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Research Article

Mitochondrial Cyt *b* and CR differentiation and in silico RFLP markers for Bangladeshi and Vietnam-originated Climbing Perch (*Anabas testudineus*)

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Abstract

This study employed mitochondrial Cytochrome *b* (Cyt *b*) and Control Region (CR) markers to investigate the genetic diversity and population structure of Bangladeshi and Vietnam-origin climbing perch (*Anabas testudineus*) populations across Bangladesh. A total of 320 individuals were collected from 16 sampling sites representing all eight administrative divisions of Bangladesh. Following quality trimming, 818 bp of Cyt *b* and 705 bp of CR sequences were analyzed, revealing 9 and 28 haplotypes, respectively. Analysis of molecular variance (AMOVA) indicated that the majority of genetic variation was attributable to differences among populations (Cyt *b*: 95.6%; CR: 89.5%). Additionally, substantial genetic differentiation was observed between Bangladeshi and Vietnam-origin groups (Cyt *b*: 67.5%; CR: 73.0%). Pairwise Fst values (Cyt *b*: 0.815; CR: 0.804) confirmed strong differentiation between Bangladeshi and Vietnam-originated groups. Bayesian phylogeny and haplotype network analyses separated Bangladeshi and Vietnam-origin stocks into distinct clades. Shared haplotypes in Dhaka and Chattogram indicated admixture with Vietnam-originated stocks. Several unique haplotypes were identified in both Bangladeshi and Vietnam-originated populations, which may serve as markers for stock identification and broodstock management. In silico RFLP analysis identified seven enzymes (*AciI, HaeIII, HhaI, MboI, MspI, RsaI,* and *StuI*) for Cyt *b* sequences as cost-effective, practical tools for stock identification, while no diagnostic enzymes were found for CR. These findings provide a genetic baseline for conservation and selective breeding, and highlight the urgent need to protect Bangladeshi *A. testudineus* from admixture with exotic strains.

Introduction

Climbing perch (*Anabas testudineus*) is an economically important freshwater fish belonging to the family Anabantidae, valued for its taste, nutritional quality, and commercial significance. The species inhabits a broad range of lowland aquatic environments, including swamps, canals, rice fields, ponds, and estuarine systems, and is widely distributed throughout Bangladesh, South Asia, and Southeast Asia (Hossain *et al.*, 2015; Mawa *et al.*, 2021). Due to its high market demand and nutritional benefits, *A. testudineus* has considerable economic importance in both domestic and international markets (Rahman *et al.,* 2022). Although captive breeding techniques have been successfully developed, aquaculture production of Bangladeshi strains remains constrained by high larval mortality, slow growth rates, and relatively small adult body size, which collectively reduce farming efficiency and profitability (Alam *et al.*, 2021; Rahman *et al.*, 2022).

The Thai strain, introduced in 2002, exhibited faster growth but gradually lost market acceptance due to its poor flavor and odor (Suraiya *et al.*, 2012). Moreover, poor hatchery management, unsystematic fry collection, and inbreeding reduced its performance over time (Habib *et al.*, 2015). In 2011, the Vietnam strain was introduced and showed approximately 50% higher growth than the Thai strain while retaining a Bangladeshi-like coloration, making it popular among farmers and consumers (Kohinoor *et al.*, 2016; Parvez *et al.*, 2020). Consequently, Vietnamese climbing perch gained widespread popularity, and its commercial culture expanded throughout Bangladesh (Amin *et al.*, 2015). Flood-prone environments increase the risk of escape of exotic strains into natural water bodies, thereby threatening native Bangladeshi populations (Islam and Yasmin, 2017). Therefore, distinguishing Bangladeshi and Vietnam-originated stocks is necessary for conserving local genetic resources and maintaining broodstock quality.

Maintaining genetic diversity is key for establishing sustainable broodstocks and future genetic improvement (Ferguson, 1994; Hayes *et al.*, 2006). Developing a base population with broad genetic variation will facilitate long-term genetic improvement. Characterizing genetic diversity within and among source populations will aid in conserving the Bangladeshi gene pool for future utilization (Tinni *et al.*, 2007; Alam *et al.*, 2010). Although morphological traits are commonly used for species identification, mitochondrial DNA (mtDNA)-based barcoding provides a more precise method for lineage differentiation and genetic diversity assessment (Hebert *et al.*, 2003; Alam *et al.*, 2008; Jannat *et al.*, 2022; Shahariar *et al.*, 2025). The cytochrome *b* (Cyt *b*) gene and the control region (CR) of mtDNA have been particularly effective in distinguishing populations of various fish species, including Asian silurid catfish, great snakeheads, striped snakeheads, and minor carp (Kanon *et al.*, 2022; Rakhi *et al.*, 2023; Kumar *et al.*, 2024; Roy *et al.*, 2024).

Earlier work using the COI gene compared Bangladeshi and introduced strains, but was restricted to populations from the Rangpur division (Parvez *et al.*, 2020). Therefore, comprehensive country-

wide genetic information on Bangladeshi and Vietnam-originated populations remains limited. This study analyzed Cyt *b* and CR sequences from 16 populations across Bangladesh to evaluate genetic differentiation between Bangladeshi and Vietnam-originated strains, assess genetic diversity and population structure, and provide baseline information for conservation and broodstock management.

Materials and methods

Sample collection and DNA extraction

Samples of *A. testudineus* were collected from natural habitats and hatcheries across the eight administrative divisions of Bangladesh. Sixteen sampling sites were selected, as illustrated in Figure 1, ensuring broad geographical coverage.

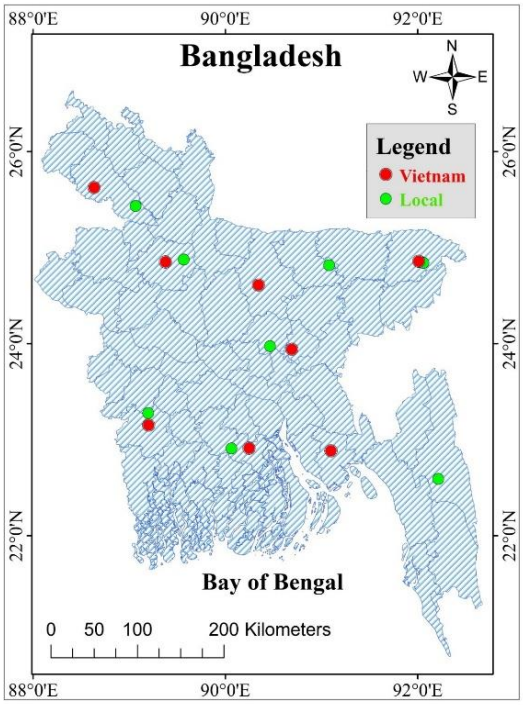

Figure 1: Illustration of sample collection sites on the Bangladesh map.

Detailed information on the sampling locations is provided in Table 1. A total of 320 individuals were collected, representing 20 specimens from each sampling site. Fin clips were collected from individual specimens for DNA extraction and preserved appropriately. The GeneJET Genomic DNA Purification Kit (Thermo Scientific) was used to extract DNA, following the manufacturer's protocol. The extracted DNA was stored at -20°C until further processing.

