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Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers

N. Nandakumar[1], A.K. Singh[1,*], R.K. Sharma[1,2], T. Mohapatra[2], K.V. Prabhu[1,3] & F.U. Zaman[1]

[1]*Division of Genetics, Indian Agricultural Research Institute, New Delhi – 12, India;* [2]*National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi – 12, India;* [3]*National Phytotron Facility, Indian Agricultural Research Institute, New Delhi – 12, India; (*author for correspondence; e-mail: aks_gene@lycos.com)*

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Summary

Microsatellite markers were used for fingerprinting of hybrids, assessing variation within parental lines and testing the genetic purity of hybrid seed lot in rice. Ten sequence tagged microsatellite sites (STMS) markers were employed for fingerprinting 11 rice hybrids and their parental lines. Nine STMS markers were found polymorphic across the hybrids and produced unique fingerprint for the 11 hybrids. A set of four markers (RM 206, RM 216, RM 258 and RM 263) differentiated all the hybrids from each other, which can be used as referral markers for unambiguous identification and protection of these hybrids. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped the hybrids into three clusters. Within the cluster all the hybrids shared a common cytoplasmic male sterile line as female parent. The genetic similarity between the hybrids ranged from 0.33 to 0.92 with an average similarity index of 0.63. The analysis of plant-to-plant variation within the parental lines of the hybrid Pusa RH 10, using informative markers indicated residual heterozygosity at two marker loci. This highlights the importance of STMS markers in maintaining the genetic purity of the parental lines. The unique value of the restorer gene linked marker for testing the genetic purity of hybrid seeds is demonstrated for the first time.

Introduction

Rice is the principal food crop, grown across 18 different countries feeding more than half of the world's population (Virmani, 1999). Ever since the report of Jones (1926), exploitation of heterosis has been contemplated as a potential strategy for yield enhancement in rice, which became a reality after the commercial success of rice hybrids in China. At present hybrid rice is commercially cultivated in China, India, Vietnam and Philippines. India, a predominantly rice growing country has released as many as 17 hybrids including a superfine grain aromatic rice hybrid for commercial cultivation.

At present, the hybrid seed production in rice is primarily based on three-line system, which involves a cytoplasmic male sterile (CMS) line or A line, a corresponding iso-nuclear maintainer (B) line and a genetically diverse restorer (R) line. The A line is maintained by crossing it with B line and hybrid seed is produced by crossing A line with R line. Assessment and maintenance of genetic purity of the parental lines and hybrids is crucial for the successful adoption of hybrid rice technology. Molecular markers have the potential in achieving this goal (Jena & Pandey, 1999; Yashitola et al., 2002). Besides, molecular markers are also useful in classifying hybrid breeding oriented source germplasm into heterotic pools and in prediction of hybrid performance (Xu et al., 2002). Genetic enhancement of parental lines for disease resistance (Chen et al., 2001), yield components and quality traits can also be achieved by marker assisted selection (Zhou et al., 2003).

Unambiguous identification of elite crop varieties and hybrids is essential for their protection and prevention of unauthorized commercial use. In India, this

is highly relevant especially in rice because the hybrid seed production and marketing of public sector bred hybrids is largely taken up by the private sector. A set of qualitative and quantitative characters known as descriptors are currently in use for variety identification and description. Some of these characters, particularly those showing quantitative inheritance, interact with the environment in which the variety is grown and thus make the process of variety identification subjective. Molecular markers, in contrast, being based on DNA sequence variation, provide an unbiased means of identifying crop varieties. The Biochemical and Molecular Techniques group of the International Union for the Protection of New Varieties of Plants (UPOV) is evaluating different DNA marker parameters prior to its routine use in establishing distinctness, uniformity and stability (DUS) of plant varieties (Bredemeijer et al., 2002; Röder et al., 2002; UPOV-BMT, 2002). Among the various DNA-based markers currently available, genetically mapped sequence tagged microsatellite sites (STMS) are the markers of choice in rice because of their abundance, co-dominant nature and uniform distribution throughout the genome (McCouch et al., 1997). Further, the nature of chromosome specificity, greater level of allelic diversity, high power of resolution, operational ease and low cost (Chen et al., 1997; Garland et al., 1999) make the STMS markers more suitable for fingerprinting. Availability of more than 2740 mapped microsatellite markers with an average density of one STMS for every 157 kb (<1 cM) of the rice genome (Cho et al., 2000; McCouch et al., 2002; Temnykh et al., 2000) has greatly improved their utility.

