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RESEARCH PAPER

Genetic Status of Exotic Chinese Carp *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* through Mitochondrial Gene Cytochrome C Oxidase Subunit I (COI) in Bangladesh

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Abstract

The use of mitochondrial gene Cytochrome C Oxidase subunit I (mtCOI) has emerged as a promising technique in study of genetic variation and phylogenetic relationship of different group of fishes. This work reports the first example of the study of genetic status of two exotic Chinese carp, silver carp, *Hypophthalmichthys molitrix* and bighead carp, *Hypophthalmichthys nobilis* in Bangladesh through the analyses of mtCOI gene. The highest nucleotide sequence divergence occurred between sequenced *H. nobilis* N3 and reference *H. molitrix* from India which was 1.208 and the lowest nucleotide divergence occurred in sequenced *H. nobilis* N1 and sequenced *H. molitrix* M3 with their reference counterparts from Bangladesh, India and China (0.002). The phylogenetic tree analysis showed that one of the *H. nobilis* (*H. nobilis* N3) formed a sister group with *H. molitrix* M2 with 100% sequence similarities. Otherwise, all sequenced and reference *H. molitrix* and *H. nobilis* showed separate clade in the tree. Therefore, we can conclude that the exotic *H. molitrix* and *H. nobilis* maintain distinct population status with lower genetic variation. However, we should take essential measures to reduce inbreeding in future.

Introduction

Over the last few decades, inclusions of exotic fishes in Bangladesh have increased rapidly. Exotic fishes are those nonindigenous fish of foreign origin that are introduced to any local area in order to serve various purposes (Rahman, 2005). Since the first introduction of tilapia (*Oreochromis mossambicus*) in Bangladesh as an exotic species in 1954, inclusion of exotic species has been evolved to the point where it is now common to introduce exotic fish for various purpose, including as food fish, for rapid growth, for biological control in natural water and for ornamental purpose. To date, a total of 92 varieties of exotic fishes of different origin have been reported in Bangladesh (Galib and Mohsin, 2011). Of these, silver carp, *Hypophthalmichthys molitrix,* and bighead carp, *Hypophthalmichthys nobilis* are two common exotic fish in Bangladesh belonging to the family Cyprinidae have been introduced from china during the last 5 decades. Both of these species have drawn considerable interest owing to being significant food fish and their ability to cope with aquaculture system in our country. However, apart from their beneficial role many exotic fishes are reported to cause severe problems in the aquatic environment. For instance, some carnivorous fishes eat valuable

indigenous species that result in loss of biodiversity. Additionally, some exotic fishes are found to be very hardy in nature and can tolerate high degree of pollution that makes them adapting with the harsh environment compare to indigenous fish (Bhakta and Bandyopadhyay, 2007). However unfortunately, many of exotic popular pond fishes entered into the country crossing the border unofficially. Having been introduced with or without authorization, most of the species established themselves and became popular among the aquaculturists. While the introduction of some of the species proved to be suitable for pond culture, the introduction of exotic fish into open waters indiscriminately is open to debate.

However, for proper management and conservation of fish species knowledge on genetic diversity and population differentiation is considered as critical component. Morphological approaches to identify species are a practical limitation towards species identification as it makes difficult to identify cryptic species. One of the promising alternatives is molecular identification. DNA barcoding techniques using mitochondrial gene Cytochrome C Oxidase subunit I (COI), sequence showed promising alternative to traditional morphological identification since it allows for access to complex, and responsive genetic diversity (Hebert et *al.* 2003*)*. COI has been widely used in identification of related species and reconstruction of phylogenetic trees within a family. Our lab has recently used this technique to identify closely related species within Cyprinidae (Alam et al., 2021), Anabantidae family (Parvez et al., 2020), and *C. batrachus*, *C. gareipinus*, and the suspected hybrids in Bangladesh (Parvez *et al.* 2022).

The study on the molecular identification of these two exotic species in Bangladesh is very few only limited to a study regarding building a DNA barcode library by Rahman et al 2019. Therefore, genetic variation study of *H. molitrix* and bighead carp, *H. nobilis* in Bangladesh has yet to be elucidated. Moreover, as these fishes were introduced a long time ago, there is the possibility of inbreeding due to limited number of stocks that may impact the growth rate and survival of these two exotic species. Hence, it is highly essential to maintain the genetic diversity of *H. molitrix* and *H. nobilis* populations. Therefore, in this study attempts were taken to reveal the genetic variation and evolution of exotic *H. molitrix* and *H. nobilis* in Bangladesh compared to their native counterpart and neighboring country.

Material and Methods

Study Area

Fish samples were collected from local fish market namely "Bura Dighi" located in Dinajpur Sadar (25.700949 N, 88.668540 E), Bangladesh during July 2017- December 2017 (Figure 1).

