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Source and Inheritance of the Within Cultivar Residual Variation Detected in an indica Variety IR64

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Abstract: The phenotypically uniform indica variety IR64 was chosen for study of the source and inheritance of within cultivar residual variation using a set of SSR markers. Residual heterogeneity in IR64 was identified on the short arm of chromosome 2 involving at least 5 SSR loci spanning nearly 30 cM. The SSR variations originated from the parental lines of IR64 (IR5657-33-2 / IR2061-465-1-5-5) and were segregating in the selfed bulk seed stock in a Mendelian manner for more than 20 years. This study verified that the within cultivar variations of SSR in a morphologically uniform variety IR64 of a selfing crop came from its parental lines, which has immediate and commercial applications including test of hybrid seed purity, varietal fingerprinting, and curation and propagation of germplasm collections.

Key words: simple sequence repeat; residual heterogeneity; within cultivar variation; rice

Seed purity is critical to economically important crops and food production. There are two main sources leading to the impurity of crop seeds: mechanical mixture and residual heterozygosity. The former is relatively easy to be detected using molecular markers that are abundant nowadays. The detection of the latter, however, is more tedious and time-consuming, and largely dependent on reliable experimental materials. Residual heterozygosity or commonly termed intra-varietal variation or within cultivar variation, a common phenomenon in crop species, has been documented in a number of DNA-marker based studies [1-5]. However, no experiment so far has been carried out to carefully trace the source and the genetic behavior of the residual variations in crops.

Among the DNA markers, SSR is especially useful because it is co-dominant, multi-allelic and can be reliably used to analyze both indica and japonica germplasm, as well as groups of AA genome *Oryza* species. Further more, the highly polymorphic nature of many SSR loci is particular valuable when analyzing closely related genotypes such as frequently used parental materials in breeding programs.

In this study, we report the detection of intra-varietal variations in the breeder IR64 seed, the sources of the variations and the genetic behavior of intra-varietal variations in the widely grown indica variety IR64 using SSR markers.

MATERIALS AND METHODS

Rice materials

Progenitors of the IR64 pedigree were grown in the greenhouse at the International Rice Research Institute (IRRI) (Table 1).

The breeder IR64 seed (S_0) was obtained from the Plant Breeding, Genetics and Biochemistry Division (PBGB), IRRI in 1998. The seeds were maintained and propagated under field conditions and selected based on phenotypic uniformity. Ninety-four individual plants were used for genotyping. Plants were bagged individually for seed increase. Forty-eight S_1 plants representing each of the 6 subtypes identified in the S_0 generation were randomly chosen for genotyping and seed increase. Bagged seeds from each of the 6 subtypes were harvested in bulk, and 48 S_2 individual plants of each subtypes were grown for genotyping.

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Table 1. List of rice materials used in present study.

Material	Seed source	Accession number	No. of plants	Marker set [a]
S_0(Breeder IR64)	Breeder, PBGB, IRRI		94	51
S_1	Selfed progenies of S_0		48×6	19
S_2	Selfed progenies of S_1		48×6	19
GRC-IR64	GRC, IRRI, 1989	66970	48	19
IR2061 (IR2061-465-1-5-5)	Male parent of IR64, GRC, 1979	39288	48	19
IR5657 (IR5657-33-2)	Female parent of IR64, GRC, 1990	77992	48	19

[a] Fifty-one markers, including seven markers (RM154, RM110, RM211, RM233A, RM279, RM174 and RM71) from the short arm of chromosome 2, 12 identifiers (RM151, RM266, RM251, RM335, RM334, RM204, RM320, RM264, RM278, RM333 RM224, RM101) on 12 chromosomes and 32 randomly distributed markers; Nineteen markers including seven from chromosomes 2 and 12 identifiers.

Seeds of IR64 (accession no. 66970, GRC-IR64 afterwards) were obtained from the Genetic Resource Center (GRC), IRRI in 2003. The seeds were originally harvested in the wet season in 1989, and have been stored and distributed by GRC. Forty-eight individual plants were used for genotyping.

