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UTILIZATION OF RAPD MARKERS FOR THE IDENTIFICATION OF CULTIVATED AND WILD RICE SPECIES

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Abstract

Random Amplified Polymorphic DNA (RAPD) markers were generated in Perkin Elmer DNA Thermal Cycler using genomic DNAs extracted from different rice varieties/species and 6 synthetic 10 mer primers of series-S. Material included in the study comprised 5 cultivated and 11 wild salt tolerant and sensitive varieties/species with different genomes and ploidy level. The scorable DNA fragments amplified by different primers ranged between 22 and 47 fragments per primer. Of the total 212 fragments that were amplified, 99 fragments were scored as potential genetic markers to be used for cultivar identification. Of the 99 markers, 50 (51%) were cultivar specific while 49 markers were genome specific. One marker appeared only in salt tolerant cultivated rice varieties while another appeared both in wild and cultivated but salt tolerant varieties. Based on these markers, 3 out of 5 cultivated and 5 out of 11 wild rice species were distinguished from each other. Inter and intraspecific variations were also detected. The markers identified in the present study can be used to check seed quality, for fingerprinting of a specific cultivar and to detect transfer of genetic material from wild species to cultivated varieties. The results showed the potential of RAPD markers for gene tagging experiments particularly tagging genes for salt tolerance.

Introduction

Identification and description of cultivars, varieties, wild species and land races are important particularly when breeders need to describe their newly produced varieties to get them registered with the seed companies. Cultivar identification is also critical for seed companies to control quality, propagation and marketing of their germplasm. Information required for cultivar identification can be obtained through morphological data (Pauksens, 1975; Troyer, 1986) largely based on phenotypic observations. It can provide unique description of cultivated varieties (Molina-Cano & Rossello,1978) however, due to interaction of genotypes with the environment, data collected under different environmental conditions (Lin & Binns, 1984) and over different years seldom serve as reliable descriptors (Stagmann, 1984). Several other methods such as isozyme analysis (Arus *et al.*, 1982; Stagmann, 1984), seed storage protein electrophoresis (Gupta & Robbelen, 1986) and High Performance Liquid Chromatography (Buchler, 1989) are also being used for cultivar identification. Since the level of polymorphism detected through these techniques is low therefore, their utilization has become limited.

Recent advancement in genetics and molecular biology has provided descriptors based on DNA molecules. While morphological data restrict only to individual identification, DNA based data such as Restriction Fragment Length Polymorphism (RFLP) can be used to get taxonomic, genetic and phylogenetic information (Doebley & Wendel, 1989; Kirby, 1990; Weir, 1990), mapping loci for characters of economic

importance such as plant height and yield in maize (Beavis *et al.*, 1991; Stuber *et al.*, 1987), seed protein and oil content in soyabean (Diers *et al.*, 1992), kernel elongation and wide compatibility in rice (Zheng *et al.*, 1994; Ahn *et al.*, 1993).

In our wide hybridization programme for improvement of salt tolerance in cultivated rice, we are using different salt tolerant accessions of *Oryza punctata* (Farooq *et al.*, 1992a), *O. grandiglumis* and *O. alta* which are morphologically similar. RAPD markers have been used to detect i) inter- and intra-specific and inter-varietal differences for cultivar identification and ii) to detect RAPD markers that can co-segregate with salt tolerance. The results obtained are presented in this paper.

Materials and Methods

Plant material: Three rice cultivars, 2 rice varieties and 11 wild rice species of different genomes and ploidy levels were used (Table I) for DNA extraction. Six synthetic 10 mer primers belonging to S-series, obtained from Operon Technologies (Alameda, California) were used for DNA amplification. Taq. polymerase together with 10X PCR buffer, $MgCl_2$ and dNTPs were purchased from Perkin Elmer Cetus. Perkin Elmer DNA Thermal cycler was used for DNA amplification.

