MOLECULAR CHARACTERIZATION OF WESTERN TRAGOPAN IN THE AVIARIES OF HIMACHAL PRADESH

Project Report submitted in partial fulfillment of the requirement for the degree of

Bachelor of Technology

In

BIOTECHNOLOGY

Under the Supervision of

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By

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to

Jaypee University of Information and Technology

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CERTIFICATE

This is to certify that project report entitled "Molecular Characterization of Western Tragopan in the Aviaries of Himachal Pradesh", **submitted by** Tanvi Saini (081766) **in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.**

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

Date: 01/06/2012

(Pradeep Kumar Naik)

Associate Professor

ACKNOWLEDGEMENT

It has been a great pleasure working under the guidance of faculty and staff in the Department of Bioinformatics and Biotechnology at Jaypee University of Information Technology,during my study as a B.tech student.Thanks a lot to Dr.Pradeep Kumar Naik,my project advisor,for his financial and scientific support;otherwise,this research work would never have been possible.

Many have also contributed to this project in a variety of ways over the past few months.To the indivisuals who have helped me,I again express my appreciation.I am indebted to all those who provided reviews and suggestions for improving the results and the topics covered in this project,and extend my apologies to anyone I may have failed to mention.

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SUMMARY

The beautiful bright-plumed Western tragopan (*Tragopan melanocephalus*) endemic to the western Himalayas is threatened by extinction due to the uncontrolled logging, indiscriminate destruction of their habitat and hunting for bush meat (Threatened Birds of Asia – The Birdlife international Red Data book, (2001), Cambridge, UK). Both Western tragopan and Cheer pheasant are considered as "vulnerable" in their native habitat (Himachal Pradesh) by the I.U.C.N. The taxa is listed on appendix 1 of the CITES and can only be transported with all necessary CITES and other documents. The bright-plumed Western tragopan is also the state bird of Himachal Pradesh, is facing an uncertain future. Inspite of various breeding program being employed to augment the depleting population in captivity the population has not increased so far. One of the reason for decreasing their populations is increased rate of unhatchability and mortality. It may be due to inbreeding depression among the populations—though the birds have been ringed to establish their parentage but still chances of sib-mating is very high as the parents used for breeding program are F_{4-5} progeny of primary stock in captivity. Although Wildlife Wing of Himachal Pradesh, Forest Department has carried out pairing of populations for breeding based on phenotyping, it would be incomplete in establishing parental lineages. In order to develop strategies for better pairing of populations for breeding purpose and establishing parental lineages, it would be essential to carry out DNA profiling of captive populations utilizing both nuclear and mitochondrial genomic resources. The study will help in developing appropriate methods for conservation management from the remaining populations with great genetic variations.

In the present study 10 RAPD and fifteen SSR markers, developed from Galliformes EST database were used for genetic diversity analysis. Polymorphism was studied using 19 individuals collected from the wild life department, Himachal Pradesh. All the loci were polymorphic with number of alleles ranging from 2 to 3 for SSRs and 4 to 19 for RAPD markers and Nei's genetic diversity was observed to be 0.136. Also, the complete mitochondrial genome of Western tragopan and Red jungle fowl was sequenced. The circular genome is of around 16 kb in size, encoding a standard set of 13 protein coding genes, two ribosomal RNA genes, and 22 transfer RNA genes, plus the putative control region, a structure very similar to that of other Galliformes. MtDNA can be used as a genetic marker because of its maternal inheritance and fast evolution for the analysis of gene flow and for the analysis of potential barcoding regions.

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CHAPTER 1
INTRODUCTION

The term pheasant is the common name for various long-tailed birds and refers to those birds mainly belonging to the Phasianinae subfamily, which includes 16 genera, with genus *Phasianus* sometimes referred to as the 'true pheasants." Five of the genera typically utilize common names other than "pheasant": *Afropavo* (Congo peafowl), *Pavo* (peafowl), *Gallus* (junglefowl), *Lophophorus* (monals), and *Tragopan* (tragopans). Pheasants are originally Old World birds, native to Asia, but are now found in Europe and have been introduced to North America. Pheasants provide numerous ecological, commercial, aesthetic, sport, and nutritional values. They are important to various food chains, consuming grains, berries, and other vegetation and being consumed by foxes and other animals. In many countries, pheasant species are hunted as game, and have been commercially raised for this purpose as well as food. Nutritionally, they are a rich source of iron, protein, niacin, and other nutrients. And aesthetically, the colorful plumage of male pheasants and the unique courtship behaviors add to the human wonder of nature.

The pheasant species: Western tragopan and Cheer pheasant are endemic to western Himalays in restricted pockets. Their populations is decreasing dramatically due to uncontrolled logging, indiscriminate destruction of their habitat, hunting for food (Threatened Birds of Asia – The Birdlife international Red Data book, (2001), Cambridge, UK) as well as low breeding rates. The Western tragopan is considered as "vulnerable" in their native habitat (Himachal pradesh) by the I.U.C.N. The taxa is listed on appendix 1 of the CITES and can only be transported with all necessary CITES and other documents. Evidently, these species are highly fragmented and reported to be declining that aids to the risk of extinction of these birds in wild. The bright-plumed Western tragopan, which is also the state bird of Himachal Pradesh, is facing an uncertain future. Inspite of various breeding program being employed by Wildlife Wing, Himachal Pradesh, Department of Forest, to augment the depleting population of both the bird species in captivity, the population has not increased. Among various reasons of such an increase in unhatchability and mortality, one could be inbreeding

depression. Though the birds have been ringed to establish their parentage but still chances of sib-mating is very high as the parents used for breeding program are F_{4-5} progeny of primary stock in captivity. For an example the department has established tragopan breeding centre at Saharan District, Shimla but the species has proved difficult to maintain and breed due to unknown reasons over the years since its inception. Since 1992, when the Saharan aviary was set up for captive breeding of the Western tragopan, this centre has bred only 20 birds and so far has not been able to release any bird into the wild. As of now there are nearly the 21 Western tragopan birds in captivity at Saharan breeding center. This year also three adult females' birds died, leaving behind a population of 11 males and 9 females. The surviving birds laid 17 fertilized eggs of which only one hatched and that too the chick died within a week. In 2009, two chicks had managed to survive out of 23 fertilized eggs that were laid.

The development of tools for the fast identification of organisms becomes a "*sine qua non*". "DNA profiling" has become popular as a rapid and reliable technique for species identification. In addition to morphological keys, a public reference library containing short DNA sequences of all known species should enable scientist to identify specimens quickly and accurately and to discover new species. Most of the researchers across the world have concentrated their efforts towards establishing the Phylogenetic relationship in galliform using mitochondrial or nuclear DNA and to some extent based on molecular markers such as SSR and SNPs (Fumihito et al., 1995; Kimball et al., 1997, 1999 and 2001; Randi et al., 2000; Armstrong et al., 2001). At present, many studies have been conducted on the reproduction, habitat, anatomy and taxonomy of these birds (Chang et al., 1998; Lei and Lu, 2006; Huang et al., 2008; Yao et al., 2008). However, there are no molecular markers studies based on SSRs or SNPs have been performed in these two bird species so far, in spite of many molecular markers that have been developed and used broadly in other gamebirds (He et al., 2009; Wang et al., 2009; Zhou and Zhang, 2009). Furthermore to establish the parental lineages in animals and birds mitochondrial genome plays an important role due to lack of recombination, low mutation rate, high copy number as well as its maternal inheritance properties. Unfortunately the mitochondrial genome sequencing from these two bird species have not been reported. Therefore it is imperative to generate mitochondrial or nuclear genomic resources for these endangered bird species.

The lack of sufficient and polymorphic SNP and SSR markers as well as mitochondrial genome resources limit research in genetic analysis for establishing parental

lineages and conservation purpose of these bird species. Thus, screening for polymorphic SSR and SNP markers is very important and necessary for analyzing genome organization, evolution and developing marker-assisted breeding technology. The present project proposal is thus originated keeping in view the importance of both the bird species, Western tragopan and Cheer pheasant, to understand population structure for better identification of mating pairs for *in situ* conservation, increasing breeding rates and to reduce the inbreeding by separating out the closely related pairs and realizing the existing lacunae in study made so far in this aspect.

Table1: Scientific Classification of Western tragopan and Red jungle fowl

CHEER PHEASANT	**RED JUNGLE FOWL**
Kingdom: Animalia	Kingdom: Animalia
Phylum: Chordata	Phylum: Chordata
Class: Aves	Class: Aves
Order: Galliformes	Order: Galliformes
Family: Phasianidae	Family: Phasianidae
Genus: *Tragopan*	Genus: *Gallus*
Species: *melanocephalus*	Species: *G. gallus*

Figure 1: The Western Tragopan and Red jungle fowl.

We are thus inquisitive to study and address following important questions in this proposed research proposal.

1. Does the protein coding genes localized in mitochondrial/nuclear genome are conserve or polymorphic among the captive populations of the bird species?

2. Does DNA profiling utilizing molecular markers (SSRs and RAPDs) could establish suitable mating pairs among captive populations?

3. Is there any relationship between the mating pairs elucidated from the genomic information with the existing mating pairs in aviaries?

We believe that the results that will be obtained with respect to polymorphism studies will provide the necessary framework for evaluating the above questions to avoiding inbreeding depression and increasing the breeding rates. Further the results will provide a proof of concept for bringing out both the bird species from the status of endangered species.

Objectives:

The present study aims to achieving the following objectives:

1. DNA Profiling using RAPD (Random Amplified Polymorphic DNA) and SSR(Simple Sequence Repeats) molecular markers in western tragopan and phylogenetic analysis.
2. Mitochondrial genome isolation and sequencing of western tragopan and Red jungle fowl.

