academicJournals

Vol. 6(12), pp. 854-860, December 2014
DOI: 10.5897/IJBC2014.0687
Article Number: B277D7D49021
ISSN 2141-243X

http://www.academicjournals.org/IJBC

International Journal of Biodiversity and Conservation

Full Length Research Paper

Assessment of genetic diversity of a critically endangered important medicinal plant *Chlorophytum borivilianum in* different agro-climatic regions of India revealed by random amplified polymorphic primer (DNA Marker)

Baby Kumari*, M. A. Mallick and Govind K. Vyas

Bhabha Atomic Research Centre, Anushakti Nagar, Trombay, Mumbai, India.

Received 26 January, 2014; Accepted 26 May, 2014

Chlorophytum borivilianum **(Family: Liliaceae), an important threatened medicinal herb is designated as 'Rare' in Red Data Book of Indian plants. In this study, random amplified polymorphic primer DNA (RAPD) markers were used to assess the genetic diversity in six genotypes of *C. borivilianum* collected from different geographical regions of Rajasthan, Madhya Pradesh and Jharkhand. Out of the 86 bands, 45 were found to be polymorphic and the level of polymorphism was 52.35% thus, revealing a high degree of polymorphism. The maximum polymorphism was obtained using primer number 108 that was 90.90% and least with primer number 102 that was 12.5%. The Jaccard's similarity coefficient ranged from 0.70 ± 0.186. The maximum similarity value was noticed between SM3 and SM5 genotypes collected from KRD (M.P) and Maa Umaya farm (M.P.), respectively.**

Key words: *Chlorophytum borivilianum*, genetic diversity, random amplified polymorphic primer DNA (RAPD), genotypes.

INTRODUCTION

World Health Organization (WHO) has estimated that more than 80% population of developing countries depends on herbal medicines for their basic healthcare needs (Canter et al., 2005). *Chlorophytum borivilianum* commonly known as "Safed Musli", a monocot belongs to liliaceae family, is a member of a special class of Ayurvedic herbs known as 'Rasayana' and falls into a group 'Vajikarna' that is Aphrodisiac (Marais and Reilly, 1978; Thakur and Dixit, 2008).

Safed Musli commands an exorbitant price for its processed products both in indigenous and global markets. It is the latest sensation in the Indian herbal medicine, gaining wide acceptance over the global neutraceutical industries. It has been listed as an endangered species in "Red data book of Indian plants" by the Botanical Survey of India (Nayar and Shastry, 1998) and it is predicted that if steps for its conservation are not taken, the Indian forest will lose this valuable plant (Oudhia, 2001). National medicinal plants board (NMPB) New Delhi, has recognized Safed Musli as sixth important herb to be protected,

*Corresponding author. E-mail: baby.rai@gmail.com.

Table 1. *Chlorophytum borivilianum* samples from different locations of India.

S/N	Location	Sample name
1	SM-1 (Udaipur, Wild)	UW
2	SM-2 (Udaipur cultivated)	UC
3	SM-3 (KRD farm, MP cultivated)	KRD
4	SM-4 (MP wild)	MW
5	SM-5 (MaaUmiya Farm, Jamli MP,cultivated)	MU
6	SM-6 (Jharkhand, wild)	JK

promoted and preserved.

The genus includes more than 300 species, which are distributed throughout the tropical and subtropical parts of the world. Tropical and subtropical Africa is the probable centre of origin of the genus, where about 85% of the species are found in India. *C. borivilianum* is mainly distributed in Southern Rajasthan, North Gujarat, Western Madhya Pradesh and some part of Jharkhand and Bihar (Maiti and Geetha, 2005). Among 300 species, 13 species are reported in India and *C. borivilianum* is producing highest yield of saponin among all species.

