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Genetic diversity and hybridization potential of wild and commercial pomegranate populations for bacterial blight resistance

Rahul Sharma¹ , Som Dev Sharma², Vikas Kumar Sharma^{2*} , Monica Sharma³, Rajat Sharma⁴ and Shireen Khatri¹

*Correspondence:

Vikas Kumar Sharma
drvks007@rediffmail.com

¹Department of Fruit Science,
College of Horticulture, Dr YSP
University of Horticulture and
Forestry, Nauni, Solan, HP
173230, India

²Department of Fruit Science,
College of Horticulture and
Forestry, Dr YSP University of
Horticulture and Forestry, Neri,
Hamirpur, HP 177001, India

³Department of Plant Pathology,
College of Horticulture and
Forestry, YSP University of
Horticulture and Forestry, Neri,
Hamirpur, HP 177001, India

⁴Department of Engineering,
Durham University,
Durham DH1 3LE, UK

Abstract

Pomegranate (*Punica granatum* L.), valued for its nutritional and antioxidant properties, faces severe yield losses due to bacterial blight caused by *Xanthomonas citri* pv. *punicae*. Given the ecological resilience and genetic potential of wild populations, identifying resistant genotypes from indigenous germplasm is vital for sustainable breeding. This study assessed genetic diversity and structure among 23 pomegranate genotypes wild Daru accessions and commercial cultivars previously screened for blight resistance. Twenty simple sequence repeats markers yielded 1119 bands, with 1036 (92.58%) being polymorphic. Primers ESP05, ESP06, ESP13, and ESP14 exhibited 100% polymorphism. Polymorphism information content (PIC) values ranged from 0.08 to 0.460 (mean = 0.321), confirming moderate informativeness. Diversity indices revealed mean alleles per locus ($N_a = 2.14$), effective alleles ($N_e = 1.906$), and Shannon's index ($H = 0.627$). Commercial cultivars displayed greater diversity ($N_a = 3.00$; $H_e = 0.52$) and private alleles (0.60) than wild populations, particularly Wild KL (from Kullu) ($N_a = 1.45$; $H_e = 0.25$). Analysis of molecular variance revealed that 91% of the genetic variation observed is within populations. Pairwise population differentiation ranged from 0.091 to 0.289, indicating moderate to high differentiation, with gene flow values between 0.615 and 2.494. Principal coordinate analysis, Neighbor joining clustering, and STRUCTURE analysis ($\Delta K = 2$) revealed two major clusters: commercial and wild types, with signs of admixture. Unique resistance-associated bands were identified in wild genotypes SH-14, SH-16, SR-15, and SL-23, linked to traits like acidity, tannins, and anthocyanins. Wild genotypes SR-11, SH-16, SL-22, and SL-23, selected as male parents, were hybridized with cultivars Bhagwa, Kandhari Kabuli, and Phule Super Bhagwa. The Bhagwa $\times$ SH-16 cross showed highest fruit set (88.90%), and Bhagwa $\times$ SL-23 had highest retention (66.67%). These F_1 progenies offer a promising genetic base for developing bacterial blight-resistant pomegranate cultivars with improved horticultural traits.

Keywords Pomegranate, Bacterial blight, Resistance breeding, SSR markers, Hybridization, Population structure



1 Introduction

Pomegranates, prized for their health benefits and nutritional richness, are characterized by their round shape, tough skin, and vibrant red or pink colour. Pomegranate juice, rich in anthocyanins, has exceptional antioxidant power, surpassing that of red wine and green tea [15, 62]. Despite its profitability, pomegranate cultivation faces challenges such as long dry spells, a lack of resistant varieties and quality planting material, environmental stress, nutritional deficiencies, and physiological disorders [10, 14]. Additionally, biotic threats, including pests and diseases, further jeopardize cultivation. Diseases like leaf spots, canker, and soft rot, caused by various pathogens, are common concerns [3]. Among these, bacterial blight, caused by *Xanthomonas citri* pv. *punicae* (Xcp), is a major threat to pomegranate production in India [17, 48, 51]. Since 2002, frequent outbreaks of this disease have severely impacted Indian growers, resulting in significant losses in both yield and quality with rising disease incidence across all pomegranate-growing regions [47, 49, 59]. Similar issues are reported in other regions like South Africa, Turkey and Pakistan, where bacterial blight also significantly hampers pomegranate production [2, 18, 41]. It primarily affects above-ground plant parts, with symptoms appearing 6–7 days post-infection under favorable conditions, eventually leading to necrotic blighting.

To manage bacterial blight, farmers rely on costly chemical sprays, which increase production costs and raise environmental and residue concerns. At present, no pomegranate cultivar in the gene pool has demonstrated resistance to bacterial blight. Consequently, plant breeders are focused on developing disease-resistant varieties that remain stable under varying environmental conditions. The selection of elite genotypes based on morphological traits from naturally growing seedling populations has traditionally driven fruit culture, with hybridization-based varietal development emerging only in the early 1900s [53]. However, breeding in perennial tree fruits is challenging due to heterozygosity and long gestation cycles, with few programs receiving sustained financial and institutional support [23, 53]. Nonetheless, plant varieties with inherent, genetically determined disease resistance are key to effective disease management [31].

Breeding for disease resistance is a cost-effective strategy that reduces the need for fungicides and mitigates environmental pollution. Despite the deployment of chemical and cultural control measures, the absence of resistant cultivars in existing germplasm leaves commercial varieties highly susceptible to bacterial blight, underscoring the urgent need for genetic improvement strategies [46, 48]. In a comprehensive screening conducted at the Indian Institute of Horticultural Research (IIHR), Bengaluru, Priya et al. [43] evaluated 105 exotic accessions from the USDA and 66 cultivated Indian types, all of which displayed varying degrees of disease susceptibility. Notably, indigenous germplasm from India, particularly the Western Himalayas, harbours wild pomegranate (locally known as Daru), which demonstrates superior tolerance to abiotic stress, natural resistance to bacterial blight, and exceptional nutritional attributes [20, 32, 56]. Traits such as thicker pericarps, elevated antioxidants, and innate defense mechanisms position these genotypes as ideal candidates for resistance breeding [43, 51]. Harnessing this germplasm through advanced breeding techniques can significantly advance the development of disease-resistant pomegranate cultivars, contributing to more sustainable and resilient production systems.

In India, wild *Punica granatum* L. is found exclusively in the Western Himalayan region, covering Jammu and Kashmir, Himachal Pradesh, and Uttarakhand [32]. It

thrives in marginal hilly tracts of Solan, Mandi, Kangra, Shimla, and Chamba districts of Himachal Pradesh at elevations between 900 and 1800 m above sea level [8]. Wild pomegranates, widely distributed in the drier Himalayan regions, differ from cultivated varieties with smaller arils, thicker rinds, and higher acidity. While tropical pomegranates are evergreen with sweet fruits ideal for fresh consumption, Daru pomegranates despite their high acidity are commercially valuable for producing anardana, a souring agent used in condiments. These hardy deciduous trees have adapted over time, showing resilience to climate variations and pests through natural selection. Singh et al. [56] identified native genotypes closely related to cultivated pomegranate, with some demonstrating resistance to bacterial blight [22]. Priya et al. [43] also highlighted the potential of Daru for resisting bacterial blight through in vivo and in vitro screening. In our previous study, the wild genotypes (Daru) collected from Himalayan region also identified resistant source for bacterial blight [51]. Therefore, Daru (wild Himalayan pomegranate) holds significant potential for breeding programs aimed at incorporating disease-resistant genes into commercial pomegranate cultivars.