The primary objective of the present study was to identify the public sector bred Indian rice hybrids and differentiation of their parental lines by employing STMS markers. The present paper describes the utility of a small set of STMS markers in establishing the identity of 11 different rice hybrids. Use of these markers in the assessment, maintenance and testing of the genetic purity of the parental lines and hybrid seed of a commercially important fine grain aromatic rice hybrid Pusa RH 10 is also demonstrated.

Materials and methods

Plant materials

For the purpose of molecular characterization, 11 public sector bred rice hybrids released for commercial cultivation in different parts of India and their parental lines (Table 1) were used. The F_1 seed of all the hybrids except Pusa RH 10 and their parental lines were obtained from Directorate of Rice Research, Hyderabad. The seeds of Pusa RH 10 and its parental lines Pusa 6A (the male sterile female), Pusa 6B (the maintainer) and PRR 78 (the restorer) were obtained from the concerned breeder, Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi. For the purpose of marker analysis, seeds were germinated using sterile media under aseptic condition in National Phytotron Facility, IARI, New Delhi. Fifteen phenotypically identical plants in case of Pusa 6A, Pusa 6B and PRR 78 were used to study plant-to-plant variation within the parental lines. A random sample of 450 seeds of Pusa RH 10 representing the commercial F_1 seed lot, produced at IARI seed production plot was used for testing their genetic purity. Out of 450, 50 randomly drawn F_1 seeds were used for marker analysis and others were used for Grow-out test (GOT). The GOT was conducted at IARI Regional Rice Breeding and Genetics Center, Aduthurai, Tamil Nadu, during *Rabi*-2002.

Table 1. Details of hybrids and their parental lines used in the study

S. No.	Hybrids	CMS lines	Restorer lines
1.	Pusa RH 10	Pusa 6A	PRR 78
2.	APHR 2	IR62829A	MTU 9992
3.	CORH 1	IR62829A	IR10198
4.	CNRH 3	IR62829A	Ajaya
5.	DRRH 1	IR58025A	IR40750
6.	KRH 2	IR58025A	KMR-3
7.	PSD 1	IR58025A	UPRI-93-133
8.	CORH 2	IR58025A	C20R
9.	ADTRH 1	IR58025A	IR66
10.	KJTRH 1	IR58025A	BR827-35
11.	NSD 2	IR58025A	NDR 30264

Molecular analysis

Plant DNA was isolated following the procedure of Doyle & Doyle (1990). For fingerprinting, DNA from the bulk of leaf samples of 10 individual plants was used. Quantification of DNA was accomplished by analyzing the DNA on 0.8% agarose gel using diluted uncut lambda DNA as standard. DNA was diluted in

$Tris_{10}$:$EDTA_1$ buffer to a concentration of approximately 25 ng/μL for PCR analysis.

Based on our earlier study where 44 STMS primers representing all the 12 chromosomes of rice were analyzed (Mishra et al., 2003), 10 most informative markers viz., RM 220 (1), RM 250 (1), RM 263 (2), RM 234 (7), RM 201 (9), RM 216 (10), RM 228 (10), RM 258 (10), RM 206 (11) and RM 247 (12) (where, the figures in the parenthesis refers to the chromosomal location of the respective markers) were selected for parental polymorphism survey. The polymorphic markers were employed for studying plant-to-plant variation within Pusa 6A, Pusa 6B and PRR 78. For testing the genetic purity of hybrid seed lot of Pusa RH 10 the STMS marker RM 258, which is linked to the fertility restorer gene at a distance of 9.5 cM (Mishra et al., 2003) in the pollen parent (PRR 78) was used. The sequence information for the primer pairs was obtained from the publications of Wu & Tanksley (1993), Chen et al. (1997) and Temnykh et al. (2000) and were synthesized from Life Technologies Inc., U.S.A. DNA amplification was carried out in a 10 μL reaction mixture containing 1 $\times$ PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$), 200 μM of each of dNTPs, 12 ng each of forward and reverse primers, 0.2 units of Taq DNA polymerase (Bangalore Genie Pvt. Ltd., Bangalore, India) and 25 ng of genomic DNA template. The amplification reaction was carried out in a thermal cycler (Perkin Elmer, Model 9600). The first cycle consisted of denaturation of template DNA at 94°C for 5 min, primer annealing (55°C) for 1 min and primer extension (72°C) for 2 min. In the next 33 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time remained as in the first cycle. The last cycle consisted only of primer extension (72°C) for 7 min.

PCR products were separated on 3% Metaphor™ agarose gel containing the nucleic acid stain Gel Star™ (MBI Fermentas, Lithuania) using 1 $\times$ TBE buffer. The size of the amplified fragments was determined by using size standards (50 bp DNA ladder, MBI Fermentas, Lithuania). DNA fragments were visualized under UV light and photographed using Polaroid photographic system.

