

Molecular Characterization of Cultivated and Wild Genotypes of *Punica granatum* L. (Pomegranate) by Using SSR Marker

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ABSTRACT

The genetic diversity among 20 pomegranate genotypes including cultivated varieties and wild germplasm by using simple sequence repeats (SSR) markers. Plant genomic DNA was isolated using the modified CTAB method. A total of 17 SSR markers were screened across the twenty selected pomegranate germplasm to understand their diversity pattern at a molecular level. Out of these twelve were found to be polymorphic and five were monomorphic. These polymorphic primers have generated 29 SSR marker alleles with an average number of 1.71 alleles per locus. The maximum number of alleles was observed for twelve markers with two alleles each. Polymorphic information content (PIC) values ranged from 0.12 to 0.38 with an average of 0.29 per marker. The observed heterozygosity value ranged from 0.12 to 0.50, with a mean value of 0.36. DARwin software was used to study the phylogenetic relationship among the selected germplasm from the scored data. Neighbor-joining cluster analysis was given three separate clusters. All the wild accessions were grouped into cluster I, while cultivated varieties in cluster II. Single accession (Ruby) was formed a unique cluster. PgSSR33, PgSSR16, and PgSSR25 marker were found highly polymorphic, can be efficiently used in future pomegranate breeding programs.

Key-words: DARwin software, PgSSR25 marker, *Punica granatum*, Polymorphic information content (PIC)

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to Lythraceae family and is widely cultivated in tropical and subtropical regions of the world. It is a highly valued delicious edible fruit crop known for its nutritional and medicinal properties. Apart from commercial cultivation, pomegranate is also cultivated for its ornamental usages^[1]. The pomegranate tree has a wide geographical distribution that spreads from Iran to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean regions of Asia, Africa and Europe^[1]. India is the world's leading country in pomegranate production. The cultivation of pomegranate has remarkably increased by more than ten folds within a short span of two decades covering an

area of 1.32 lakh hectares with the production of 13.45 lakh tonnes and productivity of 10.3 tonnes/hectare^[2].

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs) are repeating sequences of 2–5 base pairs of DNA. It is a type of Variable Number Tandem Repeat (VNTR). Microsatellites are typically co-dominant. They are used as molecular markers in STR analysis, for kinship, population, and other studies. They can also be used for studies of gene duplication or deletion, marker-assisted selection, and fingerprinting. Simple Sequence Repeats (SSR) markers have successfully proved to be a powerful tool for assessing genetic variation and establishing phylogenetic relationships in many plant species, due to their high polymorphism, abundance and co-dominance inheritance. A simple sequence repeat is an important tool for genetic variation identification of germplasm^[3,4]. SSR marker has some merits such a quickness, simplicity, rich polymorphism and stability, thus being widely applied in genetic diversity analysis, molecular map construction and gene mapping^[4,5], construction of fingerprints^[4], genetic purity test^[4], analysis of germplasm diversity^[4,6], utilization of heterosis,

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especially in identification of species with closer genetic relationship.

Although information on morphological and physiological variability among pomegranate germplasm are well documented but a few studies based on molecular markers have been performed to characterize pomegranate genotype at a molecular level so as to better understand population structure, avoid duplications and effectively utilize available germplasm for targeted breeding.

MATERIALS AND METHODS

The present study carried on titled “Molecular characterization of pomegranate (*Punica granatum* L.) by using SSR markers” was carried out for six month duration at Department of Plant Biotechnology, Lokmangal College of Agricultural Biotechnology, Wadala and Department of Plant Molecular Biology and Biotechnology, ICAR’s National Research Centre on pomegranate, Solapur, India.

Plant Materials- The experimental materials comprising twenty genotypes of pomegranate for present investigation were collected from Field Gene Banks of ICAR-NRCP, Solapur, India. Ten genotypes viz Ruby, Jyoti, Ganesh, Gulesha red, Bhagawa, Super Bhagawa, Dholka, Jodhpur collection, Kandhari, Kabuli yellow were cultivated and ten genotypes viz IC-318762, Kalpitya, IC-318733, IC-1182, IC-318734, IC-318724, ACC-8, IC-318793, IC-318716, ACC-6 were wild.

DNA Extraction- Genomic DNA was isolated from fresh leaves of each of 10 cultivated and 10 wild varieties of pomegranate following CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method given by Murray and Thompson [7] and later modified by Saghai-Marooof *et al.* [8] and Doyle and Doyle [9].

