

## DNA barcoding of mislabeled juvenile *Tenualosa* spp. as *Gudusia chapra* in the fish markets of Bangladesh

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### Abstract

Mislabeled of Juvenile hilsa, (*Tenualosa* spp.) or Jatka fish as Chapila (*Gudusia chapra*) is a recognized problem in Bangladesh resulting in fraudulence to consumers, illegal harvesting, and ultimately the degradation of hilsa fish production. We applied DNA barcoding using mitochondrial COX 1 gene together with morphometric analysis to resolve the mislabeling or misidentification of Clupeid fishes differentiating mainly hilsa fish from chapila fish. This study confirmed the identification of Jatka fish (*Tenualosa* sp.) that was mislabeled with chapila (*Gudusia chapra*). DNA barcoding identified the four species of clupeid fishes (*Tenualosa ilisha*, *Gudusia chapra*, *Sardinella jussieu* and *Tenualosa toli*) with phylogenetic placement. These cases may reflect a clear picture of admixture of locally sold mislabeled Jatka, which may warrant more comprehensive analyses. Mislabeled records of Juvenile hilsa established in the present study may also have implications for the harvesting, marketing and consumption the national fish of Bangladesh.

**Keywords:** Mislabeled; DNA barcoding; Hilsa; Chapila; Fish market

### Introduction

Hilsa shad (*Tenualosa* spp.) is the national fish of Bangladesh. It is a delicious and commercially important fish in the different parts of Asia. Bangladesh contributes around 50-60% of the annual worldwide hilsa catch which has been decreasing day by day since 2003 (Islam *et al.* 2016). Recently, its production has been obstructed due to overfishing hilsa, harvesting of juvenile hilsa, water pollution, siltation of rivers, etc. Mislabeled of Juvenile hilsa called Jatka (up to 25 cm sizes) is one of the primary causes of hilsa declination in Bangladesh because it causes underreporting of species exploitation that negatively effects on conservation efforts. Bangladesh government implements some hilsa fishery protection campaigns for a certain period in different months every year such as “Operation Maa Ilish Rokksha” during peak breeding time of hilsa and “Operation Jatka” program for the peak season of juvenile hilsa (Jatka) to protect and

increase the production of hilsa fish in Bangladesh. In Bangladesh harvesting, transportation, marketing, selling, and possessing of Jatka have been banned from 1<sup>st</sup> November to 31<sup>st</sup> March every year (Protection and Conservation of Fish act 1950). However, mislabeling or misidentification of the juvenile or Jatka hilsa hinders these types of program and leads to unexpected consumption this fish species.

Jatka is available in rivers and coastal areas more or less year-round but the period of highest abundance for harvesting is January to April and sometimes it may extend up to May. As, jatka fish looks like Indian River shad, *Gudusia chapra* / commonly called Chapila (the adult is 20 cm) (Hamilton, 1822), therefore, the juvenile hilsa is mostly mislabeled with Chapila.

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In addition, this is also mislabeled with other closely related species like Sardines (*Sardinella longiceps*), Shad (*Tenuulosa toli*) because of their similar morphological features. Because of the high restriction for selling of hilsa fish, fish traders sell these juvenile fish as Chapila which was estimated its total harvesting as 12 to 14 thousand MT Jatka in 2017-2018 (BFRI report). When law enforcement agencies are confronted with fisherman, often they show undesirables disagreement and claim that the fish are Chapila. It creates huge community misperception and clash as they repeatedly fail to differentiate between Jatka and Chapila. If Bangladesh government is able to stop or reduce jatka harvesting from rivers then it will enhance the country's overall hilsa production. Consequently, the livelihood of fishers involves with the hilsa fishery will improve and consumers will get fish at a reduced price. Therefore, there is an urgent need for avoiding the mislabeling or misidentification of the highly valuable national fish at their juvenile or Jatka stage applying effective modern techniques where molecular method called DNA barcoding could be the right option.