Wild pomegranate types can be crossed with cultivated varieties [20], making introgression feasible through DNA marker technology to enhance fruit quality and disease resistance. Compared to conventional breeding, Marker-Assisted Selection (MAS) offers significant advantages by facilitating the breeding process in horticultural crops. Molecular markers enable the identification of genes responsible for desired traits, as well as the assessment of genetic diversity and phylogenetic relationships within germplasm [36, 50, 53]. These insights help breeders develop disease-resistant plants. DNA-based markers, particularly sequence-based markers, are valuable tools in crop improvement programs [1, 9, 57]. Simple Sequence Repeats (SSRs), or microsatellites, produce codominant, multi-allelic, and reproducible bands upon amplification and have been widely utilized in *Punica* spp. [16, 25, 38, 45, 56, 60]. SSR markers are highly polymorphic and reproducible, making them effective for analyzing genetic diversity and population structure for implementing MAS programs [21, 34]. Integrating SSR markers in breeding programs can significantly advance the development of pomegranate cultivars with improved fruit quality and enhanced disease resistance [56].

The present study aimed to investigate the population genetic diversity and genetic structure of resistant wild *Punica granatum* L. populations and commercial cultivars using SSR marker analysis. This research sought to identify key genetic loci associated with disease resistance and assess their correlation with adaptive traits in diverse environmental conditions. Following the identification of potential resistant sources, hybridization was conducted for incorporation of resistance traits into commercial pomegranate cultivars (Fig. 1). The resulting F₁ hybrids, characterized for both resistance and fruit quality traits, will form the foundation for future crop improvement programs. These efforts aim to develop disease-resistant, high-yielding pomegranate cultivars with enhanced market value, thereby promoting sustainable horticultural practices and resilience against emerging pathogen threats.

2 Material and methods

Twenty-three genotypes of *Punica granatum* L., including wild accessions and commercial cultivars, were subjected to molecular analysis to assess genetic diversity and population structure. This included four wild populations from different districts of Himachal Pradesh

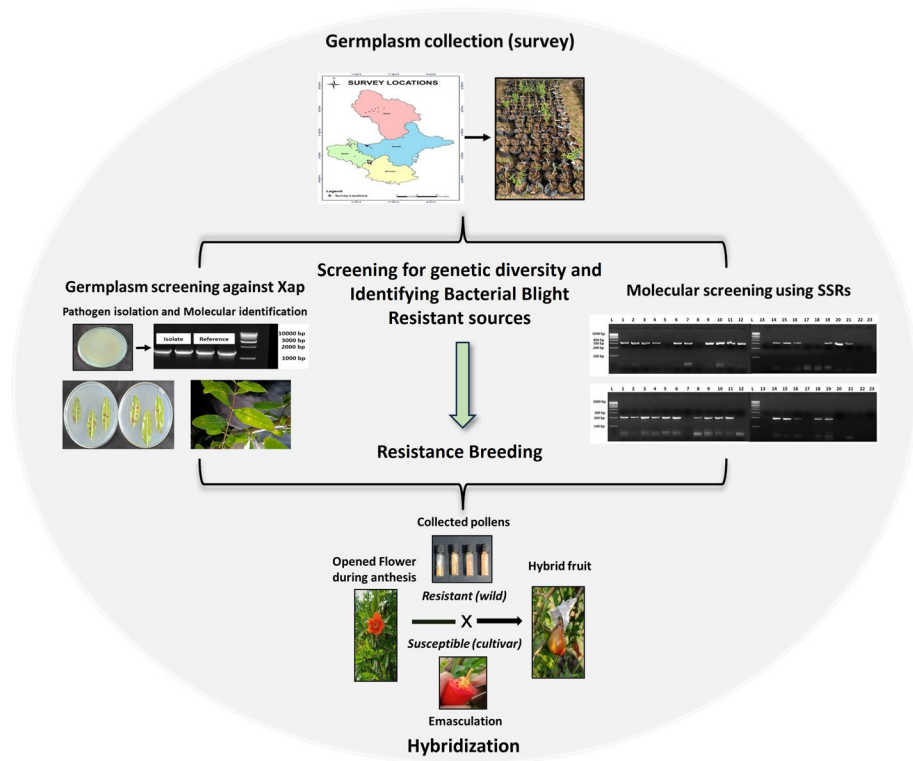


Fig. 1 Schematic illustration of the current study on screening wild populations using marker-assisted selection for resistance breeding

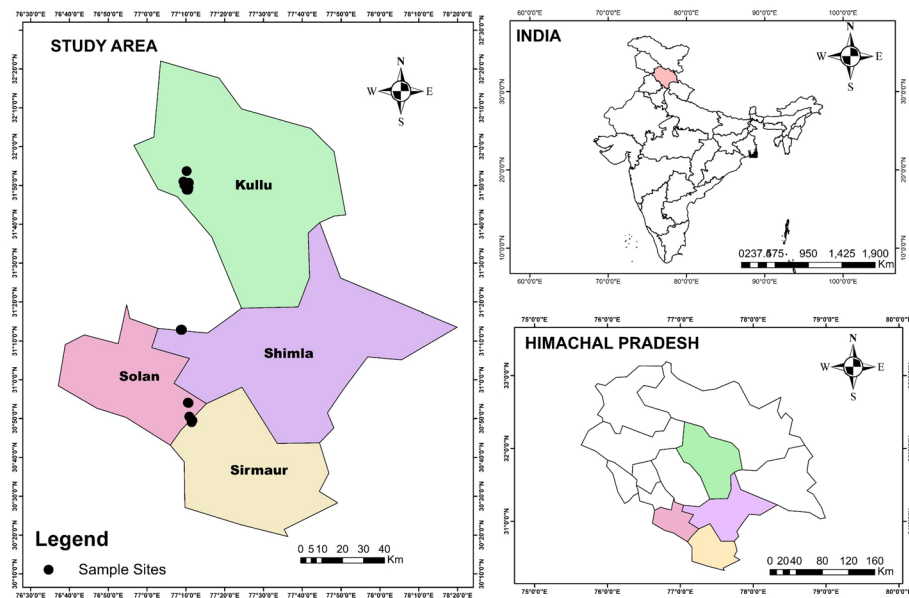


Fig. 2 Map highlighting the study area and survey locations of resistant wild genotypes and susceptible commercial pomegranate populations from Northwestern Himalayas

(Solan, Shimla, Sirmaur, and Kullu), comprising ten wild genotypes, and one commercial population with thirteen cultivars collected from the Regional Horticulture Research and Training Station (RHRTS), Bajaura, Kullu, Himachal Pradesh (Fig. 2; Table S1). The wild populations were distinguished as follows: wild SL (SL 22; 23) from Solan, wild SH (SH 14; 16;

17) from Shimla, wild SR (SR 11; 12; 15) from Sirmaur and wild KL (KL 1; 12) from Kullu. These wild genotypes were chosen based on initial pathogenicity studies and biochemical analysis conducted on 100 wild Daru pomegranate genotypes from these districts to screen for resistance to bacterial blight caused by *Xanthomonas citri* pv. *punicae* [51]. Pathogenicity assays, involving both in vitro and in vivo screening methods, were used to evaluate disease incidence and severity along with mechanistic insights to defense responses (Fig. S1). This study was conducted at the College of Horticulture and Forestry, Neri, Hamirpur, Himachal Pradesh, during 2020–2023. The research farm, situated at 650 m above sea level in a sub-montane low hill zone, features mountainous terrain and a subtropical climate.

