Genetic diversity of insectivorous bats in Uttar Pradesh

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Dedicated to my

Parents and

Teachers

DECLARATION

I hereby declare that the thesis entitled "**Genetic diversity of insectivorous bats in Uttar Pradesh**" submitted by me for the degree of **Doctor of Philosophy** in **Applied Animal Science** is the result of my original work carried out under the guidance of **Dr. V. Elangovan,** Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University and it has not been submitted for the award of any degree, diploma, associateship of any University or Institution.

Place: Lucknow Signature of candidate
Date: (Deep Narayan Prasad)

CERTIFICATE

This is to certify that the thesis titled "**Genetic diversity of insectivorous bats in Uttar Pradesh**" submitted by **Mr. Deep Narayan Prasad** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations – 1999 as amended in 2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: Supervisor

Head of the Department

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LIST OF ABBREVIATIONS

°	–	Degree
°C	–	Degree Celsius
µl	–	Microliter
µg	–	microgram
A	–	Angstorm
AFLP	–	Amplified Fragment Length Polymorphism
bp	–	Base Pair
BSA	–	Bovine Serum Albumin
dNTP	–	Deoxynucleotide triphosphates
EDTA	–	Ethlene Diamine Tetra Acetic Acid
EtBr	–	Ethidium bromide
Gs	–	Genetic similarity
ISSR	–	Inter simple sequence repeat
KDa	–	Kilo Dalton
Kb	–	Kilo base
$MgCl_2$	–	Magnesium Chloride
min	–	Minute
mM	–	Milli molar
Mg	–	Milligram
mtDNA	–	Mitochondrial DNA
Ml	–	milli liter
Mg	–	Milligram

N – Number

NaCl – Sodium chloride

$_{n}$g – Nanogram

P mol – Pico mole

PIC – Polymorphic Information Content

PCR – Polymerase Chain Reaction

RAPD – Random Amplified Polymorphic DNA

RFLP – Ristriction Fragment Length Polymorphism

rDNA – Ribosomal DNA

SDS – Sodium docecyl sulphate

Sec – Second

SDS-PAGE – Sodium dodecyl sulphate -Polyacrylamide gel electrophoresis

SSR – Simple sequence repeat

tDNA – Transfer DNA

T_m – Melting Temperature

Taq – Thermus aquiticus

TBE – Tris Boric Acid

UPGMA – Unweighted Pair Group Method with Arithmetic mean

U – Unit

V – Volt

Introduction

Introduction

Bats are the second most diverse Order among mammals. They belong to the most abundant group when measured in number of individuals (Jones *et al.*, 2009). They display an exceptionally large ecological niches and diversity (Wilson and Reeder, 2005). Along with pterosaurs and birds, bats are the only mammals to have achieved powered flight. This unique biology has enabled them to be the most widely distributed groups of mammals (Kunz *et al*., 2003).

Nevertheless, Chiropteran fauna ranks the rarest animals with least significance because of quite low abundance of several species and ethology (Hulva *et al*., 2004). Bats are considered as key stone species, since they are important for proper ecological functioning and whose lose would affect in greater consequence for other species (Kasso and Balaskrishanan, 2013). Any alteration in their population greatly affects the ecological balance and thus they represent a wider scale impact on the biota of interest. In addition, Chiropterans are considered as bioindicators of environmental change, since their sensitivity to environmental variation such as habitat destruction, environmental pollution and global climate change significantly affect the level of diversity, development and behaviour of bats (Mann *et al*., 2003; Jones *et al*., 2009).

The evolutionary history of bats still remains unresolved. As far as the late 1980s, most researchers presumed that Chiroptera was monophyletic (having single ancestral origin) and all of them shared a common flying ancestor. However, evidence from morphology of the penis and nervous system led some of the researchers to propose that bats are actually diphyletic (having two ancestral origin) (Smith and Madkour, 1980; Hill and Smith, 1984; Pettigrew, 1986). So far the earliest known evidence of Chiropteran fossil is from Eocene epoch, even before 53 million years ago (Hooker, 1996). Due to scarcity of well-preserved fossil record, the Chiropterologists have found difficult to interpret about the origin of flight and their ancestors.

However, it is presumed that bat developed by the early Eocene, they must have originated in the Paleocene or mid late Cretaceous (Jepsen, 1970). The rationale commonly cited suggests that megachiropteran members of the family Pteropodidae are more closely related to dermopterans (flying lemurs) and primates than to echolocating microchiropteran bats (Pettigrew, 1986, 1995). This prediction created intense interest among mammal systematics, resulting in a large number of independent studies of bat relationships. Practically all of these analyses have provided strong support for bat monophyletic. Molecular data supporting chiropteran monophyly include morphological data from any organ systems (Simmons, 1994). DNA hybridization data, and nucleotide sequence from numerous mitochondrial and nuclear genes (Kirsch and Pettigrew, 1998, 1996, Allard *et al*.,1996; Murphy *et al*., 2005; Teeling *et al*., 2002). However, considerable uncertainty remains concerning the relationships of bats to other mammalian orders.

Presently, bats constitute about 1232 species, almost representing one quarter of the entire mammalian species (Schipper *et al*., 2008; Kunz *et al*., 2011). Regrettably, they are speculated as least concerned mammals in regards to their evolutionary past making a significant contribution to mammalian species richness and biomass (Goodman and Benstead, 2003). In about 119 species of bats constituting into nine families, i.e Pteropodidae, Rhinopomatidae, Megadermatidae, Rhinolophidae, Hipposideridae, Molossidae, Emballonuridae, Vespertillionidae and Miniopteridae reported in India (Bates and Harrison, 1997). The Order Chiroptera is divided into two Suborders, the Megachiroptera and the Microchiroptera (834 species). Megachiroptera is represented by only one family, the pteropodidae (167 species) of Old World tropics of Africa and Asia (Smith, 1977). The modern microchiropterans show considerable variation in form and structure. Among the microchiropterans, a large number of species are from Vespertilionidae, followed by Rhinolophidae, Hipposideridae, Emballonuridae, Molossidae, Rhinopomatidae and Megadermatidae.

About 75% species of bats are insectivorous. Along with powered flight and echolocation, flight has made it possible for bats to seek shelters in many different types of structures (foliage, tree cavities, caves, rocks, crevices) that are not used by terrestrial animals and exploit a variety of food sources. The diets of these bats include (e.g. insects, spiders, scorpions, crustaceans) small vertebrates (e.g. frogs, birds, fish and other mammals) and blood (Pierson *et al*., 1995; Novacek, 1985). They primarily feed on insects as food and each bat can consume several hundreds of insects in single night (Adams, 2003). They are primary pollinators of numerous tropical plants. More than 130 genera of trees and shrubs are already known to rely on bats for pollination. There are nearly endless list of valuable products from these plants includes many fruits such as peaches, avocados, as well as kapok and hemp fibers surgical bandages.

Genetic diversity of any species is frequently studied through identification and comparison the patterns of variability in morphology, ecology, and genetic elements. A large portion of species-level taxonomy is based on a narrow array of data types, such as morphology and mitochondrial phylogenies, leaving much of evolutionary diversity and complexity yet to be discovered. In Chiroptera molecular techniques have given new strength to systematic studies as well as to acknowledge concerning the evolutionary potential of populations and species (Wright *et al*., 1999). Analysis of genetic diversity and relationship between or within different species, populations and individuals is a central task for systematic similarity according to chromosomal studies (Ray-Chaudhuri *et al*., 1968; Qumsiyeh and Baker, 1985).

Genetic diversity refers to both the vast numbers of different species as well as the diversity within the species. It is hypothesized that the number of valid mammalian species yet to be discovered remains in thousands (Baker and Bradley, 2006; Clare *et al*., 2007; Francis *et al*., 2010; Clare, 2011). Comparing genetic structure, among co-distributed species may provide significant insight to the extent to which extrinsic and intrinsic factors interact to influence the scale of population differentiation or speciation event (Arbogast *et al*., 2001). It has been complicated to determine the exact identity of species that occupy a similar ecological niche and have very close morphological characters.

Traditional morphological groupings have recently been questioned based on molecular data (Mayer and Von Helversen, 2001). Similarly, within well studied genera, morphologically cryptic and genetically divergent species have been identified (Thabah *et al.*, 2006). As a result, most comparative studies seeking to identify evolutionary patterns within Chiroptera have been carried out without reference to explicit phylogenetic frameworks (Kurta and Kunz, 1987; Barclay, 1994; Hayssen and Kunz, 1993). Due to lack of genetic information, the difficulties associated with bats in the wild become extremely difficult.

Recent advances in molecular techniques have added new insights into studies related to organismic evolution and have revealed unexpected levels of diversity in many vertebrate groups (Meyer *et al*., 2001). Current patterns of genetic variation are used to infer historical events such as population expansions, past population selection from refugia and evolutionary relationship (Miller *et al.*, 2003). In Chrioptera, new characteristics are available as a result of molecular methods that have helped to give new strength to systematic studies as well as to acknowledge the concerning, evolutionary potential of populations as species (Wright *et al*., 1999; Ditchfield, 2000; Kawai *et al*., 2003; Hoofer *et al*., 2003; Newton *et al*., 2003; Van Den Bussche and Hoofer, 2004; Dávalos and Jansa, 2004; Porter and Baker, 2004; Ferreira *et al*., 2005). Paucity of genetic information is undoubtedly due, in part, to the difficulties associated with studying them in the wild.

Application of molecular genetic techniques extracts valuable biological information to document population structure of these species. It is a PCR based marker technique, requiring only tiny amount of genomic DNA and does not require expensive material as in molecular biology techniques like blotting and radioactive material (Maruyama, 1985). DNA markers based fingerprinting can distinguish species rapidly using small amounts of DNA and therefore can assist to deduce reliable information on their phylogenetic relationships. The most commonly used marker systems are random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) (Botstein *et al*., 1980), inter simple sequence repeats (ISSRs) and microsatellites or simple sequence repeats (SSRs) (Goodwin *et al*., 1997).

Random Amplified Polymorphic DNA analysis is rapid (Tingey, 1993). PCR based RAPD approach uses arbitrary primers that requires only nanogram quantities of DNA, no radioactive probes, and is relatively simple compared to other techniques (Welsh *et al*., 1990). The number of fragments amplified and the degree of polymorphism in eukaryotic species depend on the nucleotide sequence, the secondary structure and the number of primers used for each RAPD assay. This fingerprinting is widely used in conservation biology because of quick results, less expensive and reproducibility. DNA fingerprinting techniques are very useful to correct identification of taxa. For these reasons it is widely used across an extensive range of animal and plant taxa including bats (Karuppudurai *et al*., 2007).

These features of the RAPD assay make it a suitable molecular marker to detect DNA polymorphism in the absence of specific nucleotide sequence information. Thus RAPD-PCR method has been used successfully to detect genetic diversity within and between related species and populations of different organisms, making it possible to conduct molecular phylogenetic studies (Callejas and Ochando, 1998; Almeida *et al*., 2011; Mamuris *et al*., 2002; Prioli *et al*., 2002; Hassanien *et al*., 2004). Random amplified polymorphic DNA (RAPD) based detection of genetic polymorphism has been successfully utilized on its own and in combination with other molecular markers to identify isolates, genetic diversity and population structure to demonstrate genetic variation within the species and to elucidate the distribution of genes and population structure of the species. RAPD analysis is capable of detecting differences among strains of a single species (Welsh and Mc Clelland, 1990; Williams *et al*., 1990).

The simplicity and fast processing of RAPD technique makes it useful for assessing population genetic parameters such as within-population and between populations. An additional advantage is that no prior knowledge of the DNA sequences is necessary to apply this technique (Weising *et al*., 1995). RAPD markers have been used successfully for the analysis of cultivars and identification of species in most plants due to the simplicity and speed of the method. It generates many useful genetic markers for the analysis of genetic diversity and phylogenetic relationships in closely related groups.

DNA finger prints can be generated using short nucleotide sequences of primers and does not need any prior knowledge of sequence of DNA and reveals a high level of polymorphism. RAPDs were the first PCR-based molecular markers to be employed in genetic variation analyses (Welsh, 1990). The use of short primers is necessary to increase the probability that, although the sequences are random, they are able to find homologous sequences suitable for annealing. DNA polymorphisms are then produced by rearrangements or deletions at or between oligonucleotide primer binding sites in the genome.

As this approach requires no prior knowledge of the genome analyzed, it can be employed across species using universal primers. An important feature of this method on that the profiling is dependent on reaction conditions which can vary between laboratories, even a difference of a degree in temperature is sufficient to produce different patterns. Additionally, as several discrete loci are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001).

An excellent example is the low species-level resolution and current state of knowledge of the mammalian genus is low. This poor resolution is largely due to limited genetic studies focused on species-level variation. Moreover, morphological variation within the genus is low (i.e. cryptic variation) that limits the resolving power of classical morphological studies (Laval, 1973; Ruedi, 1990; Mayer, 2001; Stadelmann, 2007; Kunz, 2011).

The current genetic status of a species occurs mainly based on the interaction of historical events and geographical structure (Emerson *et al*., 2001). Recent estimates on the rate of global climate change suggest that many species may not be able to shift their range fast enough to track suitable conditions (Loarie *et al*., 2009), and therefore species survival will depend on phenotypic plasticity or adaptive capacity (Hoffmann and Sgro, 2011). However, despite the importance of genetic diversity for species persistence and adaptive capacity, genetic effects are often ignored in climate change studies (Pauls *et al*., 2013).

The natural changes in the genetic diversity of a species create a dynamic landscape upon which influences are superimposed. The genetic structure of natural populations can result from a number of interacting factors such as recent history, dispersal, mating system and group formation (Parreira and Chikhi, 2015). Genetic diversity is affected by several ongoing natural processes. These processes are natal dispersal (flow of gene) habitat destruction and degradation, mutation, migration, genetic drift and natural selection. When facing challenges from environmental changes or stresses, organisms have to adapt to the changed environment by genetic mechanisms, physiological adaptability, phenotypic plasticity or moving to a new, more suitable area. These genetic changes may support abundant phenotypic variation in organisms for environmental adaptation, but they occur slowly and cannot keep pace with the rapidly changing environment (Bonduriansky *et al*., 2012).

Recombination of nuclear genes and genetic diversity of temperate zone species, there is a high level of gene flow among bat populations (Worthington and Wilmer, 1996). Sometimes it is observed over longer distances as in the case of migratory *Nyctalus noctuls* bat species (Petit and Mayer, 1999). Strong gene flow was also observed among the brown long-eared bat *Plecotus auritus,* which is a non-migratory species that is spread over the western Paleartic. This advocates that all individuals are potential partners. There is no restriction in mating among them, neither genetic nor behavioural upon the population and therefore all possible recombination are possible (Entwistle *et al*., 1998, 2000). In the absence of any barrier (geographical, social, climatic etc.) to gene flow, genetic distances would be expected to increase with an increase in geographical distances within the same species, commonly referred to as isolation by distance (Rousset, 1997).

The genetic structure of discrete is strongly affected by the amount of dispersal (gene flow) that occurs among them and formation of new social groups (Perrin, 2000). Majority of the mammalian population seem to exhibit a social system characterized by polygynous mating and female philopatry (Greenwood, 1980). *Cynopterus sphinx* is also a polygyous where both sexes were found to disperse completely form their natal harems (Gopukumar *et al*., 2005). *Cynopterus sphinx* was reported to be closely related to each other at the genetic level although they seem to be distant geographically. This situation could be due to habitat loss or habitat fragmentation. This could be due to the surviving individual in the remaining location and the lifespan of the species allowed them to retain diversity for long periods after habitat loss (Greenwood, 1980; Perrin, 2000).

Among the populations of migratory species, genetic diversity appears universally low and hence seasonal migration is likely to be the prevailing influence. However, for sedentary species an array of factors, including dispersal ability, extrinsic barriers to gene flow may determine the extent of genetic partitioning among populations (Burland and Wilmer, 2001). Therefore, gene flow is expected to be greater when populations are closer, and as a consequence, nearby populations should be more similar at neutral loci. This relationship is referred to as isolation by distance and it assumes a stepping-stone model of gene flow, providing sufficient time for populations to reach equilibrium conditions (Kimura and Weiss, 1964). However, levels of gene flow are not only dependent on the distance between populations, but also on the nature of the surrounding landscape between populations (Gibbs, 2001).

Classically, swarming sites were thought to serve as information pools where juveniles gain knowledge about sustainable hibernacula (Fenton 1969; Von Helversen, 1989). However, anecdotal evidence occurs for sexual interaction in *Mycotis lucifagus*. The author argues that males and females of the given roosts meet at swarming sites for pair formation and sperm transfer (Thomas *et al*., 1979). The brown long- eared bats form different colonies were observed to meet and subsequently mate at swarming sites (extra colony mating), which results in gene flow among colonies and consequently in a relaxation of colonies genetic borders (Kerth *et al*., 2003). This signifies that males and females meet at swarming sites and the colony boundaries breakdown.

In particular, stable populations that persisted from the last glacial maximum to the present harbour disproportionately large amounts of unique genetic diversity (Hampe and Petit, 2005). Many species survived periods of glaciation in southern refugia near the Mediterranean, from where they colonized their northern range during warmer interglacial periods (Hewitt, 2000). Across the rest of the range, rapid climatic cooling and warming at the beginning and end of glaciation cycles were accompanied by a loss of genetic diversity and extinction of populations that were unable to track suitable conditions (Hofreiter and Stewart, 2009). These losses provide warnings of the potential effects of future climate change, especially as projected rates of future changed dramatically exceed the past rates under which the climatic niches of vertebrates evolved (Wiens, 2013).

Extant populations of bats show a strong genetic signature of range expansion from Mediterranean glacial refugia (Rebelo *et al*., 2012). This pattern is common for temperate biodiversity, ranging from plants to mammals, and has resulted in high concentrations of genetic diversity (Hewitt, 2000). Hence, the analysis presented here is likely to be relevant for many temperate organisms that were forced to contract away from the poles into glacial refugia. The longevity and slow reproductive rates of bats suggest that they may not be able to evolve fast enough to respond to future changes because of the slow spread of favorable traits through the population (Hoffmann and Sgro, 2011).

The effects of future climate change on biodiversity have been the focus of much highlighting the potential extent of global species losses and documenting changes to the distribution of species (Parmesan and Yohe, 2003; Thomas *et al*., 2004; Bellard *et al*., 2012). Despite the importance of this radiation of mammals, their evolutionary relationships have remained poorly understood until fairly recently (Simmons, 1998; 2000). The current genetic status of species occurs mainly based on the interaction of historical events and geographical structure (Emerson *et al*., 2001).

Yet, there is a paucity of studies investigating potential effects of future climate change on bats, and in particular potential genetic consequences (Sherwin *et al*., 2013). The few published studies to date show that temperate species are likely to experience distributional shifts and range contractions, with some species losing their entire suitable niche space (Rebelo *et al*., 2010).

Review of Literature

Rajan and Marimuthu (2006) worked on the genetic diversity of the Indian false vampire bat *Megaderma lyra*. They found that habitat loss and fragmented population had serious consequence on species extinction and genetic diversity within the species. They applied a molecular marker to observe the level of diversity between four populations of *M. lyra*. The obtained results suggested that genetic variation differed considerably form 0.21-0.26 with a mean of 0.11 to 0.13. The dendrogram generated obtained by RAPD data showed that the bats were not grouped by geographical origin. The genetic variation found in *M. lyra* populations could also be due to their breeding behaviours.

Attai *et al.* (2007) studied the changes in diversity to assess the genetic behaviuor and their relationships among *Rousettus aegyptiacus* and *Taphozous nudiventris*. Random Amplified Polymorphic DNA (RAPD) was carried out to study the genetic variation and similarity coefficients. Five arbitrary random primers (A1, A12, A3, A5, and A6) were used that generated visible bands ranging from 194-1353 bp. All primers gave polymorphic bands of different molecular weights. There experiments suggest that the bands of DNA for each species may be similar at few molecular weights with the primers used. By considering the number of RAPD bands shared between these two species and the total numbers of bands for each species, the genetic similarity obtained was 0.32 corresponding to low genetic similarity and large distance between them the degree of variation in their genomic DNA between them could be due to their ecological conditions and nutrition as well.