Sample Collection and Morphological Identification

A total of 80 fish specimens were collected in aseptic and frozen condition from the study area, 40 of which were *H. molitrix* and the other 40 specimens were *H. nobilis*. The collected fishes were immediately transferred to "Fisheries Biology and Genetics Laboratory" Faculty of Fisheries, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh. Morphological identification was carried out based on the observation of morphometric and meristic character according to a previous literature (Farrington et al. 2015). Fishes were primarily identified by their distinguishing body color (silver color in case of silver carp and dark blotches in case of big-head carp) and head size (Big head carp has larger head than silver carp). After washing a part of fish muscle with 70% ethanol, tissue samples (5-10 gm) were collected carefully for DNA extraction from each fish specimen using sterile forceps, scissors and scalpels and preserved with 95% ethanol in falcon tube of 15ml capacity. All representative voucher specimens of collected fish were tagged accordingly and preserved in 10% buffered formalin solution.

DNA Extraction, PCR and Gel Electrophoresis of Mitochondrial COI Gene

The tissue samples were carried to the laboratory of Fisheries Biotechnology Division, National Institute of Biotechnology (NIB), Dhaka, Bangladesh for conducting the experimental research work such as extraction of the DNA, quantification of the extracted DNA, amplification of the targeted COI gene through polymerase chain reaction (PCR), electrophoresis of the PCR products etc. Purification of the PCR amplicons and sequencing of the purified DNA were obtained from the Biotech Concern company, Bangladesh.

A standard Phenol-Chloroform-Isoamyl alcohol (25:24:1) method including proteinase K treatment as a part of lysis was used for the genomic DNA extraction from each tissue samples (Sambrook J and Russell DW, 2001). For the amplification of the COI gene sequences (~650 bp) the primer sequences Fish-F1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and Fish-R1 (5'-TAGACTTCTGGGT GGCCAAAGAATCA-3') were used (Ward et al., 2005). The volume of PCR reaction mixture for each sample was 25 µL consisting 12.5 µl master mix (Maximo 2X premix), 0.5µlforward primer0.5 µl reverse primer, 1 µL extracted DNA template, and 11µlnuclease free water. Amplifications were performed using PRIME Thermal Cycler (5PRIMEG/02, UK) using the following protocol:initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 54°C for 40 sec and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The amplified PCR products were passed through 1.5% agarose gels, stained with ethidium bromide, and visualized using Gel

