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# Restriction analysis of Randomly Amplified Polymorphic Fragments of DNA for authentication of plants used in Ayurvedic and Siddha formulations

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**Abstract:** To authenticate, medicinal plants such as *Tridax procumbens, Curcuma longa Sesbania grandiflora* and *Premna integrifolia* having same vernacular name 'Jayanti' were subjected to Randomly Amplified Polymorphic DNA (RAPD) analysis. Analysis was carried out using twenty decamer primers (OPA1-20). Analysis revealed polymorphic bands except for OPA14 in and OPA19 in *C. longa* and OPA20 in *S. grandiflora*. A total number of 174 amplicons were observed. The gel purified amplicons were reamplified and digested with *Eco*RI, *Not*I, *Hind*III, *Sau*3AI and *Bam*HI. Among, *Sau3*AI restriction digestion patterns showed polymorphism. RAPD coupled with restriction endonuclease reaction based analysis proved to be a useful tool in authentication of plant species.
**Key words:** Random Amplified Polymorphic DNA (RAPD), restriction endonuclease, diversity, authentication.

## Introduction

Ayurveda and Siddha are the ancient systems of Indian herbal medicine[1]. For Ayurvedic and Siddha formulations collection of plants were done by people who were aware of only the vernacular names of plants; hence adulterations became a major problem. Therefore, correct identification and authentication of the plant ingredients became necessary for ensuring safety, efficacy and quality of the drugs. Intended adulterations are usually done to increase the market samples due to the difficulty in procurement of the required raw materials[1]. Plants that were having same therapeutic value were also used as part of the formulation. Because of this there were more than one species of plant belonging to different families claiming same vernacular name while scientifically identifying the ingredients of drugs in different regions[2]. Adulterant detection and authenticity of traded medicinal plants were mostly done by morphological and phytochemical assays which requires an expertise and in the finely ground powders of plants it is less applicable[3]. Moreover, phytochemical profiles may vary highly based on the processing or the environment in which the plants were grown.

Molecular tool based on DNA is much more specific and accurate. It include Random Amplified Polymorphic DNA (RAPD), Arbitrarily-Primed Polymerase Chains Reaction (AP-PCR), Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Amplified Fragment Length Polymorphism (AFLP), Direct Amplification of Length Polymorphism (DALP), Sequence Characterized Amplified Region (SCAR) and Short Sequence Repeat (SSR)[4]. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD

technique more popular[5]. Another advantage is that prerequisite knowledge of the genome is not needed as random primers can bind anywhere in the genome and amplify a particular sequence unique for that plant[4].

In this study, selected four plants namely *Tridax procumbens, Curcuma longa,*and *Sesbania grandiflorai* and *Premna integrifolia* having same vernacular name "Jayanti" in different languages in different locations of India. *T. procumbens* (Asteraceae) is a common plant found in tropical areas during rainy season. It is an official drug of Ayurvedic Pharmacopoeia and Indian Herbal Pharmacopoeia, and is used either as a single drug or in combination with other drugs. It is dispensed as "Bhringraj" by some Ayurvedic practioners well known as a therapeutic agent for liver disorders[6]. Traditionally, it is used for the treatment of bronchial catarrh, dysentery, malaria, stomach-ache, diarrhoea, high blood pressure and to check haemorrhage from cuts, bruises and wounds and to prevent falling of hair. The leaf extract has been extensively used in Indian traditional medicine as anticoagulant, anticancer, antifungal and insect repellent. Different extracts of this plant have been found to exhibit antibacterial, anti-inflammatory, hepatoprotective, anti-ulcer and, anticancer, immuno modulator[6,7] properties.

Turmeric is derived from *C. longa,* a member of Zingiberaceae. From rhizomes the main constituents of turmeric is curcumin. *C. longa* It is extensively used in ayurvedic preparations and is official (Haridra) in the Ayurvedic Pharmacopoeia of India (API 1987), Committee on herbal medicinal products (HMPC). Curcumin has antioxidant, anti-inflammatory, antiviral and antifungal actions. Curcumin exerts anti-inflammatory activity easing conditions such as bursitis, arthritis and back pain by acting as antioxidants vitamins C, E and Beta-Carotene. It is used for preventing cancer, to save liver, premature aging, post-surgical inflammation and to prevent atherosclerosis by reducing the formation of bloods clumps[8].

