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Research Article

Molecular Differentiation between Native and Vietnam Originated Striped Snakeheads (*Channa striata*) in Bangladesh Using Mitochondrial Cytochrome b Gene

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ABSTRACT

The freshwater striped snakehead, *Channa striata*, is widely distributed in several Asian countries such as China, Vietnam, Indonesia, Malaysia, India, Myanmar, and Bangladesh. The present study investigated various *C. striata* populations sequentially by the mitochondrial cytochrome *b* gene. Native populations (136) were collected from nine geographically different locations and the Vietnamese populations (50) from two other fish farms in Bangladesh. The partial sequence of Cyt *b* (836bp) was analyzed, and the results identified five haplotypes from nine natives and a single haplotype from two Vietnamese populations. This demonstrated that Vietnam-originated populations in Bangladesh had the same origin. In contrast, the pairwise highest Fst value observed between Native and Vietnamese populations shows a substantial genetic variation between them. The phylogenetic tree separated both the native and exotic populations, whereas five native haplotypes formed a cluster with the Indian *C. striata*. In contrast, the Vietnam haplotype formed a clade with the East Asian *C. striata* suggesting possible cryptic genetic diversity. Finally, the mitochondrial cytochrome b gene can also be used to identify native and Vietnam-originated *C. striata* strain available in Bangladesh, beneficial to broodstock development and conservation issues. Besides, a detailed morphological study of Vietnam-originated *C. striata* needs to be required to identify the exact morphotype available in Bangladesh.

Introduction

The striped snakehead (*Channa striata*) is a commercially important freshwater fish that belongs to the family Channidae of the order Anabantiformes. The members of Channidae are classified into two extant genera, the African *Parachanna* and Asian *Channa* (Nelson, 1994). The African genus *Parachannna* is represented by three extant species restricted to western Africa and *Channa* in Asia (Wee, 1982). The genus *Channa* has at least 40 species mainly occurring in southern Asia (Froese and Pauly, 2022). Among them, *C. striata* has the highest level of within-species divergence (Adamson et al., 2010; Nguyen et al., 2016). In Bangladesh, there are four major species of snakeheads, viz. *C. striata*, *C. marulius*, *C. gachua,* and *C. punctata*; these are also distributed in other Asian countries like India, Pakistan, Thailand, Vietnam, Malaysia, Laos, and China. *C. striata,* locally known as "shol", is the most popular and commercially important snakehead species because of its exquisite taste, high market price, and medicinal properties enriched with high antioxidants and essential albumin (Roy et al., 2020, Hidayati et al., 2017; Mustafa et al., 2012).

Although in Bangladesh, only the capture fisheries meet the demand for *C. striata* yet, the farmers made several approaches for aquaculture of this species (Roy et al., 2020), which is, instead, highly regarded in some Asian

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countries like Thailand, Vietnam, and Cambodia due to its market demand, rapid growth, easy adaptation ability, and high tolerance against adverse environmental conditions. Commercial farming of *C. striata* is presumed to be potential in Bangladesh due to having similar geographical features. Therefore, necessary steps are required for the management of the sustainable culture of *C. striata.* However, it is essential to select good broodstock with high genetic diversity (Dunham, 2011). Hence, information on population genetic structure is required to select quality broodfish from natural sources. Therefore, the study of the natural genetic variability of *C. striata* is necessary for stock-based management and conservation and genetic improvement programs. Vietnam originated *C. striata* was introduced to Bangladesh in 2014 via Innovative Agro Aid Pvt. Ltd. for small-scale breeding and aquaculture, and the phenotypic status of that strain is still unclear (Nguyen et al., 2016). The popularity of this strain as an aquaculture species grows rapidly, and commercial farming is also gradually increasing throughout the country. It has a big chance to escape from the culture to the wild, which may affect the ecosystem. Therefore, maintaining the morphological and molecular records of both wild and Vietnam-originated strains is essential for distinguishing and comparing them with each other.

