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Classification of rice germplasm: III. High-resolution fingerprinting of cytoplasmic genetic male-sterile (CMS) lines with AFLP

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Abstract The cytoplasmic genetic male-sterile (CMS) lines developed at the International Rice Research Institute are valuable in producing tropical rice hybrids. Efficient use of CMS lines in hybrid rice production will depend on their level of genetic diversity. Aside from morphological characterization, molecular analysis based on DNA markers can provide information on the genetic diversity of the germplasm. The Amplified Fragment Length Polymorphism (AFLP) technique was used to fingerprint 71 CMS lines and four rice cultivars, 'IR64', 'Azucena', 'IR74', and 'FR13A'. Eleven primer pair combinations specific to the enzymes *Pst*I and *Mse*I were used to generate 530 AFLP markers, 176 of which were polymorphic. Each CMS line revealed a distinct fingerprint. The AFLP marker-based dendrogram depicted genetic variation among the CMS lines. The CMS lines developed in japonica background grouped with 'Azucena', a japonica cultivar. None of the CMS lines clustered with 'FR13A', a flood-tolerant traditional indica variety. 'IR64' was found to be distinct from the other indica CMS lines and clustered with lines developed in its background. The grouping of CMS lines into a few groups is useful for breeders in selecting genetically diverse CMS lines for hybrid rice production and in avoiding test crossing every CMS line empirically. This study demonstrated that AFLP is a powerful and reliable tool in determining the genetic relationships and in producing distinct fingerprints of rice cultivars.

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Introduction

The cytoplasmic genetic male-sterility (CMS) system is the most effective and practical way of exploiting heterosis in rice. The yield advantage of hybrids over inbred varieties is the major factor in the adoption and cultivation of hybrid rice. Though hybrid rice technology has been exploited in China, the Chinese CMS lines were found to be unsuitable under tropical conditions (Yuan and Virmani 1988). Thus, IRRI produced a number of CMS lines in different genetic backgrounds suitable for developing hybrids for diverse agro-ecological conditions of the tropics. Characterization of these CMS lines with morphological as well as molecular markers will provide information on the genetic diversity of CMS lines, which is essential in their efficient use in hybrid production. Furthermore, the CMS lines are being shared freely with public and private research institutions operating in the tropics. The DNA fingerprints will be important to protect breeder's rights and eventually farmers' rights to access these freely available materials.

DNA-based molecular markers have been used for fingerprinting germplasm in crop plants. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) are the most commonly used techniques, and they have been used in rice (Wang and Tanksley 1989; Fukuoka et al. 1992; Yu and Nguyen 1994; Virk et al. 1995; Mackill 1995). Other techniques such as amplicon length polymorphism (Ghareyazie et al. 1995), simple sequence repeat, or hypervariable DNA sequences (Ramakrishna et al. 1994, 1995; Yang et al. 1994) and restriction landmark genomic scanning (Kawase 1994) have been proposed for fingerprinting rice. More recently, a new

PCR-based technique known as amplified fragment length polymorphism (AFLP) has been developed (Zabeau and Vos 1993, Vos et al. 1995). This technique combines the reliability and robustness of RFLP and the power of polymerase chain reaction (PCR) techniques. The AFLP technique is considered to be a powerful technique for genome mapping, genotype identification, and phylogenetic studies (Becker et al. 1995; Thomas et al. 1995; Vos et al. 1995, Cho et al. 1996, Mackill et al. 1996, Maheswaran et al. 1997). It has been used to fingerprint bacteria (Janssen et al. 1996), nematodes (Folkertsma et al. 1996), wild bean, and other plants (Tohme et al. 1996; Travis et al. 1996).

We believe that AFLP is a more suitable method for analyzing rice germplasm than other DNA marker techniques because a large number of AFLP marker loci can be rapidly scanned and fingerprint information obtained in a short period of time. In this report, we describe the application of the AFLP technique to characterize CMS lines of rice. Furthermore, we compare the AFLP fingerprintings with characterization based on qualitative and quantitative phenotypic traits.

Materials and methods

Plant materials and DNA isolation

Seventy-one CMS lines with four different sources of cytoplasm, WA, ARC, *Oryza perennis*, and *O. glumaepatula* types, developed at IRRI were used (Lin and Yuan 1980; IRRI 1986, Dalmacio et al. 1995, 1996).The CMS lines are in indica, basmati, japonica, and tropical japonica backgrounds (Table 1). The IR71564A CMS line was duplicated in our analysis to test the reproducibility of the AFLP markers. Four rice varieties, 'IR64', 'Azucena', 'IR74', and 'FR13A', were also included in the analysis as controls because they had been used earlier to develop two AFLP maps (Maheswaran et al. 1997; Nandi et al. 1997). The CMS lines and the four rice varieties were grown in the field. One leaf each from 5 plants of each line was taken, and DNA was extracted following the procedure of Dellaporta et al. (1983).

