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Phylogenetic Relationships of Mungkuih Fish (Gobiidae: *Sicyopterus macrostetholepis*) from Rivers in Padang City, West Sumatra, Indonesia

Marta Dinata[1, 2]*, Syaifullah[1], Dahelmi[1], Jabang Nurdin[1]

[1]Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, West Sumatra, 25163, Indonesia

[2]Biology Department, Faculty of Forestry and Natural Sciences, Universitas Lancang Kuning, Pekanbaru, Riau, 28265, Indonesia

***Corresponding Author: martadinata@unilak.ac.id**

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ABSTRACT

Genetic research is an important key in carrying out conservation and sustainable management of a species. *Sicyopterus macrostetholepis* (known locally as the mungkuih fish) does not yet have sufficient genetic data and is included in the IUCN Red List of Threatened Species. This research aimed to determine the phylogenetic relationship of the mungkuih fish population, thought to be endemic, in Padang City, West Sumatra. Sampling was carried out in Batang Kuranji, Batang Air Dingin and the Lubuk Hitam River during June-August 2021. Molecular analysis through the stages of extraction, PCR, electrophoresis and DNA sequencing, was conducted. DNA sequence analysis was conducted using MEGA XI (Genetic distance, Phylogenetic), DNASP (Haplotype) and arlequin (Fixation index, Amova). The research found a fragment length of 773 base pairs. Genetic distance analysis of mungkuih fish was 0.25-1.12%. The phylogenetic tree shows that the divergence between the outgroup and the Gobiidae family is 21.78-28.01%. The analysis value of the fixation index (Fst) was 0.47977 showing the high genetic heterogeneity between the mungkuih fish populations in Padang City, as can be seen from the Haplotype and AMOVA tests. The haplotypes obtained in this study were h=7 and Hdt =0.944. The results show that Haplotype and Fst indicate that the genetic structure of the mungkuih population in Padang City is not disturbed.

INTRODUCTION

Man-made activities, when uncontrolled and unregulated, threaten the existence of various animal species. This study is concerned about the mungkuih fish (*Sicyopterus macrostetholepis*), a species of fish often found in Padang City of West Sumatra, specifically in the rivers of Batang Air Dingin, Batang Kuranji and Lubuk Hitam. Currently, this fish is threatened with extinction due to environmental pressures, namely the poaching of both adult and juvenile forms, the presence of excavation mining (stone, sand and gravel), and disturbance of the rivers through the creation of irrigation and recreation areas which result in destruction, fragmentation and food chain of mungkuih

fish. After 10 years of studying the fishes in these areas, the first author of this study has found that the declining population of this particular fish over the years has made it very expensive among the public, with a price range of 100-150 thousand Rupiahs (6-9 US dollars), some even charge up to 200 thousand per pile. The researchers have tracked the numbers of mungkuih fishes; while fishermen used to be able to catch thousands of fishes per kilogram in a net in 2015, by 2019 they could only catch 10-15 fishes. In the past, there used to be a national event called mungkuih spearing, a competition event that serves as a popular tourist attraction. Nowadays, this event could not be held due to the rapid decrease of this fish's population, reducing the people's incomes according to personal communications with the Head Fisherman of Koto Tangah. Since the mungkuih fish has economic value as a source of local protein and a tourist attraction, there is a clear motive to conduct a study on *S. macrostetholepis*.

Studying the genetic diversity of the mungkuih fish is also justified by the International Union for Conservation of Nature and Natural Resources (IUCN) who is responsible for publishing the Red List of Threatened Species. In 2019, the researchers recorded that *S. macrostetholepis* is listed as Data Deficient, and this remains true in 2024 **(Jaafar, 2019)**. To this date, the data of this specific fish are still mainly rooted from local common knowledge. In particular, fish cultivation groups and the local fishermen have observed that the mungkuih fish were juveniles near the sea and became adults the moment they reach upstream, indicating that these fish lay eggs in the river. However, whether the mungkuih fish is amphidromous or not is not yet confirmed scientifically, thus this study aimed to provide proof of this as well. This specific query is beneficial because the increasing number of dams being built for the purpose of bathing tourism near the rivers of West Sumatra would cause fragmentation of this fish area, which can potentially be a dangerous threat to the existence of the mungkuih fish, as this type of fish undergoes maturity process from downstream then to the upstream of the river to spawn (reproduce), and must be able to migrate from upstream to downstream of the river (estuary) or vice versa **(Cooney, 2013)**.

