AFLP in rice

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GENETIC DIVERSITY ANALYSIS OF ORYZA USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM

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ABSTRACT

Amplified Fragment Length Polymorphism (AFLP) analysis was carried out to study the genetic diversity among eight cultivars of Oryza sativa L. and seven accessions of four wild Oryza species. UPGMA analysis of 130 AFLP loci visualized among Oryza lines was performed. At 60 per cent similarity index, all the cultivars grouped into one cluster, all the accessions of A genome into another group, whereas the only accession of O. brachyantha (FF) and O. punctata (BBCC) remained isolated. However, at 80 per cent similarity index, all cultivars grouped into one cluster. AFLP markers were able to efficiently discriminate various cultivars and species of Oryza.

INDEX WORDS: Amplified Fragment Length Polymorphism (AFLP); genetic diversity, Rice

A series of DNA based molecular markers have been used for fingerprinting germplasm in crop plants. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have been used extensively in rice (Wang and Tanksley, 1989; Fukuoka et al., 1992; Yu and Nguyen 1994; Mackill 1995). Recently a technique known as Amplified Fragment Length Polymorphism (AFLP) has been developed (Vos et al., 1995). The technique combines the reliability and robustness of RFLP and strength of PCR techniques. AFLP technique is considered powerful for genome mapping, genotype identification and phylogenetic studies (Becker et al., 1995; Thomas et al., 1995; Vos et al., 1995; Maheshwaran et al., 1996).

AFLP technology makes use of polymerase chain reaction (PCR) to amplify specific DNA sequences based on a procedure referred to as Selective Restriction Fragment Amplification or SRFA (Keygene, 1993). Using SRFA, restriction fragments derived by digesting total genomic DNA with
restriction enzymes are selected and detected following PCR amplification. First of all, the genomic DNA is cut with two restriction enzymes, one rare-cutter and the other a frequent cutter. Double-stranded adapters are then ligated to the ends of the restricted DNA fragments. The adapter sequence and the adjacent restriction site sequence serve as binding sites for primers during subsequent PCR amplification of DNA restriction fragments. Selective nucleotides are then added to the 3' ends of PCR primers leading to the recognition of only a subset of the restriction fragments. Only those fragments whose nucleotide sequence match the selective nucleotides of the primers are amplified. This study reports the use of AFLP markers for DNA fingerprinting of *Oryza* lines of cultivated and wild species.

**MATERIAL AND METHODS**

Eight varieties of rice (Pusa Basmati 1, Pusa 44, PR 103, PR 106, PR 108, PR109, PR110, PR111) released for commercial cultivation in and seven accessions of four wild *Oryza* species, *O. brachyantha* (101257); *O. nivara* (103830, 103840, 105715); *O. rufipogon* (101963, 102186) and *O. punctata* (101409) were used. Bulked leaf samples were collected and DNA was extracted from ground leaf tissue using the Potassium acetate method (McCouch et al., 1988).

**Restriction of the genomic DNA:**

Five µg of genomic DNA from each strain was used for restriction digest using five units each of EcoRI and MseI, 5 µl of 10X restriction-ligation (RL) buffer (10mM Tris-HAc, 10mM MgAc₂, 50mM KAc, 5 mM DTT, pH 7.5), and ddH₂O for a total reaction volume of 50 µl. Reactions were incubated at 37°C for 4 hrs.

**Ligation of adapters:**

Ten µl of cocktail containing 1 µl EcoRI adapter (5pMol), 1 µl MseI adapter (50 pMol), 1.2 µl 10mM ATP, 1 µl 10X RL buffer, 1 unit T4 DNA Ligase and ddH₂O. Samples were then incubated for 3 hrs, at 37°C.

