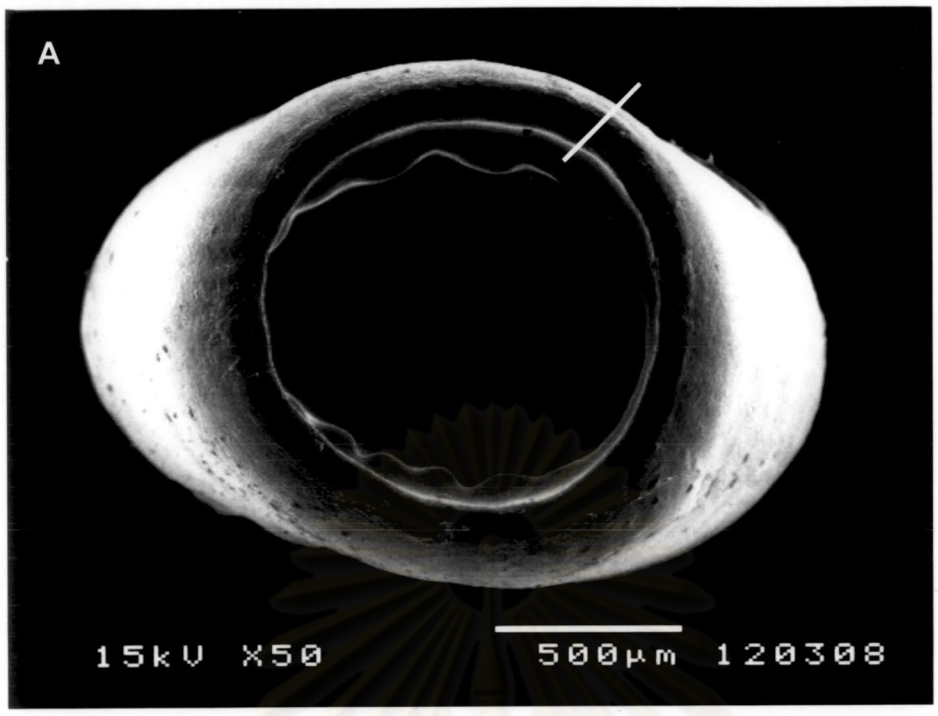
CHAPTER IV

RESULTS

4.1 Morphological characterization

Morphology of the follicular layer on feathers of *P. m. imperator* was studied by Scanning Electron Microscopy (SEM). Lots of follicular cells abide on a layer side in a calamus at the end of the feather tip and these follicular cells contain genomic DNA.

Before being morphologically compared, the feather samples of *P. m. imperator* collected from forests had been kept for two different periods of time: less than one year and more than one year. As in Figure 4.1 and 4.2, SEM photographs showed that a feather kept less than one year still had many intact layers of follicular cells in a calamus of the feather. Some tissues from dermis layers were also found on collected feathers kept less than one year (Figure 4.3A), but in the case of feathers kept more than one year, their peeled off and revealing the feather layer (Figure 4.3B). Moreover, those follicular layers were found having many small pores all over the layer surface as showing in Figure 4.3A.



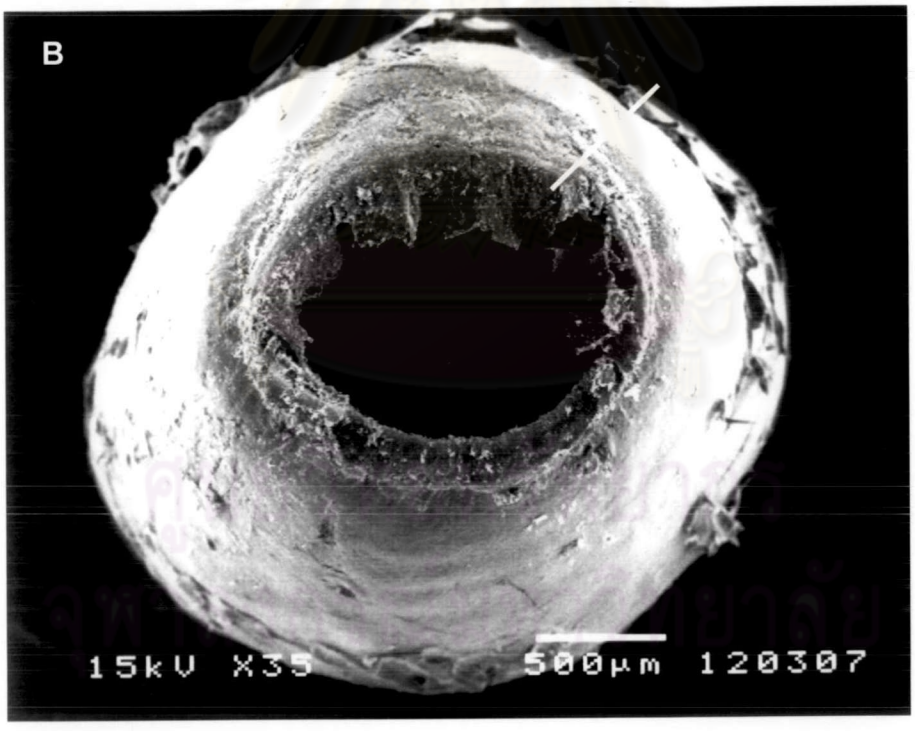
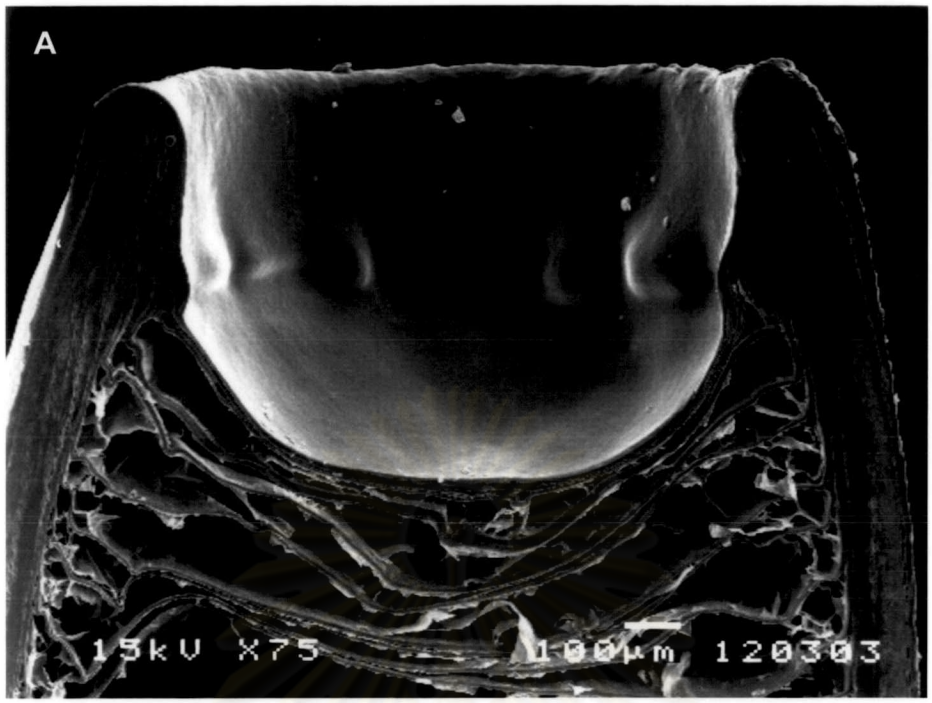


Figure 4.1 X-section of a calamus of *P. m. imperator* feathers studied by SEM (A) a feather kept less than one year (x50 magnification) (B) a feather kept more than one year (x35 magnification) and —— indicated the follicular layer.



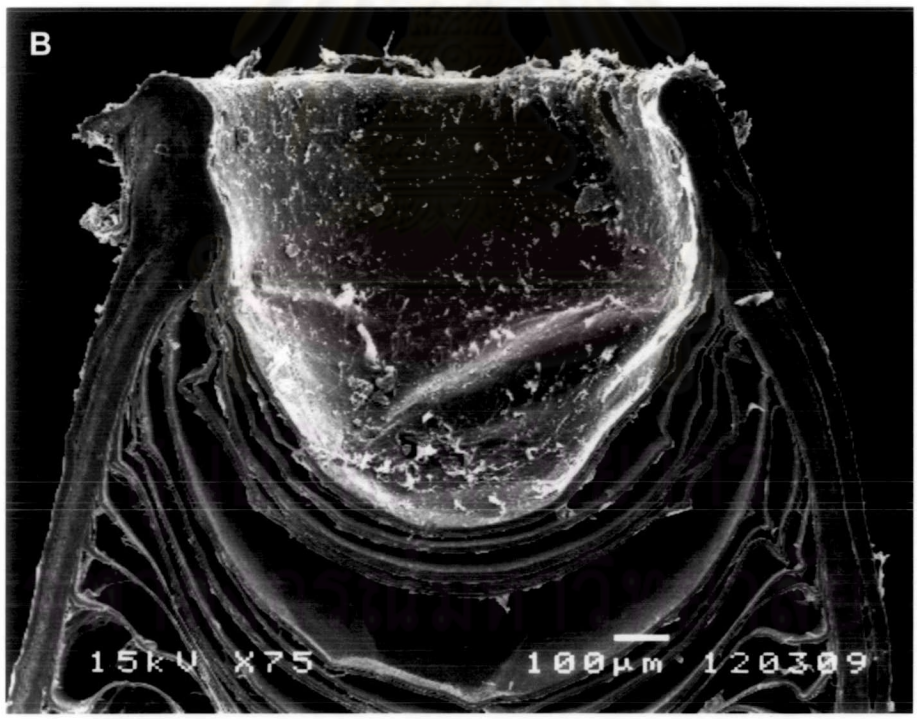
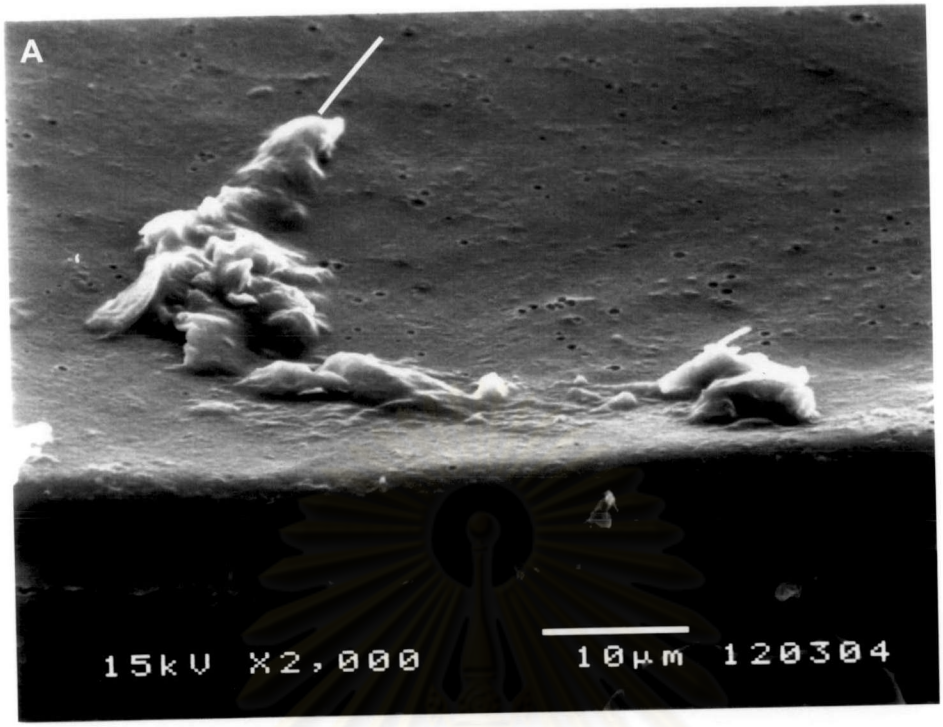


Figure 4.2 L-section of *P. m. imperator* feathers studies by SEM (x75 magnification) (A) a feather kept less than one year (B) a feather kept more than one year.



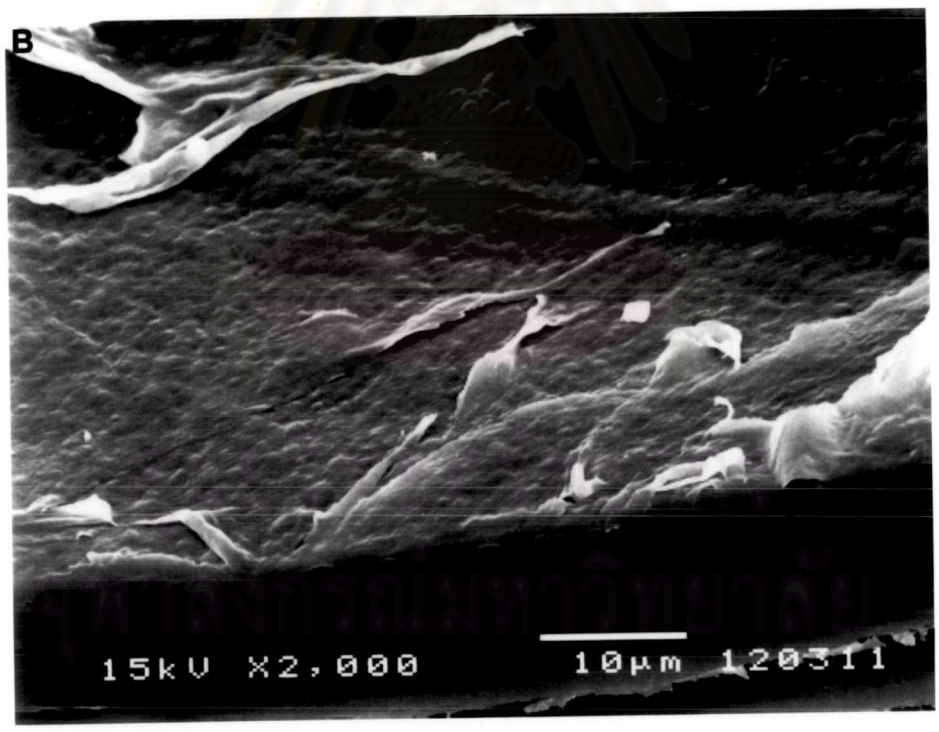


Figure 4.3 High magnification (x2,000) SEM images of follicular layers on *P. m. imperator* feathers (A) a feather which kept less than one year still has some dermal tissues (indicated by——)on its soft layer (B) a follicular layer peeled off from the feather kept over 1 year.

4.2 Genomic DNA extraction

4.2.1 DNA extraction

Genomic DNA was extracted from bloodstains and feathers of *P. m. imperator* specimens by using 5% Chelex®, proteinase K/Phenol-chloroform and QIAamp® kit method as previously described. The quality of the extracted DNA from each method was compared by using 1% agarose gel electrophoresis. The result was shown in Figure 4.4.

The concentrations of all genomic DNAs extracted from bloodstains (lane1-6 Figure 4.4) were found higher than that from feather (lane 7). When compared between different extraction methods, genomic DNAs from bloodstains extracted with proteinase K/Phenol-chloroform method (lane 5) gave better yield than those extracted with 5% Chelex® method (lane 6) and QIAamp® kit method (lane 3).

Apparently, the quality of the extracted DNA with 5% Chelex® method was found to be lowest. It appeared as smear band (lane 6), not intact band like the other. This phenomenon is often found with any DNA extracted with the 5% Chelex® method.

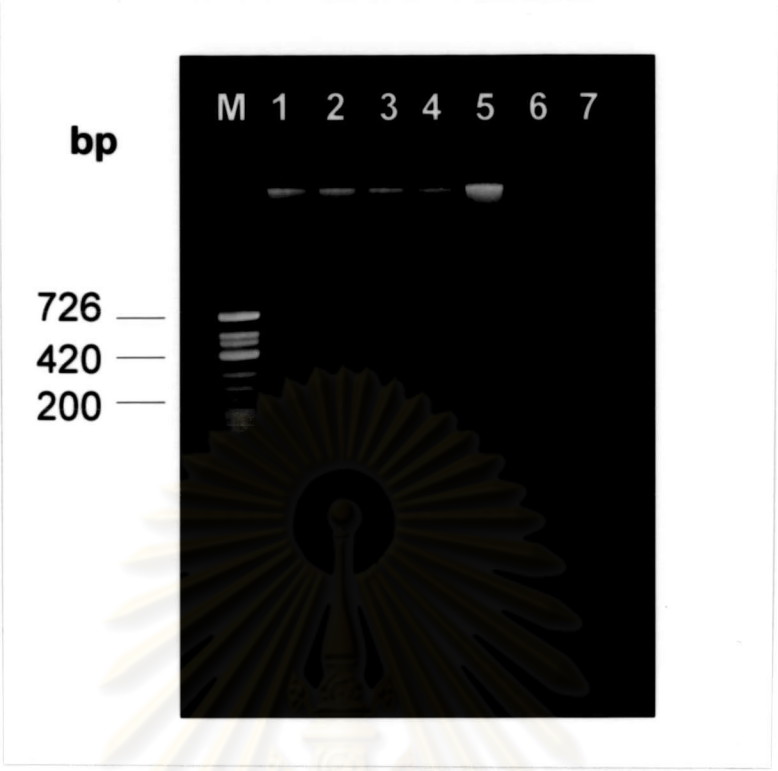


Figure 4.4 Extracted genomic DNAs from bloodstains and feathers of *P. m. imperator* using 3 different extraction methods were electrophoresed with 1% agarose gel and stained with ethidium bromide.

