Trends in Horticulture 2024, 7(1), 3491.
https://doi.org/10.24294/th.v7i1.3491

Article

True hybridity analysis using genome-wide hypervariable SSR markers in pomegranate

Prakash Patil[1,*], Dhinesh Babu Karuppannan[1], Shivani Jamma[1], Nripendra Singh[1], N Manjunatha[1], Ashutosh Murkute[2], Rajiv Marathe[1]

[1] ICAR-National Research Centre on Pomegranate (NRCP), Solapur 413255, India
[2] ICAR-Central Citrus Research Institute (CCIR), Nagpur 440033, India
*** Corresponding author:** Prakash Patil, patilbt@gmail.com

CITATION

Patil P, Karuppannan DB, Jamma S, et al. True hybridity analysis using genome-wide hypervariable SSR markers in pomegranate. Trends in Horticulture. 2024; 7(1): 3491. https://doi.org/10.24294/th.v7i1.3491

ARTICLE INFO

Received: 1 December 2023
Accepted: 3 January 2024
Available online: 4 February 2024

ABSTRACT: A total of 25 SSR primers were screened on 37 putative F_1s derived from the five different crosses. Identified cross specific highly informative SSRs primers, i.e., 14 for the first cross, 10 for the second, 12 for the third and 6 each for fourth and fifth crosses. For the first cross Bhagwa × Daru 17, four primers (HvSSRT_375, NRCP_SSR9, NRCP_SSR12 and NRCP_SSR92) were found to be highly informative with higher 100% hybrid purity index (HPI), PIC (~0.52), and observed heterozygosity (*Ho*, range 0.87–0.93) values, and two F_1s namely H_1 and H_2 were found to be highly heterotic with a heterozygosity index (HI) of 92.85%. Similarly, for Bhagwa × Nana, three primers (HvSSRT_375, HvSSRT_605 and NRCP_SSR19) had higher HPI (70%–100%), PIC (0.52–0.69), and *Ho* (0.75–0.33) values, and three F_1s H_1, H_2, and H_4 had 70% (HI). For Bhagwa × IC318712, four SSRs (HvSSRT_254, HvSSRT_348, HvSSRT_826 and NRCP_SSR95) had higher *Ho* (~0.83), HPI (100%) and PIC (~0.52) values, and four F_1s H_2, H_7, H_9, and H_{10} showed 91.66% (HI). For Bhagwa × Nayana, HvSSRT_605, HvSSRT_826, and HvSSRT_432, and for Ganesh × Nayana, HVSSRT_375, HVSSRT_605, and HvSSRT_826 were found informative. These markers will be highly useful in developing maps of populations.

Keywords: diversity; hybrid purity; heterozygosity; pomegranate; SSR markers; hypervariable

1. Introduction

Pomegranate (*Punica granatum* L.) is an important perennial fruit crop in the world and is widely cultivated in tropical and subtropical regions of Southeast Asia, Iran, China, Japan, India, the West Indies, USA (California), and Tropical America [1,2]. Being a super fruit, it has many health benefits for humans because of its diverse range of phytochemical contents such as gallotannins, ellagic acid, terpenoids, antioxidants, flavonoids, and alkaloids in its leaves, flowers, arils, seeds, rind, bark, and roots [3–6]. Globally, India ranks first with respect to pomegranate area 2.83 lakh ha and production of 30.86 lakh million tonnes in 2020 [7]. The concurrent breeding effort over the period through selections from the natural genetic variants and hybridization followed by selection has resulted in the development and release of only a few improved pomegranate varieties in India [8]. The expected productivity is still low and has no match with the productivity consistently achieved by some of the other pomegranate-producing countries like Israel, Spain, the USA, Turkey, etc. This could be mainly due to losses encountered through various biotic, i.e., bacterial and fungal diseases and insect damage, and abiotic stress-induced physiological disorders. Therefore, the currently deployment of modern genomic tools holds great promise to

address all these challenges. In this context, molecular markers represent the prerequisite in pomegranate for accelerating breeding programs through Marker assisted selection (MAS).

Since pomegranate has a high juvenile period, conventional breeding through hybridization followed by selection takes much time, and it mainly relies on the phenotypic selection of the best performing F_1 progenies as well as that of parents. This method may not be very suitable for verifying the authenticity of the progeny generated from crosses due to the possibility of outcrossing or self-pollination during hybridization. Therefore, accurate identification of progeny from crosses is critical to breeders for the integrity of a durable breeding program. Thus, the use of genomic tools that are currently in pomegranate form, such as genome-wide simple sequence repeats (gSSRs), microRNA-based simple sequence repeats (miRNA-SSRs) [9–11], expressed sequence tag-based simple sequence repeats (EST-SSRs) [3,12] holds great potential. Breeders can precisely select and grow true F_1 plants by eliminating plants that are not true heterozygotes by deploying SSR markers. The use of markers will definitely save on the costs of growing the unwanted plants during conventional methods of breeding and population development. The reliability of detecting true heterozygote plants based on SSR markers is much higher than that using conventional phenotypic markers. It is mainly because of the strong influence of environmental factors on phenotypic markers.

Genetic improvement in any crop mainly relies on mapping genes and quantitative trait loci (QTLs) through genome-wide markers, for which the development of biparental populations like F_1, and F_2 populations through hybridization is very essential. For instance, in pomegranate F_2 populations have been found very useful for mapping QTLs for fruit quality traits and plant size [13], and fine mapping of black peel colour [14] using single nucleotide polymorphism (SNP) markers. Similarly, F_2 populations have been found most suitable in other fruit crops for mapping QTLs for fruit flesh browning in apples [15] for chilling tolerance in apricots and peaches [16,17]. Therefore, quick generation of F_2 populations needs molecular assays based on SSR markers, which can quickly and accurately screen F_1 progenies coming from different crosses to identify highly heterotic true hybrids for the development of F_2 mapping populations in pomegranate. Currently, plenty of valuable genomic resources for downstream breeding applications have been developed in pomegranate. Due to the non-availability of mapping populations, trait mapping through QTL analysis and genomics enabled pomegranate improvement is still in its initial phase. The use of MAS will increase selection efficiency, reduce the breeding cycle, and enhance variety development in trees [18]. SSR markers have already been found to be very effective in hybridity analysis in many tree species, like Cassava [19], Grape [20], Mango [21], Citrus [22], and Eucalyptus [23]. Presently, considerable numbers of genome-wide SSR markers have already been reported for genetic studies in pomegranate [24,25]. Therefore, the aim of this study was to demonstrate the efficacy of these newly designed pomegranate specific hypervariable SSR markers for the identification of highly heterotic true hybrids for different crosses in the development of F_2 mapping populations.