DNA amplification and sequencing

Two mitochondrial DNA genes, Cyt *b* and the CR, were amplified using specific primer sets to assess genetic variation. The primer sequences for Cyt *b* amplification were BangladeshiK_Cytb_50_Fow (5'-CCACCCCAGTCATCTCT-3') as the forward primer and BangladeshiK_Cytb_tRNA_Rev (5'-TTACAAGACCGATGCTC-3') as the reverse primer. Besides, the CR region, Koi_CR_Fow (5'-GGTTGCGGAGGTTAGAATCC-3') served as the forward primer, and Koi_CR_Rev (5'-ATGAAGCTTTCCAGGGCTTA-3') was used as the reverse primer. These primers were designed using complete mitochondrial genome sequences of *A. testudineus* generated in our laboratory together with sequences available in public databases. A total of 320 climbing perch specimens, representing twenty individuals per sampling site, were used for mtDNA amplification. The PCR reaction mixture was prepared by combining 3 µL of template DNA, 2 µL of forward primer, 2 µL of reverse primer, 25 µL of PCR master mix, and 18 µL of PCR-grade water, resulting in a total reaction volume of 50 µL.

Table 1: Sampling sites of *Anabas testudineus* used in this study.

Population	Collection site	Sample code
Bangladeshi Rangpur	Ashura beel, Dinajpur, Rangpur	NRn
Bangladeshi Rajshahi	Jabarkandi beel, Bogura, Rajshahi	NRj
Bangladeshi Khulna	Laukhali beel, Jashore, Khulna	NK
Bangladeshi Mymensingh	Bandha beel, Netrokona, Mymensingh	NM
Bangladeshi Dhaka	Belai beel, Gazipur, Dhaka	ND
Bangladeshi Barishal	Shatla beel, Patuakhali, Barishal	NB
Bangladeshi Sylhet	Murier haor, Sylhet	NS
Bangladeshi Chattogram	Kaptai lake, Rangamati, Chattogram	NC
Vietnam-originated Rangpur	Bahadur bazar, Dinajpur, Rangpur	VRn
Vietnam-originated Rajshahi	Chasir bazar, Bogura, Rajshahi	VRj
Vietnam-originated Khulna	Rail bazar, Jashore, Khulna	VK
Vietnam-originated Mymensingh	Sharnalata fish hatchery, Mymensingh	VM
Vietnam-originated Dhaka	Kalir haat, Narsingdi, Dhaka	VD
Vietnam-originated Barishal	Batajor bazar, Patuakhali, Barishal	VB
Vietnam-originated Sylhet	Golapganj bazar, Sylhet	VS
Vietnam-originated Chattogram	Maijdee bazar, Noakhali, Chattogram	VC

The amplification was performed in a thermal cycler under the following conditions: an initial denaturation at 94°C for 2 minutes, followed by 40 cycles consisting of denaturation at 94°C for 1 minute, annealing at 49°C for 1 minute and 30 seconds, and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes, followed by a hold at 4°C. Following PCR amplification, the products were verified through electrophoresis using a Mupid-2plus system on 0.7% agarose gel at 100 volts for 20 minutes. A 1 kbp DNA ladder was included for size estimation. The PCR products were then purified using the Molecular Biology Purification Kit (Thermo Scientific) following the manufacturer's protocol and stored at 4°C for sequencing. The Big Dye Terminator Sequencing Kit (v3.1; Applied Biosystems, USA) was used for sequencing. The sequencing protocol included an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute and 30 seconds. A final extension was performed at 72°C for 8 minutes, then cooling to 4°C. The sequencing was conducted commercially using a capillary electrophoresis-based DNA analyzer (ABI Prism 3130xl Genetic Analyzer; Applied Biosystems, USA). Chromatograms were checked to resolve ambiguous bases. Cyt *b* sequences were translated to confirm absence of stop codons or frameshifts.

Data analysis

The raw DNA sequences obtained from Cyt *b* and CR genes were edited using Chromas software version 2.6.6 (Technelysium Pty Ltd., Australia) to resolve sequence ambiguities. After quality control (QC), low-quality ends were trimmed; analyses used 818 bp (Cyt *b*) and 705 bp (CR). After quality control and trimming, 148 Cyt *b* and 149 CR sequences were retained for downstream analyses. Sequence alignment was performed using ClustalW, implemented in MEGA11 (Tamura *et al.*,

2021). To compare the obtained sequences with reference sequences, BLAST searches were conducted in the NCBI database (http://www.ncbi.nih.gov). Genetic diversity indices, including nucleotide diversity, fixation index (*Fst*), and other genetic distance metrics, were calculated using DnaSP6 (Rozas *et al.*, 2017). Population genetic structure and Molecular Variance (AMOVA) were assessed using Arlequin Version 3.5.2 (Excoffier and Lischer, 2010). Tajima's neutrality test and Mismatch distribution analysis (based on the sudden expansion model) were also performed to infer population demographic history. Two genetic models were considered for AMOVA: Gene Pool-1, where all 16 populations were treated as a single pool, and Gene Pool-2, where populations were divided into two groups: Bangladeshi (Group 1) and Vietnam-originated (Group 2). Mantel tests (Mantel, 1967) were performed in R software (version 4.5.1) using the package vegan v2.7.1 (Oksanen *et al.*, 2025) to examine the relationship between genetic and geographic distances (isolation by distance) among populations. Pairwise genetic differentiation between populations was quantified using F_{st} values estimated from the mitochondrial Cyt *b* and CR genes, and the resulting pairwise F_{st} matrix was used as the genetic distance matrix. Geographic distances were calculated as the straight-line (Euclidean) distances in kilometers between sampling sites based on their geographic coordinates. The test was based on Pearson's product–moment correlation between the two distance matrices, and statistical significance was assessed with 9,999 random permutations of the data. To determine the most appropriate nucleotide substitution model for phylogenetic analysis, MrModeltest 2.0 was employed, identifying GTR+I as the best-fit model for Cyt *b* and K80+G for CR (Nylander, 2004). Phylogenetic relationships among haplotypes were inferred using the Bayesian approach implemented in MrBayes 3.2.7 (Ronquist *et al.*, 2012). The analysis was conducted with 1.5 million MCMC generations, sampling every 1,000 generations, with four chains and two independent runs to ensure convergence. The first 25% (375,000 generations) were discarded as burn-in, and a majority-rule consensus tree was constructed from the remaining trees. Convergence was confirmed by an average standard deviation of split frequencies <0.01, and posterior probabilities were used to assess clade support. The phylogenetic tree was visualized using FigTree 1.4.5. To further examine genetic relationships, median-joining haplotype networks were constructed using PopART v.1.7 (Bandelt *et al.*, 1999). This analysis helped visualize the genetic connectivity and haplotype distribution among the sampled populations. In silico RFLP analysis was conducted on Cyt *b* and CR haplotypes to identify restriction enzymes differentiating Bangladeshi and Vietnam-originated populations. In silico RFLP analysis was performed in R version 4.5.1 using custom scripts to identify restriction sites and predict fragment-size patterns for each haplotype sequence. Restriction enzyme recognition sequences were screened against aligned Cyt *b* and CR haplotypes using a panel of 44 commercially available enzymes. A panel of 44 commercially

available restriction enzymes (e.g., *EcoRI, HindIII, TaqI*), including those with IUPAC ambiguity codes (e.g., GTMKAC for AccI), was used to identify restriction sites. To test generality, we also extended the analysis by including all available Cyt *b* sequences of *A. testudineus* from INSDC (GenBank/RefSeq/ENA) and recalculated fragment-size patterns for the same enzyme panel.

