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Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers

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Abstract Genetic variation within and between five populations of *Oryza granulata* from two regions of China was investigated using RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat amplification) markers. Twenty RAPD primers used in this study amplified 199 reproducible bands with 61 (30.65%) polymorphic; and 12 ISSR primers amplified 113 bands with 52 (46.02%) polymorphic. Both RAPD and ISSR analyses revealed a low level of genetic diversity in wild populations of *O. granulata*. Furthermore, analysis of molecular variance (AMOVA) was used to apportion the variation within and between populations both within and between regions. As the RAPD markers revealed, 73.85% of the total genetic diversity resided between the two regions, whereas only 19.45% and 6.70% were present between populations within regions and within a population respectively. Similarly, it was shown by ISSR markers that a great amount of variation (49.26%) occurred between the two regions, with only 38.07% and 12.66% between populations within regions and within a population respectively. Both the results of a UPGMA cluster, based on Jaccard coefficients, and pairwise distance analysis agree with that of the AMOVA partition. This is the first report of the partitioning of genetic variability within and among populations of *O. granulata* at the DNA level, which is in general agreement with a recent study on the same species in China using allozyme analysis. Our results also indicated that the percentage of polymorphic bands (PPB) detected by ISSR is higher than that detected by RAPD. It seems that ISSR is superior to RAPD in terms of the polymorphism detected and the amplification reproducibility.

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Introduction

Over the long term, the ability of a species to respond adaptively to environmental changes depends on the level of genetic variability it contains (Ayala and Kiger 1984). As a process, genetic differentiation by natural selection to facilitate reproductive isolation involves the presupposition of the origin of geographic races, subspecies, and species (Stebbins 1999). A species without an appropriate amount of genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. Therefore, investigations of population genetic diversity and the structure of populations within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation (Schaal et al. 1991).

Oryza granulata Nees et Am. ex Watt. is one of the wild relatives of cultivated rice *Oryza sativa* L. (Vaughan 1994) which is the most important crop feeding more than half of the world's population. *O. granulata* grows in tropical and subtropical areas of South and Southeast Asia (Vaughan 1994); in China, it is distributed in the southern Yunnan Province and the southwestern Hainan Province. Within the rice genus, *O. granulata* belongs to the complex with a GG genome, and is considered as the most distinct species from those with an AA genome, occupying the most basal position in the phylogeny of the genus (Aggarwal et al. 1997; Ge et al. 1999b). During the last two decades, with the increase of human population and economic development in China, *O. granulata* has become a vulnerable species because of habitat degradation caused by human disturbance. According to our field survey from 1998 to 1999, about 12.9% of natural populations of the species have become extinct, and about 83.9% is on the verge of extinction (Qian et al., unpublished data). However, *O. granulata* is

much less well-studied compared with other wild rice species, mainly because it can not be easily crossed with cultivated rice. The genetic diversity and population genetic structure of *O. granulata* is largely unknown, which makes it difficult to conserve this important germplasm.

Gao et al. (1999) used allozymes to study the genetic variation of Chinese *O. granulata* and revealed that there was a very low level of genetic diversity within populations of this species. Meanwhile, the study also indicated that the resolving power of allozyme analyses in detecting the level of genetic diversity of the species was not adequate. In recent years, the technique of random amplified polymorphic DNA (Williams et al. 1990) has offered a promising new marker system for use in the detection of genetic diversity in population and conservation genetics (Smith and Wayne 1996; Cruzan 1998). The RAPD technique has several advantages such as a relatively unbiased portion of the genome sampled, simplicity of use, lower cost, and the use of a small amount of plant material (Fritsch and Rieseberg 1996). To-date, this technique has been successfully employed to determine genetic diversity in many species including rice (Yu and Nguyen 1994; Ge et al. 1999a), wheat (Fahima et al. 1999), barley (Bustos et al. 1998), etc. However, RAPD has several limitations including dominance, uncertain locus homology, and especially sensitivity to the reaction conditions. In order to solve some of these problems, a new technique, inter-simple sequence repeat amplification (ISSR), which involves PCR amplification of DNA using a single primer composed of a microsatellite sequence anchored at the 3' or 5' end by 2–4 arbitrary, often degenerate, nucleotides, could be used to assess genetic diversity. ISSR has been successfully employed to reveal genetic variation in dent corn and popcorn (Kantety et al. 1995), to characterize genome diversity (Yang et al. 1996), and to determine the origin of hybrids (Wolfe et al. 1998). As a dominant marker, ISSR targets simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and evolve rapidly. As a consequence, ISSR amplification reveals a much larger number of polymorphic fragments per primer than does RAPD. The technique also does not require prior knowledge of DNA sequence for primer design, and has advantages similar to those of RAPDs (Kantety et al. 1995; Yang et al. 1996; Fang and Roose 1997).

The general aim of the present study was to analyze the genetic polymorphism and population genetic structure of wild populations of *O. granulata* in China utilizing both RAPDs and ISSRs. Our particular interests were: (1) to investigate the genetic diversity within and between the natural populations of the species, which may provide important insights into the population genetics and facilitate the conservation management for this species, and (2) to compare the levels of genetic diversity detected by RAPD and ISSR, and to evaluate the utility of these two markers in population biology and conservation genetics.

Materials and Methods

Plant material

The plant materials used in this investigation consist of five populations throughout the natural distribution area of the species in China (Fig. 1). These populations could be grouped into two regions, i.e., Yunnan (M16, M22, M27) and Hainan (M5, M14, Table 1). In each of the five populations, a representative sample of 20 plants was taken. Because it is reported that *O. granulata* may have clonal growth (Academy of Agricultural Science of Yunnan provincial, unpublished data), samples were randomly collected at an interval of at least five m to prevent collecting ramets from a single genet. However, in small and endangered populations, such as populations M16 and M22 (Table 1), modules of the same genet were sampled owing to the limited number of individuals.

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 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.

