173265

DNA BARCODING OF THE INSECTIVOROUS BATS OF PARAMBIKULAM TIGER RESERVE, WESTERN GHATS, KERALA

By

PARVATHY VENUGOPAL

(2011-17-101)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN FORESTRY

Faculty of Forestry

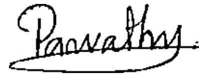
Kerala Agricultural University

DEPARTMENT OF WILDLIFE SCIENCES
COLLEGE OF FORESTRY
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2013

DECLARATION

I hereby declare that this thesis entitled **"DNA barcoding of the insectivorous bats of Parambikulam Tiger Reserve, Western Ghats, Kerala"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.



Parvathy Venugopal

(2011-17-101)

Vellanikkara

Date: 13-09-2013

Dr. P. O. Nameer
Associate Professor and Head
Department of Wildlife Sciences
College of Forestry
Kerala Agricultural University
Vellanikkara, Thrissur, Kerala

Date: 13-09-2013

CERTIFICATE

Certified that this thesis, entitled **"DNA barcoding of the insectivorous bats of Parambikulam Tiger Reserve, Western Ghats, Kerala"** is a record of research work done independently by **Miss. Parvathy Venugopal (2011-17-101)** under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellanikkara

Dr. P.O. Nameer
Chairman
Advisory Committee

CERTIFICATE

We, the undersigned members of the advisory Committee of **Miss. Parvathy Venugopal (2011-17-101)** a candidate for the degree of **Master of Science in Forestry** agree that this thesis entitled **"DNA barcoding of the insectivorous bats of Parambikulam Tiger Reserve, Western Ghats, Kerala"** may be submitted by **Miss. Parvathy Venugopal (2011-17-101)**, in partial fulfillment of the requirement for the degree.

Dr. P.O. Nameer
Associate Professor and Head
Dept. of Wildlife Sciences
College of Forestry,
Vellanikkara, Thrissur
(Chairman)

Dr. D. Girija
Professor and Head
Dept. of Agricultural Microbiology
College of Horticulture,
Vellanikkara, Thrissur
(Member)

Dr. E.V. Anoop
Assistant Professor and Head
Dept. of Wood Science
College of Forestry,
Vellanikkara, Thrissur
(Member)

Dr. T.K. Kunhamu
Associate Professor and Head
Dept. of Silviculture and Agroforestry
College of Forestry
Vellanikkara, Thrissur
(Member)

EXTERNAL EXAMINER

Dr. P.S. EASA
Scientist, Wildlife Biology
Kerala Forest Research Institute
Peechi - 680 653

ACKNOWLEDGEMENT

With at most respect and immense admiration I wish to place on record the heartfelt gratitude to my major advisor Dr. P.O. Nameer, Associate professor and Head, Department of Wildlife Sciences, for his sustained and amiable support, help, warm concern, constant evaluation and comments throughout the period of research work.

I owe my sincere thanks to my advisory committee members Dr. D. Girija, Professor and Head of Department of Agricultural Microbiology, College of Horticulture, Dr. E.V. Anoop, Associate Professor and Head, Department of Wood Science, and Dr. T.K. Kunhamu, Associate Professor and Head, Department of Silviculture and Agroforestry, College of Forestry, for their timely interventions, valuable advice and suggestions, friendly co-operation and constant encouragement during the study.

I take this opportunity to render my sincere gratitude to Dr. K. Sudhakara, Dean, College of Forestry for extending the available facilities in the college for conducting the present study. I express my deep sense of gratitude to Kerala Agricultural University for the financial assistance.

I am thankful to Mr. Vijayanandan IFS, Wildlife Warden, Parambikulam Tiger Reserve, for granting permission to access the Tiger Reserve. I also extend my sincere thanks to Mr. Jayaprakash (RFO, Karimala Range, PKTR) Mr. Manoj (RFO, Parambikulam, PKTR) Mr. Nadeshan (former RFO, Parambikulam, PKTR) Mr. Manoj (Wildlife Assistant, PKTR), for extending the facilities for field work and also for providing field staff during the strenuous field works.

Special thanks are due to forest watchers Mr. Sreenidasan, Mr. Vijayan, Mr. Shanmughan, Mr. Babu, Mr. Shelwan and the Tiger

Monitoring Team of Parambikulam Tiger Reserve who had been with me throughout the field work making the most strenuous task the least one.

Words are not enough to express my heartfelt acknowledgement towards Mrs. Soumya Sunny Llyod, Research Assistant, Wildlife Forensic Unit, Dr. Praveen Karanth, Associate Professor, Centre for Ecological Sciences, Indian Institute of Sciences, Bangaluru, and Dr. Siddharthan, Post-doctoral fellow, CES, IISc who taught me the initial lessons of the laboratory works and molecular phylogeny. They were the dragomen of my research and I am indebted to them because my work would be an incomplete one without their help, support, advice and suggestions.

Help provided by Mrs. Rincy Avarachan, Miss. Maneesha Babu, Research assistance, Wildlife Forensic Unit, College of Forestry, and Mrs. Sajitha M. S., Lab Assistant, during the laboratory studies will always be remembered with great sincerity.

I wish to thank Mr. M. Shaji, Assistant Professor, Department of Wildlife sciences, Mr, Sreehari, R., Teaching Assistant, Department of Wildlife Sciences, Mr. Adarsh Mohandas, Teaching Assistant, Department of Wood Science, for their timely support, suggestions and help.

I remember the benefaction provided by Dr. M. M. Animon, Miss. Mitra Menon, Miss. Jasica Schell, Mr. Felix Francis, Miss. Annie Anna Elias, Mrs. Parvathy Venugopal, Mr. Jomals Mathew, Mr. Vijay Menon and Mr. Aneesh Ramakrishnan and I wish to express my sincere gratitude to them for spending their valuable time for searching and sending the literatures those I wanted to write my thesis.

Words cannot really express the power of relations that I experienced from Mrs. Yesoda Bai, Miss. Remya Raghavan, Mr. Kiran Thomas and Mr. Vishnu, R. Whenever I was in need, they gave the mental strength and

support in life and that really encouraged me to successfully complete this task without facing any hurdles.

I am grateful to Mr. Sajeer, K.V., Mr. Sachin, K. Aravind, Miss. Jyothi, K.M., Mr. Jobin, K. Mathew, Mr. Venkidesh, Miss. Devika, V. S., Mr. Akhil Das, Mr. Raneesh, C., Mr. Vishnu Satheeshan, Miss. Surya Soman, Mrs. Mereena M.J., Miss. Vinu Jacob, Mrs. Ashmi Rohit, Miss. Delphy Rocha, Miss. Sukanya, S., Miss. Anju Vijayan, Mr. Anish, M.C., Mr. Iqbal, A., Miss. Samritika Takur, Miss. Saveen Takur and all other classmates for their co-operation and succour.

Since it is impossible to mention the name of all my dearest juniors who offered the stead and uninhibited moral support, I would like to remember all of them with a great gratitude.

Whatever the weakness of this book would have been far greater without the immense help to the lab care takers Mrs. Katheeja, Mrs. Prema and Mrs. Sheeja for their incredible moral support.

In this occation, I express my heartfelt love, affection and respect towards my home and menage where I come from and the source of my pride. They have always allowed me to follow my heart to do what I thought best for me. They made it possible this work to come into being and get off the ground.

I would also like to thank the countless others whom I may have forgotten.

Above all I bow my head with all due respect before THE INFINITE whose blessings and care enabled me to complete this programme with a great success.

Parvathy Venugopal

DEDICATED

TO

MY BELOVED ONES

(Who taught me the power of love and knowledge)

CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
1.	INTRODUCTION	1-2
2.	REVIEW OF LITERATURE	3-26
3.	MATERIALS AND METHODS	27-34
4.	RESULTS	35-55
5.	DISCUSSION	56-62
6.	SUMMARY	63-65
7.	REFERENCES	66-104
8.	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
1.	Sequences of primer pairs and their predicted size of amplification products	32
2.	Details of sequenced products in the present study	36
3.	Details of BLASTn results	37
4.	Genetic pairwise distance (%) among *Megaderma lyra* and *Megaderma spasma*	40
5.	Genetic pairwise distance (%) among *Hipposideros* species in Indian sub continent	45
6.	Genetic pairwise distance (%) among *Rhinolophus* species in Indian sub continent	51

LIST OF FIGURES

Fig. No.	Title	Page No.
1.	Study area – Parambikulam Tiger Reserve	29
2.	The primer positions of L14724 and H15149 on mitochondrial DNA	33
3.	Phylogenetic relationship among partial cytochrome b sequences of family Megadermatidae	38
4.	The partial sequence of cytochrome b gene of *Megaderma spasma*	41
5.	Phylogenetic relationship among partial cytochrome b sequences of family Hipposideridae	43
6.	The partial sequence of cytochrome b gene of *Hipposideros speoris*	47
7.	Phylogenetic relationship among partial cytochrome b sequences of family Rhinolophidae	49
8.	The partial sequence of cytochrome b gene of *Rhinolophus rouxii*	54

LIST OF PLATES

Page No.	Title	Between pages
1.	Water bath	32-33
2.	Centrifuge	32-33
3.	PCR machine	32-33
4.	In wildlife forensic unit	32-33
5.	Gel loading instrument	34-35
6.	UV illuminator	34-35
7.	Gel picture of mitochondrial DNA	34-35
8.	*Hipposideros speoris*	36-37
9.	*Rhinolophus rouxii*	36-37
10.	*Megaderma spasma*	36-37

Introduction

INTRODUCTION

Bats of the order Chiroptera are unique, enigmatic and second largest species rich group of mammals. This diverse group of mammals are found on every major land mass except the Polar Regions and a few oceanic islands. Chiroptera includes 1116 species in world (Simmons, 2005). But as per IUCN Red List of Threatened Species, there are 1,150 species of bats in the world (IUCN, 2010). That means one in five (21 percent) mammal species is a bat. Bats are distinguished from other mammals by their evolution of true flight, as opposed to the gliding capabilities of mammals in other orders.

Bats are sub-categorized as the Megachiroptera, which consists of 187 species of old world fruit bats in one family, with the exception of a single genus, do not echo-locate and the microchiroptera, which has 963 species of insectivorous bats, having echolocation power, in 17 families (IUCN, 2010). Recent molecular phylogenetic studies challenged this traditional subdivision and proposed that the bats be subdivided into two new suborders, Yinpterochiroptera that includes the families Pteropodidae, Rhinopholidae, Megadermatidae and Rhinopomatida and Yangochiroptera, which include all the remaining families (Teeling *et al.*, 2005).

Bats play major ecological roles in the existence of forest ecosystem as predators and pollinators. The insectivorous bats are the primary predators of nocturnal insects. Hence they play a key role in regulating prey populations. They provide enormous services and beneficial economic impacts by reducing crop pests in agricultural field. Microchiroptera are also very effective in supporting their vegetative habitats, scattering nutrients across the landscape as they fly due to their high mobility. As the fruit bats travel long distances during foraging, they distribute seeds and pollen across large areas, which is especially crucial to the regeneration of cleared areas. In cases such as islands with few wildlife species, fruit bats are thought to play a "keystone" role in forest

maintenance and community structure as the sole pollinators and seed dispersers of local plants.

Eventhough bats are this much prestigious and precious group of mammals, very little is known about their ecology and biology. Although most mammal spècies are thought to have been described, the incidence of overlooked taxa is likely to be high within bats, especially micro bats or insectivorous bats, due to their cryptic behaviour and morphology. The nocturnal habit and cryptic nature of micro bats made their classification or taxonomy difficult.

Biodiversity needs to be classified. The exercise of classifying biological diversity is of great importance because we need to know what's out there and how they are related to each other. This information in turn can be used to our benefit and moreover it is central for the management and conservation of our biological heritage. According to many field experts, classification based on morphological keys needs to be supported with automated techniques based on the analysis of DNA fragments (Bertolazzi *et al.*, 2009). Among various such techniques, DNA barcoding seeks to advance both species identification and discovery through the study of patterns of sequence divergence in a standardized gene region. Moreover, DNA barcodes can aid conservation and research by assisting field workers in identifying and describing species and by helping taxonomists delineating species groups needing more detailed analysis. The barcodes also facilitate the recognition of the appropriate units and scales for conservation planning (Francis *et al.*, 2010).

Western Ghats and Kerala has a rich diversity with 50 species of bats (Nameer *et al.*, 2001) but very little studies were carried out in various diversity aspects of this mammal especially their systematics and no studies were supported with molecular techniques, in Kerala. Hence the present study is carried out with an objective of DNA bar-coding the insectivorous bats of Parambikulam Tiger Reserve, Western Ghats, Kerala to unravel the taxonomic ambiguity.

Review Of Literature

REVIEW OF LITERATURE

2.1 ORDER CHIROPTERA

Bats belong to the order Chiroptera (*cheiros* - hand and *pteron* - wing). Bats are the second most species rich group of mammals after rodents. Chiroptera includes 1116 species in world (Simmons, 2005). But as per IUCN Red List of Threatened Species, there are 1,150 species of bats are in the world (IUCN, 2010). That means one in five (21 percent) mammal species is a bat. In some tropical areas, there are more species of bats than of all other kinds of mammals combined (Hill and Smith, 1984).

Chiroptera sub-categorized as the Megachiroptera consists of 187 species of old world fruit bats in one family and the Microchiroptera, which has 963 species of insectivorous bats in 17 families (IUCN, 2010). Recent molecular phylogenetic studies challenged this traditional classification and proposed that the bats be subdivided into two new suborders, Yinpterochiroptera includes the families Pteropodidae, Rhinopholidae, Megadermatidae and Rhinopomatida and Yangochiroptera, which include all the remaining families (Teeling *et al.*, 2005).

2.1.1 Distribution

Bats are distributed all around the world, including the higher latitudes and remote islands such as New Zealand where they are the only native mammals. Bats are widely distributed and have been recorded throughout the world excepting the Antarctic and a few Oceanic Islands (Mickleburgh *et al.*, 2002). The Microchiroptera are widespread throughout the range of bats, with the greatest diversity occurring in the tropics (Findley and Wilson, 1983) where as the Megachiroptera occurs in the subtropical and tropical regions of the Old World, from the eastern Mediterranean and the Arabian Peninsula, across Africa to Asia, Australia and the islands in the Pacific (Rainey and Pierson, 1992). Some of the

bat families are widespread and are recorded from both the Old World and the New World. Others are restricted in their range and are recorded either only from the Old World or the New World. Of the 18 families of bats, eight families (Pteropodidae, Rhinopomatidae, Nycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Myzopodidae and Mystacinidae) are restricted to the Old World; six families (Noctilionidae, Phyllostomidae, Desmodontidae, Natalidae, Furipteridae and Thyropteridae) are restricted to the New World; and three families (Emballonuridae, Molossidae and Vespertilionidae) are found both in the Old and New Worlds (Mickleburgh *et al.*, 2002; Simmons, 2005).

Only three families have representatives in both hemispheres. They are Rhinolophidae, Vespertilionidae and Molossidae. With increasing distance from the equator a very sharp decline in the number of species of bats are observed (Corbet and Hill, 1986; Koopman, 1993).

Most of the species of bats, about 88 percent are exclusively tropical. They are found everywhere in the world except in the most extreme desert and polar regions. The most important family in the temperate zone is Verspertilionidae.

2.1.2 Megachiroptera Vs. Microchiroptera

The Megachiroptera is commonly known as "fruit bats" because they feed exclusively on, flowers, nectar, pollen and fruits. While about 75% of microchiropterans feed on insects (hence the name "insectivorous bats"), although food sources may include other invertebrates, fishes, amphibians, small mammals (including other bats), blood (Altringham, 1996), fruits, and flowers (Hutson *et al.*, 2001).

As the name implies, Megachiroptera are, on average, larger in size than Microchiroptera, although considerable overlap exists: Megachiroptera weigh from 10 to 1500 g and Microchiroptera from 2 to 196g (Mickleburgh *et al.*, 1992).

With the exception of genus *Rousettus*, megachiropterans do not echolocate, but rely on vision and smell for orientation (Altringham, 1996; Nowak, 1991) while the microbats have the power of echolocation.

Megachiropteran species control their body temperature within a tight range of temperatures and none hibernates; many microchiropterans have labile body temperatures, and some hibernate (Hill and Smith, 1984; Nowak, 1991).

2.2 BAT MONOPHYLY CONTROVERSY

Bats are monophyletic (i.e., developed from a single common ancestral form) and powered flight evolved only once in mammals. This hypothesis was accepted by the bat research community until 1980s. But the findings in 1980s and 1990s contradicted the monophyly and evolution of powered flight by suggesting that primates and chiroptera (especially megabats) were sister orders and therefore the Megachiroptera and Microchiroptera may not be closely related. There was an extensive list of the features causing monophyly controversy (Pettigrew, 1995; Simmons, 1995). But later the molecular evidences and morphological studies proved that bats are monophyletic and thus there is a single origin of flight in mammals and the 'flying-primate hypothesis' must be rejected (Simmons, 1995; Simmons and Geisler, 1998; Martin, 1999). Phylogenetic analysis with diverse methods resulted in a well-resolved phylogeny, dividing the order Chiroptera into two suborders and four super-familial groups, rendering microbats paraphyletic. The two suborders in the new molecular based classification are Yinpterochiroptera (includes the families Pteropodidae, Rhinolophidae, Megadermatidae and Rhinopomatidae) and Yangochiroptera (includes all the remaining families) (Teeling *et al.*, 2005).

2.3 ECOLOGICAL IMPORTANCE OF BATS

2.3.1 Natural predators of insect pests

Bats are primary predators of vast numbers of insects that fly at night, including many that rank among worlds' most costly, agricultural and forest pests. Just a partial list of the insects these bats consume includes cucumber, potato, and snout beetles, corn-borer, corn earworm, cutworm, and grain moths, leafhoppers and mosquitoes (Ducummon, 1999). It has been found that insectivorous bats on an average, consume the equivalent of their own body weight of insects each night (Akbar *et al.*, 1999). A Florida colony of 30,000 South-eastern *Myotis* (*Myotis austroriparius*) eats 50 tons of insects annually, including more than 15 tons of mosquitoes (Zinn and Humphrey, 1981). Many species of bats acts as good friend of farmers (*Megaderma lyra* or Indian False vampire: Sinha, 1986; *Pipistrellus mimus* or Indian pygmy bat: Whitaker *et al.*, 1999; *Tadarida brasiliensis*: Romano *et al.*, 2000; *Eptesicus fuscus* or Big brown bat: Whitaker and Weeks, 2001). Some bats are depend on Lepidoptera (exclusively on moths - *Rhinolophus mehelyi*: Salsamendi *et al.*, 2008), Coleoptera, Homoptera, Diptera, Chrysomelidae, Brachycera, Chironomidae, Neuroptera and Tipulidae (Sullivan *et al.*, 1993; Waters *et al.*, 1995, Waters *et al.*, 1999; Sharifi and Hemmati, 2001; Sharifi and Hemmati, 2004; in Leisler's bat *Nyctalus leisleri*, Salsamendi *et al.*, 2008: in *Rhinolophus mehelyi*). In addition to consuming insect pests, it is suggested that bats protect crops from pests by "chasing" away insects with their echolocation calls (Ducummon, 1999). Researchers saw a 50 percent reduction in damage to corn plots by corn borers when they broadcast bat-like ultrasound over test plots (Belton and Kempster, 1962). With the growing agricultural emphasis on biological control and integrated pest management, more and more farmers are using bats as a weapon in the war against insect pests (Ducumon, 1999).

2.3.2 Pollination and dispersal of seeds

Ecological interactions between bats and plants are mutualistic, because they yield a direct positive gain in fitness to both. Bats obtain a source of nutrition from plants and in turn transport pollen and disperse seeds of plants (Fleming, 1982). Frugivorous and nectarivorous bats are found in two of the 18 families: Phyllostomidae of the New World and Pteropodidae of the Old World tropical regions. According to Law *et al.* (1999) and Medellin and Gaona (1999) bats carry six times more pollen than birds. It is also reported that bats have the capacity to carry viable pollen over great distances than birds (Southerton *et al.*, 2004).

Bats pollinate and disperse seeds of hundreds of species of plants, including many economically important species such as *Ceiba pentandra* (kapok), *Ceiba grandifolia*, *Durio zibethinus* (durian), *Ficus* sp. (fig), *Mangifera indica* (mango), *Manilkara zapota* (chicle), *Musa* sp. (wild banana), *Ochroma lagopus* (balsa), *Eucalyptus* sp., *Brisimum alicastrum*, *Cecropia* sp.(15 species), *Eugenia* sp., *Piper* sp, *Solanum* sp., *Spondias* sp., *Annona squamosa*, *Polyalthia longifolia*, *Polyalthia pendula*, *Achrus sapota*, *Calophyllum ionophyllum*, *Coccinia indica*, *Terminalia catappa*, *Syzygium nervosum*, *Agave macrocantha*, *Passiflora galbana*, *Passiflora mucronata*, *Careya arborea*, *Cullenia exarillata* and desert plants like *Agave* sp. and *Neobuxbaumia tetezo* (columnar cacti) (Howell, 1980; Fleming, 1982; Fleming, 1991; Fujitha and Tuttle, 1991; Subramanya and Radhamani, 1993; Gonzalez, 1998; Elangovan *et al.*, 1999; Shapcott, 1999; Arizaga *et al.*, 2000; Ganesh and Davidar, 2001; Varassin *et al.*, 2001; Liu *et al.*, 2002; Godinez *et al.*, 2002; Quesada *et al.*, 2003; Nassar *et al.*, 2003; Lobova *et al.*, 2003; Raju *et al.*, 2004; Theis and Kalko, 2004; Nathan *et al.*, 2005). *Cynopterus brachyotis* are found to be an important seed disperser with wide selection of fruits of more than 54 species (Tan *et al.*, 1998).

2.3.3 Seed germination and forest regeneration

Besides pollination and dispersal of seeds, bats play an important role in effecting seed germination. The frugivorous bats consume the fleshy pulp of the fruits and defecate the seeds and create soil seed bank. The separation of pulp from seeds may increase survival by reducing seed predation and microbial attack (Willson and Traveset, 2000). Seed retention time in the digestive tract is one factor affecting seed germination. The effect of the transit of seeds through the intestines of frugivorous animals has been evaluated in some mammal species (Fleming and Sosa, 1994). This intestinal treatment may cause destruction of the seeds, remove impervious layers from them or wash away germination-inhibiting substances (Williams and Arias, 1978; Vazquez-Yanes and Orozco-Segovia, 1986), thereby changing germination patterns (Schupp, 1993).

According to Gonzalez *et al.* (2000) and Shanahanz *et al.* (2001) bats are the important seed dispersers in pastures, dispersing seeds of pioneer and primary species connecting forest fragments and maintaining plant diversity. They also may contribute to the recovery of woody vegetation in distributed areas in tropical humid forests. In island ecosystems in the South West Pacific, fruit bats are considered to be key stone species, because significant declines in forest regeneration rates and diversity would accompany their extinction (Cox *et al.*, 1991). Bats are acting as dispersers for plants of early succession and contribute to the diversity in disturbed areas such as abandoned plantations (Garcia *et al.*, 2000). Bernard and Fenton (2003) have stated that a persistent biological flow may be maintaining among isolated fragments, with bats acting pollinators, seed germinators etc. Maintaining healthy bat population is critical to natural forest regeneration because they play a vital role as the primary seed dispersers in cleared areas (Evelyn and Stiles, 2003).

2.3.4 Indicators of habitat destruction and pollution

The concept and use of indicators, particularly indicator species, has received increasing attention for application in ecologically sustainable forest management (Landres *et al.*, 1988; Noss, 1990; McKenney *et al.*, 1994). Bats could be good indicators of the integrity of biological systems because of their combination of size, mobility, and longevity, as well as the variety of trophic roles they fill (Altringham, 1996; Kunz and Fenton, 2003). Bats are good indicators of environmental pollution as they are vulnerable to contaminants. One example of the potential utility of bats as environmental indicators is the annual accumulation of mercury and other metals in their fur (Hickey *et al.*, 2001). According to Senthilkumar *et al.* (2001) bats can be used for examining the organo-chloride concentration (in their body) and can be the indication of pollutant concentration of that area. Another example is the sensitivity of some species (their abundance and diversity) to habitat disturbance (Phyllostomids: Fenton *et al.*, 1992; Kalko *et al.*, 1999; Fenton *et al.*, 2000; Medellin *et al.*, 2000; Schulze *et al.*, 2000). Roosts and food are two resources that are vital to bats, suggesting that either could be used to advantage when assessing changes in habitat. A study conducted by Wikramasinghe *et al.* (2003) highlights that the position of bats as bio-indicators and victims of agricultural changes. They found that greater habitat quality in terms of prey availability and better water quality on organic farm favoured higher foraging activity by bats. The lack of details about habitat use by individual bats means that we remain unclear about the full potential of bats as indicator species to gauge the conditions of a particular habitat, community, or ecosystem (Fenton, 2003).