DNA barcoding is an important taxonomic tool for rapid and accurate identification of any species on the basis of comparison with known species of a reference database (Floyd *et al.* 2002; Tautz *et al.* 2003). The cytochrome C oxidase subunit 1 mitochondrial region (COI) is used as a standard barcode region (648 nucleotide base pairs long) for DNA barcoding of higher animals (Hebert *et al.* 2003; Lakra *et al.* 2011). This method also used to resolve the

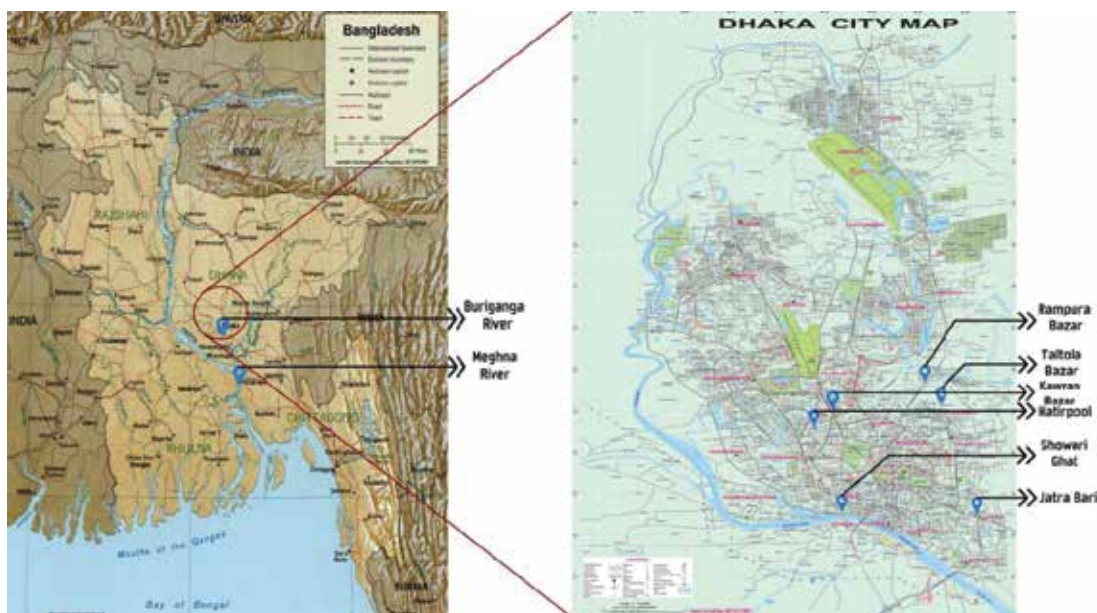
problem of cryptic species identification for conservation purposes (Hebert *et al.* 2004; Bickford *et al.* 2007). DNA barcoding has been used to identify fish in several studies including freshwater and marine fishes of Bangladesh (Smriti *et al.* 2017; Rahman *et al.* 2019; Habib *et al.* 2021). Furthermore, morphometric and meristic characters and mitochondrial DNA sequence methods were applied to resolve the taxonomic ambiguity of Punti fish, *Puntius denisonii* and *Puntius chalakkudiensis* (Menon *et al.* 1999; John, 2009). Therefore, the utilization of DNA barcoding method could be used to resolve the mislabeling of juvenile hilsa and adult chapila for accurate and reliable identification.

Therefore, in the present study, DNA barcoding technique was used to identify the Chapila and Hilsa fishes to resolve their morphological ambiguities which will be very imperative not only for the taxonomic differentiation but also for the management and conservation purposes of hilsa fish. This initiative will ultimately build awareness among the people from being fraudulence of buying mislabeled fishes and helps to take initiatives by the government policy makers.

