CHAPTER 6 – Identification of Paternal and Maternal Genetic Inheritance of the *Nycticebus c. coucang*

6.1 Introduction

Documentation of the postulated family pedigrees were drawn up based on radio-telemetry data (Ranging Patterns) as shown in Figure 6.1. The proofs of the relatedness (kinship) as observed in the field were subsequently tested with mitochondrial DNA similarities, and results were illustrated as familial pedigrees based on D-loop DNA (Figure 6.13). Consequently, a phylogenetic inference was undertaken to estimate the evolutionary past of the samples collected. This was done by statistically creating molecular trees based on the multiple sequence alignments that were obtained (Figures 14, 15, 16).

6.1.1 Relatedness or Kinship Observed through Radio Telemetry Data Pedigree

Based on the following parameters used for detailed field data collection (see Table 6.1), we inferred possibilities of relatedness of individuals within group “A”, “B”, “C”, “D” and “E”. Individuals “Linda”, “Adopt”, “Aggressive”, “Timida”, “Hermosa”, “Little”, “Cop”, “Fala”, “Gent”, “Bonita”, “ECA”, “KRO” were tagged and tracked. On the other hand, for individuals “GIA”, “Male-Boloh”, “YURI”, “ROU”, and “Baby-Born” several locations were obtained within the study but they were not tagged. Finally, “IAB” was seen but neither being tagged nor tracked for no more than few days.

6.1.2 Group A: “Linda” and “Adopted” were not related since these individuals were caught in different localities (see Figure 2.3 of Chapter 2). The individual “Adopted” was found in the morning at approximately 6 am on the few remaining trees in the deforested area, and this individual were caught right before the deforestation by machines and logging trucks continued.

Table 6.1 Parameters used from Data Collected in the Field to Infer Relatedness

Parameters to Infer Relatedness in the Wild	Section and Chapter
1) Proximity in daily movement	5.1.6 Section Chapter 5
2) Sleeping on the same trees, sleeping together on nearby tree.	5.1.7 Section Chapter 5.
3) Individuals sharing the same home range	5.2 Section Chapter 5.
4) Individuals intra-group home range overlapped	5.3.1.2 Section Chapter 5.
5) Biotope association.	5.5 Section Chapter 5.

Based on data collected in captivity, we also gained evidence of relatedness of those individuals that were kept in captivity in the Malacca Zoo, State of Melaka, namely; "1Malacca"; "2Malacca"; "3Malacca"; "4Malacca"; "5Malacca" and "6Malacca."

We decided to release the young infant with the adult female "Linda" caught in another location due the high probability that this infant would not have survived by himself if the infant has been returned back to the same location that he was originally found. In captivity, the female showed maternal behaviour towards the young infant, and which continued later on in the field as well.

We released "Linda" with the adopted baby at the border of her home range which was unknown at the time. Consequentially, the female took the infant "Adopted" a few days later to the middle of her home range by crossing through different biotopes such as Orchards and Rubber Plantations. Finally, she parked the infant in a secure place on the top of the small hill (118 meters in altitude) far from the Orchard. The area was surrounded by deep "Belukar" covered with low rattan palm. There was no any other offspring apart from the adopted Infant seen

with Linda throughout the field observation period. A male named “GIA” was seen with “Linda” on many occasions but it was never caught despite the many attempts undertaken to catch him. A possible home range was represented for him (Figure 5 .13A), hence a possible intra-group association (monogamist pair male-female without offspring) among “GIA” and “Linda” was inferred (Figure 6.1).

“Male-Boloh” and “Yuri” were caught (but not tagged) around Linda’s home range. 1008 meters was the distance of between these individuals at the time of being trapped. A few fixes were taken on them, and a possible home range was proposed from the available data (see Figure 5.13A). It was inferred that no negative kinship existed between them.

6.1.3 Group B. The individuals in this group (“Aggressive”, “Timida”, “Hermosa” and “Little”) presented during the whole study period a high association among them, intra-group overlapping of home ranges, biotopes association, sleeping association among individuals within the same group, as well as some occasional proximity in their daily movement. It was inferred a positive kinship among the individuals of this group. It is noteworthy to mention that at the time of removing the collar from the female “Timida” and released in the wild, signs of pregnancy were observed in this female, and that she was due to deliver new offspring approximately by February or March 2007.

6.1.4 Group C: The individual adult male “Cop”, adult female “Fala”, and sub-adult male “Gent”, presented in the field intra-group home range overlapping. Biotopes used were associated within the members of the group. On several occasions the sub-adult “Gent” was recorded sleeping within the same tree, or in a tree in proximity to “Fala”. It was inferred positive kinship (monogamist pair male-female and putative offspring) in the field due to parameters above which were used to establish relatedness (kinship).

Nevertheless, there was an unusual aggression by the adult “Cop” towards the sub-adult “Gent”. The event occurred at 8:45 pm (20:45 hours), when the individual “Gent” was following “Cop” alone the electrical wire on the way to an Orchard biotope, and they were within a distance of approximately 50 meters when, suddenly, the adult male turned and in quick speed walked towards “Gent” attacking him until the sub-adult fell off from the electrical wire to land on the ground. We took the sub-adult “Gent” to quarantine recovery over a period of 4 days, after which the individual was released back in the wild. The injuries recorded from this attack included skin removed from fingers, a bleeding mouth cut, a bit of hair lost on the forehead, and a small cut on the eye brow.

It is noteworthy to mention that after a week of being released this sub-adult suffered another even more vicious attack by another male, “ROU’ from group “D” along the electrical wire, forcing him to fall off again on the ground from a great height. This time “Gent” confronted the attacking adult male, but the aggression was worse than the previous one. We decided not to interfere on this occasion with nature, and hence to possibly change the cycle of nature, by bringing this sub-adult for recovery in temporary captivity. The individual, as a result, did not manage to recover from this second attack, and died a few days later in the proximity to the adult female “Fala” (Figure 6.1). Interesting additional information was observed of this female “Fala”, while removing the collar, it was concluded that she presented symptoms of pregnancy as did the female “Timida,” and she was due to deliver new offspring approximately in the period February to March 2007 as likewise recorded for the female “Timida”.

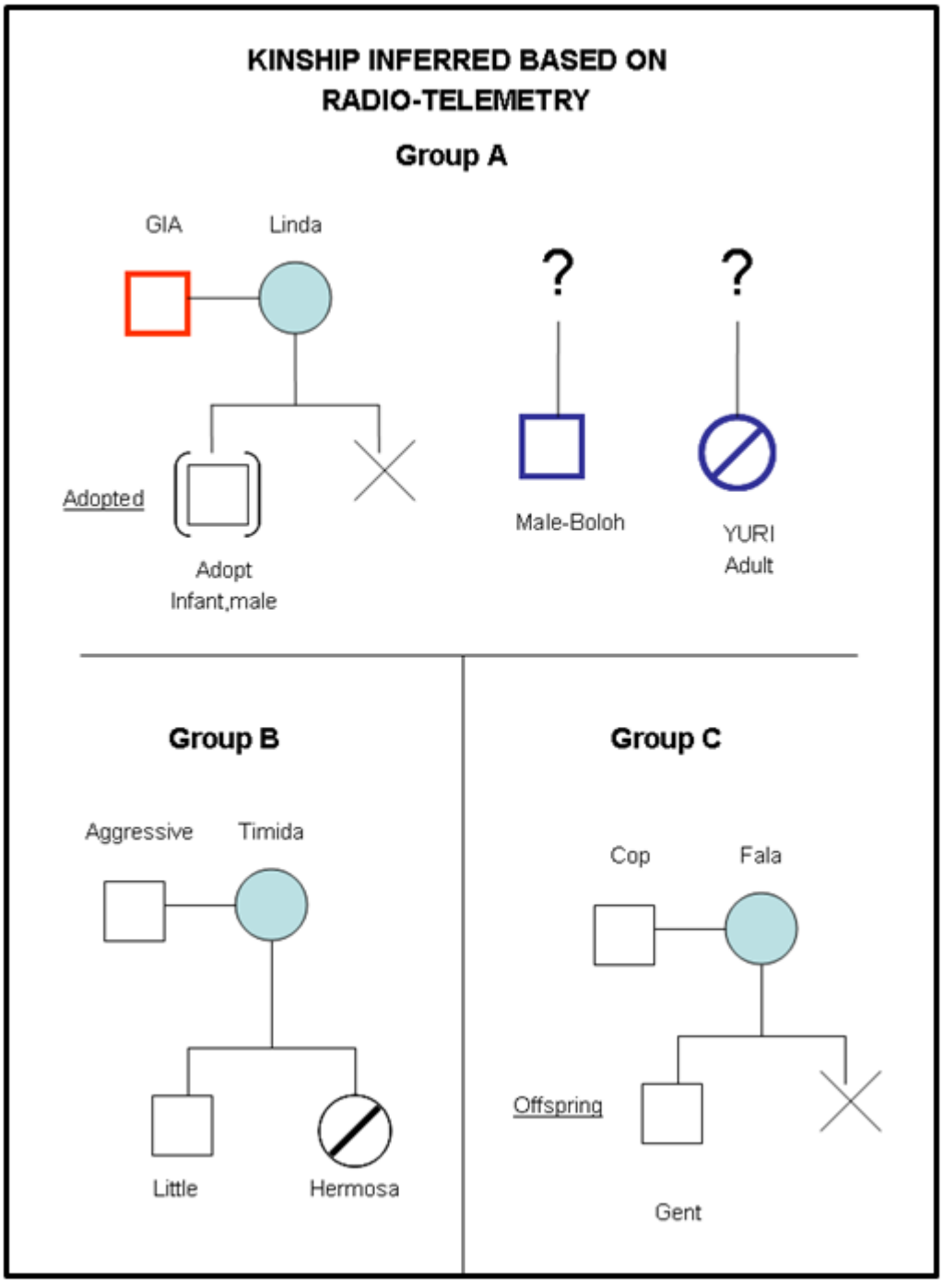
6.1.5 Group D: The kinship was expected positive among the adult female “Bonita” and the neonatal “Baby-Born” because we witnessed the delivery of the offspring in captivity on August 22, 2006 at 4:00 hours (am). Furthermore, the female during the whole study period was observed sleeping with the baby on

the same sleeping trees, maternal behaviour such as grooming was observed in several occasions and parameter established in Table 6.1 were seen. On the other hand, the adult male “ROU” and the adult female “Bonita” were seen alone on the electrical wire on a few occasions in different sites within “Bonita’s” home range. It was inferred there existed a positive kinship among individuals of this group with a monogamous pair, the putative father “ROU”, mother “Bonita” and one offspring ”Baby-Born” (Figure 6.1).

6.1.6 Group E: Inferring kinship in the field for this group was a bit more complicated, and was not clear at the start of the tracking due to gender spatial patterns and behaviour observed during the study. At the start of the tracking, we assumed no linking of kinship between these individuals because they were two adult males, so it was assumed that there was no overlapping of home range and different use of biotopes etc. Nevertheless, throughout the study field, the animals behaved differently and showed great overlapping home range, sharing some biotopes and extraordinary behaviour among them such as sharing sleeping trees, and on several occasions even sleeping close together.

