# BIODIVERSITY RESOURCES AND CONSERVATION STATUS IN PENINSULAR MALAYSIA

PERHILITAN

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# Biodiversity Resources and Conservation Status in Peninsular Malaysia

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## PHYLOGENETIC ANALYSIS OF *MACROGLOSSUS SOBRINUS* FROM PENINSULAR MALAYSIA

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**ABSTRACT**

A study on the phylogenetics of *Macroglossus sobrinus* (Greater Long-nosed Fruit Bat) from Peninsular Malaysia was done using partial cytochrome *b* (cyt-*b*) mitochondrial DNA (mtDNA) gene. The specimens of *M. sobrinus* were collected from Kedah (Weng Ulu Muda Forest Reserve), Perak (Bintang Forest Reserve), Selangor (Ulu Langat), Melaka (Asahan Catchment Area), Johor (Gunung Ledang), Kelantan (Gua Musang), and Pahang (Krau Wildlife Reserve). Thirty nine cyt-*b* sequences including eighteen sequences of *M. sobrinus*, twenty sequences of *M. minimus* taken from the GenBank, and a sequence of *Penthetor lucasi* (Lucas's Short-nosed Fruit Bat) as an outgroup were incorporated in the analyses. Three main methods namely neighbour joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) were used in the phylogenetic trees reconstruction followed by the consensus phylogenetic tree to summarize the overall findings. The four trees were reconstructed to determine the genetic relationship between *M. sobrinus* and *M. minimus*. Generally, the trees indicated genetic divergence between populations of *M. sobrinus*. The result was further supported by very low genetic distances based on Kimura 2-parameter distance (1980) calculation. There was an individual of *M. sobrinus* (Gua Musang, Kelantan)

clustered with the *M. minimus* individuals, thus indicating of possible misidentification. Apart from that, *M. sobrinus* from Kelantan gave the highest nucleotide diversity, $P_i$ (Jukes & Cantor 1969) with 0.65%, followed by Perak (0.36%), Kedah (0.27%), Selangor (0.18%), and finally Pahang and Melaka with no nucleotide diversity. Overall, the nucleotide diversity of *M. sobrinus* in Peninsular Malaysia was 0.29% indicating low level of genetic diversity. However, the sample size is too small to relate the results with the phylogeography and population genetic views. Therefore, more extensive studies incorporating other mtDNA genes with higher rate of evolution such as control region gene are highly recommended in the future study.

## INTRODUCTION

Bats form the second most diverse order of mammals. They are classified in two suborders, Megachiroptera (Old World fruitbats) and Microchiroptera. Megachiroptera, or megabats, are large animals, commonly known as Old World fruit bats. They are mainly fruit-eaters and are found only in tropical habitats of Asia, Africa, India, Oceania and Australasia (Corbet and Hill, 1992). Not all megabats are large; the smallest species is 6 cm (2 inches) long and thus smaller than some microbats. Most megabats have large eyes enabling them to orient in the twilight and inside caves. The sense of smell is excellent. Megabats are frugivorous. They eat fruits or suck nectar from flowers. Often the fruits are squashed, and only the fruit juice is consumed. The teeth are adapted to bite through hard fruit skins. In fact, the megabats lack canine teeth all together (having molars instead) and many of the nectar-sucking bats have very small almost useless teeth (Andersen, 1912).

The evolutionary and taxonomic framework of fruitbats were established by Andersen (1912) based on similarities in skull and dentition morphologies. At the suprageneric level, megabats were categorized into five sections; rousettine, epomophorine and cynopterine bats in the subfamily Pteropodinae; and eonycterine and notopterine bats in the subfamily Macroglossinae. However, recent molecular evolutionary analyses, i.e., restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA, DNA hybridization, and mitochondrial DNA (mtDNA) sequencing (Schatz, 1996), have shown that this taxonomic classification has some discrepancies.

To date, there has been no comparable attempt to describe the geographical distribution of evolutionary lineages; phylogeography of *Macroglossus sobrinus* (Greater Long-nosed Fruit Bat) in Peninsular Malaysia, nor to use molecular markers to clarify its population structure and evolutionary history. In order to devise adequate conservation and management strategies, it is important to incorporate a reliable understanding of its population structure and history; and a characterization of the distribution of its genetic diversity (Avise, 1989; O'Brien, 1994). MtDNA based phylogeographical studies have been considerable significant in the field of animal conservation where they have revealed cases of cryptic biodiversity, i.e., the identification of distinct evolutionary lineages where morphological differentiation is low (Bermingham et al., 1996). The molecular study of the Peninsular Malaysia Megachiroptera was previously done in 2004 by Polly and his co-workers (Polly et al., 2004). His study was mostly concentrated on the phylogeny and phylogeography of *Cynopterus brachyotis* complex. Since then, there was no comprehensive work on taxonomic status of the species of *M. sobrinus* specifically.

