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A low cost, high throughput gel electrophoresis method for separation of SSR markers in *Aloe vera*

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Abstract
Aloe vera is the important commercial crop used for medicinal purposes from ancient time. Because of the limitless medicinal value, it is necessary to study the genetic diversity in *Aloe vera* to detect the commonness and distinctness of the genotypes for the improvement of the traits. The main objective of the present investigation is to compare the gel-based electrophoresis methods for the separation of simple sequence repeats markers in *Aloe vera*. Proper separation of simple sequence repeats alleles is very critical that differ a little as two to five base pair. In the present study, eighteen genotypes of *Aloe vera* were screened using five di-nucleotide simple sequence repeats markers. Four methods for the separation of microsatellite markers were used *i.e.* simple agarose gel, metaphor agarose gel, polyacrylamide gel on midi assembly, polyacrylamide gel on sequencing gel apparatus. Among these, the best separation of alleles was observed in polyacrylamide gel on sequencing gel apparatus and least separation was observed in case of simple agarose gel electrophoresis. A total number of alleles detected for five microsatellite markers were 114, 151, 162 and 165 in standard agarose gel, metaphor agarose gel, polyacrylamide gel on midi assembly, polyacrylamide gel on sequencing gel apparatus respectively. Therefore, polyacrylamide gel on sequencing gel apparatus can be used as an efficient technique for the best resolution of the microsatellite markers.

Keywords: *Aloe vera*, microsatellite, polyacrylamide gel, electrophoresis, sequencing gel apparatus

1. Introduction
Aloe vera has been used since ancient times for beauty, health, skin care and many other medicinal properties. The genus Aloe comprises more than 400 species and among them, *Aloe vera* is most popular and possesses numerous medicinal properties. It belongs to family Asphodelaceae and subfamily Xanthorrhoeaceae (Klopper *et al*., 2010) [1]. Today, *Aloe vera* is a common constituent of many cosmetic formulations which include a large range of moisturizing creams, cleansers, shampoos, and soaps (Grindlay *et al*., 1986) [2]. Aloe extracts have been used to treat even AIDS (Yamaguchi *et al*., 1993) [3]. It has anti-aging effects like vitamin A derivatives (Danhof, 1993) [4]. *Aloe vera* plays an important role in the reduction of plaque and gingivitis (Namiranian and Serino, 2012) [5]. Because of the countless medicinal values of *Aloe vera*, there is a need for the improvement of qualitative and quantitative traits which can be achieved by understanding genetic variability among *Aloe vera*. Moreover, the genetic diversity evaluation will provide evidence about commonness and distinctness of genotypes, which is of vibrant significance in effective conservation of genotypic variability (Bisrat *et al*., 2000) [6]. Genetic variability in plants can be assessed by morphological, biochemical and molecular markers. However molecular markers have several advantages over other types, where they can detect the genetic difference in much more details without interference from environmental factors. Furthermore, molecular markers can be used in linkage map construction, physical and genetic map construction, evolutionary biology, gene tagging, phylogenetic analysis, and genome mapping studies.
There are different type of molecular markers available to detect polymorphisms in nuclear DNA of plants *viz*. random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), single nucleotide polymorphism (SNP), expressed sequence tags (EST) *etc.* (Mishra *et al*., 2014) [7]. Among them, SSR markers are well suited molecular markers for genetic diversity studies, marker-assisted selection (MAS), the study of genome organization and crop improvement in a wide range of plants. Because they are highly polymorphic, present abundantly, and co-dominant inheritance, they are appropriate for the assessment of genetic diversity within crop species (Gupta *et al*., 1996;

