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Genetic variations of *Cheilopogon nigricans* in the Makassar Strait, Indonesia

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Abstract. *Indrayani I, Findra MN, Jufri A, Hidayat H, Pariakan A. 2021. Genetic variations of* Cheilopogon nigricans *in the Makassar Strait, Indonesia. Indo Pac J Ocean Life 5: 22-28.* This study reports DNA Barcoding results (sequencing of cox 1 mitochondrial gene fragments) of four Makassar Strait flying fish species belonging to the Exocoetidae family. Sampling was collected from around the Makassar Strait waters in West Sulawesi. This research was carried out by molecular identification using DNA barcoding of the cytochrome oxidase 1 (COI) gene, the Wizard Promega CO1 primer kit. The molecular identification results showed that the collected fish had 100% and 99.10% genetic similarities with the species *Cheilopogon nigricans* from the South China Sea. The genetic variations of flying fish in the Makassar Strait are in the same group as flying fish originating from the South China Sea.

Keywords: Cheilopogon, DNA, Exocoetidae, flying fish, Indonesia

INTRODUCTION

Makassar Strait plays an important role related to marine science and operational fisheries oceanography. It provides the unique ocean structures such as variability of the topographic feature, the main path of the Indonesia Throughflow (ITF) (Gordon 2005; Gordon et al. 2008), important upwelling zone associated with the southeast monsoon winds (Hendiarti et al. 2005; Atmadipoera and Widyastuti 2014) and dynamics of the oceanic fronts (Hidayat et al. 2019). This area is also characterized by spatial distribution locations of both cyclonic and anti-cyclonic eddy (Hidayat et al. 2019). South Sulawesi Province, Indonesia is an exporter of single flying fish eggs *Hirundicthys oxycephalus* and *Cheilopogon cyanopterus*, thus making this commodity one of the prima donna of the sector. Fish in addition to shrimp production, since 1969 the export of *H. oxycephalus* and *C. cyanopterus* flying fish eggs from Sulawesi to Japan has been started, and until now these eggs have become increasingly popular and are considered one of the special foods. Production of flying fish eggs *H. oxycephalus* and *C. cyanopterus* in the period 1977 to 2000 ranged from 72.2 to 87.5 tons, with an average production of 308.1 tons per year. Egg collection is increasing due to higher demand, resulting in decreased egg production and fish production (Syahailatua 2006).

Flying fish (Exocoetidae) are economically important fish in the Indonesian capture fisheries sector with high species diversity. The high diversity causes difficulties in the identification process of each species. Generally, flying fish have a standard length of 38 cm. Flying fish, including pelagic fish, which live on the open ocean's surface, can jump out of the water and glide for great distances. Flying fishes are members of the family Exocoetidae (Parin 1996; Carpenter and Niem 1999; Carpenter and Niem 2001), composed of 71 valid species (Wu et al. 2017), 18 of which were found in Indonesia (Robins and Ray 1986; Shen 1993; Sommer and Poutiers 1996; Parin 1999; Carpenter and Niem 2001; Riede 2004; Febyanty and Syahailatua 2008; Ferdiansyah and Syahailatua 2010; Fricke and Wantiez 2011). Flying fish, is one of important species in South Sulawesi waters, especially in Makassar Strait and Flores Sea. This species is familiar to the local coastal communities as one of fish protein sources and its highly valued eggs for export. On the contrary, the wild stock species has been left unmanaged and tends to show signs of overfishing, indicated by the decrease of population, abundance, and catch per unit of effort (CPUE) (Nessa et al. 1993; Ali 2005). Other indications of its population stress are shown by the changing of biological reproduction such as the decrease of body length, increase the fecundity but decrease the egg diameter, and earlier spawning period (Ali 2005).

