DNA BARCODING OF PHEASANT SPECIES OF HIMACHAL PRADESH: RATE OF EVOLUTION AND IDENTIFICATION OF SPECIES

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

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In

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Under the Supervision of

Dr. Harvinder Singh

By

Shivai Gupta

Enroll: No: 111568

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN – 173 234, HIMACHAL PRADESH

CERTIFICATE

This is to certify that the work titled **"DNA Barcoding Of Pheasant species of Himachal Pradesh : Rate Of Evolution and Identification of Species"** submitted by "Ms. **Shivai Gupta"** in the partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor:

Name of Supervisor : Dr.Harvinder Singh
Designation : Assistant Professor
Date : 28-5-2015

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Shivai Gupta

SUMMARY

Phasianidae (pheasants, grouse, turkeys and partridges) is one of the five families which come under the order Galliformes. The Western Tragopan, Himalayan Monal, Kalij and Cheer Pheasant belong to the Phasianidae sub family and also are the pheasants of Himachal Pradesh.These species are endemic to Himalayas and are endangered. The aim of the present work is to perform in-silico analysis so as to develop universal primers for DNA barcoding of pheasant species especially targeting gene regions namely the internal transcribed spacer region and the cytochrome oxidase I region.

Genomic DNA was isolated by non-invasive methods from egg membranes using *Gallus gallus* as the model organism. Genomic DNA wasalso isolated from 30 blood samples of the pheasants Cheer and Western Tragopan and was amplified by Polymerase Chain Reaction for seven primers of the ITS region. The phylogenetic analysis for gene tree construction, sequence identity and homology was carried out using tools available at the National Center for Biotechnology's website, namely Nucleotide Basic Local Alignment Sequence Tool (Blast N), Protein BLAST (Blast P), Taxonomy, and Homologene. Successful barcoding of this characteristic region has been carried out in the present study.

"*The Plumage of this tragopan is beautiful beyond most birds – orange and gold, brown and black with a score of constellations of silver stars scattered over all, from neck to tail*"

From Pheasant jungles by William Beebe

Detail from a painting of Western Tragopan (*Tragopan melanocephalus*) male (on the art) and female, painted by Archibald Thorburn, in William Beebe's: A Monograph of the Pheasants (1918-22).

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LIST OF SYMBOLS AND ACRONYMS

B1- Buffer 1

B2- Buffer 2

Blast N- Basic Local Alignment Search Tool – Nucleotide

Blast P- Basic Local Alignment Search Tool- Protein

CITES-Convention on the International Trade of Endangered Species

COBOL- Consortium of Barcode of Life

COX- Cytochrome Oxidase C

Cytb- Cytochrome b

DNA- Deoxyribonucleic Acid

EDTA- Ethylene Diamine Tetra Acetic Acid

EST- Expressed Sequence Tags

ITS –Internal Transcribed Spacer

IUCN- International Union for the Conservation of Nature and Natural Resources

mM- Mili molar

mtDNA- Mitochondrial Deoxyribonucleic Acid

PCR- Polymerase Chain Reaction

SDS- Sodium Dodecyl Sulphate

TBE- Tris/Borate/EDTA

µL- Microliters

CHAPTER 1: INTRODUCTION

1. Background

Richness of endemic species is considered important for biodiversity conservation and avifaunal regionalization, but no detail studies concerning the sub-regional endemism have yet been done in Himachal Pradesh. The present study is to investigate the assemblage of Himachal Pradesh pheasants western tragopan *Tragopanmelanocephalus*, cheer pheasant *Catreus wallichii*, Himalayan monal *Lophophorus impejanus*, kalij pheasant *Lophura leucomelanos* endemics of each avifaunal sub region present as a captive stock in aviaries of Himachal Pradesh and develop the panel of de novo SSR and SNP markers which will be useful in establishing the parentage and genotyping of western tragopan, that will ultimately help the breeders to check the problem of inbreeding during conservation and breeding program. 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 sequencing of all known species.

Endangered species are important to their habitats, the food chain and to evolutionary process. It is known that throughout history species have become extinct for various reasons that generally led to evolutionary advancement but the rate at which species have become extinct and continue to become endangered over the past century is cause for concern. Species are constantly at risk of becoming extinct, but it has become much more of a problem because of loss of species biodiversity and ecosystem.Information on genetic relationships among individuals is of tremendous importance to breeders for hybrid development conservation and breeding program. The study has been to conduct DNA profiling of the captive parental stock to reduce the inbreeding by separating with closely related pairs. Endemic species have long been a key focus in conservation efforts, given that the level of endemics might be possibility correlated with species richness. A good understanding of evolutionary process such as population subdivisions, changes of effective population size and genetic connectivity of endemic species would shed light on evolutionary processes as well as conservation management (Gu et al., 2012).

1.1 Pheasants and their conservation

Pheasants are the most charismatic of all the fauna found in the Himalayan landscape. Their high endemism and brightly coloured plumage makes them the most distinctive bird family of the

Himalaya (Ali 1981). Of the 51 species of pheasants 20 (39%) are endemic to the Himalayas, which includes the genera of Ithaginis (blood pheasant), Tragopan (tragopans or horned pheasants), Lophophorus (monal pheasants), Lophura (kalij pheasant), Pucrasia (koklass pheasant), Catreus(cheer pheasant), Crossoptilon (eared pheasant) and Polypectron (peacock pheasant. The Indian Himalayas is home to 16 species of pheasants that are 94% of the total pheasant species (17) found in India. Five of the six spices of pheasants that are threatened in India are found in Himalaya (Sathyakumar & Sivakumar, 2007).

Pheasants and their Distribution

Most of the species of pheasants are found in Asia-in the montane temperate forests (of Indian Himalayas, mountains of Pakistan, Nepal, Bhutan, China, Japan, and Southeast Asia), temperate grasslands (of central Asia), and tropical forests (of India, Sri Lanka, and Southeast Asia); and one of the species, the Congo peacock *Afropavo congensis*, occurs in the tropical forests of Congo in Africa. Pheasant habitats are still some of the least-studied forest ecosystems in the world – and yet- they are one of the bird groups most seriously affected by habitat destruction and over-hunting (Johnsgard, 1999). Almost 2/3rd of the entire group of pheasant species are listed as 'Endangered', 'Vulnerable', or 'Near-threatened' in the IUCN Red List.

Indian Sub-continent

The Himalayan mountains are home to many endemic and other wildlife and pheasant species- e.g. western tragopan *Tragopanmelanocephalus,* cheer pheasant *Catreus wallichii*, koklass pheasant *Pucrasia macrolopha*, Himalayan monal *Lophophorus impejanus*, kalij pheasant *Lophura leucomelanos*, red junglefowl *Gallus gallus*, Sclater's monal Lophophorus sclateri, Blyth's tragopan Tragopan blythii, grey peacock pheasant Polyplectron bicalcaratum, and Mr. Hume's pheasant Syrmaticus humiae. (Zurick et al., 2005).

What are Pheasants?

Pheasants are moderately large birds, comparable in size to domestic chickens. They primarily feed on the ground, and eat plant matter, insects, etc. In most of the species of pheasants, the males have colourful, iridescent plumage. Pheasants build simple nests on the ground with leaf and stem-scraps, and a few species also construct nests on trees or bushes. The pheasants are generally non-migratory; and they are either monogamous or polygamous (Johnsgard, 1999).

Their courtship displays are perhaps the most spectacular and alluring among all the birds in the world. This makes them prime subjects of depiction in art and literature in their indigenous range countries. Some examples of these remarkable species are the flue peafowl, the green peafowl, the brown eared pheasants, the crested fireback pheasant, and the silver pheasant.

Ecology habitat and habits of pheasant

The pheasant species are distributed in basically 3 types of habitats (Johnsgard, 1999). A few of them occur in 2 different habitat types. For example, the blue peafowl Pavo cristatus is found in the deciduous forests of the plains, as well as the lower montane forests in India.

1. Temperate montane forest habitat: (India, Pakistan, Nepal, Bhutan, Japan, China).
2. Tropical and sub-tropical forest habitat: (India, Sri Lanka, Southeast Asia, and the Democratic Republic of Congo).
3. Temperate open-country habitat (i.e. grasslands with streams and wooded area): (China, Mongolia, Korea, Iran, and introduced in former USSR, USA, Europe, Australia, New Zealand, etc.)

Seven species of pheasants are found in Himachal Pradesh. The Red JungleFowl (*Gallus gallus*) is mostly found in the subtropical dry evergreen forest below 1200 m, and prefers dense under-growth habitats. Indian peafowl (*Pavo cristatus*) lives in habitats similar to that of red junglefowl. The kalij pheasant *Lophura leucomelanos* is found in disturbed habitats close to human habitation in lower oak and coniferous forests (Johnsgard, 1999). Himalayan monal (*Lophophouus impejanus*) prefer a less dense ground cover. For cheer pheasants (*Catreus wallichii),* the presence of dense grass appears to be animportant habitat attribute. The western tragopan Tragopan melanocephalus is associated with middle altitude meadows and temperate forests. Tree cover and slope are important to pheasants because they determine the vegetation characteristics, or the type of food available at ground level. Greater slope also prevents access by people and livestock, and perhaps also facilitates escape from predators by downhill running. Distribution of pheasants indicates a vertical segregation of pheasant species in vegetation zones (Johnsgard, 1999).

Among birds, ‘the Galliformes are one of the most threatened of taxonomic orders, in terms of the proportion of species threatened. The 17 species of Indian pheasants, which

constitute one third of the global total of 50 pheasant species, are among the most charismatic birds of the world. The colourful Indian peafowl *Pavo muticus* is India's National Bird; In fact, several States have designated different pheasant species as state birds. Himachal Pradesh has the western tragopan *Tragopan melanocephalus* as its State Bird.

The Galliformes are important indicator species and their presence in an area is a good indicator of the health of the ecosystem. As in the case of most wildlife, the single most serious threat to the survival of Galliformes is the loss of forest and grassland habitat, and its degradation and fragmentation. The other major threats are human disturbance, killing or trapping for local consumption, and the use of pesticides and other chemicals in agriculture.

The Avian order Galliforms contain about 290 species, many of which (e.g. Chicken, turkey and peacock are closely related to human society. Some of these birds are also important model system in areas as diverse as development, disease transmission and sexual selection. Thus a well resolved Galliform phylogeny is necessary to address a wide range of questions such as the geographic region of certain lineage. The possible transmission & evolution of pathogen and evolution of sexual traits. Approximately 25% of Gallifoms species worldwide are threatened (critically endangered, endangered, vulnerable based on IUCN Red list.

There is sustainability interest in using phylogenetic information to in order to have conservation priorities efforts to understand the use of phylogenetic information to establish conservation priorities has used Galliforms model system making a well resolved Galliform Phylogeny in more essential. They are a valuable resource in terms of biodiversity as they show important evolutionary relationships.The pheasants have a small and sparsely distributed population. As a result of habitat destruction (loss of living space to development or pollution), introduction of non-native organisms and direct killing for the purpose of flesh and feathers their already sparse population is declining. It is becoming increasingly fragmented due to face of continuing forest loss and degradation throughout their restricted range. Their population has decreased and it poses an increased threat to the associated biodiversity also.

Being endemic to the eastern Himalayas all the pheasant species are an important part of biodiversity. Their Population growth is either limited or does not occur. As a result of the diminishing population varied measures are taken to save the species such as captive breeding.

Further the Contemporary genetic structuring helps in understanding the long term survival of a species.

1.2 Objectives

1. To carry out morphological and genetic studies on four species of pheasants kept in captivity in Himachal Pradesh.
2. In-silico analysis to develop universal primers (ITS and COX I region) for DNA barcoding of pheasant species.
3. DNA barcoding of target regions.
4. Identification of putative single nucleotide Polymorphism (SNP's) in the genes responsible for egg shell thinning.

1.3 Study Area:

Himachal Pradesh is one of the states in the western Himalayas in India having an area of 55.673 Km^2 and population of 6.856 million (2011 Census) It is located at latitude 30^0 22' 40'' to 33^0 12' 40'' N and longitude 75^0 45' 55'' to 79^0 04' 20'' E.A total of 390 bird species have been reported from Himachal Pradesh of which many are endemic to the western Himalayan region. The State is home to seven globally threatened species classified and listed in the Red Data Book by International Union for Conservation of Nature (IUCN). The State has also been identified as an endemic Bird Area having many endemic bird species which are found only in the Western Himalayan Region. The State also has 28 important bird areas which are places of international significance for conservation of birds at the global, regional and sub-regional level. The State is especially rich in pheasants which are also colourful and intervening birds. Pheasants are birds that belong to the sub family Phasionidae characterized by colourful plumage and interesting breeding displays. The males are larger and more colourful than the females and have been introduced in many countries. The State of Himachal is home to seven pheasant species. Pheasants are considered as bio indicators by the ecologists due to their sensivity to disturbance and habitat degradation and the central position they occupy in the food web.

Worldwide, the pheasants - as a group of birds are represented by 51 species all of which, save one, have originated and thrived in Asia. India accounts for 17 species one-third of the

World's total number of pheasants. Himachal Pradesh has seven pheasants out of the seventeen, found in India. The state is home to seven globally threatened species classified and hosted in the Red Date Book by the International Union for Conservation of Nature (IUCN).

The pheasant's species Western tragopan *Tragopanmelanocephalus,* Cheer pheasant *Catreus wallichii*, Himalayan monal *Lophophorus impejanus*, kalij pheasant *Lophura leucomelanos,* Koklass pheasant *Pucrasia macrolopha*, Red junglefowl *Gallus gallus*. Seven species of pheasants are found in HP. The Red junglefowl under-grwth habitats and Indian peafowl Pavo cristatus lives in habitats similar to that of Red junglefowl.

The kalij pheasant is found in disturbed habitats close to human habitation in lower oak and coniferous forests (Johnsgard, 1999). The kalij pheasant occupies forested areas with medium tree cover and a tall shrub layer (that occurs mostly along streams) at lower altitudes, while koklass pheasant Pucrasia macrolopha occupies forested areas with lower tree cover, and shrub layer of intermediate height at middle altitudes. Himalayan monal prefer a less dense ground cover. For Cheer pheasants, the presence of dense grass appears to be an important habitat attribute. The Western Tragopan is associated with middle altitude meadows and temperate forests. Tree cover and slope are important to pheasants because they determine the vegetation characteristics, or the type of food available at ground level. Distribution of pheasants indicates vertical segregation of pheasant species in vegetation zones (Johnsgard, 1999).

Himachal Pradesh has recognized pheasants as flagships of their forests and has initiated projects to conserve them.The Wildlife Wing of the Himachal Pradesh is engaged in conservation of Himalayan pheasants through a modern conservation breeding programme of Cheer pheasant & Western Tragopan. The birds are kept in near natural conditions in carefully designed aviaries. The conservation breeding is being carried out to preserve genetic diversity of these endangered species for a possible re-introduction in the future when needed. Three ex-situ breeding projects are in different stage of progress in Sarahan (for Western Tragopan), Manali (for Himalayan Monal) and Chail (for Cheer pheasant). In addition Kufri Nature Park, near Shimla also house pheasants Fig 3). This study has been carried out on the captive population of pheasants at four locations and various samples procured for experiments from individual's pheasant's species from four sites Table 3).

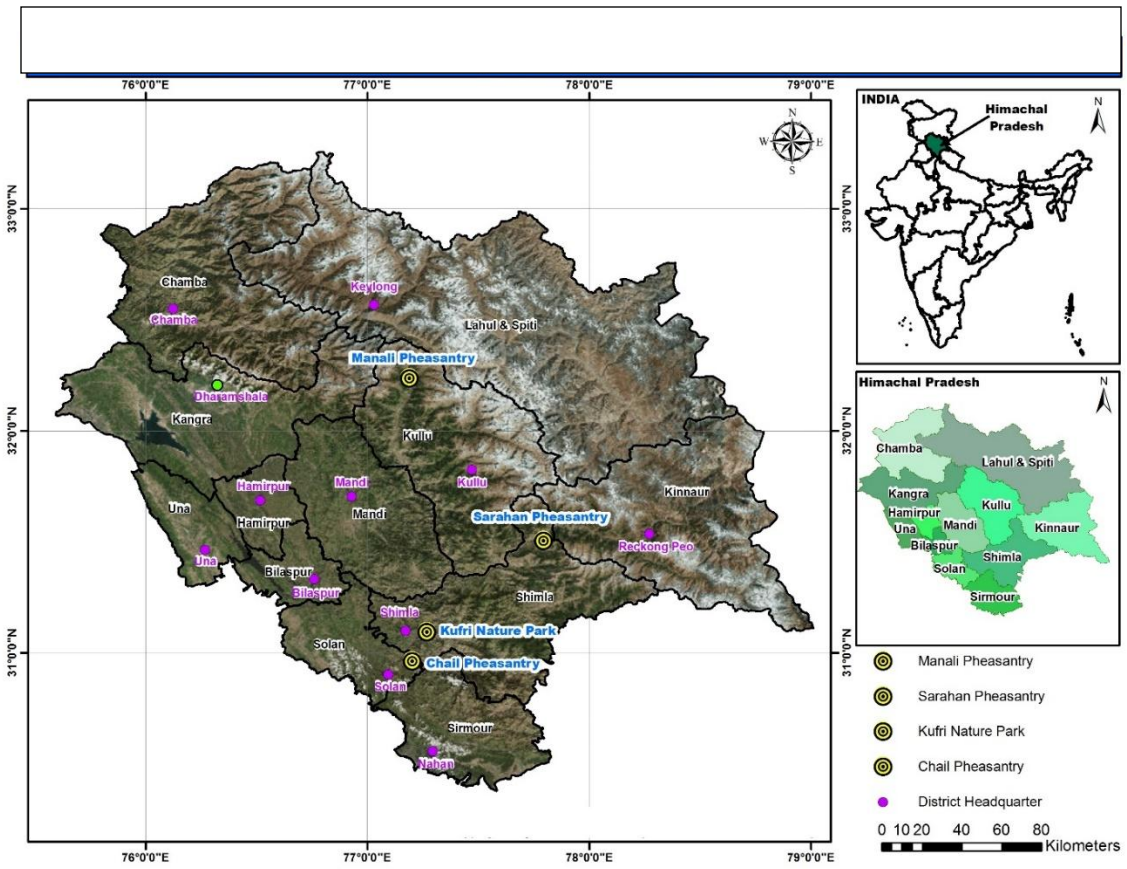
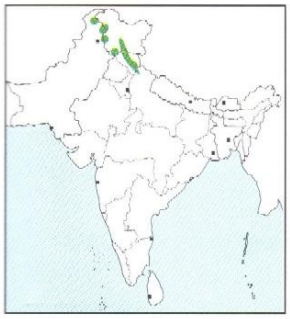


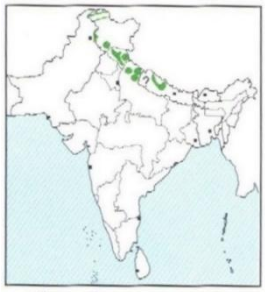
Figure1: Location of Aviaries/Pheasantries in Himachal Pradesh

Table 1: Distribution and number of pheasant species in Aviaries in Himachal Pradesh

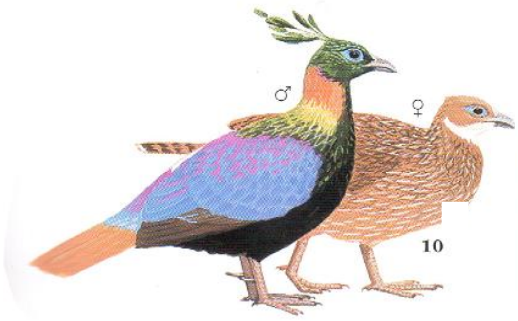
Location	Pheasant Species	Male	Female
Kufri Nature Park 31°5'46.52" N 77°15'44.29" E Altitude 2672 m	***Tragopan melanocephalus* (Western Tragopan)**	**2**	**1**
	***Catreus walichii* (Cheer)**	**7**	**19**
	***Lophophorus impejanus* (Himalayan Monal)**	**3**	**2**
	***Lophura leucomelanos* (Kalij)**	**5**	**4**
Sarahan Aviary 31°30'26.65" N 77°47'38.34" E Altitude 2195 m	***Tragopan melanocephalus* (Western Tragopan)**	**8+3***	**7+4***
	***Catreus walichii* (Cheer)**	**8**	**7**
	***Lophophorus impejanus* (Himalayan Monal)**	**2**	**-**
	***Lophura leucomelanos* (Kalij)**	**1**	**1**
Chail Aviary 30°57'49.45" N 77°12'5.07" E Altitude 2180 m	***Tragopan melanocephalus* (Western Tragopan)**	**-**	**-**
	***Catreus walichii* (Cheer)**	**13**	**18**
	***Lophophorus impejanus* (Himalayan Monal)**	**-**	**-**
	***Lophura leucomelanos* (Kalij)**	**-**	**-**
Manali Aviary 32°14'20.80" N 77°11'21.64" E Altitude 1908 m	***Tragopan melanocephalus* (Western Tragopan)**	**-**	**-**
	***Catreus walichii* (Cheer)**	**-**	**-**
	***Lophophorus impejanus* (Himalayan Monal)**	**3**	**9**
	***Lophura leucomelanos* (Kalij)**	**3**	**7**

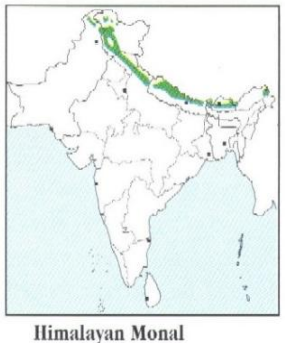


Western Tragopan (*Tragopan melanocephalus)*

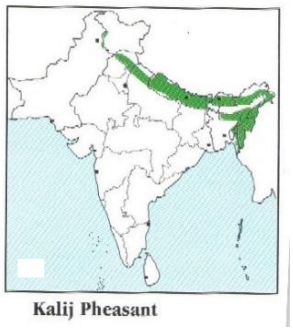


Cheer (*Catreus walichii*)





Himalayan Monal (*Lophophorus impejanus)*



Kalij *(Lophura leucomelanos)*

Figure 2: The Four Pheasants species and their Distribution Maps

Source: Kazmierczak, 2000& Grimmett et al., 2011)

1.4.1 WESTERN TRAGOPAN (*Tragopan melanocephalus*)

Distribution: India and Pakistan

India: Jammu and Kashmir and Himachal Pradesh. Presence in Uttrakhand also present

Population: Total world population is believed to be less than 3000 birds. The largest population is estimated to be in the Pallas valley in Pakistan. In India Himachal Pradesh has the maximum number of birds, making it the state bird as a result of the critically endangered nature.

Legal Status (India): Full protection provided by the virtue of inclusion in Schedule I of the Wildlife Protection Act 1972.

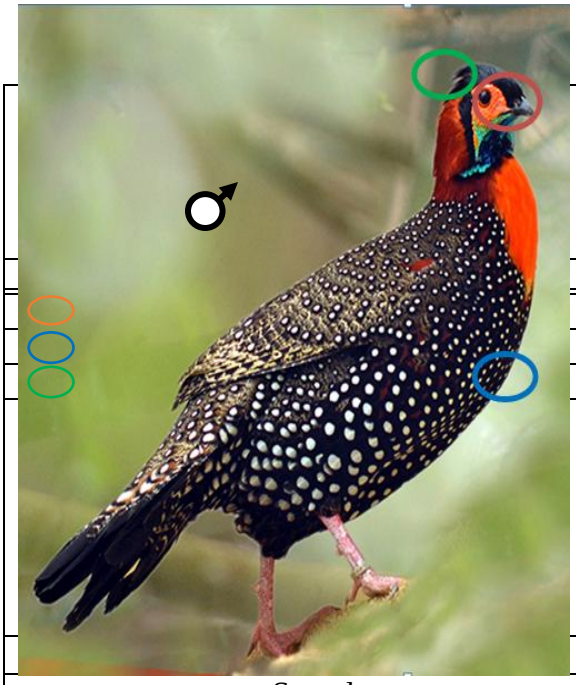
Morphology: In the male the neck, crest and forepart of the wing is red and neck downwards is predominantly black, grizzled with buff above and white spots below and above. The bare face is red, horns are blue and the dewlap purple in the middle with spots of blue at the sides. The female is mottled brown with white spots.