*S. grandiflora* (L) commonly known as "white-dragon tree", is a traditionally revered Ayurvedic medicinal plant in Southern India. It is marketed as "Hadga" in ayurvedic system for treating fever, sinus, bruises, and venereal diseases etc.[9] Sesbania leaves are known to possess antihelmintic, alixeteric, aperient, tonic, diuretic, and laxative properties. Further, they have been documented as therapeutically useful in Kaphaja disorders, pruritis, skin disorders, night blindness, epilepsy, gout, ophthalmia nasal catarrh and headache. The leaves contain a non-poisonous saponin[10].

*P. serratifolia* was previously known as *P. integrifolia*, *P. obtusifolia* or *P. corymbosa*[11]. It is used as one of the ingredients in Chyawanprash, a very popular Ayurvedic preparation in India. The roots and leaves are used in Kapha, nervine pain, indigestion and fever. Its root forms one the ten key ingredients of an Ayurvedic formulation called Dashamula or Dashamularist (Dasha-ten and mula-root).

The aim of our study is to provide a methodological approach in authenticating the plants using PCR-based RAPD markers and to analyze the polymorphism among these plants using restriction endonuclease site analysis.

## Materials and methods

### *Collection of plant samples*

*T. procumbens, S. grandiflora* and *P. integrifolia* were collected from herbal garden of Centre for Advanced Research in Indian System of Medicine (CARISM, SASTRA University, India). *C. longa* was supplied by a private cultivar and was authenticated by a botanist.

### *Genomic-DNA isolation*

### Modified CTAB protocol

Total genomic DNA was extracted from leaves of *T. procumbens*, *S. grandiflora, C. longa* and *P. integrifolia* according to modified CTAB protocol[13]. One gram of leaves was washed with 70% ethanol and double distilled water and then air dried. The leaves were ground with 2 ml of 2% CTAB buffer (1 M Tris HCl pH 8.0, 5 M NaCl, 0.5 M EDTA and 2% CTAB (Cetyl Trimethyl Ammonium Bromide) and the extract was transferred to different microfuge tubes. This was followed by incubation in water bath for 15-30 minutes at 65°C and centrifugation of samples at 12000 rpm for 15 minutes. The supernatant was transferred to fresh microfuge tubes and 500µl of buffered phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added, mixed for 5 minutes which was again followed by centrifugation at 12000 rpm for 12 minutes. This step was repeated once again. The supernatant was separated and equal volume of chloroform:isoamyl alcohol (24:1) was added

and mixed for 5 minutes. This was centrifuged at 10000 rpm for 10 minutes. To the supernatant, 0.65v of ice cold isopropanol was added. The mixture was subjected to centrifugation at 8000 rpm for 8 minutes. The supernatant was discarded and the pellet was carefully washed with 70% ethanol followed by centrifugation at 8000 rpm for 10 minutes. To the washed pellet, 100µl of autoclaved double distilled water was added for resuspension and stored at -20° C for further use.

**Modified Doyle and Doyle protocol**

Modified Doyle and Doyle protocol[14] was followed for isolation of genomic DNA from *C. longa*. 5 ml of extraction buffer (100 mM Tris HCl pH 8.0, 0.5 M EDTA pH 8.0, 500 mM NaCl, β-mercaptoethanol-7 µl) was added to 0.5 gm of leaves washed with 70% ethanol and double distilled water after grinding them in liquid nitrogen and incubated overnight at 65°C. The samples were centrifuged and to the supernatant collected , 600µl of chloroform isoamyl alcohol solution (24:1) was added. This was centrifuged at 12000 rpm for 8 minutes and to the aqueous layer 0.1v of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol were added and incubated for 2 hours. The samples were then centrifuged at 12000 rpm for 8 minutes. The supernatant was discarded and the pellet was washed with 800µl of ethanol followed by centrifugation at 8000 rpm for 8 minutes. The pellet was air-dried and 50 µl of autoclaved double distilled water was added to the pellet for resuspension. It was stored in -20° C for further use.

DNA concentration was measured spectrophotometrically using Nanodrp 2000 series Spectro photometer (Thermo Scientific, USA).

