Potential of SSR markers in hybrid purity testing of Tomato

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Abstract
The present study aimed at identifying and utilizing the molecular markers to strengthen the effectiveness of genetic purity testing of genotypes against the traditional grow-out-test. Four commercial hybrids of tomato developed by division of vegetable science, Indian Agricultural Research Institute, New Delhi and their parents were screened with 134 SSR markers. Six markers (SLM - 7, SLM - 53, TGS - 0162, TES - 1388, SSR - 63, and SSR - 212) were found to be polymorphic in distinguishing the hybrids.

A combination of more than one marker could generate distinct DNA fingerprints for three hybrids PH - 1, PH - 2 and PH - 8. Multiplexing was demonstrated using two markers in one hybrid for its unambiguous identification. Finally, the markers were validated on a large number of F_1 plants for all the hybrids by comparing them against a traditional grow-out-test.

Keywords: Tomato, molecular markers, Grow-out-test, hybrid purity.

Introduction

Cultivated tomato (*Solanum lycopersicum*) is a very important crop due to its high value as a fruit for consumption. Average tomato yields in tomato are far less in Indian scenario (24.21 t ha^{-1}) when compared with the average productivity of China (56.20 t ha^{-1}) and USA (90.29 t ha^{-1})[11]. Though the difference in the productivity can be attributed to different reasons like management and pest incidence, one of the standout factors could be availability of high quality and elite genotypes for farming community. Globally, there is an increasing trend for adoption of hybrid seed technology.

This is due to increased vigour, uniformity, pest tolerance or resistance and good horticultural traits, thus giving consistence yields. Breeders see hybrid development as a fast and convenient way to combine desirable characters. Hybrids development and farming is found to be centre of attraction in all spheres of research and farming communities. Thus, a severe competition has emerged and there are frequent cases of same hybrids available under different names and availability of spurious seed in the name of popular hybrids. Thus the need of the hour is defining identity, purity and stability of varieties for breeder rights protection and effective seed quality control program.

Among different spheres of seed quality control program, seed purity assessment is one of the vital components. Traditionally, a grow-out-test is the best practice for doing that job using conventional morphological descriptors as described by the plant breeders. Though, the morphological characters are widely used in assessing the genetic purity, the grow-out-test is marred by its time consuming, space and labour demanding lacunae. As a way through, the International Seed Testing Association (1996) has recommended the use of electrophoresis in seed purity testing.

The advent of an array of molecular markers at DNA level presently available and the new generation markers developed through spill over information from genome projects have offered an improved method of finger printing and genetic purity testing other than those involving morphological markers in a traditional Grow-out-test. Unlike morphological characters, the variations at nucleotide level remain unchanged and are more stable across environments and over a time period.

Several molecular markers such as Restricted Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), Sequence Related Amplified Polymorphism (SRAP) are successfully reported for seed genetic purity testing in field crops like rice[14], cotton[8], cabbage[13], tomato[12] and brinjal[2]. Among all the different marker systems, SSR are widely used citing their wide abundance, simplicity and rapidness in handling. More importantly, SSR markers are co-dominant in nature, thus enabling the distinct feature of hybrid to be seen as heterozygous at the locus being tested. The present study is an attempt to assess the potential of genetic purity testing in tomato using SSR markers against the traditional grow-out-test.

Material and Methods

Plant material: Four commercial hybrid tomato cultivars developed by division of vegetable science, Indian Agricultural Research Institute, New Delhi, along with their parents were used in the present study. The parents and the hybrids were raised for two reasons. During the first season,

the male and female parents of all hybrids were raised to generate the hybrids following the parentage given by the breeder. The grow-out-test was conducted during second season for all the hybrids.

Table 1
Tomato hybrids and their parental lines used in the study

Hybrid	Female	Male
PH-1	Female-1	Male-1
PH-2	Female-2	Male-2
PH-4	Female-2	Male-1
PH-8	Female-3	Male-3

DNA isolation and molecular analysis: Total genomic DNA was isolated from young leaves following the method proposed by Doyle and Doyle[9]. For screening the parental lines for polymorphic SSR markers, a total of 10 plants were sampled for their leaves and DNA was isolated. The polymorphic SSR markers were tested for their expression in heterozygous state in F_1 along with parents. To test the uniformity of the markers in the parental lines, a total of 20 plants from each parent were used for DNA isolation.