Thus, kinship was inferred with a putative father “ECA” (since his home range was extremely big compared to Kro’s). “KRO” was assumed to be ECA’s offspring and the sub-adult male “IAB” who was sharing the same sleeping sites and biotopes was considered another putative offspring of “ECA” (Figure 6.1).

6.1.7 Zoo Malacca: After observation in captivity, and from information obtained from the keepers in the Zoo on the captive individuals, it was concluded that the adult female “2Malacca” was the putative mother of “5Malacca” infant (Figure 6.1).


KINSHIP INFERRED BASED ON
RADIO-TELEMETRY
Group A
GIA
Linda
Adopted
Adopt
Infant,male
?
Male-Boloh
?
YURI
Adult
Group B
Aggressive
Timida
Little
Hermosa
Group C
Cop
Fala
Offspring
Gent

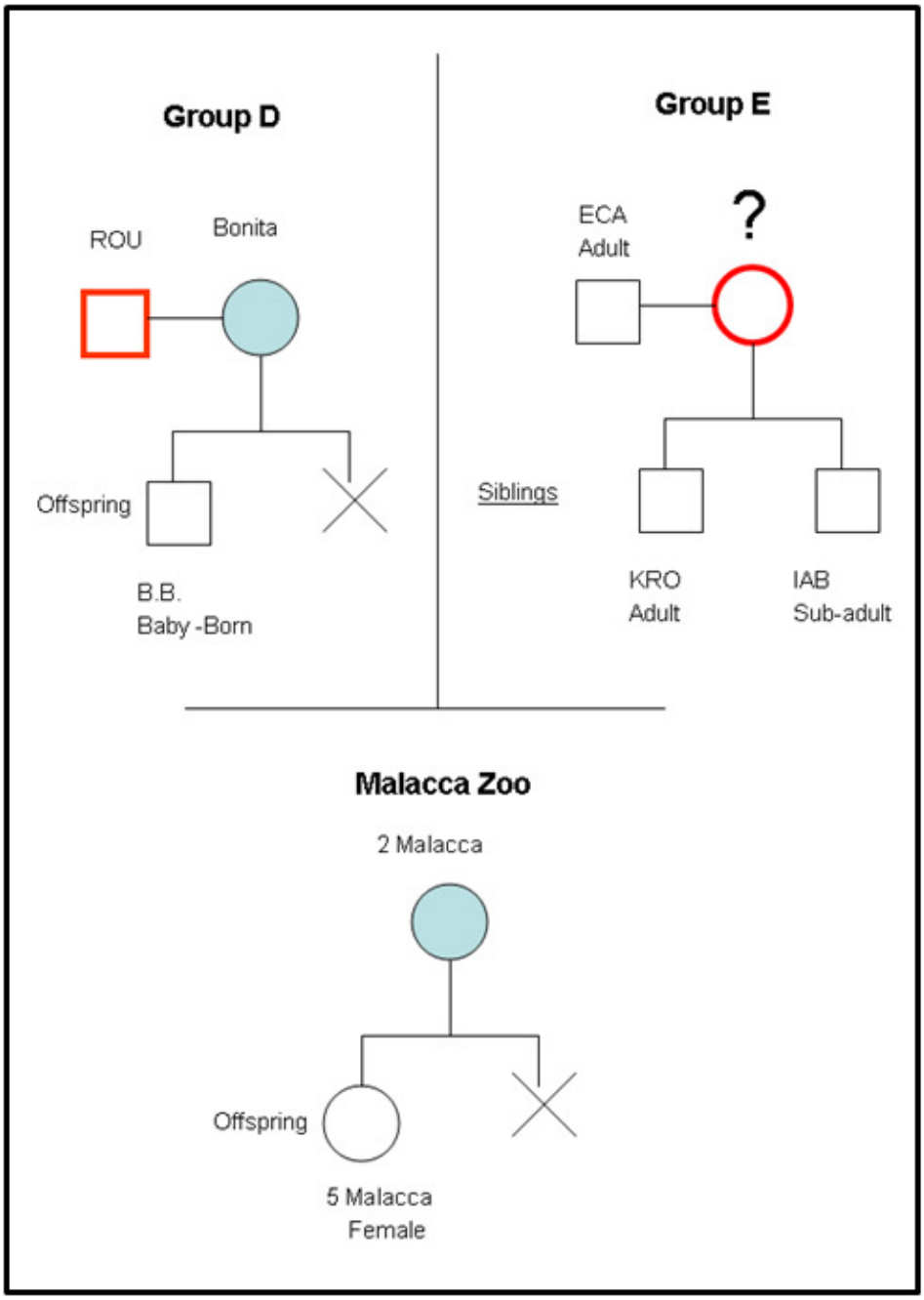


Figure 6.1: Matrilineal Pedigree of *Nycticebus c. coucang* based on Radio-Telemetry data observations. Open square- male; filled circle-female with progeny; slashed circle-female without progeny; red square-individual (male) was not caught but locations trapped was recorded; blue square-individual caught but was not tagged. (X) The X in the group “A”, “D” and Malacca Zoo represents that there was no seen another offspring with these putative parents.

6.2 Microsatellite Analysis

6.2.1 DNA Extraction Yield

The collection of sampled *Nycticebus c. coucang*'s blood on filter paper for the analysis of relatedness was carried out using two methods of DNA extractions: (a) Tris-EDTA (TE) buffer-based; and (b) Chelex® Extraction from blood spot on filter paper. Method (a) produced higher DNA yield than method (b). The sensitivity of detection also varied for the different extraction methods as shown in Table 6.2.

Table 6.2 A Comparison of Two Methods used for DNA extraction from Blood on FTA Paper.

Filter Paper	Extraction Method	PCR Positive 26 Tested
Whatman S.A	Tris -EDTA	10
	Chelex	26

6.2.2 Cross-Amplification

Amplification of 12 microsatellite markers that were previously characterized in three species of primates was performed; and thirty-three percent (4/12, or 33%) of those markers originally discovered for primates also successfully amplified the *Nycticebus c. coucang* DNA (Table 6.3). The primers used here are tetranucleotide repeat motif which could reduce the probability of typing error resulting from the stutter bands that are often associated with dinucleotide markers (Figure 6.2). Although the markers are also specific for *Nycticebus c. coucang* DNA sequence amplification, however, it did not exhibit levels of polymorphism, and did not produce reproducible genotypes to determine

relatedness between individuals, thus it was not informative since we were not able to discriminate between different individuals.

Table 6.3: Four Cross-Species Amplification of Microsatellites Markers used for *Nycticebus c. coucang* DNA (namely, “33228”, “Mm22”, “311”, “1118”) that were originally identified to amplify continent primate DNA

Primate Species	Repeat Motif	Locus	Primer Sequence (Forward)	Primer Sequence (Reverse)
Lemurs	Tetra	33228:	F-CCTGCAGCAAAACCACATC	R-ATTCCTTCTTCATATCTGGAC
		Mm22	F-GATATTTGCATGACGTCAAA	R-AACTTTGACCCTTCCCAGTA
Woolly Monkey	Tetra	311	F-CTTCCGAAAGCCATTTCTCC	R-TTAATGCCAGATGATTTTGG
		1118	F-TTTCTCCCTCTCAGATTACCAG	R-CCTTGAGGTTTTTGGGTTCC

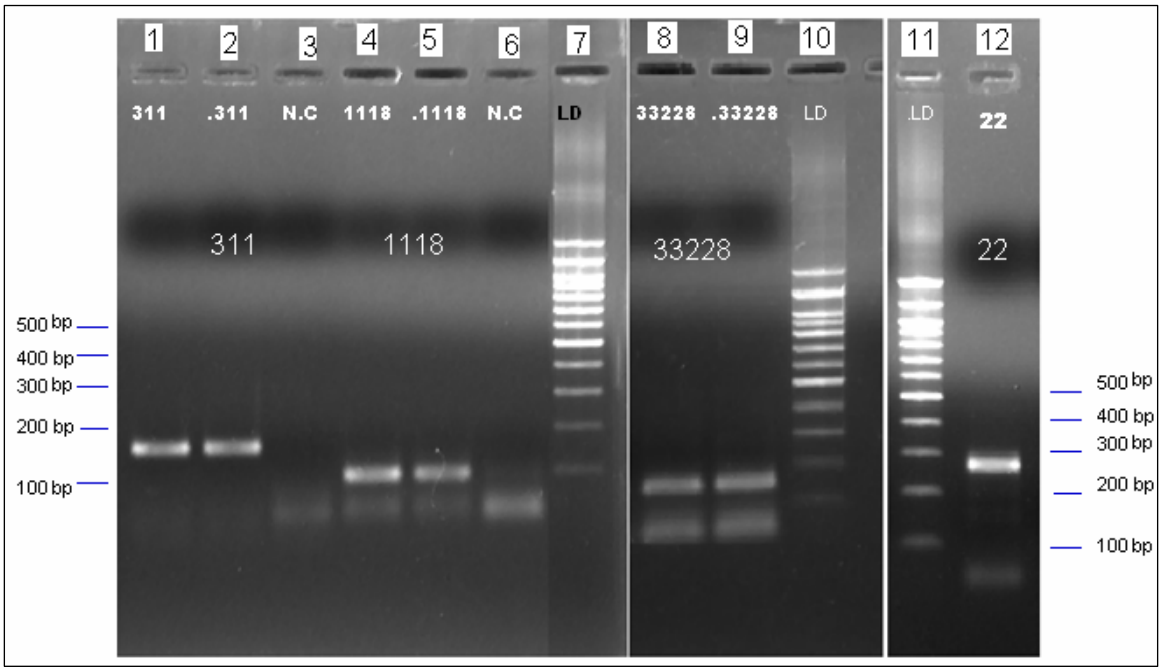


Figure 6.2: The Electrophoresis Pattern of PCR products using Microsatelites Markers for the Amplification of DNA Lanes from 1, 2, 4,5,8,9 and 12 mtDNA; NC: Template Control Lane 3, 6. and the Ladder Lanes 7, 10 and 11.

6.3 D-loop Mitochondrial DNA (mtDNA) Sequences Analysis

6.3.1 Finding D-loop Mitochondrial Sequence

The amplification of D-loop mitochondrial sequence DNA from the sampled *Nycticebus c. coucang* produced a single band and the length of PCR product was approximately 500 bp (or 466 bp) as shown on the agarose gel after electrophoresis. However, we also identified a smaller fragment of approximately 350 bp, which is known to be non-specific amplification that disappeared following optimization of the method. DNA sample were run in 3 replicates for both PCR and sequencing analysis in order to achieve high confidence of results (Figure 6.3).

