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# Genetic similarity studies of *Commiphora wightii* accessions by fluorescent-labeled RAPD primers

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**Abstract**

*Commiphora wightii* is a valuable endangered tree that produces a gum called guggul-resin, used in the alternative systems of medicine for its anti-inflammatory and cholesterol-reducing activities. RAPD analysis of the *Commiphora wightii,* taken up to record and characterize the genetic diversity of the dwindling population revealed highly variable identified markers. The study was taken up with three fluorescent-labeled RAPD primers by a high speed automated DNA fragment analysis device based upon the automated fluorescent DNA sequencer with the molecular taxonomy software FreeTree and TreeView. The program computes the distance matrix, constructs the phylogenetic or phonetic tree (dendrogram) using unweighted pair group method with arithmetic averages (UPGMA) or neighbour-joining, and computes bootstrapping values for internal branches of tree. Pairwise comparision between accessions based on the proportion of shared bands produced by the primers used, were calculated using Jaccard's similarity.

The size of the 810 amplification products produced ranged from 50.14 to 500 bp and 729 of these were polymorphic bands and 81 were monomorphic. The average polymorphism was 369.5 and percentage polymorphism was 90 %. From the 3 primers tested, only the primer-2 produced amplification products that were monomorphic across all the plants. The three primers generated high percentage of polymorphic loci, which means that genetic polymorphism in the

population, is generally high. Despite of the small size of the population, which generally exhibit lower levels of genetic diversity, our results based on the RAPD technique showed that the genetic diversity of this endangered plant species is definitely not low. The 6 accessions segregated into 2 clear clusters. Cluster-1 with the accessions [Cw-P] and [Cw-Q] and cluster-2 with [Cw-T, Cw R, Cw-S and Cw-U]. There is a similarity of 80% between [Cw-R] and [Cw-S & Cw-U] and the similarity between the [Cw-T] and [Cw-R, Cw-S & Cw-U] is 76% in the cluster-2. However, the similarity between [Cw-P] and [Cw-Q] in cluster-1 is only 47%.

**Keywords:** *Commiphora wightii,* Gum guggul-resin, accessions, RAPD, genetic similarity

## INTRODUCTION

*Commiphora wightii* (Arnold) Bhandari (known as guggul) is an endangered plant species in India and is listed in the IUCN Red Data list [1] and also listed as "Critically endangered" by UNDP [2]. *C. wightii* belonging to family Burseraceae is a shrub growing to a height of 2 to 3 m with gray-brown bark that peels off easily. The stem is thorny with small leaves. The plant has poor seed set and germination and has been subjected to indiscriminate and harsh methods of gum-harvesting leading to tree death [3]. The oleo gum (resin) is collected by tapping guggul plants in summer and the yield is about 200-800 g per plant. The guggulosterones present in the guggul gum of *C. wightii* plants have antioxidant activity [4]. The Guggulipids from the resin have been reported to be effective as anti-inflammatory, anti-bacterial, antimicrobial, anti-oxidant, anti-arthritic, anti-malarial, antimycobacterial, anti-schistomal, hepatoprotective, muscle relaxant, larvicidal, and molluscicidal and studies have reported the anti-diabetic, cardiac and neuronal protective activity of guggulsterone [5], which is used for prevention of cancer [6] and as a cholesterol lowering agent [7].

There is a need to preserve endangered natural populations and successfully and accurately assess the genetic diversity for the conservation of a species. Molecular markers have become important tools to assess the genetic variation in populations of endangered species and to address the increasing loss of biodiversity. RAPD profiling is extensively used to analyze the genetic variability in bacteria, fungi and plants because of the ease of methodology and the cost-effectiveness involving a smaller number of samples [8, 9]. RAPD is routinely used for estimation of genetic diversity in various endangered plant species and the advantages of RAPD include its simplicity, low cost, rapid, use of arbitrary primers, no need of initial genetic or genomic information, and the requirement of only tiny quantities of target DNA [10, 11, 12, 13]. The fundamental goal of conservation biology is to establish proper conservation strategies for plant species by understanding and preserving the genetic diversity (variation) of the populations. Genetic diversity needs to be maintained for the long-term survival of endangered plant species and it helps the species to adapt to the

changing environment. RAPD analysis of the populations can reveal highly variable markers which can be used for population and genetic diversity studies within and between populations [10, 11, 13]. In the above context, there is an urgent need to record and characterize the genetic diversity of the dwindling population of *C. wightii*.

