Project ID: 820

Competitive Research Grant

Sub-Project Completion Report

on

DNA barcoding of common native livestock & poultry and crossbred animals in Bangladesh: potential uses in conservation for increasing production

Project Duration

May 2017 to September 2018

Animal Biotechnology Division, National Institute of Biotechnology

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

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

AMOVA	:	Analysis of molecular variance
Cyt-B	:	Cytochrome B gene
COI	:	Cytochrome c oxidase I gene
MEGA	:	Molecular Evolutionary Genetics Analysis
mtDNA	:	Mitochondrial DNA
NIB	:	National Institute of Biotechnology
PCR	:	Polymerase Chain Reaction

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Executive Summary

DNA barcoding, recently-developed molecular tool, used widely to confirm the species or breed origin of an unknown specimen can be a reliable and practical tool with the potential to authenticate livestock food and non-food products and protect local biodiversity of livestock genetic resources and their wild relatives. The goal of DNA barcoding project was to create a barcode reference library for common livestock and poultry available in Bangladesh that will help in patenting, species/breed identification and to set priorities for the conservation of local livestock and poultry genetic resources. For DNA barcoding, a standardized molecular tag located in the mitochondrial cytochrome c oxidase I gene (COI) or cytochrome b (Cyt b) or D-loop was used to rapid and accurate discrimination of common livestock and poultry available in Bangladesh (Cattle, buffaloes, goats, sheep, chickens, pigeon and duck). According to availability, 4 to 7 regions (area) were selected for sampling for each species. About 11-67 samples were collected for different species. A total of 985 samples were collected from different regions of Bangladesh. All samples were processed, DNA was extracted, PCR amplification and sequencing was done. Out of 985 samples, 884 sequences were found to be of good quality. Success rate of sequencing was about 90%. Amplified cytochrome oxidase subunit-1 (CO1), D-loop and cytochrome b (Cyt b) genes of 8 species of common livestock and poultry were sequenced. Evolutionary analyses were conducted in MEGA5, consisting of diversity analysis, distance analysis, and phylogeny analysis. The evolutionary distance between a pair of sequences was measured by the number of nucleotide substitutions (i.e. transition and/or transversion) or differences occurring between the mAMOVA indicated that less than 80% of the genetic variation occurred between regions (geographical areas) while more than 20% of the variation occurred among individuals within regions, implying significant genetic differentiation between regions, this indicated clear mtDNA differentiation between specimens collected "not structured population". DNA Barcodes were created by the online barcode preparing software for each species analyzed against COI and Cy-B gene. The result of this study is based on only few outlines of the population and thus require further in depth study before drawing any conclusion.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**
   DNA barcoding of common native livestock & poultry and crossbred animals in Bangladesh: potential uses in conservation for increasing production

2. **Implementing organization:**
   National Institute of Biotechnology (NIB)

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: Taka 2566625
   4.2 Revised (if any): None

5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed) :08 May 2018
   5.2 End date :30 September 2018

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

Depending on the species, domestication of animals took place in different areas and periods. After domestication, livestock spread over all inhabited regions of the earth, accompanying human migrations and becoming also trade objects. This required an adaptation to different climates and varying styles of husbandry and resulted in an enormous phenotypic diversity. Approximately 200 years ago, the situation started to change with the rise of the concept of breed. Animals were selected for the same visible characteristics, and crossing with different phenotypes was reduced. This resulted in the formation of different breeds, mostly genetically isolated from other populations in different countries. The biological diversity of each country is a valuable and vulnerable natural resource. Sampling, identifying, and studying biological specimens are among the first steps toward protecting and benefiting from biodiversity. Last several hundred years, taxonomic description of

species is largely accomplished through morphological characterizations (Rosso et al., 2012). However, misidentifications occurred because of features such as phenotypic plasticity, genotypic variation, cryptic species, or differing life stages. A few decades ago, selection pressure was increased again with intensive production focusing on a limited range of types and a subsequent loss of genetic diversity. For short-term economic reasons, farmers have abandoned traditional breeds. As a consequence, during the 20th century, at least 28% of farm animal breeds became extinct, rare or endangered. Moreover, over-harvesting of natural resources is leading to species depletion, extinction and the economic collapse of industries that rely on them. The situation is alarming in developing countries, where native breeds adapted to local environments and diseases are being replaced by industrial breeds. In the most marginal areas, farm animals are considered to be essential for viable land use and, in the developing world a major pathway out of poverty (Bondoc OL, 2013b). For an example, Bangladesh is trying to meet up the huge demand of milk through inseminate native cows with high performing imported semen haphazardly. Now, inbreeding and the chance of extinction of native cattle are the serious problem where native breeds adapted to local environments and diseases are being replaced by industrial breeds. DNA barcoding, using a standardized molecular tag located in the mitochondrial cytochrome c oxidase I gene (CoxI) or cytochrome b (Cyt b) or D-loop or mitochondrial 16S rRNA has become a dominant approach for the rapid and accurate discrimination of animal species (Hebert et al., 2003; 2005). Taxonomic biases of one form or another are known to narrow the scope of both ecological and conservation research to particular taxa (Robert et al., 2008).

DNA barcoding, recently-developed molecular tool, is widely used to confirm the species or breed origin of an unknown specimen. It is practical tool with the potential to authenticate livestock food and non-food products and protect local biodiversity of livestock genetic resources and their wild relatives. A single gene sequence would be sufficient to differentiate all, or at least most, animal species, and proposed the use of the mitochondrial DNA gene Cox1 as a global bio identification system for animals (Hebert et al., 2003). The sequence was likened to a barcode, with species being delineated by a particular sequence or by a tight cluster of very similar sequences. Besides cox1, Cyt b has been considered as the most useful gene for phylogenetic work, and is probably the best known mitochondrial gene with respect to structure and function of its protein product (Esposti et al., 1993). Recent study showed that, D-loop has also been used for birds (Ramadan et al., 2011b), cattle and buffalo breeds identification (Ramadan et al., 2008). Moreover, homologous segments of mitochondrial 16S rRNA from four animal species belonging to family Bovidae, including river buffalo, cattle, sheep and goat (Ramadan et al., 2011a). Shafer et al. (2015) recently reviewed the promise of genomics in conservation science, and the usefulness and limitations of DNA barcodes for conservation broadly have been debated extensively in this journal (Rubinoff, 2006) and elsewhere (Krishnamurthy & Francis, 2012). Difficulties in species monitoring hamper assessment and enforcement efforts. For a barcoding approach to species identification to succeed, within-species DNA sequences need to be more similar to one another than to sequences in different species. Recent studies show that this is generally the case, but there are exceptions. Hybridization among species would also create taxonomic uncertainty: mitochondrial DNA is maternally inherited and any hybrid or subsequent generation would have the mtDNA of the maternal species only.

