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Mitochondrial DNA identified bat species in northeast India: electrocution mortality and biodiversity loss

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ABSTRACT

Northeast India with two biodiversity hotspots is recognized as a biodiversity-rich region. However, several extant animals including chiropterans are currently at jeopardy due to habitat loss, electrocution mortality, and other anthropogenic threats. This study examines the efficacy of mitochondrial Cytochrome b (mtCytb) sequences for species-level identification of five electrocuted bat specimens from Manipur state. The similarity search results in the global database, Kimura 2 parameter (K2P) genetic distances, and neighbor-joining (NJ) tree identified all bat specimens into two species, *Cynopterus sphinx* and *Megaerops niphanae*. The detection of *M. niphanae* is the first record of this mammal from the state. In comparison with other Pteropodidae species, the genetic distances clearly discriminate both *C. sphinx* (7.9–30.2%) and *M. niphanae* (12.2–25.7%). In addition, the combined tree analysis of present and earlier genetic information of *C. sphinx* suggested the presence of cryptic lineages and sympatric population in India. This similar approach with more sampling from a wide distribution area could assist the future genetics research on chiropterans and their precise conservation.

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Chiropterans; wildlife threats; DNA barcoding; cryptic species; conservation

1. Introduction

Chiropterans (commonly known as bats) are the second largest mammalian order with approximately 1300 species worldwide (BCT 2018). Among them, 117 species under 39 genera and nine families are known from India (Wilson and Reeder 2005; Talmale and Saikia 2018). The northeastern region of India accommodates prodigious mammalian diversity comprising more than 70 bat species (Boro et al. 2018). This nocturnal mammal plays an important role and provides many ecological and economical services (Kasso and Balakrishnan 2013). However, the population of this charismatic animal has dramatically declined in the recent past (Fujita and Tuttle 1991). Due to the intensifying of urbanization, human-wildlife interactions possess several illegitimate threats to the wild animals and their habitats. Among all, collisions and electrocutions mortality are one of the visible and emerging threats to the chiropterans and avifauna throughout the world including India (Harness et al. 2013; Loss et al. 2014; Kalam et al. 2018). The assessment of electrocutions mortality rates of animals usually are correlated with several biological, environmental, and abiotic factors (Guil et al. 2011). Owing to the widespread distribution of both, the Greater shortnosed fruit bat, *Cynopterus sphinx*, and the Ratanaworabhan's fruit bat, *Megaerops niphanae* are categorized under 'Least Concern' category in IUCN Red List (IUCN 2018). *Cynopterus sphinx* is widely distributed in South and Southeast Asian countries, and *M. niphanae* is known to be distributed in Bangladesh, Bhutan, Cambodia, India, Laos, Thailand, Viet Nam (Islam et al. 2015). However, the exact diversity and widespread population structure of *C. sphinx* and *M. niphanae* remains unknown. Further, based on the phenotypic characters the accurate identification of this group is challenging (Srinivasulu et al. 2010) and the relationships of old world chiropteran species are ambiguous (Kingston et al. 2001; Mayer and von Helversen 2001; Jones et al. 2002). Hence, the actual diversity of chiropterans is largely argued due to several anecdotal data from different regions including northeast India (Rahman and Choudhury 2017).

In recent past, the mitochondrial DNA and microsatellite data are evidenced to infer the sex determination, population structures, and phylogenetic relationships among the broadly distributed *C. sphinx* (Campbell et al. 2004, 2006; Chen et al. 2010; Sun et al. 2012). Besides, the phylogenetic position of *M. niphanae* and comparative phylogeography with African bats has been assessed to furnish new insights into medicinal avenues (Hassanin et al. 2016). The emerging DNA barcoding tool with mitochondrial and nuclear genes is largely used to identify and discriminate several faunal groups (Barratt et al. 1997; Francis et al. 2010; Neaves et al. 2018). Here, we aimed to investigate the species level identification of the electrocuted bat samples from northeast India and topological

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pattern of the studied species with other Pteropodidae Chiropterans through mitochondrial Cytochrome b (mtCytb) gene. The similar approach could be used to identify the wildlife animals at their different life stages and resolve several taxonomic dilemmas.

