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Fingerprinting Indian potato cultivars by random amplified polymorphic DNA (RAPD) markers[1]

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Summary

Random amplified polymorphic DNA markers were used to distinguish and characterize 20 Indian potato cultivars. A total of 198 scorable fragments were amplified using 10 random primers, only two of which were monomorphic. Similarity values among the cultivars ranged from 0.33 to 0.80. A primer having resolving power above 7.4 was sufficient to distinguish all 20 cultivars. Wide variations in band profiles were observed when the same template DNA was amplified using Taq DNA polymerase from four different sources. No significant difference in profile complexity was observed at 40 °C annealing temperature with a primer having 70% GC content. Prior restriction of template DNA resulted in band profiles whose complexity was similar to or higher than that of unrestricted template. However, multiplex RAPD with cleaved template DNA could not increase fingerprint complexity.

Introduction

Morphological data have traditionally been used to define a cultivar (Pauksens, 1975). But, since morphologies reflect interaction of the genotype with its environment, it is inappropriate to compare morphological data for cultivars that have been collected across different years and/or locations. Therefore, descriptors based on proteins (including isozymes) and deoxyribonucleic acid (DNA) have been developed in recent years (Tanksley & Orton, 1983; Ryskov et al., 1988). While isozyme analysis has been demonstrated to be useful in identification of potato cultivars (Stegemann & Schnick, 1985; Quiros & McHale, 1985; Douches & Ludlam, 1991), its ability to discriminate clonal variants of commercial cultivars is low, because of the small number of scorable loci available. Moreover, the isozyme profiles are also affected by environment (Higgins, 1984) and plant tissue source. More recently, restriction fragment length polymorphism (RFLP) has been used to generate cultivar specific DNA fingerprints (Powell et al., 1991; Gorg et al., 1992). However, detection of cultivars by RFLPs is time consuming, requires the development of polymorphic DNA probes, usually involves the use of radioisotopes and requires an expensive laboratory set up which is beyond economic feasibility of many developing countries. Therefore, a variety of PCR-based DNA fingerprinting techniques such as random amplified polymorphic DNA (Williams et al., 1990),

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amplified fragment length polymorphism (Vos et al., 1995) and Inter Simple Sequence Repeat-Polymerase Chain Reaction (Zietkiewicz et al., 1994) have been developed in the last decade; they do not require prior investments in terms of sequence analysis, primer synthesis or characterization of DNA probes. RAPD and ISSR-PCR are the most favoured methods for DNA fingerprinting largely because of the relative ease with which the techniques can be practiced in any molecular biology laboratory (Milbourne et al., 1997; McGregor et al., 2000). The major drawback of RAPD for genotype identification arises from the lack of reproducibility of fingerprint profiles largely due to low primer annealing temperature. Several other factors including concentration of primer, template DNA and Mg^{2+} ions in the reaction mixture, type of thermal cycler and PCR tubes are found to influence RAPD profiles (Weising et al., 1995). However, high repeatability can be achieved by standardization of the above components (Smith & Williams, 1994). In the present study we investigated the potential of RAPDs in detecting polymorphism that may be used to distinguish and identify Indian potato cultivars. Effect of Taq DNA polymerase source and annealing temperature on profile reproducibility was compared and fingerprint variability generated by standard RAPD and tec-RAPD (template endonuclease cleavage-RAPD) was also investigated.

Materials and methods

Template DNA preparation. Total genomic DNA from 20 commercial Indian potato cultivars (Table 1) was extracted from young leaf samples by a modified CTAB procedure (Doyle & Doyle, 1987). RNA contaminants in all the samples were digested with 100 μg ml^{-1} RNase A for 30 min at 37 °C, extracted once with phenol: chloroform: isoamyl alcohol (25:24:1), precipitated by adding 0.1 volume 3 M ammonium acetate and 2 volume of chilled absolute ethanol, washed with 70% ethanol and resuspended in 200 μl sterile MilliQ water. Quantity and quality of DNA preparations were checked by standard spectrophotometry (Ausubel et al., 1995) and the samples were diluted to 25 ng DNA/μl concentration.

