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Full Length Research Paper

Identification of rice hybrids using microsatellite and RAPD markers

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Having produced a series of hybrid rice varieties especially the first Iranian hybrid rice (IRH1), fingerprinting and genetic purity determination of hybrid seeds were conducted using microsatellite (SSR) and random amplified polymorphic DNA (RAPD) markers. Sixteen rice genotypes including 3 cytoplasmic male sterile (CMS) lines, 5 restorer lines and their 8 hybrid combinations were used in this study. Ten out of fourteen SSR markers produced polymorphic bands in the 16 rice genotypes. The first Iranian hybrid rice, IRH1, could be differentiated from other hybrids possessing a unique SSR fingerprint. Although the SSR markers amplified the unique fingerprints for the restorer lines, they could not differentiate between 2 CMS lines (Neda-A and Nemat-A) and their resultant hybrids. The rice CMS lines and restorer lines could be uniquely identified by RAPD multilocus amplified profile at 7 informative loci. Cluster analysis based on shared alleles and Jaccard's similarity coefficient using UPGMA algorithm, grouped the rice genotypes into 3 and 4 major clusters according to their microsatellite and RAPD fragment similarities respectively. Non-parental bands were also observed in addition to parental bands which probably correspond to heteroduplex molecules formed by two allelic sequences of different amplified fragments. This demonstrates that heteroduplex bands can be an indicative feature for identifying hybrid individuals.

Key words: Hybrid rice, fingerprinting, microsatellite, RAPD, non-parental bands, heteroduplex.

INTRODUCTION

As demands for feeding the rising world population grow, need for crop plants yield improvement is being more apparent. Rice is the main food of millions of people in the world. Hybrid rice possesses a yield advantage of 10 - 20% over the best inbred varieties (Virmani et al., 2003). Its commercial production has recently been attempted in other countries, following its success in China (Nandakumr et al., 2004). In this regard, the first aromatic hybrid rice (IRH1) in Iran was released for commercial cultivation in 2006 (Dorosti et al., 2006). The strictly self pollinating nature of rice necessitates the exploitation of a male sterility. Production of rice hybrids using a CMS system is based on cytoplasmic male sterility (CMS) and the fertility restoration system (Virmani et al., 2003), hence involves 3 lines of male sterile (A) line, maintainer (B) line, and restorer (R) line.

The fingerprinting of rice hybrids and identification of their genetic relationships are very important for plant improvement, variety registration system, DUS (distinctness, uniformity and stability) testing, seed purity testing and the protection of plant variety and breeders' rights. Accordingly, clear-cut identification of elite crop varieties and hybrids is essential for protection and prevention of unauthorized commercial use (Nandakumr et al., 2004). On the other hand, purity of hybrid seeds supplied to farmers must surpass 96% (Ichii et al., 2003). Conventional characterization of hybrids based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environ-

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Table 1. Rice hybrids and their parental lines used in this study.

Hybrids/parental lines	Type	Supplied by
Neda-A	CMS	Agricultural University, Sari, Iran
Nemat-A	CMS	Agricultural University, Sari, Iran
IR58025A	CMS	Rice Research Institute, Amol, Iran
IR42686R	Restorer	Rice Research Institute, Amol, Iran
IR62037-93-1-3-1-IR	Restorer	IRRI, Manila, The Philippines
IR69726-54-3-IR	Restorer	IRRI, Manila, The Philippines
IR28	Restorer	IRRI, Manila, The Philippines
SA4	Restorer	IRRI, Manila, The Philippines
IR58025A / IR42686R (IRHI)	Hybrid	Dasht-e-Naz, Sari, Iran
Neda-A / IR62037-93-1-3-1-IR	Hybrid	Rice Research Institute, Amol, Iran
Neda-A / IR69726-54-3-IR	Hybrid	Rice Research Institute, Amol, Iran
Neda-A / IR28	Hybrid	Rice Research Institute, Amol, Iran
Neda-A / SA4	Hybrid	Rice Research Institute, Amol, Iran
Nemat-A / IR62037-93-1-3-1-IR	Hybrid	Rice Research Institute, Amol, Iran
Nemat-A / IR69726-54-3-IR	Hybrid	Rice Research Institute, Amol, Iran
Nemat-A / IR28	Hybrid	Rice Research Institute, Amol, Iran

