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Genetic diversity among wild pomegranate (*Punica granatum*) in Azad Jammu and Kashmir region of Pakistan

Sadia Aziz [a], Syeda Firdous [a], Hifz Rahman [b], Shahid Iqbal Awan [c], Vincent Michael [d], Geoffrey Meru [d,*]

[a] Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad 1300, Pakistan
[b] Department of Horticulture, University of Poonch, Rawalakot, Azad Kashmir, Pakistan
[c] Department of Plant Breeding and Molecular Genetics, Faculty of Agriculture, University of Poonch, Rawalakot, Azad Kashmir, Pakistan
[d] Tropical Research and Education Center, Horticultural Sciences Department, University of Florida, Florida, USA

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ABSTRACT

Background: Pomegranate (*Punica granatum* L.), one of the most important tropical fruits in Azad Jammu and Kashmir regions of Pakistan, is highly valued for its nutrition and medicinal purposes. Although pomegranate is native to this region, the genetic diversity among wild pomegranate accessions is currently unknown. Such information would be vital for germplasm conservation and breeding efforts. In the current study, genetic diversity among forty-eight wild pomegranate accessions collected from different agro-ecological zones of Azad Jammu and Kashmir was assessed using 41 simple sequence repeat (SSR) markers.
Results: The markers revealed 303 alleles averaging 7.39 alleles per marker. Polymorphic information content ranged from 0.12 (PGCT093B) to 0.88 (Pom006), with a mean of 0.54. The average genetic distance (GD) across all genotypes was 0.52, and was lowest between Chattar Class and Thorar genotypes (GD = 0.27), but highest between Khun Bandway and Akhor Ban (GD = 0.74). A neighbor-joining dendrogram separated the genotypes into three major clusters, with further sub-clustering within each cluster.
Conclusions: Overall, the results presented here show significant genetic diversity among wild pomegranate accessions in Azad Jammu and Kashmir region of Pakistan. These accessions present a valuable genetic resource to breeding and cultivar improvement programs within the region.
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1. Introduction

Plant biodiversity is extensive and often occurs in biological hotspots [1,2,3]. Numerous efforts have been made to categorize diversity of fruit trees based on their anatomy, biochemical and molecular features; thereby unraveling evolutionary relationships of species and guiding conservation priorities, especially of wild or rare species [4,5,6,7,8]. Pomegranate (*Punica granatum* L.) is a tropical and subtropical tree cultivated for its delicious fruits, medicinal properties, ornamental value and religious reasons [9,10]. The primary center of origin is Iran's Transcaucasia-Caspian region [11]. The region from Iran through Turkmenistan to Western Himalayas in northern India is considered a secondary origin for pomegranate [12]. In Azad Jammu and Kashmir (Iran), wild pomegranates are well established across various agro-ecological zones. Many people depend on the high levels of important phytochemicals present in the fruit for curative purposes and as a source of nutrition [13]. However, human exploitation of natural resources in Iran has led to environmental degradation, and consequently threatened wild pomegranate populations in natural habitats [14]. Due to the absence of a germplasm conservation program for wild pomegranate, there are currently no cultivar improvement efforts utilizing wild pomegranates in Azad Kashmir. This is despite the wide biochemical and morphological diversity within wild pomegranate accessions that could be harnessed to develop superior cultivars for the pomegranate industry in the region. In contrast, neighboring countries such as India, Afghanistan and China that have established collections of wild pomegranate routinely exploit wild pomegranate germplasm to breed high yielding cultivars of superior fruit quality [15]. Characterization of genetic diversity within wild pomegranate in Azad Kashmir would inform germplasm

* Corresponding author.
E-mail address: gmeru@ufl.edu (G. Meru).
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conservation efforts and sound breeding strategies for cultivar improvement in the region.

Variability in fruit morphology and biochemical composition is evident among collections of wild pomegranates in Arab and Indian peninsula. For example, in a study examining phenotypic variation among wild and cultivated Iranian pomegranates, Zamani et al. [16] reported wide variation in fruit size, fruit peel, anthocyanin content, total soluble solids, aril juice content and seed hardness. Similar phenotypic variation has been reported within wild pomegranate collections of Pakistan [17,18] and India [19].