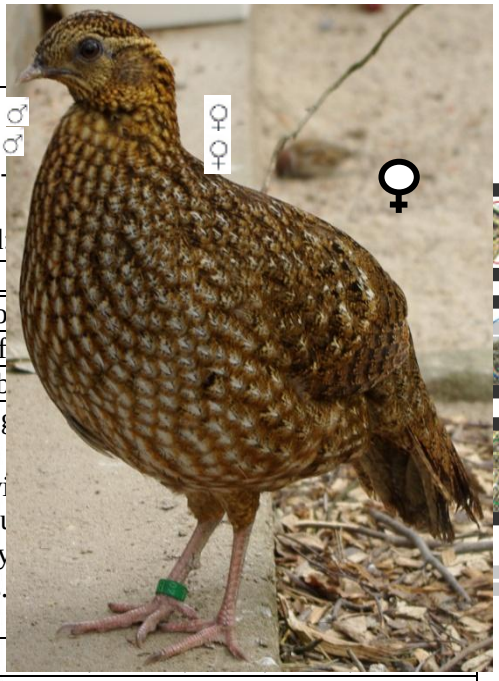
Habitat: Generally found at 1350- 3600 meters in well forested areas with precipitous mountain sides and heavy undercover. Prefers forests of coniferous species, like Silver fir (*Abies pindrow*), Blue Pine (*Pinus wallichiana*), Deodar (*Cedrus deodara*) and some broad leaved species is found to be more associated with thick understory mainly of *Skimmia lauriola*. During winter the species makes an altitudinal migration to lower altitudes. Known to be mainly vegetarians, though at times invertebrates also have been found in their diet. Mating generally takes place in the months of April-June. 3-4 eggs are laid in a nest that is rudimentary, either on the ground or in a tree.

General: The species is listed as Vulnerable by the IUCN. The threats arise from fragmentation of primary forest habitat in the western Himalaya. During winter, there is more direct confrontation with humans when the species migrates to lower altitudes. Hence, protection of the species from poaching and its habitat is vital. The Great Himalayan National Park in Himachal Pradesh affords such protection and this kind of situation is needed elsewhere.

Table 2.1 - Identification features, Distribution, reproduction and lifecycle

1. Western Tragopan *Tragopan melanocephalus (Gray, 1829)(The horned pheasant)*
   Local name: ***Jujurana*** (King of birds – Kullu), ***Fulgar*** (Chamba and Kangra)





	Body Length: Weight : 1.8- Breeding season: Incubation period
	Short, sto Slight ruf Mottled b Plumage streaks Dull gray Spots on Intricately and buffs.
	Berries, bamboo
Sound	Nasal, wailing Khuwahh; whaah
Nest site	Nest of ticks, line with grass, in thick undergrowth
Interesting feature	Shy bird, difficult to see

1.4.2CHEER PHEASANT*(Catreus wallichii)*

Distribution

Global: India, Pakistan and Nepal and Afghanistan.

India: Jammu and Kashmir, Himachal Pradesh and Uttarakhand

Population:Most Cheer populations are isolated and small, barring the certain sites Sarahan and Kufri pheasantries. The cheer pheasant is a medium sized montane pheasant in which sexual dimorphism is slight and both sexes have an occipital crest and a red orbital patch. The plumage is buffy brown and black with barring and spotting. The tail is elongated and strongly barred.

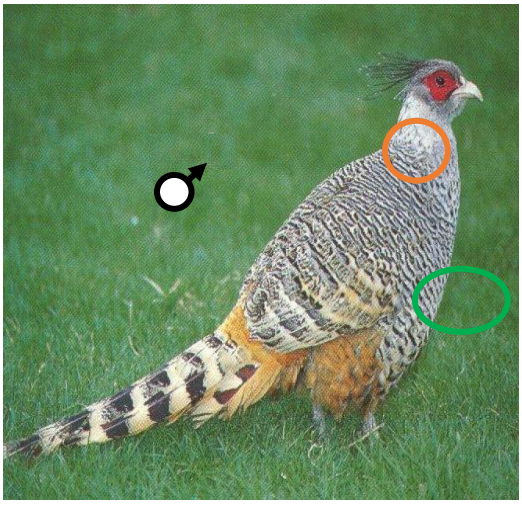
Morphology: The head of both the sexes has a long narrow pitched pointed crest with bare red skin around the eye. The male is buffy white close barred with black above. The neck and sides

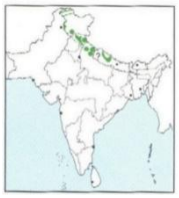
are silvery white merging with a buff barred back. The tail is long, pointed with chestnut and black bars. The middle of the belly is black and sides are rust colored. The female is drabber and smaller, the neck and breast black with pale edges, the general pattern of females being mottled than barred.

Habitat: Found between 1252-3350 meters and shows a marked lining for open grassy, scrubby areas on steep hillsides. A continuous but low level disturbance in the form of grass burning, grazing, lopping and clearing to arrest natural succession may be beneficial to the habitat of this particular pheasant. It has a primarily vegetarian diet.

The species has monogamous mating system and territories, which may be 18-25 hectares in size, are defended through calling. Nesting normally occurs in May and unsuccessful early nesters may re-nest till June, after which most of the area gets affected by monsoon. The normal clutch size is 9-14 eggs, which are laid in a hollow dug out into the ground. The birds may form larger flocks in winter with coveys of adjoining ranges grouping up to form groups of 8-10 or more birds.

Threats: Listed as ‘vulnerable’ by the IUCN/WPA. The main threats to the species arise from low numbers and isolated populations. Since grassy and scrubby habitats in the Western Himalaya are patchy, the Cheer populations are also patchy making them vulnerable to local extinctions.





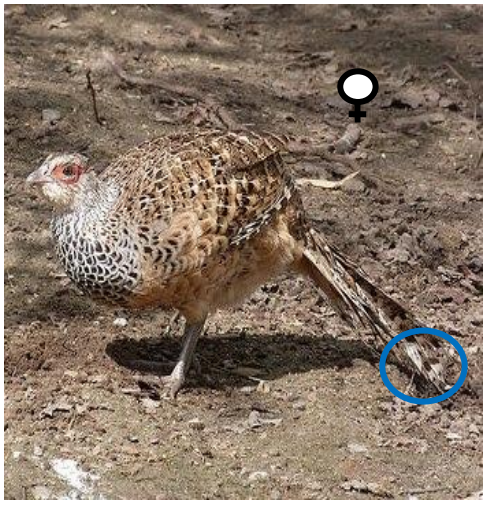
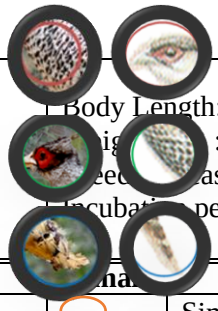


Table 2.2 - Identification features, Distribution, reproduction and lifecycle

2. Cheer Pheasant*Catreus walichii (Hardwicke, 1827) (Longitudinal himalayan pheasant)*
Local name: ***Cheera, Cheras***(Kinnaur district), ***Tana***(Shimla district)



Altitude Range: 1500 – 3050 m
Size: Medium size mountain pheasant
Status: Endemic, uncommon
Countries of occurrence: India, Nepal Afghanistan

♂ ♀
Body Length: 90-118cm 61- 76cm
ig : 1.65-1.83kg
eed ason: April-June
ncuba period: 26 days Clutch size : 9 to 14

Male		**Fema**	
	White above, white and grey below		Similar to male
	Tail-Long		Tail-Long
	Orbital patch-Bright crimson		Similar to male
	A long-tailed Himalayan Pheasant with a long narrow backward, Projecting brown crest on head, Red facial skin, Body in buffy white and pale rusty, closely barred with black		More refescent upperparts, with prominent shaft streaking.
Food		Seeds, grains, roots, shoots, berries, fruits, insects	
Sound		chir-a- pir,chir, chir,chir chirwa, chirwa; high piercing chewewoo whistles interspread with short chut calls	
Nest site		No nest, eggs are laid in undergrowth on rough grounds	
Interesting feature		Prefers legs rather than wings to escape	

1.4.3 HIMALAYAN MONAL (*Lophophorus impejanus*)

Distribution Global: India, Afghanistan, Pakistan, Nepal, Bhutan, China, and Myanmar.

India: All Himalayan States

Population: Relatively common across its range, but population estimates for most sites are not available.

LegalStatus: Full protection,Schedule I of the Wildlife (Protection) Act 1972.

Morphology: The male bird is around 72cm in length the size of a large domestic cock, and displays brilliant plumage – hence called "bird of nine colors". The male has a crest on crest on its rather large head. The feather shafts of the crest are bare and end up like lances. The crest and head are of burnished green color, back of the neck is burnished copper red, which seems golden green under different light conditions. The upper part of the back is bronze green, the lower is silver white, concealed by metallic wings, the under surface is velvety black and the tail is cinnamon. The female is mottled light brown with a short tuft on the head and a white throat. The bare skin around the eye is blue.

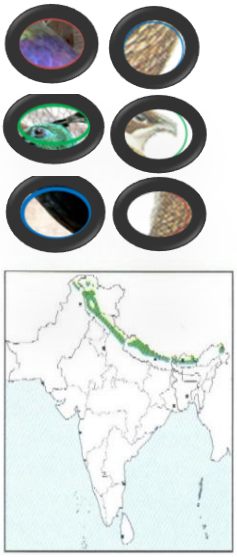
Habitat: Generally found in forested areas between 2,600 and 5,000 meters. The species prefers alpine and sub-alpine areas during summer and descends to lower altitudes during the winters. Hence habitat requirements are wide, ranging from the forested areas of oak, rhododendron, blue pine and fir to alpine grasslands on generally precipitous hillsides. The species is reported to be polygamous. Usually digging for tubers and roots which seem to form their main diet in addition to grass seeds, berries and insects. The main call of the male bird is like a fluty whistle, heard often at dawn during the breeding season. When flushed, the birds take to wing. Eggs are laid on rudimentary nests on ground during May-June, generally under boulders and are 4-6 in number. The incubation period is 28 days.

ConservationStatus: The main threat to the species arises from hunting for local consumption and for feathers, especially during winter as the bird descends to lower altitudes, closer to human habitation. Monitoring of population on a regular basis is essential especially in view of the absence of population estimates currently.

Table 2.3 - Identification features, Distribution, reproduction and life cycle

3. Himalayan Monal *Lophophorus impejanus (Latham, 1790)(Bird of nine colours)*

Local name: ***Bnal***(male), ***Bodh***(female) or***Kardi***(female) – Shimla, ***Dang***(Kinnaur)



Altitude Range: 2400–4500 m Size: Large size mountain pheasant Status: Altitudinal migrant, uncommon Countries of occurrence: *India, Nepal, Bhutan, Bangladesh, Burma, Thailand*	♂ ♀ Body Length: 70-84cm 63.5- 69cm Weight : 1.8-2.38kg Breeding season: April-August Incubation period: 27 days Clutch size : 4 to 6

Male		**Female**	
	Bronze-green back		Dark brown upperparts with streaked appearance
	Below-Jet Black		Brown underparts with variable, broad pale streaking.
	Head-Has a green crest		Mottled light brown and white throat
	Brilliant metallic green and bronze on hindneck and mantle, Iridescent blue and purple and green on wings Uppertail-coverts, cinnamon-brown tail, and velvety-black underparts		White throat, short crest, and bright blue orbit skin.
Food		Grass, shoots, seeds, roots, tubers, mushroom, insects	
Sound		Kur-lieu or kleech-vick	
Nest site		On ground under a steep, rocky & grass covered slopes	
Interesting feature		Mating dance is done by lifting its spread tail, throwing back and forward wings tucked down	

1.4.4 KALIJ (*Lophura leucomelanos*)

Distribution: India, Pakistan, Nepal, Bhutan, Bangladesh, Myanmar and Thailand. Nine sub-species recognized.

India: Five sub species distributed over Jammu & Kashmir, Himachal Pradesh, Uttarakhand, North Bengal, Sikkim, Arunachal Pradesh, Nagaland, Manipur, Assam, Meghalaya, and Tripura.

Population: No estimates are available for any of the sub species, but they are fairly commonly found.

LegalStatus: The species is on Schedule IV of the Wild Life (Protection) Act 1972.

Morphology: The male of white- crested Kalij 65-73 cm long, glossed with dark steel blue, the feathers of the rump edged white. The head is bluish black with a red face and a white crest. Feathers on the breast bone. The upper parts glossy blue black with a purple sheen except the concentric barring on the lower back and rump. The female is smaller reddish brown, with a brown crest. Tail black with the central feathers brown.

Ecology: Affects the dense undergrowth showing preference for moist ravines up to an altitude of 2,600 meters (higher in the western Himalayas). These birds keep in pairs of or small groups and feed in open areas in early mornings. They are shy and scuttle for cover at the slightest hint of alarm. Their diet is a mixture of plant and animal food, especially bamboo seeds, fruits of *Ficus spp*. And white ants. It is adaptable and found in many habitat types. Does not seem to have mating call. Breeds from February to October through mainly April and May.

General: Being usually found close to villages, it is a victim of local hunting. Like all other Kalij species, it seems to be tolerant to habitat changes and is thus, not particularly threatened by habitat loss. However, monitoring of population loss is necessary. Genus *Lophura* has 10 species & are Gallo-pheasants. The gallo-pheasants are tropical to montane pheasants. The sexes are dimorphic. The males typically have extreme purplish to greenish iridescence on upper body, black underparts, varying amount of white on tail, back and head. Erectile red or blue velvety wattles around the eyes of males and similar orbital areas in females. Females are brown to blackish with varying amounts of spotting or barring.

Table 2.4 - Identification features, Distribution, reproduction and lifecycle

4. Kalij (White Crested) *Lophura leucomelana* (Latham, 1790)
Local name :***Kalesa, Kansha or Kolsa*** (Shimla), ***Panti, Kulsus*** (Kinnaur)

Altitude Range: 245–3050 m Size: Tropical mountain pheasant Status: Altitudinal migrant, common Countries of occurrence: *India, Nepal, Afganistan, Nepal*	♂ ♀ Body Length: 63-74cm 50- 60cm Weight : 0.564-1.7kg Breeding season: March to october Incubation period: 25days Clutch size : 8

Male		Female	
	Glossy blue		Brownish in colour
	Rump-Variation		Tail feathers black
	Tail feather-Sickle shaped, Long, down-curved		Each feather tipped to a lighter shade
	Glossy blue-black on upperparts, Variable amounts of white on underparts and rump, Bright red facial skin and wattles, Long, down-curved, laterally compressed tail.		Dull brown to dark red-dish-brown in colour, Grayish-buff fringes giving scaly appearance Brownish tail.
Food		Seeds, shoots, grains, invertebrates	
Sound		Chirrup, koorchi, koorchi, koorchi, or psee, psee, psee	
Nest site		A scrape scantily lined with grass/dried vegetation amongst thick undergrowth	
Interesting feature		Visits water streams in the mornings and evenings, Six subspecies found in India	

Table 3: Conservation status, population and distribution of pheasant species

Taxon	Conservation Status (IUCN, CITES, IWPA)		Population trends (IUCN, 2013.2)	Population size (Birdlife Int.) and Habitat (Kazmierczak, 2000)	Distribution size
Western tragopan *Tragopan melanocephalus* (Gray, 1829)	IUCN	Vulnerable c2a(i)	Decreasing	3300 mature individuals. Thick undergrowth in mixed coniferous forest and alpine shrubbery on steep slopes.	21,600 km^2
	CITES	Appendix I			
	IWPA	Schedule I part-III			
Cheer pheasant *Catreus walichii (Hardwike, 1827)*	IUCN	Vulnerable c2a(i)	Decreasing	200-2700 mature individuals. Steep, rugged, south facing grassy hillsides, also scrub with scattered trees, wooded ravines.	1,49,000 km^2
	CITES	Appendix I			
	IWPA	Schedule I part-III			
Himalayan Monal *Lophophorus impejanus (Latham, 1790)*	IUCN	Least concern	Unknown	Unknown mature individual Occur in open coniferous and mixed forests, rocky slopes, gorges, grassy areas interspread with woods.	9,81,000 km^2
	CITES	Appendix I			
	IWPA	Schedule I part-III			
Kalij (White Crested) *Lophura leucomelanos* (*Latham, 1790*)	IUCN	Least concern	Decreasing	Unknown mature individual Dense evergreen and deciduous forest near stream. Tropical and montane forest habitats.	13,10,000 km^2
	CITES	Not Listed			
	IWPA	Schedule I part-III			

IUCN Red List; CITES- Convention on International trade in Endangered Species of Wild Fauna and Flora; IWPA- Indian Wildlife (Protection) Act, 1972**C.** Population size estimated to number fewer than 250 mature individuals and either. 2A.continuing decline observed projected or interested or interested in number of mature individuals and at least one of the following. (i) No subpopulation estimated to contain more than so mature individuals.

1.5 DNA Barcoding

DNA barcoding is the process of identification of species based on nucleotide diversity of short DNA segments. It is well established in animals with the introduction of cytochrome c oxidase subunit 1 (COI) as a standard barcode (Vijayan and Tsou, 2010). DNA barcoding first came to the attention in 2003 when Paul Hebert's research group at the University of Guelph published a paper titled "Biological identifications through DNA barcodes" (Hebert *et al.*, 2004). It is a relatively new concept that has been developed for providing rapid, accurate and automatable species identification using standardized DNA sequences as tags. It is well established in animals with the introduction of cytochrome *c* oxidase submit 1 (COX I) as a standard code. Therefore, these short nucleotide segments serve as a standard to which DNA barcode of any unidentified or identified specimens can be matched. DNA barcoding thus provides the taxonomist, conservationists and others who need the identification of species, a cost effective and efficient tool to identify unknown or known species with morphological characters not practicable due to extensive damage or delayed expression. Gene sequence and gene alignment data of these four pheasants of Himachal Pradesh would be made publically available after submission of gene sequence and gene alignment data to COBOL. Species taken for present study i) Western Tragopan (*Tragopan melanocephalus*); ii) Himalayan Monal (*Lophophorus impejanus*); iii) Kalij (*Lophura leucomelanos*); and Cheer Pheasant (*Catreus walichi*).

1.5.1The Internal Transcribed Spacer Region

Internal transcribed spacer(ITS) is the non-coding DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding transcribed region in the polycistronic rRNA precursor transcript. There are chiefly two ITS's in eukaryotes; **ITS1** is located between 18S and 5.8S rRNA genes, while **ITS2** is between 5.8S and 28S in animals rRNA genes.

Genes encoding ribosomal RNA and spacers occur in tandem repeats that are thousands of copies long, each separated by regions of non-transcribed DNA termed *intergenic spacer* (IGS) or *non-transcribed spacer* (NTS). Sequence comparison of the ITS region is widely used in DNA barcoding because it a) is easy to amplify even from small quantities of DNA (due to the high copy number of rRNA genes), and b) has a high degree of variation even between closely

related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences. This forms the basis and hence helps in the identification of varied organisms, and can help in understanding evolution, position of the pheasant in the evolutionary tree and help in conservation of specified species, as the closely related species can be interbred, and genetic modifications in a very narrow range can be identified to check for genetic losses causing the loss of species. As a result it is an important tool.

It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within the ITS region. Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of organisms. The nuclear ribosomal large subunit, a popular phylogenetic marker in certain groups, has superior species resolution.

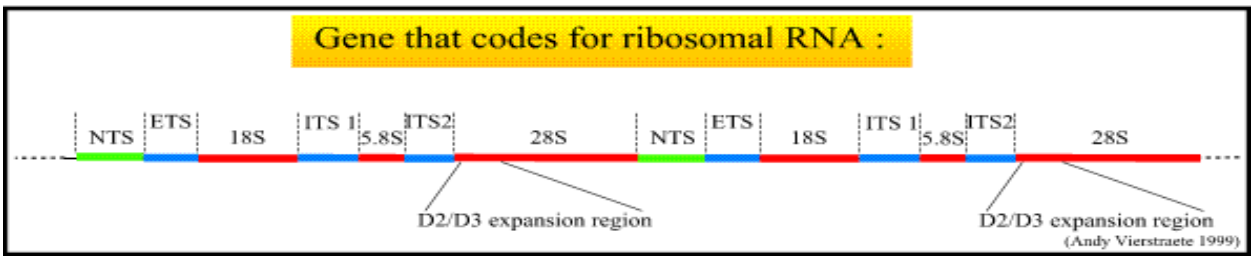


Fig. 3: Internal Transcribed Spacer Region

1.5.2 Cytochrome Oxidase I (COX I) Region

Cytochrome c oxidase subunit I is one of three mitochondrial DNA (mtDNA) encoded subunits (MT-CO1, MT-CO2, MT-CO3) of respiratory complex IV which is the third and final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation.

Cytochrome c oxidaseis a key enzyme in aerobic metabolism. Proton pumping heme-copper oxidases represent the terminal, energy-transfer enzymes of respiratory chains in eukaryotes. The CuB-heme a3 is directly involved in the coupling between dioxygen reduction and proton pumping. The enzyme complex consists of 3-4 subunits (prokaryotes) up to 13

polypeptides (mammals) of which only the catalytic subunit (equivalent to mammalian subunit I (COI)) is found in all heme-copper respiratory oxidases. COX1 has been identified as the gene with less variation among various orders and the one with the least amount of rate heterogeneity across lineages. Such findings support the choice of COX 1 among mt genes as target for developing DNA barcoding approaches in birds.

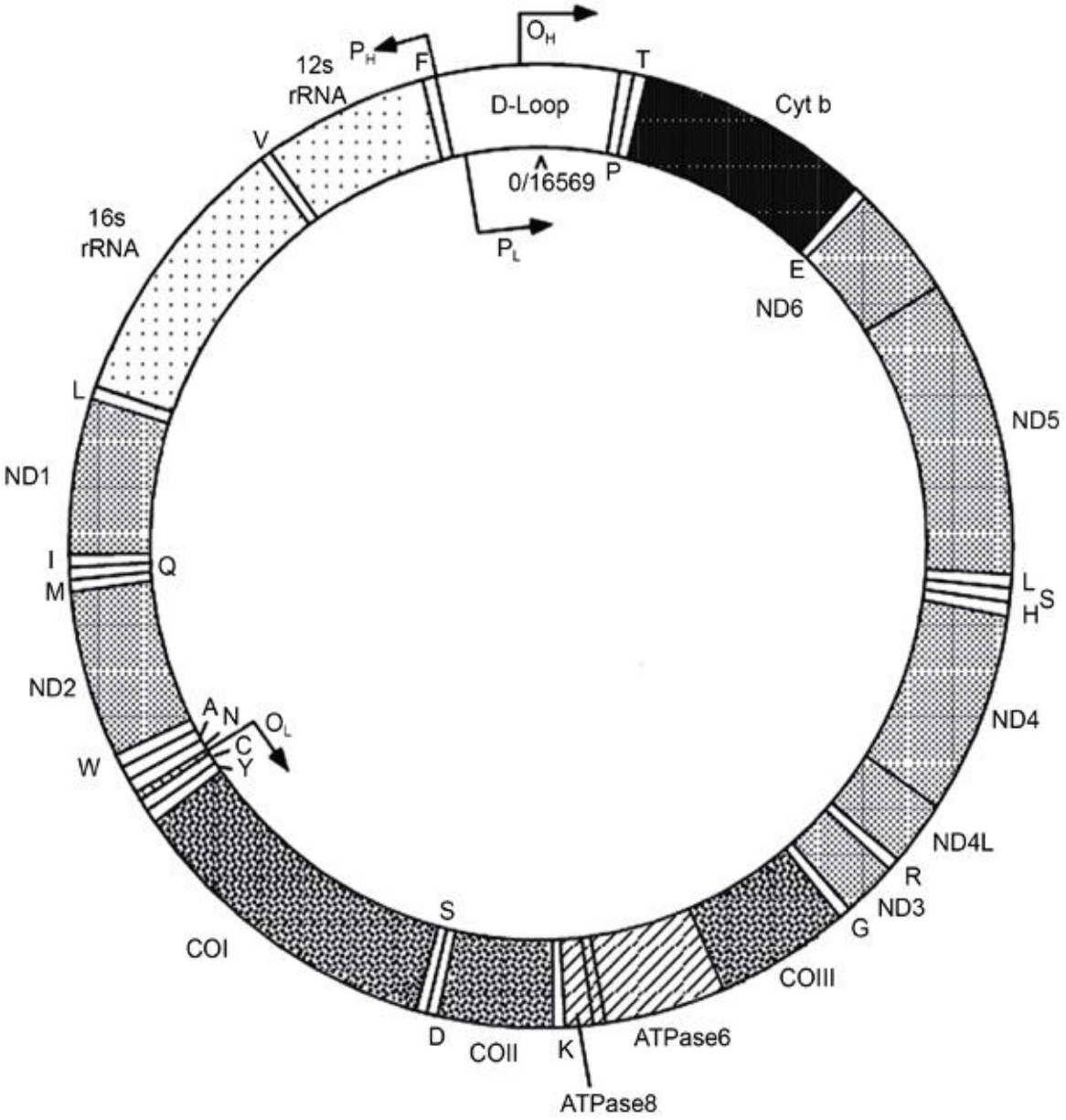


Fig 4: Cytochrome Oxidase I

CHAPTER 2: REVIEW OF LITERATURE

The main aim for carrying out this study was to carry out the barcoding of the target regionstaken the internal transcribed spacer region (ITS) and the cytochrome oxidase I (COX I) regionrespectively. Tissues from where the genomic DNA can be obtained vary. It is the pre-requisite of obtaining significant results for the purpose of amplification using the polymerase chain reaction and making the primer anneal so barcoding can be carried out. Isolation of genomic DNA can be from feathers, blood, fecal matter or any other tissue. The availability of the tissue and the organism determines the protocol followed for the process of obtaining and the yield of the DNA. Mostly noninvasive techniques are taken into account so that harm is not caused to the pheasant.

