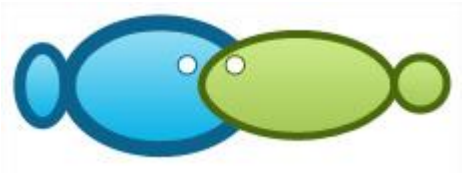

DNA barcoding of hilsa shad (*Tenualosa ilisha*) from the Barumun River, Labuhanbatu Regency, North Sumatera Province, Indonesia

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Abstract. The trade of *Tenualosa ilisha* eggs is still ongoing. This could lead to a future population decline. Molecular information regarding *T. ilisha* as an anadromous fish in the Barumun River has yet to be available. DNA barcoding utilizes the cytochrome oxidase subunit 1 (CO1) gene because this gene has been widely used to identify species. Samples were collected from the Barumun River, which is the location of the migration of *T. ilisha* for spawning. DNA was isolated using the Extragene Gene All DNA Mini Kit protocol. The results of *T. ilisha* DNA amplification contained 686 base pairs, with a composition of 29.4% T(U), C (28.6%), A (21.3%), and G (20.7%). *T. ilisha* sequences from Sungai Barumun were compared with 12 gene sequences present in the Genbank. The results of the CO1 gene analysis showed that *T. ilisha* from the Barumun River had a close kinship with *T. ilisha* from Bangladesh, with a genetic distance of 0.22%. BLAST analysis showed a 98.32% similarity between the sequences of *T. ilisha* from the Barumun River and the Genbank data. Genetic distance analysis using the Kimura Two Parameter model (K2P) showed genetic distances within species, genera, and families with an average of 0.08%, 0.28%, and 0.32%. DNA barcodes will become basic information that can be utilized to monitor migration distances and determine future *T. ilisha in situ* conservation areas. Therefore, DNA barcodes will be registered soon.

Key Words: anadromous fish, CO1 gene, mtDNA.

Introduction. Hilsa shad *Tenualosa ilisha* is a pelagic fish from the Clupeidae family, better known as herring in Europe (Whitehead 1985). Five species of the genus *Tenualosa*, namely *T. ilisha*, *T. macrura*, *T. toli*, *T. reevesii*, and *T. thibadeaui*, live in Asian waters (Suwarso 2014; Hossain et al 2019; Machrizal et al 2019a). The presence of *T. ilisha* has been reported in the estuary waters of Bangladesh (Flura et al 2015), Iran (Roomiani et al 2014), Iraq (Sarker et al 2016), India (Karim et al 2015), west Bengal (Mohanty & Nayak 2017) and Indonesia (Jihad et al 2014; Machrizal et al 2019a; Machrizal et al 2019b). Hilsa shad is an anadromous fish that spawns in fresh waters and migrates to the sea (Blaber et al 1999; Amin et al 2004). In Indonesia, the hilsa shad is also called "terubuk fish" and it can only be found in the Barumun and Bilah rivers, Labuhanbatu district (Jihad et al 2014; Machrizal et al 2019a; Machrizal et al 2019b).

T. ilisha is a species with high economic value, especially for its eggs (Efizon et al 2012; Machrizal et al 2019b). The high economic value of *T. ilisha* causes continuous fishing, which will eventually result in a decrease in population. The International Union for Conservation of Nature Red List of Threatened Species (IUCN red list) reports *T. ilisha* as a species of "least concern (LC)" status, with a tendency to decrease in population (https://www.iucnredlist.org/). Through the Decree of the Minister of Maritime Affairs and Fisheries of the Republic of Indonesia Number 43/KEPMEN-KP/2016, the government designated *T. ilisha* as a species under limited protection. Research related to *T. ilisha* in the waters of the Barumun River is minimal and is dominated by biological and ecological

studies (Jihad et al 2014; Machrizal et al 2019b; Machrizal et al 2019a), while the molecular aspects of *T. ilisha* have never been disclosed.