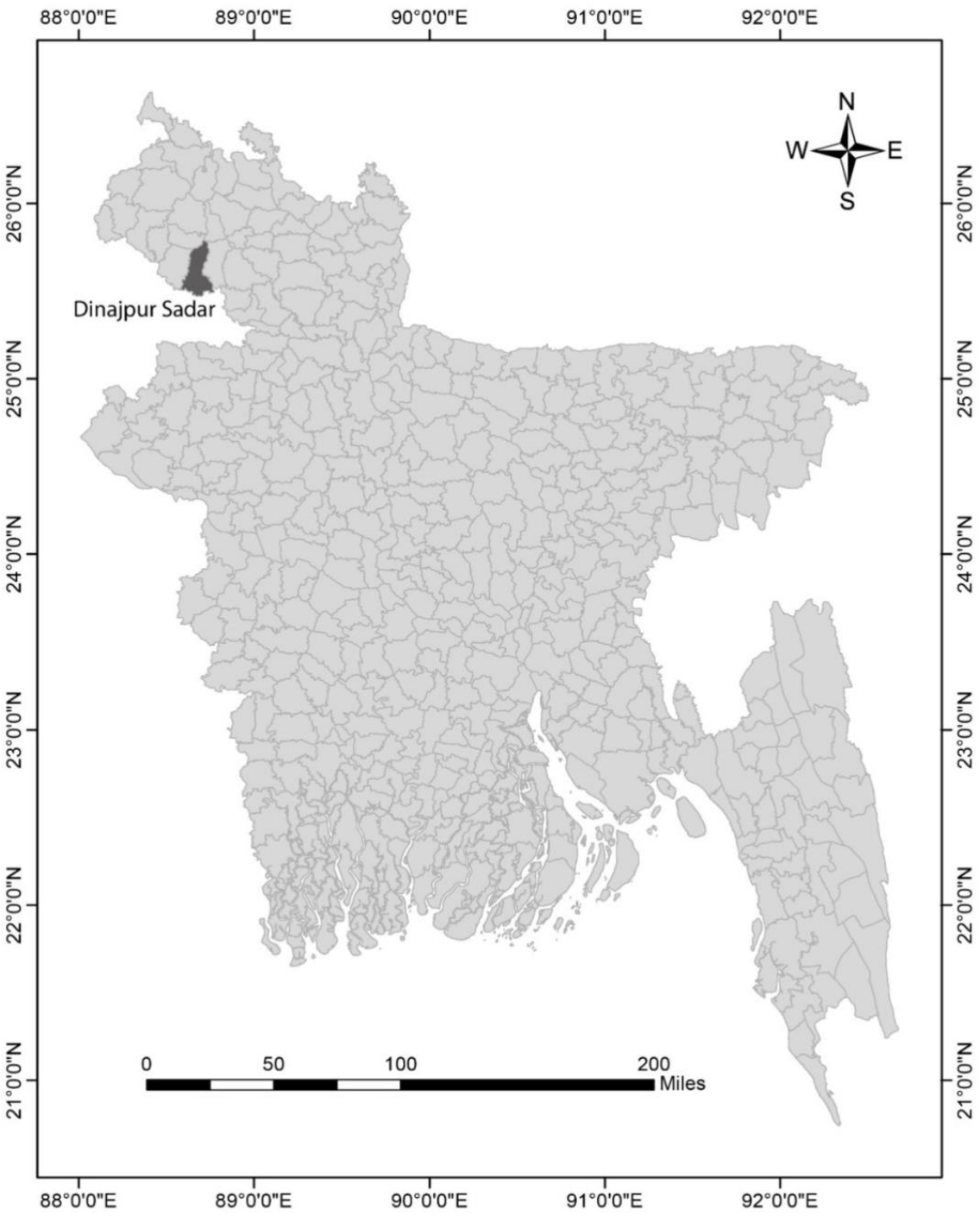

Figure 1. Sample collection sites.

documentation system (BIO-RAD, California, USA) through UV illumination.

Purification of the PCR Products and Sequencing

The PCR products were purified using Invitrogen DNA purification kit (K310001, Thermo Scientific, Waltham, Massachusetts, USA). The purified PCR products were labeled using the Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems Inc, Thermo scientific, Waltham, Massachusetts, USA) and were sequenced bi-directionally using ABI 3730 capillary sequencer.

Sequence Analysis

The raw DNA sequences of a total four (4) partial mtCOI gene sequences from the studied two fish species were aligned to acquire the consensus sequences ensuring only the quality sequences using BioEdit sequence alignment editor software (version 7.0.5.2). The size of the consensus sequences of COI gene varied from 583bp to 714 bp. The available Basic Local Alignment Search Tool (BLAST) was used to find out the identity percentages (%) of the consensus sequences with reference sequences at the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/BLAST/). The

confirmed sequences were submitted to NCBI database using BankIt submission tool (Accession Number- MG988398.1, MH176328.1, MH176327.1 and MG988397.1). Nucleotide sequences of the COI gene from *H. molitrix* and *H. nobilis* from other study of Bangladesh, India and China was retrieved from the NCBI GenBank database to include as reference sequences (Table 1). One sequence of *Clarius gariepinus* from our previous study (Parvez et al., 2022) was also retrieved from GenBank database for using as outgroup (Table 1).

After aligning the studied and retrieved sequences with ClustalW, molecular genetic analysis such as the polymorphic sites, nucleotide diversity, nucleotide composition, the disparity between sequences, net base composition bias, transitional/transversional bias were done using MEGA software version 11.0 (Tamura et. al., 2021). The probability of rejecting the null hypothesis that sequences have evolved with an equivalent pattern of substitution, as judged from the extent of differences in base composition biases between sequences was estimated using disparity Index test, (Kumar and Gangadhar, 2001, Tamura et al., 2021). A Monte Carlo test (500 replicates) was performed to estimate the P-values. A maximum composite likelihood model was used to measure the pairwise sequence divergence between species (Tamura et al., 2004, Tamura et al., 2021). The Tamura-Nei model provides an appropriate metric model once genetic distances square measures low (Nei & Kumar, 2000). Phylogenetic tree based on Maximum Likelihood Method and Tamura-Nei model (Tamura &Nei, 1993) was constructed using MEGA Version 11.0 (Tamura et al., 2021), which was validated using 1000 replicates of bootstrap values and all positions containing gaps and missing data were eliminated.

Results

Sequence Analysis of COI

A total of 4 sequences were studied and identified 757 sites where 397 (52.44%) were conserved; 323 (42.67%) were variable; 274 (36.20%) were parsimony informative sites and 44 (5.81%) were singleton. The average nucleotide composition of the COI gene was T/U=27.79, C=23.13, A=27.21, and G=21.86. The GC content was lower (45%) than the AT content (55%) in 4 sequenced samples of this study (Table 2).

The disparity index per site for all sequences was determined to observe the larger differences between the base composition biases than the expected bases of studied COI nucleotide sequences. The values greater than zero (0) indicated the larger differences in base composition biases than the expected bases on the evolutionary divergence between the studied COI

Table1. The Sequenced and reference COI gene sequences of *H. nobilis* and *H. molitrix*

Sl. No.	Species Name	Accession No.	Status	Origin
1	*H. nobilis**	MG988398.1	Sequenced	Bangladesh
2	*H. nobilis**	MH176328.1	Sequenced	Bangladesh
3	*H. nobilis*	MK572265.1	Reference	Bangladesh
4	*H. nobilis*	KF742440.1	Reference	India
5	*H. nobilis*	MF122410.1	Reference	China
6	*H. molitrix**	MH176327.1	Sequenced	Bangladesh
7	*H. molitrix**	MG988397.1	Sequenced	Bangladesh
8	*H. molitrix*	MK572264.1	Reference	Bangladesh
9	*H. molitrix*	FJ459500.1	Reference	India
10	*H. molitrix*	MF122395.1	Reference	China
11	*C. gariepinus*	MG988400.1	Reference	Bangladesh