The cytochrome b gene can be used as a genetic marker to study species diversity and kinship relationships, because its codon is based on position, has more conserved regions and more diverse regions **(Ward *et al.*, 2008)**. The cytochrome b gene for characterization and species identification has been widely used in fish, including the phylogeography of the genus *Sicyopterus* in the Indo-Pacific region inferred from the mitochondrial cytochrome b gene **(Keith *et al.*, 2005)**, in Canadian freshwater fish **(Hubert *et al.*, 2008)**, postlarvae of fish of the Gobiidae family **(Taillebois *et al.*, 2013)**, population structure and demographic history of *Sicyopterus japonicus* **(Ju *et al.*, 2013)**, identification of the black Mugilogobius in Lake Towuti **(Larson *et al.*, 2014)** and haplotypes in the eel fish **(Syaifullah *et al.*, 2022)**.

Based on National Center for Biotechnology Information's GenBank **(NCBI, 2021)**, there is a genome database of mitochondrial DNA from the same genus as the mungkuih

fish, namely, *S. lagocephalus, S. japonicus,* and *S. aiensis*. Research on the genetic characterization of Sicyopterus fish using the cytochrome b gene which was carried out by **Keith *et al*. (2005)** and **Aquino *et al*. (2011)** opened up further opportunities to study mitochondrial genes, namely by observing the nucleotide composition, especially for the mungkuih fish located in the Batang Air Dingin River, Batang Kuranji and Lubuk Hitam. However, research on the nucleotide composition of the cytochrome b gene sequence of the mungkuih fish in Indonesia, which is part of the genetic characterization of the mungkuih fish, has never been carried out. This is proven by the absence of a cytochrome b gene database for the mungkuih fish (*S. macrostetholepis*) in GenBank. It is hoped that this research can complete genetic characterization data on *S. macrostetholepis* in Indonesia, which will be useful for the mungkuih fish breeding program in Indonesia in the future. The aim of this research was to determine the phylogenetic relationships in the cytochrome b gene of the mungkuih fish as part of detecting intrapopulation, interpopulation genetic variations and population structure of the mungkuih fish in the waters of the Batang Air Dingin, Batang Kuranji and Lubuk Hitam rivers.

MATERIALS AND METHODS

1. Locations for taking and collecting Mungkuih fish samples

The mungkuih fish (*S. macrostetholepis*) samples were collected using purposive sampling in three river locations in Padang City, namely the Batang Air Dingin, Batang Kuranji and Lubuk Hitam River. Three samples of adult mungkuih fish ranging in size between 4-8cm were caught representing the upstream, middle and downstream areas of the river in the Batang Air Dingin (BAD), Batang Kuranji (BK) and Lubuk Hitam (LH) rivers. With With a total of 9 fish samples and 19 additional samples analyzed as a result of molecular genetic studies, information on the nucleotide sequence of species belonging to the genus *Sicyopterus* was obtained from the National Center for Biotechnology Information (NCBI) GenBank and compared.