DNA extraction: Seeds from 5 rice varieties/cultivars were germinated on moist filter papers. Seedlings raised in small pots containing sand were placed in net house under natural conditions required for rice cultivation. Different wild rice species growing in the glass house were cloned for vegetative propagation. The newly emerging leaves on these plants were collected alongwith the leaves of two to three week old seedlings that were germinated in Petri plates. DNAs were extracted from the leaves of all the species/varieties separately by CTAB method (Rogers & Bendich, 1988), replacing dry ice with liquid nitrogen. DNA concentration and its quality were determined on a Spectronic-21 spectrophotometer at wave lengths of 260 and 280 nm. From this measurement, a working solution of 5 ng/uL DNA was prepared. Each dilution was then repeatedly rechecked on the spectrophotometer till the exact concetration (5 ng/uL) was obtained.

DNA amplification: The reaction volume (25 μL) containing 2.5 μL (1X) PCR buffer, 2.5 μL (200 μM each) dNTPs, 2.5 μL (2.5 mM) $MgCl_2$, 0.5 μL (0.4 μM) primer, 0.2 μL (1 unit) Taq. polymerase, 5 μL (25 mg) genomic DNA and 11.8 μL water, overlaid with one drop of mineral oil was amplified (Williams *et al.*, 1990) using step cycle file programmed for 35 cycles. After amplification, one drop of (5X) RAPD dye (bromophenol blue mixed with 10% glycerol, 0.1M EDTA and 0.5% SDS) was added to the reaction mixture. Of this mixture, only 12 uL was loaded on 2% agarose gel submerged in TAE buffer. Lambda DNA digested with Hind III was used as molecular size marker. Samples were electrophorased for approximately 2 h at 100 Volts. After electrophoresis, the gels were rinsed twice with distilled water, stained in 1 ug/uL ethidium bromide for 20 minutes followed by two rinses in distilled water. The amplified products were viewed under U.V. transilluminator and photographed using the Stratagene Eagle Eye still video system. All the reactions were repeated twice using fresh DNA to confirm the reproducibility of the reaction.

Scoring of Data: The amplification of different DNA fragments in different rice varieties/species were scored from two good quality pictures obtained for two reactions.

Table 1. Description of material used in the study.

S.No.	Names of Species/ varieties	No of accession	Genome & Ploidy level		Status
1.	Basmati Pak.	cultivar	2n=2x=24	(AA)	Salt-sensitive
2.	Jhona-349	cultivar	2n=2x=24	(AA)	Moderately salt tolerant
3.	NR-I	Mutant	2n=2x=24	(AA)	Salt-tolerant
4.	Nonabokra	Variety	2n=2x=24	(AA)	Salt-tolerant
5.	Pokali	Variety	2n=2x=24	(AA)	Salt-tolerant
6.	*Oryza officinalis*	105322	2n=2x=24	(CC)	Salt-sensitive
7.	*Oryza eichingeri*	105414	2n=2x=24	(CC)	Moderately salt tolerant
8.	*Oryza rufipogon*	105463	2n=2x=24	(AA)	Moderately salt tolerant
9.	*Oryza punctata*	105158	2n=2x=48	(BBCC)	Moderately salt tolerant
10.	*Oryza punctata*	101408	2n=2x=48	(BBCC)	Salt-tolerant
11.	*Oryza punctata*	101389	2n=2x=24	(BB)	Salt-tolerant
12.	*Oryza malampuzhaensis*	105328	2n=2x=48	(BBCC)	Salt-sensitive
13.	*Oryza grandiglumis*	105669	2n=2x=48	(CCDD)	Salt- tolerant
14.	*Oryza alta*	100967	2n=2x=48	(CCDD)	Salt-sensitive
15.	*Oryza latifolia*	100891	2n=2x=48	(CCDD)	Salt- tolerant
16.	*Porteresia coarctata*	species	2n=2x=48	(?)	Salt- tolerant

All the rice species were recieved from IRRI, Phillippines and were tested for salt tolerance at NIAB.

