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Assessment of genetic diversity in *Aloe vera* L. among different provinces of H.P.

Sabina Rana and Kamlesh Kanwar

Abstract
The study was undertaken for the assessment of genetic diversity in *Aloe vera* L. genotypes from different provinces of Himachal Pradesh using morphological, biochemical, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers. On the basis of morphological and biochemical characters very low variation was found among selected genotypes. A total of 30 RAPD and 6 ISSR primers were screened and only 24 RAPD and all the 6 ISSR primers gave amplification. The similarity coefficient value ranged from 0.62 to 0.91 in RAPD. C1 genotype showed its distinct nature at 0.62 similarity index value leaving behind 65-91% genetic similarity among the remaining genotypes. Cluster analysis, using UPGMA, SAHN clustering (NTSYS-pc ver. 2.0) grouped the genotypes into 4 and 2 main clusters in RAPD and ISSR markers respectively. In ISSR markers 57-100% of genetic similarity was obtained, depicting 80% similarity in minor and 65% similarity in major cluster.

Keywords: random amplified polymorphic DNA (rapd), inter simple sequence repeats (issr), polymorphism, dendrogrm, diversity.

1. Introduction
Aloe vera L. belongs to subfamily Asphodelaceae of Liliaceae family [1]. *Aloe vera* is referred as the 'Miracle Plant' and 'Healing Plant' [2]. It grows mainly in the dry regions of Africa, Asia, Europe and America [3], in India, it is found in Rajasthan, Andhra Pradesh, Gujarat, Maharashtra, Himachal Pradesh and Tamil Nadu. *Aloe vera* contains 75 potentially active constituents: vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acids and amino acids [4]. The plant was described to contain a large amount of phenolic compounds [5], some of them were used as cathartic [6]. It has been known and used for centuries due to its health, beauty, medicinal and skin care properties [7].
Genetic polymorphism in medicinal plants has been widely studied, which helps in distinguishing plants at inter and/or intraspecific level. It also plays important role in conservation which helps to preserve the genetic variation and evolutionary process in viable populations of ecologically and commercially viable varieties/genotypes in order to prevent potential extinction. Morphological, biochemical and molecular markers have been widely used for genetic diversity studies [8].
Traditionally, diversity is estimated by measuring variation in phenotypic or qualitative traits such as growth habit, color of flower etc. Because of several defects there is a reduction in the ability of these morphological markers for the estimation of genetic diversity in plants, of which the main factor is its high dependence on the environment for expression, yet they can provide a base for genetic variation. On the other hand both biochemical and molecular markers avoid many of the environmental affects acting on characters by directly observing variation controlled by gene or by observing the genetic material. Molecular markers can give an effective tool for efficient selection of desired agronomic traits as compared to morphological and biochemical markers because they are based on the plant genotypes and also are independent of environmental variation, developmental stages and plant growth [9]. PCR–based molecular markers are widely used in many plant species for identification, phylogenetic analysis, population studies and genetic linkage mapping [10].
The genetic diversity in *Aloe* spp. was estimated by RAPD technique at different institutions [11]. But so far, very little work has been done to study the genetic variation within population of *Aloe vera* in Himachal Pradesh. RAPD and ISSR analysis can be adequately used to describe the genetic diversity of this plant species. Thus, the present study was taken to

characterize the *Aloe vera* L. genotypes collected from different agro climatic regions of Himachal Pradesh at molecular level in relation to morphological and biochemical variation.

Materials and Methods

Agro climatic locations

The plant material for the study was collected from different agro climatic regions, spread in eight districts of Himachal Pradesh *viz.*, Kangra, Hamirpur, Mandi, Solan, Bilaspur, Una, Shimla, Sirmaur. The geographical locations of twenty four selected sites along with their altitude are given in Table 1. The selected plants were given codes from A1 to H3 for further studies.

Morphological studies

The plants from every site were selected and investigated for qualitative (leaf color, leaf shape, leaf margin and leaf orientation) and quantitative morphological parameters (number of leaves, leaf length, leaf width and leaf thickness). The statistical analysis of quantitative data was done using Randomized Block Design (RBD) [12].

Biochemical studies

Quantitative biochemical estimation for phenol, proteins and carbohydrates was carried out. Protocol given by Singleton and Rossi (1965) [13] was followed for estimation of total phenols. Estimation of protein content of leaves was done using method given by Lowery *et al.* (1951) [14]. Carbohydrate content was calculated by using method by Dubois *et al.* (1951) [15].