2.1 Isolation of genomic DNA

Fresh, young, disease and insect free leaves from selected genotypes/cultivars with varying degree of resistance to bacterial blight disease were collected and total genomic DNA was isolated by using a modified cetyltrimethylammonium bromide (CTAB) method, based on the approach outlined by Doyle [13]. While Doyle [13] employed a 2% CTAB solution along with 1.4 M NaCl, 20 mM EDTA and 0.2% β -mercaptoethanol for the extraction buffer, the current protocol introduced several adjustments [50, 53]. The extraction buffer composition was modified to include 2.5% CTAB and 2.5% polyvinylpyrrolidone (PVP). The incubation time was prolonged to 60 min, and the temperature during the water bath treatment was raised to 65 °C, in contrast to the 30 min treatment at 60 °C described by Doyle. Furthermore, the cold isopropanol precipitation step was extended to an overnight duration. The DNA concentration and purity were measured by using UV–vis spectrophotometer (Thermo Scientific Evolution 200) and gel electrophoresis. DNA was dissolved in 50 μ l TE buffer and then stored at –20 °C.

2.2 SSR analysis

DNA isolated from selected genotypes/cultivars was subjected to molecular analysis using 20 primers from Singh et al. [56], Jian et al. [25] and Ono et al. [35]. These markers exhibited high polymorphism, with several reported to be associated with bacterial blight resistance and important agronomic traits such as fruit acidity, size, and weight [56]. (Table 1). The amplification reaction was performed in a reaction volume of 10 μ l containing 0.6 μ l (5 U/ μ l) Taq DNA polymerase, 1 μ l (10X) Taq Buffer, 1 μ l (1 mM) dNTP mix, 0.6 μ l (10 pmol) of primers (both forward and reverse), 4 μ l (10 ng) of genomic DNA and 2.2 μ l of autoclaved distilled water. All the chemicals and primers used in PCR reaction mixture were purchased from Genei Private Limited, Bangalore, India.

The thermal profile begins with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at the optimized temperature for 30 s, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 10 min. The reaction is then held and stored at 4 °C until further analysis. The bands of amplified PCR products were resolved along with 1 kb DNA ladder marker on 3% agarose gel in 1X Tris–borate EDTA buffer at 85 V until the loading dye reached the gel front. The gel was then viewed under the UV trans-illuminator (BIOWORLD Pvt. Ltd.) and image was taken by using GelDoc Go Imaging System (BIO RAD).

Table 1 Characteristics of microsatellite loci used for genetic analysis of *Punica granatum* L. populations

Primer code	Accession number	Sequence 5' to 3'	Repeat motifs	Expected product size (bp)	T _m (°C)
ESP01	JG771258	F "TCTATTCCACATAGAAAGAGGGG" R "ATGATGTCTATGCAATTGGCTG"	(GGA) ₇	121	57
ESP03	JG771964	F "CGCTGGTCACACTACTTACTCG" R "TTGTAGTGGAAGACACAGCAGC"	(AT) ₁₂	263	55
ESP04	JG771772	F "AAAGGGGAAAAAGACGAAGAAG" R "CCCTGTCCTTAAGTCTGAGTGG"	(CT) ₁₀	257	55
ESP05	JG771683	F "TTGTTGGGTATTCTCTTCTC" R "ACATCATACACCTTGCCCTC"	(CTT) ₁₁	197	55
ESP06	JG771472	F "AAATCGCATCCCTCCGTCT" R "CTGTTCCGCGAGGTAAAGA"	(TC) ₁₃	147	56
ESP07	JG771464	F "CATGGGAGAACAGACAACCTAA" R "AGCCAGTCTGTTAACTATCGCC"	(AGA) ₆	252	55
ESP08	JG771444	F "TTCCCGAGAAAGTTGCATATCT" R "TAGTCCGTGAGGATTTGTCTCT"	(AG) ₁₈	204	56
ESP09	JG771396	F "TAATCCCATTCCAAACAAGTCC" R "ATATTGACGGAGGCTTCACTGT"	(AG) ₁₅	199	57
ESP11	JG771255	F "AACTTCTGGTGTCTCTTCCACC" R "GTGTGGAGTTGAAGATCGATGA"	(AT) ₉	147	58
ESP12	JG771218	F "TCTCTCTCCCTCATTTCTCTG" R "GTCTCTACCATCTCAGCAGCCT"	(CT) ₉	199	55
ESP13	JG771230	F "GCTAGCGAATGAAATGTCTT" R "GAGTATAGCAGTAGGGGAGATG"	(TA) ₉	178	52
ESP14	JG771277	F "GGGACACGCTGGAGTACAAT" R "GACCCCTCTCTCTCTGCTT"	(GTTG) ₆	267	58
ESP16	JG771376	F "AGGCTTCATGACCCATCATC" R "AGGAAGAGTTCGGGTTTCGAT"	(TGG) ₈	191	57
ESP17	JG771421	F "TGTGGGTGTGGGAACATAATAA" R "TCAACAGGACAAAGGATGAAGA"	(TCC) ₅	275	58
ESP19	JG771896	F "TGGGGATTATCGTTGTCTTCA" R "TCCAAGCTGAACCTCGTTCTCT"	(CT) ₉	236	58
ESP22	JG771743	F "ATATTTGCCTCAATGGCGTC" R "AAACAGCTCCTCTTCCCAT"	(TA) ₈	300	57
ESP24	JG771352	F "CAAACGCCTTCATGAACTACA" R "GACAAAAATTCCAGTCCATC"	(GT) ₇	104	58
PGCT001	FN677521	F "AGCTCCGATTGAGAGCAGAT" R "TTGGAGCAATTGGAGAGAGA"	(TC) ₁₈	101	59
PGCT020	FN677540	F "TTCCTTTCGTTTCACTCATC" R "CCCGATCATTAAATCCACAAA"	(CT) ₁₅	152	59
PGCT043	FN677563	F "ACCACCGCACATAATAACTTC" R "TTGAATACGCCTGTTGTCTT"	(CT) ₁₉	232	56

2.3 Population structure and genetic diversity analysis

GenAlEx software 6.5 Edition [58], <https://biology-assets.anu.edu.au/GenAlEx/Download.html>) was used to estimate various genetic parameters, including observed number of alleles per locus (Na), effective number of alleles per locus (Ne), Shannon information index (I), polymorphism information content value (PIC), observed heterozygosity (Ho), expected heterozygosity (He), mean allele pattern and gene flow value across populations (Nm). Further, GenAlEx was employed to calculate pairwise genetic differentiation coefficients (Fst) and analysis of molecular variance (AMOVA). To evaluate the distribution of gene diversity and partition genetic variation, we estimated variance components both among and within populations. Following Wright's (1978) criteria, genetic differentiation was classified as low ($F_{st} < 0.05$), moderate ($0.05 < F_{st} < 0.15$), high

($0.15 < F_{st} < 0.25$), and very high ($F_{st} > 0.25$). The principal coordinate analysis (PCoA) was performed using GenAlEx based on genetic distances.