Based on previous research, there were several flying fish species found in the waters of the Makassar Strait, namely *Hirundichthys oxycephalus* (Ali 2005; Febyanty and Syahailatua et al. 2008; Indrayani et al. 2020), *Parexocoetus mento* (Febyanty and Syahailatua 2008),

Cheilopogon cyanopterus (Febyanty and Syahailatua 2008), *Cheilopogon spilopterus*, *Cheilopogon abei*, *Cypselurus poecilopterus* (Indrayani et al. 2020). The distribution of different flying fish is highly influenced by ecology. For example, one flying fish species and another are prefer a different or the same coastal ecosystem due to reproductive biology, egg, larvae morphology, etc. (Parin 1961; Parin 1968; Collette et al. 1984). However, several studies that have been carried out in the Makassar Strait waters on the types of flying fish that have been collected, have not been determined morphologically and meristically. So, errors from identifying flying fish species can occur due to the many morphologically similarities between species. The individual fish of the same species at the age stage different species often differ from each other apart from individuals of different species at the same age stage (Jayakumar et al. 2019). Research on flying fish DNA has been carried out in several countries including Jayakumar et al. (2019), this study reports the first findings of *Cypselurus opisthopus* based on DNA identification with CO1 primers in the southeast Arabian Sea based on one specimen caught by commercial trawlers (Lewallen et al. 2016) molecular identification of Exocoetidae based on Cytochrome B Mitochondria (1137bp) and RAG2 (882 bp) in Australian waters; (Gordeeva and Shakhovskoi 2017) reported the results of a study of 5 species from the Exocoetidae family in South Atlantic waters with CO1 primary. Based on this, we use molecular markers as one way to identify the types of flying fish caught in the Makassar Strait.

Genetic variation is an important feature of a population not only for short-term fitness of individuals but also for long-term survival of the population by which it allows adaptation of fish to a changing environmental condition. Genetic diversity is also similarly important for farmed populations which allows selective breeding and preventing loss of fitness due to inbreeding depression. Genetic variability can be determined by morphological characters (morphometric analysis), allozyme electro-phoresis (protein pattern), and DNA fingerprinting. The genetic variations of four-wing samples of flying fish H. affinis have been studied at molecular DNA level (Gomes et al. 1998; Gomes 2000).

Currently, DNA fingerprinting technique is extremely efficient for detection of molecular genetic markers that may be utilized in assessment of genetic variation in fish, differentiation of stocks or populations and fisheries management. In more recent development in the detection of genetic polymorphisms is random amplified polymorphic DNA (RAPD). This technique, which is based on the polymerase chain reaction (PCR), amplifies random genomic segments with a single oligonucleotide primer of arbitrary sequence (Williams et al. 1990). In contrast to isozymes, RAPD provides a more arbitrary sample of the genome and generates essentially unlimited numbers of loci for use in genetic analysis (Fritsch and Rieseberg 1996). Genetic differentiation based on RAPD analysis in various fish species has been noted in many studies (Bielawski and Pumo 1997; Coccone et al. 1997; Koh et al. 1999; Liu et al. 1999; Imron et al. 2009; Imron et al. 2010; Moria et al. 2010; Mulyasari et al. 2010; Iskandariah et al. 2011; Lante et al. 2011; Nugroho et al. 2011).

The current study aims to provide a reference for the identification and validation of flying fish from the Exocoetidae family based on the results of the DNA barcode sequencing of the mitochondrial fragment of the cytochrome oxidase 1 (cox 1) gene in flying fish from the Indonesian Makassar Strait. We used the 650 bp region of mtDNA cytochrome oxidase. Subunit I gene (COI) to equalize the CO1 fragment was used in a previous note by Jayakumar et al. (2019). These findings will be practically used for species validation and identification references in the future. The absence of a flying fish gene Exocoetidae from the genus *Cheilopogon* originating from Indonesia in GenBank provides an opportunity for this study to produce the first record. A good management strategy and the initiation of breeding program of the flying fish perhaps can be suggested as the solutions to these ever-growing problems. For that reason, the genetic data of this species is very important as baseline data for its future management.

MATERIALS AND METHODS

Study area

Flying fish specimens were collected from the local fishermen in Majene District, West Sulawesi Province, Indonesia. The location of random sampling is based on the fishing position of gillnet fishermen in the Makassar Strait.

Sample preservation procedure

Sample preservation

Twenty (20) individuals were collected from the Makassar Strait fishing area, each of them with morphological characteristics for the flying fish Exocoetidae. Five good fish specimens were randomly taken for DNA isolation. Then the whole body of specimen was stored in 96% ethanol until it reached the laboratory (Hasan and Tamam 2019). All taken samples were stored under -20º C temperature in the Ichthyology Laboratory of Brawijaya University.

