



Study Identification of Some Species of Fish Using the Partial Fragment of Mitochondrial Cytochrome Oxidase Subunit-1 Gene (COI) in Danau Panggang, South Borneo

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Abstract

Danau Panggang is a unique habitat for fish species used for food and economic purposes that live in it. This study aims to identify several important and endangered fish species in Danau Panggang using the DNA sequence method and water quality in Danau Panggang. The DNA sequence was then aligned with the RefSeq from the GenBank data library by using the BLAST program of NCBI to find the closest identity associated with several types of fish samples being analyzed. The sequence analysis of the DNA show that some fish species analyzed have specific identifications : *Trichopodus pectoralis*, *Phalacrotonotus apogon*, *Belodontichthys dinema*, *Anabas testudineus*, and *Leptobarbus hoevenii*. Based on the results of the phylogenetic tree, it is also found that the relationship between fish taxons in flooded swamps of Danau Panggang is close to one another.

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INTRODUCTION

Flooded swamps are the largest resource to support the economy, and one example of economic activities is through capture fisheries resources. Capture fisheries activities are one of the potentials and superiorities for the people of Hulu Sungai Utara Regency in developing swamplands for the welfare of the community (Rais *et al.*, 2019).

The identification of species morphology also has several limitations (Elvyra *et al.*, 2020). Fish of native species and within the same species differ in body shape, color, and growth rate, as found in the climbing perch. Morphological identification observations continue to be used to differentiate between the climbing perch of different origins. Moreover,

mitochondrial DNA-based identification systems (mt) offer higher precision in the identification of species or lineages that include amplification and sequencing of short cytochrome C oxidase subunit I (COI) mitochondrial genes (Parvez *et al.*, 2020).

The information on the identification of phylogenetic characters between species and populations is closely related because mitochondrial DNA develops faster than most core genes (Kocher and Stepien, 1997). However, information regarding fish identification using DNA sequences in the waters of Danau Panggang is still minimal, so it is necessary to study fish identification using DNA sequences in Danau Panggang, South

Kalimantan. Genetic research on the genetic conservation of Betok fish (*Anabas testudineus*) in the swamp waters of South Kalimantan has been carried out by Slamant *et al.* (2012). Research on the identification of several types of fish with DNA sequences in the flooded swamps of Danau Panggang aims to obtain a more definite identification based on information on the nucleotide sequences of fish species so that information about the phylogenetic relationships of species among fish taxons in the flooded swamps

of Danau Panggang, South Kalimantan can be obtained.

METHODOLOGY

Place and Time

This research study identified several selected fish species obtained from the catch of fishermen who used nets in fishing in Danau Panggang, South Kalimantan (Figure 1). Sampling was conducted in October 2016 (Figure 2).

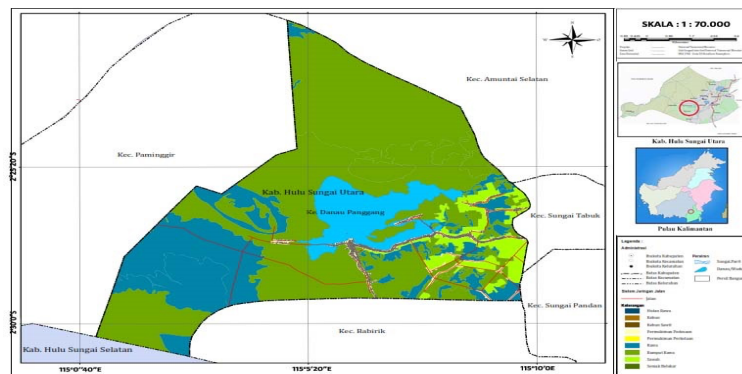


Figure 1. Research location in Danau Panggang, South Kalimantan.