Mitochondrial DNA sequences from fecal and blood samples have been used to study population structure and timing of isolation events in giant pandas in China (Zhu et al., 2011), this type of uses suggest DNA barcoding could be a useful tool for population studies. Nonlethal methods of DNA sampling for species monitoring are increasingly necessary due to ethical and legal restrictions (Powell & Proulx, 2003), and these include hair traps (e.g., for wild boar [Susscrofa] (Choi et al. 2014) and Asiatic black bear [Ursustibetanus] (Yamaguchi et al., 2014), scat collection (Ruell & Crooks, 2007), biopsies (e.g., wing punches from bats (Sing et al., 2013), claw clippings from tigers (Reddy et al., 2012), skin exuviates from snakes (Khedkar et al., 2016), and legs from butterflies (Wilson et al., 2015).

In Bangladesh, this is the first attempt in the way to identify common native animal and poultry genetic resources for their conservation and patenting for long term use & ownership through DNA barcoding. Very recently Bangladesh Livestock Research Institute studied on mtDNA diversity BLRI breed-I (Mufti et al., 2011). In another study on genetic diversity and maternal origin of Bangladeshi chicken by Bhuyan et al. (2013) using mitochondrial control region (D-loop). They stated that Bangladeshi chicken originated from Indian Subcontinent and South Asian Subcontinent. Although they started work with mtDNA, they did not set any proper identification system of different animal genetic resources.

7. Sub-project goal:

The goal of DNA barcoding project is to create a barcode reference library for common livestock and poultry available in Bangladesh that will help in patenting, species/breed identification and to set priorities for the conservation of local livestock and poultry genetic resources.

8. Sub-project objective (s):

a) To construct a DNA barcoding database for common livestock and poultry available in Bangladesh.

b) To clarify genetic relationships and breeding history of domestic breeds, crossbreeds (hybrids), and their wild relatives.

c) To determine breed traceability within some species (buffaloes, goats, sheep, chickens, pigeon and ducks)

9. Implementing location (s):

Animal Biotechnology Division, National Institute of Biotechnology (NIB)

10. Methodology in brief:

10.1. Construction of DNA barcoding database for common livestock and poultry:

Sample Collection

The samples were collected from across the country on the basis of geographical distribution and abundance of animal species & their crossbred animal and poultry species. According to availability, 4 to 7 regions (area) were selected for sampling for each species. Sample sizes were determined by "resource equation" method (Festing, 2006). About 11-17 biological specimens were collected (whole blood, tissue or hair) from each geographical area/selected region for each species of

common livestock, (cattle, sheep, goat and buffaloes) and poultry (chicken, duck and pigeon) and crossbred animals available in Bangladesh on the basis of availability and morphological characteristics (Annexure-1). Sample collection, DNA extraction and PCR amplification were completed as per target of the project. A total of 985 biological samples were collected. Samples were carried from the sample collection site to the laboratory of animal biotechnology division at National Institute of Biotechnology (NIB) immediately for DNA extraction and sequencing. Plan of sample collection, DNA extraction and PCR amplification is shown in Table 1.

Table 1: Plan of sample collection, DNA extraction and PCR amplification

Species	**Location of sampling**	**Biological specimens (Nos)**	**DNA extraction**	**PCR completion**
Cattle	Tangail, Norsingdhi, Sirajgonj, Barishal, Noakhali, Natore, Thakurgaon	155	155	155
Sheep	Khulna, Natore, Tangail, Noakhali, Thakurgaon	136	136	136
Goat	Bandarban, Manikgonj, Noakhali, Savar, Tangail, Thakurgaon	120	120	120
Buffaloes	Tangail, Jamalpur, Natore, Noakhali, Sylhet	135	135	135
Chicken	Tangail, Natore, Savar, Bandharban, Gajipur, Thakurgaon	149	149	149
Duck	Naogoan Duck Farm, Kishorgonj, Natore, Noakhali, Sunamgonj, Sylhet	184	184	184
Pigeon	Tangail, Natore, Savar, Sylhet	106	106	106
Total		985	985	985

Extraction of DNA

Total DNA were extracted from biological specimens using standardized salting out protocol and DNA extraction kit according to the instruction of manufacturer (Table 1). The extracted DNA was stored at -20°C until use for molecular analysis by Polymerase Chain Reaction (PCR). The quality and concentration of DNA was checked using Nanodrop Spectrophotometer and agarose gel electrophoresis.

PCR amplification

For the barcoding study, universal reference primers for the mitochondrial Cytochrome C Oxidase I gene (COI), Cytochrome b and D-loop gene was used for PCR amplification. Several sets of primer were designed for amplification of DNA from each species. The primer sequence and conditions for amplification of DNA has been summarized in the Table 2. Agarose gel (2%) was used to visualize the DNA banding pattern. A sample picture is shown in Figure 1.

Table 2: Summary of specific genes with primers and amplification conditions of DNA by PCR

Species	Primers	Sequences	Gene fragments for each species	Annealing Temp (°C)	Fragment size (bp)
Cattle	MT-RNR2_F	CGCCTGTTTATCAAAAACAT	Cy-B	57	550
	MT-RNR2_R	CTCCGGTTTGAACTCAGATC			
Buffalo	COIbF	TTTCAACCAACCACAAAGACATCGG	COI	59	749
	COIbR	TATACTTCAGGGTGTCCAAAGAATCA			
Goat	Cy-F	CGCCATGCTACTAATTCTTGTT	Cy-B	58	330
	Cy-R	TGTCCTCCAATTCATGTGAGTGT			
Sheep	COIbF	TTTCAACCAACCACAAAGACATCGG	COI	59	749
	COIbR	TATACTTCAGGGTGTCCAAAGAATCA			
Chicken	L16750	AGGACTACGGCTTGAAAAGC	D-loop	55	539
	H 547	ATGTGCCTGACCGAGGAACAAG			
Duck	BirdF1	TTCTCCAACCACAAAGACATTGGCAC	COI	49	749
	BirdR1	ACGTGGGAGATAATTCCAAATCCTG			
Pigeon	CO1-F	ACGCTTTAACACTCAGCCATCTTACC	COI	52	751
	CO1-R	AACCAGCATATGAGGGTTCGATTCCT			

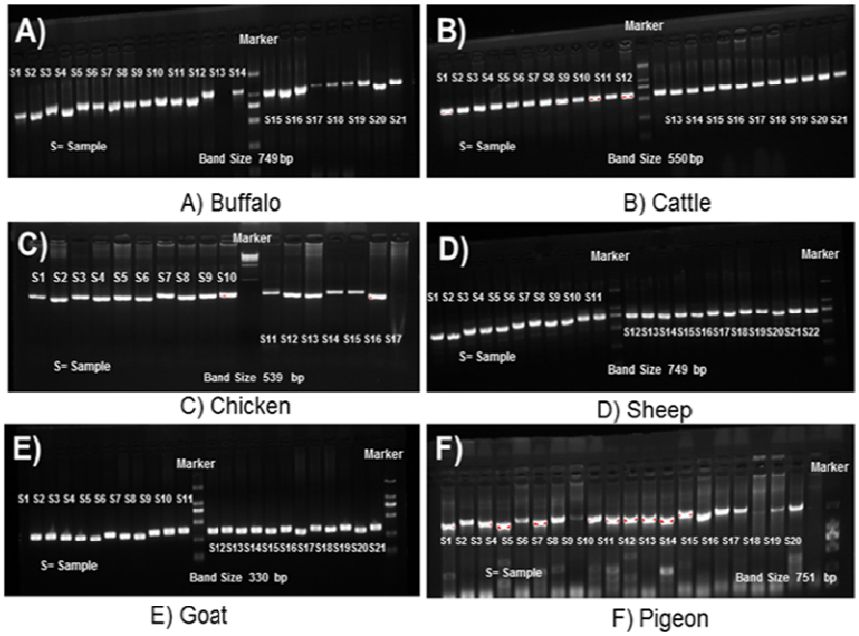

Figure 1: A representative picture of gel electrophoresis

DNA sequencing

PCR amplification and sequencing of 985 samples were completed. PCR products were purified with the QIA-quick PCR Purification Kit (Qiagen). Purified product was sent to the Molecular Biotechnology Division of NIB for sequencing. Both forward and reverse sequences were generated.

