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Assessment of molecular variations among different biotypes of *Commiphora wightii* (Arnott.) Bhandari, using RAPD markers.

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Abstract

Commiphora wightii or "Guggal" is an important medicinal plant growing in parts of Rajasthan, Gujarat and Karnataka in India. The wide arrays of medicinal uses in both ancient and modern medicinal practices have rendered the plant to a state of constant threat to extinction. Today techniques such as ISSR and RAPD not only resolve issues related to taxonomic positions of flora and cultivar identification programs, but also help characterize the genetic variations that exist among the cultivars or wild native populations. These form significant steps towards providing genetic information for future breeding and improvement programs. The present investigation is focused to analyse the molecular differentiation among the wild populations of *C.wightii* inhabiting the diverse locations of the Indian Thar desert using RAPD markers.

Key words: *Commiphora wightii*, guggul, genomic DNA, RAPD, polymorphism.

1. Introduction

Commiphora wightii (Arnott.) Bhandari, commonly known as "guggal" (family-Burseraceae) is a pharmacologically, economically and ecologically important species that grows wild, in parts of Rajasthan, Gujarat and Karnataka in India (IUCN,201). The plant has vast economic value and a wide array of medicinal uses in both ancient and modern therapeutics. Due to the presence of steroidal compound guggulsterone (forms E and Z) in the oleo-gum resin, it has been used in treating various ailments and disorders since ancient times. The exudates of the plant are a complex mixture of resin (61%), gum (29.3%) and other bio chemicals (6.1%). More than 150 compounds have been reported and new compounds continue to be reported[1]. Sushruta Samhita, a well known Ayurvedic medicinal text, describes the usefulness of the oleo-gum resin from *C. wightii* in the treatment of

ailments including obesity, lipid metabolism disorders [2, 3]. The oleo-gum resin is also prescribed to treat rheumatoid arthritis and arthrosclerosis [4, 5]. Its anti-arthritic properties were further confirmed by Chaturvedi et al., 1965 [6] and Sharma & Sharma, 1977 [7]. It works well as an antimicrobial[8], anti inflammatory [9, 10] and an anti cancerous agent [11, 12]. In recent years the use of PCR based methods such as ISSR and RAPD has become quite common for molecular analysis in order to resolve the taxonomic positions of flora and cultivar identification programs[13]. It has become necessary to characterize the genetic variations that exist among the cultivars or wild native populations and advanced selection. This is one of the significant steps towards providing genetic information for future breeding programs, and for the improvement of any plant species.

Unlike the morphological and bio chemical markers, the RAPD markers are not prone to environmental factors and they help in accurate characterization of plants, expressing the extent of genetic diversity among taxa[14, 15]. The RAPD markers are also useful in assessing the genetic variations among the different taxa at molecular level, without the requirement of prior knowledge of DNA sequence of the species[16] and can also provide significant information for conservation and improvement strategies[17, 18]. The gum resin with myriad medicinal uses is exploited without check; this is done by mechanical tapping methods which even though provide a good quantity of gum resin from each but, but at the same time also force a slow death on the plant. Heedless exploitation coupled with poor natural regeneration of this endangered plant species has resulted in a drastic decline in its populations across all the different natural habitats of Rajasthan[19].

The genetic diversity assessment among populations of such an important plant is a prerequisite for systematic conservation programs. The present investigation was focused to analyse the molecular differentiation among the wild populations of *C.wightii* inhabiting the diverse locations of the Indian Thar desert using RAPD markers.

2. Materials and Methods

2.1 Plant Material

The plant material for genomic DNA isolation was collected from identified wild populations of *C. wightii* as mentioned in table 1. Leaves were brought in the laboratory in liquid nitrogen and stored at - 20ºC in zip lock bags. The leaves were subjected to the extraction of genomic DNA by minor modifications in the CTAB method[21].

