

Genetic and morphological analysis of complex *Giuris* group species from Limboto Lake, Gorontalo Province, Indonesia

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Abstract. Lamadi A, Paricahya AF, Iranawati F, Widodo MS, Wiadnya DGR. 2023. Genetic and morphological analysis of complex *Giuris* group species from Limboto Lake, Gorontalo Province, Indonesia. *Biodiversitas* 24: 5223-5233. Wallacea, especially Sulawesi, has a high endemism level with genuine biodiversity for mammals, reptiles, freshwater, and brackish fishes. Fish biodiversity in Limboto Lake, a great natural lake of Gorontalo Province, Indonesia, still needs to be scientifically explored, in contrast with its utilization for human activities that have anthropogenic effects on their ecosystem. Eleotridae is a taxonomical complex to their identification and distributed well in Sulawesi, including Limboto Lake with genus *Giuris*, known locally as *hulu'u* fish. Ambiguous identification in *Giuris margaritaceus* morphologically causing misidentification in molecular analysis, including using Cytochrome Oxidase subunit I (COI) region's information. *Giuris* specimens from Limboto Lake were collected from June 2022 until May 2023. From 662 individuals, length and weight relationship analysis results showed the population is mildly positive allometric ($W = 0.11TL^{3.08}$). Meristic measurement result (D1 VI; D2 I.8-9; A I.9; P 14-16; V I.5; C 13-14) directing to *G. margaritaceus* with several small differences. COI analysis using six random specimens of the Limboto Lake population, comparing with four population from NCBI and BOLD System identified as *G. margaritaceus* or its synonym *Ophieleotris aporos*, indicating all populations is separated species from one another with P-distances more than 2% and made separated groups in phylogenetic tree and haplotype network. Misidentification issues in the *Giuris margaritaceus* taxonomical name for all five populations indicate they're potentially separated into five different species, with or without including *Giuris margaritaceus*, and potentially from currently described species or being new species in the future. Limboto Lake population made three haplotypes with high haplotype diversity (0.733) and low nucleotide diversity (0.001), indicating this population is facing high stressors and starting to grow. Understanding *Giuris* ambiguities could help governments, researchers, conservationists, and other stakeholders to mapping the ecological priorities.

Keywords: Cryptic species, Gorontalo, gudgeon, isolation, mitochondrial DNA

Abbreviations: W: Weight, D: Dorsal fin, A: Anal fin, P: Pectoral fin, V: Ventral fin, C: Caudal fin, NCBI: National Center for Biotechnology Information, BOLD System: Barcode of Life Data System

INTRODUCTION

The Sulawesi islands in the Wallacea region are areas of biodiversity with the highest endemism in the world; endemism in this region is unique. The Wallacea region is the dividing line between the continents of Asia and Australia; this region includes the islands of Sulawesi, Lesser Sunda, Maluku, and Timor Leste. This area has at least 62 endemic vertebrate genera, some of which are well-known are the maleo bird (*Macrocephalon* sp.), tarsier (*Tarsius* spp.), pig-deer (*Babyrousa* spp.), Anoa (*Bubalus* spp) and cuscus (*Ailurops melanotis*) originating from Sulawesi (Bashari et al. 2017; Ito et al. 2017; Froese and Mustari 2019; Repi et al. 2020; Sumampow et al. 2020; Priyono et al. 2022; Struebig et al. 2022). Endemic species in the Wallacea region are not closely linked to land animals; limiting distribution of this area also occurs in

freshwater fish. Many endemic fish species in Sulawesi, from families Adrianichthyidae, Zenarchopteridae, Telmatherinidae, and Gobiidae, but potentially including Eleotridae, especially *Giuris* from their complex *G. margaritaceus*. There are at least 22 endemic species of Adrianichthyidae in Sulawesi from Celebensis group (Sudasinghe et al. 2022), and Adrianichthyidae new species in recent years are *Oryzias kalimpaaensis* (Gani et al. 2022) and *Oryzias loxolepis* (Kobayashi et al. 2023). Furthermore, there are at least 14 endemic species from Zenarchopteridae (Kobayashi et al. 2020), four species from Telmatherinidae (Chadijah et al. 2020), and two species from Gobiidae, and one from Anguillidae (Bandjolu et al. 2021).

The limited distribution of species is the cause of the importance of study and conservation in the area, given the high threat of damage it faces. Anthropogenic damage to the Wallacea area was triggered by population growth,

including in the Limboto Lake area, Sulawesi. Limboto Lake is one of the large lakes in Sulawesi, as referred by (Yunginger et al. 2018), which is significantly degraded due to increased human populations. The anthropogenic impact of the human population has resulted in at least a reduction of 3,000 hectares of the lake area for settlement. Due to sedimentation, the lake silting makes Limboto Lake with an initial depth of 5-8 meters, and from now on, only 2-4 meters remain. Metal pollutants in Limboto Lake sediments such as Fe, Mn, Pr, Gd, La, Sc, Nd, Cu, and Zn were abundant. Closed lacustrine systems, such as Limboto Lake, tend to deposit waste in sedimentation more than open systems, such as the sea.

Eleotridae is one of Indonesia's most common families occupying the middle and lower reaches of rivers. Identification of the Eleotridae family is morphologically complex, as in Keith et al. (2020); the meristic characteristics of the Eleotridae family overlap with body shape and similar coloring. This identification difficulty also occurred in the *Giuris* genus, where several populations were later described as new species. The *Giuris* genus was initially only assigned to one species, *G. margaritaceus*. Still, as Keith et al. (2021) discussed, this species has wide distribution and may consist of several cryptic species. This species is widely distributed in lowland waters, from downstream rivers to sea estuaries. The known distribution of species from the genus *Giuris* in Indonesia is *G. aporocephalus*, *G. laglaizei*, *G. margaritaceus*, *G. tolsoni*, and *G. viator*, the five species have minor morphological differences. Furthermore, all of these species are reported in Sulawesi, making the identification of *Giuris* morphologically and genetically a critical effort to carry out a diversity inventory (Ndobe et al. 2023). This study

aimed to identify the genus *Giuris*, especially *G. margaritaceus*, in Lake Limboto using morphological and genetic approaches to obtain valid data for conservation.

