Molecular Identification of Mudskipper Fish from Bipolo and Oesapa, Kupang, East Nusa Tenggara, Indonesia

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PREFACE

Praise and gratitude to Allah SWT. who has bestowed all blessings and opportunities for the author to complete this Thesis entitled "Molecular Identification of Mudskipper Fish from Bipolo and Oesapa, East Nusa Tenggara, Indonesia. This thesis is composed in partial fulfilment of the requirements for accomplishing for the Bachelor of Science (B.Sc) Degree in Biology Department, Faculty Science and Mathematics, Diponegoro University. This thesis is discussing the molecular identification of Mudskipper fish from Bipolo and Oesapa, East Nusa Tenggara, Indonesia and their relationship with other mudskipper species all across Indonesia. This study aimed to identify mudskipper species taken from Bipolo and Oesapa, East Nusa Tenggara, Indonesia using DNA barcoding targeting the COI gene and to analyze their phylogenetic relationship with other mudskippers from GenBank. The research conducted through a molecular approach, where the mudskipper species are identified through a process including DNA extraction, PCR, Gel electrophoresis, DNA barcoding, and genetic analysis using MEGA X to analyse the phylogenetic relationship. The result includes a phylogenetic tree to know and understand the phylogenetic relationships of Mudskipper fish from Bipolo and Oesapa, East Nusa Tenggara and other Mudskippers from GeneBank. This thesis may not be a masterpiece, nor near perfection. This research is open for future interpretation and the author hopes that this thesis can be useful for fellow students and readers

ABSTRACT

Rena Galby Andadari, 24020120190038, Molecular Identification of Mudskipper Fish from Bipolo and Oesapa, East Nusa Tenggara, Indonesia. Under the supervision of Dr.rer.nat Anto Budiharjo, M.Biotech and Ni Kadek Dita Cahyani, S.Si, M.Si, PhD

Indonesia hosts a variety of unique marine and terrestrial species, including mudskippers. Despite their abundance, studies on the genetic diversity of mudskippers from Indonesia are limited and there is a noticeable lack of genetic data in GenBank. This study aims to identify mudskipper species taken from Bipolo and Oesapa, East Nusa Tenggara, Indonesia using DNA barcoding targeting the COI gene and to analyze their phylogenetic relationship with other mudskippers from GenBank. Methods used in this study includes DNA extraction using Chelex 10% and CTAB method, followed by PCR method with two sets of primers JgHCO and JgLCO as well as FishF2 and FishR2. Data analysis was conducted using BLAST and MEGA XI. BLAST analysis revealed *Periophthalmus argentilineatus* and *Periophthalmodon schlosseri* with a similarity of 99.97% and 90.67% respectively. However, phylogenetic analysis reveals a clade of initially identified *Periophthalmodon schlosseri* separated from other known mudskipper species with a notable 10% genetic distance. This suggests the presence of a potentially new, undiscovered species yet to be recorded in GenBank. Further studies incorporating morphometric analysis are needed to clarify this new potential species and a larger sampling area from a broader geographic range are necessary to understand genetic diversity of Mudskippers in Indonesia.

***Keywords**:* mudskipper, DNA barcoding, COI gene, genetic diversity

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I. INTRODUCTION

1.1.Background

Indonesia is well known as a mega biodiversity country for both marine and terrestrial animals. One of the most unique animals is Mudskipper fish, an amphibious fish known amongst local communities as ‘ikan blodo cina’, ‘ikan blodok’, ‘lunjah’, ‘glodok’, ‘layar’, ‘tembakul’, ‘tempakul’ and ‘ikan glodok’ (Saanin, 1968). Mudskippers are found along the Indo-West Pacific region, from East Africa to the Samoa and Tonga Islands, and along the Atlantic coast of Africa (Murdy, 1989). This species mostly inhabit estuaries, muds and mangrove swamps and shows their unique characteristics through skipping out of the water, climbing on mangrove trees, and sticking on rocks. The base of its pectoral fins is strongly muscular, so it can be bent and function like an arm for creeping and crawling. They have distinct flat faces with protruding eyes in the dorsal part of the head (Mukharomah, 2016).

Mudskipper fish species are cryptic species from the family Gobiidae (Thacker, 2003) which shows similar morphological features but is genetically different among the species and are often confused as one another due to their similarity of morphological characters, making their identification and differentiation a complex and time-consuming task (Arisuryanti et. al., 2018). Traditionally, fish species identification is often done based on their morphometrics and meristic characteristics, including

body shape, size and colours of scale or body, and number and type of fin (Strauss & Bond, 1990). However, in certain instances, solely relying on morphological characteristics for identification may be insufficient or unreliable due to several reasons. Some species of fish may exhibit similar characteristics or small differences between species (Teletchea, 2009). In other cases, morphological characteristics have been eliminated or altered before and during sample processing and it may become infeasible to rely on morphological characteristics alone (Strauss & Bond, 1990). For instance, a study from Callejas & Ochando (2001) showed the difficulty of identifying Barbus species of the Iberian Peninsula based on only morphological features because of the common interspecific variation. Arisuryanti et al (2018) also reported the case of identification difficulties in Mudskipper fish and even referred to it as "almost impossible task" to identify only based on morphological traits.

Several studies of Mudskipper identification using morphological traits in Indonesia have been done including from Mukharomah, et al (2016) who identified two species of Mudskipper (*Periophtalmus gracilis* and *Periopthalmus variabilis*) from Makarti Jaya and Sungsang estuaries through morphometric and meristic and descriptive methods. The result shows the two species of mudskipper have very similar morphological characteristics of body shape and scale type. The only characteristic differentiating the two species is the eye diameter of *Periophtalmus gracilis* being bigger and more prominent compared to *Periophthalmus*

variabilis. Another study from Baderan et al (2023) done in the mangrove ecosystem of coastal Bay of Tomini, Boalemo, Gorontalo Province. 561 individuals collected were identified using morphometric, meristic and comparing the results with identification keys. The result revealed five species found with high similarity in morphological features, namely *Periophthalmus argentilineatus*, *Periophthalmus kalolo*, *Periophthalmus malaccensis*, *Periophthalmus minutus*, and *Periophthalmus variabilis*.

The high similarity level of morphological features of Mudskipper emerging a problem in assigning proper names to mudskipper species, which in turn can impact conservation initiatives aimed at protecting these fish within their natural habitat. Therefore, molecular approaches offer a solution for this problem. Molecular approaches are the way forward especially in the form of DNA barcodes. The development of various molecular techniques that generate molecular markers has made it possible to accurately identify animals accurately with less time consuming. These techniques utilise differences at the level of the deoxyribonucleic acids (DNA) of the protein encoded by it. One of the widely used molecular approaches that can be used to identify the fish species quickly and accurately is DNA barcoding using the Cytochrome Oxidase Subunit I (COI) mitochondrial gene (Hogg & Hebert 2004). The COI mitochondrial gene has been accepted as a universal barcode to identify animals (Amin, 2013), and now considered highly preferable for the precise identification of fish

