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RAPD Analysis of Diversity in 'Safed Musli' (*Chlorophytum borivilianum* Sant. et Fernand.), a Rare Indian Medicinal Herb

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ABSTRACT

RAPD markers were used to assess genetic diversity in seven accessions of a rare Indian medicinal herb *Chlorophytum borivilianum* collected from different geographical regions of India. A total of 290 amplified bands were scored from 33 random decamer primers out of which 242 (83.44%) were found to be polymorphic. The average number of polymorphic bands per primer was 7.33. The Jaccard's similarity coefficient ranged from 0.108 to 0.533 with a mean of 0.338. A dendrogram generated by UPGMA analysis grouped the accessions into four clusters which were not based on their geographical distribution.

Keywords: accession, genetic variability, polymorphism, RAPD markers
Abbreviations: CTAB, cetyl trimethyl ammonium bromide; **RAPD**, random amplified polymorphic DNA; **UPGMA**, unweighted pair group method of arithmetic average

INTRODUCTION

Chlorophytum borivilianum Sant. et Fernand. (Family: Liliaceae) is an important medicinal herb. It grows wild during the rainy season on the sloppy fertile land and produces fasciculated storage roots which are reputed to possess aphrodisiac properties. In India it is mainly distributed in southern Rajasthan, north Gujarat and western Madhya Pradesh (Geetha and Maiti 2002). Known as 'Safed Musli', the roots of this plant form an important ingredient of herbal tonics prescribed in the Ayurvedic system of medicine in India (Kirtikar and Basu 1975). The plant is of great economic importance as its dried roots are sold in the market at a price of Rs 1000 (US $20) per kilogram. In recent years the demand for 'Safed Musli' on the export market has been increased substantially making it a highly prized medicinal herb. Unmindful collection of tuberous roots and poor natural regeneration has resulted in a sharp decline in natural populations of 'Safed Musli'. Nayar and Sastry (1988) have designated it as 'Rare' in the Red Data Book of Indian Plants.

A great degree of variability in growth and morphological traits (Geetha and Maiti 2002; Joshi 2005) and biochemical characteristics (Jat and Sharma 1996; Bhagat and Jadeja 2003) among natural populations of 'Safed Musli' has been reported. However, a survey of literature shows a total lack of information regarding molecular characterization and documentation of this species. The molecular characterization of its germplasm is of considerable significance as it contributes to the effective conservation by revealing the magnitude of the genetic relationship among accessions in a collection and by allowing estimation of the sample genetic diversity (Bhat *et al*. 1999). Although a wide variety of molecular markers have been used for molecular characterization their suitability depends upon their properties (Nesbitt *et al*. 1995; Weising *et al*. 2005). Random amplified polymorphic DNA (RAPD) technique has been successfully applied for identification and assessment of genetic diversity in a large number of medicinal and aromatic plants (Lim *et al*. 2007; Padmalatha and Prasad 2007; Sarwat *et al*. 2008) because of their comparative advantages.

The present investigation was undertaken to study the diversity among natural populations of *C. borivilianum* using RAPD markers.

MATERIALS AND METHODS

Collection of plant material

Field surveys in the different geographical regions of India were undertaken for the collection of *C. borivilianum* germplasm during the rainy season (July- September). Well grown (60-days old) plants along with the root tubers were removed from their natural habitats along with the soil, kept in polybags and brought to Udaipur for their establishment in the field gene bank. During the ensuing season the plants were allowed to remain in the polybags until maturity. The plants were regularly irrigated with water. Germplasm collected from each location was given an accession number (A list of accessions collected is given in **Table 1**). In the following rainy season the collected accessions were transferred to the Field Gene Bank in the nursery of University College of Science, M.L. Sukhadia University, Udaipur, India (**Fig. 1**).

Fig. 1 Field grown plant of *C. borivilianum*.

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Table 1 A list of *C. borivilianum* accessions collected from different areas and maintained in the Field Gene Bank at Udaipur.