2.1Collection of blood and tissue samples

In case of blood there are varied methods found for the purpose of blood collection namely:

- Jugular: the right jugular is the most preferred site for blood collection. It is easily visualized by applying a small amount of alcohol to the featherless tract on the right side of the neck. The vein is occluded with a thumb or forefinger at the level of the thoracic inlet prior to venipuncture.
- Basilic: this wing vein is located over the medial surface of the proximal ulna, and was convenient in some species. It was however, much more prone to hematoma requiring application of pressure upon removal of the needle.
- Metatarsal: This vein is located above the tarso-metatarsal joint (hock) on the median side of the leg.

With respect to extraction of blood as an invasive procedure, the method of extraction chosen is metatarsal as on reviewing it is found that the birds are shy organisms and allow the maximum ease of extraction from this region only.

2.2 Extraction of genomic DNA from various tissue extraction protocols and their success rate

With respect to genomic DNA isolation there Skuas have been characterized by means of extraction of DNA by blood and feathers by the Sambrook method. Silica and Guanidine isothiocyanate method of extraction has proved most efficient in DNA extraction from fecal samples by the Milligan method 1998 for the great Indian Bustard *(Otis tard*a).DNA has also been obtained from the regurgitated seeds as seen in pigeons *(Columba livia)* using noninvasive methods of DNA extraction by using the Taberlet (1999) method. Feathers yield DNA which has provided the basis of molecular sexing in Black capped Chickadees *(Poecille atricapilla).* As seen in case of the imperial eagle (*Aquila heliaca*) species identification had successfully been carried out by non- invasive methods by DNA extraction from shed feathers, species identification was using COX I gene amplification and RFLP visualization.

2.3 Extraction protocol for obtaining genomic DNA from egg membranes and shells

Eggs were used for the extraction of maternal DNA from avian eggshells, providing an alternative technique for the extraction of DNA from avian species. It can be contaminated in earlier stages by embryonic DNA but still provides significant amount of DNA as seen in the brown headed cowbird *(Molothrus ater).*The basic Sambrook et al method was identified where 50 mg of the shell and associated membranes was placed into a microfuge tube after being finely crushed. There was 10% SDS, 0.7 ml of STE and 10 µl of10mg/ml proteinase K to the shell sample. After capping and sealing with parafilm the samples were placed on a shaker for 6-12 hours at 55°C. Occasionally the samples were shaken aggressively to suspend settled matter and was placed back on the shaker. Following this 0.7 ml of phenol chloroform was added and the tube was vortexed for 10 seconds following the capping and sealing of the parafilm. Tubes were then centrifuged for 10 minutes after which the top layer was removed and placed into the new 1.5 ml tube and treated with phenol chloroform for a second time, after which the DNA was precipitated. DNA was extracted from post-hatched shells was suspended in 100µl of water, whereas DNA from pre-hatching shells was suspended in 15 µl of water. This was used for basic steps for application in the protocol standardized for extraction of DNA.

2.4 DNA Barcoding and the success rate for Avian species

Conservation genetics focuses on the effects of contemporary genetic structuring on long-term survival of a species. Within this there is a necessity of understanding the basic structural relationships and determining the success rate further. Short DNA sequences from a standardized region of the genome provide a DNA barcode for identifying species. Compiling a public library of DNA barcodes linked to named specimens could provide a new master key for identifying species, which has increased with taxon coverage and with faster, cheaper sequencing. Recent work suggests that sequence diversity in a 648-bp region of the mitochondrial gene, cytochrome c oxidase I (COX I) has served as a DNA barcode for the identification of animal species. This study has tested the effectiveness of a COX I barcode in discriminating bird species, one of the largest and best-studied vertebrate groups. Similarly it was thought that the Internal Transcribed spacer region that is also a conserved gene sequence area could provide valuable insight for the purpose of DNA barcoding. As per the literature available COX I barcodes for 260 species of North American birds and found that distinguishing species was generally straightforward. All species had a different COX I barcode(s), and the differences between closely related species were, on average, 18 times higher than the differences within species. The results identified four probable new species of North American birds, suggesting that a global survey will lead to the recognition of many additional bird species.

Double-crested Cormorant (*Phalacrocorax auritus*) numbers are increasing throughout eastern North America. We compared variation for five portions of mtDNA to determine if genetic differences existed among portions of the breeding range that would need to be considered when formulating management programs. Sequences for four mtDNA regions were identical across sample locations; frequencies of two haplotypes of the mitochondrial Control Region were similar across sampling locations. There is no evidence of restricted gene flow among breeding areas, or between subspecies with different migratory patterns.

2.5 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. Mitochondrial (mt) genes and genomes are among the major sources of data for evolutionary studies in birds. This places mitogenomic studies in birds at the core of intense debates in avian evolutionary biology. Indeed, complete mt genomes are actively been used to unveil the phylogenetic relationships among major orders, whereas single genes (e.g., cytochrome c oxidase I [COX1]) are considered standard for species identification and defining species boundaries (DNA barcoding. In Neoaves the phylogeny by analyzing 80 mt genomes, including 17 new sequences has been reported. Complete mt genomes can solve the evolutionary relationships among major bird groups. Phylogenetic hypotheses is used to estimate the time of origin of major avian orders as a way to test if their diversification took place prior to the

Cretaceous/Tertiary (K/T) boundary. Time trees are used to estimate the rate of evolution of each mt gene. A great variation is observed on the mutation rates among mt genes and within different bird groups.

CHAPTER 3: MATERIALS AND METHODS

A list of the experiments is given for the standardization of the protocol for the purpose of genomic DNA extraction from an egg membrane. As *Gallus gallus* is the closest relative to the pheasants and its eggs are available in relative abundance, it has been taken as the model organism. Varied buffers have been used for the procedure.

3.1 Study design

Around 980 bird species have been recorded in the Himalayan region (Conservation International, 2012), out of which 35 species of birds are endemic to the Himalayan region (BirdLife International, 2014). Conservation status and population trends of each species have been given according to International Union for Conservation of Nature and Natural Resources red data list (2014). Population size and distribution size have been gathered from BirdLife International data zone (2014). Habitat preference of each species have been categorized as given by Kazmierczak (2000), Grimmett *et al.* (2011), Chauhan (2014) and Thakur and Negi (2015).

Bioinformatics tools were used to carry out the phylogenetic characterization of some endemic birds found in Himalayan region. Initially, protein-gene sequences of 18 endemic species were identified using publicly available databases. The sequences were analyzed by using National Centre for Biotechnology's website (http://www.ncbi.nlm.nih.gov), following taxonomy and homologene search tools. Pubmed link was also explored for previously cited literature review, EST (Expressed Sequence Tags database) and Uniprot. Protein sequences of interest were exported in fasta text format, in order to be used later for phylogenetic analysis using Clustal X, tree view and Geneious.

After gathering sufficient information about the sequence, fasta text was extracted from NCBI. These files were used as input for Clustal X, where sequence homology and multiple alignment was performed for protein sequences. Output format options for alignment was chosen both as clustal and Nexus format, slow and accurate pairwise parameters were chosen for alignment and after choosing bootstrap labels from branch to nodes in output format options for trees, a complete alignment was performed. For such analysis multiple alignment is very critical as the bad alignment can give an incorrect phylogenetic tree and the major drawback of using Clustal X is that it does not allow users to edit the sequences manually and in order to edit the

fasta text sequences it becomes very tough and time consuming effort, therefore trial version of geneious was used alongside Clustal software. So, for a good alignment the gap regions which did not align were chopped off before performing phylogenetic analysis. Sequence homology search, multiple sequence alignment, and consensus tree construction was performed using the free trial versions of Geneious Pro 5.1.3 (Biomatters, Ltd.) It is important to note that the tree produced and analysed in this study is a gene tree not species tree. Protein sequences give better information than nucleotide so for present analysis protein sequences were taken and analyzed.

After analyzing the protein sequences, the phylogenetic analysis was done by analysing Cytochrome b gene. This gene has been chosen due to the fact that it constitutes of large amino acids, plays vital role in cell as it is involved in electron transport chain. This region is normally intolerant to mutations so hereditary record is kept safe in this region and for this reason cyt-b gene is widely used in phylogenetic studies to study and resolve divergence at various taxonomic levels.

Evaluation of threats being faced by populations of these endemics shows that habitat degradation, modifications, fragmentation, illegal hunting, etc are the main pressures being faced by these birds in the Himalayan region. According to Conservation International (2012) both legal and illegal logging often occurs on extremely steep slopes, resulting in severe erosion in the Himalayan region.

3.1.1 Biological Materials

Egg samples: Red Junglefowl(*Gallus gallus*)

Blood Samples: Cheer pheasant (*Catreus walichi*) and Red Junglefowl (*Gallus gallus*)

3.1.2 Chemical Materials

(A) EDTA- Ethylene diamine tetra acetic acid 0.5 M

It is a polyamino carboxylic acid which is extensively used in molecular biology experiments and is a chelating agent. It sequesters calcium and magnesium ions, as a result the enzymes do not function properly, as they are required for optimal activity.

Preparation: 186.12 gms dissolved in 800ml of deionized water set at pH 8.0 and 20gms of NaOH was added. Volume was adjusted and it was autoclaved.

(B) Phosphate Buffer Saline

Water based salt solution containing sodium phosphate, sodium chloride, potassium chloride, potassium phosphate. It is isotonic and nontoxic to cells.

Salt	Conc. mM/L	(500 ml)	Conc. gm/L
NaCl	137	4.0	8.0
KCl	2.7	0.1	0.2
Na2HPO4	10	0.72	1.44
KH2PO4	1.8	0.12	0.24

Table 4: Components of Phosphate Buffer Saline

(C) Lysis Buffer

Causes lysis of all the cells so that DNA can come out freely into the supernatant and facilitates ease of extraction. Two different lysis buffers were used. Their composition is given as follows.

Stock	Conc. Required
Sucrose 0.5 M	0.32 mM
MgCL2	10 mM
Tris CL	10 mM
NaCl	100 mM

Table 5: Components of *Lysis Buffer A*

Stock	Conc. Required
NaCl 0.5M	150 mM
Tris HCl 1M	15 mM
EDTA 0.5M	1 mM
SDS 20%	1%

Table 6: Components of *Lysis Buffer B*

(D) Proteinase K

It is a broad spectrum serine protease which is able to digest native keratin and is activated by Calcium. Its site of cleavage is the peptide bond adjacent to the carboxyl group of the aliphatic acid with blocked amino group. The proteins will be adjusted.

10mg/ml

(E) Sodium Dodecyl Sulphate

It is an anionic surfactant used in cleaning and acts as a detergent. It disrupts non-covalent bonds in proteins and denatures those causing molecules to lose their native shape.

20% 20 Gms in 100ml of water.

(F) Phenol

It acts a purifier of nucleic acids from cells and is a non-polar compound. Nucleic acids are highly polar and do not dissolve in presence of phenol. It has a density higher than that of water. Two phases form when phenol is added, the upper aqueous polar phase contains nucleic acids and water. The organic phase contains denatured proteins and cell components.

(G) Isoamyl Alcohol

It prevents the foaming of reagents making it easier to detect the interface between organic and aqueous phases. Alcohol acts as the dehydrating agent as DNA molecules get surrounded by water. It removes the water from the hydration shell.

(H) Chloroform

Chloroform is added along with phenol to ensure a clear separation between organic and aqueous phases.

(I)Absolute alcohol

Ethanol is used to purify and concentrate the DNA and polysaccharides. DNA being polar dissolves in water which is also polar. Adding ethanol disrupts the screen water forms around the DNA.

(J) 70% Ethanol

Causes DNA precipitation and cleaning of the DNA. Facilitates the movement of DNA outside the cell. Hence, always added chilled.

(K) TE Buffer

It is used for storing DNA as it maintains the pH.

3.2 METHODS

3.2.1 Collection of samples- Field work

- 30 samples of blood were obtained from the Manali, Sarahan, Kufri and Chail pheasantries.
- Method of extraction of blood from the pheasants:
- A 27gauge needle was taken and a micro- blood collection tube with anticoagulant was used to preserve the collected sample.
- Potassium EDTA (purple top) is the anticoagulant of choice for hematological analysis.
- Site selection for phlebotomy:
- Basilic: this wing vein is located over the medial surface of the proximal ulna, and was convenient in some species. It was however, much more prone to hematoma requiring application of pressure upon removal of the needle.
- Metatarsal: This vein is located above the tarso-metatarsal joint (hock) on the median side of the leg.
- The birds were prepared for phlebotomy.
- The bird was restrained optimally utilizing a clean towel.
- The collection site is prepared by cleaning with alcohol.
- Jugular vein is occluded with a thumb or forefinger at the level of the thoracic inlet prior to venipuncture.

- The needle may be inserted bevel-up or bevel-down, depending on operator comfort.
- Transfer of the blood from the syringe was done carefully.
- Transfer of the blood to an EDTA coated vial was carried out (Purple top). The tubes were filled a minimum of half-full to avoid significant dilution effects.
- Additional blood may be transferred to a Potassium EDTA microtainer tube for hematological analysis.

3.2.2 Designing of primers for ITS region

- Since there is no availability of sequences of *Tragopan melanocephalus, Catreus walichi, Gallus gallus* was taken as the model organism.
- NCBI Sequences were downloaded of the ITS region of *Gallus gallus.*
- Using primer 3 tool primers were made

3.2.3. Designing of primers for COX I region

- 31 closely related species to pheasants were found based on their genetic make-up of COX I.
- NCBI Sequences were downloaded
- Multiple Sequence alignment using Clustal omega was carried out
- One consensus sequence was generated
- Using Primer 3 tool, the primers were created for the flanking regions of COX 1 gene which on amplification could produce multiple copies and result in amplification of the COX 1 gene in the various bird species.
- This showed a % identity of 96% and coverage of 100%.
- Primers were designing by PRIMER-3 with a product range of 1100 to 1200.
- Primers were selected according to the criteria:
- i. Maximum product length

- ii. Melting temperature (Tm) above 50 degrees Celsius and should be approximately same for the primer pair
- iii. GC content more than 35%
- iv. Also, the primer length was taken into consideration(18 to 30bp)

3.2.4 Protocol standardization for extraction of DNA as a non-invasive method from egg membrane

Varied methods were followed for the process of extraction of genomic DNA from the egg shell as a result of the formation of a non-invasive method. The experiments carried out and the reagents are described. Initially in the first few experiments, the entire egg shell was crushed and ground and the DNA was extracted. In successive attempts of standardization, only the egg membrane was used (*Sambrook et al (1989).*

EXPERIMENT 1

DAY 1:Decalcification

- EDTA washing was carried out for 24 hours by regular change every 2-3 hours.
- PBS washing was carried out for 24 hours by regular change every 2-3 hours.

DAY 2: Lysis

- 500µl of lysis buffer A, 20 µl of 10 mg/ml of proteinase K and 30µl of 20% SDS was added. (2-3hrs at 50°C).
- 500µl of phenol was added and mixed and inverted gently for 10 minutes.
- Centrifugation at 10,000 rpm was carried out for 10 minutes.
- Supernatant was collected, to that 250µl of phenol and 250µl of chloro-isoamyl alcohol was added.
- Tubes were inverted gently for 10 minutes and centrifuged at 10,000 rpm for 10 minutes.
- Again the supernatant was collected in a fresh tube, to that 500 µl of chloroform isoamyl alcohol was added.

- It was inverted gently for 10 minutes and centrifugation was carried out at 10,000 rpm.
- Finally the supernatant was collected and 30µl sodium acetate along with 500µl of absolute alcohol was added.
- DNA was spooled by inverting tubes.
- These were kept at -30°C overnight.

DAY 3: Washing Step

- Tubes were centrifuged at 13,000 rpm for 60 minutes to pellet out DNA.
- The supernatant was discarded and 500µl of 70% alcohol and centrifugation was carried out at 13,000 rpm for 60 minutes, which was repeated.
- The supernatant was discarded and tubes were dried at room temperature till the alcohol smell was completely removed.
- 30µl of TE buffer was added and was incubated in a water bath at 48°C.
- 10ul of Proteinase K was added into a 1.5ml micro centrifuge 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$_o$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. Discarded the flowthrough.
- The above step was repeated using 250ul of buffer BW2 and centrifuged at 10,000rpm for 3minutes. Discarded the flow through and collection tube.

- The spin column was placed in fresh 1.5ml micro centrifuge tube
- Elution was done by adding 100ul of buffer BE directly to the center of the column membrane and incubated for 2minutes at room temperature
- Centrifugation was done at 10,000rpm for 1minute and DNA was eluted

EXPERIMENT 2

DAY 1: Decalcification

- PBS washing was carried out for 5 hours by regular change every 2-3 hours.

DAY 2: Lysis

- 500µl of lysis buffer A, 20 µl of 10 mg/ml of proteinase K and 30µl of 20% SDS was added. (2-3hrs at 50°C).
- 500µl of phenol was added and mixed and inverted gently for 10 minutes.
- Centrifugation at 10,000 rpm was carried out for 10 minutes.
- Supernatant was collected, to that 250µl of phenol and 250µl of chloro-isoamyl alcohol was added.
- Tubes were inverted gently for 10 minutes and centrifuged at 10,000 rpm for 10 minutes.
- Again the supernatant was collected in a fresh tube, to that 500 µl of chloroform isoamyl alcohol was added.
- It was inverted gently for 10 minutes and centrifugation was carried out at 10,000 rpm.
- Finally the supernatant was collected and 30µl sodium acetate along with 500µl of absolute alcohol was added.
- DNA was spooled by inverting tubes.
- These were kept at -30°C overnight.

DAY 3: Washing Step

- Tubes were centrifuged at 13,000 rpm for 60 minutes to pellet out DNA.

- The supernatant was discarded and 500µl of 70% alcohol and centrifugation was carried out at 13,000 rpm for 60 minutes, which was repeated.
- The supernatant was discarded and tubes were dried at room temperature till the alcohol smell was completely removed.
- 30µl of TE buffer was added and was incubated in a water bath at 48°C.

EXPERIMENT 3

DAY 1: Decalcification

- 500 mg of egg shell extract and 2.5% of Sodium oxychloride was kept for 4 hours.
- This was centrifuged at 15,000 rpm for 5 minutes.
- The tissue was then washed with water and 95% ethanol.
- It was suspended in absolute alcohol, centrifuged and dried overnight.
- The dried material is sonicated in 95% ethanol vortexed and allowed to settle.
- It is washed with 2.5% Sodium oxychloride, double distilled water and EDTA pH-8.0.
- Centrifugation at 15000 rpm is carried out for 5 minutes.

DAY 2: Lysis

- 500µl of lysis buffer A, 20 µl of 10 mg/ml of proteinase K and 30µl of 20% SDS was added. (2-3hrs at 50°C).
- 500µl of phenol was added and mixed and inverted gently for 10 minutes.
- Centrifugation at 10,000 rpm was carried out for 10 minutes.
- Supernatant was collected, to that 250µl of phenol and 250µl of chloro-isoamyl alcohol was added.
- Tubes were inverted gently for 10 minutes and centrifuged at 10,000 rpm for 10 minutes.
- Again the supernatant was collected in a fresh tube, to that 500 µl of chloroform isoamyl alcohol was added.
- It was inverted gently for 10 minutes and centrifugation was carried out at 10,000 rpm.

- Finally the supernatant was collected and 30µl sodium acetate along with 500µl of absolute alcohol was added.
- DNA was spooled by inverting tubes.
- These were kept at -30°C overnight.

DAY 3: Washing Step

- Tubes were centrifuged at 13,000 rpm for 60 minutes to pellet out DNA.
- The supernatant was discarded and 500µl of 70% alcohol and centrifugation was carried out at 13,000 rpm for 60 minutes, which was repeated.
- The supernatant was discarded and tubes were dried at room temperature till the alcohol smell was completely removed.
- 30µl of TE buffer was added and was incubated in a water bath at 48°C.

EXPERIMENT 4

DAY 1: Egg membrane separation

- The egg membrane was removed from many shells of *Gallus gallus* and kept for the purpose of genomic DNA extraction.
- Removal was carried out by soaking in formalin for 2 hours.

DAY 2: Lysis

- Only membrane was taken hence no decalcification occurred.
- It was ground in liquid nitrogen.
- Lysis buffer A was added and lysis was carried out at 50°C for 24 hours.
- Centrifugation was carried out at 10,000 rpm for 10 minutes.
- Supernatant was transferred into a separate tube and phenol, chloroform, isoamyl alcohol were added in the ratio of 25:24:1.
- The aqueous phase was taken and to that 200 µl of 70% ethanol was added.
- Finally the supernatant was collected and 30µl sodium acetate along with 500µl of absolute alcohol was added.
- DNA was spooled by inverting tubes.
- These were kept at -30°C overnight.

DAY 3: Washing Step

- Tubes were centrifuged at 13,000 rpm for 60 minutes to pellet out DNA.
- The supernatant was discarded and 500µl of 70% alcohol and centrifugation was carried out at 13,000 rpm for 60 minutes, which was repeated.
- The supernatant was discarded and tubes were dried at room temperature till the alcohol smell was completely removed.
- 30µl of TE buffer was added and was stored at 4°C.

EXPERIMENT 5

DAY 1: Lysis

- Only membrane was taken hence no decalcification occurred.
- It was ground in liquid nitrogen.
- Lysis buffer A was added and lysis was carried out at 50°C for 24 hours.
- Centrifugation was carried out at 10,000 rpm for 10 minutes.
- Supernatant was transferred into a separate tube and phenol, chloroform, isoamyl alcohol were added in the ratio of 25:24:1. The amount added was 250 µl.
- Centrifugation was carried out at 150,000 rpm for 10 minutes.
- The aqueous phase was taken and to it was added chloroform and isoamyl alcohol.
- Finally the supernatant was collected and 30µl sodium acetate along with 500µl of absolute alcohol was added.
- DNA was spooled by inverting tubes.
- These were kept at -30°C overnight.

DAY 2: Washing Step

- Tubes were centrifuged at 13,000 rpm for 60 minutes to pellet out DNA.
- The supernatant was discarded and 500µl of 70% alcohol and centrifugation was carried out at 13,000 rpm for 60 minutes, which was repeated.
- The supernatant was discarded and tube were dried at room temperature till the alcohol smell was completely removed.
- 30µl of TE buffer was added and was stored at 4°C.

EXPERIMENT 6

DAY 1: Egg membrane separation

- The egg membrane was removed from many shells of *Gallus gallus* and kept for the purpose of genomic DNA extraction.
- Removal was carried out by soaking in formalin for 2 hours.
- The sample was given EDTA washing for 5 hours continuously with change at regular intervals.
- Successively there was addition of PBS for 4 hours in which regular washing was given and it was left overnight at 50°C on an incubator shaker.

