Indian Journal of Biotechnology
Vol 15, October 2016, pp 538-549

A combined approach using RAPD, ISSR and bioactive compound for the assessment of genetic diversity in *Aloe vera* (L.) Burm.f.

Ravinder Kumar[1], Pradeep K Naik[2], Anil Kumar[1], Himanshu Aggarwal[3], Ashok Kumar[4] and Vinod Chhokar[1]*

[1]Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar 125 001, India
[2]School of Life Sciences, Sambalpur University, Jyoti Vihar Burla, Sambalpur 768 019, India
[3]Department of Biotechnology, Maharishi Markandeshwar University, Mullana 133 207, India
[4]Germplasm Evaluation Division, National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi 110 012, India

Received 11 May 2015; revised 22 October 2015; accepted 3 November 2015

Aloe vera (L.) Burm.f. is an important medicinal plant valued all over the world for its pharmacological importance. Despite limited knowledge of the levels of genetic diversity and relatedness, their cultivation as a source of valuable secondary metabolites is widespread. In order to facilitate reasoned scientific decisions on its conservation and for selective breeding programme, aloin content and genetic diversity analysis of 55 genotypes were performed. Aloin content in the leaves of 55 genotypes varied from 3.29 to 276.76 mg/g of dry wt. Twenty six RAPD and fourteen ISSR primers amplified a total of 236 and 111 scorable bands, of which 86.44 and 72.07% were polymorphic, respectively. Analysis of molecular variance (AMOVA) indicated high genetic variation among genotypes. Genetic variation among genotypes grouped into low, intermediate and high aloin content was negligible, 5.4% (RAPD) and 4.08% (ISSR). The dendrogram obtained from Neighbor-joining and STRUCTURE analysis revealed splitting of genotypes into four clusters with no clear distinction between low, intermediate and high aloin content genotypes. Results showed that genetic variability, using RAPD and ISSR, was not associated with aloin content. However, both the markers revealed high genetic variation among genotypes, which is important in the conservation and exploitation of *A. vera* genetic resources.

Keywords: *Aloe vera*, aloin content, genetic polymorphism, ISSR, RAPD

Introduction

Aloe, a genus belonging to the family *Aloaceae*, consists of perennial, evergreen and monocot crassulacean acid metabolism (CAM) plants. *Aloe* is derived from the Arabic word 'Alloeh' meaning 'shining bitter substance'. It is one of the herbaceous or woody plants characterized by stemless, large, thick, lance shaped fleshy leaves with a sharp apex and a spiny margin[1]. *Aloe vera* (L.) Burm.f. is one of approx 420 species of aloe originated in South Africa but is now indigenous to dry sub-tropical and tropical climates. In India, *A. vera* is well known as Komarika, Grithkumari, Korphad, Kattar vazha and various other provincial names. Aloe latex is a yellowish fluid derived from the inner lining of the leaves of *A. vera* plants and serves as the water and energy storage component of the plant.

A. vera shows many pharmacological activities, such as, anti-ulcer[2], anti-inflammatory[3], anti-tumor[4,5], chemo-preventive[6], anti-viral[7], anti-bacterial[8], anti-fungal[9], anti-hyperglycemic[10], anti-asthmatic[11] and healing capacity against skin burns[12]. Aloe extracts have been used to treat even AIDS[13]. It has similar anti-aging effects like vitamin A derivatives[14]. *A. vera* gel has been extensively used in the cosmetic and toiletry industries. The cosmetic formulations available include a large range of moisturizing creams, cleansers, shampoos and soaps. The world market of *A. vera* is also increasing day by day. As an example, in 2003 alone, it has been included in more than 150 products in USA[15]. The major constituents of *A. vera* include anthraquinones, polysaccharides, alkylbenzenes, dehydrabietic acid derivatives, lactin, salicylic acid, lignin, saponin and sterols. In addition to these, aloe also contains fatty acids, amino acids, minerals, vitamins and proteins and thus could also be used as food supplement[13,15,16]. Aloin, a glycosidic derivative of anthraquinone, is the main active compound in *A. vera* and is important as anti-bacterial, anti-viral and anti-fungal component. A chemotaxonomic survey of 380 species of aloe indicated the presence of the anthrones isomers aloin

*Author for correspondence:
Phone: +91- 9992793333 Fax: +91-1662-276240
vinodchhokar@yahoo.com

A and B together with the aloinoside isomers and microdontin A and B in 36 species of aloe[17]. The geographical variation of the major compounds in *A. ferox* leaf exudates showed that the composition of major compound was remarkably invariable, with aloeresin A, aloesin and aloin (both epimers A and B) contributing between 70 and 90% of total dry wt, in a ratio of approximately 4:3:2, respectively[18]. Research on this species has lagged in the rear then that of other medicinal plants. Therefore, improvement of this crop is needed through utilization of available genetic diversity.

Chemical synthesis of the aloin is not available so far and efforts to produce it in cultured cells or in modified microorganisms have not been very fruitful. Thus, the naturally growing as well as the cultivated plants is the sole source of the aloin. This has generated worldwide interest in studying the genetic diversity of *A. vera* populations, clonal variants, chemotypes, ecotypes, and in the synthesis of pure-line cultivars. Genetic diversity studies have traditionally been through morphological and biochemical markers, which are rather less effective and are influenced by environmental factors as well as developmental stage. Hence, molecular markers could be an ideal tool for germplasm characterization, because of their plasticity, ubiquity and stability. By using molecular markers, breeders can bypass traditional phenotypes based selection methods. Moreover, knowledge of genetic diversity is prerequisite towards effective utilization and protection of plant genetic resources, identification of molecular markers linked to agronomic traits and to achieve rational conservation. These molecular markers have also been used to tag the sequence associated with production of a specific secondary metabolite

In recent years, attempts have been made to study genetic diversity in *A. vera* using RAPD (random amplified polymorphic DNA) markers[19-23], but little or no attention have been paid so far on genetic diversity studies using ISSR (inter simple sequence repeats) markers. Also, the aloin content of the wide variety of genotypes has not yet been evaluated. Hence, the present study was conducted to analyze the level of aloin content and distribution of genetic polymorphism among *A. vera* genotypes using RAPD and ISSR markers, followed by its correlation with the aloin content, which is essential for the conservation and management strategies.

