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USE OF MOLECULAR MARKERS FOR DIVERSITY ANALYSIS IN RICE

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ABSTRACT

The genetic relationship between seven *japonica*, two *indica* and one tropical *japonica* rice varieties was analysed by using PCR with Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) methods. In RAPD analysis PCR with 10 arbitrary primers applied to ten rice varieties produced 84 useful markers, of which 77.4% were polymorphic. Fifteen AFLP primer combinations produced 285 markers, of which 70.8% were polymorphic. Thus, sufficient polymorphism could be detected to allow identification of individual varieties. Visual examination of electrophoresis gels and analysis of banding patterns confirmed that all the seven *japonica* types were closely related, with similarity indices of 50-85%. Two *indica* varieties were classified into separate group. However, the tropical *japonica* type was easily distinguished, producing variety specific amplification profiles and expressing a lower similarity index to all other varieties tested. Thus, both RAPD and AFLP methods offer a potentially simple, rapid and reliable method for rice genotype identification and recognition of lines that could contribute genetic diversity to new commercial varieties. AFLP was more useful than RAPD because the potential number of loci that could be assayed with AFLP far exceeds that with RAPD.

Key Words : Rice, *Oryza sativa*, RAPD, AFLP, genetic diversity

Cultivated rice has been classified into *indica*, *japonica* and tropical *japonica* (javanica). The *indica* cultivars are cultivated extensively in the tropics and sub-tropics while the *japonica* cultivars are predominantly grown in temperate regions. The javanica type is designated as tropical *japonica* based on morphological and physiological characteristics [1]. Glaszmann [2] has classified the rice cultivars into two large groups as Group I (*indica*) and Group 6 (temperate and tropical *japonica*). Zhang et al. [3] have indicated that *indica* and *japonica* cultivars are easily differentiated as compared to temperate and tropical *japonicas*. Pedigree analysis indicates that genetic diversity of modern rice cultivars has been reduced due to intensive breeding

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efforts [4]. The recently developed Random Amplified Polymorphic DNA (RAPD) based on PCR has been widely adopted in gene mapping and fingerprinting studies [5]. This technique has been applied to classify the rice cultivars [6]. In this method short oligonucleotides of arbitrary sequence are used singly to support the amplification of regions of the test plant genome and the amplification products are separated by gel electrophoresis. The differences between genotypes are reflected as differences in banding patterns. Amplified Fragment Length Polymorphism (AFLP) is a relatively new DNA fingerprinting technique [7] which selectively amplifies restriction fragments. This PCR based method is able to generate complex banding patterns, DNA fingerprints of up to at least 100 DNA fragments in each reaction. AFLP is therefore potentially very useful for the study of biological diversity, varietal identification and genetic mapping. The present study was undertaken to understand the genetic diversity based on RAPD and AFLP markers in a sample of rice cultivars involving *indica*, *japonica* and tropical *japonica* types.

MATERIAL AND METHODS

DNA isolation

Ten rice entries (seven *japonica*, two *indica* and one tropical *japonica* (Table 1) were obtained from Yanco Agricultural Institute, New South Wales, Australia. Seeds were sown in plastic pots containing black peat soil and raised in a glass house. A total of 0.3g of leaf tissue was collected by harvesting equal amounts from each of 10 seedlings within an entry. The DNA was isolated following the procedure of Murray and Thompson [8]. DNA concentration was monitored by subjecting samples to 0.8% agarose gel electrophoresis in TBE buffer [9] and by visual assessment of band intensities compared with molecular weight marker DNA standards.

