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From: Ahmad Dwi Setyawan (smujo.id@gmail.com)

To: dewi_roesma@yahoo.com

Date: Thursday, November 7, 2019, 9:38 PM GMT+7

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Thank you for submitting the manuscript, "Phylogenetic PHYLOGENETIC ANALYSIS OF TRANSPARENT GOBIES IN THREE SUMATRAN LAKES, INFERRED FROM MITOCHONDRIAL CYTOCHROME OXIDASE 1 (CO1) GENE: -" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

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[biodiv] Editor Decision

From: Smujo Editors (smujo.id@gmail.com)

To: dewi_roesma@yahoo.com

Date: Thursday, November 21, 2019, 9:19 AM GMT+7

DEWI IMELDA ROESMA:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Phylogenetic PHYLOGENETIC ANALYSIS OF TRANSPARENT GOBIES IN THREE SUMATRAN LAKES, INFERRED FROM MITOCHONDRIAL CYTOCHROME OXIDASE 1 (CO1) GENE: -".

Our decision is: Revisions Required

Smujo Editors
editors@smujo.id

Reviewer A:
Recommendation: Revisions Required

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Phylogenetic analysis of transparent gobies in three sumatran lakes, inferred from mitochondrial Cytochrome Oxidase 1 (CO1) gene

Abstract. M~~orphologically, ~~T~~t~~he transparent gobies fish found in three lakes in Sumatra island is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in Lake Siais-North Sumatra), and are morphologically very similar to the *Gobiopterus brachypterus*. T~~he fish is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in~~ L~~ake Siais-North Sumatra)~~. The phylogenetic study was carried out by analyzing 619 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene in 12 fish individuals from the three lakes. Rinuak and Badar fish in three populations have three haplotypes. The sequence divergences in and between populations are very low (0.0%-0.5%). This value indicates that Rinuak and Badar are the same species with low genetic diversity. The phylogenetic tree illustrates that this fish belongs to the group of Gobiidae and a sister taxon from *G. brachypterus*.

Keywords: Badar Fish, CO1, Genetic diversity, *Gobiopterus brachypterus*, Rinuak Fish

INTRODUCTION

Freshwater fishes biodiversity in Indonesia forms one of the largest in the world, but poorly documented. There are at least 1189 native species, 125 of them are endemic to the country (Froese and Pauly, 2014),. T~~he freshwater fish is poorly~~ d~~ocumented, with many fish are waiting to be identified.~~ Sumatra ~~is the second largest~~ island ~~on the Sundaland with~~has a high level of biodiversity of freshwater fishes with~~, characterized by a~~ high degree of endemism (Sodhi and Brook, 2006; Pfeil, 2009), including~~. In Sumatra Island, there is~~ a~~ kind of~~ small transparent fish that morphologically shows the character of the Gobiidae group.~~ That~~ The fish locally is known as Rinuak fish, with the total length range 19.91 to 22.68 mm and considered an endemic species of Maninjau Lake~~, West Sumatra. Besides, in Maninjau Lake, Rinuak fish also~~ f~~ound in~~and Singkarak Lake,~~ another big lake in Sumatra~~. These fish have an economically important value as food. Roesma and Santoso (2010)~~ also~~ reported a species suspected to be the same as Rinuak fish,~~ which named~~that locally named as Badar fish found in Siais Lake, North Sumatra. However, the scientific name of these two ~~types~~ species remained ~~is~~ unknown, and some local people consider it as a juvenile form of *Rasbora* from Maninjau Lake.

Rinuak and Badar fish can be classified as the Gobiidae group because of they have the main characters of Gobies, w~~hich consists of;~~*i.e.* a pair of pectoral fins that are fused to form suckers, two dorsal fins, rounded tail, and blunt head with big eyes (Kottelat et al.1993). ~~Morphologically,~~In addition, the Rinuak and Badar fish ~~show a~~are similar ~~feature~~ to *Gobiopterus brachypterus* (transparent gobies). *Gobiopterus* is a genus of gobies with small size, native to freshwater, marine or brackish waters, and one genus of transparent gobies (Kottelat et al. 1993). Biological and ecological information on Rinuak and Badar fish are very scanty, limited only~~ in~~ on~~ studies~~ study of processed quality of Rinuak as s food (Astuti et al. 2016). ~~In the~~A previous ~~studies~~study (~~,~~Periwaldi ~~(~~2012) reported that Rinuak fish in Maninjau Lake and Badar fish in Siais Lake have high morphological differentiations, i.e ~~(~~13 different characters of 20 characters)~~.~~

In the past, the classification of the gobies group was generally based on the external characters (Pezold 1993; Akihito et al. 2000; Nelson 2006); however, identification of species level d~~iagnostic characters are~~ was proven to be difficult and problematic~~ to identify to species level~~. Recently, the advances in molecular approaches have provided the complement to the limited of morphological data for untangling gobioid group (Aquino et al. 2011; Jeon et al. 2012; Agorreta and Ruber 2012; Agoretta et al. 2013; Tornabene et al. 2013; Taillebois et al. 2014; Thacker 2009; Thacker 2015; Wang et al. 2017). Mitochondrial DNA (mtDNA) markers have h~~istorically~~ been widely used for most systematic molecular studies because they have a large number of copies, maternally inherited, and have a higher mutation rate compared to nuclear DNA (Brown et al. 1979; Johns and Avise 1998). Mitochondrial cytochrome oxidase subunit I (COI) gene could serve as a rapid and reliable barcoding marker for identifying species and effective in delimitating species~~ in~~ of various animal~~ taxa~~s (Hebert et al. 2004; Hubert et al. 2008; Roesma et al. 2018; Roesma et al. 2019). Recently, the COI gene has been widely used to resolve the taxonomy in a gobioid group (Jeon et al. 2012; Jin et al. 2014; Taillebois et al. 2014; Wang et al. 2017; Olii et al. 2019). Further research is needed on Rinuak and Badar fish using a molecular approach. The present study aimed to investigate the phylogenetic relationship of Rinuak and Badar fish in three Sumatran Lakes using cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study ~~areas~~sites

Rinuak and Badar fish specimens were collected from three Sumatran lakes, *i.e.* c~~onsist of;~~ Maninjau Lake and Singkarak Lake in West Sumatra and Siais Lake in North Sumatra. The samples collected with cast-nets and set-nets following standard procedures by Cailliet et al. (1986). Twelve individuals out of the samples were used for phylogenetic analysis and preserved in 96% Alcohol (PA). Other individual specimens were all together fixed in 4% formalin and preserved into 70% ethanol for long term storage in the laboratory.

~~Procedures~~Molecular work

The DNA extraction was performed following the protocol Kit INVITROGEN PureLinkTM Genomic DNA Mini Kit. The COI was amplified using the following primers: forward primer Fish F1 (5' TCAACCAACCACAAAGACATTGGCAC 3') and reverse primer Fish R1 (5' TAGACTTCTGGG TGGCCAAAGAATCA3') (Ward et al. 2005). PCR reactions were conducted in 25 µl volumes, containing;3 µl of DNA template, 11 µl of PCR Supermix, 0.5 µl of each primer (0.01 mM), and ten µl of DDH20. The PCR procedure was conducted using PCR *Sensoquest* thermal cyclers with the following program: initial denaturation at 95^{0}C for 2 min; 35 cycles of denaturation at 94^{0}C for 30 s, annealing at 54^{0}C for 30 s and elongation at 72^{0}C for 1 min; followed by a final extension of 72°C for 10 min and then held at 4°C for the long term storage. PCR products were run on 2% agarose gel and visualized using GelDoc. PCR products were purified and the most powerful products selected for sequencing to1st Base, Pte. Ltd.in Malaysia.

Data analysis

The nucleotide sequences from the DNA sequencing of Rinuak and Badar fish was ~~contig and~~ edited using the DNA STAR program (Burland 2000) then. DNA sequences were obtained then compare with the data on the Genbank, NCBI (Table 1.) to investigate the similarity of all sequences in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences were aligned using the Clustal X default settings and edited using the Bioedit program (Thompson 1997; Hall 1999). DNA SP version 5.10 was used to identify haplotypes numbers (*h*), polymorphism sites (S), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Rozas 2003). Pairwise genetic distances were calculated to quantify sequence divergences among all sequences using *the p*-distance Kimura two-parameter (K2P) method in the MEGA version 6.0 program (Tamura et al. 2013). Phylogenetic analyses were performed ~~using the construction of a phylogenetic tree based on~~ the four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML), and Maximum Parsimony (MP) using 1000 bootstrapping in MEGA 6.0 program (Tamura et al. 2013). Did not do any branch support analysis?

RESULTS AND DISCUSSION

Sequence data

The mtDNA COI sequences of 32 individuals (12 sequences from the present study and 20 sequences from the GenBank) were determined for a total of 619 bp. Among them, 231 bp (37.32%) were variable sites, and 213 bp (34.42%) were parsimony informative. No stop codons, insertions, and deletions were found in its translation. The average compositions of A, T, C, and G nucleotide base was 24.4%, 30.3%, 27.5%, and 17.8%, respectively. The average compositions' value was almost similar to the previous studies of Gobiidae (Aquino et al. 2011; Aquilino et al. 2011; Jeon et al. 2012). Nucleotide sequences are AT ratio (54.7%) higher than the GC ratio, which is characteristic for the genome of mtDNA in vertebrate (Hubert et al. 2008). The GC content of the first codon is the highest, while the variable second codon has the lowest. The transitions are more common than transversion with bias ratio (Ts; Tv) is R: 3.737. A total of 22 haplotypes consisted of four haplotypes to Rinuak and Badar fish in Sumatran Lakes and 18 haplotypes to data of GenBank, NCBI. The average haplotype diversity among 22 haplotypes (Hd) was 0.938±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.16256±0.002. While among Rinuak and Badar fish in Sumatra has the haplotype diversity (*Hd*) was 0.561, and nucleotide diversity (*Pi*) was 0.00142. Genetic distance within and among populations of Rinuak and Badar fish was 0.0%-0.5% (the table of genetic distance not showed). Sequence divergence between outgroup (*Rasbora lateristriata* and *Danio rerio*) with Rinuak and Badar fish lineages ranged from 27.6% to 31.6%. That value supported that they are in a different Family.

Table 1. Species List taxonomy, Location and Genbank Accession Number of samples

No.	Family	Genus	Species	Location	Accession Numbers
1	Gobiidae	*Gobiopterus*	*Gobiopterus brachypterus*	Malaysia	KX223903.1
2			*Gobiopterus brachypterus*	Malaysia	KX223902.1
3			*Gobiopterus brachypterus*	India	MG495939.1
4			*Gobiopterus brachypterus*	Central Java	KU692539.1
5			*Gobiopterus brachypterus*	Central Java	KU692538.1
6			*Gobiopterus brachypterus*	Central Java	KU692535.1
7			*Gobiopterus chuno*	Bangladesh	MK572237.1
8			*Gobiopterus lacustris*	Philippines	HQ682693.1
9			*Gobiopterus lacustris*	Philippines	HQ682695.1
10			*Gobiopterus semivestitus*	Australia	KJ669473.1
11		*Sicyopterus*	*Sicyopterus parvei*	West Java	KU693078.1
12			*Sicyopterus lagocephalus*	Bali	KU693018.1
13		*Stiphodon*	*Stiphodon semoni*	West Java	KU693171.1
14			*Stiphodon maculidorsalis*	Banten	KU693162.1
15		*Sicyopus*	*Sicyopus zosterophorum*	Bali	KU693126.1
16			*Sicyopus ribicundus*	East Java	KU693113.1
17		*Lentipes*	*Lentipes whittenorum*	Bali	KU693014.1
18			*Lentipes ikeae*	West Java	KU692987.1
19	Cyprinidae	*Rasbora*	*Rasbora lateristriata*		KT960818.1
20		*Danio*	*Danio rerio*	India	MK714084.1

Phylogenetic relationships

The phylogenetic tree was constructed using four methods (ML/NJ/ME/MP) analysis generated almost in identical topologies, represented by the ML tree with 1000 bootstrap replicates (~~were shown in~~ Figure 1). Based on the tree constructed from COI genes, it can be observed that the Gobiidae group divided into four sublineages.

The phylogenetic tree members consist of Rinuak and Badar fish in the first sublineage, *Gobiopterus* in the second and fourth sublineage while *Sicyopterus*, *Stipodon*, *Sicyopus*, and *Lentipes* in the third sublineage. All members of Rinuak and Badar fish from three Sumatran lakes present together in the first sublineage with sequence divergences was 0.0%-0.5%. In the second sublineage, consists of three species from Gobiopterus, namely *G. brachypterus, G.chuno*, and *G. lacustris*. Among the species of *Gobiopterus* has sequence divergences was 2.0%-17.8%. The presence of small sequence divergences (2.0%) between the *G. chuno* from Bangladesh MK572237 and *G. brachypterus from* India MG495939 and (3.0%) between *G. brachypterus* from Central Java and *G. lacustris* from the Philippines is require re-identification studies in taxonomy. According to Kartavsetv et al. (2013), that value indicated for differences at the level of subspecies, semi species, or sibling species.

The phylogenetic tree showed that the *Gobiopterus* in the second Sublineage is occupying the position as a sister taxa of Rinuak and Badar fish with sequence divergences was 24.6%-27.9%. *Gobiopterus* is the only genus of transparent gobies that has the genetic data used as a comparison for Rinuak and Badar fish. Based on Kartavsetv et al. (2011), Kartavsetv et al. (2013), the value of sequence divergences between first and second sublineages showed differentiation at the level of a different genus. The third Sublineage consists of four different genera. The sequence divergences between them were 11.8%-15.3%, and with both first and second sublineages were 20.7%-29.3%. The fourth Sublineage has one member, *G. semivestitus*, which is separated from other *Gobiopterus* in the second Sublineage with sequence divergences was 27.2%-29.1%. The separation of *Gobiopterus* into two different sublineages showed that *Gobiopterus* has the non-monophyletic lineages. The value of sequence divergences between *Gobiopterus* in the second and fourth sublineages showed differentiation at the level of a different genus. Based on Kartavsetv et al. (2011), Kartavsetv et al. (2013) sequence divergences in the same genus was 11%-16%.

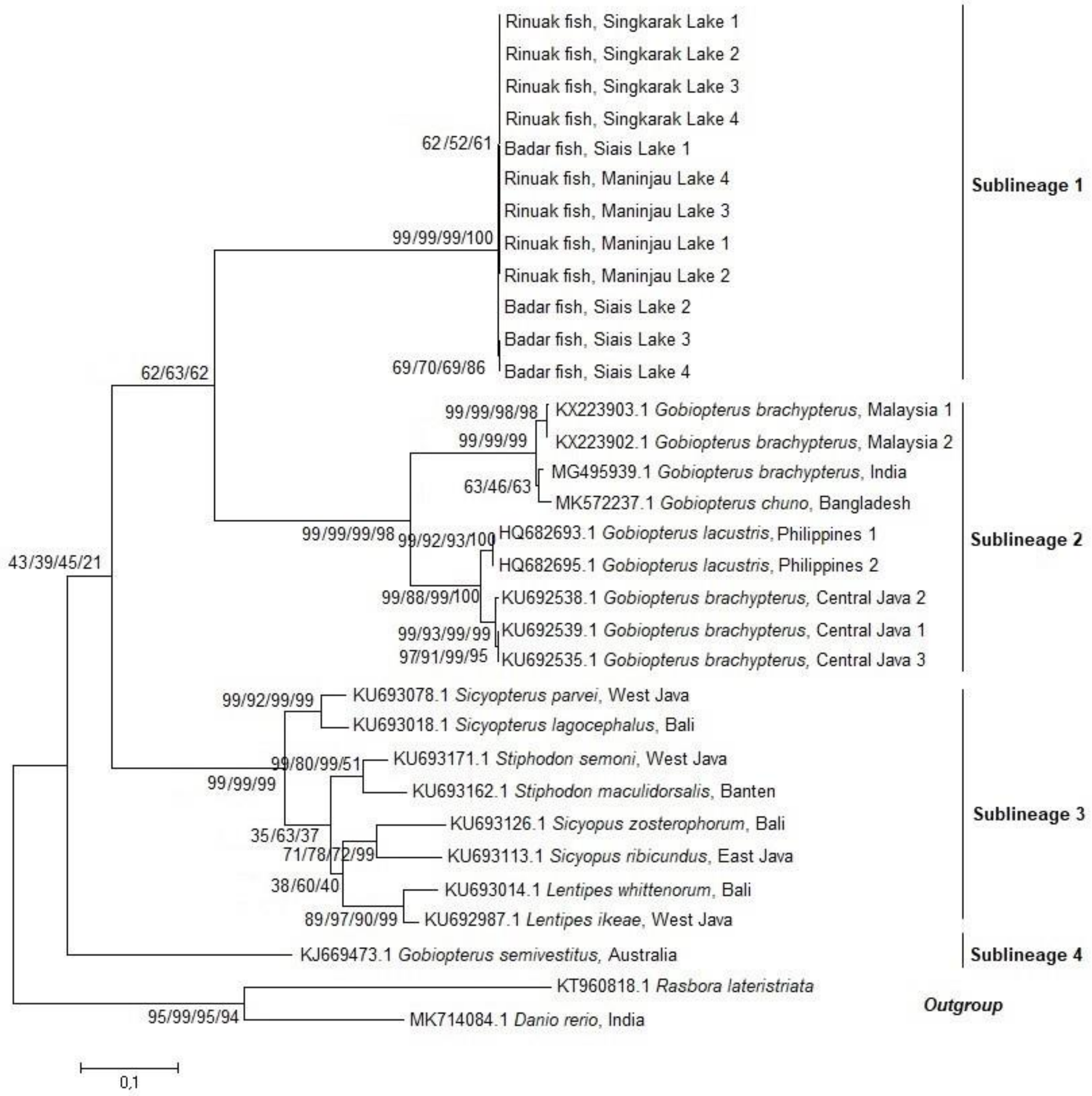

Figure 1. The ML phylogenetic tree of the COI gene with the bootstrap value of 1000 replicates (ME/NJ/ML/MP).