Materials and Methods

Plant Materials and DNA Extraction

Fifty-five genotypes of *A. vera* were collected from different geographical region of India and were grown under the same conditions at the Research Farm of National Bureau of Plant Genetic Resources, New Delhi (Table 1). The young leaf samples were collected during the period of February to September in sampling bags under aseptic conditions. The leaves were stored at −20°C prior to the aloin estimation and DNA extraction.

Estimation of Aloin Content

The aloin was extracted from frozen leaf sample (10 g) by using 20 mL methanol. The samples were homogenized with physcoton and centrifuged at 1500 g for 10 min. The supernatant was sonicated in Brason ultrasonic bath for 10 min and then again centrifuged. The above extraction procedure was repeated twice. The extract were then pooled and filtered through 0.22 µm nylon Millex-GN filters. Also, standard solution of aloin was prepared in methanol and filtered through a 0.22 µm size nylon Millex-GN filters before use. About 10 µL of both the filtrate were taken and injected into HPLC system using auto sampler. The HPLC profile was set as follows: mobile phase consisting of water:methanol (75:25) to achieve maximum separation and sensitivity at 229 nm; flow rate of 0.6 mL/min with stop and rest time of 12 and 5 min, respectively. Detection and quantification of aloin was carried out using Water series (Water Corporation USA) reverse phase high performance liquid chromatography coupled with 2996 photodiode array detector (PDA). The data thus obtained was analyzed by Empower 2 software. The peak of aloin was observed at the retention time of 4.796 min. Calibration curve was generated by plotting a graph of peak area against the concentration used to quantify the aloin content in different samples. Excellent linearity was retained for aloin between peak areas and concentration in the range of 0.1-100 µg/mL.

Based on low, intermediate and high aloin contents, all genotypes were categorized into three groups (Table 2).

Extraction of DNA

The total genomic DNA was extracted from the frozen leaves (5 g) by the CTAB method[24] with minor modifications. The yield of the extracted DNA and purity was checked on 0.8% agarose gel along with

standard (non-restriction enzyme digested) lambda DNA marker (Biogene, USA). The extracted genomic DNA was also tested for purity index (A_{260}/A_{280} absorbance ratio) on Nano drop spectrophotometer. A value of 1.8 of extracted DNA samples indicates high purity, whereas the value <1.8 or >1.8 denotes the contamination of proteins and RNA, respectively.

RAPD Analysis

Twenty six arbitrary primers obtained from IDT (Integrated DNA Technology & Alpha DNA Technology) (Table 3) were used for RAPD amplification following earlier reported protocol[25]. The primers were selected on the basis of their GC content, which ranges from 60-70%. PCR reactions were performed in volumes of 15 µL containing: 1 µL dNTP (200 µM each dNTP), 4 µL primer (5 pm μL^{-1}), 2 µL template DNA (50 ng μL^{-1}), 1.5 µL reaction buffer (10×), 0.3 µL Taq DNA polymerase (5 U μL^{-1}), 1.5 µL $MgCl_2$ (25 mM), and nuclease free water to makeup the volume. PCR reactions

Table 1—Accession numbers and aloin content of *A. vera* genotypes

No.	Acc. no.	Genotype	Aloin content (mg/g)	No.	Acc. no.	Genotype	Aloin content (mg/g)
1	1c 527160	AV-1	11.727	29	1c 111267	AV-29	9.679
2	1c 520358	AV-2	3.291	30	1c 111279	AV-30	10.470
3	1c 527342	AV-3	7.785	31	1c 471886	AV-31	11.803
4	1c 422483	AV-4	6.844	32	1c 471882	AV-32	17.160
5	1c 527334	AV-5	7.158	33	1c 471884	AV-33	15.640
6	1c 520364	AV-6	11.377	34	1c 111269	AV-34	9.710
7	1c 527336	AV-7	9.419	35	1c 471883	AV-35	42.287
8	1c527343	AV-8	4.178	36	1c112526	AV-36	11.356
9	1c 527340	AV-9	5.076	37	1c 111271	AV-37	9.185
10	1c 524161	AV-10	3.970	38	1c 112572	AV-38	12.596
11	1c 524161	AV-11	26.687	39	1c 112522	AV-39	10.134
12	1c 524163	AV-12	14.824	40	1c 112534	AV-40	14.457
13	1c 524590	AV-13	25.874	41	1c112520	AV-4 1	21.662
14	1c 520967	AV-14	12.365	42	1c 471885	AV-42	34.763
15	1c 523621	AV-15	12.077	43	1c 112532	AV-4 3	38.043
16	1c 524623	AV-16	14.206	44	1c 281340	AV-44	10.496
17	1c 519857	AV-17	9.514	45	1c 326744	AV-45	13.612
18	1c 112514	AV-18	5.099	46	1c 112530	AV-46	14.358
19	1c 112521	AV-19	54.375	47	1c 112539	AV-47	44.176
20	1c 112518	AV-20	17.047	48	1c 326744	AV-48	6.1605
21	1c 112511	AV-21	55.152	49	1c 281340	AV-4 9	31.548
22	1c 112523	AV-22	57.250	50	1c 337889	AV-50	20.440
23	1c 112513	AV-23	276.766	51	1c 436191	AV-51	38.106
24	1c 112512	AV-24	13.267	52	1c 527335	AV-52	19.839
25	1c 112516	AV-25	10.346	53	1c 520360	AV-53	15.639
26	1c 112517	AV-26	8.298	54	1c 520389	AV-54	40.154
27	1c 112527	AV-27	10.178	55	1c 527344	AV-55	7.796
28	1c 111280	AV-28	9.088				