MATERIALS AND METHODS

Study area

This research was conducted from June 2022 to May 2023 at Lake Limboto, Gorontalo Province, Indonesia, at coordinates 0°35'2" N 122°58'48"E (Figure 1), with an average temperature of 26.1 to 30.6°C, rainfall of 80.5 to 204.2 mm. Fish samples were taken from 6 observation stations using gill nets with a length of 10 m, a height of 3 m, and a mesh size of 1 cm, consisting of 6 stations; Station 1 (0°36'12.80"N 123° 0'8.03"E); Station 2 (0°34'57.00"N 123° 0'23.93"E); Station 3 (0°33'38.48"N 123° 0'18.93"E); Station 4 (0°34'4.49"N 122°58'28.91"E); Station 5 (0°35'18.00"N 122°58'48.91"E); Station 6 (0°34'35.71"N 122°59'26.37"E).

Morphology

Fish were measured for their morphometric characters using all counts and measurements taken from the left side of the fish, a total of 10 measurements of morphometric characters (Figure 2); then, we compare the results of each morphometric character measurement with the percentage of standard length. Furthermore, six parameters were counted for meristic characters as anterior dorsal fin spines/rays (D1), Posterior dorsal fin, spines/rays (D2), Anal fin spines/rays (A), Pectoral fin rays (P), Ventral fin rays (V), Caudal fin rays (C).

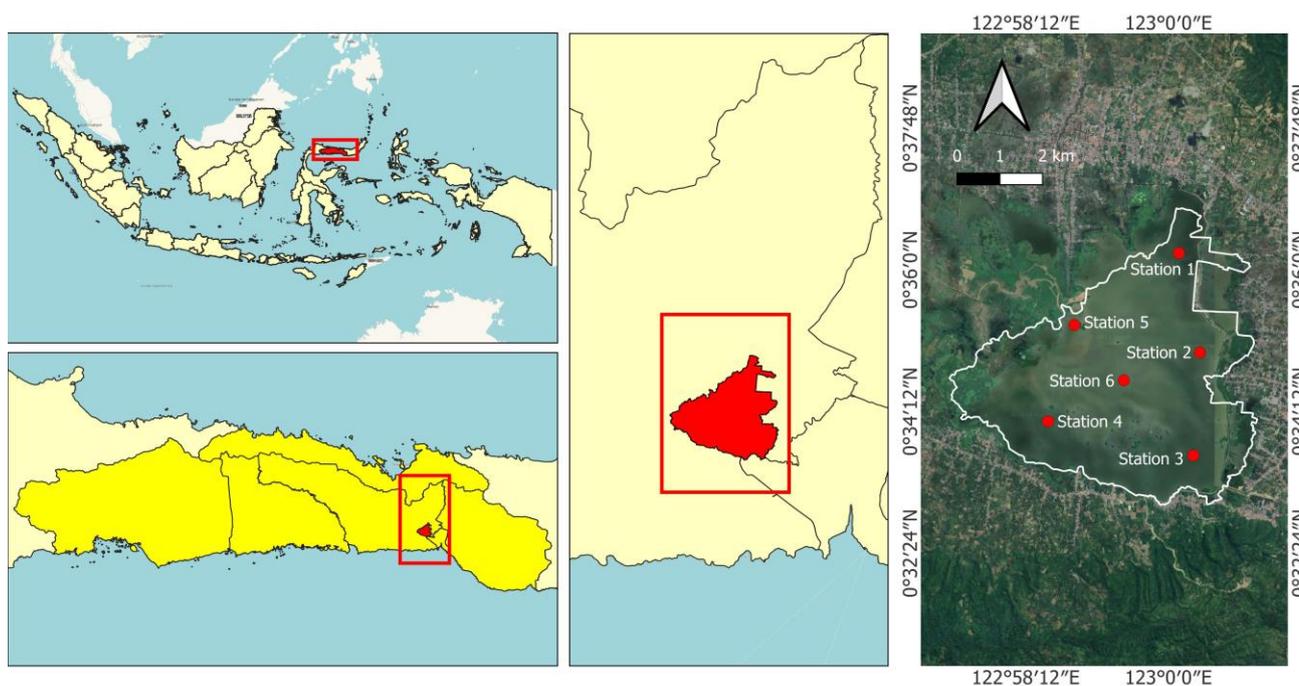


Figure 1. Observation sites surround Lake Limboto, Gorontalo Province, Indonesia

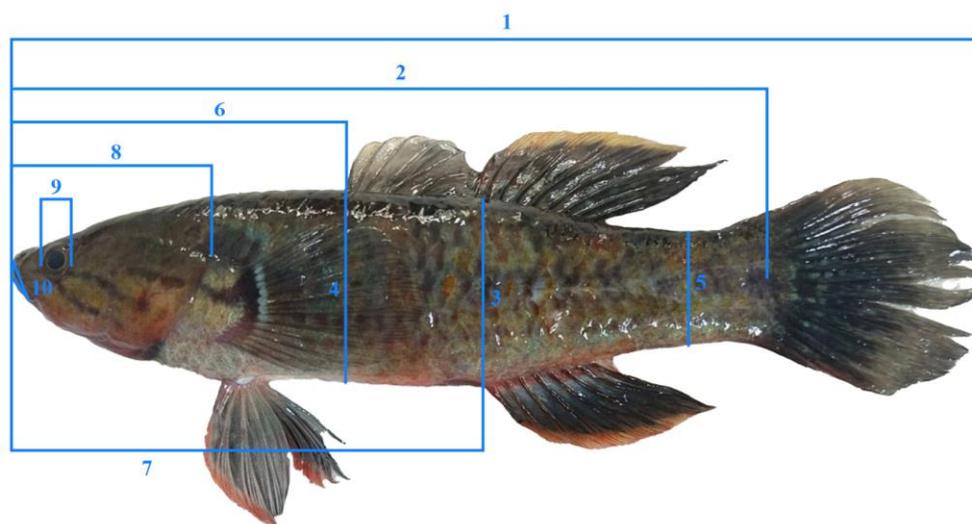


Figure 2. Sketch of the morphometric measurements used in this study: 1. Total Length (TL), 2. Standard Lengths (SL), 3. Body Depth at the Anus (BDA), 4. Body Depth at the first dorsal Fin (BDF), 5. Caudal Peduncle Depth (CPD), 6. Pre-Dorsal Length (PDL), 7. Pre-Anal Length (PAL), 8. Head Length (HL), 9. Eye Diameter (ED), 10. Jaw Length (JL)

