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A Possible Mixing Genetic Pool of Eastern Little Tuna *Euthynnus affinis* Cantor (1849) from the Western and Southern Part of Indonesian Waters

Bram Setyadji[1*], Maya Agustina[2], Raymon Rahmanov Zedta[3], Ririk Kartika Sulistyaningsih[1], Putu Viby Indriani[4], Daniar Kusumawati[5]

[1]*Centre Research for Fishery, National Research and Innovation Agency, Cibinong, Bogor, Jawa Barat, Indonesia*
[2]*Institute for Mariculture Research and Fisheries Extension, Gondol, Buleleng, Bali, Indonesia*
[3]*Research Center for Conservation of Marine and Inland Water Resources, National Research and Innovation Agency, Cibinong, Bogor, Indonesia*
[4]*Research Institute for Tuna Fisheries, Ministry of Marine Affairs and Fisheries, Denpasar, Bali, Indonesia*
[5]*Research Center for Marine and Land Bioindustry, National Research and Innovation Agency, Pemenang Barat, Mataram, Nusa Tenggara Barat, Indonesia*

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ABSTRACT

Eastern little tuna (*Euthynnus affinis*) is a commercially important species for coastal communities in the western and southern part of Indonesia. However, little is known about its characteristics. Therefore, a comprehensive and broader research of its population structure is imminent in response to conserve and manage the fishery sustainably. This study aimed to fill the gap of the lacking information on the stock separation of eastern little tuna within the Indonesian archipelagic waters and its Economic Exclusive Zone (EEZ). Population genetic approaches were used to examine population structure using the mitochondrial DNA (mtDNA) control region marker. A total of 94 samples were collected from nine landing sites between January to September 2020. Polymerase chain reaction (PCR) was used to amplify the samples, and Sanger sequencing was used to sequence them. The findings showed a mean value of 0.922 for genetic diversity (H_d) and 0.009 for nucleotide diversity (π). Both of these numbers pointed to a high level of genetic diversity. A further population analyses using Analysis of Molecular Variance (AMOVA) and Discriminant Analysis of Principal Component (DAPC) confirmed a mixing gene pool with no distinct population structure detected (Φ_{ST} value of 0.097 and p-value>0.05).

1. Introduction

Fish stock structure determination is an integral part of current fisheries management (Pita *et al.* 2016). The common concept of stock is usually explained as homogenous units of fish in discretely separated areas (Begg *et al.* 1999). Therefore, as one of the critical inputs for fisheries management, stock assessment should be conducted based on species-specific stock structure. Different stocks may possess specific biological traits affecting life processes and resilience to exploitation and environmental changes (Artetxe-Arrate *et al.* 2019). Especially in Indonesia, where fish resource utilization is still determined by estimating total catch potential for higher group species (i.e., small pelagic, large pelagic, squid, etc.). Such an approach is not ideal in the multi-species fishery. It could result in a false stock surplus or depletion detection, notably in group species that consist of many species, such as large pelagic.

Eastern little tuna, *Euthynnus affinis* (Cantor, 1849), belong to the subgroup neritic tuna under a large pelagic group. It is considered a cosmopolitan species (Collette and Nauen 1983) which can be found in open waters, but the distributin always stays close to the shoreline. Since it widespread along the entire east coast of Africa, in the Red Sea, the Gulf of Aden, the "Gulf" off Pakistan, and along India's and Sri Lanka's west coasts as well as Indonesian waters, it is regulated under RFMO (Regional Fisheries Management Organization), namely IOTC (Indian Ocean Tuna Commission). Indonesia is considered the largest producer of eastern little tuna in the

* Corresponding Author
E-mail Address: bramsetyadji@kkp.go.id

Indian Ocean in 2014-2018, encompassed around one-third of the total catch (~45,000 tons/year). Most of the catch is interacted with purse seine, followed by the line, gillnet, and other gears (IOTC–WPNT10 2020). The latest stock assessment conducted in 2020 indicates that the stock status is not overfished and shows no sign of overfishing (IOTC–WPNT10 2020). However, the assessment was heavily dependent on catch data, thus considered to be highly uncertain. Also, the stock was assumed a single population for the entire Indian Ocean.

