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Full Length Article

Availability of Two-dimensional Bulked DNA Sampling in Uniformity Assessment of 8 Inbred Rice Lines

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Abstract

Poor efficiency of current SSRs on a plant-by-plant basis makes it hesitate to accept SSRs as an alternative for routine DUS test. The present study testified the availability of two-dimensional bulked DNA sampling in uniformity assessment of 8 inbred rice lines with commonly-used SSR markers. Our results showed that, (1) bulked sampling was suggested as 10 individuals, thus in a 10-row × 10-column matrix, the bulked samples could save 80% cost compared with routine SSRs; (2) the way to identifying true off-types from putative ones was modified and optimized as that all the intersections were considered as putative off-types, and further identifications were based on bulked SSR profiles or individual ones; (3) a putative off-type could be detected using 1 markers, or in some cases, 15 markers, and each marker equally detected 12.5 true off-types, ranged from 6 to 20; (4) true off-types were generally detected using 4 or more markers, so the strategy should be based on a group of markers rather than one to reduce the interfere of residual heterozygosity; (5) the very significant correlations (0.553 equally, concluded from the data of off-types) between results of the strategy and traditional morphological test made it prospective to indicate an exclusive acceptable uniformity baseline for SSRs. The reported strategy is available to evaluate uniformity of inbred rice lines with 20% cost of routine SSRs taking advantage of two-dimensional bulked DNA sampling (in a 10-row × 10-column matrix) and 24 commonly-used SSR markers. It could also be interest for the determination of stability and distinctness of inbred rice lines, as well as other non-hybrid plant varieties. However, we found the strategy couldn't effectively discriminate self-pollinated off-types from those true hybrid individuals.

Keywords: Two-dimensional bulked DNA sampling; Uniformity assessment; Inbred rice lines; SSR markers

Introduction

Before the breeder can be granted Plant Breeders' Rights (PBR), the candidate variety should be officially declared as being distinct (D: inter-cultivar variation), uniform (U: intra-cultivar homogeneity) and stable (S: homogeneity of generations), or DUS test (UPOV, 2010). DUS test are traditionally and primarily based on morphological Characteristics (Chen *et al.*, 2009; Furones-Pérez and Fernández-López, 2009).

In China, rice ranks No. 1 with regard to PBR applications. Stronger focus on resistance, yield and quality traits has led to more morphological similarity among rice candidate varieties, making morphological differentiation more complicate than ever before. Superiorities of molecular assay over morphological assay are well acknowledged, including time-, labor-, space-saving etc. Simple sequence repeats marker (SSR) is identified as the most widely used marker system for plant variety characterization by the Biochemical and Molecular Technique (BMT) Group of International Union for the Protection of New Varieties of Plants (UPOV) (Gong and Deng, 2012; UPOV, 2013). Handbook 'Identification of Rice (*Oryza sativa* L.) Varieties Using Micro-satellite Markers' (NY/T 1433-2007, hereinafter refer as 'Handbook') was issued in 2007 (Zhang *et al.*, 2007) and revised in 2014. SSR markers in the 'Handbook' are successfully applied as an assist for rice distinctness test. However, their applications on uniformity determination are seldom reported. When it comes to uniformity assessment, large-scale molecular evaluation on a plant by plant basis (e.g., for rice, 400 individuals × a set of primers = thousands of reactions) is required to scan the intra-variety uniformity, eroding its high efficiency and low cost in DUS test.

The strategy of two-dimensional bulked DNA sampling was proposed in 2002 and was reported to assess the seed purity of certain rice parental lines using parent-specific markers (Nas *et al.*, 2002; Sundaram *et al.*, 2008).

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Other commonly-used markers as well as other rice varieties, however, haven't been reported in this regard, and application of this strategy in uniformity assessment of plant varieties hasn't been covered either.