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: DNA from a collected from PN bloodstain extracted by QIAamp® kit method

Lane 2: DNA from a collected from WL bloodstain extracted by QIAamp® kit method

Lane 3: DNA from a collected from SD bloodstain extracted by QIAamp® kit method

Lane 4: DNA from a collected from PL bloodstain extracted by QIAamp® kit method

Lane 5: DNA from a collected from SD bloodstain extracted by Phenol-chloroform method

Lane 6: DNA from a collected from SD bloodstain extracted by 5% Chelex® method

Lane 7: DNA from a collected from SD feather extracted by QIAamp® kit method

Remark : PN : Doi Phu Nang national park, **WL**: Wieng Lor wildlife sanctuary,
SD : Khao Soi Dao wildlife research and breeding station ,
PL : Phatthalung wildlife research and breeding station

4.2.2 Measurement of DNA concentration

All genomic DNA extracted from the bloodstain specimens with three different extraction methods were measured for their UV light absorption by a spectrophotometer at 260 nm UV light. Genomic DNA concentration was calculated using an assumption that an OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double-stranded DNA. (Optical density: OD). In addition, DNA purity was compared by ratio of OD_{260} and OD_{280}. The OD of the genomic DNA extracted from feather specimens could not be measured by a spectrophotometer. The ratios of OD_{260} and $OD_{280}$and concentration of genomic DNA extracted from bloodstain specimens with three extraction methods were shown in Table 4.1.

In average, genomic DNA extracted with proteinase K/phenol-chloroform method had higher DNA concentration than those extracted with QIAamp® kit method and 5% Chelex® methods. However, genomic DNA extracted with QIAamp® kit method has higher purity than those with proteinase K/phenol-chloroform and 5% Chelex® methods. Notably, an average $OD_{260/280}$ ratio of DNA extracted with 5% Chelex® method was the only one lower than 1.8. Therefore, extracted DNA from blood specimens of 5% Chelex® method was not used in the next experiment. The chosen DNA extraction method from bloodstain specimens in the PCR experiment was the QIAamp® kit method. However, the 5% Chelex® method was recommended when extracting DNA from feather due to higher PCR yields than proteinase K/phenol-chloroform and QIAamp® kit methods.

Table 4.1 The ratios of OD_{260} and OD_{280} ($OD_{260/280}$) and concentrations of genomic DNAs extracted from bloodstain specimens with three extraction methods.

Extraction Method	**$OD_{260/280}$**	**DNA concentration (ng/µl)**
Proteinase K/phenol-chloroform method	1.3 – 1.9	20.0 - 161.3
5% Chelex® method	0.9 – 1.4	6.2 – 87.4
QIAamp® kit method	1.5 – 2.1	50.0 – 133.0

4.3 Effects of long-term specimen collection on DNA amplification

Peafowl bloodstain specimens collected from Khao Soi Dao wildlife research and breeding station were stored in a desiccator on 30th February, 1994 (10 years ago) and 18th November, 2000 (3.5 years ago).

In Figure 4.5A, extracted genomic DNA of specimens collected on 30th February, 1994 (lane 1-2) appeared in degraded fragments with very low amount, compared to that of the specimen collected on 18th November, 2000 (lane 3). Moreover, PCR product of Cyt b could be amplified only from the sample collected 3.5 year ago (lane 6, Figure 4.5B).

A short-term storage of DNA specimens also affected a quality of extracted genomic DNA. Another collected bloodstain samples on 11th January, 2003 were extracted on 7th February, 2003 (27 days after specimens were taken) and also 17th March, 2003 (65 days later) As the result, the genomic DNA extracted on 17th March, 2003 (lane 1-5, Figure 4.6B) had lower quality than that extracted on 7th February, 2003 (lane 1-5, Figure 4.6A). Nevertheless, short-term collection (both 27 and 65 days collection) did not show any negative effect on PCR amplification.

From these results, bloodstain samples stored in a desiccator for about 60 days were still able to give good-quality genomic DNA. In addition, 3.5-year-old bloodstain samples stored in a desiccator could give amplified PCR product but 10-year-old bloodstain samples could not amplified PCR product.

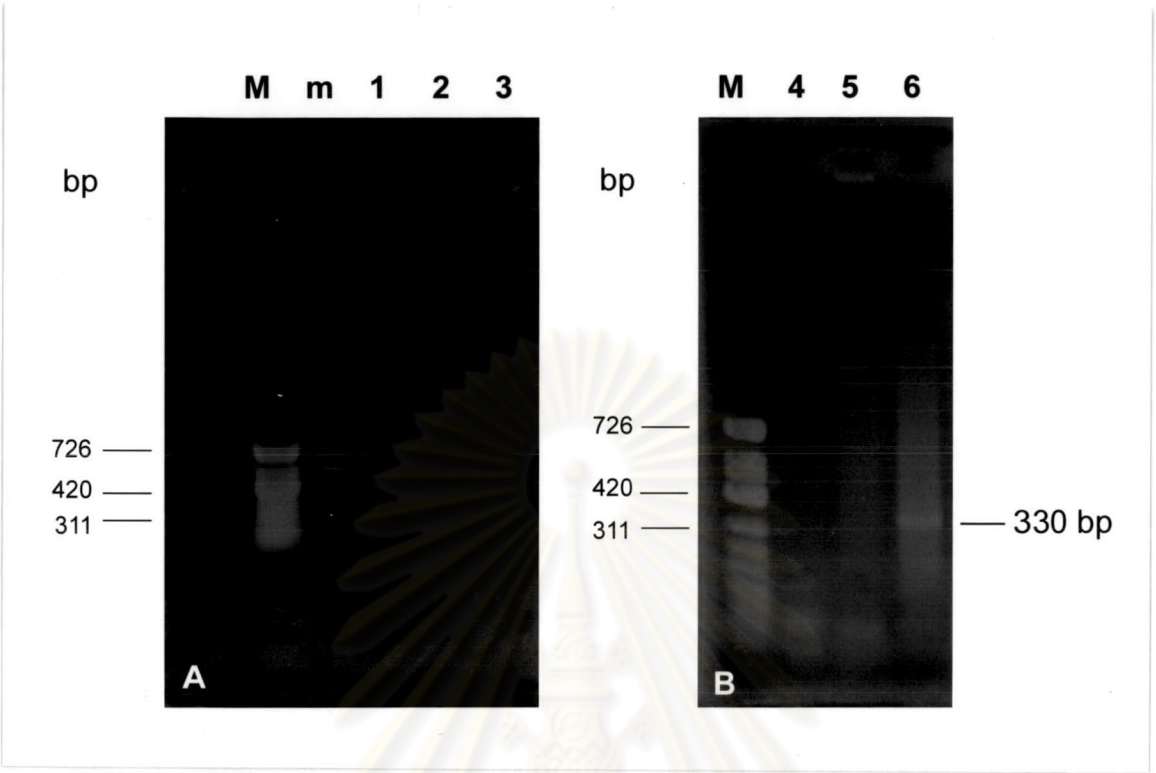


Figure 4.5 Ethidium bromide staining of 1% agarose gel showing (A) extracted genomic DNA and (B) Cytochrome b (330 bp) PCR products from blood stains of *P. m. imperator* collected from Khao Soi Dao wildlife research and breeding station.

Lane M : PhiX 174/*Hinf* I standard marker

Lane m : 100 basepair ladder standard marker

Lane 1,5 : extracted DNA and a PCR product from a male peafowl on 30th February, 1994 (10 years ago)

Lane 2,4 : extracted DNA and a PCR product from a female peafowl on 30th February, 1994 (10 years ago)

Lane 3,6 : extracted DNA and a PCR product from a male peafowl on 18th November, 2000 (3.5 years ago)

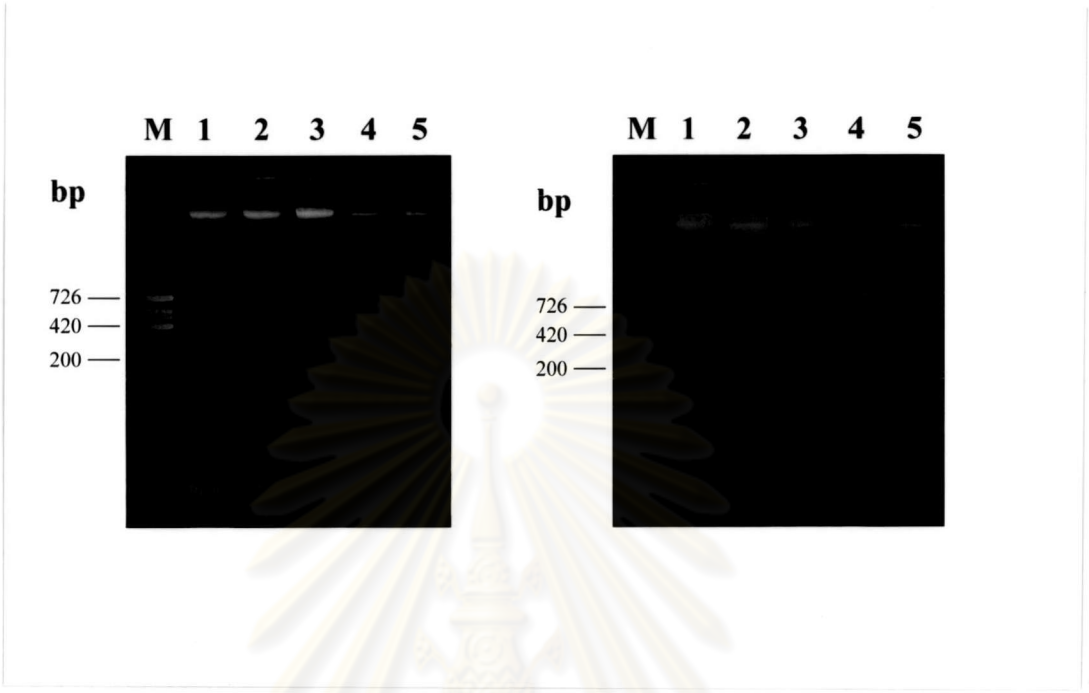


Figure 4.6 Effects of short-term collection on genomic DNA extraction from blood specimens of *P. m. imperator* (A) DNA extracted on 7th February, 2003 (27 days after specimens were taken) (B) DNA extracted on 17th March, 2003 (65 days after specimens were taken)

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: DNA extracted from a male green peafowl from Phatthalung wildlife research and breeding station (PL)

Lane 2: DNA extracted from a female green peafowl from PL

Lane 3: DNA extracted from a male green peafowl from Khao Soi Dao wildlife research and breeding station (SD)

Lane 4: DNA extracted from a female green peafowl from SD

Lane 5: DNA extracted from a female Cambodia green peafowl from SD

4.4 *In vitro* amplification of microsatellite DNA using the polymerase chain reaction (PCR)

4.4.1 PCR variables optimization

Quantity of DNA template, preincubation time, annealing temperature and time, magnesium concentration, primer concentration and dNTPs concentration were optimized for each microsatellite loci.

4.4.1.1 Quantity of template DNA

An amount of template DNA can affect on efficiency of PCR amplification. Excessive DNA in the reaction could inhibit the amplification because of higher contaminants in the DNA. In this study, PCR reaction mixture was 25 µl. The PCR product could not be amplified when using 150 ng of template DNA per reaction (lane 4, Figure 4.7) whereas a lower amount of template, as low as 20 ng, gave small amount of PCR product (lane 1, Figure 4.7). The PCR products were better amplified from either 50 or 100 ng of template DNA (lane 2 and 3, Figure 4.7). Conclusively, about 50 ng per reaction of the genomic DNA extracted from *P. m. imperator* was the most appropriate amount to give high PCR product.

4.4.1.2 Preincubation temperature and time

A 94°C preincubation (or predenaturation) temperature was found to be suitable for all mocrosatellite loci as it is the same as the denaturing temperature, Two minutes of preincubation time mostly gave equal yields of PCR products as 5 minute preincubation time. The 2-minute preincubation time was therefore chosen except several loci found more suitable with 5 minutes as previously mentioned (Crooijmans, 1997). The results were shown in Table 4.2

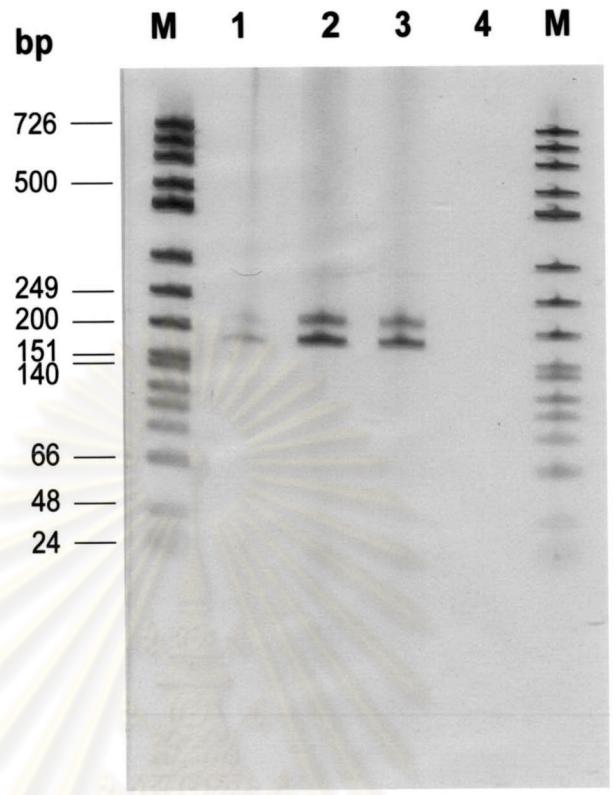


Figure 4.7 PCR products amplified from the locus ADL37 with various DNA template concentrations and constant concentrations of 2.5 mM Mg^{2+}, 0.2 µM forward and reverse primers, 0.2 mM dNTP, and annealing temperature at 52°C run in an 8% polyacrylamide gel stained with silver staining method, The DNA template was prepared from a female *P. m. imperator* in Phatthalung wildlife research and breeding station.

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: PCR products amplified with 20 ng DNA template per reaction

Lane 2: PCR products amplified with 50 ng DNA template per reaction

Lane 3: PCR products amplified with 100 ng DNA template per reaction

Lane 4: PCR products amplified with 150 ng DNA template per reaction

Table 4.2 Optimal preincubation temperatures and times chosen for 23 microsatellite loci.

Loci	Predenaturation (°C, min)
ADL 23	94, 2
MCW 87	94, 2
MCW 240	94, 2
LEI 73	94, 2
ADL 37	94, 2
HUJ 1	94, 5
HUJ 2	94, 5
HUJ 7	94, 5
LEI 92	94, 5
LEI 126	94, 5
MCW 305	94, 5
LEI 80	94, 5
LEI 136	94, 5
HUJ 12	94, 5
ADL 102	94, 2
ADL 136	94, 2
ADL 158	94, 2
ADL 171	94, 2
ADL 172	94, 2
ADL 176	94, 2
ADL 181	94, 2
ADL 210	94, 2
ADL 267	94, 2
ADL 172	94, 2
ADL 176	94, 2
ADL 181	94, 2

4.4.1.3 Annealing temperature

In Figure 4.8, an appropriate annealing temperature was found to be very important for PCR amplification. There was no PCR products amplified at the high temperature as 60°C. On the other hand, there were some nonspecific PCR products amplified with low yield at the low annealing temperature as 50°C. Nonspecific products were proved by sequencing in section 4.4.6. The annealing temperature was then the first-choice variable to optimize for PCR amplification condition. The results of annealing temperature optimization were depicted in Table 4.3 and X was the microsatellite locus that could not amplified from *P. m. imperator* DNA.

4.4.1.4 Magnesium chloride concentration

The optimal Mg^{2+} concentration were shown in Table 4.4. In the case of the ADL23 locus (Figure 4.9), Mg^{2+} concentration at 1.0 mM produced the lowest yield (lane 1). Although the reaction with 1.5 mM Mg^{2+} concentration (lane 2) gave PCR yield lower than the reaction with 2.0 mM Mg^{2+} concentration (lane 3), it was also found that the specificity could be improved by decreasing Mg^{2+} concentration from 2.0 mM to 1.5 mM; this could make the 82 bp allele disappeared. The 2.0 mM and 2.5 mM Mg^{2+} concentration gave the same stutter band-patterns of PCR products that were possibly a microsatellite pattern. Besides, there was none of product in the 3.0 mM Mg^{2+} concentration. As the result, the 2.0 mM Mg^{2+} concentration was chosen for the ADL23 amplification.