2. Materials and methods

2.1 Plant materials and DNA extraction

The six parental lines, i.e., Bhagwa, Daru 17, Nana, IC318712, Ganesh and Nayana were used earlier for crossing that have many contracting traits. The Bhagwa is the very important commercial variety of India with soft seeded type and has red rind, red arils with high export value. Ganesh is also important commercial variety of India with soft seeded type and has yellowish rind, pink arils with sweet taste. Nayana is the introduced variety from Sri Lanka and has red rind, red arils with soft seeded type. Daru-17 is the wild type, which grows naturally under Himalayan regions and has vigorous growth, free from bacterial blight disease the sour type of arils was used for anardana purpose by the tribal community. Nana is also a wild type of pomegranate, dwarf in stature, ornamental type with small sized flowers, fruits, which has sour type of arils with higher acidity and hard seeded texture. IC318712 is the indigenous collection of Daru type with small-medium size, free from fruit cracking. A total of 37 putative F_1s derived from the five different crosses involving cultivars and wild parental combinations, i.e., Bhagwa × Daru 17 (13 putative F_1s), Bhagwa × Nana (10 F_1s) and Bhagwa × IC318712 (10 F_1s), Bhagwa × Nayana (2 F_1s) and Ganesh × Nayana (2 F_1s) were used for the true hybridity testing (**Table 1**). The putative F_1 plants were confirmed as true hybrids based on SSR alleles originated from both the parents. Modified CTAB method was followed [26], to extract high quality genomic DNA from the fresh leaf samples. After determining quality and concentration of genomic DNA on 0.8% agarose gel electrophoresis in comparison to uncut Lambda, templates DNA were diluted (10 ng/µL) for polymerase chain reactions (PCR).

Table 1. Details of five crosses along with their putative hybrids used in this study.

Sl. No	Cross	No of putative F_1s tested
1.	Bhagwa × Daru-17	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13
2.	Bhagwa × Nana	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10
3.	Bhagwa × IC 318712	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10
4.	Bhagwa × Nayana	H1 & H2
5.	Ganesh × Nayana	H1 & H2
	Total	37

2.2. SSR assay

Initially, amplifications were checked for 33 hypervariable SSR primers on parental lines using NRCP_SSR and HvSSRT series that are reported recently [10,25]. Later 25 primers were shortlisted for hybridity analyses which are showing clear amplifications with polymorphism (**Table 2**). All the PCR was carried out in 10 µL reaction mixture (1 µL template DNA, 0.17 µL forward primer, 0.17 µL reverse primers (10 pmol/µL), 4 µL 2X PCR mix (Himedia, India), and 4.66 nuclease-free water) on a thermocycler (Prime-96™, Himedia, India). PCR program was run with following conditions: 94 ℃ for 5 min, then 36 cycles at 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 2 min, and a final extension at 72 ℃ for 7 min. Amplified fragments

were separated on 3% metaphor agarose gels stained with 0.5 µg/mL ethidium bromide and 1X TBE running buffer at 130 V for 4 h. Finally gels were visualized and photographed using gel documentation system (Vilbert Dourmet, France).

Table 2. Sequence details of 25 hypervariable SSR primers used for hybridity analysis.

Sl. No	SSR name	Motif	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Chromosome location	Reference
1	HvSSRT_222	(ATT)14	CACCACCCTTACATACGG	CCGATCAACATCCTTTCCC	214	2	Patil et al. [25]
2	HvSSRT_254	(AAT)14	GTCAGTGTTTGTTGTGTCC	ACTTCATTCTCACACCACC	197	2	
3	HvSSRT_348	(TC)23	ATGCCAAAAATTAGCGAGC	CTTATAAGTGAGCTCCCCC	219	3	
4	HvSSRT_375	(GA)26	TTGGGGAAGAGAAGAAAGC	GATAGCATTAATGGGCTTCC	151	3	
5	HvSSRT_431	(AC)21	TCTAGCTTTCGTGATCAGC	CATTGAGAGCCCAATTTGG	175	4	
6	HvSSRT_432	(CA)23	TTGGAACCATCGTCTATGC	CTTTAATGTTGCACTCCGG	147	4	
7	HvSSRT_437	(GAA)16	AAAGAGCTGACAACCTTCC	AACTTGTTCTGCTTCCTCC	134	4	
8	HvSSRT_592	(TC)31	TGCTCATATACATGCCAGC	ATTACACATGAAAGTGCGG	208	5	
9	HvSSRT_605	(AAG)17	TTTGGTCGGAAAGTACTGC	GGAGTCACTCGTAGTTTGG	156	5	
10	HvSSRT_628	(TC)24	AAATTCCAGTCCCCTTTGG	TTCGGTGTGTAATTAGGCC	207	5	
11	HvSSRT_695	(TC)23	TTTCGGAAATCCCAATCGG	TAGATAGGGTCGACGAAGG	210	6	
12	HvSSRT_746	(TCT)14	TGAATTTGATGGGGATGGG	CTCTATATCTGCCATGGTGG	178	7	
13	HvSSRT_812	(TAC)19	TCCTTGGTTTGCTTCTAGC	TCAGCAGCAGTAGTAGTGG	159	7	
14	HvSSRT_823	(TA)21	TCATTTAGCTAAGGCGACC	TGGCTGATTGCAATCTTCC	170	7	
15	HvSSRT_826	(AT)23	TCGTGTTTCCTTTCCTGC	AGCTGGTACAACAAACTCC	144	8	
16	HvSSRT_827	(TATCC)9	GCTGTGTGTTTGATCATCG	AAGCTCGAACCTTTTTCCC	186	8	
17	NRCP_SSR8	(AT)15	GTATTTCAGAGCGGTTGG	GTAATTGCACAAGGCACC	297	Un	Patil et al. [10]
18	NRCP_SSR9	(AT)18	AATCCGTACTATGCAGCG	CGAAATCAAGGTCGTCAG	228	Un	
19	NRCP_SSR10	(TA)14	AAAGGAGAGAAGCGCAGT	ACCCTAAAGTGGACCGAT	213	Un	
20	NRCP_SSR12	(TA)17	AGTGGGTGTAGCAGAGGTCA	GGTCGTCCAAGGGATTTG	201	Un	
21	NRCP_SSR19	(TC)22	CGATCTCCGGTTACATCA	AGGGTATAGGTCACGTTGG	271	Un	
22	NRCP_SSR31	(TA)13	GCATGACACGAGTGGATT	GTGAGTTCATTAGGCGCA	171	Un	
23	NRCP_SSR92	(TA)16	CGATTGTTGGGGAACTCT	GACAAGACCCAGCCCTTAT	296	Un	
24	NRCP_SSR93	(AT)13	AAGCCAAAGCAACTCACC	GGAGATCCCTGGTTCATAAG	294	Un	
25	NRCP_SSR95	(AT)15	ACCAAAGGTGATGGCGTA	GAGCACGCAGATATTCGAC	295	Un	

Note * Un: Unknown location.

