CHAPTER III

MATERIALS AND METHODS

The study consisted of two parts: (1) Genetic diversity of common wild rice in Cambodia, and (2) Characterization of F_2 populations between cultivated rice and common wild rice. The field survey was carried out in Cambodia and field experiment was conducted at Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University. Details of these research activities are described as follows:

3.1 Genetic diversity of common wild rice in Cambodia

3.1.1 Survey and collection of common wild rice populations in Cambodia

A survey was carried out in the central part of Cambodia covering four regions, Phnom Penh, Kandal, Takeo and Prey Veng (Figure 3.1) in October 16-25, 2007. Populations were geo-referenced, recorded for habitat conditions, i.e. canal, inside rice field, edge of rice field, abandoned fields and by the roadsides. For each population, based on wild rice survey sheet (Appendix 1), some morphological and physiological characteristics including growth stage, plant type, awning, awn color, awn length, stigma color, anther length, ecotype, hull color were recorded. Ecotypes were classified based on their reproductive systems and habitats (Oka, 1988). The perennial, mostly propagates by vegetative components and prefers stable water conditions throughout the year. In contrast, annual propagates by seeds and grows in seasonally dry habitats.

While, intermediates were hybrid between perennial and annual types. Leaf samples from ten populations (10 plants per population) with minimum distance of 5 m between plants were collected from Phnom Penh 3 populations, Kandal 1 population, Takeo 2 populations and Prey Veng 4 populations, as listed in (Table 3.1 and Figure 3.1).

In addition, leaf samples from seven cultivated rice varieties were collected from farmer fields in the same areas, including,Phnom Penh (3 varieties), Takeo (2) and Prey Veng (2) to test for the evidence of gene flow between wild and cultivated rice (Table 3.2).

