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Research Article

Genetic variation of acid lime genotypes as assessed by microsatellite markers

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Abstract
Evaluation of genetic dissimilarity is imperative to the plant breeder for improvement of high yielding varieties and hybrids. A large variability present in acid lime crop due to cross pollinated crop. In the present study, an effort was made to assess genetic diversity and their genetic relatedness of 14 acid lime genotypes collected from diferent locations and maintained at All India Coordinated Research Project on Fruits, Dr.P.D.K.V., Akola by using Simple Sequence Repeate markers.The average genetic similarity level among the 14 acid lime genotypes was 0.97 and ranged from 0.17 to 0.97. The cluster analysis separated the genotypes into three major groups.Total 52 allele were detected by ten primer pairs and size of allele ranged from 100 to 1000 bp. Average polymorphic information content (PIC) value was 0.72 whereas, the highest 0.86 and the lowest 0.38 was observed in AG-14 and CAT-01 markers, respectively.The results clearly showed that, the high level of genetic diversity was observed among the genotypes and SSR markers tested in the present study was highly polymorphic and more informative for the assessment of genetic diversity of acid lime genotypes.

Key words
Acid lime, genotypes, SSR, genetic diversity.

INTRODUCTION

Acid lime (*Citrus aurantifolia* Swingle) is one of the most important commercial fruit crop globally. India is the largest producer of acid lime in the world. Acid lime (*Citrus aurantifolia*) belongs to the genus Citrus of family *Rutaceae* with chromosome number 2n=18. It is originated in India. In India it is commonly known as nimbu. Other synonyms of nimbu are sour lime, acid lime, Indian lime, key lime and etc.The leading states of India in area and production of lime/lemon are Gujarat, Andhra Pradesh, Maharashtra, Odisha and Madhya Pradesh the area under lime/lemon is 296 thousand hectares with production of 3397 thousand tonnes and the productivity of 11.47 t/ha. (Anonymous, 2018). In India, states growing acid lime include Maharashtra, Andhra Pradesh, Telangana, Bihar, Tamil Nadu, Karnataka and Himachal Pradesh (Anonymous, 2019).

In some cases, bud mutation occurs leading to diversity in morphological characters indifferent branches of the same plant (Nicolosi *et al.,* 2000). Breeding and conservation of genetic resources necessitate the evaluation of genetic diversity in plant species. Having information on genetic diversity is essential for the acquisition of maximum relative benefits in breeding programs from germplasms (Khiavi *et al.*, 2015). Genetic dissimilarity within and among diverse populations or agro-ecological regions can be assessed using morphological, biochemical, and molecular approaches (Santos *et al.,* 2003).

The polymerase chain reaction (PCR) DNA based molecular markers technologies have been used for the effective quantification of genetic variation and cultivar identity. single nucleotide polymorphisms (SNP's) and

insertions/deletions (INDELs) have been commonly employed in citrus breeding . Genetic variations are considered to be molecular markers and improve our understanding of the genetic basis of phenotypic variations observed in many agronomic traits via linkage mapping. Qiang Li *et al.* (2020) present CitGVD (http://citgvd.cric.cn/home), a comprehensive database of citrus genomic variations that offers a publicly available and free data service for scientific studies. Presently, CitGVD includes a large sets of data on genomic variations (SNP's and INDELs) compiled from two released reference genomes for *Citrus clemientina* and Citrus *grandis* , including 84 phenotypes, gene functional annotations and informative literature. Earlier workers used various molecular markers to ascertain the variation in genotypes of citrus crops that comprise AFLP (Pang *et al.,* 2007), RFLP (Fang *et al.,* 1997), SSR (Shrestha *et al*., 2012), RAPD (EL-Mouei *et al.,* 2011). A microsatellite or Simple Sequence Repeats marker (SSR) has been usually used for genetic mapping and to study genetic diversity. It is regarded to be a more reliable marker than others because they are highly polymorphic and usually co-dominant, easy to use, and evenly distributed in the genome (Nematollahi *et al*., 2013). The major purpose of the study was, to find out the genetic diversity and assess the usefulness of SSR markers in characterizing the acid lime genotypes and select highly variable genotypes for breeding and variety development program.