**RAPD-PCR**

PCR reaction (20 µl) was carried out using 20 Operon 10-mer primers of the series OPA 1-20 containing template DNA of 5-30 ng/µl concentration, 10x buffer, 200 µM dNTPs and Taq polymerase (0.2 U) and 1 µl of primer. The PCR conditions were, initial denaturation 95°C for 2 minutes, denaturation 95°C for 1 minute, annealing 38°C for 1 minute, extension 72°C for 1 minute, step 2-4 repeated 32 cycles and final extension at 72°C for 4 minutes. The PCR products were separated in a 1.6% agarose gel at a voltage of 40V for 3 hrs. From each gel, polymorphic bands unique for that plant were selected, cut from the gel and stored at -20° C. The marker used was GeneRuler™ 1kb DNA Ladder (#SM0311, Concentration: 0.5µg/µl).

**Table 1: Operon primers of the OPA 1-20 series and their sequences**

| PRIMER NAME | SEQUENCE |
|---|---|
| OPA-01 | CAGGCCCTTC |
| OPA-02 | TGCCGAGCTG |
| OPA-03 | AGTCAGCCAC |
| OPA-04 | AATCGGGCTG |
| OPA-05 | AGGGGTCTTG |
| OPA-06 | GGTCCCTGAC |
| OPA-07 | GAAACGGGTG |
| OPA-08 | GTGACGTAGG |
| OPA-09 | GGGTAACGCC |
| OPA-10 | GTGATCGCAG |
| OPA-11 | CAATCGCCGT |
| OPA-12 | TCGGCGATAG |
| OPA-13 | CAGCACCCAC |
| OPA-14 | TCTGTGCTGG |
| OPA-15 | TTCCGAACCC |
| OPA-16 | AGCCAGCGAA |
| OPA-17 | GACCGCTTGT |
| OPA-18 | AGGTGACCGT |
| OPA-19 | CAAACGTCGG |
| OPA-20 | GTTGCGATCC |

***Extraction of DNA from cut gel***

Excised gel were suspended in 50µl of water and vortexed for 5 minutes and this was repeated after 5 minutes before centrifuging at 10,000 rpm for 10 minutes.

***Re-amplification of PCR products***

Unique bands were selected from excised bands and reamplified in a 40 µl PCR reaction under same amplification conditions as that of the previous PCR.

***Purification of PCR product***

The reaction mixture was transferred into clean tubes and 3v of binding buffer was added and mixed by inverting. This mixture was transferred to the MX-10 column (Cat no. MX-06-0501, India) and followed by incubation for 2 minutes. The samples were centrifuged at 10,000 rpm for 1 min and the flow through was discarded. 500 µl of wash solution was added followed by centrifugation at 10,000 rpm for 1 min. The flow through was discarded. The above step was repeated and spun at 10000 rpm for 10 minutes. The column was placed in a clean tube and 25 µl of pre-warmed elution buffer was added exactly at the centre of the tube followed by incubation at room temperature for 2 min and centrifugation at 10,000 rpm for 1 minute to elute DNA followed by again 25 µl elution buffer extraction. Purified DNA was stored at -20° C.

***Restriction digestion*** Restriction digestion of the purified PCR products was performed as a next level of authentication so as to strengthen the uniqueness of the polymorphic bands obtained from RAPD. *Eco*RI, *Hind*III, *Not*I, *Sau*3AI and *Bam*HI were utilised for restriction digestion of reamplified fragments. 25 µl of PCR DNA sample, 10x restriction buffer (3 µl) and 1.7 µl of restriction enzyme (10U),0.3 µl 1% BSA( if specified by the manufacturer)were used and the reaction mixture was incubated at 37°C. Digested products were electrophorised along with undigested PCR fragments as control using 1.6% agarose at 100V for 40 minutes.

## Results

A preliminary study was performed using RAPD primers from OPA1 through OPA20 to genetically authenticate *T. procumbens* and *S. grandiflora* *C. longa* and *P.integrifolia* from other "Jayanti" species by producing RAPD markers. A close study using RAPD markers and restriction site digestion with *Eco*RI, *Hind*III, *Not*I, *Sau*3AI and *Bam*HI revealed polymorphism among the selected plants. From a total of 20 decamer primers that were initially used for screening, all primers revealed faint or bright bands excepting OPA14 (*C. longa*), OPA19 (*C. longa*) and OPA20 (*S. grandiflora*). A total number of 174 amplicons were seen.The size of the amplicons ranged from 2500bp to 500 bp. Using the 20 primers, percentage of polymorphism was calculated using 14 polymorphic bands from a total of 174 amplicons. A high degree of polymorphism of about 50% was seen for the primer OPA18. Furthermore, nine bands from the RAPD amplicons were chosen for our study to digest with the *Eco*RI, *Hind*III, *Not*I, *Sau*3AI and *Bam*HI restriction enzymes. There was no restriction sites for *Eco*RI, *Hind*III and *Not*I and the sites were present for *Sau*3AI. OPA 8 and 18 were chosen for a RAPD-PCR involving all the four plants. The purified amplicons obtained through this PCR were subjected to *Sau*3AI digestion to obtain a distinct polymorphic pattern.

