# ISOLATION OF GENOMIC DNA FROM FRUIT BATS OF KELABIT HIGHLANDS FOR DNA ARCHIVING AND DETERMINATION OF GENETIC VARIATION

Mohd Azib Salleh, Edmund Sim Ui Hang, Mustaffa Abdul Rahman & Mohd Tajuddin Abdullah
Universiti Malaysia Sarawak

## ABSTRACT

*A total of 40 fruit bats belonging to five species, namely Cynopterus brachyotis (34), Aethalops alecto (3), Pipistallus tenius (1), Balionycteris maculata (1) and Megaerop ecaudatus (1) were captured from two sites during the expedition to Bario in April 1995. Whole blood and liver tissue samples were taken from freshly killed bats and stored in liquid nitrogen in the field before transferring to a - 70°C deep freezer in the laboratory. Genomic DNA was successfully isolated from whole blood samples derived from 13 bats using SDS-lysis followed by phenol-extraction methods. Pure DNA samples were dissolved in tris-EDTA buffer and stored at -20°C for DNA archiving and determination of genetic variations within and between the different species*

## INTRODUCTION

The order Chiroptera (bats) are divided into two distinct sub families, namely the Megachiropter (Megabats) and the Microchiroptera (Microbats) The former being medium to large in size and mostly frugivorous bats of the Old World tropics, while the latter are much smaller and characterized by their high-frequency echolocation. The megabats are so different from the microbats that their postulated monophyly origin is still debatable among chiroptologist (Findley, 1993).

Until the late 1980s, taxonomic classification of bats had been solely based on morphology and systematic references from established key phenotypes. Records of classification of local fruit bats had been well documented. The bats of Borneo are classified into eight families with the family Pteropodidae (fruit bats) having 11 genera and 17 species (Payne et al., 1985).
Efforts to investigate genetic variations in tropical bats to complement conventional classification systems have been carried out previously For example, Rickart et al. (1989) has karyotyped several species of pteropodid bats in the Phillipines, including Eonycteris spelaea (2n=36), Macroglossus minimus (2n=34), Pteropus hypomelanus (2n=38) and Rousattus amplexicaudatus (2n=36). Karyosystematics has enabled analysis of variations within and between a number of species. Thus, analysis of 10 species of pteropodid bats from the Phillipines (Rickart et al., 1989) and five species of African vespertilionid bats (Reudas et al., 1990) showed the absence of marked interspecific variations, suggesting close phylogenetic relationships. Chromosomal banding is another approach used, and this technique has enabled the phylogenetic tree for five genera of African long-eared vespertilionid bats to be constructed (Qumsiyeh et al., 1993).
Molecular systematics involving the determination of genetic variations at specific loci and/or

genes have also been attempted. At the highest level of Chiropteran phylogeny, comparative DNA sequence analysis of the coding sequence of a single copy nuclear gene for the interphotoreceptor retinoid binding proteins (IRBP) yielded results that strongly support a monophyletic Chiroptera, suggesting common ancestral origin among the Micro- and Megabats (Stanhope et al., 1991, Mindell et al., 1991). Restriction enzyme site polymorphism within the transcribed portion of the ribosomal-DNA gene complex enabled determination of inter generic relationships within the Neotropical bat family Phyllostomidae (Bussche, 1992). At the species level, reports from Wilkinson et al. (1991), on the length variation observed for D-loop mitochondrial DNA (due to a tandemly repeated 81 bp region) in the evening bats, Nycticeius humeralis, provided possibilities of exploring DNA data in monitoring intraspecific relationship. Efforts to obtain DNA profile unique for individual bats had not been unexplored too. The utility of Jeffrey's probes, 33.6 and 33.15, to obtain fingerprints of Pipistrelle bats in single maternity roosts had succeeded in establishing mother-pup relationships (Bishop et al., 1992). Similarly, Watt et al. (1995) were able to prove the presence of discriminate suckling and non-random mating in Myotis lucifugus after examination of the genetic similarity from DNA fingerprints of mother-pup pairs of these bats at maternity roosts.