Cluster analysis

The amplified products were scored as *present* (1) or *absent* (0) for each primer genotype combination. The data entry was done into a binary data matrix as discrete variables. Jaccard's coefficient of similarity was calculated and a dendrogram based on similarity coefficient was generated using Unweighted Pair Group Method based on Arithmetic Mean (UPGMA) through the computer package NTSYS-PC (Rohlf, 1998).

Results

Fingerprinting of rice hybrids

The 11 rice hybrids and their parental lines were analyzed for the microsatellite polymorphisms. The parental polymorphism survey identified nine informative markers (RM 201, RM 206, RM 216, RM 228, RM 234, RM 247, RM 250, RM 258 and RM 263), which were used for fingerprinting the hybrids. Markers RM 206, RM 228 and RM 263 amplified a maximum of three alleles whereas all the other polymorphic markers detected only two alleles across the genotypes. Two markers RM 201 and RM 247 amplified alleles specific to the restorer PRR 78. The nine markers together amplified a unique fingerprint for all the hybrids and therefore, were effective in distinguishing them from each other (Figure 1). The fingerprints of CMS lines and the restorer lines were also distinct, except two CMS lines Pusa 6A and IR 58025A, which had identical profiles with respect to all the informative markers used in the study (data not shown). Nevertheless, four markers RM 206, RM 216, RM 258 and RM 263 together differentiated all the 11 hybrids and the restorer lines at least with a single marker allele difference. A single marker RM 234 amplified identical patterns for hybrids sharing common CMS line and therefore could clearly classify the hybrids based on their female parent identity. The STMS profiles of the hybrids with respect to the markers RM 234 and RM 258, a restorer gene linked marker are given in the Figures 2a and 2b, respectively.

The cluster analysis grouped the 11 hybrids into three major clusters, where, within a cluster, the hybrids shared a common CMS line (Figure 3). The Jaccard's similarity coefficient based on nine STMS marker loci ranged from 0.33 to 0.92 with an average similarity index of 0.63. Cluster I consisted of a single hybrid Pusa RH 10, which had 51.8% similarity with rest of the hybrids. Cluster II consisted of IR58025A based hybrids and recorded an average similarity of 0.69 between them. Cluster III consisted of three rice hybrids having IR62829A as maternal parent with a similarity index of 0.50.

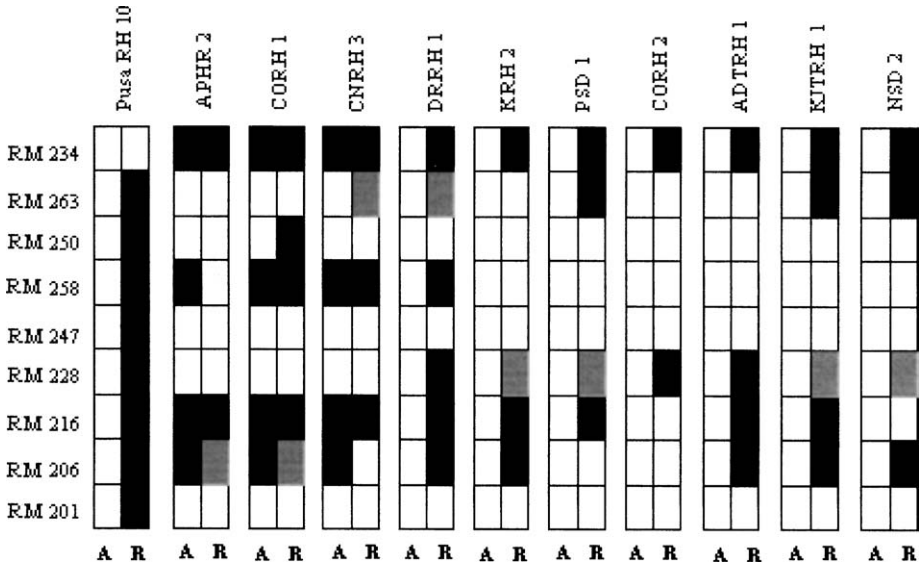

Figure 1. Diagrammatic representation of DNA fingerprints of eleven rice hybrids based on nine STMS markers. In each hybrid the column A and R represent the allelic profile of CMS (A) and restorer (R) parent, respectively. The shades indicate different alleles amplified by the respective STMS marker in the hybrids.