Simple Sequence Repeats (SSRs)- The SSRs analysis was done following the procedure was given by Singh *et al.* [10] with minor modifications. In all 17 microsatellite marker obtained from Himedia were used. The PCR reactions consisted of 1X Taq buffer, 13.5 µl sterile DDH₂O, 1.5 mM MgCl₂, 2.5 mM dNTP, 10 pmol Primer (FP&RP), 1U Taq DNA Polymerase and 20 ng DNA for 40 cycles. The cyclic condition consisted of 94°C for 40 sec, 55–65°C (Melting Temp) for 1 min and 72°C for 2 min. Amplified product was separated on 2.5% agarose gel.

Statistical Analysis- Data was scored for computer analysis on the basis of the presence or absence of the PCR products. If a product was present in a genotype, it was designated as ‘1’ and if absent; it was designated as ‘0’. The data generated by SSR loci were analyzed with the software DARwin 6.0. The PIC values were calculated with formula $PIC=1-\sum p_i^2$ (where p_i = Frequency of the i^{th} allele, where $i=1$ to $i=n$) given by Smith *et al.* [11].

RESULTS AND DISCUSSION

DNA Isolation- The Genomic DNA of good quality and quantity for all 20 germplasms were isolated. The Plant genomic DNA of all leaf samples were isolated using modified CTAB method and tested for its purity by using the gel electrophoresis [9] (Fig. 1). Total DNA yield of the selected plant material was ranged from 67.63 ng/µl to 584.50 ng/µl. The highest concentration of DNA was obtained in IC-318793. The A260/A280 ratio was in the range of 1.61 to 1.93, which indicated the purity of the genomic DNA obtained using our modified CTAB method and in-significant/ low levels of proteins and polysaccharide contamination [12].

Simple Sequence Repeat (SSR)- SSRs are the markers of choice in crop improvement programmes with more specificity, high reproducibility, multi-allelism, high polymorphic, more frequent and co-dominant nature, have been used in many types of genetic analyses such as the construction of linkage maps, diversity assessment of germplasm, and identification of molecular markers for marker-assisted selection [13-16]. Seventeen SSRs were amplified to analyze the genetic variation among 20 different genotypes of pomegranate at a molecular level. Five out of 17 SSRs tested could not be exploited due to (i) Ambiguities in allele assignment, (ii) Excessive stutter bands, and (iii) Poor quality of amplification. The remaining 12 SSRs produced allelic polymorphism at 24 loci. The results were presented in Table 1.

The 12 SSR primers earmarked for final analysis amplified 24 alleles of size varying from 100 to 300 bp. The 17 primers (both monomorphic and polymorphic) have generated 29 SSR Marker alleles with 1.71 average no. of alleles for each marker. Primer no. PgSSR7, PgSSR21, PgSSR24, PgSSR40 and PgSSR55 were monomorphic with single allele. Polymorphic primers have generated 24 SSR marker alleles. The average no. of alleles for each polymorphic marker was 2.0.

