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Phylogenetic of the Hilsa Shad (*Tenualosa ilisha*) from Labuhanbatu Indonesia and Other Asian Waters Based on Cytochrome *b* Gene Mitochondrial DNA

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ABSTRACT

Tenualosa ilisha is an anadromous fish from the *Clupidae* family that is distributed in several Asian countries. The relationship between *T. ilisha* from Indonesian waters and other waters in Asia is unknown, therefore this study was conducted to provide an overview of the relationship between *T. ilisha* from Indonesia and other waters in Asia based on the *Cytochrome b* gene (*Cyt b*) of mitochondrial DNA (mtDNA). The base sequence of *T. ilisha* from Labuhanbatu with a length of 568-572 base pairs (bp) was analyzed, then compared with Genbank data. All sequences compared showed that the composition of A+T (52.5%) was greater than G+C (47.5%). Genetic distance was calculated using Kimura two parameter (K2P); the lowest genetic distance of 0.000 was obtained between *T. ilisha* Labuhanbatu and Iraq with accession numbers LC619671.1; LC619673.1; LC619674.1. Meanwhile, the highest genetic distance is known to be 0.021 between *T. ilisha* Labuhanbatu and Bangladesh with the accession numbers MN748964.1, and MN748966.1. Neighbor-joining and maximum likelihood with 1000x repetition were the phylogenetic methods used to analyze the evolutionary relationships of *T. ilisha*. In general, both methods showed the same result that *T. ilisha* from Indonesia and other Asian waters are the same population.

INTRODUCTION

Phylogenetics is a study to understand evolution and to reconstruct the close relationships between organisms **(Lyubetsky, 2016)**. Phylogenetic studies can be conducted through molecular phylogenetic analysis **(Neapolitan, 2009)**. According to **Khan *et al*. (2017)**, the phylogenetic relationship of a species can be determined based on mitochondrial DNA (mtDNA) sequences. Research related to phylogenetics using mtDNA has been widely carried out such as the study of **Alam and Mar'ie (2021)**, **Mar'ie (2021)** and **Alyamani *et al.* (2023)**. The phylogenetic relationship of a species can be determined based on mtDNA sequences because they are maternally inherited and can produce data quickly and consistently **(Arab *et al*., 2017; Khan *et al*., 2017)**. One of

the genes in mtDNA used for molecular phylogenetic analysis is the *Cytochrome b* (*Cyt b*) gene.

The *Cytochrome b* (*cyt b*) gene is advantageous in phylogenetic studies due to its high utility in assessing inter-species variations and phylogenetic relationships **(Kaur & Singh, 2021; Emelianova *et al.*, 2022)**. It is widely used for molecular identification, offering a reliable alternative to morphological methods **(Emelianova e*t al.*, 2022)**. *Cyt b* gene sequences provide insights into genetic distances at various taxonomic levels, aiding in understanding intrageneric, interspecific, and intraspecific variations **(Hassan *et al.*, 2024)**. Additionally, the gene's sequences allow for the construction of phylogenetic trees, revealing evolutionary relationships among different species **(Kaur & Singh, 2021; Emelianova *et al.*, 2022; Hassan *et al.*, 2024)**. The gene's characteristics, such as stability, physicochemical properties, and conserved motifs, further enhance its applicability in comparative genomics and evolutionary studies **(Emelianova *et al.*, 2022)**.

The *Cytochrome b* (*Cyt b*) mtDNA gene is one of the most studied genes in fish. The size of the *Cyt b* gene in fish ranges from 1140 **(Ketmaier *et al*., 2004; Doadrio & Perdices, 2005)** to 1143bp **(Perez *et al*., 2007)**. The *Cyt b* mtDNA gene can be used as a classification parameter at various levels of taxa, as the mutation values in this gene differ based on the codon position **(Irwin *et al*., 1991)**. In fish, the *Cyt b* gene has been used to analyze biogeographic history **(Mateos *et al*., 2002)**, the genetic structure of isolated populations **(Russel, 2003)**, phylogeny **(Sullivan *et al*., 2004)**, genetic history and structure among different populations **(Aboim *et al*., 2005)**. Phylogenetic studies utilizing the *Cyt b* gene and other genes in mtDNA in fish have been widely conducted in Indonesia **(Elvyra & Sholihin, 2007; Wilujeng *et al*., 2014; Elvyra, 2023)**, but phylogenetic studies on *Tenualosa ilisha* based on the *Cyt b* gene had never been conducted.