Assessment of genetic heterogeneity within parental lines

The genetic heterogeneity, if any, within the parental lines could lead to lack of uniformity in the commercial F_1 seeds resulting into poor acceptance of the hybrid produced. Since the STMS markers are co-dominant, they can be used for unambiguous identification of both the homozygotes and the heterozygotes at a particular marker locus. Thus, these markers have the potential to test genetic heterogeneity within the parental lines. Out of 45, i.e., 15 plants each of Pusa 6A, Pusa 6B and PRR 78 (the parental lines of the hybrid Pusa RH 10) analyzed using nine polymorphic STMS markers, a single plant at the marker locus RM 228 and two plants at the marker locus RM 247 were found to be heterozygous in case of Pusa 6A. However, no plant-to-plant variation was observed within Pusa 6B and PRR 78. The experiments were repeated twice to confirm the findings from fresh genomic DNA samples of the same plants.

Testing genetic purity of hybrid seeds

Determining the genetic purity of hybrid seed is an essential requirement for its commercial use, since there is always a chance of contamination in the hybrid seed production plot because of pollen shedders, out crossing and physical mixtures during the subsequent handling of the harvested material. To test the genetic purity of Pusa RH 10 seeds, an STMS marker RM 258, which is polymorphic between Pusa 6A and PRR 78 and also linked to fertility restorer gene at a distance of 9.5 cM (Mishra et al., 2003) in PRR 78 was used. The marker amplified approximately 155 and 145 bp fragments in Pusa 6A and PRR 78, respectively. In a random sample of 50 seeds the marker identified a single pollen shedder (B line) seed, which had a CMS line specific fragment (Figure 4). This amounts to 2% off types in the hybrid seed produced. The results were confirmed using 400 seeds from the same seed lot through Grow-out test (GOT).

Discussion

In this paper we report the identification of rice hybrids and their respective parents, assessment of plant-to-plant variation within parental lines and testing genetic purity of rice hybrids using microsatellite polymorphisms. The heterotic potential of all the 11 hybrids analyzed in this paper is already established and they are being commercially cultivated in different parts of India. Therefore, the molecular fingerprinting of the hybrids and their parental lines assumes utmost importance for protecting the Plant Breeders' Rights (PBR)

