunnus tonggol*, an error rate of about 8%. Genetic distance analysis showed a close relationship between samples found in Bali, Banyuwangi, Surabaya, and Batam. Differences in nucleotide sequences between samples could occur due to environmental conditions that affect an individual's genetic material (Verawati 2015). Population genetic analysis indicates a mixing population of longtail tuna from the four fish markets.

Mitochondrial DNA has commonly been used for tuna population studies, e.g., in yellowfins (Akbar et al. 2014), bigeye (Nugraha 2009; Pertiwi et al. 2017), and longtail (Willette et al. 2016; Kasim et al. 2020). Studies of longtail tuna suggest the existence of panmictic populations among those studied, i.e., in the South China Sea by Willette et al. (2016) and Malaysian waters by Kasim et al. (2020). Wijana and Mahardika (2010) reported a similar result for yellowfin tuna, which exhibited no genetic differences in samples from the Philippines and Spain. In our analysis, longtail tuna samples nested tightly in one clade, which could be a result of the relatively narrow geographic scope in this study. The four sampling locations are connected and are along the migration path of longtail tuna.

Table 2. Distribution of nine shared haplotypes of longtail tuna (*Thunnus tonggol*) between study locations

Haplotype	Sample locations			Total sample locations
	1	2	3	
1	1	1		2
2	1		1	2
3	1		1	2
4	1	1	1	3
5	1		1	2
6	1	1		2
7	1	1		2
8	1	1		2
9	1	1		2

Table 3. Analysis of Molecular Variance (AMOVA) of longtail tuna across study locations

Source of variation	d.f	Sum of squares	Variance component	Percentage of variation
Among sample location	2	6.680	-0.01995 Va	-0.51
Within sample location	98	388.033	3.95952 Vb	100.51
Total	100	394.713	3.93957	
F_{ST}	-0.00507			
P-value	0.71652 ± 0.01300			

For future studies, expanding sampling locations and employing more sensitive genetic markers might better resolve this species' population patterns. Although little to no genetic structure has been revealed within tuna populations of the areas mentioned above, differentiation in mtDNA has been detected between wider regions, such as between longtail tuna populations of the Indian Ocean and the South China Sea (Willette et al. 2016). Next-Generation Sequencing (NGS) methods to investigate Single Nucleotide Polymorphisms (SNPs) have also uncovered genetic differentiation among Atlantic, Indian and Pacific Ocean populations (Pecoraro et al. 2018). This method also found genetic differentiation within the Atlantic Ocean (Eastern Atlantic Ocean and Western Atlantic Ocean) and the Pacific Ocean (Eastern Pacific Ocean and Western-Central Pacific Ocean) populations of yellowfin tuna (Pecoraro et al. 2018).

Our sampling of longtail tuna in Indonesia revealed a high number of haplotypes, differing in each location (Table 1); among 52 samples collected from Bali and Banyuwangi, 43 haplotypes were found; among 28 Surabaya samples there were 28 haplotypes; and among the 21 Batam samples, 21 haplotypes were detected. Only a handful of haplotypes were sampled between regions, with the haplotype diversity (Hd) value of 0.9939. Meanwhile, the nucleotide diversity value of 0.0192 shows that almost every sample has a different haplotype with a small difference in nucleotide sequences between samples. The high genetic diversity uncovered in longtail tuna obtained from four Indonesian fish markets is similar to what was reported by Willette et al. (2016) in the South China Sea. Other research on pelagic tuna fish such as yellowfin tuna (Akbar et al. 2014) and bigeye tuna (Nugraha 2009; Pertiwi et al. 2017) around Indonesia and the Indian Ocean have also shown high genetic diversities.

