CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

1. Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
2. Automatic micropipette P10, P20, P200, and P1000, Gilson Medical Electronic, France
3. Centrifuge, model: Centrifuge 5410, Eppendorf, Germany
4. Disposable gloves, Meditrate, USA
5. Disposable syringe Tuberculin® 1.0 ml. with needle gauge number 25
6. Electronic clock timer, model: CT-30, Canon Co. Ltd., Japan
7. Electronic U.V. transilluminator, Ultra Lum Inc., USA
8. Electrophoresis, model: Mupid, Advance Co. Ltd., Japan
9. Ice box, Scientific Plastic Co. Ltd., USA
10. Microcentrifuge tube 0.2, 0.5 and 1.0 ml., Treff®, Switzerland
11. Microincubator, model: M-36, Taitec, Japan
12. Micropipette tip P10, P20, P200 and P1000, Treff®, Switzerland
13. Microwave, model: Sharp carousel R7456, Sharp, Thailand
14. PCR, model: Perkin-Elmer 9700, PE Applied Biosystem, Singapore
15. Fuji film, model: Fp-3000B, Fuji Photo Film Co. Ltd., Japan
16. Polaroid camera, model: Direct screen instant camera DS 34 H-34, Peca Products, UK
17. Power supply, EC 570-90 LVD CE, E-C Apparatus Corporation, USA
18. Pyro magnestir, Lab-line Instrument, Inc., USA
19. pH meter, Cyberscan 500, Eutech Cybernetics, Singapore
20. Whatman® filter paper: number 1, England
21. Whatman® Laboratory sealing film, England
22. Surgical knife, a pair of scissors and forceps
23. -20 °C Freezer, Sanyo Co. Ltd., Japan

2.2 Chemicals

1. Absolute ethanol, Merck, Germany
2. Boric acid, Bio-RAD Laboratory, USA
3. 100 mM dATP, dGTP, dCTP, dTTP, Promega Corporation, USA
4. Ethylene diamine tetra-acetic acid (EDTA), C10H16N2O8, M.W. = 292.2, Bio-RAD Laboratory, USA
5. 95% (v/v) Ethanol, CH3CH2OH, M.W. = 46, Thailand
6. 6X Loading dye, Promega Corporation, USA
7. QIAamp DNA extraction mini kit (catalog # 51304), Qiagen, Germany
8. Tris-(hydroxymetyl)-aminomethane, NH2C(CH2OH)3, M.W. = 121.4, Pharmacia Biotech, USA

2.3 Enzymes

1. DyNAzymeTM II DNA Polymerase, Finnzymes, Finland
2. Proteinase K, Promega Corporation, USA

2.4 Oligonucleotide primers

One hundred of oligonucleotide primers were chosen from several publications which were synthesized by BioService Unit, National Science and Technology Development Agency, Bangkok, Thailand. Primers number 1-30 were selected from publications (Deepak et al., 1998) and number 31-100 were chosen from The Biotechnology Laboratory, University of British Columbia (Canada) (Table 2.1). Screening and selecting the proper primers was one of the prior steps in PCR experiment for this study. The selected primers should give high enough polymorphism and a constant pattern was chosen for further study in all gallopheasants and partridge.

Table 2.1 Sequence of oligonucleotide primers

No.	Primer	Sequence	Reference
1	OPA-01	CAG GCC CTT C	Greater Rhea
2	OPA-02	TGC CGA GCT G	Greater Rhea
3	OPA-04	AAT CGG GCT G	Greater Rhea
4	OPA-06	GGT CCC TGA C	Guinea fowl
5	OPA-07	GAA ACG GGT G	Guinea fowl
6	OPA-08	GTG ACG TAG G	Greater Rhea
7	OPA-09	GGG TAA CGC C	Greater Rhea
8	OPA-10	GTG ATC GCA G	Greater Rhea
9	OPA-14	TCT GTG CTG G	Greater Rhea
10	OPA-16	AGC CAG CGA A	Greater Rhea
11	OPA-17	GAC CGC TTG T	Guinea fowl
12	OPA-18	AGG TGA CCG T	Guinea fowl
13	OPB-19	ACC CCC GAA G	Guinea fowl
14	OPC-01	TTC GAG CCA G	Greater Rhea
15	OPC-02	GTG AGG CGT C	Greater Rhea
16	OPC-05	GAT GAC CGC C	Greater Rhea
17	OPC-06	GAA CGG ACT C	Greater Rhea
18	OPC-09	CTC ACC GTC C	Greater Rhea
19	OPC-10	TGT CTG GGT G	Greater Rhea
20	OPC-11	AAA GCT GCG G	Greater Rhea
21	OPC-18	TGA GTG GGT G	Greater Rhea
22	OPC-19	GTT GCC AGC C	Greater Rhea
23	OPI-05	TGT TCC ACG G	Guinea fowl
24	OPM-08	TCT GTT CCC C	Guinea fowl
25	OPM-09	GTC TTG CGG A	Guinea fowl
26	OPM-10	TCT GGC GCA C	Guinea fowl
27	OPP-03	CTG ATA CGC C	Guinea fowl