Molecular marker studies

DNA isolation technique: The genomic DNA was isolated from 2.5 g of leaf material from new emerging green leaves of 24 genotypes using CTAB method of Doyle and Doyle (1987) [16] and dissolved in 500 µl of TE buffer (pH 8.0). For the removal of RNA, 5 µl of RNase (10 µl/ml) was added to 500 µl of dissolved DNA and incubated at 37°C for 1 hour in a water bath. DNA purification was carried out by adding equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) to the solution and ethanol precipitation. Dried DNA was then dissolved in TE buffer (pH 8.0), depending upon the yield of DNA. The quality of genomic DNA was judged by subjecting it to electrophoresis on 0.8% agarose gel at 80 to 100 V and 70 mA for two hours in 1X TAE buffer and observing the sample DNA forming a single high molecular band under UV transilluminator (Pharmacea). The concentration and purity of DNA was checked through spectrophotometer (Nano-Drop TM Spectrophotometer). A260/A280 ratio 1.8 corresponds to pure double stranded DNA. A260/A280 ratio greater than 1.8 suggested RNA contamination whereas less than 1.8 suggested protein or phenol contamination.

DNA amplification: PCR amplifications for RAPD and ISSR analysis were performed in thermal cycler (Eppendorf). RAPD analysis was performed using 30 decamer arbitrary primers (Bangalore Genei ™, India) (Table 2). The reaction was carried out in a volume of 20 µl of reaction mixture containing 50 ng template DNA, 1× PCR buffer (Bangalore Genei Ltd., India), 1.0 mm each dNTP, 2.5 mm MgCl2, 25 pM primer, and 1 U Taq DNA polymerase (Bangalore Genei Ltd., India). The conditions for amplification were programmed as: initial denaturation at 94°C for 3 min followed by 42 cycles of denaturation at 94°C for 30s, primer annealing at 32°C for 1 min, extension at 72°C for 2.5 min, and final extension at 72°C for 4 min. For ISSR analysis 6 markers (Table 2) were used and PCR amplifications were carried out in a volume of 20 µl of reaction mixture containing 1×PCR buffer, 1.0 mm each dNTP, 2.5 mm MgCl2, 25 pm primer, 50 ng template, and 1U Taq DNA polymerase. The amplification reaction was carried out using the program of initial denaturation step at 94°C for 5 min, 35 cycles comprising denaturation at 94°C for 30s, optimum annealing temperature for particular ISSR primer for 30s, and extension at 72°C for 1 min and a final extension step at 72°C for 7 min. RAPD and ISSR amplified products were electrophoresed in 1.2% agarose in 1×TAE buffer. The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, USA).

Data analysis: RAPD and ISSR fingerprints generated were scored individually. DNA fragment profiles were scored in binary fashion, the presence of a band was coded as 1 and absence was marked as 0. The data was analyzed with SIMQUAL program of NTSYS-pc ver. 2.0 [17] and similarities between accessions were estimated using the Jaccard's coefficient. Dendrogram was created from the resultant similarity matrices using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) method [18], following SAHN function of NTSYS-pc ver. 2.0 [19].

Results and Discussion

Morphological studies: In present investigation, on the basis of observations made on qualitative characters it was found that there was only one character (leaf color) which showed variation among 24 selected genotypes of *Aloe vera* (Table 1). A total of 4 genotypes, A2 from Bilaspur, E3 from Hamirpur and F2 and F3 from Kangra were found to be dark green in color as compared to other genotypes. This may be due to the reason that Bilaspur and Hamirpur belongs to subtropical regions of Himachal Pradesh. Yet, F2 and F3 genotypes from Baijnath and Pragpur which are in temperate zone and subtropical zone respectively has also showed dark green color, which shows that there may be relatedness among these four genotypes. During four quantitative characters study leaf length, width, thickness and number of leaves were ranged from 12.67-31.00 cm, 0.74-2.77 cm, 3.27-7.31 mm and 5-10, respectively (Table 1). Narag site of Solan district possessed statistically significant maximum number of leaves (10), leaf length (31.00 cm), width (2.37 cm). Likewise, D3 genotype of Karyal site of Mandi district with 7.31 mm leaf thickness and F2 genotype of Baijnath site of Kangra with maximum leaf breadth of 2.74 cm showed variation. So these genotypes can be selected as elite genotype on the basis of quantitative traits as high leaf length and breadth are important in particular for cultivars for medicinal components. While Nejatzadeh-Barandozi *et al*. (2012) [20] observed 14-29 number of leaves, 40.40 to 45.2 cm of leaf length and 7.1 to 9.6 cm of leaf breadth while studying the genetic diversity of 10 accessions of *Aloe vera* collected from different parts of Iran on the basis of horticultural data, which is greater than our observations. Our findings are almost similar to those reported by Nayanakantha *et al.* (2010) [21] are almost similar as they identified mean leaf length and thickness among eighteen cultivars of *Aloe vera* between 14-46 cm and 0.4-1.1 cm respectively.