Total DNA extraction

Total DNA of a single leaf was isolated according to the protocol of Doyle and Doyle (1987). About 0.1 g of dried leaf was ground in a mortar with nitrogen and incubated for 1 h at 65°C in 950 µl of CTAB isolation buffer, using the following concentration: 2% CTAB, 50 mM Tris-HCl, pH 8.0, 0.7 M NaCl, 10 mM EDTA, pH 8, 20 mM mercaptoethanal and 1.5% PVP. DNA was extracted with 950 µl of chloroform-isoamyl alcohol (CI, 24: 1). After

Table 1 Populations of *Oryza granulata* for RAPD and ISSR analysis

Population no.	Location		Enviroments	Population size	Samples size
M 16	Nanla Township, Cang yuan County	Yunnan	720 m alt. NW 20, gradient 15, under bamboo forest	60	20
M 22	Laban Township, Yingjiang County	Yunnan	430 m alt. SE 10, gradient 10, under a gap in a mountain rainforest	25	20
M 27	Zhulin Township, Simao City	Yunnan	780 m alt. SE 45, gradient 25, under a second successive forest	3000–5000	20
M 5	Chongpo Town, Ledong County	Hainan	50 m alt. S, gradient 0, under shrubs at the edge of a road	150	20
M 14	Jingbo Township, Baisha County	Hainan	220 m alt., SE 20, gradient 20, under a bamboo-wood forest	300–500	20

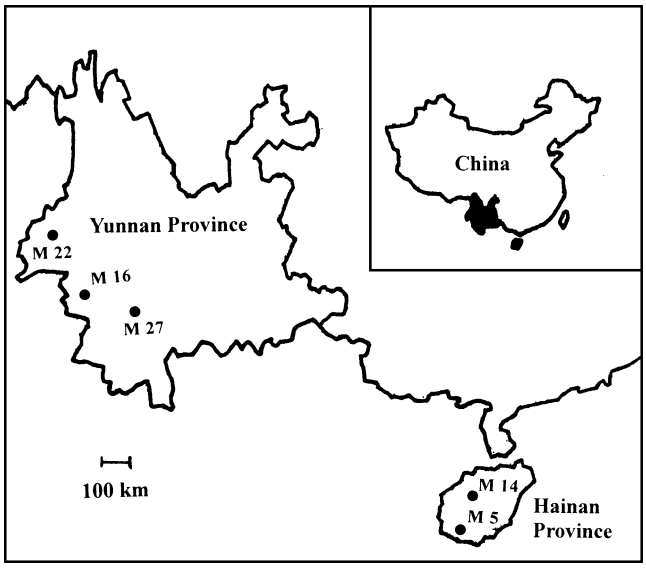

Fig. 1 Distribution of *O. granulata* populations sampled in the present study. *Numbers* correspond to populations in Table 1. The locations of the Yunnan and Hainan provinces in China are shown at the top right corner

being mixed by inversion for 5 min, the mixture was centrifuged at 5000 g for 10 min at 10°C, and the supernatant mixed with a 2/3 vol of ice-cold isopropanol. The DNA was recovered as a pellet by centrifugation at 6000 g for 10 min at 4°C, washed twice, each with 300 µl of 75% ethanol, air-dried at room temperature and re-suspended in 200 µl of 1 × TE; 2 µl of 10 g/l of RNAase was added and the solution was incubated for 1 h at 37°C. Then 200 µl of CI was added to extracted DNA; after being mixed by inversion for 5 min the mixture was centrifuged at 5000 g for 10 min at 10°C, and the supernatant was mixed with 2 vol of cold absolute ethanol and a 1/10 vol of 3 M NaAc, pH 5.2. The DNA was recovered as a pellet by centrifugation at 6000 g for 10 min at 4°C, washed twice, each with 300 µl of 75% ethanol, air-dried at room temperature and re-suspended in 200 µl of 0.1 × TE, and then stored at –20°C.

RAPD PCR amplification

A total of 79 arbitrary primers from Operon Technologies Inc. (Operon B, K, Y and Z kits, but lacking B-5) were used in the RAPD analysis. DNA amplification was performed in a Rapidcycler 1818 (Idaho Technology), programmed for an initial 120 s at 94°C, 10 s at 35°C, 20 s at 72°C for two cycles, followed by 40 cycles of 0 s at 94°C, 0 s at 35°C, and 60 s at 72°C, and a final 7 min at 72°C. Reactions were carried out in a volume of 10 µl containing 50 mM of Tris-HCl, pH 8.3, 500 µg /ml of BSA, 10% Ficoll, 1 mM of Tartrazine, 2 mM of $MgCl_2$, 200 µM of dNTP, 1 µM of primer, 5 ng of DNA template, and 0.5 U of *Taq* polymerase. Amplification products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using a 100-bp DNA ladder.

ISSR PCR amplification

A total of 30 primers from Shengong Inc. were used in the ISSR analysis. DNA amplification was performed in the same Rapidcycler as in the RAPD analysis; programmed for an initial 120 s at 94°C, followed by 38 cycles of 2 s at 94°C, 2 s at 55°C, 60 s at 72°C, and a final 5 min at 72°C. The Basic reaction components were the same as those in the RAPD analysis, but 4% DMSO was added to improve the band solutions. Bands were separated by electrophoresis on 2.0% agarose gel with ethidium bromide, and visualized in the same way as in the RAPD analysis.

Data analysis

Both RAPDs and ISSRs are dominant markers. Amplified fragments were scored for the presence (1) or absence (0) of homologous bands and two matrices of the different RAPD and ISSR phenotypes were assembled; these two matrices were then used for the following statistical analyses respectively. Genetic diversity was measured by the percentage of polymorphic bands (PPB), which was calculated by dividing the number of polymorphic bands at population, region and species levels by the total number of bands surveyed. A RAPDistance program was employed to calculate Jaccard similarity coefficients for all pairs of individuals (J.Armstrong, A. Gibbs, R. Peakall, G. Weiller, Autralian National University, Canberra, Australia). These coefficients were used as operational taxonomic units (OTUs) to construct a dendrogram using the unweighted pair group method (UPGMA) (Sneath and Sokal 1973) and the SHAN (sequential, hierarchical, agglomerative and nested clustering) routine in NTSYS software (Rohlf 1994). In order to describe genetic structure and variability among the populations, the non-parametric Analysis of Molecular Variance (AMOVA) program version 1.5 was used as described by Excoffier et al. (1992), where the variation component was partitioned among individuals within populations, among populations within regions (Yunnan and Hainan provinces), and between regions. Then a permutational procedure was used to provide significance tests for each of the hierarchical variance components based on the original inter-individual squared-distance matrix.