RAPD analysis

Oligonucleotide primers were chosen arbitrarily without prior knowledge of the presence of polymorphic markers in the amplification result. Twenty primers (OPA 01 to 20) were initially screened with three entries to identify the most promising primers for detecting polymorphism. The following 10 Operon primers were selected to screen the ten rice entries: OPA01, OPA02, OPA03, OPA05, OPA08, OPA11, OPA13, OPA18, OPA19 and OPA20. The details of 10 decamer oligonucleotide primers are listed in Table 2. The reaction consisted of 25 ng sample DNA, 1.25 m M dNTPs, 2 units of primer, 0.2 units of 5u/ul Taq DNA polymerase and 2.5 units of buffer in 25.2 ul volume. The thermocycler was programmed as follows. Cycle 1, 5 min at 94°C; cycles 2-41, 1 min at 94°C, 1 min at 36°C and 2 min at 72°C; cycle 42, 5 min at 72°C and cycle 43, 5 min at 15°C. Amplification products were resolved by

Table 1. List of rice entries included in the study

S. No.	Entry	Type	Parentage	Features
1.	Amaroo	*japonica*	Calrose/M7	semi-dwarf, medium grain
2.	Millin	*japonica*	M7*2/Somewake	semi-dwarf, medium waxy grain
3.	YRK 4	*japonica*	Bogon/Koshihikari	semi-dwarf, small grain
4.	Kyeema	*japonica*	Pelde//Della/Kulu	fragment long grain
5.	Koshihikari	*japonica*	Norin 1/Norin 22	1995 introduction into Australia
6.	Koshihikari (J)	*japonica*	Norin 1/Norin 22	Japanese Koshihikari
7.	Sasanishiki	*japonica*	Hatsunushiki/Sasashigure	semi- dwarf, medium grain
8.	IR 66160-5-2-3-2	tropical *japonica*	Multiple cross	progeny of super rice from IRRI, Philippines
9.	Dular	*indica*		drought tolerant
10.	IR 36	*indica*	IR1561-228-1-1-211//IR24*/ O. *nivara* //CR94-13	semi-dwarf, long grain

electrophoresis on 2% agarose gel in TBE buffer at 100V for 60 minutes, stained with ethidium bromide and visualized under UV illumination.

Table 2. Arbitrary Decamer primer (Operon Tech., Inc.) used in RAPD analysis

Primer	Sequence (5′ - 3′)	Primer	Sequence (5′ - 3′)
OPA 01 ...	CAGGCCCTTC	OPA 11	... CAATCGCCGT
OPA 02 ...	TGCCGAGCTG	OPA 13	... CAGCACCCAC
OPA 03 ...	AGTCAGCCAC	OPA 18	... AGGTGACCGT
OPA 05 ...	AGGGGTCTTG	OPA 19	... CAAACGTCGG
OPA 08 ...	GTGACGTAGG	OPA 20	... GTTGCGATCC

AFLP analysis

Fifteen enzyme combinations of *Eco* RI/*Mse* I were used for the double digestion of template DNA. The *Eco* and *Mse* adapters were ligated to the ends of restriction fragments. The pre selective primers have a single base overhang which selects for fragments having extra base downstream of the restriction site.

Pre selective amplification

Before selective PCR, a pre-amplification was carried out to amplify the DNA fragments non-selectively. The PCR programme was 20 cycles of 30 seconds at 94°C for DNA denaturation, 60 seconds at 56°C for DNA annealing and 60 seconds at 72°C for DNA extension with soak temperature at 4°C.

Selective amplification

The pre-amplification product was diluted 40 times as a template for selective PCR. Fifteen primer combinations were used (Table 3). The PCR programme was performed for 36 cycles with the following cycle profile: a 30 seconds DNA denaturation step at 94°C, a 30 seconds annealing step and one minute extension step at 72°C. The annealing temperature in the first cycle was 65°C, which was subsequently reduced in each cycle by 0.7°C for the next 12 cycles was continued at 56°C for the remaining 23 cycles. All amplification reactions were performed in 9700 thermocycler.