2.3. Data analysis

The DNA amplification pattern obtained for hypervariable SSR markers were scored manually for allele sizes. For the analysis, only bands that were reproducible and polymorphic were examined. NTSYS-pc version 2.02 h was used to analyze SSR data sets and compute pair-wise Jaccard's similarity coefficients using the

SIMQUAL option. The resulting similarity matrices were used to construct dendrograms using the UPGMA (Unweighted Pair Group Method with Arithmetic Average) algorithm and SAHN clustering. The software GenAlEx 6.5 was used to determine genetic diversity parameters including number of alleles (Na) and effective alleles per locus (Ne), observed (*Ho*) and expected heterozygosity (*He*), and polymorphic information content (PIC). Using the same software Principal coordinate analysis (PCoA) based on the standardized covariance of genetic distances was also performed. The hybridity measures like the hybrid purity index (HPI) of each marker locus [27] and heterozygosity of hybrid plants for each cross was identified using formulas given below [28].

Hybrid Purity Index (%) = Observed No of True hybrids (Alleles of both parents)/Total No of putative F_1s tested × 100

Heterozygosity of True hybrid (%) = No of heterozygous SSR loci observed in the hybrid plant/Total No of SSR markers screened × 100

3. Results

For parental polymorphism study, we deployed 33 hypervariable SSRs for initial screening of which total 25 primers were selected based on their ability to show clear polymorphism for parental combinations on normal agarose gels (**Figure 1a–c**). Sixteen, out of 25 HvSSRT primers had known chromosome positions on Tunisia genome (Chms 2-8) and 9 NRCP-SSR primers had unknown position on Dabenzi genome. Further, for hybridity analysis we identified cross specific informative markers, i.e., a subset of 14 informative polymorphic SSRs for first cross (Bhagwa × Daru 17) having 13 putative F_1s, 10 markers for Bhagwa × Nana and 12 markers for Bhagwa × IC318712 crosses each having 10 putative F_1s, and six markers each for Bhagwa × Nayana and Ganesh × Nayana crosses having 2 putative F_1s each.

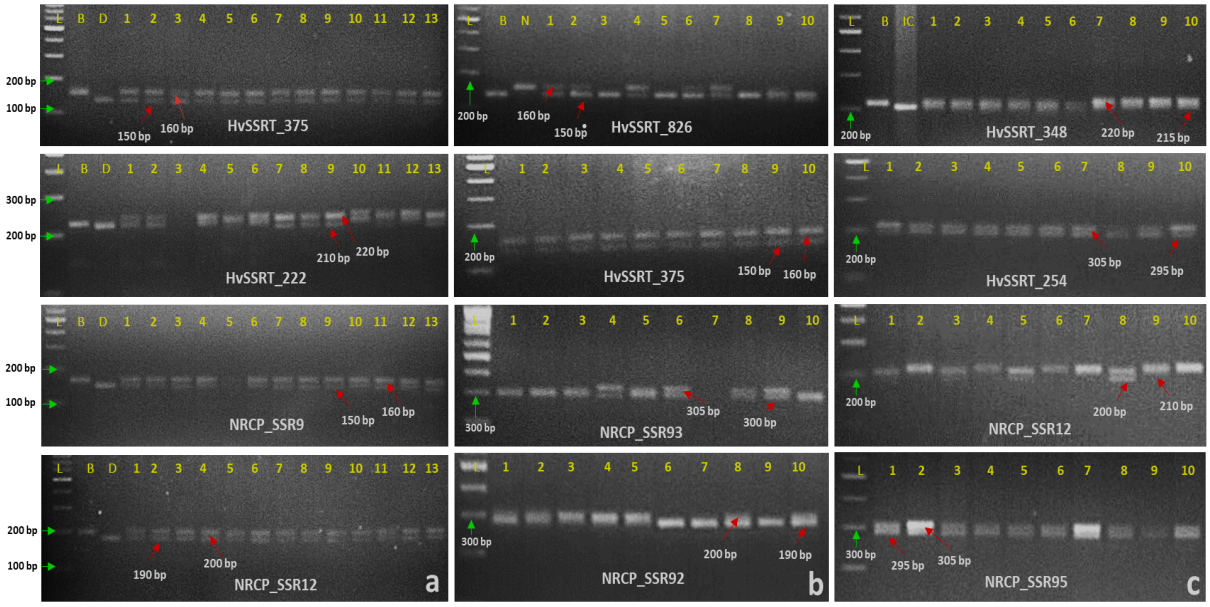

Figure 1. Profiling of HvSSRT and NRCP_SSR makers on parental lines and their hybrids, for cross **(a)** Bhagwa × Daru 17 (13 F_1 individuals) using HvSSRT_375, HvSSRT_222, NRCP_SSR9, NRCP_SSR12, **(b)** Bhagwa × Nana

(10 F_1s), using HvSSRT_826, HvSSRT_375, NRCP_SSR93, NRCP_SSR92, and **(c)** Bhagwa × IC318712 (10 F_1s) using HvSSRT_348, HvSSRT_254, NRCP_SSR12 and NRCP_SSR95 (where, L is 100 bp DNA ladder).

Based on results of genotyping experiments for different primer set on each F_1 populations, various marker parameters were calculated. For the first cross 14 polymorphic SSR loci produced a total of 31 alleles among the parents and their putative F_1s with an average of 2.21 alleles per locus. Interestingly, all these 14 markers showed higher *Ho* as compared to their *He* values (**Table 3**). With respect to HPI, four markers namely HvSSRT_375, NRCP_SSR9, NRCP_SSR12 and NRCP_SSR92 found highly informative (100%) with higher PIC (~0.52) and *Ho* (range 0.87–0.93) values.

For the second cross 10 polymorphic SSRs produced a total of 22 alleles across parents and their putative F_1s. The six primers namely HvSSRT_375, HvSSRT_826, HvSSRT_605, HvSSRT_823, NRCP_SSR19 and NRCP_SSR92, had higher *Ho* of which three markers namely HvSSRT_375, HvSSRT_605 and NRCP_SSR19 found highly informative for hybridity analysis and having higher HPI (70%–100%), PIC (0.52–0.69) and *Ho* (0.75–0.33) values.

