Research Article
SCAR Marker for Identification and Discrimination of Commiphora wightii and C. myrrha

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1. Introduction
Commiphora spp. of the family Burseraceae is being used as a medicinal plant since ancient times and now rated as an endangered plant species [1]. They are found in the arid to semiarid regions of the world, including the deserts of India, Pakistan, Africa, and Saudi Arabia, while in India, it is found in Rajasthan, Madhya Pradesh, Gujarat, Tamilnadu, Orissa, and Karnataka. About 185 species of Commiphora were found worldwide, out of them C. wightii (synonym C. mukul), C. agallocha, C. stocksiana, C. berryl, and C. myrrha were found in India [2, 3]. In earlier studies about the flora of India, the “Guggul” plant was known as Commiphora mukul (Hook ex Stocks) Engl. or Balsamodendron mukul (Hook ex Stocks). Finally, it was named as C. wightii (Arn.) by Bhandari in 1964.

C. wightii was well-documented medicinal plant since 3000 years ago [4], having exciting biological activities like being anti-inflammatory, antimicrobial, hepatoprotective, muscle relaxing, antiarthritic, hypolipidemic, hypocholesterolemic, antiobesity, antioxidant, antimalarial, antisyphilis, antischistosomal, larvicidal, and mollucidal [2, 3, 5–19].

C. wightii contains a bitter gum known as Guggul (Myrrh) in stems and leaves. The yellowish gum oozes upon making an incision and solidifies in the hot environment to a hard brownish resin. Guggul is medicinally important and is used in the treatment of hypercholesterolemia and cardiovascular diseases [9, 20]; it is also shown to have anticancerous activity [21]. The extract of gum Guggul, as gugulipid, guggulipid, or guglipid, is reported as a folk remedy in the Unani and Ayurvedic system of medicine. Two trans-isomers of Guggulsterone, namely, Guggulsterones E and Z, were reported in gum Guggul as important active steroid which are used as cholesterol-lowering agents. The pharmacological properties associated with gum Guggul include anti-inflammatory, antibacterial, anticoagulant, antirheumatic, COX inhibitory,
and hypolipidemic activities that are mostly due to the presence of these steroids [22, 23]. In 1986, Guggul lipids were granted approval in India for marketing as a lipid-lowering drug [24]. Several products of standardized formulations of \textit{C. wightii} were already in human use as cholesterol-lowering agents [22, 25].

\textit{Commiphora} species have been called “taxonomically difficult,” because of being drought-tolerant plants and they are leafless for most of the year [26]. There is resemblance of gum Guggul with gum resin of other species within and outside of the genus, which make high risk of adulteration in commercial samples either deliberately to get more profit or accidentally. Therefore, it is important to validate the \textit{C. wightii} plants and their gum Guggul in commercial samples due to its various pharmacological significances [27]. Many types of markers, namely, morphological, biochemical, and DNA based molecular markers, are commonly used in the identification of species [28]. Molecular markers were used in the identification of species and individual, their origin, and difference at the molecular level in between them [29]. During the last few decades, the use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in plant biotechnology and their genetic studies. These DNA based markers are differentiated into two types: first is non-PCR based RFLP and second is PCR based markers (RAPD, AFLP, SSR, SNP, etc.) [30]. RAPD is a PCR-based technology, based on enzymatic amplification of target or random DNA segments with arbitrary primers. The main advantage of RAPDs is that they are quick and easy to assay, had no sequence data required for primer construction, randomly distributed throughout the genome, and had a dominant nature [31]. However, RAPD marker is not suitable for the species identification, because of their low reproducibility and dominant nature [32]. A RAPD marker can be converted into a codominant and reproducible marker, that is, Sequence-Characterized Amplified Region (SCAR), which may be applicable for authentication of species.

Looking upon these problems, it is necessary to develop some molecular marker for the identification of \textit{C. wightii}. In the present study, an attempt has been made for the development of SCAR markers for \textit{C. wightii}.

### 2. Materials and Methods

Total 28 accessions of two different species of \textit{Commiphora}, that is, \textit{C. wightii} (17) and \textit{C. myrrha} (11), were collected from Bhopal, Obaidullahganj (Madhya Pradesh), Akola (Maharashtra), Anand (Gujarat), and Jaipur (Rajasthan), and conserved at MPCST Human Herbal Health Care Garden, Bhopal.