Seeds of the female parent IR5657-33-2 (accession no. 77992, IR5657 afterwards) and the male parent IR2061-465-1-5-5 (accession no. 39288, IR2061 afterwards) were obtained from GRC in 2003. IR5567 and IR2061 were harvested in the dry season in 1990 and in the wet season in 1979, respectively, both having been stored and distributed by GRC. Forty-eight individual plants of each parent were subjected to genotyping.

SSR marker genotyping

Young leaves from 94 S_0 plants of the breeder IR64, 48 S_1 and 48 S_2 plants from each of the six subtype IR64, 48 plants of GRC-IR64 and 48 plants of IR64 parental lines each at 21-day-old were harvested for DNA mini-preparation [6].

A set of 12 SSR identifiers (RM151, RM266, RM251, RM335, RM334, RM204, RM320, RM264, RM278, RM333, RM224 and RM101) that can detect 95 alleles on all chromosomes [7-8] and 7 markers (RM154, RM110, RM211, RM233A, RM279, RM174 and RM71) on the short arm of chromosome 2 were commonly used for genotyping of the breeder IR64 (S_0) and their selfed progenies (S_1 and S_2), GRC-IR64 and their parental lines. The breeder IR64 (S_0) was also subjected to genotyping by extra 33 SSR markers randomly distributed on the 12 chromosomes of rice genome (markers not listed). PCR was conducted as described by Chen et al [7] and Temnykh et al [8]. PCR products were run on 5% denaturing polyacrylamide gel and detected using silver staining following the manufacturer's instruction (Promega).

Data analysis

At each marker locus, genotypes of the individual plants were scored following the SSR band patterns. A homozygous genotype was indicated by two same characters, and a heterozygous genotype was indicated by two different characters indicating the allele composition. Different letters indicated allele differences at a given locus, based on the comparison among different individuals of all the IR64-related lines.

Frequency of a genotype group was calculated as the percentage of individuals showing the given genotype in the total number of individuals tested. Chi-square test was performed to examine the segregation of marker alleles in GRC-IR64, as well as in the S_1 and S_2 progenies of the breeder IR64.

RESULTS

Genotyping of the breeder IR64

A total of 45 SSR markers including 12 SSR identifiers and 33 randomly-distributed SSR markers were initially used for genotyping of the breeder IR64 (S_0). No polymorphisms among the 94 individual plants were found at any locus of the 12 identifiers and 32 out of the 33 random SSR markers. The only marker detected within cultivar variation in the breeder IR64 was RM154 on the short arm of chromosome 2. It was noticed that there were no morphological differences among the individuals of the breeder IR64. Based on the SSR markers surveyed,

these breeder seeds were generally high in purity except the region near RM154 locus on chromosome 2 and no mechanical mixture was evident.

To determine if RM154 was the only heterozygous locus on the short arm of chromosome 2, six SSR markers linked to RM154 (RM110, RM211, RM233A, RM279, RM174 and RM71) were selected for further test of the 94 individual plants. Results showed that the breeder IR64 were in segregation on the first four marker loci, while all of them were consistent and homozygous at both loci RM174 and RM71. We therefore inferred that there were no variations beyond RM174 up to the long arm of chromosome 2. All together five out of seven loci including RM154 were in segregation, and each locus possessed two alleles (Fig. 1). Based on the SSR band patterns of the seven markers tested on chromosome 2, there were 10 sub-genotypes among the 94 plants of the breeder IR64 (Table 2). Among them, Subtype I was the dominant genotype accounting for 70.21% while Subtypes II, III, VIII, IX and X were the smallest groups, each representing a single plant (1.06%). This result showed that the region of IR64 on chromosome 2 was highly variable but had a major genotype.