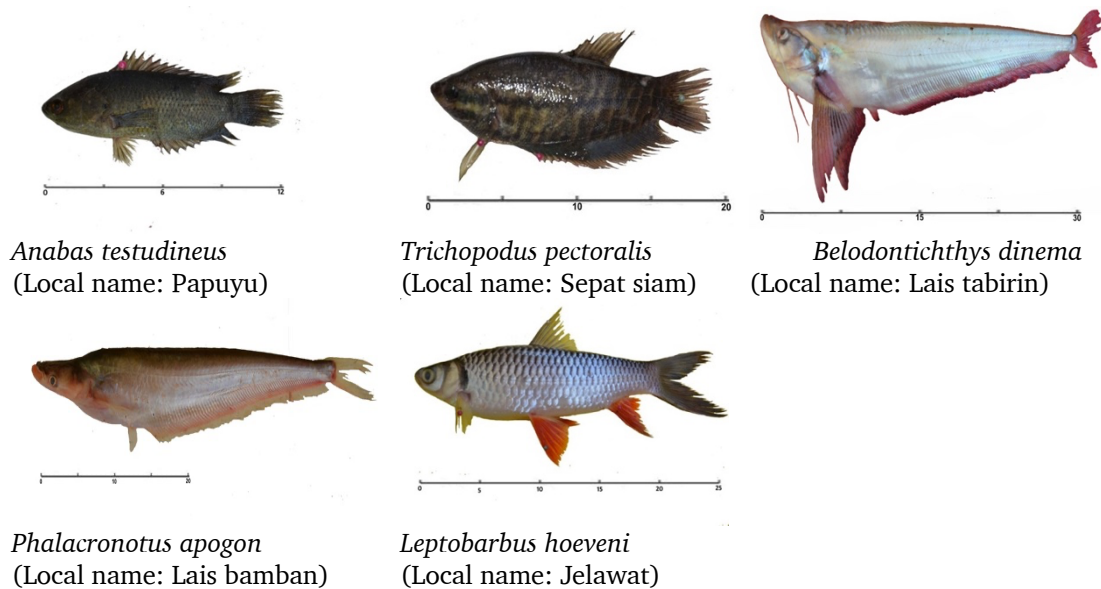


Figure 2. Samples of fish species samples obtained in Flooded Swamps of Danau Panggang, South Kalimantan.

Research Materials

The tools used in this study were 1000 μ l micropipettes (Abdos) along with 1000 μ l tips (Abdos), 200 μ l micropipettes (Abdos) along with 200 μ l tips (Abdos), 2-10 μ l micropipettes (Abdos) along with 10

μ l tips (Abdos), digital scales (Mettler), measuring flasks (Duran Schot), vortex (Genie), a centrifuge (Thermo), a PCR machine (BIOER), an electrophoresis machine, an incubator (Ecocell), 1.5 ml tubes (Abdos), 2 ml PCR Plates (Thermo),

a microwave (Panasonic), a UV Gel doc transilluminator (Alpha imager), measuring cups (Iwaki), gloves (Nitrile), and masks (Sensimask).

The materials used in this study were Genomic DNA kits (Geneaid), florosafe (First Base), myTaq red mix (Bioline), agarose (First Base), 99% absolute ethanol (Merck), nuclease water (First Base), PCR Purification Clean up (Bioline), Proteinase K (Geneaid), STE (First Base), SDS 10% (First Base), forward and reverse primers COI (IDT), DNA ladder III markers 100bp (Geneaid), loading dye (Geneaid), 5 x TBE (First Base), 1x TBE (First Base), and ethidium bromide (Invitrogen).

Research Design

Each tissue of fish sample obtained was inserted into a 1.5 ml tube filled with absolute ethanol (Merck, 99.9%) and was given a sample code. DNA sequence identification is a species identification technique that only requires a small amount of fish body tissue using the mitochondrial gene cytochrome oxidase subunit I (COI) (Wulandari *et al.*, 2018). The selected fish samples used as specimens were subjected to laboratory analysis by molecular analysis at the Fish Laboratory, molecular taxonomy section of the Public Aquatic Fisheries Research and Fisheries Extension Center, Palembang. The research was carried out experimentally in the laboratory including, DNA extraction, amplification, electrophoresis, purification of PCR products, and sequencing processes. Samples of purified PCR products were sent to Macrogen Biotechnologies Co., Ltd, Seoul, South Korea for sequencing. The method that could be used to find out genetic information and obtain the sequence of nucleotide bases on a DNA molecule could be done with DNA sequencing (Sanger *et al.*, 1977).