**Genomic DNA isolation**

Genomic DNA was extracted successfully using modified CTAB protocol for *T. procumbens* and *S. grandiflora* and Modified Doyle and Doyle protocol for *C. longa.* The samples were then separated on 1% agarose gel for the quality of DNA.

**RAPD-PCR**

RAPD-PCR was carried out with the 20 Operon 10-mer primers of the series OPA 1-20 using the conditions as mentioned above in materials and methods section. For the DNA-based analysis of the chosen plants, the 20 primers listed in Table 1 resulted in the appearance of PCR products with varied band numbers. For the three provenances *T. procumbens, S. grandiflora, C. longa* 74, 42 and 58 number of bands were detected respectively.

Upon acquiring the large number of polymorphic amplicons using primers OPA8 and OPA18, we repeated a 40 µl RAPD-PCR using these primers in *P. integrifolia*, along with the three other plants namely *T. procumbens, C. longa* and *S. grandiflora.* The conditions are same as that of the previous PCR.

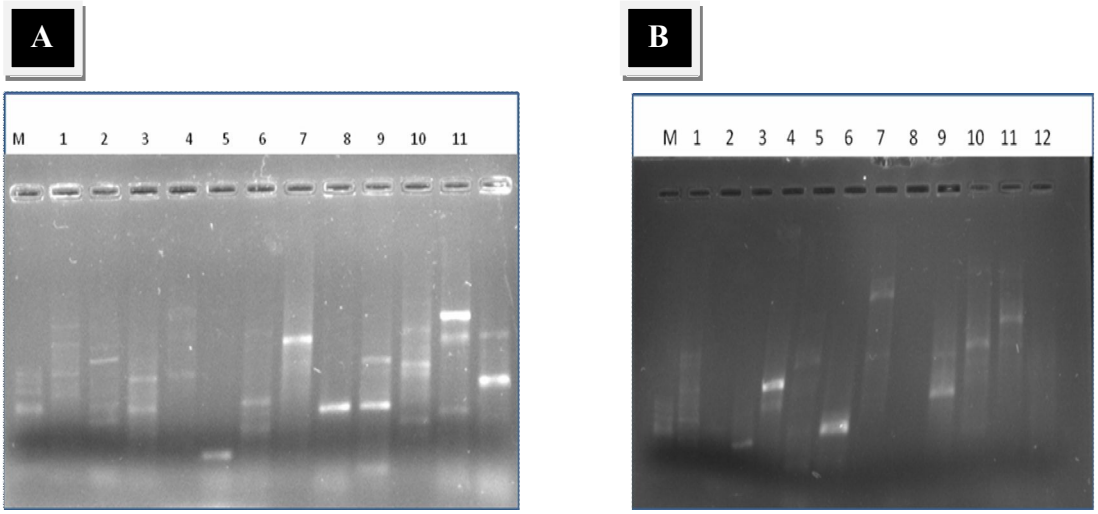


**Fig. 1. RAPD PCR-first trial using OPA 8 and OPA18 A) 1.6 % agarose gel electrophoresis of RAPD PCR run at 3V/cm using the primers OPA 5-8. Lanes: M, Marker; 1, 2 and 3 are PCR amplicons amplified by primer OPA5 in *T. procumbens, C. longa* and *S. grandiflora* respectively; similarly OPA6 for 4, 5 and 6; OPA7 for 7, 8 and 9 and OPA8 for 10, 11 and 12. B) RAPD PCR using the primers OPA 17-20. Lanes: M, Marker; 1, 2 and 3 are PCR amplicons amplified by primer OPA17 in respectively; similarly OPA18 for 4, 5 and 6; OPA19 for 7,8 and 9; OPA20 for 10, 11 and 12.**