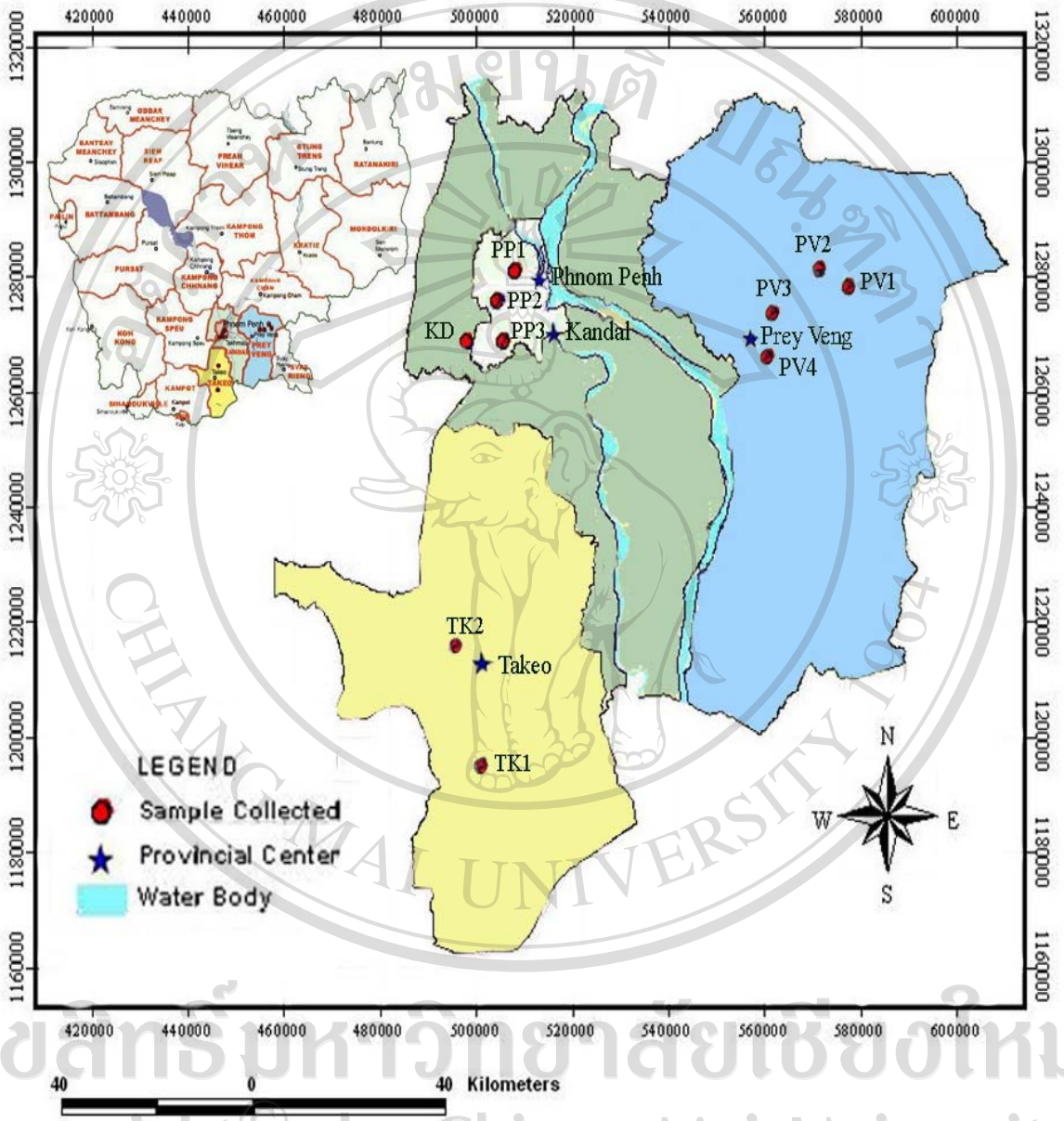

Figure 3.1 Collection sites of common wild rice in Cambodia**.**

Table 3.1 Description of common wild rice collected from four regions in Cambodia

Pop.	Habitat	Commune	District	Province/city	UTM
1	Road-side ditch	Khmuogn	Reousey Keo	Phnom Penh	48P 0486205 1281123
2	Road-side ditch	Pochen tong	DangKor	Phnom Penh	48P 0482638 1275943
3	Abandoned field	Poung teck	DangKor	Phnom Penh	48P 0483692 1268869
4	Edge rice field	Beoung Tom	Ang snuol	Kandal	48P 0475998 1268730
5	Abandoned field	Chi khnar	Trang	Takeo	48P 0479167 1194931
6	Abandoned field	Rokha knong	Doun Keo	Takeo	48P 0473717 1215890
7	Canal	Svay anthor	Prey Veng	Prey Veng	48P 0549375 1281275
8	Road-side ditch	Chrei	Prey Veng	Prey Veng	48P 0555741 1278305
9	Abandoned field	Baray	Kompong lao	Prey Veng	48P 0538709 1266155
10	Abandoned field	Takou	Kompong lao	Prey Veng	48P 0539792 1273666

Table 3.2 Description of cultivated rice collected from three regions in Cambodia used in this study

No.	Cultivar	Description	District	Province/city	Photoperiod sensitivity	Production system
1	Chhmar Prom (ChP)	Land race	DangKor	Phnom Penh	Yes	Rainfed lowland
2	Srau Krahorm (SK)	Land race	DangKor	Phnom Penh	Yes	Rainfed lowland
3	Phkar Khgney (PhK)	CARDI	DangKor	Phnom Penh	Yes	Rainfed lowland
4	Chomkoum Rumpak (ChR)	Land race	Tram Kok	Takeo	Yes	Rainfed lowland
5	Sombok Angkrorng (SA)	Land race	Tram Kok	Takeo	Yes	Rainfed lowland
6	Mrom (Mrom)	Land race	Prey Veng	Prey Veng	Yes	Rainfed lowland
7	Mong Mang (MM)	Land race	Prey Veng	Prey Veng	Yes	Rainfed lowland

3.1.2. Genetic structure of common wild rice using microsatellite markers

DNA extraction

DNA was extracted from dry leaves samples were collected individually, 10 plants per population was used by the CTAB method (Doyle and Doyle 1987.). DNA extraction buffer contained: deionized water, 4% CTAB, 100 mM Tris – Hcl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, and 0.4% β-mercapto-ethanol.

