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Allozyme variation and conservation genetics of common wild rice (*Oryza rufipogon* Griff.) in Yunnan, China

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Summary

In order to reveal levels and distribution of genetic variation within *Oryza rufipogon* Griff. of Yunnan, China, where one of the centers of genetic diversity for Asian cultivated rice *O. sativa* L. is located, allozyme variation encoded by 22 loci was electrophoretically analyzed in 149 individuals of all three existing populations as well as five from other regions (Guangxi, Hainan and Jiangxi provinces) of China. As compared to the level of genetic diversity (the mean A = 1.2, P = 24.1%, Ho = 0.045 and He = 0.079) for the populations from other regions, a rather low genetic diversity (the mean A = 1.1, P = 7.6%, Ho = 0.007 and He = 0.011) was found in Yunnan, which may originate from marginal nature of these populations, recent reduction of populations and consequent drift. The result suggests that the current center of genetic diversity for *O. rufipogon* fail to agree with that for cultivated rice in China. The genetic differentiation for all the eight populations (F_{ST} = 0.254) was slightly lower than that for three populations from Yunnan (F_{ST} = 0.302), indicating a fairly high genetic differentiation in the region. Finally, a conservation plan for sampling/preserving fewer populations but more individuals from each population for the species was given, and an appropriate strategy for conserving the three surviving populations from Yunnan was proposed.

Introduction

Common wild rice *Oryza rufipogon* Griff., the progenitor of Asian cultivated rice *O. sativa* L., is widely distributed in the tropics and subtropics of monsoon Asia (Vaughan, 1994) and in eight provinces or regions of southern China: Guangxi, Guangdong, Hainan, Yunnan, Hunan, Jiangxi, Fujiang (National Exploring Group of Wild Rice, 1984) and Taiwan (disappeared in 1978, Kiang et al., 1979).In Yunnan, it was found in Jinghong Flatland, Daganlan Flatland and Damenlong Flatland of Xishuangbannan Prefecture, as well as in Mandan of Yuanjiang County.

Extensive survey of the geographic patterns of isozyme variation among rice cultivars in Asia suggested that diversity among *indica* rice was evenly distributed in whole tropical Asia while variation among *japonica* rice showed the hilly part of continental Southeast Asia to be the region of highest genetic diversity and its probable area of origin (Glaszmann, 1987, 1988). Yunnan is one of the centers of genetic diversity for Asian cultivated rice, which is not only documented by its abundant local *indica* and *japonica* rice cultivars (Chen et al., 1993), but also strongly supported by the studies on esterase isozyme analyses (Nakaghara, 1978, 1984; Zhu et al., 1984; Zhu et al., 1985; Wang et al., 1987; Xiong et al., 1987), allozyme analyses (Nagamine et al., 1992; Huang et al., 1997) and RFLP analysis (Liu et al., 1995). Considering this, Yunnan was previously considered as one of its original centers (Liao, 1975; Watade, 1977; Nakaghara, 1978, 1985). However, the rice evolutionists (Cheng & Cai, 1993; Wang, 1993, 1997) recently proposed that Yunnan may be a secondary center of origin due to

only narrow distribution of *O. rufipogon* in Yunnan, as well as the archeological findings of ancient rice grain which are far later in the region than those in middle and lower reaches of Changjiang River of China. Does the center of genetic diversity for *O. rufipogon* agree with that for cultivated rice in China ? It is of interest to reveal the level of genetic diversity within *O. rufipogon* in the region.

Moreover, there were seven populations in 1978–1979 of 24 populations known in Xishuangbanna before (National Exploring Group of Wild Rice, 1984), but our recent detailed investigation suggested only two populations survived, where total individuals were less than 50 (Gao et al., 1996; Gao, 1997); the population growing in Yuanjiang County is regarded as one of the most primitive and typical common wild rice populations in the world (Morishima et al., 1992), but it is being destroyed due to human disturbance and unfavorable ecological conditions of Yuanjiang dry-hot Valley (Gao et al., 1996). Because little is known about population genetics of these populations, conservation management in the region has been difficult.