Results

Sequence composition and variation

The Cyt *b* gene of *A. testudineus* (818 bp) was analyzed across populations from the eight divisions of Bangladesh, resulting in the identification of nine haplotypes, which have been deposited in the GenBank database (Accession No. LC848593 - LC848601). The mean nucleotide composition across all 148 high-quality Cyt *b* sequences was estimated as adenine (A): 24.78%, thymine (T): 28.72%, guanine (G): 14.48%, and cytosine (C): 32.02%, indicating an A+T richness of 53.5%. The sequence analysis revealed four singleton variable sites and 84 parsimony-informative sites, highlighting moderate genetic variability within the Cyt *b* sequences. The control region of *A. testudineus* (705 bp) was analyzed for populations across the eight divisions of Bangladesh, identifying 28 haplotypes, which were submitted to the GenBank database (Accession No. LC848602 - LC848629). The nucleotide composition across the 149 high-quality CR sequences was adenine (A): 30.44%, thymine (T): 29.48%, guanine (G): 15.47%, and cytosine (C): 24.61%, indicating a higher A+T richness of 59.92%. The sequence analysis revealed 11 singleton variable sites and 83 parsimony-informative sites, suggesting a more diverse genetic structure in the CR sequences compared to Cyt *b*.

Genetic diversity and genetic differentiation

The highest number of polymorphic sites (19) among the 88 polymorphic sites found in the Cyt *b* sequence analysis was observed in Vietnam-originated Rajshahi and Vietnam-originated Barishal populations. Based on nucleotide variations, nine haplotypes were identified, with Vietnam-originated Khulna and Vietnam-originated Barishal having the highest haplotype count (four haplotypes each). Haplotype diversity (Hd) ranged from 0 to 0.778, with Vietnam-originated Khulna exhibiting the highest diversity (0.778). Nucleotide diversity (π) was relatively low, ranging from 0 to 0.0125 across all populations. The average number of nucleotide differences (K) for the Cyt *b* gene was 37.955, with Vietnam-originated Khulna displaying the highest value (Table 2). The AMOVA analysis revealed extremely high and significant genetic differentiation among the analyzed populations and groups. The highest Fst value (0.96959; $p = 0.000$) was observed in gene pool-2, whereas gene pool-1 also showed very high differentiation (Fst: 0.95561; $p = 0.000$), indicating strong genetic subdivision and limited gene flow among the studied populations. In gene pool-1, variation among populations (95.56%) was significantly higher than variation within populations (4.44%), whereas in gene pool-2, the highest variation (67.49%) was among groups, indicating substantial genetic divergence.

Table 2: Genetic diversity from mtDNA Cyt *b* and control region.

Population	N	S	N_h	H_d	π	K
				Cyt *b* gene		
Bangladeshi Rangpur	9	0	1	0	00	00
Bangladeshi Rajshahi	10	2	2	0.20	0.00049	0.40
Bangladeshi Khulna	9	0	1	0	0.00	0.00
Bangladeshi Mymensingh	8	0	1	0	0.00	0.00
Bangladeshi Dhaka	10	1	2	0.533	0.00065	0.533
Bangladeshi Barishal	9	1	2	0.222	0.00027	0.222
Bangladeshi Sylhet	10	2	2	0.2	0.00049	0.4
Bangladeshi Chattogram	7	0	1	0	0	0
Vietnam-originated Rangpur	9	1	2	0.5	0.00061	0.5
Vietnam-originated Rajshahi	10	18	2	0.2	0.0044	3.6
Vietnam-originated Khulna	10	19	4	0.778	0.0125	10.244
Vietnam-originated Mymensingh	9	0	1	0	0.00	0.00
Vietnam-originated Dhaka	10	1	2	0.533	0.00065	0.533
Vietnam-originated Barishal	10	19	4	0.644	0.011	8.889
Vietnam-originated Sylhet	8	1	2	0.536	0.00065	0.536
Vietnam-originated Chattogram	10	1	2	0.4667	0.00057	0.467
Overall	**148**	**88**	**9**	**0.742**	**0.046**	**37.955**
				Control region		
Bangladeshi Rangpur	9	0	1	0	0	0
Bangladeshi Rajshahi	10	1	2	0.4667	0.00067	0.4667
Bangladeshi Khulna	10	3	4	0.6444444	0.00108	0.756
Bangladeshi Mymensingh	10	5	5	0.6666667	0.00143	1.00
Bangladeshi Dhaka	9	5	3	0.417	0.0023	1.611
Bangladeshi Barishal	10	1	2	0.2	0.00029	0.2
Bangladeshi Sylhet	8	8	4	0.643	0.0029	2
Bangladeshi Chattogram	8	68	2	0.536	0.052	36.429
Vietnam-originated Rangpur	9	8	4	0.58	0.00254	1.778
Vietnam-originated Rajshahi	8	17	3	0.464	0.00634	4.428
Vietnam-originated Khulna	9	4	4	0.75	0.002	1.556
Vietnam-originated Mymensingh	9	0	1	0	0	0
Vietnam-originated Dhaka	9	4	2	0.2222	0.00127	0.889
Vietnam-originated Barishal	10	20	3	0.511	0.006	4.156
Vietnam-originated Sylhet	10	4	5	0.667	0.00162	1.133
Vietnam-originated Chattogram	10	10	4	0.644	0.00970	6.778
Overall	**149**	**94**	**28**	**0.80401**	**0.0512**	**35.812**

N, number of samples; S, number of polymorphic sites; N_h, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; K, average number of nucleotide differences.

Pairwise FST values among Bangladeshi populations averaged 0.443, ranging from 0.00 to 0.998 (Table 4). The maximum pairwise Fst value (0.998) occurred between Bangladeshi Rangpur vs. Bangladeshi Chattogram, Bangladeshi Khulna vs. Bangladeshi Chattogram, and Bangladeshi Mymensingh vs. Bangladeshi Chattogram, indicating strong genetic differentiation. The lowest FST value (0.00) was found between multiple population pairs, including Bangladeshi Rangpur vs. Bangladeshi Rajshahi, Bangladeshi Rajshahi vs. Bangladeshi Mymensingh, and Bangladeshi Barishal vs. Bangladeshi Sylhet. The pairwise FST value between Bangladeshi and Vietnam-originated populations ranged from -0.125 to 1, with an average of 0.808 (64 between-group pairs). The maximum pairwise Fst value (1.00) was recorded between Bangladeshi Rangpur vs. Vietnam-originated Mymensingh, Bangladeshi Khulna vs. Vietnam-originated

Mymensingh, and Bangladeshi Mymensingh vs. Vietnam-originated Mymensingh, while the lowest FST value (-0.125) was found between Bangladeshi Dhaka vs. Vietnam-originated Sylhet. Within Vietnam-originated populations, FST values ranged from -0.13 to 0.987, with an average of 0.291 (28 pairs within Vietnam-originated populations). The τ value for Bangladeshi populations ranged from 0.828 to 3.00, whereas for Vietnam-originated populations, it ranged from 0.695 to 18.955 in the mismatch distribution analysis (Table 5).