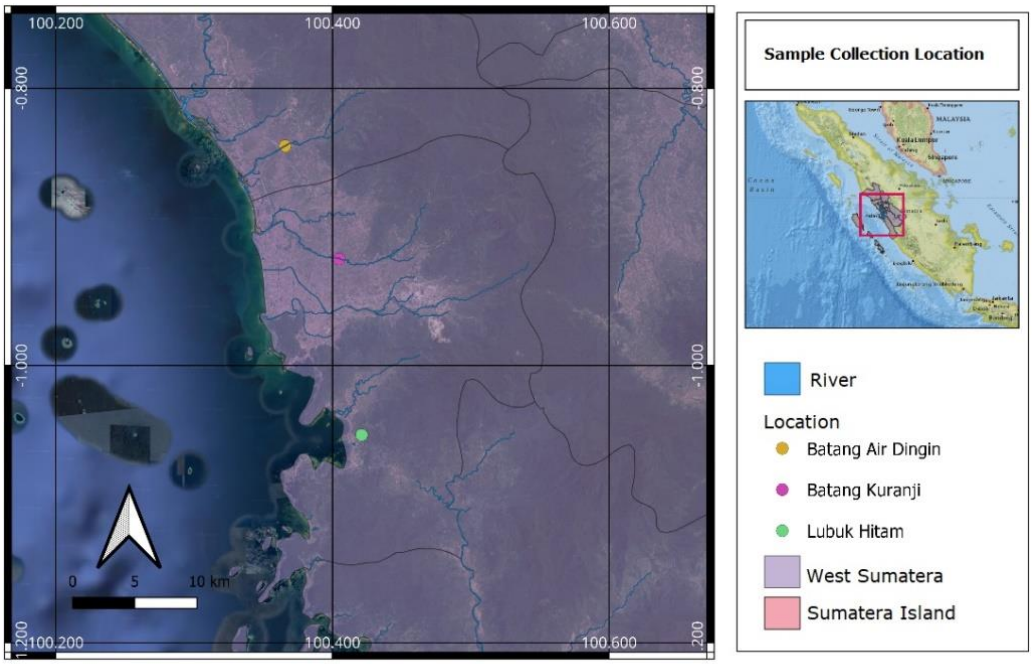

Fig. 1. Location of samples collection

Collection procedure

The mungkuih fish samples obtained were dissected by section from the base of the pharynx to the anus, then the liver was taken and put into a 1.5ml tube which was then preserved using 95% absolute ethanol, stored at 40C until used as a DNA extraction sample. To ensure that the mungkuih fish caught were of the *S. macrostetholepis* type, identification of the caught fish samples was carried out based on the studies of **Weber and de Beaufor (1916)**, **Saanin (1984)** and **Kottelat *et al.* (1993)**.

2. DNA isolation and DNA amplification

During the extraction of genomic DNA, the PureLinkTM genomic DNA kit (Invitrogen, USA) reagents were used to isolate the mungkuih fish liver DNA collected from the Batang Air Dingin River, Batang Kuranji and Batang Bungus, Padang, West Sumatra. Furthermore, the results of DNA isolation from each sample of *S. macrostetholepis* were amplified with cytochrome b encoding using primers forward F217 (5' TCGAAAYATACATGCCAATGG 3') and reverse R1043 (5'GAAGTAYAGGAAGG AYGCAATTT3') **(Keith *et al.*, 2011)**. The samples were then put into a thermal cycler (Biorad) with cycle settings: pre-denaturation at 95^{o}C for 1 minute followed by 35 cycles consisting of a denaturation stage at 95^{o}C for 15 seconds, annealing at 50^{o}C for 30 seconds, and extension at a temperature of 72^{o}C for 30 seconds and the final part was carried out in one cycle at a temperature of 72^{o}C for 5 minutes followed by a hold at a temperature of 4^{o}C for 4 minutes. Visualization of DNA fragments was carried out using a

UV lamp trans-illuminator (Daihan, Korea), and visible bands were documented with a GelDoc camera. The samples were then purified and sequenced.

3. Data analysis

The results of sequencing the cytochrome b gene of the mungkuih fish were contiqed using the SeqMan and EditSeq programs contained in DNASTAR software (DNASTAR Inc., Madison, USA). In this study, the forward and reverse lanes were carefully observed to ensure that no discrepancies were found in the consensus sequence. After the consensus sequence results matched between the forward and reverse lanes, the sequence data for each sample was calculated for its nucleotide composition using the EditSeq program on the DNA Statistics menu. The C+G composition was validated using the DnaSP v.5.10.01 program **(Librado & Rozas, 2009)**.