The present study was taken up to assess genetic diversity by evaluating significant variations (in terms of percentage polymorphism) in *C. wightii* by using the fluorescent labeled RAPD primers method to aid in its conservation.

## MATERIALS AND METHODS

Six accessions of *Commiphora wightii* in the Botanical garden, at Osmania University were taken up for the study. These 8-year-old accessions were originally sourced from different places of south India: Cw-P (Herbal garden, Rajendranagar, Hyderabad, TS); Cw-Q (Pragathi Green Nursery, Proddutur, AP); Cw-R (Karthikavanam Dhulapally, Hyderabad, TS); Cw-S (Hyderabad Urban Forestry, Erragadda, Hyderabad, TS); Cw-T (Herbal garden, Rajahmundry, AP) and Cw-U (Andhra Pradesh Medicinal Plants Board, Chilkur, Hyderabad, TS) duly authenticated by the Head, Department of Botany, Osmania University, Hyderabad.

### RAPD analysis

The main aim of this method was to evaluate the genetic similarity/diversity that exists in various accessions of *Commiphora wightii* collected from different places. The six accessions of *C. wightii* were Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T and Cw-U. The steps involved in RAPD based molecular analysis are: Isolation of plant genomic DNA (Mini Prep); Qualitative and quantitative analysis of isolated DNA; Primer selection; PCR amplification and DNA analysis. Fluorescent labeled RAPD primers were used in amplification and the amplification products were analysed by a high-speed automated DNA fragment analysis device based upon the automated fluorescent DNA sequencer (A.L.F., Pharmacia), described by [14] Ansorge *et al.,* by using the molecular taxonomy software FreeTree and TreeView.

### *Isolation of Plant Genomic DNA (Mini Prep)*

For RAPD analysis, Plant Genomic DNA was isolated by using genomic DNA isolation kit (RKN 09). DNA from fresh young leaves was isolated according to the manufacturer protocol. All centrifugation steps are carried out at 17,900xg (10,000 rpm) in a conventional table-top microcentrifuge at room temperature. For this, 100 mg of tender leaves were crushed to a fine paste in a mortar and pestle by adding 500 µl of 1X suspension buffer and transferred into a 2 ml vial (using a microtip that is cut at the bottom) and made up the final volume to 750µl**.** RNase solution (5µl) was added and mixed 5-6 times by inverting the vial and stored at 65°C for 10 min with intermittent mixing. Lysis Buffer (1 ml), was then added and vortexed for a minute and placed at 65°C for 15 min. This was centrifuged at 10,000 rpm for 2 min at room temperature. The clear supernatant was decanted into two 2 ml fresh vials (900µl in each vial).

Isopropanol (900µl) was added to each of the above vial and mixed well and spun at 10,000 rpm for 15 min at room temperature. The pellet was retained and washed twice with 1ml of 70% ethanol (spun at 10,000 rpm for 15 min at room temperature). The pellet in each of the vials was dried at 37°C for 10 min. and suspended in 50 µl of glass-distilled water by placing the vials at 65°C for 15 min or at 4°C overnight.

### *Qualitative and quantitative analysis of DNA*

The quality of the isolated DNA was checked through electrophoresis on 1% agarose gel with ethidium bromide by using Hind III+ λ DNA as standard. The yield of DNA per gram of leaf tissue extract was measured using a UV spectrophotometer. DNA yield was determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm to absorbance at 280 nm. The concentration and purity of DNA was also determined by running the DNA samples on 1% agarose gel based on intensity of band when compared with the lambda marker as standard. The ratio of OD 260 / OD 280 indicates the purity of the given sample. The range of 1.7 - 1.9 ratio is considered as pure sample.