Table 1: Geographical location and morphological leaf variation among 24 selected genotypes of *Aloe vera*

District	Site	Geno-type	Altitude (m)	Color	Shape	Margin	Orienta-tion	Number of leaves	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)
Bilas-pur	Gehravi	A1	550-610	PG	LL	SP	S	7	23.00	1.33	5.25
	Ghumarvi	A2		DG	LL	SP	S	8	21.67	1.60	5.85
	Kandraur	A3		PG	LL	SP	S	10	12.67	0.74	4.48
Una	Dangoh	B1	350-1200	PG	LL	SP	S	10	26.67	1.73	7.29
	Battkalan	B2		PG	LL	SP	S	7	15.67	1.17	3.86
	Bathu	B3		PG	LL	SP	S	7	23.33	1.37	4.22
Shimla	Bhont	C1	300-2200	PG	LL	SP	S	7	21.00	1.40	6.49
	Ragyan	C2		PG	LL	SP	S	7	25.00	1.87	6.59
	Navbhar	C3		PG	LL	SP	S	9	29.67	2.67	6.29
Mandi	Bhaglana	D1	1100-1369	PG	LL	SP	S	9	27.67	2.00	7.17
	Digho	D2		PG	LL	SP	S	9	27.67	2.57	6.79
	Karyal	D3		PG	LL	SP	S	8	23.33	1.90	7.31
Hamir-pur	Rael	E1	823-860	PG	LL	SP	S	6	27.00	1.50	5.08
	Smirpur	E2		PG	LL	SP	S	6	21.00	1.80	6.00
	Panjot	E3		DG	LL	SP	S	7	16.67	1.50	4.58
Kangra	Jwalaji	F1	937-1298	PG	LL	SP	S	7	24.33	1.70	4.75
	Baijnath	F2		DG	LL	SP	S	10	26.67	2.77	7.27
	Pragpur	F3		DG	LL	SP	S	8	19.00	1.33	4.37
Sirmaur	Daron	G1	900-3994	PG	LL	SP	S	6	19.00	1.07	3.27
	Narag	G2		PG	LL	SP	S	10	31.00	2.37	6.86
	Nahan	G3		PG	LL	SP	S	6	21.00	1.23	5.32
Solan	Nauni	H1	1365-1400	PG	LL	SP	S	8	24.33	1.43	6.10
	Nalagarh	H2		PG	LL	SP	S	5	14.67	1.77	4.42
	Arki	H3		PG	LL	SP	S	7	17.67	1.43	5.07
C.D.$_{0.05\%}$									4.42	0.61	1.23

PG: Pea green, DG: Dark green, LL: Linear, SP: Spines, S: Spiral

Biochemical estimation: Variation can also be noticed at chemical level which is due to the synthesis and accumulation of various bio-chemicals. The medicinal and cosmetic value of the *Aloe* depends upon the quantity of its biochemical constituents. The plants producing higher amount of carbohydrate, protein and phenol are considered to be of good quality. In present study the phenol content ranged from 0.624±0.003 to 2.650±0.001 mg/g (Fig.1). The maximum phenol content was observed in F1 genotype of Jwalaji site of Kangra district. While protein and carbohydrate content in leaves of 24 selected genotypes were found in the range of 0.224±0.003 to 0.561±0.002 mg/g and 0.208±0.001 to 1.458±0.001 mg/g respectively (Fig. 2 and 3). In our study B3 genotype from Bathu site of Una district and C3 genotype of Navbhar site of Shimla district showed maximum value for protein content and carbohydrate content respectively. Similar studies were carried out by Kaur and Saggoo (2010) [22] where they subjected different accessions of North Indian germplasm to biochemical analysis and found 0.96±0.0031 to 3.30±0.0018 mg/g of carbohydrate; 0.229±0.001 to 1.49±0.0017 mg/g protein and 87.2±5.40 to 149.0±2.07 mg/100g phenol content during vegetative phase. Similarly Ahmed and Hussain (2013) [23] studied chemical composition of *Aloe vera* and found 120.68±7.24 to 363.03±9.25 mg/ml carbohydrate content and 6.86±0.06% of protein content.