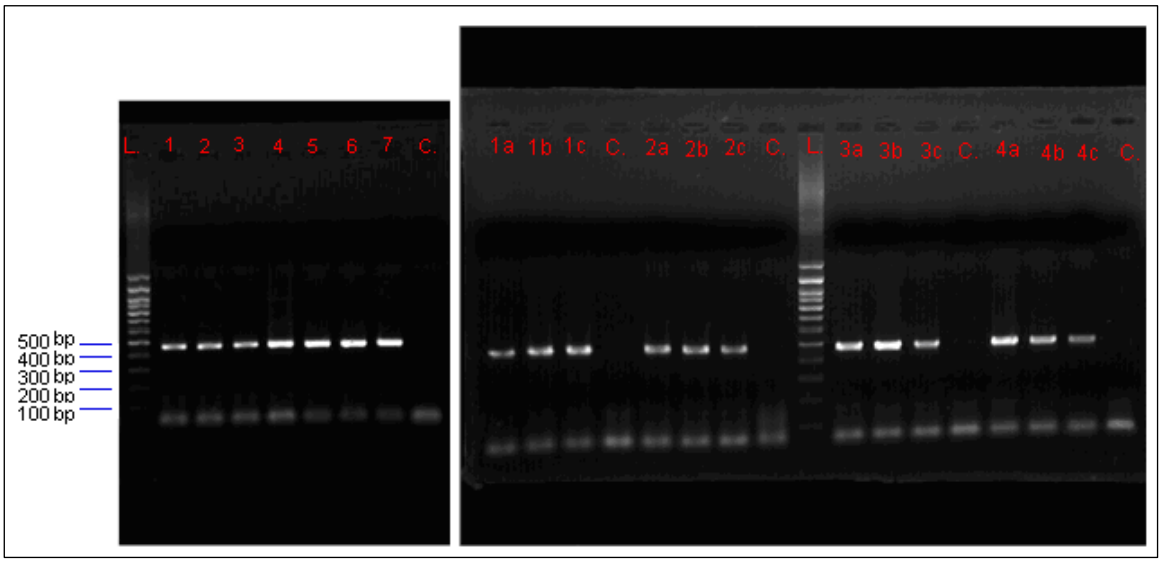


Figure 6.3: Sample of the Electrophoresis Pattern of mtDNA PCR Products using Primers (L15996) (H16498) as Template.

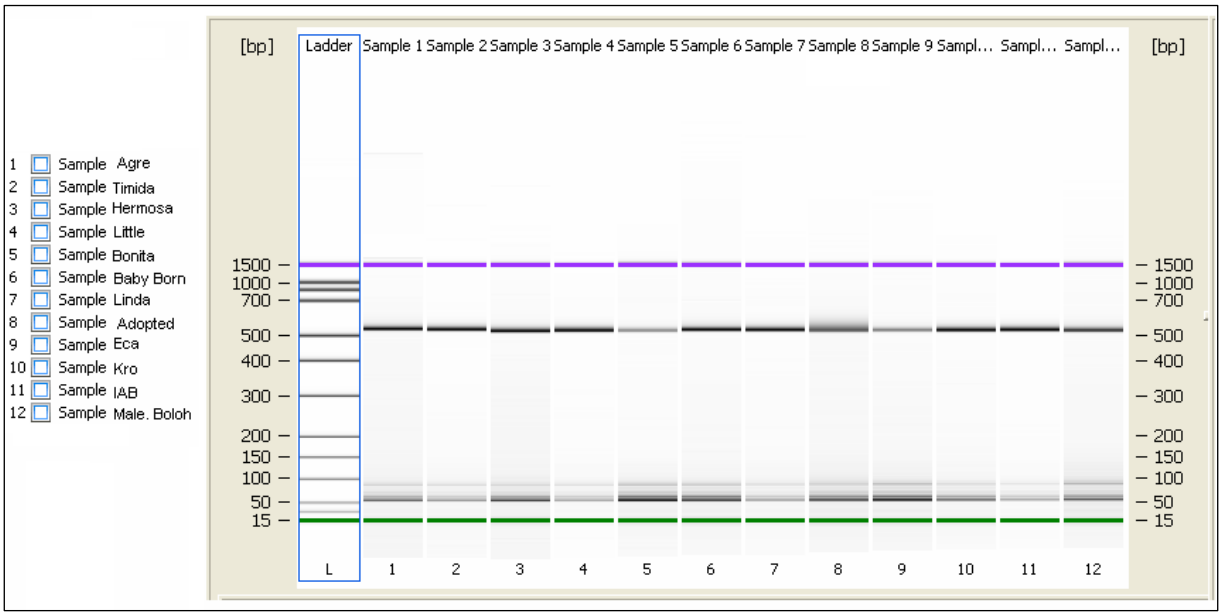


Figure 6.4: Shows the size of PCR product (in base pairs) for 12 *Nycticebus c. coucang* individuals analyzed using DNA 1000 Labchip, Agilent Bioanalyzer.

The samples were amplified using the primers L15996 and H16498. The Agilent Bioanalyzer computed more accurate data to confirm the base pair length of each sample. In general, all samples produced the same specific PCR product and the length variation is very minimal, i.e. between 529 bp to 539 bp (Figure 6.4).

The size of PCR fragment amplified for the mitochondrial D-loop region in 26 *Nycticebus c. coucang* individuals from Peninsular Malaysia was approximately 500 base pairs (bp). Figure 6.5 below shows the resulting alignment of 393-bp DNA sequence which comprised the entire hypervariable segment 1 (mt HVS1) of D-loop. The same primers L15996 & H16498 were used to successfully amplify this region in all the individuals, using the primer's loop L and loop H, overlapping with the 390-bp sequence of *Nycticebus c. coucang* D-loop (GenBank accession AY875955 and 16764-bp sequence of *Nycticebus c.*

coucang mitochondrial genome (GenBank accession AJ309867) (see Figure 6.5).

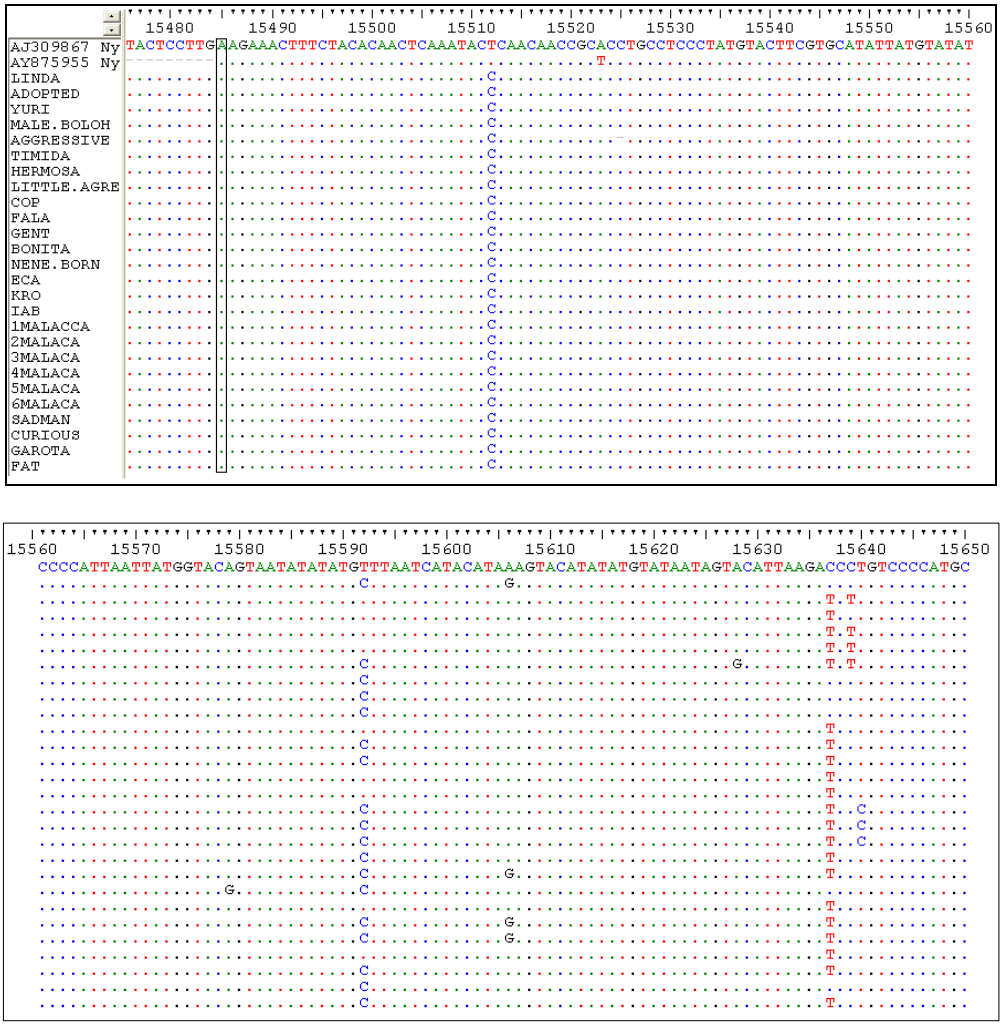


Figure 6.5 Complete Sequence Alignment 391bp of D-loop mtDNA gene, employing: Complete *Nycticebus* Mitochondrial Genome “Genbank Accession Number AJ309867”, “AY875955 *Nycticebus c. coucang* D-loop” and *Nycticebus c. coucang* Malaysian

samples obtained in different localities in Peninsular Malaysia. Note: The two vertical black squares show where the first hypervariable region of the D-loop starts (nucleotide-15485) and where it ends (nucleotide-15876)

The same alignment procedure was used to align the twenty six sequences showing 30 polymorphic sites, differences in haplotypes. Individuals were grouped by location (Table 6.4).

Table 6.4 Polymorphic Sites within the *Nycticebus c. coucang* mtDNA D-loop Sequence (Nucleotides 15 485- 15 876), according to GenBank accession number AY875955).