A Neighbor Joining (NJ) tree was generated using pairwise genetic distances in DARwin v6.0.021 [39], <https://darwin.cirad.fr/>). Population structure was assessed using the Bayesian clustering approach in STRUCTURE v2.3.4 [42], with three independent runs for each K value ($K = 1-10$), employing a burn-in of 100,000 iterations followed by 100,000 Monte Carlo Markov Chain (MCMC) replicates. The optimal K was identified based on the log probability of data [$\ln P(D)$] and its rate of change across K values, using StructureSelector [27], <https://lmme.ac.cn/StructureSelector/>).

2.4 Production of F_1 hybrids through crossing

Hybridization was performed using 12 different cross combinations, according to the method suggested in our previous study [52]. Disease-resistant genotypes (SR-11, SH-16, SL-22, SL-23) were selected as male parents and crossed with three commercial cultivars (Bhagwa, Phule Super Bhagwa, Kandhari Kabuli) serving as female parents. To conduct crosses, pollen was collected from the resistant genotypes during the flowering season (March–April, 2023), stored in glass vials, and stored at 4 °C for later use. For pollination, three branches on each selected plant were chosen, and all open flowers and undeveloped buds were removed. Unopened blossoms (about to bloom) were emasculated by removing sepals, petals, and stamens to prevent self-pollination. These emasculated flowers were covered with muslin cloth bags, which were securely tied and labelled. Pollination was carried out the following day (12:00–1:00 PM) using the stored pollen, which was applied to the stigmas with a camel hairbrush and the flowers were bagged again to avoid contamination. The bags were removed once fruit set was observed. Fruit set and fruit retention percentage were calculated as per following formulas:

$$\text{Fruit set percentage (\%)} = \frac{\text{Number of buds set into fruits}}{\text{Total numbers of buds pollinated}} \times 100$$

$$\text{Fruit retention percentage (\%)} = \frac{\text{Number of fruits retained}}{\text{Total numbers of fruit set}} \times 100$$

Fruit set and fruit retention percentage data were analyzed using a Randomized Block Design (RBD). Analysis of variance (ANOVA) for each trait was performed according to the methodology of Panse and Sukhatme [37], utilizing MS-Excel (version 2021) and OPSTAT software [55]. The critical difference (CD) was computed at the 5% significance level to determine statistically significant differences among treatments.

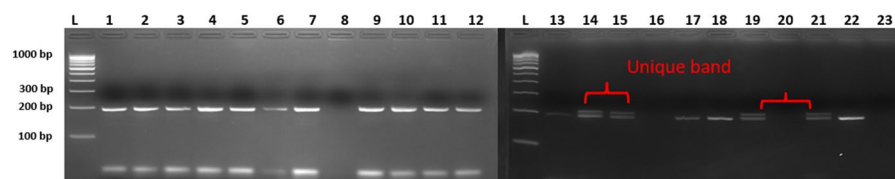
3 Results

3.1 Polymorphism of microsatellite markers

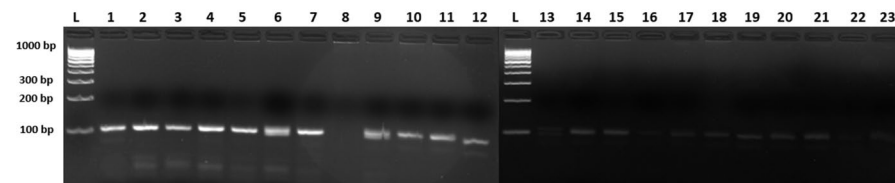
Twenty SSR primers displayed polymorphism across selected genotypes, yielding a total of 1,119 scorable bands. Among these, 1,036 bands were polymorphic, representing a polymorphism ratio of 92.58% (Table S2). Primers ESP01 and ESP04, generating bands in the 50–250 bp range, each showed a high polymorphism ratio of 94.74%. ESP03 yielded 52 bands with 94.32% polymorphism, while ESP05 and ESP06 displayed complete polymorphism (100%), producing 92 and 67 bands, respectively. ESP07 achieved 96.43% polymorphism with bands ranging from 50 to 300 bp. Further, ESP08 and ESP09

presented 89.74% and 98.74% polymorphism, respectively. Notably, ESP11, ESP13, and ESP14 displayed complete polymorphism, while ESP17 and ESP19 showed 85% and 22.22%, respectively. Primers ESP22 and ESP24 had polymorphism ratios of 87.14% and 74.29% with notable variation across genotypes. Primer ESP16 displayed lower polymorphism (34.78%), while other primers, such as PGCT001 and PGCT043, demonstrated high polymorphism (96% and 98.08%) with unique bands observed in wild Daru genotypes SH-14, SH-16, SR-15 and SL-23 (Fig. 3). The PIC values varied from 0.08 (ESP11) to 0.460 (ES12) with an average of 0.321. The PIC value of the SSR markers used in the study indicates that they were informative and polymorphic (Table 2).

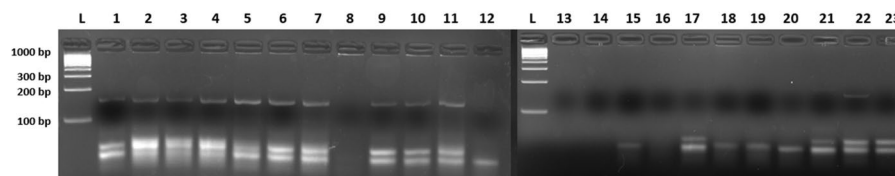
ESP16



PGCT001



PGCT020



PGCT043

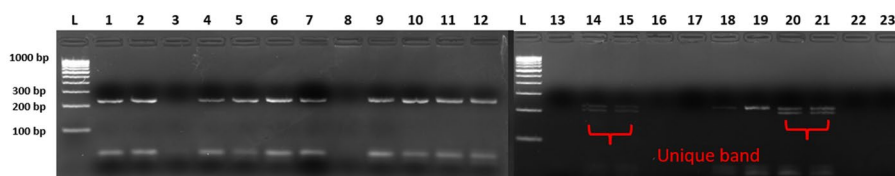


Fig. 3 Agarose gel electrophoresis PCR amplified bands of selected pomegranate genotypes/cultivars using primer ESP16, PGCT001, PGCT020 and PGCT043; L=Ladder, 1=Mridula, 2=Aarkta, 3=G-137, 4=Achikdana, 5=Jodhpur Red, 6=Nabha, 7=P-26, 8=P-23, 9=Chawla, 10=Kandhari Kabuli, 11=Bhagwa, 12=Kandhari Hansi, 13=K-75-P-5, 14=SH-14, 15=SH-16, 16=SH-17, 17=SR-12, 18=SR-11, 19=SR-15, 20=SL-22, 21=SL-23, 22=KL-1 and 23=KL-12

Table 2 Genetic diversity parameters of twenty microsatellite locus used for genetic analysis of pomegranate populations