Table 4.3 Optimal annealing temperatures chosen for some of 23 microsatellite primer loci (X = could not amplified)

Loci	Annealing Temp.(°C)	Loci	Annealing Temp. (°C)
ADL 23	55	LEI 136	55
MCW 87	X	HUJ 12	55
MCW 240	60	ADL 102	47
LEI 73	X	ADL 136	X
ADL 37	45	ADL 158	52
HUJ 1	X	ADL 171	X
HUJ 2	55	ADL 172	X
HUJ 7	X	ADL 176	52
LEI 92	55	ADL 181	X
LEI 126	55	ADL 210	X
MCW 305	55	ADL 267	50
LEI 80	55		

Table 4.4 Optimal magnesium chloride concentration chosen for some of 23 microsatellite primer loci.

Loci	$MgCl_2$ (mM)	Loci	$MgCl_2$ (mM)
ADL 23	2.0	LEI 136	1.5
MCW 87	2.5	HUJ 12	1.5
MCW 240	3.0	ADL 102	1.5
LEI 73	3.0	ADL 136	1.5
ADL 37	2.5	ADL 158	1.5
HUJ 1	1.5	ADL 171	1.5
HUJ 2	1.5	ADL 172	1.5
HUJ 7	2.0	ADL 176	1.5
LEI 92	1.5	ADL 181	1.5
LEI 126	1.5	ADL 210	1.5
MCW 305	1.5	ADL 267	1.5
LEI 80	1.5		

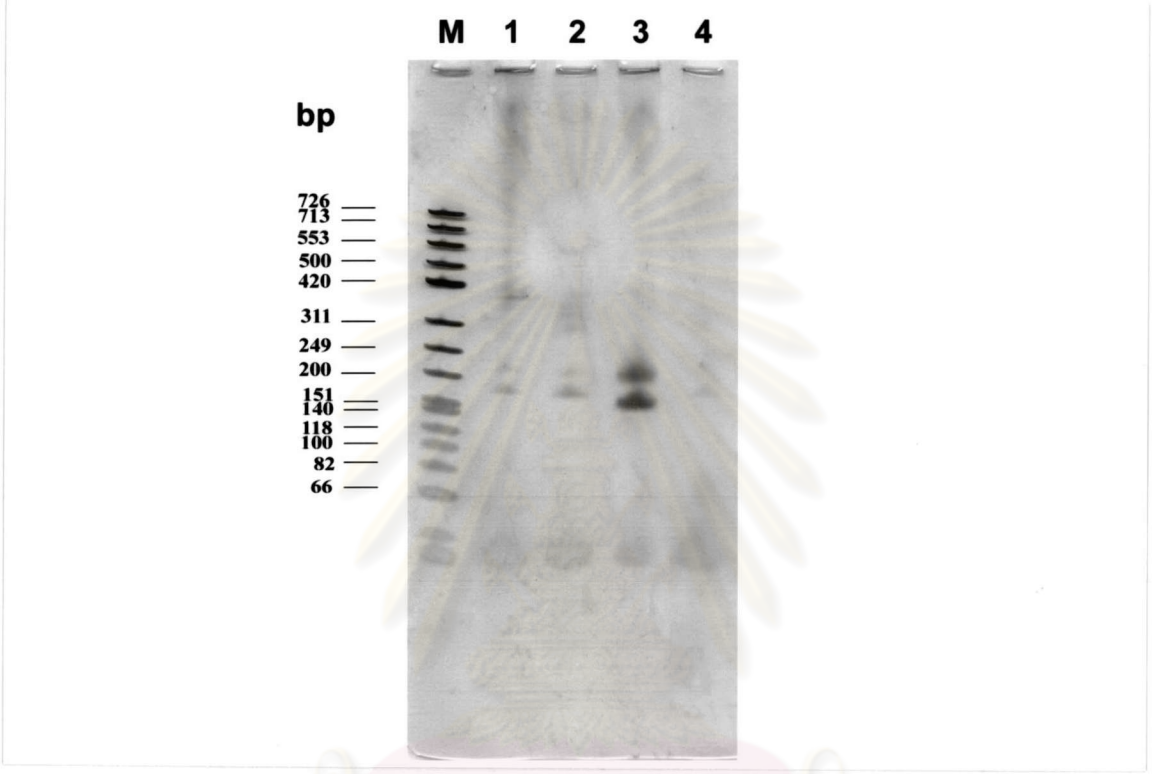


Figure 4.8 An 8% silver-stained polyacrylamide gel showing PCR products from the HUJ2 locus with various annealing temperatures and constant concentrations of 1.5 mM Mg^{2+}, 0.2 mM dNTP, 0.3 μM forward and reverse primers by using DNA template prepared from the male *P. m. imperator* no. R0326 from Khao Soi Dao wildlife research and breeding station.

Lane M: PhiX 174/*Hin*f I standard marker

Lane 1: PCR products amplified with 50 ^{0}C annealing temperature

Lane 2: PCR products amplified with 52 ^{0}C annealing temperature

Lane 3: PCR products amplified with 55 ^{0}C annealing temperature

Lane 4: PCR products amplified with 60 ^{0}C annealing temperature

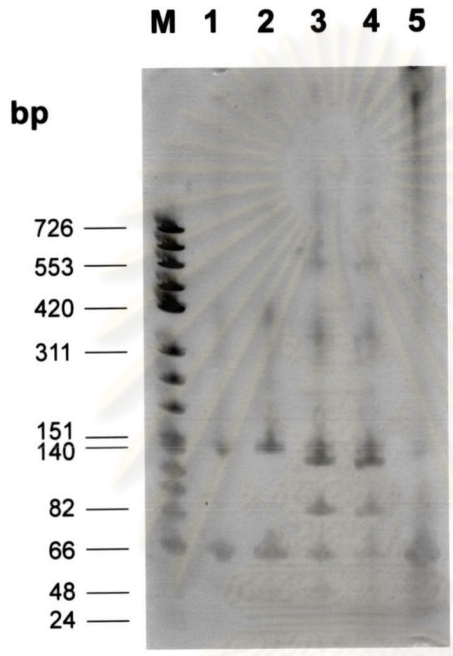


Figure 4.9 An 8% silver-stained polyacrylamide gel showing PCR products from the ADL23 locus with various magnesium concentrations and constant concentrations of 0.2 μM forward and reverse primers, 0.2 mM dNTP and annealing temperature at 55°C by using DNA template prepared from a female *P. m. imperator* at Phattalung wildlife research and breeding station.

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: PCR products amplified with 1.0 mM Mg^{2+} concentration.

Lane 2: PCR products amplified with 1.5 mM Mg^{2+} concentration.

Lane 3: PCR products amplified with 2.0 mM Mg^{2+} concentration.

Lane 4: PCR products amplified with 2.5 mM Mg^{2+} concentration.

Lane 5: PCR products amplified with 3.0 mM Mg^{2+} concentration.

4.4.1.5 Primers concentration

An example of the gel results, that of the locus ADL 102 was depicted in Figure 4.10. At 0.3 μM primer concentration (lane 4), PCR products could not be produced and primer dimers of about 20 basepairs were found. These primer dimers were about the same size as both ADL102 forward and reverse primers. Moreover, primer dimers were also found in the reaction with 0.2 μM primer concentration (lane 3) and this reaction gave lower PCR production yield than that with 0.1 μM primer concentration (lane 2). Thus, this resulted to a suggestion that the 0.1 μM concentration of both primers was an appropriate primer-concentration condition for the locus ADL102.

All optimal forward and reverse primer concentrations chosen 23 microstellite loci were shown in Table 4.5.

Table 4.5 Optimal of forward and reverse primer concentration chosen for 23 microsatellite loci.

Loci	Forward primer (μM)	Reverse primer (μM)
ADL 23	0.2	0.2
MCW 87	0.2	0.2
MCW 240	0.1	0.1
LEI 73	0.2	0.2
ADL 37	0.2	0.2
HUJ 1	0.3	0.3
HUJ 2	0.3	0.3
HUJ 7	0.2	0.2
LEI 92	0.3	0.3
LEI 126	0.3	0.3
MCW 305	0.3	0.3
LEI 80	0.3	0.3
LEI 136	0.3	0.3
HUJ 12	0.3	0.3
ADL 102	0.1	0.1
ADL 136	0.1	0.1
ADL 158	0.1	0.1
ADL 171	0.1	0.1
ADL 172	0.1	0.1
ADL 176	0.1	0.1
ADL 181	0.1	0.1
ADL 210	0.1	0.1
ADL 267	0.1	0.1

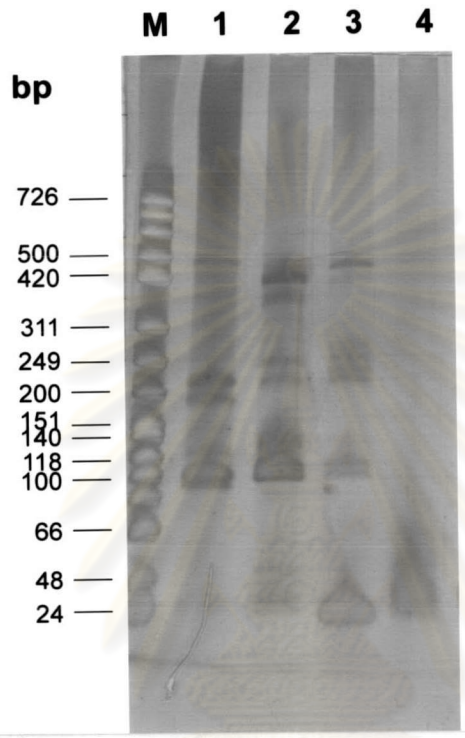


Figure 4.10 PCR products amplified from the ADL 102 locus with various forward and reverse primer concentrations and constant concentrations of 1.5 mM $MgCl_2$, 0.2 mM dNTP, and annealing temperature at 55°C, run in an 8% silver-stained polyacrylamide gel. The DNA templates were prepared from either the *Gallus gallus*(lane 1) or the male *P. m. imperator* no. R0326 (lane 2-4) from Khao Soi Dao wildlife research and breeding station. The *Gallus gallus* DNA was used as a positive control.

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: PCR products amplified with 0.1 μM of both primers

Lane 2: PCR products amplified with 0.1 μM of both primers

Lane 3: PCR products amplified with 0.2 μM of both primers

Lane 4: PCR products amplified with 0.3 μM of both primers

4.4.1.6 dNTPs concentration

Deoxynucleotide concentration for microsatellite PCR experiments normally could be lower than the standard concentrations (0.2 mM) due to for common PCR reaction the greater specificity and fidelity required for microsatellite PCR (John *et al.,* 1996). In this research, the dNTPs concentrations between 0.1 and 0.2 mM were used for PCR reaction. The yield and specificity of PCR products were then compared.

The PCR amplification results with two different dNTPs concentrations at the MCW305 and ADL102 loci were displayed in Figure 4.11 and Figure 4.12. Magnesium chloride concentration at 1.5 mM was used for both loci. In the case of MCW305 locus, the specificity of PCR amplification with 0.1 mM dNTPs (lane 3) was higher than with 0.2 mM dNTPs (lane 4) but both dNTPs concentration gave similar yields of PCR products (Figure 4.11). However, the PCR products for the ADL102 locus could not be amplified with 0.1 mM of dNTPs (lane 2, Figure 4.12).

From these results, 0.2 mM dNTPs was chosen as appropriate concentration for PCR amplification of all microsatellite loci of *P. m. imperator*. For 23 microsatellite loci screening, a dNTPs concentration was used fixed while magnesium chloride concentration was adjusted. Notably, any adjustment of the $MgCl_2$ concentration may require a compensatory adjustment of dNTPs because deoxynucleoside triphosphates quantitatively bind Mg^{2+}.

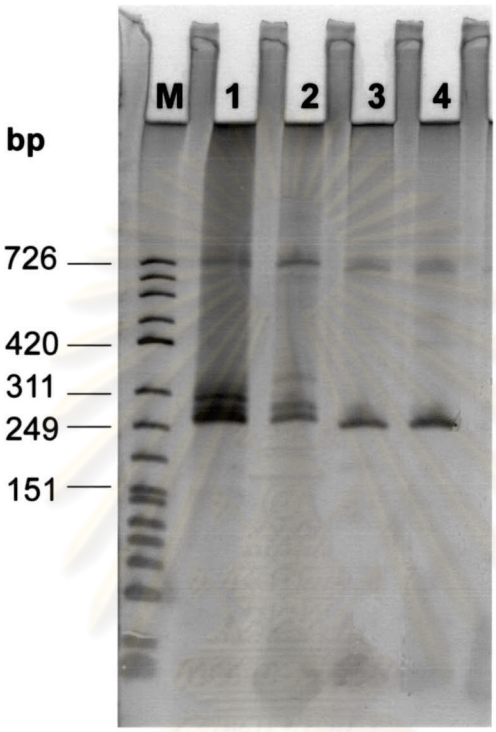


Figure 4.11 PCR products amplified from the MCW 305 locus with 2 different dNTPs concentrations and constant concentrations of 1.5 mM Mg^{2+}, 0.3 μM forward and reverse primers, 0.2 mM dNTP and annealing temperature at 55°C, run in a silver-stained polyacrylamide gel. The DNA templates were prepared from either *Gallus gallus* (lane 1 a Batum, lane 2 a fight cock), *P. m. imperator* (lane 3-4) from Phattalung wildlife research and breeding station. The *Gallus gallus* DNA was used as a positive control.

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: PCR products amplified with 0.2 mM of dNTPs

Lane 2: PCR products amplified with 0.2 mM of dNTPs

Lane 3: PCR products amplified with 0.1 mM of dNTPs

Lane 4: PCR products amplified with 0.2 mM of dNTPs

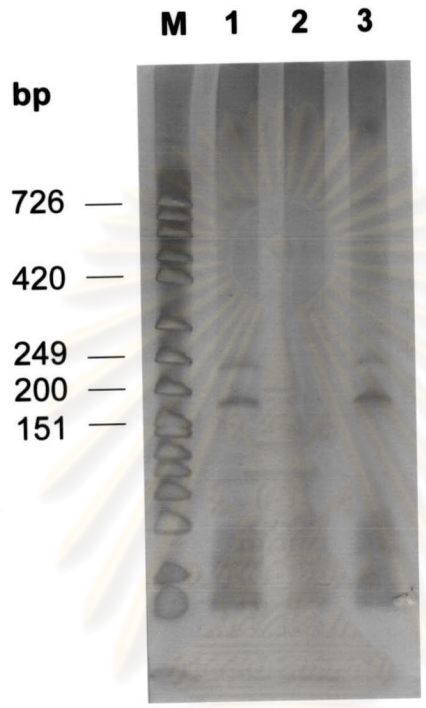


Fig 4.12 PCR products amplified from the ADL 102 locus with 2 different dNTPs concentrations and constant concentrations of 1.5 mM Mg^{2+}, 0.1 μM forward and reverse primers, 0.2 mM dNTP and annealing temperature at 47°C, run in an 8% silver-stained polyacrylamide gel. The DNA templates were prepared from either *Gallus gallus* (lane 1) or the male *P. muticus* no. R0336 (lane 2-3) from Khao Soi Dao wildlife research and breeding station. The *Gallus gallus* DNA was used as a positive control.

Lane M: PhiX 174/*Hinf* I standard marker

Lane 1: PCR products amplified with 0.2 mM of dNTPs

Lane 2: PCR products amplified with 0.1 mM of dNTPs

Lane 3: PCR products amplified with 0.2 mM of dNTPs

4.4.1.7 Microsatellite primer screening results

The optimized PCR components and PCR profiles for each microsatellite locus for *P. m. imperator* were shown in Table 4.6 and 4.7.

The annealing temperature for each locus was optimized until it provided high PCR yield with fewest nonspecific bands. In the case of some unamplifiable microsatellite loci, their annealing temperatures were followed those suggested for chicken as shown in Table 4.6.

Four types of PCR product in results were found after optimalization experiments of all 23 loci. For the first type, there were some loci, such as the ADL 181 locus, which could not be amplified at all. The second type was some loci, such as the ADL102 locus, which could be amplified from only some specimens. Some loci could be amplified from all specimens but have no allelic polymorphism; these loci were grouped in the third type. The last type was some loci which could be amplified from specimens and gave allelic polymorphism. Figure 4.13 – 4.16 showed PCR gel examples from each result type. The optimized PCR result of each locus is also reported in Table 4.6 and 4.7.

Table 4.6 Optimized values of PCR components for 23 microsatellite loci.

Loci	PCR results	Annealing temp. (°C)	$MgCl_2$ (mM)	Forward primer (µM)	Reverse primer (µM)	dNTPs (mM)
ADL 23	★	55	2.0	0.2	0.2	0.2
MCW 87	x	55	2.5	0.2	0.2	0.2
MCW 240	Δ	60	3.0	0.1	0.1	0.2
LEI 73	x	48	3.0	0.2	0.2	0.2
ADL 37	✵	45	2.5	0.2	0.2	0.2
HUJ 1	x	55	1.5	0.3	0.3	0.2
HUJ 2	★	55	1.5	0.3	0.3	0.2
HUJ 7	x	57	2.0	0.2	0.2	0.2
LEI 92	Δ	55	1.5	0.3	0.3	0.2
LEI 126	✵	55	1.5	0.3	0.3	0.2
MCW 305	✵	55	1.5	0.3	0.3	0.2
LEI 80	✵	55	1.5	0.3	0.3	0.2
LEI 136	✵	55	1.5	0.3	0.3	0.2
HUJ 12	✵	55	1.5	0.3	0.3	0.2
ADL 102	Δ	47	1.5	0.1	0.1	0.2
ADL 136	x	52	1.5	0.1	0.1	0.2
ADL 158	✵	52	1.5	0.1	0.1	0.2
ADL 171	x	46	1.5	0.1	0.1	0.2
ADL 172	x	49	1.5	0.1	0.1	0.2
ADL 176	Δ	52	1.5	0.1	0.1	0.2
ADL 181	x	48	1.5	0.1	0.1	0.2
ADL 210	x	46	1.5	0.1	0.1	0.2
ADL 267	✵	50	1.5	0.1	0.1	0.2

Remark: x No PCR products , Δ PCR products could be amplified in only some samples, ✵ PCR products could be amplified in all samples but without allelic polymorphism, ★ PCR products could be amplified in all samples with allelic polymorphism.

