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Full Length Research Paper

Genetic diversity of Myanmar rice and their implementation on management methods

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Myanmar has diverse agronomic landscape and potentially preserves high level of genetic resources for important crop species. However, little study on rice landrace diversity in Myanmar has been done. Genetic and phenotypic variation to characterize rice genetic resource in Myanmar was analyzed using molecular markers as well as common garden experiments. Two populations of rice landraces, a seed-bank population maintained by seed-propagation in a genebank for several generations and an "on-farm" population collected from agricultural lands were used. A functional (cytochrome P450 related PBA) and neutral (SSR) markers were used in this study. Phenotypic characteristics of representative agronomic traits in rice, such as culm length, panicle length, number of tillers and days to heading, were measured in both populations. Multivariate analysis suggested that the seed-bank and on-farm population had different genetic bases with both functional and neutral markers. There was no significant relationship between the functional and neutral markers based on Mantel test. In addition, PCA analyses of agronomic traits showed that a variation in the seed-bank population had narrower genetic bases than the on-farm population. Genetic bias caused by 'unconscious selection' during the genebank management processes may have occurred in the landraces. The importance of the conservation on on-farm landraces of *Oryza sativa* and its wild relatives was proposed in order to ensure the genetic resources for further breeding and conserve biological diversity.

Key words: *Oryza sativa*, rice, landrace, on-farm, diversity, conservation.

INTRODUCTION

The union of Myanmar has diverse landscapes and contains continuous geographic variation from the delta area of the Ayeyarwaddy River in the southern region, to the mountainous areas in the northern region. This landscape diversity resulted in the diverse agricultural system for examples, deep water fields in the delta areas, irrigated and rain-fed paddy fields in plain areas and slash and burn fields in the mountain areas. Geographic and crop diversity, coupled with diverse traditional agricultural systems, contributes to the diversity of crop genetic resources in Myanmar (Garcia et al., 2003; San San Yi et al., 2008). However, the rice genetic resources of Myanmar have so far been unexploited in terms of their genetic relationships among different landraces (Garcia et al., 2003). Information based on systematic observations and evaluations of these resources is still very limited. Both cultivated rice and their wild relatives, play an important

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Figure 1. Rice germplasm collections in Myanmar: A) various seed phenotypes of accessions in the seed-bank; B) *Ex-situ* management in the seed-bank field.

role in contributing to the entire rice gene pool.

Recent social and economic development in developing countries has led to the introduction of modern and improved cultivars in agriculture. This however, has also partly contributed to genetic erosion in traditional landraces. In the case of Myanmar rice populations, modern high yielding varieties such as the hybrid rice have been introduced, thus have replaced traditional landraces (Anonymous, 2001). As a result, genetic erosion and reduction in the genetic diversity of rice might have accelerated. To prevent such genetic erosion and the resource from the loss of genetic diversity, genebank management is now being promoted in many countries. Ministry of Agriculture and Irrigation (MOAI) in Myanmar has established the seed-bank as a part of genebank management since 1990. The seed-bank collected over one thousand accessions of rice and is maintaining them by seed-based propagation (Anonymous, 2001) (Figure 1). Such '*ex situ* conservation' in genebank is generally believed to be the most effective conservation strategies for genetically homozygous, self-pollinated and seed-based propagated plant species such as rice. Despite the implementation of such ex-situ management procedures, it is still very possible that both genetic drift and unconscious selection during regeneration may occur and affect the diversity of these original corrections. To further clarify

Table 1. Origin of Myanmar rice landraces used in the study.

Collection region (Division or State)	Number of strains used in this study	
	Seed-bank	On-farm
Ayeyarwaddy	2	0
Bago	6	2
Kachin	1	2
Kayah	2	6
Kayin	3	4
Magway	0	5
Mandalay	4	4
Mon	0	2
Rakhine	5	4
Sagaing	7	5
Shan	0	4
Tanintharyi	1	0
Yangon	8	4
Unknown	2	0
Total	41	42

and address this problem, rapid and precise evaluations of genetic resources from multi-dimensional view points are required.