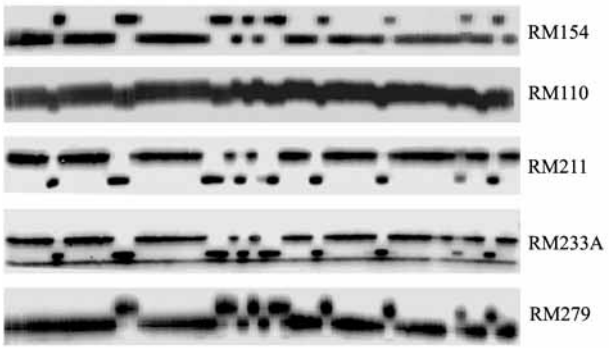

Fig. 1. SSR band patterns of 48 out of 94 breeder IR64 at loci RM154, RM110, RM211 RM233A and RM279 on the short arm of chromosome 2. Each locus showed two alleles.

Genotyping of GRC-IR64

Forty-eight IR64 plants from the stock center were tested for SSR variations both on chromosome 2 and the whole genome. In the genome wide scanning with the set of 12 SSR identifiers, all the 48 plants were identical to each other and to the breeder IR64. However, variations were found in the same region on the short arm of chromosome 2 when the seven markers were tested. Unlike the breeder IR64, there were only four subtypes of IR64 (Subtype I, IV, VI and VII) identified among the GRC stocks, and none of them were hetrozygous at the SSR loci (Table 3). The results indicated that lower heterogeneity was presented in GRC-IR64 than in the breeder IR64. GRC-IR64 also differed from the breeder IR64 by showing absence of major genotype groups. As a matter of fact, the GRC-IR64 was in segregation for two alleles and fitted the ratio of 1:1 at each of the five SSR loci showing variations. This suggested that over the course of propagating, 'genetic drift' might occur leading to the predominance of certain genotypes in the breeder IR64.

Although there were genotypic differences

Table 2. IR64 subtypes identified in breeder seeds based on SSR pattern of chromosome 2.

Subtype	Genotype at the SSR loci [a]							No. of plants	% [b]
	RM154	RM110	RM211	RM233A	RM279	RM174	RM71		
I	BB	BB	BB	BB	BB	BB	BB	66	70.21
II	BB	BB	AB	AB	BC	BB	BB	1	1.06
III	CC	BB	AA	AA	CC	BB	BB	1	1.06
IV	CC	AA	AA	AA	CC	BB	BB	6	6.38
V	BC	AB	AB	AB	BC	BB	BB	4	4.26
VI	CC	AA	AA	AA	BB	BB	BB	8	8.51
VII	BB	BB	BB	BB	CC	BB	BB	5	4.26
VIII	CC	AA	AA	AA	BC	BB	BB	1	1.06
IX	BB	BB	BB	BB	BC	BB	BB	1	1.06
X	BC	AA	AB	AB	BC	BB	BB	1	1.06

[a] AA, BB and CC refer to homozygotes of the SSR loci; AB and BC refer to heterozygotes of the SSR loci. A refers to the smallest allele in size, B is bigger than A and C is the largest.

[b] Frequencies of subtypes among 94 individual plants of the breeder IR64.

Table 3. Four subtypes of IR64 identified in the GRC-IR64.

Subtype	Genotype at the SSR loci							No. of plants	%
	RM154	RM110	RM211	RM233A	RM279	RM174	RM71		
I	BB	BB	BB	BB	BB	BB	BB	13	27.08
IV	CC	AA	AA	AA	CC	BB	BB	17	35.42
VI	CC	AA	AA	AA	BB	BB	BB	11	22.92
VII	BB	BB	BB	BB	CC	BB	BB	7	14.58
Subtotal	BB(20)	BB(20)	BB(20)	BB(20)	BB(24)				
	CC(28)	AA(28)	AA(28)	AA(28)	CC(24)				
$P_{(1:1)}$	0.25	0.25	0.25	0.25	1.00				

between the GRC-IR64 and the breeder seeds, the same alleles were found in the two sources of IR64. As can been seen from Tables 2 and 3, a cross between Subtype I and Subtype IV would generate all other subtypes detected in the breeder IR64. Therefore, we inferred that the heterogeneity in the breeder IR64 was originated from their GRC ancestor through crossing between the two subtypes after many generations of propagation, while both of them have remained phenotypically uniform possibly under selection. It appears that the heterogeneity at these loci has no apparent effects on their morphology or common agronomic attributes.