To date, allozyme electrophoresis has been widely used to study the population genetic structure of endangered species (Soltis & Soltis, 1991; Gottlieb & Edwards, 1992; Soltis et al., 1992; Gao 1997; Gao et al., 1999, 2000a,b; Gao et al., 2001a,b). In the present study, allozyme analyses were conducted for all the surviving populations of *O. rufipogon* from Yunnan. In comparison, five from other regions of China were collected and analyzed. The specific questions we hoped to answer were the following: (1) What are the levels and distribution of genetic variability within and among populations of *O. rufipogon* in Yunnan? (2) Would Yunnan be one of the possible centers of genetic diversity of the species in China? and (3) What conservation strategies for the species, especially in the region should we develop?

Materials and methods

Materials

Living samples were taken from the three surviving populations of *O. rufipogon* from Yunnan, as well as five from other major parts of its range in China (Table 1; Figure 1). Of them, three populations, Yuanjiang, Dongxiang and Guilin, which are commonly considered as the typical ones (Wang, 1993), were included. Because *O. rufipogon* is a perennial with colonizing ability, care was taken to prevent collecting multiple samples from a single genetic individual. Individual live ratoons were randomly collected at intervals of at least 5 meters in the field in October, 1994, numbered, transplanted to pots and maintained in Xishuangbanna Tropical Botanical Garden (Mengla County, Yunnan) and South China Botanical Garden (Guangzhou City), respectively (Table 1). Young leaves were individually collected in March, 1995, stored in plastic bags on ice and transported to the laboratory by airplane. For each individual, 0.05 g of fresh leaf materials were crushed in 100 ul of Tris-HCl buffer (pH 7.5; see Soltis et al., 1983). The extract was absorbed into 3 × 8 mm^2 paper wicks and stored at –70 °C until electrophoresis was conducted.

Table 1. The sample sizes and localities of eight populations of *O. rufipogon* from China according to number assigned in the data analysis

Population No.	Population localities	Sample sizes
1	Zhoujiacun, Guilin City, Guangxi	15
2	Fushui County, Guangxi	20
3	Donxiang County, Jiangxi	19
4	Chongpo, Ledong County, Hainan	15
5	Qianjia, Ledong County, Hainan	15
6	Mandan, Yuanjiang County, Yunnan	88
7	Gasa, Jinhong City, Yunnan	29
8	Meiting, Jinhong City, Yunnan	32

Starch-gel electrophoresis

Fourteen enzymes were resolved and scored using starch-gel electrophoresis (Table 2). The electrophoretic methods followed Glaszmann et al. (1988) and Soltis et al. (1983) with 12% starch gels. A modification of buffer system 1 (S1#)was used to resolve FBA, MDH, ME and 6PGD(Electrode buffer was diluted 2 times before use); AAT, DIA, PGI and TPI were resolved on buffer system 6 (S6); ADH, G3PDH, IDH, LAP, PGM and SKD were resolved on buffer system I of Glaszmann et al. (1988) (G_I). Staining procedures for all enzymes followed Soltis et al. (1983). When more than one isozyme were observed for an enzyme, isozymes were numbered sequentially with the most anodally migrating enzyme designated 1. Allelic variation at a locus was alphabetically coded with the most anodally migrating allozyme designated a.