2. Materials and methods

2.1. Sample collections

Five electrocuted dead bat samples were encountered during the recent survey besides the Zeilad Lake, Tamenglong district, Manipur state in northeast India (25.13 N 93.61 E). No bat specimens were arrested by mesh netting or sacrificed, hence no prior permission was required. We adopted molecular techniques for species-level identification and topological assessment. The samples were preserved in 70% molecular grade alcohol with proper voucher ID (ZSI_BAT1: ZSI/28331, ZSI_BAT2: ZSI/28332, ZSI_BAT3: ZSI/28333, ZSI_BAT4: ZSI/28334, ZSI_BAT5: ZSI/28340) in the National Zoological collections at Mammals and Osteology section, Zoological Survey of India (ZSI), Kolkata. A meager amount of tissue was collected from each sample by using a sterile surgical blade and rigorously washed by 70% ethanol for the downstream experiment.

2.2. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted by standard phenol-chloroform-isoamyl alcohol (PCI) protocol (Sambrook and Russell 2001), checked in 1% agarose gel electrophoresis, and stored –30 °C at Centre for DNA Taxonomy laboratory, Zoological Survey of India, Kolkata. The published primer pair (mcb 398: 5′TACCATGAGGACAAATATCATTCTG3′ and mcb 869: 5′CCTCCTAGTTTGTTAGGGATTGATCG3′) (Verma and Singh 2002) was used to amplify the partial fragment of mtCytbgene in a Veriti® Thermal Cycler (Applied Bio systems, Foster City, CA, USA) with the standard thermal profile. The 25 µl PCR mixture contains 10 pmol of each primer, 20 ng of DNA template, 1X PCR buffer, 1.0–1.5 mM of MgCl2, 0.25 mM of each dNTPs, and 1U of Taq polymerase (Takara BIO Inc., Shiga, Japan). The PCR products were checked in 1% agarose gel and purified using a QIAquickR Gel extraction kit (QIAGEN Inc., Germantown, MD, USA). The bidirectional sequencing of each sample was carried out by 48 capillary array 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) following Sanger sequencing methods available at ZSI, Kolkata.

2.3. Sequence quality control, dataset preparation and analysis

The generated forward and reverse sequences were checked by using SeqScanner Version 1.0 (Applied Biosystems Inc., CA, USA) and assembled through BioEdit v7.2.5 (Hall 1999). Each consensus sequence was further checked through the online nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/), and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The final sequences were submitted in both GenBank and BOLD database. The DNA sequences were preliminarily checked through online identification systems in the global databases. Based on the similarity search, 113 database sequences of 113 species (Mammalia: Pteropodidae) were acquired from GenBank to make a combined dataset (425 bp). Further, to check the cryptic diversity within *C. sphinx* in India, 51 published sequences (Chattopadhyay et al. 2016) were also acquired from the GenBank. The datasets were aligned by using ClustalX software (Thompson et al. 1997). The genetic distance and topology were estimated through Kimura 2 parameter (K2P) and neighbor-joining (NJ) tree by using MEGAX (Kumar et al. 2018). Sequence of *Pteronotus pusillus* (suborder Microchiroptera) was used as an out-group.