Discussion

The present study represents the first molecular data of Rinuak and Badar fish in Sumatra using mitochondrial markers (COI gene). Rinuak and Badar fish in Sumatra present in one sublineage with low haplotype diversity, which is some individual shared the same haplotype. Refers to Kartavtsev et al. (2011); Kartavtsev et al. (2013), our analysis revealed that the Rinuak and Badar fish are the same species, with low genetic diversity (0.0%-0.5%). While previously, the morphological study by Periwaldi (2012) showed the substantial morphological differences between Rinuak Maninjau Lake and Badar Siais Lake. Geographically, the three lakes are not connected, no gene flow between the population of the lakes, ~~which~~ that causes the geographical isolation. Roesma et al. (2018) explained that the freshwater fauna in Maninjau Lake isolated because of no connection with other waters. Water in the Maninjau Lake comes from small rivers, which is filled by water catchment in the surrounding area and only has one outlet. The low genetic divergences of Rinuak and Badar fish between Lakes indicated that geographic distance and geographical isolation do not correlate their sequence divergences.

Using mtDNA, Mcglasan, and Hughes (2001) ~~revealed~~ considered that there was no significant relationship between geographical distance and gene flow on *Hypseleotris compressa* from different drainages with low genetic differentiation on the populations. Keith et al. (2005) also reported that the *Sicyopterus lagocephalus* from different distant archipelagos has minimal genetic distances and suggests they as the same species. The study by Arisuryanti et al. (2018) showed that *Periophthalmus argentilineatus* in Bogowonto Lagoon (Middle Java) and population in Pandeglang (West Java) clustered together with low sequence divergences. *H. compressa, S. lagocephalus*, and *P. argentilineatus* are the species from other Gobies. Referring to study Mcglasan and Hughes (2001); Keith et al. (2005), the possibilities conditions of present study are: 1) natural selection is able to maintain the balance of allele frequencies, 2) there is a geographic connection in the past at these distant locations, and 3) the ability of the fish to change.

Morphologicaly~~y~~ and genetic studies showed that environmental conditions play an essential role in determining the phenotype of a species. The phenotype shows the existence of high adaptability to environmental conditions without changing their genetic makeup. Akihito et al. (2000) reported that the adaptations of Gobiidae fishes to habitats had developed various morphological specializations. It is difficult to estimate the evolutionary scenario using only morphological information, so that needed the genetic data. The phylogenetic tree showed that the *Gobiopterus* is a sister taxon of Rinuak, and Badar fish with sequence divergences was 24.6%-27.9%. Based on Kartavsetv et al. (2011); Kartavsetv et al. (2013), these values show the different of the genus level. The same case also found in other studies of Gobiidae. Jeon et al. (2012) reported that the average sequence divergences of intragenus of more than 20 genera with multiple species of Gobies higher (21.09%) than among species (9.93%-9.54%) in the study of Ward et al. (2005); Ward and Holmes (2007).

According to Kottelat (2013), the transparent gobies consist of two genera; *Gobiopterus* and *Mistichthys*. *Mistichthys* only has one species, *Mistichthys luzonensis*. Referring to Kottelat et al. (1993), Rinuak and Badar fish has similar in morphology with *G. brachypterus*. The similarity of the main characters between Rinuak and Badar fish with *G. brachypterus* consists of cheek naked, cheeks without raised ridges and flaps, pelvic thin without fleshy lobes, head pores absent, transparent body, cheek and margins of preopercle usually with a dark vertical mark, dorsal midline in front of the first dorsal usually with black pigments, mouth of males is more extensive than female and almost vertical and distance between eyeballs 0.5-1.0 times eyeball diameter. However, Rinuak and Badar fish have high sequence divergences (24.6%-27.9%) against *G. brachypterus* in Central Java, Malaysia, and India. Despite having the similarity in morphology, the high sequence divergences do not support the grouping of Rinuak and Badar fish as the same species to *G. brachypterus* even to the genus *Gobiopterus*.

Previously, the transparent gobies were separated into several genera (Munro,1949) consists of Gobiopterus Bleeker (1874), *Mistichthys* Smith (1901), *Micrapocryptes* Hora (1923), *Mirogobius* Herre (1927), *Gobiella* Smith (1931), and *Herreolus* Smith (1931). However, previous authors (Hora ~~(~~1934~~)~~; Mukerji ~~(~~1936~~)~~; Aurich ~~(~~1938~~)~~; Koumans (I940), demonstrated that *Gobiopterus* is a synonym of the re-examined holotype of *Micrapocryptes*, *Gobiella*, *Mistichthys*, and *Mirogobius*. While, Kottelat (2013) has reported that the valid of transparent gobies consists of two genera (*Mistichthys* and *Gobiopterus*), where is *Gobiopterus* has ten species. However, only four species of them have morphological and genetic data (*G. brachypterus*, *G. lacustris*, *G. chuno*, and *G. semivestitus*). Unfortunately, there is no information explained in the published article about *G. brachypterus* in Rawa Pening lake, Central Java, which was used as a comparison species in the present analysis of the study.

According to (Kottelat, 2013), the type locality of *G. brachypterus* (Bleeker,1955) in Pasuruan, Ranu Grati Lake, East Java, Indonesia. Based on the several sources, *G. brachypterus* in East Java are known with the local name as Lempuk fish. The local peoples consider Lempuk fish only found in Ranu Grati Lake (East Java). Lempuk fish also has high economic value as of fresh food and processed food like Rinuak fish from Maninjau Lake. The genetic similarity study of Lempuk fish and *G. brachypterus* using the 12s rRNA gene by Candra (2012) showed the high sequence divergences (8.2%) between them and concluded that Lempuk fish was different genus to *G. brachypterus*. These results supported our study, which showed Rinuak and Badar fish as different from *G. brachypterus*. The lack of morphological and genetic studies on the *Gobiopterus* group makes it difficult for further identification. Studies on *Gobiopterus* are limited to the first records *G.brachypterus* in Sri Lanka (Ott, 2011), sexual dimorphism of *G.semivestitus* (Macdowall and David, 2008), and genetic differentiation of *G.lacustris* (Aquino et al. 2011; Wang et al. 2017).).

The data of IUCN (year?) and FishBase (year?) s~~howed~~ suggested that species of *Gobiopterus* are endemic to certain islands/countries; some are also found in other regions such as *G. lacustris* is endemic in the Philippines. However, Wang et al. (2017) reported that *G. lacustris* also found in China. Aquino et al. (2011) explained that *G. lacustris* divided into two distinct clusters with deep genetic divergence at 23.80% and proposed that they are a cryptic species. Wang et al. (2017) confirmed that no cryptic species on *G. lacustris* in the Philippines and supported that *G. lacustris* in China should not be an invasive species from the Philippines.

There are no photo vouchers, and the availability of the data of DNA sequences on GenBank and BOLD System to all *Gobiopterus* species causes the difficulties in identified and verified their taxonomy status. The taxonomy of Gobiidae has been studied extensively for the last decades, but confusion still exists (Jeon et al. 2012). The Present study revealed that Rinuak fish is not an endemic of Maninjau Lake. Rinuak and Badar exist as a different genus with *Gobiopterus*. T~~he~~ F further comprehensive studies using the morphology and other molecular markers on Rinuak and Badar are ongoing.

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Phylogenetic analysis of transparent gobies in three sumatran lakes, inferred from mitochondrial Cytochrome Oxidase 1 (CO1) gene

Abstract. ~~Morphologically, T~~The transparent gobies fish found in three lakes in Sumatra island is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in Lake Siais-North Sumatra), and are morphologically very similar to the *Gobiopterus brachypterus*. ~~The fish is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in Lake Siais-North Sumatra)~~. The phylogenetic study was carried out by analyzing 619 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene in 12 fish individuals from the three lakes. Rinuak and Badar fish in three populations have three haplotypes. The sequence divergences in and between populations are very low (0.0%-0.5%). This value indicates that Rinuak and Badar are the same species with low genetic diversity. The phylogenetic tree illustrates that this fish belongs to the group of Gobiidae and a sister taxon from *G. brachypterus*.

Keywords: Badar Fish, CO1, Genetic diversity, *Gobiopterus brachypterus*, Rinuak Fish

INTRODUCTION

Freshwater fishes biodiversity in Indonesia forms one of the largest in the world, but poorly documented. There are at least 1189 native species, 125 of them are endemic to the country (Froese and Pauly, 2014),. ~~The freshwater fish is poorly documented, with many fish are waiting to be identified.~~ Sumatra ~~is the second largest~~ island ~~on the Sundaland with~~ has a high level of biodiversity of freshwater fishes with~~, characterized by a~~ high degree of endemism (Sodhi and Brook, 2006; Pfeil, 2009), including~~. In Sumatra Island, there is~~ a~~ kind of~~ small transparent fish that morphologically shows the character of the Gobiidae group.~~ That~~ The fish locally is known as Rinuak fish, with the total length range 19.91 to 22.68 mm and considered an endemic species of Maninjau Lake~~, West Sumatra. Besides, in Maninjau Lake, Rinuak fish also found in~~and Singkarak Lake,~~ another big lake in Sumatra.~~ These fish have an economically important value as food. Roesma and Santoso (2010)~~ also~~ reported a species suspected to be the same as Rinuak fish,~~ which named~~ that locally named as Badar fish found in Siais Lake, North Sumatra. However, the scientific name of these two ~~types~~ species remained ~~is~~ unknown, and some local people consider it as a juvenile form of *Rasbora* from Maninjau Lake.

Rinuak and Badar fish can be classified as the Gobiidae group because of they have the main characters of Gobies, ~~which consists of;~~*i.e.* a pair of pectoral fins that are fused to form suckers, two dorsal fins, rounded tail, and blunt head with big eyes (Kottelat et al.1993). ~~Morphologically,~~In addition, the Rinuak and Badar fish ~~show a~~are similar ~~feature~~ to *Gobiopterus brachypterus* (transparent gobies). *Gobiopterus* is a genus of gobies with small size, native to freshwater, marine or brackish waters, and one genus of transparent gobies (Kottelat et al. 1993). Biological and ecological information on Rinuak and Badar fish are very scanty, limited only~~ in~~ on ~~studies~~ study of processed quality of Rinuak as s food (Astuti et al. 2016). ~~In the~~A previous ~~studies~~study (~~,~~Periwaldi ~~(~~2012) reported that Rinuak fish in Maninjau Lake and Badar fish in Siais Lake have high morphological differentiations, i.e ~~(~~13 different characters of 20 characters)~~.~~

In the past, the classification of the gobies group was generally based on the external characters (Pezold 1993; Akihito et al. 2000; Nelson 2006); however, identification of species level ~~diagnostic characters are~~ was proven to be difficult and problematic~~ to identify to species level~~. Recently, the advances in molecular approaches have provided the complement to the limited of morphological data for untangling gobioid group (Aquino et al. 2011; Jeon et al. 2012; Agorreta and Ruber 2012; Agoretta et al. 2013; Tornabene et al. 2013; Taillebois et al. 2014; Thacker 2009; Thacker 2015; Wang et al. 2017). Mitochondrial DNA (mtDNA) markers have ~~historically~~ been widely used for most systematic molecular studies because they have a large number of copies, maternally inherited, and have a higher mutation rate compared to nuclear DNA (Brown et al. 1979; Johns and Avise 1998). Mitochondrial cytochrome oxidase subunit I (COI) gene could serve as a rapid and reliable barcoding marker for identifying species and effective in delimitating species~~ in~~ of various animal~~ taxa~~s (Hebert et al. 2004; Hubert et al. 2008; Roesma et al. 2018; Roesma et al. 2019). Recently, the COI gene has been widely used to resolve the taxonomy in a gobioid group (Jeon et al. 2012; Jin et al. 2014; Taillebois et al. 2014; Wang et al. 2017; Olii et al. 2019). Further research is needed on Rinuak and Badar fish using a molecular approach. The present study aimed to investigate the phylogenetic relationship of Rinuak and Badar fish in three Sumatran Lakes using cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study ~~areas~~sites

Rinuak and Badar fish specimens were collected from three Sumatran lakes, *i.e.* c~~onsist of;~~ Maninjau Lake and Singkarak Lake in West Sumatra and Siais Lake in North Sumatra. The samples collected with cast-nets and set-nets following standard procedures by Cailliet et al. (1986). Twelve individuals out of the samples were used for phylogenetic analysis and preserved in 96% Alcohol (PA). Other individual specimens were all together fixed in 4% formalin and preserved into 70% ethanol for long term storage in the laboratory.

~~Procedures~~Molecular work

The DNA extraction was performed following the protocol Kit INVITROGEN PureLinkTM Genomic DNA Mini Kit. The COI was amplified using the following primers: forward primer Fish F1 (5' TCAACCAACCACAAAGACATTGGCAC 3') and reverse primer Fish R1 (5' TAGACTTCTGGG TGGCCAAAGAATCA3') (Ward et al. 2005). PCR reactions were conducted in 25 µl volumes, containing;3 µl of DNA template, 11 µl of PCR Supermix, 0.5 µl of each primer (0.01 mM), and ten µl of DDH20. The PCR procedure was conducted using PCR *Sensoquest* thermal cyclers with the following program: initial denaturation at 95^{0}C for 2 min; 35 cycles of denaturation at 94^{0}C for 30 s, annealing at 54^{0}C for 30 s and elongation at 72^{0}C for 1 min; followed by a final extension of 72°C for 10 min and then held at 4°C for the long term storage. PCR products were run on 2% agarose gel and visualized using GelDoc. PCR products were purified and the most powerful products selected for sequencing to1st Base, Pte. Ltd.in Malaysia.

Data analysis

The nucleotide sequences from the DNA sequencing of Rinuak and Badar fish was ~~contig and~~ edited using the DNA STAR program (Burland 2000) then. DNA sequences were obtained then compare with the data on the Genbank, NCBI (Table 1.) to investigate the similarity of all sequences in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences were aligned using the Clustal X default settings and edited using the Bioedit program (Thompson 1997; Hall 1999). DNA SP version 5.10 was used to identify haplotypes numbers (*h*), polymorphism sites (S), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Rozas 2003). Pairwise genetic distances were calculated to quantify sequence divergences among all sequences using *the p*-distance Kimura two-parameter (K2P) method in the MEGA version 6.0 program (Tamura et al. 2013). Phylogenetic analyses were performed ~~using the construction of a phylogenetic tree based on~~ the four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML), and Maximum Parsimony (MP) using 1000 bootstrapping in MEGA 6.0 program (Tamura et al. 2013). Did not do any branch support analysis?

RESULTS AND DISCUSSION

Sequence data

The mtDNA COI sequences of 32 individuals (12 sequences from the present study and 20 sequences from the GenBank) were determined for a total of 619 bp. Among them, 231 bp (37.32%) were variable sites, and 213 bp (34.42%) were parsimony informative. No stop codons, insertions, and deletions were found in its translation. The average compositions of A, T, C, and G nucleotide base was 24.4%, 30.3%, 27.5%, and 17.8%, respectively. The average compositions' value was almost similar to the previous studies of Gobiidae (Aquino et al. 2011; Aquilino et al. 2011; Jeon et al. 2012). Nucleotide sequences are AT ratio (54.7%) higher than the GC ratio, which is characteristic for the genome of mtDNA in vertebrate (Hubert et al. 2008). The GC content of the first codon is the highest, while the variable second codon has the lowest. The transitions are more common than transversion with bias ratio (Ts; Tv) is R: 3.737. A total of 22 haplotypes consisted of four haplotypes to Rinuak and Badar fish in Sumatran Lakes and 18 haplotypes to data of GenBank, NCBI. The average haplotype diversity among 22 haplotypes (Hd) was 0.938±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.16256±0.002. While among Rinuak and Badar fish in Sumatra has the haplotype diversity (*Hd*) was 0.561, and nucleotide diversity (*Pi*) was 0.00142. Genetic distance within and among populations of Rinuak and Badar fish was 0.0%-0.5% (the table of genetic distance not showed). Sequence divergence between outgroup (*Rasbora lateristriata* and *Danio rerio*) with Rinuak and Badar fish lineages ranged from 27.6% to 31.6%. That value supported that they are in a different Family.

Table 1. Species List taxonomy, Location and Genbank Accession Number of samples

No.	Family	Genus	Species	Location	Accession Numbers
1	Gobiidae	*Gobiopterus*	*Gobiopterus brachypterus*	Malaysia	KX223903.1
2			*Gobiopterus brachypterus*	Malaysia	KX223902.1
3			*Gobiopterus brachypterus*	India	MG495939.1
4			*Gobiopterus brachypterus*	Central Java	KU692539.1
5			*Gobiopterus brachypterus*	Central Java	KU692538.1
6			*Gobiopterus brachypterus*	Central Java	KU692535.1
7			*Gobiopterus chuno*	Bangladesh	MK572237.1
8			*Gobiopterus lacustris*	Philippines	HQ682693.1
9			*Gobiopterus lacustris*	Philippines	HQ682695.1
10			*Gobiopterus semivestitus*	Australia	KJ669473.1
11		*Sicyopterus*	*Sicyopterus parvei*	West Java	KU693078.1
12			*Sicyopterus lagocephalus*	Bali	KU693018.1
13		*Stiphodon*	*Stiphodon semoni*	West Java	KU693171.1
14			*Stiphodon maculidorsalis*	Banten	KU693162.1
15		*Sicyopus*	*Sicyopus zosterophorum*	Bali	KU693126.1
16			*Sicyopus ribicundus*	East Java	KU693113.1
17		*Lentipes*	*Lentipes whittenorum*	Bali	KU693014.1
18			*Lentipes ikeae*	West Java	KU692987.1
19	Cyprinidae	*Rasbora*	*Rasbora lateristriata*		KT960818.1
20		*Danio*	*Danio rerio*	India	MK714084.1

Phylogenetic relationships

The phylogenetic tree was constructed using four methods (ML/NJ/ME/MP) analysis generated almost in identical topologies, represented by the ML tree with 1000 bootstrap replicates (~~were shown in~~ Figure 1). Based on the tree constructed from COI genes, it can be observed that the Gobiidae group divided into four sublineages.

