Genetic Diversity of Wild Rice (*Oryza* spp.) in the Northern Region of Thailand

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Abstract

Very little in Thailand is known about population structure and genetic diversity of wild rice species (*Oryza.* spp.), which are needed to be investigated for their potential use in breeding programs. A study using 136 polymorphic Amplified Fragment Length Polymorphism (AFLP) markers was conducted to examine the genetic structure and diversity of natural populations of wild rice collected from the northern region of Thailand. One hundred twenty five wild rice accessions were taxonomically classified as 21 *O.officinalis,* 12 *O.nivara* and 92 *O.rufipogon.* Different clustering analyses clearly differentiated *O. officinalis* accessions and grouped the majority of *O.rufipogon* and *O.nivara* accessions according to neighbor collection sites. On the basic of cluster analyses as well as geographic distances, 104 accessions of *O. rufipogon* and *O. nivara* were divided into 6 populations. An analysis of molecular variance (AMOVA) was performed among 104 accessions of combined data from *O. rufipogon* and *O. nivara* to examine the distribution of genetic diversity. The majority of genetic diversity (75.18%) was within populations, which was expected in these outcrossing common wild rice populations. Significant differences of genetic diversity (18.52%) among six populations were detected, whereas a non-significant difference of genetic diversity (6.30%) between the upper and lower northern regions was revealed. Appropriate strategies for sampling common wild rice in the collection sites for conservation and management of *O. rufipogon* and *O.nivara* were discussed.

Keywords: wild rice, AFLP, AMOVA, *O.officinalis, O.nivara* and *O.rufipogon*

1. Introduction

The genus *Oryza* spp. consists of 22 wild species and two cultivated rice (*O. sativa* and *O. glaberrima*) providing food for more than half of the world's population. Due to its useful traits, adaptation to environmental stresses, pest and disease tolerance, the wild relatives of cultivated rice with AA genome have been used as genetic resources for developing modern rice cultivars. In Thailand, five wild species of *Oryza, O. rufipogon, O. nivara, O. officinalis, O. granulata* and *O. ridleyi* are known [4]. *O. rufipogon* is more widespread than the others. Most investigations so far have been based on a field survey and collections. These works have neglected population genetic studies, which could contribute to knowledge about genetic diversity, genetic structure and relationships among natural wild rice populations.

A detailed understanding of the extent and structure of genetic variation of wild plants is important for the development of appropriate and efficient strategies for collection, conservation, and preservation of wild relatives. In addition, the efficient use of genetic resources in all plant breeding programs requires knowledge about genetic diversity. Interest in the genetic structure of the natural plant populations has increased recently due to the necessity to broaden the genetic base of cultivated crops. Molecular markers have played important roles for gene introgression from wild species to cultivated varieties [20]. The assessment of genetic variability in natural populations of wild rice can provide knowledge of the amount and apportionment of genetic variation within species.

Molecular markers are an efficient method for determining genetic relationships because they are not affected by environmental or epistatic interactions which may influence morphological traits. In the present, various molecular marker methods are available, each with their own advantages and disadvantages.

The AFLP technique developed by Vos et al. (1995) [21] is a PCR based fingerprinting method which relies on the selective amplification of restriction fragments. It has the capacity to detect large number of polymorphisms with single primer pairs. Results are reproducible due to stringent reaction conditions for primer annealing. This technique has been widely used to determine genetic diversity and relationships both among [1] and within species [7, 13, 15] in various crops and their wild relatives.

The general aim of the present study was to explore the distribution and genetic diversity of *O. rufipogon, O. nivara and O. officinalis* which are widespread in different geographic collection sites in the northern region of Thailand. The particular interest was to investigate the genetic diversity and population genetic structure of the natural populations of common wild rice, *O. rufipogon* and *O. nivara*, by means of AFLPs. Knowledge about the genetic diversity and population genetic structure may provide information to develop conservation strategies and germplasm collection and utilization.