CHAPTER 2
REVIEW OF LITERATURE

2.1 REVIEW OF LITERATURE IN THE PROPOSED AREA

Molecular characterization of Western tragopan has not been done so far. Being a state bird of Himachal Pradesh and highly endangered species, the state government had started a conservation breeding programme. The department has created several phesantaries for the breeding purpose of Western tragopan however; the success rate is not so successful. The Saharan aviary was set up in Shimla district in 1992 as a captive breeding centre for the Western tragopan. But till date the centre has bred only 20 birds of this species, of which none have been released in the wild. In 2009, the birds at the centre laid about 23 fertilized eggs of which only two chicks managed to survive. Earlier this year in March- April 2010, all the 23 birds in captivity had bacterial infection, of which three adult female birds died. This left behind only 11 males and 9 females. These surviving birds laid 17 fertilized eggs, but only one hatched and later this chick too died due to bacterial E-coli infection. Most of the researchers across the world have concentrated their effort towards establishing the phylogenetic relationship in galliforms using mitochondrial or nuclear DNA (Fumihito et al., 1995; Kimball et al., 1997,1999 and 2001;Randi et al., 2000; Armstrong et al., 2001) and to some extent the molecular markers SSR and SNP's has been used for the same study. At present, many studies have been conducted on the reproduction, habitat, anatomy and taxonomy of this bird (Chang et al., 1998; Lei and Lu, 2006; Huang et al., 2008; Yao et al., 2008). However, there are no molecular markers, such as SSR's isolated and applied in this species to date in spite of many molecular markers that have been developed and used broadly in the other game birds (He et al., 2009; Wang et al., 2009; Zhou and Zang 2009).

2.2 GENETIC DIVERSITY OF WESTERN TRAGOPAN POPULATIONS

For the purpose of efficient conservation and successful breeding program, it will be prudent to study the populations of Western tragopan at genetic and molecular levels. Study within and between populations variations at the molecular level provides an efficient tool for taxonomic and evolutionary studies and for devising strategies to protect the genetic diversity

of this species. Genetic variability also can be exploited to select useful mating pairs for breeding process to avoid inbreeding.

2.3 USE OF MOLECULAR MARKERS FOR STUDYING GENETIC DIVERSITY

In simple terms, genetic diversity is a statistical concept referring to the variations within the individual gene loci among alleles of a gene or gene combinations, between individuals or between populations. The classical methods of diversity studies are based on physiological characters which are influenced by various environmental factors. However, the molecular markers which are unrestricted in number and not influenced by the environment have the ability of sampling diversity directly at the genome level. They provide increased accuracy and expanded scope of inferring genetic variability within and between populations of species. The drawbacks of biochemical markers led many workers to shift to nuclear DNA markers such as RFLPs, RAPDs, ISSRs, AFLPs, SSRs, ILPs, CAPs etc. DNA-based molecular-marker techniques have been proved powerful in genetic diversity estimations. Different types of marker systems have been used for biodiversity and phylogenetic analyses. These include restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Selection of a correct marker system depends upon the type of study to be undertaken and whether that marker system would fulfill at least a few of the mentioned characteristics such as easy availability, highly polymorphic nature, Mendelian inheritance, frequent occurrence in genome, selective neutral behavior, easy and fast assay, high reproducibility, free of epistasis and pleiotropy etc.

2.4 RAPDS AS MOLECULAR MARKERS

Random amplified polymorphic DNA (RAPD) is a PCR-based technique that has been applied to the study of populations. RAPDs are one of a family of techniques that produce arbitrary fragment length polymorphism and are collectively described as multiple arbitrary amplicon profiling. The RAPD technique utilizes single, arbitrary, decamer DNA oligonucleotide primers to amplify regions of the genome using PCR. Priming sites are thought to be randomly distributed throughout a genome and polymorphism in these regions results in different amplification products. The methodology is simple and has been widely used for the assessment of genetic diversity, genetic variation within species, determining relationships between closely related species and genotypes within a species to identify particular genotypes. RAPD technique has also been used to study and investigate clonal and

population structure. The simplicity of the technique and the speed of data generation have attracted many researchers, particularly those interested in either genetic fingerprinting or the patterns and levels of genetic diversity. In addition to the studies of genetic diversity there have been an increasing number of papers concerned with population genetics, phylogenetics and hybridisation/introgression. The greatest attraction of this method is that it generates DNA data that are, theoretically at least, randomly scattered across the genome. Such markers are attractive for studies that involve differentiation of similar species and identification of patterns of variation. Rieseberg suggested that RAPDs may be useful for investigation within species or between closely related species. Many reports are available on inter and intra generic genetic diversity and molecular phylogeny using RAPDs. All the above studies confirmed the efficiency of RAPD markers for systematic investigations. Thus, it has been suggested that RAPD fingerprinting method is simple and so powerful that one primer can distinguish between different clones while the use of multiple primers reduces fingerprint similarity and resolves discrepancies. RAPD analysis is a very good starting point for studies of relationships within and among closely related species. No report was so far available on RAPD characterization of Western tragopan.

2.5 SSRS MOLECULAR MARKERS

Simple sequence repeats (SSR) or microsatellite form an important class of molecular markers for genomic and animal breeding applications. SSR markers quickly became the markers of choice for plant and animal genomes during the last decade because of the small sample size (genomic DNA) requirement for their analysis and their suitability for automation and high-throughput analysis. The presence of SSR in the transcript of genes suggests that they may have a role in gene expression or function. While di-, tri- or tetra-nucleotide SSR are most commonly used for the construction of linkage maps of nuclear genomes, single nucleotide repeats have been used in the population genetic analysis of mitochondrial genomes.

SSR can be assayed using PCR technology and can be screened using high-throughput platform for molecular genetic linkage and population studies. The number of repeats at a locus can change by mutation and the rate of mutation depends on the number of tandem units within the repeat. Another area where SSR markers are extremely valuable and are increasingly becoming popular is comparative genomics where SSR markers developed

from one species could be utilized in a related or heterologous species towards genetic mapping, characterization, gene cloning, diversity, evolutionary studies of genetic variation, linkage mapping, gene tagging, establishment of genetic maps, integration of physical and genetic maps, determination of evolutionary relationships and comparative genome analyses. SSRs, once developed are extremely valuable, although their development is time consuming, laborious and expensive. SSRs derived from ESTs essentially represent expressed gene sequences and hence are potential candidates for markers for comparative genomic studies. ESTs are particularly attractive for marker development since they represent coding regions of the genome and are also being developed at an extremely faster pace for many genomes.

2.6 MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA analysis (mtDNA) can be used to examine the DNA from samples that cannot be analyzed by RFLP or STR. Nuclear DNA must be extracted from samples for use in RFLP, PCR, and STR; however, mtDNA analysis uses DNA extracted from another cellular organelle called a mitochondrion. While older biological samples that lack nucleated cellular material, such as hair, bones, and teeth, cannot be analyzed with STR and RFLP, they can be analyzed with mtDNA. In the investigation of cases that have gone unsolved for many years, mtDNA is extremely valuable.

Mitogenomics is a high-resolution molecular genetic approach that includes the study of the complete genome of the mitochondrion of an organism. Mitochondria are structures within cells that convert the energy from food into a form that cells can use. In addition to energy production, mitochondria play a role in several other cellular activities.

Although most DNA is packaged in chromosomes within the nucleus, mitochondria also have a small amount of their own DNA. This genetic material is known as mitochondrial DNA or mtDNA. It is a supercoiled, circular molecule that lacks histones and is present in very high copy number. The size of the mitochondrial genome is around 16kb in most animals. Mitochondrial DNA can be regarded as the smallest chromosome, and was the first significant part of the human genome to be sequenced. In most species, mtDNA codes for 13 or 14 proteins involved in the electron transfer chain, 2 rRNA subunits and 22 tRNA molecules (all necessary for protein synthesis).

In total, the mitochondrion hosts about 3000 proteins, but only about 13 of them are coded on the mitochondrial DNA. Only about 3% of them code for ATP production proteins. This means most of the genetic information coding for the protein makeup of mitochondria is in chromosomal DNA.

In most species, including humans, mtDNA is inherited solely from the mother (exceptions with biparental inheritance in marine mussels, Zouros *et al.* 1992). The DNA sequence of mtDNA has been determined from a large number of organisms and individuals (including some organisms that are extinct), and the comparison of those DNA sequences represents a mainstay of phylogenetics, that it allows biologists to elucidate the evolutionary relationships among species.

2.7 AVIAN MITOCHONDRIAL DNA

Mitochondrial DNA as a genetic marker has been successfully applied to the study of molecular evolution of birds. The apparently maternal inheritance of mitochondrial DNA and its fast evolution in primary sequence has made it attractive in population and evolutionary genetics. Mitochondrial DNA of birds displays two characteristics not seen in other vertebrates' mtDNA, that is, a novel gene order and the absence of an equivalent to the light-strand replication origin.

This uniparental mode of inheritance is one of the great advantages of mtDNA, as it enables researchers to trace related lineages back through time, highlighting the maternal ancestry of a population, without the confounding effects of biparental inheritance and recombination inherent in nuclear DNA.

As a molecular marker, mitochondrial DNA has many **advantages.**

- It evolves faster than nuclear DNA (Brown *et al.* 1982), probably due to inefficient replication repair (Clayton 1984). Different regions of the mitochondrial genome evolve at different rates (Saccone *et al.* 1991) allowing suitable regions to be chosen for the study.
- Mitochondrial DNA does not recombine (Hayashi *et al.* 1985), though some evidence of recombination events has recently been reported (Eyre-Walker *et al.* 1999, Hagelberg *et al.* 1999).