## Materials and methods

### Sampling schedule and sites

Fresh twenty fish samples as chapila were collected from different habitats and markets are shown in Figure 1, and



**Fig. 1. Sampling sites in the two rivers (left): Buriganga and Meghna River and six markets (right): Rampura Bazar, Taltoal Bazar, Kawran Bazar, Hatirpul Bazar, Showari ghat and Jatrabari**

**Table I. Sampling schedule, habitat, sites and sampling name with number, code and sampling picture**









Sl no	Sampling date	Types of sampling sites	Name of sampling site	Sample name	Number of fish individual	Voucher specimen code	Sampling picture
01.	02.02.2015	Main River	Meghna River (Chandpur)	Chapila	03	CCF	
02.	23.02.2015	Tertiary River	Buriganga River	Chapila	03	BLF	
03.	18.02.2015	Whole sale Fish Market	Jatrabari Bazar	Chapila	01	JNF	
04.	19.02.2015		Kawran Bazar	Chapila	02	MF	
05.	18.02.2015		Suarighat Bazar	Chapila	03	SEF	
06.	18.02.2015	Retail Market	Rampura Bazar	Chapila	02	RSF	
07.	13.04.2015		KhilgoanTaltola Bazar	Chapila	03	TCF	
08.	11.03.2015		Hatirpul Bazar	Chapila	03	EF	

Table I with their voucher specimen code. One sample fish was collected from the main river named the Meghna River (specimen code: CCF) which was confiscated from fishermen by Coast Guard. Other samples were from a catching point of Buriganga river (specimen code: BLF), three wholesale fish markets (where trade among fishermen and fish merchants and fish retailer): Jatrabari (specimen code: JNF), Kawran bazar (specimen code: MF), Suarighat (specimen code: SEF) bazar and three retailer fish markets (where consumers direct buy fishes): Rampura (specimen code: RSF), Khilgoan-taltola (specimen code: TCF), Hatirpul (specimen code: EF) bazar at early morning. The specimens were preserved in a cool box with sufficient ice and trans-

ferred at -20°C freezer in the Fisheries Laboratory, Department of Zoology, Jagannath University, Dhaka until further study. All specimens were kept in the museum of the Zoology Department, Jagannath University as voucher specimens until completing the study.

#### *Taxonomic procedure*

#### *Morphometrics identification*

The fish length was measured in centimeter (cm) to the nearest 0.01, and weight was measured in gram. Morphometrics and meristics methods were similar to those described by

Allen and Talbot (1985). A total of eight meristic and 20 morphometrics characters were considered and some descriptive characters such as body and fin coloration were observed. The morphomeristics study was carried out in the Fisheries Laboratory, Department of Zoology, Jagannath University, Dhaka, Bangladesh.

#### Molecular identification

The molecular experiment was carried out in the Zoology Section, Biological Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka. For the molecular study, 8 fish were selected randomly, including a single individual from each of the two rivers (CCF and BLF), five wholesale fish markets (JNF, MF, TCF, RSF, and SEF), and one retailer fish market (EF).

For each sample, about 20-100 mg of tissue was collected from the selected part (below dorsal fin) of fish with a sterile scalpel. Genomic DNA was extracted following phenol-chloroform method (Sambrook *et al.* 1989). The extracted DNA was measured on Gel electrophoresis and also by UV-Spectrophotometry (Nanodrop spectrophotometer ND-2000, Thermo Scientific, USA). To amplify the target DNA segment of mitochondrial Cytochrome c Oxidase subunit I (COI) gene, Go Taq PCR master mix (Promega, USA) was used with template DNA and different combination of specific primer for fish species (Ward *et al.* 2005): Fish F1: TCAACCAACCACAAAGACATTGGCAC and Fish R1: TAGACTTCTGGGTGGCCAAAGAATCA, Fish F2: TCGACTAATCATAAAGATATCGGCAC and Fish R2: ACTTCAGGGTGACCGAAGAATCAGAA (primer 2). PCR thermal cycler was run in following the cycle: initialization step consists of heating the reaction to a temperature of 96°C for 1 min followed by a 40 cycle of denaturation at