Table 1. Details of *Giuris* sequences from Limboto Lake

Specimen tag code	Deposit number	Accession number	TL	SL	BDA
LMB_GMA 001	DIB FISH.1.457.1518	OQ788245.1	19.22	14.10	3.22
LMB_GMA 002	DIB FISH.1.457.1518	OQ788246.1	15.79	12.01	2.84
LMB_GMA 003	DIB FISH.1.457.1518	OQ788247.1	11.32	8.82	1.95
LMB_GMA 004	DIB FISH.1.457.1518	OQ788248.1	8.47	6.40	1.35
LMB_GMA 005	DIB FISH.1.457.1518	OQ788249.1	13.44	10.52	2.49
LMB_GMA 006	DIB FISH.1.457.1518	OQ788250.1	18.78	14.46	3.16

Genetic

DNA extraction, amplification and sequencing

DNA extraction used the Wizard® Genomic DNA Purification Kit protocol as stated in (Kurniawan et al. 2022); the initial stage was that the 20 mg tissue specimen was crushed manually using sterile surgical scissors on a 1.5 mL microtube. A mixture of 500 µL nuclei lysis solution and 120 µL of 0.5 M EDTA was cooled for 10 minutes and then added to a 600 µL microtube, then 17.5 µL proteinase K was added. Microtubes were then incubated overnight at 55°C. After cooling to room temperature, the microtube was added with 200 µL of protein precipitation solution, vortexed for 2 seconds, and centrifuged for 4 minutes at 14,500 rpm. 600 µL of the supernatant was transferred to a new 1.5 mL microtube, then 600 µL of isopropyl alcohol was added and centrifuged again for 1 minute at the same speed. The supernatant was discarded, 600 µL 70% EtOH was added, and then centrifuged at the same speed. The supernatant was discarded, and the pellet air-dried for 10-15 minutes; 100 µL of DNA rehydration solution was added and incubated for 1 hour at 65°C with gentle tapping every 30 minutes. Isolates were stored at -4°C.

DNA amplification in PCR tubes as in Wiadnya et al. (2023) with slight modifications, using 15 µL of a mixture of taq DNA polymerase, MgCl₂, and dNTPs in GoTaq® Green Master Mix, 11 µL of ddH₂O, 2 µL of DNA template (isolate), and 1 µL of F1 primer and 1 µL of R1 primer. Partial amplification of the Cytochrome Oxidase

subunit I (COI) region of 655 bp as in Ward (2012), using the universal primers F1 and R1 for fish with the F1: 5'TCAACCAACCACAAAGACATTGGCAC3' and R1: 5'TAGACTTCTGGGTGGCCAAAGAATCA3'. The thermal cyclor was set for 35 cycles with 2 minutes of predenaturation time at 96°C, 15 seconds of denaturation at 94°C, 1-minute annealing at 55°C, 5 minutes of elongation at 72°C, and 10 minutes of final elongation at 72°C. Amplicon was tested qualitatively by electrophoresis, gel density 1.5%, mixed with 2 µL ethidium bromide and buffer solution using 1x TBE. Running electrophoresis was conducted within 30 minutes at 90 Volt and 60 Ampere settings. Amplicons with good qualitative test results were then sent to PT. Genetics Science Indonesia for sequencing.

Data analysis

Genetic analysis applications

Chromatogram processed as in Wiadnya et al. (2023) with a few modifications first used Chromas 2.6.6 to eliminate noise in both forward and reverse sequences. Sequences were then converted into consensus by combining the forward and reverse sequences to become contig using UGENE 46.0. Consensus was included in the data set with other sequences after identification using the BLAST feature on NCBI (www.ncbi.nlm.nih.gov). Limboto Lake population sequences submitted to NCBI, deposit number at Brawijaya University, and accession number on NCBI can be seen in Table 1. The data set was added with

collected data from NCBI, both ingroup and outgroup. In this study, the outgroup is the *Ophiocara porocephala* species. The data set was aligned, cut, and confirmed to have no stop codons using the Mesquite 3.70 application, exported, and continued to make phylogenetic estimates. The alignment result data set was opened in the Molecular Evolutionary Genetic Analysis 11 (MEGA 11) application to create a phylogenetic. The statistical algorithm in this study uses Maximum Likelihood (ML), 1,000 times replication with the Kimura 2-parameter model, and combined with rates among sites I (has invariant sites). The haplotype network in this study was created using the Network 10.2.0.0 application, as was also used with the previous version in the article by Corrigan et al. (2018); the aligned data set format was changed using the DnaSP v6.12.03 application to an RDF file. Haplotype network construction using the median-joining network. Genetic variation can be seen in the DnaSP analysis as in the study by Dasuki et al. (2023) to see the polymorphism of fish COI data sets.

RESULTS AND DISCUSSION

This study showed that *G. margaritaceus* in Limboto Lake had a cylindrical body shape and a combination of blackish brown, green, and orange colors with black spots almost all over his body except for the abdomen to the anus. The color is greenish brown, with black spots on the pectoral fins. This fish has a small head with a superior mouth position and cycloid-type inserts, two dorsal fins, one pair of pelvic and pectoral fins, anal fins, and a rounded caudal fin.

Length and weight relationship

The relationship between length and weight is very important to evaluate the growth and population of fish populations and assess fisheries management (Dinh et al. 2022). In addition, growth patterns are also critical in understanding the ecological adaptation of fish. The measurement results of the relationship between the total length and total weight of fish provide important information in formulating fisheries management tools for wise exploitation (Al-Otaibi et al. 2022). This relationship can also indicate the welfare and suitability of a particular fish species with the environment. The relationship between length and weight in fish is allometric and isometric. Isometric growth is defined as the increase in the length of the fish along with the increase in body weight, while allometric growth is the increase in length faster or slower than the increase in body weight (Ndobe et al. 2023).