Tenualosa ilisha is an anadromous fish of the *Clupidae* family like salmon (*Salmonidae*) that spawns in freshwater and then migrates to the sea to spend its life **(Blaber *et al*., 1999; Amin *et al*., 2004)**. *T. ilisha* is widely distributed in the waters of Indonesia **(Jihad *et al*., 2014; Machrizal *et al*., 2019)**, Iran **(Roomiani *et al*., 2014)**, Bangladesh **(Flura *et al*., 2015; Nima *et al*., 2021)**, the Indian Bay of Bengal **(Karim *et al*., 2015; Mohanty and Nayak, 2017)**, and Iraq **(Sarker *et al*., 2016)**. In Indonesia, only the Barumun and Bilah rivers in Labuhanbatu Regency are migratory areas for *T. ilisha* **(Jihad *et al*., 2014; Machrizal *et al*., 2019)**. Until now, research related to *T. ilisha* from the Barumun and Bilah rivers in Labuhanbatu Regency is still limited. Several studies related to *T. ilisha* in Labuhanbatu only explore biological and ecological information, while research related to molecular aspects is still not revealed. This research is a preliminary study to reveal the relationship of *T. ilisha* from the Barumun and Bilah Rivers of Labuhanbatu with *T. ilisha* from various waters in Asia based on genetic markers of the *Cytochrome b* gene, which has been unknown.

MATERIALS AND METHODS

Samples were collected from two rivers, namely Barumun and Bilah, Labuhanbatu Regency, North Sumatra Province in April 2023. *T. ilisha* specimens were caught using gill nets with a size of 1-3 inches (25.4-76.2mm). Captured fish were immediately photographed, and their morphological characteristics were recorded. Samples were then preserved using 10% formalin for collection. Captured *T. ilisha* samples were matched for morphological characteristics using identification books **(Kottelat *et al.*, 1993; Kottelat, 2012; Nelson *et al.*, 2016)**. The liver of *T. ilisha* fish taken for DNA isolation was stored in a microtube with the addition of 96% ethanol pro analyst.

The extraction of mitochondrial DNA (mtDNA) Cyt b was conducted using the Polymerase Chain Reaction (PCR) method with universal primers: forward (5'-CTAACGACGCAGTAGTTGATCTCCCA-3') and reverse (5'-CTGAGTTTAGCCCCGCAGGGTTGTT-3'), as reported by **Abdullah *et al.* (2019)**. The PCR mixture consisted of 12.5µl of master mix (containing 1.0 mM $MgCl_2$, 0.4 µM of each dNTP, 0.1 U/µl Taq polymerase, 20 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, and 20 mM Tris-HCl, pH 8.8). The final reaction volume was 25µl, which included the primers, 100 ng of template DNA, and 8.5µl of nuclease-free water.

Amplification was performed using the following cycle parameters: initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 48°C for 1 minute, and extension at 72°C for 45 seconds. A final extension step at 72°C for 5 minutes concluded the last cycle. The PCR products were visualized on a 1.5% agarose gel. PCR products (700bp) were purified using the Invitrogen™ PureLink™ PCR Purification Kit (Fisher Scientific) according to the manufacturer's instructions (Invitrogen, Germany).

The forward and reverse DNA sequences were assembled into a contig using DNA STAR software **(Burland, 2000)** to obtain the complete DNA sequence. The DNA sequences of *T. ilisha* were then compared with those of other fish samples from the same family using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi to assess the level of similarity. All sequences were aligned using Clustal X 2.0 **(Thompson *et al.*, 1997)** and edited with BIOEDIT software **(Hall, 1999)**. Nucleotide sequences were translated into amino acids using the online tool at http://insilico.ehu.es/translate.

The genetic distance for each sequence was calculated using molecular evolutionary genetics analysis (MEGA) XI with the Kimura two-parameter (K2P) model and 1000 bootstrap replicates **(Tamura *et al.*, 2021)**. Phylogenetic trees were constructed based on the neighbor-joining (NJ) and maximum likelihood (ML) methods, also using 1000 repetitions in MEGA XI **(Tamura *et al.*, 2021)**.

RESULTS

The alignment results of five *Cyt b* gene sequences of *T. ilisha* from Barumun and Bilah rivers obtained 568-572bp to be analyzed with the composition of A (23.5%), T(U) 29.0%, G (19.9%), and C (27.6%) (Table 1).