28	OPP-05	CCC CGG TAA C	Guinea fowl
29	OPP-09	GTG GTC CGC A	Guinea fowl
30	OPP-10	TCC CGC CTA C	Guinea fowl
31	UBC-001	CCT GGG CTT C	UBC set 1
32	UBC-002	CCT GGG CTT G	UBC set 1
33	UBC-003	CCT GGG CTT A	UBC set 1
34	UBC-006	CCT GGG CCT A	UBC set 1
35	UBC-013	CCT GGG TGG A	UBC set 1
36	UBC-049	TTC CCC GAG C	UBC set 1
37	UBC-050	TTC CCC GCG C	UBC set 1
38	UBC-098	ATC CTG CCA G	UBC set 1
39	UBC-100	ATC GGG TCC G	UBC set 1
40	UBC-101	GCG GCT GGA G	UBC set 2
41	UBC-102	GGT GGG GAC T	UBC set 2
42	UBC-103	GTG ACG CCG C	UBC set 2
43	UBC-104	GGG CAA TGA T	UBC set 2
44	UBC-105	CTC GGG TGG G	UBC set 2
45	UBC-106	CGT CTG CCC G	UBC set 2
46	UBC-107	CTG TCC CTT T	UBC set 2
47	UBC-108	GTA TTG CCC T	UBC set 2
48	UBC-109	TGT ACG TGA C	UBC set 2
49	UBC-110	TAG CCC GCT T	UBC set 2
50	UBC-111	AGT AGA CGG G	UBC set 2
51	UBC-112	GCT TGT GAA C	UBC set 2
52	UBC-113	ATC CCA AGA G	UBC set 2
53	UBC-114	TGA CCG AGA C	UBC set 2
54	UBC-115	TTC CGC GGG C	UBC set 2
55	UBC-116	TAC GAT GAC G	UBC set 2
56	UBC-117	TTA GCG GTC T	UBC set 2
57	UBC-118	CCC GTT TTG T	UBC set 2

58	UBC-119	ATT GGG CGA T	UBC set 2
59	UBC-120	GAA TTT CCC C	UBC set 2
60	UBC-121	ATA CAG GGA G	UBC set 2
61	UBC-122	GTA GAC GAG C	UBC set 2
62	UBC-123	GTC TTT CAG G	UBC set 2
63	UBC-124	ACT CGA AGT C	UBC set 2
64	UBC-125	GCG GTT GAG G	UBC set 2
65	UBC-126	CTT TCG TGC T	UBC set 2
66	UBC-127	ATC TGG CAG C	UBC set 2
67	UBC-128	GCA TAT TCC G	UBC set 2
68	UBC-129	GCG GTA TAG T	UBC set 2
69	UBC-130	GGT TAT CCT C	UBC set 2
70	UBC-131	GAA ACA GCG T	UBC set 2
71	UBC-132	AGG GAT CTC C	UBC set 2
72	UBC-133	GGA AAC CTC T	UBC set 2
73	UBC-134	AAC ACA CGA G	UBC set 2
74	UBC-135	AAG CTG CGA G	UBC set 2
75	UBC-136	TAC GTC TTG C	UBC set 2
76	UBC-137	GGT CTC TCC C	UBC set 2
77	UBC-138	GCT TCC CCT T	UBC set 2
78	UBC-139	CCC AAT CTT C	UBC set 2
79	UBC-140	GTC GCA TTT C	UBC set 2
80	UBC-141	ATC CTG TTC G	UBC set 2
81	UBC-142	ATC TGT TCG G	UBC set 2
82	UBC-143	TCG CAG AAC G	UBC set 2
83	UBC-144	AGA GGG TTC T	UBC set 2
84	UBC-145	TGT CGG TTG C	UBC set 2
85	UBC-146	ATG TGT TGC G	UBC set 2
86	UBC-147	GTG CGT CCT C	UBC set 2
87	UBC-148	TGT CCA CCA G	UBC set 2