	Samples	Nucleotide position																																		
		15512	15523	15525	15592	15605	15628	15637	15639	15640	15651	15665	15677	15693	15694	15695	15696	15698	15712	15719	15739	15740	15748	15761	15764	15778	15783	15809	15813	15816	15841	15843	15855	15856	15861
	AY875955	T	T	C	C	G	A	C	C	T	G	C	A	G	A	A	C	C	A	C	A	A	A	T	C	C	G	A	G	A	T	A	A	C	A
A	LINDA	C	A	.	T	A	.	T	T	.	A	T	.	A	.	.	.	T	.	.	.	.	.	C	.	T	A	G	A	G	.	.	G	.	G
A	ADOPTED	C	A	.	T	A	.	T	.	.	A	T	.	A	.	.	.	.	.	T	C	.	.	C	.	T	.	G	.	.	.	G	G	.	.
A	YURI	C	A	.	T	A	.	T	T	.	A	T	G	A	G	.	.	.	G	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
A	MALE.BOLOH	C	A	.	T	A	.	T	T	.	A	T	G	A	G	.	.	.	G	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
B	AGGRESSIVE	C	A	-	.	A	G	T	T	.	A	T	.	A	.	.	.	T	.	.	.	G	.	C	.	T	.	G	.	G	C	.	G	.	.
B	TIMIDA	C	A	.	.	A	.	.	.	.	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	G
B	HERMOSA	C	A	.	.	A	.	.	.	.	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	G
B	LITTLE.AGRE	C	A	.	.	A	.	.	.	.	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	G
C	COP	C	A	.	T	A	.	T	.	.	A	T	G	A	G	.	.	.	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
C	FALA	C	A	.	.	A	.	T	.	.	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	.	.	.	.	.	G	.	.
C	GENT	C	A	.	.	A	.	T	.	.	A	T	.	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	G
D	BONITA	C	A	.	T	A	.	T	.	.	A	T	G	A	G	.	.	.	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
D	NENE.BORN	C	A	.	T	A	.	T	.	.	A	T	G	A	G	.	.	.	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
E	ECA	C	A	.	.	A	.	T	.	C	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
E	KRO	C	A	.	.	A	.	T	.	C	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
E	IAB	C	A	.	.	A	.	T	.	C	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	.	.	.	G	.	.
1	FAT	C	A	.	.	A	.	T	.	.	.	T	G	A	.	.	.	T	.	.	.	.	.	C	T	T	.	.	.	.	.	.	G	.	.
2	1MALACCA	C	A	.	.	A	.	T	.	.	A	T	.	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	G	.	.	G	.	.
2	2MALACA	C	A	.	.	.	.	T	.	.	A	T	.	A	.	.	.	T	.	.	.	.	T	C	.	T	.	G	.	G	.	.	G	.	.
2	3MALACA	C	A	.	.	A	.	.	.	.	A	T	G	A	.	.	.	T	.	.	.	.	T	C	.	T	.	G	.	G	.	.	G	.	.
2	4MALACA	C	A	.	T	A	.	T	.	.	A	.	.	A	.	.	.	T	.	.	.	.	T	C	.	T	.	G	.	G	.	.	G	.	.
2	5MALACA	C	A	.	.	.	.	T	.	.	A	T	.	A	.	.	.	T	.	.	.	.	T	C	.	T	.	G	.	G	.	.	G	.	.
2	6MALACA	C	A	.	.	.	.	T	.	.	A	T	.	A	.	.	.	T	.	.	.	.	T	C	.	T	.	G	.	G	.	.	G	A	.
3	CURIOUS	C	A	.	.	A	.	T	.	.	A	T	G	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	G	.	.	G	.	.
3	GAROTA	C	A	.	.	A	.	.	.	.	A	T	G	A	.	G	T	.	.	.	.	.	.	C	.	T	.	G	.	G	.	.	G	.	.
3	SADMAN	C	A	.	T	A	.	T	.	.	A	T	.	A	.	.	.	T	.	.	.	.	.	C	.	T	.	G	.	G	.	.	G	.	.

Note. View conservation by Plotting Identities to a Standard as a Dot (.); Nucleotide Substitutions is indicated by a dash (-). Localities/Groups DNA samples: A. Bukit Boloh (Group A); B. Cempaka (Group B); C. Cempaka (Group C); D. Cempaka (Group D); E. Cempaka (Group E); 1. Kampung Pasu (Pahang), 2. Zoo Malacca, and 3. Seremban Perhilitan Sanctuary.

In order to confirm sequencing for the 393-bp sequences in the mitochondrial control region and the d-loop region of the pro-simian, BLAST pairwise alignment tool (GenBank) was used. Results showed that *Nycticebus c. coucang* has a score 98% similarity with the canonical sequence in the database.

6.3.2 Genetic Variation

From the 30 polymorphic sites, 18 haplotypes were found within 26 individuals with a length of 466 (bp). The gene diversity (h) and Nucleotide diversity (π) of all individuals sampled were 0.9662 ± 0.0196 and 0.011740 ± 0.006503, respectively. The extracted information for standard diversity indices (intra-population) and molecular indices are presented in the Table 6.5 and the Distance method: Pairwise Difference is shown in Table 6.6.

Table 6.5 Computational Analyses carried out in 26 mtDNA sequences (Haplotypic Data)

Standard Diversity Indices:	**Molecular Diversity Indices**
No. of gene copies : 26 No. of sequences : 18 No. of loci : 466 No. of usable loci : 466 loci with less than 5.00 % missing data No. of polymorphic sites : 30	Sample size : 26 No. of haplotypes : 18 No. of original haplotypes in sample : 18 Deletion weight : 1 Transition weight : 1 Transversion weight : 1 Allowed level of missing data : 5 % Number of observed transitions : 25 Number of observed transversions : 3 Number of substitutions : 28 Number of observed indels : 2 Number of polymorphic sites : 30 Number of observed sites with transitions : 25 Number of observed sites with transversions : 3 Number of observed sites with substitutions : 28 Number of observed sites with indels : 2 Number of observed nucleotide sites : 466 Number of usable nucleotide sites : 466

Table 6.6 Inter-haplotypic Pairwise Distance Matrix between each haplotype of *Nycticebus c. coucang* used in this study (S.D. above the diagonal):

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		1.4112	2.2240	2.8040	2.4337	3.1282	1.9914	1.9914	2.2240	1.9914	2.2240	2.2240	2.6258	2.6258	2.4337	2.8040	2.2240	2.4337
2	2.0000		2.6258	2.8040	2.8040	3.1282	2.4337	2.4337	2.6258	2.4337	2.6258	2.6258	2.9709	2.9709	2.8040	3.1282	2.6258	2.8040
3	5.0000	7.0000		2.6258	2.2240	2.9709	1.7265	2.2240	1.4112	1.7265	2.8040	1.9914	2.4337	1.9914	2.6258	2.6258	1.9914	2.2240
4	8.0000	8.0000	7.0000		3.1282	2.8040	2.8040	1.9914	2.2240	2.8040	2.9709	2.2240	2.6258	2.9709	2.4337	2.8040	2.6258	3.1282
5	6.0000	8.0000	5.0000	10.0000		3.1282	2.4337	2.4337	2.6258	2.4337	2.9709	2.2240	2.6258	2.2240	2.8040	2.8040	2.2240	2.8040
6	10.0000	10.0000	9.0000	8.0000	10.0000		2.8040	2.4337	2.6258	2.8040	3.2772	2.2240	2.6258	2.9709	2.8040	2.8040	2.6258	3.1282
7	4.0000	6.0000	3.0000	8.0000	6.0000	8.0000		1.9914	1.7265	1.4112	2.6258	1.7265	2.2240	2.2240	2.4337	2.4337	1.7265	1.9914
8	4.0000	6.0000	5.0000	4.0000	6.0000	6.0000	4.0000		1.7265	1.9914	2.2240	0.9989	1.7265	2.2240	1.4112	1.9914	1.7265	2.4337
9	5.0000	7.0000	2.0000	5.0000	7.0000	7.0000	3.0000	3.0000		1.7265	2.4337	1.4112	1.9914	2.4337	2.2240	2.2240	1.9914	2.2240
10	4.0000	6.0000	3.0000	8.0000	6.0000	8.0000	2.0000	4.0000	3.0000		2.6258	1.7265	2.2240	2.2240	2.4337	2.4337	1.7265	1.4112
11	5.0000	7.0000	8.0000	9.0000	9.0000	11.0000	7.0000	5.0000	6.0000	7.0000		2.4337	2.8040	3.1282	2.6258	2.9709	2.8040	2.9709
12	5.0000	7.0000	4.0000	5.0000	5.0000	5.0000	3.0000	1.0000	2.0000	3.0000	6.0000		1.4112	1.9914	1.7265	1.7265	1.4112	2.2240
13	7.0000	9.0000	6.0000	7.0000	7.0000	7.0000	5.0000	3.0000	4.0000	5.0000	8.0000	2.0000		1.9914	1.7265	0.9989	1.9914	2.6258
14	7.0000	9.0000	4.0000	9.0000	5.0000	9.0000	5.0000	5.0000	6.0000	5.0000	10.0000	4.0000	4.0000		2.2240	2.2240	1.9914	2.6258
15	6.0000	8.0000	7.0000	6.0000	8.0000	8.0000	6.0000	2.0000	5.0000	6.0000	7.0000	3.0000	3.0000	5.0000		1.9914	2.2240	2.8040
16	8.0000	10.0000	7.0000	8.0000	8.0000	8.0000	6.0000	4.0000	5.0000	6.0000	9.0000	3.0000	1.0000	5.0000	4.0000		2.2240	2.8040
17	5.0000	7.0000	4.0000	7.0000	5.0000	7.0000	3.0000	3.0000	4.0000	3.0000	8.0000	2.0000	4.0000	4.0000	5.0000	5.0000		2.2240
18	6.0000	8.0000	5.0000	10.0000	8.0000	10.0000	4.0000	6.0000	5.0000	2.0000	9.0000	5.0000	7.0000	7.0000	8.0000	8.0000	5.0000	

The statistical results of each individual (relative frequency, standard deviation) and nucleotide composition of the haplotypes are shown in Table 6.7.

In summary, the following *Nycticebus c. coucang* haplotypes were found in 5 different areas within Peninsular Malaysia : We found haplotypes 1A, 3C, 6F, 7G, 9I, and 10J in the **Cempaka** individuals; 2B, 4D and 11K haplotypes found in the area of **Bukit Boloh**; 5E, 8H, and 17R found in those individuals at **Seremban Perhilitan Sanctuary**, at **Kampung Pasu** one haplotype 18S was found; those individuals sampled in **Zoo Malacca** presented 12L, 13M, 14N, 15P and 16Q haplotypes.

In the Bukit-Boloh, Group A Adult Female “Yuri” and Adult “Male-Boloh” both had the same haplotype (2B), while the rest of individuals of this group an adult female and baby grouped together (Adult female “Linda” and Infant male “Adopted”) presented haplotype (4D) and (11K) respectively.

Area of Cempaka Group “B”, three individuals (Adult Female “Timida”, Subadult Female “Hermosa” and Infant Male “Little”) presented the same haplotype (3C), and the other individual of this group (Male adult Aggressive) showed a different haplotype (6F).

All the individuals in Cempaka Group “C”, (Adult Male Cop, Adult Female Fala and Subadult male Gent) showed different haplotypes, (1A), (10J) and (9I) respectively. Within the group of Cempaka Group “D”, both individuals the mother and its infant born (Adult Female “Bonita” and “Baby-Born”) shared the same haplotype (1A). In the Cempaka Group “E”, all the three individuals (Adult Male “Eca”, Adult Male “Kro” and Sub-adult “IAB”) presented the same haplotype (7G). The haplotype found for the individual (Subadult Male “Fat”) from the area Kampung Pasu, was (18S).

