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Molecular characterization to assess genetic diversity in wild pomegranate of H.P

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Abstract
Genetic diversity in wild pomegranate selections of H.P was carried out using randomly amplified polymorphic DNA (RAPD) markers. Out of 25 random decamer primers used, only 19 produced polymorphism. Total number of bands amplified was 142, out of which 116 were polymorphic and 19 were specific RAPD markers. The amplified fragments ranged in size from 178-3895 bp and percentage of total polymorphic bands was 70. All the 24 selections of the six sites were distinguished with the combination of 19 primers selected in this study. Similarity matrix was constructed using Dice and Jaccard coefficient. It ranged from 0.42 -0.91 (Jaccard coefficient) and 0.60-0.92 (Dice coefficient). Low similarity value was obtained between Rajgarh-3 and Kandaghat-2 and high similarity was between Badiyal-2 and Shoghi-4 selections. Dendrogram was constructed by using UPGMA method for the clustering for all the selections. All the selectionss were grouped together except for Rajgarh-3, which formed separate cluster. Relationship between individual site and its selections were not clear from the dendrogram, for this purpose individual dendrogram between single site and its selections was constructed. From the data obtained in this study it can be concluded that RAPD studies can be useful in breeding programmes allowing the identification of different selections and assessing the genetic similarity among them, which would facilitate their use as identified genetic stock in future breeding programmes.

Keywords: molecular characterization, genetic diversity, wild pomegranate

Introduction
Wild pomegranate, *Punica granatum* L. belongs to the family Punicaceae and has acquired few commonly recognized vernacular names apart from its many regional epithets in India, most of which are *dadima, dalim*, daru or darim. To improve the crop with our desirable character it is important to evaluate the genetic diversity present in the nature. Traditionally genetic diversity in fruit crop is tested through morphological methods, time consuming physiological assay that is greatly influenced by environmental factors at different stages of plant growth. In contrast, molecular markers based on DNA sequence polymorphism are independent of environmental conditions. DNA based molecular markers has two broad categories, first is nucleic acid restricted fragment length polymorphism (Beckman and Soller, 1983) [1] the second group is PCR based on DNA amplification techniques (Rafalski and Tingey, 1993) [8]. These markers are extensively used in DNA fingerprinting and genetic diversity studies as they provide imambigous reliable, fast and cost effective assessment which is quite important for determining uniqueness and distinctiveness of species. Durgac *et al.* (2008) evaluated molecular and pomological diversity among pomegranate cultivars of Turkey. 18 quantitative fruit characteristics were studied and it was found that these characters successfully discriminate the cultivars. Twenty-two RAPD primers generated 106 bands, of which 22% were polymorphic. Dendrogram showed that the Tathnar and Gerife were closely related while Gncekabuk was distinct from other cultivars. Kanwar *et al.* (2008) [6] used RAPD method to study genetic variability among wild and cultivated pomegranate (viz., Ganesh, G-137, Mridula, Musket and Kandhari Kabuli) growing in Western Himalyan region of India. They found 76.26% polymorphism. Jaccard coefficient and UPGMA analysis showed 0.62 to 0.83% similarities among these genotypes. Kandhari and Mridula showed maximum similarity (0.83) and Ganesh and G-137 showed minimum similarity (0.64). In the dendrogram there was one main cluster and four subclusters. Wild pomegranate was found to be distant from the cultivars and grouped alone at 63% similarity level.

Materials and Methods

The plant material for present studies was collected from different geographical regions of Himachal Pradesh. Three districts from Himachal Pradesh were selected viz., Solan, Shimla and Sirmour. From each district, two locations were selected and from each location, four plants were selected which were at least 200 meters apart.

Different locations selected with their altitudes are tabulated in Table1.

Table 1: Geographical location of six different sites of *Punica granatum*

S. No	District	Location	Altitude (meter)
1	Sirmour	Rajgarh	1345
2		Narag	1250
3	Solan	Kandaghat	1150
4		Darlaghat	1250
5	Shimla	Shoghi	950
6		Badiyal	1050

Different sites were given different code numbers to maintain their distinct individuality during the course of present investigation.