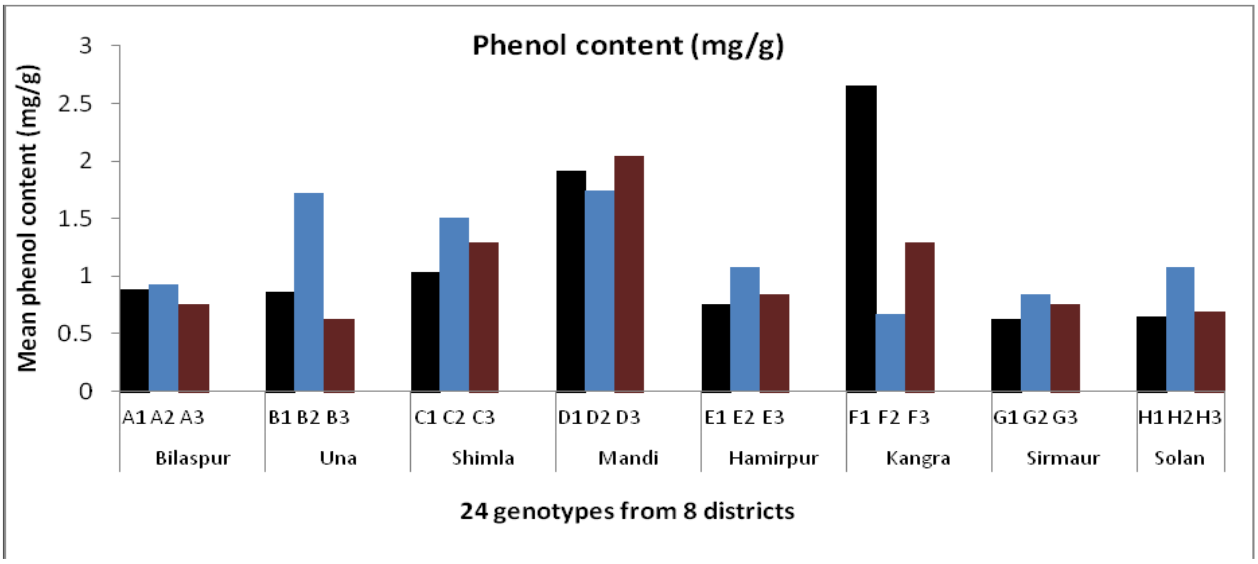

Fig 1: Phenol content (mg/g) among 24 *Aloe vera* genotypes

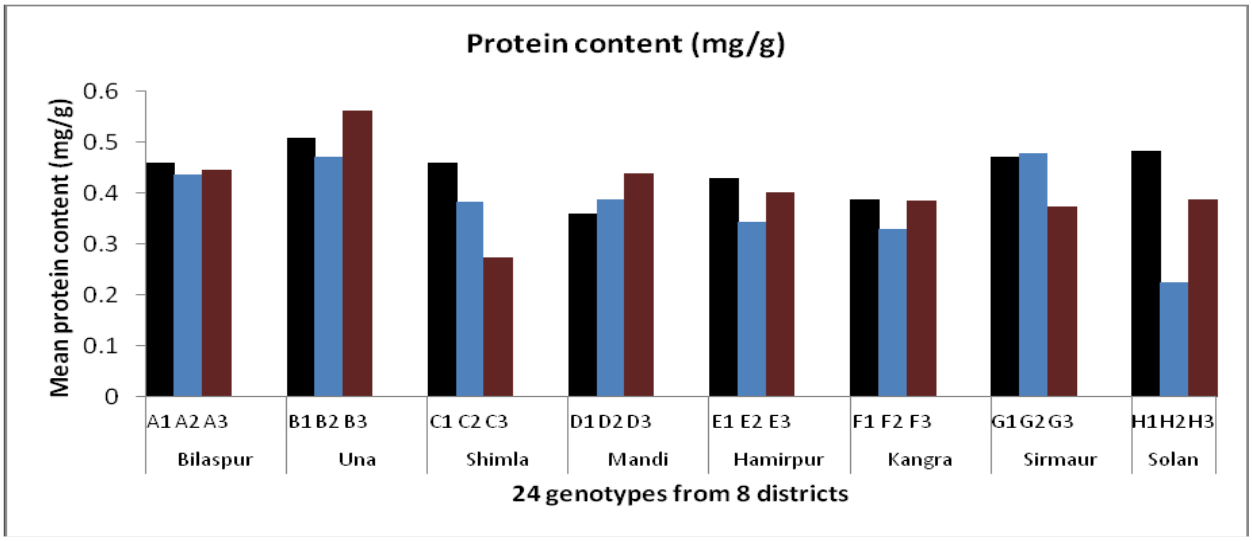

Fig 2: Protein content (mg/g) among 24 *Aloe vera* genotypes

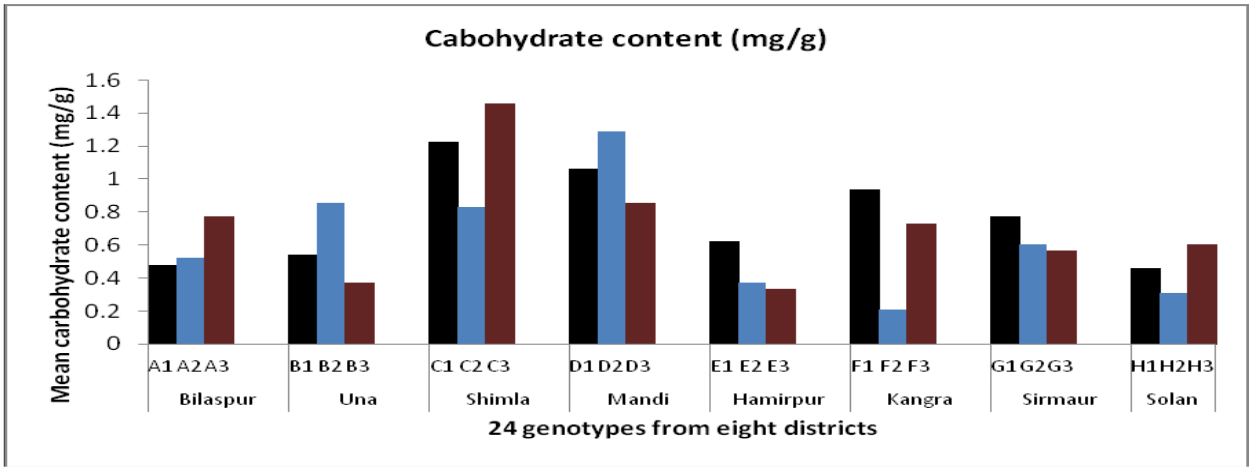

Fig 3: Carbohydrate content (mg/g) among 24 *Aloe vera* genotypes

RAPD and ISSR Studies: During the present investigation good quality DNA was isolated from the leaves of selected genotypes of *Aloe vera* using CTAB method developed by Doyle and Doyle (1987) [16]. Two marker systems were employed RAPD and ISSR. Both the marker systems being employed to assess genetic diversity were quite informative and were able to generate adequate polymorphism and unique DNA fingerprints for identification of few genotypes. Thirty random decamer primers were used. Out of these, 24 primers gave amplification. A total of 91 bands were amplified. 82 bands were polymorphic (90.11%) and only 9 (9.89%) were found to be monomorphic in nature. Average number of amplified bands per polymorphic primer was 3.79. Eight unique bands were recorded, which can be used for specific genotypic identification. G3 genotype from Nahan could be identified specifically by OPA-04, OPE-15, similarly C3 genotype (Navbhar) by OPA-19, OPF-07, OPS-17 and F1 genotype of Jwalaji by OPF-07. Number of amplified fragments per primer varied from 21 (OPV-05) to 107 (OPE-10) with 61.95 average number of amplified fragments per primer. On the other hand a total of 6 ISSR primers were examined all were able to amplify DNA. A total of 24 bands were amplified. Out of which 21 (87.5%) were polymorphic and 3 (12.5%) were monomorphic. Only one unique band was observed which could be used for genotypic identification of "Navbhar" genotype of Kangra district. Average number of amplified bands per polymorphic primer was 4. Nayanakantha *et al.* (2010) [21] observed 27.4 bands per primer which are higher than the present study with both the primers. Similarly, in the work done by Tripathi *et al.* (2011) [24] average 51.25 bands were generated with each primer combination of AFLP marker. The percent polymorphism observed in the study made by Panwar *et al*. (2013) [25] was 96.42% which is higher than our results but produced average 5.4 bands per primer which is in accordance with our results. Our results are showing higher polymorphism than the AFLP markers (48.5%) used by Tripathi *et al.* (2011) [24].

