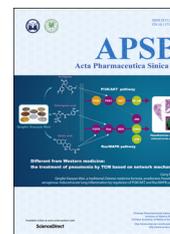




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ORIGINAL ARTICLE

Internal transcribed spacer guided multiplex PCR for species identification of *Convolvulus prostratus* and *Evolvulus alsinoides*



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DNA barcoding

Abstract Shankhpushpi is a reputed drug from an Indian system of medicine for treating mental disorders and enhancing memory. Two herbs, namely *Convolvulus prostratus* Forssk. and *Evolvulus alsinoides* (L.) L., are commonly known as Shankhpushpi. Ambiguous vernacular identity can affect the scientific validity of the Shankhpushpi-based herbal drug therapy. In the present investigation, a novel and sensitive multiplex PCR method based on polymorphism in the internal transcribed spacer (ITS) region was developed to establish the molecular identity of *C. prostratus* and *E. alsinoides*. DNA was isolated and the ITS region was amplified, sequenced and assembled. Sequences were aligned to identify variable nucleotides in order to develop plant-specific primers. Primers were validated in singleplex reactions and eventually a multiplex assay was developed. This assay was tested for sensitivity and validated by amplifying DNA isolated from the simulated blended powdered plant material. Primers developed for *C. prostratus* resulted into a 200 bp amplicon and 596 bp for *E. alsinoides*. The assay was found to be sensitive enough for amplification of low quantities of DNA. The method can detect 10% of the mixing of plants with each other in blended material. This PCR assay can be used for rapid botanical identification of Shankhpushpi plant materials and will improve evidence-based herbal drug therapy.

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1. Introduction

Convolvulus prostratus Forssk. (CP) syn *Convolvulus microphyllus* Sieb. ex Spreng syn *Convolvulus pluricaulis* Choisy, vernacularly known as Shankhpushpi^{1,2}, is reputed to be “medhya rasayna” (“wisdom drug”) in the Ayurvedic system of medicine. The whole plant is used as a drug to boost memory, improve cognitive functions and to treat central nervous system (CNS) disorders like psychosis, epilepsy and Alzheimer's disease^{3–5}. The major chemical constituents of the plant are carbohydrates, fatty acids, alkaloids and coumarins which are believed to work in synergy to produce desired effects in these disorders. The term “Shankhpushpi” has plurality and is also equated with *Evolvulus alsinoides* (L.) L.³. The whole plant of *E. alsinoides* is also reported to have therapeutic properties to increase intellect, improve learning, and enhance memory⁶, along with other medical applications^{7,8}. Controversial and ambiguous vernacular identity of Shankhpushpi can present a challenge for correct plant identification, which is the first step for preparation of safe and efficacious herbal drugs. Market examination of crude drugs sold as Shankhpushpi, indicated the presence of *E. alsinoides* (EA) as the major substituent and mixing material⁹. Moreover, extensive and systematic information about the bioactive compounds of these two plants is needed to check comparable therapeutic effects when *E. alsinoides* is used as substituent⁵. However, comparative pharmacological evaluation of plant extracts for CNS activities from these two plants species have established the superiority of *C. prostratus*^{5,9}. In order to unequivocally identify the original botanicals and to maintain the scientific validity of Shankhpushpi-based drugs, various approaches have been reported^{3,10,11}. These methods were based on conventional pharmacognosy and phytochemical analytical techniques. Utilization of these methods is restricted by their dependency on personal expertise and low sensitivity for the processed material (powder, capsules and tablets) in which taxonomic characteristics can degrade. In addition, identification with chemical techniques can fluctuate with mutable environmental and geographical conditions¹². These limitations have raised the need for developing additional DNA-based approaches to identify the Shankhpushpi plant species. These methods can be used independently and also as auxiliary tools in conjunction with the conventional methods to enhance the validity of plant identification^{13,14}.