Table 2: Sites, District and codes of collections of *Punica granatum*

Sr. No	Location	District	Code
1	Rajgarh-1	Sirmour	R-1
2	Rajgarh-2	Sirmour	R-2
3	Rajgarh-3	Sirmour	R-3
4	Rajgarh-4	Sirmour	R-4
5	Narag-1	Sirmour	N-1
6	Narag-2	Sirmour	N-2
7	Narag-3	Sirmour	N-3
8	Narag-4	Sirmour	N-4
9	Kandagaht-1	Solan	K-1
10	Kandagaht-2	Solan	K-2
11	Kandagaht-3	Solan	K-3
12	Kandagaht-4	Solan	K-4
13	Darlaghat-1	Solan	D-1
14	Darlaghat-2	Solan	D-2
15	Darlaghat-3	Solan	D-3
16	Darlaghat-4	Solan	D-4
17	Shoghi-1	Shimla	S-1
18	Shoghi-2	Shimla	S-2
19	Shoghi-3	Shimla	S-3
20	Shoghi-4	Shimla	S-4
21	Badiyal-1	Shimla	B-1
22	Badiyal-2	Shimla	B-2
23	Badiyal-3	Shimla	B-3
24	Badiyal-4	Shimla	B-4

Isolation of genomic DNA of *Punica granatum* from fresh young leaves was carried out using CTAB method (Doyle and Doyle, 1987) [3] with certain modifications. DNA amplification was done using 25 random 10 base oligonucleotide primers by RAPD PCR technology (William *et al.,* 1990) in Perkin Elmer Gene Amp 9700 PCR system version 3.03. The polymerase chain reaction contained in a reaction volume of 20 µl, 7.9 µl of sterile distilled water, 2.0 µl Taq DNA polymerase buffer (1× containing 1.5 mM $Mgcl_2$), 1.6 µl dNTPs mixture (200 µM of each dNTPs), 2.00 µl of random primer (10 pmole), 1.5 µl Taq DNA polymerase (1.5 U) and 5.0 µl genomic DNA (50 ng). PCR was carried out in a thermal cycler with a total of 45 cycles. Each cycle consisted of 1 minute of denaturation at 94 °C, 1 minute of annealing at 40 °C and 2 minutes of extension at 72 °C. All the PCR samples were given 5 minutes pre-amplification at 94 °C and 10 minutes post-amplification at 72°C. The amplification products were separated by electrophoresis in agarose gel (1.6%) under submerged conditions. The gel and tray buffer used were 50× TAE buffer. To each PCR amplified DNA, 4 µl of 6× loading dye was added and about 20 µl of sample was loaded into each well. 1 Kbp marker (DNA ladder, Fermentas) was used as standard and gel was run at 80 V constant voltage until the loading dye reached the gel front. After the run was over, the gel was viewed under UV light in a trans illuminator and the image was taken through Bio-Rad, Fluoro-S Multi Imager.

Amplified DNA fragments (bands) were scored for their presence or absence. Co-migrating bands were considered to represent the same locus and thus considered as same band while scoring. DNA fragment profiles were scored in binary fashion with '0' indicating absence and '1' indicating the presence of a band. Intensity of amplified products were not taken into account while scoring. Similarity matrix was constructed from the binary data using Dice and Jaccard coefficients (Dice, 1945 [2]; Jaccard, 1908) [5]. This was further subjected to UPGMA cluster analysis and dendrograms were constructed. All the above analysis was done using SIMQUAL programme of NTSYS (Numerical Taxonomy and Multivariate Analysis System) pc (Version 2.0).

Result

Genetic diversity study among the 24 selections of wild pomegranate was carried out using *25* random decamer oligonucleodtide primers of Operon series. Only 19 random primers were able to amplify the genomic DNA successfully. All the primers were found to be polymorphic in nature and they generated unique set of amplification products ranging in size from 178-3895 bp. Number of bands for each primer ranged from 1 to 13. Total number of bands obtained with all the primers was 142. Out of these 142 bands 116 were polymorphic and 26 monomorphic. Maximum numbers of bands (13) were obtained with primer OPA-14 and minimum number of bands (1) was obtained with primer-OPC-17.

RAPD pattern with OPA primer series

Twelve random primers of OPA series were used out of which nine primers viz., OPA-01, OPA-07, OPA-08, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14 and OPA-15 amplified the genomic DNA.