Over the last two decades, several genetic studies have investigated the population structure of eastern little tuna. Santos *et al.* (2010) suggested panmixia of eastern little tuna in Southeast Asian water. Further evidence of its low genetic variation revealed a single stock in Indian waters (G. Kumar, Kunal, Menezes, *et al.* 2012), a possible indication of localized stock in the Indian Ocean. A recent finding by Feutry *et al.* (2020) detected the presence of at least two distinct genetic groups between the northeastern (western part of Indonesia) and eastern central Indian Ocean (southern part of Indonesia and northern part of Australia). However, although the latest study showed a promising result, the low number of sampling locations within Indonesian territory might hamper the detailed information about its genetic flow and the presence of ongoing mixing genetic pools. Therefore, this study aimed to address this gap by incorporating more sampling locations and the western and southern parts of Indonesian waters. Hopefully, the result could be utilized as a vital data reference for scientists and policymakers, especially in conducting species-specific stock-based assessments within the Indonesian territory.

2. Materials and Methods

A total of 94 samples were collected from three landing sites in western part of Sumatra (Lampulo, Sibolga, and Padang), and six across southern part of Java (Binuangen, Pacitan, Prigi, and Muncar), Bali (Kedonganan) and Nusa Tenggara (Tanjung Luar). Samples were collected in thin tissue slices between pectoral and dorsal fins and preserved in 70% alcohol. All the materials were taken in January and September 2020, following a tight Covid-19 prevention protocol. The origin of fishes was confirmed thoroughly with the respective fishermen to ensure they came from the northeastern Indian Ocean (FMA-572 and FMA-573) and prevented any potential mixing from other areas (i.e., South China Sea, Java Sea). Detailed sampling sites are shown in Figure 1.

