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Quantitative assessment of genomic DNA isolated from different populations of *Commiphora wightii* (Arnott.) Bhandari, in Rajasthan

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ABSTRACT

Commiphora wightii commonly known as "Guggal" is found in arid and semi-arid regions including the deserts of India, Africa and Pakistan. The present study was aimed to investigate the quantity as well of quality of genomic DNA extracted from the different wild populations present in the state of Rajasthan. Samples were collected from different eco climatic regions in the state. An optimized protocol was used for the extraction of genomic DNA of the species, owing to the high content of polysaccharides and phenols present in it. The use of 3M NaCl proved to be especially beneficial during the extraction process and helped yield pure and high quality DNA samples that were verified through the ISSR profiles generated and were deemed fit for research downstream. The study also revealed that diverse edaphic and climatic conditions have had no major effect on the quantity and quality of genomic DNA of C.wightii.

Key words: *Commiphora wightii*, Guggul, Genomic DNA, ISSR, Gum resin, CTAB

INTRODUCTION

Commiphora wightii (*Arnott.*) Bhandari (Family- Burseraceae) commonly known as "Guggal" is found in arid and semi-arid regions including the deserts of India, Africa and Pakistan. A very vast natural population of guggal inhabits the different ecological conditions of Rajasthan. The species has been included in IUCN Red data list under Data deficient. UNDP has listed this species as "critically endangered" [1]. The Government of India has banned the export of the species because of its unchecked use and exploitation. Guggul comprises of several plant sterols, diterpenes, steroids and alcohols [2], however, its main components are guggulsterones E ($C_{21}H_{28}O_2$) and Z ($C_{21}H_{28}O_2$) [3] which are involved in anticholesterol activity [4]. After successful experiments, the ayurvedic formulation of this species was approved for marketing in India as a hypolipidemic drug [5], [6]. It is considered to be a threatened plant [7] and an over-exploited species in India [8].Observations have revealed that this species is under threat in its entire range of distribution, in all the regions it inhabits in Rajasthan and Gujarat [9]. According to a report by Parmar (2003) [10], within a period of 10 years or so, the populations of *C. wightii* have shrunk to less than 50%, and have been scattered and divided into smaller subpopulations of fewer individuals. A recent study by Reddy *et al.* (2012) [9], conducted through a survey in Rajasthan during 2007-2009, showed that the species was present only in 2% of the areas that were samples, affirming its rarity.

In general, the classification of plant groups are based on comparative assessment in morphological characters more specifically floral characters. It has been already established that morphological traits are the expression of gene interactions, which are often greatly affected by climatic and edaphic factors, therefore, plants of the same species can exhibit enormous variability growing in diverse locations.

Molecular techniques are very useful for exploiting genetic diversity and also for authentication and identification of genotypes. With regards to this, the isolation of high purity genomic DNA is an important pre-requisite to achieve a logical conclusion for molecular biological applications. The present investigation was, therefore, undertaken to

evaluate the quality and quantity of genomic DNA isolated from different populations of this highly useful medicinal plant in Rajasthan, India.

MATERIALS AND METHODS

Plant Material

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 CTAB method [11].

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

Area	Terrain	Population Code	Sample size	Sample code	Climatic Conditions
Ajmer Region					
Ajmer	Hilly	A	7	A3	Hot, Semi Arid
Pushkar	Hilly	PB	5	PB1	Hot, Semi Arid
Srinagar	Rocky	SN	5		Hot, Semi Arid
Mangaliyawas	Hilly	M	10	423	Hot, Semi Arid
Jaipur Region					
Jobner	Plains	JOB	6	JOB6	Hot, Semi Arid
Jodhpur Region					
Kailana	Plains	J	7	J6	Hot, Arid climate
Rajasamand Region					
Bheem	Rocky	BH	1	BH	Sub-tropical dry climate
Gomti Chouraha	Rocky	H	6	H2	Sub-tropical dry climate
Udaipur Region					
Kirwa Ghat	Hilly	KG	4	KG2	Tropical climate
Kavita	Hilly	KVT	2	KVT2	Tropical climate
Neemach Mata	Hilly	NM	2	NM1	Tropical climate
Thoor	Hilly	TH	5	TH2	Tropical climate

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 10,000rpm 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 ethanol. The sample was centrifuged at 10,000 rpm for 15 minutes and 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 amplifiable in PCR as compared to other methods.

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:

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

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 best in quality as evident on agarose gel electrophoresis and had an OD_{260}/OD_{280} ratio nearer to 1.7-1.8 were used for further analysis.