Table 3. Details of primer combinations used in AFLP analysis

Primer combinations	
E-AC & M-CTG	E-TA & M-CTA
E-AT & M-CAT	E-TC & M-CAC
E-AT & M-CTC	E-TG & M-CAC
E-AG & M-CAC	E-TG & M-CTC
E-AG & M-CTA	E-TT & M-CAT
E-AG & M-CTG	E-TT & M-CTC
E-TA & M-CAA	E-TT & M-CTT
E-TA & M-CAG	

Gel analysis

Following amplification reaction, products were mixed with 10 ul of loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.1% bromophenol blue and xylene cyanol). Each sample (30 µl) was loaded on a 7% denaturing polyacrylamide gel. The gel matrix was prepared using 40% acrylamide, 7M urea and 10x TBE buffer. To 100 ml of gel solution, 700 µl of 10% APS and 100 µl of TEMED were added and gels were casted in gel apparatus. 1.0% TBE was used as running buffer. Electrophoresis was performed at constant power (250 V) for 2 hr. 30 minutes. After electrophoresis gels were fixed for 10 minutes in 10% acetic acid and washed with

milli Q water for 2 minutes (3 washes) and kept in silver stain (1g $AgNO_3$; 1.5 ml 37% H_2CO) for 30 minutes. The gel was washed in milliues water for 10 seconds only and kept in developer solution (30 g $NaCO_3$; 1.5 ml 37% H_2CO; 200 µl $NaSO_3$ 10 µg/ml) until the bands developed. Then the gel was fixed in 10% acetic acid for 5 minutes and washed in milliues water to remove acid and to prevent gel cracking. The gel was stored between plastic sheets and photographed in Novaline unit.

Data analysis

Only data generated from the detection of polymorphic fragments were analysed. Specific amplification products were scored as present (1) or absent (0) for each of the 10 varieties with 10 primers and 15 primer combinations for RAPD and AFLP analysis, respectively. The genetic similarity index was calculated as per Jaccard's coefficient method.

$$F = N_{AB1}/(N_T - N_{AB0})$$

Where,

F = Similarity index

N_{AB1} = Number of bands present (scored 1) in both accessions A and B

N_{AB0} = Number of bands present in all test entries but not present in accessions A or B

N_T = Total number of bands scored in the study

Similarity index was used to construct a cluster diagram using the complete linkage method. The Phylip software package (DRAWTREE) was used to produce a phenogram.

RESULTS AND DISCUSSION

RAPD

The ten random primers produced a total of 84 useful markers. Of these 84 PCR products generated, 22.6% (19 bands) were monomorphic across all varieties. Many bands appeared in most of the varieties and were absent in only a few varieties. The remaining 65 bands (77.4% of the total products scored) were polymorphic among the varieties tested. This amounts to an average of 6.5 polymorphic bands per primer. This is a relatively high level of polymorphism expressed by arbitrary primers compared to reports of other RAPD studies in rice [10-12]. In general, sufficient polymorphism exists to allow distinction between the genotypes tested. Each primer produced between 4 (OPA 2) and 10 (OPA 18) amplification products,

which ranged in size between 375 and 2600 bp. The arbitrary primer OPA 13 was useful for discriminating varieties of distinct characteristics (Fig. 1). The varieties and their banding patterns are described in Table 4. Some varieties could be distinguished from all other varieties with selection of these primers. For instance, IR 66160-5-2-3-2 and Kyeema gave specific banding profiles with primers OPA 01, 02, 05, 08 and 11;