Cytochrome b gene sequence analysis was carried out to see the phylogenetic relationship of the mungkuih fish (*S. macrostetholepis*) in each population. Haplotype diversity (h) was analyzed using PopART v.1.7 **(Leigh & Bryant, 2015)** and the DnaSP v.6.12.03 **(Rozas *et al.*, 2017)** applications. Phylogenetic trees were processed with Itol (https://itol.embl.de/) **(Letunic & Bork, 2021)**.

RESULTS AND DISCUSSION

1. Sequence Data

Cytb mtDNA sequences from 28 individuals (9 sequences from this study and 19 sequences from GenBank) were determined for a total of 773bp (Table 1). Conserved sites: 477; singleton sites: 34 bp; variable sites: 296 bp (37.32%); and parsimonious informative sites: 262 bp (34.42%). No stop codons, insertions, or deletions were found in translation. The average composition of A, T, C, and G nucleotide bases was 29.03, 25.09, 30.13, and 15.75%, respectively. This average composition is almost identical to previous research on Gobiidae **(Slynko *et al.*, 2014; Jakšić *et al.*, 2016; Tserkova *et al.*, 2017; Fu'adil-Amin *et al.*, 2020)**. The nucleotide sequence shows an AT ratio of 54.12%, which is higher than the GC ratio, a typical characteristic of mtDNA genomes in vertebrates **(Hubert *et al.*, 2008)**.

Table 1. Taxonomic list of species, locations and accession numbers of GenBank samples

Family	Genus	Species	Location	Accession number
Eleotridae	*Eleotris*	*E. sandwicensis*	Hawaii	AY722186
		Eleotris_sp.	England	KF415547
		E. picta	Panama	AY722204
		E. amblyopsis	Panama	AY722205
		E. fusca	Sulawesi	AY722245
Oxudercidae	*Sicyopus*	*S. zosterophorum*	New Kaledonia	KF669049
		S. zosterophorum	New Kaledonia	KF669048
		S. zosterophorum	Southwest Pacific Region	KC407364
		S. zosterophorum	Southwest Pacific Region	KC407363

		S. zosterophorum	Southwest Pacific Region	KC407362
Gobiidae	*Sicyopterus*	*S. parvei*	Papua New Guinea	NC044145
		S. longifilis	Indonesia	MK496959
		S. parvei	Indonesia	MK496965
		S. parvei	Indonesia	MK496966
		S. squamosissimus	Sumatera	MK496981
		S. squamosissimus	Sumatera	MK496982
		S. squamosissimus	Sumatera	NC 044151
	Smilosicyopus	*S. leprurus*	Jepang	KF668998
		S. leprurus	Jepang	KF668997

2. Relationship between the mungkuih fish (*S. macrostetholepis*)

A phylogenetic tree was constructed using ML method analysis with 1000 bootstrap replications (Fig. 1). Based on the tree built from the Cytb gene, it can be observed that the Gobiidae group is divided into 2 clades. Members of the phylogenetic tree consist of *Sicyopus* fish in the first clade (A), *Smilosicypus* in clade (B) sublineage 1, and *S. longifilis* and *S. squamosissimus* in the second sublineage, while in the third sublineage there were *S. parvei* and *S. macrostetholepis*. The phylogenetic tree showed that the divergence between the outgroup and the Gobiidae family is 21.78-28.01%. In the Gobbidae family the divergence level was 6.57-17.98%, in the *Sicyopterus* genus the divergence level was 6.57-12.02%. The genetic distance of the *S. macrostetholepis* population in Padang City was 0.25-1.12%. Based on **Kartavtsev (2011, 2013)** sequence divergence in the same genus was 11-16%.