Despite the high rate of biodiversity amongst freshwater fish and high abundance of Mudskipper in Indonesia, studies investigating the DNA barcoding Mudskipper fish using COI gene marker in Indonesia is very limited and considered new as it just started less than 10 years ago by Dahruddin, et. al (2016). This study was conducted from the concern about the limited and uncertainty of Indonesian DNA barcodes library and database, especially for ichthyofauna. They noted that at that time Indonesia had very limited, unorganised, and non-inclusive databases, while the presence of proper, accurate, and inclusive databases are important in the development of taxonomic and phylogenetic studies in the future. Despite facing challenging difficulties, 1046 sequences belong to 159 species, 107 genera and 50 families of freshwater fishes from several locations along Java and Bali were successfully identified and deposited to GenBank, and 30 sequences of them includes 4 species of Mudskipper namely *Periophthalmus argentilineatus*, *Periohthalmus kalolo*, *Periophthalmus novemradiatus*, and *Boleophthalmus boddarti*. These sequences are notably being the first sequences of Mudskipper fish COI region from Indonesia deposited in GenBank, and helped initiate the development of DNA barcoding, taxonomic and phylogenetic studies of Mudskipper in Indonesia. However, difficulties persisted due to the limited availability of locations available for conducting phylogenetic studies

Until years later, Arisuryanti, et al. (2018) conducted a study on mudskipper fish collected from Bogowonto Lagoon and identified 2 species of Mudskipper from the location, namely *Periophthalmus argentilineatus* and *Periophthalmus kalolo*. Further phylogenetic analysis revealed that *P. argentilineatus* from Bogowonto Lagoon is closely related to *P. argentilineatus* from Pandeglang, and *P. kalolo* is closely related from *P. kalolo* from Cilacap. Phylogenetic studies of Mudskipper fish in Indonesia continue revealing a lot of new findings, as one from Arisuryanti, et. al. (2021) investigated Mudskipperfish from Tekolok Estuary, West Nusa Tenggara and suspected the presence of cryptic species in *P. argentilineatus* from the separation of two distinct clades with a relatively huge genetic distance that showed a distinct geographicpatterns between eastern and western area of Indonesia. However, as the database kept increasing, Arisuryanti et. al (2023) investigated *P. argentilineatus, P. kalolo* and *P. novemradiatus* from Baros Beach and Pasir Mendit Beach, Jogjakarta and found other evidence that did not support the initial theory of separation by geographical region happening in the population structure of *P. kalolo* and *P. argentilineatus*.

Despite the growing interest in DNA barcoding and phylogenetic studies of Mudskipper fish in Indonesia, it still can be considered very limited and there are still gaps to be understood and fill, such as further analysis for reconfirmation of suspected cryptic species existed in

Periophthalmus argentilineatus, and greater coverage of sampling areas need to be done to give more understanding about the probability of geographic patterns of Mudskipper fish in Indonesia. Most importantly, greater coverage of sampling areas and greater total of individuals identified can help develop a proper and better database of Mudskipper in Indonesia that can help provide an advanced understanding of the species, evolutionary relationships, and conservation plan in the future.

1.2. Problem Formulation

1.2.1. How is the process of identifying mudskippers using DNA barcoding technique to ensure accurate species identification?

1.2.2. How is the molecular diversity of mudskipper species based on the conducted phylogenetic analysis?

1.3. Objective

1.3.1. To implement the process of identifying mudskipper species using DNA barcoding techniques.

1.3.2. To analyze the molecular diversity of mudskipper species in Indonesia using a phylogenetic tree approach.

II. LITERATURE REVIEW

2.1.Mudskipper

Mudskippers belong to the Oxudercinae subfamily, which is a subgroup of the Gobiidae family. The Oxudercinae family comprises 40 species of elongate gobiid fishes that spend most of their life on land (Murdy, 2011). Many Oxudercinae are endemic to geographic areas. They can be found in bottom intertidal areas and mangrove swamps in the Indo-West Pacific and tropical west Africa (Graham, 1997). Some but not all can be referred as Mudskipper. Amongst 40 species distributed in between 10 genera, only 32 species found in 5 genera are typically referred to as "mudskippers." These five genera are *Boleophthalmus, Periophthalmodon, Periophthalmus, Scartelaos, and Pseudapocryptes* (Steppan, 2022).

Figure 2.1. Mudskippers resting on mudflats in Banyuasin peninsular, South Sumatera, Indonesia. (Pormansyah, et al., 2019)

According to Jaafar and Murdy (2017), mudskippers have been attracting a lot of fascination from scientists due to their unique

characteristics. They spend most of their life outside the water moving with speed and agility over the muddy substrate, which earned them the common name "Mudskippers". They are morphologically characterised by having rounded and elongated body shapes with length vary from 7 to 30 cm (Purwaningsih et al., 2013), protruding eyes equipped with a good aerial vision (You et. al., 2018) that can be used to stick up out of the water while their body hidden laying waiting for their prey (Ansari et al., 2014), muscular base of bendable pectoral fins functioned like arms for 'walk', creeping and crawling on land (Swanson and Gibb 2004), two dorsal fins and pelvic fins that are placed forward under the body (Murdy, 1989), and gills used to store some water that can help them to stay moist in and outside of the water (Polgar & Crosa, 2009). These morphological characteristics acquired them the ability to successfully adapt to terrestrial environments.

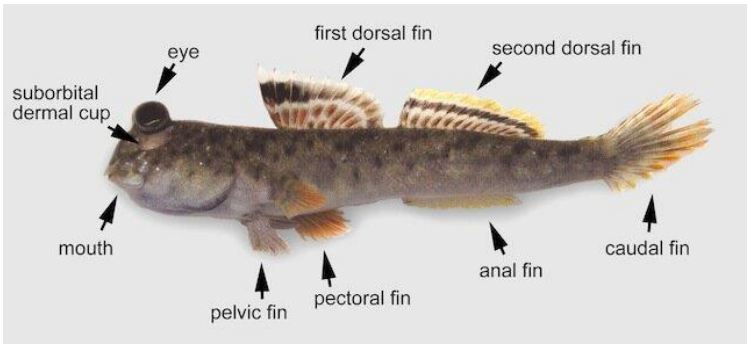

Figure 2.2. External anatomy of mudskipper *Periophthalmus sp* (Polgar, 2010)

Mudskipper fish are unique inhabitants of coastal regions and mangrove swamps. According to Kordi & Ghufran (2012), in the mangrove ecosystem, scientists classified fish inhabitants into four categories: true residents, high tide

high-tide visitors, and seasonal visitors. The only true resident species is the mudskipper. They complete their entire life cycle within the mangrove environment, often sharing their habitat with digging crabs. They tend to live in made up polygonal territories of about one meter in size, aimed to reduce an aggression between neighbouring animals, searching for planktons to feed (Al-Behbehani & Ebrahim, 2010) and protect themselves from threats and predators including fishes, shorebirds and water snakes (Clayton, 1993).

The behaviour of mudskippers is intricately related to the fluctuations of the tides. These fish have adapted to living in areas that are sometimes submerged during high tide and exposed during low tide (Muhtadi et al., 2016). They are highly active when out of the water, engaging in activities like feeding, social interaction, and defense (Chen et al, 2008). When mudskippers sense danger, they can quickly hurdle into the open sea or swiftly move onto muddy land using their strong pectoral fins. They are capable of digging burrows up to one and a half meters deep, often right beside the walls of their territory. According to Polgar et al (2011), some mudskipper species have adopted terrestrial habits and spend about 90% of their time out of the water. Others emerge only at night to graze on algae, which allows them to evade predators and avoid oxygen-poor conditions that can occur in pools during very low tides.

