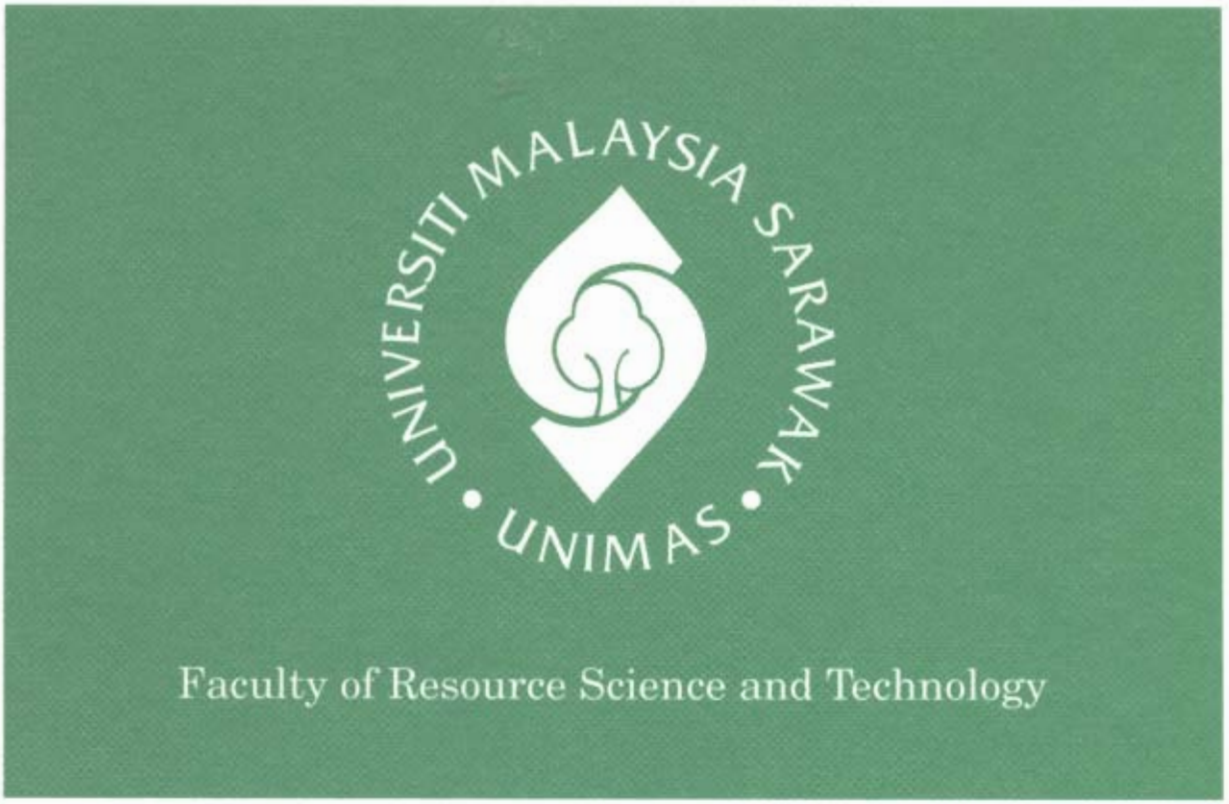

IDENTIFICATION OF GENETIC MARKER IN THE FAMILY RHINOLOPHIDAE USING PARTIAL CYTOCHROME *b* GENE

Shida Zulia Sidek

Bachelor of Science with Honours
(Animal Resource Science and Management)
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This project is submitted in partial fulfilment of the requirements for the degree of
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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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Identification of Genetic Marker in the Family Rhinolophidae Using Partial Cytochrome *b* Gene

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ABSTRACT

The analysis of family Rhinolophidae using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of the partial mitochondrial cytochrome *b* (450bp) gene was conducted as the alternative tool to DNA sequencing. PCR-RFLP is an inexpensive and easy tool to discriminate between species. The samples were collected from Sarawak, Sabah and Pahang. Eleven restriction enzymes were used in this study but only five enzymes namely, *Bam*HI *Csp*6I, *Sal*I *Alu*I and *Rsa*I has shown the haplotypes for certain species and only *Bam*HI is diagnostic to discriminate *R. trifoliatus*, *R. borneensis* and *R. affinis* of this family. It is concluded that the partial cytochrome *b* is not suitable to be used as the genetic marker for discriminating some species in the family Rhinolophidae. Thus, further study should be conducted by using complete cytochrome *b* (1140bp) gene in order to reveal the phylogenetic relationship among species of this family.