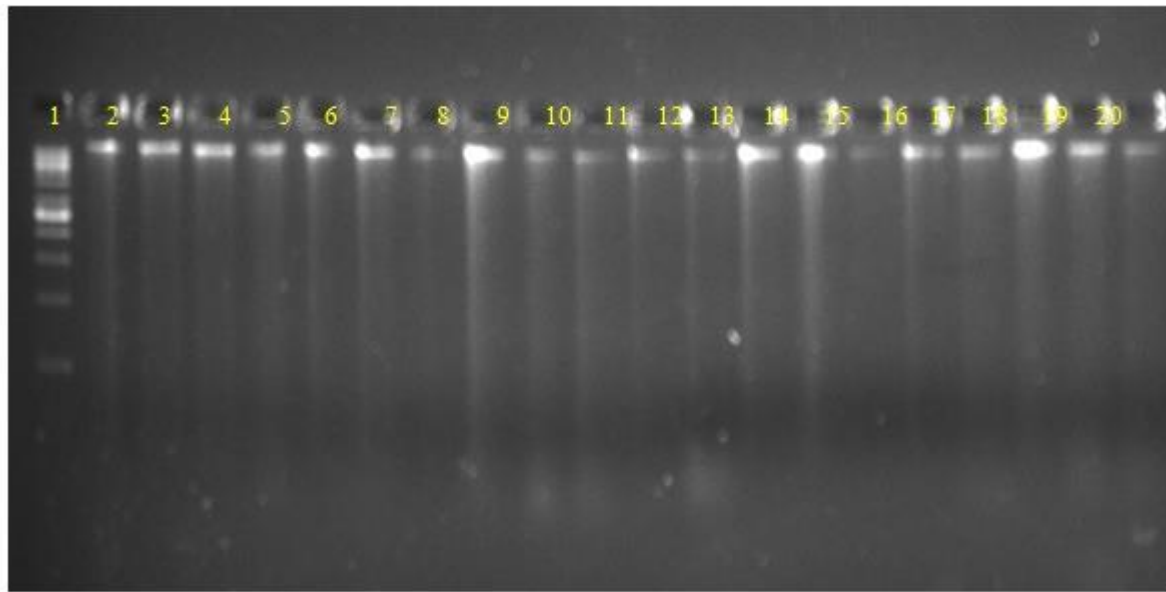


Fig. 1: Genomic DNA of good quality and quantity for all 20 pomegranate germplasm
L= Ladder (100 bp)

Germplasm (1- Ruby; 2- Jyoti; 3- Ganesh; 4- Gulesha red; 5- Bhagawa; 6- Super Bhagawa; 7- Dholka; 8- Jodhpur collection; 9- Kandhari; 10- Kabuli yellow; 11- IC-318762; 12- Kalpitya; 13- IC-318733; 14- IC-1182; 15- IC-318734; 16- IC-318724; 17- ACC-8; 18- IC-318793; 19- IC-318716; 20- ACC-6)

The maximum number of alleles was observed in primer no. PgSSR6, PgSSR8, PgSSR16, PgSSR17, PgSSR19, PgSSR22, PgSSR23, PgSSR25, PgSSR26, PgSSR30, PgSSR33 and PgSSR38 were polymorphic with bi-allelic (Fig. 2 & Fig. 3). A similar value was also reported in watermelons with 2.0 alleles per locus ^[17] and 2.46 in pigeon pea ^[16] but it was too lower than in the other crops like aromatic rice (3.3) ^[18], grapes (4.6) ^[19]. Polymorphic information content (PIC) values ranged from 0.12 to 0.38 with an average of 0.29 per marker (Table 1), which was similar to findings of Noormohammadi *et al.* ^[20], and slightly higher than those of Hasnaoui *et al.* ^[21]. The observed heterozygosity value was ranged from 0.12 to 0.50, with the mean value of 0.36. Among twelve polymorphic markers, PgSSR16, PgSSR25 and PgSSR33 were found to be very informative and highly polymorphic. These informative markers could be in future crop breeding programmes to aid in the marker-assisted selection of desirable genotypes.

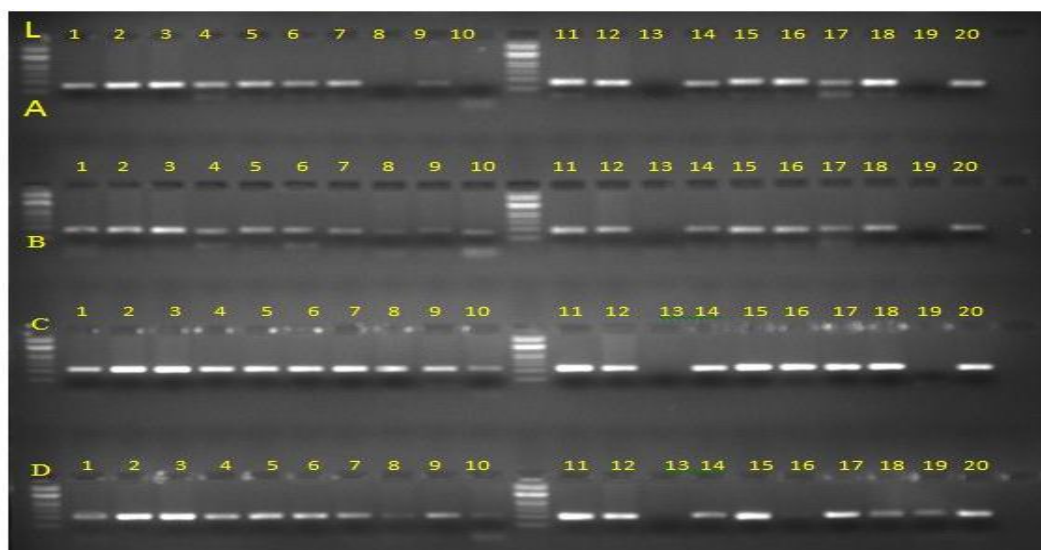
In consonance to the present finding Singh *et al.* ^[10] have also reported similar results while characterizing 88 genotypes of pomegranate by using 44 SSR markers of different crop species origin and also similar to findings of Sainjare *et al.* ^[22] studied a set of 12 simple sequence repeat (SSR) markers to evaluate the genetic diversity of 11 pomegranate cultivars.