The mitochondrial cytochrome b (Cyt b) gene is used as a species identifying marker (Parson et al., 2000). It was also employed in detecting genetic distances in vertebrates such as sheep, amphibians, and fish (Johns et al., 1998; Xin et al*.,* 2006; Alam et al., 2008; Alves et al., 1997; Farias et al*.,* 2001; Dowling et al*.,* 2002;). However, among mitochondrial DNA genes, cytochrome b (Cyt b) is the most frequently used for analyzing population genetic structure in fishes across taxonomic orders such as Squaliformes (Murray et al., 2008), Clupeiformes (Lecomte et al., 2004), Scorpaeniformes (Myoung et al., 2016), and Perciformes (Hsu et al., 2007; Habib et al., 2011; Wang et al., 2013; Ju et al., 2021). Some authors used the Cyt b gene for molecular research of *C. striata* (Adamson et al., 2010; Wang and Yang, 2011). In Bangladesh, partial sequence analysis of mitochondrial Cyt b gene to identify genetic diversity of *Labeo calbasu* (Begum et al*.,* 2019) and *Pangasius* spp. (Ha et al*.,* 2020) was also reported. However, sequence analysis of mtDNA Cyt *b* gene is an effective and convenient method used to analyze the molecular phylogenetic relationship, genetic distance analysis in various species, including snakeheads in different regions of the world (Adamson et al.; 2010; Aquilino et al.; 2011). Therefore, in the present investigation, we used the mtDNA Cyt b gene to differentiate the native and exotic strains of *C. striata* in Bangladesh by sequence analysis. In Bangladesh, few reports on genetic characterization of *Channa* species using the mtDNA cytochrome c oxidase 1 (COI) gene (Ahmed et al., 2018) and genetic variation assessment of Bangladeshi and Vietnamese C. *striata* using PCR-RFLP analysis of the CO1 gene were also reported (Alam et al., 2021).

However, mitochondrial cytochrome b gene could also be a powerful tool to differentiate the native and Vietnam-originated *C. striata*. From this perspective and to obtain fundamental knowledge for broodstock development and conservation, we collected *C. striata* from across Bangladesh, including introduced populations, and performed genetic analysis.

Materials and Methods

Sample collection

Native and Vietnam-originated striped snakeheads (*C. striata)* were collected from natural habitats and hatcheries. Native 136 samples were collected from natural sources of 9 districts of Bangladesh viz. Rangamati, Patuakhlai, Dinajpur, Natore, Mymensingh, Netrokona, Kishoreganj, Sylhet and Gazipur, which covered almost all the divisions of Bangladesh and the Vietnam-originated 50 samples were collected from two local hatcheries- Reliance Aqua Farms and Biswas agro Fisheries, located in Trishal, Mymensingh (Table 1, Fig. 1).

DNA extraction and amplification

DNA was extracted using Gene JET genomic DNA purification kit (Thermo Scientific) following the manufacturer's protocol. We used fin tissues for DNA extraction. PCR amplification of mtDNA Cyt *b* gene was performed using universal primers, forward Cyt *b* GLUDG-L (5’-TGA CTT GAA RAA CCA YCG TTG -3'), and reverse CB3H (5’-GGC AAA GAG AAA RTA TCA TTC -3') primer (Palumbi et al., 2002) and the PCR amplificon size was about 800bp. , The final volume of the PCR mixture was 50 µL, and the mixture contains 5µL template DNA, 2 µL forward and 2 µL reverse primer, 25 µL PCR master mixture (Thermo Scientific), and 16 µL double distilled water. PCR was performed in a Master cycler by 40 cycles. Each cycle consisted of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute 10 seconds, and extension at 72°C for 1 minute 10 sec. The cycle was started with 1 cycle at 94°C for 2 minutes and ended with 1 cycle of 72°C for 7 minutes. Finally, the PCR product was held at 4°C in the thermal cycler.