Table 2—Groupings of *A. vera* genotypes on basis of their aloin contents

No.	Groups	*A. vera* genotypes
1.	Group 1 (Low aloin content)	AV-1, AV-2, AV-3, AV- 4, AV-5, AV-6, AV-7, AV- 8, AV- 9, AV-10, AV- 12,AV- 14, AV-15, AV- 16, AV-17, AV-18, AV-20, AV- 24, AV-25, AV-26, AV-27, AV-28, AV-29, AV- 30, AV- 31, AV-32, AV- 33, AV-34, AV- 36, AV-37, AV-38, AV-39, AV- 40, AV-44, AV- 45, AV-46, AV-48, AV- 52 AV-53, AV- 55,
2.	Group 2 (Intermediate aloin content)	AV- 11, AV-13, AV- 35, AV-41, AV-42, AV-43, AV-47, AV-49, AV- 50, AV-51, AV-54
3.	Group 3 (High aloin content)	AV-19, AV- 21, AV-22, AV-23

Table 3—Characteristics of RAPD primers and genetic diversity assessed in 55 genotypes of *A. vera*

Primer	Primer sequence	% GC content	T_A	TL	NPL	PPL	TF	Rp	PIC
OPT-1	GGGCCACTCA	70	42	8	7	87.5	164	5.96	0.83
OPT-2	GGAGAGACTC	60	41.7	12	11	91.7	207	7.53	0.89
OPT-4	CACAGAGGGA	60	41.7	5	4	80.0	139	5.06	0.77
OPT-5	GGGTTTGGCA	60	38	8	6	75.0	191	6.95	0.85
OPT-6	CAAGGGCAGA	60	45.5	10	10	100.0	151	5.49	0.88
OPT-7	GGCAGGCTGT	70	42	9	8	77.7	127	4.62	0.76
OPT-8	AACGGCGACA	60	41	7	5	42.8	211	7.67	0.85
OPT-11	TTCCCCGCGA	70	41	9	9	100.0	156	5.67	0.99
OPT-13	AGGACTGCCA	60	45.5	5	5	100.0	65	2.36	0.78
OPT-16	GGTGAACGCT	60	43.8	8	6	75.0	130	4.73	0.79
OPT-17	CCAACGTCGT	60	42	8	6	75.0	190	6.91	0.85
OPH-1	GGTCGGAGAA	60	36	15	12	66.7	423	15.38	0.92
OPH-2	TCGGACGTGA	60	36	6	6	100.0	97	3.53	0.79
OPH-3	AGACGTCCAC	60	36	9	9	100.0	134	4.87	0.85
OPH-5	ACGCATCGCA	60	36	15	15	100.0	268	9.75	0.92
OPH-10	CCTACGTCAG	60	36	6	6	100.0	53	1.93	0.80
OPH-15	AATGGCGCAG	60	48.8	13	13	100.0	116	4.22	0.90
OPH-18	GAATCGGCCA	60	48.8	11	9	72.7	226	8.22	0.87
OPBH-1	CCGACTCTGG	70	44.5	12	9	58.3	359	13.06	0.91
OPBH-2	GTAAGCCGAG	60	42.5	9	8	88.0	187	6.80	0.86
OPBH-3	GGAGCAGCAA	60	41.7	9	6	66.7	270	9.82	0.88
OPBH-5	GTAGGTCGCA	60	36.9	11	9	72.7	280	10.18	0.89
OPBH-10	GTGTGCCTGG	70	44	4	1	25.0	152	5.53	0.74
OPBH-12	TCGCCTTGTC	60	43.8	11	11	100.0	161	5.86	0.86
OPBH-15	GAGAACGCTG	60	41	9	9	100.0	170	6.18	0.85
OPBH-16	CTGCGGGTTC	70	36.1	7	4	57.1	180	6.55	0.81
	Total			236	204		4807		

T_A, Annealing temperature; TL, Total no. of loci; NPL, No. of polymorphic loci; PPL, Percentage of polymorphic loci; TF, Total fragments amplified; Rp, Resolving power; & PIC, Polymorphic information content

were performed with PTC-100 thermal cycler (M J Research, USA). The first cycle consisted of denaturation of template DNA at 94°C for 7 min, primer annealing at 37°C for 1 min, and primer extension at 72°C for 1 min. For the next 35 cycles, the period of denaturation was reduced to 1 min at 92°C, while the primer annealing and primer extension time remained the same as in the first cycle. The last cycle consisted of only primer extension (72°C) for 10 min.

ISSR Analysis

Fourteen ISSR primers were obtained from Applied Biosciences, India (Table 4) and PCR amplification was performed in reaction cocktail similar to RAPD. Initial denaturation for 4 min at 94°C was followed by next 40 cycles of denaturation at 94°C for 45 second, 30 second at specific annealing temperature (±5°C of Tm), 2 min at 72°C and a 5 min final extension step at 72°C. PCR products were stored at 4°C until further analysis.

The amplification for each primer was performed twice independently with same procedure in order to ensure the fidelity of RAPD and ISSR markers. Amplified PCR products were electrophoresed on 1.8% agarose gel at constant voltage (70 V) in 1× TAE buffer for approx 2 h, visualized by staining with ethidium bromide (0.5 µg mL^{-1}). A total of 3 µL loading buffer (6×) was added to each reaction before electrophoresis. After electrophoresis, the gels were documented on a gel documentation system (Syngene, USA). Molecular sizes of amplicons were estimated using a 1 Kb and 100 bp DNA ladders (Fermentas, USA).