Table 2. Nucleotide frequencies (%) of the COI sequences in the study

Sl. No.	Species Name	T/U	C	A	G	Total	A+T	G+C
1	*H. nobilis*_N1*(MG988398.1)	29.50	27.96	25.73	16.81	583.00	55.23	44.77
2	*H. nobilis*_N3*(MH176328.1)	25.63	19.58	27.46	27.32	710.00	53.10	46.90
3	*H. nobilis*_China(MF122410.1)	28.69	27.88	26.28	17.15	624.00	54.97	45.03
4	*H. nobilis*_Bangladesh(MK572265.1)	29.31	27.48	25.65	17.56	655.00	54.96	45.04
5	*H. nobilis*_India(KF742440.1)	29.14	27.45	25.77	17.64	652.00	54.91	45.09
6	*H. molitrix*_M2*(MH176327.1)	25.49	18.07	29.41	27.03	714.00	54.90	45.10
7	*H. molitrix*_M3*(MG988397.1)	30.53	26.93	26.24	16.30	583.00	56.78	43.22
8	*H. molitrix*_China(MF122395.1)	29.97	26.60	26.76	16.67	624.00	56.73	43.27
9	*H. molitrix*_Bangladesh(MK572264.1)	30.23	26.56	26.11	17.10	655.00	56.34	43.66
10	*H. molitrix*_India(FJ459500.1)	30.08	26.56	26.26	17.10	655.00	56.34	43.66
	Average	28.83	25.36	26.73	19.08	639.82	55.56	44.44

nucleotide sequences. In our study, the highest differences (6.111) were found between the sequenced *H. molitrix* M3 and sequenced *H. molitrix* M2, reference *H. molitrix* from China and Bangladesh. However, higher differences were observed between the sequence pair of sequenced *H. nobilis* N1 and sequenced *H. nobilis* N3 (5.023), sequenced *H. molitrix* M2 (6.104), sequenced *H. molitrix* M3 (0.042), reference *H. molitrix* from China (0.042), Bangladesh (0.042) and India (0.039), *H. nobilis* N3 and other sequenced and reference *H. molitrix* and *H. nobilis* samples, sequenced *H. molitrix* M2 and sequenced *H. nobilis* N1 (6.104), reference *H. nobilis* from China (6.104), Bangladesh (6.060) and India (6.060), Sequenced *H. molitrix* M2 and reference *H. molitrix* from India (6.007) (Table 3).

Transition/Transversion Bias

The transition/transversion biases of COI gene sequences substitution were estimated using Tamura-Nei model (Tamura 1993). The estimated transition/transversions bias (R) among the COI gene sequences of the *Hypophthalmichthys* genus from different countries was 1.11. The rate of transitional and transversional substitutions are presented in bold and italics respectively (Table 4). The nucleotide frequencies were A=26.79%, T/U=29.28%, C=25.90%, and G=18.03%. The rates of transitional substitution from A to G, T/U to C, C to T/U, and G to A were 8.81%, 14.24%, 16.10% and 13.10% respectively (Table 4). The rates of transversional substitution from A to T/U, A to C, T/U to A, T/U to G, C to A, C to G, G to T/U and G to C were 6.99%, 6.18%, 6.40%, 4.30%, 6.40%, 4.30%, 6.99%, and 6.18% respectively (Table 4). The rate of transitional substitution among the COI nucleotide sequences was found little bit higher (52.25) than the transversional substitution (47.74).

Homogeneity of Substitution Patterns of COI Sequences

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Kumar and Gadagkar 2001). A Monte Carlo test (500 replicates) was used to estimate the *p*-values and *p*-values smaller than 0.05 are considered significant (Table 5). It was found that *p*-values were smaller than 0.05 between sequenced *H. nobilis* N3 and sequenced *H. molitrix* M2 with all sequenced and reference *H. molitrix* and *H. nobilis*. Therefore, sequenced *H. nobilis* N1 and sequenced *H. molitrix* M3 showed significant results only with *H. nobilis* N3 and sequenced *H. molitrix* M2.

Genetic Distance

The pair-wise genetic distance values (Nucleotide sequence divergences) using the Tamura-Nei model showed that there was no genetic distance (0.00) between reference China vs sequenced *H. nobilis* N1, reference *H. nobilis* India vs reference *H. nobilis* Bangladesh COI gene sequences and reference *H. molitrix* Bangladesh vs sequenced *H. molitrix* M3, reference *H. molitrix* Bangladesh vs reference *H. molitrix* China and reference *H. molitrix* China vs sequenced *H. molitrix* M3 COI gene sequences (Table 6). The highest genetic distance (1.208) was found between sequenced