The dendrogram based on UPGMA analysis grouped 20 genotypes in three major clusters (Cluster I- Wild types, Cluster II- Cultivated, and Cluster III- Solitary cluster with single genotype). Cluster I consisted of 12 genotypes, cluster II with 7 genotypes and cluster III was the solitary cluster with only one cultivated genotype (Ruby). Maximum genetic dissimilarity (0.44) was observed between IC-318733 and Jyoti as well as 'IC-318733' and 'Jodhpur collection' among all the genotypes. As Ruby variety is known to be developed from a complex hybridization programme, molecularly it has been separated in a separate cluster due to complexity in the genomic content (Fig. 4).

Jaccard's pair-wise dissimilarity similarity coefficient values for 20 different genotypes were calculated using DARwin 6.0 and were presented in Table 2. The genetic dissimilarities ranged from 0.00 to 44.00. The clustering analysis was well supported by principle component analysis (PCA). The first two axes of PCA with positive Eigen values accounted for 75.85% of the total variations, respectively (Fig. 5). The first axis has accounted for 59.71%, whereas the second axis covered 16.14% of the variance.

Table 1: Allele number, Heterozygosity value and PIC values of Polymorphic SSR loci in pomegranate genotypes

S. No.	Name of primer	Primer Sequence 5'-3'	No. of Marker alleles	Het	PIC
1	PgSSR6	ATTCAGCAGATTTTCAGGTC-F GATGAGGTGTGAGTTTGATG-R	2	0.426	0.3353
2	PgSSR8	ACCGACACACAAACCCGC-F AGGAGAGGTGGAGGAGGAT-R	2	0.4012	0.3207
3	PgSSR16	TTCCTTCGCTTTCACATC-F CCCGATCATTAAATCCACAAA-R	2	0.4983	0.3741
4	PgSSR17	GATGGCGAAGTGTGTCCTCT-F TTGGGACTGTGTTGACTGCT-R	2	0.4297	0.3374
5	PgSSR19	ATCTCTCATCTCTGCTTCCC-F GCACACTTTCCTCCCTATGT-R	2	0.4444	0.3457
6	PgSSR22	CCTCATGTCAGATTGTTGG-F GTTATGAGAGGGAGGCAGGA-R	2	0.1884	0.1706
7	PgSSR23	AGTTTGATCGACTGAGGAATG-F CACTCGAGAAGCTCTGTGAA-R	2	0.3878	0.3126
8	PgSSR25	TAATAAGCTGCCCCGAAGTC-F CGGTGATGTCCCTATTGGAG-R	2	0.4995	0.3748
9	PgSSR26	ATTCGTGCTCTGTGCCTCT-F GTGTTGGGAAGAAGAACGGAAAA-R	2	0.1244	0.1167
10	PgSSR30	TCCGACGATATAATCCCAAT-F ATTGCTTTCTTTTGCACCTC-R	2	0.255	0.2225
11	PgSSR33	ACCACCCACATAAATACTTC-F TTGAATACGCCTGTTGTTCT-R	2	0.5	0.375
12	PgSSR38	CCTTCACCTCCCCACATAGA-F TCGACCGTTCATCTCTTTC-R	2	0.18	0.1638