Lamb *et al.* (2006) studied the large-eared free-tailed bat *Otomops martiensseni* which is sparsely distributed throughout Africa. They carried out Random Amplification of Polymorphic DNA (PCR-RAPD) technique, to investigate evolutionary development diversification of a species in geographically distant populations of Kenya, Ethiopia and South Africa. PCR-RAPD data generated by the molecular marker showed slight nucleotide variation between East and South African populations (0.9%), with constant overlap of individuals between the colonies and maximum intra-colony divergence of (0.19%). They conclude that both nucleotide and haplotype diversity was higher in the southern than in the northeastern clade, that could be possibly due to differences in roosting biology (several dispersed home roosts in Durban versus single large cave roosts in northeastern Africa). The genetic data analyzed also may be due to a combination of high-altitude, long-distance flight capabilities of these species, migration events, and female philopatry resulting from stable harems.

Rossiter *et al*. (2000) studied that population of the endangered greater horseshoe bat *Rhinolophus ferrumequinum* that were highly reported to be fragmented. To assess the patterns of genetic diversity and variation because of habitat fragmentation and limited dispersal, they used a sophisticated molecular marker (microsatellite) to screen bats from around half of the known maternity colonies. Their results strongly implied that the colonies were genetically isolated from each other. These effects could also be either through lower variability from genetic drift, or the species colonization history. The results showed that gene flow among most neighboring colonies generally restricted, with one or few exception. Their findings could be important implications for the conservation management of this species.

Hua *et al*. (2013) studied the genetic variation that in evolutionary process and conservation genetics of lesser flat headed bat, *Tylonycteris Pachypus*. The genetic makeup of these populations depended on the patterns of landscape, permanent movement of individuals for genetic exchange, and the dispersal movement of their genes during temporary mating events. Microsatellite and mt. DNA molecular marker to assess the limits and nature of gene flow in the same population they utilized among the flat headed bats. Statistical data revealed significant genetic differentiation among the localities. Global spatial autocorrelation showed positive genetic correlation for up to certain distance, indicating the role of fragmented habitat and the barriers that alters the movement of individuals. Their findings also suggested that male biased dispersal pattern showed weaker spatial genetic structure between the colonies. Further finer scale analysis strongly confirms that interrelatedness levels within internodes were higher among females than males. The results confirm that that natal female philopatry were more related to the same sex roost mate than to individual for neighboring roosts.

Brown *et al*. (2011) studied the Mariana flying fox, *Pteropus mariannus* that suffered substantial decline in recent decades. Taxonomic classification of *P. mariannus* had been inconsistent, with subspecies designations based mainly on geography and morphological variation within small sample sizes. They examined relationships of *P. mariannus* across two island groups in the western Pacific Ocean. They used various molecular marker microsatellite, from D-loop, cytochrome oxidase I, and cytochrome b. There results implies that population on the islands of Palau is genetically isolated from the populations in the Mariana Islands. It further strengthens that the bats of Palau should be considered a separate conservation unit. Finally their findings concluded that there may be gene flow among island population within the Mariana archipelago. The results showed that the bats on these islands, currently classified into two subspecies. It also suggests that it should be managed as a single conservation unit. Although they refrain from suggesting that taxonomic revisions, until genetic and morphological data become available from geographically intermediate populations.

Mayer *et al*. (2001) studied the cryptic diversity of European bats *Plecotus austriacus austriacus* and *Plecotus austriacus austriacus kolombatovici*. It is clearly visible that different species of bat can be morphologically very similar. A molecular marker (NADH dehydrogenase I) used to estimate the hidden diversity of these European bats. They screened inter and intra-specific genetic variation in vespertilionid bat species from several individual of bat which form different geographical locations. There results showed a high divergence of mitochondrial lineages, in the two taxa which differed in at least by 11%. The results strongly indicate that convergent adaptive evolution might have contributed to the morphological similarity among distantly related species even if they occupy same ecological niches. However closely related species may differ in their ecology but does not necessarily in there morphology also.

Halczok *et al*. (2017) studied the evidence for genetic co-variation in Natterer's bat, *Mycotis nattereri* across regions in Germany. They used several advanced molecular techniques to analyze the genetic population size within and among the population at different geographical regions. Their results concluded that roosts can be grouped into three distinct genetic clusters, corresponding to the three geographic regions isolated. It was also seen that an overall isolation-by-distance pattern, with no major pattern was observed within a geographic location. Moreover, no correlation between the genetic distances among the bat populations was found. The genetic population structure of this bat suggests that mating sites where several local breeding colonies meet may act as a stepping-stone for gene flow. Nevertheless, high genetic similarity of some of the haplotypes found in several different regions implies that occasional transmission across regions does occur.

Moreira *et al*. (2006) worked on the genetic variability in species of bat *Molossus molossus*, *M. rufus*, *Eumops glaucinus*, *E. perotis*, *Myotis nigricans*, *Eptesicus furinalis*, and *Artibeus planirostris.* Random amplified polymorphic DNA (RAPD), molecular marker was utilized as a means of analyzing genetic diversity. Determination of genetic diversity was based on bands produced by random primers. Nei’s genetic diversity index of all species was considered. Nei’s unbiased genetic distance values and the UPGMA clustering pattern show that *M. molossus* and *M. rufus* have a close genetic relationship, unlike that observed between *E. perotis* and *E. glaucinus*. They clustered with *A. planirostris* and *E. furinalis*. The results showed low values for genetic diversity and the distance observed indicated a means of genetic conservation of all the species studied.

Karuppudurai *et al*. (2007) studied the genetic diversity among the 40 different zones in the Indian short-nosed fruit bat, *Cynopterus sphinx*. RAPD- PCR technique was carried out to find the level of polymorphism at the molecular level. RAPD-PCR with 30 random primers was applied to the 40 zones samples of different geographical regions. The genetic similarity displayed by dendrogram between the zones ranged from 0.42 to 0.92. The results showed that considerable genetic diversity was observed in species from different zonal populations, the results also implies that there might be possibly due to complete dispersal of juveniles of both sexes from their natal groups and gene flow between the zones. Hence it suggests that it is not only a predictive framework for future studies, but also to use the genetic data in the management and meaningful conservation of this species.

Tereba *et al*. (2011) studied the polymorphism level by using the microsatellite in a greater false vampire bat, *Megaderma lyra*. They used new microsatellite markers. Of the 10 loci produced, only one locus showed statistically significant, and one was found to be X-linked. All these loci were tested in 189 individual representing four populations from southern India, and all the loci were found to be highly polymorphic. Expected heterozygosity values ranged from 0.54 to 0.91. Their results suggest that these polymorphic markers could be used to examine population structure, mating and dispersal behaviour of *M. lyra* in the field.

Kanagaraj *et al.* (2010) studied the genetic variation and population structure of the leaf-nosed bat, *Hipposideros speoris*. They estimated using 16S rRNA sequence and microsatellite markers. They analyzed about twenty seven distinct mitochondrial haplotypes which were identified from 186 individuals, isolated from eleven populations. In addition, haplotype network and analysis of molecular variation analysis consistently suggest the prevalence of genetic structure in the isolated populations. However, the Mt. DNA data was not significantly different in few closely located urban populations of bats, but there were significant difference observed with the use of molecular data. Clustering analysis identified eight clusters among the populations; the clustering pattern also suggests to the haplotype networks. Overall, the study also suggests a macrogeographic genetic isolation-by-distance and possibility of gene flow among closely located populations.

Levin *et al*. (2008) studied genetic and phylogeography of the greater mouse-tailed bat, *Rhinopoma microphyllum*. They covered a large geographical range covering most of the arid and warm areas. Genetic variability was carried out of this species using two mitochondrial markers, from several colonies and from over most of the species range. Their results signify that the cytochrome *b* sequences, unlike those of the control region, are much conserved to separate among *R. microphyllum* populations. Three clades were seen over the species zone. Intermediate and Palaearctic. This sort of separation advocates most of the traditional taxonomy of the species. The Israeli population, which belongs to the Palearctic clade, was seen to be isolated from the Oriental and Intermediate clades. We suggest that the colonization of the greater mouse-tailed bat in the Levant occurred from African populations during the late Pleistocene, when many Saharan animals penetrated the northern part of the Great Rift Valley.

Fox *et al*. (2012) studied the genetic differentiation in the cryptic bat. They assessed that during the late Pleistocene there was a reduced habitat available to organisms that depended on forest landscape. The bioclimatic modeling of rainforest in northern Queensland indicated the region was subject to massive change during quaternary glaciations. The results of supported that rainforest bats were severe especially for organisms with limited mobility or adaptability. It also indicated that present day regional scale genetic structure in the speculated bat generally assumed to be a rainforest specialist and behalf of this modern day processes, it may provide understanding responses of an extremely mobile animal to habitat contraction and fragmentation.

Vanhof *et al*. (2015) studied the genetic approaches of conserving migratory bats. They figured out those fatalities at wind turbines that had serious concern about the future impacts of increased power stations of populations of migratory bat species. They utilized the genetic data of *Lasiurus boreialis* that are one of the highly most affected by wind power. To evaluation the population structure across the landscape and decline in population size, molecular marker techniques like mitochondrial and nuclear DNA were used. There results suggested that these species forms a single, panmictic population across their wide range with no evidence of divergent migratory pathways by any portion of the population. It also showed a high level of gene flow and connectivity across the populations of eastern red bats indicating and integrating information across the range of these species.

Asher (2009) studied the patterns of genetic diversity in the populations of *Sturnira ludovici* and *Artibeus toltecus*. They examined whether habitat disturbance, are primarily due to the cultivation. They carried out genetic diversity using molecular marker approach as a measure of population health. Genetic diversity was assessed using inter simple sequence repeats, a technique similar to random amplification of polymorphic DNA (RAPD). They measured various habitat variables including foliage height diversity, fruit availability. They found that fruit availability differed significantly between the two localities, with the buffer zone having higher values for both. Despite these differences in habitat, they found no significant differences in the level of genetic diversity between the two locations for either species bat. This could be because of effective population size of the bats that do not differ significantly between the sites. There results suggested that there was a lag between disturbance and population decline because migration is more frequent to homogenize allele frequencies between the localities studied.

Carstens *et al*. (2004) worked on population genetic structure in phyllostomid bats *Ardops nichollsi*, *Brachyphyllac avernarum* and *Artibeus jamaicensis*. These island populations are genetically differentiated. They hypothized that island populations are genetically distinct because of a number of combination of founding events, limited levels of migration and genetic drift or by catastrophic-induced fluctuations in population size is derived from a priori hypothesis erected. There results predicted that within each species in island populations are monophyletic which was even verified using a parametric bootstrap approach. There results also, supported that molecular variance is partitioned among islands. A conservative approach of the molecular data is that island populations of *A. jamaicensis*, although isolated geographically are not isolated genetically.

Ali (2011) studied the molecular phylogenetic relationship between and within the fruit bat, *Rousettus aegyptiacus* and the lesser tailed bat, *Rhinopoma Hardwickii*. RAPD-PCR was carried out to determine the genetic variation within and among two bat species, *R. aegyptiacus* and *R. hardwickii.* A total of 39 bands were amplified by three primers with an average 13 bands per primer at molecular weights ranged from 1409 to 107 bp. The polymorphic loci between both species were with percentage 87.18 %. The numbers of monomorphic bands in *R. aegyptiacus* and *R. hardwickii* were 14 and 9 bands. Cluster analysis indicated that, the two genotypes were separated into two clusters. Their results showed more variation among members of *R. hardwickii* in comparison to those of *R. aegyptiacus*. It can be concluded that, the similarity coefficient value between the two bat species may have the same origin but are not identical and separated into two clusters.

Larsen *et al*. (2013) studied the genetic diversity of Neotropical (Chiroptera: Vespertilionidae). They studied the morphological variation in the Chiropteran genus that limits the understanding of species boundaries and species richness within the genus. They carried out an assessment of the genetic diversity in New World by analyzing cytochrome-b gene. Their results provided a baseline genetic data for researchers investigating phylogeographic and phylogenetic patterns of bats in these regions. They used a molecular marker Cytochrome-b that generated and phylogenetically analyzed from 215 specimens. Their findings provide evidence that the perception of lower species richness in *Myotis* is largely due to a combination of cryptic morphological variation. Further the results also imply that more accurate assessment of the level of diversity and species richness is needed. However is not only helpful for delimiting species boundaries, but also for understanding evolutionary processes within this globally distributed bat genus.

Wilson *et al*. (2014) studied the DNA barcoding for rapid and accurate assessment of bat diversity. They incorporated DNA barcoding into surveys that revealed numerous species-level taxa overlooked by conventional method previously. Their results discuss whether a barcode library provides a means of recognizing and recording these taxa across biodiversity inventories. The nuclear DNA was extracted and the COI barcode region amplified and sequenced. They identified 9 species-level taxa within our samples, based on analysis of the DNA barcodes. Further their study confirms that the high diversity of bats within Peninsular Malaysia and DNA barcoding allows for inventory to document known taxa lacking formal taxonomic status.

Seim *et al*. (2013) studied genome analysis that revealed insights into physiology and longevity of the Brandt's bat *Myotis brandtii*. They studied genome and transcriptome, which suggested that adaptations consistent with echolocation and hibernation, as well as altered metabolism. Their research also confirms that unique sequence changes in growth hormone and insulin-like growth factor 1 receptors. The data also highlights that an altered growth hormone/insulin-like growth factor 1 axis, which may be common to other long lived bat species, together with an adaptations such as hibernation and low reproductive rate, contribute to the exceptional lifespan of the Brandt's bat.

Francis *et al*. (2010) studied the role of DNA barcodes in conserving mammalian diversity (chiropteran) in Southeast Asia. They examined the value of DNA barcode, sequences of mitochondrial COI gene, to enhance understanding of mammalian diversity in the region and to aid conservation. Their findings suggested that DNA barcodes obtained from various species including 165 species of bats. It is currently recognized that species contains multiple barcode lineages, often with deep divergence suggesting unrecognized species. However most widespread species showed substantial genetic differentiation across their distributions. Their results suggested that bat species richness within the region may be underestimated across the distributions and there are higher levels of endemism and greater intra–specific population structure than previous recognized.

Wang *et al*. (2003) studied five species of Hipposideridae and seven species of Rhinolophidae. The molecular phylogenetic of hipposiderids were based on mitochondrial cytochrome *b* sequences. Analysis of cytochrome *b* sequences of

Hipposideridae and Rhinolophidae suggest that each formed a monophyletic group. The genetic distance values indicate that *H. larvatus* and *H. armiger* diverged from each other approaximately 1.7 – 4.3 million year ago, and *H.pratti* diverged from the larvatus-armiger clade approximately 2.1 – 5.2 million years ago.

Haig (1998) found that several molecular methods could be used collectively to contribute to molecular conservation. Random amplified polymorphic DNA (RAPD), MHC, minisatellite, microsatellite, procedures allow for identification of percentage, more distant relatives, founders of new populations, unidentified indivuduals, population structure, effective population size, populations specific markers etc. PCR (polymerase chain reaction) that provide more sophisticated analysis of metapopulation structure, delineation of species, subspecies, and races, all of which aid in setting for species recovery priorities. RAPD technique can be powerful on its own but is most credible when used in conjunction with other molecular techniques and, most importantly, with ecological and demographic data collected from the field. The author suggests broad range of cases in which molecular techniques have been used to provide insight into conservation effort.

Sinclair *et al*. (1996) studied the genetic diversity of the Red flying fox *Pteropus Scapulatus*. Allozyme electrophoresis and Random Amplified Polymorphic DNA (RAPD) were carried out to determine the genetic diversity within and among six populations form widely separated locations on the continent. Both the allozyme and DNA techniques showed very little genetic structure among the subpopulations samples. Analysis of molecular variance on the RAPD data showed only 5% of variance among the populations. Although this difference was shown to be significant. The result obtained that the level of gene flow detected by these genetic analysis indicates that *P. scapulatus* is effectively panmictic. This assumes that there is no mating restriction, neither genetic nor behavioral.

Giannini and Simmons (2003) studied the phylogeny of megachiropteran bats that had been investigated using several several molecular markers, collected from 5 loci i.e (12S rDNA, 16S rDNA, tDNA-valine, cytochrome b, and the nuclear gene c-mos) for (n = 43) species of megachiropterans and six microchiropteran species. Analysis of individual loci suffer 70% loss in the number of compatible groups recovered across all analyses with respect to combined analyses. Their findings indicate that megachiroptera and all recognized genera, including *Pteropus* are monophyletic, and that melonycteris is the sister group of the clade containing all the other genera. The clades previously proposed using molecular markers, as well as many new and traditional groups, were well-supported, there results also indicates that indicate that, within the megachiroptera, nectarivory and cave-dwelling originated several times, but echolocation evolved only once.

Hulva *et al*. (2004) investigated the molecular analysis of *Pipistrelle pygmaeus* Species. Although they are almost identical morphologically. They performed phylogenetic analysis based on a 402 bp portion of the cytochrome b gene. They concluded that distinctive allopatric haplotypes from the two regions. The difference of about 6 – 7% in the population suggests the occurrence of a new species in the Mediterranean region. The phylogeographic patterns obtained and analysis of fossil records supports the hypothesis of expansion of both species into Europe from the Mediterranean region during the Holocene. The allopatric speciation model fits their data best. The paleobiographic scenario observed is corroborated also by molecular clock estimations and correlations with environmental changes in the Mediterranean region.

Vonhof *et al*. (2008) worked on patterns of genetic diversity within and among maternity roosts of *Eptesicus fuscus* using microsatellites and mitochondrial molecular markers. Their objective was to explore the genetic linkage between the temperate bats, and finding maternity roosts represent philopatric females connected to other maternity roosts via male-mediated gene flow. They examined critically the patterns of matrilineal diversity within and between roosts, and clear evidence of strict philopatry. Their findings suggest that no differentiation among colonies using nuclear microsatellite markers were reported. They observed groups of colonies were identified in analyses of molecular variance, among which variation was high (21.2%), but within which variation was low (1.4%), and significant matrilineal isolation by distance was seen. They reported that maternity colonies were characterized by high haplotype diversity and a large number of matrilines (5 – 15) per colony. Even a small number of maternities within colonies, and only 5 – 17% females were found roosting with a mother or daughter. Finally the outcome of their study confirms that strict female philopatry are not constant to the theory earlier reported. The potential for female dispersal in temperate bats prompts new and exciting questions about social and dispersal dynamics.

Koubínová *et al*. (2013) workerd on genetic diversity of vespertilionid bats. They collected (n = 213) vespertilionid bats and karyotyped with multi-locus sequence data and analysed with maximum likelihood and Bayesian methods. They analyzed and concluded that (80%) representatives were of closely related groups. The systematic position of several taxa differed from previous studies and the tribes Pipistrellini and Vespertilionini were redefined. The subfamily Scotophilinae was confirmed as one of the basal branches of Vespertilionidae. Their findings suggested that, still new taxa and new systematic arrangements are to be resolved in the vespertilionid bats.

Levin *et al*. (2008) worked on the genetic diversity on *Rhinopoma microphyllum*. They implemented the study by using two mitochondrial markers (the cytochrome *b* gene and the control region) from several colonies. Their outcome confirms that the cytochrome *b* sequences, unlike other control regions, are too conserved to be separated among *R. microphyllum* populations. High level of sequence similarity was found within the populations. Three clades were recorded over the species range: Oriental, Intermediate and Palaearctic. This division supports most of the traditional taxonomy of the species. The population, which belongs to the Palaearctic clade, was found to be isolated from the Oriental and Intermediate clades. Their results suggested that the colonization of *R. microphyllum* bat in the Levant occurred from African populations during the late Pleistocene, somewhere when many Saharan plants and animals penetrated the northern part of the Great Rift Valley.