2.4 THREATS

There is a general decline in bats population all over the world. The reasons for that, include habitat loss due to distruction and modification (Hutson *et al.*, 2001), disturbance of roosting sites (Tuttle, 1979; Stebbings, 1988; Ransome,

1990; Hutson, 1993; Walsh and Harris, 1996a; Walsh and Harris, 1996b; Ducummon, 1999; Mayen, 2003; Lane *et al.*, 2006), loss of feeding habitats, particularly due to the deforestation of the rain forest (Carroll, 1984; Fujitha and Tuttle, 1991; Evelyn and Stiles, 2003), conflict between bat and fruit growers (Fujitha and Tuttle, 1991; Korine *et al.*, 1999; Ducummon, 1999) and pesticide use associated with agricultural intensification, over exploitation for trade and over hunting (Fugitha and Tuttle, 1991; Craig *et al.*, 1994; Ducummon, 1999), natural disasters like hurricane (Craig *et al.*, 1994) and bad public image due to misleading information about bats often affect their survival near human settlement (Fenton, 2003). An increasing population of humans and their demands for land, resources, and food, often results in the degradation or destruction of several habitat types of bats (Hutson *et al.*, 2001).

2.5 DNA BARCODING

The description and identification of species are very basic to biology. Without taxonomy, biologists in different disciplines would be unable to report their empirical findings or to access available information on their target organisms because they would not be sure of their identities. Taxonomy laid the foundations for the construction of tree of life, makes baseline information available for ecology and conservation studies, and affords man the possibility to take advantage of the less utilized resources offered by the earths' biological diversity (Wilson, 2004). As Dayrat (2005) clearly expressed that the correct and accurate delineation of species boundaries and identification of species are crucial to the discovery of life's diversity because it determines whether different individuals are belonging to the same entity or not. The identification of an organism depends on the knowledge held by taxonomists. But a taxonomists' work cannot cover all taxon identification requested by non-specialists. Taxonomy is one of the most avoided fields of research, suffering from low financial investment and because of that low interest from students (Godfray, 2002; Wilson, 2003). To deal with these difficulties, the 'DNA Barcode of Life'

project aims to develop a standardized, faster and low cost species identification method accessible to non-specialists (i.e. non-taxonomists) (Frezal and Leblois, 2008).

2.5.1 Through the time line of Barcoding

Two and a half centuries after the Carl Linnaeus, there are about 1.5 to 1.8 millions of described species, with an estimate that between five and 100 million species in queue for discovery, identification and description (Wilson, 2003; Blaxter, 2003). Due to this reason, the advent of novel approaches to boost taxonomy, both in terms of investment and popularity, were unavoidable (Godfray, 2002; Hebert *et al.*, 2003a; Tautz *et al.*, 2003; Wheeler, 2007; laSalle *et al.*, 2009).

DNA methods aiming to modernize taxonomy were then proposed (Pires and Marinoni, 2010). The build-up of DNA databases has great potential for the identification and classification of organisms and for supporting ecological and biodiversity research programmes (Savolainen *et al.*, 2005). The idea of a standardized molecular identification system emerged progressively during the 1990s with the development of PCR-based approaches for species discovery and identification. Molecular technique has largely been applied to bacterial studies, microbial biodiversity surveys (e.g. Woese, 1996; Zhou *et al.*, 1997) and routine pathogenic strains diagnoses (e.g. Maiden *et al.*, 1996; Sugita *et al.*, 1998; Wirth *et al.*, 2006). PCR-based methods have also been frequently used in fields related to taxonomy, food and forensic molecular identification (Teletchea *et al.*, 2008) and for identification of eukaryotic pathogens and vectors (e.g. Walton *et al.*, 1999).

One of the first conferences exploring these issues was the DNA Taxonomy Workshop at the Deutsche Staatssammlung in Munich in April 2002, with a funding by the German Science Association (DFG) with the participation of some

100 scientists mainly from European countries (Tautz *et al.*, 2002). At this early stage, the issues much in focus were the most useful markers for the so-called DNA taxonomy (i.e. a universal DNA-based classification system across all groups of organisms), the difficulties in connecting already established names to entities within a DNA-based system (Tautz *et al.*, 2003), and the implications in naming organisms (Minelli, 2003). With a different viewpoint from the German meeting, a group of scientists headed by Paul Hebert at University of Guelph in Canada developed the use of part of one mitochondrial gene as a universal 'identification' marker for animal species (Hebert *et al.*, 2003a; Hebert *et al.*, 2003b). Based on the idea of the 'universal product code', known as 'barcodes' in the retail industry (Brown, 1997), a few DNA nucleotides (e.g. the sequences of a short DNA fragment) may well provide an immediate diagnosis for species.

Several universal systems for molecular-based identification have been used for lower taxa (e.g. nematodes, Floyd *et al.*, 2002) but were not successfully implemented for broader scopes. The Barcode of Life project soon after began that attempt, aiming to create a universal system for a eukaryotic species inventory based on a standard molecular approach (Frezal and Leblois, 2008). As with commercial barcodes, the use of these 'species barcodes' first requires the assembly of a comprehensive library that links the barcodes to organisms. Realizing the potential of molecular identification approach, the Alfred P. Sloan Foundation funded two meetings at Cold Spring Harbor, in March and September, 2003. From these meetings brought the idea that important natural history museums should take the lead in connecting diagnostic DNA sequences both to specimen vouchers in collections and to the existing taxonomic system (Linnean system). In 2004, the Sloan Foundation provided yet another substantial award to establish a secretariat for the 'Barcode of Life', based at the Smithsonian's National Museum of Natural History in Washington, DC, USA. The first international scientific conference on Barcoding of Life was held at the Natural History Museum in London in February 2005, attended by over 200 participants from about 50 countries, and a portable device for DNA sequencing to identify all

life was claimed to now be within reach (Marshall, 2005). The wide acceptance of the barcode of life reflects its scientific success since it was first proposed (Hebert *et al.*, 2004a; Ward *et al.*, 2005; Cywinska *et al.*, 2006; Hajibabaei *et al.*, 2006; Smith *et al.*, 2007; Borisenko *et al.*, 2008; Kerr *et al.*, 2009).

The Consortium for the Barcode of Life (CBOL) was also created and joined by many natural history museums and herbaria, private partners and research organizations (www.barcoding.si.edu) (Savolainen *et al.*, 2005). Today, the CBOL involves cooperation between 200 organizations worldwide. The organization aims to explore and develop the barcode as global standard for species identification (www.barcoding.si.edu) (Pires and Marinoni, 2010). Besides having attracted large amount of money, DNA barcoding research has been facilitated by the Barcode of Life Data System (BOLD), an online resource available to the scientific community which is developed in 2004 and officially established in 2007 (www.boldsystems.org/views/login) (Ratnasingham and Hebert, 2007). This resource offers tools that allow researchers to perform neighbour-joining clustering, in order to store information on the different groups studied, and to identify organisms using an updated sequence library, among other things (Pires and Marinoni, 2010).

2.5.2 DNA Barcoding: definition and objectives

DNA barcoding is a sexy name for an old DNA fingerprinting technology. A DNA barcode is a short gene sequence taken from standardized portions of the genome, used to identify species. The DNA barcode project was initially conceived as a standard system for fast and accurate identification of different species of animals. The scope of this project is now that of all eukaryotic species (Hebert *et al.*, 2003b; Miller, 2007). Usually it is the mitochondrial genome in animal species and chloroplast genome in plant species. This barcoding technique is based on the assumption that DNA sequences are conserved within a species and are diverged between species (Hebert *et al.*, 2003a; Hebert *et al.*, 2004a).

The two main applications of DNA barcoding are to (i) assign unknown specimens to species and (ii) enhance the discovery of new species and facilitate identification, especially in organisms having cryptic, microscopic and those with complex or inaccessible morphology (Hebert *et al.*, 2003b; deSalle *et al.*, 2005; Frezal and Leblois, 2008). But these two activities require different type and amount of data (deSalle *et al.*, 2005).

2.5.3 Morphological Vs. Molecular taxonomy

Taxonomy, the science of discovering, describing, classifying and naming of organisms, had been a subject of many debates in the past few years (Lee, 2002; Blaxter and Floyd, 2003; Lipscomb *et al.*, 2003; Moritiz and Cicero, 2004; Prendini, 2005; Meier *et al.*, 2006; Carvalho *et al.*, 2008; Pires and Marinoni, 2010). These debates centred the use of revolutionary ideas (Godfray, 2002; Hebert *et al.*, 2003b; Tautz *et al.*, 2003) that help to overcome the "taxonomic crisis" of the last decades (Wilson, 1985). This crisis is mainly characterized by a lack of specialists in several groups and by insufficient funding for taxonomic work (Godfray, 2002; Mallet and Willmott, 2003; Pires and Marinoni, 2010). The limitations of morphology-based taxonomy were mentioned by Hebert *et al.* (2003a) are phenotypic plasticity in the characters which lead to incorrect identifications, existence of morphologically cryptic species, insufficient taxonomic keys to identify immature specimens of many species and requirement of high levels of expertise and specialists.

Along with this others like Stoeckle (2003) sighted that the main limiting factor in distinguishing closely related species is likely to be the rate of accumulation of new mutation.

Several alternative and complementary approaches like molecular taxonomy (Tautz *et al.*, 2003; Hebert *et al.*, 2003a), development of investment funds,

information technology, (Wheeler, 2007) and increased utilization of cyber tools (Pyle *et al.*, 2008; laSalle *et al.*, 2009) have been proposed to revitalize traditional taxonomy and help it rise above the taxonomic crisis (Pires and Marinoni, 2010). Among those proposals, DNA barcoding has received increased acceptance because it is simple and affordable (Padial and de la Riva, 2007). Genomic barcodes have only four alternate nucleotides at each position, but the array of nucleotides available for inspection is huge. That is with a possible nucleotide variation of four nitrogenous bases (A, T, C, G) at each site, there are 4^n (where "n" corresponds to the number of nucleotides surveyed) possible codes for any given sequence, making it possible to identify every taxon (Hebert *et al.*, 2003a; Pires and Marinoni, 2010). The survey of just 15 of these nucleotide positions creates the possibility of 4^{15} (1 billion) codes, 100 times the number that would be required to discriminate life if each taxon was uniquely branded (Hebert *et al.*, 2003a). The DNA barcoding can be performed quickly and at low cost (Stoeckle, 2003) without any taxonomic specialists and it helps to identify individuals at any stage of development and cryptic species (Pires and Marinoni, 2010).

Both molecular and morphological data have their own advantages and disadvantages. The superposition of intra and inter specific variation is a serious problem (Meyer and Paulay, 2005; Cognato, 2006; Meier *et al.*, 2006; Whitworth *et al.*, 2007) not only to molecular data but also to morphological, ecological and other sources of data ((Will *et al.*, 2005). Here comes the importance of integrative taxonomy which is an integration of molecular as well as morphological taxonomy (Will *et al.*, 2005; Dayrat, 2005; Pires and Marinoni, 2010). Cryptic species are a good example of the importance of using integrated datasets whenever possible. The use of DNA in addition to morphology helps the recognition of cryptic species that consequently become distinguished based on both sources of characters (Fisher and Smith, 2008; Wiedenbrug *et al.*, 2009; Hamada *et al.*, 2010). There are many articles published from 2003 onwards on the acceptance (Pires and Marinoni, 2010) and rejection of integrated taxonomy (Ebach and Carvalho, 2010).

2.5.4 The choice of genome regions for barcoding

One of the major problem or rather limitation in DNA barcoding discussed so far is the finding of a universal gene region especially for animal kingdom (Frezal and Leblois, 2008). Despite the broad utility of DNA, the choice, length and combination of markers best suited for different questions is highly variable (Rubinoff, 2006). Genomic approaches to taxon diagnosis exploit diversity among DNA sequences to identify organisms (Kurtzman, 1994; Wilson, 1995). Moreover the efficacy of barcoding is centred by the selection of a suitable segment of DNA (Waugh, 2007). In DNA barcoding we usually depend on either mitochondrial genome or nuclear genome as markers.

Use and shortcoming of mtDNA in barcoding

Unlike nuclear DNA, which is inherited from both parents and in which genes are rearranged in the process of recombination, there is usually no change in mitochondrial DNA or mtDNA from parent to offspring. This particular genome is haploid with a maternally inherited pattern and has a low effective population size (one- quarter that of nuclear genome) (Rubinoff *et al.*, 2006). Because of this and rapid mutation rates (Moore, 1995) in animals than that in nuclear DNA, makes mtDNA for assessing genetic relationships of individuals or groups within a species and also for identifying and quantifying the evolutionary history (phylogeny) among different species, provided they are closely related (Rubinoff, 2006). There are multiple copies of mitochondrial gene in all cells whereas there are only two copies of nuclear genes in each cell. Multiples copies of mitochondrial genes make it easier to obtain DNA for PCR and sequencing. The entire mitochondrial DNA codes for protein. There are few non-coding sequences called introns. This makes mtDNA genes shorter and easier to work with. Mitochondrial genes are five to ten times more variable between species than nuclear genes.

Hebert *et al.* (2003b, 2004a, 2004b) established that the mitochondrial gene cytochrome *c* oxidase I (COI) can serve as the core of a global bio-identification system for animal. Hebert *et al.* (2003) used universal primers for amplifying approximately a 650 bp region of the COI gene (Tob *et al.*, 2009). He also describes 100% success when using this technique with Lepidoptera (Hebert *et al.*, 2003b). Several studies have shown that >95% of species possess unique COI barcode sequences (Hebert *et al.*, 2003b; Hebert *et al.*, 2004a; Ward *et al.*, 2005; Hajibabaei *et al.*, 2006; Smith *et al.*, 2006). Along with COI the other mitochondrial DNA markers used most commonly in mammalian species identification are the cytochrome b (cyt b) (Parson *et al.*, 2000; Arif and Khan, 2009) and ND2 (for avian species also, Boonseub *et al.*, 2009).

There are several arguments focusing on the selection of COI and cytochrome b for DNA barcoding. Traditionally the cytochrome b gene was used for species identification (Tobe *et al.*, 2009) but Hebert *et al.* (2003a) demonstrated the biological identification through COI and called it as the "universal barcode" for animals. Actually this started the ongoing debates as to which gene offers the best. The COI amplification does not always ensure the success of the specimen identification (Frezal and Lblois, 2008). Along with this a study conducted by Tobe *et al.* (2009) demonstrated that, for mammalian samples cytochrome b gene will offer greater informative value in smaller fragment. But they also said that for intra-specific variations for similar species one is not better than other. There are several proponents for the partial use of COI in barcoding studies (Armstrong and Ball, 2005; Blaxter *et al.*, 2005; Janzen *et al.*, 2005; Lorenz *et al.*, 2005; Smith *et al.* 2005) because of several reasons. One among them is the nucleotides of the gene that codes for COI show sufficient variation to differentiate between species. And another is the intra-specific variation in this gene is generally greater than ten percent of that observed between species. Moreover, insertions and deletions are rare (Blaxter, 2004; Waugh, 2007).

The mtDNA is not adequate as a sole source of species-defining data due to the following factors: reduced effective population size (Patton and Smith, 1994), introgression followed by hydridization (Funk and Omland, 2003), maternal inheritance, recombination, mutation rate, heteroplasmy, inconsistent and compounding evolutionary processes (Bensasson *et al.*, 2001; Ballard and Whitlock, 2004; Rubinoff *et al.*, 2006). In this situation the nuclear subunit ribosomal RNA genes emerged as an important additional molecular marker because of their abundance in the genome and their relatively conserved flanking regions (Frezal and Leblois, 2008), its use allows efficient species distinction (e.g. for amphibians, Vences *et al.*, 2004; Vences *et al.*, 2005; for truffles, Karkouri *et al.*, 2007; Frezal and Leblois, 2008) and can sometimes provide classifications into MOTU or molecular taxonomic units (Floyd *et al.*, 2002; Blaxter *et al.*, 2005; Frezal and Leblois, 2008). Eventhough the above mentioned problems is existing we can't forget the fact that mtDNA, the genome has long been extremely valuable to a vast array of studies. So the scientists like Rubinoff are not advocating the exclusion of mtDNA data from systematic use but they underlying the need of using mtDNA in conjunction with other sources of data such as nuclear ribosomal DNA (Markmann and Tautz, 2005; Monaghan *et al.*, 2005), morphology or ecology (Rubinoff and Holland, 2005; Rubinoff, 2006).

Plant genome region for DNA barcoding

In plants, especially in higher plants the mitochondrial genome evolves very slowly than that in animals. So because of the limited variations in mtDNA the use of COI in identification is applicable only in (Frezal and Leblois, 2008) some macroalgae (*Rhodophyta*, Saunders, 2005), few groups of fungi (*Penicillium* sp., Seifert *et al.*, 2007), and two ciliophoran protists genera (*Paramecium* and *Tetrahymenas*, Barth *et al.*, 2006; Lynn and Struder-Kypke, 2006; Chantangsi *et al.*, 2007). In angiosperms, several contain non-coding intergenic spacers (e.g. *trnH-psbA*, Kress *et al.*, 2005; Chase *et al.*, 2005; Kress and Erickson, 2007) , plastidial coding sequences (e.g. *matK*, Chase *et al.*, 2007) and typical

phylogenetic markers such as *rbcL* and *trnL-F* (Chase *et al.*, 2005) are used to discriminate species (Savolainen *et al.*, 2005; Frezal and Leblois, 2008). According to Pennisi (2007) at present there is no candidate markers best suited to plant DNA barcoding region. Some others argue that multiple genetic loci might be necessary to account for the common hybridization and polyploidy events in angiosperms (Savolainen *et al.*, 2005). The CBOL Plant Working Group (PWG) also agreed for this multiple genetic locus barcoding system in plants, with one 'anchor' (i.e. universal across the plant kingdom) and 'identifiers' to distinguish closely related species. Lahaye *et al* (2008) advocated the *matL* locus as the best universal 'anchor' for DNA barcoding of plant taxa. They also agreed with the need for an extra locus (i.e. 'identifier') to resolve lower taxon identification (Frezal and Leblois, 2008).

2.5.5 Benefits of DNA barcoding

Renaissance of systematic

The traditional morphological taxonomy is in a crisis (Godfray, 2002; Mallet and Willmott, 2003; Waugh, 2007; Pires and Marinoni, 2010). Into this environment comes DNA barcoding which is not a solution to this crisis but a tool that may help to deal with this crisis. According to its proponents, barcoding neither replaces taxonomy nor reconstruct the phylogeny (Schindel and Miller, 2005; Savolainen *et al.*, 2005). It might help the taxonomists as well as the taxonomy interested people in specimen identification to a species level (Waugh, 2007; Hajibabaei *et al.*, 2007). Barcoding provide a relief from the identification burden to a taxonomist so that they can focus more on other duties (Savolainen *et al.*, 2005). Moreover DNA barcoding can be used before conventional taxonomic work to quickly sort specimens into genetically divergent group in poorly studied taxonomic groups. For example the assessment of ants in Madagascar by Smith *et al.* (2007) showed that DNA barcoding is a valuable addition to the taxonomic tool box (Smith *et al.*, 2005; Hajibabaei *et al.*, 2007; Miller, 2007). The utility of

DNA barcoding can be applied in the identification of cryptic species, extant and extinct and species regardless to life stage or maturity (Waugh, 2007; e.g. Dinoflagellate taxonomy, Litaker *et al.*, 2007; diatomea, Evans *et al.*, 2007; earthworms, Huang *et al.*, 2007).

"DNA taxonomy" is an entirely different approach from DNA barcoding and barcoding is not a part of taxonomy (Waugh, 2007). It is most useful for cryptic species and groups that lack a detailed taxonomic system. DNA taxonomy does not aim to link the genetic entities recognised through sequence analysis with Linnean species (Hajibabaei *et al.*, 2007).

In molecular phylogeny and population genetics

Molecular phylogeny depicts the evolutionary history of organisms. The DNA sequences generated through barcoding is used to assemble the 'Tree of Life' which in turn useful in identifying clades and evolutionary relationships (Blaxter, 2003; Cracraft and Donoghue, 2004; Savolainen *et al.*, 2005; Hajibabaei *et al.*, 2007). By paraphrasing Hajibabaei *et al.* (2007) the number of distinct DNA sequences in environmental sampling and reconstruction of phylogenetic trees to place these sequences into an evolutionary context have been used in several inventories of cryptic biodiversity (e.g. soil bacteria or marine/freshwater micro-organisms).

The typical DNA sequences obtained through the barcoding is not sufficient for rigorously addressing the population level questions (Moritz and Cicero, 2004; Bazin *et al.*, 2006; Hajibabaei *et al.*, 2007). It can be a powerful tool to facilitate comparative studies of genetic diversity in different species.

In ecological studies

DNA barcoding techniques will be increasingly used by ecologists. They will be able to identify a single species from a specimen or an organism's remains and to determine the species composition of environmental samples. Short DNA fragments persist in the environment and might allow an assessment of local biodiversity from soil or water. Even DNA-based diet composition can be estimated using faecal samples (Valentini *et al.*, 2008).

2.5.6 Criticisms on DNA barcoding

Eventhough the DNA barcoding is a widely accepted method due to many of its advantages (Padial and de la Riva, 2007), the scientific world had its own proponents and opponents for this technique in its foundation aspects like the accessibility to general public (Cameron *et al.*, 2006), cost involved (Hajibabaei *et al.*, 2005; Hebert and Gregory, 2005; Meyer and Paulay, 2005; Cameron *et al.*, 2006; Pires and Marinoni, 2010), use of DNA barcoding to delimit new species (Moritz and Cicero, 2004; Lee 2004; Ebach and Holdrege, 2005; Hebert and Gregory, 2005; Rubinoff *et al.*, 2006), the use of molecular distances to construct the neighbour-joining trees used in DNA barcoding (de Salle *et al.*, 2005; Hebert and Gregory, 2005; Cognato, 2006), lack of a well-defined species concept that can be used consistently in DNA barcoding (Rubinoff *et al.*, 2006) and technical aspects like the use of a mitochondrial gene to delineate species boundaries (Blaxter, 2004; Blaxter, 2006; Rubinoff *et al.*, 2006).

On top of all other caveats already mentioned, the restrictions imposed by the very nature of the mitochondrial genome (Rubinoff, 2006; Rubinoff *et al.*, 2006) and the possible loss of the intellectual content of taxonomy (Lipscomb *et al.*, 2003; Pires and Marinoni, 2010) has triggered a series of criticisms to DNA barcoding.

2.6 DNA BARCODING IN BATS

The absence of distinct morphological differences between species obstructs the identification of species in many groups of organisms. There for DNA-based approaches are increasingly used to survey biological diversity (Mayer *et al.*, 2007).

Sequence diversity in the cytochrome *c* oxidase subunit 1 gene has been shown to be an effective tool for identifying and discovering species in various groups of animals, but has not been extensively used in mammals (Clare *et al.*, 2006). So, Clare *et al.* addressed this gap by examining the performance of DNA barcodes in 87 species of bats from Guyana. In their study 81species showed both low intraspecific variation and clear sequence divergence from their congeners, while the other six showed deeply divergent intraspecific lineages suggesting that they represent species complexes. Their study validated the effectiveness of barcoding for the identification of regional bat assemblages and even in highly diverse tropical faunas.

Mayer *et al.* (2007) proved that the molecular species identification increases bat diversity, through their study in Western Palaeartic region. By sequencing the mitochondrial protein-coding gene NADH dehydrogenase, subunit 1 (*nd1*) gene of 534 bats, they corroborated the promise of DNA barcodes in too major respects. First, the genetic identification (with only a few exceptions) of species those were described with classical taxonomic tools. Second, substantial sequence divergence suggests an unexpected high number of undiscovered species.

The mammal species richness within the Southeast Asia may be underestimated by at least 50 percent, and there are higher levels of endemism and greater intra-specific population structure than previously recognized (Francis *et al.*, 2010). In their study, all morphologically or acoustically distinct species,

based on classical taxonomy, discriminated with DNA barcodes except four closely allied species pairs. Many of the currently recognized species contained multiple barcode lineages, frequently with deep divergence and thus suggested the existence of unrecognized species. They also observed that, most widespread species shows substantial genetic differentiation across their distributions.

Recently, Nesi *et al.* (2011) tested the DNA barcoding approach on African fruit bats of family Pteropodidae. The study was the first kind in that sense. They used CO1, complete cytochrome b and the intron 7 of the nuclear b-fibrinogen (FGB) gene as markers. But their results revealed an unexpected discordance between mitochondrial and nuclear genes. So their work showed the failure of DNA barcoding to discriminate between two morphologically distinct fruit bat species and highlights the importance of using both mitochondrial and nuclear markers for taxonomic identification.

