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Genetic Diversity and Population Differentiation of Liaoning Weedy Rice Detected by RAPD and SSR Markers

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Weedy rice refers to populations of usually annual Oryza *species that diminish farmer income through reduction of grain yield and lowered commodity value at harvest. The genetic diversity and population genetic structure of weedy rice in Liaoning Province were studied by RAPD and SSR markers. The results indicate that the level of genetic diversity of Liaoning weedy rice is very low, with polymorphic loci being only 3.70% (RAPDs) and 47.62% (SSRs). On the other hand, high genetic differentiation was found among populations, in particular between two regions (Shenyang and Dandong), with* Fst *values of 0.746 (RAPDs) and 0.656 (SSRs), suggesting that more than two thirds of the genetic variation resides among regions. Combined with our investigations of cultural traditions, the low level of genetic diversity in Liaoning Province is attributed to its narrow genetic background enhanced by exchanges of cultivar seeds, whereas the high genetic differentiation between the two regions is most likely the result of different founding parents and gene flow from local rice varieties to weedy rice. The rice cultivars in the two regions are all local varieties and are different genetically. A comparison of the two marker systems demonstrates that SSR is more informative*

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and powerful in terms of the assessment of genetic variability, although both RAPD and SSR provide useful genetic information on weedy rice.

KEY WORDS: genetic diversity; RAPD; SSR; weedy rice.

INTRODUCTION

Weedy rice, which is also called red rice in America, occurs in temperate regions, especially where direct seeding and intensive production prevail (Bres-Patry *et al*., 2001). Weedy rice shows weedy traits such as phenotypic plasticity, high seed dispersal ability, and seed dormancy (Federici *et al*., 2001). Seeds of weedy rice usually have a red pericarp (thus the common name, "red rice"), display earlier tillering and flowering than cultivated rice, and show anthocyanin pigmentation of different plant parts, such as the collar, ligule, grain apiculus, stigma, and awns (Cho *et al*., 1995; Suh *et al*., 1997). The term "weedy rice" refers to populations of usually annual *Oryza* species that diminish the farmer's income both quantitatively through reduction of grain yield and qualitatively through lowered commodity value at harvest. Weedy rice occurs in all the major rice growing areas in the tropics, being a particular problem in the direct-seeded rice agriculture of Latin and North America, the Caribbean, Africa, South and Southeast Asia (Oka, 1988).

Studies on genetic diversity of weedy rice have been reported. Federici *et al*. (2001) investigated Uruguayan weedy rice using AFLP markers and found that weedy rice adapts either to the natural environment or to cultivation. Several studies, based on morphological and physiological traits, isozymes, RFLP, and RAPD markers, indicate that weedy rice strains appear to be differentiated into *indica* and *japonica* types (Cho *et al*., 1995; Suh *et al*., 1997). A recent study using SSRs shows that some weedy rice is closely related to *O*. *sativa* while others are related to *O*. *rufipogon* (Vaughan *et al*., 2001). Thus, as pointed out by Watanabe *et al*. (2000), different rice-growing locations often show different patterns of genetic diversity, depending on the specific combination of germplasm from which weedy rice emerges.

Liaoning Province is one of the main regions for rice production in China. Recently, however, weedy rice is becoming a more serious problem in rice fields in Liaoning where direct seeding and simple transplanting technologies are used, which not only diminishes yield production but also the commodity value of rice. To date, almost nothing is known about the origin and genetic background of weedy rice in Liaoning. Random amplified polymorphic DNA (RAPD; Williams *et al*., 1990) and microsatellites (SSR; Tautz, 1989) are two widely used markers for estimating genetic diversity in many species, including wild, weedy, and cultivated rice (Powell *et al*., 1996; Suh *et al*., 1997; Ge *et al*., 1999; Watanabe *et al*., 2000; Vaughan *et al*., 2001). RAPD has advantages, such as a relatively unbiased portion of the genome sampled, simplicity of use, and lower cost, while SSR samples

abundant, codominant markers and has a high power of genetic resolution. In the present paper, we have used both markers to investigate the level and pattern of genetic diversity of Liaoning weedy rice. Such information may contribute to a better understanding of the origin of weedy rice and may help to develop recommendations for its control in rice fields. In addition, we are also interested in comparing and evaluating the utility of the RAPD and SSR techniques in terms of the study of population genetics of weedy rice.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