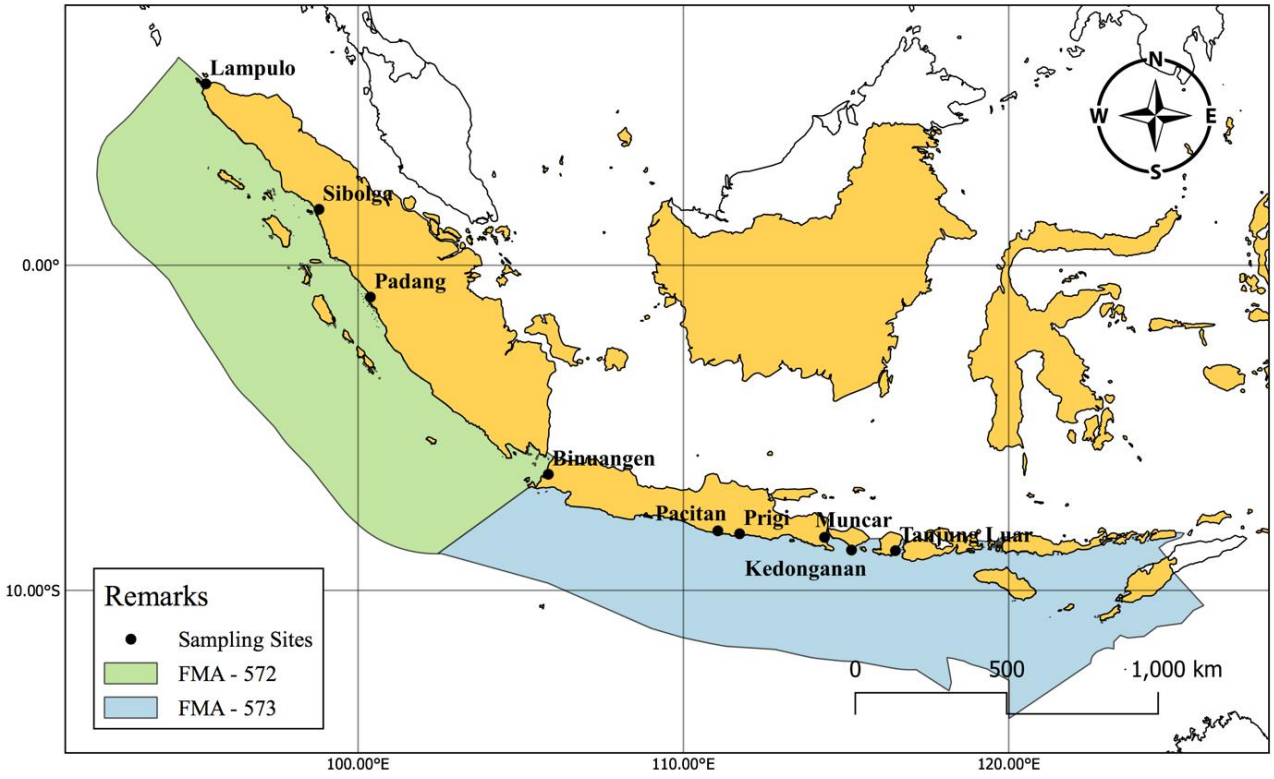

Figure 1. Sampling locations for eastern little tuna (*E. affinis*) in the western and southern part of Indonesian waters

The mitochondrial DNA (mtDNA) of the control region (D-loop) locus was used in this study as a molecular genetic approach. It is specified as a non-coding area capable of controlling the expression of certain genes (Falkenberg *et al.* 2002). In marine species, D-loop haplotypes can differentiate mitochondrial lineages that correspond to certain geographic areas, allowing for the determination of origin. (Ogden and Linacre 2015). It has a hypervariable area, contains a high polymorphism level, providing insight into an intraspecific variety (Wu *et al.* 2006). The reason is that the biodiversity (number and genetic proximity of species in a catch) would be better represented if more preserved sequences were available (Ardura *et al.* 2013).

The DNeasy Blood and Tissue® kit from QIAGEN was used to extract DNA. Polymerase Chain Reaction was used to amplify a fragment of the mitochondrial (mtDNA) control region (PCR) according to Menezes *et al.* (2006). Both PCR reaction and thermocycling profiles were based on Menezes *et al.* (2006) with some modifications, whereas the composition of PCR reaction consists of 12.50 µL My TaqTM HS Red Mix (Bioline), 6.00 µL nucleus free water, 1.25 µL forward primer, 1.25 µL reverse primer, and 3.00 µL DNA template. PCR was performed at the following thermocycling conditions: using the Mastercycler Eppendorf Gradient, the sample was first denaturated at 94°C for 3 minutes, followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, first extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes (Eppendorf, Germany). With the aid of a UV trans-illuminator, PCR products were separated on 1% agarose gels in 1 TEA buffer for 40 minutes. The microfluidic QIAxcel capillary electrophoresis system (Qiagen, Valencia, CA, USA) was then used to separate the PCR products for 30 minutes at 6 kV using 25 L of each product added to the capillary tubes. Using the QIAxcel software, the resulting electropherograms and DNA patterns were examined. Successfully amplified PCR products (Table 1) were sent to 1st Base Laboratories (www.base-asia.com) for further analysis using Sanger methods.

Sequencing editing and alignment were conducted using MEGA X software (Kumar *et al.* 2018). A BLAST (Basic Local Alignment Search Tools) comparison with the Genbank database (www.ncbi.nlm.nih.gov) was also performed to accurately identify the sample as *E. Affinis* species. The identity cover value of 99-100% and query cover value of 98-100% were used as the cut-off values for similar species in the BLAST result.