Russell *et al*. (2005) studied the genetic variation and migration of *Tadarida brasiliensis*. They used mt. DNA sequence and allozyme to evaluate hypotheses regarding the relationship between migration and genetic structure. From their work, they assumed that the migratory behavior in bats is associated with structured gene pool. Finally they concluded that there were no significant genetic structural changes related to migratory behavior. Their analysis clearly states that it do not take such processes into account (migratory group, demographic analysis) can lead to false conclusions regarding a species phylogeographical history**.**

Trujillo *et al*. (2009) studied the molecular phylogenetic pattern of the genus *Scotophilus* form maternally and paternally inherited genomes. They carried out using (mt.DNA) and Y-chromosome (paternal) sequence data from (n =11) bats. There findings indicate that *S. kuhlii* and *S. heathii* were of two separate continents and embedded by a complex biogeographical multiple continental exchangers. Study also confirms that, Malagasy taxa are most closely related to 2 different African species, suggesting independent colonizations from the continental mainland. The measurements of interspecific genetic distances ranged from 4.2% to 19.2% for mt.DNA data and 0.18% to 2.14% for Y-chromosome shows that members of the genus *Scotophilus* are highly divergent from one another.

Chen *et al*. (2006) studied the genetic diversity and demography of the endemic *Rhinolophus monoceros* species. Their objective was to assess the population genetic structure and colonization history of this species. They used mitochondrial DNA control region in (n=203) bats sampled at 26 different sites. They observed very high haplotype and nucleotide diversity, which decreased from the center to the south and north. They followed a pattern of isolation by distance, though most regional genetic variance. Mismatch distributions used confirms a past population expansion predating the last glacial maximum, and a neighbour-joining tree showed that *R. monoceros* formed a monophyletic grouping with respect to its sister taxa. Finally they suggested that t that this taxon arose from a single period of colonization. Even the genetic structure reflects limited gene flow, probably coupled with stepwise colonization in the past.

Roehrs *et al*. (2010) used nuclear marker and Mt. sequence data to reveal the phylogenetic analysis in vesper bats. In the present study, they used 8,500 nucleotide base pairs of digenomic DNA for (n = 111) taxa were sequenced and analyzed using maximum-parsimony and phylogenetic methods to construct evolutionary relationships in Vespertilioninae. Their findings confirms the monophyletic nature of Vespertilioninae with the exclusion of *Myotis* and support recognition of six tribes, and two new unnamed tribal clades, further the tree topologies indicates a *Nycticeiini – Eptesicini* group, but this clade is not supported. The results provided further supports the greater resolution for previously proposed hypotheses of Vespertilioninae evolution based on mitochondrial DNA, although deep branching patterns are not fully resolved, these data increase our understanding of the evolution of this ecologically important and diverse group of bats.

Kruskop *et al*. (2012) studied the genetic diversity of palaearctic bats through DNA barcodes. To assess the pattern of genetic diversity, they used DNA barcode region of the cytochrome oxidase subunit I gene (COI) obtained from 38 bats. The outcome of their work supports the earlier findings of deep phylogeographic splits in four pairs of vicariant species *Myotis daubentonii M. nattereri*/ *bombinus*, *Plecotus auritus*/ *ognevi* and *Miniopterus schreibersii*/ *fuliginosus*. Their work also suggested that DNA barcodes confirms that all taxa raised to species rank in the past 25 years and confirms an additional species *Myotis sibiricus* should be separated from *Myotis brandtii*. The present study also supports that utility of DNA barcodes as a taxonomic assessment tool for bats.

Salazar *et al*. (2018) studied the genetic diversity in tropical bats in relation to ecological function. In the present study they used D-Loop gene of random samples collected from (n = 21) bat species. The objective was to study the clustering pattern of species based on nucleotide variation, genetic distances. They found that genetic differences between the, localities, and species. About 20 species analyzed maintained genetic diversity. The results confirm that genetic distances, with species with the same ecological function shared a greater number of nucleotide substitutions, with some exceptions. Thus our findings confirm that no association between the diversity of the D-Loop gene and ecological function was observed. Nonetheless, it also shows the importance of bat species richness and genetic diversity.

Sayed (2011) worked on the phylogenetic analysis between *Rousettus aegytiacus* and *Rhinopoma hardwickii*. To find the phylogeny between them G-banding technique and chromosomal SDS-PAGE were implemented. They displayed obvious alternations of white and dark bands. This facilitated ideogram construction. The molecular weight of the bands ranges from 118-26 kDa and from 118-24 kDa in the *R. aegyptiacus* and *R. hardwickii,* respectively. Bands of molecular weight 70,60,50,42,36,31,29 and 27 kDa are characteristic to the *R. aegyptiacus* and are missed in the *R. hardwickii.* Also, bands of molecular weight 113,105, 65 and 24 KDa are characteristic to the *R. hardwickii*. His work confirms that unique band at molecular weight 113 is characteristic to the *R. hardwickii*. The statistical analysis also confirms that degree of similarity between the two species is only (9.5%). As a result, *R. aegytiacus* and *R. hardwickii* are separate species.

Lack *et al* (2010) attempted to find the confounding factors related to phylogenetic relationships within Vespertilionidae. In the present study they used mt.DNA 12s rRNA, tRNAVAL, 16s rRNA, and the nuclear exon RAG2. Study confirms that the data of all subfamilies and tribes within Vespertilionidae and Phyllostomidae, radiated at approximately the same time as Vespertilionidae. They found that substitution rates for Vesper bats were significantly higher than those for Phyllostomid bats, with the fast-evolving lineages found within Vespertilioninae. The cladogram confirms that vespertilionid radiations were compressed toward the root, with a rapid initial diversification, while phyllostomid diversification was much more gradual rate.

Study sites in Uttar Pradesh

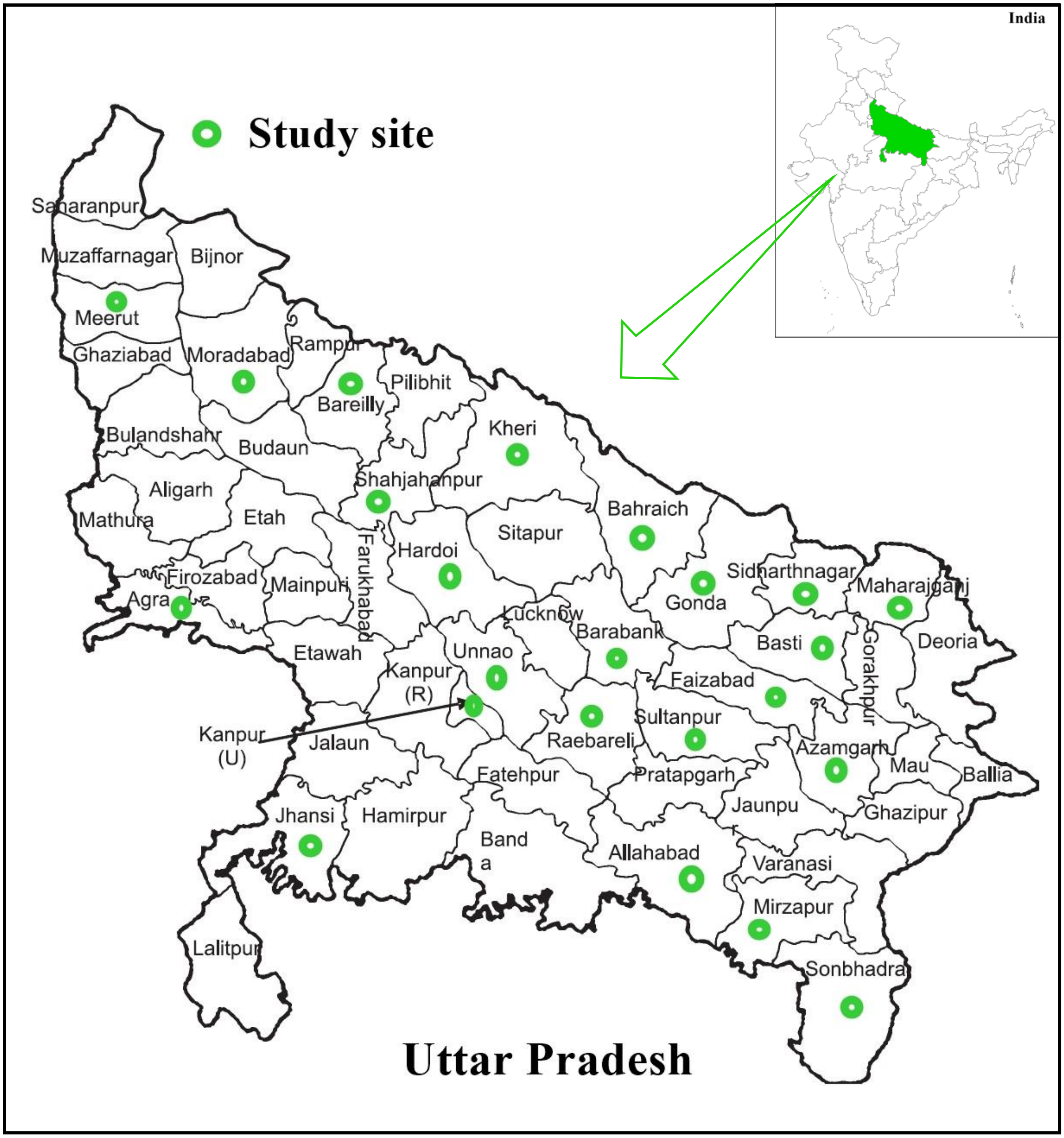

Figure 1. Map of study sites in Uttar Pradesh.

Chapter I

Genetic diversity among the populations of *Rhinopoma hardwickii*

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INTRODUCTION

Nowadays, molecular genetic techniques have turned to be a noble approach to reveal population genetics and evolutionary ecology. Rhinopomatidae bats are represented by single genus and regarded as most primitive (Koopman *et al*., 1993; Eicki *et al*., 2005). Despite the fact that almost all molecular experts have challenged to characterize mitochondrial DNA, the RAPD still holds the best marker that gathers maximum information about many vertebrate species, chiefly concerned with the evaluation of genetic diversity. Genetic markers have the potential to easily detect the level of variation of any population. Application of RAPD markers have been largely used to assess the patterns of descent within the organisms (Cornuet *et al*., 1999). RAPD data, produced by Polymerase Chain Reaction (PCR) have been routinely used since 1990's to study the differences within the species at the chromosomal level (Garrigan *et al*., 2002). It extracts all biological information from the population complexity of the species. Usage of this technique in population biology and the processes that governs them to exist is now prevalent, which is extensively practiced and bats are no exception. PCR-based RAPD uses a 10 decamer primer with nonogram amount of template (Welsh, 1990; Williams, 1993). Many investigators have reported that application of RAPD are beneficial to discriminate and resolve the differences between individuals or populations of bats (Moreira and Morielle-Versuate, 2006; Karuppudurai and Sripathi, 2007). The identity of any species as discrete (cryptic) species is a landmark in population biology.

The RAPD analysis was performed in order to characterize the genetic diversity among five populations of *Rhinopoma hardwickii* in Uttar Pradesh.

MATERIALS AND METHODS

Sample collection

The bats were trapped using hoop net with an extensible aluminum pole. A 6m x 9m Nylon mist-net (Avinet-Dryden, USA) was also used for trapping the bats. A small portion of patagium tissue (3 sq. mm) from each pieces was collected using a sterile biopsy-punch (patagium got repaired on its own within 15-20 days). The samples were collected from five different locations of Uttar Pradesh, namely Jhansi Fort, Jhansi (JHS [1], 25° 27′27.28″ N; 78° 34′ 32.23″E), Gupt Godavari, Chirtakoot (CKTD [2], 25° 05′24.09″N; 80° 46′.02.38″E), Baba Vasil Shah Sulemani Tomb, Chunar (CAR [3], 25° 06′55.95″N; 82° 52′09.23″E), Buland Darwaja, Fatehpur Sikhri (FTS [4], 27° 05′29.33″N; 77° 39′48.11″E) and Faizabad Maqbara, Faizabad (FBD [5], 26° 46′18.61″N; 82° 08′33.86″E). After each collection of samples, the punched patagium and the biopsy punch were sanatized with 70% ethanol. Bats were handled with extreme care and not scarified. Precautions were also taken in capturing them to minimize the possibility of stress and accidental fatalities.

DNA isolation and primer screening

The tissue samples were homogenized using mortor and pistle containing 100 µl of lysis buffer 1% SDS, 10 mM Tris-HCl (pH 8.5), 125 mM NaCl, 5 mM EDTA and 0.5 mg/ml proteinase K (Sigma Aldrich). Followed by homogenization, the DNA extraction was carried out by following phenol:chloroform method (Sambrook *et al.*, 1989). Both the quality and quantity of extracted DNA were confirmed using 0.7% agarose gel electrophoresis and spectrophotometrically at A260 and A280 nm (Perkin-Elmer USA).

Polymerase chain reaction

RAPD-PCR was designed using a set of five primers such as OPA-02, OPA-03, OPB-08, OPB-18, and OPD-02. Conditions for amplification were set by following Williams *et al*. (1990). Optimization was done by taking different concentrations of template, primer, $MgCl_2$, and *Taq* DNA polymerase in order to avoid primer dimer (Banglore Genei, India). Primer optimization was also done by calculating the (T_m) value and annealing temperature. The reactions were carried out in a total volume of 15 µl containing 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 50 pmol of a single decamer primer (Banglore Genei, India), 0.5 µg/mL BSA, and 1 U *Taq* DNA polymerase. PCR was carried out in a thermocycler (Gene Amp PCR system 2400, Perkin Elmer) programmed at 94 ºC for 3 min followed by 43 cycles of 30 sec at 94 ºC, 1 min at 35 ºC, and two min at 72 ºC. Finally at last cycle, there was a 5-min of extension at 72 ºC. Initially investigation was carried out with all 13 primers using genomic DNA from five different locations. RAPD-PCR analysis was operated twice so that only primers that gave strong bands were used in the investigation of all the five locations. Gels with amplification fragments were visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad).

RAPD Profile analysis

NTSYS-pc version 2.0 software was exploited to evaluate the RAPD data. All calculations related regarding genetic similarity (GS) was done with the help of Jaccard's coefficient of similarity GS(ij) = a/(a+b+c). GS(ij) signify the amount of genetic similarity between the species i and j, b denotes the frequency of bands present in the i while absent in j, whereas c indicates the number of bands present in j while absent in i, respectively. Each RAPD patterns was considered as a single character and was scored as 1/0. This binary matrix was constructed for all the bands scored to generate similarity coefficients. Finally the dendrogram using UPGMA was created by data provided by Jaccard's coefficient. The Polymorphism information content (PIC) a measure of informativeness of these RAPD markers was calculated by following Botstein *et al*. (1980).

Plate 1.1. A colony of *Rhinopoma hardwickii* at Kakori, Lucknow.

Plate 1.2. A fully stretched mist net at sampling site.

Plate 1.3. The frontal view of *Rhinopoma hardwickii*.

Results

Among 13 primers screened with the samples collected from five different zones of Uttar Pradesh, most polymorphism were obtained with OPA-02, OPA-03, OPB-08, OBP-18, OPD-02 as shown in Table 1.1.

Table 1.1. List of primers, nucleotide sequences and percent GC content.

S. No.	Primer	Sequence (5'-3')	% G+C content
1	OPA-02	GGACCCAACC	70
2	OPA-03	AGTCAGCCAC	60
3	OPB-08	GTCCACACGG	70
4	OPB-18	CCACAGCAGT	60
5	OPD-02	GGACCCAACC	70

These short oligonucleotides of random sequence gave different level of polymorphism. The primers OPB-08 and OPA-02 were more reproducible and produced a more distinct RAPD profile than primer OPA-03, OPB-18 and OPD-02. A sum of 51 bands were generated using these primers and among them 29 bands (56.86%) were found to be polymorphic and the 22 bands (43%) were found to be monomorphic in nature. The Jaccard’s coefficient similarity matrix constructed among the five populations was presented in Table 1.2.

The similarity coefficients values among the populations of *R. hardwickii* ranged from 0.60 to 1.00, indicating that the two zones did not show 100% similarity. UPGMA clustering of RAPD data indicated that the five zones of the *R. hardwickii* population comprise two different clusters. A dendrogram analysis indicated that the five zones formed two major clusters, A and B. Cluster A comprised of two populations namely (JHS and CKTD). Cluster B is further divided into two sub group representing population (FTS) and (FBD) while in Cluster B population (CAR) consisted of only segregated genotype, as shown in Figure 1.1.

Table 1.2. Jaccard's coefficient among five morphotypes of *Rhinopoma hardwickii.*

	1	**2**	**3**	**4**	**5**
1	1				
2	0.70	1			
3	0.62	0.60	1		
4	0.72	0.66	0.66	1	
5	0.60	0.64	0.68	0.82	1

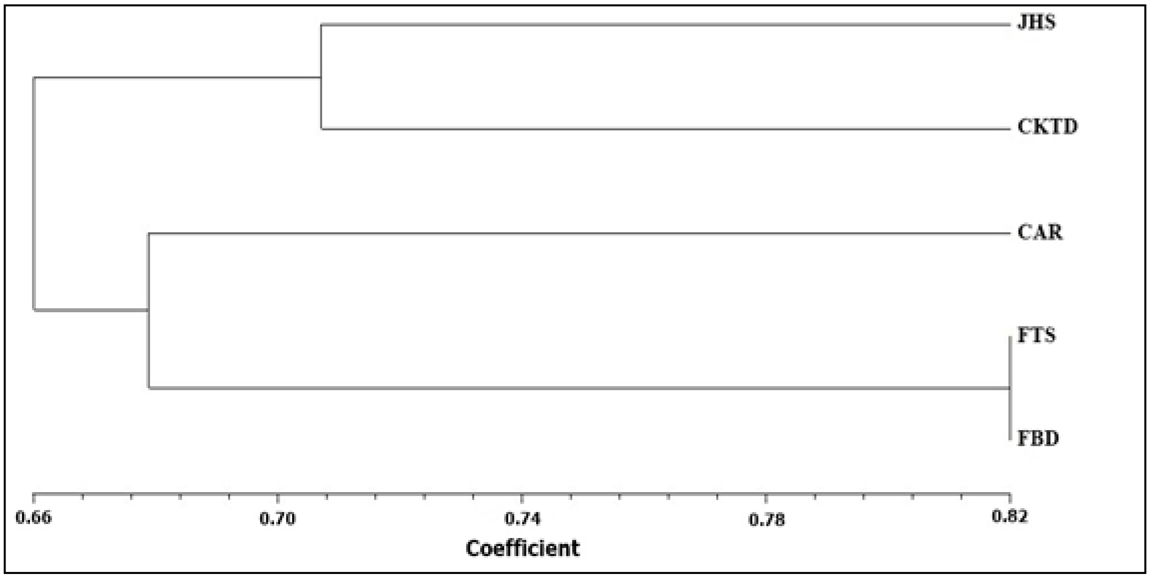

Figure 1.1. UPGMA based dendrogram showing the phylogenetic relationship among five populations of *Rhinopoma hardwickii*.

Maximum similarity was observed between zones (FTS) and (FBD) clustering together with a value of 0.82 and zone (CKTD) and (CAR) were found to be clustering separately and with a minimum similarity value of 0.60. The RAPD profile generated from these primers was utilized to estimate the gene frequency as shown in Figure 1.2 (a – e).

