The molecular phylogenetic of payangga (*G. margaritacea*), manggabai (*G. giuris*) and hulu'u from Limboto Lake based on cytochrome B sequences

U Nuha[1], M Amin[2] and U Lestari[2]

[1]Faculty of Teacher Training and Education, University Jember, Jalan Kalimantan no. 37, Jember, Indonesia
[2]Faculty of Mathematic and Science, Universitas Negeri Malang, Jalan Semarang no.5, Malang, Indonesia

ulin.fkip@unej.ac.id

Abstract. Payangga (*G. margaritacea),* manggabai (*G. giuris*), and hulu'u are native fishes that live in Limboto Lake, Gorontalo. The aim of this research was to describe the molecular phylogenetic of payangga (*G. margaritacea),* manggabai (*G. giuris*), and hulu'u based on *cytochrome b* sequences. The phylogenetic three was reconstructed using MEGA 6.0 with Neighbour Joining (NJ) and Minimum Evolution (ME) methods. The result indicated that hulu'u was included in the same cluster with three comparable species, namely *P. microps, P. minutus*, and *C. chanos*, while *G. giuris* belongs to the same cluster as *G. margaritacea*.

1. Introduction

Manggabai, payangga, and hulu'u are common fish found in Limboto Lake, Gorontalo [1]. These fish are usually consumed by the people lived around the lake. Manggabai (*Glossogobius giuris*) belongs to family Gobiiidei of order Perciformes [2], while payangga (*G. margaritacea*) belongs to family Eleotridae of order Perciformes[3]. Both fish belong to suborder Gobioidei [2]. While manggabai and payangga have been widely studied and their scientific names are has been known, hulu'u is not known for its scientific name. Some people around the lake said that hulu'u is the mature phase of payangga, but some people said that hulu'u is different from payangga. The limited information about hulu'u is caused by the lack of literature review of this fish.

The phylogeny of gobioid fishes is still poorly understood [4]. This is because those species are morphologically reduced or it can be said that they lose some morphological aspect [4,5]. As a result, there are many morphological variations in species that make the process of identification has become confusing. This is confirmed by information obtained from the International Union for Conservation of Nature (IUCN) that further researches are needed to determine the taxonomy of those fish [3,6,7].
The phylogenetic study not only can be done based on morphology but also can be done by a molecular approach. Molecular phylogenetic uses DNA sequences as a source of character to study the line of descent and relationship among a broad group of organisms. DNA sequences data can explain the relationship of Gobioid and does not consider morphological reduction that can confuse as in morphological character analysis [6]. The molecular marker used in this research is cytochrome b (Cyt b). Cyt b is one of the protein-coding genes in mtDNA that is used as a marker for genetic analysis at low category level [8].

Manggabai generally lives in clear to turbid freshwater and also could live in estuarine habitat in sandy or gravel river flows that lots of stone [6]. Manggabai has an elongated body shape, thickened flat head with a prominent lower jaw. It has wide fins, has two dorsal fins, and the fins on the abdomen are fused. This type of fish is found in fresh and estuarine waters, usually living in muddy waters, rocky sand [9]. Figure 1 shows the ethanol-preserved specimen of manggabai.

Figure 1. Ethanol-preserved sample of manggabai (*Glossogobius giuris*).

Figure 2. Ethanol-preserved sample of payangga (*Giuris margaritacea*).

Figure 3. Ethanol-preserved sample of hulu'u.

Payangga (*Giuris margaritacea*) belongs to the Eleotridae family. This fish inhabits rocky estuaries and freshwater. It has a cylindrical body with flattened head in the dorsal part, generally greenish-brown with a transverse border on the edge with black spots on the pectoral fin. This species is often referred to as *Ophieleotris aporos* in some literature and there are approximately three species with this name, so further research is needed [3]. Figure 2 shows the ethanol-preserved sample of payangga *Giuris margaritacea*.

Hulu'u is usually found in rivers and estuaries, most commonly in freshwater. Information from this species is still confusing with other species that have not been described. According to the International Union for Conservation of Nature [7], this species is listed as "Data Deficient" so further research is needed. The ethanol-preserved sample of hulu'u is shown in figure 3.