3. Results and discussion

The similarity search results of the generated sequences revealed 98–99% similarity search with the database sequences of two Pteropodidae species. Hence, the four samples (ZSI_BAT1-4) were confirmed to be *Cynopterus sphinx* and a single sample (ZSI_BAT5) was *Megaerops niphanae*. The present study detected the occurrence of *M. niphanae* from Manipur in northeast India, which is the first record of this mammal from the state. The overall mean genetic distance of family Pteropodidae with 115 species was 18.7% with the maximum of 30.7% in the studied dataset. Further, the mean genetic distance between two subfamilies was 20.6%, with the maximum of 30.6% (Pteropodinae) and 25.1% Macroglossinae. The subfamily Pteropodinae was further classified into three tribes, Epomophorini, Myonycterini, and Stenonycterini. Due to the unavailability of sequences of Stenonycterini species in the global database, the genetic distance of this tribe was not estimated. The Epomophorini tribe with two genera, Epomops, and Epomophorus shows 1.4–9.2% genetic distance. The Myonycterini tribe with two genera, Megaloglossus, and Myonycteris shows 6.5–16.9% genetic distance. Further, the genetic divergence between these two tribes (Epomophorini and Myonycterini) was 15% in the studied dataset. The previous study recommended that the species under these tribes share a common ancestor and formed distinct 'Epomophorini-Myonycterini Lineage' (Almeida et al. 2011, Chattopadhyay et al. 2011). The Epomophorini-Myonycterini lineage maintained 18.2% genetic distance with other Pteropodidae species. Both *C. sphinx* and *M. niphanae* showed interspecies genetic distance ranging from 7.9 to 30.2% and 12.2 to 25.7% respectively with the other Pteropodidae members. The NJ tree shows close relationship of *C. sphinx* with other *Cynopterus* species (*C. horsfieldi*, *C. brachyotis*) and *M. niphanae* with other *Megaerops* species (*M. kusnotoi*, *M. wetmorei*) (Figure 1). Both the genus *Cynopterus* and *Megaerops* revealed close association with the nearest-neighbor genus *Ptenochirus* with two species, *P. jagori*, and *P. minor*. Further, few Pteropodidae species shows ambiguous cladding in the NJ tree; *Rousettus lanosus* and *Megaerops ecaudatus* shows different clustering from the other *Rousettus* and *Megaerops* congeners. Furthermore, seven species of *Epomops* and two species of