These studies provide the impetus for the development of an comprehensive molecular systematics of bats. Towards this end, there is a need to establish an exhaustive archive of genetic material which can be utilized for various molecular analysis. The present study describes our initial step towards establishing a genomic DNA collection of the various frugivorous bat species in Borneo which can subsequently be used for studies on genetic variations, and as a source of certain genes whose structure and functions can be analysed for possible application in animal biotechnology.

## METHODS

**Sample Collection:** Fruit bats were captured using mist nets with mesh size of 36 millimeter and overall size of 2 meter by 6-36 meter. The mist nets were set at ground level and across forest trails and water bodies in submontane rainforest at Bario (Kelabit Highlands), a highland area situated in the Miri Division of Sarawak. Two sites of capture with different vegetation types were chosen. The first site, Bario salt well (Lubang Garam Pa Umor) is a semi- wetland area of sub-montane secondary rainforest habitat, and the second site, the Bario new dam (Lelang Baru) area, was an area of sub-montane primary rainforest.

Mist nets were opened for capture from 7.00 till 9.00 p.m. and were checked once every hour. Species identification through morphological characteristics were based on data and measurements of body parts.

Whole blood and liver tissue samples collected from freshly chloroform-killed bats were put into sterile cryogenic vials. Whole blood (200 to 500μl) was drawn from the heart using a sterile syringe, and mixed with about 20μl of the anticoagulant, 0.5M $Na_2EDTA$, before storage. The tissue and blood samples were stored in liquid nitrogen in the held and later transferred to a -70$^{\circ}$c freezer upon returning to the laboratory until ready for DNA extraction.

**DNA Extraction:** Isolation of genomic DNA from whole blood was performed as follow: The blood samples (stored at -70°C in $Na_2EDTA$ in cryogenic vial) were first thawed at room temperature and then 200 to 500µl was transferred into sterile microfuge tubes. The tubes were then added with 600µl of standard citrate buffer, mixed by a few inversions, then centrifuged at 12,000 rpm (1-2 mins, room temperature). The top portion of the supernatant was discarded and the washing with citrate buffer was repeated once. After discarding the supernatant, the cell pellet was lysed in 0.4ml 10% solution of SDS detergent and proteinase-K by incubating at 55°C for one hour. The lysed mixture was then phenol-extracted once with a phenol/chloroform/isoamyl alcohol solution, and after centrifugation, the aqueous layer containing genomic DNA was transferred to a fresh microcentrifuge tube. The DNA was precipitated in 0.7ml 70% ethanol and pelleted by centrifuging at 12,000 rpm for 10 minutes. The pellet obtained was dissolved in 40µl buffer (Tris-EDTA) and then ethanol-precipitated a second time. After the final centrifugation, the pellet recovered was air-dried and redissolved in 40µl Tris-EDTA buffer by incubation at 55°C overnight. The genomic DNA solution was then stored at -20°C.

**Gel Electrophoresis:** A horizontal agarose gel electrophoresis system was used to estimate the yield and purity of the genomic DNA isolated. 0.8% agarose was used and ethidium bromide was incorporated in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light. 20µl DNA samples were mixed with loading/tracking dye and loaded into the sample wells. Electrophoresis was carried out at 90 Volts (48mA) for 2 hours at room temperature. Estimation of concentration of extracted DNA samples was done by comparing DNA band intensity (Fig. 1) with an app ropriate molecular weight marker (DNA marker VI: Bgl I - Hinf I dige sted pBR 328 DNA) of known concentration.

## RESULTS

**Trapping and Tissue Collection:** A total of 40 fruit bats (Table 1) were captured in this study. These bats, comprising of five species based on morphological characteristics, were collected from the locations which differ in terms of forest types. The most prevalent species captured was C. brachyotis. Whole blood and liver tissue samples collected could be stored in liquid nitrogen in the field for up to two weeks in 5-litre tanks. However, the number of samples that can be kept was relatively small (up to 34 per tank), and the bulkiness and and weight of the tanks tended to restrict transportation.