Work Procedure

DNA samples were extracted from fish tissue samples by referring to the

Genomic DNA mini kit (Geneaid) procedure. The steps of the work procedure included DNA extraction, amplification, purification of PCR products, and DNA sequencing, described as follows:

MtDNA Extraction

The coded fish tissue DNA samples were extracted using a genomic DNA mini kit for tissue (Geneaid). Fish tissue samples stored in 99% absolute ethanol (Merck) were washed with distilled water (molecular grade) twice and were then suspended in an STE buffer (First Base) up to a volume of 250 μ l. Muscle tissue of the fish was lysed (broken/removed) with SDS (Firstbase, 10%) as much as 50 μ l and proteinase K (Geneaid) as much as 20 μ l and then put in an incubator (EcoCell) at a temperature of 42°C for 14 hours. The DNA extraction method later follows the instructions for the genomic DNA mini kit for tissue (Geneaid).

Amplification

Amplification used COI (Integrated DNA Technologies) primers to amplify DNA sequences. Some fragments of the mitochondrial COI gene were amplified using universal primers based on Ivanova *et al.* (2007): Fish COI-F (5'ACT TCA AAC TTC CAY AAA GAY ATY GG-3 ') and COI-Fish-R (5'- TAG ACT TCT GGG TGG CCR AAR AAY CA-3 '). The composition of the PCR reaction was carried out with a final volume of 50 μ l consisting of 2 μ l of DNA samples, 21 μ l of sterile nuclease water (Firstbase), primer of 1 μ l each and also the addition of 25 μ l of PCR premix (My Taq Bioline red mix). The PCR reaction was carried out using a thermocycler (BIOER) machine with the following conditions: the pre-denaturation stage at a temperature of 95 °C for 1 minute, the second stage consisting of 35 cycles which included a denaturation stage at temperature of 95 °C for 15 seconds for each cycle, the primary attachment (annealing) at a temperature of 55 °C for 15 seconds, extension at a temperature of

72 °C for 30 seconds, and the last stage in the form of final extension at a temperature of 72 °C for 3 minutes and the introduction to renaturation at a temperature of 6 °C for 6 minutes (Wulandari *et al.*, 2019).

Electrophoresis

Electrophoresis was carried out on PCR products to be tested using agarose gel (Firstbase) which was previously added with 10 µl of ethidium bromide (Invitrogen) in 10x TBE (Firstbase, 1%) buffer. Following this, the gel was poured into gel molds that were ready to be injected into each well of 2 µl of PCR DNA samples according to the sequence of sample numbers, negative control, positive control and 5 µl markers. Afterward, the electrophoresis process was carried out at conditions of 120 v, 250A for 35 minutes. After the electrophoresis had been completed, the gel was put into gel documentation (Protein Simple Alpha Imager), and the gel doc UV lamp was turned on. The analysis of the presence of DNA bands was done by comparing ladder III 100 bp (Firstbase) DNA markers, positive control, and negative control (Wulandari *et al.*, 2019).

Purification of PCR products

PCR products on agarose gel set in size according to the primary design were purified using a purification kit (Bioline isolate II PCR and gel) following the manufacturer's protocol.

Data Analysis

The results of the nucleotide sequences were then manually edited using Bioedit software based on a chromatogram (Hall, 1999). The edited nucleotide sequences were then aligned using Clustal W (Thompson *et al.*, 1997) using the MEGA 6.0 program (molecular evolutionary genetics analysis) (Tamura *et al.*, 2013). The DNA sequences were then aligned with the reference sequences from the gene bank data library from NCBI using the BLAST application to find the identity that was most closely related to the type of fish sample analyzed.