2. Materials and Methods

Plant Materials

Material used in this study comprised 125 accessions of wild rice collected from the northern region of Thailand. In this study, the northern region was divided into two subregions on the basis of geographic areas which were referred to the northern part and the upper central part of the country. The northern part (or upper northern region) comprises ChiangRai (CR), Phayao (PY), ChiangMai (CM), Lampang (LP), Lamphum (LPu) and Phrae (PR). The upper central part (or lower northern subregion) includes Sukhothai (ST), Tak (TK), Phitsanulok (PL), KamphaengPhet (KP), Phichit (PC) and NakhonSawan (NS). Of the 125 accessions, 92, 12 and 21 accessions were taxonomically classified as *O. rufipogon*, *O. nivara* and *O. officinalis*, respectively. The number of accessions collected from each province within subregion are presented in Table 1. Live plants were randomly collected in the field, transplanted to pots, and maintained in Lampang Agricultural Research and Training Center, Lampang province. Young leaves were collected individually, stored in plastic bags on ice, and transported to the department of Biotechnology, Thammasat University (Rangsit campus). One individual per accession was used for AFLP analysis.

Table 1. Material used in the present study

Sub Regions	Provinces	Species		
		O.rufipogon	*O.nivara*	*O.officinalis*
The Northern part (The upper northern subregion)	ChiangRai (CR)	12	5	-
	Phayao (PY)	4	-	-
	ChiangMai (CM)	10	-	-
	Lampang (LP)	15	4	-
	Lamphum (LPu)	-	3	-
	Phrae (PR)	3	-	-
The Upper Central part (The lower northern subregion)	Sukhothai (ST)	13	-	3
	Tak (TK)	7	-	18
	Phitsanulok (PL)	7	-	-
	KamphaengPhet (KP)	2	-	-
	Phichit (PC)	13	-	-
	NakhonSawan (NS)	6	-	-
	Overall Total	92	12	21

DNA Extraction

Fresh leaves of a single plant of each accession were ground in liquid nitrogen. DNA was extracted from the ground tissues using the procedure of Gawel and Jarnet (1991) [10] with minor modifications.

AFLP analysis

AFLP analysis was done as described by Vos et al. (1995) [21]. A 250 ng genomic DNA accession was digested with restriction enzymes, *Eco*RI and *Mse*I. Adapters were ligated to the digested DNA with T4 DNA ligase in a total volume of 50 µl. A 2 µl aliquot of the adapter-ligated DNA fragments was preamplified using the primers 5′-GAC TGC GTA CCA ATT CA-3′ (*Eco*RI) and 5′-GAT GAG TCC TGA GTA AC-3′ (*Mes*I) with one selective nucleotide (underline) in a volume of 25 µl, Twenty cycles were performed at 94 °C for 30 S, 56 °C for 60 S and then 72 °C for 60 S. The preamplified products were diluted approximately twenty-fold with water for use as a template for selective amplification.