Accession №	Place of Collection
PBL-1	Jhadol, Rajasthan
PBL-2	Dariba, Rajasthan
PBL-3	Dhar, Madhya Pradesh
PBL-4	Mandsaur, Madhya Pradesh
PBL-5	Kotda, Rajasthan
PBL-6	Mandsaur, Madhya Pradesh
PBL-7	Mandsaur, Madhya Pradesh

Genomic DNA isolation and RAPD analysis

Total genomic DNA was extracted from the fresh leaves of seven accessions of *C. borivilianum* maintained in the field gene bank using the CTAB method described by Saghai-Maroof *et al*. (1984) with minor modifications. A high concentration (1%) of PVP was added to the extraction buffer to remove phenolics. DNA was quantified by a Versa Flour^{TM} Flourometer (Bio-Rad, USA) using Hoechst 33258 as the dye and calf thymus DNA as the standard (Brunk *et al*. 1979). All the DNA samples were diluted to a final concentration of 5 ng/µl with 1XTE buffer for ready use. Sixty arbitrary decamer primers (Operon Technologies, California, USA) were screened for PCR amplification based on the protocol described by Williams *et al*. (1990). Amplification reactions were performed in a 25 µl reaction mixture containing 2.5 mM $MgCl_2$, 200 µM each of dNTP (Promega, Madison, WI, USA), 1U of Taq DNA polymerase (Bangalore Genei, India), 0.2 µM decamer random primers (Operon Technologies, California, USA) and 25 ng of genomic DNA in 1X reaction buffer. DNA amplification was performed in a Thermal Cycler-9600 (Perkin-Elmer, Boston, MA, USA) programmed as: an initial denaturation step of 4 min at 94°C, followed by 40 cycles of 1 min at 94°C (denaturation), 1 min at 37°C (annealing) and 2 min at 72°C (extension). The last cycle was followed by a final extension step of 7 min at 72°C. After amplification, the samples were loaded and electrophoresed on 1.6% agarose gel (Sigma, USA) containing 0.5 µg/ml ethidium bromide. A 1 kb ladder (MBI Fermantas, USA) was used as the size marker.

Data analysis

All reactions were repeated in triplicate and only the consistently reproducible RAPD bands were scored for the presence (1) or absence (0) across the accessions for each primer. Pairwise comparisons were performed using the Jaccard's coefficient (Jaccard 1908) to calculate variability between different accessions. The similarity matrix was subjected to UPGMA clustering to generate a dendrogram using NTSYS-pc, version 1.80 Software (Rohlf 1995).

Primer efficiency

Primer Resolving Power (Prevost and Wilkinson 1999) was used to identify the primers that would distinguish cultivars most efficiently. Resolving Power (Rp) of a primer was calculated as the sum of "band informativeness" of all bands produced by a primer.

$$\text{Band Informativeness (Ib)} = 1 - \left(2 \times \left| 10.5 - P \right|\right);$$

$$\text{Rp} = \sum \text{Ib}$$

where 'P' is the proportion of accessions containing the band.

Polymorphism Information Content (PIC) (Botstein *et al*. 1980) was calculated for each marker as genetic diversity measure (Anderson *et al*. 1993). PIC was used to identify primers that would distinguish cultivars most efficiently.

$$\text{PIC} = \frac{\Sigma\left(1 - \Sigma P_i^2\right)}{n}$$

where 'P_i' is the frequency of i^{th} allele and 'n' is the number of cultivars/accessions.