PCR (Polymerase Chain Reaction) using microsatellite markers

PCR fingerprinting was performing follow by description of (Panaud et al., 1996). Five microsatellite markers that consist of RM20, RM164, RM225, RM341, and RM588 were used (Table3.3). Amplification of DNA was performed in 20 µl PCR per reaction consist of 1µl DNA, deionized water 16 µl, 10X buffer 2 µl, 50 mM $MgCL_2$ 1 µl, 25 mM dNTP 0.16 µl,, 0.2µM of each primers and 0.1 µl unit of Taq DNA polymerase (Invitrogen) in reaction buffer [10mM of Tris-HCl pH 8.5, 50mM KCl, 1.5mM $MgCl_2$, 0.1mM EDTA, 50%(v/v) glycerol]. Amplified polymorphism was distinguishable with the electrophoresis in 10% Polyacrylamide Gel Electrophoresis (PAGE).

Table 3.3 Primer sequences, repeat motif, expected PCR product size, annealing temperature and chromosomal locations of five microsatellite primers.

Name	Primer sequences (5′→3′)	Repeat	Annealing Temperature (◦c)	Chromosomal location	References
RM20	F: TGTATGCACAGCTGCTCTACTCC R: GCACGACCAGAAATTAACAAGG	(ATT)14	55	12	Panaud et al. (1996.)
RM164	F: CTTGCCCGTCACTGCAGATATCC R: CAGCCCTAATGCTACAATTCTTC	(GT)16TT(GT)4	58	5	Wu et al. (1993.)
RM225	F: TGCCCATATGGTCTGGATG R: GAAAGTGGATCAGGAAGGC	(CT)18	55	6	Chen et al. (1997.)
RM341	F: CAAGAAACCTCAATCCGAGC R: CTCCTCCCGATCCCAATC	(CTT)20	55	2	Temnykh et al. (2000.)
RM588	F: TTGCTCTGCCTCACTCTTG R: AACGAGCCAACGAAGCAG	(TGC)9	55	6	Temnykh et al. (2001.)

F = Forward Primer, R = Reverse Primer

Data analysis

Resulted banding pattern of the gels were scored manually and transferred to the present-absent scale (1 or 0 for each allele and genotype). This data were subjected to analysis using FSTAT version 2.9.3 (Goudet 2001). Population relationships were assumed using the UPGMA clustering method on the basis of pairwise genetic differentiation using FSTAT version 2.9.3 (Goudet 2001). Allele frequencies and genetic diversities within and between populations was calculated (Nei *et al*., 1983 and 2000) for each microsatellite locus as follows.

1. Average gene diversity or heterozygosity (h)

$$h = 1 - \sum P_i^2$$

P_i = is the i^{th} allele frequency

h = genetic diversity

2. Effective number of alleles (A_e)

$$A_e = \frac{1}{(1-h)} = \frac{1}{\sum P_i^2}$$

P_i = is the i allele frequency

h = genetic diversity

3. Average number of alleles per locus (N_a)

$$N_a = (\frac{1}{k})\sum_{i=1}^{k} n_i$$

k = number of loci

n_i = number of alleles were found in locus

4. Expected heterozygosity (H_e)

(H_e) is the expected heterozygosity deviation from Hardy-Winberg Equilibrium (HWE) in randomly mating population.

5. Observed heterozygosity (H_o)

(H_o) is the observed heterozygosity in randomly mating population.

6. Inbreeding coefficient (F_{IS})

$$F_{IS} = (H_e - H_o)/H_e$$

H_e is the expected heterozygosity in randomly mating population.

H_o is the observed heterozygosity in randomly mating population.

7. Out crossing rate; *t*

$$t = \frac{(1 - F_{ST})}{(1 + F_{ST})}$$

F_{IS} is the inbreeding coefficient, measures the reduction in heterozygosity of each population from Hardy-Winberge Equilibrium (HWE).

t is the out-crossing rate of each subpopulation.