Table 3. Estimates of Net Base Composition Bias Disparity between Sequences

Sl. No.	Species Name	1	2	3	4	5	6	7	8	9	10
1	*H. nobilis*_N1*(MG988398.1)										
2	*H. nobilis*_N3*(MH176328.1)	5.023									
3	*H. nobilis*_China (MF122410.1)	0.000	5.023								
4	*H. nobilis*_Bangladesh (MK572265.1)	0.000	5.000	0.000							
5	*H. nobilis*_India (KF742440.1)	0.000	5.000	0.000	0.000						
6	*H. molitrix*_M2*(MH176327.1)	6.104	0.232	6.104	6.060	6.060					
7	*H. molitrix*_M3*(MG988397.1)	0.042	5.270	0.042	0.025	0.025	6.111				
8	*H. molitrix*_China (MF122395.1)	0.042	5.270	0.042	0.025	0.025	6.111	0.000			
9	*H. molitrix*_Bangladesh (MK572264.1)	0.042	5.270	0.042	0.025	0.025	6.111	0.000	0.000		
10	*H. molitrix*_India (FJ459500.1)	0.039	5.188	0.039	0.023	0.023	6.007	0.000	0.000	0.000	

Table 4. Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	*6.99%*	*6.18%*	**8.81%**
T/U	*6.40%*	-	**14.24%**	*4.30%*
C	*6.40%*	**16.10%**	-	*4.30%*
G	**13.10%**	*6.99%*	*6.18%*	-

*The rates of different transitional substitutions are presented in bold, and transversions substitutions are presented in italics.

H. nobilis N3 and reference *H. molitrix* from India while the lowest genetic distance (0.002) was observed among sequenced *H. nobilis* N1 vs reference *H. nobilis* from Bangladesh, India, and China; and sequenced *H. molitrix* M3 vs reference *H. molitrix* from Bangladesh, India, and China.

Similar genetic distance (1.199) found among sequenced *H. molitrix* M3, *H. molitrix* from China and Bangladesh, and sequenced *H. nobilis* N3. Similarly, sequenced *H. molitrix* M2, sequenced *H. molitrix* M2, reference *H. molitrix* from China, India, and Bangladesh showed similar (1.189) genetic distances, whereas reference *H. nobilis* from Bangladesh and India showed the similar genetic distances with sequenced *H. nobilis* N3 (1.153) and *H. molitrix* M2 (1.137).

Phylogenetic Analysis Using Maximum Likelihood Methods

The phylogenetic tree using COI gene sequences based on the maximum likelihood method showed that all the sequenced and reference *H. molitrix* and *H. nobilis* were grouped into three clades (clade 1, clade 2, and clade 3 (Figure 1). Interestingly, sequenced *H. nobilis* N1 and *H. nobilis* N3 clustered in two different clades (clade 1 and clade 2 respectively) whereas sequenced *H. nobilis* N3 formed a sister group with sequenced *H. molitrix* M2 with 100% sequence similarities (clade 3). On the other hand, sequenced *H. nobilis* N1 formed a single clade (clade 1) with reference *H. nobilis* from China, Bangladesh, and India. All the reference *H. molitrix* showed sequence similarities with sequenced *H. molitrix* M3, however, formed a sister group with reference H. molitrix from China (Clade 3) (Figure 2).

Discussion

DNA barcoding serves as a systematic tool for molecular identification of species using the mitochondrial COI gene sequence (Pugedo et al., 2016). It is indisputable from more than a decade of studies that the barcoding can discriminate fish species inhabiting different water bodies (Hebert, 2003). However, in our study, we used the 650 bp barcode COI gene to identify two exotic fish species *H. molitrix* and *H. nobilis* currently available in Bangladesh and determine the level of genetic variation among them. The sequence analysis identified a total of 757sites, where 397(52.44%) were conserved; 323 (42.67%) were variable; 274 (36.20%) were parsimony informative sites and 44 (5.81%) were singletone. These results were found to be similar with previous studies of different fishes. For instance, in a study where DNA barcoding was used to identify *Anabas testudineus* of Bangladesh sequence analysis provided a total of 691sites, where 160 (23.15%) were conserved; 530 (76.70%) were variable; 399 (57.74%) were parsimony informative and 127 (18.38%) were singleton (Parvez et al. 2020). In another study of *C. batrachus* and *C. gariepinus* a total

Table 5. Test of the Homogeneity of Substitution Patterns Between Sequences

Sl. No.	Species Name	1	2	3	4	5	6	7	8	9	10
1	*H. nobilis*_N1*(MG988398.1)		0.000*	1.000	1.000	1.000	0.000*	0.142	0.150	0.138	0.204
2	*H. nobilis*_N3*(MH176328.1)	0.000*		0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
3	*H. nobilis*_China(MF122410.1)	1.000	0.000*		1.000	1.000	0.000*	0.120	0.136	0.126	0.166
4	*H. nobilis*_Bangladesh(MK572265.1)	1.000	0.000*	1.000		1.000	0.000*	0.194	0.212	0.232	0.222
5	*H. nobilis*_India(KF742440.1)	1.000	0.000*	1.000	1.000		0.000*	0.206	0.236	0.214	0.230
6	*H. molitrix*_M2*(MH176327.1)	0.000*	0.000*	0.000*	0.000*	0.000*		0.000*	0.000*	0.000*	0.000*
7	*H. molitrix*_M3*(MG988397.1)	0.142	0.000*	0.120	0.194	0.206	0.000*		1.000	1.000	1.000
8	*H. molitrix*_China(MF122395.1)	0.150	0.000*	0.136	0.212	0.236	0.000*	1.000		1.000	1.000
9	*H. molitrix*_Bangladesh(MK572264.1)	0.138	0.000*	0.126	0.232	0.214	0.000*	1.000	1.000		1.000
10	*H. molitrix*_India(FJ459500.1)	0.204	0.000*	0.166	0.222	0.230	0.000*	1.000	1.000	1.000	

