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Population genetic structure of *Kawakawa* (*Euthynnus affinis* Cantor, 1849) in Malaysian waters based on COI gene

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Abstract. *Binashikhbubkr K, Setyawan AD, Naim DM. 2023. Population genetic structure of* Kawakawa *(*Euthynnus affinis *(Cantor, 1849)) in Malaysian waters based on COI gene. Nusantara Bioscience 15: 258-268. Kawakawa* (*Euthynnus affinis* Cantor, 1849) is widely distributed in the subtropical and tropical waters of the Indo-Pacific region. Still, insufficient data about its stock, management, and protection in Malaysia and nearby waters raises concerns about overfishing and depletion. Therefore, to ensure effective and successful management of a species, it is imperative to conduct a molecular-based assessment of the stock structure. The present study investigated the population genetic structure of *E. affinis* in Malaysian waters using the mtDNA COI gene. Furthermore, the 632 bp segment of the COI region was sequenced in 372 individuals from 19 distinct populations in Malaysian waters. The results revealed that the genetic divergence varied from low to high. The average Haplotype diversity (Hd) and nucleotide diversity (π) were calculated to be 0.5401 and 0.0045, respectively. Examining haplotype distribution unveiled the presence of 22 unique haplotypes within the COI gene of *E. affinis*. The analysis of the Neighbor Joining (NJ) tree and the Minimum Spanning Network (MSN) revealed the formation of three distinct clades among *E. affinis* samples. Analysis of Molecular Variance (AMOVA) showed a significant genetic structure among the 19 populations of *E. affinis* [(F_{ST} = 0.5354 (P < 0.05)]. The neutrality test and mismatch distribution analysis indicated that the specimens underwent a period of population expansion. This study is a significant milestone, providing the first comprehensive documentation of the genetic structure of *E. affinis* in Malaysia.

Keywords: COI, *Euthynnus affinis*, fisheries stock management, mitochondrial DNA, population genetics

INTRODUCTION

Assessing animal population distribution and genetic structure is important in developing efficient conservation strategies, particularly for species that have undergone overexploitation. Maintaining species biodiversity and populations and safeguarding them against commercial exploitation are crucial objectives (Turan et al. 2005; Ha et al. 2020). The stock composition of a species holds significance, as its geographical distribution and genetic structure influence it. Hence, comprehending this framework will yield numerous advantages, including recognizing populations with distinct ecological, genetic, and management attributes. Furthermore, acquiring this knowledge is important in developing suitable conservation and management strategies tailored to various demographic cohorts.

Additionally, it is advantageous to pinpoint a region characterized by substantial genetic diversity, as this is a crucial attribute to safeguard the enduring welfare of a species (Khan et al. 2012). Utilizing this information will facilitate the evaluation of interrelatedness and genetic exchange between populations, thereby enabling a comprehensive evaluation of genetic well-being. Overall, the comprehension of species stock structure is important in management, conservation, and species' sustained survival and resilience (Cronin-Fine et al. 2013; El Mghazli et al. 2021).

Researchers have proposed various techniques to ascertain the presence of marine and freshwater fish stocks. The earlier mentioned techniques encompass morphometric techniques (Griffiths et al. 2017), geometric morphometrics (Imtiaz and Naim 2018; Mohammadi-Sarpiri et al. 2021; Binashikhbubkr et al. 2022), meristic analysis (Gain et al. 2017), otolith shape analysis (Wujdi et al. 2022), and DNA analysis techniques, specifically focusing on mitochondrial genes (Kumar et al. 2012; Johnson et al. 2016; Kasim et al. 2020; Jamaludin et al. 2022). DNA coding, particularly utilizing the COI gene in mitochondrial DNA, has been widely used since the early 2000s in various research, including forensics, population, ecology, phylogenetics, and biodiversity (Hürkan 2020). These genes are useful in several research objectives, such as ensuring seafood safety, managing fisheries resources, finding new species, and documenting animal biodiversity (Seyhan and Turan 2016). The conservation of COI genes in mitochondrial genomes is important for classifying and identifying different animal species (Hebert et al. 2009; Kamal et al. 2020). Recent studies on marine fish have proven that using COI genes can be an effective tool for species identification, population genetic analysis, and fisheries management (Kunal and Kumar 2013; Halim et al. 2022).

Kawakawa, scientifically known as *Euthynnus affinis* (Cantor, 1849), is a type of tuna found in the Indo-Pacific region, particularly in warm seas. It is a crucial species for Malaysia and nearby waters but faces threats from overfishing and illegal fishing activities (Samsudin et al. 2015; Mardlijah et al. 2022). To ensure its sustainable use, strong surveillance and management strategies are essential. Despite these challenges, *E. affinis* remains significant for Malaysia's fishing industry and local economy. Mitochondrial DNA (mtDNA) methods have been used in previous studies in Malaysia and nearby waters to understand the genetic diversity and stock structure of *E. affinis*. Santos et al. (2010) used DNA markers from the control region (specifically D-loop) to study 35 *E. affinis* samples from the Philippines and 13 samples from Peninsular Malaysia (Pangkor Island and Penang Island). Another study by Masazurah et al. (2012) focused on the population structure of *E. affinis* in two states in Peninsular Malaysia (Perlis and Penang) using cytochrome b (Cyt b) marker. These investigations revealed a lack of understanding of the stock structure, genetic diversity, and phylogenetic connections of *E. affinis* in Malaysian waters. Therefore, this research is crucial for fisheries managers to create effective regional and international policies to address this fish resource's decline. Additionally, the findings can contribute to better management and conservation strategies for the *E. affinis* population in Malaysian waters.

This study evaluated the genetic variability and organization of 19 distinct populations of Malaysian *E. affinis* by utilizing the mitochondrial cytochrome oxidase I (COI) marker. This study represents a pioneering effort in utilizing the COI gene to examine the genetic makeup and population composition of *E. affinis* in Malaysian waters, thereby yielding significant insights.