DNA isolation

A small portion of the right pectoral fin was excised and retained in TNESU8 buffer for molecular studies. DNA extraction used TNESU8 buffer containing a high concentration of urea and SDS from DNase to digest DNA in the sample. Four hundred (400) l of TNESU8 was added with 100 mg of fish fin samples in a sterile microtube (Kusuma et al. 2016). Genomic DNA was extracted using the phenol method, a procedure described by Asahida et al. (1996). Twenty (20) µl of 20 mg/ml proteinase K slock solution was added to the DNA sample suspension, and the sample was shaken at 150 rpm at 37°C overnight. Subsequently, 50 µl of NaCl5M and 500 µl of phenol/chloroform (1:1) were added, and sample vortexed for 5 minutes. The suspension was centrifuged at 12000 rpm at cold temperature for 10 minutes. Three hundred (300) µl of chloroform/isoamyl alcohol (24:1) was added to 100 µl of the supernatant, and the result was centrifuged for

10 minutes at 12000 rpm at cold temperature. DNA binding was carried out by adding 1000 µl of cold 96% ethanol to 100 µl of supernatant. The suspension was vortexed for 5 minutes and inserted into a GD column, which was already attached to the collection tube. The suspension was centrifuged for 1 minute at 5000 rpm at a cold temperature. Precipitation was completed by removing the filtrate from the collection tube. The collection tube was washed with 800 µl of cold 70% ethanol and dried before reattaching to the GD column. Subsequently, the collection tube was centrifuged at 12000 rpm at a cold temperature for 5 minutes. In the results of this last centrifugation, remaining liquid was taken by removing the collection tube. The resulting pellets were dissolved in 200 µl Tris- EDTA buffer and stored in a freezer.

DNA sequencing

Molecular confirmation of the species identification was carried out by using partial mtDNA cytochrome oxidase sub-unit I gene (COI). Genomic DNA was extracted by using Wizard Promega Purification Kit as per manufacturer's protocol. The partial COI gene was amplified by using universal primers COI F (5'-TCAACCAACCACAAAGACATTGGC AC-3') and COI R (5'-TAGACTTCTGGG TGGCCAAAGAATCA-3') (Ward et al. 2005). The amplifications were performed in 25 µL reactions containing 10× assay buffer (100 mM Tris, 500 mM KCl, pH 9.0) with 20 mM MgCl2, 10 pmoles of each primer, 200 µM of each dNTP, 0.25 U TaqDNA polymerase and 25 ng of template DNA. PCR conditions were used as follows: initial denaturation at 95°C for 5 min, denaturation 94°C for 30s, annealing 54°C or 45s, extension 72°C for 1 min (30 cycles) followed by a final extension for 10 min at 72°C. PCR products were sequenced bidirectionally.

Data analysis

The sequence data were translated into amino acids to confirm the absence of premature stop codons. The forward and reverse sequences were edited by using the Chroma 2.6.6 program, the consensus was drawn up by using the Ugene 1.32 program, and the comparison sequences were applied by using the Mesquite program. DNA sequences developed in the present study and already available sequences for the species and related species in NCBI were aligned and edited by using MEGA version 10 (Kurniawan et al. 2021). Phylogenetic and molecular evolutionary were analyzed by using Kimura 2-parameter method (Kimura 1980) and were conducted by using MEGA version 7.0 (Kumar et al. 2016). The sequence of Decapterus ruselli was used as outgroup for phylogenetic analysis.

