CHAPTER III

MATERIALS AND METHODS

1. Studied Population

Five completed elephant families (father, mother and calf) with clear pedigree were used in this study. They were altogether 22 elephants, consisting of 5 fathers, 7 mothers and 10 calves, as shown in Table 2. One female (Mae Noi 2 - CM1-C-NO2) mated with two males (Kher - CM1-B-KH and Yai - CM1-B-YA). The calves were 6 males and 4 females. The families were selected from Mae Sa elephant camp, Chiang Mai and Thai Elephant Conservation Center (TECC), Nation Elephant Institute, Lampang. The elephants were given a code as the following.

Elephant code

Code example : CM1-B-XX, LP1-C-YY

Code detail :

- Place CM1 = MSEC : Mae Sa elephant camp, Chiang Mai

  LP1 = TECC : Nation Elephant Institute, Lampang
- Sex B = Bull (father)

  C = Cow (mother)

  Cb = Calf (bull)

  Cc = Calf (cow)
- Elephant's name = XX, YY

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Table 2 Family and individual data of elephants.

Place and Family	ID-Name	Sex	Code	Sample	Remark
TECC-01	Tadang	male	LP1-B-TD	whole blood	
	Singkhorn	female	LP1-C-SK	whole blood	
	Nuer-un	male	LP1-Cc-NU	hair follicle	
TECC-02	Pamae	male	LP1-B-PM	whole blood	
	Pumpoung	female	LP1-C-PP	whole blood	
	Alena	female	LP1-Cc-AN	hair follicle	
MSEC-01	Boonpeng	male	CM1-B-BP	whole blood	
	Nid	female	CM1-C-ND	hair follicle	
	Duanpen	female	CM1-Cc-DP	whole blood	
MSEC-02	Kher	male	CM1-B-KH	whole blood	
	Mae-Noi 2*	female	CM1-C-NO2	whole blood	
	Cometool	female	CM1-Cc-CT	whole blood	
MSEC-03	Yai (Sidor)	male	CM1-B-YA	whole blood	
	Mae-Noi 2*	female	CM1-C-NO2	whole blood	mother 1
	Wanpen 2	female	CM1-Cc-WP2	hair follicle	
	Mho	female	CM1-C-MH	hair follicle	mother 2
	Lancome	male	CM1-Cb-LC	whole blood	
	Tongpoon	male	CM1-Cb-TP	whole blood	
	Souy	female	CM1-C-SY	hair follicle	mother 3
	Songpan	male	CM1-Cb-SP	whole blood	
	Wengping	male	CM1-Cb-WP	whole blood	
	Somnok	female	CM1-C-SN	hair follicle	mother 4
	Tongtawee	male	CM1-Cb-TT	whole blood	

* = same animal

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2. Sample Collection

Two types of samples, whole blood and hair follicular, were collected depending on elephant's age (young or baby) and attitude (fear or aggressive) as well as their owner's permission. The list of samples is shown in Table 2.

2.1 Whole blood samples

Approximate 5 ml of venous peripheral blood from ear vein of each elephant was collected by veterinarian using disposable 1 inch, 18G needle and 10 ml syringe with aseptic procedure. The blood was transferred into a vacuum tube coated with an anti-coagglulant, ethylenediaminetetracetic acid (EDTA), and then transported under the cooling condition to the laboratory. After that, the whole blood was centrifuged at 2500 rpm, 10 min and buffy coat were separated for further DNA extraction. The samples were preserved in 1.5 ml micro-centrifuge tube at -20°C.

2.2 Hair follicle samples

When the whole blood could not be collected, the hair with its follicle was taken instead. Thirty hairs with follicles were extracted from each elephants and kept in a sterile plastic bag during transportation to laboratory. Around 10 hair follicles were separated from hair shafts and put into a 1.5 ml sterile micro-centrifuge tube. After that the samples were stored in -20°C freezer for further DNA extraction.

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3. DNA Extraction

3.1 DNA extraction from WBC in buffy coat

Frozen buffy coat was thawed and centrifuged at 3,000 rpm for 5 minutes, and the supernatant was discarded. The commercial DNA extraction kit, QIAamp® DNA

Blood Mini Kit, was used for DNA extraction (Appendix B). Then the extracted DNA was re-suspended in 150 µl eluted buffer, and stored in -20°C freezer. DNA quality was checked with 1% agarose gel electrophoresis.