Data Collection and Analysis

The genetic relationship among the entire genomic DNA under study was assessed by comparing the

Table 4—Characteristics of ISSR primers and genetic diversity assessed in 55 genotypes of *A. vera*

Primer	Primer sequence	% GC content	T_A	TL	NPL	PPL	TF	Rp	PIC
S1	AGAGAGAGAGAGAGAGT	47	53	11	5	45.5	325	11.82	0.89
S2	AGAGAGAGAGAGAGAGC	53	57.6	5	4	80.0	171	6.22	0.80
S3	AGAGAGAGAGAGAGAGG	53	54.5	9	4	44.4	295	10.73	0.88
S4	GAGAGAGAGAGAGAGAT	47	57.2	6	4	66.7	208	7.56	0.81
S5	GAGAGAGAGAGAGAGAC	53	58	11	9	81.8	297	10.80	0.89
S6	GAGAGAGAGAGAGAGAA	47	53	7	6	85.7	181	6.58	0.83
S7	CTCTCTCTCTCTCTCTG	53	59	10	10	100.0	232	8.44	0.89
S8	ACACACACACACACACT	47	58	7	5	71.4	171	6.22	0.82
S9	AGAGAGAGAGAGAGAGTA	44	54.3	7	3	42.8	243	8.84	0.84
S10	GAGAGAGAGAGAGAGAAT	44	55.3	9	7	77.8	238	8.66	0.88
S11	GAGAGAGAGAGAGAGAGC	53	57.7	10	6	60.0	320	11.64	0.89
S12	CCGCCGCCGCCGCCGCCG	100	55	4	2	50.0	121	4.40	0.71
S13	CTCCTCCTCCTCCTCCTC	67	58	8	8	100.0	202	7.35	0.87
S14	GGCGGCGGCGGCGGCGGC	100	59	7	7	100.0	141	5.13	0.83
	Total			111	80		3145		

T_A, Annealing temperature; TL, Total no. of loci; NPL, No. of polymorphic loci; PPL, Percentage of polymorphic loci; TF, Total fragments amplified; Rp, Resolving power; & PIC, Polymorphic information content

RAPD and ISSR fragments separated according to their size. The banding pattern of each of the primer was scored as present (1) or absent (0), each of which was treated as an independent character. Only the reproducible bands were observed for scoring and the light bands were omitted as they were not reproducible. The Jaccard's dissimilarity coefficient (J) was calculated, subjected to cluster analysis by bootstrapping and neighbor-joining method using the program DARWIN (version 5.0.158). Statistically unbiased clustering of collected genotypes was performed using STRUCTURE (version 2.3.1)[26]. POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage of polymorphic loci (PPL) across all the 55 genotypes were analyzed[27]. Within group diversity (Hs) and total genetic diversity (Ht) were calculated within the species and within three major groups (based on the aloin content of genotypes) by using POPGENE software[28]. The RAPD and ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA)[29], using three hierarchical levels, such as, at individual, at population and by grouping of genotypes based on their aloin content. The non-parametric AMOVA was done *via* Gen Alex[29], where the variation component was partitioned among individuals within populations, among populations within groups and among groups. The resolving power of the RAPD and ISSR primers was calculated according earlier reported method[30]. The resolving power (*Rp*) of a primer is: $Rp=\Sigma\ IB$ where *IB* (band informativeness) takes the value of: 1–[2*(0.5–P)], P being the proportion of the 55 genotypes containing the band.

In order to determine the utility of each of the marker systems, diversity index (DI), effective multiple ratio (EMR) and marker index (MI) were calculated[31]. DI for the genetic markers was calculated from the sum of squares of allele frequencies: $DI_n=1-\sum pi^2$ (where pi is the allele frequency of the i^{th} allele). The arithmetic mean heterozygosity, DI_{av}, was calculated for each marker class: $DI_{av}=\sum Di_{n/n}$, [where n represents the number of the markers (loci) analyzed]. The DI for the polymorphic marker is: (DI_{av}) $p=\sum Di_n/n_p$ (where n_p is the number of polymorphic loci and n is the total number of loci). EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay, EMR (E) = n_p (n_p/n). MI is defined as the product of the average diversity index for polymorphic bands in any assay and the EMR for that assay, $MI=DI_{avp} \times E$.

PIC (Polymorphism Information Content)

The frequency of the polymorphism obtained in the genotypes was calculated on the basis of presence (1) and absence (0) of the bands amplified. The PIC was calculated based on the allele pattern of all the aloe accessions by employing the following formula[32]:

$$PIC_i=1-\sum_{j=1}^{n} Pij^2$$

Where, p_i is the frequency of an individual genotype generated by a given primer and summation extends over *n* alleles.

Results and Discussion

Aloin Contents in *A. vera*

Aloin was extracted from the leaves and analyzed in triplicate from 55 genotypes. It was found that aloin content varied widely among all the genotypes ranging from 3.291 to 276.766 mg/g of dry wt (Table 1). Based on aloin contents, all the genotypes were categorized into three groups, *viz.*, low (< 20 mg/g), intermediate (20-50 mg/g) and high (>50 mg/g) aloin genotypes (Table 2). In a previous study, it was revealed that, in young leaf L3 of *A. hereroenses*, the average content of aloin reached 44.9% of the dry weight of the exudate[33]. A similar distribution of aloin was also found in *A. arborescens*, which showed that young leaves had the highest content. Even the top part of each leaf had the highest content aloin, while the basal part had the lowest content. Similarly, the aloin content of *A. vera, A. ferox* and *A. perryi* were found to be 30, 10 and 25%, respectively[34]. Thus, the aloin content reported in the present study was found to be higher in comparison to earlier reports. The possible reasons for this variation could be differences in agro-climatic conditions, genotypes in question, methods used for analysis, *etc*. Further, all these studies constitutively reported aloin to be the major bioactive compound responsible for the pharmacalogical activities of the *A. vera*. The aloin can be used as possible marker compound for standardization of Kumariasava, a marketed ayurvedic formulation containing *A. vera* as one of the main ingredients[35].

