# CHAPTER V

# DISCUSSION

## 5.1 Sample collection and preservation

In this study, *Pavo muticus imperator* samples were taken in form of bloodstain and feather pulps because they could be obtained while not being necessary to sacrifice this endangered species. In the case of feces, although they are readily animal materials that can be collected completely non-intrusively while *P. m. imperator* is a difficult animal to trap or encounter in the wild, the feces of *P. m. imperator* were not used in this study. That is because Gerloff (1995) reported that some heterozygous individuals falsely appeared to be homozygous following microsatellite PCR when re-extracting the same feces sample and performing PCR.

Bloodstains of each sample were collected instead for DNA extraction because it is convenient for specimen collection in wildlife research and breeding stations or other fields. Bloodstains from the field can be easily managed in the laboratory. They do not need dry ice or liquid nitrogen for preservation. However, in natural sources feather samples are easier to be collected along their trails.

A bloodstain is also appropriate for avian sample collection, because red blood cells of avian contain genomic DNA in their nuclei and bloodstains can be kept for a long time (Karnsomdee, 1999). However, there was no previous report about any effect of long-term collected bloodstain specimens to DNA amplification. As the PCR results revealed in this study, PCR products of *P. m. imperator* samples could not be amplified if the bloodstain samples had been kept for 10 years, but could be done with the 3.5-year-old bloodstain samples. The exact

time still suitable for PCR amplification between 3.5 to 10 years old could estimated because there were no specimen samples in that period for PCR testing. Although, bloodstains are convenient in sample storaging in desiccator they are not suitables for long-term collection, only for short-term. The quality of genomic DNA was distinctively degraded after bloodstain samples had been storaged in a desiccator for 60 days. Thus, DNA should be extracted from bloodstains immediately after collected and it should be kept as a DNA solution in the dark at -20$^0$C rather than a bloodstain form. This could stabilize the extracted DNA during long-term storage which was report to be for approximately 42 years (Hillis, 1996).

Although genomic DNA extracted from bloodstains had higher quality and yield than that from feathers, the blood sample could not be taken easily in the wild. In the other way round, there were also several problems found from genomic DNA extracted from a feather pulp. All genomic DNA from a feather could not be determined by 1% agarose gel or measured by a spectrophotometer. Microsatellite DNA could not be amplified possibly because of a low DNA concentration obtained a feather pulp. According to the morphological analyses follicular layers of the feather collected from the forest more than 1 year were incomplete and some tissues from dermis layer disappeared. Those follicular layers and tissues contain genomic DNA the loss of these cells then also means to lose of the DNA. Moreover, feathers should be collected from the natural source after breeding season and before rainy season. This is the only period of the year which peafowls can produce good dropped feathers. Most of feathers taken from any other period would be dilapidated because they remained grounded in the wild for too long. To solve this problem of in proper collecting-period, DNA must be extraction from

those feathers using a suitable extraction method and then concentrated using sodium acetate and ethanol. Thereafter, PCR products could be amplified. This finding result is different from Plubcharoensook's report (2000) which stated that D-loop region of mitochondria DNA could be amplified from unconcentrated DNA template after extraction *P. m. imperator* feathers with Chelex® method.

## 5.2 DNA extraction method

There are many DNA extraction protocols reported, such as CTAB precipitation, SDS precipitation, GuSCN (guanidium isothiocyanate), PEX (potassium ethyl xanthogenate), Chelex®100 and proteinase K/phenol-chloroform extraction. They are best suitable for difference tissue types and useful for a variety of analytical techniques. Thus, one may first need to try different protocols to determine which one provides a suitable for extraction in specific sample and application (Miligan *et al.*, 1998). Previously, Chelex®100 and proteinase K/phenol-chloroform methods were used for avian DNA extraction (Karnsomdee, 1999; Boripat, 1997). Hence, they were chosen to test on bloodstain and feather DNA extraction of *P. m. imperator*. Moreover, another recent extraction method, QIAamp® DNA extraction kit, was also used in this study.

