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Genetic Diversity of Flying Fish (Exocoetidae) in Southeast Sulawesi, Indonesia

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ABSTRACT

Flying fish (Exocoetidae) is a pelagic fishery commodity that holds economic value among the people of eastern Indonesia. Flying fish are advantageous as they are abundant in Indonesian sea waters, making them an affordable source of omega-3, which is beneficial for human health. However, the high demand for flying fish has resulted in overfishing, which poses a risk of reducing their natural population. Proper management of this commodity is necessary to minimize these risks. Effective management requires adequate information, including genetic diversity data. This study aims to determine the genetic diversity of flying fish in Southeast Sulawesi, specifically in the Banda Sea and Bone Bay populations. Tissue samples were collected from flying fish in the two populations during the study in March 2022. The results show that the genetic diversity values of flying fish in Bone Bay and the Banda Sea were 0.984 and 1.00, respectively, while the nucleotide diversity values were 0.021 and 0.018. High genetic diversity values and nucleotide diversity values were found between the two populations, with values of 0.990 and 0.020, respectively. Based on phylogenetic analysis, the two populations in Southeast Sulawesi are genetically similar.

1. Introduction

Flying fish (Exocoetidae), in general, can be found in tropical and sub-tropical seas, usually live on the surface offshore and in coastal areas, and are components of the pelagic ecosystem food chain. This fish is abundant in certain waters, especially in tropical waters, as a source of people's fisheries, such as in Indonesia, the Philippines, Vietnam, Thailand, and Kiribati (Parin 1996). The potential for flying fish resources in the Banda Sea and Bone Bay waters is supported by geographical location and entry in the coral reef triangle area, which has a high diversity of biodiversity.

The intensity of fishing for flying fish *Hirundichthys oxycephalus* (Bleeker 1852) in Southeast Sulawesi waters is high, and fish eggs are also a targeted catch. This condition can lead to overfishing (Anwar 2023) which can decrease flying fish populations in the area. As is the condition in the Flores Sea, where catches have dropped sharply for three consecutive years, with the greatest catch reaching 260,525 kg before dropping by 70,703 kg, this condition may lead to overfishing, which may diminish flying fish populations in the area (Ali *et al.* 2004). In addition, the Tual waters have also witnessed a decline in the catches of flying fish in the past three years, as mentioned by Anwar (2023). Furthermore, Risa and Wahyuni (2020) have reported that the fishing of flying fish in the Makassar Strait has exceeded the MSY limit.

To prevent this, a conservation plan is necessary to protect flying fish from extinction. Their genetic diversity must be protected and preserved. This can be accomplished by protecting surviving flying fish populations (Frankham *et al.* 2002). This genetic diversity information can be used as a basis for establishing management policies and genetic conservation of flying fish in the region. Previous

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research on the genetic diversity of flying fish has been conducted in various locations worldwide; the first finding of flying fish *Cypselurus opisthopus* in southeastern waters of India (Jayakumar *et al.* 2019), identification of flying fish DNA in Australian waters using cytochrome b found that the genus *Cheilopogon* is not monopiletic (Lewallen *et al.* 2011, 2016; Lewallen 2012; Gordeeva and Shakhovskoi 2017), identification of flying fish DNA down to the genus level has been carried out with CO1 (Parenrengi *et al.* 2016), identification of flying fish morphology in the Makassar Strait found six species (Indrayani *et al.* 2020), and the use of DNA analysis for identification of flying fish is much better than using morphological analysis which often results in errors (Gordeeva and Shakhovskoi 2017). Since limited research has been done on the genetic diversity of flying fish in the waters of Southeast Sulawesi, the findings of this study are useful information. Therefore, this study aimed to study the genetic diversity of flying fish populations to protect genetic conservation.

2. Materials and Methods

Flying fish sampling was conducted in March 2022 at two different locations: the fishing base of Gaya Baru Village in South Buton and Anawai Kolaka Village (Figure 1). Sample analysis was carried out at the Genetics Science Jakarta laboratory. Each sample was photographed and its length measured. A 3 cm-long pectoral fin was then taken and stored in a tube containing 96% ethanol for preservation.