Table 1. Nucleotide composition of *T. ilisha Cyt b* gene from Barumun and Bilah rivers, Labuhanbatu Regency

Species	Sampling location	Nucleotide (%)					
		A	T(U)	G	C	AT	GC
T. ilisha	Barumun River (BR01)	23.5	29.0	19.9	27.6	52.5	47.5
T. ilisha	Barumun River (BR01)	23.5	29.0	19.9	27.6	52.5	47.5
T. ilisha	Bilah River (BL01)	23.5	29.0	19.9	27.6	52.5	47.5
T. ilisha	Bilah River (BL01)	23.5	29.0	19.9	27.6	52.5	47.5
T. ilisha complete genome	Barumun River	23.5	29.0	19.9	27.6	52.5	47.5

BLAST analysis of five samples of *Cyt b* gene sequences from Barumun River and Bilah River had a similarity level of 100.00% with Genbank data (Table 2).

Table 2. The similarity of *Cyt b* gene sequence of *T. ilisha* from Barumun and Bilah rivers with Genbank database based on blast analysis

Species	Country	Similarity (%)	Accession No
T. ilisha	Iraq	100	LC619671.1
T. ilisha	Iraq	99.47	LC619673.1
T. ilisha	Iraq	99.64	LC619674.1
T. ilisha	India	99.64	KC816530.1
T. ilisha	India	98.92	MK608838.1
T. ilisha	India	98.92	MN101849.1
T. ilisha	Malaysia	98.92	KX859109.1
T. ilisha	Malaysia	98.92	KX859108.1
T. ilisha	Malaysia	98.92	KU888658.1
T. ilisha	Bangladesh	98.92	EU552622.1
T. ilisha	Bangladesh	98.92	MN748966.1
T. ilisha	Bangladesh	98.92	MN748964.1

Genetic distance analysis was conducted using the Kimura 2-parameter (K2P) model (Fig. 1). The results of genetic distance analysis showed that 4 individuals of *T. ilisha* collected from the Barumun and Bilah rivers had a genetic distance of 0.000. Genetic distance of 0.000 was also obtained between samples from the Barumun and Bilah rivers with samples of *T. ilisha* from Iraq (LC619671.1; LC619673.1; LC619674.1). The longest genetic distance of *T. ilisha* collected from Barumun and Bilah rivers was with *T. ilisha* samples from Bangladesh (MN748966.1; MN748964.1), with a genetic distance of 0.021.

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*T.ilisha* Cytb (complete genome) Labuhanbatu	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2	*T.ilisha* BR02	0.007	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	*T.ilisha* BR01	0.007	0.000	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
4	*T.ilisha* BL02	0.007	0.000	0.000	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
5	*T.ilisha* BL01	0.007	0.000	0.000	0.000	*	*	*	*	*	*	*	*	*	*	*	*	*	*
6	*T.ilisha* Bangladesh (MN748966.1)	0.014	0.021	0.021	0.021	0.021	*	*	*	*	*	*	*	*	*	*	*	*	*
7	*T.ilisha* Bangladesh (MN748964.1)	0.014	0.021	0.021	0.021	0.021	0.000	*	*	*	*	*	*	*	*	*	*	*	*
8	*T.ilisha* Bangladesh (EU552622.1)	0.003	0.010	0.010	0.010	0.010	0.010	0.010	*	*	*	*	*	*	*	*	*	*	*
9	*T.ilisha* India (MN101849.1)	0.000	0.007	0.007	0.007	0.007	0.014	0.014	0.003	*	*	*	*	*	*	*	*	*	*
10	*T.ilisha* India (MK608838.1)	0.000	0.007	0.007	0.007	0.007	0.014	0.014	0.003	0.000	*	*	*	*	*	*	*	*	*
11	*T.ilisha* India (KC816530.1)	0.003	0.003	0.003	0.003	0.003	0.017	0.017	0.007	0.003	0.003	*	*	*	*	*	*	*	*
12	*T.ilisha* Iraq (LC619674.1)	0.007	0.000	0.000	0.000	0.000	0.021	0.021	0.010	0.007	0.007	0.003	*	*	*	*	*	*	*
13	*T.ilisha* Iraq (LC619673.1)	0.007	0.000	0.000	0.000	0.000	0.021	0.021	0.010	0.007	0.007	0.003	0.000	*	*	*	*	*	*
14	*T.ilisha* Iraq (LC619671.1)	0.007	0.000	0.000	0.000	0.000	0.021	0.021	0.010	0.007	0.007	0.003	0.000	0.000	*	*	*	*	*
15	*T.ilisha* Malaysia (KX859109.1)	0.000	0.007	0.007	0.007	0.007	0.014	0.014	0.003	0.000	0.000	0.003	0.007	0.007	0.007	*	*	*	*
16	*T.ilisha* Malaysia (KX859108.1)	0.003	0.010	0.010	0.010	0.010	0.010	0.010	0.000	0.003	0.003	0.007	0.010	0.010	0.010	0.003	*	*	*
17	*T.ilisha* Malaysia (KU888658.1)	0.000	0.007	0.007	0.007	0.007	0.014	0.014	0.003	0.000	0.000	0.003	0.007	0.007	0.007	0.000	0.003	*	*
18	*T.toli* Malaysia (KR824837.1)	0.178	0.178	0.178	0.178	0.178	0.166	0.166	0.172	0.178	0.178	0.184	0.178	0.178	0.178	0.178	0.172	0.178	*