*Table 1: Species types and frequencies of fruit bat specimens collected at the two trapping sites in Bario.*

| Site of Capture | Species | No. of Samples |
|---|---|---|
| Salt Well (Lubang Garam Pa Umor) | Cynopterus brachyotis | 23 |
| | Pipistallus tenius | 1 |
| New Dam (Lelang Baru) | Balionycteris maculata | 1 |
| | Cynopterus brachyotis | 11 |
| | Megaerops ecaudatus | 1 |

| | | |
|---|---|---|
| | Aethalops alecto | 3 |
| | TOTAL | 40 |

**DNA Isolation:** Genomic DNAs were successfully isolated from 13 of the 40 fruits bats. Of these, 10 were from the species C. brachyotis - eight from the first trapping site (Bario salt well) and two from the second site (Bario new dam). The other three were two different species, A. alecto (2) and M. ecaudatus (I) caught at the second site. The failure to obtain DNA from the other samples could be attributed to the low sample volume and inefficient extraction steps. Unfortunately each of the single specimen species P. tenius and B. maculata failed to yield any DNA. Figures 1 and 2 show the genomic DNA bands of 8 samples isolated from the first site and 5 samples from the second site, respectively. The bands obtained indicated the presence of high molecular weight DNA, confirming successful isolation of genomic DNA. A comparison of DNA band intensities with the marker DNA of known concentration suggested that the concentration of the extracted DNA was approximately 0.5 to 1μg/μl. Low band intensity of two DNA samples (Figure 1, lane 6) indicated relatively lower DNA concentration, estimated to be 0.25μg/μl.

## DISCUSSION

In this study the most common fruit bat captured was the pteropodid species C. brachyotis (85%). The dominance of C. brachyotis at the two locations in Bario probably suggest that this species has adapted very well to the area, and is in agreement with Peterson et al. (1993) which showed a relative abundance and widespread distribution of the eurytopic genus Cynopterus in Southeast Asia. Their studies in the Phillipines Indicated the occurrence of high genetic variability within population of Cynopterus. There are, in fact, nine subspecies of C. brachyotis recognized to date. The subspecies C. b. brachyotis is distributed in Borneo, Lombok, Peninsu lar Malaysia, Phillipines and Sulawesi; and is widespread from sea level up to 1,600 meters (Mickleburge et al., 1992).

The abundance of C. brachyotis in submontane habitats could be attributed to their distribution over a wide spectrum of foraging range in Borneo, and the failure of other frugivorous bats genera to exploit habitats of higher altitude. From an ecological aspect a large distribution of C. brachyotis at Bario would give an indication that this species is an important fauna in the ecosystem, possibly playing the role of a vector for pollination and seed dispersal (Marshall, 1985). This would seem logical considering the fact that continued forest regeneration through seed dispersal would mean adequacy of existing reserves for their conservation. Nonetheless the fact that our total sample size is only 40 does not allow any meaningful conclusion to be made. Trapping of more bats from more sites throughout Bario would be essential, and a study of the species migratory habits must also be carried out.

Of the 40 bats' blood specimens, only 13 samples proved useful for genomic DNA isolation although all samples were subjected to the same extraction procedure. A factor that could have contributed to this is the fact that most of the blood samples collected were in small volumes.