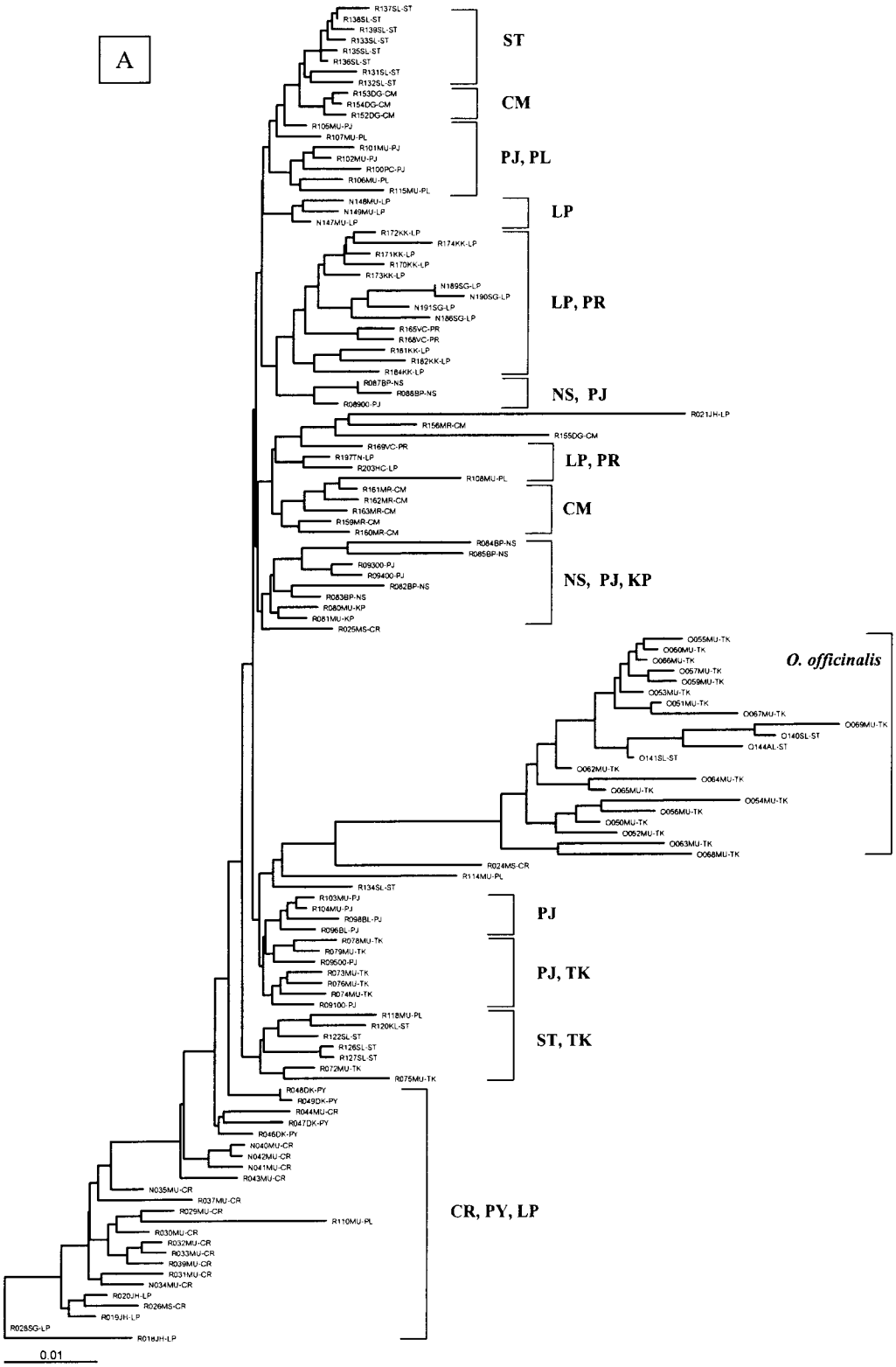
Selective amplification was performed using sixteen primer pair combinations. Four *Eco*RI primers (5′-GAC TGC GTA CCA ATTC + AAC, ATG, ACG and AAT-3′) were each combined separately with four *Mse*I primers (5′-GAT GAG TCC TGA GTAA + CAC, CTG, CCG and CAT-3′). Each primer contained three selective nucleotides at the 3′ end. Selective amplification was carried out in 25 µl of diluted preamplified product as the template. The PCR amplification was performed for 35 cycles, beginning with the profile of 94 °C for 30 S, 65 °C for 60 S, 72 °C for 60 S and followed by 12 cycles with a stepwise reduction of annealing temperature by 0.7 °C to 56 °C that was subsequently maintained for the next 23 cycles. PCR products were then separated on 6% denaturing polyacrylamide gel. The gel was silver stained as described by Bassum et al. (1991) [2].