mental condition and inefficient. Molecular marker assisted identification with high power of genetic resolutions has emerged as a robust technique for cultivar fingerprinting, identity profiling, estimating and comparing genetic similarity, and variety protection. Several types of molecular marker viz. allozymes (Devanand et al., 1999), RAPD (Wang and Lu, 2006; Ichii et al., 2003), SSR (Yashitola et al., 2002; Nandakumar et al., 2004) and STS (Yashitola et al., 2002) have been used in this term. Having produced several rice CMS lines/hybrids in our project during 1994 - 2006 years (Nematzadeh et al., 2006; Dorosti et al., 2006), the objectives of this study were DNA profiling of hybrids rice and their parental lines as well as identifying specific primers for genetic purity testing. Also, application of non-parental extra band for hybrid detection has been addressed.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation

Sixteen rice genotypes, including 3 cytoplasmic male-sterile (CMS) lines, 5 restorer lines and their 5 hybrid combinations were used in this study (Table 1). Genomic DNA was isolated from young leaves of 10 plants of each parent lines while the leaves of individual plant were used for hybrids. DNA was extracted according to Dellaporta et al. (1983) procedure with minor modification. The quantity and quality of DNA was assessed with 0.7% agarose gel electrophoresis using diluted uncut lambda phage DNA as size standard.

SSR and RAPD analyses

Fourteen microsatellite markers including RM1, RM1108, RM154, RM164, RM171, RM206, RM216, RM258, RM263, RM264, RM337, RM443, RM6344 and RM9 were selected based on Panaud et al. (1996), Chen et al. (1997) and McCouch et al. (2002) reports and used in this study. The sequences of the polymorphic primer pairs and their chromosomal locations are presented in Table 2. Polymerase chain reaction (PCR) was performed in a volume of 12.5 µl reaction mixture containing 40 ng of template DNA, 240 µM of each dNTPs, 1× *Taq* buffer PCR (10 mM Tric-HCl, 50 mM KCl (pH 8.8), 0.08 Nonidet P40), 2.5 mM $MgCl_2$, 1 U of *Taq* polymerase and 0.2 µM each of the SSR primer pair (Metabion International AG, Germany) using a Techne Genius thermal cycler (Techne Ltd., U.K.).

Amplification reactions were initiated by 5 min predenatutation at 95˚C and followed by 38 cycles each at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A final extension step at 72°C for 7 min was performed after 38 cycles. PCR amplified products were separated by electrophoresis in 2% agarose gels at 50 V in 0.5× TBE buffer and in a 7% polyacrylamide gel (29:1 acrylamide:bis) at 200 V in 1× TBE buffer. Gels stained with ethidium bromide and then were imaged in Biometra (UV-solo model) gel documentation system.

Twenty-nine randomly decamer primers (Primm, Italy) from different series (A, B, C, E, G, H and I) were tested and 15 polymorphic primers were selected (Table 3). PCR was carried out in 12.5 µl reaction mixture containing 40 ng of template DNA, 200 µM of each dNTPs, 1× *Taq* buffer PCR (10 mM Tric-HCl, 50 mM KCl (pH 8.8), 0.08 Nonidet P40), 2.5 mM of $MgCl_2$, 1.2 U of *Taq* polymerase, and 1 µM each of the RAPD primers using a Techne Genius thermal cycler (Techne Ltd., U.K.). Amplification reaction was started by initial denaturation of template DNA at 94°C for 4 min and followed by 40 cycles at 92°C for 1 min, 35°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 5 min. PCR amplified products were separated by electrophoresis in 1.8% agarose gels at 50 V in 0.5× TBE buffer. Gels stained with ethidium bromide, were imaged in Biometra (UV-solo model) gel documenttation system. Each reaction was repeated twice and only reproducible bands were considered for analysis.

Data analysis

RAPD reproducible fragments were scored as present or absent (1, 0). The RAPD matrices were then analyzed using (NTSYS) version 2.02 (Rohlf, 1998). Similarity for RAPD data was computed using the Jaccard's similarity index and similarity estimates were ana-

Table 2. Details of selected primers used in microsatellite DNA profiling analysis.