MATERIAL AND METHODS

In this study 14 acid lime genotypes (**Table 1**) were used from the existing mother block of AICRP on Fruits at Dr.Panjabrao Deshmukh Krishi Vidyapeeth, Akola. From the selected genotypes the young and newly emerging leaves were taken from the plants for DNA isolation and further molecular characterization of acid lime genotypes were perform at Biotechnology Centre, Dr.P.D.K.V., Akola.

Total genomic DNA was isolated using CTAB method (Doyle and Doyle,1990) with a minor modification. Instandard protocol contaminating polysaccharide were removed by using PCI and extracted. The pellet of DNA washed with 70% ethanol and dissolved in 50µl of TE Buffer. The DNA concentration was determined on agarose gel electrophoresis using a known amount of standard 100 bp ladder.

The total 12 SSR primers (Barkley *et al.,* 2006) were amplified with the minor modification in the standard PCR reaction. The details of SSR primers used in the present investigation is mentioned in **Table 2**. PCR was carried out on a Eppendorf using 12 SSR markers. Each 20 µl PCR reaction contained 2 µl 10 X PCR buffer, 17.5 mm $MgCl_2$, 10 mM$dNTP_S$, 0.3 µl taq polymerase (Himedia). Ten pmol of oligonucleotide primer and 10 ng/µl DNA template. For amplification of DNA for SSR markers, Each up the 40 PCR cycles consisted of 30 cycles at 94˚C for template denaturation, 30 cycles at 55˚C to 60˚C for primer annealing and 30 cycles at 72˚C for primer extension. The PCR reaction was completed with 5 minutes incubation at 72˚C. The SSR amplified products were separated on 10 % Polyacrylamide Gel Electrophoresis (PAGE) and stained with silver nitrate staining (Khelurkar *et al*., 2018.) The DNA fragments were photographed and documented using Gel doc system. Electrophoresis was conducted in 1X TBE buffer at 100 Volt for 2 hours.

The presence of an amplicon on polyacrylamide gel was identified as (1) and (0) for the absence and the similarity matrix was constructed based on Jaccard's coefficient as described by Sneath and Sokel (1973). The selected genotypes were grouped by cluster analysis using (UPGMA). Molecular weights of the amplicons were estimated using 100 bp DNA ladder (Hi-media) as standard.

These computations were performed using the program XLSTAT software (www.xlstat.com). The total number of alleles, monomorphic alleles and polymorphic alleles were calculated and per cent polymorphic were also calculated using formula (Blair *et al.,* 1999). The polymorphic information contents were calculated using PIC calculator which mentioned in **Table 3**.

RESULTS AND DISCUSSION

Twelve SSR primers were used to detect the genetic diversity of acid lime genotypes. Out of 12 SSR markers,

Table 1. List of acid lime genotypes used in the investigation

S. No.	Genotypes	Source	S. No.	Genotypes	Source
1	PDKV lime	Dr. PDKV, Akola	8	Akola lime 4	Dr. PDKV, Akola
2	PDKV Bahar	Dr. PDKV, Akola	9	Akola lime 5	Dr. PDKV, Akola
3	PDKV Chakradhar	Dr. PDKV, Akola	10	Sai Sarbati	MPKV, Rahuri
4	PDKV Trupti	Dr. PDKV, Akola	11	Vikram	VNMKV, Parbhani
5	Kagzi lime local	Dr. PDKV, Akola	12	Tenali	AICRP Fruits, Tirupati
6	Akola lime 1	Dr. PDKV, Akola	13	Mangalipattu	TNAU, Coimbtore
7	Sriganganagar local	Dr. PDKV, Akola	14	Pramalini	VNMKV, Parbhani

