CHAPTER 2 – Research Materials and Methods

2.1 Introduction:

This chapter shall present the materials and methods used by the researcher in the course of achieving the research objectives, specifically, biotope mapping of the study areas, studying ranging patterns, and finally, evaluating genetic relationships.

2.2 Objective 1:

2.2.1 Material and Methods for Biotope Map of the Study Areas

The materials and software that were utilized in this study are listed in Table 2.1 below. Mapping and assignment of each study area was undertaken using both direct observations and digitizing of a project area satellite image. These Biotopes showed the extent of the area, major and key features in and around the site.

Table 2.1: Materials and Software used to undertake Objective 1

Materials	Software
GPS (Garming 12 XL)	Cartalinx 1.2 Version 1.2
Extended and amplified antenna	
Laptop	ArcView GIS software
Map of the Area obtained in JUPEM	
Satellite SPOT-5 Pansharp Supermode PNC 2007. Image Projected of the area obtained from (Department of Remote Sensing and Satellite imagery Peninsular Malaysia)	FGIS Software
	Google Earth Software
Digital Lot map of obtained from Federal Land Development Authority (FELDA)	

The methodology consisted of collecting point and line data of desired features using the GPS (Garmin 12XL) and integrating these into a satellite image and Digital Lot Map using ArcView GIS 3.2 Software (Figure 2.1 A and B). While in the field, point and line data was collected using the GPS instrument. Point data was used to identify tagged trees, flagging of the trail network (identifying changes in the trail's direction) borders of clear-cut land and so on.

Line data was used to collect information on features that had an unobstructed view of sky, such as roads and swamp fresh water rubber plantation etc. All points were averaged to an accuracy of 3 to 5 meters; the majority of points were collected while the GPS unit was in 3-D data collection mode.

Data collection was imported into the GIS program and integrated into a developing comprehensive and informative geospatial databases for the various features of the map, so that future analysis and management of the features, and their assorted attributes, could be easily conducted.

With the development of the field site map in a GIS program the information was able to be used for more that mere display purposes. Information collected and the satellite image were imported into Cartalinx 1.2 software in order to be digitized and to build map polygons for each Biotope. Consequently, each polygon (Biotope) was named and colored according to its geographical and physical characteristic.

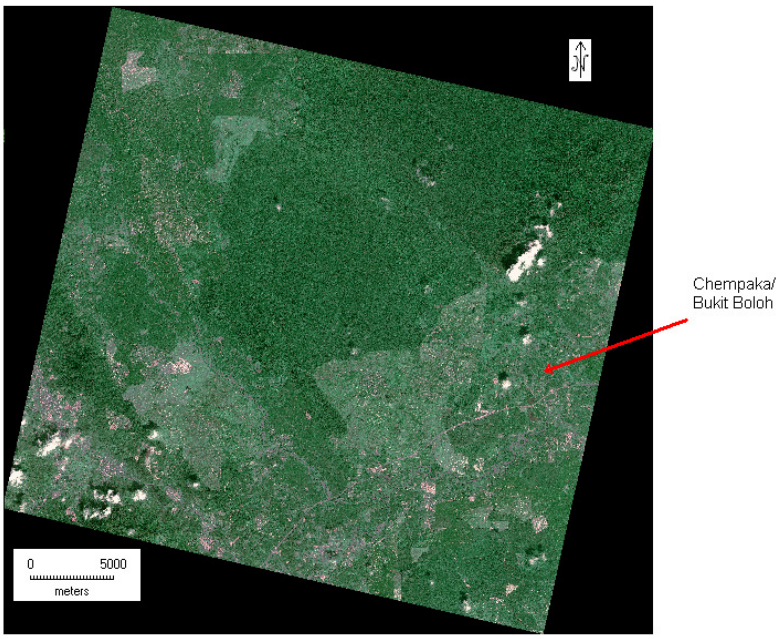


Figure 2.1A Satellite SPOT5 Pansharp Supermode PNC 2007 (Lat/Lon, WGS84)