DNA amplification was achieved by setting up a reaction volume of 20 µl (molecular biology grade water - 15 µl, DNA - 1 µl, primer (both forward and reverse) - 1 µl, 10X buffer-2 µl, Taq polymerase(Genei make) - 0.25 µl, $MgCl_2$ - 0.25 µl, dNTP (10 mM) - 0.5 µl) (Chemicals supplied by GCC Pvt. Ltd.). Conditions for polymerase chain reaction were achieved by resorting to Touch down PCR. PCR products were separated on 3.5% agarose gels by running the loaded gels in 1X TAE buffer at 120 V for 3 hours. The size of DNA amplicon was determined by using a 100 bp ladder (BR Biochem make). A total of 132 SSR markers selected from different studies[1,3-5,10] were used in the present study which were synthesized from IDT and GCC Pvt. Ltd.

Grow-out-test: The hybrids along with their parental lines were grown under open field conditions at experiment farm, Division of Seed Science and Technology, Indian Agricultural Research Institute, New Delhi during Rabi, 2015. A total of 175, 364, 182 and 162 plants were maintained in PH1, 2, 4 and 8 hybrids respectively and their integrity was maintained in the field from transplanting to harvest.

The plants were observed for their characters like stem anthocyanin on upper 1/3rd portion, leaf blade margin, fruit shape and any deviation from the prescribed characters was treated as offtypes/selfed plants/admixtures from males. Standard management practices and time to time plant protection measures were taken up. DNA was isolated from all the plants and was subjected to SSR analysis with the procedure described above.

Results and Discussion

Identification of polymorphic markers to identify hybrids: Out of 132 markers tested, six markers (SLM - 7, SLM - 53, TGS - 0162, TES - 1388, SSR - 63, SSR - 212) (Table 2) were found to be polymorphic in distinguishing the hybrids under study (Fig. 1). These markers formed a distinct fingerprint profile in different combinations for all the hybrids under study. Thus, the hybrids could be identified separately using a single or a combination of more than one marker (Table 3).

Table 2
List of polymorphic markers

S.N.	Primer	Forward primer	Reverse primer
1	SLM-7	5'-caattgaagattggggcttt-3'	5'-agcagctcacctcacgtttt-3'
2	SLM-53	5'-cccgcaattttaatagtataaccaa-3'	5'cggaatccatgaatgagagc-3'
3	TGS 0162	5'-ggcaacactcaatggtgaaa	5'-aggggtgggattggaaatta
4	TES 1388	5'-gcataccctatgcctttgga-3'	5'-cacacactgtgaaaccattttct-3'
5	SSR 63	5'-ccacaaacaattccatctca-3'	5'-gcttccgccatactgatacg-3'
6	SSR 212	5'-acgaaaacgaaatctcgactataac-3'	5'-aggcttcgttagtgaactagaat-3'
7	SSR 212-1	5'-acgaaaacgaaatctcgactataac-3'	5'-aggcttcgttagtgaactagaat-3'

Table 3
Different markers with their amplification profile in tomato hybrids

Hybrid	SLM-7	SLM-53	TGS-0162	TES-1388	SSR-63	SSR-212
PH-1	+	-	+	-	-	+
PH-2	+	+	-	-	-	-
PH-4	+	-	-	-	-	-
PH-8	-	-	+	+	+	-

(+ indicates polymorphic; - indicates monomorphic)

