RAPD STUDY OF *CASSIA ANGUSTIFOLIA*

V.E. VIJAYA SEKHAR, T. SUJATHA

Abstract: Among the globe humans prefer herbal medicines. The World Health Organization estimates that 80% of the world's population utilizes traditional medicines for healing and curing diseases Medicinal plants. There is an increasing international market for medicinal plants, which are used both for herbal medicine and for pharmaceutical products. Accurate and rapid authentication of medicinal plants is difficult to increasetheir marketing. Since the natural medicines are safer than synthetic drugs, they are preferred for phyto-pharmaceutical usage. Due to the popularity of herbal drugs globally, their adulteration/substantiation aspects are gaining importance at the commercial level. Pharmaceutical companies are procuring materials from traders, who are getting these materials from untrained persons from rural and/or forest areas. This has given rise to wide-spread adulteration/substitution, leading to poor quality of herbal formulations.

Introduction: Among the globe humans prefer herbal medicines. The World Health Organization estimates that 80% of the world's population utilizes traditional medicines for healing and curing diseases Medicinal plants. There is an increasing international market for medicinal plants, which are used both for herbal medicine and for pharmaceutical products. Accurate and rapid authentication of medicinal plants is difficult to increasetheir marketing. Since the natural medicines are safer than synthetic drugs, they are preferred for phyto-pharmaceutical usage. Due to the popularity of herbal drugs globally, their adulteration/substantiation aspects are gaining importance at the commercial level. Pharmaceutical companies are procuring materials from traders, who are getting these materials from untrained persons from rural and/or forest areas. This has given rise to wide-spread adulteration/substitution, leading to poor quality of herbal formulations.

Misidentification of herbs may be non-intentional or intentional. Adulteration can occur due to ignorance or intentional substitution with cheaper plant material and may cause damage to humans. Therefore, authentication at various stages, from the harvesting of the plant material to the final product, is a need of the hour. The general approaches to herb identification are dependent on morphological chemical and molecular techniques. However, traditional taxonomic studies require expertise of experienced professional taxonomists. In the case where diagnostic morphological traits of the given specimen are lacking, it becomes difficult even for specialists to recognize a species correctly. Genetic analysis has a promising role in resolving disputes of taxonomic identities, relations and authentication of the species in question, as the genetic composition is unique for each species and is not affected by age, physiological conditions and environmental factors. Therefore, DNA-based methods have gained wide acceptance in quality control to authenticate crude materials.

Cassia angustifolia (family: Caesalpiniaceae) is widely used in Unani medicine and also has been adopted by the pharmacopoeias of the world It is found in abundance throughout south India and other parts of the country. It is a branching shrub with a height up to 1.8 m. The seeds are creamish to brown in color, obovate–oblong, dicotyledonous and medium size (3–4 × 1.5–2.0 × 4–5 mm) with weight 2.65 g per 100 seeds approximately. It has complex mixture of several active constituents such as dianthrone glycosides, free anthraquinones (aloe-emodin, chrysophanol and rhein) and anthraquinone glycosides*C. angustifolia* contributes significantly to commercial drugs and has been investigated for various therapeutic preparations in several parts of the world in various ways such as anti-mutagenic, anti-genotoxic and anti-fungal.

Other species such *Cassia sophera*, *Cassia acutifolia and Cassia tora* h ave medicinal value and are found in Indian subcontinent, and widely used as folk medicine for the treatment of numerous diseases. The phytochemicals of various species of *Cassia* are different and some are common to each other which depend on its genotype and environmental conditions. However, their identification based on these markers is difficult as they are influenced by environmental conditions. The search for and development of herbal medicines is rapidly increasing worldwide, therefore, practical and accurate authentication methods are needed to

maintain quality and efficacy of herbal formulations.

Random Amplified Polymorphic DNA (RAPD) is a simple and cost ineffective PCR based method as compared to other DNA based markers. Due to such property of this technique, it has been widely used for the differentiation of a large number of medicinal species from their close relatives or adulterants, including *Echinacea* species turmeric *Dendrobium officinale*, *Astragali radix* and *Typhonium* etc.

The *Cassia* species viz., *C. angustifolia*, *C. acutifolia* and *C. tora* are immensely used in Ayurvedic prescription and their accurate identification is urgent need in herbal industry to maintain the efficacy and quality of herbal formulations. Our major objective therefore, was to develop DNA based marker "RAPD" for accurate identification of *Cassia* species in the local markets.

Materials and Methods:

Plant Materials: The genuine sample of *C. angustifolia* was provided by Central Council for Research in Unani Medicine (CCRUM) Hyderabad. The samples for authentication were purchased from the local markets of Delhi, India. The material was identified at Acharya NG Ranga Agricultural University (ANGRAU), Hyderabad.