In the third cross, 12 polymorphic SSRs generated a total of 27 alleles for parents and their putative F_1s, of which except two SSRs (HvSSRT_628 and HvSSRT_812) all other markers were found informative. The four SSRs namely HvSSRT_254, HvSSRT_348, HvSSRT_826 and NRCP_SSR95, had higher *Ho* (~0.83), HPI (100%) and PIC (~0.52) values suggesting highly useful for hybridity analysis.

However, our main objective of this study was to identify highly heterotic F_1 hybrid plants for developing trait specific F_2 mapping populations with high variability for the traits. Therefore, based on the percent heterozygosity values calculated for true hybrids of each cross, H1 and H2 showed ~92.85% heterozygosity in the first population (**Table 4**). Similarly, hybrids H1, H2 and H4 showed highest heterozygosity (~70%) in the second population. Lastly, H2, H7, H9 and H10 revealed heterozygosity of ~91.66% in the third population.

Cluster analysis and PCoA was performed for three mapping populations separately based on SSR marker data (**Figures 2** and **3**). As a result, in all the three populations we found clear discrimination between parental lines and their respective hybrids. Interestingly, all the three wild male parents (Daru 17, Nana and ICP318712) and their corresponding female parent (Bhagwa) used were found more diverse in comparison to their hybrid progenies (**Tables S1–S3**). With respect to clustering of hybrid progenies, for the second cross single group with high level of diversity 47% (0.53–0.93) was observed among 10 hybrids, followed by first cross having two subgroups with moderate level of diversity 38% (similarity range 0.62–1) for 13 hybrids, and third cross with moderate level of diversity 37% (0.63–1) for 10 hybrids having single group with H1 as a more distinct hybrid. Factorial analysis was also performed for three populations to understand the genetic relationships. The results of PCoA were found well corroborated with that of grouping as observed using UPGMA based clustering (**Figure 3**). Furthermore in the PCoA, it was interesting to note that axis 1 explained higher variation (>47%) by separating the parental lines

used in the three crosses. However, axis 2 explained lesser variations (>20%) with distributions of hybrids in single or separate groups.

Table 3. Markers statistics and hybrid purity index of SSR loci for three populations.

Population	Primers	Na	Ne	*Ho*	*He*	PIC	HPI (%)
Bhagwa × Daru 17	HvSSRT_375	2.000	2.000	0.867	0.500	0.517	100
	HvSSRT_222	2.000	1.960	0.714	0.490	0.508	77
	HvSSRT_695	3.000	2.778	0.800	0.640	0.662	46
	HvSSRT_432	2.000	1.724	0.467	0.420	0.434	54
	HvSSRT_823	2.000	1.867	0.600	0.464	0.480	69
	HvSSRT_746	2.000	1.923	0.667	0.480	0.497	77
	HvSSRT_437	3.000	1.685	0.467	0.407	0.421	46
	HvSSRT_605	3.000	2.866	0.933	0.651	0.674	69
	NRCP_SSR9	2.000	2.000	0.867	0.500	0.517	100
	NRCP_SSR12	2.000	1.991	0.933	0.498	0.515	100
	NRCP_SSR92	2.000	2.000	0.867	0.500	0.517	100
	NRCP_SSR8	2.000	1.991	0.667	0.498	0.515	77
	NRCP_SSR10	2.000	1.990	0.786	0.497	0.516	85
	NRCP_SSR31	2.000	1.923	0.667	0.480	0.497	77
	Mean	**31 (2.214)**	**2.050**	**0.736**	**0.502**	**0.519**	
Bhagwa × Nana	HvSSRT_375	2.000	2.000	0.833	0.500	0.522	100
	HvSSRT_826	2.000	1.882	0.583	0.469	0.489	70
	HvSSRT_605	3.000	2.969	0.833	0.663	0.692	70
	HvSSRT_823	3.000	2.160	0.667	0.537	0.569	50
	HvSSRT_695	2.000	1.492	0.083	0.330	0.344	10
	HvSSRT_812	2.000	1.492	0.250	0.330	0.344	30
	HvSSRT_827	2.000	1.800	0.333	0.444	0.464	40
	NRCP_SSR19	2.000	1.986	0.750	0.497	0.518	90
	NRCP_SSR92	2.000	1.882	0.583	0.469	0.489	70
	NRCP_SSR93	2.000	1.541	0.273	0.351	0.368	40
	Mean	**22 (2.200)**	**1.921**	**0.519**	**0.459**	**0.480**	
Bhagwa × IC318712	HvSSRT_254	2.000	2.000	0.833	0.500	0.522	100
	HvSSRT_222	2.000	2.000	0.818	0.500	0.524	90
	HvSSRT_348	2.000	2.000	0.833	0.500	0.522	100
	HvSSRT_628	2.000	1.385	0.000	0.278	0.290	90
	HvSSRT_431	2.000	1.986	0.750	0.497	0.518	90
	HvSSRT_592	2.000	1.976	0.667	0.494	0.523	60
	HvSSRT_826	2.000	2.000	0.833	0.500	0.522	100
	HvSSRT_605	3.000	2.504	0.833	0.601	0.627	90
	HvSSRT_812	2.000	1.492	0.250	0.330	0.344	30
	HvSSRT_695	3.000	2.796	0.917	0.642	0.670	50
	NRCP_SSR95	2.000	2.000	0.833	0.500	0.522	100
	NRCP_SSR12	3.000	2.743	0.917	0.635	0.663	60
	Mean	**27 (2.250)**	**2.073**	**0.707**	**0.498**	**0.520**	

Note* Na-Number of alleles; Ne-Number of effective alleles; *Ho*-observed heterozygosity, *He*-expected heterozygosity; PIC-Polymorphism information content; HP-Hybrid purity percentage.

Table 4. Heterozygosity percent for hybrid progenies of three populations based co-dominant SSR loci.

Cross	Total number of markers screened	Number of heterozygous SSR loci observed in hybrid individuals and Heterozygosity percentage (%)												
		H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**	**H13**
Bhagwa × Daru 17	14	13 (92.85)	13 (92.85)	10 (71.42)	10 (71.42	10 (71.42)	11 (78.57)	10 (71.42)	10 (71.42)	12 (85.72)	11 (78.57)	7 (50)	13 (92.85)	11 (78.57)
Bhagwa × Nana	10	7 (70)	7 (70)	4 (40)	7 (70)	5 (50)	5 (50)	5 (50)	5 (50)	6 (60)	6 (60)			
Bhagwa × IC318712	12	7 (58.33)	11 (91.66)	9 (75)	10 (83.33)	8 (66.66)	9 (75)	11 (91.66)	9 (75)	11 (91.66)	11 (91.66)			

Note* H1–H13: cross specific putative F_1s used; values in bracket are Heterozygosity percentage (%).

