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RAPD variation within and between natural populations of the wild rice *Oryza rufipogon* from China and Brazil

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Genetic variation within and between eight natural populations of *Oryza rufipogon* from China and Brazil was investigated at the DNA level by analysis of RAPD fragments. Out of 60 random primers, which were initially screened against DNA from four individuals, 20 generated highly reproducible RAPD fragments which were then used for further population analysis. With these primers, 95 discernible DNA fragments were produced and 78 (82.1%) were polymorphic, which indicated that high levels of genetic variation existed in these natural populations. In addition, the Chinese populations showed greater polymorphism than those from Brazil at both the population and regional levels. This is noteworthy considering that the Chinese populations are from a relatively restricted area of China. The factors responsible for these findings include the contrasting mating systems in the Brazilian and Chinese populations, and gene flow from annual cultivated rice to perennial natural populations in China. An Analysis of Molecular Variance (AMOVA) was used to apportion the variation between individuals within populations, between populations within regions, and between regions. Results showed that 61.8% of the total genetic diversity resided between the two continents, whereas only 14.9% and 23.3% was attributable to population differences within regions and to individual differences within a population, respectively. The great genetic differentiation between the Chinese and Brazilian populations is in agreement with recent treatment of the American form of *O. rufipogon* as a separate species, *O. glumaepatula*.

Keywords: genetic variation, *Oryza glumaepatula, Oryza rufipogon*, RAPDs.

Introduction

The common wild rice, *Oryza rufipogon* Griff., is the ancestor of cultivated rice, *O. sativa* L., which constitutes an important part of the diet of more than half of the world's population. As indicated by many workers (Morishima *et al*., 1984; Second, 1985; Barbier, 1989; Vaughan, 1989, 1994; Ishii *et al*., 1996), *O. rufipogon* is distributed throughout the tropical and subtropical regions of the world, and is divided into Asian, American, African and Oceanian forms as well as perennial, annual and intermediate types. To date, most studies have concentrated on cultivated rice, assessing genetic variation, varietal classification, germplasm identification and domestication processes (Chang, 1976; Second, 1982, 1985; Glaszmann, 1988; Oka, 1988; Wang & Tanksley, 1989; Yu & Nguyen, 1994; Virk *et al*., 1995; Ishii *et al*., 1996). In contrast, the genetic variability and population genetic structure of natural populations of wild rice are less well known (Morishima *et al*., 1984; Barbier, 1989; Akimoto *et al*., 1994; Gao, 1997; Buso *et al*., 1998). Because of the great geographical range of *O. rufipogon*, its ecological and morphological diversity and the frequent introgression between cultivated and wild species, there has been much controversy on the origin of cultivated rice and the taxonomic classification of cultivated and wild species (Tateoka, 1962; Second, 1982, 1985; Oka, 1988; Vaughan, 1989; Ishii *et al*., 1996). Historically, the American, African and Oceanian forms of *O. rufipogon* were treated as *O. glumaepatula*, *O. longistaminata* and *O. meridionalis*, respectively (Vaughan, 1989). Although the species status of *O. longistaminata* and *O. meridionalis* is generally accepted, the taxonomic status of the

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American form of *O. rufipogon* has been the subject of contention in recent years (Vaughan, 1994; Morishima & Martins, 1994; Ishii *et al*., 1996; Juliano *et al*., 1997).

The assessment of genetic variability in natural populations can provide new insights into the evolutionary history and phylogenetic relationships of *Oryza* species, and address issues of cultivar classification and domestication of crop plants (Second, 1985; Vaughan, 1989). In addition, the development of appropriate strategies for the conservation and exploitation of plant genetic resources requires a detailed knowledge of the amount and apportionment of genetic variation within the species (Vaughan, 1994). The RAPD technique has several advantages over isozyme and other DNA marker methodologies, such as speed, low cost, and the use of small amounts of plant material (Huff *et al*., 1993; Heun *et al*., 1994). In recent years, RAPD analysis has become a popular method for estimating genetic diversity and relatedness in plant populations, cultivars and germplasm accessions (Huff *et al*., 1993; Yu & Nguyen, 1994; Virk *et al*., 1995; Ishii *et al*., 1996; Stewart *et al*., 1996; Martin *et al*., 1997; Buso *et al*., 1998). In the present study, we have used RAPD markers to determine the pattern and extent of genetic variation within and between natural populations of *O. rufipogon* from China and Brazil. Moreover, it is of great interest to determine the degree of genetic differentiation between populations of *O. rufipogon* from China (the Asian form) and from Brazil (the American form), because the taxonomic status of the American forms of *O. rufipogon* is unclear.