**Table II. The morphomeristic measurements of the collected fish sample from six markets and two rivers**

Morphomeristics Variables (cm)	CCF ( <i>T. ilisha</i> )	SEF ( <i>T. toli</i> )	JNF ( <i>T. ilisha</i> )	RSF ( <i>T. ilisha</i> )	BLF ( <i>T. ilisha</i> )	TCF ( <i>G.chapra</i> )	EF ( <i>T. ilisha</i> )	MF ( <i>G.chapra</i> )
Total length	20.4	18.5	17.5	15.1	8.8	10.3	11.2	14
Fork length	17	15.8	14.2	12.3	7.3	8.7	10.3	12.2
Standard length	16	13	12.1	10.3	5	5.1	8.5	11
Predorsal length	6.6	6.6	6.6	4	2.9	4	3.9	5.8
Head length	4.2	3.2	4.3	2	1.6	1.6	2.5	3.5
Preorbital length	0.9	1.3	0.9	0.9	0.4	0.3	0.6	0.7
Post orbital length	2.5	1.3	2.6	1.5	0.8	0.8	1.2	1.8
Eye diameter	0.8	1.1	0.7	0.6	0.3	0.5	0.5	0.8
Body depth	1.8	1.7	4.1	2.5	0.6	0.8	0.7	1.2
Dorsal fin base	2.1	1.8	2.4	1.2	1	1	1.3	1.6
Peduncle depth	0.7	0.4	0.6	1	0.2	0.3	0.2	0.5
Peduncle length	1.1	0.8	1	0.9	0.5	0.7	0.5	1.1
Length of upper jaw	1.9	1.8	1.9	0.8	0.9	0.7	1.4	1.3
Length of lower jaw	1.5	1.6	1.7	0.9	0.8	0.8	1.1	1.6
Jaw gape	1.3	0.7	1.3	0.3	0.7	1	1.3	1.6
Pectoral fin base	2.5	1.4	2.6	1.5	1	1.3	1.5	1.3
Pelvic fin base	1.5	0.7	1.6	1	0.7	0.8	1	1.2
Anal fin base	2.1	3.8	2.2	2	1	1	1.3	2.2
Length of caudal fin	1.5	1.3	1.5	1.8	0.7	0.6	1	1.2
Dorsal fin ray	19	17	19	19	17	13	19	15
Pectoral fin ray	15	14	16	14	12	12	16	12
Pelvic fin ray	8	8	8	7	8	8	8	7
Anal fin ray	24	21	23	18	21	24	23	24
Branchiostegal ray	5 pair	5 pair	5 pair	4 pair	5 pair	6 pair	5 pair	6 pair
Scutes	31	29	30	32	32	27	25	26

96°C for 30 seconds, annealing at 50°-65°C for 40 second and extension at 72°C for 1 min. The final extension was at 72°C for 5 mins and the final hold step at 4°C for the short-term storage of the reaction. After that, the PCR product was checked by running agarose gel electrophoresis and banding pattern was used for qualitative and quantitative analysis. Approximately 655 bp were amplified from the COI in mitochondrial DNA. Following PCR amplification, the COI PCR product was cleaned up by PCR purification Kit (ExoSap, Thermo Fisher, USA). For samples showing clean, discrete PCR product proceeded directly to sequencing.

In this study, the sequencing has been done from First BASE Laboratories, Selangor, Malaysia by using a Genetic Analyzer (M:3031, Applied biosystems, USA). The analysis of the sequenced barcode segment (COI gene) of target fish species were done by using the Bioinformatics tools - Chromas Lite and Geneious R8. Chromas Lite was used to viewing the chromatogram figure and the sequence data were transferred to FASTA format. All sequences were proofread and assembled using the software SeqMan (DNASTar, USA). All sequences were blasted within the nucleotide database for the authentication of the morphological identification at the National Centre for Biotechnology Information databases (NCBI) to determine the highest homology and thus to identify the species. The software MEGA 6.0 (Tamura *et al.* 2013) was used for estimation of genetic P distance and to form the Neighbor-joining (NJ) tree based on the Kimura 2 parameter model (K2P) and 1000 bootstrap replications. Finally, the sequences were submitted to the GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide/>) with accession numbers (MW286124- MW286132)

## Results and discussion

Fraud labeling of jatka fish was investigated as a role of wholesaler and retailer fish markets within the capitals and

rivers where the number of samples was collected. In the present study, 20 fish samples from 6 local different types of fish markets and 2 river sites (SEF, JNF, RSF, TCF, MF, EF, CCF, and BLF) were collected and showed consistency in both morphological and molecular investigation.

