CHAPTER V

DISCUSSION

The successfully extracted DNA from blood leucocytes and hair follicles were amplified at the microsatellite loci, FH94, FH102 and LafMS03, and at the cytochrome *b* region of mtDNA. Only a few hair follicle samples could not be amplified on the first try, but when more DNA template was used the amplification was successful. This failure of hair follicle DNA amplification might be caused by the low yield of DNA, DNA degradation or the left over of hair debris. When the PCR products were examined using the agarose gel electrophoresis, the fragment size of all products comparing to DNA marker were as expected. DNA fragment size determination could be done in all DNA samples, but the DNA sequencing using the DNA from hair follicles had to be repeated in a few samples, due to poor DNA quality.

As for the parentage test, the bi-parental inheritance, which means an elephant calf in each family inherited one allele from the father and another allele from the mother (Randi *el al*, 2002), was observed, except in TECC-02 and MSEC-03 families at the locus FH102. The LP1-Cc-AN (TECC-02) inherited only 203 allele from the mother (LP1-C-PP) and CM1-Cb-SP (MSEC-03) inherited only 223 allele from the father (CM1-B-YA).

The non-matched alleles in the two families might be due to the effect of the null alleles, the alleles that fail to produce a visible product caused by the mutation at

a primer binding site (Devoody *et al*, 2006, Kalinowski *et al*, 2006). This sensitive point will easily lead to the failure of the polymerase chain reaction, since it does not perfectly match those at the priming site on the DNA template anymore. Therefore, no amplified DNA is observed in agarose gel electrophoresis, or in case of heterozygous the homozygote mistyping can occurred.

These null alleles will bias allelic frequency by overestimating the visible alleles and underestimating the observed heterozygosity (Devoody *et al*, 2006). The null allele effect can be proved by multiplex- PCR using two primer pairs (Siripunkaw , 2003). to amplify the DNA fragments derived from null allele primer and another allele at the same locus. The PCR product is then subjected to electrophoresis or electrophorogram. If the result shows a clear absence of amplified product at that locus, then it is confirmed that the locus is true null allele locus. Actually the locus with null alleles is not good to be used as genetic marker, therefore the results from this locus should be excluded. Generally, in animal population the probability of null allele occurs around 2P (Phupat, 1994).

There was an interesting observation at the locus FH102 in this study. The largest observed fragment (allele 223) is different from the next smaller fragment (allele 209) by 7 repeats. That means the mutation had to occur 7 times in the past, due to step-wise mutation hypothesis, in order to create the allele 223. Since the mutation rate of the microsatellite in mammal is very high, approximately 10^4 per locus per generation (Frankham, 2002), and the existing elephants diverged from a common ancestor some 5 million years ago with long evolution, the allele 223 should be the real occurrence in the studied population. The finding of allele 223 was also reported by Thitaram *et al* (2007), analyzing microsatellite FH102 from 19 Asian

elephants. The alleles locating between alleles 209 and 223 were also discovered in another study of FH102 in 128 Asian elephants from 3 populations (Thitaram's unpublished data). Siripunkaw (2003) reported the FH102 alleles analyzing from 20 Asian elephants to be 179, 183, 185, 187, 191, 193, 213, 217 and 225, which also showed the large gap of alleles between 193 and 213 (by 10 repeats). Thus, the FH102 locus seems to be highly polymorphic with the total of 23 alleles, ranging from 179 to 225, but only 17 alleles were observed in the present study (Siripunkaw,2003 and this study).

The eletrophorograms showed stutter bands and stray bands that located prior to the true alleles. The stutter band peak is smaller than the peak of true allele consisting of one peak or series of peaks. Stutter band is arising from the slippage of *Taq* polymerase activity, while stray band is arising from non-specific PCR amplification (Walsh *et al*, 1996 and Siripunkaw , 2003). These kinds of bands are particularly prominent in dinucleotide microsatellite (Armour *et al*, 1999). Thus, the occurrence of stutter bands helped to ensure the microsatellite amplification product (Siripunkaw, 2003).

In this study the Liz 500 size standard (ABI), that covered all expected size of three microsatellte loci PCR products of 22 elephants listing in Table 1, was used. The genotypes of all elephants were determined and the allele size ranging between 230-238 bp at LafMS03, 197-223 bp at FH94 and 153-171 bp at FH102 were observed. The results were quite similar to a previous studies that reported by Nyakaana (1998), Comstock (2000) and Siripunkaw (2003). Besides the presence of microsatellite PCR products, the electropholograms also detected peaks of stutter bands and stray bands. The stray bands were non-specific amplification products

presenting at farther distance from the true, high peak, alleles, and the stutter bands were those with much lower peak locating just prior to the true alleles (Siripunkaw 2003). However, the stutter bands, which were longer than the main alleles, were observed at low levels at some dinucleotide repeats loci (Walsh *et al*, 1996).