Germplasm conservation is the major prospective of this rare medicinal herb along with other prospects. The focus of this study was to achieve the molecular diversity found in *C. borivilianum* for the selection of vigorous germplasm which produces highest quantity of saponin that is its major bioactive compound. The study was focused on major biodiversitical region producing Safed Musli (wild and cultivated). The characterization of *C. borivilianum* germplasm has been based mainly on phenotypic characteristics. *C. borivilianum* shows wide morphological and agronomic variations for roots (Geetha and Maiti, 2002). However, divergence studies made on the basis of molecular markers provide precise information about phylogenetic descriptive and hierarchical account of individuals or groups of individuals.

The method of polymerase chain reaction with a set of arbitrary primers (RAPD-PCR) was used to determine the genetic variations and to estimate genetic diversity in collected germplasm of *C. borivilianum* sant and frend.

RAPD markers have caught the fancy of many individuals in the field of applied plant breeding. RAPD due to their low cost and high reproducibility have been used for different purpose, such as assessment of genetic variability, genetic relationship, identification of cultivars etc. in number of crop plants. This molecular marker is based on the PCR amplification of random locations in the genome of the plant. The number of amplification products is directly related to the number and orientation of the sequences that are complementary to the primers in the genome. RAPD was found to be simple and efficient among the available DNA based techniques (Welsh and McClelland, 1990; Williams et al., 1990) and furthermore sequence information is not needed (Gepts, 1993; Karp et al., 1997). With the availability of this genetic tool, genetic diversity and genetic analysis can also be estimated (Chapco et al., 1992; Landry et al., 1993; Demeke et al., 1996; Li et al., 2004) Furthermore, RAPD techniques are advantageous because of their simple requirement of a small quantity of DNA and their ability to uncover a large number of polymorphisms (Cheng et al., 1997; Carelli et al., 2006). In this study, *C. borivilianum* accessions collected from the wild forests and organic farms of Madhya Pradesh, Rajasthan and Jharkhand were characterized using RAPD-random amplified polymorphic DNA markers, to study the molecular diversity among the collected accessions.

MATERIALS AND METHODS

Plant material

Six accessions of *C. borivilianum* (Safed Musli) from three different agro-climatic regions of India that is Madhya Pradesh (MP), Rajasthan (Udaipur) and Jharkhand (three cultivated and three wild) were used (Table 1).

DNA extraction

Plantlets grown from seeds on filter paper bridge were used for DNA extraction: 3 g leaves obtained from each variety of plants were homogenized in liquid nitrogen. DNA was isolated by Doyle and Doyle (1990) method with some modifications. Pellets were washed with 70% alcohol and dissolved in TE (Tris- Cl-EDTA) buffer after drying.

DNA quantification and dilution

The Quantification of DNA was carried out using Nano Drop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The DNA was again re-quantified by running it through agarose gel (0.8%), 150 ml in 0.5 X TBE (Tris borate EDTA) buffer containing 0.5 μg/ml of ethidium bromide. 5 μl of DNA sample was loaded with a mixture of DNA loading dye, after electrophoresis gel was viewed under UV. Presence of single compact band at the corresponding position to λ phase DNA (Control) indicated high molecular weight of isolated DNA. The dilution was done with sterile distilled water to ensure that all of the DNA samples have equal concentration of 25 ng/μl.

RAPD polymerase chain reaction

For RAPD analysis, 50 primers from set # 1 obtained from the University of British Columbia, Vancouver, Canada were screened. Out of 50 primers, 15 primers were selected for the study and four primers were removed because of their monomorphic nature and poor reproducibility. PCR reactions were performed in final volume of 25 μl containing 2.5 μl of 10 X Assay Buffer (“Puregene, Gband”); 0.5 units of Taq DNA polymerase (“Bangalore Genei”), 0.5 μl (200 μM) each of dNTPs (“Puregene, Gband”), 10 pmols/reaction of random primers (British Columbia) and 1 μl (50 ng of template DNA). The PCR was performed in ‘Biometra Thermo cycler’ using the standard cycling parameters. Following the amplification, the PCR products were loaded on 1.2% agarose gel (Himedia, molecular grade), which was prepared in 1X TBE buffer containing

Table 2. DNA yield based on absorbance (260nm) obtained from various Safed Musli samples.

