Faculty of Resource Science and Technology

DEVELOPMENT OF MICROSATELLITE MARKER FOR THE CRYPTIC *Cynopterus brachyotis*

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This project is submitted in partial fulfillment of the requirements for the degree of
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LIST OF ABBREVIATIONS

µl	microliter
%	percentage
ml	milliliter
L	liter
mM	milimolar
M	molar
g	gram
bp	base pair
rpm	rotation per minute
ºC	degree Celsius
min	minute
mm	millimeter
mmol	milimol
N	normality
V	volt
CTAB	cetyltrimethyl ammonium bromide
DNA	deoxyribonucleotide acids
dNTPs	deoxyribonucleotide triphosphate
EDTA	ethylene diaminetetraacetate
HCl	hydrochloric acid
$MgCl_2$	magnesium chloride
$NaCl_2$	natrium chloride
UV	ultraviolet
dH_2O	distilled water
ddH_2O	double distilled water
Taq	*Thermus aquaticus*
SDS	sodium dodecyl sulfate

Development of microsatellite marker for the cryptic *Cynopterus brachyotis*

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ABSTRACT

Cynopterus brachyotis is one of the widely dispersed Megachiroptera in Indo Malayan region. Recently, a study has shown the presence of cryptic species within *C. brachyotis* populations through the analysis of mitochondrial DNA (mtDNA). The study has identified two types of this species, the large-sized *C. brachyotis* and small-sized *C. brachyotis*. Therefore, the development of microsatellite molecular marker was done in order to identify the suitable marker for each form of *C. brachyotis*. Samples from three different habitats of *C. brachyotis* were used in accomplishing this study; from the open area, which occupied by the large-sized *C. brachyotis*, the close area of the small-sized *C. brachyotis* and the fringe area, the contact area of these two cryptic species . However, only one sample of small-sized *C. brachyotis,* utilizing the primer $(AAG)_8$ has successfully sent for DNA sequencing. No microsatellite primer has yet been successfully designed for the cryptic *C. brachyotis* through this study.

Keywords: *Cynopterus brachyotis*, cryptic, microsatellite

ABSTRAK

Cynopterus brachyotis adalah salah satu jenis kelawar Megachiroptera yang mempunyai taburan luas di kawasan Indo Malayan. Baru-baru ini, suatu kajian telah membuktikan kewujudan spesis kriptik di dalam C. brachyotis di sekitar kepulauan Borneo melalui analisis yang dilakukan ke atas DNA mitokondria (mtDNA). Kajian tersebut telah mengenalpasti spesis berkenaan yang terbahagi kepada dua jenis, iaitu C. brachyotis yang bersaiz besar dan C. brachyotis yang bersaiz kecil. Oleh itu, penghasilan penanda molekular mikrosatelit telah dilakukan untuk mengenalpasti penanda yang sesuai untuk digunakan ke atas dua jenis C. brachyotis tersebut. Sampel daripada tiga habitat C. brachyotis digunakan untuk tujuan kajian ini; dari kawasan terbuka yang didiami C. brachyotis bersaiz besar, kawasan tertutup untuk C. brachyotis bersaiz kecil dan kawasan pinggir yang menjadi kawasan pertembungan bagi kedua-dua spesis kriptik ini. Walau bagaimanapun, hanya satu sampel untuk kawasan tertutup yang menggunakan primer $(AAG)_8$ *berjaya dihantar untuk penjujukan DNA. Masih belum terdapat sebarang primer mikrosatelit yang berjaya direka untuk C. brachyotis kriptik melalui kajian ini.*

Kata kunci: Cynopterus brachyotis, kriptik, mikrosatelit

CHAPTER 1
INTRODUCTION

Bats or Chiroptera have been identified to exist since 50 million years ago. Being the second largest group of identified mammalians after rodents, bats are discovered to be the only mammals which have develop the true ability to fly (Altringham, 1996; Feldhamer *et al*, 1999). Due to this, they are reported to be widely distributed all over the regions depending on their feeding nature, especially in the tropical and subtropical areas (Corbet and Hill, 1992). Having the ability to adapt on various kind of environment, yet this organism is negatively affected due to the environment disturbance such as deforestration and habitat loss (Kunz, 1988). Thus, to keep their existence, habitat isolation and recolonization of this organism has lead them to live in small colonies (Feldhamer *et al.*, 1999).