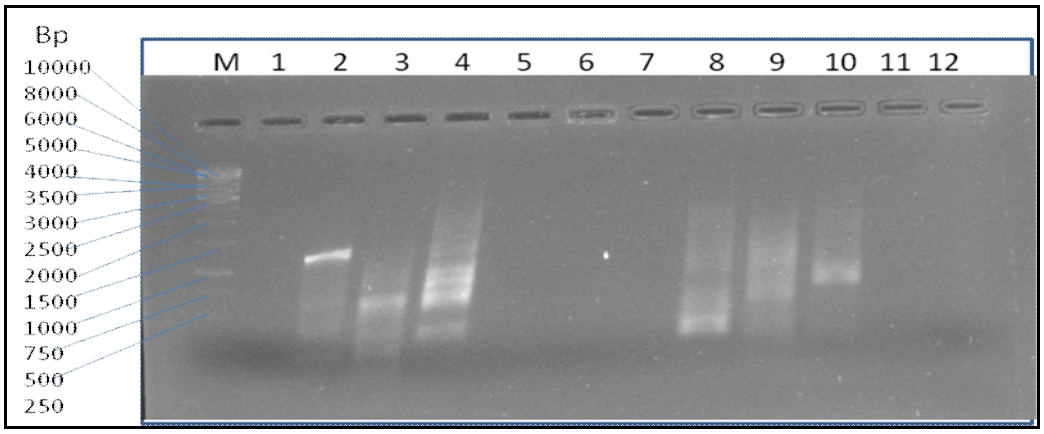


**Fig. 2. Second trial of PCR using OPA8 and OPA18. 1.6 % agarose gel electrophoresis of RAPD PCR at 3V/cm using the primers OPA 8 and 18 in *T.procumbens, C.longa, S.grandiflora and P.integrifolia.* Lanes M, marker; 1, 2, 3 and 4 are PCR amplicons amplified by OPA8 in *T.procumbens, C.longa, S.grandiflora and P.integrifolia* respectively; 7,8,9,10 amplified by OPA18 in *T.procumbens, C.longa, S.grandiflora and P.integrifolia* respectively.**

***Re-amplification of gel purified PCR products***

The RAPD amplicons showing the highest degree of polymorphism were selected and excised from the gel. Reamplification of the extracted PCR products were performed in a 40µl reaction using the primers OPA8 and OPA18 for all the four plants followed by purification of the amplicons. . A 250 bp fragment amplified by OPA8 in *T.procumbens* was able to distinguish it from all the other plants. A 300 bp fragment amplified by OPA18 in *S.grandiflora* distinguishes it from *P.integrifolia.* A single 1000 bp fragment in *C.longa* amplified by OPA8 distinguishes it from all the other plants.