Hence, the aim of this study was to characterize the genetic diversity in Myanmar rice genetic resources and compared its trends in two populations from different origins, (one from the seed-bank and another taken directly from agricultural fields). Genetic diversity in these populations was evaluated from both genotypic and phenotypic variation. Two types of molecular markers were employed; functional marker (P450-related PBAs, Yamanaka et al., 2003a) and neutral markers (SSR markers), and phenotypic variations was observed based on representative agronomic traits in rice.

MATERIALS AND METHODS

Plant materials and DNA extraction

A total of 83 different rice landraces (*Oryza sativa* L.) from various regions in Myanmar were used (Table 1), two typical *indica* (Ac.130 and Ac. 419) and *japonica* (T65 and Ac. 221) strains were employed as control samples. 41 out of 83 strains were obtained from the seed-bank at the Department of Agricultural Research (DAR) and Ministry of Agriculture and Irrigation (MOAI), Myanmar. The other 42 strains were directly sampled from agricultural fields in the areas where the seed-bank materials were originally derived. In this study, these populations were designated as seed-bank and On-farm, respectively. Total DNA was extracted from 100 mg of fresh leaf tissues from each strain, following the method described in Fulton et al. (1995). The accessions were not common between the two groups due to the unavailability of the on-farm materials by the loss of conservation. However, similar group of materials were employed from on-farm for comparison with the past collections conserved at the genebank.

PBA analysis

Fifteen combinations of 3 forward primers (CYP1A1F, CYP2B6F, and CYP2C19F) and 5 reverse primers (CYP1A1R, CYP2B6R, CYP2C19R, heme2B6, heme2C19) to estimate the diversity related to PBA (Yamanaka et al., 2003a) (Table 3) were utilized. PCR amplification was done using approximately 10 ng of extracted DNA in a total volume of 25 µl containing 1X PCR Ex *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 0.5 units of Ex *Taq* Polymerase (TaKaRa, Japan). A total reaction of 32 cycles was programmed for 1 min at 94°C, 2 min at each annealing temperature and 3 min at 72°C in a Thermal Cycler from Applied Biosystems. Annealing temperatures for each primer set are previously described in Yamanaka et al. (2003a). PCR products were electrophoresed in 1.5 % agarose gels.

SSR analysis

Twelve SSR markers, corresponding to each rice chromosome were assessed by PCR amplification (Panaud et al., 1996; Temnykh et al., 2000), (Table 4), using 10 ng of extracted DNA in a total volume of 20 µl containing 1X PCR Ex *Taq* buffer, 0.2 M dNTPs, 0.5 µM of each primers and 0.5 units of Ex *Taq* DNA polymerase (TaKaRa, Japan). A total reaction of 40 cycles was programmed for 30 s at 94°C, 30 s at 55°C and 1 min at 72°C in a thermal cycler from Applied Biosystems. PCR products were electrophoresed in 8.0 to 12.0% polyacrylamide (29:1) gels.

indica-japonica classification

To evaluate *indica-japonica* differentiation among the materials used in this study, chloroplast and nuclear DNA variation were assessed. Two PCR markers, ORF/ORF2 and CMN-A32 were employed. The ORF/ORF2 marker detects a 69 bp deletion, specific to *indica* varieties at the ORF100 region in rice chloroplast DNA (Chen et al., 1993; Kanno et al., 1993; Yamanaka et al., 2003b). DNA fingerprinting (RAPD) analysis suggests that the primer CMN-A32 clearly shows a polymorphism between the *indica* and *japonica* varieties of *O. sativa* (Suh et al., 1997; Yamanaka et al., 2003b). Most *japonica* varieties have a ca. 300 bp fragment, unlike most *indica* varieties. It has been confirmed using an *indica-japonica* hybrid population that this 300 bp polymorphic fragment is from nuclear DNA. These two markers are therefore effective for classifying *indica-japonica* differentiation using both chloroplast and nuclear DNA.

Phenotypic characteristics

To assess genetic diversity based on phenotypic variations, four representatives of agronomical traits in rice which includes days to heading; culm length; panicle length and tiller number were measured.

Data analyses

Nei's gene diversity (*H*) was calculated for PBAs and SSRs (Nei, 1973). Multivariate analyses were conducted using Quantification theory III (QTIII, Hayashi, 1956) for PBAs (1 - 0 based category data) and principal component analysis (PCA) for both SSRs and phenotypic data. The association between the PBA, SSR and phenotype based similarity matrices was determined with the Mantel test (Mantel, 1967), using NYSYS-pc (version 2.11T) software.