Pairwise similarity matrix was generated among 24 selected genotypes using Jaccard's coefficient. The similarity coefficient values ranged from 0.52-0.91 in case RAPD and 0.38-1.00 with ISSR primers. Among all the combinations the maximum similarity (91%) was found between E3 and H3 genotypes of Panjot and Arki sites of Hamirpur and Solan districts. However

Table 2: Nucleotide sequences of RAPD and ISSR primers which showed amplification in *Aloe vera*

S. No.	Primer	Nucleotide sequence (5'-3')
1.	OPA-01	CAGGCCCTTC
2.	OPA-04	AATCGGGCTG
3.	OPA-12	TCGGCGATAG

4.	OPA-17	GACCGCTTGT
5.	OPA-18	AGGTGACCGT
6.	OPA-19	CAAACGTCGG
7.	OPB-05	TGCGCCCTTC
8.	OPB-11	GTAGAGCCGT
9.	OPE-02	GGTGCGGGAA
10.	OPE-05	TCAGGGAGGT
11.	OPE-08	TCACCACGGT
12.	OPE-10	CACCAGGTGA
13.	OPE-15	ACGCACAACC
14.	OPF-03	CCTGATCACC
15.	OPF-07	CCGATATCCC
16.	OPS-02	CCTCTGACTG
17.	OPS-07	TCCGATGCTG
18.	OPS-15	CAGTTCACGG
19.	OPS-17	TGGGGACCAC
20.	OPV-04	CCCCTCACGA
21.	OPV-05	TCCGAGAGGG
22.	OPV-08	GGACGGCGTT
23.	OPV-10	GGACCTGCTG
24.	OPV-14	AGATCCCGCC

the minimum similarity of 52% was seen in A2 and G2 of Ghumarvi and Narag of Bilaspur and Sirmaur district respectively which could be explained on the basis of large genetic and geographical distance. While among all the combinations with ISSR primers the 100% similarity was found among A1, B2, C2, and D3 and between D2 and E2. The minimum 38% similarity was seen in C3 and E1, C1 and G1, G1 and H1 along with E1 and G2 with ISSR primers. ISSR showed very less variation among the genotypes studied while RAPD markers showed large variation among the genotypes. Darokar *et al.* 2003 [11] have reported similarity of 78.8% to 99% in A*loe vera* accessions revealed by RAPD and AFLP. Nayanakantha *et al.* (2010) [21] reported similarity values ranging from 6.8% to 61% indicating remarkable genetic variation among selected *Aloe* accessions.

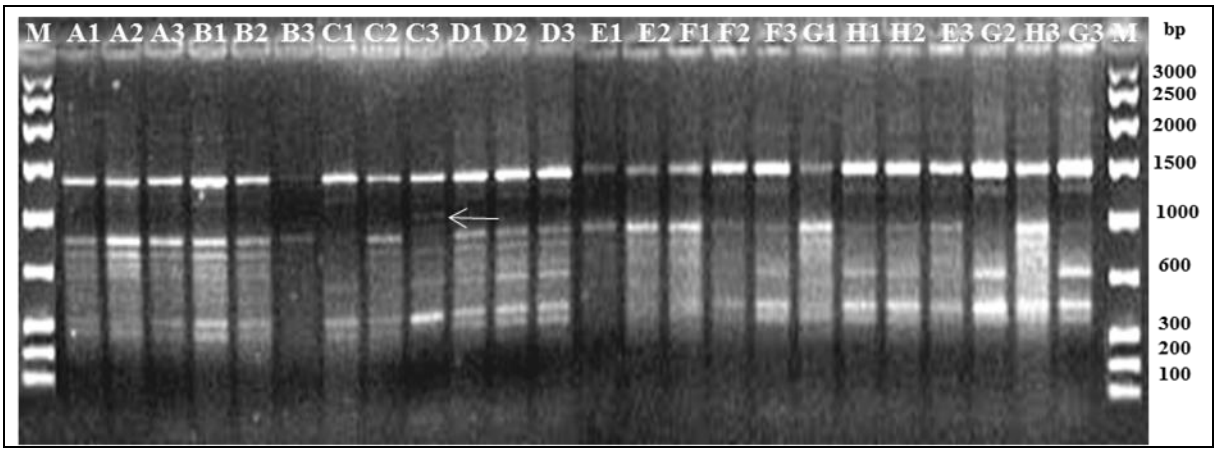

Fig IV: RAPD pattern of twenty four genotypes of *Aloe vera* generated by primer OPA-19. The lane M represents the molecular size marker used (100bp -3kb ladder, Gene Ruler ™)