One of the largest surveys to employ DNA barcoding strategy on any animal group was carried out by Clare *et al.* (2011). In that study they surveyed current and potential species diversity using DNA barcodes with a collection of more than 9000 individuals from 163 species of Neotropical bats. The study was certainly the largest to date for land vertebrates. Their analysis documented the utility of DNA barcoding tool over great geographic distances and across extraordinarily diverse habitats. And that provides strong support for the continued assembly of DNA barcoding libraries and ongoing taxonomic investigation of bats.

Very recently, Zhang *et al.* (2012) proposed two new methods (DV-RBF and FJ-RBF) to address the problems of non-coding region alignment for species assignment by both coding and non-coding sequences that take advantage of the power of machine learning and bioinformatics. A 100% success rate of species identification was achieved with the two new methods for the 4,122 bats.

DNA barcoding technique is widely used for identifying taxonomically poorly studied group of organisms (Baker *et al.*, 2004; Hebert *et al.*, 2004a; Monaghan *et al.*, 2005; Smith *et al.*, 2005; Smith *et al.*, 2006). Eventhough the mammals, especially the group of bats, are considered as well-known taxonomic group, many aspects of them are poorly known. Similarly many of the biodiversity rich regions of the world are still waiting for their diversity documentation and exploration (Francis *et al.*, 2010). Considering the above facts, the applicability of DNA barcoding should be tested in groups like bats in diversity rich regions for facilitating the conservation planning programmes.

2.6 MOLECULAR PHYLOGENY

A molecular phylogeny is the evolutionary history of a group of entities revealed by the use of molecular data either DNA or Protein. Given that this can only truly be known in exceptional circumstances, the main aim of phylogeny reconstruction is to describe the evolutionary relationships in terms of relative recency of common ancestry. The primary objective of molecular phylogeny is to reconstruct the evolutionary history and represent that in a tree-like structure graphically among genes and species over time. This is an extremely complex process, further complicated by the fact that there is no one right way to approach all phylogenetic problems (Blaxter, 2003; Cracraft and Donoghue, 2004; Savolainen *et al.*, 2005; Hajibabaei *et al.*, 2007).

Phylogenetic diversity is one of indicators of species diversity and it gives useful information for developing conservation strategies (Krishnamurthy and Francis, 2012). This suggests the integration of web-based barcoding databases such as CBOL and iBOL, and phylogenetic diversity analysis tools in helping conservation policy makers (Faith and Baker, 2006; Krishnamurthy and Francis, 2012).

2.6.1 Cryptic species and molecular phylogeny

Cryptic species, two or more distinct species that are erroneously classified and or hidden under one species name (Bickford *et al.*, 2007), have been recognised for nearly 300 years (Sun *et al.*, 2009). It is very difficult to identify those using morphological characters (Chattopadhyay *et al.*, 2012). With the advance of PCR technology and DNA sequencing, research on this kind of species has increased exponentially over the past two decades (Bickford *et al.*, 2007). Since the cryptic species represent undiscovered biodiversity, their identification increases our knowledge on species diversity and their conservation (Bickford *et al.*, 2007; Sun *et al.*, 2009). Ambiguity on the distribution of cryptic species exists across the biogeographical regions as well as across taxa (Bickford *et al.*, 2007; Pfenninger and Schwenk, 2007). Avise (2004) reported the use of molecular markers in identifying closely related species. The discovery of cryptic species can solve many problems in biodiversity and conservation and helps formulate more efficient conservation management policies (Chattopadhyay *et al.*, 2012).

2.6.2 Cryptic diversity in bats

The order Chiroptera is one of the most extensively studied groups within mammals and is replete with examples of cryptic species (Sun *et al.*, 2009; Chattopadhyay *et al.*, 2012) mainly from the temperate zone. In tropics also the diversity is observed within Old World families Hipposideridae and Rhinolophidae (superfamily Rhinolophoidea) (Kingston *et al.*, 2001). Cryptic diversity has been a topic of great interest (Mayer and von Helversen, 2001; Jones and Barlow, 2003; Ibanez *et al.*, 2006; Mayer *et al.*, 2007) and many species of bats from different regions have been identified by molecular techniques (Sun *et al.*, 2009). Recently described cryptic species include, for example, *Plecotus austriacus* and *Myotis mystacinus* from Europe (Mayer and von helversen, 2001), *Hipposideros bicolor* (Kingston *et al.*, 2001), *Eptesicus serotinus* and *M. natterei*

from Europe (Ibanez *et al.*, 2006), *Scotophilus dinganii* from South Africa (Jacobs *et al.*, 2006) *Hipposideros larvatus* (Thabah *et al.*, 2006), *Emballonura alecto* from Asia (Hulva and Horacek, 2006), *Hipposideros khaokhouayensis* (Guillen and Francis, 2006), *Hipposideros khasiana* (Thabah *et al.*, 2006), *Hipposideros boeadii* (Bates *et al.*, 2007) *Rhinolopus macrotis* from China (Sun *et al.*, 2008), *Miniopterus petersoni* from lowland South- eastern Madagascar (Goodman *et al.*, 2008) and *Hipposideros griffini* from Vietnam (Thong *et al.*, 2012).

To accurately assess the number of species both locally and globally, and to set conservation priorities, it is essential to identify and describe cryptic diversity (Murray *et al.*, 2012).

Under all these background, realizing the importance and need of molecular techniques and molecular phylogeny in this era in order to assist and support the classical morphological taxonomy, I attempted the DNA barcoding of insectivorous bats of Parambikulam Tiger Reserve.

Materials And Methods

MATERIALS AND METHODS

3.1 STUDY AREA

3.1.1 Name, Location and Extent

Parambikulam Tiger Reserve (PKTR), lies within the geographical extremes of latitudes $10^0 20'$ and $10^0 26'$N and longitudes $76^o 35'$and $76^0 50'$ E (Fig. 1) in the southern part of Western Ghat, immediately south of Palghat gap. PKTR is the second Tiger Reserve of the state and also the 38th Tiger Reserve of India came into existence in 2010 with an extent of $643.66km^2$ with a core area of $390.89Km^2$ and buffer area of $252.77km^2$. The present study was carried out in Parambikulam Wildlife Sanctuary with an extent of $285km^2$ (Kaler, 2011).

It lies between the Anamalai hills and Nelliampathy hills. The Altitude ranges between 300m and 1438m above MSL. There are seven major valleys and three major river systems. Major peaks in the sanctuary are Karimala (1438m), Pandaravarai (1290m), Kuchimudi, Vengoli (1120m) and Puliyarapadam (1010m). Apart from the natural rivers and streams, the sanctuary possesses three man-made reservoirs namely Parambikulam, Thunacadavu and Peruvaripallam.

A unique forest tramway was in existence at Kuriarkutty at PKTR from 1907 exclusively meant for timber transport from Parambikulam to Chalakudy. Extensive extraction of timber took place during this period, but the tramway was abandoned in 1951. The first plantation in this area was raised in 1912. All the plantations after 1932 were raised under taungya system (Vijayan, 1979).

3.1.2 Habitat and Vegetation

Natural vegetation of this reserve is a combination of Malabar and Deccan elements. Micro climatic fluctuations coupled with edaphic, topographic and

biotic factors have endowed this reserve with rich floral diversity. The PKTR has a variety of habitats, both natural and man-made. Natural habitats include moist deciduous forests to tropical wet evergreen rain forests. Grasslands are seen on the upper reaches of Karimalagopuram and Vengoli hills above 1000m. The man-made habitats are primarily teak plantations, which have an extent of about 90km^2, and were first introduced in the year 1912. In addition to this, a small area of the Tiger Reserve bordering Tamil Nadu is planted with eucalyptus (Menon, 1991).

According to Champion and Seth (1968), the natural vegetation of this reserve can be classified into west coast tropical evergreen forests (1a/c4), west coast tropical semi-evergreen forests (2a/c2), southern moist mixed deciduous forests (3b/c2), southern dry mixed deciduous forests (5a/c3), moist bamboo brakes (2/e3), Ochlandra reed brakes (8a/c1/e1), southern montane wet grasslands (11a/c1/ds2), low altitude marshy grassland-vayals, teak plantations and eucalypts plantations.

3.1.3 Fauna

The sanctuary is endowed with very rich and diverse wildlife due to the mosaic pattern of vegetation. The ecotones (edge effects) created at the interspersion of different vegetation types are the vital wildlife habitats. Such junctions are plenty in this sanctuary and are well distributed as well. Same is the case with water resources constituted by the reservoirs, rivers and streams. The abundant herbivore population present in the sanctuary in turn supports a substantial population of predators like tiger and leopard. And also harbour animals such as sloth bear, gaur, sambar deer, elephant, small cats etc. Nameer and Praveen (2006) recorded 230 species of birds from PKTR. Sivaperuman *et al.* (2005) recorded 51 species of spiders belonging to 19 families and 34 genera from various habitats of PKTR. Jahas and Easa (2008) recorded 19 species of Amphibians and 51 species of reptiles from PKTR.

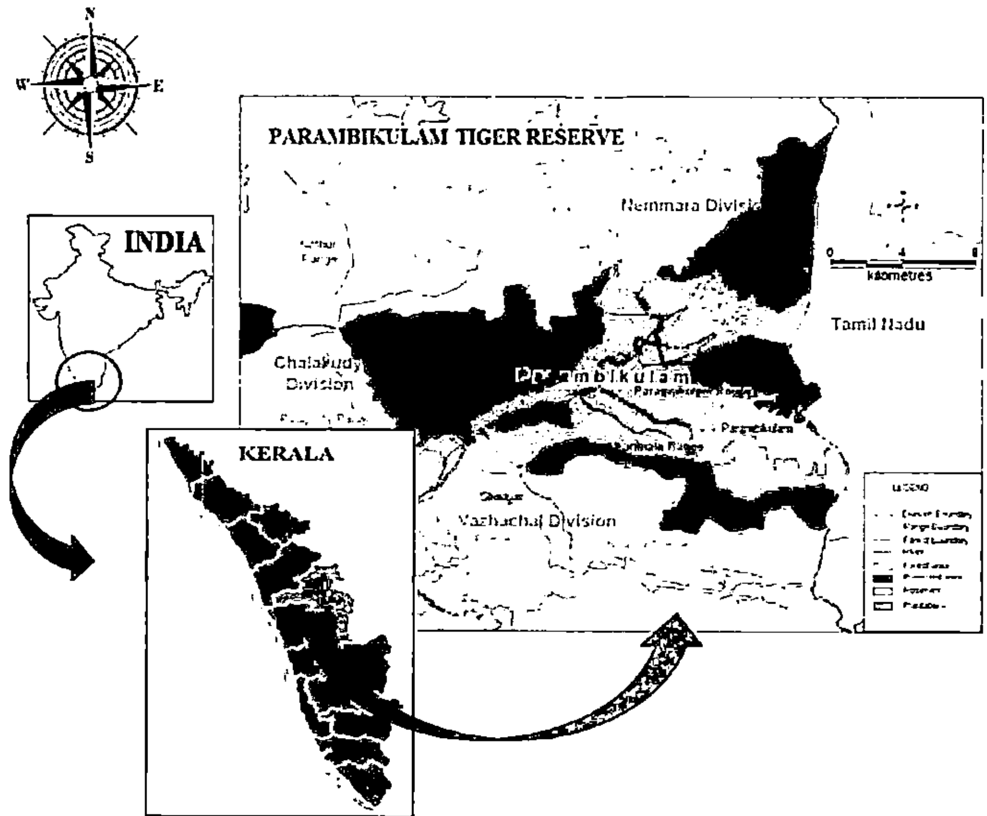


Fig. 1. Study area - Parambikulam Tiger Reserve

3.2 METHODS

3.2.1 Selection of Sites

Representative sample plots in different habitats of Parambikulam Tiger Reserve such as moist deciduous, evergreen, semi evergreen, rocky areas, settlement areas, water bodies, stream sides and altitudinal variant sites were selected and mist netting was done in all these representative habitats. Day transects to the roosting places were also carried out.

3.2.2 Capturing Technique

Most commonly used devices for capturing flying bats are the mist nets (Tuttle, 1976). Mist nets can be used successfully at almost any site where bats shows a high rate of activity like places near roosts, at water holes, feeding sites, along flyways such as animal or human made trials, and natural forest gaps. For survey and census work, to get the least biased estimates of species richness and relative abundance mist nets are placed randomly along transects. Depending on the habitat the configuration in which mist nets are deployed will vary (Kunz and Kurta, 1988). Mist nets set along ridge top and above waterfalls also were effective in capturing flying bats, because individuals often fly close to the ground or near the water in these situations.

3.2.3 Field Study

Mist nets were used to capture bats during this study. The mist nets are made of monofilament nylon with a usual mesh size of 36 mm and an overall size of 10 x 1.5m. Mist nettings were done on the specific sites selected. Capture success depends on the position of the mist nets in the field. Net should be erected up to the height, where the bat activity is high. For decreasing the chance of bat avoiding the net across the water body, it should be raised such that the

lowest edge of the net is as near to the water surface (Kunz and Kurta, 1988). These nets are set across water bodies, caves, streams or trek path. From dusk onwards bats starts their activity. Based on the species the time of bats leaving the roosts varies. Hence dusk is the best time to capture bats. During the present study the nets were erected between 5.30 pm and 6.00 pm and were kept opened for two to four hours after dusk.

In case of dense vegetation, the nets were mostly erected across the corridors (gap between rows of trees) so that the capture success was high. During the netting, two nets were used and raised right angle to each other, which decrease the chance of bats to avoid the nets (Kunz and Kurta, 1988). This definitely increased the capture success. Nets were usually erected on long poles at specific heights.

Nettings were not done continuously on the same site more than two days, as it would affect the capture success (Laval and Fitch, 1977). Nets are watched continuously, if left unattended, the captured bats struggles and become completely entangled that they cannot be removed easily and can also lead to injury to bats and damages to nets. It can also result in the small sized bats to chew out of the net if left unattended for long.

3.2.4 Tissue Sampling and DNA Extraction

We obtained tissues from each bat. DNA was isolated using Phenol-chloroform extraction method (Sambrook *et al.*, 1989) and GeNei™ Mammalian DNA purification kit. The detailed phenol-chloroform method was as follows:

Phenol-Chloroform technique was used for the extraction. For that we weighed 1g of wing tissue and grinded it into a paste in mortar and pestle by adding liquid nitrogen. After this process we added lysis buffer, SDS and

Proteinase K to the ground tissue and incubated the sample at 55°C for overnight for digestion. Then on the second day we centrifuged the digested samples at 10,000 rpm for 10 minutes at 4°C by adding saturated phenol: chloroform: isomyl alcohol in the ratio 25:24:1. After centrifugation, supernatant was transferred and chloroform: isoamyl alcohol were added in the ratio 24:1. Again the samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred and repeated the centrifugation. After the centrifugation add 1/10 volume of 3M sodium acetate and 1ml of Isopropanol to the supernatant. Then incubate the supernatant at -20°C for one hour and after that the samples again centrifuged at 10000 rpm for 10 min at 4°C. Then discard supernatant from the pellet. Removed the supernatant and allowed the pellet to dry. Re-suspend the pellet containing DNA in sufficient distilled water.

3.2.5 Polymerase Chain Reaction (PCR)

The universal primers for PCR amplification were L14724 and H15149 (Fig. 2), as described by Kocher *et al.* (1989) and Irwin *et al.* (1991). The sequences of primers were listed in Table 1.

Table 1. Sequences of primer pairs and their predicted size of amplification products

Primers*	**Sequences**	**Size**
L14724	5'-CGAAGCTTGATATGAAAAACCATCGTTG-3'	486bp
H15149	5-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'	

* Numbering is according to the human mtDNA sequence (Anderson *et al.*, 1981)

Plate 1. Water Bath

Plate 2. Centrifuge

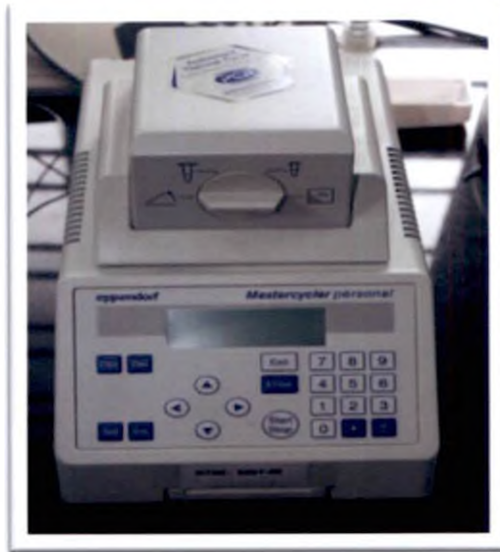


Plate 3. PCR Machine

Plate 4. In Wildlife Forensic Lab

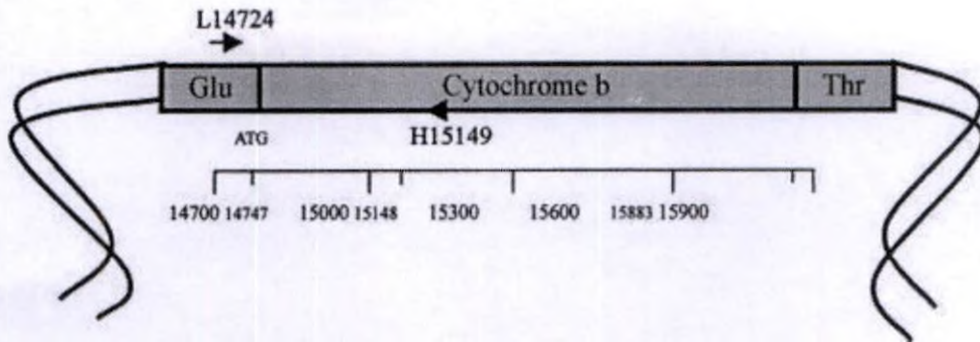


Fig. 2. The primer positions of L14724 and H15149 on mitochondrial DNA

PCR amplifications were performed in a reaction mixture of 25µl, which contained 1µl of isolated genomic DNA, 2µM of primers in 1:10 ratio, 2.5µl reaction buffer (10mM Tris-HCl, pH 8.3, 2.5mM $MgCl_2$, 50mM KCl, 0.01% (w/v) gelatin), 1µM dNTP, and 2 µl of 0.5unit of Taq DNA polymerase and 16.5 µl of distilled water. The PCR conditions were: 29 cycles of 94^0C for one minute, 56^0 C for 45 seconds and final extension at 72^0 C for two minutes. PCR products were visualised on one percent agarose gel containing ethilium bromide and run for about 30 minutes at 100V.

3.2.5 Purification and Sequencing

Column purification and sequencing were done by a private lab, SciGenome Labs Pvt. Ltd.

3.2.6 Sequence Analysis

The sequences were compared with those registered in NCBI databank (blast.ncbi.nlm.nih.gov). Sequences from the cytochrome b gene obtained during this study were aligned through ClustalW (Thompson *et al.*, 1994) implemented in BioEdit ver 7.0.5.3 (Hall, 1999). Multiple sequence alignment allowing gaps was performed using default parameters.

3.2.7 Phylogeny Reconstruction

The phylogenetic trees were generated through neighbour joining method (Saitou and Nei, 1987) using the software MEGA 5.0 (Tamura *et al.*, 2011). Confidence values for internal lineages were assessed with the bootstrapping option (Felestein, 1985). Pairwise distances between all sequences were calculated using the Kimura two parameter model (Kimura, 1980) in MEGA 5.0 (Tamura *et al.*, 2011). This model corrects for multiple hits, taking into account transitional and transversional substitution rates, whilst assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites (Nei and Kumar, 2000). This model was used as it can provide direct comparison with distance measures reported by Bradley and Baker (2001).

Plate 5. Gel Loading Instrument

Plate 6. UV Illuminator

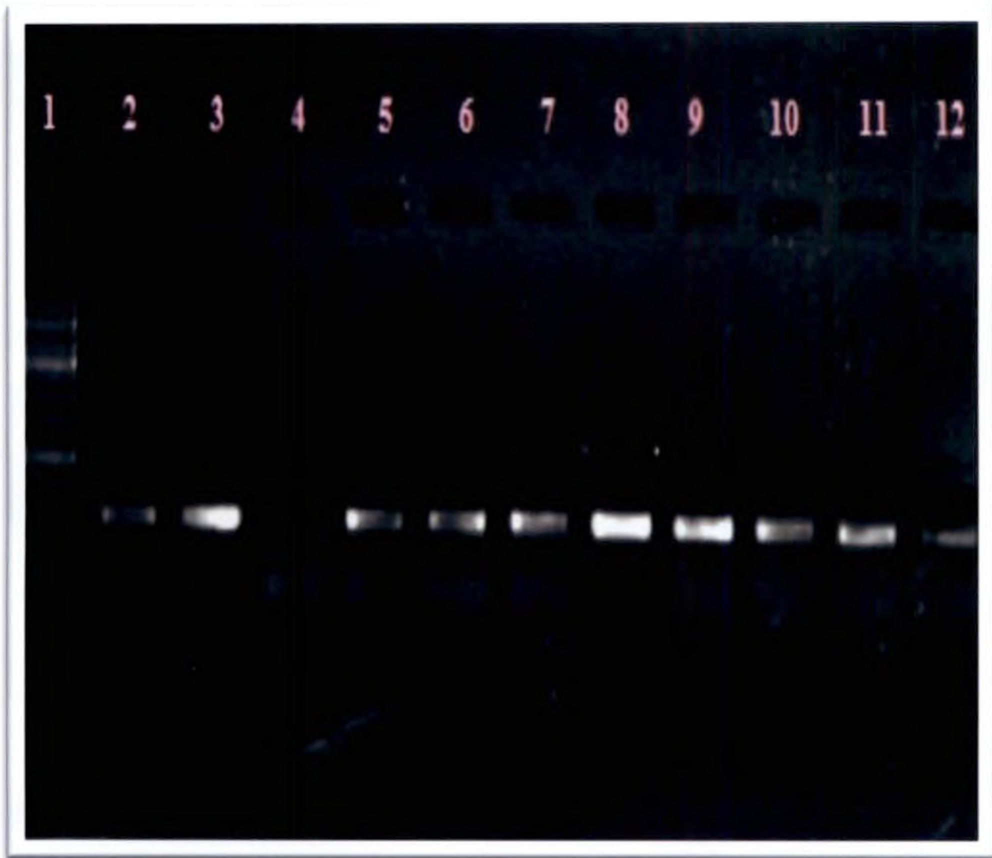


Plate 7. Gel Picture of Mitochondrial DNA

Results

RESULTS

4.1 DNA ISOLATION AND PCR AMPLIFICATION OF PARTIAL CYTOCHROME B GENE WITH UNIVERSAL PRIMERS

The DNA of five species of bats such as *Megaderma spasma, Hipposideros speoris, Rhinolophus rouxii, Rhinolophus beddomei,* and *Rhinolophus lepidus* were isolated using either Phenol-Chloroform extraction (Sambrook *et al*., 1989) method or the GeniPure™ Mammalian Genomic DNA Purification Kit (GeNei™) The universal primers L14724 and H15149 developed by Kocher *et al*. (1989) were used to amplify part of the cytochrome b gene from the collected tissues of bats from the study area. The PCR produced a single amplification product for each genomic template. The size of all PCR products from the bat DNA studied were approximately 400bp when they separated on a one percent agarose gel.

4.2 SEQUENCING OF PCR PRODUCTS

The two universal primers L14724 (forward primer) and H15149 (reverse primer), used in the PCR were used to sequence the PCR products. Sequences obtained from forward and reverse primers were assembled to obtain a contig with the help of DNA Baser version 3.5.4. The size of the cytochrome b gene for the PCR products from the bat tissues varied between 450 to 465 bp including the primer sequences. All the PCR products were successfully sequenced. Table 2 shows the details of sequenced products.

Table 2. Details of sequenced products in the present study

Sl. No.	Specimen number	Location	Sequence length (bp)
1	KAUNHM2012313	Thellikkal	457
2	KAUNHM2012314	Kuriyarkutty	459
3	KAUNHM2012317	Kuriyarkutty	454
4	KAUNHM2012319	Kottayaly	459
5	KAUNHM2012310	Vengoly	460
6	KAUNHM201185	Anapady	459
7	KAUNHM20114	Poopara	458
8	KAUNHM2012318	Kuriyarkutty	459
9	KAUNHM2012320	Vengoly	459
10	KAUNHM2012309	Kuriyarkutty	463
11	KAUNHM2012315	Thellikkal	461
12	KAUNHM201176	Kuriyarkutty	459

4.3 BLASTn ANALYSIS

The sequences were compared with those registered in NCBI databank (blast.ncbi.nlm.nih.gov). The results are shown in Table 3. In the case of KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319, the BLASTn search of cytochrome b gene showed 98% sequence similarity and an E value of 0.0 with *Hipposideros speoris* (Plate 8). The KAUNHM2012310, KAUNHM201183 and KAUNHM2011 displayed 97% similarity with the cytochrome b gene of *Rhinolophus rouxii* (Plate 9) with the highest homology. In this case also the E value was 0.0. Whereas the KAUNHM2012318 and KAUNHM2012320 showed 91% identity and an E value of $2e^{-149}$ with the registered cytochrome b sequence of *Megaderma spasma* (Plate 10).