### Morphological identification

The morphometric and meristic characters of these fishes were given in Table II. According to morphological and meristic analysis, collected fishes from CCF, JNF, RSF, BLF, and EF site were matched to *Tenulosa ilisha*, SEF site matched to *Tenulosa toli*, and TCF and MF sites matched to *Gudusia chapra*.

### Molecular identification of sample fish

#### Blast results of COI gene sequences

Among 16 sequences of 8 samples, 4 different species were identified after the blast in the NCBI reference database (Table III). Among 7 samples amplified by primer-1, three samples were detected as *T. ilisha* (CCF, JNF, and BLF). Two samples were detected as *G. chapra* (MF and TCF). One was *T. toli* by molecular identification (SEF). Another one was also *T. ilisha* (EF) but less similarity within the species. RSF was not identified by primer-1 (Table III). Among 7 samples amplified by primer-2, two samples were detected as *T. ilisha* (CCF and JNF) and one was contaminated (BLF) (Table III). Two samples were detected as *G. chapra* (MF and TCF). One was *T. toli* by molecular identification (SEF). Only one sample was *Sardinella jussieu* (Lacepède 1803) (RSF). EF was not identified by primer-2 but morphometrically identified as *T. ilisha* (Table I, II and III).

**Table III. Molecular identification of samples using DNA barcoding with conventional morphological identification.**

Sample ID	Morphological Identification	Molecular Identification (Primer -1)	Identity by NCBI blast result (%)	Molecular Identification (Primer -2)	Identity by NCBI blast result (%)
1. CCF	<i>T. ilisha</i>	<i>T. ilisha</i>	99%	<i>T. ilisha</i>	99%
2. SEF	<i>T. toli</i>	<i>T. toli</i>	99%	<i>T. toli</i>	99%
3. JNF	<i>T. ilisha</i>	<i>T. ilisha</i>	99%	<i>T. ilisha</i>	99%
4. RSF	<i>T. ilisha</i>	Not Identified	-	<i>S. jussieu</i>	91%
5. BLF	<i>T. ilisha</i>	<i>T. ilisha</i>	99%	Not identified	-
6. TCF	<i>G. chapra</i>	<i>G. chapra</i>	100%	<i>G. chapra</i>	100%
7. EF	<i>T. ilisha</i>	<i>T. ilisha</i>	94 %	Not identified	-
8. MF	<i>G. chapra</i>	<i>G. chapra</i>	100%	<i>G. chapra</i>	100%

Table IV. Comparison of pairwise genetic p distance of sequenced samples of Ilish and Chapila. Higher p distance value indicated the higher differentiation among populations

	T_illisha_BLF1	T_illisha_CCF1	T_illisha_CCF2	T_illisha_JNF1	G_chapra_MF1	G_chapra_MF2	T_toli_SEF2	G_chapra_TCF1	G_chapra_TCF2
T_illisha_BLF1									
T_illisha_CCF1	0.000								
T_illisha_CCF2	0.002	0.002							
T_illisha_JNF1	0.000	0.000	0.002						
G_chapra_MF1	0.141	0.141	0.139	0.141					
G_chapra_MF2	0.142	0.142	0.140	0.142	0.000				
T_toli_SEF2	0.213	0.213	0.211	0.213	0.190	0.184			
G_chapra_TCF1	0.141	0.141	0.139	0.141	0.000	0.000	0.190		
G_chapra_TCF2	0.141	0.141	0.139	0.141	0.000	0.000	0.190	0.000	