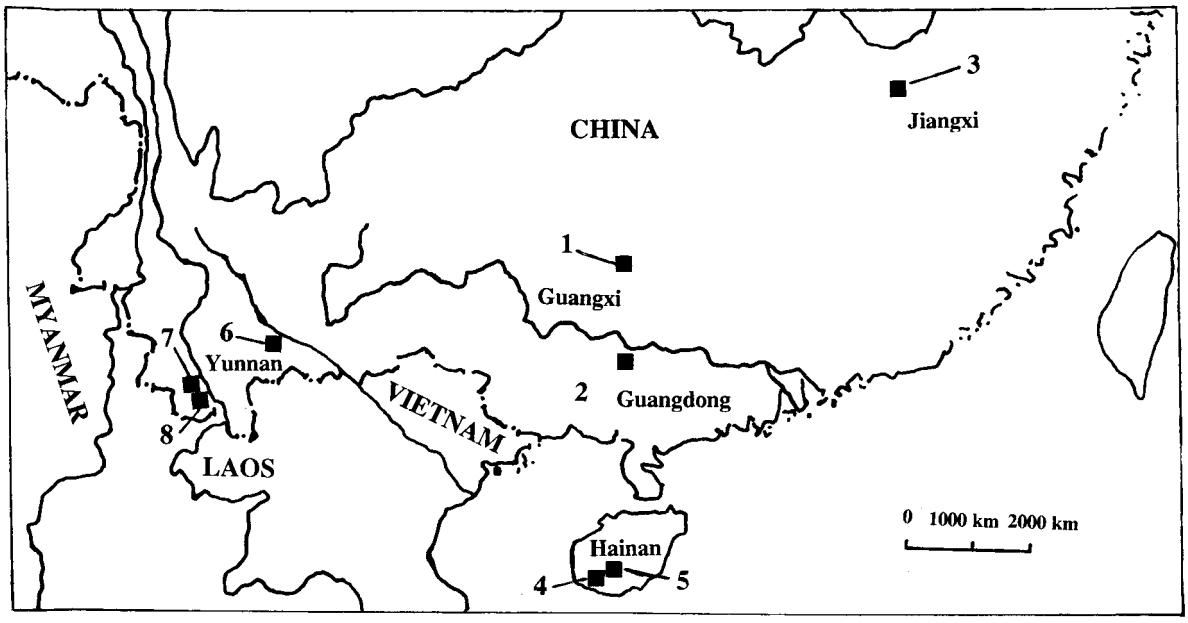

Figure 1. Geographical localities of eight populations of *O. rufipogon* sampled in China.

Table 2. Enzyme systems assayed, gel buffers and the number of loci scored

Enzyme system	Abbreviation	EC No.	Gel buffer	No. of loci
Aspartate aminotransferase	AAT	EC 2.6.1.1	S6	2
Alcohol dehydrogenase	ADH	EC 1.1.1.1	G_I	1
Diaphorase	DIA	EC 1.6.2.2	S6	2
Fructose-bisphosphate aldolase	FBA	EC 4.1.2.13	S1#	1
Glutamate dehydrogenase	G3PDH	EC 1.4.1.2	G_I	1
Isocitrate dehydrogenase	IDH	EC 1.1.1.42	G_I	1
Leucine-amino peptidase	LAP	EC 3.4.11.1	G_I	1
Malate dehydrogenase	MDH	EC 1.1.1.37	S1#	3
Malic enzyme	ME	EC 1.1.1.40	S1#	1
Phosphogluconate dehydrogenase	6PGD	EC 1.1.1.44	S1#	2
Phosphoglucoisomerase	PGI	EC 5.3.1.9	S6	3
Phosphoglucomutase	PGM	EC 2.7.5.1	G_I	1
Shikimate dehydrogenase	SKD	EC 1.1.1.25	G_I	1
Triosephosphate isomerase	TPI	EC 5.3.1.1	S6	2

Data analysis

Electrophoretic data were analyzed using the computer program Biosys-1 (Sworfford & Selander, 1989) version 1.7 for the IBM-PC. Data were entered as genotype numbers from which allele frequencies were calculated. Genetic variability, Nei's unbiased genetic identity (I) (Nei, 1978) and F-statistics were calculated. The populations were analyzed for mean number of alleles per locus (A), percentage of polymorphic loci (P), observed heterozygosity (Ho) and expected heterozygosity (He).Genetic identity values measure the similarity of allele frequencies between pairs of populations and range from 0, indicating no shared alleles between populations, to 1, indicating the two populations have the same alleles in identical frequen-