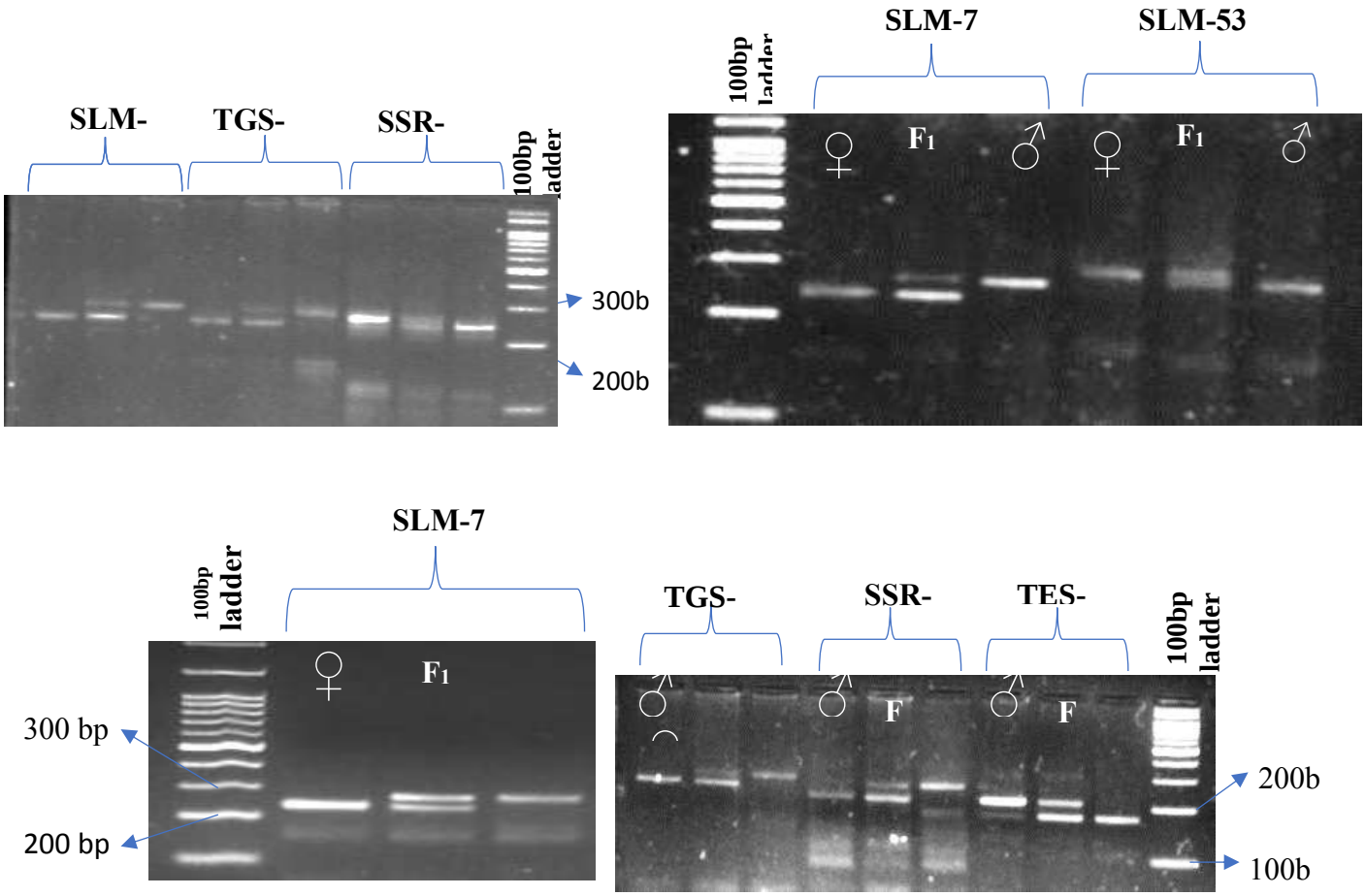

Figure 1: SSR profiles of four tomato hybrids and respective parental lines obtained with different markers. (a) PH-1, (b) PH-2, (c) PH-4 and (d) PH-8

SLM - 7 amplified two alleles with a size of 210 bp and 220 bp in female and male parents respectively in all the three hybrids PH - 1, PH - 2 and PH - 4. TGS - 0162 produced a heterozygous profile having alleles of 220 bp and 240 bp in both PH - 1 and PH - 8. SSR - 212 also had a unique amplification pattern in PH - 1 with allele size of 230 bp and 220 bp. SLM - 53 could distinctly identify the hybrid PH - 2 from others with allele sizes 270 bp and 250 bp in female and male parents, respectively. Similarly, TES - 1388 and SSR - 63 also produced a distinct fingerprints in PH - 8.

TES - 1388 generated allele sizes of 210 bp and 160 bp whereas as SSR - 63 amplified 230 bp and 210 bp alleles in female and male parents respectively (Fig. 1). Though a single marker is enough to establish hybridity in the F_1, it may fail to establish the distinctiveness of a hybrid from others. Thus, when these markers are used in combination, it may result in a full proof method of detecting the hybrid.

Multiplex PCR which can simultaneously amplify different primer mixtures can reduce the cost and overcome the weakness of single PCR reaction and has been applied successfully in many areas since its inception in 1988 by Chamberlain et al[6]. Multiplexing of two polymorphic markers was done in PH - 1. Citing the same size of one of the allele generated by all the three markers TGS - 0162, SLM - 7 and SSR – 212, we decided to multiplex TGS - 0162 and SSR - 212. The sequence of the marker SSR - 212 was elucidated by a simple primer blast and a different marker was developed from this amplicon to reduce the amplicon size to a range of 180 – 190 bp. This marker is renamed as SSR - 212 - 1. The resultant two markers TGS - 0162 and SSR - 212 - 1 were used to identify the hybrid PH - 1 proving to be a cheaper and one go approach of identifying PH - 1 (Fig.2).