Therefore, it is unknown whether two-dimensional bulked DNA sampling can be applied in uniformity assessment of inbred rice lines with commonly-used SSR markers. In order to improve the unbearable efficiency of routine SSRs on a plant by plant basis, two-dimensional bulked DNA sampling and commonly-used SSR markers were jointly used in this study to evaluate uniformity of inbred rice lines with 20% cost of routine SSRs. In the process of identifying true off-types from putative ones, modifications and optimizations were first proposed in our study to improve accuracy and efficiency. The strategy may also be interest for the determination of stability and distinctness of inbred rice lines and may be extended to other non-hybrid plant varieties alike. For the first time, the strategy was announced unable to effectively discriminate self-pollinated off-types from those true hybrid individuals.

Materials and Methods

Materials

Eight inbred rice lines were used for this study, including 5 *japonica* varieties and 3 *indica* varieties (Table 1). Field trials were carried out in paddy field ensuring satisfactory growth (Wei *et al*., 2010). Seeds were sown in April, 2012, and 4-weeks-old seedlings were transferred to paddy field at the four-leaf stage, in randomized complete plot designs with four replications. Each plot was composed of 100 plants, in a '10-row × 10-column' grow-out matrix. Individual plant was coded as 'Variety No. – Plot No. – Row No. – Column No.' At the time of heading, leaf tissue was sampled using a punch with a diameter of 1 cm for DNA extraction.

Availability of the Way of Bulked DNA Sampling in Detecting Diluted Heterogeneity

To find the optimal ratio of bulked samples, DNA pools of stepwise mixtures of 3-, 5-, 7-, 10-, 12-, 15- and 20-component were prepared (i.e., leaf of Fengdao26 was mixed with Chujing27 to concentration percentages of 1/3, 1/5, 1/7, 1/10, 1/12, 1/15, 1/20 respectively) and the sensitivity of SSR assay to gradually diluted heterogeneity was investigated. DNA from individual plant was involved as control.

DNA extractions were conducted according to a modified CTAB protocol (Smýkal, 2006), then treated with 1 mg/mL RNase for 1 h at 37°C. DNA quality and quantity was assessed on a 1% (w/v) agarose gel stained with ethidium bromide. DNA quantification was conducted using a NanoDrop®ND-1000 Spectrophotometer.

DNA was amplified using 24 primer pairs (Table 2). Reaction mixtures (20 μL) contained 0.2 μM of primer, 400 μM of deoxyribonucleotides, 100 mM KCl, 20 mM Tris HCl (pH 8.3), 3 mM $MgCl_2$, 20 ng of DNA, and 0.5 unit of *Taq* DNA polymerase. The amplification conditions involved an initial step of 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final extension at 72°C for 10 min.

The PCR products (3 μL) were separated on 6% acrylamide gels and electrophoresed at 1,700 V for 2 h. The gel was then placed in a 10% glacial acetic acid fixation solution for 20 min with gentle shaking, silver-stained for 30 min and immediately developed in a 3% sodium carbonate solution as described in the DNA silver staining kit (Promega Cat. Q4132).

All analyses were performed at least twice to ensure reproducibility of the results. Allele sizes were determined for each SSR locus using the Beckman CEQ fragment analysis software.

Uniformity Assessment of 8 Inbred Rice Lines using the Strategy of Two-dimensional Bulked DNA Sampling and the 24 SSR Markers

The strategy of two-dimensional bulked DNA sampling was introduced to identify putative off-types of Plot 1 of Chujing27 using above markers and optimal bulked sampling. Rows (or columns) with SSR profiles different from the typical one of Chujing27 were considered as impure rows (or columns). Plants located on intersections of the impure row(s) and column(s) were considered as putative off-types (Sundaram *et al.*, 2008). Putative off-types were further verified either through the size of heterozygous alleles of bulked DNA, or through individual SSR profile of suspected plant alone.

In the same way, putative off-types of the rest 3 plots of Chujing27 and the other 7 varieties were identified using the strategy.

Routine DUS test to Validate Availability of the Strategy

Grow-out test was also carried out to identify off-types. Fifty-one characteristics provided in DUS test Guidelines (Wei *et al.*, 2010) were applied. Details regarding to off-types were set down, including their codes, their differences in appearance from typical plants. Results were compared with those obtained above.