Table 12: Total number of amplified bands and number of polymorphic bands generated by PCR using 19 random decamer primers

Primer	Sequence 5'-3'	No. of amplified bands	No. of polymorphic bands	Size (bp)
OPA-01	-CAGGCCCTTC-	8	4	327-1394
OPA-07	-GAAACGGGTG-	12	12	457-2284
OPA-08	-GTGACGTAGG-	7	6	178-920
OPA-10	-GTGATCGCAG-	7	6	214-2159
OPA-11	-CAATCGCCGT-	10	8	318-2457
OPA-12	-TCGGCGATAG-	5	4	702-1281

OPA-13	-CAGCACCCAC-	5	4	526-1809
OPA-14	-TCTGTGCTGG-	13	13	304-2021
OPA-15	-TTCCGAACCC-	4	3	541-2306
OPB-10	-CTGCTGGGAC-	9	9	503-1536
OPB16	-TTTGCCCGGA-	7	6	389-2454
OPB-19	-ACCCCCGAAG-	111	Q	406-3895
OPC-13	-AAGCCTCGTC-	5	1	250-1267
OPC-17	-TTCCCCCCAG-	1	1	1023
OPD-03	-GTCGCCGTCA-	7	6	367-1913
OPD-06	ACCTGHACGG-	5	5	590-2283
OPD-11	AGCGCCATTG-	9	4	317-2174
OPD-13	-AAGGGTCGTC-	9	6	641-3503
OPD-14	-GAGAGCCAAC-	8	7	725-2289
Total		142	116	

20. Summary of RAPD amplified products obtained from 24 collections of *Punica granatum* using 19 random decamer primers

Description	Number of bands scored	Number of monomorphic bands	Number of polymorphic bands	Average number of fragments per primer	Average no. of polymorphic bands per primer	Percentage of polymorphic bands	Number of unique bands
Rajgarh-1	98	26	72	5.15	3.78	0.73	1
Rajgarh-2	86	26	60	4.52	3.15	0.69	0
Rajgarh-3	62	26	36	3.26	1.89	0.57	0
Rajgarh-4	87	26	61	4.57	3.21	0.70	0
Narag-1	99	26	73	5.21	3.84	0.73	0
Narag-2	100	26	74	5.26	3.89	0.74	1
Narag-3	107	26	81	5.63	4.26	0.75	4
Narag-4	103	26	77	5.42	4.05	0.74	1
Kandaghat-1	88	26	62	4.63	3.26	0.70	0
Kandagaht-2	79	26	53	4.15	2.78	0.66	0
Kadagaht-3	100	26	74	5.26	3.89	0.73	3
Kandagaht-4	94	26	68	4.94	3.57	0.72	4
Darlaghat-1	94	26	68	4.94	3.57	0.72	1
Darlaghat-2	96	26	70	5.04	3.68	0.73	0
Darlaghat-3	94	26	68	4.94	3.57	0.73	1
Darlaghat-4	104	26	78	5.47	4.10	0.74	0
Shoghi-1	105	26	79	5.52	4.15	0.75	3
Shoghi-2	71	26	45	3.73	2.36	0.63	1
Shoghi-3	83	26	57	4.36	3.00	0.68	3
Shoghi4	106	26	80	5.57	4.20	0.75	1
Badiyal-1	104	26	78	5.47	4.10	0.74	6
Badiyal-2	112	26	86	5.89	4.52	0.76	2
Badiyal-3	107	26	81	5.63	4.26	0.75	6
Badiyal-4	105	26	79	5.52	4.15	0.75	1

Similarity Matrix

Similarity coefficient was calculated by using Dice coefficient and Jaccard coefficient. Similarity value ranged from 0.42 - 0.91 (Jaccard coefficient) and 0.60-0.92 (Dice coefficient). Minimum similarity was obtained between Rajgarh-3 and Kandaghat-2 and maximum similarity was observed between Badiyal-2 and Shoghi-4, which could be explained on the basis of differences in geographical distances between different locations which support the presence of diversity in wild pomegrante. Similarity coefficient calculated by Sarkhosh *et al.* (2009) [9] in twenty one Pomegranate accessions ranged from 0.13 to 1.00, Zamani *et al.* (2007) also calculated similarity matrix and it ranged from 0.30 – 0.88 in twenty four Iranian Pomegranate genotypes and Narzary *et al.* (2009) [7] calculated similarity coefficient and it varied from 0.08 and 0.79 across different accessions of wild pomegranate of Western Himalayas.