Safed Musli samples	Ratio of A260/A280	Concentration of DNA (ng/μl)
SM-1 (Udaipur Wild,UW)	1.97	2595.40
SM-2 (Udaipur cultivated, UC)	1.89	532.37
SM-3 (KRD farm, Indore)	1.8	5043.08
SM-4 (MP wild, MW)	1.8	805.30
SM-5 (MaaUmiya Farm, Jamli MP, MU)	1.86	707.16
SM-6 (Jharkhand, JK)	2.1	671.62

0.5 μg/ml of the Ethidium Bromide. The amplified products were electrophoresed for 3 - 3.5 h at 100 V with cooling. After separation, the gel was viewed under UV trans-illuminator and photographed by digital camera.

Gel analysis and scoring of RAPD product

In order to score and preserve banding pattern, photograph of the gel was taken by a digital camera, under UV transilluminator. RAPD bands were designated on the basis of their molecular sizes (length of polynucleotide amplified). 200 bp ladders were loaded simultaneously with primer and product in the gel was used to estimate the molecular sizes. The distance run by amplified fragments from the well was translated to molecular sizes with reference to molecular weight marker. The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored, but a major band corresponding to faint bands was considered for scoring.

Similarity matrix and cluster analysis

The statistical calculations were done using Free Tree programme version, 0.9.1.50 (Pavlicek et al. 1999). Similarity matrices, was constructed using the Jaccard's similarity coefficient values (Rholf et al., 2000) to find out genotypic relationship. The average distance of a single variety from the rest of the genotypes was also calculated. The 0/1 matrix data obtained from RAPD primers was arranged to get separate similarity matrix which was subjected to unweighted pair-group method with arithmetic averages (UPGMA) analysis. This analysis generated dendrogram which was further compared using the Mantel matrix correspondence test (MxComp module of NTSyS).

RESULTS AND DISCUSSION

The present investigation included six accessions of *C. borivilianum* (Safed Musli) from three different agro-climatic regions of India that is Madhya Pradesh (MP), Rajasthan (Udaipur) and Jharkhand. In this work, we report on the results of genetic diversity in the collected *C. borivilianum* genotypes (cultivated and wild), using RAPD marker.

DNA isolation and RAPD analysis

A single sharp band corresponding to λDNA was observed for all the samples of DNA on 0.8% agarose gel. The quality of DNA was determined as the ratio A_{260}/A_{280} which ranged from 1.8 to 2.0, which is indicative of good quality plant DNA and ration was almost consistent, irrespective of the *C. borivilianum* samples. The concentration of DNA preparation varied from 0.6 to 5.04 μg/μl for SM-3 and SM-6 respectively (Table 2). This is because the stochastic nature of the band and banding pattern of DNA amplification with RAPD, reproducibility of the banding pattern has been found to change. Finally, only those bands were considered as polymorphic, which did not amplify in certain samples on repetition. The banding pattern generated by each primer was primer and species dependent and varied from 3-15 at 37°C annealing temperature. A total of 86 amplicons were obtained with 11 primers with an average of 7.8 bands per primer (Table 3).

Out of 86 bands, 45 were found to be polymorphic and the level of polymorphism was 52.35% (Table 3).The most informative primers were 101 and 108 with maximum polymorphic bands and least informative primer was 102 and 111 with 1 polymorphic band out of total 8 and 6 bands. The maximum polymorphism was obtained using primer number 108 that was 90.90 % and least with primer number 102 that was 12.5 %. None of the primer produced all monomorphic bands (Table 4).

This probability was calculated by Simpson's index as described by Hunter and Gaston (1988). The discriminatory power ranged from 0.35 to 1.0 for pattern generated by various primers. The discriminatory index of value one was obtained for the primers 129 and 160 which produced unique banding pattern for all the varieties (Table 4).