10.2. Analysis of genetic relationships and breeding history of domestic breeds-crossbreeds and determination of breed traceability within some species:

Analysis of the information/data

The sequence data was analyzed using Bioedit version 7.2. Sequence alignment was done using ClustalW of MEGA version 6 (Molecular Evolutionary Genetics Analysis). Phylogenetic analysis was conducted using MEGA version 6 (Tamura et al., 2011). Sequence divergence values within and between species were calculated in Arlequen software, using the Kimura two parameter (K2P) distance model (Kimura et al., 1980), hereafter referred to as distance (D). A phenogram (neighbour joining tree of D) was created in Arlequen to display the similarities and differences in the barcode sequences of animal and poultry included in the study. Analysis of molecular variance (AMOVA) and F-st were calculated using Arlequen. DNA barcodes were prepared using online barcode preparing software. Sequence data have been submitted to the Barcode of Life Database (BOLD, http://www.boldsystems.org) and to GenBank.

11. Results and discussion:

11.1. Construction of DNA barcoding database for common livestock and poultry:

Sequence features:

A total of 985 PCR products were sent for sequencing. Out of 985 PCR products, 884 sequences produced good results. Success rate of sequencing was 90%. In this study, we analyzed seven different species of common livestock and poultry. Amplified cytochrome oxidase subunit-1 (CO1), D-loop and cytochrome b (Cyt b) genes of 7 species of common livestock and poultry available in Bangladesh were sequenced. Sequence length of CO1 averaged 700 bp and 90% of the amplified sequences were larger than 600 bp. In the case of Cyt b, the sequence length was 550 bp for cattle and 330 bp for goat. D-loop was used only to sequence chicken DNA. Based on 700 COI positions, average genetic distance (in d units) was highest among sheep breeds (d>1), followed by buffalo (d = 0.78), duck (0.40) and lowest among pigeon breeds (d = 0.009). Present analyses indicated that DNA barcodes can separate individuals into families and most genera or species. As a promising alternative to the traditional species identification based on morphological characters, partial cytochrome c oxidase subunit I (COI) sequences (DNA barcodes) have been suggested for standardized and routine species identification (Hebert et al., 2003). DNA barcoding can distinguish individuals of a species because genetic variation between species exceeds that within species. It (generally) contributes powerfully to taxonomic and biodiversity research. It (specifically) complements taxonomy, molecular phylogenetics (deal with evolutionary relationships among deeper clades), and population genetics (deal with variation within and among populations of a single species).

In biodiversity assessment studies, DNA barcoding may be used in facilitating species identification, highlighting cases of range expansion for known species, flagging previously overlooked species, and enabling identifications where traditional methods cannot be applied. DNA barcodes are especially useful to (1) distinguish species differences in animals by overcoming the deficits of

morphological approaches to species discrimination, (2) quantify intra-specific diversity, (3) recognize sibling species, (4) be objective in taxonomic decisions in all life stages, and (5) allow single laboratories to execute taxon diagnoses (Hebert et al., 2003a).

While the taxonomy of animals remains largely reliant on dated morphological studies, molecular data such as DNA barcodes promise to expedite a newly detailed understanding of this fauna. DNA barcoding may be used to identify animal species that cannot be distinguished by traditional methods or require expensive equipment or advanced training. Although levels of genetic differentiation do not dictate taxonomic status, barcode analysis illuminates those taxa and those segments of their ranges where further research is justified. Furthermore, taxonomic decisions cannot be based simply on COI or Cy-B or D-loop sequences, but barcode surveys are a powerful tool for rapidly identifying those species in need of further investigation. DNA barcoding as a uniform, practical method for species identification could benefit the biological scientific community, educators, and interested public.

Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007), consisting of diversity analysis, distance analysis, and phylogeny analysis. The sequences were aligned using ClustalW (Thompson et al., 1994), (http://www.ebi.ac.uk/clustalw/). The evolutionary distance between a pair of sequences was measured by the number of nucleotide substitutions (i.e. transition and/or transversion) or differences occurring between them. Groups of taxa within a farm species (congeneric) were created based on their evolutionary relatedness or based on the geographic origin of the breed/strain.

11.2. Analysis of genetic relationships and breeding history of domestic breeds-crossbreeds and determination of breed traceability within some species:

i) Diversity analysis

Diversity analysis involved the calculation of sequence divergence using the Kimura 2-parameter or K2P model (Kimura, 1980) and the standard errors of the estimates using the bootstrap method. Standard error estimate(s) were obtained by a bootstrap procedure (1000 replicates), Nei and Kumar (2000). Kimura's two parameter model of base substitution (1980) corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites. A genetic diversity within the taxa (intra-specific divergence) of 2% may justify the effectiveness of COI barcodes as an identification tool to discriminate among species of mammals and birds (Hebert et. al., 2003a). A sequence threshold of ten times the average intraspecific variation could be used to identify those cases where a current species might represent more than one taxon (Hebert et al., 2004a). The average nucleotide diversity was observed to be 0.0002 ± 0.0005 in all populations. Inter population nucleotide divergences (dxy) was low, average 0.0664, intra population divergences (dx or dy) were 0.0650. Average evolutionary divergence over all sequence pairs (CO1) was 0.084±0.01. Relatively higher haplotype (0.923 ± 0.008) and nucleotide (0.015 ± 0.001) diversities were observed in chicken population. This was consistence with Bhuyan *et al. (*2013). Haplotype diversity and nucleotide diversity of mtDNA are the important indices for assessing

population polymorphism and genetic differentiation. The value of haplotype diversity and nucleotide diversity of mtDNA are bigger, the population polymorphism is higher. The numbers of polymorphisms were only 5 in sheep, 104 in pigeon, 31 in goat, 63 in duck, 143 in chicken, 211 in cattle, 16 in buffalo, which suggests population expansion in cattle and chicken, other specie population analyzed were reducing.

ii) Distance analysis

To test whether genetic distances within species (or group) are less than those between species (or group), the evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) with their variances estimated by a bootstrap approach. The average distance between sequence pairs are in the units of the number of base substitutions per site (i.e. d units). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Within group mean distance was estimated as the average evolutionary divergence over sequence pairs within groups. Between group difference was used to estimate the evolutionary divergence over sequence pairs between groups. Net between group mean distances was an estimate of net evolutionary divergence between groups of sequences. The net average distances between groups of taxa was given by dA = dXY – ((dX + dY)/2), where, dXY is the average distance between groups X and Y, and dX and dY are the mean within-group distances.