Restriction Digestion and Gel Electrophoresis

Genomic DNA (1 µg) was digested overnight with 10 units of each of the three restriction enzymes such as *Eco* RI, *Hind* III and *Bam* HI individually. The reaction was carried out in buffered condition at 37ºC following manufacturer's instructions (*Bangalore Genei*, Bangalore, India). The digested DNA samples were electrophoresed in 1XTAE buffer for 1h at 80 V on 0.8% agarose gel matrix. Gels were photographed under UV light using a Gel documentation system (Bio-vision, Mumbai, India). All reactions were repeated thrice to confirm results.

PCR Amplifications

For the optimization of ISSR reactions, oligonucleotide primers from 800P and UBC series were used for amplification to standardize the PCR conditions. PCR amplification was performed in 25 µl reaction volumes containing 2.5 µl of 10X assay buffer. All the reagents of PCR amplification reaction mixtures used were procured from *Invitrogen Bioservices*, Bangaluru, India. The amplification reaction was carried out in a DNA Thermal Cycler (T100, Bio-Rad, USA). The quantities of thermal cycler reaction mixtures are shown in table-2 and the thermal cycler programs for genomic DNA amplification are shown in table-3. After completion of the PCR amplification, 5.0 µl of the amplified products were electrophoresed in a 1.5% (m/v) agarose gels (Bangalore Genei, Bangalore, India) with 1X TAE buffer, stained with ethidium bromide, and were later photographed and documented by a gel documentation system. The sizes of the amplification products were estimated by comparing them to a standard DNA ladder. All the reactions were repeated three times.

Table 2: Optimized reaction mixture for PCR amplification by ISSR primers

Component	Concentration
1. Template DNA	50 ng
2. PCR assay buffer	1X
3. $MgCl_2$	2.0 mM
4. dNTPs (dATP, dGTP, dCTP and dTTP)	200 µM
5. Taq DNA Polymerase	1.0 U
6. ISSR primer	20 pmol

Table 3: Thermal Cycle for PCR amplification by ISSR primers

Step	Cycles	Temperature	Time (mm:ss)
Initial denaturation	1	94ºC	04.00
Denaturation-	35	94ºC	01.00
Annealing-		TmºC*	01.00
Extension-		72ºC	02.00
Final extension-	1	72ºC	07.00

**Tm: The melting temperature of the ISSR primer being used for amplification*

RESULTS

The isolation and purification of genomic DNA of all the samples of *C.wightii* was followed by its quantification by UV – Viz spectrophotometer. The results obtained are described here. Of the 7 samples selected from Ajmer region (Fig. 1A) sample A3 yielded maximum amount of DNA (645 ng/µl) which was also most befitting for further analysis, whereas sample A4 yielded the least amount of DNA (585 ng/µl). DNA samples obtained from Pushkar (Pandu Beri) Region are shown in Fig.1B. Sample PB2 yielded the maximum amount of DNA at 712 ng/µl and was chosen for further analysis; it was also the sample with maximum yield in comparison to all the samples analysed throughout all regions; sample PB4 on the other hand yielded the least amount of DNA at 468 ng/µl among all the 5 samples.

Out of the 10 samples collected from the protected area situated at Mangaliya was (Fig.2C) sample M419 yielded the maximum amount of DNA- 600 ng/µl, yet sample M423 at 262 ng/µl was observed to give most optimum results on analysis through gel electrophoresis and hence was chosen for further assessment instead of sample 419. On the other hand the least amount of DNA yield was recorded in sample M102 at 103 ng/µl. (Note that the sample M423 is designated as 423 in the gel profiles and tables, for the ease of representation). Quantitative data of DNA yields from the 5 samples obtained from Sri Nagar village (Fig. 2D). Sample SN2 and SN4 yielded the maximum amount of DNA- 643 ng/µl, while SN3 yielded the least- 318 ng/µl. SN2 showed the most acceptable results and hence was selected for further study.

