CHAPTER III

MATERIALS AND METHODS

3.1 Field work

The area of investigation covers about 550 km^2 (343,500 rai) in Doi Saket district, Chiang Mai province, called *Khun Mae Kuong* Forest, which is part of the state conservation program *Huai Hong Khrai* Royal Development Study Centre established in 1982. Three different types of forest were selected, two sites each: Hill evergreen forest (KPA & KNK, Figure 3.1a) and Hill evergreen forest with pine (KBA & KRD, Figure 3.1b) at relatively high elevation (1000-1800 m) and Dry deciduous forest at lower altitudes below 800 m (KPS & KHH, Figure 3.1c), some 50 km from the hill sites (Figure 3.2). The field work was organized with the help of local staff of the *Huai Hong Khrai* centre. At each site, 20 - 25 trees in the family Fagaceae (*Castanopsis, Lithocarpus* and *Quercus*, Table 3.1), which were 5 - 30 m tall and 30 - 100 m apart, were selected for this investigation. Tracking routes into the forests followed those occasionally used by the locals who collected herbs and edible nuts, but starting usually from the highest point of a forest. The first Fagaceae tree encountered after about 50 m distance along the route downhill would be selected and so on with the next trees. The trees were marked and numbered, local names recorded together with their relative positioning.

Twigs with fully grown leaves, fruits (acorns) and flowers were collected for taxonomic identification and kept as voucher specimen at Botany Department, Chulalongkorn University. Taxonomic identification followed Flora Malesiana (Soepadmo, 1972) and the revision of the family Fagaceae for the Flora of Thailand (C. Phengklai, unpublished). Young leaves were collected for DNA isolation, while shoot buds and root tips were collected for chromosome isolation.