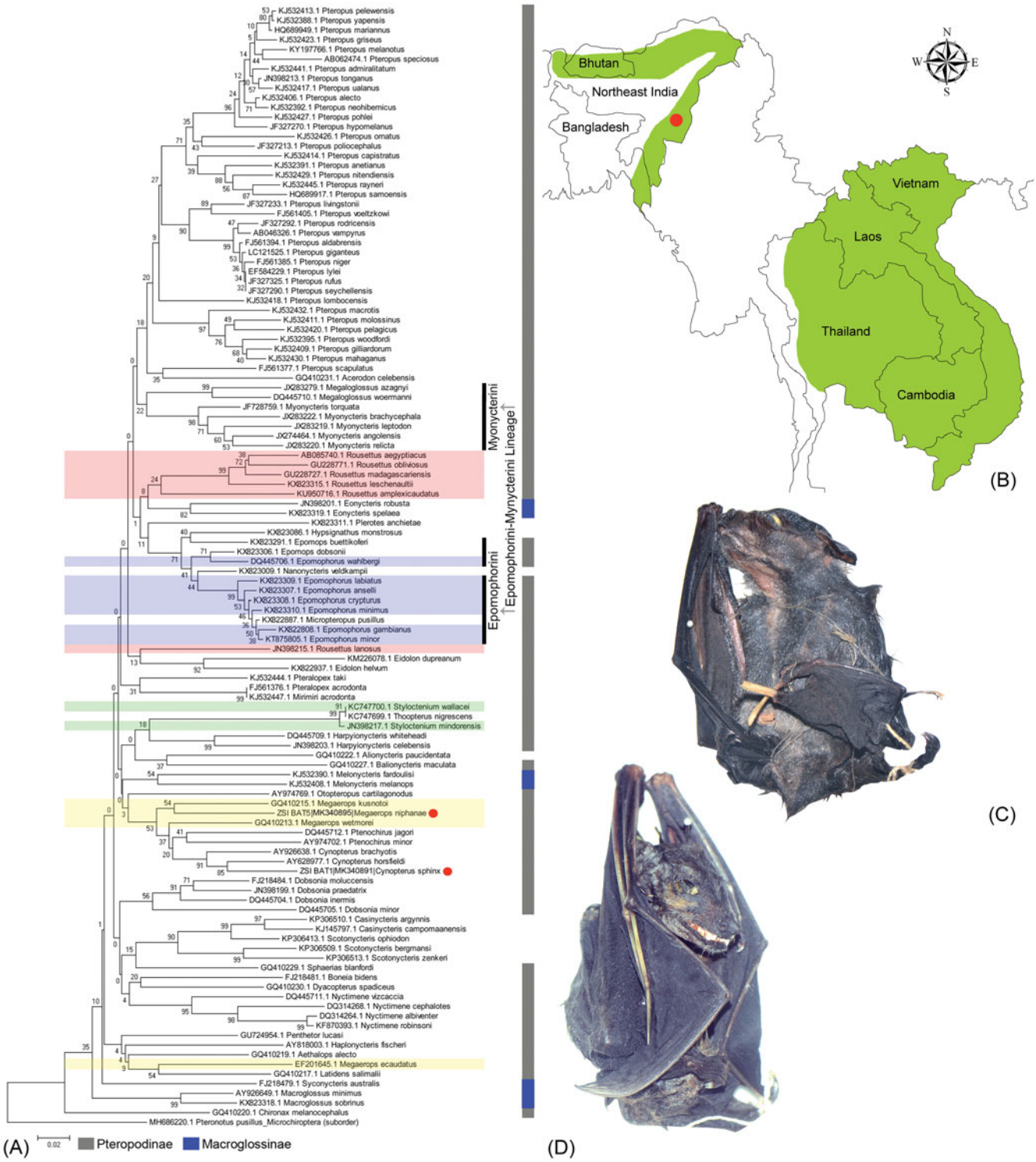

Figure 1. (A) Neighbor-joining tree illustrates the relationship between *M. niphanae* and *C. sphinx* with other Pteropodidae species. Generated accession numbers were superimposed with the voucher IDs and species names. Different color shades marked in the tree taxon shows the ambiguous cladding of different congeners. Taxonomic ranks (subfamilies) were marked by color bars beside the respective clades. (B) Map showing the distribution pattern of *M. niphanae* and collection locality of bats specimens in northeast India. Photograph of collected bat samples (C) *M. niphanae* (D) *C. sphinx*.

Styloctenium shows equivocal clustering in the NJ tree, which needs further investigation with more molecular markers.

The earlier study also examined the genetic variability of *C. sphinx* and *C. brachyotis* from two natural contact zones and 17 allopatric regions in India to investigate the population structure, evolutionary pattern, and possible cryptic diversity (Chattopadhyay et al. 2016). The past molecular data suggested the separate cryptic lineages of fruit bat in northeastern India and furnished the evidence of admixture and introgression within *C. sphinx*. The present *C. sphinx* dataset with the generated and database sequences furnished three distinct clades in NJ tree with significant bootstrap support (Figure 2). The Clade-1 resulted cohesive clustering of *C. sphinx*, collected from different locations of