PCR reaction condition and temperature profile (Basic protocol). The polymerase chain reaction was performed in a reaction volume of 25 μl containing 1× Taq DNA polymerase buffer with 1.5 mM $MgCl_2$ (Perkin Elmer), 100 μM of each dNTP (Promega), 25 pmole random primer (Operon Technology, USA), 100 ng genomic DNA (Nadeau et al., 1992) and 1.0 unit Taq DNA polymerase (AmpliTaq, Perkin Elmer). Ten primers, known to produce good amplification products in our earlier experiments, were used in this study. Amplification was performed in a Perkin Elmer Thermal Cycler (GeneAmp PCR System 2400). In total, 45 cycles were used, each cycle consisting of 1.0 min denaturation at 94 °C, 1.0 min annealing at 35.5 °C and 2 min extension at 72 °C. The samples were maintained at 94 °C for 5 min before the start of the first cycle and after the final cycle all amplified products were completed with a 10 min extension at 72 °C. Amplification products were separated by electrophoresis on 1.6% agarose gels (20 cm length) with 0.5 μg ml^{-1} ethidium

Table 1. List of potato cultivars used.

Serial number	Name	Year of release	Parentage
1	K. Sindhuri	1967	K. Red × K. Kundan
2	K. Lauvkar	1972	Serkov × Adina
3	K. Bahar	1980	K. Red × Gineke
4	K. Giriraj	1999	SLB/J-132 × EX/A-680-16
5	K. Chipsona 1	1998	CP 2416 × MS/78-79
6	K. Chipsona 2	1998	F-6 × QB/B-92-4
7	K. Lalima	1982	K. Red × AG149(Wis.X37)
8	K. Neela	1963	Katahdin × Shamrock
9	K. Shakti	NR	Kufri Red × Kufri Kundan
10	K. Red	1958	Clonal selection of DRR
11	K. Safed	1958	Clonal selection of DRR
12	K. Chamatkar	1968	Ekishirazu × Phulwa
13	K. Kuber	1958	(*S. curtilobum* × *S. tuberosum*) × *S. tuberosum* ssp. *andigena*)
14	K. Khasigaro	1968	Taborky × Sd. 698-D
15	K. Pukhraj	1998	Craig's Defiance × JEX/B-687
16	Gulmarg Special		Old *andigena* variety
17	*Burma Special*		-do-
18	Lalmutti 1		-do-
19	Phulwa		-do-
20	Gulabia		-do-

bromide for 5 h at 4 V cm^{-1} constant voltage. The gels were scanned in FluorS MultiImager (Bio-Rad) and the images were analyzed by the Diversity Database software package (Bio-Rad). Sensitivity for detection of bands under this programme was kept at 25.9 for all the gels.

Effect of Taq DNA polymerase source and annealing temperature. To study the effect of Taq DNA polymerase from different sources on DNA fingerprinting, AmpliTaq (Part No. N801-0060) of M/S Perkin Elmer, USA, Taq from M/S Promega, USA (Cat. No. 1861), GenTaq (Cat. No. LB-8) from M/S Genetix, Kirtinagar, New Delhi, India, and Taq (Cat. No. MME 5J) from M/S Bangalore Genei Pvt. Ltd., Bangalore, India were used to amplify genomic DNA of 3 potato cultivars (Kufri Jyoti, Kufri Badshah and Kufri Chipsona 1) with five different primers (OPB-03, OPC-04, OPA-03, OPA-04, and OPB-04). All PCR reactions were repeated twice. The data was analyzed as a three-way factorial design with 2 replications. Effect of annealing temperature on DNA fingerprints of Kufri Badshah was studied with the above four Taq DNA polymerases and the random primer OPB-03 at 35.5, 37 and 40 °C annealing temperatures. Data on the total number of RAPD fragments was analyzed as a 2-way factorial design with two replications.