- They are present in large numbers in each cell, so fewer samples is required.
- Each mitochondrion has several copies of its own genome, and there are several hundred to several thousand mitochondria per cell, thus the mitochondrial genome is highly amplified.
- The genome is highly economized with few sections of noncoding DNA, intergenic regions or repetitive sequences, except for one major control region.
- Because of its compactness, maternal inheritance, fast evolutionary rate compared to nuclear DNA, and short coalescence time, mtDNA is useful for population genetic studies such as the analysis of gene flow, hybridization and introgression.

The first complete sequence of an avian mitochondrial genome was published from chicken by Desjardins and Morais (1990). It showed highly conserved features when compared to other vertebrate mtDNAs.

2.8 RESEARCH GAP

The Western tragopan is included under Schedule 1 of Wildlife Protection Act. Western tragopan and Red Jungle fowl are beautiful plumed birds threatened by extinction due to indiscriminate destruction, uncontrolled logging of their habitat and their hunting for food. Among various reasons of such an increase in unhatchability and mortality one could be inbreeding depression. Though the birds have been ringed to establish their parentage but still chances of sib-mating is very high as parents used for breeding program are F4-5 progeny of primary stock in captivity. The lack of sufficient and polymorphic SNP and SSR markers limits research in genetic analysis for conservation purpose of these species (CITES, 2001; IUCN, 2006).

CHAPTER 3

MATERIAL AND METHODS

3.1. IN SILICO SSR ANALYSIS

- EST sequences were downloaded from NCBI data base (http://www.ncbi.nlm.nih.gov) for all the pheasant species available so far.
- SSR analysis were performed utilizing MISA (http://wsmartins.net/websat/) and Primer's were designed.

3.2 PRIMER DESIGNING FOR SSR MARKERS.

A set of 15 Primers flanking different motif and of variable size, were designed based on the EST sequence from the different species of Pheasant based on the following criteria.

1. Minimum repeat length for motifs.
2. Primers were selected with Tm more than 57°C and minimum GC% to be 40%.

The primers designed were custom synthesized from IDT pvt. Ltd. and used for PCR amplification.

3.3 BIOLOGICAL MATERIALS

Blood samples used in the present investigation were collected from the Wildlife Department at Sarahan, Himachal Pradesh. We have obtained samples from 19 birds (10 male and 9 female) and one sample form red jungle fowl.

3.4 CHEMICALS AND REAGENTS

3.4.1 WHOLE DNA EXTRACTION

Lysis buffer: It is used for the purpose of lysing cells for use in experiments that analyze the compounds of the cells. Generally, lysis buffers have tris-HCl, EDTA, SDS, deoxycholate, and tritonX or NP-40. In some cases it may also contain NaCl(150 mM). It is comprised of Buffer A and Buffer B.

Buffer A: It was prepared by 10mM NaCl, 50mM Tris Cl (pH=8.0) and 10mM EDTA. Further it was autoclaved at 15 psi for 20 minutes.

Buffer B: Equal volumes of Buffer A and 2% SDS (2g of SDS was dissolved in 100 ml of autoclaved distilled water) were added to prepare Buffer B.

TE buffer: It was prepared by10 mM Tris (pH 8.0) and 1 mM EDTA in distilled water and was further autoclaved.

Other chemicals: Tris saturated phenol, PCI, 70% Ethanol, Absolute ethanol, Proteinase K

50X TAE : In 1000ml.

Tris (hydroxymethyl) aminomethane	242.28 gm.
Acetic acid (glacial)	57.1 ml.
0.5M EDTA (pH 8.0)	100ml. (50 mM.)

1X : 10 ml. of 50X TAE buffer and 490 ml. distilled water.

3.4.2 MITOCHONDRIAL DNA EXTRACTION

TKM1: A low salt solution composed of 10mM Tris-HCl pH=7.6, 10mM $MgCl_2$ and 2mM EDTA.

TKM2: A high salt solution composed of 10mM Tris-HCl pH=7.6, 10mM KCl, 10mM $MgCl_2$, 0.4M NaCl and 2mM EDTA.

Other chemicals: NP- 40(Nonidet 40), 10% SDS, 6M NaCl, 70% Ethanol, Absolute ethanol

3.4.3 DNA EXTRACTION

Whole DNA isolation from blood

- 10ul of Proteinase K was added into a 1.5ml microcentrifuge tube
- 100ul of blood sample was added to the tube and mixed well by pipetting up and down
- 2ul of RNase A was from 100mg/ml stock was added and mixed well
- 150ul of buffer BL was added to the sample and mixed thoroughly by vortexing
- The sample was incubated at 56°C for 15minutes and mixed well
- 100ul of ethanol was added and briefly centrifuged
- The spin column was placed in collection tube and the sample was passed through the spin column

- Centrifugation was done at 10,000rpm for 1minute. Discard the flowthrough
- Column was washed with 250ul of buffer BW1 and centrifuged at 10,000rpm for 1minute. Discard the flowthrough
- The above step was repeated using 250ul of buffer BW2 and centrifuged at 10,000rpm for 3minutes. Discard the flow through and collection tube
- The spin column was placed in fresh 1.5ml microcentrifue tube
- Elution was done by adding 100ul of buffer BE directly to the centre of the column membrane and incubated for 2minutes at room temperature
- Centrifugation was done at 10,000rpm for 1minute and DNA was eluted

Mitochondrial DNA isolation

- 100ul of blood sample was taken in a centrifuge tube.
- To it, an equal volume of a low salt solution, TKM1 was added.
- To that 3ul of NP-40(Nonidet P40) was added and the contents were mixed thoroughly and incubated for 10min at room temperature until transparent red solution comes, indicating complete lysis of erythrocytes was observed.
- This was then centrifuged at 800g for 20 min.
- The residual pellet was resuspended in 100ul of TKM1 buffer with 3ul of NP-40 and centrifuged again. The additional wash with NP-40 gives a better enrichment of mitochondrial fraction by ensuring complete lysis of cell.
- The supernatant from the first and the second centrifugation was pooled and retained in a sterile microfuge tube and centrifuged at 15000g at 4^0C for 30 minutes to sediment the mitochondrial pellet.
- This pellet was washed thrice with TKM1 ensuring complete elimination of lysed erythrocytes.
- The mitochondrial pellet was then suspended in 20ul of high salt buffer, TKM2.
- Then, 3ul of 10% SDS was added and incubated at 55^0C in heating block for complete denaturation and solubilisation of protein.
- Salting out of protein was done by addition of 10ul of 6M NaCl and centrifuged at 11300g for 20min.
- The supernatant was then transferred to a 2ml tube and twice the volume 100% ethanol was added for complete precipitation of MtDNA pellet.

- The MtDNA pellet was washed twice with 70% ethanol, dried and finally dissolved in 50ul of sterile water.

Electrophoresis of purified DNA

Preparation of 0.8% agarose gel :

- 0.56 gm. of agarose was weighed and 70ml. of 1 X TAE was added and heated in a microwave oven for about 1-2 min.
- It was cooled and 3 ul. of EtBr was added.
- This solution was poured onto a casting tray, comb was inserted and the gel was left for about 20-30 min. for solidification.
- After the gel was solidified comb was removed.
- Gel was placed in the electrophoresis tank and 1X TAE was poured into it. Samples were loaded in the wells with the help of a loading dye.
- Voltage was applied to the tank and the gel was allowed to run for about 1 hr.
- Gel was visualized in GelDoc.

3.5 PCR AMPILIFICATION:

RAPD Amplification:

- The primers were dissolved in autoclaved distilled water to make a total volume of 50ul. by vortexing for 30 sec. The primer solutions were stored at 4 degrees overnight and then stored at -20 degrees till their use.
- The stock solutions of primer was diluted to a concentration of 50 pM for PCR amplification.
- Universal primers were used for the RAPD study of genetic diversity among the *pheasant species*.
- Amplification reactions were performed in volumes of 25ul. containing the components listed in the table below.

Table 2: Reaction Mixture for PCR Amplification using RAPD primers

Components	Stock Concentration	Used Concentration	Amount(in µl)
DNA template	-	-	1.0
Autoclaved Water	-	-	19.75
Primer	50pM	5pM	1
dNTPs	2mM	.2mM	0.5
PCR Buffer	10X	1X	2.5
Taq Polymerase	5U	.5U	0.25
Total	-	-	25 µl

- The amplification products were resolved in 1.5% agarose gel (1X TAE Buffer) followed by ethidium bromide staining and visualization in GelDoc.

SSR Amplification :

- The primers were dissolved in autoclaved distilled water to make a total volume of 50ul. by vortexing for 30 sec. The primer solutions were stored at 4 degrees overnight and then stored at -20 degrees till their use .
- The stock solutions of primer was diluted to a concentration of 50 pM for PCR amplification.
- 7 primers were used for the study of genetic diversity among the *pheasant* species.
- Amplification reactions were performed in volumes of 25ul. containing the components listed in table no. 8.

Table 3: Reaction Mixture for PCR Amplification using SSR primers

Components	Stock Concentration	Used Concentration	Amount(in µl)
DNA template	-	-	1.0
Autoclaved Water	-	-	18.25
Forward Primer	50pM	5pM	1.25
Reverse Primer	50pM	5pM	1.25
dNTPs	2mM	.2mM	0.5
PCR Buffer	10X	1X	2.5
Taq Polymerase	5U	.5U	0.25
Total	-	-	25 µl

- DNA amplification was performed in a thermal cycler. Conditions for the amplification:

- The amplification products were resolved in 1.5% agarose gel (1X TAE Buffer) followed by ethidium bromide staining and visualization in GelDoc.