2.1. Extraction, Polymerase Chain Reaction, Electrophoresis, and DNA Sequencing

The dorsal flesh was cut into 2 mm pieces, and DNA was extracted using the gSYNC DNA Extraction Kit (Geneaid, GS300). The extracted DNA was then amplified through polymerase chain reaction (PCR) using primers targeting the mitochondrial DNA Cytochrome Oxidase subunit I (mtDNA COI) gene. For each PCR reaction, 1 µL DNA template was mixed with 12 µL My Taq HS Red Mix 2x, 10.5 µL ddH_2O, and 0.5

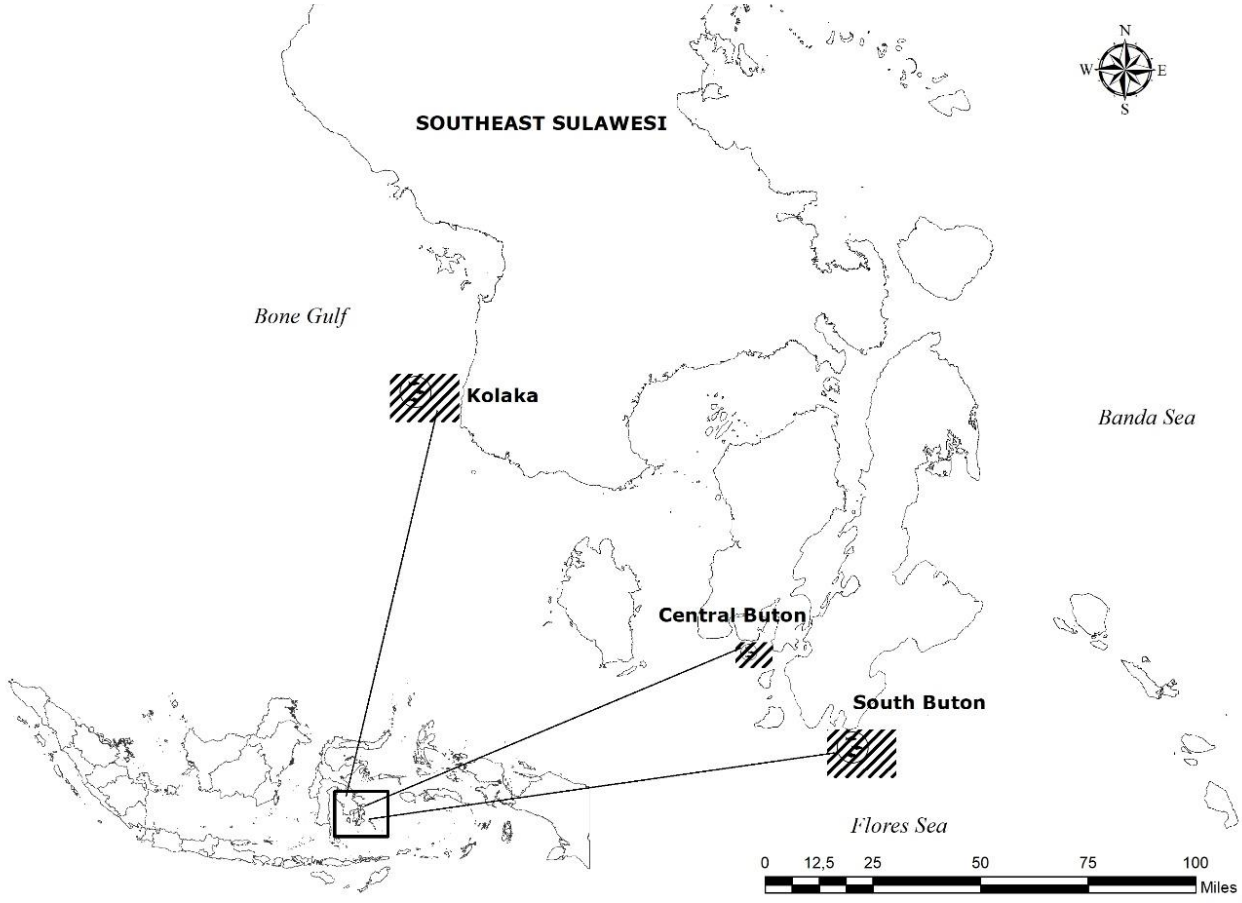

Figure 1. Map of sampling sites in Kolaka, Central Buton, and South Buton, Indonesia