MATERIALS AND METHODS

Sampling and study area

The specimens belonging to the species *E. affinis* were collected from November 2019 to September 2021. The collection encompassed a total of 19 distinct commercial fish landing sites. The sites mentioned above comprised a total of 11 landing sites located in Peninsular Malaysia and 8 landing sites situated in Malaysian Borneo (specifically Sabah and Sarawak). These landing sites are distributed across four main geographical regions: Straits of Malacca, South China Sea, Sulu Sea, and Celebes Sea. In this study, samples of *E. affinis* were obtained from 5 distinct landing sites situated in the Straits of Malacca: Batu Maung (Penang), Lumut (Perak), Sungai Besar and Kuala Selangor (Selangor), and Kukup (Johor). In addition, a total of 10 landing sites have been designated in the South China Sea, namely Pasir Puteh and Tok Bali (Kelantan), Pulau Tenggol and Pantai Kijal (Terengganu), Kuantan (Pahang), Endau (Johor), Bintawa, Pulau Bruit, and Mukah (Sarawak), and Kota Kinabalu (Sabah); 2 landing sites from the Sulu Sea: Kudat and Sandakan (Sabah), and 2 landing sites from the Celebes Sea: Lahad Datu and Tawau (Sabah) (Table 1, Figure 1). All samples were morphologically identified and validated based on Collette and Nauen (1983). The specimens were relocated to the Molecular Ecology Laboratory at Universiti Sains Malaysia's School of Biological Sciences. Therefore, to enhance visibility, the fish samples underwent rinsing with running water, gentle tapping, and subsequent placement on the left side of a flat surface featuring a black background. For each specimen obtained, a segment measuring approximately 1-2 cm from the right pectoral fin was excised and stored in 95% ethanol for DNA extraction.

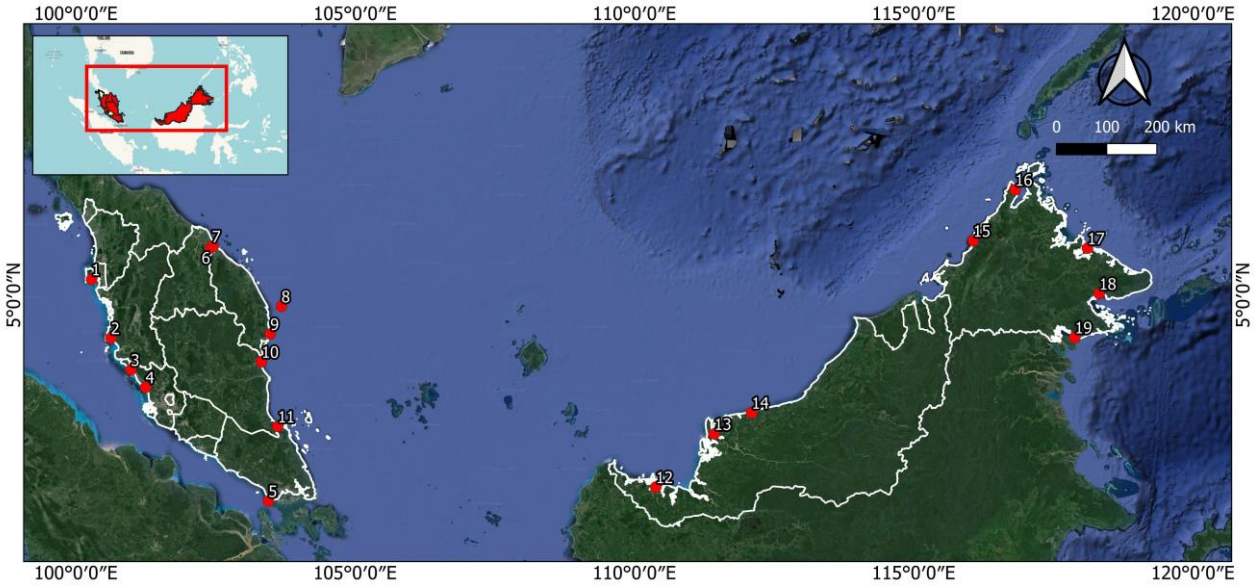

Figure 1. The specimen sampling areas of *Euthynnus affinis* obtained across the Straits of Malacca (M), South China Sea (SCS), Sulu Sea (SS), and Celebes Sea (CS) are as follows: 1. Batu Maung, 2. Lumut, 3. Sungai Besar, 4. Kuala Selangor, 5. Kukup, 6. Pasir Puteh, 7. Tok Bali, 8. Pulau Tenggol, 9. Pantai Kijal, 10. Kuantan, 11. Endau, 12. Bintawa, 13. Pulau Bruit, 14. Mukah, 15. Kota Kinabalu, 16. Kudat, 17. Sandakan, 18. Lahad Datu, 19. Tawau

Table 1. Sampling areas, sample size (N), and geographic coordinates of 19 *Euthynnus affinis* populations were determined through COI gene analysis