The high genetic diversity exhibited by longtail tuna may be due to several factors, i.e., large population size, migration, and high adaptability of the species. Large population size allows an individual to freely breed (interbreeding) with other individuals from that population. This can help increase the frequency of alleles (gene couples located at the corresponding locus on the homologous chromosomes that make up gene arrangement) of an offspring. A large-sized population can prevent a population from declining. Detecting high genetic diversity of longtail tuna suggests that localized catches do not selectively remove sub-populations or genetic diversity of the overall species. Similar results were reported by Nugraha (2009), who reported that 190 samples of bigeye tuna from two populations of the Indian Ocean and the Pacific Ocean were broken into 5 broadly distributed sub-groups. Grewe and Hamptom (1998), who conducted a study of 800 samples of the bigeye tuna using mtDNA and microsatellite DNA analysis, also found sub-populations within the bigeye tuna populations in the Pacific Ocean.

A second factor that could cause high genetic diversity in longtail tuna is migration, which influences the flow of genes in a population. This is supported by Nishida et al. (1998), who discovered deep oceanic migration of several tuna species by using tagging methods. A third factor would be widespread adaptive response by longtail tuna to environmental conditions, such as temperature, turbidity, or chlorophyll-a presence. Many factors, including temperature pattern and chlorophyll-a, are influenced by upwelling. Upwelling can affect the abundance and distribution of phytoplankton in water (Barata et al. 2014), which determines local productivity. Phytoplankton, in turn, will attract small fish, which is the food source for tuna (Padmaningrat 2017).

High genetic diversity in longtail tuna populations indicates that nucleotide changes are still occurring in their mtDNA control region locus and, that all else being equal, increases the survival chance of the population. High genetic diversity should be maintained to preserve its sustainability in the face of having high commercial value. But note that Hughes et al. (2008) have argued that genetic diversity may or may not directly impact individual species, populations, communities, and ecosystems. Overfishing a tuna species may decrease the genetic diversity of those species, affecting its population structure. Therefore, a strategy to preserve biodiversity through genetic conservation is needed. Several efforts that can be undertaken to maintain sustainable tuna fisheries are (1) controlling the minimum size limit on every catch, and (2) limiting the catch time, where fishing tuna is only allowed at the peak of the tuna season.

A recent review (Griffiths et al. 2020) posited four putative stocks across the entire range of longtail tuna: Western Indian, Northern Indian, Oceania and Southeast Asia. The hypothesis of a single southeastern Asia stock is based on prior studies by Willette et al. (2016) and Malik et al. (2020) that detected no genetic structure in longtail tuna populations in the South China Sea and the Java Sea. In our Indonesia-focused study, we also derived a fixation index (Fst) from AMOVA analysis indicating that there is no population genetic structure in the longtail tuna population between the three populations we sampled. Thus, our work corroborates the idea that longtail tuna populations within the South China Sea and Indonesia constitute a single stock of population. Similarly, Kunal et al. (2014) found that the longtail tuna population inhabiting northwest Indian waters constitutes a single stock population. Further genetic studies of longtail tuna from Oceania and the Western Indian Ocean are needed to assess their respective population structures more fully.

Although longtail tuna are known to be neritic and not to undergo high levels of migration, this species has a wide distribution with little known population differentiation other than that between populations in the South China Sea and Indian waters (Kunal et al. 2014). Islands and the general complexity of Indonesia's marine ecosystem may help explain differentiation flow through current between Southeast Asian waters and northern Australia (Lee et al. 2002), which were made separate stock populations between those locations (Griffiths et al. 2020). Moreover, morphometric, meristic, and electrophoretic observations of longtail tuna (Abdulhaleem 1989) have also indicated that there are two distinct populations between Indian and Pacific. As a result, the two locations should be managed as different population stocks (Kumar and Kocour 2015).

Like others focusing on the South China Sea (Willette et al. 2016) and Java Sea (Malik et al. 2020), this study covers only a narrow, small-scale region of the overall geographic distribution of *Thunnus tonggol*. However, this data could help the government evaluate the policy for sustainable fisheries of this species. In sum, the genetic diversity of longtail tuna landed in four fish markets (Bali, Surabaya, Banyuwangi, and Batam) is high and there is no population structure revealed across the three sampling areas covered by this study.

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