DAY 2: Lysis

- Only membrane was taken hence no decalcification occurred.
- It was ground in liquid nitrogen.
- Lysis buffer A along with 10µl of 10mg/ml proteinase K was added and lysis was carried out at 50°C for 24 hours.
- Centrifugation was carried out at 10,000 rpm for 10 minutes.
- Supernatant was transferred into a separate tube and phenol, chloroform, isoamyl alcohol were added in the ratio of 25:24:1.
- To this further there was addition of chloroform isoamyl alcohol in the ratio of 24:1 (300 µl).
- Centrifugation was carried out at 10,000 rpm for 10 minutes.
- Without the pellet being disturbed the aqueous phase was taken and to that 200 µl of 70% ethanol was added.
- Finally the supernatant was collected and 30µl sodium acetate along with 500µl of absolute alcohol was added.
- DNA was spooled by inverting tubes.
- These were kept at -30°C overnight.

DAY 3: Washing Step

- Tubes were centrifuged at 13,000 rpm for 60 minutes to pellet out DNA.

- The supernatant was discarded and 500µl of 70% alcohol and centrifugation was carried out at 13,000 rpm for 60 minutes, which was repeated.
- The supernatant was discarded and tube were dried at room temperature till the alcohol smell was completely removed.
- 40µl of TE buffer was added and was stored at 4°C.

EXPERIMENT 7

DAY 1: Lysis

- Only membrane was taken hence no decalcification occurred.
- It was ground in liquid nitrogen.
- The ground tissue was given a PBS wash and was centrifuged at 5,000 rpm twice.
- Lysis buffer was added (Lysis buffer B).
- 20 µl of Proteinase K was added.
- This was kept at incubation overnight at 50°C.

DAY 2: Extraction of DNA

- Equal volume of Phenol, Chloroformand Isoamyl alcohol was added in the ratio of 24:25:1 which was freshly prepared.
- This was centrifuged at 16,000 rpm for 10 minutes.
- The aqueous layer was carefully removed and transferred to a clean labelled tube.
- This step was repeated twice.
- There was addition of equal volumes of Chloroform, Isoamyl alcohol in the ratio of 24:1.
- This was centrifuged at 16,000 rpm for 10 minutes.
- Carefully the aqueous layer was removed to a clean labelled tube.
- DNA was precipitated with ice cold 100% ethanol (which was twice the volume of the aqueous phase) and 3M Sodium acetate($1/10^{th}$) the volume.
- This was incubated overnight at -20°C. To allow precipitation of DNA.

DAY 3: Washing Step

- Centrifuged at 16,000 rpm for 20 minutes and then decant the supernatant.

- Did not disturb the pellet.
- Washed the pellet with 70% ethanol 500µl.
- Centrifuged at maximum speed for 15 minutes.
- Decanted the supernatant without disturbing the pellet.
- Left tube till ethanol evaporated.
- Dissolved in 40 µl of TBE buffer.
- DNA was loaded onto the agarose gel and checked for the result.

EXPERIMENT 8

(QIAGEN Kit method)

- 10ul of Proteinase K was added into a 1.5ml micro centrifuge tube.
- 500mg of tissue sample was added to the tube.
- 2ul of RNase A was from 100mg/ml stock was added and mixed well.
- 150ul of buffer AL was added to the sample and mixed thoroughly by vortexing.
- 220 µl of PBS was added.
- The sample was incubated at 56◦C for 15minutes and mixed well.
- 200ul of ice cold ethanol was added and briefly centrifuged at 10,000 rpm.
- The spin column was placed in collection tube and the supernatant was passed through the spin column.
- Centrifugation was done at 10,000rpm for 1minute. The flow through was discarded.
- Column was washed with 250ul of buffer B1 and centrifuged at 10,000rpm for 1minute. The flow through was discarded.
- The above step was repeated using 250ul of buffer B2 and centrifuged at 10,000rpm for 3minutes. Discarded the flow through and collection tube.
- The spin column was placed in fresh 1.5ml micro centrifuge tube.

- Elution was done by adding 20ul of bufferTBE directly to the center of the column membrane and incubated for 2minutes at room temperature.
- Centrifugation was done at 10,000rpm for 1minute and DNA was eluted.

3.2.5 Genomic DNA isolation from blood of 30 samples of Cheer pheasant and Western Tragopan

- 10ul of Proteinase K was added into a 1.5ml micro centrifuge tube.
- 100µl of tissue sample was added to the tube.
- 2ul of RNase A was from 100mg/ml stock was added and mixed well.
- 150ul of kit lysis buffer was added to the sample and mixed thoroughly by vortexing.
- 220 µl of PBS was added (volume was raised).
- The sample was incubated at 56◦C for 15minutes and mixed well.
- 200ul of ice cold ethanol was added and briefly centrifuged at 10,000 rpm.
- The spin column was placed in collection tube and the supernatant was passed through the spin column.
- Centrifugation was done at 10,000rpm for 1minute. The flow through was discarded.
- Column was washed with 250ul of buffer B1 and centrifuged at 10,000rpm for 1minute. The flow through was discarded.
- The above step was repeated using 250ul of buffer B2 and centrifuged at 10,000rpm for 3minutes. Discarded the flow through and collection tube.
- The spin column was placed in fresh 1.5ml micro centrifuge tube.
- Elution was done by adding 20ul of buffer TBE directly to the center of the column membrane and incubated for 2minutes at room temperature.
- Centrifugation was done at 10,000rpm for 1minute and DNA was eluted.

3.2.6 Checking DNA by means of Gel Electrophoresis

3.2.6 (A) Genomic 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 3ul of Ethidium Bromide 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 Gel Visualizing System.

3.2.6 (B) PCR Product

Preparation of 1.8% agarose gel:

- 1.8 gm. of agarose was weighed and 100ml. of 1 X TAE was added and heated in a microwave oven for about 1-2 min.
- It was cooled and 3ul of Ethidium Bromide 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 Gel Visualizing System.

3.2.7 Standardization of primers of ITS region by PCR 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 10 pM for PCR amplification.
- The designed primers were used for the amplification of genomic DNA based on the sequence obtained of *Gallus gallu*s
- Amplification reactions were performed in volumes of 12.5ul containing the components listed in the table below.

Reaction Mixture for PCR Amplification using ITS primersComponents	**Stock Concentration**	**Used Concentration**	**Amount(in µl)**
DNA template	-	-	1.5
Autoclaved Water	-	-	7.75
Primer (f)		10pM	1
Primer (r)		10pM	1
dNTPs	2mM	.2mM	0.625
PCR Buffer	10X	1X	1.25
Taq Polymerase	5U	.5U	0.125
Total	-	-	13.25µl

Table 7: PCR components used in standardization

- DNA amplification was performed in a thermal cycler. Conditions for the amplification:
- The amplification products were resolved in 1.8% agarose gel (1X TAE Buffer) followed by ethidium bromide staining and visualization in Gel Visualizing System.
- A gradient PCR was set up to analyze the annealing temperatures of primers for ITS primers 1,2,3,4,5,6 and 7 at temperatures 53°C, 54°C, 55°C, 56°C, 57°C and 58°C.

3.2.8 Amplification of DNA sample of Cheer pheasant using PCR with the ITS primers

- 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 10 pM for PCR amplification.
- The designed primers were used for the amplification of genomic DNA of sample number 032.
- Amplification reactions were performed in volumes of 13.25ul approximately 13µl containing the components listed in the table below.
- DNA amplification was performed in a thermal cycler. Conditions for the amplification:
- The amplification products were resolved in 1.8% agarose gel (1X TAE Buffer) followed by ethidium bromide staining and visualization in Gel Visualizing System.
- A PCR was set up as per the analyzed annealing temperatures of individual ITS primers 1, 2, 3, 4, 5, 6 and 7 at specific temperatures observed as per standardization.

Reaction Mixture for PCR Amplification using ITS primers Components	**Stock Concentration**	**Used Concentration**	**Amount(in µl)**
DNA template	-	-	1.5
Autoclaved Water	-	-	7.75
Primer (f)		10pM	1
Primer (r)		10pM	1
dNTPs	2mM	.2mM	0.625
PCR Buffer	10X	1X	1.25
Taq Polymerase	5U	.5U	0.125
Total	-	-	13.25µl

Table 8: PCR Components used in ITS primer amplification

3.2.9 Determining evolutionary relationships of Cheer pheasant, Monal, Western Tragopan and Kalij

- Sequence identity and homology was determined using tools available at the National Center for Biotechnology's website, namely Nucleotide Basic Local Alignment Sequence Tool (BlastN), Protein BLAST (BlastP), Taxonomy, and Homologene.
- The BlastN search for the given unknown nucleotide sequence was restricted to Nucleotide collection database (nr/nt). The search was made more stringent by increasing the Expect value (E-value) to 1000 and increasing the word size to 11.
- The rest of the parameters were accepted as default values.
- The nucleotide sequence was then translated into amino acid sequence by using the tool ExPasy, which is another bioinformatics website.
- Expasy Translate tool was used to get the protein sequence from previously obtained nucleotide sequence.
- The best frame (5'-3' Frame 2) was selected from the 6 resulting reading frames. This sequence was then used for the BlastP search.
- The parameters were the same as BlastN.
- For each of the resulting homologous sequence from BlastP and BlastN, genomic information and expression profiles were explored whenever available.
- This information were gathered using the corresponding links for UniGene, EST and EntrezGene. A Homologene search was also performed for the conserved domain resulting from BlastP.
- Finally the phylogenetic tree was constructed using Clustal X for alignment and then the alignment results using Clustal X were used for gene tree construction using Fig Tree and Tree View.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Primers for ITS region

Designed from *Gallus gallus*	Similarity Index	bp	Primer (5'-3')
ITS 1F	59.45	615	GTCGAGAAGACGGTCGAACT
ITS 1R	62.85		CTTCCCTCCGAGCGGACT
ITS 2F	62.85	664	AGTCCGCTCGGAGGGAAG
ITS 2R	61.86		CGCCCCGAGTCTTTAAACC
ITS 3F	62.89	441	GAGGAACGCGAGGTGGTG
ITS 3R	59.57		GGGGACGAAGGAGAGGAC
ITS 4F	62.11	560	CGCGTCCTCTCCTTCGTC
ITS 4R	60.40		AACGACCCTGTCTCGCTTC
ITS 5F	59.56	594	GGGGGAGGAAGGTGAGAG
ITS 5R	61.34		CGAGTGATCCACCGCTAAGA
ITS 6F	60.81	591	CAAGGCGAGAGAGAACGAGA
ITS 6R	60.41		GCTCACGGGTCTCTGGTAGA
ITS 7F	58.62	601	TGAATTGCAGGACACATTGA
ITS 7R	60.54		GGAATCCTGGTTAGTTTCTTTTCC

Table 9: Primer sequences for ITS Region

4.2 Primers for COX I region

GCTTTAATTAAACTAAGGCCTTCACCTAGGCAGATGGGCCTCGATCCCATACAATTCTAG
TTAACAGCTAGATGCCGTAACCCATTGGCTTCTGCCTACAAGACCCCGGCATACTTTAAT
ATGCATCAATGAGCTTGCAACTCACCATGAACTTCACTACAGGGTCGATAAGAAGAGGAA
TTGAACCTCTATAAAAAGGACTACAGCCTA ACGCTTTAACATTCAGCCATCTTACCT
GTGACCTTCATCAACCGATGATTATTCTCAACCAACCACAAAGACATTGGCACTCTTTAC
CTAATTTTCGGCACATGGGCGGGCATAGCCGGCACAGCACTTAGCCTTCTAATTCGCGCA
GAACTAGGACAGCCCGGAACTCTCTTAGGAGACGATCAAATTTACAATGTAATCGTCACA
GCCCATGCTTTCGTCATAATCTTCTTTATAGTTATACCCATCATGATCGGTGGCTTCGGA
AACTGACTAGTCCCACTTATAATCGGTGCCCCAGACATAGCATTCCCCCGCATAAATAAC
ATAAGCTTCTGACTCCTCCCTCCCTCCTTCCTTCTCCTACTAGCCTCATCTACCGTAGAA
GCTGGGGCCGGCACAGGATGGACAGTTTACCCCCCTTTAGCCGGCAACCTAGCCCACGCT
GGCGCATCAGTAGACCTAGCCATCTTTCATTACTTAGCAGGTGTTTCCTCCATTCTAGGA
GCCATCAACTTTATCACTACCATCATCAACATAAAACCCCCCGCACTGTCACAATACCAA
ACACCCCTATTCGTATGATCCGTCCTCATTACTGCCATCCTACTACTCCTCTCCTTACCC
GTCCTAGCAGCTGGGATTACCATACTACTTACCGACCGCAACCTTAACACCACATTCTTC
GACCCAGCTGGAGGAGGAGACCCAATCCTATACCAACACCTATTCTGATTCTTCGGTCAC
CCCGAAGTTTACATCCTCATCCTCCCAGGTTTCGGAATAATTTCCCACGTAGTAGCATAC
TATGCAGGAAAAAAAGAACCATTCGGATACATAGGAATAGTCTGAGCCATACTGTCAATC
GGATTCCTTGGCTTCATTGTATGAGCCCACCATATATTCACAGTCCGAATGGACGTAGAC
ACCCGAGCCTACTTTACATCAGCCACAATAATCATCGCCATCCCAACTGGTATTAAAGTC
TTCAGCTGACTAGCAACCCTGCACGGAGGAACAATTAAATGAGACCCCCCTATGCTATGA
GCCCTAGGATTCATCTTCCTCTTCACTATCGGAGGCCTAACGGGAATCGTCCTTGCTAAC
TCATCACTAGATATTGCCCTTCATGACACCTACTATGTAGTCGCCCACTTCCACTATGTC
CTCTCAATGGGGGCAGTTTTTGCCATTCTAGCAGGATTTACCCACTGATTTCCCCTCTTC
ACAGGCTTTACCCTACACCCATCATGAACCAAGGCACATTTCGGAGTAATATTTACCGGA
GTTAACCTAACCTTTTTCCCCCAACATTTCCTGGGCCTAGCTGGAATACCCCGACGATAC
TCAGATTACCCAGACGCCTACACACTATGAAACACACTATCCTCAATCGGCTCCTTAATT
TCAATAACAGCCGTAATCATACTCATATTCATCGTCTGAGAAGCCTTCTCAGCAAAACGA
AAAGTACTCCAACCCGAATTAACTGCCACTAATATCGAATGAATTCATGGCTGCCCACCC
CCATACCACACCTTCGAAGAACCAGCCTTTGTACAAGTGCAAGAAAGG
AAGGAATCGAACCCTCACATGCTGGTTTCAAGCCAACCGCATCAAACCATTTAATGCTTCTT
TCTTATGAGATGTTAGTAAACAAATTACATAGACTTGTCAAGACTAAATCACAGGTGCAAAC
CCTGTACACCTCACATGGCAAACCACTCCCAACTAGGATTTCAAGATGCCTCATCCCCAATC
ATAGAA

Fig 5: Consensus Sequence for 31 species COX I region

Fig. 6: Multiple sequence alignment for the 31 related species

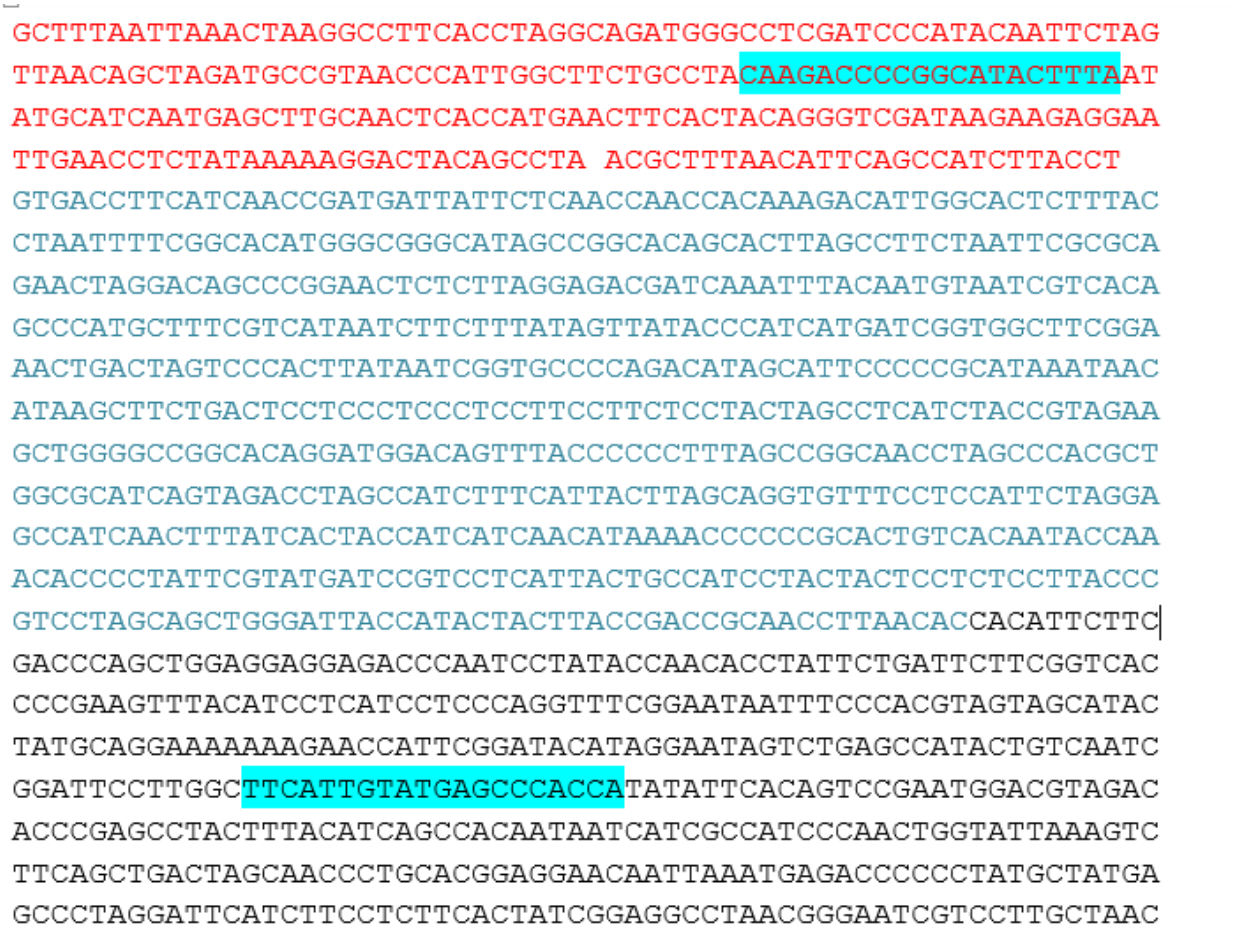


Fig 7: The Primer of the specified region.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER         99   20   59.95    50.00  4.00  2.00 CAAGACCCCGGCATACTTTA
RIGHT PRIMER      1109   20   59.92    45.00  5.00  3.00 TGGTGGGCTCATACAATGAA
SEQUENCE SIZE: 1197
INCLUDED REGION SIZE: 1197