#### 2.1. Selection of RAPD Primers and Amplicons

Genomic DNA was isolated from fresh young stem \textit{C. wightii} and \textit{C. myrrha} using the method of Saiarkar et al. (unpublished). The yield of DNA was measured using a NanoDrop UV-Spectrophotometer (ND-1000). Genomic DNA was amplified by the 20 primers (Table 1). A cocktail of 40 \(\mu\)L reaction volumes was made with 20 \(\mu\)L, 2x red dye PCR mix (Merck), 1\(\mu\)L primer (10 pM), and 1\(\mu\)L template DNA (25 ng/\(\mu\)L) and amplification was performed on the gradient automatic thermal cycler (Eppendorf) following Saiarkar et al. [33]. The PCR products were separated electrophoretically on 1.5% agarose gel at 5–10 volts/cm of the gel and visualized by ethidium bromide. The specific amplicon, which discriminates between \textit{C. wightii} and \textit{C. myrrha}, was selected and processed for the development of SCAR marker.

#### 2.2. Cloning of Selected RAPD Amplicon

The selected amplicons were eluted using Medox-Easy Spin Column Cleanup Minipreps kit and ligated with the TA cloning vector (pGEM5Z, Promega). The ligated TA vector was transformed into competent cells of \textit{E. coli} (DH5\(\alpha\)), which was prepared using single step ultracompent cell preparation kit (Medox). The first selection of recombinant clones was based on developed blue and white colonies on LB (Luria Burtani) agar plates containing 0.5 mg/mL ampicillin, 24 \(\mu\)g/mL IPTG, and 30 \(\mu\)g/mL X-gal. The plasmid of white and blue colonies was isolated through Medox-Easy ultrapure spin column plasmid DNA minipreps kit. Three selection steps, that is, clone retardation, restriction digestion, and amplification of plasmid, were adopted to identify positive insert within the plasmid. In retardation step, plasmids were separated electrophoretically to observe the presence of insert within plasmid, while in restriction digestion, plasmids were digested with PvuI enzyme for insert release. In the final step, the plasmids were amplified through the PCR reaction using 50 \(\mu\)L that consist of 25 \(\mu\)L 2x red dye PCR mix (Merck), 1\(\mu\)L each of forward and reverse M-13 primers (10 pM each), 1\(\mu\)L of plasmid DNA (25 ng/\(\mu\)L) with a PCR profile of 94°C for 12 minutes, 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, and final extension on 72°C at 10 minutes using the gradient automatic thermal cycler (Eppendorf).

#### 2.3. Designing and Screening of SCAR Marker

Plasmid having desired amplicon was sequenced by Aristogene Pvt. Ltd., Bangalore, India, using M13 reverse and forward sequencing primers and consensus sequence of amplicons was developed. The homology search of consensus sequences was performed by the NCBI BLAST tool. The primer pairs were designed for these sequences by using PRIMER 3 software [34] and

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Table 1: Sequence of SCAR markers designed using 1 kb amplicon.

| Name of SCAR | Name of fragments | Sequence (5’-3’) | Total length | Temp | Size (bp) |
|--------------|------------------|-----------------|--------------|------|-----------|
| ScIP         | ScIP (F)         | CTGTGAGGCATTGTATTTAA | 23 bases | 60°C | 631       |
|              | ScIP (R)         | CTTGTGCTTTCAGTCAATAG | 22 bases | 62°C |           |
| ScIPm        | ScIP (F)         | CTGTGAGGCATTGTATTTAA | 23 bases | 60°C | 910       |
|              | ScIPm (R)        | CTTGAGAACGAAATCTAAACAG | 22 bases | 60°C |           |
Figure 1: Selection and elution of desired amplicon. (a) PCR product of sample Commiphora wightii on low melting agarose gel. Lanes 1 and 2 amplified by primers OPD-02 and OPD-08. (b) Eluted desired amplicon run on agarose gel. Lanes 1 and 2, fragment sizes 1kb and 0.6kb, respectively.

used as a candidate for SCAR primer. Four accessions of each species of C. wightii and C. myrrha were amplified through these primer pairs (synthesized by Aristogene) with a cocktail of 40μL containing 20μL of 2x red dye PCR mix (Merck), 1μL of each of the SCAR primer pair (10pM each), and 1μL of template DNA (25ng/μL). Amplification was performed on the gradient automatic thermal cycler (Eppendorf) with PCR conditions: 94°C for 5 minutes, 30 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 1 minute at 72°C, and final extension on 72°C at 10 minutes. Among the all designed primer pairs, suitable primer pair was selected which discriminate the both species of Commiphora and further screened in all the accessions for validation of SCAR marker.