Plate 8. ***Hipposideros speoris***

Plate 9. ***Rhinolophus rouxii***

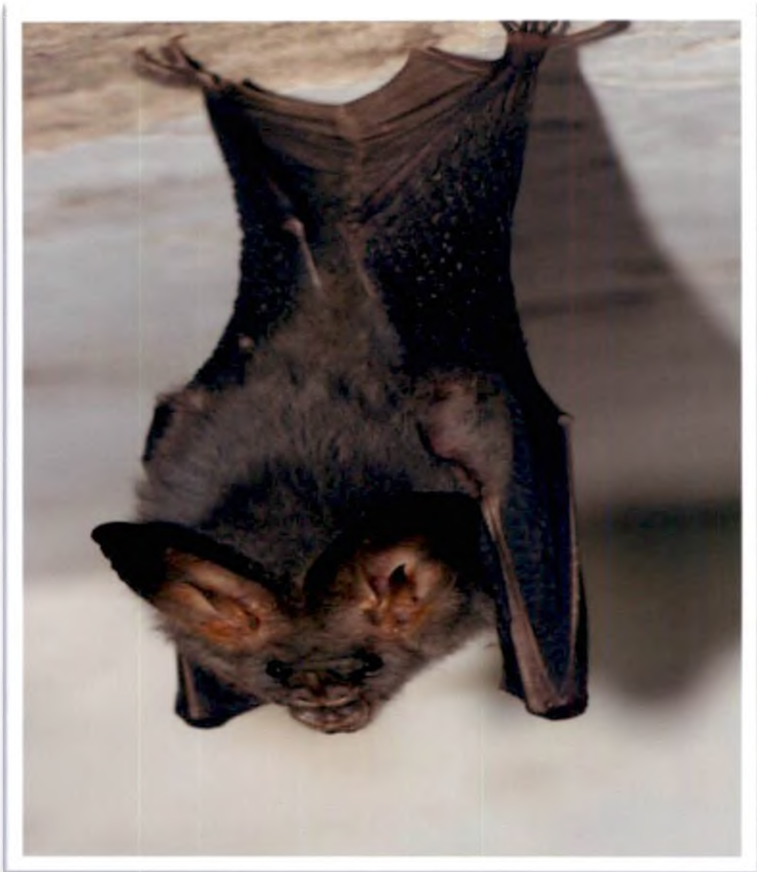


Plate 10. ***Megaderma spasma***

But the sequence comparison of the rest two sequences gave wrong results. One had the maximum identity with a fruit bat and another had maximum identity with a deer species.

Table 3. Details of BLASTn results

<table>
<tr><th rowspan="2">Sample ID</th><th colspan="2">Details of accessions showing homology</th><th rowspan="2">Maximum Identity (%)</th><th rowspan="2">E value</th></tr>
<tr><th>Species</th><th>Accession No.</th></tr>
<tr><td>KAUNHM2012313
KAUNHM2012314
KAUNHM2012317
KAUNHM2012319</td><td>Hipposideros speoris
Schneider, 1800</td><td>DQ680823</td><td>98</td><td>0.0</td></tr>
<tr><td>KAUNHM2012310</td><td rowspan="2">Rhinolophus rouxii
Temminck, 1835</td><td>JQ316214
JQ316213
JQ316212
JQ316211
JQ316206</td><td rowspan="2">97</td><td rowspan="2">0.0</td></tr>
<tr><td>KAUNHM201185
KAUNHM20114</td><td>JQ316210
JQ316209
JQ316202
JQ316201
HM590049</td></tr>
<tr><td>KAUNHM2012318
KAUNHM2012320</td><td>Megaderma spasma
Linnaeus, 1758</td><td>EU521606
AY057942</td><td>91</td><td>$2e^{-149}$</td></tr>
</table>

4.4 PHYLOGENETIC ANALYSIS

4.4.1 *Megaderma spasma* Linnaeus, 1758 (Lesser False Vampire)

Two cytochrome b sequences of *Megaderma lyra* from GenBank (DQ680822, India and DQ888678), two sequences of *Megaderma spasma* from GenBank (AY057942, Central Java and EU521606, Malaysia) and two sequences of *Megaderma spasma* from the present study (KAUNHM2012318 and KAUNHM2012320) were used for the phylogenetic analysis. The two species belong to the family Megadermatidae. These six sequences were aligned in Clustal W (Thompson *et al*., 1994) using BioEdit ver 7.0.5.3 (Hall, 1999) and corrected manually. After the final alignment; the sequence length was 387 bp. Phylogenetic position of the *Megaderma spasma* sequences from the present study (KAUNHM2012318 and KAUNHM2012320) was determined using the neighbour-joining (NJ) tree of Kimura-2-parameter distance model (Kimura, 1980) using MEGA ver. 5.0 (Tamura *et al*., 2011). The branch support was evaluated using 1000 bootstrap replicates (Felestein, 1985). The resultant clustering patterns are given in the Fig. 3. And all the branches showed 100% bootstrap value. The tree was rooted with the sequences of *Hipposideros speoris* (KAUNHM2012313 and KAUNHM2012314) in the present study.

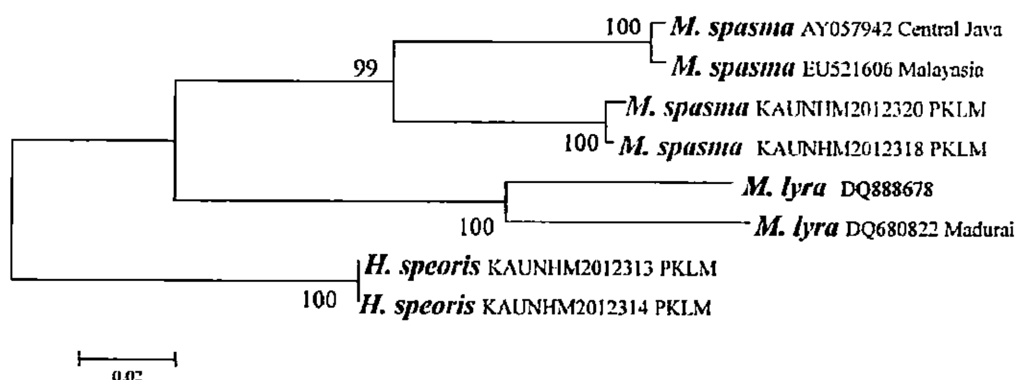


Fig. 3. Phylogenetic relationship among partial cytochrome b sequences of family Megadermatidae. NJ tree was constructed using MEGA ver. 5.0 (Tamura *et al*., 2011). Digits on the nodes indicate the bootstrap values.

The pairwise genetic distance between two cytochrome b sequences of *Megaderma lyra* from GenBank (DQ680822, India and DQ888678), two sequences of *Megaderma spasma* from GenBank (AY057942, Central Java and EU521606, Malaysia) and two sequences of *Megaderma spasma* from the present study (KAUNHM2012318 and KAUNHM2012320) were also analysed (Table 4). In that, the *Megaderma spasma* sequences from the present study (KAUNHM2012318 and KAUNHM2012320) showed more than 20% genetic distance between the available GenBank sequences of *Megaderma lyra* (DQ680822 and DQ888678). But the genetic distance between the available GenBank sequences of *Megaderma spasma* (AY057942 and EU521606) and the *Megaderma spasma* sequences from the present study (KAUNHM2012318 and KAUNHM2012320) was 10.8%. The pairwise genetic distance analysis showed that the two *Megaderma spasma* sequences from the present study (KAUNHM2012318 and KAUNHM2012320) genetically differed from each other by only one percent.

Table 4. Genetic pairwise distance (%) among *Megaderma lyra* and *Megadrma spasma*

Species	*Megaderma lyra* (DQ888678)	*Megaderma lyra* (DQ680822, India)	*Megaderma spasma* (AY057942,Central Java)	*Megaderma spasma* (EU521606, Malayasia)	*Megaderma spasma* (KAUNHM2012318)	*Megaderma spasma* (KAUNHM2012320)
Megaderma lyra (DQ888678)						
Megaderma lyra (DQ680822, India)	9.3					
Megaderma spasma (AY057942, Central Java)	21.0	20.5				
Megaderma spasma (EU521606, Malayasia)	21.4	20.9	0.5			
Megaderma spasma (KAUNHM2012318)	21.5	23.7	10.8	10.8		
Megaderma spasma (KAUNHM2012320)	20.5	22.7	10.8	10.8	1.0	

The two sequences of *Megaderma spasma* retrieved from GenBank (AY057942, Central Java and EU521606, Malayasia) and the two *Megaderma spasma* sequences from the present study (KAUNHM2012318 and KAUNHM2012320) were aligned in BioEdit Sequence Alignment Editor. The final lengths of the aligned sequences were 386bp (Fig. 4). When compared to *Megaderma spasma* from GenBank (AY057942, Central Java and EU521606, Malayasia), the two sequences of *Megaderma spasma* in the present study (KAUNHM2012318 and KAUNHM2012320) showed 38 variable sites (Fig. 4).

```
                                           10        20        30        40        50
M. spasma (AY057942, CJ)          ATGACCCACATCCGGAAATCCCACCCACTATTCAAAATCATCAATGACTC
M. spasma (EU521606, Malayasia    ..................................................
M. spasma (KAUNHM2012320)         ...T...G......A............T..........T...........
M. spasma (KAUNHM2012318)         ..CTG..G......A............T..........T...........

                                           60        70        80        90       100
M. spasma (AY057942, CJ)          ATTCATTGATCTCCCAGCACCATCAAGTATCTCCTCCTGATGAAATTTCG
M. spasma (EU521606, Malayasia    ..................................................
M. spasma (KAUNHM2012320)         G.....C......................................C..T.
M. spasma (KAUNHM2012318)         G.....C......................................C..T.

                                          110       120       130       140       150
M. spasma (AY057942, CJ)          GCTCCCTCCTAGGGATCTGCTTGGCCATCCAAATCCTAACAGGACTATTC
M. spasma (EU521606, Malayasia    ..................................................
M. spasma (KAUNHM2012320)         .............T......C.A...........T.........T.G...
M. spasma (KAUNHM2012318)         .............T......C.............T...........G...

                                          160       170       180       190       200
M. spasma (AY057942, CJ)          CTAGCAATACACTATACCTCAGACACTGCTACAGCCTTCCACTCCGTAAC
M. spasma (EU521606, Malayasia    ....................G.............................
M. spasma (KAUNHM2012320)         ..............C..............C..G......T..........
M. spasma (KAUNHM2012318)         ..............C..............C..G......T..........

                                          210       220       230       240       250
M. spasma (AY057942, CJ)          CCACATTTGCCGAGACGTAAACTACGGCTGAATCCTGCGCTACCTCCACG
M. spasma (EU521606, Malayasia    ..................................................
M. spasma (KAUNHM2012320)         T.................T..............T..A.....T..A....
M. spasma (KAUNHM2012318)         T.................T..............T..A.....T..A....

                                          260       270       280       290       300
M. spasma (AY057942, CJ)          CTAACGGCGCCTCTATGTTTTTTATCTGCCTGTTCCTACACGTAGGACGA
M. spasma (EU521606, Malayasia    ........................................T.........
M. spasma (KAUNHM2012320)         .C...........C..A.....C........A........T.........
M. spasma (KAUNHM2012318)         .C...........C..A.....C........A........T.........

                                          310       320       330       340       350
M. spasma (AY057942, CJ)          GGAATTTACTACGGCTCCTACACATTCACGGAAACATGAAACGTCGGGAT
M. spasma (EU521606, Malayasia    ..................................................
M. spasma (KAUNHM2012320)         ..C....................G.....A....................
M. spasma (KAUNHM2012318)         ..C....................G.....A....................

                                          360       370       380
M. spasma (AY057942, CJ)          CATCCTACTTTTCGCCGTCATAGCCACCGCATTCAT
M. spasma (EU521606, Malayasia    ....................................
M. spasma (KAUNHM2012320)         ...TT....C...........G..............
M. spasma (KAUNHM2012318)         ...TT....C...........G..............
```

Fig. 4. The partial sequence of cytochrome b gene of *Megaderma spasma*. 'Dot' indicates the same base as the first sequence (AY057942; Central Java). All the sequences were 386 bp lengths.

Alignment of *Megaderma spasma*, KAUNHM2012318, with the GenBank deposition, AY057942 from Central Java, showed 31 transitions at nucleotide positions, such as eight, 16, 29, 40, 52, 58, 97, 100, 122,136, 148, 166, 181, 184, 191, 202, 235, 238, 244, 253, 265, 268, 274, 283, 292,325, 331, 355, 356, 361, and 373 and seven transversions at positions, four, three, five, 115, 220, 247 and 304. When compared with *Megadema spasma*, AY057942, the KAUNHM2012320 showed 33 transitions at positions such as eight, 16, 29, 40,

52, 58, 97, 100, 122, 124, 136, 146, 148, 166, 181, 184, 191, 202, 235, 238, 244, 253, 265, 268, 274, 283, 325, 331, 355, 356, 361, and 373 and five transversions at positions four, 115, 220, 247 and 304.

Alignment of *Megaderma spasma*, KAUNHM2012320, with the GenBank deposition, EU521606 from Malaysia, showed 33 transitions at positions, eight, 16, 29, 40, 52, 58, 97, 100, 122, 124, 136, 146, 148, 166, 172, 181, 184, 191, 202, 235, 238, 244, 253, 265, 268, 274, 283, 292, 325, 331, 355, 356, 361, and 373 and five transversions at positions four, 115, 220, 247 and 304. When compared with *Megadema spasma*, EU521606, the KAUNHM2012318 showed 31 transitions at positions eight, 16, 29, 40, 52, 58, 97, 100, 122, 136, 148, 166, 172, 181, 184, 191, 202, 235, 238, 244, 253, 265, 268, 274, 283, 325, 331, 355, 356, 361, and 373 and seven transversions at positions four, three, five, 115, 220, 247 and 304.

The sequences of *Megaderma spasma*, KAUNHM2012318 and KAUNHM2012320, varied by two transversions at third and fifth position and two transitions at 124th and 146th positions.

4.4.2 *Hipposideros speoris* Schneider, 1800 (Schneider's Leaf nosed bat)

Four sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319) from the present study and two sequences of other members of Hipposideridae seen in Indian subcontinent such as *Hipposideros armiger* (JN247016, Vietnam and AF451332, China), *Hipposideros diadema* (EF108149, Malaysia and JQ915899, Philippine), *Hipposideros ater* (JQ915691, Philippine and EF108139, Malaysia), *Hipposideros larvatus* (AF451333, China and JQ915903, Philippine), *Hipposideros galeritus* (JQ915902, Philippine and EU521621, Malaysia), *Hipposideros cineraceus* (DQ054809, Central Laos and JQ915700, Philippine), *Hipposideros pomona* (DQ054810, Central Laos and EU434950, China), *Asellia tridens* (JF439018 and JF439003 from South Arabia), *Coelops frithii* (EU434955,

China and DQ888674, Taiwan) and one sequence of *Triaenops persicus* (DQ005807, Madagasgar) and *Hipposideros speoris* (DQ680823, Madurai, India) from GenBank and were used for the phylogenetic tree reconstruction. The tree rooted with sequences of *Megaderma spasma* (KAUNHM2012318 and KAUNHM2012320) from the present study.

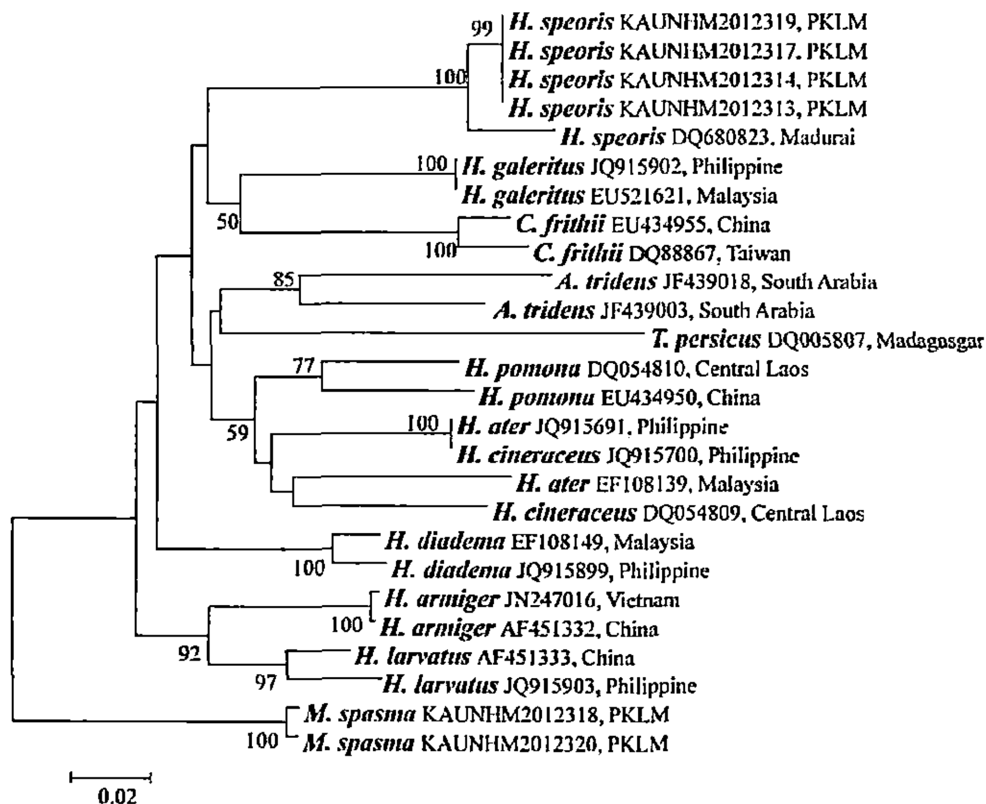


Fig. 5. Phylogenetic relationship among partial cytochrome b sequences of family Hipposideridae. NJ tree was constructed using MEGA ver. 5.0 (Tamura *et al.*, 2011). Digits on the nodes indicate the bootstrap values.

In the neighbour-joining tree the sequences of *Hipposideros speoris* in the present study (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317, and KAUNHM2012319) clustered with the *Hipposideros speoris* (DQ680823; Madurai, India) sequence from GenBank (Fig. 5). The bootstrap value for that branch was 100%. The clustering of other species, except *Hipposideros ater* (JQ915691, Philippine and EF108139, Malaysia) and *Hipposideros cineraceus*

(DQ054809, Central Laos and JQ915700, Philippine), of Hipposideridae considered in this study was with the same species itself. JQ915691 (*Hipposideros ater*) and JQ915700 (*Hipposideros cineraceus*) were clustered together with a bootstrap support of 100% where as EF108139 (*Hipposideros ater*) and DQ054809 (*Hipposideros cineraceus*) were in one group with a very low bootstrap value (41%).

Table 5. Genetic pairwise distance (%) among *Hipposideros* species in Indian subcontinent

Species	*H. armiger* (AF451332, China)	*H. diadema* (EF108149, Malaysia)	*H. ater* (EF108139, Malaysia)	*H. larvatus* (AF451333, China)	*H. galeritus* (EU521621, Malaysia)	*H. cineraceus* (DQ054809, C. Laos)	*H. pomona* (EU434950, China)	*A. tridens* (JF439018, South Arabia)	*C. frithii* (EU434955, China)	*T. persicus* (DQ005807, Madagascar)	*H. speoris* (DQ680823, India)	*H. speoris* (KAUNHM2012313)	*H. speoris* (KAUNHM2012314)	*H. speoris* (KAUNHM2012317)	*H. speoris* (KAUNHM2012319)	*M. spasma* (KAUNHM2012320)
H. armiger (AF451332, China)																
H. diadema (EF108149, Malaysia)	10.9															
H. ater (EF108139, Malaysia)	13.9	14.7														
H. larvatus (AF451333, China)	8.5	12.0	14.0													
H. galeritus (EU521621, Malaysia)	13.7	13.8	12.8	12.4												
H. cineraceus (DQ054809, C. Laos)	14.9	13.6	10.3	11.5	15.3											
H. pomona (EU434950, China)	13.8	14.3	13.0	12.9	15.3	11.1										
A. tridens (JF439018, South Arabia)	16.4	13.4	15.4	16.1	16.1	13.4	12.4									
C. frithii (EU434955, China)	16.1	14.1	15.9	14.4	12.4	15.2	13.8	14.4								
T. persicus (DQ005807, Madagascar)	19.1	17.0	19.1	17.3	16.1	19.5	16.3	18.0	19.8							
H. speoris (DQ680823, India)	15.4	14.5	18.4	15.8	15.7	15.2	15.5	16.4	16.1	22.3						
H. speoris (KAUNHM2012313)	14.8	13.2	17.4	13.8	14.1	12.8	13.9	15.0	14.1	20.9	3.0					
H. speoris (KAUNHM2012314)	14.8	13.2	17.4	13.8	14.1	12.8	13.9	15.0	14.1	20.9	3.0	0.0				
H. speoris (KAUNHM2012317)	14.8	13.2	17.4	13.8	14.1	12.8	13.9	15.0	14.1	20.9	3.0	0.0	0.0			
H. speoris (KAUNHM2012319)	14.8	13.2	17.4	13.8	14.1	12.8	13.9	15.0	14.1	20.9	3.0	0.0	0.0	0.0		
M. spasma (KAUNHM2012320)	16.4	16.7	20.7	17.1	18.4	20.3	17.7	20.6	21.6	21.0	19.5	19.5	19.5	19.5	19.5	

The pairwise genetic distance between different *Hipposideros* species from Indian subcontinent was calculated (Table 5). Sequences of *Hipposideros armiger* (AF451332, China), *Hipposideros diadema* (EF108149, Malaysia), *Hipposideros ater* (EF108139, Malaysia), *Hipposideros larvatus* (AF451333, China), *Hipposideros galeritus* (EU521621, Malaysia), *Hipposideros cineraceus* (DQ054809, Central Laos), *Hipposideros pomona* (EU434950, China), *Asellia tridens* (JF439018, South Arabia), *Coelops frithii* (DQ888674, China), *Triaenops persicus* (DQ005807, Madagasgar) *Hipposideros speoris* (DQ680823, Madurai) from GenBank, four sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317, and KAUNHM2012319) and one sequence of *Megaderma spasma* (KAUNHM2012320) from the present study were used for calculating the genetic distance. And it is observed that the *Hipposideros speoris* (DQ680823) sequence from Madurai, India had a genetic distance ranged from 14.5% [with *H. diadema* (EF108149, Malaysia)] to 22.3% [with *T. persicus* (DQ005807, Madagascar)]. All the five sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317A, KAUNHM2012317B and KAUNHM2012319) from the present study had a genetic distance ranged from 12.8% [with *H. cineraceus* (DQ054809, Central Laos)] and 20.9% [with *T. persicus* (DQ005807, Madagascar)]. There was a three percent genetic distance between *Hipposideros speoris* (DQ680823) sequence from Madurai, India and all the five sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317A, KAUNHM2012317B and KAUNHM2012319) from the present study. There was no genetic difference observed between all the five sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317A, KAUNHM2012317B and KAUNHM2012319) from the present study. The average genetic distance between *Megaderma spasma* (KAUNHM2012320) and the other *Hipposideros* species considered for calculating the genetic distance was 19.22%.

```
                                       10        20        30        40        50
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) ATGACCAACATTC-GAAATCCCACCCACTTTTTAAAATTATCAACGACTC
H. speoris (KAUNHM2012313)     -..........C.GA.....T.............................
H. speoris (KAUNHM2012314)     ....T........-A.....T.............................
H. speoris (KAUNHM2012317)     ...........C.GA.....T.............................
H. speoris (KAUNHM2012319)     ...........C.GA.....T.............................

                                       60        70        80        90       100
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) CTTCATTGACCTACCCGCCCCCTCAAGCATCTCTTCCTGATGAAACTTTG
H. speoris (KAUNHM2012313)     .................................C................
H. speoris (KAUNHM2012314)     .................................C................
H. speoris (KAUNHM2012317)     .................................C................
H. speoris (KAUNHM2012319)     .................................C................

                                      110       120       130       140       150
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) GTTCACTACTGGGCGTATGCCTAGCACTACAGATCCTAACAGGCCTATTC
H. speoris (KAUNHM2012313)     ..........A....................A..................
H. speoris (KAUNHM2012314)     ..........A....................A..................
H. speoris (KAUNHM2012317)     ..........A....................A..................
H. speoris (KAUNHM2012319)     ..........A....................A..................

                                      160       170       180       190       200
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) CTAGCAATACACTACACATCCGACACCGCTACGGCCTTCTACTCCGTCAC
H. speoris (KAUNHM2012313)     .............................C....................
H. speoris (KAUNHM2012314)     .............................C....................
H. speoris (KAUNHM2012317)     .............................C....................
H. speoris (KAUNHM2012319)     .............................C....................

                                      210       220       230       240       250
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) CCATATCTGCCGAGATGTCAACTACGGCTGAATCCTACGCTACCTTCATG
H. speoris (KAUNHM2012313)     ..................................................
H. speoris (KAUNHM2012314)     ..................................................
H. speoris (KAUNHM2012317)     ..................................................
H. speoris (KAUNHM2012319)     ..................................................

                                      260       270       280       290       300
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) CCAACGGAGCATCAATATTCTTCATCTGTCTGTTCCTACATGTCGGCCGA
H. speoris (KAUNHM2012313)     ..................................................
H. speoris (KAUNHM2012314)     ..................................................
H. speoris (KAUNHM2012317)     ..................................................
H. speoris (KAUNHM2012319)     ..................................................

                                      310       320       330       340       350
                               ....|....|....|....|....|....|....|....|....|....|
H. speoris (DQ680823, Madurai) GGTATCTACTACGGCTCTTATACATATACAGAGACATGAAACATCGGGGT
H. speoris (KAUNHM2012313)     ..................................................
H. speoris (KAUNHM2012314)     ..................................................
H. speoris (KAUNHM2012317)     ..................................................
H. speoris (KAUNHM2012319)     ..................................................

                                      360       370       380
                               ....|....|....|....|....|....|....|.
H. speoris (DQ680823, Madurai) CATTCTTCTCTTCGCCGTCATAGCCACTGCCTTTAT
H. speoris (KAUNHM2012313)     ..................T.....A..G..A..C..
H. speoris (KAUNHM2012314)     ..................T.....A..G..A..C..
H. speoris (KAUNHM2012317)     ..................T.....A..G..A..C..
H. speoris (KAUNHM2012319)     ..................T.....A..G..A..C..
```

Fig. 6. The partial sequence of cytochrome b gene of *Hipposideros speoris*. 'Dot' indicates the same base as the first sequence (DQ680823; Maduari, India). All the sequences were 386 bp sizes.