Template endonuclease cleavage RAPD (tec-RAPD). Genomic DNA of Kufri Badshah was completely digested with Sau 3A and Eco R1 (Promega) and the digested samples were diluted to 25 ng DNA/µl concentration. PCR amplification

with uncut and cut samples was performed twice with five random primers viz. OPA-17, OPB-04, OPB-02, OPB-14, and OPB-19. Data was analyzed as a 2-way factorial design with 2 replications. Multiplexing of cut and uncut templates was done with 1:1 mixture of OPA-17 and OPB-04.

Similarity analysis and clustering. The amplified DNA fragments were scored for each cultivar as 1 (band present) and 0 (band absent) with band number 1 being the biggest fragment. Resolving power (Rp) of a primer was calculated as ΣIb. Band informativeness (Ib) was represented into a 0–1 scale by the formula: $Ib=1-(2 \times |0.5-p|)$, where p is the proportion of the 20 samples containing the band (Prevost & Wilkinson, 1999). A similarity matrix was generated on NTSYSpc 2.0 h (Rohlf, 1998) using Dice coefficient (Dice, 1945). The similarity matrix thus generated was used for cluster analysis by unweighted pair-group method with arithmetic average (UPGMA) under SAHN program of NTSYSpc. The output data was graphically represented as a phenetic tree.

Identification of cultivar. The binary data matrix generated for individual primers was analyzed separately by NTSYSpc to work out their ability to distinguish 20 potato cultivars. To find out the minimum number of polymorphic bands required to distinguish all 20 cultivars, the binary matrices of only those bands having Ib values ≥ 0.9 were analyzed by NTSYSpc.

Results and discussion

Band reproducibility. Resolution of PCR amplified product in 1.6% agarose gel often gives rise to a few faint bands that are difficult to reproduce. It is, therefore, necessary to repeat a particular set of amplifications at least twice and consider only reproducible bands for scoring. In our experiment repetition of 75 PCR reactions twice yielded 96.5% band reproducibility. Difference in band profile between two replications was, however, statistically nonsignificant. Therefore, amplification with each primer was performed twice and only reproducible bands were included in analysis.

Cultivar identification and genetic similarity analysis. Amplification of total genomic DNA from the 20 tetraploid potato cultivars with 10 decamer primers yielded a total of 198 scorable fragments (ca. 19.8 per primer) ranging in size from 700 to 2300 bp, only two of which were present in all potato cultivars (Table 2). The number of fragments produced by different primers ranged from 6 (OPD-04) to 31 (OPD-03). But more fragments per primer were detected in our experiment in comparison with earlier reports (Hosaka et al., 1994; Oganisyan et al., 1996; Sosinski & Douches, 1996). This could be because: (i) only selected primers were used for PCR amplification, or (ii) we used sensitive image analysis system (FluorS MultiImager) for detecting and scoring bands. The fragment analysis software (Diversity Database fingerprinting software of M/S Bio-Rad) used in this experiment differentiated bands

Table 2. Total number and size range of amplified fragments generated by 10 random decamer primers along with their band informativeness.

Serial number	Name	Sequence (5′-3′)	Number of amplified fragments	Size range (bp)	Resolving power (Rp)	Discriminatory ability[a]
1	OPA 03	AGTCAGCCAC	22	780–2035	11.59	1.00
2	OPA 04	AATCGGGCTG	16	732–2031	6.92	0.90
3	OPB 01	GTTTCGCTCC	15	1034–2160	5.93	0.85
4	OPB 04	GGACTGGAGT	25	745–2272	11.25	1.00
5	OPC 03	GGGGGTCTTT	20	810–2300	7.41	1.00
6	OPC 04	CCGCATCTAC	22	725–1835	9.83	1.00
7	OPD 01	ACCGCGAAGG	19	837–2116	7.16	0.95
8	OPD 03	GTCGCCGTCA	31	700–2180	13.58	0.95
9	OPD 04	TCTGGTGAGG	6	1106–1652	2.73	0.40
10	OPE 20	AACGGTGACC	22	812–1939	10.44	1.00
Total	10		198			

[a] Discriminatory ability of the primer is the proportion of cultivars distinguished to total number of cultivars.

that were very close to each other. It also detected several faint bands which were not perceptible by naked eye. Usually 20–50% of the random primers used in RAPD analysis do not give rise to any PCR products (Caetano-Anolles, 1994). In the present experiment, however, we used only those primers already known to produce good banding patterns in our laboratory. RAPD fragments produced by the random primer OPB-04 are shown in Fig. 1.

