

Competitive Research Grant

Sub-Project Completion Report

on

**Molecular Identification of Local and Exotic
Strains of Koi (*Anabas testudineus*) for Strategic
Conservation Management**

Project Duration

July 2017 to September 2018

**Department of Genetics and Fish Breeding,
Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur**

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215



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Citation

Molecular Identification of Local and Exotic Strains of Koi (*Anabas testudineus*) for Strategic Conservation Management

Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

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Published in: September 2018

Printed by:

Acronyms

BSMRAU	: Bangabandhu Sheikh Mujibur Rahman Agricultural University
QC	: Quality Control
NGS	: Next Generation Sequencing
DNA	: Deoxyribonucleic Acid
mtDNA	: Mitochondrial DNA
PCR	: Polymerase Chain Reaction
CR	: Control Region

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Executive Summary

The climbing perch, *Anabas testudineus*, is an important anabantoid fish widely distributed in Southeast Asia. In Bangladesh, *A. testudineus* (Local Koi) is available including exotic Thai and Vietnamese Koi, which is one of the largest aquacultural species in Bangladesh. Information regarding genetic variability is very important for both proper management and conservation of this species. Mitochondrial genome information can be used to know the genetic structure and development of molecular marker. Considering this issue complete mtDNA sequence of both local and exotic Koi (*A. testudineus*) were analyzed and subsequently genetic structure of local and exotic Koi populations in Bangladesh were estimated. Firstly, complete mtDNA of local and Vietnamese Koi (*A. testudineus*) were determined by NGS method. It reveals as usual 37 mtDNA genes (16603bp) corresponding to Indian and Chinese *A. testudineus* when compared with online databases. After assembling the NGS data ten short gaps (1-97bp) were found in local Koi whereas single gap (43bp) was found in Vietnamese Koi. The high sequence similarity of Vietnamese Koi was observed with Indian/Chinese *A. testudineus* compared to local Koi. However, after studying complete mtDNA genes of local and Vietnamese Koi, control region (CR) and Cytochrome *b*(Cytb) genes were selected to study genetic structure of local and exotic Koi populations in Bangladesh. A total of 640 fish samples of local (natural habitat) and Vietnamese Koi (fish market/fish hatcheries) were collected from eight aqua-ecological regions of Bangladesh. At first, samples were used for morphometric and meristic analysis, and the results did not show major dissimilarities among different populations. Subsequently, samples were amplified using PCR reaction and the fragment size of CR and Cytb were found around 900 to 1000bp. Then sequence analysis was performed and 36 haplotypes were observed from all populations. In case of local Koi (*A. testudineus*), the highest haplotype diversity (0.66) was found in Netrakona population and lowest haplotype diversity (0.00) was found in Chottogram population. In case of Vietnamese Koi (*A. testudineus*), the highest haplotype diversity (0.86) was found in Jashore population and lowest haplotype diversity (0.25) was found in Mymensingh population. The pair wise F_{st} values ranged from 0.0 to 0.99. Within local *A. testudineus*, the highest F_{st} value (0.66) was observed in Chottogram vs. Bogura population and lowest (0.00) was observed in Sylhet vs. Khulna population. Within Vietnamese *A. testudineus*, the highest F_{st} value (0.96) was observed in Mymensingh vs. Dinajpur population and lowest (0.00) was observed in Gazipur vs. Dinajpur population. Between local and exotic *A. testudineus*, the highest F_{st} value ranged from 0.99 to 0.93 indicating these two population are completely separated from one another. The preliminary phylogenetic tree with online data produced three major clades whereas Bangladeshi Koi (*A. testudineus*) produced distinct and separate group in one clade than Vietnamese koi with other exotic Koi (*A. testudineus*) populations. Thus, mtDNA CR and Cytb genes and designed primer sets and PCR protocol could be used as molecular marker for the identification of local Koi (*A. testudineus*) from exotic Koi (*A. testudineus*) in Bangladesh. This molecular identification process will be helpful in future stock identification of Koi fish (*A. testudineus*) from natural habitats in Bangladesh. However, quality broodstock development of local koi (*A. testudineus*) is not possible without proper identification and subsequent artificial breeding and conservation.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:** Molecular identification of local and exotic strains of Koi (*Anabas testudineus*) for strategic conservation management

2. **Implementing organization:** Department of Genetics and Fish Breeding, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**

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4. **Sub-project budget (Tk):**

4.1 Total: **36,59,760.00**

4.2 Revised (if any):

5. **Duration of the sub-project:**

5.1 Start date (based on LoA signed): 11 July, 2017

5.2 End date: 30 September 2018

6. **Justification of undertaking the sub-project:**

Koi (*A. testudineus*) is a popular indigenous fish in Bangladesh, and the availability of local Koi drastically reduced in the canals, small rivers, swamps and inundated lands throughout Bangladesh. However, Thai Koi was introduced in 2002 and the stock faced several ecological, genetical and physiological problem especially growth retardation. Later fast growing Vietnamese Koi was introduced in 2011 for aquaculture, again this species also lost its acceptance due to odd flavor and outbreak of infectious diseases causing mortality. Remarkably, indiscriminate crosses of these exotic Koi become available in the market of Bangladesh. Besides, it is very possible to escape cultured exotic Koi in the natural water bodies which might facilitate crossing with local variety and result genetic corruption in indigenous species. There are very limited studies so far done on the molecular genetic structure of Koi fish in Bangladesh. Therefore, molecular characterization of local and exotic Koi strain should be carried out and recorded. Otherwise, once upon a time it will be very difficult to identify the pure strain

of local Koi from Bangladesh. Moreover, to develop quality broodstocks and increase Koi production, it is important to develop a base population with genetic purity because local Koi has high demand in the market than exotic Koi available in Bangladesh. Therefore, quality broodstock of Koi strains in Bangladesh should be detected using molecular marker. Sequence analysis of mitochondrial and nuclear DNA is one of the most advanced genetic marker to investigate genetic distinction of a population, strain and species. In the present study, these three issues will be studied covering the samples collected from all over the Bangladesh. Thus, the findings from this study will be helpful to make conservation management strategies and broodstock development in future.

7. **Sub-project goal:** Genetic structure and molecular identification of Koi fish (*A. testudineus*) in Bangladesh by marker developed in the present study

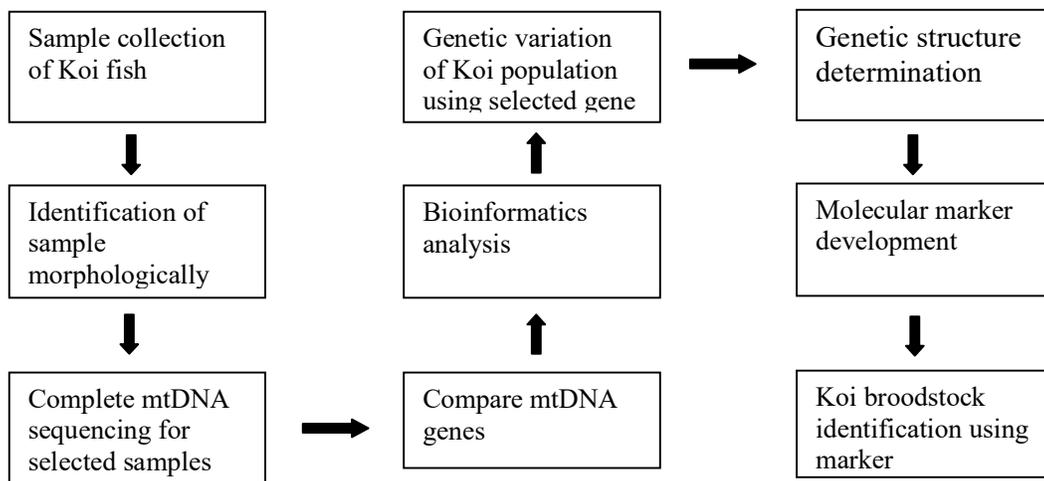
8. **Sub-project objective (s):**

- i). To study complete mitochondrial genome of local and exotic Koi (*A. testudineus*) in Bangladesh
- ii) To study genetic structure of local and exotic Koi population in Bangladesh
- iii) Molecular marker development for the prompt identification of quality broodstock of local and exotic Koi for breeding program

9. **Implementing location (s):** Department of Genetics and Fish Breeding, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur

10. **Methodology in brief:**

Following schematic diagram indicating the total approach of the research carried out:



10.1 **Sample collection:**

A total of 800 (50 × 8 × 2) fish sample of local and exotic Koi (Vietnam) was collected from eight aqua-ecological region of Bangladesh such as Barisal, Chittagong, Dhaka, Khulna, Mymensingh, Rajshahi, Rangpur and Sylhet. Local Koi was collected from the open water bodies of respective

regions of the country. The Vietnam Koi was collected from fish market and hatcheries. Thai Koi sample could not be collected due to unavailability. But a few samples were collected for mtDNA complete genome sequencing. At least 50% fish samples were used for collecting morphometric and meristic data and then preserved at -26°C in the Laboratory, Dept. of Genetics and Fish Breeding, Faculty of Fisheries, BSMRAU, Gazipur. Fin clips were done from the samples using sterilized scissors and stored in 1.5 ml eppendorf tube for DNA extraction.

10.2 DNA extraction

A pectoral fin was cut from each specimen of *A. testudineus* and preserved in 99.5% ethanol for DNA extraction. DNA extraction was performed using commercial DNA extraction kit following manufacturer's protocol. Finally, the extracted DNA from each sample were stored at -26°C for further use. The quality of the DNA was checked using 0.7% agarose gel electrophoresis stained with ethidium bromide.

10.3 Study of complete mitochondrial genome of local *A. testudineus*

Representative samples from local (Sylhet) and exotic Koi (Thai and Vietnam) were selected after morphological examination for the study of complete mitochondrial genome. The complete genome was done by second generation sequencing system. Bioinformatics analysis was carried out after sequencing. Then the complete mitochondrial genome features (13 protein genes, 2 rRNA genes, 22 tRNAs genes, CR regions) of local and exotic *A. testudineus* were compared and most suitable genes were selected (CR and Cytb) for further study of genetic variation of Koi fishes. The sequence result was compared with the online databases of Indian and Chinese *A. testudineus*. Finally, specific molecular marker of local and exotic were developed and suggested.

10.4 Study of genetic structure of local and exotic Koi population

After the complete genome sequences, mtDNA CR and Cytb genes were used to study the genetic variation of local and exotic Koi (Vietnam) available in Bangladesh.

10.5 PCR primers and amplification

The suitable gene from the first experiment along with other primer sets (Table 1) were used for test amplification of different regions of mitochondrial DNA (ND2, Cyt *b*, D-loop region, CO, 16S rRNA etc.) and the best amplification resolution was used to amplify the fish samples. For amplification, the following reagents were added to each microtube: 3 μl of template DNA; 2 μl Primer (F); 2 μl Primer (R); 25 μl PCR master mixture; 18 μl PCR grade water. PCR condition was optimized by manipulating different parameters before performing the final amplification reactions. PCR was performed in a thermal cycler with the following cycle parameters: 40 cycles of denaturation at 94°C for 1 minute annealing at 49°C for 1 minute 30 seconds and extension at 72°C for 1 minute. The cycle started with one cycle at 94°C at 2 minutes and ended with 1 cycle of 72°C for 10 minutes, followed by holding at 4°C .