Leaves of 42 individuals representing seven populations were collected from the rice fields of Liaoning Province and dried by silica gel. These populations were grouped into two regions, Shenyang and Dandong. Four populations (F01, F02, F03, F04) were collected from the rice fields of Shenyang, where the cultivated rice varieties are Liaojing 294 and Liaojing 454; three populations (F05, F06, F07) were collected from Dandong, where the cultivated rice varieties were Danjing 9 and Liaojin 151. All these rice varieties belong to the subspecies Japonica (*O. sativa* ssp. *japonica*). Nearly uniform morphology of weedy rice was observed in each rice field (population), thus six individuals separated by a distance of more than 10 m were sampled for each population. Locations of the seven populations are shown in Fig. 1. DNA was extracted as described by Ge *et al*. (1999) from silica-gel-dried leaves. The concentration of DNA was quantified using 1.0% agarose gel (containing EB).

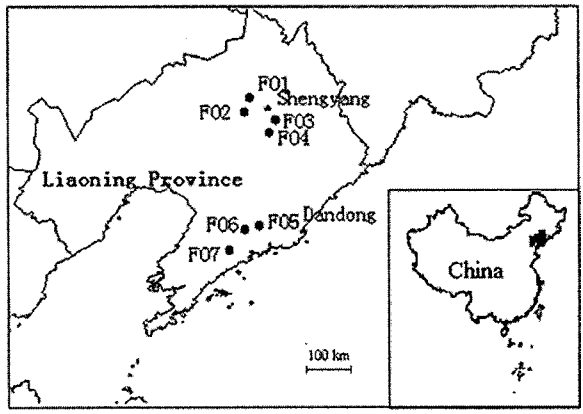

Fig. 1. Distribution of weedy rice populations sampled in the present study. Inset shows the location of Liaoning Province.

RAPD and SSR Assays

Forty 10-mer RAPD primers from Shengong Inc. were evaluated, and 20 (S4, S60, S87, S91, S99, S179, S226, S237, S246, S291, S300, S303, S337, S338, S361, S369, S370, S375, S462, S501) were selected for final study because of their strong and reproducible amplification. RAPDs were performed in capillaries of 10 μL total volume containing about 10 ng template, 1 μM of each primer, 50 mM Tris-HCl (pH 8.3), 5 μg BSA, 2 mM $MgCl_2$, 0.5 U *Taq* polymerase, 0.2 mM of each dNTP, 1 mM Tartrazine, and 10% Ficoll. Reactions were performed on a Biometra-2000 thermocycler programmed to include two cycles each at 94°C for 1 min, 37°C for 10 s, and 72°C for 20 s, and 40 cycles each at 94°C for 2 s, 37°C for 10 s, and 72°C for 70 s. A final extension step at 72°C for 5 min was performed after the 40 cycles. The PCR products were electrophoresed on 1.5% agarose gels (containing EB) for about 180 min at 100 V. The gels were imaged on Bio-Rad imaging devices (Gel Doc 2000 Gel Documentation System) supported by Quality One (Version 4.2).

Fifty-five SSR primers developed in *Oryza sativa* were used for primer screening for their utility in studying weedy rice, including 48 RM, 6 OSR, and one OS primer pairs (Davierwala *et al*., 2001; Zhou *et al*., 2003). Twenty-one of them (OSIA6, OSR2, OSR16, OSR20, OSR22, OSR32, OSR34, RM3, RM4A, RM13, RM22, RM26, RM29, RM212, RM215, RM228, RM230, RM238B, RM249, RM253, RM263) were selected for final study because of their good amplification. The SSR reactions were performed in a volume of 25 μL, and each assay contained about 50 ng of template DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 3 U *Taq* polymerase. PCR was performed in a Biometra-2000 thermocycler and included a 5 min predenature at 94°C, 35 cycles of 60 s denaturation at 94°C, 50 s annealing at 55°C, and 60 s elongation at 72°C, followed by a final 5 min elongation step at 72°C. The PCR products were electrophoresed on 6% polyacrylamide denaturing gels for about 120 min at 1500 V and silver stained as described in the manufacturer's instructions (Promega, USA).