Numerous genetic markers have been utilized to characterize diversity of wild pomegranates including dominant markers such as random amplified polymorphic DNA, amplified fragment-length polymorphism [20], inter simple sequence repeat [21], directed amplification of minisatellite DNA [22] and sequence-related amplified polymorphism markers [23]. However, codominant markers such as simple sequence repeats markers (SSR) are easier to use, more informative and reproducible than dominant markers [24,25,26,27,28], thus are preferred for diversity analyses of pomegranate germplasm [29]. For example, SSR markers have been widely used to elucidate variation within and among collections of wild and domesticated pomegranate genotypes in Iran [30,31], India [32] and Pakistan [17]. However, genetic characterization of wild pomegranates from Azad Jammu and Kashmir region of Pakistan is currently lacking.

The goal of current study was to determine the genetic diversity among wild pomegranate accessions in Azad Jammu and Kashmir using SSR markers to aid germplasm conservation efforts, as well as inform best strategies for incorporating beneficial traits into pomegranate breeding programs.

2. Materials and methods

2.1. Collection of plant material

Forty-eight wild pomegranate accessions from 24 localities in Azad Jammu and Kashmir, Pakistan were used in the current study. The sampling locations were selected to represent variable micro-climatic conditions in the region [33]. Two young leaves from each accession were sampled in the field, preserved in silica gel in a zipper bag, and stored at −80°C until DNA extraction.

2.2. DNA isolation

Genomic DNA extraction was done using GeneJET® Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham MA) following manufacturer's protocol. Briefly, 100 mg of frozen leaf tissue was ground with liquid nitrogen using a mortar and pestle and the powder placed in a 2-ml microtube. Lysis buffer was added, and the samples were incubated at 65°C for 10 min. Plant debris and other soluble impurities were removed by precipitation and centrifugation before a DNA binding buffer was added to the samples. Finally, the DNA was bound to a spin column, washed twice and eluted with 50 μl of elution buffer. The quantity and quality of the extracted DNA was determined using a Nanodrop One® spectrophotometer (Thermo Fisher Scientific) and on a 0.8% (*w*/*v*) agarose gel, respectively.

2.3. PCR and SSR data analysis

A total of 59 SSR markers were selected based on marker polymorphism and allele richness from previously published sources [32,34,35,36,37,38]. All forward primers were tagged with a M13 sequence to incorporate a fluorescent dye and allow for capillary electrophoresis of PCR products [39]. For each primer pair, PCR was performed in a 15-μl reaction containing 25 ng of template DNA, 0.32 mM of a fluorescently (either 6-FAM, VIC, or PET) labeled M13 forward primer (GCCTCCCTCGCGCCA) [40], 0.08 mM of M13-tagged forward primer, 0.4 mM unlabeled reverse primer, and 1-μl PROMEGA Colorless GoTaq® master mix (Promega, Madison, WI). Depending on band intensity for each primer pair on an agarose gel (2% *w*/*v*), products were diluted appropriately for capillary electrophoresis. Amplification products for three primer pairs, each labeled with a different fluorescent dye, were multiplexed and combined with a GeneScan-600 ROX internal-lane size standard and Hi-Di Formamide before analysis on a ABI 3730 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA) at the Gene Expression and Genotyping Core facility, University of Florida. Peak Scanner™ v2.0 software (Thermo Fisher Scientific) was used for size estimation and allele calling.

Allelic data was analyzed with PowerMarker V3.25 [41] to obtain summary statistics for each SSR marker that included; major allele frequency, number of alleles, heterozygosity and polymorphic information content (PIC). Additionally, a dissimilarity matrix calculated by simple matching coefficient [42] was used to generate a weighted neighbor-joining dendrogram and perform principle coordinate analysis in DARWin V6.0 software [43]. Confidence limits of different clades were tested by bootstrapping 1000 times to assess the repetitiveness of genotype clustering [44].

Table 1
Summary statistics of 41 SSR markers used for genetic diversity analysis among 48 wild pomegranate accessions.