Evaluation of morphological traits

Observations were taken on 10 randomly selected plants from each CMS line with respect to 12 qualitative and six quantitative traits. The qualitative traits studied were blade pubescence, coloral leaf sheath, ligule, collar, auricle, internode, apiculus, seed, stigma, panicle exertion, awn pubescence, and the varietal group. The varietal group was determined as japonica or indica based on the grain characteristics, as indicated in Table 1. The squantitative traits were length of clum, panicle, grain, grain width, 1000 grain weight, and maturity (days).

AFLP analysis

The original AFLP protocol developed by Zabeau and Vos (1993) was followed with minor modifications. Five hundred nanograms of genomic DNA of each CMS line and the four rice cultivars was digested with 5 U of *Pst*I and 5 U of *Mse*I in a reaction volume of

Table 1 List of CMS lines analyzed by AFLP

Sl. no.	Lines	Cytoplasm	Characteristics
1	IR58025A	WA	Irrigated, indica
2	IR62829A	WA	Irrigated, indica
3	IR68885A	WA	Irrigated, indica
4	IR64608A	WA	Irrigated, indica
5	IR68886A	WA	Irrigated, indica
6	IR68888A	WA	Irrigated, indica
7	IR68892A	WA	Irrigated, indica
8	IR58893A	WA	Irrigated, indica
9	IR68894A	WA	Irrigated, indica
10	IR68895A	WA	Irrigated, indica
11	IR68896A	WA	Irrigated, indica
12	IR68897A	WA	Irrigated, indica
13	IR68898A	WA	Irrigated, indica
14	IR68899A	WA	Irrigated, indica
15	IR68900A	WA	Irrigated, indica
16	IR68901A	WA	Irrigated, indica
17	IR68902A	WA	Irrigated, indica
18	IR69616A	WA	Irrigated, indica
19	IR69618A	WA	Irrigated, indica
20	IR69619A	WA	Irrigated, indica
21	IR69620A	WA	Irrigated, indica
22	IR69621A	WA	Irrigated, indica
23	IR69622A	WA	Irrigated, indica
24	IR69623A	WA	Irrigated, indica
25	IR69625A	WA	Irrigated, indica
26	IR69626A	WA	Irrigated, indica
27	IR69627A	WA	Irrigated, indica
28	IR70362A	WA	Irrigated, indica
29	IR70365A	WA	Irrigated, indica
30	IR70366A	WA	Irrigated, indica
31	IR70368A	WA	Irrigated, indica
32	IR70369A	WA	Irrigated, indica
33	IR70371A	WA	Irrigated, indica
34	IR70959A	WA	Irrigated, indica
35	IR70960A	WA	Irrigated, indica
36	IR70963A	WA	Irrigated, indica
37	IR71564A(1)	WA	Irrigated, indica
38	IR71564A(2)	WA	Irrigated, indica
39	IR68275A	WA	Rainfed, indica
40	IR68887A	WA	Rainfed, indica
41	IR68889A	WA	Rainfed, indica
42	IR68890A	WA	Rainfed, indica
43	IR69624A	WA	Rainfed, indica
44	IR69628A	WA	Rainfed, indica
45	IR70363A	WA	Rainfed, indica
46	IR70364A	WA	Rainfed, indica
47	IR70367A	WA	Rainfed, indica
48	IR71562A	WA	Rainfed, indica
49	IR71563A	WA	Rainfed, indica
50	IR68279A	WA	Rainfed, indica
51	IR68280A	WA	Irrigated, Basmati
52	IR68281A	WA	Irrigated, Basmati
53	IR69617A	WA	Irrigated, Basmati
54	IR70372A	WA	Irrigated, Basmati
55	IR67701A	WA	Irrigated, WC, tro jap[a]
56	IR68277A	WA	Irrigated, WC, tro jap[a]
57	IR68884A	WA	Irrigated, WC, tro jap[a]
58	IR68276A	WA	Irrigated, japonica
59	IR68283A	WA	Irrigated, japonica
60	IR54755A	ARC	Irrigated, indica
61	IR68273A	ARC	Irrigated, indica
62	IR66707A	*O. perennis*	Irrigated, indica
63	IR70961A	*O. perennis*	Irrigated, indica
64	IR70962A	*O. perennis*	Irrigated, indica
65	IR70964A	*O. perennis*	Irrigated, indica
66	IR69700A	*O. glumaepatula*	Irrigated, indica

Table 1 Continued.

