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Comparisons of microsatellite variability and population genetic structure of two endangered wild rice species, *Oryza rufipogon* and *O. officinalis*, and their conservation implications

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Abstract. Conserving endangered wild rice species requires a thorough understanding of their population genetic structure and appropriate approaches. We applied six and seven microsatellite loci to study the genetic structure of six populations throughout the range of Chinese *Oryza rufipogon* and *Oryza officinalis*, respectively. The results showed that *O. rufipogon* possesses higher levels of genetic diversity but lower differentiation ($R_S = 3.2713$, $P = 100.0\%$, $H_O = 0.1401$, $H_S = 0.5800$, $F_{ST} = 0.271$) than *O. officinalis* ($R_S = 2.0545$, $P = 57.14\%$, $H_O = 0.0470$, $H_S = 0.2830$, $F_{ST} = 0.554$). Mean population F_{IS} was slightly larger for *O. officinalis* ($F_{IS} = 0.844$) than that for *O. rufipogon* ($F_{IS} = 0.755$), indicating that *O. officinalis* has slightly higher departures from Hardy–Weinberg expectations and heterozygosity deficits than *O. rufipogon*. In addition to different origins and evolutionary histories, *O. officinalis* has restricted gene flow, high inbreeding, isolated small populations and fewer opportunities of hybridization with other taxa, which may determine major differences in population genetic structure from *O. rufipogon*. Our results suggest the adoption of a plan of involving fewer populations but more individuals within populations for *O. rufipogon*, while both the number of populations and the individuals for a sampled population should be almost equally considered for *O. officinalis*. The known high degree of inbreeding in the populations of both species implies that conservation and restoration genetics should particularly focus on the maintenance of historically significant processes such as high levels of outbreeding, gene flow and large effective population sizes. We finally proposed to further estimate the role of rice gene flow in the conservation of *O. rufipogon*, and to perform detailed analysis of mating systems in both species for better conservation perspectives of their ecological and evolutionary processes.

Introduction

Many conservation programs aim at maintaining existing levels of genetic variation in rare or threatened species (Simberloff 1988; Barret and Kohn 1991; Frankel et al. 1995) because it increases the chance of population long-term survival. As for genetic studies on these rare species, Gitzendanner and Soltis (2000) asserted that the genetic data collected solely in a rare species are usually uninformative for conservation markers, and emphasized the necessity to compare genetic diversity

between a rare species and its widespread congeners. Indeed, such population genetic studies on rare endangered plant species in comparison with their more common congeners have increasingly enhanced our knowledge of population genetic structure and conservation efficiency (e.g., Young and Brown 1996; Gustafsson and Sjögren-Gulve 2002; Maki et al. 2002;). To better evaluate possible differences in the characteristics of genetic data obtained, however, comparisons between endangered relatives, which historically had extensive geographic distribution but currently become endangered, are of potential significance in conservation biology. This comparative approach may reveal evolutionary and ecological factors underlying the differences of population genetic structure observed, and further provide specific guidelines for their conservation.

The rice genus *Oryza*, including about 21 wild species, is widely distributed in tropical and subtropical regions (Vaughan 1989). They have been proven an invaluable gene pool for the rice genetic improvement (Chang 1984; Oka 1988; Yuan et al. 1989; Jackson 1994, 1997; Xiao et al. 1996; Tanksley and McCouch 1997). However, almost all wild species are currently under extreme pressure of habitat destruction and human disturbance on our planet, and therefore, the development of appropriate and efficient strategies for *in situ* conservation and germplasm collection is urgently required (Vaughan and Chang 1980; Vaughan and Sitch 1991; Vaughan and Chang 1992; Gao et al. 1996, 1998; Gao 2003; Gao et al. unpublished data). Up-to-date population genetic studies provided information about genetic structure within natural populations of *Oryza* species (Oka 1988; Barbier 1989a,b; Akimoto et al. 1998; Buso et al. 1998; Ge et al. 1999; Gao et al. 2000a,b; Gao et al. 2001; Gao et al. 2002a,b). Genetic data available implied that population genetic structure of endangered wild rice species may be different from each other, as detected by allozyme and RAPD DNA analysis (Barbier 1989a,b; Akimoto et al. 1998; Buso et al. 1998; Ge et al. 1999; Gao et al. 2000a,b; Gao et al. 2001; Gao et al. 2002a,b). However, questions to answer how genetic structure is shaped within different wild rice species and what are those affected evolutionary factors are essential for a deep understanding of their conservation concerns but have not been completely resolved. Genetic resources management of wild rice species particularly require a full understanding of their conservation concerns for effectively planning *in situ* and *ex situ* conservation practices.