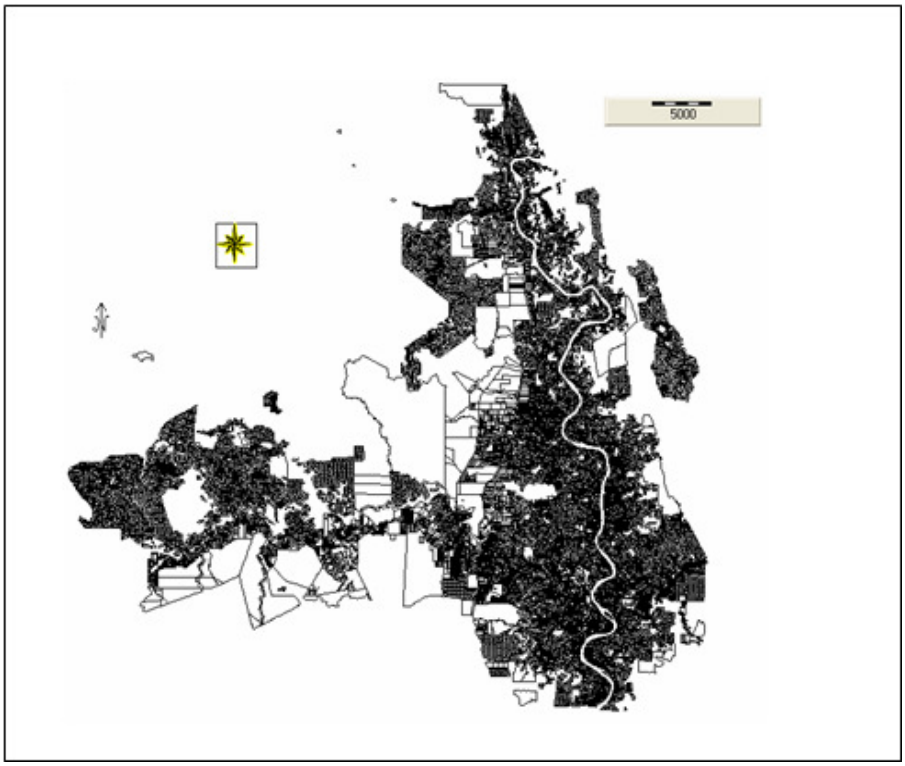


Figure 2.1B Digital Lot Map of Temerloh District, Pahang State

2.3 Objective 2

2.3.1 Material and Methods for Studying the Ranging Patterns of *Nycticebus c. coucang*.

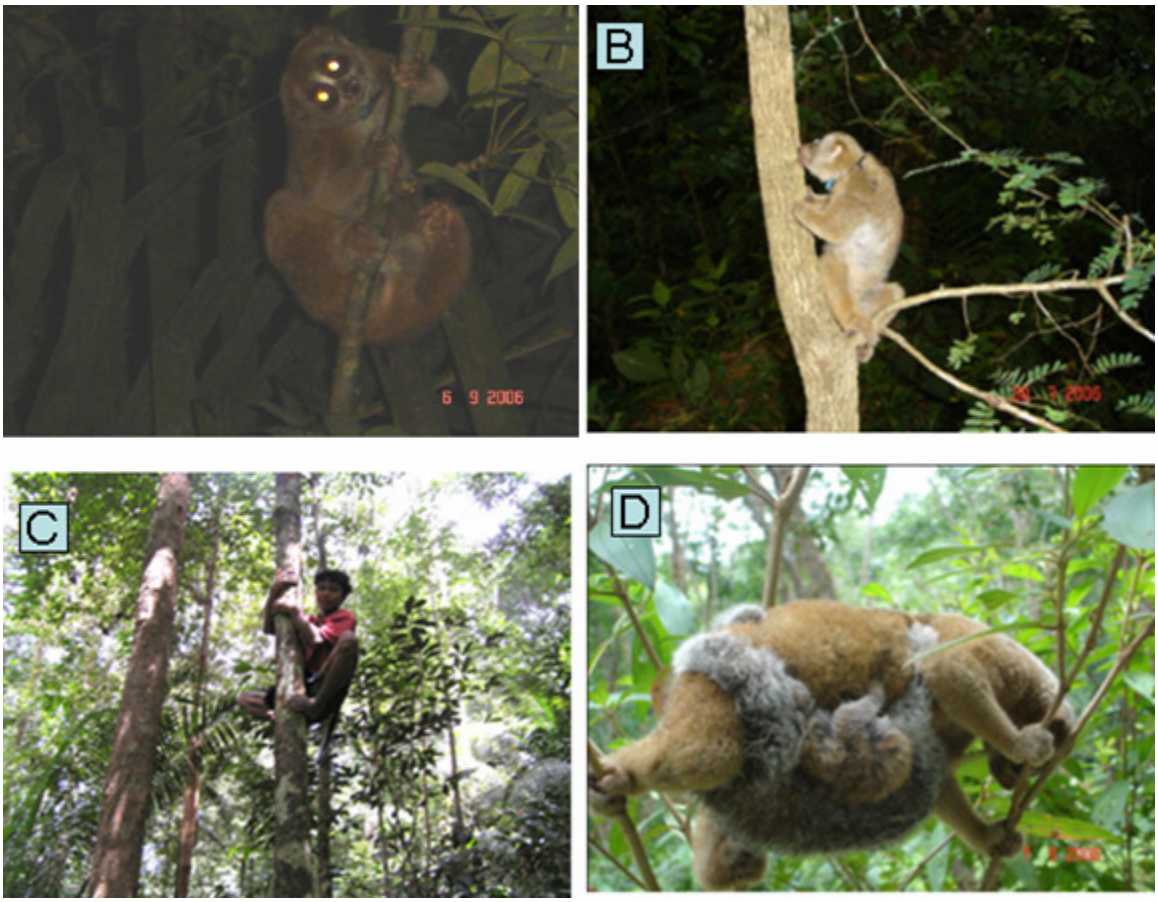
2.3.2 Capture, Handling, and Radio Collars

Selection of areas to trap was based upon local knowledge of where habitat and terrain factors suggested it was most likely to capture the *Nycticebus c. coucang*. Animals were sighted by waving with 6 volts torch-light in the darkness and with the assistance of the local villagers who were informed of this study and who assisted in capturing the animal by hand.

Table 2.2 Technology and Items used for Radio- Tracking

Materials	Software
GPS (Garmin 12 XL)	ArcView GIS software
Extended and amplified antenna	
Laptop	Diva GIS
Map of the Area published by JUPEM (Department of Geography, Univ Malaya)	
Satellite Image Projected of the area obtained from the Department of Remote Sensing and Satellite Imagery, Malaysia)	FGIS Software
TR-4 Telonics receiver	Google Earth Software
Gloves	Animal Movement Analyst Extension (AMAE) version 1.0
Munsell Color Table	
Syringe	
Tiletamine/Zolazepam	
Data Sheet	
RA-2AK"H" antenna Yagi	
headphones	
cable-tie from Biotrack Ltd	
ATN (Night Vision)	
Spring Balance 1kg	
Sliding Caliper	
Headlamp/ 6v Torch-light	
Rechargeable Batteries	

Once the distinctive golden-yellow eye shine of the *Nycticebus c. coucang* was detected, some during day time observation and the rest by night-vision which enabled the researcher to confirm presence, then the individuals were captured by expert tree climbers from the local community using a pole net, or physically by hand (see Photo 2.1 A,B,C,D below). Individuals were then anesthetized by injection of *Zoletil* 50 [Tiletamine/Zolazepam] (10-17 mg/ kg body mass).



(Photo 2.1 A & B) showing one *Nycticebus c. coucang* golden-yellow eye reflection detected by the use of torching white light. C) Expert tree climbers from the local community. D) Adult Female Bonita with her born infant; pictures taken in the field by Jaime Castillo G.

The immobilized individual was then given a complete physical examination, and body measurements were taken, and sex and age class was recorded onto a Data-Sheet (see Appendix 1). Classification was as follows: Adult (1) (Infant) <350g first permanent teeth, long hairs with white tips 2) (Juvenile) a few permanent teeth erupt (Juvenile) II all later phases of dental eruption 4) (Subadult) >350g teeth white and unworn, may already be sexually mature, nipples short 5) (Adult) >500g short fur without white tips, elongated nipples or signs of pregnancy with complete permanent dentition physically mature, sexually mature (Wiens, 2002).

Within the whole study period, a total of 12 individuals were selected for tracking purposes and fitted with VHF radio tag cable-tie (Biotrack Ltd) (refer to Table 2.3. for Radio-tag specifications). Individuals 1 and 2 were tracked in the locality of Kampung Bukit Boloh and 3 to 12 in Kampung Cempaka (refer to Table 2.4 and Photo 2.2). Each individual was kept in-house for observations during a 4 to 10 day period, and later on released at the same site where it was captured, the sole exception being of one infant, collected from another area due to the high logging activity in that area, which was then released with an adult female (who was not its mother and which had adopted it).

Authorization for collecting and the sampling of specimens and procedures were carried out with permission of the provincial and national authorities of the Malaysian Wildlife Department (PERHILITAN). Recommendations of animal care and handling procedures by the American Society of Mammalogists were strictly followed.

Table 2.3 Radio-Transmitter Unit used with its Programmed Channel and Frequencies

TR-4 Channels (CH)	**Biotrack Tag Frequencies (MHz) Type (Collar cable-tie)**	**Specifications**
27	165.079	
28	165.104	Silver cell battery (Ag357)
29	165.157	Tag Range (300 m)
31	165.233	Life-Span (6 to 7 months)
33	165.318	Aprox 3/5 g
34	165.360	Length/mm 22
37	165.474	Width/mm 13
39	165.559	Height/mm 9
41	165.649	
43	165.721	

Table 2.4 Radio-Tracking Data for 12 *Nycticebus c. coucang* in two Localities in Pahang, Temerloh District within the Buffer of the KWR (Krau Wildlife Reserve).

	Name (given by this researcher)	**Sex**	**Age**	**Body weight before collaring (gm)**	**Body weight after Removing Collar (gm)**	**Tracking Period (dd/mm/yy)**	**Number of GPS Readings Taken**
1	Linda (LI)	F	Adult	700	750	8/06-27/11/06	316
2	Adopted (ADA)	M	Infant	280	-	8/06-14/07/06	126
3	Aggressive (AG)	M	Adult	750	750	6/07/06-18/01/07	257
4	Hermosa (HE)	F	Sub adult	500	600	6/07/06-16/01/07	250
5	Timida (TIM)	F	Adult	750	750	1/08/06-18/01/07	215
6	Little Aggressive (LIAG)	M	Infant	320	-	1/11-29/11/06	76
7	Bonita (BO)	F	Adult	740	750	17/09/06-16/01/07	213
8	Cop (CO)	M	Adult	750	650	9/11/06-16/01/07	71
9	ECA	M	Adult	750	750	6/11/06-18/01/07	73
10	KRO	M	Adult	650	600	26/11/06-15/01/07	39
11	Gent (GE)	M	Sub adult	500	-	10/11/06-6/01/07	72
12	Fala (FA)	F	Adult	900	900	12/12/06-18/01/07	40

Photo 2.2 Showing an individual tagged for Tracking Purposes
[Photo taken by Jaime Castillo G.]