Diversity indices, i.e., the number of alleles, gene diversity, observed and expected heterozygosity, were calculated locus by locus from each area using Arlequin Ver. 3.5 (Excoffier and Lischer 2020). In each significance test (p-values 0.05), 10,000 permutations were used to analyze genetic diversity, haplotype distribution, and genetic differentiation analysis between locations such as K_S^*, K_{ST}^*, Z^*, S_{nn}, and F_{ST}, which were analyzed using DNASp 6 (Rozas *et al.* 2017; Al Malik *et al.* 2020). Analysis of Molecular Variance (AMOVA) was used to examine the coefficient of genetic differentiation between populations (FST), with 10,000 replicates per permutation apex (Schliep *et al.* 2020), and poppr (Kamvar *et al.* 2014, 2015) under R version 4.0.2 (R Core Team 2020). The significance level for AMOVA analysis was 5% (p-value 0.05). Further, Discriminant Analysis of Principal Component (DAPC) (Jombart *et al.* 2010) under the adegenet package (Jombart 2008; Jombart and Ahmed 2011), was utilized to visualize any stock disaggregation within pre-determined populations. The process

Table 1. Diversity indices for 76 genotypes of eastern little tuna (*E. affinis*) for each population in the western and southern part of Indonesian waters. For each metric, the mean and standard deviation (SD) was calculated locus by locus from each area using Arlequin Ver. 3.5 (Excoffier and Lischer 2020). The following metrics are shown: the number of alleles (No. alleles), gene diversity (Avg. gene diversity) calculated from Tajima (1983), and observed (Obs.) and expected (Exp.) heterozygosity

Area	No alleles		Avg. gene diversity		Obs. heterozygosity		Exp. heterozygosity	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lampulo	2.000	0.000	0.105	0.061	0.000	0.000	0.252	0.053
Sibolga	2.000	0.000	0.006	0.004	0.000	0.000	0.307	0.088
Padang	2.000	0.000	0.006	0.004	0.000	0.000	0.277	0.094
Binuangeun	2.062	0.250	0.012	0.007	0.000	0.000	0.321	0.107
Pacitan	2.000	0.000	0.008	0.005	0.000	0.000	0.267	0.084
Prigi	2.000	0.000	0.005	0.003	0.000	0.000	0.275	0.077
Muncar	2.000	0.000	0.008	0.005	0.000	0.000	0.278	0.104
Tanjung Luar	2.000	0.000	0.007	0.004	0.000	0.000	0.303	0.116
Kedonganan	2.040	0.200	0.014	0.008	0.000	0.000	0.244	0.090

involved a machine learning technique by dividing the data into two sets: training and validation set, with proportions each 90% and 10%, respectively. The sampling and DAPC procedures were repeated 1,000 times for each level of PC retention in order to determine the ideal number of PCs to keep. It is best to keep the number of PCs with the lowest root mean square error.

3. Results

3.1. Genetic Variability and Diversitys

Among the number of samples collected and amplified, only eight samples per location, except Kedonganan (twelve), were delivered for further analysis (sanger sequencing). Allelic variation-based genetic diversity indices of *E. affinis* were similar across all nine areas; the mean number of alleles ranged from 2,000 to 2,062 and did not differ significantly across sites (Table 1). In the western part of Sumatra, average gene diversities ranged from 0.006 to 0.105, and in the southern parts of Jawa, Bali, and Nusa Tenggara, they ranged from 0.005 to 0.014 (Table 1). Average observed heterozygosity was lower than average heterozygosity expected. The observed and expected heterozygosity was zero and from 0.244 (Kedonganan) to 0.321 (Binuangeun), respectively (Table 1). Overall, around 60-100% haplotype (H_n) can be identified from each location. Haplotype (H_d) diversity value was relatively high, ranging from 0.7857 (Sibolga) to 1.0000 (Binuangeun). In contrast, the nucleotide diversity (π) was very low, ranged 0.0069 (Sibolga and Padang) to 0.0148 (Kedonganan) (Table 2).