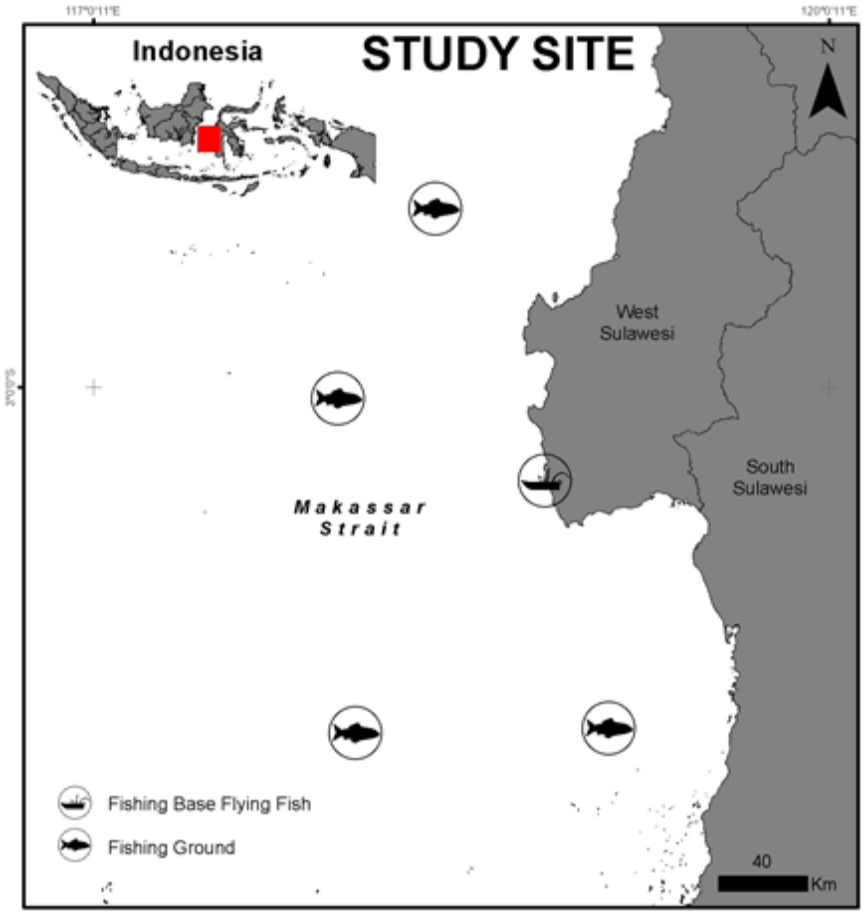

Figure 1. Location of sampling in Majene District, West Sulawesi Province, Indonesia

RESULTS AND DISCUSSION

Molecular identification and barcoding DNA

The molecular identification of flying fish originating from the Makassar Strait showed that the collected samples had 99.10% and 100% similarities with the species *Cheilopogon nigricans* (Table 1) originating from the South China Sea. The alignment analysis result showed that the amplicon sample sequence had similarities with the *C. nigricans* CO1 gene sequences of 100% (Accession no. MH638695.1) and 99.10% (Accession no. KU360275.1). Based on these similarity figures, it can be concluded that it is true that the sample used in this study is the CO1 oxidase gene from flying fish (*C. nigricans*).

Tree phylogeny

Reconstruction of the phylogeny tree was made from the gene *control region* mtDNA sequences downloaded from *GenBank* (Table 2). The data which were downloaded from *GenBank* came from various locations. This was done to determine the distribution of flying fish caught in the waters of the Makassar Strait, and the reconstruction of phylogeny trees was also carried out to determine the genetic relationship between species. Genetic and phylogenetic identification of the CO1 gene from each fish specimen was successfully amplified, resulting in a clear and band-specific appearance. There are currently seven nucleotides for the species *C. nigricans* recorded on NCBI, with four records from the South China Sea and three records from the South Atlantic (Table 2). This suggests that *C. nigricans* nucleotide records from the Makassar Strait are the first records. The CO1 sequences were truncated to the same length as those from GenBank, so the final dataset for phylogenetic reconstructions consisted of sequences of 650 bp for 8 specimens.

To determine the level of kinship between flying fish samples which was used in this study, tree analysis was carried out phylogenic. Phylogenic Tree can represent hypothesized evolutionary relationships among groups of organisms. Phylogenic tree was reconstructed by aligning the study sample sequences (2 samples). The reconstruction of phylogenic tree using the MEGA program with the UPGMA model (Figure 3). The phylogenic tree analysis results showed that the MKS1 and MKS2 samples were in the same group with a genetic distance of 0.0062, which showed that the two isolates had a very close kinship.