Table 1. Name of the primer used in the present study

Primer name	Sequence	Reference
L4438-Met	5'-AAG CTT TTG GGC CCA TRC CC-3'	Yamanouee <i>et al.</i> , 2006
H5669-Asn	5'-AAC TGA GAG TTT GWA GGA TCG AGG CC-3'	Yamanouee <i>et al.</i> , 2006
MTA-01	5'-AAG CCA GGA TTC TAAATT AAA-3'	Hidayat and Senanan, 2010
MTA-02	5'-TCT TCA GTG TTA TGC TTT GA-3'	Hidayat and Senanan, 2010
DonGlu F	5'-AACCACCGTTGTATTCAACTACAA-3'	Ruberet <i>et al.</i> , 2004
DonThr R	5'ACCTCCGATCTTCGGATTACAAGACCG-3'	Ruberet <i>et al.</i> , 2004

To quantify the size of amplified DNA, the PCR products were subjected to electrophoresis in 0.7% agarose gel at 100 volt for 30 minute and the migration distance were compared in a gel with DNA fragments of known molecular size (1 Kbp Gene ruler). The gel was stained with ethidium bromide for about 40 minutes and washed in water before visualizing on a UV transluminator (Major science-UVDI).

10.6 PCR product purification

PCR products were purified using the Molecular Biology Purification Kit according to the manufacturer's protocol. Finally, the purified PCR products were checked again using 0.7% agarose gel electrophoresis. The purified PCR products were stored at 4°C until further use.

10.7 Sequencing

Purified PCR products of each sample from all the populations were stored and later used for sequencing analysis. At least 20 samples from each population were sequenced for further comparison and analysis. Sequencing reactions was carried out using the BigDye 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 commercially on a capillary electrophoresis DNA analyzer (ABI Prism 3130xl Genetic Analyzer; Applied Biosystems, USA).

10.8 Data analysis

Sequence editing were resolved using Chromas and then the sequence data were aligned and edited using CLUSTALW as implemented in MEGA. The haplotype were determined and homology search were done from the online gene bank and genetic distances were determined for showing relationship among local and exotic Koi (*A. testudineus*) strains using sequences by MEGA software. Nucleotide diversity, fixation index and other genetic distance index were calculated by Dnasp5. Phylogenetic analysis were conducted using MEGA (version 6).

10.9 Molecular Marker development for detection of broodstock of local Koi

The genetic structure of local and exotic Koi population were analyzed using CR and Cytb genes. Subsequently, local and exotic Koi could be differentiated observing separate phylogenetic clade and which suggest that this information could be used as molecular marker.

11. Results and discussion:

11. 1 Complete mitochondrial genome of local and exotic Koi (*A. testudineus*)

Three representative samples (Local-Sylhet; Exotic-Vietnam and Exotic-Thai from Mymensingh) were used for complete mtDNA sequence by NGS technique. The following Fig shows the extracted DNA quality and concentration of the samples. The DNA was not good in case of Thai Koi and could not proceed further NGS experiment.

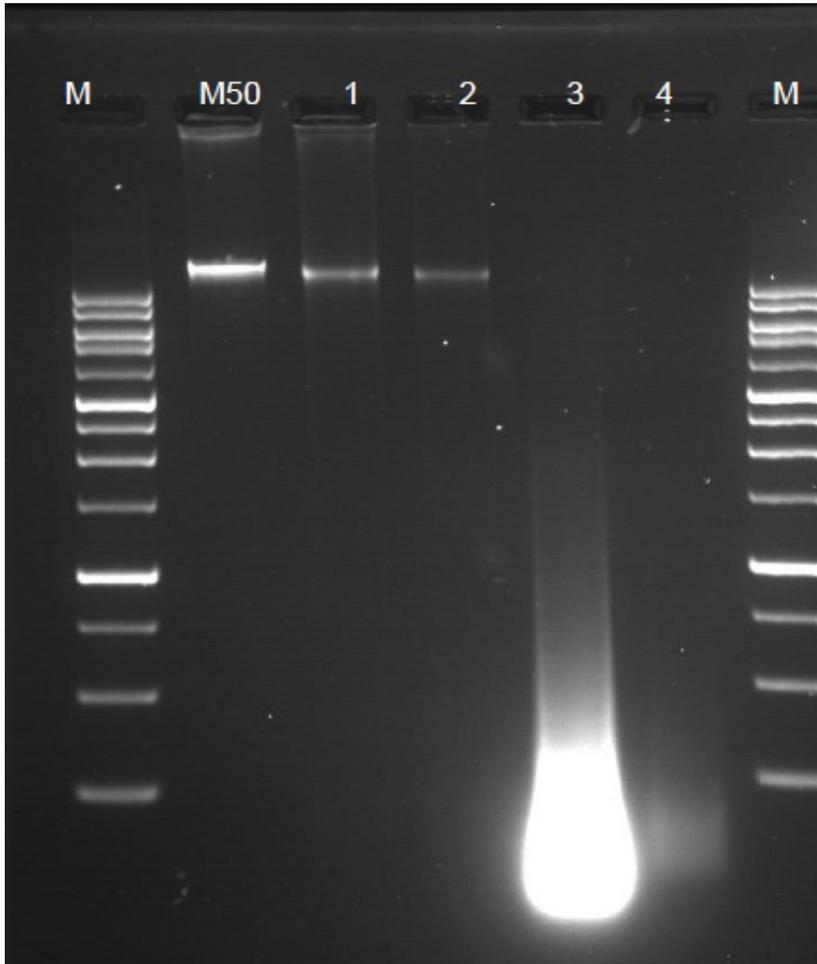


Figure No. 1. Photograph showing extracted DNA quality of three samples of Local (1), Vietnamese (2) and Thai (3) Koi used for whole genome sequencing by NGS technique. Here M50 is used for control and M is for ladder.

Table 2. The Nanodrop concentration of thee (DNA1a-Local, DNA2a-Viet, DNA3a and DNA3b- Thai Koi) samples are given as follows:

Nanodrop (water as blank)						Fluorometric Qualification			Comment	
No.	Sample	A260/280	A260/230	Conc. (ng/μl)	Volume (μl)	Total amount (μg)	Conc. (ng/μl)	Dilution factor		Total amount (μg)
1	DNA1a	1.935	1.026	156.00	85	13.26	60.59	4	20.60	OK
2	DNA2a	1.933	1.150	120.00	85	10.20	12.64	4	4.30	OK
3	DNA3a	1.750	1.241	4.90	85	0.42	40.22		3.42	Degraded DNA
4	DNA2b	1.998	0.938	55.45	85	4.71	9.07		0.77	Degraded DNA

11.2 QC and variant calling

Paired-end reads sequences were first removed of sequence adaptors and reads with low quality scores usingbbduk of the BBToolsPackages(<https://jgi.doe.gov/data-and-tools/bbtools/>). QC-reads were mapped against thereference template (KT001153.1) using bowtie 2 (1). Variant calling was done using FreeBayes (2).

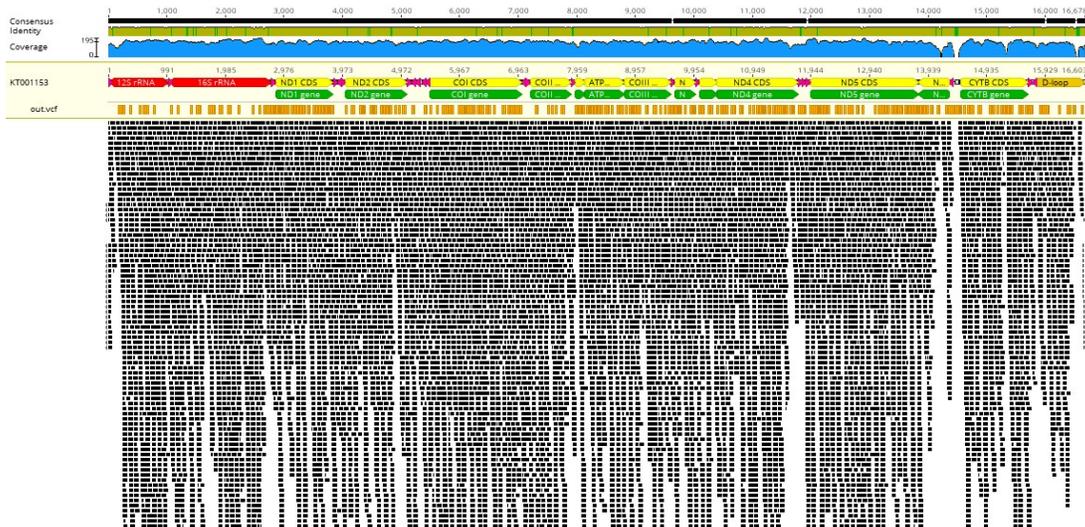


Figure No. 2. Photograph showing the map of QC-ed of local Koi(dna1a) unassembled reads onto KT001153.

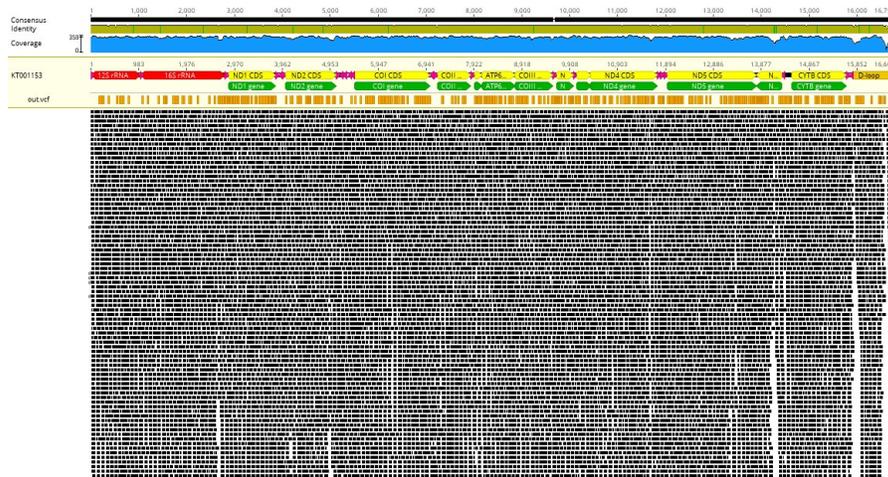


Figure No.3. Photograph showing the map of QC-edof Vietnamese Koi (*dna2a*) unassembled reads onto KT001153.

11.3 De novo assembly

QC reads were assembled *de novo* using SPAdes (3) 3.11.1. Norgal (4) was not used as paired end reads are needed for the program to run (data is present in single end format). Resulting scaffolds were subjected to MEGABLAST searches against the NCBI nucleotide database to identify for closest relative and sequence contaminant. Assembled contigs were mapped onto the reference genome using Lastz version 1.02.00 (http://www.bx.psu.edu/miller_lab/dist/README.lastz-1.02.00/README.lastz-1.02.00a.htm). The resulting Figs (below; Located in the Lastz folder) show the contigs that are mapped to the reference. Contigs not mapped likely represent sequence contaminants. The full description of the ORF and other annotations was recorded.