Appreciable amount of DNA could be extracted from blood samples of volume 300 to 500µl. Obviously better methods of tissue collection have to be employed for effective DNA extraction as it has proven to be difficult to obtain vol umes of blood in this range from small fruit bats. However, Wilk inson et. al. (1991) reported successful extraction of bat genomic DNA (50-1000ng) from approximately 1-3 mg of pectoral muscle. Ethylene chloride was applied topically as local anasthetic while muscle biopsies were procured. This is an attractive alternative as the bats captured need not be killed and would represent a more humane method of sample collection

The relative purity of our extracted DNA samples suggeste that further molecular analyses like DNA cloning, Polymerase Chain Raction (PCR), Restriction Fragment Length Polymorphism (RFLP) determination and DNA fingerprinting may be carried out. These molecular techniques would certainly help towards resolving certain uncertainties in the classification, migratory pattern and breeding habits of tropical fruit bats. The application of such molecular and biotechnological approaches will be the focus of our follow-up research activites as data from such studies will be vital towards effective protection strategies and conservation of this important fauna.

## ACKNOWLEDGEMENTS

We thank Besar Ketol, Isa Sait, Wahap Marni and Amin Manggi for their expert assistance in trapping and identification of specimens. This research is supported by Universiti Malaysia Sarawak Research Grants Nos. 8/94 and 2 1/94.

## REFERENCES

**Bishop, C.M., G.James, C.M. Lazarus & P.A. Racey**
[1992] Discriminate suckling in pipistrellus bats is supported by DNA fingerprinting. Molecular Ecology 1(4): 255-258.

**Bussche, R.A.V.D.**
[1992] Restriction-site variation and molecular systematics of new world leaf nosed bats. J. Mammal. 73(1):29-42.

**Findley, J.S.**
[1993] Bats : A Community Perspective. Cambridge University Press, Cambridge.

**Marshall, A.G.**
[1985] Old world phytophagus bats (Megachiroptera) and their food plants: a survey Zool. J. Linnean Soc. 83 : 351-369.

**Mickleburg, S.P, A.M. Hutson & Paul A. Racey.**
[1992] Old world fruit bats: an action plan for their conservation. IUCN/FFPS/ZSL/WWF/JWPT/NWF /Sultanate of Oman.

**Mindell, D.P, C.W Dick & R.J. Baker**
[1991] Phylogenetic relationships among megabats, microbats, and primates Proc. Natl. Acad. Sci. USA 88 (22):10322-1 0326.

**Payne,J., C.M. Francis & Phillipps**
[1985] A held guide to the mammals of Borneo. The Sabah Society and World Wildlife Fund Malaysia, Kota Kinabalu and Kuala Lumpur.

**Peterson, A.T. & L.R. Heaney**
[1993] Genetic differentiation in Philippines bats of the genera Cynopterus and Haplonycteris. Biol. J. Linnean Soc. 49(3): 203 -218.

**Qumsiyeh, M.B. & J.W. Bickham**
[1993] Chromosomes and relationships of long-eared bats of the genera Plecotus and Otonycteri. J. Mammal. 74 (2):3 76-382.

**Rickart, E.A., L.R. Heaney & M.J. Rosenfeld**
[1989] Chromosome of ten species of Phillipine fruit bats (Chiroptera: Pteropodidae). Proc. Biol. Soc. Wash. 102(2) : 520-531.

**Ruedas, L.A., T.E. Lee Jr.,J.W Bickham & D.A. Schlitter**
[1990] Chromosomes of five species of vespertriolionid bats from Africa. J. Mammal. 71 (1):94-100.

**Stanhope, M.J.,J. Czelusniak, J.S. Si,J. Nickerson & M. Goodman**
[1992] A molecular perspective on mammalian evolution from the gene encoding interphotoreceptor retinoid binding protein, with convincing evidence for bat monophyly Molecular Phylogenetics and Evolution 1 (2):41 6-160.

**Watt, E.M. & M.B. Fenton**
[1995] DNA fingerprinting provides evidence of discriminate suckling and non-random mating in little brown bats Myotis lucifugus. Molec. Ecol. 4(2): 261-1 64.

**Wilkinson, G.S. & A.M. Chapman**
[1991] Length and sequence variation in evening bat D-Loop mtDNA. Genetics 128: 607-617.