All the four sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319) from the present study and *Hipposideros speoris* (DQ680823; Maduari, India) were aligned using ClustalW Multiple Sequence Alignment (Thompson *et al.*, 1994) of BioEdit programme. When compared to *Hipposideros speoris* (DQ680823; Maduari,

India), all the *Hipposideros speoris* sequences, except KAUNHM2012314, from the present study showed 12 variable sites including nine transitions at nucleotide positions 12, 15, 21, 84, 111, 132, 180, 369, and 384 and three transversions at positions, 375, 378 and 381. The sequence, KAUNHM2012314, had an additional transition at position fifth. A missing data was observed at 14th nucleotide position in KAUNHM2012314 and DQ680823, where as all other sequences, KAUNHM2012313, KAUNHM2012317 and KAUNHM2012319, showed a base Guanine (Fig. 6).

4.4.3 *Rhinolophus rouxii* Temminck, 1835 (Rufous Horseshoe bat)

Three sequences of *Rhinolophus rouxii*, KAUNHM2012310, KAUNHM201185 and KAUNHM20114, from the present study and the GenBank sequences of 80kHz phonic type of *Rhinolophus rouxii* (JQ316201 MDB, HM590049 SRP, JQ316202 MDB, JQ316209 SRP, and JQ316210 SRP) 90kHz phonic type of *Rhinolophus rouxii* (JQ316214 MKM, JQ316213 MKM, JQ316212 MKM, JQ316211 MKM, and JQ316206 YRCD) *Rhinolophus ferrumequinum* (EU360630, Arabia and AB085729, Japan), *Rhinolophus affinis* (EF108160, Malaysia and JN106274), *Rhinolophus hipposideros* (EU360635, Arabia and DQ120922), *Rhinolophus pusillus* (JX465361, China and EF108171, Malaysia), *Rhinolophus luctus* (EF108165, Malaysia and DQ178987, Borneo) *Rhinolophus lepidus* (AF451338, China and FJ185202, Myanmar), *Rhinolophus macrotis* (AF460976, China and EU434957, China) *Rhinolophus trifoliatus* (EF108176, Malaysia and EU521614, Malaysia) *Rhinolophus pearsonii* (AF451340, China and JN106282) from GenBank and were used for the phylogenetic analysis. All the above species belong to the family Rhinolophidae. The phylogenetic tree was rooted with the sequences of *Megaderma spasma*, KAUNHM2012320 and KAUNHM2012318.

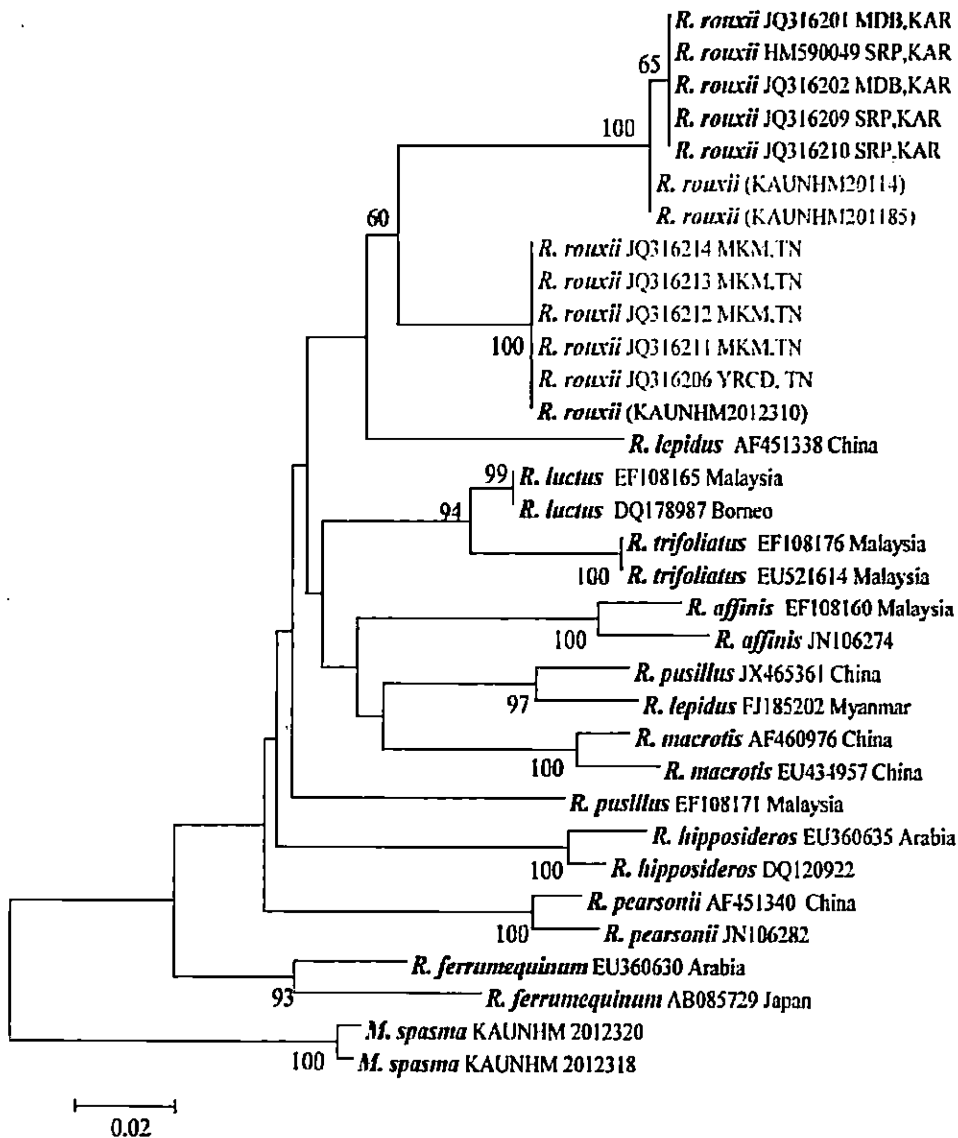


Fig. 7. Phylogenetic relationship among partial cytochrome b sequences of family Rhinolophidae. NJ tree was constructed using MEGA ver. 5.0 (Tamura *et al.*, 2011). Digits on the nodes indicate the bootstrap values.

In the neighbour-joining tree (Fig. 7) the two sequences of *Rhinolophus rouxii* from the present study, KAUNHM20114 and KAUNHM201185 were clustered together in one branch and they again clustered to the GenBank sequences of 80 kHz phonic type of *Rhinolophus rouxii*, JQ316201 MDB, HM590049 SRP, JQ316202 MDB, JQ316209 SRP, and JQ316210 SRP with a bootstrap value of 100%. But the third sequence of *Rhinolophus rouxii* from the

present study, KAUNHM2012310, clustered with the sequences of 90 kHz phonic type of *Rhinolophus rouxii,* JQ316214 MKM, JQ316213 MKM, JQ316212 MKM, JQ316211 MKM, and JQ316206 YRCD with a bootstrap value of 100%. All other sequences of different *Rhinolophus* species clustered sensibly except that of *Rhinolophus lepidus* (AF451338, China and FJ185202, Myanmar) and *Rhinolophus pusillus* (JX465361, China and EF108171, Malaysia). *Rhinolophus pusillus,* JX465361 from China and *Rhinolophus lepidus,* FJ185202 from Myanmar were clustered in the same branch with a high bootstrap value (97%) whereas *Rhinolophus lepidus,* AF451338, from China and *Rhinolophus pusillus,* EF108171, from Malaysia were in two single separate branch with very low bootstrap values of 45% and 15% respectively.

Table 6. Pairwise Genetic Distance (%) among *Rhinolophus* species in Indian sub continent

Species	*R. ferrumequinum* (AB085729, Japan)	*R. affinis* (EF108160, Malaysia)	*R. hipposideros* (DQ120922)	*R. pusillus* (DQ297589, China)	*R. luctus* (EF108165, Malaysia)	*R. lepidus* (AF451338, China)	*R. macrotis* (AF460976, China)	*R. trifoliatus* (EF108176, Malaysia)	*R. pearsonii* (AF451340, China)	*R. rouxii* [JQ316214, MKM TN(90kHz)]	*R. rouxii* [JQ316210,SRP KAR(80kHz)]	*R. rouxii* [KAUNHM20114]	*R. rouxii* [KAUNHM201185]	*R. rouxii* [KAUNHM2012310]
R. ferrumequinum(AB085729, Japan)														
R. affinis (EF108160, Malaysia)	12.0													
R. hipposideros (DQ120922)	16.8	13.6												
R. pusillus (DQ297589, China)	16.8	11.7	16.0											
R. luctus (EF108165, Malaysia)	12.3	11.6	13.9	11.3										
R. lepidus (AF451338, China)	12.3	13.0	13.2	12.3	11.7									
R. macrotis (AF460976, China)	13.7	9.8	15.0	9.2	11.3	12.7								
R. trifoliatus (EF108176, Malaysia)	13.9	10.0	14.9	11.3	3.8	13.3	10.7							
R. pearsonii (AF451340, China)	12.7	11.8	12.3	13.1	12.0	11.7	13.7	12.6						
R. rouxii [JQ316214, MKM TN(90kHz)]	12.9	12.3	13.2	14.3	11.3	9.4	11.9	12.2	11.0					
R. rouxii [JQ316210,SRP KAR(80kHz)]	15.0	14.3	17.0	12.9	12.2	13.0	14.0	13.2	16.4	8.0				
R. rouxii [KAUNHM20114]	16.0	14.3	17.3	12.2	11.6	12.9	13.9	12.5	16.7	9.5	1.9			
R. rouxii [KAUNHM201185]	16.0	14.6	17.3	13.2	12.5	13.3	14.3	13.5	16.7	9.5	1.9	1.1		
R. rouxii [KAUNHM2012310]	13.5	12.9	13.8	14.9	11.9	10.0	12.6	12.8	11.6	1.3	9.4	10.1	9.5	

Table 6 shows the pairwise genetic distance between different *Rhinolophus* species from Indian subcontinent. Three sequences of *Rhinolophus rouxii* (KAUNHM20114, KAUNHM201185 and KAUNHM2012310), from the present study and the GenBank depositions of *Rhinolophus rouxii* [JQ316214 TN(90 kHz) and JQ316210 KAR(80 kHz)], *Rhinolophus ferrumequinum* (AB085729, Japan), *Rhinolophus affinis* (EF108160, Malaysia), *Rhinolophus hipposideros* (DQ120922,), *Rhinolophus pusillus* (DQ297589, China), *Rhinolophus luctus* (EF108165, Malaysia), *Rhinolophus lepidus* (AF451338, China), *Rhinolophus macrotis* (AF460976, China), *Rhinolophus trifoliatus* (EF108176, Malaysia), and *Rhinolophus pearsonii* (AF451340, China), were used for the genetic distance calculation. It is observed that the *Rhinolophus rouxii* [KAUNHM20114] from the present study showed a range of genetic distance 11.6% [with Rhinolophus luctus (EF108165, Malaysia)] to 17.3% [with Rhinolophus hipposideros (DQ120922)]. When compared with other Rhinolophus species sequences, the *Rhinolophus rouxii*, KAUNHM201185, gave almost the same range of genetic distance as that of KAUNHM20114. The sequence *Rhinolophus rouxii*, KAUNHM20114 and KAUNHM201185, compared with the 80kHz, JQ316210KAR, and 90 kHz, JQ316214TN, phonic types of *Rhinolophus rouxii*, showed a genetic distance of 1.9% and 9.5% respectively. But the genetic distance between both the *Rhinolophus rouxii* sequences, KAUNHM20114 and KAUNHM201185, was only 1.1%.

Interestingly the third sequence of *Rhinolophus rouxii* (KAUNHM2012310) from this study had a genetic distance of 1.3% and 9.4% with the 90 kHz (JQ316214, TN) and 80 kHz (JQ316210 SRP KAR) of *Rhinolophus rouxii* respectively. And this particular sequence showed a distance of more than or equal to 9.5% with the other two *Rhinolophus rouxii* sequences, KAUNHM20114 and KAUNHM201185, in the study. Moreover, the *Rhinolophus rouxii* (KAUNHM2012310) had a range of genetic distance from 10.0% [with *Rhinolophus lepidus* (AF451338, China)] to 14.9% [with *Rhinolophus pusillus* (DQ297589, China)].

When compared with other species of *Rhinolophus*, the 90kHz phonic type of *Rhinolophus rouxii* [JQ316214, TN] showed a genetic distance ranged from 9.4% [with *Rhinolophus lepidus* (AF451338, China)] to 14.3% [with *Rhinolophus pusillus* (DQ297589, China)] where as the 80kHz phonic type of *Rhinolophus rouxii* JQ316210 SRP, KAR had a genetic distance ranged from 12.2% [with *Rhinolophus luctus* (EF108165, Malaysia) to 17% [with *Rhinolophus hipposideros* (DQ120922,)]. The two phonic type of *Rhinolophus rouxii* [JQ316214, TN (90 kHz) and JQ316210 SRP, KAR (80 kHz)] had a genetic distance of 8%.

```
                                         10        20        30        40        50
R. rouxii (JQ316210, SRP, 80kH    ATGACCAACATTCGCAA-GTCCCACCCATTATTCAAGATCATCAACGACT
R. rouxii (KAUNHM20114)           GCT.T..TG. --.AT.GCC..............................
R. rouxii (KAUNHM201185)          GAT.T..TG.---.A.T-C...............................
R. rouxii (JQ316214, MKM, 90kH    .................-A..........C.......A...........G
R. rouxii (KAUNHM2012310)         GCAG..CTA --....T-C..........C.......-...........G

                                         60        70        80        90       100
R. rouxii (JQ316210, SRP, 80kH    CGTTCGTAGACTTACCAGCCCCATCAAGTATCTCTTCCTGATGAAACTTT
R. rouxii (KAUNHM20114)           ..................................................
R. rouxii (KAUNHM201185)          ........A.........................................
R. rouxii (JQ316214, MKM, 90kH    .A.....T.........................................C
R. rouxii (KAUNHM2012310)         .A.....T.........................................C

                                        110       120       130       140       150
R. rouxii (JQ316210, SRP, 80kH    GGATCCCTTCTAGGAGTCTGCTTAGCCGTACAAATTATTACAGGCCTTTT
R. rouxii (KAUNHM20114)           ..................................................
R. rouxii (KAUNHM201185)          ..................................................
R. rouxii (JQ316214, MKM, 90kH    ........C.....GA......................C...........
R. rouxii (KAUNHM2012310)         ........C.....GA......................C...........

                                        160       170       180       190       200
R. rouxii (JQ316210, SRP, 80kH    CCTAGCCATACATTACACATCAGATACCGCCACAGCCTTTTACTCGGTGA
R. rouxii (KAUNHM20114)           ..................................................
R. rouxii (KAUNHM201185)          ..................................................
R. rouxii (JQ316214, MKM, 90kH    ......T.....C...........C..............C.....A..A.
R. rouxii (KAUNHM2012310)         ......T.....C...........C..............C.....A..A.

                                        210       220       230       240       250
R. rouxii (JQ316210, SRP, 80kH    CCCACATCTGCCGAGACGTCAATTATGGCTGAATCCTGCGCTACCTTCAT
R. rouxii (KAUNHM20114)           .....................................A............
R. rouxii (KAUNHM201185)          .....................................A............
R. rouxii (JQ316214, MKM, 90kH    ...................T..C..C...........A............
R. rouxii (KAUNHM2012310)         ...................T..C..C...........A............

                                        260       270       280       290       300
R. rouxii (JQ316210, SRP, 80kH    GCCAACGGCGCCTCCATATTTTTCATCTGCTTATTCCTGCACGTAGGACG
R. rouxii (KAUNHM20114)           ..................................................
R. rouxii (KAUNHM201185)          ..................................................
R. rouxii (JQ316214, MKM, 90kH    ........T...........C..T......C...................
R. rouxii (KAUNHM2012310)         ........T...........C..T......C...................

                                        310       320       330       340       350
R. rouxii (JQ316210, SRP, 80kH    AGGAATCTACTATGGTTCCTATACATTCTCAGAAACATGAAATATCGGAA
R. rouxii (KAUNHM20114)           ..................................................
R. rouxii (KAUNHM201185)          ..................................................
R. rouxii (JQ316214, MKM, 90kH    .....................C.....................C..T...
R. rouxii (KAUNHM2012310)         .....................C.....................C..T...

                                        360       370       380       390       400
R. rouxii (JQ316210, SRP, 80kH    TTATTCTCCTCTTCGCCGTTATAGCCACAGCATTCATGGGCTACGTACTT
R. rouxii (KAUNHM20114)           ..................................................
R. rouxii (KAUNHM201185)          ..................................................
R. rouxii (JQ316214, MKM, 90kH    ....C...........T..........................T......
R. rouxii (KAUNHM2012310)         ....C...........T..........................T......

                                        410       420       430
R. rouxii (JQ316210, SRP, 80kH    CCATGAGGCCAAATATCCTTCTGAGGAGCAACCGTCAT
R. rouxii (KAUNHM20114)           ........A........A........G..TG.A..T--
R. rouxii (KAUNHM201185)          ........A........A........G..TG.A..TTA
R. rouxii (JQ316214, MKM, 90kH    ................................A.....
R. rouxii (KAUNHM2012310)         ........A........A........G..TG.A..TTA
```

Fig. 8. The partial sequence of cytochrome b gene of *Rhinolophus rouxii*. 'Dot' indicates the same base as the first sequence (JQ316210; TN). All the sequences were 437 bp sizes.

The sequences of *Rhinolophus rouxii*, KAUNHM20114, KAUNHM201185 and KAUNHM2012310 obtained by this study and JQ316210 (from KAR), JQ316214 (from TN) of 80 kHz and 90 kHz phonic types respectively from

GenBank were aligned for comparison. While comparing with the sequence of 80 kHz phonic type, the sequence, KAUNHM20114, showed 18 variable sites. Among the 18 variable sites, 10 were transitions, at nucleotide positions one, two, five, 14, 15, 17, 238, 427, 431, 436 and eight were transversions, at positions three, eight, nine, 19, 409, 418, 430, 433. When compared to the 80 kHz phonic type, the sequence KAUNHM201185 showed eight transitions at positions one, five, 14, 59, 238, 427, 431 and 436 and 11 transversions at positions two, three, eight, nine, 16, 409, 418, 430, 433, 437and 438. At 18th nucleotide position of 80 kHz phonic type, a missing data was observed while in that of KAUNHM20114 and KAUNHM201185 sequence, showed a nitrogen base, Cytosine. The sequences, KAUNHM20114 and KAUNHM201185 differed at four nucleotide positions by two transitions at positions 15 and 59 and by two transversion at positions two and 16. At 437th and 438th positions of KAUNHM20114, missing data were observed while in that of KAUNHM201185, showed bases Thymine and Adenine respectively. Similarly a missing data was observed at 17th nucleotide position of KAUNHM201185 sequence, while in that of KAUNHM20114 showed Guanine (Fig. 8).

While comparing with the sequence of 90 kHz phonic type, the sequence, KAUNHM20114, showed 44 variable sites. Among the 43 variable sites, 34 were transitions, at nucleotide positions one, five, 14, 15, 29, 37, 52, 100, 109, 115, 116, 139, 157, 163, 175, 190, 196, 199, 220, 223, 226, 259, 271, 274, 281, 322, 343, 346, 355, 367, 394, 427, 431, 436, and 10 were transversions, at positions three, eight, nine, 18, 19, 50, 58, 409, 418, 430. When compared to the 90 kHz phonic type, the sequence KAUNHM201185 showed 34 transitions at positions one, five, 14, 29, 37, 52, 59, 100, 109, 115, 116, 139, 157, 163, 175, 190, 196, 199, 220, 223, 226, 259, 271, 274, 281, 322, 343, 346, 355, 367, 394, 427, 431, 436 and 13 transversions at positions two, three, eight, nine, 16, 18, 50, 58, 409, 418, 430, 437, 438. The sequence KAUNHM2012310 differed at 14 sites. Among the 14 variable sites, seven transitions at positions one, two, three, four, 427, 431, 436 and at positions seven, eight, nine, 16, 18, 409, 418, 430, 437, 438 (Fig. 8).

Discussion

DISCUSSION

Taxonomic ambiguity is a characteristic of the suborder microchiroptera due to the presence of 'cryptic species'. Since they are very difficult to identify using the traditional taxonomic tools, its presence always create a practical problem for biodiversity assessment, conservation and in developing management policies and approaches (Bickford *et al.*, 2007; Pfenninger and Schwenk, 2007; Chattopadhyay *et al.*, 2012). It is accepted that the molecular markers are very efficient in identifying closely related taxa (Avise, 2004). In bats DNA barcoding technique can be used as an efficient and effective tool for differentiating, identifying and understanding cryptic species (Francis *et al.*, 2010). Applying the same principle in the present study, we have made an attempt to unravel the taxonomic ambiguity in insectivorous bats of Parambikulam Tiger Reserve.

5.1 DNA ISOLATION, PCR AMPLIFICATION AND SEQUENCING OF PARTIAL CYTOCHROME B GENE

Eventhough the Cytochrome Oxidase I gene of mitochondrial DNA (mtDNA) is considered as the 'barcode' of animals (Hebert *et al.*, 2003a); we used another mtDNA, cytochrome b. This is because the cytochrome b gene has extensively been used in species identification (Kocher *et al.*, 1989; Irwin *et al.*, 1991; Alvarez *et al.*, 2000; Weisrock and Janzen, 2000; Hsieh *et al.*, 2001; Ross *et al.*, 2003; Dalebout *et al.*, 2004; Hsieh *et al.*, 2006; Herath, 2007; Sholl *et al.*, 2008; Jayasankar *et al.*, *2009*; George *et al.*, 2011; Bijukumar *et al.*, 2012), taxonomy, systematic and phylogenetic studies (Kuwayama and Ozawa, 2000; Bastian *et al.*, 2001; Bradley and Baker, 2001; Matthee *et al.*, 2001; Matthee *et al.*, 2004; Eick *et al.*, 2005; Piaggio and Perkins, 2005; Li *et al.*, 2006; Stoffberg *et al.*, 2010; Sazali *et al.*, 2011). Since the complete sequencing of the cytochrome b gene is time-consuming and laborious (Hsieh *et al.*, 2001) due to the size of this locus being about 1140bp, in this study we report the use of a partial cytochrome b sequence for species identification and genetic analysis.