Right lane of the gel was considered as lane 1 (except for Fig.3) and in the present study it is always the opposite side of the lane that shows DNA size marker. Amplified fragments were scored by starting from the top of the lane 1 (fragment No.1) to its bottom (last fragment). All easily visible and unambiguously scorable fragments amplified by one primer were scored under the heading of total scorable fragments. The fragments that were repeatedly present in one particular variety or species were scored as polymorphic fragments. These amplified fragments were considered as cultivar specific markers. All the amplified fragments which appeared specifically in one genome irrespective of the primers and species were scored as genome specific markers. These markers were identified as primer number followed by fragment number. For example S-16,5 means fragment number 5 produced by primer S-16 is present only in cultivated rice varieties was therefore termed as marker specific for cultivated rice varieties. Similarly, S-12,21-27 means fragment numbers 21-27 all produced by primer S-12 are monomorphic within CCDD genome species. Positive (+) sign indicates presence of a particular fragment, negative (-) sign indicates absence of that fragment and zero (0) sign indicates amplfication not attempted.

Results

All the 6 primers used in the present study amplified different DNA fragments in all the rice species and varieties except primer S-17 and 18 which repeatedly failed to

Table 2. Description and distribution of polymorphic DNA markers identified by different primers in different wild and cultivated rice species.

Primer	Fragments*		Fragment no.	Distribution of polymorphic fragments															
	A	B		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
OPS-12	43	12(28%)	1	+	-	0	0	-	0	-	-	0	-	0	0	-	-	-	-
CTGGTGAGT			2-4,8,																
			11,12	-	-	0	0	-	0	-	-	0	-	0	0	-	-	-	+
			14-16	-	+	0	0	-	0	-	-	0	-	0	0	-	-	-	-
OPS-16	42	10(24%)	16-20	0	-	-	-	-	-	0	0	-	-	-	-	-	0	-	+
AGGGGGTTCC			21	0	-	-	-	-	-	0	0	-	-	-	+	-	0	-	+
			24-27	0	-	-	-	-	-	0	0	-	-	-	+	-	0	-	-
OPS-17	24	4(17%)	2,3	0	-	-	-	-	-	0	0	-	-	-	-	-	0	+	-
TGGGGACCAC			12,14	0	-	-	-	-	-	0	0	-	-	-	-	-	0	-	+
OPS-18	48	8(17%)	6-8	0	-	-	-	-	-	0	0	-	-	-	-	+	0	-	-
CTGGCGAACT			35,40,41	0	-	-	-	-	-	0	0	-	-	-	-	-	0	-	+
			42	0	-	+	-	-	-	0	0	-	-	-	-	-	0	-	-
			48	0	-	-	-	-	-	0	0	-	-	+	-	-	0	-	-
OPS-19	34	5(15%)	16,17	0	-	-	-	-	-	0	0	-	-	-	-	+	0	-	-
GAGTCAGCAG			21	0	-	-	-	-	-	0	0	-	-	-	-	-	0	-	+
			31,32	0	-	+	-	-	-	0	0	-	-	-	-	-	0	-	-
OPS-20	21	11(52%)	1	0	-	-	-	-	-	0	0	-	-	-	-	+	-	-	-
TCTGGACGGA			7,8,10	0	-	-	-	-	-	0	0	-	-	-	+	-	0	-	-
			12,13	0	-	-	-	-	-	0	0	-	-	-	-	-	0	-	+
			15,19	0	-	-	-	-	-	0	0	-	-	+	-	-	0	-	-
Total	212	50(24%)																	

* A and B refer to total and polymorphic fragments, respectively.

amplify any DNA fragment in *O. officinalis*, Nonabokra, Pokali (S-17) and *O. officinalis* (S-18) respectively. Most of the amplified fragments appeared to be between 2000 and 400 base pairs. Depending upon the primer used for amplification, the number of fragments amplified in a particular variety/species varied and ranged between one fragment (Basmati Pak., Jhona-349 and *O. punctata* acc: 101308 with primer S-12 and S-18 respectively) and 8 fragments (*P. coarctata* with S-12). Of the 212 different DNA fragments that were amplified only 50 (24%) fragments were polymorphic.