2.3.3 Finding Animals with Radio Tracking

The samples of *Nycticebus c. coucang* were manually tracked, mainly on foot. Animal tracking using radiotelemetry began the 8th of June 2006, immediately after fitting the two first individuals with a radio collar.

Nycticebus c. coucang were radio-tracked each night from dusk until dawn. A Telonics receiver (model TR-4; frequency: 165.079 -165.721MHz), a three-element Yagi antenna (See Photo 2.3 A and B), headphones and a hand-held GPS (Garmin 12 XL) were used for radio tracking.

Photo 2.3A TR-4 Telonics Receiver

Photo 2.3B RA-2AK"H" Antenna Yagi

Locations of marked individuals were made by homing in on the signal and by multiple triangulations when the tracker was close to the animals. This was followed by a direct exact visualization of the animal in order to take the GPS-reading. At each radio-location, a positioning measurement was recorded as the latitude and longitude using the GPS. The waypoints were considered sufficiently accurate within a range of ± 3 /4 m. If the satellite signal was not picked up in the immediate vicinity of the individual (s) due to high trees or weather etc., the GPS was situated in a more open location nearby to obtain the GPS reading. It was not recorded as waypoints unless the estimated positional error displayed on the GPS unit was < 4/5 m. When the animal was visually spotted, a distance

between 10 to 15 meters away from the animal was considered. This was done in order to avoid any behavioral disturbances towards the animal. The individuals were monitored from June-2006 to Jan-2007, taking a minimum of 4 to 5 waypoints of each animal during observation nights. The interval between consecutive radio-tracking days was irregular. The distance of approximately 5 km between the two study areas was covered by car in order to be able to track all the individuals in a single night.

Each day the radio was used immediately upon entering the individual's area. The search began unassisted, stopping frequently to look, listen, and use the radio considering the possible home range of the animal and the limited range of the radio signals (from 100-300 + m, depending on vegetation terrain, and weather). Throughout the study, radio tracking was used in conjunction with knowledge of movement patterns, core areas, and feeding trees. The search was always initiated on the sleeping site where the animal was left after dawn. It was possible to use the radio to locate and follow individuals under almost all conditions, including rain.

2.3.4 Finding Animals before Radio Failure

Once an individual was contacted, it was followed continuously for as long as possible. Nevertheless, it was not uncommon to lose the individual due to poor visibility. In the final month of the study, the signals of radio transmitters worn by the individuals began to weaken, and we started losing the signal, forcing the tracker to stop, recapture and remove collars.

2.3.5 Mapping Techniques: Definitions and Analysis

Two Biotope maps of each study area were developed using a geo-referenced Satellite image (UTM coordinates, WGS 1984 datum) with known ground locations (see Objective-1). The image was obtained from the Malaysian Remote Sensing Agency (Taken by the Satellite SPOT-5 Pansharp Supermode PNC 2007 and provided in GeoTIFF format). Each set of geolocations for individual *Nycticebus c. coucang* that were tracked was plotted on digital sitemaps and Biotopes maps of the study area with the aid of the ArcView Geographic Information System (GIS) program. Movement patterns were examined using Animal Movement Analyst Extension for the Arcview® GIS 3.2 software (AMAE version 1.0 developed by Environmental Systems Research Institute, Inc., Redlands, California, U.S.A. Hooge, 1999) (Hooge & Eichenlaub, 1999).

To evaluate spatial overlap of *Nycticebus c. coucang*, the points collected throughout the study period to delineate home range and core areas for each individual were used. Home range has been defined as the area traversed by the individual in its normal activities (Burt, 1943). Home range can be defined as the smallest sub-region which accounts for a specified proportion of its total utilization; a range thus defined will be that habitually used (Jenrich, 1969; Seaman, 1996).

Non-parametric methods were chosen to calculate the home-range size and the centre activity of the animal; the fixed kernel method known as Kernel Density Estimation (Worton, 1989) and the Minimum Convex Polygon (MCP %) (Mohr, 1947) because a combination of polygon and contouring methods is often recommended to account for the potential limitations and shortcomings of each of these techniques (Kernohan, 2001).

Probability Kernels are regions around each point location containing some likelihood that the individual is present; the width of the kernel is based on a smoothing parameter (Kernohan, 2001; Mitchell 2006). Home-Range data for each *Nycticebus c. coucang* were primarily analyzed using Kernel Density Estimation (KDE), a method that regarded as reliable and robust (Börger, 2006), and less prone to overestimation of home-range size than other methods and with increased spatial resolution (Wray, 1992; Cresswell & Rogers, 1992; Worton,1989; Seaman,1996; Hooge, 2000).

Kernel Home Range highlights areas used most frequently by animals and thus provides higher resolution information on the use of habitat and space than convex polygon based techniques. The adaptive Kernel Method was used since it is not sensitive to sample size and less sensitive to user-set values than the most widely used harmonic mean estimator (White, 1990; Kie, 1984). The Kernel Method is highly flexible and able to fit a non-convex, multimodal irregularly shaped distribution it can produce density estimate directly and; it is not influenced by grid size or placement (Seaman, 1996; Silverman, 1986; Worton, 1987; Seaman 1998).

Computed Kernel Analysis of home range estimates are presented as the smallest area that incorporates a set percentage most commonly 95% or 50% or both of the utilization distribution of the area (Kernohan, 2001). Animal positions within the 95% contour represent “the actual are a used by the animal” and those within the 50% contour represent “the core area of activity” (Hooge, 1999). The width of the kernel, called the ‘smoothing parameter (H)’, determines the influence that more distant points have on the density estimate at each location. Wide kernels produce a model which emphasizes the general shape of the distribution. Narrow kernels produce a model which emphasizes structure (Seaman, 1996; Kenward 1987).

During analysis, the smoothing parameter is applied in one of three ways: *Fixed* (smoothing is the same for all areas), *Adaptive* (more smoothing for low density areas, less for high density areas), or *Inverse Adaptive* (Seaman, 1996; Kenward, 1987). The value of the smoothing parameter (H) can be determined objectively by the 'Least-squares Cross-Validation' method (LSCV) (Silverman, 1986; Worton, 1989).

Least-squares Cross-Validation (LSCV) is interpreted as a jackknife method which uses an interactive approach in selecting the smoothing width, and minimizes the estimated error for a given sample (Silverman, 1986; Seaman, 1998). The Animal Movement Analyst extension (AMAE) for ArcView ® GIS 3.2 automatically calculates the LSCV for the smoothing parameter (H). Hooge (1997) found this method to provide a less biased Kernel estimator. Because the choice of the smoothing parameter (*H*) is the most crucial variable influencing estimates of kernel density, it is critical to report the smoothing parameter chosen for a fixed Kernel Analysis (Kernohan , 2001).

For this study, Kernel Home Range was set to yield 50 to 95 percent Minimum Area Probabilities (MAP) contours, respectively, as indications of animal and groups core area and home range boundaries (Figure 2.2). The AMAE program was allowed to choose the most appropriate smoothing factor for the data of *Nycticebus c. coucang* using Least-squares Cross-validation (LCSV). The calculation of territory or home range requires that the locations for each individual show fidelity to a given area over the course of the sampling period (Seaman, 1999). The evaluation of site fidelity was done visually inspecting each individual *Nycticebus c. coucang* set of locations for overlap among its sessions. Furthermore, it evaluated the absence of a move or shift, where individuals did not abandon one area for an area elsewhere (Wiens, 1969).