The successful isolation of DNA from *Megaderma spasma, Hipposideros speoris, Rhinolophus rouxii, Rhinolophus beddomei,* and *Rhinolophus lepidus* using either Phenol-Chloroform extraction (Sambrook *et al.*, 1989) method or the GeniPure™ Mammalian Genomic DNA Purification Kit (GeNei™) prove the efficiency of these two methods as reported by others (Jayasankar *et al.*, 2007) earlier.

5.2 BLASTn (BASIC LOCAL ALIGNMENT SEARCH TOOL-NUCLEOTIDE) ANALYSIS

The sequences of *Hipposideros speoris* (KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319), *Rhinolophus rouxii* (The KAUNHM2012310, KAUNHM201185 and KAUNHM20114) and *Megaderma spasma* (KAUNHM2012318 and KAUNHM2012320) showed a sequence similarity of 98%, 97% (both with an E value of 0.0) and 91% (with an E value of $2e^{-149}$) respectively. Since it is reported that the lower E-value (Expect value), or closer it is to zero, indicate that the query sequence has a more "significant" match with the retrieved sequence (Hillis and Bull, 1993; Harrison and Langdale, 2006), the results of species identification for bat species such as *Hipposideros speoris, Rhinolophus rouxii, Megaderma spasma* obtained by this method are definite, accurate and reliable.

When compared with *Hipposideros speoris* and *Rhinolophus rouxii*, the sequences of *Megaderma spasma* (KAUNHM 2012 318 and KAUNHM 2012 320) showed only 91% sequence similarity with an E value $2e^{-149}$. As no other species had a higher percentage match with the sequences of *Megaderma spasma*, this result can be best explained by the very low E value or greater intra-species variation. A very similar case was reported by Lee *et al.* (2009) in the identification of commercial turtle shell.

The sequence similarity from the BLASTn search indicates that the method used in this study can be used to identify the above mentioned insectivorous bats to species level.

5.3 SEQUENCE AND PHYLOGENETIC ANALYSIS

5.3.1 *Megaderma spasma* Linnaeus, 1758 (Lesser False Vampire)

The phylogenetic analysis unequivocally showed that the query sequences, KAUNHM2012318 and KAUNHM2012320 belong to *Megaderma spasma* (Fig. 3). The very high bootstrap values (99%) for the clade indicate reliable grouping of the species of interest (Hillis and Bull, 1993).

High sequence divergence (10.8%) in *Megaderma spasma* were observed between the two sequences, KAUNHM2012318 and KAUNHM2012320, in this study and two sequences from GenBank, AY057942 from Central Java and EU521606 from Malaysia (Table 4). Bradly and Baker (2001) considered that a genetic distance more than 10% or between two percent and 11% at cytochrome b is an indication of species level divergence. The high sequence divergence may possible as they were collected from two different geographic locations and they result from geographic barriers separating genetic flow (Sun *et al.*, 2009). To identify this complexity more specimen should be collected from each geographical location and merit additional study concerning specific status (Bradly and Baker, 2001; Sun *et al.*, 2009). May be because of this high genetic distance or sequence divergence, the two sequences, KAUNHM2012318 and KAUNHM2012320, in this study and two sequences from GenBank, AY057942 from Central Java and EU521606 from Malaysia, were clustered in two different clades as sister taxon (Fig. 3).

The two sequences of *Megaderma spasma* (KAUNHM2012318 and KAUNHM2012320) from the present study are found to be genetically very

similar with a genetic divergence of one percent. But the two samples of *Megaderma spasma* (KAUNHM2012318 from Kuriyarkutty and KAUNHM2012320 from Vengoli) collected from different locations in Parambikulam Tiger Reserve. The less than two percent genetic divergence (Bradly and Baker, 2001) and the difference in collection localities may result in intra-population or intra-species variation.

Two haplotypes were identified in this study (KAUNHM2012318 and KAUNHM2012320) while aligning with the GenBank sequences of *Megaderma spasma*, AY057942 from Central Java and EU521606 from Malaysia. The point mutations or single nucleotide substitutions were observed at 38 nucleotide positions (Fig. 4). Since the two sequences, KAUNHM2012318 and KAUNHM2012320, have point mutations at four positions, they can be designated as two haplotype sequences of *Megaderma spasma*.

5.3.2 *Hipposideros speoris* Schneider, 1800 (Schneider's Leaf nosed bat)

The phylogenetic tree obtained with neighbour-joining (NJ) method showed a reliable grouping of query sequences, KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319, with that of *Hipposideros speoris* (DQ680823, Madurai) and the branch was supported with 100% bootstrap value. This confirms the identification of query sequences, KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319, as *Hipposideros speoris*, in agreement with BLASTn analysis (Table 3 and Fig. 5).

The grouping of *Hipposideros armiger* (JN247016, Vietnam and AF451332, china), *Hipposideros diadema* (EF108149, Malaysia and JQ915899, Philippine) and *Hipposideros larvatus* (AF451333, China and JQ915903, Philippine) was similar with that reported by Sazali *et al.* (2011). This was also supported by Payne *et al.* (1985), Khan (1992), Koopman (1994) and Kingston *et*

al. (2006), as these species have three or more lateral leaflets. However, *Hipposideros larvatus* can be easily identified by the forearm length, which ranges from 52 to 65mm, whereas *Hipposideros armiger* (FA: 85-97mm) and *Hipposideros diadema* (FA: 76-87mm) can be recognised using their body coloration (Payne *et al.*, 1985; Khan, 1992; Bates and Harrison, 1997; Kingston *et al.*, 2006).

The GenBank depositions of *Hipposideros ater*, JQ915691 from Philippine, and that of *Hipposideros ciraceus*, JQ915700 from Philippine, were clustered in one branch with a bootstrap support of 100%. In that sequence of *Hipposideros ciraceus*, JQ915700 was confirmed as *Hipposideros ater* after BLASTn search. A similar case was reported by Bijukumar *et al.* (2012) in the case of *Balaenoptera edeni* sequence having an accession number X75583.

The four sequences of *Hipposideros speoris*, KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319, from the present study showed 3% genetic distance or divergence with the GenBank deposition of *Hipposideros speoris*, DQ680823, collected from Madurai. The species is extensively distributed in south India and some variations are reported (Bates and Harrison, 1997). The sequence divergence may be due to geographic distances between sampling locations (Li *et al.*, 2007). According to Bradly and Baker (2001) genetic distance values between two percent and 11% had a high probability of being indicative of conspecific populations or valid species. More specimens should be collected from all the localities in future to explain the causes of sequence divergence.

The sequence, KAUNHM2012314, is identified as a haplotype as it has single nucleotide substitutions or nucleotide polymorphisms at 13 sites when compared with all other *Hipposideros speoris* sequences from the present study and GenBank.

5.3.3 *Rhinolophus rouxii* Temminck, 1835 (Rufous Horseshoe bat)

Chattopadhyay *et al.* (2012) proposed the existence of two phonic types (80kHz and 90kHz) of *Rhinolophus rouxii* on the basis of acoustic, morphological and genetic differences between populations of this species between Southern India. They also proposed that the two phonic types be called sibling species and thus recommend a new name, *Rhinolophus indorouxii*, for the 90kHz phonic type.

In the neighbour joining tree (Fig. 7) the two sequences of *Rhinolophus rouxii* from the present study, KAUNHM20114 and KAUNHM201185 were clustered together in one branch and them again clustered to the GenBank sequences of 80 kHz phonic type of *Rhinolophus rouxii*, with a bootstrap value of 100%. But the third sequence of *Rhinolophus rouxii* from the present study, KAUNHM2012310, clustered with the sequences of 90 kHz phonic type of *Rhinolophus rouxii,* with a bootstrap value of 100%. The clustering pattern and high bootstrap value (Hillis and Bull, 1993) confirm those two sequences from the present study, KAUNHM20114 and KAUNHM201185, as 80 kHz phonic type of *Rhinolophus rouxii* and KAUNHM2012310 as 90 kHz phonic type of *Rhinolophus indorouxii*.

It is observed that the sequence of *Rhinolophus lepidus* with accession number AF451338 from China clustered with the Rhinolophus rouxii (or *Rhinolophus indorouxii*) clade during the phylogenetic analysis of family Rhinolophidae (Fig. 7). But the sequence of *Rhinolophus lepidus* was confirmed as *Rhinolophus sinicus* after BLASTn search. In addition, Chattopadhyay *et al.* (2012) suggested that 90 kHz bats (or *Rhinolophus indorouxii*) are generally closer to *Rhinolophus sinicus* relative to the 80 kHz bats.

The high genetic distance of more than 9.5% (Table 6) between the sequences of 80 kHz and 90 kHz (KAUNHM20114, KAUNHM201185 and KAUNHM2012310) is same as that observed among cryptic species. In four

mammalian orders including Chiroptera, genetic divergence over five percentages for the cytochrome b gene are generally considered as an indicator of cryptic taxonomic diversity (Bradly and Baker, 2001; Baker and Bradly, 2006). And thereby we can suggest the presence of distinct species in this present study.

The present study could successfully DNA barcoded three species of insectivorous bats such as *Megaderma spasma*, *Hipposideros speoris* and *Rhinolophus rouxii*. But the species, *Megaderma spasma* and *Hipposideros speoris* showed a genetic distance between two percent and 11% and according to Bradly and Baker (2001) that distance range was an indicator of existence of species level divergence. Whereas in the case of *Rhinolophus rouxii* all the analysis suggested the existence of 80kHz *Rhinolophus rouxii* and 90 kHz phonic type of *Rhinolophus rouxii*, ie. *Rhinolophus indorouxii*. The study could be considered as an indicator that might be able to raise questions on current taxonomic status of insectivorous bats and thus proposing additional study on that.

Summary

SUMMARY

There exists a taxonomic ambiguity in the case of insectivorous bats and because of that reason very little is known about their ecology, biology, habits, conservation problems etc. This is particularly true in Kerala. The nocturnal habit, small body size and cryptic nature make their taxonomy difficult. So, in such cases the conventional morphological classification need be supported with novel molecular techniques such as DNA barcoding. There are no other taxonomic studies on insectivorous bats of Kerala backed by molecular techniques. The present study on "DNA barcoding of insectivorous bats of Parambikulam Tiger Reserve, Western Ghats, Kerala", thus is the first-ever study of this kind in Kerala. The important findings are summarized below.

1. The insectivorous bats collected from Parambikulam Tiger Reserve having the following species ids', KAUNHM2012318 and KAUNHM2012320, showed 91% identity with the registered cytochrome b sequence of *Megaderma spasma.*

2. The phylogenetic analysis of construction of neighbour-joining (NJ) tree showed that the query sequences, KAUNHM2012318 and KAUNHM2012320 belong to *Megaderma spasma* with a very high bootstrap values (99%) at the node. Because of the high genetic distance or sequence divergence, the two sequences on the specimens KAUNHM2012318 and KAUNHM2012320, in this study and two sequences from GenBank, AY057942 from Central Java and EU521606 from Malaysia, were clustered in two different clades as sister taxon.

3. Bradly and Baker (2001) considered that a genetic distance between two percent and 11% at cytochrome b gene is an indication of species level

divergence. In the present study, high sequence divergence (10.8%) was observed between the two sequences of *Megaderma spasma* in this study and two sequences of same species retrieved from GenBank (AY057942 from Central Java and EU521606 from Malaysia). This clearly indicates the existence of species divergence in the South Indian and South Asian *Megaderma spasma*.

4. To identify the above said complexity in genetic distance, I recommend that, more specimens should be collected from each geographical location and merit additional study concerning specific status.

5. The two sequences of *Megaderma spasma* (KAUNHM2012318 and KAUNHM2012320) collected from Parambikulam Tiger Reserve are found to be genetically very similar with a genetic divergence of one percent.

6. Since the two sequences of *Megaderma spasma*, KAUNHM2012318 and KAUNHM2012320, have point mutations at four positions, they can be designated as two haplotype sequences of *Megaderma spasma*.

7. The bat tissue samples collected from Parambikulam Tiger Reserve such as, KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319, showed 98% sequence similarity with the registered sequences of *Hipposideros speoris* during the BLASTn search of cytochrome b gene.

8. The phylogenetic tree obtained with neighbour-joining (NJ) method showed a reliable grouping of query sequences, KAUNHM2012313, KAUNHM2012314, KAUNHM2012317 and KAUNHM2012319, with that of *Hipposideros speoris* (DQ680823, Madurai) and the branch was supported with 100% bootstrap value.

9. The three percent genetic distance between the *Hipposideros speoris* sequences in the current study and that of *Hipposideros speoris* (DQ680823, Madurai) is an indication of existence of two genetically different species and also merit additional study.

10. The sequence of *Hipposideros speoris* of present study with a species id KAUNHM2012314, is identified as a haplotype as it has single nucleotide substitutions or nucleotide polymorphisms at 13 sites when compared with all other *Hipposideros speoris* sequences from the present study and GenBank.

11. The sequences of bats collected from Parambikulam Tiger Reserve with a species id of KAUNHM2012310, KAUNHM201183 and KAUNHM2011 displayed 97% similarity with the cytochrome b gene of *Rhinolophus rouxii* with the highest homology.

12. In the case of *Rhinolophus rouxii* sequences, the phylogenetic analysis put KAUNHM20114 and KAUNHM201185 together in one branch and they again clustered to the GenBank sequences of 80 kHz phonic type of *Rhinolophus rouxii*. The genetic distance also confirms this.

13. But the third sequence of *Rhinolophus rouxii* from the present study, KAUNHM2012310, clustered with the sequences of 90 kHz phonic type of *Rhinolophus rouxii* and that showed a genetic distance of 9.5% when compared with two other *Rhinolophus rouxii* sequences of present study.

14. So, the study clearly indicates the existence of sibling species of *Rhinolophus rouxii* and *Rhinolophus indorouxii* in the study area and this support the study of Chattopadhyay *et al.* (2012).

References

REFERENCES

Akbar, Z., Hong, L. W., and Sastroutomo, S. S. 1999. Insectivorous bats as natural predators of insect pests. Biological control in tropics: towards efficient biodiversity and bioresource management for effective biological control. Proceedings of the Symposium on biological control in the tropics, March 18-19, Serdang, Malaysia. CABI Publishing, Willingford, U.K, pp. 34-37.

Altringham, J. D. 1996. *Bats: Biology and Behaviour*. Oxford University Press, London, United Kingdom, 262p.

Alvarez, Y., Mateo, J. A., Andreu, A. C., Diaz-Paniagua, C., Diez, A., and Bautista, J. M. 2000. Mitochondrial DNA haplotyping of Testudo graeca on both continental sides of the Straits of Gibraltar. *J. Hered.* 91: 39-41.

Anderson, S., Bankier, A. T., Barrel, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. 1981. Sequence and organization of the human mitochondrial genome. *Nature*. 290: 457-65.

Arif, I. A. and Khan, H. A. 2009. Molecular markers for biodiversity analysis of wildlife animals: a brief review. *Anim. Biodiver. Conserv.* 32 (1): 9-17.

Arizaga, S., Escurra, E., Peters, E., de Arellano, R. F., and Vega, E. 2000. Pollination ecology of Agave macrocantha in a Mexican tropical desert. *Am. J. Bot.* 87:1011-1017.

Armstrong, K. F. and Ball, S. L. 2005. DNA barcodes for biosecurity: invasive species identification. *Phil. Trans. R. Soc.* 360: 1813-1823.

Avise, J. C. 2004. *Molecular Markers, Natural History, and Evolution* (2nd Ed.). .Sinauer, Sunderland, MA, 684p.

Baker, A. M., Hughes, J. M., Dean, J. C., and Bunn, S. E. 2004. Mitochondrial DNA reveals phylogenetic structuring and cryptic diversity in Australian freshwater macro invertebrate assemblages. *Mar. Freshw. Res.* 55: 629-640.

Baker, R. J. and Bradley, R. D. 2006. Speciation in mammals and the genetic species concept. *J. Mammal.* 87: 643-662.

Ballard, J. W. O and Whitlock, M. C. 2004. The incomplete natural history of mitochondria. *Mol. Ecol.* 13: 729-744.

Barth, D., Krenek, S., Fokin, S. I., and Berendonk, T. U. 2006. Intraspecific genetic variation in Paramecium revealed by mitochondrial cytochrome c oxidase 1 sequences. *J. Eukaryot. Microbiol.* 53: 20-25.

Bastian, S. T. Jr., Tanaka, K., Anunciado, R. V. P., Natural, N. G., Sumalde, A. C., and Namikawa, T. 2001. Phylogenetic relationships among megachiropteran species from the two major islands of Philippines, deduced from DNA sequence of cytochrome b gene. *Can. J. Zool.* 79: 1671-1677.

Bates, P. J. J. and Harrison, D. L. 1997. *Bats of the Indian Sub-continent.* Harrison Zoological museum publications, London, 258p.

Bates, P. J. J., Rossiter, S. J., Suyanto, A., and Kingston, T. 2007. A new species of Hipposideros (Chiroptera: Hipposideridae) from Sulawesi. *Acta Chiropterol.* 9: 13-26.

Bazin, E., Glemin, S., and Galtier, N. 2006. Population size does mitochondrial genetic in animals. *Science,* 312: 570-572.

Belton, P. and Kempster, R. H. 1962. A field test on the use of sound to repel the European corn borer. *Entomol. Exp. Appl.* 5: 281-288.

Bensasson, D., Zhang, D. X., Hartl, D. L., and Hewitt, G. M. 2001. Mitochondrial pseudogenes: Evolution's misplaced witnesses. *Trends. Eco.l Evol.* 16: 314-321.

Bernard, E. and Fenton, M. B. 2003. Bat mobility in a fragmented landscape in Central Amazonia, Brazil. *Biotropica,* 35: 262-277.

Bertolazzi, P., Felici, G., and Weitschek, E. 2009. Learning to classify species with barcodes. *BMC Bioinformatics.* 10 (suppl. 14): S7.

Bickford, D., Lohman, D. J., and Sodhi, N. S. 2007. Cryptic species as a window on diversity and conservation. *Trends. Ecol. Evol.* 22: 148-155.

Bijukumar, S. S., Jijith, U., Kumar, S., and George, S. 2012. DNA barcoding of the Bryde's Whale *Balaenoptera edeni* Anderson (Cetacea: Balaenopteridae) washed ashore along Kerala coast, India. *J. Threatened Taxa.* 4(3): 2436-2443.

Blaxter, M. 2003. Counting angels with DNA. *Nature,* 421: 122-124.

Blaxter, M. and Flyod, R. 2003. Molecular taxonomics for biodiversity surveys: already a reality. *Trends. Ecol. Evol.* 18: 268-269.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., and Abebe, E. 2005. Defining operational taxonomic units using DNA barcode data. *Phil. Trans. R. Soc.* 360: 1935-1943.

Blaxter, M. L. 2004. The promise of DNA taxonomy. *Philos. Trans. R. Soc.* 359: 669-679.

Blaxter, M. L. 2006. Will DNA barcoding advance efforts to conserve biodiversity more efficiently than traditional taxonomic methods (two views). *Front. Ecol. Environ.* 4: 268-273.

Boonseub, B., Tobe, S. S., and Linacre, A. M. T. 2009. The use of mitochondrial DNA genes to identify closely related avian species. *Forens. Sci. Inter.: Genet. Supplem. Ser.* 2: 275-277.

Borisenko, A. V., Burton, K. L., Ivanova, N. V., Hanner, R. H., and Hebert, P. D. N. 2008. DNA barcoding in surveys of small mammal communities: a field study in Suriname. *Mol. Ecol. Resour.* 8: 471-479.

Bradley, R. D. and Baker, R. J. 2001. A test of the genetic species concept: cytochrome-b sequences and mammals. *J. Mammal.* 82: 960-973.

Brown, S. A. 1997. *Revolution at the checkout counter*. Cambridge, MA: Harvard University Press, 320p.

Cameron, S., Rubinoff, D., and Will, K. 2006. Who will actually use DNA barcoding and what will it cost. *Syst. Biol.* 55: 844-847.

Carroll, J. B. 1984. The conservation and wild status of the Rodrigues fruit bat *Pteropus rodricensis*. *Myotis*. 21: 148-154.

Carvalho, M. R., Bockmann, F. A., Amorim, D. S., and Brandao, C. R. 2008. Systematics must embrace comparative biology and evolution, not speed and automation. *Evol. Biol.* 35: 150-157.

Champion, H. G. and Seth, P. K. 1968. *A revised survey of the forest types of India*. Government of India Press, Nasik, New Delhi, India, 404p.

Chantangsi, C., Lynn, D. H., Brandl, M. T., Cole, J. C., Hetrick, N., and Ikonomi, P. 2007. Barcoding ciliates: a comprehensive study of 75 isolates of the genus *Tetrahymena*. *Int. J. Syst. Evol. Microbiol.* 57: 2412-25.

Chase, M. W., Cowan, R. S., Hollingsworth, P. M., van den Berg, C., Madrinan, S., Petersen, G., Seberg, O., Jorgsensen, T., Cameron, K. M., Carine, M., Pedersen, N., Hedderson, T. A. J., Conrad, F., Salazar, G. A., Richardson, J. E., Hollingsworth, M. L., Barraclough, T. G., Kelly, L.,

and Wilkinson, M. 2007. A proposal for a standardised protocol to barcode all land plants. *Taxon*. 56: 295-299.

Chase, M. W., Salamin, N., Wilkinson, M., Dunwell, J. M., Kesanakurthi, R. P., Haidar, N., and Savolainen, V. 2005. Land plants and DNA barcodes: short-term and longterm goals. *Philos. Trans. R. Soc.* 360: 1889-1895.

Chattopadhyay, B., Garg, K. M., Kumar, V. A. K., Doss, P. S. D., Ramakrishnan, U., and Kandula, S. 2012. Sibling species in South Indian populations of the rufous horse-shoe bat *Rhinolophus rouxii*. *Conserv. Genet.* 13(6):1435-1445.

Chattopadhyay, B., Garg, K. M., Doss, P. S., Ramakrishnan, U., and Kandula, S. 2011. Molecular genetic perspective of group-living in a polygynous fruit bat, *Cynopterus sphinx*. *Mamm. Biol.* 76:290-294.

Clare, E. L., Lim, B. K., Engstrom, M. D., Eger, J. L., and Hebert, P. D. N. 2006. DNA barcoding of Neotropical bats: species identification and discovery within Guyana. *Mol. Ecol. Notes*. 7(2): 184-190.

Clare, E. L., Lim, B. K., Fenton, M. B. and Hebert, P. D. N. 2011. Neotropical bats: Estimating species diversity with DNA barcodes. *Plos One*. 6(7): e22648.

Cognato, A. I. 2006. Standard percent DNA sequence difference for insects does not predict species boundaries. *J. Econ. Entomol.* 99: 1037-1045.

Corbet, G. B. and Hill, J. E. 1986. *A World List of Mammalian Species.* British Mus. (Nat. Hist.), London, 226p.

Cox, P. A., Elmqvist, E. D., Pierson, E. D. and Rainey, W. E. 1991. Flying foxes as strong interactions in South Pacific Island ecosystems: a conservation hypothesis. *Conserv. Biol.* 5: 448-454.

Cracraft, J. and Donoghue, M. J. 2004. *Assembling the tree of life.* Oxford University Press. New York, 576p.

Craig, P., Trail, P., and Morrell, T. E. 1994. The decline of fruit bats in American Samoda due to hurricanes and over hunting. *Biol. Conserv.* 69: 261-266.

Cywinska, A., Hunter, F. F., and Hebert, P. D. N. 2006. Identifying Canadian mosquito species through DNA barcodes. *Med. Vet. Entomol.* 20: 413-424.

Dalebout, M. L., Baker, C. S., Mead, J. G., Cockroft, V. G., and Yamada, T. K. 2004. A comprehensive and validated molecular taxonomy of beaked whales, family Ziphiidae. *J. Hered.* 95: 459-473.

Dayrat, B. 2005. Towards integrative taxonomy. *Biol. J. Linn. Soc.* 85: 407-415.

deSalle, R., Egan, M. G., and Siddall, M. 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Phil. Trans. R. Soc.* 360: 1905-1916.

Ducummon, S. L. 1999. The north american bats and mines project: a cooperative approach for integrating bat conservation and mine-land reclamation, *Proceedings of the 23rd Annual British Columbia Mine Reclamation Symposium* in Kamloops, BC, 1999. The Technical and Research Committee on Reclamation.

Ebach, M. C. and Carvalho, M. R. 2010. Anti-intellectualism in the DNA barcoding enterprise. *Zoologia.* 27: 165-178.