RESULTS AND DISCUSSION

DNA isolation of several types of fish in Danau Panggang was amplified using a pair of universal primers, Fish-COI-F and COI-Fish-R based on Ivanova *et al.* (2007) which was then continued with electrophoresis observed from the formation of DNA bands on the UV transilluminator (Figure 2). Initially, DNA extracts of fish tissue were taken from 22 fish species and were amplified on the 530bp target DNA fragment, but only 16 fish specimens were successful. The identification of several fish species using DNA sequences was carried out on 16 fish samples and five NCBI Genbank as comparisons. The amplification of the cytochrome oxidase subunit I (COI) gene produced a COI gene fragment measuring 640-702 bp.

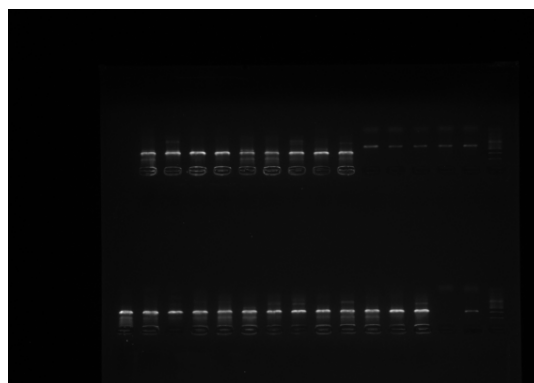


Figure 3. A photo of fish COI-F and COI-Fish-R gel electrophoresis amplification.

DNA Sequencing

The mitochondrial cytochrome c oxidase I (COI) gene serves as the core of a global bio-identification system for animals and aids the diversity resolution (Hebert *et al.*, 2003).

Based on the results of BLASTN gene sequences of partial mitochondrial cytochrome oxidase subunit I (COI) genes, the fish species analyzed on a molecular basis as a whole have similarities ranging from 89% to 100% (Table 1). Fish with

EC08 FCOI samples have 89% similarity with *P. apogon* (voucher FNP108), ED07 FCOI has 94% similarity with *L. hoevenii* (voucher YR033701), EA08FCOI and EB08FCOI have 100% similarity with *T. pectoralis* (voucher Tpec1). Fish with sample code EH07 FCOI, EG07 FCOI, ED08 FCOI have 100% similarity with *B. dinema* (voucher SLM-BD (PH) -01), and the other nine fish samples have 99% similarity with *A. testudineus* (voucher BIF2107).

Table 1. Results of BLASTN of fish species in the flooded swamps of Danau Panggang, South Kalimantan.

Sample	Species	% Identity	Access No.
EA08 FCOI; EB08FCOI	<i>Trichopodus pectoralis</i>	100%	KX817191.1
EE07 FCOI; EF07 FCOI; EA09 FCOI; EB09 FCOI; EC09 FCOI; EE08 FCOI; EF08 FCOI; EG08 FCOI; EH08 FCOI	<i>Anabas testudineus</i> (voucher BIF2107)	99%	KU692243.1
EH07 FCOI; EG07 FCOI; ED08 FCOI	<i>Belodontichthys dinema</i> (voucher SLM-BD(PH)-01)	100%	JF781186.1
EC08 FCOI	<i>Phalacronotus apogon</i> (voucher FNP108)	89%	MK448160.1
ED07 FCOI	<i>Leptobarbus hoevenii</i> (voucher YR033701)	94%	KC242349.1

The percentage of the nucleotide composition of fish species analyzed based on molecular sequencing on the four fish species, namely *P. apogon*, *B. dinema*, *A. testudineus*, and *L. hoevenii* has a DNA G + C base composition that is higher than the base composition of DNA A + T, whereas fish with the species *T. pectoralis* (n = 2) have a lower G + C composition than the composition of A + T. The value of the composition of the G + C content is higher

in the nucleotide, which means it is more difficult for the double-stranded DNA molecules to separate (Yuwono, 2008). *A. testudineus* COI nucleotide sequences taken from the NCBI Bank genes in India, Thailand, and Vietnam were analyzed with higher AT content (53.6%) and lower GC content (46.4%) (Parvez *et al.*, 2020). The percentage composition of the nucleotide species of fish analyzed by molecular analysis is presented in Table 2.