**A= PgSSR17, B= PgSSR19, C= PgSSR21, D= PgSSR23**

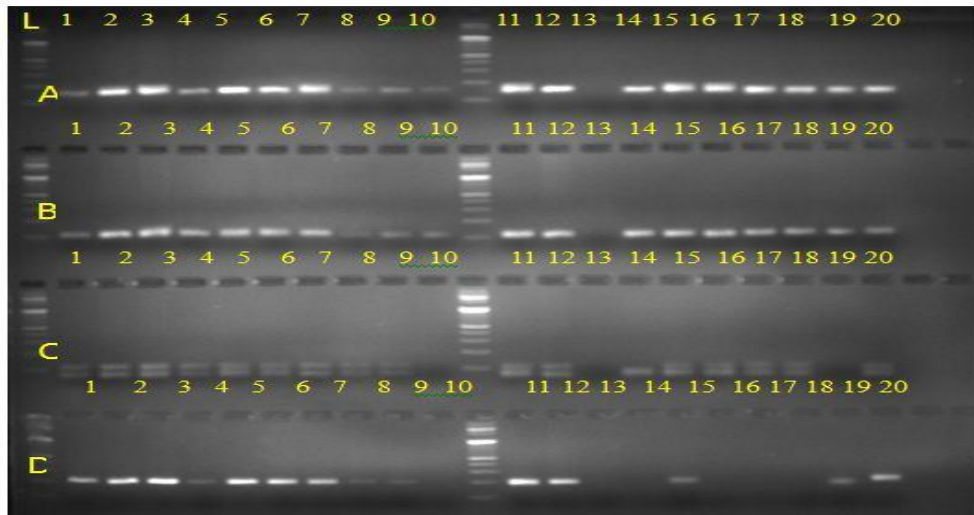
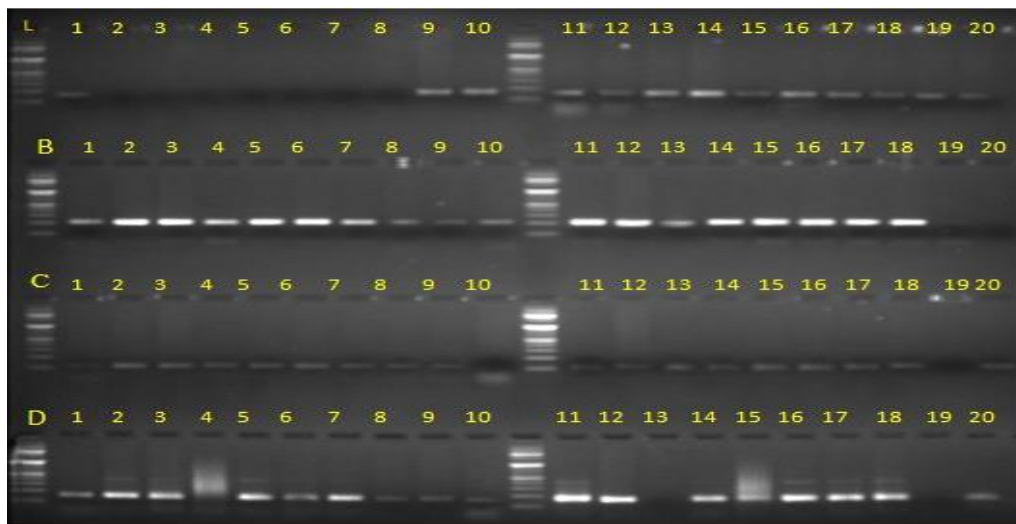
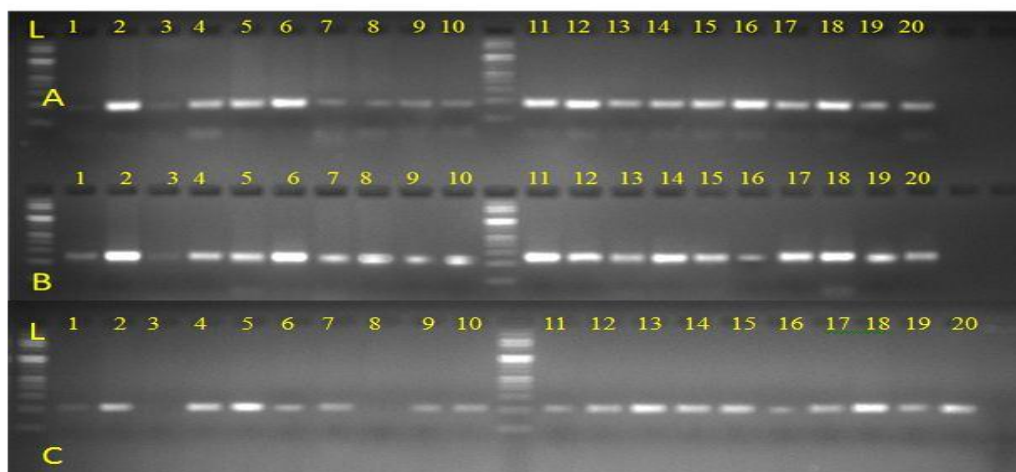