In the present study we have developed a PCR-based method for the rapid molecular identification of individual species and to differentiate two Shankhpushpi plant species CP and its closely related substituent EA^{15,16}. The assays were developed for both processed and unprocessed material using sequences of the internal transcribed spacer (ITS) locus of nuclear DNA. The ITS region represents a nonfunctional sequence present between highly conserved rRNA genes. It can be divided into two regions namely ITS1 and ITS2 which are interrupted by a 5.8s rRNA gene sequence. This array ITS1-5.8s rRNA gene-ITS2 is flanked by 18s rRNA and a 26s rRNA gene sequences. This tandem array is spread randomly in the nuclear genome and is recommended as one of the DNA barcoding tools for differentiation between closely related species¹⁷.

2. Material and methods

2.1. Plant collection

Whole plant samples of CP and EA were collected from seven different locations in India during their flowering season (Table 1). At

least three samples of each species were collected from each location. Plants were authenticated with the help of taxonomist and voucher specimens were submitted to the herbarium division of our institute. The samples were stored at -20°C prior to analysis (unprocessed material). Some amounts (2–5 g) of the samples were heat dried at 45°C for 48 h and then powdered (considered as processed material).

2.2. DNA isolation, amplification and sequencing of ITS locus

DNA was isolated from leaf tissue of both the plants using the CTAB method¹⁸ and was used for amplification of the ITS locus. ITS regions from CP and EA were amplified using universal primer pairs¹⁹. Amplification was carried out in 25 μL reaction volume with $1 \times$ Taq buffer, 2 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ dNTP, 10 pmol of the forward (ITS5) and reverse primers (ITS4), 1U of Taq polymerase and 40–60 ng of DNA template, 5% DMSO (dimethyl sulphoxide) and 0.5 $\mu\text{g}/\mu\text{L}$ BSA (bovine serum albumin). The reaction was performed as follows: initial denaturation 94°C (10 min), 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, polymerization at 72°C 1 min which was finally extended at 72°C for 5 min. The amplicons thus obtained were subjected to bidirectional sequencing.

2.3. Sequence assembly, analysis and characterization

Sequences of ITS loci of both plants were analyzed for quality and assembled using Sequence analysis vs 5.2 (Applied Biosystem, USA) and Codon Code Aligner v.6.0.1 (Codon Code, Dedham, Massachusetts, USA) software respectively. Determination of sequence boundaries and verification of the contigs were done using BLAST search²⁰. The sequences thus obtained were submitted to NCBI (Accession numbers CP-KC688313, EA-KC688314). The sequences were also characterized in terms of length, aligned length, guanine-cytosine (GC) content, conserved and variable sites using MEGA software v.5.1²¹.

2.4. Selection of plant-specific primers

Plant-specific primers were designed manually using variable sites, determined within the aligned ITS sequences of both the plants using the ClustalW tool (MEGA vs 5.1)²¹. Groups of feasible primers were subjected to OligoCalc²² and Primer3 software²³ for *in silico* selection of best suitable primers on the basis of optimal length, optimum GC content, melting temperature compatibility, hairpin formation, secondary structure and species specificity. *In silico* verified primers were experimentally validated in various singleplex PCR in order to identify

Table 1 Description of plant samples and their geographical origin.

Plant species	Location
<i>C. prostratus</i>	Anand, Gujarat (Vouchered-BVPPERD/PP/04/13/01)
	Udaipur, Rajasthan
	Boriavi, Gujarat
	Dholka, Ahmedabad, Gujarat
<i>E. alsinoides</i>	Kota, Rajasthan
	Pune, Maharashtra (Vouchered-BVPPERD/PP/08/12/10)
	Anand, Gujarat.

optimum annealing temperatures, to check the amplifiability of the primers for their respective species samples and cross amplifiability of the primers with opposite species. The primer finally selected for CP (5' TTGGCCTAAATGCGAGTCTT 3') was 20 bp long with a melting temperature of approximately 56 °C. The calculated melting temperature of 21 bp long EA specific primer (5' TGTTTAAACAC-CATACCGCGG 3') was approximately 59 °C. Primer3 software was also used to determine approximate lengths of the amplicons which were 200 bp for CP and 596 bp for EA.