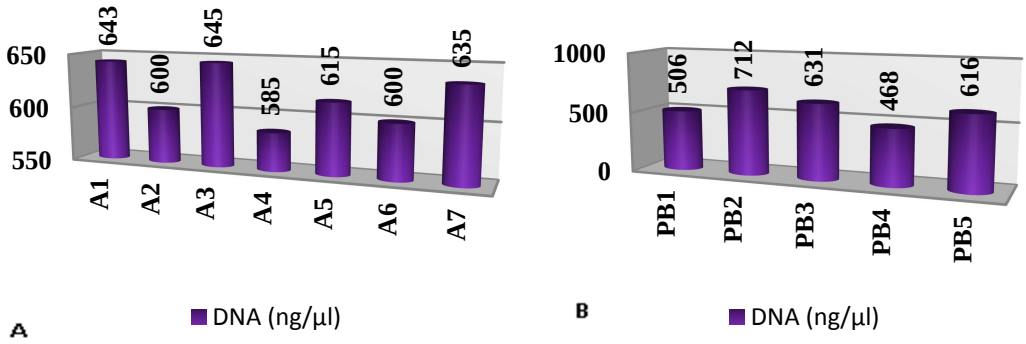

Fig. 1 (A&B): Amount (ng/µl) of Genomic DNA in different samples of *C. wightii* collected from A. Ajmer (A1 to A7) and B. Pushkar (PB1 to PB5)

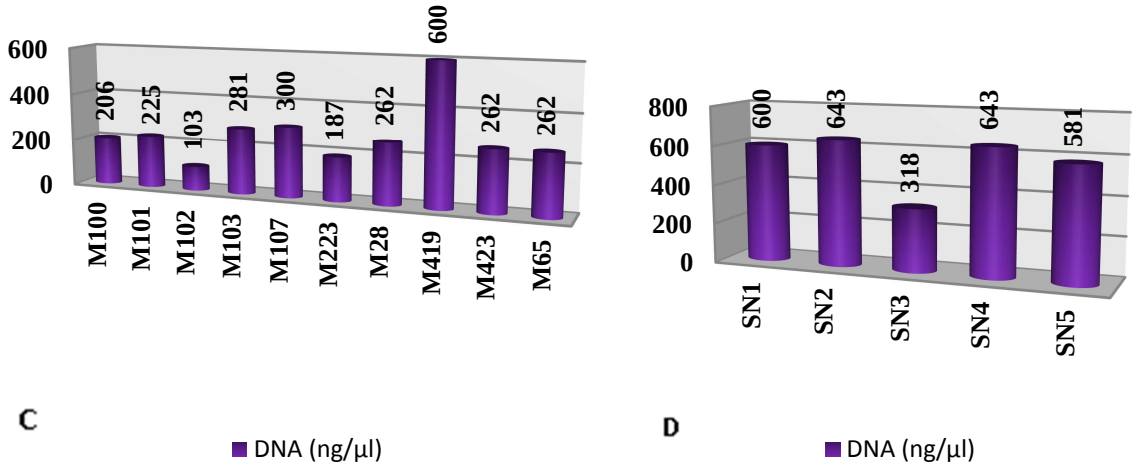

Fig. 2 (C&D): Amount (ng/µl) of Genomic DNA in different samples of *C. wightii* collected from C. Mangaliawas (M100 to M65) and D. Srinagar (SN1to SN5)

DNA yields from the Jaipur zone- Jobner. Of the 6 samples initially acquired only 5 were deemed fit for extraction (Fig.3E). Sample JOB1 was discarded. From the 5 samples left, sample JOB4 yielded maximum amount of DNA - 639 ng/µl, while JOB3 yielded the least amount- 281 ng/µl. However sample JOB6 which yielded 605 ng/µl DNA and was the sample of choice for further study owning to the optimum results it showed. The DNA from the 7 samples acquired from the Jodhpur region as shown in Fig. 3F, maximum amount was yielded from sample J6 which was 675 ng/µl. The DNA yield was least in sample J3- 525 ng/µl.

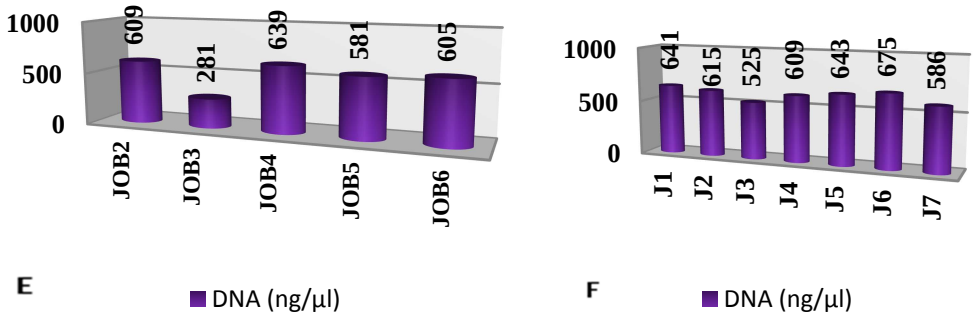

Fig. 3 (E&F): Amount (ng/µl) of Genomic DNA in different samples of *C. wightii* collected from E. Jobner (JOB2 to JOB6) and F. Jodhpur (J1 to J7)

In Rajsamand region, only one plant was obtained from the sub-region Bheem(BH) which yielded 637 ng/µl of DNA(Fig.4G). Six samples were procured from the Gomti Chouraha area, designated H1- H6. Sample H3 yielded the maximum amount (575 ng/µl) of DNA. The poorest amount of DNA obtained in the lot was from sample H4- 375 ng/µl.