SSR	Major allele frequency	Genotype number	Allele number	Gene diversity	Heterozygosity	PIC[a]
PGCT093B	0.94	3	3	0.12	0.00	0.12
EPS17	0.90	4	4	0.18	0.04	0.17
PgAER121	0.43	6	6	0.68	0.00	0.62
PgAER138	0.88	5	5	0.23	0.08	0.22
PgAER154	0.61	8	8	0.59	0.31	0.56
PgAER154	0.53	8	7	0.67	0.06	0.64
PgAER194	0.41	16	11	0.75	0.47	0.72
PgAERB3	0.55	7	6	0.64	0.60	0.60
PgAERB7	0.31	18	12	0.81	0.36	0.79
PGCT015	0.58	18	13	0.65	0.26	0.64
PGCT016	0.64	4	4	0.53	0.00	0.48
PGCT021	1.00	1	1	0.00	0.00	0.00
PGCT023	0.63	16	15	0.58	0.29	0.57
PGCT025	0.81	7	7	0.33	0.17	0.32
PGCT028	0.38	14	10	0.74	0.52	0.70
PGCT031A	0.33	10	8	0.78	0.29	0.75
PGCT032	0.23	14	9	0.82	0.31	0.80
PGCT037A	0.51	7	5	0.65	0.21	0.60
EPS01	1.00	1	1	0.00	0.00	0.00
PGCT046	0.75	4	4	0.41	0.02	0.37
PGCT057	0.39	12	6	0.74	0.37	0.70
PGCT059	0.74	5	5	0.43	0.04	0.40
PGCT062	0.48	14	12	0.70	0.21	0.67
PGCT080	0.70	10	8	0.48	0.14	0.47
PGCT087	0.50	9	6	0.67	0.12	0.62
EPS06	0.56	3	3	0.50	0.02	0.39
PGCT093Ab	0.22	24	13	0.88	0.38	0.87
PGCT093B	0.77	10	7	0.39	0.24	0.38
PGCT097	0.78	6	6	0.38	0.40	0.36
PGCT104	0.86	3	3	0.24	0.27	0.23
PGCT109A	0.41	9	7	0.74	0.18	0.71
PGCT109B	0.68	6	5	0.50	0.13	0.47
PGCT110	0.17	9	9	0.87	0.00	0.86
PGCT112	0.27	20	11	0.84	0.40	0.82
EPS08a	0.80	5	4	0.34	0.04	0.30
PgSSR16	0.51	9	6	0.66	0.10	0.61
PgSSR19	0.37	18	10	0.77	0.65	0.74
PgSSR23	0.52	11	9	0.69	0.26	0.66
PgSSR30	0.56	10	8	0.64	0.64	0.62
Pom006	0.20	18	14	0.89	0.23	0.88
Pom021	0.20	15	12	0.87	0.25	0.86
Mean	**0.56**	**9.68**	**7.39**	**0.57**	**0.22**	**0.54**

[a] PIC = polymorphic information content.

3. Results and discussion

Following amplification and allelic scoring, markers with more than 60% missing data were dropped, leaving 41 SSR loci for further analyses. Interestingly, SSR marker PGAER154 generated alleles at two different loci. SSR amplification at multiple loci has been previously reported in pomegranate [45] and *Prunus* [46]. In total, the markers revealed 303 alleles ranging from one allele per loci (PGCT021 and EPS01) to 15 alleles per loci (PGCT023), with an average of 7.39 alleles per loci across all markers (Table 1). This number is slightly higher than that (6.31 alleles per marker) reported for 13 SSR markers used to determine genetic diversity among 136 pomegranate accessions collected across seven countries [47]. Ravishankar et al. [48] reported a lower average number of alleles per loci (5.56 alleles) across 171 SSR markers while examining the genetic diversity among 12 Indian pomegranate accessions. Polymorphic information content ranged from 0.12 (PGCT093B) to 0.88 (Pom006) with an average PIC of 0.54 across all markers. The discrimination power (average PIC) of the markers observed in the present study was higher than that (0.19–0.43) reported for SSR markers across many genetic diversity studies incorporating wild, landraces and cultivated pomegranate accessions [31]. The higher PIC observed in the current study support evidence of greater allelic richness and gene diversity in wild pomegranate than in cultivated or landrace genotypes [21,22,49]. This is expected due to high cross pollination levels in wild pomegranate populations compared to cultivated pomegranates which are primarily clonally propagated [29].