Among *Oryza* species, *O. rufipogon* (AA genome) shares similar geographical distribution to another wild rice species, *O. officinalis* (CC genome). The former is distributed in tropical and subtropical regions, such as China, Nepal, India, Sri Lanka, Bangladesh, Myanmar, Laos, Thailand, Cambodia, Vietnam, Malaysia, Indonesia, Philippines, Papua New Guinea, and Australia. The latter has almost same range without occurring in Australia (Vaughan 1994). They show relatively different ecological preference. *O. rufipogon* is usually found in swamps, marshes, open ditches, rivers, swampy grassland, and rice fields (sea level to 1400 m in elevation), but *O. officinalis* prefers full or partial-shaded seasonally wet habitats at the edge of or in forests, evergreen or deciduous forests (sea level to 1500 m in elevation) (Vaughan 1994). Serious destruction and fragmentation of their habitats have brought about the decrease in population number and size of two wild rices, and thus

they have become endangered plant species (Vaughan and Chang 1992). In China, for example, *O. rufipogon* grows in eight provinces and autonomous regions during 1978–1982 (National Exploring Group of Wild Rices 1984). The species historically had such a large population system that, only in Guangdong and Hainan provinces, 1182 populations were documented (Liang and Wu 1993). Our recent field surveys, unfortunately, suggested that this species was at the edge of extinction due to the loss and fragmentation of natural habitats. Among 193 populations revisited, only 80 and 75 populations were surviving during 1994–1995 and in 1999, respectively (Gao et al. 1996; Gao 1997; Gao et al. 1998; Gao 2003; Gao et al. unpublished data). In addition, remaining populations have largely declined in size. *O. officinalis*, which grows in four provinces (Guangdong, Guangxi, Hainan and Yunnan) of China, is also at high risk of extinction (Gao et al. 1996; Gao et al. unpublished data). Chinese Conservation Committee of Biodiversity and Chinese Ministry of Agriculture listed these two wild rice species as endangered species in China (Fu 1992). Considering the threat of extinction to the wild rice species is extremely serious (Gao and Hong 1999; Gao 2003), intensive conservation efforts are necessary to secure their survival.

In this study, we attempted to address the extent of genetic diversity within and differentiation among populations of Chinese *O. rufipogon* and *O. officinalis* through microsatellite DNA analysis. This molecular maker has many positive attributes including hypervariability, abundance, and tolerance to sample quality and quantity. Analysis of microsatellite loci has effectively resolved many questions such as effective population size, population genetic structure, migration and colonization rates, mating system and conservation (e.g., Rossetto et al. 1999; Simon et al. 1999; Gibbs et al. 2000; Neraas and Spruell 2001; King et al. 2001). This approach, in comparison to allozyme analysis, seemed particularly well suited to the study of population genetic structure of wild rice species (Gao et al. 2002b). Specific objectives of this study were to (1) define the extent and pattern of microsattelite variability among Chinese populations of *O. rufipogon* and *O. officinalis*; (2) determine evolutionary factors that may affect their differences in population genetic structure; and (3) provide data crucial to the development of appropriate conservation strategies for long-term sustainability of different endangered wild rice species.

Methods

Sample collection

In this study, we included six populations of *O. rufipogon* and *O. officinalis*, respectively, throughout the geographical ranges in China (Figure 1). At least 25 plants were collected in each of the six populations. Because it was reported that *O. rufipogon* may have clonal growth (Gao et al. 1998; Xie et al. 2001), samples were randomly taken at an interval of at least 5 m for both species to prevent collecting ramets from a single genet. About 4 g of fresh leaves per plant was collected and immediately stored in a ziplock plastic bag with about 50 g of silica gel. In order