Fig. 2: SSR Patterns of different pomegranate genotypes

1-Ruby; 2-Jyoti; 3-Ganesh; 4-Guleshared; 5-Bhagawa; 6-Super Bhagawa; 7-Dholka; 8-Jodhpur collection; 9-Kandhari; 10-Kabuli yellow; 11-IC-318762; 12-Kalpitya; 13-IC-318733; 14-IC-1182; 15-IC-318734; 16-IC-318724; 17-ACC-8; 18-IC-318793; 19-IC-318716; 20-ACC-6
L= Ladder, A= PgSSR22, B= PgSSR24, C= PgSSR25, D= PgSSR26



A= PgSSR6 , B= PgSSR17, C= PgSSR8, D= PgSSR16



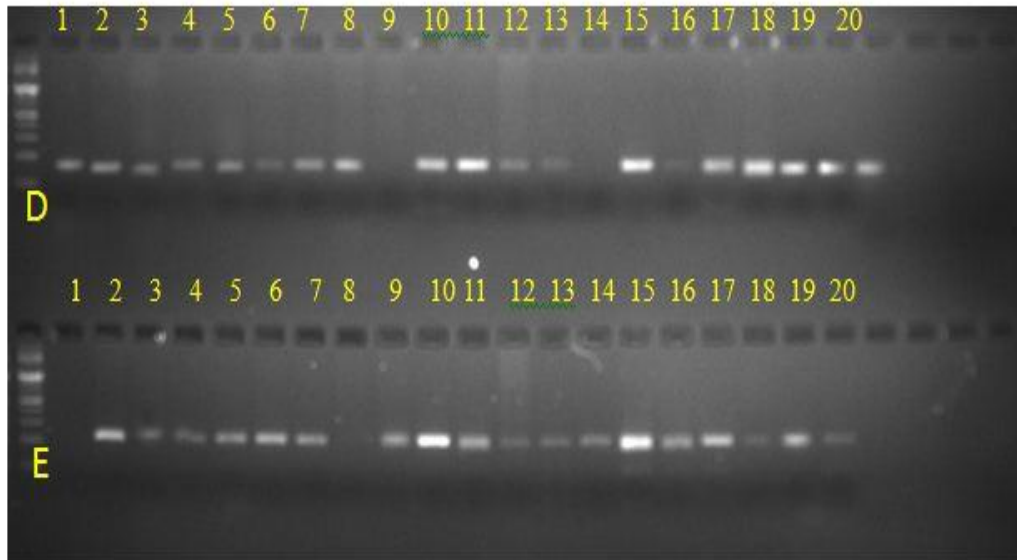


Fig. 3: SSR patterns of different pomegranate genotypes

1-Ruby; 2-Jyoti; 3-Ganesh; 4-Guleshared; 5-Bhagawa; 6-Super Bhagawa; 7-Dholka; 8-Jodhpur collection; 9-Kandhari; 10-Kabuli yellow; 11-IC-318762; 12-Kalpitya; 13-IC-318733; 14-IC-1182; 15-IC-318734; 16-IC-318724; 17-ACC-8; 18-IC-318793; 19-IC-318716; 20-ACC-6)