Primer S-12 amplified 43 scorable fragments of which 12 (28%) fragments were polymorphic. Of these 12 fragments, 8 (67%) were found in *P. coarctata*, 1 (8%) in rice cultivar Basmati Pak., while 3 (25%) fragments were found in Jhona 349 (Table 2). Similarly, 10 polymorphic markers were generated by primer S-16 in *O. malampuzhaensis* and *P. coarctata*; 4 by primer S-17 in *O. latifolia* and *P. coarctata*; 8 by primer S-18 in NR-I, *O. punctata*, *O. grandiglumis* and *P. coarctata*; 5 by primer S-19 in NR-I, *O. grandiglumis* and *P. coarctata* while 11 polymorphic markers were generated by primer S-20 in *O. punctata* acc.:101389, *O. malamphuzhaensis*, *O. grandiglumis* and *P. coarctata*. Of the 50 potential genetic markers, one marker (2%) was specific for Basmati Pak., 3 (6%) each were specific for Jhona 349 and NR-I, 6(12%) each for *O. punctata* acc: 101389 and *O. grandiglumis*, 2 (4%) for *O. latifolia* while 21 (42%) markers were found specific for *P. coarctata* (Table 2).

In addition to 50 cultivars specific polymorphic marker, 49 other markers were also identified that were monomorphic in a particular genome (Table 3) of which 22 were specific for BBCC genome species. This included 13 markers that were monomorphic in two accessions (105158 and 101408) of *O. punctata* and 4 markers were monomorphic in another 2 accession (101408 and 101389), while 4 markers were monomorphic in all the three accessions of *O. punctata* used in the present study. Likewise, 8 markers were found monomorphic in CCDD genome species, 3 markers between CCDD genome and *P. coarctata*, one marker was monomorphic between BBCC genome species and *P. coarctata* while 7 markers were found monomorphic between different BBCC and CCDD genome species particularly *O. latifolia*. Among the remaining markers, marker S-16-2 appeared particularly in salt tolerant wild and cultivated rice species/varieties while S-16-3 appeared only in salt tolerant cultivated rice varieties. Eight (8) markers were monomorphic between Jhona-349 (AA), 3 accessions of *O. punctata* (BBCC), *O. malampuzhaensis* (BBCC), *O. grandiglumis* (CCDD), *O. latifolia* (CCDD) and *P. coarctata* (unknown).

Fig. 1 shows amplification profile of different wild and cultivated rice varieties obtained by primer S-12. In this profile, 4 rice species/varieties (Lanes 1-4) can be clearly separated from each other by the polymorphic nature of most of the fragments. However, three wild rice species viz., *O. latifolia* (Lane 5), *O. alta* and *O. grandiglumis* (Lanes 6 and 7 respectively) showed almost similar amplification profile and hence are difficult to be distinguished from each other. However, presence of marker S-16-2 (Fig. 2) in *O. latifolia* (Lane 2) helps differentiating *O. latifolia* from *O. grandiglumis* (Lane 11) where this particular marker is absent. Fig.2 also exhibit amplification profile of three different accessions of *O. punctata* (Lanes 4, 9 and 10) that is almost identical and does not help in differentiating the three accessions from each other. Fig. 3 on the other hand, exhibited entirely different profile for one of the *O. punctata* acc:

Table 3. Distribution of genome specific monopmorphic markers identified with different primers.

Markers specific	Primer	Fragment no.	Distribution of monomorphic markers															
for:			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AA genome	S-16	3	0	+	+	+	+	-	0	0	-	-	-	-	-	0	-	-
BBCC genome	S-16	6-9	0	-	-	-	-	-	0	0	+	+	+	-	-	0	-	-
	S-17	8-11	0	-	-	-	-	-	0	0	-	+	+	-	-	0	-	-
	S-18	9-10,																
		15-18	0	-	-	-	-	-	0	0	+	+	-	-	-	0	-	-
	S-19	3-8	0	-	-	-	-	-	0	0	+	+	-	-	-	0	-	-
	S-20	4	0	-	-	-	-	-	0	0	+	+	-	-	-	0	-	-
		22 (45%)																
CCDD genome	S-12	21-27	-	-	0	0	-	0	-	-	0	-	0	0	+	+	+	-
	S-17	6	0	-	-	-	-	-	0	0	-	-	-	-	+	0	+	-
		8 (16%)																
CCDD genome and	S-12	9-10	-	-	0	0	-	0	-	-	0	-	0	0	+	+	-	+
P. coarctata	S-18	1	0	-	-	-	-	-	0	0	-	-	-	-	+	0	-	+
		3 (6%)																
BBCC genome and	S-20	3	0	-	-	-	-	-	0	0	+	+	-	-	-	0	-	+
P. coarctata		1 (2%)																
BBCC and CCDD	S-17	4-5	0	-	-	-	-	-	0	0	-	-	-	+	-	0	+	-
genome		6	0	-	-	-	-	-	0	0	-	-	+	-	+	0	+	-
	S-19	5-8	0	-	-	-	-	-	0	0	+	+	-	-	-	0	+	-
		7 (14%)																