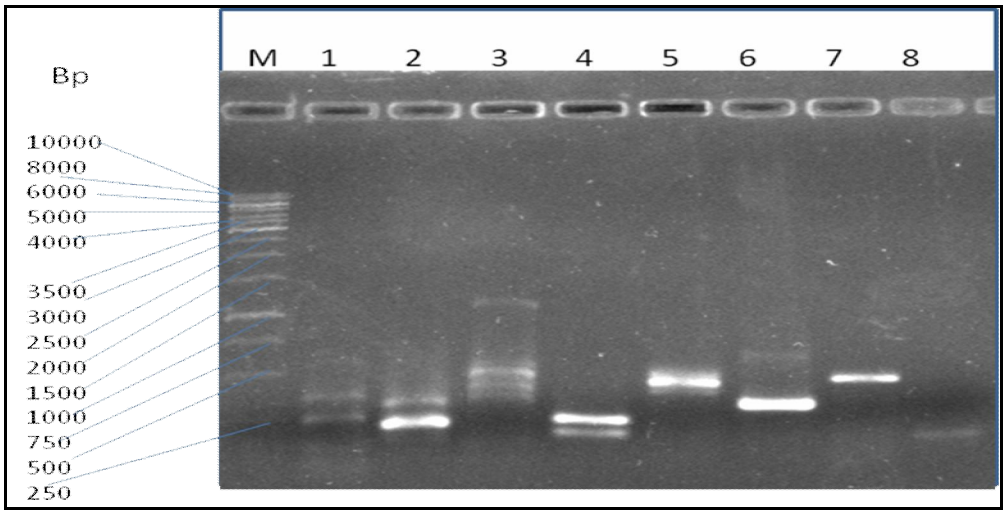


**Fig.3 Re-amplification. 1.6% Gel electrophoresis of Re-amplified RAPD-PCR amplicons using primers OPA8 and OPA18 in *T. procumbens, C. longa, S. grandiflora* and *P. integrifolia.* Lane M-marker, lanes 1, 3, 5 and 7 are OPA8 amplified amplicons in *T. procumbens, C. longa, S. grandiflora* and *P. integrifolia* respectively and lanes 2, 4, 6 and 8 are OPA18 amplified amplicons in *T. procumbens, C. longa, S. grandiflora* and *P. integrifolia* respectively.**

***Restriction digestion***

Among the restriction enzymes used, the frequent cutter namely *Sau*3AI showed distinct restriction digestion pattern in all of the three plants. The rare cutters, *Eco*RI, *Hind*III and *Not*I did not show any restriction sites in the RAPD amplicons. This was followed by repetition of restriction digestion using *Sau*3AI in RAPD-amplicons of OPA8 and OPA18 in *P. integrifolia* along with the other three plants. Using *Sau*3AI digestion of OPA8 amplicons, we can distinguish *T.procumbens* and *P.integrifolia* clearly by the 250 bp band in the digested well of *T.procumbens* and the excessive digestion in *P.integrifolia.*

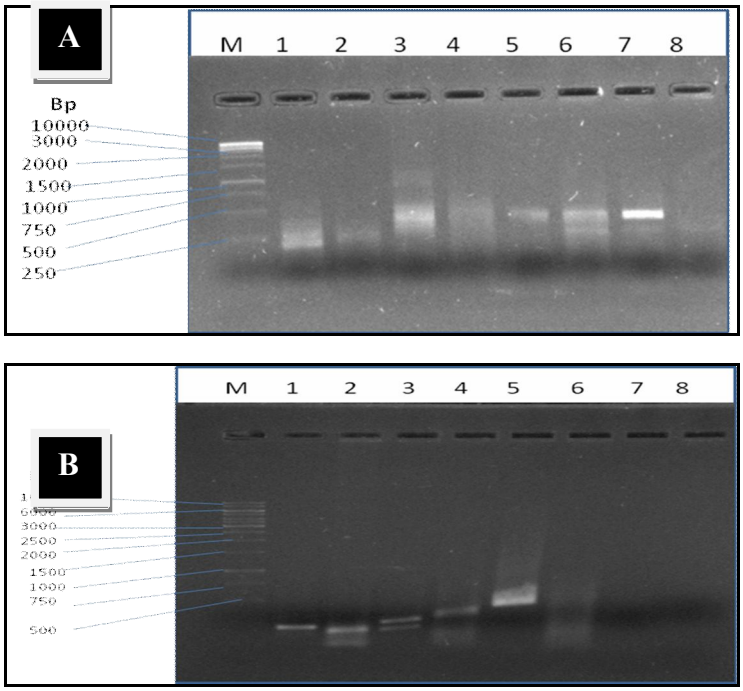


**Fig.4. Restriction digestion with Sau3AI A) 1.6% agarose gel of restriction digestion pattern using Sau3AI in OPA8 amplified-amplicons. B) 1.6% agarose gel of restriction digestion pattern using *Sau3*AI in OPA18 amplified-amplicons. Lanes: M, marker; 1, 3, 5 and 7 are undigested RAPD-amplicons (used as a control) in *T. procumbens, C. longa, S. grandiflora and P. integrifolia* respectively, 2, 4, 6 and 8 are Sau3AI digested RAPD-amplicons in *T. procumbens, C. longa, S.grandiflora* and *P. integrifolia* respectively.**

## Discussion

The present study focuses on identification of RAPD marker for the four plants namely *T. procumbens, C. longa* and *S. grandiflora* and *P. integrifolia* which share the same vernacular name "Jayanti" and commonly used in Ayurvedic and Siddha systems of medicine. RAPD analysis is a well-proven tool, widely used in differentiating medicinal plant species, close relatives or adulterants[15]. An RAPD is derived from two short DNA stretches in a genomic region which are in inverted orientation located in opposite strands and are complementary to the forward and reverse primers that are responsible for amplification[4]. The simplicity, the low amount of genomic DNA required and the fact that prior genetic information is not required[4] makes RAPD-PCR analysis ideal for genetic authentication. RAPD- PCR proved to be a more rapid and less laborious replacement for the digestion of genomic DNA by restriction enzymes for the characterization of RFLP[16]. RAPD has been widely used and often coupled with other methods such as AP-PCR, DALP, SCAR, Sequencing[17]. In our study, RAPD-PCR was coupled with restriction digestion based identification of polymorphisms among the digested RAPDs.