Sampling locations	Coordinates	Marine region	N
Batu Maung, Penang (BM)	5° 16' 60.00" N, 100° 16' 60.00" E	M	24
Lumut, Perak (LUM)	4° 13' 56.28" N, 100° 37' 47.28" E	M	24
Sungai Besar, Selangor (SB)	3°39′50.4″N, 100° 59′ 16.6″E	M	14
Kuala Selangor, Selangor (KS)	3° 21' 0.00" N, 101° 15' 0.00" E	M	24
Kukup, Johor (KUK)	1° 18' 60.00" N, 103° 26' 59.99" E	M	12
Pasir Puteh, Kelantan (PP)	5° 49' 58.28"N, 102° 24' 19.02"E	SCS	18
Tok Bali, Kelantan (TB)	5°52′35.5″N, 102° 27′29.9″E	SCS	14
Pulau Tenggol,Terengganu (PT)	4° 47' 59.99" N, 103° 40' 59.99" E	SCS	21
Pantai Kijal, Terengganu (PK)	4°18'20.2"N, 103°28'57.2"E	SCS	6
Kuantan, Pahang (KUA)	3° 48' 27.72" N, 103° 19' 33.60" E	SCS	24
Endau, Johor (END)	2° 38' 59.99" N, 103° 36' 59.99" E	SCS	24
Bintawa, Sarawak (BIN)	1°33'50.96"N, 110°23'15.6"E	SCS	13
Pulau Bruit, Sarawak (PB)	2° 30' 59.99" N, 111° 25' 59.99" E	SCS	14
Mukah, Sarawak (MUK)	2° 53' 45.5784" N, 112° 6' 13.4820" E	SCS	24
Kota Kinabalu, Sabah (KK)	5° 58' 29.64" N, 116° 04' 20.64" E	SCS	24
Kudat, Sabah (KU)	6° 53' 14.35" N, 116° 49' 25.10" E	SS	24
Sandakan, Sabah (SAN)	5° 50' 24.72" N, 118° 07' 4.44" E	SS	20
Lahad Datu, Sabah (LD)	5° 01' 36.48" N, 118° 19' 37.20" E	CS	24
Tawau, Sabah (TA)	4° 14' 41.35" N, 117° 53' 28.14" E	CS	24
Total			372

Note: M: Straits of Malacca, SCS: South China Sea, SS: Sulu Sea, CS: Celebes Sea

Isolation of DNA extraction and PCR amplification

The DNA samples obtained from the clipped fins were isolated using the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method, as described by Bakar et al. (2018), with minor changes to the proteinase K concentration to enhance the quality of the extracted DNA. Subsequently, the pelleted DNA underwent purification and elution using ddH_2O, then visualization through electrophoresis in an agarose gel (1.7%) stained with ethidium bromide (EtBr). Utilizing a spectrophotometer Q3000 (Quawell, Korea), the DNA purity was evaluated and fixed at -20°C until further use. Polymerase Chain Reaction (PCR) amplifications were used to amplify the cytochrome oxidase I (COI) gene. The expected length of the COI gene was 632 bp, and the primer sequences used for DNA amplification were FishF1 (5'TCAACCAACCACAAAGACATTGGCAC3') and FishR1 (5'TAGACTTCTGGGTGGCCAAAGAATCA3') (Ward et al. 2005). The PCR amplification was conducted in 25 µL volume including 16.25 µL molecular grade water, 2.5 µl 10X PCR buffer, 2.0 µl $MgCl_2$, 1.0 µL dNTPs, 0.5 µL of each primer (10 mM), 0.25 µL i- Taq (Intron, South Korea), and 2.0 µL DNA template. Gradients PCR were initially utilized to obtain the best annealing temperature using a T100TM Thermal-cycler (BIO-RAD, USA). The PCR reaction was performed in a Major Cycler, CyCLER-25 (Major Science, USA). The temperature profile was: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, annealing temperature 47.9-48.5°C for 50 sec, extension step 72°C for 1 min, followed by a final extension at 72°C for 7 min, and final hold at 4°C. PCR products were visualized in a 1.7% agarose gel (Vivantis Sdn.Bhd.) and stained with ethidium bromide to check for the presence of DNA. The PCR materials were cleaned using the Intron Purification Kit (Intron, South Korea) to confirm that no impurities or extra suppressors were present. The purified products of PCR were later delivered to NHK Bioscience (Korea) for the sequencing process.

Data analysis

The sequences were processed using MEGA ver. 11 software (Tamura et al. 2021) for trimming and alignment, utilizing the ClustalW algorithm. The nucleotide composition was determined using MEGA ver. 11 (Tamura et al. 2021). The DNA sequences acquired were effectively identified through the utilization of the Basic Local Alignment Search Tool (BLAST) within the National Centre for Biotechnology Information (NCBI) database, which can be accessed at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The *E. affinis* samples were assigned using homogeneity/similarity threshold values exceeding 99%. The pairwise genetic disparities within and between populations were computed using the Kimura 2-parameter (K2P) model in MEGA version 11 software. The variance of genes, haplotype variation (Hd), and nucleotide composition (π) was measured using DnaSP ver. 6.12 (Rozas et al. 2017). The reconstruction of the phylogenetic tree was carried out using the Neighbour Joining (NJ) tree method. The investigation involved the analysis of evolutionary relationships and sequence variation using the Kimura 2-parameter method, which was implemented in MEGA ver. 11 (Tamura et al. 2021). The estimation of the values of all phylogenetic nodes was conducted using 1000 replicates, and only bootstrap values exceeding 50% are presented. The phylogenetic analysis incorporated the sequence of *Lutjanus erythropterus* Bloch, 1790 (Accession no: GU673202.1) retrieved from GenBank (https://blast.ncbi.nlm.nih.gov) as an outgroup. In addition, a sequence of *E. affinis* obtained from GenBank (Accession

no: KY371510.1) was included in the analysis to verify the identification of the specimens as *E. affinis*.