Pair-wise genetic distance of each species was done based on pair-wise distance analysis using Maximum Composite Likelihood method in the current study. All genetic distance was summarized as shown in Table 3 to 9. Population three and five had genetically lesser distance (0.09) in case of pigeon. In case of sheep, population one and two were found same i.e no distance. Lesser distance was found in population one and three in the goat population. Highest distance was observed in population two and five of buffalo. Lesser distance was observed in population two and five of cattle. Highest distance was observed in population two and four in chicken (0.78) and two and seven in duck (0.40).

Table 3: Genetic distance between each pair among the pigeon population

	1	2	3	4	5	6	7
1	-						
2	0.19682	-					
3	0.05441	-0.19653	-				
4	-0.04188	0.06008	-0.06739	-			
5	0.36532	0.08943	0.09818	0.35697	-		
6	0.19609	-0.34672	-0.23077	0.04012	0.17562	-	
7	0.22624	-0.16038	-0.11628	-0.01316	0.50992	-0.24000	-

Table 4: Genetic distance between each pair among the sheep population

	1	2	3	4
1	-			
2	0.0000	-		
3	-0.03448	-0.21992	-	
4	-0.03448	0.00339	-0.0000	-

Table 5: Genetic distance between each pair among the goat population

	1	2	3	4
1	-			
2	0.10714	-		
3	-0.05263	0.45205	-	
4	-0.03694	-0.03205	-0.07958	-

Table 6: Genetic distance between each pair among the buffalo population

	1	2	3	4	5
1	-				
2	0.0000	-			
3	0.0000	-0.02564	-		
4	0.43023	0.42402	0.07178	-	
5	0.95585	0.97689	0.63415	0.17051	-

Table 7: Genetic distance between each pair among the cattle population

	1	2	3	4	5	6
1	-					
2	0.49698	-				
3	0.49645	0.30380	-			
4	0.49380	0.18919	0.24051	-		
5	-0.00502	0.06147	0.06254	0.05640	-	
6	-0.15930	0.53610	0.53722	0.53519	0.11087	-

Table 8: Genetic distance between each pair among the chicken population

	1	2	3	4	5
1	-				
2	0.38927	-			
3	0.52629	-0.18719	-		
4	0.78648	0.06693	-0.05984	-	
5	0.38618	-0.19568	-0.18820	0.06629	-

Table 9: Genetic distance between each pair among the duck population

	1	2	3	4	5	6	7	8
1	-							
2	-0.00742	-						
3	-0.17230	-0.04590	-					
4	-0.24979	-0.00761	-0.17243	-				
5	-0.18079	-0.09939	-0.16703	-0.18087	-			
6	-0.06216	-0.04566	-0.12434	-0.06217	-0.23313	-		
7	0.14003	0.40477	0.05957	0.14041	0.22486	0.19270	-	
8	-0.17610	-0.04595	-0.18803	-0.17607	-0.16516	-0.12067	0.06693	-

iii) Analysis of molecular variance (AMOVA)

AMOVA has been used to calculate level of genetic differentiation among different populations this was to see whether or not there was a population structuring. Population wise AMOVA was calculated for each species (Table 10 to 16).

Table 10: Analysis of molecular variance (AMOVA) for chicken population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	4	805.563	21.30440 Va	21.46	0.21460	0.05
Within populations	24	1871.333	77.97222 Vb	78.54		

Table 11: Analysis of molecular variance (AMOVA) for duck population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	7	902.232	-7.88097 Va	5.01	-0.05007	0.72
Within populations	29	4793.200	165.28276 Vb	105.01		

Table 12: Analysis of molecular variance (AMOVA) for pigeon population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	6	294.958	4.00801 Va	10.15	0.10154	0.13
Within populations	17	602.917	35.46569 Vb	89.85		

Table 13: Analysis of molecular variance (AMOVA) for cattle population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	5	996.050	29.49426 Va	34.17	0.34169	0.01
Within populations	23	1306.950	56.82391 Vb	65.83		

Table 14: Analysis of molecular variance (AMOVA) for buffalo population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	4	33.191	1.48022 Va	43.71	0.43715	0.02
Within populations	17	32.400	1.90588 Vb	56.29		

Table 15: Analysis of molecular variance (AMOVA) for sheep population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	3	0.306	-0.00638 Va	-4.89	-0.04886	0.91
Within populations	18	2.467	0.13704 Vb	104.89		

Table 16: Analysis of molecular variance (AMOVA) for goat population

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index (FST)	P-value
Among populations	3	15.300	-0.30705 Va	-4.86	-0.04860	0.79
Within populations	16	106.000	6.62500 Vb	104.86		

For mtDNA, 10% (among) and 90% within was Sharply not structured populations, this means that was not mtDNA variation in the population but if higher percentage in "among" 80% and 20% within, AMOVA indicated that 80% of the genetic variation occurred between regions (geographical areas) while only 20% of the variation occurred among individuals within regions, implying significant genetic differentiation between regions, this indicated clear mtDNA differentiation between specimens collected "structured population". The analysis results showed that except pigeon population all other species population under this study was not in "structured population". A study was conducted by Bhuyan *et al.* (2013), the AMOVA calculation showed the genetic variation among five populations was 11.0 % while the remaining 89 % of the total variation was accounted for within population diversity with an overall UST value of 0.1084 in case of chicken in Bangladesh. Our result was behind from their results. This may be happened because of lower sample size or sampling error.

iv) Phylogeny analysis

Phylogeny analysis to infer the evolutionary history used the Neighbour-Joining (NJ) method. To more stringently test the discriminatory power of COI barcodes, we examined the nearest-neighbour distance, the minimum genetic distance between a species and its closest congeneric relative. The NJ method has the advantage of speed, and performs strongly when sequence divergences are low, so it is generally appropriate for recovering intra- and interspecies phylogeny (Hebert et al, 2004a). High bootstrap support for species nodes suggests neighbour-joining analysis of COI barcode sequences will be widely effective (e.g., Ward et al., 2005; Hajibabaei et al., 2006). Another way of identifying species in need of taxonomic scrutiny involves the search for taxa whose specimens from two or more distinct clusters with high bootstrap support (i.e. 98%) in a neighbour-joining tree.

A NJ tree of K2P distances was created to provide a graphic representation of the pattern of divergences among taxa or animal breeds/strains (Saitou and Nei, 1987). The NJ method uses distance measures to correct for multiple hits at the same sites, and chooses a topology showing

the smallest value of the sum of all branches as an estimate of the correct tree. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). As a general rule, if the bootstrap value for a given interior branch is 95% or higher, then the topology at that branch is considered "correct". The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. While the NJ method does not require the assumption of a constant rate of evolution so it produces an unrooted tree, MEGA, for ease of inspection, displays NJ trees in a manner similar to rooted trees.