PRODUCT SIZE: 1011, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00
```

Fig. 8: Primer output as given by primer 3, with product size 1011, included size 1197.

After obtaining the consensus sequence and the multiple sequence alignment of all the 31 sequences, whose COX I region was available, Primer 3 provided the following output. As a result of this, based on a 45% GC content, the primer was sent to be made. This was obtained to achieve desired PCR results, which would lead in a significant amount of DNA, which would further lead to amplification and thus result in the sequencing of the organism. From that we

would be able to identify the unique barcode of the organism based on the COX I gene. The result was obtained as follows.

4.3 Genomic DNA isolation from egg membrane

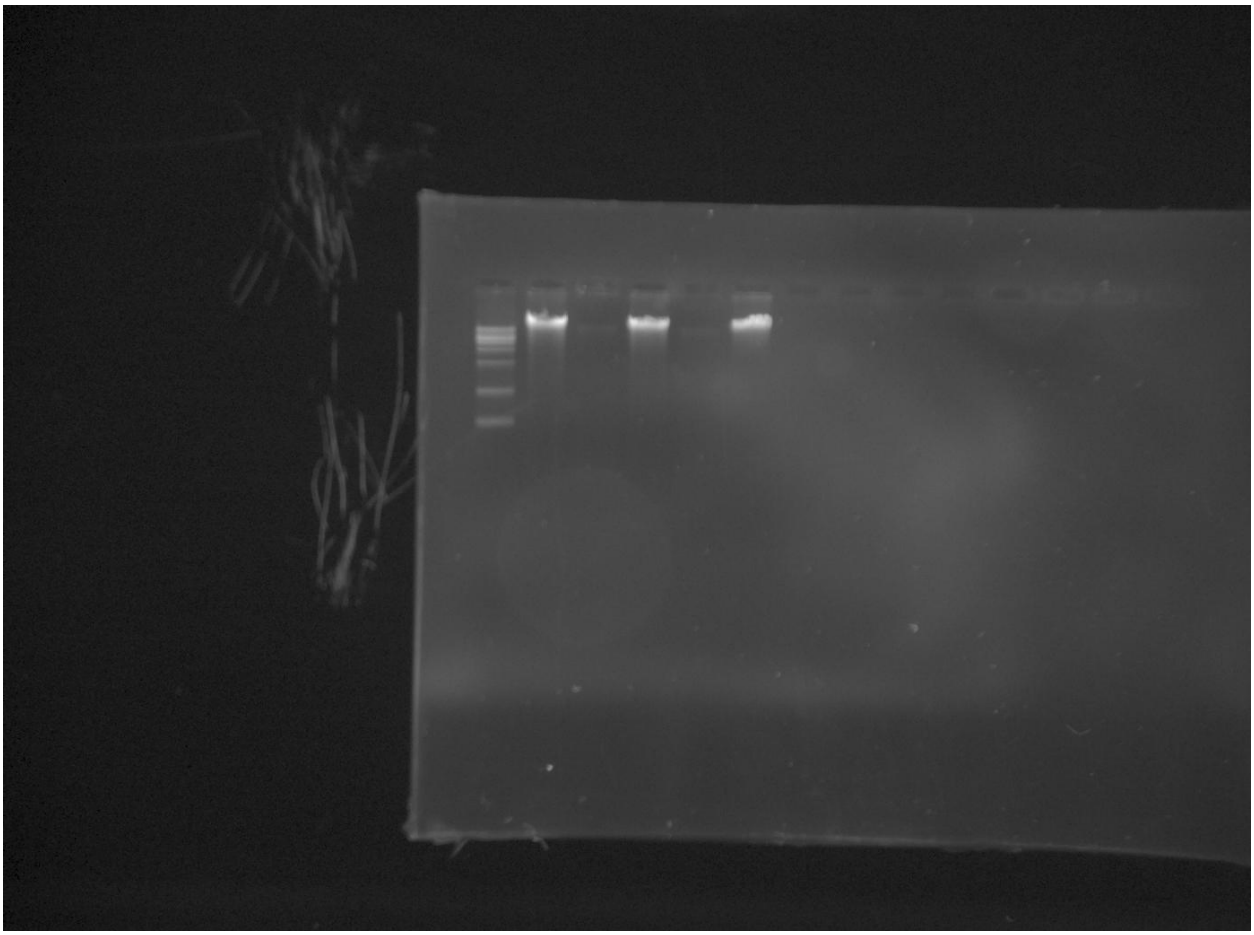


Fig. 9: Picture indicating the DNA bands as a result of the protocol standardization (experiments 6 and 7) in wells 1, 3 and 5.

The above picture indicates the various wells which have the presence of DNA as indicated by the bands seen on the gel. As per the experiments indicated above, experiments 6 and 7 were successful in achieving the result. The lysis buffer and the protocol yielded result leading to a defined protocol for the extraction of DNA by the egg membrane.

4.4 Genomic DNA isolation from 30 blood samples

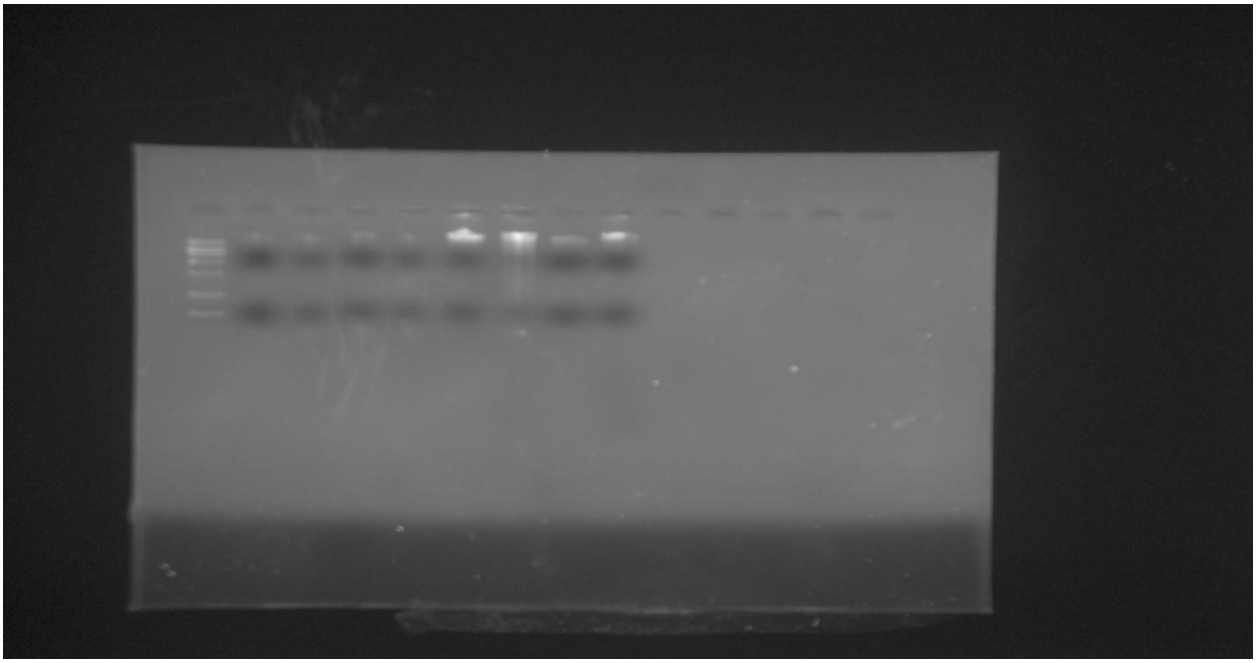


Fig. 10: Gel picture of samples 001, 011, 5206, 038, 2295.

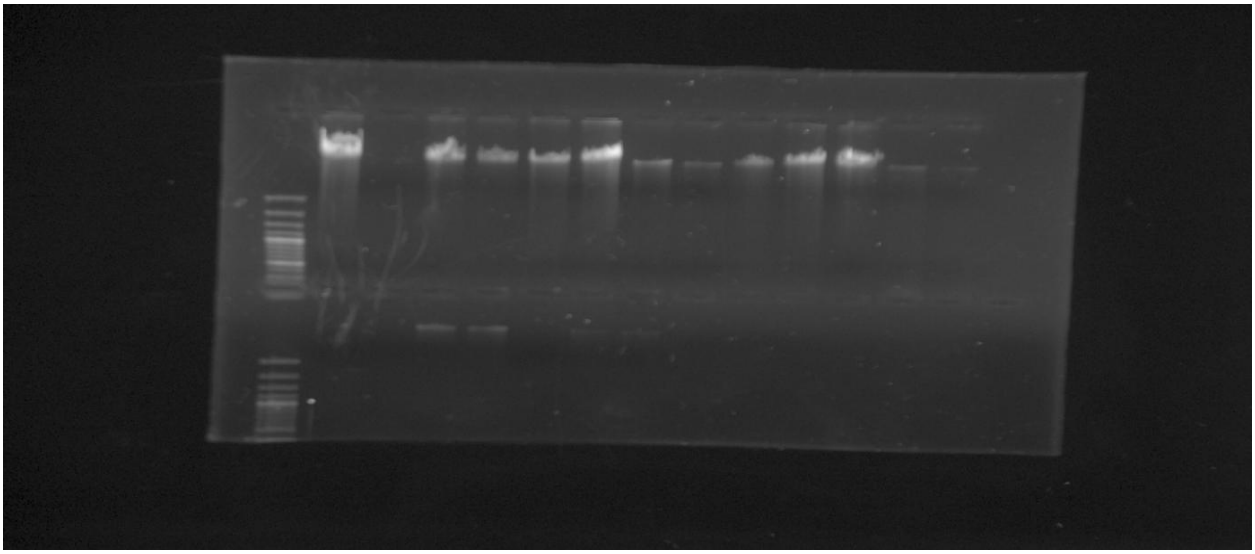


Fig. 11: Gel picture of samples 008, 034, 032, 2296, 003, 014, 2293.

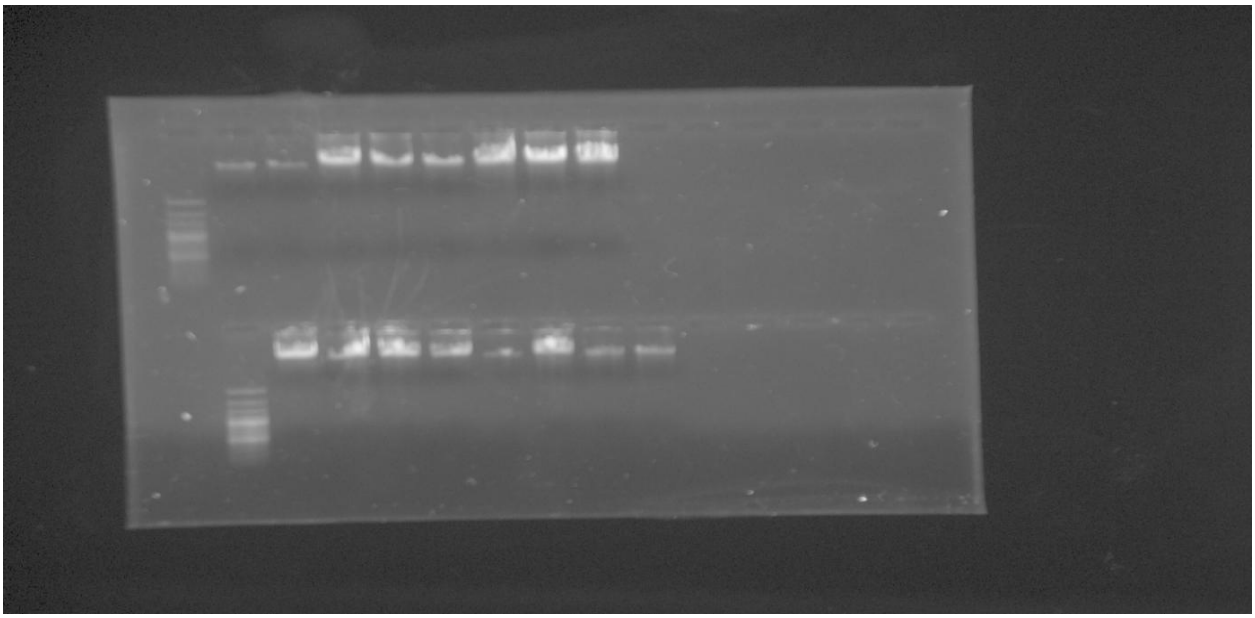


Fig. 12: Gel Picture of 806, 036, 5209, 5205, 033, 5201, 010.

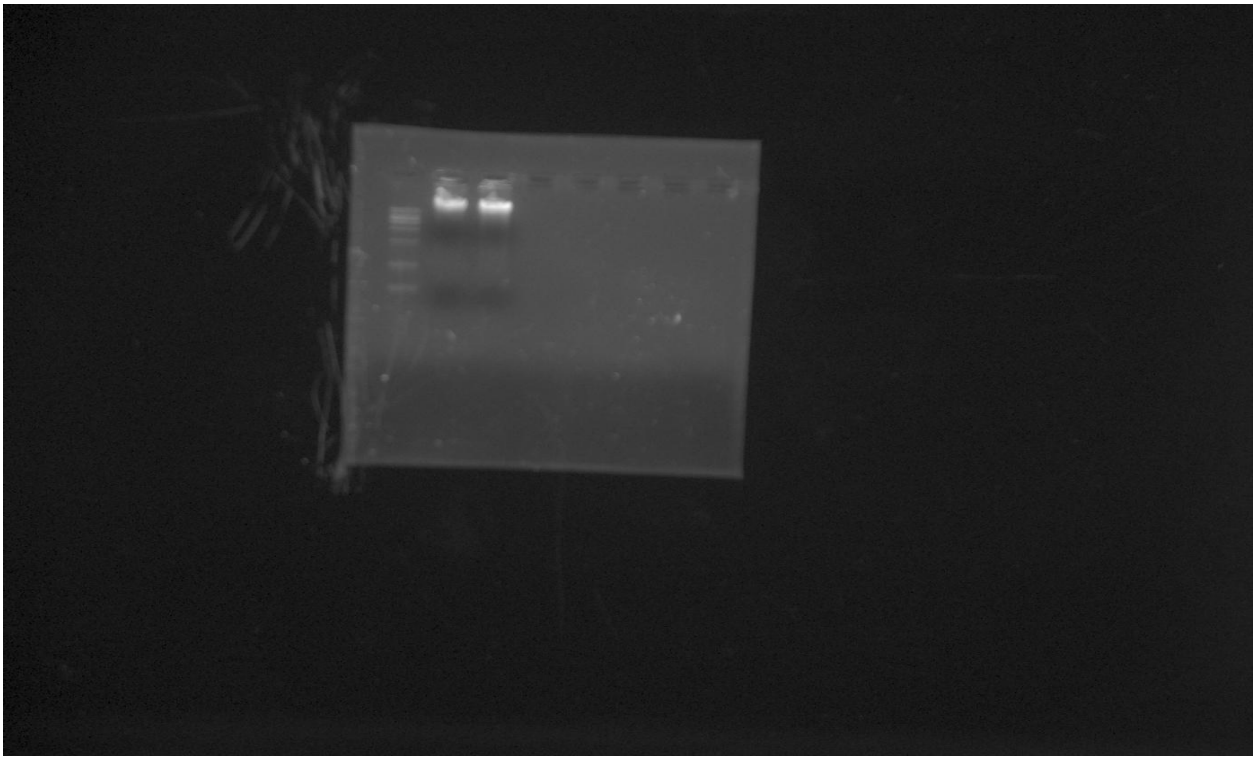


Fig 13: Gel picture of sample 2295.

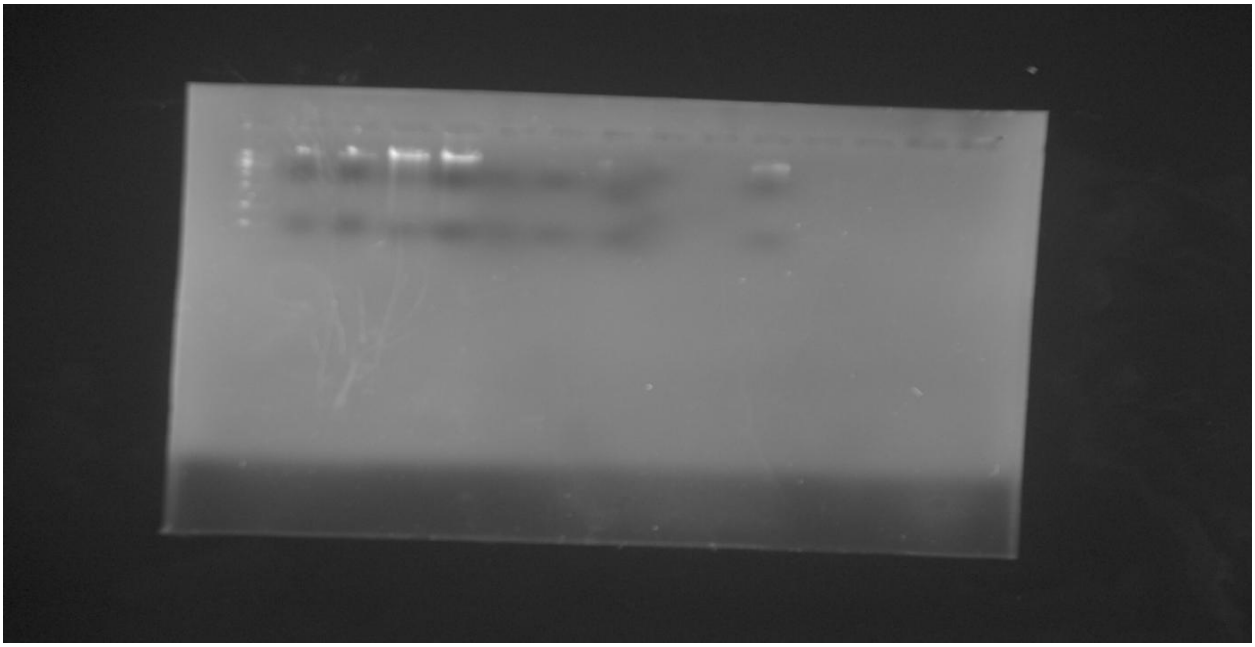


Fig. 14: Gel picture of sample hybrid, Kalij, Monal.

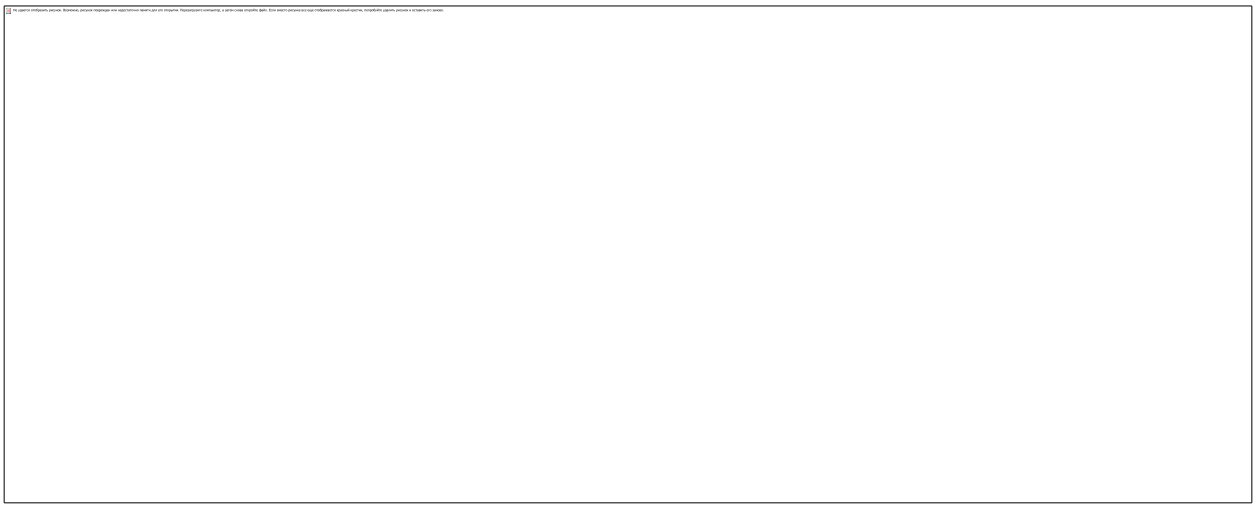


Fig. 15: Gel Picture of sample Tannu, 049, 2299, 839P.

In all the given pictures above it is observed that there was a small quantity of DNA as we see faint bands in samples 001, 011, 0839P. There was a significant amount of DNA as sharp bands were obtained in the case of sample 5206, 038, 008, 034, 032, 2296, 003, 014, 2293, 806, 036, 5209, 5205, 033, 5201, 010, 2295. Kalij, Hybrid and 004. These were used to further set up

Polymerase Chain Reactions to carry out the process of DNA sequencing of the ITS and COX regions. As a result to generate a barcode for the species.

4.5 Standardization of ITS Primers

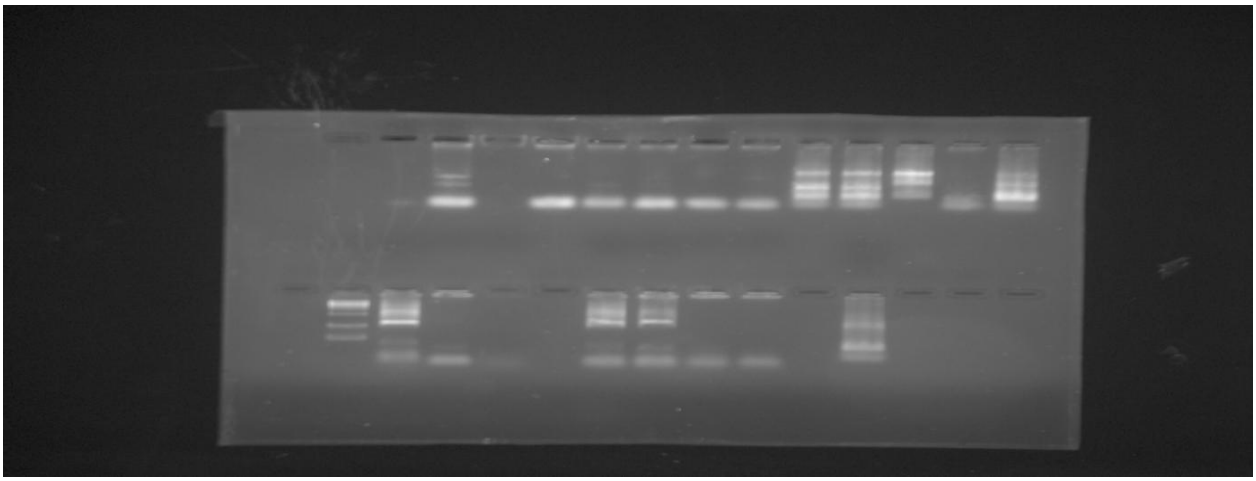


Fig 16: Gel picture indicating the standardization for identifying the annealing temperature as observed by the gradient PCR reaction for ITS primers 1 and 2.

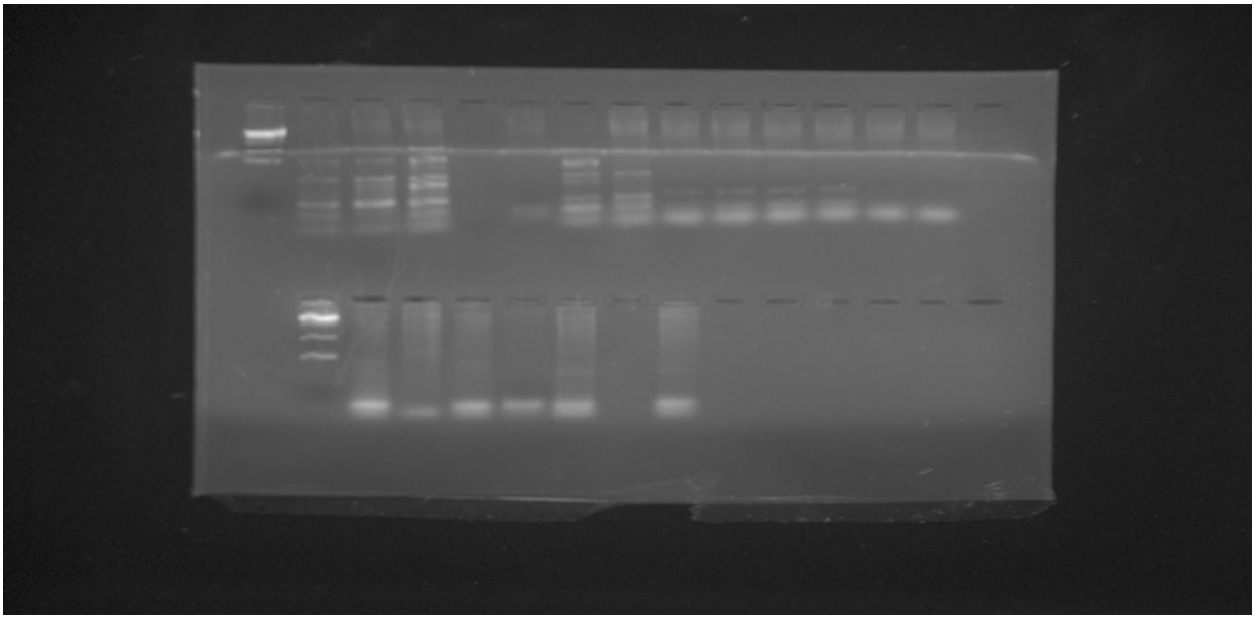


Fig 17: Gel picture indicating the standardization for identifying the annealing temperature as observed by the gradient PCR reaction for ITS primers 3 and 4.

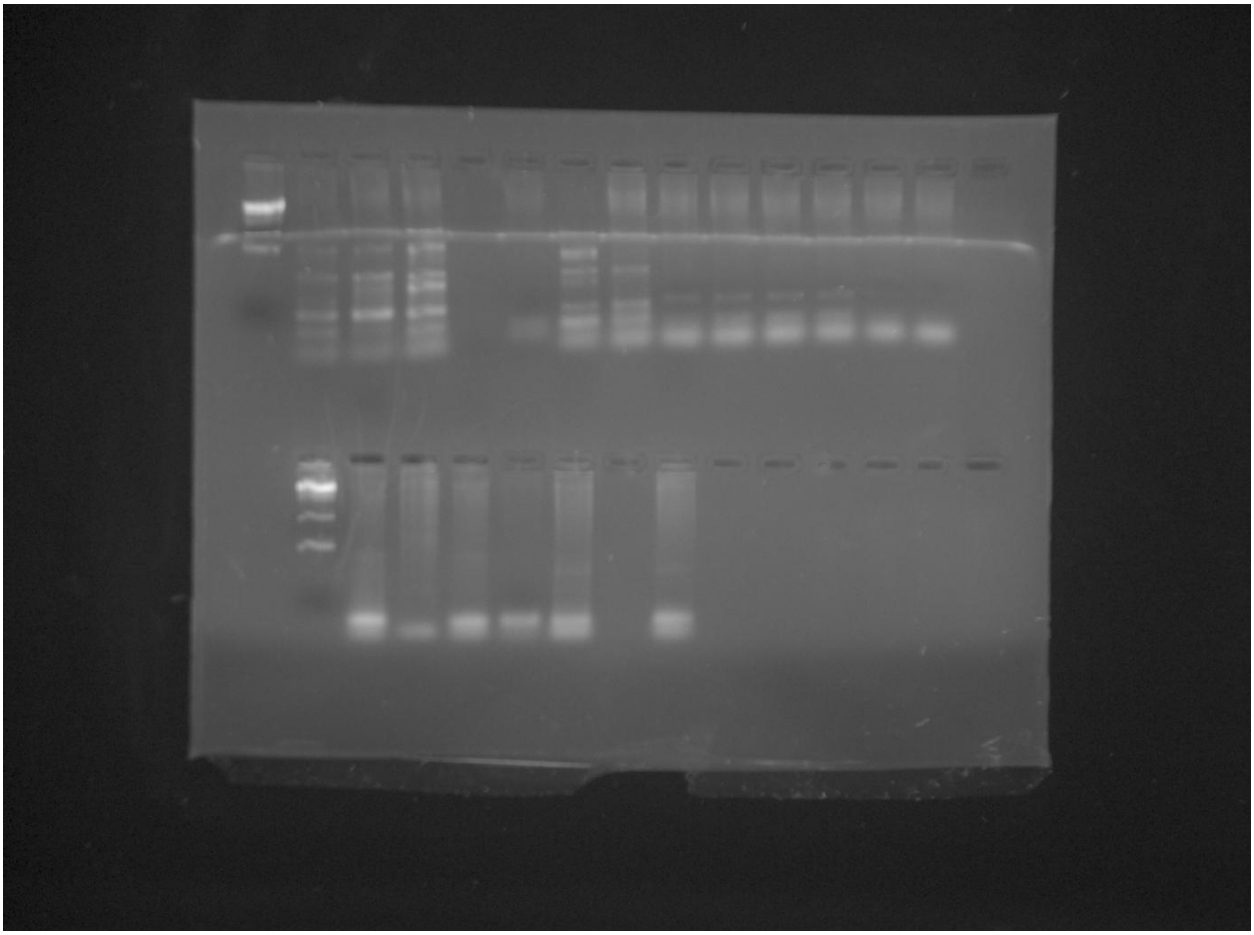


Fig 18: Gel picture indicating the standardization for identifying the annealing temperature as observed by the gradient PCR reaction for ITS primers 5, 6 and 7.

As per the bands observed of the PCR product on a 1.8% gel, it has been observed that there is formation of a large number of primer-dimers. Based on the sharpest band observed, or in case of primer 4 the only band observed, the final PCR reaction was carried out. Gradient PCR reactions were set up in which the temperature was set for the temperatures 53°C, 54°C, 55°C, 56°C, 57°C and 58°C. As per the citations viewed it was understood that this range of temperature is most suited for the annealing of primers. The specific temperature was observed for every single primer that indicates the annealing temperature and is indicated by a sharp band of DNA observed behind the faint band of primer dimers visible. The specific temperatures which were set are indicated as follows:

Primer	Annealing Temperature
ITS Primer 1	53°C
ITS Primer 2	54°C
ITS Primer 3	57°C
ITS Primer 4	57°C
ITS Primer 5	57°C
ITS Primer 6	55°C
ITS Primer 7	54°C

Table 10: ITS Primers and their annealing temperature

4.6 Amplification of Cheer pheasant DNA by PCR using 7 ITS region primers

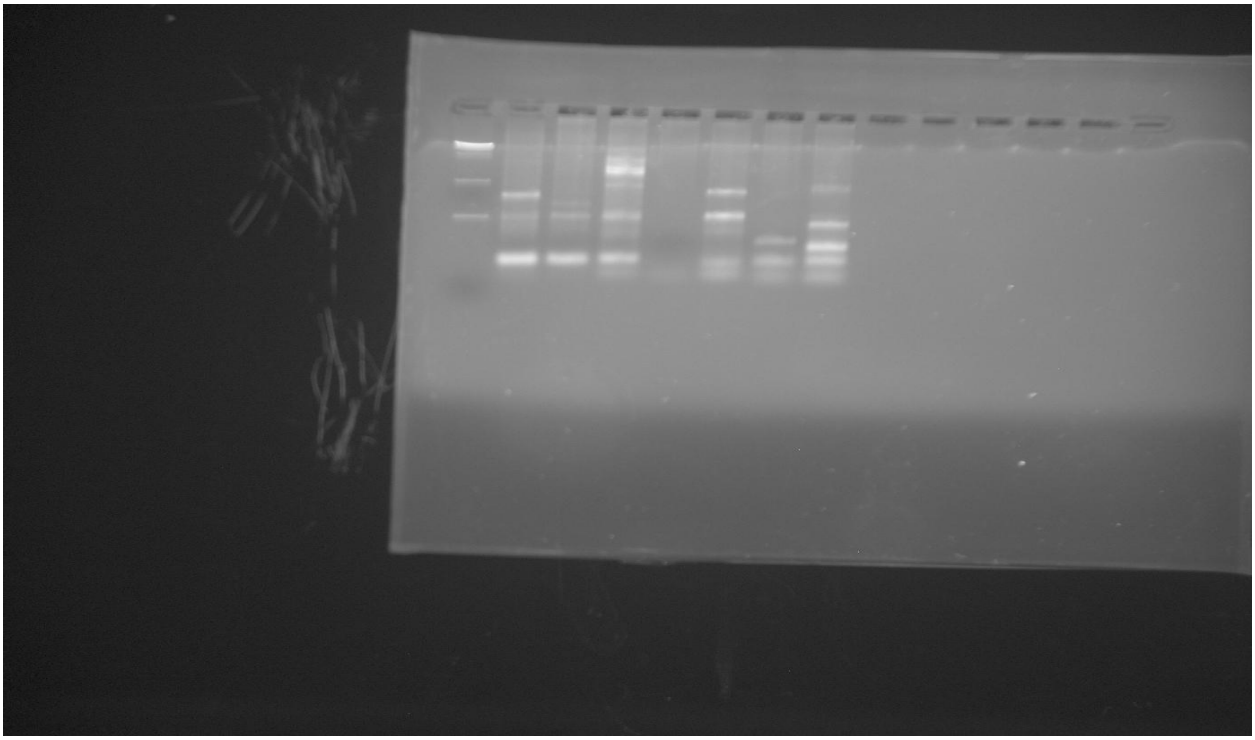


Fig. 19: Gel picture indicating the 7 primers at which the amplification is carried out. Well 1-ladder, well 2- primer 1, well 3-primer 2, well 4- primer 3, well 5- primer 4, well 6- primer 5, well 7-primer 6, well 8- primer 7.

As per the observations made we see that primers 1, 2, 3, 5, 6and 7 contain DNA bands indicating a significant level of DNA, that is the amplified product. These can be used for the purpose of sequencing which would lead to a significant result and can be sent for sequencing.

4.7 Determining evolutionary relationships

Primarily bioinformatic tools were used for phylogenetic analysis and for gene tree construction. Sequence identity and homology were determined using tools available at the National Center for Biotechnology's website, namely Nucleotide Basic Local Alignment Sequence Tool (BlastN), Protein BLAST (BlastP), Taxonomy, and Homologene. Wet lab procedures were followed for gene isolation followed by PCR amplification of target regions. Evolutionary relationships were determined among these four species.

Sequences were first analysed by using National Center for Biotechnology's website (http://www.ncbi.nlm.nih.gov) following bioinformatic tools like:Basic Local Alignment Sequence Tool (BLAST). Various other tools that were used for the analysis of this sequence were Basic Local Alignment Sequence Tool (BlastN), Protein BLAST (BlastP), ExPASY including Expasy Translate Tool and ExPASY BLAST, Taxonomy, and Homologene, Pubmed for previously cited literature review, EST (Expressed Sequence Tags database) Uniprot, Geneontology and Conserved Domains Search to know about the functionality and conserved domains of proteins associated with this gene. For structural analysis another database known as Predict Protein (http://www.predictprotein.org/) was also used.

Sequence Homology search, Genomic profile and Phylogeny

Sequence identity and homology was determined using tools available at the National Center for Biotechnology's website, namely Nucleotide Basic Local Alignment Sequence Tool (BlastN), Protein BLAST (BlastP), Taxonomy, and Homologene. The BlastN search for the given unknown nucleotide sequence was restricted to Nucleotide collection database (nr/nt). The search was made more stringent by increasing the Expect value (E-value) to 1000 and increasing the word size to 11. The rest of the parameters were accepted as default values. The nucleotide sequence was then translated into amino acid sequence by using the tool ExPasy, which is another bioinformatics website. Expasy Translate tool was used to get the protein sequence from previously obtained nucleotide sequence. The best frame (5'-3' Frame 2) was selected from the 6 resulting reading frames. This sequence was then used for the BlastP search. The parameters were the same as BlastN. For each of the resulting homologous sequence from BlastP and BlastN, genomic information and expression profiles were explored whenever available.

These informations were gathered using the corresponding links for UniGene, EST and EntrezGene. A HomoloGene search was also performed for the conserved domain resulting from BlastP.

Finally the phylogenetic tree was constructed using Clustal X for alignment and then the alignment results using Clustal X were used for gene tree construction using Fig Tree and Tree View.

Western Tragopan (*Tragopan melanocephalus*)

Tragopan melanocephalus haplotype mitochondrial control region:

>gi|12642412|gb|AF230311.1| Tragopan melanocephalus haplotype TME1 mitochondrial control region, partial sequence
AATAATTTTTTTTAACCTAACCCCCCTATTGAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTAT
GTATAATCGTGCATACATTTATATACCACATACATTATGGTACCAATACTATATATTATATACGTACTAA
ACCCATTATATGTAGACGGACATAACACCTTGACCCCCTTCTACCAATGTACATATCATGCAACGTCCCA
GACTAATCCTGCTCAACTACCCCTAAGCTGCCAATACAACATCCAAGGCACCATAACTATGAATGGTTAC
AGGACATACTTCTAAATCCTTATGTTCTAACCCCATTTGGTTATGCTCGTCGTATCAGATGGATTTATTG
ATCGTACACCTCACGAGA

FNLTPLLSVPPLSPPGGVYYYV-SCIHLYTTYIMVPILYIIYVLNPLYVDGHNTLTPFYQCTYHATSQTNPAQLPLSCQYNIQGTITMNGYRTYF-ILMF-
PHLVMLVVSDGFIDRTPHE

Himalayan Monal (*Lophophorus impejanus*)

Lophophorus impejanus ovomucoid-G gene:

>gi|112491877|gb|DQ832075.1| Lophophorus impejanus ovomucoid-G gene, intron G
GTACGTACAGCCTTGGTGCATTCACGTTGTCGGCTGCCTCCTACAGGCACCAACTTGCACAGTTCCTGCT
TTCGTTGCTGATTGCTGGCCAGGATTTGGGGGCAGAAAAGAACACCAGGCATCGCGCCAGCCATTCATTT
TATTTTTCACCAGAGCATGTCTGGTTTGTTAGGATGGATGTTTTGAACATCATTAACCTTAAGGGAAGTT
TTCCTTGCTTGCTGCAAAGAAAATCAAATTTGGTGTTTCATTATAGTTTTCGAAGGGGTTAAACGATTTC
ACTCATCTCTTAATAATCAGGTAAATCAGAAGATGCTGAGTCTGCCAGTTCTTGGGCTCTGGGCAGGATC
CCATCTCTTGCCTTCTCTAGGCAGAGCGCAGCAGGCAGGGCTCTGTGGCTCTGTGTCTAACCCACTTCTT
TCTCTCCTCGCTTTCAG

TYSLGAFTLSAASYRHQLAQFLLSLLIAGQDLGAEKNTRHRASHSFYFSPEHVWFVRMDVLNIINLKGSFPCLLQRKSNLVFHYSFRRG-TISLIS--
SGKSEDAESASSWALGRIPSLAFSRQSAAGRALWLCV-PTSFSPRFQ

Kalij(*Lophura leucomelanos*)

Lophura leucomelanos cytochrome b (cytb) gene:

>gi|14348648|gb|AF314643.1| Lophura leucomelanos cytochrome b (cytb) gene, complete cods; mitochondrial gene for mitochondrial product
ATGGCACCCAATATCCGAAAATCACACCCCCTACTAAAAATAATCAATAACTCCCTAATTGACCTACCCA
CCCCATCCAACATCTCTGCTTGATGAAACTTCGGCTCCCTGCTAGCAGTATGCCTTGCCACTCAAATCCT
CACCGGTCTCCTACTAGCCATACATTACACTGCAGATACCTCCTTAGCTTTCTCCTCCGTAGCCCACACA
TGTCGCAACGTACAATACGGCTGACTCATCCGAAATCTTCATGCAAACGGCGCCTCATTCTTCTTCATCT
GCATCTTCCTCCACATCGGACGCGGCCTCTACTACGGCTCCTACTTATACAAAGAAACATGAAACACCGG
AGTCATCCTACTCCTCACACTCATAGCAACCGCCTTCGTGGGATACGTCCTTCCATGAGGACAAATATCA
TTTTGAGGAGCTACCGTCATCACAAACCTATTCTCAGCAATCCCCTACATCGGACAAACCCTAGTAGAAT
GAGCTTGAGGCGGATTCTCAGTTGACAACCCAACCCTTACCCGATTCTTCGCCCTACACTTCCTCCTCCC
CTTCGTAATTGCAGGAATTACCATCATCCACCTCCTATTCCTGCACGAATCAGGCTCAAACAACCCACTA
GGCATCTCATCTAACTCTGACAAAATCCCATTCCACCCCTACTACTCTTTCAAAGACATCCTAGGCCTAG
CACTTATATTTACCCCATTCCTAACACTAGCCCTATTCTCACCAAACCTTCTAGGTGATCCAGAAAACTT
CACCCCAGCAAATCCTCTAGTAACCCCTCCCCACATTAAACCAGAATGGTACTTCCTATTTGCTTACGCC
ATCCTACGCTCAATCCCTAATAAACTAGGTGGTGTCCTGGCCCTGGCAGCCTCAGTACTCATCCTCCTCC
TCATCCCTTTCCTTCACAAATCTAAACAACGAACCATAACCTTTCGCCCACTTTCCCAAACCCTATTCTG
ATTCCTAGTCGCTAACCTCCTCATCCTAACTTGAGTAGGAAGCCAACCAGTAGAACACCCATTCATCATC
ATTGGCCAAATAGCATCATTCTCTTACTTCACTATTCTACTAATCCTCTTCCCCGCAATCGGGACCCTAG
AAAACAAAATACTCAATTACTAG

>gi|14348649|gb|AAK61329.1| cytochrome b (mitochondrion) [Lophura leucomelanos]
MAPNIRKSHPLLKMINNSLIDLPTPSNISAWWNFGSLLAVCLATQILTGLLLAMHYTADTSLAFSSVAHT
CRNVQYGWLIRNLHANGASFFFICIFLHIGRGLYYGSYLYKETWNTGVILLLTLMATAFVGYVLPWGQMS
FWGATVITNLFSAIPYIGQTLVEWAWGGFSVDNPTLTRFFALHFLLPFVIAGITIIHLLFLHESGSNNPL
GISSNSDKIPFHPYYSFKDILGLALMFTPFLTLALFSPNLLGDPENFTPANPLVTPPHIKPEWYFLFAYA
ILRSIPNKLGGVLALAASVLILLLIPFLHKSKQRTMTFRPLSQTLFWFLVANLLILTWVGSQPVEHPFII
IGQMASFSYFTILLILFPAIGTLENKMLNY

Cheer Pheasant (*Catreus walichi*)

Catreus walichi cytochrome b (cytb) gene, mitochondrial gene:

>gi|3687600|gb|AF028792.1| Catreus walichi cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, complete cds
ATGGCACCCAACATCCGAAAGTCCGACCCCCTACTAAAAATAATCAATAACTCCCTAATCGACCTACCCG
CCTCATCCAACATCTCTGCTTGATGAAACTTCGGTTCCCTACTAGCAGTATGCCTCACCACTCAAATCCT
CACCGGTCTCCTACTAGCCATACACTACACTGCAGATACCTCCCTAGCCTTCTCCTCCGTAGCCCACACA
TGTCGAAACGTACAATACGGCTGACTCATCCGAAATCTCCATGCAAACGGCGCCTCATTCTTTTTCATCT
GCATCTTCCTTCACATTGGACGCGGCCTCTACTACGGCTCCTACTTATACAAAGAAACATGAAACACCGG
AGTCGTCTTACTCCTCACACTCATAGCAACCGCCTTCGTAGGATACGTCCTTCCATGGGGACAAATATCA
TTTTGAGGGGCTACTGTCATCACAAATCTATTCTCAGCAATCCCTTACATCGGACAGACCCTAGTAGAAT
GAGCCTGAGGAGGATTCTCAGTTGACAATCCAACTCTCACCCGATTCTTCGCCCTGCACTTCCTCCTTCC
CTTCGTAATTGCAGGAATCACCATCACCCATCTCATATTCCTACATGAATCAGGCTCAAATAACCCCCTA
GGCATCTCATCTAACTCCGACAAAATCCCATTCCACCCATACTACTCCCTCAAAGATATCCTAGGCCTAG
CACTTATATTCACCCCATTCCTAACACTAGCCCTATTCTCACCAAATCTTCTGGGCGACCCAGAAAACTT
CACCCCAGCAAATCCATTAGTAACCCCACCACACATTAAACCAGAATGGTACTTCTTATTTGCCTACGCT
ATCCTACGCTCAATCCCAAATAAACTCGGAGGTGTCCTAGCACTAGCAGCTTCAGTACTAATTCTCCTCC
TCATCCCCTTCCTTCATAAATCCAAACAACGAACCATAACCTTCCGCCCACTTTCCCAAGCCCTATTTTG
ATTACTAGTCGCCAACCTTCTCATCCTGACTTGAGTGGGAAGCCAACCAGTAGAACACCCGTTCATCATC
ATTGGCCAAATAGCATCATTTTCATACTTCACCATTCTATTAGTCCTCTTCCCCACAATCGGGACTCTAG
AAAACAAAATACTCAACTACTAG

>gi|3687601|gb|AAC62182.1| cytochrome b (mitochondrion) [Catreus walichi]
MAPNIRKSDPLLKMINNSLIDLPASSNISAWWNFGSLLAVCLTTQILTGLLLAMHYTADTSLAFSSVAHT
CRNVQYGWLIRNLHANGASFFFICIFLHIGRGLYYGSYLYKETWNTGVVLLLTLMATAFVGYVLPWGQMS
FWGATVITNLFSAIPYIGQTLVEWAWGGFSVDNPTLTRFFALHFLLPFVIAGITITHLMFLHESGSNNPL
GISSNSDKIPFHPYYSLKDILGLALMFTPFLTLALFSPNLLGDPENFTPANPLVTPPHIKPEWYFLFAYA
ILRSIPNKLGGVLALAASVLILLLIPFLHKSKQRTMTFRPLSQALFWLLVANLLILTWVGSQPVEHPFII
IGQMASFSYFTILLVLFPTIGTLENKMLNY

Sequences extracted for final sequencing procedure:

Tragopan melanocephalus

AATAATTTTTTTTAACCTAACCCCCCTATTGAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTAT
GTATAATCGTGCATACATTTATATACCACATACATTATGGTACCAATACTATATATTATATACGTACTAA
ACCCATTATATGTAGACGGACATAACACCTTGACCCCCTTCTACCAATGTACATATCATGCAACGTCCCA
GACTAATCCTGCTCAACTACCCCTAAGCTGCCAATACAACATCCAAGGCACCATAACTATGAATGGTTAC
AGGACATACTTCTAAATCCTTATGTTCTAACCCCATTTGGTTATGCTCGTCGTATCAGATGGATTTATTG
ATCGTACACCTCACGAGA

Lophophorus impejanus

GTACGTACAGCCTTGGTGCATTCACGTTGTCGGCTGCCTCCTACAGGCACCAACTTGCACAGTTCCTGCT
TTCGTTGCTGATTGCTGGCCAGGATTTGGGGGCAGAAAAGAACACCAGGCATCGCGCCAGCCATTCATTT
TATTTTTCACCAGAGCATGTCTGGTTTGTTAGGATGGATGTTTTGAACATCATTAACCTTAAGGGAAGTT
TTCCTTGCTTGCTGCAAAGAAAATCAAATTTGGTGTTTCATTATAGTTTTCGAAGGGGTTAAACGATTTC
ACTCATCTCTTAATAATCAGGTAAATCAGAAGATGCTGAGTCTGCCAGTTCTTGGGCTCTGGGCAGGATC
CCATCTCTTGCCTTCTCTAGGCAGAGCGCAGCAGGCAGGGCTCTGTGGCTCTGTGTCTAACCCACTTCTT