Key words: PCR-RFLP, Rhinolophidae, Cytochrome *b* gene.

Abstrak

Analisis dan perbandingan terhadap famili Rhinolophidae menggunakan teknik polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) menggunakan jujukan sebahagian gen cytochrome b (450 base pairs) sebagai kaedah alternatif kepada DNA sequencing. PCR-RFLP lebih murah dan lebih mudah untuk membezakan antara spesies. Sampel yang digunakan dalam kajian ini dikumpulkan dari Sarawak, Sabah dan Pahang. Sebelas enzim pembatasan telah digunakan dalam kajian ini tetapi hanya lima enzim iaitu BamHI, Csp6I, SalI, AluI dan RsaI di dapati sesuai untuk membezakan spesies sesetengah spesies. Hanya BamHI merupakan enzim pembatasan terbaik yang dapat membezakan R. trifoliatus, R. borneensis dan R. affinis untuk kajian ini. Kesimpulannya jujukan sebahagian gen cytochrome b tidak sesuai untuk membuat kajian penanda genetik untuk membezakan spesies dari famili Rhinolophidae. Kajian lanjut perlu dilakukan dengan menggunakan jujukan penuh gen cytochrome b (1140bp) bagi mengkaji hubungan filogenetik di kalangan spesies dalam famili ini.

Kata kunci: PCR-RFLP, Rhinolophidae, gen cytochrome b.

1.0 Introduction

Chiroptera is the second-largest order in mammals after rodent (Altringham, 1996). They are distinguished from other mammals by having wings for true flight and widely distributed because their ability to fly (Payne *et al.* 1985; Feldhamer *et al.*, 1999). Chiroptera includes 188 modern genera and about 977 modern species (Corbet and Hill, 1992). The order is divided into two suborders, namely, Megachiroptera or known as Old World fruit bats, which are frugivorous and lack laryngeal echolocation; and Microchiroptera or insect eating bats which are carnivorous species which possess laryngeal echolocation (Altringham, 1996; Bastian *et al.*, 2001; Springer *et al.*, 2001).

According to Feldhamer *et al.* (1999), the family Rhinolophidae consists of 130 species in ten genera in the world and divided into two subfamilies of Rhinolophinae (horse-shoe bats) and Hipposiderinae (Old World leaf-nosed bats). According to Payne *et al.* (1985), the genera *Rhinolophus* and *Hipposideros* are classified as two distinct families. In addition to that, some authors agreed that the Rhinolophidae and Hipposideridae are sister-taxa (Corbet and Hill, 1992; Koopman, 1993; Bussche and Hoofer, 2001). However, Hand *et al.* (1994) stated that the morphological problem among these species can be resolved using phylogenetic study.

Rhinolophidae distribution ranges from Europe, Africa, Middle East, Asia, Japan, the East India and Australia with wide variety of habitats ranging from desert to tropical forest (Corbet and Hill, 1992; Feldhamer *et al.*, 1999). Besides that, they also roost singly

or in small group in caves, crevices, hollow trees or houses (Payne *et al.*, 1985; Corbet and Hill, 1992).

According to Payne *et al.* (1985) and Mohd Azlan *et al.* (2005), there are 77 species of Microchiroptera recorded in Sarawak. Rhinolophidae or horseshoe bats consist of ten species namely, *Rhinolophus philippinensis, R. creaghi, R. acuminatus, R. arcuatus, R. borneensis, R. pusillus, R. affinis, R. luctus, R. sedulus,* and *R. trifoliatus*.

The association between species and the habitat ranging is hard to find because of their diversity and mobility (Fenton, 1997). Bats have high capacity for dispersal and phylogeographic pattern similar to other birds and different to other mammals (Ditchfield, 2000). According to Payne *et al.* (1985), all species in this family have almost similar external characteristic that make the identification process harder. Mohd Azlan *et al.* (2005) stated that bats play an important role in regulating tropical rainforest ecosystem and the degraded forest will decrease the number of individual because lack of food source.