Table 6.7 Haplotypes, Frequencies and Nucleotide Composition of each Individuals coming from Different Locations

Haplotype	Individuals	Shared Haplotypes	Relative Freq	S.D	Nucleotide Composition			
					T(U)	C	A	G
1A	Bonita, Baby-Born, Cop	3	0.115385	0.063897	27.7	26.9	31.6	13.8
2B	Male-Boloh, Yuri	2	0.076923	0.053294	28	26.7	31.4	14
3C	Timida, Little Agre, Hermosa	3	0.115385	0.063897	27.5	27.1	31.6	13.8
4D	Linda	1	0.038462	0.038462	28.2	26.5	32	13.3
5E	Garota	1	0.038462	0.038462	27.5	27.1	31.4	14
6F	Aggressive	1	0.038462	0.038462	27.8	26.7	31.5	14
7G	Kro, Eca, IAB	3	0.115385	0.063897	27.5	27.1	31.8	13.5
8H	Sadman	1	0.038462	0.038462	28	26.7	31.8	13.5
9I	Gent	1	0.038462	0.038462	27.7	26.9	31.8	13.5
10J	Fala	1	0.038462	0.038462	27.7	26.9	32	13.3
11K	Adopted	1	0.038462	0.038462	28	26.9	31.6	13.5
12L	1 Malacca	1	0.038462	0.038462	27.7	26.9	31.8	13.5
13M	2 Malacca, 5 Malacca	2	0.076923	0.053294	28	26.9	31.4	13.8
14N	3 Malacca	1	0.038462	0.038462	27.7	27.1	31.2	14
15P	4 Malacca	1	0.038462	0.038462	28	26.9	31.6	13.5
16Q	6 Malacca	1	0.038462	0.038462	28	26.7	31.6	13.8
17R	CURIOUS	1	0.038462	0.038462	27.7	26.8	31.8	13.7
18S	FAT	1	0.038462	0.038462	28	26.7	31.8	13.5
				Average	27.81	28.86	31.65	13.6

Note: Haplotypes (1A), (2B), (3C), (7G) and (13M) sharing identical mtDNA sequence, in agreement with the general rule that mtDNA is inherited maternally.

Those samples obtained from Seremban Perhilitan Sanctuary (Adult Male "Sadman", Adult Male "Curious" and Adult Female "Garota") showed different haplotypes, (8H), (17R) and (5E) respectively. Although there was no historical record from these animals, they were expected to have some ancestral relatedness due to they could have been brought from villages nearby the Seremban area.

Meanwhile, within those 6 individuals (1, 2, 3, 4, 5, and 6 Malacca) examined in Zoo Malacca, only two individuals the mother (Adult Female 2Malacca) and its infant (Infant Female 5Malacca) presented the same haplotype (13M). The other individuals 1, 3, 4 and 6 showed the haplotypes (12L), (14N), (15P) and (16Q) respectively.

6.3.3 Observation of the Genetically Maternal Inheritance.

6.3.3.1 Presence of Close Kinship: Intra-Groups and Inter-Groups

The results shown here are consistent with the social structure model of the species obtained in the field as well as the background from where the individual samples were obtained.

In total, 13 distinct mitochondrial DNA sequences were found to have similar nucleotides with two or three individuals from the group or outside the group. In those groups, the adult males, or females, infants, and subadults living in the group or in a nearby area, shared the same DNA sequence.

This is in agreement with the general rule that mtDNA is inherited maternally (Hutchinson et al. 1974). Although the sample size in this study is small, the results support the assumption inferred from the study field of these groups or

from where the sample were taken in the case of the Zoo Malacca and the Perhilitan Center.

The parallel use of PCR methods using genomic DNA and sequencing of the mitochondrial DNA segments amplified from blood samples, showed clearly interpretable results obtained for “Cop”, “Bonita”, “Baby-Born”, “Male-Boloh”, “Yuri”, “Timida”, “Hermosa”, “Little”, “ECA”, “Kro”, “IAB” and “2Malacca” and “5 Malacca”.

These 13 sequences are presented as case histories in order to emphasize the relatedness observed in this study. The protocol used in this study was tested on a relatedness cases below.

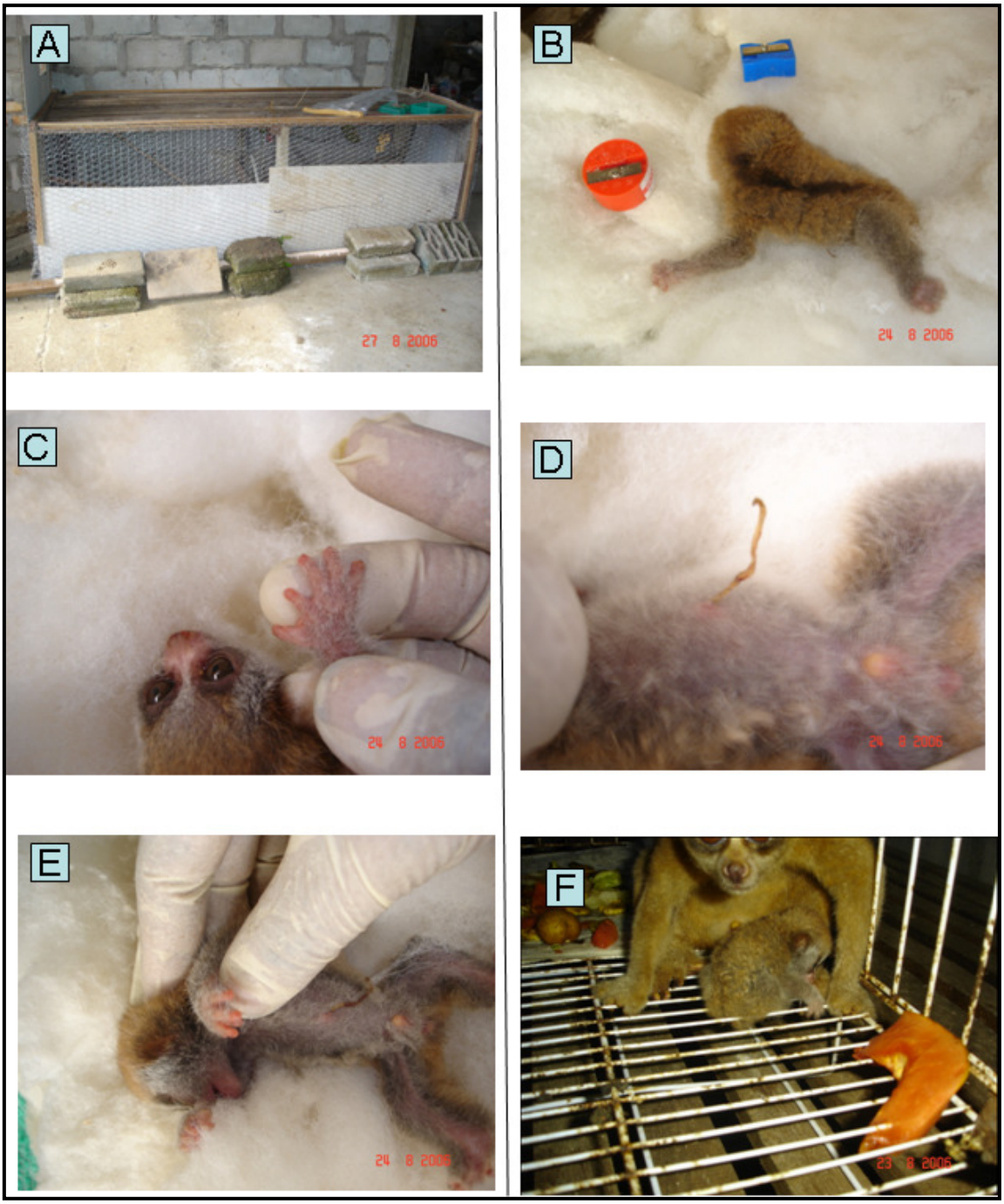
6.3.3.2 Case of Group “D” Cempaka Comprising “Bonita”, “Baby-Born” and Group C “Cop”

This female delivered a baby when observations were carried out in captivity (see Photo 6.1), and because this event was personally witnessed by the researcher, this special case was used as a control sample. Maternal inheritance of the mitochondrial genome was positive as expected.

On the other hand, “Bonita” and adult “Cop” from (Group “C”) shared the same sequences. It can be assumed from data observed in the field, such as “home range overlapping,” that this adult male knew well the home range of female “Bonita”, raising the assumption that it was previously its natal area, and hence explaining this direct maternal link (Figure 6.6).

```
COP          C A . T A . T . . A T G A G . . . . . . . C . T . G . . . . G . .
BONITA       C A . T A . T . . A T G A G . . . . . . . C . T . G . . . . G . .
NENE.BORN    C A . T A . T . . A T G A G . . . . . . . C . T . G . . . . G . .
```

Figure 6.6: The Matrilineal Sequence of 3 individuals in the Cempaka Groups C and D



Photos 6.1: (A) Temporary captivity place where “Bonita” and the baby were brought for few days to being observed before days later being release in the exact place where “Bonita” was trapped; (B) Sample comparison after hours later the baby was born, using soft cotton in order not to harm the fragile neonatal; (C) Comparison individual hand size, (D) and (E) Showing the size and indication of the cordon umbilical; (F) “Bonita” and “Baby-born” resting after few days before being released in the wild. Refer to Appendix 6.1 to see the baby after 6 months old.

6.3.3.3 Case of Group "A" Bukit Boloh Comprising "Male-Boloh", "Linda", "Yuri", and "Adopted"

"Male-Boloh" was observed moving within Linda's home range, although it was not radio-collared as mentioned in the previous chapter. A blood sample was taken from this individual "Male-Boloh". DNA sequence of this individual is the same as the Female Yuri. This female Yuri was trapped approximately 100 meters away from Linda's home range. It could be possible that natal's "Male-Boloh" home range was previously within Yuri's movement area and when he reached subadult age, he moved to its current area, thus explaining their maternal relationship (Figure 6.7).

Figure 6.7: The Matrilineal Sequence of 2 individuals in the Bukit Boloh Group A

As for the other two individuals "Linda" and "Adopted" that were tracked in the field (see Photos 6.2), although from the observations obtained the female displayed a great deal of maternal care towards the infant, also sharing sleeping sites (see chapter 5 ranging patterns), these individuals did not show any genetic relatedness.

It was expected their DNA sequence differ because they were trapped approximately four kilometer away from each other, and at the time of releasing them in the wild, Adopted was released together with Linda within Linda's home range, and it was very unlikely these two individuals were related (Figure 6.8).

Figure 6.8: The Matrilineal Sequence of 2 individuals in the Bukit Boloh Group A

Photos 6.2: Photos (A) and (B) showing Infant “Adopted” (Adopt) by the female “Linda” Color Comparison and Size. (C) and (D): “Adopt” infant being held by “Linda” before being released in the wild.