Table 3 (Cont'd)

Markers specific	Primer	Fragment no.	Distribution of polymorphic fragments															
for:			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
BBCC and CCDD	S-16	2**	0	+	+	-	-	-	0	0	+	+	+	-	-	0	+	+
genomes, *P. coarctata*	S-17	1	0	+	+	-	-	-	0	0	+	+	+	+	+	0	+	+
and cultivated rice	S-18	2-3	0	-	+	-	-	-	0	0	+	+	-	+	+	0	-	+
varieties		4	0	-	-	-	-	-	0	0	-	-	-	+	+	0	-	+
		5	0	-	+	-	-	-	0	0	+	+	-	+	+	0	-	+
	S-19	15	0	-	+	-	-	-	0	0	+	+	-	+	+	0	-	+
	S-20	11	0	+	+	-	-	-	0	0	-	-	-	-	-	0	-	+
		8(16%)																

*Underlined figures refer to total number of fragments.

**DNA fragment amplified in salt tolerant wild and sultivated rice species.

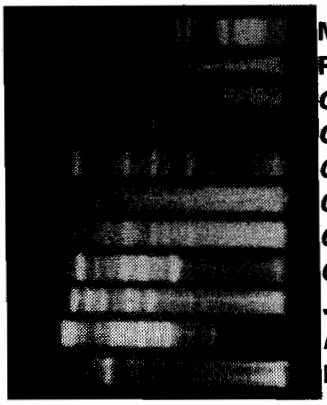

Fig. 1. Amplification profile of cultivated and wild rice species produced by primer S-12. Three wild rice species; *Oryza grandiglumis, O. alta* and *O. latifolia* all of CCDD genome are exhibiting identical profile.

101389 (Lane 9) compared to accessions 105158 and 101408 (Lanes 3 and 4). Both these accessions can be differentiated from each other by the presence of marker S-18-9 which is present in accession 105158 and absent in 101408 (Table 3). Fig. 3 also exhibited markers S-18, 2-5 that are monomorphic in *O. grandiglumis* (Lane 2), *O. punctata* accessions 105158 and 101408 (Lane 3 and 4), *O. malampuzhaensis* and *P. coarctata* (Table 3).

Discussion

DNA based markers such as Restriction Fragment Length Polymorphism (RFLP) markers (McCouch *et al.*, 1988; Weber & Helentjaris, 1989) and Random Amplified Polymorphic DNA (RAPD) markers (Rafalski *et al.*, 1991; Williams *et al.*, 1991) are being extensively used in plant breeding programmes. There large scale utilization in tomato (Martin *et al.*, 1991), Brassica, broccoli, cauliflower (Quirose *et al.*, 1991; Hu & Quirose 1991) and wheat (D'Ovidio *et al.*, 1990; Weining & Langridge, 1991; Devos & Gale 1992) have successfully demonstrated the effectiveness of RAPD markers in identification of strains and varieties. In the present study, we have generated RAPD markers in 16 different wild and cultivated salt tolerant and sensitive rice varieties to detect cultivar specific and genome specific markers and also the markers that particularly appear in salt tolerant germplasm. Cultivar specific markers were generated by each primer used. Although, their frequency was low (24%) nevertheless, it was possible to identify markers for 8 out of 16 (50%) species/ varieties. These markers can thus be used for quality control and fingerprinting of specific cultivars. Of the 50 culti-

