Phylogeny and systematics of the genus *Cynopterus* (Chiroptera: Pteropodidae) in Kerala

By

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(2013-17-101)

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
2015

DECLARATION

I hereby declare that this thesis entitled **"Phylogeny and systematics of the genus *Cynopterus* (Chiroptera: Pteropodiade) in 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.

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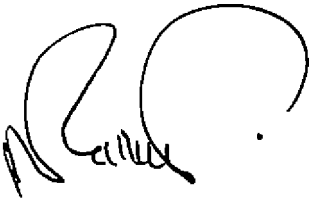
Certified that this thesis, entitled **"Phylogeny and systematics of the genus *Cynopterus* (Chiroptera: Pteropodiade) in Kerala"** is a record of research work done independently by **Miss. Devika, V. S. (2013-17-101)** under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.



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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, encouragement, constant evaluation and guidance 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. Siby Philip,*** *Assistant Professor, Department of Zoology, Nirmalagiri College, Kannur, for their timely interventions, valuable advice and suggestions, friendly co-operation and constant encouragement during the study.*

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

I am extremely grateful to ***Dr. Sverup John****, Dean, College of Agriculture, Vellayani,* ***Dr. M. Govindan,*** *Associate Dean, College of Agriculture, Padannakad,* ***Dr. M. Sivaswami,*** *Dean, Kelappaji College of Agricultural Engineering and Technology, Tavanur,* ***Dr. P. Rajendran****, Associate Director of Research Regional Agricultural Research Station, Ambalavayal, Associate Director, Regional Agricultural Research Station, Kumarakom,* ***Dr. P. Rajendran****, Professor and Head, Agricultural Research Station, Anakkayam,* ***M. Murugan****, Professor and Head, Cardamom Research Station, Pampadumpara, for granting permission to access the representative study locations and for extending the facilities for field work.*

Words are not enough to express my heartfelt acknowledgement towards ***Miss. Parvathy Venugopal*** *who taught me the initial lessons of the DNA barcoding and molecular phylogeny and whose words has been a constant source of inspiration to me.*

With all regards I sincerely acknowledge ***Mr. Nikhil, S.*** *for his constant support and valuable advice.*

Words cannot really express my gratefulness to ***Miss. Abha Manohar, Miss. Syamili, M. S., Miss. Abhirami, M. J., Miss. Remya Raghavan, Mr. Bill Nelson Paul, Mr. Anand, R., Miss. Akshaya Ajith, Miss. Gayathri, G. S. Miss. Anu Sagaran, K., Miss. Devipriya, K. S., Miss. Reshma, M. Raj, Mr. Sudeesh, Mr. Pradheep, Miss. Sini, S., Mrs. Mareena, M. J., Mr. Alfred,*** *and* ***Mr, Santhosh*** *who had been with me in the field making the most strenuous task the least one. My work would be an incomplete one without their help and support.*

I accord my sincere thanks to ***Miss. Nimisha Cherian*** *and* ***Mrs. Saleena, V. A.,*** *Research Assistant, Wildlife Forensic Unit, for their help, support, advice and suggestions throughout my laboratory work. I wish to express my gratitude to* ***Dr. Praveen Karanth,*** *Associate Professor, Centre for Ecological Sciences, Indian Institute of Sciences, Bangaluru and* ***Dr. Neelesh Dahanukar,*** *Indian Institute of Science Education and Research, Pune, for the timely help in analysis part. I wish to thank* ***Mr. M. Shaji,*** *Assistant Professor, Department of Wildlife sciences, for his timely support, suggestions and help.*

Help provided by ***Miss. Jyothi Krishnan*** *and* ***Mrs. Jayasree K.,*** *Teaching Assistant, Department of Wildlife Sciences in will always be remembered with great sincerity for their valuable guidance and timely help to carry out statistical analysis.*

Words cannot really express the power of relations that I experienced from ***Miss. Anju, S. Vijayan , Mr. Nithin Lal, Mr. Adarsh, C. K., Mr, Sreehari, R., Mr. Vishnu, R., Mr. Kiran Thomas , Mr. Anoob, P., Mr. Sachin, K. Aravind, Miss. Aswathy Chandran, U. B., Miss. Devika Sanghamitra, Miss. Leela. V., Mr. Sreekumar, E. R.*** *and* ***Mr. Ajay, K. G.*** *Whenever I was in need, they gave the mental strength and support and that really encouraged me to successfully complete this task without facing any hurdles. I am also grateful to* ***all other classmates, juniors and seniors*** *for their co-operation and moral support.*

The support and help rendered by ***Mrs. Mini, J.***, ***Mrs. Jyothy Haridas***, ***Mrs. Sheeja***, ***Mrs. Sindhu*** *and* ***Mrs. Prema*** *will always be remembered.*

In this occation, I express my heartfelt love, affection and respect towards ***my father, mother and Devan,*** *who gave me the strength to get through all tedious circumstances.*

A word of apology to those have not mentioned in person and a note of thanks to one and all who worked for the successful compilation of this endeavor.

Above all I bow my head before ***Lord Almighty*** *whose blessings and care enabled me to complete this programme with a great success.*

Devika, V. S.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

Introduction

INTRODUCTION

Bats (Order Chiroptera), the only mammals capable of powered flight and sophisticated laryngeal echolocation, represent one of the most species-rich and ubiquitous orders of mammals. They are the second most numerous groups after the order Rodentia (Simmons, 2005), with 1150 species (IUCN, 2015) under two suborders Megachiroptera (Frugivorous bats) and Microchiroptera (Insectivorous bats). Bats originated in Laurasia, most probably in North America in the early Eocene (Teeling *et al.*, 2005). Now this unique group of mammals is diversified and distributed all over the world except the Polar regions and a few oceanic islands.

The suborder Microchiroptera consist of 963 species in 17 families and is widespread throughout the range of bats, with the greatest diversity in the tropics (Findley and Wilson, 1983; Simmons, 2005) and the suborder Megachiroptera includes 187 species of bats in a single family Pteropodidae which occurs in the subtropical and tropical regions of the Old World (Rainey and Pierson, 1992; Simmons, 2005). But the recent molecular phylogenetic studies challenged the monophyletic nature of the order Chiroptera and proposed new classification with two suborders, Yinpterochiroptera and Yangochiroptera. The suborder Yinpterochiroptera includes the families Pteropodidae, Rhinolophidae, Megadermatidae, Craseonycteridae and Rhinopomatidae and Yangochiroptera consists of all the remaining families such as Molossidae, Emballonuridae, Nycteridae, Phyllostomidae, Mormoopidae, Noctillonidae, Furipteridae, Thyropteridae, Mystacinidae, Myzopodidae, Natalidae and Vespertilionidae (Teeling *et al.*, 2005).

Bats play an important role in pollination, seed dispersion (Marshall, 1985; Fujita and Tuttle, 1991), biological seed treatment and germination (Izhaki *et al.*, 1995), biological pest control (Akbar *et al.*, 1999) and also a good indicator of

pollution as they are vulnerable to contaminants (Hickey *et al.*, 2001). Even though bats are extremely important in performing various ecosystem services, very little is known about their ecology and biology. Incidence of overlooked taxa is very high within bats due to its nocturnal habit and cryptic nature, which makes their classification difficult.

The genus *Cynopterus* comes under the family Pteropodidae, which is geographically distributed from India to Malaysia, Thailand, Indonesia and Philippines (Koopman, 1993) with a total of seven species. Out of the 15 species of frugivorous bats within eight genera of Pteropodids from Indian subcontinent (Bates and Harrison, 1997) two species belong to the genus *Cynopterus* viz., *Cynopterus sphinx* (Greater Short-nosed Fruit Bat) and *Cynopterus brachyotis* (Lesser Short-nosed Fruit Bat) (Plate 1 and Plate 2). These species have been reported from Kerala too. While the *Cynopterus sphinx* is known from many parts of Kerala, *Cynopterus brachyotis* is known only from Silent Valley National Park in Kerala (Das, 1986; Bates and Harrison, 1997). But the taxonomic relationship of these two species is not clearly understood yet (Bates, 2013) and it is a subject of confusion and controversy, especially about the taxonomic status of *C. sphinx-brachyotis* species complex in the Indo-Malayan region. This is mainly because of the apparent overlap in the size between *C. sphinx* and *C. brachyotis* throughout the Southeast Asia. It is also related with the problem of selecting appropriate morphological characters to differentiate these two species (Francis, 1990) and also recent studies discovered genetically distinct lineages within *C. sphinx-brachyotis* complex (Campbell *et al.*, 2004).

Loss and deterioration of biodiversity is one of the major environmental crises in the modern society. This is due the lack of knowledge of global, regional and local biodiversity and actual rate of loss of biodiversity. A majority of biological diversity remains to be under-described or undiscovered (Wilson, 2000). Under estimation of biodiversity may also lead to loss of diversity through mismanagement of

geographically or ecologically isolated population. So classification of biodiversity is essential which in turn helps in the management and conservation of our biological heritage.

The modern integrated approaches of taxonomic study through the analysis of DNA fragment from each species (or population) in conjunction with traditional morphological analysis helps to define species more accurately. The genetic relatedness between species, subspecies and sibling species can be assessed by comparing mitochondrial DNA sequence divergence. It is more successful and provides better resolution than the studies based on only a few samples and morphological characters (Mapatuna *et al.*, 2002).

Since the evolutionary history of bats is much complex and is still not completely revealed (Teeling *et al.*, 2005), the broad application of molecular techniques to phylogenetic reconstruction will help to reveal unrecognized diversity (Gleeson *et al.*, 1999). Moreover, the molecular phylogenetic studies of Megachiroptera have provided considerable insight into intrafamilial relationships (Almeida *et al.*, 2011). Only little studies have been carried out on the genus *Cynopterus* of India and no studies on the taxonomic status of the genus *Cynopterus* in Kerala. Hence the present study, based on morphometrics as well as molecular data will help to reveal the taxonomic status, clarify any taxonomic ambiguity and find the systematic position of the genus *Cynopterus* in Kerala.

Plate 1. ***Cynopterus sphinx***

Plate 2. ***Cynopterus brachyotis***

Review of literature

REVIEW OF LITERATURE

2.1 ORDER CHIROPTERA BLUMENBACH, 1779

Bats, of the order Chiroptera are the unique, mysterious and second largest group of mammals which comprise 1116 species in 202 genera under 18 families globally (Simmons, 2005). The word Chiroptera was derived from two Latin words '*cheiros*' means 'hand' and '*pteron*' means 'wing'. They are distinguished from other mammals by the unique character of true flight, as compared to the gliding capability of mammals in other orders. As per most recent taxonomic assessment, out of 5,514 mammal species 1,150 species belongs to the order Chiroptera (IUCN, 2015). This means approximately 21 percent of mammals are bats.

The order is categorized into two- Megachiroptera (frugivorous bats) and Microchiroptera (insectivorous bats). The suborder Megachiroptera consists of 187 species of bats in a single family, Pteropodiae and Microchiroptera includes 963 species in 17 families (IUCN, 2015). But the recent molecular study based on biogeography and fossil record challenged the traditional classification based on monophyly of bat and proposed new classification which categorize order Chiroptera into suborders Yinpterochiroptera and Yangochiroptera. The suborder Yinpterochiroptera includes the families Pteropodidae, Rhinolophidae, Megadermatidae, Craseonycteridae and Rhinopomatida and Yangochiroptera, consist of all the remaining families such as Molossidae, Emballonuridae, Nycteridae, Phyllostomidae, Mormoopidae, Noctillonidae, Furipteridae, Thyropteridae, Mystacinidae, Myzopodidae, Natalidae and Vespertilionidae (Teeling *et al.*, 2005).

2.1.1 Origin and distribution

Bats are considered to be originated in Laurasia, most possibly in North America in the early Eocene (52-50 million years ago [Mya]) (Teeling *et al.*, 2005). The divergence in Oligocene (35 Mya) led to the evolutionary development of two distinct suborders, Megachiroptera which 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) and Microchiroptera which are widespread throughout the range of bats, with the greatest diversity in the tropics (Findley and Wilson, 1983). Hence now this group of mammals is diversified and distributed all over the world except the Polar Regions and a few oceanic islands (Mickleburgh *et al.*, 2002).

Of the 18 families, nine families such as Pteropodidae, Rhinopomatidae, Nycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Myzopodidae, Craseonycteridae and Mystacinidae are restricted to the Old World. The six families- Noctilionidae, Phyllostomidae, Mormoopidae, Natalidae, Furipteridae and Thyropteridae are restricted to the New World. The remaining three families- Emballonuridae, Molossidae and Vespertilionidae are found both in the Old and New Worlds (Mickleburgh *et al.*, 2002). The families Rhinolophidae, Vespertilionidae and Molossidae have representatives in both hemispheres. There is a very sharp decline in the number of species of bats with increase in distance from the equator (Corbet and Hill, 1986; Koopman, 1993).

2.1.2 Bats in India

India, one of the 17 mega diversity countries of the world is renowned for its rich biodiversity. It accounts 2.4% of total world area with 8.86% of the recorded species of the world. Of the 410 known mammal species in India, 117 species are of

the order Chiroptera (representatives of eight bat families which includes 39 genera), which means more than a quarter of Indian mammals are bats (Simmons, 2005; Hedge *et al.*, 2013) (Table 1).

India support more than 90% of the bat species in the South Asian region, while the other South Asian countries like Bhutan (51%), Nepal (40%), Pakistan (33%), Bangladesh (29%), Afghanistan (28%), Sri Lanka (23%) and Maldives (2%) had only less than 50% of total bat diversity (Srinivasulu and Srinivasulu, 2001).

Table 1. Family wise classification of bats in India

Sl. No.	Family	Number of Genera	Number of Species
1	Pteropodidae	8	14
2	Rhinolophidae	1	17
3	Hipposideridae	2	13
4	Megadermatidae	1	2
5	Rhinopomatidae	1	3
6	Emballonuridae	2	6
7	Molossidae	3	4
8	Vespertilionidae	21	58
	Total	39	117

2.2 MEGACHIROPTERA AND MICROCHIROPTERA

The Megachiroptera or the megabats are commonly known as "fruit bats" because they feed exclusively on flowers, nectar, pollen and fruits. While about 75% of Microchiropterans feed on insects and known as "insectivorous bats", the food sources may also include other invertebrates, fishes, amphibians, small mammals

(including other bats), blood (Altringham, 1996), fruits, and flowers (Hutson *et al.*, 2001).

As the name indicates, Megachiroptera are larger in size than Microchiroptera (considerable overlap exists). On an average, Megachiroptera weighs between 10g and 1500g and Microchiroptera (micro bats) between 2g and 196g (Mickleburgh *et al.*, 1992). With the exception of genus *Rousettus*, megachiropterans do not echolocate, but have light-sensitive eyes which help in navigation and they also use smell for orientation (Nowak, 1991; Altringham, 1996) while the microbats have the power of echolocation. Most fruit bats are helpless in total darkness but can see very well in dim light. Megachiropterans have a claw on the second finger of the wing and have longer muzzles than microchiropterans (Gaikwad *et al.*, 2012).

Megachiropterans control their body temperature within a tight range of temperatures and they do not hibernates, but many microchiropterans have labile body temperatures, and some hibernate (Hill and Smith, 1984; Nowak, 1991).

There is considerable debate concerning the evolutionary history of the two suborders i.e., are they monophyletic or paraphyletic? There are much evidences which supports both hypotheses which includes morphological, neurological, developmental, biochemical and molecular (Teeling *et al.*, 2005; Bates, 2013).

2.2.1 Pteropodidae Gray, 1821

Pteropodidae, the one and only family under the suborder Megachiroptera are characterized by more primitive ears and shoulder joints. They are restricted to the Old World. Most of them still retain a claw on the second digit which is absent in Microchiroptera and few have developed an echolocation (sonar) mechanism. The

teeth, however, are highly modified for eating fruit or nectar. They have strong muzzle and jaws and do not possess nose leaf or tragus. Their eyes are large and possess keen sense of smell. Tail is small or absent (Bates and Harrisson, 1997). Fourteen species of pteropodids belonging to eight genera had been reported from India (Srinivasulu *et al.*, 2010; Johnsingh and Nameer, 2015).

2.2.2 Genus *Cynopterus* Cuvier, F., 1824

The genus *Cynopterus* includes medium sized fruit bats (Forearm length – 57.0-79.0mm) with short tail (2.0-19.0mm) which is half enclosed within the interfemoral membrane. There is deep emargination between the projecting nostrils and their muzzles are short. Both the first and second digits have distinct claws. The ears usually have well defined pale anterior and posterior border. The neck tufts of male comprise of semi-rigid ruff of hairs. Rostrum is short and the ventral profile is nearly straight. They have primitive form of skull and two pairs of lower and upper incisors (Bates and Harrison, 1997).

The distribution of the genus *Cynopterus* spans more than 40^0 of latitude and 60^0 of longitude in the Indomalayan region (Corbet and Hill, 1992). Seven species of *Cynopterus* had been reported from the world (IUCN, 2015) and the geographical range extends from India to Malaysia, Thailand, Indonesia and the Philippines (Koopman, 1993). They are *C. brachyotis* (Lesser dog-faced fruit bat), *C. horsefieldii* (Horsefield's fruit bat), *C. luzoniensis* (Peter's fruit bat), *C. minutes* (Minute fruit bat), *C. nusatenggara* (Nusatenggara short nosed fruit bat), *C. sphinx* (Greater short nosed fruit bat) and *C. titthaecheilus* (Indonesian short nosed fruit bat). Two species: *C. sphinx* Vahl, 1797 and *C. brachyotis* Muller, 1838, are present in India and both species had been reported from Kerala also (Nameer, 2008).

2.2.3 *Cynopterus sphinx* and *Cynopterus brachyotis*

C. sphinx is a medium - sized fruit bat with an average forearm length of 70.2mm (range: 64-79mm) and characterized by a short tail half enclosed within the interfemoral membrane (Bates and Harrisson, 1997). It has larger ears with paler anterior and posterior borders than its close relative *C. brachyotis* (Storz and Kunz, 1999). Males are orange on the chin, sides of the chest, belly and thighs, whereas females have a paler grey belly and a tawny brown collar. It can be distinguished from its smaller cousin *C. brachyotis* by dark brown wings with pale fingers (Menon, 2014). *C. brachyotis* is similar in appearance to *C. sphinx* with an average forearm length of 60.3mm (range: 57- 63mm) and the ear length was not found to be greater than 18mm (Bates and Harrisson, 1997). The comparison of various external, cranial and dental measurements of *C. sphinx* and *C. brachyotis* are given in the Table 2 (Bates and Harrisson, 1997).

Table 2. Comparison of external, cranial and dental measurements of *C. sphinx* and *C. brachyotis*

Variables	*C. sphinx*		*C. brachyotis*	
	Mean (mm)	Range (mm)	Mean (mm)	Range (mm)
HB	98.8	76.0 – 113.0	87.5	80.0 - 96.0
T	10.9	4.5 -19.0	7.2	2.0 – 13.0
HF	15.6	12.6 – 18.0	13.6	11.0 – 15.0
FA	70.2	64.0 – 79.0	60.3	57.3 – 63.3
WSP	380.4	309.0 – 436.0	-	-
5MT	45.4	41.1 – 52.1	39.1	34.8 – 41.2
4MT	44.4	40.7 – 51.1	38.5	34.5 – 42.3

3MT	47.0	43.2 – 53.4	40.8	36.7 – 44.8
E	20.6	17.5 – 24.0	16.7	14.5 - 18.0
GTL	32.4	30.2 - 34.9	28.9	27.5 – 30.5
CBL	30.9	28.4 – 33.3	27.6	26.0 – 28.8
ZB	20.6	18.8 – 23.1	18.8	17.6 – 19.8
BB	13.5	11.1 – 14.8	12.2	9.9 – 13.3
M	24.9	22.7 – 27.5	22.2	20.5 – 23.8

The *C. sphinx* is distributed from Pakistan, India, Sri Lanka to southern China, Hong Kong, Malaysia, Java, Lesser Sunda Island, Sulawesi and Borneo and the *C. brachyotis* has a distribution that extends from southern India and Sri Lanka to Myanmar, Thailand, Malaysia, Sumatra, Borneo, Sulawesi and Philippines (Bates and Harrisson, 1997).

