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Semi-automated simple sequence repeat analysis reveals narrow genetic base in Indian potato cultivars

V.P. CHIMOTE, S.K. CHAKRABARTI, D. PATTANAYAK and P.S. NAIK*

Division of Crop Improvement, Central Potato Research Institute, Shimla-171001, HP, India

Abstract

A study was conducted to generate fingerprints of thirty-two Indian potato cultivars using capillary electrophoresis based semi-automated simple sequence repeat analysis. Five fluorescent-tagged primer pairs (STPOACUTR, LEGAST1, STPOAC58, STM0030 and STM0031) used in this study yielded 43 alleles at 16 different loci, showing multi-loci resolving character. The estimated similarity between the cultivars was in the range of 0.72 to 0.98 indicating narrow genetic relationship. None of the primer set alone could differentiate all 32 cultivars included in this study. However, two primer sets STM-0031 and STPOAC58 amplifying 12 and 9 polymorphic alleles, respectively, could together distinguish all of them. The results indicated usefulness of semi-automated capillary electrophoresis in quick and reproducible SSR genotyping of potato cultivars.

Additional key words: genetic relationship, microsatellites, simple sequence repeats polymorphism, *Solanum tuberosum*.

Introduction

Identification of a cultivar is essential for its registration and protection of plant breeder's rights. Conventional characterization of cultivars using morphological data is time consuming, difficult to access and sometimes environmentally variable. Therefore, various DNA based molecular markers have been developed during past few years for use in cultivar identification. Some of these are, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP).

Microsatellite repeats based markers known as simple sequence repeats (SSRs), simple sequence length polymorphism (SSLP) or sequence tagged microsatellite sites (STMS), have also been successfully used for studying polymorphism in potato (Kawchuk *et al*. 1996, Provan *et al*. 1996, Milbourne *et al*. 1997, 1998, Schneider and Douches 1997). These markers are randomly distributed along the genome both in intragenic as well as intergenic regions (Weber and May 1989). SSR markers are based on a one to six nucleotide tandem repeats, two to many thousand times with conserved flanking regions that are used for designing primers. SSR amplification provides highly reproducible, polymorphic and co-dominant markers from a small amount of starting material. Amplified fragments sizes are then precisely determined by agarose/ polyacrylamide gel electrophoresis with detection by ethidium bromide, silver staining, α (S^{35})-dATP or α (P^{33})-dATP labeling. In recent years use of non-radiolabeled detection techniques are preferred to avoid biohazard risks associated with radiolabeling. Semi-automated fluorescent label detection based on capillary electrophoresis is one such technique that can be safely and efficiently used to detect SSR polymorphism (Mitchell *et al*. 1997).

In view of their reproducibility, multi-allelism, co-dominance, and ease in exchange of data between laboratories, semi-automated SSR analysis was used to generate DNA fingerprints of thirty-two Indian potato cultivars in the present study. The data were also used to analyze genetic relationship among these cultivars.

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Abbreviations: AFLP - amplified fragment length polymorphism; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; RFLP - restriction fragment length polymorphism; SSLP - simple sequence length polymorphism; SSR - simple sequence repeat; STMS - sequence tagged microsatellite sites.

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* Author for correspondence; fax: (+91) 177 2624460, e-mail: naikps@excite.com

Materials and methods

Plants: Disease-free tubers of 32 potato (*Solanum tuberosum* L.) cultivars (Table 1) developed by Central Potato Research Institute, Shimla, India, were used in this study. The plants were grown from these tubers in glasshouse under long summer days of Shimla (latitude: 31.60-N, longitude: 77.13-E, altitude: 2202 msl).

Table 1. Indian potato cultivars used in present study.