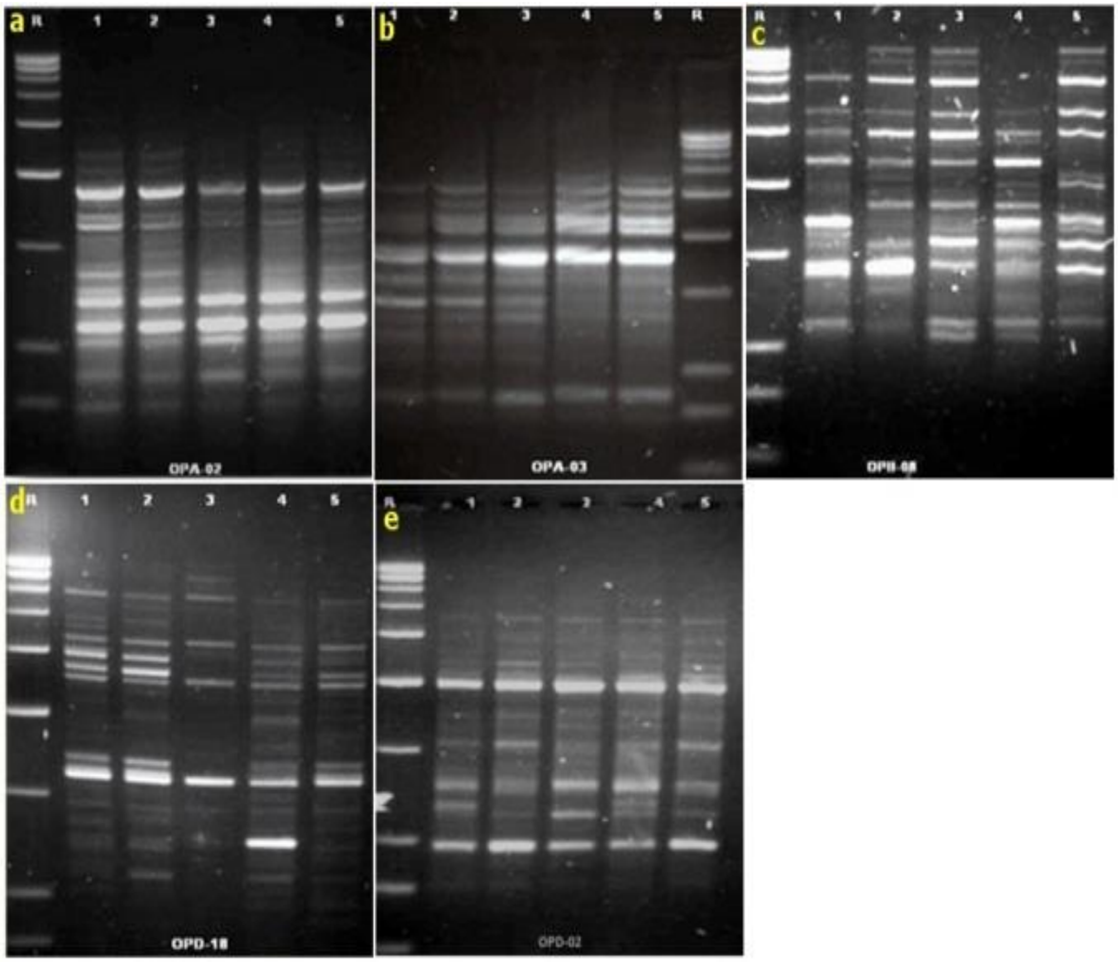

Figure 1.2. Genetic variations among the populations of *Rhinopoma hardwickii*. The number of corresponds to the serial of the populations with reference to RAPD primer OPA-01 (a), OPA-03 (b), OPB-08 (c), OPB-18 (d) and OPD-02 (e). R is a 1000bp DNA ladder. The serial numbers in the Figure (a – e) are similar.

The five random primers produced a PCR product for the investigation of the genetic variation among the populations of *R. hardwickii*. The primers such as OPA-02, OPA-03, OPB-08, OPB-18 and OPD-02 produced 63.63%, 80.0%, 50.0%, 50.0% and 37.5% polymorphic loci, respectively.

The primer OPA-03 produced 10 bands where monomorphic ones lie at 316 and 281 bp while polymorphic ones lie at 562 to 144 bp. observed in all populations. Similarly, the primers OPA-02 and OPA-08 produced 11 and 14 bands, where monomorphic ones located at 407, 251, 208, 199 and 794, 676, 316, 223 bp. Furthermore, primers OPB-18 and OPD-02 produced 8 bands each while the former one produced four monomorphic bands situated at 712, 650, 600 and 300 bp while the later one produced five monomorphic bands located at 741, 446, 380, 309, and 251. All the polymorphic bands ranged from 199 to 800 bp. However, some (polymorphic) unique bands were also detected with primer OPA-02 and OPA-08 with molecular weight 144, 262, 223, 316, 676 and 794 bps.

The discrimination power of each locus was estimated by the PIC (Polymorphic information content) value. PIC value ranged from 0.303 (OPB-18) to 0.557 (OPB-080) with an average PIC value of 0.43. High PIC value indicates enormous heterozygosity which is associated with a high degree of polymorphism (Table 1.3). This indicates that RAPD are well suited for determining the genetic diversity present in *R. hardwickii* populations.

Table 1.3. Shows the well amplified primers with number of monomorphic bands, polymorphic bands, percent of polymorphism and PIC value.

S. No.	Primer code	No. of bands	No. of monomorphic bands	Size of monomorphic bands (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	Polymorphism (%)	PIC
1	OPA-02	11	4	600-200	7	407-199	63.63	0.352
2	OPA-03	10	2	700-200	8	562-144	80	0.504
3	OPB-08	14	7	900-300	7	912-223	50	0.557
4	OPB-18	8	4	900-250	4	771-251	50	0.303
5	OPD-02	8	5	750-300	3	800-300	37.5	0.405
	Total	51	22	43%	29	56%	56.86	

Discussion

In the present study, the intra-specific genomic polymorphisms among the bat populations were analyzed using RAPD-PCR technique. Genetic analysis of the five markers in the present study showed reasonable level of diversity. Among the observed bands, 29 (56%) bands were polymorphic which show relatively high diversity among the populations of different zones. The genetic structure of distant populations is affected by the amount of dispersal (resulting in gene flow) that occurs among them (Perrin and Mazalov, 2000). Intermediate levels of gene flow can also maintain genetic diversity which is the raw substrate for natural population (Petren *et al*., 2005). Habitat fragmentation can result in driving the mammalian populations to genetic variation. These habitat configuration and structural connectivity can modify dispersal which is crucial for re-colonization. Furthermore, analyzing genetic variation in a particular landscape shows how landscape elements influence populations, either alone or in combination with other processes (Angers *et al*., 1999; Mendez *et al*., 2010). Hence all these factors enhance genetic variation. The relatively high level of genetic diversity presently observed could be explained by these three factors; (1) dispersal resulting in gene flow, (2) habitat fragmentation, (3) landscape. This may probably be the reason that zones FTS (Fatehpur Sikri) and FBD (Faizabad) appeared closely related at the genetic level, although geographically they are from zones of highly distinct locations in Uttar Pradesh. In addition, recent habitat fragmentation / degradation or habitat loss, which might have led to the density of the surviving populations in the existing habitat and gradually the lifespan of these species allowed populations to retain diversity for long periods after habitat loss (Greenwood, 1980; Hanski 1999; Perrin, 2000; Prugh *et al*., 2008). In this study maximum similarity was observed between zones JHS and FTS, JHS and CKTD, CAR and FBD, FHS and FBP with similarity coefficient index of 0.72, 0.70, 0.68 and 0.66, respectively and the zones were closer to each other. Therefore, gene flow among the populations can be expected to be much higher when they reside closely. This kind of relationship is known as isolation by distance, providing ample time for populations to reach equilibrium conditions (Kimura and Weiss, 1964). Moreover, levels of gene flow are not exclusively dependent on the distance between populations, but also the nature of the surrounding landscape between populations (Gibbs, 2001).

However, *R. hardwickii* is an insectivorous bat that is highly distributed throughout Uttar Pradesh. They often roost in caves, tunnel and deserted houses. They undergo seasonal migration of up to 900 km. Therefore, the most genetic variation among the population assigned is not astonishing and mainly due to high level of gene flow (dispersal) because *R. hardwickii* population is continuous. Thus, maintaining gene flow may be important for the long term existence of the populations of *R. hardwickii* in Uttar Pradesh.

Chapter II

Genetic diversity within the populations of *Megaderma lyra*

Chapter II

Genetic diversity within the populations of *Megaderma lyra*

INTRODUCTION

Megadermatidae bats belong to a large family of carnivorous bats and restricted to the Old World tropics (Bates *et al*., 2006). Indian false vampire bat, *Megaderma lyra* is a heterogeneous group of echolocating bat. *Megaderma lyra* scattered in patches in different regions of Uttar Pradesh. Like many other tropical bats, *M. lyra* also show its roost fidelity for several years and exhibit no seasonal migration. The sexes of philopatric species exhibit differences in foraging, the male forage about 500 m form the roost while female makes a flight for about 5 km (Audet *et al*., 1991). Female *M. lyra* gets an opportunity to acquire new roosting sites since their home range is relatively larger. It has specially become important for species with isolated distribution, small neighboring colonies and fragmented populations are more prone to inbreeding, genetic drift, and ultimately leading to loss of genetic diversity (Avise, 1998). Gene flow may aid adaptation to local conditions by the introduction of either maladaptive or advantageous alleles into a population (Slatkin, 1987). Gene flow is often considered to be an important factor to bring genetic diversity within these populations. For instance, it counters drift and may prevent inbreeding depression in fragmented populations (Young and Brown, 1996). Even within the populations, spatially restricted gene flow may lead to a genetic substructure and may allow selection to lead to micro-site adaptation (Prentice *et al*., 1995). Although, IUCN lists *M. lyra* as a least concerned (LC), but conserving this species is of significant importance. Genetic analyses have revealed the consequence of current behavioral and demographic status often supporting evidence for conservation.

The physical integrity of *M. lyra* makes them a champion gleaners among bats and often predating insects, pests and even rodents that destroys paddy and crops plants. They are well adapted in caves, old buildings, thatched huts, old disused wells, temples, forts, tunnels, mines, cow sheds in caves, and artificial underground (Brosset, 1962). Several studies were carried out to investigate the behaviour and genetic diversity of *M. lyra* but the exact reasons are still not certain (Balasingh *et al*., 1994; Goymann *et al*., 2000). Now molecular genetic techniques provide easy way to understand the complex behavior and genetic structure (Sugg *et al*., 1996; Hughes, 1998). Here, the genetic diversity of *Megaderma lyra* via RAPD marker within the neighboring roosts to assess population variation, gene flow, and behavioural information to establish valuable guideline for conservation measures were studied.

MATERIALS AND METHODS

The tissue samples of bats were collected from the neighbouring colonies of Barabanki, Uttar Pradesh (latitude 26°53'18.72"N; longitude 81°33'24.84"E), India. The roost of *Megaderma lyra* were located at the basement of a storehouse, demolished temple, thatched hut, Pillar crevices and old disused well. They were trapped using a hoop net with an extensible aluminum pole. Bats were also trapped using mist nets. The tissues samples were collected from five neighboring populations located at Barabanki, i.e. [1] FTP (Fatehpur Population – 01), [2] RNR (Ramnagar population – 02), [3] NBJ (Nawabganj population – 03), [4] GSP (Gauspur population – 04) and [5] HDH (Haidergarh population – 05). All the neighboring populations were located between 7 and 9 Km without any physical barriers (Figure 2.1).

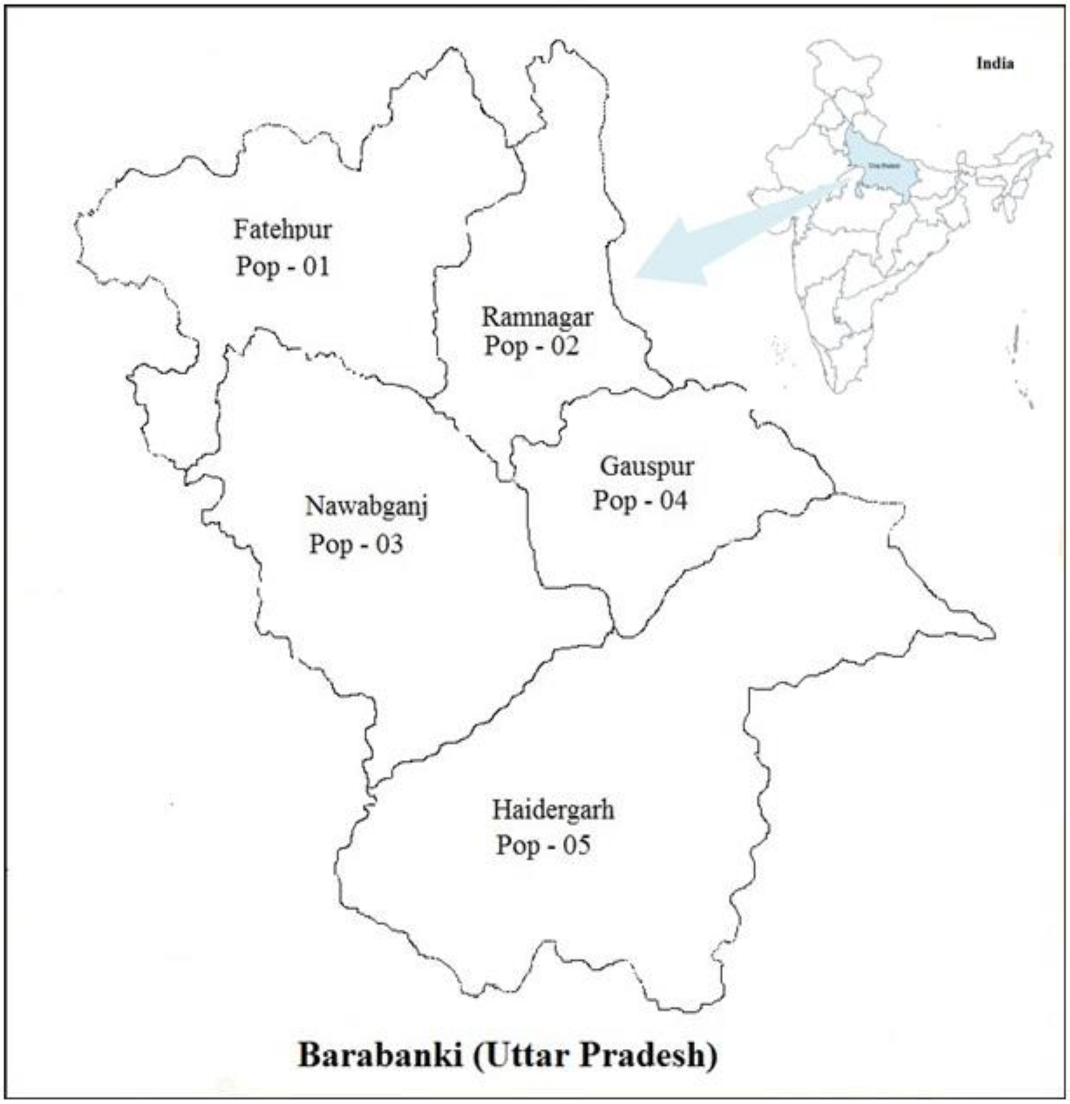

Figure 2.1. Represents the study sites in Barabanki district, Uttar Pradesh.

Plate 2.1. A frontal view of *Megaderma lyra*.

Plate 2.2. A colony of *Megaderma lyra* at Dariyabad, Barabanki district.

A small bit of patagium tissue (of 3 mm^2) from individual bat was collected using a sterile biopsy-punch and the sampled bats were collared for recognition. After collection of each sample, the punched patagium and the biopsy punch were sanitized with 70% ethanol. No biopsy mark was found during the subsequent observation made after a fortnight. The tissues were preserved at –20 °C in 25% DMSO for further DNA extraction. Bats were handled with extreme care and not sacrificed. Precautions were also taken in capturing them to minimize the possibility of stress and accidental fatalities.

DNA isolation screening

The tissue samples were homogenized using mortor and pistle containing 100 µl of lysis buffer 1% SDS, 10 mM Tris-HCl (pH 8.5), 125 mM NaCl, 5 mM EDTA and 0.5 mg/ml proteinase K (Sigma Aldrich). Followed by homogenization, the DNA extraction was carried out by following phenol:chloroform method (Sambrook, 1989). Both the quality and quantity of extracted DNA were confirmed using 0.7% agarose gel electrophoresis and spectrophotometrically at A260 and A280 nm (Perkin-Elmer USA).

Polymerase chain reaction

RAPD-PCR was designed using a set of six primers such as OPA-11, OPAA-09, OPW-02, OPB-18, OPR-08 and OPA-13. Conditions for amplification were set by following Williams (1990). Optimization was done by taking different concentrations of template, primer, $MgCl_{2,}$ and *Taq* DNA polymerase in order to avoid primer dimer (Banglore Genei, India). Primer optimization was also done by calculating the (T_m) value and annealing temperature. The reactions were carried out in a total volume of 15 µl containing 25 ng of genomic DNA, 1.5 mM $MgCl_{2,}$ 200 µM of each dNTP, 50 pmol of a single decamer primer (Banglore Genei, India), 0.5 µg/mL BSA, and 1 U *Taq* DNA polymerase. PCR was carried out in a thermocycler (Gene Amp PCR system 2400, Perkin Elmer) programmed at 94 ºC for 3 min followed by 43 cycles of 30 sec at 94 ºC, 1 min at 35 ºC, and two min at 72 ºC. Finally at last cycle, there was a 5-min of extension at 72 ºC. Initially investigation was carried out with all 13 primers using genomic DNA from five different locations. RAPD-PCR analysis was operated twice so that only primers that gave strong bands were used in the investigation of all the five locations. Gels with amplification fragments were visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad).

RAPD Profile analysis

NTSYS-pc version 2.0 software was exploited to evaluate the RAPD data. All calculations regarding genetic similarity (GS) were done with the help of Jaccard's coefficient of similarity GS(ij) = a/(a+b+c). GS(ij) signifies the amount of genetic similarity between the species i and j, b denotes the frequency of bands present in the i while absent in j, whereas c indicates the number of bands present in j while absent in i, respectively. Each RAPD patterns was carried as a single character and was scored as 1/0. This binary matrix was constructed for all the bands scored to generate similarity coefficients. Finally a dendrogram using UPGMA was created by data provided by Jaccard's coefficient. The Polymorphism information content (PIC) a measure of informativeness of these RAPD markers was calculated by following (Botstein *et al.*, 1980).

RESULTS

The sample collected from the Population site showed noticeable changes in genetic diversity from barabanki, Uttar Pradesh (Figure 2.1). Out of 10 primers screened, most polymorphisms were obtained with OPA-11, OPAA-09, OPW-02, OPB-18, OPR-08, and OPA-13 (Table 2.1). The RAPD fingerprinting data for five populations of *M. lyra* showed significant level of genetic variation (polymorphism) within the neighboring colonies (Figure 2.2).

Table 2.1 List of primers, decamer sequences and percent of G+C content.