The main objective of this study was to use polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique to discriminate among species in the family Rhinolophidae.

2.0 Literature review

2.1 Characteristic of the Rhinolophidae

Rhinolophids are small to moderately large sized bats and can be distinguished by their elaborate noseleaf structure (Payne *et al.*, 1985). Sella is the raised portion behind the middle of the nostrils. Behind the sella, there is posterior noseleaf which rises to a long lancet-shaped point. The sharp of ears are large with the prominent fold on the outside edge, called the antitragus. The eyes are tiny and almost concealed by the noseleaf (Figure 1a and 1b) (Payne *et al.*, 1985).

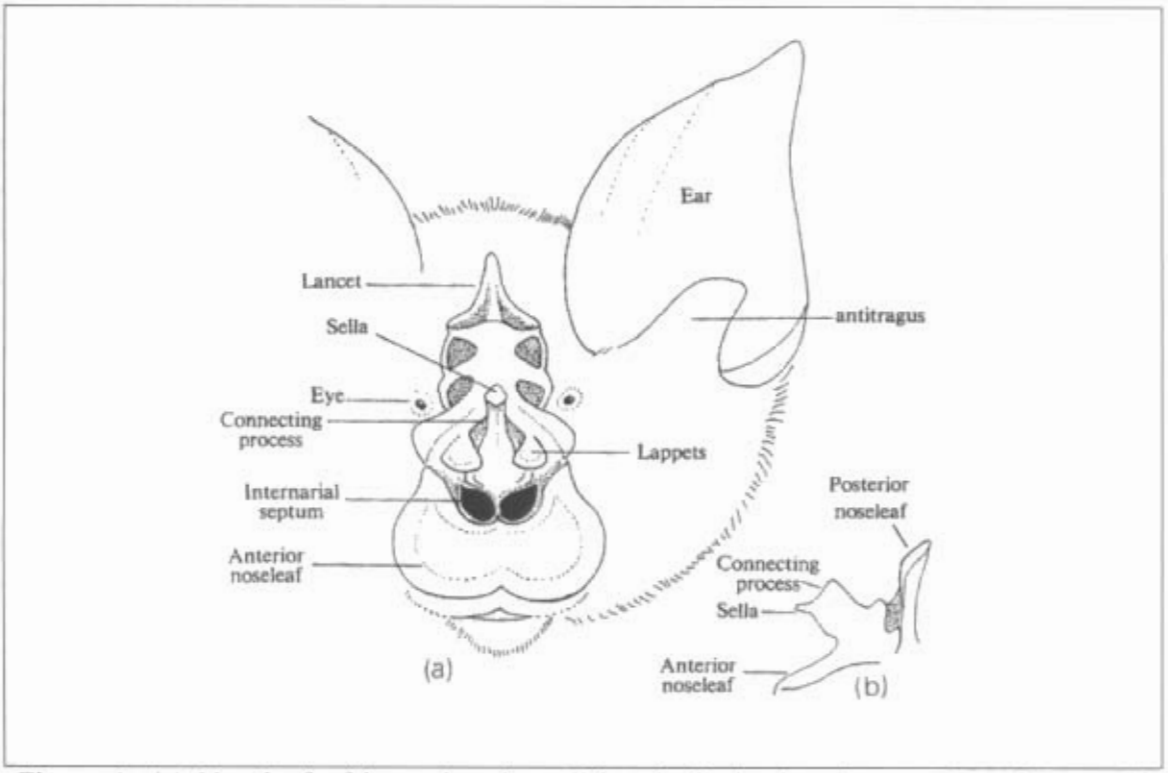

Figure 1: (a) Noseleaf of horseshoe bats (*Rhinolophus*), showing naming of parts. (b) Side view of the noseleaf (Source: Payne *et al.*, 1985)

Rhinolophidae are insectivorous, catching prey during flight or gleaning insects from vegetation where the females always consume vast quantities of prey (Altringham, 1996). Because of their diet, rhinolophids are important as the biological controller and suited as indicators of environment condition (Fenton, 1997).

According to Hand *et al.* (1994), the one of oldest bat fossil was found from the Eocene period approximately 54.6 million years ago. Rhinolophoid fossils found in Australia dates back at the Oligocene period (Hand *et al.*, 1994).