The phylogenetic tree members consist of Rinuak and Badar fish in the first sublineage, *Gobiopterus* in the second and fourth sublineage while *Sicyopterus*, *Stipodon*, *Sicyopus*, and *Lentipes* in the third sublineage. All members of Rinuak and Badar fish from three Sumatran lakes present together in the first sublineage with sequence divergences was 0.0%-0.5%. In the second sublineage, consists of three species from Gobiopterus, namely *G. brachypterus, G.chuno*, and *G. lacustris*. Among the species of *Gobiopterus* has sequence divergences was 2.0%-17.8%. The presence of small sequence divergences (2.0%) between the *G. chuno* from Bangladesh MK572237 and *G. brachypterus from* India MG495939 and (3.0%) between *G. brachypterus* from Central Java and *G. lacustris* from the Philippines is require re-identification studies in taxonomy. According to Kartavsetv et al. (2013), that value indicated for differences at the level of subspecies, semi species, or sibling species.

The phylogenetic tree showed that the *Gobiopterus* in the second Sublineage is occupying the position as a sister taxa of Rinuak and Badar fish with sequence divergences was 24.6%-27.9%. *Gobiopterus* is the only genus of transparent gobies that has the genetic data used as a comparison for Rinuak and Badar fish. Based on Kartavsetv et al. (2011), Kartavsetv et al. (2013), the value of sequence divergences between first and second sublineages showed differentiation at the level of a different genus. The third Sublineage consists of four different genera. The sequence divergences between them were 11.8%-15.3%, and with both first and second sublineages were 20.7%-29.3%. The fourth Sublineage has one member, *G. semivestitus*, which is separated from other *Gobiopterus* in the second Sublineage with sequence divergences was 27.2%-29.1%. The separation of *Gobiopterus* into two different sublineages showed that *Gobiopterus* has the non-monophyletic lineages. The value of sequence divergences between *Gobiopterus* in the second and fourth sublineages showed differentiation at the level of a different genus. Based on Kartavsetv et al. (2011), Kartavsetv et al. (2013) sequence divergences in the same genus was 11%-16%.

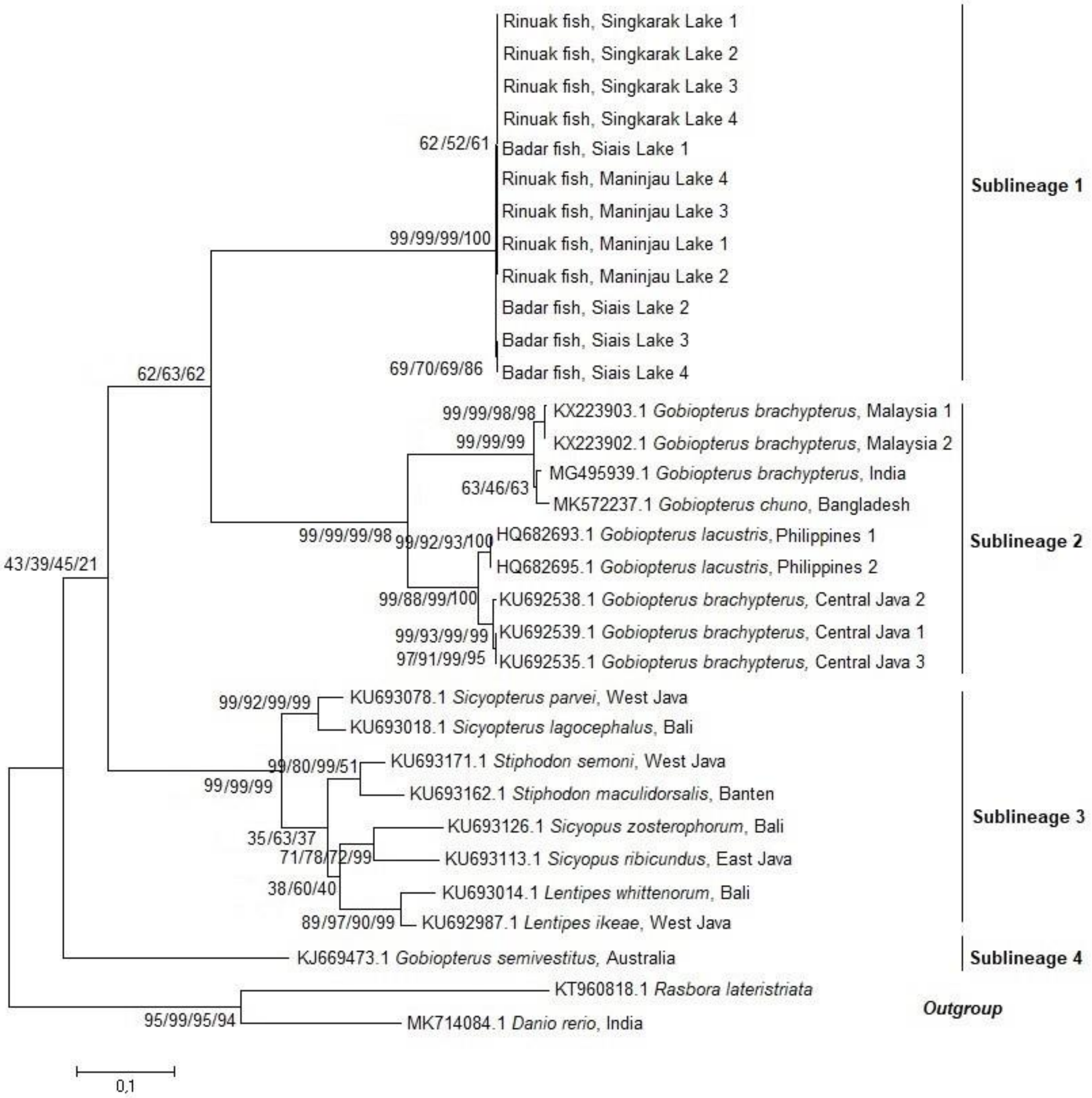

Figure 1. The ML phylogenetic tree of the COI gene with the bootstrap value of 1000 replicates (ME/NJ/ML/MP).

Discussion

The present study represents the first molecular data of Rinuak and Badar fish in Sumatra using mitochondrial markers (COI gene). Rinuak and Badar fish in Sumatra present in one sublineage with low haplotype diversity, which is some individual shared the same haplotype. Refers to Kartavtsev et al. (2011); Kartavtsev et al. (2013), our analysis revealed that the Rinuak and Badar fish are the same species, with low genetic diversity (0.0%-0.5%). While previously, the morphological study by Periwaldi (2012) showed the substantial morphological differences between Rinuak Maninjau Lake and Badar Siais Lake. Geographically, the three lakes are not connected, no gene flow between the population of the lakes, ~~which~~ that causes the geographical isolation. Roesma et al. (2018) explained that the freshwater fauna in Maninjau Lake isolated because of no connection with other waters. Water in the Maninjau Lake comes from small rivers, which is filled by water catchment in the surrounding area and only has one outlet. The low genetic divergences of Rinuak and Badar fish between Lakes indicated that geographic distance and geographical isolation do not correlate their sequence divergences.

Using mtDNA, Mcglasan, and Hughes (2001) ~~revealed~~ considered that there was no significant relationship between geographical distance and gene flow on *Hypseleotris compressa* from different drainages with low genetic differentiation on the populations. Keith et al. (2005) also reported that the *Sicyopterus lagocephalus* from different distant archipelagos has minimal genetic distances and suggests they as the same species. The study by Arisuryanti et al. (2018) showed that *Periophthalmus argentilineatus* in Bogowonto Lagoon (Middle Java) and population in Pandeglang (West Java) clustered together with low sequence divergences. *H. compressa, S. lagocephalus*, and *P. argentilineatus* are the species from other Gobies. Referring to study Mcglasan and Hughes (2001); Keith et al. (2005), the possibilities conditions of present study are: 1) natural selection is able to maintain the balance of allele frequencies, 2) there is a geographic connection in the past at these distant locations, and 3) the ability of the fish to change.

Morphologicaly~~y~~ and genetic studies showed that environmental conditions play an essential role in determining the phenotype of a species. The phenotype shows the existence of high adaptability to environmental conditions without changing their genetic makeup. Akihito et al. (2000) reported that the adaptations of Gobiidae fishes to habitats had developed various morphological specializations. It is difficult to estimate the evolutionary scenario using only morphological information, so that needed the genetic data. The phylogenetic tree showed that the *Gobiopterus* is a sister taxon of Rinuak, and Badar fish with sequence divergences was 24.6%-27.9%. Based on Kartavsetv et al. (2011); Kartavsetv et al. (2013), these values show the different of the genus level. The same case also found in other studies of Gobiidae. Jeon et al. (2012) reported that the average sequence divergences of intragenus of more than 20 genera with multiple species of Gobies higher (21.09%) than among species (9.93%-9.54%) in the study of Ward et al. (2005); Ward and Holmes (2007).

According to Kottelat (2013), the transparent gobies consist of two genera; *Gobiopterus* and *Mistichthys*. *Mistichthys* only has one species, *Mistichthys luzonensis*. Referring to Kottelat et al. (1993), Rinuak and Badar fish has similar in morphology with *G. brachypterus*. The similarity of the main characters between Rinuak and Badar fish with *G. brachypterus* consists of cheek naked, cheeks without raised ridges and flaps, pelvic thin without fleshy lobes, head pores absent, transparent body, cheek and margins of preopercle usually with a dark vertical mark, dorsal midline in front of the first dorsal usually with black pigments, mouth of males is more extensive than female and almost vertical and distance between eyeballs 0.5-1.0 times eyeball diameter. However, Rinuak and Badar fish have high sequence divergences (24.6%-27.9%) against *G. brachypterus* in Central Java, Malaysia, and India. Despite having the similarity in morphology, the high sequence divergences do not support the grouping of Rinuak and Badar fish as the same species to *G. brachypterus* even to the genus *Gobiopterus*.

Previously, the transparent gobies were separated into several genera (Munro,1949) consists of Gobiopterus Bleeker (1874), *Mistichthys* Smith (1901), *Micrapocryptes* Hora (1923), *Mirogobius* Herre (1927), *Gobiella* Smith (1931), and *Herreolus* Smith (1931). However, previous authors (Hora ~~(~~1934~~)~~; Mukerji ~~(~~1936~~)~~; Aurich ~~(~~1938~~)~~; Koumans (I940), demonstrated that *Gobiopterus* is a synonym of the re-examined holotype of *Micrapocryptes*, *Gobiella*, *Mistichthys*, and *Mirogobius*. While, Kottelat (2013) has reported that the valid of transparent gobies consists of two genera (*Mistichthys* and *Gobiopterus*), where is *Gobiopterus* has ten species. However, only four species of them have morphological and genetic data (*G. brachypterus*, *G. lacustris*, *G. chuno*, and *G. semivestitus*). Unfortunately, there is no information explained in the published article about *G. brachypterus* in Rawa Pening lake, Central Java, which was used as a comparison species in the present analysis of the study.

According to (Kottelat, 2013), the type locality of *G. brachypterus* (Bleeker,1955) in Pasuruan, Ranu Grati Lake, East Java, Indonesia. Based on the several sources, *G. brachypterus* in East Java are known with the local name as Lempuk fish. The local peoples consider Lempuk fish only found in Ranu Grati Lake (East Java). Lempuk fish also has high economic value as of fresh food and processed food like Rinuak fish from Maninjau Lake. The genetic similarity study of Lempuk fish and *G. brachypterus* using the 12s rRNA gene by Candra (2012) showed the high sequence divergences (8.2%) between them and concluded that Lempuk fish was different genus to *G. brachypterus*. These results supported our study, which showed Rinuak and Badar fish as different from *G. brachypterus*. The lack of morphological and genetic studies on the *Gobiopterus* group makes it difficult for further identification. Studies on *Gobiopterus* are limited to the first records *G.brachypterus* in Sri Lanka (Ott, 2011), sexual dimorphism of *G.semivestitus* (Macdowall and David, 2008), and genetic differentiation of *G.lacustris* (Aquino et al. 2011; Wang et al. 2017).).

The data of IUCN (year?) and FishBase (year?) ~~showed~~ suggested that species of *Gobiopterus* are endemic to certain islands/countries; some are also found in other regions such as *G. lacustris* is endemic in the Philippines. However, Wang et al. (2017) reported that *G. lacustris* also found in China. Aquino et al. (2011) explained that *G. lacustris* divided into two distinct clusters with deep genetic divergence at 23.80% and proposed that they are a cryptic species. Wang et al. (2017) confirmed that no cryptic species on *G. lacustris* in the Philippines and supported that *G. lacustris* in China should not be an invasive species from the Philippines.

There are no photo vouchers, and the availability of the data of DNA sequences on GenBank and BOLD System to all *Gobiopterus* species causes the difficulties in identified and verified their taxonomy status. The taxonomy of Gobiidae has been studied extensively for the last decades, but confusion still exists (Jeon et al. 2012). The Present study revealed that Rinuak fish is not an endemic of Maninjau Lake. Rinuak and Badar exist as a different genus with *Gobiopterus*. T~~he~~ F further comprehensive studies using the morphology and other molecular markers on Rinuak and Badar are ongoing.

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Phylogenetic analysis of transparent gobies in three sumatran lakes, inferred from mitochondrial Cytochrome Oxidase 1 (CO1) gene

Abstract. Morphologically, the transparent gobies fish found in three lakes in Sumatra island are very similar to the *Gobiopterus brachypterus*. The fish is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in Lake Siais-North Sumatra). The phylogenetic study was carried out by analyzing 619 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene in 12 fish individuals from the three lakes. Rinuak and Badar fish in three populations have three haplotypes. The sequence divergences in and between populations are very low (0.0%-0.5%). This value indicates that Rinuak and Badar are the same species with low genetic diversity. The phylogenetic tree illustrates that this fish belongs to the group of Gobiidae and a sister taxon from *G. brachypterus*.

Keywords: Badar Fish, CO1, Genetic diversity, *Gobiopterus brachypterus*, Rinuak Fish

INTRODUCTION

Freshwater fishes biodiversity in Indonesia forms one of the largest in the world. There are at least 1189 native species, 125 of them endemic to the country (Froese and Pauly, 2014). The freshwater fish is poorly documented, with many fish are waiting to be identified. Sumatra is the second largest island on the Sundaland with a high level of biodiversity, characterized by a high degree of endemism (Sodhi and Brook, 2006; Pfeil, 2009). In Sumatra Island, there is a kind of small transparent fish that morphologically shows the character of the Gobiidae group. That fish locally is known as Rinuak fish, with the total length range 19.91 to 22.68 mm and considered an endemic species of Maninjau Lake, West Sumatra. Besides, in Maninjau Lake, Rinuak fish also found in Singkarak Lake, another big lake in Sumatra. These fish have an economically important value as food. Roesma and Santoso (2010) also reported a species suspected to be the same as Rinuak fish, which named locally as Badar fish in Siais Lake, North Sumatra. However, the scientific name of these two types is unknown, and some local people consider it as a juvenile of *Rasbora* from Maninjau Lake.

Rinuak and Badar fish can be classified as the Gobiidae group because of they have the main characters of Gobies, which consists of; a pair of pectoral fins are fused to form suckers, two dorsal fins, rounded tail, and blunt head with big eyes (Kottelat et al.1993). Morphologically, Rinuak, and Badar fish show a similar feature to *Gobiopterus brachypterus* (transparent gobies). *Gobiopterus* is a genus of gobies with small size, native to freshwater, marine or brackish waters, and one genus of transparent gobies (Kottelat et al. 1993). Biological and ecological information on Rinuak and Badar fish are very scanty, limited only in studies of processed quality of Rinuak as s food (Astuti et al. 2016). In the previous studies, Periwaldi (2012) reported that Rinuak fish in Maninjau Lake and Badar fish in Siais Lake have high morphological differentiation (13 of 20 characters).

In the past, the classification of the gobies group generally based on the external characters (Pezold 1993; Akihito et al. 2000; Nelson 2006); however, diagnostic characters are difficult and problematic to identify to species level. Recently, the advances in molecular approaches have provided the complement to the limited of morphological data for untangling gobioid group (Aquino et al. 2011; Jeon et al. 2012; Agorreta and Ruber 2012; Agoretta et al. 2013; Tornabene et al. 2013; Taillebois et al. 2014; Thacker 2009; Thacker 2015; Wang et al. 2017). Mitochondrial DNA (mtDNA) markers have historically been used for most systematic molecular studies because they have a large number of copies, maternally inherited, and have a higher mutation rate compared to nuclear DNA (Brown et al. 1979; Johns and Avise 1998). Mitochondrial cytochrome oxidase subunit I (COI) gene could serve as a rapid and reliable barcoding marker for identifying species and effective in delimitating species in animal taxa (Hebert et al. 2004; Hubert et al. 2008; Roesma et al. 2018; Roesma et al. 2019). Recently, the COI gene has been widely used to resolve the taxonomy in a gobioid group (Jeon et al. 2012; Jin et al. 2014; Taillebois et al. 2014; Wang et al. 2017; Olii et al. 2019). Further research is needed on Rinuak and Badar fish using a molecular approach. The present study aimed to investigate the phylogenetic relationship of Rinuak and Badar fish in three Sumatran Lakes using cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study area

Rinuak and Badar fish specimens were collected from three Sumatran lakes consist of; Maninjau Lake and Singkarak Lake in West Sumatra and Siais Lake in North Sumatra. The samples collected with cast-nets and set-nets following standard procedures by Cailliet et al. (1986). Twelve individuals out of the samples were used for phylogenetic analysis and preserved in 96% Alcohol (PA). Other individual specimens were all together fixed in 4% formalin and preserved into 70% ethanol for long term storage in the laboratory.

