Australian Journal of Crop Science AJCS
AJCS 6(4):717-723 (2012) ISSN:1835-2707

Genetic diversity and population structure of wild rice, *Oryza rufipogon* from Northeastern Thailand and Laos

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Abstract

Northeastern Thailand and Laos are the two most important habitats of the wild, (*Oryza rufipogon*). To examine the genetic diversity of this wild rice species, seven rice microsatellite markers were used to evaluate the genetic diversity of 94 accessions. In the entire sample a total of 83 alleles were detected, with an average of 11.85 allele per locus. The total gene diversity was an average of 0.733. The Laos population showed higher measures of genetic diversity (i.e., mean of allele number per locus, mean of genetic diversity, and mean of PIC value) than the northeastern Thailand population. Clustering analysis, using STRUCTURE to determine the true number of populations (*k*), reveals occurrences of different gene pools of wild rice among the examined populations. This observation support the hypothesis of the presence of subgroups in wild rice from northeastern Thailand and Laos.

Keywords: SSR markers; genetic diversity; wild rice.
Abbreviations: *SSR*: Simple Sequence Repeat, *RM*: Rice microsatellite.

Introduction

The wild rice species, *O. rufipogon* Griff. is a perennial species with a wide distribution that extends from southern part of China (Jiangxi province) through south and southeast Asia down to Papua New Guinea and northern Australia (Vaughan, 1994). This wild rice species contain the AA genome and is recognized as a progenitor of cultivated rice (*O. sativa* L.) (Khush, 1997). As a progenitor of cultivated rice, this species has proven to be a valuable reservoir of genes for genetic improvement in cultivated rice. Notably, those improvements include: resistance to blast (Ram et al., 2007); resistant to brown plant hopper (Rongbai et al., 2001). While this wild rice species is the most agriculturally important, most populations, which represent the primary gene pool of rice, are seriously endangered by introgression (gene flow from cultivated rice) and human activity that has led to the extinction of the species throughout the areas of distribution (Gao et al., 2000; Gao et al., 2002; Gao, 2004). Today, the species exists in only a few severely fragmented populations that may be remnants of a formerly more continuous widespread range. Thailand and Laos are rich resources for wild rice germplasm (Vaughan, 1989). In Laos, the Lao-IRRI Project and National Genebank collaborated and collect wild rice species in the south and central part of Laos (Rao et al., 2002). In Thailand, there were 807 accessions of five wild rice species that collected between 1981 and 2002 from all regions of the country including the North, Lower North, Central Plain, Northeast I and II; Western, Eastern South I and II and Bangkok area (Vutiyano et al., 2010). Five existing species of wild rice in Thailand, namely, *Oryza rufipogon*, *O. nivara*, *O. officinalis*, *O. ridleyi* and *O. granulata* , were collected and stored in a gene bank. Wild rice *O. officinalis* was a genetic resource for early-morning flowering trait to reduce high temperature-induced sterility at anthesis (Ishimaru et al., 2010). To address of the question is that how much of genetic diversity of wild rice has been preserved is of concern and cannot be adequately understood without knowledge of the genetic diversity of natural populations. Conservation programs must be developed that ensure the long-term survival of the species and maintain uninhibited ecological and evolutionary processes that lead to the preservation of genetic diversity (Hamrick and Godt, 1996; Frankham et al., 2002). An understanding of the genetic structure of *O. rufipogon* is required if an appropriate conservation scheme is to be designed. The development of such schemes rely on the genetic structure of this wild rice species, such as Chen et al. (2004) and Wang et al. (2008). As such, it becomes necessary to draw the basic information of genetic structure and to further determine the critical factors needed to bring an evolutionary perspective to *in situ* or *ex situ* conservation and germplasm management of wild rice in Thailand and Laos.