The availability of DNA sequence data from mitochondrial DNA (mtDNA) genes has revolutionized phylogenetic research. Among mammals, mtDNA data frequently are used to test hypotheses of relationship among closely related taxa (Ferris et al., 1982). While in higher animals, mtDNA usually shows maternal inheritance (Gyllesten et al., 1991). It evolves, on average, faster than nuclear structural genes (Brown et al., 1982) and this makes them particularly suitable for population levels studies. Different regions of the mitochondrial genome evolve at different rates (Saccone et al., 1991) allowing suitable regions to be chosen for the question under study. Another important feature of mtDNA is that it does not undergo genetic recombination. Hence it is derives from a single ancestral molecule. Thus signature sequences can be used to place any organisms in the proper group.

Using a phylogeographic approach, this project attempted to address the following questions; 1) What is the evolutionary relationship between *M. sobrinus* and *M. minimus* based on cytochrome *b* (cyt-*b*) mtDNA gene? 2) Is their current taxonomy adequate? By defining the framework within which evolution takes place, we hope to understand the broad evolutionary perspective of the Peninsular Malaysian Macrochiroptera. This preliminary study primarily intended to describe genetic diversity; and to reconstruct and clarify the preliminary genetic relationship of *Macroglossus sobrinus* in Peninsular Malaysia. Plus, this research was also chiefly forced by the need to establish the patterns of biodiversity of Macrochiroptera in Peninsular Malaysia to underpin the implementation of wildlife management and conservation efforts by Department of Wildlife and National Parks (DWNP) Peninsular Malaysia or aka PERHILITAN.

## METHODOLOGY

### Samples collection and identification

The tissue samples of *Macroglossus sobrinus* were taken from the Department of Wildlife and National Parks (DWNP) museum at the Institute of Biodiversity (IBD), Bukit Rengit, Lanchang, Pahang Darul Makmur, Malaysia. Originally, the fruit bat specimens were caught from various localities in Peninsular Malaysia during several inventory programs. The inventory programs were conducted by the IBD to update current species distribution in Malaysia. This study covered several populations of *M. sobrinus* in Peninsular Malaysia as shown in Table 1.

Table 1: The details of sampling sites and sample size of *M. sobrinus*

| State | n | Sampling Locality | GenBank, NCBI, US Accession No. |
|---|---|---|---|
| Kelantan | 5 | Gua Musang | FJ226478 – FJ226482 |
| Selangor | 3 | Ulu Langat | FJ226483 – FJ226485 |
| Melaka | 2 | Asahan Area | FJ226486 – FJ226487 |
| Kedah | 2 | Weng Ulu Muda F.R. | FJ226488 – FJ226489 |
| Johor | 2 | Gunung Ledang | FJ226490 |
| Terengganu | 1 | Ulu Terengganu F.R. | - |
| Perak | 3 | Bintang F.R. | FJ226491 – FJ226493 |
| Pahang | 2 | Krau Wildlife Reserve | FJ226494 – FJ226495 |

* F.R. stands for Forest Reserve; n = sample size

In brief, identification of the species was made based on the characteristics that have been described by Medway (1983). After the process of identification, the dead samples were dissected in order to take out small portions of tissues. The tissues were placed in cryo vials containing 70% ethanol and then stored at -20°C in order to maintain the quality of the tissue.

**DNA extraction**

Total genomic DNA extraction was done using kits. The total genomic DNA was extracted from muscle tissue. Quantification of extracted DNA was done using spectrophotometer and 1% (w/v) agarose gel electrophoresis.

**DNA amplification**

Approximately 450 base pairs (bp) section of the cytochrome b (cyt-*b*) mtDNA region was amplified using standard polymerase chain reaction (PCR) procedures. The following primers from Palumbi et al. (1991) were used for the amplification of cyt-*b*.