Phylogenetics is the study of the relationship among groups of the organism based on evolutionary relationship and life history of a species [10]. Phylogeny is a hypothesis about the relationship among organisms that are described as the phylogeny tree. Phylogeny tree can be constructed from qualitative and quantitative data. Molecular phylogenetics is the study of evolutionary relationships among organisms based on molecular data such as DNA sequences and protein, insertion from a transposable element or another molecular marker [11].

There are some reasons why molecular data more suitable for evolutionary studies than morphological and physiological data. First, DNA and protein are strictly heritable entities. Second, the description of molecular characters and character states is unambiguous. Third, molecular traits generally evolve in a much more regular manner than do morphological and physiological characters and therefore can provide a clearer picture of the relationships among organisms. Fourth, molecular data are often much more amenable to quantitative treatments than are morphological data [11].

Cyt b is one of the mtDNA genes that is often used for phylogeny analysis. This gene is one of nine to ten genes that is involved in the oxidative phosphorylation system [12]. The position of the codon in cyt b develops quickly and slowly so that there are parts that are conservative and there are parts that are changed [13]. The cyt b gene also has many variations and is widely used because it undergoes rapid evolution that makes it suitable for knowing variations at the species level or below [14].

2. Methods

The research method is showed in figure 4. The first step was the DNA isolation. Total genomic DNA was isolated from ethanol-preserved of payangga, manggabai, and hulu'u. As much as 30 mg of ground fish fin tissue is placed in 5 µl of buffer STE and homogenized. Then 40 µl of Proteinase K and 50 µl of SDS were added and homogenized with the vortex. Samples were incubated in a shaking waterbath incubator at 55°C for 2 hours. As much as 50 µl NaCl 5M, 400 µl phenol and 400 µl chloroform isoamyl alcohol (CIA) were added into the sample and homogenized with vortex for 1,5 hours. Samples were centrifuged at 3000 rpm for 5 minutes. The supernatant was placed in a new tube and then 50 µl of 5M NaCl was added and incubated in the freezer for 1 hour. After incubated, the samples were centrifuged at 8000 rpm for 5 minutes. The supernatant was removed, 50 µl of TE buffer is added and stored at 4°C.

The second step was the DNA purification. As much as 50 µl of RNAse was added into 50 µl of DNA sample and then it was homogenized with the vortex. Samples were incubated at 37°C for 3 hours. After 3 hours, 200 µl of sterile aquadest, 200 µl of phenol, and 200 µl of chloroform were added into the samples. The samples were centrifuged at 8000 rpm for 10 minutes. The supernatant was removed into a new tube and then 25 µl of 5 M NaCl and 500 µl absolute were added. Samples were incubated at -20° C for 1 hour. After 1 hour, samples were centrifuged at 8000 rpm and 4°C for 10 minutes. Supernatant in the tube was removed and then 50 µl of TE buffer was added into the tube.

The third step was the DNA Amplification. The primers that were used in amplification are L1481 and H1549.As much as 5 µl forward primer L14841 (5'AAAGCTTCCATCCAACATCTCAGCATGATGAAA3') and reverse H15149 (5'AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA3'), 25 µl of PCR mix, 10 µl of dH2O and 5 µl of DNA template were added into the tube. This solution was homogenized with a vortex. The PCR tubes were put in the thermal cycler. The temperature of each stage was set at the thermal cycler. The PCR cycles were set for 35 cycles.