8. Gene diversity for all population (H_T)

The total genetic diversity (H_T) is partitioned into within population diversity (H_S) and between population diversity (D_{ST}) components were analysis using:

$$H_T = H_S + D_{ST}$$

$$D_{ST} = H_T - H_S$$

H_S = average of genetic diversity between subpopulation

9. Gene differentiation among population (F_{ST})

Gene diversity between populations was expressed as relative to total population diversity or genetic differentiation index (F_{ST}), where,

$$F_{ST} = \frac{D_{ST}}{H_T}$$

10. Genetic relationships

Population relationships were inferred using the UPGMA (Unweighted Pair-group Method Using Arithmetic Averages) clustering method on the basis of pairwise genetic differentiation using FSTAT version 2.9.3 (Goudet 2001). The tree subsequently visualizes with MEGA4 (Kumar et al. 2001).

11. STRUCTURE software

The detection of gene flow among common wild and cultivated rice were tested the proportion of genetic admixture among the clusters using Structure (Pritchard *et al.*, 2000) and Falush *et al.,* (2003) code by Pritchard and Falush version 2.2 (March 2007). One to ten *K* (number of clusters) was applied to infer the number of clusters for common wild and cultivated rice. For each run, the admixture model, without prior population information, was applied under the condition of 100,000 Markov Chain Monte Carlo (MCMC) replication followed by 100,000 burn-in (iteration) period. Number of clusters (*K*) was inferred when a consistent result was obtained among five independent runs of the admixture analysis.

3.2 Characterization of F_2 populations between cultivated rice and wild rice

3.2.1 Genetic materials

Two improved rice varieties, Sen Pidao and IR66 released by CARDI were used to cross with two common wild rice biotypes, collected from Takeo and Kompong Thom provinces (Table 3.4 and Figure 3.2). Two crosses were made (Sen pidao x Takeo and IR66 x Kompong Thom). The F_1s were grown at Cambodian Agricultural Research and Development Institute (CARDI) for F_2 seeds multiplication. F_2 seeds were kept at 4 C until used.

Table 3.4 Description of cultivated rice varieties and common wild rice populations were used in the experiment.

No	Genotype	Year released	Description	Photoperiod sensitivity	Origin	Growing area (Habitat)
Cultivated rice						
1	IR 66	1990	IR32307-107-3-2-2	No	IRRI	Upper spot of the fields or where water can be supplemented or recession rice.
2	Sen pidao	2002	IR65610-105-2-5-2-2-2	No	IRRI	Upper spot of the fields or where water can be supplemented or recession rice.
Wild rice						
1	Kompong Thom	-	Abandoned field	Yes	Cambodia	Shallow swamp
2	Takeo	-	By the roadsides	Yes	Cambodia	Shallow swamp