TCTCTCCTCGCTTTCAG

Lophophorus leucomelanos

ATGGCACCCAATATCCGAAAATCACACCCCCTACTAAAAATAATCAATAACTCCCTAATTGACCTACCCA
CCCCATCCAACATCTCTGCTTGATGAAACTTCGGCTCCCTGCTAGCAGTATGCCTTGCCACTCAAATCCT
CACCGGTCTCCTACTAGCCATACATTACACTGCAGATACCTCCTTAGCTTTCTCCTCCGTAGCCCACACA
TGTCGCAACGTACAATACGGCTGACTCATCCGAAATCTTCATGCAAACGGCGCCTCATTCTTCTTCATCT
GCATCTTCCTCCACATCGGACGCGGCCTCTACTACGGCTCCTACTTATACAAAGAAACATGAAACACCGG
AGTCATCCTACTCCTCACACTCATAGCAACCGCCTTCGTGGGATACGTCCTTCCATGAGGACAAATATCA
TTTTGAGGAGCTACCGTCATCACAAACCTATTCTCAGCAATCCCCTACATCGGACAAACCCTAGTAGAAT
GAGCTTGAGGCGGATTCTCAGTTGACAACCCAACCCTTACCCGATTCTTCGCCCTACACTTCCTCCTCCC
CTTCGTAATTGCAGGAATTACCATCATCCACCTCCTATTCCTGCACGAATCAGGCTCAAACAACCCACTA
GGCATCTCATCTAACTCTGACAAAATCCCATTCCACCCCTACTACTCTTTCAAAGACATCCTAGGCCTAG
CACTTATATTTACCCCATTCCTAACACTAGCCCTATTCTCACCAAACCTTCTAGGTGATCCAGAAAACTT
CACCCCAGCAAATCCTCTAGTAACCCCTCCCCACATTAAACCAGAATGGTACTTCCTATTTGCTTACGCC
ATCCTACGCTCAATCCCTAATAAACTAGGTGGTGTCCTGGCCCTGGCAGCCTCAGTACTCATCCTCCTCC
TCATCCCTTTCCTTCACAAATCTAAACAACGAACCATAACCTTTCGCCCACTTTCCCAAACCCTATTCTG
ATTCCTAGTCGCTAACCTCCTCATCCTAACTTGAGTAGGAAGCCAACCAGTAGAACACCCATTCATCATC
ATTGGCCAAATAGCATCATTCTCTTACTTCACTATTCTACTAATCCTCTTCCCCGCAATCGGGACCCTAG
AAAACAAAATACTCAATTACTAG

Catreus walichi

ATGGCACCCAACATCCGAAAGTCCGACCCCCTACTAAAAATAATCAATAACTCCCTAATCGACCTACCCG
CCTCATCCAACATCTCTGCTTGATGAAACTTCGGTTCCCTACTAGCAGTATGCCTCACCACTCAAATCCT
CACCGGTCTCCTACTAGCCATACACTACACTGCAGATACCTCCCTAGCCTTCTCCTCCGTAGCCCACACA
TGTCGAAACGTACAATACGGCTGACTCATCCGAAATCTCCATGCAAACGGCGCCTCATTCTTTTTCATCT
GCATCTTCCTTCACATTGGACGCGGCCTCTACTACGGCTCCTACTTATACAAAGAAACATGAAACACCGG
AGTCGTCTTACTCCTCACACTCATAGCAACCGCCTTCGTAGGATACGTCCTTCCATGGGGACAAATATCA
TTTTGAGGGGCTACTGTCATCACAAATCTATTCTCAGCAATCCCTTACATCGGACAGACCCTAGTAGAAT
GAGCCTGAGGAGGATTCTCAGTTGACAATCCAACTCTCACCCGATTCTTCGCCCTGCACTTCCTCCTTCC
CTTCGTAATTGCAGGAATCACCATCACCCATCTCATATTCCTACATGAATCAGGCTCAAATAACCCCCTA
GGCATCTCATCTAACTCCGACAAAATCCCATTCCACCCATACTACTCCCTCAAAGATATCCTAGGCCTAG
CACTTATATTCACCCCATTCCTAACACTAGCCCTATTCTCACCAAATCTTCTGGGCGACCCAGAAAACTT
CACCCCAGCAAATCCATTAGTAACCCCACCACACATTAAACCAGAATGGTACTTCTTATTTGCCTACGCT
ATCCTACGCTCAATCCCAAATAAACTCGGAGGTGTCCTAGCACTAGCAGCTTCAGTACTAATTCTCCTCC
TCATCCCCTTCCTTCATAAATCCAAACAACGAACCATAACCTTCCGCCCACTTTCCCAAGCCCTATTTTG
ATTACTAGTCGCCAACCTTCTCATCCTGACTTGAGTGGGAAGCCAACCAGTAGAACACCCGTTCATCATC
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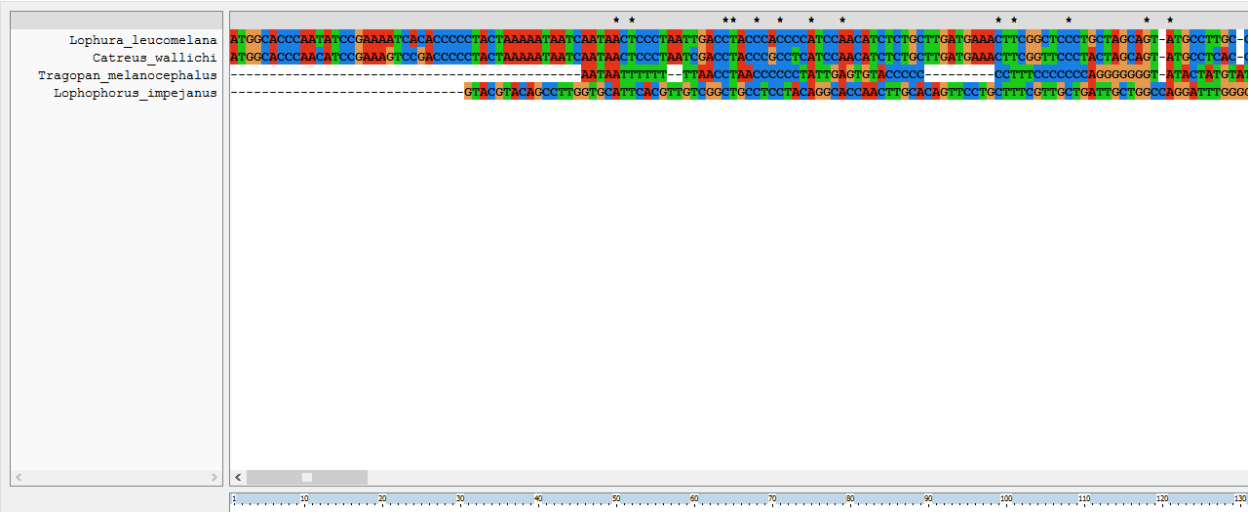

Fig. 20: Sequence Alignment from 1bp to 130 bp

Gene similarity shown for *Lophura leucomelanos* and *Catreus walichi*, *Tragopan melanocephalus* and *Lophophorus impejanus* showed less similarity with rest of the group but showed some similarity with each other from 1bp to 130 bp

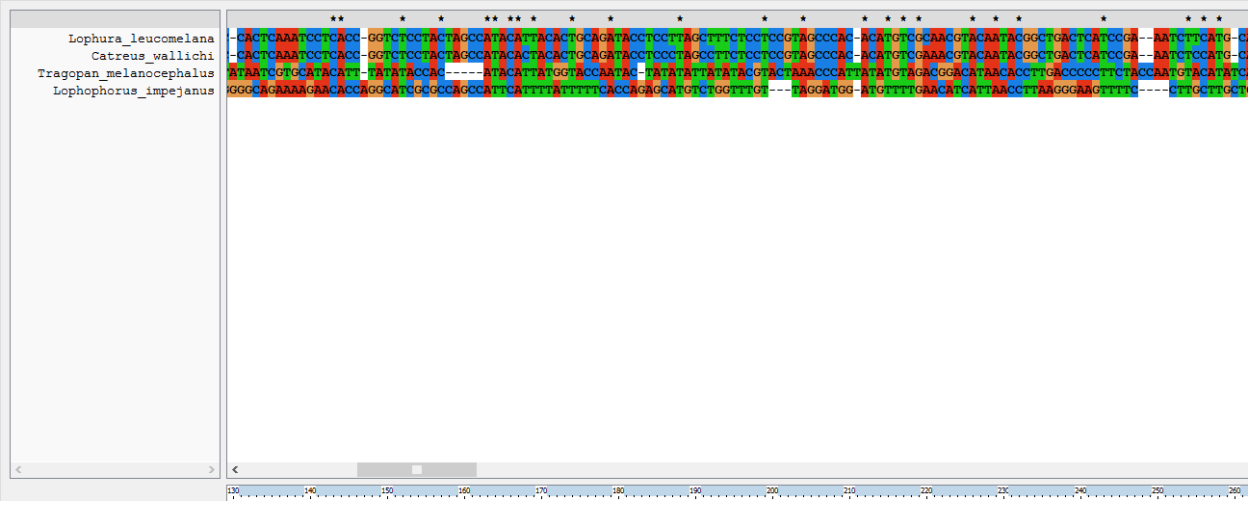

Fig. 21: Sequence Alignment from 130bp to 260 bp

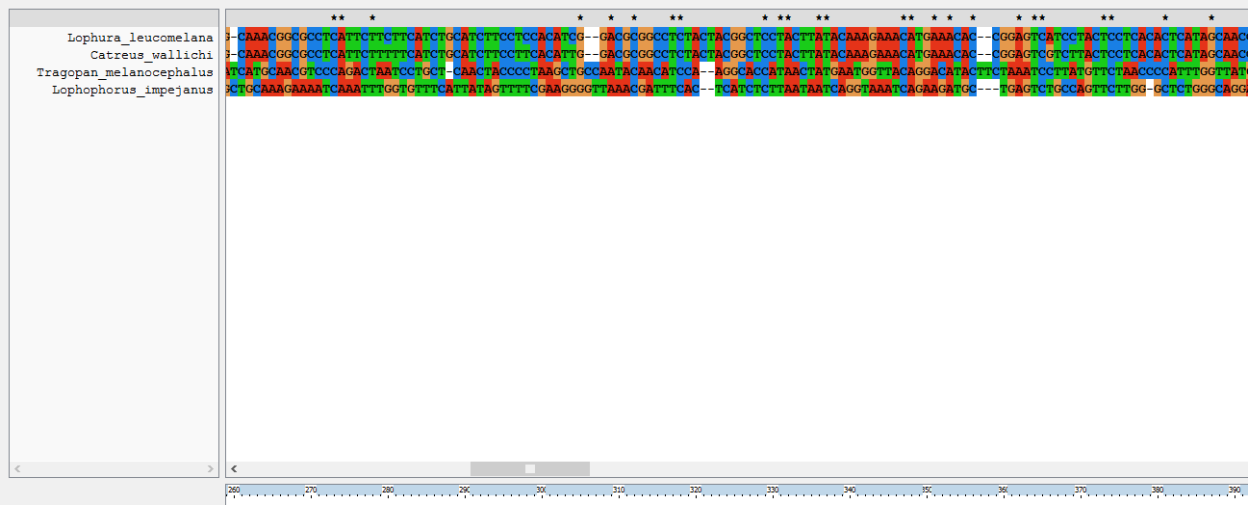

Fig. 22: Sequence Alignment from 260bp to 390 bp

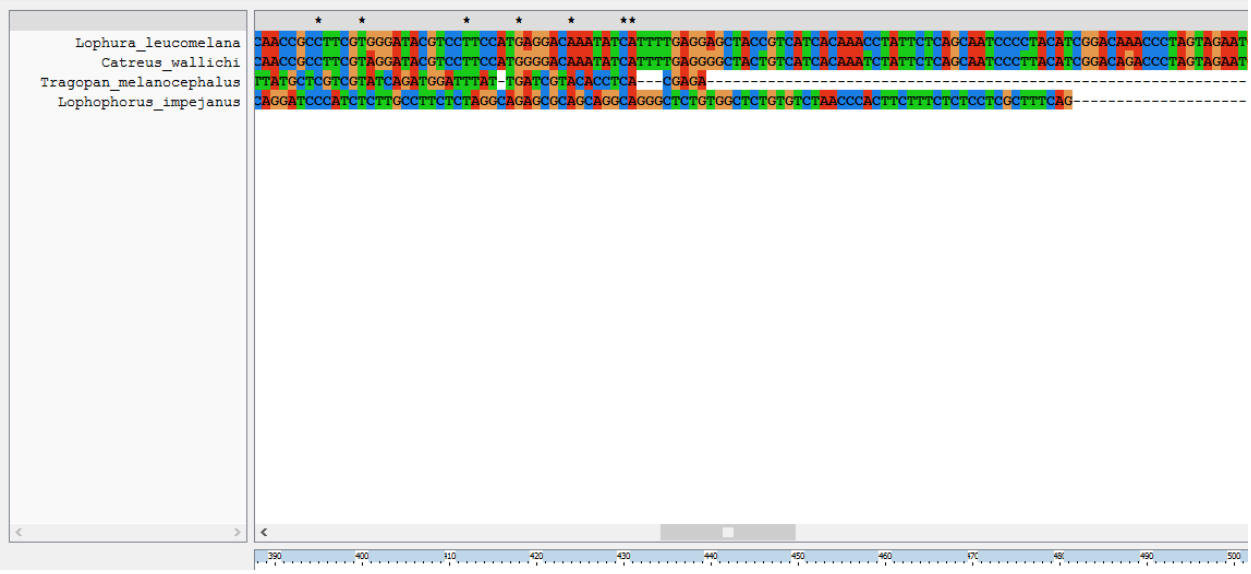

Fig. 23: Sequence Alignment from 390bp to 500 bp

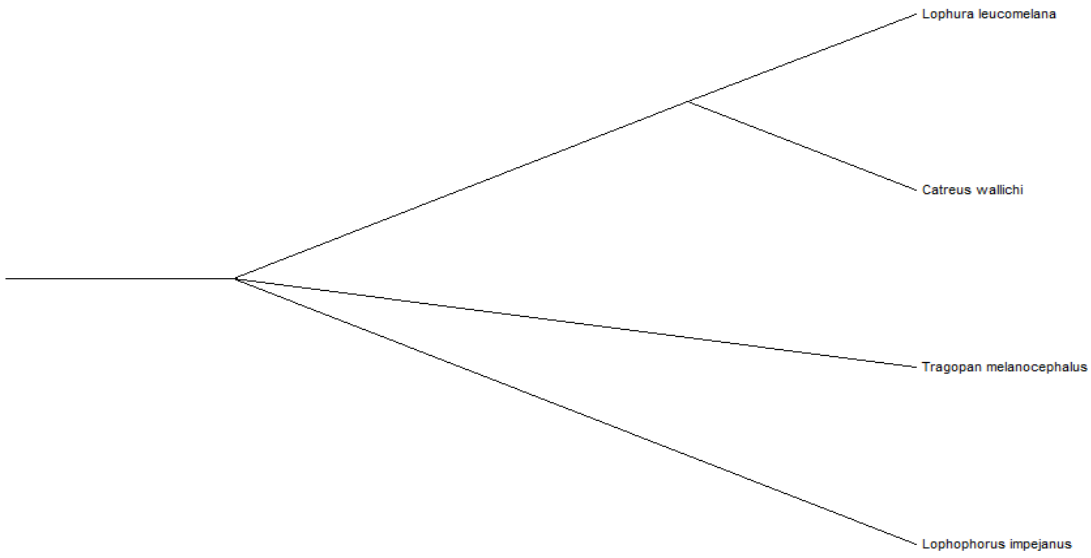

Fig. 24: Clustal X output

Description:

- Clustal X alignment data was used to get this output.
- Sequence alignment was performed using Clustal X.
- Then the sequences were imported to fig tree and MEGA finally MEGA results were taken for analysis.

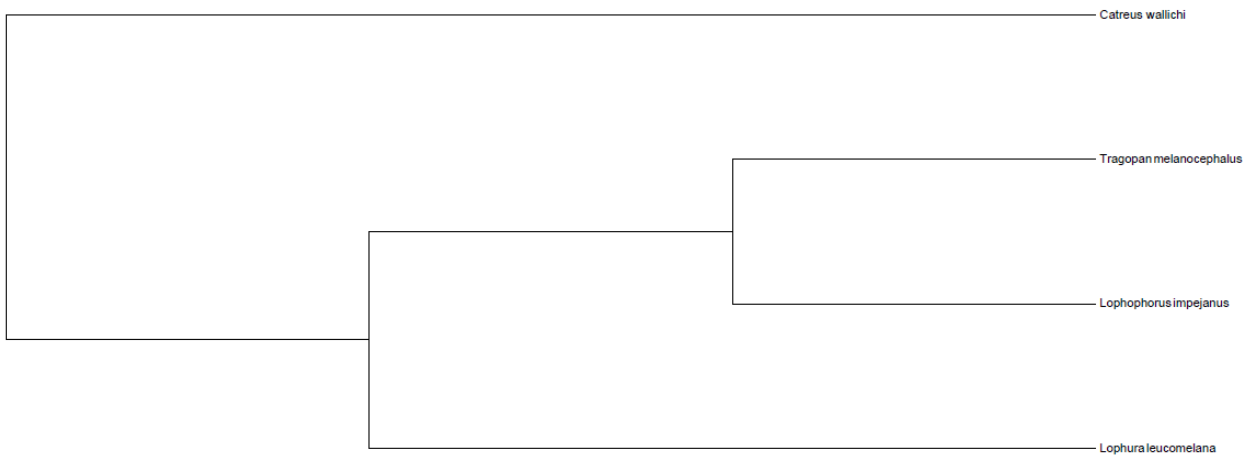

Fig. 25: Mega Tree Output

Outgroup: *Catreus wallichi*

Catreus walichi was chosen as an outgroup. This species came out to be quite unique with single genera and it is evident from MEGA result that *Catreus walichi* had followed an independent line of evolution when compared with rest of the group but from alignment data it is also evident that genetically it is quite similar to *Lophura leucomelanos*.On the other hand *Tragopan melanocephalus* and *Lophophorus impejanus* were very closely related to each other sharing the same clade.

Discussion:

A tree is an acyclic connected graph that consists of a collection of nodes (internal and external) and branches connect them so that every node can be reached by a unique path from every other branch. In the area of phylogenetic inference, trees are used as visual displays that represent hypothetical, reconstructed evolutionary events. Phylogenetic tree building methods presume particular evolutionary models. For a given dataset, these models can be violated because of occurrences such as the transfer of genetic material between organisms. Thus, when interpreting a given analysis one should consider the model used similarly after interpreting the tree output in the present study. It was observed that gene similarity shown for *Lophura leucomelanos and Catreus wallichi, Tragopan melanocephalus and Lophophorus impejanus* showed less similarity with rest of the group but showed some similarity with each other from 1bp to 130 bp (see appendix). High level of genetic similarity was observed from 130 bp to 390 bp. Based on gene

sequence alignment data tree was constructed using MEGA Tree which is publically available free software for phylogenetic construction. Nucleotide sequences were taken for tree construction (see appendix) and phylogenetic tree was constructed. In the tree construction *Catreus wallichi* was chosen as an outgroup. This species came out to be quite unique with single genera and it is evident from MEGA result that *Catreus wallichi* had followed an independent line of evolution when compared with rest of the group. From alignment data it is also evident that it is quite similar to *Lophura leucomelanos*. On the other hand *Tragopan melanocephalus* and *Lophophorus impejanus* were closely related to each other sharing the same clade. The results obtained from this study would be further used for DNA barcoding of pheasant species for conservation and identification purpose.

CONCLUSION

Phasinidae (pheasants, grouse, turkeys and partridges) is one of the five families which come under the order Galliformes. The western Tragopan, Himalayan Monal, Kalij and Cheer Pheasant belong to the Phasianidae subfamily and also are the pheasants of Himachal Pradesh. These species are endemic to Himalayas and are endangered. Throwing light onto their critically endangered condition, this project was undertaken so that there would be a significant amount of work carried out within this area leading to the conservation of the species preventing further loss.By the means of studies conducted as per discussed in this one year project, there are a significant number of results that are obtained. The evolutionary relationship is discussed on the basis of the mitochondrial gene structure can lead to significant results in the process of conservation of the bird species. Wet laboratory experimentation has been able to provide a standardized method of genomic DNA extraction, which can be utilized as a non-invasive procedure. As per the results obtained in case of the PCR products we observe that amplification of the target regions is carried out significantly leading to the possibility of sequencing and ultimately getting results leading to DNA barcoding of the individual organism.

In the tree construction *Catreus wallichi* was chosen as an outgroup. This species came out to be quite unique with single genera and it is evident from MEGA result that *Catreus wallichi* had followed and independent line of evolution when compared with rest of the group. From alignment data it is also evident that it is quite similar to *Lophura leucomelanos*. On the other hand *Tragopan melanocephalus* and *Lophophorus impejanus* were closely related to each other sharing the same clade.This will serve many objectives as there can be identification of inbreeding depression which is a possible cause of extinction. Individual species can be identified and cross breeding can be carried out depending on the compatibility with the nearest possible relative which is indicated by the evolutionary map. Though it is said that research is a never- ending endeavor, this project gives a significant contribution to Wildlife and conservation genetics, so that there is no more loss of valuable species.

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GLOSSARY

Allopatric: Refers to species occurring in geographically separated areas, i.e. ranges not overlapping.

Altitudinal migrant: A species found at different altitudes at different times of the year, especially breeding higher in the mountains and wintering lower down.

Aviary: An enclosure constructed to house and rear birds. Unlike cages, aviaries allow birds a larger living in space in which to fly and simulate a natural environment.

Clutch size: The number of eggs laid by one female in a nest at one time.

DNA: Abbreviation for Deoxyribonucleic acid. It is a polynucleotide molecule which comprises the genetic material of organisms consisting of the sugar-deoxyribose, phosphate groups and nitrogenous bases. DNA is usually double stranded with each strand coiled alongside the other in a characteristic double helix. The structural basis for helix is hydrogen bonding between specific pairs of bases in each strand. The nitrogenous bases are the purines-adenine (A) and guanine (G) and Pyrimidines-thymine (T) and cytosine (C). In double-stranded DNA, A pairs with T and G pairs with C for base pairing.

DNA barcoding is the process of identification of species based on nucleotide diversity of short DNA segments. It is well established in animals with the introduction of *cytochrome c oxidase subunit 1* (COI) as a standard barcode.

Dimorphic (Dichromatic): Occurring in two distinct plumage types, usually related to gender, e.g. species where males and females appear different are said to be sexually dimorphic (dichromatic if the difference is only one of colour).

Electrophoresis: A laboratory technique used to separate mixtures of molecules, such as proteins and nucleic acids in a suspension, by their charge-to-mass ratio. The mixture is added to an inert medium such as an agarose or acrylamide gel in an appropriate buffer solution and is subjected to an electric field. The charged molecules then move through the gel towards the appropriate electrode. Gel electrophoresis of fragments of DNA is routinely used to produce genetic fingerprints and to carry out DNA sequencing.

Endangered (EN): A taxon is Endangered when the best available evidence indicates that it meets any of the criteria A to E for Endangered, and it is therefore considered to be facing a very high risk of extinction in the wild (*Category in the IUCN Red List*).

Endemic: Indigenous; exclusively confined to a defined geographic area.

Eukaryotes: Organisms in which the cells have a true nucleus bounded by a nuclear membrane, and also possess membrane- bound cellular structures.

Ex-situ: Removed from its natural habitat.

Flagship species: Popular charismatic species that serve as symbols to stimulate conservation awareness and action locally, nationally, regionally or globally.

Galliform: Species belonging to the Order Galliformes.

Gene: Gene unit of a chromosome that codes for a specific protein.

Genetic Code information which is the basis of all gene functions encoded as a liner sequence of the four nitrogenous basis that form of the structures of DNA. After transcription when DNA is copied into RNA the information is encoded as sequences of three basis called triplets or codons. The 64 possible 3-base triples/codons drive from combinations of the four bases present on RNA and the amino acids by them. The code is degenerate in that some amino acids recoded for by more than one codon. The codons UAA.UAG and UCA are termed 'stop' or termination codons 'and they signal the end of end of an open reading frame. The codon AUG coding for methionine is the initiation, codons starting an open reading frame.

Gene sequencing techniques that allow to identify distinct species with a high degree of precision.

Genome: The total genetic complement of an organism. **Genomic DNA** is the sum total of all an individual's DNA present in one cell.

IUCN Red List: A comprehensive inventory of the global conservation status of species, using a set of criteria to evaluate extinction risk (IUCN, 2001).

Least Concern (LC): A taxon is Least Concern when it has been evaluated against the criteria and does not qualify for CR, EN, VU or NT. Widespread and abundant taxa are included in this category (*Category in the IUCN Red List*).

Mitochondrial DNA: Mitochondrial DNA (mtDNA) is the DNA located in organelles called mitochondria, structures within eukaryotic cells that convert the chemical energy from food into a form that cells can use, ATP. Most other DNA present in eukaryotic organisms is found in the cell nucleus. The mtDNA is useful for studying the evolutionary relationships (phylogeny) of organisms. Biologists can compare mtDNA sequences among different species, and use the comparisons to build evolutionary tree for the species examined.

Polymerase chain reaction (PCR): A technique enabling multiple copies to be specific of sections of DNA molecules. It allows isolation and amplification of such from large heterogeneous mixtures of DNA and has many diagnostic applications. The technique has revolutionized many areas of molecular biology. Polymerase chain reaction starts with:

1. A double standard DNA fragment with known ends sequence, which is to be copied.
2. The two DNA strands are separated by heating to 95^{o} C.
3. Two primers are added that have complementary base pairs to end sequence in the DNA. Cooling to about 50^{o} C allows the primer to bond each strand.
4. DNA nucleoside triphosphates and the heat-stable Taq polymers become involved, and the temperature is raised to 72^{o} C.