Table 2. Classification of *indica-japonica* types using ORF100 and CMN-A32 markers that is chloroplast and nuclear DNA markers, respectively. Both markers had two genotypes that are specific to either *O. japonica* or *O. indica.*

CMN-A32 (nuclear)	ORF100 (chloroplast)	
	ND (*japonica*)	D (*indica*)
J type (*japonica*)	6	0
I type (*indica*)	21	56

Table 3. Genetic diversity of Myanmar rice landraces estimated by PBAs.

Primer	Gene diversity[a]		
	Seed-bank	On-farm	Total
Primer-set	(n = 41)	(n = 42)	(n = 83)
CYP1A1F/CYP2B6R	0.402	0.379	0.394
CYP1A1F/heme2B6	0.428	0.382	0.411
CYP2B6F/CYP1A1R	0.283	0.278	0.280
CYP2C19F/heme2C19	0.475	0.472	0.473
CYP2B6F/heme2C19	0.456	0.452	0.465
CYP2C19F/CYP2C19R	0.414	0.384	0.400
CYP1A1F/CYP2C19R	0.195	0.171	0.191
CYP2B6F/CYP2C19R	0.272	0.375	0.331
Average	0.366	0.362	0.378

[a] from Nei, (1973).

RESULTS

Genetic features of Myanmar rice landraces

The results of *indica-japonica* classification analysis in Myanmar rice landraces, using nuclear and chloroplast DNA markers, are shown in Table 2. Out of the 83 strains tested, 56 (67%) were classified as *indica* and six (7%) as *japonica,* suggesting that *indica* cultivars are preferentially cultivated and predominantly distributed in Myanmar. The remaining 21 rice strains (26%) were classified as recombinant types with *indica* nuclear DNA and *japonica* chloroplast DNA. This indicates the presence of *indica-japonica* substitutions between nuclear and chloroplast DNA. This high frequency of recombinant type rice strains suggests that, there is a significant probability of natural hybridization at an individual level, which may be asymmetric gene flow from *indica* to *japonica* and of gene flow at a population level in rice grown in the field. This is possibly caused by diversity of varieties and heterogeneity in the farmers' fields depending on the concept for recognition of 'variety'.

Assessment of genetic diversity using both functional and neutral markers

In the case of PBAs, Nei's gene diversity in both seed-bank and on-farm populations based on the selected eight primer-sets were 0.366 and 0.362, respectively (Table 3). The gene diversity using 12 SSR markers were 0.809 in seed-bank and 0.826 in on-farm (Table 4). For both PBAs and SSRs, there was no statistical difference in the gene diversity levels between seed-bank and on-farm populations. The genetic diversity in terms of Nei's gene diversity was not affected by genebank management.

Genetic structures of seed-bank and on-farm populations

Multivariate analyses were conducted to further characterize the variation detected using different molecular marker sets. Quantification theory III (QTIII, Hayashi, 1956) to analyze the PBA data (1-0 based category data) and principal component analysis (PCA) for the SSR data was employed. Our analysis of the PBA results indicated differences in the distribution patterns for the two population collections, with a scatter plot using axis 1 and 2 contribution: Axis 1 = 19.7%; Axis 2 = 12.1% (Figure 2). The relatively low contribution rates from the first two axes may be attributable to the heterogeneity of the collections from on-farm. Indeed, the on-farm population had much greater variation in the direction of axis 1 (the most pronounced element of axis 1 is a polymorphism in the 1000 bp CYP1A1F/CYP2C19 fragment). The data suggested

Table 4. Genetic diversity of Myanmar rice landraces estimated by SSRs.