Mitochondrial DNA cytochrome oxidase subunit 1 (CO1) gene is used as a DNA barcode in species identification and biodiversity studies (Hebert et al 2003; Ward et al 2005; Kartavtsev & Lee 2006; Dawnay et al 2007; Thu et al 2019). The CO1 gene is an effective DNA marker in fish from various rivers (Ward et al 2009; Roesma et al 2019; Roesma et al 2022). DNA barcoding has been widely applied in various studies, including identifying freshwater, marine, and estuarine fish species from Pakistan and Bangladesh waters (Ghouri et al 2020; Ahmed et al 2021), identification of freshwater fish species from Siberut Island, Mentawai Islands, Indonesia (Roesma et al 2022) and new records of *Puntius* species from West Sumatra (Roesma et al 2018). However, information on the DNA barcode of *T. ilisha* originating from the Barumun River has yet to be available. The aim of this paper was to analyze the characteristics of the CO1 gene of *T. ilisha* from the Barumun River that can be used in molecular identification and for monitoring its presence in the future. This DNA barcode could later become basic information for the monitoring, conservation, and management of *T. ilisha* in the Barumun River.

Material and Method

Sample collection. Samples were collected from the estuary of the Barumun River, Labuhanbatu District, North Sumatra Province, using a survey method. Fish sampling was carried out in June 2022 outside the fishing ban period (Figure 1). A sampling of *T. ilisha* was carried out using gill nets with a size of 25.4-76.2 mm. The samples were preserved using 4% formalin (Roesma et al 2022). The captured *T. ilisha* samples were morphologically identified based on Kottelat (2013), Kottelat et al (1993) and Nelson et al (2016). The liver of *T. ilisha* was collected for DNA isolation and stored in a microtube with 96% ethanol pro-analysis. DNA isolation was performed at the Laboratory of Genetics and Molecular Biology, Department of Biology, FMIPA, Andalas University, Indonesia.

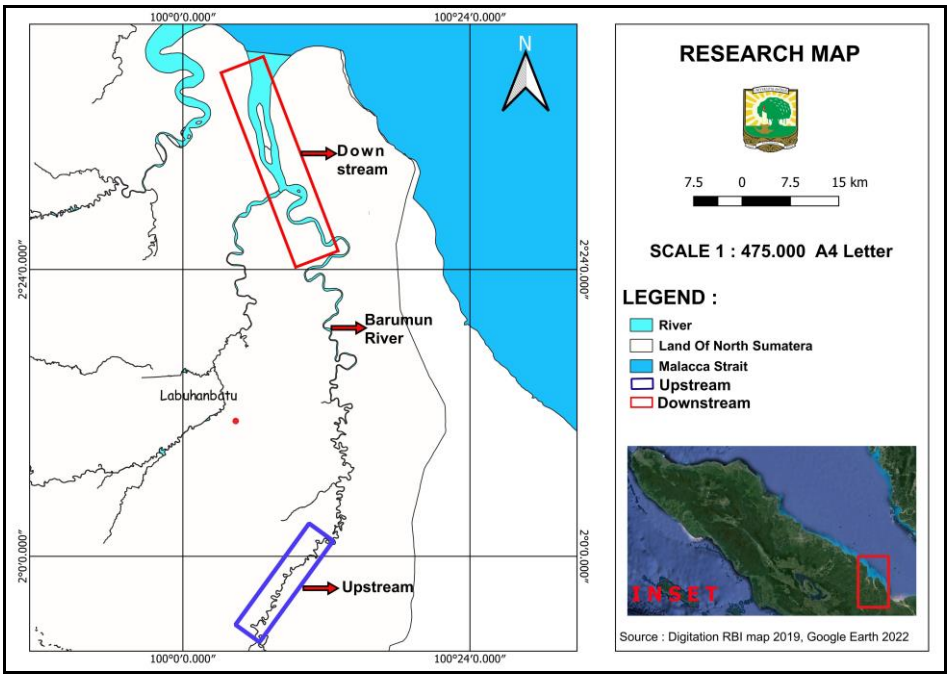

Figure 1. Sampling location of *Tenualosa ilisha* in Barumun River, Indonesia.

DNA extraction, PCR, and sequencing. DNA extraction followed the KIT Extragene Gene All DNA Mini Kit protocol. The quality of DNA isolates was checked using 1.2% agarose gel. DNA samples were amplified using universal fish primers Forward F1 (5'TCAACCAACCAAAAAGACATTGGCAC3') and reverse R1

(5'TAGACTTCTGGGTGGCCAAAGAATCA 3') (Ward et al 2005). The PCR amplification process used a 25 µL DNA isolate sample consisting of 11 µL PCR Supermix, 3 µL DNA isolate, 10 µL DDH20, 0.5 µL forward (F1), and Reverse (R1) primers each. The PCR amplification cycle has begun with denaturation at 95°C for 2 min. This process continued with 34 cycles of denaturation (94°C for 0.5 min), annealing (55.6°C for 0.5 min), extension (72°C for 1 min), the final extension of one cycle (72°C for 10 min), and 12°C for long term storage (Ward et al 2005). The DNA fragments were visualized using 2% agarose gel in the ultraviolet (UV) light and stained using ethidium bromide. Furthermore, the PCR product was purified with an agarose gel extract kit (Promega) and sent to First Base Malaysia for sequencing.