Procedures

The DNA extraction was performed following the protocol Kit INVITROGEN PureLinkTM Genomic DNA Mini Kit. The COI was amplified using the following primers: forward primer Fish F1 (5' TCAACCAACCACAAAGACATTGGCAC 3') and reverse primer Fish R1 (5' TAGACTTCTGGG TGGCCAAAGAATCA3') (Ward et al. 2005). PCR reactions were conducted in 25 µl volumes, containing;3 µl of DNA template, 11 µl of PCR Supermix, 0.5 µl of each primer (0.01 mM), and ten µl of DDH20. The PCR procedure was conducted using PCR *Sensoquest* thermal cyclers with the following program: initial denaturation at 95^{0}C for 2 min; 35 cycles of denaturation at 94^{0}C for 30 s, annealing at 54^{0}C for 30 s and elongation at 72^{0}C for 1 min; followed by a final extension of 72°C for 10 min and then held at 4°C for the long term storage. PCR products were run on 2% agarose gel and visualized using GelDoc. PCR products were purified and the most powerful products selected for sequencing to1st Base, Pte. Ltd.in Malaysia.

Data analysis

The nucleotide sequences from the DNA sequencing of Rinuak and Badar fish was contig and edited using the DNA STAR program (Burland 2000). DNA sequences were obtained then compare with the data on the Genbank, NCBI (Table 1.) to investigate the similarity of all sequences in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences were aligned using the Clustal X default settings and edited using the Bioedit program (Thompson 1997; Hall 1999). DNA SP version 5.10 was used to identify haplotypes numbers (*h*), polymorphism sites (S), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Rozas 2003). Pairwise genetic distances were calculated to quantify sequence divergences among all sequences using *the p*-distance Kimura two-parameter (K2P) method in the MEGA version 6.0 program (Tamura et al. 2013). Phylogenetic analyses were performed using the construction of a phylogenetic tree based on the four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML), and Maximum Parsimony (MP) using 1000 bootstrapping in MEGA 6.0 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

Sequence data

The mtDNA COI sequences of 32 individuals (12 sequences from the present study and 20 sequences from the GenBank) were determined for a total of 619 bp. Among them, 231 bp (37.32%) were variable sites, and 213 bp (34.42%) were parsimony informative. No stop codons, insertions, and deletions were found in its translation. The average compositions of A, T, C, and G nucleotide base was 24.4%, 30.3%, 27.5%, and 17.8%, respectively. The average compositions' value was almost similar to the previous studies of Gobiidae (Aquino et al. 2011; Aquilino et al. 2011; Jeon et al. 2012). Nucleotide sequences are AT ratio (54.7%) higher than the GC ratio, which is characteristic for the genome of mtDNA in vertebrate (Hubert et al. 2008). The GC content of the first codon is the highest, while the variable second codon has the lowest. The transitions are more common than transversion with bias ratio (Ts; Tv) is R: 3.737. A total of 22 haplotypes consisted of four haplotypes to Rinuak and Badar fish in Sumatran Lakes and 18 haplotypes to data of GenBank, NCBI. The average haplotype diversity among 22 haplotypes (Hd) was 0.938±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.16256±0.002. While among Rinuak and Badar fish in Sumatra has the haplotype diversity (*Hd*) was 0.561, and nucleotide diversity (*Pi*) was 0.00142. Genetic distance within and among populations of Rinuak and Badar fish was 0.0%-0.5% (the table of genetic distance not showed). Sequence divergence between outgroup (*Rasbora lateristriata* and *Danio rerio*) with Rinuak and Badar fish lineages ranged from 27.6% to 31.6%. That value supported that they are in a different Family.

Table 1. Species List taxonomy, Location and Genbank Accession Number of samples

No.	Family	Genus	Species	Location	Accession Numbers
1	Gobiidae	*Gobiopterus*	*Gobiopterus brachypterus*	Malaysia	KX223903.1
2			*Gobiopterus brachypterus*	Malaysia	KX223902.1
3			*Gobiopterus brachypterus*	India	MG495939.1
4			*Gobiopterus brachypterus*	Central Java	KU692539.1
5			*Gobiopterus brachypterus*	Central Java	KU692538.1
6			*Gobiopterus brachypterus*	Central Java	KU692535.1
7			*Gobiopterus chuno*	Bangladesh	MK572237.1
8			*Gobiopterus lacustris*	Philippines	HQ682693.1
9			*Gobiopterus lacustris*	Philippines	HQ682695.1
10			*Gobiopterus semivestitus*	Australia	KJ669473.1
11		*Sicyopterus*	*Sicyopterus parvei*	West Java	KU693078.1
12			*Sicyopterus lagocephalus*	Bali	KU693018.1
13		*Stiphodon*	*Stiphodon semoni*	West Java	KU693171.1
14			*Stiphodon maculidorsalis*	Banten	KU693162.1
15		*Sicyopus*	*Sicyopus zosterophorum*	Bali	KU693126.1
16			*Sicyopus ribicundus*	East Java	KU693113.1
17		*Lentipes*	*Lentipes whittenorum*	Bali	KU693014.1
18			*Lentipes ikeae*	West Java	KU692987.1
19	Cyprinidae	*Rasbora*	*Rasbora lateristriata*		KT960818.1
20		*Danio*	*Danio rerio*	India	MK714084.1

Phylogenetic relationships

The phylogenetic tree was constructed using four methods (ML/NJ/ME/MP) analysis generated almost in identical topologies, represented by the ML tree with 1000 bootstrap replicates were shown in Figure 1. Based on the tree constructed from COI genes, it can be observed that the Gobiidae group divided into four sublineages.

The phylogenetic tree members consist of Rinuak and Badar fish in the first sublineage, *Gobiopterus* in the second and fourth sublineage while *Sicyopterus, Stipodon, Sicyopus*, and *Lentipes* in the third sublineage. All members of Rinuak and Badar fish from three Sumatran lakes present together in the first sublineage with sequence divergences was 0.0%-0.5%. In the second sublineage, consists of three species from Gobiopterus, namely *G. brachypterus, G.chuno,* and *G. lacustris*. Among the species of *Gobiopterus* has sequence divergences was 2.0%-17.8%. The presence of small sequence divergences (2.0%) between the *G. chuno* from Bangladesh MK572237 and *G. brachypterus from* India MG495939 and (3.0%) between *G. brachypterus* from Central Java and *G. lacustris* from the Philippines is require re-identification studies in taxonomy. According to Kartavsetv et al. (2013), that value indicated for differences at the level of subspecies, semi species, or sibling species.

The phylogenetic tree showed that the *Gobiopterus* in the second Sublineage is occupying the position as a sister taxa of Rinuak and Badar fish with sequence divergences was 24.6%-27.9%. *Gobiopterus* is the only genus of transparent gobies that has the genetic data used as a comparison for Rinuak and Badar fish. Based on Kartavsetv et al. (2011), Kartavsetv et al. (2013), the value of sequence divergences between first and second sublineages showed differentiation at the level of a different genus. The third Sublineage consists of four different genera. The sequence divergences between them were 11.8%-15.3%, and with both first and second sublineages were 20.7%-29.3%. The fourth Sublineage has one member, *G. semivestitus*, which is separated from other *Gobiopterus* in the second Sublineage with sequence divergences was 27.2%-29.1%. The separation of *Gobiopterus* into two different sublineages showed that *Gobiopterus* has the non-monophyletic lineages. The value of sequence divergences between *Gobiopterus* in the second and fourth sublineages showed differentiation at the level of a different genus. Based on Kartavsetv et al. (2011), Kartavsetv et al. (2013) sequence divergences in the same genus was 11%-16%.

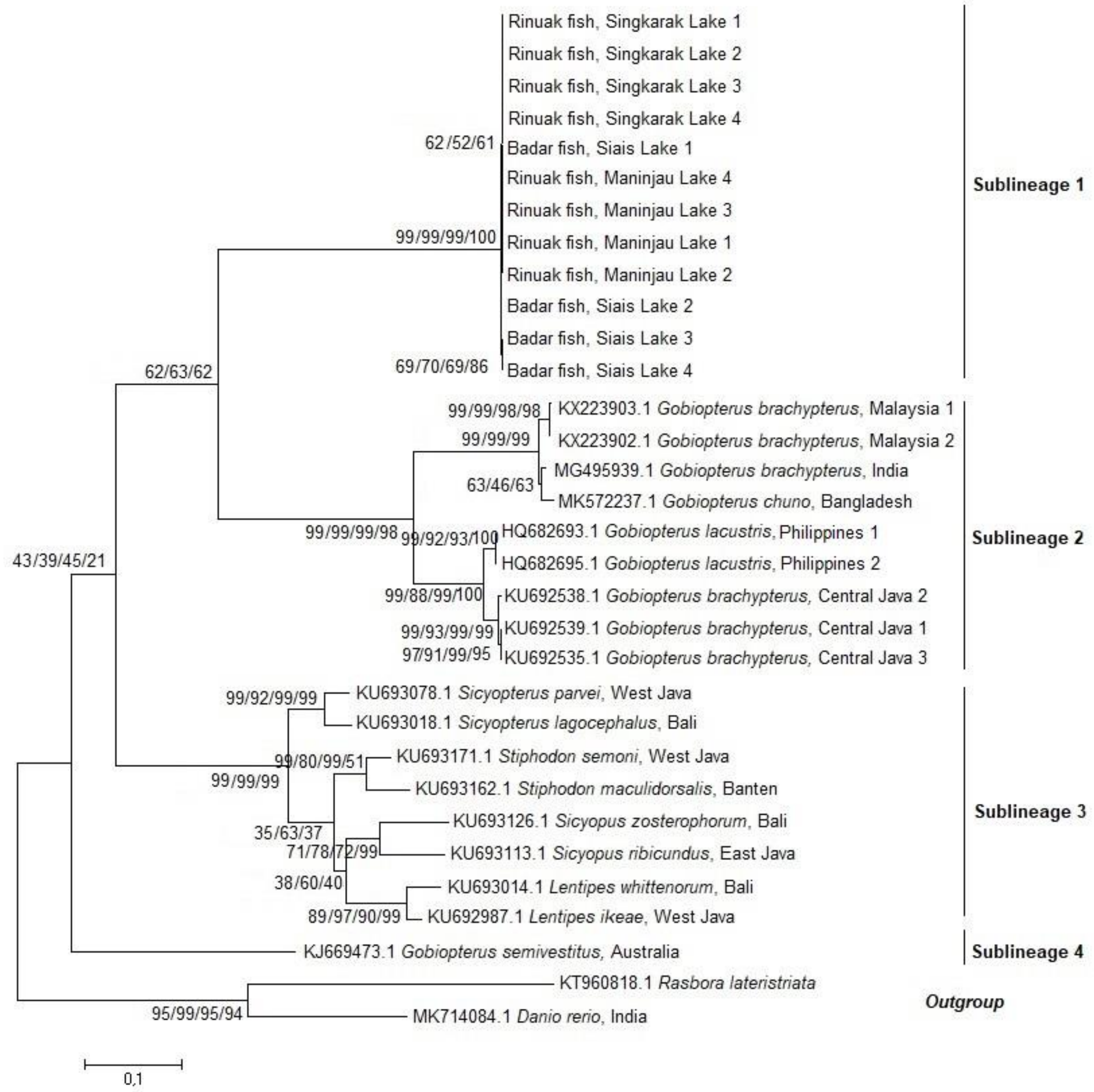

Figure 1. The ML phylogenetic tree of the COI gene with the bootstrap value of 1000 replicates (ME/NJ/ML/MP).

Discussion

The present study represents the first molecular data of Rinuak and Badar fish in Sumatra using mitochondrial markers (COI gene). Rinuak and Badar fish in Sumatra present in one sublineage with low haplotype diversity, which is some individual shared the same haplotype. Refers to Kartavtsev et al. (2011); Kartavtsev et al. (2013), our analysis revealed that the Rinuak and Badar fish are the same species, with low genetic diversity (0.0%-0.5%). While previously, the morphological study by Periwaldi (2012) showed the substantial morphological differences between Rinuak Maninjau Lake and Badar Siais Lake. Geographically, the three lakes are not connected, no gene flow between the population of the lakes, which causes the geographical isolation. Roesma et al. (2018) explained that the freshwater fauna in Maninjau Lake isolated because of no connection with other waters. Water in the Maninjau Lake comes from small rivers, which is filled by water catchment in the surrounding area and only has one outlet. The low genetic divergences of Rinuak and Badar fish between Lakes indicated that geographic distance and geographical isolation do not correlate their sequence divergences.

Using mtDNA, Mcglasan, and Hughes (2001) revealed that there was no significant relationship between geographical distance and gene flow on *Hypseleotris compressa* from different drainages with low genetic differentiation on the populations. Keith et al. (2005) also reported that the *Sicyopterus lagocephalus* from different distant archipelagos has minimal genetic distances and suggests they as the same species. The study by Arisuryanti et al. (2018) showed that

Periophthalmus argentilineatus in Bogowonto Lagoon (Middle Java) and population in Pandeglang (West Java) clustered together with low sequence divergences. *H. compressa, S. lagocephalus,* and *P. argentilineatus* are the species from other Gobies. Referring to study Mcglasan and Hughes (2001); Keith et al. (2005), the possibilities conditions of present study are: 1) natural selection is able to maintain the balance of allele frequencies, 2) there is a geographic connection in the past at these distant locations, and 3) the ability of the fish to change.

Morphology and genetic studies showed that environmental conditions play an essential role in determining the phenotype of a species. The phenotype shows the existence of high adaptability to environmental conditions without changing their genetic makeup. Akihito (2000) reported that the adaptations of Gobiidae fishes to habitats had developed various morphological specializations. It is difficult to estimate the evolutionary scenario using only morphological information, so that needed the genetic data. The phylogenetic tree showed that the *Gobiopterus* is a sister taxon of Rinuak, and Badar fish with sequence divergences was 24.6%-27.9%. Based on Kartavsetv et al. (2011); Kartavsetv et al. (2013), these values show the different of the genus level. The same case also found in other studies of Gobiidae. Jeon et al. (2012) reported that the average sequence divergences of intragenus of more than 20 genera with multiple species of Gobies higher (21.09%) than among species (9.93%-9.54%) in the study of Ward et al. (2005); Ward and Holmes (2007).

According to Kottelat (2013), the transparent gobies consist of two genera; *Gobiopterus* and *Mistichthys*. *Mistichthys* only has one species, *Mistichthys luzonensis*. Referring to Kottelat et al. (1993), Rinuak and Badar fish has similar in morphology with *G. brachypterus*. The similarity of the main characters between Rinuak and Badar fish with *G. brachypterus* consists of cheek naked, cheeks without raised ridges and flaps, pelvic thin without fleshy lobes, head pores absent, transparent body, cheek and margins of preopercle usually with a dark vertical mark, dorsal midline in front of the first dorsal usually with black pigments, mouth of males is more extensive than female and almost vertical and distance between eyeballs 0.5-1.0 times eyeball diameter. However, Rinuak and Badar fish have high sequence divergences (24.6%-27.9%) against *G. brachypterus* in Central Java, Malaysia, and India. Despite having the similarity in morphology, the high sequence divergences do not support the grouping of Rinuak and Badar fish as the same species to *G. brachypterus* even to the genus *Gobiopterus*.

Previously, the transparent gobies were separated into several genera (Munro,1949) consists of Gobiopterus Bleeker (1874), *Mistichthys* Smith (1901), *Micrapocryptes* Hora (1923), *Mirogobius* Herre (1927), *Gobiella* Smith (1931), and *Herreolu*s Smith (1931). However, Hora (1934); Mukerji (1936); Aurich (1938); Koumans (I940), demonstrated that *Gobiopterus* is a synonym of the re-examined holotype of *Micrapocryptes, Gobiella, Mistichthys*, and *Mirogobius.* While, Kottelat (2013) has reported that the valid of transparent gobies consists of two genera (*Mistichthys* and *Gobiopterus*), where is *Gobiopterus* has ten species. However, only four species of them have morphological and genetic data (*G. brachypterus*, *G. lacustris, G. chuno,* and *G. semivestitus*). Unfortunately, there is no information explained in the published article about *G. brachypterus* in Rawa Pening lake, Central Java, which was used as a comparison species in the present analysis of the study.

According to (Kottelat, 2013), the type locality of *G. brachypterus* (Bleeker,1955) in Pasuruan, Ranu Grati Lake, East Java, Indonesia. Based on the several sources, *G. brachypterus* in East Java are known with the local name as Lempuk fish. The local peoples consider Lempuk fish only found in Ranu Grati Lake (East Java). Lempuk fish also has high economic value as of fresh food and processed food like Rinuak fish from Maninjau Lake. The genetic similarity study of Lempuk fish and *G. brachypterus* using the 12s rRNA gene by Candra (2012) showed the high sequence divergences (8.2%) between them and concluded that Lempuk fish was different genus to *G. brachypterus*. These results supported our study, which showed Rinuak and Badar fish as different from *G. brachypterus*. The lack of morphological and genetic studies on the *Gobiopterus* group makes it difficult for further identification. Studies on *Gobiopterus* are limited to the first records *G.brachypterus* in Sri Lanka (Ott, 2011), sexual dimorphism of *G.semivestitus* (Macdowall and David, 2008), and genetic differentiation of *G.lacustris* (Aquino et al. 2011; Wang et al. 2017).).

The data of IUCN and FishBase showed that species of *Gobiopterus* are endemic to certain islands/countries; some are also found in other regions such as *G. lacustris* is endemic in the Philippines. However, Wang et al. (2017) reported that *G. lacustris* also found in China. Aquino et al. (2011) explained that *G. lacustris* divided into two distinct clusters with deep genetic divergence at 23.80% and proposed that they are a cryptic species. Wang et al. (2017) confirmed that no cryptic species on *G. lacustris* in the Philippines and supported that *G. lacustris* in China should not be an invasive species from the Philippines.