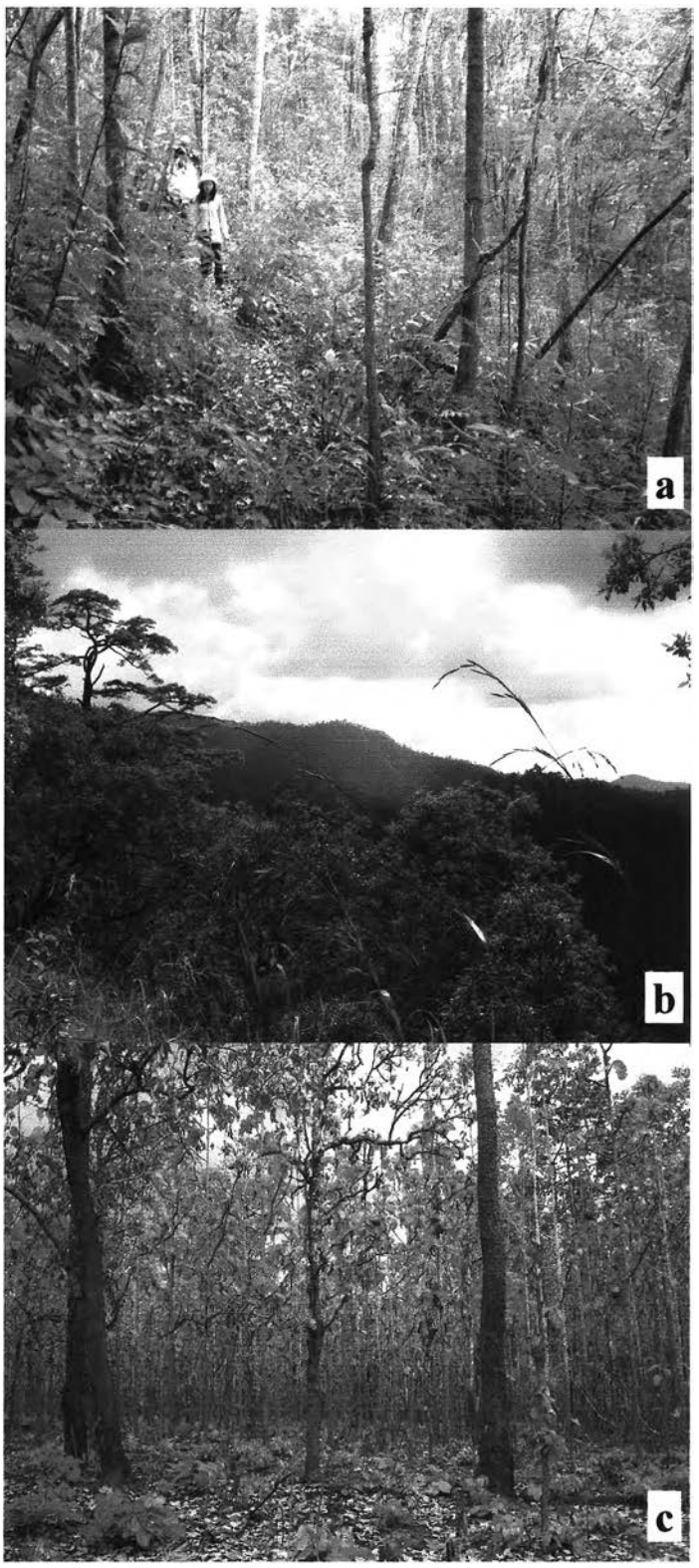

Figure 3.1: Three different types of forest: Hill evergreen forest (a), Hill evergreen forest with pine (b), and Dry deciduous forest (c).

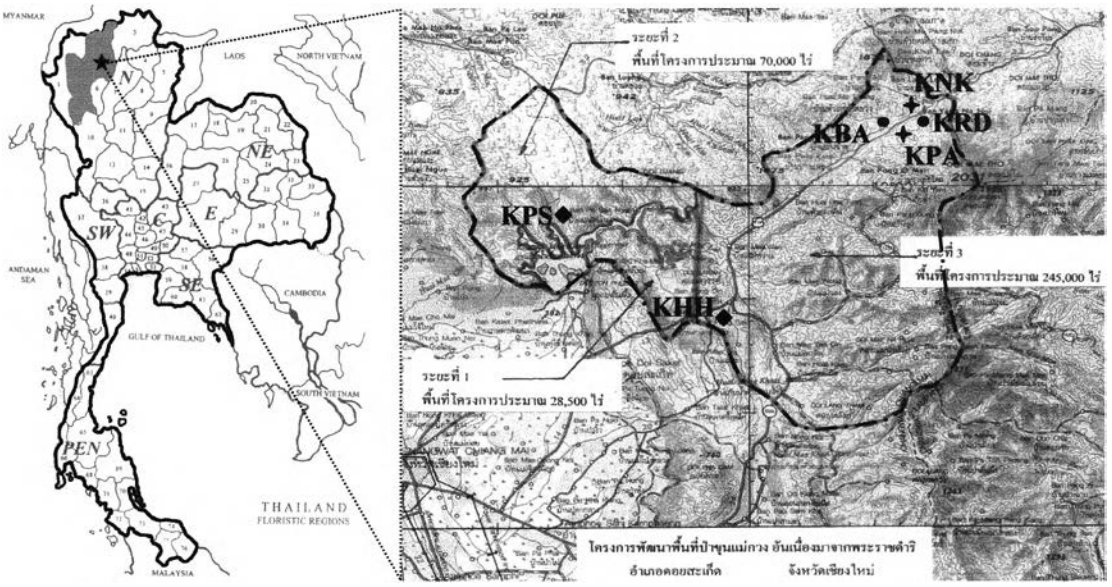

Figure 3.2: Map of the study site, *Khun Mae Kuong* Forest, at Doi Saket district, Chiang Mai province, with total area of about 550 km^2 (343,500 rai). Three different types of forest were selected, two sites each: Hill evergreen forest (KPA & KNK) and Hill evergreen forest with pine (KBA & KRD) at high elevation (1000 - 1800 m) and Dry deciduous forest at lower altitudes below 800 m (KPS & KHH).