Marker	Gene diversity		
	Seed-bank	On-farm	Total
Marker (chromosome number)	(n = 41)	(n = 42)	(n = 83)
RM1 (1)	0.920	0.912	0.932
RM154 (2)	0.886	0.853	0.873
RM135 (3)	0.677	0.704	0.718
RM131 (4)	0.836	0.794	0.831
RM153 (5)	0.835	0.871	0.875
RM190 (6)	0.903	0.909	0.919
RM125 (7)	0.604	0.717	0.710
RM72 (8)	0.838	0.754	0.810
RM278 (9)	0.650	0.747	0.741
RM171 (10)	0.811	0.875	0.873
RM287 (11)	0.869	0.850	0.890
RM117 (12)	0.874	0.920	0.923
Average	0.809	0.826	0.846

[a] from Nei,(1973).

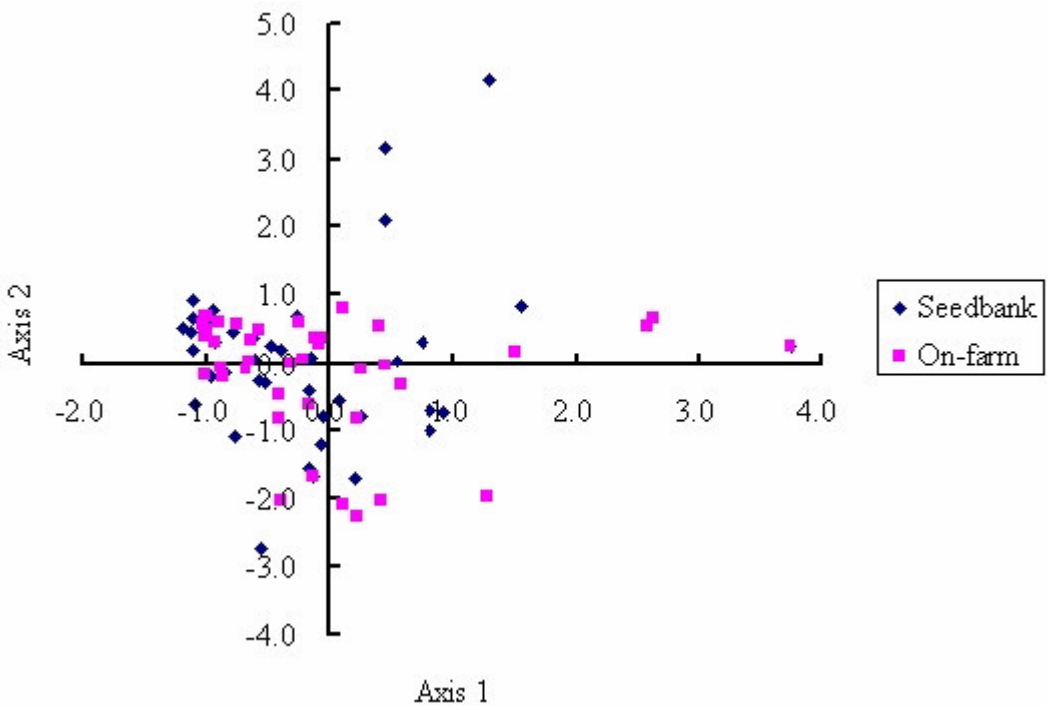

Figure 2. Multivariate analysis of Myanmar rice landraces using Quantification Theory III (Hayashi, 1956) based on 18 polymorphic fragments amplified with PBA-based eight P450 primer-sets (Yamanaka et al., 2003a) (contribution rates: axis 1 = 19.7%, axis 2 = 12.1%, respectively).

that, there were more unique genotypes in the on-farm population than in the seed-bank collections, even though similar gene diversity levels was calculated for both.

The PCA based on SSR data (Contribution rates: PC 1 = 40.6%; PC 2 = 21.5%), displayed a different distribution patterns of both populations, having greater variation and more unique genotypes again in on-farm population (Figure 3). The genetic variation for both marker systems (PBA and SSR) indicated that, on-farm material was comparatively more diverse than the seed-bank samples. This suggested the possibility that genetic diversity of the collected materials in the seed-bank have been originally