Table 1. List of the samples of *C. striata* used in the present study

Population	Source	Location	Sample code	Sample number
Native	Kaptai Lake, Rangamati	22°35'34"N 92°12'49"E	R	24
	Jamla beel, Patuakhali	22°26'35"N 90°23'12"E	P	20
	Halti beel, Natore	24°30'49"N 88°59'30"E	N	20
	Ashura beel, Dinajpur	25°26'3"N 89°4'2"E	D	22
	Tanguar haor, Sylhet	25°09'01"N 91°03'37"E	S	15
	Chamra Bondor, Kishoreganj	24°28'49"N 90°57'27"E	K	10
	Kangsa River, Mohonganj, Netrokona	25°0'50"N 90°32'40"E	Ne	10
	Bayhe beel, Mymensingh	24°42'21"N 90°19'37"E	M	5
	Balu River, Gazipur	23°58'27"N 90°27'49"E	G	10
Vietnam-originated aquaculture	Biswas Agro Farms, Trishal, Mymensingh	24°39'0"N 90°21'13"E	BV	25
	Reliance Aqua Farms, Trishal, Mymensingh	24°39'2"N 90°24'7"E	RV	25
	Total sample			186

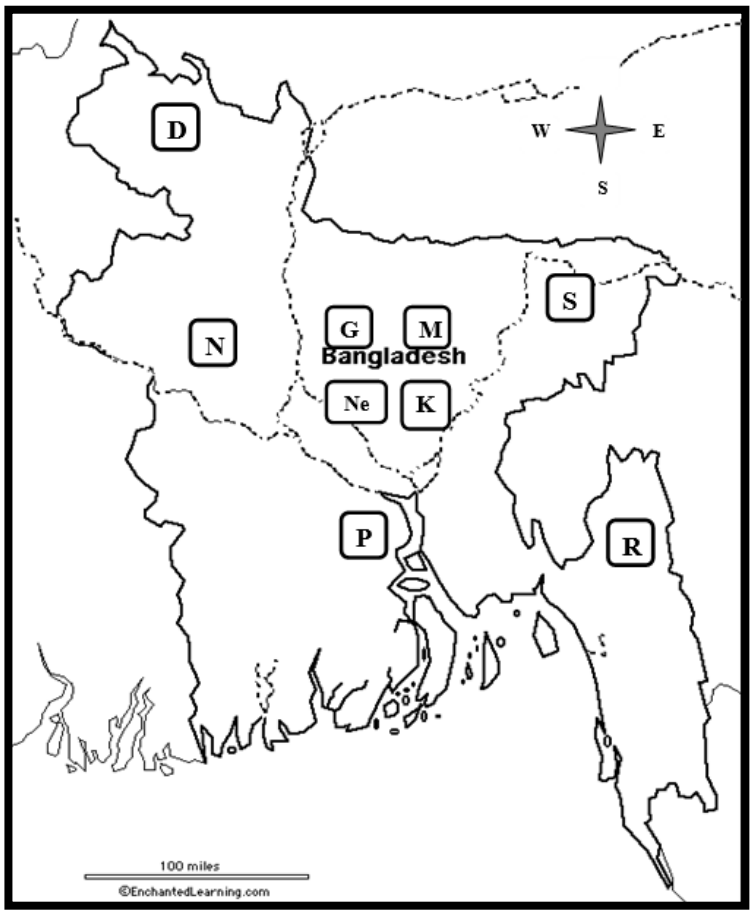

Figure 1. The map shows the sampling locations used in the present study. D: Dinajpur, N: Natore, S: Sylhet, P: Patuakhali, R: Rangamati, M: Mymensingh, G: Gazipur, Ne: Netrokona, K: Kishoreganj

To determine the size of amplified DNA, the PCR products were subjected to electrophoresis (Mupid-2 plus, Advanced) on 0.7% agarose gel at 100 volts for 30 minutes and compared the migration distance with DNA fragments of known sizes ladder (1 Kb Gene ruler). The gel was stained with ethidium bromide for about 40 minutes and washed in water before being visualized on a UV transilluminator (Major science-UVDI).