Table 3.1: Plant materials used in this study.

Plant species			Number of trees from each species at each study site					
			Hill evergreen forest		Hill evergreen forest with pine		Deciduous Dipterocarp forest	
			KPA	KNK	KBA	KRD	KPS	KHH
Castanopsis	*C. acuminatissima* (Blume) A.DC.	Ca	-	12	3	3	-	-
	C. argentea (Blume) A.DC.	Cag	1	1	-	1	-	-
	C. armata (Roxb.) Spach	Car	1	1	-	-	-	-
	C. calathiformis (Skan.) Rehder & Wilson	Cc	3	2	-	-	-	-
	C. cerabrina (Hickel & A. Camus) Barnett	Cce	1	1	-	1	-	-
	C. crassifolia Hickel & A. Camus	Ccr	1	1	-	-	-	-
	C. diversifolia (Kurz) King & Hook.f.	Cd	4	2	-	-	-	-
	C. echinocarpa A.DC.	Ce	-	-	1	1	-	-
	C. ferox (Roxb.) Spach	Cfe	4	1	-	1	-	-
	C. fissa (Champ) Rehder & Wilson	Cfi	2	-	-	-	-	-
	C. indica (Roxb.) A.DC.	Ci	6	-	-	-	-	-
	C. tribuloides (Sm.) A.DC.	Ct	-	-	-	8	-	-
Lithocarpus	*L. ceriferus* (Hickel & A. Camus) A. Camus	Lc	-	-	1	1	4	-
	L. elegans (Blume) Hatus ex Soepadmo	Le	-	1	1	1	-	-
	L. harmandianus (Hickel & A. Camus) A. Camus	Lh	1	1	3	-	-	-
	L. polystachyus (A. DC.) Rehder	Lp	-	-	1	2	-	-
	L. recurvatus Barnett	Lr	-	-	1	-	-	-
	L. sootepensis (Craib) A. Camus	Ls	-	-	2	2	2	-
	L. vestitus (Hickel & A. Camus) A. Camus)	Lv	1	1	-	-	-	-
Quercus	*Q. brandisianus* Kurz	Qb	-	-	3	2	4	-
	Q. fleuryi Hickel & A.Camus	Qf	-	-	-	-	-	1
	Q. helferianus A.DC.	Qh	-	-	-	-	2	-
	Q. kerrii Craib	Qk	-	-	4	-	9	9
	Q. kingianus Craib	Oki	-	-	2	-	-	2
	Q. lenticellatus Barnett	Ql	-	-	-	1	-	1
	Q. lineatus Blume	Qli	-	-	-	-	-	3
	Q. mespilifolius Wall. ex DC.	Qm	-	-	-	-	4	2
	Q. myrsinaefoius Blume	Qmy	-	-	1		-	-
	Q. quangtriensis Hickel & A. Camus	Qq	-	-	-	-	-	5
	Q. rex Hemsl.	Qr	-	-	-	-	-	2
	Total number of trees (146)		**25**	**24**	**23**	**24**	**25**	**25**

3.2 Molecular genetics

3.2.1 DNA isolation

Total genomic DNA was extracted using method modified from Anamthawat-Jónsson and Heslop-Harrison (1995). Dried leaves were ground to powder in a mortar in liquid nitrogen. The powder was transferred to 7 ml of pre-warmed (65°C) isolation buffer in a 15 ml polypropylene centrifuge tube, mixed thoroughly, and incubated at 65°C for 2 h with occasional mixing by inversion. The isolation buffer contained 2% (w/v) CTAB (hexadecyl-trimethylammonium bromide), 2% (w/v) PVP (Polyvinylpyrrolidone), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 2% (v/v) 2-mercaptoethanol. The leaf lysate was extracted with 7 ml chloroform-isoamyl alcohol (24:1) by continuous mixing for 5 min and centrifugation at 3000 rpm for 10 min. The aqueous phase was transferred to a new tube using a wide-bore pipette, and the DNA was precipitated with two-third volumes of cold ethanol for 30 min. The DNA pellet was washed with 5 ml 70% ethanol, precipitated again at 3000 rpm for 5 min, air dried, and resuspended in 1 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA was removed by digestion with RNase-A (20 µg/ml) at 37°C for 1 h, after which the sample was diluted to 3 ml with TE buffer, the DNA was precipitated by adding 0.3 ml of 3 M sodium acetate and 7 ml of cold absolute ethanol and recovered by centrifugation for 10 min. Air-dried DNA pellet was resuspended in TE buffer, quantified by gel electrophoresis using 0.7% agarose. The DNA was stored at -20°C.