Genomic DNA from bloodstains of *P. m. imperator* from Khao Soi Dao and Phatthalung wildlife research and breeding station were successfully extracted by using all three methods. But the suitable extraction method from bloodstain sample for amplification was QIAamp® kit. The quality and yield of genomic DNA extracted from the QIAamp® kit method was high and suitable for PCR amplification. In the case of proteinase K/phenol-chloroform method, it gave high quality and high yield of extracted genomic DNA but lower yield of PCR products

than the QIAamp® kit. Although, it costs less than the QIAamp® kit method, the proteinase K/phenol-chloroform method could cause interferce to PCR its organic solvents and other detergents (Table 5.1) left the in DNA solution (Stephen, 1996) This method is also not recommend for DNA extraction from feather specimen because of its high affect on PCR amplification. The last method, a DNA extraction from bloodstain specimens using 5% Chelex® method, although Meckvichai (1997) succeeded in using the mothod for *cytochrome b* amplification, it was found not suitable in this experiment for microsatellite amplification. This because the Chelex® method gave the lowest yield and purity of the DNA template extracted from *P. m. imperator* samples

When extracting DNA from feather, DNA concentration and purity could not be investigated using both 1% agarose gel electrophoresis and an ultraviolet absorption spectrophotometry. For this reason, the most suitable DNA extraction method was chosen considering its ability to give amplifiable DNA template. Noted that, all extracted DNA from feathers must be concentrated before PCR to avoid too low amount of DNA template to amplify.

The 5% Chelex® 100 method was found in this study to be appropriate for genomic DNA extraction from wild *P. m. imperator* feathers. This extraction method is easy, cheap, and less time-consuming. It involves fewer opportunities for DNA concentration and loss of DNA than the traditional phenol/chloroform extraction (Sambrook, 1989). Furthermore, this method involves no organic solvent (Table 5.2) and does not aliquot into several tubes (Walsh *et al.*, 1991) to protect loss of small amount DNA from feathers. Another advantage of the Chelex® method is its ability to extract small amount of degraded DNA in samples such as feathers from *P. m. imperator*. These small amount of extracted DNA by

this method was reported to be successfully amplified (Cooper, 1994). In this study, proteinase K/phenol-chloroform extraction was successful in recovering high molecular-weight DNA from bloodstains and gave higher purity than the 5% Chelex®100 method because of protein and other substance (e.g. hemoglobin) removal ability prior to PCR amplification (Davis *et al.*, 1994), it does not suitable for feather DNA extraction. It is because this method uses a large amount of salt, organic solvents, detergents and excess proteinase K (Walsh *et al.*, 1991) which can inhibit PCR amplification (Stephen, 1996). These reasons explain why proteinase K/phenol-chloroform extracted DNA from only some feather specimens could be amplified. The levels above which various solvents inhibit PCR reaction are shown in Table 5.1.

**Table 5.1** Level above which various solvents inhibit polymerase chain reaction

| Solvent | Inhibitory level |
|---|---|
| Ethanol | 10% |
| DMSO | 1% |
| SDS | 0.01% |
| Formamide | 10% |

**Remark** : Table from Stephen (1996), ethanol and SDS were used in proteinase K/phenol-chloroform method in this research.

Unlike the results from bloodstain specimens, the QIAamp® DNA extraction method gave low PCR product yield, presumably by a very low concentration of DNA template extracted from the feathers. This may have caused from a lysis buffer (buffer ATL in a kit) which not design to digest feather specimens and then could not release DNA from the cells. Other reason may be a binding of cell debris directly to a silica-gel membrane in the QIAamp® column and then obstruct a DNA binding step. Such problem would not have occur in the Chelex® method because its polyvalent chelating-agent resins in the solution were dispersed throughout small pieces of feathers.

To choose an appropriate DNA extraction method for bloodstain and feather samples in *P. m. imperator*, we need to consider its quality, yield of extracted DNA and PCR product yield. Other reasons such as consuming time, cost, contaminated substances, safety and convenience should also be considered. All details in this experiment were shown in Table 5.2.

In conclusion after investigating various factors of all three extraction methods, the QIAamp® kit method was recommended for DNA extraction from bloodstain specimens while the 5% Chelex® 100 method was suggested for recovering DNA template from feather specimens for polymerase chain reaction.

**Table 5.2** Overview details of each DNA isolation protocols.

| **Extraction method** | | **5% Chelex® 100** | **ProteinaseK/Phenol -chloroform** | **QIAamp®** |
|---|---|---|---|---|
| **Tissue disruption** | | Grinding | Protein digestion | Protein digestion |
| **Tissue solubilization** | | $H_20$ | $H_20$, SDS | Lysis buffer |
| **1st purification** | | None | Phenol:chloroform | 1st wash by AW1 |
| **2nd purification** | | None | Ethanol precipitation | 2nd wash by AW2 |
| **Purification** | | Low ✓ | High ✓✓ | **High ✓✓✓** |
| **Quantity** | | Low ✓ | **High ✓✓✓** | High✓✓ |
| **Cost per tube (baht), cheapness** | | **21** ✓✓✓ | 50 ✓✓ | 120 ✓ |
| **Time** | | 2 hrs 25 min. ✓✓ | 1 day 3 hrs ✓ | **1 hr 27 min** ✓✓✓ |
| **Contaminated organic solvents** | | None ✓✓✓ | High ✓ | None ✓✓✓ |
| **PCR product yield** | **Blood stain** | ✓ | ✓✓ | **High ✓✓✓** |
| | **Feather** | **High ✓✓✓** | ✓ | ✓✓ |
| **Equipments** | | Incubator, centrifuge, waterbath | Hood, centrifuge, freezer | Incubator, Centrifuge **Least equipment** ✓✓✓ |