Materials and methods

Population sampling

During 1992–95, several extensive collecting trips for wild rice species were conducted in China and Brazil. The seed samples used in this study were obtained during those trips, representing eight natural populations of *O. rufipogon* in China and Brazil. The four Chinese populations, located ≈25–65 km apart, were sampled from Guangxi Autonomous Region of southern China, a region of high genetic diversity for *O. rufipogon* in China (Gao, 1997). The remaining four populations, located ≈100–1100 km apart, were sampled from the flood plains adjacent to the Amazon and Solimoes rivers of Brazil, where the highest population densities of natural *O. rufipogon* are found (Oliveira, 1993). The sampling localities and sizes (in parentheses for the eight natural populations) are: B01, Periquitinho Bay, Pará State, Brazil (10); B02, Ressaca de Sao Tomé, Amazonas State, Brazil (10); B03 Cuiucuiu Lake, Amazonas State, Brazil (11); B04, Mamiá Lake, Amazonas State, Brazil (9); P01, Yuling City, Guangxi, China (10); P02, Guixian County, Guangxi, China (9); P03, Guipin County, Guangxi, China (10); P04, Rongxian County, Guangxi, China (11). Each population was derived from half-sib seeds collected individually from over 20 plants, separated by distances of more than 5 m. After one week of heat shock at 50–55°C, seeds were grown and maintained under conditions of 28°C day/25°C night in the greenhouse of the Biology Department, Washington University. When the seedlings were two months old, one was randomly chosen from the half-sib offspring of each plant. As a result, 9–11 seedlings representing 9–11 plants of each population were used for the RAPD survey in the present work.

DNA isolation

Leaves were harvested, dried with silica gel, and then stored in paper bags until DNA isolation. Genomic DNA was extracted using a modification of the protocol of Doyle & Doyle (1987). Dried leaf materials were ground to fine powder in a 1.5-mL Eppendorf tube, and then mixed with 750 μL of preheated 2×CTAB extraction buffer containing 0.3% mercaptoethanol. The homogenate was incubated at 65°C for 30 min prior to adding 750 μL of chloroform:isoamyl alcohol (24 : 1, v/v). After mixing by inversion for 5 min the mixture was centrifuged at 9400 **g** for 5 min at room temperature, and the supernatant reserved and mixed with 2/3 vol. ice-cold isopropanol. The DNA was recovered as a pellet by centrifugation at 6000 **g** for 5 min, washed with 500 μL of 70% ethanol, dried, and dissolved in 100 μL of 1×TE buffer. DNA quality and quantity were determined in 1.5% agarose gels.

Polymerase chain reaction

A total of 60 decamer primers were used in the RAPD analysis. Primers were obtained either from the Oligonucleotide Synthesis Laboratory of the University of British Columbia (UBC 331~UBC 360) or from Operon Technologies Inc. (Kit A, OPA-10–20 and Kit B, OPB-1–20). DNA amplification was performed in a Hybaid OmniGene Thermal Cycler, and commenced with 2 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C. Reactions were carried out in a volume of 25 μL containing 10 mM Tris-HCl (pH 9.2), 25 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of random primer, 25 ng of genomic DNA and 1.25 units of *Taq* polymerase. RAPD fragments were separated electrophoretically in 1.4% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light using

Polaroid 667 film. Molecular weights were estimated using a 1 kb DNA ladder (Gibco-BRL).

Data analysis

RAPD bands were scored as present (1) or absent (0) for each DNA sample, and used to compute the measures of genetic distance for all pairs of individuals. In addition, genetic diversity was measured by the percentage of polymorphic bands (*PPB*), which was calculated by dividing the number of polymorphic bands at population, region or species levels, by the total number of bands surveyed. In order to describe population structure and variability among populations, the nonparametric Analysis of Molecular Variance (AMOVA) procedure was used as described in Excoffier *et al.* (1992), where the variation was partitioned among individuals within populations, among populations within regions, and between regions (China and Brazil). All the analyses were performed with the RAPDISTANCE program version 1.04 (Armstrong *et al.*, 1994) and WINAMOVA program version 1.5 (Excoffier, 1993). We also conducted an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) to produce a dendrogram as a visual aid.