Chemicals: The stock solution concentration were CTAB 3% (w/v), 1 M Tris–HCl (pH 8), 0.5 M EDTA (pH 8), 5 M NaCl, absolute ethanol, chloroform-IAA (24:1 [v/v]), Polyvinylpyrrolidone(PVP) (40,000 mol. wt.) (Sigma) and β-mercaptoethanol. The extraction buffer consisted of CTAB 3% (w/v), 100 mMTris–HCl (pH 8), 25 mM EDTA (pH 8), and 2 M NaCl, respectively. PVP and β-mercaptoethanol were added freshly prepared in the extraction buffer at the time of genomic DNA extraction.

DNA Extraction: DNA was isolated from dried leaves using a modified CTAB method .The dried leaves were ground into 800 µl extraction buffer in pestle-mortar and taken into micro-tube. The suspension was gently mixed and incubated at 65 °C for 20 min with occasional mixing. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 12,000 rpm for 5 min. The clear upper aqueous phase was then transferred to a new tube and added 2/3 volume of ice-cooled isopropanol and incubated at –20 °C for 30 min. The nucleic acid was collected by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 80% ethanol. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE buffer (10 mMTris buffer pH 8 and 1 mM EDTA) at room temperature and stored at 4 °C until used. The RNA from crude DNA was eliminated by treating the sample with RNase A (10 mg/ml) for 30 min at 37 °C.. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

RAPD Analysis: RAPD reaction was performed according to the method developed by McClelland et al. (1995). PCR reaction for RAPD analysis consisted of 15 mM $MgCl_2$, 2.5 µl 10× buffer, 2 µl 2 mMdNTPs (mix), 0.5 U *Taq* polymerase in buffer, 25 ng/µl of each primer, 30 ng/µl plant DNA and sterile water up to 18 µl. For DNA amplification a Techne thermal cycler was programmed for 1 cycle of 3 min at 94 °C, 30 s at 36 °C, and 1 min at 72 °C followed by 45 cycles of 1 min at 94 °C, 30 s at 36 °C, and 1 min at 72 °C, then terminating with 5 min at 72 °C. The RAPD fragments were separated on 1.2% agarose gel by electrophoresis in 1× TAE buffer for 2 h 30 min at 60 V. The gel was stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light using a chemiluminescence Imaging System

Data Analysis: The RAPD bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Only prominent and reproducible bands obtained for each RAPD primer were considered. By comparing the banding patterns of species for a primer, species-specific bands were identified. Faint or unclear bands were not considered. A dendrogram was constructed using with arithmetic average (UPGMA) with the SAHN module of NTSYS-pc to show a phenetic representation of genetic relationships as revealed by the similarity coefficient.

Results: Three species of *Cassia* were chosen to test the reliability of quality control using RAPD technique. In the local market samples, the adulteration was found among these species. The dried leaves of these species are more or less similar in morphology to each other and easily adulterated (Fig.1). RAPD technique was carried out in replicates (three) using genomic DNA with 32 decamer primers for reproducibility of the results. The selected primers amplified DNA fragments across the 4 species studied, with the number of amplified fragments varied from 3

(OPC-3) to 10 (OPC-4, OPC-17, OPC-19, OPC-19 and OPC-20), and the amplicon size varied from 500 to 2500 bp. Out of 42 loci detected, the polymorphic, monomorphic and unique loci were 24, 2 and 16, respectively. All *Cassia* species were discriminated by the presence or absence of unique fragment in RAPD profile.

Figure1. Morphological slides of 3 *Senna* species: (a) *S. angustifolia*, (b) *S. acutifolia*, (c) *S. tora*,

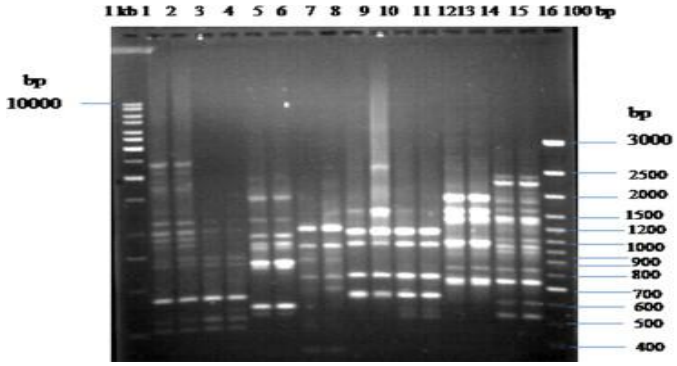

Figure 1. RAPD analysis carried out with primers: OPC-3 (lanes: 1–8), OPC-4 (lanes: 9–16). *C. angustifolia* (lanes:1,2,9,10), *S. acutifolia* (lanes:3,4, 11, 12), *S. tora* (lanes: 7, 8, 15, 16). 1 kb and 100 bpare DNA ladder.