Table 6. Estimates of evolutionary divergence between sequences and/or pairwise genetic distance

Sl. No.	Species Name	1	2	3	4	5	6	7	8	9	10
1	*H. nobilis*_N1*(MG988398.1)										
2	*H. nobilis*_N3*(MH176328.1)	1.147									
3	*H. nobilis*_China(MF122410.1)	0.000	1.147								
4	*H. nobilis*_Bangladesh(MK572265.1)	0.002	1.153	0.002							
5	*H. nobilis*_India(KF742440.1)	0.002	1.153	0.002	0.000						
6	*H. molitrix*_M2*(MH176327.1)	1.130	0.032	1.130	1.137	1.137					
7	*H. molitrix*_M3*(MG988397.1)	0.051	1.199	0.051	0.049	0.049	1.189				
8	*H. molitrix*_China(MF122395.1)	0.051	1.199	0.051	0.049	0.049	1.189	0.000			
9	*H. molitrix*_Bangladesh(MK572264.1)	0.051	1.199	0.051	0.049	0.049	1.189	0.000	0.000		
10	*H. molitrix*_India(FJ459500.1)	0.053	1.208	0.053	0.051	0.051	1.198	0.002	0.002	0.002	

of 751 sites were identified: 76 (10.11%) of them were conserved, 670 (89.21%) were variable, 670 (89.21%) were informative for parsimony sites, 653 (86.95%) were singleton sites (Parvez et al.2022). These results also corroborate with other similar previous studies of different fishes (Falade et al 2016). The average nucleotide composition of the COI gene was for T/U=27.79, C=23.13, A=27.21, and G=21.86 which was similar of Parvez et al. 2020 that described nucleotide frequencies were 22.84% for A, 31.03% for T, 28.57% for C, and 17.56% for G. The GC content was lower (45%) than the AT content (55%) in 4 sequenced samples of this study which was similar with the study of Falade et al 2016, Parvez et al.2020, Parvez et al.2022. The AT content was higher than GC content of these exotic species. Since AT content positively relates to the rate of evolution, a high level of mutation rate has been expected based on the results in this study (Parvez et al.2020). The base composition bias was estimated through the disparity index between the studied COI sequences of *H. molitrix and H. nobilis*. Larger differences were observed in base composition biases than the expected bases among the sequences. In our study, the highest differences (6.111) were found between the sequenced *H. molitrix* M3 and sequenced *H. molitrix* M2, reference *H. molitrix* from China and Bangladesh. The estimated transition/transversions bias (R) among the COI gene sequences of the present study was 1.11. The rate of transitional substitution among the COI nucleotide sequences was found little bit higher (52.25) than the transversional substitution (47.74).

The pair-wise genetic distance between sequenced *H. nobilis* N1 vs reference *H. nobilis*, sequenced *H. molitrix* M3 vs reference *H. molitrix* Bangladesh and China was 0.00 %. Similarly genetic distance was 0.00% between sequenced vs reference Thai *Anabas testudineus*, and sequenced vs reference Vietnamese *A. testudineus* (Parvez et al., 2020). Chandra et al. (2012) found the inter-specific genetic distance of *Schizothorax richardsonii* was 0.00% and *S. progastus* was 0.00%. The genetic distance between sequenced *H. molitrix* M2 vs *H. molitrix* M3 was 1.189; sequenced *H. nobilis* N1 vs sequenced *H. nobilis* N3 was 1.147; sequenced *H. nobilis* N1 vs *H. molitrix* M2 was 1.13; sequenced *H. nobilis* N1 vs *H. molitrix* M3 was 0.051; sequenced *H. nobilis* N3 vs *H. molitrix* M2 was 0.032; sequenced *H. nobilis* N3 vs *H. molitrix* M3 was 1.199. The results of genetic distance values are almost aligned with the phylogenetic group formation and predict that *H. molitrix* and *H. nobilis* are two separate species. The substitution pattern of sequences was tested to test whether the COI gene sequences of *H. molitrix* and *H. nobilis* are evolved in the same pattern or not. We found that the sequences of the COI gene in *H. molitrix* and *H. nobilis* were evolved with the same pattern of substitution with significant *p*-value.