Tajima's D test was significant for Bangladeshi Rajshahi, Bangladeshi Sylhet, and Vietnam-originated Rajshahi, while Fu's Fs values were insignificant for all populations. Mantel tests were conducted separately for Bangladeshi populations, Vietnam-originated populations, and for the combined dataset including all populations. In the combined dataset, no significant correlation was observed between genetic and geographic distances (r = – 0.102, p=0.881) (Fig 2A). Similarly, when only Bangladeshi populations were analyzed, the correlation was weak and non-significant (r= 0.091, p= 0.398) (Fig 2B). In the Vietnam-originated dataset, a negative correlation was obtained, but it was also not significant (r=– 0.326, p=0.904) (Fig 2C). Collectively, these results suggest that geographic distance does not significantly explain the observed genetic differentiation among the sampled populations for the Cyt *b* gene (Fig. 2).

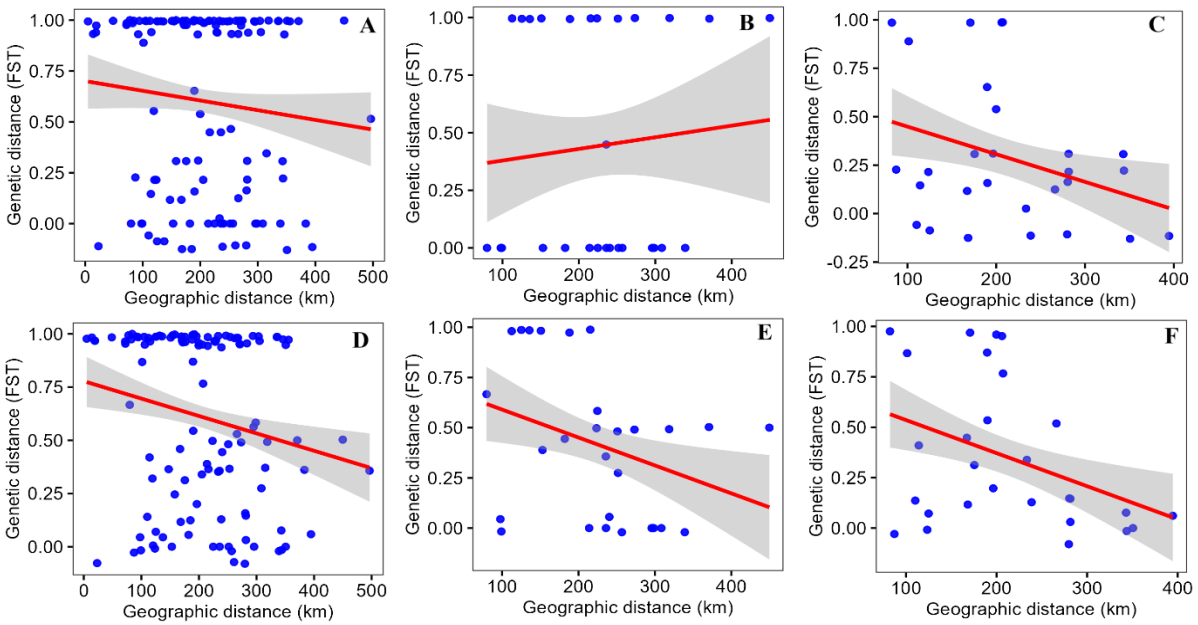

Figure 2: Scatter plots showing the relationship between pairwise FST values and geographic distance among *Anabas testudineus* populations. (A) All populations, Cyt *b*; (B) Bangladeshi populations, Cyt *b*; (C) Vietnam-origin populations, Cyt *b*; (D) All populations, Control region (CR); (E) Bangladeshi populations, CR; (F) Vietnam-originated populations, CR.

Bangladeshi Chattogram showed a high polymorphism within the control region (CR), with 68 polymorphic sites out of 94, despite containing only two haplotypes. In

contrast, Bangladeshi Mymensingh and the Vietnam-origin population from Sylhet showed the most haplotypes (five haplotypes each). Haplotype diversity (Hd) ranged from 0 to 0.75, with the highest value (Hd = 0.75) observed in the Vietnam-origin Khulna population. The lowest haplotype diversity (Hd = 0.00) was recorded in Bangladeshi Rangpur and the Vietnam-origin Mymensingh population. Nucleotide diversity (π) among populations ranged from 0.000 to 0.052, with an overall alignment-wide nucleotide diversity of 0.0512. The highest genetic diversity was observed in Bangladeshi Chattogram, whereas Bangladeshi Rangpur and the Vietnam-origin Mymensingh population exhibited the lowest genetic diversity. The AMOVA analysis confirmed significant hierarchical genetic variation (Table 3).

Table 3: AMOVA based on Cyt *b* and control region.

	Source of variation	df	Sum of squares	Variance components	Percentage variation (%)	Fixation indices
				Cyt *b* gene		
Gene pool-1	Among populations	15	2672.159	19.17466 Va	95.56	*Fst*: 0.95561
	Within populations	132	117.564	0.89064 Vb	4.44	-
Gene pool-2	Among groups	1	1543.102	19.76713 Va	67.49	F_{SC}: 0.90648
	Among populations within groups	14	1129.057	8.63322 Vb	29.47	F_{ST}: 0.96959
	Within populations	132	117.564	0.89064 Vc	3.04	F_{CT}: 0.67485
				Control region		
Gene pool-1	Among populations	15	2543.305	17.98653 Va	89.48	*Fst*: 0.89483
	Within populations	133	281.158	2.11397 Vb	10.52	-
Gene pool-2	Among groups	1	1716.254	22.24040 Va	72.98	F_{SC}: 0.74330
	Among populations within groups	14	827.051	6.12127 Vb	20.09	F_{ST}: 0.93063
	Within populations	134	281.158	2.11397 Vc	6.94	F_{CT}: 0.72978

AMOVA was performed at a significance level of $p<0.001$. Fst, Fsc, and Fct values were obtained from hierarchical AMOVA implemented in Arlequin v3.5.2.

In gene pool-1, variation among populations (89.48%) was higher than within populations (10.52%). In gene pool-2, variation among groups (72.98%) was the highest, indicating clear genetic differentiation. Pairwise FST values among Bangladeshi populations ranged from -0.021 (Bangladeshi Khulna vs. Bangladeshi Mymensingh) to 0.988 (Bangladeshi Rangpur vs. Bangladeshi Dhaka), with an average of 0.415 (28 pairs), indicating moderate genetic divergence (Table 4). The FST value between Bangladeshi and Vietnam-originated populations ranged from -0.077 (Bangladeshi Dhaka vs. Vietnam-originated Dhaka) to 1 (Bangladeshi Rangpur vs. Vietnam-originated Mymensingh), averaging 0.804 (64 pairs). Within Vietnam-originated populations, FST values ranged from -0.08 to 0.974, with an average of 0.356 (28 pairs). Mismatch distribution analysis revealed τ values ranging from 0.00 to 43.250 for Bangladeshi populations, while for Vietnam-originated populations, they ranged from 0.027 to 19.268 (Table 5).