Genotyping of IR64 parental lines

In order to trace the sources of heterogeneity of IR64 in the GRC stock, we investigated the SSR band patterns of its parental lines. Forty-eight plants from each of the parental line were firstly subjected to scanning for variations using the set of 12 identifiers. For the male parent IR2061, 10 out of the 12 loci were in segregation and only two loci (RM224 and RM278) were homozygous. Among the ten heterozygous loci, RM151 had 4 alleles, RM335 had 3 alleles and the remaining 8 loci had 2 alleles each. For the female parent IR5657, 3 (RM151, RM224 and RM335) out of the 12 loci were in segregation and the remaining 9 loci were homozygous. All three heterozygous loci had two alleles each. Unlike IR64, it was noticed that the phenotypes of both parental lines were in segregation especially IR2061 on traits including plant height and heading date. IR5657 were more uniform in plant height. This phenotypic variation seemed to correspond to the genotypic variation observed at the loci.

For variations of SSR on the short arm of chromosome 2, among the 48 IR2061 individuals tested, 24 plants belonged to Subtype IV, the remaining 24 plants did not belong to any of the 10 subtypes. Here, we referred them as offtype. Nineteen (Offtype I) of these 24 plants showed a larger band at locus RM174 than the band detected in all the IR64 individuals, the remaining 5 plants (Offtype II) showed a new allele at locus RM154 which was not detected in any of the IR64 individuals. For the female parent IR5657, 45 out of the 48 plants belonged to Subtype I, the remaining 3 plants (Offtype III) showed a smaller band at locus RM154 (Table 4). The results

Table 4. Five subtypes detected in the parental lines of IR64.

Parental line	Subtype [a]	Genotype at the SSR loci [b]							%
		RM154	RM110	RM211	RM233A	RM279	RM174	RM71	
IR2061	IV (24)	CC	AA	AA	AA	CC	BB	BB	50.00
	Offtype I (19)	CC	AA	BB	BB	BB	CC	BB	39.58
	Offtype II (5)	DD	BB	AA	AA	CC	BB	BB	10.42
IR5657	I (45)	BB	BB	BB	BB	BB	BB	BB	93.75
	Offtype III (3)	AA	BB	BB	BB	BB	BB	BB	6.25

[a] The number in the brackets indicate the number of plants observed.

[b] D indicates the band was larger than B but smaller than C in Offtype II.

Table 5. Segregation of heterozygous loci in Subtypes II and V at S_1 generation.

Subtype	Locus	Genotype at S_0	No. of plants showing the genotype at S_1				$P_{(1:2:1)}$
			BB	AB or BC	AA or CC	Total	
II	RM211	AB	13	24	9	46	0.68
	RM233A	AB	13	22	10	45	0.80
	RM279	BC	10	23	13	46	0.82
V	RM154	BC	8	28	10	46	0.31
	RM110	AB	18	19	9	46	0.09
	RM211	AB	16	23	7	46	0.17
	RM233A	AB	16	22	8	46	0.24
	RM279	BC	15	20	11	46	0.48

indicated that the two IR64 parental lines were genetically heterogeneous in chromosome 2 and other parts of the genome. IR64 was originated from a cross between a Subtype I female plant of IR5657 and a Subtype IV male plant of IR2061.