Figure 2.3. Natural habitat and territorial behaviour of mudskippers at Red Sea coast of Duba, Tabuk, Saudi Arabia. (Ansari et al, 2014)

Since mudskippers are very active in the coastal environment and always in direct contact with various pollutants directly discharged in coastal waters, they are commonly used as biomarker and bioindicator of pollution in coastal ecotoxicology studies (Polgar, 2011). The first study done in this topic using Mudskipper as biomarker can be traced back to Uchida et al (1971) who investigated heavy metals content in the Ariake Sea Japan through mudskipper *Boleophthalmus pectinirostris* and other organisms. Prior to that, a lot of studies in the same topic emerged including by Ahmed et al (2011) who investigated metal content in Sundarbans mangrove forest, Southwest Coast of Bangladesh and found metal (Fe, Cu, Zn, Cd) accumulated in Mudskippers *Periophthalmus sp* taken from that area. Another one by Nwakarma & Hart (2012) investigated the levels of barium content from oil-based drilling fluid on the muscle tissue of Niger Delta Mudskipper (*Periophthalmus barbarus*).

Besides being a significant ecological value as biomarkers, mudskippers also hold economic value as they are consumed and farmed in a lot of places in the world. Although they are not commonly considered a primary food source, people from several countries including Indonesia (Arisuryanti et al, 2018), China, Taiwan, India, Japan, Korea, Philippines, and Vietnam consume and process mudskippers as medicine and food sources to fulfil daily protein needs (Saritha et al, 2014). While in Nigeria, besides being sources of food, mudskippers are widely used by fishermen as bait to catch other commercially valuable species (Akinrotimi, 2013). However, the consumption of mudskippers should be limited and monitored thoroughly considering the probability of contaminants contents due to their role as bioindicators in the coastal ecosystem.

2.2.Molecular Identification Technique

Species identification is essential for researchers and people interested in expanding knowledge, recognizing and describing biodiversity, and establishing hierarchical relationships among different species. This necessity in biological investigations has led scientists to constantly developing numerous species identification methods that can vary depending on the type of organism (Vidal et. al, 2021). In animals, species identification traditionally used to rely on meristic and external morphological features. For instance, in fish the features include body shape, body pattern and colour, scale size and colour, and fins number and location.

Some characteristics like behavioural and physiological character such as habitat preferences, breeding seasons and growth rates were needed if necessary (Saritha et al, 2013). However, species identification has some limitations due to the lack of morphological features, the organisms showing considerable intraspecific variations or small differences between species (Strauss and Bond, 1990).

In the beginning part of the 20th century, along with the advancement of technology and genetics, species identification underwent a transformation from morphological features to leaning towards exploring molecular identification using information within cells (Saritha et. al, 2013). Early molecular techniques predominantly revolved around the separation and characterization of specific proteins utilising electrophoretic methods like isoelectric focusing (IEF) (Rehbein, 1990), capillary electrophoresis (CE) (Kvasnicˇka, 2005), high-performance liquid chromatography (HPLC) (Hubalkova et al. 2007), and immunoassay systems such as the Enzyme-Linked ImmunoSorbent Assay (ELISA) (Asensio et al. 2008). While these techniques possess distinct advantages, limitations arise due to factors like the rapid degradation of proteins under adverse conditions and their irreversible denaturation during food processing or extreme conditions (Saritha et al, 2013). As a result, attention has shifted towards harnessing DNA as a valuable source of information for species identification. In contrast to protein analysis, DNA-based methods have prominently emerged over the past decade (Teletchea. 2009) as a promising alternative.

Compared to proteins, DNA molecules have advantages due to some characteristics including: DNA molecules are relatively stable and have long-life that can remain intact despite having to withstand extreme conditions such as food processing or extreme weather (Nsubuga et al, 2004). Additionally, DNA are convenience to harvest on almost all biological tissues or fluids with both nucleated and non-nucleated cells with plastids or mitochondria (Smith et al, 2004), allowing it to be found from almost all kinds of biological substrates such as tissues, blood, saliva, faeces, and milk (Rogers et al, 2007). DNA also can offer more comprehensive information compared to proteins, primarily because of the redundancy in the genetic code and the existence of significant non coding sections (Teletchea, 2009). The discovery regarding DNA advantages over protein has sparked ongoing advancements in molecular biology, notably contributing to the widespread utilisation of PCR as a species identification method in recent years (Saritha et. al, 2013).

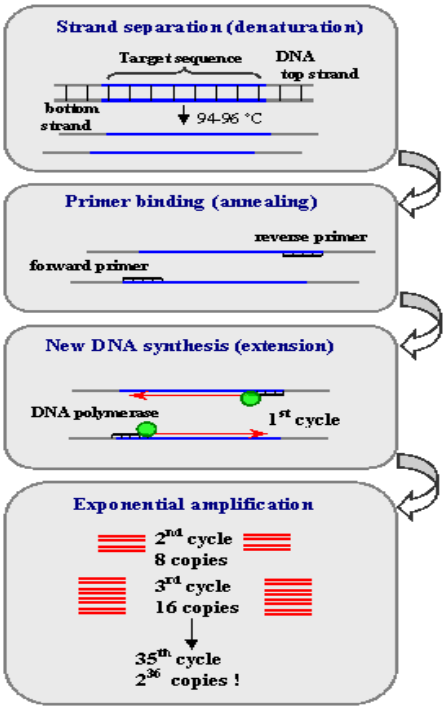

Figure 2.4. Process of PCR that includes 3 or more steps, namely denaturation, annealing, extension and amplification. (NIH, 2022)

PCR is the primary method of animal species identification that allows the amplification of any selected DNA sequence. According to Canene-Adams (2013), PCR involves four main components: a double-stranded DNA template, primers that bind to the template, deoxyribonucleotides (dATP, dCTP, dGTP, dTTP), and a stable DNA-dependent DNA polymerase. The PCR process consists of several steps, including denaturation, annealing, and extension, which are repeated through more than 25 cycles using an automated thermal cycler (Verma et al, 2014). During denaturation at 94°C, the double-stranded DNA template is separated into single-stranded DNA. Annealing, usually occurring at a lower temperature (between 45 to 60°C), allows primers to bind to their complementary sites on the template DNA.

Finally, the extension phase, typically at 72°C, varies based on the primers used. Following primer attachment, DNA-dependent DNA polymerase synthesises a new DNA fragment. Each three-step cycle results in the production of two daughter molecules from a single parent DNA molecule (Malewski et. al, 2021).

2.3.DNA Barcoding

PCR-based methods are valuable for confirming whether a given sample belongs to a specific species; however, they are limited in their ability to precisely identify the species in question (Malewski et. al, 2021). To achieve species level identification, DNA barcoding is necessary and considered as one of the most robust and effective approaches. DNA barcoding is a growing concept coined by Hebert et. al (2003) that offers a highly accurate means of determining the exact species to which a sample belongs by primarily relying on the utilisation of molecular operational taxonomic units to differentiate variations intra species and interspecies. In the field of biodiversity and taxonomy, it has the potential to identify and discover species yet to be named, acknowledge undescribed cryptic species (Saritha et al, 2013) help assessing community structure and phylogenetic diversity (Fierer et al, 2012), and helps conservation management effort (Francis et al. 2010) making it an invaluable and effective tool.