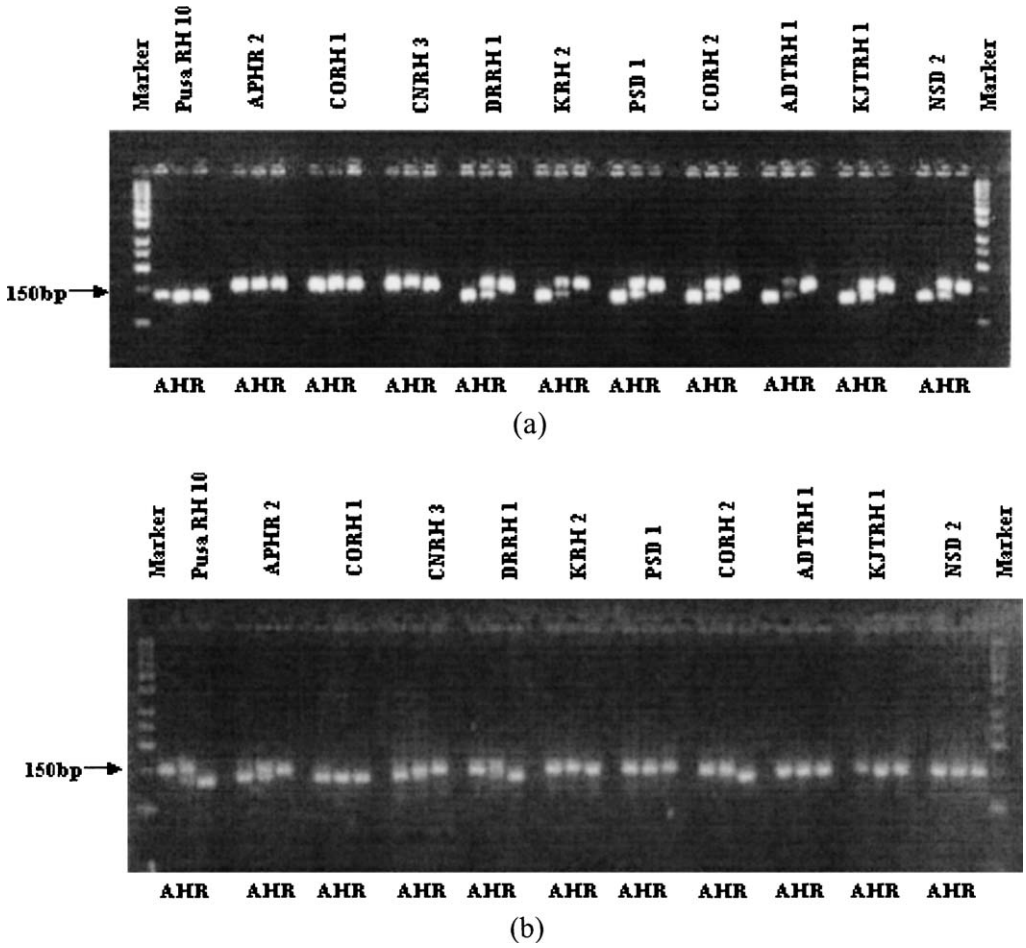

Figure 2. The molecular profiles of 11 rice hybrids obtained with (a) RM 234 and (b) RM 258. Marker = 50 bp ladder, A = CMS line, H = hybrid, R = restorer line.

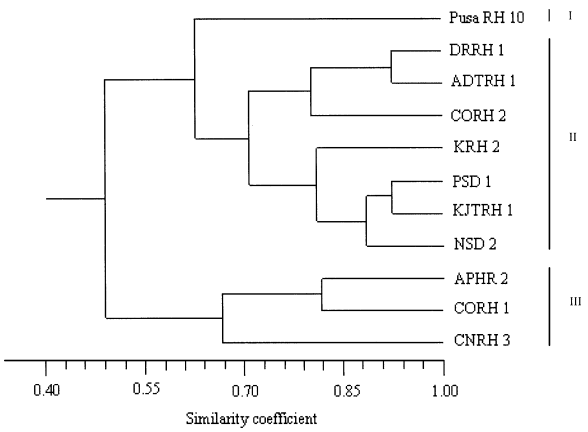

Figure 3. Dendrogram of commercially cultivated Indian rice hybrids constructed using UPGMA based on nine STMS markers. Scale at the bottom is Jaccard's coefficient of similarity.

on them and ensuring their genetic purity. Even though the utility of RAPD fingerprints for plant variety protection is known in case of rice hybrids (Wang et al., 1994), the microsatellite markers are considered more reliable because of their ability to produce high fidelity profiles as a result of their co-dominant nature and chromosome specificity. The nine polymorphic markers clearly distinguished all the hybrids and the restorer lines used in the study. Further it was found that four STMS markers (RM 206, RM 216, RM 258 and RM 263) alone could precisely distinguish all the 11 hybrids from each other, even though all the nine markers were informative. Thus, RM 206, RM 216, RM 258 and RM 263 can be used as a set of referral STMS markers for identification of the 11 hybrids analyzed. But the entire set of nine markers is still relevant and can be used to differentiate future rice hybrids from these existing hybrids.

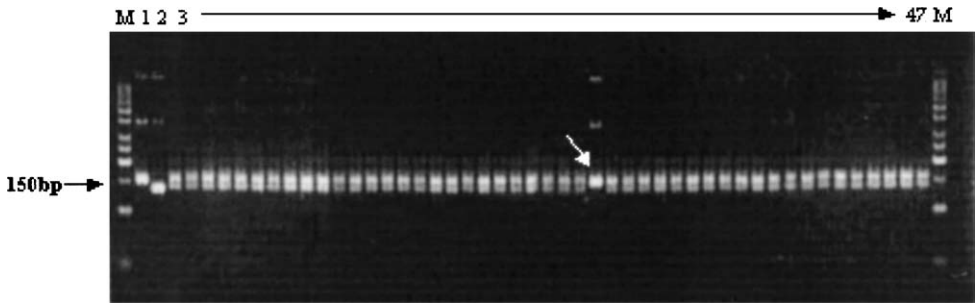

Figure 4. Testing genetic purity of hybrid seeds of Pusa RH 10 using the STMS marker RM 258 linked to restorer gene in PRR 78, the male parent of the hybrid. M = 50 bp ladder, Lane 1 = Pusa 6A (CMS line), Lane 2 = PRR 78 (restorer line) and Lane 3–47 = individual F_1 plants representing a random sample from hybrid seed lot of Pusa RH 10. Lane 29 (arrow) represents a B line plant, a contaminant.

Among the restorer lines, the overall marker profile of PRR 78 suggests that it is diverse from all other restorers, which could be because PRR 78 is a Basmati quality iso-cytoplasmic restorer line developed through selective intermating in F_2 and subsequent generations of a partially restored Basmati quality rice hybrid. Because of the rarer alleles contributed by PRR 78, the markers RM 201, RM 234 and RM 247 amplified specific patterns for the hybrid Pusa RH 10, enabling the identification of the hybrid using any of these markers. Since the marker RM 234 is monomorphic, unlike the other two markers it cannot differentiate the hybrid from its parental lines, even though it is useful in differentiating Pusa RH 10 from other hybrids. Identification and use of such hybrid specific markers can effectively reduce the cost and simplify the procedure of hybrid identification.