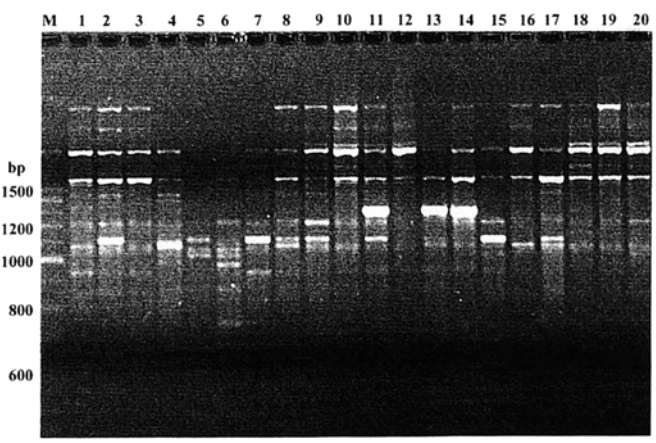

Fig. 1. RAPD profiles of 20 tetraploid potato cultivars generated by the random decamer primer OPB-04. M denotes 100 bp DNA step ladder; lane numbers conforms to the serial number in the Table 1.

Pairwise similarity values among 20 cultivars ranged from 0.33 to 0.80 with standard deviation 0.08. A dendrogram of 20 potato cultivars derived from the similarity matrix is presented in Fig. 2. The goodness of fit of the dendrogram with the similarity data was tested by matrix comparison module using cophenetic correlation procedure. Cophenetic matrix of the SAHN cluster was calculated by using 'coph' program of the NTSYSpc. Matrix correlation value was 0.95 indicating a very good fit between similarity values and the dendrogram. Maximum similarity (0.80) was observed between two old andigena cultivars Lalmutti and Phulwa. These were clonal selections done by the farmers. Kinship relationship among different cultivars could not be predicted from the similarity data. For example, lower similarity values (0.50–0.66) were observed with 3 half-sib combinations (K Sindhuri vs. K Bahar, K Bahar vs. K Lalima, K Sindhuri vs. K Lalima) and 3 progenitor vs. progeny combinations (K Red vs. K Sindhuri, K Red vs. K Bahar, K Red vs. K Lalima) in comparison with a pair of unrelated cultivars K. Safed vs. K. Khashigaro. Conflicting results are also found in the literature regarding the relationship between similarity values and kinship among potato cultivars. Hosaka et al. (1994) studied the genetic relationship of 73 Japanese potato cultivars and reported that RAPD banding patterns of closely related cultivars were clustered together and they concluded that banding patterns are reflections of the pedigree relationship. However, Demeke et al.