Data analysis. The results of the forward and reverse DNA sequences were edited using the DNA STAR program (Burland 2000) to obtain intact DNA sequences. The DNA sequence of *T. ilisha* was compared to the DNA sequence of samples from fish of the same family using the Basic Local Alignment Search Tool (BLAST) program on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi to measure the degree of similarity. All sequences were aligned with Clustal X 2.0 (Thompson et al 1997) and edited with the BIOEDIT program (Hall 1999). Nucleotide bases are translated into amino acids using the online website http://insilico.ehu.es/translate. Molecular diversity analysis consisted of the number of haplotypes (h), polymorphism sites (S), haplotype diversity (Hd), and nucleotide diversity (Pi), calculated with the DNA polymorphism program (DNA SP) version 5.10 (Rozas et al 2003). The genetic distance in each sequence was calculated by Molecular Evolutionary Genetics Analysis (MEGA) XI using the Kimura two-parameter (K2P) model with 1000 bootstraps (Tamura et al 2021). A phylogenetic tree was created using the Neighbors Joining (NJ) method with 1000 repetitions using MEGA XI (Tamura et al 2021). Phylogenetics is the grouping of species based on their evolutionary history, using DNA sequence analysis. Mathematical models are used to analyze molecular data to infer the evolutionary history of species (Yuan et al 2014). The phylogenetic reconstruction used the Neighbor Joining (NJ) method with a 2-parameter Kimura model, with a 1000x bootstrap. *Rhynchorhamphus malabaaricus* (MF170953.1) and *Squalidus gracilis* (JN003351.1) were used as outgroups. The aligned sequences were registered to the Barcode of Life Data (BOLD) system to obtain DNA barcodes and accession sequence codes.

Results and Discussion

Sequence analysis. The results of the alignment of the two *T. ilisha* CO1 gene sequences from the Barumun River presented 686 bp with the following compositions: A - 21.3%; T(U) - 29.4%; G - 20.7%; and C - 28.6% (Table 1). These results are not much different from those obtained by Ghouri et al (2020) for *T. ilisha* in Bangladesh: A - 23.35%; T - 28.3%; G - 17.62%; and C - 30.7%). The results of Ahmed et al (2021) found the following in the Clupeiformes order originating from Bangladesh: T - 28.6%; C - 27.8%; A - 24.2%; G - 19.4%; AT - 52.8%; and GC - 47.2%.

Table 1

Nucleotide composition of *Tenualosa ilisha* from Barumun River

Species	*Sampling location*	*Nucleotide*					
		A (%)	*T (%)*	*G (%)*	*C (%)*	*AT (%)*	*GC (%)*
T. ilisha	Downstream of Barumun River	21.3	29.4	20.7	28.6	50.7	49.3
T. ilisha	Upstream of Barumun River	21.3	29.4	20.7	28.6	50.7	49.3

BLAST analysis. BLAST analysis of the two CO1 gene sequences showed that the sample from the Barumun River was *T. ilisha*, with a similarity rate of 98% to Genbank data (Table 2).

Table 2
Sequence similarity of *Tenualosa ilisha* from Barumun River to Genbank database within the BLAST analysis

Species name	*Country*	*Similarity (%)*	*Accession no.*
Tenualosa ilisha	Bangladesh	98	KX657721.1
Tenualosa ilisha	Bangladesh	97.81	AP011611.1
Tenualosa ilisha	Bangladesh	97.5	KY802073.1

Genetic distance. Genetic distance is a basis for studying molecular evolution, phylogenetic reconstruction, and evolutionary time estimates (Sohpal et al 2013). Genetic distance analysis was performed using the Kimura 2-Parameter model (Table 3).