A minimum spanning network was created using the median-joining method performed in PopART ver.1.7 (Leigh and Bryant 2015) to infer the relationships among haplotypes. Next, to estimate the population genetic structure of 19 *E. affinis* populations, an analysis of molecular variance (AMOVA) was conducted in Arlequin ver.3.5 (Excoffier and Lischer 2010). The isolation by distance (IBD) or Mantel test was performed in Arlequin ver.3.5 to verify the association between geographical and genetic distances. Population pairwise genetic distance values represented the gene distances, while geographical intervals between populations were estimated using the ruler tool in Google Earth. The neutrality test was conducted utilizing DnaSP version 6.12 (Rozas et al. 2017), which incorporates Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) analyses. Tajima's D statistic can identify departures from neutrality that arise due to factors such as population bottleneck, population expansion, directional selection, or introgression. This is achieved by analyzing data on the frequency of mutations or segregation sites. Tajima's D is a statistical measure that provides insights into various evolutionary processes; a positive value of Tajima's D indicates balancing selection, population sub-structuring, or a recent population bottleneck. Conversely, negative values of Tajima's D suggest recent directional selection or population growth beyond the presence of rare alleles (Tajima 1989). On the other hand, Fu's (1997) Fs analyses employ haplotype distribution to detect historical variations in population size. Additionally, the mismatch distribution was computed using DnaSP ver. 6.12 (Rozas et al. 2017). Analyzing the pattern can provide insights into the population's past demographic changes (Chen et al. 2015). An unimodal distribution pattern indicates a population recently experienced extension, whereas a multimodal dispersal model suggests a stable population (Rogers and Harpending 1992). The accession numbers OP595397-OP595523 were utilized to deposit all sequences into the GenBank database.

RESULTS AND DISCUSSION

Sampling data

Moreover, 372 specimens of *E. affinis* were sequenced for the COI gene. The specimens were obtained from a total of 19 distinct locations situated within four primary geographic regions: Straits of Malacca, South China Sea, Sulu Sea, and Celebes Sea (Figure 1 and Table 1).

Genetic distance and genetic diversity

The DNA sample sequence successfully produced fragments measuring 632 base pairs (bp) for the COI gene. The findings of this study demonstrate a 100% success rate in amplification for the COI gene. The intra and inter-population genetic distances of all *E. affinis* populations are displayed in Table 2. The intrapopulation genetic distance exhibited a range of values from 0.000 to 0.029. The population of TA demonstrated the highest level of intrapopulation genetic distance (0.029), whereas the populations of TB, PK, PT, SB, KS, KUK, and SAN exhibited the lowest intrapopulation genetic distance (0.000).

Conversely, the interpopulation genetic distance ranged from 0.000 to 0.082. The interpopulation genetic distance between KUA and MUK populations was the highest at 0.082. On the other hand, the populations of PK and TB, PT and TB, SB and TB, SB and PK, SB and PT, KUK and TB, KUK and PK, KUK and PT, KUK and SB, SAN and TB, SAN and PK, SAN and PT, SAN and SB, SAN and KUK exhibited the lowest interpopulation genetic distance at 0.000 (Table 2). Overall, the genetic divergence among 19 populations of *E. affinis* varied from low to high.

The nucleotide sequences of COI in *E. affinis* exhibited a high A+T content, with percentages of 51.7%. The observed values exceeded the G+C content, consistent with the range observed in vertebrates (Nei and Kumar 2000). The haplotype diversity (Hd) values varied between 0.0000 and 0.8961, with an average value of 0.5401. Similarly, the nucleotide diversity (π) values spanned between 0.0000 to 0.0137, with a mean value of 0.0045 (Table 3). The findings suggest that each of the 19 populations of *E. affinis* exhibited significant values of haplotype variations (Hd >0.5) and comparatively low values of nucleotide diversity (π <0.5).

Phylogenetic analysis and Minimum Spanning Network (MSN)

The Neighbor Joining (NJ) tree analysis demonstrated the formation of three distinct clades among the *E. affinis* samples collected from Malaysian waters (Figure 2). The first clade consisted of individuals from the following locations: END, LUM, KUA, and KU. The second clade comprised samples from PK, PT, KUK, SAN, LUM, SB, PP, TB, KS, BM, TA, and KK, whereas the third clade encompassed individuals from LD, TA, BIN, PB, TB, PK, PT, KUK, SAN, and MUK. The NJ tree analysis also demonstrated that the GenBank sequence of *E.affinis* exhibited a closer relationship with the *E.affinis* specimens studied, as opposed to the outgroup species (*L. erythropterus*). This finding confirms that the collected individuals belong to the *E. affinis* species. The analysis of the Minimum Spanning Network (MSN) revealed that Hap 2 had the most frequent occurrence among the haplotypes. The remaining haplotypes exhibited limited distribution across a few populations or exclusive to a specific population. The presence of three distinct clades (1, 2, and 3) was further supported by the MSN analysis, revealing a nucleotide divergence of over 5 differences between them (Figure 3).

Haplotype distribution

The analysis of haplotype distribution reveals that 22 distinct haplotypes were identified for the COI gene across 19 populations. Haplotype 2 (N=277) emerged as the dominant haplotype, exhibiting authority across all populations except for KS and BM. Haplotype 1 was present in two distinct populations, namely PP and KS. Conversely, Haplotype 5 was identified in three separate populations: TA, KUA, and LUM. The remaining haplotypes were private or specific to a particular population (Figure 3).

Table 2. Pairwise genetic distance within and between *Euthynnus affinis* populations from Malaysian waters for COI gene based on Kimura 2 parameter model.