A number of studies have resulted the discovery of cryptic morphology and species divergence within the bat taxa, including the well-studied genera such as *Myotis* and *Hipposideros* (Arelatez *et al.*, 1997; Barrat *et al.*, 1997; Kingston *et al.*, 2001). Geographical isolation, which exists between species, may have been one of the contributing reasons for the presence of these cryptic species (Hartl and Clark, 1989).

Within the recent years, rapid advancement of technology has brought human the ability to explore molecular level of organism. Integration of these molecular technologies within the population study has proven to give a different perspective in species identification and thus,

benefit the field of phylogenetics (Sugg *et al*., 1996; Hughes, 1998; Rossiter *et al*., 1999). Successful microsatellite primers were designed for *Cynopterus sphinx* of peninsular India by Storz (2000) in the population study of the species. Thus, microsatellite marker development for the cryptic *Cynopterus brachyotis* species, a closely related species of *C. sphinx*, is done in compliment to the ongoing effort of broader study to define species boundary.

There are two main objectives of this study. The first aim is to develop microsatellite marker for both large and small sized *C. brachyotis* and then to identify, select and sequence the microsatellite DNA clones.

CHAPTER 2

LITERATURE REVIEW

2.1 Chiroptera

Generally bats are divided into the suborder Megachiroptera (fruit bats) and Microchiroptera (insect eating bats) (Springer *et al.*, 1995; Alvarez *et al.*, 1999). Most microchiroptera feed on insects as their food. They are also known as echolocation bats since they depend on ultrasound as their navigator and sight in the total darkness. They will emit a high frequency of sound pulses through their oral or nasal cavity and detect things on their surroundings from the returning echoes (Feldhamer *et al.*, 1999). Microchiroptera consist of 16 families and approximately 759 species that are well distributed around the world. Compared to the Megachiroptera, these insect bats are smaller in size, as their name implies 'micro' (Salleh *et al.*, 1998). Many of the insect bats are heterothermic and some hibernate for a long period of time (Vaughan, 1986). Being heterothermic, these bats are able to occupy the only seasonally productive to adequate food area, which have been the main reason of the cosmopolitan distribution of this bat. Their low metabolism activity during hibernation has really aid them in their life longetivity. For example, *Myotis lucifugus*, a bat weighing only about 10 grams, is found to be able to live as long as 30 years (Keen and Hitchcock, 1980; Vaughan, 1986).

According to Myers (2001), fruit bats are found in the Old World tropics environment. Having only one family, the Pteropodidae, the fruit-bats are reported to consist of 977 modern species (Corbet and Hill, 1992). In Borneo, this family can be further divided into the

subfamily of Pteropodinae, the fruit eating bats, consisting 36 genera and 154 species, and subfamily Macroglossinae, which feed on pollen and nectars, consisting of six genera and 12 species (Payne *et al.*, 1985). Pteropodids are also known to have large eyes, which can see very well for navigation, but only the representative of the genus *Rousettus* are able to perform a primitive form of echolocation (Vaughan, 1986; Myers, 2001). Their body size range from medium to large (Salleh *et al.*, 1998; Feldhamer *et al.*, 1999). In terms of reproduction, fruit bats have low reproductive rates and commonly, the female bats will not breed until they are one or two years old (Thomas and Marshall, 1984). In Borneo, it is reported that there are about eight bat families. In Pteropodidae, there are 11 genera and 17 species (Payne *et al.*, 1985; Salleh *et al.*, 1998), which make up of 40% of the existing mammal on this island (Abdullah and Hall, 1997; Lim, 1998). In contrast to the insect bats, the fruit bats are not known to hibernate (Vaughan, 1986). This explains their limited area of distribution as they only occupy the tropic and subtropics area (Corbet and Hill, 1992).