6.3.3.4 Case of Group "B" Cempaka Comprising "Aggressive", "Timida", "Hermosa", and "Little"

The results of spatial data obtained in the field and the patterns of genetic relatedness from (Group "A" & Group "C"), raised questions as to whether or not infant and subadult individuals were genetically related to the adult female "Timida". Field data analysis carried out on *Nycticebus c. coucang* patterns, inferred that individuals among this group were related; however, to accurately discern this "kinship", this researcher sought to prove this genetically.

Due to the maternal inheritance of the mitochondrial genome, it was expected that the sequences of the infant "Little", subadult "Hermosa" and adult "Timida" would be identical if "Timida" was indeed the mother. On the other hand, the mitochondrial sequence of the male father of the two young individual should differ from these sequences, and was therefore used as the control sample.

Figure 6.9 shows part of the sequence of H-strand of the *Nycticebus c. coucang* mitochondrial genome (GenBank accession AJ309867) of the infant, "Little" subadult Hermosa, female adult "Timida" and the adult male "Aggressive".

For the analysis, we compared the individuals sequence to the hypervariable segment 1 (mt HVS1) of D-loop *Nycticebus c. coucang* d-loop (GenBank accession AY875955) sequence. The results have shown that the sequences of the infant "Little", subadult "Hermosa" and female adult "Timida" have 34 identical base variations explaining their maternal relationship with "Timida".

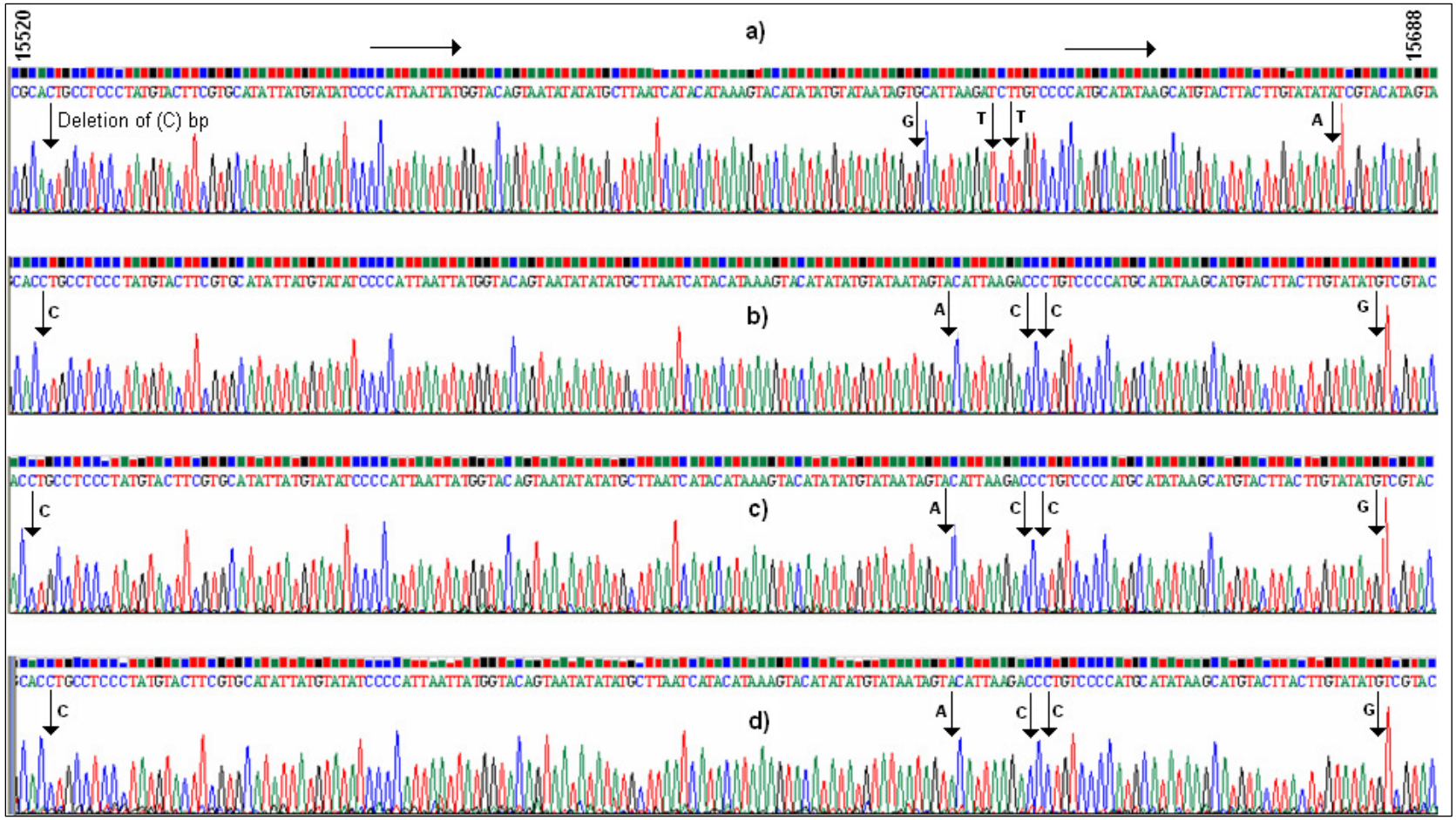


Figure 6.9: H–Strand DNA Sequence Plot in the first Hypervariable D-loop of mitochondrial DNA with 5 different base variations as compared to the *Nycticebus c. coucang* mitochondrial genome (GenBank accession No. AJ309867). This fragment corresponds to position 15520 to 15688 according to access number mentioned above. (a) Sequence plot for male adult father aggressive; (b) Sequence plot for the adult mother Timida; (c) sequence plot for the sub-adult female Hermosa; (d) Sequence plot for the infant male "Little". Different Nucleotide A,G,C or T and degree nucleotide confidence are represented by color. The horizontal arrow represents the direction of the strand.

In contrast, the sequence of the adult male “Aggressive” has 25 variable positions, and differed by nine other base variations (Figure 6.10). The remaining positions of the male father were identical with the GenBank accession AY875955 sequence.

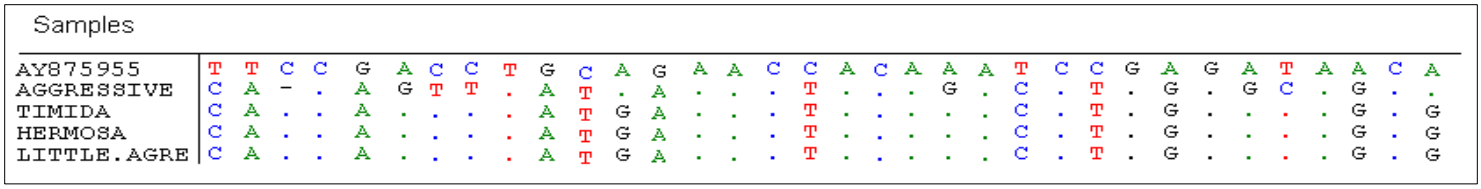


Figure 6.10: The Matrilineal Sequence of 3 Individuals in the Cempaka Group B

6.3.3.5 Case of Group “E” in Cempaka Comprising “Eca”, “Kro” and “IAB”

Two adult males “Eca”, “Kro” and one sub-adult male showed identical DNA sequences. These individuals were moving with the same home range and trapped within a few meters from one another. Male adults shared habitat and occasionally sleep on the same tree. Field data showed these two adult males sleeping in body contact on the same branch of the tree, sleeping behavior never observed among other groups.

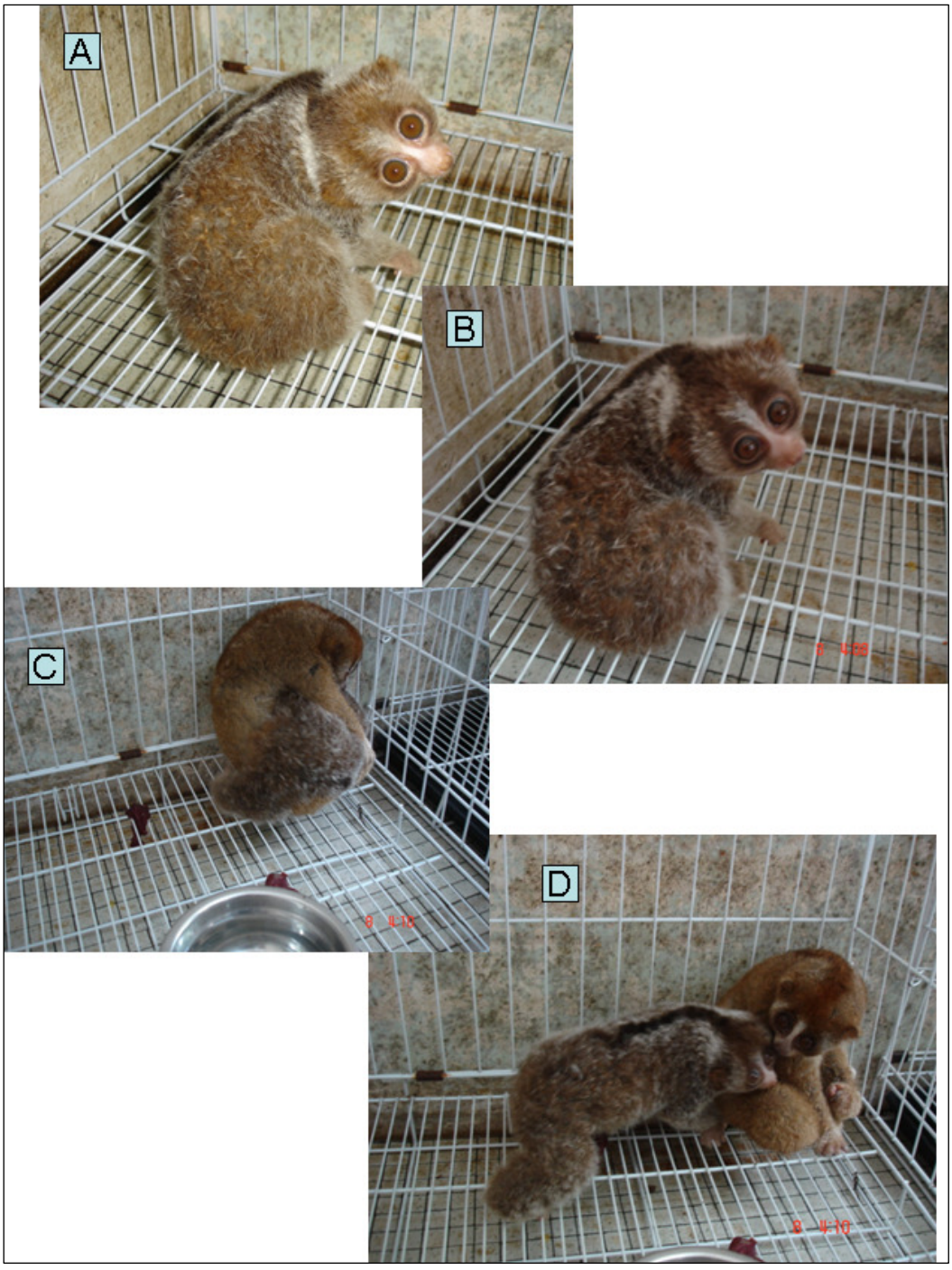
Moreover, the field showed 100% home range overlapping among these two adult males. Hence, the similarities in their maternal relationship may help to explain the field data mentioned. Since those individuals are genetically related, they have the tendency to be more tolerant and less aggressive, making them more likely to share the habitat, home range and sleep together (Figure 6.11).