RESULTS AND DISCUSSION

Out of the sixty primers screened, thirty three primers (**Table 2**) gave scorable amplification and band resolution. The size of the amplification products generated by these primers ranged from 250 to 3500 bp. A total of 290 bands were amplified by 33 primers. The number of bands per primer ranged from 4 (OPG-9, OPX14) to 15 (OPK 16, OPK-14) (**Fig. 2**) with an average of 8.78 bands per primer. Out of 290 bands generated 242 were polymorphic (83.44% polymorphism) (**Table 2**). The average number of polymorphic bands per primer was 7.33. Eight out of the 33 primers displayed 100% polymorphism. Primer OPK-16 showed the maximum number of polymorphic bands (15) followed by OPG-1 and OPK-14 (12).

Pooled band informativeness of all bands generated by a primer represents its resolving power (Rp). Rp was found to be highest in OPK-14 followed by OPK-16 and OPG-1 and lowest in OPG-9 (**Table 2**). Polymorphic Information Content (PIC) was highest for primer OPC-11 followed by OPK-19 and OPG-8.

Jaccard's similarity coefficient values ranged from 0.108 to 0.533 with a mean value of 0.338. The similarity

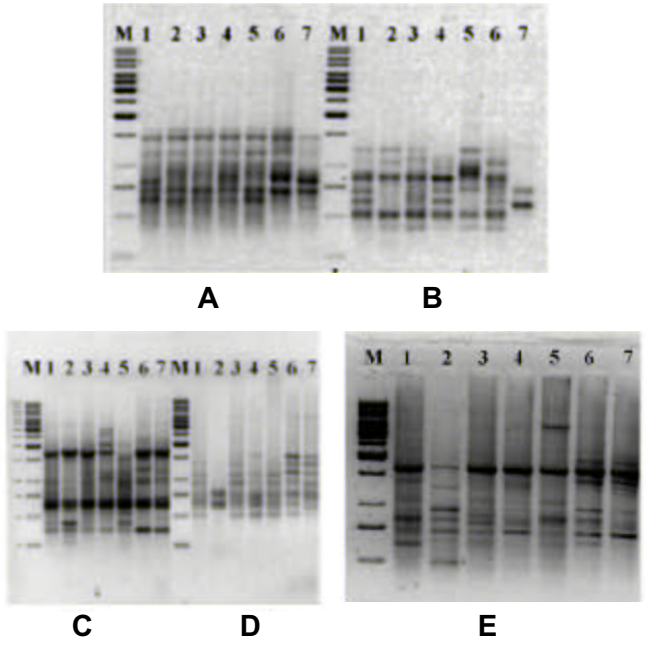

Fig. 2 RAPD profiles generated from seven accessions of *C. borivilianum* using selected primers (A, OBG2; B, OPK 20; C, OPK14; D, OPK11; E, OPK1). Lanes: 1, PBL-1; 2, PBL-2; 3, PBL-3; 4, PBL-4; 5, PBL-5; 6, PBL-6; 7, PBL-7); M = molecular weight marker 1 kb ladder (MBI Fermentas).

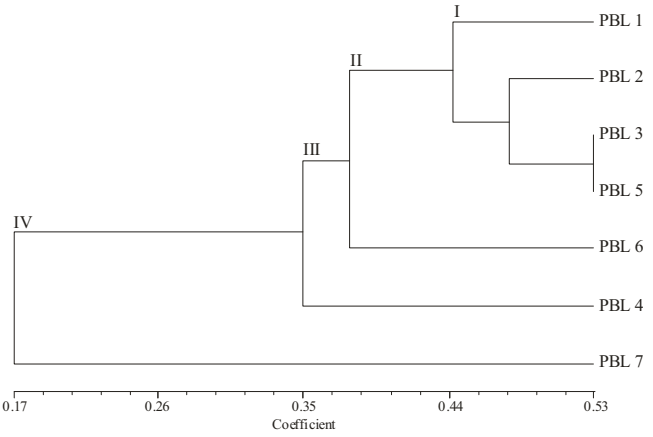

Fig. 3 Dendrogram obtained from RAPD analysis using UPGMA demonstrating relationship between different accessions of *C. borivilianum*.

Table 2 Details of RAPD bands generated by 33 random primers for 7 natural accessions of *C. borivilianum*.