Wunsch and Hormaza, 2002) [8, 9]. Although many studies have been conducted to characterize the genetic diversity in *Aloe vera* using RAPD markers **(**Darokar *et al*., 2003; Nayanakantha *et al*., 2010; Nejatzadeh-Barandozi *et al*., 2012, Panwar *et al*., 2013; Kumar *et al.*, 2016) [10-14] but little or no attempts have been made so far to study the genetic diversity in *Aloe vera* using locus-specific primers flanking EST- or genic-SSR markers. The generation of genic- SSR markers is relatively easy and economical because they are designed using the sequence data from genes or ESTs that is already available in public database. The SSR markers are DNA fragments in the size ranges of 100-300 bp in repeating units of 2, 3, 4 or 5 bases. Because of the small size and small difference in the size of alleles of amplified fragments, the separation of the fragments on the electrophoretic system is crucial. There is a number of methods available for the separation of the SSR markers *i.e.* capillary electrophoresis, DNA sequencer, simple agarose gel, metaphor agarose gel, polyacrylamide gel electrophoresis on the midi PAGE unit and sequencing gel apparatus (Wang *et al.*, 2007) [16]. Most of the genotyping techniques rely on either capillary electrophoresis or automated DNA sequencer, in order to get reliable genotyping information in terms of allele polymorphism. But, the capillary electrophoresis and automated DNA sequencer is not affordable for all the laboratory of the developing countries, so, many researchers go for polyacrylamide gel electrophoresis to get better resolution (Brondani *et al*., 2000, Liao *et al.*, 2014, Patil *et al*., 2015) [17-19]. It is very difficult to resolve on simple agarose gel system. Some researcher even tried specialized agaroses like super fine agarose and metaphor agarose for better separation of SSR markers (Mondal *et al*., 2010, Hipparagi *et al.*, 2017) [20, 21]. But, this type of agarose was found to be very costly than normal agarose and provides less resolution than polyacrylamide (Wang *et al*., 2003) [22]. In case of polyacrylamide gel electrophoresis, different size of the gel can be prepared like mini gel and midi gel and gel on sequencing gel apparatus as per the number of samples. There is the advantage of using the sequencing gel apparatus because of the big size of the gel, more number of samples (20-100) can be run at a single time than that of mini and midi PAGE gel. In view of the above, the present study was designed to compare the relative efficiency of simple agarose gel electrophoresis, metaphor agarose gel electrophoresis, polyacrylamide gel electrophoresis on midi PAGE unit and polyacrylamide gel electrophoresis on sequencing gel apparatus, for the better resolution of the SSR markers in *Aloe vera.*

2. Material and methods

2.1 Plant material and DNA extraction

The plant material included eighteen genotypes of *Aloe vera* collected from the different geographical region of India and grown under the natural conditions at the Research Farm of National Bureau of Plant Genetic Resources, New Delhi (Table 1). The young leaves were collected under aseptic conditions and stored at -20 ºC for the extraction of DNA.

Table 1: Details of the aloe genotypes with accession number

Sr. No.	Accession No.	Sr. No	Accession No
1	IC 112514	10	IC 112517
2	IC 112521	11	IC 112531
3	IC 112519	12	IC 112527
4	IC 112518	13	IC 111280
5	IC 112511	14	IC 111267
6	IC 112523	15	IC 111279
7	IC 112513	16	IC 471886
8	IC 112512	17	IC 471882
9	IC 112516	18	IC 111269

Total genomic DNA was extracted from the frozen leaves by CTAB method (Saghai-Maroof *et al*., 1984) [23] with minor modifications *i.e.* 0.2% 2-mercaptoethanol, 0.2% polyvinylpyrrolidone and incubation at 60 ºC overnight. The extracted genomic DNA was also tested for purity index (A_{260}/A_{280} absorbance ratio) on Nanodrop spectrophotometer. Purification of DNA was done by RNase-A treatment at 37 ºC for 30 min. The yield and purity of the extracted DNA was checked on 0.8% agarose gel.

2.2 PCR amplification of SSR markers

Five Di-nucleotide SSR markers were chosen from the SSR markers developed from the transcriptome sequencing data of *Aloe vera*. The primers were selected on the basis of their GC content which ranges from 40-60%, annealing temperature 40 °C–60 °C, and PCR product ranges from 276 to 314 bp. Details of the primers is mentioned in Table 2.

