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*Full Length Research Paper*

# Reproducibility testing of RAPD and SSR markers in Tomato

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**Large no of PCR-based techniques can be used to detect polymorphisms in plants. For their wide-scale usage in germplasm characterization and breeding it is important that these marker technologies can be exchanged between laboratories, which in turn require that they can be standardized to yield reproducible results, so that direct collation and comparison of the data are possible. This article describes a network experiment involving screening of 60 RAPD primers and was used to confirm the reproducibility. Every experiment was performed 50 times by 10 candidates in which the reproducibility of two popular molecular marker techniques was examined: random-amplified fragment length polymorphism (RAPD), and sequence-tagged micro satellites (SSR). For each technique, an optimal system was chosen, which had been standardized and routinely used by one individual. This system (genetic screening package) was distributed to different candidates in the laboratory and the results obtained were compared with those of the original generator or sender. Different experiences were gained in this exchange experiment with the different techniques. RAPDs proved difficult to reproduce. Whilst SSR alleles were amplified by all candidates, but small differences in their sizing were obtained.**

**Key words:** DNA markers, RAPD, SSR, micro-satellite, reproducibility.

## INTRODUCTION

Advances in DNA sequencing, data analysis and PCR have resulted in powerful techniques, which can be used for the characterization and evaluation of germplasm and genetic resources, and for the identification of markers for use in breeding programmes (Lin et al., 1996; Thomas et al., 1994). For the wide-scale application of these techniques, it is important that they are suitable for use in network activities in which many laboratories may be involved in coordinated actions and in which common data-bases are continually fed with data. It is essential for such activities that the different screening techniques employed can be standardized to yield reproducible results across laboratories, so that direct collation and comparison of the data are possible. In the present study, an account is given of reproducibility testing of two popular molecular marker systems. Random-amplified polymorphic DNA (RAPDs) involve the use of a single arbitrary primer (purchasable from commercial companies) in a PCR reaction and result in the amplification of several discrete DNA products (Devos et al., 1992; Büscher et al., 1993 ). Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands that are complementary to the primer and sufficiently close together for the amplification to work. In RAPDs, the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light (Welsh et al., 1990). It is now widely recognised that to obtain reproducible band profiles on the gels it is absolutely essential to maintain consistent reaction conditions. Numerous studies have reported the separate effects of altering different parameters, ratio of template DNA primers, concentration of *Taq* polymerase and Mg concentration.

A summary of these experiments is that RAPD profiles

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**Abbreviations:** RAPDs, random-amplified polymorphic DNA; SSR, Micro-satellites or simple sequence repeats; GSP, Genetic Screening Package.

should be reproducible among laboratories provided that all details of the reaction conditions are standardised and strictly adhered to. Microsatellites or simple sequence repeats (SSRs) are highly mutable loci (Bowers et al., 1996), which may be present at many sites in a genome (Morgante et al., 1993). As the flanking sequences at each of these sites may be unique, once SSR loci are cloned and sequenced, primers can be designed to the flanking sequences. The resultant sequence tagged microsatellite usually identifies a single locus, which, because of the high mutation rate of SSRs, is often multi-allelic. Alleles which differ in many base pairs of length can be resolved on agarose gels but often SSRs are visualized on sequencing gels where single repeat differences can be resolved and, thus, all possible alleles detected. SSRs provide highly informative markers because they are co-dominant (unlike RAPDs and generally highly polymorphic. The nature of the PCR-based assay used in their amplification and detection (i.e. the use of specifically designed primers based upon the flanking sequences) suggests that they should be highly reproducible between laboratories. To test the standardization and reproducibility of RAPD and SSR markers, network experiments were undertaken by a number of different candidates in laboratory. For each technique, an optimal system was chosen, i.e. a system, which had been standardized and used routinely by one of the participating candidate, and used to construct a genetic screening package (GSP). The 'sender' or generator candidate distributed the GSP to different participating candidates (recipients) in the network and the results obtained were compared with those of the original sender. Different experiences were gained in the network experiments for RAPDs and SSRs.

## MATERIALS AND METHODS

A genetic screening package (GSP) was prepared for each technique (RAPDs, and SSRs) by different 10 candidates through out the year in different times to check the reproducibility of said techniques as follows.