2.3 DEBATE ON TAXONOMIC STATUS OF *C. sphinx* AND *C. brachyotis*

The taxonomic relationship of these two species, *C. sphinx* and *C. brachyotis* is not clearly understood (Bates, 2013). According to Kitchener and Maharadatunkamsi (1991), the taxonomy of this genus is a subject of confusion and controversy, especially about the taxonomic status of *Cynopterus sphinx-brachyotis* species complex in the Indo-Malayan region. This is mainly because of the apparent overlap in the size between *C. sphinx* and *C. brachyotis* throughout the Southeast Asia (Andersen, 1912; Chasen, 1940; Hill, 1961). It is also related with the problem of selecting appropriate morphological characters to differentiate these two species (Francis, 1990; Campbell *et al.,* 2004). A better way identified was to analyze the DNA fragments from each species (or population) in conjunction with morphological characters for taxonomic studies (Mapatuna *et al.*, 2002; Bertolazzi *et al.*, 2009).

(Tan *et al.*, 1998; Vanitharani *et al.*, 2011). The genus *Cynopterus* are proved to be contributing to the revegetation in different areas, for example, Krakatau Island (Whittaker and Jones, 1994).

2.5 THREATS

According to IUCN status 2015 both the species *C. sphinx* and *C. brachyotis* are categorized as Least Concern and their trend is increasing for *C. sphinx* and unknown in the case of *C. brachyotis*. But certain threats had been identified such as habitat loss and loss of food source due to urbanization, disturbances at roosting site by man, conflict with fruit growers, unavailability of quality water, use of pesticides and natural calamities (Fujitha and Tuttle, 1991; Mickleburgh *et al.*, 1992; Craig *et al.*, 1994; Korine *et al.*, 1999; Mapatuna *et al.*, 2002; Gaikwad *et al.*, 2012).

2.6 INTEGRATIVE TAXONOMY

Taxonomy is the science of discovering, describing, classifying and naming of organisms (Aravind *et al.*, 2007). Unfortunately, over the past few years, the traditional taxonomy, morphological taxonomy was completely overshadowed by glamorous branches of biology. This was due to 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 include 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 (Hebert *et al.*, 2003). These taxonomic crises were overcome by the entry of molecular taxonomy along with other approaches like development of investment funds, information technology, (Wheeler, 2007) and increased utilization of cyber tools (Pyle *et al.*, 2008; laSalle *et al.*, 2009).

The study by Campbell *et al.*, (2004) supports the importance of molecular study along with morphological study, in which six genetically distinct lineages within *C. brachyotis* were identified. Two divergent mitochondrial lineages were identified in Sri Lanka, in an investigation of the systematic relationship between *C. brachyotis* and *C. sphinx* (Mapatuna *et al.*, 2002). The deep divergence identified between the Indian/Sri Lankan *C. brachyotis* lineage and all other in group taxa strongly suggests that this lineage represents an additional unrecognized species within the genus (Campbell *et al.*, 2004). Detailed study on the Indian *Cynopterus* may help to raise the subspecies identified in Sri Lanka to species status since they are geographically more identical areas and also the Indian/Sri Lankan lineage was identified as distinct from lineages from Philippine, Sulawesi and Myanmar (Mapatuna *et al.*, 2002; Campbell *et al.*, 2004).

Campbell *et al.*, (2004) suggested that the taxonomy of the genus *Cynopterus* requires substantial revision to recognize the evolutionary diversity that exists within the *C. brachyotis* complex. Additional taxon sampling will help to resolve basal relationships of these taxa. Since the initial radiation of *Cynopterus* took place over an evolutionarily brief time span, the geographic origin of extant lineages may prove difficult to reconstruct, regardless of the addition of taxa (Schmitt *et al.*, 1995).

2.4 ECOLOGICAL IMPORTANS OF CYNOPTERUS

Pollination and seed dispersal are mutualistic population interactions in which plants provide a nutritional reward (nectar, pollen, and fruit pulp) for a beneficial service: pollen and seed dispersal. Bats, along with many other flower visiting and fruit-eating animals, provide important mobility for plant gametes and propagules (Kunz *et al.*, 2011).

While considering the *C. sphinx* many plant species both economically and ecologically important are found to be pollinated and dispersed by them, such as *Ceiba pentandra, Bombax ceiba, Parkia spp., Adansonia digitata, Kigelia pinnata, Oroxylum indicum, Bauhinia spp., Musa spp., Anacardium occidentale, Careya arborea, Madhuca indica, Radermachera xylocarpa, Heterophragma roxberghi, Mimusops hexandra, Grevillia robusta, Durio zibethinus, Cullenia exarillata, Alangium salvifolium, Annonas squamosa, Polyalthia longifolia, Alstonia scholaris, Calophyllum inophyllum, Anogeissus latifolia, Terminalia catappa, Actephilla excels, Diospyros ebenum, Prosopis glandulosa, Pithicolobium dulce, Ficus bengalensis, Syzgium cuminii, Erythrina variegata, Morinda tinctoria, Glycomis pentaphylla, Mangifera indica, Psidium guajava* and *Manilkara zapota* (Fleming, 1982; 1991; Fujitha and Tuttle, 1991; Subramanya and Radhamani, 1993; Gonzalez, 1998; Elangovan *et al.*, 1999; Ganesh and Davidar, 2001; Liu *et al.*, 2002; Godinez *et al.*, 2002; Nassar *et al.*, 2003; Lobova *et al.*, 2003; Corlett, 2004; Raju *et al.*, 2004; Nathan *et al.*, 2005; Vanitharani *et al.*, 2011).

C. brachyotis are found to be an important seed disperser with wide selection of fruits of more than 54 species (Tan *et al.*, 1998). This include *Alangium salvifolium, Capparis zeylanica, Careya arborea, Dillenia indica, Diospyros malabarica, Erythroxylum monogynum, Actephilla excels, Mallotus phillipensis, Strychynos cinnamifolia, Agalia elaeagnoidea, Artocarpus communis, Ficus tajahela, Ficus bengalensis, Ficus racemesa, Ensete superba, Syzygium mundagam, Syzygium rubicndum, Eugenia calcadensis, Erythrina variegate, Canthium parviflorum, Pavetta indica, Atalantia monophylla, Pallaquium ellipticum, Madhuca longifolia, Mimusops elengi, Calophyllum inophyllum, Eleocarpus stipularis, Eugenia grandis and Gordonia obtuse* (Tan *et al.*, 1998; Vanitharani *et al.*, 2011).

As compared to other frugivores, these bats, ate ripe fruits by swallowing seeds and excrete unharmed dropping seeds far away, a biological seed treatment

(Tan *et al.,* 1998; Vanitharani *et al.*, 2011). The genus *Cynopterus* are proved to be contributing to the revegetation in different areas, for example, Krakatau Island (Whittaker and Jones, 1994).

2.5 THREATS

According to IUCN status 2015 both the species *C. sphinx* and *C. brachyotis* are categorized as Least Concern and their trend is increasing for *C. sphinx* and unknown in the case of *C. brachyotis.* But certain threats had been identified such as habitat loss and loss of food source due to urbanization, disturbances at roosting site by man, conflict with fruit growers, unavailability of quality water, use of pesticides and natural calamities (Fujitha and Tuttle, 1991; Mickleburgh *et al.,* 1992; Craig *et al.*, 1994; Korine *et al.*, 1999; Mapatuna *et al.,* 2002; Gaikwad *et al.*, 2012).

2.6 INTEGRATIVE TAXONOMY

Taxonomy is the science of discovering, describing, classifying and naming of organisms (Aravind *et al.,* 2007). Unfortunately, over the past few years, the traditional taxonomy, morphological taxonomy was completely overshadowed by glamorous branches of biology. This was due to 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 include 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 (Hebert *et al.*, 2003). These taxonomic crises were overcome by the entry of molecular taxonomy along with other approaches like development of investment funds, information technology, (Wheeler, 2007) and increased utilization of cyber tools (Pyle *et al.*, 2008; laSalle *et al.*, 2009).

Molecular taxonomy based on the DNA barcoding has received increased acceptance because it is tool that helps to obtains species specific signature which is based on the simple concept that a small stretch of organism's genome can represent that organism and enable identification at species level (Aravind *et al.,* 2007). The genetic relatedness between species, subspecies and sibling species can be assessed by comparing mtDNA sequence divergence; it is more successful and provides better resolution than the studies based on only a few samples and morphological characters (Mapatuna *et al.*, 2002). Genomic barcodes have only four alternate nucleotides at each position. But 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.*, 2003; Pires and Marinoni, 2010). The DNA barcoding can be performed quickly and at low cost (Stoeckle, 2003) without any taxonomic specialists and it helps to identify cryptic species and individuals at any stage of development (Pires and Marinoni, 2010).

As morphological taxonomy, molecular taxonomy also invited several criticisms in last decade. In certain cases DNA barcodes based on a few specific genes failed to distinguish closely related species due to the existence of ancestral polymorphism (Mallet and Willmott, 2003), mitochondrial introgression (Nesi *et al.,* 2011), 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; 2006; Rubinoff *et al.*, 2006). Here comes the importance of integrative taxonomy which is an incorporation of molecular as well as morphological taxonomy. In the case of *Cynopterus* genus also the integrative taxonomy could find many genetically distinct lineages under *C. sphinx-brachyotis* complex (Mapatuna *et al.,* 2002; Campbell *et al.,* 2004; Bates

2013). 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; Wiedenburg *et al.*, 2009; Hamada *et al.*, 2010).

2.7 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.

2.7.1 Nuclear DNA and Mitochondrial DNA

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 (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 *et al*, 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.

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 *et al.*, 2006).

2.7.2 Cytochrome B and Cytochrome C Oxidase 1

Hebert *et al.* (2004) 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 (Tobe *et al.*, 2009). Several studies have shown that more than 95% of species possess unique COI barcode sequences (Hebert *et al.*, 2004; 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.* (2003) 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).

2.8 PHYLOGENY AND SYSTEMATICS

Phylogeny depicts the evolutionary history of organismsin the form of a tree. Taxonomy is the formal system for identifying, naming and classifying species. Systematics is the broader science of classifying organisms based on similarity, biogeography, etc.

2.8.1 Molecular Phylogeny

Phylogeny is the evolutionary tree that shows how different species are related to each other. 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 such as identification of cryptic species (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).

2.8.2 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). 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).

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). 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, *C. sphinx and C. brachyotis* (Mapatuna *et al.*, 2002 and Campbell *et al.*, 2004), *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).

Identifying and describing cryptic diversity is essential for accurately assessing the number of species and to set conservation priorities (Campbell *et al.*, 2004). Considering all these facts, I attempted the research on "Phylogeny and systematic of the genus *Cynopterus* in Kerala".

Materials and methods

MATERIALS AND METHODS

3.1 STUDY AREA

3.1.1 Name, location and extent

Kerala, a small landmass between the hill ranges of the Western Ghats in the east and the Arabian Sea in the west within the geographical extremes of latitudes, 8°17'30" N and 12° 47'40" N and longitudes, 74°27'47" E and 77°37'12" E and has an area of 38,863 sq. km. Kerala shares its state borders with Tamil Nadu on the east and south and Karnataka on the north. Although Kerala lies close to the equator, its proximity with the sea and the presence of the fort like Western Ghats, provides it with an equable climate which varies little from season to season. The temperature varies from 27°C to 32°C. Southwest Monsoon and Northeast Monsoon are the main rainy seasons through which Kerala receives an average rainfall of 3,000 mm annually.

Biogeographically Kerala is divided into three climatically distinct regions: the eastern highlands, the central midlands, and the western lowlands. The highlands slope down from the Western Ghats which rise to an average height of 900m. It accounts for 48 percent of the total land area of Kerala. The Midlands, lying between the mountains and the lowlands, is made up of undulating hills and valleys. It is about 40 percent of the total land area with an intensive cultivation of cashew, coconut, arecanut, tapioca, banana and vegetables. Lowlands covers an area of almost 4000 sq.km. It is made up of numerous shallow lagoons, river deltas, backwaters and shores of the Arabian Sea and is essentially a land of coconuts and rice.

The samples of bats were collected from different biogeographical regions of Kerala (Figure 1). Based on elevation range, two locations from highland (> 700m),

three locations each from midland (50 – 700m) and lowland (<50m) were selected. The sampling locations from highland include, Ambalavayal and Pampadumpara. In the midland, the sampling was done at Vellanikkara, Vellayani, and Anakkayam while the sampling locations at the lowland were Kumarakom, Tavannur and Padanakkad. The research stations and institutions under Kerala Agricultural University were selected for sampling due to difficulty in proceedings of bat collection from other areas.

3.1.1.1 Ambalavayal, Wayanad

Ambalavayal is located in the Wayanad district at an altitude of about 974m above MSL. The region enjoys a mild subtropical climate. The Regional Agricultural Research Station, Ambalavayal has an area of 87.3 ha and grows a wide variety of crops like coffee, pepper, rice including scented varieties, spices like ginger, turmeric, clove, cinnamon, tropical and subtropical fruits, summer and cool season vegetables.

3.1.1.2 Pampadumpara, Idukki

The Pampadumpara village is situated in Udumbanchola taluk of Idukki district at an average altitude of 1000m above MSL. The Cardamom Research Station in Pampadumpara primarily grows cardamom, pepper and tree spices.

3.1.1.3 Anakkayam, Malappuram

The Agricultural Research Station was started in 1963. The station situated at Anakkayam village in Malappuram district at an average altitude of 60m above MSL and has an area of 9.92ha of which 8ha is under cashew and 0.5ha under coconut.

Figure1. Study locations in Kerala

3.1.1.4 Kumarakom, Kottayam

The RARS, Kumarakom is situated at 9° 3' latitude and 76° 3' longitude in the Kumarakom village of Kottayam district on the southern side of the Kavanar River. It lies at an altitude of 0.6 m below MSL. The total geographical area of the farm attached to the RARS is 44.76ha. Kumarakom enjoys a humid tropical climate. The bulk of the land area (19.5ha) is planted with coconut. The other important crops grown are, banana, vegetables, clove, nutmeg, cocoa, pepper and fodder grass. An area of about 150ha is utilized for aquaculture of fish and prawns.

3.1.1.5 Padannakkad, Kasargode

The College of Agriculture, Padannakad, was started in 1994, as the third agricultural college under the Faculty of Agriculture of the Kerala Agricultural University. The 27ha campus was formerly a coconut research station established in 1916. A beautiful pond (Theerthamkara pond) with an area of 4.2ha is also a peculiarity of the campus. The average altitude of the area is 20m above MSL.

3.1.1.6 Tavanur, Malappuram

The Kelappaji College of Agricultural Engineering and Technology (KCAET) located in Tavanur Village in Malappuram District of Kerala. The campus consists of 99 acres on the bank of the river Bharathapuzha, which host a number of fruit trees like *Mangifera indica, Manilkara zapota* etc.

3.1.1.7 Vellayani, Thiruvananthapuram

The College of Agriculture, Vellayani is situated 12km away from Thiruvananthapuram. The campus is situated at an altitude of 60m above MSL and is rich in biodiversity with the presence of large number of cultivated crops including fruit trees and is situated on the banks of Vellayani Lake.

3.1.1.8 Vellanikkara, Thrissur

Vellanikkara is the location of the main campus of Kerala Agricultural University. It is situated at an altitude of 60m above MSL and is a shelter for a wide range of flora and fauna. The presence of fruit orchards and water bodies make the campus a healthy habitat of different bat species.

Plate 3. Mist netting

Plate 4. Bat trapped in the mist net

3.2 METHODS

3.2.1 Field Study period

The capturing of bats from the above mentioned study locations were carried out during a time span from April 2014 to December 2014. A total of 92 hours of mist netting was done during this period at different selected locations of Kerala, which means 9.2 mist- netting hours per month (Table 3).

3.2.2 Selection of Sites

Representative sample plots were selected from each location based on observations such as habitat, availability of food and water and proximity to roosting site. Day transects to the roosting places were also carried out.

3.2.3 Capturing Technique

Mist-netting was the standard methodology adopted for the capture (Tuttle, 1976; Kunz and Kurta, 1988). The mist-nets were placed in sites where bats showed a high rate of activity such as near bat roosts, water bodies, feeding sites of the bats, along flyways such as trails, and openings in the natural forest. The mis-tnets are made of monofilament nylon with a usual mesh size of 36mm and an overall size of 10 x 1.5m. Mist-nettings were done on the specific selected sites at a height of 3-4m because the capture success depends on the placing of the mist-nets. The mist-nets should be kept open and ready prior to the dusk, before the bats actually leave the roost, and was kept open for two to four hours, depending on the capture success. Based on the species the time of bats leaving the roosts also varies (Plate 3 and Plate 4).

Plate 3. Mist netting

Plate 4. Bat trapped in the mist net

Mist-nettings were not done continuously on the same site for more than two days, as it would affect the capture success (Laval and Fitch, 1977). Nets were 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 Field Study

Habitat parameters such as GPS location, altitude, habitat type, micro-habitat parameters such as canopy cover, undergrowth cover, proximity to water and habitation and distance from the forests were recorded for the sites from where the specimens were collected. The date of specimen collection, duration of mist netting, number of mist nets used was also recorded.

During the study 66 individuals of bats of three species were captured in the mist-nets from orchards of sapota, mango and banana. The proximity to water source from the mist-nets varied from 0.5 m to 100 m (Table 3).

Table 3. Details of mist- netting at each study locations.

Sl. No.	Locations	Total duration of mist netting (hrs)	Habitat	Proximity to water (m)
1	Vellanikkara	11.20	Mango orchard Cocoa orchard	50.00
2	Vellayani	25.35	Mango orchard	50.00
3	Kumarakom	19.00	Banana orchard Coconut plantation	0.50

4	Tavanur	3.00	Sapota orchard Coconut plantation	5.00
5	Anakkayam	2.45	Banana orchard Coconut plantation	30.00
6	Padanakkad	9.00	Banana orchard Coconut plantation	50.00
7	Pambadumpara	16.00	Banana orchard Guava	50.00
8	Ambalavayal	6.00	Sapota orchard Vegetables	30.00

The captured bats were sexed and different external, cranial and dental measurements were taken using Mitutoyo digital calliper with a precision of 0.01 mm (Plate 5). Fresh weight of each bats were measured with 0.01gm precision using Persola balance (Plate 6). The major external measurements measured on the bats were head to body length (HB), tail length (T), hind foot length (HB), length of tibia (TIB), forearm length (FA), wing span length (WSP), length of ear (E), thumb, third metacarpal (3MT), fourth metacarpal (4MT) and fifth metacarpal (5MT), and first (1PH3MT) and second (2PH3MT) phalanx of the third metacarpal (Plate 7). The samples (specimens or tissues) were preserved in 100 percent ethyl alcohol for further laboratory study and analysis as museum collection.