Results

Optimization of RAPD protocol

Because the RAPD PCR technology is sensitive to changes in experimental parameters, a total of 60 primers were initially screened against four plants selected from two Chinese and two Brazilian populations. The effects of magnesium and template DNA concentrations, pH values, and duration of time during the denaturation stage of the amplification were examined. Under the optimized conditions described in the Materials and methods, 43 (71.1%) primers out of 60 generated RAPD fragments, with typically one to 10 major bands amplified along with a number of bands of lesser intensity. From the initial 60 primers, a subset of 20 primers (UBC 333, 335, 340, 341, 345, 349, 350, 354, 356, 358, 359; OPA-10, -11, -12, -18; OPB-05, -07, -10, -11, -17) was selected for further analyses based on the following criteria: (i) consistent production of strong amplification products; (ii) production of uniform, reproducible fragments between replicate PCRs; and (iii) lack of sensitivity to DNA template concentrations varying from 8 to 15 ng/μL.

RAPD polymorphism

A total of 95 bands ranging in size from 300 to 2800 bp was scored, corresponding to an average of 3.9 bands per primer, and 82.1% (78 in total) of these were polymorphic among 80 plants. Each plant within the eight populations had a unique genotype, except for two individuals from the Brazilian population (B04), which had identical genotypes. An example of the polymorphism detected with primers OPA-10, OPB-11 and UBC 341 is shown in Fig. 1. A data matrix is available from the first author upon request.

Percentages of polymorphic bands (*PPB*) for each population and each region are shown in Table 1. Population P04 exhibits the greatest level of variability ($PPB = 43.2$), whereas population B04 exhibits the lowest level of variability ($PPB = 6.3$) with only four primers (UBC 335, UBC 356, OPA-10 and OPB-11) detecting six polymorphic amplification bands. It is evident that the Chinese populations show a higher variability ($PPB = 55.8$) than those populations from Brazil ($PPB = 41.1$), and the within-population variation is significantly greater in China ($PPB = 33.7$–43.2) than in Brazil ($PPB = 6.3$–27.4). An example of comparative polymorphism between the Chinese and Brazilian populations is shown in Fig. 1(c). Overall, eight bands showed fixed differences between China and Brazil; primer OPB-11 did not yield any shared band between the regions (Fig. 1b).

The genetic structure of populations

To assess the overall distribution of diversity within and between populations, an AMOVA was performed from the distance matrix. AMOVA showed highly significant ($P < 0.001$) genetic differences between Chinese and Brazilian regional collections, as well as among populations within either region (Table 2). In addition, substantial variation was observed in each of the eight populations, which is consistent with the results above. Of the total genetic diversity, 61.8% is attributable to regional divergences, 14.9% to population differences within regions, and 23.3% to differences between individuals within a population. For the within-region analyses, most of the genetic variation (71.3%) resided between individuals within a population for the Chinese collections, whereas less than half of the genetic variation (47.3%) was attributable to differences between individuals within a population for the Brazilian collections.

In order to represent the relationships among populations and regions, a cluster analysis (UPGMA) was used to generate a dendrogram based on pairwise Φ_{ST} distances between populations (Fig. 2). The Chinese and Brazilian populations are clearly separated into two main clusters, which corroborates the AMOVA by indicating considerable genetic differentiation between the two regions.