Locus	Na	Ne	I	PIC	Ho	He
ESP 01	2.600	2.278	0.851	0.310	0.933	0.551
ESP 03	2.800	2.217	0.797	0.330	0.764	0.477
ESP 04	2.600	2.282	0.869	0.160	0.933	0.556
ESP 05	3.000	2.513	0.945	0.270	1.000	0.583
ESP 06	2.000	2.000	0.693	0.290	1.000	0.500
ESP 07	1.800	1.800	0.555	0.290	0.800	0.400
ESP 08	2.400	2.080	0.705	0.440	0.667	0.433
ESP 09	1.200	1.075	0.187	0.410	0.182	0.116
ESP 11	2.000	2.000	0.693	0.080	1.000	0.500
ESP 12	2.200	2.045	0.685	0.460	0.644	0.447
ESP 13	3.600	3.028	1.149	0.340	1.000	0.653
ESP 14	2.200	1.967	0.703	0.240	0.759	0.474
ESP 16	1.600	1.477	0.367	0.290	0.467	0.256
ESP 17	2.400	2.098	0.728	0.330	0.587	0.458
ESP 19	1.000	0.836	0.057	0.290	0.033	0.031
ESP 22	1.800	1.615	0.380	0.440	0.340	0.235
ESP 24	1.600	1.437	0.363	0.290	0.383	0.249
PGCT001	2.200	1.888	0.626	0.330	0.733	0.413
PGCT020	2.200	1.888	0.635	0.430	0.482	0.405
PGCT043	1.600	1.597	0.554	0.390	0.782	0.399
Overall mean	2.140	1.906	0.627	0.321	0.674	0.407

Here; Na: Number of different alleles, Ne: Number of different alleles with a frequency $\geq 5\%$, I: Shannon's Information Index, PIC: Polymorphic Informative Content value, Ho: Observed heterozygosity, He: Expected heterozygosity

3.2 Assessment of genetic diversity among SSRs and populations

Statistical results of genetic diversity analysis of 20 SSR loci are shown in Table 2. This table provides a comprehensive analysis of genetic diversity across loci based on microsatellite markers, reflecting key metrics of genetic variation within a population with a total of 42.800 alleles (Na) were detected. The number of alleles (Na) is a direct indicator of genetic variability, with values ranging from a low of 1.0 for ESP19 to a high of 3.6 for ESP13. The overall mean of 2.14 suggests moderate allelic richness across loci. Loci with higher Na, such as ESP05 and ESP13, are likely more polymorphic and contribute significantly to genetic diversity. The effective number of alleles (Ne) accounts for allele frequency distribution, capturing the alleles' relative contribution to diversity. With an overall mean of 1.906, Ne values are consistently lower than Na due to the unequal frequencies of alleles. Loci such as ESP13 (Ne = 3.028) exhibit high diversity, while others like ESP19 (Ne = 0.836) demonstrate limited effective diversity.

The Shannon's Information Index (I) measures both richness and evenness of alleles, with values ranging from 0.057 (ESP19) to 1.149 (ESP13). The mean value of 0.627 reflects moderate genetic complexity across loci. Loci like ESP13 and ESP05, with high I value, highlight greater allelic variation and even allele distribution. Observed heterozygosity (Ho) varies significantly across loci, from 0.033 (ESP19) to 1.0 (e.g., ESP05, ESP06, ESP11, ESP13), with an overall mean of 0.674 (Table 2). The higher-than-expected Ho values at several loci may indicate excess heterozygosity. At all loci, the observed heterozygosity was significantly lower than the expected heterozygosity pointing towards the existence of population structure. Conversely, loci with low Ho values, such as ESP19, suggest inbreeding or allele fixation. The expected heterozygosity (He) ranges from 0.031 (ESP19) to 0.653 (ESP13), with a mean of 0.407. He reflects the genetic variation

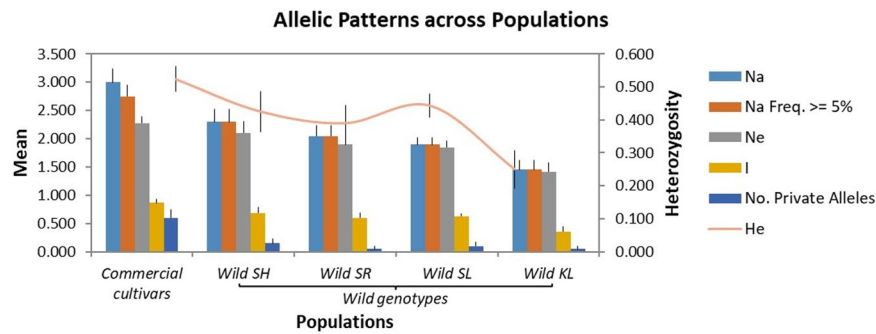


Fig. 4 Mean allelic patterns across wild and commercial pomegranate populations

Table 3 Pairwise population Fst values (below diagonal) and Gene flow Nm (above diagonal) among the commercial and wild populations of *Punica granatum* L

Populations	Commercial cultivars	Wild SH	Wild SR	Wild SL	Wild KL
Commercial cultivars	0	1.668	1.786	1.422	0.913
Wild SH	0.130	0	2.494	1.070	0.700
Wild SR	0.123	0.091	0	0.892	0.732
Wild SL	0.150	0.189	0.219	0	0.615
Wild KL	0.215	0.263	0.255	0.289	0

Here, Wild SH, Wild SR, Wild SL and Wild KL represents wild genotypes from Shimla, Sirmaur, Solan and Kullu districts of Himachal Pradesh

expected under Hardy–Weinberg equilibrium and shows moderate genetic diversity. Overall, the data reveals varying levels of genetic diversity across loci, with some (e.g., ESP13, ESP05) being highly polymorphic and others (e.g., ESP19) exhibiting low diversity. These findings underline the importance of selecting loci with higher Na, Ne, and I for robust genetic studies and highlight the potential impacts of evolutionary forces such as selection, drift, and population structure on genetic variability.

Figure 4 graphically represents mean allelic patterns across pomegranate populations, highlighting distinct genetic diversity parameters among commercial cultivars and wild populations (Wild SH, Wild SR, Wild SL and Wild KL). Commercial cultivars demonstrate the highest genetic diversity, characterized by the largest mean number of alleles (Na = 3.00) and expected heterozygosity (He = 0.52). In contrast, wild populations exhibit a progressive decline in diversity, with Wild KL showing the lowest Na (1.45), Shannon’s Information Index (I = 0.36), and He (0.25) (Table S3).

The number of private alleles, representing unique genetic markers, is highest in commercial cultivars (0.60) but markedly low in Wild KL and Wild SR (0.05). Similarly, effective alleles (Ne) and allele richness (I) reveal reduced genetic variability in wild populations. These trends suggest limited diversity within Wild KL, while commercial cultivars maintain a broader genetic base, likely due to selective breeding and genetic mixing.

3.3 Population differentiation, gene flow, genetic identity and distance for population genetic diversity

Pairwise population differentiation (Fst values) and gene flow (Nm) among commercial and wild pomegranate populations provide insights into their genetic relationships (Table 3). Fst values (below diagonal) range from moderate value of 0.091 (Wild SH and Wild SR) to very high value of 0.289 (Wild SL and Wild KL), with lower values indicating

Table 4 Pairwise Nei's genetic distance D (below diagonal) and genetic identity (above diagonal) among the five populations of *Punica granatum* L.

Populations	Commercial cultivars	Wild SH	Wild SR	Wild SL	Wild KL
Commercial cultivars	–	0.750	0.766	0.706	0.721
Wild SH	0.288	–	0.873	0.680	0.702
Wild SR	0.267	0.136	–	0.619	0.721
Wild SL	0.348	0.386	0.480	–	0.637
Wild KL	0.327	0.353	0.327	0.450	–

Here, Wild SH, Wild SR, Wild SL and Wild KL represents wild genotypes from Shimla, Sirmaur, Solan and Kullu districts of Himachal Pradesh

Table 5 Analysis of molecular variance (AMOVA) in five populations of *Punica granatum* L.