Cultivars	Parentage	Year of release
Kufri Jyoti	3069d(4) × 2814a(1)	1968
Kufri Chandramukhi	Sd. 4485 × Kufri Kuber	1968
Kufri Muthu	3046 (1) × M109-3	1971
Kufri Badshah	Kufri Jyoti × Kufri Alankar	1979
Kufri Sherpa	Ultimus × Adina	1983
Kufri Swarna	Kufri Jyoti × $(VTn)^2$ 62.33.3	1985
Kufri Sutlej	Kufri Bahar × Kufri Alankar	1996
Kufri Jawahar	Kufri Neelmani × Kufri Jyoti	1996
Kufri Jeevan	M109-3 × 698-D	1968
Kufri Kanchan	SLB/Z-405(a) × Pimpernel	2000
Kufri Alankar	Kennebec × ON 2090	1968
Kufri Kundan	Ekishirazu × Katahdin	1958
Kufri Kumar	Lumbri × Katahdin	1958
Kufri Megha	SLB/K-37 × SLB/Z-73	1989
Kufri Dewa	Craig's Defiance × Phulwa	1973
Kufri Ashoka	EM/C-1021 × CP-1468	1996
Kufri Sheetman	Craig's Defiance × Phulwa	1968
Kufri Naveen	3070d(4) × 692 D	1968
Kufri Sindhuri	Kufri Red × Kufri Kundan	1967
Kufri Lauvkar	Serkov × Adina	1972
Kufri Bahar	Kufri Red × Gineke	1980
Kufri Giriraj	SLB / J-132 × EX/A 680-16	1998
Kufri Pukhraj	Craig's Defiance × JEX/B-687	1998
Kufri Chipsona2	F-6 × QB/B92-4	1998
Kufri Lalima	Kufri Red × AG 14 (Wis.X 37)	1982
Kufri Neela	Katahdin × Shamrock	1963
Kufri Shakti	Kufri Red × Kufri Kundan	-
Kufri Red	clonal selection of Darjeeling Red Round	1958
Kufri Safed	clonal selection of Phulwa	1958
Kufri Chamatkar	Ekishirazu × Phulwa	1968
Kufri Kuber	(*S. curtilobum* × *S. tuberosum*) × *S andigenum*	1958
Kufri Khasigaro	Taborky × 692-D	1968

DNA isolation procedure: Leaf samples were collected from 45 d old glasshouse grown plants. A modified CTAB method (Doyle and Doyle 1987) was used to isolate genomic DNA from 2 g of fresh leaves. The amount of DNA was quantified by spectrophotometry (*SmartSpec*^{TM} *3000*, *Bio-Rad Laboratories*, Hercules, USA) and quality was checked both by A_{260}/A_{280} ratio and by gel-electrophoresis.

PCR amplification: Five primer sets used in this study (Table 2) were custom synthesized. Two primer pairs, LEGAST1 and STM0030 were labeled with JOE and remaining three pairs, STPOACUTR, STPOAC58 and STM0031 with FAM. PCR reactions were carried out in 0.01 cm^3 final volume comprising 1 u AmpliTaq DNA polymerase (*PE Applied Biosystems*, Foster City, USA), 1×PCR buffer (without $MgCl_2$), 1.5 mM $MgCl_2$, 0.2 mM each dNTP mix, 10 pmol of both forward and reverse primers and 20 ng of template DNA. Amplification was done on a *GeneAmp* PCR system *9700* (*PE Applied Biosystems*) with following temperature regimes: to hold at 94 °C for 5 min, 30 cycles of 1 min at 94 °C, 1 min at annealing temperature (Tm) and 1 min elongation at 72 °C, and final extension at 72 °C for 10 min.

Working samples for SSR analysis: To achieve peaks of uniform height during multiplexing, JOE labeled (LEGAST1 and STM0030) amplified samples were diluted 100 fold while those with FAM (STPOACUTR, STPOAC58 and STM0031) were used undiluted. For multiplexing, 0.001 cm^3 of LEGAST1 amplified sample was mixed with 0.004 cm^3 of STPOACUTR sample. Similarly 0.002 cm^3 of STM0030 amplified DNA and 0.002 cm^3 of STM0031 amplified samples were mixed together. The STPOAC58 amplified samples were not included in multiplexing. Working samples for semi-automated SSR analysis were prepared by mixing 0.012 cm^3 deionized formamide, 0.0004 cm^3 *GeneScan 500 ROX* size standard (*PE Applied Biosystems*) and 0.001 cm^3 of PCR amplified samples in each PCR tube. Before capillary electrophoresis the working mixtures were denatured at 95 °C for 5 min and kept on ice. All samples were analyzed twice to check reproducibility of results.

Capillary electrophoresis: In the present study microsatellite analysis was done with the *Performance Optimized Polymer 4* (*POP-4*^{TM}, *PE Applied Biosystems*) on the *ABI Prism 310* genetic analyzer (*PE Applied Biosystems*). The *ABI Prism 310* was operated in sequential order as suggested in its manual using *GeneScan* analysis capillary. The autosampler was calibrated after setting temperature at 60 °C. Denatured working samples were transferred to 0.5 cm^3 sample tubes and covered with septa before placing them on the sample tray. The injection time was 5 s at 15 kV and run time was kept 24 - 36 min at 15 kV.