3.2.2 rDNA-RFLP

Total genomic DNA from individual plants was digested with restriction enzymes *Eco*RI, *Bam*HI, and *Hind*III. The digests were size fractionated by gel electrophoresis using 0.6 - 0.65% agarose, together with a *Hind*III digest of λ DNA as a size marker. The DNA in gel was depurinated with 0.25 M HCl for 20 min before Southern transfer to HybondN+ nylon membrane (Amersham-Biosciences) using 0.4

M NaOH as a transfer and denaturation buffer. The blot was then hybridised with labelled rDNA probe from the plasmid clone pTa71 (9-kb *Eco*RI fragment of wheat 18S-25S ribosomal genes (Gerlach and Bedbrook, 1979). The labelling, hybridization and detection was performed using ECL chemilluminescent system (Amersham-Biosciences) following the manufacturer's protocol. The stringency of hybridization was 78% (0.5 M NaCl) and the exposure time on film was 5 - 60 min.

3.2.3 ISSR

The inter-simple sequence repeat (ISSR, or anchored SSR) markers, which amplify variable microsatellites or flanking regions, were performed using method modified from Zietkiewicz et al. (1994). After an initial screening using 32 ISSR markers provided in primer set #9 from the University British Columbia Biotechnology Laboratory (UBC, Vancouver, Canada), 10 UBC-primers were selected (Table 3.2). The amplifications were performed in a final volume of 30 µl containing 10-20 ng total DNA, 200 µM of each dNTPs, 0.25 µM of each primer, 3.0 mM of $MgCl_2$, 1xPCR buffer and 1 unit Taq DNA polymerase (Fermentas). The amplifications were generated in a Techne Genius Thermocycler under the following conditions: an initial denaturation step at 94°C for 3 min, 45 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 90 sec, and the last extension step at 72°C for 10 min. DNA amplification fragments were separated in a 1.7% agarose gel and the size of amplified bands was estimated using DNA size marker (GeneRuler 100 bp DNA Ladder Plus, Fermentas).

Table 3.2: ISSR primer sequences. *Y stands for pyrimidine.

Primer from UBC set 9	
Code	Sequence*
810	$(GA)_8T$
825	$(AC)_8T$
834	$(AG)_8YT$
835	$(AG)_8YC$
840	$(GA)_8YT$
857	$(AC)_8YG$
868	$(GAA)_6$
873	$(GACA)_4$
876	$(GATA)_2(GACA)_2$
881	$(GGGTG)_3$

3.2.4 Genetic distant analysis

The rDNA-RFLP fragments and the ISSR amplification products were scored from each plant sample as present (1) and absent (0). These scores were used to generate distance matrix and to construct an UPGMA (unweighted pair group method with arithmetic average) phenogram, using the NTSYS-PC version 2.1 (Rohlf, 1998).