Additionally, Minimum Convex Polygons (MCP %) were used to describe the maximum area covered by *Nycticebus c. coucang*. A Minimum Convex Polygon (MCP %) is still the most frequently used technique. The range is constructed by drawing a line around the outermost locations recorded for an animal.

This limits its utility, since range size is strongly influenced by peripheral fixes, and the nominal range area may include large areas which are never visited (White, 1990). Although this method is not generally recommended to estimate home range because it does not assess probability of occurrence, it is useful to visually assess the potential area visited by each *Nycticebus c. coucang*. Seaman et al. suggested the usage of a minimum of 30 location points, preferably 50 or more, for kernel analysis of home range. In this study we attempted to record 50 points for each individual.

The overlap in MCP areas was calculated using Animal Movement Extension for ArcView (AME) in conjunction with the XTools application extension for ArcView (Delaune, 2003).

To determine habitat selection and line corridor within the habitat used by this species, the researcher used the Biotope-map which he developed (see Objective 1). *Nycticebus c. coucang* locations were plotted in the Forestry GIS (fGIS) software (2005 University of Wisconsin) and each location was assigned a habitat type; generating ‘observed' habitat types used by each *Nycticebus c. coucang* individual. Line corridors (movement paths) (per individual-night were examined to determine routes followed by each *Nycticebus c. coucang* on a given night. Nightly path lengths (or night ranges) were calculated by summing all distances between consecutive locations, which were obtained during overnight tracking as well as measuring the distances between sleeping sites.

For the identification of repeatedly used pathways, location records were entered into ArcView GIS 3.2, and successive records for the same focal animal joined to create daily paths or partial daily paths for less than full-day samples. Trajectories that suggested a mapping error in the field were omitted.

Initial determination of repeatedly used routes was carried out by overlaying all recorded daily paths and then identifying, by visual inspection, all paths that showed to have been used by an individual *Nycticebus c. coucang* multiple times. These initial routes were sketched and digitized and were then confirmed by superimposing, one at a time, the set of individual paths recorded in each dataset.

The result of this process was a method of overlapping and intersecting travel paths, resembling a system of connections similarly to a network. A route was permanently included in the map when sections of the paths followed by the same individual on at least two different days were clearly concordant with the proposed route. Consequently, crossing-structures used by the *Nycticebus c. coucang* within its home-range as passage through different patches of forest were identified, digitized and analyzed using field data, GPS tracking data, line corridors and a Biotope map.

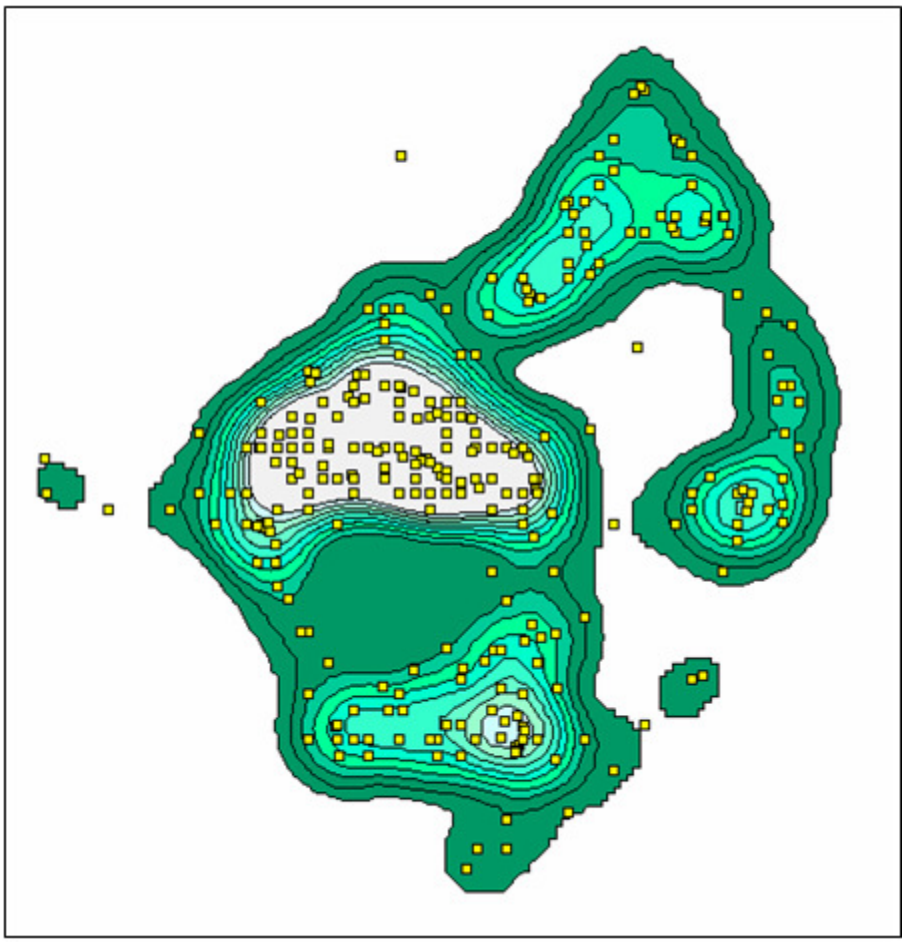


Figure 2.2 Example of spatial utilization distribution for an individual *Nycticebus c. coucang (*Adult female Linda) tracked in Bukit Boloh area, generated from a Fixed Kernel Density Estimator. Dots in yellow represent the location collected for this individual during the sampling period. The outermost line represents the 95th density isopleths (values that describe the likelihood of encountering this individual in a particular area) and territory boundary. The grey area represents the core area, which resolved at the 50th density isopleth for this particular individual.

2.4 Objective 3.

2.4.1 Material and Methods for Evaluation of the Paternal Genetic Inheritance Using Cross-species Microsatellite Markers.

2.4.2 Chemicals / Reagents

2.4.2.1 DNA Extraction

Table 2.5: Materials for DNA Extraction

Chemicals	Sources
"FTA Cards" with blood samples stored	Watman S.A
Chelex®	Bio-Rad Laboratories.
Sterilize water	---

2.4.2.2 Amplification –Polymerase Chain Reaction

Table 2.6: Materials for Cross-Species Microsatellites Amplification

Materials	Sources
Taq DNA polymerase	Biotools
(12 Primers Pair) 45HDZ016,45HDZ052,1110,1115,1118,157,311,312,113 3328, Mm22,WOOL682,	Bio Basic Inc

2.4.2.3 Agarose Gel Electrophoresis

Table 2.7: Materials for Agarose Gel Electrophoresis

Chemicals	Sources
Seakem ® LE agarose	Cambrex Bio Science
ethidium bromide (10mg)	Promega
6X loading solution	Fermentas Life Sciences
GeneRuler™ 100 bp DNA ladder	Fermentas Life Sciences
TRIS Base	J.T Baker Inc.
EDTA	Bio-Rad Laboratories Inc.
Boric Acid	Bio-Rad Laboratories Inc.

2.4.2.4 Polyacrylamide Gel Electrophoresis

Table 2.8: Materials for Polyacrylamide Gel Electrophoresis

Chemicals	Sources
Plus one Bind Saline	Amersham Pharmacia Biotech
Plus one Repel Silane – dimethyldichlorosilane solution 2% in octamethyl-cyclotetrasiloxane.	Amersham Pharmacia Biotech
TEMED	Bio-Rad Laboratories Inc.
Ammonium persulphate	Promega
Absolute ethanol	Hamburg
30% (19:1) acrylamide:bisacrylamide	Bio-Rad Laboratories Inc.