Table 4: Genetic differentiation metrics among populations.

Population	Fst Average (Min-Max)	Nst Average (Min-Max)	Gst Average (Min-Max)	Da Average (Min-Max)
	Cyt *b* gene			
Within Bangladeshi populations	0.443 (0 – 0.998)	0.443 (0 – 0.998)	0.368 (-0.027 - 1)	0.04 (0 - 0.093)
Between Bangladeshi and Vietnam-originated populations	0.808 (-0.125 - 1)	0.806 (-0.125 - 1)	0.473 (-0.057 - 1)	0.069 (-0.00007 - 0.093)
Within Vietnam-originated populations	0.291 (-0.13 - 0.987)	0.290 (-0.13 - 0.987)	0.209 (-0.061 - 0.791)	0.005 (-0.0006 - 0.0217)
	Control region			
Within Bangladeshi populations	0.415 (-0.02067 - 0.988)	0.415 (-0.0207 - 0.9888)	0.192 (-0.034 - 0.655)	0.027 (0.00004 - 0.097)
Between Bangladeshi and Vietnam-originated populations	0.804 (-0.077 – 1)	0.805 (-0.077 – 1)	0.329 (-0.038 – 1)	0.076 (-0.00016 - 0.1094)
Within Vietnam-originated populations	0.356 (-0.08 - 0.974)	0.355 (-0.08 - 0.975)	0.212 (-0.02998 - 0.8)	0.0064 (-0.00021 - 0.025)

Fst = fixation index; Ns_t = coefficient of nucleotide differentiation considering sequence divergence; Gst = coefficient of genetic differentiation; Da = net nucleotide divergence between populations.

Table 5: Neutrality tests and mismatch distribution parameters.

Population	τ	θ_o	θ_1	SSD	RI	Tajma's D value	Fu's F_S
	Cyt *b* gene						
Bangladeshi Rangpur	-	-	-	-	-	0.00000 (1.00)	-
Bangladeshi Rajshahi	3	0	0.180	0.052 (0.1)	0.72 (0.55)	-1.40085 (0.037)	0.58622 (0.406)
Bangladeshi Khulna	-	-	-	-	-	0.00000 (1.00)	-
Bangladeshi Mymensingh	-	-	-	-	-	0.00000 (1.00)	-
Bangladeshi Dhaka	0.828	0.00	99999.00	0.030 (0.0)	0.289 (0.20)	1.30268 (0.936)	1.02917 (0.597)
Bangladeshi Barishal	2.93	0.90	3.60	0.307 (0.15)	0.358 (0.25)	-1.08823 (0.196)	-0.26348 (0.177)
Bangladeshi Sylhet	3.00	0	0.180	0.052 (0.00)	0.72 (0.60)	-1.40085 (0.036)	0.58622 (0.423)
Bangladeshi Chattogram	-	-	-	-	-	0.00000 (1.00)	-
Vietnam-originated Rangpur	0.764	0.00	99999.00	0.022 (0.25)	0.25 (0.20)	0.98627 (0.88600)	0.84950 (0.515)
Vietnam-originated Rajshahi	3.00	0.00	0.133	0.058 (0.05)	0.72 (0.70)	-2.02713 (0.002)	6.11910 (0.99)
Vietnam-originated Khulna	18.955	0.000	2.888	0.22570164 (0.10)	0.30913580 (0.20)	2.46140 (1.00)	5.66899 (0.99)
Vietnam-originated Mymensingh	-	-	-	-	-	0.00000 (1.00)	-

Table 5 (continued):

Population	τ	θ_o	θ_1	SSD	RI	Tajma's D value	Fu's F_S
				Cyt *b* gene			
Vietnam-originated Dhaka	0.828	00	99999.00	0.03 (0.2)	0.28888889 (0.05)	1.30268 (0.934)	1.02917 (0.596)
Vietnam-originated Barishal	18.703	0.000	1.500	0.15920675 (0.25)	0.20839506 (0.55)	1.51572 (0.96)	5.07112 (0.982)
Vietnam-originated Sylhet	0.832	0.00	99999.00	0.031 (0.15)	0.292 (0.35)	1.16650 (0.929)	0.86637 (0.567)
Vietnam-originated Chattogram	0.695	0.000	99999.00	0.01554637 (0.35)	0.22222222 (0.25)	0.81980 (0.86600)	0.81801 (0.54300)
				Control region			
Bangladeshi Rangpur	-	-	-	-	-	0.00000 (1.00)	-
Bangladeshi Rajshahi	0.695	0.000	99999	0.016 (0.25)	0.222 (0.5)	0.81980 (0.868)	0.81801 (0.521)
Bangladeshi Khulna	0.961	0.000	99999	0.033 (0.30)	0.22 (0.30)	-1.03446 (0.203)	-1.46598 (0.033)
Bangladeshi Mymensingh	1.074	0.011	99999.00	0.001 (1.00)	0.067 (0.6)	-1.74110 (0.016)	-2.25975 (0.009)
Bangladeshi Dhaka	0.000	0.00	99999.00	0.326 (0.00)	0.644 (0.95)	-0.52629 (0.355)	1.30416 (0.766)
Bangladeshi Barishal	2.930	0.9	3.600	0.331 (0.10)	0.40 (0.05)	-1.11173 (0.20)	-0.33931 (0.14)
Bangladeshi Sylhet	7.684	0.000	1.499	0.068 (0.5)	0.15 (0.5)	-1.70118 (0.022)	0.15498 (0.50)
Bangladeshi Chattogram	43.250	0.000	0.999	0.395 (0.05)	0.815 (0.15)	2.61700 (1.00)	19.74868 (1.00)
Vietnam-originated Rangpur	5.049	0.004	1.720	0.053 (0.70)	0.149 (0.70)	-1.79752 (0.004)	0.14326 (0.508)
Vietnam-originated Rajshahi	0.027	0.00	99999.00	0.274 (0.00)	0.228 (1.00)	-1.68765 (0.02)	4.13437 (0.97)
Vietnam-originated Khulna	2.375	0.007	7.22	0.028 (0.30)	0.113 (0.45)	0.23146 (0.647)	-0.13343 (0.417)
Vietnam-originated Mymensingh	-	-	-	-	-	0.00000 (1.00)	-
Vietnam-originated Dhaka	3	0	0.157	0.071 (0.00)	0.704 (0.65)	-1.60974 (0.037)	1.84369 (0.785)
Vietnam-originated Barishal	0.611	0.00	99999	0.040 (0.10)	0.167 (0.65)	-1.94842 (0.007)	4.68165 (0.98)
Vietnam-originated Sylhet	1.945	0.002	5.214	0.07 (0.2)	0.307 (0.20)	-0.76195 (0.252)	-1.94393 (0.028)
Vietnam-originated Chattogram	19.268	0.002	2.924	0.151 (0.20)	0.282 (0.30)	0.04230 (0.554)	4.57245 (0.968)

τ, time in several generations; θo, pre-expansion population size; θ1, post-expansion population size; SSD, the sum of squared deviations; RI, raggedness index; -, the variance of the mismatch distribution is too slight that no demographic parameters can be estimated.