3.2.5 Laboratory study

From each of the specimens, the skull was pulled out using standard procedure according to Bates and Harrison (1997) and the skull was processed and was made ready for making standard measurements on the skull (Plate 8). The cranial and dental measurements were taken which included greatest length of the skull

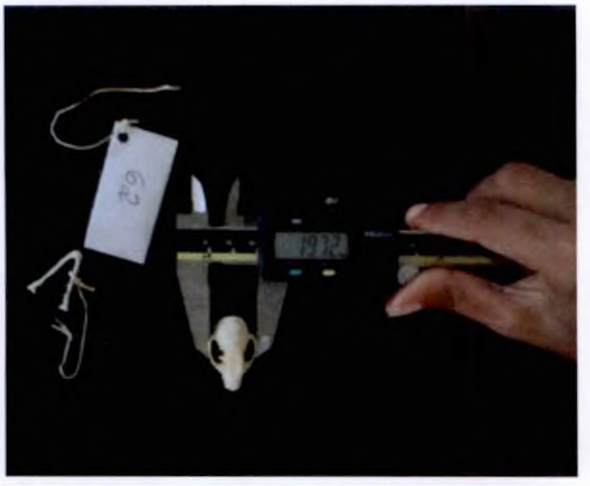


Plate 5. Recording of cranial and dental measurements using vernier caliper

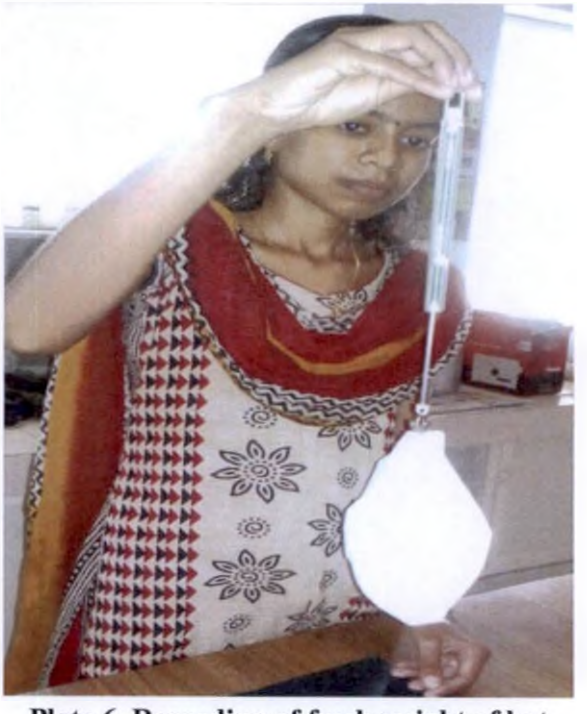
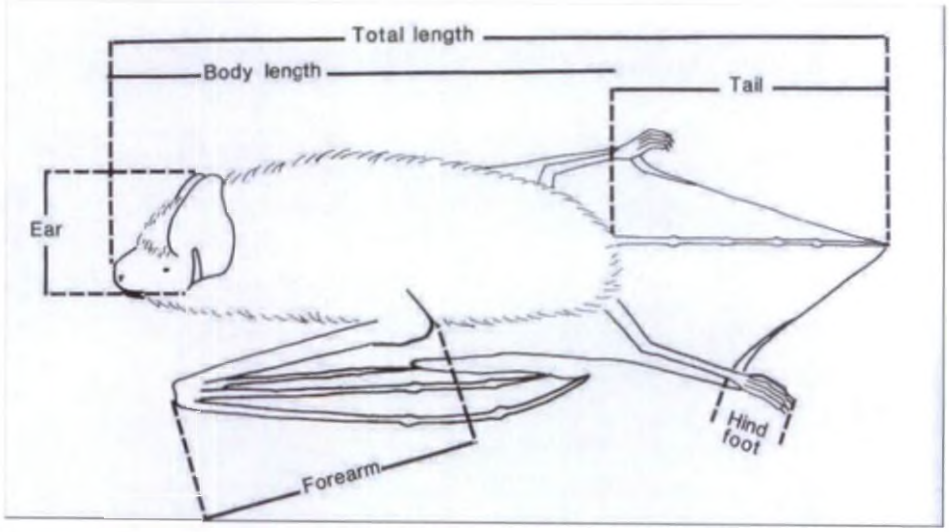


Plate 6. Recording of fresh weight of bat



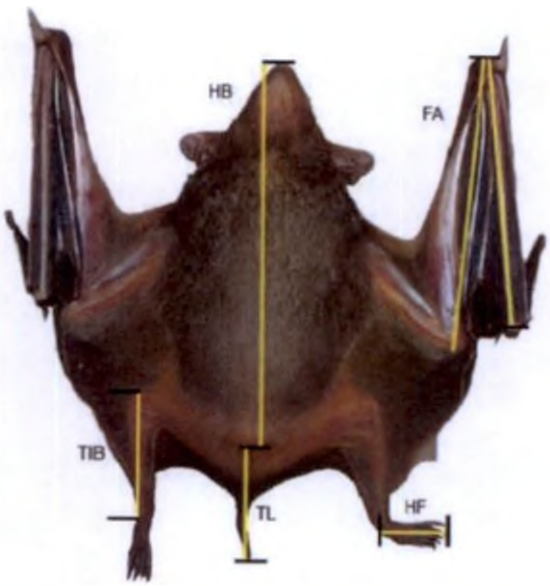


Plate 7. Morphometric measurements

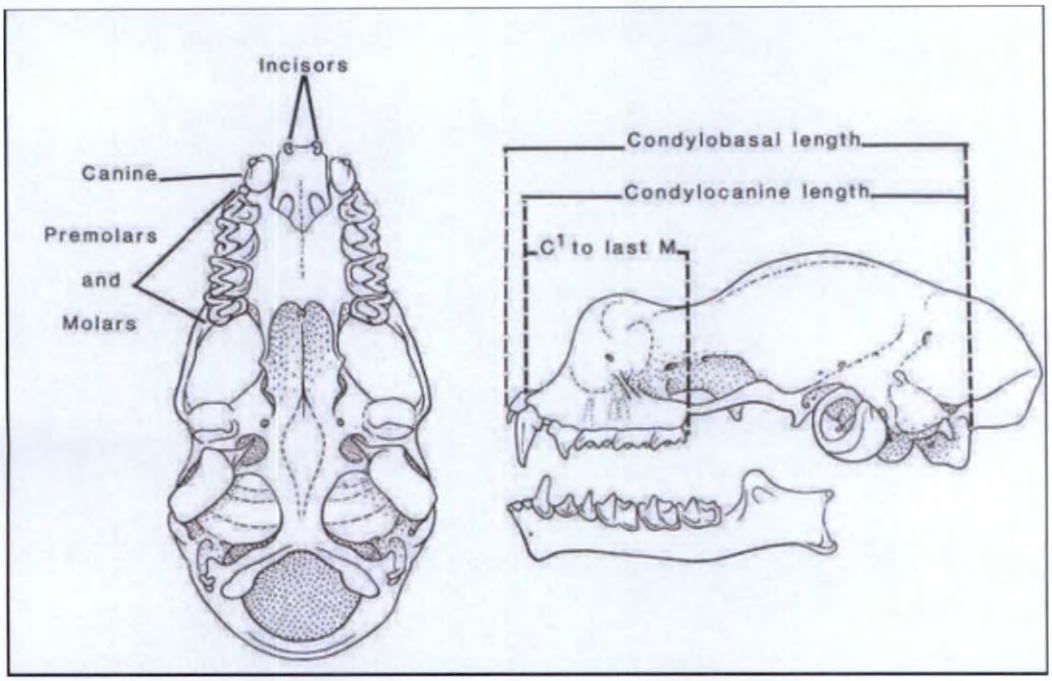


Plate 8. Cranial and dental measurements

(GTL), condylo-basal length (CBL), condylo- canine length (CCL), breadth of braincase (BB), zygomatic breadth (ZB), mandible length (M), maxillary toothrow ($C\text{-}M^n$), mandibular toothrow ($C\text{-}M_n$), posterior palatal width ($M^n\text{-}M^n$) and anterior palatal width ($C^1\text{-}C^1$).

3.2.6 Morphometric analysis

Fourteen external and 10 cranial and dental dimensions were measured using a digital calliper accurate to 0.01mm. Statistical analysis of the morphometric data was performed on size adjusted measurements by taking all measurements as percent of head to body length except for weight and wingspan. Principal Component Analysis (PCA) and Permutation Multivariate Analysis of Variance (PERMANOVA) were performed to understand whether related species of *Cynopterus* form significantly different clusters. Statistical analysis was performed in PAST version 2.14 (Hammer *et al.,* 2001) and Microsoft Excel version 2007.

3.2.7 Tissue Sampling and DNA Extraction

The DNA was isolated from tissues obtained from each bat either using Phenol-chloroform extraction method (Sambrook *et al.*, 1989) or GeNei Mammalian DNA purification kit.

In the Phenol-Chloroform extraction technique, 10 – 20 mg of tissue was ground into fine powder using mortar and pestle. The homogenate was transferred into a 2ml polypropylene centrifuge tube (Oakridge tube). Lysis buffer (250µl) and 10% SDS (250µl) was added to the tube and mixed by inversion. To this mixture 50µl Proteinase K was added and mixed by inversion and the sample was incubated for two to three hours at 55°C. Then the digested samples were centrifuged at 10,000

rpm for 10 minutes at 4°C by adding TRIS saturated phenol of pH 7.8 (Plate 10). After centrifugation, the aqueous phase was transferred to new Oakridge tube and centrifuged at 10,000 rpm for 10 minutes at 4°C by adding saturated phenol: chloroform: isoamyl alcohol mixture in the ratio 25:24:1. After centrifugation, supernatant was transferred and a mixture of chloroform: isoamyl alcohol (24:1) was added. The samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. After the centrifugation a 1/10 volume of 3M 40µl sodium acetate and 500µl of Isopropanol were added to the supernatant. Then the supernatant was incubated at -20°C overnight. Next day the samples were again centrifuged at 10000 rpm for 10 minutes at 4°C. Then supernatant was discarded and the DNA pellet (which was most of the times clearly visible) was allowed to dry. The pellet containing DNA was re-suspended in sufficient distilled water and stored at -20°C.

GeneiPure™ Mammalian Genomic DNA Purification Kit is a simple and rapid method for purification of genomic DNA from mammalian tissues. In this 10-20 mg of tissue was cut into small pieces. It was placed in a fresh 1.5 ml vial (sterile) and 180 µl of Lysis Buffer 1was added. The tissue was mechanically ground using the tissue grinder. Then 20 µl Proteinase K was added and mixed thoroughly. It was incubated at 55°C until complete lysis was observed (1-3 hours) and vortexed occasionally during incubation. Then 200 µl of Lysis Buffer II was added and mixed thoroughly by vortexing and incubated at 70 °C for 15-20 minutes. It was centrifuged for 10 minutes at 10000 rpm to remove any debris that might clog the GeneiPure™ Column. The supernatant was transferred to a fresh 1.5 ml vial. To this supernatant 4 µl of RNase A (100mg/ml) was added and mixed by vortexing and incubated at room temperature for 5-10 minutes. Then 200 µl of absolute ethanol was added to the sample and vortexed vigorously. The GeneiPure™ Column was placed in a collection tube and sample-ethanol mixture was added. The mixture was centrifuged for 1 minute at 11000 rpm. The Collection Tube with the flow through was discarded and

Plate 9. Working in Laminar Air Flow Chamber

Plate 10. Centrifuge

Plate 11. Mini centrifuge

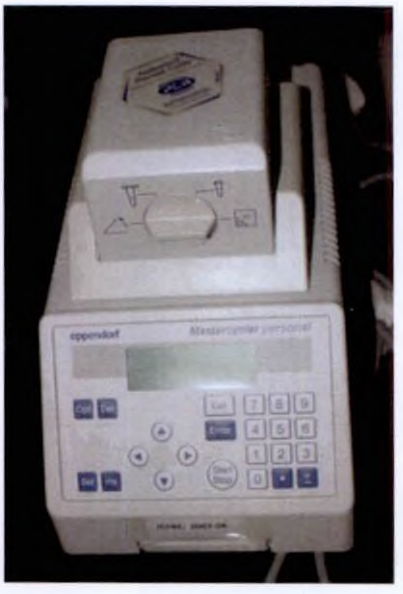


Plate 12. Thermo cycler

the GeneiPure™ Column placed into a new collection tube. One volume of Wash Buffer 1 was diluted with 3 volumes of absolute ethanol just before use. Then 500 µl of Wash Buffer I – Ethanol mix was added and centrifuged for 1 minute at 11000 rpm. The collection tube with flow through was discarded and placed the GeneiPure™ Column in a new collection tube. The column was centrifuged for 2 minutes at 11000 rpm. Required amount of Elution Buffer was warmed at 70°C dry bath for 5 minutes. And 100 µl of pre-warmed Elution Buffer (70°C) added to the center of GeneiPure™ Column. It was incubated at room temperature for five minutes and centrifuged for 1-2 minutes to elute the DNA. The eluted DNA was stored at -20°C.

3.2.8 Polymerase Chain Reaction (PCR)

The primers with sequence 5'-CCHCCATAAATAGGNGAAGG-3' (forward) and 5'-WAGAAYTTCAGCTTTGGG-3' (reverse) were used for amplifying the complete Cytochrome b (Cyt b) DNA fragments (Naidu *et al.* 2012) in the Polymerase Chain Reaction (PCR).

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. Amplification was done in a thermo cycler under following conditions: initial denaturation at 95^{0}C for 10 min followed by 29 cycles of 95^{0}C for 45s, 53^{0}C for one min (annealing) and 72^{0}C for two min (extension) (Plate 12). PCR products were visualized on one percent agarose gel containing ethidium bromide and run for about 20 minutes at 110V.

Plate 11. Mini centrifuge

Plate 12. Thermo cycler

Plate 13. Hot Plate

Plate 14. Water Bath

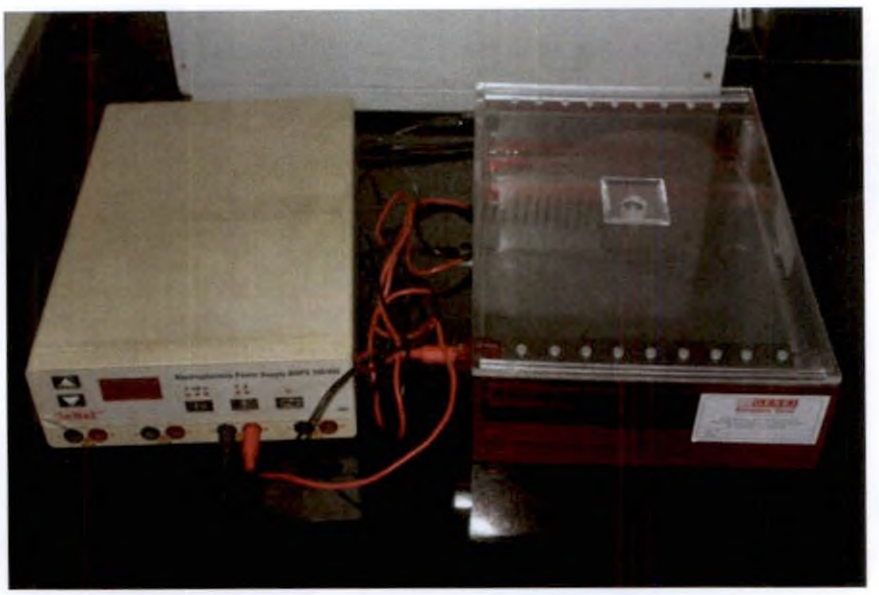


Plate 15. Gel Loading Instrument

Plate 16. UV Illuminator

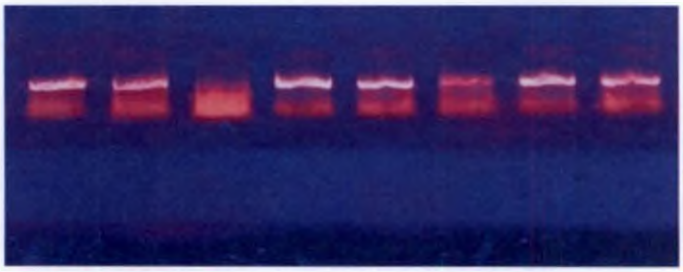


Plate 17. Gel Picture of Mitochondrial DNA

3.2.9 DNA sequencing

Amplified PCR product was subjected to Agarose Gel Electrophoresis to assess the amplicon size and to confirm the absence of non-specific amplification (Plate 17). The products were purified and sequenced (outsourced to) at a commercial sequencing facility. These sequences were compared with sequences of bat species from the GenBank (www.ncbi.nlm.nih.gov/nuccore) to confirm the species identity.

3.2.10 Phylogenetic analysis

The sequences were visualised and edited in BioEdit software (Hall, 1999). The edited sequences were aligned using MUSCLE (Edgar, 2004) algorithm, as implemented in MEGA 5.2 (Tamura *et al.*, 2011) and SeaView 4 (Gouy *et al.*, 2010). The maximum likelihood phylogeny construction the sequences generated during the course of this study was joined together with another published dataset (Campbell *et al.*, 2004) for adequate comparision and since it comprised of fruit bats (of *Cynopterus* genera) from throughout the range except the Kerala region of the Western Ghats. Maximum likelihood phylogeny was constructed using IQ- TREE (Nguyen *et al.*, 2015). Before finding the maximum likelihood tree the best nucleotide substitution model and the best partition regimen was found using the same software. The software, for finding the best partition regimen, uses a 'greedy algorithm' implemented in the software Partition Finder (Lanfear *et al.*, 2012). The confidence values at nodes were generated using an ultra-fast bootstrapping procedure developed recently (Minh, *et al.*, 2013). The HKY+R (Hasegawa *et al.*, 1985; Soubrier *et al.*, 2012) model was found to fit the data best with all the codon positions considered as a single partition being the best partition regimen. The Free Rate model used here has been shown to be less complicated than the +G+I models (Soubrier *et al.*, 2012), and it was chosen as the best rate heterogeneity model from among all the +G+I models and Free Rate models.

Pairwise distances between all sequences were calculated using the Kimura two parameter model (Kimura, 1980) in MEGA 5.2 (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).

Twenty four sequences of *C. sphinx* and seven sequences of *C. brachyotis* from the present study were used for the phylogenetic analysis. Since a total of 54 sequences each of *C. sphinx* and *C. brachyotis* from different part of its geographical range are used for analysis, the difference in the sample size will not affect the result.

Results

RESULTS

4.1 DISTRIBUTION AND SPECIES COMPOSITION OF BATS

A total of 66 individuals of frugivorous bats were captured, out of which 51 individuals belongs to the species *C. sphinx,* 13 were *C. brachyotis* and two individuals belongs to *Rousettus leschenaultii*. The species *C. sphinx* was captured from all the eight locations. Seven, four, eight, eight, nine, one, nine and five individuals were captured from Vellanikkara, Ambalavayal, Anakkayam, Kumarakom, Padanakkad, Pampadumpara, Tavanur and Vellayani respectively. Ambalavayal was the only place from where *C. brachyotis* was captured. Two individuals of *R. leschenaultii* were got from Vellayani (Appendix I).

4.2 MORPHOMETRIC ANALYSIS

Measurements of taxonomic characters

The 14 external and 10 cranial and dental measurements of each individuals captured and analysed are shown in Appendix II and Appendix III. The weight of *C. sphinx* varied from 29g to 56.50g with a mean of 43.87g. In the case of *C. brachyotis* the weight varied from 30g to 37g, with a mean weight of 34.18g. The forearm length and ear length, which are the two crucial measurements that distinguishes *C. sphinx* and *C. brachyotis,* ranged between 63.66mm to 74.91mm (forearm length for *C. sphinx*) and 60.46mm to 67.66mm (forearm length for *C. brachyotis*). The range for ear length ranged between 14.43mm to 20.43mm (for *C. sphinx*) and 14.95mm to 18.3mm (for for *C. brachyotis*). The mean anterior palatal width was 6.98mm and 6.58mm for the *C. sphinx* and *C. brachyotis* respectively. The range of all the 24 parameters, that is, morphological characters was overlapping (Table 4).

Table 4. Mean external, cranial and dental measurements of *C. sphinx* and *C. brachyotis* and the range of measurements.