Table 1: Location of the sampled population of *C. wightii* with terrains and average climatic conditions

S.No.	Area	Terrain	Population Code	Sample size	Sample code	Climatic Conditions
Ajmer Region						
1.	Ajmer	Hilly	A	7	A3	Hot, Semi Arid
2.	Pushkar	Hilly	PB	5	PB1	Hot, Semi Arid
3.	Srinagar	Rocky	SN	5	SN2	Hot, Semi Arid
4.	Mangaliyawas	Hilly	M	10	423	Hot, Semi Arid
Jaipur Region						
1.	Jobner	Plains	JOB	6	JOB6	Hot, Semi Arid
Jodhpur Region						
1.	Kailana	Plains	J	7	J6	Hot, Arid climate
Rajasamand Region						
1.	Bheem	Rocky	BH	1	BH	Sub-tropical dry climate
2.	Gomti Chouraha	Rocky	H	6	H2	Sub-tropical dry climate
Udaipur Region						
1.	Kirwa Ghat	Hilly	KG	4	KG2	Tropical climate
2.	Kavita	Hilly	KVT	2	KVT2	Tropical climate
3.	Neemach Mata	Hilly	NM	2	NM1	Tropical climate
4.	Thoor	Hilly	TH	5	TH2	Tropical climate

2.2 DNA Isolation Protocol

Fresh green leaves weighing 0.5 g were de-veined and grinded to a fine powder in mortar pestle using liquid N_2. A 60 ml homogenization buffer stock was prepared by adding 9ml 150 mM Tris-Cl, 3 ml 25mM EDTA, 18ml 1.5 M NaCl (all at pH 8.0) to 30 ml of DDW, and warmed at 65ºC. 2.1g CTAB and 1.8g PVP was added to the pre-warmed solution, 180 µl Beta Mercaptoethanol was added prior to the process of homogenization. The fine leaf powder was then suspended in 3 ml of pre-warmed CTAB solution. This 3 ml suspension was transferred to a sterile centrifuge tube & 20 µl of RNase was added to it. The solution was

incubated for 45 minutes at 65ºC with gentle inversions. The tube was then cooled to room temperature & 3 ml of Chloroform: IAA ratio (24:1) was added to it. The tube was inverted gently 20-25 times to form an emulsion. The emulsion was centrifuged at 10,000 rpm for 10 min. at RT. The upper aqueous layer was pipette out, transferred into sterile centrifuge tubes without disturbing the interphase. 3 ml of 3M NaCl was added to the aqueous phase and once again subjected to centrifugation at 10,000 rpm at RT. 0.6 volumes (1.8 ml) Isopropyl alcohol was added to the aqueous phase, mixed well and incubated for 30 min. at RT. The solution was centrifuged at 10,000 rpm for 15 min. at RT. The supernatant obtained was gently poured off. The pellets obtained were washed thoroughly with 750 µl of 70% ethanol & spun at 10,000 rpm for 5 minutes. The supernatant was discarded and the white pellet obtained was air dried (~45 min), & then re-suspended in 30 µl of TE (10 mM Tris HCl+ 0.1 mM EDTA; pH 8.0) at 4ºC. 3M sodium chloride solution was again added to the T.E. Buffer + DNA solution and re-precipitation was done with 70% ethanol. The sample was centrifuged at 10,000 RPM for 15 minutes and the pellet was re-dissolved in TE buffer. The process was repeated three times. This method allowed recovery of good quality DNA, suitable for complete restriction digestion and was amplifiable in PCR as compared to other methods.

2.3 DNA Quantification

DNA concentration was estimated using spectrophotometric method (UV-Vis Spectrophotometer, Pharmaspec UV-1700, Shimadzu, Japan). Absorbance of the solution was measured at wavelengths 260 nm and 280 nm. The DNA concentrations were calculated using following formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{50 \times OD_{260} \times \text{Dilution Factor}}{1000}$$

The ratio of OD_{260}: OD_{280} was calculated. The same procedure was followed for quantifying all the samples. DNA samples were diluted to final concentration of 5ng/µl and 10ng/µl for use in PCR analysis. DNA samples which were the best in quality as evident on agarose gel and an OD_{260}/OD_{280} ratio nearer to 1.7-1.8 were used for further analysis.