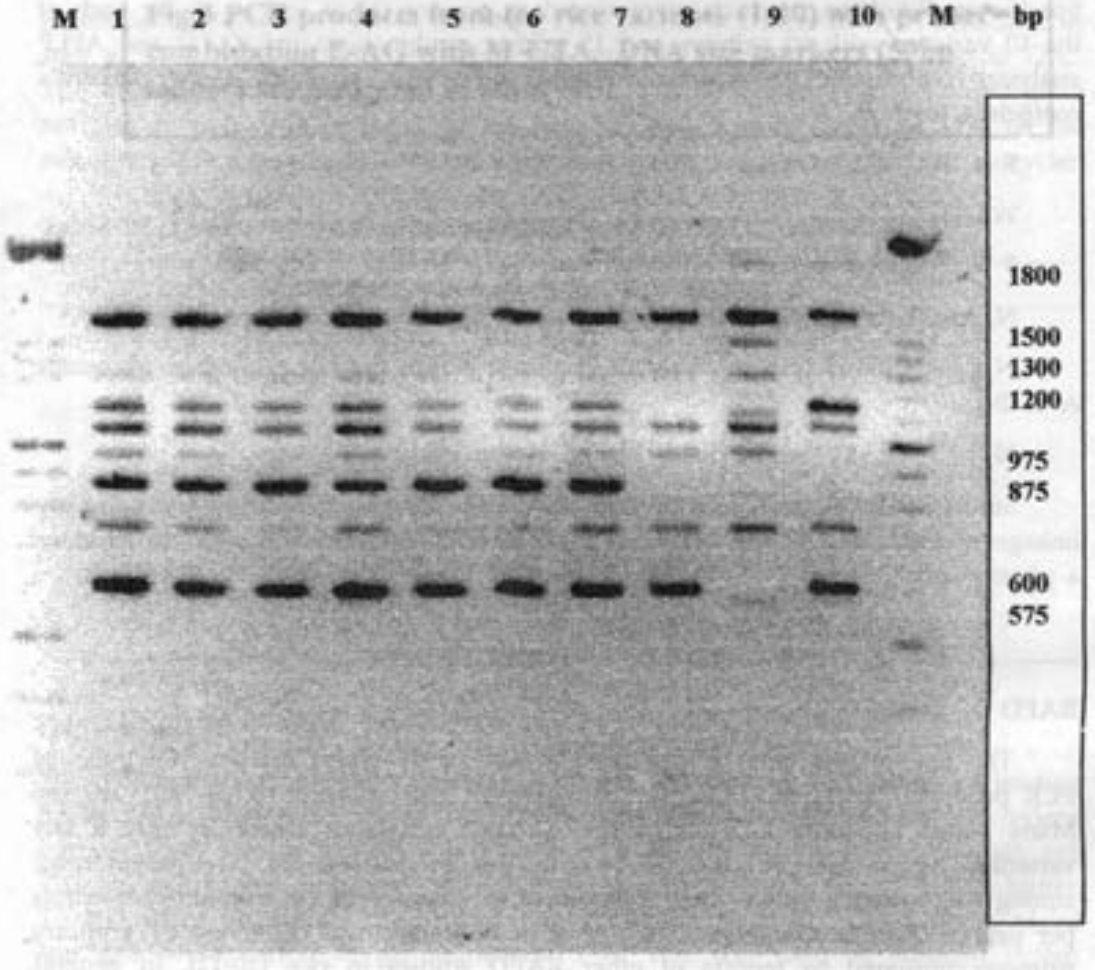

Fig. 1. PCR products from ten rice varieties (1-10) with primer OPA-13. DNA size markers (100 bp ladder) are indicated in lane M

Table 4. Distinct varieties discriminated with primer OPA 13, illustrated in Fig. 1

Variety	Origin	Specific banding pattern
Dular	*indica*	bands of 600, 875 and 1200 bp missing, presence of 1500, 1300, 1150 and 575 bp bands
IR 36	*indica*	bands of 1500, 1300, 1150; 975 and 575 missing, presence of 1800, 1200 ad 600 bp bands
IR66160-5-2-3-2	tropical *japonica*	bands of 1200, 875 and 575 missing; presence of 975 and 600 bp bands
YRK 4	*japonica*	bands of 975 missing
Kyeema	*japonica*	presence of 975 bp

while IR 36 and Dular gave unique banding patterns with primers OPA 05, 08, 11, 13, 18 and 19. In contrast all other *japonica* types could not be readily distinguished among themselves.