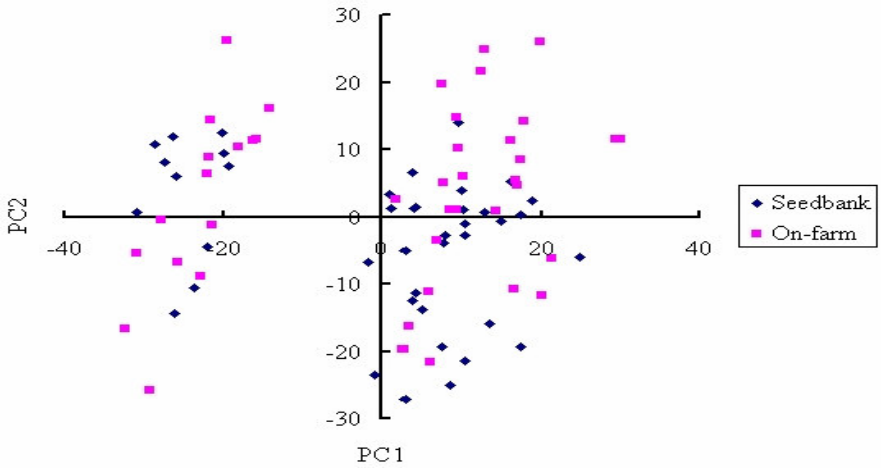

Figure 3. The plot of Principal Component Analysis (PCA) of Myanmar rice landraces based on 12 microsatellite (SSR) markers. (Contribution rates: PC 1 = 40.6% and PC 2 = 21.5%, respectively).

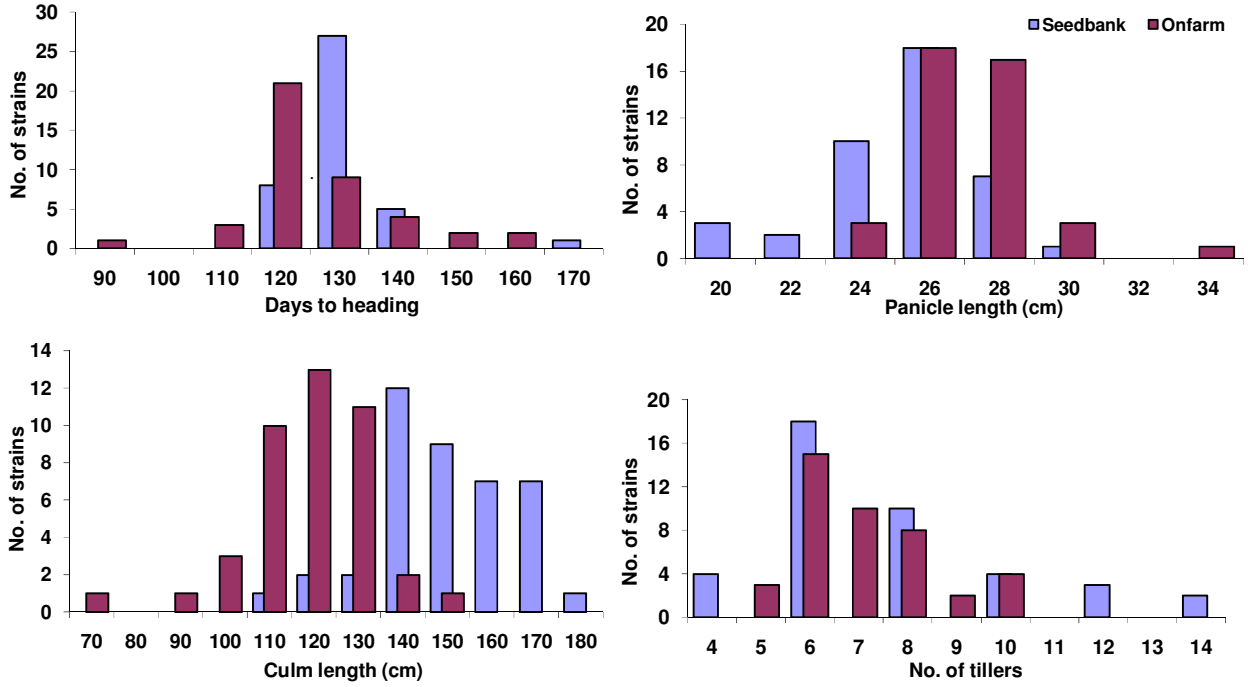

Figure 4. Variation among four representative agronomical traits in Myanmar rice.

biased or forced to decrease during the genebank management by genetic drift and unconscious selection.