At present the DUS testing for the purpose of registration of new plant varieties as per UPOV guidelines is primarily based on essential characters and in certain crops isozyme markers are also being used. The DUS testing based on phenotype and isozyme expression suffers from the limited number of target traits and genotype × environment interactions when the candidate variety is evaluated across the environments. Therefore, the use of DNA markers for establishing distinctness, uniformity and stability (DUS) of plant varieties is being contemplated (Bredemeijer et al., 2002; Röder et al., 2002; Singh et al., 2004; UPOV-BMT, 2002). If accepted, the DNA-marker-based DUS testing will effectively augment the process of discrimination of the candidate varieties and hybrids. The efficacy of the STMS markers in discretely identifying the rice hybrids has been clearly brought out by the present study.

The 11 rice hybrids studied have been released for commercial cultivation in diverse geographical locations across India and therefore should be genetically diverse. This is obvious from the range of similarity coefficient between the hybrids (0.33–0.92) obtained in the dendrogram analysis. However, a maximum similarity of 92% was observed between DRRH 1 and ADTRH 1 and also between PSD 1 and KJTRH 1, which share a common maternal parent IR58025A. Therefore, diversification of the nuclear background of the CMS lines is important for enhancing the level of heterosis. The hybrid Pusa RH 10 formed a separate cluster owing to the diverse nature of its restorer parent PRR 78. Although based on limited number of markers, this is the first report regarding genetic relatedness among the commercial Indian rice hybrids. The study should be logically extended with more number of markers providing genome wide coverage.

Keeping in view the need to maintain the genetic purity of the parental lines in hybrid breeding, their molecular fingerprinting using reliable markers such as STMS assumes significance. Pusa RH 10 developed at IARI, New Delhi, is the latest and the only superfine grain aromatic rice hybrid released for commercial cultivation, which occupies substantial area in the Basmati growing regions of India. This hybrid matures 20 days early with 40% higher yield as compared to the best Basmati check variety Pusa Basmati 1 and has excellent grain and cooking quality. Considering the commercial significance of the hybrid in domestic and export market, Pusa RH 10 and its parental lines were selected for this study as a model case. Further, the fertility restorer gene linked marker RM 258, in PRR 78, the pollen parent of Pusa RH 10 was already known (Mishra et al., 2003), which we could use for testing genetic purity of hybrid seeds.

The molecular marker profiling of individual plants of Pusa 6A, Pusa 6B and PRR 78 indicated the existence of plant-to-plant genetic variation at two marker loci, viz., RM 228 and RM 247 in case of

Pusa 6A, although all the plants selected for molecular profiling were phenotypically similar. Any variation in the A line has to come from the corresponding B line as the former is maintained by crossing with the latter. However, no plant-to-plant variation at respective marker loci (RM 228 and RM 247) was observed within Pusa 6B and PRR 78. This could be due to limited sample size analyzed. Such variation at the molecular level are, expected to be there even after several generations of maintenance since it is primarily based on phenotype, which is perhaps not influenced by variations for marker alleles in the non-coding region. The residual heterozygosity or the heterogeneity detected at the molecular level are, however, likely to result in the breakdown of CMS lines in longer run (Jena & Pandey, 1999). Therefore, it is recommended that the A and B line plants having similar marker profile should be used for paired mating to produce genetically homogenous seeds of the A line. Eventhough this study was conducted using the parental lines of only one hybrid; the results are expected to be applicable to the parental lines of other hybrids as well. However, the extent of heterogeneity and the marker loci under question may vary depending upon pedigree and maintenance of the parental lines.