3. Result

3.1. Identification of RAPD Primer and Amplicon. Out of 20 RAPD primers, 1kb amplicon of OPD-02 and 0.6 kb amplicon of OPD-08 discriminate both Commiphora species as it was present only in C. wightii accessions (Figure 1(a)). Due to specificity of these amplicons, they were cloned, sequenced, and used for SCAR marker development. These bands were elicted from agarose gels and gel electrophoresis revealed that they were appropriate for cloning (Figure 1(b)).

3.2. Cloning and Selection of Positive Clone. White colony of competent cells (E. coli) having T vector with 1kb and 0.6 kb insert was undertaken for plasmid isolation and three selection criteria were performed for the conformation of positive clone. The screening for retardation checking reveals that 17 positive plasmids for 1kb insert and 5 positive plasmids for 0.6 kb may have proper insert (Figure 2). These positive plasmids were digested with the restriction endonuclease (PvuII) for insert release. A total of 6 positive plasmids of 1kb insert and 4 positive plasmids of 0.6 kb insert release their respective insert fragment (Figure 3). In the third stage of selection, 4 positive plasmids for 1kb insert 3 positive plasmids for 0.6 kb insert were finalized for sequencing after amplify with M-13 primer (Figure 4).

3.3. Sequencing and In Silico Application. The clones were sequenced and 979bp and 590bp consensus sequences were formed for 1kb insert and 0.6 kb insert respectively (Figures 5 and 6). The BLAST search was performed for the obtained sequences and no significant homologous sequence was found in the NCBI database. This DNA sequences were deposited in the NCBI gene bank database with accession numbers K90051 and K90052. Two candidate SCAR primer pairs for each DNA sequences were designed, that is, primers Sc1P and Sc1Pm from 979 bp sequences and primer Sc2P and Sc1Pm from 590 bp sequences (Tables 1 and 2). These primers were deposited in the NCBI Prob database with accession number Pr031905450 to Pr031905453.
Table 2: Sequence of SCAR markers designed from 0.6 kb amplicon.

| Name of SCAR | Name of fragments | Sequence (5'→3') | Total length | Temp. | Size (bp) |
|--------------|------------------|------------------|--------------|-------|-----------|
| Sc2P         | Sc2P (F)         | GTACCCAATGTAGTAAATTTCC | 22 bases     | 60°C  | 491       |
|              | Sc2P (R)         | TAGTTAGTTTGGATGACCATACACA | 23 bases     | 62°C  | 570       |
| Sc2Pm        | Sc2P (F)         | GTACCCAATGTAGTAAATTTCC | 22 bases     | 60°C  | 491       |
|              | Sc2Pm (R)        | GTGTGCCCCATTCAACCAAT | 20 bases     | 60°C  | 570       |

3.4. Development of SCAR Marker. The candidate SCAR primer pairs were screened with three accessions of each of C. wightii and C. myrrha which revealed that the primer Sc1Pm is highly specific for C. wightii and amplified 910 bp amplicon, while primer Sc1P had a similar banding pattern in all the samples. Primer Sc2P discriminated both the Commiphora species as it gave 491 bp amplicon for C. wightii, and 1200 bp for C. myrrha, while primer Sc2Pm gives 491 and 570 bp amplicon for C. wightii and C. myrrha, respectively (Figure 7).

Based on the above results, primers Sc1P and Sc2P were authenticated through amplification of eight accession of each species, that is, C. wightii and C. myrrha (Figure 8). The similar results were observed during this screening as they were discriminated both the species of Commiphora.

4. Discussion

Identification of plants at the species level traditionally is a feverish job and needs special care during identification. This...
Molecular markers allow the detection of specific DNA sequences and are unlimited in number and are not affected by environmental factors and developmental stages of the plant [41]. The discovery of PCR technology changed the entire molecular biology and a single random oligonucleotide primer (10-bp long) was discovered in 1990 as a universal marker technology called RAPDs [42]. The main advantages of RAPD markers are the following: they are universal and cost-effective and for application of these markers they did not need any genetic information of the target organism and they can map almost completed genomic DNA of the target organism [43]. However, each method analyses different aspects of DNA sequence variation and different regions of the genome. RAPD and AFLP markers appear to frequently target repetitive regions of the genome.