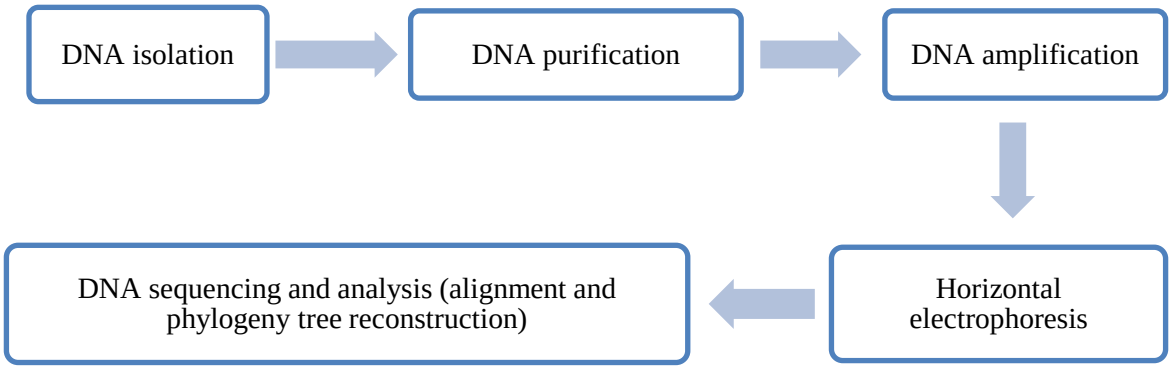

Figure 4. Research method.

The fourth step was the horizontal electrophoresis. The compositions of agarose gel were 1 ml of agarose, 30 ml of ETBR, and 1x20 ml of TBE. The gel composition was put into an Erlenmeyer flask and it was heated in a microwave for 3 minutes until homogenous and then it was cooled. As much as 30 µl of ETBR was put in Erlenmeyer flask and it was homogenized by gently shaking the flask. The

mixture was poured into the electrophoresis gel tray and was awaited until the gel is formed. The gel was placed into the electrophoresis tube and then the TBE 1x solution was poured until the gel is submerged. The DNA sample was inserted into the well.

The fifth step was sequencing and analysis. Cyt b gene amplicon was sent to PT Genetika science for sequencing. Cyt b sequences were aligned with cyt b sequences of 3 fishes, namely *Pomatoschistus microps* (*P. microps*), *Pomatoschistus minutus* (*P. minutus*), dan Chanos *chanos* (*C. chanos*). The cyt b sequences of those fish were obtained from Genebank (http://www.ncbi.nlm.nih.gov). The purpose is to determine the taxon position of sample fish. The phylogenetic tree was constructed using MEGA 6 software by Neighbour Joining (NJ) method [15] and Minimum Evolution (ME) method [16].

3. Research Finding

The results obtained from the process of DNA isolation, purification, PCR, horizontal electrophoresis, and sequencing were cyt b gene sequence data. Figure 5 shows the amplification result of the three sample fish Cyt b gene. The horizontal electrophoresis was done to detect the cyt b fragment from the process of PCR. The result show that cyt b sequences length of hulu'u, *Glossogobius giuris* dan *Giuris margaritacea* were respectively 287 bp, 286 bp, and 294 bp.

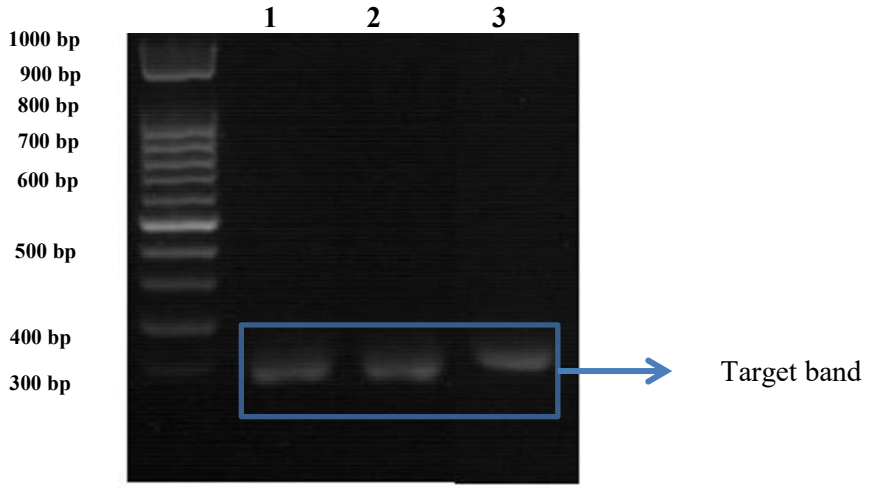

Figure 5. PCR amplification result of Cyt b gene by PCR: (1) Hulu'u, (2) *Glossogobius giuris*, and (3) *Giuris margaritacea*.