Results

Evaluation of primers

Because RAPD PCR is sensitive to reaction parameters, 79 primers were initially screened against four plants selected from Yunnan and Hainan. The effects of Mg^{2+} and template DNA concentrations, *Taq* polymerase concentrations, and different times and temperatures during the annealing stage of amplification were examined. Under the optimized condition described in Materials and methods, 38 out of 79 primers generated strong amplification products. Then a subset of 20 primers which were insensitive to DNA template concentrations and produced reproducible bands were selected for further analysis. The decamer primers were B-6, B-7, B-12, K-3, K-6, K-8, K-9, K-11, K-12, K-13, K-15, Y-1, Y-2, Y-9, Y-14, Y-15, Y-18, Y-19, Z-3 and Z-4.

A total of 30 ISSR primers were screened with the four plants mentioned above. After comparing the effects of Magnesium concentration, template DNA concentration, and temperature during the annealing stage of amplification, 12 primers (Table 2) which produced clear and reproducible fragments were selected for further analysis.

RAPD polymorphism

A summary of the genetic data for each of the five populations of *O. granulata* is given in Table 3. Very low genetic variation of the species was detected using RAPDs:

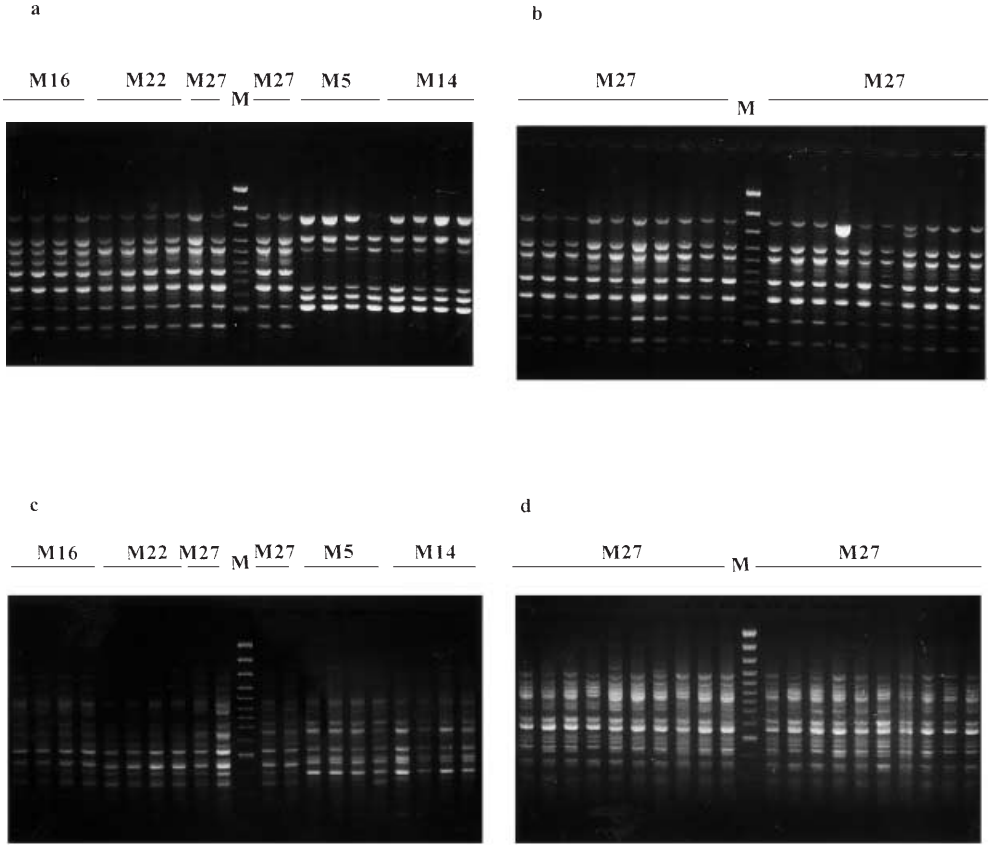

Fig. 2 RAPD and ISSR amplification products generated from *O. granulata* genomic DNA: (**a**) from four individuals of each of five populations obtained with RAPD primer Z-3; (**b**) from 20 individuals of population M27 from the Yunnan Province obtained with RAPD primer Z-3; (**c**) from four individuals of each of five populations obtained with ISSR primer ISSR-21; (**d**) from 20 individuals of population M27 from the Yunnan Province obtained with ISSR primer ISSR-21. M, 100-bp ladder

Table 2 Primers for ISSR analysis

Primer	Sequence[a]
ISSR-4	BDBCACACACACACA
ISSR-5	VHVGTGTGTGTGTGTGT
ISSR-6	DBDGAGAGAGAGAGAGA
ISSR-7	CTCTCTCTCTCTCTCTRG
ISSR-9	CTCTCTCTCTCTCTCTRC
ISSR-15	CCCGTGTGTGTGTGT
ISSR-16	GSGGTGTGTGTGTGT
ISSR-17	CSCGAGAGAGAGAGA
ISSR-18	GCWGAGAGAGAGAGAG
ISSR-20	CCAGTGGTGGTGGTG
ISSR-21	GCGACACACACACACA
ISSR-22	SSWNGACAGACAGACA

[a] B = C/G/T, D = A/G/T, V = A/C/G, H = A/C/T, S = G/C, W = A/T, N = A/G/C/T

Table 3 Percentage of polymorphic RAPD bands (PPB) and polymorphic ISSR bands (PPB) in each *Oryza granulata* population and region

Population No.	No. of RAPD polymorphic bands[a]	PPB of RAPDs	No. of ISSR polymorphic bands[a]	PPB of ISSRs
M 16	13	6.53	8	7.08
M 22	9	4.52	9	7.96
M 27	26	13.06	18	15.93
Yunnan province	41	20.60	20	26.55
M 5	25	12.56	30	26.55
M 14	9	4.52	11	9.73
Hainan province	31	15.58	34	30.09
Total	61	30.65	52	46.02