Determining the uniformity of markers in parental lines:
A marker was found to be effective only when it is expressed uniformly and stably in the parental lines. This holds ground only when the marker lies in homozygous region of genome. Tomato, a self-pollinated crop is reported to have residual heterozygosity in its genome. So, if the marker belongs to this region, it tends to segregate causing false positives and false negatives in identifying the F_1 in the ensuing seasons, thus severely defeating the purpose of its reliability. To cull out this problem, each polymorphic marker was tested on twenty individual plants of each parent during both the seasons.

Though, no variation was found for all the agronomic traits in the parents of all hybrids, TES - 1388 was found to be in heterozygous state in female parent of PH - 8. The profile of

this marker in female of PH - 8 was found to have both alleles 210 bp and 160 bp in homozygous and heterozygous condition in 20 plants tested. Thus, the utility of this marker TES - 1388 was curtailed from the present study (Fig. 3).

Purity testing of hybrids through markers in comparison with field grow-out-test: Assessing the genetic purity of hybrid seed is essential prior to its commercialization, as there is always a chance of contamination during hybrid seed production either by selfing, out-crossing or by admixtures due to improper handling during harvesting or post-harvest operations.

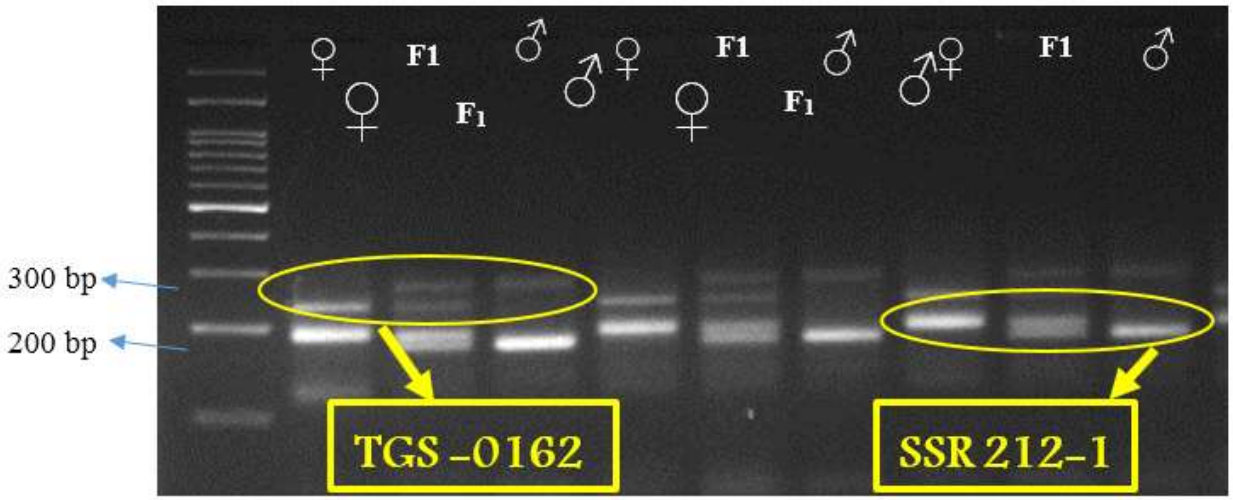

Figure 2: Multiplexing of TGS-0162 and SSR-212-1 in PH-1 and their parental lines