Simple sequence repeats (SSRs) or microsatellites are stretches of DNA consisting of tandemly repeating mono-, di-, tri-, tetra- or penta-nucleotide units widely distributed across all eukaryotic genomes (Powell et al., 1996). In addition, the uniqueness and value of microsatellites arises from their multiallelic nature, codominant transmission, and ease of detection by PCR. Microsatellite markers for cultivated rice have been developed which are available of 2240 microsatellite markers (McCouch et al., 2002). Several studies have shown that microsatellite loci developed in cultivated rice can be successfully amplified in related wild species and thus provide powerful tools for their population genetic studies. For example, rice species with AA genome

microsatellite markers have been applied to detect genetic variations in the perennial wild rice (*Oryza rufipogon*) (Kuroda et al., 2007; Gao, 2004; Song et al., 2003), in cultivated rice (*O. sativa*) (Garris et al., 2005), in weedy rice (*O. sativa f. spontanea*) (Yu et al., 2005; Cao et al., 2006; Prathepha, 2011). The ease of typing microsatellite alleles and the availability of large numbers of such highly informative loci across the rice genome make them the markers of choice. The aims of this study were to (1) investigate the genetic diversity of natural populations of wild rice *O. rufipogon* from northeastern Thailand and Laos, and (2) to examine the population structure of wild rice by using a model-based approach.

Results

Genetic diversity of wild rice populations

Example of genotyping of wild rice samples for RM 11 and RM 84 shown in Fig. 1. Genotyping of seven SSR loci in 94 individuals from northeastern Thailand and Laos populations revealed a total of 83 alleles, with the number of alleles per locus ranging from 8 to 20 (mean 11.85). Estimates of major allele frequency, gene diversity, heterozygosity, and PIC value for each SSR locus are presented in Table 2. For each population, the mean number of alleles per locus within northeastern Thailand and Laos populations was 8.57 and 9, respectively. In addition, genetic diversity was high in the two populations, with 0.6185 and 0.749, respectively. A similar pattern was observed for the PIC value, with 0.5929 and 0.7234, respectively. Result from Mantel test (Fig. 2) showed that the two genetic distance matrices of wild rice from northeastern Thailand and Laos (A and B) were correlated with significant differences between the two populations (r_{AB}= 0.187, P = 0.001). Each SSR locus, the relative low mean fixation index (F_{is}=0.2298) were found in 94 accessions of wild rice used in this study, and all were positive (Table 2).

Cluster analysis

An analysis of the population structure of the 100 accessions of wild rice (Table 1) using STRUCTURE and STRUCTURE HARVESTER, the magnitude change of LnP(D) relative to the standard deviation, called ΔK by Evanno et al. (2005), showed the highest peak at *k=2*, and there was one smaller peak at *k=5* (Fig. 3). Fig. 4 shows the membership of accessions to the populations identified by STRUCTURE. The membership indicates that the inferred genetic structure of these wild rice populations is accorded with the sampling origins. Each accession was assigned to one of the five clusters (*k)*, as follows: Nong Harn Lake, Sakon Nakhon Province (6 accessions), Nong Harn Lake and the Vientiane plain, Vientiane Province (30 accessions), Siem Reap Province, Cambodia (6 accessions), Nong Harn Lake and Mahasakham (23 accessions), and the Vientiane plain and Savannakhet Province (35 accessions). Among these five clusters, some accessions assigned to each cluster had genotyped profile which were identified as admixture. In addition, cluster analysis revealed that two accessions (accession no. 59 and 61), identified as wild rice carrying a recessive allele of fragrance gene (*fgr)*.This is probably a resulted of gene introgression from a cultivated aromatic rice (Prathepha, 2009) being assigned to one cluster (cluster 1). These two accessions showed a unique genetic constitution when compared to some wild rice accessions which were identified as without gene introgression of the fragrance locus. This would be imply that some wild rice accessions used in this analysis showed a primary gene pool of wild rice (i.e., without gene introgression from cultivated rice to wild rice population). Results from this study support a previous study which found that cultivated rice alleles cryptically persisted in natural populations of wild rice on the Vientiane plain (Kuroda et al., 2007).