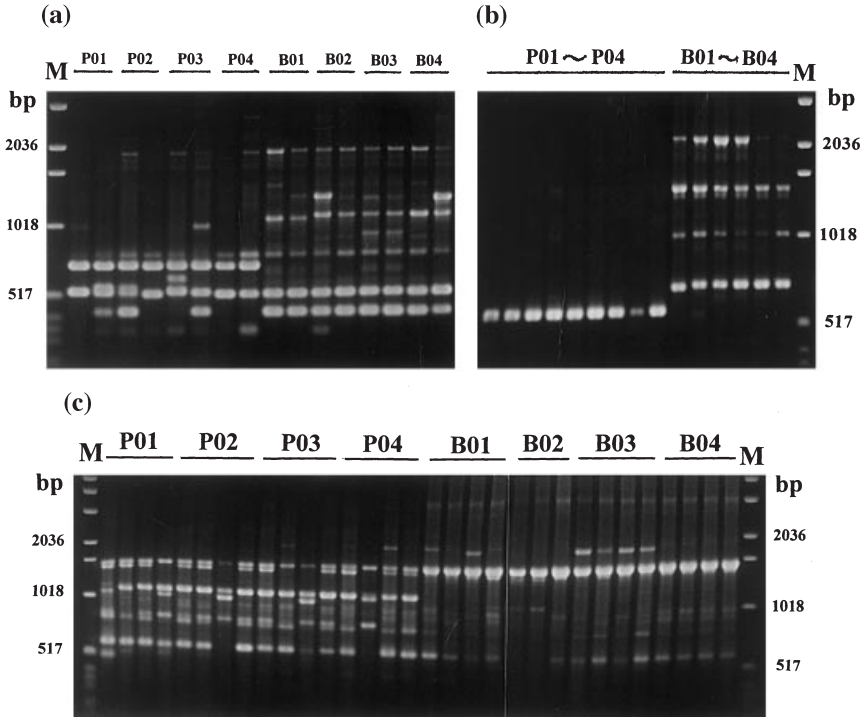

Fig. 1 RAPD amplification products generated from *Oryza rufipogon* genomic DNA: (a) from two individuals of each of eight populations obtained with primer OPA-10; (b) from nine individuals of Chinese populations and six of Brazilian populations obtained with primer OPB-11, indicating no band shared; (c) from three or four individuals of eight populations obtained with primer UBC 341. M, 1 kb ladder.

Discussion

Genetic variation within and between populations

Our RAPD survey of eight natural populations of *O. rufipogon* from China and Brazil indicates a high level of genetic variation with 82.1% of bands being polymorphic. Yu & Nguyen (1994) detected similar levels of variation from nine upland and four lowland rice cultivars (80% of polymorphism in a sample of 260 RAPD fragments). These studies indicate that RAPDs are sufficiently informative and powerful to assess genetic variability of both natural populations of *O. rufipogon* and derived rice cultivars. Thus RAPD markers will provide a useful tool in the future design of collection strategies for germplasm conservation.

The levels and distribution of genetic diversity detected by RAPDs here are in overall agreement with recent allozyme and RAPD studies on the South American form of *O. rufipogon* (*O. glumaepatula*). Akimoto *et al.* (1994), for example, studied 37 natural populations from central Amazon and found significantly lower average gene diversity within populations compared with that of 29 populations from Asia (0.074 vs. 0.158). More recently, Buso *et al.* (1998) investigated four natural populations collected from the Amazon forest and western Brazil rivers using isozyme and RAPD markers, and revealed a pattern of greater variation between than within populations. By an AMOVA analysis of RAPD data, they showed that most of the genetic diversity resulted from differences between populations. In contrast, allozyme studies on natural populations of *O. rufipogon* throughout China have shown that most of the genetic diversity resided within populations (Gao, 1997). Likewise, in the current study, Chinese populations show higher polymorphism for RAPDs at both

Table 1 Percentage of polymorphic RAPD bands (*PPB*) in each *Oryza rufipogon* population and region

Population	No. of polymorphic bands†	*PPB*
P01	32	33.7
P02	29	30.5
P03	31	32.6
P04	41	43.2
China	53	55.8
B01	23	24.2
B02	26	27.4
B03	18	18.9
B04	6	6.3
Brazil	39	41.1
Total	78	82.1

†A total of 95 bands was scored in the present study.

Table 2 Analysis of molecular variance (AMOVA) for 80 individuals of *Oryza rufipogon*

Source of variation	d.f.	SSD	MSD	Variance component	Percentage total	*P*-value
China vs. Brazil	1	568.49	568.49	13.28	61.80	< 0.001
Populations/region	6	221.47	36.91	3.19	14.86	< 0.001
Individuals/population	72	361.20	5.02	5.02	23.34	< 0.001
Populations/China	3	101.76	33.92	2.72	28.75	< 0.001
Individuals/population	36	242.89	6.75	6.75	71.25	< 0.001
Populations/Brazil	3	119.71	39.90	3.67	52.74	< 0.001
Individuals/population	36	118.32	3.29	3.29	47.26	< 0.001

the population and regional levels but lower genetic variation between populations than do South American populations (Table 1, Fig. 2). The AMOVA analysis corroborated these findings with the within-population variance component being 6.75 and 3.29, and the percentage genetic diversity between populations being 28.75 and 52.74 in the Chinese and Brazilian populations, respectively (Table 2). Considering the fact that the Chinese populations are from a relatively restricted area of Guangxi Autonomous Region, it is noteworthy that higher genetic diversity existed in the Chinese populations than in the Brazilian ones, although lower genetic differentiation between the Chinese populations is expected.