Genetic variation and % GC content using COI gene

Total 9 COI sequences among *T. ilisha* (JNF1, CCF1, BLF1 by primer 1 and CCF2 by primer 2), 4 for 2 individuals of *G. chapra* (MF1, TCF1 by primer 1 and MF2, TCF2 by primer 2) and one for a single individual of *T. toli* (SEF2 by primer

2) were selected as good sequences during the present study and submitted to GenBank with accession number (MW286124-MW286132). The overall mean P distance diversity in the entire population, within-population (species) and inter-population (genus) was 0.11, 0.04 and 0.06, respectively. Interspecies mean P distance between ilisha and

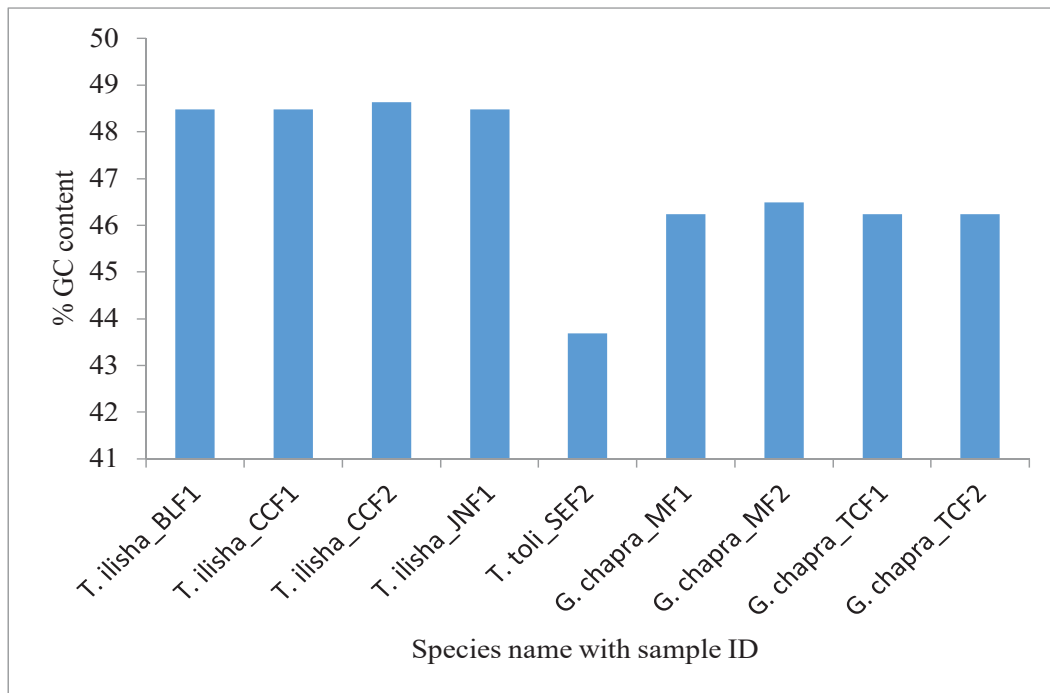


Fig. 2. The % GC content variation for 9 COI gene sequences of *T. ilisha*, *T. toli* and *G. chapra*

Chapila group was 0.150 whereas intraspecies (within ilisha) mean p distance was 0.09. The pair-wise comparison of P distance showed that genetic differential of *T. ilisha* was highest with *T. toli* species than *G. chapra* species (Table IV). Besides, intraspecies K2P distances for *T. ilisha*, *T. toli*, and *G. chapra* ranged from 0.000 to 0.004 and interspecies distances ranged from 0.166 to 0.254, the threshold of species delimitation (0.035) distant exceeding (Ward *et al.* 2005; Ward *et al.* 2009) based on the metric of 10× the average intra-species genetic variation (Hebert *et al.* 2004). Different ranges of % GC content were observed among three different species *T. ilisha*, *T. toli*, and *G. chapra* with higher content in the *Tenualosa* genus (average 47.55%) than *Chapila* genus (average 46.30%)(Figure 2).