Amplification of overlapping primers

Table 4: Reaction Mixture for PCR Amplification using overlapping primers

Components	Stock Concentration	Used Concentration	Amount(in µl)
DNA template	-	-	2.4
Autoclaved Water	-	-	29.2
Forward Primer	50pM	5pM	1.6
Reverse Primer	50pM	5pM	1.6
dNTPs	2mM	.2mM	0.8
PCR Buffer	10X	1X	4.0
Taq Polymerase	5U	.5U	0.4
Total	-	-	40 µl

RAPD ANALYSIS:

- Genomic DNA was amplified by Polymerase chain reaction using universal primers.
- Polymorphism was observed.

Table 5: List of primers used for RAPD analysis

ID	Sequence
OPN-16	AAGCGACCTG
OPG-18	GGCTCATGTG

OPL-20	TGGTGGACCA
OPT-17	CCAACGTCGT
OPT-20	GACCAATGCC
OPU-13	GGCTGGTTCC
OPF-14	TGCTGCAGGT
OPX-03	TGGCGCAGTG
OPF-5	CCGAATTCCC
OPX-20	CCCAGCTAGA

SSR ANALYSIS:

- There are no complete genome sequences available for *Caterus wallichii* and *Tragopan malenocphlus*. Mitochondrion complete genome sequence of *Tragopan caboti* and *Tragpan temminckii* (related species belonging to the same family) were downloaded from NCBI.
- Further, the sequence of the *cyt b* gene (common to both species) was downloaded and both of these were BLAST together to check percentage identity, similarity and coverage.
- This showed similarity between 14844 to 15984 bp. So the sequences with 200 bases up and down were downloaded i.e. from 14644 to 16184 of both *Tragopan caboti* and *Tragopan temminckii* and were BLAST together to design primers.
- This showed a % identity of 96% and coverage of 100%.
- We went for primer designing by PRIMER-3 with a product range of 1100 to 1200.
- We selected primers according to the criteria:
    i. Maximum number of repetitions of SSR lying between the forward and reverse primer
    ii. Maximum product length
    iii. Melting temperature (Tm) above 50 degrees Celsius and should be approximately same for the primer pair
    iv. GC content more than 35%
    v. Also, the primer length was taken into consideration(18 to 25bp)
- All the primers were designed by this method and used for PCR amplification.

Table 6: List of primers for SSR analysis

Id	Forward	Motif	Reverse	Ta
ADL268	CTCCACCCCTCTCAGAACTA	(GT)12	CAACTTCCCATCTACCTACT	60
ADL136	TGTCAAGCCCATCGTATCAC	(TG)20	CCACCTCCTTCTCCTGTTCA	56
MCW0016	ATGGCGCAGAAGGCAAAGCGATAT	(TG)16	TGGCTTCTGAAGCAGTTGCTATGG	62
MCW067	GCACTACTGTGTGCTGCAGTTT	(TA)6+(TG)11	GAGATGTAGTTGCCACATTCCGAC	56
MCW069	GCACTCGAGAAAACTTCCTGCG	(CA)11	TTGCTTCAGCAAGCATGGGAGGA	58
MCW0111	GCTCCATGTGAAGTGGTTTA	(CA)7	ATGTCCACTTGTCAATGATG	57
MCW0216	GGGTTTTACAGGATGGGACG	(GT)9	AGTTTCACTCCCAGGGCTCG	60
MCW222	GCAGTTACATTGAAATGATTCC	(GT)8	TTCTCAAAACACCTAGAAGAC	62
MCW0295	ATCACTACAGAACACCCTCTC	(AC)10	TATGTATGCACGCAGATATCC	60
LEI0192	TGCCAGAGCTTCAGTCTGT	(TTTC)12	GTCATTACTGTTATGTTTATTGC	56
WT(T)10-	CAGGTCTCACTCCTAGAGCCAT	(T)10	GACAGCATGAATTGCAGGTTTA	51
WT(TTAT)3	TATTGGTGGGTGTTGAAAAGTG	(TTAT)3	GTTTCCTTAATAGGCAGAGGCA	53
WT(AGGA)3	CAGGTCTCACTCCTAGAGCCAT	(AGGA)3	CAAGTCCCACTACAAGCATTTG	53
WT(A)10	TAGCGATTGGAGACTTTCAACA	(A)10	AGAGATGCTCCTCAGCTCAAGT	53
WT(AT)6	TACTACAGAAGGA AAATCAGCC	(AT)6	ACACTGAAACCTCAAGAGAAGT	52.5

3.6 DESIGNING OF OVERLAPPING PRIMERS FOR MtDNA SEQUENCING

- The complete mitochondrial genome sequence of *Tragopan caboti* (16,727bp) was downloaded from NCBI.
- The entire sequence was fragmented into 17 parts of 2000bp each in the following way: 1-2000, 1000-3000, 2000-4000 and so on till 15000- 16727.
- A list of overlapping primers was generated using each fragmented part with the help of Primer 3.
- Product range provided was 800-1000bp.
- The final primers showing the maximum coverage were selected and thus a total of 26 primers were designed which covered the entire mitochondrial genome.

Table 7: List of overlapping primers

S NO		PRIMER- SEQUENCE	COVERAGE	PRODUCT SIZE	TM
1	L	ACACCTTGACCCCATTTCTG	165-989	825	59.82
	R	TGTGGTTCTGGGGTTTTAGG			59.82
2	L	CCCTCTTTAGTCCGTGATCG	541-1313	773	59.69
	R	ACTGGAATGCGGATACTTGC			60.1

3	L	TTCAAAGCATGGCACTGAAG	1193-2020	828	59.99
	R	CACGTCCTTTTTCGTTTGGT			60.01
4	L	ACCAAACGAAAAAGGACGTG	2001-2791	791	60.01
	R	TTTTCGATGGAGCTGTACCC			60.07
5	L	CGCTGGTAGAGGTGAAAAGC	2635-3357	723	60.02
	R	CGTTTTTGCACGGGAATACT			60
6	L	GAACGCCCACATAGACGATT	3088-4255	1168	59.96
	R	GGCTAGGATGGGAGTTGTGA			60.07
7	L	GGGCCCTTTTGGTCTACTTC	4141-4924	784	59.94
	R	GCATAGGGCTAGGGTTAGGG			59.95
8	L	CCCTAACCCTAGCCCTATGC	4905-5754	850	59.95
	R	CCTAGGTGGGAGATGGATGA			59.88
9	L	TAGCCTGAACGGGCTTAGAA	5274-6039	766	59.97
	R	GTGATGATTGTGGCTGTTGG			59.97
10	L	CCAAAACACCCATACCCAAC	5895-6618	724	59.95
	R	TCATCGGTTGATGAAGGTCA			60.05
11	L	GAGCTTGCAACTCACCATGA	6491-7322	832	59.99
	R	GGGTGACCGAAGAATCAGAA			60.05
12	L	AGGAGGAGACCCAATCCTGT	7272-8039	768	59.93
	R	CGTTTTGCTGAAAAGGCTTC			60
13	L	AGCCTTTTCAGCAAAACGAA	8022-8822	801	60
	R	TTTAGTCGTCCTGGGATTGC			60.07
14	L	CCTCCCCTCCTTACAGATCC	8514-9524	1011	59.89
	R	AGTGGGATGGCTAGGGCTAT			59.95
15	L	CGATGGGTCAATAACCGTCT	9307-10058	752	59.81
	R	TCGCCATCATTGGAGTATGA			60.03
16	L	GGCGAGACGTAATTCGAGAG	10054-10899	846	59.98
	R	TCGGATTGAGAATGGGAGTC			60.01
17	L	CACATCAAACCACCACTTCG	10562-11629	1068	60
	R	GAGATTTGGTCGATGCCTGT			60.08
18	L	AAACCAAACTCCATCGTTCG	11304-12152	849	59.97
	R	TAGTAGTAGGGCGGCGAGAA			60
19	L	GCCCATGTAGAAGCACCAAT	12099-12824	726	59.96
	R	GCATATGGGGTTTCCTGAGA			59.89
20	L	TCTCCCATGGACTCACATCA	12394-13166	773	60.05
	R	AGGAAGGCGGTTTTAGTGGT			60
21	L	ACCACTAAAACCGCCTTCCT	13147-13968	822	60
	R	TATAAGGCCCAGTTGGCTTG			60.09
22	L	AAGGTCCAACCCCAGTCTCT	13742-14497	756	59.97
	R	CTGCAGTTTTGGTGATGGTG			60.15
23	L	ACTGCAGCCATCACTGTCAC	14491-15321	831	59.9

	R	TTTGGCCAATGTATGGGATT			60.02
24	L	AGCAAAGCCTCAACCACACT	14749-15704	956	59.91
	R	GTTTGGGATTGAGCGTAGGA			60.07
25	L	CCACCCGTACTACTCCCTCA	15506-16319	814	59.98
	R	AGGGGTGGCTACTGTTGATG			59.99
26	L	CATAACATTCCGCCCACTCT	15788-16704	917	59.96
	R	TGGCTTTTCAAGCCGTAGTC			60.39

3.7 Data Collection and Analysis

The banding patterns obtained from gene specific primers were scored as present (1) or absent (0), each of which was treated as an independent character. Dice coefficient was used to calculate the similarity between pairs of accessions. The similarity matrix was subjected to cluster analysis by unweighted pair group method with arithmetic means (UPGMA) and a dendogram was generated using the program NTSYS pc (Rohlf FJ, 1992) POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across all the three populations were also analyzed. (Zhao WG et al., 2006) Within species diversity (Hs) and total genetic diversity (Ht) (Nei M, 1978) were calculated within the species and within three major groups (as per their collection site) using POPGENE software.