Table 4.7 Optimized values of PCR profiles for 23 microsatellite primer. Original conditions follow those of Karnsomdee, 1999 (dark blue), Faculty of Agricultural Sciences, Chiang Mai University, 1996 (green), Khatip, 1993 (orange), Crooijmans, 1997 (blue), and Cheng, 1997 (pink).

Loci	Predenaturation (^{0}C,min)	Denaturation (^{0}C,min)	Annealing (^{0}C,min)	Extension (^{0}C,min)	Final extension
ADL 23	94, 2	94,3	55,1	72,1.30	
MCW 87	94, 2	94,3	55,1	72,1.30	
MCW 240	94, 2	94,3	60,1	72,1.30	
LEI 73	94, 2	94,3	48,1	72,1.30	
ADL 37	94,2	94,1	45,1	72,1	
HUJ 1	94,5	94,1	55,1	72,1	
HUJ 2	94,5	94,1	55,1	72,1	
HUJ 7	94,5	94,1	57,1	72,1	
LEI 92	94,5	94,30 sec	55,45 sec	72,1.30	
LEI 126	94,5	94,30 sec	55,45 sec	72,1.30	72 ^{0}C
MCW 305	94,5	94,30 sec	55,45 sec	72,1.30	10 min
LEI 80	94,5	94,30 sec	55,45 sec	72,1.30	
LEI 136	94,5	94,30 sec	55,45 sec	72,1.30	
HUJ 12	94,5	94,30 sec	55,45 sec	72,1.30	
ADL 102	94,2	94,1	47,1	72,1	
ADL 136	94,2	94,1	52,1	72,1	
ADL 158	94,2	94,1	52,1	72,1	
ADL 171	94,2	94,1	46,1	72,1	
ADL 172	94,2	94,1	49,1	72,1	
ADL 176	94,2	94,1	52,1	72,1	
ADL 181	94,2	94,1	48,1	72,1	
ADL 210	94,2	94,1	46,1	72,1	
ADL 267	94,2	94,1	50,1	72,1	

Type 1: No PCR products

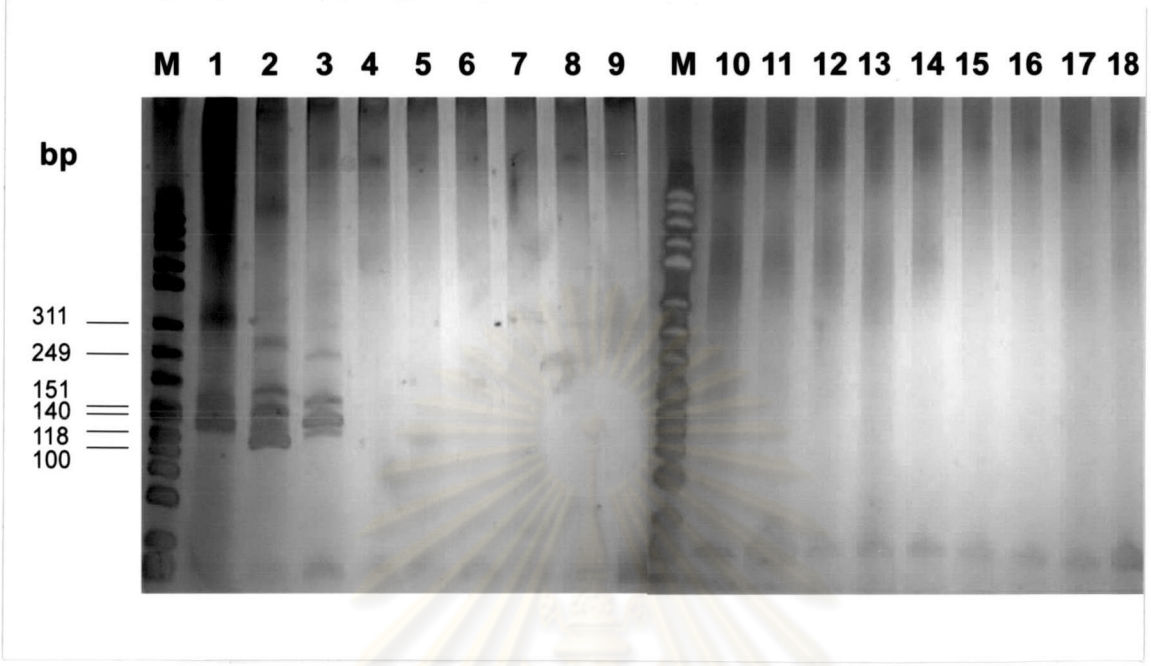


Figure 4.13 PCR products amplified from the ADL 181 locus under the optimal PCR condition with an annealing temperature at 48^0C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker
Lane 1 - 3 : Male jungle fowl, Batum and fighting cock (positive controls)
Lane 4 - 5 : Male and female Thai green peafowl from PL, respectively
Lane 6 - 7 : Male and female Thai green peafowl from SD, respectively
Lane 8 : Female Cambodia green peafowl from SD
Lane 9 : Male Thai green peafowl from PN
Lane 10-13: 2 male and 2 female Thai green peafowl from SD, respectively
Lane 14 : Male Thai green peafowl from WL
Lane 15 : Male Thai green peafowl from SN
Lane 16 : Male Thai green peafowl from HH
Lane 17-18 : Male and female Thai green peafowls from HK, respectively

Remark (PL) Phatthalung wildlife research and breeding station, (SD) Khao Soi Dao wildlife research and breeding station , (PN) Doi Phu Nang national park, (WL) Wieng Lor wildlife sanctuary, (SN) Sri Nan national park, (HH) Huay hong Krai royal project, (HK) Huay Kha Kaeng wildlife sanctuary

Type 2 : Amplified PCR products in only some samples

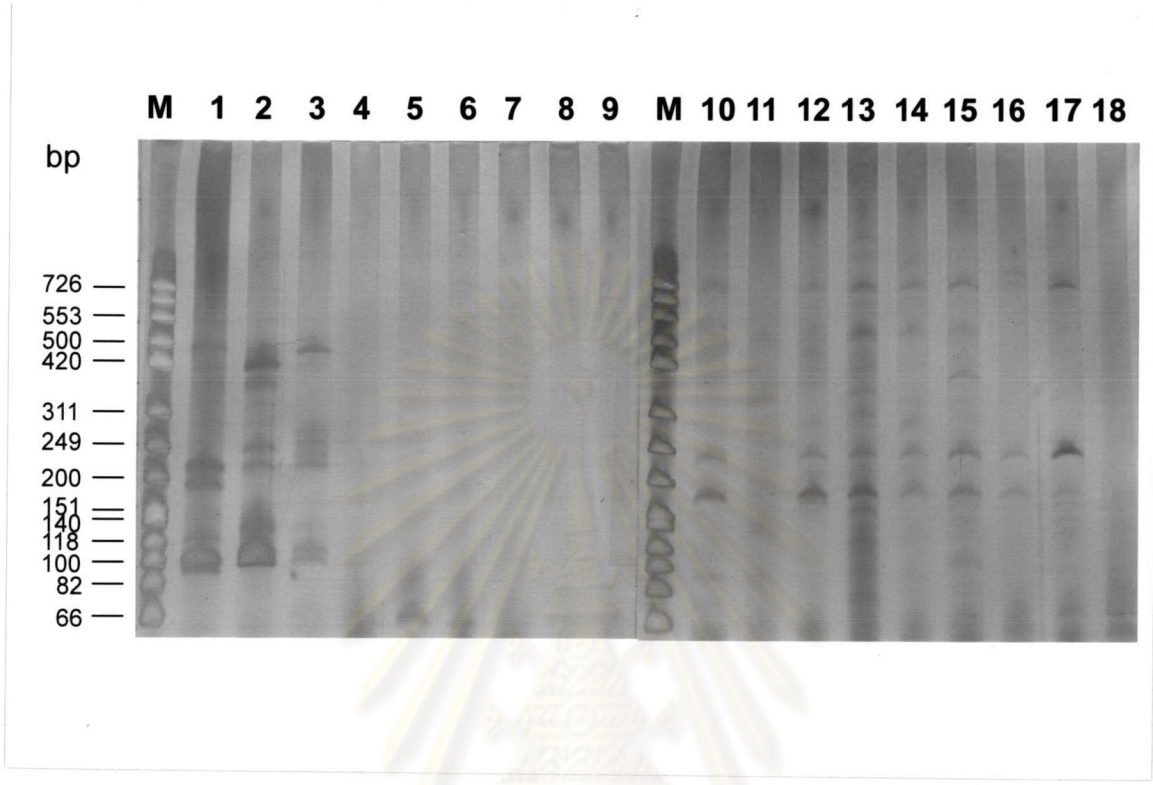


Figure 4.14 PCR products amplified from the ADL102 locus under the optimal PCR condition with annealing temperature at 47^0C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker
Lane 1 - 3 : Male jungle fowl, Batum and fighting cock (positive control)
Lane 4 - 5 : Male and female Thai green peafowls from PL, respectively
Lane 6 - 7 : Male and female Thai green peafowls from SD, respectively
Lane 8 : Female Cambodia green peafowl from SD
Lane 9 : Male Thai green peafowl from PN
Lane 10-13: 2 male and 2 female Thai green peafowls from SD, respectively
Lane 14 : Male Thai green peafowl from WL
Lane 15 : Male Thai green peafowl from SN
Lane 16 : Male Thai green peafowl from HH
Lane 17-18: Male and female Thai green peafowls from HK, respectively

Type 3: Amplified PCR products in all samples but without allelic polymorphism

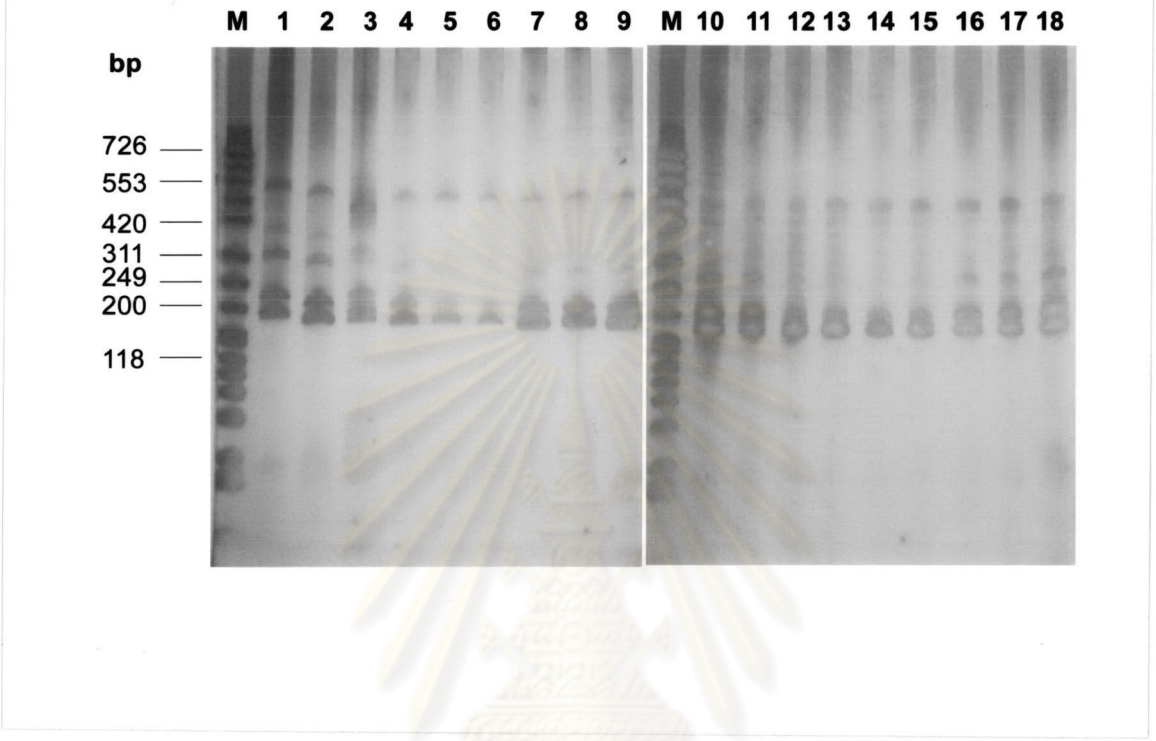


Figure 4.15 PCR products amplified from the LEI 80 locus under the optimal PCR condition with annealing temperature at 55^0C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker
Lane 1 - 3 : Male jungle fowl, Batum and fighting cock (positive control)
Lane 4 - 5 : Male and female Thai green peafowls from PL, respectively
Lane 6 - 7 : Male and female Thai green peafowls from SD, respectively
Lane 8 : Female Cambodia green peafowl from SD
Lane 9 : Male Thai green peafowl from PN
Lane 10-13: 2 male and 2 female Thai green peafowl from SD, respectively
Lane 14 : Male Thai green peafowl from WL
Lane 15 : Male Thai green peafowl from SN
Lane 16 : Male Thai green peafowl from HH
Lane 17-18: Male and female Thai green peafowl from HK, respectively

Type 4 : Amplified PCR products in all samples with allelic polymorphism

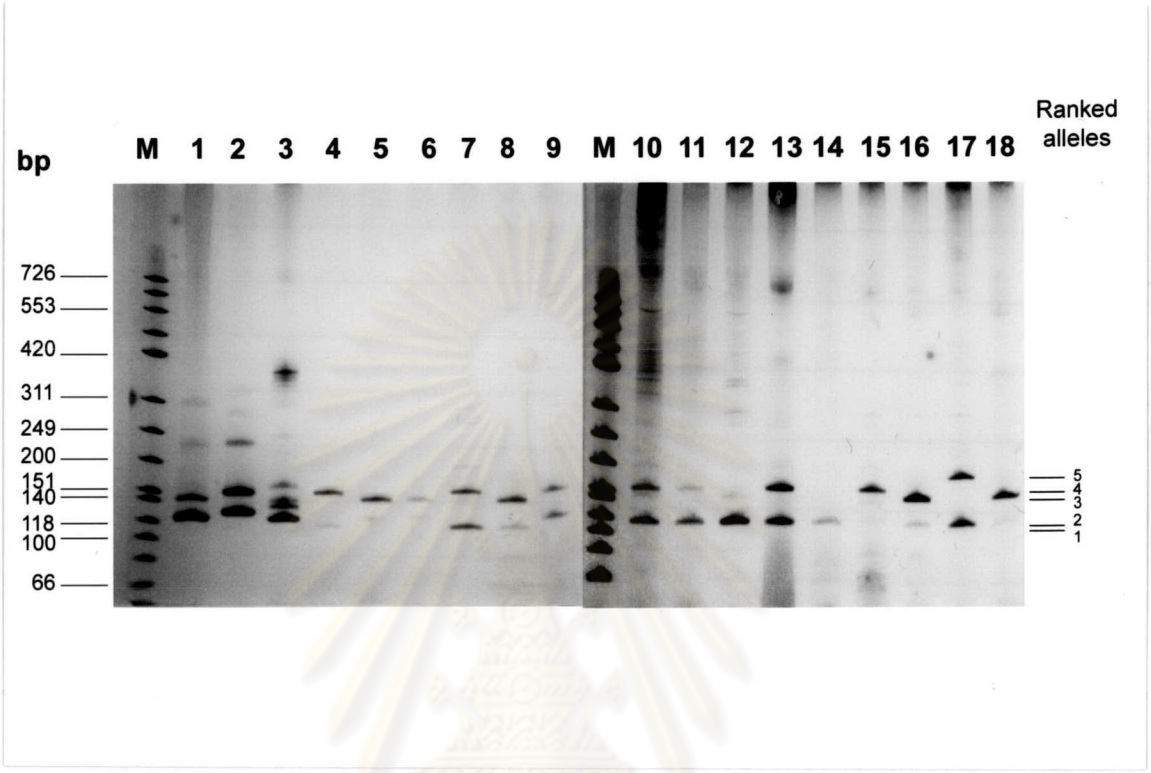


Figure 4.16 PCR product from the locus HUJ 2 under the optimal PCR condition with annealing temperature at 55^0C and using different DNA template as followed. 5 alleles were found in *P. m. imperator*.