Cluster analysis based on RAPD profile

In the dendrogram two clusters i.e. A and B were obtained. Cluster A included all the collections except Rajgarh-3. Collection Rajgarh-3 was included in another cluster i.e. B. The dendrogram showed that geographical diversity of the collections is not corroborating with genetic diversity, and wild pomegranate plants are independent of their geographical affiliation. This could be because of human intervention, which has made partitioning and distribution of variability complex. Dendrogram obtained by Sarkhosh *et al.* (2009) [9] grouped 21 accessions into 4 groups, 16 accessions in one group, 3 accessions in one group and other 2 in separate group. Narzary *et al.* (2009) [7] obtained 2 main clusters, while studying genetic diversity in wild pomegranate of Western Himalayan region, one had grouped all the accessions from Uttrakhand and second main cluster contained accessions from Himachal Pradesh. Kanwar *et al.* (2008) [6] characterized and filled the gap and obtained one main cluster and four sub clusters, wild pomegranate was found to be distant from rest of the cultivars (Ganesh, G-137, Mridula, Musket, Kandhari Kabuli).

Intersite cluster analysis

In the dendrogram two clusters i.e. A and B were obtained. Cluster A included all the collections except Rajgarh-3. Collection Rajgarh-3 was included in another cluster i.e. B.

Intra site cluster analysis

Genetic relatedness among four collections of *Punica granatum* in Rajgarh (district Sirmour)

The pair wise genetic distances obtained based on Dice coefficient by combined scores of all the 19 informative primers were used for clustering collections of Rajgarh site by using UPGMA methods. In the dendrogram two clusters i.e. A and B were obtained. Collection Rajgarh-3 formed one cluster i.e. A and Rajgarh-1, Rajgarh-2 and Rajgarh-4 formed another cluster i.e. B (Fig- 9).