3.2. Genetic Differentiation

The K_S^*, K_{ST}^*, Z^*, and S_{nn} values obtained from each locus comparisons among possible population pair permutations, there are no notable variations (p-value>0.05), except for Muncar against the other eight locations (Table 3), which means these populations are closely related to each other and should be assumed as a single cluster. The lowest FST value was discovered between Sibolga and Pacitan (-0.058), whereas the highest was a permutation of Lampulo and Pacitan (0.202) (Table 3). However, in general, all FST values were considered low (<0.3), which indicated low genetic differences between the related populations. In addition, some negative FST values were likely a result of low sample representation. Hence increasing the sample size could eliminating it.

3.3. Population Structure

Table 4 shows that genetic variability within populations (98.33%) was greater than variability between populations (1.67%), indicating that the population is genetically diverse. Low population differentiation statistics (F_{ST} = 0.0971) and the observed distribution that didn't fall within the

Table 2. Genetic diversity estimates of eastern little tuna (*E. affinis*) based on nine populations in the western and southern part of Indonesian waters. The metrics are as follows: N is the number of samples, H_n is the number of haplotypes, H_d is haplotype diversity, and π is nucleotida diversity

Populations	N	H_n	H_d	π
Lampulo	8	7	0.9643	0.0113
Sibolga	8	5	0.7857	0.0069
Padang	8	7	0.9643	0.0069
Binuangeun	8	8	1.0000	0.0128
Pacitan	8	6	0.9286	0.0086
Prigi	8	7	0.9643	0.0055
Muncar	8	6	0.8929	0.0083
Tanjung Luar	8	6	0.8929	0.0076
Kedonganan	12	9	0.9091	0.0148

Table 3. Genetic differentiation estimates of eastern little tuna (*E. affinis*) from possible population pair permutations in the western and southern part of Indonesian waters. Metrics shown are: K_S^*, K_{ST}^*, K_S^*, K_{ST}^*, and S_{nn} = test statistics of the genetic differentiation based on Hudson *et al.* (1992), F_{ST} = coefficient of the gene differentiation, which measures the inter-population diversity, P-value = levels of significance at 0.05

Location	K_S^*	K_{ST}^*	$K_S^*.K_{ST}^*$ P-value	Z^*	P-value	S_{nn}	P-value	F_{ST}
Lampulo/Sibolga	1.386	-0.017	0.829	3.869	0.674	0.425	0.688	-0.061
Lampulo/Padang	1.426	0.001	0.384	3.852	0.513	0.490	0.367	0.022
Lampulo/Binuangen	1.688	-0.007	0.599	3.844	0.537	0.458	0.525	-0.023
Lampulo/Pacitan	1.491	-0.001	0.458	3.836	0.486	0.508	0.317	-0.008
Lampulo/Prigi	1.360	-0.003	0.546	3.862	0.671	0.452	0.564	0.005
Lampulo/Muncar	1.471	0.096	0.001	3.546	0.001	0.879	0.001	0.202
Lampulo/Kedonganan	1.707	-0.010	0.741	4.309	0.762	0.526	0.300	0.006
Lampulo/TanjungLuar	1.442	0.001	0.368	3.849	0.474	0.488	0.402	0.036
Sibolga/Padang	1.230	-0.020	0.793	3.886	0.818	0.431	0.672	-0.020