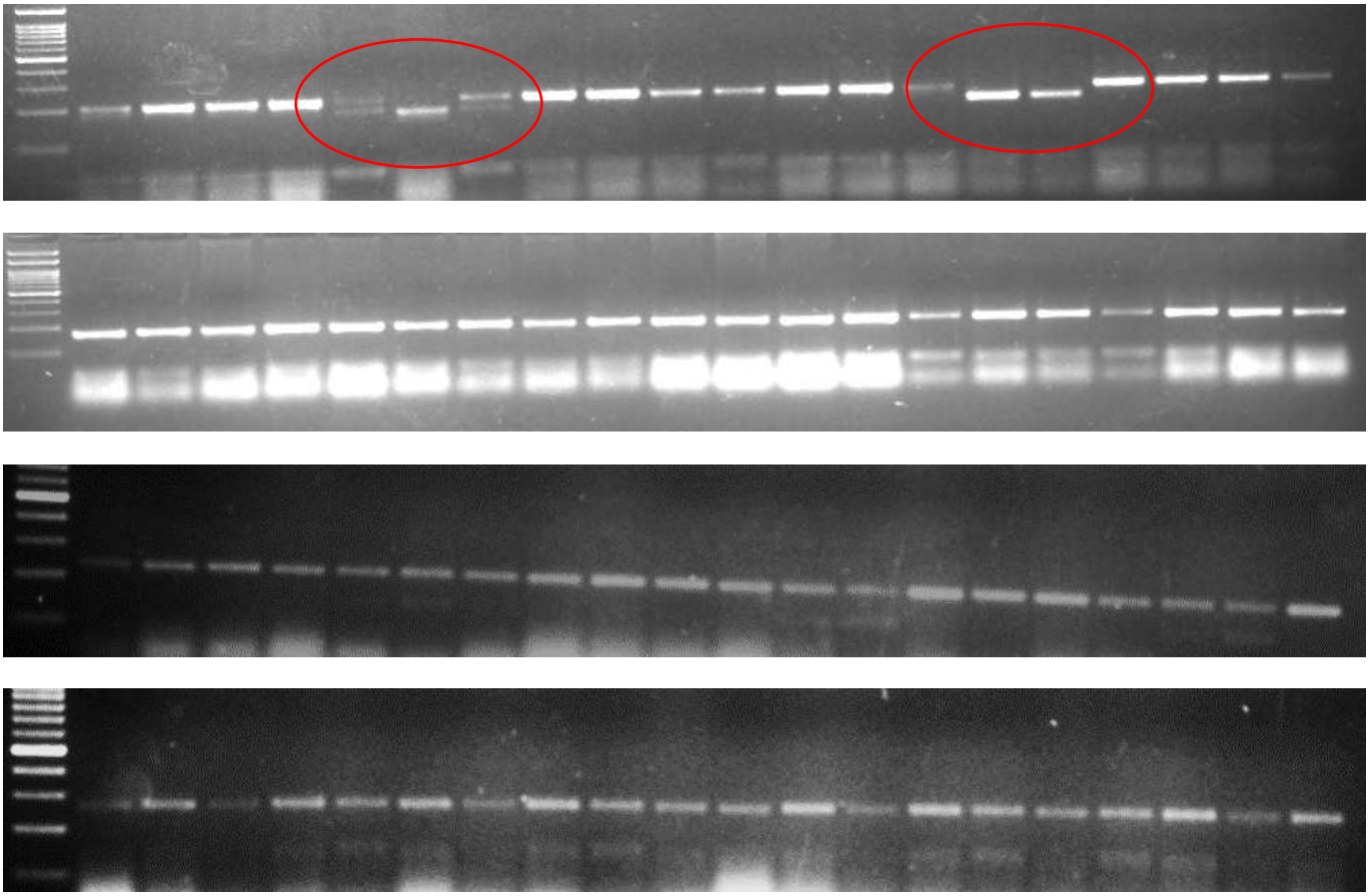

Figure 3: Testing for uniformity of marker in parental lines (a) TES-1388 in female parental line of PH-8 (b) TES-1388 in male parental line of PH-8 (c) TGS-0162 in female parental line of PH-8 (d) TGS-0162 in male parental line of PH-8

Since GOT is mandatory for certified seeds of hybrids in Indian Scenario, which suffers by its time consuming, labour and land intensive criteria, an alternative testing strategy of genetic purity using molecular markers can be adopted.

The utility and efficacy of these molecular markers in genetic purity testing were compared against conventional field grow-out-test. Grow-out-test was conducted for all the chosen hybrids in the field, and all the plants were subjected to marker analysis by all respective polymorphic markers. The plants identified as selfed based on molecular markers indeed turned out to be so based on morphological screening in the grow-out-test. Genetic purity analysis of all the four hybrids was conducted using all the polymorphic markers identified. In PH - 1, out of 175 plants, plant no. 129 was found to be offtype with the help of SSR - 212 by virtue of different amplicon size it generated. For other two markers TGS - 0162 and SLM - 7, the allele size (220 bp and 210 bp, respectively) indicated it as selfed plant.