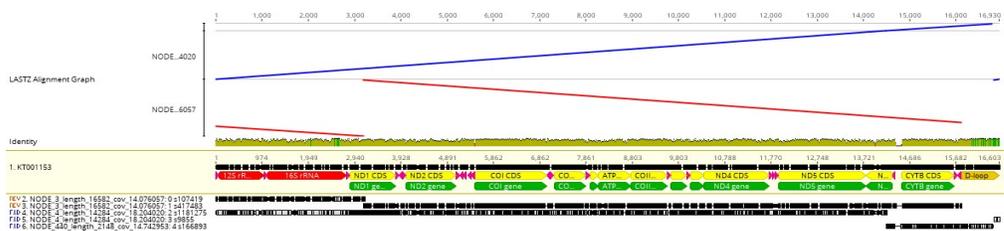


Figure No.4. Photograph showing the map of local Koi (*dna1a*) assembled contigs onto KT001153.

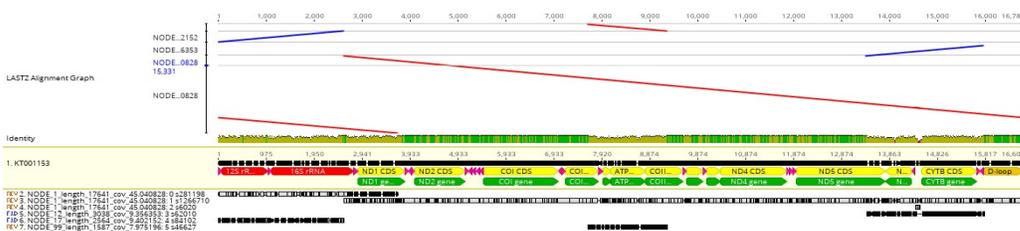


Figure No.5. Photograph showing the map of Vietnamese (*dna2a*) assembled contigs onto KT001153.

Two datasets (Local and Vietnamese Koi) were assembled to the reference genome of KT001153 (Indian Koi). Variant calling and mapping of assembled contigs suggest that the two datasets were sequence variants of KT001153 (Indian Koi).

11.4 Genetic structure of local and exotic Koi (*A. testudineus*) populations in Bangladesh

Climbing perch (*A. testudineus*) were collected from natural habitat of eight aqua-ecological regions like Barishal, Chottogram, Dhaka, Khulna, Mymensingh, Rajshahi, Rangpur and Sylhet respectively (Table 3). The eight sampling stations are showed in Figure 6 and 7. A total of 320 samples were collected. The detail information of samples used in this research is given in Table 3. The fish samples were transported to the laboratory in fresh condition by icebox. After collection, fish samples were used to take morphometric and meristic data and preserved at -20°C in the laboratory of Dept. of Genetic and Fish Breeding, BSMRAU, Gazipur until further research work.

Table 3. List of the samples used in the present study

Strain	No	Division	District	Haor/Beel
Local Koi	1	Barishal	Patuakhali	Shatla beel
	2	Chottogram	Rangamati	Kaptai lake
	3	Dhaka	Gazipur	Belai beel
	4	Khulna	Jessore	Laukhali beel
	5	Mymensingh	Netrakona	Bandha beel
	6	Rajshahi	Bogura	Jabarkandi beel
	7	Rangpur	Dinajpur	Ashura beel
	8	Sylhet	Sylhet	Murier haor
Vietnam Koi	9	Barishal	Barishal	Batajor bazar
	10	Chottogram	Noakhali	Maijdee bazar
	11	Dhaka	Narsingdi	Kalir bazar
	12	Khulna	Jashore	Rail bazar
	13	Mymensingh	Mymensingh	Sharnalata fish hatchery
	14	Rajshahi	Bogura	Chasir bazar
	15	Rangpur	Dinajpur	Bahadur bazar
	16	Sylhet	Sylhet	Golapganj bazar

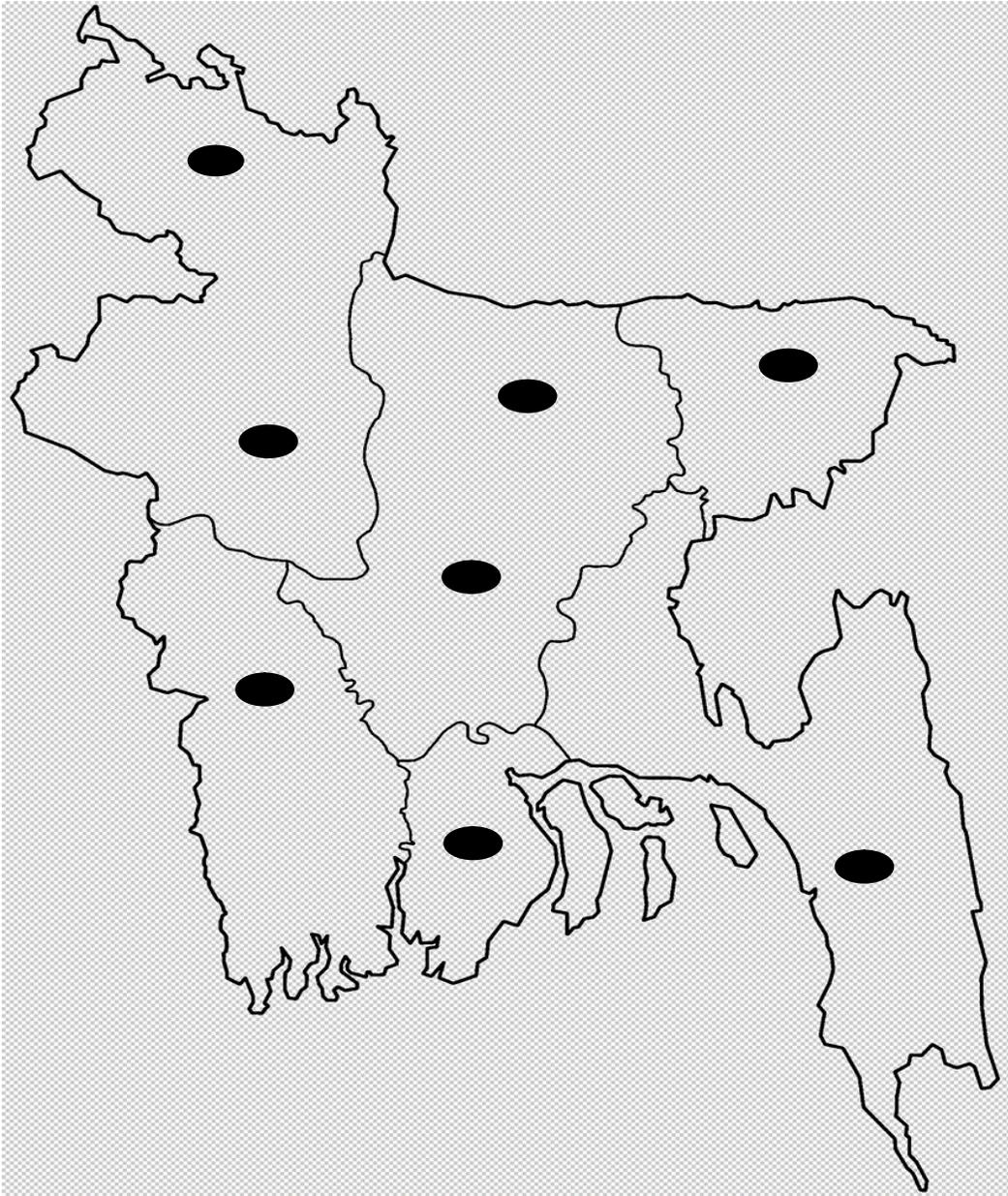


Figure No. 6. Map shows the sampling locations used in the present study.



Figure No.7. Some representative photograph of Koi fish sampling in Bangladesh.



Figure No.8. Photograph of some representative samples (Frozen sample) of Local *A. testudineus*. B=Barishal, C=Chottogram, D=Dhaka, K=Khulna, M=Mymensingh, R=Rajshahi, Rp=Rangpur, S=Sylhet.



Figure No. 9. Photograph of some representative samples (Frozen sample) of Vietnamese *A. testudineus*. B=Barishal, C=Chottogram, D=Dhaka, K=Khulna, M=Mymensingh, R=Rajshahi, Rp=Rangpur, S=Sylhet.

11.5 Morphology

At first samples were used for morphometric and meristic characteristics to check whether there was any deviation from the published reports of *A. testudineus* or not. A total 160 samples out of collected 320 samples were used for morphometric and meristic study. The samples were measured followed by Rahman (2005) and Doherty and McCarthy (2004) with some modification (Fig10). The morphological and meristic characteristics were measured by digital slide calipers and average data are shown in Table4 and 5, respectively. Detail morphometric and meristic data were recorded.

11.6 Morphometric and meristic data analysis

In case of Morphometric analysis, the highest and lowest average total length of local *A. testudineus* was found in both Bogura and Sylhet (13.91) and Khulna populations (8.37), respectively. On the other hand, the highest and lowest average total length of Vietnamese *A. testudineus* was found in Narsingdi (18.88) and Barishal populations (13.85), respectively. In case of meristic analysis, Caudal Fin Ray (CFR) was observed 15-18 for local *A. testudineus*. On the other hand, for Vietnamese *A. testudineus* the range of Caudal Fin Ray (CFR) was observed 16-17. But there is no previous information about CFR of *A. testudineus* in Bangladesh. After measuring, data were compared with published literature and no major deviations found. Thus the samples used in the present study were confirmed as *A. testudineus*.

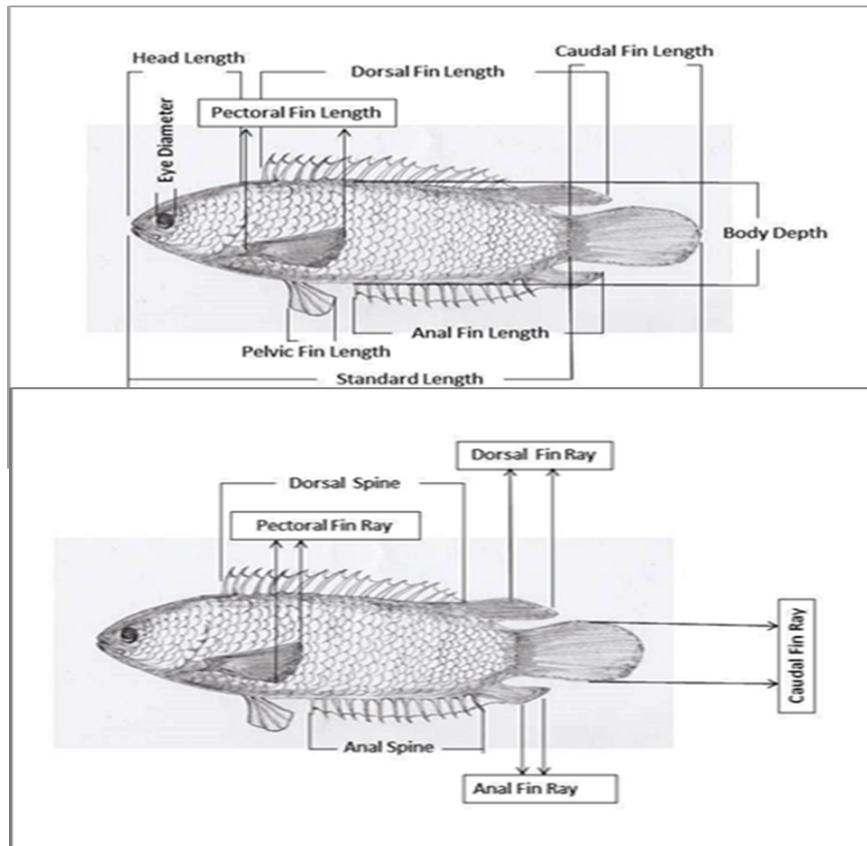


Figure No. 10: Morphometric (A) and Meristic (B) characteristics of *A. testudineus*.