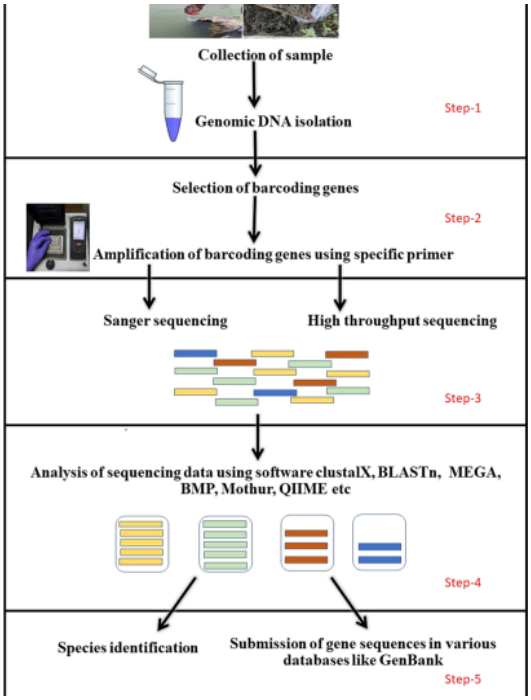

Figure 2.5. Schematic representation of the methodology for DNA barcoding (Antil et. al, 2022)

DNA barcoding seeks to identify a wide range of species using only a few selected gene regions typically consisting of short DNA sequences ranging from 400 to 800 base pairs in length (Malewski et. al, 2021), and then distinguishing it from other molecular methods that rely on specific markers like COI for animals, rbcL, matK, and ITS for plants, ITS for fungi, and 16S rRNA gene for bacteria and archaea (Lethcuman, 2018). According to Shektosov et al (2019), DNA barcoding technique involves several steps, including sample collection from the field, DNA extraction, gene amplification using universal primers, sequencing through methods like Sanger or High-throughput sequencing, assessing diversity, and analysing data with software such as MEGA, Mothur, DNAsp, and Qiime2.

After a library of DNA barcodes from known species is built, the DNA barcodes from unidentified samples are compared to the known barcodes using a matching algorithm typically used to find the most similar sequence in the database and match the unknown sample to a known species (Mir et al, 2021). The Basic Local Alignment Search Tool (BLAST), provided through GenBank, is one such tool for searching for similarities between a query sequence and a sequence in the library (Shekhovtsov, 2019). Two other commonly used methods for measuring similarity are the Kimura-2-Parameter Distance and the Smith-Waterman Algorithm, which work similarly to BLAST for local alignment comparisons (Srivathsan & Meier 2012).

2.4. COI Gene Marker

Cytochrome c oxidase I (COI) also known as mitochondrial encoded cytochrome c oxidase I (MT-CO1) is a protein that is encoded by the mt-CO1 gene. In other eukaryotes, the gene is called COX1, or COI. Cytochrome c oxidase subunit I (CO1 or MT-CO1) is one of three mitochondrialDNA (mtDNA) encoded subunits (MT-CO1, MT-CO2, MT-CO3) of respiratory complex IV. Complex IV is the third and final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation (Kosakyan et al. 2012).

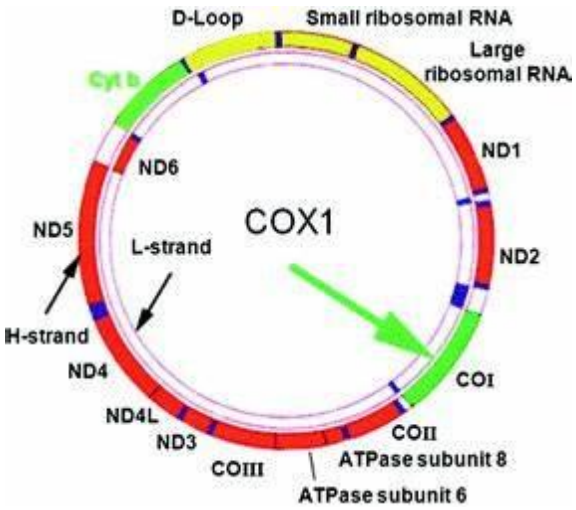

Figure 2.4 Location of COI gene in Mitochondrial Genome (Dhar et al., 2016)

The Cytochrome c Oxidase subunit I, often abbreviated as COI, is a crucial gene marker present in mitochondrial genes of animals that is frequently used in molecular biology and taxonomic studies. COI was first proposed by Hebert (2003) and has been accepted by CBOL (Consortium for the Barcode of Life) as a universal barcode to identify animal life on this planet (Malewski et al, 2021; Saritha et al, 2013; Kaur, 2015). Mitochondrial genes are maternally inherited and characterised by a very high substitution rate (several times higher as compared with the nuclear genome), as well as the absence of recombination, which make it easily distinguishable to track closely related species and makes it a perfect candidate for investigating genetic diversity and evolution. Moreover, the region of COI gene has a very robust universal primer and is approximately limited for 650 base pairs (bp) which is exactly the right length for a reliable reading of a standard Sanger sequencing (Folmer et al, 1994).

III. METHODS

3.1. Sample Collection and Preservation

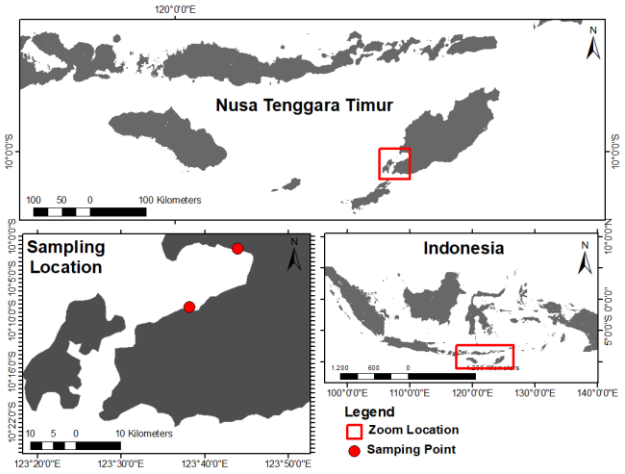

Figure 3.1 Sampling location of mudskipper fish. Samples were taken from Bipolo and Oesapa, East Nusa Tenggara, Indonesia

Total eight samples of unidentified mudskipper fish were collected from two different locations, Oesapa West Coast Mangrove Ecotourism, Kupang City, East Nusa Tenggara and Bipolo Village, Sulamu District, Kupang Regency, East Nusa Tenggara. The fresh mudskipper fish were captured using a net during low tide. Subsequently, sections of dorsal fin tissues were excised and placed into tubes containing 95% ethanol, closed tightly, labelled and stored at room temperature.

3.2. DNA Extraction

3.2.1. CTAB Methods

The CTAB method of DNA extraction started by rinsing off the ethanol from the sample with double distilled water (ddH2O) and

then drying it by gently patting on a tissue. Following this, the samples were dissected into smaller pieces, placed into microtubes, and combined with 600µL CTAB buffer along with 20µL proteinase K. The mixture was then incubated on a heating block set at 65°C overnight. After the incubation period, the samples were thoroughly mixed using a vortex until an even distribution was achieved. Next, cold chloroform (-4°C) was equally added to the sample volume, followed by mixing using a vortex and centrifugation for 20 minutes at 12,000 rpm. This centrifugation process resulted in the formation of three distinct liquid layers, comprising the supernatant, protein layer, and chloroform. The supernatant layer was transferred into a new microtube for further processing. Last step includes DNA precipitation that is carried out by introducing 600µL of isopropanol, followed by centrifugation for 15 minutes. Subsequently, the isopropanol layer was completely removed. To purify the DNA, 600µL of 70% ethanol (ETOH) was added, and the mixture was centrifuged for an additional 15 minutes. The ethanol was then removed, leaving the DNA pellet inside the microtubes. The tubes were air-dried by inverting them on a tissue. The final step involved elution by adding 50µL of AE buffer to the tube.