CHAPTER 4

Result and Discussion

4.1 WHOLE DNA ISOLATION FROM BLOOD

In order to characterize isolated microsatellite markers, twenty five samples of cheer pheasant were collected from bird sancturies in chail Sarahan and Chail, district Shimla of Himachal Pradesh. Feather follicles were taken from birds and stored in 100% ethanol immediately after removal. Blood samples were collected in EDTA (7 µg/µl blood), kept in ice and shifted to the lab for examination. Genomic DNA was extracted from blood samples using the standard phenol/chloroform method.

4.2 RAPD analysis

A total of 353 fragments were amplified of which 182 were polymorphic loci with allele frequency ranging from 4(OPT 17) to 19 (OPF14) loci per primer, which may be attributed to the randomness of RAPD markers as compared to SSR markers that are developed from expressed part as expressed portion is under high selection pressure and highly conserved regions as compared to the intergenic regions (Scott KD et al., 2000) (Rungis D et al., 2004)

Ten RAPD primers generated reproducible, informative and easily scorable RAPD profiles were preselected. These primers produced multiple band profiles (Figure 2) with a number of amplified DNA fragments varying from 4 to 9, with a mean of 6.2 bands per primer. All the amplified fragments varied in size from 200-1000 bp. Out of 353 amplified bands, 182 were found polymorphic. The observed high proportion of polymorphic loci suggests that there is a high degree of genetic variation in the Western tragopan individuals. A dendrogram analysis

based on neighbor joining (NJ) method grouped all the 20 genotypes into two main clusters (Figure 3).

A relatively high genetic variation was detected among the individuals. Genetic diversity analysis in terms of Na, Ne, H, I, Ht, Hs, and PPL revealed higher values, indicating more variability among the genotypes (Table 8). This is helpful in making strategy for breeding pairs.

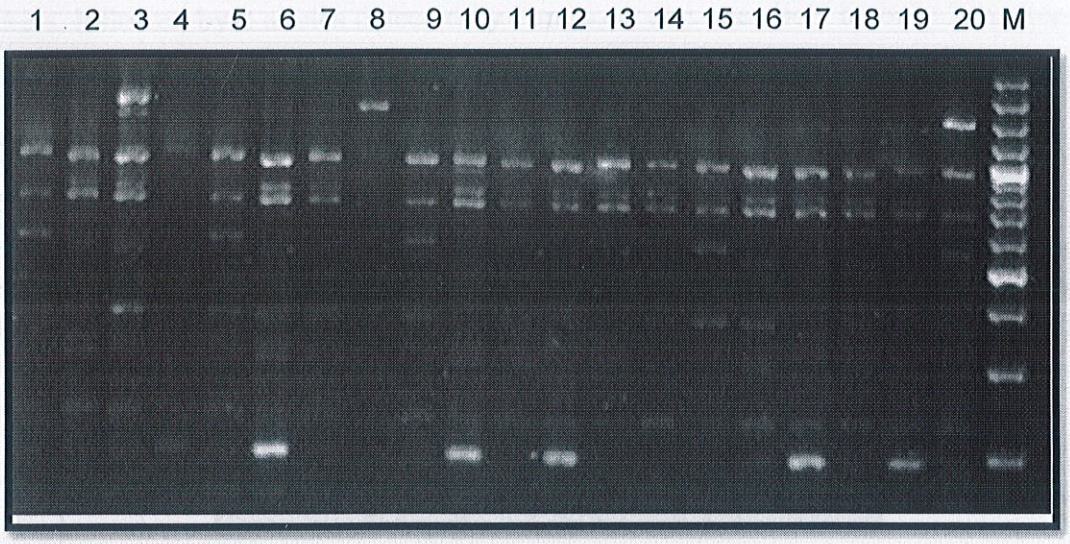

Figure 2: Representative gel showing different levels of polymorphism for coding regions in 19 Western tragopan (1 to 19) and one red Jungle Fowl (20) using RAPD primer OPF 14 M= 100 bp+ ladder.

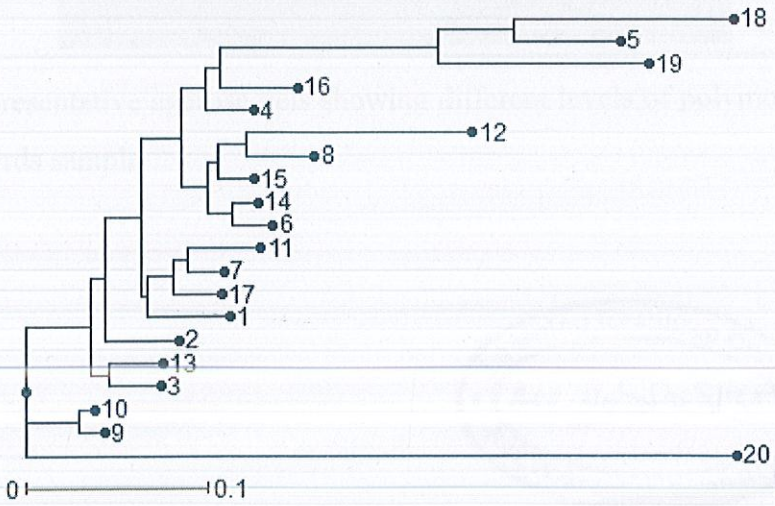

Figure 3. Dendrogram generated utilizing RAPD molecular markers illustrating genetic relationships among 20 *Pheasant* sp. The dendrogram was generated by NJ cluster analysis.

4.3 SSR POLYMORPHISM

Using DNA samples isolated from 20 birds sample as templates, polymorphic DNA fragments were amplified from 15 SSR primer pairs selected in the study. The sizes of these fragments ranged from 90 to 600 bp. A total of 38 alleles with the average alleles per locus of 2.92 were detected at 13 loci. More than one allele was detected at 12 out of 13 loci, with the polymorphic markers ratio of 92%. The maximum of 3-7 alleles were detected with five markers, followed by 2 alleles detected by 7 markers and one allele by only 3 marker. Figure 4 shows the representative amplified products from 3 different SSRs marker.

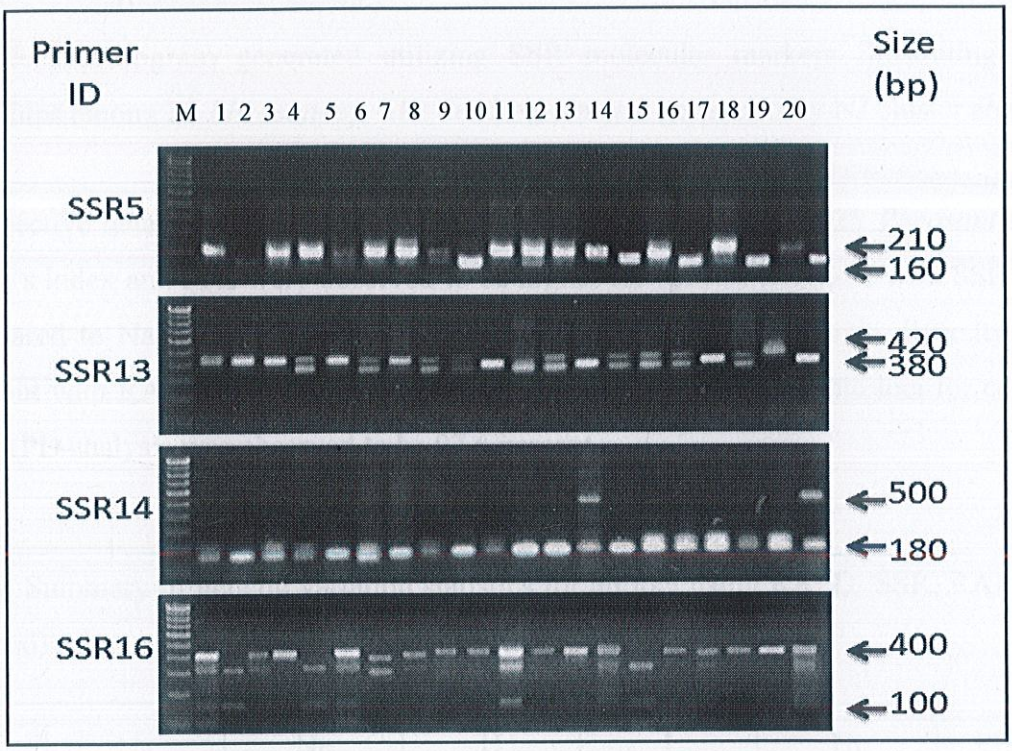

Figure 4: Representative agarose gels showing different levels of polymorphism for coding regions in 20 birds sample.

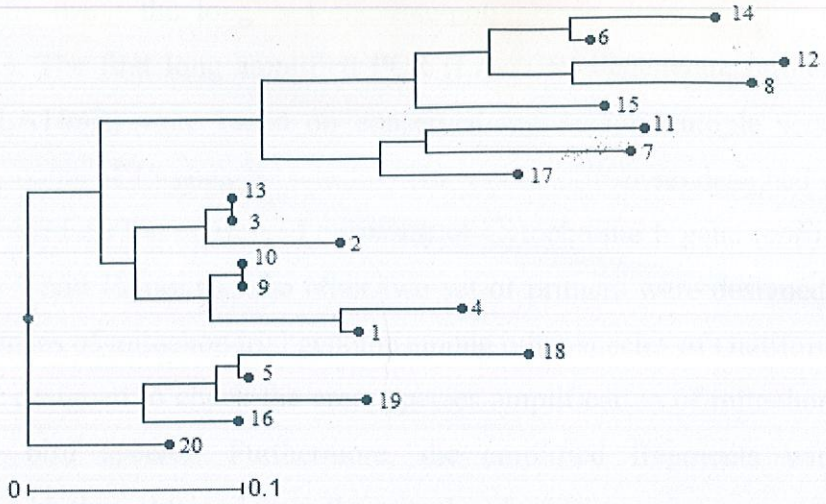

Figure 5. Dendrogram generated utilizing SSR molecular markers illustrating genetic relationships among 20 *Pheasant* sp. The dendrogram was generated by NJ cluster analysis.