Primer	F/R Primer	CL	NA	NG	PIC
RM171	AACGCGAGGACACGTACTTAC ACGAGATACGTACGCCTTTG	10	2	3	0.37
RM1	GCGAAAACACAATGCAAAAA GCGTTGGTTGGACCTGAC	1	2	3	0.35
RM1108	GCTCGCGAATCAATCCAC CTGGATCCTGGACAGACGAG	10	2	3	0.19
RM154	ACCCTCTCCGCCTCGCCTCCTC CTCCTCCTCCTGCGACCGCTCC	2	3	6	0.52
RM164	TCTTGCCCGTCACTGCAGATATCC GCAGCCCTAATGCTACAATTCTTC	5	2	3	0.28
RM337	GTAGGAAAGGAAGGGCAGAG CGATAGATAGCTAGATGTGGCC	8	3	4	0.43
RM6344	ACACGCCATGGATGATGAC TGGCATCATCACTTCCTCAC	7	2	3	0.37
RM263	CCCAGGCTAGCTCATGAACC GCTACGTTTGAGCTACCACG	2	2	3	0.35
RM216	GCATGGCCGATGGTAAAG TGTATAAAACCACACGGCCA	10	2	3	0.16
RM206	CCCATGCGTTTAACTATTCT CCCATGCGTTTAACTATTCT	11	3	5	0.47

F/R Primer: forward/reverse primer; CL: chromosomal location; NA: number of allele; NG: number of genotype; PIC: polymorphic information content.

lyzed by the UPGMA algorithm. The resulting clusters were expressed as dendrogram. The microsatellite matrices were analyzed by Power-Marker V3.25 (Liu and Muse, 2005). Similarity for microsatellite data was calculated using the shared allele similarity index, and similarity estimates were analyzed by the UPGMA algorithm. The resulting clusters were expressed as dendrogram with the MEGA 3.1 program (Kumar et al., 2004).

RESULTS

Microsatellite profiling analysis

Ten out of the fourteen SSR loci amplified polymorphic bands in the 16 genotypes (Table 2). The RM258, RM443 and RM264 loci were monomorphic, and RM9 showed ambiguous pattern. The average numbers of band per primers and per genotypes were 1.92 and 1.68, respectively. The maximum of alleles at a locus were 3 for the RM154, RM206, RM337 markers, with a mean of 2.3 alleles. The polymorphic information content (PIC) of SSR loci ranged from 16% for RM216 to 52% for RM154 markers, with a mean of 35%. The number of genotypes possessing a particular locus ranged from 3 (most SSR loci) to 6 for RM154 markers with a mean of 3.6. RM154 microsatellite marker was the most-informative locus for genotypic DNA profiling and differentiation, having 6 genotypes. IRH1, the first hybrid rice with the commercial cultivation at north of Iran, had unique DNA profiling. Figure 1 shows the schematic presentation of microsatellite profiling.

RAPD profiling analysis

Fifteen informative primers out of Twenty-nine RAPD primers were chosen for DNA profiling (Table 3). A total of 155 reproducible amplifications products was observed, and 102 (65.8) polymorphic bands were scored. The highest and lowest numbers of polymorphic products obtained with primers OPH04 and OPG13 with 1 and 17, respectively. The average number of bands per primer and genotypes were 10.3 and 9.7, respectively. The rice CMS lines and restorer lines could be uniquely identified by RAPD multilocus amplified profile at seven informative loci: OPA04, OPA08, OPA17, OPB14, OPC04, OPG11 and OPG13. Figure 2 shows the schematic presentation of amplification pattern in these loci.

Identification of a non-parental band specific to hybrid lines

A common feature of the minority of SSR markers, in this study, was the presence of a non-parental extra band in hybrid individuals. These bands could correspond to the slower movement than either of respective parental bands. To check whether the extra band(s) present in some SSR reactions are heteroduplex molecules created between two allelic sequences of different fragments amplified, RM337 PCR template with combining of equal amounts of PCR product of parental lines were mixed. The mixture of single parent products was heated to near

Table 3. Details of selected primers used in RAPD profiling analysis.