2.4 RAPD Amplification

Twenty random decamer primers (Eurofins Genomics, Bangalore, India) (MAP-11P, 13P,17P; OPA-11,17,20; OPG 17; OPH4, 14,17; OPJ 18, OPN 06,10,16,20; OPU-02,08,11,13,17) were used for RAPD analysis. RAPD amplifications were performed in a final volume of 25 µl, containing 50 ng of template DNA, 2.5 µl of 10X assay buffer, 25mM

(2.0 µl) $MgCl_2$, 200 µM dNTPs, 2.0 µl (20 pmol) of primer and 1 unit of Taq polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The RAPD amplifications were performed as using a thermal cycler (T-100, Bio-Rad, U.S.A.)

The first cycle consisted of initial denaturation of template DNA at 94ºC for 04 minutes, followed by 40 cycles of denaturation at 94ºC for 01 minute, primer annealing at the melting temperature, TmºC for the particular RAPD primer (TmºC range- 30ºC to 36ºC) for 01 minute, primer extension at 72ºC for 02 minutes and a final extension at 72ºC for 07 minutes.

2.5 Data Analysis

The data were scored as "1" for the presence and "0" for the absence of band for each primer sample combination for RAPD analysis. A dendrogram was constructed using the un-weighted pair group method with arithmetic average (UPGMA) with the SAHN model of the NTSys-PC software to show a phenotypic representation of the genetic relationships as revealed by similarity coefficient (Sneath and Sokal, 1972)[18].

3. Results & Discussion

A total of 20 RAPD primers were screened, of which 11 primers showed reliable banding patterns with high reproducibility and clear band resolution (Fig. 1) DNA samples of *C.wightii* were collected from 12 locations.

Table 2: Extent of polymorphism determined in different samples of *C. wightii* using RAPD primers, and their sequence used.

Primer name	Sequence 5'–3'	Loci amplified	Polymorphic Loci	Percentage polymorphism	Approx. band range size (bp)
OPU 17	ACCTGGGGAG	06	00	00	1500-200
OPU 13	GGCTGGTTCC	03	00	00	1200- <500
OPU 11	AGACCCAGAG	02	00	00	700-200
OPU 08	GGCGAAGGTT	03	01	33.33	900-400
OPU 02	CTGAGGTCTC	01	00	00	300
OPN-06	GAGACGCACA	03	00	00	700-300
OPN 10	ACAACTGGGG	03	00	00	700-300
OPH 14	ACCAGGTTGG	03	00	00	700-300
OPH 04	GGAAGTCGCC	05	01	20	700-100
OPG-17	TTGTCTCAGG	06	00	00	3000-400
OPA-11	CAATCGCCGT	02	00	00	400-300
Total		37	02	5.405	-

These 11 primers produced a total of 37 amplified loci ranging between 100 to 3000 bp. The maximum percentage ~33.33% polymorphism was exhibited by random decamer OPU-08; with the band size ranging between 400-900bp. OPH-04 produced a total of 05 loci with the band size between ranging 700-1000 bp, out of these 05 loci, only one locus was polymorphic, showing a polymorphism of 20%. The average percentage of polymorphic loci across the RAPD primers was 5.405%, the range was- 20% produced by the primer OPH-04 to 33.33% obtained in primer OPU-08, where as the rest of the primers did not show polymorphism.