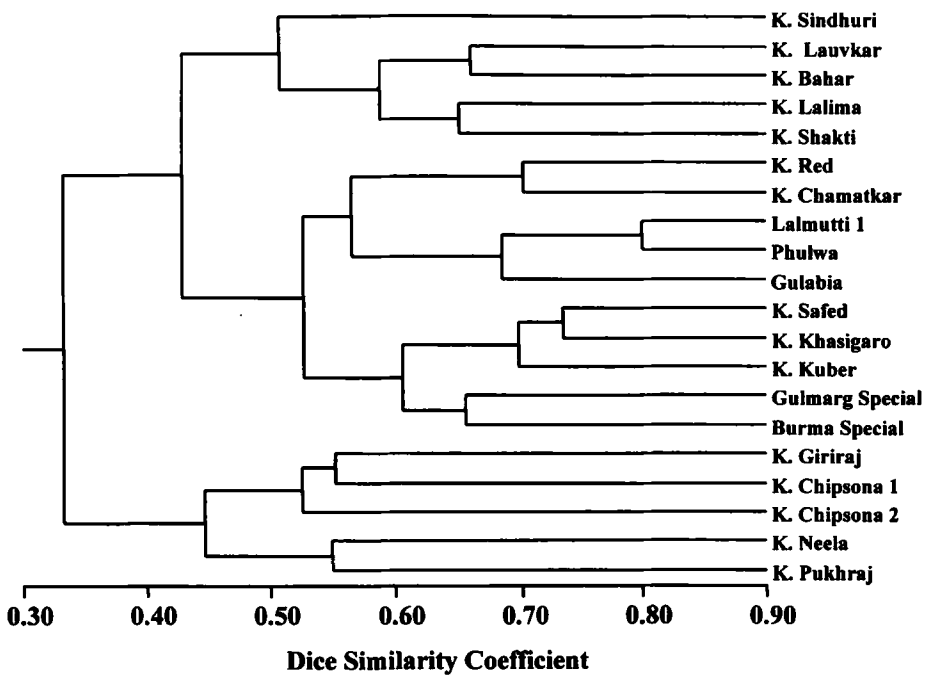

Fig. 2. Dendrogram showing relationship between 20 potato cultivars.

(1996) studied genetic diversity of 28 North American potato cultivars and observed exceptions to this observation and recorded that cultivars with close kinship can often be as genetically diverse as those with no immediate relationship. Our observation also demonstrated that kinship relationship could not be reflected in the similarity of the banding pattern. This is probably due to highly heterozygous nature of the tetraploid genome.

Resolving power of each primer was calculated as described by Prevost & Wilkinson (1999). Binary matrices, generated by using qualitative band information for each primer, were individually analyzed to find their discriminatory ability. Resolving power of the 10 primers used in this study ranged from 2.73 to 13.58 (Table 2). Each of five random primers (OPA-03, OPB-04, OPC-03, OPC-04 and OPE-20) could distinguish all 20 potato cultivars individually. A primer having resolving power above 7.4 was sufficient to distinguish all the cultivars. To find out the minimum number of RAPD bands necessary to distinguish all 20 cultivars, the binary matrix of only those bands having band informativeness (Ib) value of ≥0.9 was then analyzed by NTSYSpc. Only 3 bands out of 196 were found to have Ib value of 1.0. Fingerprint profiles obtained by these 3 bands were inadequate to resolve all 20 cultivars since they could generate at the most 8 (2^3) different patterns. Therefore, the binary data of bands having Ib value of 0.9 were included step by step with these 3 bands in all possible combinations and the resulting matrices were analyzed by NTSYSpc. Theoretically it is possible to distinguish 20 samples using a minimum of 5 bands (2^5=32). However, in our experiment it was observed that at least 8 bands were required to distinguish all the cultivars. These bands were generated by using six random primers. For achieving more stringency in fingerprint profile these 8 bands can be converted into Sequence Characterized Amplified Region (SCAR) markers.

Effect of Taq DNA polymerase brand and annealing temperature. Taq DNA polymerase is the most important component of polymerase chain reaction. Therefore, four different sources of Taq DNA polymerase were tried separately in PCR reaction twice to assess their effect on fingerprint pattern. Wide range of variations in band profiles was observed when the same template DNA was amplified using Taq DNA polymerase from four different sources (Fig. 3A). Three-way analysis of variance (ANOVA) table (Table 3) revealed highly significant differences in the number of RAPD fragments due to differences in sources of Taq polymerase, genotypes, and primer either singly or in any two-way combinations. However, a non-significant difference in band numbers was observed when a three-way interaction among the variables was considered. Differences in RAPD band numbers due to variation in genotype and primer is expected, but our data also demonstrated a significant effect of Taq polymerase source on band numbers (Fig. 4). Maximum numbers of RAPD bands were amplified by the Taq DNA polymerase marketed by M/S Bangalore Genei and least by that of M/S Promega, USA. AmpliTaq of M/S Perkin Elmer, USA and GeneTaq of M/S Genetix, New Delhi produced almost similar number of fragments (Fig. 4). It is not always possible to use Taq DNA polymerase from the same source in a particular laboratory. For example, we use Taq