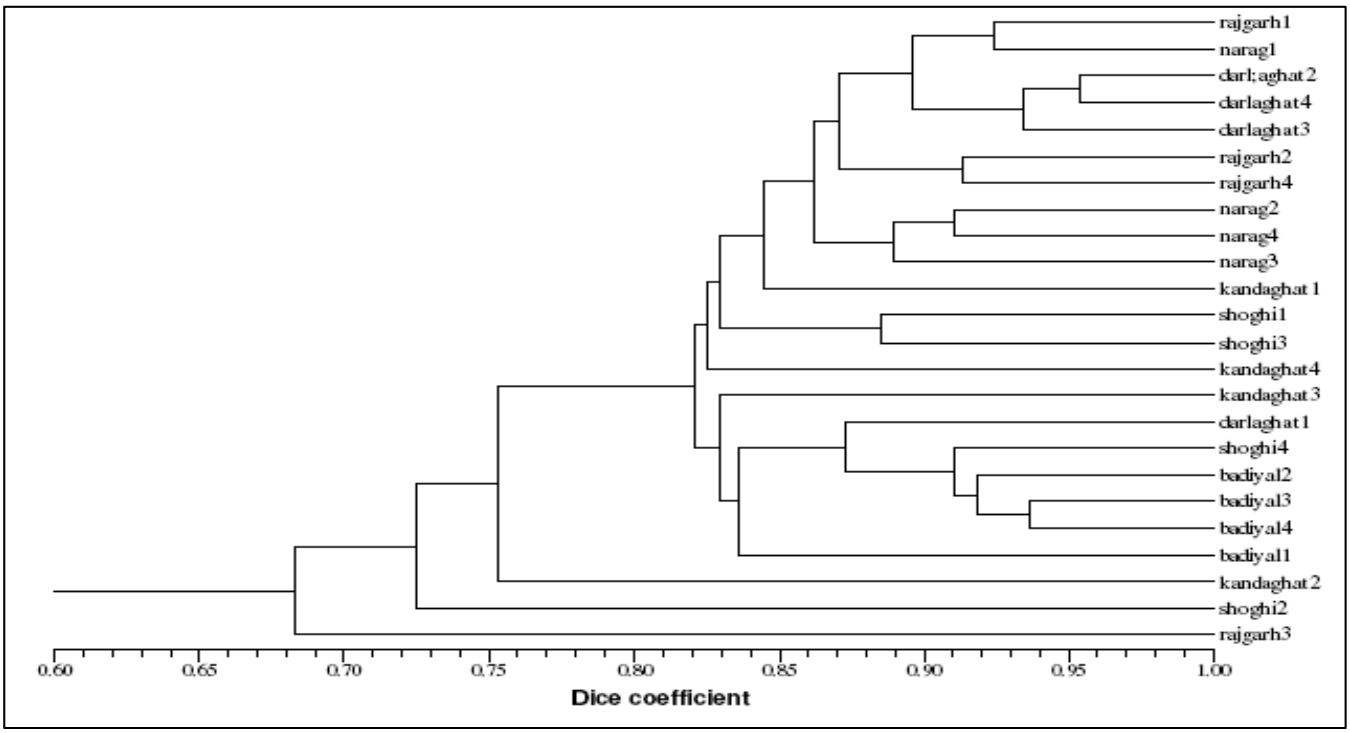

Fig 9: RAPD derived dendrogram by using dice coefficient showing relationship among 24 collection of *Punica granatum*

Genetic relatedness among four collections of *Punica granatum* in Narag (district Sirmour)

The pair wise genetic distances obtained based on Dice coefficient by combined scores of all the 19 informative primers were used for clustering collections of Narag site by using UPGMA methods. In the dendrogram two clusters i.e. A and B were obtained. Narag-1 and Narag-3 formed one cluster i.e. A and Narag-2 and Narag-4 formed another cluster i.e. B (Fig-10).

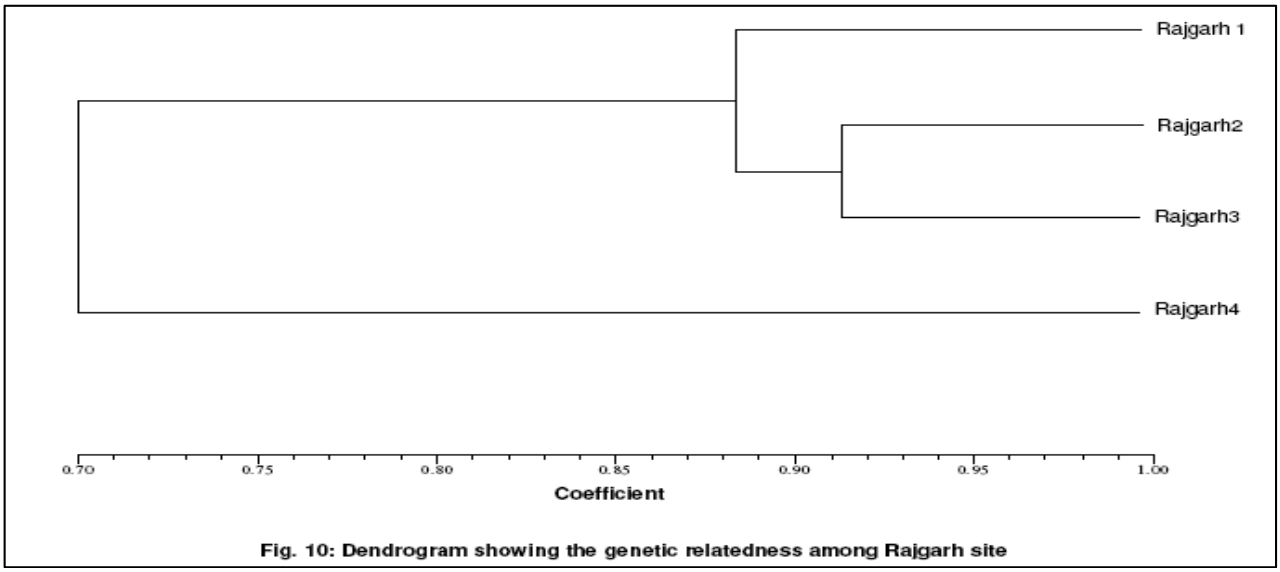

Fig. 10: Dendrogram showing the genetic relatedness among Rajgarh site

Genetic relatedness among four collections of *Punica granatum* in Kandaghat, (district Solan)

The pair wise genetic distances obtained based on Dice coefficient by combined scores of all the 19 informative primers were used for clustering collections of Kandagaht site by using UPGMA methods**.** In the dendrogram two clusters i.e. A and B were obtained. Kandagaht-1 and Kandagaht-2 formed one cluster i.e. A and Kandagaht-3 and Kandaghat-4 formed another cluster i.e. B (Fig-11).