Based on four enzyme systems and 20 RAPD primers, Buso *et al.* (1998) found high values of inbreeding coefficients and suggested that the natural populations of the South American form of *O. rufipogon* (*O. glumaepatula*) were typically autogamous (Buso *et al.*, 1998). In contrast, the natural populations of Chinese *O. rufipogon* were primarily outcrossing (Gao, 1997). Consequently, the higher genetic diversity of the Chinese populations may be explained by high levels of outcrossing rates. In addition, compared with the well-isolated populations of the Amazon Basin, the Chinese populations were obtained from an area where rice is widely cultivated. As pointed out by Morishima *et al.* (1984), the major direction of pollen flow in the *O. sativa* complex was from annual to perennial because the outcrossing rates were much higher in perennial than in annual types. Therefore, gene flow from cultivated rice (annual) to the natural populations of Chinese *O. rufipogon* (perennial) may be a factor that has led to higher genetic diversity within Chinese populations. Moreover, southern China (including the Guangxi Autonomous Region) is one of four centres of genetic diversity for Chinese cultivated rice, and the natural populations from Guangxi have the highest genetic diversity (Gao, 1997), which may also have contributed to the findings in the present study.

Relationship between the Asian and American forms of O. rufipogon

In the present study, the AMOVA partition indicates that 61.8% of genetic diversity is distributed between the populations of China and those of Brazil, whereas less than 40% of genetic diversity resides between populations within regions (14.9%) and between individuals within populations (23.3%). The genetic diversity between China and Brazil is greater than that seen between species of some other genera. Stewart *et al.* (1996) analysed the genetic structure of five populations representing two related species (*Iliamna corei* and *I. remota*) by the same AMOVA procedure and found only 24.2% of the total genetic variation between species. In a study of the grass *Buchloe dactyloides*, Huff *et al.* (1993) used an AMOVA, based on 98 polymorphic RAPD bands, to apportion the variation of four natural populations and found that 58.4% of the total genetic diversity was attributable to two regional ecotypes, and only 9.7% to population differences within regions, and 31.9% to individual differences within a population.

In the genus *Oryza*, the *O. sativa* complex consists of species with AA genomes, including two cultivated

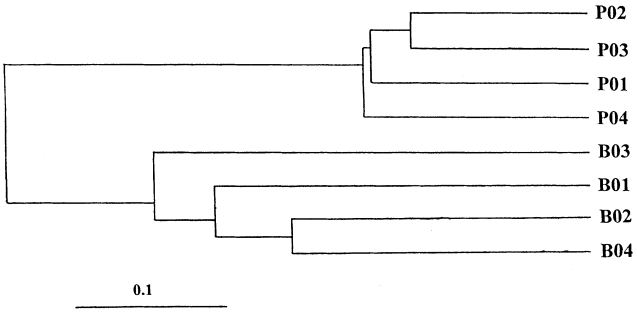

Fig. 2 Dendrogram of eight natural populations of *Oryza rufipogon* from China and Brazil, constructed using UPGMA based on differences at 78 polymorphic RAPD bands.

species (*O. sativa* and *O. glaberrima*), *O. barthii* and *O. rufipogon sensu lato*. Of four wild *Oryza* species in South America, the only diploid member with an AA genome was originally classified as *O. glumaepatula* by Steudel in 1854, and was considered an American form of *O. rufipogon* Griff. because of a lack of morphological distinction from *O. rufipogon* (Tateoka, 1962; Second, 1985; Vaughan, 1994). However, by an analysis of nuclear RFLPs, Wang *et al.* (1992) examined the phylogenetic relationships among *Oryza* species and found that the American form of *O. rufipogon* had slightly closer affinity with two African species (*O. barthii* and *O. glaberrima*) than with Asian *O. rufipogon*. Recently, Martin *et al.* (1997) used RAPDs to examine four wild species with AA genomes and found that four out of 22 accessions designated as *O. glumaepatula* had been misidentified. The remaining accessions were clearly clustered in a well-defined group, suggesting a clear distinction between the true *O. glumaepatula* and the other A genome species. Similarly, an extensive morphological study of Asian and American forms of *O. rufipogon* revealed distinct morphological variation in the accessions from South America, particularly the lower Amazon River basin (Juliano *et al.*, 1997). These studies and the recent finding that the American form of *O. rufipogon* (*O. glumaepatula*) is autogamous (Buso *et al.*, 1998) suggest a separate taxonomic status of *O. glumaepatula* from *O. rufipogon*. In this study, it is evident from AMOVA partitioning and cluster analysis that large genetic differences occur between Chinese and Brazilian populations, which is in agreement with treating the American forms of *O. rufipogon* as a separate species, *O. glumaepatula*. Nevertheless, the phylogenetic relationships between the Asian and South American taxa remain unclear, and further study encompassing the other A genome species is needed in order to understand the evolutionary relationships among taxa.

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