Phenotypic variation

Four agronomical traits, which are considered to be important for rice production, were assessed (Figure 4) using a data set from the seed-bank population. According to the PCA based on these four traits (Contribution: PC 1 = 79.0%; PC 2 = 19.5%), the variation in the seed-bank population was also found to be less than the on-farm samples (Figure 5). There was an obvious trend in whichthese two populations could be clearly separated by PC1, with the seed-bank population showing lower variation along PC2. The most effective traits, showing the biggest principal component load in PC1 and PC2, were culm length and days to heading, respectively. This

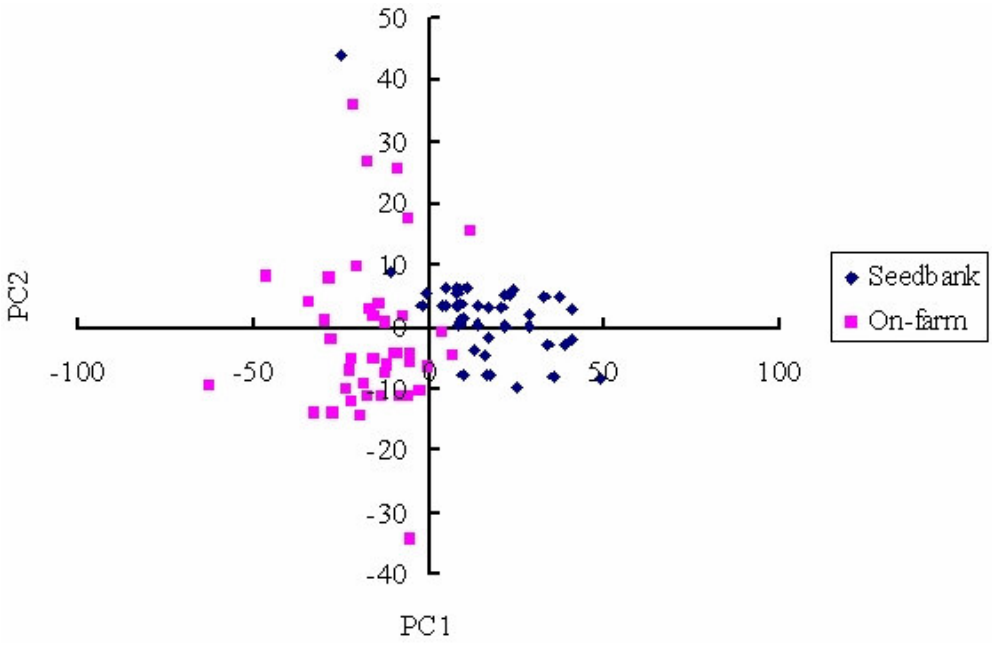

Figure 5. The plot of Principal Component Analysis (PCA) of Myanmar rice landraces based on four agronomical characters. (Contribution rates: PC 1=79.0% and PC 2=19.5%, respectively).

pressure suggested that, these two traits may be under selection through genebank management. This would have resulted to the shift in the phenotypic variation between the seed-bank and on-farm collections, even though it is unintentionally.

DISCUSSION

Efficacy of specific polymorphism detection systems in rice

SSR markers are widely used for fingerprinting and diversity studies on rice cultivars and wild relatives due to its high polymorphic rates, which is identified even at individual levels (Colowit and Mackill, 2002; Nagaraju et al., 2002). In this study, SSRs provided an efficient polymorphism identification system, while PBA markers detected even high polymorphism using only 15 primer combinations. The PBA markers were initially designed for crop species with little genetic information. In addition, the PBA polymorphisms were easily detectable on agarose gels and may be cost effective with limited budgets, compared to SSR markers, which principally depend on acrylamide gels to detect small differences in the allelic sizes.

Comparison of the different approaches for assessment of genetic variation

The correlation of the estimated distances between individuals based on molecular and phenotypic data were determined by a Mantel test (Mantel, 1967). There was no significant correlation between the estimations based on the PBA and SSR data (r = 0.032), suggesting that they highlighted different characteristics of genetic variation in the materials used in this study. Furthermore, the correlations between PBA and phenotypic data, SSR and phenotypic data, were also not found to be statistically significant (r = 0.016 and -0.102, respectively). Thus, the different distribution patterns in multivariate analyses between PBA, SSR and phenotypic variation were revealed. It was speculated that, the different types of molecular markers might feature in different aspects of the genetic structure. Evaluating genetic diversity with different assessment methods would be worthwhile, especially, as both genetic and phenotypic data will provide detailed information for both breeding and conservation purposes.