Parameters	*C. sphinx* (n= 51)		*C. brachyotis* (n= 13)	
	Mean	**Range**	**Mean**	**Range**
W (gm)	43.87	29.00 - 56.50	34.18	30.00 - 37.00
HB (mm)	84.74	65.89 - 103.13	75.48	67.72 - 86.98
FA (mm)	68.54	63.66 - 74.91	63.60	60.46 - 67.66
T (mm)	8.91	5.37 - 13.80	6.99	5.31 - 11.20
E (mm)	17.53	14.43 - 20.43	15.72	14.95 - 18.3
HF (mm)	14.11	9.57- 19.03	12.44	10.52 - 14.97
WSP (mm)	433.36	379.00 - 473.00	405.75	398.00 - 422.00
TIB (mm)	27.01	21.22 - 30.03	23.53	21.73 - 25.21
THU (mm)	18.78	16.07 - 21.68	17.17	15.60 - 19.30
3MT (mm)	47.26	43.52 - 52.71	43.66	41.66 - 45.61
1PH3MT (mm)	30.59	26.14 - 34.79	27.99	26.33 - 31.52
2PH3MT (mm)	39.76	34.88 - 47.05	37.15	32.18 - 41.05
4MT (mm)	44.35	40.26 - 49.53	40.51	37.21 - 43.14
5MT (mm)	45.77	41.66 - 50.96	42.27	40.04 - 43.50
ZB (mm)	19.90	17.19 - 21.73	19.03	17.58 - 21.08
BB (mm)	13.36	12.29 - 14.29	12.78	12.30 - 14.03
CBL (mm)	29.72	26.15 - 33.07	27.93	25.84 - 30.08
CCL (mm)	28.99	26.00 - 32.04	27.28	25.46 - 29.19
GTL (mm)	31.70	28.18 - 35.36	30.06	28.92 - 33.36
CM^3 (mm)	11.03	9.38 - 12.21	10.00	8.82 - 11.38
CM_3 (mm)	12.16	10.64 - 13.68	11.31	9.35 - 13.43
M (mm)	24.09	20.54 - 26.65	22.48	21.22 - 25.52
M^3-M^3 (mm)	9.82	9.01 - 10.70	9.30	8.71 - 9.84
C^1-C^1 (mm)	6.98	6.06 - 8.10	6.58	6.21 - 7.34

The Figure 2 illustrates the mean of length of different measurements of *C. brachyotis* and *C. sphinx.* The error bar indicates their variation. In the case of *C. sphinx* variation was highest for weight (43.87 ± 10.42g) and lowest for anterior palatal width (6.98 ± 0.42mm). For *C. brachyotis* head to body length showed the highest variability (75.48 ± 5.65mm) followed by length of second phalanx of the third metacarpal (37.15 ± 2.64mm), weight (34.18 ± 2.46g), forearm length (63.60 ± 1.72mm) etc. As in the case of *C. sphinx,* the variability of anterior palatal width (6.58 ± 0.28mm) was lowest for *C. brachyotis.* Wing span length (WSP) was not considered in these cases because it is more susceptible to human error while stretching the wings when the measurements of the wings are taken.

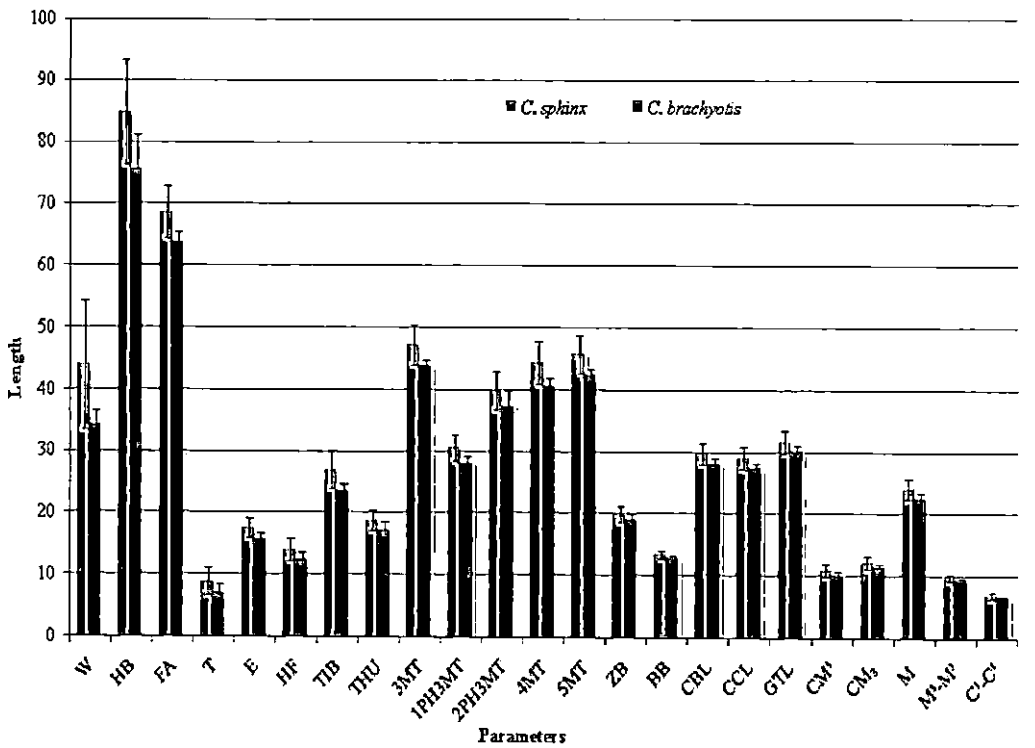


Figure 2. Mean external, cranial and dental measurements of *C. sphinx* (n=51) and *C. brachyotis* (n=13).

Identification of diagnostic character to distinguish between *C. sphinx* and *C. brachyotis*

When the measurements of the forearm length and ear length were analyzed for identifying, diagnostic character for distinguishing the two species of *Cynopterus,* the ear length didn't show any considerable difference. However, the forearm length showed distinctive difference between the *C. sphinx* and *C. brachyotis*. It is clear that in the case of ear length the range is overlapping between 15mm and 18.2mm for both species. While for forearm length, the maximum value was around 63.5mm for *C. brachyotis* and the minimum value for *C. sphinx* was greater than 63.5mm (Figure 3).

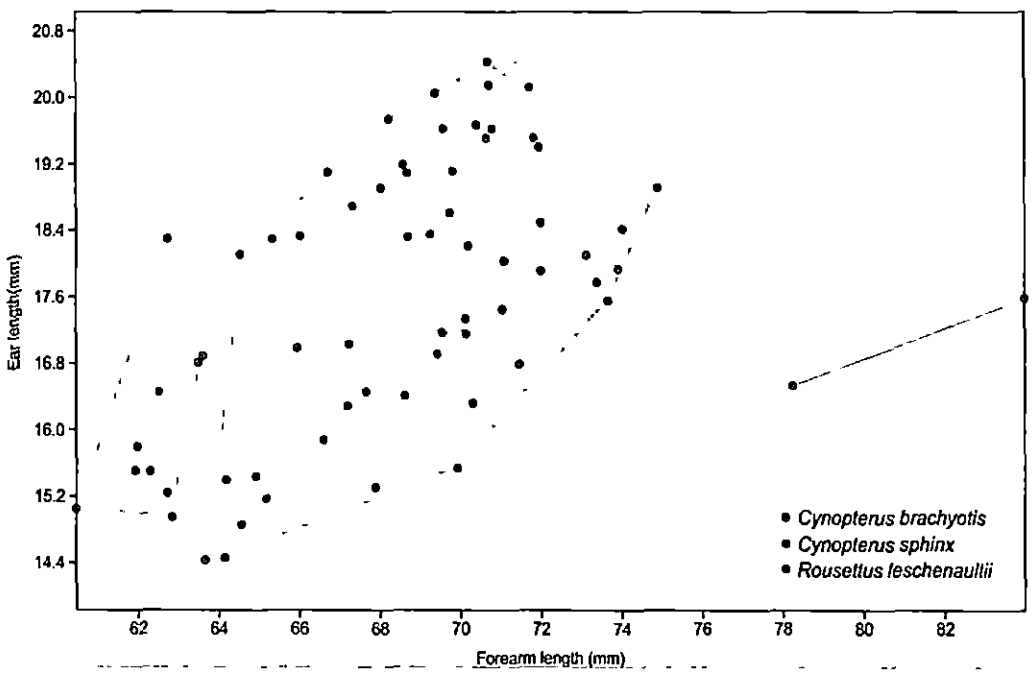


Figure 3. Scatter plot for identifying diagnostic character for distinguishing *C. brachyotis* and *C. sphinx.*

Mulivariate morphometric analysis for differentiating *C. sphinx* and *C. brachyotis*

Principal Component Analysis (PCA) was performed to investigate the presence of one or more natural groupings, that is, to differentiate *C. sphinx* and *C. brachyotis* population in Kerala. The PCA without size corrected morphometric data identified two informative components. These components together explained 100% of variability. The first component accounted for 93.02% of the total variance and the second component for 6.98% (Table. 5). But it was found that there was overlapping between *C. sphinx* and *C. brachyotis*. One more fruit bat species (*R. leschenaultii)* was also used in the analysis, which was found to be separated from the two species of *Cynopterus* (Figure 4).

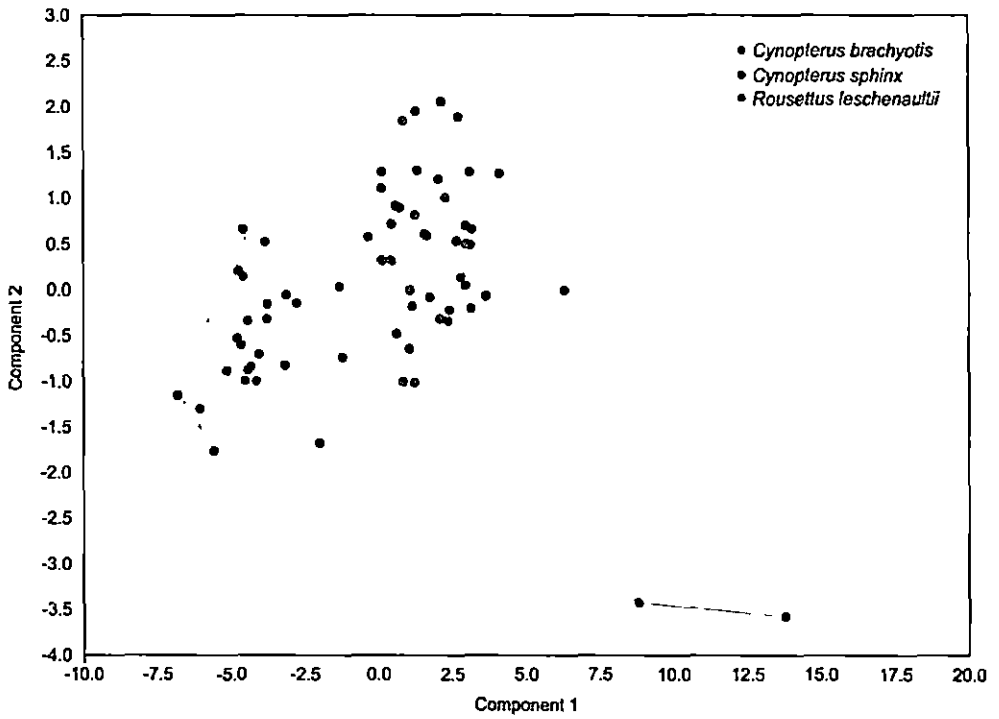


Figure 4. Scatter plot of PCA analysis without size corrected morphometric data to differentiate *C. sphinx* and *C. brachyotis*.

Table 5. PCA statistics and factor loadingfor multivariate morphometric analysis without normalization.

PC	Component 1	Component 2
Eigenvalue	21.40	1.61
% variance explained	93.02	6.98
Head to Body length (mm)	0.22	0.05
Forearm length (mm)	0.22	-0.002
Ear length (mm)	0.09	0.71
Tail Length (mm)	0.21	-0.11
Hind foot length (mm)	0.19	0.37
Wingspan (mm)	0.21	-0.21
Tibia (mm)	0.22	-0.09
Thumb (mm)	0.21	0.11
3^{rd} metacarpals (mm)	0.22	-0.04
1^{st} ph of 3^{rd} mt (mm)	0.22	0.06
2nd ^{ph} of 3^{rd} mt (mm)	0.20	0.34
4^{th} metacarpals (mm)	0.22	-0.07
5^{th} metacarpals (mm)	0.22	0.01
ZB (mm)	0.22	-0.05
BB (mm)	0.21	-0.14
CBL (mm)	0.22	-0.06
CCL (mm)	0.21	-0.06
GTL (mm)	0.21	-0.14
CM^3 (mm)	0.21	-0.10
CM_3(mm)	0.21	-0.16
M (mm)	0.21	-0.13
M^3-M^3 (mm)	0.21	-0.01
C^1-C^1 (mm)	0.21	0.22

The normality of the data was checked by using Doornik and Hansen omnibus test for normality. The result shows a rejection of the null hypothesis of normality at 1% level (Ep = 135.6; $p < 0.0001$). This means that the data is not

multivariate normal. Hence, a non-parametric multivariate analysis of variance (PERMANOVA) using Euclidian distance was performed. The result is given in the form of a matrix table (Table. 6).Values above the diagonals are F values and below are Bonferroni corrected *p* values. The analysis showed a significant difference between the species *Rouseteus leschenaultii* and *Cynopterus sphinx* (p=0.0012) and *R. leschenaultii* and *C. brachyotis* (p=0.0444). However, the F-value was found to be non significant in the case of C. *sphinx* and *C. brachyotis* (p= 0.1131).

Table 6. Permutation Multivariate Analysis of Variance for distinguishing C. *sphinx* and *C. brachyotis*.

Species	***C. brachyotis***	***C. sphinx***	***R. leschenaultii***
C. brachyotis		4.004	53.8
C. sphinx	0.1131^{ns}		11.99
R. leschenaultii	0.0444*	0.0012*	

* = significant at 5% level; ns = non significant at 5% level

To find whether the non-significant result between the species *C. sphinx* and *C. brachyotis* in PERMANOVA is due to the effect of size of the individuals, all the characters were divided by measurement 'head to body length' and performed Principle Component Analysis (PCA). In PCA two informative components were identified. These components together explained 100% of variability. The first principal component explains 74.94% of the overall variability; the second one explains 25.06% of it. (Table.7). It is evident from the scatter plot of principal components that the two species, *C. sphinx* and *C. brachyotis* were overlapping (Figure 5).

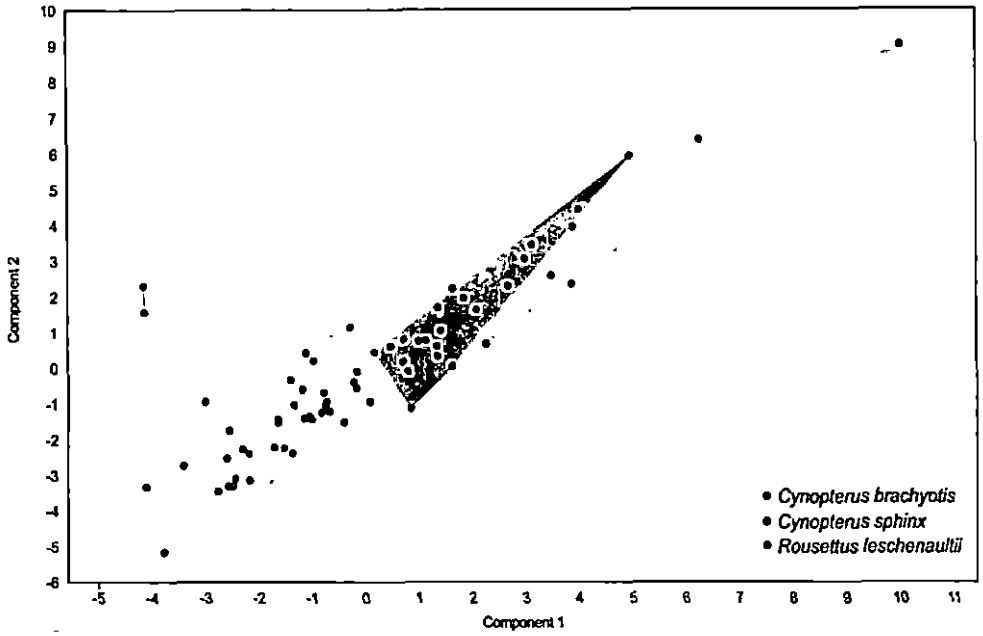


Figure 5. Scatter plot of PCA analysis with size corrected morphological data

Table 7. PCA statistics and factor loading for multivariate morphometric analysis with size corrected morphological data.

	PC 1	PC 2
Eigenvalue	16.71	5.29
% variance	75.94	24.06
Forearm length (mm)	0.22	0.19
Tail Length (mm)	-0.24	0.11
Ear length (mm)	0.24	-0.12
Hind foot length (mm)	0.23	-0.17
Wingspan (mm)	0.23	-0.15
Tibia (mm)	-0.23	0.14
Thumb (mm)	0.24	-0.04
3rd metacarpals (mm)	0.14	0.35
1st ph of 3rd mt (mm)	0.25	0.02
2nd ph of 3rd mt (mm)	0.24	-0.1
4th metacarpals (mm)	-0.17	0.31
5th metacarpals (mm)	0.24	0.11

ZB (mm)	0.24	0.10
BB (mm)	0.23	0.16
CBL (mm)	0.23	0.16
CCL (mm)	0.23	0.17
GTL (mm)	0.18	0.30
CM^3 (mm)	-0.17	0.32
CM_3(mm)	-0.11	0.39
M (mm)	0.03	0.43
M^3-M^3 (mm)	0.24	0.07
C^1-C^1 (mm)	0.25	-0.001

4.3 DNA ISOLATION AND PCR AMPLIFICATION OF CYTOCHROME B GENE

The DNA of 31 individuals under two species of bats, C. *sphinx* (24) and *C. brachyotis* (seven) were isolated using either Phenol-Chloroform extraction (Sambrook *et al.*, 1989) method or the GeniPure™ Mammalian Genomic DNA Purification Kit (GeNei™). The PCR produced a single amplification product for each genomic template.

4.4 SEQUENCING OF PCR PRODUCTS

The size of the complete cytochrome b gene for the PCR products from the bat tissues varied between 700 to 1480bp including the primer sequences (Table 8). All the PCR products were successfully sequenced.

Table 8. Details of sequenced products in the present study

Sl. No.	KAUNHM No.	Sequence length (bp)
1	201403	1480
2	201404	1206
3	201411	1226

4	201412	1254
5	201413	843
6	201415	1239
7	201416	1241
8	201417	1232
9	201420	1244
10	201422	1257
11	201423	1270
12	201425	1226
13	201426	1229
14	201427	1235
15	201428	1240
16	201429	1246
17	201432	1242
18	201433	1228
19	201438	1260
20	201443	1243
21	201445	1237
22	201450	702
23	201452	1221
24	201453	1274
25	201454	1280
26	201455	1261
27	201457	1241
28	201460	1268
29	201466	810
30	201467	1243
31	201472	1262

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

The sequences were compared with those registered in NCBI databank (http:\\blast.ncbi.nlm.nih.gov/Blast.). The details are shown in Table 9. The samples with KAUNHM201413 and KAUNHM201466 showed 99% similarity with an E value 0.0 during the BLASTn search of complete cytochrome b gene with NCBI deposits having Accession number KF042249.1 and KF042248.1 respectively. The similarity between *C. brachyotis* with KAUNHM201450 and NCBI accession number KF042232.1 was only 84%, but the E value was low as 1e - 124. For the sample KAUNHM201404 also the maximum identity was less than 90% with E value 0.0. All other 29 samples showed more than 90% similarity with any of the NCBI deposit. NCBI sequences with maximum similarity to the samples in the present study were deposited by NCBS, Bengaluru.