Molecular Analysis Using RAPD Markers

A total of 55 genotypes were fingerprinted using 26 RAPD makers. These primers produced multiple band profiles with a number of amplified DNA fragments varying from 4 to 15. The size of amplified fragments varied from 100-2000 bp. A representative gel figure for one of the RAPD marker is included in Fig. 1. Of 236 amplified bands, 204 were found polymorphic (86.44%) (Table 3). The resolving power of the 26 RAPD primers ranged from 1.93 for primer OPH-10 to a maximum of 15.38 for primer OPH-1. Polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a population or set of genotypes by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus. As revealed in Table 3, RAPD marker 'OPT-11' showed the highest level of polymorphism with PIC value of 0.99, whereas the PIC values for rest of the RAPD markers were in the range of 0.74-0.92. A dendrogram analysis based on bootstrapping and neighbor joining (NJ) method grouped all the 55 genotypes into four main clusters, which were further extensively divided into mini clusters: cluster-1 (18 genotypes), cluster-2 (14 genotypes), cluster-3 (9 genotypes) and cluster-4 (14 genotypes) (Fig. 2a). An unbiased clustering of genotypes based on STRUCTURE program without prior knowledge about the populations clustered all the 55 genotypes into four major groups. Under the admixed model, STRUCTURE calculated that the estimate of likelihood of the data [LnP(D)] was greatest when K=4. For K > 4, LnP(D) increased slightly but more or less plateued (Fig. 2b), *i.e.*, ΔK reached its maximum value when K=4 (Fig. 2c), suggesting that all the populations fell into one of the 4 clusters albeit small interference (Fig. 2d). These results are almost

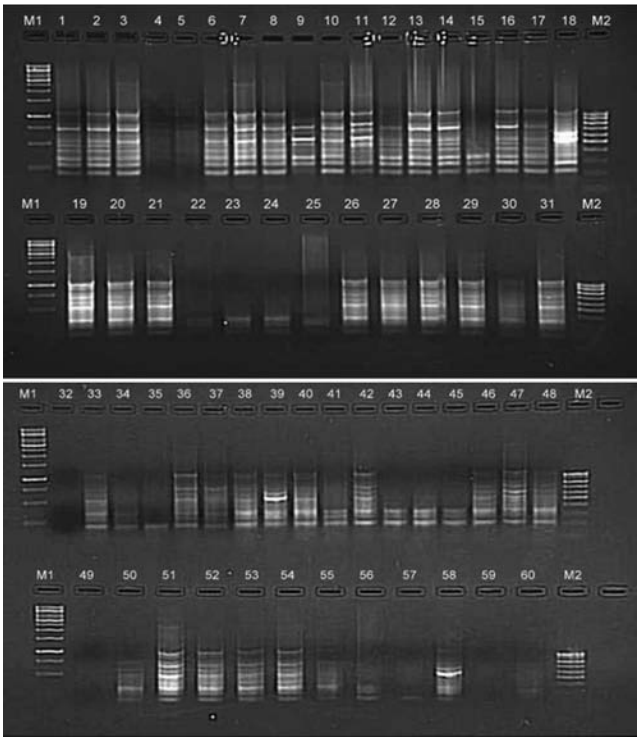

Fig. 1—A representative gel picture showing amplified products obtained from 55 genotypes of *A. vera* using OPBH-1 RAPD primer. [M1and M2 indicates the DNA ladder of 1 kb and 100 bp, respectively

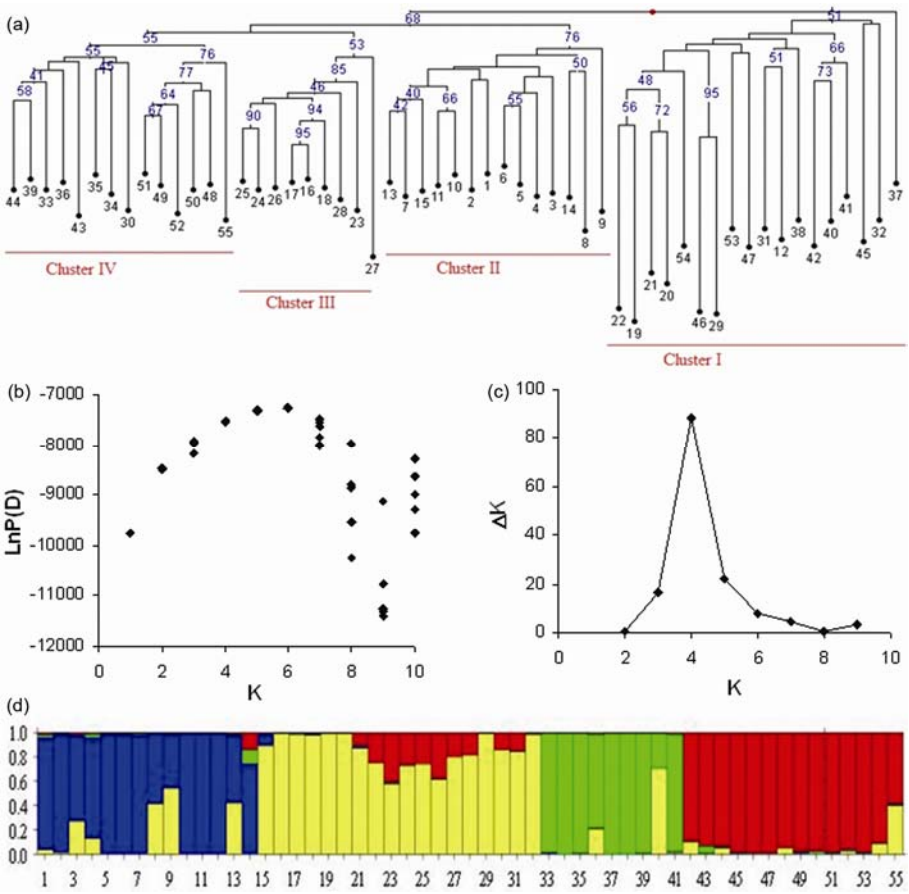

Fig. 2 (a-d)—(a) NJ tree representing clustering of *A. vera* genotypes along with supported bootstrap values based on RAPD profiling; (b) Relationship between the number of cluster (K) and the estimated likelihood of data (LnP(D)); (c) Relationship between K and ΔK based on STRUCTURE analysis; & (D) STRUCTURE analysis of *A. vera* genotypes based on RAPD data showing grouping of genotypes when K=4.

similar to the splitting in the NJ tree. Overall the cluster analysis strongly suggested that the 55 sampled genotypes can be divided into four clusters.