In terms of diet, most bats are known to be insectivorous (feed on insect), but then there are also frugivorous (feed on fruits) or nectarivorous (feed on nectar) bats and carnivorous, which feed on small vertebrates, frogs, lizards, rodents, fish or even other bats. These bats build their roost on trees, foliage, tree hollows or holes, caves, ruins or even in mines, culverts and buildings (Corbet and Hill, 1992).

The species which will be studied, C. *brachyotis* is a fruit-bat; belong to the suborder Megachiroptera, family of Pteropodidae and the subfamily of Pteropodinae. This species is

reported to be widely distributed in mainland Southeast Asia, the Malay peninsula, Borneo and Sumatra, and in India and Sri Lanka (Campbell and Kunz, 2004; Corbet and Hill, 1992).

C. brachyotis is characterized by the ability to locate food by using their acute smelling sense. Physically, it has a fox-like face, large dark eyes, short brown hair and dark spotted wings (Feldhamer et al., 1999). According to Schultes (2003), an adult *C. brachyotis* have an average body weight of 30-100 g, body length of 70-127 mm and wingspan in the range of 305-457 mm. They feed mainly on small fruits, by sucking out the juices and soft pulps. They also consume nectar and pollen for nourishment (Payne *et al.*, 1985). Due to this, these fruit bats is typically found in the tropical rainforests, where it is warm and wet the whole years and food are easily available (Feldhamer *et al.*, 1999). They occur in most habitats in Borneo including the lower montane forest, dipterocarp forest and mangrove (Payne *et al.*, 1985). According to Corbet and Hill (1992), they roost preferably vary, including trees, foliage, tree hollows and holes, caves, rock shelters or other man-made structures such as mines and buildings. While some of these bats live in large colonies, there are others which prefer to live in smaller groups or solitary (Corbet and Hill, 1992).

C. brachyotis can be physically differentiated from other species of bats from their appearance. They usually are large in size, having dark orange collar in adult males and yellowish in females. As for the immature *C. brachyotis*, greyer fur with indistinct collar was observed. Their ear and wing bones edge are white in color and also posses 2 pairs of lower incisors (Payne *et al.*, 1985).

This species has an indirect economic importance for humans, in the seed dispersion and pollination of plants (Feldhamer *et al.*, 1999).

2.2 Cryptic *C. brachyotis*

Cryptic species indicates the presence of more than one species which are morphologically similar yet differ in terms of their genetic properties. In short, cryptic species are morphologically indistinguishable species. Such phenomenon occurs as the species are reproductively isolated (Knowlton, 1993; Perkins, 2000; Shaw, 2001). Both species have an independent population dynamic which can be explained by speciation processes (allopatric, parapatric or sympatric).

Allopatric speciation is the term used to indicate the formation of two or more species caused by geographical isolation such as rivers or mountains or any physical separation which results in the prevention of gene flow between populations (Mayr, 1963, 1970). A different species will arise due to the great geographical isolation of species. Parapatric speciation refers to formation of two or more descendant species in adjacent populations with gene flow (White, 1968). In other words, the species face a very strong environmental change and caused adjacent populations undergo the evolution of reproductive isolation. This especially affects those organisms with low vagility and exists in numerous small subpopulations (Hartl and Clark, 1989). It is noted by Hartl and Clark (1989) that low vagility (ability of organisms to disperse) and small subpopulations will promote random genetic drift, thus results in

genetic differentiation. As for sympatric speciation (Smith, 1966), it refers to the formation of two or more species from the same ancestral species within the same geographic location. This might occur due to temporal isolation (Hartl and Clark, 1989). For example, if the mating season or times for two species do not overlap, this might lead to the effective premating isolation. Thus, although they share the same geographic range, it is possible that different species might arise (Hartl and Clark, 1989).

Recently, Abdullah *et al.* (2001) has proven the presence of cryptic species within *C. brachyotis* of Borneo through the analysis of mitochondrial DNA (mtDNA) cytochrome *b* (cyto *b*) sequences. This is supported by the analysis of five external body measurements (forearm length, tibia length, ear length, tail length and weight) which indicate the Bornean *C. brachyotis* can be further divided into large and small, according to their body sizes.