Table 9. Details of BLASTn results

SL No	KAUNHM	Details of accessions showing maximum similarity		Maximum Identity	E Value
		Species	Accession No		
1	201403	*C. sphinx*	KF042248.1	97%	0
2	201404	*C. sphinx*	KF042173.1	87%	0
3	201411	*C. sphinx*	KF042248.1	96%	0
4	201412	*C. sphinx*	KF042248.1	97%	0
5	201413	*C. sphinx*	KF042248.1	99%	0
6	201415	*C. sphinx*	KF042249.1	91%	0
7	201416	*C. sphinx*	KF042248.1	97%	0
8	201417	*C. sphinx*	KF042248.1	97%	0
9	201420	*C. sphinx*	KF042248.1	98%	0

10	201422	*C. sphinx*	KF042249.1	98%	0
11	201423	*C. sphinx*	KF042212.1	98%	0
12	201425	*C. sphinx*	KF042249.1	98%	0
13	201426	*C. sphinx*	KF042248.1	98%	0
14	201427	*C. sphinx*	KF042174.1	97%	0
15	201428	*C. sphinx*	KF042248.1	97%	0
16	201429	*C. sphinx*	KF042249.1	98%	0
17	201432	*C. sphinx*	KF042248.1	98%	0
18	201433	*C. sphinx*	KF042248.1	98%	0
19	201438	*C. brachyotis*	KF042221.1	98%	0
20	201443	*C. brachyotis*	KF042223.1	98%	0
21	204145	*C. brachyotis*	KF042252.1	97%	0
22	201450	*C. brachyotis*	KF042232.1	84%	1e - 124
23	201452	*C. brachyotis*	KF042192.1	97%	0
24	201453	*C. brachyotis*	KF042250.1	87%	0
25	201454	*C. brachyotis*	KF042225.1	95%	0
26	201455	*C. sphinx*	KF042248.1	97%	0
27	201457	*C. sphinx*	KF042248.1	98%	0
28	201460	*C. sphinx*	KF042248.1	97%	0
29	201466	*C. sphinx*	KF042248.1	99%	0
30	201467	*C. sphinx*	KF042248.1	97%	0
31	201472	*C. sphinx*	KF042249.1	97%	0

4.6 PHYLOGENETIC ANALYSIS

4.6.1 Variability among sequences

***Cynopterus sphinx* Vahl, 1797**

The sequence of *C. sphinx* retrieved from GenBank (KF042249.1, Karnataka) and the 24 sequences from the present study (KAUNHM201403, 201404, 201411, 201412, 201413, 201415, 201416, 201417, 201420, 201422, 201423, 201425, 201426, 201427, 201428, 201429, 201432, 201433, 201455, 201457, 201460, 201466, 201467, 201472) were aligned in BioEdit Sequence Alignment Editor ver. 7.2.5. The final lengths of all the aligned sequences were 671bp. When compared to *C. sphinx* from GenBank, the sequences of present study showed 572 conserved sites and 99 variable sites which include both transitions and transversions (Figure 6).

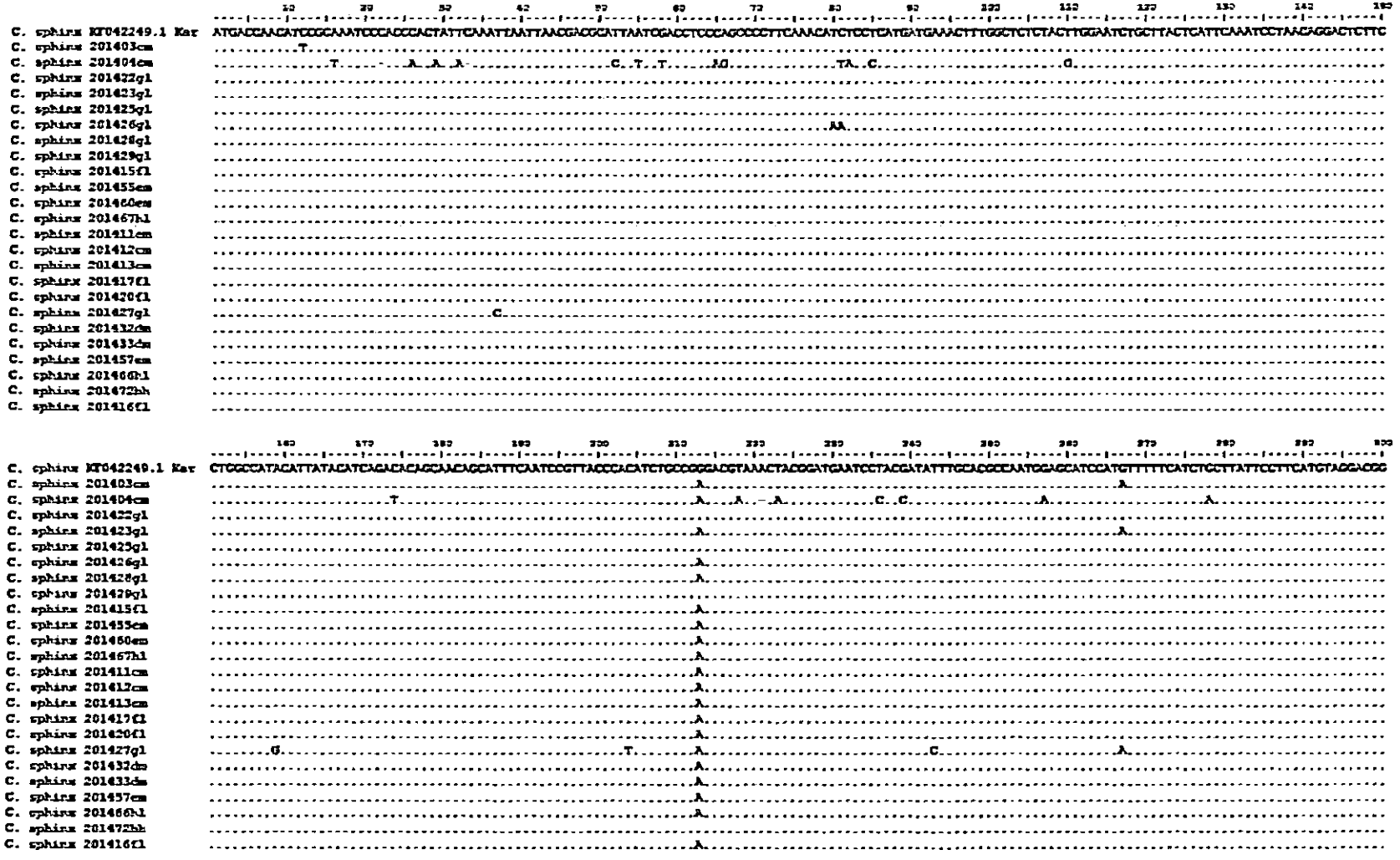
There was no variable sites in the case of aligned sequences of KAUNHM 201422, 201425 and 201429 with the sequence of *C. sphinx* from GenBank with accession number KF042249.1 from Karnataka. When the sequences of KAUNHM 201412, 201415, 201416, 201417, 201428, 201432, 201433, 201455, 201457 and 201467 were aligned with sequence of *C. sphinx* from GenBank (KF042249.1, Karnataka), only one site was found to be variable. The nucleotide position 213 showed transition in all these sequences. While comparing KAUNHM201472 a transversion was found at nucleotide position 324. Alignment of *C. sphinx*, KAUNHM201403 showed 3 transitions at nucleotide positions, 12, 213 and 267.

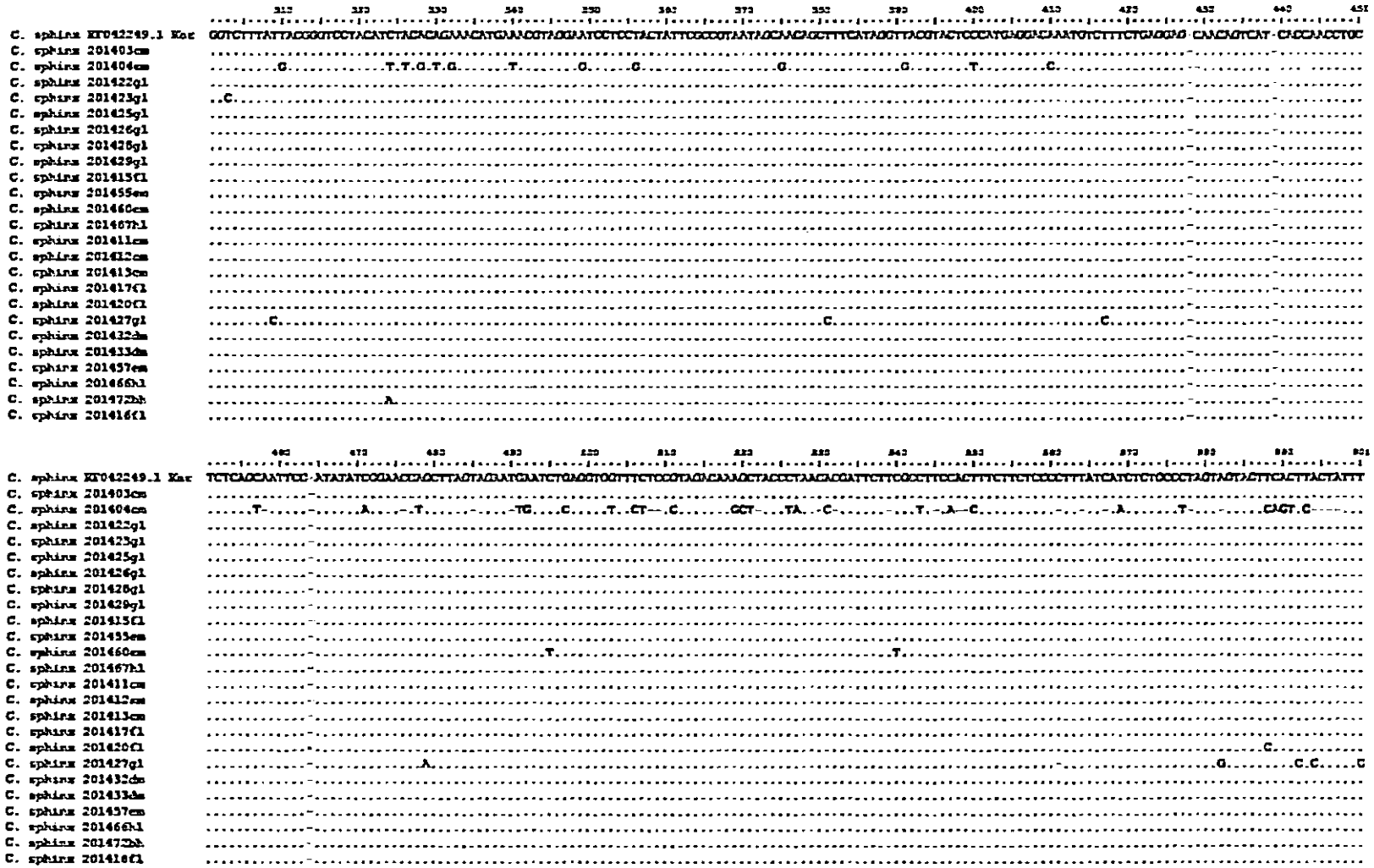
When compared with NCBI sequence KF042249.1 from Karnataka, the KAUNHM201404 showed 43 transitions at positions 52, 55, 58, 66, 81, 85, 174, 213, 236, 257, 278, 310, 324, 328, 332, 349, 375, 400, 457, 492, 506, 507, 519, 521, 526, 543, 550, 569, 577, 588, 590, 591, 593, 601, 602, 603, 607, 616, 624,

631, 633, 663, 664 and 667 and 30 transversions at positions 16, 26, 29, 32, 65, 82, 110, 218, 223, 239, 326, 330, 340, 356, 391, 410, 471, 478, 491, 497, 503, 511, 520, 527, 531, 547, 589, 630, 645 and 651.

For KAUNHM201423 the alignment showed three transitions at nucleotide positions 213, 267 and 303. In the case of KAUNHM201460 also there were three transitions at nucleotide positions 213, 495 and 540. The aligned sequence of KAUNHM201426 showed two transversions at positions 80 and 81 and a transition at position 213. Alignment of KAUNHM20111 and 201420, with the GenBank deposition, KF042249.1 from Karnataka, showed two transitions at positions, 213 and 664 and 213 and 588 respectively and for KAUNHM201413 there was a transition at position 666 and transversion at 665.

Fifteen transition and three transversion sites in KAUNHM201427 while aligned with GenBank deposition, KF042249.1 were 37, 159, 204, 213, 243, 267, 309, 381, 417, 479, 582, 592, 600, 660 and 666 and 594, 645 and 651 respectively. Aligned sequence of KAUNHM201466 showed three transitions at nucleotide positions 664, 669 and 213 and five transversions at positions 627, 630, 660, 661 and 672.


C. sphinx KT042249.1 Kar ATGACCAACATCCGCAAATCCCACCCACTATTCAAATTAATTAACGACGCATTAATGACCTCCCAGCCCCTTCAAACATCTCCTCATGATGAAACTTTGGCTCTCTACTTGGAATCTGCTTACTCATTCAAATCCTAACAGGACTCTTC
C. sphinx 201403cm
C. sphinx 201404cm
C. sphinx 201422gl
C. sphinx 201423gl
C. sphinx 201425gl
C. sphinx 201426gl
C. sphinx 201428gl
C. sphinx 201429gl
C. sphinx 201415fl
C. sphinx 201455cm
C. sphinx 201460cm
C. sphinx 201467hl
C. sphinx 201411cm
C. sphinx 201412cm
C. sphinx 201413cm
C. sphinx 201417fl
C. sphinx 201420fl
C. sphinx 201427gl
C. sphinx 201432dm
C. sphinx 201433dm
C. sphinx 201457cm
C. sphinx 201466hl
C. sphinx 201472hh
C. sphinx 201416fl
C. sphinx KT042249.1 Kar CTCGCCATACATTATACATCAGACACAGCAACAGCATTTCAATCCGTTACCCACATCTGCCGGGACGTAAACTACGGATGAATCCTACGATATTTGCACGCCAATGGAGCATCCATGTTTTTCATCTCCTTATTCCTTCATGTAGGACGG
C. sphinx 201403cm
C. sphinx 201404cm
C. sphinx 201422gl
C. sphinx 201423gl
C. sphinx 201425gl
C. sphinx 201426gl
C. sphinx 201428gl
C. sphinx 201429gl
C. sphinx 201415fl
C. sphinx 201455cm
C. sphinx 201460cm
C. sphinx 201467hl
C. sphinx 201411cm
C. sphinx 201412cm
C. sphinx 201413cm
C. sphinx 201417fl
C. sphinx 201420fl
C. sphinx 201427gl
C. sphinx 201432dm
C. sphinx 201433dm
C. sphinx 201457cm
C. sphinx 201466hl
C. sphinx 201472hh
C. sphinx 201416fl


C. sphinx KT042249.1 Kar
C. sphinx 201403cm
C. sphinx 201404cm
C. sphinx 201422gl
C. sphinx 201423gl
C. sphinx 201425gl
C. sphinx 201426gl
C. sphinx 201428gl
C. sphinx 201429gl
C. sphinx 201415fl
C. sphinx 201455em
C. sphinx 201460cm
C. sphinx 201467hl
C. sphinx 201411cm
C. sphinx 201412cm
C. sphinx 201413cm
C. sphinx 201417fl
C. sphinx 201420fl
C. sphinx 201427gl
C. sphinx 201432dm
C. sphinx 201433dm
C. sphinx 201457em
C. sphinx 201466hl
C. sphinx 201472bh
C. sphinx 201416fl

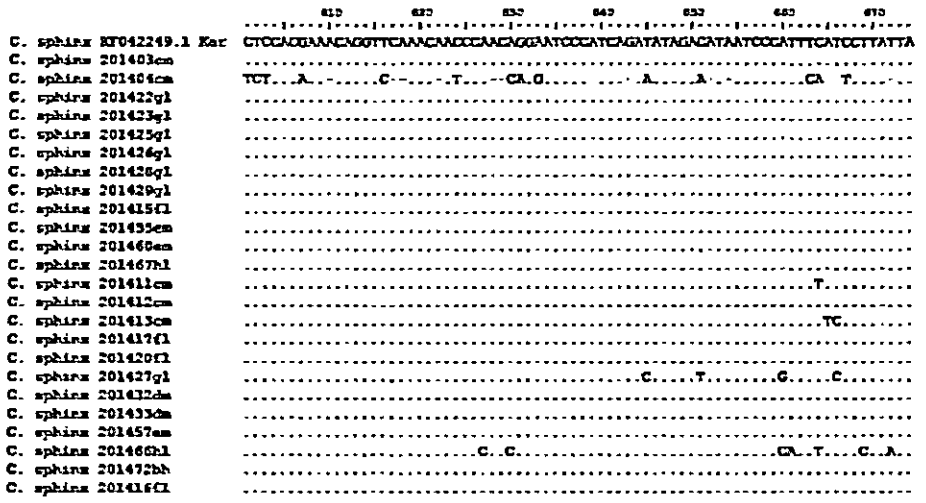


Figure 6. The aligned sequence of cytochrome b gene of *C. sphinx*. 'Dot' indicates the same base as the first sequence (KF042249.1, Karnataka). All the sequences were 671bp lengths.

***Cynopterus brachyotis* Muller, 1838**

The sequence of *C. brachyotis* retrieved from GenBank (KF042221.1, Karnataka) and the seven sequences from the present study (KAUNHM201438, 201443, 201445, 201450, 201452, 201453 and 201454) were aligned using ClustalW Multiple Sequence Alignment (Thompson *et al.*, 1994) of BioEdit Ver. 7.2.5. The final lengths of all the aligned sequences were 584bp. When compared to *C. brachyotis* from GenBank, the sequences of present study showed 428 conserved sites and 147 variable sites which include both transitions and transversions (Figure 7).

The sequence KAUNHM201438 showed a variable site, that is a transversion at the nucleotide position 305. This position was found to be variable for all other sequences, where Adenine was replaced by Cytosine. For KAUNHM201443, except this seven transitions were also found in nucleotide positions 82, 122, 346, 394, 499, 532 and 546 and for KAUNHM201454 eight transitions at positions 82, 112, 160, 304, 346, 394, 499 and 532. When KAUNHM201450 was aligned, nucleotide positions 69, 73, 74, 75, 77, 78, 80, 81, 82, 85, 87, 89, 95,102, 110, 132, 135, 152, 153, 165, 173, 180, 198, 204, 215, 256, 268, 280, 305, 340, 360, 387, 392, 401, 406, 412, 419, 436, 438, 445, 450, 454, 482, 484, 498, 506, 507, 517, 519, 520, 526, 528, 544, 545, 546, 553,554, 564, 567, 568, 571, 574, 581, 582 and 584 showed transversions and 68, 72, 119, 122, 140, 156, 166, 182, 207, 220, 230, 258, 285, 303,354, 374, 376, 389, 394, 407, 431, 446, 457,459, 470, 479, 483, 496, 497, 499, 505, 508, 511, 539, 541, 542, 549, 551, 552, 555, 560, 565, 566, 573, 575, 578 and 583 showed transitions.

Aligned sequence of KAUNHM201452 showed transitions at nucleotide positions 82, 122, 346, 394, 499 and 532 and transversions at 73, 74, 75, 77, 78, 80, 81 and 305. In the case of KAUNHM201453, 55 variable sites were identified. Transition was shown by the sites 39, 86, 122, 156, 160, 167, 178, 343, 346, 376, 394, 431, 445, 496, 510, 511, 517, 523, 529, 532, 556, 573, 582 and 583

and the nucleotide positions 33,34, 52, 77, 82, 110, 130, 158, 169, 171, 173, 175, 224, 305, 311, 392, 419, 437, 481, 488, 495, 499, 501, 507, 533, 545, 553, 570, 575 and 584 showed transversions. In the case of KAUNHM201445 seven transitions were shown at nucleotide positions 56, 211, 319, 346, 394, 412 and 532 and five transversions were found at nucleotide positions 74, 80, 81, 82 and 305.