88	UBC-149	AGC AGC GTG G	UBC set 2
89	UBC-150	GAA GGC TCT G	UBC set 2
90	UBC-151	GCT GTA GTG T	UBC set 2
91	UBC-152	CGC ACC GCA C	UBC set 2
92	UBC-153	GAG TCA CGA G	UBC set 2
93	UBC-154	TCC ATG CCG T	UBC set 2
94	UBC-155	CTG GCG GCT G	UBC set 2
95	UBC-156	GCC TGG TTG C	UBC set 2
96	UBC-157	CGT GGG CAG G	UBC set 2
97	UBC-158	TAG CCG TGG C	UBC set 2
98	UBC-159	GAG CCC GTA G	UBC set 2
99	UBC-160	CGA TTC AGA G	UBC set 2
100	UBC-161	CGT TAT CTC G	UBC set 2

2.5 Sample collection

Blood sample and feathers of gallopheasants were collected from various parts of Thailand at least three different locations and each were collected location least 3 individuals. The detail of specimens were shown in Table 2.2 and Appendix A.

Blood sample was collected by radial venipuncting from a branchial vein with a new Tuberculin® syringe with needle gauge number 25. About 0.1-0.2 ml of blood was dropped on a piece of autoclaved Whatman® filter paper, air dried and placed into labeled autoclaved paper bag for each sample. Every step must use clean technique and complete air dried to avoid fungal contaminate. A feather was collected by cutting at the end and placed into a labeled paper bag. Both blood stains and feathers were kept in desiccators or room temperature.

Table 2.2 Shown species, locations and number of specimens used in this studied.

Species	Location	Number of individual
Great Argus	Hala Bala wildlife sanctuary (from the wild)	3
	Khao Pratabchang wildlife research and breeding station	3
	Khao Soi Dao wildlife research and breeding station	3
	Individual farm in Amphoe Sattaheep, Chonburi Province	2
Red Junglefowl	Huay Kha Kaeng Wildlife Sanctuary	6
	Huay Yang Parn wildlife research and breeding station	1
	Phatthalung wildlife research and breeding station	3
	Satun	3
Siamese Fireback	Phu Khieu wildlife research and breeding station	3
	Individual farm in Amphoe Sattaheep, Chonburi Province	3
	Khao Pratabchang wildlife research and breeding station	3
	Khao Soi Dao wildlife research and breeding station	3
Green Peafowl	Khao Pratabchang wildlife research and breeding station	3
	Huay Yang Parn wildlife research and breeding station	1
	Pong from the wild, Payao province	1
	Lumpang from the wild, Lumpang province	1
	Chiang Khong Wild, Chiang Rai province	1
	Surin from the wild	3
	Phu Khieu wildlife research and breeding station	2
	Khao Soi Dao wildlife research and breeding station	3
	Mae Ping Watrershed, Chiang Mai (from the wild)	3
Creasted Wood Partridge	Individual Farm in Bangkok	1
	Khok Mai Rua wildlife research and breeding station	2
	Amphoe Sattaheep, Chonburi Province	3
	Khao Pratabchang wildlife research and breeding station	3

2.6 DNA extraction

Total DNA of gallopheasant were extracted from bloodstain and feather; papilae. In this study was used QIAamp DNA extraction mini kit method to prepare DNA template for PCR (Dried blood spot protocol, QIAamp® DNA Mini Kit, QIAGEN, Germany), according to the manufacturer's instructions for tissues.

QIAamp® Kit extraction method

Genomic DNA was also extracted by QIAamp® DNA Mini Kit (Qiagen, Germany) and shown in Appendix C. The kit together with its buffer set, are designed to isolate pure genomic DNA direct from various samples including the dried-blood spot samples. Its procedure bases on the a selectivity of QIAGEN anion-exchange resin, which allows isolation of high yields of pure genomic DNA ranges in size from 20-150 kb.