There are no photo vouchers, and the availability of the data of DNA sequences on GenBank and BOLD System to all *Gobiopterus* species causes the difficulties in identified and verified their taxonomy status. The taxonomy of Gobiidae has been studied extensively for the last decades, but confusion still exists (Jeon et al. 2012). The Present study revealed that Rinuak fish is not an endemic of Maninjau Lake. Rinuak and Badar exist as a different genus with *Gobiopterus.* The further comprehensive studies using the morphology and other molecular markers on Rinuak and Badar are ongoing.

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Phylogenetic analysis of transparent gobies in three sumatran lakes, inferred from mitochondrial Cytochrome Oxidase I (COI) gene

Abstract. The transparent gobies fish found in three lakes in Sumatra island is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in Lake Siais-North Sumatra), and are morphologically very similar to the *Gobiopterus brachypterus*. The phylogenetic study was carried out by analyzing 619 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene in 12 fish individuals from the three lakes. Rinuak and Badar fish in three populations have four haplotypes. The sequence divergences in and between populations are very low (0.0%-0.5%). This value indicates that Rinuak and Badar fish are the same species with low genetic diversity. The phylogenetic tree illustrates that this fish belongs to the group of Gobiidae and a sister taxon from *G. brachypterus*.

Keywords*:* Badar Fish, COI, Genetic diversity, *Gobiopterus brachypterus*, Rinuak Fish

INTRODUCTION

Freshwater fishes biodiversity in Indonesia forms one of the largest in the world, but poorly documented. There are at least 1189 native species, 125 of them are endemic to the country (Froese and Pauly 2014). Sumatra island a high level of biodiversity of freshwater fishes with high degree of endemism (Sodhi and Brook 2006; Pfeil 2009), including a small transparent fish that morphologically shows the character of the Gobiidae group. The fish locally is known as Rinuak fish, with the total length range 19.91 to 22.68 mm and previously considered as an endemic species of Maninjau Lake. The fish have an economically important value as food. Roesma and Santoso (2010) reported a species suspected to be the same as Rinuak fish, that locally named as Badar fish found in Siais Lake, North Sumatra. However, the scientific name of these two species remained unknown, and some local people consider it as a juvenile form of *Rasbora* from Maninjau Lake.

Rinuak and Badar fish can be classified as the Gobiidae group because of they have the main characters of Gobies, i.e. a pair of pectoral fins that are fused to form suckers, and blunt head with big eyes (Kottelat et al. 1993). In addition, the Rinuak and Badar fish are similar to *Gobiopterus brachypterus* (transparent gobies). *Gobiopterus* is a genus of gobies with small size, native to freshwater, marine or brackish waters, and one genus of transparent gobies (Kottelat et al. 1993). Biological and ecological information on Rinuak and Badar fish are very scanty, limited only on study of processed quality of Rinuak as s food (Astuti et al. 2016). A previous study (Periwaldi 2012) reported that Rinuak fish in Maninjau Lake and Badar fish in Siais Lake have high morphological differentiations i.e. 13 different characters of 20 characters.

In the past, the classification of the gobies group was generally based on the external characters (Pezold 1993; Akihito et al. 2000; Nelson 2006); however, identification of species level was proven to be difficult and problematic. Recently, the advances in molecular approaches have provided the complement to the limited of morphological data for untangling gobioid group (Aquino et al. 2011; Jeon et al. 2012; Agorreta and Ruber 2012; Agoretta et al. 2013; Tornabene et al. 2013; Taillebois et al. 2014; Thacker 2009; Thacker 2015; Wang et al. 2017). Mitochondrial DNA (mtDNA) markers have been widely used for most systematic molecular studies because they have a large number of copies, maternally inherited, and have a higher mutation rate compared to nuclear DNA (Brown et al. 1979; Johns and Avise 1998). Mitochondrial cytochrome oxidase subunit I (COI) gene could serve as a rapid and reliable barcoding marker for identifying species and effective in delimitating species of various animals (Hebert et al. 2004; Hubert et al. 2008; Imtiaz, Mohd-Nor, and Naim, 2017; Roesma et al. 2018; Roesma et al. 2019). Recently, the COI gene has been widely used to resolve the taxonomy in a gobioid group (Jeon et al. 2012; Jin et al. 2014; Taillebois et al. 2014; Wang et al. 2017; Olii et al. 2019). Further research is needed on Rinuak and Badar fish using a molecular approach. The present study aimed to investigate the phylogenetic relationship of Rinuak and Badar fish in three Sumatran Lakes using cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study sites

Rinuak and Badar fish specimens were collected from three Sumatran lakes, i.e. Maninjau Lake and Singkarak Lake in West Sumatra and Siais Lake in North Sumatra. The samples collected with cast-nets and set-nets following standard procedures by Cailliet et al. (1986). Twelve individuals out of the samples were used for phylogenetic analysis and preserved in 96% ethanol (PA). Other individual specimens were all together fixed in 4% formalin and preserved into 70% ethanol for long term storage in the laboratory.

Molecular Work

The DNA extraction was performed following the protocol Kit INVITROGEN PureLink^{TM} Genomic DNA Mini Kit. The COI was amplified using the following primers: forward primer Fish F1 (5' TCAACCAACCACAAAGACATTGGC AC 3') and reverse primer Fish R1 (5' TAGACTTCTGGGTGGCCAAAGAATCA 3') (Ward et al. 2005). PCR reactions were conducted in 25 µl volumes, containing ; 3 µl of DNA template, 11 µl of PCR Supermix, 0.5 µl of each primer (0.01 mM), and 10 µl of DDH20. The PCR procedure was conducted using PCR *Sensoquest* thermal cyclers with the following program: initial denaturation at 95^{0}C for 2 min; 35 cycles of denaturation at 94^{0}C for 30 s, annealing at 54^{0}C for 30 s and elongation at 72^{0}C for 1 min; followed by a final extension of 72°C for 10 min and then held at 4°C for the long term storage. PCR products were run on 2% agarose gel and visualized using GelDoc. PCR products were purified and the most powerful products selected for sequencing to1st Base, Pte. Ltd.in Malaysia.

Data analysis

The nucleotide sequences from the DNA sequencing of Rinuak and Badar fish was edited using the DNA STAR program (Burland 2000). Then, DNA sequences were obtained compare with the data on the Genbank, NCBI (Table 1.) to investigate the similarity of all sequences in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences were aligned using the Clustal X default settings and edited using the Bioedit program (Thompson 1997; Hall 1999). DNA SP version 5.10 was used to identify haplotypes numbers (*h*), polymorphism sites (S), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Rozas 2003). Pairwise genetic distances were calculated to quantify sequence divergences among all sequences using the *p*-distance Kimura two-parameter (K2P) method in the MEGA version 6.0 program (Tamura et al. 2013). Phylogenetic analyses were performed the four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML), and Maximum Parsimony (MP) using 1000 bootstrapping in MEGA 6.0 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

Sequence data

The mtDNA COI sequences of 32 individuals (12 sequences from the present study and 20 sequences from the GenBank) were determined for a total of 619 bp. Among them, 231 bp (37.32%) were variable sites, and 213 bp (34.42%) were parsimony informative sites. No stop codons, insertions, and deletions were found in its translation. The average compositions of A, T, C, and G nucleotide base was 24.4%, 30.3%, 27.5%, and 17.8%, respectively. The average composition value was almost similar to the previous studies of Gobiidae (Aquino et al. 2011; Aquilino et al. 2011; Jeon et al. 2012). Nucleotide sequences are AT ratio (54.7%) higher than the GC ratio, which is characteristic for the genome of mtDNA in vertebrate (Hubert et al. 2008). The GC content of the first codon is the highest, while the variable second codon has the lowest. The transitions are more common than transversion with bias ratio (Ts; Tv) is R: 3.737. A total of 22 haplotypes consisted of four haplotypes to Rinuak and Badar fish in Sumatran Lakes and 18 haplotypes to data of GenBank, NCBI. The average haplotype diversity among 22 haplotypes (Hd) was 0.938±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.16256±0.002. While among Rinuak and Badar fish in Sumatra has the haplotype diversity (*Hd*) was 0.561, and nucleotide diversity (*Pi*) was 0.00142. Genetic distance within and among populations of Rinuak and Badar fish was 0.0%-0.5% (the table of genetic distance not showed). Sequence divergence between outgroup (*Rasbora lateristriata* and *Danio rerio*) with Rinuak and Badar fish lineages ranged from 27.6% to 31.6%. That value supported that they are in a different Family.

Table 1. Species List taxonomy, Location and Genbank Accession Number of samples

No.	Family	Genus	Species	Location	Accession Numbers
1	Gobiidae	*Gobiopterus*	*Gobiopterus brachypterus*	Malaysia	KX223903.1
2			*Gobiopterus brachypterus*	Malaysia	KX223902.1
3			*Gobiopterus brachypterus*	India	MG495939.1
4			*Gobiopterus brachypterus*	Central Java	KU692539.1
5			*Gobiopterus brachypterus*	Central Java	KU692538.1
6			*Gobiopterus brachypterus*	Central Java	KU692535.1
7			*Gobiopterus chuno*	Bangladesh	MK572237.1
8			*Gobiopterus lacustris*	Philippines	HQ682693.1
9			*Gobiopterus lacustris*	Philippines	HQ682695.1
10			*Gobiopterus semivestitus*	Australia	KJ669473.1
11		*Sicyopterus*	*Sicyopterus parvei*	West Java	KU693078.1
12			*Sicyopterus lagocephalus*	Bali	KU693018.1
13		*Stiphodon*	*Stiphodon semoni*	West Java	KU693171.1
14			*Stiphodon maculidorsalis*	Banten	KU693162.1
15		*Sicyopus*	*Sicyopus zosterophorum*	Bali	KU693126.1
16			*Sicyopus ribicundus*	East Java	KU693113.1
17		*Lentipes*	*Lentipes whittenorum*	Bali	KU693014.1
18			*Lentipes ikeae*	West Java	KU692987.1
19	Cyprinidae	*Rasbora*	*Rasbora lateristriata*		KT960818.1
20		*Danio*	*Danio rerio*	India	MK714084.1

Phylogenetic relationships

The phylogenetic tree was constructed using four methods (ML/NJ/ME/MP) analysis generated almost in identical topologies, represented by the ML tree with 1000 bootstrap replicates (Figure 1). Based on the tree constructed from COI genes, it can be observed that the Gobiidae group divided into four sublineages.

The phylogenetic tree members consist of Rinuak and Badar fish in the first sublineage, *Gobiopterus* in the second, and fourth sublineage while *Sicyopterus*, *Stipodon*, *Sicyopus*, and *Lentipes* in the third sublineage. All members of Rinuak and Badar fish from three Sumatran lakes present together in the first sublineage with sequence divergences was 0.0%-0.5%. In the second sublineage, consists of three species from *Gobiopterus*, namely *G. brachypterus*, *G. chuno,* and *G. lacustris*. Among the species of *Gobiopterus* has sequence divergences was 2.0%-17.8%. The presence of small sequence divergences (2.0%) between the *G. chuno* from Bangladesh MK572237 and *G. brachypterus from* India MG495939 and (3.0%) between *G. brachypterus* from Central Java and *G. lacustris* from the Philippines is require re-identification studies in taxonomy. According to Kartavsetv et al. (2013), that value indicated for differences at the level of subspecies, semi species, or sibling species.

The phylogenetic tree showed that the *Gobiopterus* in the second sublineage is occupying the position as a sister taxa of Rinuak and Badar fish with sequence divergences was 24.6%-27.9%. *Gobiopterus* is the only genus of transparent gobies that has the genetic data used as a comparison for Rinuak and Badar fish. Based on Kartavtsev et al. (2011), Kartavtsev et al. (2013), the value of sequence divergences between first and second sublineages showed differentiation at the level of a different genus. The third Sublineage consists of four different genera. The sequence divergences between them were 11.8%-15.3%, and with both first and second sublineages were 20.7%-29.3%. The fourth Sublineage has one member, *G. semivestitus,* which is separated from other *Gobiopterus* in the second sublineage with sequence divergences was 27.2%-29.1%. The separation of *Gobiopterus* into two different sublineages showed that *Gobiopterus* has the non-monophyletic lineages. The value of sequence divergences between *Gobiopterus* in the second and fourth sublineages showed differentiation at the level of a different genus. Based on Kartavtsev et al. (2011), Kartavtsev et al. (2013) sequence divergences in the same genus was 11%-16%.

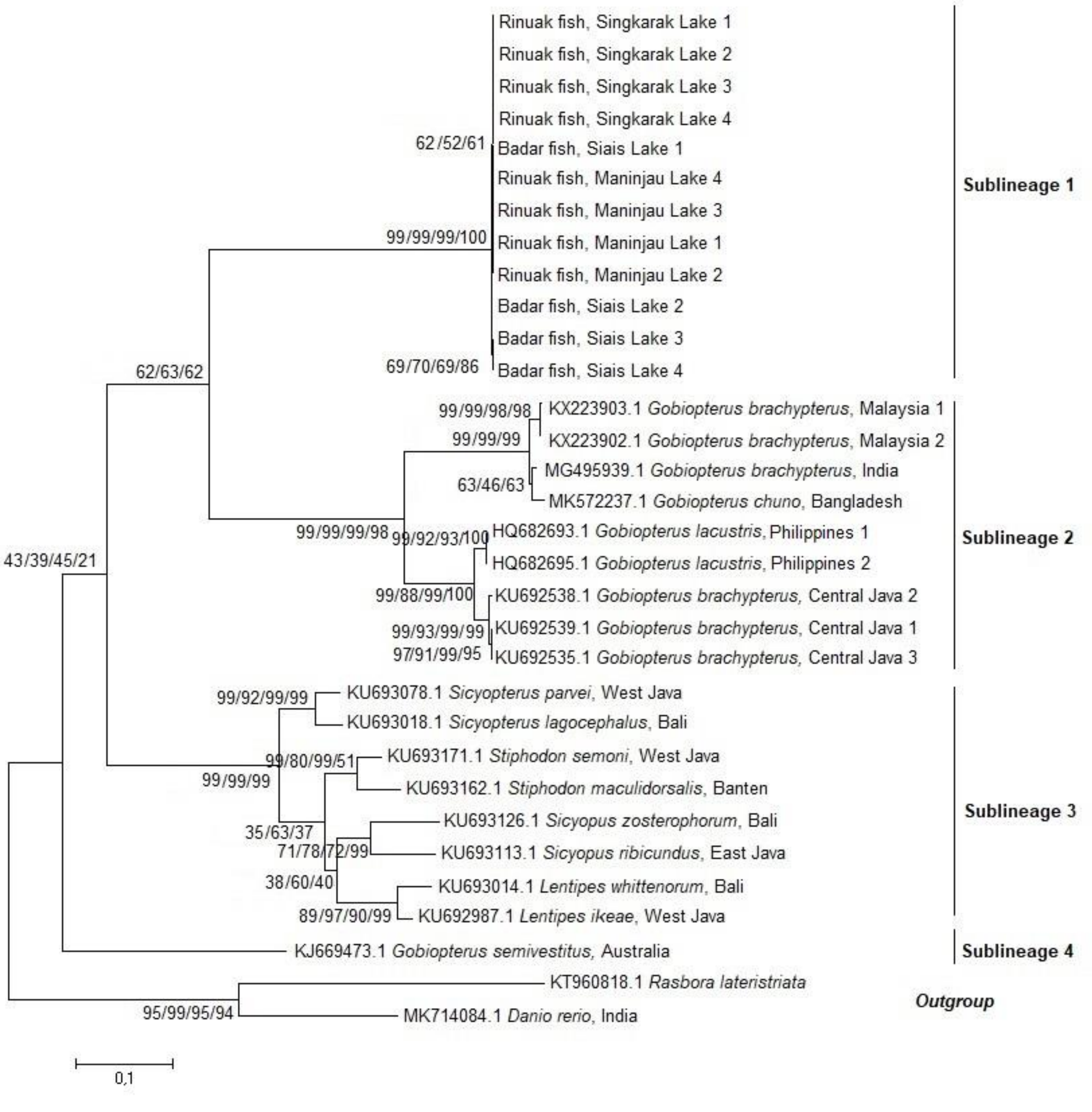

Figure 1. The ML phylogenetic tree of the COI gene with the bootstrap value of 1000 replicates (ME/NJ/ML/MP).

Discussion

The present study represents the first molecular data of Rinuak and Badar fish in Sumatra using mitochondrial markers (COI gene). Rinuak and Badar fish in Sumatra present in one sublineage with low haplotype diversity, which is some individual shared the same haplotype. Refers to Kartavtsev et al. (2011); Kartavtsev et al. (2013), our analysis revealed that the Rinuak and Badar fish are the same species, with low genetic diversity (0.0%-0.5%). While previously, the morphological study by Periwaldi (2012) showed the substantial morphological differences between Rinuak Maninjau Lake and Badar Siais Lake. Geographically, the three lakes are not connected, no gene flow between the population of the lakes, that causes the geographical isolation. Roesma et al. (2018) explained that the freshwater fauna in Maninjau Lake isolated because of no connection with other waters. Water in the Maninjau Lake comes from small rivers, which is filled by water catchment in the surrounding area and only has one outlet. The low genetic divergences of Rinuak and Badar fish between Lakes indicated that geographic distance and geographical isolation do not correlate their sequence divergences.