The respective details of Na, Ne, H, I, Ht, and PPL were studied for 25 *Pheasant* species. Shannon's index and PPL were observed to be higher for species analyzed with SSR marker as compared to Na, Ne, H and Ht which was on higher side for genetic diversity studies carried out with RAPD (Table 8). The overall percentage of polymorphic loci for combined SSR+RAPD analysis was observed to be 97.6 percent.

Table 8: Summary of genetic variation statistics for all loci using RAPD, SSR, RAPD+SSR (combined) primers among the *Pheasant* species.

	Na	Ne	H	I	Ht	PPL(%)
RAPD	2.0±0.00	1.4559±0.30	0.2848±0.14	0.2848±0.14	0.2848±0.02	28
SSR	1.9333±0.25	1.4108±0.32	0.2551±0.16	0.3995±0.21	0.2551±0.02	14 (93.33%)
Combined	1.9767±0.15	1.4401±0.31	0.2745±0.14	0.4293±0.18	0.2745±0.02	42 (97.67%)

Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index; Ht = Total genetic diversity; PPL = percentage of Polymorphic Loci.

4.4 MITOGENOMICS

In order to isolate and amplify the whole mitochondrial genome from both the bird species two set of PCR primers were designed. Two long overlapping fragments (~16 kb in length)

amplified initially using the long and accurate-polymerase chain reaction (LA-PCR) kit (Qiagen pvt.ltd.). The first long amplified PCR (LA-PCR) degenerate primer set designed (LA16SF and LA16SR) were based on conserved regions of multiple sequence aligned mitochondrial genome of 15 pheasant species. The second primer set designed was Cytb605F and Cytb252R based on the conserved sequence of Cytochrome b gene (*cob*) from Cabot's tragopan (Table 9 and Figure 6). The other two set of primers were designed based on the conserved sequences of mitochondrial genome among other species of Galliformes. These set of primers were designed to check the cross species amplification of mitochondrial genome from both the bird species. Furthermore, the amplified fragments with a size of approximately 16 kb thus obtained from the mitochondrial genome was amplified using a set of 26 overlapping primers followed by sequencing and compared with the available mitochondrial genome sequences from other species such as Red Jungle Fowl.

Table 9: List of primers designed to amplify the whole mitochondrial genome of both Western tragopan and Cheer pheasant. The long amplified PCR (LA-PCR) primers are LA16SF and LA16SR. The other two set of mitochondrial specific primers were designed to check whether the mitochondrial genome has amplified or not.

NAME	SEQUENCE(5'-3')	LENGTH	MW	Tm	GC%	OD	TUBE	nmol	WATER/ TUBE	PURIFICATION
LA16SF	CCTACGTGATCTGAGTTCAGAC CGGAGYAATCCAG	35	10748.53	69.10-70.27	51.43%-54.29%	2	1	6.14	61.4	OPC
LA16SR	TRCACCATTAGGWTGTCCTGA TCCAACATCGAGGT	35	10718.52	66.76-69.10	45.71%-51.43%	2	1	6.16	61.58	OPC
CYTB605F	AYGAATCAGGCTCWAACAACC CWCTRGGCATC	32	9746.89	65.89-71.02	46.88%-59.38%	2	1	6.77	67.71	OPC
CYTB252R	GATGCAGATGAAGAAGAATGA GGCGCCRTTTGC	33	10283.76	67.03-68.27	48.48%-51.52%	2	1	6.42	64.18	OPC
AVMT2F	AACCCATTATATGTAKACGG	20	6112.56	51.65-53.70	35.00%-40.00%	2	1	10.8	107.97	OPC
AVMT2R	TTACTGCTGARTACCCGTGG	20	6116.03	57.80-59.85	50.00%-55.00%	2	1	10.79	107.91	OPC
AVMT3F	RCAAAAGACTYAGTCCTAACC	21	6367.72	54.11-58.01	38.10%-47.62%	2	1	10.36	103.65	OPC
AVMT3R	CTTTTGCRACAGAGAYGGGTT	21	6476.78	56.06-59.97	42.86%-52.38%	2	1	10.19	101.9	OPC

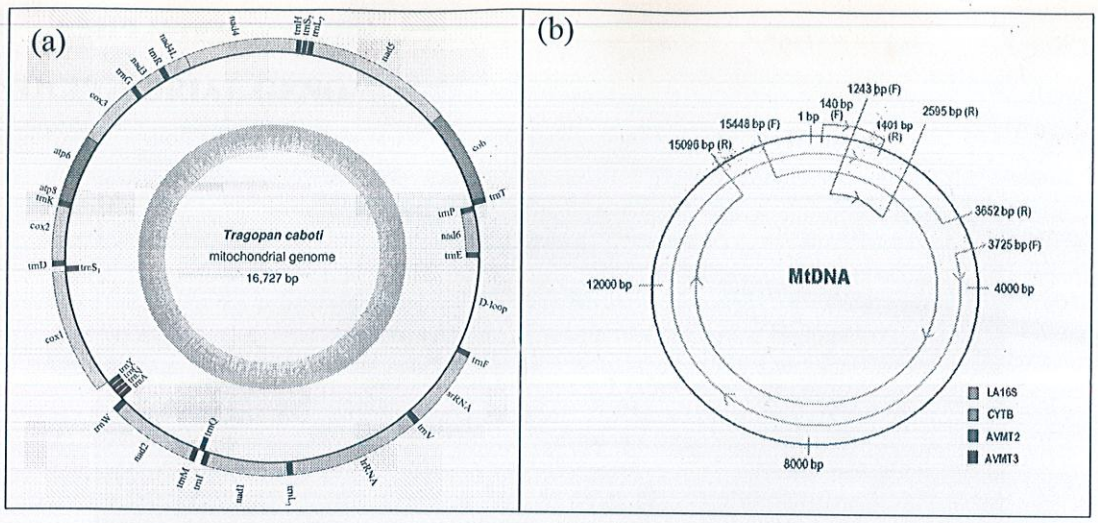

Figure 6. The schematic diagram demonstrating (a) the organization of mitochondrial genome of *Tragopan caboti* and (b) the localization of designed primers and their expected amplified product size.

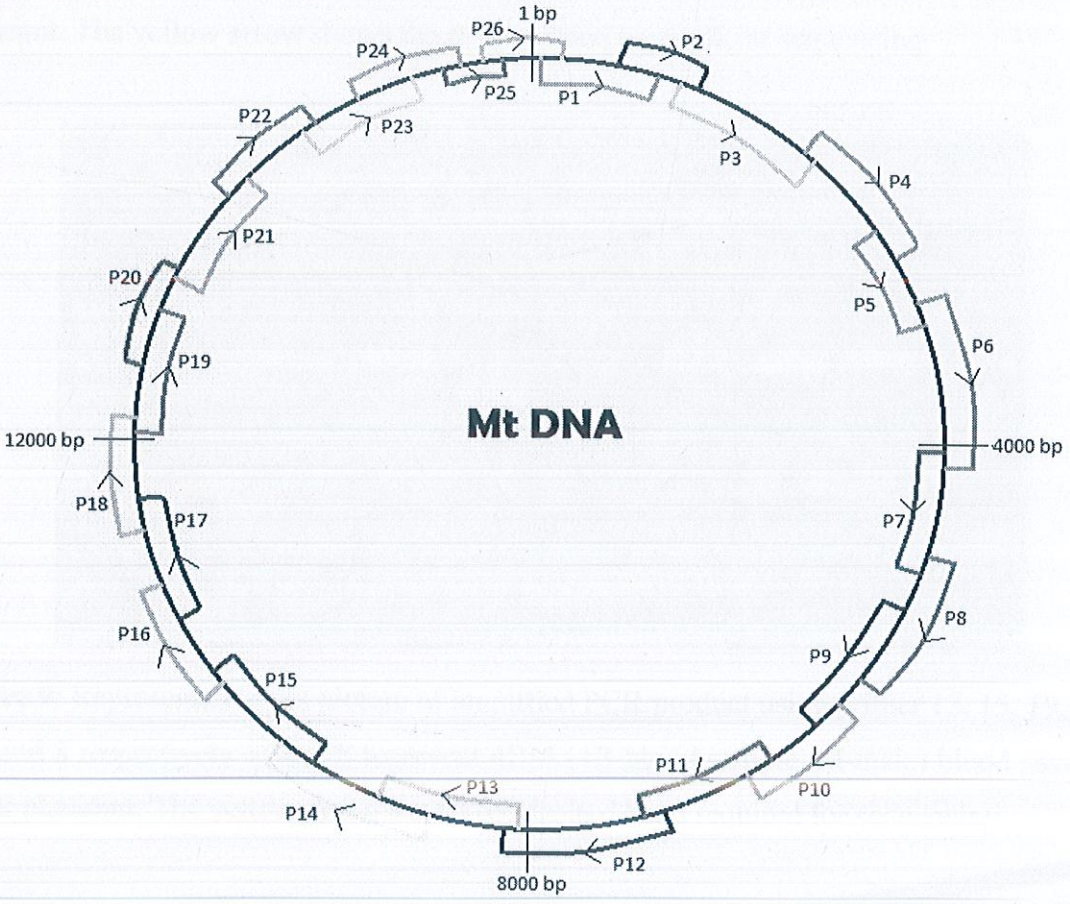

Figure 7: Representation of the coverage of overlapping primers on the entire mitochondrial genome.