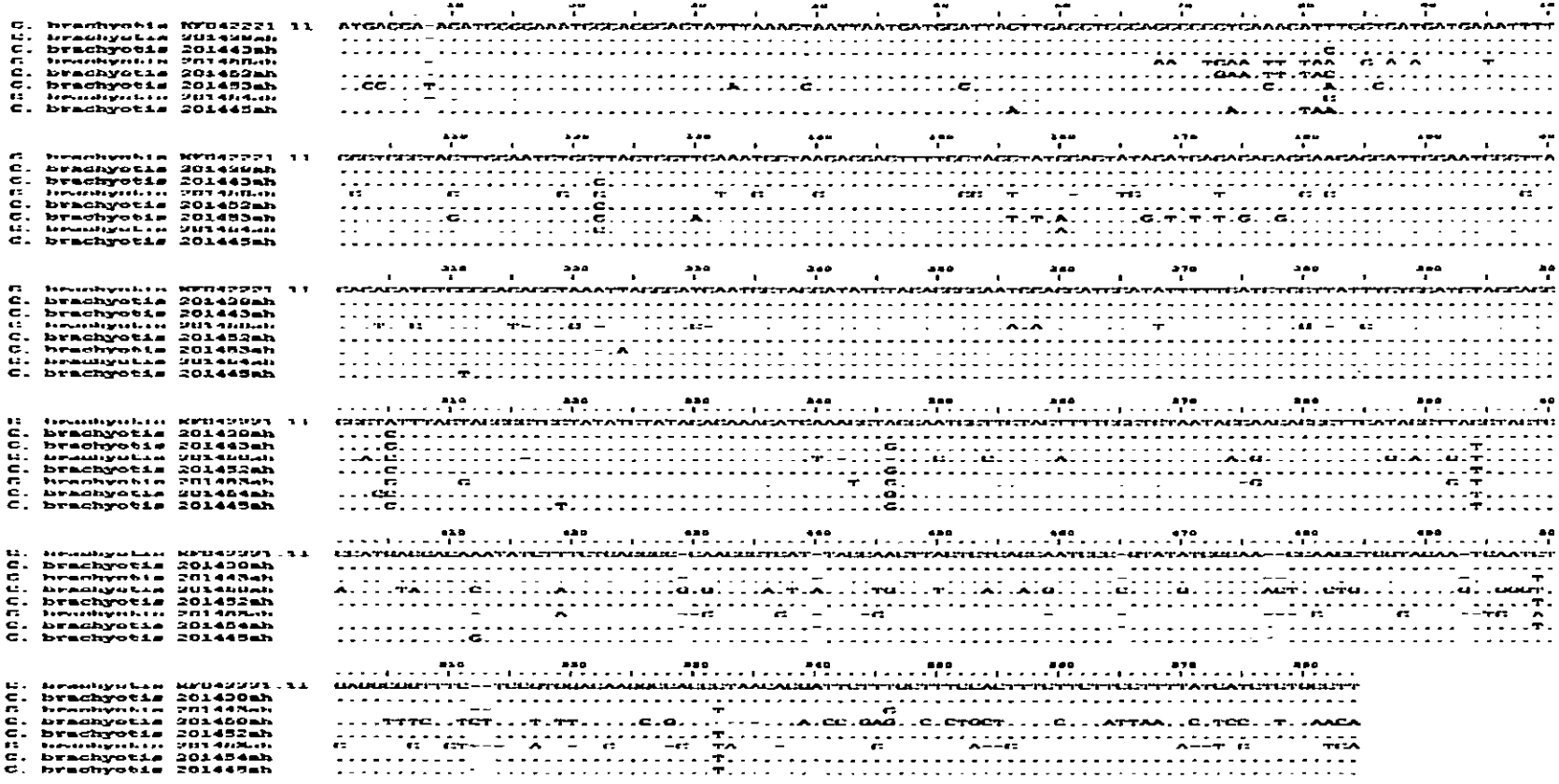


Figure 7. The aligned sequence of cytochrome b gene of *C. brachyotis*. 'Dot' indicates the same base as the first sequence (KF042221.1, Karnataka). All the sequences were 584bp lengths.

4.6.2 Genetic distances

The sequences were aligned in ClustalW Multiple Sequence Alignment (Thompson *et al.*, 1994) of BioEditVer. 7.2.5. and pairwise genetic distance was calculated using MEGA Ver. 5.2.

Genetic distance between *Cynopterus sphinx* from different locations

The pairwise genetic distance between individuals of *C. sphinx* from different locations within its geographical range was calculated (Table 10). Sequences used includes *C. sphinx*, AY628989, Thailand, *C. sphinx*, AY628996, Tamil Nadu, *C. sphinx*, AY628997, Vietnam, *C. sphinx*, KAUNHM201411, *C. sphinx*, KAUNHM201412, *C. sphinx*, KAUNHM201413, *C. sphinx*, KAUNHM201416, *C. sphinx*, KAUNHM201417, *C. sphinx*, KAUNHM201420, *C. sphinx*, KAUNHM201427, *C. sphinx* KAUNHM201432, *C. sphinx*, KAUNHM201433, *C. sphinx*, KAUNHM201457, *C. sphinx*, KAUNHM201466, *C. sphinx*, KAUNHM201472, *C. sphinx*, KAUNHM201422, *C. sphinx*, KAUNHM201423, *C. sphinx*, KAUNHM201425, *C. sphinx*, KAUNHM201426, *C. sphinx*, KAUNHM201428, *C. sphinx*, KAUNHM201429, *C. sphinx*, KAUNHM201403, *C. sphinx*, KAUNHM201460, *C. sphinx*, KAUNHM201467, *C. sphinx*, KF042249, Karnataka and *C. sphinx*, AY009889, Sri Lanka.

It was observed that *C. sphinx* from Kerala, except KAUNHM201427 (2.2% and 2.5% respectively) showed less than 2% genetic distance with sequences from Tamil Nadu and Karnataka. Most of the Indian specimens showed a genetic distance greater than 3% and 2% with sequence from Thailand and Vietnam respectively. When the sequences from Kerala were compared with that from Sri Lanka the genetic distance ranged between 2.2% to 4.1%. The genetic distance was less than 1% between sequences from Kerala, except for KAUNHM201427 (range: 1.9% - 2.8%).

Table 10. Pairwise Genetic Distance (%) among *C. sphinx* from different locations in its geographical range.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1																										
2	3.1																									
3	0.9	2.2																								
4	3.1	0.0	2.2																							
5	3.1	0.0	2.2	0.0																						
6	3.1	0.0	2.2	0.0	0.0																					
7	3.1	0.0	2.2	0.0	0.0	0.0																				
8	3.1	0.0	2.2	0.0	0.0	0.0	0.0																			
9	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0																		
10	1.5	2.2	0.6	2.2	2.2	2.2	2.2	2.2	2.2																	
11	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2																
12	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0															
13	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0														
14	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0													
15	3.8	0.6	2.8	0.6	0.6	0.6	0.6	0.6	0.6	2.8	0.6	0.6	0.6	0.6												
16	3.4	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.3	2.5	0.3	0.3	0.3	0.3	0.3											
17	3.1	0.6	2.2	0.6	0.6	0.6	0.6	0.6	0.6	2.2	0.6	0.6	0.6	0.6	1.2	0.9										
18	3.4	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.0	0.9									

19	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.6	0.3	0.6	0.3								
20	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.6	0.3	0.6	0.3	0.0							
21	3.4	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.0	0.9	0.0	0.3	0.3						
22	2.8	0.3	1.9	0.3	0.3	0.3	0.3	0.3	0.3	1.9	0.3	0.3	0.3	0.3	0.9	0.6	0.3	0.6	0.3	0.3	0.6					
23	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.6	0.3	0.6	0.3	0.0	0.0	0.3	0.3				
24	3.1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.6	0.3	0.6	0.3	0.0	0.0	0.3	0.3	0.0			
25	3.4	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.3	2.5	0.3	0.3	0.3	0.3	0.3	0.0	0.9	0.0	0.3	0.3	0.0	0.6	0.3	0.3		
26	5.1	2.2	4.1	2.2	2.2	2.2	2.2	2.2	2.2	4.1	2.2	2.2	2.2	2.2	2.8	2.5	2.8	2.5	2.2	2.2	2.5	2.5	2.2	2.2	2.5	

1.*C. sphinx*, AY628989, Thailand, 2.*C. sphinx*, AY628996, Tamil Nadu, 3.*C.sphinx*, AY628997, Vietnam, 4.*C. sphinx*, KAUNHM201411, 5.*C. sphinx*, KAUNHM201412, 6.*C. sphinx*, KAUNHM201413, 7.*C. sphinx*, KAUNHM201416, 8.*C. sphinx*, KAUNHM201417, 9.*C. sphinx*, KAUNHM201420, 10.*C. sphinx*, KAUNHM201427, 11.*C. sphinx*, KAUNHM201432, 12.*C. sphinx*, KAUNHM201433, 13. *C. sphinx*, KAUNHM201457, 14.*C. sphinx*, KAUNHM201466, 15.*C. sphinx*, KAUNHM201472, 16.*C. sphinx*, KAUNHM201422, 17.*C. sphinx*, KAUNHM201423, 18.*C. sphinx*, KAUNHM201425, 19.*C. sphinx*, KAUNHM201426, 20.*C. sphinx*, KAUNHM201428, 21.*C. sphinx*, KAUNHM201429, 22.*C. sphinx*, KAUNHM201403, 23.*C. sphinx*, KAUNHM201460, 24.*C. sphinx*, KAUNHM201467, 25.*C. sphinx*, KF042249, Karnataka, 26.*C. sphinx*, AY009889, Sri Lanka.

Genetic distance between *C. brachyotis* from different locations

Five sequences (KAUNHM201438, KAUNHM201443, KAUNHM201445, KAUNHM201452, KAUNHM201454) of *C. brachyotis* from Kerala were used for computing pairwise genetic distance with sequences from Borneo (AY628952), Karnataka (AY628921), Sulawesi (AY628943) and Sri Lanka (AY009894). Borneo is the type locality of *C. brachyotis*. The genetic distance of sequences from Kerala varied between 10.6% and 11.4% with sequence from Borneo. The sequence from Sulawesi also showed greater than 9% genetic divergence with sequences from Kerala (Range: 9.6% to 13.6%). The percentage genetic distance between sequences from Kerala and Sri Lanka showed a variation within the range of 4.7% and 5.4%. The genetic divergence among the sequences from Kerala was found to be less than 2%. There genetic distance observed between the sequences AY628921 and KAUNHM201443 and KAUNHM201452 and that between KAUNHM201443 and KAUNHM201452 was zero (Table 11).

Genetic distance between *C. sphinx* and *C. brachyotis* from Kerala

Twenty two sequences of *C. sphinx* and five sequences of *C. brachyotis* in the present study were aligned and pairwise genetic distances were calculated (Table 12). The C. *brachyotis* showed 8.5% to 10.2% variation when compared to *C. sphinx*. The within species variation for *C. sphinx* was between 0% and 2.2% and for *C. brachyotis* was in a range 0.5% and 1.7%.

Table 11. Pairwise Genetic Distance (%) among *C. brachyotis* from different locations in its geographical range.

Species	*C. brachyotis*AY628921 Karnataka	*C. brachyotis*AY628943 Sulawesi	*C. brachyotis*AY628952 Borneo	*C. brachyotis*KAUNHM 201445	*C. brachyotis*KAUNHM 201438	*C. brachyotis*KAUNHM 201443	*C. brachyotis*KAUNHM 201452	*C. brachyotis*KAUNHM 201454	*C. brachyotis*AY009894 Srilanka
*C. brachyotis*AY628921 Karnataka									
*C. brachyotis*AY628943 Sulawesi	12.6								
*C. brachyotis*AY628952 Borneo	10.6	9.6							
*C. brachyotis*KAUNHM201445	1.2	13.3	11.4						
*C. brachyotis*KAUNHM201438	0.9	11.4	11.0	1.5					
*C. brachyotis*KAUNHM201443	0.00	12.6	10.6	1.2	0.9				
*C. brachyotis*KAUNHM201452	0.00	12.6	10.6	1.2	0.9	0.00			
*C. brachyotis*KAUNHM201454	0.6	12.6	11.4	1.8	1.5	0.6	0.6		
*C. brachyotis*AY009894 Srilanka	4.7	13.6	12.0	5.4	5.0	4.7	4.7	4.7	

Table 12. Pairwise Genetic Distance (%) between *C. sphinx* and *C. brachyotis* from Kerala.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1																											
2	0.0																										
3	0.0	0.0																									
4	0.0	0.0	0.0																								
5	0.0	0.0	0.0	0.0																							
6	0.0	0.0	0.0	0.0	0.0																						
7	1.9	1.9	1.9	1.9	1.9	1.9																					
8	0.0	0.0	0.0	0.0	0.0	0.0	1.9																				
9	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0																			
10	10.2	10.2	10.2	10.2	10.2	10.2	9.8	10.2	10.2																		
11	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2																	

12	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2	0.0																	
13	0.5	0.5	0.5	0.5	0.5	0.5	2.5	0.5	0.5	10.8	0.5	0.5																
14	0.3	0.3	0.3	0.3	0.3	0.3	2.2	0.3	0.3	10.5	0.3	0.3	0.3															
15	0.5	0.5	0.5	0.5	0.5	0.5	1.9	0.5	0.5	10.2	0.5	0.5	1.1	0.8														
16	0.3	0.3	0.3	0.3	0.3	0.3	2.2	0.3	0.3	10.5	0.3	0.3	0.3	0.0	0.8													
17	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2	0.0	0.0	0.5	0.3	0.5	0.3												
18	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2	0.0	0.0	0.5	0.3	0.5	0.3	0.0											
19	0.3	0.3	0.3	0.3	0.3	0.3	2.2	0.3	0.3	10.5	0.3	0.3	0.3	0.0	0.8	0.0	0.3	0.3										
20	8.5	8.5	8.5	8.5	8.5	8.5	8.2	8.5	8.5	1.4	8.5	8.5	9.1	8.8	8.5	8.8	8.5	8.5	8.8									
21	0.3	0.3	0.3	0.3	0.3	0.3	1.7	0.3	0.3	9.8	0.3	0.3	0.8	0.5	0.3	0.5	0.3	0.3	0.5	8.2								
22	9.5	9.5	9.5	9.5	9.5	9.5	9.2	9.5	9.5	1.1	9.5	9.5	10.1	9.8	9.5	9.8	9.5	9.5	9.8	0.8	9.2							
23	9.5	9.5	9.5	9.5	9.5	9.5	9.2	9.5	9.5	1.1	9.5	9.5	10.1	9.8	9.5	9.8	9.5	9.5	9.8	0.8	9.2	0.0						
24	9.5	9.5	9.5	9.5	9.5	9.5	9.8	9.5	9.5	1.7	9.5	9.5	10.1	9.8	8.8	9.8	9.5	9.5	9.8	1.4	9.2	0.5	0.5					

25	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2	0.0	0.0	0.5	0.3	0.5	0.3	0.0	0.0	0.3	8.5	0.3	9.5	9.5	9.5			
26	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2	0.0	0.0	0.5	0.3	0.5	0.3	0.0	0.0	0.3	8.5	0.3	9.5	9.5	9.5	0.0		
27	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	10.2	0.0	0.0	0.5	0.3	0.5	0.3	0.0	0.0	0.3	8.5	0.3	9.5	9.5	9.5	0.0	0.0	

1. *C. sphinx*, KAUNHM201411, 2. *C. sphinx*, KAUNHM201412, 3.*C. sphinx*, KAUNHM201413, 4.*C. sphinx*, KAUNHM201416, 5.*C. sphinx*, KAUNHM201417, 6.*C. sphinx*, KAUNHM201420, 7.*C. sphinx*, KAUNHM201427, 8.*C. sphinx*, KAUNHM201432, 9.*C. sphinx*, KAUNHM201433, 10.*C. brachyotis* KAUNHM201445, 11.*C. sphinx*, KAUNHM201457, 12.*C. sphinx*, KAUNHM201466, 13.*C. sphinx*, KAUNHM201472, 14.*C. sphinx*, KAUNHM201422, 15.*C. sphinx*, KAUNHM201423, 16.*C. sphinx*, KAUNHM201425, 17.*C. sphinx*, KAUNHM201426, 18.*C. sphinx*, KAUNHM201428, 19.*C. sphinx*, KAUNHM201429, 20.*C. brachyotis*, KAUNHM201438, 21.*C. sphinx*, KAUNHM201403, 22.*C. brachyotis*, KAUNHM201443, 23.*C. brachyotis*, KAUNHM201452, 24.*C. brachyotis*, KAUNHM201454, 25.*C. sphinx*, KAUNHM201455, 26.*C. sphinx*, KAUNHM201460, 27.*C. sphinx*, KAUNHM201467.

4.6.2 Phylogenetic relationship between *C. sphinx* and *C. brachyotis*.

One hundred and twenty eight sequences including 54 *C. brachyotis*, 54 C. sphinx, 13 *C. horsfieldi* and seven species of out groups were aligned together for the construction of a (Maximum Likelihood) phylogenetic tree (Fig. 8.). This included 22 sequences of *C. sphinx* and five species of *C. brachyotis* from the present study. The dataset included sequences generated as part of this study and a previous one (Campbell *et al.*, 2004) which studied the phylogenitics of fruit bats of South East Asia. In addition five sequence of *C. sphinx* (KF042249, KF042248, KF042198, KF042184 and KC248379) from south India was used for the study. The outgroup taxa used were other members of the family Pteropodidae such as, *Pteropus giganteus* (KJ532397), *Eonycteris spelaea* (AB062476), *Rousettus leschenaultii* (FJ549337), *Chironax melanocephalus* (AY629005), *Latidens salimalii* (GQ410217), *Aethalops alecto* (AY629006) and *Megaeropse caudatus* (AY629007). One thousand ultra-fast bootstrap replicates (Minh *et al.*, 2013) were run to check for the node support and to ascertain the robustness of the topology. The final alignment was 585 base pairs in length.

Five sequences of *C. brachyotis* (KAUNHM201438, KAUNHM201443, KAUNHM201445, KAUNHM201452 and KAUNHM201454) from Kerala together with three sequences (AY628921, AY628933 and AY628932) from Karnataka formed an independent clade separate from the other *C. brachyotis* sequences (Fig. 8). *C. sphinx* sequences generated as part of the study formed a clade along with sequences from other parts of south India. But the sequence KAUNHM201427 formed another group with sequences from Myanmar (AY629000), Thailand (AY628991) and Vietnam (AY628999).