Scoring of AFLPs and data analysis

Sixteen primer combinations were tested for their ability to generate a number of unambiguously polymorphic bands. Three primer combinations of E-AAC/M-CCG, E-AAT/M-CAC, E-AAC/M-CAC were selected to fingerprint all accessions in this study. Polymorphic bands generated by these three primer combinations were scored as present (1) or absent (0). No monomorphic bands were scored. Pair-wise genetic distances between accessions were calculated based on the dissimilarity coefficients following Nei and Li (1979) [16]. The resulting distance matrices were used to construct dendrograms by the Neighbor Joining (NJ) method compared to unweighted pair-group method with arithmetic means (UPGMA) using software packages PHYLIP version 3.57C (J. Felsenstein, University of Washington, Seattle, USA) and NTSYS-PC 1.8 (F.J. Rohlf, State University of New York, Stony Brook, USA), respectively. The resulting clusters and close geographic distances were considered to group accessions which were referred to as populations. In order to describe population genetic structure and variability among populations, the Analysis of Molecular Variance (AMOVA) described by Excoffier et al. (1992) [6] in the ARLEQUIN 2.00 software [17] was used. The total variance was partitioned among individuals within populations, among populations within subregions and between regions (the northern part and the upper central part). Then a permutational procedure was used to provide significant tests for each of the hierarchical variance components based on the original distance matrices. The phi-statistic (ϕ_{st}), calculated by AMOVA, was used as an interpopulation genetic distance measurement as described by Huff (1997) [12]. The UPGMA clustering procedure was used to construct a dendrogram on the basis of the interpopulation distance matrices. Within a population, mean squares were calculated to examine the different levels of population variance [8].

3. Results

The AFLP primer combinations of E-AAC/M-CCG, E-AAT/M-CAC, E-AAC/M-CAC yielded 52, 54 and 30 polymorphic AFLPs respectively. These 136 polymorphic bands were used to analyze 125 wild rice accessions. AFLP fragment sizes ranged from approximately 50 to 1,000 base pairs. Polymorphic fragments were distributed across the entire size range.