#### Phylogenetic analysis using Neighbor-Joining tree

To study the phylogenetic origin of collected samples, 9 COI sequences of ilisha and Chapila were selected from the present study. Three conspecies sequences were downloaded from GenBank and their accession number provided in the associated figure. The NJ tree based on COI gene sequences (Figure 3) revealed that the three different species *T. ilisha*, *T. toli*, and *G. chapra* formed monophyletic groups with reference sequences from NCBI of each.

Mislabeled fish is a big issue now a days in Bangladesh which must be addressed by our government to deal with taking necessary steps. Fish species substitution ultimately cheats consumers who fall victim to bait and hurts honest fishermen and fish businesses. It is also critical to certify scientifically that all fish sold in the market should be correctly labeled for ethical fishing and business practices. Therefore, the purpose of the present study is to differentiate jatka from chapila for identification of mislabeling through morphometric and molecular tools.

The most common substitute species for jatka (*T. ilisha*) was chapila which has been reported in the wholesale, retailer market and the main river of Bangladesh (Dof, 2018). Furthermore, the vast majority of the consumers were not able to distinguish jatka from chapila. Our study observed around 80% of hilsa has been mislabeled as chapila by fisherman and seller of different fish market. Similarly, the mislabeling of fish and many seafood items has been reported frequently in the local market of many countries of the world, causing deleterious impacts on human health, environment, the economy and the society (Munguia-Vega *et al.* 2021; Ryburn *et al.* 2022; Cundy *et al.* 2023). Furthermore, the majority of exchanges recognized in our samples were, on