L=Ladder, A= PgSSR38 , B= PgSSR40, C= PgSSR43, D= PgSSR30 , E= PgSSR33

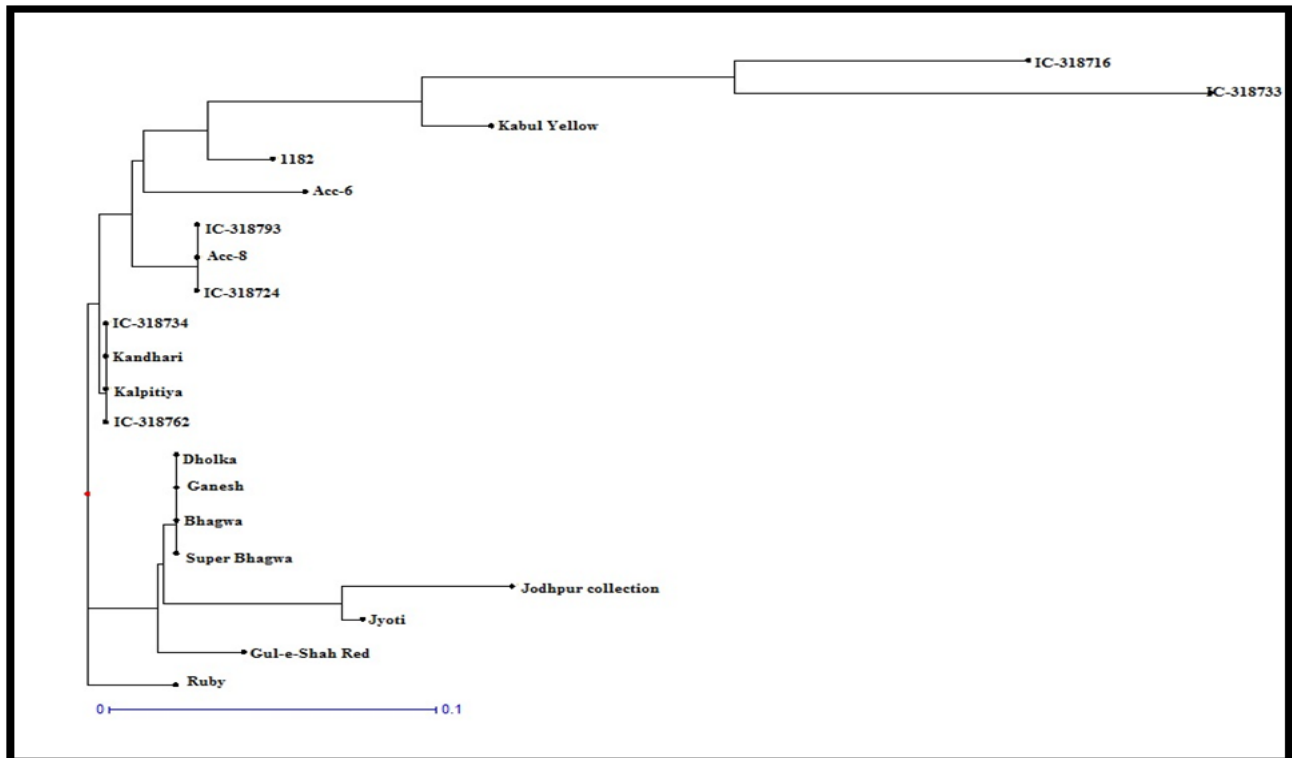


Fig. 4: Dendrogram showing clustering of twenty pomegranate genotypes constructed using UPGMA based on Jaccard's similarity coefficient obtained from SSR primers

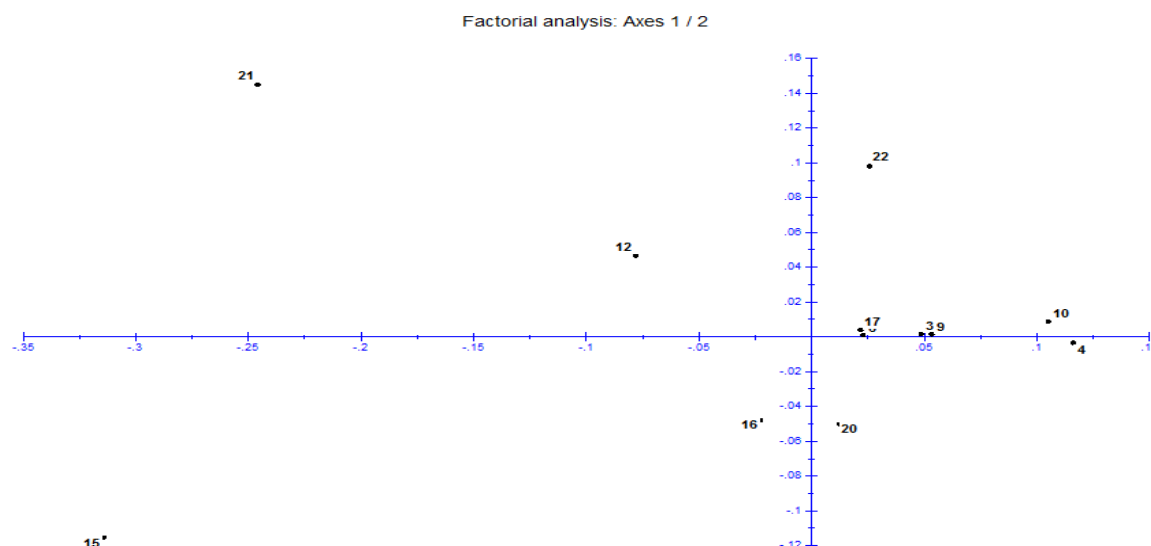


Fig. 5: Principal Component Analysis (PCA) of twenty selected pomegranate germplasm using DARwin 6.0 version