2.2 Mitochondrial DNA (mtDNA) and Cytochrome *b*

Mitochondrial DNA (mtDNA) is cytoplasmic DNA with many characters like the clonal inheritance; haploid and non-recombining and evolves rapidly than nuclear genes, maternally inherited and have selectively neutral marker (Irwin *et al.*, 1991). According to Alexeve (2004), MtDNA encodes two rRNAs, 22 tRNAs and 13 polypeptides, of which seven are components of complex I (NADH dehydrogenase), three are components of complex IV (cytochrome *c* oxidase), two are subunits of complex V (ATP synthase) and cytochrome *b* (a subunit of complex III) and the structure is a closed circle DNA with approximately 17,000 base pairs (Avise, 1994).

According to Lovette *et al.* (1999), the study of four hybridizing taxa in a North American *Dendroica* warblers (*Dendroica occidentalis, D. townsendi, D. virens* and *D. nigresens*) using mtDNA sequence from cytochrome oxidase *I* (COI), ATP-synthase 6

(ATPase 6) and ATP-synthase 8 (ATPase 8) provided insight on the mitochondrial evolution in the related taxa. According to Rodriguez and Ammerman (2004), *Myotis calirnicus* and *M. ciliolabrum* have the complex taxanomic history due to intraspecific geographic variation which has the overlapping ranges in the North America. Thus, this study used sequences of mtDNA from cytochrome *b* and control region fragment to determine the species boundary between two closely related species and reconstruct the phylogenetic tree.

The evolutionary relationship among individuals, species and population can be analyzed by using mtDNA and PCR technique where the method is used to amplify mtDNA sequences directly from many taxa which are present in the large number in cells (Palumbi, 1996). According to Brown (1979 as cited in Palumbi 1996) animal mtDNA evolved at about two percent sequence divergence per million years between pairs of taxa and According to Walker *et al.* (2004), the advantage of mitochondrial-based DNA analysis is that there are many mitochondria per cell making mtDNA as a naturally amplified source of genetic variation.

One of the region in mtDNA is designated as cytochrome *b* (cyt *b*) (Figure 2) gene which was used in this study for approximately 450 bp. According to Palumbi (1996) cyt *b* is the protein in electron transport chain and the only protein product of mitochondrial genome that is fully functional monomer. This gene is easier to align for protein coding sequence in mammals. The cyt *b* protein of mammals does not evolve at uniform rate and the level of acid amino conservation varies significant in different part of cyt *b* gene

(Irwin *et al.*, 1991). Cyt *b* is useful to resolve the organism until species level because it has moderate evolutional rate and is appropriate to study intraspecific and interspecific variation in evolutionary relationship of mammals (Demboski *et al.*, 1998).