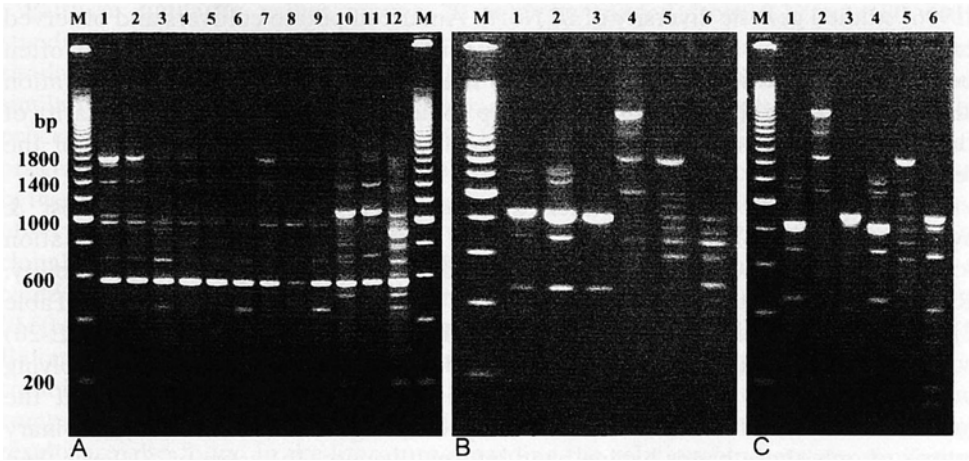

Fig. 3. Effect of Taq DNA polymerase brands, annealing temperature, and cleavage of template DNA on RAPD profile of potato cultivar Kufri Badshah. 'M' denotes 200 bp DNA step ladder. 3A: Genomic DNA was PCR amplified using the random primer OPB-03 and four different brands of Taq DNA polymerase, viz. AmpliTaq of M/S Perkin Elmer (lanes 1,2,3); Taq of M/S Promega (lanes 4,5,6); GenTaq of M./S Genetix (lanes 7,8,9); and Taq of M/S Bangalore Genei (lanes 10,11,12). In total 45 cycles were used for amplification, each cycle consisting of 1 min at 94 °C; 1 min at either 35.5 °C (lanes 1,4,7,10), 37 °C (lanes 2,5,8,11) or 40 °C (lanes 3,6,9,12); 2 min at 72 °C. 3B: Genomic DNA was restricted with EcoR1 (lanes 2,5) and Sau 3A1 (lanes 3,6). Restricted as well as uncut DNA (lanes 1,4) samples were PCR amplified using random primers OPA-17 (lanes 1,2,3) and OPB-04 (lanes 4,5,6). 3C: Uncut genomic DNA (lanes 1,2,3) and its EcoR1 restriction digest (lanes 4,5,6) were PCR amplified with either OPA-17 (lanes 1,4), OPB-04 (lanes 2,5) or 1:1 mixture of both (lanes 3,6).

Table 3. Analysis of variance table for three-way interaction between sources of Taq polymerase, genotypes, and random primers.

Source	Df	Sum of squares	Mean square	F value	P
Taq polymerase	3	432.40	144.13	104.19	0.000
Genotype	2	125.42	62.71	45.33	0.000
Primer	4	413.92	103.48	74.80	0.000
Taq × Genotype	6	25.45	4.24	3.07	0.011
Taq × Primer	12	49.02	4.08	2.95	0.002
Genotype × Primer	8	288.83	36.104	26.10	0.000
Taq × Genotype × Primer	24	34.63	1.44	1.04	0.431
Error	60	83.00	1.38		

DNA polymerase from M/S Bangalore Genei, Promega and Perkin Elmer according to their availability. The above result, however, suggests that it is necessary to use Taq DNA polymerase from the same source when comparing RAPD fingerprint