The field appearance of the plant had indeterminate growth habit, deeply lobed dark green coloured leaves with densely hairiness and was not resembling PH - 1. Also, two plants numbered 142 and 154 were found to be selfed plants amplifying female corresponding alleles for all the three markers tested (Fig.4a and 4b). In PH - 2, out of 364 plants tested, plant number 134 and 158 were found to be selfed plants as revealed by both markers (SLM - 7 and SLM - 53) generating female corresponding amplicons. The plants were resembling the female parent of PH - 2 with respect to leaf serration, and fruit characters. In PH - 4, out of 182 plants tested, plant no. 174 was found to be selfed by SLM – 7 (Figure 5a).

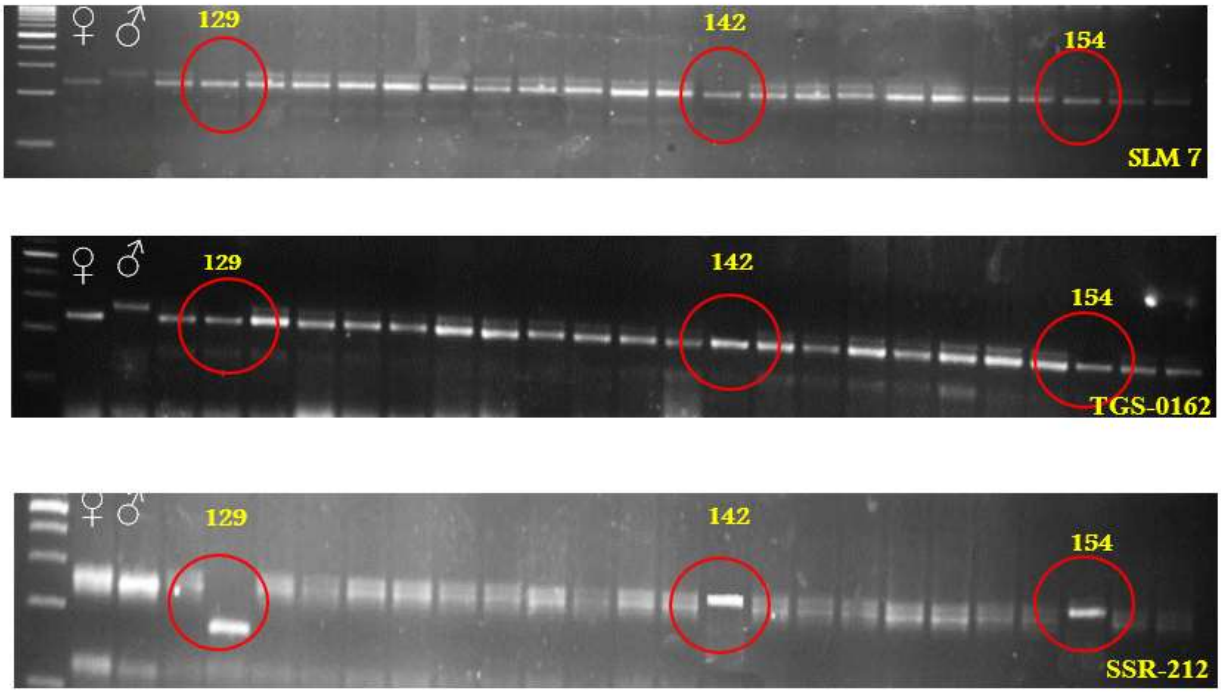

Figure 4a: Genetic purity testing of PH-1 using three polymorphic markers

Figure 4b: Morphological differences for plant and leaf characters between PH-1 and offtype (#129)

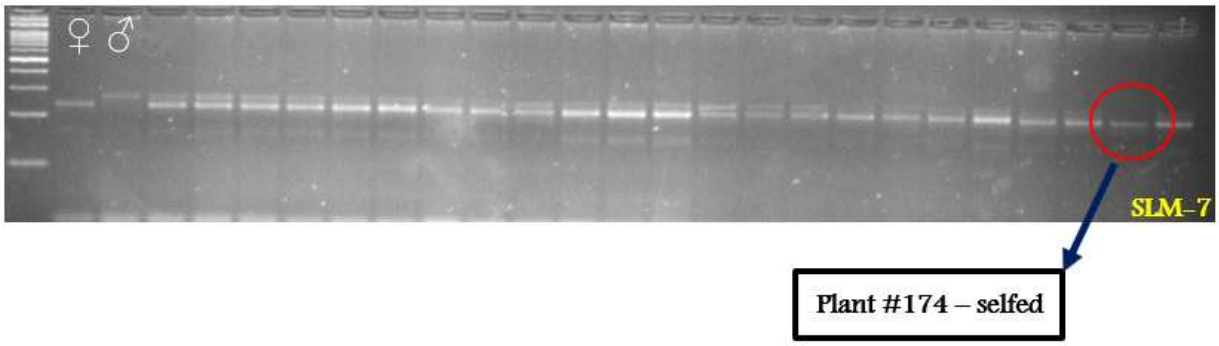

Figure 5a: Genetic purity testing in tomato hybrid PH-4 using marker SLM-7

Figure 5b: Visual differences for leaf character between PH-4 and selfed plant (#174)