Table 2: Primers Sequence, GC Content and Tm value of SSR primers

Sr. no.	Primer Name	Primer Sequences	GC Content (%)	Tm
1	VKC 56	5’-ATTAACGCAGCCAGCATAAG -3’	45.0	55.3
		3’-TCTCTTTCTCTTTCTTCCTCCAC-5’	43.5	58.9
2	VKC 57	5’-CTCCGGAAATGAAAGGAAAT-3’	40.0	53.2
		3’-TGAGAGCAAGATGGTGATTG-5’	45.0	55.3
3	VKC 58	5’-CATTCAAAGCCCAACAGAGT-3’	45.0	55.3
		3’-CTGGTAAGATGTAAGAGTTGATTGC-5’	40.0	59.7
4	VKC 59	5’-CTTGCTGATAGTATGACTTTGTCC-3’	41.7	59.3
		3’-CTACTTGCCAACCCTAGATCC-5’	52.4	59.8
5	VKC 60	5’-AGGATGTGATTATGGTGCTGT-3’	42.9	55.9
		3’-AATTAGAGTGGAGTTGCGTGAT-5’	40.9	56.5

PCR amplification using SSR primers was carried out in volume of 15 µL containing 0.2 mM each dNTP (Promega, India), 15 pM each primer (Integrated DNA Technology, India), 100 ng template DNA, 1X Taq buffer (Promega, India), 1 unit Taq DNA polymerase (Promega, India), 2 mM $MgCl_2$ (Promega, India) and nuclease free water (Amresco, USA) to make-up the volume. Amplification was carried out in 96-well block Applied Biosystems 2720 Thermal Cycler. The thermal profile included one cycle of denaturation of DNA at 94 °C for 7 min, for the next 30 cycle, denaturation at 94 °C for 1 min, primer annealing at Tm of the primers, extension at 72 °C for 1 min and last cycle of extension at 72°C for 10 min. The annealing temperature for each primer pair was standardized using gradient PCR.

2.3 Electrophoretic separation of SSR markers using different gel methods

2.3.1 Simple agarose gel electrophoresis

For the preparation of 100 ml gel, 3% standard agarose was slowly added to the pre-chilled 1X TBE buffer with continuous swirling. Agarose was soaked in cold buffer for 10 min to avoid foaming and over boiling of the gel. Before pouring the gel 5 ul of ethidium bromide (10 mg/ml) was added to the molten gel and mixed. After casting of gel it was kept at 4 °C for 15 min for better resolution (Anonymous, 2004) [24]. The amplified product was loaded on to the horizontal submerged gel in 1X TBE buffer after the addition of 1X loading dye and electrophoresed for about 2 h at 5 V/cm. After electrophoresis, the resolved SSR alleles were visualized on trans-illuminator and photographed with a camera. Good quality images were used for scoring of the fragments.

2.3.2 Metaphor agarose gel electrophoresis

Metaphor agarose was used in place of simple agarose and other conditions remained the same as in the simple agarose gel electrophoresis.

2.3.3 Non-denaturing polyacrylamide gel electrophoresis on midi PAGE unit.

Non-denaturing polyacrylamide gel electrophoresis was performed on PROTEAN II xi (BIORAD) polyacrylamide gel electrophoresis unit. Both plates were washed with laboratory reagent and rinsed properly with water. Then the outer plate was treated with gel repel solution and the inner plate was treated with gel bind solution and then wipe with double distilled water followed by 70% ethanol to remove the excess gel repel and gel bind solutions.

Eight percent non-denaturing polyacrylamide gel solution was prepared, which included acrylamide/bis-acrylamide mix (29:1), 1X TBE buffer, 10% (w/v) ammonium persulfate and 0.15% (w/v) TEMED. After the addition of an appropriate volume of ammonium persulfate and TEMED, the gel solution was mixed and poured between glass plates. The comb was placed on top side of the glass plates and the gel was allowed to polymerize at room temperature for 30-40 min. After polymerization, the comb was removed and the electrophoresis unit was assembled. 1X TBE buffer was filled as running buffer. The amplified product was loaded on to the gel, after the addition of 1X loading dye and electrophoresed for 5 h at 150 volts and stopped when backward dye (xylene cyanol) reaches to 3/4th of the total gel size. 100 bp DNA ladder (New England BioLabs) was used as the standard size marker. After completion, the glass plates were separated and subjected to the silver staining.