3.2.2. Chelex 10%

Chelex 10% extraction process started with preparing the solution by mixing double distilled water (ddH20) with chelating

resin in 1:10 ratio. Following this, sample tissue approximately 2 mm was broken down into smaller cuts and put into the tube containing 10% chelex and heated at 95°C for 45 min on a waterbath. The Chelex was then mixed using a vortex and centrifuged to separate the pellet and the supernatant. Supernatant was taken to another tube and ready to be used.

3.3. PCR (Polymerase Chain Reaction)

Genomic DNA was PCR amplified with the COI gene primer pair of Fish2 Forward and Fish2 Reverse (Ward, 2005) and a set of JgLCO1490 Forward and JgHCO2198 (Geller et. al., 2013). Master mix for the PCR reaction mixture consisted of 12.5 µL 2x MyTaq Mix Bioline, each 0.5µL of forward and reverse primer, 9.5µL nucleus free water, and 2.0µL DNA Template. 1 tube of negative control was added excluding the DNA template from the reaction mixture. Total volume for each PCR reaction was 25µL. DNA samples were amplified using BioRad Thermal Cycler started with pre-denaturation at 95°C for 4 minutes, continued by denaturation at 95°C for 30 seconds, annealing at 50°C for 4 minutes, extension at 72°C for one minute, post-extension at 72°C for 1 minute.

3.4. Gel Electrophoresis

The PCR products were run on a 2% agarose gel consisting of 40 ml Tris-acetate EDTA (TAE) 1x buffer, 0,8 g agarose powder, and Bioline SYBRSafe Gel Stain. The electrophoresis process was done at 100 volts for 45 minutes and the gel result then visualised under UV light for observation.

3.5. DNA Sequencing

The final DNA products were sent for DNA sequencing to First BASE Laboratories Sdn Bhd, Kuala Lumpur, Malaysia and P.T Genetika Science Jakarta, Indonesia using forward and reverse primers.

3.6. Genetic Analysis

Each sample was sequenced using both forward and reverse primers. The chromatograph results were observed manually using Chromas to check ambiguous bases and peaks. Then the COI sequence data was converted to FASTA format through Notepad++ and aligned in the MEGA X program using ClustalW in the default setting. The resulting sequences were compared to databases by the Basic Logical Alignment Search Tool (BLAST) program on GenBank at http://blast.ncbi.nlm.nih.gov/ to determine the species.

The construction of the phylogenetic tree was carried out using the MEGA X software, as described by Kumar et al. (2018). In this study, 10 COI mitochondrial sequences data from Bipolo and Oesapa, East Nusa Tenggara, Indonesia and 16 COI sequences of other Mudskippers sequences from Indonesia consists of *Periophthalmus argentilineatus*, *Periophthalmodon schlosseri*, and *Periophthalmus kalolo* were retrieved from GenBank. Total sequences with 597 bp alignment were analyzed using vertebrate mitochondrial code and built into a phylogenetic tree using Maximum Likelihood with HKY+G (Hasegawa-Kishino-Yano) method as recommended by MEGA best DNA model finder. *Boleophthalmus boddarti* accession number KU692382 was chosen as an outgroup.

IV. RESULT AND DISCUSSION

4.1. Molecular Identification of Mudskipper Using COI Gene Marker

Molecular identification is a process of identifying the unknown to known samples of specimens into a certain known taxon. Molecular identification methods include sampling, DNA Extraction, PCR amplification, electrophoresis and finally DNA Sequencing (Vijayakumar et al., 2019; Vidal et. al, 2021).

The DNA extraction isolate quality was assessed using a Nanodrop spectrophotometer to assess the quality of DNA samples before continuing to DNA amplification through PCR (*polymerase chain reaction*) process.

Table 4.1 Quality assessment of DNA isolate using nanodrop spectrophotometer

Sample ID	Extraction method	Nucleic Acid Conc.	Unit	260/280	Sample type
DBP012261	CTAB	33,2	ng/µl	0,99	DNA
DBP012261	Chelex 10%	47,8	ng/µl	2,52	DNA
DBP012262	CTAB	103,3	ng/µl	1,56	DNA
DBP012243	CTAB	93,52	ng/µl	0,90	DNA
DBP012245	CTAB	32,44	ng/µl	1.32	DNA
DBP012245	Chelex 10%	114,1	ng/µl	1,45	DNA
DBP012291	CTAB	17,0	ng/µl	1,70	DNA

DBP012259	CTAB	33,3	ng/µl	1,88	DNA
DBP012260	Chelex 10%	26,3	ng/µl	1,26	DNA

The assessment of DNA quality was done with a nanodrop spectrophotometer and DNA purity was determined by calculating the absorbance ratio 260/280. Nanodrop spectrophotometer works by corresponding to the absorbance at the wavelengths 230, 260 and 280 nm. This is in accordance with García-Alegría et al. (2020) and Gupta (2019) who stated that The 260/280 absorbance ratio is used to determine DNA purity and the presence of contaminants in the biological samples during the DNA extraction process. Absorption peak for nucleic acids is at ~260 nm. The A260/A280 standard good quality ratio is ~1.8 for double strand DNA (dsDNA).

Samples DBP012291 and DBP012259 had ratios closest to the ideal 260/280 for DNA purity, at 1.70 and 1.88, respectively. However, the other samples showed lower DNA quality. Specifically, DBP012261 (CTAB) and DBP012243 had the lowest ratios, at 0.90 and 0.99. This low DNA quality includes DBP012261 (Chelex 10%) despite having the highest ratio of 2.52. Low quality of DNA isolate can happen for several reasons such as degraded DNA from the biological samples. In this study, mudskipper samples were stored in 96% ethanol for preservation in a long period of time. Ethanol can preserve DNA and the specimen for a long time but it was found to be able to hinder the DNA extraction process if not cleaned properly. A study by Turan, et. Al (2015) showed that ethanol preservation long

term before DNA extraction can cause DNA from the biological samples to degrade and affect the DNA extraction quality and a study from stated that additional cleaning steps are required to clean the samples before DNA extraction which in this study have been done using double distilled water (ddH20).

Another case that might contribute to the low quality of DNA samples is contamination. According to Gupta (2019), RNA, phenol, carbohydrates, and other organic substances can contribute to the purify level of DNA. In addition, Setiaputri et al. (2020) stated that DNA samples with 260/280 ratio higher than 2,0 contain RNA and less than 1,8 shows the presence of phenol or protein contamination. Based on that it can be stated that samples DBP012261 (chelex 10%) was contaminated by RNA and the rest of the samples were contaminated by protein. This protein came from the remnants of undegraded cell components during the extraction

In terms of nucleic acid concentration, DBP012245 and DBP012261 had the highest with 114,1 and 103,3 ng/ul respectively. DBP012243 closely followed behind with 93,52 ng/ul and the rest of the samples had lower quality with average being under 50 ng/ul. According to Dewanata and Muslih (2021), the overall quality of DNA isolate can be accepted as good if passed the minimum concentration of ng/ul. This suggests that only DBP012245 and DBP012261 are good overall. However, according to (Dewanata and Muslih), the minimum standard of nucleic acid concentration for PCR process is 10 ng/ul. According to this, most of the samples except DBP012245 (Chelex 10% method) are under standard quantity, but can still be continued to the PCR process. In addition, also stated that PCR actually

does not require native high concentration of DNA in order to amplify target sequences, as long as the target sequence is still present. Therefore, all the samples extracted can still be used for the PCR process.