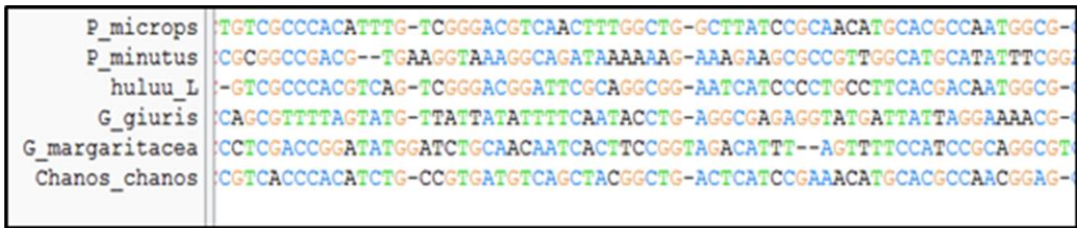

Figure 6. The alignment of sample fishes (Hulu'u, *G. giuris,* dan *G. margaritacea*) cyt b sequences with comparison fishes (*P. microps*, *P. minutus*, and *C. chanos*) using ClustalX.

Figure 6 shows the alignment result of cyt b sequences in sample fishes and comparison fishes using ClustalX software. ClustalX is used for carrying out multiple alignment of nucleotide sequences. The result of alignment is used to reconstruct phylogenetic tree to determine the taxon position of each species. The dashes (-) in the alignment result represents gaps in the sequence of one genome relative to its counterparts in the pairwise view. The gaps show the genetic mutation occurring from insertion or deletion (indel).

The results of genetic distance calculation using MEGA 6 software with the pairwise distance method are presented in Table 1. Genetic distance is the level of gene differences (genomic differences) between species or populations calculated based on the average number of differences in codons or nucleotides per gene. Table 1 shows the value of genetic distance between every two species that had been compared using MEGA 6 software. Calculation of genetic distance values is done by comparing phylogenetic trees into numerical values so that the genetic distance between individuals tested is known. Genetic distance is determined based on the number of nitrogen bases that change. Based on the results, the genetic distance between hulu'u and *G. giuris* is 1,781. The genetic distance between hulu'u and *G. margaritacea* is 1,812. The genetic distance between *G. margaritacea* and *G. giuris* is 1,354. The genetic distances between hulu'u and *G. giuris* is 1,172. The smaller the genetic distance, the greater the nucleotide base similarity between two species.

Tabel 1. The value of genetic distance between sample species (Hulu'u, *G. giuris,* and *G. margaritacea)* with comparison species (*P. microps, P. minutus,* and *C. chanos*)

	1	2	3	4	5	6
1. *P. Microps*						
2. *P. Minutus*	1,351					
3. Hulu'u	0,656	1,251				
4. *G. Giuris*	1,668	1,781	1,172			
5. *G. Margaritacea*	1,410	1,812	1,243	1,354		
6. *C. Chanos*	0,291	1,142	0,604	1,429	1,202	

Tabel 2. The value of similarity and genetic variation

Species 1	Species	Similarity	Varian
P. microps	*P. minutus*	-35,085	135,085
P. microps	Hulu'u	34,414	65,586
P. minutus	Hulu'u	-25,052	125,052
P. microps	*G. giuris*	-66,760	166,760
P. minutus	*G. giuris*	-78,081	178,081
Hulu'u	*G. giuris*	-24,299	124,299
P. microps	*G. margaritacea*	-41,041	141,041
P. minutus	*G. margaritacea*	-81,231	181,231
Hulu'u	*G. margaritacea*	-35,379	135,379
G. giuris	*G. margaritacea*	-17,200	117,200
P. microps	*C. chanos*	70,925	29,075
P. minutus	*C. chanos*	-14,234	114,234
Hulu'u	*C. chanos*	39,580	60,420
G. giuris	*C. chanos*	-42,875	142,875
G. margaritacea	*C. chanos*	-20,200	120,200

The value of similarity and genetic variation can be calculated based on the genetic distance. Table 2 shows the value of similarity and genetic variation between two species. The values of similarity and

genetic variation are used to determine the percentage of the relationship between species being studied. The higher similarity value, the higher similarity degree of the nucleotide sequences possessed by the two species. The lower value of variance between two species, the higher similarity degree of the nucleotide sequences.