GludG-L (forward) 5'- **TGA CTT GAA RAA CCA YCG TTG** -3'
CB2H (reverse) 5'- **CCC TCA GAA TGA TAT TTG TTC TCA** – 3'

The PCR reaction mixture was prepared in 1.5 ml microcentrifuge tube and then dispensed into 0.2 ml PCR tubes with the addition of 4 µL DNA template. Standard thermal cycle amplification was performed in 50 µL reaction volume containing 30.0 µl of sterilized $dH_2O$, 5.0 µl of 10X PCR reaction buffer, 3.0 µl of magnesium chloride ($MgCl_2$, 25 mM ), 2.5 µl of each primer (10 µM), 1.0 µl of nucleotide/dNTP mix (10 mM), 1.0 µl of Acetylated Bovine Serum Albumin (BSA, 10 µg/µl) , 0.5 µl of Glycerol, 4 µL of DNA template and 0.5 µl of 5 u/µl *Taq* DNA polymerase.

DNA amplification was then performed in the Mastercycler (Eppendorf), which at first had been programmed according to the desired temperature cycling profile. The PCR cycle was then repeated for 30 cycles. Cycle parameters for PCR were 7 min at 96°C for initial denaturation, 1 min at 95°C for denaturation, 45 s at optimized temperature for annealing, 1 min 30 s at 72°C for extension, repetition of step 2-4 for another 29 cycles (total: 30 cycles) and 10 min at 72°C for final extension. Quantification of the PCR products was analyzed by 1% (w/v) agarose gel electrophoresis. Purification kits from manufacturer were used for direct purification.

**DNA sequencing**

The purified products were directly sent for sequencing using BigDye® Terminator v3.1 Sequencing Kit and analysed on ABI PRISM® 377 Genetic Analyzer. Cycle sequencing reaction was done in a programmable cycler. Cycle sequencing reaction was done for 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 min at rapid.

### Phylogenetic analysis

Chromas Lite (Version 2.1) program was used to display the results of fluorescence-based DNA sequence analysis. Multiple sequence alignment for forward reaction sequences was done using ClustalX program (version 1.81; Thompson et al., 1997), and subsequently aligned by eyes. During the multiple sequence alignment, a sequence of *Penthetor lucasi* (Lucas's Short-nosed Fruit Bat) taken from the GenBank was assigned as an outgroup. The multiple sequence alignment was made against the outgroup. Sequences with short length and the one with bad sequences were excluded from the multiple sequence alignment i.e. "JOH 1 f" and "TER 1 f". PAUP* version 4.0b10 (Swofford, 1998) was used to reconstruct rectangular cladogram or topology of neighbour-joining tree (Saitou and Nei, 1987) and then topology of maximum parsimony tree. Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 (Kumar et al., 2001) was subsequently used to reconstruct original tree or rectangular phylogram of neighbour-joining method. Kimura 2-parameter distance method was incorporated to reconstruct the neighbourjoining trees based on equal base frequencies and unequal ratio of transition to transversion (*ti/tv*).

Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets. The reconstruction of rectangular cladogram of maximum likelihood method and topology of consensus phylogenetic tree summarizing the clusterings inferred from neighbour-joining tree, maximum parsimony tree and maximum likelihood tree was done by using PHYLIP version 3.6b (Felsenstein, 2004) with 1000 sequence replications and 100 data sets. TreeView (Win32) version 1.6.6 by Page (1996) was used to display and edit the reconstructed phylogenetic trees. PAUP* program was also utilised to examine other relevant analyses such as base frequency, proportion of sites differing and genetic distance.

## RESULTS AND DISCUSSION

### DNA extraction and PCR

The expected length of bands obtained for total genomic DNA were near the first band of DNA marker with size more than 10,000 bp. The desired bands for partial cyt-*b* mtDNA region were observed with different quality of PCR products. The position of bands was observed between 250bp and 500bp while expected length is approximately 450bp. All the positive PCR products were proceeded to direct purification.

### DNA sequencing and sequence alignment

Eighteen cyt-*b* PCR products of *M. sobrinus* showed results with good signal strength from DNA sequencing. Whilst 2 samples of *M. sobrinus* labeled as Sample 17 and Sample 19 produced noisy data and Sample 15 showed early signal loss. There was no corresponding sequence of *M. sobrinus* for partial cyt-*b* mtDNA gene in the GenBank showing no study on the cyt-b mtDNA gene of *M. sobrinus* was done in the past. Beside that, 20 cyt-*b* mtDNA sequences of *M. minimus* were taken from the GenBank.

**Phylogenetic analysis**

In this study, approximately 450 bp of cyt-*b* of the samples were amplified and sequenced. 374 aligned bases of cyt-*b* mtDNA gene were preceded for further analyses.