3.2 DNA extraction from hair follicle samples

DNA from hair follicle sample was extracted following Chaleamchat's protocol (Somgird *et al*, 2005) adapting from Srikummool (1998) and Siripunkaw (2003), as follows. Hair follicles were digested by 500 µl of solution D (4 M guanidine thiocyanate, 25 mM sodium citrate , 0.5% N – lauroylsarcosine, pH 7.0) and 20 µl of 20 mg/ml proteinase K, incubated at 37°C by using shaking water bath for at least 5 hours or until the tissue was completely lysed. The solution appeared viscous and blackens with hair pigment. Five-hundred µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the lysed sample. The tube was shaken till the emulsion was formed. After centrifugation at 10,000 rpm for 5 minutes, the supernatant was collected and transferred to a new 1.5 micro-centrifuge tube. The phenol-chloroform-isoamyl alcohol extraction was repeated. After that, the equal volume of chloroform-isoamyl alcohol (24:1) was added to the collected supernatant to remove the phenol residual. The tube was shaken till the emulsion was formed. The supernatant was collected and transferred to new tube after centrifugation at 10,000 rpm for 5 minutes. DNA was precipitated by adding an equal volume of pre-cooled isopropanal, centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and DNA pellet was washed with 70% ethanol. DNA pellet was allowed to dry at room temperature, and then re-suspended in 150 µl TE buffer and stored in -20°C freezer. DNA quality was checked with 1% agarose gel electrophoresis.

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4. DNA Amplification

4.1 Oligonucleotide Primers

Three highly polymorphic microsatellite loci reported by Siripunkaw (2003): LafMS03, FH94 and FH102 were used in this study. The selected oligonucleotide primers are listed in Table 3.

Table 3 Oligonucleotide primers for 3 microsatellite markers

Locus	**Repeat motif**	**Primer**	**Sequence from 5′ to 3′**	**Primer Length**	**Tm**
LafMS03	$(TG)_{15}$	MS03 F	CATATGAACATACCGGAAC	19	54
		MS03 R	GAAACTCCTCGAGTAGTAGAA	21	60
FH94	$(CA)_{16}$	FH94 F	TTCCTCCCACAGAGCAGC	18	58
		FH94 R	ATTGGTTAATTTGCCAGTCCC	21	60
FH102	$(CT)_{11}(CA)_{14}$	FH102 F	TTCATTACTGACCTAAACGAG	22	58
		FH102 R	GGACAGGGCTGGAGAAATATG	21	64

Tm (°C) = (A+T) x 2 + (G+C) x 4

Two regions of mtDNA, partial D-loop (Srikummool, 1998) and 5′ end cytochrom *b* (Lertwatcharasarakul *et al*, 2003) were selected for sequencing, using the following primers:

partial D-loop ELE 31 ; ACCCAAAGCTGAAATTCTCTTTAAA

ELE 32 ; TGAAGTAGGAAGCAGATGCCAGGTA

Tm is 55 °C and product size is ~500 bp

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5′ end cytochrome b Mito-CB1 ; TCCAACATCTCAGCATGATGAA

Mito-CB2 ; CTCAGAATGATATTTGTCCTCA

Tm is 53 °C and product size is 352 bp

All primers were purchased from Pacific Science®, Bio Basic Inc.

4.2 DNA amplification

4.2.1 For microsatellite DNA

Polymerase Chain Reaction (PCR) was performed using a PTC-2000 DNA Engine Cycler, DNA Engine®. The reaction was carried out in 25 µl volume containing 2 µl DNA solution, 50 mM $MgCl_2$, 5 mM of each dNTP, 5 pmol of each primer and 2.5 units *Taq* DNA polymerase (Sigma®) in a 1x PCR buffer. The thermal profile was as follows: initial denature at 95°C for 10 min, followed by 35 cycles of; 95°C, 30 sec of denature, 30 sec of annealing at different temperatures, FH94 and FH102 at 60°C and LafMS03 at 50°C, and 72°C, 30 sec of extension. After finishing the last cycle, the reaction tube was incubated at 72°C for 10 minutes. PCR products were checked by electrophoresis using 1% agarose gel in 1x TBE buffer at 80 volts for 60 minutes, and stored at -20°C prior to fragment size analysis.