Giuris margaritaceus males have a total length range of 6.6-20.7 cm with an average of 10.5 ± 2.2 cm and a total weight range of 1.8-65.9 g with an average of 10.4 ± 9.2 g. Meanwhile, female *G. margaritaceus* fish have a total length range of 6.5-20.9 cm with an average of 11.8 ± 2.9 cm and a weight range of 2.2-68.0 g with an average of 16.2 ± 12.23 g. Looking at the relationship between length and weight and also the graphs that have been obtained, it can be concluded that the number of fish observed in female fish with a total of 215 specimens, the relationship between length and weight is $W = 0.14 \cdot TL^{2.92}$ with a value of $R^2 = 0.88$ (Figure 3A). The equation for the relationship between length and weight obtained a value of 0.14 and b value of 2.92, which indicates that the growth pattern in female fish is negative allometric, that is the growth in length is faster than the growth in weight.

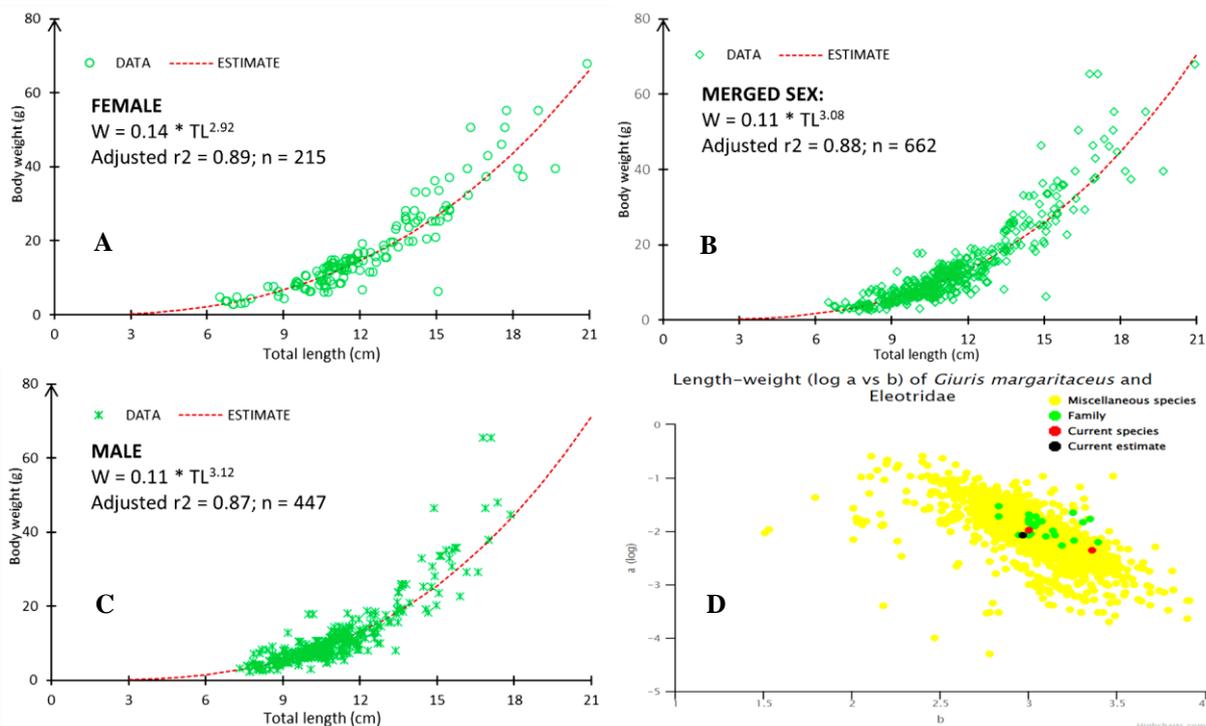


Figure 3. Length and weight relationship of A. Female n=215, B. Merged sex=662, C. Male n=447, D. Plot of length-weight relationships (log a vs. b) available for *G. margaritaceus*

Moreover, in 447 males (Figure 3C), fish had a relationship between length and weight, namely $W = 0.11TL^{3.12}$, with a value of $R^2 = 0.87$. The equation for the relationship between length and weight obtained a value of 0.11 and b value of 3.12, which indicates that the growth pattern in fish is positive allometric; that is, the growth in weight is faster than the growth in length.

Analysis of the relationship between length and weight of the fish as a whole had (Figure 3B) a total length range of 6.5-20.9 cm with an average of 10.9 ± 2.5 cm and a total weight range of 1.8-68.0 g with an average of 12.3 ± 10.7 g. Based on the model of the relationship between length and weight and also the graph that has been obtained, it can be concluded that the number of fish observed, with a total of 662 fish, has a relationship between length and weight, namely $W = 0.11TL^{3.08}$ with a value of $R^2 = 0.88$ and a value of $r = 0.94$. The equation for the relationship between length and weight obtained a value of 0.11 and b value of 3.08, which indicates that the growth pattern in fish is positive allometric; that is, the growth in weight is faster than the growth in length.

The analysis of the relationship between length and weight of *G. margaritaceus* based on sex and overall (merged sex) showed different growth patterns, namely negative and positive allometric. The allometric growth pattern is temporary due to changes related to gonadal maturity, while the isometric growth pattern is a proportional change that occurs continuously (Phan et al. 2021). In the study of the relationship between length and weight, *G. margaritaceus* from Lake Bolano Sau had a positive allometric growth pattern (Ndobe et al. 2023). Likewise, the research by *G. margaritaceus* from Lake Tondano has a positive allometric growth pattern, which means the growth rate in body weight is faster than in length (Makmur et al. 2019).

Based on the study results of the length and weight relationship of *G. margaritaceus* fish in Lake Limboto, namely positive and negative allometric. The analysis showed that the condition factor for *G. margaritaceus* fish ranged from 0.12-1.91, meaning that *G. margaritaceus* fish had good averageness (Auliyah 2019). Fish flatness is influenced by the environment, food availability, and diseases that attack fish.