µL of each primer pair. The thermocycler conditions included denaturation at 98°C, annealing at 57°C, and extension at 72°C for 35 cycles. The PCR products were then electrophoresed in 1% agarose at 100 Volts for 30 minutes. The separated DNA molecules were visualized under UV light and documented. DNA extraction is intended to destroy cells and separate the DNA in the sample. In this study, 25 mg of tissue was taken for DNA extraction using the gSYNC DNA Extraction Kit. The mitochondrial cytochrome c oxidase subunit I (COI) gene locus was used for analysis.

DNA amplification using the Polymerase Chain Reaction (PCR) method is an enzymatic DNA replication reaction. The primers used for fish amplification were FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward *et al.* 2005). The components in the PCR mix were 12.5 µl MyTaq HS Red Mix 2x, 1.25 µl of each primer, 9 µl ddH_2O, and 1 µl DNA template. The DNA amplification process used the DIAB Mastercycler DNA Engine Thermal Cycler. The initial stage of DNA amplification is the denaturation stage at 94°C for 30 seconds, followed by primer annealing at 50°C for 1 minute, DNA segment extension at 72°C for 1 minute, and a final extension stage at 72°C for 7 minutes with 38 cycles.

The extracted DNA was amplified via polymerase chain reaction (PCR) using primers CO1 F1 and R1 targeting the mitochondrial DNA cytochrome oxidase subunit I (mtDNA COI) gene (Ward *et al.* 2005).

One µL DNA template was added to 12 µL My Taq HS Red Mix 2x, 10.5 µL ddH_2O, and 0.5 µL of each primer pair. The thermocycling conditions used were denaturation at 98°C, annealing at 57°C, and extension at 72°C for 35 cycles. The PCR product was then electrophoresed on 1% agarose gel at 100 volts for 30 minutes. The separated DNA molecules were visualized using UV light and documented.

Electrophoresis is a method used to purify molecules by separating chemical compounds based on their rate of movement in an electric field, which is commonly used to assess the quality of DNA from PCR results (Isbir *et al.* 2013). In this study, a 1% agarose gel was prepared using GelRedTM (Biotium®) dye as the electrophoresis medium. The PCR products were loaded into the agarose wells, and electrophoresis was conducted at a voltage of 120 volts for 20 minutes. The separated PCR products were visualized using a UV transilluminator and documented using Geldoc. Finally, the PCR products were sent to FirstBase Singapore for further analysis.

2.3. Data Analysis

The control region mtDNA sequence area was analyzed using the MEGA10 software (Tamura *et al.* 2011). The software was used to align the sequences and determine their real similarities using the DNA Weight Matrix ClustalW (1.6) and Translation Weight (0.5) methods. Species identification was done through the Blast (Basic Local Alignment Tools) application. A phylogenetic analysis was performed using the neighbor-joining method, the 2-parameter Kimura evolution model, and bootstrap 1,000 replications with the MEGA10 application, involving all samples of flying fish found.

3. Results

In the following table, we briefly describe genetic divergence between species of 14 flying fish specimens and one specimen out group. The support values for the reconstructed tree, obtained from the molecular datasets of each subfamily, tribe, genus, and sub-genus, are displayed in Figure 2.

The length of the PCR-amplified fragments obtained from the mtDNA control region was determined to be 720 bp (base pairs) from the analyzed samples. The fragment length in this study was longer than the base length (bp) of 600 bp for flying fish from the Exocoetidae family.

The DNA sequence obtained from the molecular analysis was found to be 720 bp in length. The sequence was identified using BLAST on the GenBank data provider, which identified six species collected from the Banda Sea with the South Buton fishing base and 3 from Bone Bay with the Kolaka fishing base.