**Remark :** ✓✓✓ mean good and suitable for DNA extraction.

## 5.3 Storage extracted DNA for PCR

A degradation of genomic DNA would have started soon after the bloodstain was collected. Therefore, sampled DNA should be extracted immediately after the specimens were collected to avoid the degradation of DNA. Only in the case that DNA could not be extract immediately, they can be stored as in a bloodstain form in a desiccator, possibly for at least 3.5 years, and still be used as DNA template for polymerase chain reaction as found in this research. However, for long-term, the extracted DNA should be kept in a solution form and stored in -20$^0$C (Hillis, 1996).

## 5.4 Microsatellite PCR amplification

Developments of cross-species amplification for microsatellite markers have been reported to be sucessful in many publications. Kayang (2002) studied cross-species amplification of microsatellite loci between in Japanese quail, chicken while guinea fowl and Baratti (2001) used chicken primers to amplify microsatellite markers in common pheasants. Both of studies, microsatellite DNA could be amplified with cross-species amplification because the range of divergence between two species over which positive amplification of orthologous microsatellite remains possible (Schlötterer, 1998). In this study, cross-species amplification of microsatellite loci in green peafowls (*P. m. imperator*) using chicken primers was successful. Although, chicken (*G. gallus*) and green peafowls (*P. m. imperator*) are in the same avian family, they are in different genera and that gives difficulty to the cross-species amplification experiment. Optimization of each PCR component is therefore very essential. From the PCR variable optimization results, the optimized annealing-temperature was found to be more important than

others. Adjustment of annealing temperature had most effects to the PCR product yield. For example, in the LEI 92 locus, the PCR products were absent when an annealing temperature was increased by two degree Celsius (55°C to 57°C). An adjustment of magnesium concentrations between 1.5 mM and 2.0 mM in this Locus did not significantly affect the yield when using 55°C annealing temperature, 1.5 mM magnesium concentration gave only little higher yield than 2.0 mM concentration. However, at of 57°C annealing temperature, PCR products were not obtained at all in both concentrations. In this research, an annealing temperature at $T_m$-5 °C could amplify PCR product better than other temperature. From These reasons, the annealing temperature was suggested to be the first-choice variable to optimize for PCR amplification condition and conclusively recommended to be $T_m$-5 °C.

In the case of magnesium chloride concentration, optimization experiments for those twenty-three microsatellite loci revealed that a specificity of PCR could be improved by decreasing $Mg^{2+}$ concentration. Although increasing $Mg^{2+}$ concentration could increase yields of PCR amplification excess $Mg^{2+}$ tends to cause nonspecific reactions (Alkami, 1999).

For primer concentration, excess primers in the reaction could cause primer dimers can interfere the sequencing step when testing microsatellite DNA. Optimization of the primer concentration therefore could reduce primer dimers and give higher PCR yield. In this study, the primer dimers problem was avoid by excising PCR bands from a Metaphor gel to prepare for sequencing.

Both normal PCR and touchdown PCR conditions and cycles were also optimized. Nine chicken primers (MCW87, LEI73, HUJ1, HUJ7, ADL136, ADL171, ADL172, ADL181 and ADL210) certainly could not give microsatellite

PCR products. These cross-species failure occurred from different sequence of flanking regions those loci between chicken and *P. m. imperator*.

In this study, a touchdown PCR was used to reduce non-specific PCR products of the HUJ2 locus. Although, the HUJ2 locus revealed high polymorphism in *P. m. imperator*, it also gave non-specific bands in 55°C optimal annealing temperature. Before using the touchdown technique, PCR conditions had been already adjusted by increasing an annealing temperature from 55°C to 57°C and 60°C. The higher annealing temperatures could not reduce those non-specific bands. In the other hand, and any higher annealing temperature could fail PCR amplification. Likewise, reducing magnesium chloride concentrations did not help decreasing non-specific bands at 55°C annealing temperature. Thus, these touchdown PCR were experimented and could reduce some non-specific bands. That finding is the same as that in according to Pongsomboon's study (2002), reporting that a touchdown PCR could reduce non-specific band in a multiplex PCR reaction. The touchdown PCR technique allows several loci having different optimal annealing-temperatures to be amplified simultaneously while suppressing the production of spurious artifact bands. This allowed only expected allele to be amplified (although, some non-specific is still present in small quantities).