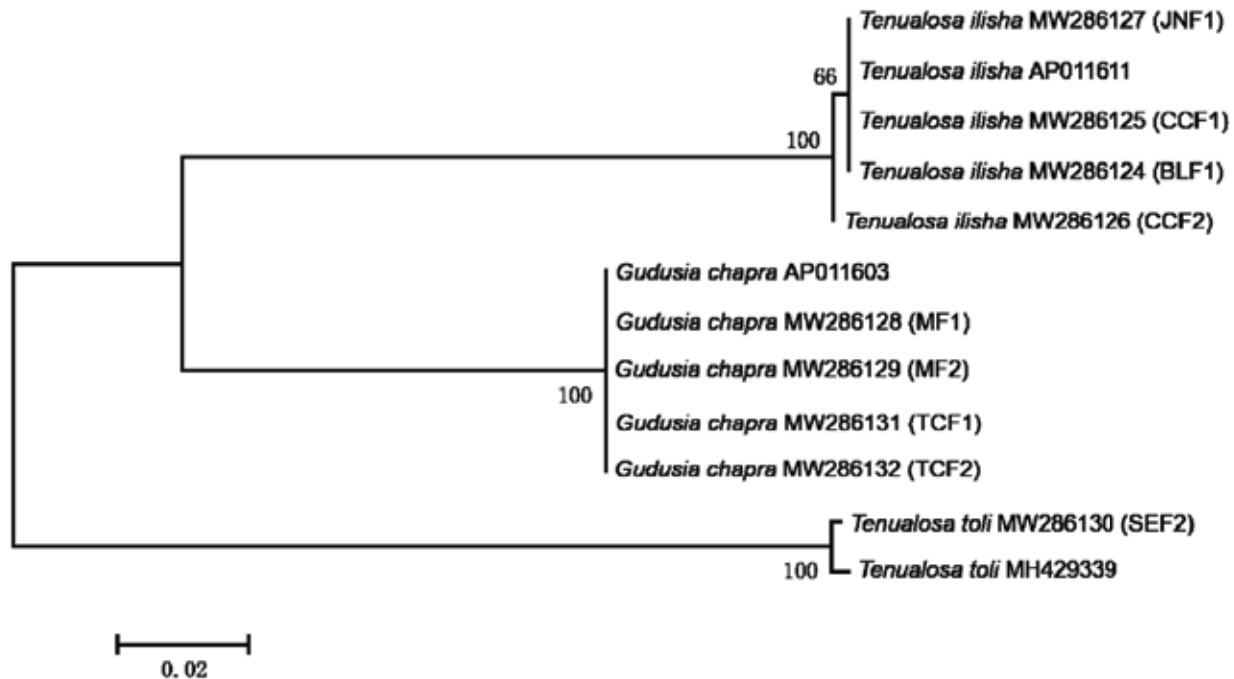


Fig. 3. The neighbor-joining tree was constructed using the K2P model for 12 COI gene sequences of *T. ilisha*, *T. toli*, and *G. chapra* including 3 retrieved data from NCBI

average, fewer costly and apparently less wanted alternatives to jatka. These results suggested a financial motivation because the alternative signifies lower-rated replacements and impede consumer choices.

Molecular analysis has been utilized for many years for fish species identification. Initially, allozyme differences were used (Awise 1989), followed by mtDNA examination (Awise 1994). DNA barcoding is becoming an increasingly popular method for the identification of animal species (Hebert *et al.* 2003; Costa and Carvalho 2007). The differentiated four species of tuna (*Thunnus* spp.) were identified by mtDNA sequencing (Bartlett and Davidson 1991). The results of the present investigation clearly indicates that DNA barcoding is a dominant method and correctly detecting collected samples of different sources such as vender, fish markets, or rivers as different species instead of mislabeled chapila. Phylogenetic tree reconstruction methods such as NJ were used to justify the result of DNA barcode sequences. NJ tree was constructed for understanding the distance relationship among the sampling species. In the present study, *T. ilisha*, *G. chapra*, and *T. toli* had close relationship with each other but a large distance relationship between hilsa and chapila. Therefore, this relationship confirmed the presence of different species mislabeled as one species. The samples except for 5 individuals of *G. chapra* (TCF and MF) collected from different sites were mislabeled, with one species named jatka (*T. ilisha*) being sold as chapila. We have collected all the fish samples as a name of chapila but after morphological and DNA barcoding study we found 3 different species among them, most of the individuals were jatka (hilsa) which was mislabeled with chapila. The result indicated that substantial amounts of jatka are being mislabeled for trading every day and it causes great loss to the economy of our country.

During “Jatka Operation” Coast Guard seized the harvested jatka and the setting of current jal (net) from Meghna rivers indicates that the harvesting of undersized hilsa fish is going on yet it’s a violation of the Protection and Conservation of Fish act 1950 (Rayhan *et al.* 2021). Furthermore, this result also indicates that the illegal setting of nets and the misreporting of the catch was confirmed in those habitats. This activity also indicates few fishermen do not respect the fishery act 1950 still now. Hilsa is transported through one or further transitional steps and later offers several chances for the legally and illegally sourced fish mixing, where the unlawful jatka are basically legalized and later move in general trade as a lawful product. Considering the opportunity, identification of jatka mislabeling is significant for customers, fisheries administrators, and in the hilsa fish supply chain. In the present study, the result of the %GC content variation and neighbor-joining tree clearly indicated

the separation of the different root of commonly called chapila/jatka in our local trade which badly impacts the future stock of our royal fish hilsa. Selling and purchase of such fish species establish severe financial fake, and consequences raised the unlawful dealing of our national fish from both economic and management topics of vision. Circulating mislabeling records may inspire, fisherman, sellers, and consumers could motivate to check that suppliers offer the right product. Therefore, the authority of quality control and identifying the species frequently trade in our country is unconditionally vital.

### Conclusion

Mislabeleding of jatka was confirmed at different stages of the supply chain in the present study. It causes a great hamper to our economy and loyalty. Along with the government, we should take proper steps by providing more data on mislabeling to save hilsa fisheries. This was a preliminary study based on small number of data considering the cost of sequencing during study period. However, it will provide a base-line data for the further large-scale research on mislabeling of fishes based on DNA barcoding method.

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