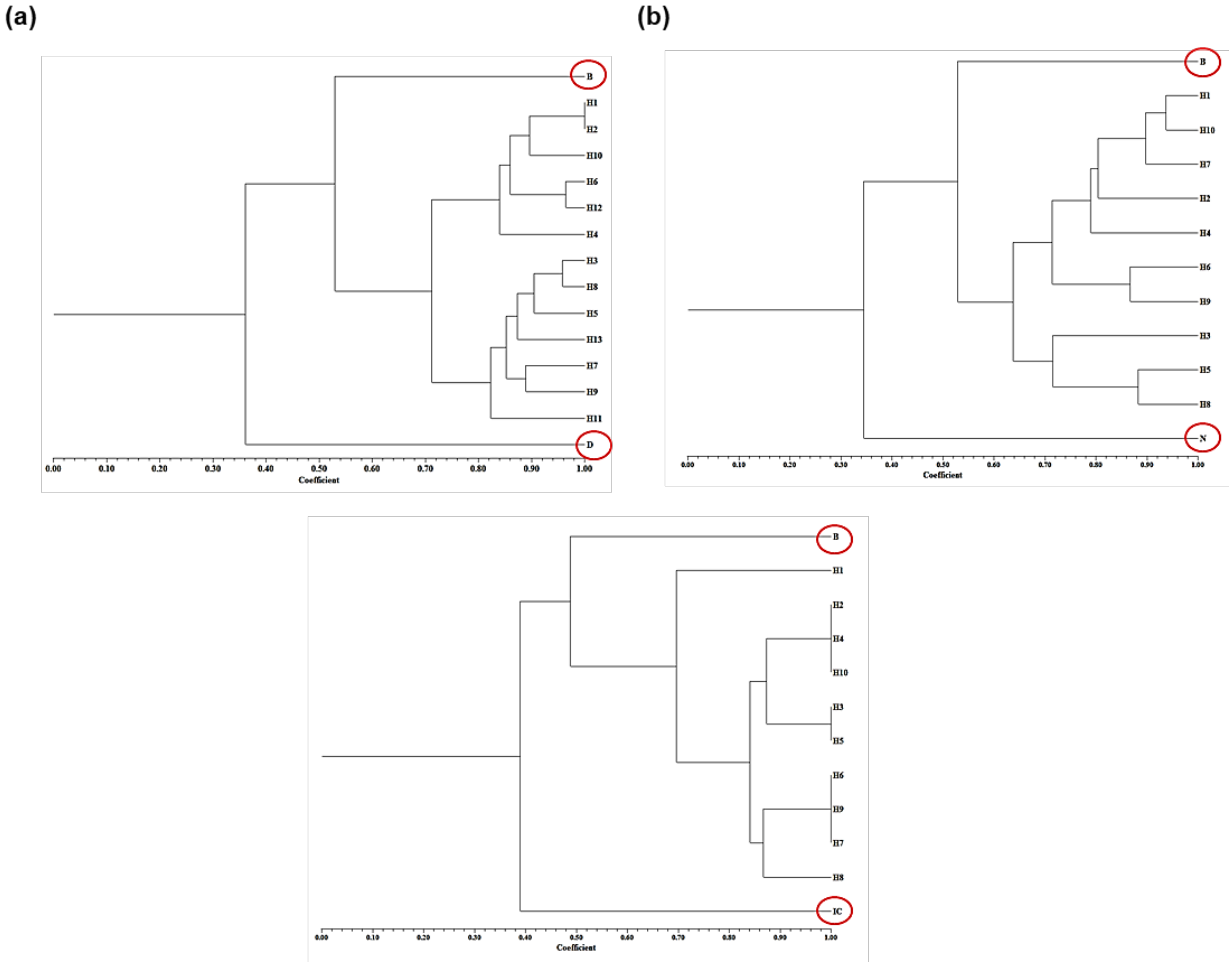

Figure 2. UPGMA dendrogram showing the ability of SSR markers to differentiate hybrids from their parental lines for crosses **(a)** Bhagwa × Daru 17, **(b)** Bhagwa × Nana, and **(c)** Bhagwa × IC318712.