Discussion

Wild rice (O. rufipogon) in northeastern Thailand and Laos and its conservation

Wild rice, *O. rufipogon* is considered to be a potential source of agronomic traits improvement for Asian cultivated rice (*O. sativa*). This wild rice species has been widely used in rice breeding (Zhao et al., 2003). Wild rice, which inhabits the Vientiane plain of Vientiane province and other parts of Laos and northeastern Thailand, is a germplasm resource for genetic improvement. The geographic locations of wild rice in these areas demonstrate the close-genetic relationship to each other. Conservation of this wild rice species is important due to their tremendous agronomic values and the critical deterioration of their habitats. In northeastern Thailand, Nong Harn Lake in Sakon Nakhon province is very large area inhabited by *O. rufipogon*, and should be considered to be in *situ* conservation of this wild rice species. The important strategy for *in situ* conservation is to preserve the genetic integrity and diversity of the conserved populations (Wang et al., 2008). In the SSR analysis of wild rice from northeastern Thailand and Laos, using seven rice SSR markers, a relatively high level of genetic variation was detected in *O. rufipogon* populations. This indicates that rice SSR markers have a good cross-amplified motif with close relatives, such as *O. rufipogon*, and that SSR assay provides a useful tool in estimating the genetic variations of wild rice populations as suggested by Wang et al. (2008). Results from this SSR analysis supports the trend of an overall genetic variation pattern resided within populations of this wild rice species, which was reported by Gao et al. (2002); Song et al. (2003); Londo et al. (2006). Veasey et al. (2011) reported that American wild rice, *Oryza latifolia* and *O. grandiglumis* showed high intra-population variability. These results suggest that higher degree of genetic diversity was observed in these wild rice species. To consider for their possible use in rice breeding programs. It is therefore valuable to begin the *in situ* conservation of *O. rufipogon* at the population level in Nong Harn Lake, Sakon Nakhon province. Meanwhile, for the wild rice distributed in the Vientiane plain and other parts of Laos, it was found that some habitats (i.e., swamp) are well suited for *in situ* conservation based on the high value of genetic diversity existed in these populations and the fact that these populations receive limited disturbances by human activity.

Genetic structure of wild rice in northeastern Thailand and Laos

An assessment of genetic diversity and an examinination of the population structure of wild rice germplasm is important for the efficient organization of genetic resources. The genetic structure of wild rice, *O. rufipogon* from China has

Table 1. Description on of wild rice (*Oryza rufipogon* Griff.) populations collected from Northeastern Thailand and Laos used in this study

Population/code (N)	Location (N/E)	Habitat and population status
The Vientiane plain, Laos LAOS (no. 1-8)	Na Phaeng village (18°21.74'/102° 38'), Vientiane Province	Marsh, < 5 m apart from rice fields
The Vientiane plain, Laos LAOS (no. 9-18)	Forest swamp (18°10.25'/102° 37.89'), Vientiane Province	Isolated population from rice fields
The Vientiane plain, Laos LAOS (no. 19-28)	Nong Vai swamp, Naxaythong District, Vientiane Province (18°05.18'/102° 31.79')	Swamp, >15 m apart from rice fields
The Vientiane plain, Laos LAOS (no. 29-37)	Ban Hai village, (18°13.78'/102° 40.12') Vientiane Province	Roadside canal, <5 m apart from rice fields
Savannakhet Province, Laos LAOS (no. 38-47)	Forest swamp at National Park of Savannakhet Province, (16°45.25'/105° 28.9')	Isolated population from rice fields
Sakon Nakhon Province, Thailand		
SKN1 (no.48-59)	Lake (Nong Han Lake), (17°07'/104° 13')	Lake, >100 m apart from rice fields
SKN2 (no.60-74)	Lake (Nong Han Lake), (17°07'/104° 13')	Lake, >100 m apart from rice fields
SKN3 (no.75-84)	Mountain swamp, (17°03'/103° 42')	Swamp, <10 m apart from rice fields
Maha Sarakham Province, Thailand MSK (no. 85-94)	Pond, (15°27'/103° 24')	Pond, <5 m apart from rice fields
Siem Reap Province, Cambodia CBD (no. 95-100)	Marsh, (13°41'/103° 81')	Marsh, surrounded by roads and houses

Table 2. SSR primers used in this study.