S. no.	**Primer**	**Sequence (5' - 3')**	**% G+C content**
1	OPA-11	CAATCGCCGT	60
2	OPAA-09	AGATGGGCAG	60
3	OPW-02	ACCCCGCCAA	70
4	OPB-18	CCACAGCAGT	60
5	OPR-08	CCCGTTGCCT	60
6	OPA-13	CAGCACCCAC	70

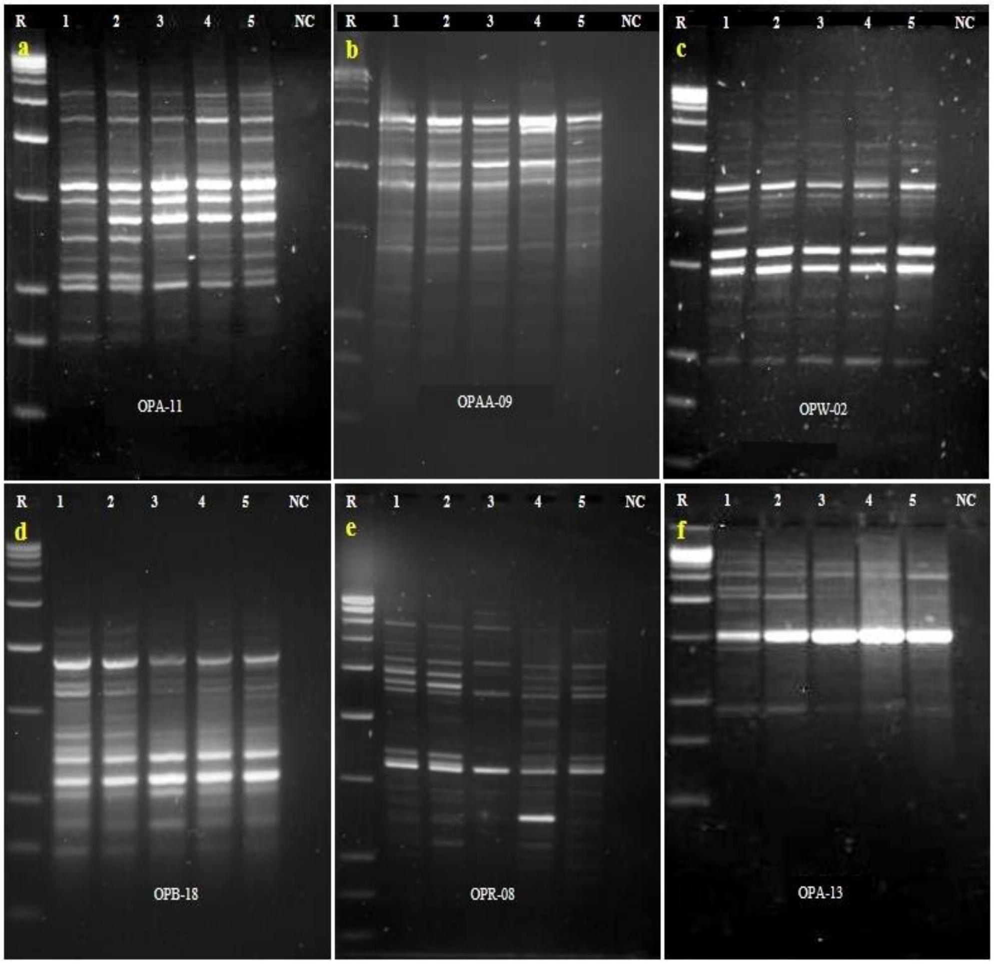

Figure 2.2. Genetic diversityof five neighbouring colonies of *M. lyra*: [1] FTP (Fatehpur Colony – 01), [2] RNR (Ramnagar Colony – 02), [3] NBJ (Nawabganj Colony – 03), [4] GSP (Gauspur Colony – 04) and [5] HDH (Haidergarh Colony – 05) with reference to primer OPA-11 (a), OPAA-09 (b), OPW-02 (c), OPB-18 (d), OPR-08 (e) and OPA-13 (f). R is a 1000 bp DNA ladder. NC represents the negative control.

All the primers used were polymorphic in nature and a total of 59 loci were obtained. Out of which 25 bands were polymorphic and 34 bands detected were monomorphic. The highest polymorphic loci was obtained with primer (OPA-11) and lowest with 2 (OPB-13) and (OPB-18) each. The amplification product size value ranged from 100 to 900 bp. The genetic similarity values based on RAPD banding patterns were evaluated by using the method Jaccard’s coefficient analysis (Table 2.2).

Table 2.2. Jaccard's coefficient among five Populations of *Megaderma lyra*.

	1	**2**	**3**	**4**	**5**
1	1.00				
2	0.728	1.00			
3	0.423	0.627	1.00		
4	0.543	0.576	0.677	1.00	
5	0.745	0.677	0.610	0.661	1.00

The cluster constructed through NTSys (2.02 Pc) that is represented in the form of dendrogram (Figure 2.3).

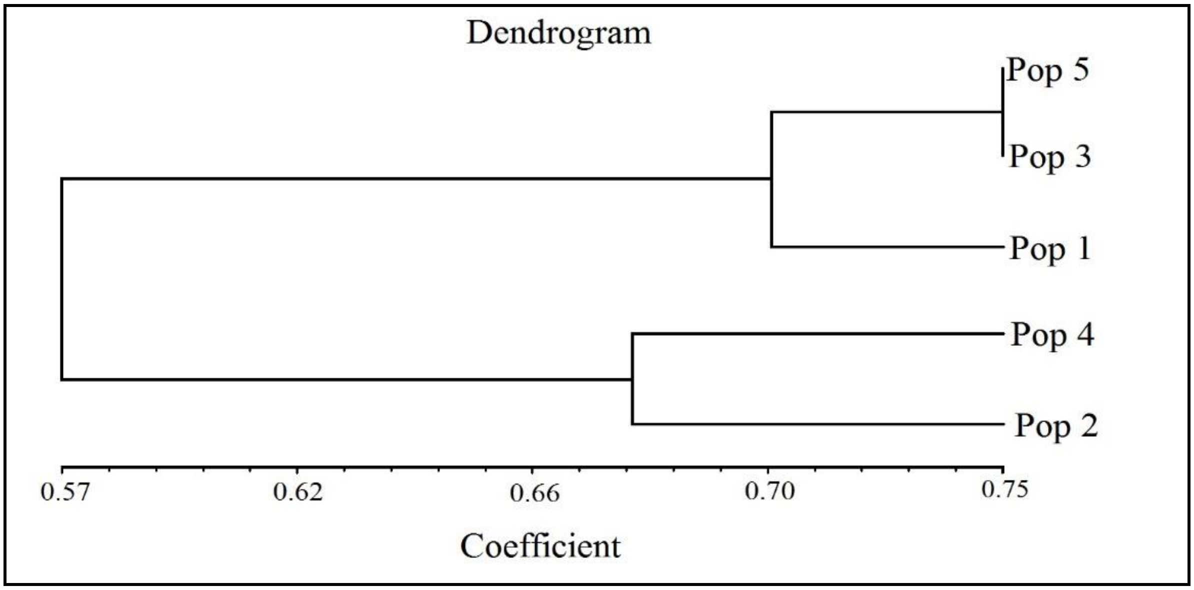

Figure 2.3. UPGMA based dendrogram showing the phylogenetic relationship within the populations of *Megaderma lyra*.

The PIC value ranged from 0.656 (OPA-13) to 0.720 (OPA-11) with a mean PIC value of 0.64 the discrimination power of each locus was evaluated by the (polymorphic information content) value (Table 2.3).

The UPGMA clustering generated form the binary matrix of the studied populations comprised of two groups (group A and group B). Group A is further separated into two sub groups representing three morphotypes (pop 05 and pop 03) in one group and (pop 01) as segregated genotype. Group B comprises of two morphotypes (pop 02 and pop 04), respectively. The % similarity within the colony was 72% between [1] FTP and [2] RNR, 42% [1] FTP and [3] NBJ, 54% [1] FTP [1] and [4] GSP, 74% [1] FTP and [5] HDH, 62% [2] RNR and [2] NBJ [2], 57% [2] RNR and [4] GSP, 67% [2] RNR and [5] HDH and [3] NBJ and [4] GSP each, NBJ [3] and HDH [5], 66% [4] GSP and [5] HDH respectively.

Table 2.3. The well amplified primers with number of monomorphic bands, polymorphic bands, and percent of polymorphism value.

S. no.	Primer code	No. of bands	No. of monomorphic bands	Size of monomorphic bands (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	Polymorphism (%)	PIC
1	OPA-11	13	6	600-200	7	407-199	46.15	0.72
2	0PAA-09	10	6	700-200	4	562-144	60	0.651
3	OPW-02	10	5	900-300	5	912-223	50	0.66
4	OPB-18	8	6	900-250	2	771-251	25	0.568
5	OPR-08	12	7	750-300	5	800-300	58.33	0.585
6	OPA-13	6	4	100-230	2	300-550	40	0.656
	Total	59	34		25			

DISCUSSION

The RAPD-fingerprinting showed a high percentage of genetic similarity within the populations of *Megaderma lyra*. Increase in similarity percentage also predicts that the colonies maintain high roost fidelity because of long term intra population selection history. However, the low genetic variation limits the species ability to responds to threats in both long and short term (Amos and Harwood, 1998). Inbreeding leads to low level of heterozygosity. Heterozygosity may lead to greater probability of the two alleles at a loci being identical. The polymorphic information content (PIC) deduced from the RAPD – fingerprinting ranged from 0.651 to 0.720 suggests less heterogygosity. Decrease in heterozygosis or polymorphism within a colony also causes higher mortality, lower fecundity, reduced mating ability, slower growth, more prone to disease, lower ability to withstand stress, leading to intra and inters specific competitive ability (Allendorf and Leary, 1986; Darwin, 1986; Falconer, 1989; Ledig, 1986). *Mederma lyra*, a non-migratory insectivorous species maintains high degree of roost fidelity and rarely migrate, or disperse over longer distance (Advani, 1981). It has been reported that the bats equipped with slow manoeuvrable flight and specialized gleaners are likely to make small colonies and maintains site fidelity compared to long range aerial dispersers (Entwistle *et al*., 2000).

It is evident that RAPD markers may also be useful for systematic investigations at the species level as well (Dinesh *et al*., 1993). More advanced molecular techniques is needed to determine that under what conditions populations could be purged of the genetic loads. Genetic problems are adding to the decline and vulnerability. The contribution of genetic variability to population viability is basically an interaction with physiological, behavioral and ecological processes. For more productive management, options should involve actions to restore genetic variability by reestablishing gene flow by selective translocation.

Chapter III

Genetic variation among the colonies of Scotophilus heathii

Chapter III

Genetic variation among the colonies of *Scotophilus heathii*

INTRODUCTION

Today chiroptera is one of the most diversified orders amongst placental mammals, extending as much as 20% of all mammalian species (IUCN, 2009). In the context of mammalian species diversity, bat holds an exceptional rank, covering 1232 species representing almost one fifth of all extant mammals and are among the animals that echolocate (Kunz *et al*., 2011). Diversity of chiropteran faunal richness walks together with other mammals in general, showing a strong elevation towards the equator (Patterson *et al*., 2003). Along the tropics, bats occupy a large variety of tropical niches and are often seen to occupy the most species richness and abundant mammalian assemblages (Zubaid *et al*., 2006; Rex *et al*., 2008; Fahr and Kalko, 2011). They display a great variety of behavioural, morphological and ecological attributes in the ecosystem, as pollination, insect predation and seed dispersal (Cosson *et al*., 1999; Burns and Boarders, 2014; Mayer *et al*., 2016). Microchiropterans particularly vespertilionid bats feed on insects and may play an important role in the agro-ecosystems. These bats are also among the smallest of mammals, thus challenging the observed positive correlation between the bodymass and maximum lifespan (Kirkwood *et al*., 2000). Their adaptability to occupy several ecological niches makes their distribution around the earth and in nearly every geographic provinance, except the Polar Regions and some oceanic islands (Hill and Smith, 1984; Marandat, 1991; Harrison and Hooker, 2010).

Vesper bats are usually ubiquitous and are known to occupy the highest number of bats in the chiropteran family occupying 44 genera with more than 350 species of the small insectivorous chiropteran domain (Corbet and Hill, 1991).

Due to the existence of convergent, parallel or mosaic evolution and the retention of plesiomorphic conditions in several vespertilionid groups, it is difficult to determine the generic status and estimate phylogenetic relationships of many species. Earlier, the phylogenetic relationships of vespertilionid bats were studied mainly through analyses of traditional morphological or cytogenetic characters (Koopman, 1994; Simmons, 1998; Volleth *et al.,* 1994; Volleth *et al.,* 2001; Zima and Horacek, 1985).

Scotophilus heathii choose to reside in the temperate areas throughout Europe and Asia, resting during the day, feeding at night hours and often hibernating during the winter season. Although these animals have high potential for dispersal, they can display unexpected levels of genetic differentiation and strong geographic structure (Ruidi and McCrakenn, 2009).

Genetic diversity basically determines the evolutionary potential of a species and its ability to cope with changes in its environment (Frankham *et al*., 2002). Community genetics focuses at understanding how within species variation, species diversity and environmental factors interact to shape community assemblages (Lamy *et al*., 2017).

Genetic diversity can correlate with species diversity, both between and within trophic levels in at least three possible ways (Avolio *et al*., 2017). First intraspecific genetic diversity can influence the species diversity, structure and functioning of community of these through genetic feedback mechanisms (Genung *et al*., 2011;

Whitlock, 2014). Second genetic diversity and ecological function can be associated through parallel responses to selection pressure and eco evolutionary dynamics over time (Hughes *et al*., 2008; Whitlock, 2014). Third ecological interactions (e.g., competition, depredation, reproduction) determine demographic changes (birth, death and movement of different biological types affecting the dynamics of both populations and communities, maintaining the level of species diversity and genetic structure (Moreira *et al*., 2014; Vellend and Geber, 2005).

As of yet, however, these highly specialized animals have escaped the detailed genome characterization. However molecular phylogenetic methods using DNA markers (RAPD) have been extensively applied and are changing many traditionally recognized groups (Hoofer *et al*., 2003; Lack *et al*., 2010; Roehrs *et al*., 2011). RAPD fingerprinting have revolutionized our approach to understand the social system of animals by analyzing the kinship relationships including bats (Jones *et al*., 1991; Burke *et al*., 1991; Schlotteröer *et al*., 1991). RAPD markers have added new technology in the field of DNA fingerprinting including molecular analysis of relatedness between genotypes. RAPD is one of the methods in the study of population genetics to estimate genetic variability, relatedness, inbreeding level, species verification and pedigree analysis (Jayasankar and Dharmalingam, 1997). It is used to measure the genetic diversity in population studies, based on which numerous conservation efforts have been undertaken in desert fishes (Vrijenhoek 1996), sea turtles (Avise, 1998) and whales (Palumbi and Baker 1994).

Unfortunately, bats are often victimized as reservoirs of several sporadic infectious diseases whose transmission may be related to habitat declination and climate change (Jones *et al*., 2009). Hence a broader understanding of the biology of bats may be essential for the development of future management or conservation strategies. The present study was attempted to analyze the genetic relatedness among the populations of *S. heathii* in Uttar Pradesh using RAPD-PCR technique.

MATERIALS AND METHODS

Unlike the conventional population genetics studies, which mainly focus on collecting large volume of sample size of different populations, we collected one population form either sites of the study area. Mist net (6m x 9m Nylon mist-net) Avinet-Dryden, USA was spread around each site and the tissue was collected from the uropatigium for genetic analysis. The mist nets were stretched out just after the sunset for four hours form (18:00 - 22:00 h:m). For each sample the biological characteristics i.e body size, sex, reproductive status and geographical locations were recorded. The specimen was collected from different districts of Uttar Pradesh (India). They are Balrampur (BLR [1] 27°25'51.00"N; 82°18'6.38"E), Mirzapur (MZR [2] 25°8'1.32"N; 82°33'51.96"E), Allahabad (ALD [3] 25°26'8.88"N; 81°50'46.72"E), Sultanpur (STR [4] 26°15'53.19"N; 82°4'21.74"E), Maharajganj (MRJ [5] 26°14'4.31"N; 81°14'27.13"E), Gorakhpur (GKP [6] 26°45'38.00"N; 83°22'23.40"E), Raebareli (REB [7] 26°14'4.31"N; 81°14'27.13"E), Azamgarh (AZH [8] 26°14'4.31"N; 81°14'27.13"E), Unnao (UNO [9] 26°32'21.64"N; 80°29'16.15"E, Fatehpur (FTR) [10] 26°11'21.06"N; 80°41'48.49"E). The bats were released immediately after taking morphology measurements and tissue samples without giving them much stress. The tissue samples were preserved in 1.5ml vial tube containing 96% ethyl alcohol.

DNA extraction and Amplification

Genomic DNA was extracted through the cellular lysis method followed by purification with phenol:chroloform method following the treatment of tissue homogenates with proteinase K (Sambrook *et al*., 1989). Both the quality and quantity of extracted DNA were confirmed using 0.7% agarose gel electrophoresis and spectrophotometrically at A260 and A280 nm (Perkin-Elmer USA).

Polymerase Chain Reaction

RAPD-PCR was designed using a set of primers such as OPR-08, OPA-11, OPAA-09, OPB-08, OPC-07, and OPA-17. Conditions for amplification were set by following Williams *et al*. (1990). Optimization was done by taking different concentrations of template, primer, $MgCl_{2,}$ and *Taq* DNA polymerase in order to avoid primer dimer (Banglore Genei, India). Primer optimization was also done by calculating the (T_m) value and annealing temperature. A similar method called ramped cycle was used in order to let setting of the primers only at the best site. This was optimized by allowing a small time at the annealing phase but longer phase at the extension with slower temperature as usual. This technique allows even the poorly fitting primers to be locked up onto the template DNA. The reactions were carried out in a total volume of 15 μl containing 25 ng of genomic DNA 1.5 mM $MgCl_{2,}$ 200 μM of each DNTP, 50 pmol of a single decamer primer (Banglore Genei, India), 0.5 μg/mL BSA, and 1 U *Taq* DNA polymerase. PCR was carried out in a thermocycler (Gene Amp PCR system 2400, Perkin Elmer) programmed at 94 ^{O}C for 3 min followed by 43 cycles of 30 sec at 94 ^{O}C, 1 min at 35 ^{O}C, and two min at 72 ^{O}C. Finally at last cycle, there was a 5-min of extension at 72 $^{\circ}$C. Initially investigation was carried out with all primers using template DNA from 10 different populations. RAPD-PCR analysis was operated twice so that only primers that gave strong bands were used in the investigation of all the 10 locations.

A 2% agarose gels in 0.5X TBE buffer were used. Again the gels were placed in a (running buffer) containing ethidium bromide (EtBr) at 100 V constant voltages. For evaluating the base pair length of bands, DNA ladder (1 Kb) was loaded on the beginning lane of each gel. Finally the PCR product were visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad).

RAPD Profile analysis

NTSYS-pc version 2.0 software was exploited to evaluate the RAPD data. All the calculations related to regarding genetic similarity (GS) was done with the help of Jaccards coefficient of similarity GS(ij) = a/(a+b+c). GS(ij) signifies the amount of genetic similarity between the species i and j, b denotes the frequency of bands present in the i while absent in j, whereas c indicates the number of bands present in j while absent in i, respectively. Each RAPD patterns was considered as a single character and was scored as 1/0. This binary matrix was constructed for all the bands scored to generate similarity coefficients. Finally the dendrogram using UPGMA was created by data provided by Jaccards coefficient. The Polymorphism information content (PIC) a measure of informativeness of these RAPD markers was calculated by following Botstein *et al* (1980).

RESULTS

All the populations (ten) of *Scotophilus heathii* collected from various regions of Uttar Pradesh showed noticeable level of genetic variation. Out of the fifteen primers used in the reaction initially, most of the polymorphism was obtained with the OPR-08, OPA-11, OPB-08, OPC-07, OPAA-09, OPC-07 and OPA-17 (Table 3.1).

Plate 3.1. An individual of *Scotophilus heathii*.

Table 3.1. List of primers in decamer sequence and percent G+C content.

S. no.	Primer	Sequence (5'-3')	% G+C content
1	OPR-08	CCCGTTGCCT	70
2	OPA-11	CAATCGCCGT	60
3	OPAA-09	AGATGGGCAG	60
4	OPB-08	GTCCACACGG	70
5	OPC-07	GTCCCGACGA	70
6	OPA-17	GACCGCTTGT	60

The RAPD Fingerprinting as shown in the gel image denotes clear patterns of banding (Figure 3.1 – 3.6).