Lane M : PhiX 174/*Hinf* I standard marker

Lane 1 - 3 : Male jungle fowl, Batum and fighting cock (positive control)

Lane 4 - 5 : Male and female Thai green peafowls from PL, respectively

Lane 6 - 7 : Male and female Thai green peafowls from SD, respectively

Lane 8 : Female Cambodia green peafowl from SD

Lane 9 : Male Thai green peafowl from PN

Lane 10-13: 2 male and 2 female Thai green peafowls from SD, respectively

Lane 14 : Male Thai green peafowl from WL

Lane 15 : Male Thai green peafowl from SN

Lane 16 : Male Thai green peafowl from HH

Lane 17-18 : Male and female Thai green peafowls from HK, respectively

Status 4 : Amplified PCR products in all samples and polymorphism

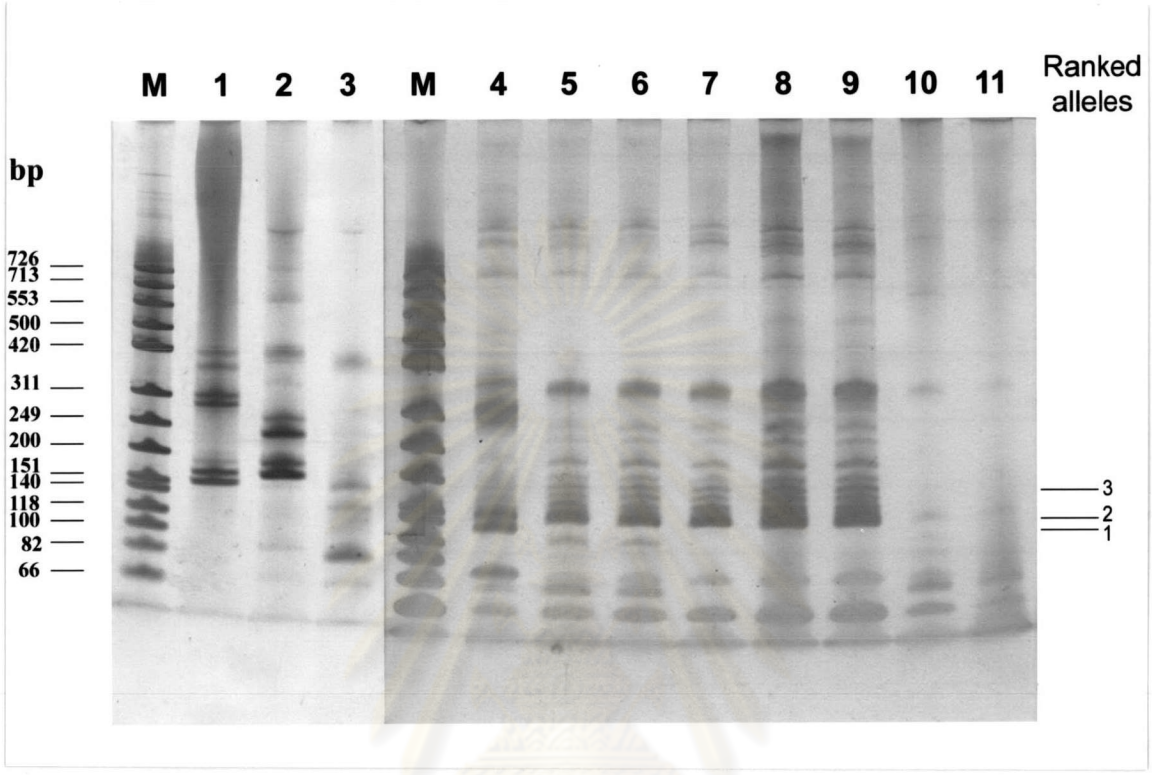


Figure 4.17 PCR product amplified from the locus ADL 23 under the optimal PCR condition with annealing temperature at 55^0C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker
Lane 1 - 2 : Male jungle fowl and Batum (positive control)
Lane 3 - 4 : Male and female Thai green peafowl from PL, respectively
Lane 5 - 6 : Male and female Thai green peafowl from SD, respectively
Lane 7 : Female Cambodia green peafowl from SD
Lane 8 : Male Thai green peafowl from PN
Lane 9 : Male Thai green peafowl from HH
Lane 10 : Male Thai green peafowl from SN
Lane 11 : Male Thai green peafowl from WL

Remark: All alleles were sequenced. Only 3 alleles from DNA template of *P. m. imperator* were microsatellite DNA.

4.4.2 Touchdown PCR

For the HUJ 2 locus, amplification of various specimens showed allelic polymorphism and touchdown PCR technique could reduce non-specific products. Figure 4.18 depicts the comparison of non-specific product obtained from touchdown PCR in Figure 4.18A and conventional PCR in Figure 4.18B. In addition, lane 7 and 8 are the same DNA template from Khao Soi Dao wildlife research and breeding station.

Nine microsatellite loci (MCW87, LEI73, HUJ1, HUJ7, ADL136, ADL171, ADL172, ADL181 and ADL210) previously found unamplified were tested by using touchdown PCR. Nine loci could not really amplified microsatellite PCR product in both of PCR techniques. The touchdown PCR result of the LEI73 locus was shown in Figure 4.18C.

4.4.3 Percentage of cross-species microsatellite amplification

Fourteen microsatellite primers (60.87%) out of twenty-three *Gallus gallus* microsatellite primers could successfully cross-species amplified *P. m. imperator* DNA. Nine microsatellite loci (39.19 %) were tested in conventional PCR and touchdown PCR that could not amplify PCR products from *P. m. imperator* DNA.

The patterns of amplified PCR results were grouped into 4 types as shown in Table 4.8. Only 2 loci (8.70%) gave allelic polymorphic PCR products.

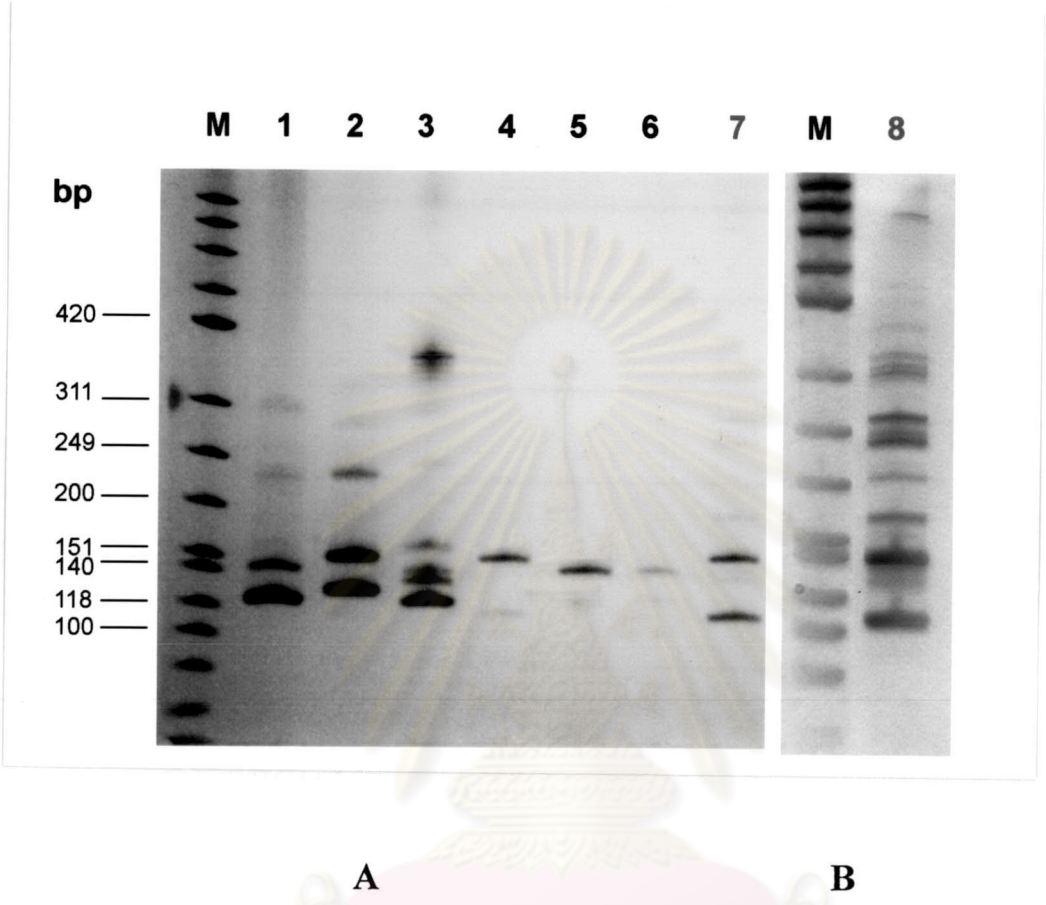


Figure 4.18 Touchdown PCR (A) and conventional PCR (B) products amplified from the HUJ2 locus and using different DNA template as followed. Lane 7 and 8 were the same DNA template

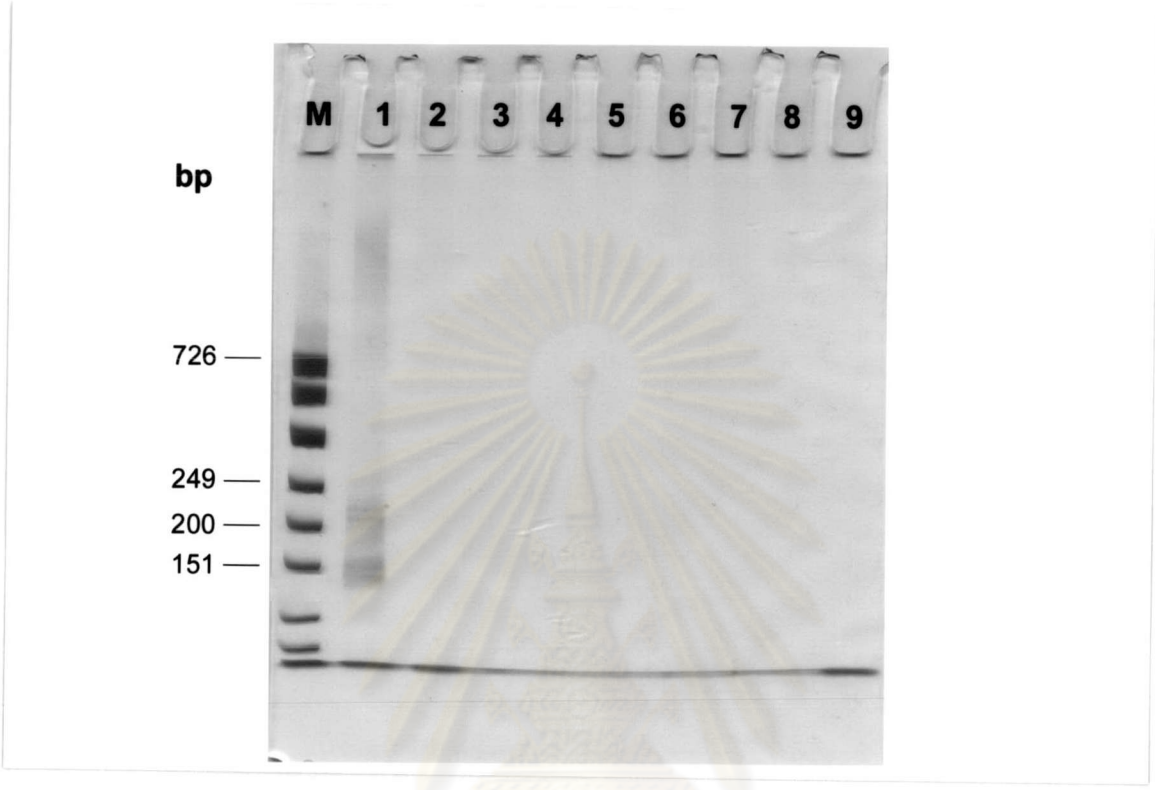
Lane M : PhiX 174/*Hinf* I standard marker

Lane 1 - 3 : Male jungle fowl, Batum and fighting cock (positive control)

Lane 4 - 5 : Male and female Thai green peafowls from PL, respectively

Lane 6 : Female Thai green peafowls from SD

Lane 7 - 8 : Same a female Thai green peafowl from SD



C

Figure 4.18C Touchdown PCR products amplified from the locus LEI73 under annealing temperature reducing from 94^{0}C to 48^{0}C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker
Lane 1 - 2 : Batum and fighting cock (positive control), respectively
Lane 3 - 4 : Male and female Thai green peafowls from PL, respectively
Lane 5 - 6 : Male and female Thai green peafowls from SD, respectively
Lane 7 : Female Cambodia green peafowl from SD
Lane 8 : Male Thai green peafowl from PN
Lane 9 : Male Thai green peafowl from WL

Table 4.8 Amount and percentage of four microsatellite loci types

Type	Primer	Amount		%
No PCR products	MCW87, LEI73, HUJ1, HUJ7,ADL136, ADL171, ADL172,ADL181,ADL210	9		39.13
Amplified PCR products in some samples	MCW240, LEI92, ADL102, ADL176	4		17.39
Amplified PCR products in all samples but no polymorphism	MCW305, LEI126, LEI80, LEI136, HUJ12, ADL37, ADL158, ADL267	8	= 14	34.78
Amplified PCR products in all samples and polymorphism	ADL23, HUJ2	2		8.70
		23		100

4.4.4 Sequencing by an automated sequencer

The PCR products were investigated by sequencing to confirm and identify microsatellite bands. Both ADL23 locus and HUJ2 locus were selected for sequencing because they gave allelic polymorphism of PCR product. In addition, from 8 loci that gave no polymorphism was selected one locus for sequencing. LEI80 locus was selected because it gave very high yield of PCR product.

For sequencing, 20 µl of DNA template was used to amplify PCR products and PCR mixtures were 500 µl. The size of PCR products were estimated and selected bands by using 8% denaturation polyacrylamide gel at 150 volts, 2 hours. Then all PCR products were run in 3.5% Metaphor agarose gel at 60 volts, 8 hours to separate adjacent PCR product bands. The selected bands were excised and purified. After they were purified by QIAquick gel extraction kit, quantity of purified PCR products were estimated by 1.5% agarose gel at 100 volts for 1 hour. The concentration of purified PCR products was about 10 ng/µl (300 ng in 30 µl). They are low concentration for sequencing. Low PCR product yield of ADL102 locus was shown in Figure 4.14 and low purified PCR products from that locus disappeared in 1.5% agarose gel. They were sequenced by BSU (Bioservice unit) and could not read their nucleotide sequences because it was very low template concentration for sequencing. BSU required 5-10 µg (500-1,000 ng) per reaction of PCR product. For this result, some loci could be sequenced. The locus selection for sequencing was important, the locus should give high PCR product as LEI80 locus (Figure 4.15).

Some nucleotides in sequence data of the ADL23 locus from Macrogen Inc., Korea and sequence data of the LEI80 locus and the HUJ2 locus from Bioservice unit, Thailand were identified using either Chromas or Acrobat program. Some nucleotides in unclear sequences could not identify so some alphabets were used. R in cleared sequences represented A or G nucleotide and S represented C or G (Figure 4.20). In the case of unidentified nucleotide, N was used (Figure 4.21). After that, all nucleotide sequences in FASTA file form were aligned by using Clustal X program.

For the ADL23 locus, PCR products were amplified by using DNA template from male and female *P. m. imperator* in Patthalung wildlife research and breeding station, male *P. m. imperator* number R0119 in Khao Soi Dao wildlife research and breeding station (SD), female *P. m. imperator* number R0490 in SD and male jungle fowl. The selected bands for sequencing of PCR products were shown in Figure 4.19. After purification and sequencing, all sequences from jungle fowl, *G. gullas* from GenBank and *P. m. imperator* were aligned. Sequences of jungle fowl (14-, 15- *G. gallus* in Figure 4.20) were compared to that sequence of chicken from GenBank (CHKMICBA-G) for confirming fidelity of microsatellite amplification. Most of the nucleotides of jungle fowl were similar in chicken from GenBank (labelled with yellow bar in Figure 4.20) but some nucleotides were different because jungle fowl is *G. g. gallus* but chicken from GenBank is *G. g. domesticus* (strain White leghorn). They stay in different subspecies. Moreover, microsatellite motif in chicken from GenBank was $(CA)_5(CG)_4(CA)_9$ and microsatellite motif in jungle fowl was $(CA)_5(CG)_3(CA)_7$ (bold alphabet in Figure 4.20). In the case of *P. m. imperator* sequences, large size PCR product bands were not selected to sequence, because microsatellite size was between 60-300

basepairs (Hearne *et al.*, 1992). Selected PCR product bands from male *P. m. imperator* in Patthalung wildlife research and breeding station (circle number 1 in Figure 4.19) could not read sequence (Appendix VIII). However, nucleotide sequences from female *P. m. imperator* in Patthalung wildlife research and breeding station (circle number 4 and 5 in Figure 4.19) and from male *P. m. imperator* number R0119 in Khao Soi Dao wildlife research and breeding station (circle number 4 and 5 in Figure 4.19), female *P. m. imperator* number R0490 in Khao Soi Dao wildlife research and breeding station (circle number 2 and 3 in Figure 4.19) could read for alignment. Sequences of number 4 and 5 were aligned with jungle fowl and chicken from GenBank in Figure. 4.20. Microsatellite motif in *P. m. imperator* was $(CA)_4TA(CA)_2$. This motif was shown in underline pink alphabets in Figure 4.20. In addition, only one motif was found throughout the *P. m. imperator* sequences like one motif in both chickens (bold alphabet in Figure 4.20). Moreover the motif, there were 2 periods of nucleotide sequences in *P. m. imperator* that was same in chicken. First period was shown by green bar and the second periods were shown by red rectangles (Figure 4.20). For observation, all sequences of *P. m. imperator* were arranged by three parts (one set). A set composed the blue bar, green bar and red rectangle in Figure 4.20. In sequence number 4, there were 1.75 sets and in sequence number 5, there were about 2.75 sets. Besides, no other nucleotide interrupted between the sets. Sequence number 4 and number 5 were from a same *P. m. imperator* so the size of both sequences differed from amounts of set. Both sequences could separate in 8%denaturation polyacrylamide gel (lane 4 in Figure 4.22).