Sl. no.	Lines	Cytoplasm	Characteristics
67	IR72078A	WA	Rainfed, indica
68	IR72079A	WA	Irrigated, indica
69	IR72080A	WA	Irrigated, indica
70	IR72081A	WA	Irrigated, indica
71	IR72082A	WA	Irrigated, indica
72	IR72083A	WA	Irrigated, tro jap
73	1R64		Irrigated, indica
74	Azucena		Upland, tro jap
75	IR74		Irrigated, Indica
76	FR13A		Flood prone

[a] tro jap = tropical japonica

40 µl at 37°C for 4 h in a restriction ligation buffer (10 m*M* TRIS-HAc, pH 7.5, 10 m*M* $MgAc_2$, 50 m*M* KAc, 5 mM DTT, and 0.005% BSA). The digested DNA was then mixed with 10 µl of solution containing *Pst*I adapter (5 pMol) and *Mse*I adapter (50 pMol), 1 µl 10 m*M* ATP, 1 U T4 DNA ligase, and 2 µ l of 5 × restriction ligation buffer and incubated again at 37°C for another 3 h. The adapter sequences specific for the *Pst*I and *Mse*I sites, the primer sequences used for preamplification of adapter-ligated restriction fragments, and the PCR conditions were the same as those in Maheswaran et al. (1997). The preamplified PCR products were run on a 2% agarose gel in which a low-molecular weight smear was visible indicating the presence of abundant PCR products. Then the PCR products were diluted 20 times with sterile water.

Selective restriction fragment amplification was performed using *Pst*I primers end-labeled with [^{33}P]ATP and an unlabeled *Mse*I + 3 primer. The *Pst*I primers were end-labeled in a 30-µl reaction volume containing 150 ng primer, 50 µCi (185° Bq) [^{33}P]ATP, 5U T4 polynucleotide kinase and 3 µl 10 × kinase buffer (100 m*M* TRIS-acetate, pH 7.6, 100 m*M* $MgAc_2$ 50 m*M* KAc, 5 m*M* β-mercaptoethanol). The mixture was incubated at 37°C for 30 min followed by an incubation at 70°C for 10 min. Thirty microliters of labeled mixture is enough for 30 selective amplification reactions.

A 25-µl PCR mixture, containing 5 µl of preamplified DNA, 5 ng of labeled *Pst*I + 3 primer, 50 ng of unlabelled *Mse*I + 3 primer, 0.2 m*M* of each dNTP, 10 m*M* TRIS-HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 1U of *Taq* DNA polymerase was run in a Techne thermocycler with the same profile of thermal cycling as mentioned above. The gel analysis was performed following the method described in Maheswaran et al. (1997). The 11 primer pair combinations used in this study are listed in Table 2, along with the selective nucleotides. The sequences of the primers are the same as in Maheswaran et al. (1997), except for P3 (*Pst*I), which is the primer sequence of 5′-GACTGCGTACATGCAG **ACC**-3′.

AFLP marker scoring and data analysis

Only clear and unambiguous bands were scored. Markers were scored for the presence and absence of the corresponding band among the lines. AFLP bands of different electrophoretic mobilities were assumed to be nonallelic, while bands with the same mobility were assumed to be allelic. Each marker was then treated as a unit character, and a pair-wise similarity matrix was calculated using the Dice coefficient (Sokal and Sneath 1963). Similarly, the qualitative traits were converted into binary characters before estimating the Dice similarity coefficients. For the quantitative traits, the data was standardized, and Euclidean distances were determined using SIMMINT subroutine of the NTSYS-pc. In all cases, cluster analysis was carried out with the UPGMA method using NTSYS-pc (Rohlf 1990).

Results and discussion

Morphological markers

The dendrograms were constructed using the 12 qualitative (Fig. 1) and six quantitative traits (Fig. 2) of the 71 CMS lines. For qualitative traits, a large number of lines clustered together at 100% similarity in 10 different groups. However, quantitative traits, which are commonly used by breeders in studying genetic diversity and similarity, provided better resolving power than qualitative traits. While all of the CMS lines were clearly separated from each other, the japonica and indica lines could not be distinguished clearly and only 2 japonica lines, IR67701A and IR68277A, were

Table 2 Polymorphism of AFLP markers in 11 primer pair combinations used in CMS line fingerprinting