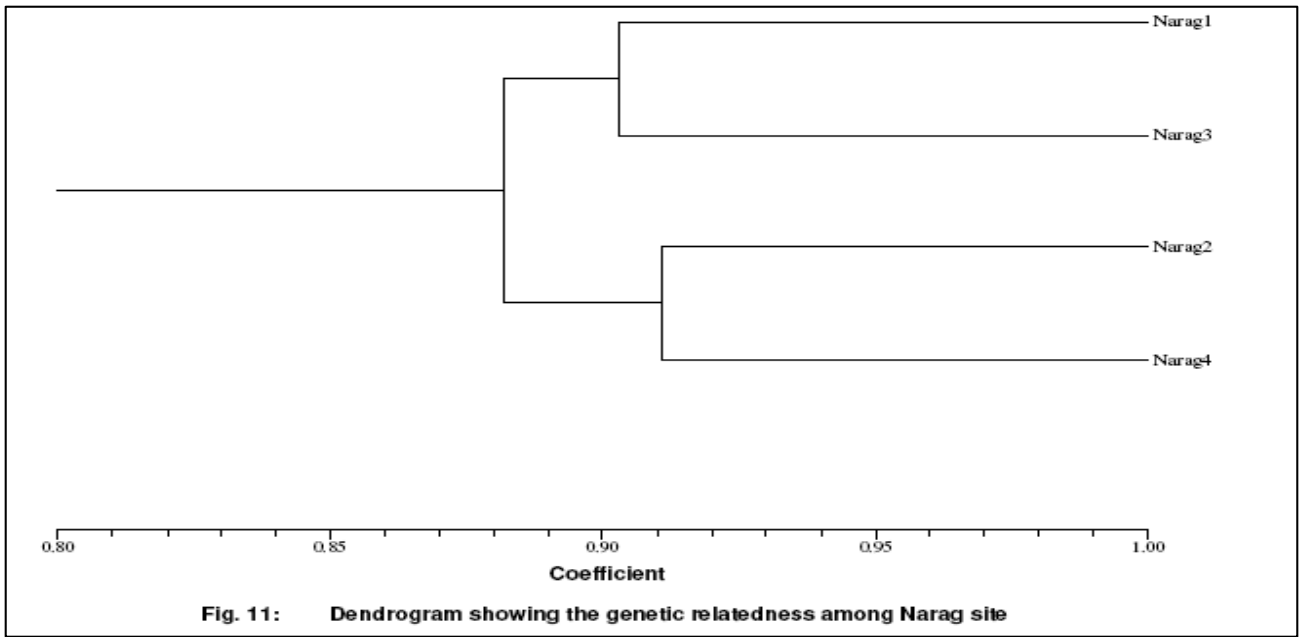

Fig. 11: Dendrogram showing the genetic relatedness among Narag site

Genetic relatedness among four collections of *Punica granatum* in Darlaghat (District Solan).

The pair wise genetic distances obtained based on Dice coefficient by combined scores of all the 19 informative primers were used for clustering collections of Darlaghat site by using UPGMA methods. In the dendrogram two clusters i.e. A and B were obtained Darlagaht-1 formed one cluster i.e. A and Darlagaht-2, Darlagaht-3 and Darlagaht-4 formed another cluster i.e. B (Fig-12).