The BLAST searches by each sample sequence in GenBank revealed the closest matches with the same species and nearest neighbour (NN) of same or different genus. COI barcode sequence of Sample of our buffaloes and Egyptian buffaloes showed 99% identity. COI sequences of sample sheep, duck and pigeon showed 99%-100% identity with *Ovis aris*, *Anasplatyrhynchos* voucher and Columba livia respectively and below 97% NN similarity with same or different genus was also recorded. Cy-B sequences barcode sequence of Sample of our cattle and *Bos indicus* crossbred showed 99% identity below 97% NN similarity with *Bos frontalis* and *Bos indicus* shahiwal breed was also recorded. All phylogenetic analyses are shown in Figures 2 (Buffalo), 3 (Sheep), 4 (Duck), 5 (Pigeon), 6 (Cattle) and 7 (Chicken).

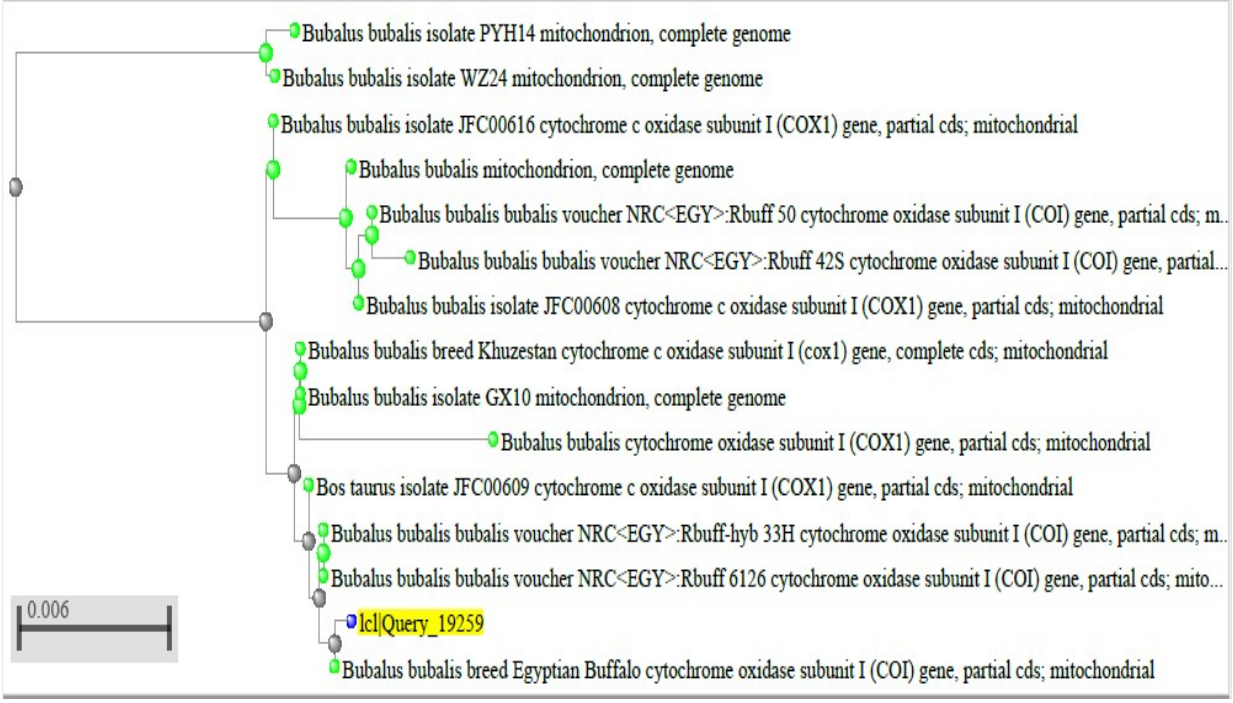

Figure 2: K2P distance neighbour-joining tree of COI sequences from several species of Buffalo

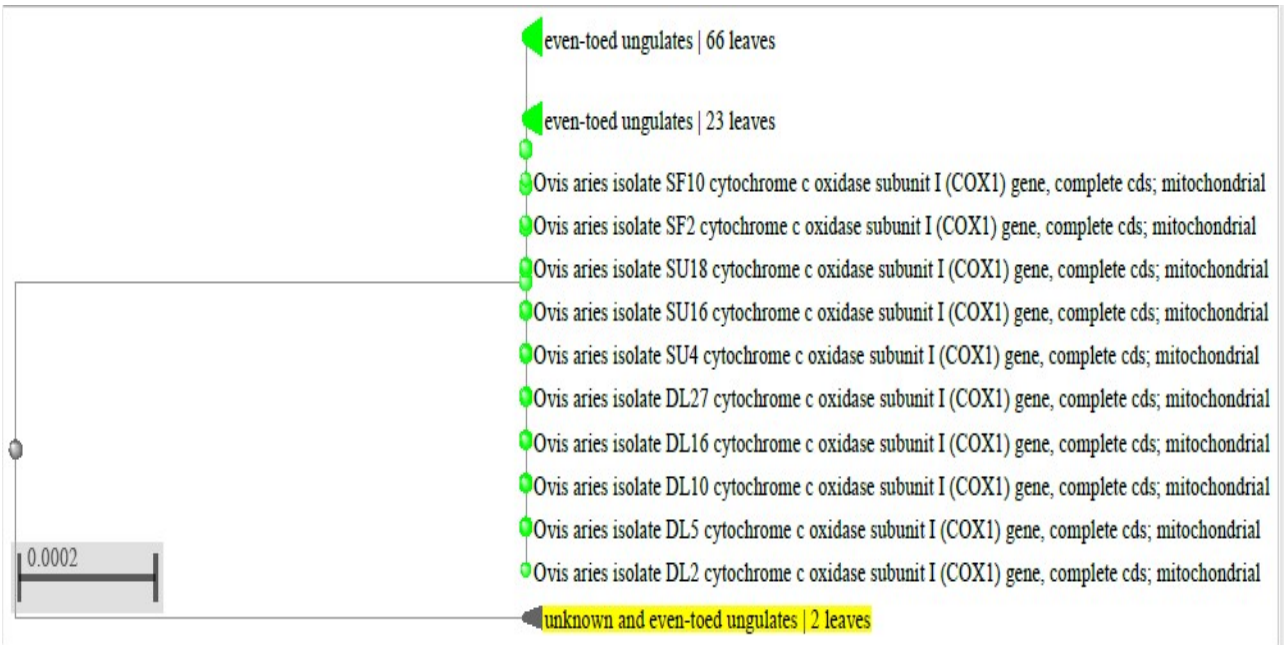

Figure 3: K2P distance neighbour-joining tree of COI sequences from several species of Sheep.