	PP	**TB**	**PK**	**PT**	**SB**	**KS**	**KUK**	**SAN**	**LD**	**TA**	**KU**	**KK**	**BIN**	**PB**	**MUK**	**KUA**	**LUM**	**END**	**BM**
PP	**0.001**																		
TB	0.001	**0.000**																	
PK	0.001	0.000	**0.000**																
PT	0.001	0.000	0.000	**0.000**															
SB	0.001	0.000	0.000	0.000	**0.000**														
KS	0.002	0.003	0.003	0.003	0.003	**0.000**													
KUK	0.001	0.000	0.000	0.000	0.000	0.003	**0.000**												
SAN	0.001	0.000	0.000	0.000	0.000	0.003	0.000	**0.000**											
LD	0.005	0.004	0.004	0.004	0.004	0.007	0.004	0.004	**0.008**										
TA	0.022	0.021	0.021	0.021	0.021	0.025	0.021	0.021	0.024	**0.029**									
KU	0.008	0.007	0.007	0.007	0.008	0.011	0.007	0.007	0.011	0.018	**0.005**								
KK	0.021	0.020	0.020	0.020	0.020	0.024	0.020	0.020	0.023	0.030	0.017	**0.009**							
BIN	0.028	0.027	0.027	0.027	0.027	0.031	0.027	0.027	0.033	0.044	0.032	0.051	**0.014**						
PB	0.040	0.039	0.039	0.039	0.039	0.044	0.039	0.039	0.044	0.051	0.040	0.057	0.064	**0.016**					
MUK	0.060	0.058	0.058	0.058	0.058	0.064	0.058	0.058	0.064	0.071	0.060	0.079	0.081	0.015	**0.004**				
KUA	0.023	0.022	0.022	0.022	0.022	0.026	0.022	0.022	0.026	0.040	0.030	0.038	0.052	0.063	0.082	**0.020**			
LUM	0.019	0.018	0.018	0.018	0.018	0.022	0.018	0.018	0.023	0.036	0.026	0.036	0.049	0.060	0.079	0.022	**0.015**		
END	0.018	0.017	0.017	0.017	0.017	0.021	0.017	0.017	0.021	0.036	0.025	0.038	0.053	0.061	0.080	0.028	0.019	**0.008**	
BM	0.014	0.016	0.016	0.016	0.016	0.013	0.016	0.016	0.020	0.048	0.025	0.038	0.048	0.059	0.079	0.046	0.039	0.041	**0.013**

Note: Bold indicates intrapopulation values

Population genetic structure

The genetic compositions of 19 populations of *E. affinis* originating from Malaysia were examined by analyzing the COI gene. In comparison, the Analysis of Molecular variance (AMOVA) results indicate a significant genetic variation level observed among populations, accounting for 53.54% of this variation. Conversely, genetic variation within populations is relatively low, accounting for 46.46%. The results revealed significant genetic structure among all populations of *E. affinis* examined. This was indicated by the values of F_{ST} and *P*, showing an F_{ST} value of 0.5354 and a *P* value of less than 0.05 (Table 4). The Mantel test results reveal a significant correlation between genetic differentiation (measured by the F_{ST} value) and geographical distance (r = 0.6815, P = 0.0000) among the examined populations.

Neutrality test and mismatch distribution

The neutrality assessment of the COI gene revealed a statistically significant negative Tajima's D statistic (-0.6416, P >0.05) across all populations. However, the Fs statistic calculated by Fu (1997) yielded a positive value, although it did not reach statistical significance (0.4203, P >0.05) (Table 3); this mismatch distribution analysis exhibited an unimodal pattern (Figure 4).

Table 3. Haplotype diversity, nucleotide diversity, Tajima's D, and Fu's Fs statistics for 19 *Euthynnus affinis* populations from Malaysian waters based on COI gene

Population	N	h	Hd	π	Tajima's D	Fu's Fs
PP	18	2	0.7994	0.0009	1.5476	1.4280
TB	14	1	0.0000	0.0000	–	–
PK	6	1	0.0000	0.0000	–	–
PT	21	1	0.0000	0.0000	–	–
SB	14	1	0.0000	0.0000	–	–
KS	24	1	0.0000	0.0000	–	–
KUK	12	1	0.0000	0.0000	–	–
SAN	20	1	0.0000	0.0000	–	–
LD	24	3	0.8961	0.0047	-2.3034*	-4.3770
TA	24	3	0.8833	0.0110	0.0275	4.6530*
KU	24	2	0.8419	0.0036	-1.7842	0.2360
KK	24	1	0.8806	0.0070	-1.2180	-0.7560
BIN	13	2	0.8508	0.0105	-2.2971*	0.7870
PB	14	1	0.8891	0.0132	-0.9982	0.0140
MUK	24	6	0.8452	0.0033	-2.1814*	-3.2320
KUA	24	2	0.8788	0.0137	1.5295	6.2490*
LUM	24	2	0.7849	0.0089	-0.9948	3.5950
END	24	8	0.8503	0.0054	-2.3601*	-2.5950
BM	24	2	0.8614	0.0033	-1.1581	1.9840
Total	372	22	0.5401	0.0045	-0.6416*	0.4203

Notes: N: Number of samples, h: Number of haplotypes, Hd: Haplotype diversity, π: Nucleotide diversity. *Significant at P >0.05