[a] A total of 199 RAPD and 113 ISSR bands were scored in the present study

a total of 199 bands ranging from 220 to 2000 bp were scored, corresponding to an average of 9.95 bands per primer; of these 30.65% (61 in total) were polymorphic among 100 plants. Of 20 decamer primers, primer OPK-8 produced a monomorphic band in all populations. OPZ-4 showed no variation in populations M16, M22 and M27 from Yunnan, whereas OPB-6, OPB-7, OPK-6, OPY-3, and OPY-19 produced monomorphic bands in populations M5 and M14 from Hainan. Figure 2 shows an example of the polymorphic bands of primer Z-3 between regions and within populations. The percentage of polymorphic bands (PPB) for each population and each region is shown in Table 3, in which population M27 exhibited the highest level of variability (PPB = 13.06), while populations M22 and M14 gave the lowest (PPB = 4.52). Within regions, the PPB of the populations from Yunnan was 20.60, a little higher than that from Hainan (15.58).

ISSR polymorphism

Table 3 summarizes the genetic data for each of the five populations of *O. granulata* using ISSRs. A total of 113

Table 4 Analysis of molecular variance (AMOVA) for 100 individuals of *Oryza granulata* using RAPD

Source of variance	*df*	SSD	MSD	Variance component	Percentage total	*P*-value[a]
Yunnan vs Hainan	1	7.6233	7.6233	14.29	73.85	<0.001
Among populations/regions	3	2.2967	0.7660	3.76	19.45	<0.001
Within populations	95	1.2319	0.0130	1.30	6.70	<0.001
Among populations	4	9.9200	2.4800	12.34	90.49	<0.001
Within populations	95	1.2319	0.0130	1.30	9.51	<0.001
Among regions	1	7.6233	7.6233	15.81	81.45	<0.001
Within regions	98	3.5286	0.036	3.60	18.55	<0.001

[a] Significance tests after 3000 permutations

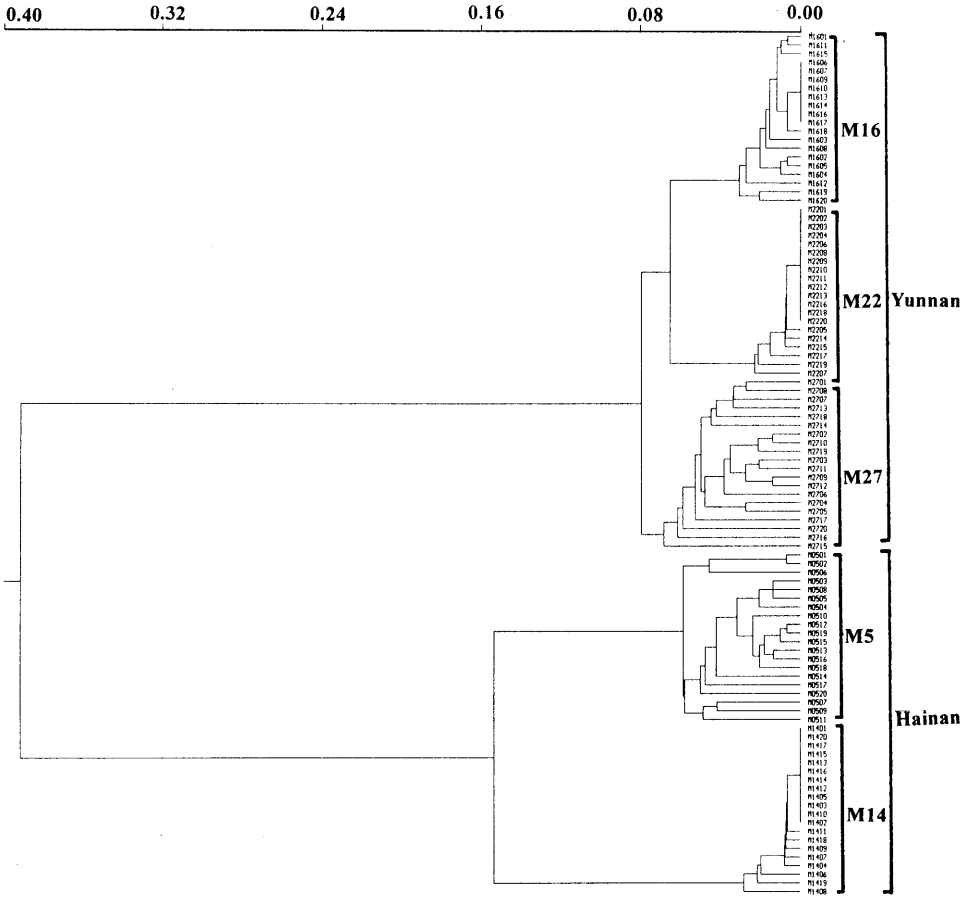

Fig. 3 Dendrogram illustrating genetic relationships among 100 individuals of five populations of *O. granulata*, generated by the UPGMA cluster analysis (NTSYS) calculated from 199 RAPD markers produced by 20 primers

bands ranging from 230 to 1500 bp were scored, corresponding to an average of 9.42 bands per primer, of which 46.02% (52 in total) were polymorphic across the 100 plants. Every primer produced polymorphic bands when all of the five populations were analyzed, whereas primer ISSR-17 was invariable in the three populations from Yunnan, while ISSR-6 and ISSR-18 were monomorphic in the two populations from Hainan. Figure 2 shows an example of the polymorphic bands amplified by primer ISSR-21 between the two regions and within populations. The percentage of polymorphic bands (PPB) for each population and each region is shown in Table 3. Population M5 exhibited the highest level of variability (PPB = 26.55), whereas population M16 was the lowest (7.08). Within populations, the PPB of the populations from Yunnan was 26.55, but that from Hainan was about 30.09.

The genetic structure of populations revealed by RAPDs

The AMOVA program was used to partition the genetic variation by hierarchical analysis from the distance matrix. AMOVA showed a highly significant (P <0.001) genetic difference between Yunnan and Hainan as well as among populations within each region (Table 4). An examination of the proportion of diversity between regions, among populations within regions, and within populations indicated that, on average, most of the variance (73.85%) occurred between Yunnan and Hainan. The variances among populations within regions and within populations, however, were only 6.70% and 19.45%, respectively. For the among-population analysis, the great majority of genetic variation (90.46%) resided between populations, and only a small amount of variation (9.51%) presented differences within populations.