Genotyping of progenies from the breeder IR64

Based on the subtypes identified in the breeder seeds, 48 plants from each of the six subtypes (Subtype I, II, III, IV, V and VI) at each generation were genotyped for the variations of SSR loci on chromosome 2. Results indicated that the genotypes of S_1 (the first generation of selfed breeder IR64) and S_2 (the second generation of selfed breeder IR64) were the same as the breeder IR64 at all 7 loci (RM154, RM110, RM211, RM279, RM233A, RM174 and RM71). No new alleles were detected in the progenies. Uniform homozygotes at RM174 and RM71 were detected in all the progenies. At the remaining five loci, homozygotes detected in individual subtypes of the breeder IR64 were retained in their progenies, including all loci for Subtypes I, III, IV and VI, and RM154 and RM110 for Subtypes II. On the other hand, heterozygous loci detected in the breeder IR64 resulted in 1:2:1 allele segregation at S_1 generation, including RM211, RM233A and RM279 for subtype II, and RM154, RM110, RM211, RM233A and RM279 for Subtype V (Table 5). Similar results were observed at S_2 generation (data not shown). The results showed that alleles of SSR loci on chromosome 2 were inherited in a Mendelian manner both in S_1 and S_2, as well as in GRC-IR64. Since the stocks of IR2061 and IR5657 were from 1979 and 1990, respectively, we concluded that the alleles were stably inherited at least for 16-27 years.

DISCUSSION

In this study, SSR variation within rice cultivar was analyzed in a set of IR64-related lines. Although variations of SSR in a presumably pure-breeding variety could be generally resulted from mutation and outcrossing, our data suggest that the segregation of residual heterozygous loci from the parents might also contribute to the within cultivar variation. The main reason of mutation for microsatellite comes from replication slippage [9]. Variation could come from a repeat itself via gain or loss of repeats [11-13] or indels (inserts and deletions) in regions flanking the repeat motif. In present study, we did not investigate mutations on sequences of microsatellite loci and their flanking sequences. Instead, by tracing the genotypic variation back to the GRC-IR64 stock from 1989 and its parental lines from 1979 and 1990, we identified a region on the short arm of chromosome 2 in IR64 that explained the genotypic variations found in breeder seeds currently used as commercial variety. Variations involved in the region defined by 5 SSR loci covering approximately 30 cM . The source of variation came from its parental lines, IR5657 and IR2061.

While both the breeder IR64 and the GRC-IR64 were identical morphologically, but their parental lines were obviously in segregation for plant height and heading date. During the process of selection after crossing between the two parents, uniformity in phenotype was gradually achieved before it was

commercially released to farmers in 1985. The genotypic data showed that IR64 was a product between Subtype IV of IR2061 as male parent and Subtype I of IR5657 as female parent. The hybrid was heterozygous on at least five loci on the short arm of chromosome 2. Although the alleles at all five loci were inherited in a Mendelian manner in the early generations, Subtype I has become the dominant type. It appears that subsequent selfing and selection for phenotypic uniformity after many generations has made IR64 Subtype I as the dominant genotype that is homozygous for the chromosomal region. This genotype accounts for more than 70% in the sample of breeder seeds examined. Was Subtype I favored over the course of seed increase and line purification? Whether this was due to genetic drift or selection needs to be further studied.

In the breeder IR64 displaying heterogeneity, no phenotyical differences were shown. This was also true that SSR variations were detected in another indica variety IR8, but no phenotypic differences were found between IR8 plants from current seeds and the seeds which have been stored in GRC for 30 years, on many agronomic and physiological traits including plant height, days to heading, spikelet number per panicle, grain yield and its components, biomass yield, harvest index and photosynthetic rate (Dr. Peng Shaobing, personal communication, IRRI).

The present study indicated that within cultivar variation is common in rice. It changes the usual concepts of pure lines to which it can be explained phenotypically uniform with molecular heterogeneity that usually does not affect the phenotypes of cultivars unless a microsatellite is located within a functional gene associated with phenotypic changes. We also conclude that the SSR variations in the breeder IR64 are derived from residual heterogeneity preexisted in its original form same as to the GRC stock including both parental lines, rather than resulted from spontaneous mutation or outcrossing. Results from this study may have commercial application such as test of hybrid seed purity, varietal fingerprinting, and curation and propagation of germplasm collections.

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