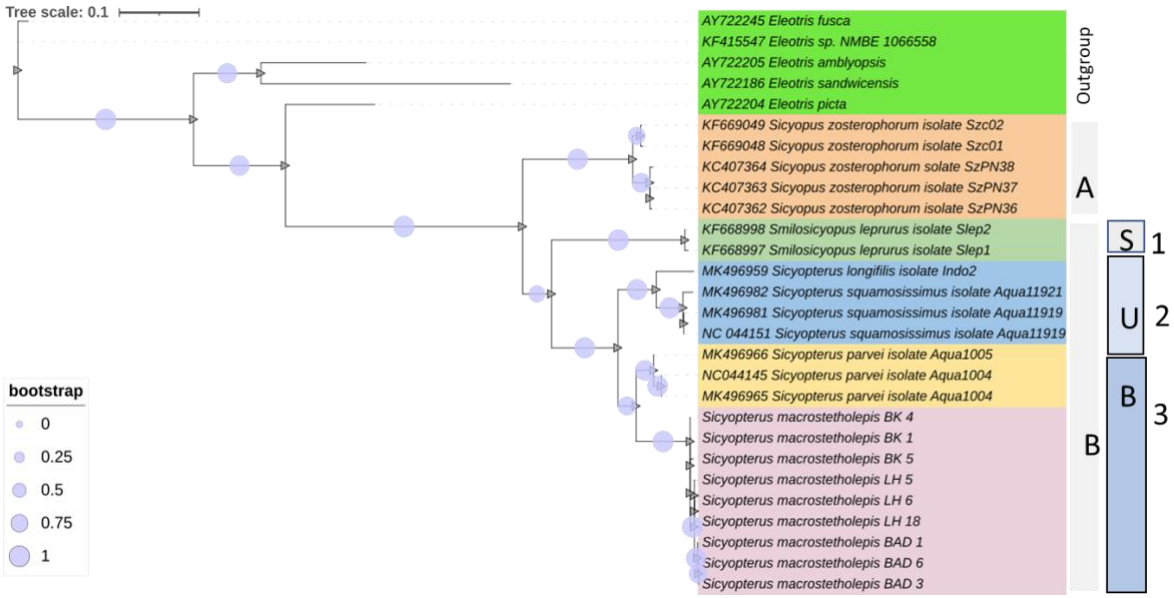

Fig. 2. Phylogenetic tree of the mungkuih fish (*S. macrostetholepis*)

E. fusca (AY7222245) is an early species on the phylogenetic tree (Fig. 2). In evolutionary history, *E. fusca* is the ancestral species for several other groups. *Eleotris* sp. (KF415547) has a fairly close evolutionary relationship to *E. fusca*, with short branches

indicating a close relationship. *E. amblyopsis* (AY722205) and *E. sandwicensis* (AY722186) form a closer group in the tree. They have a common ancestor and are separate from the *Eleotris* sp. branch. *E. picta* (AY722204) has a more distant evolutionary relationship than the previous group, indicated by longer branches. Based on the phylogenetic tree, *S. macrostetholepis* is the most recently evolved species among the four species. *S. parvei* (MK496966, NC044145, MK496965) is the most closely related species, followed by *S. longifilis* (MK496959) and *S. squamosissimus* (MK496982, MK496981, NC 044151).

Based on morphological and molecular characteristics, *S. macrostetholepis* and *S. parvei* have several similarities, such as long and slender bodies, continuous lateral lines, and long dorsal spines. This similarity shows that these two species are closely related. *S. longifilis* and *S. squamosissimus* share several morphological similarities, such as shorter, stubbier bodies, interrupted lateral lines, and shorter dorsal spines. This similarity shows that these two species are also closely related. *S. macrostetholepis* and *S. parvei* have a limited distribution in Southeast Asia, while *S. longifilis* and *S. squamosissimus* have a wider distribution, from Southeast Asia to Australia. This suggests that *S. parvei* and *S. macrostetholepis* have evolved more recently.

3. Haplotypes of mungkuih fish (*S. macrostetholepis*)

Based on this research, there were 7 haplotypes recorded for the mungkuih fish species from Padang City. A total of 3 haplotypes came from Batang Kuranji, 2 from Batang Air Dingin and 2 from Lubuk Hitam (Table 2). Hap1 consists of 1 sample, namely *S. macrostetholepis* LH 18; hap2 contains 2 samples, namely *S. macrostetholepis* LH 6 and *S. macrostetholepis* LH 5; Hap3 consists of 1 sample, namely *S. macrostetholepis* BK 5; hap4 consists of 1 sample, namely *S. macrostetholepis* BK 4; hap5 consists of 1 sample, namely *S. macrostetholepis* BK 1. While, Hap6 consists of 2 samples, namely *S. macrostetholepis* BAD 6 and *S. macrostetholepis* BAD 3, and hap7 consists of 1 sample, namely *S. macrostetholepis* BAD 1, haplotype network formed in the mungkuih fish species in Padang City (Fig. 3).