It was previously highlighted in a report by Mohammadi and Prasanna, (2003) that there were no relevant examples of a comparison between different measurements of genetic diversity on alternative types of molecular markers and phenotypes. However, such comparisons in this report and a case study of the integration of diversity measurement methods and statistical approaches have been demonstrated.

The importance of on-farm conservation

The diversity that was found in the on-farm collection suggested bilateral gene flow between cultivars and wild

relatives (Chen et al., 2004; Yamanaka et al., 2003b). Indeed, wild relatives such as *O. rufipogon* and *O. nivara* were often found close to cultivated rice fields in Myanmar (Yamanaka et al., 2003b). The genetic erosion and shifts in seed-bank management caused by genetic drift and unconscious selection through the maintenance and accumulation of mutations during long-term seed storage, has caused serious concerns (Schoen et al., 1998; Schoen and Brown, 2001). In this study, the results of both molecular and phenotypic variation analyses also suggested a genetic erosion and genetic shift in the seed-bank collection. Genetic resources can be described as the total genetic diversity of cultivated species and their wild relatives (Ford-Lloyd, 2001) and the proper conservation and utilization of genetic resources on genetic diversity estimated from a wide range of technical and scientific disciplines (Siddiqui et al., 2007a, b; Rabbani et al., 2008). Information that will support conservation strategies should be obtained from not only biological disciplines but also from sociological, anthropological, economical and geographical disciplines (Hodgkin and Rao, 2002). To complement *ex situ* strategies, *in situ* approaches are also required and this was focused on in the studies on wild rice populations (Vaughan and Chang, 1992). The concept of *in situ* conservation (genetic reserve conservation) is defined as 'the location, management and monitoring of genetic diversity in natural wild populations within defined areas designated for active, long-term conservation' (Maxted et al., 1997). This method also aims to conserve environmental heterogeneities and evolutionary potentials within a species. Primitive rice cultivars or landraces would thus provide genetic materials for plant breeders, for example, to promote greater resistance to the environ-mental stresses imposed on modern cultivars (Khush, 1995; 1999).

To sustain the genetic diversity of traditional cultivars, the concept of "on-farm conservation" has been proposed for crop species (Altieri and Merrick, 1987; Oldfield and Alcorn, 1987; Brush, 1991; Maxted et al., 1997; Jana, 1999). This concept is defined as 'the sustainable management of genetic diversity of locally developed traditional crop varieties with associated wild and weedy species or forms, by farmers within traditional agricultural, horticultural or agri-silvicultural cultivation systems' (Maxted et al., 1997). The approach based on the concept would be quite effective for the genetic conservation of crop species including native cultivars and landraces.

However, effective collaborations between farmers and conservationists would be required to fulfill the concept (Bellon, 1996). It should be emphasized that, crops are not only the results of naturally occurred mutations and natural selections, but of human selections and manage-ments also (Bellon et al., 1997; Yamanaka et al., 2004). Taking this into account in the development of a multi-disciplinary approach, both rapid and precise evaluations of artificial and natural factors affecting the genetic diversity will also be necessary and will contribute to conservation efforts (Newbury and Ford-Lloyd, 1997; Haig, 1998; Hughes, 1998; Smouse and Chevillon, 1998; Rabbani et al., 2010; Siddiqui, et al. 2010).

The value of genetic diversity

Both wild and exotic germplasm can be of great interests as sources for plant breeding (Tanksley and McCouch, 1997). With appropriate information using genome data-bases, molecular marker tools and holistic germplasm enhancement schemes could be used to organize breeding materials and consequently innovative rice cultivars for Myanmar and other developing countries (Zamir, 2003; Cyranoski, 2003). The diversity and the unique features of the Myanmar rice-landrace collections examined in this study could be quite relevant to both domestic and global rice development, as the rice gene pool in Myanmar has not been yet utilized in such a manner.

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