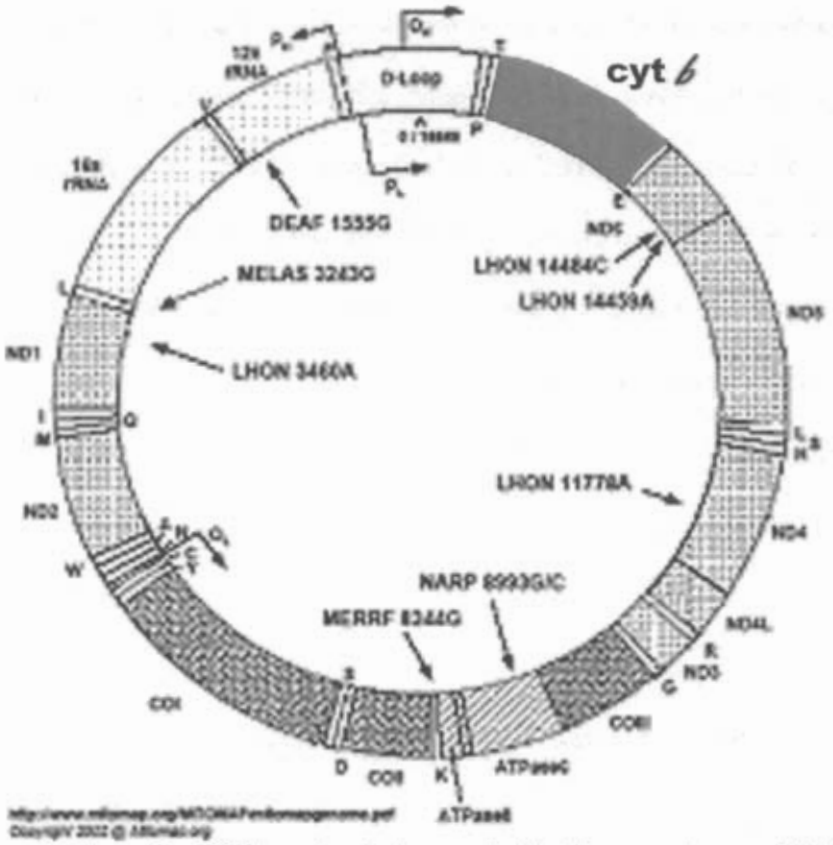

Figure 2: Map of mtDNA and cytochrome *b* site (Source: Anon., 2002)

Primers used to prime DNA synthesis is included in the reaction to initiated DNA synthesis that take place on DNA template (Palumbi, 1996). PCR requires deoxyribonucleotide (dNTP) and *Taq* polymerase; a thermostable DNA polymerase from *Thermos aquaticus*, a thermophilic bacterium collected at a hot spring in Yellowstone National Park (Hoy, 1994). Usually, at least a million-fold increase of a specific section

of a DNA molecule can be realized and the PCR product can be detected by gel electrophoresis (Palumbi, 1996).

According to Palumbi (1996), there are three steps in PCR; denaturation of double-stranded DNA by heating; annealing of extension primers to be amplified and primer extension. Primers are short, synthetic, single-stranded DNA molecules of 20-30 nucleotides long. PCR product is produced at an exponential rate in every cycle. According to Walker (2004), PCR has the ability to accurately detect known quantities of species from mixed DNA sources. It also stated that the most common method in the species identification currently is using PCR analysis from species- specific mitochondrial DNA sequence. This technique can be used to identify a very high-probability, disease-causing viruses and/or bacteria, a deceased person, or criminal suspect (Palumbi, 1996).

2.3 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

There are many analysis which use polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique where the organism can be differentiated by analysis of pattern derived from cleavage of their DNA. This method is cheaper compared to DNA sequencing and it is less time consuming (Pfeiffer *et al.*, 2004). Pfeiffer *et al.* (2004) discriminate different species such as cattle, sheep, goat, roe buck

and deer using cytochrome *b* and the result showed that the species can be differentiated either from fresh or degraded samples.

PCR-RFLP technique is successfully used to identify the specific identification of several organisms. It is used to determine the maternal parent of hybrid individual *Lymnaeid* snails using first and second Internal Transcribed Spacer (ITS1 and ITS2) rDNA and 16 rDNA. Twelve restriction enzymes were used and the molecular result is concordant to morphological result (Carvalho *et al.,* 2004).Because of its sensitivity, PCR-RFLP is the best method to detect contamination (Simsek *et al.,* 2001).

PCR-RFLP is now widely used (Preiffer *et al.*, 2004). This method has not only been employed in cyt *b* gene but also in COI, ATPase 6 and ATPase 8 (Lovette *et al.,* 1999), internal transcribed spacer one and two (ITS1 and ITS2) (Carvalho *et al.,* 2004; Ping Xiang *et al.,* 2004) and mitochondrial 16S ribosomal gene (16S rDNAmt) (Carvalho *et al.,* 2004) and chloroplast DNA (Chang *et al.*, 2000; Korzun, 2000; Ando *et al.,* 2005). According to Ferreira *et al.* (2005), the overlapping measurement in two closely related species of bats in genus *Platyrrhinus* was solved using PCR-RFLP technique. From the result, no shared haplotypes were found and they conclude that these species is different from each other.

3.0 Material and method

3.1 Sampling sites

Fourty-two samples of selected *Rhinolophus* species were used in this study (Table 1). The fresh bats samples were captured during field work conducted at Bako National Park, Kubah National Park and Matang Wildlife Center using four-bank harp traps. Harp trap work on the principle that wires could not be easily detected by the echolocation cries of bats and the bank of wires was sufficient to stop the flight momentum of bats (Kunz, 1988). Other samples were taken from zoological museum at Universiti Malaysia Sarawak (UNIMAS) and Institute of Biological Diversity, Bukit Rengit, Lanchang, Pahang (Table 1).