Figure No. 11. Photographs of Morphometric data and DNA extraction.

Table 4. Morphometric characteristics of *A. testudineus*

SL No.	Morphometric Characteristics	Local Koi								Vietnam Koi							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	TL	12.22	13.15	13.49	8.37	11.23	13.91	13.18	13.91	13.85	15.25	18.88	15.46	16.57	16.01	14.83	16.56
2	SL	9.76	10.38	10.72	6.61	8.92	10.98	10.47	11.54	11.23	12.29	15.32	12.82	13.48	12.97	12.13	13.39
3	HL	3.22	3.41	3.53	2.19	2.95	3.52	3.31	3.33	3.61	3.78	4.78	3.76	3.88	4.07	3.54	4.42
4	BD	3.3	3.6	4.17	1.97	3.08	3.88	3.7	3.45	4.21	5	5.97	4.52	4.22	4.87	5.06	4.84
5	ED	0.5	0.5	0.5	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.59	0.5	0.5	0.5	0.5	0.58
6	DFL	6.53	6.97	7.62	3.88	6.12	7.8	3.79	7.64	8.06	8.73	11.2	8.99	9.8	9.23	8.6	9.78
7	PCFL	1.54	1.72	2.18	1.07	1.45	2.4	2.1	1.81	1.81	2.49	3.17	2.37	2.14	2.45	2.26	2.86
8	PVFL	2.05	2.33	1.7	1.54	1.85	1.75	1.65	2.24	2.21	1.84	2.45	2.05	2.53	1.91	1.85	2.3
9	AFL	4.49	4.65	4.71	2.84	4.15	5.37	4.81	5.29	5.31	5.58	6.88	5.55	6.37	5.68	5.58	6.15
10	CFL	2.46	2.77	2.77	1.76	2.31	2.93	2.71	2.37	2.62	2.86	3.56	2.62	3.08	3	2.7	3.17

1-8= Local Koi; 9-16= Vietnam Koi. TL = Total length, SL = Standard length, HL = Head length, BD = Body depth, ED = Eye diameter, DFL = Dorsal fin length, PCFR = Pectoral fin length, PVFL = Pelvic fin Length, AFL = Anal fin length, CFL = Caudal fin length.

Table 5. Meristic characteristics of *A. testudineus*

SL No.	Meristic Characteristics	Local Koi								Vietnam Koi							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	PFR	14-15	13-14	13-14	14	14	13-14	14	14-16	14-15	14-15	14-16	14-15	14-15	15	14-15	14-15
2	CFR	16	14-16	16	15-16	16-17	16-17	16	16-18	16-17	16	16-17	16-17	16-17	16-17	16-17	16-17
3	DS	16-17	16-17	16-17	14-17	16-17	16-17	16-17	17-18	17-18	17-18	16-17	17	17-18	16-17	17	17-18
4	AS	10	9-10	9-10	9-10	9-10	10-11	09-11	10-11	9	9-10	9-11	9-11	9	8-9	9	09-10
5	PVS	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
6	LLS	26-27	26-28	25-28	24-25	25-28	25-27	25-27	27-28	26-28	24-28	26-30	26-30	27-29	24-29	26-30	25-29

1-8= Local Koi; 9-16= Vietnam Koi. PFR = Pectoral fine ray, CFR = Caudal fin ray, DS = Dorsal spine, AS = Anal spine, PVS = Pelvic spine, LLS = Lateral line scale.

11.7 Molecular study

Polymerase chain reaction was done for 160 samples (In case of mtDNA CR-10 individuals from each local and Vietnamese climbing perch population) out of 320 samples. To quantify the size of PCR product of 10 individuals from each local and Vietnamese climbing perch population was subjected to electrophoresis on 0.7% agarose gel at 100 volts for 30 min. The original size of the amplified mtDNA control region for all samples of *A. testudineus* was approximately 900bp.

11.7.1 Total DNA extraction

Fin clips of *A. testudineus* were used for extracting DNA for further study. DNA extraction was performed using Gene JET genomic DNA purification kit (Thermo Scientific) by following manufacturer's protocol with slight modification.

11.7.2 DNA Extraction from fin tissue

At first, 20 mg tissue from each specimen was sliced into small pieces and placed in a 1.5 ml centrifuge tube and 180 µl of Digestion solutions were added. Then, 20 µl of proteinase K were added and mixed by vortexing and incubated at 55°C in thermomixer until the tissue was completely lysed. The eppendorf tubes were used to vortex and spin down by mini centrifuge occasionally during incubation to disperse the sample and lysis properly. Thus, the samples were lysed within 3-4 hours and then 2-4 µl of RNase A (10 mg/ml) were added and mixed by vortexing, and incubated for 10 min at room temperature (15-20). Then, vortex was done for 20 s. and centrifuge 12,000 rpm for 30s. After that, 200 µl lysis solution was added and mixed thoroughly by vortexing for 15s until a homogeneous mixture is obtained. Then 400 µl of 50% ethanol was added to the eppendorf tube and then mixed by pipetting or vortexing. Then the prepared lysate was transferred to a Gene JET genomic DNA purification column inserted in a collection tube. The column was then centrifuged for 1 min at 8000rpm. The collection tube containing the flow-through solution was discarded. Then 500 µl of Wash buffer AW1 was added and centrifuged for 1 min at 12,000 rpm. Again, 500 µl of Wash buffer AW2 was added and centrifuged for 3 min at 14,500 rpm. Again flow-through and collection tube were discarded. The Gene JET genomic DNA purification column was placed in a clean 1.5 ml eppendorf tube (not provided) and 200 µl Elution Buffer was added directly onto the Gene JET genomic DNA purification column membrane to elute genomic DNA. Then samples were incubated at room temperature for 2 min and centrifuged for 1 min 12000 rpm to elute and repeat elution to increase the volume of DNA. Finally, the samples were stored at -20°C for further use. The quality of the DNA was determined using 0.7% agarose gel electrophoresis stained with ethidium bromide.

11.7.3 PCR primers and amplification

After trials of several amplification using designed primers from the sequence of complete genome of local and Vietnam *A. testudineus* two set of primer (Table 6) was selected in the present study. However, DNA of 320(8 × 20 × 2) individuals of climbing perch, 20 from each location was used for the amplification of the mtDNA control region (CR) and Cytb. For amplification, the following reagents were added to each microtube: 3 µl of template DNA; 2 µl Primer (F); 2 µl Primer (R); 25 µl PCR master

mixture; 18 µl PCR grade water. PCR conditions were optimized by manipulating different parameters before performing the final amplification reactions. PCR was performed in a thermal cycler with the following cycle parameters: 40 cycles of denaturation at 94°C for 1 minute annealing at 49°C-55°C for 1 minute 30 seconds and extension at 72°C for 1 minute. The cycle started with one cycle at 94°C at 2 minutes and ended with 1 cycle of 72°C for 10 minutes, followed by holding at 4°C.

Table 6. List of the primer designed and used as molecular marker in the present study

Primer name	Sequence	Gene name	Reference
Koi_CR_Fow	5'-GGTTGCGGAGGTTAGAATCC-3'	Control region (1080bp)	This study
Koi_CR_Rev	5'-ATGAAGCTTTCCAGGGCTTA-3'		
LocalK_Cyb__50_Fow	5'-CCACCCAGTCATCTCT-3'	Cytb (1160bp)	This study
LocalK_Cytb_tRNA_Rev	5'-TTACAAGACCGATGCTC-3'		

To quantify the size of amplified DNA, the PCR products were subjected to electrophoresis (Mupid-2plus, Advance) in 0.7% agarose gel at 100 volt for 20 minute and the migration distance was compared in a gel with DNA fragments of known molecular size (1 Kbp Gene ruler). The gel was stained with ethidium bromide for about 40 minutes and washed in water before visualizing on a UV transilluminator (Major science- UVDI).

11.7.4 PCR product purification

PCR products were purified using the Molecular Biology Purification Kit (Thermo Scientific) according to the manufacturer's protocol. At first, PCR product: Binding buffer = 1:1, were mixed thoroughly and color of the sample was checked, yellow is optimum color. But if orange color showed, add 10µl of 3M sodium acetate could be added. Then total volume products were transferred to the gene get purification column. Centrifugation were done at 14000 rpm for 1 min, flow through were discarded. Then 700µl wash buffer were added and centrifuged at 14000 rpm for 1 min, flow through were discarded. The empty buffer were centrifuged at 14000 rpm for 1 min, flow through were removed. The column was transferred to 1.5 ml eppendorf tube and 45µl elution buffer were added and centrifuged at 14000 rpm for 1 min, gene get purification column were discarded. Finally, the yield of PCR products (45 µl) was analyzed using 0.7% agarose gel electrophoresis. The purified PCR products were stored at 4 until further use.

11.7.5 Sequencing

Purified PCR products of each sample was stored and later used for sequencing analysis. Twenty samples from each population were sequenced for further comparison and analysis. Sequencing reactions was 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 commercially on a capillary electrophoresis DNA analyzer (ABI Prism 3130xl Genetic Analyzer; Applied Biosystems, USA).

11.8 Molecular data analysis

The mtDNA CR and Cytb gene sequences were aligned and edited using CLUSTAL W (version 1.6) as implemented in MEGA (version7), followed by manual adjustments. Sequence ambiguities were resolved by checking chromatograph using Chromas software (2.1.1) and searches for sequence similarity to other sequences, which were available in the NCBI database (<http://www.ncbi.nih.gov>), using the BLAST search to confirm the identity of the sequence. The haplotype distances and UPGMA dendrogram was determined for showing relationship among population using sequences by MEGA software. Nucleotide diversity, fixation index and other genetic distance index were calculated by DnaSP6. Some Phylogenetic analysis and evolutionary distances were computed using the NJ method as implemented in MEGA (Version 7).