Primer combination	Selective nucleotide[a]	Number of visible bands	Number of polymorphic bands	Polymorphism (%)
P1M2	CCA/ACC	42	13	31.0
P1M5	CCA/ACG	47	19	40.4
P1M6	CCA/CAG	44	18	40.9
P1M7	CCA/CAT	61	19	31.1
P1M10	CCA/CCT	49	17	34.7
P2M1	GTT/CAC	39	18	46.2
P2M2	GTT/ACC	49	12	24.5
P2M3	GTT/CCA	44	8	18.2
P2M4	GTT/CAA	54	23	42.6
P2M10	GTT/CCT	43	10	23.3
P3M3	ACC/CCA	58	19	32.8
Total		530	176	33.2
Mean		48.2	16.0	–

[a] The selective nucleotides for *Pst*I/*Mse*I primers

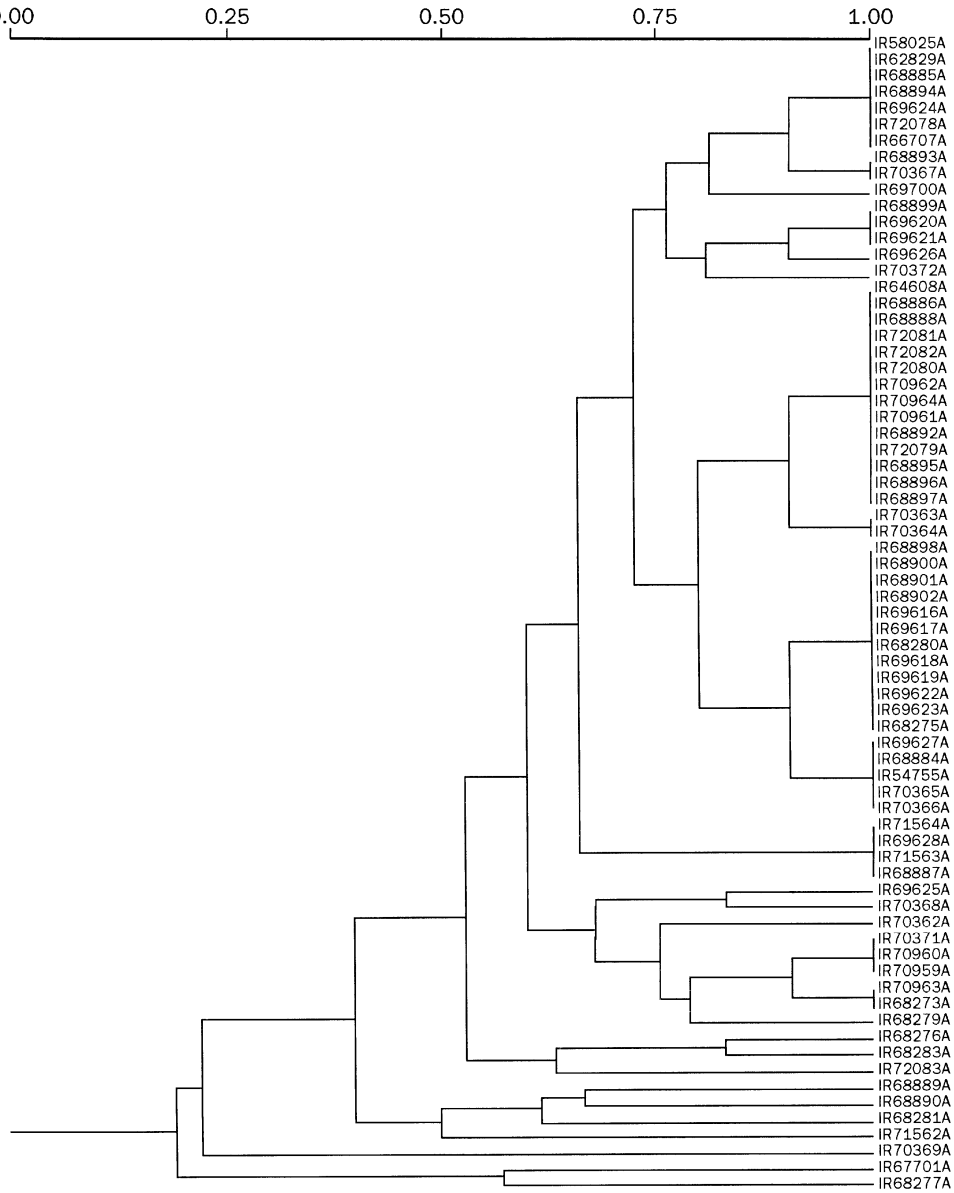

Fig. 1 Dendrogram of 71 CMS lines based on morphological data on 12 qualitative traits, constructed using UPGMA based on Dice similarity coefficients. Scale on *top* of dendogram is Dice coefficient of similarity

separated from the rest (Fig. 2). Because of the strong interaction between quantitative trait loci and the environment, the observations made here may be different from any made later in a different environment and location. Consequently, morphological markers provide insufficient characterization of CMS lines.