Table 1: Samples and the localities

Sample	Localities	No. sample	Reference no
R. philippinensis	Niah NP	3	NNP094, NNP106, NNP126
R. sedulus	Kubah NP	3	KNP031, 00101, 00095
	Krau, Pahang	1	KWR161
	IKB Bukit Rengit	1	IKB004
R. creaghi	Niah NP,	2	NNP093, NNP136
	P. Bangi Sabah,	4	00344, 00345, 00346, 00347
R. acuminatus	Tawau Hills	2	00486, 000487
	Lahad Datu	1	00232
	Lembah Danum	1	00098
R. borneensis	Niah NP	4	NNP051, NNP040, NNP023, NNP057
	Kubah NP	1	KNP042
R. pusillus	Kg. Asah, P.Tioman	1	C0815
R. affinis	Matang WC	2	MWL119, MWC003
	Pahang NP	1	TM013
	Kg. Asah, P.Tioman	4	TM007, TM011, TM013, TM018
	G. Berumput Semantan	3	1045, 1049, 1051
R. luctus	Kg. Asah, P.Tioman	1	TM006, C0812
	Bako NP	1	BNP 008
R. trifoliatus	Bako NP	4	BNP016, BNP017 BNP124, BNP125
	Bakun	2	00012, 00013
Total		42	

3.2 DNA Extraction

DNA was extracted using a modified cetyltrimethylammonium bromide (C-TAB) protocol with the presence of the Proteinase-K (Grewe *et al.*, 1993). The major reason for conducting DNA extraction was to lyses the cell and to remove cellular protein and other cellular component (Scoot and Graham, 2001). Seven hundred micro-liters of C-TAB was added into a 1.5 ml sterile microcentrifuge tube. Thirty milligrams of bat muscle was minced up and put into the microcentrifuge tube. Five to eight µl of Proteinase-K (100 mg/ml) were added into the microcentrifuge tube and incubated at 55°C for one to two hours until the entire sample were digested.

Six hundred µl chloroform-isoamyl alcohol were added and shaked for two minutes. The sample was then vortexed and later centrifuged in 13,000 rounds per minute (rpm) for 10 minutes. The sample was then carefully handled and 550µl upper aqueous layer was then transferred to a new 1.5ml sterile microcentrifuge tube. Equal amounts of cold absolute ethanol were added to bind the DNA and for better result, the sample was kept in -20 °C for one night to ensure the entire DNA isolated bind together. The mixture was centrifuged in 13,000 rpm for 10 minutes. A pellet was formed in the microcentrifuge tube after centrifugation. The supernatant was discharged carefully leaving the formed pellet in the microcentrifuge tube. Then 25 µl of 70% cold ethanol and 25 µl of 3M sodium chloride (NaCl) were added to wash the pellet. The sample was centrifuged again in 13,000 rpm for 10 minutes. The ethanol was pipetted out and the liquid still present was dry off in room temperature. The pellet was then redissolved in 30 µl to 100 µl sterilize deionized water or depend on the pellet size.

One micro-litre of bromophenol blue dye was mixed with three µl of DNA extraction product and electrophoresized on 1% agarose gel mixed with one µl of ethidium bromide and run at 90V for 45 minutes. A 1 kb DNA ladder was used as a standard size marker. The gels were visualized under the UV-transilluminator and the photograph of the gel was taken using Polaroid film. The extracted DNA was kept in –20°C and for future use.

3.3 Polymerase Chain Reaction (PCR)

Table 2 shows the primers for partial cyt *b* and its sequence was used in this study. Table 3 shows the reagents used to perform a 25 µl reaction volume. PCR was done by using a

thermocycler (BIOMETRA) and the parameter and annealing temperature is shown in Table 4. One µl of bromophenol blue dye was mixed with three µl of PCR product and electrophoresized on 1% agarose gel mixed with one µl of ethidium bromide and run at 90V for 45 minutes. MassRuler low range DNA ladder was used as a standard size marker. The gels were visualized under the UV-transilluminator and the photograph of the gel was taken using Polaroid film.