Primer name	Forward primer (5'→3')	Reverse primer(5'→3')
RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTT
RM17	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA
RM21	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG
RM84	TAAGGGTCCATCCACAAGATG	TTGCAAATGCAGCTAGAGTAC
RM164	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC
RM180	CTACATCGGCTTAGGTGTAGCAACCACG	ACTTGCTCTACTTGTGGTGAGGGACTG
RM212	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG

Table 3. Microsatellite diversity at the seven SSR loci in 94 accessions of wild rice populations (Northeastern Thailand and Laos).

Marker	Major.Allele.Frquency	Genotype No.	Sample Size	Allele No.	Gene Diversity	Heterozygosity	PIC	F_{is}
RM11	0.3670	16.0000	94	11	0.7922	0.5319	0.7690	0.3333
RM17	0.4681	18.0000	94	9	0.7230	0.5745	0.6953	0.2106
RM21	0.1543	34.0000	94	14	0.8934	0.7234	0.8839	0.1955
RM84	0.5426	12.0000	94	9	0.6508	0.6489	0.6171	0.0082
RM164	0.2553	24.0000	94	12	0.8533	0.5106	0.8378	0.4061
RM180	0.3617	29.0000	94	20	0.8018	0.7340	0.7813	0.0898
RM212	0.7553	11.0000	94	8	0.4172	0.2553	0.4030	0.3925
Mean	0.4149	20.5714	94	11.8571	0.7331	0.5684	0.7125	0.2298

been previously reported (Gao et al., 2002; Wang et al., 2008; Xie et al., 2010), but meaningful information pertaining to the genetic structure of the wild rice that inhibits these two areas is limited. The results of cluster analysis using STRUCTURE, revealed the presence of five subgroups or clusters ($k=5$) in the all accessions examined. This observation supports the hypothesis regarding the presence of subgroups in wild rice from northeastern Thailand and Laos. Among the identified five clusters, only cluster 2 composed of wild rice accessions from northeastern Thailand and Laos, while four remaining cluster (cluster 1, 3, 4 and 5) were composed of accessions from its original sampling. Based on this finding it might hypothesize that there would be four main gene pools of wild rice used in this analysis. A uniform genetic constitution was apparent for the population from Siem Reap province, Cambodia (cluster 3), while populations from northeastern Thailand and Laos exhibited a highly admixture genetic constitution (cluster 1, 2, 4 and 5). Interestingly, some wild rice accessions from the two isolated populations (a forest swamp of the Vientiane plain and a forest swamp of Savannakhet National Park) from Laos exhibited admixed genetic constitution, i.e., accession no. 9, 12, 38, 41, 43, 44 and 45. This is probably explained by either these accessions have a unique genetic constitution of

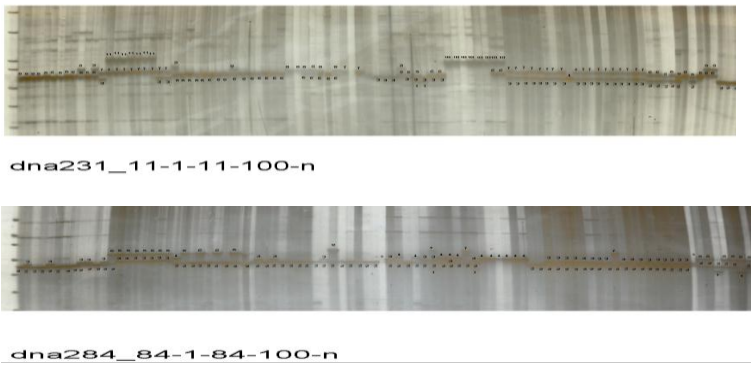

Fig 1. Example of gels after resolution in 6% polyacrylamide gel and scored for each genotype for RM 11 (upper) and RM 84 (lower) of wild rice examined.