Table 2. SSR primers used for characterization of acid lime genotypes

S. No.	Primer Name	Sequence Forward – Reverse	Annealing Temp ^{0}C (Tm)	Amplicon Size (bp)
1	TAA45	F-GCACCTTTTATACCTGACTCGG R-TTCAGCATTTGAGTTGGTTACG	56.5	80-150
2	TAA15	F-GAAAGGGTTACTTGACCAGGC R-CTTCCCAGCTGCACAAGC	57.5	160-200
3	CAC15	F-TAAATCTCCACTCTGCAAAAGC R-GATAGGAAGCGTCGTAGACCC	57.5	180-225
4	TC26	F-GATAGGAAGCGTCGTAGACCC R-GATAGGAAGCGTCGTAGACCC	55.5	130-145
5	CAT01	F-GCTTTCGATCCCTCCACATA R-GATCCCTACAATCCTTGGTCC	55.5	50-180
6	TAA3	F-AGAGAAGAAACATTTGCGGAGC R-GAGATGGGACTTGGTTCACACG	57.5	130-170
7	CT19	F-CGCCAAGCTTACCACTCACTAC R-GCCACGATTTGTAGGGGATAG	57.5	50-155
8	TAA27	F-GGATGAAAAATGCTCAAAATG R-TAGTACCCACAGGGAAGAGAGC	55.5	80-125
9	AG14	F-AAAGGGAAAGCCCTAATCTCA R- CTTCCTCTTGCGGAGTGTTC	55.5	125-140
10	GT03	F-GCCTTCTTGATTTACCGGAC R- TGCTCCGAACTTCATCATTG	56.3	120-180
11	TAA52	F-GATCTTGACTGAACTTAAAAG R-ATGTATTGTGTTGATTACG	46.6	80-150
12	TAA41	F-AGGTCTACATTGGCATTGTC R-ACATGCAGTGCTATAATTGAAGT	52	80-170

Table 3. Characterization of acid lime genotypes by using SSR Primers

Primer	Total number of amplicons	Monomorphic alleles	Polymorphic alleles	PIC value	Polymorphism (%)
AG-14	8	0	8	0.86	100
TAA-45	5	1	4	0.77	80
CAC-15	4	1	3	0.59	75
TAA-27	5	1	4	0.70	80
TAA-15	4	0	4	0.70	100
TC-26	4	0	4	0.77	100
GT-03	6	0	6	0.84	100
CT-19	4	0	4	0.77	100
TAA-3	5	0	5	0.84	100
CAT-01	7	0	7	0.38	100
Total	**52**	**3**	**49**	**7.22**	**935**
Average	**5.2**	**3**	**4.9**	**0.72**	**93.5**

10 were amplified in the 14 acid lime genotypes and the remaining two were not amplified. Out of 10 amplified SSR markers, all markers were found polymorphic. **Table 3**. Showed that the primers AG-14, TAA-15, TC-26, GT-03, CT-19, TAA-3, CAT-01 shown 100% polymorphism (**Plate 1-3**) whereas, the 80 per cent polymorphism shown in primer TAA-27 and in TAA 45, 75 per cent polymorphism observed in CAC-15. Total alleles per locus was 5.2 whereas, the average number of monomorphic and polymorphic alleles was 3 and 4.9, respectively. The PIC value of 10 SSRs ranged from 0.38 to 0.86 with an average value of 0.72. The maximum PIC value was observed in marker AG-14 (0.86) and GT-03 (0.84) and the minimum was in CAT-01 (0.38). In a null allele frequency analysis, all SSRs had null allele frequencies close to zero. From (**Table 2**) it can be concluded that the