Genetic relationship among the varieties/lines

The pair wise genetic similarity estimates (Jaccard's coefficient) based on RAPD banding pattern were used for cluster analysis to present genetic relationship in the form of dendrogram (Figure 1). The similarity coefficient matrix was subjected to algorithm unweighted pair group method for arithmetic average analysis (UPGMA) to generate clusters. The Jaccard's pair wise similarity coefficient value for six Safed musli samples are presented in Table 5. The range of genetic similarity was found to

Table 3. List of arbitrary primers with total polymorphic amplicons and polymorphism generated for six Safed Musli samples.

Primers	Sequence 5'-3'	Total number of bands (a)	Total number of polymorphic band (b)	Polymorphism (%) b/a × 100
101	GCGGCTGGAG	8	7	87.5
102	GGTGGGGACT	8	1	12.5
105	CTCGGGTGGG	7	4	57.14
108	GTATTGCCCT	11	10	90.90
111	AGTAGACGGG	6	1	16.66
125	GCGGTTGAGG	6	3	50.00
127	ATCTGGCAGC	3	1	33.33
129	GCGGTATAGT	11	5	45.45
160	CGATTAAGAG	15	8	53.33
165	GAAGGCACTG	6	3	50.00
170	ATCTCTCCTG	5	2	40.00
		86	45	52.35

Table 4. Primer discriminative power (D).

Primer	D at 37°
101	0.54
102	0.60
105	0.88
108	0.74
111	0.35
125	0.88
127	0.74
129	1.00
160	1.00
165	0.88
170	0.61

Table 5. Jaccard's similarity coefficient for six Safed Musli genotypes based on RAPD profiling

Parameter	UW	UC	KRD	MW	MU	JK
UW	1.00					
UC	0.67	1.00				
KRD	0.73	0.71	1.00			
MW	0.74	0.68	0.71	1.00		
MU	0.76	0.69	0.80	0.79	1.00	
JK	0.68	0.68	0.64	0.71	0.67	1.00

be between 0.64 (KRD and JK) to 0.80 (KRD and MU). The average genetic similarity coefficient observed was 0.70 ± 0.186.

Dendrogram based on UPGMA analysis spitted into three clusters, in which one cluster contains four Safed musli genotypes while two other clusters contain single genotype each. Cluster containing four genotypes were UW, KRD, MU and MW while UC and JK clustered themselves as a single entry. UC spitted as a distinct entry at 0.68 Jaccard's similarity coefficient while JK spitted at 0.67 similarity coefficient. Sixteen genotype specific allelic positions were generated through seven primers out of 11 RAPD primers used. Maximum four allelic positions were obtained using the primer no. 108