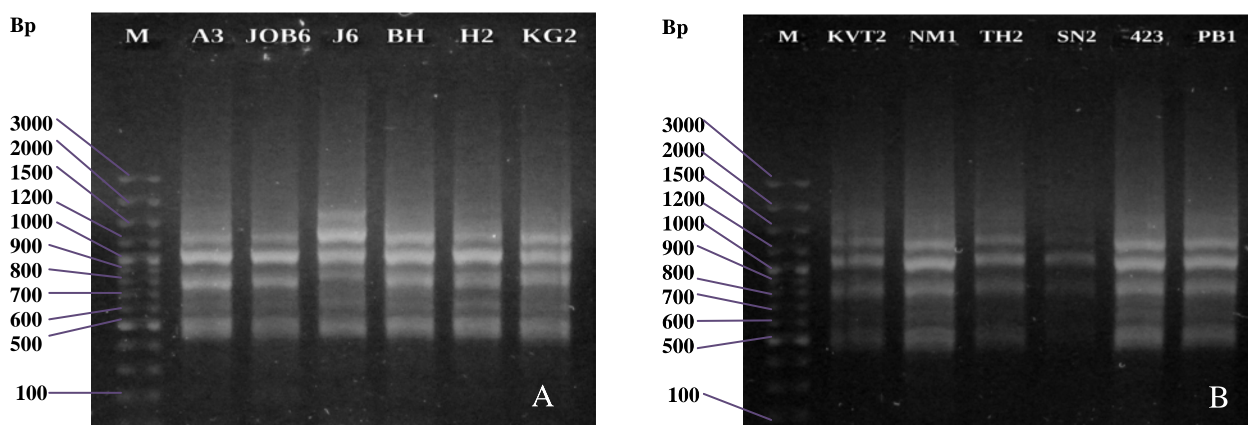

Figure 1: Results of 1.5% agarose gel electrophoresis of PCR products obtained by using RAPD primer OPU-13 M - Represents 100bp ladder. A: Ajmer (A3), JOB6 (Jobner), Jodhpur (J6), Bheem(BH), Gomati Chouraha (H2), Kirwa Ghaat (KG2), B: Kavita village (KVT2), Nimach Mata (NM1), Thoor Village (TH2), Sri Nagar Village(SN2), Mangaliyawas (423) and Pandu beri Pushkar (PB1)

The dendrogram constructed using the SAHN clustering separated the 12 samples, into two major clusters. The first cluster consists of 9 sample populations- A3, KG2, H2, NM1, JOB6, BH, J6, M423, PB1, second consist of 3 sample populations- KVT2, TH2 and SN2. These two clusters shared a common node at 53% similarity level (Fig. 2). The larger first cluster was further subdivided into three sub clusters separating the 09 sample populations from the second cluster of 03 sample populations; they shared a common node at 64% similarity level. The second large cluster with 2 sub clusters comprised of 03 sample populations sharing a node at 73% similarity level.

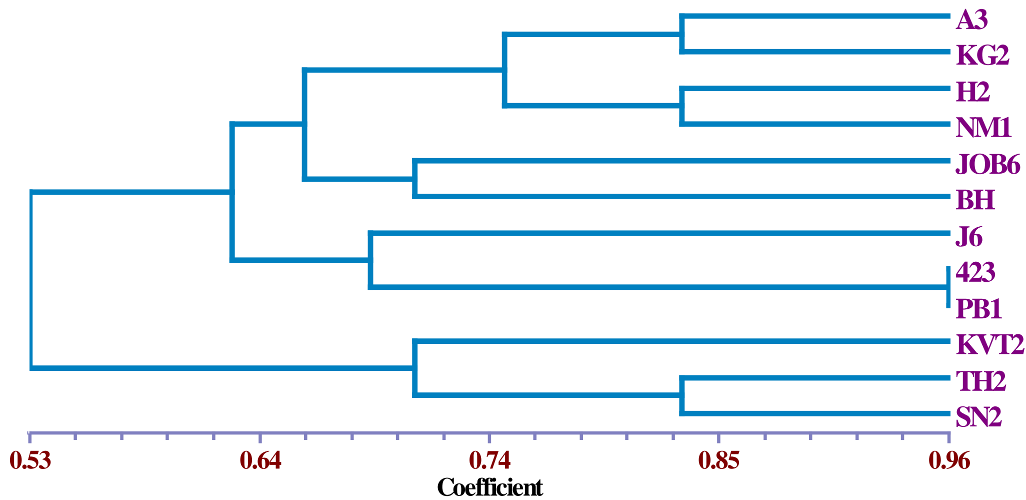

Figure 2: Dendrogram showing clustering patterns among 12 sample populations of C. wightii as revealed through RAPD markers.