Udaipur region was sub divided into 4 sub regions, namely- Kirwa Ghat (KG), Kavita Village (KVT), Neemach Maata area (NM), and Thoor (TH). In terms of totality, the maximum yield of DNA was recorded in sample TH1- 661 ng/µl and the minimum yield was in sample TH5 94 ng/µl (Fig.4H).

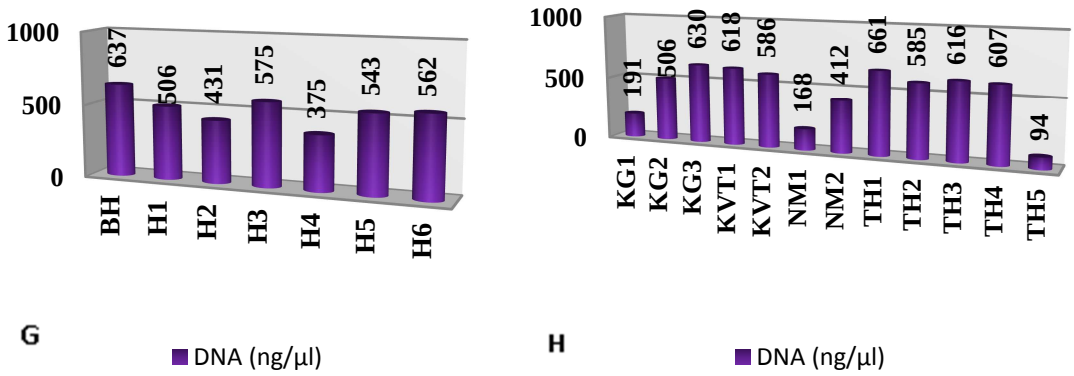

Fig. 4(G&H): Amount (ng/µl) of Genomic DNA in different samples of *C. wightii* collected from G. Rajsamand region (BH1 to H6) and H. Udaipur region (KG1 to TH5)

The isolated genomic DNA from all the samples were electrophoresed in 1x TAE buffer at 80V on a 0.8% agarose matrix and clear bands of genomic DNA from all the samples were visualized (Fig.5). None of the bands showed evidence of RNA contaminations.

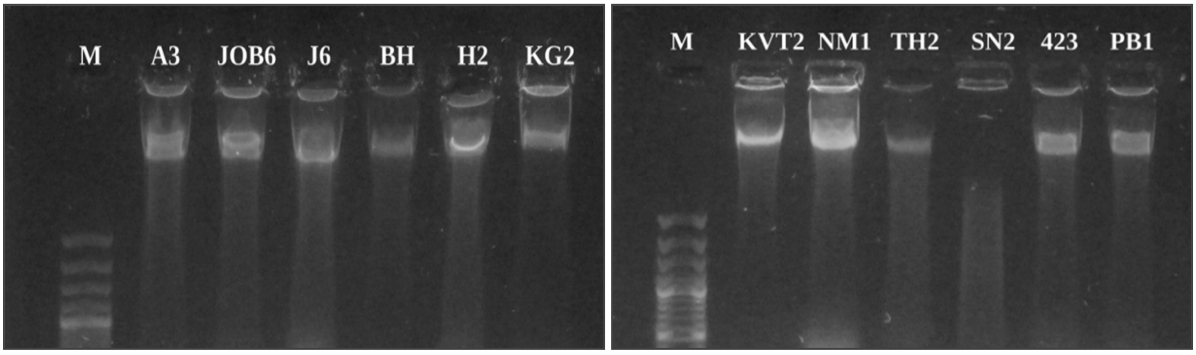

Fig.5: Electrophoresis profile of genomic DNA extracted from different samples of *C. wightii*

A comparative study involving the analysis of the quantities of DNA extracted from different samples in all the eco-climatic zones using the modified CTAB methods was undertaken. The quantity of DNA (ng/µl) in all the samples acquired, varied; and the samples which showed the most optimum results were selected for further analysis.