The results of genetic distance analysis showed that two individuals of *T. ilisha* collected from the Barumun River in Labuhanbatu Regency have the same haplotype, with a genetic distance of 0.000 between them. The lowest genetic distance was 0.15, between *T. ilisha* samples from the Barumun River and Bangladesh (KY802073.1 and AP011611.1). The farthest genetic distance (0.024) was obtained between a sample of *T. ilisha* from the Barumun River and a sample of *T. ilisha* (MF588659.1) from Bangladesh. Several factors can cause genetic differences, for example, different habitat conditions, lack of connectivity between locations, or geographic distances. The short genetic distance between *T. ilisha* from the Barumun River and *T. ilisha* from Bangladesh indicates similarities in the habitat conditions of the Barumun River and the rivers in Bangladesh. Several possibilities cause species from different locations to be genetically similar, for example, genetic sharing (Leatemia et al 2018), connectivity between regions (Díaz-Ferguson et al 2010), habitat similarities, and migration processes (Saleky et al 2016). The genetic distance between species showed that *T. ilisha* from the Barumun River had a low genetic distance (0.018) compared to *T. revesii* (MF123318.1) from China. The highest genetic distance (0.128) was obtained between *T. ilisha* from the Barumun River with *T. macrura* (KY570294.1) from Malaysia. The average genetic distance increases with increasing taxonomic levels; this is consistent with the statement that genetic divergence will increase significantly due to differences in taxa (Hebert et al 2003).

The phylogenetic tree (Figure 2) formed consists of 2 large clades with high bootstrap values. The higher the bootstrap value formed is, the better the phylogenetic tree reconstruction is. *T. ilisha* from the Barumun River forms the same clade as *T. ilisha* from Bangladesh and the United Kingdom, due to the low genetic distance between *T. ilisha* from the Barumun River and *T. ilisha* from various regions. Referring to Cai et al (2016), a genetic distance of less than 2% indicates that the samples are from the same species. The distinct clade consists of three species belonging to the genus *Tenualosa*, namely *T. macrura*, *T. toli*, and *T. reevesii*, native to Malaysia and China. According to Roesma et al (2022), the same species will gather in the same branch and be separated from other species on the phylogenetic tree. The joining of *T. ilisha* fish from several different areas indicates the occurrence of genetic sharing or gene flow between regions. The phylogenetic reconstruction showed connectivity between *T. ilisha* of Indonesia and Bangladesh through the Malacca Strait. The migration process of *T. ilisha* from the Barumun River towards the Malacca Strait to the Andaman Sea allows gene flow between *T. ilisha* from the Barumun River, and *T. ilisha* from Bangladesh. Referring to the statement of Jefri et al (2015), migrating species or larval dispersal can cause gene flow. According to Saleky et al (2016), gene flow in marine organisms can occur due to migration.

Table 3

The genetic distance of *Tenualosa ilisha* from Barumun River and *T. ilisha* from different regions (Genbank data) based on the Kimura 2-parameter model

No	*Species*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
1	*T. ilisha*- Barumun River- TP	*	*	*	*	*	*	*	*	*	*	*
2	*T. ilisha* Barumun River- LB	0.000	*	*	*	*	*	*	*	*	*	*
3	*T. ilisha* (MF588659.1) Bangladesh-2	0.024	0.024	*	*	*	*	*	*	*	*	*
4	*T. ilisha* (KY802073.1) Fish trade Bangladesh	0.015	0.015	0.013	*	*	*	*	*	*	*	*
5	*T. ilisha* (MN972485.1) Bangladesh-1	0.022	0.022	0.006	0.011	*	*	*	*	*	*	*
6	*T. ilisha* (AP011611.1) Bangladesh	0.015	0.015	0.013	0.000	0.011	*	*	*	*	*	*
7	*T. ilisha* (KY802061.1) FishTrade-UK-Liverpool	0.017	0.017	0.015	0.006	0.013	0.006	*	*	*	*	*
8	*T. reevesii* (MF123318.1) China	0.118	0.118	0.124	0.122	0.125	0.122	0.125	*	*	*	*
9	*T. toli* (KX786677.1) Malaysia	0.122	0.122	0.128	0.125	0.128	0.125	0.128	0.011	*	*	*
10	*T. macrura* (KY570294.1) Malaysia	0.128	0.128	0.141	0.122	0.138	0.122	0.132	0.124	0.121	*	*
11	*Rhynchorhamphus malabaricus* (MF170953.1) Bangladesh	0.219	0.219	0.218	0.204	0.211	0.204	0.196	0.248	0.244	0.230	*
12	*Squalidus gracilis* (JN003351.1)	0.202	0.202	0.213	0.202	0.210	0.202	0.202	0.213	0.209	0.229	0.198

Note: TP - Teluk Panji; LB - Labuhan Bilik.