Genetic diversity analysis of 55 genotypes in terms of Na, Ne, H, I, Ht, and PPL with respect to three different groups, such as, low, intermediate and high aloin content revealed higher values, indicating more variability among the genotypes (Table 5a). Percentage of polymorphic loci using POPGENE was calculated that varied from 63.14% in high aloin content genotypes to 97.88% in low aloin content genotypes. Three groups containing genotypes with low, intermediate and high aloin contents showed Nei's genetic diversity (H): 0.380, 0.365 and 0.286, and of Shannon's information index (I): 0.557, 0.537 and 0.407, respectively. The respective values for overall genetic variability for observed number of alleles (Na) , effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), heterozygosity (Ht), homozygosity (Hs), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), genetic differentiation (Gst), gene flow (Nm), effective multiplex ratio (EMR) and marker index(MI) across all the 55 genotypes were are included in Table 6. Gst value of 0.388 indicated that 61.2 % of the genetic diversity resided within the population. The rate of Nm estimated using Gst value was found to be 0.788, which is very low. AMOVA among genotypes based on three major groups with respect to aloin content indicated that majority of genetic variation (94.6%) occurred among genotypes, while the variation between the three groups was 5.4% (Table 7).

Molecular Analysis Using ISSR markers

Fourteen ISSR primers selected in the study generated a total of 111 ISSR bands (an average of 7.93 bands per primer), of which 80 were polymorphic (72.07%) (Table 4). Number of bands varied from 4 to 11 with sizes ranged from 807-867 bp (Fig. 3). Amplification result of 14 primers seems

Table 5—Summary of genetic variation statistics for the combination of (a) RAPD only (b) ISSR only and (c) combination of both RAPD and ISSR profiling among the genotypes of *Aloe vera* with respect to their distributions into 3 groups based on aloin content

(a) RAPD markers

Aloin content	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Low	41	1.979	1.672	0.380	0.557	0.380	231	97.88
		(0.144)	(0.291)	(0.129)	(0.161)	(0.017)		
Intermediate	10	1.945	1.637	0.365	0.537	0.365	223	94.49
		(0.229)	(0.298)	(0.136)	(0.176)	(0.018)		
High	4	1.631	1.538	0.286	0.407	0.286	149	63.14
		(0.483)	(0.440)	(0.225)	(0.316)	(0.051)		

(b) ISSR markers

Aloin content	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Low	41	2.0	1.755	0.417	0.603	0.417	111	100
		(0.0)	(0.239)	(0.099)	(0.116)	(0.010)		
Intermediate	10	1.991	1.729	0.408	0.593	0.408	110	99.10
		(0.095)	(0.244)	(0.099)	(0.117)	(0.010)		
High	4	1.658	1.521	0.286	0.411	0.286	73	65.77
		(0.477)	(0.411)	(0.213)	(0.303)	(0.046)		

(c) Combination of RAPD and ISSR markers

Aloin content	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Low	41	2.00	1.679	0.385	0.564	0.385	347	100
		(0.00)	(0.281)	(0.121)	(0.145)	(0.015)		
Intermediate	10	1.911	1.601	0.344	0.508	0.344	316	91.07
		(0.286)	(0.332)	(0.155)	(0.205)	(0.024)		
High	4	1.231	1.164	0.094	0.138	0.094	80	23.05
		(0.422)	(0.312)	(0.175)	(0.254)	(0.031)		

Na, Observed number of alleles; Ne, Effective number of alleles; H, Nei's gene diversity; I, Shannon's Information index; Ht, Heterozygosity; NPL, Number of polymorphic loci; & PPL, Percentage of polymorphic loci

Table 6—Overall genetic variability across all the 55 genotypes of *A. vera* based on RAPD only, ISSR only and combination of both RAPD and ISSR markers

	Sample size	Na	Ne	H	I	Ht	Hs	NPL	PPL	Gst	Nm	EMR	MI
RAPD	55	2.00	1.641	0.367	0.543	0.367	0.225	236	100	0.388	0.788	6.49	0.69
		(0.00)	(0.299)	(0.131)	(0.159)	(0.017)	(0.010)						
ISSR	55	2.00	1.768	0.423	0.610	0.423	0.278	111	100	0.341	0.965	3.72	0.55
		(0.00)	(0.226)	(0.092)	(0.107)	(0.008)	(0.009)						
RAPD+ISSR	55	2.00	1.682	0.385	0.564	0.385	0.242	347	100	0.371	0.846		
		(0.00)	(0.283)	(0.123)	(0.148)	(0.015)	(0.010)						

Na, Observed number of alleles; Ne, Effective number of alleles; H, Nei's gene diversity; I, Shannon's Information index; Ht, Heterozygosity; Hs, Homozygosity; NPL, Number of polymorphic loci; PPL, Percentage of polymorphic loci; Gst, Genetic deifferentiation; Nm, Gene flow; EMR, Effective multiplex ratio; & MI, Marker index

to indicate that microsatellites in *A. vera* contain more frequently the repeated di-nucleotides (AG)n and (GA)n. The number of bands produced with different repeat nucleotide were more with the (AG)n and (GA)n, followed by (CT)n and (AC)n, and tri-nucleotide (CTC)n primers. The primers that were based on the (AG)n, (GA)n and (CT)n motif produced more polymorphism (on average 8.5 bands per primer) than the primers based on any other motifs used in the present investigation. We obtained good amplification products from primers based on (AG)n and (GA)n repeats. The resolving power (Rp) of 14 ISSR primers ranged from 4.40 to 11.82 (Table 4). Similarly the PIC value ranges from 0.71 to 0.89,