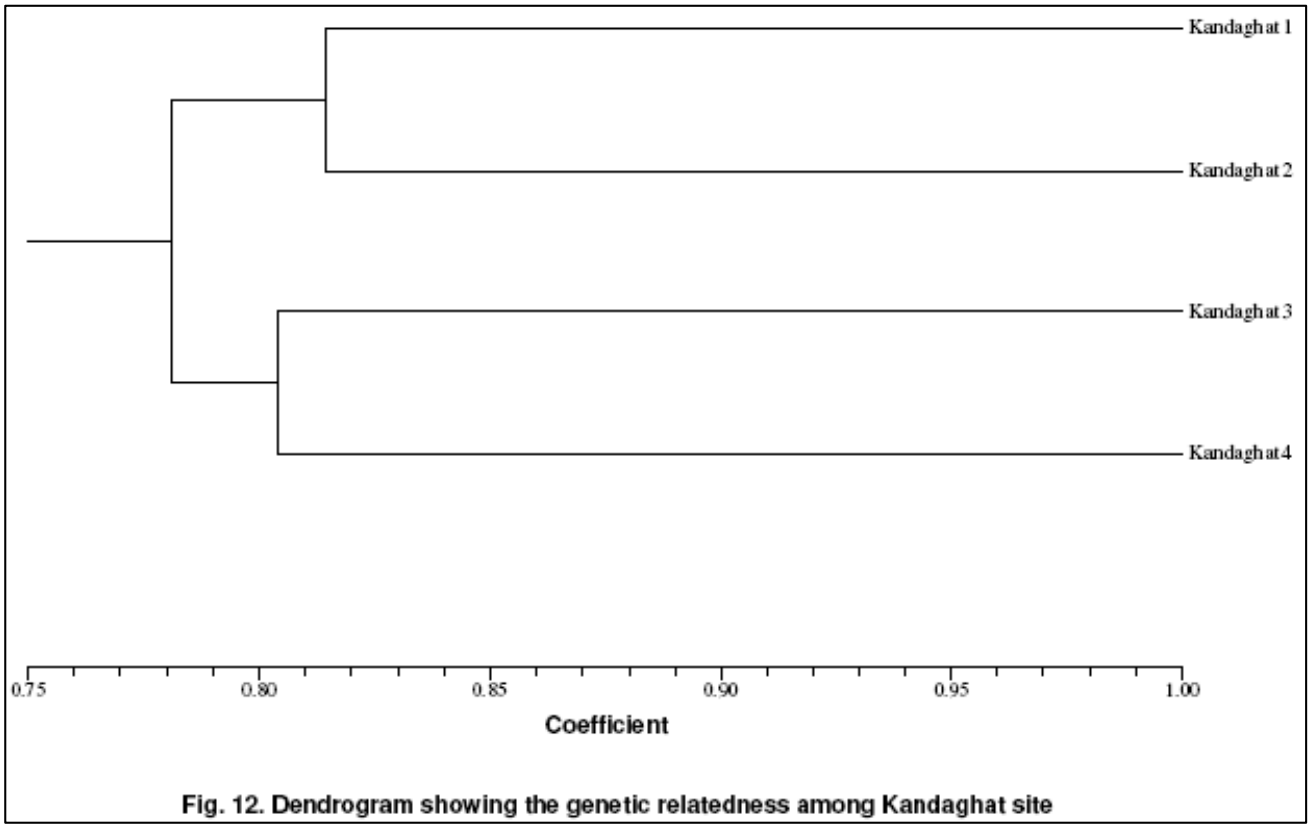

Fig. 12. Dendrogram showing the genetic relatedness among Kandaghat site

Genetic relatedness among four collections of *Punica granatum* in Shoghi (district Shimla)

The pair wise genetic distances obtained based on Dice coefficient by combined scores of all the 19 informative primers were used for clustering collections of Shoghi site by using UPGMA methods. In the dendrogram two clusters i.e. A and B were obtained. Shoghi-2 formed one cluster i.e. A and Shoghi-1, Shoghi-3 and Shoghi-4 formed another cluster i.e. B (Fig-13).