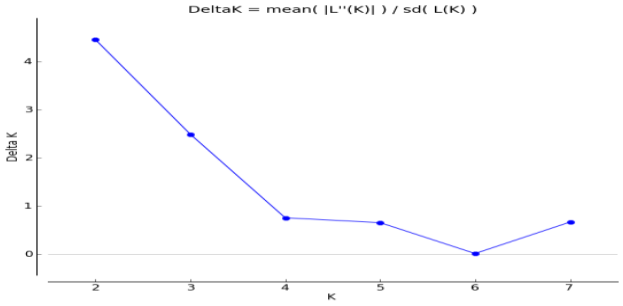

Fig 2. The magnitude change of LnP(D) relative to the standard deviation for each K value, indicating that the highest peak at *k=2* and a smaller peak at *k=5*

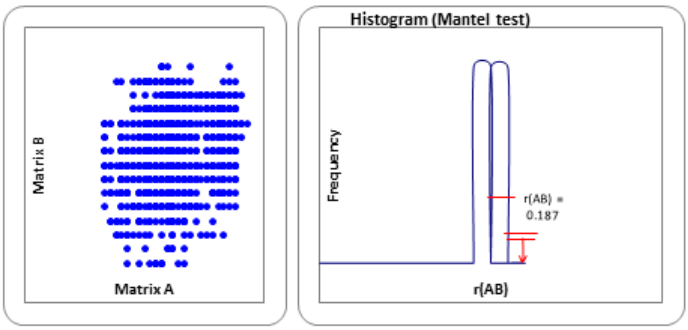

Fig 3. Correlation between genetic distance matrix of wild rice from Northeastern Thailand and Laos and histogram obtained from the Mantel test.

primary wild rice population or these accessions were a result of gene introgression from cultivated rice to two wild rice populations sometime in the past as suggested by several studies (Gao et al., 2000; Song et al., 2003; Isshiki et al., 2005;Song et al., 2006).

Materials and methods

Rice samples and DNA extraction

During a 2006-2008 field survey of natural populations of wild rice in Thailand, Laos and Cambodia, a total of 100 individual plant samples from 10 populations of wild rice from the three countries was collected and used in this work. The collection sites are listed in Table 1. The exact location of each site was documented using a global positioning system (GPS) receiver from GARMIN (iQue 3600, Garmin Co. Ltd.). Young leaves were collected individually from at least six individuals per population. Total individual plant samples were collected randomly at an interval of at least 5 to 12 m from one another to prevent the collection of multiple sample from a single plant. This collection procedure was employed because this wild rice species propagates mainly by vegetative growth (ratooning) (Xie et al., 2001). Genomic DNA extractions of each individual leaf sample of wild rice were performed based on the CTAB method as described by Doyle and Doyle (1987). Seven SSR primer pairs (Table 2) were selected as a subset of SSR markers previously used to assay genetic diversity of wild rice and weedy rice by specific polymerase chain reaction (PCR) conditions (Song et al., 2003; Song et al., 2006; Cao et al., 2006). Detailed information of primer sequences is available at http://www.gramene.org/microsat/ssr.txt. The PCR reactions were carried out in a volume of 20 µL containing 1x buffer, 1 mM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 10 mM of SSR primer, 50 ng of genomic DNA and 1 unit of *Taq* polymerase (Promega). The polymerase chain reactions were performed using the following cycle: 94 °C for 4 min and followed by 36 cycles of 40 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C, and 10 min at 72 °C for the final extension. The PCR products were separated on 6 % polyacrylamide denaturing gels and silver stained. Migration distance of each allele was determined by comparing with a known molecular-weight standard (PUC 19 DNA digested by *Msp*I) after performing electrophoresis.