Table 3. Continued

Location	K_S^*	K_{ST}^*	$K_S^*.K_{ST}^*$ P-value	Z^*	P-value	S_{nn}	P-value	F_{ST}
Sibolga/Binuangen	1.492	0.012	0.201	3.807	0.183	0.530	0.205	0.004
Sibolga/Pacitan	1.294	-0.023	0.805	3.898	0.857	0.429	0.611	-0.058
Sibolga/Prigi	1.164	-0.024	0.855	3.897	0.923	0.428	0.649	-0.038
Sibolga/Muncar	1.274	0.074	0.030	3.705	0.034	0.702	0.015	0.150
Sibolga/Kedonganan	1.560	-0.014	0.777	4.322	0.666	0.452	0.736	-0.019
Sibolga/TanjungLuar	1.245	-0.016	0.732	3.889	0.781	0.425	0.689	0.001
Padang/Binuangen	1.532	0.021	0.088	3.784	0.131	0.516	0.210	0.079
Padang/Pacitan	1.334	-0.021	0.966	3.884	0.841	0.417	0.943	-0.037
Padang/Prigi	1.204	-0.032	0.995	3.920	0.997	0.329	0.970	-0.064
Padang/Muncar	1.314	0.072	0.017	3.667	0.024	0.706	0.021	0.183
Padang/Kedonganan	1.590	-0.007	0.584	4.307	0.642	0.421	0.853	0.029
Padang/TanjungLuar	1.285	-0.020	0.962	3.887	0.970	0.388	0.901	-0.030
Binuangen/Pacitan	1.596	0.007	0.303	3.805	0.290	0.458	0.506	0.017
Binuangen/Prigi	1.466	0.027	0.066	3.763	0.079	0.531	0.229	0.076
Binuangen/Muncar	1.576	0.070	0.010	3.605	0.010	0.706	0.038	0.171
Binuangen/Kedonganan	1.786	-0.010	0.723	4.303	0.689	0.416	0.778	-0.004
Binuangen/TanjungLuar	1.547	0.021	0.101	3.794	0.152	0.530	0.143	0.085
Pacitan/Prigi	1.268	-0.019	0.803	3.887	0.796	0.421	0.814	-0.033
Pacitan/Muncar	1.379	0.042	0.072	3.763	0.101	0.657	0.046	0.137
Pacitan/Kedonganan	1.638	-0.015	0.850	4.319	0.813	0.401	0.866	-0.035
Pacitan/TanjungLuar	1.350	-0.017	0.808	3.882	0.839	0.452	0.493	-0.022
Prigi/Muncar	1.248	0.084	0.015	3.665	0.025	0.738	0.014	0.229
Prigi/Kedonganan	1.541	-0.003	0.485	4.305	0.591	0.463	0.679	0.033
Prigi/TanjungLuar	1.219	-0.023	0.950	3.896	0.993	0.398	0.862	-0.037
Muncar/Kedonganan	1.623	0.060	0.005	4.116	0.004	0.729	0.010	0.141
Muncar/TanjungLuar	1.330	0.072	0.011	3.664	0.018	0.750	0.011	0.157
Kedonganan/TanjungLuar	1.602	-0.006	0.533	4.306	0.524	0.491	0.502	0.035

Table 4. Results of analysis of molecular variance (AMOVA) and F-statistics for the whole population of eastern little tuna (*E. affinis*) in the western and southern part of Indonesian waters. Metrics shown are: Df = degrees of freedom; SS = Sum of squares; MS = Mean square; Est.Var. = Estimated variance; % = Percentage of variation

Source of variation	Df	SS	MS	Est. Var	%	F_{ST}	p-value
Among populations	8	8.6974	1.0872	0.0161	1.67	0.0971	0.0000
Within populations	67	63.7500	0.9515	0.9515	98.33		
Total	75	72.4474	0.9660	0.9676	100.00	0.0971	0.0000

distribution expected from the permutation (Figure 2) signified no differentiation in population structure. Further, a follow-up DAPC analysis confirmed the prior AMOVA result. The samples were clumped into the center of the graph. They indicated low admixture and no segregation among the population for eastern little tuna across the western and southern part of Indonesian waters (Figure 3).

4. Discussion

Low observed (H_o) and expected (H_e) heterozygosity suggesting a possible genetic uniformity among locations. In contrast, the haplotype diversity index (H_d) was considered high (>0.8) for all areas, according to Nei (1978). At the same time, high indices were strongly linked with high genetic diversity (Smith and Chesser 1981). Previous studies in Indian waters (Kumar *et al.* 20212a, 2012b), the coastal area of Taiwan (Chiou and Lee 2004), and broad southeast Asia populations (Philippine and Malaysia) (Santos *et al.* 2010) also indicated similar characteristics. Thus, it could be inferred that the null hypothesis of the single stock structure of eastern little tuna (*E. affinis*) for all samples cannot be rejected.