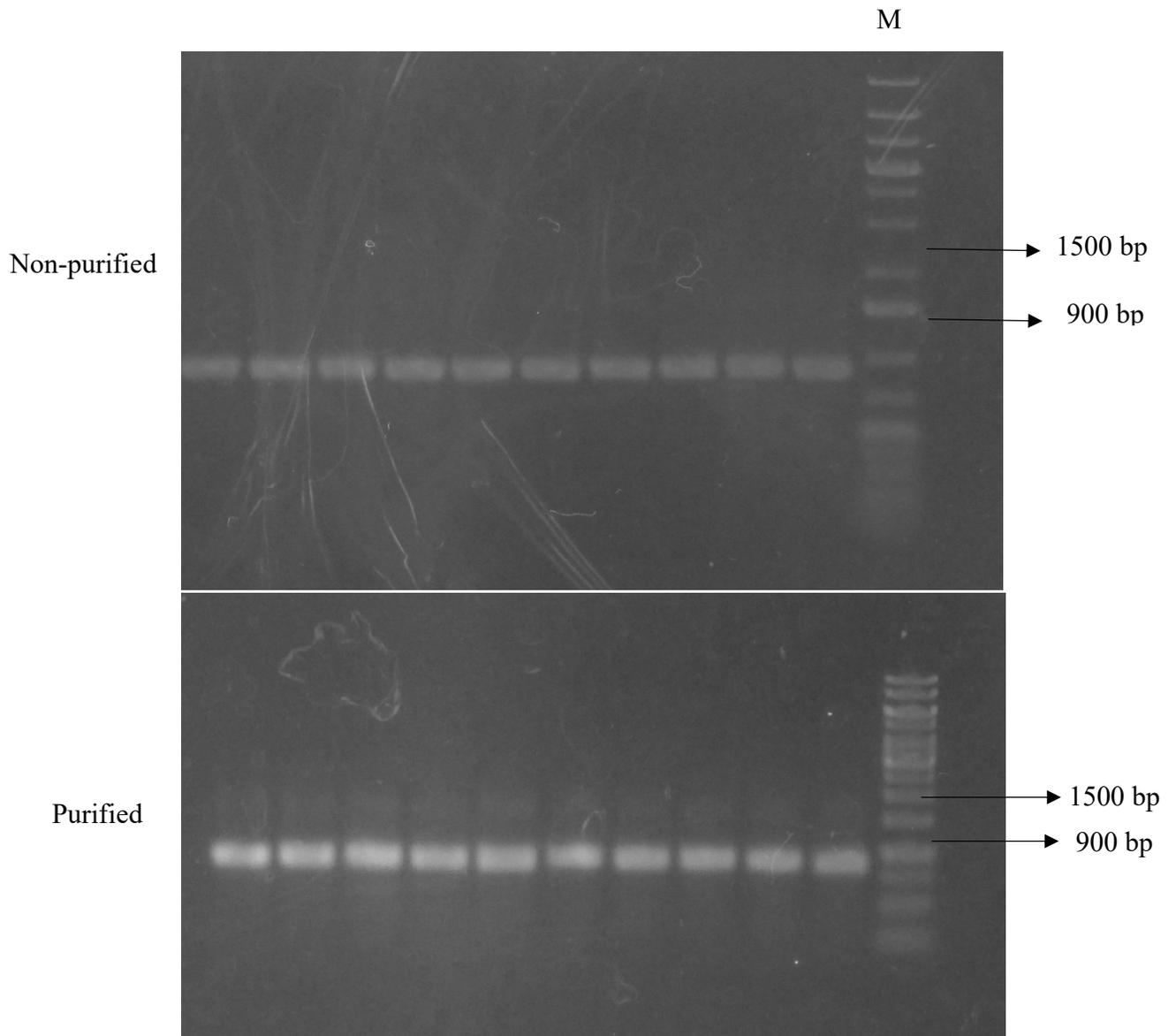


Figure No.12. Electrophoregram view of some amplification of the Control region (CR)

11.9 Sequence analysis

Representative twenty individuals from each population and a total of 320 individuals were selected for sequence analysis. Sequence data were edited by Chromas, bio-edit and the edited sequence had 700bp from the original amplified Control region sequence (900bp). Sequence data of 18 individuals (one from Noakhali viet, five from Rangamati local, three from Bogura viet, two from Sylhet viet, two from Narsingdi viet, three from Sylhet local and two from Mymensingh viet) were not good and discarded for further analysis. Therefore, total 142 sequence data were used in further analysis. Any changes of base pair in any site of sequences were considered as a haplotype. Thus, a total of 36 haplotypes were observed from 142 sequence data of the mtDNA Control region and named as h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12, h13, h14, h15, h16, h17, h18, h19, h20, h21, h22, h23, h24, h25, h26, h27, h28, h29, h30, h31, h32, h33, h34, h35, h36 and these sequence information are now processing to submit in the online Gene Bank for getting accession number. However, the Haplotype h29 was the most common in all population and represented in individuals of Rangamati, Bogura, Khulna, Patuakhali, Gazipur, Dinajpur, Sylhet and Netrakona local climbing perch population. On the other hand Haplotype h12 was the second most common in all population and represented in individuals of Noakhali, Barishal, Jashore, Dinajpur, Sylhet and Narsingdi Vietnamese climbing perch population. The list of haplotypes is showed in Table 7.

Table 7. List of population wise haplotypes and haplotypes summary

Population	Haplotypes information	Haplotypes summary
Noakhali (viet Koi)	H5,H12,h12,h8,h12,h6,h12,h12,h8	H5,h12,h8,h6
Rangamati (local Koi)	H29,h29,h29,h29,h29	H29
Bogura(local Koi)	H27,h29,h27,h27,h27,h27,h29,h27,h29	H27,h29
Khulna (local Koi)	H30,h29,h29,h34,h34,h29,h29,h26,h29,h29	H29,h30,h34,h26
Patuakhali (local Koi)	H29,h29,h29,h29,h29,h29,h29,h33,h29,h29	H29,h33
Barishal (viet Koi)	H12,h3,h14,h12,h12,h13,h12,h12,h14,h12	H12,h3,h14,h13
Jashore (viet Koi)	H7,h12,h12,h19,h21,h12,h20,h18,h17,h12	H7,h12,h19,h21,h20,h18,h17
Dinajpur (viet Koi)	H12,h12,h12,h12,h12,h12,h8,h12,h12,h12	H12,h8
Bogura (viet Koi)	H8,h4,h8,h9,h8,h10,h8	H8,h4,h9,h10
Gazipur (local Koi)	H36,h36,h36,h36,h36,h29,h29,h29,h29,h29	H36,h29
Sylhet (viet Koi)	H23,h22,h12,h17,h12,h16,h12,h23	H23,h22,h12,h17,h16
Dinajpur (local Koi)	H29,h29,h32,h29,h29,h31,h31,h29,h29,h29	H29,h32,h31
Gazipur (viet Koi)	H12,h12,h12,h12,h11,h12,h8,h15	H11,h12,h8,h15
Sylhet (local Koi)	H29,h32,h29,h29,h29,h34,h29	H29,h32,h34
Mymensingh (viet Koi)	H1,h1,h1,h1,h1,h2,h1,h1	H1,h2
Netrakona (local Koi)	H29,h29,h25,h29,h35,h24,h29,h29,h29,h28	H29,h25,h35,h24,h28

Table 8. List of haplotype variable sites found in the present study

	10	20	30	40	50	60	70	80	90	100								
Hap_1	AGTATTCCTAATTTACTTAATTGAACCCAGACGCTTCGCTCTCATCCACTCTGACGTTTTATTGCATAATAGCACTGCCTCCGCCGGGCTAGGTATCTACACC																	
Hap_2									A									
Hap_3		C					T		C	A								
Hap_4		G								A								
Hap_5			A		C					A								
Hap_6					C					A								
Hap_7		AG	G	T	CTC	G		G		A								
Hap_8	C	T	G	G	G	C	ATA	T	C	C	A	C	T	A	C	AA		
Hap_9	C	T	G	G	G	C	ATA	T	G	C	C	A	C	T	A	C	AA	
Hap_10	C	T	G	G	G	C	ATA	T	C	C	C	A	C	T	A	C	AA	
Hap_11	C	T	G	G	G	C	ATA	T	C	C	C	A	T	A	C	AA	A	
Hap_12	C	T	G	G	G	C	ATA	T	C	C	C	A	T	A	C	AA		
Hap_13	C	A	T	G	G	G	C	ATA	T	C	C	A	T	A	C	AA	A	
Hap_14	C	T	G	G	G	C	ATACT		C	C	A	T	A	C	AA			
Hap_15	TT	T	G	G	G	C	ATA	T	C	C	A	T	A	C	AA			
Hap_16	C	T	A	G	G	G	C	ATA	T	C	C	A	T	A	C	AA		
Hap_17	C	T	G	G	G	AG	C	ATA	T	G	C	C	A	T	A	C	AA	
Hap_18	C	T	G	G	AG	C	ATA	T	A	G	A	C	C	A	T	A	AA	
Hap_19	C	G	T	GA	G	G	AG	C	ATACT	G	C	C	A	T	A	C	AA	G
Hap_20	T	G	T	A	G	G	AG	C	ATA	T	G	C	A	C	A	T	A	AA
Hap_21	T	T	A	G	G	AG	C	ATA	T	G	C	C	A	T	A	C	AA	
Hap_22	C	T	A	G	G	G	C	ATA	T	G	C	C	A	T	A	C	AA	
Hap_23	C	T	G	G	G	C	ATA	T	G	C	C	A	T	A	C	AA		
Hap_24	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAGTC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_25	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.AT.GAACGA.ATAC..																	
Hap_26	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CCC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_27	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.CGAACGA.ATAC..																	
Hap_28	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.G.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_29	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_30	.ACGCCT.ACTGC.C.CC.T.GAC.A.AG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_31	.ACGCCT.ACTGC.C.GC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_32	.A.GCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_33	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_34	.ACGCCT.ACT.C.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_35	.ACGCCT.ACT.C.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.AC.G.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	
Hap_36	.ACGCCT.ACTGC.C.CC.T.GAC.A.GAG.TC.GTA.TC.GAGTCTCTCAG.ACCG.CC.T.T.CGATGTAATTCTTATTA.A.GAACGA.ATAC..																	

Table 9. List of population wise highest and lowest genetic distance among haplotypes

Population	Haplotypes	Genetic distance	Remarks
Local vs. Local	H35 vs. h24	0.004	Highest value
	H35 vs. h25	0.004	Highest value
	H35 vs. h26	0.004	Highest value
	H35 vs. h27	0.004	Highest value
	H35 vs. h28	0.004	Highest value
	H35 vs. h30	0.004	Highest value
	H35 vs. h31	0.004	Highest value
	H35 vs. h32	0.004	Highest value
	H35 vs. h33	0.004	Highest value
	H36 vs. h35	0.004	Highest value
	H29 vs. h24	0.001	Lowest value
	H29 vs. h25	0.001	Lowest value
	H29 vs. h26	0.001	Lowest value
	H29 vs. h27	0.001	Lowest value
	H29 vs. h28	0.001	Lowest value
	H30 vs. h29	0.001	Lowest value
	H31 vs. h29	0.001	Lowest value
	H32 vs. h29	0.001	Lowest value
	H33 vs. h29	0.001	Lowest value
	H34 vs. h29	0.001	Lowest value
H35 vs. h34	0.001	Lowest value	
H36 vs. h29	0.001	Lowest value	
H36 vs. h31	0.001	Lowest value	
Vietnam vs. Vietnam	H8 vs. h7	0.037	Highest value
	H10 vs. h7	0.037	Highest value
	H13 vs. h7	0.037	Highest value
	H19 vs. h2	0.037	Highest value
	H19 vs. h3	0.037	Highest value
	H19 vs. h7	0.037	Highest value
	H2 vs. h1	0.001	Lowest value
	H6 vs. h4	0.001	Lowest value
	H6 vs. h5	0.001	Lowest value
	H9 vs. h8	0.001	Lowest value
	H12 vs. h11	0.001	Lowest value
	H14 vs. h12	0.001	Lowest value
	H23 vs. h12	0.001	Lowest value
H23 vs. h17	0.001	Lowest value	
H23 vs. h22	0.001	Lowest value	
Local vs. Vietnam	H24 vs. h7	0.130	Highest value
	H25 vs. h7	0.130	Highest value
	H26 vs. h7	0.130	Highest value
	H27 vs. h7	0.130	Highest value
	H28 vs. h7	0.130	Highest value
	H35 vs. h8	0.100	Lowest value
	H35 vs. h9	0.100	Lowest value
H35 vs. h10	0.100	Lowest value	

The Neighbour-Joining (NJ) tree showed the evolutionary relationship among haplotypes produced by the mtDNA Control region analysis (Fig 13). As in Fig 13, two clusters were formed whereas haplotype h1- h23 produced one cluster and haplotype h24-h36 produced another cluster. First cluster again subdivided into 2 sub-clusters as h1- h7, and h8- h23.