In terms of base frequencies, there was a slight mean excess of A residues (30.53%) while G residues showed the least mean of 13.58%. Furthermore, T residues showed a mean of 29.54% while 26.35% was presented by C residues. Across all species, GC content averaged about 39.93%. The average value for base count was approximately 374 base positions. On the whole, the frequencies for all bases i.e. Adenine (A), Cytosine (C), Guanine (G) and Thymine (T) were considered equal as all the sequences incorporated were partial. Such consideration was one of the important assumptions used for Kimura 2-parameter (1980) distance method.

The neighbour joining (NJ) method in overall likely resolved the clustering at species level as it showed two main species clusters of fruit bat namely *M. minimus* and *M. sobrinus*. The cluster that contains *M. sobrinus* from Peninsular Malaysia showed no regional fragmentation as all the individuals mingled together. An individual of *M. sobrinus* (KEL 2 f) formed the basal in the major cluster of *M. minimus* from the Philippines archipelago and Africa, with 73% bootstrap value showing the possibility of species misidentification. The major cluster of *M. minimus* showed regional fragmentations as the cluster formed four separate subclusters which represent *M. minimus* from the Philippines archipelago and remarkably, two individuals of *M. minimus* obtained from Africa were separated from *M. minimus* of the Philippines archipelago.

Bootstrap method with heuristic search was used to search for the most optimal tree from maximum parsimony (MP) analysis. The number of bootstrap replicates incorporated was 1000 with starting seed of 2333. Of 374 total characters, 134 characters were constant, 184 variable characters were parsimony-uninformative. In terms of maximum likelihood (ML) analysis, Hasegawa, Kishino and Yano (HKY)-gamma model was used with the assumptions of unequal base frequencies and unequal ratio of transition to transversion (ti/tv). 1000 sequence replication, 100 datasets and 10 times of jumbles were incorporated in order to maximize the level of confidence in conducting the bootstrap analysis. Global rearrangements and empirical base frequencies were also applied. The ratio of transition to transversion was fixed to 2.0 with the starting seed of 2333. However, both MP and ML trees did not support the formation of two major clusters separating *M. minimus* from *M sobrinus* due to the status of GeneBank19 (the sample of *M. minimus*) which became basal to *M. minimus* and *M. sobrinus*. Likewise, the consensus phylogenetic tree (Figure 1.0) unsuccessfully resolved the population of fruit bats at species level as the tree also showed that individual GeneBank19 has formed basal to *M. minimus* and *M. sobrinus* with 100% bootstrap value.

**Genetic variation within the *M. sobrinus***

The average sequence divergence when using the Kimura 2-parameter model indicated that genetic distance within the *M. sobrinus* group is 5.1%. Unfortunately, small samples were available from each locality – even though the data were representing Peninsular Malaysia. At present, the genetic variation within the samples of *M. sobrinus* did not reflect any major geographical partitions within the species. Generally, the genetic distances

between samples of *M. sobrinus* were postulated very low, showing genetically close relationship among individuals thus enforcing their taxonomic validation as belonging to a single taxon (*M. sobrinus*).

Many factors could have influenced the results of this study. The sampling strategy may influence average sequence divergence at any level of comparison. Samples from isolated populations or population representing geographical extreme of a species distribution could result in the overestimations of average genetic divergences for within species comparison (Bradley and Baker, 2001).

## CONCLUSION AND RECOMMENDATIONS

More studies on *M. sobrinus* in Peninsular Malaysia are needed to fully resolve the taxonomic status in this study. This will require re-evaluation of morphology and use of other characters, especially nuclear gene that may provide resolution at the higher level of relationships. Among other mitochondrial genes available that can be used for further studies are 12S, 16S, and cytochrome *c* oxidase I (COI). Future studies on the phylogeography in local *M. sobrinus* are recommended with broad geographical sampling for *M. sobrinus* (e.g. inclusion of Sabah and Sarawak regions).