### *Primer Selection*

RAPD primers were labeled with 6-carboxyfluorescein (6-FAM) from Applied Biosystems, USA, which gives blue color in Genescan analysis. Three fluorescent labeled RAPD primers used for amplification are given below:

Primer-1: 5'AGGHCTCGATAHCMGVY3'
Primer-2: 5'CCCHGCAMCTGMTCGCACHC3'
Primer-3: 5'MTGTAMGCTCCTGGGGATTCHC3'

### *PCR amplification*

The PCR amplification reactions of the fluorescent labeled RAPD primers method were conducted in 50 µl volume reaction mixture comprising DNA(1 µl), RAPD fluorescent primer (4 µl), dNTPs (10mM) (2 µl), PCR assay buffer (10X)(5 µl), Taq DNA polymerase (0.5 µl) and nuclease free water (37.5 µl). PCR amplification was carried out in a ABI 3130 Genetic analyzer with the following conditions: Initial denaturation was done at 94°C for 5 minutes, followed by denaturation for 40 cycles at 94° C for 1 minute, annealing for 1 minute at 50° C followed by 2 minutes for extension at 72°C and final extension at 72° C for 2 minutes. The size marker was the 100-bp ladder and internal size standards were added (Please note that blue colored peaks and RAPD PCR products peaks correspond to the size as sized by internal size standard). Orange peaks are internal size standard (LIZ 500, ABI). The GeneScan™ 500 LIZ® Size Standard is a fifth dye-labeled size standard for the reproducible sizing of fragment analysis data. This size standard was used for fragments between 35 and 500 bp. Amplification products were analyzed by a high-speed automated DNA fragment analysis device

based upon the automated fluorescent DNA sequencer (A.L.F., Pharmacia), described by [14] by using a molecular taxonomy software.

### *DNA analysis*

RAPD-PCR amplified fragments were scored as 1 for present and 0 for absent. The binomial data generated was used to estimate the level of polymorphisms by dividing the polymer bands by the total number of scored bands. The RAPD data was analyzed using the FreeTree and TreeView software. The program computes the distance matrix, constructs the phylogenetic or phonetic tree (dendrogram) using unweighted pair group method with arithmetic averages (UPGMA) or neighbour-joining, and computes bootstrapping values for internal branches of tree. The data was pasted into the spreadsheet of the "New analysis" of FreeTree and the Nei-Li distance [15] with 7 different similarities/distance and the method of tree construction, e.g. neighbour-joining and UPGMA [16] were selected to get the distance matrix. The Reference tree was checked on the basis of this distance matrix. This form of the tree was pasted into the program for drawing phylogenetic trees (dendrograms) [17]. Pairwise comparision between accessions based on the proportion of shared bands produced by the primers used, were calculated using Jaccard's similarity [18]. Polygenetic Tree (dendrogram) was constructed by UPGMA (Unweighted Pair Group Method with Arithmetic mean) method to measure the resulting phenotypic groups.

## RESULTS AND DISCUSSION

The results obtained in the study concerning the molecular analysis of six accessions of *C. wightii,* Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T and Cw-U for genetic diversity (fluorescent labeled RAPD primers method using an automated fluorescent DNA sequencer and special software) as an aid in its conservation are discussed.

### RAPD analysis

#### *Polymorphism in the six accessions of Commiphora wightii*

DNA samples were checked by running on agarose gel and then amplified. Twelve primers were scored initially but only 3 primers resulted in good banding patterns. However, only the primer-2: (5'AGGHCTCGATAHCMGVY3') elicited good amplification with reliable and clear band resolution (Fig. 1). The other two primers i.e. primer-1: 5`CCCHGCAMCTGMTCGCACHC3' and primer-3: 5'MTGTAMGCTCCTGGGGATTCHC3' elicited moderate amplification which were not as clear.