A
R137SL-ST
R138SL-ST
R139SL-ST
R133SL-ST
R135SL-ST
R136SL-ST
R131SL-ST
R132SL-ST
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R154DG-CM
R152DG-CM
R105MU-PJ
R107MU-PL
R101MU-PJ
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N189SG-LP
N190SG-LP
N191SG-LP
N186SG-LP
R165VC-PR
R168VC-PR
R181KK-LP
R182KK-LP
R184KK-LP
R087BP-NS
R088BP-NS
R08900-PJ
LP
LP, PR
NS, PJ
R021JH-LP
R156MR-CM
R155DG-CM
R169VC-PR
R197TN-LP
R203HC-LP
R108MU-PL
R161MR-CM
R162MR-CM
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R160MR-CM
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R09300-PJ
R09400-PJ
R082BP-NS
R083BP-NS
R080MU-KP
R081MU-KP
R025MS-CR
LP, PR
CM
NS, PJ, KP
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O060MU-TK
O066MU-TK
O057MU-TK
O059MU-TK
O053MU-TK
O051MU-TK
O067MU-TK
O069MU-TK
O140SL-ST
O144AL-ST
O141SL-ST
O062MU-TK
O064MU-TK
O065MU-TK
O054MU-TK
O056MU-TK
O050MU-TK
O052MU-TK
O063MU-TK
O068MU-TK
O. officinalis
R024MS-CR
R114MU-PL
R134SL-ST
R103MU-PJ
R104MU-PJ
R098BL-PJ
R096BL-PJ
R078MU-TK
R079MU-TK
R09500-PJ
R073MU-TK
R076MU-TK
R074MU-TK
R09100-PJ
R118MU-PL
R120KL-ST
R122SL-ST
R126SL-ST
R127SL-ST
R072MU-TK
R075MU-TK
PJ
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N041MU-CR
R043MU-CR
N035MU-CR
R037MU-CR
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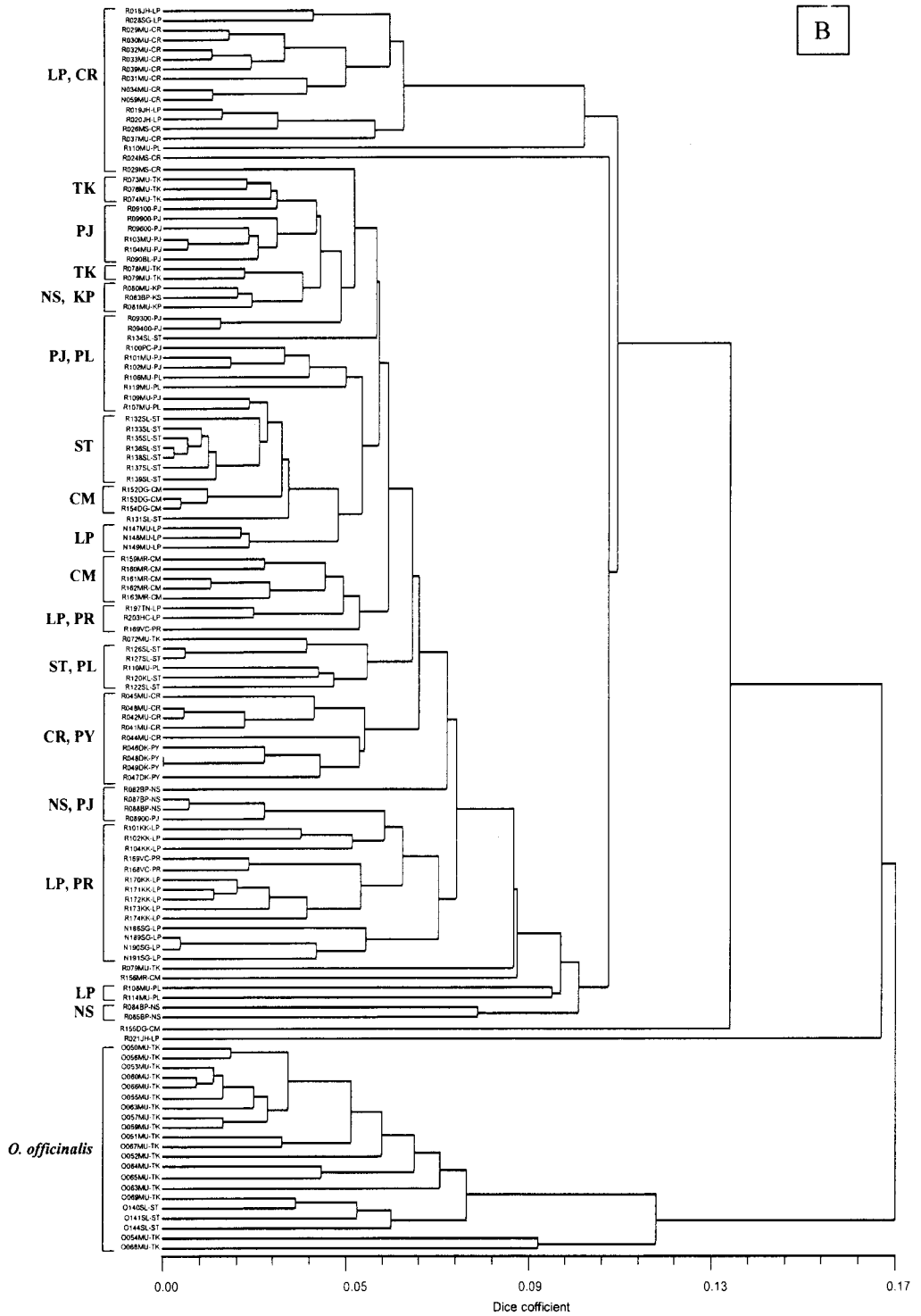

Fig. 1 Comparison of the two dendrograms constructed by NJ method (A) and UPGMA method (B) based on 136 AFLP polymorphic bands illustrating genetic relationship among 21 *O. officinalis,* 12 *O. nivara* and 92 *O. rufipogon* (The first letter of the accession name indicates species of the accessions, O = *O.officinalis*, N = *O. nivara*, and R = *O. rufipogon*. The later three digits with the two letters indicate a collection site. The last two letters are the abbreviation of the province presented in Table 1.)