**Selective pre-amplification of primary template DNA**

The first PCR amplification of adapter-ligated restriction fragments (primary template) was used to generate large quantities of secondary template DNA suitable for AFLP reactions with radioactively labelled selective primers; the primer sequence appropriate to prime the EcoRI end, with the additional 3' nucleotide (E+A); the sequences of the selective M+1 primer fitting the MseI ends was 5'-GATGAGTCCT GAGTAAC (M+C). Five µl of primary template was mixed with 30 ng E+A and 30 ng M+C primer, 0.8 µl mM dNTPs, 1.0 U Taq polymerase, 2 µl 10 x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂ in a final volume of 20 µl. PCR reaction was performed in a PE9600 thermal cycler programmed for 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 60 Sec at 72°C followed by 72°C for 5 min.
SRFA Reactions

For the reproducible and selective amplification of a limited number of DNA restriction fragments primer combinations were used with three additional 3' nucleotides (E+3 and M+3 primers). Such primer pairs would theoretically recognize only one out of 4096 of the initial number of restriction fragments. The following E+3 primers in combination with M+3 primers were used in the AFLP fingerprinting:

E+AAC/(5'-GACTGCGCGTACCAATTCAAC),
M+ACG/(5'GATGAGTCCTGAGTAAAC)

The PCR reaction mixture comprised of 5 µl secondary template, primed with 5 ng 32P labelled E+3 primer and 30 ng unlabelled M+3 primer, whereas the other components and the temperature profile used for the amplification was 30 sec at 94°C, 30 sec at 65°C, 1 min at 72°C with the annealing temperature for next 11 cycles lowered by 1.0°C, followed by 24 cycles at 30 sec at 94°C, 30 Sec at 45°C, 1 min at 72°C and a final soak temperature of 4°C.

Separation of labelled fragments and autoradiography:

Reaction products were loaded on 6 percent polyacrylamide gel in 1xTBE electrophoresis buffer using a Bio Rad sequence gel system. The gels were dried on Whatmann 3 MM paper and X-ray films were exposed for 1-4 days at room temperature.

Data analysis

AFLPs were scored as dominant and recessive markers based on presence/
absence of bands, respectively. Only clearly scorable bands were included in the data set. Similarity coefficients were derived through pair-wise comparison of the genotypes based on the presence or absence of shared polymorphic bands. These coefficients, computed using Nei’s method (Nei and Li, 1979) were used to perform cluster analysis and draw a dendogram using the UPGMA method and the computer program NTSYS-PC (Rohlf, 1990).

RESULTS AND DISCUSSION
A total of 131 major AFLP loci were detected using one selective nucleotide in the first amplification and a EcoRI (E+AC/ (5'-GACTGCGCGTACCAATTCAC)/MseI (M + ACG (5' GATGAGTCCTGAGAAACG)) primer combinations with three selective nucleotides. Five AFLP loci are specific to particular varieties and are indicated by arrow in Fig. 1.

Genetic distance coefficients based on Nei’s formula (Nei and Li, 1979) were used for performing cluster analysis. The dendogram showed two clusters at 60 per cent similarity index and outgrouped O. brachyantha accession 101257 and O. punctata acc. 101409 (Fig. 2). All the cultivars including Pusa Basmati 1 grouped into one cluster whereas all the accessions of A genome species of O. nivara and O. rufipogon into another cluster. However, at 80 per cent similarity index,

Fig. 2. Clustering of eight rice varieties and seven accessions of four wild species of rice based on 130 AFLP markers using UPGMA analysis. Scale on the top indicate Dice coefficient of similarity.
all the rice cultivars grouped into one cluster except Pusa Basmati 1 suggesting low level of genetic diversity among cultivars. It is also evident from the dendrogram that there is high diversity among wild species as compared with the cultivars. The F genome species O. brachyantha appeared to be relatively closer to a genome Oryza species and indica rice cultivars as compared to O. punctata (BB). The germplasm of related wild Oryza species is a rich reservoir of genetic diversity for resistance to biotic and abiotic stresses and other useful traits some of which have already been transferred to cultivars and commercially exploited (Khush, 1984).

A high degree of polymorphism between the Oryza sativa cultivars and the wild species will further facilitate the transfer, tagging and pyramiding of useful traits from the wild species for rice improvement.

REFERENCES
system. Applied Biostatistics Inc. New York NY, USA.