PCR product purification and Sequencing

PCR products were purified by using the Molecular Biology Purification Kit (Thermo Scientific) according to the manufacturer's protocol. The purified PCR product of each sample was stored and used for sequencing analysis. Sequencing reactions were carried out using the Big Dye Terminator Sequencing Kit (v3.1; Applied Biosystems, USA). Cycle sequencing was carried out for 30 cycles with the following temperature profile: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min 30 s, preceded by 3 min at 94 °C and followed by 8 min at 72 °C after cycling completion, and the sample cooled to 4 °C. Sequencing analysis was conducted on a capillary electrophoresis DNA analyzer (ABI Prism 3130xl Genetic Analyzer; Applied Biosystems, USA). In the procedure, bidirectional sequencing was carried out, and the sequencing data was curated and converted into FASTA format, and the size of the final useable sequences was 648 bp.

Molecular analysis

The mtDNA Cyt *b* gene sequences were aligned and edited using CLUSTAL W (version 1.6) as implemented in MEGA (version 6), followed by manual adjustments. Sequence ambiguities were resolved by checking chromatograph using Chromas (2.1.1) and searching for sequence similarity to other sequences available in the NCBI database (http://www.ncbi.nih.gov), using the BLAST search to confirm the identity of the sequence. The sequence determined in this study were deposited in the online GenBank databases under Accession No. LC513895 to LC513900. The haplotype distances and UPGMA dendrogram were determined to show the population's relationship using sequences by MEGA (Version 6). Haplotype networking was done by popart1.7. Nucleotide diversity, fixation index, and other genetic distance index were calculated by Dnasp5. To infer the historical demographic change, the sum of squared deviations (SSD) and raggedness index for mismatch distribution and Fu's F_s and Tajima's *D* were calculated by ARLECORE version 3.5.2.2 (Excoffier and Lischer, 2010). Phylogenetic analyses were performed by the neighbor-joining (NJ) method and the maximum likelihood (ML) method. In addition to the Cyt *b* sequences obtained in this study, we downloaded the sequence data for *C. striata*, *C. micropeltes* (GenBank accession number: MN057103), *C. punctata* (NC042213), and *C. asiatica* (KJ930190) from GenBank. The NJ NJ and ML analyses were performed by MEGA version 10.1.7 (Kumar et al., 2018) with 1,000 bootstrap replicates. For ML analysis, HKY+I model was used as it was selected as the best fit model.

Results

Any changes of base pair in any site of sequences were considered as a haplotype. Thus, six haplotypes were identified, and they were named as H1, H2, H3, H4, H5, and H6, respectively, where H6 was found to be specific for the Vietnamese population, other five were of Native (Table 2). Among the six haplotypes, one haplotype (H1) was typical for eight native populations except Kishoreganj. Two haplotypes (H1 and H4) were shared by the Patuakhali, Netrokona, and Gazipur populations, and additionally, the Netrokona population has one more H5 haplotypes. Besides, haplotype H2 was specific for Sylhet, and H3 was specific for Dinajpur populations. The highest haplotype diversity was found in the Netrokona population (0.80), and moderate haplotype diversity was found in Patuakhali, Dinajpur, Kishoreganj, Gazipur, and Sylhet populations. The Rangamati, Natore, and Mymensingh populations had no haplotype diversity (Table 3). The overall haplotype diversity within all native populations was Hd: 0.3391, whereas haplotype diversity among all native and Vietnamese populations was Hd: 0.6049. The nucleotide diversity among Rangamati, Natore, Mymensingh and Vietnamese populations was 0.00. The nucleotide diversity of other populations was like Patuakhali-0.00248, Dinajpur-0.00055, Sylhet-0.250, Kishoreganj-0.00186, Netrokona-0.00341 and Gazipur-0.00186. The moderate to highest nucleotide diversity was found in Sylhet and Netrokona populations (Table 3). The overall nucleotide diversity, number of segregating sites, number of parsimony sites was 0.0216021, 40, and 3.