Microsatellite scoring and data analysis

For each microsatellite locus, the amplified SSR DNA bands representing different alleles were scored as different genotypes. As a result, the bands were recorded as homozygous genotypes or heterozygous genotypes. Based on SSR genotypes data, standard measures of genetic diversity of each population were calculated. The number of alleles per locus, major allele frequency, gene diversity (or expected heterozygosity), polymorphism information content (PIC values), and classical F_{is} value, were included and calculated using PowerMarker version 2.3.2 (Liu and Muse 2004). Relationships between northeastern Thailand and Laos *O. rufipogon* populations were estimated from the matrix of pairwise genetic distance which was generated by PowerMarker using the Mantel test (Mantel, 1967) with 1,000 random mutation by using XLSTAT (http://www.xlstat.com). The population structure of wild rice , as shown in Table 1, was examined using the model-based approach of Pritchard et al. (2000) implemented in the software package STRUCTURE version 2.2 (http://www.pritch.bsd.uchicago.edu) and the program Structure Harvester (http://taylor0.biology.ucla.edu) that is used with STRUCTURE.

Table 4. Genetic parameter at the seven SSR loci in wild rice (*O. rufupogon*) from Northeastern Thailand and Laos.

Population	Sample size	Mean no. alleles per locus	Mean gene diversity	Mean PIC value
Northeastern Thailand	47	8.57	0.6185	0.5929
Laos	47	9.0	0.749	0.7234