2.3.4 Non-denaturing polyacrylamide gel electrophoresis on sequencing gel apparatus.

In this process, Sequi-Gen GT electrophoresis systems (Bio-Rad) was used. Eight percent non-denaturing gel of 38x30 cm and 0.4 mm thickness was used for the separation of SSR markers. The inner notched plate is fixed to the system and outer plates were properly washed with the laboratory reagent and then rinsed with distilled water. Then the outer plate was treated with gel repel solution and the inner plate was treated with gel bind solution and then wipe with double distilled water followed by 70% Ethanol to remove the excess gel repel and gel bind solutions. The glass plates were assembled into the precision caster and placed horizontally. Eight percent non-denaturing polyacrylamide gel solution in a total volume of 70 ml was prepared, which includes acrylamide/bis-acrylamide mix (30%), 1X TBE buffer, 10% (w/v) ammonium persulfate (APS) and 0.15% (w/v) TEMED. After the addition of APS and TEMED, the gel was injected with the help of syringe between the glass plates assembled into the precision caster with a constant flow rate. The comb was inserted on the top side of the glass plates and the gel was allowed to polymerize at room temperature for 30-40 min. After polymerization, the comb was removed and the sequencing gel apparatus was assembled. 1X TBE buffer was filled as running buffer. The amplified product was loaded on to the gel, after the addition of 1X loading dye and electrophoresed for about 2 h at 30 W and stopped when backward dye (Xylene cyanol) reaches to 3/4th of the total gel size. 100 bp DNA ladder (New England Biolabs) was used as the standard size marker. After completion, the glass plates were separated. The outer plate containing the gel subjected to silver staining.

2.4 Detection of the polymorphism in non-denatured polyacrylamide gel by silver staining.

Both the non-denatured polyacrylamide gel was silver stained by using the method suggested by Kumar *et al*., (2015) [25]. After electrophoresis, the gel was placed in a plastic tray reserved for silver staining very carefully (gel still attached to one plate) and wash with distilled water twice to remove electrophoresis buffer and gel pieces. After washing, the gel was impregnated with the staining solution at room temperature for 5-30 min. The staining solution includes 10% ethanol, 0.7% nitric acid, 0.22% $AgNO_3$. After staining the gel, washing is very important to remove the extra silver nitrate. The gel was developed with the developing solution includes (Per liter: 22.9 g Na_2CO_3, 1.25 ml 37% HCOH, 2 mg Na_2SO_3. $5H_2O$ (4 °C for 3-5 min). The reaction was stopped by the stop solution which contains 250 ml of 3% acetic acid (4 °C) 10% ethanol. After removing the stop solution the bright polymorphic bands were seen on a white background and photographed.

3. Results and Discussion

Total genomic DNA of the eighteen genotypes of *Aloe vera* was extracted using modified CTAB method. The quality and quantity of the extracted DNA was checked on 0.8% agarose gel. Good quality genomic DNA was used for the amplification of SSR markers. After the amplification of the SSR markers, the amplified products were subjected to the separation on different gels. The comparison of the gel pictures obtained from simple agarose, metaphor agarose, and

polyacrylamide gel on midi and the polyacrylamide gel on sequencing gel apparatus of PCR products with SSR primer pair from 18 genotypes of *Aloe vera* was shown in Figure 1. A total of five SSR primers were used for amplification and number of alleles observed were 114, 151, 162 and 165 on simple agarose (AGE), metaphor agarose (MAGE), PAGE on midi and PAGE on sequencing gel apparatus respectively.