Primer	5‘ to 3’ Sequence	TNB	NPB	PPB
OPA01	CAGGCCCTTC	6	2	33
OPA04	AATCGGGCTG	11	6	54
OPA08	GTGACGTAGG	14	12	85
OPA09	GGGTAACGCC	11	6	54
OPA17	GACCGCTTGT	13	9	77
OPB14	TCCGCTCTGG	9	6	66
OPC04	CCGCATCTAC	8	3	37.5
OPG06	GTGCCTAACC	11	7	63
OPG11	TGCCCGTCGT	7	6	86
OPG13	CCACACTACC	20	17	85
OPH04	GGAAGTCGCC	5	1	20
OPH07	CTGCATCGTG	6	2	33
OPH12	ACGCGCATGT	12	6	50
OPH14	ACCAGGTTGG	10	8	80
OPI04	CCGCCTAGTC	12	9	75

TNB: Total number of bands; NPB: number of polymorphic bands; PPB: percent of polymorphic bands.

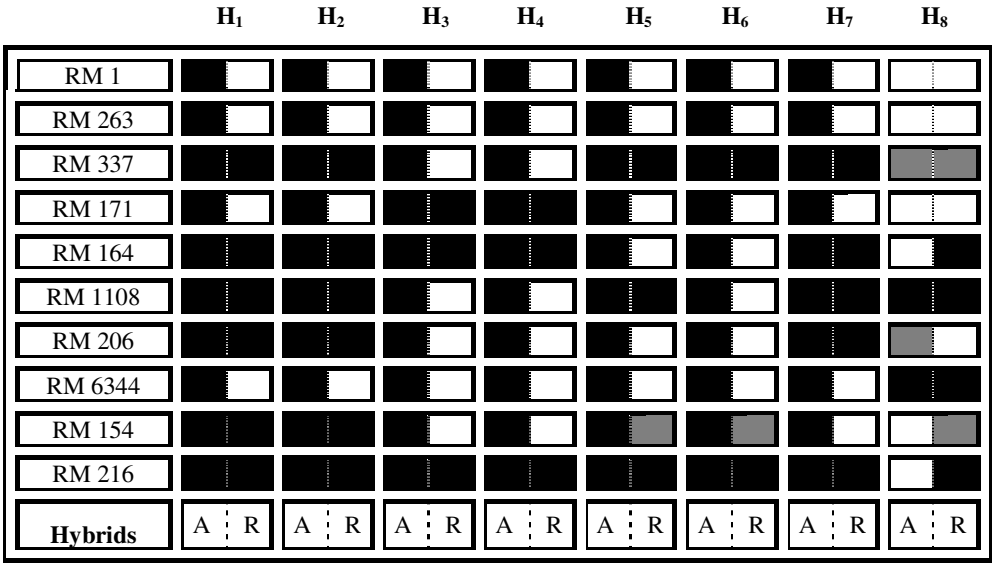

Figure 1. Schematic presentation of microsatellite DNA profiling for hybrids and their parents. Each column (H) corresponds to a hybrid (above) and consists of two subcolumns, A and R lines. Each row shows the allelic pattern of each SSR marker in eight hybrids. The shaded blocks correspond to the presence of different allele in respected marker. H_1: Nemat-A / IR28; H_2: Neda-A / IR28; H_3: Neda-A / IR69726-54-3-IR; H_4: Nemat-A / IR69726-54-3-IR; H_5: Nemat-A / IR62037-93-1-3-1-IR; H_6: Neda-A / IR62037-93-1-3-1-IR; H_7: Neda-A / SA4; H_8: IR58025A /R42686R.

boiling for 5 min and slowly cooled to allow annealing and hybridization. Then, they were analyzed by electrophoresis with usual PCR products in other lanes. Snap-chilled heated PCR products served as control.

The non-parental band(s) were formed in the lane where the mixture of PCR products was heated and slow cooled and it was present with any other treatments. The result of product mixing experiments (data not shown) confirmed the high degree of homology between two DNA segments and showed that extra band(s) were the consequence of heteroduplex formation by allelic sequences. Figure 3a and 3b shows the representative RM337 profiles in 2% agarose gel (a) and 7% acrylamide gel (b).