Data analysis: The analysis was done using *310GeneScan*® analysis software (version 3.1, *PE Applied Biosystem*). Data points coinciding with 50 and 500 bp peaks were selected for analysis. The peak positions of SSR alleles were identified using

Table 2. Primers used for SSR amplification.

Primer	Source	Tm [°C]	Orientation	Sequence
STPOACUTR	Provan *et al.* 1996	46.2	forward	5'FAM-TGTGTTTGTTTTTCTGTA T-3'
			reverse	5'-AATTCTATCCTCATCTCTA-3'
LEGAST1	Provan *et al.* 1996	51.5	forward	5'JOE-GTTCTTTTGGTGGTTTTCCT-3'
			reverse	5'-TTATTTCTCTGTTGTTGCTG-3'
STPOAC58	GenBank Database	56.3	forward	5'FAM-TCATGAAAGGAATGCAGCTTGTG-3'
			reverse	5'-ACGTTAAAGAAGTGAGAGTACGAC-3'
STM0030	Milbourne *et al.* 1998	54.3	forward	5'JOE-AGAGATCGATGTAAAACACGT-3'
			reverse	5'-GTCGCATTTTGATGGATT-3'
STM0031	Milbourne *et al.* 1998	60.1	forward	5'FAM-CATACGCACGCACGTACA C-3'
			reverse	5'-TTCAACCTATCATTTTGTGAGTCG-3'

Genotyper®2.5 (*PE Applied Biosystem*) and data were recorded as 1 (peak present) and 0 (peak absent). This binary data was fed to *NTSYSpc* (Rohlf 1998) and analyzed using a symmetrical similarity matrix (*SIMQUAL* module) that uses Dice coefficient (Nei and Li 1979). Genetic similarity (S_{ij}) between two individuals i and j was calculated by following equation: $S_{ij} = 2a/(2a+b+c)$, where a is the number of peaks present in both i and j, b is the number of peaks present in i but absent in j, and c is the number of peaks present in j but absent in i.

SAHN module that performs clustering algorithms using UPGMA clustering method was used to generate a tree matrix (dendrogram). The goodness of fit of the dendrogram with the similarity data was tested by cophenetic correlation analysis. To measure utility of each SSR marker, genotype index (GI) and diversity index (DI) were calculated for each primer set. Genotype index reveals the proportion of genotype profiles to the total cultivars studied per assay (McGregor *et al*. 2000). Diversity index (DI) of polymorphic bands in each assay was calculated as $DI = 1 - \sum(pg^2)$, where *pg* is the frequency of an individual genotype (Powell *et al*. 1996).

Results

Amplification of the genomic DNA from the 32 potato cultivars with five SSR primer sets produced a total of 43 alleles ranging in size from 70 to 279 bp from 16 different loci. The number of amplified fragments was dependent on the cultivar and primer set. Total number of microsatellite alleles varied from lowest of 14 in Kufri Dewa to highest of 22 in Kufri Sherpa, with the mean number of alleles per cultivar being 18.3 (≈18). The number of amplified fragments detected by individual primer set varied from 5 to 12 with the mean number of 8.6 (≈9) (Table 3). A minimum of 5 alleles were amplified with primer set LEGAST1, while primer set STM0031 amplified maximum 12 alleles from 4 loci. The patterns of majority of alleles (33 out of 43, *i.e*. 76.74 %) varied between cultivars. The number of polymorphic alleles for each primer set varied from 0 to 12 with average 7 polymorphic alleles per primer set (Table 3). These include 5 poorly informative alleles (unique in one cultivar or absent only in one cultivar) and 4 highly informative alleles (present in 40 - 60 % cultivars). STM0031 amplified 12 alleles of which 3 were highly informative. On the other hand all the 5 alleles

Table 3. Informative value of SSR primers. Each parenthesis represents a locus and figures in it indicate sizes (bp) of alleles amplified at that locus.

Primer set	Loci	Alleles	Polymorphic alleles	Genotype index	Diversity index
STPOACUTR	4	9: (141), (186, 188), (260, 262, 266, 268, 270), (278)	8	0.41	0.866
LEGAST1	3	5: (70), (86, 88), (107, 109)	0	0.03	0.000
STPOAC58	2	9: (229, 231, 233), (236, 238, 242, 244, 246, 248)	9	0.53	0.904
STM0030	3	8: (74, 76, 78, 80), (91, 93), (110, 113)	4	0.25	0.736
STM0031	4	12: (118, 121, 127), (144, 150), (164, 166, 170), (184, 186, 188, 192)	12	0.84	0.957
Total	16	43	33		

amplified by LEGAST1 primer set were monomorphic and thereby least informative. Amplification of loci also varied from primer to primer. STPOAC58 amplified only 2 loci whereas, STPOACUTR and STM0031 amplified 4 loci.