Figure No. 13. Evolutionary relationship among 36 haplotypes of *A. testudineus*.

Haplotype distribution (%) and diversity of Local and Vietnamese Climbing perch was showed in Fig 14 and Figure No. 15, respectively. In case of Local Climbing perch, Rangamati population had only one haplotype, so haplotype diversity was zero (0). On the other hand, highest five haplotypes were found in Netrakona population and haplotype diversity was 0.66. In case of Vietnamese Climbing perch,

Dinajpur population had two haplotypes and so haplotypes diversity was 0.20. On the other hand, highest seven haplotypes were found in Jashore population and haplotype diversity was 0.86.

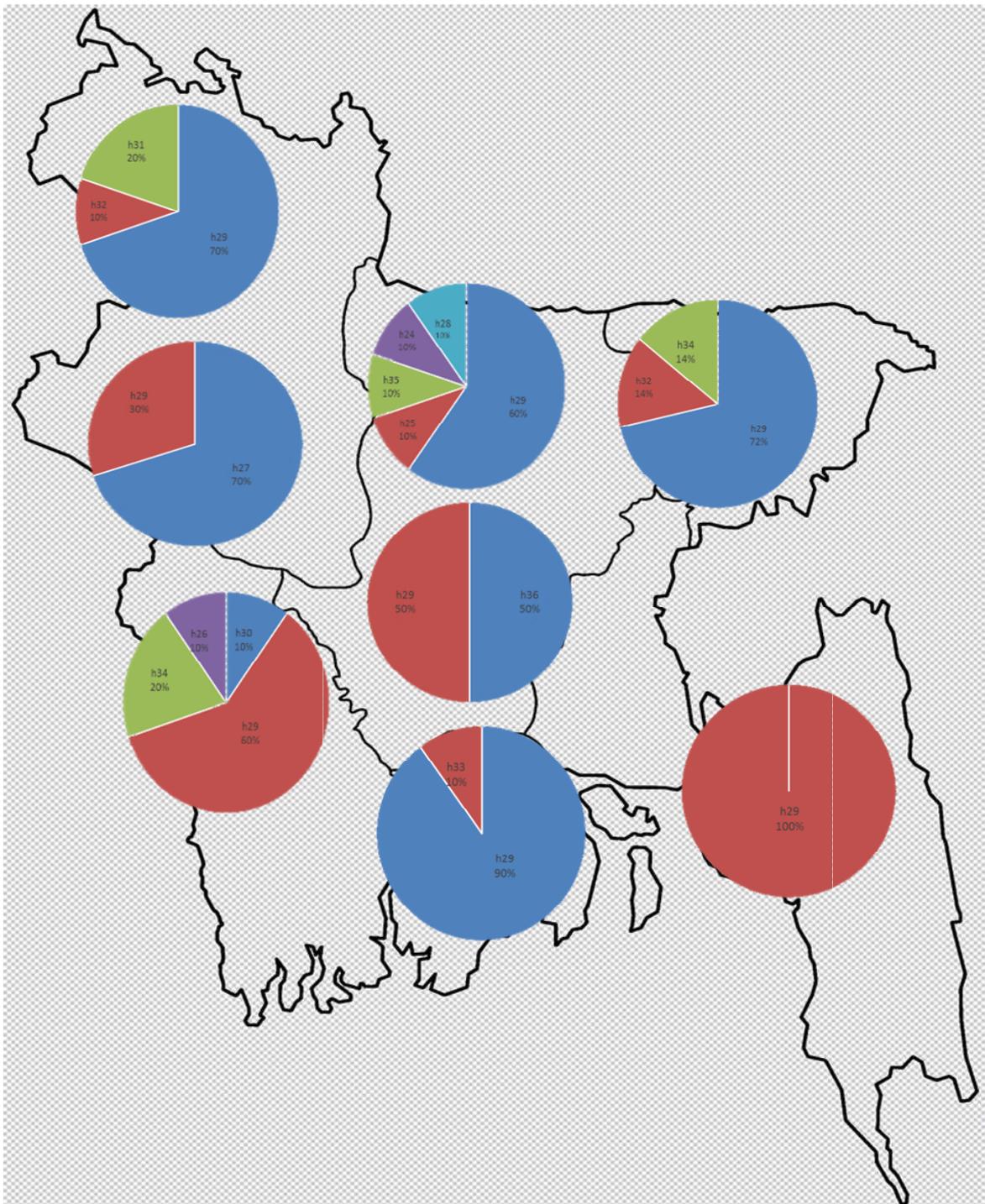


Figure No.14. Distribution of haplotypes (in percentage) for Local *A. testudineus* in eight sampling locations

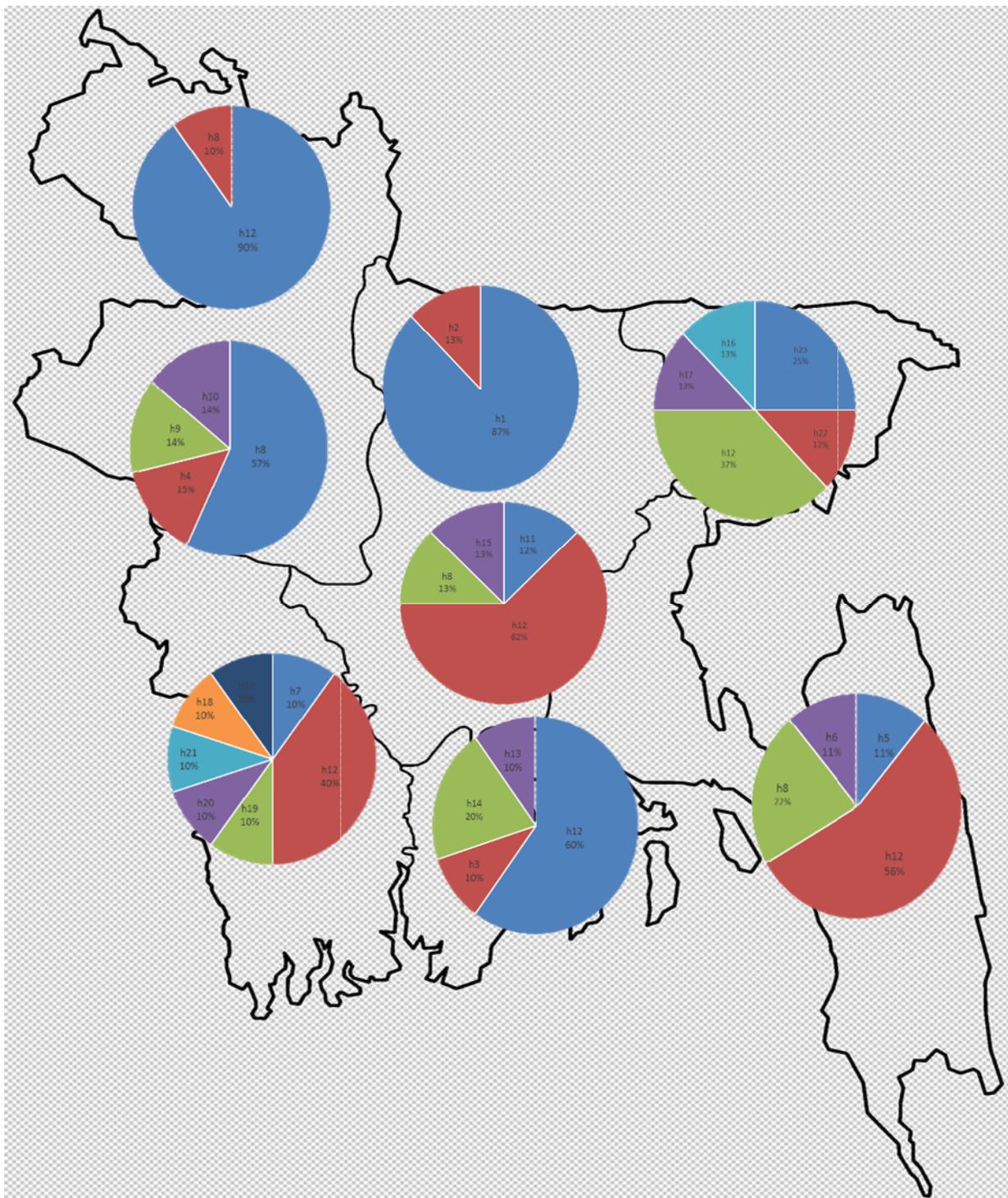


Figure No.15. Distribution of haplotypes (in percentage) for Vietnamese *A. testudineus* in eight sampling locations

11.10 Genetic variation within populations

Sequence data were obtained for 700 bases of the mtDNA Control regions for total 142 individuals. From this 142 individuals, total 36 variable mt DNA Control regions were identified. So sequence result of mtDNA control region produced 36 haplotypes. In case of local Climbing perch, Rangamati population showed single haplotype represented as h29 haplotype from five individuals. Therefore, haplotype diversity within the individuals of Rangamati was zero (0). Accordingly, haplotype diversity, nucleotide diversity was also zero (0). Therefore, Rangamati samples were genetically alike. Bogura population showed two haplotypes represented as h27, h29. So haplotype diversity within the individuals of Bogura was 0.46 and nucleotide diversity was 0.0006. So, Bogura population is genetically distinct. Similarly, Khulna population showed four types of haplotypes represented as h26, h29, h30, h34. So haplotype diversity within the individuals of Khulna was 0.64 and nucleotide diversity was 0.0001. So, Khulna population is genetically distinct. Patuakhali population showed two haplotypes represented as h29, h33. So haplotype diversity within the individuals of Patuakhali was 0.20 and nucleotide diversity was 0.0002. So, Patuakhali population is genetically distinct. Gazipur population showed two haplotypes represented as h29, h36. So haplotype diversity within the individuals of Gazipur was 0.55 and nucleotide diversity was 0.0008. So, Gazipur population is genetically distinct. Dinajpur population showed two haplotypes represented as h29, h36. So haplotype diversity within the individuals of Dinajpur was 0.51 and nucleotide diversity was 0.0008. So, Dinajpur population is genetically distinct. Sylhet population showed three haplotypes represented as h29, h32, h34. So haplotype diversity within the individuals of Sylhet was 0.52 and nucleotide diversity was 0.0008. So, Sylhet population is genetically distinct.