Genotype / RAPD loci	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
OPA04(250)															■	
OPA04(300)	■	■	■	■	■	■	■	■	■		■	■		■	■	■
OPA04(800)							■			■			■			■
OPA08(1904)							■	■								
OPA08(1150)	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
OPA08(200)		■	■	■			■	■								
OPA17(1250)																■
OPA17(1100)			■													■
OPB14(940)	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■
OPB14(900)	■	■		■	■	■		■			■	■				
OPC04(850)																■
OPC04(400)														■	■	■
OPG11(1310)	■								■	■	■			■	■	■
OPG11(750)	■	■	■	■	■	■	■	■	■	■	■	■	■			■
OPG11(560)													■			
OPG13(1350)							■					■				
OPG13(1300)								■								
OPG13(830)							■									■
OPG13(900)																■

Figure 2. Schematic representation of RAPD profiling of eight hybrids rice and their parental lines. The numbers at the top table are serial number of 16 genotypes (below). Shaded block indicate the presence of allelic bands by the unique respective RAPD loci. (1): Nemat-A; (2): Nemat-A / IR28; (3): IR28; (4): Neda-A / IR28; (5): Neda-A; (6): Neda-A/ IR69726-54-3-IR; (7): IR69726-54-3-IR; (8): Nemat-A / IR69726-54-3-IR; (9): Nemat-A / IR62037-93-1-3-1-IR; (10): IR62037-93-1-3-1-IR; (11): Neda-A / IR62037-93-1-3-1-IR; (12): Neda-A / SA4; (13): SA4; (14): IR58025A; (15): IR58025A / IR42686R (IRHI), (16): IR42686R.

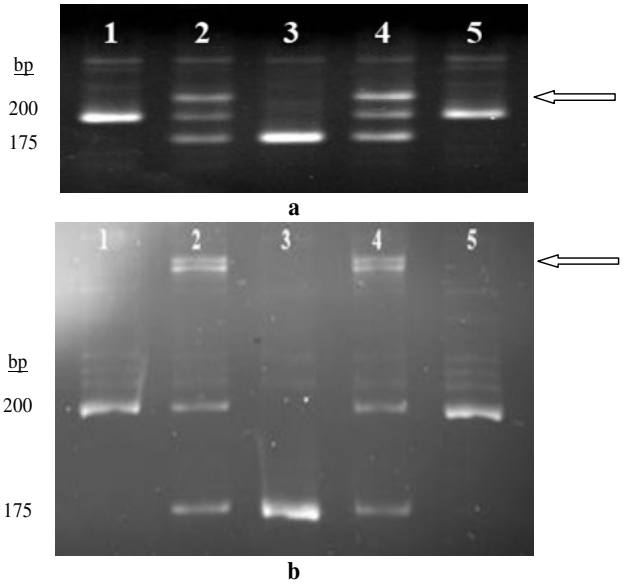

Figure 3. The SSR profile of DNA amplification products generated from RM337 on a 2% agarose (a) and 7% acrylamide gel (b). Non-parental extra band(s) (arrow) in F_1 genotypes is a heteroduplex SSR, as could be specific feature for hybrid individual detection.

Genetic purity testing

Being codominant, polymorphic SSR markers were used to test the seed purity of rice hybrids. As expected, when polymorphic bands detected for two parent (A and R lines), two alleles were observed in F_1 hybrid individuals, while off type seeds had only one allele. In this study, we carried out seed purity test with individual seedlings in each hybrid by respected polymorphic markers. Figure 4 shows the PCR electrophoretic profiles of single plants of hybrid rice for genetic purity test with RM171 marker linked to *Rf*$_4$ gene (Ahmadikhah et al., 2007). Out of 6 seeds tested with RM171 in Neda-A/IR28 hybrid, 3 off-type seeds were detected.