Large pelagic fishes have vast distribution since larval stages and usually form large population sizes (Kumar *et al.* 2012b). In this study, the presence of Sunda, Bali, and Lombok straits along the sampling locations was allegedly played an insignificant role as a genetic barrier that diminishes variation across populations, making it challenging to distinguish discrete populations (Palumbi 1992; Menezes *et*

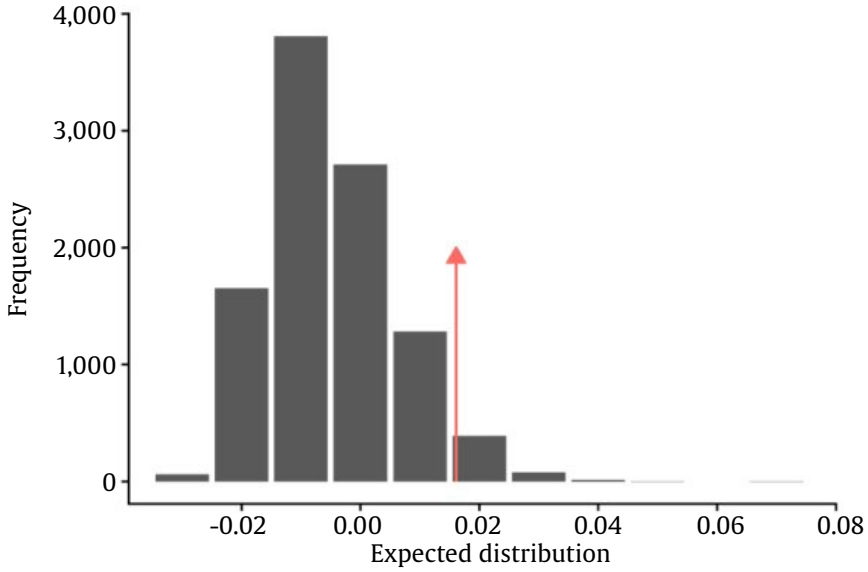

Figure 2. Null distributions of the molecular variance components obtained through a random permutations (bar = expected distribution, arrow = observed distribution)