5. New DNA fragments are produced by the nucleotides bonding to the primers, the single strands acting as templates.
6. The process is repeated with the two new double strands. Each cycle of heating and cooling double the number of copies of DNA template, so that after 20 cycles there are over a million copies of original DNA.

RNA polymers: An enzyme that initiates and elongates RNA chain by assembling > ribose-containing nucleotide in a sequence determined by a > DNA template.

Tarsus (plural tarsi): Part of leg of a bird below the thigh.

Vulnerable (VU): A taxon is Vulnerable when the best available evidence indicates that it meets any of the criteria A to E for Vulnerable, and it is therefore considered to be facing a high risk of extinction in the wild (*Category in the IUCN Red List*).

Biodiversity Conservation and Management in India through Biodiversity Management Committees: Institutional Mechanism Approach

Biodiversity

Biodiversity includes variety and variability among different organism on earth and the processes in which they occur

Convention on Biological Diversity, 1992

Convention on Biological Diversity

Objectives

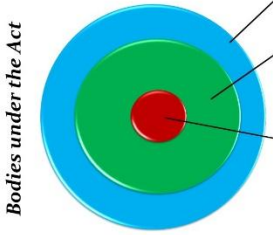
- Conservation of Biological diversity
- Sustainable use of components of biodiversity
- Fair and equitable sharing of benefits arising out of the utilization genetic resources

Biological Diversity Act, 2002

Implementation of Biological Diversity Act, 2002

Three Tier System

Bodies under the Act



- *National Biodiversity Authority* [Central Government]
- *State Biodiversity Boards* [State Governments]
- *Biodiversity Management Committees* [Local Bodies]

Salient Features

- To regulate access to biological resources of the country equitable share in benefits arising out of the use of biological resources.
- To conserve and sustainable use of biological diversity.
- To respect and protect knowledge of local communities related to biodiversity.
- To secure sharing of benefits with local people as conservers of biological resources and holders of knowledge and information relating to the use of biological resources.
- Conservation and development of areas of importance from the standpoint of biological diversity by declaring them as biological diversity heritage sites.
- Protection and rehabilitation of threatened species.
- Involvement of institutions of State Government in the broad scheme of the implementation of the Biological Diversity Act through constitution of committees.

Role of National Biodiversity Authority

(Section 8C)

State Biodiversity Board

(Section 22)

Functions:

- Regulation
- Collection, Compilation, Documentation and Publication
- Trainings
- Grants/Funds
- Procedure and Guidelines
- Prohibition or restriction
- Update and Implementation of Biodiversity Action Plan

Constitution of Biodiversity Management Committees (BMCs)

(Section 41)

Role of BMCs

Documentation of local biodiversity

Chronicling of Traditional Knowledge

Custodian and Trustee (PBRs)

Conservers of local biodiversity

Functions and Duties of BMC's

Mandate of BMC's

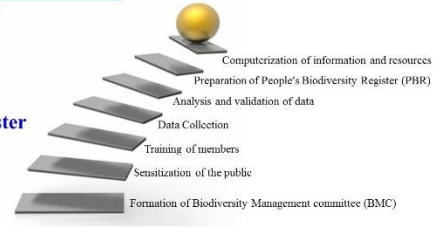
Power of BMCs

Decision relating to use of Biological resources and associated traditional knowledge.

Levy charges by way of collection fees from any person for accessing or collecting any biological resources for commercial purposes

Restrict Access to biological resources within its territorial jurisdiction by mean of reporting to the Board

Preparation of People's Biodiversity Register



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Shivai Gupta
B. Tech. (VI Sem) Biotechnology
Jaypee University of Information Technology (JUIT), Waknaghat-Solan-173234, Himachal Pradesh, India
E-mail: shivai.gup@gmail.com

Value addition through biotechnological approaches for the conservation of medicinal and aromatic plants: a case study

- Any technology application that uses biological systems, living organisms, or derivatives there of, to make or modify products or processes for specific use
- Covers tools and techniques commonly used in agriculture and food production, processing and utilization
- Encompasses DNA techniques, molecular biology, and reproductive technological applications dealing primarily with gene splicing and recombination, and genomics

Helps in number of ways:

- Plant tissue culture
- Genetic Engineering/Plant Transformation
- Molecular Markets
- DNA Fingerprinting
- Gene Cloning
- Gene Therapy
- Genome Sequencing
- Functional Genomics
- Bioinformatics
- Mass production/cultivation

Biotechnology

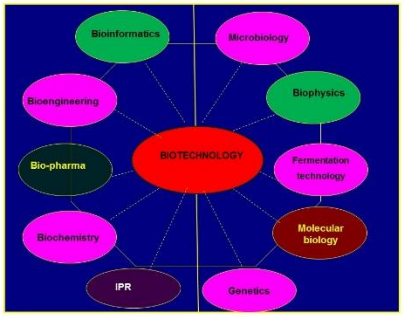
Applications:

- Medicinal & Aromatic Plants Tissue Culture
- Genetic Engineering/Plant Transformation
- Molecular Markers
- DNA Fingerprinting
- Gene Cloning
- Gene Therapy
- Genome Sequencing
- Functional Genomics
- Bioinformatics
- Mass production/cultivation

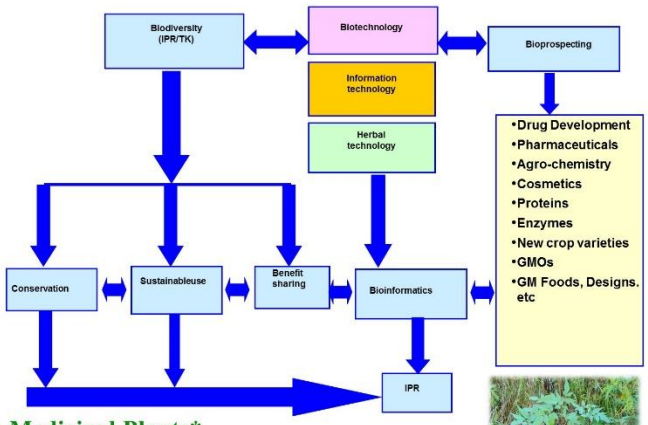
Approaches:

- Tissue Culture: (Collection, multiplication, storage of plant germplasm) provides propagating plant material with high multiplication rates in aseptic environment.
- Cryopreservation: To ensure the safe and cost-efficient long-term conservation of species.
- In-vitro Production of Embryos: Include splitting and cloning of embryos, marker-assisted selection, sexing of embryos and transfer of new genes into an embryo.
- Transgenic Farm Animals: Production of transgenic crops and animals.

BASIC AND APPLIED SCIENCES
FOR BIOBUSINESS FROM BIOTECHNOLOGY



Technological Interventions



Conclusions:

- Indian Himalayan Region (IHR) is the rich repository of Medicinal and Aromatic Plants with over 1740 species with various traditional and modern therapeutic uses. The unique diversity of MPs in the region is manifested by the presence of a number of native (31%), endemic (15.5%) and threatened elements: MPs comprise 14% of total Red data plant species of IHR. In addition, the MPs of the region are highly valued because of their potential to deliver novel biomolecular and larger quantity of active compounds .
- In-vitro propagation of medicinal and aromatic plants with enriched bioactive principles and cell culture methodologies for selective metabolite production is highly useful for commercial production of medicinally important compounds. Study on mass scale propagation of thigh value species would help in ex situ and in situ conservation.
- Advances in plant cell culture systems would provide improved understanding of the secondary metabolite pathway in Medicinal and aromatic plants. Plant cell culture would provide new means for the cost-effective, commercial production of rare or exotic plants, their cells and the chemicals that they will produce. These new technologies will serve to extend and enhance the continued usefulness of higher plants as renewable sources of chemicals, especially medicinal compounds.
- In addition, educational and awareness programmes on status, conservation and management of these high value species for different stakeholder would help in their conservation and management.

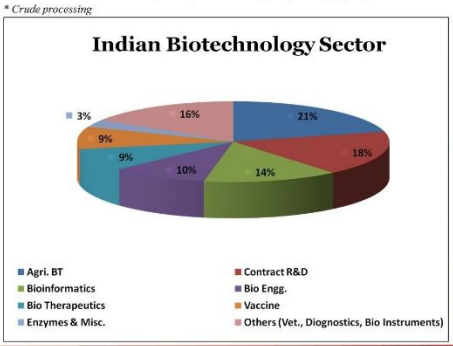
❖ **Medicinal Plants***

Picrorrhiza, Aconitum, Tagetus, Swertia, Pelargonium, Taxus, Podophyllum, Dactylorhizha

❖ **Aromatic Plants***

Lavender, Lavendin, Rose, Rosemarry, Cymbopogon, Geranium

* Crude processing



Shivai Gupta
B. Tech. (VI Sem) Biotechnology
Jaypee University of Information Technology (JUIT), Waknaghat-Solan-173234, Himachal Pradesh, India
E-mail: shivai.gup@gmail.com

Breeding activity pattern, DNA barcoding and reproductive hormone profiling of Indian rock lizard *Psammophilus dorsalis* (Agamidae, Reptilia)

ABSTRACT

Field observations and lab studies were carried out on the **peninsular rock agama** or **South Indian rock agama** (*Psammophilus dorsalis*). Ecological and behavioural studies were carried out on rock Agamas found within a 5 hectare area of LaCONES (CCMB), Hyderabad during its breeding season in June-July 2014. Egg laying sites were identified followed by collection and incubation of eggs. Faecal pellets were collected to analyze the quantity of male hormone testosterone. Molecular genetic studies were carried out from DNA extracted from egg shells. Polymerase Chain Reaction (PCR) was carried out for amplifying specific regions of DNA using MCb, 16S, C-mos and COX primers followed by DNA sequencing.

INTRODUCTION

Population dynamics, microhabitat use and activity pattern are important components of animal ecology that lead to enhancing a better resource base for a specific species. The tools and techniques of biotechnology are further utilized with this analysis that would lead to developing a better and more effective system to manage species. Agamas are most visible and commonly seen lizards throughout south India. Most of them are diurnal lizards that are active during the day. They are sexually dimorphic, males being more colourful and larger than the females. Six genera occur in South India. ***Psammophilus dorsalis***, is a common species of agama found on rocky hills in south India. In-situ population and activity patterns were studied during its breeding season, which included a ten day survey to identify frequency of occurrence and noting their specific activity time and pattern during the May- July months. Behaviour was studied by taking observations on five individuals within a five hectare region. Their behavioural patterns were noted and classified into five states and seven events respectively. Male hormone analysis and gene sequencing was undertaken for *Psammophilus dorsalis*. Sequencing of genes of the organism was carried out using MCb, 16S, C-mos and COX1 primers.

MATERIALS AND METHODS

Population density and frequency of occurrence of *P. dorsalis* was studied in areas selected by point sighting and scan sampling. Hourly observations were made while walking along a predetermined path around each study site. From 7:00 AM to 6:00 PM hourly observations were carried out for a ten day time period. During breeding season, the males develop brilliant yellow or orange stripe over a jet black lateral and ventral mid dorsal region. A total of five male Agamas were captured for the study. Two specific marks were made on the body for counting the number of scales. The scales were counted in a circular manner by viewing the picture on camera. Tagging was done to provide unique identification marks to all individuals. After tagging the individuals were released to the places from where they were captured. Twenty four hours later it was observed that all the individuals were normal and carrying out normal activity. Five tagged males were studied further for behavioural analysis. In *Psammophilus* species the standard weight of adult males is from 30 gm to 35 gm. The snout vent length is approximately 13.5 cm and tail length is approximately 20.0 cm. To isolate DNA from. *P. dorsalis* egg yolk and egg shell procedure of Sambrook et al (1989) was followed. The extracted DNA was dissolved in TE buffer and then it was subjected to quality and quantity analysis. Enzyme Linked Immunosorbent Assay (ELISA) was performed on faecal samples collected from all the five male lizards.

Map of LaCONES (CCMB) Hyderabad

***Psammophilus dorsalis* (Peninsular/South Indian rock agama)**

RESULTS AND DISCUSSION

From all the samples taken there was a variable amount of testosterone present in the samples collected indicating presence of varied concentration during the breeding season further resulting in varied behaviours of individuals with respect to mating, as the females are very specific in choosing males for insemination depending upon the color. Brighter color indicates a significant amount of testosterone present.

The present study gives us an insight into its activity patterns, behaviour, genetic and hormonal aspects. The species exhibits a clear sex specific niche separation. Males occupied higher perches than females". Barcoding provides sufficient insight to all the matches available. No variation was found in the MCb, 16S rRNA, C-mos and COX1 sequences obtained from the five animals studied. The testosterone based hormone profile has provided us with valuable information regarding the amount of testosterone present within the collected samples. Activity pattern and behavioural studies will lead to development of better management systems for species. This study provides us with basic raw data that can be used for developing a methodology for better management of this species.

102nd INDIAN SCIENCE CONGRESS
3-7 January 2015, Mumbai

Name of Section: New Biology (including Biochemistry, Biophysics, Molecular Biology & Biotechnology)

Shivai Gupta
B. Tech. Biotechnology (2011-15)
Jaypee University of Information Technology (JUIT), Waknaghat-Solan-173234, Himachal Pradesh, India
E-mail: shivai.gup@gmail.com

Curriculum Vitae: Shivai Gupta

PERSONAL DETAILS

Name : **Shivai Gupta**

Date of Birth : **19.04.1993**

Father's Name : Dr. Hemant K Gupta

Mother's Name : Dr. Anu Gupta

Sex : Female

Nationality : Indian

Passport Details : **H 7695213;**

Date of Issue 19/01/2010 Date of Expiry: 18/01/2020

Current Address : Student B. Tech, Biotechnology (2011-15 Batch)

Jaypee University of Information Technology (JUIT),

Waknaghat, P.O. Dumehar Bani, Kandaghat, Solan,

Himachal Pradesh, India PIN 173215

E-mail : shivai.gup@gmail.com, Mobile: +918894569550

Corresponding Address : C/o Dr. Hemant K Gupta, IFS

Chief Conservator of Forests (Env., S&T)

Evergreen House, 1 New Road, Chhota Shimla, Shimla-2, Himachal Pradesh, India Pin 171002