Cluster analysis

The coefficient of similarity for microsatellite markers among cultivars and lines ranged from 1% for Neda-A and Nemat-A to 11% for IR58025A with Neda-A and Nemat-A. Neda-A and Nemat-A resultant hybrids could not be differentiated with an average similarity index of 0.6. Neda-A and Nemat-A are sister lines developed by

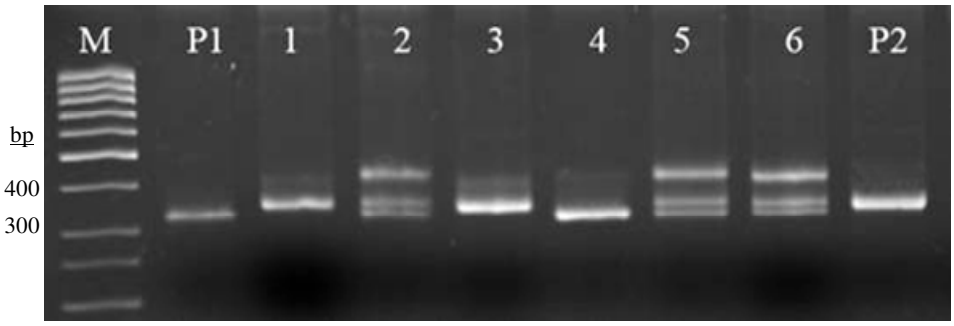

Figure 4. PCR electrophoretic profile of hybrid individuals for genetic purity test with RM171 linked to Rf4 gene. M = 100 bp DNA size marker, lane P1 = Neda-A (CMS line), lane P2 = IR28 (R line), and hybrid individuals in lanes 1-6. Hybrid individuals had an extra band addition to both parental bands.

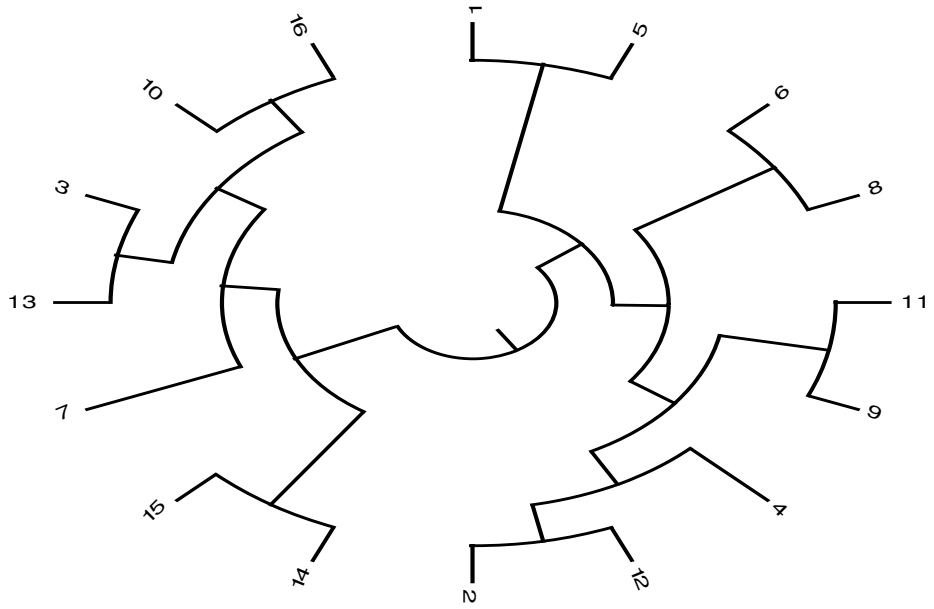

Figure 5. Dendrogram of 16 rice genotypes based on shared allele similarity index by microsatellite markers. (1): Nemat-A; (2): Nemat-A / IR28; (3): IR28; (4): Neda-A / IR28; (5): Neda-A; (6): Neda-A/ IR69726-54-3-IR; (7): IR69726-54-3-IR; (8): Nemat-A / IR69726-54-3-IR; (9): Nemat-A / IR62037-93-1-3-1-IR; (10): IR62037-93-1-3-1-IR; (11): Neda-A / IR62037-93-1-3-1-IR; (12): Neda-A / SA4; (13): SA4; (14): IR58025A; (15): IR58025A / IR42686R (IRHI), (16): IR42686R.