Data Analysis

Fragments amplified by RAPDs were scored for the presence (1) or absence (0) of homologous bands, and bands of SSRs were entered in the form of single-individual genotypes. These two matrices were then used for the following statistical analyses. Genetic diversity for both RAPDs and SSRs was measured by the mean number of alleles per locus (N_a), and the percentage of polymorphic loci (P). In addition, the mean expected heterozygosity (H_e) and observed heterozygosity (H_o) were calculated for SSR markers. These parameters were computed using the POPgene program (Yeh *et al*., 1999).

In order to assess the overall distribution of genetic diversity, *Fst* values (Nei, 1978) were calculated by POPgene for both RAPD and SSR datasets. Also, the nonparametric analysis of molecular variance (AMOVA) calculated by the WinAmova program (Excoffier, 1993) and the θ value (Weir and Cockerham, 1984) calculated by the FStat program (Goudet, 2001) were used to describe population structure for RAPDs and SSRs, respectively. In addition, population relationships were inferred using the UPGMA clustering method on the basis of Nei's (1978) unbiased genetic distance with POPgene. The tree was subsequently visualized with Treeview (Page, 2001).

RESULTS AND DISCUSSION

Genetic Diversity

Twenty-one RAPD primers that were employed in the study yielded a total of 108 bands, but only four generated by primer S303 showed polymorphism. Of the seven weedy rice populations studied, two (F05, F07) had polymorphic loci, with the mean number of alleles being a little more than 1, while the other five populations shared monomorphic loci. Overall, the percentage of polymorphic loci (3.70%) was very low (Table I). Of the 20 pairs of SSR primers used in the study, 11 showed polymorphism. Of them, 7 primers (OSIA6, OSR20, OSR22, RM13, RM215, RM249, OSR2) possessed two alleles, while each of the primers OSR32, OSR34, and RM228 had three alleles. Genetic parameters for the seven

Table I. Genetic Characteristics of Seven Liaoning Weedy Rice Populations Based on SSR and RAPD Data

	RAPD		SSR			
Name of population	*Na*	*P* (%)	*Na*	*P* (%)	*Ho*	*He*
F01	1.000	0.000	1.240	23.810	0.008	0.087
F02	1.000	0.000	1.290	28.570	0.016	0.098
F03	1.000	0.000	1.100	9.520	0.016	0.027
F04	1.000	0.000	1.100	9.530	0.032	0.025
Mean for Shengyang	1.000	0.000	1.180	17.860	0.018	0.059
Shenyang	1.000	0.000	1.380	38.100	0.018	0.080
F05	1.010	0.930	1.100	9.520	0.079	0.046
F06	1.000	0.000	1.140	14.290	0.071	0.052
F07	1.010	0.930	1.100	9.520	0.060	0.034
Mean for Dandong	1.010	0.620	1.110	11.110	0.070	0.044
Dandong	1.020	1.850	1.140	14.290	0.071	0.050
Total mean	1.003	0.266	1.153	14.966	0.040	0.053
Liaoning	1.037	3.700	1.620	47.620	0.040	0.053

Note. *Na*, mean number of alleles per locus; *P*, percentage of polymorphic loci; *Ho*, observed heterozygosity; *He*, expected heterozygosity.

populations are given in Table I. The mean number of alleles is less than 2 in all the populations. H_o ranged from 0.008 (F01) to 0.079 (F05) and H_e from 0.027 (F03) to 0.087 (F01). Population F04 exhibited the lowest level of polymorphism (P = 9.52, $H_e = 0.025$), whereas population F02 exhibited the highest ($P = 28.57$, $H_e = 0.098$). Higher diversity was found in Shenyang than in Dandong at both regional (P: 38.1 vs. 14.29; H_e: 0.080 vs. 0.050) and population (P: 17.86 vs. 11.11; H_e: 0.059 vs. 0.044) levels.