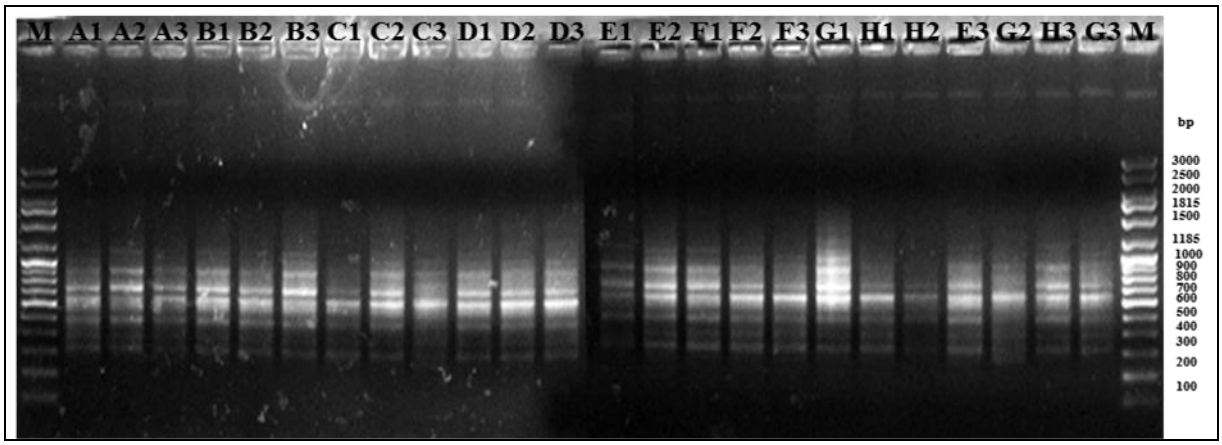

Fig V: ISSR pattern of twenty four genotypes of *Aloe vera* generated by primer hb-13. The lane M represents the molecular size marker used (100 bp -3kb ladder, Gene Ruler Plus™)

The Jaccard's similarity matrix was used for constructing dendrogram using UPGMA analysis. In dendrogram constructed from RAPD primers at similarity index value 0.62, C1 genotype of Bhont (Shimla district) was separated from rest of the 23 genotypes giving information about its diverse nature. The rest 23 genotypes were further divided into 4 Clusters. There was random clustering of the genotypes in three clusters, clearing there intersite diversity. Only three genotypes from Bilaspur district were grouped in one cluster showing intrasite relationship among three although they were not 100% similar. This may be because majority of the genome is composed of non-coding regions; one would expect number of RAPDs from them. RAPD has been employed to screen germplasm for several higher plant species including cross pollinated plants and clonally propagated plants [26-27].

While dendrogram constructed on the basis of ISSR primers divided 24 genotypes into 2 clusters. Since distribution of various genotypes in clusters, sub clusters and sub-sub clusters was somewhat random although some of the genotypes belonging to the different districts were placed in the same cluster and were very close to each other. This may be due to human intervention and also due to similarity in climate. Like A1, B2 and D3 from Bilaspur, Una and Mandi district were showing 100% similarity, also D2 and E3 from Mandi and Hamirpur were 100% similar. This may be because all these genotypes were from hot climatic regions. Similar results were shown by Bahmani *et al.* (2012) [28] in Iranian fennels, where by ISSR primers grouping among fennel ecotypes were somewhat congruent with the geographical distribution and similarity in climate. Likewise there are species where relationship between genetic diversity, geographic distribution and climatic similarity has been observed, as in *Achillea* species [29] and *Achillea millefolium* [30]. It may be suggested that ISSR showed more relatedness among genotypes as compared to RAPDs. Although *Aloe vera* is propagating only vegetatively but showing this much of variation which may be due to genotype environmental interaction.