4.5 AMPLIFIED OVERLAPPING PRIMERS ON THE COMPLETE MITOCHONDRIAL GENOME

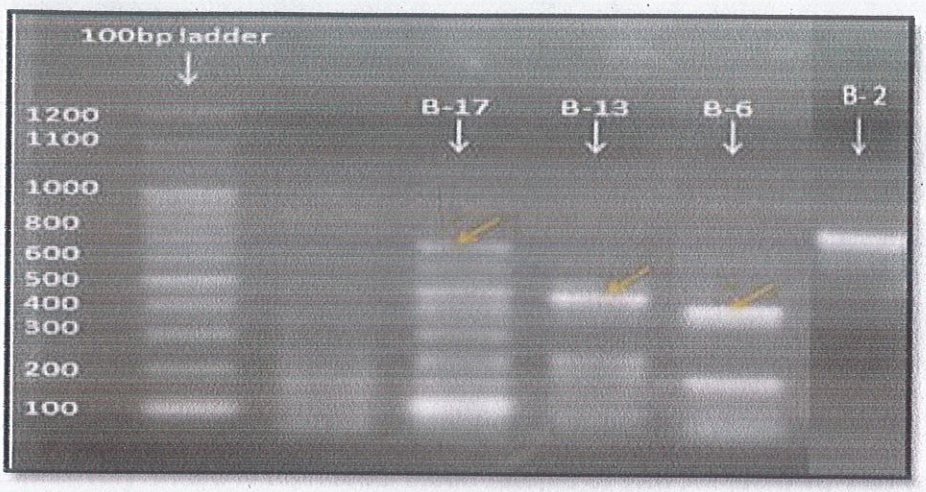

Figure 8: Representative gel picture of amplified PCR product using primer 17, 13, 6 and 2 respectively where B represents 4BM (4B-blood sample, M-male) blood sample of Cheer pheasant. The yellow arrow shows the desired band required for sequencing.

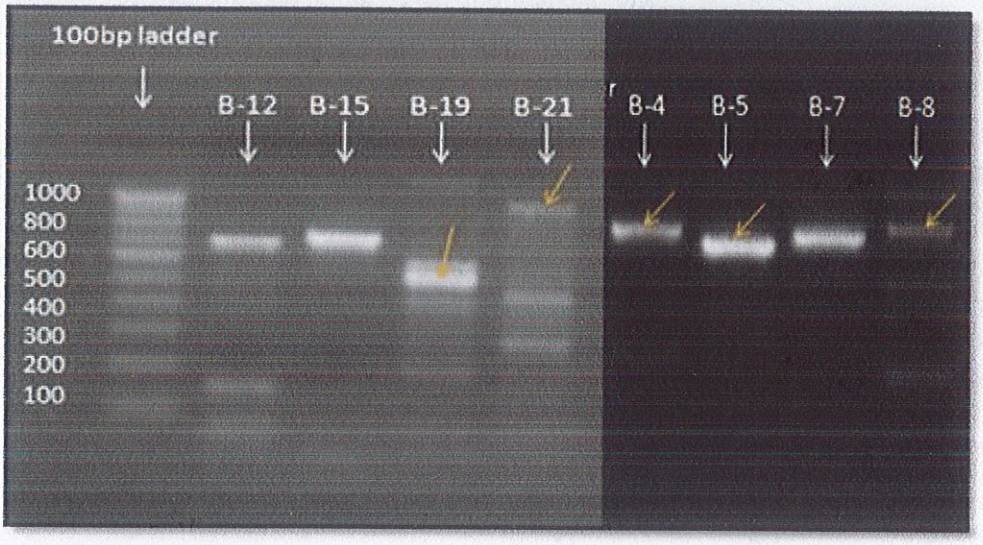

Figure 9: Representative gel picture of amplified PCR product using primer 12, 15, 19, 21, 4, 5, 7 and 8 respectively where B represent 4BM (4B-blood sample, M-male) blood sample of Cheer pheasant. The yellow arrow shows the desired band required for sequencing.

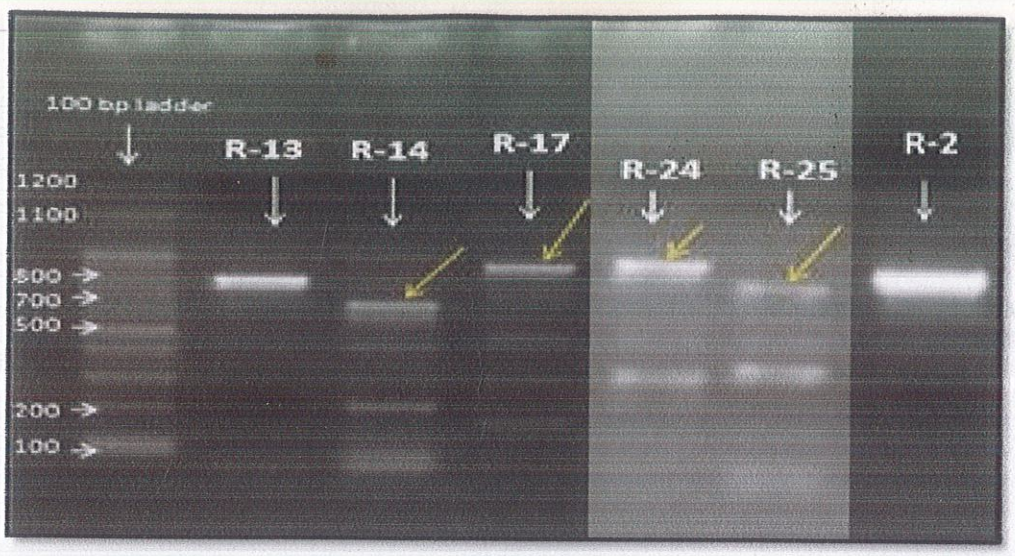

Figure 10: Representative gel picture of amplified PCR product using primer 13, 14, 17, 24, 25 and 2 respectively where R represents blood sample of Red Junglefowl. The yellow arrow shows the desired band required for sequencing.

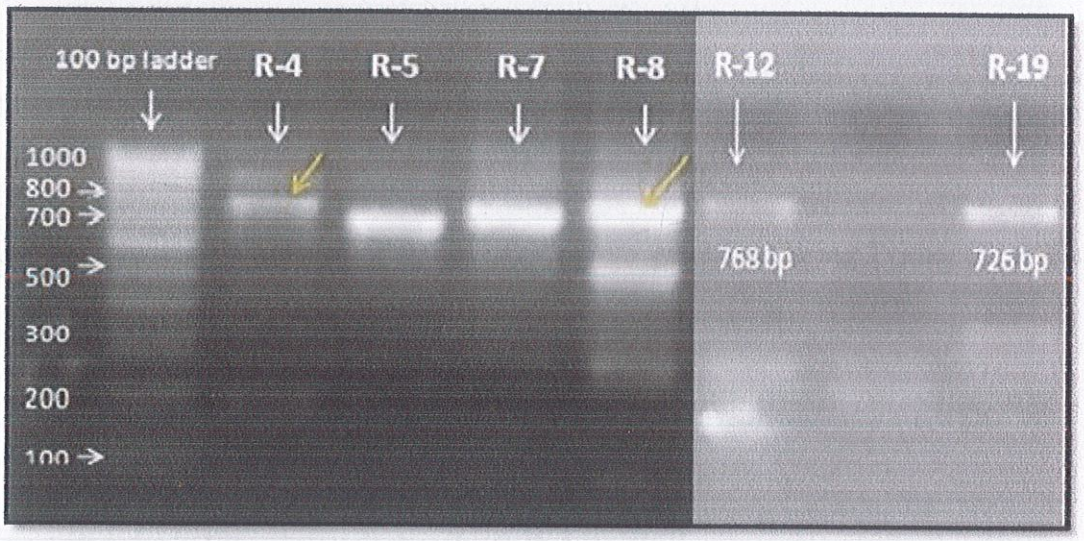

Figure 11: Representative gel picture of amplified PCR product using primer 4, 5, 7, 8, 12 and 19 respectively where R represents blood sample of Red Junglefowl. The yellow arrow shows the desired band required for sequencing.

4.6 SEQUENCING OF WHOLE MITOCHONDRIAL GENOME OF WESTERN TRAGOPAN AND RED JUNGLE FOWL

The amplified PCR products of overlapping primers were sent for sequencing and the following chromatograms were generated using DNA Baser software v3.5.3 which depicts the consensus sequences generated by DNASTAR for both the Western tragopan and Red Jungle Fowl.

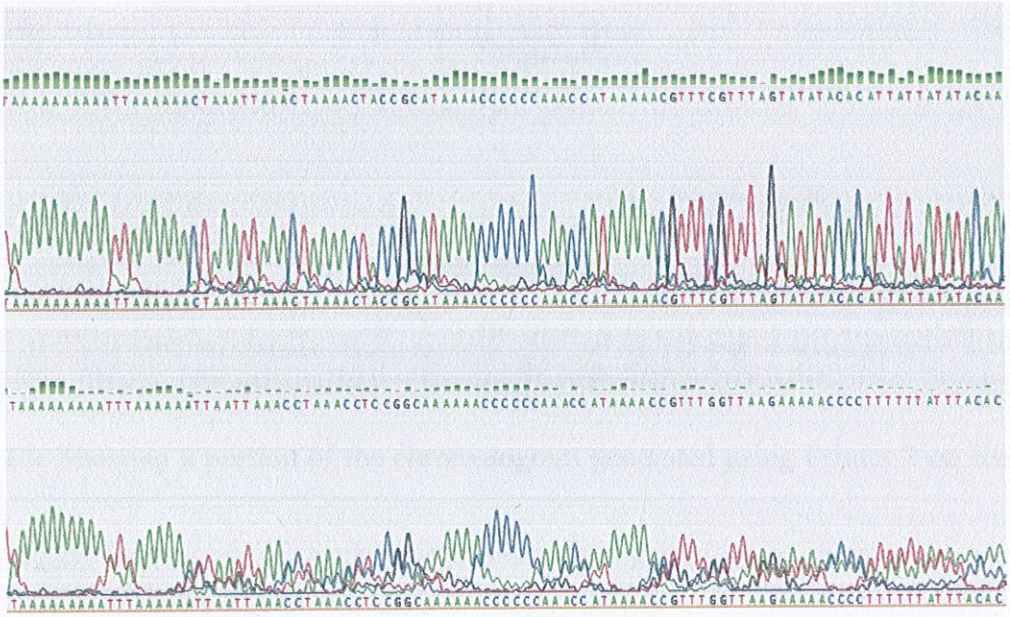

Figure 12: Showing a portion of the chromatogram generated using Primer 2 on Western tragopan.