Each fish must have a different length and weight because it is influenced by the season and sex. Also, the length-weight relationship between male and female fish can differ due to gonad development in each individual (Dinh et al. 2022). Apart from differences in species, factors that can affect growth patterns include environmental factors, differences in fish stocks, fish development, sex, gonadal maturity levels, and differences in time of day related to changes in stomach contents (Famoofo and Abdul 2020). Furthermore, several factors affect the growth pattern of fish, including gobies, namely sexual, intraspecific, and spatiotemporal variables (Phan et al. 2021).

Meristic count

Morphology is determined by morphometric measurements and meristic characteristics, which are important characters in species identification (Yanti et al.

2022). Meristic is a word primarily used to quantify the character of each organism and plays a major role in observing differences between fish populations in different regions of the world (Masood et al. 2021).

The meristic character of *G. margaritaceus* consists of 6 spines on the anterior dorsal fin, 1 spine and 8-9 rays on the posterior dorsal fin, 1 spine and 9 rays on the anal fin, 14-16 rays on the pelvic fins, 1 spine and 5 rays on pectoral fins, and 13-14 rays on the caudal fin. The results of this study did not have any striking differences. Compared to research conducted on similar fish, the results were relatively the same in the parameters measured, namely 6 spines on the anterior dorsal fin, 1 spine, and 7-9 rays on the posterior dorsal fin, 1 spine and 8-10 rays on the anal fin, 12-16 rays on the pelvic fins, 1 spine and 4-5 rays on pectoral fins, and 11-17 rays on the caudal fin (Makmur et al. 2019; Keith et al. 2020; Ndobe et al. 2023).

In addition to Table 2, counts are also carried out on the lateral row scale, which found 26-28 scale series and 16 predorsal scales in the counts. In contrast (Kottelat and Whitten 1996) states that the lateral row scale is 30 scale rows, and the predorsal scale is 13-18. From the difference in meristic counts, the morphology of *G. margaritaceus* is still not defined because many species variations have yet to be revealed (Kottelat 2013).

Differences in morphometric characteristics are caused by many variables, including temperature, dissolved oxygen, ammonia, salinity, photoperiod, competition, quantity and quality of food available, age, mortality rate, and interactions between these variables to influence fish growth, which can cause differences in morphometric characteristics (Bertin et al. 2020; Sintondji et al. 2020; Yulianto et al. 2020). Each species will have a different absolute size from each other. Differences in fish size are caused by age, gender, environment, and environmental factors such as food, temperature, pH, and salinity. Morphometric parameters in fish are important in determining whether there are differences between the same species from various geographic regions (Khanom et al. 2020).

Morphology

We tried to compare the results of measuring the morphometric characters in this study with those obtained by Keith et al. 2021. The results of measuring the morphometric truss ratio (% SL) in this study and compared with Keith et al. 2021, showed there are slight differences in the morphometric characters, including PDL 43.1-49.7% (compared with 43-46%), PAL 58.1-67.0% (compared with 59-66%), HL 29.1-33.2% (compared with 30-33%), while other measurements are relatively the same, such as BDA 20.6-23.8% (compared with 20-24%), BDF 21.1-24.8% (compared with 20-25%), CPD 12.6-15.7% (compared with 13-16%), ED 5.9-8.5% (compared with 6-8%), JL 9.7-10.4% (compared with 10-11%). Furthermore, mean and deviation standard values showed in Table 3.

Principal Component Analysis (PCA) results on the correlation matrix of morphometric character data from 40 specimens of hulu'u fish and 8 characters yielded variance in the main components. The first principal component (C1) was found to be 3.15 and could explain the data

variance of 52.45% of the total variance. The second principal component (C2) was obtained at 1.30 and could explain the variety of data by 21.67%. The first and second characteristic roots could explain the diversity of the data by 74.13% and were sufficient to explain all the data because they were >70%. The first principal component (C1) is in the BDA, BDF, CPD, and PDL landmarks, while the second principal component (C2) is in HL and JL.

Many variables, such as water temperature, dissolved oxygen, ammonia, salinity, photoperiod, competition, the amount and quality of food available, age, mortality rate, and the interactions between these variables to affect fish growth, might cause differences in morphometric features (Bertin et al. 2020; Sintondji et al. 2020; Yulianto et al. 2020). Each species will have a different absolute size from one another. Differences in fish size are caused by age, sex, environment, and environmental factors such as food, temperature, pH, and salinity. Morphometric parameters in fish have a significant role in determining whether there are differences between the same species from various geographical areas (Khanom et al. 2020).

Genetic

The data set for all populations has been aligned, and the length of each sequence is 600 bp with the content of the pair Adenine+Thymine (A+T), which is always more dominant in each population than the pair Guanine and Cytosine (G+C). The highest A+T pair in the *Giuris* genus was in the Limboto Lake population, 52%, and the lowest in the Bali Island population, 50.6%. The G+C pair is the opposite of the A+T pair. The composition comparison percentage, including the outgroup, is 51.4% A+T and 48.6% (Table 4).

Giuris spp. population genetic variation for Hd ranges from 0.700 to 1.000, while for Pi, it ranges from 0.00133 to 0.00958 (Table 5). The highest Hd value is for the Java Island population, with a value of 1, and the lowest is 0.700 for the Central Sulawesi population. The Filipino population

owns the highest Pi value, 0.00958, and the lowest is 0.00133 for the Central Sulawesi population.

The Limboto Lake population formed 3 haplotype groups from 6 sequences for population representation, the Philippines population formed 8 haplotype groups from 17 sequences, the Bali Island population formed 7 haplotype groups from 10 sequences, the Java Island population formed 3 haplotype groups from 3 sequences, and the Central Sulawesi population formed 3 groups haplotypes from 5 sequences. Usually, minimum number of individuals for variety analysis in genetics is 5 to 10 specimens, but occasionally 20 (Lande 1981). Nucleotide site mutations (Table 6) in the Limboto Lake population from this study's 600 bp data set are present at the 236th and 464th sites.

The Limboto Lake population forms a separate clade from the GenBank comparison population, namely the Philippines, Java Island, and Bali Island populations (Figure 4). The Philippines population is the closest to the Limboto Lake population from phylogenetic estimation using a maximum likelihood of 1000 times. Both have a monophyletic relationship, but from the haplotype network, it can be seen that the differences in bases occur at more than 23 sites.