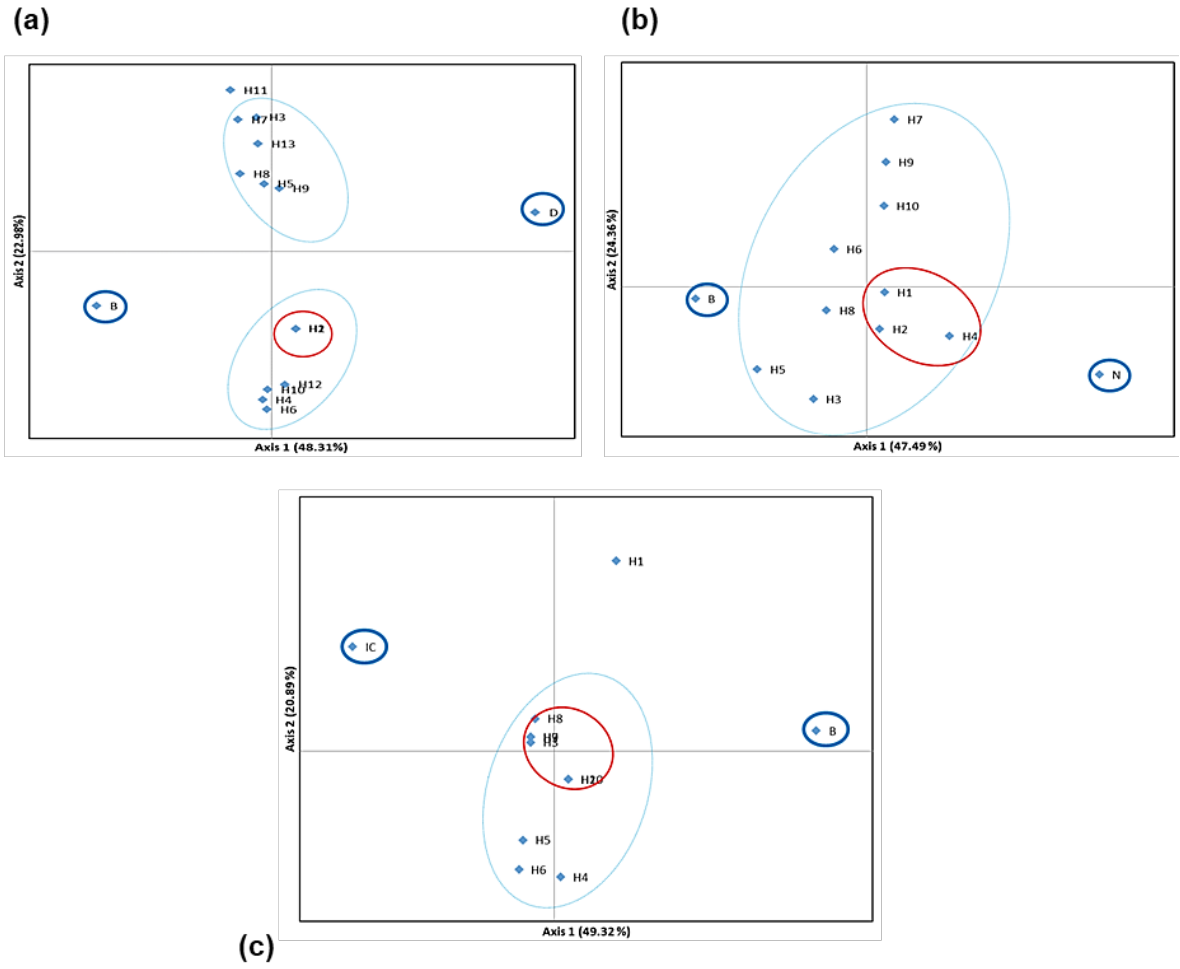

Figure 3. Principal coordinates analysis based on hypervariable SSR markers among the hybrids and their parental genotypes for a cross **(a)** Bhagwa × Daru 17, **(b)** Bhagwa × Nana, and **(c)** Bhagwa × IC318712. Dark blue circles represent parents, light blue circles for number of hybrid groups formed and red circle represents highly heterotic F_1 hybrid plants selected for selfing.

4. Discussion

The integration of conventional and marker-assisted breeding methods in order to combine phenotyping and genotyping information promotes the development of novel horticultural crop varieties [29]. Due to long duration of tree species, unlike field crops Grow Out Tests (GOTs) for morphological assessment cannot be practiced. Therefore, DNA markers such as SSRs could be of excellent choice for such varietal characterization. Due to codominant inheritance, multi-allelic nature, widely distributed throughout genome, easily scored with automation, microsatellite markers have proven useful in plant breeding [21]. Wherein, hybrid purity assessment in trees using SSR marker technologies has already been found highly useful, which would support the requirements of intellectual property for varietal registration through plant protection of varieties and farmer's right authority of India (PPV & FRA) [23]. Jalikop et al. [30] reported in pomegranate following hybridization larger segregation takes place in the F_1 progeny itself and selection for the desired genotypes can be practiced in this generations. However, he has also

reported occurrence of more recombinants in F_2 than in F_1 and breeders should look new recombinants in F_2 population [31]. Further, in pomegranate F_2 populations were also found most suitable for mapping genes/QTLs. Harel-Beja et al. [13] identified 25 QTLs for fruit traits and plant size traits using F_2 population derived from Nana × Black. Trainin et al. [14] fine mapped *ANR* gene responsible for black peel colour phenotype in pomegranate using F_2 population. Therefore, while developing F_2 populations identification of true to type F_1 hybrid plants resulting from cross-pollination is very crucial.

In our study we deployed 25 hypervariable SSRs for hybridity analysis and identified cross specific informative markers, i.e., 14 SSRs for first cross (Bhagwa × Daru 17), 10 markers for Bhagwa × Nana and 12 markers for Bhagwa × IC318712 crosses and 6 markers each for Bhagwa × Nayana and Ganesh × Nayana crosses. Similarly, hybridity analysis was performed in progenies obtained from different cross combinations of seven mango parental genotypes using 13 SSR primers and identified 3 informative primer pairs, i.e., LMMA 11, ESTD 9 and ESTD 10 to confirm the true hybridity in the mango progenies [21]. In our study we used hypervariable SSRs, which are known to show high polymorphism on metaphor agarose gels as previously we demonstrated through diversity study in pomegranate genotypes [10,11,25]. In contrast to the morphological method of hybrid identification which usually takes 3–4 years, SSR analysis takes only 1–3 months depending on the number of populations or sample size in trees [18]. Hypervariable SSRs were already found highly useful for hybrid authentication or purity assessment and parentage confirmation in other crops [32].

Further based on the higher marker parameter values HPI, PIC and *Ho*, we identified four informative SSR markers, i.e., HvSSRT_375, NRCP_SSR9, NRCP_SSR12 and NRCP_SSR92 for the first cross, three markers HvSSRT_375, HvSSRT_605 and NRCP_SSR19 for second cross and four HvSSRT_254, HvSSRT_348, HvSSRT_826 and NRCP_SSR95 for third cross that are highly useful. Similarly, in Bhagwa × Nayana and Ganesh × Nayana crosses three SSRs each, i.e., HvSSRT_605, HvSSRT_826 and HvSSRT_432, and HvSSRT_375, HvSSRT_605 and HvSSRT_826 respectively were identified for hybridity analysis. Earlier, Corley [33] also stated that the number of markers necessary for hybrid confirmation might be fewer than five due to the high polymorphism of microsatellites at the locus level. Similarly, best SSR markers were selected for hybrid purity analysis in many studies based on the higher *Ho*, HPI and PIC values [18,23,34]. SSR markers were used to determine the purity of a eucalyptus hybrid (*Eucalyptus camaldulensis* × *Eucalyptus tereticornis*) and confirmed parentage of hybrids with an 85–100 hybrid purity index [23].

Since, our ultimate aim of this study was to identify highly heterotic cross specific true F1s, therefore based on the percent heterozygosity values we identified H1 and H2 individuals as highly heterotic in the first population, H1, H2 and H4 in the second population, and H2, H7, H9, H10 in the third population. These hybrid plants can be used for selfing to develop trait specific high resolution F_2 mapping population in pomegranate to identify superior donors, varieties and or to map genes/QTLs. Haynes et al. [28] reported higher levels of heterozygozity within an accession should lead to more phenotypic variation, resulting in both superior and

inferior phenotypes. However, lower levels of heterozygosity within an accession, on the other hand, would result in less phenotypic diversity and a narrower range of phenotypic values. In forest trees there are many reports where inter-specific hybrids are generated and then highly heterotic individuals have been selected for mass multiplication through clonal propagation [35]. Since, our ultimate aim of this study was to identify highly heterotic cross specific true F_1s, therefore based on the percent heterozygosity values we identified H1 and H2 individuals as highly heterotic in the first population, H1, H2 and H4 in the second population, and H2, H7, H9, H10 in the third population.