### RAPD

The RAPD GSP was assembled by one of the candidate and distributed to all of the other 9 candidates in the laboratory. The RAPD GSP comprised the following: (1) genomic DNA isolation: we have taken two lines BSSD-2 & BSS6112B (Lycopersicon hirsutum) developed a very simple DNA isolation protocol for PCR analysis which does not require liquid nitrogen and can be done very quickly. The quality of DNA isolated with the protocol is sufficient for PCR based analysis and are as satisfactory as those of the conventional large scale procedure. Samples from two lines of tomato were extracted by the method. 60 decamers RAPD primers: 20 from each kit OPA,OPB and OPC, *Taq* polymerase and 10X PCR buffer (both from BANGLORE GENEI, INDIA), agarose (HIMEDIA, INDIA) were used in the experiment. Primers (diluted in TE) and ethanol-precipitated genomic DNA were sent in 1.5 ml microfuge tubes to all participants. PCRs were performed as described in (Zhang et al., 1995) PCR reaction mixes consisted of 1 2.5 ul of 10X reaction buffer (100 mM Tris pH 9, 15 mM $MgCl_2$, 500 mM KCL and 0.1% Geletin, 3 ul dNTPs (200 µM), 2 ul of 5 pica moles/µl primer, 1 ul of 50 ng/µl genomic DNA 0.8 U/µl *Taq* polymerase and remaining water. Only one DNA sample and one primer were added to any single reaction. All reaction volumes were 25 µl overlaid with a drop of mineral oil. A standard thermocycling programme was used by all candidates in the laboratory: 1 cycle (an initial denaturing step) of 3 min at 94°C, 35 cycles of 1 min at 94°C, 30 s at 32.1°C, 1 min 30 sec at 72°C 1 cycle (final extension) 7 min at 72°C, then kept at 4°C. Only one PCR machine was used by all candidates. Electrophoresis was performed in 5 mm thick agarose gels (2% HIMEDIA) with 0.5 X TBE buffer for 2 h at 5 V/cm, constant voltage. The gels were stained with 10 mg/ml ethidium bromide, visualized on a 302 nm UV transilluminator and photographed with a Gene Genus gel documentation system (SYNGENE).

### SSR

The SSR primers were selected from the published data and used for the genetic screen package .The SSR GSP was prepared by one of the candidate and the recipients were asked to amplify the products, run them on a gel and size the alleles. The GSP contained: (1) genomic DNA from two cultivars of tomato BSSD-2 and BSS6112B (2) PCR primers: PCR reaction mixes were 25 µl of 2.5ul 10X reaction buffer contain (100 mM Tris pH 9, 15 mM $MgCl_2$, 500 M KCL and 0.1% Geletin, 3 ul dNTPs (200 µM), 2ul of each forward and reverse primers 5 pica moles/µl primer, 1ul of 50 ng/µl genomic DNA and 0.8 U/µl *Taq* polymerase and remaining water. Only one DNA sample and both forword and reverse primer were added to any single reaction (Table 1). All reaction volumes were 25 µl overlaid with a drop of mineral oil. The thermocycling programme used was: one cycle (an initial denaturing step) at 94°C for 3 min; 35 cycles at 94°C for 1 min (denaturing); 55°C for 1 min; 72°C for 1 min 30 sec and one cycle (final extension) at 72°C for 7 min, kept at 4°C. The PCR amplification products were stored at –20°C until used. For the determination of bands on the gel the agarose percentage was increased upto 3%. The length of the alleles was determined by comparison with marker loaded on adjacent gel tracks.

## RESULTS

Initially we had taken 60 primers taken for the PCR amplification, only 8 primers were found polymorphic (Table 2). Results were exchanged among 10 candidates in the laboratory for the two procedures RAPDs, and SSRs. In the case of RAPDs, all recipient candidates performed the reactions in same manner as like the original sender. All had some previous experience with this technique. Few of the candidates which received the SSR GSPhad experienced difficulties when performing the reactions for the first time in case of annealing temperature standardization. Eventually, all candidates were able to carry out the procedure satisfactorily. Most of the recipient for the SSR GSP had some experience of sequence tagged micro satellites. A major difficulty experienced was in obtaining satisfactory results with the high percentage agarose gel as compare to silver staining method and some candidates were only able to