Table 2: Primers for partial cyt *b* and its sequence (Palumbi *et al.*, 1996)

Primer		Sequence
Glud G-L	(forward)	5' TGA CCT GAA RAA CCA YCG TTG 3'
CB2H	(reverse)	5' CC TCA GAC TGA TAT TTG TCC TCA 3'

Table 3: Ingredient of master mix

Component	1 reaction (µl)	Master mix x 10(µl)
dH_2O	16.30	163.0
10 x reaction buffer	2.50	25.0
dNTP mix (10mM)	0.50	5.0
Primer Glud (10mM)	1.25	12.5
Primer CB2-H (10mM)	1.25	12.5
$MgCl_2$	1.00	10.0
DNA template	2.00	**
Taq polymerase (5 units' µl)	1.00	**
Total	25.00	

** were not included in master mix and put into tube separately.

Table 4: PCR parameter

Parameter	Temperature (°C)	Time (min)	No of cycle
Initial denaturation	94	1	1
Denaturation	94	1	29
Annealing	56	1	
Extension	72	2	
Final extension	72	5	1
Soaking	4	8	

3.4 Restriction Fragment Length Polymorphism (RFLP)

Generally there are two kinds of REs; six-base cutters which are usually used for evolutionary studies resolving closely related species and four based cutters for detailed information. In this study both six-based cutter and four-base cutter enzymes were used to discriminate relationship among the representatives of family Rhinolophidae. In this study, the PCR products were digested with restriction enzyme *Alu*I, *Csp*6I, *Rsa*I, *Sal*I, *Knp*I, *Xho*I, *Hpa*I, *Ssp*I, *Bam*HI, *Pst*I and *Msp*I from Promega (Table 5). The mixtures were incubated in water bath for three to four hours and for better result it was incubated overnight at 37 °C. The RFLP product was run on 3% agarose gels containing 1.0 µl of ethidium bromide.

Table 5: Restriction digests for RFLP

Component	1 reaction (µl)	10 reaction (master mix)
ddH_2O	4.00	40.00
RE buffer	1.00	10.00
Restriction enzyme	1.00	**
PCR product	4.00	**
Total	10.00	

** were not included in master mix and put into tube separately.

Table 6: Restriction enzymes and it cleavage

Restriction Enzymes	Recognition sites
*Bam*HI	G^GATCC
*Pst*I	CTGCA^G
*Sal*I	G^ TCGAC
*Kpn*I	GGTAC^C
*Rsa*I	GT^AC
*Alu*I	AG^ CT
*Hpa*II	C^CGG
*Csp*6I	G^TAC
*Msp*I	C^CGG
*Ssp*I	AAT ^ATT
*Xho*I	C^TCGAG

^ cutting site

4.0 Result

4.1 DNA extraction

Out of 42 samples only 27 samples DNA were successfully extracted. The appearance of single and bright bands above the position 1kb ladder is shown in Figure 3.