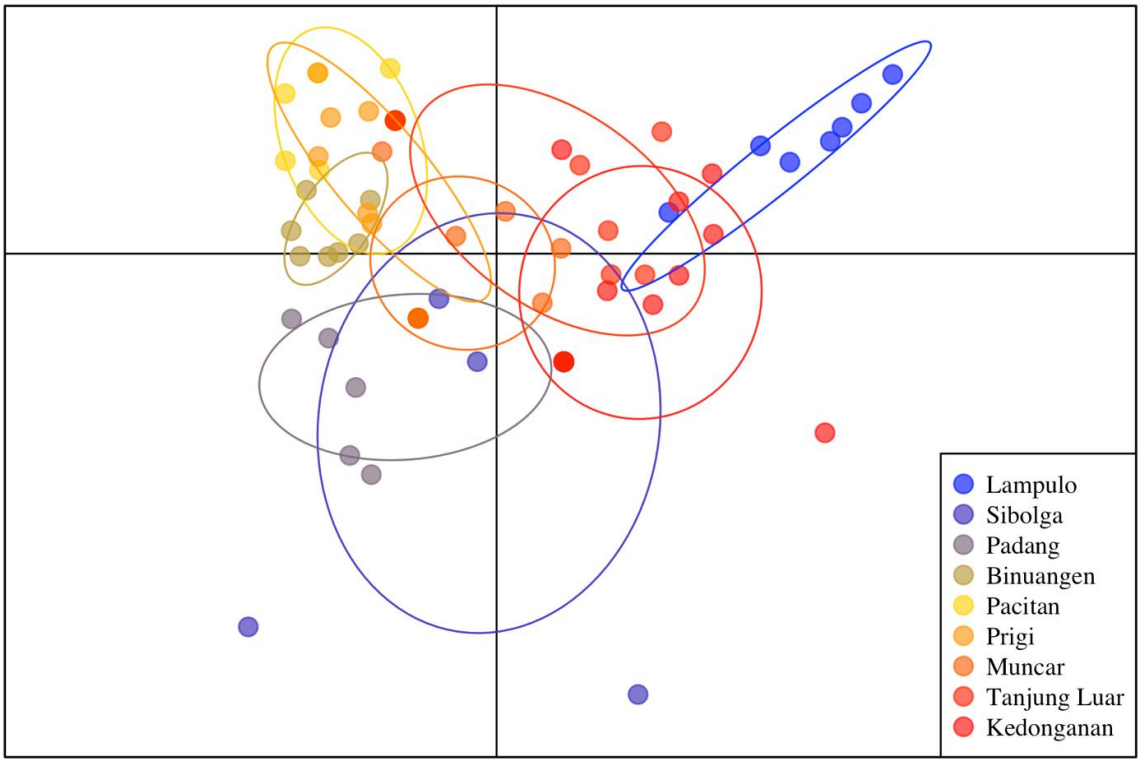

Figure 3. Discriminant analysis of principal components (DAPC) for 76 genotypes of eastern little tuna (*E. affinis*) from the western and southern part of Indonesian waters

al. 2008). The nonsignificant value of pairwise F_{ST} (<0.05) as well as AMOVA and DAPC analysis, which found no geographically important group, further supported this argument. The presence of the Indo-Malay archipelago's significant marine connection and the influence of oceanographic condition, i.e., Indonesian throughflow may favor large levels of gene flow between populations (Rohfritsch and Borsa 2005) instead of limiting it. Therefore, some previous studies on the population structure of large and small pelagic fishes based on microsatellite and mitochondrial markers within Indonesian waters

mostly resulted as a single group (Jackson *et al.* 2014; Pertiwi *et al.* 2014; Jatmiko *et al.* 2018, 2019).

Although the finding in this study was coherent with most of the previous results, Feutry *et al.* (2020) suggested a different idea, which the lack of identification of population structure could be owing to either the genuine absence of obstacles to geneflow or simply be due to the method's insufficient resolution. Through examination of Single Nucleotide Polymorphisms (SNPs), they resolved a distinct separation of eastern little tuna in the Indian Ocean, the gene flow barrier located in-between the western and south part of Indonesian waters. However, there were some uncertainties, such as a possible mixing of the genetic pool and incomplete lineage sorting between two groups still occurred (Feutry *et al.* 2020). However, with only two sampling locations within Indonesian territory (compared to nine in this study) could inflict loss of information on its genetic diversities. As shown in Table 3. the differentiation between Lampulo and Muncar was statistically significant, as Muncar with other locations, but not if the results were lumped together. Therefore, a more representative sampling location was likely a better approach to understand eastern little tuna's genetic flow and population structure in the region.

No distinct population structure was detected for eastern little tuna within western and southern parts of Indonesian waters. Therefore, for future consideration, the species-specific stock-based assessments for eastern little tuna within the Indonesian territory, especially from western and eastern part of Indonesian waters should be considered as a single stock. So, the right harvest strategy should be implemented based on their respective stock structure and it will no longer treated merely a guess, as it has been in the past. These steps are necessary in order to ensure the sustainability of the fisheries resource. Similar approaches should also be deployed not only for neritic tuna species but also for other highly valued fishes, e.g., red snapper, grouper, eel, etc. before any assessment begun. Further, developing a Next-Generation Sequencing (NGS) technique for population structure is advisable for a higher resolution insight into the population structure of this species.

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