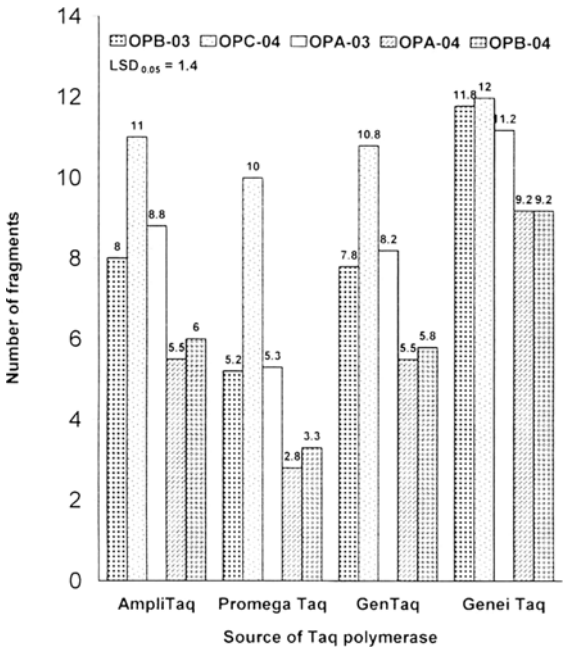

Fig. 4. Average number of RAPD fragments amplified by five random primers using Taq polymerase of four different sources.

profiles. If the source of Taq DNA polymerase is changed in a laboratory, it will be impossible to use a RAPD database generated with a different brand of enzyme for cultivar identification. This observation constitutes a major drawback of the RAPD technique in cultivar identification.

The annealing temperature in a PCR cycle is an important parameter for successful DNA amplification in RAPD analysis. We used three different temperatures (35.5, 37 and 40 °C) for amplification of genomic DNA of a single potato cultivar (Kufri Badshah) with the random primer OPB-03 (70% GC content) and four different Taq DNA polymerase brands. Although a significant difference in number of RAPD bands was observed among four different Taq polymerases used, no difference was observed among three different annealing temperatures (Table 4). More RAPD bands were detected at 40 °C than at 37 °C annealing temperature only with Taq DNA polymerase from M/S Bangalore Genei (Fig. 3A; Fig. 5). Complexity of RAPD profile decreased at higher annealing temperatures only when Taq DNA polymerase from M/S Promega was used (Fig. 5). With two other enzymes (M/S Perkin Elmer and M/S Genetix) the band complexity was similar at three different annealing temperatures (Fig. 5). Usually an annealing temperature of 36 °C is selected for polymerase chain reaction with random primers. Non-specific amplification of DNA fragments often is a problem at this low temperature. It is generally believed that at

Table 4. Analysis of variance table for two-way interaction between Taq polymerase and annealing temperature on RAPD band numbers.

Source	Df	Sum of squares	Mean square	F value	P
Taq	3	187.46	62.49	214.24	0.000
Annealing temperature	2	0.58	0.29	1.00	NS
Taq × annealing temperature	6	11.42	1.90	6.52	0.002
Error	12	3.50	0.29		

NS: Non-significant difference.

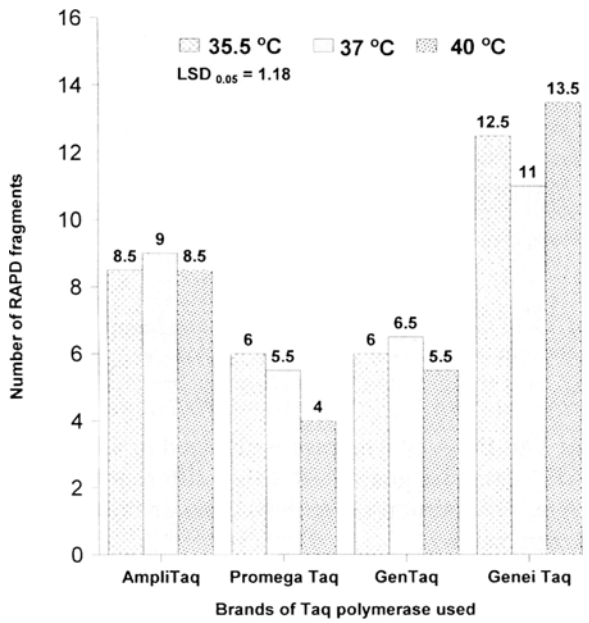

Fig. 5. Average number of RAPD fragments amplified from the genomic DNA of Kufri Badshah by the random primer OPB-03 at 35.5, 37 and 40 °C annealing temperature.

higher annealing temperature the random primer (with 60–70% GC content and 80–90% homology with the template) might slip out of the template and consequently DNA polymerase cannot synthesize during the extension step. On the contrary, it has been reported that at higher annealing temperatures fewer and more distinct bands are amplified that are fairly reproducible (Weising et al., 1995). By contrast our results suggested that it is possible to generate RAPD profile with similar complexity at a higher annealing temperature of 40 °C with a primer having 70% GC content. This preliminary evidence needs to be confirmed with several primers.