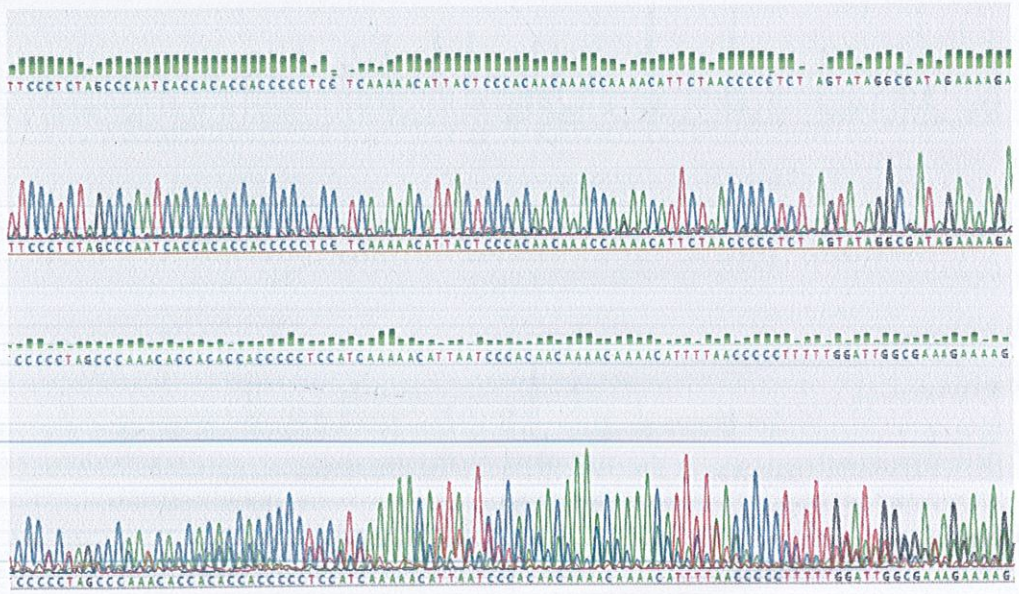

Figure 13: Showing a portion of the chromatogram generated using Primer 4 on Western tragopan.

Red jungle fowl (RJF)

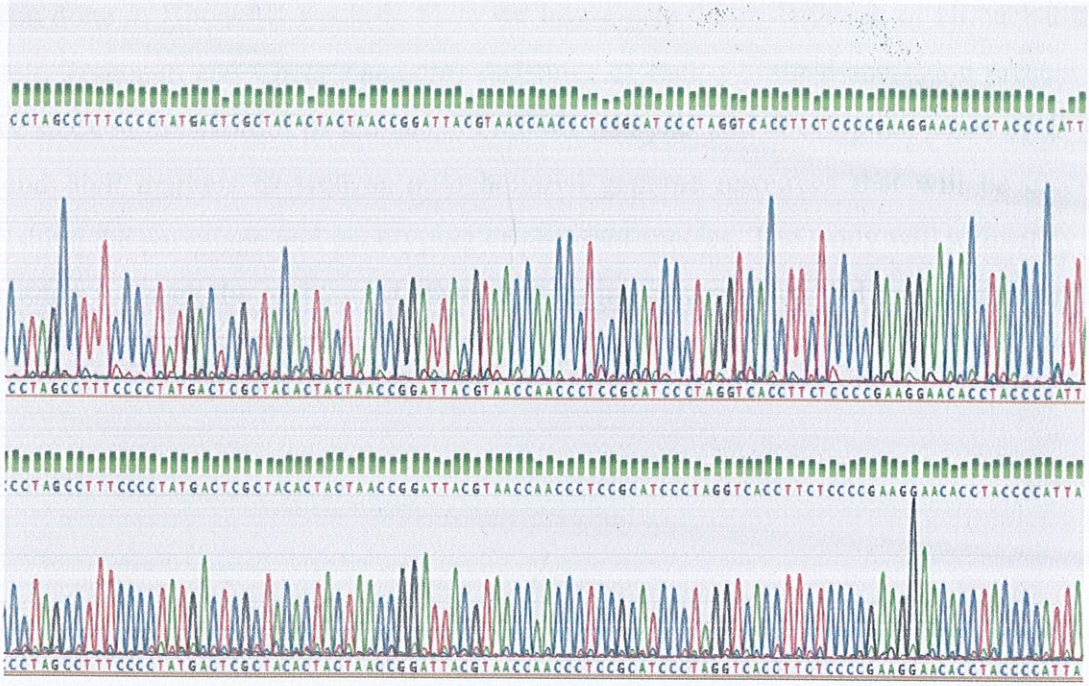

Figure 14: Showing a portion of the chromatogram generated using Primer 2 on Red jungle fowl.

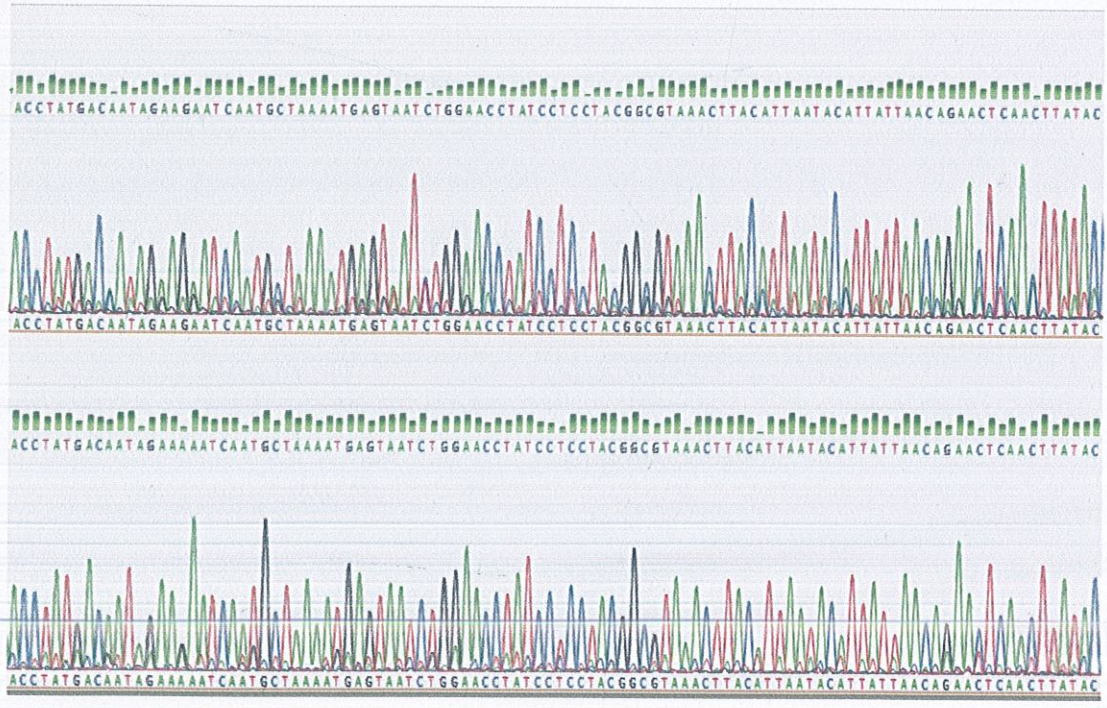

Figure 15: Showing a portion of the chromatogram generated using Primer 5 on Red jungle fowl.

Richness of endemic species is considered important for biodiversity conservation and avifaunal regionalization, but no detailed studies concerning the subregional endemism have yet been done in Himachal Pradesh. Here we investigate the assemblage of Himachal avian (Western Tragopan and Cheer Pheasant) endemics of each avifaunal subregion present as a captive stock in the aviaries of Himachal Pradesh, analyze and develop the panel of *de novo* SSR and SNP markers as well as mitochondrial genome resources that will be useful in establishing the parentage and genotyping of pheasant species. The study will ultimately help the breeders to check the problem of inbreeding during conservation and breeding program.

Conclusion

Our results indicate that the molecular analysis of both RAPD and SSR markers were extremely useful for studying the genetic relationship of cheer pheasants. Seven of the fifteen microsatellite loci were highly polymorphic. The observed numbers of alleles ranged from 2 to 3 with an average of 2.14 per locus. The total genetic diversity for SSR was observed to be 0.28. In contrast to SSR only 3 out of 10 RAPD primers gave consistent amplification and among three only one was polymorphic with comparatively low degree of polymorphism (51.5%). Cluster 1(Acc1 and Acc5), cluster 5 (Acc 17) and cluster 8 (Acc 9 and Acc 13), the mentioned common clusters should be avoided from selection as parental breeding stock as they can lead to inbreeding depression in the offspring.

Because of low availability of sequence specific markers (SSR) to search for nucleotide variation and for further validation, mitochondrial genome sequencing and analysis is also done to understand interspecific evolutionary process, and to improve/barcode the present captive stock i.e. to avoid inbreeding depression by separating out the pheasant birds with high homozygosity and pairing the birds which are showing genetic variation.

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