Email/ Contact no. : hemantgifs@gmail.com; hemant.gupta@fulbrightmail.org

Tel. +91 177 2620469 (Res), Mobile: +91 9418020469

EDUCATIONAL QUALIFICATIONS

Examination	**Board /University**	**Year**	**Subjects**	**Marks (%)**	**Division**
High School	I.C.S.E., New Delhi	2009	English, Hindi, Environmental Education, History, Civics and Geography, Mathematics, Science-	89.9%	I

	Loreto-Convent Tara Hall, Shimla		{Physics, Chemistry, Biology}, Commercial Application		
Senior Secondary	C.B.S.E., New Delhi Dayanand Public School, Shimla	2011	English, Biology, Physics, Chemistry, Physical Education	82.9%	I
B.TECH (Biotech) Ist Year Semester 1	Jaypee University of Information Technology (JUIT), Waknaghat, Kandaghat, Solan - 173215	2011-2012	Computers and Programming, Electrical Circuit Analysis, Basic Mathematics, Physics-1, Computer Programming Lab, Electrical Circuits Lab, Physics Lab 1, Presentation and Communication Skills.	67.0%	I
Semester 2	Jaypee University of Information Technology	2011-2012	Data Structures, Basic Electronic Devices and Circuits, Basic Mathematics -2, Group and Cooperative Processes, Biophysical Techniques, Basic Biosciences Lab, Data Structures and Computer Programming Lab, Basic Electronics Lab.	70.0%	I
IInd Year Semester 3	Jaypee University of Information Technology	2012-2013	Thermodynamics and Chemical Processes, Biochemistry, Microbiology, Probability and Statistics, Thermodynamics and Chemical Processes Lab, Biochemistry Lab, Microbiology Lab, Managerial Economics	73.0%	I
Semester 4	Jaypee University of Information Technology	2012-2013	Biomaterial Sciences, Cell Tissue Culture Techniques, Molecular Biology, Genetics, Environmental Sciences, Genetics Lab, Molecular Biology Lab, Animal Tissue Culture Lab, Plant Tissue Culture Lab, Financial Management	76%	I
IIIrd Year Semester 5	Jaypee University of Information Technology	2013-2014	Process Engineering, Genetic Engineering, Immunology, Bioinformatics Lab, Genetic Engineering Lab, Immunology Lab, GLP & Instrumentation, GLP & Instrumentation Lab	77%	I

Semester 6	Jaypee University of Information Technology	2013-2014	Comparative and Functional Genomics, Food and Agricultural Biotechnology, Cell and Developmental Biology, Fermentation Technology and Downstream Process, Comparative and functional Genomics Lab, Food and Agricultural Biotechnology Lab, Cell and Developmental Biology Lab, etc.	78%	I
Semester 7	Jaypee University of Information Technology	2014-2015	Human Resource Management, Bioenergy and Biofuels, Stem Cells and Regenerative Medicine, Bioresources and Industrial Products.	79%	I

SGPA/CGPA REPORT: Shivai Gupta [Enrolment No. 111568] Examination details of 7 Semesters

Semester	Grade Points	Course Credit	Earned Credit	Points Secured SGPA	Points Secured CGPA	SGPA	CGPA	Disciplinary Grade
1	51.0	23.0	23.0	138.0	138.0	6.0	6.0	-
2	56.0	23.0	23.0	152.0	152.0	6.6	6.3	A^+
3	61.0	23.0	23.0	169.0	169.0	7.4	6.7	-
4	84.0	26.0	26.0	212.0	212.0	8.2	7.1	A^+
5	87.0	28.0	28.0	213.0	213.0	7.6	7.2	-
6	90.0	28.0	28.0	220.0	220.0	7.9	7.3	A^+
7	43.0	22.0	22.0	192.0	192.0	8.7	7.5	_
8								

- Disciplinary grade: A+ for year 2011-12, 2012-13 & 2013-14.
- The CGPA of 7.5 on the scale of 10 equates to 79 percent as per Conversion Table – 2005, approved by the Academic Council of the University.
- Examination details of 7 Semesters (Transcripts; Annexure I)

TRAININGS AND PROJECT EXPERIENCE

- **3 Weeks Summer Training (26th June to 13th July, 2012)** *"Plant Tissue Culture"* in the Crop Improvement Division, at Central Potato Research Institute (ICAR) Shimlaunder the supervision of **Dr. Vinay Bhardwaj**, *Acting Head & Sr. Scientist*, Crop Improvement Division, Horticulture (Vegetable Science), E-mail: vinaycpri@gmail.com. Tel: 0177 2625182 Ext: 501. Mob. +91 94180 46415.
- **4 Weeks Summer Training (10th June to 4th August 2013)***"Advance techniques in Biotechnology"* at Department of Biotechnology at CSIR- Institute of Himalayan Bioresources Technology (IHBT) - Palampur, District Kangra, Himachal Pradesh under supervision of **Dr. Anil Sood**, *Chief Scientist, Biotechnology & Co-ordinator*, Business Development & Marketing Unit, CSIR-IHBT, E-mail: asood@ihbt.res.in; Tel.: +91 1894 233337; Mob. +91 94180 42984.
- **3 Months Online Training** (**15th October, 2013 to 15th January, 2014)** GBioFin Entrepreneurship and Innovation Certificate North Board Leadership, USA in *"Developing innovative ideas and learning entrepreneurship in biotechnology in India"*.
- **8 Weeks Summer Training** (**26th May to 25th July 2014)** "*Breeding activity pattern, DNA barcoding and reproductive hormone profiling of Agamidae, Reptila Species Psaminophilus dorsalis"* under the supervision of **Dr. Kartikayan Vasudevan**, *Sr. Principal Scientist*, CSIR-Centre for Cellular & Molecular Biology, Laboratory for the Conservation of Endangered Species (LaCONES), CCMB Annexe 1, Attapur, Hderguda, Hyderabad, E-mail: karthik@ccmb.res.in; Tel.: +914024006403, Mob.: +91 9491036403.
- Presently working on the Project **(2014-2015)** entitled "*DNA Barcoding of Pheasant of Himachal Pradesh: Rate of Evolution and Identification of Species"* under the supervision of **Dr. Harvinder Singh** at Jaypee University of Information Technology, Waknaghat, Distt. Solan-173234, Himachal Pradesh, India, E-mail: harvinder.singh@juit.ac.in, harvinder_07@yahoo.com; Tel.: +91-1792-2579999; Mob.: +91 9816979794.

ABSTRACTS IN CONFERENCE PROCEEDING:

- Gupta, A., **Gupta, S.** and Gupta, H. **2010**. *Heritage trees of India: Ambassadors for Global Biodiversity*. Under Theme 'D': Asia's Forest for the future in **XIII IUFRO World Congress**, Korea, Forests for the future: sustaining Society and the Environment, **August 23rd to 28th, 2010.***The International Forestry Review* 12(5): 232.
- **Gupta, S.2014.A)** *Biodiversity Conservation and Management in India through Biodiversity Management Committees: Institutional Mechanism Approach.*p.17.
  B)*Value addition through biotechnological approaches for the conservation of medicinal and aromatic plants: a case study.* National Conference on "Perspectives & trends in Plant Sciences and Biotechnology. Organized by Department of Botany, Panjab University, Chandigarh and Society for Plant Research (SPR) India at Department of Botany, Panjab University Chandigarh from **21st to 23rd February, 2014**p. 132-133**.**

- Sharma, P., **Gupta, S.** and Gupta, H.K. **2014**. *Conservation, Sustainable Utilization and Management of Orchids in India.* National Conference on **"Orchids: Conservation, Improvement and Sustainable Development"** organized by The Orchid Society of India (TOSI), Botany Department, Panjab University, Chandigarh, India and College of Horticulture, Kerala Agriculture University, Vellenikkara, Thrissur (Kerala) India from **13th - 15th March, 2014.** p. 38.

- **Gupta, S.2014**. *Isolation of Genomic DNA and barcoding of Pheasants of Himachal Himalayas for Conservation*. **1st Himachal Pradesh Science Congress"**Role of Science & Technology in Sustainable Development**"** organized by Himachal Pradesh State Council for Science, Technology and Environment at Hotel Peterhoff, Shimla from **October 15-16, 2014**. p. 93 **(Best Poster Award).**
- P. Sharma, **Shivai Gupta,** S.S. Samant and H.K. Gupta. **2014**. Diversity, floristic composition and status of the communities in and around the dam submergence areas of Malana II Hydroelectric Project in Himachal Pradesh, Northwestern Himalaya. The Tropical Ecology Congress - 2014 on **"Tropical ecosystems in a changing world"** at Convention Centre, Jawaharlal Nehru University, New Delhi-110067, India from **December 10-12, 2014.**
- **Gupta, S.** 2015. A) Breeding activity pattern, DNA barcoding and reproductive hormone profiling of Indian rock lizard *Psammophilus dorsalis* (Agamidae, Reptilia);
  B)Conservation and sustainable utilization of medicinal plants through biotechnological approaches **102nd Indian Science Congress**, hosted by University of Mumbai, Mumbai, India from **January 3-7, 2015.**

CO-CURRICULAR ACTIVITIES

a) During B. Tech. (Biotech.) Course in JUIT:

- Editor in Chief JUIT Youth Parliament – 2015.
- Editor in Chief- World Affairs - JUIT Model United Nations 2015.
- Certificate of Appreciation, Manager-Entertainment Training, Entrepreneurship and Development Conference (TEDxJUIT) "*Defining the Redefined*"–2014.
- Certificate of achievement for being *Editor-World Affairs* and *Best Press Correspondent* at JUIT Model United Nations, The Winter Summit, **18th -19th January, 2014**, having shown exemplary skills of oratory, diplomacy and crisis resolution.
- Certificate of recognition as *Correspondent* at JUIT Model United Nations Conference 2013 held on **16th -17th March 2013.**
- Certificate of appreciation for outstanding contribution as the **Member** for *Reverié* 2011-12 & 2012-13 the **College Magazine** of Jaypee University of Information Technology (JUIT).
- **Co-ordinator** JUIT Youth Club's Entertainment Club: 2014-2015.
- Certificate of appreciation for outstanding performance as **Deputy Co-ordinator** Entertainment Club for the session 2012-2013.
- Posters presented at National Conference on *"Perspectives & Trends in Plant Sciences and Biotechnology"* held from **21st-23rd February, 2014** organized by Department of Botany, Panjab University, Chandigarh and Society for Plant Research, India at Department of Botany, Panjab University, Chandigarh.
- Participant in *"Biorhythm"* organized by The Department of Science and Technology in S.D. College Chandigarh 2013.
- Winner of third prize in Poster making Bio Rhythm February 11th-2014.
- Certificate of participation in *"IP Connect"* a workshop on *"Intellectual Property Rights"* organized by IP Cell, JUIT, Waknaghat, on **3rd August, 2013**.
- Resource Person during *21st National Children's Science Congress-2013 (NCSC-2013)* from **27th- 31st December, 2013** at M.P. Council of Science & Technology (MPCOST) Bhopal, Madhya Pradesh, India.
- Member of Synapse - The JUIT Biotechnology Club 2011-2012, 2012-1013.

- 1st position in Start Rolling Camera Cut event in **Murious** Technical Festival JUIT during **1st – 3rd, February, 2013**.
- Certificate of merit for 2nd position in Balloon Brawl ever ***Le-Fiestus*** 2012 held on **22nd - 24th March, 2012.**

b) During Senior Secondary & High School:

- Bonafide Student of Dayanand Public School (DPS), The Mall, Shimla, Himachal Pradesh-171003 from **16th April, 2009 to 23rd May,** 2011. Website : www.dpsshimla.org; e-mail dpsshimla01@gmail.com
- Bonafide Student of Loreto Convent School Tara Hall, Shimla-171003 (H.P.), India from **8th March, 1999 to 3rd April 2009**. Website: www.loretoshimla.org; e-mail: contact@loretoshimla.org
- Certificate of Merit among the top 0.1% of successful candidates of All India Senior Secondary Certificate Examinations 2011 in **Physical Education** by CBSE Board 2011.
- Participated and volunteer in National Service Scheme (NSS), Ministry of Youth & Sports, NSS Unit, Dayanand Public School (DPS) for Environmental Education and Plantation activities during **2010-2011**.
- Participant in 12th& 13th National Science Olympiads held on **19th November, 2009, 2010.**
- House Captain (Nehru House) for the Academic Session of **2008-09**, Loreto Convent School, Tara Hall, Shimla.
- 1st prize of the 12th Dr. Mahbub - Ul - Haq Memorial Inter School Debate Competition **2009.**
- Winner of Inter School debate on "United Nations Development Programme & Rajiv Gandhi Institute for Contemporary Studies **2009**, (Best Speaker; supporting the Motion).
- Certificate of Appreciation for the participation in the "**Forest Aid Campaign to Maintain Ecological Balance in Shimla"** from **30th July to 6th August, 2008** organised by Department of Youth Services & Sports, Govt. of Himachal Pradesh.
- Member of the Loreto Green Eco-club under National Green Corps Programme for Environment Audit of School during **2006 and 2007**.
- Winner of 9th Annual Rotary Inter School Declamation Competition on Environment and Population held on **16th September, 2007**.
- Winner of 10th Annual Rotary Inter School Poster Painting and Slogan Competition.
- 2nd prize winner of Inter-State Paper Presentation Competition organized by the Ministry of Non-Conventional Energy Sources on *Rajiv Gandhi Akshay Urja Divas* on **20th August, 2007.**
- Participated in 5th Inter School Painting Competition on Drug Abuse and Illicit Trafficking organized by the District Shimla Police on **26th June, 2004.**
- General Proficiency from 1st to 8th Standard and Sustained Excellence obtained in English, Hindi, Geography, Environmental Education, Biology in 9th to 10th Standard Exams.
- Kindergarten and initial Primary education received in Aberdeen, Scotland, UK.

c) Countries visited:

- Visited United States of America (USA) from **December 10, 2010 to January 06, 2011** on J-2 Visa with my father under academic exchange programme of Fulbright Environment Leadership Fellow at USDA Forest Service, Northern Research Station, St Paul, Minnesota.

- Resident in Aberdeen, Scotland, United Kingdom for 4 years and two months **(January, 1995- February, 1999).**
- Visited France, Germany, Belgium, and Netherland (stayed for holidays for 9 to 15 days) during visit to USA & United Kingdom.

d) Language proficiency:

- English (Reading, Speaking & Writing - Excellent)
- Hindi (Reading, Speaking & Writing - Excellent)
- Sanskrit (Reading, Speaking & Writing - Good)

Declaration

I hereby declare that the above said details/information given above is true to the best of my knowledge and belief.