The size range of amplified products was 276-314 bp for all the analysis methods. There was a great difference in a number of presumed alleles observed on AGE and MAGE. Alleles were separated in case of MAGE but no clear separation was observed in AGE because metaphor agarose is specially designed agarose for small size fragments. The analysis of DNA fingerprinting using AGE revealed a lower level of polymorphism, because of its large pore size. MAGE had good resolution than the AGE but lower resolution than the polyacrylamide gel. MAGE is easy to use than that of PAGE but the cost of MAGE is about five times higher than PAGE (Gomez *et al*., 2008) [26]. Agarose-based separation of SSR alleles can be used if the difference between the alleles is more than 10 bp if the difference is less than 10 bp, polyacrylamide gel is better for allele separation (Mishra *et al*., 2014) [7].

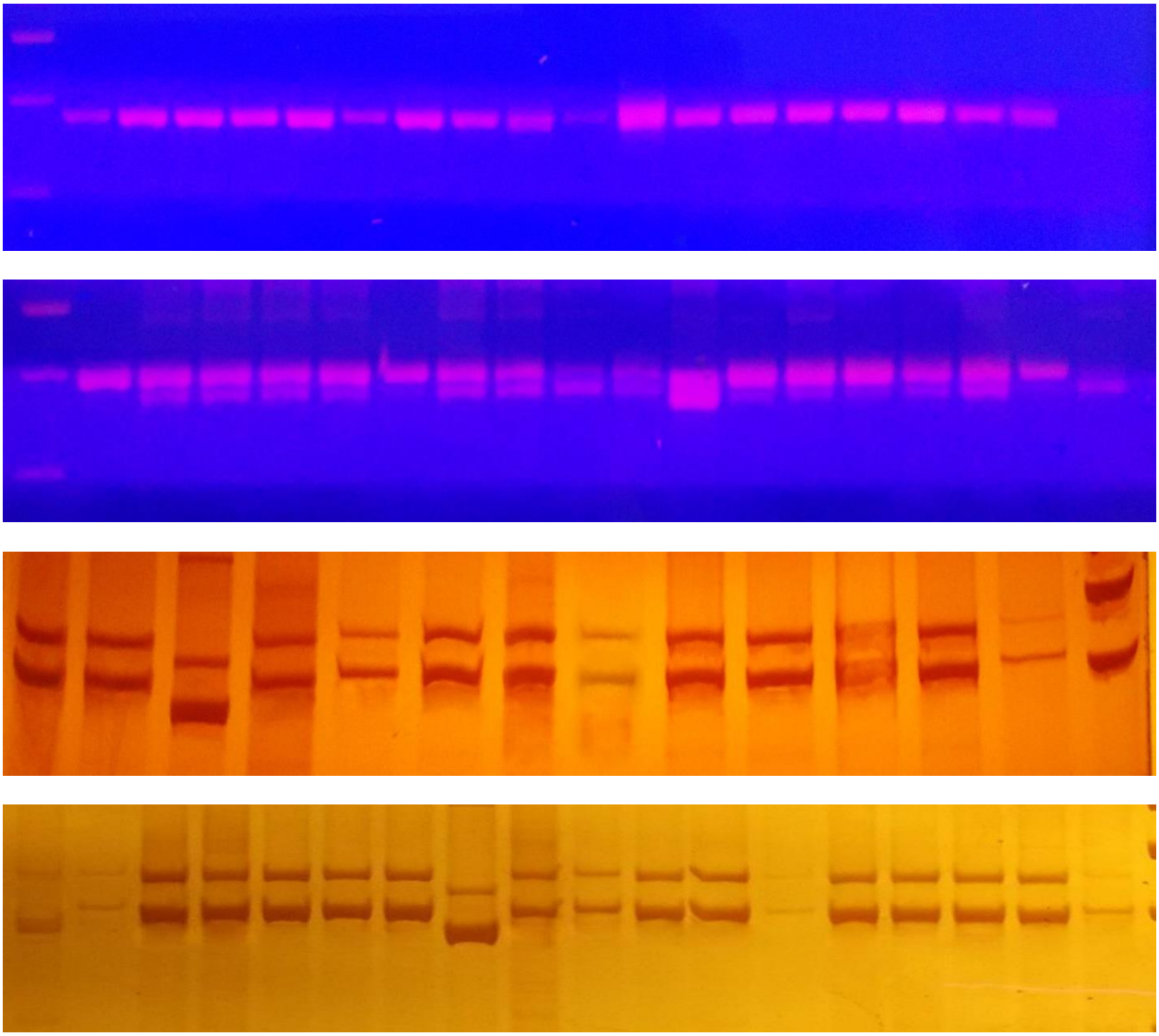

Fig 1: Representative gel pictures showing amplified products obtained from 18 genotypes of *A. vera* using primer VKC 58 and separation of alleles on simple agarose, metaphor agarose, polyacrylamide gel on midi gel assembly and polyacrylamide gel on sequencing gel apparatus respectively.

A number of alleles observed in midi PAGE and sequencing gel PAGE are almost similar in all the five markers but sequencing gel PAGE revealed better resolution than midi PAGE. In case of sequencing PAGE, more number of sample (20-100) can be run in a single time. But, in case of midi PAGE maximum 15 samples can be run at one time and time required to run the samples was almost double than the sequencing gel PAGE. Among all the gels, PAGE on sequencing apparatus revealed the best resolution.

Others method for the separation of SSR markers are capillary electrophoresis methods, Applied Biosystems 3730 Genetic Analyzer and the CEQ 8000 Genetic Analysis system. These methods can detect 2.4 fold more alleles than that of agarose gel electrophoresis. Although these methods are much more costly than the normal agarose or polyacrylamide gels (Ochsenreither *et al*., 2006, Stewart *et al*., 2011) [27, 28]. In case of these capillary electrophoresis fluorescent labeling of primers also costs additionally. QIAxcel System is a new technique that is also employed for genotyping using SSR markers, which is less expensive than the capillary electrophoresis (Wang *et al*., 2009) [29] but very costly than AGE and PAGE. These techniques are very effective for the