The average genetic distance (GD) across the genotypes was 0.52 (Fig. 1; Table S1). The most genetically similar genotypes were Chattar Class and Thorar (GD = 0.27), while the most dissimilar (GD = 0.74) were Khun Bandway and Akhor Ban. A dendrogram constructed based on the genetic distances produced three distinct clusters (Fig. 2). Cluster I contained 25 accessions, which further diverged into four sub-clusters. Chatiyan and Nambal shared more alleles than with other samples in sub-cluster I. Rawalkot, included as a check, grouped with Chattar Class while Punjgaran and Narouri grouped with Langarpura and Rarah, respectively. In the second sub-cluster, Saran, Gojra and Deerkot formed a separate group from Androot and Chaman Kot. Dar, Dhalkot and Kohala accessions were closer to Rerrah and Sarlay, than to Khun Bandway, Mang and Kotera in sub-cluster III. Sub-cluster IV had three samples from Darh Bazar, Prat and Chota Gala. Cluster II had two sub-clusters, wherein sub-cluster I showed tighter grouping of Tandali and Patan compared to other members, while sub-cluster II consisted of Hatiyan Bala, Thorar, Arja and Rangla. Cluster III also had two sub-clusters, the first showing closer association among five accessions (Chinari, Garhi Dupatta, Patika, Daaana and Chatiyan), while the second showing grouping of three accessions (Paniola, Ghazi Abad and Khai Gala).

Pomegranate accessions in the dendrogram clustered independent of their geographical origin. Wild pomegranate populations established a long time ago in Azad Jammu and Kashmir, as these regions lie along ancient human migratory/trade routes responsible for distribution of pomegranate seeds [50]. The whole region from Iran to northern India is regarded as a center of origin for pomegranate and existing natural forests contain many wild pomegranate populations [51,52]. Consequently, analyses of diversity in wild pomegranate from nearby countries such as India [22,49], China [53] and Iran [16] report clustering patterns unrelated to geographical origin of samples. High dissimilarity values between neighboring localities may signify low possibility of identity by descent [49], underscoring uniqueness of the genetic resources present in each region.

These results reveal considerable genetic diversity among wild pomegranate accessions in Azad Jammu and Kashmir. It is therefore essential to pay attention to distinct accessions from each locality for germplasm conservation initiatives. Furthermore, combining this genetic information with various consumer-oriented fruit traits observed in these accessions (data not shown) will be beneficial for the improvement of existing pomegranate cultivars through breeding.

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Conflict of interest

The authors declare no conflict of interest.

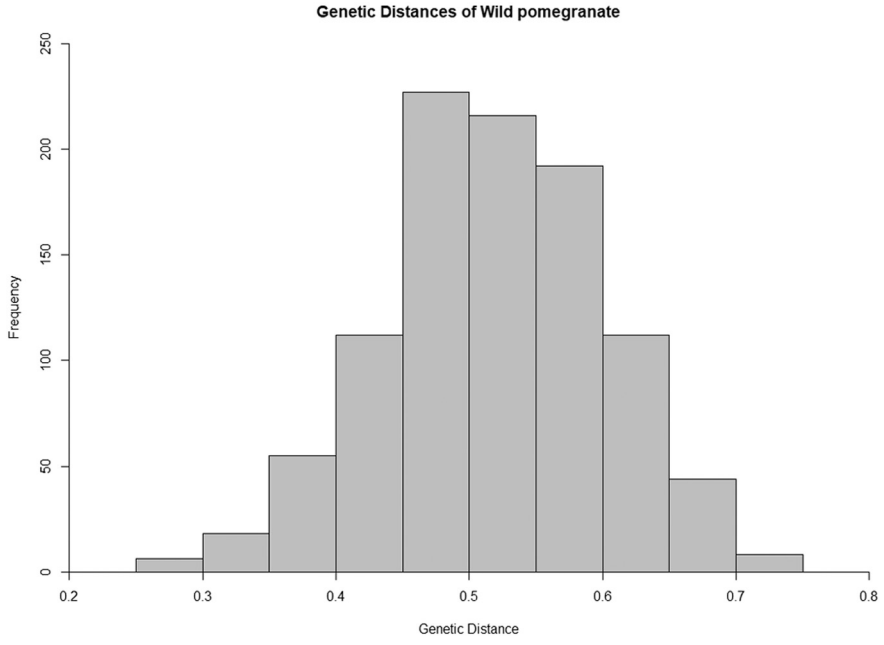

Fig. 1. Frequency distribution of pairwise genetic distances between wild pomegranate accessions used in the study.