Using mtDNA, Mcglasan, and Hughes (2001) considered that there was no significant relationship between geographical distance and gene flow on *Hypseleotris compressa* from different drainages with low genetic differentiation on the populations. Keith et al. (2005) also reported that the *Sicyopterus lagocephalus* from different distant archipelagos has minimal genetic distances and suggests they as the same species. The study by Arisuryanti et al. (2018) showed that *Periophthalmus argentilineatus* in Bogowonto Lagoon (Middle Java) and population in Pandeglang (West Java) clustered together with low sequence divergences. *H. compressa, S. lagocephalus,* and *P. argentilineatus* are the species from other Gobies. Referring to study Mcglasan and Hughes (2001); Keith et al. (2005), the possibilities conditions of present study are: 1) natural selection is able to maintain the balance of allele frequencies, 2) there is a geographic connection in the past at these distant locations, and 3) the ability of the fish to change.

Morphological and genetic studies showed that environmental conditions play an essential role in determining the phenotype of a species. The phenotype shows the existence of high adaptability to environmental conditions without changing their genetic makeup. Akihito et al. (2000) reported that the adaptations of Gobiidae fishes to habitats had developed various morphological specializations. It is difficult to estimate the evolutionary scenario using only morphological information, so that needed the genetic data. The phylogenetic tree showed that the *Gobiopterus* is a sister taxon of Rinuak, and Badar fish with sequence divergences was 24.6%-27.9%. Based on Kartavtsev et al. (2011); Kartavtsev et al. (2013), these values show the different of the genus level. The same case also found in other studies of Gobiidae. Jeon et al. (2012) reported that the average sequence divergences of intragenus of more than 20 genera with multiple species of Gobies higher (21.09%) than among species (9.93%-9.54%) in the study of Ward et al. (2005); Ward and Holmes (2007).

According to Kottelat (2013), the transparent gobies consist of two genera; *Gobiopterus* and *Mistichthys*. *Mistichthys* only has one species, *Mistichthys luzonensis*. Referring to Kottelat et al. (1993), Rinuak and Badar fish has similar in morphology with *G. brachypterus*. The similarity of the main characters between Rinuak and Badar fish with *G. brachypterus* consists of cheek naked, cheeks without raised ridges and flaps, pelvic thin without fleshy lobes, head pores absent, transparent body, cheek and margins of preopercle usually with a dark vertical mark, dorsal midline in front of the first dorsal usually with black pigments, mouth of males is more extensive than female and almost vertical and distance between eyeballs 0.5-1.0 times eyeball diameter. However, Rinuak and Badar fish have high sequence divergences (24.6%-27.9%) against *G. brachypterus* in Central Java, Malaysia, and India. Despite having the similarity in morphology, the high sequence divergences do not support the grouping of Rinuak and Badar fish as the same species to *G. brachypterus* even to the genus *Gobiopterus*.

Previously, the transparent gobies were separated into several genera (Munro 1949) consists of *Gobiopterus* Bleeker (1874), *Mistichthys* Smith (1901), *Micrapocryptes* Hora (1923), *Mirogobius* Herre (1927), *Gobiella* Smith (1931), and *Herreolu*s Smith (1931). However, previous authors (Hora 1934; Mukerji 1936; Aurich 1938; Koumans I940), demonstrated that *Gobiopterus* is a synonym of the re-examined holotype of *Micrapocryptes, Gobiella, Mistichthys*, and *Mirogobius.* While, Kottelat (2013) has reported that the valid of transparent gobies consists of two genera (*Mistichthys* and *Gobiopterus*), where is *Gobiopterus* has ten species. However, only four species of them have morphological and genetic data (*G. brachypterus*, *G. lacustris*, *G. chuno,* and *G. semivestitus*). Unfortunately, there is no information explained in the published article about *G. brachypterus* in Rawa Pening lake, Central Java, which was used as a comparison species in the present analysis of the study.

According to (Kottelat 2013), the type locality of *G. brachypterus* (Bleeker 1955) in Pasuruan, Ranu Grati Lake, East Java, Indonesia. Based on the several sources, *G. brachypterus* in East Java are known with the local name as Lempuk fish. The local peoples consider Lempuk fish only found in Ranu Grati Lake (East Java). Lempuk fish also has high economic value as of fresh food and processed food like Rinuak fish from Maninjau Lake. The genetic similarity study of Lempuk fish and *G. brachypterus* using the 12s rRNA gene by Candra (2012) showed the high sequence divergences (8.2%) between them and concluded that Lempuk fish was different genus to *G. brachypterus*. These results supported our study, which showed Rinuak and Badar fish as different from *G. brachypterus*. The lack of morphological and genetic studies on the *Gobiopterus* group makes it difficult for further identification. Studies on *Gobiopterus* are limited to the first records *G. brachypterus* in Sri Lanka (Ott 2011), sexual dimorphism of *G. semivestitus* (Macdowall and David 2008), and genetic differentiation of *G. lacustris* (Aquino et al. 2011; Wang et al. 2017).

The data of (IUCN 2010; IUCN 2019) and FishBase (2013) suggested that species of *Gobiopterus* are endemic to certain islands/countries; some are also found in other regions such as *G. lacustris* is endemic in the Philippines. However, Wang et al. (2017) reported that *G. lacustris* also found in China. Aquino et al. (2011) explained that *G. lacustris* divided into two distinct clusters with deep genetic divergence at 23.80% and proposed that they are a cryptic species. Wang et al. (2017) confirmed that no cryptic species on *G. lacustris* in the Philippines and supported that *G. lacustris* in China should not be an invasive species from the Philippines.

There are no photo vouchers, and the availability of the data of DNA sequences on GenBank and BOLD System to all *Gobiopterus* species causes the difficulties in identified and verified their taxonomy status. The taxonomy of Gobiidae has been studied extensively for the last decades, but confusion still exists (Jeon et al. 2012). The Present study revealed that Rinuak fish is not an endemic of Maninjau Lake. Rinuak and Badar exist as a different genus with *Gobiopterus.* Further comprehensive studies using the morphology and other molecular markers on Rinuak and Badar are ongoing.

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Phylogenetic analysis of transparent gobies in three Sumatran lakes, inferred from mitochondrial Cytochrome Oxidase I (COI) gene

DEWI IMELDA ROESMA♥, DJONG HON TJONG, DYTA RABBANI AIDIL
Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University.Jl. Limau Manis, Padang, West Sumatra, Indonesia.
♥email: dewiroesma@sci.unand.ac.id

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Abstract. *Roesma D. I, Tjong D. H, Aidil D. R. 2019. Title. Biodiversitas 20: xxxx.* The transparent gobies fish found in three lakes in Sumatra island is known as Rinuak fish (in Lake Maninjau and Lake Singkarak-West Sumatra) or Badar fish (in Lake Siais-North Sumatra), and are morphologically very similar to the *Gobiopterus brachypterus*. The phylogenetic study was carried out by analyzing 619 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene in 12 fish individuals from the three lakes. Rinuak and Badar fish in three populations have four haplotypes. The sequence divergences in and between populations are very low (0.0%-0.5%). This value indicates that Rinuak and Badar fish are the same species with low genetic diversity. The phylogenetic tree illustrates that this fish belongs to the group of Gobiidae and a sister taxon from *G. brachypterus*.

Keywords: *Badar Fish, COI, Genetic diversity,* Gobiopterus brachypterus, *Rinuak Fish*

INTRODUCTION

Freshwater fishes biodiversity in Indonesia forms one of the largest in the world, but poorly documented. There are at least 1189 native species, 125 of them are endemic to the country (Froese and Pauly 2014). Sumatra island a high level of biodiversity of freshwater fishes with high degree of endemism (Sodhi and Brook 2006; Pfeil 2009), including a small transparent fish that morphologically shows the character of the Gobiidae group. The fish locally is known as Rinuak fish, with the total length range 19.91 to 22.68 mm and previously considered as an endemic species of Maninjau Lake. The fish have an economically important value as food. Roesma and Santoso (2010) reported a species suspected to be the same as Rinuak fish, that locally named as Badar fish found in Siais Lake, North Sumatra. However, the scientific name of these two species remained unknown, and some local people consider it as a juvenile form of *Rasbora* from Maninjau Lake.

Rinuak and Badar fish can be classified as the Gobiidae group because of they have the main characters of Gobies, i.e. a pair of pectoral fins that are fused to form suckers, and blunt head with big eyes (Kottelat et al. 1993). In addition, the Rinuak and Badar fish are similar to *Gobiopterus brachypterus* (transparent gobies). *Gobiopterus* is a genus of gobies with small size, native to freshwater, marine or brackish waters, and one genus of transparent gobies (Kottelat et al. 1993). Biological and ecological information on Rinuak and Badar fish are very scanty, limited only on study of processed quality of Rinuak as s food (Astuti et al. 2016). A previous study (Periwaldi 2012) reported that Rinuak fish in Maninjau Lake and Badar fish in Siais Lake have high morphological differentiations i.e. 13 different characters of 20 characters.

In the past, the classification of the gobies group was generally based on the external characters (Pezold 1993; Akihito et al. 2000; Nelson 2006); however, identification of species level was proven to be difficult and problematic. Recently, the advances in molecular approaches have provided the complement to the limited of morphological data for untangling gobioid group (Aquino et al. 2011; Jeon et al. 2012; Agorreta and Ruber 2012; Agoretta et al. 2013; Tornabene et al. 2013; Taillebois et al. 2014; Thacker 2009; Thacker 2015; Wang et al. 2017). Mitochondrial DNA (mtDNA) markers have been widely used for most systematic molecular studies because they have a large number of copies, maternally inherited, and have a higher mutation rate compared to nuclear DNA (Brown et al. 1979; Johns and Avise 1998). Mitochondrial cytochrome oxidase subunit I (COI) gene could serve as a rapid and reliable barcoding marker for identifying species and effective in delimitating species of various animals (Hebert et al. 2004; Hubert et al. 2008; Imtiaz, Mohd-Nor, and Naim, 2017; Roesma et al. 2018; Roesma et al. 2019). Recently, the COI gene has been widely used to resolve the taxonomy in a gobioid group (Jeon et al. 2012; Jin et al. 2014; Taillebois et al. 2014; Wang et al. 2017; Olii et al. 2019). Further research is needed on Rinuak and Badar fish using a molecular approach. The present study aimed to investigate the phylogenetic relationship of Rinuak and Badar fish in three Sumatran Lakes using cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study sites

Rinuak and Badar fish specimens were collected from three Sumatran lakes, i.e. Maninjau Lake and Singkarak Lake in West Sumatra and Siais Lake in North Sumatra. The samples collected with cast-nets and set-nets following standard procedures by Cailliet et al. (1986). Twelve individuals out of the samples were used for phylogenetic analysis and preserved in 96% ethanol (PA). Other individual specimens were all together fixed in 4% formalin and preserved into 70% ethanol for long term storage in the laboratory.

Molecular Work

The DNA extraction was performed following the protocol Kit INVITROGEN PureLink^{TM} Genomic DNA Mini Kit. The COI was amplified using the following primers: forward primer Fish F1 (5' TCAACCAACCACAAAGACATTGGC AC 3') and reverse primer Fish R1 (5' TAGACTTCTGGGTGGCCAAAGAATCA 3') (Ward et al. 2005). PCR reactions were conducted in 25 µl volumes, containing ; 3 µl of DNA template, 11 µl of PCR Supermix, 0.5 µl of each primer (0.01 mM), and 10 µl of DDH20. The PCR procedure was conducted using PCR *Sensoquest* thermal cyclers with the following program: initial denaturation at 95^{0}C for 2 min; 35 cycles of denaturation at 94^{0}C for 30 s, annealing at 54^{0}C for 30 s and elongation at 72^{0}C for 1 min; followed by a final extension of 72°C for 10 min and then held at 4°C for the long term storage. PCR products were run on 2% agarose gel and visualized using GelDoc. PCR products were purified and the most powerful products selected for sequencing to1st Base, Pte. Ltd.in Malaysia.

Data analysis

The nucleotide sequences from the DNA sequencing of Rinuak and Badar fish was edited using the DNA STAR program (Burland 2000). Then, DNA sequences were obtained compare with the data on the Genbank, NCBI (Table 1.) to investigate the similarity of all sequences in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences were aligned using the Clustal X default settings and edited using the Bioedit program (Thompson 1997; Hall 1999). DNA SP version 5.10 was used to identify haplotypes numbers (*h*), polymorphism sites (S), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Rozas 2003). Pairwise genetic distances were calculated to quantify sequence divergences among all sequences using the *p*-distance Kimura two-parameter (K2P) method in the MEGA version 6.0 program (Tamura et al. 2013). Phylogenetic analyses were performed the four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML), and Maximum Parsimony (MP) using 1000 bootstrapping in MEGA 6.0 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

Sequence data

The mtDNA COI sequences of 32 individuals (12 sequences from the present study and 20 sequences from the GenBank) were determined for a total of 619 bp. Among them, 231 bp (37.32%) were variable sites, and 213 bp (34.42%) were parsimony informative sites. No stop codons, insertions, and deletions were found in its translation. The average compositions of A, T, C, and G nucleotide base was 24.4%, 30.3%, 27.5%, and 17.8%, respectively. The average composition value was almost similar to the previous studies of Gobiidae (Aquino et al. 2011; Aquilino et al. 2011; Jeon et al. 2012). Nucleotide sequences are AT ratio (54.7%) higher than the GC ratio, which is characteristic for the genome of mtDNA in vertebrate (Hubert et al. 2008). The GC content of the first codon is the highest, while the variable second codon has the lowest. The transitions are more common than transversion with bias ratio (Ts; Tv) is R: 3.737. A total of 22 haplotypes consisted of four haplotypes to Rinuak and Badar fish in Sumatran Lakes and 18 haplotypes to data of GenBank, NCBI. The average haplotype diversity among 22 haplotypes (Hd) was 0.938±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.16256±0.002. While among Rinuak and Badar fish in Sumatra has the haplotype diversity (*Hd*) was 0.561, and nucleotide diversity (*Pi*) was 0.00142. Genetic distance within and among populations of Rinuak and Badar fish was 0.0%-0.5% (the table of genetic distance not showed). Sequence divergence between outgroup (*Rasbora lateristriata* and *Danio rerio*) with Rinuak and Badar fish lineages ranged from 27.6% to 31.6%. That value supported that they are in a different Family.

Phylogenetic relationships

The phylogenetic tree was constructed using four methods (ML/NJ/ME/MP) analysis generated almost in identical topologies, represented by the ML tree with 1000 bootstrap replicates (Figure 1). Based on the tree constructed from COI genes, it can be observed that the Gobiidae group divided into four sublineages.

The phylogenetic tree members consist of Rinuak and Badar fish in the first sublineage, *Gobiopterus* in the second, and fourth sublineage while *Sicyopterus, Stipodon, Sicyopus,* and *Lentipes* in the third sublineage. All members of Rinuak and Badar fish from three Sumatran lakes present together in the first sublineage with sequence divergences was 0.0%-0.5%. In the second sublineage, consists of three species from *Gobiopterus*, namely *G. brachypterus, G. chuno,* and *G. lacustris*. Among the species of *Gobiopterus* has sequence divergences was 2.0%-17.8%. The presence of small sequence divergences (2.0%) between the *G. chuno* from Bangladesh MK572237 and *G. brachypterus from* India MG495939 and (3.0%) between *G. brachypterus* from Central Java and *G. lacustris* from the Philippines is require re-identification studies in taxonomy. According to Kartavsetv et al. (2013), that value indicated for differences at the level of subspecies, semi species, or sibling species.

The phylogenetic tree showed that the *Gobiopterus* in the second sublineage is occupying the position as a sister taxa of Rinuak and Badar fish with sequence divergences was 24.6%-27.9%. *Gobiopterus* is the only genus of transparent gobies that has the genetic data used as a comparison for Rinuak and Badar fish. Based on Kartavtsev et al. (2011), Kartavtsev et al. (2013), the value of sequence divergences between first and second sublineages showed differentiation at the level of a different genus. The third Sublineage consists of four different genera. The sequence divergences between them were 11.8%-15.3%, and with both first and second sublineages were 20.7%-29.3%. The fourth Sublineage has one member, *G. semivestitus*, which is separated from other *Gobiopterus* in the second sublineage with sequence divergences was 27.2%-29.1%. The separation of *Gobiopterus* into two different sublineages showed that *Gobiopterus* has the non-monophyletic lineages. The value of sequence divergences between *Gobiopterus* in the second and fourth sublineages showed differentiation at the level of a different genus. Based on Kartavtsev et al. (2011), Kartavtsev et al. (2013) sequence divergences in the same genus was 11%-16%.