Jaccard's pair-wise similarity coefficient values among all the populations of *Commiphora wightii* using all the ISSR primer profiles generated vary between 0. 20 and 0.95 as shown in table 3. 20% similarity which happens to be the least in terms of similarity coefficient data for all RAPD profiles has been observed between A3 and SN2. NM1-SN2 and TH2-NM1 show 33% similarities while 37% similarity is observed betweenKG2-SN2 and A3-TH2. A similarity of 50% has been recorded between BH and TH2.

Table 3: Similarity matrix for Nei and Li's Coefficient of twelve populations of *C.wightii*

Sample	A3	JOB6	J6	BH	H2	KG2	KVT2	NM1	TH2	SN2	423	PB1
A3	1.00											
JOB6	0.58	1.00										
J6	0.58	0.58	1.00									
BH	0.54	0.70	0.62	1.00								
H2	0.62	0.62	0.62	0.75	1.00							
KG2	0.83	0.58	0.75	0.70	0.79	1.00						
KVT2	0.58	0.66	0.66	0.62	0.54	0.75	1.00					
NM1	0.79	0.79	0.54	0.66	0.83	0.79	0.54	1.00				
TH2	0.37	0.45	0.62	0.50	0.41	0.54	0.79	0.33	1.00			
SN2	0.20	0.54	0.54	0.58	0.41	0.37	0.62	0.33	0.83	1.00		
423	0.58	0.66	0.66	0.45	0.70	0.66	0.66	0.70	0.62	0.54	1.00	
PB1	0.54	0.70	0.70	0.50	0.66	0.62	0.62	0.66	0.58	0.58	0.95	1.00

70 to 75% similarity has been seen in many sample populations like JOB6-BH, H2-BH, BH-

KG2, PB1-JOB6, J6-KG2, M423-H2, PB1-J6, NM1-M423, and KG2-KVT2. 79% is observed in A3-NM1, KVT2-TH2, JOB6-NM1, KG2-NM1, and KG2-NM1. 83% similarity is observed in A3-KG2, H2-NM1, and TH2-SN2. The highest similarity coefficient of 0.95 or 95% similarity is observed between M423-PB1

The existence of genetic variants represented by the numbers of alleles at a locus and their frequency of distribution in a population often causes polymorphism in a given population. This study is an attempt to reveal the natural genetic diversity among different populations of *C. wightii* in order to frame efficient conservation strategies. Polymorphism studies on *C. wightii* and other species utilizing different RAPD primers have been reported [22, 23]. Similarly a considerable amount of literature is available on assessment of genetic diversity of many plant species using molecular markers, and they have established a correlation between geographical distance and genetic similarity between individuals [24, 25, 26].

The genetic polymorphism detected by RAPD markers in this study, is in agreement with the assumption that natural populations of cross breeding plant species carry high level of genetic diversity as compared to self breeding ones.

The 11 RAPD primers in the present study yielded 37 bands out of which only two bands were polymorphic and an average of 5.4% polymorphism was observed and a maximum 33.33% polymorphism was detected in decamer OPU-08. Cluster analysis in the present investigation suggest a strong genetic differentiation among all the populations of *C.wightii*; similar results were reported by Harish et al., 2013 [19] and Verma & Rana, 2011[26].

The Jaccard's similarity coefficient of *C.wightii* by RAPD primer analyses in the present study also revealed that a maximum 95% similarity level was observed between M423 and PB2; this may be attributed to the eco-climatic conditions for both these sample populations being nearly same with a geographical distance of 25 km. separating them. In contrast the minimum similarity level at 33.33% was observed between sample populations SN2 and NM1, which are located in diverse ecological zones. SN2 is present in a hot semi arid zone where as NM1 inhabits a zone with tropical climate.

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