There is not much of a noticeable difference in DNA concentration between samples extracted using the CTAB method and Chelex 10%. Both methods could produce relatively good quality and quantity of DNA isolate. A study from Turan, et al. (2015) encountered findings that samples extracted with CTAB extraction were having low concentration compared to samples from the Chelex method. The study stated that contamination might be more likely to happen in the CTAB method because of the chemicals usage such as chloroform or direct surface contact between sample tissue with pestle, mortar and other equipment used. However, mortar or pestle were not used in the DNA extraction process. Furthermore, it is important to note that in this study only two of the total samples were extracted using both methods and can be taken into comparison. Therefore, even if there is evidence to support this statement, there is insufficient data to justify it conclusively. Other factors also can be taken into consideration such as personal laboratory skills, sample age, and the overall DNA quality of the samples. To summarize, both the Chelex 10% and CTAB methods are applicable for DNA extraction from Mudskipper fish as they are able to extract and purify DNA from the samples. However, differences exist between the two approaches. Both methods are meeting the basic principles of DNA extraction (lysis, precipitation, and purification), but the CTAB method requires a longer total processing time compared to the Chelex 10% method that can be completed in just 3 hours.

Additionally, the CTAB method involves the use of risky chemicals e.g., chloroform, while the Chelex method does not have such risks. The preparation of CTAB mixture also needed more ingredients than Chelex 10% which can cause more expenses and CTAB method required more advanced skills to do than Chelex 10% methods. All of these advantages and disadvantages can be seen accordingly depending on available resources, time constraints, and budget considerations. Another thing to note is that being attentive throughout the extraction steps and having proper laboratory skills are always necessary to produce high quality DNA and to minimalize contaminations regardless of the method used.

The DNA isolate exhibited relatively low nucleic acid concentrations observed through Nanodrop. The DNA isolate was run into gel electrophoresis showing no bands were visible.

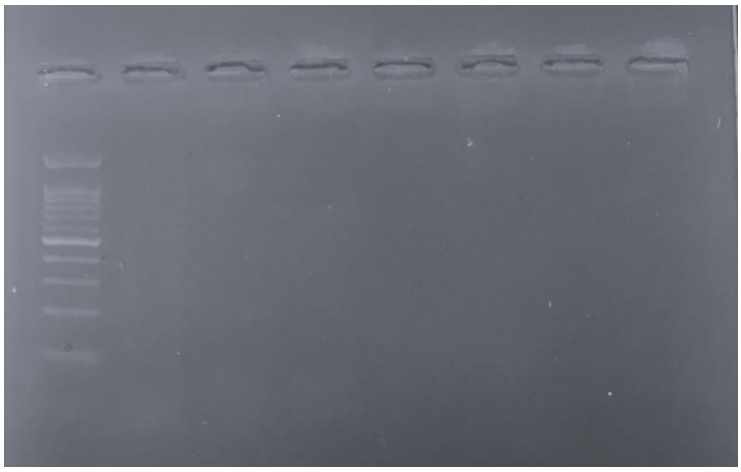

Figure 4.2 Pictures showing the result of gel electrophoresis from DNA isolate. DNA ladder positioned on the left and there were no visible bands observed rather than very faint streaks.

However, in contrast to the DNA quantification results, the PCR result samples showed clear and bright bands (Figure 4.1), suggesting that the PCR

process was successfully amplified targeted DNA from the COI region of mudskipper DNA isolate. The observed bands fell within the range of 600-700 base pairs, indicative of the amplified DNA fragments meeting the necessary criteria for subsequent Sanger sequencing analyses, as according to Crossley et al. (2020) sequencing procedures are most efficient for amplicons ranging from 100 to 800 bp.

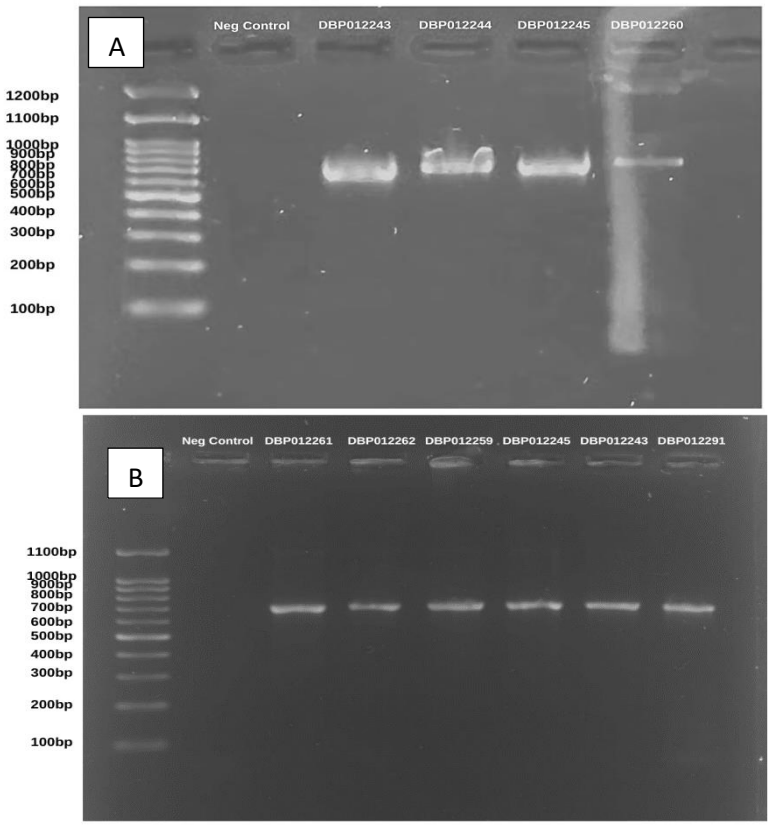

Figure 4.2 Pictures showing the result of gel electrophoresis. A) includes (from left to right) negative control, DBP012243, DBP012244, DBP012245, DBP012260. B) includes negative control, DBP012261, DBP012262, DBP012259, DBP012245, DBP012243, DBP012291. All samples exhibit clear and bright bands, with base pairs aligning between 600 and 700 on the DNA ladder positioned on the left.

4.2.BLAST Analysis

Sequencing results revealed that all the 12 samples of Mudskipper fish from Bipolo and Oesapa, East Nusa Tenggara, Indonesia produced sequences with lengths ranged from 602-685 bp. Subsequent analysis of chromatograms reveal the consensus results as 591 bp alignment or equal with 197 amino acids translations. BLAST analysis result revealed that samples DBP012261, DBP012262, DBP012259, and DBP012260 held a 99.54-100% similarity with *Periophthalmus argentilineatus* accession number MW514019. Samples DBP012291, DBP012245, and DBP012244 held 90.67% similarity with *Periophthalmodon schlosseri* accession number NC_030766. According to Triandiza and Maduppa (2018), the most similar GenBank sequence is characterized by the same maximum and total score, query coverage close to 100%, E value close to 0, and percentage of identity or similarity close to 100%. Mudskipper samples in this study were close to these criterias and showed distinguishable similarity to the GenBank sequence. Thus, through BLAST identification, twelve individual samples from this study were identified as two species, namely *Periophthalmus argentilineatus* and *Periophthalmodon schlosseri*.