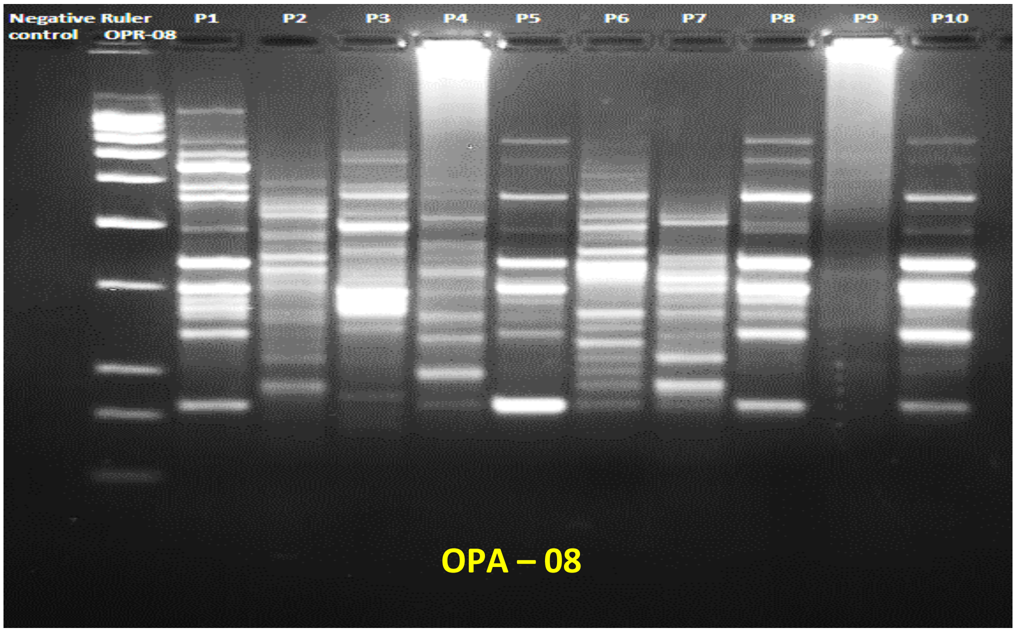

Figure 3.1. Genetic variation among ten populations of *Scotophilus heathii* amplified by OPA-08 [1] BLR (Balrampur population - P1), [2] MZR (Mirzapur population - P2), [3] ALD (Allahabad population - P3), [4] STR (Sultanpur population - P4), [5] MZR (Mirzapur population - P5), [6] GKP (Gorakhpur population - P6), [7] REB (Raebareli population - P7), [8] AZH (Azamgarh population - P8), [9] UNO (Unnao population - P9), and [10] FTR (Fatehpur population - P10), the first lane of the gel is Negative control. The Ruler is located in the second lane of 1000 bp.

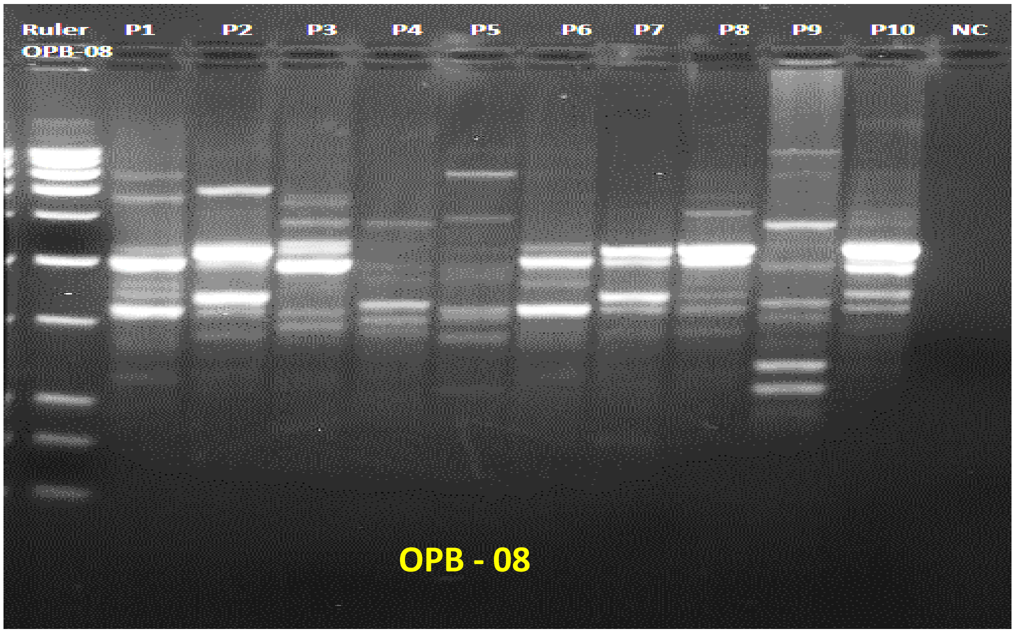

Figure 3.2. Genetic variation among ten populations of *Scotophilus heathii* amplified by OPB-08. [1] BLR (Balrampur population -P1), [2] MZR (Mirzapur population - P2), [3] ALD (Allahabad population - P3), [4] STR (Sultanpur population - P4), [5] MZR (Mirzapur population - P5), [6] GKP (Gorakhpur population - P6), [7] REB (Raebareli population - P7), [8] AZH (Azamgarh population - P8), [9] UNO (Unnao population - P9), and [10] FTR (Fatehpur population - P10), the last lane of the gel is Negative control. The Ruler is located in the second lane of 1000 bp.

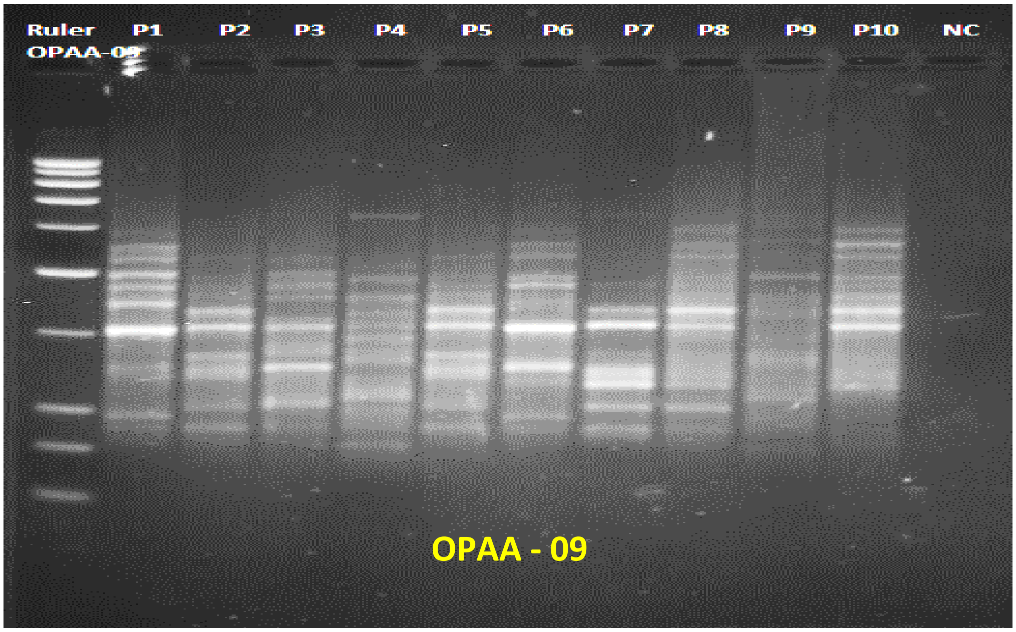

Figure 3.3 Genetic variation among ten populations of *Scotophilus heathii* amplified by OPAA-09. [1] BLR (Balrampur population -P1), [2] MZR (Mirzapur population - P2), [3] ALD (Allahabad population - P3), [4] STR (Sultanpur population - P4), [5] MZR (Mirzapur population - P5), [6] GKP (Gorakhpur population - P6), [7] REB (Raebareli population - P7), [8] AZH (Azamgarh population - P8), [9] UNO (Unnao population - P9) , and [10] FTR (Fatehpur population - P10), the last lane of the gel is Negative control. The Ruler is located in the second lane of 1000 bp.

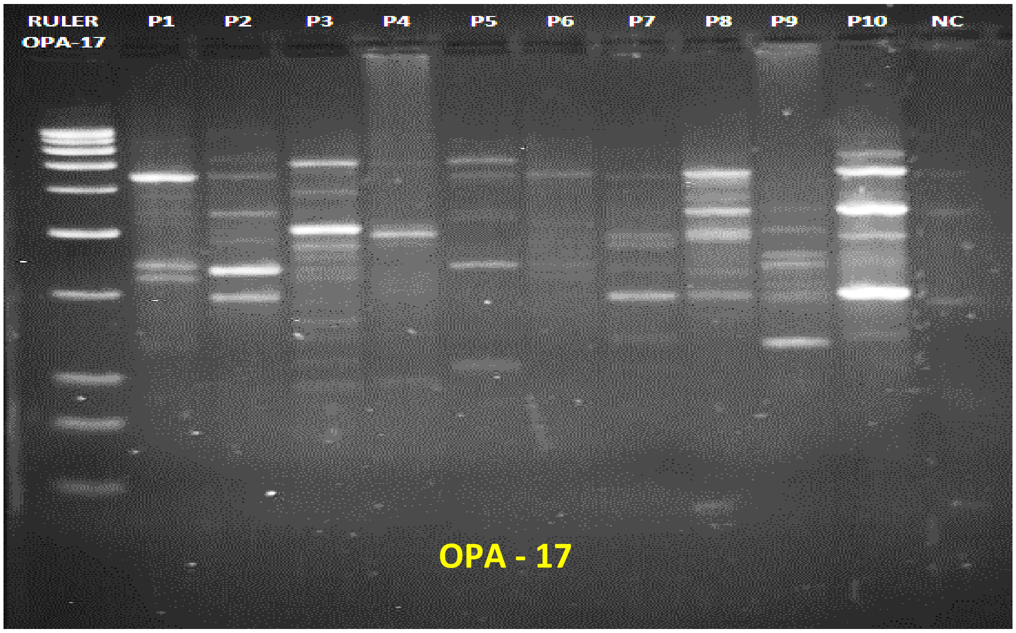

Figure 3.4. Genetic variation among ten populations of *Scotophilus heathii* amplified by OPA-17.[1] BLR (Balrampur population -P1), [2] MZR (Mirzapur population - P2), [3] ALD (Allahabad population - P3), [4] STR (Sultanpur population - P4), [5] MZR (Mirzapur population - P5), [6] GKP (Gorakhpur population - P6), [7] REB (Raebareli population - P7), [8] AZH (Azamgarh population - P8), [9] UNO (Unnao population - P9) and [10] FTR (Fatehpur population - P10), the last lane of the gel is Negative control. The Ruler is located in the second lane of 1000 bp.

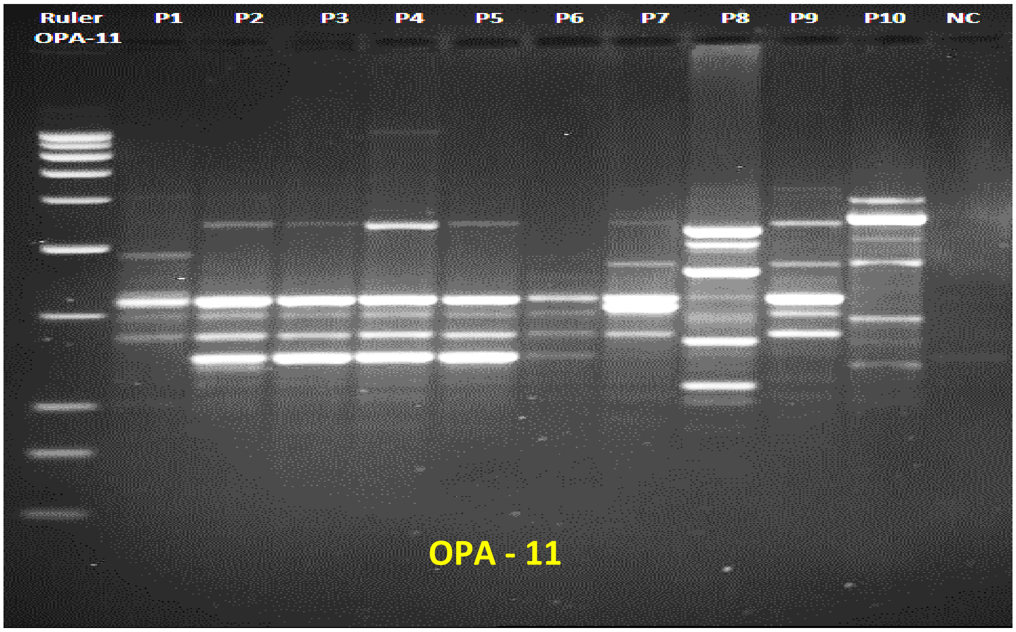

Figure 3.5. Genetic variation among ten populations of *Scotophilus heathii* amplified by OPA-11. [1] BLR (Balrampur population - P1), [2] MZR (Mirzapur population - P2), [3] ALD (Allahabad population - P3), [4] STR (Sultanpur population - P4), [5] MZR (Mirzapur population - P5), [6] GKP (Gorakhpur population - P6), [7] REB (Raebareli population - P7), [8] AZH (Azamgarh population - P8), [9] UNO (Unnao population - P9), and [10] FTR (Fatehpur population - P10) the last lane of the gel is Negative control. The Ruler is located in the second lane of 1000 bp.

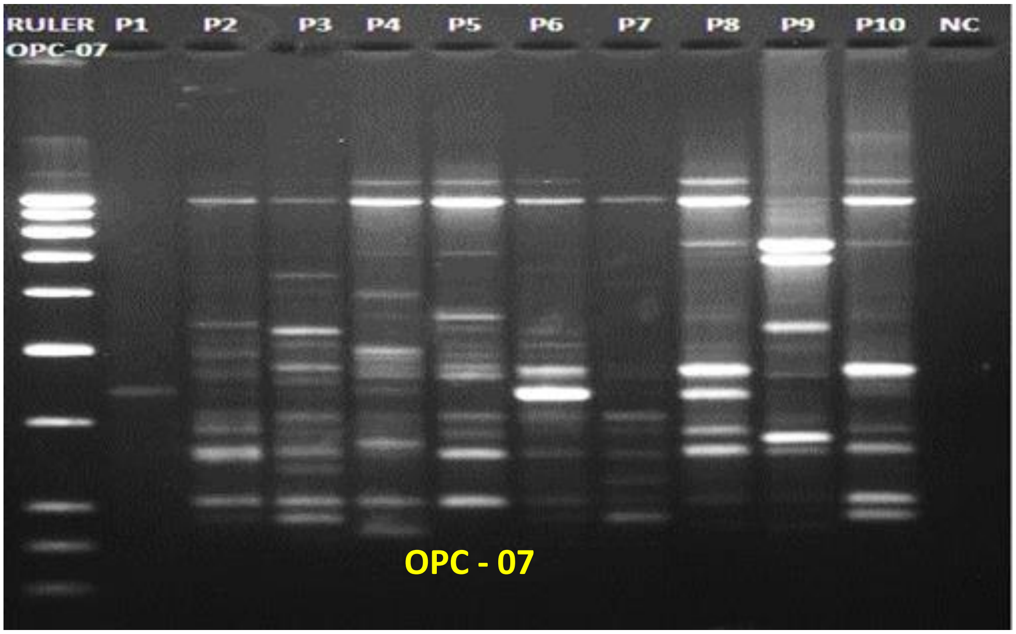

Figure 3.6. Genetic variation among ten populations of *Scotophilus heathii* amplified by OPC-07. [1] BLR (Balrampur population -P1), [2] MZR (Mirzapur population - P2), [3] ALD (Allahabad population - P3), [4] STR (Sultanpur population - P4), [5] MZR (Mirzapur population - P5), [6] GKP (Gorakhpur population - P6), [7] REB (Raebareli population - P7), [8] AZH (Azamgarh population - P8), [9] UNO (Unnao population - P9), and [10] FTR (Fatehpur population - P10), the last lane of the gel is Negative control. The Ruler is located in the second lane of 1000 bp.

The RAPD profile generated for all the ten populations produced significant level of polymorphism (Table 3.2.). All the primers expressed polymorphism (bands) and a total of 115 bands were observed. All the faint and dark bands were counted to check the similarity among the populations. Out of the total bands observed 73 (63%) were found to be monomorphic in nature while 42 (36%) of them were polymorphic (unique bands) among the populations studied. The highest polymorphic bands were obtained with the primer (0.893) OPR-08 and the lowest was recorded with the primer (0.498) OPA-17 with a mean of 0.70. The amplification product values ranged between 150 to 950 base pair. The discrimination power of each locus was evaluated with the help of PIC value (Table 3.2).

The amount of genetic relatedness among the population of *S. heathii* of the population was deduced by using the jaccard coefficient (Table 3.3).

The jaccard coefficient data were evaluated by the number of bands present (+) or absent (-) of all the primers used. Once the binary matrix was prepared, it was imported to a software NTsys (1.02 Pc) that is represented in the dendrogram (Figure 3.7).

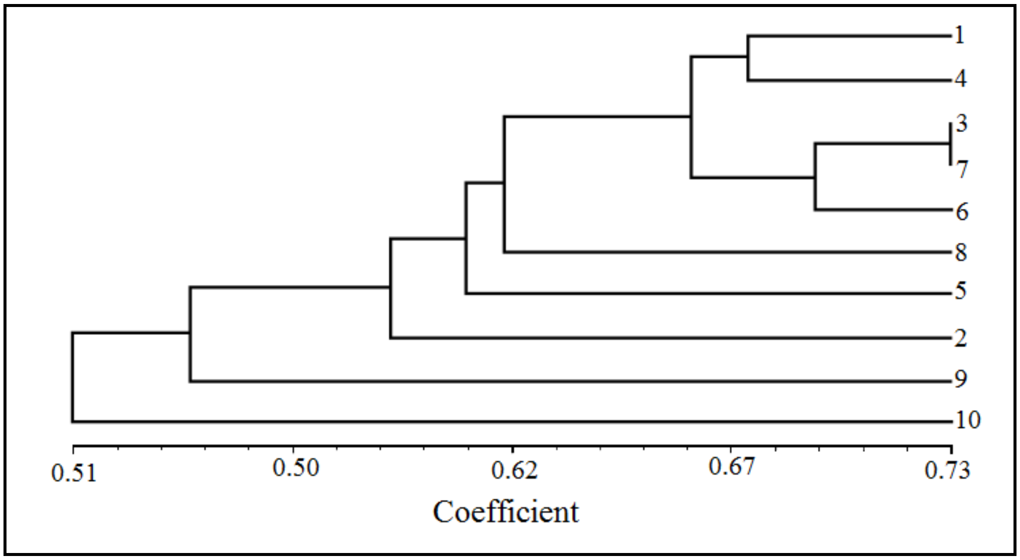

Figure 3.7. UPGMA based dendrogram showing the phylogenetic relationship among the colonies of *Scotophilus heathii*.

Table 3.2. The well amplified primers with number of monomorphic bands, polymorphic bands and percent polymorphism value.