Results

Availability of the Way of Bulked DNA Sampling in Detecting Diluted Heterogeneity

Allele sizes of the two independent plants of Chujing27 and Fengdao26 (Fig. 1, Lane 1 and Lane 2) corresponded with those of the admixed samples (Fig. 1, Lane 3 – Lane 9), indicating diluted heterogeneity can be detected where DNA

of several plants was pooled. Visibility of the amplified fragments of Fengdao26 decreased gradually as its genomic DNA was diluted with increasing DNA of Chujing27 (Fig. 1). Among the 7 sets of stepwise mixtures, alleles were clear at a dilution ratio of 1/10 or less (Fig. 1). Although allele was still vaguely detectable at a dilution ratio of 1/20 in a DNA pool, the ratio of 1/10 was recommended here considering the precision requirement of DUS test.

Uniformity Assessment of 8 Inbred Rice Lines using the Strategy of Two-dimensional Bulked DNA Sampling and the 24 SSR Markers

Uniformity of Plot 1 of Chujing27 (V1) was assessed at first. Genomic DNA of bulked leaves of 10 plants in the same row or same column was extracted. Totally, 20 pooled DNA samples (10 rows + 10 columns = 20 samples) could represent 100 individuals (10 plants per row × 10 plants per column = 100 individuals) and were amplified using the 24 SSR markers (Table 2). It was observed that, Row 6 and Column 9 had heterozygous amplifications generated using the SSR markers of RM336 and RM471 (Fig. 2), indicating the intersection of R6C9 of the matrix would be putative off-type (Fig. 3A). RM219 and RM71 detected two impure rows and two impure columns (Fig. 2), and thus the four intersections of Row 2, Row 6 and Column 3, Column 9 (R2C3, R2C9, R6C3 and R6C9) could be putative off-types (Fig. 3A). R2C9 and R6C3 can be excluded from off-types in that Row 2 and Column 9, Row 6 and Column 3 showed different heterozygous allele sizes with respect to RM71 (Fig. 2). Such hints can't be applied to the cases of identical profiles of heterozygous alleles e.g., those amplified using RM219 (Fig. 2). To double-check, DNA extracted from the four plants individually was amplified using the above four pairs of primers respectively. Two plants (R2C3 and R6C9) were revealed as off-types with distinct allele patterns, while the other two plants (R2C9 and R6C3) not (Fig. 3B, C, D, E). Besides the above four pairs of primers in Group A, other 10 markers in Group B also gave similar results.

There was variation among different varieties as well as different markers in terms of the degree of heterogeneity observed (data not shown). A putative off-type was in some cases detected using 1 marker, or sometimes, 15 markers (data not shown). On average, each marker detected 12.5 true off-types, with the minimum of 6 off-types (RM190) and the maximum of 20 off-types (RM471).

Routine DUS Test to Validate Availability of the Strategy

Grow-out test on Plot 1 of Chujing27 (V1) was conducted in this regard to verify the putative off-types and their locations. Based on the 51 descriptors provided in 'Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability – Rice' (Wei *et al.*, 2010), two plants (coded as 'V1-P1-R2-C3' and 'V1-P1-R6-C9') were determined as putative off-types, identical to the molecular assay.

Table 1: Rice inbred lines used in study

Code	Inbred lines	Type	Code	Inbred lines	Type
V1	Chujing27	*Japonica*	V5	Fengdao26	*Japonica*
V2	Xiujing12	*Japonica*	V6	Diantun502	*Indica*
V3	Yunjingyou1	*Japonica*	V7	Dianlong201	*Indica*
V4	Hexi35	*Japonica*	V8	Banna21	*Indica*

Table 2: SSR markers employed

†C	Group A	Group B	†C	Group A	Group B
1	RM583	RM1195	7	RM336	RM481
2	RM71	RM208	8	RM72	RM339
3	RM85	RM232	9	RM219	RM278
4	RM471	RM119	10	RM311	RM258
5	RM274	RM267	11	RM209	RM224
6	RM190	RM253	12	RM19	RM17