The presence of polysaccharides, polyphenols, and other secondary metabolites in the leaves of Commiphora species creates complications in the DNA process. Haque et al. 2008 [44] and Samantaray et al. [45] used various methods and described the process for DNA isolation. Their isolated DNA showed good PCR amplification; therefore, it can further be used in molecular downstream applications. Molecular variations among accessions collected from different localities of Rajasthan and Gujarat were described by Suthar et al. [46]. Intraspecific variation in Commiphora wightii populations was described by Haque et al. [47] using Internal Transcribed Spacer (ITS1-5.8S-ITS2) Sequences while Harish et al. [48] studied genetic variations on accessions collected from Indian Thar Desert using RAPD and ISSR markers. Molecular variations among different biotypes of Commiphora wightii were done by Vyas and Joshi in 2015 [49] using RAPD markers. Genetic variability among the C. wightii germplasm collected from Rajasthan and Haryana was studied by Kulhari et al. [50]. Samantaray et al. [51] used sixty different random decamer primers and identified three primers which produced specific fragment in the female plant of C. wightii but failed to do so from the male plant DNAs. Their finding was helpful for the breeding practice of C. wightii and our SCAR markers may be useful for identification of C. wightii at species level.

The developed SCAR markers by us were used for identification of C. wightii and discrimination among C. wightii and C. myrrha. SCAR markers maybe are developed using sequence of RAPD fragments which are characterized by many advantages, including their specificity, low cost, ease, fast use, reproducibility, abundance, and being polymorphic in nature targeting specific regions of the genomes [52–54] employed with success in plant and animal species identification [30, 55–58].

In this study, RAPD amplicons were selected for cloning, sequencing, and final development of SCAR markers. Specific characters of RAPD markers entice researchers and usually SCAR markers have been developed from RAPD amplicons [58–61]. Amplicon of other fingerprinting methods like AFLP [62–64] and ISSR (Inter Simple Sequence Repeat) [52] was also used to develop SCAR markers.

Developed Sc1Pm marker in this study produced a 910 bp amplicon with C. wightii, while in other samples no amplification was observed. These results revealed that this SCAR marker might be used in identification and authentication of C. wightii. Many reports are available in which SCAR markers have been used for authentication of medicinal plant species like Panax ginseng [65], bent-grass [66], Bamboo [67], Piper longum [68], Artemisia princeps and A. argyi [69], Phyllanthus emblica [70], strawberry [71] Jatropha curcas [72, 73], Ganoderma lucidum [74], Pueraria tuberosa [75], Dendrobium candidum [76], Sinapis arvensis [77], Cornus officinalis [78], and Scrophularia ningpoensis [79]. Three
SCAR markers of *Phyllanthus* species were developed from three specific RAPD sequences that can identify and differentiate the morphologically similar *Phyllanthus* species [80].

SCAR markers have been also developed for breeding programs of crops like Rice [81], *Citrus tristeza* [82], *Brassica napus* L. [83], Grapevine [84], Wheat [85], Buckwheat [86], Grape [87], Barley [88], *Atractylodes japonica* and *A. macrocephala* [89], *Diplocarpon rosae* [90], *Puccinia coronata* [91], *Puccinia striiformis* [92], *Thinopyrum elongatum* [93], *Liriope* and *Ophiopogon* [94], *Medicago sativa* [95], *Triticum turgidum* [96], and *Miscanthus sacchariflorus* [97].

Our marker Sc2P produced a prominent amplicon of 491 bp in the *C. wightii*, and 1.2 kb in the *C. myrrha* while other plant samples did not show amplification. The result revealed that this SCAR primer might be used for the discrimination among *C. wightii* and *C. myrrha*. Only few reports are present with a single primer discrimination among two closely related species.

**Conflict of Interests**

The authors declare that there is no conflict of interests.

**Authors’ Contribution**

Dr. P. K. Sairkar performed wet and dry laboratory work under the supervision of Dr. N. P. Shukla and Professor Anjana Sharma. Professor A. Sharma was cosupervisor of this work and all microbiology related work was performed under her supervision, while Dr. N. P. Shukla was supervisor of this work and probes were developed under his supervision.

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