Table 3. The results of measuring the morphometric truss of *G. margaritaceus*

Truss morphometrics	Average truss morphometrics ratio (% SL)	
	<i>This study</i> (n=40)	<i>G. margaritaceus</i> (n=24)
BDA	22.0±0.4	20-24
BDF	22.8±0.5	20-25
CPD	14.5±0.4	13-16
PDL	46.0±0.8	43-46
PAL	62.1±1.3	59-66
HL	30.9±1.1	30-33
ED	7.3±0.7	6-8
JL	10.6±0.6	10-11

Note: average truss morphometrics ratio % of SL and standard deviation

Table 2. Meristic count for *Giuris* from this study and from several references. Specimens number in this study was adjusted to references used

Species	N	Fin spines and rays					
		D1	D2	A	P	V	C
<i>Giuris</i> sp. (This study)	40	VI	I.8-9	I.9	14-16	I.5	13-14
<i>G. margaritaceus</i> ¹	10	VI	I.9	I.9	16		
<i>G. margaritaceus</i> ²	12	VI	I.8	I.9	14-15	I.5	13-14
<i>G. aporocephalus</i> ²	12	VI	I.8	I.9	14-15	I.5	13-14
<i>G. laglaizei</i> ²	42	V-VI	I.7-8	I.8-10	12-15	I.4-5	11-17
<i>G. tolsoni</i> ²	11	VI	I.8	I.9	14	I.5	13-15
<i>G. viator</i> ²	10	VI	I.8	I.9	14	I.5	13-14

Note: ¹Makmur et al. 2019; ²Ndobe et al. 2023

Table 4. Nucleotide content from all populations of *Giuris* in the data set. Combination of Adenine+Thymine always dominant than Guanine+Cytosine

Nucleotide content	General	Limboto Lake	Philippines	Bali Island	Java Island	Central Sulawesi	Outgroup
Guanine+Cytosine	48,6%	48,0%	48,1%	49,4%	48,9%	49,2%	47,9%
Adenine+Thymine	51,4%	52%	51,9%	50,6%	51,1%	50,8%	52,1%

The base differences in the two populations align with their genetic p-distance between the Philippines and Limboto Lake, which reaches 3.3%. Furthermore (Table 7), the Limboto Lake population has paraphyletic relationship with the Java Island population, with a genetic p-distance of 6.3%. The Bali Island population has the farthest relationship from the Limboto Lake population, with a polyphyletic relationship. The genetic p-distance between the two populations is 11.8%. The genetic p-distance of the entire *Giuris* population with the outgroup, namely *Ophiocara porocephala*, was the lowest at 21.9% (Limboto Lake population) and the highest at 23.2% (Philippine

population). The highest intrapopulation genetic p-distance was in the Philippine population, namely 1%.

Table 5. Genetic variation of *Giuris* spp. from several populations analyzed per 600 bp

Population	n	H	Hd	Pi
Limboto Lake	6	3	0.733	0.00144
Philippines	17	8	0.875	0.00958
Bali Island	10	7	0.733	0.00489
Java Island	3	3	1.000	0.00222
Central Sulawesi	5	3	0.700	0.00133

Note: The symbols used are as follows: n is the number of sequences, H is the number of haplotypes formed, Hd is haplotype diversity, and Pi is nucleotide diversity

Table 6. Nucleotide variation per site of Limboto Lake population specimens, alignment in 600 bp with two mutated sites