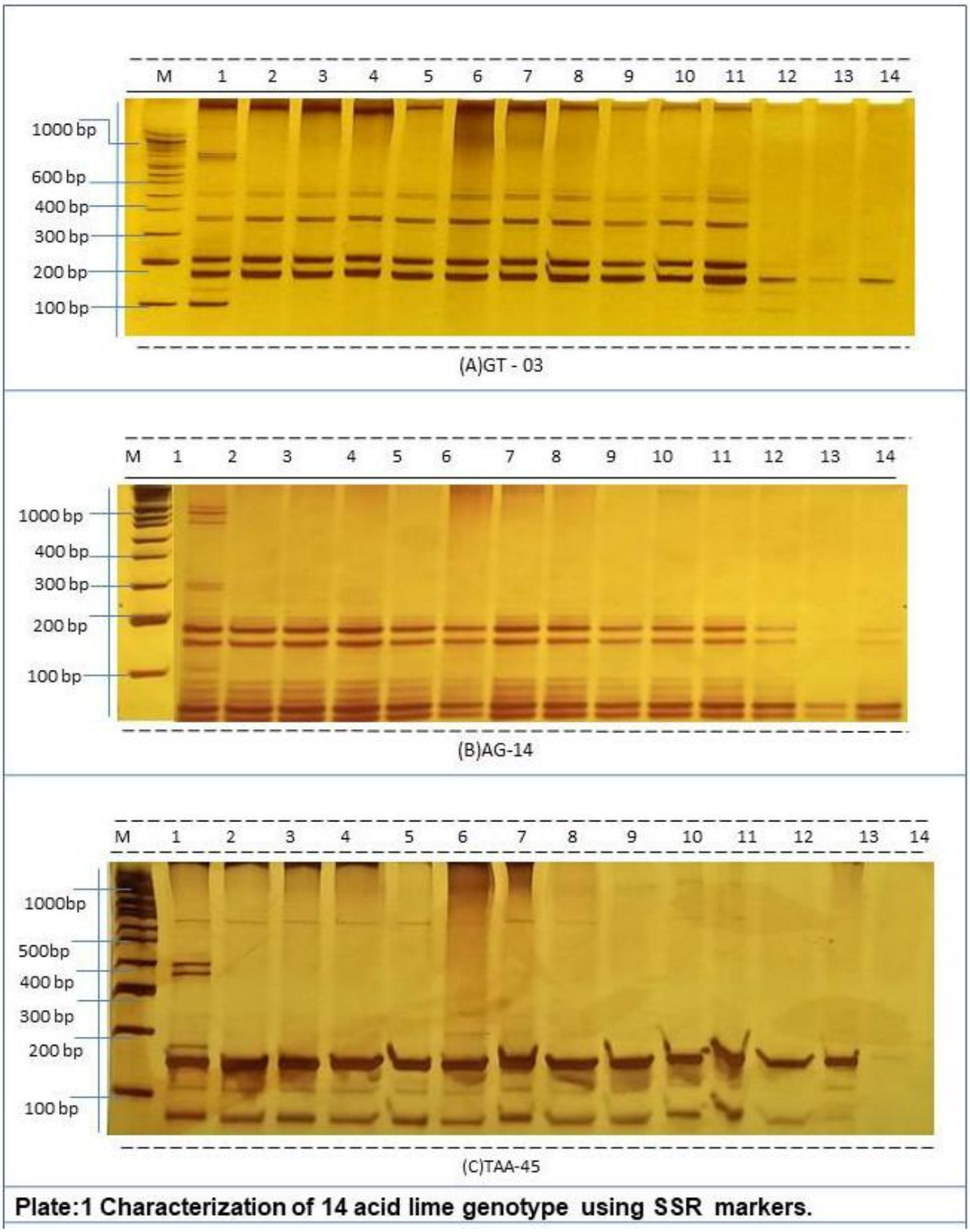

Plate:1 Characterization of 14 acid lime genotype using SSR markers.

(A)GT-03, (B)AG-14, (C)TAA-45, 1-PDKV Chakradhar, 2- PDKV Bahar, 3-Sai Sarbati, 4-Kagzi lime local, 5-mangallipattu, 6-Pramalini, 7-Vikram, 8-PDKV Trupti, 9- Sriganganagar, 10-PDKV Lime, 11-Tenali, 12-PDKV Lime 4, 13-PDKV Lime1, 14-PDKV Lime5