proper separation of the alleles but expensive to use, time-consuming and require special facilities and not affordable for the laboratory of the tropical countries.

Polyacrylamide gel on a sequencing gel using silver staining is a reliable and appropriate approach for better separation of the SSR allele (Lagoda, 1998, Korolija *et al*., 2008) [30, 31]. This method is very useful because of simple chemicals used in the gel preparation and high sensitivity of silver staining. Ultraviolet light chamber, molecular imager, and gel documentation systems are not required for the PAGE with silver staining. Simple X-ray film viewer is sufficient for the imaging and it reduces the cost of SSR analysis and makes the method most effective and efficient (Vuylsteke *et al*., 2007, Fountain *et al*., 2011) [32, 33]. In case of metaphor agarose large amount of PCR amplified product is required, because ethidium bromide is not much sensitive to detect low amplified product in comparison to PAGE with silver staining.

The main drawback of this system is strong background development occur sometimes, which may cause a problem in the allele scoring. But with practices and standardization of the run time, gel concentration, voltage supply and use of good quality reagents for silver staining these issues can be minimized. Polyacrylamide gel electrophoresis on sequencing gel apparatus with silver staining is a good alternative to the high-cost instruments used for genotyping.

Conclusion

Microsatellite markers are the great tools for the assessment of genetic diversity in plants. The separation of the fragments on the electrophoretic system is an important aspect. In the present investigation, among the four gel base methods, polyacrylamide gel on sequencing gel apparatus with silver staining revealed the best resolution for the alleles. In case of PAGE on midi assembly, it is useful if the population size is limited to 10-15 only. Polyacrylamide gel on sequencing gel apparatus is very cost effective and efficient method than metaphor agarose gel, capillary electrophoresis, and other DNA sequencer based techniques. Allelic separation was not clear on metaphor agarose and on simple agarose no separation was observed. The observed results indicate polyacrylamide gel with silver staining is the best method for genotyping.

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