AFLP markers

A typical AFLP fingerprint using the primer combination P2/M4 is shown in Fig. 3. DNA from 1 CMS line, IR68892A, (Fig. 3, lane 7) was poorly amplified for unknown reasons. It is evident from Fig. 3 that 54 loci can be scanned in a single PCR analysis and that 23 loci are polymorphic. The number of bands for each primer pair ranged from 39 to 61 (Table 2). The number of polymorphic bands for each primer pair ranged from 8 to 23 with an average of 16. With 11 primer pairs, 530 AFLP markers were scored and 176 were polymorphic. The last 4 lanes in Fig. 3 were those of the four rice varieties which were the parents of the two mapping populations previously analyzed using AFLP markers (Maheswaran et al. 1997; Nandi et al. 1997). These 4 lanes served as controls to ensure the reproducibility of the AFLP markers. The banding patterns of the AFLP markers were remarkably consistent. The duplicated CMS lines (Fig. 3, lanes 37, 38) shared AFLP

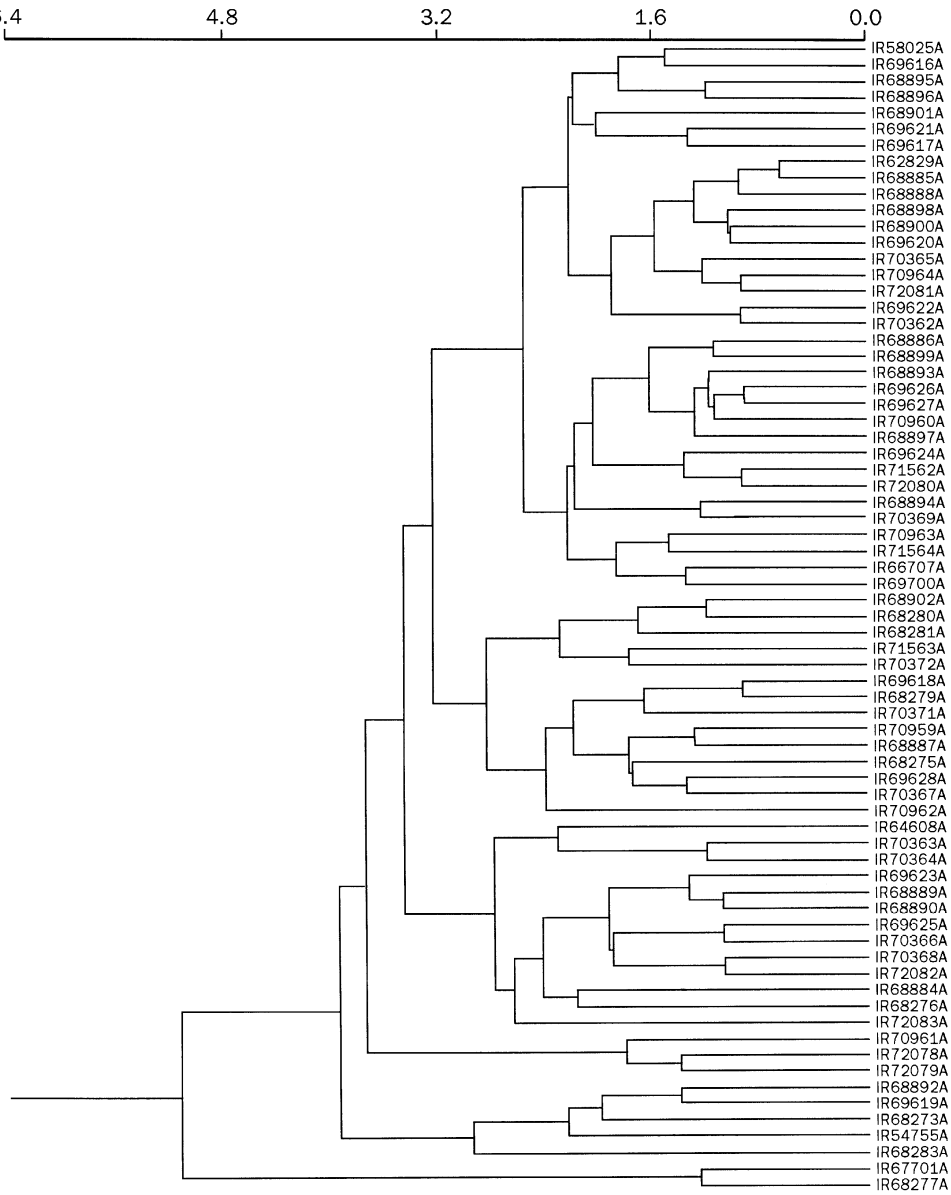

Fig. 2 Dendrogram of 71 CMS lines based on morphological data on 6 quantitative traits, constructed using UPGMA based on Euclidean distance of standardized data. Scale on *top* of dendogram indicates Euclidean distance