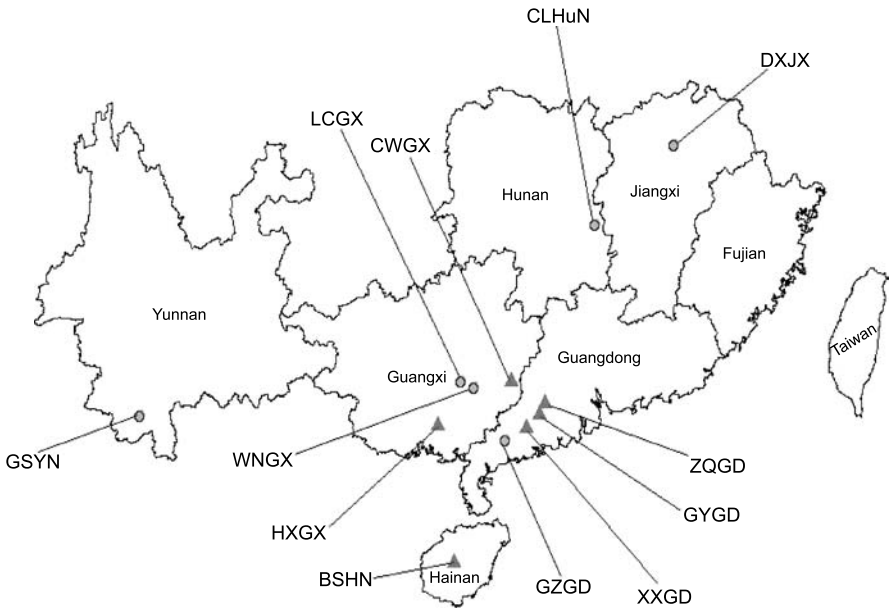

Figure 1. Sampling locations of *O. rufipogon* and *O. officinalis* populations from China (● indicates the *O. rufipogon* population, and ▲ shows the *O. officinalis* population).

to dry the material within 8 h, 2–3 additional batches of silica gel were used to substitute those in which the color turned to pale purple, then the samples were taken back to the laboratory and stored at room temperature once thoroughly dried.

DNA extraction and microsatellite assay

DNA was isolated from fresh or silica-dried leaf tissues according to the method of Edwards et al. (1991). A total of 12 primer pairs, which are randomly distributed throughout the 12 rice chromosomes (Wu and Tanksley 1993; Akagi et al. 1996; Panaud et al. 1996; Chen et al. 1997), were used in this study. Six (RM164, RM241, RM211, RM253, OSR28, RM222) and seven (RM211, RM225, RM60, RM220, RM224, RM231, RM233A) microsatellite loci were randomly chosen for assaying samples of *O. rufipogon* and *O. officinalis*, respectively. Detailed information of primer sequences is now available at http://www.gramene.org/microsat/microsats.txt). Of them, RM211 was the only locus used to assay samples in both species. Microsatellite polymorphisms were analyzed by specific PCR conditions as described in Panaud et al. (1996). PCR products were run on 4% polyacrylamide denaturing gels, and marker bands were revealed using the silver staining as described by Panaud et al. (1996).

Statistical analysis

Genetic diversity for each population and overall was estimated by R_S, P (percentage of polymorphic loci), H_O (the observed heterozygosity) and H_S (gene diversity within the sample). H_O was calculated using GENEPOP version 3.1c (GENEPOP on the web http://wbiomed.curtin.edu.au/genepop; Raymond and Rousset 1995) while R_S and H_S were calculated by FSTAT version 2.9.3 (Goudet 2001). Allelic richness (R_S) is a measure of the number of alleles independent of sample size, hence allowing to comparing this quantity between different sample sizes (El Mousadik and Petit 1996). Unbiased estimates and standard deviations of gene diversity or expected heterozygosity were calculated following Nei (1973). For each population-locus combination, departure from Hardy–Weinberg expectations was assessed by exact tests (Guo and Thompson 1992), with unbiased P values estimated through a Markov chain method (Guo and Thompson 1992); a global test across loci and populations was constructed using Fisher's method (Raymond and Rousset 1995). In order to test specifically the hypothesis of heterozygote deficiency, the multiscore (U) test of Raymond and Rousset (1995) was applied. Tests for genotypic linkage disequilibrium among pairs of loci in each population were performed using Fisher's exact tests (Raymond and Rousset 1995), with unbiased P values again derived by a Markov chain method. We set the significance value for multiple significance tests using the sequential Bonferroni procedure (Rice 1989). Weir and Cockerham (1984) estimators of F_{IT}, F_{ST} and F_{IS} (Capf, theta and smallf, respectively in FSTAT output) were estimated for each locus and population by FSTAT version 2.9.3 (Goudet 2001). F-statistics are a hierarchical series of fixation indices where F_{IS} represents the deviation from Hardy–Weinberg expectations 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. Bootstrapping over loci was automatically performed for the statistics CapF, theta and smallf. Tests for the presence of population differentiation were also made using an unbiased estimated P value for a log-likelihood (G)-based exact test (Goudet et al. 1996) with FSTAT version 2.9.3 (Goudet 2001). The differences of population genetic mean values between the two species were tested assuming each estimate follows a normal distribution. The t-test was conducted by using the SAS system (Der and Everitt 2001).