Source of variation	Degree of freedom (df)	Sum of squares (SS)	Estimated variance	Percentage of variation (%)
Among populations	4	42.189	0.607	9%
Within populations	41	255.006	6.283	91%
Total	45	297.196	6.890	100%

genetic similarity and higher values reflecting divergence. Commercial cultivars show moderate to high differentiation from wild populations (0.123–0.215), likely due to shared ancestry or human-mediated gene flow. Wild KL is the most genetically distinct, with highest F_{st} values (0.263 with Wild SH), indicating isolation and possible environmental adaptation. Gene flow (N_m , above diagonal) inversely correlates with F_{st} , ranging from 0.615 (Wild SL and Wild KL) to 2.494 (Wild SH and Wild SR).

Nei's genetic distance and genetic identity among five populations of *Punica granatum* L., (one commercial cultivars and four wild) highlighting their genetic relationships. Genetic identity ranges from 0.619 (Wild SR and Wild SL) to 0.873 (Wild SH and Wild SR), indicating the latter are the most genetically similar populations (Table 4). Commercial cultivars exhibit moderate genetic identity with wild populations (0.706–0.766), while Wild SL and Wild KL show the lowest identity (0.637), reflecting significant differentiation. Genetic distance values range from 0.136 (Wild SH and Wild SR) to 0.480 (Wild SR and Wild SL), with smaller distances indicating closer genetic relationships. Analysis of molecular variance (AMOVA) conducted on five populations of *Punica granatum* L., highlighting the distribution of genetic variation among and within population (Fig. S2). The analysis reveals that most genetic variation is found within population, accounting for 91% of the total variation (Table 5). In contrast, only 9% of the genetic variation occurs among population, as indicated by an estimated variance of 0.607.

3.4 Genetic structure and genetic relationship among wild and cultivated *Punica granatum* populations

Principal Coordinate Analysis (PCoA) effectively reveals differences between two or more sample groups by intuitively comparing linear distances between them on coordinate axes. Shorter linear distances signify smaller genetic differences. Table 6 summarizes the PCoA results, including eigenvalues, percentage variance explained, and eigenvectors for selected pomegranate populations. The first three principal axes collectively explain 43.35% of the total genetic variation, with Axis 1 accounting for the highest proportion (19.70%), followed by Axis 2 (12.48%) and Axis 3 (11.17%). These axes capture

Table 6 Percentage of variation along with eigen values and sample eigen vectors explained by the first 3 axes among selected pomegranate populations using 20 Simple Sequence Repeat markers

Axis	1	2	3
Eigen value	12.830	8.123	7.275
Variance (%)	19.70	12.48	11.17
Cumulative variance (%)	19.70	32.18	43.35
Eigen vectors			
Mridula	−0.709	−0.307	0.340
Aarkta	−0.692	−0.130	−0.547
G 137	−0.580	−0.317	0.292
Achikdana	−0.801	−0.149	−0.606
Jodhpur Red	−0.548	−0.265	0.339
Nabha	−0.322	−0.505	−0.149
P 26	−0.112	−0.572	−0.488
P 23	1.847	−1.127	−1.165
Chawla	−0.806	−0.436	0.059
Kandhari Kabuli	−0.654	−0.392	−0.114
Bhagwa	−0.728	−0.151	−0.485
Kandhari Hansi	−0.601	−0.091	0.137
K-75-P-5	0.768	−1.000	1.215
SH 14	−0.233	0.390	−0.072
SH 16	0.369	1.085	0.287
SH 17	1.407	0.504	−0.405
SR 11	0.192	0.264	1.045
SR 12	0.087	1.062	−0.348
SR 15	−0.380	0.993	−0.427
SL 22	0.776	0.553	−0.216
SL 23	0.175	0.570	−0.116
KL 1	0.390	0.322	1.033
KL 12	1.157	−0.299	0.389

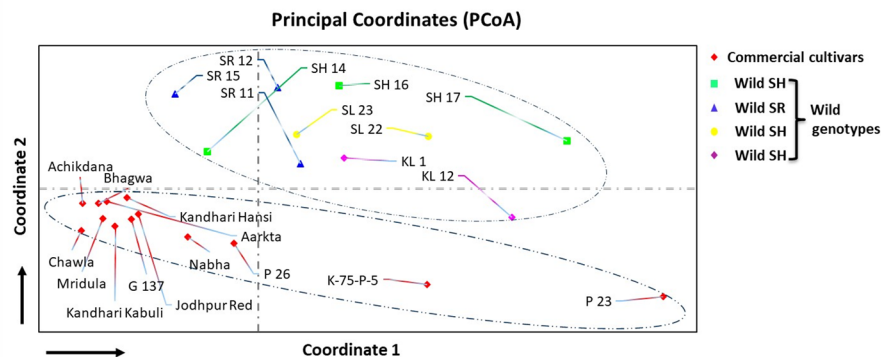


Fig. 5 Principal coordinate analysis of wild and commercial populations of pomegranate based on SSR profiles

the major genetic relationships among populations. The eigenvectors indicate how individual populations contribute to these axes, identifying distinct genetic clusters. Based on PCoA, it divided 23 genotypes into two main clusters (Fig. 5). Commercial cultivars such as Bhagwa, Chawla, Achikdana and Mridula cluster closely, indicating high genetic similarity. Conversely, populations such as P-23 and K-75-P-5 exhibit extreme positive or negative values on Axis 1, highlighting their genetic divergence. Wild genotypes from all wild populations (e.g., SR-11, SL-22, KL-1 etc.) display unique contributions across

axes and cluster together, reflecting their genetic distinctiveness and potential ecological adaptations.

The NJ tree delineated two major clusters (Fig. 6A): Cluster 1 comprising 10 genotypes predominantly representing commercial cultivars such as Mridula, Bhagwa, Kandhari Hansi, Aarkta, G-137, Achikdana, Jodhpur Red, Nabha, and Chawla. Cluster 2 consisted of 12 genotypes primarily representing indigenous wild accessions (SH 15, 16, 17; SR 11, 12, 15; SL 22; KL 1, 12) collected from four districts of Himachal Pradesh, India. Notably, genotypes P-23 and P-26 (selections from the exotic line Muscat) and K-75-P-5, though cultivated, grouped within Cluster 2, indicating divergence from mainstream commercial types. The cultivar Kandhari Kabuli appeared as an outlier, forming a distinct lineage separate from both major clusters. Population structure of the 23 pomegranate genotypes was assessed using SSR data under a model-based approach. A clear peak in ΔK was observed at $K = 2$ ($\Delta K = 8.2$), indicating the presence of two major genetic clusters (Fig. 6B). This clustering pattern aligned with the NJ tree, dividing the genotypes into cultivated varieties (Cluster 1) and wild accessions (Cluster 2) (Fig. 6C). The analysis also revealed admixture among genotypes, suggesting that cultivated pomegranates have inherited genomic segments from wild types in varying proportions, contributing to their genetic diversity.

For hybridization, four wild genotypes from Cluster 2 (SH-16, SL-22, and SL-23) were chosen as male parents, while three commercial cultivars (Bhagwa, Kandhari Kabuli, and Phule Super Bhagwa) were selected as female parents. These findings are elaborated in the subsequent section.