Our studies showed clear discrimination of *T. procumbens ,S. grandiflora and C. longa* based on unique fragments generated using 9 RAPD primers OPA10, OPA8, OPA11, OPA18, OPA19, OPA1, OPA7, OPA9, OPA13 and two frequent cutters namely *Sau*3AI and *Bam*HI. RAPD-PCR was repeated using primers OPA8 and OPA18 in all the four plants together. A fair amount of 50% polymorphism was achievable particularly in OPA18.

For large scale authentication the primers OPA18 and OPA8 can be used, because they showed an appreciable level of polymorphism in RAPD-PCR as well as their amplicons can be used for restriction-site based identification using *Sau*3AI. The bands obtained in restriction digestion were of different sizes for each RAPD amplicon of the corresponding plant.

Review of literature revealed that Bisoyi et.al(2010)[3] had presented a genetic diversity studies on six species of the *Sesbania* genus namely *S. javanica, S. speciosa, S. rostrata, S.grandiflora, S. aculeata* using RAPD and ISSR markers. 243 polymorphic bands were detected in the study, among which primer OPAF3 gave the most number of polymorphic bands (17) and the least number of polymorphic bands was given by OPAD03 (1). However, there is no mention of a specific marker for *S.grandiflora* in the previous literature. So in our study we developed a specific RAPD marker amplified by the primer OPA18 and OPA8 and *Sau*3AI digestion also showed polymorphism among the chosen plants including *S.grandiflora*.

As there has been no previous research undertaken on germplasm studies of *T.procumbens,* we reviewed the work done on the plants under the family Asteraceae of which *T.procumbens* belongs to. An AFLP analysis has been performed[18] to analyse the genetic structure of the nDNA(nuclear DNA) among two plants of 55 different accessions belonging to Asteraceae family. Furthermore, they have also distinguished three chloroplast (cp) DNA types in each of the species. Apart from the above studies, biochemical and morphological assays have been conducted to identify the plant species. However these physical/chemical evaluation methods is of considerable value in identifying local plant samples, they are not convenient for adulterant detection because there is a possibility of overlapping of marker compounds with those in related but unwanted species[19].

S. Nayak *et* al.(2006)[20]performed a genetic diversity studies on different cultivars of *C.longa.* In their study, seventeen cultivars of *C.longa* were undertaken and 4C nuclear DNA content and RAPD analysis were carried out using 20 random primers and the results showed high genetic variations with OPN06 and OPA04 showing high polymorphism. The genetic distances and similarities were studied using Nei's Coefficient. In a similar study by Jan et al (2011)[21], genetic diversity analysis was conducted on *C.longa* germplasm from Pakistan using RAPD markers. RAPD was performed using 10 primers and high polymorphism was detected. Nei's and Li's similarity coefficient and UPGMA were used to construct dendrogram. Till date studies pertaining to genetic diversity have been done among different cultivars of C.*longa*.

Various chemical investigations of its compounds like alkaloids, irridoid glysoid had been done so far[22] on *P.integrifolia*, but there is a lack of genetic based authentication studies performed on these species. Although , genetic diversity using ISSR and RAPD markers were studied on *Lippia alba*, which belongs to the same family Verbenaceae. It is important to note that the results in this study showed that just two primers were enough to discriminate all the other possible pair-wise comparisons between *Lippia alba* accessions.[23]

Thus from previous studies we found that there was no prior authentication done in the chosen plants based on the ambiguity arising from the same vernacular name deigned on unrelated species.

Our study using RAPD-PCR showed polymorphism among the different species and has affirmed the ability of specific RAPD markers as a reliable and cost effective authentication tool. Restriction digestion analysis was performed and the resulting restriction site based polymorphism further aided in distinguishing the plant species, thereby improving the efficacy and congruent nature of our results.

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