The divergence was also said to be resulted from the factors of differential selection of their habitat gradient. According to Abdullah *et al.* (2001), *C. brachyotis* which occupies the open area is larger as this will provide a powerful flight against its predator, such as owl, whereby the one which live in the close forest assemble a smaller-sized body in order to adjust themselves for flight between the forested or dense habitat.

2.3 Molecular marker

According to McDonald (2000), molecular marker is an inheritable trait such as phenotypic traits, protein products and segments of DNA which can be applied as marker of genetic variation. These markers are found to be useful in determining diagnostic trait, in detecting heritable genetic disease and correlation between species as in molecular level. However, the absence of a suitable tool to identify these variable genetic traits which termed as polymorphism had been one of the main limitations for genetic study within organisms. Realizing this, several molecular techniques have been worked out to overcome this limitation (McDonald, 2000).

A few years before, restriction fragment length polymorphisms (RFLP) is one of the first widely used techniques in genetic analysis (Botstein *et al.*, 1980). Based on its capability on detecting variation at sequence level, it is well applied in parental and forensic analysis. Later, polymerase chain reaction (PCR) based methods were introduced. With the advantages on sensitivity of DNA quality and its reaction conditions, methods such as random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA amplification fingerprints (DAF) (Caetano-Anolles *et al.*, 1991) were developed. More efficient in giving the total product through PCR amplification, amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995) was then introduced.

According to Mueller and Wolfenbarger (1999), to explore and discover more of the genetic diversity, a technique which enables to fulfill the following criteria would be the ideal technique ever. A technique which is cheap and time efficient, enable to generate multiple and independent marker, provide researchers with adequate resolution of genetic differences, replicable, need no pre-information on the studied organism, utilize only a small amount of DNA samples, even the ancient ones and requires little molecular expertise are the suggested criteria. However, there is no such marker deriving technique yet to be discovered. Of the entire molecular biology techniques listed, RFLP, RAPD and AFLP are the three most popular markers utilizing technique exist nowadays. Summary for the comparison of these are listed in Table 1.

Table 1. Comparison of RFLP, RAPD and AFLP.

Criteria	RFLP	RAPD	AFLP
Degree of polymorphism	Moderate to high	Moderate to high	Moderate to high
Dominance	Codominant	Dominant	Dominant
Quantity of information	Low	High	High
Reproducibility	Very high	Low to medium	Medium to high
Ease of assay	Difficult	Easy	Moderate
Development time	Long	Short	Short

(Hillis *et al.*, 1996; Karp and Edwards, 1997; Mueller and Wolfenbarger, 1999; Glaubitz and Moran, 2000).

According to Glaubitz and Moran (2000), all these three molecular techniques have a moderate to high degree of polymorphism. RAPD and AFLP constitute a dominant nature which somehow have resulted a lower precision of population genetic statistic compared to RFLP, which shows codominant trait. However, this in turn has made both RAPD and AFLP have a high number of polymorphic loci availability which in other word provides high

quantity of information. On the basis of reproducibility, RAPD is the weakest of all as it is really sensitive in terms of DNA quality and reaction condition (Glaubitz and Moran, 2000). In the other hand, AFLP is moderately reproducible as RFLP is the most reproducible of all. In terms of technique development, RAPD is the easiest to construct and require a shorter development time. Although developing AFLP is also time efficient, it is reported to be moderately difficult to do the assay. As for RFLP, it is difficult to develop and consume time labour (Glaubitz and Moran, 2000; Mueller and Wolfenbarger, 1999).

2.4 Microsatellite genetic marker

In the recent years, another molecular application has been widely used to study the genetic structure of organisms; microsatellite marker. It is reported that this marker has been proven as a powerful marker and can be very useful in studying fine-scale population structures and tracing their evolutionary history genetically (O'Reilly and Wright, 1995; De Garcia Leon *et al.*, 1998).

Microsatellite marker is also known as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs). It consists of a simple sequence of nucleotides in tandem repeat of 2-6 basepairs. For example, dinucleotide sequence of $(CA)_n$, where n is number of repeats, is a very common repetitive motif found in human genome (n can range from 5 to 50)(Klug and Cummings, 2005). It can also be found in form of tri- and tetranucleotide tandem repeats. In this case it is quite rare, but is very useful in developing a highly polymorphic marker (Strachan and Read, 1999). These microsatellites are found to be naturally occurring due to

errors during slippage replication whereby the repeats can be either excised or added during the replication. This has made microsatellite tend to be hypervariable (McDonald, 2000).