Results

Microsatellite polymorphism across populations and intra-population genetic variation

Microsatellite allele frequencies for all loci in *O. rufipogon* and *O. officinalis* populations are available from the first author upon request. The 158 individuals, representing six different *O. rufipogon* populations, produced a total of 61 alleles at

the six loci tested. The number of alleles ranged from five alleles at RM164 to 13 at RM241. R_S, H_O and H_S varied among the loci tested (data not shown). The mean number of observed alleles per locus ranged from 1.6667 (RM164) to 6.1667 (OSR28). Across the populations, OSR28 had the highest mean observed heterozygosity ($H_O = 0.2257$) while the least variable locus was RM164, which had mean observed heterozygosity of 0.0192. Observed gene diversity (H_S) ranged from 0.1493 at RM164 to 0.7393 at RM253. As given in Table 1, all the values varied among the six populations, with R_S ranging from 2.3890 in DXJX to 4.4570 in GZGD; P ranging from 83.33% in LCGX, GZGD and DXJX to 100% in other three populations; H_O ranging from 0.0556 in DXJX to 0.2419 in GZGD and H_S ranging from 0.4905 in DXJX to 0.8040 in GZGD. Differences in genetic diversity among *O. rufipogon* populations were evident. The populations from Guangxi and Guangdong provinces showed relatively high levels of genetic diversity. Among six populations assayed, GZGD from Guangdong Province had the highest levels of microsatellite variability, while two northern marginal populations, DXJX and CLHuN from Jiangxi and Hunan provinces, respectively, exhibited low genetic diversity. Moreover, these two northern populations, DXJX ($H_O = 0.0556$) and CLHuN ($H_O = 0.0750$), were less heterozygous than other populations (mean $H_O = 0.1912$).

As shown in Table 1, 70 alleles at seven loci were examined in 150 individuals of six *O. officinalis* populations. R_S, H_O and H_S varied among the loci tested (data not shown). The mean number of observed alleles per locus varied from 1.167 (OSR15) to 2.833 (RM231). The locus RM233A was the most polymorphic with eight alleles, while the least polymorphic locus was OSR15, which only had two alleles. Across *O. officinalis* populations detected, RM26 had the highest mean observed heterozygosity (mean $H_O = 0.2827$) while OSR20, OSR27 and RM233A were the least variable (mean $H_O = 0$). Observed gene diversity (H_S) ranged from 0.03 at OSR15 to 0.4501 at RM233A. All the values varied among the populations examined, with R_S ranging from 1.3333 in XXGD to 3.1967 in CWGX, P ranging from 28.57% in XXGD to 100% in CWGX, H_O ranging from 0.0000 in ZQGD and XXGD to 0.065 in CWGX, and H_S ranging from 0.139 in HXGX to 0.540 in CWGX. It is noteworthy that, of the six populations studied, CWGX and BSHN had higher levels of genetic diversity than other populations. Two populations (ZQGD and XXGD) were clearly the least heterozygous with $H_O = 0.000$.

Population genetic structure

Tests for genotypic heterogeneity among six *O. rufipogon* populations were highly significant for each of the six loci individually and for all loci combined ($P < 0.001$ following sequential Bonferroni adjustment) (data not shown). The estimates of F_{IS} for all populations ranged from 0.6300 (GSYN) to 0.8840 (DXJX), suggesting nonrandom mating and heterozygosity deficits within populations ($P < 0.001$) (Table 1). At the intra-population level, F_{IS} was positive ($F_{IS} = 0.755$ across all populations of the species, $P < 0.01$; Table 2), a pattern consistent with the het-