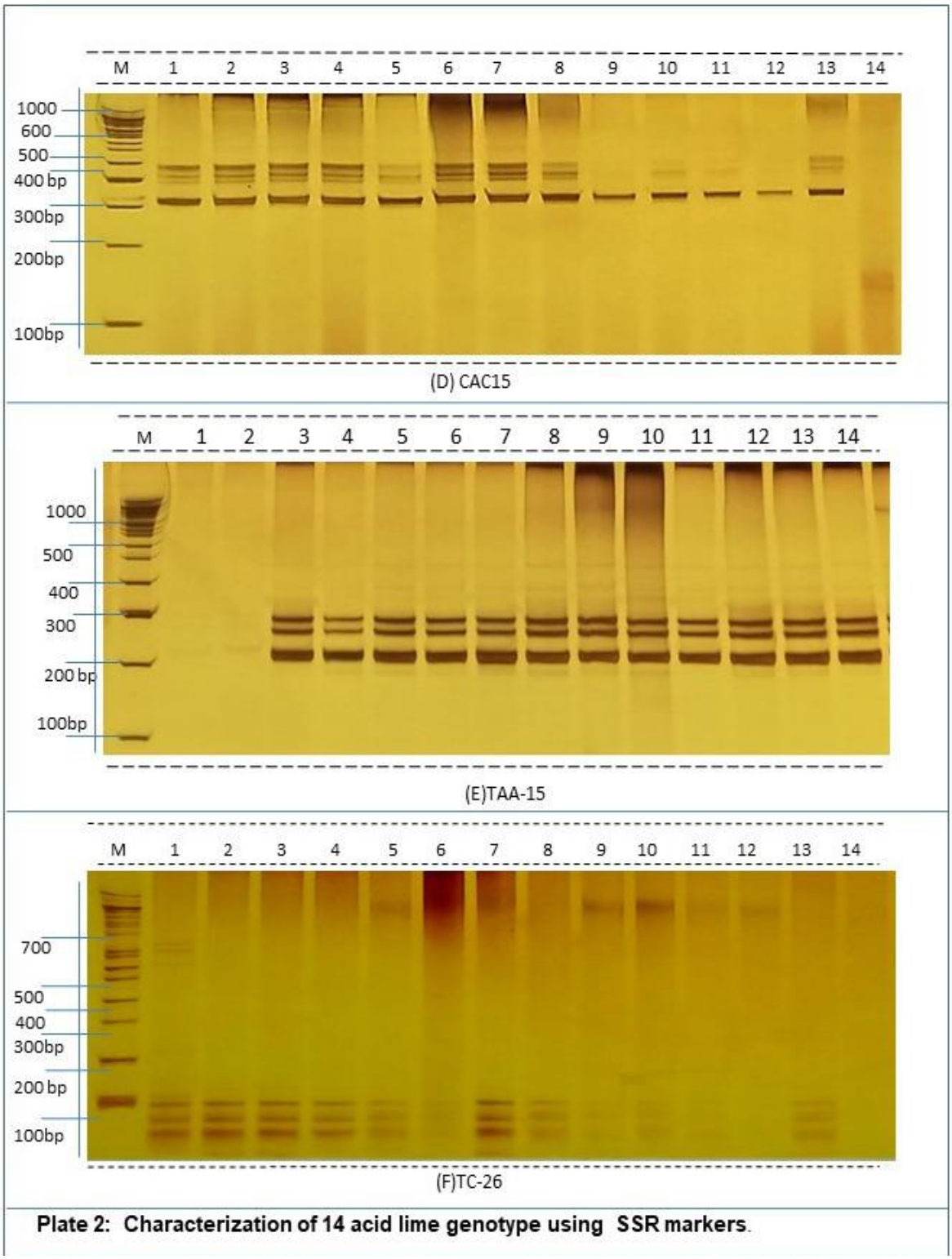

Plate 2: Characterization of 14 acid lime genotype using SSR markers.

(A)CAC-15, (B) TAA-15, (C)TC-26, 1-PDKV Chakradhar, 2- PDKV Bahar, 3-Sai Sarbati, 4-Kagzi lime local, 5-mangallipattu, 6-Pramalini, 7-Vikram, 8-PDKV Trupti, 9- Sriganganagar, 10-PDKV Lime, 11-Tenali, 12-PDKV Lime 4, 13-PDKV Lime1, 14-PDKV Lime5