Table 3. Allele frequencies in *O. rufipogon* populations

Locus	Population							
	1	2	3	4	5	6	7	8
Aat-1a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Aat-3a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Adh- a	0.000	0.000	0.000	0.000	0.000	0.040	0.000	0.000
- b	1.000	1.000	1.000	1.000	1.000	0.960	1.000	1.000
Dia-1a	0.111	0.150	0.237	0.000	0.000	0.017	0.000	0.000
-1b	0.889	0.800	0.763	0.950	1.000	0.948	1.000	1.000
-1c	0.000	0.050	0.000	0.050	0.000	0.034	0.000	0.000
Dia-2a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Fba- a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Gdh- a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Idh- a	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000
- b	0.833	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Lap-1a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-1a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-2a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-3a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Me - a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgd-1a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgd-2a	0.556	1.000	1.000	1.000	1.000	1.000	1.000	1.000
-2b	0.444	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgi-1a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgi-2a	0.222	0.225	0.184	0.400	0.400	0.000	0.000	0.000
-2b	0.778	0.750	0.816	0.550	0.600	1.000	1.000	1.000
-2c	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000
-2d	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000
Pgi-3a	0.222	0.000	0.000	0.400	0.000	0.000	0.000	0.000
-3b	0.778	0.625	0.816	0.600	0.650	1.000	1.000	1.000
-3c	0.000	0.375	0.184	0.000	0.350	0.000	0.000	0.000
Pgm- a	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000
- b	0.889	1.000	1.000	1.000	1.000	1.000	0.983	1.000
- c	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Skd- a	0.222	0.000	0.053	0.000	0.000	0.000	0.000	0.000
- b	0.111	0.200	0.316	0.000	0.000	0.006	0.000	0.000
- c	0.111	0.450	0.632	0.000	0.500	0.494	0.017	0.000
- d	0.556	0.350	0.000	1.000	0.500	0.500	0.983	1.000
Tpi-1a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Tpi-2a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

cies. Nei's (1978) unbiased genetic identities were computed to alleviate any bias caused by small sample sizes (e.g., 50 fewer individuals). Wright's F-statistics are a hierarchical series of fixation indices where F_{IS} represents the deviation from Hardy-Weinberg expectation within populations (approximately equal to the mean F across populations), F_{ST} measures the fixation of different alleles in different populations, and F_{IT} measures deviations from Hardy-Weinberg expectation across the population system as a whole.

Table 4. Genetic variability at 22 loci in eight populations of *O. rufipogon*

Population No.	A	P*	Ho	He**
6	1.2	13.6	0.017	0.031
7	1.1	9.1	0.003	0.003
8	1.0	0.0	0.000	0.000
The means for Yunnan	1.1	7.6	0.007	0.011
1	1.4	31.8	0.015	0.119
2	1.3	18.2	0.073	0.085
3	1.2	18.2	0.050	0.068
4	1.2	13.6	0.009	0.053
5	1.1	13.6	0.032	0.069
The mean for other regions	1.2	24.1	0.045	0.079
Total mean	1.2	14.8	0.025	0.054

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.99.
** Unbiased estimate (see Nei, 1978).

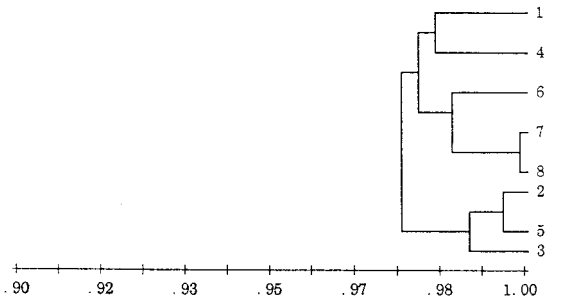

Figure 2. Cluster analysis of the eight populations of *O. rufipogon* using unweighted pair group method and Nei's (1978) unbiased genetic identity values.

Results

Allelic isozyme variation

Enzyme electrophoresis resulted in clear staining for 14 enzymes encoded by 22 putative loci. All enzymes migrated anodally. Fourteen loci, Aat-1, Aat-3, Dia-2, Fba, G3pdh, Lap-1, Mdh-1, Mdh-2, Mdh-3, Me, 6Pgd-1, Pgi-1, Tpi-1 and Tpi-2 were monomorphic, with all individuals from eight populations scored possessing a single enzyme band with an identical mobility band for each locus, and remaining eight loci were polymorphic in at least one population. Adh, Idh and 6Pgd-2 had two alleles, Dia-1, Pgi-3 and Pgm each had three, and Pgi-2 and Skd each had four. Although two isozymes of PGM are typically present in diploid seed plants (Gottlieb, 1982), only one PGM isozyme was observed in *O. rufipogon*; two loci of G3PDH were typically reported (Second, 1982), but only one was observed in this study. The banding patterns of Pgi-2 and Pgi-3 seemed to be apparent gene duplication. Allele frequencies for all loci in eight populations are presented in Table 3.