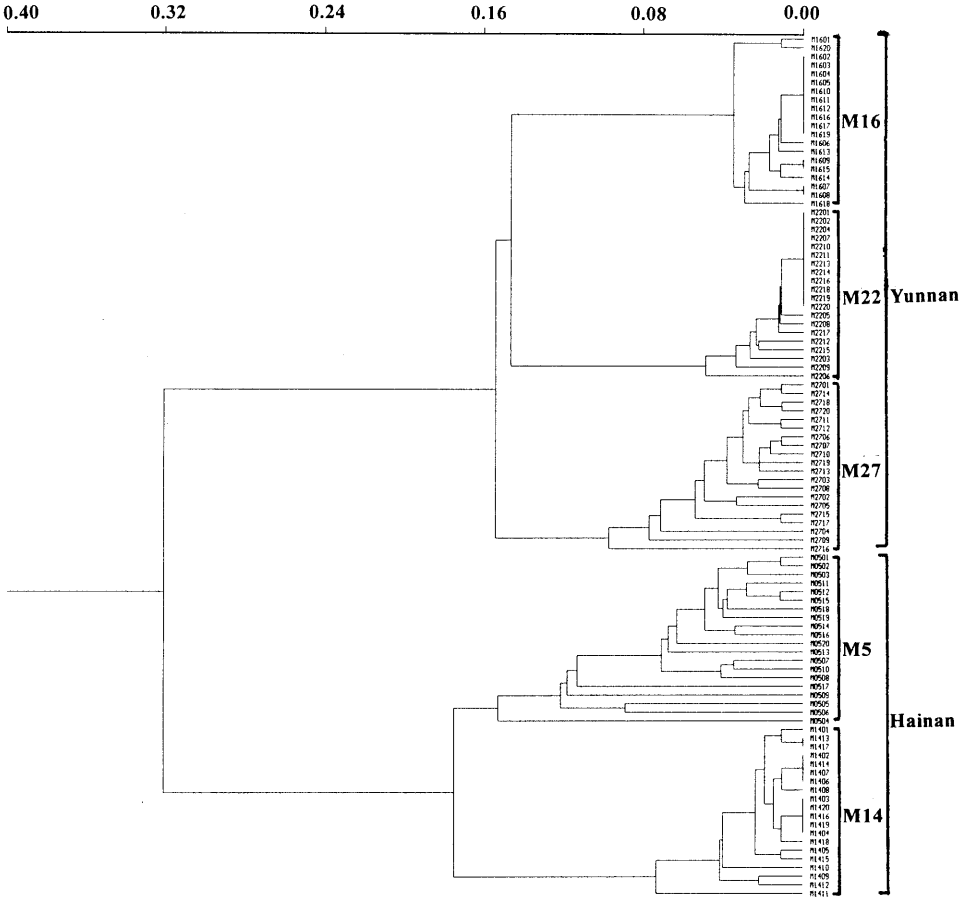

Fig. 4 Dendrogram illustrating genetic relationships among 100 individuals of five populations of *O. granulata*, generated by the UPGMA cluster analysis (NTSYS) calculated from 113 ISSR markers produced by 12 primers

Table 5 Matrix based on pairwise Φ_{st} distance between populations using RAPD

Pop.	M 16	M 22	M 27	M 5	M 14
M 16	0.0000				
M 22	0.8109	0.0000			
M 27	0.6087	0.6095	0.0000		
M 5	0.9257	0.9358	0.8866	0.0000	
M 14	0.9641	0.9768	0.9176	0.8205	0.0000

An UPGMA dendrogram produced using the Jaccard coefficient between all studied individuals is shown in Figure 3. The tree obtained indicated that the populations from Yunnan and Hainan were clearly separated into two major clusters. In each cluster, individuals from the same population formed a distinct group, and genetic similarity between these individuals was high (>0.90). Moreover, in populations M16, M22 and M14, there were 10, 12 and 12 individuals respectively; no variation was detected by RAPD analysis. The relationships among populations are presented in Table 5, which displays the genetic distance the based on a pairwise Φ_{st} between populations. The genetic distances among the populations from Yunnan and Hainan were lower than those between the two regions, and hence the result corroborated the UPGMA cluster.

The genetic structure of populations detected by ISSRs

Similar to the result of the RAPD analysis, AMOVA analysis of ISSR bands showed highly significant (P<0.001) genetic differences between Yunnan and Hainan, and among populations within either region (Table 6). Of the total genetic variance, 49.26% existed between regions, lower than that based on RAPDs (73.85%), 38.07% between population within regions, and 12.66% within populations. In addition, a 84.23% variance was detected among populations, whereas only 15.77% occurred within populations based on an among-population analysis.

A cluster analysis (UPGMA) was used to generate a dendrogram based on the Jaccard coefficient between all 100 individuals (Fig. 4). The dendrogram also indicated that the Yunnan and Hainan populations were distinctly separated into two major clusters. In each of the clusters, individuals from the same population formed a group. Genetic similarity between individuals within a population was over 0.88, and an identical band pattern was detected within populations M16 and M22. In population M14, three genotypes containing 11 individuals were ascertained by ISSR analysis. In order to reveal the relationships among populations and regions, a matrix was generated based on the pairwise Φ_{st} distance between populations (Table 7); this indicated that those populations from Yunnan and Hainan are divided into two

Table 6 Analysis of molecular variance (AMOVA) for 100 individuals of *O. granulata* using ISSRs

Source of variance	*df*	SSD	MSD	Variance component	Percentage total	*P*-value[a]
Yunnan vs Hainan	1	4.9897	4.990	7.832	49.26	<0.001
Among populations/regions	3	3.6917	1.231	6.052	38.07	<0.001
Within populations	95	1.9125	0.020	2.013	12.66	<0.001
Among populations	4	8.6814	2.170	10.75	84.23	<0.001
Within populations	95	1.9125	0.020	2.013	15.77	<0.001
Among regions	1	4.9897	4.990	10.28	64.25	<0.001
Within regions	98	5.6042	0.057	5.720	35.75	<0.001

[a] Significance tests after 3000 permutations

Table 7 Matrix based on the pairwise Φ_{st} distance between populations using ISSRs

Pop.	M 16	M 22	M 27	M 5	M 14
M 16	0.0000				
M 22	0.8807	0.0000			
M 27	0.7858	0.7694	0.0000		
M 5	0.8443	0.8534	0.7792	0.0000	
M 14	0.9230	0.9303	0.8660	0.6706	0.0000

groups, in agreement with the results of the UPGMA cluster analysis.