3.3 Karyotype analysis and molecular cytogenetics study

3.3.1 Chromosome preparation

Chromosomes of each sample were isolated using a method modified from Anamthawat-Jónsson (2003b). Leaf buds were collected and placed in ice-water (4

°C) for 23 - 27 h. The buds were then removed from ice-water and were quickly blot-dried excess water on filter paper before placing the buds in fresh cold fixative (3:1 absolute ethanol: glacial acetic acid). These fixed buds were kept at -20 °C until ready to begin the chromosome preparation. Rinse fixed buds with distilled water and keep in water for 30 min. Trim away outer leaves and use the small bud in side, which is about 1-2 mm. Place 2-3 buds in 100 µl of cellulose/pectinase enzyme mixture. Incubate at room temperature for at lease 3 h. Break the tissue in its enzyme mixture into suspension, filtered the suspension onto nylon mesh, which is places over 1.5 ml microtube. Discard the nylon mesh and cell debris. Add 1.5 ml of cold 75 mM KCl solution into the microtube containing protoplast suspension. Invert gently to mix and let stand for 15 min. Spin down the protoplast suspension at 7000 rpm for 5 min, discard the supernatant. Add 1.5 ml of fresh and cold fixative to the protoplast pellet, resuspend the protoplasts very gently and let stand at room temperature for 5 min. Spin down the protoplast at 7000 rpm for 5 min, discard the supernatant. Repeat this fixative treatment twice more, by adding another 1 ml of cold fixative, mixing and spinning down the protoplast pellet. Add 50 µl of fresh and cold fixative to the protoplast pellet and gently mix into suspension. Drop the protoplast suspension onto an ice-cold and wet slide from 10-20 cm height. When the drop has just dried up, dip the slide briefly in absolute ethanol, and air-dry the slide. The slides can be stored at 4 °C in a dry place. Examine chromosome preparations with phase contrast microscope, the preparations should contain evenly distributed nuclei, well-spread metaphases, and there should be no cell wall or cytoplasm. They can be used for chromosome count and karyotype analysis after staining with the fluorochrome DAPI (4, 6-diamidino-2-phenylindole).

3.3.2 Karyotype analysis.

Chromosomes on a microscopic slide were stained with DAPI and examined by epifluorescent microscope with filters for visualization of DAPI. Only well spread chromosomes were captured and used for karyotype analysis. The karyotypes were constructed from at least five metaphases in each sample. Chromosome pairs were

identified and arranged on the basis of chromosome length and arm ratio following Vij et al (1982).

3.3.3 Fluorescence *in situ* hybridization.

The fluorescence *in situ* hybridization (FISH) was performed using a method modified from Anamthawat-Jónsson (2001) and Schwarzacher and Heslop-Harrison (2000).

Probe labelling: Two ribosomal DNA probes were used for double-target FISH. Clone pTa71, 9-kb fragment, which contained a part of 18S and the entire 5.8S and 25S coding region together with non-transcribed spacers of wheat (Gerlach and Bedbrook, 1979) was used as 18S-25S rDNA probe. Clone pTa794, which contained complete 410-bp *Bam*HI fragment of the 5S rRNA gene and spacer regions of wheat (Gerlach and Dyer, 1980), was used as 5S rDNA probe. The rDNA probes were labelled by nick translation. The probe labelling were performed in a final volume of 50 µl consisting 1 µg of double strand template DNA, 2 µl of labelled nucleotide mixture (see note*) and unlabelled dTTP (0.5 mM stock)), 5 µl of 10x unlabelled nucleotide mixture (a mixture of dATP, dCTP and dGTP, 0.5 mM each, in 100 mM Tris-HCl, pH 7.5), 5 µl of 10x nick translation buffer (0.5 M Tris-HCl, pH 7.5, 50 mM MgCl2 and 0.5 mg/ml nuclease free bovine serum albumin (BSA)), 1 µl of 100 mM DTT (1,4-dithiothreitol, D-9779, Sigma) and distilled water to make 45 µl volume. After mixing all ingredients well, add 5 µl of the labeling enzyme mixture (3 µl of DNaseI and 2 µl of DNA polymeraseI) and mix well. Incubate the reaction mixture at 16 °C for 2h, avoid exposure to light. Stop the reaction with 3 µl of 0.5 M EDTA. The labeling probe was purified using ProbeQuant G-50 Micro Colum (Amersham-Biosciences) following the manufacturer's protocol. Labelled probes can be stored at -20 °C. The labelled probe was examined for incorporation of labeled nucleotides by dot-blot analysis. The fluorescently labelled probes fixed on blot and washed in and estimated on UV transilluminator abainst reference series of pure nucleotides in dots of 20, 10, 4 and 1 nM. Labelled probes can be stored at –20 °C.