Although female *P. m. imperator* in Phattalung wildlife research and breeding station (circle number 4 and 5) were partially similar to the sequences in

jungle fowl and chiken from GenBank but both sequences of R0119 and R0483 in Khao Soi Dao wildlife research and breeding station (circle number 2 and 3) were absolute different with chicken (blue asterisk in Figure 4.21). Their motif were $(A)_n$ (Figure 4.21) so microsatellite pattern differed from microsatellite *P. m. imperator* in Phattalung wildlife research and breeding station $(CA)_4TA(CA)_2$. As the alignment result, sequence of *P. m. imperator* number R0119 in Khao Soi Dao wildlife research and breeding station were similar in R0483. That was shown by pink asterisk in Figure 4.23.

For the LEI80 locus, PCR products were amplified by using DNA template from male *P. m. imperator* number R 0500 in Khao Soi Dao wildlife research and breeding station. The selected bands for sequencing of PCR products were shown in Figure 4.22 (circle green). Sizing of the sequence was 160 base pairs. The alignment of the nucleotide of a sample together with microsatellite DNA of *G. gallus* (GGMIC68H4) from GenBank was present in Figure 4.23. The (CA) motif of microsatellite DNA was found in *P. m. imperator* $(CA)_7$ (blue bar in Figure 4.23) and chicken in GenBank $(CA)_{18}$ (bold alphabet in Figure 4.23). Furthermore, the similarity of both sequences was 75 %.

For the HUJ2 locus, PCR products were amplified by using DNA template from male *P. m. imperator* number R 0500 in Khao Soi Dao station as template as LEI80 locus. The selected bands for sequencing of PCR products were shown in Figure 4.22 (circle number 1-7 black and red). The dominant band was number 5 and 7 circles that were labeled by red circle. Sizing of the selected PCR bands was 311- 103 base pairs for identification microsatellite bands. As the results, only sequence from selected bands number 5 and 7 were microsatellite

DNA and they were dominant bands (Figure 4.16). The sequence of number 1- 4 and 6 were not microsatellite DNA (Appendix VIII). Thus, sequence number 5 and 7 were aligned with CHKP551MS (chicken from GenBank). The (CA) motif of microsatellite DNA was found in *P. m. imperator* ($(CA)_5GA$) (blue bar in Figure 4.24) and chicken in GenBank $(CA)_{10}$. Both sequences of the number 5 and 7 in same *P. m. imperator* DNA template were different size. Because number 5 had three repeat units and number 7 had one repeat unit. Amount of repeat unit was shown by blue bar in red rectangle in Figure 4.24). In addition, nucleotide bases behind the repeat units in number 5 and 7 were similar at 89% (pink alphabet in Figure 4.24).

Electrofluorograms of all sequences in this section were shown in Appendix VII.

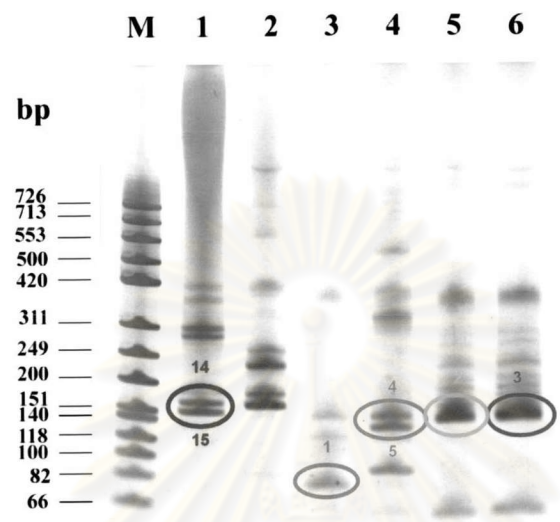


Figure 4.19 Selected PCR products of the ADL 23 locus for sequencing. (lane M) PhiX 174/*Hin*f I standard marker, (lane 1) male jungle fowl (positive control; circle number 14-15), (lane 2) male Batum (positive control), (lane 3) male (circle number 1) and (lane 4) female Thai green peafowl from Patthalung wildlife research and breeding station(circle number 4-5), (lane 5) R0119 male Thai green peafowl from Khao Soi Dao wildlife research and breeding station (SD) (circle number 2), (lane 6) R0483 female Thai green peafowl from SD (circle number 3).

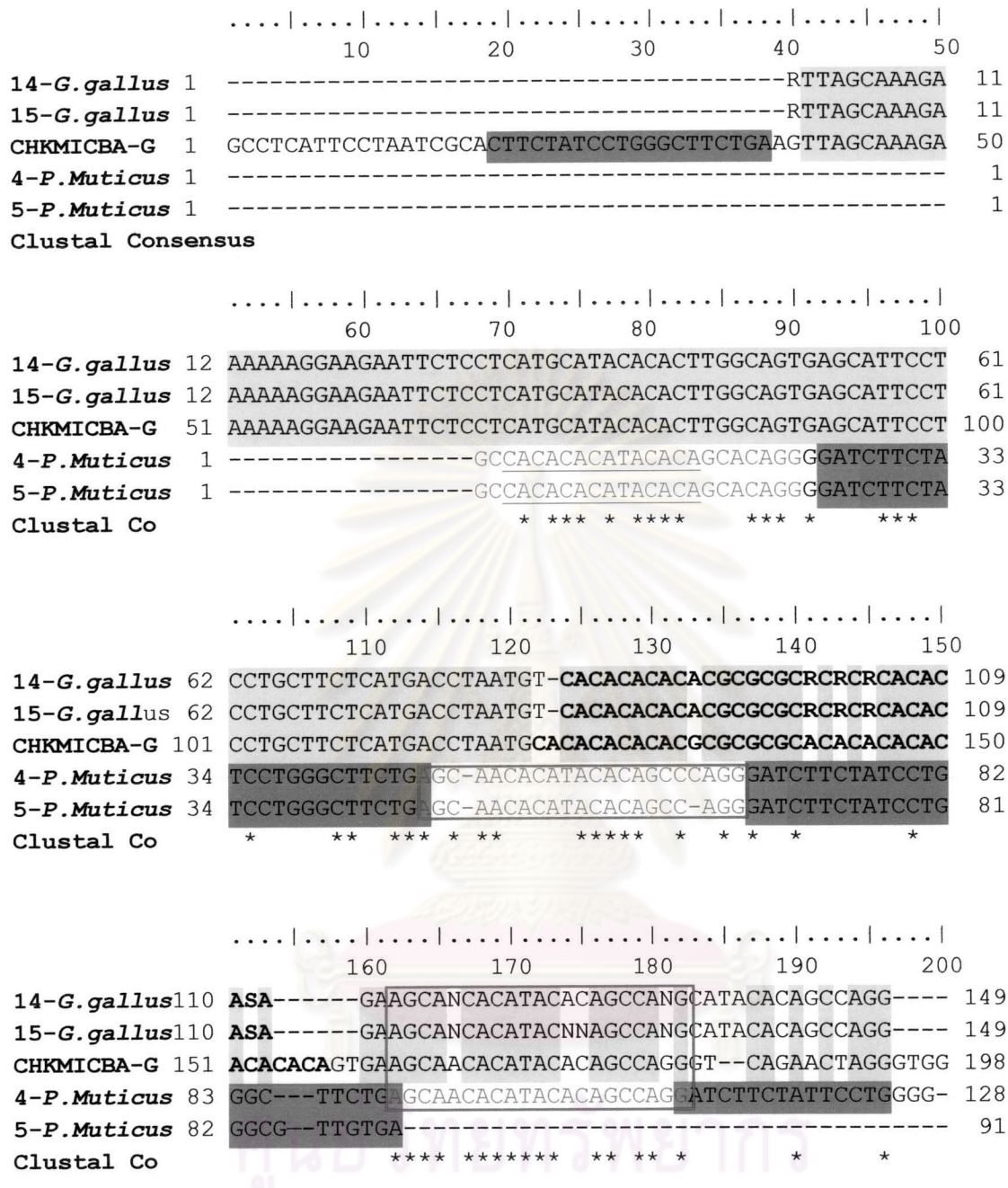


Figure 4.20 The multiple alignment of the DNA sequences of PCR products at the ADL23 locus. (14, 15) Jungle fowl sequences, (CHKMICBA) sequence of *G.g. domesticus* microsatellite from GenBank and (4-, 5-*P. muticus*) sequences of female *P.muticus* from Patthalung station. R represents A or G nucleotide, S represents C or G, bold alphabet: reported microsatellite $(CA)_5(CG)_n(CA)_n$ of chicken, underline alphabet:microsatellite in *P.muticus*, consensus bases of chicken and GenBank chicken, consensus bases of *P.muticus* and GenBank chicken, sequence before only in *P.muticus*, other same sequence of *P.muticus* and chicken and stay behind in *P. muticus*, same sequence in *P. muticus*. * indicates the consensus bases.

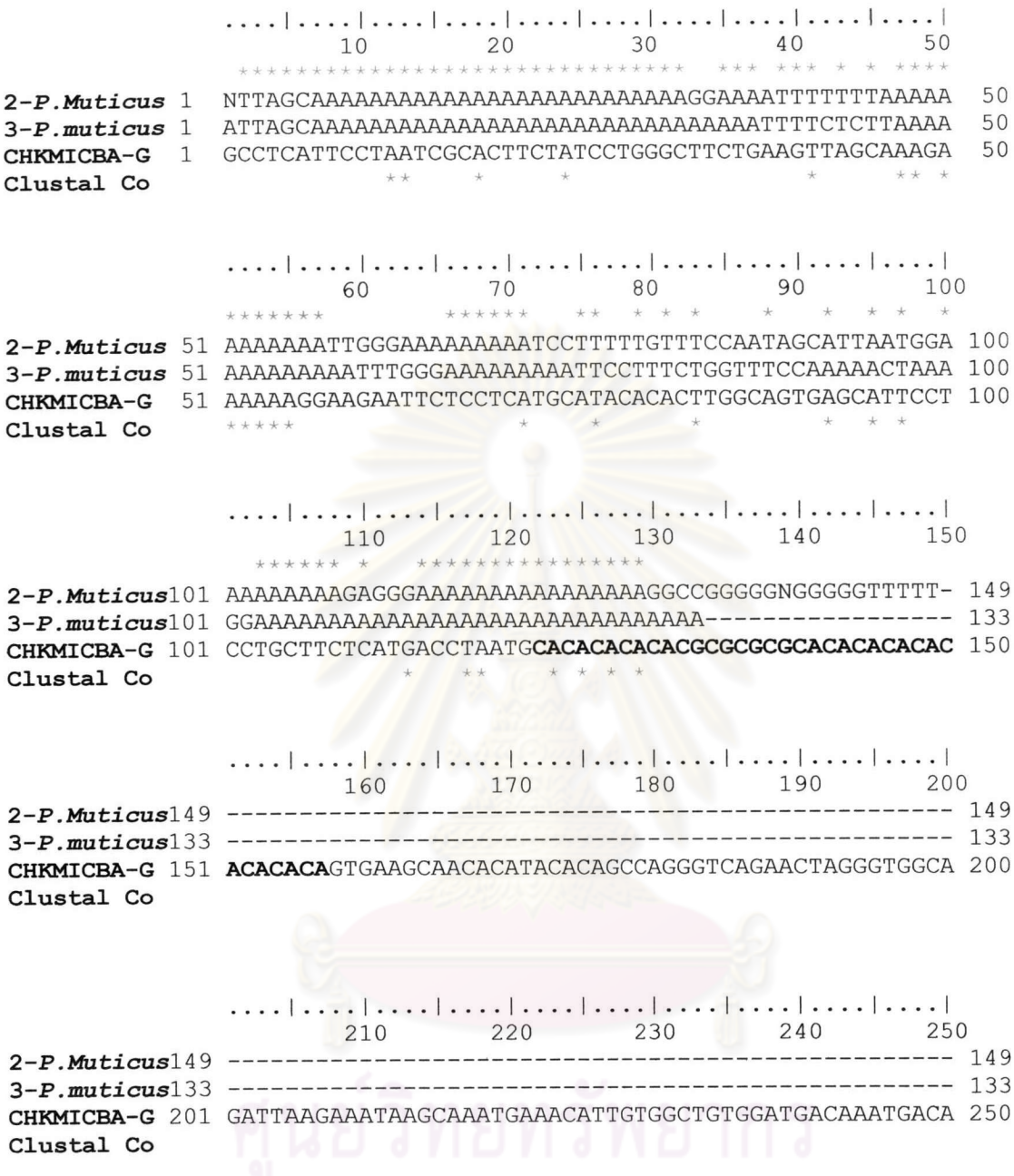


Figure 4.21 The multiple alignment of the nucleotide sequence of PCR products at the ADL23 locus and sequence of 2-*P. muticus* using template from R0119 male Thai green peafowl from Khao Soi Dao wildlife research and breeding station (SD) (circle 2 in Figure 4.19) and sequence of 3-*P. muticus* from R0483 female Thai green peafowl from SD (circle 3 in Figure 4.19). * consensus bases between 2-*P. muticus* and 3-*P. muticus* and * consensus bases for 2-*P. muticus*, 3-*P. muticus* and CHKMICBA-G (chicken from GenBank) and bold alphabet reported microsatellite $(CA)_5(CG)_4(CA)_9$ of CHKMICBA-G.

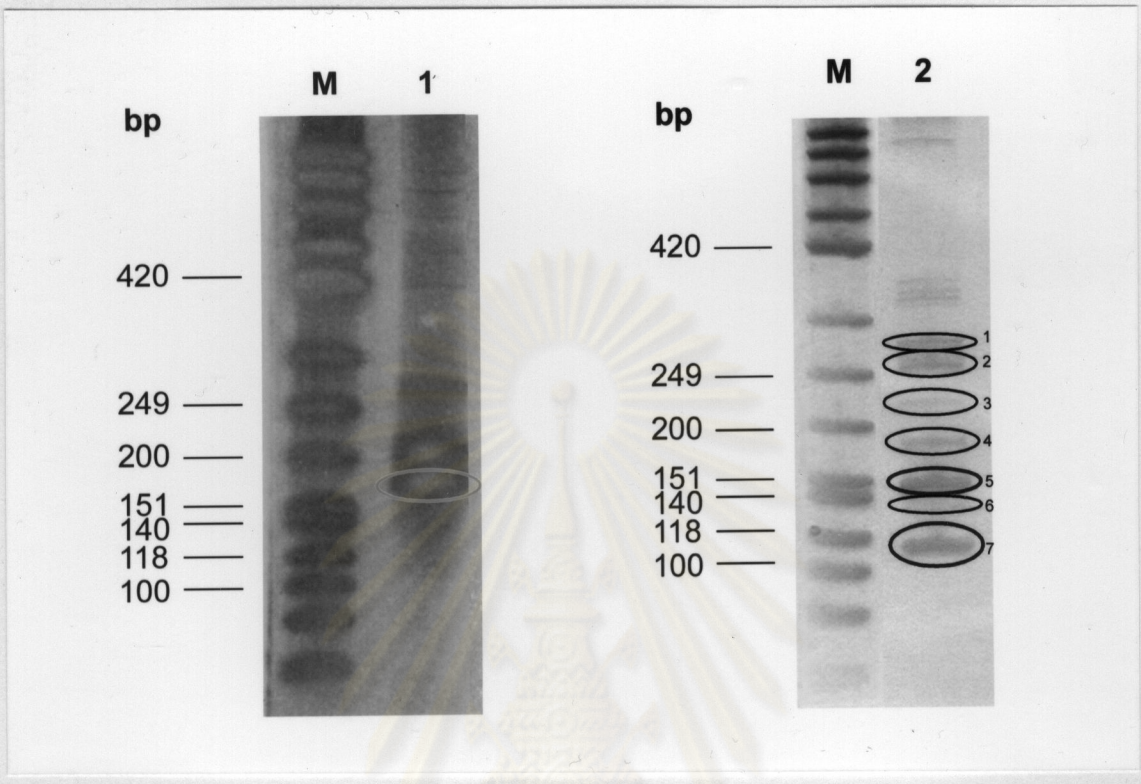


Figure 4.22 Selected microsatellite PCR products of the LEI 80 locus (circle green) and the HUJ 2 locus (circle 1-7) for sequencing. (lane M) PhiX 174/*Hinf* I standard marker, (lane 1-2) PCR products at the LEI80 locus and the HUJ2 locus from male *P. muticus* number R0500 from Khao Soi Dao wildlife research and breeding station.

```
                 ....|....|....|....|....|....|....|....|....|....|
                         10        20        30        40        50
GGMIC68H4      1 GATCACACAAGCTTTCTTCCTGTAAGTGCAGCACAGCAGACC-TGGCAGC  50
P.muticus      1 ----------------------GTTAGGNNAGCGCAGCAGACCCTGGCAGC  29
Clustal Co                           ** **   *** ********* *******

                 ....|....|....|....|....|....|....|....|....|....|
                         60        70        80        90        100
GGMIC68H4     51 ATGTN-CTAAACAAACCAAACAACAAAGTGAGCAGAGCCCTTAGCACAAA 100
P.muticus     30 ACG-TACTAAACAAACCAAACAACGAAGTGAACAGAGCCCTTAGCACAAA  78
Clustal Co       * *   ****************** ****** ******************

                 ....|....|....|....|....|....|....|....|....|....|
                         110       120       130       140       150
GGMIC68H4    101 TCCTATGGAACTGTTATTTTATCCAGCATCACACACACACACACACACAC 150
P.muticus     79 TCCTATGGAACTGTTATTTTTTCCAGCATAATACACACACACACACAC-- 126
Clustal Co       ******************** ******** * ***************

                 ....|....|....|....|....|....|....|....|....|....|
                         160       170       180       190       200
GGMIC68H4    151 ACACACACACACACAAAGTACAGTGCTGAGAAGTTTCTGTATGGCTCTAA 200
P.muticus    127 -------------TACAGTGCGGC---GAAATGTCTGTGTGGGGCTCTAA 160
Clustal Co                   * *** * *    ** * ** * **   ********

                 ....|....|....|....|...
                         210       220
GGMIC68H4    201 CTTTAAATGTGGTTAAGAAATAAGATC  227
P.muticus    161 CA-------------------------  162
Clustal Co       *
```

Figure 4.23 The multiple alignment of the nucleotide sequence of PCR products at the LEI80 locus. (GGMIC68H4) sequence of *G. g. domesticus* microsatellite from GenBank and sequences of male *P. muticus* number R0500 from Khao Soi Dao wildlife research and breeding station.$(CA)_7$ motif of microsatellite DNA in *P. muticus*. Bold alphabet are reported $(CA)_{18}$ microsatellite of chicken in GenBank. Asterisks indicate the consensus bases.