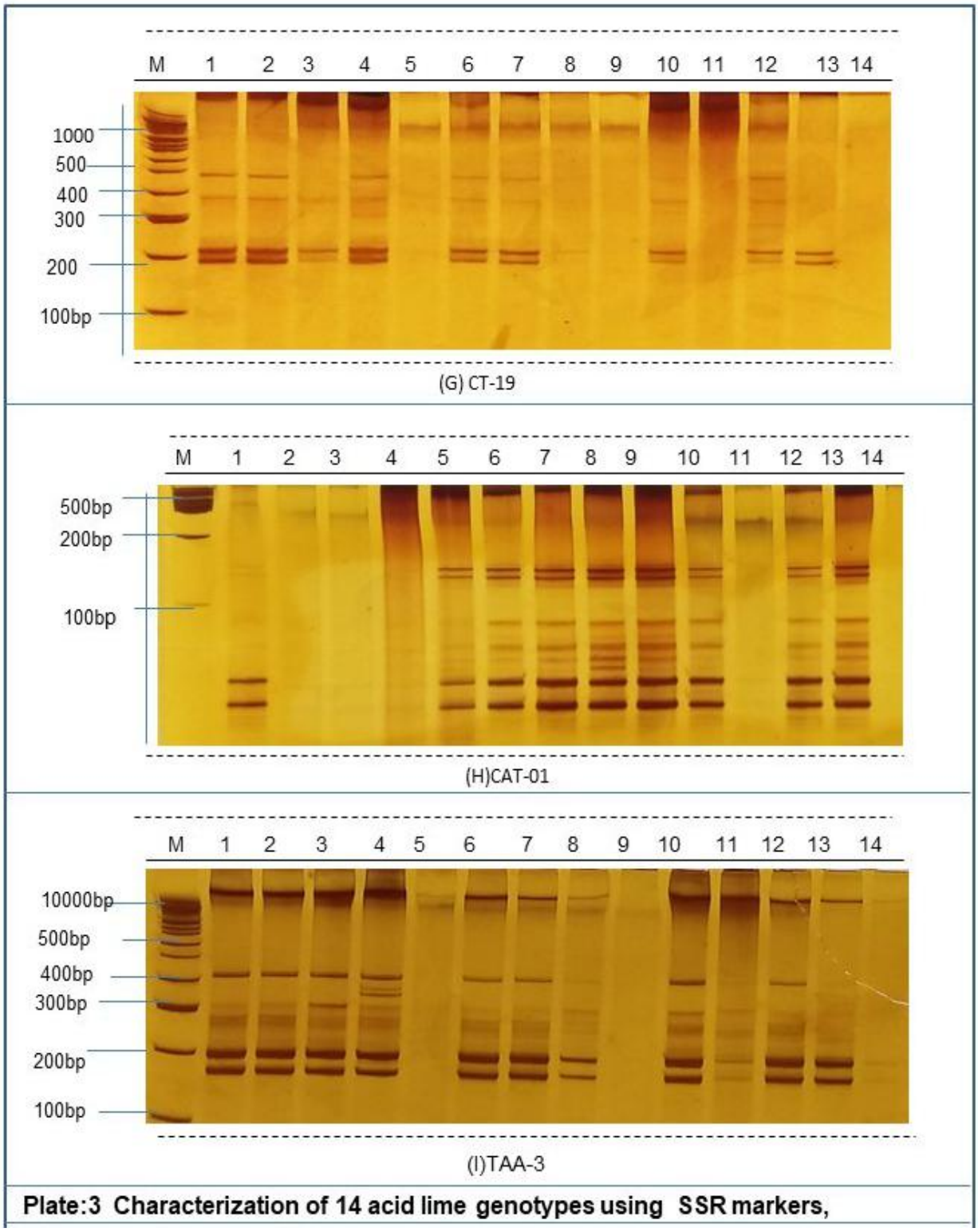

Plate:3 Characterization of 14 acid lime genotypes using SSR markers,

(A)CT-19, (B) CAT-01, (C)TAA-3, 1-PDKV bahar, 2- Kagzi lime local, 3-Vikram, 4-PDKV Chakrdhar, 5-Akola Lime5, 6-Mangalipattu, 7-Tenali, 8-PDKV Lime, 9- Akola Lime4, 10-Sai Sarbati, 11-Pramalini, 12-PDKV Trupti 4, 13-Akola Lime, 14-Sriganganagar local

primer AG-14, TAA-45, TAA-27, TAA-15, TC-26, GT-03, CT-19, and TAA-3 showed 100 per cent polymorphism and showed the higher PIC values. The size of the amplified 10 primer pair was ranged from 100-1000bp.