Table 7—Summary of analysis of molecular variance (AMOVA) based on (a) RAPD only (b) ISSR only and (c) combination of both RAPD and ISSR markers among the genotypes of *A. vera*

Source of variation	Degree of freedom	Variance component	Percentage of variation	P-value
(a) Based on RAPD profiling				
Among groups	2.0	2.46	5.4	-
Among genotypes	52.0	43.13	94.6	< 0.001
(b) Based on ISSR profiling				
Among groups	2.0	1.0	4.08	-
Among genotypes	52.0	23.49	95.92	< 0.001
(c) Based on combination of both RAPD and ISSR profiling				
Among groups	2.0	11.0	14.8	-
Among genotypes	52.0	63.48	85.2	< 0.001

Levels of significance are based on 1000 iteration steps

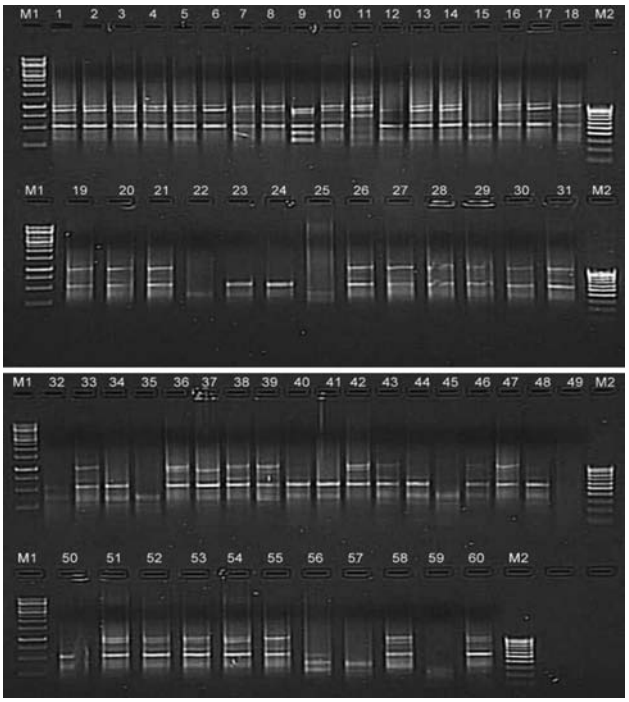

Fig. 3—A representative gel picture showing amplified products obtained from the 55 genotypes of *A. vera* using ISSR primer. [M1 and M2 indicate the DNA ladder of 1 kb and 100 bp, respectively]

demonstrating uniform polymorphism rate among all the fourteen ISSR primers.

The complete data set of 3145 bands was used for cluster analysis based on bootstrapping and NJ method. The genotypes were clustered into four major clusters, well supported by bootstrap value of > 40 (Fig. 4a). The estimated likelihood [LnP(D)] of the clustering of data using STRUCTURE was found to be optimal when K=4, LnP(D) increased slightly for K > 4, but more or less plateued (Fig. 4b). ΔK reached its maximum value when K=4 (Fig. 4c), suggesting that all the populations were distributed with high probability into one of the 4 clusters (Fig. 4d). The clustering pattern of the genotypes were almost similar to the splitting in the NJ tree; however, there was no distinct clustering of genotypes based on their low, intermediate and high aloin content.

A relatively high genetic variation was detected among the genotypes grouped into 3 different groups. Genetic diversity analysis in terms of Na, Ne, H, I, Ht, NPL, and PPL reveals higher value for the group with low aloin content in comparison to the group having high aloin content. This disparity may be because of more number of genotypes included in the group with low aloin content (Table 5b). Overall genetic variability across all the 55 genotypes in terms of Na, Ne, H, I, Ht, Hs, NPL, PPL, Gst, Nm, EMR and MI are also included in Table 6. The H index was 0.423 and I was 0.610, demonstrating high rate of genetic variability. AMOVA for among groups (4.08%) and among genotypes (95.92%) indicated that there are more variations across the genotypes and not among the groups in terms of aloin content (Table 7). The estimated Nm was 0.965.

RAPD and ISSR Combined Data for Custer Analysis

Based on combined data set of RAPD and ISSR markers, the dendrogram obtained gave similar clustering pattern like RAPD and ISSR (Fig. 5a). These results are also corroborative with STRUCTURE analysis. The estimated likelihood of distribution [LnP(D)] for all the 55 genotypes was highest when K=4 (Fig.. 5b), ΔK was maximum with K=4 (Fig. 5c), reveals that all the genotypes were clustered better (with high likelihood probability) with 4 clusters (Fig. 5d). Other genetic variation analyses were also performed on RAPD and ISSR combined data, which are represented in Tables 5-7.

Previously, both the molecular markers had been successfully used in a variety of taxonomic and genetic diversity studies and it was found suitable for use with *A. vera* genotypes because of their ability to generate reproducible polymorphic markers. High amplification was detected using both the RAPD and ISSR markers, which provided enough genomic data to assess the genetic variation among the *A. vera*