Note* labelled nucleotide mixture

A) Red lebel:

1:1 mixture of red-fluorescing rhodamine-4-dUTP (RPN2122, Amersham-Biosicences, 1 mM stock) and unlabelled dTTP (0.5 mM stock); or

1:1 mixture of SpectrumRed dUTP (Vysis 30-803400, Molecular Probes USA, 1 mM stock) and unlabelled dTTP (0.5 mM stock); or

1:1 mixture of ChromaTide Alexa Fluor 568-5-dUTP (C11399, Molecular Probes USA, 1 mM stock) and unlabelled dTTP (0.5 mM stock); or

1:1 mixture of Cy3-dUTP (Amersham-Biosicences, 1 mM stock) and unlabelled dTTP (0.5 mM stock)

B) Green lebel:

2.5:1 mixture of fluorescein-11-dUTP (RPN2121, Amersham-Biosicences, 1 mM stock) and unlabelled dTTP (0.5 mM stock); or

1:1 mixture of ChromaTide Alexa Fluor 488-5-dUTP (C11397, Molecular Probes USA, 1 mM stock) and unlabelled dTTP (0.5 mM stock); or

1:1 mixture of Fluorescein-12-dUTP (Enzo-Roche 1-373-242, Roche Applied Science, 1 mM stock)

Pre-treatment of chromosomes on a microscopic slide: The slide was treated with fresh fixative for 10 min at room temperature then washed twice with 96% ethanol, 10 min each, and air dry. The slide was treated with 5 μg/ml of RNase at 37°C for 1 h, and washed twice with 2xSSC at 37 ° C, 5 min each. The slide was incubated in protinase K buffer at 37 °C for 5 min, treated with 4-10 μg/ml protinase K for 10-20 min, treated with stop buffer at room temperature for 1 min, and wash twice with 2xSSC at 37 ° C, 5 min each. The concentration and time for protinase K treatment are depended on the quality of chromosome preparation. High concentration and/or long time of protinase K were used if there is more cytoplasm on the chromosome preparation. The slide was treated with 4% paraformaldehyde at room temperature for 20 min, and wash twice with 2xSSC, 5 min each. The slide was dehydrated through an ethanol series (70, 90, 100%), 2 min each, and air dry. Air-dried slides can be kept at 4 °C until ready for in situ hybridization.

Preparation of probe mixture: The probe mixture were performed in a final volume of 20 µl containing labelled probe 50 ng, 50% formamide, 20% dextran sulphate, 2xSSC, 0.5% SDS, and distil water to make 20 µl total volume, and mix well.

Denaturation and hybridization: The probe mixture was boiled for 6 min and keeps on ice for 5 min, and then applied onto the pre-treated slide and place with coverslip. The probe mixture and chromosomes were denatured together in in situ thermocycle chamber at 89 °C for 20 min, and then the temperature was dropped to 37 °C. The slide was incubated in humid chamber at 37 °C for overnight.

Washing and detection: The cover slip was removed using warm 2xSSC. The slide was washed with 0.1X SSC at 60 °C for 3 times, 5 min each, treated with 2xSSC from 37 °C to room temperature (about 5-10 min), and treated with 4X SSC Tween for 5 min at room temperature. The 50 µl of counterstain, DAPI, was applied onto the slide, rinse briefly with distilled water after 1 min and air dry for few min. A drop of antifade mountant was placed onto the slide, and a cover slip was placed. The slide was kept in a dark and cool place until ready for examination. The chromosomes were examined with epifluorescence microscope with 1000x magnification using appropriate filter.

Reprobing: The slide can be hybridised again. The cover slip was removed using 2xSSC, and the slide was dehydrated with 96% ethanol. The slide was repeated the pre-treatment slide, if necessary, and proceed to the denaturation and hybridization.