Discussion

Genetic diversity and population structure

By using RAPD and ISSR primers, we demonstrated that there was very low genetic variation within populations of *O. granulata*. For RAPDs, only 30.65% of bands were polymorphic in all five populations. The percentage of polymorphic bands (PPB) in each population ranged from 4.52% to 13.06%. For ISSRs, although the PPB was 46.02%, much higher than that detected by RAPDs, ranged was from 7.08% to 26.55% in each population, also higher than that from RAPD, the genetic variation detected was still low compared with other species in the same genus. By using 20 RAPD primers, Ge et al. (1999a) detected the genetic diversity of *Oryza rufipogon* for eight populations from China and Brazil, and found that the PPB values were 55.8% and 41.1% for the Chinese and Brazilian populations, respectively. Buso et al. (1998) also revealed a high genetic diversity of *Oryza glumaepatula* from the Amazon forest and western Brazil by using allozymes and RAPDs. Yu and Nguyen (1994) detected 13 *O. sativa* cultivars from upland and lowland rice cultivars with RAPDs and found that 208 out of 260 bands (80.0%) were polymorphic. Recently, based on 17 allozyme loci encoded by 12 enzymes, Gao et al. (1999) found that very low genetic diversity existed within populations of *O. granulata*. The percentages of polymorphic loci (P) ranged from 5.6 to 16.7 within populations, with the average expected heterozygosity (He) being only 0.015. In comparison, *O. granulata* presents a low genetic diversity both within populations and among populations within regions, but with a considerable amount of genetic differentiation between regions. The level and pattern of genetic diversity detected by RAPDs and ISSRs in the present study were in overall agreement with those studies.

In China, *O. granulata* is distributed separately in the Yunnan and Hainan provinces. It grows in deciduous forests such as bamboo thickets, spinney, and especially at edges, gaps and hedges of forests formed by natural and human disturbance. Populations of the species are subdivided into demes or clumps of varying size, always with no more than 100 individuals in each patch. Allozyme analysis indicated a high genetic differentiation between populations (F_{st} = 0.859) (Gao 1997). Similarly, the present study revealed that the levels of genetic differentiation between populations of *O. granulata* increased with geographical distance. Of the total genetic variance component, only 19.45% (RAPD) and 38.07% (ISSR) of the genetic variance occurred in populations within regions, whereas 73.85% (RAPD) and 49.26% (ISSR) existed between Yunnan and Hainan. Gao (1997) has proposed that founder effect, gravit dispersal of seeds, and clonal growth are responsible for the low diversity within populations and the considerable differentiation between populations of *O. granulata.* However, based on our field survey of 90% reported habitats in China during 1998 to 1999, we considered that the special pattern of genetic variance was the consequence of metapopulation structure, gene flow, and habitat fragmentation under disturbance.

In a study on populations of the cheetah (*Acinonyx jubatus*), Pimm et al. (1989) suggested that the low level of genetic variation in this species may not be the result of population bottlenecks as originally hypothesized by Menotti-Raymond and O'Brien (1993), and that the metapopulation dynamics (Hanski 1998), extinction and re-colonization of patches by a few individuals, could result in low genetic variation (Harrison and Hastions 1996; Hedrick 1996a). Gilpin (1991) demonstrated that even in a large population, with a high turnover of extinction and colonization among its local populations, small effective population sizes should accelerate the loss of genetic variation. In addition, the genetic differentiation between local populations may be enhanced during the process. Giles and Goudet (1997) studied 52 populations of *Silene dioica* using allozyme analysis and found that the turnover of local populations along with environmental heterogeneity and spatial restriction might increase the genetic variances among populations. A typ-

ical metapopulation structure was found in *O. granulata* based on our field-survey of over 40 natural populations in China (Qian et al., unpublished data). In a study on the spatial pattern of three Yunnan populations, we found that *O. granulata* showed an aggregate distribution within a community, with a density as low as 2.11 individuals /m^2 (computed in 240 entries of 1×1 m^2 quadrats). Evidently, either the habitat exposed to intensive sunlight or intensive shade was not suitable for the growth of this species. In addition to forming clumps by gravity dispersal of seeds, *O. granulata* is able to invade and grow rapidly at the edges of communities (personal observation), perhaps by the transmitted force of insects and rodents. All of these aspects determine a turnover of extinction and colonization between clumps (local populations) of *O. granulata* during a succession of communities. As described by Pimm et al. (1989), Hanski and Gilpin (1998) and Hedrick (1996b), the metapopulation process may lead to a decrease of genetic variation within a whole metapopulation, while the total population size could remain fairly large. In addition, the small effective population size caused by the founder effect occurring in these clumps may be another factor to accelerate the loss of genetic variation of demes and to increase the differentiation between them. Therefore, we suggest that metapopulation structure dynamics is the main factor in shaping the genetic diversity and genetic structure of populations.

O. granulata exchanges its genes through three channels between populations: seeds, pollen, and indirectly by clone growth. It disperses seeds mainly by gravity (Qian et al., unpublished data). Hamrick and Godt (1989) summarized that the population diversity of these kinds of species (He = 0.101) is lower than that of other seed-dispersed types (0.123–0.137), except those dispersing its seeds by an explosive mechanism (0.062). The effect of gravity dispersal was observed in populations M16 and M22, which were in decline owing to human disturbance so that only a small number of individuals had survived and began to reproduce. Based on the fact that a considerable number of individuals with identical RAPD and ISSR genotypes were found within the two populations (Figs. 3 and 4), we are of the opinion that the special genetic pattern was attributable to a result of clumping, with offspring distributed around the ma ternal plants due to gravity dispersal.