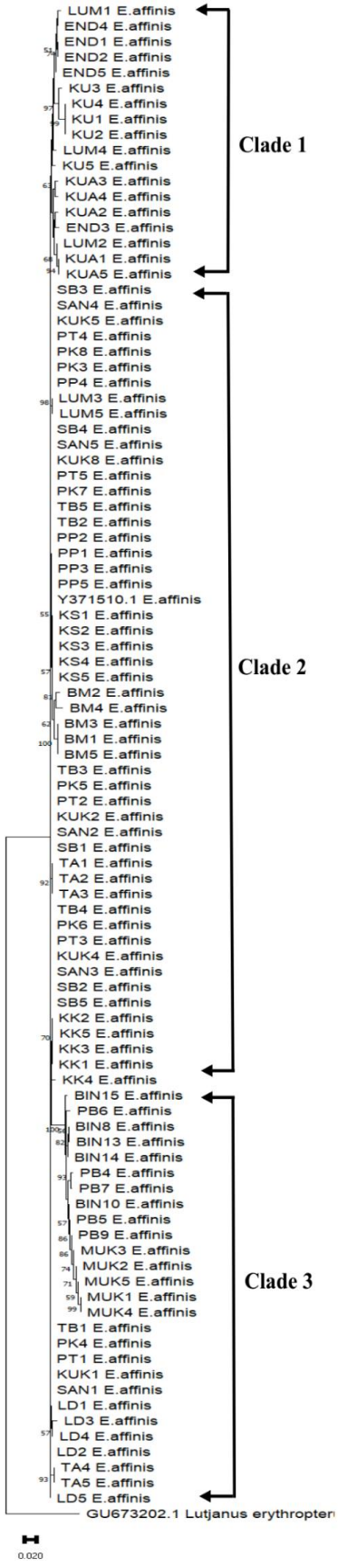

Figure 2. Neighbor Joining (NJ) tree illustrating the correlation among COI haplotypes of *Euthynnus affinis* specimens obtained from Malaysian waters. Only bootstrap values greater than 50% are displayed

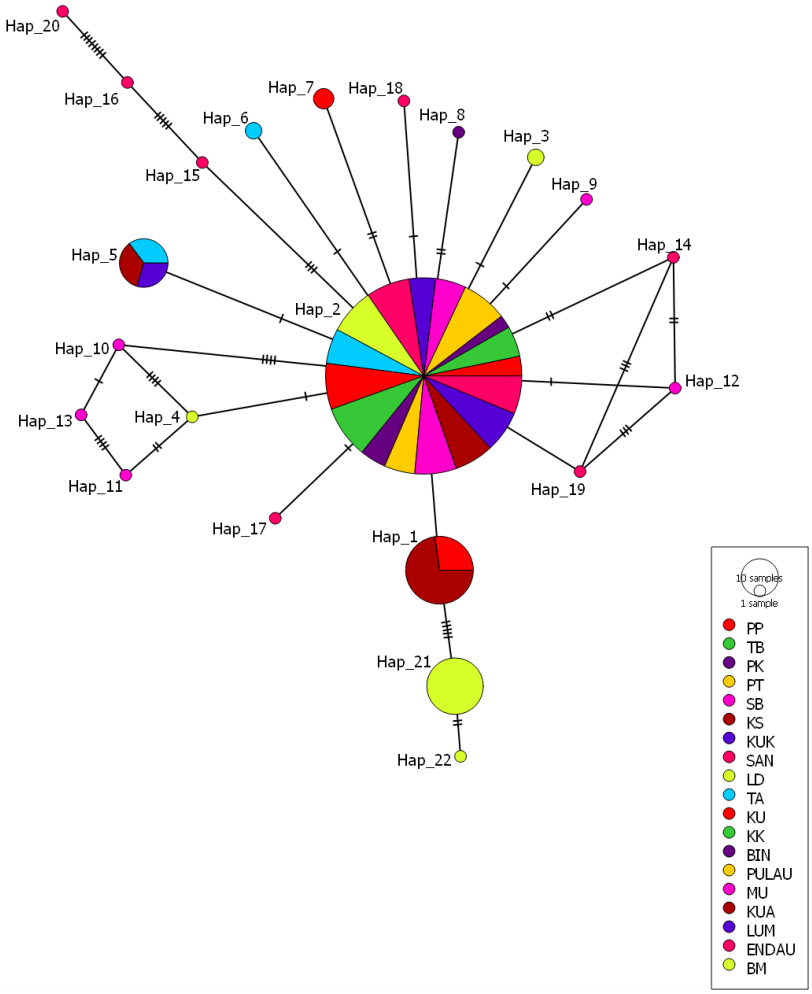

Figure 3. Minimum spanning network (MSN) using 22 COI haplotypes of *E. affinis* obtained from 19 distinct populations from Malaysian waters

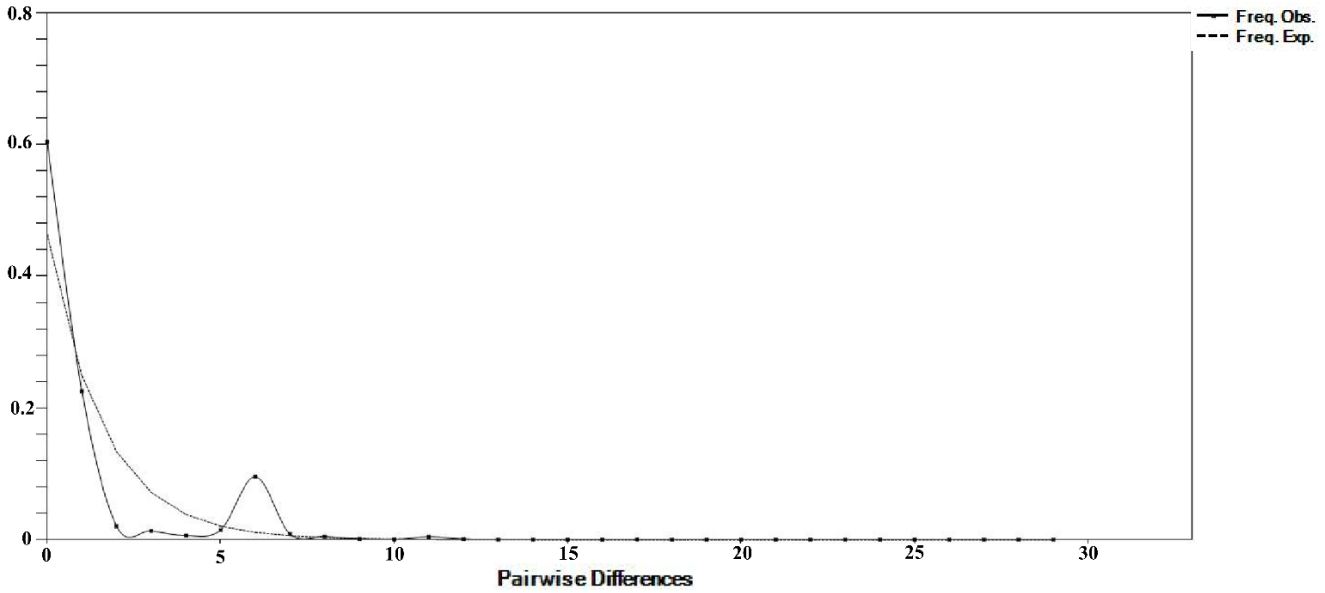

Figure 4. Mismatch distributions (pairwise number of differences) for the COI gene of *E. affinis* showing the expected and observed pairwise differences between sequences with the respective frequencies