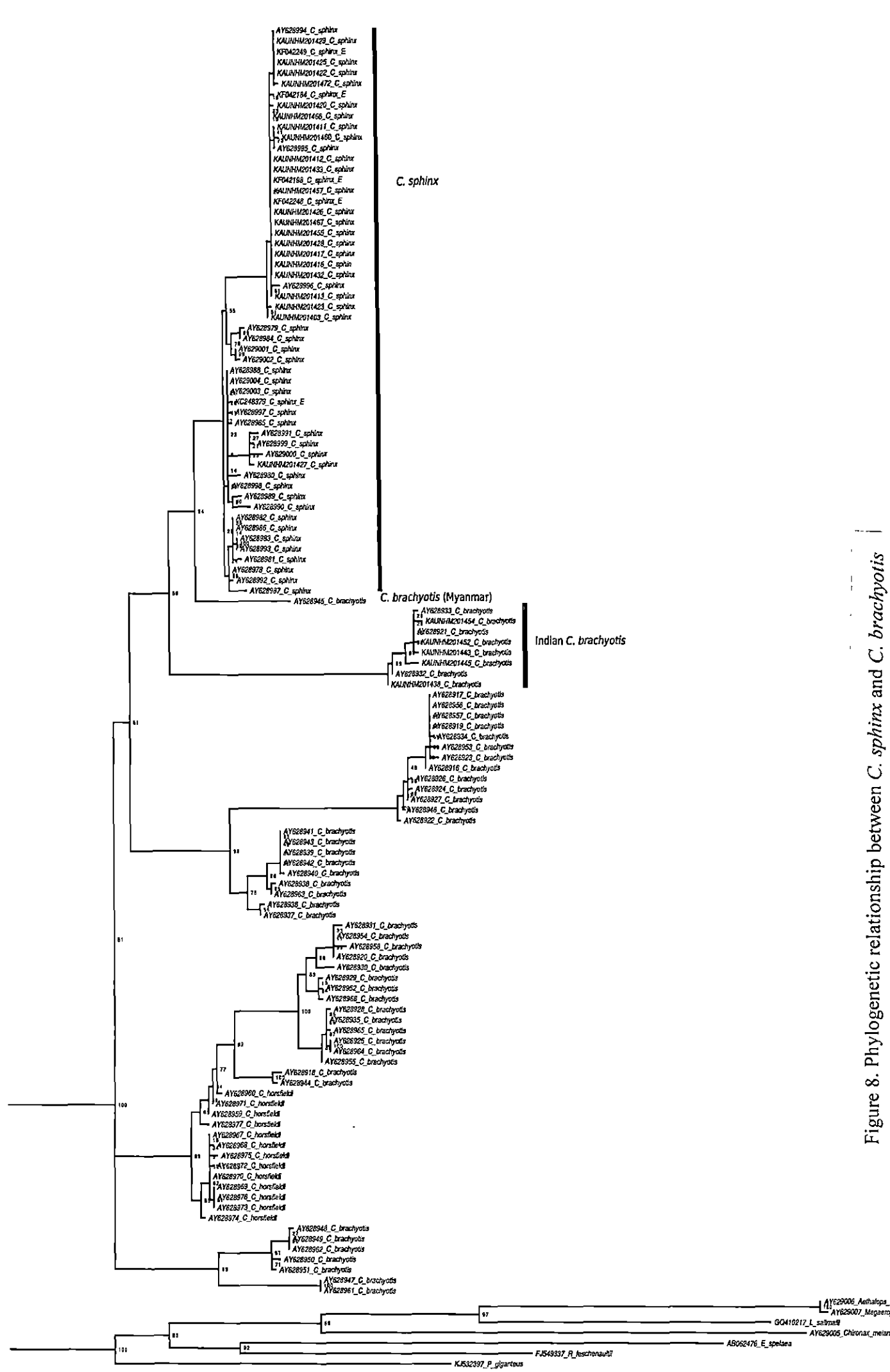


Figure 8. Phylogenetic relationship between *C. sphinx* and *C. brachyotis*

Discussion

DISCUSSION

The taxonomic status of the species *C. sphinx* and *C. brachyotis* is a subject of confusion and controversy (Campbell *et al.,* 2004). This is mainly because of the apparent overlap in the size between *C. sphinx* and *C. brachyotis* throughout the Southeast Asia (Andersen, 1912). It is also related with the problem of selecting appropriate morphological characters to differentiate these two species (Francis, 1990; Campbell *et al.,* 2004). And also the relationship of these two species is not clearly studied throughout its geographical range (Campbell *et al.,* 2004; Bates, 2013). A better way is to analyze the DNA fragments from each species (or population) in conjunction with morphological characters for taxonomic studies (Mapatuna *et al.*, 2002; Bertolazzi *et al.*, 2009; Padhye *et al.,* 2014) in an integrative taxonomic approach. Here we aimed to solve the issue related to the systematic position of these aforementioned species.

5.1 MORPHOMETRIC ANALYSIS

A total of 24 morphometric measurements (14 external and 10 cranial and dental measurements) were used for analysis according to Bates and Harrison (1997). All the measurements showed a mean and range as mentioned in the Bates and Harrison (1997). As reported by Bates and Harrison (1997), Mapatuna *et al.* (2002) and Storz and Kunz (1999) the *C. sphinx* averages larger than *C. brachyotis.* The diagnostic characters for differentiating *C. sphinx* and *C. brachyotis* identified by Bates and Harrison (1997) were forearm length with a range of 57.3mm – 63.3mm for *C. brachyotis* and 64mm – 79mm for *C. sphinx,* ear length of 14.5mm – 18 and 17.5mm – 24mm and lighter and darker ear edges respectively. Bumrungsri and Racey (2005) also supports the same approach that a combination of forearm length and ear length can be used as most reliable characters to distinguish the *C. sphinx* and

C. brachyotis. But in the present study only the forearm length was found to be diagnostic character in distinguishing *C. brachyotis* and *C. sphinx* (Fig. 3).

A Principal Component Analysis of 23 measurements failed to differentiate the two species; *C. brachyotis* and *C. sphinx* (Fig. 4 and Fig. 5). The result was also same in Permutation MANOVA (PERMANOVA). That means the two species could not be separated based on morphometric parameters. This may be due the overlapping characters exhibited by both species. However, it is interesting to note that previous studies of the same species (Mapatuna *et al.*, 2002) from Sri Lanka have been successful in differentiating them based on morphometric measurements and multivariate statistics. Low sample numbers may be an appropriate explanation for the inability to differentiate (based on morphometry) these species during the tenure of this study. An intensive sampling of both species from different regions may be helpful in this regard.

5.2 DNA ISOLATION, PCR AMPLIFICATION AND SEQUENCING OF COMPLETE CYTOCHROME B GENE

In the present study DNA from 24 samples of *C. sphinx* and seven samples of *C. brachyotis* were isolated, appropriate gene fragment amplified and sequenced successfully.

The mitochondrial cytochrome b gene has been extensively used for species identification, taxonomic and phylogenetic study of mammals (Hsieh *et al.*, 2001) and has been proved very effective in the case of bats (Irwin *et al.*, 1991; Juste *et al.*, 1999; Campbell *et al.*, 2004; Almeida *et al.*, 2011) in addressing various evolutionary questions. Even though the complete sequencing of the cytochrome b gene is time-consuming and laborious (Hsieh *et al.*, 2001), it had been used in molecular studies on taxonomic work because of its efficiency (Irwin *et al.*, 1991; Bastian *et al.*, 2001).

Since the size of complete cytochrome b gene is 1140bp, the size of the PCR products from the present study varied between 700 to1480 bp including the primer sequences (Table 4).

5.3 BLASTn ANALYSIS

BLASTn searches (Altschul *et al.*, 1990) against the non-redundant GenBank database was used to assess the integrity of the sequences generated. The identity values with database sequences ranged from 84% to 100% and E values of 0.0 to 1e – 124 (Table 5). E-value is a measure of "chance or probability of a random match", this value decreases with increase in the pairwise alignment score employed in the BLAST algorithm (Hillis and Bull, 1993; Harrison and Langdale, 2006). Thus the lower e-values and high identity scores that retrieved aids confidence that the sequences are of good quality and bats are of from the same species. Hence the results of species identification for the bat species obtained are definite, accurate and reliable.

5.4 PHYLOGENY AND TAXONOMY

The phylogenetic analysis of the genus *Cynopterus* from Kerala shows that the species identified as *C. sphinx* and *C. brachyotis* are indisputably two different taxa. The samples analyzed from Kerala formed two different clusters. An earlier study by Mapatuna *et al.*, (2002) had similar results about these same two species from Sri Lanka.

The *C. sphinx* from Kerala grouped along with those sequences of *C. sphinx* from other parts of India such as Tamil Nadu, Karnataka and Andhra Pradesh (Figure 8). Since the Tranquebar in Tamil Nadu is the type locality of *C. sphinx*. The clustering of sequences in present study with those sequences mentioned above

indicates that the species that exist in Kerala constitutes the true *C. sphinx*. When the genetic distance was calculated the sequences from Kerala showed less than 2% variation with other Indian sequences (Table 10). But one of the sequences (KAUNHM201427) was clustered separately with sequences from Myanmar (AY629000), Thailand (AY628991) and Vietnam (AY628999) with a low bootstrap value and the genetic distance between these sequences and other Indian sequences varied between 2.2% to 2.8%. These results of low genetic variation (less than 3% in *C. sphinx*) indicate that the species is widespread (in peninsular India and South East Asia) and the genetic distances is due to intra-specific variations (Bradley and Baker, 2001).

Since the Indian *C. sphinx* and *C. brachyotis* clustered separately with a bootstrap value of 86% (Figure 8) and showed a genetic distance of 8.5% to 10.2% (Table 12). This could indicate that *Cynopterus* genera in India comprise of more than one species. The *C. brachyotis* collected during this study formed a monophyletic group along with sequences AY628921, AY628933 and AY628932 (from the study by Campbell *et al.,* 2004) from other parts of southern India. The type locality of *C. brachyotis* is Borneo and sequences of specimen from the type locality (from Campbell *et al.*, 2004) had high genetic distance compared to the *C. brachyotis* collected during this study and formed a separate clade. The genetic distance ranged from 10.6% to 12% between the Borneo sequence and Kerala sequences (Table 11). This indicates that, our *C. brachyotis* is a distinct lineage which should receive proper taxonomic and conservation attention. More over the *C. brachyotis* from Kerala showed a genetic distance of 4.7% to 13.3% with sequences from Sri Lanka and Sulawesi and clustered separately in phylogenetic tree. This means the species recognized as *C. brachyotis* in different countries are different lineages, or in other words, the *C. brachyotis* species in the present form is a species complex with several "evolutionary distinct lineages". This study hence supports the argument and findings by Campbell *et al.,* (2004) that the *C. brachyotis* is a complex of lineages. They

identified six divergent mitochondrial lineages from its current geographical range and found that *C. sphinx* and *C. horsfieldi* haplotypes formed monophyletic group nested within the *C. brachyotis* species complex.

5.5 SYSTEMATIC STATUS OF *CYNOPTERUS* SPECIES COMPLEX IN KERALA

The result of combined morphometric and molecular taxonomic study clarify a number of systematic problems concerning the genus *Cynopterus* in Kerala, which were hitherto unresolved. This study reiterates the importance of integrated taxonomic study using both morphological and molecular tools as raised by Mapatuna *et al.,* (2002), Moratelli and de Oliveira (2011) and Bates (2013).

Since the multivariate morphometric analysis could not separate the two species of *Cynopterus* in Kerala, the phylogenetic tree and the difference in the pairwise genetic distance based on molecular analysis undoubtedly clustered *C. sphinx* and *C. brachyotis* separately. Phenotypic characteristics are 'plastic' and hence are inadequate to resolve systematic questions in many cases (Thorpe *et al.,* 1994). The present study highlights the case of morphological plasticity. The two species focused in this study are cryptics, they are confused to be a single species. The morphological characteristics separating them are evident (although only) to a trained taxonomist.

C. sphinx samples from the peninsular India and South East Asia formed a monophyletic group. These sequences had very low genetic divergence values (<2.8%). Thus this species is widely distributed, and has not undergone evolutionary diversification. All *C. sphinx* studied forms a single species.

However, the cryptic species, *C. brachyotis*, is separate from *C. sphinx*, because they form a different clade, and also due to a higher genetic divergence (~10%) which is a five-fold difference when compared to *C. sphinx*. However the case of *C. brachyotis* is intriguing, since there are several lineages of *C. brachyotis*, as per the present identification systems. The C. *brachyotis* from the type locality formed a distinct clade well separated from the C. *brachyotis* from South India.

Campell *et al.* (2004) had identified six lineages of *C. brachyotis* from its area of geographical distribution. One among them was the Indian lineage. In this study the Indian *C. brachyotis* show high genetic separation from *C brachyotis* from Borneo (type locality). In this case also the species from Kerala clustered separately with sequences from other area of its geographical distribution. The average genetic distance was also higher than 10% with other sequences. This confirms that the Indian *C. brachyotis* is a distinct lineage. The C. *brachyotis* showed 8.5% to 10.2% variation when compared to *C. sphinx* from Kerala. According to Bradly and Baker (2001) the average genetic distance between sister taxa was 8.13%, hence *C. sphinx* and *C. brachyotis* from Kerala are sister species. To raise the status of Indian *C. brachyotis* to a new species, extensive study based on both morphometric as well as molecular techniques should be carried out all over India and necessary conservation plans should be taken.

This study highlights different adaptive strategies used by sister species. While *C. brachyotis* has radiated as independent lineages throughout the range, the genetic separation in *C. sphinx* is very low (compared to the earlier), although they co-exist in some of the location within their range. This could indicate that the species *C. brachyotis* (as recognized now) has diversified independently at multiple instances (in different locations) during the past. For this scenario to be proved, rigorous molecular analysis with both nuclear and mitochondrial markers (several base pairs), phylogeographic analyses and divergence time analyses are required. The

same strategy is required for a taxanomic treatment of the genus. The present integrative taxonomic approach to resolve this issue is a right step in this direction and should be expanded in 'space and time' to solve the taxonomic puzzle, evolutionary conundrum and to provide adequate conservation attention to the valuable biodiversity of the region.

Summary

SUMMARY

There exists confusion and controversy about taxonomic status of the genus *Cynopterus* throughout its geographical range. The nocturnal habit, cryptic nature, overlapping morphological characters and addition of genetically distinct lineages in the last decade made their taxonomy ambiguous. So, to unravel ambiguity, the conventional morphological classification need be supported with novel molecular techniques such as DNA barcoding. There are no other taxonomic studies on the genus *Cynopterus* of Kerala. The present study on "Phylogeny and systematics of the genus *Cynopterus* (Chiroptera: Pteropodidae) in Kerala", thus is the first-ever study of this kind in Kerala. The important findings are summarized below.

1. Principal Component Analysis showed that the forearm length is the diagnostic character in distinguishing between the two species of *Cynopterus* in Kerala.

2. All the morphometric measurements except forearm length were found to be overlapping in the case of *C. sphinx* and *C. brachyotis* in the present study.

3. The range forearm length and ear length, which are the crucial measurements for distinguishing *C. sphinx* and *C. brachyotis,* were 63.66 mm to 74.91 mm and 60.46 mm to 67.66 mm and 14.43 mm to 20.43 mm and 14.95 mm to 18.3 mm respectively. Except ear length of *C. brachyotis*, all other values ranges as mentioned by Bates and Harrison (1997).

4. In the case of both species of *Cynopterus*, such as the *C. sphinx* and *C. brachyotis*, the variability in the measurement of anterior palatal width was found to be lowest.

5. Multivariate morphometric analysis doesn't result in the separation of the two species, *C. sphinx* and *C. brachyotis*. Overlapping was observed between the two species.

6. DNA isolation and PCR amplification of 24 *C. sphinx* and seven *C. brachyotis* were successfully done.

7. Except four, all other sequences showed a high similarity of 97% to 99% with the query sequences with an E value of 0.0. Since the E value of the four sequences was zero or close to zero, their similarity is reliable.

8. When the *C. sphinx* sequences from Kerala were compared with the sequence having accession number KF042249, 14.75% variability in nucleotide base pairs were observed. The alignment of *C. brachyotis* from Kerala with sequence KF042221 showed a variability of 25.56%.

9. Phylogenetic analysis and pairwise genetic distance calculation undoubtedly identified two different species in the genus *Cynopterus* in Kerala.

10. Since *C. sphinx* samples from the peninsular India and South East Asia formed a monophyletic group with very low genetic divergence values (<2.8%). All *C. sphinx* studied forms a single species.

11. The *C. brachyotis* identified from Kerala may be a genetically different lineage, since it differ genetically from those species from Borneo, the type locality of *C. brachyotis* and also formed a sister taxa of *C. sphinx*.

12. This study proposes the presence of more number of species under the genus *Cynopterus* in its geographical range.

13. Extensive sampling and study of Indian *Cynopterus* should be done to confirm the systematic position and identity of each species.

14. The study throughout its geographical range is inevitable to revise the taxonomic status of this genus.

15. Special conservation and management plans for the *Cynopterus* genus should be implemented considering the present findings.

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Phylogeny and systematics of the genus *Cynopterus* (Chiroptera: Pteropodidae) in Kerala

By

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(2013-17-101)

ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Forestry

Faculty of Forestry

Kerala Agricultural University

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2015

ABSTRACT

The taxonomic status of the genus *Cynopterus* is subject of debate in the scientific world due to the cryptic nature, overlapping morphological characters and addition of six new genetically distinct lineages in the *Cynopterus* in the last decade. The present study was carried out with the objective of resolving the taxonomic ambiguity of the genus *Cynopterus* in Kerala and to infer the phylogenetic affinity of the genus *Cynopterus* within order Chiroptera. This is the first study which address the taxonomic status of the genus *Cynopterus* in Kerala and second one in India.

The bats were studied from eight different locations in three biogeographical regions of Kerala during April 2014 to December 2014. A total of 67 bats were captured from these locations. On each of these bats the 24 morphometric, dental and cranial variables were measured. Apart from that a molecular analysis was also carried out on 31 samples. Phenol-chloroform extraction or the GeniPure™ Mammalian Genomic DNA Purification Kit (GeNei™) was employed for DNA extraction and PCR amplification was done for complete cytochrome b gene. The sequences were compared with those registered in NCBI databank. The phylogeny reconstruction and the calculation of genetic distances were done using the IQ-TREE and MEGA 5.2.

The study reiterates the importance of integrative taxonomy using morphological and molecular techniques. Fifty one samples of *C. sphinx* and 13 samples of *C. brachyotis* were used for the multivariate morphometric analysis. This proved that the forearm length has been the only distinctive morphological character to distinguish between the *C. sphinx* and *C. brachyotis*. However, the other morphometric parameters were found to be overlapping.

Twenty four sequences of *C. sphinx* and 7 sequences of *C. brachyotis* were used for molecular analysis. The phylogenetic tree constructed and the pairwise genetic distance has proved that the *C. sphinx* from Kerala constitutes the 'true' *C. sphinx,* since they are genetically more identical to *C. sphinx* from its type locality and all *C. sphinx* studied forms a single species

The phylogenetic analysis indicated that the *C. brachyotis* from Kerala may be a different species, since it differ genetically from those species from Borneo, the type locality of *C. brachyotis*. The presence of genetically distinct lineages within *C. brachyotis* indicates that the species *C. brachyotis* has diversified independently at multiple instances in different locations during the past. The clustering of *C. brachyotis* from Kerala with *C. sphinx* confirms that they are sister taxa. Extensive sampling from throughout the geographical range of the genus *Cynopterus* should be done to confirm their systematic position and revise their taxonomic status.