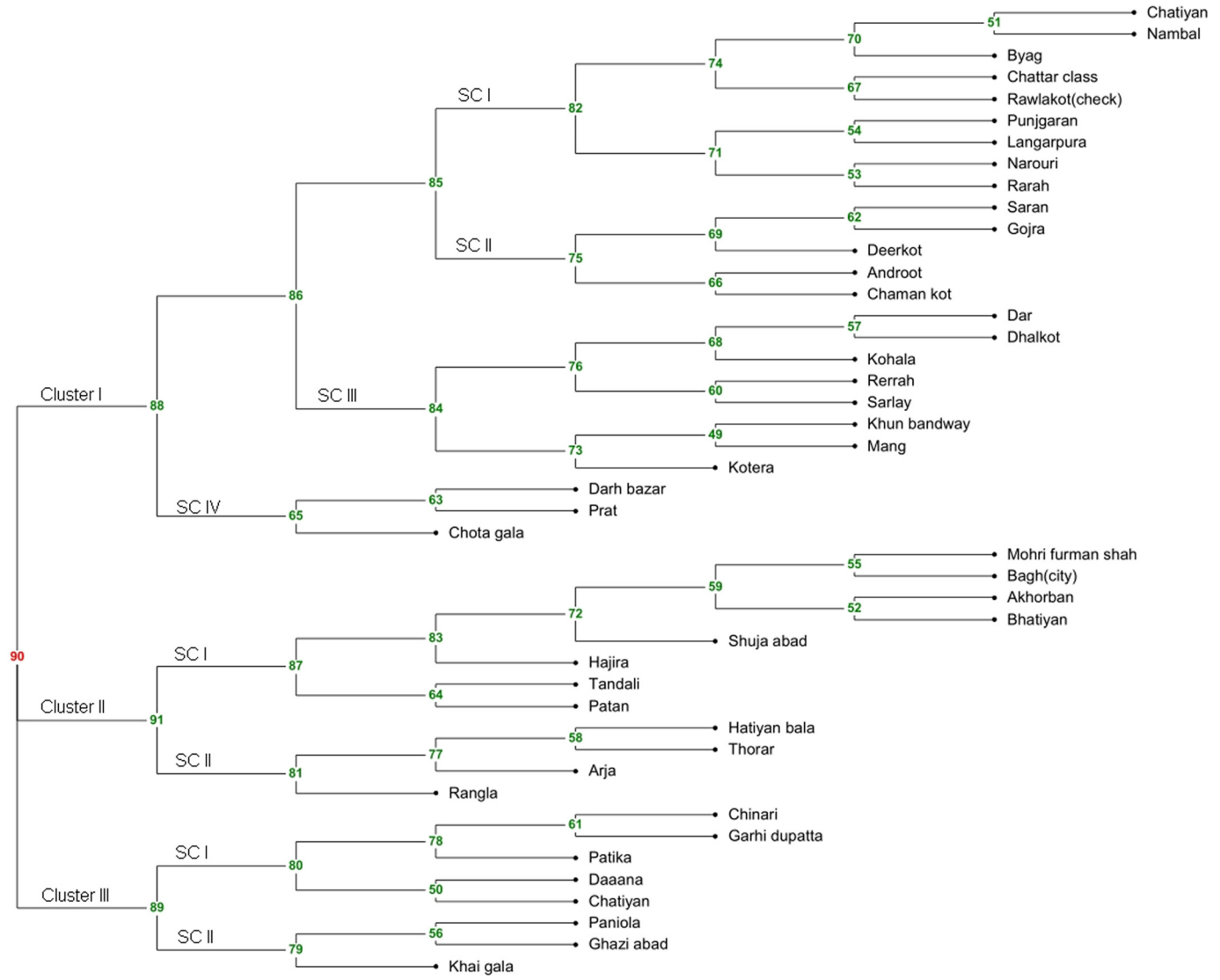

Fig. 2. Neighbor-joining dendrogram displaying separation of wild pomegranate genotypes into three major clusters and sub-clusters. Numbers at the nodes represent confident intervals obtained from bootstrap resampling. SC = Sub-cluster.

Supplementary material

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