Table 1. Geographical locations, sample sizes, mean values of allelic richness (R_S), percentage of polymorphic loci (frequency of most common allele < 0.95) (P), observed heterozygosity (Ho), mean values of the gene diversity within sample (Hs), and fixation index (F_{IS}) for 6 populations of *O. rufipogon* and *officinalis*, respectively. Statistically significant deviations from Hardy–Weinberg expectations are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Species	Population localities	Sample size	R_S	$P(\%)$	H_O	H_S	F_{IS}
O. rufipogon	Linci, Silin, Wuxuan County, Guangxi Province (**LCGX**)	25	3.9540	83.33	0.1467***	0.7398	0.7980***
	Wangnong, Xianfeng, Guiping County, Guangxi Province (**WNGX**)	26	3.2340	100.00	0.1594***	0.6210	0.7420***
	Zhengjiang, Gaozhou County, Guangdong Province (**GZGD**)	27	4.4570	83.33	0.2419***	0.8040	0.6990***
	Dongyuan, Dongxiang County, Jiangxi Province (**DXJX**)	29	2.3890	83.33	0.0556***	0.4905	0.8840***
	Gasa, Jinghong City, Yunnan Province (**GSYN**)	26	3.0520	100.0	0.2167***	0.5852	0.6300***
	Huli, Caling County, Hunan Province (**CLHuN**)	25	2.5480	100.0	0.0750***	0.5046	0.8440***
	Overall	26	3.2723	100.0	0.1401***	0.5800	0.7517***
O. officinalis	Hengxian County, Guangxi Province (**HXGX**)	25	1.6423	42.86	0.0130***	0.1390	0.9060***
	Cangwu County, Guangxi Province (**CWGX**)	25	3.1967	100.0	0.0650***	0.5400	0.8790**
	Baisa County, Hainan Province (**BSHN**)	25	2.3832	71.43	0.1900***	0.3880	0.5090***
	Zhaoqing County, Guangdong Province (**ZQGD**)	25	2.0800	42.86	0.0000***	0.2340	1.0000***
	Xingxin County, Guangdong Province (**XXGD**)	25	1.3333	28.57	0.0000***	0.1710	1.0000**
	Gaoyao County, Guangdong Province (**GYGD**)	25	1.6915	57.14	0.0160***	0.2040	0.9220***
	Overall	25	2.0545	57.14	0.0470***	0.2830	0.8440***

Table 2. Genetic differentiation within and among populations of *O. rufipogon* and *O. officinalis*.

O. rufipogon				*O. officinalis*			
Locus	F_{IT}	F_{ST}	F_{IS}	Locus	F_{IT}	F_{ST}	F_{IS}
RM211	0.820	0.272**	0.753	RM211	0.979	0.814**	0.887
RM164	0.881	0.381**	0.809	RM225	0.919	0.634**	0.778
RM241	0.837	0.341**	0.752	RM60	0.918	0.510**	0.833
RM253	0.752	0.135**	0.714	RM220	0.500	0.004**	0.498
OSR28	0.739	0.217**	0.667	RM224	0.916	0.557**	0.810
RM222	0.903	0.289**	0.863	RM231	0.881	0.495**	0.764
				RM233A	0.974	0.324**	0.962
All	0.821	0.271**	0.755	All	0.930	0.554**	0.844

**$P < 0.01$.

erozygosity deficits observed in tests of Hardy–Weinberg equilibrium. However, $F_{IS} = -0.069$ was detected in our previous survey, indicating that *O. rufipogon* populations may not deviate from Hardy–Weinberg equilibrium (Gao et al. 2002b). The different population samples and loci used may be probable reasons for such a discrepancy. Recently, assaying the same six microsatellite markers in 47 populations yielded similar results ($F_{IS} = 0.683$) (Gao 2004) as this study ($F_{IS} = 0.755$). The F_{IS} values in two northern marginal populations (DXJX and CLHuN) were obviously greater than other populations. Overall mean F_{ST} was 0.289, indicating 28.90% of the total genetic variation resided among populations.

In *O. officinalis*, tests for genotypic heterogeneity among the six populations studied were also highly significant for each of the six loci individually and for all loci combined ($P < 0.001$ following sequential Bonferroni adjustment) (data not shown). The estimates of F_{IS} for all populations varied from 0.5090 (YJYN) to 1.0000 (ZQGD and XXGD), indicating nonrandom mating and heterozygosity deficits within populations ($P < 0.001$). At the intra-population level, F_{IS} was positive ($F_{IS} = 0.844$ across all populations studied, $P < 0.01$; Table 2). The observation seems consistent with the heterozygosity deficits observed in tests of Hardy–Weinberg equilibrium. Overall mean F_{ST} of 0.554 suggests 55.40% of the total genetic variation partitioned among populations.