The presence of non-specific fragments and stutter bands caused greater or less problem in genotyping. Siripunkaw (2003) reported that the non-specific fragment could be ensured by their locations that located outside the expected size range of the microsatellite PCR product. If the non-specific amplified product was located within the expected size range, one should look for the presence of stutter bands first, since the presence of stutter bands was expected when microsatellite was dinucleotide repeats. If there was no sign of stutter band, the peak would be recognized as a non-specific amplification. The stutter bands might lead to genotyping problem with a slippage mechanism by *Taq* polymerase, especially in the homozygous cases. If the slippage occurred in the early stage of PCR cycle, the stutter band might be yielded in high content and could be detected as relatively the same peak height as the true allele. Therefore, it would be recognized as a heterozygote instead of homozygote. However, in this study almost non-microsatellite PCR products were evaluated as stutter bands, and a few were non-specific amplification that might cause by contamination of skin normal flora or organism in dust that cover the skin which occurred only in hair follicle samples.

The power of discrimination (PD) and the power of exclusion (PE) of all loci, as ssummarized in Table 8, showed the high value. The PD values were 0.7830, 0.9043 and 0.8675 at the loci FH94, FH102 and LafMS03 respectively. The PD value which is nearly to 1 means that the locus has strong discrimination power, thus that

locus is suitable to use for discriminating one individual from another (Phupat, 1994). When only one locus or a single marker, such as FH94, was used, two animals would have the probability of having different genotype around 78.30 percent, or the probability of those two animals having the same genotype would be 22.70 percent. However, the PD value can be increased when the poly-marker system is used (Boeling *et a*l, 1997, Chakraborty *et a*l, 1998 and Mommens *et al*, 1998). The PD value of FH94 is lower than the other two loci due to its small number of alleles (Chun Xu *et al*, 2005), not like the FH102 which is highly polymorphic.

The combined PD, calculated by using the poly-marker system of three markers or loci (FH94, FH102 and LafMS03) was 0.9972. The combined PD is very high in this study, comparing to 0.9999 percent in the study in tigers using 7 loci of microsatellites (Chun Xu *et al*, 2005). The PE value of each locus is low, i.e., 0.2789 at FH94, 0.4604 at FH102 and 0.3654 at LafMS03. Such a low PE value might due to the closed relationship between all samples used in this study, since they are from five elephant families, including father, mother and calf (n=22). Similar result was found in Mommens, *et al* (1998) who reported that the PE value at each locus of bisons study were generally low with an alleged parents (cows and calves). However, the culmulative PE (0.7531) is necessary in this study, since it demonstrated the probability that the elephant bull, the cow and the calf were closely related. In this study, only 3 microsatellite markers were used, comparing to the 31 loci used in the bisons, and 10 loci in the horses studies (Mommens *et al*, 1998, Boeling *et a*l, 1997).

Parentage test in elephants were reported by Whitehouse and Herley (2001) and Hollister-Smith *et al* (2004) using microsatellite genotyping of African elephants to analyze a mating monopoly by dominant bull. They were successfully analyzed by

confirming the bulls and their calves and showed that the bulls in musth were more likely to father the calves than non-musth bulls. In Thailand, Siripunkaw (2003) reported the usage of cross-species primer of 8 microsatellite loci from African elephant to amplify these loci in 20 Asian elephants. The report suggested that the 4 highly polymorphic loci (LafMS03, FH60, FH94 and FH102) could be used for individual identification. These loci were used in this study, except FH60, for parentage test. The results of genotyping showed a successful parentage test in all five elephant's families and confirmed by statistic analysis of combined PD and culmulative PE values. The combined PD and culmulative PE are the efficient statistical methods that usually be performed when the observed alleles in eletrophorogram cannot be clearly evaluated (Phupat, 1994, Mommens *et al*, 1998, Randi *el al*, 2002). However, the observed null alleles in two elephant calves should be rechecked, as recommended by Hollister-Smith *et al* (2004) who did the analyses twice, at a minimum, for the heterozygote and seven times for the homozygotes.

The elephant calves in Thailand are commonly found living with only their mothers, especially in the remote area. Therefore, it is usually unclear about the mating background, i.e, which elephant bull is a father and when was the mating time. Moreover, in most of the cases there is no record of the pedigree. Thus, it is easier to trace the elephant's mother that the father, and mitochondrial DNA sequencing is the best technique for indicating the mother and calf (Phupat, 1994 and Randi *el al*, 2002). Mitochondrial DNA is maternally inherited and wildly used to assess taxonomic relationship and differences among populations within species (Frankham, 2002 and Randi *el al*, 2002). All calves have got the mitochondrial DNA from their mothers'

ova and their sequences are the same as their mother's sequence. Somehow, more loci of mitochondrial DNA should be used in order to get high efficient identification.

The CITES has recommended to assess the parentage test using genotype information from DNA fingerprint or fragment analysis, such as electrophoresis or electrophorogram (Randi *el al*, 2002). Although the recommended technique can be used to solve the elephant registration problem in Thailand, the main organization responsible for the problem solving and the supported regulation are still unclear. The active organization and the strong regulation are the essential clues and the urgent task for the government to help creating the good management to conserve both domesticated and wild elephants in Thailand.