4.2.2 For mitochondrial DNA

PCR reaction was performed as the above protocol for microsatellite DNA using the aforementioned pair of primers in section 4.1. The thermal profile was as follows: initial denature at 95°C for 10 min, followed by 35 cycles of; 95°C, 30 sec of denature, 60°C, 30 sec of annealing, and 72°C, 30 sec of extension. After finishing the last cycle, the reaction tube was incubated at 72°C for 10 minutes. PCR products were checked by electrophoresis using 1% agarose gel in 1x TBE buffer at 80 volts for 60 minutes, and stored at -20°C prior to sequencing.

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5. Fragment analysis (GeneScan)

Three di-nucleotide repeated microsatellite loci (LafMS03, FH94 and FH102) of 22 elephant individuals were genotyped at the Molecular Laboratory of Utrecht University, The Netherlands, using the following procedure. The forward primers were tailed with M13 sequencing tags (5'-GTT TTC CCA GTC ACG AC-forward primer), and indirectly labelled with FAM (FAM-GTT TTC CCA GTC ACG AC-3'), which allowed the analysis on an automated ABI PRISM 3130XL Genetic Analyser. As for the M13-tailed primers, a reaction mix used containing 25 ng template DNA, 0.8 μM forward primer, 1 μM reverse and labelled-M13 primer, 3 mM dNTP, and 0.5 unit AmpliTaq Gold (Perkin Elmer), in 1x PCR buffer II with 37.5 mM $MgCl_2$. DNA was initially denaturated at 95°C for 5 min and was then subjected to 35 cycles of denature at 95°C for 30 sec, annealing for 40 sec at different temperatures, FH94 and FH102 at 60°C and LafMS03 at 50 °C, and extension at 72°C for 60 sec, followed by a final extension step for 5 min at 72°C. PCR products were run with Liz 500 (ABI) size standards on an automated ABI 3130XL DNA Analyzer, using the protocol describing in Appendix C. Genescan 4.0 software (ABI) was used for genotype assessment.

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The microsatellite loci were delineated a genotype based on the size of amplified PCR products. The alleles were scored using GeneScan 4.0 (ABI), ignoring stutter bands and stray amplified products, identifying the highest peak (for homozygote) or the two highest peaks (for heterozygote). The stutter bands, representing by smaller peaks and locating just prior to the real alleles, are typical feature occurring with dinucleotide microsatellite amplification by a slippage of *Taq* polymerase activity. The stray bands arise from a non-specific PCR amplification.

Due to the enzyme specific property, *Taq* DNA polymerase often adds a non-template adenine to the 3' end of amplified fragments during PCR amplification. Thus, each size scored was reduced by 1 base pair to give the actual nucleotide length of the microsatellite allele size.

6. DNA sequencing

PCR products of mitochondrial DNA from female elephants and their calves were sequenced by cycle sequencing (BigDye, Applied Biosystems) or automatic gel electrophoresis (ABI Prism 3130XL), followed the protocol describing in Appendix D.

7. Data analysis

DNA fragments of polymorphic microsatellite loci of each elephant in each family were compared and the parentage test and kinship relation were checked. The calf should have one fragment figure similar to its father and another one similar to its mother for each locus. The results were also compared between other elephants and families.

Frequency distribution of alleles at each locus were summarized. The power of discrimination (PD) and power of exclusion (PE) were calculated as follows,

$PD = 1-\sum(P_i)^2$

P_i : frequency distribution of phenotype

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$$PE = \sum_{i=1}^{n} P_i^2(1-P_i)^2 + \sum_{\substack{i=1 \\ j=1}}^{n} 2P_iP_j(1-P_i-P_j)^2$$

n : number of allele

P_i, P_j : frequency distribution of allele

$$Combined\ PD = 1-(1-PD_1)(1-PD_2)....(1-PD_n)$$

$$Culmulative\ PE = 1-(1-PE_1)(1-PE_2)....(1-PE_n)$$

n : number of combined markers

The sequences of mitochondrial DNA were analyzed using the multiple sequence alignment program, LaserGene biocomputing software package (SeqMan 5.08; DNA Star Inc.) and Clustal X. DNA sequences between mother and calf were compared in order to determine the maternal inheritance and indicate the mother and calf relation.

8. Location of research operation and data collection

Elephants' pedigrees and their samples were collected from two elephant camps, the Thai Elephant Conservation Center (TECC), Nation Elephant Institute, Lampang province and the Mae Sa Elephant Camp, Chiang Mai province.

Laboratory works, the DNA extraction and DNA amplification, were done at Central Laboratory, Faculty of Veterinary Medicine, Chiang Mai University. The fragment analysis and DNA sequencing were performed at the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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