Urea	Univar
TRIS Base	J.T Baker Inc.
EDTA	Bio-Rad Laboratories Inc.
Boric Acid	Bio-Rad Laboratories Inc.

2.4.2.5 Silver Staining

Table 2.9: Materials for Silver Staining

Chemicals/Materials	Sources
Silver nitrate	Sigma
37% formaldehyde in methanol	Sigma
Sodium thiosulfate	Sigma
Glacial acetic acid	Ajax Chemicals
Sodium carbonate anhydrous	ICN Biomedicals Inc.
0.2 micron sterile syringe filter	Nalgene® Sartorius

2.4.3 Instruments

2.4.3.1 DNA Extraction

Table 2.10: Instruments for DNA Extraction

Instruments	Sources
Centrifuge	Kubota 2420, Japan
Biofuge fresco - refrigerated microcentrifuge	Heraeus, Germany
Water bath	Memmert, Germany
UV/Vis biophotometer	Eppendorf AG, Germany

2.4.3.2 Microsatellites Amplification –Polymerase Chain Reaction

Table 2.11: Instruments for Cross - Amplification

Instruments	Sources
PTC200 Thermo Cycler (BC-MJPC200)	Applied Biosystem
Mini centrifuge, 220V	Cole Palmer

2.4.3.3 Agarose Gel Electrophoresis

Table 2.12: Instruments for Agarose Gel Electrophoresis

Instruments	Sources
Gel imaging system – Alpha DigiDoc RT	Alpha Innotech
MJ-105 MINI Horizontal Gel Electrophoresis System	Medigene
MIDI 250 Volt Power Supply	Medigene

2.4.3.4 Polyacrylamide Gel Electrophoresis

Table 2.13: Instruments for Polyacrylamide Gel Electrophoresis

Instruments	Sources
Vertical DNA sequencing unit TVS1000 - Plain glass plates TVS1000-PG - Notched glass plates TVS1000-NG - Set 0.35mm Melinex Spacers - Comb, 0.35mm, 36 sample lanes	Scie-Plas, England
Power supply unit – 3000V, 150 mAmp	Consort
Peltier Thermal Cycler – 200 DNA Engine	MJ Research, USA

2.4.3.5 Silver Staining

Table 2.14: Instruments for Silver Staining

Instruments	Sources
Orbital shaker (Innova 2000)	New Brunswick Scientific
Magnetic hotplate stirrer	Harmony LMS, Japan
White light box	-
Perspex staining tubs (8cm X 24cm X 54cm)	-
Lab water purifier	Sartorius
Desktop scanner	*hp* officejet 6110

2.5 Study Subjects

2.5.1 Sample Collection

Blood was obtained from 26 individuals from five localities in Peninsular Malaysia (Table 3.15) and (Figure 2.3). Animals were anaesthetized using tiletamine/zolazepam (10-17 mg/ kg body mass). Less than one-milliliter of blood was obtained from limb vessels; and dropped onto FTA Classic Card for record keeping.

FTA (Watman FTA Cards) cards were used following protocol to preserve blood samples collected in the field. Authorizations for the sampling of specimens were obtained from the provincial and national wildlife authorities (PERHILITAN).

Animal handling procedures were performed according to the American Society of Mammalogists recommendations. The trapping and sampling of Slow Loris was carried out between 2006 and 2007. Individuals were trapped by hand by expert tree climbers from the local community.

Table 2.15 Collection Sites and Sample Sizes of *Nycticebus c. coucang* by Locality

No. Samples	**Locality**
12	Kampung Chempaka (Pahang)
4	Kampung Bukit Boloh (Pahang)
6	Zoo Melaka Malaysia
3	SerembanPerhilitan Office
1	Kampung Pasu (Pahang)

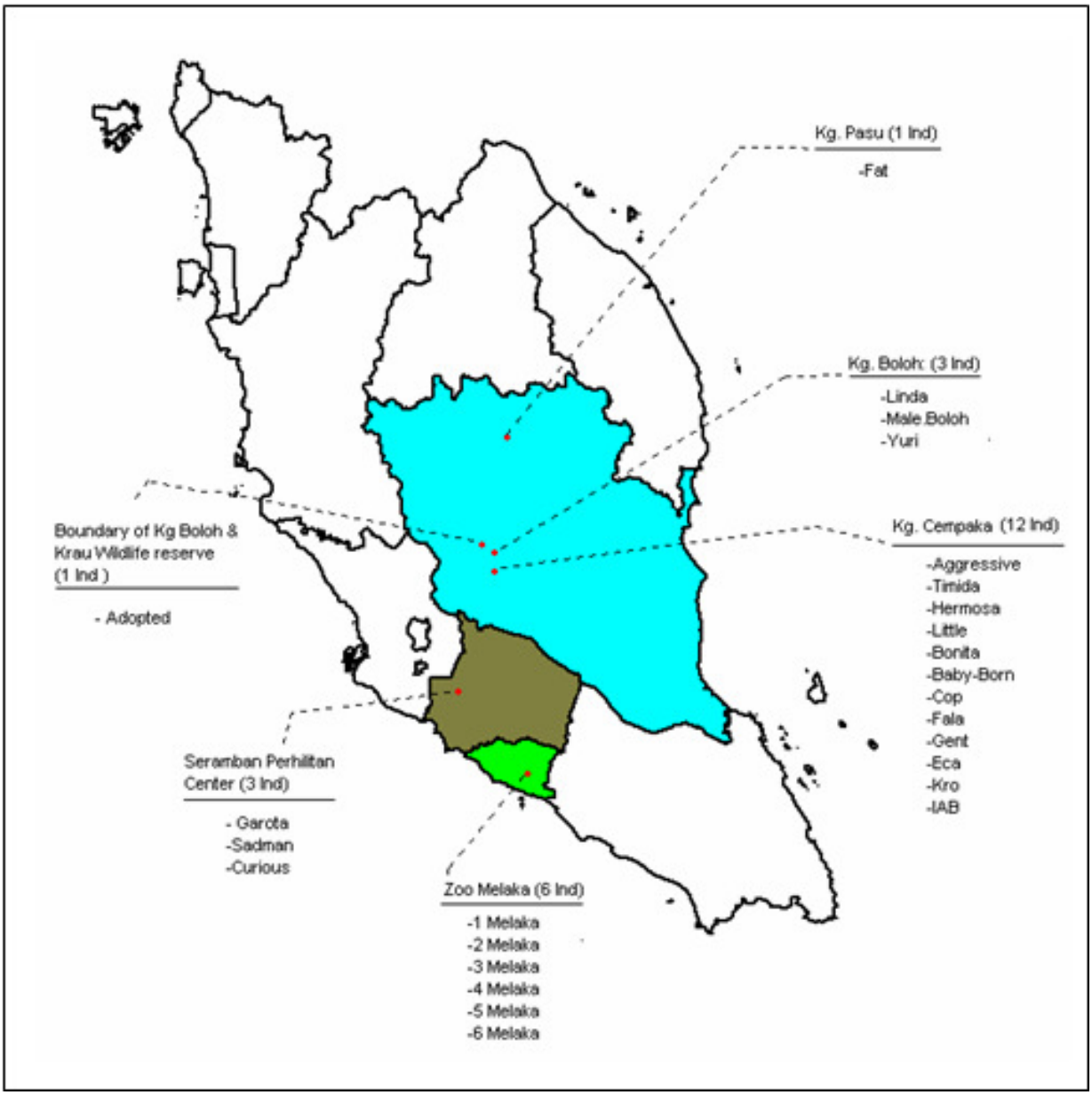


Figure 2.3 Map of Peninsular Malaysia showing Sampling Site States in Color, and the number of individuals sampled and the names given to each Slow Loris to facilitate the study.