After being discovered by Weber and May in 1989, microsatellite markers have been used numerously in marker development of organisms, especially in the field of livestock conservation and organisms phylogenetic studies (Vogel and Motulsky, 1997; Montaldo and Meza-Herrera, 1998; Ni *et al.*, 2002). According to Weber and Wong (1993), microsatellite has a high mutation rate of 10^{-2} to 10^{-3} per locus per gamete per generation. A high level of polymorphism, codominance of alleles, locus specific and applicable in fine scale studies are some of the reasons for preferable of utilizing this marker. Microsatellite would be the best marker available for analyses that require a highly differentiating stage such as analyzing parentage, DNA fingerprinting and cross verification (Glaubitz and Moran, 2000).

This marker can be derived by either of two methods which are the hybridization of DNA fragment with synthetic oligonucleotides (Rassman *et al.*, 1991) and by using polymerase chain reaction (PCR) (Jones *et al.*, 2000; Storz, 2000). Utilizing the first method would be more labor consuming with a lot of cloning to be done (Jarne and Lagoda, 1996). By applying the technology of PCR, developing this marker is found to be more efficient with high reproducibility. However, this marker is quite costly due to primer development and is labor intensive. Thus, a developed primer from a closely related species can be use to reduce the cost.

CHAPTER 4

METHODOLOGY

4.1 DNA extraction

Isolation of total genomic DNA was done using CTAB (cetyltrimethylammonium bromide) extraction method (Grewe *et al.*, 1993) with Proteinase K. Samples were taken from muscle tissue of 15 different *C. brachyotis* individuals preserved in ethanol in the Universiti Malaysia Sarawak Zoological museum.

About 0.1 to 0.2 g tissue sample was cut and minced into fine pieces using sterile scalpel blade. The minced tissue was then transferred into a 1.5 ml microcentrifuge tube containing 700 μl of 2X CTAB buffer. Five μl (20 μg/ml) of Proteinase K was then added into the microcentrifuge tube. Then the suspension was incubated at 55°C for one hour or longer until tissue is completely lysed. Next, a total of 700 μl of chloroform:isoamylalcohol (24:1) was added to the suspension and the tube containing the mixture was shaked for two minutes before centrifuged at 13,000 rpm for 10 minutes. The upper aqueous phase containing DNA was transferred into a new microcentrifuge tube. Absolute ethanol of the same volume was added. Next, the sample was centrifuged for 10 minutes at 13,000 rpm. The supernatant was then discarded. Six hundred microliter of 70% ethanol and 25 μl of 3M $NaCl_2$ were added to the tube and were shake for two minutes. Then, the mixtures were centrifuged at 13,000 rpm for 10 minutes. Next, supernatant was again discarded and the pellet was air-dried. The pellet was then redissolve in 30-50 μl ultrapure water, depending on the size of pellet and was

stored at -20°C until further use. Next, the samples were treated using RNase to lyse any RNA present in samples. For 50 µl of DNA, two µl RNase was added, as for 100 µl of DNA, five µl RNase was added. Before adding the RNase, the sample must be in liquid form. The mixture of DNA sample and RNase were then let in room temperature for about 15-30 minutes. The quality and approximate yield was determined by electrophoresis of five µl of genomic DNA mixed up with one µl of 6X loading dye with one kb DNA ladder (Gene Ruler ™, Fermentas) on 1% agarose gel electrophoresis. One µl of ethidium bromide (EtBr) was used for staining. The electrophoresis was run at 90 V for 45 minutes and visualized by using UV transilluminator. List of samples which have been extracted are listed on Table 2.

Table 2. Samples of large-sized and small-sized *C. brachyotis* which have been extracted.