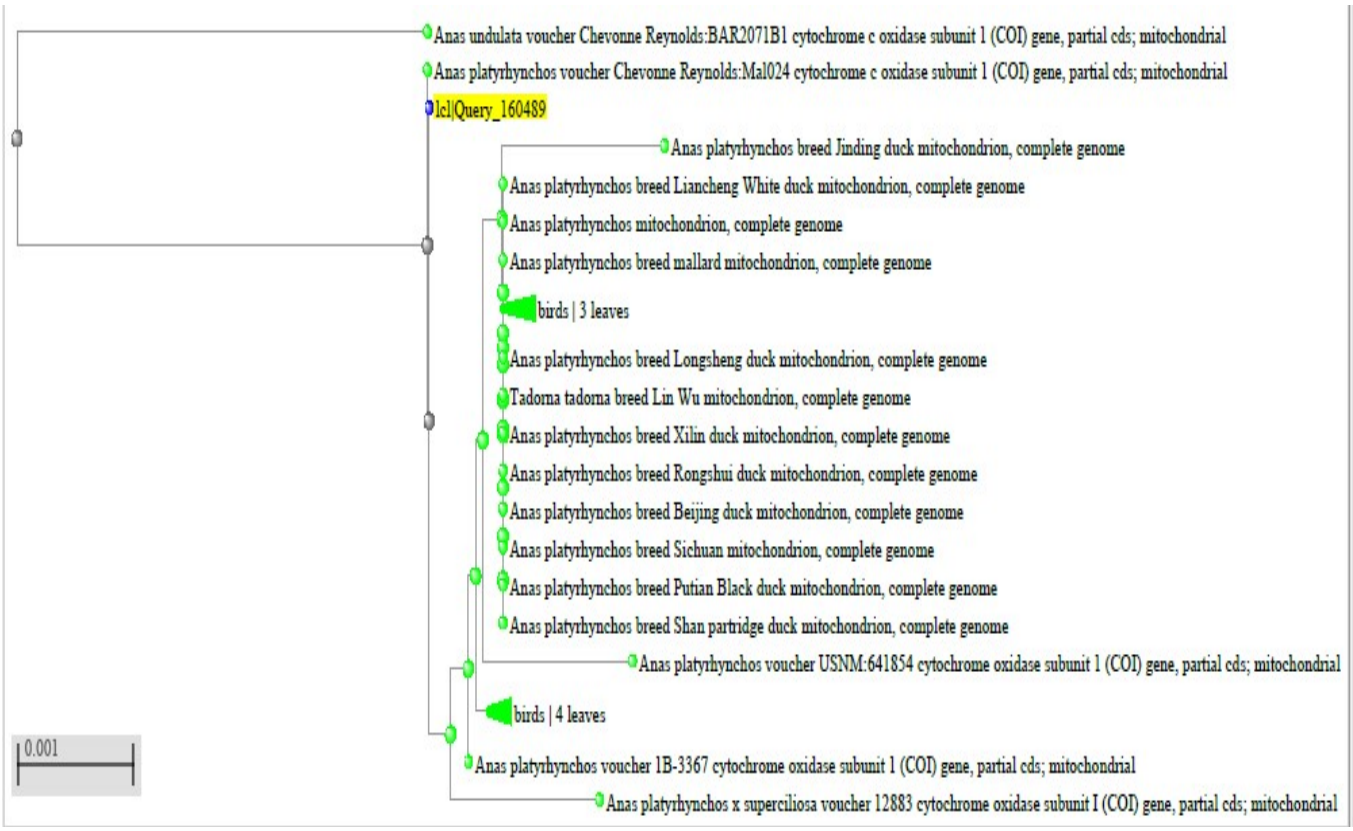

Figure 4: K2P distance neighbour-joining tree of COI sequences from several species of Duck.

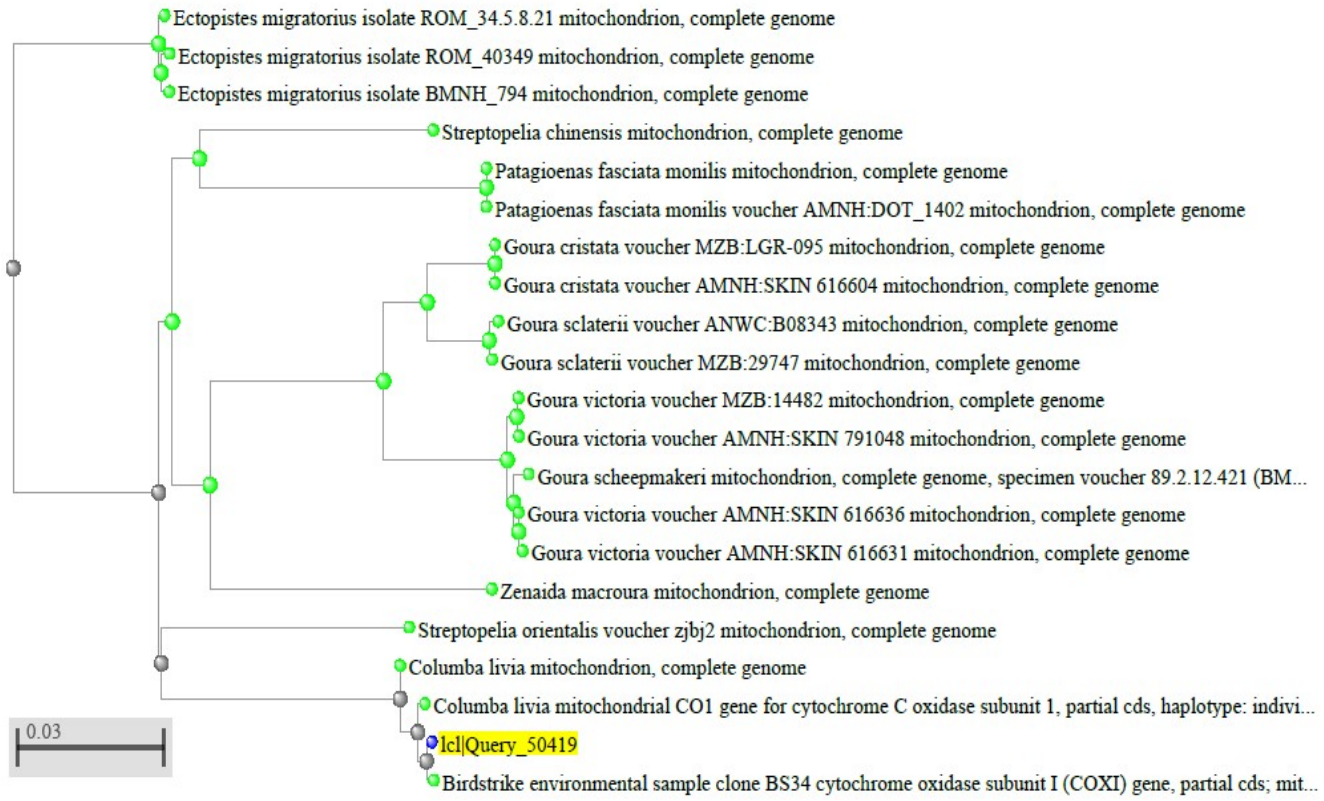

Figure 5: K2P distance neighbour-joining tree of COI sequences from several species of pigeon.