The fidelity of microsatellite amplification can be confirmed by comparing amplified sequences with microsatellite sequences of chicken retrieved from GenBank. Most of the nucleotides of jungle fowls (one of positive controls used in this study) at the ADL 23 were similar to those of chicken in GenBank but some nucleotides were different. That is because a jungle fowl is a subspecies *G. g. gallus* but the chicken sequence submitted in GenBank is from a subspecies *G. g. domesticus* (strain white leghorn). Moreover, a microsatellite motif of chicken from

GenBank is $(CA)_5(CG)_4(CA)_9$ (Cheng, *et al.*, 1994) while that of a jungle fowl is $(CA)_5(CG)_3(CA)_7$. In this thesis, the microsatellite motif of the female *P. m. imperator* number R0490 in Khao Soi Dao wildlife research and breeding station was found to be $(CA)_4TA(CA)_2$. There were only two nucleotides, T and A, differing from G and A of the chicken motif. Notably, different size of allelic PCR product in this female peafowl came from different amounts of 43-nucleotide tandemly repeated units after its microsatellite motif. This repeat unit may be instead a minisatellite DNA because a minisatellite DNA is a tandemly repeated nucleotide sequence which commonly ranges between 9-65 bp per repeat unit (Wright, 1993). Moreover, a motif $(A)_n$ was also found in this locus , having various sizes among the *P. m. imperator* in Khao Soi Dao wildlife research and breeding station.

In the case of the LEI80 locus, $(CA)_n$ motif of *P. m. imperator* was found as in chicken from GenBank. In the case of the HUJ2 locus, PCR products from optimal annealing temperature at 55°C were investigated and non-specific bands could confirm by sequencing. Only two dominant alleles were microsatellite. This result is according to the result from touchdown PCR because the non-specific products were the disappeared-PCR-product bands by using touchdown PCR. Moreover, the motif of this locus was $(CA)_5GA$ and sizing of two alleles in the same *P. m. imperator* were differed from copy numbers of repeat unit. The $(CA)_n$ repeats have been repeated as the most abundant microsatellite in avian (Hillis, 1996) and in this research, (CA) motif was found in all three sequenced microsatellite loci.

Twenty-three *Gallus gallus* microsatellite primers were cross-species used in PCR amplification with *P. m. imperator* DNA. Only two microsatellite primers

(ADL23 and HUJ2) could successfully amplify PCR products and gave allelic polymorphic PCR products.

Microsatellite PCR product could amplified by using DNA template from Huay Hong Krai royal project, Sri Nan national park, Wieng Lor wildlife sanctuary, Doi Phu Nang national park, Phatthalung wildlife research and breeding station, Khao Soi Dao wildlife research and breeding station and Huay Kha Kaeng wildlife sanctuary. As the result, at the ADL23 locus, allelic polymorphic PCR products were occurred in only male and female *P. m. imperator* from Phattalung wildlife research and breeding station. But at the HUJ2 locus, polymorphic PCR products were occurred in all *P. m. imperator* samples. Thus the HUJ2 locus was chosen investigated genetic variation in *P. m. imperator* from many mentioned location. From this reason, many *P. m. imperator* from all sampled location were still found genetic polymorphism. Thus, the HUJ2 primer was high polymorphism primer for further population or other study in *P. m. imperator*.

For application microsatellite DNA markers, ten *P. m. imperator* from Khao Soi Dao wildlife research and breeding were testing for genetic polymorphism by using the ADL23 and HUJ2 loci. There was no polymorphism in the ADL23 and HUJ2 loci. Thus, one reason of frailty in *P. m. imperator* found previously in Khao Soi Dao wildlife research and breeding may assume from an inbreeding phenomenon in a population. This result must be confirmed by calculation the inbreeding coefficient of Wright ($\Delta F$) (Wright, 1921; Hillis, 1996). In the case of $\Delta F$ will be above 1%, which is in the danger zone of inbreeding. This coefficient was calculated from heterozygous and homozygous from ancestor. But in this study have not enough information about pedigree for calculation.