The estimated similarity between the cultivars as calculated using Dice co-efficient ranged from 0.72 between Kufri Jyoti and Kufri Dewa to 0.98 between Kufri Jawahar and Kufri Neela (Table 3). High similarity values suggested a close similarity or narrow genetic base of the thirty-two cultivars tested in the present study. All thirty-two cultivars could be grouped into two major groups as shown in the dendrogram (Fig. 1). The reasons for the groupings obtained are not obvious from co-ancestry, since kinship relationship among different cultivars could not be predicted from the similarity data. Similarity value between the full-sibs Kufri Dewa and Kufri Sheetman (Craig's Defiance × Phulwa) was 0.72, which was lower than 0.85 that was recorded between half-sibs Kufri Sheetman and Kufri Pukhraj (common parent Craig's Defiance). The genetic similarity coefficient among seven half-sib combinations varied from 0.72 to 0.86 and among parent-progeny combination it ranged from 0.72 to 0.89.

None of the primer set could distinguish between all 32 cultivars singly. Therefore, the binary data matrix was analyzed by the software NTSYSpc to find out the minimum number of primer combinations required to distinguish all cultivars. It was observed that two primer sets STM-0031 and STPOAC58 together could distinguish all 32 cultivars. The primer sets STPOACUTR, LEGAST1, STPOAC58, STM0030 and STM0031 could identify 13, 1, 17, 8 and 27 cultivars, respectively. The genotype index (GI) and diversity index (DI) calculated for all markers are in Table 3.

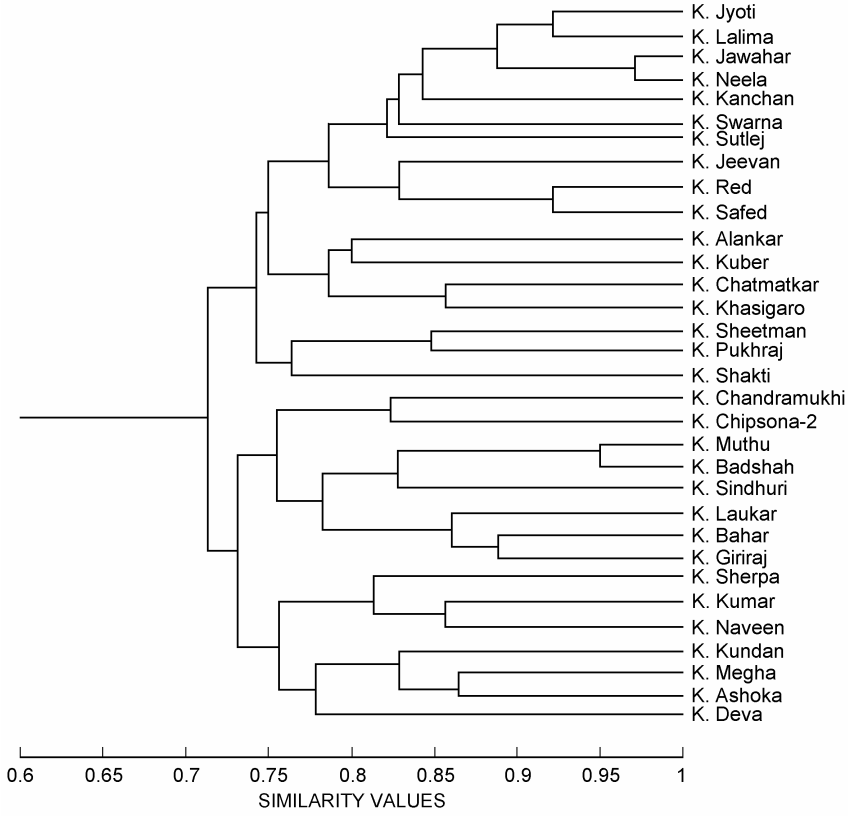

Fig. 1. Dendrogram showing relationship between thirty-two Indian potato cultivars.