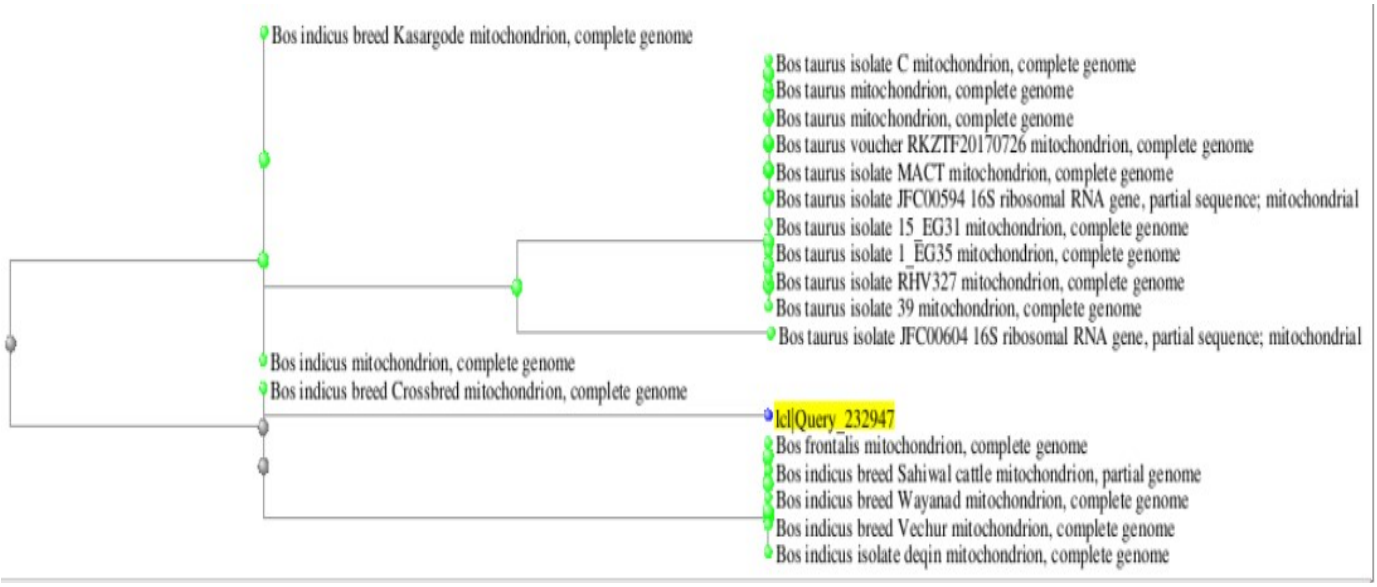

Figure 6: K2P distance neighbour-joining tree of Cy-B sequences from several species of cattle.

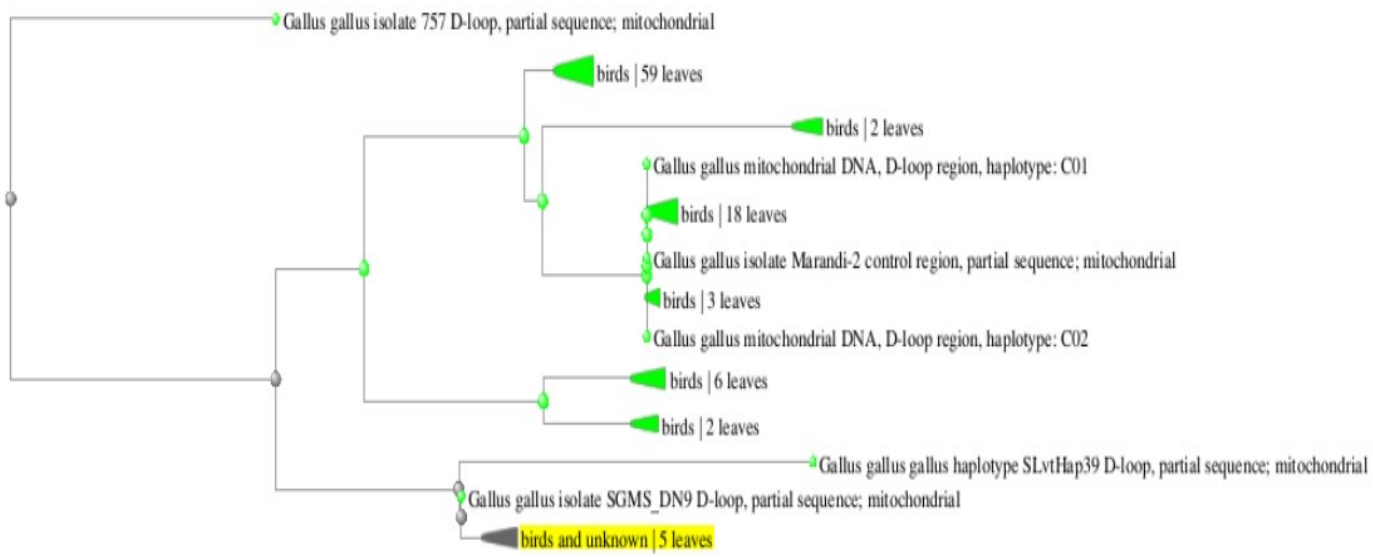

Figure 7: K2P distance neighbour-joining tree of D-loop sequences from several species of chicken

Preparation of DNA Barcode

Drafts of the DNA Barcodes were created by the online barcode preparing software for each species analyzed against COI and Cy-B gene (Figure-8 & 9). DNA barcoding may be used in facilitating species identification, highlighting cases of range expansion for known species, flagging previously overlooked species, and enabling identifications where traditional methods cannot be applied. DNA barcodes are especially useful to (1) distinguish species differences in animals by overcoming the deficits of morphological approaches to species discrimination, (2) quantify intra-specific diversity, (3) recognize sibling species, (4) be objective in taxonomic decisions in all life stages, and (5) allow single laboratories to execute taxon diagnoses (Hebert et al., 2003a). This barcode may be used to discriminate species under this study. This result needs to validate with another study including more samples.

Figure 8: Preparation of DNA barcode against COI gene.