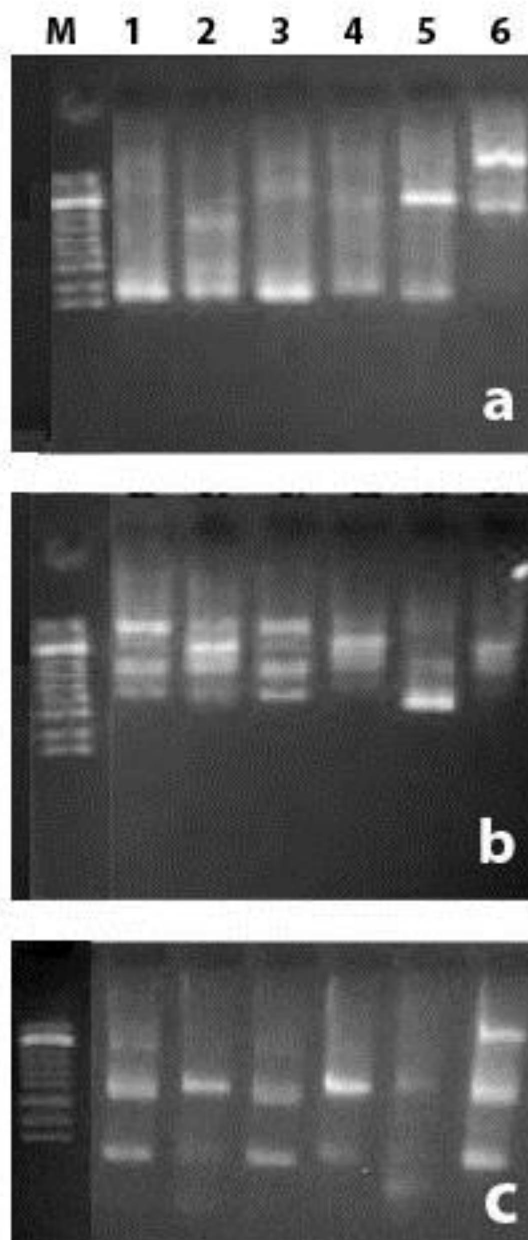



**Fig. 1. (a-c)** RAPD profiles of 6 accessions of *C. wightii* using three primers.

Lane M: 100 bp ladder; Lanes 1 to 6: PCR products for the accessions [Cw-P], [Cw-Q], [Cw-R], [Cw-S], [Cw-T] and [Cw-U]

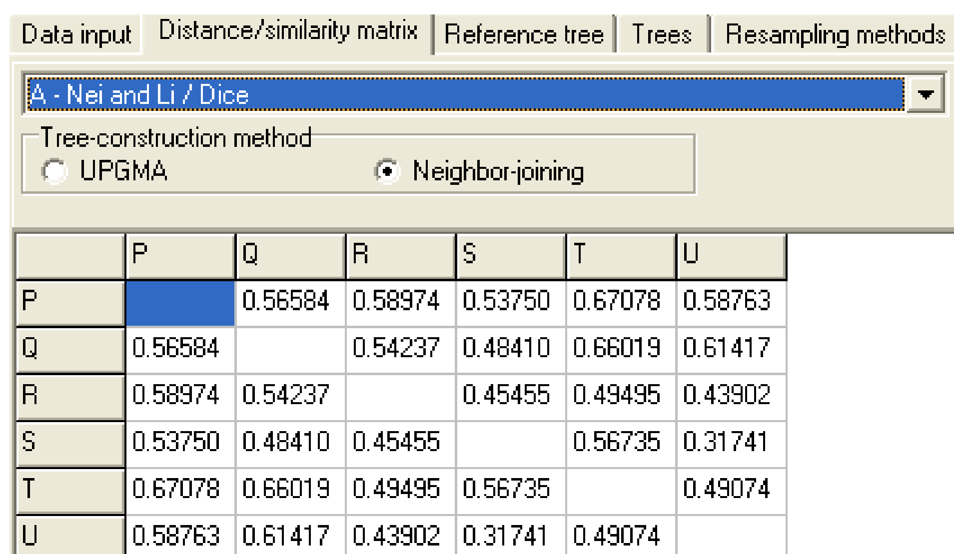
(a) with primer-1, (b) with primer-2 and (c) with primer-3.