The indices of genetic similarly among 10 rice genotypes are presented in Table 5 and as a phenogram in Fig. 2. This analysis clearly distinguished the two *indica* varieties namely Dular and IR 36 from all other varieties. They showed only less than 20% similarity to the remaining varieties. This result was not unexpected, considering the diverse origin (i.,e., *indica* types) and characteristics of these varieties

Table 5. Similarity index for 10 rice varieties by RAPD method

	1	2	3	7	6	5	4	8	9	10
1	1.00	0.85	0.75	0.73	0.67	0.62	0.58	0.49	0.20	0.13
2		1.00	0.85	0.85	0.67	0.70	0.54	0.52	0.20	0.13
3			1.00	0.81	0.74	0.67	0.52	0.50	0.19	0.12
7				1.00	0.80	0.67	0.55	0.50	0.19	0.13
6					1.00	0.61	0.60	0.51	0.18	0.11
5						1.00	0.48	0.37	0.17	0.15
4							1.00	0.49	0.24	0.20
8								1.00	0.21	0.05
9									1.00	0.19
10										1.00

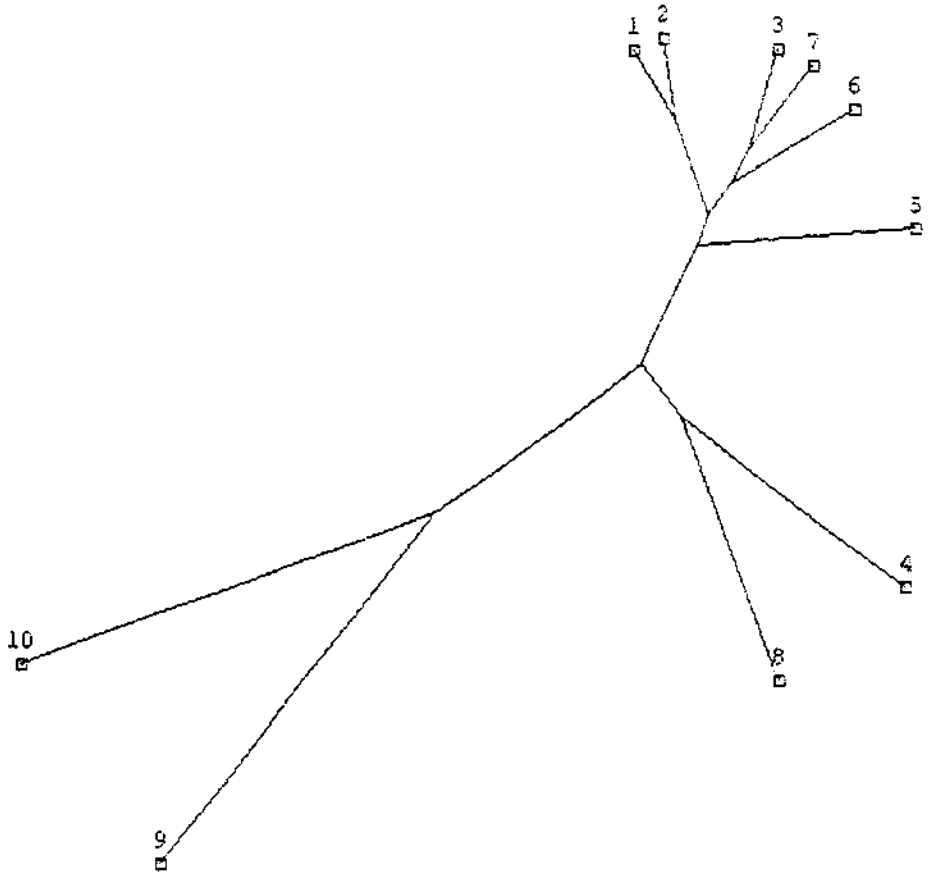

Fig. 2. Phenogram showing clustering of ten rice varieties through RAPD analysis

compared to others. Among the other eight varieties, tropical *japonica* IR 66160-5-2-3-2 and *japonica* long grain variety Kyeema are distinctly different from the remaining six varieties with only around 50% similarity. The other six *japonica* types showed high level of genetic similarity with values ranging from 60 to 85%.