Tajima's D test was significant for Bangladeshi Mymensingh, Bangladeshi Sylhet, Vietnam-originated Rangpur, Vietnam-originated Rajshahi, Vietnam-originated Dhaka, and Vietnam-originated Barishal. Fu's Fs values were significant only for Bangladeshi Khulna, Bangladeshi Mymensingh, and Vietnam-originated Sylhet. For the CR gene dataset, Mantel tests revealed no significant isolation by distance (IBD) in any of the analyses. When all populations were considered together, a

weak negative correlation was detected, but it was not significant (r= – 0.195, *p*=0.993) (Fig 2D). Similarly, when populations were analyzed separately, both the Bangladeshi dataset (r=– 0.331, *p*=0.921) and the Vietnam-originated dataset (r= –0.385, *p*=0.942) showed non-significant negative correlations (Fig 2E and Fig 2F). Thus, geographic distance did not significantly explain the genetic variation observed in the CR region.

Evolutionary and phylogeographic relationships

The phylogenetic tree constructed from Cyt *b* sequences clustered haplotypes into well-supported clades (Fig. 3).

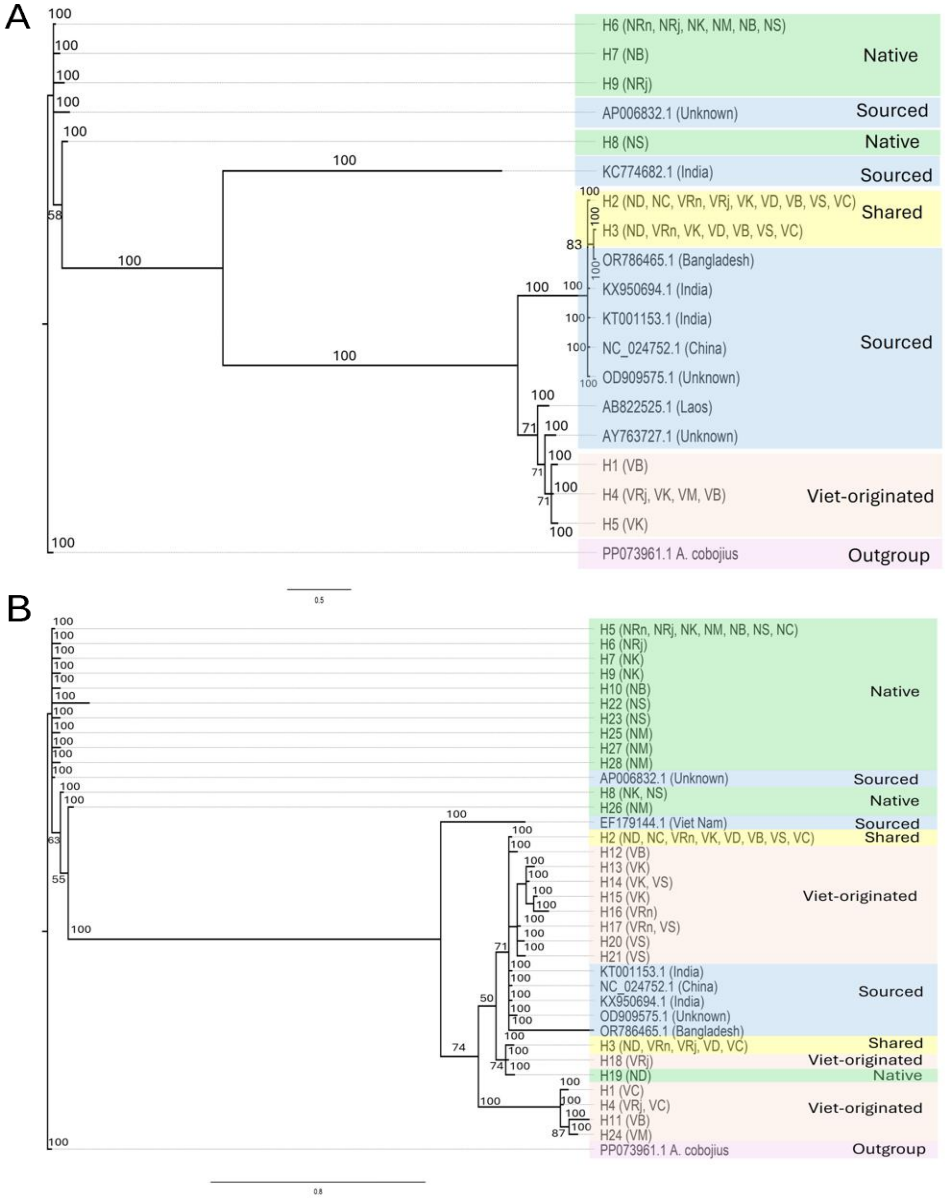

Figure 3: Phylogenetic tree of *Anabas testudineus* constructed from Cyt *b* gene (A) and CR region (B).

Trees were rooted with *Anabas cobojius* (GenBank accession: PP073961.1). Posterior probabilities (PP) are shown at nodes; PP ≥ 0.90 supports the main clades.

Haplotypes H6, H7, and H9 from Bangladeshi populations formed a distinct cluster, indicating strong genetic relatedness. H8 was positioned on a separate branch, suggesting minor genetic divergence. Vietnam-originated haplotypes H1, H4, and H5 clustered together, highlighting their shared ancestry. The haplotype network (Fig. 4) showed variations in haplotype distribution, with H2 being the most common haplotype across nine populations.