S. No.	Primer code	No. of bands	No. of monomorphic bands	Size of monom orphic bands (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	Polymorphism (%)	PIC
1	OPR-08	24	14	900-200	10	950-199	41	0.893
2	OPA-11	17	11	700-200	6	562-144	35.29	0.811
3	OPAA-09	18	11	900-300	7	912-223	38.88	0.656
4	OPB-08	20	13	900-250	7	771-251	35	0.66
5	OPC-07	24	16	750-300	8	800-300	57	0.697
6	OPA-17	12	8	150-250	4	150-200	33	0.498
	Total	115	73		42			

Table 3.3. Jaccard coefficient among the ten populations of *S.heathii*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1.00									
2	0.475	1.00								
3	0.559	0.559	1.00							
4	0.661	0.508	0.576	1.00						
5	0.492	0.508	0.712	0.627	1.00					
6	0.407	0.475	0.678	0.593	0.661	1.00				
7	0.559	0.559	0.492	0.576	0.610	0.678	1.00			
8	0.593	0.542	0.610	0.627	0.695	0.661	0.610	1.00		
9	0.390	0.542	0.644	0.593	0.729	0.695	0.644	0.695	1.00	
10	0.441	0.610	0.678	0.525	0.559	0.627	0.610	0.661	0.559	1.00

From the obtained results the populations of the *S. heathii* showed diverse range of similarities among the studies area. The maximum similarity 72% among the populations was observed in Maharajganj MRJ [5] and Unnao UNO [9]. Next to that the second highest 71% was found to be between the populations of Maharajganj MRJ [5] and Allahabad ALD [3]. The minimum similarity value 39% was obtained between population of Unnao UNO [9] and Balrampur BLR [1]. It can be concluded that majority of the similarity value 20 ranged between 60% to 70%. About 16 populations gave a similarity value of ranged from 50% to 60%, and only 5 populations similarity value ranged from 40% to 50%.

DISCUSSION

All the populations of *Scotophlus heathii* observed through RAPD fingerprinting showed significant level of diversity. Although the populations were sampled form different geographical locations of Uttar Pradesh (India). The nucleotide diversity was closely observed, even if the population of the same species resided, the geographical distance did not matter much among the ten populations. The reasons that lead to such slight changes in these organisms may be of the following reasons. (1) The evolutionary potential of a species is certainly determined by the species ability to come up with variations in its environment (Frankhann *et al*., 2002). The genetic diversity is often correlated with the community. It can influence the species diversity, structure and functioning through genetic feed bank mechanism (Genung *et al*., 2011; Whitlock, 2014). The ecological interactions e.g. competition, depredation, reproduction may determine the demographic changes affecting the dynamics of species diversity and genetic structure (Moreira *et al*., 2014; Vellend and Geber, 2005). All these conditions with the life history of the species including social sturucture and mating behavior may help in defining the levels of genetic diversity (Lamy *et al*., 2017). The RAPD fingerprinting as represented by the cluster analysis of sub group I showed to contain eight morphotypes suggesting that they all are more genetically similar than the others. Sub group II contains only one species (9) suggesting that the populations of this place show more genetic variation. Group B also contained only one morphotype (10) suggesting that the population of *S. heathii* is genetically more diverse among all the population studied. A Similar studies were conducted by using the same RAPD marker to study the species of buffalo (*Bubalus bubalis*) and it was reported that species of the same population can show population variation (Rao *et al*., 1996). Another study was carried out by the same RAPD marker with the breeds of chicken showing sufficient level of polymorphism (variation) between and between the chicken populations (Sharma *et al*., 2001).

However this insectivorous bat species is not commonly found everywhere, and the females species of *S. heathii* have got the capacity to store sperm for about five months, they may disperse for longer distance, and flow of gene may be archived. Thus the level of diversity is maintained among the colonies.

Summary

Summary

Presently, more than 1300 species of bats are reported, by simply following morphology and commonly observed biogeographic pattern. Bat diversity maximizes as one progresses towards the tropics while minimizes towards the poles. India alone accounts about 11% of the total bats diversity around the globe. Among the two sub orders of chiroptera, the microchiropterans are mostly studied and most diverse group of bats. They are generally the insectivorous bats as the food and mode of feeding in bats often used as characterization insectivorous, piscivorous, sangivouous, frugivorous, omnivorous, and carnivorous. India is enshrined with almost 119 speceis of bats constituting into nine families. These include pteropodidae, Rhinopomatidae. Megadermatidae, Rhinolophidae, Hipposideridae, Molossidae, Emballonruidae and Vespertilionidae. About 75% species of bats are insectivorous with true powered flight and echolocation which has made them to seek shelters in many different types of structure. It has been complicated to determine the exact identity of species that occupy a similar ecological niche and have very close morphological characters.

Despite a large portion of species level taxonomy is based on a narrow array of data types like patterns of variability in morphology, ecology and genetic elements, still much of the complexity and evolutionary biology is yet to be revealed. Due to scarcity of appropriate genetic information. It becomes extremely difficult to study them in the wild. Thereby the proposed work of my research was to assess the genetic diversity of a few insectivorous bats in Uttar Pradesh.

Summary

Random Amplified Polymorphic DNA (RAPD) was used to study the genetic diversity of insectivorous bats. As this technique extracts valuable biological information to document the population structure of different locations. This technique is widely used to molecular biology since it requires tiny amount of genomic DNA to assist reliable information on their phylogenetic relationships.

Assessment of genetic diversity among the populations of mouse tailed bats (Rhinopotatidae) was carried out in the district of Uttar Pradesh. The purpose of the work was to find the genetic diversity form the five populations of different geographical regions using RAPD-PCR technique. A total of 51 bands were detected by the five amplified primer (OPA-02, OPA-03, OPB-08, OPB-18 and OPD-02). They produced 10.2 bands per primer with an average and the molecular weight ranged from 912 to 158 base pair. The polymorphic loci among the populations of *R. hardwickii* varied considerably with (56.86%). Among all the primer used, OPB-08 produced the maximum amplified fragments.

The similarity coefficient values among the populations deduced by the Jaccard coefficient with the help of (NTSYS-pc version 2.0 software) ranged from 0.60 to 1.00. The polymorphic information content values also ranged from 0.303 (OPB-18) to 0.557 (OPB-080), with a mean PIC value of 0.43. The PIC value suggests that enormous heterozygosity was associated with a high degree of polymorphism among the population, despite the increasing fragmentation of its habitat. So the high genetic diversity observed in the study suggests less threat to the population of *R. hardwickii*.

Another study was conducted to assess the genetic variation in the populations of the Indian false Vampire bat, *Megaderma lyra* by using the same molecular marker. *Megaderma lyra* is a heterogeneous group of echolocating bat and is scattered in patches in different regions of Uttar Pradesh. This species of bat is a non-migratory carnivorous and maintains high level of roost fidelity and its dispersal ability is still obscure. The bat population was collected from the neighboring colonies of Barabanki, Uttar Pradesh. All the neighboring colonies were located at a distance of 7-9 Km without any physical barrier. The data expressed quite low level of diversity within the five populations studied. A total of 59 bands were generated with the six primers used, (OPAA-09, OPW-02, OPB-18, OPR-08 and OPA-13) with an average of 9.8 bands per primer. Among the primers used OPA-11, produce the maximum number of bands. The molecular weight ranged from 100 – 900 base pair. The Polymorphic information content varied over 46.5%. The similarity coefficient values ranged from 0.432 to 1.00 while the polymorphic information content (PIC) ranged from 0.651 (OPAA-09) to 0.720 (OPA-11). Thus the alleviated level of heterogygosity reveals that the populations will be less resilient to threats and less adaptive.

Study was also conducted to assess the genetic diversity among the colonies of *Scotophilus heathii* form ten different location of Uttar Pradesh. The aim of this study was to see the level of heterozygosity (Polymorphism) among the populations studied. This vesper bat usually is ubiquitous and known to occupy the highest number of bats species in the chiropteran family. This insectivorous choose to reside in the temperate areas of Europe and Asia resting during the day, feeding at night hours and often hibernating during the winter season. Although *S. heathii* have high potential for dispersion, yet they can display unexpected levels of genetic differentiation and strong geographic structure. RAPD markers were used to analyze the genetic relatedness among the ten populations of *S. heathii* in different geographical regions of Uttar Pradesh. The specimens were sampled from They are Balrampur BLR [1], Mirzapur MZR [2], Allahabad ALD [3], Sultanpur STR [4], Maharajganj MRJ [5], Gorakhpur GKP [6], Raebareli REB [7], Azamgarh AZH [8], Unnao UNO [9], and Fatehpur FTP [10].

RAPR-PCR reaction was designed using a set of six primers OPR-08, OPA-11, OPAA-09, OPB-08, OPC-07 and OPA-17. During the optimization of the RAPD-PCR reaction a similar method called ramped cycle was used for the amplification. This manipulation allows a small time at the annealing phase and a longer time at the extension with slower temperature than usual. This method allows the even poorly fitting primers to be locked up onto the template DNA. All the ten samples of *S. heathii* collected showed noticeable level of genetic variation. All the primers expressed polymorphism and a total of 115 bands were detected. Out of the total bands observed 63% were found to be monomorphic while 36% of them gave polymorphic bands. The highest polymorphic information content were obtained with primer 0.0893 (OPR-03) and lowest with the primer 0.498 (OPA-17) with a mean of 0.70. The amplification product size ranged from 150 – 950 base pair. The discrimination power of each locus was evaluated with the help of PIC value. The amount of genetic relatedness among the population of *S. heathii* spread over different regions was deduced by Jaccard coefficient analysis. Among the results obtained from all the populations studied the maximum similarity 72% was observed between the population of Raebareli REB [7] and Maharajganj MRJ [5]. Minimum similarity value 39% was obtained between the populations of Balrampur BLR [1] and Unnao UNO [9]. From the above result it can be concluded that even if the species of *S. heathii* far or close by geographical distance did not play a major role in separating them at a genetic level. Further these species changes the habitat according to their suitable niche and space, and of course the evolutionary potential of any species help them to cope with the changing environment.

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Assessment of Genetic Diversity among the Populations of Mouse-tailed Bats (Rhinopomatidae) using RAPD-PCR

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ABSTRACT

This study was aimed to assess the genetic diversity among the populations of *Rhinopoma hardwickii* from five different regions of Uttar Pradesh using RAPD-PCR. A total of 51 bands were amplified by the five primers (OPA-02, OPA-03, OPB-08, OPB-18 and OPD-02) with an average 10.2 bands per primer and the molecular weight ranged from 912-158bp. The polymorphic loci among the populations of *R. hardwickii* varied considerably (56.86%). Among the primers used, OPB-08 produced more amplified fragments. The similarity coefficient values among the populations ranged from 0.60 to 1.00. Polymorphic information content value ranged from 0.303 (OPB-18) to 0.557 (OPB-080), with a mean PIC value of 0.43. The PIC suggests an enormous heterozygosity associated with a high degree of polymorphism among the population, despite the increasing fragmentation of its habitat. Thus, the high genetic diversity observed in this study supports the less threat to the populations of *Rhinopoma hardwickii.*

Key words *Genetic diversity, Jaccard's coefficient, Microchiroptera, Polymorphism, Rhinopoma hardwickii*

Nowadays, molecular genetic techniques have turned to be anoble approach to reveal population genetics and evolutionary ecology. Rhinopomatidae bats are represented by single genus and regarded as most primitive (Koopman *et al*., 1993; Eicki *et al*., 2005). Despite the fact that almostall molecular experts have challenged to characterize mitochondrial DNA, the RAPD still holds the best marker that gathers maximum information about many vertebrate species, chiefly concerned with the evaluation of genetic diversity. Genetic markers have the potential to easily detect the level of variation of any population. Application of RAPD markers have been largely used to assess the patterns of descent within the organisms (Cornuet *et al*., 1999). RAPD data, produced by Polymerase Chain Reaction (PCR) have been routinely used since 1990's to study the differences within the species at the chromosomal level (Garrigan *et al*., 2002). It extracts all biological information from the population complexity of the species. Usage of this technique in population biology and the processes that governs them to exist is now prevalent, which is extensively practiced and batsare no exception. PCR-based RAPD uses a 10 decamer primer with non ogram amount of template (Welsh, 1990; Williams, 1993). Many investigators have reported that application of RAPD are beneficial to discriminate and resolve the differences between individuals or populations of bats (Moreira and Morielle-Versuate, 2006; Karuppudurai and Sripathi, 2010). The identity of any species as discrete (cryptic) species is a landmark in population biology. The RAPD analysis was performed in order to characterize the genetic diversity among five populations of *Rhinopoma hardwickii* in Uttar Pradesh.

MATERIALS AND METHODS

Sample collection

The bats were trapped using hoop net with an extensible aluminum pole. A 6 m x9 m Nylon mist-net (Avinet-Dryden, USA) was also used for trapping the bats. A small portion of patagiumt issue (>3 sq. mm) from each pieces was collected using a sterile biopsy-punch (patagium got repaired on its own within 15-20 days). The samples were collected from five different locations of Uttar Pradesh, namely Jhansi Fort, Jhansi (JHS [1], 25 272 27.283 N; 78 342 32.233 E), Gupt Godavari, Chirtakoot (CKTD [2], 25 052 24.093 N; 80 462 .02.383 E), Baba Vasil Shah Sulemani Tomb, Chunar (CAR [3], 25 062 55.953 N; 82 522 09.233 E), Buland Darwaja, Fatehpur Sikhri (FTS [4],27 052 29.333 N; 77 392 48.113 E) and Faizabad Maqbara, Faizabad (FBD [5], 26 462 18.613 N; 82 082 33.863 E). After each collection of samples, the punchedpatagium and the biopsy punch were sanatized with 70% ethanol. Bats were handled with extreme care and not scarified. Precautions were also taken in capturing them to minimize the possibility of stress and accidental fatalities.

DNA isolation and primer screening

The tissue samples were homogenized using mortor and pistle containing 100 µl of lysis buffer 1% SDS, 10 mM Tris-HCl (pH 8.5), 125mM NaCl, 5 mM EDTA and 0.5 mg/ml proteinase K (Sigma Aldrich). Followed by homogenization, the DNA extractionwas carried out by following phenol : chloroform method (Sambrook *et al*., 1989). Both the quality and quantity of extracted DNA were confirmed using 0.7% agarose gel electrophoresis and spectrophotometrically at A260 and A280 nm (Perkin-Elmer USA).

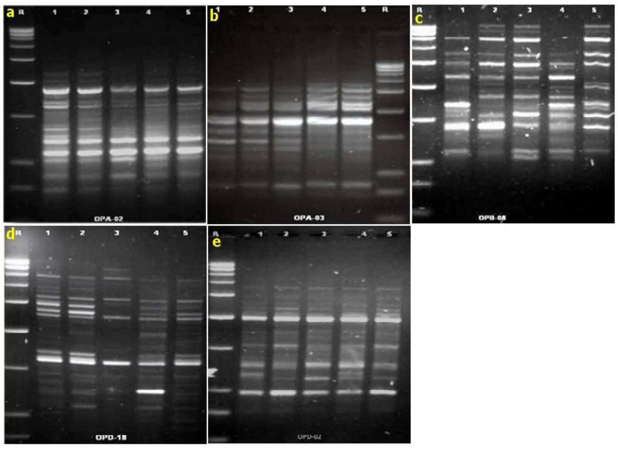

Fig. 1. Shows the number of corresponds to the serial of the genotypes with reference to RAPD primer OPA-01 (a), OPA-03 (b), OPB-08 (c), OPB-18 (d) and OPD-02 (e). R is a 1000bp DNA ladder. The serial numbers in the Figure (a – e) are similar.

Polymerase chain reaction

RAPD-PCR was designed using a set of five primers such as OPA-02, OPA-03, OPB-08, OPB-18, and OPD-02. Conditions for amplification were set by following Williams *et al.* (1990). Optimization was done by taking different concentrations of template, primer, $MgCl_2$ and *Taq* DNA polymerase in order to avoid primer dimer (Banglore Genei, India). Primer optimization was also done by calculating the (T_m) value and annealing temperature. The reactions were carried out in a total volume of 15 µ l containing 25 ng of genomic DNA, 1.5 mM $MgCl_2$ 200 µM of each DNTP, 50 pmol of a single decamer primer (Banglore Genei, India), 0.5 µg/mL BSA, and 1 U *Taq* DNA polymerase. PCR was carried out in a thermocycler (Gene Amp PCR system 2400,Perkin Elmer) programmed at 94 ºC for 3 min followed by 43 cycles of 30 sec at 94 ºC, 1 min at 35 ºC, and two min at 72 ºC. Finallyat last cycle, there was a 5-min of extension at 72 ºC. Initially investigation was carried out with all 13 primers using genomic DNA from five different locations. RAPD-PCR analysis was operated twice so that only primers that gave strong bands were used in the investigation of all the five locations. Gels with amplification fragments were visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad).

RAPD Profile analysis

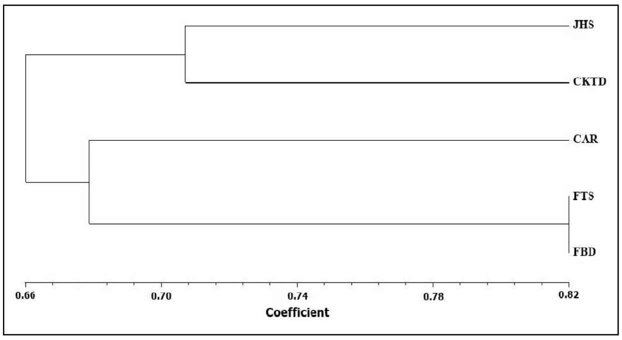

Fig. 2. UPGMA based dendrogram showing the phylogenetic relationship among five populations of *Rhinopoma hardwickii.*

NTSYS-pc version 2.0 software was exploited to evaluate the RAPD data. All calculations related regarding genetic similarity (GS) was done with the help of Jaccard's coefficient of similarity GS(ij) = a/(a+b+c). GS(ij) signify the amount of genetic similarity between the species i and j, b denotes the frequency of bands present in the iwhile absent in j, whereas c indicates the number of bands present in j while absent in i, respectively. Each RAPD patterns was considered as a single character and was scored as 1/0. This binary matrix was constructed for all the bandsscored to generate similarity coefficients. Finally the dendrogram using UPGMA was created by data provided by Jaccard's coefficient. The Polymorphism information content (PIC) a measure of informativeness of these RAPD markers was calculated by following Botstein *et al.* (1980).

RESULTS AND DISCUSSION

In this present study, the RAPD-PCR data showed intra-specific genomic polymorphism among the bat populations. Earlier this marker was widely used to identify genetic diversity (Welsh and Mc Celland,1990;Bowditch *et al.*,1993; Karuppudurai and Sripathi,2010). The five markers in the present study showed reasonable level of diversity (Table 1).Most polymorphism were obtained with OPA-02, OPA-03, OPB-08, OBP-18, OPD-02 as shown in (Table 3). These short oligonucleotides of random sequence gave different level of polymorphism. The primers OPB-08 and OPA-02 were more reproducible and produced a more distinct RAPD profile than primer OPA-03, OPB-18 and OPD-02. A sum of 51 bands were generated and among them 29 bands (56.86%) were polymorphic and 22 bands (43%) found to be monomorphic in nature. The above result showed relatively high genetic diversity among the populations of

Table 1. List of primers, sequences and per cent GC content.

S. No.	Primer	Sequence (5'-3')	% G+C content
1	OPA-02	GGACCCAACC	70
2	OPA-03	AGTCAGCCAC	60
3	OPB-08	GTCCACACGG	70
4	OPA-18	CCACAGCAGT	60
5	OPD-02	GGACCCAACC	70

Table 2. Jaccard's coefficient among five morphotypes of *Rhinopoma hardwickii.*

	1	2	3	4	5
1	1				
2	0.7	1			
3	0.62	0.6	1		
4	0.72	0.66	0.66	1	
5	0.6	0.64	0.68	0.82	1

Table 3. Shows the well amplified primers with number of monomorphic bands, polymorphic bands, per cent of polymorphism and PIC value.

S. No.	Primer code	No. of bands	No. of monomorphic bands	Size of monomorphic bands (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	Polymorphism (%)	PIC
1	OPA-02	11	4	600-200	7	407-199	63.63	0.352
2	OPA-03	10	2	700-200	8	562-144	80	0.504
3	OPB-08	14	7	900-300	7	912-223	50	0.557
4	OPB-18	8	4	900-250	4	771-251	50	0.303
5	OPD-02	8	5	750-300	3	800-300	37.5	0.405
	Total	51	22	43%	29	56%	56.86	

different zones. The Jaccard's coefficient similarity matrix constructed among the five populations was presented in (Table 2). The similarity coefficients values among the populations of *R. hardwickii* ranged from 0.60 to 0.82. UPGMA clustering of RAPD data indicated that the five zones of the *R. hardwickii* populations comprise two different clusters. Cluster A comprised of two populations namely JHS and CKTD, while the cluster B was further divided into two sub groups representing populations FTS and FBD as one group and CAR as a segregated genotype (Fig. 2).