Based on the table, hulu'u has the highest similarity with *C. chanos* (similarity = 39.58%), while *G. giuris* has the highest similarity with *G. margaritacea* (-17.20%). The genetic variation of hulu'u and *C. chanos* is 60,420, while the genetic variation of *G. giuris* and *G. margaritacea* is 117,200. So it can be said that hulu'u is closest relatives to C. chanos while G. giuris is the closest related to G. margaritacea.

4. Discussion

The sequences of three sample fish was aligned with cyt b sequences of *P. microps*, *P. minutus*, dan *C. chanos*.The cyt b sequences of those comparison fishes was obtained from Genebank. Alignment is the process of comparing homologous sequences by identifying the location of insertions or deletions that may occur in two lineages since splitting from a common ancestor [11]. The result of alignment is used to reconstruct phylogenetic tree to determine the taxon position of each species.

Based on phylogenetic trees formed, both by the NJ and ME methods, *G. giuris* and *G. margaritacea* are sister groups and hulu'u is outgroup of both species. It means that *G. giuris* and *G. margaritacea* are two species that are descended from the same last ancestor. Two descendants that separate from the same node are called sister groups, while outgroups are taxon outside the sister group that is used to provide a clearer picture of the evolutionary path of the taxon being studied [15].

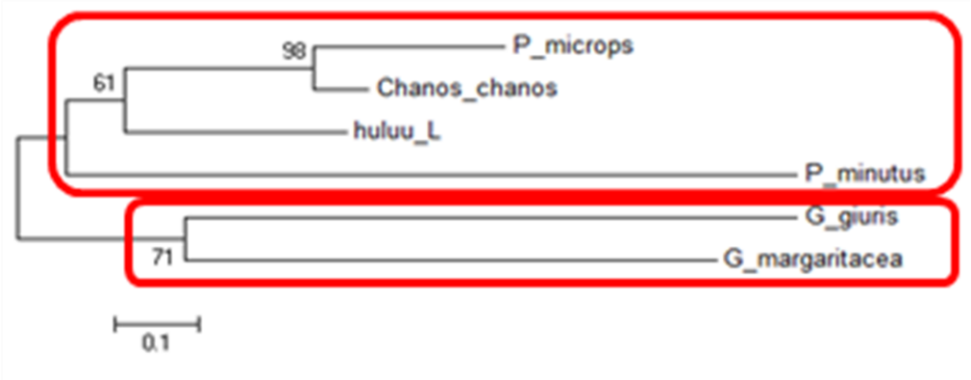

Figure 7. The phylogenetics tree topology of hulu'u, *G. giuris* dan *G. margaritacea* with comparison fishes based on cyt b sequences using NJ method.

The phylogenetic tree reconstruction using the NJ method produces one type of tree, whereas with the ME method all the possibilities of trees will be searched and compared to produce the best phylogeny tree [16]. The results of phylogenetic tree reconstruction using the NJ method showed the formation of two groups or clusters (figure 7). The taxon position can be determined by reconstructing the phylogenetic tree.

The phylogenetic tree that is reconstructed by the ME method is not much different from the phylogenetic tree that is reconstructed by the NJ method. Based on figure 6, phylogenetic trees that are formed are also divided into two clusters. The grouping is the same as the grouping of phylogenetic trees reconstructed using the NJ method. Hulu'u is belonged to the same cluster with three comparison species, namely *P. microps, P. minutus*, and *C. chanos*, while *G. giuris* and *G. margaritacea* belong to the same cluster.