Netrakona population showed five haplotypes represented as h24, h25, h28, h29, h35 from ten individuals. So haplotype diversity within the individuals of Netrakona was 0.66 and nucleotide diversity was 0.0014. Therefore, there was higher genetic variation in Netrakona population than the other seven populations.

In case of Vietnamese Climbing perch, Narsingdi population showed four haplotype represented as h5, h6, h8, h12 haplotype from nine individuals. Therefore, haplotype diversity within the individuals of Narsingdi was 0.69 and nucleotide diversity was also 0.0106. Therefore, Narsingdi samples were genetically distinct.

Bogura population showed four haplotypes represented as h4, h8, h9, h10 from seven individuals. So haplotype diversity within the individuals of Bogura was 0.714 and nucleotide diversity was 0.0086. So, Bogura population is genetically distinct.

Jashore population showed seven types of haplotypes represented as h7, h12, h17, h18, h19, h20, h21. So haplotype diversity within the individuals of Jashore was 0.86 and nucleotide diversity was 0.011. So, Jashore population is genetically distinct.

Barishal population showed four haplotypes represented as h3, h12, h13, h14. So haplotype diversity within the individuals of Barishal was 0.64 and nucleotide diversity was 0.006. So, Barishal population is genetically distinct.

Narsingdi population showed four haplotypes represented as h8, h11, h12, h15. So haplotype diversity within the individuals of Narsingdi was 0.64 and nucleotide diversity was 0.0025. So, Narsingdi population is genetically distinct.

Dinajpur population showed two haplotypes represented as h8, h12. So haplotype diversity within the individuals of Dinajpur was 0.20 and nucleotide diversity was 0.0011. So, Dinajpur population is genetically distinct.

Sylhet population showed five haplotypes represented as h12, h16, h17, h22, h23. So haplotype diversity within the individuals of Sylhet was 0.85 and nucleotide diversity was 0.002. So, Sylhet population is genetically distinct.

Mymensingh population showed two haplotypes represented as h1, h2 from eight individuals. So haplotype diversity within the individuals of Mymensingh was 0.25 and nucleotide diversity was 0.0003. So, Mymensingh population is genetically distinct. Therefore, there was higher genetic variation in Jashore population than the other seven populations.

11.11 Genetic variation between populations

A total of 36 haplotypes were observed after sequence analysis of 142 individuals. In case of local Climbing perch, Nucleotide diversity was zero in Rangamati population. So, there is no sign of interbreeding or random breeding of Rangamati population with other population. In the Patuakhali population nucleotide diversity 0.0002 indicates very few sign of interbreeding status of this population with other populations. Similarly, nucleotide diversity of Bogura population was 0.0006. Dinajpur, Gazipur, Sylhet populations showed nucleotide diversity 0.0008. Khulna and Netrakona populations showed nucleotide diversity 0.001. So, there is some status of interbreeding or random breeding of Khulna and Netrakona populations with other populations.

In case of Vietnamese Climbing perch, highest nucleotide diversity was found 0.01 for Jashore and Narsingdi populations. So, there is some status of interbreeding or random breeding of Jashore and Narsingdi populations with other populations. Lowest nucleotide diversity was found 0.0003 for Mymensingh population. This indicates very few sign of interbreeding status of this population with other populations.

Pairwise F_{st} value ranged from 0.0 to 1.0. In case of local vs. local climbing perch, highest F_{st} value was found 0.66667 for Rangamati local vs. Bogura local climbing perch. Lowest F_{st} value was found 0.00000 for Sylhet local vs. Khulna local climbing perch. In case of Vietnamese vs. Vietnamese climbing perch, highest F_{st} value was found 0.96970 for Mymensingh Vietnamese vs. Dinajpur Vietnamese climbing perch. Lowest F_{st} value was found 0.00000 for Narsingdi Vietnamese vs. Dinajpur Vietnamese climbing perch. In case of local vs. Vietnamese climbing perch, highest F_{st} value was found 0.99835 for Rangamati local vs. Mymensingh Vietnamese climbing perch. Lowest F_{st} value was found 0.9372 for Netrakona local vs. Jashore Vietnamese climbing perch.

Table 10. List of pairwise Fst value of *A. testudineus* in the present study

Location	Fst value	Remarks
Local Koi vs. local Koi		
Rangamati local vs. Bogura local	0.66667	Highest value
Sylhet local vs. Khulna local	0.00000	Lowest value
Vietnamese Koi vs. Vietnamese Koi		
Mymensingh Viet vs. Dinajpur Viet	0.96970	Highest value
Narsingdi Viet vs. Dinajpur Viet	0.00000	Lowest value
Local Koi vs. Vietnamese Koi		
Rangamati local vs. Mymensingh Viet	0.99835	Highest value
Netrakona local vs. Jashore Viet	0.93720	Lowest value

Sequence analysis of mtDNA Control region showed 36 haplotypes. In case of Local Climbing Perch, Netrakona population showed higher genetic diversity and Rangamati population showed lower genetic diversity. The genetic variation between Rangamati vs. Bogura showed lower genetic diversity indicating low migration rate, and Sylhet vs. Khulna showed higher genetic diversity indicating maximum migration rate for Local vs. Local Climbing Perch populations. In case of Vietnamese Climbing Perch, Jashore population showed higher genetic diversity and Dinajpur population showed lower diversity. The genetic variation between Mymensingh vs. Dinajpur showed lower genetic diversity indicating low migration rate and Narsingdi vs. Dinajpur showed higher genetic diversity indicating maximum migration rate for Vietnamese vs. Vietnamese Climbing Perch populations. On the other hand, genetic variation between Rangamati Local vs. Mymensingh Vietnamese populations showed lower genetic diversity indicating low migration rate, and Netrakona Local vs. Jashore Vietnamese populations also showed lower genetic diversity indicating lower migration rate for Local vs. Vietnamese Climbing Perch populations (Table 8).

11.12 Homology search to Phylogenetic tree

Phylogenies were constructed by the mtDNA Control region sequences with the sequences data obtained from the gene bank after homology blast search. The detailed sequence information of homology search used to construct phylogenetic tree is given in the Table 11 and Fig 9. Shows the relationships among taxa resolved in mtDNA Control region phylogenetic analysis.

Table 11.List of sequence data of *A. testudineus* used to draw phylogenetic tree

Location of collection	Accession Number
India	KT001153
India	KX950694
China	KJ808811
Vietnam	EF179144
Malaysia	GU570698
Malaysia	GU570699
Malaysia	GU570700
Malaysia	GU570701
Malaysia	GU570702
Malaysia	GU570703

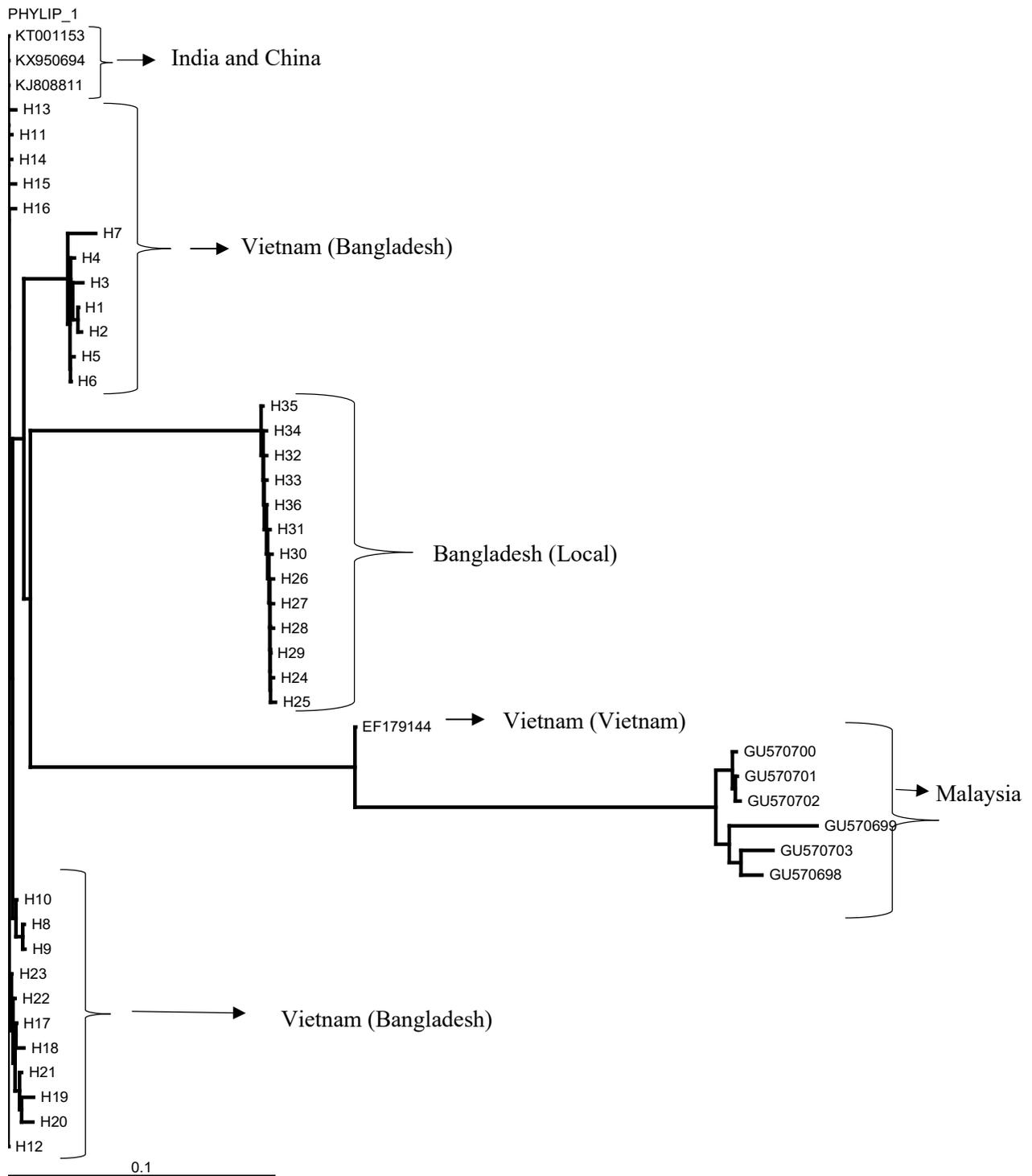


Figure No.16. Phylogenetic relationships constructed by mtDNA Control region of different populations of *A. testudineus* from different countries.

The mtDNA is often used in genetic diversity and population genetic structure studies as it contains abundant intra and interspecific phylogenetic information. It is also a good marker to infer the impact of genetic differentiation among species within the same genus or the same family (Rahim *et al.*, 2011).

A total of 160 Local and Vietnamese *A. testudineus* samples were used for morphometric and meristic analysis. After measuring, data were compared with some published book and did not show any major deviation. Thus, the samples used in the present study were confirmed as *A. testudineus*.

According to sequence analysis of mtDNA Control region, 36 haplotypes were observed after sequence analysis of 142 individuals from eight locations. In case of Local Climbing perch, Maximum number of haplotypes was found in Netrakona population whereas single haplotype was found in Rangamati population. In case of Vietnamese Climbing perch, Maximum number of haplotypes was found in Jashore population whereas only two haplotypes were found in Mymensingh and Dinajpur population. According to Rahim *et al.* (2011), the sharing or overlapping of haplotypes is probably based on geographical proximities and similar climatic condition of location where the impact of migration could be high.