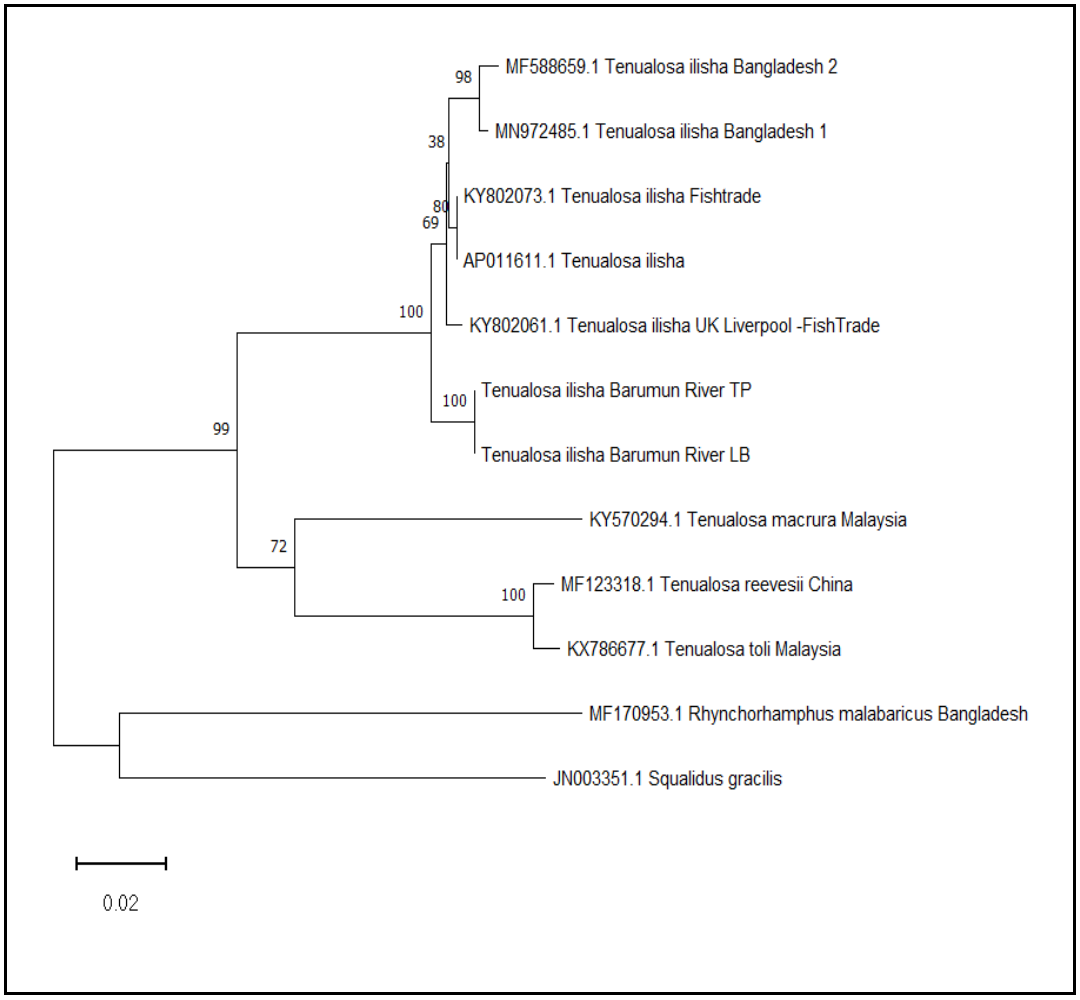

Figure 2. Reconstruction of a phylogenetic tree using the Neighbor-Joining method using 10 DNA sequences from GenBank with *Rhynchorhamphus malabaricus* and *Squalidus gracilis* as out groups.

Conclusions. DNA barcoding analysis showed that the species being analyzed was Hilsa Shad (*Tenualosa ilisha*, Hamilton, 1822) with a sequence length of 686 bp. Sequence similarities between *T. ilisha* from Sungai Barumun and *T. ilisha* from Bangladesh indicate gene flow caused by migration. The phylogenetic tree forms two clades with a bootstrap value of 100. All sequences are formed based on sequence similarity and genetic distance.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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