2.5. Multiplex PCR assay for CP and EA

Molecular identity of the individual plants (CP and EA) and their mixtures (both processed and unprocessed) was established by developing multiplex PCR assay. The optimized final reaction conditions were as follows: a total 25 µL multiplex reaction mixture was added with 1 × Taq buffer, 2.50 mmol/L MgCl₂, 200 µmol/L dNTP, 20 pmol primer of CP, 2 pmol primer of EA, reverse primer (ITS4) 10 pmol, Taq Polymerase 1 U and the reaction was supplemented with BSA (0.5 µg/µL). Concentration of primer specific for EA was increased up to 6 pmol for amplification from dried samples. PCR amplifications were carried out in ABI light thermal cycler and performed according to following PCR conditions as initial denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 20 s and polymerization at 72 °C for 1 min subsequently final extension was carried out at 72 °C for 5 min.

2.6. Specificity and sensitivity of the assays

The specificity in multiplex assay was verified by including two cross controls *i.e.* in control-1 DNA of CP was mixed with both the primers and in control-2 DNA of EA was mixed with both the primers. All the PCR assays were tested for their sensitivity. Different dilutions of DNA of each plant were made (2, 5, 10, 25, 50, 75 and 100 ng) to test the limit of amplification and detection on agarose gel.

2.7. Validation of multiplex PCR assay and analysis of PCR products

The developed multiplex PCR method was further validated by amplifying DNA isolated from the mixture of processed material. The mixing was done in various ratios to imitate possibility of commercial adulteration and substitution. Plants material was mixed in various ratios *viz.* 10:90, 30:70, 50:50, 70:30 and 90:10 (CP:EA). DNA was isolated from each mixture and subjected to amplification with developed multiplex PCR assay. PCR products were analyzed using agarose gel electrophoresis.

3. Results and discussion

The use of authentic botanicals is the very first level of quality control for herbal drugs. Lacking of originals can pose negative impact on the efficacy of finished products and hence, on consumer's belief. Therefore, use of authentic species is essential to maintain the growth and development of medicinal plant sectors. DNA diagnostics have become the gold standard for scientific

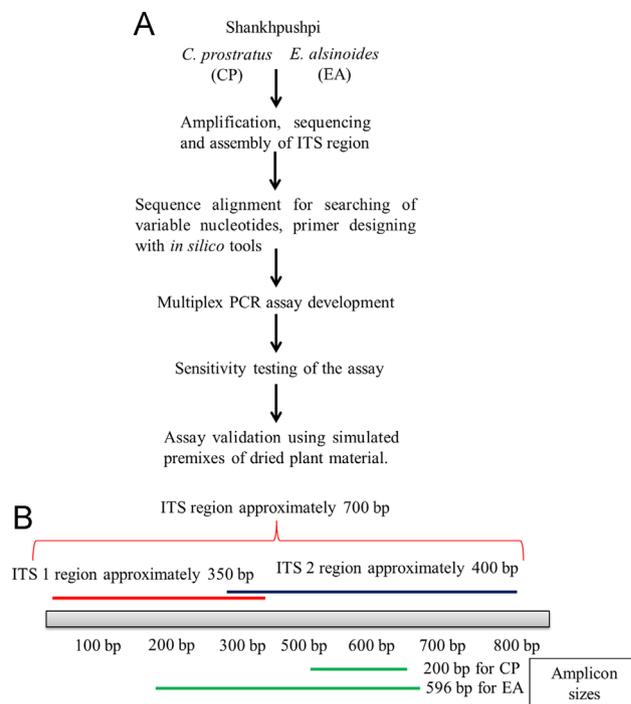


Figure 1 (A) Schematic representation of the method development. (B) Representation of different amplicon sizes.