Fig. 1. Distribution of sequence divergence values based on *Cyt b* gene in *T. ilisha* from Barumun and Bilah rivers with other species in %

Phylogenetic reconstruction using neighbour-joining (Fig. 2) and maximum likelihood (Fig. 3) methods with a Kimura 2 parameter (K2P) model, bootstrap value 1000x. *T. toli* species was used as an outgroup for comparison. The phylogenetic tree of *T. ilisha* (Fig. 2) forms a branching where the analyzed species form groups according to genetic distance and DNA sequence similarity with a bootstrap value of 100%. The higher the bootstrap value, the better the phylogenetic tree reconstruction. Four samples of *T. ilisha* from the Barumun and Bilah rivers were in the same branch with *T. ilisha* from Iraq and India (KC816530.1). Meanwhile, one other sample from the Barumun River formed another branch with *T. ilisha* from India, Malaysia, and Bangladesh.

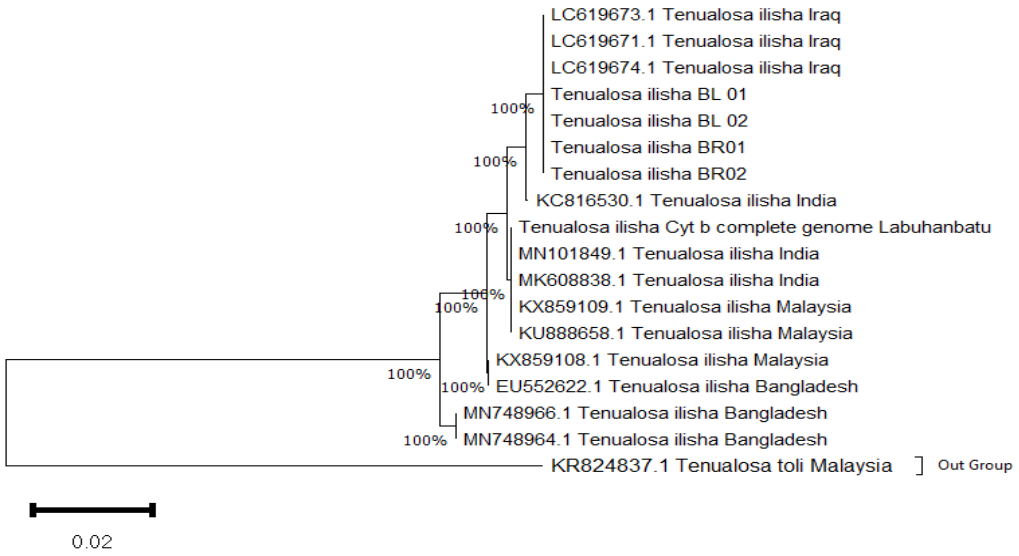

Fig. 2. Reconstruction of phylogenetic tree by neighbor-joining method using 13 DNA sequences from GenBank with *Tenualosa toli* as out group