Table 4.2 Species identification of samples based on GenBank database through BLAST

Sample Code	GenBank Species Identified	Similarity (%)	Query Cover (%)	Accession Number
DBP012261	*Periophthalmus argentilineatus*	100.00%	100%	MW514019
DBP012262	*Periophthalmus argentilineatus*	100.00%	100%	MW514019
DBP012259	*Periophthalmus argentilineatus*	100.00%	100%	MW514019
DBP012291	*Periophthalmodon schlosseri*	90.67%	100%	NC_030766
DBP012245	*Periophthalmodon schlosseri*	90.67%	100%	NC_030766
DBP012243	*Periophthalmodon schlosseri*	90.67%	100%	NC_030766
DBP012260	*Periophthalmus argentilineatus*	99.54%	96%	MW514019
DBP012244	*Periophthalmodon schlosseri*	90.67%	100%	NC_030766
DBP012279	*Periophthalmus argentilineatus*	100.00%	100%	MW514019
DBP012278	*Periophthalmus argentilineatus*	100.00%	100%	MW514019
DBP012277	*Periophthalmus argentilineatus*	100.00%	100%	MW514019
DBP012276	*Periophthalmus argentilineatus*	100.00%	100%	MW514019

4.1. Phylogenetic Analysis

Phylogenetic tree built using Maximum Likelihood reveals six distinct clades named clade A, B C, D, E, and F (Figure 4.2). This grouping

appears to be influenced by geographical patterns, as demonstrated by *Periophthalmus argentilinatus*, which divided into two clades: Clade A from eastern Indonesia (Lombok and Bali) and Clade B from the western region of Indonesia (Jogjakarta). A similar geographical separation is observed in *Periophthalmus kalolo*, separating into two clades including Clade C and Clade D.

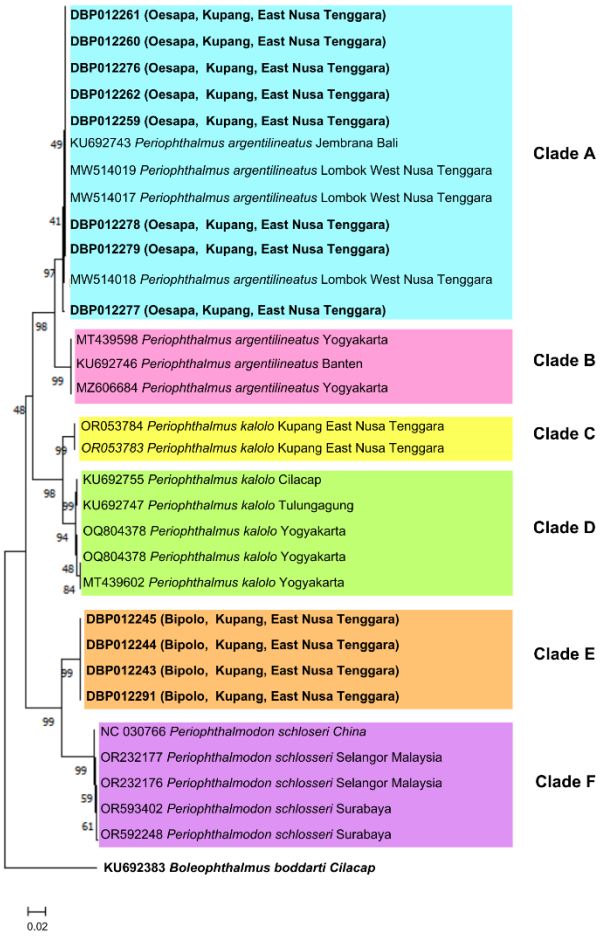

Figure 4.2 The phylogenetic tree of various mudskipper species from Indonesia, including specimens from this study, was constructed using the Maximum Likelihood method in MEGA XI with the HKY model, 1000 bootstrap replicates, and a scale bar representing 0.02 substitutions per site.

The samples DBP012262, DBP012261, DBP012260, and DBP012259, DBP012276, DBP012277, DBP01278, DBP012279 were clustered together with *P. argentilineatus* from Tekolok Estuary, Lombok (MW514017) and *P. argentilineatus* from Jembrana, Bali (KU692743) therefore confirmed previous identification through BLAST. The clustering of samples from this study with *P. argentilineatus* from Bali and Lombok appear to be consistently influenced by geographical patterns between western and eastern areas of Indonesia. This finding is in line with the finding of previous study from Arisuryanti et al. (2021). This also indicates a high genetic similarity within individuals and also genetic divergence from these different locations.

While the rest of the samples including DBP012243, DBP012244, DBP012245, and DBP012291 (Clade E) which previously identified as *Periophthalmodon schlosseri* using BLAST shown to be separating from other *Periophthalmodon schlosseri* from other region clustered in Clade F. Separation of clades within the same species might suggest genetic differences between different geographical areas. However, Clade F consists of samples from various locations that are far separated geographically from each other (China, Malaysia, and Indonesia), yet they are genetically closely related. It indicates that individuals in Clade E might not be as closely related to *Periophthalmodon schlosseri* as previously believed, or might not be *Periophthalmodon schlosseri* at all.

4.2. Genetic Distance

Genetic distance table (Table 4.4) was computed through MEGA based on the assumption that every clade represents a distinct species. The result shows that Clade A and Clade B exhibit the closest genetic distance with 6%, followed by Clade C and Clade D with 7%. This indicates the possibility presence of a cryptic species in both of *P. argentilineatus* and *P. kalolo*, aligning with the threshold proposed by Zemlak (2009) which stated that genetic divergence greater than 3.5% is most likely to be congeneric species rather than population-level variation within a single species.

This possible presence of cryptic species in *P. argentilineatus* and *P. kalolo* is also in line with the findings from Arisuryanti et al. (2018) and Rha'ifa et al. (2021), as they discovered that P. argentilineatus in Indonesia was divided into two clades separated by a genetic distance, followed by further study from Arisuryanti et al (2021) who a formation of two clades between each of *P. kalolo* and *P. argentilineatus* in the phylogenetic tree, followed by a geographical pattern within the formed clades.

Table 4.4. This table shows the genetic distances between various clades (A to F) and an outgroup from the phylogenetic tree. The smaller the values, the closer the genetic relationships between clades.

	Clade A	Clade B	Clade C	Clade D	Clade E	Clade F	outgroup
Clade A							
Clade B	6%						
Clade C	13%	14%					
Clade D	13%	13%	7%				
Clade E	14%	16%	16%	16%			
Clade F	15%	14%	18%	18%	11%		
outgroup	22%	21%	21%	20%	21%	23%	

The specimens of *P. argentilineatus* from this study clustered closely with those from Lombok and Bali, further reinforcing the idea that geographical separation plays a significant role in the genetic divergence observed within these species. This pattern of separation suggests that *P. argentilineatus* and *P. kalolo* populations may have been isolated for extended periods, possibly due to the geographic distance and natural barriers between regions such as between Lombok or Bali and Java Island. Such isolation could have limited gene flow between populations, leading to the significant genetic divergence observed in this study.

Furthermore, Clade E exhibits higher genetic distances compared to other clades: 16% with both Clades C and D, then 14%, 16%, and 11% with Clades A, B, and E respectively. The high genetic divergence among Clade

A, B, C, and E is expected, but not with Clade E. The significant genetic distance of 11% between Clade E and Clade F needs to be highlighted, as both clades were initially identified as *Periophthalmodon schlosseri* through BLAST analysis. Although *Periophthalmodon sp*. exhibit the closest genetic distance among other species in the phylogenetic tree, this distance is still nearly equal compared to other interspecies distance such as between *Periophthalmus argentilineatus* and *Periophthalmus kalolo*. This provides additional evidence to support that *Periophthalmodon* sp. is a distinct species from *Periophthalmodon schlosseri*.

However, although BLAST and phylogenetic analysis provide an initial insight on a new potential species from genus *Periophthalmodon* that has yet to be recorded in Genbank, there is no morphological data and insufficient to no data of mudskipper species from the same genus that can help to identify the exact species and its taxonomic status. Therefore, further investigation incorporating morphological and morphometrical identification needs to be applied to thoroughly identify and determine this species. It is also important to broaden the sampling area to include a wider geographic range in order to enrich the genetic database of mudskippers in Indonesia and provide a more comprehensive study of their phylogenetic relationships.