transferring of wild abortive (WA) cytoplasm from IR58025A to them via backcrossing. Cluster analysis based on shared allele similarity coefficient using UPGMA grouped the rice genotypes into three clusters, each containing 12.5, 43.75 and 43.75% of genotypes (Figure 5). Cluster I consisted of two cytoplasmic male sterile, Nada-A and Nemat-A lines. Cluster II consisted of hybrids having Nada-A and Nemat-A as their parents. IRH1 hybrid, their parents and all restorer lines allocated to cluster III. RAPD genetic similarity among cultivars and lines ranged from 0.25 to 0.91 with an average similarity index of 0.58. Based on these markers all hybrids were similar to their seed parents. Cluster analysis based on Jaccard's similarity coefficient using UPGMA procedure grouped the cultivars and lines into four clusters, each containing 75, 6.25, 12.5 and 6.25% of genotypes (Figure 6).

DISCUSSION

Fifteen microsatellite and twenty nine RAPD markers were used for DNA profiling of hybrids and their parental lines in this investigation. Ten SSR loci and 15 RAPD

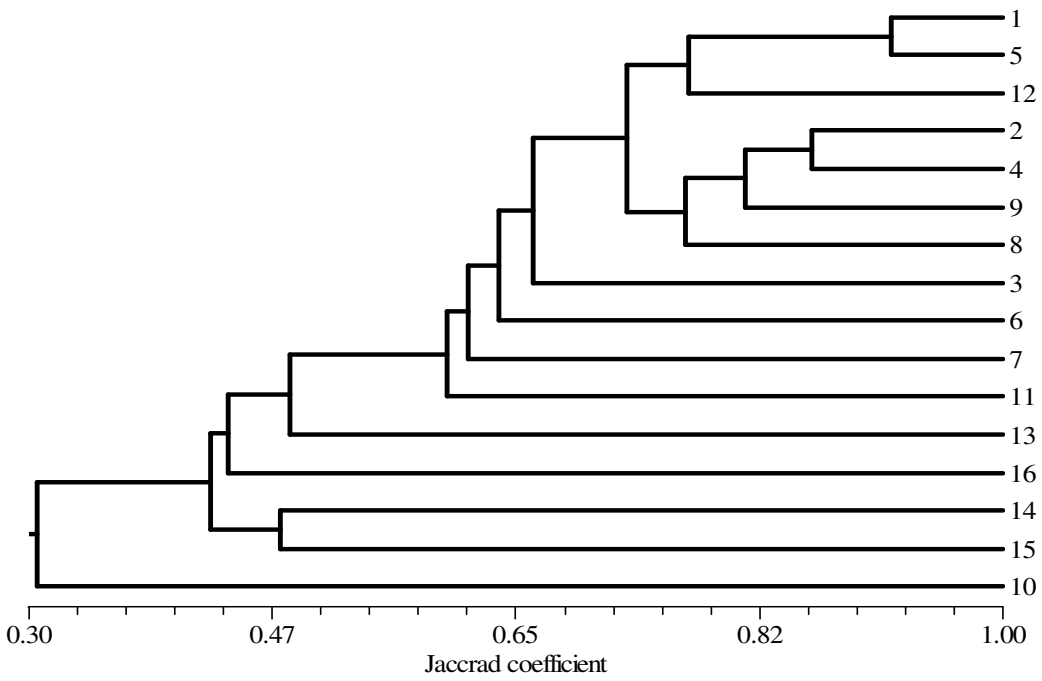

Figure 6. Dendrogram of 16 rice genotypes based on Jaccard's similarity index by RAPD markers. (1): Nemat-A; (2): Nemat-A / IR28; (3): IR28; (4): Neda-A / IR28; (5): Neda-A; (6): Neda-A/ IR69726-54-3-IR; (7): IR69726-54-3-IR; (8): Nemat-A / IR69726-54-3-IR; (9): Nemat-A / IR62037-93-1-3-1-IR; (10): IR62037-93-1-3-1-IR; (11): Neda-A / IR62037-93-1-3-1-IR; (12): Neda-A / SA4; (13): SA4; (14): IR58025A; (15): IR58025A / IR42686R (IRHI), (16): IR42686R.