The size of the 810 amplification products produced ranged from 50.14 to 500 bp (Fig. 1). Out of the 810 bands, 729 were polymorphic bands and 81 bands were monomorphic. The average polymorphism was 369.5 and percentage polymorphism was 90 %. From the 3 primers tested, only the primer-2 produced amplification products that were monomorphic across all the plants. The three primers generated high percentage of polymorphic loci, which means that genetic polymorphism in the population, is generally high. An amplified product is considered polymorphic if it occurs in less than 95% of the total individuals assayed. The scale indicates the genetic distance between individuals. Despite of the small size of the population, which generally exhibit lower levels of genetic diversity, our results based on the RAPD technique showed that the genetic diversity of this endangered plant species is definitely not low.

***Distance Matrix and Genetic Similarity Index***

Distance matrix was calculated by Nei and Li/Dice Tree Construction with Neighbor joining method and presented in Table 1.

**Table-1:** Distance Matrix Table of the 6 accessions of *C. wightii* calculated by Nei and Li/Dice Tree Construction with Neighbor joining method



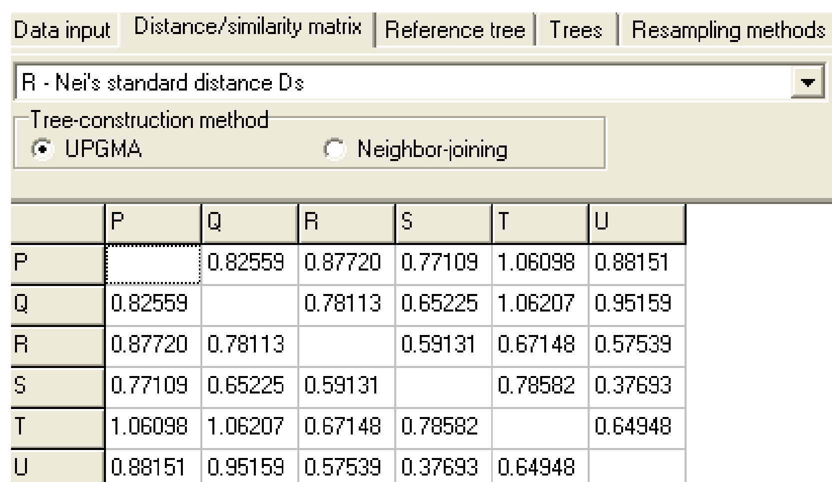

| | P | Q | R | S | T | U |
|---|---|---|---|---|---|---|
| P | | 0.56584 | 0.58974 | 0.53750 | 0.67078 | 0.58763 |
| Q | 0.56584 | | 0.54237 | 0.48410 | 0.66019 | 0.61417 |
| R | 0.58974 | 0.54237 | | 0.45455 | 0.49495 | 0.43902 |
| S | 0.53750 | 0.48410 | 0.45455 | | 0.56735 | 0.31741 |
| T | 0.67078 | 0.66019 | 0.49495 | 0.56735 | | 0.49074 |
| U | 0.58763 | 0.61417 | 0.43902 | 0.31741 | 0.49074 | |

**Note:** P, Q, R, S, T and U represent the 6 accessions of *C. wightii* [Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T and Cw-U].

This shows the similarity index/distance matrix between the six accessions of *C. wightii.* Maximum similarity was seen between [Cw-P] and [Cw-T]. The [Cw-U] and [Cw-S] were the least similar. The distance matrix was also calculated by the Nei's standard distance Ds Tree construction method with UPGMA (Unweighted Paired Group Method with Arithmetic Mean), which shows the genetic diversity among the

six accessions of *C. wightii* (Table 2). There is highest similarity between [Cw-Q] and [Cw-T] and the [Cw-U] and [Cw-S] have the least similarity.

**Table-2:** Distance matrix calculated by R-Nei's standard distance Ds coefficient with UPGMA of the 6 accessions of *C. wightii*



Data input | Distance/similarity matrix | Reference tree | Trees | Resampling methods