V. CONCLUSION AND SUGGESTIONS

5.1. CONCLUSION

5.1.1. BLAST analysis reveals 2 species, namely *Periophthalmus argentilineatus* and *Periophthalmodon schlosseri* with the average 90-100% similarity to MW514019 and NC_030766 from Genbank, respectively.

5.1.2. Samples from this study grouped with *P. argentilineatus* from Lombok and Bali. Genetic distance analysis revealed possible cryptic species: Clades A and B had a 6% distance, Clades C and D a 7% distance, and Clades E and F, both identified as *P. schlosseri*, showed an 11% distance, suggesting Clade E might be a separate species.

5.2. SUGGESTIONS

5.2.1. Further investigation with broader sampling and morphological analysis is needed to confirm this and improve the understanding of mudskipper biodiversity in Indonesia.

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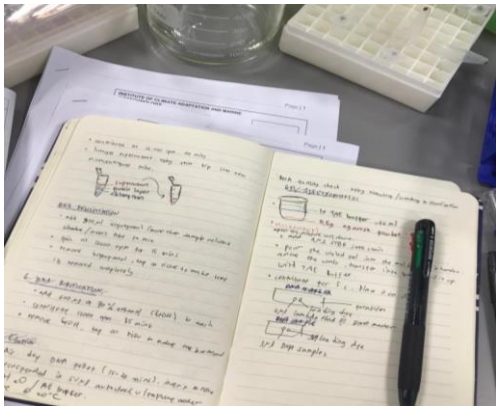
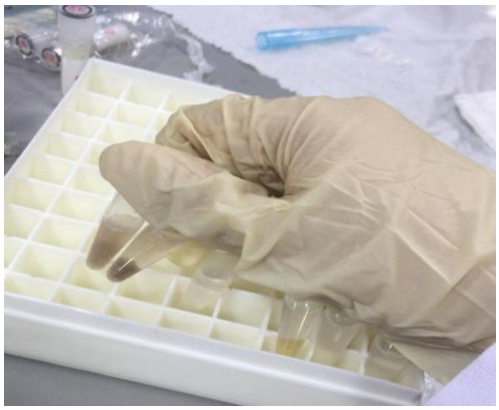
ATTACHMENTS

Attachment 1. Preparation of methods

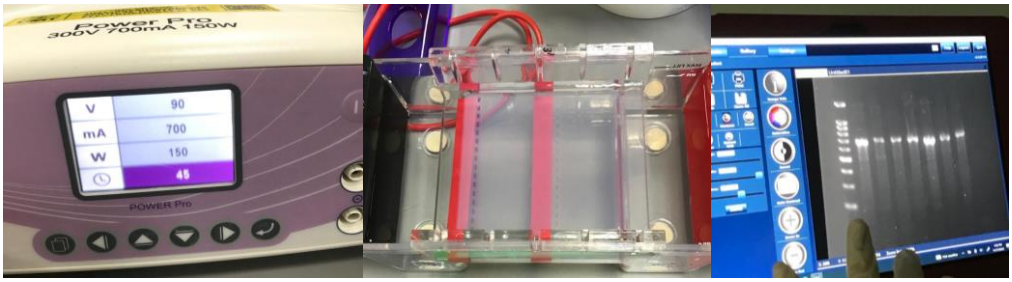


Attachment 2. DNA extraction of mudskipper fish samples

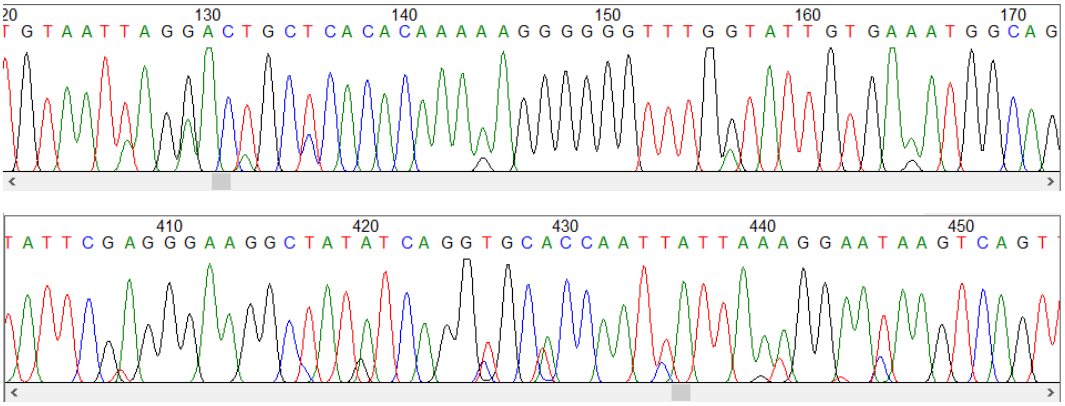


Attachment 3. PCR (*polymerase chain reaction*) of Mudskipper fish samples

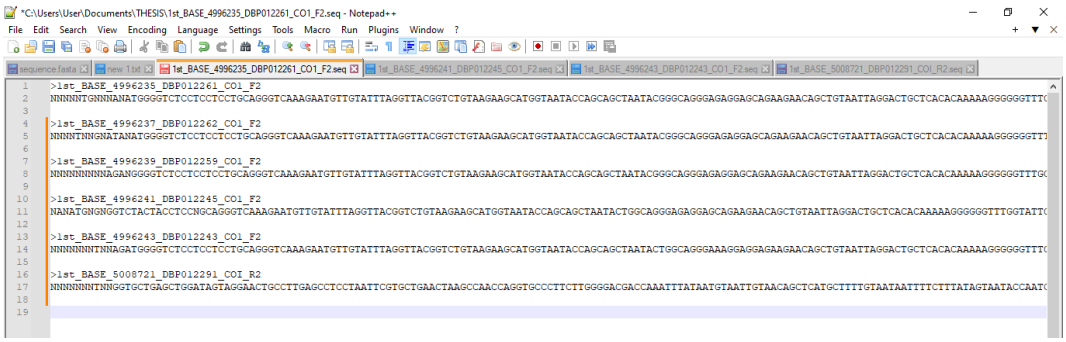
Attachment 4. Gel Electrophoresis of Mudskipper fish samples



Attachment 5. Chromatograph of Mudskipper Fish DNA sequences



Attachment 6. DNA Sequencing of Mudskipper fish sample



Attachment 7. BLAST analysis of mudskipper fish samples

Succesful	Sample ID	Primer	Species Name BLAST	Similarity	Assesion no. NCBI
	DBP012261_CO1_F2	CO1 LCO1490	Periophthalmus argentilineatus	99.70%	MW514019
	DBP012262_CO1_F2	CO1 LCO1490	Periophthalmus argentilineatus	99.85%	MW514019.1
	DBP012259_CO1_F2	CO1 LCO1490	Periophthalmus argentilineatus	99.70%	MW514019.1
	DBP012245_CO1_F2	CO1 LCO1490	Periophthalmus argentilineatus	98.93%	MW514019.1
	DBP012243_CO1_F2	CO1 LCO1490	Periophthalmus argentilineatus	98.93%	MW514019.1
	DBP012291_CO1_F2	CO1 LCO1490	Periophthalmodon schlosseri	99.84%	OR053775.1

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PRACTICUM	YEAR
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