Appendices

Appendix I. Details of the geographical locations of individual bats recorded

KAUNHM No	*Species*	Location	Latitude	Longitude	Altitude (m)
201403	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201404	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201405	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201406	*Rousettus leschenaultii*	Vellayani	N8°25.033'	E077°01.829'	63
201407	*Rousettus leschenaultii*	Vellayani	N8°25.033'	E077°01.829'	63
201408	*Cynopterus sphinx*	Vellayani	N8°25.563'	E076°59.357'	64
201409	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201411	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201412	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201413	*Cynopterus sphinx*	Vellanikkara	N10°32.865'	E076°16.709'	62
201414	*Cynopterus sphinx*	Kumarakom	N9°37.370'	E076°25.618'	11
201415	*Cynopterus sphinx*	Kumarakom	N9°37.370'	E076°25.618'	11
201416	*Cynopterus sphinx*	Kumarakom	N9°37.370'	E076°25.618'	11
201417	*Cynopterus sphinx*	Kumarakom	N9°37.655'	E076°25.874'	11

201418	*Cynopterus sphinx*	Kumarakom	N9°37.655'	E076°25.874'	11
201419	*Cynopterus sphinx*	Kumarakom	N9°37.655'	E076°25.874'	11
201420	*Cynopterus sphinx*	Kumarakom	N9°37.655'	E076°25.874'	11
201421	*Cynopterus sphinx*	Kumarakom	N9°37.655'	E076°25.874'	11
201422	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201423	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201424	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201425	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201426	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201427	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201428	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201429	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201430	*Cynopterus sphinx*	Tavanur	N10°51.273'	E075°59.321'	26
201432	*Cynopterus sphinx*	Vellayani	N8°25.008'	E077°01.828'	69
201433	*Cynopterus sphinx*	Vellayani	N8°25.008'	E077°01.828'	69
201434	*Cynopterus sphinx*	Vellayani	N8°25.008'	E077°01.828'	69
201435	*Cynopterus sphinx*	Vellayani	N8°25.008'	E077°01.828'	69
201438	*Cynopterus sphinx*	Ambalavayal	N11°36.913'	E076°12.675'	920

201439	*Cynopterus sphinx*	Ambalavayal	N11°36.913'	E076°12.675'	920
201440	*Cynopterus brachyotis*	Ambalavayal	N11°36.913'	E076°12.675'	920
201441	*Cynopterus brachyotis*	Ambalavayal	N11°36.913'	E076°12.675'	920
201442	*Cynopterus brachyotis*	Ambalavayal	N11°36.913'	E076°12.675'	920
201443	*Cynopterus brachyotis*	Ambalavayal	N11°36.913'	E076°12.675'	920
201444	*Cynopterus brachyotis*	Ambalavayal	N11°36.913'	E076°12.675'	920
201445	*Cynopterus sphinx*	Ambalavayal	N11°36.913'	E076°12.675'	920
201446	*Cynopterus sphinx*	Ambalavayal	N11°36.913'	E076°12.675'	920
201447	*Cynopterus sphinx*	Ambalavayal	N11°36.952'	E076°12.652'	928
201448	*Cynopterus brachyotis*	Ambalavayal	N11°36.952'	E076°12.652'	928
201449	*Cynopterus sphinx*	Ambalavayal	N11°36.952'	E076°12.652'	928
201450	*Cynopterus brachyotis*	Ambalavayal	N11°36.952'	E076°12.652'	928
201451	*Cynopterus brachyotis*	Ambalavayal	N11°36.952'	E076°12.652'	928
201452	*Cynopterus sphinx*	Ambalavayal	N11°36.952'	E076°12.652'	928
201453	*Cynopterus brachyotis*	Ambalavayal	N11°36.952'	E076°12.652'	928
201454	*Cynopterus brachyotis*	Ambalavayal	N11°36.952'	E076°12.652'	928
201455	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	58
201456	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	59

201457	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	60
201458	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	61
201459	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	62
201460	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	63
201461	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	64
201462	*Cynopterus sphinx*	Anakkayam	N11°05.471'	E076°07.064'	65
201463	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201464	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201465	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201466	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201467	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201468	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201469	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201470	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201471	*Cynopterus sphinx*	Padanakkad	N12°15.150'	E075°07.014'	17
201472	*Cynopterus sphinx*	Pambadumpara	N09°47.880'	E077°09.738'	1066

Appendix II. External measurements of individual bats collected from different study location in Kerala.

KAUNHM No	W (gm)	HB (mm)	FA (mm)	T (mm)	E (mm)	HF (mm)	WSP (mm)	TIB (mm)	THU (mm)	3MT (mm)	1PH3MT (mm)	2PH3MT (mm)	4MT (mm)	5MT (mm)
201403	56.50	93.47	71.82	10.29	19.51	19.03	415.00	29.20	21.02	47.10	31.99	40.65	44.87	46.74
201404	49.50	88.46	68.04	9.69	18.91	15.32	379.00	29.17	21.63	47.45	31.07	43.44	45.55	47.21
201405	46.50	98.51	69.82	6.37	19.11	18.76	410.00	28.86	19.04	47.15	30.24	40.90	43.99	46.16
201406	98.00	103.43	83.98	15.15	17.58	15.34	497.00	40.80	22.05	59.70	34.23	42.89	58.69	55.10
201407	70.00	98.32	78.23	14.72	16.53	14.30	466.00	35.90	19.78	54.13	35.68	40.26	53.82	50.76
201408	49.00	81.27	68.62	8.78	16.41	9.57	389.00	27.93	17.10	47.22	31.84	35.47	44.96	37.61
201409	49.50	94.70	73.12	9.29	18.10	14.90	470.00	29.56	20.89	51.47	32.30	44.59	47.63	49.06
201411	37.50	80.38	69.74	7.39	18.61	13.45	436.00	27.40	19.90	46.57	30.87	38.85	43.67	45.48
201412	43.50	78.91	69.42	9.06	16.91	15.36	451.00	26.33	19.00	46.52	30.87	40.82	44.29	46.94
201413	51.00	83.55	70.31	9.27	16.32	15.22	434.00	28.68	19.44	48.42	31.20	42.01	45.75	47.85
201414	46.50	88.89	69.55	13.80	17.17	16.20	404.00	30.03	19.50	47.72	30.22	34.88	45.25	46.21
201415	53.00	103.13	74.91	13.01	18.91	14.15	454.00	28.73	19.88	51.73	34.33	43.98	48.62	50.48
201416	40.00	88.06	70.19	11.80	18.21	15.38	429.00	27.30	21.68	47.84	31.18	40.53	44.77	47.16
201417	43.00	90.04	71.95	12.05	19.40	15.21	440.00	27.69	21.07	50.43	33.08	41.81	46.76	49.32
201418	49.00	90.61	68.23	10.52	19.74	12.49	445.00	28.87	21.34	47.00	31.81	42.88	44.66	46.22
201419	29.00	81.29	64.17	10.81	15.40	16.10	415.00	24.40	19.71	44.48	28.89	36.05	42.66	43.74
201420	47.00	92.77	68.70	10.83	18.32	14.16	453.00	28.66	20.80	48.59	32.14	41.59	45.73	48.09
201421	51.00	79.94	73.92	6.77	17.93	14.84	438.00	29.04	19.20	52.71	34.79	43.09	49.06	50.41
201422	48.00	88.96	71.09	9.97	18.03	13.54	460.00	28.55	20.34	49.27	31.27	40.82	44.42	47.67
201423	54.00	85.16	74.02	8.50	18.41	15.61	403.00	28.21	20.21	51.47	32.79	45.61	47.47	49.39
201424	50.00	91.66	70.40	10.13	19.67	13.25	400.00	28.27	18.21	49.18	31.57	43.18	47.31	47.47
201425	56.00	89.89	73.39	8.10	17.77	14.28	425.00	29.75	19.98	51.06	32.78	41.50	47.69	49.70
201426	50.20	94.97	73.67	10.22	17.55	15.49	415.00	29.93	18.76	50.42	34.04	43.87	46.92	49.78

201427	50.35	95.82	71.99	10.05	18.49	15.57	444.00	28.87	18.54	52.35	32.44	47.05	49.53	50.96
201428	47.50	89.08	70.69	8.95	20.43	14.79	420.00	27.79	17.11	48.52	32.31	41.12	45.42	48.34
201429	46.20	86.89	67.23	6.25	17.03	18.13	417.00	27.40	18.07	47.29	30.14	41.58	44.33	45.95
201430	50.10	93.04	70.14	10.38	17.34	15.13	412.00	29.40	17.68	48.51	31.34	41.25	45.54	47.44
201432	48.50	87.00	71.05	9.22	17.45	13.99	439.00	28.18	20.79	47.94	31.68	41.61	45.77	46.69
201433	34.00	74.53	65.94	6.42	16.99	13.83	423.00	25.07	17.91	44.50	28.30	35.60	41.12	43.20
201434	45.00	84.05	67.88	9.92	15.31	14.45	435.00	27.66	18.57	46.52	30.14	39.55	43.49	44.16
201435	39.00	77.25	68.59	10.03	19.19	16.67	444.00	25.29	19.87	47.80	30.53	39.04	45.40	46.90
201438	35.00	75.94	66.61	5.57	15.88	10.52	422.00	24.83	17.79	45.41	31.52	35.88	43.14	43.31
201439	35.00	75.90	63.66	5.37	14.43	10.99	402.00	22.67	16.41	44.18	26.90	37.92	40.97	41.66
201440	35.00	78.35	62.84	7.25	14.95	11.91	398.00	24.19	19.30	43.29	28.34	35.66	40.94	42.29
201441	37.00	78.56	61.92	6.84	15.51	12.49	401.00	24.06	15.98	43.50	29.95	35.65	41.33	42.91
201442	36.00	76.98	63.49	6.19	16.81	12.48		23.44	16.62	43.70	26.58	36.50	41.28	43.50
201443	35.00	82.14	62.29	5.75	15.50	12.86		22.77	15.96	41.66	26.42	37.96	37.21	40.04
201444	30.00	69.24	62.73	7.86	18.30	14.97		23.87	16.42	42.68	27.49	33.58	40.25	42.18
201445	37.00	86.98	64.91	7.13	15.44	11.59		23.28	17.22	42.07	26.33	37.29	39.00	40.63
201446	36.00	76.23	64.56	6.11	14.86	12.20		24.50	17.17	43.52	28.03	41.29	41.62	42.27
201447	33.00	65.89	64.15	8.19	14.46	15.26		21.22	16.37	43.67	26.14	36.21	40.26	41.83
201448	33.00	75.62	62.52	11.20	16.46	13.12		22.58	18.84	42.61	28.51	38.63	40.31	41.95
201449	35.00	72.59	65.17	7.81	15.17	13.10		24.35	16.07	44.78	29.34	37.85	40.65	44.38
201450	30.00	71.66	61.99	8.70	15.80	12.80		23.77	17.58	44.25	27.83	36.69	38.93	42.03
201451	35.00	78.80	63.6	6.08	16.89	12.14		25.21	19.14	45.61	28.56	41.15	41.88	42.60
201452	36.00	78.80	67.66	6.08	16.46	12.14		25.21	19.14	45.61	28.56	41.15	41.88	42.60
201453	30.00	71.80	60.46	7.34	15.05	11.37		22.37	16.24	43.28	27.57	35.99	40.34	42.73
201454	33.00	67.72	62.72	5.31	15.24	11.56		21.73	15.60	42.39	27.81	32.18	38.72	41.67
201455	45.00	91.01	69.38	8.64	20.05	15.24	435.00	27.82	16.78	44.30	30.22	40.41	42.41	44.16
201456	43.00	83.56	70.79	8.66	19.62	15.65	455.00	28.75	18.62	47.03	33.71	39.58	45.02	46.23
201457	35.00	82.60	65.33	11.07	18.29	11.85	415.00	25.30	19.08	44.58	29.30	36.19	42.75	43.41
201458	36.00	83.75	64.53	8.69	18.11	13.86	417.00	24.72	16.78	44.24	29.67	34.97	41.84	44.00
201459	38.00	89.82	66.02	11.36	18.33	12.78	430.00	25.06	18.48	46.34	29.04	37.20	44.23	45.41

201460	38.00	83.63	66.72	11.73	19.10	13.53	413.00	26.26	19.09	46.04	30.95	37.85	43.70	44.91
201461	43.00	85.78	69.25	10.37	18.35	13.43	438.00	27.35	19.54	46.55	30.88	40.63	42.73	44.97
201462	43.00	87.59	71.71	9.66	20.12	14.10	456.00	29.00	18.20	49.90	30.78	40.30	46.91	48.25
201463	50.00	89.67	68.69	7.05	19.09	14.51	450.00	28.01	19.49	48.29	31.00	40.75	45.27	45.41
201464	45.00	83.05	67.33	9.22	18.69	13.26	445.00	25.95	19.26	49.38	29.81	41.40	45.63	47.11
201465	40.00	67.72	69.58	7.96	19.62	16.33	440.00	27.68	20.50	47.41	31.69	42.50	44.61	46.01
201466	50.00	92.24	71.46	6.01	16.79	14.14	440.00	28.19	18.45	48.36	30.62	39.08	45.48	46.74
201467	48.00	91.16	72	8.66	17.91	14.12	470.00	28.32	20.04	49.85	31.82	43.49	46.89	48.36
201468	45.00	88.20	69.92	8.75	15.54	14.19	437.00	26.85	20.07	48.64	29.43	40.68	43.12	45.85
201469	43.00	83.43	70.71	9.62	20.15	13.67	460.00	27.72	17.93	48.78	31.79	40.87	46.26	48.00
201470	43.00	90.50	70.65	5.98	19.51	15.97	449.00	27.17	19.31	48.26	31.66	36.65	43.79	45.79
201471	51.00	92.70	70.13	8.17	17.16	15.06	473.00	28.73	18.11	49.72	33.23	42.59	47.29	48.74
201472	50.00	89.06	67.2	10.08	16.29	13.26	460.00	26.66	18.75	48.23	31.63	41.02	45.34	46.57

Appendix III. Dental and cranial measurements of individual bats collected from different study location in Kerala.

KAUNHM No	ZB (mm)	BB (mm)	CBL (mm)	CCL (mm)	GTL (mm)	CM^3 (mm)	CM_3 (mm)	M (mm)	M^3-M^3 (mm)	C^1-C^1 (mm)
201403	21.3	13.45	29.83	29.15	32.48	11.32	11.9	25.58	9.35	7.09
201404	20.54	13.27	30.68	30.47	30.49	10.93	11.9	25.03	9.31	6.92
201405	20.99	13.66	29.97	28.86	30.98	11.14	12.17	25.56	10.21	6.94
201406	23.89	15.4	35.02	34.86	38.75	14.95	16.35	30.54	11.11	7.53
201407	20.89	14.87	33.6	32.33	37.07	13.56	15.48	29.36	10.7	7.04
201408	20.06	12.84	27.8	26.95	30.29	10.2	10.77	23.1	10.02	7.08
201409	20.09	13.5	29.4	29.1	30.73	11.78	12.15	24.03	10.16	7.16
201411	18.88	13.96	28.87	28.39	31.92	11.13	12.25	24.48	10.35	7.14
201412	20.88	13.96	30.3	29.54	31.82	10.91	11.8	25.64	10.07	7.28
201413	21.49	14.02	30.67	30.41	32.52	11.45	12.64	25.04	9.87	7.61
201414	17.19	13.78	30.44	28.64	33.27	11.68	11.51	24.39	10.09	6.83
201415	21.68	13.44	33.07	31.71	35.36	12.17	13.68	26.65	10.61	7.71
201416	20.42	13.77	31.24	30.9	32.7	11.69	12.13	24.82	10.35	7.42
201417	20.48	14.29	31.87	28.67	33.31	11.33	11.88	25.1	10.7	8.1
201418	19.72	13.42	33	32.04	35.22	10.96	12.71	25.27	10.3	7.37
201419	17.41	13.21	26.77	26.01	29.47	10.17	11.38	22.43	9.26	6.06
201420	18.45	12.8	29.93	29.75	32.09	10.9	11.89	23.37	9.62	7.3
201421	20.48	13.62	29.76	29.55	32.25	11.92	12.64	24.35	10.55	7.66
201422	19.98	13.6	31.28	30.58	32.01	10.96	12.83	24.11	10.29	6.95
201423	21.39	12.85	30.13	29.19	32.49	12.11	12.05	24.31	10.19	7.7
201424	21.3	13.72	31.25	30.27	33.66	10.81	12.19	25.23	10.44	7.31
201425	21.3	13.4	31.34	30.79	32.85	11.78	13.59	25.31	9.99	7.02
201426	20.07	13.64	31.9	31.18	32.93	11.7	12.28	23.32	10.28	7
201427	21.02	13.33	31.31	29.35	32.18	11.05	11.27	24.61	9.86	7.15
201428	19.97	13.64	30.77	30.44	32.4	11.71	12.85	25.03	10.57	7.17

201429	18.35	13.44	30.3	30.1	31.19	11.66	11.31	24.79	9.73	7.39
201430	21.09	14.29	31.26	30.84	33.66	11.24	12.7	25.14	9.86	7.35
201432	19.89	13.91	30.62	30.15	32.46	11.56	12.17	23.92	9.98	7.04
201433	17.67	13.27	27.93	27.54	30.31	10.53	12.18	22.3	9.17	6.72
201434	20.02	12.72	28.36	28.21	29.9	11.76	12.44	23.17	9.07	6.9
201435	20.04	13.27	29.29	29.05	32.5	10.76	11.81	22.62	9.76	6.74
201438	18.99	12.76	27.6	26.84	29.71	9.74	9.35	21.96	9.38	6.51
201439	17.97	12.53	27.66	27.02	29.71	9.82	12.41	20.54	9.25	6.12
201440	19.15	12.7	28.55	27.73	30.56	9.89	11.38	22.52	9.43	6.79
201441	19.87	12.35	28.13	27.51	30.54	10.73	11.74	22.8	9.84	6.93
201442	19.57	12.75	27.86	27.15	29.88	10.3	11.13	22.95	9.32	6.72
201443	21.08	14.03	30.08	29.19	33.36	11.38	13.43	25.52	9.83	7.34
201444	17.58	13.57	26.47	25.99	29.72	10.67	11.6	21.83	9.78	6.44
201445	19.69	12.75	27.59	27.17	30.11	10.68	11.55	23.32	9.5	6.85
201446	19.04	12.54	26.15	26	29.01	9.38	12.17	22.55	9.13	6.61
201447	19.51	12.29	28.95	27.98	29.39	9.73	11.18	22	9.28	6.45
201448	19.2	12.83	27.91	27.01	28.92	8.9	11.13	21.22	8.88	6.56
201449	19.24	12.46	28.05	27.46	30.05	9.7	10.64	23.11	9.1	6.37
201450	17.65	13.42	29.05	27.36	30.61	10.13	10.94	22.61	9.13	6.4
201451	18.74	12.3	27.73	27.15	29.55	9.54	9.89	22	8.86	6.42
201452	19.66	12.55	28.5	28.4	30.4	10.3	10.87	22.72	9.42	6.41
201453	17.98	12.55	25.84	25.46	29.2	8.82	11.17	21.9	8.71	6.21
201454	18.58	12.81	28.75	28.26	30.26	10.25	11.63	22.67	9.32	6.68
201455	20.41	13.57	30.95	30.65	32.66	11.75	13.15	24.2	9.58	7.05
201456	19.57	14	30.89	30.41	32.58	11.66	11.96	23.76	9.92	6.78
201457	18.05	13.06	29.01	28.37	30.5	10.19	12.3	23.16	9.01	6.57
201458	17.75	13.02	26.73	26.05	28.18	10.14	12.11	22.68	9.81	6.67
201459	18.12	12.92	29.29	28.06	30.2	11.59	12.62	22.39	9.77	6.89
201460	18.42	13.21	30.56	29.61	32.53	12.21	12.63	25.21	10.16	7.27
201461	20.99	13.42	31.14	30.34	32.04	11.37	12.84	24.53	9.71	7.09

201462	20.46	12.93	28.99	28.13	31.5	10.97	11.91	24.46	9.05	6.52
201463	20.92	13.67	30.18	29.25	32.39	11.11	13.64	24.75	9.79	7.43
201464	20.66	13.3	27.92	27.34	31.15	10.14	11.6	24.4	9.97	6.79
201465	20.47	13.69	30.21	29.75	32.27	11.28	12.46	25.16	10.14	6.93
201466	20.41	13.81	30.33	29.53	32.26	11.35	12.64	24.37	10.03	7.06
201467	21.73	14.18	30.11	29.42	33.18	11.52	12.98	24.97	10.53	7.58
201468	21.37	13.77	31.32	30.13	32.11	11.43	11.99	24.68	10.09	7.01
201469	21.22	14	30.99	30.5	33.17	11.76	13.24	25.6	10.17	7.25
201470	20.98	12.73	29.53	28.02	31.73	10.83	13.34	24.8	9.91	6.97
201471	20.67	13.44	30.69	30.26	31.76	11.7	11.63	25.44	10.47	7.65
201472	20.77	13.33	29.53	29	31.65	11.04	12.22	24.85	10.02	6.87