Table 2. Percentage of nucleotide composition.

No	Sample	Composition of Nucleotides	
		G+C (%)	A+T (%)
1	<i>Trichopodus pectoralis</i> (n=2)	48.9	51.1
2	<i>Anabas testudineus</i> (n=9)	54.5	45.5
3	<i>Belodontichthys dinema</i> (n=3)	54.8	45.2
4	<i>Phalacronotus apogon</i> (n=1)	54.0	46.0
5	<i>Leptobarbus hoevenii</i> (n=1)	52.0	48.0

Phylogenetic Tree and Genetic Distance

The molecular marker, region 570 bp of the mitochondrial cytochrome c oxidase I (COI) gene, has been successful

in finding specific species, and it is also found that there are more variations between species than within species (Wibowo and Merlia, 2017). This research is an important step to understand the kinship of fish living in the flooded swamps of Danau Panggang in South Kalimantan. The creation of COI sequences from the Danau Panggang system also supports the global DNA barcode libraries.

The identification of DNA sequences results in five types of fish. The DNA sequences were then aligned with the RefSeq from the GenBank data library to see the phylogenetic tree using the BLAST program from NCBI to find the identities that are most closely related to the analyzed fish samples. In general, the position of phylogenetic tree organisms is based on genetic similarities between one species and another. The five types of fish are obtained from the catch of fishermen in the flooded swamps of Danau Panggang, South Kalimantan, including *T. pectoralis*, *P. apogon*, *B. dinema*, *A. testudineus*, and *L. hoevenii* (Figure 2). The position of organisms on the phylogenetic tree shows the close kinship between adjacent taxons. Organisms of different species of the same genus will be close to each other in trees, and the further

distance in the phylogenetic tree is found in different genera (Wulandari *et al.*, 2019).

A. testudineus belongs to the Anabantidae family (one of the 30 species in this family) and is the main component of ichthyofauna Sundaland. This species is widely distributed in Africa and Asia and is the only member of the Anabantidae family in Asia. *A. testudineus* is one of the foreign fish species scattered in the waters of Sulawesi and Sundaland (Weber and de Beaufort, 1922; Kottelat and Whitten, 1996; Hubert *et al.*, 2015). However, the actual data on their occurrence or even impacts on native fauna are very limited and scattered in literature.

The collection of basic genetic information and data on a species is an initial condition needed to determine its kinship (Nugroho *et al.*, 2003). Molecular species identification techniques have the potential for rapid and precise labeling assessment (Wong *et al.*, 2011). The COI gene sequence produced in the *T. pectoralis* sample in the flooded swamps of Danau Panggang ranges from 510 bp-585 bp. Research by Syaifudin *et al.* (2019) states that the resulting *T. pectoralis* COI gene sequence is at 604 bp and has 100% similarity to the same species in Indonesia and the Philippines.