2.6.2 Methods

2.6.2.1 Extraction of Genomic DNA

2.6.2.2 Chelex ® Extraction (FTA Cards -Blood DNA Samples).

Filter paper discs (7mm) were cut out from the FTA cards (Whatman S.A) using a sterile paper punch. Those discs were punched directly into 1 ml of sterile millipore water, and left at room temperature for 30 minutes. Samples were then centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and 200 µL 5% and Chelex was added. The tubes were incubated at 56°C for 35 minutes. After being vortexed at high speed for 5-10 seconds, the samples were heated at 99 degrees Celsius for 8 minutes to elute the DNA.

Consequently, samples were vortexed for 10,000 rpm for 2 minutes, the upper layer was discarded and the remainder of the supernatans was kept (70 µL) which kept later and were transferred into new tubes ready to be used for PCR reactions (Walsh, 1991).

2.6.2.3 Measurement of DNA Concentration and Purity

All extracted DNA samples were subjected to a purity check and quantization prior to subsequent testing using an automated spectrophotometer (Eppendorf AG, Germany) and quartz cuvette with reference set to sterile double distilled water in 20X dilution of the DNA (5 µl DNA + 95 µl sdH2O). Purity and concentration was estimated from the double stranded DNA optical density ratio (OD_{260}/OD_{280}). The range of DNA purity obtained was 1.6 – 1.9.

2.6.2.4 Polymerase Chain Reaction (PCR) Set Up for Microsatellites Genotyping

A panel of 12 cross-species microsatellites isolated from various species of primates was used to amplify the corresponding and poliphormic loci in the study species *Nycticebus c. coucang* (Table 2.16). Microsatellites markers were amplified using two different amplification programs. PCR reagents were commercially obtained, and PCR amplifications were performed in an automated MJ Research PTC200 Thermo Cycler (BC-MJPC200).

Table 2.16: Sequences and Amplicon Sizes for those Isolated Microsatellites (Huang, 1995; Masibay, 2000)

Primate Species	Locus	Primer Sequence (5'-3')	Repeat Motif	Annealing Temp.	GenBank Accession no.
Woolly Lemur	WOOL682	F: CGAGTGGAGTAACGACCCC R: GGATTTTGGTGAGAGAACG	(GT)14CT(GT)6CT(GT)3	50	AY515535
Cheirogalous medius	33228	F:CCTGCAGCAAAACCACATC R:ATTCCTTCTTCATATCTGGAC	(TC) 6 (TC)7 (CA)3(CA)11	58	AY227662
Microcebus murinus	Mm22	F: GATATTTGCAGTGACGTCAAA R:AACTTTGACCCTTCCCAGTA	(CA) 16	58	AY154670
Hapalemur griseus	45HDZ016	F: GAATCAATCTAAATCAAATGCCC R: TTCACCTTTTTCTCCCCAGTC	(CA)11	50	AY045527
	45HDZ052	F: TGAATAAACCAAGAAACTCCCC R: CAATAGAATACCTGGCTCACTGG	(GCAC)4GC(CA)11	54	AY045534
Woolly Monkey	1110	F GGTGAATGAGAGAATCAAAG R TATGTTCCACAGTAGAAAGC	[GT]20	53	AY450288
	1115	F GCTCATATTCATACATCCCTTGG R TTTGCTTGCTCATTCATTGC	[GT]3[GA]1[GT]5 [CT]1[GT]11	53	AY405289
	1118	F TTTCTCCCTCTCAGATTACCAG R CCTTGAGGTTTTTGGGTTCC	[CA]2[TA]1[CA]17	50	AY405290
Woolly Monkey	157	F* TGGCAAGTCTGGTTTCAAGC R TTCCAGACTGAGCTAGGATGC	[GA]4[GT]4[CT]1 [GT]2[AT]1[GT]13 [GA]9[GT]5	53	AY405291
	311	F CTTCCGAAAGCCATTTCTCC R TTAATGCCAGATGATTTTGG	[GAA]3[GAG]9 [CAG]6	52	AY405292
	312	F GAGACAACGACATTAACAATGC R GCTTCTGGTTTCTGATTGAGG	[GCT]10	50	AY405293
	113	F CAAAACTCCCCTGTGACTG R* CCCACTCTCCTCCACAAAGG	[GT]15	50	AY405294

The amplification condition for the 12 microsatellites markers was done nd according to the method described by Huang (1995) and Masibay (2000) from

where the sequence was obtained. Nevertheless, after optimization, only two PCR conditions were used (Table 2.17A and 2.17B).

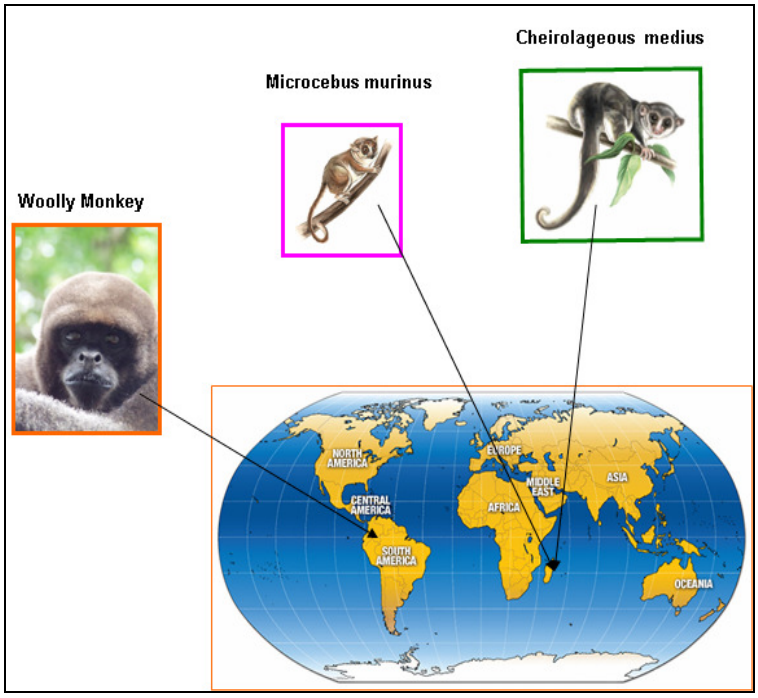


Figure 2.4 Map showing the Ecological Distribution of the Cross-Species Primates used.

Table 2.17A Parameters of PCR conditions (1) (33 cycles) for amplifying Cross-Species Microsatellite Loci species the Woolly Monkey (311 and 1118) (Figure 2.4)

Cycling condition	**Temperature (°C)**	**Duration**
Initial denaturation	95	3 minutes
Denaturation	95	1 minute
Annealing	50	1 minutes
Extension	72	1 minutes
Final Extension	72	5 minutes

Table 2.17B Parameters of PCR conditions (2) (33 cycles) for amplifying Cross-Species Microsatellite Loci species *Cheirogalous medius* and *Microcebus murinus* (3328 and Mm22) respectively (Figure 2.4).