Ebach, M. C. and Holdrege, C. 2005. DNA barcoding is no substitute for taxonomy. *Nature*, 434:697.

Eick, G. N., Jacobs, D. S., and Matthee, C. A., 2005. A nuclear DNA phylogenetic perspective on the evolution of echolocation and historical biogeography of extant bats (Chiroptera). *Mol. Biol. Evol.* 22: 1869-1886.

Elangovan, V., Marimuthu, G., and Kunz, T. H. 1999. Temporal patterns of individual and group foraging behaviour in the Short nosed fruit bats, Cynopterus *sphinx* in South India. *J. Trop. Ecol.* 15: 681-687.

Evans, K. M., Wortley, A. H., and Mann, D. G., 2007. An assessment of potential diatom "barcode" genes (cox1, rbcL, 18S and ITS rDNA) and their effectiveness in determining relationships in Sellaphora (Bacillariophyta). *Protist,* 158: 349-364.

Evelyn, M. and Stiles, D. 2003. Roosting Requirements of Two Frugivorous Bats (*Sturnira lilium* and *Arbiteus intermedius*) in Fragmented Neotropical Forest. *Biotropica*, 35 (3): 405-418.

Faith, D. P. and Baker, A. 2006. Phylogenetic Diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evol. Bioinform.* 2: 121-128.

Felsenstein, J. 1985. Confidence limits on phylogenies, an approach using bootstrap. *Evolution,* 39: 783-791.

Fenton, M. B. 2003. Eavesdropping on the echolocation and social calls of bats. *Mammalian Rev.* 33: 193-204.

Fenton, M. B., Acharya, L., Audet, D., Hickey, M. B. C., Merriman, C., Obrist, M. K., Syme, D. M., and Adkins, B. 1992. Phyllostomid bats (Chiroptera: Phyllostomidae) as indicators of habitat disruption in the neotropics. *Biotropica,* 24: 440-446.

Fenton, M. B., Vonhof, M. J., Bouchard, S., Goli, S. A., Johnston, D. S., Reid, F. A., Riskin, D. K., Standing, K. L., Taylor, J. R., and Wagner, R. 2000. Roosts used by *Sturnira lilium* (Chiroptera: Phyllostomidae) in Beliz. *Biotropica,* 32: 729-733.

Findley, J. S. and Wilson, D. E. 1983. Are bats rare in tropical Africa? *Biotropica,* 14: 299-303.

Fisher, B. L. and Smith, M. A. 2008. A revision of Malagasy Species of *Anochetus Mayr* and *Odontomachus Latreille* (Hymenoptera: Formicidae). *Plos One,* 3(5): e1787.

Fleming, T. H. 1982. Foraging strategies of plant-visiting bats. In: Kunz, T. H. (ed.), *Ecology of Bats* Plenum Press, New York, pp. 287-325.

Fleming, T. H. 1991. Following the Nectar Trail. *Bats*. 9(4):4-7.

Fleming, T. H. and Sosa, V. J. 1994. Effects of nectarivorous and frugivorous mammals on reproductive success of plants. *J. Mammal.* 75: 845-851.

Floyd, R., Abebe, E., Papert, A., and Blaxter, M. 2002. Molecular barcodes for soil nematode identification. *Mol. Ecol.* 11: 839-850.

Francis, C. M. and Kunz , T. H. 2012. Molecular phylogeny of hipposiderid bats from Southeast Asia and evidence of cryptic diversity. *Mol. Phylogenet. Evol.* 62: 597-611.

Francis, C. M, Borisenko, A. V, Ivanova, N. V, Eger, J. L, Lim, B. K, Guillen-Servent, A., Kruskop, S. V., Mackie, I., and Hebert, P. D. N. 2010. The role of DNA barcode in understanding and conservation of mammal diversity in Southeast Asia. *Plos One*, 5(9):12575.

Frezal, L. and Leblois, R. b2008. Four years of DNA barcoding: Current advances and prospects. *Infect. Genet. Evol.* 8: 727-736.

Fujita, M. S. and Tuttle, M. D. 1991. Flying foxes (Chiroptera: Pteropodidae) Threatened animals of key ecological and economic importance. *Conserv. Biol.* 5:455- 463.

Funk, D. J and Omland, K. E. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annu. Rev. Ecol Syst.* 34: 397-423.

Ganesh, T. and Davidar, P. 2001. Dispersal modes of tree species in the wet forests of southern Western Ghats. *Curr. Sci.* 80:394-399.

Garcia, Q. S., Rezende, J. L. P., and Aguiar, L. M. S. 2000. Seed dispersal by bats in a disturbed area of South eastern Brazil. *Rev. Biol. Trop.* 48: 125-128.

George, S., Meenakshi, K., and Bijukumar, A. 2011. Molecular taxonomy of marine mammals stranded along Kerala coast, India. *Curr. Sci.* 100: 117-120.

Godfray, H. C. J. 2002. Challenges for taxonomy. *Nature.* 417: 17-18.

Godinez, A. H., Valiennte, B. A. and Rojas, M. A. 2002. The role of seed dispersers in the population dynamics of the columnar cactus *Neobuxbaumia tetezo*. *Ecology.* 83: 2617-2629.

Gonzalez, G. J. 1998. Seed dispersal by bats: its importance in the conservation and regeneration of tropical forest. *Acta Zool. Mexicana.* 73: 57-74.

Gonzalez, G. J., Guevara, S., and Sosa, V. J. 2000. Bat and bird generated seed rains at isolated trees in pastures in a tropical rainforest. *Conserv. Biol.* 14: 1693-1703.

Goodman, S. M., Bradman, H. M., Maminirina, C. P., Ryan, K. E., Cristidis, L. L., and Appleton, B. 2008. A new species of *Miniopterus* (Chiroptera: Miniopteridae) from lowland southern Madagascar. *Mamm. Bio.* 73: 199-213.

Guillen, A. and Francis, C. M. 2006. A new species of bat of the Hipposideros bicolor group (Chiroptera: Hipposideridae) from Central Laos, with evidence of convergent evolution with Sundaic taxa. *Acta Chiropterol.* 8: 39-61.

Hajibabaei, M., Singer, G. A. C., Hebert, P. D. N., and Hickey, D. A. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends Genet.* 23: 167-172.

Hajibabaei, M., Waard, J. R., Ivanova, N. V., Ratsasingham, S., Dooh, R. T., Kirk, S. L., Mackie, P. M., and Hebert, P. D. N. 2005. Critical factors for assembling a high volume of DNA barcodes. *Phil. Trans. R. Soc. B* 360:1959-1967.

Hajibabei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., and Hebert, P. D. N. 2006. DNA barcodes distinguish species of tropical Lepidoptera. *P. Natl. Acad. Sci.* 103: 968-971.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95-98.

Hamada, N., Pepinelli, M., Mattos gloria, A., and Luz, S. L. B. 2010. A new black fly species from Brazil, closely related to *Simulium guianense* Wise

(Diptera, Simuliidae), revealed by morphology and DNA barcoding. *Zootaxa*. 2428 :22-36.

Harrison, J. C. and Langdale, J. A. 2006. Techniques for molecular analysis: A step by step guide to phylogeny reconstruction. *Pl. J. 45*: 561-572.

Hebert, P. D. N. and Gregory, T. R. 2005. The promise of DNA barcoding for taxonomy. *Syst. Biol.* 54: 852-859.

Hebert, P. D. N., Cywinska, A., Ball, S. L., and DeWaard, J. R. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc.* B 270: 313-321.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H. and Hallwachs, W. 2004a. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. U.S.A.* 101: 14812-14817.

Hebert, P. D. N., Ratnasingham, S. and Dewaard, J. R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. B.* 270: S96-S99.

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S., and Francis, C. M. 2004b. Identification of birds through DNA barcodes. *Plos Biol.* 2: 1657-1663.

Herath, D. R. 2007. Identification of a stranded whale by mitochondrial DNA analysis-www.DNA-surveillance programme in action. *Asian Fisheries Sci.* 20: 319-324.

Hickey, M. B. C., Fenton, M. B., MacDonald, K. C., and Soulliere, C. 2001. Metal contaminants in the fur of bats (Chiroptera: Verspertilionidae) from Ontario and Quebec. *Bull. Environ. Contamin. Toxicol.* 66: 699-706.

Hill, J. E. and Smith, J. D. 1984. *Bats: A natural history.* Austin: University of Texas Press. Pp.243.

Hillis, D. M. and Bull, J. J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analyses. *Syst. Biol.* 42: 182-192.

Howell, D. R. 1980. Adaptive variation in diets of desert bats has implications for evolution of feeding strategies. *J. Mammal.* 61: 730.

Hseih, H. M., Huang, L. H., Tsai, L. C., Liu, C. L., Kuo, Y. C., Hsiao, C. T., Linacre, A., and Lee, J. C. I. 2006. Species identification of *Kachua tecta* using the cytochrome b gene. *J. Forensic Sci.* 51: 52-56.

Hsieh, H. M., Chiang, H. L., Tsai, L. C., Lai, S. Y., Huang, N. E., and Linacre, A. 2001. Cytochrome b gene for species identification of the conservation animals. *Forensic Sci. Int.* 122: 7-18.

Huang, J., Qin Xu, Q., Sun, Z. J., Tang, G. L., Su, Z. Y., 2007. Identifying earthworms through DNA barcodes. *Pedobiologia,* 51: 301-309.

Hulva, P. and Horacek, I. 2006. Cryptic sympatric diversity in *Emballonura alecto*: further bat species? *Acta Chiropterol.* 8: 537-542.

Hutson, A. M. 1993. *Action plan for the Conservation of Bats in the United Kingdom*. The Bat Conservation Trust, London.

Hutson, A. M., Mickleburgh, S. P., and Racey, P. A. 2001. *Microchiropteran bats: global status survey andconservation action plan*. IUCN/SSC Chiroptera Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK, 258 p.

Ibanez, C., Garcia-Mudarra, M., Ruedi, B., Stadelmann and Juste, J. 2006. The Iberian contribution to cryptic diversity in European bats. *Acta Chiropterol.* 8: 277-297.

Irwin, D. M., Kocher, T. D., and Wilson, A. C. 1991. Evolution of the cytochrome b gene of mammals. *J. Mol. Evol.* 32: 128-44.

IUCN. 2010. *IUCN Red List of Threatened Species,* version 2010.3. www.iucnredlist.org/initiatives/mammals.

Jacobs, D. S., Eick, G. N.., Schoeman, M. C., and C. A. Matthee. 2006. Cryptic species in an insectivorous bat, *Scotophilus dinganii*. *J. Mammal.* 87: 161-170.

Jahas, S. and Easa, P. S. 2008. *Abundance estimation of reptiles and amphibians of Parambikulam WildlifeSanctuary*. Research Centre for Environment and Social Sciences Thiruvananthapuram.

Janzen, D. H., Hajibabaei, M., Burns, J. M., Hallwachs, W., Remigio, E., and Hebert, P. D. N. 2005.Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Phil. Trans. R. Soc.* 360: 1835-1845.

Jayasankar, P., Anoop, B., Peter, R., Afsal, V. V. and Rajagopalan, M. 2007. Species of a whale and an unknown fish sample identified using molecular taxonomy. *Indian J. Fish.* 54: 339-343.

Jayasankar, P., Anoop, B., Rajagopalan, M., Yousaf, K. M. M., Renold, P., Krishnakumar, P. K., Afsal, V. V., and Krishnan, A. A. 2009. Indian efforts on the inventorization of marine mammal species for their conservation and management. *Asian Fisheries Sci.* 22: 143-155.

Jones, G. and Barlow, K. E. 2003. Cryptic species of echolocating bats. In: Thomas, J. A. (ed.) Echolocation in bats and dolphins. University of Chicago Press, Chicago, IL. pp. 345-349.

Kaler, O. P. 2011. *Management Plan for Parambikulam Tiger Reserve* (2011-2021). Kerala Forest Department. 282p.

Kalko, E. K. V., Friemel, D., Handley, C. O., and Schitzler, H. U. 1999. Roosting and foraging behaviour of two Neotropical gleaning bats, *Tonatia silvicola* and *Trachops cirrbosus* (Phyllostomatidae). *Biotropica*, 31: 344-353.

Karkouri, E. K., Murat, C., and Zampieri, E. 2007. Identification of internal transcribed Spacer sequence motifs in truffles: a first step toward their DNA barcoding. *Appl. Environ. Microbiol.* 73 (16): 5320-5330.

Kerr, K. C. R., Lijtmaer, D. A., Barreira, A. S., Hebert, P. D. N., and Tubaro, P. L. 2009. Probing evolutionary patterns in neotropical birds through DNA barcodes. *Plos One,* 4: e4379.

Khan, M. M. 1992. *Mamalia Semenanjung Malaysia.* Kuala Lumpur: Jabatan Perlindungan Hidupan Liar dan Taman Negara.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Kingston, T., Lim, B. L., and Akbar, Z. 2006. *Bats of Krau wildlife reserve.* Bangi: Penerbit Universiti Kebangsaan, Malaysia, 145p.

Kingston, T., Lara, M. C., Jones, G., Akbar, Z., Kunz, T. H., and Schneider, C.J. 2001. Acoustic divergence in two cryptic *Hipposideros* species: a role for social selection? *Proc. R. Soc. B-Biol. Sci.* 268: 1381-1386.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., and Villablanca, F.X. 1989. Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci.* U. S. A. 86: 6196-6200.

Koopman, K. F. 1993. Chiroptera. In: Wilson, D. E., and Reeder, D. M. (eds.) *Mammalian species of the world.* Washington, DC, Smithsonian Institution Press. pp. 137-241.

Koopman, K. F. 1994. *Handbuch der zoologie.* Berlin: Walter de Gruyter and Co.

Korine, C., Izhaki, I. and Arad, Z. 1999. Is the Egyptian fruit bat *Rousettus egyptiacus* a pest in Israel? An analysis of the bat's diet and implications for its conservation. *Biol. Conserv.* 88: 301-306.

Kress, W. J. and Erickson, D. L. 2007. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnHpsbA spacer region. *Plos One,* 2 (6): e508.

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A. and Janzen, D. H. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci.* U.S.A. 102: 8369-8374.

Krishnamurthy, P. K. and Francis, R. A. 2012. A critical review on the utility of DNA barcoding in biodiversity conservation. *Biodivers. Conserv.* 21: 1901-1919.

Kunz, T. H. and Kurta, A. 1988. Capture methods and holding devices. In: Kunz, T.H, (ed.). Ecological and Behavioural methods for the study of bats, Smithsonian Institution Press, Washington DC, pp.1-30.

Kunz, T. H. and Fenton, M. B. 2003. *Ecology of bats.* University of Chicago Press, Chicago, Illinois, USA.

Kurtzman, C. P. 1994. Molecular taxonomy of the yeasts. *Yeast* 10: 1727-1740.

Kuwayama, R. and Ozawa, T. 2000. Phylogenetic relationships among European red deer, wapiti, and sika deer inferred from mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* 15: 115-123.

Lahaye, R., van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, Barraclough, T.G., and Savolainen, V., 2008. DNA barcoding the floras of biodiversity hotspots. *Proc. Natl. Acad. Sci. U.S.A.* 105 (8): 2923-2928.

Landres, P. B., Verner, J., and Thomas, J. W. 1988. Ecological use of vertebrate indicator species: a Critique. *Conserv. Biol.* 2: 316-328

Lane, D. J. W., Kingston, T., Benjamin, P., and Lee, Y. H. 2006. Dramatic decline in bat species richness in Singapore, with implications for Southeast Asia. *Biolo. Conserv.* 131: 584-593.

la Salle, J., Wheler, Q., Jackway, P., Winterton, S., Hobern, D., and Lovell, D. 2009. Accelerating taxonomic discovery through automated character extraction. *Zootaxa.* 2217: 43-55.

Laval, R. K. and Fitch, H. S. 1977. Structure, movements and reproduction in three Coasta Rican bat communities. *Occasional Pap. Mus. Nat. Hist.* 69:1-28.

Law, B. S., Lean, M., Laurence, W. F. and Gascon, C. 1999. Common blossom bats (*Syconycteris australis*) as pollinators in fragmented Australian

tropical rain forest: Ecology and management of fragmented tropical landscapes. *Biol. Conserv.* 91: 201-212.

Lee, J. C. I., Tsai, L. C., Liao, S. P., Linacre, A., and Hsieh, H.M. 2009. Species identification using the cytochrome b gene of commercial turtle shells. *Forensic Sci. Int: Genet.* 3: 67-73.

Lee, M. S. Y. 2002. Online database could end taxonomic anarchy. *Nature,* 417: 787-788.

Lee, M. S. Y. 2004. The molecularisation of taxonomy. *Invertebr. Syst.* 18: 1-6.

Li, G., Jones, G., Rossiter, S. J., Chen, S. F., Parsons, S., and Zhang. 2006. Phylogenetics of small horseshoe bats from East Asia based on mitochondrial DNA sequence variation, *J. Mammal.* 87(6): 1234-1240.

Li, G., Liang, B., Wang, Y. N., Zhao, H. B., Helgen, K. M., Lin, L. K., Jones, G., and Zhang, S. Y. 2007. Echolocation calls, diet, and phylogenetic relationships of Stoliczka's trident bat, *Aselliscus stoliczkanus* (Hipposideridae). *J. Mammal.* 88: 736-744.

Lipscomb, D., Platnik, N. and Wheeeler, Q. 2003. The intellectual content of taxonomy: a comment on DNA taxonomy. *Trends Ecol. Evol.* 18: 65-66.

Litaker, R. W., Vandersea, M. W., Kibler, S. R., Reece, K. S., Stokes, N. A., Lutzoni, F. M., Yonish, B. A., West, M. A., Black, M. N. D., and Tester, P. A., 2007. Recognizing dinoflagellate species using ITS rDNA sequences. *J. Phycol.* 43: 344-355.

Liu, A. Z., Li, D. Z., and Wang, H. 2002. Ornithophilous and chiropterophilous pollination in *Musa itinerans* (Musaceae), a pioneer species in tropical rainforests of Yunnan, south eastern China. *Biotropica*. 34: 254-260.

Lobova, T. A., Mori, S. A., Balachard, F., Peckham, H. and Charles, D. P. 2003. *Cecropia* as a food resource for bats in French Guiana and the significance of fruit structure in seed dispersal and longevity. *Am. J. Bot.* 90: 388-403.

Lorenz, J. G., Jackson, W. E., Beck, J. C., and Hanner, R. 2005. The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. *Phil. Trans. R. Soc.* 360: 1869-1877.

Lynn, D. H. and Struder-Kypke, M. C. 2006. Species of Tetrahymena identical by small subunit rRNA gene sequences are discriminated by mitochondrial cytochrome c oxidase I gene sequences. *J. Eukaryot. Microbiol.* 53: 385-387.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I. M., Achtman, M., and Spratt, B. G. 1996. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci.* U.S.A. 95 (6): 3140-3145.

Mallet, J. and Willmott, K. 2003. Taxonomy: renaissance or tower of babel. *Trends Ecol. Evol.* 18: 57-59.

Markmann, M. and Tautz, D. 2005. Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. *Phil. Trans. R. Soc.* 360: 1917-1924.

Marshall, E. 2005. Will DNA bar codes breathe life into classication. *Science.* 307: 1037.

Martin, B. 1999. 'Firing up the power plant in our heads', *Nature*, 399: 426-427.

Matthee, C. A., Burzlaff, J. D., Taylor, J. F., and Davis, S. K. 2001. Mining the mammalian genome for artiodactyls systematics. *Syst. Biol.* 50: 367-390.

Matthee, C. A., Vuuren,J. V. B., Bell, D., and Robinson, T. J. 2004. A molecular supermatrix of the rabbits and hares (Leporidae) allows for the identification of five intercontinental exchanges during the Miocene. *Syst. Biol.* 53: 433-447.

Mayen, F. 2003. Haematophagous bats in Brazil, their role in rabies transmission, impact on public health, livestock, industry and alternatives to an indiscriminate reduction of bat population. *J. Vet. Med.* 50: 469-472.

Mayer, F. and von Helversen, O. 2001. Cryptic diversity in European bats. *Proc. R. Soc. B-Biol. Sci.* 268: 1825-1832.

Mayer, F., Dietz, C. and Kiefer, A. 2007. Molecular species identification boosts species diversity. *Front. Zool.* 4(4): 1742-9994.

McKenney, D. W., Sims, R. A., Soulea, M. E., Mackey, B. G., and Campbell, K. L. 1994. Towards a set of biodiversity indicators for Canadian forests. *Proc. Forest Biodiversity Indicators Workshop.* Natural Resources Canada, Canadian Forest Service.

Medellin, R. A. and Gaona, O. 1999. Seed dispersal by bats and birds in forest and disturbed habitats in Chiapas, Mexico. *Biotropica*, 31: 432-441.

Medellin, R. A., Equihua, M. and Amin, M. A. 2000. Bat diversity and abundance as indicators of disturbance in neo-tropical rainforests. *Conserv. Biol.* 14: 16666-1675.

Meier, R., Shiyang, K., Vaidya, G., and Ng, P. K. L. 2006. DNA Barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Syst. Biol.* 55(5): 715-728.

Menon, A. R. R. 1991. Vegetation analysis and mapping of Parambikulam Wildlife Sanctuary. KFRI Research Report No. 79: 28p.

Meyer, C. P. and Paulay, G. 2005. DNA barcoding: error rates based on comprehensive sampling. *Plos Biol.* 3 (12): e422.

Mickleburgh, S. P., Hutson, A. M., and Racey P. A. 2002. A review of the global conservation status of bats. *Oryx,* 36(1):18-34.

Mickleburgh, S. P., Hutson, A. M., and Racey,P. A. 1992. Old World fruit bats. An action plan for their conservation. International Union for

Conservation of Nature and Natural Resources, Chiroptera Specialist Group (IUCN/SSC). Gland, Switzerland, 252p.

Miller, S. E. 2007. DNA barcoding and the renaissance of taxonomy. *Proc. Natl. Acad. Sci.* U.S.A. 104: 4775-4776.

Minelli, A. 2003. The status of taxonomic literature. *Trends Ecol. Evol.* 18: 75-76.

Monaghan, M. T., Balke, M., Gregory, T. R., and Vogler, A. P. 2005. DNA-based speciesdelineation in tropical beetles using mitochondrial and nuclear markers. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 360(1462): 1925-1933.

Moore, W. S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution*, 49: 718-726.

Moritz, C. and Cicero, C. 2004. DNA barcoding: promise and pitfalls. *Plos Biol.* 2: 1529-1531.

Murray, S. W., Campbell, P., Kingston, T., Zubaid, A., Francis, C. M., and Kunz, T. H. 2012. Molecular phylogeny of hipposiderid bats from Southeast Asia and evidence of cryptic diversity. *Mol. Phylogenet. Evol.* 62: 597-611.

Nameer, P. O., Molur, S., and Walker, S. 2001. Mammals of Western Ghats; A simplistic overview. *J. Zoos' Print.* 16: 629-639.

Nameer, P.O. and Praveen, J. 2006. Checklist of Birds in Parambikulam Wildlife Sanctuary. Kerala Forest Department, 45p.

Nassar, J. M., Hamrick, J. L., and Fleming, T. H. 2003. Population genetic structure of Venezuelan chiropterophilous columnar cacti (Cactaceae). *A. J. Bot.* 90:1628-1637.

Nathan, P. T., Raghuram, H., Elangovan, V., Karuppudurai, T., and Marimuthu, G. 2005. Bat pollination of kapok tree, *Ceiba pentandra*, *Curr. Sci.* 88(10): 509-515.

Nei, M. and Kumar, S. 2000. *Molecular evolution and phylogenetics*. Oxford University Press, New York. 333p.

Nesi, N., Nakoune, E., Cruad, C., and Hassanin, A. DNA barcoding of African fruit bats (Mammalia, Pteropodidae). The mitochondrial genome does not provide a reliable discrimination between *Epomophorus gambianus* and *Micropteropus pusillus*. *Comptes Rendus Bioligies*. 334: 544-554.

Noss, R. F. 1990. Indicators for monitoring biodiversity: A hierarchical approach. *Conserv. Biol.* 44:355-364.

Nowak, R. 1991. Order Chiroptera. In: Walker. (ed). *Mammals of the World* (5th Ed.). Baltimore, Johns Hopkins University Press. pp.190-194.

Padial, J. M. and de la Riva, I. 2007. Integrative taxonomists should use and produce DNA barcodes. *Zootaxa,* 1586: 67-68.

Parson, W., Pegoraro, K., Niederstatter, H., Foger, M., and Steinlechner, M. 2000. Species identification by means of the cytochrome b gene. *Int. J. Legal Med.* 114: 23-28.

Patton, J. L. and Smith, M. F. 1994. Paraphyly, polyphyly, and the nature of species boundaries in pocket gophers (genus *Thomomys*). *Syst Biol.* 43: 11-26.