Further, in order to understand genetic relationships between parents and their progenies, the cluster and factorial analysis was performed for each population. All the three populations showed clear cut discrimination, relationships between parental lines and their respective hybrids in the dendrograms. Nadeem et al. [36] also performed cluster analysis based on 10 polymorphic microsatellites markers for nine hip-bearing rose parents and their 22 F_1 hybrids, and found all the hybrid progeny sharing common bands with their parents. In our study the results of PCoA were well corroborated with that of grouping as observed in UPGMA based clustering for each population. Similarly, Ben Romdhane et al. [34] also carried out genetic purity analysis of four putative F_1 barley hybrids and their parents using 11 SSR markers, they found results of neighbor joining tree were well correlated with the factorial analysis.

Genetic linkage maps derived from mapping populations are the powerful tools for mapping genes for economically important traits. The saturated linkage maps developed could help in identification of effective markers that are linked to genes/QTLs for trait of interest to deploy in marker-assisted breeding program [13]. Therefore, development of trait specific F_2 populations are required to identify QTLs governing biotic, abiotic stress tolerance and fruit quality traits in pomegranate. For instance, in trees species such as in apple QTL for fruit flesh browning [15], and in apricot and peach QTL for chilling tolerance were identified using F_2 populations [16,17]. Similarly, in pomegranate 25 QTLs were identified for fruit traits using F_2 population derived from Nana × Black cross through SNP genotyping [13]. More recently, Trainin et al. [14] deployed SNP markers to map phenotype of “black” fruit colour using F_2 population; they discovered a few markers that were intimately connected to the “black” peel of pomegranate. Due to co-dominant allelic patterns, SSR markers were found highly informative for hybridity testing in many horticultural fruit crops. Our findings confirmed that the pomegranate specific hypervariable SSR markers deployed in this study are highly useful for gel based assay to confirm true hybridity. We assume such informative markers could speed up the process of development of new varieties in pomegranate by reducing the number of generations of selection through marker assisted selection.

5. Conclusion

In this study, we have offered highly informative cross-specific hypervariable SSR markers for hybridity analysis in pomegranate. We could also identify highly heterotic cross specific F_1 hybrid plants, which could help us in developing high

resolution F_2 mapping populations for mapping important genes or QTLs for fruit quality traits in pomegranate.

Supplementary materials: Table S1, **S2** and **S3** are the Jaccard's similarity matrices as obtained for F_1s of three biparental crosses (Bhagwa into Daru-17, Nana and IC318712) using SSR markers.

Author contributions: Conceptualization, PP and DBK; methodology, PP, DBK and SJ; investigation, PP, SJ and NS; data curation, PP and NM; writing-original draft preparation, PP, NM and NS; writing-review and editing, RM, AM and DK; supervision, RM. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are highly grateful to the Indian Council of Agricultural Research (ICAR), New Delhi for extended financial support through ICAR-National Research Centre on Pomegranate, Solapur under institute-funded project.

Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Conflict of interest: The authors declare no conflict of interest.

References

1. Holland D, Bar-Ya'akov I. Pomegranate: aspects concerning dynamics of health beneficial phytochemicals and therapeutic properties with respect to the tree cultivar and the environment. In: Yaniv Z, Dudai N (editors). Medicinal and Aromatic Plants of the Middle-East. Springer Netherlands; 2014. doi: 10.1007/978-94-017-9276-9
2. Bar-Ya'akov I, Tian L, Amir R, et al. Primary Metabolites, Anthocyanins, and Hydrolyzable Tannins in the Pomegranate Fruit. Frontiers in Plant Science. 2019; 10. doi: 10.3389/fpls.2019.00620
3. Ophir R, Sherman A, Rubinstein M, et al. Single-Nucleotide Polymorphism Markers from De-Novo Assembly of the Pomegranate Transcriptome Reveal Germplasm Genetic Diversity. PLoS ONE. 2014; 9(2): e88998. doi: 10.1371/journal.pone.0088998
4. Bellesia A, Verzelloni E, Tagliazucchi D. Pomegranate ellagitannins inhibit α-glucosidase activityin vitroand reduce starch digestibility under simulated gastro-intestinal conditions. International Journal of Food Sciences and Nutrition. 2014; 66(1): 85-92. doi: 10.3109/09637486.2014.953455
5. Aslan A, Can M, Boydak D. Anti-Oxidant effects of pomegranate juice on *Saccharomyces cerevisiae* cell growth. African Journal of Traditional, Complementary and Alternative Medicines. 2014; 11(4): 14. doi: 10.4314/ajtcam.v11i4.3
6. Ahmed MM, Samir ESA, El-Shehawi AM, et al. Anti-obesity effects of Taif and Egyptian pomegranates: molecular study. Bioscience, Biotechnology, and Biochemistry. 2015; 79(4): 598-609. doi: 10.1080/09168451.2014.982505
7. NHB. Available online: http://nhb.gov.in/PDFViwer.aspx?Enc=3ZOO8K5CzcdC/Yq6HcdIxC0U1kZZenFuNVXacDLxz28 (accessed on 20 August 2020).
8. Jalikop SH, Rawal RD, Kumar R. Exploitation of sub-temperate pomegranate Daru in breeding tropical varieties. Acta Horticulturae. 2005; (696): 107-112. doi: 10.17660/actahortic.2005.696.18
9. Ravishankar KV, Chaturvedi K, Puttaraju N, et al. Mining and characterization of SSRs from pomegranate (*Punica granatum* L.) by pyrosequencing. Plant Breeding. 2015; 134(2): 247-254. doi: 10.1111/pbr.12238
10. Patil PG, Singh NV, Parashuram S, et al. Genome-wide characterization and development of simple sequence repeat markers for genetic studies in pomegranate (*Punica granatum* L.). Trees. 2020; 34(4): 987-998. doi: 10.1007/s00468-020-01975-y
11. Patil PG, Singh NV, Parashuram S, et al. Genome wide identification, characterization and validation of novel miRNA-based SSR markers in pomegranate (*Punica granatum* L.). Physiology and Molecular Biology of Plants. 2020; 26(4): 683-696. doi: 10.1007/s12298-020-00790-6