bands that were highly reproducible. Earlier, RAPD analysis was used for duplicate identification (Virk et al. 1995). Because AFLP markers are more stable (Vos et al. 1995), the AFLP technique provides a better approach for eliminating duplicate accessions in germplasm banks. The stability and speed of the AFLP marker system make it suitable for DNA fingerprinting of rice and other crops, as indicated previously (Vos et al. 1995; Janssen et al. 1996; Folkertsma et al. 1996; Tohme et al. 1996; Travis et al. 1996).

After analyzing 11 primer combinations, we were able to distinguish all CMS lines at the DNA level, even though some were closely related advanced sister lines of the same cross. The power of AFLP technology to reveal such small differences among germplasms is due to its ability to scan many loci. The 530 loci scanned with AFLP was the largest number of loci ever scanned in a rice germplasm study.

Cluster analysis

Cluster analysis was performed on similarity coefficient matrices calculated from AFLP markers to generate a dendrogram (Fig. 4). The similarity coefficients ranged from 0.89 to 1.0. The dendrogram separated the 71 CMS lines and the four rice cultivars into two broad groups. The two rice varieties, 'Azucena' and 'FR13A'

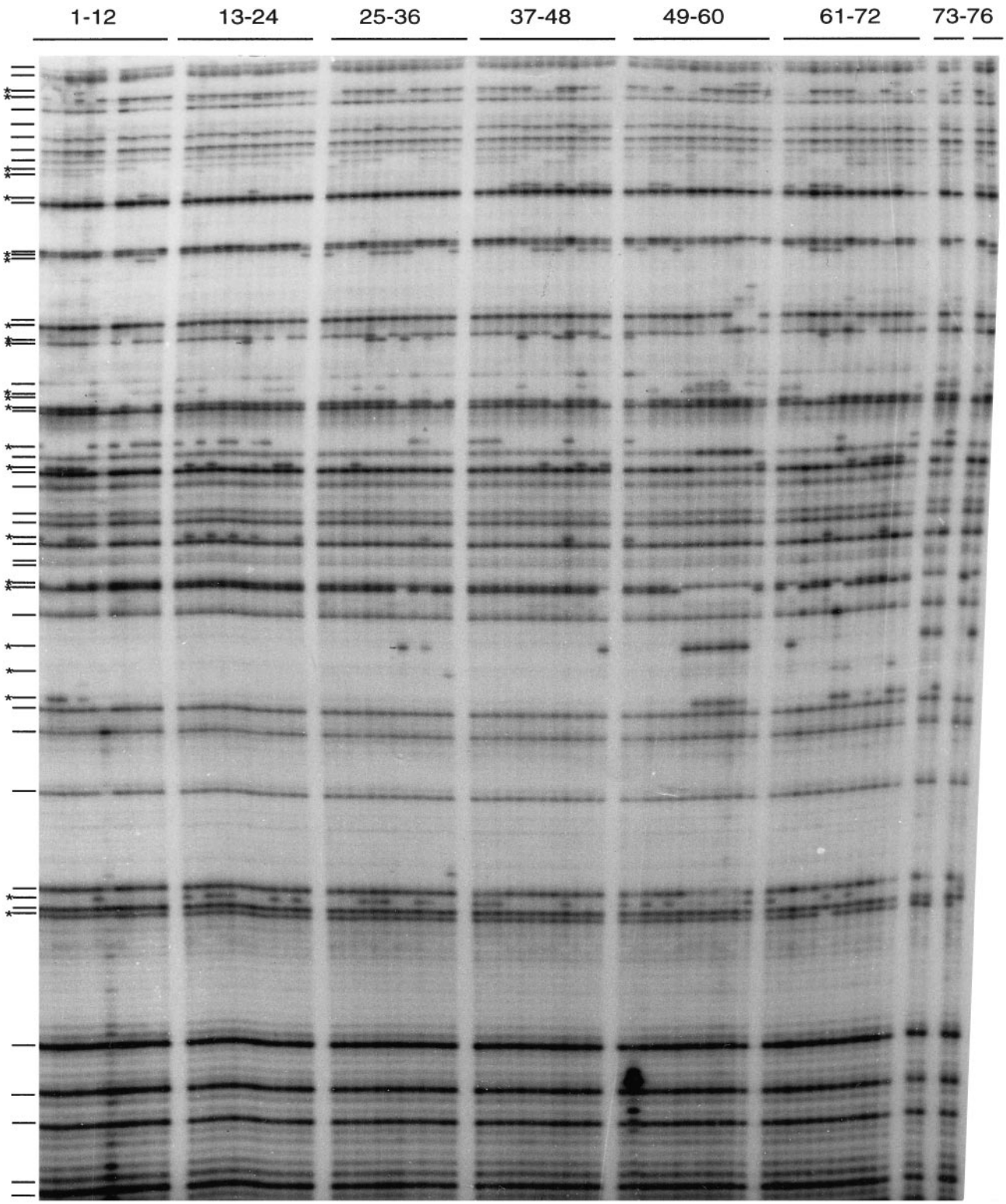

Fig. 3 AFLP fingerprint pattern of the 72 CMS lines (*lanes 1–72*) and four rice varieties (*lanes 73–76*) using the primer pair combination P2/M4. The serial number of the lanes corresponds to the serial number of the individual CMS line listed in Table 1. *Lanes 37 and 38* are the duplicates of IR71564A showing identical AFLP fingerprints. The bands scored are indicated by a *short horizontal bar*. Polymorphic bands are labeled by a *star* (∗)