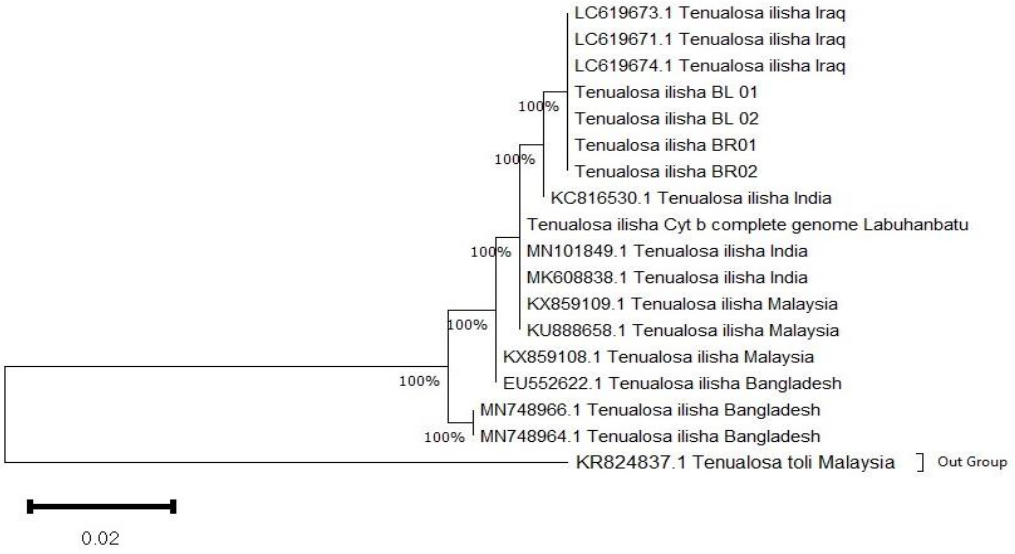

Fig. 3. Reconstruction of phylogenetic tree by maximum likelihood method using 13 DNA sequences from GenBank with *Tenualosa toli* as out group

DISCUSSION

The nucleotide base composition of *T. ilisha* from the Bilah and Barumun rivers of Labuhanbatu Regency is almost similar to that obtained by **Ghouri *et al.* (2020)** on *T. ilisha* in Bangladesh with a nucleotide base composition of Adenine (A) 23.35%, Thymine (T) 28.3%, Guanine (G) 17.62% and Cytosine (C) 30.70%. A previous research by **Ahmed *et al.* (2021)** found the following nucleotide composition in the order Clupeiformes from Bangladesh: T (28.60%), C (27.80%), A (24.20%), and G (19.40%), with AT content at 52.80% and GC content at 47.20%.

The average composition of each nucleotide base obtained is almost the same as the research of **Satoh (2016)**, that the composition of nucleotide bases in 250 fish is A 24.8%, T 29.5%, C 30.5%, and G 15.3%. According to **Belle *et al*. (2005)** in vertebrate groups, the number of bases (A + T) is higher than the bases (G + C) because the mutation rate (transitions and transversions) in mitochondrial DNA is relatively high.

Genetic distance is used as a basis for studying molecular evolution, phylogenetic reconstruction, and estimation of evolutionary time **(Sohpal, 2013)**. Genetic distance analysis was conducted using the Kimura 2-parameter model (Table 1). Based on the results of the genetic distance analysis, it is known that the genetic distance is 0.000-0.021, this means that *T. ilisha* from Barumun River and Bilah River come from the same population. This is in accordance with the statement of **Kartavtsev (2011, 2013)** based on the mitochondrial *cytochrome b* gene in vertebrates; the sequence difference at the species level in the population is 1.38 ± 0.30%; the level of subspecies, semispecies, sibling species is 5.10 ± 0.91%; the species level in the same genus is 10.31 ± 0.93%; the genus level in the same family is 17.86 ± 1.36, and in different families is 26.36 ± 3.88. The low genetic distance of *T. ilisha* from various waters is because this species is an anadromous species that migrates from the Malacca Strait to the Andaman Sea. This is in line with the findings of **Leatemia *et al*. (2018)**, who explained that several possibilities cause species from different locations to be genetically similar. These factors include genetic sharing, connectivity between regions **(Díaz-Ferguson *et al*., 2010)**, habitat similarity, and in marine organisms who tend to go through the process of migration and ocean currents as a medium of transfer **(Saleky *et al*., 2016)**.

Phylogenetics is the taxonomic classification of species based on evolutionary history, the analysis of which is based on the analysis of species DNA sequences. Mathematical models are used to infer the evolutionary history of species through molecular data **(Yuan *et al*., 2014)**. The results showed that the bootstrap value on each branching of the phylogenetic tree was 100%. The high bootstrap value affects the phylogenetic reconstruction formed, the higher the bootstrap value, the better the tree formed **(Horiike *et al*., 2009)**. The high bootstrap value is due to the high morphological similarity of all locations (Fig. 3). This morphological similarity is also due to the physical and chemical factors of the waters in each habitat occupied by *T. ilisha* (Table 3). The phylogenetic reconstruction shows connectivity between locations in Indonesia, Malaysia, Bangladesh, India, and Iraq forming the same connectivity. The geographical distance between Indonesia and Malaysia can cause gene flow which in marine organisms, gene flow occurs through the process of species migration or through the process of larval dispersal **(Jefri *et al*., 2015)**.