Table 2: Estimate of genetic distance between twenty pomegranate genotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1	0																				
2	0.12	0																			
3	0.06	0.06	0																		
4	0.09	0.09	0.03	0																	
5	0.06	0.06	0	0.03	0																
6	0.06	0.06	0	0.03	0	0															
7	0.06	0.06	0	0.03	0	0	0														
8	0.12	0.06	0.12	0.15	0.12	0.12	0.12	0													
9	0.03	0.09	0.03	0.06	0.03	0.03	0.03	0.15	0												
10	0.15	0.21	0.15	0.18	0.15	0.15	0.15	0.21	0.12	0											
11	0.03	0.09	0.03	0.06	0.03	0.03	0.03	0.15	0	0.12	0										
12	0.03	0.09	0.03	0.06	0.03	0.03	0.03	0.15	0	0.12	0	0									
13	0.03	0.44	0.38	0.35	0.38	0.38	0.38	0.44	0.35	0.29	0.35	0.35	0								
14	0.09	0.15	0.09	0.11	0.09	0.09	0.09	0.21	0.06	0.12	0.06	0.06	0.29	0							
15	0.03	0.09	0.03	0.06	0.03	0.03	0.03	0.15	0	0.12	0	0	0.35	0.06	0						
16	0.06	0.12	0.06	0.09	0.06	0.06	0.06	0.18	0.03	0.15	0.03	0.03	0.32	0.03	0.03	0					
17	0.06	0.12	0.06	0.09	0.06	0.06	0.06	0.18	0.03	0.15	0.03	0.03	0.32	0.03	0.03	0	0				
18	0.06	0.12	0.06	0.09	0.06	0.06	0.06	0.18	0.03	0.15	0.03	0.03	0.32	0.03	0.03	0	0	0			
19	0.32	0.38	0.32	0.29	0.32	0.32	0.32	0.38	0.29	0.18	0.29	0.29	0.24	0.29	0.29	0.32	0.32	0.32	0		
20	0.09	0.15	0.09	0.12	0.09	0.09	0.09	0.21	0.06	0.18	0.06	0.06	0.41	0.12	0.06	0.09	0.09	0.09	0.2	0	

Label: 1- Ruby; 2- Jyoti; 3- Ganesh; 4- Guleshared; 5- Bhagawa; 6- Super Bhagawa; 7- Dholka; 8- Jodhpur collection; 9- Kandhari; 10- Kabuli yellow; 11- IC-318762; 12- Kalpitya; 13- IC-318733; 14- IC-1182; 15- IC-318734; 16- IC- 318724; 17- ACC-8; 18- IC-318793; 19-IC- 318716; 20- ACC-6

CONCLUSIONS

Based on 17 SSR marker analyses, 20 different genotypes of pomegranate were grouped in three major clusters. The genetic dissimilarity ranged from 0.00 to 0.44. Maximum genetic dissimilarity (0.44) was observed between IC-318733 and Jyoti as well as 'IC-318733' and 'Jodhpur collection' among all the genotypes so here we can be concluded that these genotypes were more favorable for the breeding program. Three markers namely PgSSR16, PgSSR25 and PgSSR33 were identified as highly polymorphic markers, which can be efficiently used in future pomegranate breeding.

The present work focused to study genetic diversity and phylogenetic relationship between diverse pomegranate genotypes. Highly informative markers could be used in future crop breeding programmes to aid in marker assisted selection of desirable genotypes and this study reconfirmed SSR as a powerful marker tool to study a wide variety of pomegranate genotypes.

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CONTRIBUTION OF AUTHORS

Mahajan Sagar R: Concept, Data collection, Design of the work, Data collection, Data analysis and work interpretation, article drafting, revision of the article.

Mahajan Vaishali: Data Analysis and Interpretation for the work, Drafting of the article.

Bhosale SS: Experimental work design, data collection and data analysis.

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