4. Discussion

In traditional taxonomy, species identification can be challenging as there may be no clear criteria for character selection or coding, and morpho-meristic data sets can be subjective. Genetic analysis can be an additional tool to determine taxonomic identity, and the COI gene has been demonstrated to be useful for species identification and resolving taxonomic ambiguity (Hebert *et al.* 2003). In this study, three species of flying fish were characterized to produce

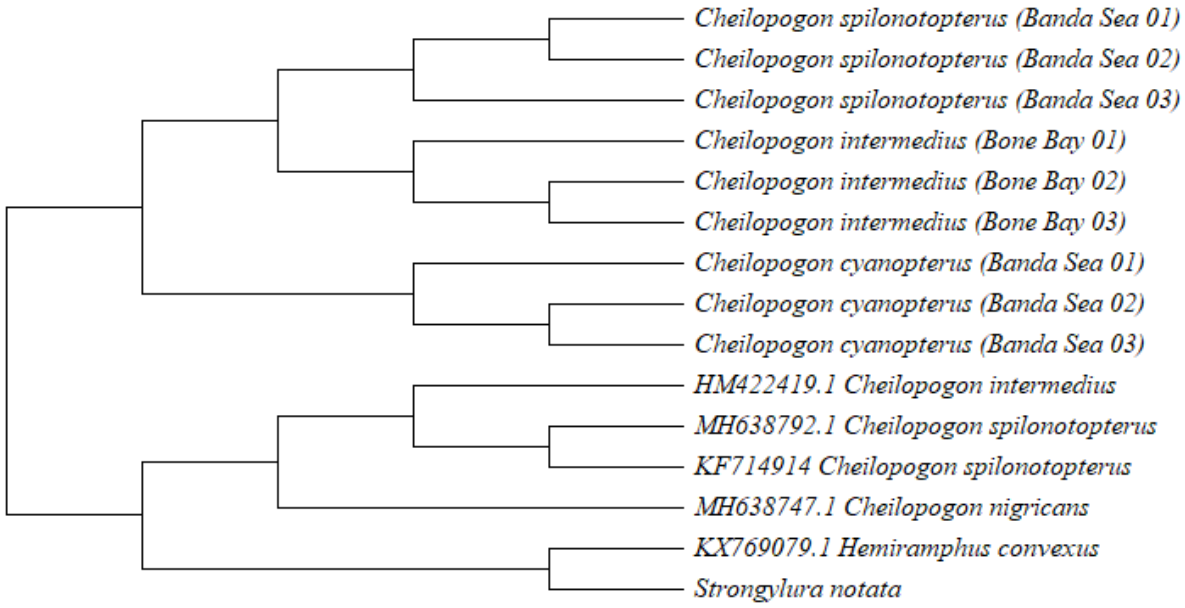

Figure 2. Reconstructed phylogenetic tree of flying fish from Bone Bay and Banda Sea

COI mtDNA sequences to determine the types of flying fish species found in the Southeast Sulawesi region. DNA barcoding using the COI mtDNA gene was performed on Cheilopogon spilonotopterus, *C. intermedius*, and *C. cyanopterus* from Southeast Sulawesi waters. The results of this study can facilitate efforts to standardize the identification of species diversity and expedite the process of identifying cryptic species through DNA barcoding. The study found that the average total nucleotide length in the COI region is 700 bp for the three species. However, it was slightly different from the total base length of 590 bp obtained by Jayakumar *et al.* (2019). The difference in primary length was due to the use of a different number of samples, but it did not affect the results of the sequence analysis for each sample.

The phylogenetic reconstruction based on the CO1 gene sequence (Figure 2) is generally in agreement with the results obtained in previous studies using other nuclear and mtDNA markers, thereby supporting the potential use of DNA barcoding as a promising approach for phylogenetic studies of the family Exocoetida. The study identified one genus and three species based on the obtained results. The phylogenetic tree showed that species from the genus Cheilopogon formed their respective groups. The genetic diversity between the two populations was found to be 0.990. High levels of genetic diversity have been reported in flying fish populations in several other studies, including those by Gordeeva and Shakhovskoi (2017), Jayakumar *et al.* (2019), Lewallen *et al.* (2011, 2016), and Rathipriya *et al.* (2019) indicating the consistent occurrence of high genetic diversity in flying fish.