Table 2. List of haplotypes and a corresponding individual from eleven populations of *C. striata*

Haplotype (H)	Location	Accession number
H1	Rangamati, Patuakhali, Natore, Dinajpur, Sylhet, Netrokona, Mymensingh, Gazipur	LC513895
H2	Sylhet	LC513896
H3	Dinajpur	LC513897
H4	Kishoreganj, Netrokona, Patuakhali, Gazipur	LC513898
H5	Netrokona	LC513899
H6	Vietnam	LC513900

Table 3. Haplotype distribution

Haplotype	Native									Vietnamese
	Rangamati	Patuakhali	Natore	Dinajpur	Sylhet	Mymensingh	Kishoreganj	Netrokona	Gazipur	
H1	1	1	1	1	1	1	1	1	1	
H2					1					
H3				1						
H4		1					1	1	1	
H5								1		
H6										1
Haplotype diversity	0.00	0.5333	0.00	0.35556	0.25	0.00	0.40	0.80	0.40	0.00
Nucleotide diversity	0.00	0.00248	0.00	0.00055	0.00154	0.00	0.00186	0.00341	0.00186	0.00

The present study revealed the inter-population genetic distance in 11 populations of *C. striata* based on the Nei (1983) and Jost (2008) genetic distance. In the present study, summarized genetic differentiation patterns between and within populations pairwise Fst, Nst, Gst, and Da values are presented in Table 4. The pairwise highest Fst values were observed between native and Vietnamese populations ranging from 0.97090 to 1.0 (average 0.9903). In contrast, the Fst value among the native population pairs was 0.0 to 0.75, with an average of 0.2378. The Fst values were observed 0.00 in the following population pairs - Rangamati vs. Natore, Rangamati vs. Sylhet, Natore vs. Mymensingh, Natore vs. Gazipur, Sylhet vs. Mymensingh, Sylhet vs. Gazipur, Mymensingh vs. Gazipur, and Biswas Viet vs. Reliance Viet. Similarly, the higher Da values were found to be 0.5681 to 0.05728 (average 0.0571) between the native and Vietnamese population pairs than only the native pairs ranging from 0.00279 to 0.00 (average 0.0007) (Table 4).

Table 4. Pairwise average Fst, Nst, Gst, and Da values of 11 populations of *C. striata*

Location	Fst Average (Min-Max)	Nst Average (Min-Max)	Gst Average (Min-Max)	Da Average (Min-Max)
Native vs. native	0.2378 (0.0 - 0.75)	0.2256 (0.0 - 0.75)	0.3159 (0.6 - 1.0)	0.0007 (0.00279 - 0.0)
Native vs. Vietnamese	0.9903 (0.97090 – 1.0)	0.9889 (0.97-1.0)	0.8367 (1-0.56)	0.0571 (0.5681 - 0.05728)
Vietnamese vs. Vietnamese	0 - 0	0 - 0	1 - 1	0 – 0

The minimum spanning network between haplotypes (Fig. 2) showed the evolutionary relationship among haplotypes produced by the mtDNA Cyt *b* gene analysis. As in Fig. 2, two groups were formed, whereas haplotype H1, H2, H3, H4, and H5 produced one group, and haplotype H6 produced another group. It also showed the considerable mutation changes between the native (H1 to H5) and the Vietnamese haplotypes (H6). The native haplotype (H1 to H5) is less mutated among the native haplotypes (1-2 mutation) than the Vietnamese populations (mutation 36).

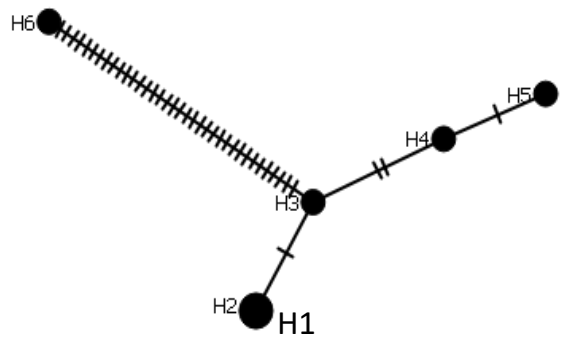

Figure 2. Minimum spanning network between haplotypes

Tajima's *D* calculated based on native populations in Bangladesh was 0.957 which was not significantly deviated from zero (p value=0.7940) implying stable population size whereas Fu's F_S was -2.862 which was significantly negative (p value=0.0140) indicating population expansion. Both SSD and RI were not significant in demographic expansion (SSD (p value) = 0.042 (0.550), RI (p value) = 0.170 (0.700)), spatial expansion (0.044 (0.450), 0.170 (0.500)), and sudden expansion models (0.042 (0.550), 0.170 (0.700)). Non-significant SSD and RI indicate that historical expansion was not statistically rejected.