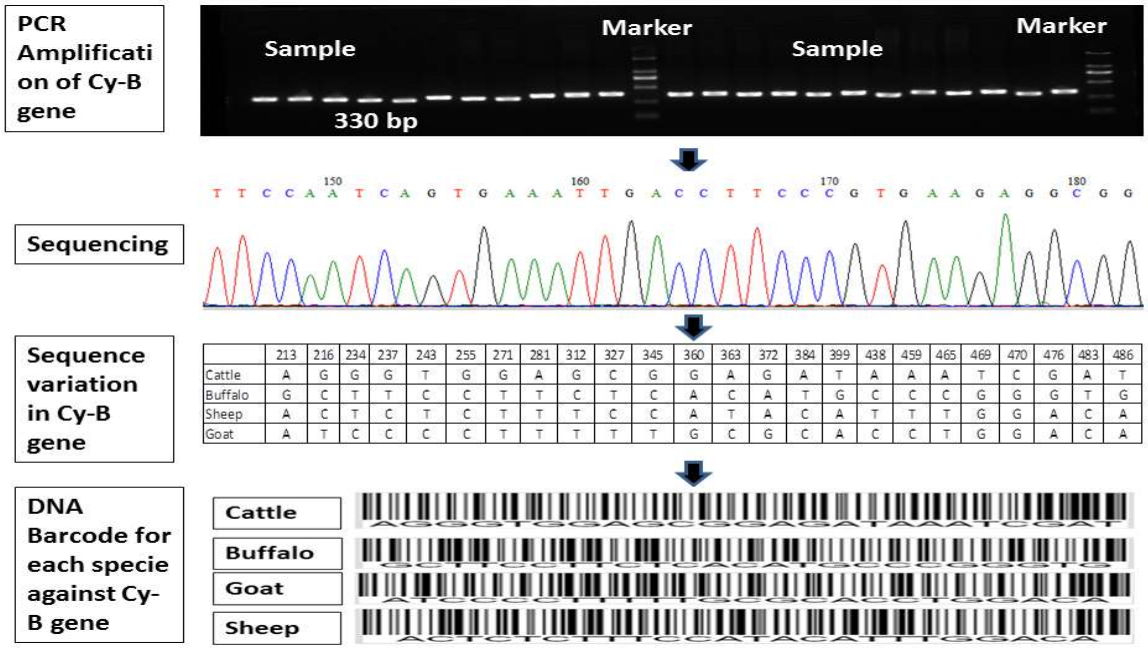

Figure 9: Preparation of DNA barcode against Cy-B gene

Conclusion

In conclusion, the results of this study suggest that the mtDNA diversity of Bangladeshi common livestock and poultry populations are relatively moderate. In contrast, the genetic distances among the populations are relatively low. We have barcoded 7 species of Bangladeshi common livestock and poultry populations for the first time. These barcode data corroborate the presence of seven species in Bangladesh as new record. However, our data are based on only few outline of the population and thus require further in depth study.

12. Research highlight/findings:

- A total of 985 biological specimens from cattle, sheep, goats, buffalo, chicken, duck and pigeon were collected from different regions of the country.

- DNA extraction and PCR amplification of all the specimens done.

- A draft DNA barcode for common livestock and poultry i.e. cattle, buffalo, sheep, goat, duck, chicken and pigeon has been prepared.

- mAMOVA indicated that less than 80% of the genetic variation occurred between regions (geographical areas) while more than 20% of the variation occurred among individuals within regions, implying significant genetic differentiation between regions, this indicated clear mtDNA differentiation between specimens collected "not structured population".

- Proper identification system of species, diversity assessment aiming to conserve native animal genetic resources has been developed.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment					
Executive Table	1	18000.00	1	18000.00	
Executive Chair	1	8000.00	1	8500.00	
Steel Almira	1	24000.00	1	24000.00	
Visitor/Front Chair	1	4000.00	1	4200.00	
Computer Table	1	5000.00	1	3500.00	
Computer Chair	1	3500.00	1	4200.00	
Total	6	62500.00	6	62400.00	
(b) Lab &field equipment					
Chest type deep freeze	2	120000.00	2	120000.00	
Desktop computer, UPS (offline)	1	60000.00	1	60000.00	
Laser Printer	1	20000.00	1	19000.00	
Scanner	1	8000.00	1	8000.00	
Digital Camera	1	25000.00	1	25000.00	
Total	6	233000.00	6	232000.00	
(c) Other capital items					

2. Establishment/renovation facilities:

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	
-	-	-	-	-	-

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

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	279241.00	279241.00	279241.00	0.00	100%	
B. Field research/lab expenses and supplies	1748547.00	1748547.00	1748547.00	0.00	100%	
C. Operating expenses	107873.00	107873.00	107873.00	0.00	100%	
D. Vehicle hire and fuel, oil & maintenance	0.00	0.00	0.00	0.00	0.00%	
E. Training/workshop/ seminar etc.	0.00	0.00	0.00	0.00	0.00%	
F. Publications and printing	87106.00	0.00	0.00	87106.00	0.00%	Fund was not released
G. Miscellaneous	48458.00	48458.00	48458.00	0.00	100%	
H. Capital expenses	294400.00	294400.00	294400.00	0.00	100%	

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)**
a) To construct a DNA barcoding database for common livestock and poultry available in Bangladesh.	1. Sample collection 2. Extraction of DNA 3. Primer design 4. PCR amplification 5. DNA Sequencing 6. Sequence analysis	1. A total of 985 biological specimens of cattle, sheep, goat, buffalo, chicken, duck and pigeon from different region of the country have been collected. 2. DNA extraction of all the samples has been completed. 3. PCR and sequencing has been done.	The knowledge of genetic relationship (between and within population) of common livestock and poultry in Bangladesh will help proper identification of species (cattle, buffaloes, goats, sheep, chickens, pigeon and ducks) and diversity
b) To clarify genetic relationships and breeding history of domestic breeds, crossbreeds (hybrids), and their	1. Sequence alignment with Bioedit 2. Phylogenetic analysis ClustalW of MEGA 3.Sequence	Evolutionary analyses, diversity analysis, distance analysis, and phylogeny analysis completed.	

wild relatives.	divergence values within and between species with Arlequen software		assessment of common livestock and poultry in the country.
c) To determine breed traceability within some species (e.g., buffaloes, goats, sheep, chickens, and ducks)	1. Sequence alignment with Bioedit 2. Phylogenetic analysis ClustalW of MEGA 3.Sequence divergence values within and between species with Arlequen software 4. Barcode preparation with barcode creator.	Sequence divergence, distance and phylogenetic relationship of COI, Cy-B and D-loop genes in cattle, buffaloes, goats, sheep, chickens, pigeon and ducks population in Bangladesh. mAMOVA indicated that less than 80% of the genetic variation occurred between regions (geographical areas) while more than 20% of the variation occurred among individuals within regions, implying significant genetic differentiation between regions, this indicated clear mtDNA differentiation between specimens collected "not structured population".	

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	2	-	-
Information development	-	-	-
Other publications, if any	-	-	-

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

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

None

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

DNA barcode technology will help to estimate the biodiversity aiming to conserve native animal genetic resources

iii. Technology transferred that help increased agricultural productivity and farmers' income
None

iv. Policy Support

This technology will help proper identification of species, diversity assessment aiming to conserve native animal genetic resources.

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring:

Desk monitoring helped us about financial management, reporting, group discussion and lesson to manage farmers during sample and data collection.

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

Field monitoring was done one time by a group of specialist. They suggested us about how to deal with farmers, area and number of samples and sample collection, processing and preservation.

H. Lesson Learned (if any)

This type of project related to molecular genetics and biodiversity assessment require more time and money.

I. Challenges (if any)

a) Area and species wise blood sample collection was a big challenge.
b) Blood sample collection from buffalo was very tough especially from the coastal areas.
c) Sometimes farmers did not allow collecting blood sample from their animals and birds.

Signature of the Principal Investigator
Date:
Seal

Counter signature of the Head of the organization/authorized representative
Date:
Seal

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