The commercial success of hybrid rice technology depends to a large extent on the quality of hybrid seed supplied, especially the genetic purity. Conventionally the genetic purity of the hybrids is assessed by the GOT. However, GOT requires one full season thus precluding the immediate cultivation of the hybrid seed produced. In addition, locking up of the capital invested on hybrid seed production and additional expenditure incurred on storage of hybrid seed ultimately increases the hybrid seed cost. This limitation and the environmental dependence of the entire procedure can be managed effectively by employing the molecular markers. Yashitola et al. (2002) have demonstrated the use of STMS markers for testing the genetic purity of hybrid seeds in rice. But the number of such markers not linked to any specific trait of the pollen parent, required in determining the genetic purity of hybrids remains as a question to be answered. Here, we report the utility of a single, restorer gene linked co-dominant marker in testing the genetic purity of the rice hybrids. The microsatellite marker RM 258, which is linked to the fertility restorer gene in PRR 78 (Mishra et al., 2003), the pollen parent of the hybrid Pusa RH 10, effectively identified the true hybrids from the pollen shedders (B line plants) present in the hybrid seed lot. Detection of B line seeds is important because the presence of pollen shedders in the female rows of the hybrid seed production plot is a major cause of contamination of the hybrid seed. Since the marker is co-dominant and linked to the fertility restorer gene, the heterozygosity at the marker locus confirms the hybridity right at the fertility restorer locus and thus the hybrid nature of the plant. Although very rare, the mixture of R line seed in F_1 seed lot can also be easily detected by homozygosity for the marker allele linked to the restorer gene. Testing the genetic purity of hybrid seeds using a combination of markers (Yashitola et al., 2002), would be laborious and costly when compared to the use of a single, restorer gene linked co-dominant DNA marker like RM 258 reported in the present study. The results presented here and our subsequent study (data not presented) has shown plant-to-plant allelic variation at unlinked STMS marker loci within the A, B and R lines of rice in comparison to no variation at marker locus linked to specific trait of the pollen parent, making the latter reliable and efficient for testing the genetic purity of hybrid seed.

Another important point that needs discussion here is the sample size required for testing the genetic purity of hybrid seeds using molecular markers. The GOT is conducted using a random sample of 400 F_1 seeds representing one seed lot of a maximum size of 20 MT. The same sample size, therefore, can be used for determining the genetic purity using STMS markers. However, there is a scope for further optimizing the number of seeds required in case of marker-based hybrid purity testing as the percentage of off types identified by the marker RM 258 using 50 seeds corresponded well with the results of GOT conducted from the same seed lot.

Employing a single restorer gene linked marker such as RM 258 for testing genetic purity of hybrid seeds is the first report of its kind, which will substantially reduce the time, space, labor and ultimately the cost involved in testing the genetic purity of hybrid seeds using molecular markers. Identifying restorer gene linked markers in the pollen parent of other commercial rice hybrids will pave the way for large-scale use of these markers in testing hybrid seed genetic purity. This concept of using fertility restorer gene linked markers for testing genetic purity of hybrid seeds can be extended to other crops where hybrid seed production is based on the cytoplasmic genic male sterility (CGMS) system and where restorer gene linked markers are already reported such as *Brassica* (Janeja et al., 2003), cotton (Liu et al., 2003), *Sorghum* (Wen et al., 2002) and sunflower (Horn et al., 2003). This will greatly augment the entire process of testing

genetic purity of hybrid seeds by saving one full crop season and thus reducing the cost of hybrid seed.

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References

Bredemeijer, G.M.M., R.J. Cook, M.W. Ganal, R. Peeters, P. Issac, Y. Noordijk, S. Rendell, J. Jackson, M.S. Röder, K. Wendehake, M. Dijcks, M. Amelaine, V. Wickaert, L. Bertrand & B. Vosman, 2002. Construction and testing of microsatellite database containing more than 500 tomato varieties. Theor Appl Genet 105: 1019–1026.

Chen, S., C.G. Xu, S.H. Lin & Q. Zhang, 2001. Improving BB resistance of "6078", an elite R line of hybrid rice by molecular marker assisted selection. Plant Breeding 120: 133–137.

Chen, X., S. Temnykh, Y. Xu, Y.G. Cho & S.R. McCouch, 1997. Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). Theor Appl Genet 95: 553–567.

Cho, Y.G., T. Ishii, S. Temnykh, X. Chen, L. Lipovich, S.R. McCouch, W.D. Park, N. Ayres & S. Cartinhour, 2000. Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100: 713–722.

Doyle, J.J. & J.L. Doyle, 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13–14.

Garland, S.H., L. Lewin, M. Abedinia, R. Henry & A. Blakeney, 1999. The use of microsatellite polymorphisms for the identification of Australian breeding lines of rice (*Oryza sativa* L.). Euphytica 108: 53–63.

Horn, R., B. Kusterer, E. Lazarescu, M. Prüfe & W. Friedt, 2003. Molecular mapping of the Rf 1 gene restoring pollen fertility in PET1-based F_1 hybrids in sunflower (*Helianthus annuus* L.). Theor Appl Genet 106: 599–606.

Janeja, H.S., S.S. Banga & M. Lakshmikumaran, 2003. Identification of AFLP markers linked to fertility restorer genes for *tournefortii* cytoplasmic male-sterility system in *Brassica napus*. Theor Appl Genet 107: 148–154.