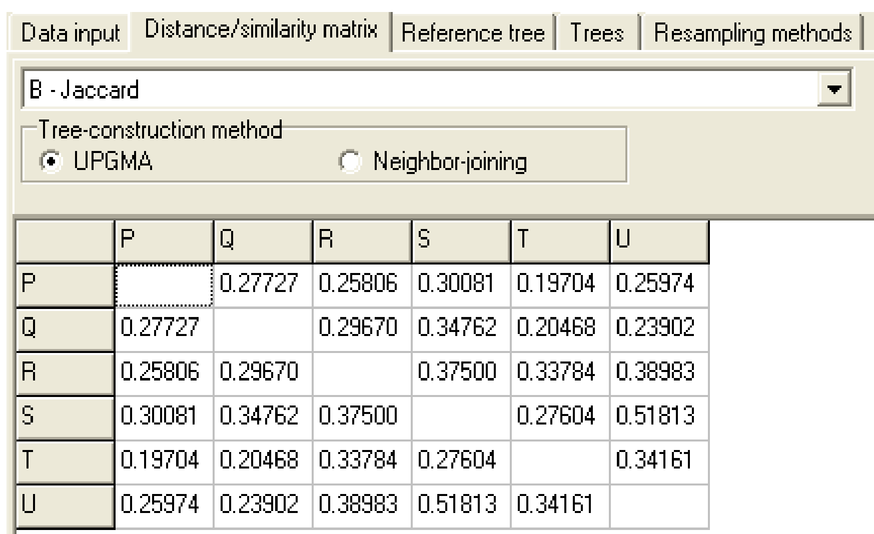
R - Nei's standard distance Ds

Tree-construction method: (•) UPGMA ( ) Neighbor-joining

| | P | Q | R | S | T | U |
|---|---|---|---|---|---|---|
| P | | 0.82559 | 0.87720 | 0.77109 | 1.06098 | 0.88151 |
| Q | 0.82559 | | 0.78113 | 0.65225 | 1.06207 | 0.95159 |
| R | 0.87720 | 0.78113 | | 0.59131 | 0.67148 | 0.57539 |
| S | 0.77109 | 0.65225 | 0.59131 | | 0.78582 | 0.37693 |
| T | 1.06098 | 1.06207 | 0.67148 | 0.78582 | | 0.64948 |
| U | 0.88151 | 0.95159 | 0.57539 | 0.37693 | 0.64948 | |

**Note:** P, Q, R, S, T and U represent the 6 accessions of *C. wightii* [Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T and Cw-U].

**Table-3:** Similarity matrix calculated by Jaccard's coefficient of the 6 accessions of *C. wightii* with UPGMA method



Data input | Distance/similarity matrix | Reference tree | Trees | Resampling methods

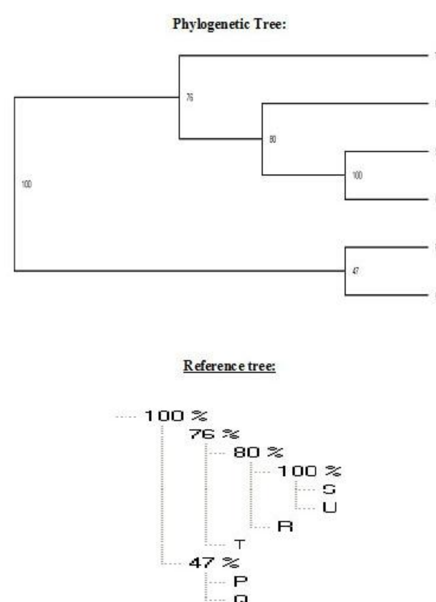
B - Jaccard

Tree-construction method: (•) UPGMA ( ) Neighbor-joining

| | P | Q | R | S | T | U |
|---|---|---|---|---|---|---|
| P | | 0.27727 | 0.25806 | 0.30081 | 0.19704 | 0.25974 |
| Q | 0.27727 | | 0.29670 | 0.34762 | 0.20468 | 0.23902 |
| R | 0.25806 | 0.29670 | | 0.37500 | 0.33784 | 0.38983 |
| S | 0.30081 | 0.34762 | 0.37500 | | 0.27604 | 0.51813 |
| T | 0.19704 | 0.20468 | 0.33784 | 0.27604 | | 0.34161 |
| U | 0.25974 | 0.23902 | 0.38983 | 0.51813 | 0.34161 | |

**Note:** P, Q, R, S, T and U represent the 6 accessions of *C. wightii* [Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T and Cw-U].