Cycling condition	**Temperature (°C)**	**Duration**
Initial denaturation	94	2 minutes
Denaturation	94	30s
Annealing	57	30s
Extension	72	1 minutes
Final Extension	72	5 minutes

2.6.2.5 DNA Amplification for Microsatellite Genotyping

PCR reactions were performed in a final volume of 25 µl, in 0.5 ml thin wall PCR tubes containing 9,7 µl master mix components, 0.5ng to 200ng (1.2 to 2.5 µl) template DNA depending on the concentration of the DNA yield from the sample, and sterile water. Usually the DNA concentration obtained varied from the sample, and the amount of PCR sterile water was adjusted accordingly so that the final volume per reaction was 25 µl. PCR master mix preparation for multiplex reactions containing microsatellite isolated loci was done according to Table 2.18.

Table 2.18: PCR master Mix Preparation for Microsatellite Genotyping

Ingredients	Initial Concentration	Volume added per samples (µl)	Final concentration
dH2O	-	-	-
Buffer	10X	2.5	1x
$MgCl_2$	50mM	1.0	2.0 µM
dNTP	5 mole/each	4.0	200mM
Primer Forward	10 p/mol	1.0	10 p/mol
Primer Reverse	10 p/mol	1.0	10 p/mol
Hot Star Taq™ DNA polymerase (QIAGEN)	5u / µl	0.15	0.75u
Total volume of PCR master mix	9.65 µl		

2.6.2.6 Negative Control

Negative controls are amplification reactions without genomic DNA to ensure that there was no DNA contamination when the test was carried out.

2.6.3 Agarose Gel Electrophoresis and Photography

The aim was to confirm the success of the amplification reaction before performing Polyacrylamide Gel Electrophoresis (PAGE). The microsatellites were electrophoresed in a 1% (w/v) agarose gel. Gel casting was prepared by addition of 0.3 g of agarose gel to 30 ml 1X Tris borate EDTA (TBE) buffer and heated to boiling until all the agarose powder melted. The mixture was then allowed to cool down before 1 μl of ethidium bromide (10 μg/ml) was added.

The gel mixture was then transferred into a gel mould with combs for well formation. For each sample, 1 μl of the amplicons was mixed with 1 μl 6X loading dye. The voltage was set at 120 V and electrophoresis was performed for 20 minutes. 1.0 μl of a 100 bp ladder was electrophoresed together to enable determination of the size range of the amplified products. DNA bands were visualized using a UV transilluminator (Alphaimager, 2200) and polaroid photographs were taken for a permanent record.

2.6.4 Denaturing Polyacrylamide Gel Electrophoresis – Fragments Resolution

Polyacrylamide gel, which is much thinner than agarose, was used to improve resolution and to discriminate alleles, as a 2-5 bp difference in length should be distinguished. The vertical Scie-Plas DNA sequencing unit TVS1000 (England), dimensions of 20 cm wide X 50 cm high X 0.35 mm thick, with a set of 0.35 mm melinex spacers and shark tooth comb was used to resolve the alleles of the STR polymorphisms.

2.6.4.1 Preparation of Glass Plates

The procedures for preparation of the plain plate and notched plate were done according to the method routinely performed in the lab. Both the plain and notched glass plates must be meticulously cleaned. The plain glass plate was cleaned with 70% ethanol, followed by 95% ethanol. Then, a fresh bind silane mixture was prepared in a 1.5 ml sterile microcentrifuge tube containing the following solutions:-

- 3.5 µl bind silane
- 250 µl 10% acetic acid
- 1000 µl absolute alcohol

The bind silane mixture was poured in the middle of plain plate and wiped until the plate is completely covered by the solution followed by rinsing with 95 % ethanol. The binding solutions will chemically crosslink the gel to the plain glass plate after gel polymerization. Changing gloves is necessary before preparing the notched glass plate to avoid cross contamination with the binding solution. The notched glass plate was cleaned thoroughly with 70% ethanol. Then, the plate was treated with 2 ml of repel – silane solution, followed by rinsing with 95% ethanol.

2.6.4.2 Preparation of the Polyacrylamide Gel

Premixed solutions of a 30% (19:1) acrylamide:bisacrylamide was used to prepare the 4% polyacrylamide gel. A fresh gel mixture was prepared according to the ingredients listed below (Table 2.19): -

Table 2.19: Preparation of the 4% Polyacrylamide Gel

Component	4% gel	Final Concentration
Urea	21 g	7 M
Sterile distilled water	24 ml	-
5X TBE	6 ml	0.6X
30% acrylamide:bis (19:1)	6.7 ml	4%
Total Volume	50 ml	

.

The acrylamide solution was filtered through a 0.2 micron sterile syringe filter (Nalgene® Sartorius). Then, 300 µl 10% ammonium persulfate and 30 µl TEMED were added to the acrylamide mixture and gently mixed before the solution was poured between the glass plates horizontally. Polymerization was allowed for at least 1 hour. The PAGE apparatus was assembled according to the TVS 1000 DNA sequencing unit (Scie-Plas) instruction manual.

2.6.4.3 Sample Loading and Electrophoresis Parameters

A 2.5 µl portion of PCR product was mixed with 2.5 µl of 2X loading solution containing 95% formamide. 2.5 µl of the molecular weight ladder (100bp) was loaded in parallel with PCR products to provide visual standards to confirm allelic size ranges for the loci. All samples were denatured at 95℃ for 2 minutes using a thermal cycler (MJ Research,USA) and chilled immediately in an ice water bath.

Before gel pre-run, the upper and lower buffer tanks were filled with 0.6X TBE. After gel pre-run at 1500 V for 30 minutes, 3 µl of each sample mixture was loaded into their respective wells after no longer than 20 minutes to prevent the

gel from cooling. Allelic ladders were loaded next to sample lanes in order to provide feasible assignment of the alleles of each locus. After completion of sample loading, the gel was electrophoresed at 2500 V for 1 ½ hours (50-60 ℃).

2.6.5 Silver Staining Procedure

Following electrophoresis, the gel was subjected to silver staining for genotype determination. The protocol described by the manufacturer was adapted from Bassam et al (1991). The water used for preparation of all staining solutions was ultrapure water (Mili-Q purified). Poor results were obtained if this consideration was not followed. The solutions used for silver staining are listed below:-

- Fix/stop solution
- Developer solution
- Staining solution

The gel was fixed in fix/stop solution containing 10% acetic acid to remove electrophoresis buffer and urea from the gel and stained in a solution consisting of silver nitrate and formaldehyde for 30 minutes. Subsequently, the gel was rinsed very briefly in ultrapure water to remove excess silver and immediately developed in ice-cold alkaline sodium carbonate solution containing formaldehyde and sodium thiosulfate. Under these conditions, the silver ions are reduced to metallic silver by the formaldehyde (Bassam, 1991; Sambrook, 2001 see Table 3.20). The speed of the reaction is temperature dependent and is stopped by the addition of 10% acetic acid.

Table 2.20: Silver Stain Protocol (Bassam, 1991).

Step	Solution	Time
1	Fix/Stop solution	20 minutes
2	Ultrapure water	2 minutes
3	Repeat Step 2, twice	2 x 2 minutes
4	Staining solution	30 minutes
5	Ultrapure water	10 seconds
6	Developer solution (4 °C)	5 minutes
7	Fix/Stop solution	5 minutes
8	Ultrapure water	2 minutes

2.6.6 Polyacrylamide Gel Visualization and Data Analysis

After staining, the DNA fragments were viewed directly in the gel with the aid of a white light box. A permanent copy of the gel was obtained by scanning the gel using a desktop scanner.