12. Ono NN, Britton MT, Fass JN, et al. Exploring the Transcriptome Landscape of Pomegranate Fruit Peel for Natural Product Biosynthetic Gene and SSR Marker Discovery. Journal of Integrative Plant Biology. 2011; 53(10): 800-813. doi: 10.1111/j.1744-7909.2011.01073.x
13. Harel-Beja R, Sherman A, Rubinstein M, et al. A novel genetic map of pomegranate based on transcript markers enriched with QTLs for fruit quality traits. Tree Genetics and Genomes. 2015; 11(5). doi: 10.1007/s11295-015-0936-0
14. Trainin T, Harel-Beja R, Bar-Ya'akov I, et al. Fine Mapping of the "black" Peel Color in Pomegranate (*Punica granatum* L.) Strongly Suggests That a Mutation in the Anthocyanidin Reductase (*ANR*) Gene Is Responsible for the Trait. Frontiers in Plant Science. 2021; 12. doi: 10.3389/fpls.2021.642019
15. Di Guardo M, Tadiello A, Farneti B, et al. A Multidisciplinary Approach Providing New Insight into Fruit Flesh Browning Physiology in Apple (*Malus domestica* Borkh.). PLoS One. 2013; 8(10): e78004. doi: 10.1371/journal.pone.0078004
16. Olukolu BA, Trainin T, Fan S, et al. Genetic linkage mapping for molecular dissection of chilling requirement and budbreak in apricot (*Prunus armeniaca* L.). Genome. 2009; 52(10): 819-828. doi: 10.1139/g09-050
17. Fan S, Bielenberg DG, Zhebentyayeva TN, et al. Mapping quantitative trait loci associated with chilling requirement, heat requirement and bloom date in peach (*Prunus persica*). New Phytologist. 2009; 185(4): 917-930. doi: 10.1111/j.1469-8137.2009.03119.x
18. Okoye MN, Singh R, Uguru MI, et al. Application of microsatellite markers for hybrid verification and genetic analysis of oil palm (*Elaeis guineensis* Jacq.). Nigerian Journal of Biotechnology. 2021; 37(2): 1-12. doi: 10.4314/njb.v37i2.1
19. Vincent K, Robert K, Morag F, et al. Identification of F_1 Cassava (*Manihot esculenta* Crantz) Progeny Using Microsatellite Markers and Capillary Electrophoresis. American Journal of Plant Sciences. 2014; 5(1): 119-125. doi: 10.4236/ajps.2014.51015
20. Chi ZJ, Shan GY, Dong LZ, et al. Identification of the F1 hybrids of grape using SSR molecular markers. Journal of Shenyang Agricultural University. 2016; 47: 148-152.
21. Kumar K, Srivastav M, Singh S, et al. Ascertaining hybridity of progenies in mango (*Mangifera indica* L.) using microsatellite (SSR) markers. Journal of Agriculture and Ecology. 2016; 2(2): 1-10. doi: 10.53911/jae.2016.2201
22. Kaur K, Kumar K, et al. Allelic pattern of SSRs with high hybrid detection efficiency and inheritance of leaf petiole wing in interspecific Citrus crosses. Fruits. 2021; 76(1): 30-38. doi: 10.17660/th2021/76.1.4
23. Subashini V, Shanmugapriya A, Yasodha R. Hybrid purity assessment in Eucalyptus F1 hybrids using microsatellite markers. 3 Biotech. 2013; 4(4): 367-373. doi: 10.1007/s13205-013-0161-1
24. Patil PG, Jamma SM, Singh NV, et al. Assessment of genetic diversity and population structure in pomegranate (*Punica granatum* L.) using hypervariable SSR markers. Physiology and Molecular Biology of Plants. 2020; 26(6): 1249-1261. doi: 10.1007/s12298-020-00825-y
25. Patil PG, Singh NV, Bohra A, et al. Comprehensive Characterization and Validation of Chromosome-Specific Highly Polymorphic SSR Markers From Pomegranate (*Punica granatum* L.) cv. Tunisia Genome. Frontiers in Plant Science. 2021; 12. doi: 10.3389/fpls.2021.645055
26. Ravishankar KV, Anand L, Dinesh MR. Assessment of genetic relatedness among mango cultivars of India using RAPD markers. The Journal of Horticultural Science and Biotechnology. 2000; 75(2): 198-201. doi: 10.1080/14620316.2000.11511223
27. Bohra A, Dubey A, Saxena RK, et al. Analysis of BAC-end sequences (BESs) and development of BES-SSR markers for genetic mapping and hybrid purity assessment in pigeonpea (*Cajanus* spp.). BMC Plant Biology. 2011; 11(1). doi: 10.1186/1471-2229-11-56
28. Haynes KG, Zaki HEM, Christensen CT, et al. High Levels of Heterozygosity Found for 15 SSR Loci in *Solanum chacoense*. American Journal of Potato Research. 2017; 94(6): 638-646. doi: 10.1007/s12230-017-9602-4
29. Patella A, Scariolo F, Palumbo F, et al. Genetic Structure of Cultivated Varieties of Radicchio (*Cichorium intybus* L.): A Comparison between F_1 Hybrids and Synthetics. Plants. 2019; 8(7): 213. doi: 10.3390/plants8070213
30. Jalikop SH, Venugopalan R, Kumar R. Association of fruit traits and aril browning in pomegranate (*Punica granatum* L.). Euphytica. 2010; 174(1): 137-141. doi: 10.1007/s10681-010-0158-3
31. Jalikop SH, Kumar PS, Rawal RD, et al. Breeding pomegranate for fruit attributes and resistance to bacterial blight. Indian Journal of Horticulture. 2006; 63(4): 352-358.

32. Bohra A, Pareek S, Jha R, et al. Modern genomic tools for pigeonpea improvement: status and prospects. In: Varshney RK, Saxena RK, Jackson SA, et al. The Pigeonpea Genome. Springer International Publishing; 2017. doi: 10.1007/978-3-319-63797-6
33. Corley RHV. Illegitimacy in oil palm breeding—A review. Journal of Oil Palm Research 2005; 17: 64-69.
34. Ben Romdhane M, Riahi L, Jardak R, et al. Fingerprinting and genetic purity assessment of F_1 barley hybrids and their salt-tolerant parental lines using nSSR molecular markers. 3 Biotech. 2018; 8(1). doi: 10.1007/s13205-017-1080-3
35. Jansson S, Bhalerao R, Groover A, et al. Genetics and Genomics of Populus. Springer New York; 2010. doi: 10.1007/978-1-4419-1541-2
36. Nadeem M, Wang X, Akond M, et al. Hybrid identification, morphological evaluation and genetic diversity analysis of' Rosa x hybrid by SSR markers. Australian Journal of Crop Science 2014; 8(2): 183-190.