5. Conclusion

This research results implies that the relationship of *G. giuris, G. margaritace* and hulu'u can be determined by reconstructing the phylogenetic tree based on cyt b sequences. Based on cyt b sequences, the hulu'u is closely related to the comparison fishes (*P. microps, P. minutus*, and *C. chanos*) while *G. giuris* is closely related to *G. margaritacea* which both originate from Limboto Lake. The similarity between hulu'u and *C. chanos* is 39,58%, while the similarity between *G. giuris and G. margaritacea* is -17,20%. It is expected that the reconstruction of this phylogenetic tree can be developed into learning material that can be used in learning Evolution.

Acknowledgment

We would like to thank Mohamad Amin and Umie Lestari, Faculty of Mathematic and Science, State University of Malang for the support and guidance during this study. We gratefully acknowledge the support from The Faculty of Teacher Training and Education, University of Jember.

References

[1] Environmental Information System Of Gorontalo Province 2015 Biodiversity http://sil.blhrd.gorontaloprov.go.id/ buku-laporan/13-keanekaragaman-hayati#typkabgor.
[2] Bayot F A A, Joya K N A and Jacinto W R 2014 Phylogenetic analysis and barcoding of gobies in Maragondon River, Cavite, Philippines *Current Sciences Journal* Vol 12: 50-56
[3] Larson H 2012 *Giuris margaritacea,* Snakehead Gudgeon The IUCN Red List of Threatened Species 2012: T196317A2444751 http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T188101A1854106.en
[4] Argorreta A, Mauro D S, Schliewen U, Tassel J L, Kovacic M, Zardoya R, and Ruber L 2013 Molecular phylogenetics of Gobioidei and phylogenetic placement of Euoropean gobies. *Molecular Phylogenetics and Evolution Vol 69: 619 – 633.*
[5] Thacker C E and Hardman M A 2005 Molecular phylogeny of basal gobioid fishes: Rhyacichthyidae, Odontobutidae, Xenisthmidae, Eleotridae (Teleostei: Perciformes: Gobioidei) *Molecular Phylogenetics and Evolution* Vol: 37: 858-871.
[6] Larson H and Britz R 2012 *Glossogobius giuris* The IUCN Red List of Threatened Species 2012 http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T166533A1136011.en
[7] Larson H 2012 *Glossogobius celebius* The IUCN Red List of Threatened Species http://dx.doi.org//10.2305 IUCN.UK.2012.RLTS.T196317A2444751.en.pdf
[8] Arif I A and Khan H A 2009 Molecular markers for biodiversity analysis of wildlife animal: a brief review. *Anim. Biodiv and Conserv* Vol 32 (1): 9-17
[9] Suryandari A and Krismono 2011 Some Biological Aspects of Manggabai Fish (*Glossogobius giuris*) in Limboto Lake *BAWAL* Vol.3. No. 5. Hal 329-336.
[10] Brown T A 2002 Genomes 2nd. Magdalen Road Oxford UK: BIOS Scientific Publisher Ltd.
[11] Graur D and Li W H 2000 Fundamentals of Molecular Evolution Second Edition USA: Sinauer Associates, Inc.
[12] Hatefi Y 1985 The mitochondrial electron transport and oxidative phosphorylation system. *Annu. Rev. Biochem.* 54: 1015 – 1069.
[13] Farias I P G, Ortı I, Sampaio H, Schneider A M 2001 The Cytochrome *b* Gene as a Phylogenetic Marker: The Limits of Resolution for Analyzing Relationships Among Cichlid Fishes *Journal Molecular Evolution* 53:89-103.
[14] Bruford M W, Bradley D G and Luikart G 2003 DNA markers reveal the complexity of livestock domestication *Genetics*, Vol 4:900-910.
[15] Saitou N and Nei M 1987 The Neighbor-Joining Method: A New Method for Constructing Phylogenetic Tree *Molecular Biology Evolution.* 4: 406-425.
[16] Rzhetsky A and Nei M 1992 A Simple Method for Estimating and Testing Minimum Evolution Tree *Molecular Biology Evolution,* 9: 945-967.