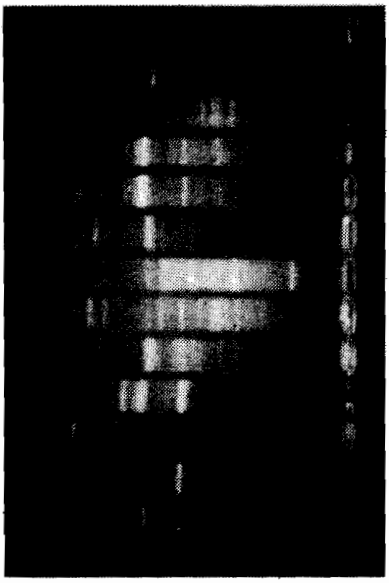

Fig.2. Amplification profile of primer S-16. *Oryza latifolia* and *O. grandiglumis* both of CCDD genome are exhibiting different profile while three different accessions of *O. punctata* are exhibiting nearly identical profile.

var specific markers, 21 (42%) were identified only in *P. coarctata* which may be due to the fact that *Porteresia* is a different genus than the genus *Oryza*.

Among the genome specific markers, frequency of markers specific for BBCC genome was the highest (49%). These markers not only differentiated species with BBCC genome from species with CCDD genome but also differentiated two tetraploid (4X) accessions of *O. punctata* (105158 and 101408) from each other and from diploid (2X) accession. The markers appearing monomorphic in BBCC, CCDD, AA genome species (or cultivated varieties) and *P. coarctata* (unknown genome) indicated that *P. coarctata* might have CC genome as one of its genomes. This is further supported from the fact that first living intergeneric hybrid of *P. coarctata* was produced with tetraploid accession (BBCC) of *O. punctata* (Farooq *et al.*, 1992b). However, to confirm this hypothesis, it is imperative to do meiotic chromosome analysis of the hybrid.

Among the remaining markers, marker S-16-2 that appeared only in salt tolerant cultivated and wild rice species and S-20-3; a single marker present in three salt tolerant accessions of *O. punctata* are the most important. Salt tolerance is a polygenically controlled trait which is highly influenced by the environment and it is difficult to select gene(s) for salt tolerance through conventional breeding techniques (Tanksley *et al.*,

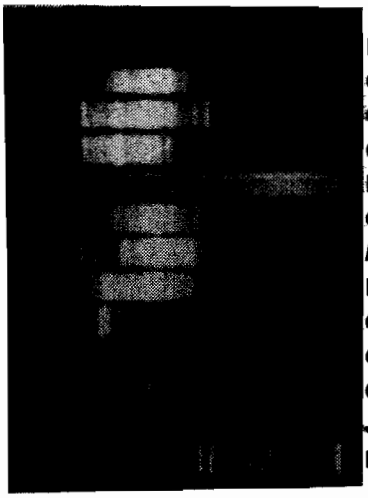

Fig.3. Amplification profile of primer S-18. *Oryza punctata* accession 101389 (BB genome) is exhibiting entirely different profile compared to *O. punctata* accessions: 105158 and 101408 (BBCC genome).

1989). These markers can therefore, be used as probe for screening against salt tolerance in plant breeding programme, to monitor the transfer of salt tolerant gene(s) from *O. punctata* to cultivated rice varieties in the F_1 hybrids, and to check the co-segregation of these markers with the level of salt tolerance in F_2 segregating population.

The earlier attempts of using RAPD markers in rice were made to dectect polymorphism in Indica, Japonica and Javanica varieties (Mackil, 1993; Ibrahim *et al.*, 1994), for tagging genes for quality traits (Mridula *et al.*, 1994) and to detect genetic variation in upland and lowland rice cultivars (Yu & Nguyen, 1994). In the present study cultivar specific and genome specific markers were identified in 5 cultivated rice varieties and 11 wild rice species. Markers for salt tolerance were also identified. RAPD markers can thus be used for fingerprinting as well as for gene tagging experiments particularly tagging genes for traits which are highly influenced by the environment like plant height and aroma of rice.

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