Maximum similarity was observed between the populations of FTS and FBD with similarity coefficient of 0.82. The minimum similarity coefficient (0.60) was observed between the populations of CKTD and CAR. Although, the FTS and FBD populations geographically located apart each other but showed high level of genetic similarity. The genetic makeup of distant populations may be affected by the amount of dispersing nature (resulting in gene flow) among the zones (Perrin and Mazalov, 2000). Nevertheless, a few landscape parameters like rivers, mountains and environmental factors may also impose their effect on genetic variation among mammals (Manel, 2003; Storfer, 2007). In the present study a maximum similarity was observed between the zones JHS and FTS, JHS and CKTD, CAR and FBD, FHS and FBP with similarity coefficient values of 0.72, 0.70, 0.68 and 0.66, respectively. Therefore, gene flow among the populations can be expected to be much higher when they appear closely. The above results reflects a kind of relationship known as isolation by distance, as the geographic distance between the population increases, the genetic similarity decreases (Wright, 1938). Hence the relationship between pattern of gene flow and distance between the populations are not completely dependent on each other but surrounding landscape should also be considered (Gibbs, 2001).

The RAPD profile generated from these primers was utilized to estimate the gene frequency as shown in (Fig. 1 a – e). The primers such as OPA-02, OPA-03,OPB-08,OPB-18 and OPD-02 produced 63.63%, 80.0%, 50.0%, 50.0% and 37.5% polymorphic loci, respectively. However, some unique bands (polymorphic) were also detected with primer OPA-02 and OPA-08 with molecular weight 144, 262, 223, 316, 676 and 794 bps. These unique bands indicate the gene that may be responsible for their existence in different environmental heterogeneity and therefore shows variation among the populations. PIC value ranged from 0.303 (OPB-18) to 0.557 (OPB-08) with an average PIC value of 0.43.A high PIC value indicates enormous heterozygosity which was associated with a high degree of polymorphism (Table 3). This indicates that RAPD are well suited for determining the genetic diversity present in *R. hardwickii* populations.

However, *R. hardwickii* is an insectivorous bat that highly distributed throughout Uttar Pradesh. They often roost in caves, tunnel and deserted houses and the genetic variation among the population assigned is not surprising as the population of *R. hardwickii* was observed over the arid region of Uttar Pradesh which facilitated a high level of gene flow (dispersal). Thus, maintaining the gene flow may be crucial for the longer existence of the populations of *R. hardwickii* in Uttar Pradesh.

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Evaluation of genetic diversity within the populations of the Indian false vampire bat, *Megaderma lyra* deduced by RAPD – PCR

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Abstract

Megaderma lyra is a non-migratory carnivorous bat and maintains high level of roost fidelity and its dispersal ability is still obscure. The present work was conducted to examine genetic diversity within the populations of *M. lyra*. RAPD-PCR approach was used to assess the genetic diversity within five colonies located at Barabanki, Uttar Pradesh, India. The RAPD-PCR data illustrates quite a low level of diversity within five populations. A total of 59 bands were amplified with the 6 primers OPA-11, OPAA-09, OPW-02, OPB-18, OPR-08, and OPA-13 with an average of 9.8 bands per primer. The molecular weight ranged from 100-900 bp. The polymorphic loci within the populations of *M. lyra* varied over 46.5%. Among the primers used OPR-08 produced a maximum of 12 bands. The similarity coefficient values ranged from 0.432 to 1.00 while the polymorphic information content (PIC) ranged from 0.651 (OPAA-09) to 0.720 (OPA-11). The alleviated level of heterozygosity reveals that the population will be less resilient to threats and less adaptive.

Keywords: gene flow, genetic diversity, *Megaderma lyra*, polymorphism, RAPD-PCR

Introduction

Megadermatidae bats belong to a large family of carnivorous bats and restricted to the Old World tropics [1]. The Indian false vampire bat, *Megaderma lyra* is a heterogeneous group of echolocating bat. *Megaderma lyra* scattered in patches in different regions of Uttar Pradesh. Like many other tropical bats, *M. lyra* also show its roost fidelity for several years and exhibit no seasonal migration. The sexes of philopatric species exhibit differences in foraging, the male forage about 500 m form the roost while female makes a flight for about 5 km [2]. Female *M. lyra* gets an opportunity to acquire new roosting sites since their home range is relatively larger. It has specially become important for species with isolated distribution, small neighbouring colonies and fragmented populations are more prone to inbreeding, genetic drift, and ultimately leading to loss of genetic diversity [3]. Gene flow may aid adaptation to local conditions by the introduction of either maladaptive or advantageous alleles into a population [4]. Gene flow is often considered to be an important factor to bring genetic diversity within these populations. For instance, it counters drift and may prevent inbreeding depression in fragmented populations [5, 6]. Even within the populations, spatially restricted gene flow may lead to a genetic substructure and may allow selection to lead to micro-site adaptation [7]. Although, IUCN lists *M. lyra* as a least concerned (LC), but conserving this species is of significant importance. Genetic analyses have revealed the consequence of current behavioral and demographic status often supporting evidence for conservation. The physical integrity of *M. lyra* makes them a champion gleaners among bats and often predating insects, pests and even rodents that destroys paddy and crops plants. They are well adapted in caves, old buildings, thatched huts, old disused wells, temples, forts, tunnels, mines, cow sheds in caves, and artificial underground [8]. Several studies were carried out to investigate the behaviour and genetic diversity of *M. lyra* but the exact reasons are still not certain [9, 10]. Now molecular genetic techniques provide easy way to understand the complex behavior and genetic structure [11, 12]. Here, we tried to report the genetic diversity of *Megaderma lyra* via RAPD marker within the neighboring roosts to assess population variation, gene flow, and behavioral information to establish valuable guideline for conservation measures.

Materials and Methods

The tissue samples of bats were collected from the neighbouring colonies of Barabanki, Uttar Pradesh (latitude 26°53'18.72"N; longitude 81°33'24.84"E), India. The roost of *Megaderma lyra* were located at the basement of a storehouse, demolished temple, thatched hut, Pillar crevices and old disused well. They were trapped using a hoop net with an extensible aluminum pole. Bats were also trapped using mist nets. The tissues samples were collected from five neighboring populations located at Barabanki, i.e. FTP [1] (Fatepur Population – 01), RNR [2] (Ramnagar population – 02), NBJ [3] (Nawabganj population – 03), GSP [4] (Gauspur population – 04) and HDH [5] (Haidergarh population – 05). All the neighboring populations were located between 7 and 9 Km without any physical barriers (Fig. 1).

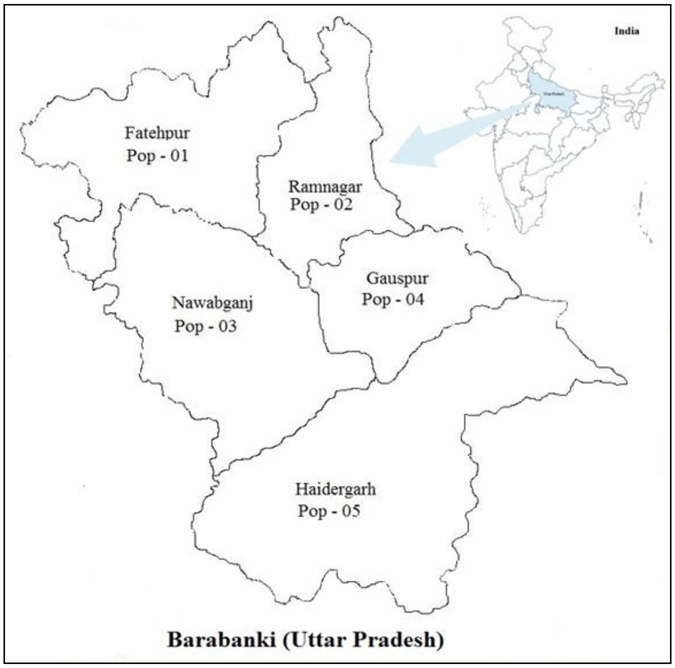

Fig 1: Represents the study sites in Barabanki district, Uttar Pradesh.

A small bit of patagium tissue (>3 sq. mm) from individual bat was collected using a sterile biopsy-punch and the sampled bats were collared for recognition. After collection of each sample, the punched patagium and the biopsy punch were sanitized with 70% ethanol. No biopsy mark was found during the subsequent observation made after a fortnight. The tissues were preserved at –20 °C in 25% DMSO for further DNA extraction. Bats were handled with extreme care and not scarified. Precautions were also taken in capturing them to minimize the possibility of stress and accidental fatalities.

DNA isolation screening

The tissue samples were homogenized using mortor and pistle containing 100 µl of lysis buffer 1% SDS, 10 mM Tris-HCl (pH 8.5), 125 mM NaCl, 5 mM EDTA and 0.5 mg/ml proteinase K (Sigma Aldrich). Followed by homogenization, the DNA extraction was carried out by following phenol:chloroform method [13]. Both the quality and quantity of extracted DNA were confirmed using 0.7% agarose gel electrophoresis and spectrophotometrically at A260 and A280 nm (Perkin-Elmer USA).

Polymerase chain reaction

RAPD-PCR was designed using a set of six primers such as OPA-11, OPAA-09, OPW-02, OPB-18, OPR-08 and OPA-13. Conditions for amplification were set by following [14]. Optimization was done by taking different concentrations of template, primer, $MgCl_2$, and *Taq* DNA polymerase in order to avoid primer dimer (Banglore Genei, India). Primer optimization was also done by calculating the (T_m) value and annealing temperature. The reactions were carried out in a total volume of 15 µl containing 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 µM of each DNTP, 50 pmol of a single decamer primer (Banglore Genei, India), 0.5 µg/mL BSA, and 1 U *Taq* DNA polymerase. PCR was carried out in a thermocycler (Gene Amp PCR system 2400, Perkin Elmer) programmed at 94 ºC for 3 min followed by 43 cycles of 30 sec at 94 ºC, 1 min at 35 ºC, and two min at 72 ºC. Finally at last cycle, there was a 5-min of extension at 72 ºC. Initially investigation was carried out with all 13 primers using genomic DNA from five different locations. RAPD-PCR analysis was operated twice so that only primers that gave strong bands were used in the investigation of all the five locations. Gels with amplification fragments were visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad).

RAPD profile analysis

NTSYS-pc version 2.0 software was exploited to evaluate the RAPD data. All calculations related regarding genetic similarity (GS) were done with the help of Jaccard's coefficient of similarity GS(ij) = a/(a+b+c). GS(ij) signifies the amount of genetic similarity between the species i and j, b denotes the frequency of bands present in the i while absent in j, whereas c indicates the number of bands present in j while absent in i, respectively. Each RAPD patterns was carried as a single character and was scored as 1/0. This binary matrix was constructed for all the bands scored to generate similarity coefficients. Finally a dendrogram using UPGMA was created by data provided by Jaccard's coefficient. The Polymorphism information content (PIC) a measure of informativeness of these RAPD markers was calculated by following Botstein *et al*. (1980) [15].

Results

The sample collected from the Population site showed noticeable changes in genetic diversity from barabanki (Uttar Pradesh) (Fig. 1). Out of 10 primers screened, most polymorphisms were obtained with OPA-11, OPAA-09, OPW-02, OPB-18, OPR-08, and OPA-13 (Table 1).

Table 1: List of primers, decamer sequences and percent of G+C content.

S. No.	Primer	Sequence	% G+C content
1	OPA-11	CAATCGCCGT	60
2	OPAA-09	AGATGGGCAG	60
3	OPW-02	ACCCCGCCAA	70
4	OPB-18	CCACAGCAGT	60
5	OPR-08	CCCGTTGCCT	60
6	OPA-13	CAGCACCCAC	70

The RAPD fingerprinting data for the five populations of *Megaderma lyra* showed significant level of genetic variation (polymorphism) within the neighboring colonies (Fig. 2). All the primers used were polymorphic in nature and a total of 59 loci were obtained. Out of which 25 bands were polymorphic and 34 bands detected were monomorphic. The highest polymorphic loci was obtained with primer (OPA-11) and lowest with 2 (OPB-13) and (OPB-18) each. The amplification product size value ranged from 100 to 900 bp. The PIC value ranged from 0.656 (OPA-13) to 0.720 (OPA-11) with a mean PIC value of 0.64 the discrimination power of each locus was evaluated by the (polymorphic information content) value (Table 2).

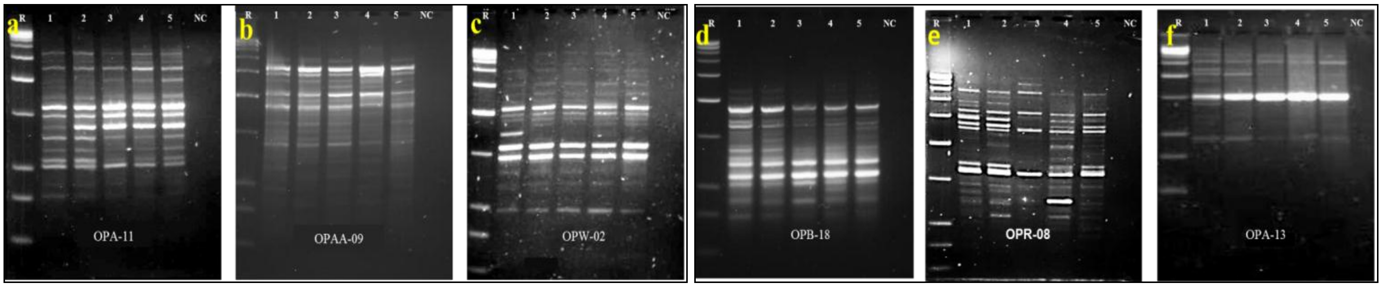

Fig 2: The five neighbouring populations of *M. lyra*: [1] FTP (Fatepur Population – 01), [2] RNR (Ramnagar population – 02), [3] NBJ (Nawabganj population – 03), [4] GSP (Gauspur population – 04) and [5] HDH (Haidergarh population – 05) with reference to primer OPA-11 (a), OPAA-09 (b), OPW-02 (c), OPB-18 (d), OPR-08 (e) and OPA-13 (f). R is a 1000bp DNA ladder. NC represents the negative control.

Table 2: The well amplified primers with number of monomorphic bands, polymorphic bands, and percent of polymorphism value.

S. No	Primer code	No. of bands	No. of monomorphic bands	Size of monomorphic bands (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	Polymorphism (%)	PIC
1	OPA-11	13	6	600-200	7	407-199	46.15	0.720
2	0PAA-09	10	6	700-200	4	562-144	60.00	0.651
3	OPW-02	10	5	900-300	5	912-223	50.00	0.660
4	OPB-18	8	6	900-250	2	771-251	25.00	0.568
5	OPR-08	12	7	750-300	5	800-300	58.33	0.585
6	OPA-13	6	4	100-230	2	300-550	40.00	0.656
	Total	59	34		25			

The genetic similarity values based on RAPD banding patterns were evaluated by using the method Jaccard's coefficient analysis (Table 3). The cluster constructed through NTSys (2.02 Pc) that is represented in the form of dendrogram (Fig. 3). The UPGMA clustering generated form the binary matrix of the studied population comprised of two groups (group A and group B). Group A is further separated into two sub groups representing three morphotypes (pop 05 and pop 03) in one group and (pop 01) as segregated genotype. Group B comprises of two morphotypes (pop 02 and pop 04) respectively. The % similarity within the populations was 72% between FTP [1] and RNR [2], 42% FTP [1] and NBJ [3], 54% FTP [1] and GSP [4], 74% FTP [1] and HDH [5], 62% RNR [2] and NBJ [3], 57% RNR [2] and GSP [4], 67% RNR [2] and HDH [5] and NBJ [3] and GSP [4] each, NBJ [3] and HDH [5], 66% GSP [4] and HDH [5] respectively.

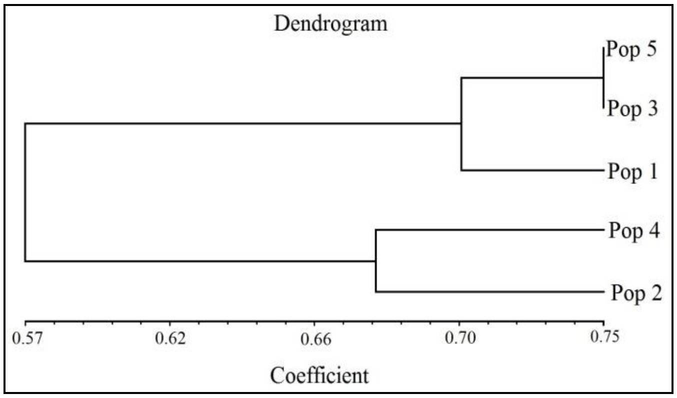

Fig 3: UPGMA based dendrogram showing the phylogenetic relationship within the populations of *Megaderma lyra*.

Table 3: Jaccard's coefficient among five populations of *Megaderma lyra*

	1	2	3	4	5
1	1.00				
2	0.728	1.00			
3	0.423	0.627	1.00		
4	0.543	0.576	0.677	1.00	
5	0.745	0.677	0.610	0.661	1.00

Discussion

The RAPD-fingerprinting showed a high percentage of genetic similarity within the populations of *Megaderma lyra*. Increase in similarity percentage also predicts that the populations maintain high roost fidelity because of long term intra population selection history. However, the low genetic variation limits the species ability to responds to threats in both long and short term [16]. Inbreeding leads to low level of heterozygosity. Heterozygosity may lead to greater probability of the two alleles at a loci being identical. The polymorphic information content (PIC) deduced from the RAPD – fingerprinting ranged from 0.651 to 0.720 suggests less heterogygosity. Decrease in heterozygosis or polymorphism within a population also causes higher mortality, lower fecundity, reduced mating ability, slower growth, more prone to disease, lower ability to withstand stress, leading to intra and inters specific competitive ability [17, 18, 19, 20]. *Mederma lyra*, a non-migratory insectivorous species maintains high degree of roost fidelity and rarely migrate, or disperse over longer distance [21]. It has been reported that the bats equipped with slow manoeuvrable flight and specialized gleaners are likely to make small colonies and maintains site fidelity compared to long range aerial dispersers [22].

It is evident that RAPD markers may also be useful for systematic investigations at the species level as well [23]. More advanced molecular techniques is needed to determine that under what conditions populations could be purged of the genetic loads. Genetic problems are adding to the decline and vulnerability. The contribution of genetic variability to population viability is basically an interaction with physiological, behavioral and ecological processes. For more productive management, options should involve actions to restore genetic variability by reestablishing gene flow by selective translocation.

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when measured in number of individuals (Jones et al., 2009). They

display an exceptionally large ecological niches and diversity (Wilson and Reeder, 2008). Along with pterosaurs and birds, bats are the only mammals to have achieved powered flight. This unique biology has enabled them to be the most widely distributed groups of mammals (Kunz and Fenton, 2003). They wean their young ones when they reach an adult size

that is rear among other mammals (Hayssen et al., 1993).

Nevertheless, chiropteran fauna ranks the rarest animals with least significance because of quite low abundance of several species and ethology (Hulva

et al., 2004). Bats are considered as key stone species, since they are important for proper ecological functioning and whose lose would affect in greater consequence for other species

display an extraordinarily large ecological niches and genetic diversity (Wilson and Reeder 2008). They are the only mammals that have evolved true sustained flight. This unique biology has enabled them to be one of the most widely distributed groups of mammals (Kunz and Fenton, 2003).They are the only mammals that suckle their young ones until they become are nearly adult size (