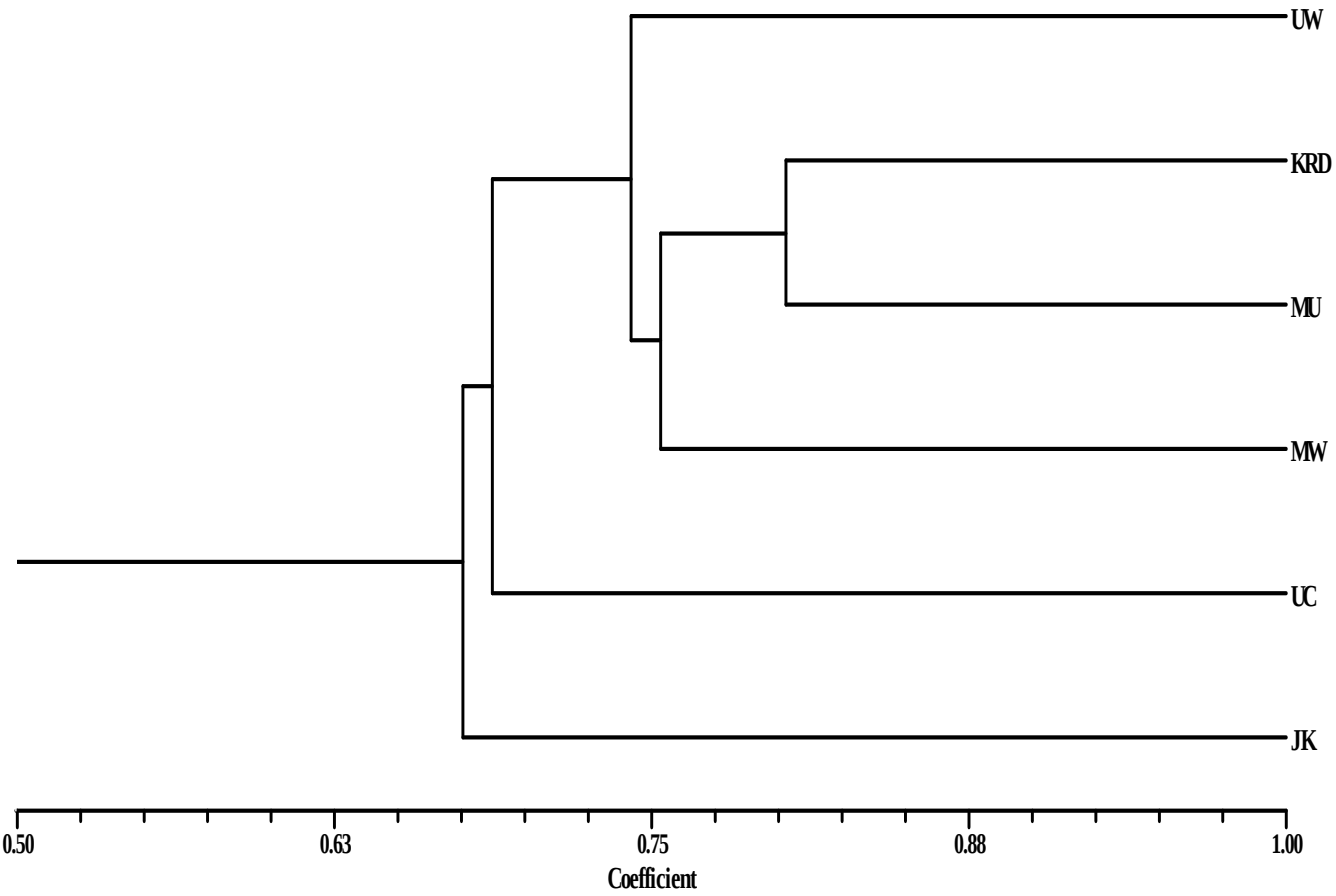

Figure 1. Dendrogram generated for six Safed musli genotypes using UPGMA cluster analysis based on Jaccards' similarity coefficient for RAPD data.

and 160. All the obtained specific allelic positions can be used for the variety identifications.

In this study, the extent of genetic diversity in cultivated and wild variety of *C. borivilianum*, obtained from central, western and eastern parts of India was determined using RAPD markers (Figure 2). Several reports are available to demonstrate the use of RAPD markers for determination of genetic variation in plants. Genetic diversity assessment of nine accessions of *C. borivilianum* from central India was studied by PCR based Molecular marker RAPD (Dwivedi and Sharma, 2011). Identification and assessment of genetic relationship in three *Chlorophytum* species of and two high yielding genotypes of *C. borivilianum* through RAPD markers were done (Sanghamitra et al., 2011). Tripathi et al. (2012) also observed genetic similarity using RAPD markers among *C. borivilianum* accessions collected from different places of Central India. Our results are also in agreements with other researchers. Shi et al. (2008) studied on the genetic diversity among cultivated and wild variety of *C. chinensis* using ISSR markers and found similar results as we found in our results that there is very less diversity among cultivated and wild variety of plant, although it can be said that geographical and agro climatic factors plays important role in the genetic diversity.

Conclusion

The level of RAPD variation in the population of *C. borivilianum* has been investigated. RAPD variation was found in accessions of different agro climatic regions of India. It can be suggested that RAPD markers have the potential to detect genetic variability among different populations of *C. borivilianum*. RAPD marker will have impact on vigorous germplasm selection and its presser-vation. However, this study is not complete in terms of sample size and area coverage; hence a more extensive genetic diversity investigation including areas not covered yet should be done representing a population by as many collections as possible and awareness of the public about the importance, value of conservation and sustainable utilization of this plant genetic diversity should be promoted.