Figure 6.11: The Matrilineal Sequence of 3 individuals in the Cempaka Group E

6.3.3.6 Case of Zoo Malacca Individuals “2Malacca” and “5Malacca”

From 6 samples collected in the Zoo Malacca only two individual showed genetic maternal inheritances (Figure 6.12). This is the case of one adult female “2 Malacca” and one infant female “5Malacca” which were in the quarantine room sharing the same cage (Photos 6.3). Villagers from Malacca donated these pair of individual for the welfare of the animal according to the local wildlife authorities.

Figure 6.12: Matrilineal Sequence of 2 individuals in the Zoo Malacca



Photos 6.3: (A) and (B) Infant female (“5Malacca”); (C) Mother (“2Malacca”) and its baby (“5 Malacca”) mother protecting the baby before blood sample being taken for genetics evaluation; (D) Infant looking for mother’s protection in the cage.

6.4 Pedigrees Demonstrating the Inheritance of Maternal Mitochondrial DNA among Eight Groups of Slow Loris.

In this section, we are attempted to see the correlation in the pedigrees seen by field work and molecular findings. We tested the presumed maternal pedigrees seen by direct observations (Figure 6.1) with a distinct method using genetic data from D-loop mitochondrial sequencing. We then correlated both field observations with genetic data to resolve the kinship among individuals in each group tracked. The genetics findings can be further grouped based on their degree of genetic relationships; namely parent-offspring or siblings (Figure 6.13).

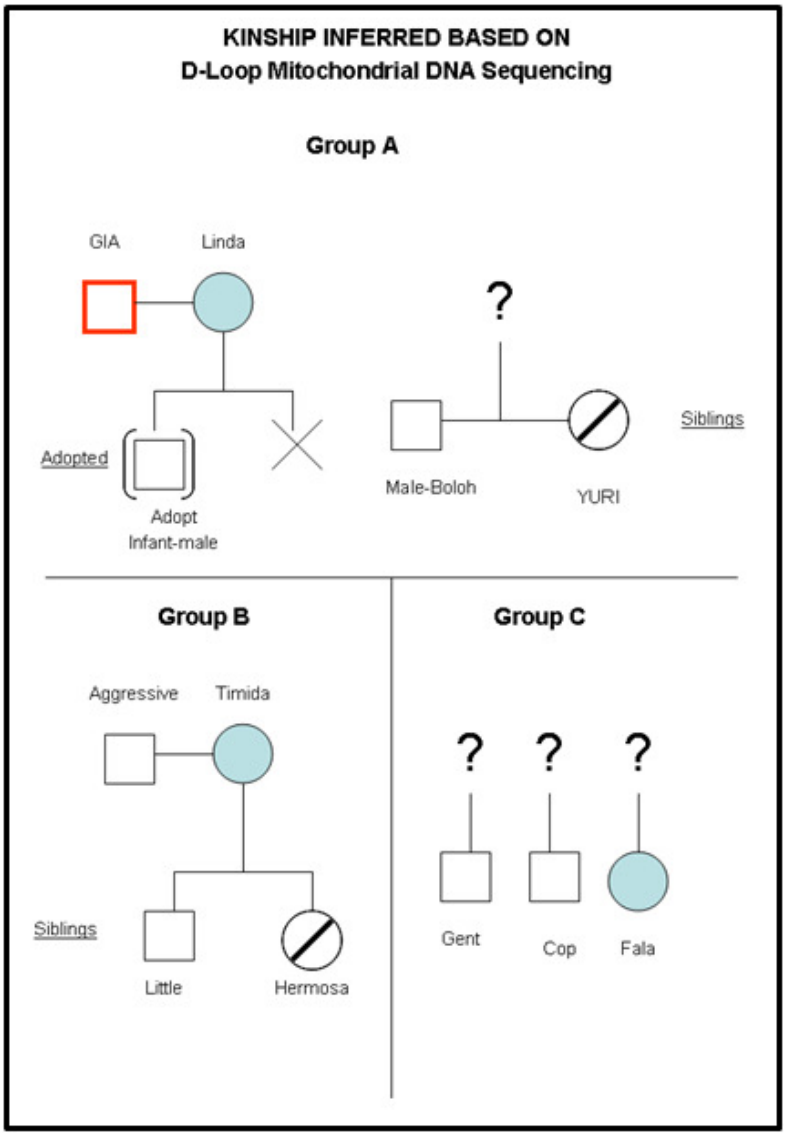
6.4.1 Parent and Offspring

First degree relationship for parent and offspring relationship (i.e: maternal pedigrees) were established for several groups (Figure 6.13). Group A ("Linda" and its adopted infant) different haplotypes are shown. Group "B", Group "D", Group "E" and "Malacca Zoo" have identical haplotypes at multiple alleles by descent (Table 6.3). However, we could not establish the maternal relationship for individuals in Group C as were inferred previously in Section 6.1 by their behavior in the wild. In summary, perfect correlations with field study were seen in the mother and offspring first degree of genetic relationship for the following families: "Linda" (Group A), "Timida" (Group B), "Bonita" (Group D) and "2Malacca" (Group Malacca Zoo) (Figure 6.13).

6.4.2 Siblings

Confirmation of sibling relationship between all 3 individuals in Group "E" was achieved by a defined inheritance of their maternal DNA which was previously assumed from the field observation differently (see Figure 6.1 from this chapter). For "Male-Boloh" and "Yuri" in Group "A", there was no expected sibling

relationship between them based on field tracking but we could see sibling's genetic linkage by the similarities at mitochondrial DNA in which they share the same biological parent of unknown identity. Furthermore, the same case of sibling relationship were observed among the individual "Bonita" and "Cop" were was not inferred any type of relatedness after radio-telemetry study but genetically it was proved these individuals were related and was inferred sibling relationship among them (Figure 6.13).



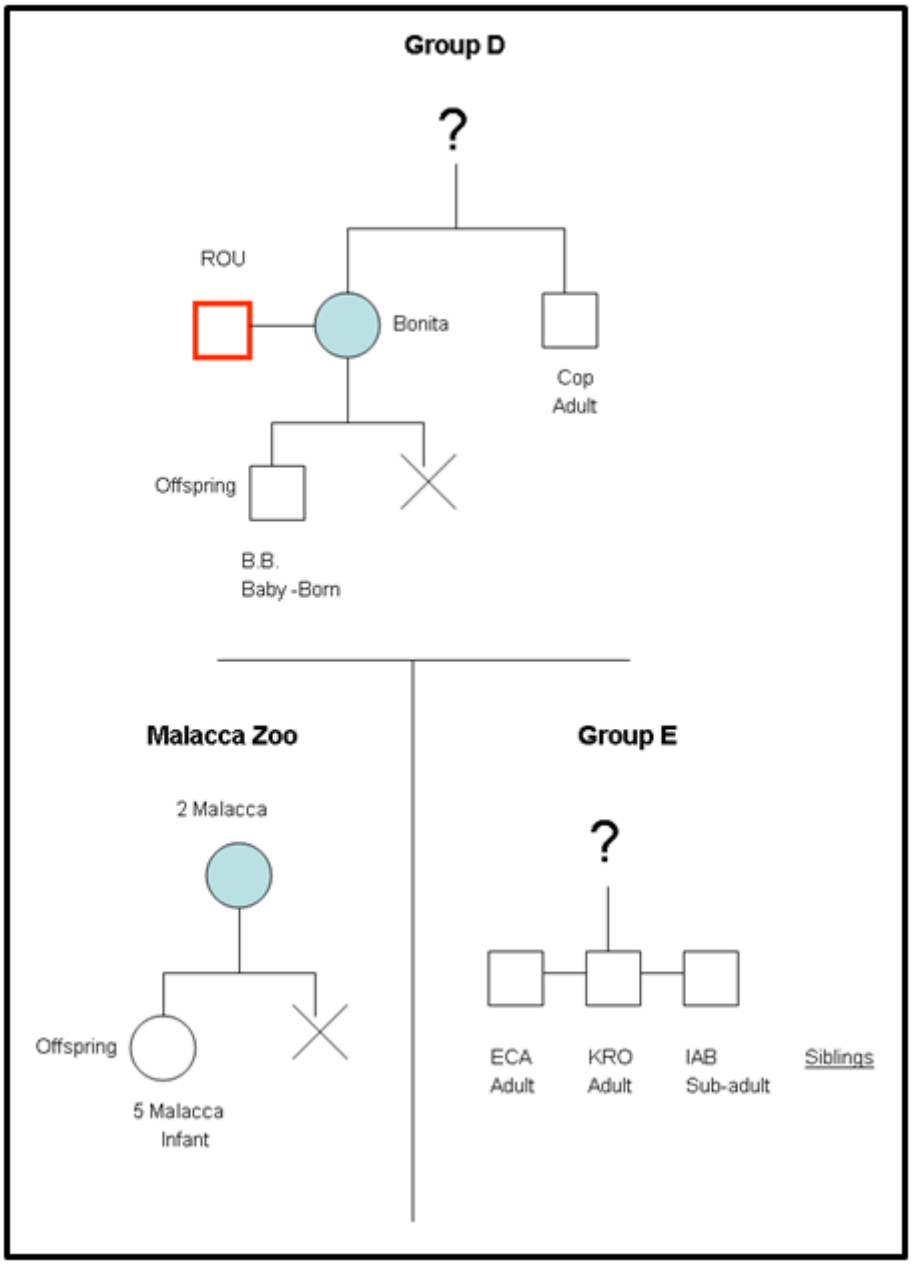


Figure 6.13: Pedigrees for Group A – Group E were drawn for the same selected animals as in Figure 6.1 based on haplotype uniformity within the mtDNA D-Loop region. Open square- male; filled circle- female with progeny; slashed circle- female without progeny; red square- individual (male) was not caught but locations trapped was recorded; blue square- individual caught but was not tracked. (X) The X in group A,D and Malacca Zoo represents that there was no seen another offspring with these putative parents.

6.5 Phylogenetic Trees

Phylogenetic analyses of the sequence data of the mtDNA D-loop containing the first variable region were done with the following methods: Maximum Parsimony (MP); UPGMA Maximum Composite Likelihood (ML); and Neighbor-Joining (NJ). The same results were consistently obtained when the three MP, ML NJ, methods were used.

We used the reference sequences of 'outgroup' (*Nycticebus c coucang*, GeneBank No. AY875955) and the D-loop sequences of the *Nycticebus c. coucang* samples obtained in Peninsular Malaysia for reconstruction of phylogenetic trees. The analysis involved 27 nucleotide sequences. There were a total of 391 positions in the final dataset.