No.	Label	Collection No.	Size	Habitat	Forearm Length (mm)	Locality
1	L1	BA011	L	Open	62.29	Batang Ai National Park
2	L2	BA024	L	Open	62.21	Batang Ai National Park
3	L3	KNP029	L	Fringe	60.46	Kubah National Park
4	L4	KNP047	L	Fringe	60.17	Kubah National Park
5	L5	KNP050	L	Fringe	60.87	Kubah National Park
6	L6	KNP051	L	Fringe	62.24	Kubah National Park
7	L7	KNP030	L	Open	60.69	Kubah National Park
8	L8	BA005	L	Open	60.98	Batang Ai National Park
9	L9	BA009	L	Open	62.78	Batang Ai National Park
10	L10	KNP022	L	Open	60.75	Kubah National Park
11	L11	KNP044	L	Fringe	62.97	Kubah National Park
12	S1	KNP045	S	Fringe	59.90	Kubah National Park
13	S2	BA010	S	Close	56.21	Batang Ai National Park
14	S3	BA026	S	Close	54.90	Batang Ai National Park
15	S4	KNP064	S	Close	59.61	Kubah National Park
16	S5	KNP042	S	Fringe	58.77	Kubah National Park
17	S6	BA008	S	Close	56.68	Batang Ai National Park
18	S7	KNP069	S	Close	59.73	Kubah National Park

L= large; S= small

4.2 PCR amplification

The primers that were used in this method is designed by First Base Company; $(AAC)_8$, $(ATC)_8$, $(AC)_{12}$, $(AAT)_{12}$, $(ACT)_8$, $(AG)_{12}$, $(CCG)_8$, $(AGG)_8$, $(AAG)_8$. With total reaction of 25 µl, Polymerase Chain Reaction (PCR) was done using 3.5 µl of 10X buffer, 1.25 µl primer, 1.5 µl magnesium chloride, 0.5 µl oligonucleotide consisting of dATP, dGTP, dCTP and dTTP, 17.05 µl of distilled water, 0.2 µl of *Taq polymerase* and 1 µl of DNA per tube. The reaction mixtures used were summarized in Table 3.

Table 3. PCR reaction mixtures used

Mastermix	**1x (µl)**
10x PCR buffer (Fermentas)	3.50
25 pmol/µl Primer	1.25
25 mM $MgCl_2$ (Fermentas)	1.50
10 mM dNTP (Fermentas)	0.50
Steriled distilled water	17.05
Taq Polymerase (Fermentas)	0.20
Template DNA	1.00
Total	**25 µl**

The initial denaturation was done at 94°C for 2 minutes. Thirty cycles was applied, with each step was done according to the following parameters: denaturation at 94°C for 1 minute, annealing at 40-70°C, varies according to primer used for 1 minute and extension at 72°C for 2 minutes. Amplification product was resolved in a 1.5% agarose gel electrophoresis and this percentage may increase depending on the size and the sharpness of PCR product. Visualization was done using ethidium bromide (EtBr) staining (Storz, 2000).

Table 4. Temperatures used in PCR

Step	Temperature (°C)	Time (min)
Pre-denaturation	94	2
Denaturation	94	1
Annealing	40-70	1
Extension	72	2
Final Extension	72	5

4.3 Cloning

Ligation of DNA and plasmid vector and transformations of plasmid with XL-1 Blue (Stratagene) was done using pGEM®-T Easy Vector Systems kit (Promega).

4.4 Plasmid Isolation

Lysis method was used for isolation of plasmid from bacterial culture (Sambrook *et al.*, 1989). The bacterial cells containing the plasmid to be isolated were harvested in Luria broth with ampicillin overnight at 37°C with shaking 250 rpm. Two ml of the harvested bacterial cells were transferred into a two ml centrifuge tube and was centrifuged at 8000 rpm for two minutes. The supernatant which was the culture media was carefully removed and the pellet was recentrifuged for one minute. Any traces of liquid media were removed completely. The cell pellet was resuspend using 100 µl solution I (50 mM of 40% of sterile glucose, 10 mM of 0.5M EDTA pH 8.0, 25 mM of 1M Tris HCl pH 8.0, sterile ddH_2O) and was vortexed briefly for 10 seconds. The tube was then kept on ice. Another 100 µl of solution II (0.2 N of 2N NaOH, 1% of 10% SDS, sterile ddH_2O) were added to the cell suspension and was mixed