Conflict of Interests

The author(s) have not declared any conflict of interests.

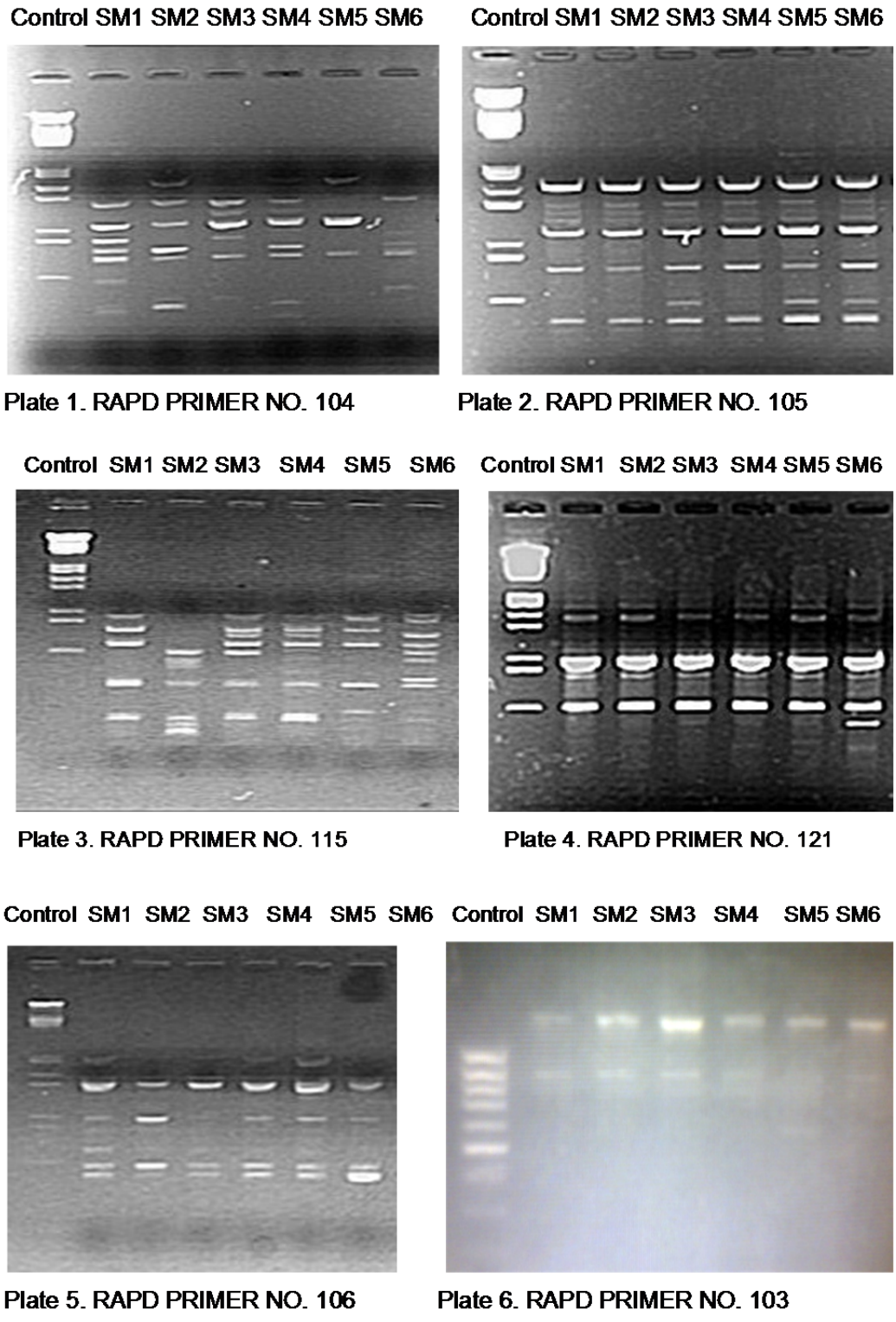

Figure 2. RAPD profiles of six *C. borivilianum* genotypes.

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