In phylogenetic tree analysis, sister group formation was found between sequenced *H. molitrix* (M2 and M3) and sequenced *H. nobilis* N3 and reference *H. molitrix* from China, Bangladesh and India. Since, they are from the same family (Cyprinidae), therefore, it is obvious that they both (*H. molitrix* and *H. nobilis*)

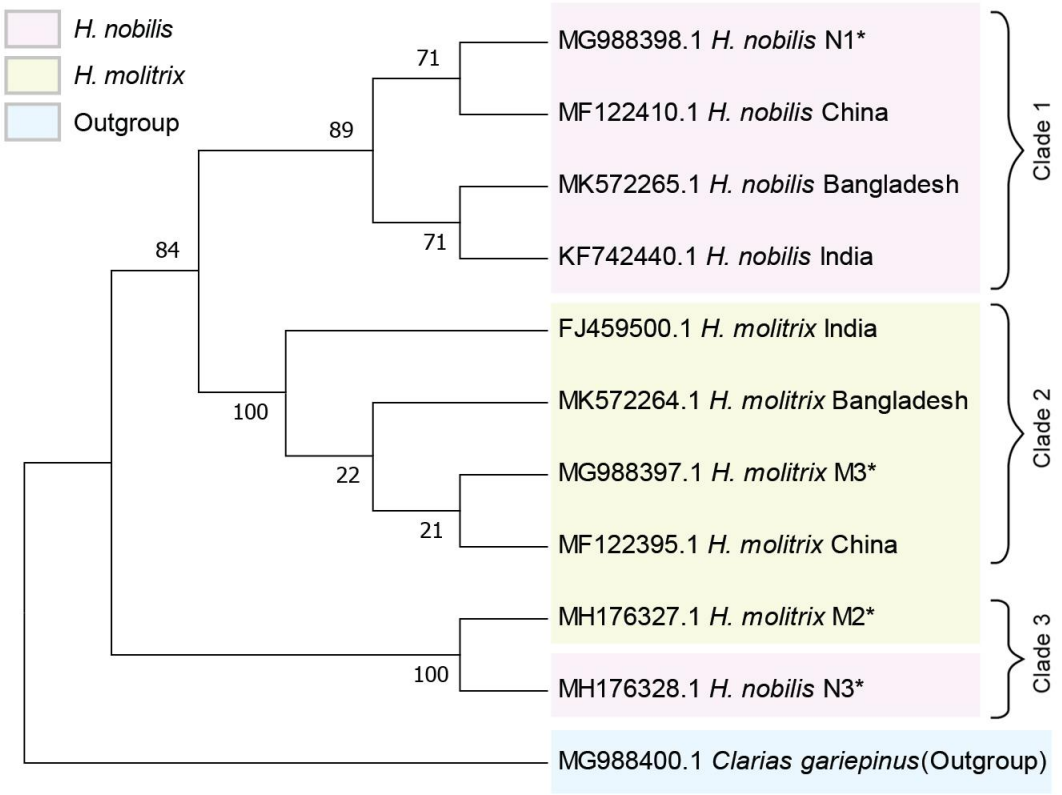

Figure 2. Phylogenetic relationship of exotic and native *H. molitrix* and *H. nobilis* using Maximum Likelihood Method.

formed a sister group. The results of group formation were aligned with the pairwise genetic distance. In several study, it was found that closely related species form a sister group with their counterparts. For example, Boidya et al. (2015) found cluster groups between *Plicofollis tenuispinis* and *P. polystaphylodon*; *Arius venosus* and *A. venosus*; *Netuma thalasinus* and *N. thalasinus*. Parvez et al. (2020) also showed that native (Bangladesh) and Indian *Anabas testudineus* formed a sister group. However, we cannot overlook the feasibility of hybridization between species of same family which may be a future study with a large sample size. In our study, we used only two samples from both species. Except for *H. nobilis* N3 all other sequenced and reference *H. nobilis* formed an independent group of *H. molitrix*. Hence, it indicates that though both *H. molitrix* and *H. nobilis* are from the same family but they are distinctly separate from each other.

Conclusion

We have demonstrated that the exotic fish *H. molitrix* and *H. nobilis* maintain distinct species status with lower genetic variation from their native counterpart. This knowledge of intraspecific genetic diversity and evolution of fish species is a critical component for their proper management and culture practice. Therefore, the present study suggested that proper measures should be taken for the re-introduction of these fish from China and India to maintain inbreeding-free broodstocks.

Ethical Statement

Since this study did not disturb environmental populations and did not use recombinant DNA technology to genetically engineer the organisms, ethical approval was not required.

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Author Contribution

Conceptualization: IP, RAA; Data Curation: RP, IP; Formal Analysis: RAA; Funding Acquisition: IP; Investigation: MNK, IH; Methodology: RAA, RP, IP; Project Administration: IP; Resources: MNK, MRA; Supervision: IP, IH; Visualization: MAA, NG; Writing-orginal draft: RP, NG; Writing-review and editing: SA, MRA.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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References

Alam, M.A., Parvez, I., Ara, Y., Khan, M.N., Nehrin, S., Mahajebin, T., & Hassan, M.M. (2021). Phylogenetic relations of the cyprinid fishes (Cyprinidae) in Bangladesh inferred from morphological traits and cytochrome b gene sequences. AACL Bioflux, 14(3), 1631-1644.

Bhakta, J.N., & Bandyopadhyay, P.K. (2007). Exotic fish biodiversity in Churni River of West Bengal, India. Electronic Journal of Biology, 3(1), 13-17.

Boidya, P., Haque, W., & Rahman, M.M. (2015). Molecular identification and phylogenetic assessment of some marine catfishes of the Bay of Bengal. International Journal of Pure Applied Zoology, 3(4), 279-86.