Phylogenetic relationships between *C. striata* from different localities were not clear based on our dataset (Fig. 3). However, haplotypes observed from Bangladesh-native populations (from H1 to H5) were closely located with Indian haplotypes whereas the haplotype H6 observed from aquaculture individuals in Bangladesh were close to those from Vietnam and Mekong.

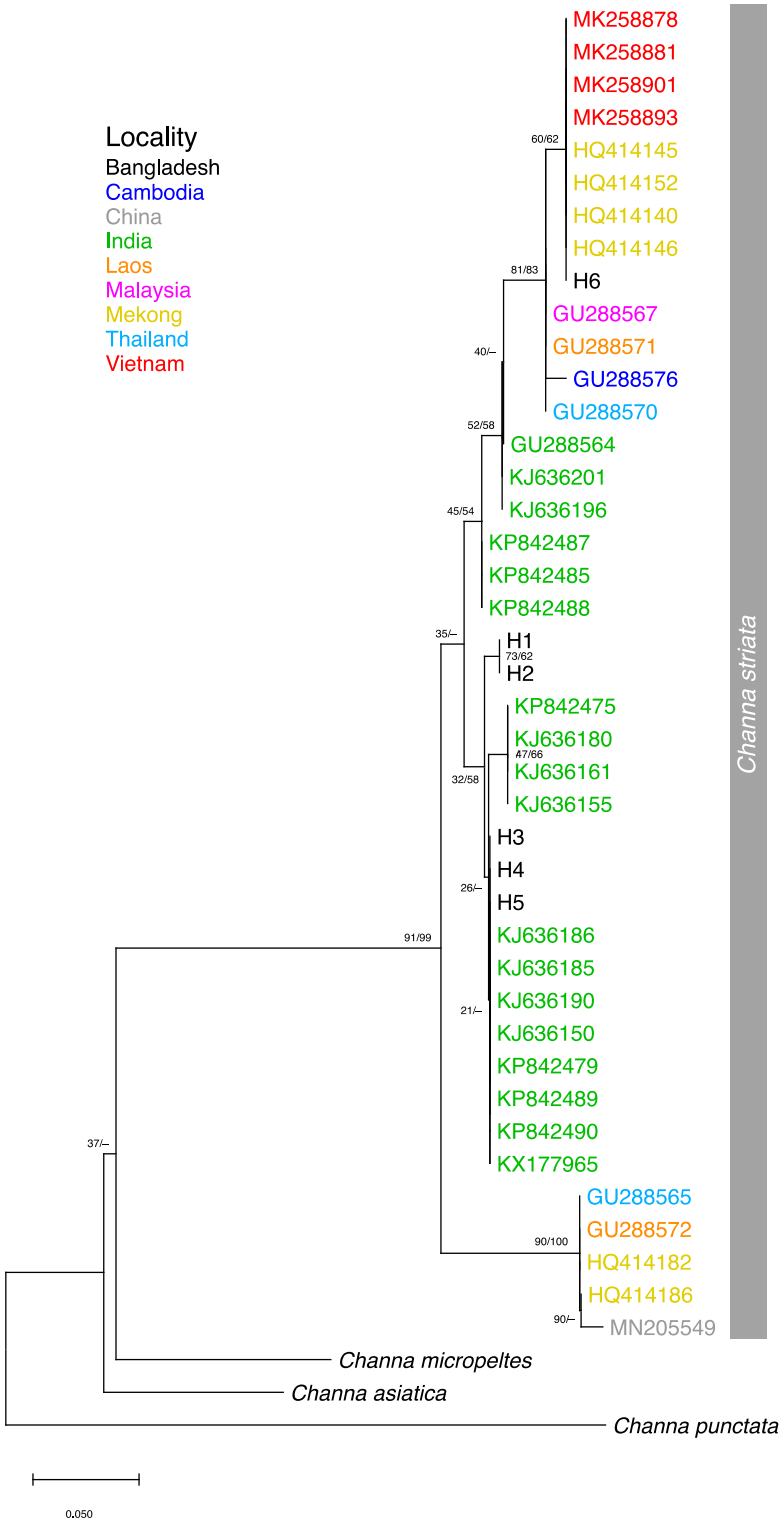

Figure 3. Neighbour joining tree showing phylogenetic relationship within *C. striata*. Values on each node represent bootstrap values of neighbour joining and maximum likelihood methods. Dashes indicate that the node was not supported by the method