†C: chromosome distribution of each marker

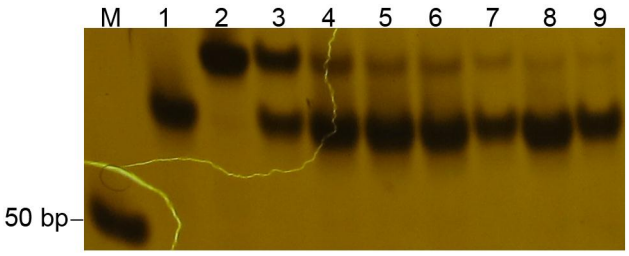

Fig. 1: Availability of the way of bulked DNA sampling in detecting diluted heterogeneity using SSR marker RM85

DNA from leaf material(s) of individual plant or plants was amplified using RM85. M - DM500, Lane 1 - DNA from leaf material of individual plant of Chujing27, Lane 2 - DNA from leaf material of individual plant of Fengdao26, Lane 3～Lane 9: DNA from bulked leaf materials where leaf of Fengdao26 was mixed with that of Chujing27 to concentration percentages of 1/3, 1/5, 1/7, 1/10, 1/12, 1/15 and 1/20 respectively

Differences between the two plants (R2C9 and R6C3) and the typical plants involved lemma color, grain length, time of heading etc. The results indicated the strategy of two-dimensional bulked DNA sampling is available to evaluate the uniformity of inbred rice lines.

However, results of this strategy and morphological assays were not always in total agreement with each other. Heterozygous alleles were amplified e.g., using RM258 (Group B) in Row 3 and Column 5 of Plot 1 of Chujing27, but R3C5 was not off-type under the judgment of robust morphological characteristics. Such disparity can also be found in the rest 3 plots of Chujing27 and the other 7 varieties (data not shown). Molecular markers were too sensitive to mistake some typical plants as off-types due to residual heterozygosity (Vanniarajan *et al.*, 2012; Ye *et al.*, 2013). Plants with residual heterozygosity can't always be counted as true off-types since only distinctness in appearance can be acknowledged in DUS test (UPOV, 2013). In our study, true off-types were generally detected using 4 or more markers, while false off-types showed heterogeneous profiles in only 1 or 2 marker(s). Therefore, different from

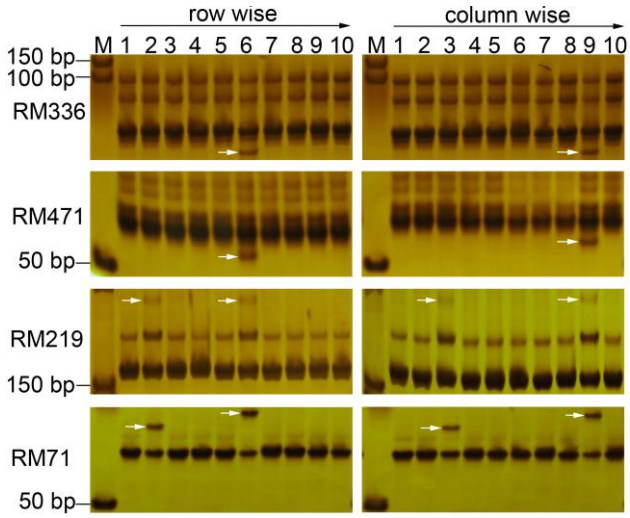

Fig. 2: Identification of contaminated rows and columns of 100 plants of Chujing27 grown in a 10 × 10 matrix using SSR markers RM336, RM471, RM219 and RM71

DNA extracted from bulked leaf materials of 10 plants of the same row or column was applied for SSR assay. A total of 20 pooled samples can represent 100 plants: 10 rows (row-wise) × 10 columns (column-wise). Row-wise Lane 2 & lane 6, and column-wise Lane 3 & Lane 9 (indicated by arrows) represent putative contaminated ones. M - DM500