Boonkusol, D., & Tongbai, W. (2016). Studied genetic variation of striped snakehead fish (*Channa striata*) in river basin of central Thailand inferred from mtDNA COI gene sequences analysis. Journal of Biological Sciences, 16(1-2), 37-43.

Chandra, S., Barat, A., Singh, M., Singh, B.K., & Matura, R. (2012). DNA bar-coding of Indian coldwater fishes of genus *Schizothorax* (family: Cyprinidae) from Western Himalaya. World Journal of Fish and Marine Sciences, 4(4), 430-435.

DoF. Yearbook of Fisheries Statistics of Bangladesh, 2015-2016: Department of Fisheries, Bangladesh: Ministry of Fisheries and Livestock.

Falade, M.O., Opene, A.J., & Benson, O. (2016). DNA Barcoding of *Clarias gariepinus*, *Coptodon zillii* and *Sarotherodon melanotheron* from Southwestern Nigeria. F1000Research, 5, 1268.

Farrington, H., Edwards, C., Guan, X., Carr, M., Baerwaldt, K., & Lance, R. (2015). Mitochondrial genome sequencing and development of genetic markers for the detection of DNA of invasive bighead and silver Carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in environmental water samples from the United States. PLOS ONE, 10(2), e0117803.

Galib, S.M., & Mohsin, A.B.M. (2011). Cultured and ornamental exotic fishes of Bangladesh past and present. LAP–Lambert Academic Publishing, Germany, 65–66.

Hebert, P.D., Ratnasingham, S., & Waard, J.R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. In*:* Proceedings of the Royal Society of London, Series B: Biological Sciences, 270 (suppl_1), 96-99.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H., & Herbert, P.D.N. (2007). Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes, 7(4), 544-548.

Jiaguang, S., Wang, L., Song, R., & Yanga, S. (2016). Enhancing pyridinic nitrogen level in graphene to promote electrocatalytic activity for oxygen reduction reaction. Nanotechnology*,* 27(5), 055404.

Kumar, S., & Gadagkar, S.R. (2001). Disparity Index: A simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. Genetics, 158, 1321-1327.

Nei, M., Kumar, S. (2000). Molecular evolution and phylogenetics. Oxford University Press: New York.

Parvez, I., Mahajebin, T., Clarke, M., Chhanda, M.S., & Sultana, S. (2020). Genetic variation of native and introduced climbing perch, *Anabas testudineus* (Bloch, 1792) derived from mitochondrial DNA analyse. Ecological Genetics and Genomics, 17, 100067.

Parvez, I., Rumi, R.A., Ray, P.R., Hassan, M.M., Sultana, S., Pervin, R., Suwanno, S., & Pradit, S. (2022). Invasion of African *Clarias gariepinus* drives genetic erosion of the indigenous *C. batrachus* in Bangladesh. Biology, 11, 252.

Pereira, L.H., Hanner, R., Foresti, F., & Oliveira, C. (2013). Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna. BMC Genetics, 14, 1-14.

Persis, M., Reddy, A.C.S., Rao, L.M., Khedker, G.D., & Nasruddin, K. (2009). COI (Cytochrome Oxidase-I) sequence based studies of Carangid fishes from Kakinanda coast, India. Molecular Biology Reports, 36, 1733-1740.

Pugedo, M.L., Neto, D.A, F.R., Pessali, T.C., Birindelli, J.L., & Carvalho, D.C. (2016). Integrative taxonomy supports new candidate fish species in a poorly studied neotropical region: the Jequitinhonha river basin. Genetica, 144(3), 341–349.

Rahman, A.K.A. (2005) Freshwater Fishes of Bangladesh, 2nd edition, Zoological Society of Bangladesh, Department of Zoology, University of Dhaka, Dhaka–1000. 26.

Rahman, M.M., Norén, M., Mollah, A.R., & Kullander, S.O. (2019). Building a DNA barcode library for the freshwater fishes of Bangladesh. Scientifc Reports, 9(1), 9382.

Rasmussen, R.S., Morrissey, M.T., & Hebert, P.D.N. (2009). DNA barcoding of commercially important salmon and trout species (*Oncorhynchus* and *Salmo*) from North America. Journal of Agricultural and Food Chemistry, 57, 8379-8385.

Sambrook, J., Russell, D.W. (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Shen, Y., Guan, L., Wang, D., & Gan, X. (2016). DNA barcoding and evaluation of genetic diversity in Cyprinidae fish in the midstream of the Yangtze River. Ecology and Evolution, 6, 2702–2713.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10, 512-526.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *In:* Proceedings of the National Academy of Sciences, USA, 101, 11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725-2729.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. Molecular biology and evolution, 38, 3022-3027.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., & Hebert, P.D.N. (2005). DNA barcoding of Australia's fish species. Philosopical Transactions of the Royal Society B: Biological Sciences, 360, 1847–1857.

Wu, X., Golden, K., & Bodmer, R. (1995). Heart development in Drosophila requires the segment polarity gene wingless. Developmental Biology, 169, 619-628.