Table 4. Analysis of molecular variance (AMOVA) of 19 populations of *Euthynnus affinis* collected from Malaysian waters based on the COI gene

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	F_{ST}	*P*
Among populations	18	2633.509	7.18584 Va	53.54	0.5354	0.0000
Within populations	353	2201.244	6.23582 Vb	46.46		
Total	371	4834.753	13.42166	100		

Discussion

The presence of skilled taxonomists is crucial in ensuring the accurate identification of species using morphological identification techniques or traditional methods, as there is a significant risk of misidentification resulting from phenotypic plasticity. Therefore, DNA barcoding has demonstrated significant utility in the identification of species, particularly for specimens that are imperfect, damaged, or at distinct morphological stages (Wang et al. 2018). The COI barcoding enables the precise identification of nearly 98% of documented marine fish species. This approach has been widely adopted across diverse geographic regions to classify and record fish populations (Becker et al. 2015). The current investigation effectively identified over 99% of *E. affinis* samples obtained using the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) database utilizing the COI gene. This study demonstrates the efficacy of the COI gene in distinguishing *E. affinis* specimens and assessing the genetic diversity and population pattern of *E. affinis* collected from four important oceanic areas in Malaysia, encompassing a total of 19 landing sites.

The genetic distance within and between 19 populations of *E. affinis* exhibited a range from low to high values (0.000-0.082) (Table 2). The findings indicate that there exists a genetic differentiation among a total of 19 populations of *E. affinis*. The assessment of genetic distance is frequently employed to assess the taxonomic classification of closely related species and for evolutionary investigation. According to Hebert et al. (2003), a higher genetic distance indicates a substantial decrease in relatedness between the samples, accompanied by extensive variations in their nucleotide bases. Marine species commonly exhibit a substantial population size, the capacity to disperse extensively during their pelagic larval stages, and a wide geographical distribution (Palumbi 1992). For example, the limited genetic diversity observed among tuna populations across different oceans can be attributed to a consistent and extensive pelagic habitat and abundant spawning areas (Durand et al. 2005). In contrast, the physical obstacles in the marine environment, such as ocean currents, reefs, and other land formations, are thought to be why marine fish display genetic diversity and population structure. The presence of these barriers has the potential to impede the movement of genes between populations, resulting in genetic differentiation (Ward et al. 1994).

Furthermore, it is important to note that various fish species exhibit distinct environmental preferences, including but not limited to water temperature, salinity, and depth. When populations of a given species inhabit distinct habitats, they have the potential to undergo distinct genetic adaptations in response to their respective environments. It is important to acknowledge that genetic differentiation is an inherent and customary phenomenon in all species. However, human activities such as overfishing, pollution, and habitat degradation may also lead to genetic differentiation and population fragmentation among marine fish species (Conover et al. 2006).

These present findings are consistent with prior studies that have examined the genetic structure of *E. affinis* in various marine regions, including the North Indian Ocean (Kumar et al. 2012), the Straits of Malacca (Masazurah et al. 2012), and the coastal waters of Tanzania (Johnson et al. 2016). These studies have identified discrete clades of *E. affinis*. Similarly, the findings of the current investigation align with the findings presented by Kasim et al. (2020), wherein a minimal genetic differentiation was observed among 11 distinct populations of *Thunnus tonggol* (Bleeker, 1851) (Longtail Tuna) in Malaysia. In their study, Akbar et al. (2018) provided findings on the genetic distance observed in *Thunnus obesus* (Lowe, 1839) (Bigeye Tuna). Their research focused on two distinct populations inhabiting Indonesia's North and South Moluccas Seas. The reported genetic distance values ranged from 0.023 to 0.027, indicating the level of genetic differentiation within and between these populations. Furthermore, evaluating the GC content in commercial fish helps understand nucleotide diversity and assess evolutionary lineages, leading to better interpretations of mutation pressures on taxa by selecting substitution models (Figuet et al. 2014). For instance, higher GC content corresponds to more nucleotide diversity and a greater likelihood of mutations in a population (Imtiaz 2018).

An examination of haplotype diversity (Hd) and nucleotide diversity (π) within populations can offer valuable insights into the demographic history of a population (Grant and Bowen 1998). Low nucleotide diversity and high haplotype diversity indicate that the population is immense, has recently expanded, and contains unique genetic variations. Nevertheless, this also implies that there has been an insufficient duration for the accumulation of supplementary nucleotide substitutions across the various haplotypes (Grant and Bowen 1998; Kasim et al. 2020). As stated by Kumar et al. (2012), it is widely accepted that the substantial genetic diversity observed in marine fishes can be attributed to their large population sizes. The extensive haplotype diversity observed in this study can be attributed to the wide geographic range and substantial population sizes of *E. affinis*. The present findings are consistent with previous

studies conducted on various species of tuna, including *Kawakawa* (*E. affinis*) (Hd = 0.67, π = 0.0124) (Santos et al. 2010), Longtail Tuna (*T. tonggol*) (Hd = 0.990, π = 0.0195) (Kasim et al. 2020), bigeye tuna (*T. obesus*) (Hd = 0.981, π = 0.028) (Pertiwi et al. 2017). According to Vandewoestijne et al. (2004), the substantial diversity of haplotypes can be attributed, at least in part, to the elevated mutation rate observed in mitochondrial DNA. This assertion is corroborated by the analysis of MSN (Figure 3), which indicates that a minimum of three mutations occurred before a novel haplotype emerged.