**Table 1.** SSR primers used for the screening of two tomato cultivars.

| S/ N | SSR Repeat | Forward primer 5` 3` | Reverse primer 5` 3` |
|---|---|---|---|
| 1 | Tom 8-9 ATT7 | GCA TTG ATT GAA CTT CAT TCT CGT CC | ATT TTT GTC CAC CAA CTA ACC G |
| 2 | Tom 11-28 CTT5/CT5 | ATT GTA ATG GTG ATG CTC TTC C | CAG TTA CTA CCA AAA ATA GTC AAA CAC |
| 3 | Tom 31A-32A TA11 | AAT GTC CTT CGT ATC CTT TCG T | CTC GGT TTT AAT TTT TGT GTC T |
| 4 | Tom 39A-40A AATT4 | TAA CAC ATT CAT CAA AGT ACC | TTG CGT GAT AAT CCA GTA AT |
| 5 | Tom 41- 42 TCC6 | GAA ATC TGT TGA AGC CCT CTC | GAC TGT GAT AGT AAG AAT GAG |
| 6 | Tom 43- 44 TCC6 | GCA GGA GAT AAT AAC AGA ATA AT | GGT AGA AGC CCG AAT ATC ATT |
| 7 | Tom 47- 48 AT10 | CAA GTT GAT TGC ATT ACC TAT TG | TAC AAC AAC ATT TCT TCT TCC TT |
| 8 | Tom 49- 50 AT10 | AAG AAA CTT TTT GAA TGT TGC | ATT ACA ATT TAG AGA GTC AAG G |
| 9 | Tom 55-56ATTT5 | ATT TCT GTA ACT CCT TGT TTC | TGA CTT CAA CCC GAC CCC TCT T |
| 10 | Tom 57- 58 CT8 | TCT AAG TGG ATG ACC ATT AT | GCA GTG ATA GCA AAT GAA AAC |

**Table 2.** RAPD primers found polymorphic in screening of Dirty 2 and CLN2116B tomato cultivars.

| RAPD Primer Name | Sequence (5'-3') |
|---|---|
| OPA02 | 5'-TGCCGAGCTG-3' |
| OPA05 | 5'-AGGGGTCTTG-3' |
| OPA12 | 5'-TCGGCGATAG-3' |
| OPB04 | 5'-GGACTGGAGT-3' |
| OPB10 | 5'-CTGCTGGGAC-3' |
| OPC02 | 5'-GTGAGGCGTC-3' |
| OPC08 | 5'-TGGACCGGTG-3' |
| OPC18 | 5'-TGAGTGGGTG-3' |

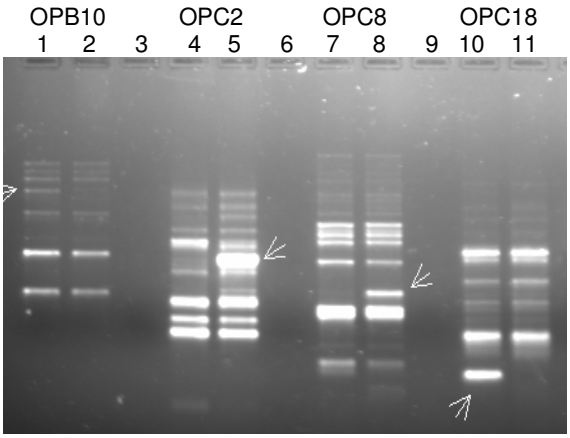


**Figure 1**.showing the polymorphic bands in both the cultivars, from left Lane =:BSSD-2,2=BSS6112B;3 =Blank;4=BSSD2 ;5=BSS6112B; 6=-blank;7=BSSD-2,8=BSS6112B;9=blank;10= Dirty ;11=BSS6112B

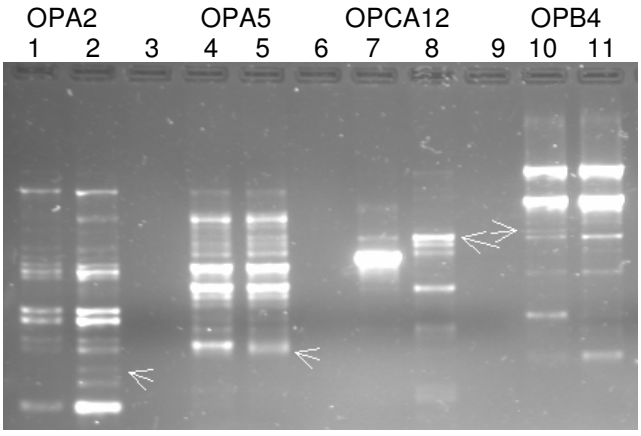


**Figure 2.** showing the polymorphic bands in both the cultivars, from left Lane =BSSD-2,2=BSS6112B;3 =Blank;4=BSSD2 ;5=BSS6112B; 6=-blank;7=BSSD-2,8=BSS6112B;9=blank;10= Dirty ;11=BSS6112B