Table 1. Similarity specimen

Sample	Query cover	Identity	Species validation	Accession
Makassar 1	95%	100%	*Cheilopogon nigricans*	MH638695.1
Makassar 2	96%	99.10%	*Cheilopogon nigricans*	KU360275.1

Table 2. Genetic record of *Cheilopogon nigricans* in GenBank (NCBI 2020)

GenBank accession number	Gene	NCBI Released time	Location
MH638695.1	cytochrome oxidase subunit I (COI)	02 June 2019	South China Sea
MH638713.1	cytochrome oxidase subunit I (COI)	02 June 2019	South China Sea
KU360275.1	cytochrome oxidase subunit I (COI)	28 February 2017	South Atlantic
JF493131.1	cytochrome oxidase subunit I (COI)	25 July 2016	South Atlantic
MH638747.1	cytochrome oxidase subunit I (COI)	02 June 2019	South China Sea
KU360277.1	cytochrome oxidase subunit I (COI)	28 February 2017	South Atlantic

Table 3. The distance of *Cheilopogon nigricans* sequences from Makassar strait with other species in *Cheilopogon* genus on CO1 gene

Spesies	1	2	3	4	5	6	7	8
*C.nigricans*_Mks (1)								
*C.nigricans*_Mks (2)	0.0062							
MH638695.1 *C.nigricans*	0.0046	0.0031						
KU360275.1 *C.nigricans*	0.0062	0.0046	0.0015					
MN392929.1 *Decapterus russelli*	0.1973	0.1953	0.1913	0.1933				
MH638713.1 *C.nigricans*	0.0062	0.0046	0.0015	0.0031	0.1933			
KU360277.1 *C.nigricans*	0.0078	0.0062	0.0031	0.0046	0.1893	0.0046		
JF493131.1 *C.nigricans*	0.0093	0.0078	0.0047	0.0031	0.1892	0.0062	0.0078	
MH638747.1 *C.nigricans*	0.0093	0.0078	0.0047	0.0062	0.1892	0.0062	0.0078	0.0093

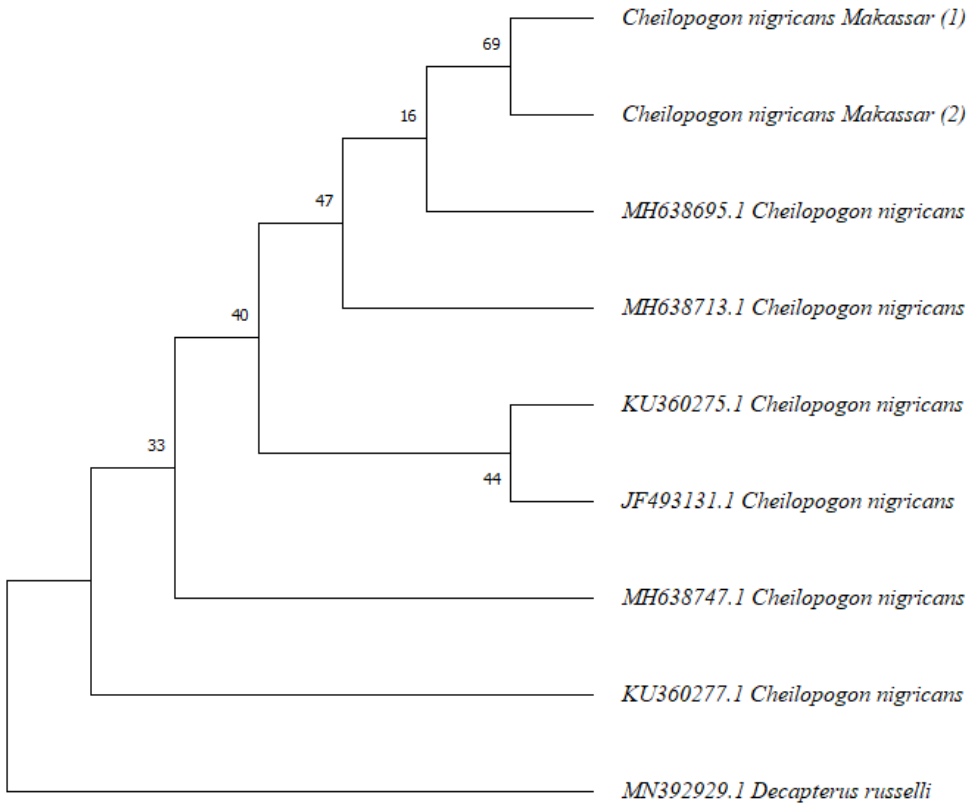

Figure 2. Maximum likelihood phylogenetic tree with 1000 bootstrap replicates. Specimens from the Makassar Strait were grouped together with a strong bootstrap value (100%). Species *Decapterus ruselli* used as an outgroup