2.7 Materials: Evaluation of the Maternal Genetically Inheritance by Using D-loop Mitochondrial Sequences

2.7.1 Chemicals / Reagents

2.7.1.1 DNA Extraction

Materials used in this section for DNA extraction are mentioned in Table 2.21 of this chapter.

2.7.1.2 Amplification –Polymerase Chain Reaction

Table 2.21: Materials for D-loop Mitochondrial DNA Sequences

Materials	Sources
Taq DNA polymerase	Biotools
D-loop mitochondrial primer pairs **(L15996) (H16498)**	Bio Basic Inc
PCR reagents and DNA Purification kit	Biotools

2.7.1.3 Agarose Gel Electrophoresis

Materials for Agarose Gel Electrophoresis see Table 2.7.

2.7.1.4 D-loop Mitochondrial DNA Sequences Amplification – Polymerase Chain Reaction

Table 2.22: Instruments for D-loop Mitochondrial Amplification

Instruments	Sources
PTC200 Thermo Cycler (BC-MJPC200)	Applied Biosystem
Applied Biosystems ABI 7700 DNA Sequence detector	Applied Biosystems
Mini centrifuge, 220V	Cole Palmer

2.7.1.5 Polymerase Chain Reaction (PCR) Set up for D-loop Mitochondrial DNA Sequences

The HV-I region of the D-loop was amplified using a pair of human primers L15996 (Vigilant, 1989) and H16498 (Kocher, 1989; Table 2.23). This region in the D-loop generated a fragment of 392 bp of the mtDNA control region amplified by using L15996 primers and fragment from the H16498 primer.

This primer pair for D-Loop was used to identify the DNA strand (H or L) and the position of the 3' end of the oligonucleotide according to the numbering system for the human sequence (Anderson, 1981).

Table 2.23: Primer Sequences for the mtDNA Amplification, Hypervariable Region of the D-loop

Name of Primer	Primer Sequence 5'- 3'	Primer Length (bp)
(L15996)	5'-CTCCACCATGAGTAGCACCCAAAGC-3'	25
(H16498)	5'-CCTGAAGTAGGAACCAGATG-3'	20

2.7.1.6 DNA Amplification for the Hypervariable Region of D-Loop Mitochondrial DNA

PCR reaction amplifications were performed in 25 µl reactions using 1- 2 µl DNA extract, 2.5 µl PCR buffer (Biotools), 1.5µl 10 mM $MgCl_2$, 4µl 1mM each dNTP, 5U of Taq polymerase (Biotools) and 1.0 µl each primer. Usually the DNA concentration obtained varied from the sample, and the amount of PCR sterile

water was adjusted accordingly so that the final volume per reaction was 25 µl. PCR master mix preparation for the D-loop mitochondrial region was done according to Table 2.24.

Table 2.24: PCR Master Mix Preparation for D-loop Mitochondrial Sequences Amplification

Ingredients	Initial Concentration	Volume Added per Sample (µl)	Final Concentration
dH2O	-	-	-
Buffer	10X	2.5	1x
$MgCl_2$	50mM	1.5	2.0 µM
dNTP	5 mole/each	4.0	200mM
Primer Forward	10 p/mol	1.0	10 p/mol
Primer Reverse	10 p/mol	1.0	10 p/mol
Taq polymerase	5u / µl		5U
Total volume of PCR Master mix	10.2 µl		

The PCR profile was carried out under the following conditions: initial denaturation (2 min at 94℃), followed by 35-38 cycles of denaturation (1 min at 94℃) annealing (1 min at 51℃), extension (1 min at 72 ℃) and a final extension (10 min at 72 ℃) (Table 3.25). The PCR products were visualized

electrophoretically using 1.0% agarose-TBE buffer and the purified for sequencing.

Table 2.25 Parameters of PCR Conditions (35 cycles) for Amplifying HV-1 Region of D-Loop Mitochondrial

Cycling Condition	**Temperature (°C)**	**Duration**
Initial denaturation	94	2 minutes
Denaturation	94	1 minutes
Annealing	50	1 minutes
Extension	72	1 minutes
Final Extension	72	10 minutes

2.7.1.7 PCR Product Purification

The amplification reaction products were purified using QIAquick PCR Purification Kit QIA# 28104 (Qiagen) prior to sequencing. DNA was purified using a simple and fast bind-wash-elute procedure and an elution volume of 30–50 µl. Fragments were purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge according to manufacturer recommendations.

The volume of Buffer PB was added to the PCR sample and mixed in a 5:1 ratio mix (eg. 500 µl of Buffer PB to 100 µl PCR sample). The QIAquick spin column was placed in a 2 ml collection tube. To bind DNA, the mixture of PCR product was applied to the QIAquick column and centrifuged for 30–60 sec. Flow-through was discarded and the QIAquick column was placed back into the same tube. In order to wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for another 30–60 sec. Flow-through was discarded again and the QIAquick column was placed back in the same tube and centrifuged for an additional 1 min at maximum speed. QIAquick column was finally placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or distilled RNAse free water was added to the center of the QIAquick membrane and centrifuged for 1 min. The purified PCR product solution collected in the 1.5ml microtube was kept for further sequencing analysis.

2.7.1.8 Mitochondrial DNA Sequencing

Purified PCR fragments were subsequently subjected to direct sequencing using DNA Sequencer ABI 377 (Applied Biosystems). DNA sequencing was carried out using dye-terminator chemistries; both strands forward and reverse were sequenced to confirm reliability. All PCR products were sequenced in triplicates in order to minimize error due to inefficient Taq Polymerase proof reading or unequal PCR efficiency in different PCR runs. The primers used for sequencing were similar to the primers used in mitochondrial DNA PCR amplification as indicated in Table 24.

2.7.1.9 D-loop Mitochondrial Sequences Alignment and Data Analysis

Nucleotides sequenced from the 26 individuals were analyzed using Chromas Technelysium Pty Ltd version 2.33. Each was checked in parallel to the

electropherogram peak generated by the DNA sequencer and double-checked by visual inspection of the chromatograms.

For comparisons within all Slow Loris mtDNA, the curated sequences for all individuals were aligned using BioEdit Sequence Alignment Editor Software (Version 7.0) and double- checked by visual scoring. Intra population and inter-population methods were used to analyze the aligned the D-loop DNA sequences.

The following Molecular diversity indices were calculated:

(a) Inter-haplotypic Distance Matrix (Distance Method Pair-Wise Distance Analysis) among D-loop haplotypes of *Nycticebus c. coucang* was calculated using Mega v2.0 (Kumar et al., 2001).

(b) With this program, it was also estimated nucleotide compositions (% of A, C, T and G bases) for each haplotype.

(c) The Arlequin v3.01 software (ExcoYer et al., 2005) was used to calculate indices of nucleotide (π) and haplotype (h) diversity (Nei, 1987) to estimate levels of intra-population genetic diversity within the samples obtained in the field and other localities in Peninsular Malaysia

(d) Using this software (above), haplotype frequencies were also estimated.

Phylogenetic trees were constructed and analyzed, utilizing unweighted Maximum Marsimony (MP), UPGMA (Sneath, 1973) computed by the Maximum Composite Likelihood method (ML) and neighbor-joining (nj) (Saitou, 1987) methods implemented in PAUP (Ver. 4.B 10; Swofford, 2001) and MEGA5

(Tamura, 2007). Phylogenetic confidence was estimated by bootstrapping (Felsenstien, 1985) with 1000 replicate data sets.

2.8 Statistical Analysis for Data Collected for Ranging Patterns

Data were subjected to statistical “T-Test” using the software STATISTICA. P1 values of less than 0.005 were considered significant, otherwise higher values were not significant.