Clonal growth and mating systems may also influence genetic structure. However, as *O. granulata* grows in forests, it is rather difficult to observe directly whether the species has clonal growth or what other characteristics it has (Silvertown 1982). As inferred from the fixation index F = 0.733 based on allozyme data (Gao 1997), the species seems to be selfing and inbreeding to a certain extent.

Habitat fragmentation under human disturbance is another factor leading to low genetic variation (Soulè 1986; Hunter 1996). Both RAPD and ISSR analyses revealed higher levels of genetic diversity in large populations than in small ones. For population M16, which survived after de-forestation of growing rubber trees, the level of genetic diversity was low. The PPB of M16 detected by RAPDs was 6.53% and that by ISSRs was 7.08%. Population M14 was similar to M16. Studies on habitat fragmentation indicated that the process would lead to population extinction and the loss of genetic variation, by not only minimizing suitable habitats, but also increasing the mating opportunity between genetically closely related individuals. Innes et al. (1988) also found that when a few individuals re-colonized in a newly established habitat, *Solidago sempervirens* could convert its mating system from outcrossing to selfing. From an evolutionary point of view, loss of genetic variation means a decrease of adaptation to changing environments, and hence *O. granulata* would be in a more-endangered situation with habitat fragmentation caused by human disturbance.

The RAPD and ISSR analyses gave rise to a consistent result, which indicates that the levels of genetic diversity of the three populations from Yunnan were lower than those from Hainan. AMOVA analysis of RAPD data shows that the sums of squared deviation (SSD) in Yunnan was 0.236 but 0.262 in Hainan. Similarly, 0.272 of the SSD in Yunnan and 0.548 in Hainan were detected by ISSRs. These results are inconsistent with allozyme data: P 0.0781 and He 0.016 for the populations from Yunnan, with P 0.0360 and He 0.016 for those from Hainan (Gao 1997). Further analysis of genetic diversity with more representative populations from both regions is currently under way.

Comparison of RAPD and ISSR data

Our RAPD and ISSR surveys on the five populations of *O. granulata* revealed 30.65% and 46.02% of polymorphic bands respectively, which are higher than those from the allozyme analysis (A = 1.06, P = 6.33%) (Gao 1997). When the levels of variation detected by RAPDs and ISSRs were compared, the PPB detected by ISSRs was higher than that by RAPDs at every level of hierarchy structure, although 20 decamer primers were used in the RAPD analysis whereas only 12 primers were used in the ISSR analysis. This discrepancy is mainly due to the DNA segments targeted by the two methods. First, the greater polymorphism detected by RAPDs and ISSRs than by allozymes may be partially explained by the conservative nature of the coding sequences, primarily housekeeping genes, sampled by allozymes in contrast to the non-coding sequences sampled by RAPDs and ISSRs (Fritsch et al. 1996; Fahima et al. 1999.). Second, microsatellites are short tandem repetitive DNA sequences with a repeat length of only a few base pairs. These sequences are abundant, dispersed throughout the eukaryotic genome and highly polymorphic due to DNA slippage (Hamada and Kakunaga 1982; Weber and May 1989). Therefore, by using a single primer composed of a microsatellite sequence anchored by two to four arbitrary, often degenerate, nucleotides at the 3’ or 5’ ends

(Zietkiewicz et al. 1994; Ratmaparkhe et al. 1998; Gilbert et al. 1999), ISSRs could detect many more polymorphic bands per primer than RAPDs. Although the evidence from Figs. 3 and 4 indicates that ISSRs may detect a higher genetic polymorphism than RAPDs at all levels of the hierarchy structure, RAPDs resolved more genetic differentiation between Yunnan and Hainan because the genetic distance detected by RAPDs and ISSRs respectively was 0.38 vs 0.32. Therefore, the inconsistency between RAPD and ISSR patterns resulted from the fact that the genomic regions sampled by the two markers maintain a different evolutionary process under selection forces. The present study used a 2% agarose gel to separate amplification products and obtained a reasonable result. If a polyacrylamide non-denaturing gel were to be used, the resolution would be enhanced, but with a greater expense of technique and time.

If reproducible ability in amplification is considered, ISSR is more reliable than RAPD as our experiment showed. RAPD is highly sensitive to the reaction conditions, including the concentrations of Mg^{2+}, *Taq* polymerase, dNTP, primers, DNA purity, etc (Skroch and Nienhuis 1995; Fritsch and Rieseberg 1996). The length of primers for RAPD is always 10 bp, and thus the annealing temperature is always under 42°C. As a consequence, it is possible to generate non-specific products because of primer mismatching in PCR amplification, so that the standard procedure should be established and carried out with more caution to obtain good-quality and reproducible bands. ISSR is robust, however, because it is less sensitive to the reaction conditions than RAPD. For example, during the PCR optimization we found that some of the RAPD primers, such as B-6, K-11 and Z-3, were sensitive to the DNA template purified by proteinase K. Expected bands were lost and 1–4 unexpected bands were obtained even for the same individuals (data not shown). In contrast, this phenomenon did not occur in ISSR optimization no matter whether a proteinase k-purified template or the control template was used. We believe that the cause of this was that protein would disturb the annealing process, or else that remaining ions introduced by the proteinase K treatment would influence the PCR process.

In conclusion, as two dominant DNA markers, RAPDs and ISSRs are effective and promising marker systems for detecting genetic variation. Furthermore, ISSR is superior to RAPD in terms of the polymorphic bands detected per primer and the reproducibility involved.