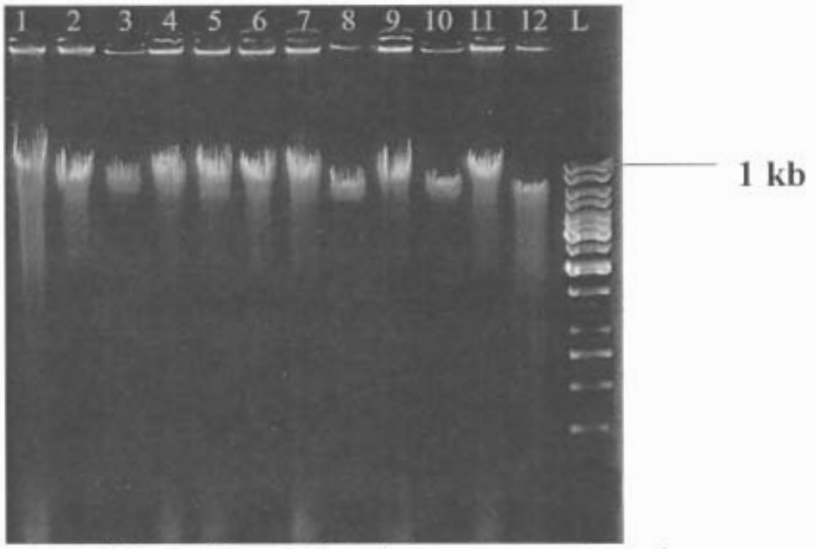

Figure 3: DNA extraction of 12 samples of *Rhinolophus*; Lane 1: DNA from *R. trifoliatus* (BNP016); Lane 2: DNA from *R. trifoliatus* (BNP017); Lane 3: DNA from *R. trifoliatus* (BNP124); Lane 4: DNA from *R. trifoliatus* (BNP125); Lane 5: DNA from *R. luctus* (BNP008); Lane 6: DNA from *R. luctus* (TM006); Lane 7: DNA from *R. sedulus* (KNP031); Lane 8: DNA from *R. borneensis* (NNP057); Lane 9: DNA from *R. borneensis* (NNP040); Lane 10: DNA from *R. borneensis* (NNP051); Lane 11: DNA from *R. affinis* (MWL119); Lane 12: DNA from *R. affinis* (TM007); and Lane 13 (L): GeneRuler^{TM} 1 kb.

4.2 Polymerase Chain Reaction

There were good amplified products where all samples show single bands positioned at below 500 bp (Figure 4). The negative control showed there was no contamination during the preparation of master mix. All the amplified products were then used for RFLP work.

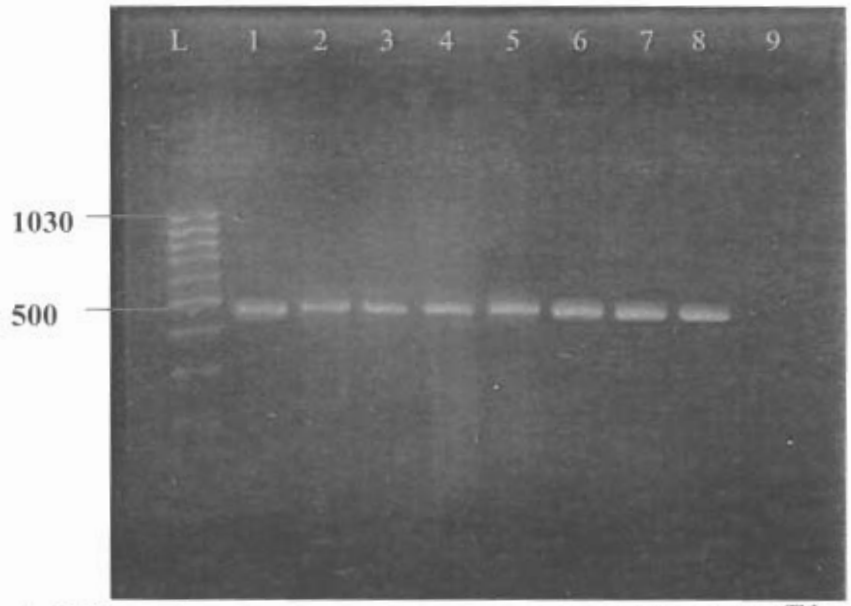

Figure 4: PCR product of eight samples; Lane 1 (L): Mass Ruler™ Low range ladder Lane 1: PCR product for *R. trifoliatus* (BNP016); Lane 2: PCR product for *R. trifoliatus* (BNP017); Lane 3: PCR product for *R. trifoliatus* (BNP124); Lane 4: PCR product for *R. trifoliatus* (BNP125); Lane 5: PCR product for *R. luctus* (BNP008); Lane 6: PCR product for *R. affinis* (); Lane 7: PCR product for *R. luctus* (TM006); Lane 8: PCR product for *R. borneensis* (NNP057); and Lane 9: negative control.

4.3 Restriction Fragment Length Polymorphism

Eleven restriction endonucleases were tested and only five showed restriction sites namely, *Alu*I, *Csp*6I, *Rsa*I, *Sal*I, and *Bam*HI. Six restriction endonucleases namely *Kpn*I, *Xho*I, *Hpa*II, *Ssp*I, *Pst*I and *Msp*I failed to produce restriction sites.

4.3.1 *Bam*HI

This four base cutter restriction endonuclease was a good restriction endonuclease for partial cyt *b* where the result show that the enzyme generate polymorphic cutting profile