Discussion

In the present study, the haplotype diversity between native and Vietnamese populations was found to be 0.6049, whereas it was 0.80 for the Netrokona native populations. However, Rahim et al. (2012) reported haplotype diversity as 0.8 in Malaysia using Cyt b for the same species by PCR-RFLP analysis. Very recently, Alam et al. (2021) also found haplotype diversity as 0.888

between Bangladesh and Vietnam populations of *C. striata* using PCR-RFLP methods of a CO1 gene fragment. Big, stable population sizes, environmental variability, and life-history traits that enable rapid population expansion are all recommended by high haplotype diversity (Liu et al., 2008). Therefore, the Netrokona population has a significant population size and is possibly connected to other populations as all populations shared H1 haplotype. The haplotype H4 shared by the Kishoreganj, Netrokona, Patuakhali and Gazipur populations indicating these populations have a particular origin and maintain comparative better diversity than others. The private haplotypes found in Dinajpur (H3), Sylhet (H2), and Netrokona (H5) populations might be due to the distinctive natural habitat of these populations. However, the highest haplotype found in Netrokona was also reported by Alam et al. (2021).

According to Wright (1978), the Fst value ranging from 0 - 0.05, 0.05 - 0.15, 0.15-0.25, and >0.25 indicate little, moderate, considered, and more prominent level of genetic differentiation, respectively. Fst values based on the Cyt *b* gene are generally higher than those found in the D-loop region because Cyt *b* sequences are more conservative (Broughton et al., 2001). The lower the Nst, Fst, and the values of separate native population pairs and the maximum Gst values of other population pairs, the fewer separated they were. The lowest Da values of various population pairs indicated that they were less segregated, that individuals might openly interbreed, and more migration. On the other hand, higher values indicated lower gene flow, lower allelic frequency, and lower interbreeding (e.g., Patuakhali vs. Sylhet, Sylhet vs. Dinajpur, Natore vs. Sylhet, Patuakhali vs. Dinajpur, and Rangamati vs. Sylhet populations). The highest Nst, Fst, Gst, and Da levels were observed between the native and the Vietnamese populations, indicating distinct differentiation between the populations and no gene flow (Table 4). Though the Fst value ranged from low to high (0.0 to 0.75) within the native populations, the average value was 0.2378, indicating gene flow is going on. Some populations still maintaining isolation, and interbreeding may occur between native populations.

The sequence review showed that the native groups had minimal to moderate genetic variations (Table 4). While being collected from two different sites, both Vietnamese populations displayed a lower degree of genetic diversity. They shared the same haplotype (H6) with the lowest pairwise Fst, Nst, and Da values (0.0) denoted interbreeding populations freely between themselves and the distinct population. However, low genetic diversity within the Vietnamese populations may result in inbreeding depression soon, which can be improved by increasing the breeding population from the source periodically for culture with proper care. We recommend the development of native broodstock for seed production and other cultural purposes. Comparative genetic study of Vietnamese and native strains revealed significant genetic diversity, resulting in distinct genetic properties. These results show that, since the introduction of the Vietnamese strain in Bangladesh, no interbreeding has existed, and the genetic profiles of both groups are identical. When there are few genetic variations, a limited number of migrants would not significantly impact allele frequencies. Vietnamese populations are genetically distinct from native populations, and even slight immigration could cause significant shifts in allele frequencies in the future.