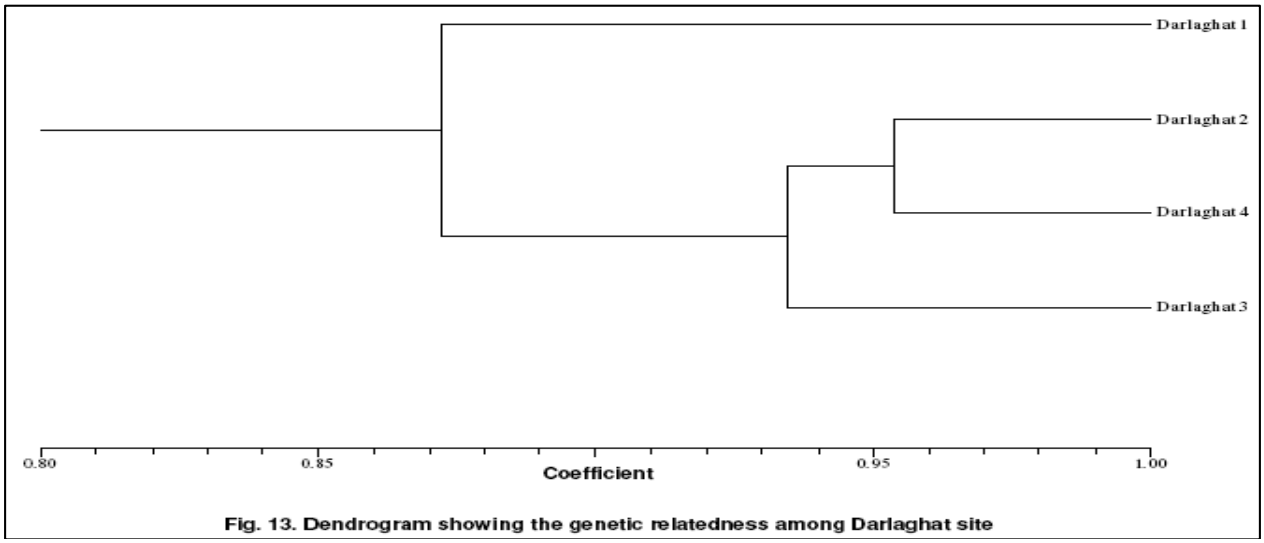

Fig. 13. Dendrogram showing the genetic relatedness among Darlaghat site

Genetic relatedness among four collections of *Punica granatum* in Badiyal (Distt Shimla).

The pair wise genetic distances obtained based on Dice coefficient by combined scores of all the 19 informative primers were used for clustering collections of Badiyal site by using UPGMA methods. In the dendrogram two clusters i.e. A and B were obtained. Badiyal-1 formed one cluster i.e. A and Badiyal-2, Badiyal-3 and Badiyal-4 formed another cluster i.e. B (Fig-14).

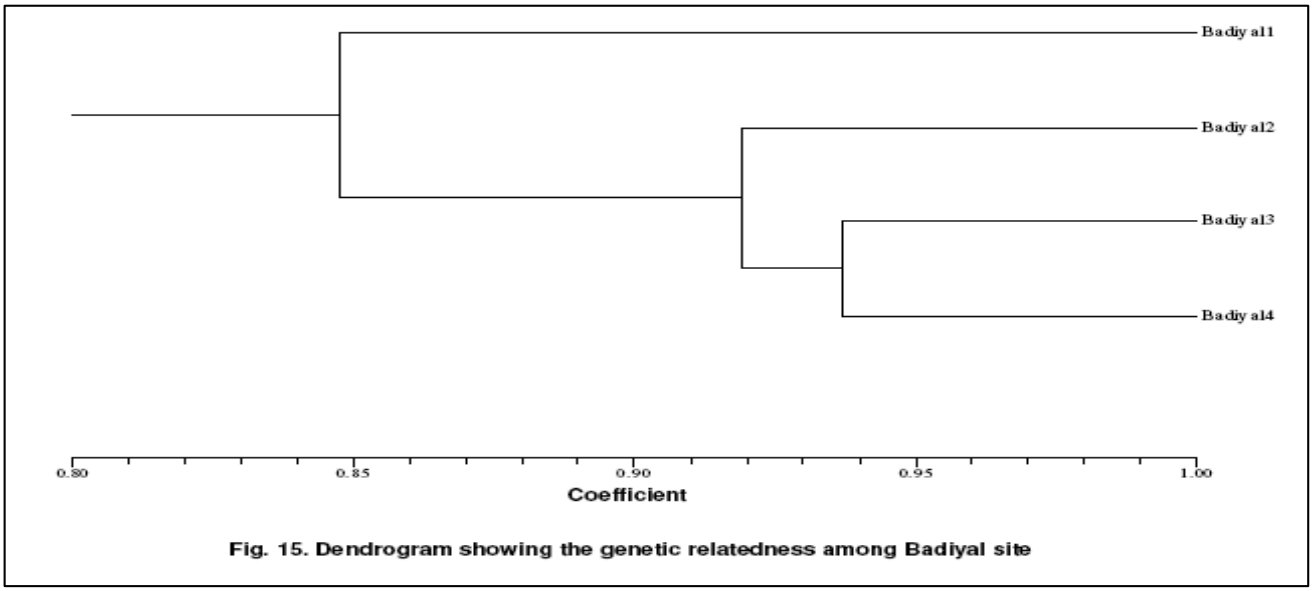

Fig. 15. Dendrogram showing the genetic relatedness among Badiyal site

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