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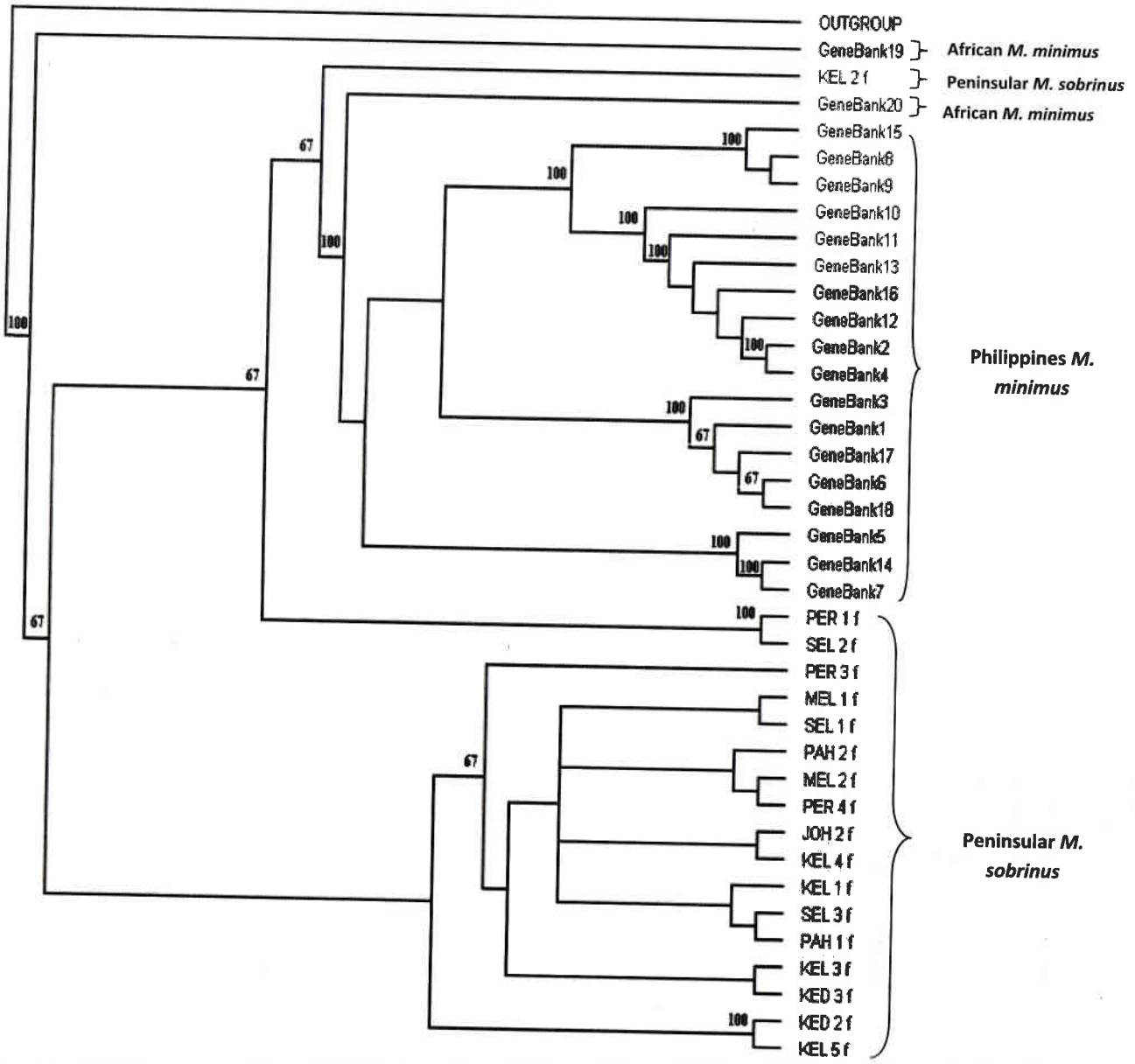
OUTGROUP
GeneBank19
KEL 2 f
GeneBank20
GeneBank15
GeneBank8
GeneBank9
GeneBank10
GeneBank11
GeneBank13
GeneBank16
GeneBank12
GeneBank2
GeneBank4
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GeneBank18
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PER 1 f
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PAH 2 f
MEL 2 f
PER 4 f
JOH 2 f
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KEL 1 f
SEL 3 f
PAH 1 f
KEL 3 f
KED 3 f
KED 2 f
KEL 5 f
African M. minimus
Peninsular M. sobrinus
African M. minimus
Philippines M. minimus
Peninsular M. sobrinus

Figure 1: Topology of consensus phylogenetic tree of fruit bat species from subfamily of Macroglossinae inferred from cyt-*b* mtDNA gene using PHYLIP version 3.6b (Felsenstein 2004). Three types of phylogenetic trees namely neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) trees were incorporated. Abbreviation of GeneBank refers to corresponding sequences obtained from GenBank. The tree is rooted with a sequence of *Penthetor lucasi*, also known as Lucas's short-nosed fruit bat (GenBank accession number: EF105544). Kimura 2-parameter distance (1980) with 1000 sequence replications and 100 data sets were used. Numbers at nodes indicate the bootstrap values in percentage (%).