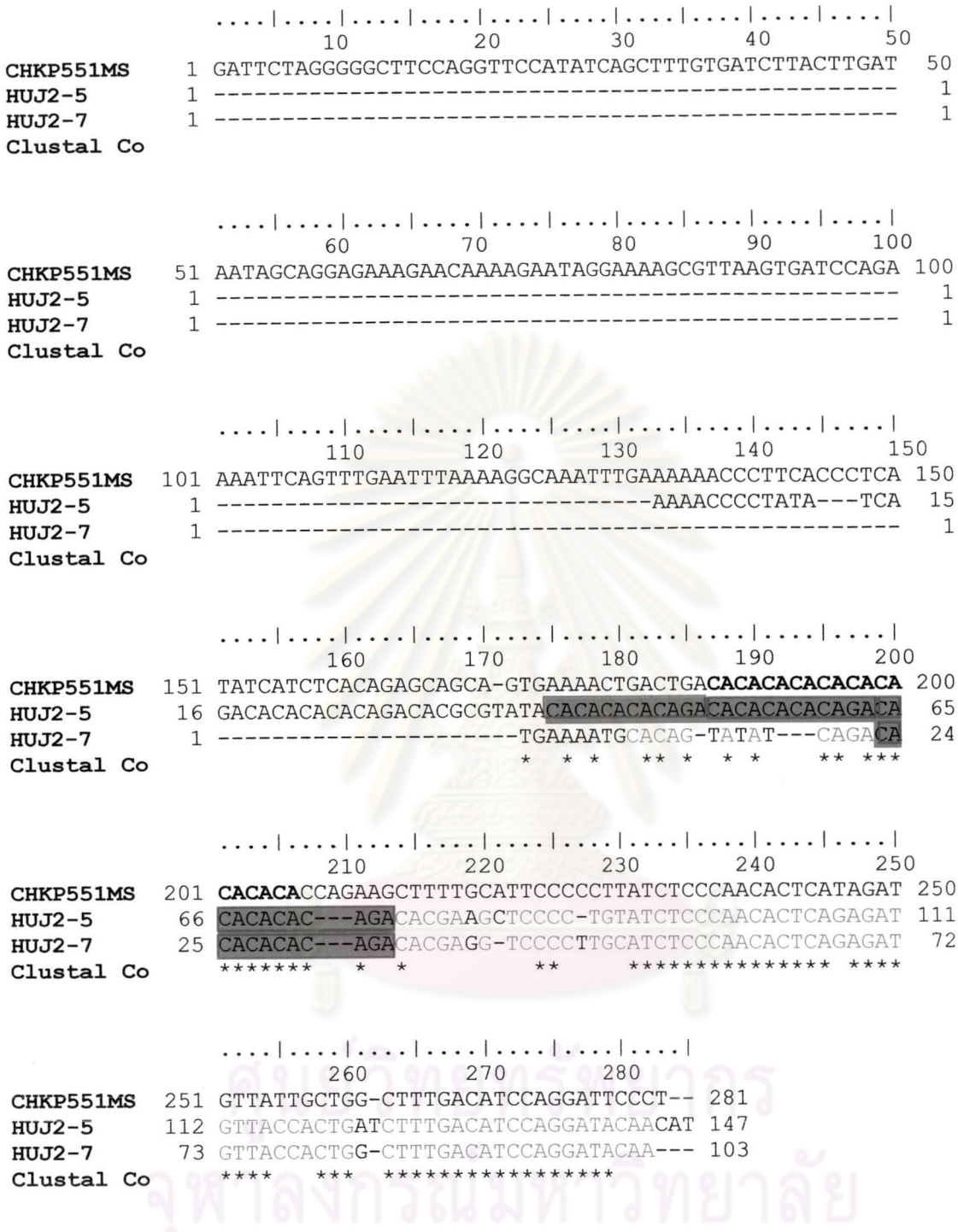


Figure 4.24 The multiple alignment of the nucleotide sequence of PCR products at HUJ2 locus. (CHKP551MS) sequence of *G. g. domesticus* microsatellite from GenBank and (HUJ2-5, HUJ2-7) sequences of male *P. muticus* number R0500 from Khao Soi Dao wildlife research and breeding station.$(CA)_5GA$ motif of microsatellite DNA in *P. muticus.* Bold alphabet are reported $(CA)_{10}$ microsatellite of chicken in GenBank. ■ Consensus bases in both sequences of *P. muticus.* Asterisks indicate the consensus bases.

4.4.5 Application of some microsatellite primers

Microsatellite can be used in a wide variety of applications including pedigree analysis, identification of individual animals, determining parentage, and selective breeding to minimize effects of inbreeding.

Many green peafowls from Khao Soi Dao wildlife research and breeding station at Chantaburi province have been found delibitated and some chicks were died by unknown reason. Thus microsatellite primers at the ADL 23 locus and HUJ 2 locus were investigated for testing polymorphism of ten *P. m. imperator*.

The results were found that allelic variation of *P. m. imperator* in the ADL23 locus was very low in 10 *P. m. imperator* from Khao Soi Dao wildlife research and breeding station (lane 5-14 in Figure 4.25). But allelic variation occurred in *P. m. imperator* from Phattalung wildlife research and breeding station (lane 3-4 in Figure 4.25).

At the HUJ2 locus, allelic variation appeared only in male *P. m. imperator* number R0324 (lane 5 in Figure 4.28) but in 9 *P. m. imperator* remaining from Khao Soi Dao wildlife research and breeding station was not found allelic variation of microsatellite bands. However, some bands (about 249 basepairs) of female *P. m. imperator* in Khao Soi Dao wildlife research and breeding station (lane 12, 13 and 14 in Figure 4.26) differed from male *P. m. imperator* in the same station. But these bands were not microsatellite marker DNA (results from section 4.4.4 sequencing at the HUJ2 locus).

From both loci, most of *P. m. imperator* in Khao Soi Dao wildlife research and breeding station had very low allelic variation.

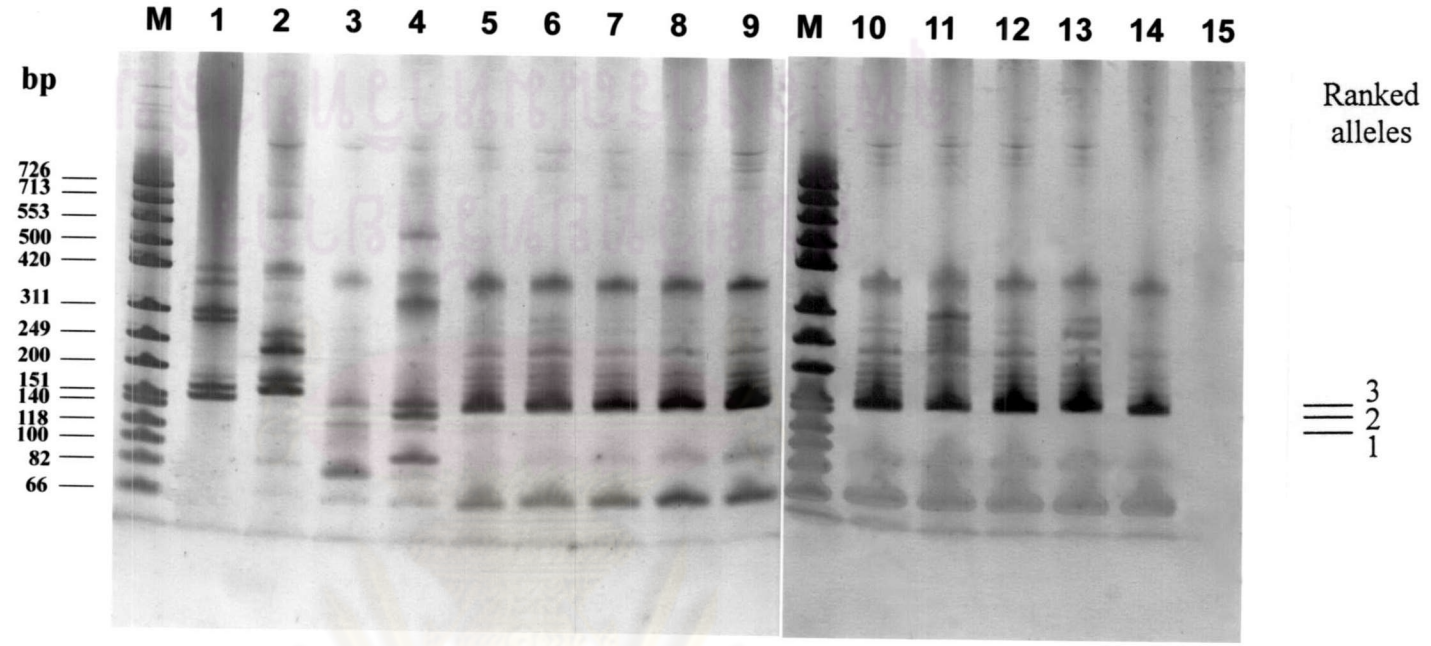


Figure 4.25 Silver staining of 8% polyacrylamide gels showing alleles distribution of the ADL 23 locus under the optimal PCR condition with an annealing temperature at 55^0C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker

Lane 1 - 2 : Male jungle fowl and male Batum, respectively (positive controls)

Lane 3 - 4 : Male and female Thai green peafowls from Patthalung wildlife research and breeding station , respectively (PL)

Lane 5 - 9 : Male Thai green peafowl from Khao Soi Dao wildlife research and breeding station(SD)

Lane 10-14: Female Thai green peafowl from SD

Lane 15 : Negative control

Remark: All alleles were sequenced. Only 3 alleles from DNA template of *P. m. imperator* (lane 3-14)were microsatellite DNA.

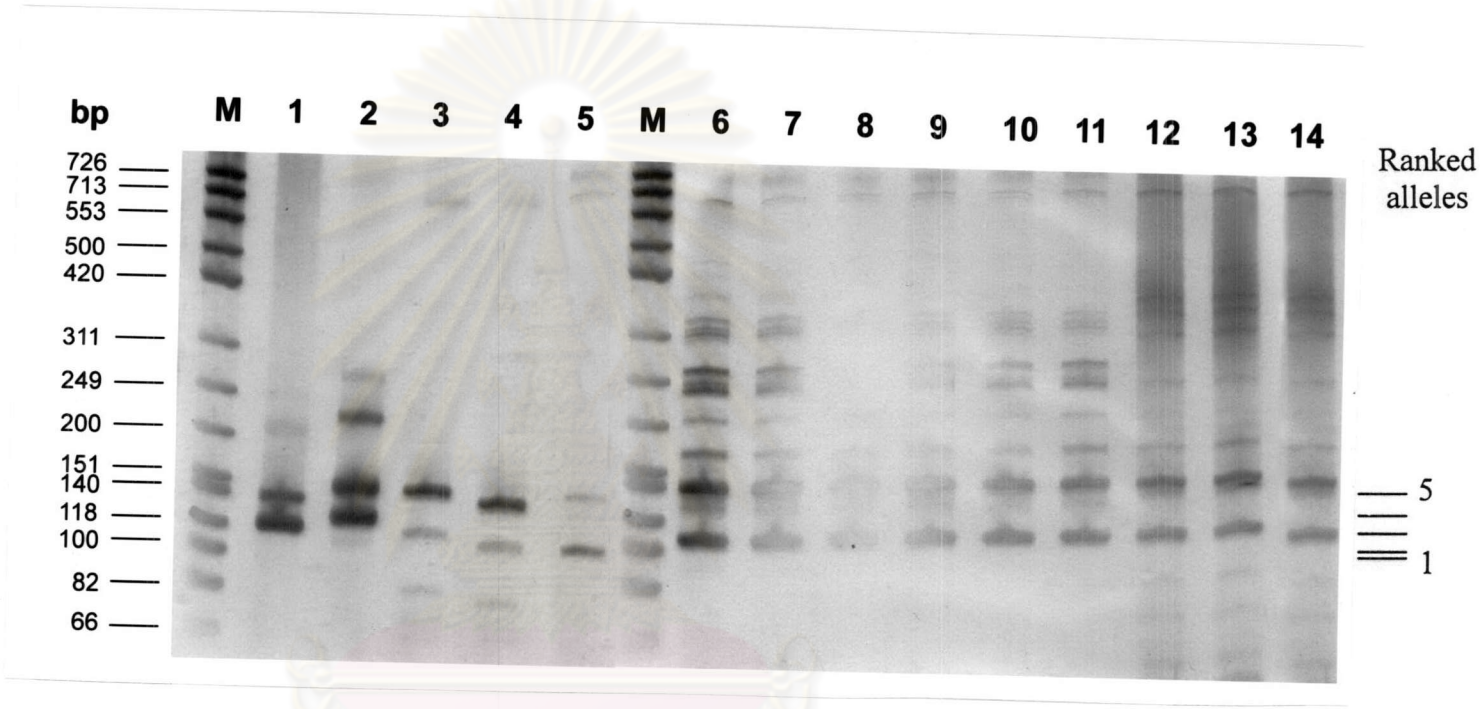


Figure 4.26 Silver staining of 8% polyacrylamide gel showing alleles distribution of the HUJ 2 locus under the optimal conventional PCR condition with an annealing temperature at 55^0C and using different DNA template as followed.

Lane M : PhiX 174/*Hinf* I standard marker

Lane 1 - 2 : Male jungle fowl and male Batum, respectively (positive controls)

Lane 3 - 4 : Male and female Thai green peafowl from Patthalung wildlife research and breeding station(PL) , respectively

Lane 5 - 9 : Male Thai green peafowl from Khao Soi Dao wildlife research and breeding station(SD)

Lane 10-14: Female Thai green peafowl from SD

Remark: All alleles were sequenced. Only 5 alleles from DNA template of *P. m. imperator* (lane 3-14) were microsatellite DNA.

4.4.6 Highest percentage in each component and condition PCR optimization

All results of PCR components optimization with 23 microsatellite loci screened in this study were summarized in circle chart (Figure 4.27). Each PCR component in the list below has value which got the highest percentage, for example, the T_m-5°C annealing temperature was chosen for the list because it could successful amplify PCR products more than other temperatures.

The list

Components

Reaction volume	:	25 µl
P. muticus DNA template	:	50 ng
Taq	:	1 unit
Magnesium chloride	:	1.5 mM
Forward primer	:	0.1 µM
Reverse primer	:	0.1 µM
dNTPs	:	0.2 mM

Conditions

Preincubation	:	94°C for 2 min.
Denaturation	:	94°C for 1 min.
Annealing temperature	:	T_m-5°C for 1 min.
Extension	:	72°C for 1 min.
Final extension	:	72°C for 10 min.