The phylogenetic analysis showed that the Bangladeshi native populations produced a cluster with only Indian *C. striata*. On the other hand, the Vietnam-originated populations clustered with partial Indian, Vietnam, Thailand, Laos, and Malaysian samples of *C. striata*. (Fig. 3). This result indicates that Bangladeshi native *C. striata* are closely related to Indian populations and maintain genetic differentiation. This distinct clade of *C. striata* may be due to the possible cryptic diversity of the snakehead species (Adamson et al., 2010) belonging to the same species within their distribution from South to East Asian countries. The population of the originated Vietnamese population in Bangladesh belongs to the East Asian group. The result suggests that the indigenous *C. striata* populations maintain integrity, producing in the same clade. Tan et al. (2012) reported three distinct lineages of *C. striata* in Malaysia using the mtDNA ND5 gene. Phylogenetic reconstruction was also suggested by Wang and Yang (2011) for *C. striata*. Cryptic diversity of the genus *Channa*, especially *C. gachua*, *C. marulius*, *C. punctata,* and *C. striata,* is also suggested by Serrao et al. (2014) after studying 36 putative species of the genus *Channa*. Nguyen et al. (2016) discussed phylogenetic trees using CO1. They noticed that the triangle head morphotype is closer to Malaysian and Indonesian *C. striata,* and the wild and square head morphotype is closer to the Indian sample. In the present study, the wild Bangladeshi type is closer to the Indian sample than the East Asian sample, and it can be said that Bangladeshi native *C. striata* is closer to the square and/or wild Vietnam morphotype. And Vietnam originated *C. striata* in Bangladesh may be closer to the triangle head morphotype, and it needs further study with more samples for detailed clarification.

Haplotype network construction is a widely used approach for analyzing and visualizing the relationships among DNA sequences within or between populations

or species. We formed Median-joining networks of mtDNA Cyt b haplotypes of *C. striata* of Bangladesh and Vietnam origin. The haplotype network revealed two separate lineages, implying the evolution of two lineages from genetically similar ancestors. The Vietnamese haplotype (H6) revealed a distinct cluster distinct from Bangladeshi root populations. It is possible that the stock of imported Vietnamese *C. striata* populations in Bangladesh will encounter specific adaptation problems in the future and that the community will eventually become bottlenecked. As reported for the Thai strain of *Anabas testudineus* introduced in Bangladesh before (Habib et al., 2015), its distinctness may appear in the question.

Tests for neutrality and mismatch distribution supported population expansion. The samples were collected from the low land ecosystem called *beel* in Bangladesh. The natural habitat of that low land has unique characteristics where native *C. striata* have been adapted and thriving well. When overflow and flooding are standard during the rainy season, this low ground is connected to the river and other water bodies. As a result, there could be a possibility of connecting the various population streams.

Furthermore, native fish translocation for marketing purposes could be feasible during transport. Many fish hatcheries have recently attempted to breed native *C. striata* using natural broodfish on their premises. Thus, population expansion may have occurred, implying that if introgression between native and Vietnamese lineages occurs, genetic colonization of imported *C. striata* will propagate rapidly throughout Bangladesh. However, we do not want to detail this since it is not our primary focus. As a result, further studies with a larger sample size would be needed in the future for this reason.

Conclusion

We used the mtDNA Cyt b gene to distinguish the native and Vietnam-originated *C. striata* in Bangladesh though introduced Vietnamese *C. striata* morphotypes is still unknown. As a consequence of the translocation, the Vietnam-originated *C. striata* in Bangladesh could put the Bangladeshi native populations at risk of genetic mixing, leading to genetic attrition, reduced natural adaptability, and increased extinction risk. However, it may also be possible to find more diversified morphotypes in Bangladesh due to different aquaculture conditions that still are within-species variation. Therefore, comparative morphological variation of the snakehead strains in Bangladesh needs to be addressed by incorporating molecular identification.

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Authors contribution

KFK and BJ collected the samples, performed the laboratory work, generated the data, and prepared the initial manuscript. SK performed partial data analysis and partial manuscript reviewed critically and improved the manuscript. MShahanoorA reviewed and edited the manuscript critically and improved the manuscript. MSA generated original ideas, finalized the data and analysed, reviewed, improved, and finalized the manuscript, and received research project funds. Finally, all authors read the article and approved the final version to be published.

Competing interests

The authors have declared that no competing interests exist.

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