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References

Aggarwal RK, Brar DS, Khush, GS (1997) Two new genomes in the *Oryza* complex identified on the basis of molecular divergence analysis using total genomic DNA hybridization. Mol Gen Genet 254: 1–12

Ayala FJ, Kiger JA (1984) Modern genetics, 2nd (edn) Benjamin-Cumings, Menlo Park, USA

Buso GSC, Rangel PH, Ferreira ME (1998) Analysis of genetic variability of South American wild rice populations (*Oryza glumaepatula*) with isozymes and RAPD markers. Mol Ecol 7: 107–117

Bustos AD, Casamova C. Soler C. Jouve N (1998) RAPD variation in wild populations of four species of the genus Hordeum (Poaceae). Theor Appl Genet 96: 101–111

Cruzan MB (1998) Genetic markers in plant evolutionary ecology. Ecology 79: 400–412

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Focus 12: 13–15

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491

Fahima T, Sun GL, Beharav A, Krugman T, Beiles A, Nevo E (1999) RAPD polymorphism of wild emmer wheat populations, *Triticum dicoccoides*, in Israel. Theor Appl Genet 98: 434–447

Fang DQ, Roose ML (1997) Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theor Appl Genet 95: 408–417

Fritsch P. Rieseberg LH (1996) The use of random amplified polymorphic DNA (RAPD) in conservation genetics. In: Smith TB, Wayne RK (eds) Molecular genetic approaches in conservation. Oxford University Press, London, pp 54–73

Gao LZ (1997) Studies on genetic variation of three wild rices (*Oryza spp.*) in China and their conservation biology. PhD dissertation, Institute of Botany, Chinese Academy of Sciences, Beijing

Gao LZ, Ge S, Hong DY, Zhang JW, Luo QY, Tao GD, Xu ZF (1999) A study on the population genetic structure of *Oryza meyeriana* (Zoll. et Mor. ex Steud.) Baill. from Yunnan and its in situ conservation significance. Science in China (Series C) 42: 102–108

Ge S, Oliveira GC, Schaal BA, Gao LZ, Hong DY (1999a) RAPD variation within and between natural populations of the wild rice *Oryza rufipogon* from China and Brazil. Heredity 82: 638–644

Ge S, Sang T, Lu BR, Hong DY (1999b) Phylogeny of rice genomes with emphasis on the origins of allotetraploid species. Proc Natl Acad Sci USA 96: 14400–14405

Gilbert JE, Lewis RV, Wilkinson MJ, Caligari PDS (1999) Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theor Appl Genet 98: 1125–1131

Gilpin M (1991) The genetic effective size of a metapopulation. Biol J Linn Soc 42: 165–175

Giles BG, Goudet J (1997) Genetic differentiation in *Silene dioica* metapopulations: estimation of spatiotemporal effects in a successional plant species. Am Nat 149: 507–52

Hamada H, Kakunaga T (1982) Potential Z-DNA forming sequences are highly dispersed in the human genome. Nature 298: 396–398

Hamrick JL, Godt MJ (1989) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (eds) Plant population genetics, breeding, and genetic resources. Sinauer Associates Publishers, Massachusetts, USA, pp 43–63

Hanski I (1998) Metapopulation dynamics. Nature 369: 41–49

Hanski I, Gilpin M (eds) (1998) Metapopulation biology: ecology, genetics, and evolution. Academic Press, London

Harrison S, Hastions A (1996) Genetic and evolutionary consequences of metapopulation structure. Trends Ecol Evol 11: 180–183

Hedrick PW (1996a) Conservation genetics and molecular techniques: a perspective. In: Smith JB, Wayne RK (eds) Molecular genetic approaches in conservation. Oxford University Press, London, pp 459–477

Hedrick PW (1996b) Genetics of metapopulations: aspects of a comprehensive perspective. In: McCullough DR (ed) Metapopulations and wildlife conservation. Island, Press, Washington D.C., pp 29–51

Hunter ML (1996) Fundamentals of conservation biology. Blackwell Science, London

Innes J, Hermanutz IA (1988) The mating system and genetic structure in a disjunt population of the seaside golden rod *Solidago sempervirens* (Asteraceae). Heredity 61: 447–454

Kantetky RV, Zhang X, Bennetzen JL. Zehr BZ (1995) Assessment of genetic diversity in dent and popcorn (*Zea mays* L.) inbred lines using inter-simple sequence repeat (ISSR) amplification. Mol Breed 1: 365–372

Menotti-Raymond M, O'Brien SJ (1993) Dating the genetic bottleneck of the African cheetah. Proc Natl Acad Sci USA 90: 3172–3176

Pimm SL, Gittlemam JL, McCracken GF, Gilpin M (1989) Plausible alternatives to bottlenecks to explain reduced genetic diversity. Trends Ecol Evol 4: 176–178

Ratnaparkhe, MB, Tekeoglu M, Fuehlbauer FJ (1998) Inter-simple-sequence-repeat (ISSR) polymorphisms are useful for finding markers associated with disease resistance gene clusters. Theor Appl Genet 97: 515–519

Rohlf FJ (1994) NTSYS-pc, numerical taxonomy and multivariate analysis system, v. 1.80. Exeter Software, New York

Schaal BA, Leverich WJ, Rogstad SH (1991) Comparison of methods for assessing genetic variation in plant conservation biology. In: Falk DA, Holsinger KE (eds) Genetics and conservation of rare plants. Oxford University Press, New York, pp 123–134

Silvertown JW (1982) Introduction to plant population ecology. Longman, London

Skroch P, Nienhuis J (1995) Impact of scoring error and reproducibility of RAPD data on RAPD based estimates of genetic distances. Theor Appl Genet 91: 1086–1091

Smith TB, Wayne RK (1996) (eds) Molecular genetic approaches in conservation. Oxford University Press, London

Sneath, P, Sokal, R (1973) Numerical taxonomy. W.H. Freeman and Company, San Francisco

Soulè ME (1986) Conservation biology, an evolutionary ecology perspective. Sinauer Associates, Massachusetts, USA

Stebbins GL (1999) A brief summary of my ideas on evolution. Am J Bot 86: 1207–1208

Vaughan DA (1994) The wild relatives of rice. IRRI, Manila

Weber JL, May PE (1989) Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. Am J Hum Genet 44: 388–396

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6531–6535

Wolfe AD, Xiang QY, Kephart AS (1998) Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). Proc Natl Acad Sci USA 95: 5112–5115

Yang WP, Oliveira AC, Godwin I, Schertz K, Bennetzen JL (1996) Comparison of DNA marker technologies in characterizing plant genome diversity: variability in Chinese sorghums. Crop Sci 36: 1669–1676

Yu LX, Nguyen NT (1994) Genetic variation detected with RAPD markers among upland and lowland rice cultivars (*Oryza sativa* L.). Theor Appl Genet 87: 668–672

Zietkiewicz E, Rafalske A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genome 20: 178—183