Site number	LMB_GMA 001	LMB_GMA 002	LMB_GMA 003	LMB_GMA 004	LMB_GMA 005	LMB_GMA 006
236	A	A	T	A	T	A
464	T	T	T	T	T	C

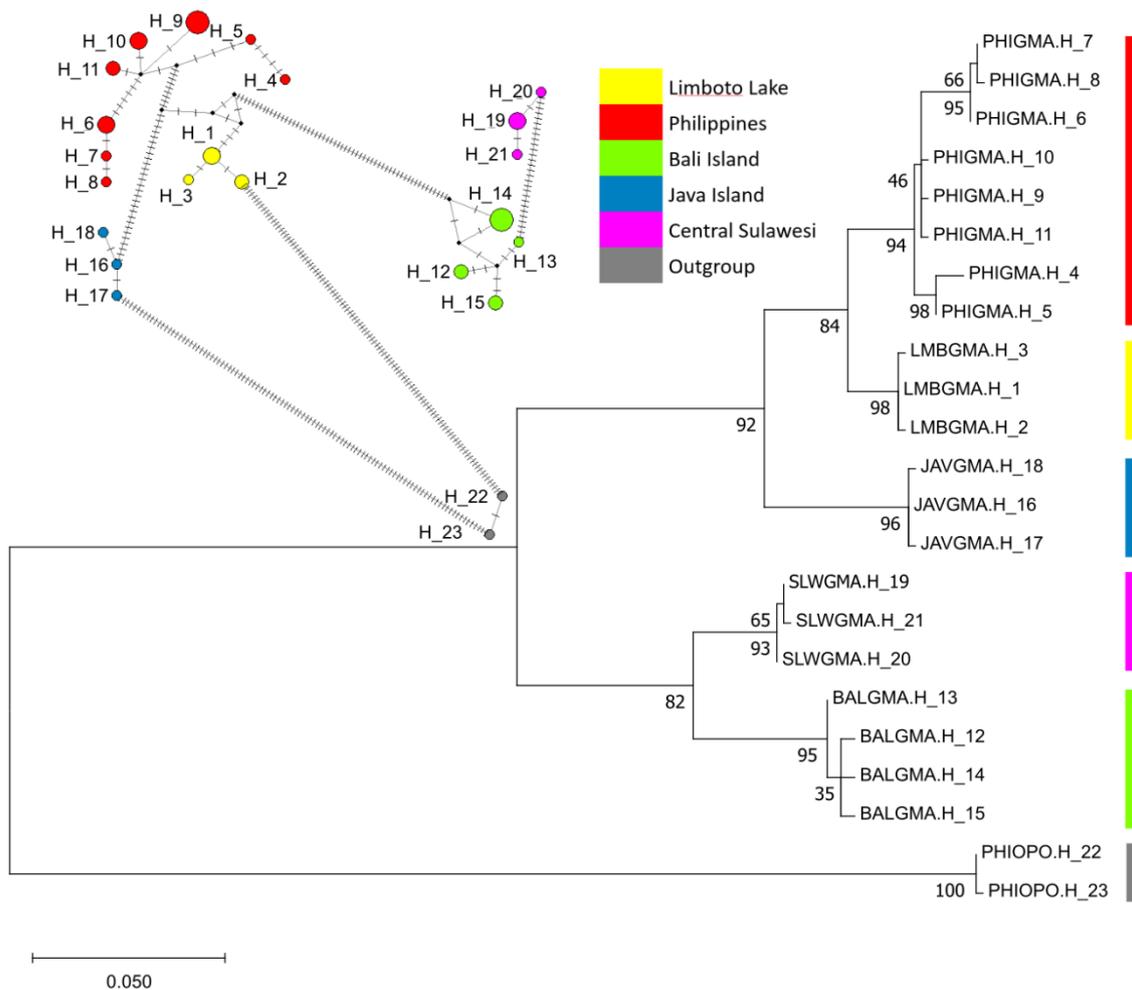


Figure 4. Phylogenetic and haplotype network of *Giuris* with *Ophiocara porocephala* as an outgroup of the genus. All populations form separate clades in their phylogenetics, with the number of sites differing between populations by more than 20 sites in their haplotype network

Table 7. P-distance between the *Giuris* population with *Ophiocara porocephala* as outgroup of the genus

Population	Limboto Lake	Philippines	Bali Island	Java Island	Central Sulawesi	Outgroup
Limboto Lake	0.0014					
Philippines	0.0331	0.0097				
Bali Island	0.1178	0.1239	0.0049			
Java Island	0.0629	0.0720	0.1231	0.0022		
Central_Sulawesi	0.1232	0.1250	0.0555	0.1200	0.0013	
Outgroup	0.2193	0.2331	0.2268	0.2200	0.2199	0.0017

Note: P-distance was the interpopulation and intrapopulation of *Giuris* with *Ophiocara porocephala* as an outgroup of the genus. All populations show a rate above 2%, indicating the possibility that the entire population is a distinct species. The numbers in bold are the intrapopulation P-distance numbers

Table 8. Data set information for secondary sequences from NCBI and BOLD System of *G. margaritaceus* and synonym of this species, *Ophieleotris aporos*

Accession number/BIN ID	Species	Voucher	Internal code (Haplotype group)	Population locality	Source
HQ682712.1	<i>Giuris margaritacea</i>	Oapo1-LdB	PHIGMA.001 (H_4)	Philippines	NCBI
HQ682711.1	<i>Giuris margaritacea</i>	Oapo2-LdB	PHIGMA.002 (H_5)	Philippines	NCBI
MG407392.1	<i>Giuris margaritacea</i>	BLF-HA1	PHIGMA.003 (H_6)	Philippines	NCBI
MG407391.1	<i>Giuris margaritacea</i>	BLF-HA2	PHIGMA.004 (H_7)	Philippines	NCBI
MG407390.1	<i>Giuris margaritacea</i>	BLF-HA3	PHIGMA.005 (H_6)	Philippines	NCBI
MG407388.1	<i>Giuris margaritacea</i>	BLF-HA4	PHIGMA.006 (H_6)	Philippines	NCBI
MG407389.1	<i>Giuris margaritacea</i>	BLF-HA5	PHIGMA.007 (H_8)	Philippines	NCBI
HQ654741.1	<i>Ophieleotris aporos</i>	Oapo4	PHIGMA.008 (H_9)	Philippines	NCBI
HQ654740.1	<i>Ophieleotris aporos</i>	Oapo5	PHIGMA.009 (H_10)	Philippines	NCBI
HQ654739.1	<i>Ophieleotris aporos</i>	Oapo7	PHIGMA.010 (H_9)	Philippines	NCBI
HQ654738.1	<i>Ophieleotris aporos</i>	Oapo8	PHIGMA.011 (H_9)	Philippines	NCBI
HQ654737.1	<i>Ophieleotris aporos</i>	Oapo10	PHIGMA.012 (H_11)	Philippines	NCBI
HQ654736.1	<i>Ophieleotris aporos</i>	Oapo1	PHIGMA.013 (H_9)	Philippines	NCBI
HQ654735.1	<i>Ophieleotris aporos</i>	Oapo2	PHIGMA.014 (H_10)	Philippines	NCBI
HQ654734.1	<i>Ophieleotris aporos</i>	Oapo3	PHIGMA.015 (H_11)	Philippines	NCBI
HQ654733.1	<i>Ophieleotris aporos</i>	Oapo6	PHIGMA.016 (H_10)	Philippines	NCBI
HQ654732.1	<i>Ophieleotris aporos</i>	Oapo9	PHIGMA.017 (H_9)	Philippines	NCBI
KU692515.1	<i>Giuris margaritacea</i>	BIF2849	BALGMA.001 (H_12)	Bali Island	NCBI
KU692514.1	<i>Giuris margaritacea</i>	BIF2283	BALGMA.002 (H_13)	Bali Island	NCBI
KU692512.1	<i>Giuris margaritacea</i>	BIF2847	BALGMA.003 (H_14)	Bali Island	NCBI
KU692511.1	<i>Giuris margaritacea</i>	BIF2846	BALGMA.004 (H_14)	Bali Island	NCBI
KU692510.1	<i>Giuris margaritacea</i>	BIF2962	BALGMA.005 (H_14)	Bali Island	NCBI
U692509.1	<i>Giuris margaritacea</i>	BIF2850	BALGMA.006 (H_14)	Bali Island	NCBI
KU692507.1	<i>Giuris margaritacea</i>	BIF2757	BALGMA.007 (H_15)	Bali Island	NCBI
KU692506.1	<i>Giuris margaritacea</i>	BIF2756	BALGMA.008 (H_15)	Bali Island	NCBI
KU692505.1	<i>Giuris margaritacea</i>	BIF2848	BALGMA.009 (H_12)	Bali Island	NCBI
KU692504.1	<i>Giuris margaritacea</i>	BIF2851	BALGMA.010 (H_14)	Bali Island	NCBI
KU692513.1	<i>Giuris margaritacea</i>	BIF1558	JAVGMA.001 (H_16)	Java Island	NCBI
KU692503.1	<i>Giuris margaritacea</i>	BIF1555	JAVGMA.002 (H_17)	Java Island	NCBI
KU692508.1	<i>Giuris margaritacea</i>	BIF2148	JAVGMA.003 (H_18)	Java Island	NCBI
BOLD:ADM7170	<i>Giuris margaritacea</i>	BIF10287	SLWGMA.001 (H_19)	Central Sulawesi	BOLD
BOLD:ADM7170	<i>Giuris margaritacea</i>	BIF10293	SLWGMA.002 (H_19)	Central Sulawesi	BOLD
BOLD:ADM7170	<i>Giuris margaritacea</i>	BIF10296	SLWGMA.003 (H_20)	Central Sulawesi	BOLD
BOLD:ADM7171	<i>Giuris margaritacea</i>	BIF10303	SLWGMA.004 (H_21)	Central Sulawesi	BOLD
BOLD:ADM7170	<i>Giuris margaritacea</i>	BIF10692	SLWGMA.005 (H_19)	Central Sulawesi	BOLD
JN021236.1	<i>Ophiocara porocephala</i>	Opor3	PHIOP0.001 (H_22)	Philippines	NCBI
JN021235.1	<i>Ophiocara porocephala</i>	Opor4	PHIOP0.002 (H_23)	Philippines	NCBI