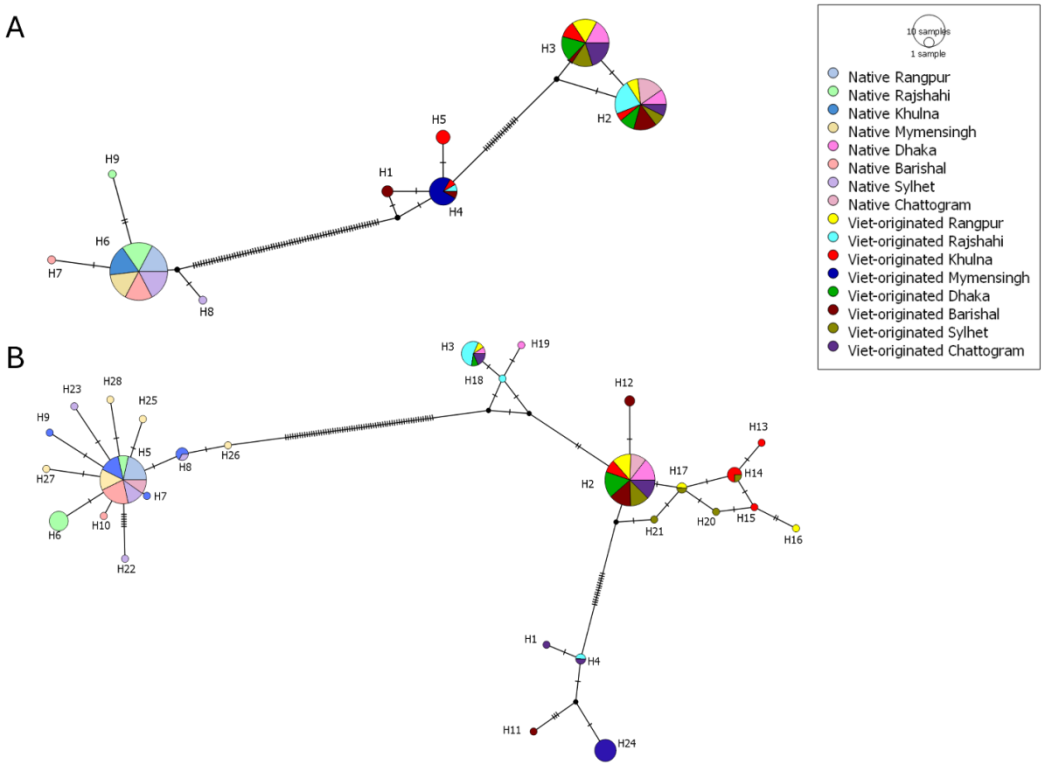

Figure 4: The median-joining network of *Anabas testudineus* (A) Cyt *b* gene and (B) CR region. Each circle represents a distinct haplotype, with the circle size corresponding to the frequency of that haplotype. Colors denote the locality, as the legend shows, and hatch marks indicate the number of mutations separating different haplotypes.

Phylogenetic analysis based on CR sequences revealed distinct clades (Fig. 3B). Trees were rooted with *A. cobojius* (GenBank accession: PP073961.1). Posterior probabilities (PP) are shown at nodes; PP ≥ 0.90 supports the main clades. Haplotypes H5-H9 and H22-H28 represented Bangladeshi populations, while H1, H4, and H11-H21 were predominantly Vietnam-originated. Shared haplotypes in Dhaka and Chattogram indicate an mtDNA admixture signal with Vietnam-originated stocks. The haplotype network (Fig. 4B) confirmed these findings, showing significant differences in haplotype distributions and population structure.

In silico RFLP analysis

For Cyt *b*, seven restriction enzymes (*AciI, HaeIII, HhaI, MboI, MspI, RsaI*, and *StuI*) fully differentiated Bangladeshi and Vietnam-originated populations by producing non-overlapping fragment patterns. Fragment numbers ranged from 1 to 4, with distinct patterns such as AciI (529–289 bp for Bangladeshi; 552–266 or

818 bp for Vietnam-originated) and MspI (100–718 bp for Bangladeshi; 100–184–534 or 100–184–94–440 bp for Vietnam-originated). Based on fragment numbers alone, Bangladeshi and Vietnam-originated populations could also be differentiated using HaeIII, HhaI, MboI, MspI, and StuI. For CR, no enzymes among the 44 tested differentiated the two groups, likely due to shared fragment patterns or limited restriction site variation. Detailed fragment patterns for differentiating enzymes are presented in Table 6. However, when all Cyt *b* sequences of *A. testudineus* from INSDC were included, overlapping fragment-size patterns were observed between groups for every enzyme tested, indicating that none were universally diagnostic across the species range (Table 6).

Table 6: Restriction enzymes differentiating Bangladeshi and Vietnam-originated populations of *Anabas testudineus* based on Cyt *b* sequences. Fragment numbers and sizes shown. No diagnostic enzymes were found for CR. Inclusion of all INSDC Cyt *b* sequences removed universal diagnostics

Enzyme	Bangladeshi fragment number/sizes	Viet-originated fragment number/sizes
AciI	2 → 529, 289	1 → 818 2 → 552, 266
HaeIII	3 → 57, 327, 434	2 → 384, 434
HhaI	3 → 13, 168, 637	2 → 13, 805
MboI	1 → 818	2 → 601, 217 3 → 601, 28, 189
MspI	2 → 100, 718	3 → 100, 184, 534 4 → 100, 184, 94, 440
RsaI	1 → 818 2 → 794, 24	2 → 572, 246 3 → 572, 184, 62
StuI	2 → 56, 762	1 → 818

Discussion

Genetic diversity plays a crucial role in the adaptability of fish populations to environmental changes (Sahoo *et al.*, 2019; Das *et al.*, 2024; Gwak and Roy, 2024). This study revealed strong genetic variation between Bangladeshi and Vietnam-originated *A. testudineus*. The base composition showed A+T richness (Cyt *b*: 53.5%; CR: 59.9%), in agreement with earlier studies on COI (Parvez *et al.,* 2020) and other fish mtDNA (Sahoo *et al.*, 2019; Das *et al.*, 2024).

Bangladeshi populations showed lower haplotype diversity than Vietnam-originated stocks (Table 2). Vietnam-originated Khulna had the highest Hd (0.778, Cyt *b*; 0.75, CR). This pattern reflects multiple maternal lineages in introduced hatchery stocks, while Bangladeshi populations may have evolved under more isolated conditions. Similar to haplotype diversity, low nucleotide diversity was detected in most Bangladeshi populations, in contrast to Vietnam-originated populations, which showed greater variability. A comparable pattern has been observed in other freshwater fish species, where higher haplotype diversity indicated rapid population expansion following a bottleneck event (Dutta *et al.*, 2020; Roy *et al.*, 2024). Low nucleotide

diversity with higher haplotype diversity is typical of populations that expanded recently after a bottleneck (Sahoo *et al.*, 2020; Das *et al.*, 2024; Roy *et al.*, 2024).

The CR marker showed more polymorphic sites and haplotypes than Cyt *b*, consistent with its faster evolutionary rate. This result indicates that the mitochondrial control region provides a more detailed representation of mutations and genetic variation in this study. Several populations showed negative Tajima's D and Fu's Fs values. Although many were not significant, these results suggest population expansion or selection pressures. Very large θ1 values (Table 5) are a known outcome of sudden-expansion models (Roy *et al.*, 2024). The insignificant negative values of Fu's Fs and Tajima's D suggest an excess of rare nucleotide variants and haplotypes compared to expectations under neutrality. The combined effects of high haplotype diversity, low nucleotide diversity, and negative neutrality test values suggest that recent population expansion followed a genetic bottleneck or historically small effective population size.

The AMOVA analysis revealed a high proportion of genetic variation among populations in gene pool-1 and among groups in gene pool-2, indicating strong separation between Bangladeshi and Vietnam-originated stocks. A similar study on the control region of *A. testudineus* collected from Sumatra and Peninsular Malaysia reported 84.72% variation among populations and 15.28% within populations (Jamsari *et al.*, 2010). The predominance of among-population variance suggests limited contemporary gene flow, consistent with low dispersal and site fidelity supporting the classification of *A. testudineus* as a non-migratory species. (Vrijenhoek, 1998).

Higher FST and NST within Bangladeshi groups suggest restricted connectivity among natural populations, while Vietnam-originated hatchery stocks appear more uniform. Low Da within Vietnam-originated groups indicates shared ancestry and inter-hatchery mixing. In contrast, the higher Da values observed between Bangladeshi and Vietnam-originated populations indicate reduced gene flow, distinct allelic frequencies, and limited genetic exchange between the populations. Similar genetic differentiation patterns have been reported in a previous study comparing Bangladeshi and Vietnam-originated *Channa striata* in Bangladesh (Kanon *et al.*, 2022).