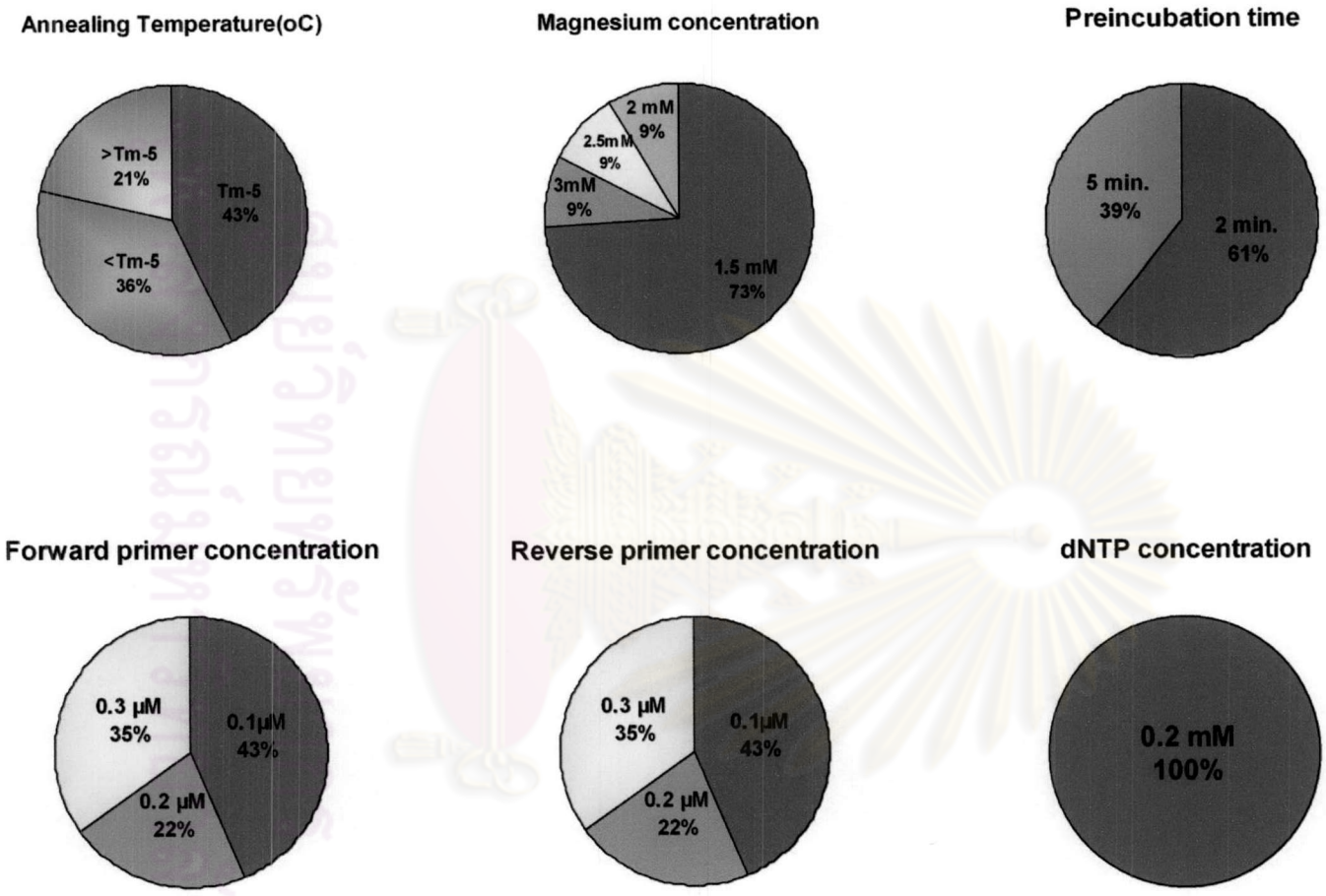


Figure 4.27 Percentages of optimized annealing temperatures, component concentrations and preincubation times of 23 microsatellite loci screened on *P. m. imperator*

4.5 RAPD analysis

4.5.1 Effect of different *Taq* polymerase on RAPD-PCR

RAPD-PCR amplification using enzyme *Taq* polymerase from Promega Corporation (with buffer B) was compared with that using DyNAzyme™ II DNA polymerase from Finnzymes. RAPD-PCR products were not able to be amplified when using *Taq* polymerase from Promega but successfully amplified when using DyNAzyme™ II DNA polymerase. Therefore, DyNAzyme™ II DNA polymerase were chosen for RAPD amplification in all further study.

4.5.2 Effect of different sources of DNA templates on RAPD-PCR

Genomic DNA from blood and feather samples gave different RAPD-PCR product patterns. The result was shown in Figure 4.28.

4.5.3 Template concentrations

Before RAPD primers screening, diferrent amount of DNA templates were tested ie. 10, 50, 100, 150 and 300 ng per each reaction (15 µl). The result was shown in Figure 4.31. The PCR products had lowest yield when using 10 ng of template DNA per reaction. Some RAPD bands disappeared when using 150 ng per reaction whereas there was no PCR product at all when using 300 ng DNA template per reaction. In the conclusion, the least amount of DNA template from *P. m. imperator* giving high-yield PCR products was found to be 50 ng per reaction which then was used in all further RAPD experiments..

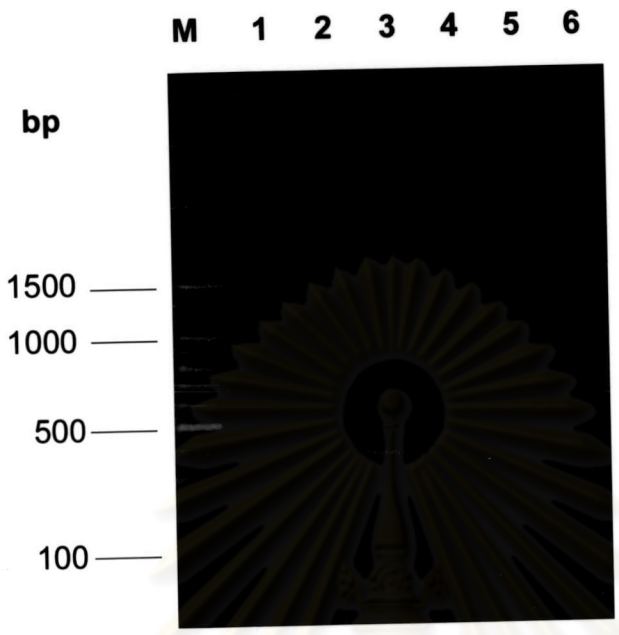


Figure 4.28 A 1.5% ethidium-bromide stained agarose gel showing RAPD-PCR product patterns from different DNA template sources. This RAPD-PCR used the primer number 6 with DNA templates from bloodstains and feathers of the male *P. m. imperator* number R0019 from Khao Soi Dao wildlife research and breeding station. PCR product amplified with different template concentration shown in lane2-5.

Lane M: 100 bp DNA ladders marker (0.1 μg)

Lane 1 : 50 ng DNA template from blood stain of *G. gallus* (positive control)

Lane 2 : 200 ng template from blood stain of *P. m. imperator*

Lane 3 : 50 ng template from blood stain of *P. m. imperator*

Lane 4 : 50 ng template from feather of *P. m. imperator*

Lane 5 : 20 ng template from blood stain of *G. gallus*

Lane 6 : Negative control

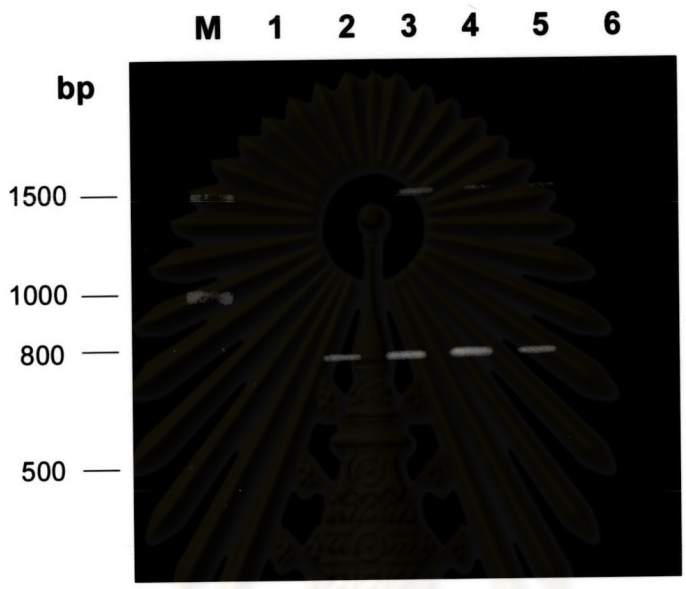


Figure 4.29 A 1.5% ethidium-bromide stained agarose gel showing RAPD-PCR product patterns resulted from various DNA template concentrations. This RAPD reaction used the primer number 100 with DNA templates from bloodstains of the male *P. m. imperator* number R0019 from Khao Soi Dao wildlife research and breeding station.

Lane M: 100 bp DNA ladders marker
Lane 1: Negative control
Lane 2: PCR products amplified with 10 ng DNA template
Lane 3: PCR products amplified with 50 ng DNA template
Lane 4: PCR products amplified with 100 ng DNA template
Lane 5: PCR products amplified with 150 ng DNA template
Lane 6: PCR products amplified with 300 ng DNA template

4.5.4 PCR Condition for RAPD primer screening

After primarity testing with DNA templates and DNA polymerase enzymes, 60 RAPD primers were screened using the same PCR condition as in Table 4.9. However, some of RAPD amplifications could not give the same RAPD patterns even when the same primer and PCR condition were used. The reproducible problem of RAPD was reported in the topic 4.5.7.

4.5.5 RAPD-PCR products

The patterns of RAPD-PCR products from sixty screened primers were grouped into three result types. The results were shown in Table 4.10.

4.5.6 Selection RAPD primers

Only 2 primers (the primer number 1 and primer number 13) from sixty screened primers showed genetic polymorphism in RAPD-PCR products. However, the genetic variation among samples was not high (Figure 4.30 and 4.31). The low genetic polymorphism was found in the primer number 1(Figure 4.30). On the other hand, the primer number 13 was found it could amplify some samples (Figure 4.31).

Table 4.9 Concentration of each chemical component in a RAPD-PCR reaction

Component	Final Concentration
DNA template	50 ng*
DNA polymerase	0.15 unit of DyNAzyme™ II* DNA Polymerase.
10X Mg^{2+} free buffer	1X†
Mg^{2+}	1.5 mM†
Primer	0.2 µM††
dNTP	200 µM†
Reaction volume	15 µl

*Adjusted components for *P. muticus imperator*

† Standard concentration of RAPD amplification (Piyachokanakul, 2002)

††Recommended concentration from the Biotechnology Laboratory, University of British Columbia.

Table 4.10 Amounts and percentages of 3 RAPD-PCR result types

Type	Primer number	Amount	%
No RAPD-PCR products	4, 5, 7, 8, 9, 10, 11, 12, 14, 17, 19, 20, 22, 26, 27, 28, 29, 30, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 56, 57, 58, 99	39	65
-Amplified PCR products without genetic polymorphism	2, 3, 6, 15, 16, 18, 23, 24, 25, 31, 32, 33, 34, 51, 52, 53, 54, 98, 100	19	31.67
-Amplified PCR products with genetic polymorphism	1,13	2	3.33
		60	100

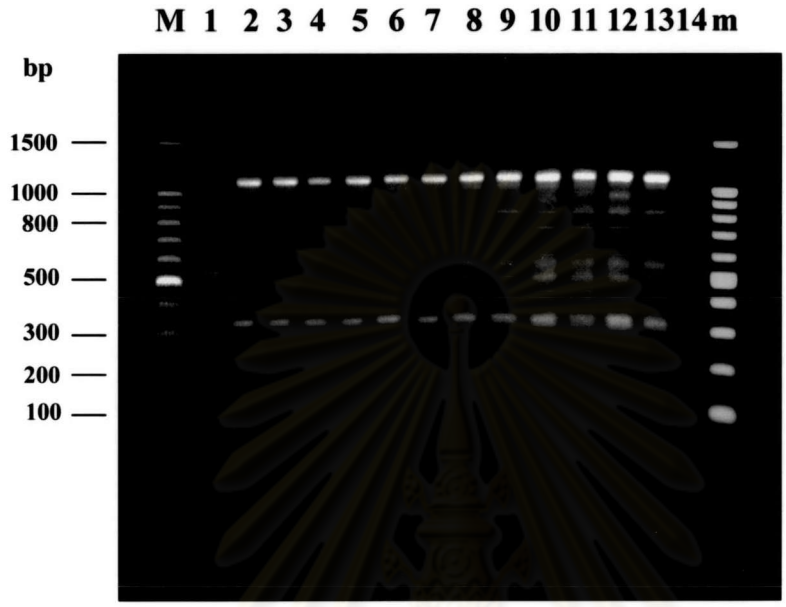


Figure 4.30 A 1.5% ethidium bromide stained agarose gel showing RAPD-PCR product patterns from the primer number 1. Different DNA templates were shown in lane 1-13.

Lane M : 100 bp DNA ladders marker (0.2 μg)
Lane 1 : Positive control
Lane 2 - 3: Male and female Thai green peafowls from PL, respectively
Lane 4 - 6: Male and female Thai green peafowl from SD, respectively
Lane 7 : Female Cambodia green peafowl from SD
Lane 8 : Male Thai green peafowl from PN
Lane 9 : Male Thai green peafowl from WL
Lane 10 : Male Thai green peafowl from SN
Lane 11 : Male Thai green peafowl from HH
Lane 12-13: Male and female Thai green peafowl from HK, respectively
Lane 14 : Negative control
Lane m : 100 bp DNA ladders marker (0.4 μg)

Figure 4.31 A 1.5% ethidium bromide stained agarose gel showing RAPD-PCR product patterns from the primer number 13. Different DNA template from (lane1-13).

Lane M : 100 bp DNA ladders marker (0.2 μg)
Lane 1 - 2 : Male and female Thai green peafowl from PL
Lane 3 - 4: Male and female Thai green peafowl from SD
Lane 5 : Female Cambodia green peafowl from SD
Lane 6 : Male Thai green peafowl from PN
Lane 7 : Male Thai green peafowl from WL
Lane 8-9: Male and female Thai green peafowl from HK
Lane 10 : Male Thai green peafowl from SN
Lane 11 : Male Thai green peafowl from HH
Lane 12-13: Male and female Thai green peafowl from SD
Lane 14 : Positive control
Lane 15 : Negative control

4.5.7 Reproducibility of RAPD

Some RAPD-PCR amplifications were found the different RAPD patterns when using the same primers and PCR conditions. Among 60 decanucleotide primers, the primer number 2, 3, 15 and 25 showed low reproducible rates whereas the RAPD primer number 1 and 6 were found to be highly reproducible.

4.5.8 Ghost bands

Among 60 RAPD primers, no fault-positive band (ghost band) appeared in negative controls in the reaction 59 primers. However, there was some ghost bands occurred only in the primer number 6. When RAPD-PCR was repeated for three times, ghost bands appeared for two times in the same pattern. Ghost bands were shown in Figure 4.32.

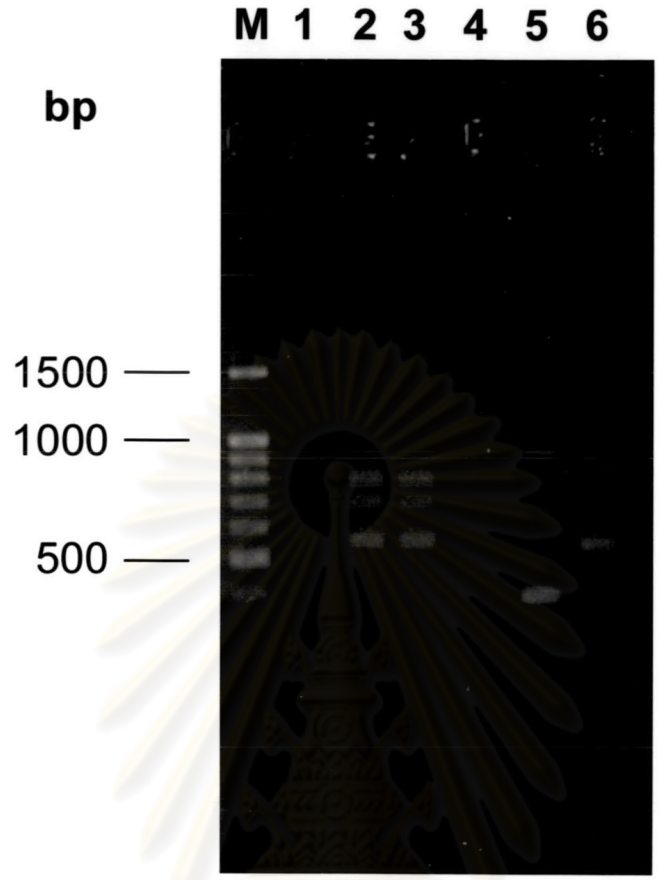


Figure 4.32 A 1.5% ethidium bromide stained agarose gel showing RAPD-PCR ghost bands only in (lane 2-3) from primer number 6. In the negative control in 1st, 2nd and 3rd RAPD-PCR amplification (lane1-3), in the RAPD products from DNA of *P. m. imperator* (lane 4-5), in the RAPD product from DNA of *G. gallus* (lane6).