Using D-loop sequences, we observed 13 of these sequences of *Nycticebus c. coucang* samples to be monophyletic with 100 % bootstrap support in all analysis. The rest of the sample does share a common ancestor, showing to some extent an evolutionary relationship, depending on the localities from where the sample was taken.

6.5.1 Maximum Parsimony

The tree shown in Figure 6.14 reveals that individual sequences shared characters in different entities resulting from a common descent, there being 156 parsimonious trees (length = 51). Molecular data strongly suggest that individuals from the Cempaka and Bukit Boloh areas are more closely related to each other than individuals from coming from the more distant locations in Seremban and Melaka. By using the Maximum Parsimony method, all positions containing gaps and missing data were eliminated. The consistency index is 0.666667 (0.468750), the retention index is 0.738462 (0.738462), and the composite index is 0.492308 (0.346154) for all sites and parsimony-informative sites (in parentheses).

Figure 6.14: Unrooted Maximum Parsimony tree with bootstrap values, showing relationships among 27 partial mtDNA D-loop sequences from *Nycticebus c. coucang* within several localities of Peninsular Malaysia: (1) Bukit Boloh (Group A); (2) Cempaka (Group B); (3) Cempaka (Group C); (4) Cempaka (Group D); (5) Cempaka (Group E); (6) Kampung Pasu (Pahang); (7) Zoo Malacca; (8) Seremban Perhilitan Sanctuary.and *Nycticebus c. coucang* haplotype (GenBank No. AY875955).

6.5.2 UPGMA (Unweighted Pair Group Method with Arithmetic Mean) Computed with (ML)

Using the UPGMA method, a phylogenetic tree for a 27 *Nycticebus c. coucang* aligned sequences was digitally created.

This dendrogram recreates an ancestor to one of these subbranches, showing the distance relationships among these partial mtDNA D-loop sequences from individuals within several localities of Peninsular Malaysia. The optimal tree with the sum of branch length = 0.12208916 is shown (Figure 6.15). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 391 positions in the final dataset.

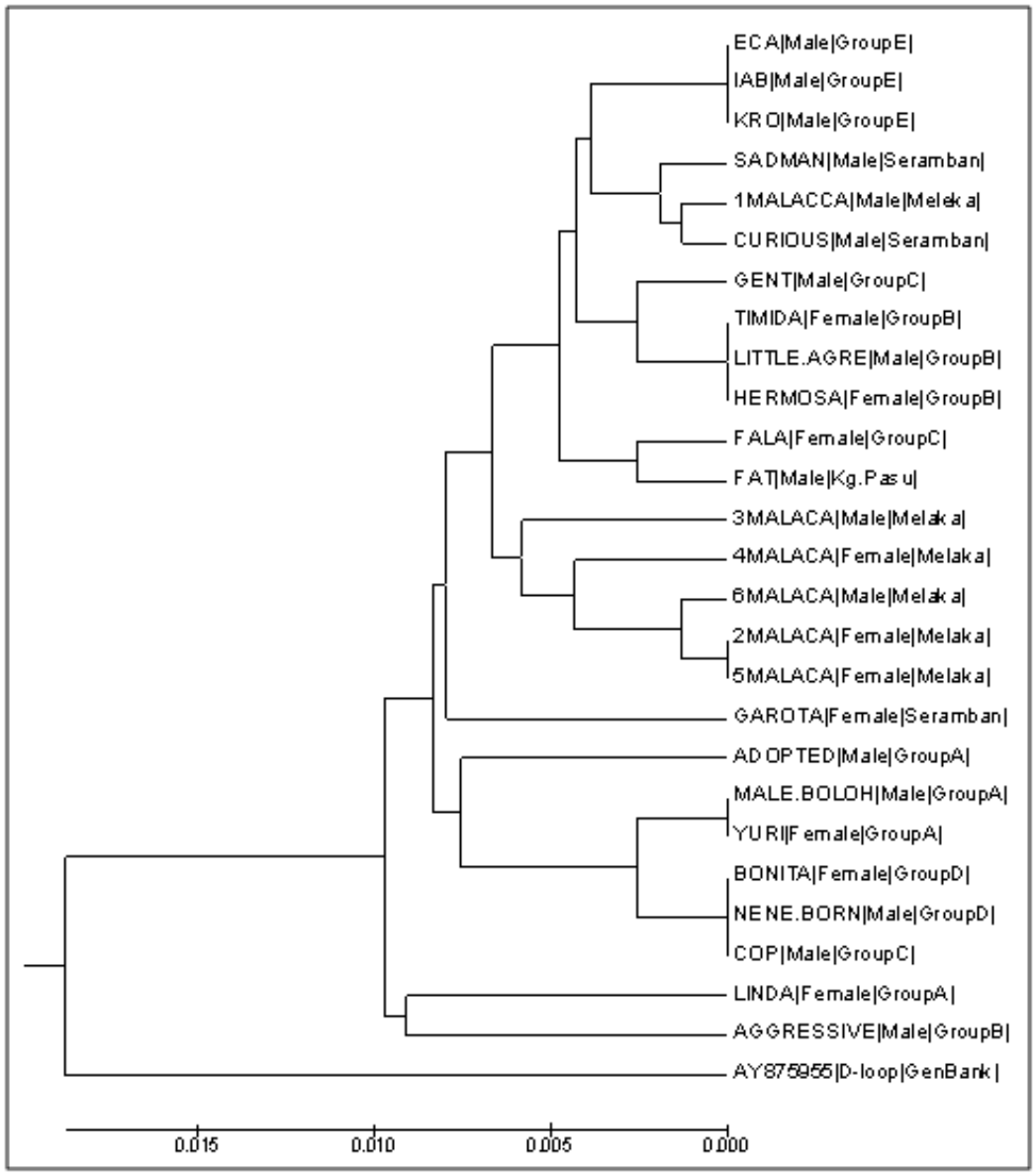


Figure 6.15 Rooted UPGMA (ML) Phylogenetic Tree showing Distance Relationships between 27 partial mtDNA D-loop sequences from *Nycticebus c. coucang* from several localities in Peninsular Malaysia, and an outgroup (*Nycticebus c. coucang* GenBank accession No. AY875955) .

6.5.3 NJ Computed with (ML)

Evolutionary distances between sequences from these 27 animals that were sampled within several localities of Peninsular Malaysia were computed using the “Neighbor-Joining” method.

In this phylogenetic tree, neighbors were joined by a branch to the same node as well as finding the correct branches lengths, thus inferring a common ancestor. The core of this tree is similar with that of the UPGMA tree, and, therefore, confirming the relationships as correct.

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The optimal tree with the sum of branch length = 0.12245568 is shown (Figure 6.16). The evolutionary distances were computed using the Maximum Composite Likelihood method choosing the best tree and are in the units of the number of base substitutions per site.

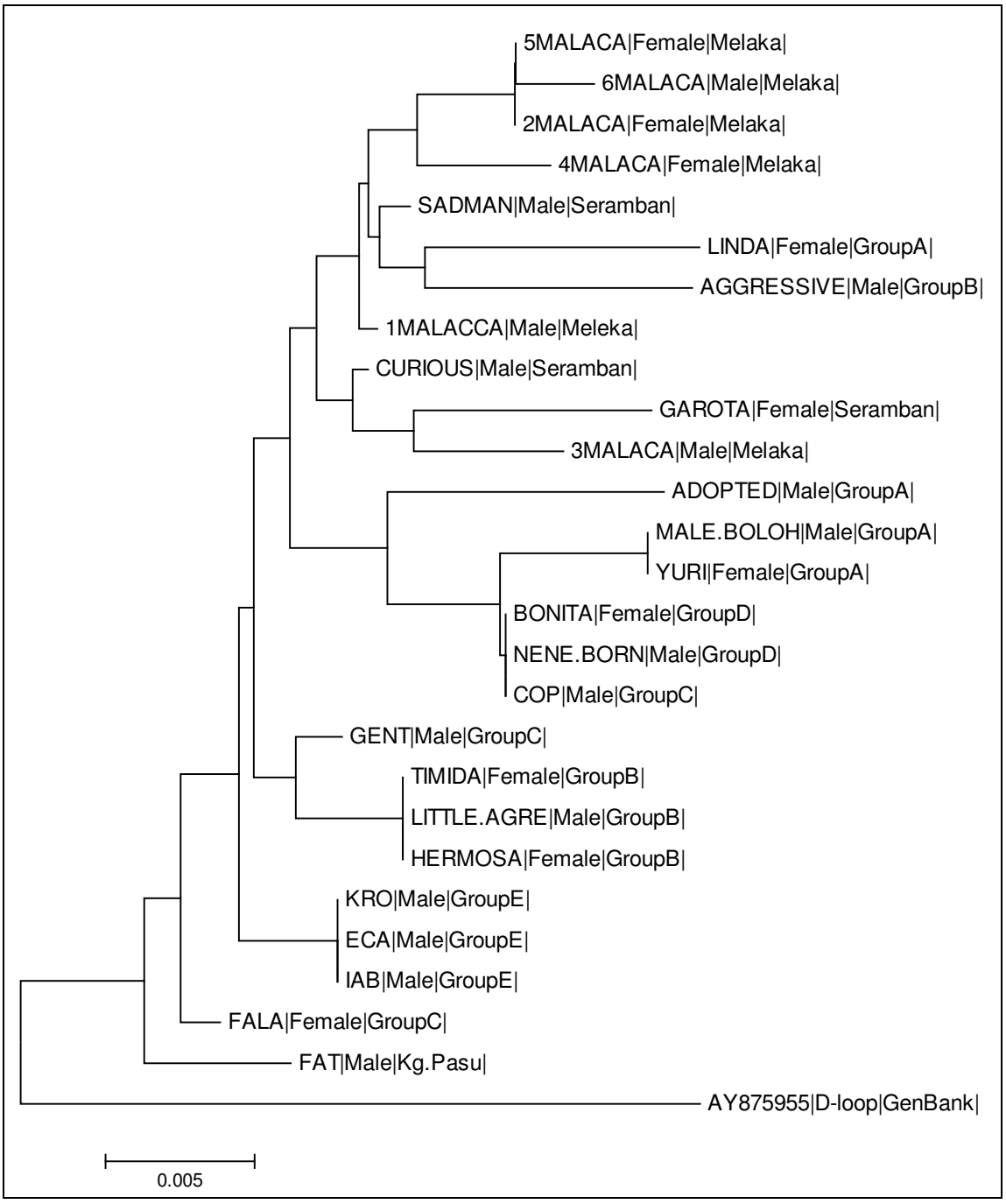


Figure 6.16: Rooted NJ Tree showing Evolutionary Distances among 27 partial mtDNA D-loop sequences from *Nycticebus c. coucang* from several localities in Peninsular Malaysia, and an outgroup (*Nycticebus c. coucang* GenBank accession No. AY875955).