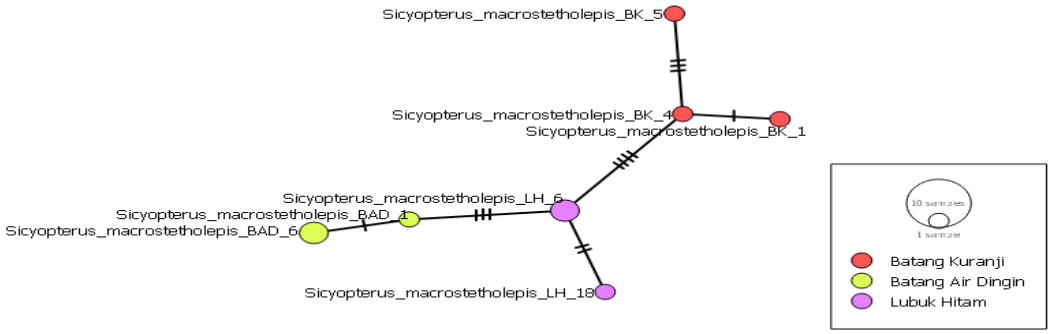

Fig. 3. Haplotype network of mungkuih fish (*S. macrostetholepis*)

Genetic diversity is an important basis of biodiversity. It is the result of long-term survival, evolution and adaptation of species **(Vida, 1994; Lande & Shannon, 1996)**. Two important indicators for measuring genetic diversity are haplotype diversity and nucleotide diversity. The *S. macrostetholepis* population as a whole is characterized by low nucleotide diversity (π = 0.00676), accompanied by high haplotype diversity (h = 0.944). Referring to **Nei (1973)**, this haplotype is included in the high category. This genetic diversity provides the potential for species to adapt to environmental changes and is key to the long-term development of species. Higher genetic diversity usually indicates that the species is more adaptable to the environment, while low genetic diversity results in lower fitness **(Provan & Bennett, 2008)**.

Genome analysis of different populations of *S. macrostetholepis* revealed that both haplotype diversity and nucleotide diversity were high in the LH (h = 0.66667, π = 0.00173) and BAD (h = 0.66667, π = 0.000860) populations. The BK population exhibited the highest values (h = 1.0000, π = 0.00345).

The BAD population (K = 0.66667) showed a lower K value compared to other populations, indicating a greater level of genetic similarity with the LH and BK populations. These lower values suggest that the BAD population may be genetically closer to other populations.

In contrast, the BK population (K = 2.66667) had a higher K value compared to other populations, indicating greater genetic differences from the LH and BAD populations. This could suggest significant genetic variation between the BK population and the other populations.

Table 2. Haplotype diversity analysis of the mungkuih fish (*S. macrostetholepis*) in Padang City

Population	Number of sequences	Number of segregating sites (S)	Number of haplotypes (h)	Haplotype diversity (Hd)	Average number of differences, (K)	Nucleotide diversity (π)
LH	3	2	2	0.66667	1.33333	0.00173
BAD	3	1	2	0.66667	0.66667	0.00086
BK	3	4	3	1.0000	2.66667	0.00345
Total	9	14	7	0.944	5.2222	0.00676

*LH (Lubuk Hitam), BAD (Batang Air Dingin), BK (Batang Kuranji).