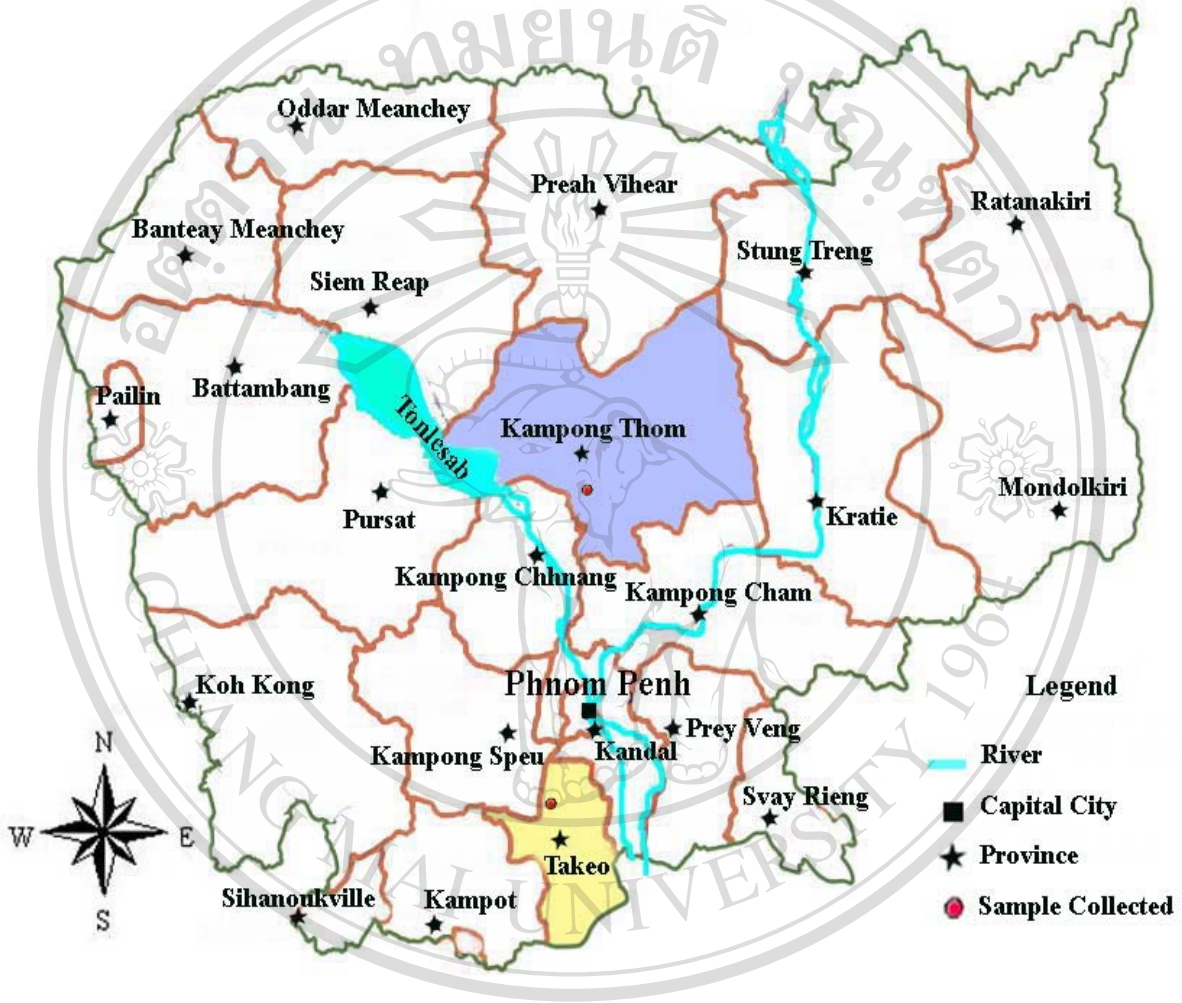

Figure 3.2 Collection sites of two common wild rice biotypes crossed with two improved rice varieties.

3.2.2 Experimental procedure

The experiment was set up in pots at Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University in August, 2007 – February, 2008. Seeds of F_2 and parents of each population were germinated on August 11, 2007 in petri-dish for 7 days then transferred to pots, 5 plants per pot. Two hundred plants per each population and twenty plants of each parent were grown. Morphological and physiological characteristics were recorded individually based on IRRI-IBPGR (1996). Plant was recorded at different plant parts including leaf blade color, leaf sheath color, ligule color, auricle color, internode color, stigma color, apiculus color, awn and awn color, hull color, plant type, tillering ability, days to flowering and plant height. Each plant was harvested separately. At harvesting, two panicles from each plant was randomly collected and measured for panicle length, branch number, spikelet number, seed fertility (%), seed shattering (%) and pericarp color.

3.2.3 Data analysis

Segregation patterns of morphological characters were determined based on the frequency distribution. The goodness of fit of the observed segregation ratios to the expected ones was examined by chi-square test.

$$\chi^2 = (O - E)^2/E$$

O = Segregation observed

E = Expected

Physiological characters were calculated for mean and standard deviation (sd).