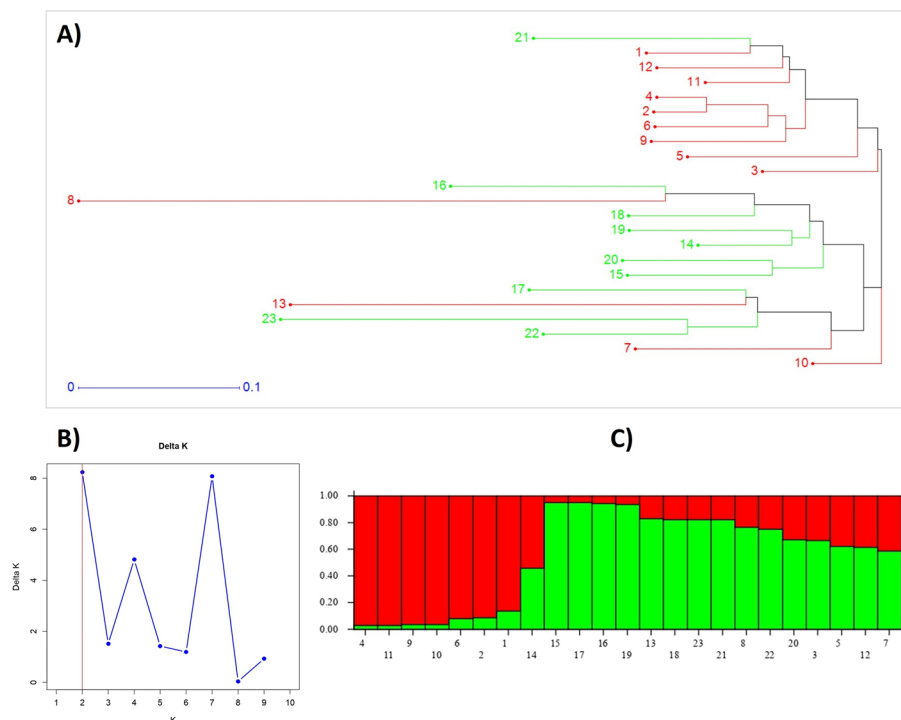


Fig. 6 Neighbor Joining tree (A), K plot (B) and population structure analysis (C) depicting the genetic relationships among wild and commercial pomegranate populations based on 20 SSR markers. Here, 1=Mridula, 2=Aarkta, 3=G-137, 4=Achikdana, 5=Jodhpur Red, 6=Nabha, 7=P-26, 8=P-23, 9=Chawla, 10=Kandhari Kabuli, 11=Bhagwa, 12=Kandhari Hansi, 13=K-75-P-5, 14=SH-14, 15=SH-16, 16=SH-17, 17=SR-12, 18=SR-11, 19=SR-15, 20=SL-22, 21=SL-23, 22=KL-1 and 23=KL-12

Table 7 Hybridization fruit set (%) and fruit retention (%) in various cross combinations derived between wild and commercial pomegranate varieties

S. No	Crosses	Fruit set (%)	Fruit retention (%)
1	Bhagwa × SR-11	41.67 (39.98)	50.00 (44.99)
2	Bhagwa × SL-23	66.70 (54.73)	66.67 (59.99)
3	Bhagwa × SH-16	88.90 (78.25)	61.13 (51.48)
4	Bhagwa × SL-22	55.57 (48.23)	33.33 (29.99)
5	Phule super Bhagwa × SR-11	19.43 (21.74)	0.00 (0.00)
6	Phule super Bhagwa × SL-23	55.57 (48.23)	50.00 (44.98)
7	Phule super Bhagwa × SH-16	25.00 (24.99)	16.67 (14.99)
8	Phule super Bhagwa × SL-22	44.43 (41.73)	33.33 (29.99)
9	Kandhari Kabuli × SR-11	27.77 (31.74)	66.67 (60.00)
10	Kandhari Kabuli × SL-23	11.10 (11.74)	0.00 (0.00)
11	Kandhari Kabuli × SH-16	13.33 (17.70)	33.33 (30.00)
12	Kandhari Kabuli × SL-22	19.43 (21.74)	0.00 (0.00)
	CD	28.09	N/A

*Figures in the parenthesis are angular transformed values

**Fig. 7** Hybrid fruit set obtained for the respective crosses between resistant genotypes (male) and susceptible cultivars (female)

3.4.1 Hybridization

Significant variations were observed for fruit set among different cross combinations (Table 7). The highest fruit set percentage was observed in the cross Bhagwa × SH-16 (88.90%), which was statistically similar to the cross Bhagwa × SL-23 (66.70%). This was followed by the crosses Bhagwa × SL-22 and Phule Super Bhagwa × SL-23, which had fruit set percentages of 55.57% (Fig. 7). On the other hand, the low fruit set was observed in the cross combinations of Kandhari Kabuli × SL-23 (11.0%), Kandhari Kabuli × SH-16 (13.33%), Kandhari Kabuli × SL-22 (19.43%), Phule Super Bhagwa × SR-11 (19.43%), and Phule Super Bhagwa × SH-16 (25.00%). In terms of fruit retention, the lowest fruit retention percentage (16.67%) was observed in the cross Phule Super Bhagwa × SH-16. On the other hand, the highest fruit retention percentage (66.67%) was recorded in the cross combinations of Bhagwa × SL-23 and Kandhari Kabuli × SR-11. However, no fruit

retention was observed in the crosses Phule Super Bhagwa $\times$ SR-11, Kandhari Kabuli $\times$ SL-23, and Kandhari Kabuli $\times$ SL-22 (0%).

4 Discussion

Bacterial blight is a highly destructive airborne disease that causes significant losses in pomegranate crops, making management increasingly challenging [30]. To develop resistant pomegranate cultivars, identifying sources of resistance by exploring genetic variation in both cultivated and wild germplasm is crucial. The screening of pomegranate wild genotypes and commercial cultivars for bacterial blight resistance through both detached and attached leaf assay methods has provided valuable insights into the resistance potential of various genotypes [19, 43]. The findings from our previous study [51] reinforce the potential of wild germplasm as a valuable resource for breeding programs aimed at enhancing bacterial blight resistance in pomegranate, offering a path forward for developing cultivars with improved disease resilience.

The use of molecular markers is instrumental in evaluating the genetic diversity of plant species, and this study specifically focused on assessing the molecular diversity among pomegranate genotypes using 20 microsatellite markers (SSRs), revealing significant polymorphism (Table 2). This highlights the considerable genetic variation within the populations, crucial for developing cultivars with improved resistance to bacterial blight. Similar findings were reported by Wang et al. [60] and Perveen et al. [40], who also observed significant genetic diversity among pomegranate genotypes using SSR markers. Additionally, Madadi et al. [29] confirmed a significant level of genetic diversity within commercial Iranian pomegranate cultivars using inter simple sequence repeat (ISSR) and SSR markers. High polymorphism (Table S2), particularly with some primers emphasizes the rich genetic pool available for distinguishing genotypes and identifying traits like pathogen resistance.