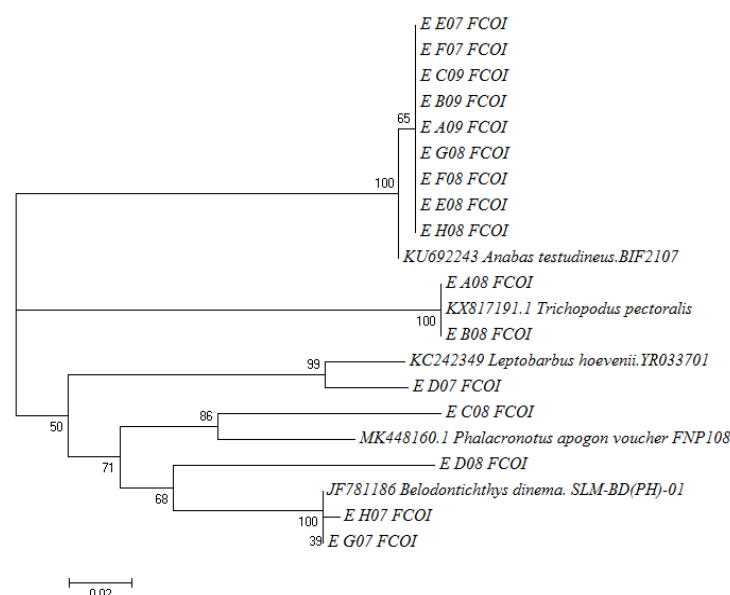


Figure 4. Analysis of neighbor-joining trees from COI sequencing of fish in Danau Panggang, South Kalimantan.

Gene sequencing (DNA sequencing) for phylogenetic analysis is rarely performed because the cloning of homologous genes from several samples still needs high cost (Kocher and Stepien, 1997). The COI profile correctly identifies the specimen. When the identification systems using COI are fully developed, they can provide reliable, cost-effective, and accessible solutions to current species problems (Hebert *et al.*, 2003).

The introduction of primary sequences with broad phylogenetic uses such as with the use of universal primers allows the rapid amplification of a specific sequence from a large number of samples

and helps to create a rapid number of studies using DNA sequences to examine phylogenetic questions (Kocher and Stepien, 1997). DNA sequencing data has several inherent advantages over other types of systematic data. First, an unlimited number of potentially available sequence characters. Second, this character is useful for studying the relationship between close and distant relatives. Based on the phylogenetic tree, evolutionary relationships are obtained between several fish species, genera and families. Organisms of different species of the same genus will be adjacent to each other in the phylogenetic tree.

Table 3. Average genetic distances between groups of fish species.

Name of Fish Species	1	2	3	4
<i>Trichopodus pectoralis</i>				
<i>Phalacrotonotus apogon</i>	0.264			
<i>Belodontichthys dinema</i>	0.291	0.170		
<i>Anabas testudineus</i>	0.262	0.248	0.229	
<i>Leptobarbus hoevenii</i>	0.234	0.215	0.199	0.254

The most common analysis used to estimate genetic distance values is the genetic distance (Nei, 1972; Kocher and Stepien, 1997). The phylogenetic relationship was deduced from this distance using the Neighbour-Joining method (Saitou and Nei, 1987). The result of measuring the genetic distance was an evolutionary estimate of the scatter among the fish group sequences. The number of base substitutions per site from the mean of all sequences between groups was later shown. Moreover, the analysis was performed using a 2-parameter Kimura model using the MEGA6 program (Kimura, 1980; Tamura *et al.*, 2013). The analysis was performed on 21 nucleotide sequences. Codon positions included the first, second and third positions. All positions had gaps and the lost data could be eliminated.

The nucleotide sequence data were compared by aligning them with each other and checking for differences. Closely related taxa would have few differences, and these differences were easy to find and evaluate, whereas for far related taxa

larger differences would be noticeable, which could even make alignment impossible (Beaumont *et al.*, 2010).

Organisms of different species and genera have longer distances, such as in the phylogenetic trees of *T. pectoralis*, *P. apogon*, *B. dinema*, *A. testudineus* and *L. hoevenii* than in other groups with node spacing ranging from 0.170 to 0.254 (Table 3). The NJ estimate and the bootstrap estimate in this study show the minimum genetic distance (0.262) between these two species (*T. pectoralis* and *A. testudineus*) which emphasizes the evolutionary relationship as well as their recent differences. When the order of individual fish is compared with the same species, it shows that the profile is almost the same. Therefore, it can be concluded that the population of each species is almost homogeneous (Meffe and Vrijenhoek, 1988). The average genetic distance between fish species is found to be high enough, showing a separate gene pool for these species.

CONCLUSION

Organisms of different species and genera have longer distances, such as in the phylogenetic trees of *Trichopodus pectoralis*, *Phalacronotus apogon*, *Belodontichthys dinema*, *Anabas testudineus* and *Leptobarbus hoevenii* than in other groups with node spacing ranging from 0.170 to 0.254

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