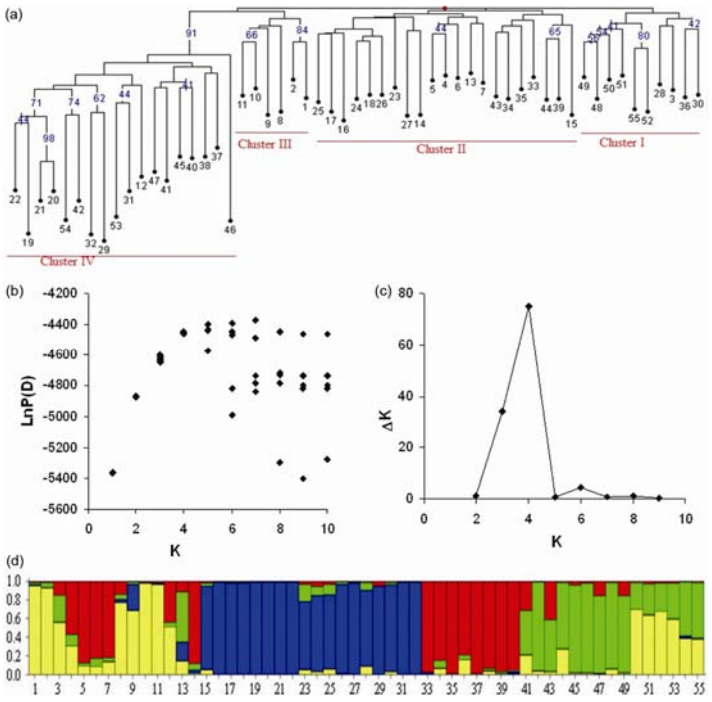

Fig. 4 (a-d)—(a) NJ tree representing clustering of *A. vera* genotypes along with supported bootstrap values based on ISSR profiling; (b) Relationship between the number of cluster (K) and the estimated likelihood of data [LnP(D)]; (c) Relationship between K and ΔK based on STRUCTURE analysis; & (D) STRUCTURE analysis of *Aloe vera* genotypes based on ISSR data showing grouping of genotypes when K=4.

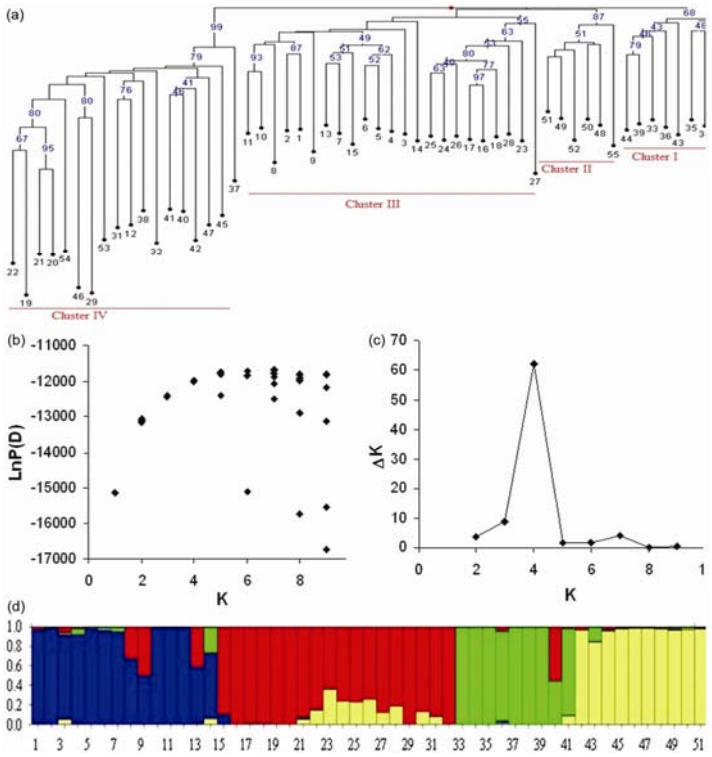

Fig. 5 (a-d)—(a) NJ tree representing clustering of *A. vera* genotypes along with supported bootstrap values based on combination of both RAPD and ISSR profiling; (b) Relationship between the number of cluster (K) and the estimated likelihood of data [LnP(D)]; (c) Relationship between K and ΔK based on STRUCTURE analysis; & (d) STRUCTURE analysis of *A. vera* genotypes based on combination of both RAPD and ISSR data showing grouping of genotypes when K=4.

genotypes. ISSR markers indicate that microsatellites more frequent in *A. vera* genome contain the repeated di-nucleotides, (AG)n and (GA)n, despite the fact that (AT)n di-nucleotide repeats are thought to be the most abundant motifs in plant species[36]. Similar results were obtained in wheat[37], chickpea[38,39] and *Vigna*[40]. A possible explanation of these results is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity, and would form dimers during PCR amplification or it may be due to its-non annealing with template. The observed high proportion of polymorphic loci using both the molecular markers suggests that there is a high degree of genetic variation in the *A. vera* genotypes. Similarly the genetic diversity analysis with respect to three different groups, such as, low, intermediate and high aloin content revealed higher values, indicating more variability among the genotypes. However, no association between genetic variation and aloin content among the genotypes was detected. As a result all the 55 genotypes were clustered into four major clusters and there is no distinct clustering of genotypes based on their low, intermediate and high aloin content. The small differences found among the distribution of genotypes across the four major clusters generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (4807 for RAPDs and 3145 for ISSRs), reinforcing again the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships as observed in barley[41]. Another explanation could be the low reproducibility of RAPDs[42]. The genetic similarity of these genotypes is probably associated with their similarity in the genomic and amplified region. Overall, RAPD markers were found more efficient with respect to polymorphism detection (based on average PPL value) compared to ISSR markers. This is in contrast to the results obtained for several other plant species like wheat[37] and *Vigna*[40]. More polymorphism in case of RAPD than ISSR markers might be due to the fact that 14 ISSR primers used in the study producing less number of amplified fragments, while in case of RAPD, 26 primers were used that produced more number of amplified fragments. Similar polymorphism pattern was also observed in case of Jatropha[43] and Podophyllum[44]. A possible explanation for the difference in resolution of RAPD and ISSR is that the two-marker techniques target different portions of the genome.

With this study, we can conclude that the molecular analyses of both RAPD and ISSR markers were extremely useful for studying the genetic relationships of *A. vera* genotypes. The results indicates the presence of high genetic variability, which should be exploited for the future conservation and breeding of the species. Since no single, or even a few plants, represent the whole genetic variability, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *A. vera* to avoid genetic erosion.

Acknowledgement

Authors are thankful to University Grants Commission, New Delhi, for the financial assistance in the form of Major Research Project.

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