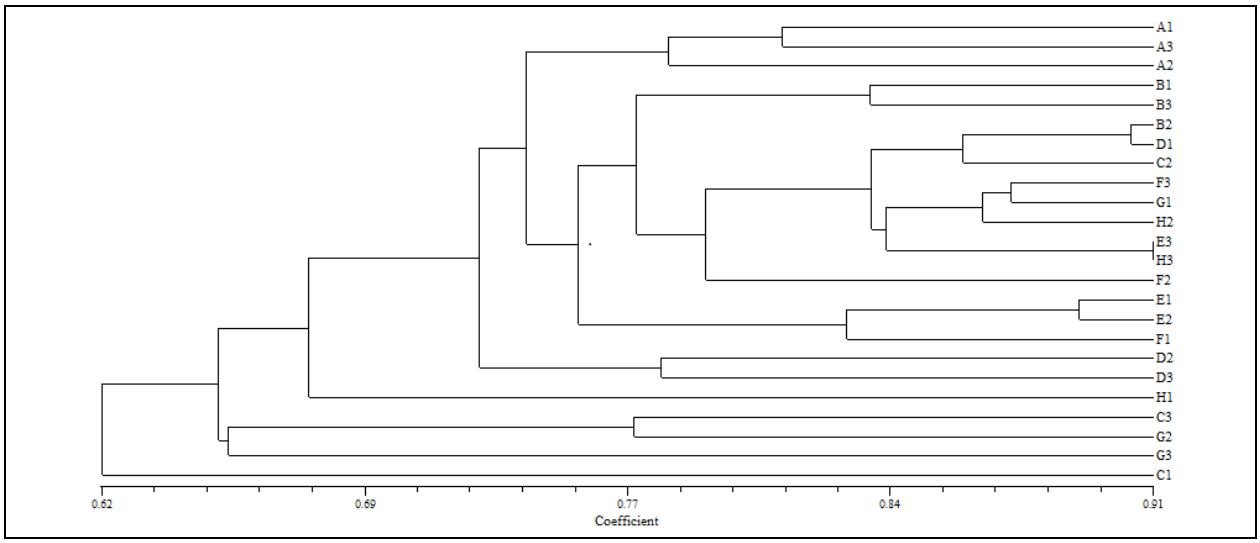

Fig 6: Dendrogram showing genetic relatedness among 24 genotypes of *Aloe vera* based on 24 RAPD primers

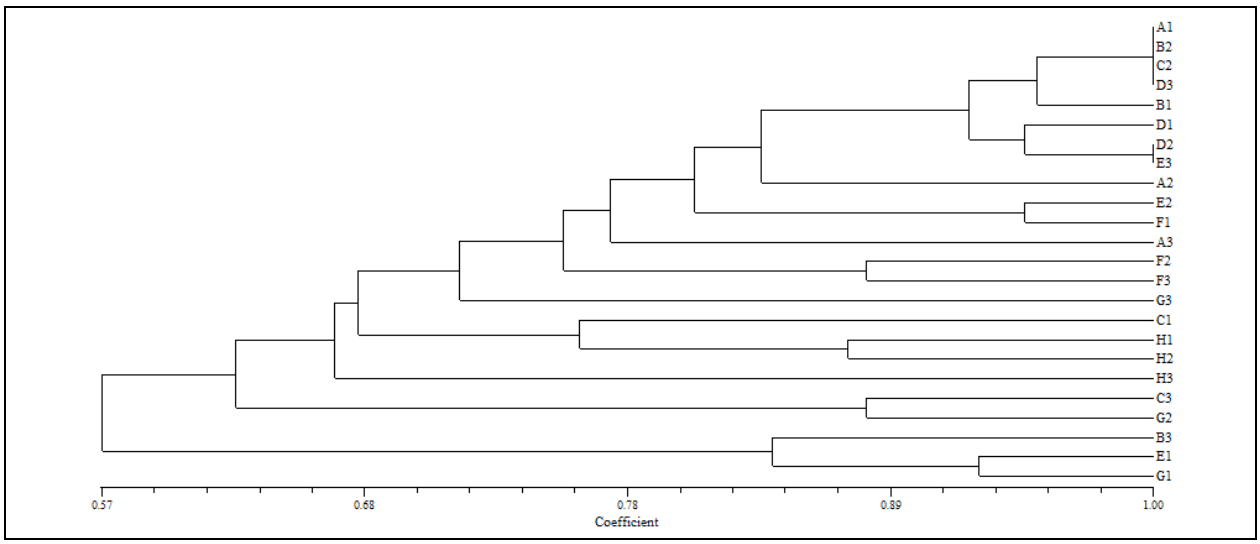

Fig 7: Dendrogram showing genetic relatedness among 24 genotypes of *Aloe vera* based on 6 ISSR primers

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