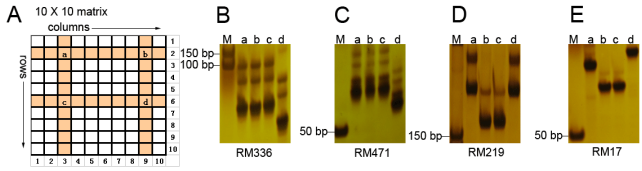

Fig. 3: Identification of putative off-types based on individual SSR profile using RM336, RM471, RM219 and RM71

(A): Schematic of 10 rows × 10 columns matrix, where colored rows and columns represent contaminated ones, and their intersections indicate putative off-types, marked as a, b, c and d respectively. (B)~(E): individual SSR profiles of the four putative off-types, where M - DM500, Lane a - plant 'a' marked in schematic, Lane b - plant 'b' marked in schematic, Lane c - plant 'c' marked in schematic, Lane d - plant 'd' marked in schematic

morphological characteristics, where one obvious difference is enough to distinguish varieties, SSR assay should be based on a group of markers rather than one.

Despite the disparity, there existed significant correlation between markers of Group A and Group B (correlation coefficient = 0.365). Both groups of markers also gave very significant correlated results with routine DUS test (field trial) (0.626 and 0.491, respectively). Correlations here were concluded from the data of off-types. Given the data of whole sample, such correlations would be more significant (data not shown). On account of the great correlation, it is desired to indicate an acceptable uniformity baseline for molecular assay based on practical experience of a large set of varieties.

Discussion

Here, 20 bulked samples (10 rows + 10 columns) can represent the original 100 individuals, thus cutting the cost by 80%. Compared with previous researches, which paid great attention to multiplex PCR or simplifying DNA extraction to improve the efficiency (Ali *et al.*, 2011; Monden *et al.*, 2014), the strategy of two-dimensional bulked DNA sampling (in a 10-row × 10-column matrix) can save time and labor dramatically without any simplification. To gain ideal SSRs, the assays were optimized to avoid weak amplification, e.g. increasing amplification cycles to 40 to improve PCR yield and removing RNA for the sake of precise DNA quantification.

It is worth mentioning that, we found the way to identifying true contaminants from putative ones in previous research were improper (Sundaram *et al.*, 2008). We proposed that all of intersections of impure row(s) and column(s) should have been considered as putative off-types, and further discrimination should have based on the size of heterozygous amplification of bulked samples or on the individual SSR profile of the putative off-type alone.

Inbred rice lines can be efficiently analyzed for uniformity using the said strategy. Given seed samples from consecutive generations of a variety, stability could likewise be assessed. It may also be possible to use bulked samples of a variety to produce an overall SSR profile for distinctness determination, in which case residual heterozygosity arguably becomes less of an issue. And thus, DUS test might be expectedly accomplished in this context. Besides rice, the present study can also be extended to other vegetatively propagated or self-pollinated crops. However, our strategy is not applicable to distinguishing self-pollinated contaminants from hybrid plants, which hasn't been mentioned in previous researches. The reason could be explained as follows: the true hybrid individuals in the bulked samples may produce MPS (male parent specific) and FPS (female parent specific) allelic types simultaneously, which will mask the absence of MPS or FPS alleles of self-pollinated plants. Such confusion may be avoided with future consideration in accurate quantification of DNA content and PCR output in SSR assay.

Conclusion

Aside from the availability of two-dimensional bulked DNA sampling in evaluating seed purity of certain rice parental lines using parent-specific markers, our study found that two-dimensional bulked DNA sampling can assess uniformity of inbred rice lines with commonly-used markers, which can cut the unbearable cost of routine SSRs by 80% (in a 10-row × 10-column matrix). Due to residual heterozygosity, off-type determination in this regard should be based on a group of markers rather than one, which is

different from morphological characteristics where one obvious difference is enough. In view of the great correlation between results of this strategy and routine morphological assessment, it is desired to indicate an acceptable uniformity baseline for this strategy exclusively. The strategy could also be interest for the determination of stability and distinctness of inbred rice lines, as well as other non-hybrid plant varieties, but it couldn't effectively discriminate self-pollinated off-types from those true hybrid individuals.

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