The plant was less vigorous than the F_1 and was resembling the female parent in terms of its fruit shape and leaf blade serration (Figure 5b). When 162 PH - 8 plants were subjected to marker analysis by TGS - 0162 and SSR - 63, two plants numbered 67 and 76 were detected as selfed plants by generating female corresponding alleles for both the markers(Figure 6a). Both the plants were less vigorous than F_1. Morphologically, PH - 8 plant closely resembles the female parent making it difficult to identify in a traditional grow-out-test (Figure 6b). Use of molecular markers simplifies the process of identifying a selfed plant in such cases. Till date conventional morphological markers are in place for assessing the hybrid purity using grow-out-test. Apart from the disadvantages it hosts, with increasing number of public and private players releasing more and more number of hybrids, the morphological evaluation is getting more difficult.

Use of molecular markers for hybrid purity evaluation is demonstrated in many crops; few reports are available in tomato[16,17], the present study has emphasized more on using more than one molecular marker for hybrid purity testing considering the narrow genetic base with which the parental lines were bred and the inability of a single morphological character or a single molecular marker unable to differentiate the parental lines, hybrid among themselves and from the offtypes.

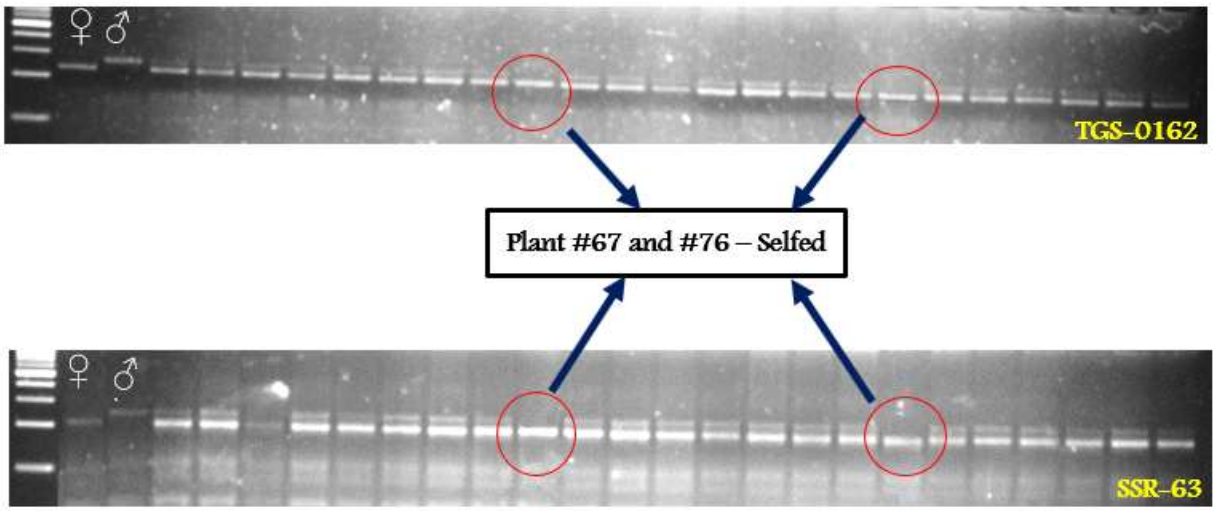

Figure 6a: Genetic purity testing of tomato hybrid PH-8 using polymorphic markers