Comparison of population genetic structure

In comparison, *O. rufipogon* populations showed approximately two times higher levels of genetic diversity ($R_S = 3.2723$, $P = 100.00\%$, $H_O = 0.1401$ and $H_S = 0.5800$) than *O. officinalis* populations ($R_S = 2.0545$, $P = 57.14\%$, $H_O = 0.047$ and $H_S = 0.283$) (R_S: $p = 0.017$; P: $p = 0.019$; H_o: $P = 0.038$; H_S: $P = 0.001$), suggesting that *O. rufipogon* maintains significantly higher genetic diversity than *O. officinalis*. However, genetic variation for two northern marginal populations of *O.*

rufipogon was almost as large as two *O. officinalis* populations (CWGX and BSHN) which were abundant in genetic diversity.

Although sampling range of *O. rufipogon* in this study was wider than that of *O. officinalis* (Figure 1), population divergence measured by F_{ST} for the former populations was approximately two times lower than that for *O. officinalis* populations. Values for *O. officinalis* at most loci assayed were significantly greater than those for *O. rufipogon*. For example, RM211 is the only locus that was assayed in both species, at which F_{ST} for *O. officinalis* ($F_{ST} = 0.814$) was significantly larger than that for *O. rufipogon* ($F_{ST} = 0.272$). Only RM220 assayed in *O. officinalis* exceptionally exhibited low value of $F_{ST} = 0.004$.

Mean population F_{IS} was slightly larger for *O. officinalis* ($F_{IS} = 0.844$) than that for *O. rufipogon* ($F_{IS} = 0.755$), indicating that *O. officinalis* has slightly higher departures from Hardy–Weinberg expectations and heterozygosity deficits than *O. rufipogon*. Most of *O. officinalis* populations, except BSHN ($F_{IS} = 0.5090$), showed more heterozygosity deficits than *O. rufipogon* populations (mean $F_{IS} = 0.7517$).

Discussion

Microsatellite variation and population genetic structure

The most significant finding of this study is that the populations of *O. rufipogon* possess higher levels of genetic diversity but a lower genetic differentiation than *O. officinalis* populations. The differences observed in *O. rufipogon* and *O. officinalis* with microsatellite analysis reasonably agrees with our previous allozyme studies (*O. rufipogon*: $A = 1.33$, $P = 22.7\%$, $H_O = 0.033$, $H_e = 0.068$, and $F_{ST} = 0.310$; *O. officinalis*: $A = 1.13$, $P = 12.49\%$, $H_O = 0.029$, $H_e = 0.0290$, and $F_{ST} = 0.882$) (Gao et al. 2000a, 2001). Therefore, this study highlights that rice microsatellite loci may offer reliable estimates on intra-specific DNA variation and its inter-population structuring. Using wide germplasm collections of these two species, our previous investigation suggested that *O. rufipogon* obviously harbor higher microsatellite variation than *O. officinalis* at 60 loci assayed (Gao et al. unpublished data). This suggests that, although the numbers of loci are small and only one locus is shared between the two species in the present study, the result obtained here would not affect the general trends observed.

Since *O. rufipogon* (AA genome) and *O. officinalis* (CC genome) have relatively distant phylogenetic relationship in the genus *Oryza*, different origins and evolutionary history may have mainly determined their population genetic structure observed in the present study. Moreover, based on several main factors that may impact on population genetic structure, there are more likely hypotheses to interpret the differences of genetic structure observed between two wild rices. *O. rufipogon* maintains high within-population variation probably because its populations are naturally larger than *O. officinalis* populations. Comparing with historical records (National Exploration Group of Wild Rices 1984), *O. rufipogon* has been suffering more loss of populations in number and size than *O. officinalis* in recent years (Gao

et al. 1996; Gao et al. unpublished data). Nevertheless, remnant populations of *O. rufipogon* are still larger than those of *O. officinalis* populations in size, and thus the species is not subject to significant loss of variation through small-population effects such as random genetic drift and inbreeding coupled with natural selection. Microsatellite analysis for 47 populations from wider range of Chinese *O. rufipogon* suggested little impact on population genetic structure due to recent habitat fragmentation (Gao 2004). Population genetic theory predicts that the subdivision of a population into small, isolated subpopulations brings about a loss of genetic diversity through drift (Templeton 1991). It is particularly true for the case of *O. officinalis* populations, which declined to some severely isolated habitats in mountain valleys.