Primer	Total № of bands	№ of polymorphic bands	Percentage polymorphism	Molecular size of bands (kb)	Resolving power (Rp)	Polymorphic information content (PIC)
OPA12	9	9	100	0.40-1.30	3.14	0.32
OPB16	5	3	60.0	0.50-1.25	1.5	0.19
OPB19	10	10	100	0.63-2.40	4.28	0.35
OPB20	8	6	75.0	0.45-2.50	4.0	0.30
OPC1	10	10	100.0	0.38-2.70	6.0	0.39
OPC2	7	4	57.4	0.35-2.20	2.0	0.22
OPC11	10	10	100	0.40-3.50	6.67	0.42
OPF7	9	6	66.66	0.32-1.80	3.71	0.30
OPG1	14	12	85.71	0.35-1.60	7.71	0.32
OPG2	6	5	83.33	0.60-1.47	2.57	0.23
OPG8	9	9	100	0.45-1.30	4.28	0.41
OPG9	4	2	50.0	0.60-1.30	1.33	0.24
OPG11	7	4	57.4	0.67-2.50	3.0	0.26
OPG13	10	6	60.0	0.60-1.00	4.57	0.26
OPG19	9	8	88.88	0.45-2.00	3.43	0.35
OPK1	7	4	57.4	0.50-3.60	2.86	0.27
OPK4	9	9	100	0.45-2.00	3.67	0.26
OPK11	8	7	87.5	0.50-2.00	4.57	0.33
OPK14	15	12	80.0	0.25-3.00	12.57	0.36
OPK16	15	15	100	0.40-3.00	7.71	0.40
OPK17	11	10	90.90	0.30-2.25	4.86	0.28
OPK19	11	10	90.90	0.35-3.50	5.71	0.41
OPK20	9	9	100	0.40-1.35	4.0	0.31
OPL8	5	4	80.0	0.60-2.30	2.0	0.23
OPR2	10	8	80.0	0.55-1.80	4.28	0.34
OPS20	7	5	71.4	0.45-1.00	3.43	0.31
OPT5	7	6	85.71	0.40-1.80	4.28	0.34
OPW10	8	7	87.5	0.45-1.20	3.14	0.38
OPW19	9	7	77.77	0.55-2.40	3.43	0.28
OPX8	8	7	87.5	0.40-1.30	3.6	0.39
OPX14	4	3	75.0	0.67-1.90	2.33	0.35
OPY5	9	6	66.66	0.40-1.50	4.0	0.25
OPZ11	11	10	90.90	0.50-1.80	4.67	0.36

Table 3 Distribution of Jaccard's similarity coefficient values of seven field-grown accessions of *C. borivilianum*.

	PBL-1	PBL-2	PBL-3	PBL-4	PBL-5	PBL-6	PBL-7
PBL-1	1.0000						
PBL-2	0.4733	1.0000					
PBL-3	0.4328	0.5141	1.0000				
PBL-4	0.3600	0.3186	0.4340	1.0000			
PBL-5	0.4286	0.4460	0.5333	0.3619	1.0000		
PBL-6	0.4000	0.4074	0.3750	0.2793	0.3381	1.0000	
PBL-7	0.2069	0.1081	0.1409	0.1524	0.1643	0.2366	1.0000

values obtained between pairs of accessions are presented in **Table 3**. The matrix was subjected to UPGMA clustering to generate a dendrogram. The dendrogram obtained through cluster analysis revealed 4 clusters I, II, III, and IV (**Fig. 3**). PBL-1, PBL-2, PBL-3 and PBL-5 were grouped into cluster I. PBL-4, PBL-6 and PBL-7 did not form any group and remained as separate clusters. Accessions PBL-3 and PBL-5 shared almost 53% similarity which was maximum of all the similarity values among all the accessions. Accession PBL-2 shared 51% and 44% similarity with PBL-3 and PBL-5, respectively and formed a subgroup of cluster I. Accession PBL-1 was more similar to PBL2, PBL3, PBL-5 and PBL-6 as compared to PBL-4 and PBL-7. Accession PBL-6 was more diverse from PBL-4 and PBL-7 as compared to rest of the accessions. Accession PBL-7 was found to be significantly diverse from the rest displaying a maximum similarity coefficient of 0.23 with PBL-6 and minimum of 0.108 with PBL-2.