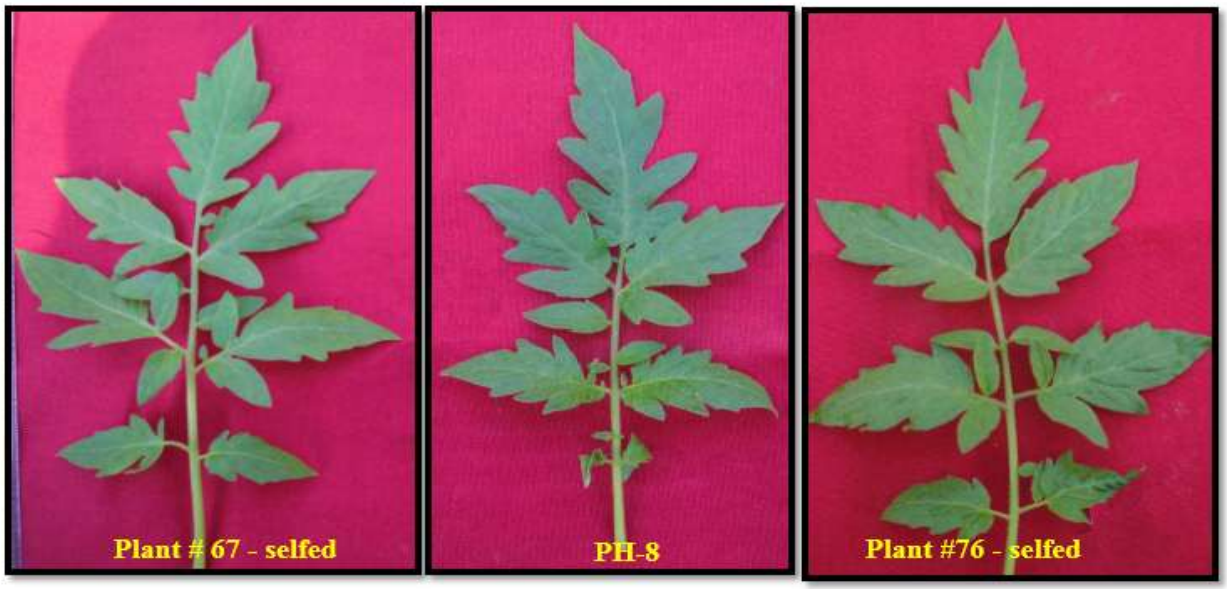

Figure 6b: Visual differences for leaf character between tomato hybrid PH-8 and selfed plants #67 & #76

Multiplexing is a cost saving approach wherein one can use 2-3 molecular markers in a single reaction with negligible addition to the cost of conducting the experiment. Multiplexing is more efficient in identifying a hybrid from other hybrids and also to identify an offtype from a lot of single hybrid. An attempt has been made to multiplex two markers (SSR-212-1 and TGS-0162) to identify PH-1. The marker combination was highly specific to identify PH-1 from rest of the four hybrids included in the present study. Nanda Kumar et al[15] proposed the utility and sufficiency of single marker for hybrid purity testing. However, in the present study we used of more than one molecular marker for hybrid purity testing and accurate determination of offtypes from the selfed plants (plant #129 of PH-1).

Reports from Corbet et al[7] and Bredemeijer et al[4] state that the gene diversity values of the tomato STMS markers range from 0.01 to 0.70 which is low compared to compared to the values of other crops. Also, the number of alleles per locus ranged from 2 to 8 with an average of 4.7 in tomato. Thus, usefulness of more than one marker in hybrid purity testing is demonstrated in the present study to identify an off type, selfed or male parent admixture which is as effective as grow-out-test. Thus, it is always recommended to use more than one marker in assessing the hybridity of plants under question as reported by Yashitola et al[20] and Sundaram et al[18]. Considering the innate disadvantages of grow-out-test, the marker based approach for hybrid purity testing can be recommended by optimizing the sample size, cheaper DNA extraction protocols and standardized multiplexing techniques[17].

Conclusion

Looking at the innate disadvantages of the conventional grow-out-test, the present study was undertaken to identify various molecular markers for distinguishing all the four tomato hybrids and their parental lines. Of the 132 SSR markers, five markers viz. SLM-7, SLM-53, TGS-0162,

SSR-63 and SSR-212 were polymorphic among the respective parental lines of four studied tomato hybrids. These five polymorphic markers using individually or in combination thereof distinguished the tomato hybrids from one another, and can be used as distinct finger prints.

Multiplexing PCR was standardized with two markers (SSR-212-1 and TGS-0162) for fast track identification of PH-1. These five polymorphic markers using individually or in combination there of, distinguished the tomato hybrids from one another and can be used as distinct finger prints. Molecular markers have the potential to distinguish all the four tomato hybrids and their respective parental lines during seed and seedling stages itself, and also to spot offtypes and/or selfed plant as a fast track, cost-effective (labour saving) and easy alternative to conventional grow-out-test.

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