Table 1. Species List taxonomy, Location and Genbank Accession Number of samples

No.	**Family**	**Genus**	**Species**	**Location**	**Accession Numbers**
1	Gobiidae	*Gobiopterus*	*Gobiopterus brachypterus*	Malaysia	KX223903.1
2			*Gobiopterus brachypterus*	Malaysia	KX223902.1
3			*Gobiopterus brachypterus*	India	MG495939.1
4			*Gobiopterus brachypterus*	Central Java	KU692539.1
5			*Gobiopterus brachypterus*	Central Java	KU692538.1
6			*Gobiopterus brachypterus*	Central Java	KU692535.1
7			*Gobiopterus chuno*	Bangladesh	MK572237.1
8			*Gobiopterus lacustris*	Philippines	HQ682693.1
9			*Gobiopterus lacustris*	Philippines	HQ682695.1
10			*Gobiopterus semivestitus*	Australia	KJ669473.1
11		*Sicyopterus*	*Sicyopterus parvei*	West Java	KU693078.1
12			*Sicyopterus lagocephalus*	Bali	KU693018.1
13		*Stiphodon*	*Stiphodon semoni*	West Java	KU693171.1
14			*Stiphodon maculidorsalis*	Banten	KU693162.1
15		*Sicyopus*	*Sicyopus zosterophorum*	Bali	KU693126.1
16			*Sicyopus ribicundus*	East Java	KU693113.1
17		*Lentipes*	*Lentipes whittenorum*	Bali	KU693014.1
18			*Lentipes ikeae*	West Java	KU692987.1
19	Cyprinidae	*Rasbora*	*Rasbora lateristriata*		KT960818.1
20		*Danio*	*Danio rerio*	India	MK714084.1

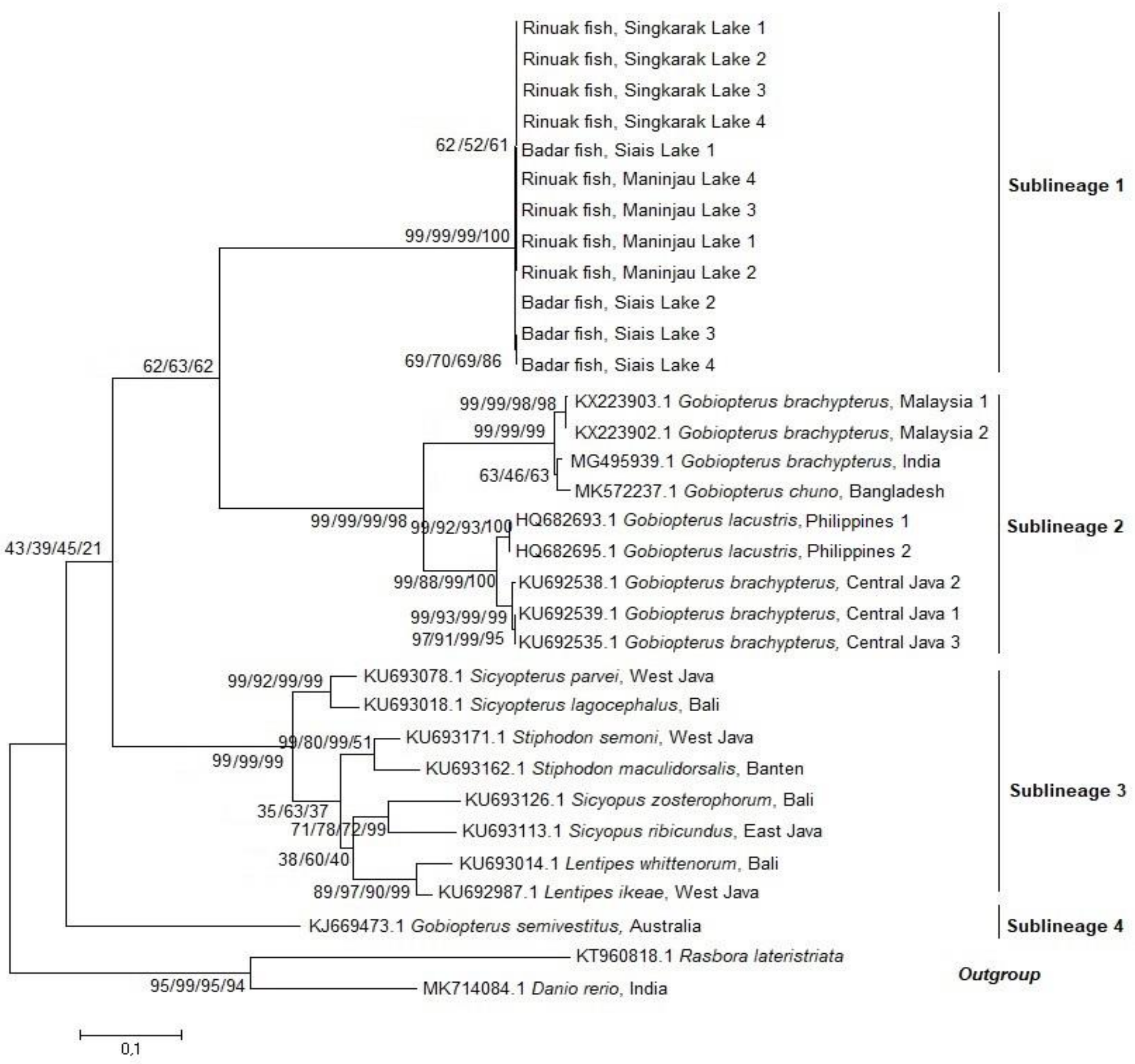

Figure 1. The ML phylogenetic tree of the COI gene with the bootstrap value of 1000 replicates (ME/NJ/ML/MP).

Discussion

The present study represents the first molecular data of Rinuak and Badar fish in Sumatra using mitochondrial markers (COI gene). Rinuak and Badar fish in Sumatra present in one sublineage with low haplotype diversity, which is some individual shared the same haplotype. Refers to Kartavtsev et al. (2011); Kartavtsev et al. (2013), our analysis revealed that the Rinuak and Badar fish are the same species, with low genetic diversity (0.0%-0.5%). While previously, the morphological study by Periwaldi (2012) showed the substantial morphological differences between Rinuak Maninjau Lake and Badar Siais Lake. Geographically, the three lakes are not connected, no gene flow between the population of the lakes, that causes the geographical isolation. Roesma et al. (2018) explained that the freshwater fauna in Maninjau Lake isolated because of no connection with other waters. Water in the Maninjau Lake comes from small rivers, which is filled by water catchment in the surrounding area and only has one outlet. The low genetic divergences of Rinuak and Badar fish between Lakes indicated that geographic distance and geographical isolation do not correlate their sequence divergences.

Using mtDNA, Mcglasan, and Hughes (2001) considered that there was no significant relationship between geographical distance and gene flow on *Hypseleotris compressa* from different drainages with low genetic differentiation on the populations. Keith et al. (2005) also reported that the *Sicyopterus lagocephalus* from different distant archipelagos has minimal genetic distances and suggests they as the same species. The study by Arisuryanti et al. (2018) showed that *Periophthalmus argentilineatus* in Bogowonto Lagoon (Middle Java) and population in Pandeglang (West Java) clustered together with low sequence divergences. *H. compressa, S. lagocephalus,* and *P. argentilineatus* are the species from other Gobies. Referring to study Mcglasan and Hughes (2001); Keith et al. (2005), the possibilities conditions of present study are: 1) natural selection is able to maintain the balance of allele frequencies, 2) there is a geographic connection in the past at these distant locations, and 3) the ability of the fish to change.

Morphological and genetic studies showed that environmental conditions play an essential role in determining the phenotype of a species. The phenotype shows the existence of high adaptability to environmental conditions without changing their genetic makeup. Akihito et al. (2000) reported that the adaptations of Gobiidae fishes to habitats had developed various morphological specializations. It is difficult to estimate the evolutionary scenario using only morphological information, so that needed the genetic data. The phylogenetic tree showed that the *Gobiopterus* is a sister taxon of Rinuak, and Badar fish with sequence divergences was 24.6%-27.9%. Based on Kartavtsev et al. (2011); Kartavtsev et al. (2013), these values show the different of the genus level. The same case also found in other studies of Gobiidae. Jeon et al. (2012) reported that the average sequence divergences of intragenus of more than 20 genera with multiple species of Gobies higher (21.09%) than among species (9.93%-9.54%) in the study of Ward et al. (2005); Ward and Holmes (2007).

According to Kottelat (2013), the transparent gobies consist of two genera; *Gobiopterus* and *Mistichthys*. *Mistichthys* only has one species, *Mistichthys luzonensis*. Referring to Kottelat et al. (1993), Rinuak and Badar fish has similar in morphology with *G. brachypterus*. The similarity of the main characters between Rinuak and Badar fish with *G. brachypterus* consists of cheek naked, cheeks without raised ridges and flaps, pelvic thin without fleshy lobes, head pores absent, transparent body, cheek and margins of preopercle usually with a dark vertical mark, dorsal midline in front of the first dorsal usually with black pigments, mouth of males is more extensive than female and almost vertical and distance between eyeballs 0.5-1.0 times eyeball diameter. However, Rinuak and Badar fish have high sequence divergences (24.6%-27.9%) against *G. brachypterus* in Central Java, Malaysia, and India. Despite having the similarity in morphology, the high sequence divergences do not support the grouping of Rinuak and Badar fish as the same species to *G. brachypterus* even to the genus *Gobiopterus*.

Previously, the transparent gobies were separated into several genera (Munro 1949) consists of *Gobiopterus* Bleeker (1874), *Mistichthys* Smith (1901), *Micrapocryptes* Hora (1923), *Mirogobius* Herre (1927), *Gobiella* Smith (1931), and *Herreolu*s Smith (1931). However, previous authors (Hora 1934; Mukerji 1936; Aurich 1938; Koumans I940), demonstrated that *Gobiopterus* is a synonym of the re-examined holotype of *Micrapocryptes, Gobiella, Mistichthys,* and *Mirogobius.* While, Kottelat (2013) has reported that the valid of transparent gobies consists of two genera (*Mistichthys* and *Gobiopterus*), where is *Gobiopterus* has ten species. However, only four species of them have morphological and genetic data (*G. brachypterus, G. lacustris, G. chuno,* and *G. semivestitus*). Unfortunately, there is no information explained in the published article about *G. brachypterus* in Rawa Pening lake, Central Java, which was used as a comparison species in the present analysis of the study.

According to (Kottelat 2013), the type locality of *G. brachypterus* (Bleeker 1955) in Pasuruan, Ranu Grati Lake, East Java, Indonesia. Based on the several sources, *G. brachypterus* in East Java are known with the local name as Lempuk fish. The local peoples consider Lempuk fish only found in Ranu Grati Lake (East Java). Lempuk fish also has high economic value as of fresh food and processed food like Rinuak fish from Maninjau Lake. The genetic similarity study of Lempuk fish and *G. brachypterus* using the 12s rRNA gene by Candra (2012) showed the high sequence divergences (8.2%) between them and concluded that Lempuk fish was different genus to *G. brachypterus*. These results supported our study, which showed Rinuak and Badar fish as different from *G. brachypterus*. The lack of morphological and genetic studies on the *Gobiopterus* group makes it difficult for further identification. Studies on *Gobiopterus* are limited to the first records *G. brachypterus* in Sri Lanka (Ott 2011), sexual dimorphism of *G. semivestitus* (Macdowall and David 2008), and genetic differentiation of *G. lacustris* (Aquino et al. 2011; Wang et al. 2017).

The data of (IUCN 2010; IUCN 2019) and FishBase (2013) suggested that species of *Gobiopterus* are endemic to certain islands/countries; some are also found in other regions such as *G. lacustris* is endemic in the Philippines. However, Wang et al. (2017) reported that *G. lacustris* also found in China. Aquino et al. (2011) explained that *G. lacustris* divided into two distinct clusters with deep genetic divergence at 23.80% and proposed that they are a cryptic species. Wang et al. (2017) confirmed that no cryptic species on *G. lacustris* in the Philippines and supported that *G. lacustris* in China should not be an invasive species from the Philippines.

There are no photo vouchers, and the availability of the data of DNA sequences on GenBank and BOLD System to all *Gobiopterus* species causes the difficulties in identified and verified their taxonomy status. The taxonomy of Gobiidae has been studied extensively for the last decades, but confusion still exists (Jeon et al. 2012). The Present study revealed that Rinuak fish is not an endemic of Maninjau Lake. Rinuak and Badar exist as a different genus with *Gobiopterus.* Further comprehensive studies using the morphology and other molecular markers on Rinuak and Badar are ongoing.

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Phylogenetic analysis of transparent gobies in three Sumatran lakes, inferred from mitochondrial Cytochrome Oxidase I (COI) gene

DEWI IMELDA ROESMA♥, DJONG HON TJONG, DYTA RABBANI AIDIL
Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University.Jl. Limau Manis, Padang, West Sumatra, Indonesia.
♥email: dewiroesma@sci.unand.ac.id

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Abstract. *Roesma D. I, Tjong D. H, Aidil D. R. 2019. Phylogenetic analysis of transparent gobies in three Sumatran lakes, inferred from mitochondrial Cytochrome Oxidase I (COI) gene. Biodiversitas 20: xxxx.* The transparent gobies fish found in three lakes in Sumatra island is known as Rinuak fish (in ~~Lake~~ Maninjau Lake and ~~Lake~~ Singkarak Lake -West Sumatra) or Badar fish (in ~~Lake~~ Siais Lake-North Sumatra), and are morphologically very similar to the *Gobiopterus brachypterus*. The phylogenetic study was carried out by analyzing 619 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene in 12 fish individuals from the three lakes. Rinuak and Badar fish in three populations have four haplotypes. The sequence divergences in and between populations are very low (0.0%-0.5%). This value indicates that Rinuak and Badar fish are the same species with low genetic diversity. The phylogenetic tree illustrates that this fish belongs to the group of Gobiidae and a sister taxon from *G. brachypterus*.

Keywords: Badar Fish, COI, Genetic diversity, *Gobiopterus brachypterus*, Rinuak Fish

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INTRODUCTION

Freshwater fishes biodiversity in Indonesia forms one of the largest in the world~~,~~ but poorly documented. There are at least 1189 native species, whereas 125 of them are endemic to the country (Froese and Pauly 2014). Sumatra island a high level of biodiversity of freshwater fishes with a high degree of endemism (Sodhi and Brook 2006; Pfeil 2009), including a small transparent fish that morphologically shows the character of the Gobiidae group. The fish locally is known as Rinuak fish, with the total length range 19.91 to 22.68 mm and previously considered as an endemic species of Maninjau Lake. The fish have an economically important value as food. Roesma and Santoso (2010) reported a species suspected to be the same as Rinuak fish, that locally named as Badar fish found in Siais Lake, North Sumatra. However, the scientific name of these two species remained unknown, and some local people consider it as a juvenile form of *Rasbora* from Maninjau Lake.

Rinuak and Badar fish can be classified as the Gobiidae group because of they have the main characters of Gobies, i.e. a pair of pectoral fins that are fused to form suckers~~,~~ and blunt head with big eyes (Kottelat et al. 1993). In addition, the Rinuak and Badar fish are similar to *Gobiopterus brachypterus* (transparent gobies). *Gobiopterus* is a genus of gobies with small size, native to freshwater, marine or brackish waters, and one genus of transparent gobies (Kottelat et al. 1993). Biological and ecological information on Rinuak and Badar fish are very scanty, limited only on the study of processed quality of Rinuak as ~~s~~ food (Astuti et al. 2016). A previous study (Periwaldi 2012) reported that Rinuak fish in Maninjau Lake and Badar fish in Siais Lake have high morphological differentiations, i.e. 13 different characters of 20 characters.

In the past, the classification of the gobies group was generally based on the external characters (Pezold 1993; Akihito et al. 2000; Nelson 2006); however, identification of the species level was proven to be difficult and problematic. Recently, the advances in molecular approaches have provided the complement to the limited of morphological data for untangling gobioid group (Aquino et al. 2011; Jeon et al. 2012; Agorreta and Ruber 2012; Agoretta et al. 2013; Tornabene et al. 2013; Taillebois et al. 2014; Thacker 2009; Thacker 2015; Wang et al. 2017). Mitochondrial DNA (mtDNA) markers have been widely used for most systematic molecular studies because they have a large number of copies, maternally inherited, and have a higher mutation rate compared to nuclear DNA (Brown et al. 1979; Johns and Avise 1998). Mitochondrial cytochrome oxidase subunit I (COI) gene could serve as a rapid and reliable barcoding marker for identifying species and effective in delimitating species of various animals (Hebert et al. 2004; Hubert et al. 2008; Imtiaz et al. ~~, Mohd Nor, and Naim,~~ 2017; Roesma et al. 2018; Roesma et al. 2019). Recently, the COI gene has been widely used to resolve the taxonomy in a gobioid group (Jeon et al. 2012; Jin et al. 2014; Taillebois et al. 2014; Wang et al. 2017; Olii et al. 2019). Further research is needed on Rinuak and Badar fish using a molecular approach. The present study aimed to investigate the phylogenetic relationship of Rinuak and Badar fish in three Sumatran Lakes using cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study sites

Rinuak and Badar fish specimens were collected from three Sumatran lakes, i.e. Maninjau Lake and Singkarak Lake in West Sumatra and Siais Lake in North Sumatra. The samples collected with cast-nets and set-nets following standard procedures by Cailliet et al. (1986). Twelve individuals out of the samples were used for phylogenetic analysis and preserved in 96% ethanol (PA). Other individual specimens were all together fixed in 4% formalin and preserved into 70% ethanol for long term storage in the laboratory.

Molecular Work

The DNA extraction was performed following the protocol Kit INVITROGEN PureLink^{TM} Genomic DNA Mini Kit. The COI was amplified using the following primers: forward primer Fish F1 (5' TCAACCAACCACAAAGACATTGGC AC 3') and reverse primer Fish R1 (5' TAGACTTCTGGGTGGCCAAAGAATCA 3') (Ward et al. 2005). PCR reactions were conducted in 25 µl volumes, containing ; 3 µl of DNA template, 11 µl of PCR Supermix, 0.5 µl of each primer (0.01 mM), and 10 µl of DDH20. The PCR procedure was conducted using PCR *Sensoquest* thermal cyclers with the following program: initial denaturation at 95^0C for 2 min; 35 cycles of denaturation at 94^0C for 30 s, annealing at 54^0C for 30 s and elongation at 72^0C for 1 min; followed by a final extension of 72°C for 10 min and then held at 4°C for the long term storage. PCR products were run on 2% agarose gel and visualized using GelDoc. PCR products were purified and the most powerful products selected for sequencing to1st Base, Pte. Ltd.in Malaysia.