Measures of genetic variability

Genetic variability in *O. rufipogon* was quantified using standard measures of genetic variation to estimate allelic diversity, levels of polymorphism, and heterozygosity. These values (Table 4) varied among populations. The highest value of A = 1.4, P = 31.8%, Ho = 0.015 and He = 0.119 was shown in the Guilin population from Guangxi, while the Meiting population from Yunnan was the lowest (A = 1.0, P = 0.0%, Ho = 0.000 and He = 0.000). Among three populations from Yunnan, the Yuanjiang population had the highest genetic diversity (A = 1.2, P = 13.6%, Ho = 0.017 and He = 0.031). It is clear that the mean value of genetic diversity for Yunnan (A = 1.1, P = 7.6%, Ho = 0.007 and He = 0.011) was significantly lower than that for the other regions (A = 1.2, P = 24.1%, Ho = 0.045 and He = 0.079).

Distribution of genetic variation

The distribution of genetic variation among populations may be an important consideration in the management of endangered species concerning the populations that should be enhanced and/or preserved. Typically, inbreeding species maintain higher genetic diversity among populations than outcrossers (Brown, 1979). In the eight populations of *O. rufipogon* studied, F_{IS} was 0.517, suggesting that most of the populations deviated from Hardy-Weinberg expectation within populations (Table 5); F_{IT} was 0.640, indicating nonequilibrium conditions across populations of the species and a deficiency of heterozygotes. F_{ST} was 0.254, suggesting that 25.4% of the total genetic variation existed among populations. A slightly higher value of F_{ST} = 0.302 for the three populations from Yunnan (Table 5) suggests a fairly high genetic differentiation in the region.

Table 5. Summary of F-statistics at all the polymorphic loci

Locus	China			Yunnan		
	F_{IS}	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}
Adh	0.553	0.569	0.035*	0.553	0.566	0.027**
Dia-1	0.171	0.255	0.101***	0.884	0.887	0.027*
Idh	0.600	0.660	0.149***	—	—	—
Pgd-2	1.000	1.000	0.412***	—	—	—
Pgi-2	0.332	0.447	0.172***	—	—	—
Pgi-3	0.552	0.649	0.217***	—	—	—
Pgm	0.851	0.864	0.086***	–0.018	–0.006	0.012*
Skd	0.639	0.768	0.358***	0.340	0.585	0.371***
Mean	0.517	0.640	0.254***	0.418	0.593	0.302***

* $P < 0.5$; ** $P < 0.1$; *** $P < 0.001$.

Table 6. Matrix of Nei (1978) unbiased genetic identity values

Population	1	2	3	4	5	6	7	8
1	*****	0.982	0.974	0.983	0.982	0.983	0.982	0.982
2		*****	0.996	0.976	0.998	0.990	0.976	0.975
3			*****	0.955	0.987	0.987	0.962	0.960
4				*****	0.984	0.973	0.986	0.986
5					*****	0.988	0.978	0.977
6						*****	0.989	0.989
7							*****	1.000
8								*****

Genetic identity measures

In the present study, genetic identity values ranged from 0.955 between the Dongxiang population and the Chongpo population to 1.000 between the Gasa population and the Meiting population, with a mean of all pairwise comparisons of 0.981 (Table 6). Cluster analysis (UPGMA) produced a phenogram to show the genetic identity of all populations studied (Figure 2). In addition, standard genetic identities estimated between four geographical regions of *O. rufipogon* are presented in Table 7. There is no significant correlation between geographical distances and genetic identity values among eight populations.

Discussion

The mean values of genetic diversity for Yunnan are much lower than those for other regions of China, indicating that the center of genetic diversity for *O. rufipogon* fails to agree with that for cultivated rice in China. Comparatively speaking, the populations from Guangxi (the mean A = 1.4, P = 25.0%, Ho = 0.044 and He = 0.102) possessed a high genetic diversity in the present study. It seems supported the results derived from studies on seed samples of 21 populations over China (Gao et al., 2000b).Therefore, our result implies that the current center of genetic diversity for cultivated rice is not related to that for its wild progenitor, probably because little knowledge about the early stages of rice domestication has been obtained, as well as continuous evolutionary events have afterwards been involved.