AFLP

Fifteen AFLP primer combinations produced 285 bands. Of these 202 were clearly polymorphic among the ten varieties with 70.8% of the total products scored. This amounts to an average of 13.5 polymorphic bands per primer combination. This is a high level of polymorphism expressed by AFLP primer combinations

Table 6. Distinct varieties discriminated with primer combination E-AG with M-CTA, illustrated in Fig. 3

Variety	origin	Specific banding pattern
IR 66160-5-2-3-2	tropical *japonica*	presence of 1100, 1900 and 2000 bp bands and absence of all other bands
Dular	*indica*	absence of 100, 140, 200, 310 and 375 bp bands and presence of 330 bp
IR 36	*indica*	presence of 330 and 375 bp and absence of 110, 140, 160, 165, 200 and 310 bp
Millin	*japonica*	absence of 140 and 440 bp bands; presence of 120 bp.

compared to the results already reported in rice [13-15]. The number of polymorphic markers from the 15 primer combinations ranged from 9 to 20 and the size of amplification products varied from 105 to 1500 bp. The primer combination E-AG with M-CTA was particularly useful for discriminating varieties of distinct characteristics (Fig. 3). The varieties and their banding patterns are described in Table 6. Some varieties could be clearly distinguished from all other varieties with selection of the primers. For instance the tropical *japonica* type IR 66160-5-2-3-2 gave specific banding patterns with almost all the primer combinations. It revealed very few bands mostly of above 1000 bp. Hence the tropical *japonica* may need new primer combinations to reveal polymorphism for bands with less than 1000 bp. The *indica* types Dular and IR 36 had unique banding profiles with the primer combinations 2, 4, 7, 13 and 15. However, all *japonica* types could not be readily distinguished among themselves. In general, it can be concluded that sufficient polymorphism exists to allow distinction between the rice genotypes tested.

The indices of genetic similarity among 10 rice genotypes are presented in Table 7 and as a phenogram in Fig. 4. This analysis clearly distinguished the tropical *japonica* genotype IR66110-5-2-3-2 from all other varieties. It showed negligible similarity values with all other varieties tested. This result is quite expected, considering the diverse origin and characteristics of this tropical *japonica* variety compared to others. Among the other genotypes, two *indica* types namely Dular and IR 36 are distinctly different from the remaining seven temperate *japonica* types with less than 50% genetic similarity values. Such a clear distinction between *indica* and *japonica* types separated by AFLP primer combinations has already been reported earlier [13, 15]. The other seven *japonica* types showed high level of genetic similarity with values