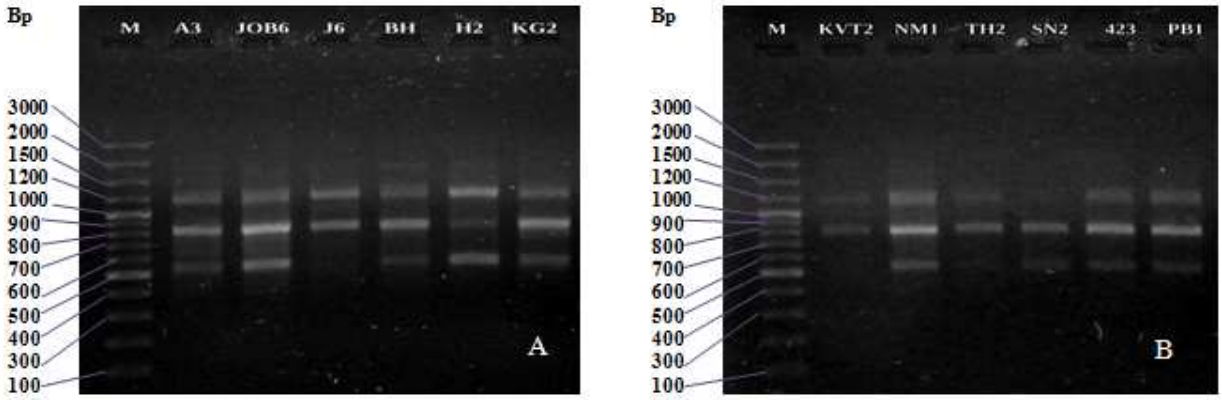

Fig. 6: ISSR profile of *C. wightii* generated by the primer UBC 881. M – Represents 100bp ladder. Samples collected from A- Ajmer (A3), JOB6 (Jobner), Jodhpur (J6), Bheem(BH), Gomti Chouraha (H2), Kirwa Ghaat (KG2), Kavita village (KVT2), B - Nimach Mata (NM1), Thoor village (TH2), Sri Nagar village(SN2), Mangaliyawas (423) and Pandu beri Pushkar (PB1)

DISCUSSION

Polysaccharide contamination is a common problem in genomic DNA extraction in many plant species [12]. Though in a number of plant species the yield of genomic DNA has been very high [13], modifications in the standard protocols are imperative to remove polysaccharides, gum and resins. The CTAB protocol, the protocols devised by Doyle & Doyle [14] and a few others have been studied and utilized extensively in different plant species including *Commiphora* wightii[15], [16] and *Boswelia serrata* [17]. The major issue is that the polyphenols bind covalently with the isolated DNA and reduce its purity and yield [18], [19]. High yield and purity of DNA are the two fundamental requirements for a wide range of molecular biology applications. In the present study, many commonly used protocols (CTAB, Doyle & Doyle) were used for the isolation of polysaccharide free genomic DNA of *C. wightii*. The genomic DNA of *C. wightii* isolated in our laboratory using the above mentioned protocols was viscous, of glue like texture and was unmanageable at the time of pipetting and PCR amplification. The DNA precipitated in the presence of a high salt concentration however, is generally good in quality and perfectly fit to be used as template for PCR amplifications [20]. In the present study, the issue of high viscosity of genomic DNA in *C.wightii* was resolved by adding 3M NaCl in the T.E. Buffer+ DNA solution followed by precipitation. Marked improvement was observed when 3 M NaCl treatments were repeated twice, this resulted in the recovery of translucent and non sticky genomic DNA. Quality of genomic DNA was also confirmed through spectrophotometric analysis, which showed absorbance ration between 1.75-1.85 when measured at two different wavelengths (A_{260}/A_{280}). To further confirm the purity, the DNA isolated from all the samples were electrophoresed on 0.8 % agarose (containing ethidium bromide) gel. All the samples showed intensely fluorescent bands without RNA contamination. The genomic DNA of *C. wightii* extracted by a partially modified CTAB protocol in our laboratory was then subjected to ISSR analysis; the oligo primers produced clear, scorable and reproducible bands which indicated that the isolated DNA was suitable for molecular biological applications like genetic diversity. The study was also aimed to evaluate the comparative quantitative values of genomic DNA between the different eco climatic zones of Rajasthan, such as hot semi arid, hot arid, sub tropical dry and tropical climate. The quantitative analysis of genomic DNA of *C.wightii* in these different climatic zones revealed that an average of 600-700 ng/µl genomic DNA was measured from all the zones almost uniformly. Thus the quantity of genomic DNA of *C.wightii* was not affected by diverse edaphic and climatic conditions.

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