Discussion

The taxonomy of flying fishes is ambiguous due to limits of diagnostic characters which distinguish genera, overlapping diagnostic characters between certain species, morphological differences of juveniles from adults of the same species, especially in color pattern and presence of barbels and probability of species yet to be described (Gordeeva and Shakhovskoy 2017). In order to support the limited information on taxonomic features of the species, molecular analysis is incorporated. Mitochondrial DNA (mtDNA) is widely used for phylogenetic studies because its evolution is faster than evolution of nuclear DNA, and it can be used for differentiating closely related species (Tamura et al. 1993). Hebert et al. (2003) demonstrated the utility of the COI gene in species delineation. COI gene sequence is appropriate for this role because, its mutation rate is often fast enough to differentiate closely related species and also because its sequence is conserved among congeners. It should be noted, however, that Gordeeva and Shakhovskoy (2017) were not able to distinguish two closely related species of flying fishes using COI. In the present study, molecular analysis using mitochondrial COI gene revealedthat the collected specimen belongs to *C. opisthopus*. The genetic divergence values of present specimen and GenBank specimens of *C. opisthopus* are within well acceptable level for intraspecific variation in fish species (Jayakumar et al. 2019).

The morphological characters of flying fish in Makassar Strait have a total length which is not different too much from those in other waters. De Croos (2009) found the length distribution of flying fish with an average total length of 19.5 cm to 39.2 cm in Kandakuliya, Sri Lanka. The maximum total length of flying fish can reach 45 cm and generally reaches 38 cm (Carpenter and Niem 1999). If we look further, the total length of the flying fish in the Makassar Strait is still small fish that are experiencing growth and development. The flying fish in the waters of the Makassar Strait in this study proved to be fish from the Exocoetidae family. The value of the genetic distance between species with data from GenBank is still very low, so it can be concluded that the used samples of isolate flying fish still have a very close relationship with *C. nigricans* species from the South China Sea. According to Tallei et al. (2016), the less the value of the genetic distance between two organisms, the closer the relationship between them. Akbar and Aris (2018b) said that the linked genetics, among other things, shows that all populations are closely related. The closeness of the kinship between populations may be caused by interpopulation having the same parentage and genetic proximity (Kusuma et al. 2016; Akbar and Aris 2018b). The evolutionary distance is calculated using the Maximum Composite Likelihood method (Tamura et al. 2011) and is in units of the number of basic substitutions per site. The phylogeny tree analysis results show that the Indonesian Makassar Strait specimens are in the same group as *C. nigricans* from the South China

Sea while *C. nigricans* from the North Atlantic are in a different group. The difference in genetic distance between the two sample groups is presumed because geographically, there are boundaries that allow it to occur. Separation of groups from one another, for example, straits or islands (Kurniawan et al. 2021) or perhaps migration due to environmental suitability and food availability. The cytochrome gene's success was amplified from flying fish samples with a nucleotide length of 650 bp. The amplicon sample sequence was similar to the CO1 gene sequence of *C. nigricans* isolates by 99.10% and 100% through BLAST alignment analysis and the close relationship between flying fish samples. Flying fish obtained in the Makassar Strait showed low genetic distance values in the phylogenic tree analysis, namely 0.000-0.005. The genetic variation of flying fish (*C. nigricans*) in the Indonesian Makassar Strait waters consists of the same population group as the *C. nigricans* specimens from the South China Sea. Regarding the results of this study, this flying fish species is included in the IUCN Red list database in a near-threatened status so that it requires immediate action in capture fisheries management and conservation actions. Domestication can be done to support efforts to save endangered species through ex-situ conservation strategies. In addition, further studies on habitat and biological characteristics are also urgently needed to support fisheries management efforts and in-situ conservation activities.

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