High genetic differentiation of *O. officinalis* may be due to restricted gene flow among remaining populations. Gene flow among plant populations is a result of pollen dispersal, seed dispersal and clonal spread (Gliddon et al. 1987). For wind-pollinated species such as *O. rufipogon*, pollen dispersal is considered as the main form of gene flow (Oka and Morishima 1967). However, pollen flow of *O. officinalis* may easily occur within populations, but it could be hard to move to neighboring populations due to geographical restriction. Most probably, *O. officinalis* disperses many small seeds by scattering large number in the water and floating down along the stream. Therefore, seed dispersion may act as a main form of gene flow in *O. officinalis* rather than pollen flow in *O. rufipogon*. Limited gene flow between isolated populations may well explain high genetic divergence in *O. officinalis*. Previous allozyme analysis also provided evidence that different alleles could be well fixed in different populations of *O. officinalis* (Gao et al. 2001). In contrast, *O. rufipogon* produces heavier but fewer seeds than *O. officinalis*. They hardly move out the habitats after shattering into the water and swamps. Thus pollen dispersion serves as the main component. Moreover, as observed in natural populations of Amazonian *O. glumaepatula* growing along or close to rivers (Akimoto et al. 1998), broken culms of *O. rufipogon* often are another important form of long-distance gene flow by floating on the surface of water and moving from one population to another (Gao et al. 1998).

Fewer opportunities of hybridization with other taxa, as reported in Amazonian *O. glumaepatula* (Akimoto et al. 1998), may also lead to relatively low genetic variability and high genetic differentiation observed in *O. officinalis*. Besides two wild rice species under study, there are two other *Oryza* species, *O. granulata* (G genome) and cultivated rice *O. sativa* (AA genome) occurring in the studied region. Many literatures reported the introgression between cultivated rice and its wild progenitor *O. rufipogon* (e.g., Morishima, Oka and Chang 1961; Oka 1988) as they are actually the same biological species. The introgression may help *O. rufipogon* to maintain considerable genetic diversity but relatively low differentiation by potentially extending its population size. In some natural habitats, *O. officinalis* did exist sympatrically with the above *Oryza* species. But natural hybrids are hardly produced due to different genome types. Additionally, variation on reproductive system (sexual vs. asexual reproduction) may be an important contributor to their population genetic structure since two wild rice species propagate both by clones

and seeds. Therefore, the influence of clonal (asexual) reproduction on population structure should not be given full consideration in further studies.

Microsatellite variation observed in *O. rufipogon* suggested that the populations with high levels of genetic diversity are from South China, where the center of genetic diversity is located based on previous allozyme and RAPD analyses (Xie 1999; Gao et al. 2000a). In agreement with those findings with allozyme analysis (Gao et al. 2002a), low levels of genetic diversity for the surviving population from Yunnan and northern populations from Jiangxi and Hunnan are not unexpected because of the nature of either marginal populations or highly fragmented populations in small sizes (Gao et al. unpublished data). In *O. officinalis*, high microsatellite diversity detected in two large populations, CWGX and BSHN, is almost equal to marginal populations of *O. rufipogon*. One likely explanation is that they are remnant individuals of larger populations as a result of recent decline (Gao et al. field observation, unpublished data). Rather low genetic variation observed within two populations (ZQGD and XXGD) is probably due to serious human disturbance and the destruction of their habitats. These sites might be colonized by few individuals and have experienced numerous population bottlenecks caused by adverse conditions such as extended drought due to the deforestation (Gao et al. field observation, unpublished data).