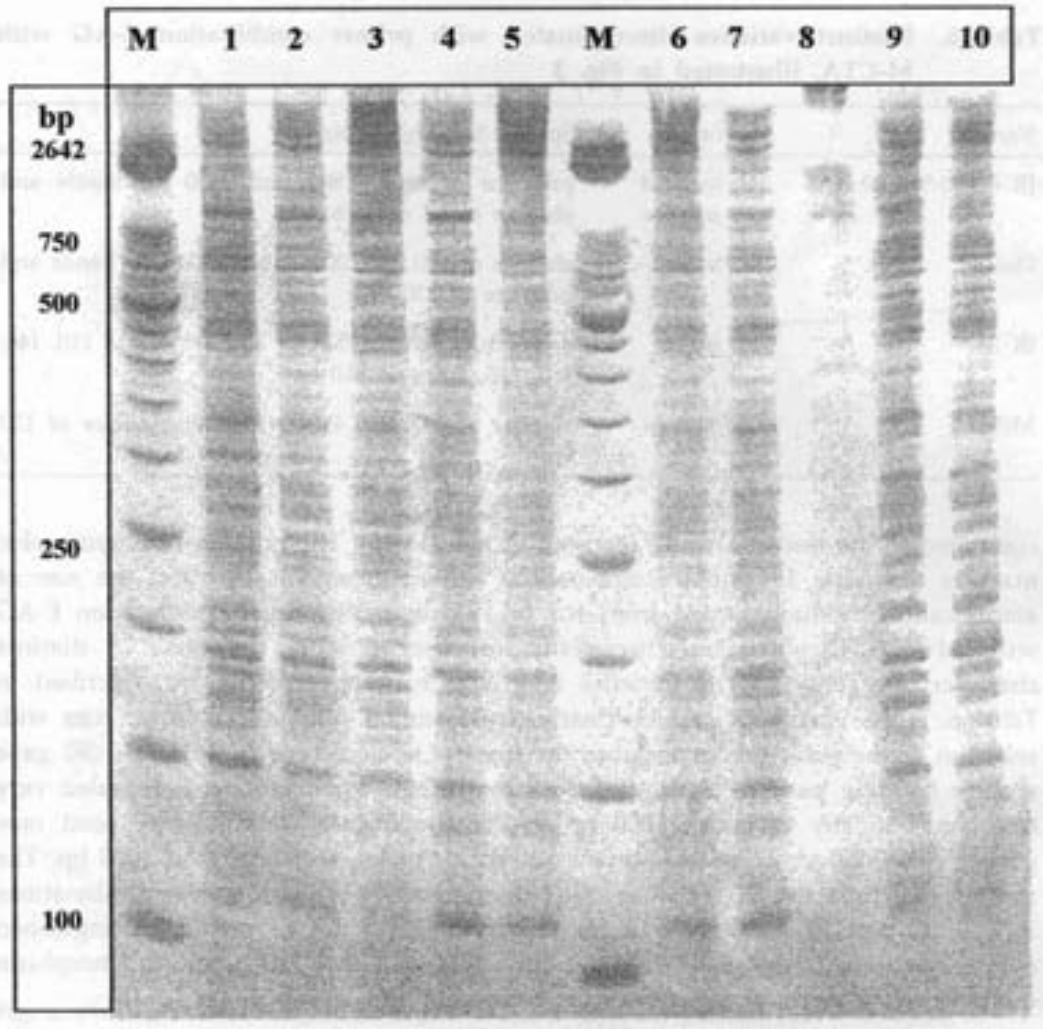

Fig. 3. PCR products from ten rice varieties (1-10) with primer combination E-AG with M-CTA. DNA size markers (50bp ladder) are indicated in lanes M

ranging from 51 to 79%. Zhang *et al.* [3] also indicated that *japonica* rices reflect lower genetic diversity than *indica* in RFLP analysis.

Comparison of RAPD wit AFLP

Given the proliferation of genetic markers, comparison between techniques are inevitable. There is a need for such comparison in order to decide on which technique is best suited to the issues being examined. Both the techniques used in this study