To date, RAPD markers have been widely used in genetic diversity screening in many crop species, and moderate to high diversity was identified in rice and its close relatives such as rice cultivars ($P = 80\%$, Yu and Nguyen, 1994; Davierwala *et al*., 2000), *O. rufipogon* ($P = 82.1\%$, Ge *et al*., 1999). Even in *O. granulata*, which has been considered a species with the lowest genetic variation in wild rice, 30.7% of bands (8.2% at the population level) were found to be polymorphic (Qian *et al*., 2001). Based on RAPD polymorphism, Watanabe *et al*. (2000) analyzed weedy rice from three different regions in Malaysia and one in Surinam and found that 90% of the 87 samples from Tanjung Karang and MUDA of Malaysia and 68% of 60 samples from Surinam could be uniquely identified by their DNA polymorphism patterns, with 23, 15, and 21 polymorphic bands identified for the three regions, respectively. In comparison, our 20 primers detected only 4 polymorphic bands ($P = 3.7\%$), and out of 42 samples only 2 (4.8%) samples were uniquely identified, indicating unusually low genetic diversity of weedy rice in the two regions of Liaoning Province.

Basically, SSR markers detect more diversity than RAPDs (see reviews in Zhou *et al*., 2003) and thus reveal high diversity in both the cultivated rice ($P = 98.2\%$, $H_e = 0.882$, Davierwala *et al*., 2000) and its wild relatives ($P = 100\%$, $H_e = 0.787$ for *O. rufipogon*, Zhou *et al*., 2003). Our present survey also found significantly low genetic variation, with P of 47.6% and H_e of 0.053 (Table I). Therefore, the present study indicates that Liaoning weedy rice has a very low level of genetic variation compared to the previous results based on morphological, physiological, and molecular surveys (Cho *et al*., 1995; Suh *et al*., 1997; Vaughan *et al*., 2001; Federici *et al*., 2001), implying a narrow genetic base for the origin of weedy rice in Liaoning Province.

Oka (1988) suggested that weedy rice can be classified into two categories, one occurring together with common wild rice and the other distributed in the region where no wild rice occurs. Our case obviously belongs to the latter, because the northernmost population of common wild rice (*O. rufipogon*) in the world is in Jiangxi Province of China, which is far from the regions involved in the present study (about 1300 km). Our investigation showed that weedy rice in Liaoning Province became a serious problem in conjunction with the direct seeding and simple transplanting used in recent years. Therefore, it is most likely that weedy rice was introduced from other places by being sown together with the cultivar seeds, because seed dispersal is one of its main reproductive methods (Oka,

Table II. Population Genetic Structure of Liaoning Weedy Rice

Source of variance	RAPD		SSR	
	Fst	AMOVA (%)	Fst	θ
Shengyang vs. Dandong	0.746	95.490***	0.656	0.787
Individual/regions	0.254	4.510***	0.344	0.213
Among populations	0.885	92.310***	0.72	0.698
Individual/populations	0.115	7.690***	0.28	0.302

*** $P < 0.001$.

1988), and exchanges of cultivar seeds are common practice in Liaoning Province (personal observation). In addition, the limited sampling area may also contribute to the low genetic diversity detected in this study. A more comprehensive study of weedy rice in China is currently under way, which may contribute to a better understanding of the origin of weedy rice both in Liaoning Province and in China as a whole.