Deviation from the Hardy–Weinberg expectations

Hardy–Weinberg expectations are affected by several factors such as inbreeding, natural selection, and genetic differentiation of populations (Crow and Kimura 1970). Heterozygote deficits for most of locus-population combinations and positively high F_{IS} values for all studied populations in *O. rufipogon* and *O. officinalis* showed drastic departures from Hardy–Weinberg expectations and strong linkage disequilibrium. It is clear that *O. officinalis* had slightly higher departures from Hardy–Weinberg expectations and stronger linkage disequilibrium than *O. rufipogon*. The discrepancy also indicated that *O. officinalis* might have larger inbreeding than *O. rufipogon*. Either nonrandom mating or genetic differentiation among populations, or both may be the cause of low levels of heterozygotes observed in two wild rice species. Effective population sizes of *O. officinalis* may be smaller than those of other outcrossing plants such as *O. rufipogon* because of highly fragmented habitats, very limited gene flow and strong colonization (Gao et al. 1996, 1998, 2000b). As mentioned above, most Chinese populations of *O. officinalis* grow in humid habitats beside streams along mountain valleys. Water flow may act as the most possible dispersal agent of seeds. Gene flow occurs along the same stream, but the populations along different streams are relatively isolated. Additionally, although Chinese *O. officinalis* once had large populations in size (Wu 1981; Liang and Wu 1993), most surviving ones have become fairly small and seriously isolated among each other due to recent extinction events (Gao et al. 1996; Gao unpublished data). Therefore, small isolated populations in separate streams may also reduce chances of migration among populations. Even without any epistatic selection,

small effective population sizes would result in strong linkage disequilibrium in *O. officinalis* (Hill and Robertson 1968; Ohta and Kimura 1969).

Implications for conservation

An important objective of conservation genetics is to estimate levels and apportionment of genetic variation in endangered species (Fritsch and Rieseberg 1996; Cardoso et al. 1998). Accurate estimates of genetic diversity are useful for optimizing sampling strategies driven at the conservation and management of genetic resources (Hamrick et al. 1991; Schaal et al. 1991; Chalmers et al. 1992; Cardoso et al. 1998). In the present study, microsatellite markers revealed differences of population genetic structure of *O. rufipogon* and *O. officinalis* and thus provide useful information for their genetic management.

The primary concerns of the conservation programs for endangered plant species have been allelic diversity, heterozygosity and inbreeding in order to insure their long-term survival and maintain their ecological and evolutionary processes. The observation that up to 71.1% of microsatellite variation was partitioned within populations of *O. rufipogon* is instructive for adopting a plan of involving fewer populations but more individuals within populations. Large remnant populations with high levels of genetic variation, such as GZGD from South China, should be more attractive for both *in situ* conservation and *ex situ* germplasm expeditions. However, the result that 44.6% of genetic diversity was resided within populations of *O. officinalis*, suggests that both the number of populations and the individuals for a sampled population should be almost equally considered for taking a proper conservation strategy in the species. In order to capture the utmost allelic variation harbored among populations, an appropriate strategy of sampling those populations with higher variation from the different geographic regions and/or valleys should be considered for *O. officinalis*.

High degree of inbreeding in the populations of both species implies that conservation and restoration genetics should particularly focus on the maintenance of historically significant processes such as high levels of outbreeding, gene flow and large effective population sizes in these two wild rice species. Considering the fact that *O. officinalis* has a high extent of inbreeding because of severely isolated habitats, ways should be sought to strengthen gene flow by transplanting among different small fragmented populations and thus increasing their effective sizes.

For the restoration conservation of both species, genetically diverse and large populations would be appropriate to select as donor ones in order to maximize genetic diversity within reintroduced populations. In *O. rufipogon*, however, the genetic diversity within small marginal populations should not be diffused with larger populations by means of increasing their own sizes and thus allele frequencies. Promoting interchange with larger populations will make these small precious populations themselves ultimately lost.

Conservation concerns of gene flow from cultivated rice are particularly critical in *O. rufipogon*. As suggested above, rice gene flow may enhance to maintain high

levels of intra-population genetic diversity and decrease genetic differentiation among populations. On the other hand, however, frequent introgression will inevitably lead to conservation genetic problems such as 'genetic assimilation', as observed and reported in many other crop wild relatives (e.g., Colwell et al. 1985; Snow and Palma 1997; Ellstrand et al., 1999). The extinction of Taoyuan Population of *O. rufipogon* in Taiwan, China, might be due to long-term gene flow from the surrounding rice fields (Kiang et al. 1979; Oka and Chang 1961). Our ongoing gene flow experiments and population genetic analysis could enhance to understand the role of introgression for conserving genetic diversity of *O. rufipogon*.

Further detailed analysis of mating system patterns in *O. rufipogon* and *O. officinalis*, such as an assessment of paternity to individual plants will be required. The information could help to determine outcrossing, inbreeding and outbreeding depression, and the importance of genetic system adaptation in resisting the loss of genetic resources due to small population size. Understanding this ecological and evolutionary process may thus be important for gaining new insights into and opportunities for the study and genetic management of endangered wild rice species.

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