multiloci were chosen for further molecular analyses. The results revealed that the SSR loci RM 154, RM 206, RM 337 (with 6, 5 and 4 observed genotypes, respectively) and RAPD multiloci OPA04, OPA08, OPA17, OPB14, OPC04, OPG11 and OPG13 have the highest efficacy for DNA profiling and discrimination of rice hybrids and lines. The primer pair for RM264 locus did not generate any PCR amplicon in some of the restorer lines (SA4, IR62037-93-1-3-1-IR, IR69726-54-3-IR, data not shown). The lack of PCR products of an allele (null allele) in certain genotypes can be the consequence of divergence in the microsatellite flanking sequences. Deletions or point mutation in the primer complementary region result in inhibiting or preventing primer binding and amplification (Masi et al., 2003).

F_1 hybrid genotypes were characterized by non-parental band(s) having slower migration through gel than the allelic bands. Presumably, these slower-migrating bands are heteroduplex molecules. Although, they were observed as a single band in 2% agarose gels, they produced two distinct bands in 7% acrylamide gels. Heteroduplexes are double-stranded DNA molecules formed between two different alleles and, therefore, they contain mismatches (Perez et al., 1999).

Heteroduplexes also have an aberrant, distorted structure with bubbles or bulges at the sites of mismatched bases, and generally move more slowly in gel than homoduplexes (Kozlowski and Krzyzosiak, 2001). The slow migration is due to the fact that there is a loop (insertion or deletion) in one strand that makes a bubble between the two strands of the DNA helical bend at that site. Novy and Vorsa (1996) showed that presence of an artifactual heteroduplex band in heterozygotes may well be diagnostic of RAPD marker allelism/codominance in general. Davis (1995) stated that non-parental, heteroduplex bands in PCR are consistent and useful feature of codominant RAPD markers. Wu et al. (2002) revealed that the presence of non-parental band(s) is a supplementary evidence for codominancy of the markers. Similar features were also reported in other markers and plants viz: RAPD codominant markers in soybean (Zheng et al., 2003), rice (Wu et al., 2002), *Chrysanthemum* hybrids (Huang et al., 2000) and SSR markers in maize heterozygous DH lines (Heckenberger et al., 2002). The presence of slower migrating heteroduplex DNA is, therefore, useful for detection of heterozygous individuals.

Maintenance of hybrid seeds conformity in high level is essential for exploitation of hybrid vigor. Therefore, testing the hybrid seed purity is necessarily required before its release into the market. We recommend RM1, RM263 and RM6344 markers for genetic purity test in hybrids except IRH1.

Variety identification is critical importance of worldwide in view of its application in plant variety protection (PVP) (Kwon et al., 2005). Garg el al. (2006) suggested the RM258 linked to a single *Rf* gene that possesses co-

dominant status can provide a precise and quick alternative to grow out test (GOT), for testing the hybrid seed purity. Hence, determination of seed genetic purity in hybrid crops is crucial to confirm the hybridization and resulted increased grain yields through hetrosis (Jianhua et al., 1997). In rice, it was reported that 1% impurity in the hybrid seeds caused the yield reduction of 100 kg per hectare (Mao et al., 1996). Yashitola et al. (2002) indicated that confirmation of genotypes by an unlinked marker is a reliable approach for assessing hybrid seed purity. A single polymorphic marker should suffice to ascertain hybrid seed purity in rice. Also Nandakumar et al. (2004) successfully employed a single restorer gene-linked marker assessment for testing genetic purity of hybrid seeds that substantially reduced the time, space and labor.

Concisely, concordant with results of the present study, a non-parental extra band is not only useful for fingerprinting the rice hybrids and unambiguous hybrid identity, but could also be used as a specific feature for characterization and genetic purity test and detection of off-type seeds in the hybrids based on monomorphic markers. In this experiment, heterozygous individual, IRH1, in monomorphic locus (RM171) was successfully recognized as hybrid and was discriminated from its parental lines (with same alleles) (data not shown).

Contrasting SSR markers which could not discriminate two CMS lines (Neda-A and Nemat-A), RAPD markers could differentiate all CMS and restorer lines. The dendrograms obtained using these two sets of molecular markers are in agreement with their known origin.

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