Population Differentiation

The *Fst* values measured by RAPDs and SSRs were 0.885 and 0.720, respectively (Table II), suggesting that a large proportion of genetic variation resided among populations within a region. The AMOVA partition based on RAPDs shows that the population differentiation is highly significant ($P < 0.001$) with 92.31% of genetic variation residing among populations. The θ statistic based on SSRs also shows the similar trends (0.698) with almost 70% genetic variation residing among populations (Table II). It is noteworthy that the differentiation between two regions was very high (RAPD: Fst = 0.746, AMOVA = 95.49%; SSR: Fst = 0.656, θ = 0.787), implying that the population differentiation actually originated from the genetic variation between two regions (Table II). The test of pairwise population differentiation based on SSRs showed that the four pairs of populations with significant differentiation ($P < 0.01$) were exclusively from those between the two regions (Table III). The dendrogram produced by the UPGMA method also shows two major clusters, and the populations from the same region are clustered together (Fig. 2).

Both SSRs and RAPDs reveal that the differentiation between Shenyang and Dandong populations is high, although the level of genetic diversity of weedy rice in Liaoning Province is very low in general. This phenomenon could be explained by an isolation-by-distance model (Zhou *et al*., 2003). It is most unlikely that gene flow of weedy rice takes place between Shenyang and Dandong since the two regions are separated by about 300 km (Fig. 1). Shenyang and Dandong are the two main rice-growing areas in northeastern China. Our investigation of the

Table III. Pairwise Population Differentiation (Measured by θ).

Name of population	F01	F02	F03	F04	F05	F06
F02	0.004					
F03	0.354	0.080				
F04	0.425	0.176	0.029			
F05	0.774	0.740*	0.878	0.886*		
F06	0.751	0.713	0.859	0.869*	0.071	
F07	0.706	0.672	0.850*	0.863	0.240	0.140

Note. Lower triangle matrix of θ calculated for all population pairwise comparisons, tested by permutation of genotypes among populations (1000 randomizations). Significances were corrected by a sequential Bonferroni technique. $^*P < 0.01$.

cultural traditions indicated that the rice cultivars in the two regions are all local varieties and thus are unable to be cultivated outside the region. Consequently, the rice varieties cultivated in Shenyang, such as Liaojing 294 and Liaojing 454, are different from those cultivated in Dandong, such as Danjing 9 and Liaojin 151. As indicated by previous studies, gene flow from cultivated rice to weedy rice frequently occurs (Chen *et al*., 2001). Therefore, another potential cause of high genetic differentiation between Shenyang and Dandong is that weedy rice in the two regions gained unique alleles from the local rice varieties through gene flow.

Comparison of SSR and RAPD Marker Systems

The present study demonstrates that both RAPD and SSR methods obtain concordant results that genetic variation of Liaoning weedy rice is low and differentiation between Shenyang and Dandong is high. However, polymorphism obtained using RAPDs is far lower than that obtained using SSRs (3.70% vs. 47.62%) (Table I),

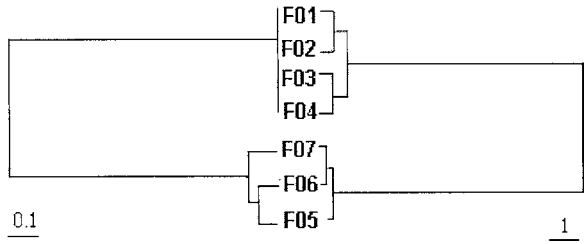

Fig. 2. Dendrograms of seven Liaoning weedy rice populations, constructed using UPGMA based on Nei's (1978) unbiased genetic distance by RAPD data (*left*) and SSR data (*right*).

although almost the same number of RAPD and SSR primers (21 vs. 20) was used in this study. Therefore, SSR is more informative and powerful in terms of the assessment of genetic variability, although both RAPD and SSR methods provide useful genetic information on weedy rice. As pointed out by Milbourne *et al*. (1997), SSR polymorphism is due to a particular mechanism, namely slippage. This mechanism occurs more frequently than point mutation or insertion-deletion events that are responsible for the polymorphism of RAPDs. This explains why a higher level of polymorphism associated with SSR was evident in many other studies related to a variety of plant species (Davierwala *et al*., 2000; Palombi and Damiano, 2002; Belaj *et al*., 2003; Zhou *et al*., 2003). In essence, SSRs offer a better marker system with more powerful discrimination capacity than RAPDs, especially in plants where the level of diversity is not high.

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