The objective of this study was to assess genetic diversity existing in *C. borivilianum* using RAPD markers. The present study and similar studies on *Oroxylum indicum* (Jayaram and Prasad 2008) suggested that RAPD is more appropriate for analysis of genetic variability in closely related genotypes. The evaluation of RAPD fingerprints for natural accessions of *C. borivilianum* by 33 random primers resulted in a data set of 290 bands, out of which 242 bands were polymorphic. The percentage polymorphism was as high as 83.44%, higher than reported in *Feronia limonia* (80%) by Vyas (2006), *Magnolia officinalis* (78.22%) by He *et al*. (2008), *Citronella* (57.0%) and Palmarosa (63.0%) by Sangwan *et al*. (2001), *Vetiveria zizaniodes* (29-35%) by Shasany *et al*. (1998) and *Crotalaria longipes* (33%) by Jayanthi and Mandal (2001). The observed high level of polymorphism suggests profound genetic heterogeneity in the species which is in agreement with the results obtained from morphological and biochemical evaluation in *C. borivilianum* by Bhagat and Jadeja (2003). The resolving power (Rp), calculated as the sum of band informativeness of all bands produced by a primer, was found to be highest in OPK-14 followed by OPK-16. Therefore it can be concluded that OPK-14 and OPK-16 are the most informative primers which can assist in distinguishing all the accessions. The high Rp values of these primers like OPC-1 and OPC-11 were also correlated with a maximum number of polymorphic loci and 100% polymorphism. A dendrogram was constructed on the basis of genetic similarity values calculated according to Jaccard's coefficient to reveal similarity between the accessions. The coefficient similarity value ranged from 0.108 to 0.533 with a mean of 0.338 which indicated that the distribution of variation in *C. borivilianum*

was moderately diverse. The lowest Jaccard's similarity value represents maximum diversity. Four clusters obtained through dendrogram were not in agreement with the geographical distribution of the genotypes of *C. borivilianum*. Moderate variability distribution has also been reported in *Andrographis paniculata* (Padmesh *et al*. 1999). This association where accessions belonging to different geographical regions are grouped together may be attributed to the unique and broad genetic base of the species.

During the present study a large number of RAPD primers (33 out of 60 screened primers) were used to ensure that a larger portion of the genomes were covered in the analysis. RAPD analysis employing 30 primers has been reported to be sufficient to provide better estimates of genetic relationships (Singh *et al*. 2002b). The level of genetic variation detected among *C. borivilianum* accessions with RAPD analysis proved it to be a rapid, precise and sensitive technique for delineating genetic relationships among genotypes.

In another study (Joshi 2005) a significant degree of genetic variability among the accessions in terms of morphological traits was observed. However, no relationship among the clusters/groups formed on the basis of morphological characteristics such as leaf number, leaf length, number of flower per inflorescence, etc. could be established based on molecular markers in the present study. Occurrence of differences in molecular and morphological characters is not uncommon (Khanuja *et al*. 2000; Rao and Hodgkin 2002) as all the loci responsible for expression of morphological traits may not be covered by the molecular markers. Singh *et al*. (2002a) have reported such a lack of similarity in *Azadirachta indica* clones.

The results obtained during the present investigation provide evidence for the occurrence of variation in *C. borivilianum* germplasm. Further, they demonstrated that it would be advantageous to acquire and assess more accessions from different locations for wider and more comprehensive information about the pattern of genetic variation distribution in this species.

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