complete. Only two bands were found in SSR amplification one of 200 bp was found polymorphic in above two plants. Aside from this, all candidates were able to carry out the SSR GSP reactions satisfactorily. In terms of the reproducibility of the results obtained, different experiences were gained with the different techniques and details are thus given separately for the different genetic screening package

One of the the representative RAPD profile obtained from the different candidatesis given in Figures 1 and 2 in which arrowhead shown the polymorphic band considered for further reproducibility experiments by

other 9 candidates. The original sender had sent the profile in which a total of 8 primer polymorphic banding pattern was obtained in which primer OPA2 and OPA5 shown the polymorphic band in BSS6112B but out t of 10 candidates, 2 candidates were not able to reproduce the same polymorphism in the given same plant DNA. In Figure 2, the results from the two DNA samples BSSD-2 and BSS6112B are shown. Any bands, which were polymorphic between them, are both represented as arrowheads. The original profile obtained by original sender has number of bands with different primers. Primer OPA02 amplified 12 bands of variable intensity, which ranged in size from 200 to 3000 bp of which a single band was polymorphic between BSSD-2 and BSS6112B (Figures 1 and 2). The majority of recipient candidates were able to amplify the same bands as the sender and to observe the same polymorphism but none reproduced the profile exactly. In general, many candidates found some primers which were polymorphic by original sender as not having amplification, amplification in only one plant, polymorphic but with different band intensities. Only 8 candidates were able to produce all the bands of brightest intensity in the series of experiments. All recipient candidates reproduced the amplification of the all brightest bands.

In case of SSR screening package, candidates reproduced the profile exactly. Four candidates produced of all SSR profiles of same molecular weight but with could reproduce the bands with intermediate bright intensity.

## DISCUSSION

A number of reproducibility experiments have been reported in the literature, in which the robustness of DNA marker systems was tested. In the majority of instances, this has involved reproducibility tests within a laboratory, although experiments testing the ability of different laboratories to achieve identical RAPD profiles have also been described (Penner et al., 1993). In the present article, the reproducibility of RAPDs, and SSRs marker have been compared in network experiments involving several candidates from same laboratory. Different experiences were gained with RAPDs and SSRs markers.

RAPDs were found to be easy to perform by all candidates but reproducibility was not achieved to a satisfactory level that out of 10 candidates, two candidates was not able to reproduce the results exactly. Systematic tests on the effects of some factors influencing RAPD patterns have been carried out by several research groups (Meunier and Grimont, 1993) and there are many reports on the importance of *Taq* polymerases and thermocycling machines in the optimisation of PCR reactions conditions for RAPDs (Yu and Pauls, 1992). Variability in RAPD profiles may be due to the use of different PCR chemicals, dilution with different pippets and thermal cyclers. Our results support earlier findings of irreproducibility in RAPD profiles performed by a number of laboratories (Penner et al., 1993). Skroch and Nienhaus (1995) have further examined the impact of this irreproducibility on the scoring of RAPDs. When expressed as the percentage of RAPD bands scored that were also scored in replicate data, only 80% reproducibility was obtained for 60 RAPD primers. Since RAPD may sometimes depend on the quality of the extracted DNA and the method of extraction could affect the profiles obtained. However, in the present study, all the DNA was extracted by the sender and the differences observed were introduced at the template preparation step. Although RAPD is insensitive to template concentration, differences may occur when templates are excessively diluted (Vos et al., 1995; MacPherson et al., 1993 ). This was not the case here, however, and the absence of the polymorphic band can only be placed down to experimental errors incurred in performing the RAPD procedure (Huys et al., 1996).

In the case of the SSR reproducibility experiment, some recipients experienced difficulties with the standardization of annealing temperature but the alleles of the original GSP were reproduced by all candidates as have been previously observed (Smulders et al., 1997). These results are in general keeping with previous claims on the robustness of microsatellites (Schwengel et al., 1994)

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