Ju *et al.* (2013) also stated the same thing in the *S. japonicus* population. Moreover, this was also found by **Aboim *et al.* (2005)** in the fish *Helicolenus dactylopterus* and **Han *et al.* (2008)** on the fish *Plectorhinchus flavomaculatus*. Nucleotide and haplotype diversity provide historical information about the *S. macrostetholepis* population. **Grant**

and Bowen (1998) interpreted four basic scenarios for population history based on haplotype and nucleotide diversity. To explain that high haplotypicity in the population could be due to the large population size, environmental heterogeneity, and life history traits that support rapid population increases. *S. macrostetholepis* inhabits ecosystems with high environmental heterogeneity, experiencing specific life histories in freshwater and marine habitats. Haplotype is a signal that a fish has high immunity (less prone to carrying diseases and dying). However, since the results confirm that this particular fish is amphidromous, this also means that the mungkuih fish can only survive in a specific marine habitat: a fast-flowing river floor. Moreover, since he mungkuih fish is endemic in the rivers of Padang City, West Sumatra, the dams currently being built may cut off the fish's route from upstream to downstream, decreasing the area of river where this fish is able to live. The researchers urge for further studies on the management policies for the mungkuih fish in order to allow it to mature downstream.

4. Analysis of molecular variance (AMOVA)

AMOVA was carried out at the three research locations (Fig. 1), the results of the AMOVA analysis (Table 3) showed that the majority of genetic variation (47.98%) occurred between populations within groups (Inter Population). This shows that there are significant genetic differences between these populations. These differences can be caused by various factors, such as geographic isolation, environmental differences, or natural selection. Meanwhile, another 52.02% of genetic variation occurs within populations (Intra Population). This shows that there is quite high genetic variation within each population. Overall, the results of AMOVA analysis show that genetic variation in the three study populations is high. This variation is important for the survival of a population, because it can help the population adapt to environmental changes.

The Fst value obtained, amounting to 0.47977, indicates that there is a high level of genetic heterogeneity between the mungkuih fish populations in three different locations. The population differentiation index (Fst) is commonly used to express the level of genetic differentiation and genetic exchange between populations. According to Wright's criteria **(Wright, 1978)**, the genetic differentiation index can indirectly reflect the status of genetic differentiation and the level of gene exchange between populations (Fst < 0.05, mild genetic differentiation; 0.05 < Fst < 0.15, moderate inheritance differentiation; Fst > 0.15, high genetic differentiation). In this study, genetic divergence between *S. macrostetholepis* individuals was significant (P=0.00293, <0.05), and this was confirmed by the AMOVA analysis (Table 3). From AMOVA analysis it is known that most of the genetic variation in *S. macrostetholepis* comes from within the percentage of variation with population (52.02%).

Table 3. Analysis of molecular variance of *S. macrostetholepis* populations

Source of Variation	df	Sum of squares	Variance Component	Percentage of Variation	Fixation Index
Among Populations	2	12.556	1.53704	47.98 Va	Fst: 0.47977
With Population	6	10.000	1.66667	52.02 Vb	P-value = 0.00293
Total	8	22.556	3.20370		

Higher Fst values indicate that there are more genetic differences between populations. There are several possible causes for this genetic difference. One possibility is that the mungkuih fish populations in the three locations have been geographically isolated for some time. Geographic isolation can cause genetic differences because these populations cannot exchange genes with each other. Another possibility is that the mungkuih fish populations in the three locations have adapted to different environmental conditions. These adaptations can lead to genetic differences because populations have developed different genes to help them survive in their environments.

CONCLUSION

From the results, it can be concluded that the phylogenetic tree shows that the divergence between the outgroup and the Gobiidae family is 21.78-28.01%. In the Gobbidae family, the divergence level is 6.57-17.98%, in the *Sicyopterus* genus the divergence level is 6.57-12.02%. The genetic distance of the *S. macrostetholepis* population in Batang Air Dingin, Batang Kuranji, and Lubuk Hitam Rivers is 0.25-1.12%. *S. macrostetholepis* and *S. parvei* are the closest related species, followed by *S. longifilis* and *S. squamosissimus*. AMOVA shows that genetic variation (47.98%) occurs between populations within groups (Inter Population), and 52.02% of other genetic variation occurs within populations (Intra Population). This shows that there are significant genetic differences between Mungkuih fish populations in the large rivers of Padang City. It is hoped that the genetic differences in these three locations can become the basis for other genetic research that requires more samples as a domestication effort.

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