The dendrograms constructed using the two different algorithms (NJ method compared to UPGMA method) based on dissimilarity coefficients following Nei and Li (1979) [16] also known as Dice coefficients [3] were examined. The two different clustering methods clearly grouped *O. officinalis* accessions but were unable to differentiate *O. rufipogon* and *O. nivara* (Fig. 1). *O. officinalis* was considered as an outgroup because this species contains a different genome from the other two species. Due to a few number of accessions of *O. officinalis*, the population genetic structure of *O. officinalis* was not analyzed. The similar results from the two clustering analyses indicated that the majority of *O. rufipogon* and *O. nivara* accessions were clustered according to neighbor collection sites within neighbor provinces. Prior to conducting this experiment, there was no information about natural populations of *O. rufipogon* and *O. nivara*. These results facilitated clustering of accessions which were grouped as the same population genetic structure. Since *O. rufipogon* and *O. nivara* contains the same AA genome, 92 accessions of *O. rufipogon* and 12 *O. nivara* were combined for further analysis, and referred to as common wild rice.

According to clustering analyses based on both NJ and UPGMA methods as well as close geographic distances, 104 accessions of common wild rice were separated into six populations as presented in Fig 2. Population 1 consisted of 12 accessions from TK and ST. Population 2 was the cluster of 15 accessions from the lowest collection sites, NS, PJ and KP. Population 3 which was an inter-geographic distance between population 1 and population 2 comprised 21 accessions from ST, PL and PJ. These 3 populations were grouped in the sub-region of the upper central part. Population 4 was a cluster of 15 accessions from the lower part of LP, 3 accessions from PR and 3 accessions from LPu. Population 5 comprised 10 accessions from 2 collection sites in CM province. Population 6 was the cluster of uppermost accessions which consisted of 4 accessions from the upper part of LP, 17 accessions from CR, 4 accessions from PY. The later 3 populations were grouped in the subregion of the northern part.

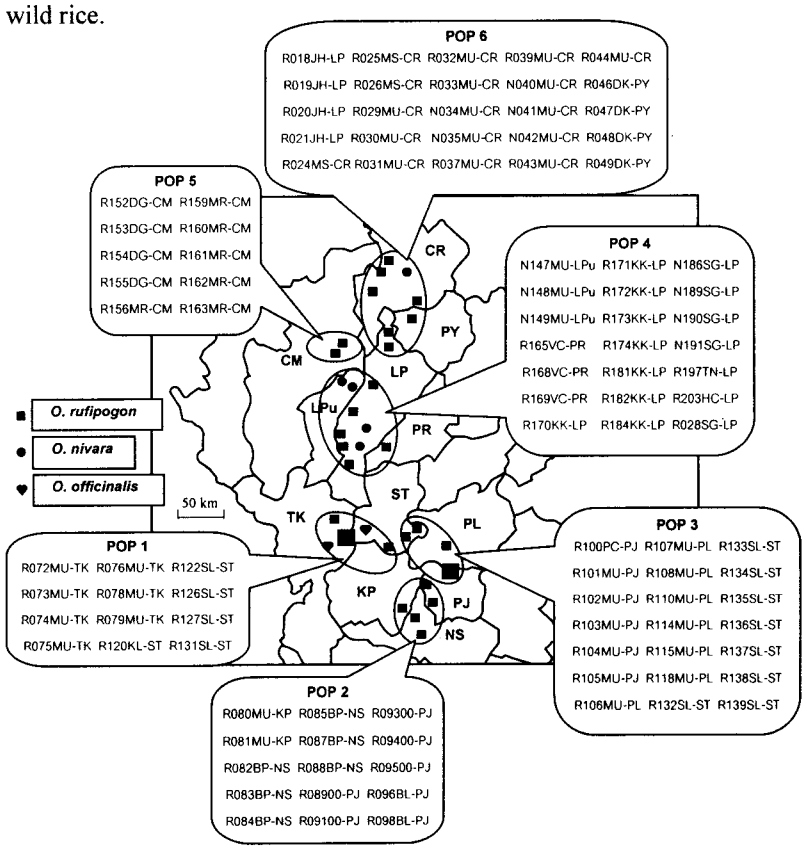

Fig.2 Geographic distribution of six common wild rice populations collected in the northern region of Thailand. Population 1, 2, 3 and population 4, 5, 6 were grouped in the upper central part (the lower northern subregion) and the northern part (the upper northern subregion), respectively. (Accession names are described as in Fig. 1