Diversity indices revealed higher genetic diversity in commercial cultivars (Fig. 4), reflected by a greater number of alleles (N_a) and expected heterozygosity (H_e). In contrast, wild populations, particularly from Kullu (KL), displayed reduced genetic diversity, likely due to genetic bottlenecks or geographic isolation, emphasizing the urgent need for conservation efforts to protect these genetic resources [56]. The allelic number and allelic diversity detected for SSR markers in this study were nearly the same as those reported in previous studies [12, 25, 38, 60]. Pairwise population differentiation and gene flow (N_m) analysis showed significant genetic similarity and connectivity between certain wild populations (Table 3), such as Wild SH and Wild SR, likely due to shared ancestry or ongoing gene flow. However, marked differentiation between populations like Wild SL and Wild KL highlights the influence of geographic and ecological barriers on genetic divergence. The close genetic relationship between Wild SH and Wild SR underscores their potential as reservoirs of genetic diversity, while the distinctiveness of Wild SL and Wild KL reflects their adaptation to specific environments [28]. Commercial cultivars exhibited moderate genetic connectivity with wild populations, possibly due to shared genetic origins or historical human-mediated gene flow, suggesting their role as genetic intermediaries [60]. These findings reinforce the importance of conserving both diverse and distinct populations to enhance breeding programs and support adaptation to environmental challenges. Nei's genetic identity and distance metrics revealed high divergence between wild populations and commercial cultivars, yet moderate overlap

suggests historical gene flow. Analysis of molecular variance (AMOVA) revealed that 91% of the total genetic variation was distributed within populations, while only 9% was found among populations (Table 5), underscoring the critical role of within-population diversity in promoting adaptability and resilience [33]. The highest genetic diversity was observed in the Himalayan germplasm, likely reflecting both intensive sampling and historical gene flow among populations in this region [38, 56]. Despite being collected from distinct geographic locations, the populations are situated in adjoining districts of the northwestern Himalayas, where shared environmental conditions and close proximity promote frequent gene exchange. This explains the limited inter-population differentiation. Local adaptation, mutation, and recombination further enhance within-population variability, while the absence of strong geographic or ecological barriers limits divergence among populations [11, 54].

Principal coordinate analysis (Fig. 5) and neighbor joining clustering (Fig. 6A) further supported these findings, differentiating commercial cultivars from wild populations but revealing some genetic overlap, consistent with historical gene flow. The tree identified two main clusters: one comprised predominantly of highly susceptible commercial cultivars and one consisting of wild Daru germplasm known for moderate to full bacterial blight resistance. These patterns are congruent with those of principal component analysis explaining high variation among pomegranate wild genotypes and commercial cultivars [43, 51]. Similarly, population structure analysis also revealed the same pattern of clustering by dividing whole populations into 2 clusters. These patterns align with prior studies [38, 56, 60], highlighting the genetic potential of wild germplasm for resistance breeding.

These findings underscore the extensive genetic variation in the collected germplasm, presenting valuable opportunities for allele discovery and trait improvement in pomegranate breeding programs. Specifically, primers PGCT043 and ESP16 identified alleles at 200 bp and 210 bp, respectively (Fig. 3), in the resistant genotypes (wild types), which were associated with higher fruit acidity and the presence of natural products like hydrolyzable tannins and anthocyanins [35, 56]. Elevated fruit acidity and increased levels of hydrolyzable tannins and anthocyanins in pomegranates are linked to enhanced resistance to bacterial blight. Acidic environments suppress bacterial growth, while tannins and anthocyanins exhibit antimicrobial properties, directly interfering with pathogen colonization [24, 43, 51]. Ellagitannins, a type of hydrolyzable tannin, have been shown to have bactericidal effects by disrupting bacterial cell membranes and inhibiting essential bacterial enzymes [7, 61]. Anthocyanins contribute to bacterial blight resistance by scavenging reactive oxygen species (ROS) produced during pathogen invasion, thereby protecting the fruit from oxidative stress and pathogen-induced damage [63]. Singh et al. [56] further reported that a 153-bp allele, commonly found in wild types, is linked to smaller fruit sizes, lower bacterial blight severity, and higher fruit acidity. However, as Basaki et al. [6] highlighted, an effective marker-assisted selection (MAS) strategy necessitates the use of a broad range of SSR markers to comprehensively cover the pomegranate genome and generate valuable insights for pomegranate improvement. Therefore, future studies should employ a more extensive set of SSRs and single nucleotide polymorphism marker (SNPs) linked to these traits to achieve significant advancements in pomegranate breeding programs.

Our hybridization studies revealed significant variation in fruit set among pomegranate genotypes, governed by both genetic makeup and environmental factors [4]. Among 12 crosses (Table 7), the highest fruit set was recorded in Bhagwa × SH-16, Bhagwa × SL-23, and Phule Super Bhagwa × SL-23, whereas crosses involving Kandhari Kabuli as the female parent showed notably lower fruit set (Kandhari Kabuli × SL-23, Kandhari Kabuli × SH-16, Kandhari Kabuli × SL-22). Bhagwa, when used as a female parent, consistently exhibited superior fruit set performance compared to other cultivars, while Kandhari Kabuli showed reduced compatibility. These variations were largely attributed to genotypic effects and differential responses to temperature fluctuations [4]. Furthermore, stigma receptivity, pollen viability, and germination during anthesis were key determinants of hybridization success [52]. Based on these findings, Bhagwa appears to be a more suitable female parent for genetic improvement through crosses with wild genotypes.

Consistent with earlier studies [4, 26, 44, 52], our results emphasize the critical role of genetic compatibility and environmental interactions in influencing fruit set. Strategic selection of parental combinations is therefore vital to optimize hybridization success [5]. Notably, the use of disease-resistant wild genotypes as male parents enhanced fruit set and retention, highlighting their relevance in resistance breeding. These genetically diverse wild populations harbour alleles conferring resistance to bacterial blight and represent valuable reservoirs for crop improvement.

The integration of wild genetic material into commercial breeding programs, as demonstrated by our hybridization outcomes, provides a feasible pathway for developing Xcp resistant cultivars. F₁ hybrids derived from such crosses can be refined through molecular breeding techniques, including QTL mapping, transcriptome analysis, and gene tagging, to localize resistance-associated loci. These tools will accelerate the development of elite pomegranate cultivars with stable disease resistance and high fruit quality. The synergistic application of conventional breeding and genomic technologies thus holds promise for ensuring sustainable pomegranate cultivation under biotic stress conditions.

5 Conclusion

This study identified promising bacterial blight resistant genotypes (SH-16, SL-23, and SL-22) from wild Himalayan pomegranate populations, which clustered distinctly from susceptible commercial cultivars like Bhagwa, Mridula, and Aarkta. Molecular diversity analysis using SSR markers revealed high genetic variation within populations, and specific alleles were associated with higher acidity and elevated levels of hydrolyzable tannins and anthocyanins (traits linked to disease resistance). Hybridization studies further confirmed that crosses such as Bhagwa × SH-16 and Bhagwa × SL-23 yielded higher fruit set, underscoring cultivar Bhagwa's utility as a female parent. These findings demonstrate the value of integrating wild germplasm into breeding programs and provide a foundation for marker-assisted selection and genomic-assisted improvement of bacterial blight resistance in pomegranate.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44372-025-00388-w>.

Supplementary Material 1.

Supplementary Material 2.

Author contributions

All authors contributed to the study conception and design. Rahul Sharma: Writing—review & editing, Writing—original draft, Visualization, Validation, Software, Resources, Investigation, Data handling & analytical work. SD Sharma: Conceptualization, Preparation of research protocol, Editing & Refinement. VK Sharma: Conceptualization, Writing—review & editing, Preparation of research protocol, Editing & Compilation. Monica Sharma: Methodology, Software, Analysis & refinement. Rajat Sharma: Data handling, Analytical work, Writing—original draft & Writing—review & editing. Shireen Khatri: Writing—review & editing.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Competing Interests

The authors declare no competing interests.

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