A dried blood spot was cut 3 mm (1/8 inch) diameter punches with a pair of sterile scissors. The blood punch was transferred into a new 1.5 ml centrifuge tube containing 180 µl of buffer ATL. Then it was incubated at 85 °C for 10 minutes and briefly centrifuged to remove solution drops from inside of the lid. Twenty microlitres of proteinase K were added to the sample, mixed thoroughly by vortex, followed by incubation it at 56 °C for 1 hour or until the blood was dissolved out from the paper. After that, 200 µl of buffer AL was added to the sample, mixed well by vortex, and incubated at 70 °C for 10 minutes. In order to ensure an efficient lysis, it was essential that the sample and buffer AL was mixed immediately and thoroughly. Two hundred microlitres of 96 to 100% ethanol were added, mixed thoroughly by vortex and briefly centrifuged to remove drops from inside the lid. The mixture was carefully applied to the QIAamp spin column without wetting the rim. The sample was centrifuged at 8,000 rpm for 1 minute and the filtrate was discarded. The QIAamp spin column was washed first time with 500 µl buffer AW1, centrifuged at 8,000 rpm for 1 minute, and the collection tube containing filtrate was discarded. Then 500 µl of buffer AW2 was added for second washing and centrifuged at full speed or 14,000 rpm for 3 minutes. The column

was centrifuged again at maximum speed for 1 minute to completely remove any buffer AW2 because residual buffer AW2 in the elute may cause problems in downstream applications. After centrifugation, the bound genomic DNA was eluted with 150 µl of buffer AE or distilled water to a new 1.5 ml centrifuge tube. The QIAamp spin column was incubated at room temperature for 1 minute, and then at 8,000 rpm for 1 minute. DNA extracts were kept at -20 °C (shown in Appendix C).

For DNA extraction of feather, single feather tip was washed with 70% ethanol and sterile water. Then 5-10 mm of the end of the tips was divided to 2 sides, sliced off with a sterile razor blade, and transferred to a 1.5 microcentrifuge tube. They were chopped with a pair of sterile scissors. This step was importance for DNA extraction from feathers. One hundred and eighty microlitres of buffer ATL were added to the microcentrifuge tube. After lysis complete, other buffers were added respectively as suggested. The later extraction steps for feathers are like those for blood spots.

2.7 Quality determination of genomic DNA

Electrophoresis is a standard method used for estimation of DNA quality on the basis of its direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining. DNA was run in a 0.8% agarose gel prepared in 1X TBE buffer (89 mM Tris HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) at 100 V. After electrophoresis, the gel was stained with ethidium bromide. In addition, used 100 bp bioladder as a DNA standard molecular size marker.

2.8 Screening of primers used in this study

From one hundred RAPD primers were screened for amplification of five different breeds in gallopheasants and partridge DNA. Fifteen primer (OPA-18, OPC-02, OPC-10, OPM-10, OPP-03, OPP-09, OPP-10, UBC-02, UBC-98, UBC-101, UBC-104, UBC-132, UBC-133, UBC-135 and UBC-137) were gave high enough polymorphism and a constant pattern for the amplification reactions. Only appropriate five primers (OPA-18,

OPC-02, OPP-03, UBC-133 and UBC-135) were chosen for this study because it generated easy scoring patterns and appeared in all species.

2.9 *In vitro* amplification of Randomly Amplified Polymorphic DNA primer using the polymerase chain reaction (PCR)

RAPD analysis. PCR amplification was carried out in a 15 µl reaction mixture, containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 5 pmole of the primers, 50 ng of genomic DNA and 0.01 unit of DyNAzymeTM II DNA Polymerase. The reaction mixture was performed in a DNA Thermal Cyclic (Perkin Elmer Model 9700) programmed for 40 cycles consisting of 94 °C predenaturation for 3 minutes, a 94 °C denaturation for 1 minute, a 37 °C annealing for 1 minute and a 72 °C extension for 2 minutes. The final extension was carried out at 72 °C for 5 minutes (modified from Peyachoknagul, 2002). After amplification, the resulting RAPD-PCR products were electrophoretically analyzed as soon as possible. If not, RAPD-PCR products were kept at 4 °C.