Discussion

Identification of the genus *Giuris* still lacks systematic references, even for the species *G. margaritaceus*, the most studied species from the genus. Systematic morphological comparison is needed in this genus, as in the identification results, the Limboto Lake population is biased towards *G. margaritaceus* in the current systematical identification as

Ndobe et al. (2023). The relationship between length and weight also shows a tendency towards *G. margaritaceus*. However, some characteristics, such as the lateral row scales are only 26-28, the predorsal scales are consistently 16, and the maxilla does not extend below the front of the eye. These results differ from the description of (Kottelat et al. 1996), which states a lateral row scale of 30, a predorsal

scale of 13-18, and the upper jaw extending below the front of the eye. The taxonomy of *G. margaritaceus* has a problem with the extent of the distribution of this species. This distribution allows many species other than *G. margaritaceus* to experience misidentification due to updates in the taxonomic literature and the possibility that cryptic species have yet to be described (Kottelat 2013).

All populations form separate clades according to their distribution area with a genetic p-distance above 2%; a p-distance above 2% is generally assumed to be a distinct species of teleost fish (Bañón et al. 2022). Each population may be different species from the Limboto Lake population genetically, compared with the farthest population, namely Central Sulawesi, and with the closest population, namely the Philippines. The pattern of speciation is thought to be closely related to the isolation of Sulawesi Island, which occurred 45 million years ago (Triandiza et al. 2020; Utama et al. 2022). The separation of taxa, including the ancestors of the Eleotridae, especially *Giuris*, causes gene flow between the Limboto Lake population and other populations in the comparative data to be geographically separated. The cessation of gene flow caused the *Giuris* population in Limboto Lake to grow independently; from the p-distance value obtained, the Limboto population was farthest apart from the Central Sulawesi population and then followed sequentially by the Bali Island population and the Java population. The Limboto Lake and Philippines populations had the lowest between population p-distance value, but were still above 2%. Potential *Giuris* species in Philippines has been mentioned by Ndobe et al. (2023) as *G. laglaizei*, *G. tolsoni*, and/or *G. margaritaceus*; the three species are recorded as distributed in Sulawesi. However, species of Philippines population in data set, also for Limboto Lake and other population too, is still questionable. Perhaps, *G. margaritaceus* is still included in data set as a population, or not included because of the high ambiguity and misidentification potential for every populations in data set.

The conservation status of the genus *Giuris*, especially the species *G. margaritaceus*, which is the only assessed species on the IUCN Red List, still does not represent the entire conservation status of the genus *Giuris*, even for *G. margaritaceus*. The *G. margaritaceus* distribution map covers various countries, from Mozambique in the West to the Oceania region in the East and Taiwan in the North (Ndobe et al. 2023). According to the phylogenetic results in this study, these species have more than one species and at least four species in the Indonesian archipelago genetically. Conservation can be supported using genetic variation, especially concerning population health. Populations and genetic uniformity in populations causes conservation of population vulnerability (Phadphon et al. 2019). The genetic variation of the Limboto Lake population has an Hd value above 0.5 and a Pi value below 0.005, so the Limboto Lake population can be grouped as having high Hd and low Pi. Populations with high Hd and low Pi indicate that the population is thought to have experienced a bottleneck effect. However, population growth is starting to show in the mutations in the population. This condition is the same as that of the population of Java Island, Bali

Island, and Central Sulawesi. The Philippines population has high Hd and Pi values, which are the best conditions for genetic variation, assuming that this population has experienced a long evolutionary history and has been well-adapted or connected with other populations (Grant and Bowen 1998). Genetic variation pressure on the Limboto Lake population is possible due to environmental degradation around the Limboto Lake area, population growth, and the construction of dams that can block the gene flow of aquatic animals, including fish from the genus *Giuris*.

Furthermore, the waters in the Sulawesi region are also facing pressure from invasive species from other zoogeographical areas, such as *Anabas testudineus*, *Channa striata*, *Barbonymus gonionotus*, *Aplocheilichthys armatus*, and *Osteochilus vittatus*. Other introduced species that are disturbances in various places in the Indonesian Archipelago are also found, namely *Oreochromis niloticus*, *Oreochromis mossambicus*, *Poecilia reticulata*, *Pterygoplichthys pardalis*, *Cyprinus carpio*, *Melanochromis auratus*, etc (Bandjolu et al. 2021).

The Limboto Lake population of *Giuris* sp. represents how complex the genus is and how far misidentification is at the species level nowadays. Morphologic and genetic methods for identifying *Giuris*, especially *G. margaritaceus*, still lack valid data and affect conservation plans for *Giuris* species, as in Limboto Lake, that estimated starts growing after their population health quality somehow decreases. The Limboto Lake *Giuris* population is separated from Java, Bali, Philippines, and Central Sulawesi, demanding separate and specific conservation plans.

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