To evaluate the overall distribution of diversity within and between populations, an AMOVA was performed from the distance matrix (Table 2). The majority of the AFLP variations (75.18%) observed among the 104 individuals from the 6 populations was accounted for by individuals within population variance. The variation among the six populations (18.52%) is relatively small but is statistically significant ($P<0.001$). The remaining variation between two subregions is statistically non-significant. This result indicated that the genetic structure of common wild rice populations could not be separated between the northern part and the upper central part based on geographic differences. The highest percentage of within population variance is expected in these two wild rice species since they are outcrossing species. In the current study, the AMOVA analysis showed similar results to RAPD studies on natural populations of *O. rufipogon* in China, that most of the genetic diversity resided within populations (71%) [11].

Table 2 Analysis of molecular variance (AMOVA) for 104 individuals of common wild rice from six populations based on 136 AFLP markers.

Source of Variation	df	Sum of squares	Variance Components[1]	*P*-value[2]
Between sub-regions	1	176.898	1.50 (6.30%)	0.18084
Populations/sub-regions	4	361.813	4.38(18.52%)	< 0.001
Individuals/Populations	98	1743.981	17.80 (75.18%)	< 0.001

[1]Number in the bracket indicates percentage total
[2]Probability computed by nonparametric randomization from 1023 permutations

The relationships among populations were presented in Table 3 and Fig. 3 which showed the genetic distances based on a pairwise ϕ_{ST} between populations. The genetic distances among the populations from the upper central part were lower than those from the northern part. This result indicated that accessions collected from the northern part had a higher genetic variation. The pairwise test between population 1 and 3 was non-significant at $P<0.01$, meaning that the genetic diversity of these two populations was very close together. A similar result was obtained between population 4 and 5. The genetic variation of population 6 was unexpectedly close to that of populations from the upper central part resulting that a variance component between subregions is statistically non-significant.

Table 3 Matrix based on pairwise ϕ_{ST} distance between 6 populations of common wild rice

	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6
Pop 1	-					
Pop 2	0.095	-				
Pop 3	0.058^{ns}	0.061	-			
Pop 4	0.359	0.330	0.369	-		
Pop 5	0.172	0.168	0.189	0.120^{ns}	-	
Pop 6	0.128	0.119	0.162	0.327	0.207	-

^{ns} statistically non-significant at $P< 0.01$

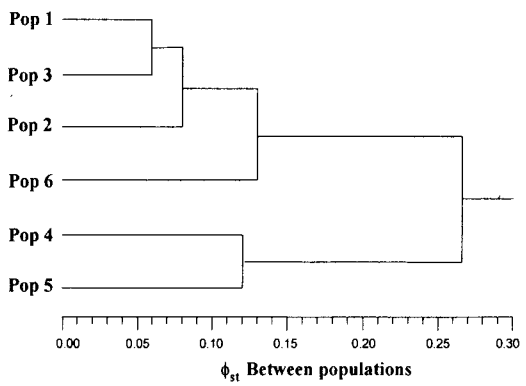

Fig. 3 Cluster analysis of six common wild rice populations based on pairwise ϕ_{ST} interpopulation distances

To examine the different levels of within population variance, within population mean squares were calculated (Table 4). Population 5 had the largest within population mean square even though the smallest the numbers of sample size were collected. This may be due to the fact that population 5 comprises two groups of accessions which are clustered in different groups according to the result of cluster analysis (see Fig 1). This result leads to speculation that habitat fragmentation under human disturbance

in this population (CM province) increased the mating opportunity among accessions in the same collection sites and differentiated groups of accessions between the two collection sites (DG-CM and MR-CM). The within population mean square per accession of population 3 was lowest reflecting of the lowest genetic variation of individuals within population. The within population mean squares of the other four populations were likely to increase with increasing number of accessions.