Jena, K.K. & S.K. Pandey, 1999. DNA markers for purification of A and B lines for hybrid rice improvement. Hybrid Rice News Lett 2(1): 13–14.

Jones, J.W., 1926. Hybrid vigor in rice. J Am Soc Agron 18: 424–428.

Liu, L., W. Guo, X. Zhu & T. Zhang, 2003. Inheritance and fine mapping of fertility restoration for cytoplasmic male sterility in *Gossypium hirsutum* L. Theor Appl Genet 106: 461–469.

McCouch, S.R., X. Chen, O. Panaud, S. Temnykh, Y. Xu, Y.G. Cho, N. Huang, T. Ishii & M. Blair, 1997. Microsatellite marker development, mapping and applications in rice genetics and breeding. Plant Molec Biol 35: 89–99.

McCouch, S.R., L. Teytelman, Y. Xu, K.B. Lobos, K. Clare, M. Walton, B. Fu, R. Maghirang, Z. Li, Y. Xing, Q. Zhang, I. Kono, M. Yano, R. Fjellstrom, G. DeClerck, D. Schneider, S. Cartinhour, D. Ware & L. Stein, 2002. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Res 9: 199–207.

Mishra, G.P., R.K. Singh, T. Mohapatra, A.K. Singh, K.V. Prabhu, F.U. Zaman & R.K. Sharma, 2003. Molecular mapping of gene for fertility restoration of wild abortive (WA) cytoplasmic male sterility using a basmati rice restorer line. J Plant Biochem Biotech 12: 37–42.

Röder, M.S., K. Wendehake, V. Korzun, G.M.M. Bredemeijer, D. Laborie, L. Bertrand, P. Issac, S. Rendell, J. Jackson, R.J. Cook, B. Vosman & M.W. Ganal, 2002. Construction and analysis of a microsatellite-based database of European wheat varieties. Theor Appl Genet 106: 67–73.

Rohlf, F.J., 1998. NTSYS-PC Numerical Taxonomy and Multivariate Analysis System, version 2.0. Exeter Software, Setauket, New York.

Singh, R.K., R.K. Sharma, A.K. Singh, V.P. Singh, N.K. Singh, S.P. Tiwari & T. Mohapatra, 2004. Suitability of mapped sequence tagged microsatellite site markers for establishing distinctness, uniformity and stability in aromatic rice. Euphytica 135(2): 135–143.

Temnykh, S., W.D. Park, N. Ayres, S. Cartinhour, N. Hauck, L. Lipovich, Y.G. Cho, T. Ishii & S.R. McCouch, 2000. Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100: 697–712.

UPOV-BMT, 2002. BMT/36/10 Progress Report of the 36th Session of the Technical Committee, the technical working parties and working group on biochemical and molecular techniques and DNA-profiling in particular, Geneva.

Virmani, S.S., 1999. Exploitation of heterosis for shifting the yield frontier in rice. In: J.G. Coors & S. Pandey (Eds.), The Genetics and Exploitation of Heterosis in Crops, pp. 423–438. CIMMYT, ASA, CSSA, Madison, WI.

Wang, G., S. Castiglione, J. Zhang, R. Fu, J. Ma, W. Li, Y. Sun & F. Sala, 1994. Hybrid rice (*Oryza sativa* L): Identification and parentage determination by RAPD fingerprinting. Plant Cell Rep 14: 112–115.

Wen, L., H.V. Tang, W. Chen, R. Chang, D.R. Pring, P.E. Klein, K.L. Childs & R.R. Klein, 2002. Development and mapping of AFLP markers linked to the sorghum fertility restorer gene *rf4*. Theor Appl Genet 104: 577–585.

Wu, K.S. & S.D. Tanksley, 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241: 225–235.

Xu, W., S.S. Virmani, J.E. Hernandez, L.S. Sebastian E.D. Redona & Z. Li, 2002. Genetic diversity in the parental lines and heterosis of the tropical rice hybrids. Euphytica 127: 139–148.

Yashitola, J., T. Thirumurugan, R.M. Sundaram, M.K. Naseerullah, M.S. Ramesha, N.P. Sarma & R.V. Stone, 2002. Assessment of purity of rice hybrids using microsatellite and STS markers. Crop Sci 42: 1369–1373.

Zhou, P.H., Y.F. Tan, Y.Q. He, C.G. Xu & Q. Zhang, 2003. Simultaneous improvement for four quality traits of Zhenshan 97, by molecular marker assisted selection. Theor Appl Genet 106: 326–331.