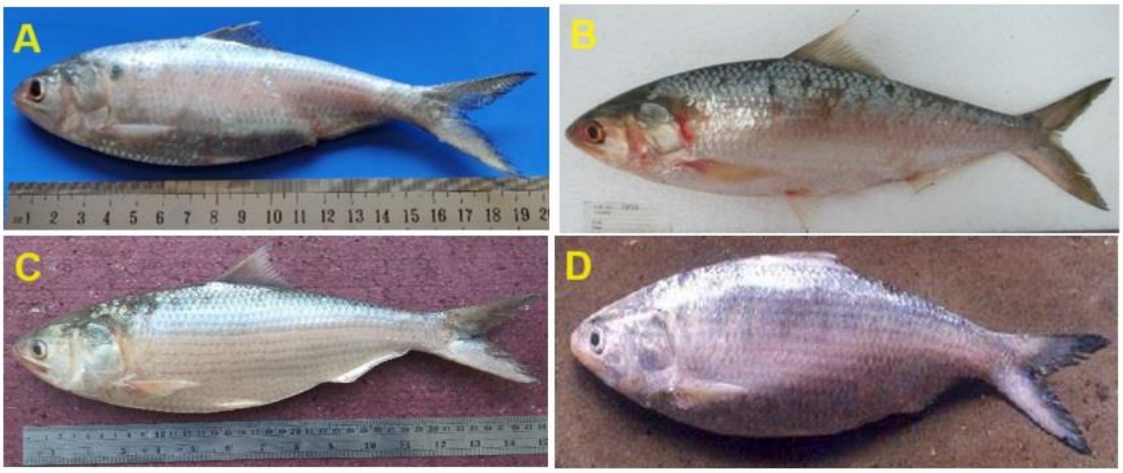

Fig. 4. Morphology of *Tenualosa Ilisha* from Indonesia and various waters in Asia

Note : A=Bangladesh (Bold System); B= Malaysia **(Arai & Amalina, 2014)**; C= Indonesia (Primary Data); D= India **(Bhaumik & Sharma, 2011)**

Table 3. Water quality parameters in different areas of *T. ilisha* fish habitat

Parameter	Indonesia[a*]	Bangladesh [b*]	Iraq [c*]	India [d*]
Temperature (°C)	27-31	21-27	13-29.80	28-30
pH	6.9-7.7	7.2-7.5	7.26-7.98	7.9-8.32
DO (mg L^{-1})	10.1-10.7	4.2-6.71	5.80-11.50	6.14-7.09
BOD (mg L^{-1})	2.34-5.93	0.67-3.71	2.20-6.10	-
NO_3^- N (µ L^{-1})	2.10-8.18	0.90-0.135	7.98-15.98	-
PO_4^- P (µ L^{-1})	0.01-2.01	0.11-0.90	0.01-2.11	-

Note : [a*](primary data); [b*]**(Bhuyan *et al.*, 2017)**; [c*]**(Moyel&Hussain 2015)**; [d*]**(Bhakta *et al.*, 2018)**

The aquatic environment's physical and chemical factors significantly impact fish DNA morphology and structure. Studies suggest that environmental conditions like salinity and migration affect the genomic GC content of teleostean fishes, influencing DNA bendability and stability **(Tarallo *et al.*, 2016)**. Additionally, exposure to heavy metals like nickel, mercury, lead, and zinc induces genetic mutations and affects genomic stability in fish species, leading to differential variations in DNA structure **(Pal & Choudhury, 2014)**. Furthermore, changes in environmental temperature conditions can impact the cellular level adaptation of fish embryos, potentially causing disturbances in embryogenesis and genetic changes in somatic cells, reflecting environmental stressors **(Ejikeme *et al.*, 2022)**. Overall, the interplay of physicochemical factors such as salinity, temperature, and pollutants in the aquatic ecosystem plays a crucial role in shaping fish DNA morphology and structure.

CONCLUSION

The phylogenetic analysis illustrates that *T. ilisha* from Indonesia is very closely related to *T. ilisha* from all waters in Asia; this is due to gene flow between populations during the migration process. We suggest phylogenetic studies of *T. ilisha* with other species of the *Clupidae* family.

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