The phylogenetic tree and MSN analysis demonstrate that the 19 populations of *E. affinis* obtained from Malaysian waters are classified into three distinct clades (Figures 2 and 3). The first clade comprises END, LUM, KUA, and KU samples. The second clade, comprising 12 populations (PK, PT, KUK, SAN, LUM, SB, PP, TB, KS, BM, TA, and KK), is notably dominated by haplotype Hap 2, which is the most prevalent. In comparison, the third clade encompasses individuals from LD, TA, BIN, PB, TB, PK, PT, KUK, SAN, and MUK. The remaining haplotypes are encompassed by the first and third clades (Figures 2 and 3).

Moreover, various genetic groups inside a species may be linked to secondary interaction and continuous interbreeding amongst populations that have been geographically apart for many generations (Durand et al. 2005). The results of MSN analysis indicate that a significant proportion of the populations exhibited shared genetic characteristics with Hap 2. This finding suggests that there has been a recent exchange of genetic material between these populations, as reported by Koopman et al. (2007). Furthermore, Horne et al. (2008) demonstrated that over a long period, significant genetic exchange and movement of individuals between geographically distant populations resulted in the sharing of haplotypes. In addition, LD, TA, BIN, MUK, END, and BM populations exhibited unique COI haplotypes. The findings of this study indicate a potential decrease in the exchange of genetic material between the studied locations, most probably caused by oceanic currents (Halim et al. 2022). Oceanic currents can affect gene flow among regions by creating barriers that isolate populations and increase differentiation levels or promoting long-distance dispersal (Gouvêa et al. 2023). The direction and strength of ocean currents can influence the movement of marine species and their larvae, which affects the distribution of genetic variation among populations (Snead et al. 2023). Therefore, oceanic currents are crucial in shaping marine populations' genetic structure by promoting or hindering gene flow among different regions. These currents are a key factor in the evolution and adaptation of marine species and have significant ecological and conservation implications (White et al. 2010).

The AMOVA analysis corroborated the findings derived from the analyses of the phylogenetic tree and MSN, thus emphasizing the presence of a genetic structure among the populations of *E. affinis*. Using F_{ST} values is a common practice in estimating gene flow, whereby a greater F_{ST} value signifies a substantial level of genetic differentiation. In comparison, a lesser F_{ST} value indicates a minimal level of genetic differentiation (Jose et al. 2023). AMOVA analysis indicated a substantial level of F_{ST} values (0.5354) with a statistically significant difference (P <0.05, 0.0000) (Table 4). Furthermore, the Mantel test or isolation by distance (IBD) demonstrates a substantial relationship between genetic difference and geographical distance. The findings of the present investigation confirm the emergence of a structural framework within *E. affinis* populations. Moreover, earlier studies have reported the existence of a genetic framework within tuna species (e.g., Kunal et al. 2013; Li et al. 2015).

The analysis of demographic history examines fluctuations in the size of a population over time, using nucleotide sequences to infer historical variations in mutation patterns (Tajima 1989). The present study employed an independent demographic history test to ascertain that populations of *E. affinis* obtained from Malaysia experienced a period of population expansion. The neutrality test yielded a statistically significant negative Tajima's D statistic for all populations (-0.6416, P >0.05). Additionally, Fu's Fs statistic was positive, although it did not reach statistical significance (0.4203, P >0.05) (Table 3). The findings show that Fu's Fs' effectiveness in detecting significant population changes surpasses Tajima's D, thereby addressing the discrepancy between the two measures (Fu 1997). The results of this study indicate the occurrence of a population change in the past, suggesting a population expansion based on Tajima's D (Tajima 1989). Tajima's D is a widely employed method for assessing whether the nucleotide sequence is in a state of mutation-drift equilibrium. According to Korneliussen et al. (2013), a negative value of Tajima's D suggests the possibility of population growth, expansion, or the occurrence of purifying selection. A positive value of Tajima's D indicates balancing selection, population sub-structuring, or a recent population bottleneck. As stated by Fu (1997), the Fs test is susceptible to detecting evolutionary forces resulting from an abundance of new mutations within a population. Moreover, it is considered a potent tool for identifying instances of population expansion. Furthermore, the appearance of an unimodal mismatch distribution pattern, as shown in Figure 4, supports the concept of a dramatic population increase in the taxa's current state. These findings align with prior studies conducted on pelagic species, including *Thunnus albacares* (Bonnaterre, 1788) (Kunal et al. 2013; Li et al. 2015), *T. tonggol* (Kasim et al. 2020), and *Scomberomorus commerson* (Lacepède, 1800) (Johnson et al. 2021). In general, the findings of this study indicate that the *E. affinis* specimens collected from different geographical locations underwent a period of population expansion.

In conclusion, this study represents the inaugural documentation encompassing multiple locations in Malaysian waters, examining the genetic diversity and population structure of *E. affinis* through the utilization of the COI gene. The mitochondrial DNA gene, specifically COI, has been effectively utilized to discern 19 distinct populations of *E. affinis* in the waters of Malaysia. This analysis has unveiled the presence of three distinct lineages

within these populations, which are distributed across four marine regions: Straits of Malacca, South China Sea, Sulu Sea, and Celebes Sea. Moreover, the study findings will build a solid basis for successfully administering and protecting neritic tuna stocks in Malaysia. Further studies on *E. affinis* may necessitate the application of various DNA markers and an increased amount of sampling locations and specimens.

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