Discussion

Random amplified polymorphic DNA (RAPD) markers has been used extensively for cultivar identification in barley (Reddy and Soliman 1997), chickpea (Banerjee *et al*. 1999), peas (Samec *et al*. 1998), soybean (Taylor and Soliman 1999) and many other crops. However, the ability to detect large number of discrete alleles repeatedly, accurately, and efficiently makes microsatellite marker most appropriate for cultivar identification. As compared to AFLPs and RAPDs, SSR alleles of tomato were found to be more reproducible

with minor differences in sizing when analyzed in different European laboratories (Jones *et al*. 1997). The results obtained using α (P^{33})-dATP or α (S^{35})-dATP were more reproducible than those of silver staining. Milbourne *et al*. (1997) did comparative analysis of SSRs, AFLPs and RAPDs of 16 northwestern European potato cultivars. They found that SSRs detected highest polymorphism (diversity index, or DI) and AFLPs revealed the highest number of loci (effective multiplex ratio, or EMR). McGregor *et al*. (2000) studied 39 South African potato cultivars and found that on the basis of genotype index per primer (or primer pair) the order of merit was AFLPs, a multi-locus SSR, RAPDs, ISSRs and single locus SSRs. Schneider and Douches (1997) could distinguish only 24 genotypes out of 40 North American potato cultivars using 5 SSR primer sets. Studies on identification of grape cultivars have shown that SSRs are the most promising molecular method for reproducible and inter-changeable cultivar identification than RFLPs and AFLPs (Sanchez-Escribano *et al*. 1999). Recent improvements in capillary electrophoresis (with ability to detect single base differences) have been employed for high efficiency semi-automated microsatellite genotyping. Fluorescence based systems utilize the ability to detect multiple fluorescent dyes simultaneously to increase the number of loci that can be detected at any one stage (Ziegle *et al*. 1992). Capillary electrophoresis has the advantages of rapid DNA separation, high sensitivity, automatic sample loading and elimination of the need to pour gels (Wenz *et al*. 1998).

The present study indicated usefulness of semi-automated capillary electrophoresis and four SSR primer sets in cultivar genotyping. All the 32 cultivars were distinguished from each other with as few as two primer sets (STPOAC58 and STM-0031). These two primer sets have the highest genotype (0.53 and 0.84) and diversity (0.904 and 0.957) indices. The narrow genetic base observed in present study is in contrast to the wide genetic base of Indian potato cultivars estimated using RAPD analysis in our laboratory (Chakrabarti *et al*. 2001, Pattanayak *et al.* 2002). This may perhaps be due to non-specific amplification (due to lower annealing temperature), smaller primer size and involvement of entire genomic DNA in RAPD amplification. However, the results obtained in present study were in conformity with those obtained by Naik (unpublished) when Indian potato cultivars were analyzed using 8 SSR primer pairs and silver staining of denaturing polyacrylamide gels at the International Potato Centre, Lima, Peru. The kinship relationship among different cultivars could not be predicted from the similarity data because potato is highly heterozygous tetraploid. Similar observation on kinship relations in Indian potato cultivars were also reported by Chakrabarti *et al*. 2001, Pattanayak *et al.* 2002 and Naik (unpublished). The genetic diversity revealed by microsatellite amplification may not present actual genetic relationship in a polyploid crop. This is because the similarity coefficient is based on presence or absence of a peak and the potato genotype having an allele in single, double, triple or quadruple doses will be considered same for estimation of similarity value. Thus, there is a need of some software package to estimate allele dosage after considering height and area of each peak.

SSR markers STPOACUTR**,** LEGAST1, STM 0030 and STM 0031 were also used by Provan *et al*. (1996) and Milbourne *et al*. (1998) to study polymorphism in European potato cultivars. They reported amplification of 2, 1, 2 and 2 loci, respectively with above primers. In our studies these primers amplified 4, 3, 3, and 4 loci, respectively. Better resolution of microsatellite loci in our studies may be due to fluorescent labeling combined with capillary electrophoresis. This shows that multi-locus resolving microsatellite primers are extremely powerfull tools in differentiating samples as they reveal high level of polymorphism. Multiple loci have also been reported in other crops such as sugarcane (Cordeiro *et al*. 2000), wheat (McLauchlan *et al*. 2001) and *Brassica* (Westman and Kresovich 1998). The genetic profiles produced by multiplexing specific combinations of microsatellite markers can be used to document ownership and protect intellectual property rights. It will also facilitate generation of information on molecular-based genetic relationship among the cultivars.

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