Payne, J., Francis, C. M., and Phillipps, K. 1985. *A field guide to the mammals of Borneo.* Kota Kinabalu: The Sabah Society and World Wildlife Fund Malaysia.

Pennisi, E. 2007. Wanted: a barocde for plants. *Science.* 318 (2): 190-191.

Pettigrew, J. D. 1995. Flying primates: crashed, or crashed through? In: '*Ecology, evolution and behaviour of bats*', Oxford University Press.

Pfenninger, M. and Schwenk, K. 2007. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evol. Biol.* 7: 121-127.

Piaggio, A. J. and Perkins, S. L. 2005. Molecular phylogeny of North American long-eared bats (Vespertilionidae: Corynorhinus); inter- and intraspecific relationships inferred from mitochondrial and nuclear DNA sequences. *Mol. Phylogenet. Evol.* 37: 762-775.

Pires, A. C. and Marinoni, L. 2010. DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. *Biota Neotrop.* 10 (2): 339-346.

Prendini, L. 2005. Comment on "Identifying spiders through DNA barcodes". *Can. J. Zool.* 83: 481-491.

Pyle, R. L., Earle, J. L., and Greeene, B. D. 2008. Five new species of the damselfish genus *Chromis* (Perciformes: Labroidei: Pomacentridae) from deep coral reefs in the tropical western Pacific. *Zootaxa,* 1671: 3-31.

Quesada, M., Stoner, K. E., Rosa, G. V., Palacios, G. C., and Lobo, J. A. 2003. Effects of habitat disruption on the activity of nectarivorous bats (Chiroptera:Phyllostomidae) in a dry tropical forest: implications for the reproductive success of the neotropical tree *Ceiba grandiflora. Oecologia*, 135: 400-406.

Rainey, W. E. and Pierson, E. D. 1992. Distribution of Pacific Island flying foxes. *Pacific Island FlyingFoxes: Proceedings of an International Conservation Conference.* Washington DC, UnitedStates Department of the Interior Fish and Wildlife Service.

Raju, A. J. S., Rao, S. P., and Ezradanam, V. 2004. Pollination by bats and passerine birds in a dry season blooming tree species, *Careya arborea* in the Eastern Ghats. *Curr. Sci.* 86: 509-511.

Ransome, R. D. 1990. *The Natural History of Hibernating Bats*. Christopher Helm, Bromley, 282p.

Ratnasingham, S. And Hebert, P. D. N. 2007. BOLD: The Barcode of Life Data System. *Mol. Ecol. Notes*. 7: 355-364.

Romano, M. C., Maidagan, J. I. and Pire, E. F. 2000. Behaviour and demography in an urban colony of *Tadarida brasilliensis* (Chiroptera: Mollosidae) in Rosario, Argentina. *Revista-de-Biologica. Tropical*. 47: 1121-1127.

Ross, H. A., Lento, G. M., Dalebout, M. L., Goode, M., McLaren, P., Rodrigo, A. G., Lavaery, S., and Baker, C. S. 2003. DNA surveillance: web-based molecular identification of whales, dolphins and porpoise. *J. Hered*. 94: 111-114.

Rubinoff, D. 2006. Utility of Mitochondrial DNA barcodes in species conservation. *Conserv. Biol.* 20(4): 1026-1033.

Rubinoff, D. and Holland, B. S. 2005. Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. *Syst. Biol.* 54: 952-961.

Rubinoff, D., Cameron, S. and Will, K. 2006. A Genomic Perspective on the Shortcomings of Mitochondrial DNA for ''Barcoding'' Identification. *J. Hered*. 97(6): 581-594.

Saitou, N. and Nei, M. 1987. The neibour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Salsamendi, E., Garin,I.,Almenar, D., Goiti, U., Napal, M., and Joxerraaihartza. 2008. Diet and prey selection in Mehelyi's horseshoe bat *Rhinolophus mehelyi* (Chiroptera, Rhinolophidae) in the south-western Iberian Peninsula. *Acta Chiropterol.* 10(2): 279-286.

Sambrook, E., Fritsch, F., and Maniatis, T. 1989. *Molecular Cloning*. Cold Spring Harbour, Newyork, Cold Spring Harbour Press.

Saunders, G. W. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Phil. Trans. R. Soc.* B 360: 1879-1888.

Savolainen,V., Cowan, R. S., Vogler, A. P., Roderick, G. K., and Lane, R. 2005. Towards writing the encyclopaedia of life: an introduction to DNA barcoding. *Phil. Trans. R. Soc.* 360: 1805-1811.

Sazali, S. N., Besar, K., and Abdullah, M. T. 2011. Phylogenetic Analysis of the Malaysian *Rhinolopus* and *Hipposideros* Inferred from Partial Mitochondrial DNA Cytochrome *b* Gene Sequences. *Pertanika J. Trop. Agric. Sci.* 34 (2): 281-294.

Schindel, D. E. and Miller, S. E. 2005. DNA barcoding a useful tool for taxonomists. *Nature,* 435: 17.

Schulze, M. D., Seavy, N. E. and Whitacre, D. F. 2000. A comparison of the phyllostomid bat assemblages in undisturbed Neotropical forest and in forest fragments of a slash and burn farming mosaic in Peten Gautemala. *Biotropica.* 32: 174-184.

Schupp, E. W. 1993. Quantity, quality and the effectiveness of seed dispersal by animals. *Vegetatio,* 107/108: 15-29.

Seifert, K. A., Samson, R. A., Dewaard, J. R., Houbraken, J., Levesque, C. A., Moncalvo, J. M., Louis-Seize, G., and Hebert, P. D. N. 2007. Prospects for fungus identification using C01 DNA barcodes, with Penicillium as a test case. *Proc. Natl. Acad. Sci.* U.S.A. 104: 3901-3906.

Senthilkumar, K., Kannan, K., Subramanian, A., and Tanabe, S. 2001. Accumulation of organochlorine pesticides and polychlorinated biphenyls in aquatic organisms, birds and bats collected from South India. *Environ. Sci. Pollut. Res.* 8(1): 35-47.

Shanahanz, M., Harrison, R. D., Yamuna, R., Boen, W. and Thronton, I. W. B. 2001. Colonization of an island volcano, Long island and an emergent island, Motmot and colonization by figs, their dispersers and pollinators. *J. Biogeography.* 28: 1365-1377.

Shapcott, A. 1999. Vagility and the monsoon rainforest archipelago of northern Australia: patterns of genetic diversity in *Syzygium nervosum* (Myrtaceae). *Biotropica.* 31:579-590.

Sharifi, M. and Hemmati, Z. 2001. Food of Mehelyi's horseshoe bat *Rhinolophus mehelyi* in a maternity colony in western Iran. *Myotis*, 39: 17-20.

Sharifi, M. and Hemmati, Z. 2004. Variation in the diet of Mehely's horseshoe bat, *Rhinolophus mehelyi,* in three contrasting environments in western Iran. *Zool. Middle East*, 33: 65-72.

Sholl, T. G. C., Nascimento, F. F., Leoncini, O., Bonvicino and Sciliano, S. 2008. Taxonomic identification of dolphin love charms commercialized in the Amazonian region through the analysis of cytochrome b DNA. *J. Marine Biol. Associ. UK.* 88: 1207-1210.

Simmons, N. B. 1995. Bat relationships and the origin of flight. In P. A. Racey and S.M. Swift (eds.) *Ecology, Evolution, and Behavior of Bats. Symposium Zool.Society*, 67: 27-43.

Simmons, N. B., and J. H. Geisler. 1998. Phylogenetic relationships of *Icaronycteris, Archaeonycteris, Hassianycteris,* and *Palaeochiropteryx* to extant bat lineages, with comments on the evolution of echolocation and foraging strategies in Microchiroptera. *Bull. Brit. Mus. Nat. Hist.* 235: 1-182.

Simmons, N. B. 2005. Order Chiroptera. In: Wilson, D. E. and Reeder, D. M. (eds.), *Mammal Species of the World* (3rd Ed.)., Vol. 1. The John Hopkins University Press, Baltimore, MD, USA, 2142 p.

Sinha, Y. P. 1986. The bats of Bihar; taxonomy and field ecology. *Rec. Zool. Surv. India. Occ. Pap.* 77:67

Sivaperuman, C., Karthikeyan, M., and Ravikumar, R. 2005. Divesity of spiders in Parambkulam Wldlife Sanctuary. *Tiger pap.* 32(4): 18-23.

Smith, M. A., Fisher, B. L., and Hebert, P. D. N. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Phil. Trans. R. Soc.* B. 360: 1825-1834.

Smith, M. A., Wood, D. M., Janzen, D. H., Hallwachs, W., and Hebert, P. D. N. 2007. DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *P. Natl. Acad. Sci.* 104: 4967-4972.

Smith, M. A., Woodley, N. E., Janzen, D. H., Hallwachs, W., and Hebert, P. D. N. 2006: DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera:Tachinidae). *Proc Natl Acad Sci* .103(10): 3657-3662.

Southerton, S. G., Birt, P., Porter, J., and Ford, H. A. 2004. Review of gene movement by bats and birds in its potential significance for eucalypt plantation forestry. *Aus. For.* 67: 44-53.

Stebbings, R. E. 1988. *Conservation of European Bats.* Christopher Helm, London, 246p.

Stoeckle, M. 2003. Taxonomy, DNA, and the Bar Code of Life. *BioScience, 53:9.*

Stoffberg, S., Jacobs, D. S., Mackie, I. J., and Matthee, C. A. 2010. Molecular phylogenetics and historical biogeography of Rhinolophus bats. *Mol. Phylogenet. Evol.* 54: 1-9.

Subramanya, S. and Radhamani, T. R.1993. Pollination by birds and bats. *Curr. Sci.* 65: 201-209.

Sugita, T., Nishikawa, A., and Shinoda, T. 1998. Identification of trichosporon asahii by PCR based on sequences of the internal transcribed spacer regions. *J. Clin. Microbiol.* 2742-2744.

Sullivan, C. M., Shiel, C. B., McAney, C. M., and Fairley, J. S. 1993. Analysis of the diets of Leisler's Nyctalus leisleri and pipistrelle Pipistrellus pipistrellus bats in Ireland. *J. Zool.* Lond. 231: 656-663.

Sun, K., Feng, J., Zhang, Z., Xu, L., and Liu, Y. 2009. Cryptic diversity in Chinese rhinolophids and hipposiderids (Chiroptera: Rhinolophidae and Hipposideridae). *Mammalia.* 73: 135-141.

Sun, K. P., Feng, J. T. L., Jiang, J., Ma, Z. Z., Zhang and Jin, L. R.. 2008. A new cryptic species of *Rhinolophus macrotis* (Chiroptera: Rhinolophidae) from Jiangxi Province, China. *Acta Chiropterol.* 10: 1-10.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28(10): 2731-2739.

Tan, K. H., Zubaid, A. and Kunz, T. H. 1998. Food habits of Cynopterus brachyotis in Penisular Malaysia. *J. Trop. Ecol.* 14: 299-307.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H., and Vogler, A. P. 2002. DNA points the way ahead in taxonomy in assessing new approaches, it's time for DNA's unique contribution to take a central role. *Nature,* 418: 479.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H., and Vogler, A. P. 2003. A plea for DNA taxonomy. *Trends Ecol. Evol.* 18: 70-74.

Teeling, E. C., Springer, M. S., Madsen, O., Bates, P., O'Brien, S. J., and Murphy, W. J. 2005. A Molecular Phylogeny for Bats Illuminates Biogeography and the Fossil Record. *Science*, 307: 580-584.

Teletchea, F., Bernillon, J., Duffraisse, M., Laudet, V., and Hanni, C. 2008. Molecular identification of vertebrate species by oligonucleotide microarray in food and forensic samples. *J. Appl. Ecol.* 45 (3): 9 67-975.

Thabah, A., Rossiter, S. J., Kingston, T., Zhang, S., Parsons, S., Mya, K., Zubaid, A., and Jones, G. 2006. Genetic divergence and echolocation call frequency in cryptic species of *Hipposideros larvatus s.l.* (Chiroptera: Hipposideridae) from the Indo- Malayan region. *Biol. J. Linn. Soc.* 88: 119-130.

Thies, W. and Kalko, E. K. V. 2004. Phenology of neotropical pepper plants (Piperaceae) and their association with their main dispersers, two short-tailed fruit bats, *Carollia perspicillata* and *Carollia castanea* (Phyllostomidae). *Oikos*, 104: 362-376.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weigh matrix choice. *Nucleic Acids Res.* 22: 4673-4680.

Thong, V. D., Puechmaille, S. J., Denzinger, A., Dietz, C., Csorba, G., Bates, P. J. J., Teeling, E. C., and Schnitzler, H. U. 2012. A new species of *Hipposideros* (Chiroptera: Hipposideridae) from Vietnam. *J. Mammal.* 93(1):1-11.

Tobe ,S. S., Kitchener , K., and Linacre, A. 2009. Cytochrome b or cytochrome c oxidase subunit I for mammalian species identification- an answer to the debate. *Forensic Sci. Internat. Genet. Supplim. Ser.* 2: 306-307.

Tuttle, M. D. 1976. Status, Causes of decline, and management of endangered gray bats. *J. Wildl. Manage.* 43: 1-17.

Valentini, A., Pompanon, F., and Taberlet, P. 2008. DNA barcoding for ecologists. *Trends. Ecolo. Evol.* 24(2): 110-117.

Varassin, I. G., Trigo, J. R., and Sazima, M. 2001. The role of nectar production, flower pigments and odour in the pollination of four species of Passiflora (Plassifloraceae) in south eastern Brazil. *Bot. J. Linn. Soc.* 136: 139-152.

Vazquez-Yanes, C. and Orozco-Segovia, A. 1986. Dispersal of seeds by animals: effect on light controlled dormancy in *Cecropia obtusifolia*. Frugivores and seed dispersal, 15: 71.

Vences, M., Thomas, M., Bonett, R. M., and Vieites, D. R. 2005. Deciphering amphibian diversity through DNA barcoding: chances and challenges. *Philos. Trans. R. Soc. Lond.* 360: 1859-1868.

Vences, M., Thomas, M., Van der Meijden, A., Chiari, Y., and Vieites, D. R. 2004. Performance of 16S rRNA in DNA barcoding of amphibians. *Integr. Comp. Biol.* 44: 657-1657.

Vijayan, V. S. 1979. Parambikulam Wildlife Sanctuary and its adjacent areas. *J. Bombay Nat. Hist. Soc.* 75: 888-901.

Walsh, A. L. and Harris, S. 1996b. Foraging habitat preferences of vespertilionid bats in Britain. *J. Appl. Ecol.* 33: 508-518.

Walsh, A. L. and Harris, S. 1996a. Factors determining the abundance of vespertilionid bats in Britain: geographical, land class and local habitat relationships. *J. Appl. Ecol.* 33: 519-529.

Walton, C., Sharpe, R. G., Pritchard, S. J., Thelwell, N. J., and Butlin, R. K. 1999. Molecular identification of mosquito species. *Biol. J. Linn. Soc.* 68: 241-256.

Ward, R. D., Zemilak, S. T., Innes, B. H., Last, P. R., and Hebert, P. D. N. 2005. DNA barcoding Australia's fish species. *Philos. T. Roy. Soc.* 360: 1847-1857.

Waters, D., Jones, G., and Furlong, M. 1999. Foraging ecology of Leisler's bat *(Nyctalus leisleri*) at two sites in southern Britain. *J. Zool.* 249:173±180.

Waters, D. A., Rydell, J., and Jones, G. 1995. Echolocation call design and limits on prey size: a case study using the aerial-hawking bat *Nyctalus leisleri. Behav. Ecol. Sociobiol.* 37: 321-328.

Waugh, J. 2007. DNA barcoding in animal species: progress, potential and pitfalls. *BioEssays*, 29: 188-197.

Weisrock, D. W. and Janzen, F. J. 2000. Comparative molecular phylogeography of North American softshell turtles (Apaone): implications for regional and wide-scale historical evolutionary forces. *Mol. Phylogenet. Evol.* 14: 152-164.

Wheeler, Q. D. 2007. Invertebrate systematics or spineless taxonomy? *Zootaxa.* 1668: 11-18.

Whitaker, J. O., Jr. and Weeks, H. P., Jr. 2001. Food of Eptesicus fuscus, the big brown bat, in Indiana in the absence of cultivated fields and agricultural pests. *Proc. Indiana Acad. Sci.* 110: 123-125.

Whitaker, J. O., Jr., Issac, S. S., Marimuthu, G., and Kunz, T. H. 1999. Seasonal variation in the diet of the Indian Pygmy bat, Pipistrellus mimus, in southern India. *J. Mammal.* 80:60-70.

Whitworth, T. L., Dawson, R. D., Magalon, H., and Baudry, E. 2007. DNA barcoding cannot reliably identify species of the blowfly genus Protocalliphora (Diptera: Calliphoridae). *Phil. R. Soc. B.* 274: 1731-1739.

Wickramasinghe, L. P., Harris, S., Jones, G., and Vaughan, N. 2003. Bat activity and species richness on organic and conventional farms: impact of agricultural intensification. *J. Appl. Ecol.* 40: 984-993.

Wiedenburg, S., Mendes, H. F., Pepinelli, M., and Trivinhostrixino, S. 2009. Review of the genus *Onconeura* Andersen et Saether (Diptera: Chironomidae), with the description of four new species from Brazil. *Zootaxa,* 2265: 1-26.

Will, K. W., Mishler, B. D., and Wheeler, Q. D. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Syst. Biol.* 54: 844-851.

Williams, P. M. and Arias, I. 1978. Physio-ecological studies on plant species from the arid and semi-arid regions of Venezuela. I. The role of endogenous inhibitors in the germination of the seeds of *Cereus griseus* (Haw.). *Acta Cienti'fica Venezol-ana.* 29:93-97.

Willson, M. F. and Traveset, A. 2000. The ecology of seed dispersal. In: Fenner, M. (ed.), *Seeds: the ecology of regeneration in plant communities* (2nd Ed.). CAB International, UK, pp. 85-110.

Wilson, E. O. 1985. The Biological Diversity Crisis. *BioScience.* 35: 700-706.

Wilson, E. O. 2003. The encyclopedia of life. *Trends Ecol. Evol.* 18: 77-80.

Wilson, E. O. 2004. Taxonomy as a fundamental discipline. *Phil. T. Roy. Soc.* 359:739.

Wilson, K. H. 1995. Molecular biology as a tool for taxonomy. *Clin. Infect. Dis.* 20: 192-208.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., Karch, H., Reeves, P. R., Maiden, M. C. J., Ochman, H., and Achtman, M. 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol. Microbiol.* 60 (5): 1136-1151.

Woese, C. R. 1996. Phylogenetic trees: whither microbiology. *Curr. Biol.* 1060-1063.

Zhang, A., Feng, J., Ward, R. D., Wan, P., Gao, Q., Wu, J., and Zhao, W. 2012. A new method for species identification via protein-coding and non- coding DNA barcodes by combining machine learning with bioinformatic methods. *Plos One*, 7(2): e30986.

Zhou, J., Davey, M. E., Figueras, J. B., Rivkina, E., Gilichinsky, D., and Tiedje, J. M., 1997. Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. *Microbiology*, 143: 3913-3919.

Zinn, T. L. and Humphrey, S. R. 1981. Seasonal food resources and prey selection of the south eastern brown bat (*Myotis austroriparius*) in Florida. *Florida Sci.* 44(2):81-90.

POSTER PRESENTED IN FOURTHEENTH STUDENT CONFERENCE ON CONSERVATION SCIENCE, CAMBRIDGE, 19-21 MARCH, 2013P

CAN DNA- BARCODING UNRAVEL THE TAXONOMIC AMBIGUITY IN INSECTIVOROUS BATS IN WESTERN GHATS?

Parvathy Venugopal & P.O. Nameer.
College of Forestry, Kerala Agricultural University KAU (PO), 680 656. Thrissur., Kerala, India.
E.mail: pvparvathycof@gmail.com, nameerpo@gmail.com

ABSTRACT

We identified 4 different insectivorous bat species from the Parambikulam Tiger Reserve, Western Ghats. The present study agreed with the morphological description of each species.

BACKGROUND

Bats of the Order Chiroptera are one of the species-rich groups of mammals. 20% of all mammal groups belongs this order (Wilson and Reeder,2005). Although most mammal species are thought to have been described, the incidence of overlooked taxa is likely to be high within bats due to their cryptic behaviour and morphology. According to many field experts, classification based on morphological keys needs to be supported with automated techniques based on the analysis of DNA fragments (Bertolazzi et al., 2009).

METHODS

Capturing of bat: Using mist nets
Preservation of tissues: 70% ethyl alcohol
DNA Isolation: Phenol-Chloroform technique (Sambrook et al. 1989)
[illegible]
[illegible]
[illegible]

STUDY AREA

RESULTS

Sl. No	Species
1	*Rhinolophus rouxii*
2	*Megaderma spasma*
3	*Hipposideros speoris*
4	*Rhinolophus beddomei*

CONCLUSIONS

The Cyt b gene was sequenced from 12 bats belonging to 4 different species collected from the PTR, Western Ghats. The present study agreed with the morphological description of each species. *This study highlights the value in combining genetics with morphological measurements in the discovery of cryptic species.*

CONSERVATION MEASURES

The roosts identified during the study are conserved and managed through tribal watchers and forest officials.
Nature education camps for students and local people have been carrying out to aware them about the ecological importance, conservation issues and importance of conservation of bats

REFERENCES

Bertolazzi, P., Felici, G. and Weitschek, E. 2009. Learning to classify species with barcodes. BMC Bioinformatics. 10 [illegible]
Wilson D.E. and Reeder D.M. 2005. Mammal Species of the World [illegible]

ACKNOWLEDGMENT

DNA BARCODING OF THE INSECTIVOROUS BATS OF PARAMBIKULAM TIGER RESERVE, WESTERN GHATS, KERALA

By

PARVATHY VENUGOPAL
(2011-17-101)

ABSTRACT

Submitted in partial fulfillment of the

requirement for the degree of

MASTER OF SCIENCE IN FORESTRY

Faculty of Forestry

Kerala Agricultural University

DEPARTMENT OF WILDLIFE SCIENCES
COLLEGE OF FORESTRY
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2013

ABSTRACT

The study was carried out at Parambikulam Tiger Reserve during 2012-2013 with an objective of DNA barcoding the insectivorous bats to unravel the taxonomic ambiguity. The methods employed were the phenol-chloroform extraction (Sambrook *et al.*, 1989) or the GeniPure™ Mammalian Genomic DNA Purification Kit (GeNei™). The sequences were compared with those registered in NCBI databank (blast.ncbi.nlm.nih.gov). The phylogeny reconstruction and the calculation of genetic distances were done using the MEGA 5.0 (Tamura *et al.*, 2011).

In this study, three bat species such as *Hipposideros speoris*, *Megederma spasma* and *Rhinolophus rouxii*, were identified using the partial cytochorme b sequence from nine samples of individuals. But the species, *Megaderma spasma* and *Hipposideros speoris* showed a genetic distance between two percent and 11% while compared with the Genbank sequences of those species. Bradly and Baker (2001) considered that a genetic distance more than 10% or between two percent and 11% at cytochrome b is an indication of species level divergence or valid species. So the high sequence divergence in *Megaderma spasma* and *Hipposideros speoris* of present study with the same species from South Asia and South India gives an idea on the existence of conspecific population or valid species in these localities. The high sequence divergence may possible as they were collected from two different geographic locations and they result from geographic barriers separating genetic flow. To identify this complexity, the present study recommends additional study concerning specific status and for that more specimens should be collected from each geographical location. Because of this high genetic distance or sequence divergence between the sequences of *Megaderma spasma* and *Hipposideros speoris* of present study and that of retrieved from Genbank were clustered as sister clades in their respective phylogenetic neighbour-joining tree. The two sequences of *Megaderma spasma*,

KAUNHM2012318 and KAUNHM2012320, and one sequence of *Hipposideros speoris*, KAUNHM2012314, were identified as a haplotypes.

In the case of *Rhinolophus rouxii* sequences, the phylogenetic analysis put KAUNHM20114 and KAUNHM201185 together in one branch and they again clustered to the GenBank sequences of 80 kHz phonic type of *Rhinolophus rouxii*. The genetic distance also confirms this. But the third sequence of *Rhinolophus rouxii* from the present study, KAUNHM2012310, clustered with the sequences of 90 kHz phonic type of *Rhinolophus rouxii* and that showed a genetic distance of 9.5% when compared with two other *Rhinolophus rouxii* sequences of present study. So, the study clearly indicates the existence of sibling species of *Rhinolophus rouxii* and *Rhinolophus indorouxii* in the study area and this support the study of Chattopadhyay *et al.* (2012).