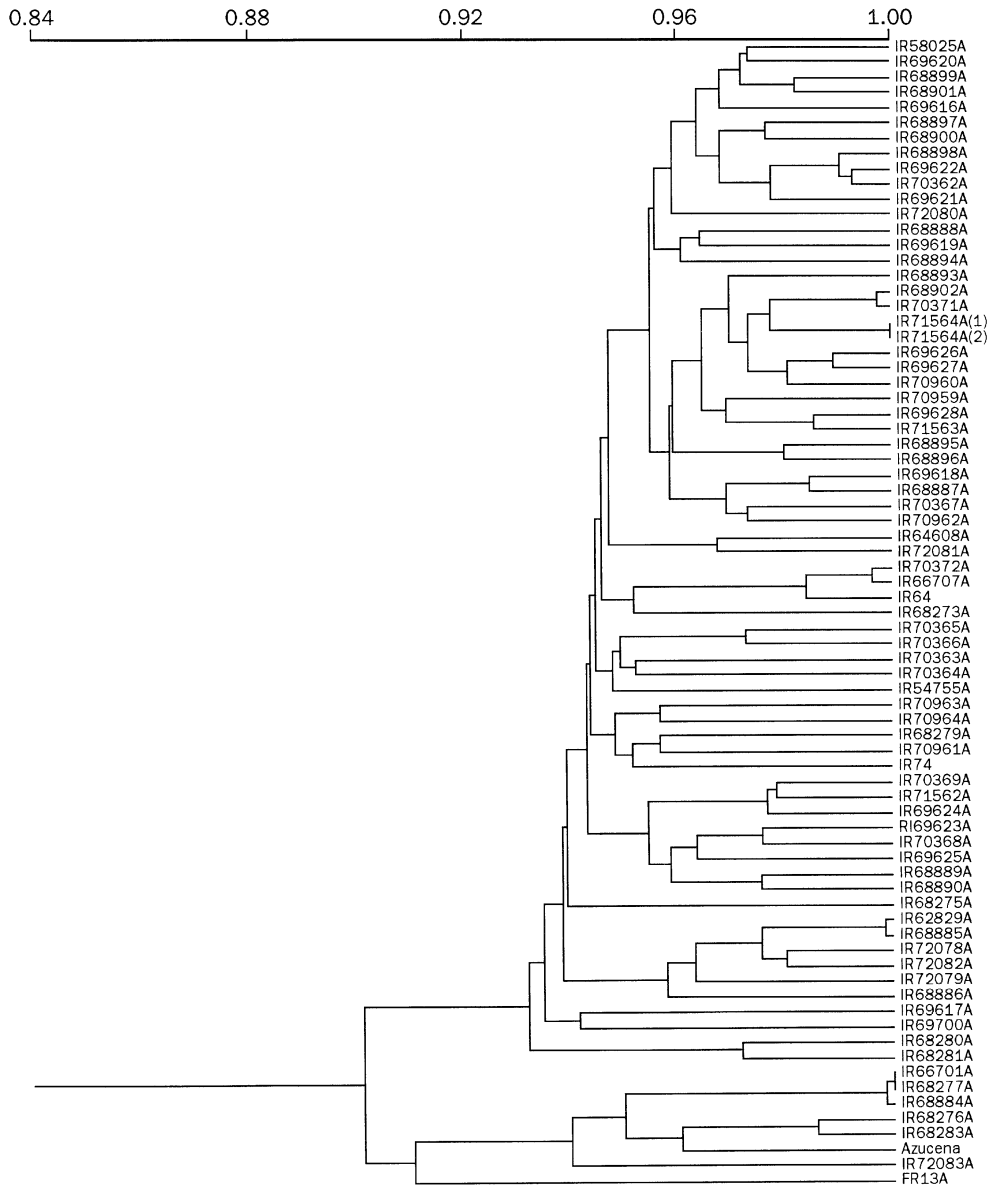

Fig. 4 Clustering of the CMS lines and four rice varieties as described in Table 1 based on 530 AFLP markers using UPGMA analysis based on Dice coefficient of similarity. Scale on top of figure indicates Dice coefficient of similarity

and 6 CMS accessions were clustered together. This group is called the japonica group because most of these lines are considered to be japonica based on grain characteristics (Table 1 and S. S. Virmani, unpublished observations). The CMS lines IR67701A, IR68277A, and IR68884A were with the wide-compatibility (WC) genes (Table 1). The first 2 lines could not be distinguished individually, and the pedigree record also indicated the same parentage (IRRI 1995). The CMS lines, IR68276A and IR68283A, were of japonica type and very close to 'Azucena', an upland japonica variety. IR72083A was in a tropical japonica background but found to be slightly distant from other japonica lines.

'FR13A', a submergence-tolerant landrace from India was distinctly separated from the rest of the lines but found to be more closely related to japonica type than indica. 'FR13A' was classified in group II by Glaszmann (1987). It was neither indica nor japonica according to the Classification of International Rice Germplasm Center at IRRI. Moreover, none of the CMS lines analyzed was bred in this background, suggesting a need to breed new CMS lines for the flood-prone environment.

Most of the CMS lines fell into the indica group. Overall, the CMS lines were found to be closely related to each other (more than 95% similarity). However, each CMS line in this cluster could be distinguished

individually. The three pairs of lines – IR62829A and IR68885A, IR68902A and IR70371A, IR70372A and IR66707A – could be distinguished from each other by 1, 2, or 3 AFLP markers. Pedigree information supported the closeness between two pairs of CMS lines: IR62829B was used as recurrent parent in a cross to produce IR68885A, while IR68902A and IR70371A shared the same female parent, IR19807-21-2–2. However, it was not clear why IR70372A, developed from IR62829A/'Pusa Basmati' came so close to IR66707A, which was developed from the *O. perennis*/IR64R//IR64R combination.