Pairwise comparision between accessions based on the proportion of shared bands produced by the primers used, were calculated using Jaccard's similarity [18]. The similarity matrix (Table 3) showed the extent of similarity between the six accessions. The lowest similarity was between [Cw-P] and [Cw-T] (0.19704) and the highest similarity was between [Cw-U] and [Cw-S] (0.51813) (Table 3). Whereas there is maximum similarity between the accessions [Cw-S] and [Cw-U] according to the Jaccard's Coefficient, they recorded the least similarity values according to the other two methods. There is least similarity between [Cw-P] and [Cw-T] with Jaccard's Coefficient in contrast to the results observed with the Nei and Li/Dice Tree Construction with Neighbor joining method. This can be studied in depth through other more refined molecular analyses. The Polygenetic Tree (dendrogram) was constructed by UPGMA method to measure the resulting phenotypic groups among the six accessions of *C. wightii* based on the genetic distance generated by three fluorescent labeled RAPD primers (Fig. 2), shows that there are 2 clear clusters. Cluster-1 with the accessions [Cw-P] and [Cw-Q] and cluster-2 with [Cw-T, Cw-R, Cw-S & Cw-U]. Further, the Reference Tree (Fig. 2), which was checked on the basis of the distance matrices obtained, reflects a 100% similarity between [Cw-S] and [Cw-U] which also reflects the results obtained with the Jaccard's Coefficient. There is a similarity of 80% between [Cw-R] and [Cw-S & Cw-U] and the similarity between the [Cw-T] and [Cw-R, Cw-S & Cw-U] is 76% in the cluster-2. However, the similarity between [Cw-P] and [Cw-Q] in cluster-1 is only 47%.




Note: P, Q, R, S, T & U refer to accessions Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T & Cw-U

**Fig. 2.** Phylogenetic Tree (Dendrogram) and Reference Tree of 6 accessions of *C. wightii* based on genetic distance generated by three fluorescent (FAM) labeled RAPD primers.

***Note**: P, Q, R, S, T and U represent the 6 accessions of C. wightii [Cw-P, Cw-Q, Cw-R, Cw-S, Cw-T and Cw-U].*

RAPD is one of the important molecular markers for identification of the individual species and provides the polygenetic information of the population. The RAPD technique is used successfully in a variety of taxonomic and genetic diversity studies [19, 20]. The technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationship in several genera [20]. RAPD markers have the greatest advantage of its capability to scan across all regions of genome hence suited for phylogeny studies at species level [21, 22]. The report on the genetic similarity of the samples slightly correlated with their close geographical locations [23]. Sequencing based molecular techniques provide better resolution at intra-genus and above level, while frequency data from markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites provide the means to classify individuals into nominal genotypic categories and are mostly suitable for intra-species genotypic variation study. This distinction is important to grasp for population studies, particularly when the diversity data is used as a basis for making decisions about conservation of plant resources. For instance, a recent study on Napier grass (*Pennisetum purpureum*) has showed that RAPD is compatible with morphological data [12]. The source of polymorphism observed may be due to deletion, addition or substitution of base within the priming site sequence [24]. The present study confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of 6 different accessions of *C. wightii* collected from different regions. This method can be used for identification of the original *C. wightii* plant. This method can also help distinguish *Commiphora wightii* from other species or adulterants. Despite the small size of the population, which generally exhibit lower levels of genetic diversity, our results based on the RAPD technique showed that the genetic diversity of this critically endangered plant species is high. Further, protection of this critically endangered plant species is very essential and *in situ* and *ex situ* conservation through micropropagation would be a good strategy.

## CONCLUSION

The current endeavor has accomplished the study of genetic diversity of the endangered plant *C. wightii* through molecular analysis of different accessions. The fundamental goal of conservation biology is to establish proper conservation strategies for plant species by understanding and preserving the genetic diversity (variation) of the populations. Genetic diversity needs to be maintained for the long-term survival of endangered plant species and it helps the species to adapt to the changing environment. RAPD analysis of the *C. wightii* populations revealed highly variable identified markers which can be used for population and genetic diversity studies within and between populations. This greatly helps in the identification of superior accessions, or for spotting adulterants and also for taking up major conservation measures of this endangered plant.

## ACKNOWLEDGEMENTS

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