Tec-RAPD vs. standard RAPD. Large-sized DNA template (>25 Kbp) may intertwine after denaturation and as a result some of the amplicons may be lost during annealing and extension reaction in PCR. This may reduce repeatability of the RAPD profile. Restriction digestion of template DNA into smaller sizes could minimize this problem, so improving repeatability. With this in mind an experiment was conducted to investigate the effect of cleaving template DNA prior to PCR amplification. Restriction of template DNA resulted in amplification of several de novo fragments. Analysis of variance (Table 5) showed significant effects of primer as well as template digestion on the number of RAPD bands. No significant difference in the average number of amplified fragments was observed from that of uncut template when a 6-cutter enzyme (Eco R1) was used, except with the primer OPB-14 (Fig. 3B; Fig. 6). Template digestion with a 4-cutter enzyme (Sau 3A1), however,

Table 5. Analysis of variance table for two-way interaction between random primers and template cleavage on RAPD band numbers.

Source	Df	Sum of squares	Mean square	F value	P
Primer	4	87.20	21.80	163.50	0.000
Cleavage of template	2	9.87	4.93	37.00	0.000
Primer × template cleavage	8	32.80	4.10	30.75	0.000
Error	15	2.00	0.13		

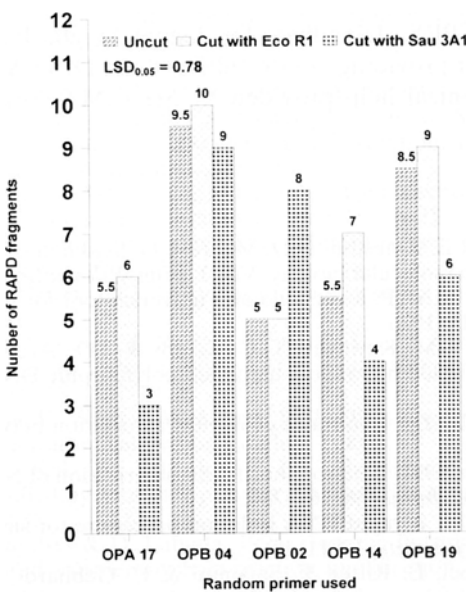

Fig. 6. Average number of RAPD fragments amplified by using DNA template either uncut or cut with EcoR1 or Sau 3A1.

resulted in a significant loss of profile complexity (Fig. 6). Only with the primer OPB-02 did the average number of amplified fragments increase when the template was restricted with Sau 3A1 before PCR amplification (Fig. 6). Loss of profile complexity due cleavage of template with frequent cutter is expected. This is probably because of non-specific destruction of amplicons. Enhancement of profile complexity by using cleaved template DNA could be because of selective destruction of silent amplicons by restriction endonuclease, thereby decreasing inter-amplicon competition during the first few PCR cycles. Therefore, it is concluded that prior restriction of template DNA with a six-cutter restriction endonuclease may produce a RAPD profile that is as complex as that generated by using uncut template. Improvement in profile reproducibility, if any, due to template cleavage is yet to be confirmed. Multiplex RAPD with cleaved (Eco R1) template DNA, however, failed to increase profile complexity (Fig. 3C). The number of fragments amplified by a mixture of two random primers (OPA-17 and OPB-04) reduced significantly as compared with those generated by an individual primer. With multiplex RAPD, more amplified fragments are usually expected (Micheli et al., 1993). But with potato we found a large reduction of profile complexity when two random primers were mixed for PCR amplification. The reason for such reduction is not apparent.

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