The average haplotype diversity was 0.84 in the present study whereas Hidayat and Senanan (2010) found average haplotype diversity as 0.4 in *A. testudineus* in Thailand. Rahim *et al.*, (2011) found haplotype diversity as 0.8 in *C. striata* in Malaysia. Brykovet *al.* (2002) found average haplotype diversity as 0.6 in *Carassius auratus*, and Weiss *et al.* (2000) found the value as 0.3 in *Salmo trutta*. According to Xiao (2008), high haplotype diversity (0.94) was observed in willow flounder (*Tanakius kitaharai*) which suggests large, stable, and effective population sizes, environmental heterogeneity, and life-history traits that enable rapid population expansion (Liu *et al.*, 2008). Therefore, the haplotype diversity values at present study are seems to be similar from some previous studies.

Haplotype diversity (HD) in Netrakona Local population was around 0.66, which was found highest value in the findings of above study. Similarly, higher haplotype values were also found from Netrakona Local population. Whereas, haplotype diversity was lowest (0.00) in Rangamati Local population. Lowest haplotype frequency was 10% in Khulna, Patuakhali, Dinajpur and Netrakona Local population and higher haplotype frequency (100%) was observed in Chottogram Local population.

Haplotype diversity (HD) in Jashore Vietnamese population was around 0.86, which was found highest value in the findings of above study. Whereas, haplotype diversity was lowest (0.25) in Mymensingh Vietnamese population. Lowest haplotype frequency was 10% in Barishal, Jashore Vietnamese population and higher haplotype frequency (90%) was observed in Dinajpur Vietnamese population.

The present study also revealed the inter-population genetic distance in 16 populations of *A. testudineus* based on the Nei's (1983) and Jost's (2008) genetic distance. In present study, pair wise F_{st} values (Table 10) were used to test the pattern of genetic differentiation between populations. According to Wright

(1978), F_{st} value ranging from 0 - 0.05 indicates little genetic differentiation, 0.05 - 0.15, 0.15-0.25 and >0.25 indicate moderate level, considered level and large level of genetic differentiation, respectively.

Therefore, the little genetic differentiation was observed in Sylhet vs. Khulna population and large level of genetic differentiation was observed in Rangamati vs. Bogura for the case of Local Climbing Perch. On the other hand, the little genetic differentiation was observed in Narsingdi vs. Dinajpur population and large level of genetic differentiation was observed in Mymensingh vs. Dinajpur for the case of Vietnamese Climbing Perch. Here, no little genetic differentiation was observed in Local vs. Vietnamese Climbing Perch population and large level of genetic differentiation was observed in Rangamati local vs. Mymensingh Vietnamese Climbing Perch.

Lower F_{st} value of Sylhet Local vs. Khulna Local Climbing Perch, Narsingdi Vietnamese vs. Dinajpur Vietnamese Climbing Perch indicated that they were less isolated. The individual can freely inter breed and higher migration also occurred between them. On the contrary, higher values denoted gene flow, lower allelic frequency and lower inter breeding between Rangamati Local vs. Bogura Local Climbing Perch, Mymensingh Vietnamese vs. Dinajpur Vietnamese Climbing Perch, Rangamati local vs. Mymensingh Vietnamese Climbing Perch (Table 10).

Phylogenetic tree revealed that all Bangladeshi populations as same from the other population that means they are closely related with each other.

In the present study, preliminary phylogeny was constructed by the partial sequence of mtDNA Control region with the similar sequences of *A. testudineus* obtained from Gene Bank including Bangladeshi *A. testudineus* (Fig 16). Phylogenetic analysis showed significant distinction of Bangladeshi population compared to others. Clade analysis also revealed the Vietnamese populations as distinct from the others (Fig 16). Furthermore, Indian population of *A. testudineus* was closer to Bangladeshi Vietnamese than the others. Bangladeshi samples in the present study represent specific area and also related with Malaysian samples. Therefore, the preliminary phylogeny from the present study indicates diversified cryptic findings from *A. testudineus* and interesting evolutionary relationship will be identified when studied with more samples from all over the country and abroad in future.

11. 13 Marker development

The local and exotic Koi (*A. testudineus*) populations in Bangladesh could be isolated using mtDNA CR and Cyb genes and the designed primer set with PCR protocol and sequence information obtained can be used as molecular marker for the prompt identification of samples.

12. Research highlight/findings:

- i. The present study has provided complete mtDNA sequence information of local and exotic Koi (*A. testudineus*) populations in Bangladesh
- ii. Mitochondrial gene can be used as molecular marker for Koi fish in Bangladesh
- iii. Local and exotic Koi in Bangladesh can be identified using mtDNA CR and *Cytb* gene sequences
- iv. Genetically problematic wild stock of Koi fish can be identified
- v. Information regarding cross breed Koi from the field can be found
- vi. Quality broodstock of local Koi will be identified and developed
- vii. Artificial breeding of local Koi using quality broodstock for conservation and commercial propagation will be possible
- viii. Long term and short term conservation management strategy of Koi fish can be adopted in Bangladesh

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	1. Visitor chair (3) 2. Computer Table (1) 3. Computer Chair (1) 4. File cabinet (1)	40500	1. Visitor chair (3) 2. Computer Table (1) 3. Computer Chair (1) 4. File cabinet (1)	40400	Procurement method and other details are in the RFQ document
(b) Lab & field equipment	1. Micropipette (0.1 to 1000 μ L)-3 set 2. Deep Freezer (1) 3. Mini gel chamber with power supply (1) 4. Minicentrifuge (2) 5. Thermomixer (1) 6. Distill water plant (1) 7. Laptop Computer (1)	875000	1. Micropipette (0.1 to 1000 μ L)-3 set 2. Deep Freezer (1) 3. Mini gel chamber with power supply (1) 4. Minicentrifuge (2) 5. Thermomixer (1) 6. Distill water plant (1) 7. Laptop Computer (1)	8716000	Procurement method and other details are in the RFQ document
(c) Other capital items					

2. Establishment/renovation facilities:

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	
Laboratory renovation for molecular work			Cabinet lock system developed and Thai work, lighting etc. for facilities developed for molecular work	100%	

3. Training/study tour/ seminar/workshop/conference organized: N/A

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	289260	269982	283102	-13120	98	Fund remain for PI Honorium
B. Field research/lab expenses and supplies	1945000	1910645	1944493	-33848	100	Vat, tax and other purposes
C. Operating expenses	195000	192481	192870	-389	100	
D. Vehicle hire and fuel, oil & maintenance	140000	140063	127920	12143	91	
E. Training/workshop/seminar etc.	0	0	0	0	0	
F. Publications and printing	125000	0	15000	-15000	12	Fund not received for publication from NATP-2 office
G. Miscellaneous	50000	50707	44900	5807	90	
H. Capital expenses	915500	913545	912000	1545	100	
Total	3659760	3477423	3520285	-42862		Fund not released

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output(i.e. product obtained, visible, measurable)	Outcome(short term effect of the research)
To study complete mitochondrial genome of local and exotic Koi (<i>A. testudineus</i>) in Bangladesh	i) High quality DNA extraction of Local, Vietnam and Thai Koi ii) Check DNA quality iii) NGS for complete mtDNA sequence of two samples iv) Analysis done for assembling NGS sequence	i) Complete mtDNA sequence of local Koi with minor gaps (97bp-1bp) in ten places compare to Indian/Chinese Koi fish ii) Complete mtDNA sequence of Vietnam Koi with minor gap (43bp) in one place compare to Indian/Chinese Koi fish Sample of Thai Koi was not good and DNA was not good and could not proceed for NGS	i) Gene by gene comparison of mtDNA of local and exotic Koi in Bangladesh is possible now
To study genetic structure of local and exotic Koi population in Bangladesh	i) Sample collection from eight division of Bangladesh ii) Amplification of several targeted genes iii) Suitable primer designed, amplification, sequence and analysis was done	i) Genetic structure of local and exotic Koi population was studied	i) Local and exotic Koi population in Bangladesh can be identified by knowing genetic structure
Molecular marker development for the prompt identification of quality broodstock of local and exotic Koi for breeding program	i) Analysis of complete mtDNA and several genes ii) Suitable primer designed	i) Prompt identification of local and exotic Koi population in Bangladesh	i) Local and exotic Koi population in Bangladesh could be identified by the marker developed from the present study

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication	1		Complete mitochondrial genome sequences of local and exotic climbing perch (<i>Anabas testudineus</i>) available in Bangladesh (Submitted in the 4th IPFS-ICBHA-2019 Conference will be held on 11-13 November, 2019 in Dhaka University, Dhaka)
Information development			
Other publications (MS Thesis)		2	<p>1. Genetic variation of Climbing Perch (<i>Anabas Testudineus</i>) populations in Bangladesh revealed by sequence analysis of mitochondrial control region (MS Thesis)</p> <p>2. Genetic variation of Climbing Perch (<i>Anabas Testudineus</i>) populations in Bangladesh revealed by sequence analysis of mitochondrial Cytb gene (MS Thesis)</p>

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

Molecular marker and genetic makeup of mtDNA of local and Exotic Koi were developed in Bangladesh

ii. Generation of new knowledge that help in developing more technology in future

Molecular identification of Koi fish in Bangladesh for quality brood stock development and conservation

iii. Technology transferred that help increased agricultural productivity and farmers' income

Molecular laboratory can be set up and prompt identification of quality brood fish can be ensured

iv. Policy Support

- i) Record keeping of molecular documentation of any exotic fish species eg. Koi should be mandatory for Bangladesh, when enter into Bangladesh for culture or any other purpose
- ii) It will protect local Koi fish for any contamination via gene introgression by exotic Koi and purity of Local fish will be maintained

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring[description & output of consultation meeting, monitoring workshops/seminars etc.):

- A. Participated in the Progress Review Workshop was held on 10-11 April, 2018 in BARC and presented the research result.
- B. Participated in the Projectwise monitoring workshop was held on 15-16 May, 2018 and received the comment and suggestion
- C. Participated in the Annual Review workshop was held on 3-3 October, 2018 and nicely presented the research result.

ii) Field Monitoring (time& No. of visit, Team visit and output): N/A

I. Lesson Learned

- i) High quality DNA extraction (for NGS work) is very challenging though ordinary DNA extraction is enough for normal molecular work
- ii) Morphological identification of immature Vietnam Koi fish is sometimes difficult
- iii) Sample collection is time specific not all the year round

J. Challenges

- i) Sudden damage of any laboratory molecular instrument and subsequent repair is time consuming and delay the whole work and also need additional financial help otherwise it's very difficult to complete work in time
- ii) Assembling of complete mtDNA sequence from NGS requires many sophisticated software and lacks of full continuous sequence and need further amplification and sequencing to fill up the gaps and it need extra time and fund
- iii) Some molecular chemicals (especially primer) order and delivery in hand was time consuming and finally hampered the research to be completed in time.

Signature of the Principal Investigator

Date

Seal

Counter signature of the Head of the organization/authorized representative

Date

Seal

J References

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