Cluster analysis was carried out using the UPGMA clustering algorithm. The similarity matrix and dendrogram were constructed using the XLSTAT software (www.xlstat.com). The genetic relationship ofthe 14 acid lime genotypes were studied . Total 14 acid lime genotypes separated into major 3 cluster groups. The majority of the 12 genotypes are grouped under cluster I , clusters 2 and 3 contain only one genotype each, Akola lime- 1 and Akola lime-5, respectively (**Table 4, Fig. 1**.). The results showed that the genotypes PDKV Chakradhar, PDKV Bahar, Sai-Sarbati, Kagzi lime local, Mangallipattu,Pramalini, Vikram,PDKV Trupti, Sriganganagar Local, PDKV Lime, Akola lime-4 were grouped in the same genetic level group under cluster 1. Markers found no differentiation among genotypes present in the cluster 1. Madhumathi *et al.* 2015 perform genetic diversity by UPGMA amongs the sweet orange and found the similarity indices based on Jaccard similarity coefficient ranged from 0.52 to 0.80, which showed the presence of low to moderate diversity among 27 sweet orange accessions.

A few unique SSR alleles were found in one acid lime genotype i.e., PDKV Chakradhar. PDKV Chakradhar showed two specific SSR fragment using primers GT-03 (700-900 bp), AG-14 (800-1000 bp), TAA-45 (450-500 bp) and TAA-3 (300-350 bp), TC-26 (600-700 bp). In another genotype, PDKV lime showed a unique allele in Primer CAT-01 amplified on the range of (100-150 bp) ladder. Such markers used to differentiate closely related genotypes.

Table 4. Clustering of genotypes

Group	Cluster	Genotypes
1	C1	PDKV Chakradhar, PDKV Bahar, Sai-Sarbati, Kagzi lime local, Mangallipattu, Pramalini, Vikram, PDKV Trupti, Sriganganagar Local, PDKV Lime, Akola lime4
2	C2	Akola lime 1
3	C3	Akola lime 5