The most likely explanation for low genetic diversity of *O. rufipogon* in Yunnan is that the populations may be a subgenepool of marginal nature according to the range of the species. Furthermore, low levels of genetic diversity for Yunnan may stem from recent reduction of population size and consequent drift. The species was widely distributed in the Xishuangbanna Prefecture before, but most of the populations have fallen into extinction due to human activities and the

Table 7. Matrix of Nei (1978) unbiased genetic identity averaged by regions

Region	1	2	3	4
Guangxi	0.982			
	(0.982–0.982)			
Jiangxi	0.985	*****		
	(0.974–0.996)	(*****–*****)		
Hainan	0.985	0.971	0.984	
	(0.976–0.998)	(0.955–0.987)	(0.984–0.984)	
Yunnan	0.981	0.970	0.981	0.993
	(0.975–0.990)	(0.960–0.987)	(0.973–0.988)	(0.989–1.000)

farming construction in the past years. The reduction in the population size is a cause of inbreeding through mating between relatives (Fowler, 1965; Lewis, 1973), selfing and genetic drift. Therefore, a fairly low diversity was observed in the two existing populations, especially within the Meiting population, which consisted of only few clones. A loss of genetic variation might have occurred in the Yuanjiang population even if it maintained a much higher level of genetic diversity than the other two. All types of tropical rain forest had grown in Southern Yunnan five thousands years ago (Xu et al., 1985), and thus the species might be distributed more widely in Yuanjiang dry-hot valley and geographically connected the populations from the Xishuangbannan Prefecture. The fact that only one isolated population is surviving there suggests that historical events such as bottlenecks have probably occurred due to destruction of flora and regional climatic changes, and thus led to the loss of ample genetic variation. Genetic drift certainly results in high genetic differentiation, and therefore, a larger value of F_{ST} for Yunnan was observed. In addition, F_{ST} is a relative value when used to assess genetic differentiation, and low genetic diversity within the region might produce an increased estimate.

The population genetic structure of the species revealed by this study has implications for its conservation. An estimation of F_{ST} of 0.254 shows that 25.4% of the total genetic variation existed among populations. Therefore, a general criteria of sampling fewer populations but more individuals from each population for *O. rufipogon* in China, should be taken. The populations with high variation such as Guilin, Dongxiang, Qianjia and Fushui populations, which represent the main parts of its range in China, should be preserved and/or sampled. In combination with other detailed studies on population genetics (Gao, 1997; Gao et al., 2000a,b; Gao et al., 2001a, 2001b), conservation genetics of the species in China is fully discussed in another paper (Gao, 2001c). As far as Yunnan is concerned, it is urgent in the short time to conserve the existing populations. Since the Meiting population was sampled from the natural populations about 3 miles apart from the Gasa population and is preserved in Xishuangbanna Prefecture Institute of Agricultural Sciences (Jinghong City) by *ex situ* conservation, it may be recently founded by a few individuals from the populations that had shared the same gene pool with the Gasa population several years before (Wang Wenhua 1994, personal communication). As a result, the highest genetic identity value (I = 1.000) was found between them. Therefore, the management efforts should certainly focus on the Yuanjiang population and at least one population from the Xishuangbanna Prefecture. As far as two populations from Xishuangbanna are concerned, a little higher level of genetic diversity for the Gasa population than for the Meiting population indicates that genetic variability within the Gasa population has not totally been represented in the Meiting population by means of *ex situ* conservation. However, there were differences in allele frequencies between them and such the alleles as Pgm-a, Skd-c and some genetic diversity underestimated may be harboring in the Gasa population. Hence, further sampling is needed from the Gasa population, because it was located at a ditch beside the road and confronted by extinction due to human disturbance. The Yuanjiang population may be paid attention for *in situ* conservation of the species in China. First, it is the typical population of *O. rufipogon* for exploring the origin of Asian cultivated rice; and second, it is also being threatened seriously by increasing human disturbance and unfavorable ecological conditions of Yuanjiang dry-hot Valley.

In conclusion, low levels of genetic diversity of *O. rufipogon* for Yunnan may originate from marginal nature of populations and recent reduction of the population size. As further studies using molecular techniques are completed, they will enable us take deep insight into current center of genetic diversity of *O. rufipogon* in China and probably provide some other valuable implication on the origin of Asian cultivated rice.

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