'IR64' grouped with 3 CMS lines, IR70372A, IR66707A and IR68273A, and 'IR74' clustered with 4 different CMS lines at an approximate 95% similarity level. The lines with *perennis* cytoplasm, ARC cytoplasm, and Basmati grain quality were represented in different clusters of indica type (Fig. 4). No association between AFLP markers and CMS type was observed. Most of the fingerprints generated from the CMS lines were from the nuclear genome, because the nuclear genome is much larger than the genomes in the cytoplasm, it is reasonable to assume that the source of the cytoplasm scarcely played a role in cluster analysis.

Discrimination ability of AFLP markers and morphological markers

Comparison of the results of cluster analysis based on morphological and AFLP markers showed that AFLP marker system has distinct advantages in fingerprinting CMS lines. It is known that morphological markers are limited in number and that they do not often reflect genetic relationships because of interactions with environment, epistasis, and the largely unknown genetic control of the traits (Smith and Smith 1989). In contrast, AFLP markers show genetic variation at the DNA level, allowing an estimation of the degree of relatedness between lines without the influence of environment. In this study, 11 pairs of primers were used. If more detailed characterization is needed, more AFLP primer pairs can be used to increase the number of markers. For an initial characterization, 8–10 markers should be sufficient. In this study, the selective primers have a total of six selective nucleotides (Table 2). If this is reduced to 5, more AFLP markers would be generated and fewer primer pairs will be needed. The ability of AFLP fingerprints to discriminate CMS lines will be very useful in future varietal identification.

In conclusion, the AFLP technique is a useful tool for fingerprinting very closely related rice cultivars in a more stable and reliable manner. This study also demonstrates that the CMS lines developed at IRRI are quite different from each other, even though most of them have the same WA cytoplasm. These CMS lines will be suitable for hybrid production in tropical countries. The AFLP technique should be the method of choice in varietal identification, phenetic, and genome composition studies in rice as well as in other crops.

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