validation of herbal products^{14,24}. Previously, random amplification polymorphic DNA (RAPD) markers were developed to authenticate Shankhpushpi species²⁵ as well as for genetic diversity analysis^{1,26}. However, reproducibility and differential expertise of various laboratories are the major limitations of this technique²⁷. Molecular authentication processes based on the DNA sequencing are comparatively more effective and economical²⁷. Among the sequence-based methods, DNA barcoding is the most recent^{28,29}. Application of standard DNA barcoding has been extended further in various investigations by developing various methodologies like conventional and quantitative PCR, Bar-HRM (DNA barcode high-resolution melting curve analysis), LAMP (loop mediated isothermal amplification) and high throughput next generation sequencing, to identify plant species^{30,31}. In the present investigation a multiplex PCR was developed for molecular identification (Fig. 1) of CP and EA³². The method employs the universality and precision of DNA barcoding with the added advantage of rapidity of PCR. This procedure is based on variability in the sequence of the ITS region. The assays developed here are suitable to identify both the plants in the original and the dried (processed) forms.

DNA isolated from both plants was subjected to qualitative and quantitative analysis with agarose gel electrophoresis and UV spectrophotometry. Prominent bands of DNA were observed on the gel (Supplementary Fig. 1). UV analysis showed 260/280 ratio as 1.4–1.8 indicating DNA purity. ITS region from both the species were amplified, sequenced and assembled into consensus. The lengths for CP and EA, ITS consensus were 676 bp and 686 bp, respectively. BLAST search of these contigs revealed their novelty and authenticity as first 100 BLAST outputs were matched with the same species or the genus. Sequences characteristics of the ITS loci of both the plants are given in Supplementary Table 1.

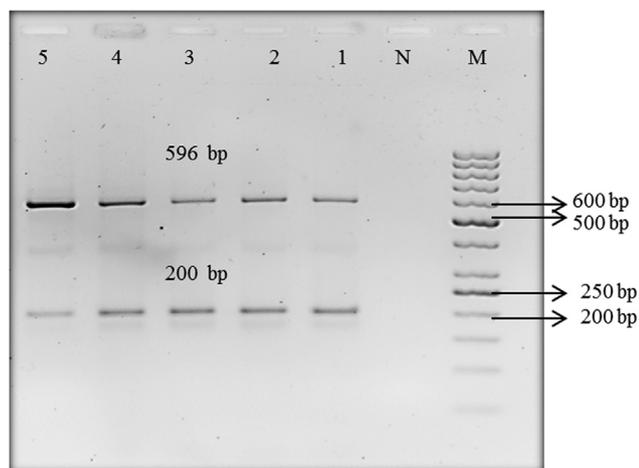


Figure 5 1.8% agarose gel is showing results of validation of the single tube multiplex PCR assay. 200 bp amplicon specific to CP and 596 bp amplicon specific to EA can be observed in each lane. Simulated mixture contained both species in different ratios, Lanes 1 to 5 species specific amplification from the dried powder material having CP: EA in ratio of 90:10, 70:30, 50:50, 30:70 and 10:90, respectively; M: GeneRuler 50 bp DNA ladder (Thermo Scientific); N: no template control.

validation showed the assay is sensitive enough to detect the mixing of both the species with each other as low as at 10% level (Fig. 5).

4. Conclusion

In the present study we developed a DNA-based assay to identify and distinguish between medicinal preparations of two Shankhpushpi plants. DNA-based tools can be used independently or in combination with conventional methods. The developed assays were able to detect the presence of either single plant or a variety of mixtures of the two plants. Successful amplification of DNA from dried and powdered material proves that the assays were not affected by the physical or physiological conditions of the DNA. The assays were able to detect very low quantities of the DNA even in the mixture. The present method will help to ensure quality and molecular standardization of these two plant species and contribute to the commercial identification of genuine Shankhpushpi starting material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.apsb.2016.02.003>.

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