Data analysis

The nucleotide sequences from the DNA sequencing of Rinuak and Badar fish was edited using the DNA STAR program (Burland 2000). Then, DNA sequences were obtained compare with the data on the Genbank, NCBI (Table 1.) to investigate the similarity of all sequences in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences were aligned using the Clustal X default settings and edited using the Bioedit program (Thompson 1997; Hall 1999). DNA SP version 5.10 was used to identify haplotypes numbers (*h*), polymorphism sites (S), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Rozas 2003). Pairwise genetic distances were calculated to quantify sequence divergences among all sequences using the *p*-distance Kimura two-parameter (K2P) method in the MEGA version 6.0 program (Tamura et al. 2013). Phylogenetic analyses were performed the four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML), and Maximum Parsimony (MP) using 1000 bootstrapping in MEGA 6.0 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

Sequence data

The mtDNA COI sequences of 32 individuals (12 sequences from the present study and 20 sequences from the GenBank) were determined for a total of 619 bp. Among them, 231 bp (37.32%) were variable sites, and 213 bp (34.42%) were parsimony informative sites. No stop codons, insertions, and deletions were found in its translation. The average compositions of A, T, C, and G nucleotide base was 24.4%, 30.3%, 27.5%, and 17.8%, respectively. The average composition value was almost similar to the previous studies of Gobiidae (Aquino et al. 2011; Aquilino et al. 2011; Jeon et al. 2012). Nucleotide sequences are AT ratio (54.7%) higher than the GC ratio, which is characteristic for the genome of mtDNA in vertebrate (Hubert et al. 2008). The GC content of the first codon is the highest, while the variable second codon has the lowest. The transitions are more common than transversion with bias ratio (Ts; Tv) is R: 3.737. A total of 22 haplotypes consisted of four haplotypes to Rinuak and Badar fish in Sumatran Lakes and 18 haplotypes to data of GenBank, NCBI. The average haplotype diversity among 22 haplotypes (*Hd*~~Hd~~) was 0.938±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.16256±0.002. While among Rinuak and Badar fish in Sumatra has the haplotype diversity (*Hd*) was 0.561, and nucleotide diversity (*Pi*) was 0.00142. Genetic distance within and among populations of Rinuak and Badar fish was 0.0%-0.5% (the table of genetic distance not showed). Sequence divergence between outgroup (*Rasbora lateristriata* and *Danio rerio*) with Rinuak and Badar fish lineages ranged from 27.6% to 31.6%. That value supported that they are in a different ~~Family~~family.

Phylogenetic relationships

The phylogenetic tree was constructed using four methods (ML/NJ/ME/MP) analysis generated almost in identical topologies, represented by the ML tree with 1000 bootstrap replicates (Figure 1.). Based on the tree constructed from COI genes, it can be observed that the Gobiidae group divided into four sublineages.

The phylogenetic tree members consist of Rinuak and Badar fish in the first sublineage, *Gobiopterus* in the second, and fourth sublineage while *Sicyopterus, Stipodon, Sicyopus,* and *Lentipes* in the third sublineage. All members of Rinuak and Badar fish from three Sumatran lakes present together in the first sublineage with sequence divergences was 0.0%-0.5%. In the second sublineage, consists of three species from *Gobiopterus*, namely *G. brachypterus, G. chuno,* and *G. lacustris*. Among the species of *Gobiopterus* has sequence divergences was 2.0%-17.8%. The presence of small sequence divergences (2.0%) between the *G. chuno* from Bangladesh MK572237 and *G. brachypterus from* India MG495939 and (3.0%) between *G. brachypterus* from Central Java and *G. lacustris* from the Philippines is require re-identification studies in taxonomy. According to Kartavsetv et al. (2013), that value indicated for differences at the level of subspecies, semi species, or sibling species.

The phylogenetic tree showed that the *Gobiopterus* in the second sublineage is occupying the position as a sister taxa of Rinuak and Badar fish with sequence divergences was 24.6%-27.9%. *Gobiopterus* is the only genus of transparent gobies that has the genetic data used as a comparison for Rinuak and Badar fish. Based on Kartavtsev et al. (2011), Kartavtsev et al. (2013), the value of sequence divergences between first and second sublineages showed differentiation at the level of a different genus. The third Sublineage consists of four different genera. The sequence divergences between them were 11.8%-15.3%, and with both first and second sublineages were 20.7%-29.3%. The fourth Sublineage has one member, *G. semivestitus*, which is separated from other *Gobiopterus* in the second sublineage with sequence divergences was 27.2%-29.1%. The separation of *Gobiopterus* into two different sublineages showed that *Gobiopterus* has the non-monophyletic lineages. The value of sequence divergences between *Gobiopterus* in the second and fourth sublineages showed differentiation at the level of a different genus. Based on Kartavtsev et al. (2011), Kartavtsev et al. (2013) sequence divergences in the same genus was 11%-16%.

Table 1. Species List taxonomy, Location and Genbank Accession Number of samples

No.	Family	Genus	Species	Location	Accession Numbers
1	Gobiidae	*Gobiopterus*	*Gobiopterus brachypterus*	Malaysia	KX223903.1
2			*Gobiopterus brachypterus*	Malaysia	KX223902.1
3			*Gobiopterus brachypterus*	India	MG495939.1
4			*Gobiopterus brachypterus*	Central Java	KU692539.1
5			*Gobiopterus brachypterus*	Central Java	KU692538.1
6			*Gobiopterus brachypterus*	Central Java	KU692535.1
7			*Gobiopterus chuno*	Bangladesh	MK572237.1
8			*Gobiopterus lacustris*	Philippines	HQ682693.1
9			*Gobiopterus lacustris*	Philippines	HQ682695.1
10			*Gobiopterus semivestitus*	Australia	KJ669473.1
11		*Sicyopterus*	*Sicyopterus parvei*	West Java	KU693078.1
12			*Sicyopterus lagocephalus*	Bali	KU693018.1
13		*Stiphodon*	*Stiphodon semoni*	West Java	KU693171.1
14			*Stiphodon maculidorsalis*	Banten	KU693162.1
15		*Sicyopus*	*Sicyopus zosterophorum*	Bali	KU693126.1
16			*Sicyopus ribicundus*	East Java	KU693113.1
17		*Lentipes*	*Lentipes whittenorum*	Bali	KU693014.1
18			*Lentipes ikeae*	West Java	KU692987.1
19	Cyprinidae	*Rasbora*	*Rasbora lateristriata*		KT960818.1
20		*Danio*	*Danio rerio*	India	MK714084.1

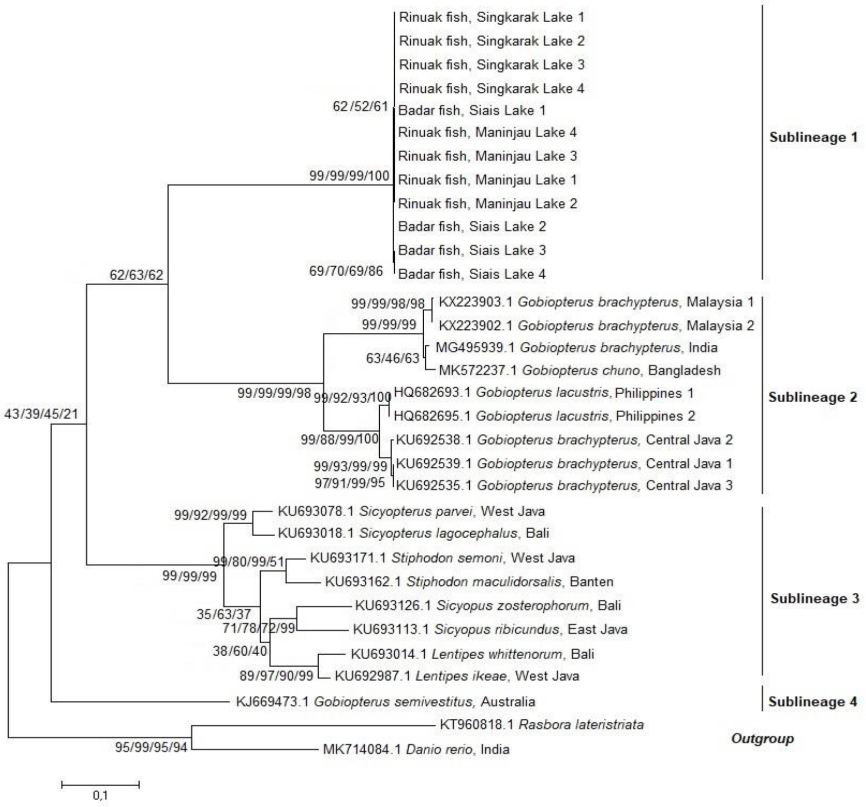

Figure 1. The ML phylogenetic tree of the COI gene with the bootstrap value of 1000 replicates (ME/NJ/ML/MP).

Discussion

The present study represents the first molecular data of Rinuak and Badar fish in Sumatra using mitochondrial markers (COI gene). Rinuak and Badar fish in Sumatra present in one sublineage with low haplotype diversity, which is some individual shared the same haplotype. Refers to Kartavtsev et al. (2011); Kartavtsev et al. (2013), our analysis revealed that the Rinuak and Badar fish are the same species, with low genetic diversity (0.0%-0.5%). While previously, the morphological study by Periwaldi (2012) showed the substantial morphological differences between Rinuak Maninjau Lake and Badar Siais Lake. Geographically, the three lakes are not connected, no gene flow between the population of the lakes, that causes the geographical isolation. Roesma et al. (2018) explained that the freshwater fauna in Maninjau Lake isolated because of no connection with other waters. Water in the Maninjau Lake comes from small rivers, which is filled by water catchment in the surrounding area and only has one outlet. The low genetic divergences of Rinuak and Badar fish between Lakes indicated that geographic distance and geographical isolation do not correlate their sequence divergences.

Using mtDNA, Mcglasan~~,~~ and Hughes (2001) considered that there was no significant relationship between geographical distance and gene flow on *Hypseleotris compressa* from different drainages with low genetic differentiation on the populations. Keith et al. (2005) also reported that the *Sicyopterus lagocephalus* from different distant archipelagos has minimal genetic distances and suggests they as the same species. The study by Arisuryanti et al. (2018) showed that *Periophthalmus argentilineatus* in Bogowonto Lagoon (Middle Java) and population in Pandeglang (West Java) clustered together with low sequence divergences. *H. compressa*, *S. lagocephalus*, and *P. argentilineatus* are the species from other Gobies. Referring to study Mcglasan and Hughes (2001); Keith et al. (2005), the possibilities conditions of present study are: 1) natural selection is able to maintain the balance of allele frequencies, 2) there is a geographic connection in the past at these distant locations, and 3) the ability of the fish to change.

Morphological and genetic studies showed that environmental conditions play an essential role in determining the phenotype of a species. The phenotype shows the existence of high adaptability to environmental conditions without changing their genetic makeup. Akihito et al. (2000) reported that the adaptations of Gobiidae fishes to habitats had developed various morphological specializations. It is difficult to estimate the evolutionary scenario using only morphological information, so that needed the genetic data. The phylogenetic tree showed that the *Gobiopterus* is a sister taxon of Rinuak, and Badar fish with sequence divergences was 24.6%-27.9%. Based on Kartavtsev et al. (2011); Kartavtsev et al. (2013), these values show the different of the genus level. The same case also found in other studies of Gobiidae. Jeon et al. (2012) reported that the average sequence divergences of intragenus of more than 20 genera with multiple species of Gobies higher (21.09%) than among species (9.93%-9.54%) in the study of Ward et al. (2005); Ward and Holmes (2007).

According to Kottelat (2013), the transparent gobies consist of two genera; *Gobiopterus* and *Mistichthys*. *Mistichthys* only has one species, *Mistichthys luzonensis*. Referring to Kottelat et al. (1993), Rinuak and Badar fish has similar in morphology with *G. brachypterus*. The similarity of the main characters between Rinuak and Badar fish with *G. brachypterus* consists of cheek naked, cheeks without raised ridges and flaps, pelvic thin without fleshy lobes, head pores absent, transparent body, cheek and margins of preopercle usually with a dark vertical mark, dorsal midline in front of the first dorsal usually with black pigments, mouth of males is more extensive than female and almost vertical and distance between eyeballs 0.5-1.0 times eyeball diameter. However, Rinuak and Badar fish have high sequence divergences (24.6%-27.9%) against *G. brachypterus* in Central Java, Malaysia, and India. Despite having the similarity in morphology, the high sequence divergences do not support the grouping of Rinuak and Badar fish as the same species to *G. brachypterus* even to the genus *Gobiopterus*.

Previously, the transparent gobies were separated into several genera (Munro 1949) consists of *Gobiopterus* Bleeker (1874), *Mistichthys* Smith (1901), *Micrapocryptes* Hora (1923), *Mirogobius* Herre (1927), *Gobiella* Smith (1931), and *Herreolu*s Smith (1931). However, previous authors (Hora 1934; Mukerji 1936; Aurich 1938; Koumans I940), demonstrated that *Gobiopterus* is a synonym of the re-examined holotype of *Micrapocryptes*, *Gobiella*, *Mistichthys*, and *Mirogobius.* While, Kottelat (2013) has reported that the valid of transparent gobies consists of two genera (*Mistichthys* and *Gobiopterus*), where is *Gobiopterus* has ten species. However, only four species of them have morphological and genetic data (*G. brachypterus*, *G. lacustris, G. chuno*, and *G. semivestitus*). Unfortunately, there is no information explained in the published article about *G. brachypterus* in Rawa Pening lake, Central Java, which was used as a comparison species in the present analysis of the study.

According to (Kottelat 2013), the type locality of *G. brachypterus* (Bleeker 1955) in Pasuruan, Ranu Grati Lake, East Java, Indonesia. Based on the several sources, *G. brachypterus* in East Java are known with the local name as Lempuk fish. The local peoples consider Lempuk fish only found in Ranu Grati Lake (East Java). Lempuk fish also has high economic value as of fresh food and processed food like Rinuak fish from Maninjau Lake. The genetic similarity study of Lempuk fish and *G. brachypterus* using the 12s rRNA gene by Candra (2012) showed the high sequence divergences (8.2%) between them and concluded that Lempuk fish was different genus to *G. brachypterus*. These results supported our study, which showed Rinuak and Badar fish as different from *G. brachypterus*. The lack of morphological and genetic studies on the *Gobiopterus* group makes it difficult for further identification. Studies on *Gobiopterus* are limited to the first records *G. brachypterus* in Sri Lanka (Ott 2011), sexual dimorphism of *G. semivestitus* (Macdowall and David 2008), and genetic differentiation of *G. lacustris* (Aquino et al. 2011; Wang et al. 2017).

The data of (IUCN 2010; IUCN 2019) and FishBase (2013) suggested that species of *Gobiopterus* are endemic to certain islands/countries; some are also found in other regions such as *G. lacustris* is endemic in the Philippines. However, Wang et al. (2017) reported that *G. lacustris* also found in China. Aquino et al. (2011) explained that *G. lacustris* divided into two distinct clusters with deep genetic divergence at 23.80% and proposed that they are a cryptic species. Wang et al. (2017) confirmed that no cryptic species on *G. lacustris* in the Philippines and supported that *G. lacustris* in China should not be an invasive species from the Philippines.

There are no photo vouchers, and the availability of the data of DNA sequences on GenBank and BOLD System to all *Gobiopterus* species causes the difficulties in identified and verified their taxonomy status. The taxonomy of Gobiidae has been studied extensively for the last decades, but confusion still exists (Jeon et al. 2012). The Present study revealed that Rinuak fish is not an endemic of Maninjau Lake. Rinuak and Badar exist as a different genus with *Gobiopterus.* Further comprehensive studies using the morphology and other molecular markers on Rinuak and Badar are ongoing.

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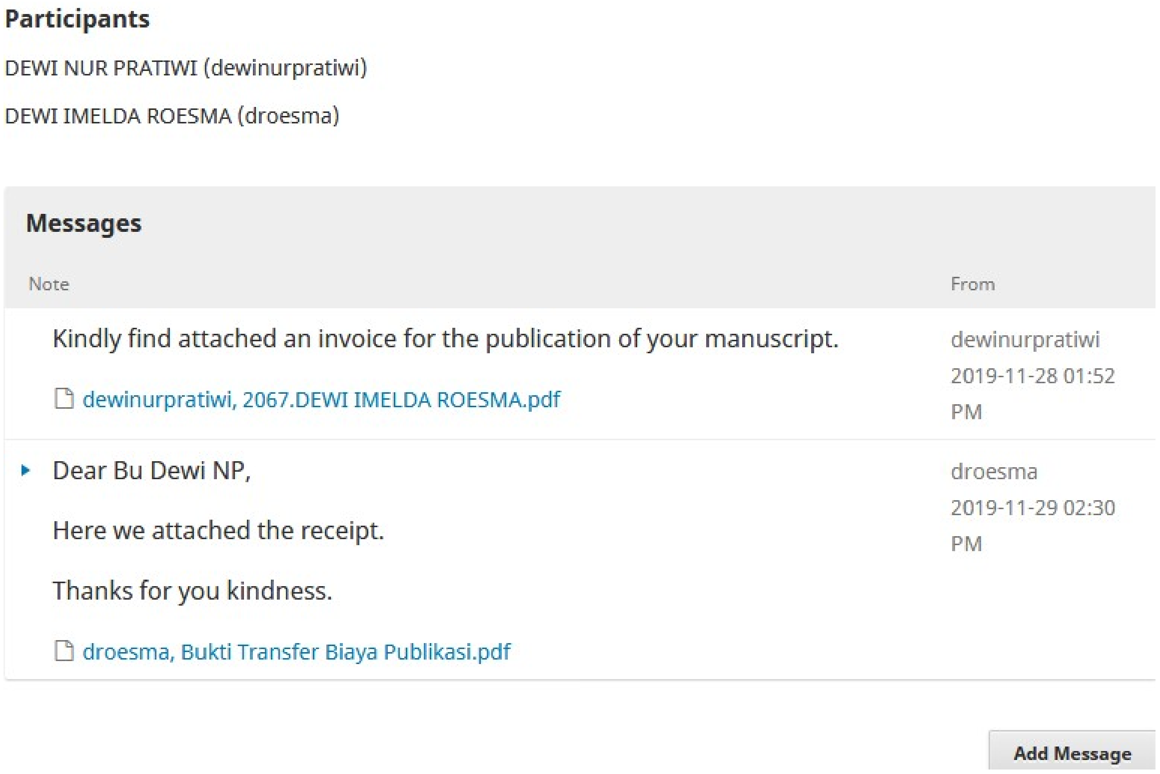

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