Table 4 Within population mean squares for six populations with an unequal number of individuals per population

Population	Mean squares	Number of accessions	Mean squares per accession
Pop 1	14.61	12	1.22
Pop 2	16.57	15	1.10
Pop 3	15.16	21	0.72
Pop 4	24.41	21	1.16
Pop 5	28.09	10	2.81
Pop 6	21.50	25	0.86

4. Discussion and Conclusions

This research represents the comprehensive study of genetic diversity of natural populations of common wild rice in the northern region of Thailand. The AFLP technique was used to describe the distribution of genetic diversity across the collection sites of the wild rice. Knowledge about the distribution of genetic diversity in the natural areas where the species of interest appear may provide relevant information to develop sampling strategies that will maximize the probability of collecting genetically distinct accessions.

The high genetic diversity of common wild rice in Thailand may be explained by high level of outcrossing rates. Common wild rice populations were collected where rice is widely cultivated. Morishima et al. (1984) [14] pointed out that the major direction of pollen flow in the *O. sativa* complex was from annual to perennial because the outcrossing rates were much higher in perennial than in annual types. Consequently, gene flow to from cultivated rice from the natural populations of Thailand common wild rice may be a factor that had led to higher genetic variation within populations [9, 11, 19]. For the opposite consequence, genetic introgression resulting from gene flow from *O. sativa* to common wild rice may lead to the extinction of wild populations [5]. This result is useful for making decisions for *in situ* conservation strategies for common wild rice populations. Avoiding gene flow from cultivated rice to in situ conserved common wild rice germplasm should be carefully considered. A safe isolation distance between *in situ* conservation sites of common wild rice and cultivated rice should be required in order to maintain genetic integrity of the conserved common wild rice populations.

The results of clustering analysis displays the clusters of accessions collected from neighbor collection sites in neighbor provinces. One of the factors that shape a population genetic structure is the pollen flow among outcrossing plants. Song et al. (2003) [19] had experimentally estimated that the range of pollen flow of *O. rufipogon* was approximately 110 m. Base on pollen movement ability, the relatively high rate of gene flow may lead to low differentiation among clusters of accessions collected from the neighbor collection sites. On the other hand, the limited gene flow through cross pollination may be responsible for the high level of differentiation among populations.

The three populations from the upper central part had relatively close genetic distances (Table 3 and Fig. 3) among them and each population had small genetic variance within the population (Table 4). This could be explained in part by the fact that during the rainy season, this plain area may be flooded and many rivers are linked, probably increasing the gene flow by seed dispersion.

The results presented in this study are important for determining strategies for future collection of common wild rice from the collection sites of the northern region of Thailand. As a rule for outcrossing species, sampling fewer populations but more individuals in each collection sites within population should be done. This method of collection should be applied for population 1, 2, 4 and 6 since genetic variances within these populations are likely increased with increasing number of accessions. The lowest genetic variation of individuals within population 3 was revealed. Sampling few individuals but more collection sites in this area should be adopted. Habitat fragmentation may have occurred in

population 5. As a result, population 5 of *O. rufipogon* in CM province would have become fragmented into two collection sites. The isolated cluster accessions resulted in more inbreeding, which in turn increased genetic differentiation among accessions collected in different collection sites. For future studies, sampling more collection sites and more individuals within each collection site should be done to test whether different clusters of accessions collected from different collection sites have different genetic structures. Extensions of population genetic structures of common wild rice from other regions of Thailand are also needed for future studies.

In summary, considerable genetic diversity exists within the natural common wild rice populations in the northern region of Thailand. The remaining diversity that is attributed to variation among populations is relatively small. The diversity level varied among populations, which is likely due to different conditions, under which the populations are grown. A safe isolation distance between *in situ* conservation sites of common wild rice and cultivated rice is recommended. To maximize genetic variation of common wild rice accessions, sampling strategies used for outcrossing plants should be adopted.

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