The genetic distance analysis revealed that *C. spilonotopterus* and *C. cyanopterus* samples collected from the Banda Sea shared the same haplotype with a genetic distance of 0.0000 (Table 1). Similarly, a genetic distance of 0.0000 was observed between samples collected from Bone Bay. The flying fish samples with the farthest genetic distance in the Banda Sea were from (KF714914.1) Philippines and (MH6387471) South China Sea waters. Different factors, such as disparate living conditions, lack of connectivity, and geographical distances, can lead to genetic differences. The minimal genetic distance between flying fish from Bone Bay and those from the Banda Sea suggests a connection between the regions via ocean currents. The similarity in genetic makeup of species from different locations could result from genetic sharing (Stern 2013), connectivity between regions (Díaz-Ferguson *et al.* 2010), habitat similarity, and the tendency of marine organisms to migrate using ocean currents as transportation (Saleky *et al.* 2016).

The findings of this study clearly differentiate the three flying fish species based on their taxonomic distribution. Additionally, this study reinforces the

Table 1. Genetic distance between individuals of flying fish in Bone Bay and Banda Sea waters

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0.0000														
0.0000	0.0000													
0.0238	0.0238	0.0238												
0.0238	0.0238	0.0238	0.0000											
0.0238	0.0238	0.0238	0.0000	0.0000										
0.4994	0.4994	0.4994	0.4984	0.4984	0.4984									
0.5094	0.5094	0.5094	0.5084	0.5084	0.5084	0.0201								
0.5587	0.5587	0.5587	0.5639	0.5639	0.5639	0.1160	0.1099							
0.0288	0.0288	0.0288	0.0337	0.0337	0.0337	0.4962	0.5080	0.5512						
0.0288	0.0288	0.0288	0.0337	0.0337	0.0337	0.4962	0.5080	0.5512	0.0000					
0.0288	0.0288	0.0288	0.0337	0.0337	0.0337	0.4962	0.5080	0.5512	0.0000	0.0000				
0.5050	0.5050	0.5050	0.5040	0.5040	0.5040	0.0285	0.0258	0.1147	0.5056	0.5056	0.5056			
0.5094	0.5094	0.5094	0.5084	0.5084	0.5084	0.0201	0.0000	0.1099	0.5080	0.5080	0.5080	0.0258		
0.5248	0.5248	0.5248	0.5300	0.5300	0.5300	0.0813	0.0718	0.0915	0.5235	0.5235	0.5235	0.0750	0.0718	

(1) Cheilopogon spilonotopterus (GB01); (2) Cheilopogon spilonotopterus (GB02); (3) Cheilopogon spilonotopterus (GB03); (4) Cheilopogon intermedius (GB01); (5) Cheilopogon intermedius (GB01); (6) Cheilopogon intermedius (GB01); (7) MH638747.1 Cheilopogon nigricans; (8) Cheilopogon spilonotopterus KF714914; (9) Strongylura notata; (10) Cheilopogon cyanopterus GB01; (11) Cheilopogon cyanopterus; (12) Cheilopogon cyanopterus; (13) HM422419.1 Cheilopogon intermedius; (14) MH638792.1 Cheilopogon_spilonotopterus; (15) KX769079.1 Hemiramphus convexus

effectiveness of COI barcoding for investigating allopatric variation among flying fish species in Southeast Sulawesi waters. DNA barcoding has the potential to provide contemporary genomic solutions to taxonomic uncertainties, and it can complement traditional taxonomic studies of significant taxa, allowing for the identification of biological units to develop conservation and protection measures for flying fish in Southeast Sulawesi. Utilizing molecular identification methods is expected to provide more conclusive fish species identification results. Overall, this study revealed that there are three species of flying fish belonging to the genus Cheilopogon, Exocoetidae in the waters of Southeast Sulawesi. One of the species has a close relationship with the species found earlier in the South China Sea and waters southeast of India. The results of identification using DNA barcoding techniques have broad potential in various areas of study, such as identification studies in various life stages, conservation status, taxonomy (Dailami *et al.* 2021), biogeography, and biodiversity inventory (Achmad *et al.* 2020; Ran *et al.* 2020).

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