The Mantel test analyses for both the mitochondrial *Cyt b* and control region (CR) genes consistently revealed no significant isolation by distance (IBD) among Bangladeshi, Vietnam-origin, or combined datasets. These findings parallel recent research demonstrating the erosion of natural spatial genetic patterns due to human-mediated stocking and introductions. For example, Masuda *et al.* (2024) observed significant IBD in Bangladeshi white-spotted charr but none in stocked populations. Similarly, Radinger *et al.* (2023) reported that large-scale fish stocking disrupted Bangladeshi genetic structures. The absence of IBD in invasive species has also been documented (Johansson *et al.*, 2018), highlighting how rapid human-facilitated movement can homogenize genetic variation. In this study,

Cyt b and CR analyses indicate that geographic distance alone fails to explain genetic differentiation in *A. testudineus*, suggesting a dominant role of non-geographic factors. Among the most plausible drivers are human-mediated dispersal, including aquaculture-related introductions of Vietnam-originated stocks, historical translocations, and admixture events following stocking. These processes likely disrupted pre-existing IBD signals that might otherwise remain intact in isolated or unmanaged Bangladeshi populations. While natural dispersal mechanisms may facilitate some level of gene flow in *A. testudineus*, the dominant influence of human-mediated translocations likely plays a central role in shaping its current genetic landscape. Bayesian phylogeny (rooted with *A. cobojius*) placed Bangladeshi and Vietnam-originated haplotypes in separate clades with high posterior probability (PP > 0.95). This confirms their distinct maternal origins. Parvez *et al.* (2020) also reported Thai and Vietnam-originated koi forming separate clusters, supporting current findings. In contrast, Bangladeshi and Indian climbing perch were placed in a different clade, supporting their genetic distinction. Shared haplotypes in Dhaka and Chattogram indicate an mtDNA admixture signal between Bangladeshi and Vietnam-originated stocks. These hubs are major centers of fish trade and seed distribution, which may explain the genetic mixing. Such findings explain the importance of managing Bangladeshi *A. testudineus* stocks carefully, as hybridization or sample misidentification could complicate conservation efforts to preserve genetically pure Bangladeshi populations.

The median-joining haplotype network analysis of mtDNA Cyt *b* and CR regions revealed two genetically distinct lineages, strengthening the idea that Bangladeshi and Vietnam-originated populations diverged even longer ago than recently. Based on Cyt *b*, haplotype H4, widely found among Vietnam-originated populations, appears to be the ancestral haplotype, suggesting its presence prior to geographic divergence. In contrast, H1, H2, H3, and H5 likely emerged more recently, influenced by stock introductions, genetic drift, and local adaptation. For Bangladeshi populations, H6 was identified as the ancestral haplotype, while H7, H8, and H9 seem to have evolved through recent mutations, indicating limited gene flow and possible founder effects. Based on the CR gene haplotype network, haplotype H2, predominantly observed in Vietnam-originated populations, appears to represent the ancestral lineage, suggesting its establishment prior to geographic dispersal. In contrast, haplotypes such as H1**,** H3, and H4 likely originated more recently, shaped by stock introductions, genetic drift, and regional adaptation processes. For the Bangladeshi populations, haplotype H5 forms the central node, pointing to its role as the ancestral haplotype within these groups, while H6 – H10, H22, and H23 seem to have emerged through recent mutational events, reflecting restricted gene flow and potential founder effects across locations. A similar pattern was observed in Bangladeshi *C. striata*, where Bangladeshi and Vietnam-originated groups formed separate clusters, further supporting the

genetic differentiation between introduced and indigenous stocks (Kanon *et al.*, 2022). In silico RFLP analysis of Cyt *b* sequences identified seven restriction enzymes that fully differentiated Bangladeshi and Vietnam-originated *A. testudineus* populations, complementing the genetic differentiation observed in mitochondrial analyses. The absence of shared fragment patterns reflects significant sequence variation in restriction sites, likely driven by geographic isolation or historical introductions of Vietnam-originated populations. No diagnostic RFLP enzymes were found for CR sequences, which likely reflects high nucleotide variability but a lack of fixed restriction-site differences. The Cyt *b* RFLP markers offer a cost-effective tool for rapid identification of Bangladeshi and Vietnam-originated *A. testudineus* populations, particularly for aquaculture management and conservation in Bangladesh. Nevertheless, they are not globally diagnostic when all available Cyt *b* sequences are considered, reinforcing their use as population-specific tools for aquaculture management in Bangladesh. Enzymes like *HaeIII, HhaI, MboI, MspI,* and *StuI* could be implemented in PCR-RFLP assays to monitor stock mixing in hatcheries or assess introgression in natural habitats. Such applications are critical for maintaining genetic integrity of Bangladeshi populations, given the widespread use of Vietnam-originated stocks in Bangladeshi aquaculture. Experimental validation of these in silico markers will enable routine monitoring of broodstock integrity and hybridization events in Bangladeshi hatcheries.

This study provides the first country-wide comparison of Bangladeshi and Vietnam-originated *A. testudineus* across Bangladesh using Cyt *b* and CR markers. In silico RFLP analysis of Cyt *b* identified seven restriction enzymes (e.g., *AciI, HaeIII, MspI*) that fully differentiate Bangladeshi and Vietnam-originated populations, offering a cost-effective tool for stock identification in hatcheries and conservation programs. The results confirm strong genetic differentiation between the two groups. Bangladeshi Khulna, Mymensingh, and Sylhet, and Vietnam-originated Rangpur and Khulna populations showed the highest genetic diversity, making them important sources for broodstock development. In contrast, Bangladeshi Rangpur and Vietnam-originated Mymensingh showed genetic isolation, indicating limited gene flow that needs careful monitoring. Phylogeny confirmed Bangladeshi populations as a distinct lineage, related to populations from India, China, and Laos. In addition, Shared haplotypes in Dhaka and Chattogram indicate admixture with Vietnam-originated stocks, raising concern for the loss of Bangladeshi genetic identity. The protection of Bangladeshi populations is recommended through the establishment of broodstock reserves, strict hatchery certification, and monitoring of high-risk hubs such as Dhaka and Chattogram. This work establishes a genetic baseline for conservation planning, selective breeding, and sustainable aquaculture of *A. testudineus* in Bangladesh. It also highlights the urgent need to limit further admixture between Bangladeshi and exotic lineages; mtDNA data, enhanced by RFLP

markers, indicate an admixture signal. These findings offer valuable insights for policymakers and researchers working towards the long-term sustainability of climbing perch populations and their role in aquaculture development.

To ensure the conservation of genetic integrity, emphasis should be placed on several key management priorities. First, populations with high haplotype diversity, including Khulna, Mymensingh, and Sylhet, should be protected and utilized for broodstock development. Quarantine and certification of hatchery seed are required to prevent unintended genetic mixing between Bangladeshi and introduced lineages. In addition, routine mtDNA screening should be conducted in hatcheries located in Dhaka and Chattogram, where admixture is most likely to occur. Through these measures, the integrity of Bangladeshi gene pools can be safeguarded, and a reliable foundation for selective breeding programs can be established.

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Conflicts of interest

The authors have declared that they have no conflicts of interest.

Ethical approval

The Gazipur Agricultural University (GAU) Animal Research Ethics Committee (AREC) approved the research, and the approval number is FVMAS/AREC/2023/39.

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