## 5.5 RAPD analysis

From the RAPD results, genomic DNA templates from bloodstains and feathers gave different RAPD-PCR product pattern. The DNA templates from feather were low quality than DNA template from bloodstains. This could have caused by the fact that the DNA template from feathers were extracted by the Chelex® method. This finding suggested that could come to do RAPD experiment, DNA template should come from the same source with the same genomic DNA extraction method. Hoelzel and Green (1998) and Hillis (1996) have reported that the RAPD banding patterns normally are very dependent on template quality and reaction condition.

Considering using different bands of DNA polymerase enzymes, some of RAPD-PCR products were able to be amplified by using DyNAzyme ™ II DNA polymerase from Finnzymes but not able to be amplified when using *Taq* polymerase from Promega. These two DNA polymerase enzymes were isolated from different sources. DyNAzyme ™ II DNA polymerase from Finnzymes was isolated and purified from an *E.coli* strain carrying a plasmid with the cloned from DyNAzyme™ II DNA polymerase gene from *Thermus brockianus* (strain F) while *Taq* polymerase from Promega was natively isolated from *thermus aquaticus* (strain YT1). From the results, different DNA polymerase sources would affect RAPD-PCR patterns, as the reported previously by Gamow (1954). Among RAPD publications, *Taq* polymerase (promega) and Ampli*Taq* (Perkin Elmer) were the most frequently used in RAPD publication. However, they could give completely different RAPD profiles. The initial choice of polymerase is therefore extremely

important because changing of polymerase from one to another type of enzyme is unlikely to generate comparable results (Gamow, 1954).

Only two primers (the primer 1 and the primer 13) from 60 screened primers showed genetic polymorphism in RAPD-PCR product. However, the primer 1 gave very low allelic polymorphism from prepared DNA templates of *P. m. imperator* from all locations (Huay Hong Krai royal project, Sri Nan national park, Wieng Lor wildlife sanctuary, Doi Phu Nang national park, Phatthalung wildlife research and breeding station, Khao Soi Dao wildlife research and breeding station and Huay Kha Kaeng wildlife sanctuary). Some alleles were not clearly seen in 1.5% agarose gel and they were hard to identify their differences. Problems were often from the thinckness and blurriness of bands in RAPD-PCR pattern, making it difficult to resolve differences among bands migrating at similar rates (Grosberg *et al.*, 1996). Ferraris and Palumbi (1996) advised to use polyacrylamide gel to be an alternative medium. The polyacrylamide gel is preferred by some for RAPD-PCR because of its apparently superior ability to resolve differences in molecular weight.

The reasonable cause of how fault-positive bands in negative controls result, they were found in two times of three RAPD amplification with primer 6 is still unknown. Contamination of solutions by unwanted foreign DNA was probably not the case. Repeated amplification of negative control reactions usually provides non-reproducible patterns. It is not considered to be serious if bands in negative control reaction are different each time and not similar to major bands (Gamow, 1954).

Comparing between the results of screening microsatellite and RAPD primers screening, cross-species screening of chicken microsatellite primers to

peafowls spent much more time for PCR optimization than RAPD screening condition. However, after optimization finished, a microsatellite primer can give high reproducible and all alleles can be discriminated in 8% polyacrylamide gel for further scrolling. Some microsatellites as the HUJ2 locus in this research, exhibited high levels of allelic polymorphism and could be used for variety of research objectives, including determine a genetic diversity determination in any species that shows low levels of variation, a genetic variation determination at intraspecific level when populations of such a species are inbred or experienced severe bottleneck effects, and a pedigree analysis in selective breeding programmes for *P. m. imperator*. On the other hand, RAPD primer screening requires no foreknowledge about any particular gene in a target template and no limited primers can be amplified in one time. In addition, RAPD amplification is very fast to optimize because all primers all primers use the same reaction condition, even thought RAPD gives low producibility and it is hard to score RAPD bands. Conclusively, both methods have different advantages and disadvantages.

Nowadays, the royal Thai government have given permission to set an engrossing *P. m. imperator* farm for breeding. These birds, however, can not be bought and sold before approved by the government, just like the case of *P. critatus*. In the future, there could be a new plan from the government to develop *P. m. imperator* as commercial domestic animals. If such, these microsatellite markers suggested from this research can help tagging any *P. m. imperator* pedigree for planning of a better breeding program. In another case that could happen soon, the government would plan to release *P. m. imperator* from wildlife research and breeding stations to the natural sources. Again, microsatellite markers can check genetic variation of *P. m. imperator* before and after released them. The last but

not least, other genetic markers, such as RAPD marker, screened more to find specific-species markers. These markers should be useful for illegally animal-larcenous investigation across the country border.