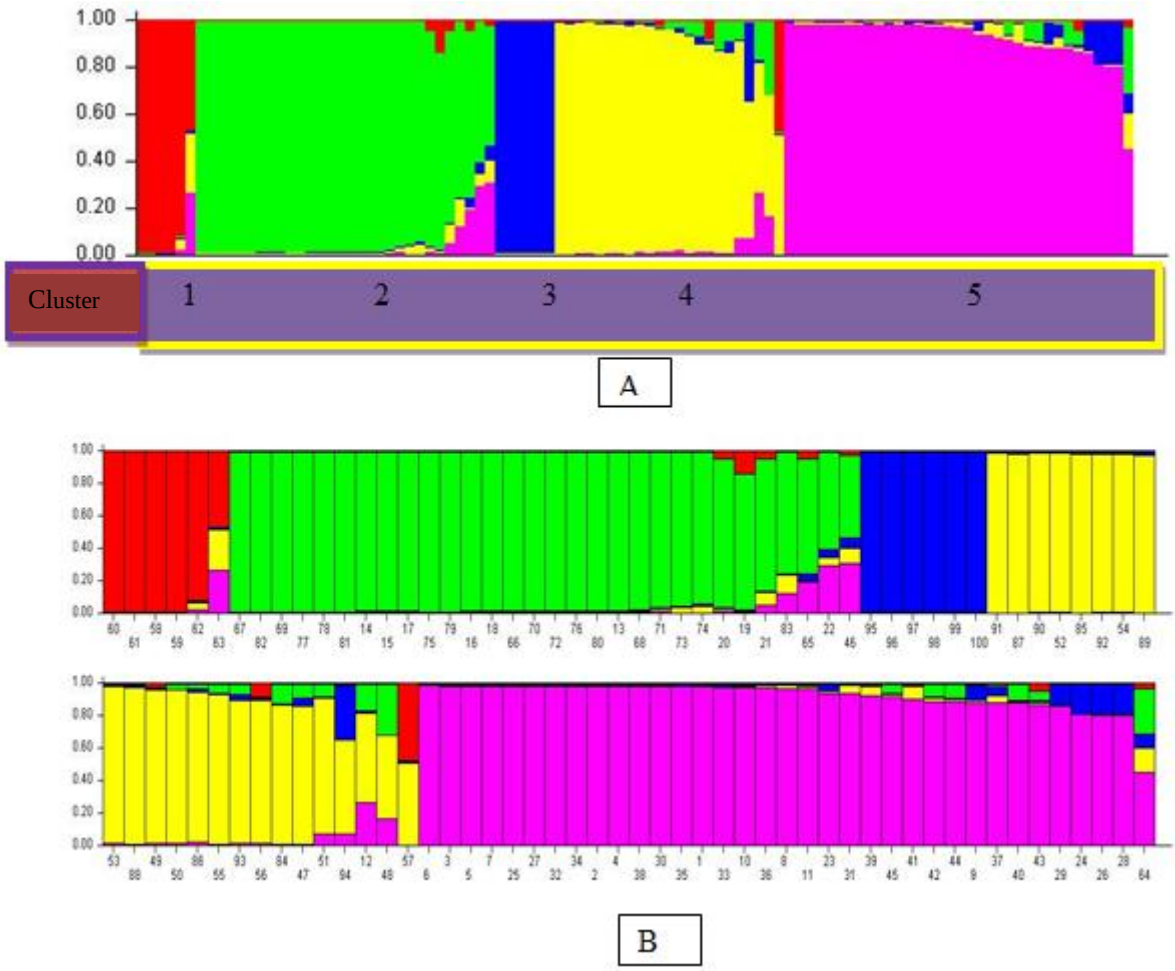

Fig 4. Model-based population structure plot for each accession with *k=5*, using STRUCTURE. Color codes are as follows: *Nong Harn Lake and the Vientiane plain* (red); *Nong Harn Lake and the Vientiane plain* (light green); *Siem Reap* (blue); *Nong Harn Lake and Mahasarakham* (yellow); and *the Vientiane plain and Savannakhet*, light purple (A). Code of each accession corresponds to description in Table 1 (B)

In this method, a number of populations (*K*) are assumed to be present and to contribute to the genotypes of wild rice sample examined. A series of tests was performed using a different number of population (*K*=1 to 10, each with five independent runs) to guide an empirical estimate of number of identifiable populations without consideration of sampling origins. In each run, the admixture model, without prior population information, was applied under the condition of 5,000 Markov Chain Monte Carlo (MCMC) replication followed by 10,000 burn-in (iteration) period. This study used the method of Evanno et al. (2005) and to estimate the number of clusters (K-value). Briefly, they argued that an *ad hoc* statistic Δ*K* based on the rate of change in the log probability of data between successive *K*-values could accurately detect true *K*.

The suggested $\Delta k = m(|L(k+1) - 2L(k) + L(k-1)|)/s[L(k)]$, where L(*k*) represents the k^{th} LnP(D), m is to the mean of five runs and their standard deviation. The graphical display of the STRUCTURE results was generated and illustrated that the genotype of each individual is a junction of the allele frequencies in these *K* populations and a proportion of its genotype drawn from each of the *K* populations. Individuals were assigned probabilistically to a population if their genotype profile indicated admixture (Weckworth et al., 2005).

Conclusion

The wild ancestor of cultivated rice, *O. rufipogon*, grows a broad geographic range spanning eastern India, Indochina, and

portions of southern China. Genetic and ecological studies with wild rice natural populations are necessary to provide information for both *ex situ* or *in situ* conservation programs for wild rice species. This study analyzed the genetic diversity and genetic structure of wild rice, *O. rufipogon* which collected from Thailand and Laos. In summary, the present SSR analysis of wild rice shows that a high level of genetic diversity exists in the populations of *O. rufipogon* from northeastern Thailand, the Vientiane plain, and Savannakhet province of Laos. In addition, the population structuring analysis using STRUCTURE reveals the occurrences of different gene pools of wild rice among the examined populations. In order to effectively implement an *in situ* conservation strategy of this wild rice species in Thailand and Laos, several effective approaches need to be recommended by all stakeholders.

Acknowledgements

I thank P. Alexander Dulfer for helpful and improvement of the English readability of manuscript, V. Pilap for technical assistant. This project was supported by Mahasarakham University, Thailand.

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