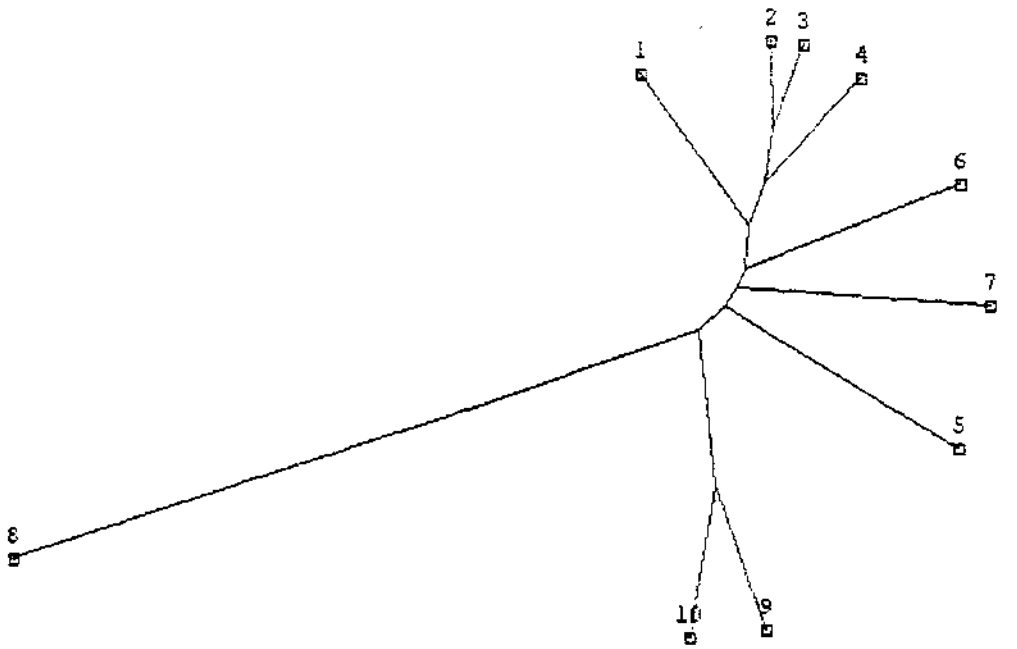

Fig. 4. Phenogram showing clustering of ten rice varieties through AFLP analysis

not only differs in principle, but also in the type and amount of polymorphism detected. Both the molecular approaches were able to uniquely fingerprint each of the ten rice genotypes. The total number of bands scored ranged from 84 for RAPDs to 285 for AFLPs. The percentage of polymorphic bands for each assay did not correlate to the total number of bands. For RAPD only 65 bands were Polymorphic with 77.4%. In contrast 202 polymorphic bands were scored with 70.8% for AFLP. The level of polymorphism was high in RAPD when compared to AFLP as reported by Mackill *et al.* [15] in rice and Russel *et al.* [14] in barley. The average number of polymorphic bands per primer/primer combination is more in AFLP (13.5) than for RAPD (6.5). However, although AFLPs do not offer the highest level of polymorphism, they are the most efficient because they have the capacity to reveal many polymorphic bands in a single lane [14, 16].

The genetic similarity values between the genotypes tested ranged from 0.05 to 0.85 for RAPD and from 0.02 to 0.79 for AFLP (Tables 4 and 6). Classification of genotypes using cluster analysis for RAPD and AFLP is depicted in Fig. 3 and 4 respectively. Both the molecular techniques have classified the ten rice genotypes into distinct sub species viz., *indica*, *japonica* and tropical *japonica*. Such a clear separation between the sub species of rice [15] and between the spring and winter

Table 7. Similarity index of 10 rice varieties by AFLP method

	1	2	3	4	5	6	7	9	10	8
1	1.00	0.75	0.68.	0.62	0.53	0.53	0.56	0.45	0.48	0.02
2		1.00	0.76	0.67	0.53	0.56	0.59	0.48	0.49	0.03
3			1.00	0.79	0.55	0.62	0.64	0.52	0.51	0.04
4				1.00	0.58	0.57	0.65	0.48	0.47	0.03
5					1.00	0.57	0.51	0.39	0.39	0.02
6						1.00	0.57	0.42	0.43	0.04
7							1.00	0.48	0.52	0.02
9								1.00	0.70	0.03
10									1.00	0.03
8										

barley types [14] was already reported. In clustering the genotypes, the AFLPs have three distinct groups. In one group, both the *indica* types namely Dular and IR 36 were included. In the other group the tropical *japonica* type IR66160-5-2-3-2 alone was included. In the third group, all temperate *japonica* types were included. Though the RAPDs also had the same clustering pattern, the long grain *japonica* type kyeema was included with tropical *japonica* type. Several previous studies have compared the use of RFLPs, RAPDs and SSRs [17-19] and suggested that higher band sharing would make RAPDs more suitable for genetic relatedness studies. From the present study, it could be concluded that AFLPs could be more useful than RAPDs. Mackill *et al.* [15] has already reported that the potential number of loci that could be assayed wit AFLPs far exceeds that with RAPDs or other markers in rice.

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