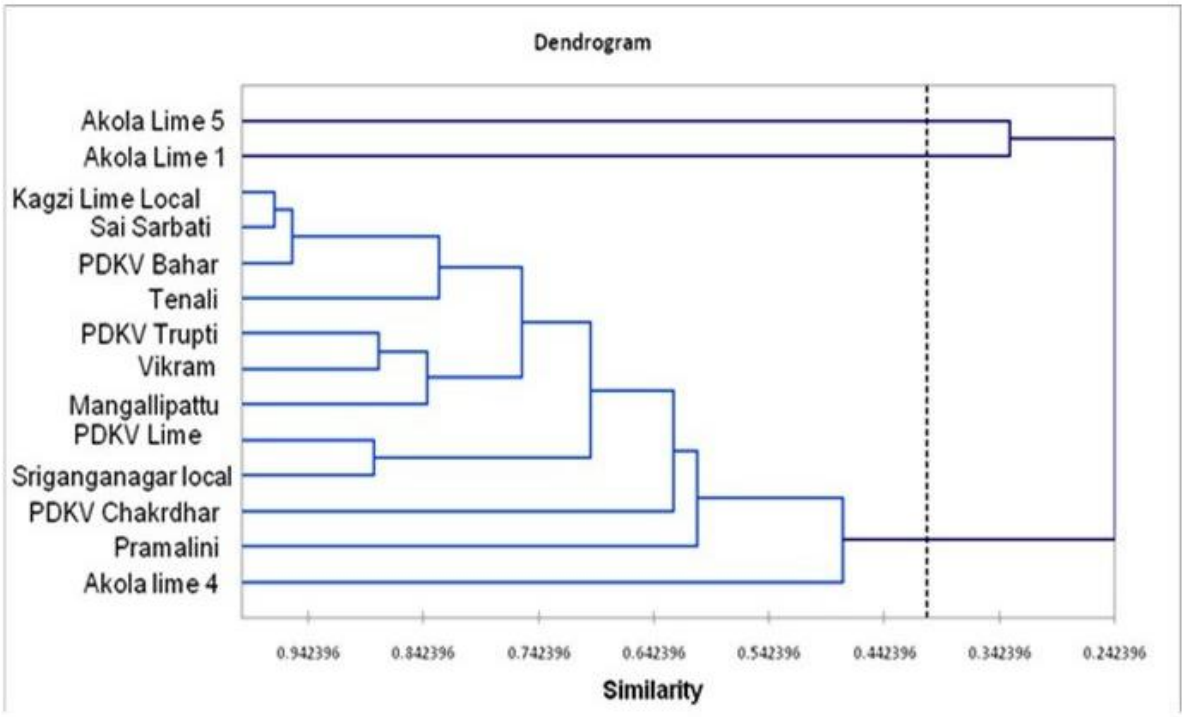

Fig.1. Clustering of genotyes based on UPGMA

Molecular markers are commanding and suitable tools for estimating genetic diversity, determining different percentages, and revealing phylogenetic relationship

among various citrus genotypes (Sharma *et al.,* 2015). The time and cost-effectiveness, replicability and resolution of SSR marker are better or equivalent to those of other markers (Jannati *et al.,* 2009). A total 12 SSR primer pair were used for the evaluation of genetic diversity in 14 acid lime genotypes. Among them ten primer pair were amplified computable bands; primer TAA-52 and TAA-41 not amplified in any of the genotypes. The average number of alleles per locus provide complementary information of polymorphism and more adequate to co-dominant markers, because dominant markers did not permit to detection of all alleles. In this study, a total of 52 alleles were detected with an average number of alleles per locus was 4-8 and total 49 polymorphic amplicons were obtained. The highest number of alleles was detected AG-14 loci (8) (**Plate 1**) and the lowest was in CAC-15 (**Plate 3**), TAA-45, TAA-27, TAA-15, and CT-19. A maximum offive alleles were observed in TAA-3 (**Plate 2**). Average 6alleles were observed in Mandarin and lemon accessions (Li *et al.,* 2006), while 4.4 alleles per locus was observed in the diversity of citrus species by SSR markers (Santos *et al.,* 2003). In Nepal Shrestha *et al.* (2012) observed total of 33 alleles with an average of 3 alleles per locus. In his studies, the highest number of alleles was 4 and it was observed in CAT01, TAA14 and TAA47 loci and the lowest were 2 observed in TC26, AG14 and GT03 loci.

The average value of PIC was 0.72. The markers GT-03, AG-14, and TAA-45 and TAA-3 which having unique banding pattern in the sets of acid limes genotype i.e. PDKV Chakradhar. These bands were specific to the genotypes which can be utilized for genotype/cultivar identification. A setof SSR markers detect the considerable level of genetic variability within the acid lime genotypes may be based on morphological traits i.e., seeded to less seeded, shy bearing habit, thorn less nature of genotype. The results showed the high level of genetic similarity within the acid lime genotypes. These results are coinciding with the results of Ahmed *et al.* 2003 and Uzun *et al.* (2009). In the previous studies (Shrestha *et al.,* 2012), 12 SSR primers were used for assessment of genetic diversity in 62 acid lime landraces in Nepal region and it was found that the applied primer combinations were suitable and efficient in investigating genetic diversity in the given samples, according to polymorphic percentage and polymorphic information content. The results of Jaccard coefficient method and UPGMA algorithm were the superlative tools for designing dendrogram. In the similarity matrix of Jaccard coefficient, calculated similarities were in the range of 0.24 to 0.94. Munankarmi *et al.* (2014) reported the same range of similarity in acid limes. The dendrogram shows that 14 acid lime genotypes were separated into three cluster groups. Cluster 2 and 3 had a high level of genetic distance at the molecular level might be due to morphological variation in characters. This is in agreement with the Jannati *et al*. (2009) who reported that morphologically different acid lime accessions showed the higher level of genetic distance. This variation allows identifying different genotypes with molecular markers. Molecular markers may provide information on the past and biology of genotypes, but it does not necessary to reflect what may be observed in the morphological traits. Present results demonstrate that;the genotypes collected from orchards were morphologically similar but genetically separate within the genotypes.

The results of this investigation proved that SSR markers are more useful to differenciate acid lime genotypes of a single specie. Among SSR markers tested, AG-14, TAA-15,TC-26,GT-O3,CT-19,TAA-3 and CAT-01 showed the high polymorphism and PIC value, are more helpful for diversity analysis in acid lime.

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