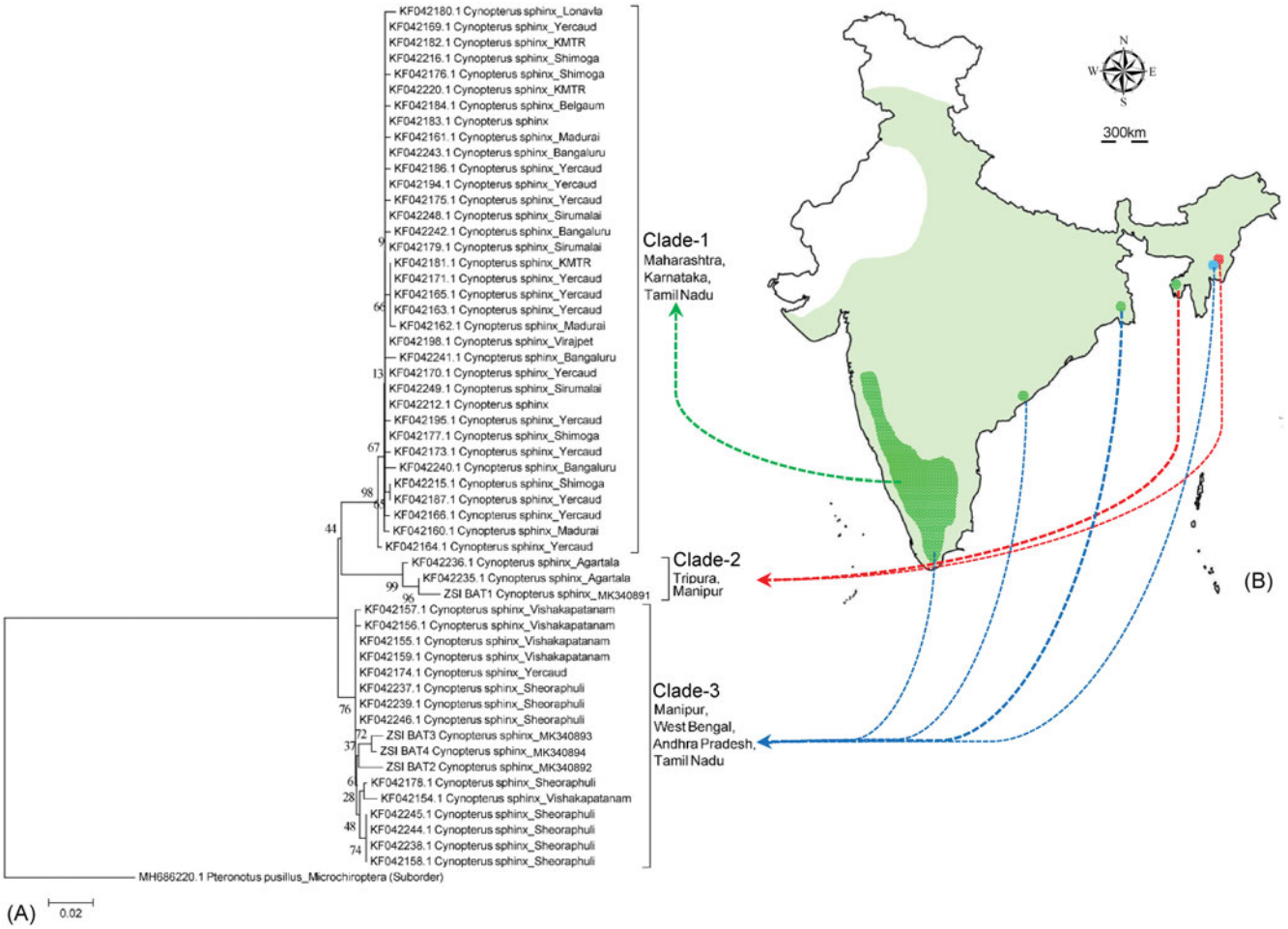

Figure 2. (A) Neighbor-joining tree shows the cryptic diversity and different lineages of *C. sphinx* from the different geographical region in India. (B) Map with light green shade represents the distribution pattern of *C. sphinx* in India. Red and blue pattern dots denote the collection localities of *C. sphinx* in the present study. Deep green pattern and dots denote the collection localities of *C. sphinx* from the previous study (Chattopadhyay et al. 2016). The color dotted arrows represent the distinct lineages of *C. sphinx* correspond with their different collection localities.

Maharashtra, Karnataka and Tamil Nadu state in southern India. Further, Clade-2 represents the samples from Tripura, Manipur and Clade-3 from Manipur, West Bengal, Andhra Pradesh, and Tamil Nadu. The generated sequences of *C. sphinx* from Manipur state shares two clades, Clade-2 and Clade-3 in the NJ topology. The mean genetic distance of *C. sphinx* dataset was 2.2% with ranging from 0 to 7%. The intraclade genetic distance of Clade-1 (Maharashtra + Karnataka + Tamil Nadu) was 0 to 1%, Clade-2 (Tripura + Manipur) was 0.9 to 1.9%, and Clade-3 (Manipur + West Bengal + Andhra Pradesh + Tamil Nadu) was 0 to 2.2%. Further, the Clade-1 and Clade-2 revealed 5.8%, Clade-2 and Clade-3, 5.2%, and Clade-1 and Clade-3, 3.5% genetic distances between the clades.

The present outcome showed that one single specimen of *C. sphinx* is genetically close to the previously described cryptic population from Tripura and other three samples are close to the other cryptic population from West Bengal, Andhra Pradesh, and Tamil Nadu. Hence, the coexistence of genetically diverge population in the same geographical regions depicts the sympatric population of *C. sphinx* in northeast India. Moreover, additional sampling from broad geographical regions and the generation of more molecular data can be estimated to acquire the accurate phylogeny of this group. Since the availability of genetic information of Indian chiropterans is scanty till now, the present effort and generated molecular data will help for further research, especially on population structure, and conservation genetics to formulate the conservation action plans of Indian chiropterans.

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No potential conflict of interest was reported by the authors.

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