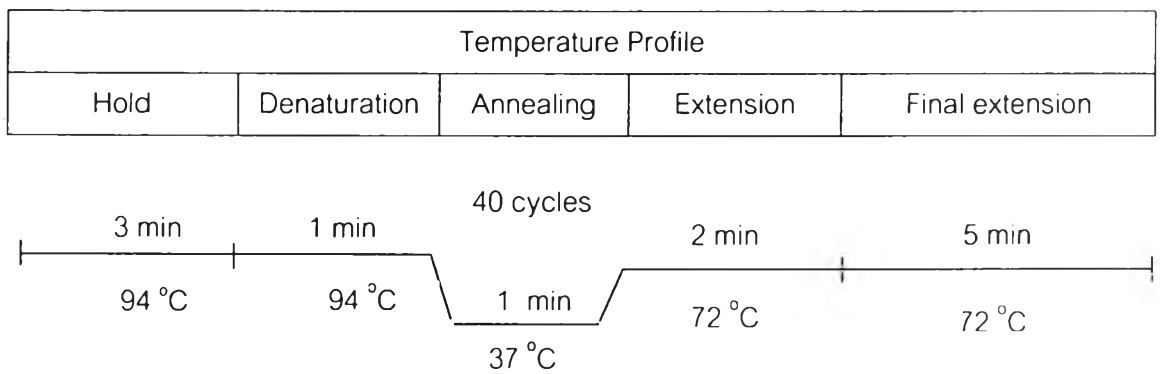

Figure 2.1 Temperature profile for all RAPD primers

2.10 Agarose gel electrophoresis analysis

RAPD PCR products were analyzed with 1.8% agarose gel electrophoresis (Appendix D). The 1.8% agarose was weighed out and heated to dissolve in 100 ml of 1X TBE buffer. Melted agarose was poured into the gel mould that the comb was already inserts to gel mould. When the melted agarose were left at room temperature to cool to approximately about 40-50 °C (the gel had cooled and solidified), the comb was gently removed. The gel submerged in the gel chamber containing an enough of 1X TBE buffer that covered the gel to a depth about 1-2 mm. Each of RAPD PCR product was mixed with 6x loading dye by ratio 5:1; that is, 15 microlitres of PCR products were mixed with 2 µl of 6x loading dye (0.03% bromophenol blue, 0.03 % xylene cyanol FF, 0.4 % orange G, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA and 15 % ficoll® 400: Promega). The samples were applied into the wells slowly that used 100 bp DNA ladders, was loaded into a well for served as a DNA standard. A gel chamber was connected to a power supply and turned on a power supply (the gel was run at 80 volt), then DNA migrated into the gel toward the anode. When bromophenol blue moved to approximately 0.5 mm from the bottom of the gel (about 1.10 hrs), turned off the power supply and stain the gel with 0.25 µg/ml ethidium bromide for 3 minute. The gel was destained in a bath of deionise distilled water for 10-20 minutes to leach out unbound ethidium bromide from the gel. The RAPD bands were visualized under a UV transilluminator. The gel was directly photographed using a Polaroid camera with Fuji 3000B Polaroid film. The exposure time was usually about 10-15 seconds.

2.11 Statistical procedures of determination of genetic variation by RAPD analysis

1.5 kb+100 bp standard markers were used to assign the size of each RAPD fragment. Only fragments that were 200 bp–1.5 kb in size and dark bands that could be accurately scored throughout all lanes were chosen to score. Each RAPD fragment was assigned a molecular length and recorded in binary matrix for each individual as presence (1) or absence (2) of a given band by naked eyes. The percentages of polymorphic and monomorphic bands were evaluated. The RAPD pattern of individuals

were compared between interspecies and intra-specific taxa among five gallopheasants and partridge.

For example species A and species B

RAPD pattern		locus	Scoring results	
A	B		A	B
		1	1	1
		2	1	2
		3	2	1

2.11.1 Genetic distance

A Genetic distance between pairs of genotypes or population is a quantitative estimate of genetic divergence between two compared operational taxonomic unit (Avis, 1994) and used Tools for Population Genetic Analyses (TFPGA) version 1.3 (Miller, 2000) and selected distance measurement method of Nei (1972, 1978) for calculated genetic distance.

2.11.2 Phylogentic reconstruction

The phylograms of interspecies and intraspecies were analyzed base on genetic distance obtained was constructed by using an exactly tests for population differentiation (Raymond and Rousset, 1995). Neighbor-joining was implemented by averages of genetic distance in Phylip version 3.6 (alpha2) (J. Felsenstein, Dept. of Genetics, Univ. of Washington, Seattle, 2001).