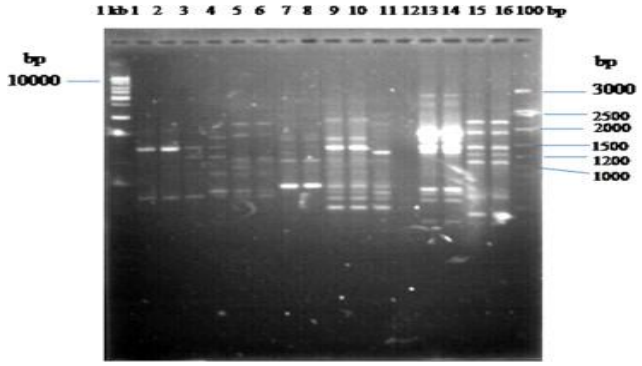

Figure 2. RAPD analysis carried out with primers: OPC-17 (lanes: 1–8), OPC-18 (lanes: 9–16). *S. angustifolia* (lanes:1,2,9,10), *S. acutifolia* (lanes: 3, 4, 11, 12), *S. tora* (lanes: 7, 8, 15, 16). 1 kb and 100 bpare DNA ladder.

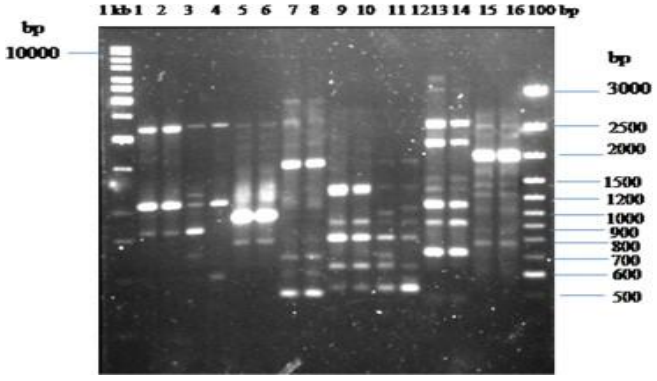

Figure 3. RAPD analysis carried out with primers: OPC-19 (lanes: 1–8), OPC-20 (lanes:916). *C. angustifolia* (lanes:1,2,9,10), *C. acutifolia* (lane;3,4,11,12), *S. tora* (lanes: 7, 8, 15, 16). 1 kb and 100 bpare DNA ladder.

Cluster Analysis: A dendrogram based on UPGMA analysis, the 3 *Senna* species clustered into two groups (group I and II), with Jaccard's similarity coefficient ranging from 0.1034 to 0.7273 (Fig. 5, Table 2). First group comprised*C. anustifolia* and *S. acutifolia* which had 72.73% similarity whereas second group comprised *C. tora* which had 23.68% similarity to each other.

Table 2. Genetic similarity matrix based on
RAPD data among 3 Cassia species estimated according to Jaccard's method.

Empty Cell	***C. angustifolia***	***C. acutifolia***	***C. tora***
C. angustifolia	1.0000		
C. acutifolia	0.7273	1.0000	
S. tora	0.1875	0.1034	1.0000

Discussion: According to WHO general guidelines for methodologies on research and evaluation of traditional medicines, first step is assuring quality, safety and efficacy of traditional medicines for correct identification. In the present article *C. angustifolia* was chosen for the identification through RAPD technique.

In the samples, *C. tora*, and *C. acutifolia* were found as adulterant when identified at NISCAIR, New Delhi. For accuracy of the results, the high quality and purity of genomic DNA free from secondary metabolites was isolated from these species by modified CTAB method. For RAPD reaction, it was necessary to standardize the following variables for successful amplification with PCR: RAPD amplification is not reproducible below a certain concentration of genomic DNA and produces 'smears' or results in poor resolution, if DNA concentration is high, series of dilutions were made to check good amplification. PCR trials were undertaken with different concentrations of $MgCl_2$ (0.5 mM, 1 mM and 1.5 mM) keeping all other parameters constant. $MgCl_2$ of 1.5 mM concentration was proved best in 25 µl reaction volume. Therefore the quantity was reduced to 0.5 units of *Taq* polymerase per 25 µl reaction volume, which gave better amplification. In all PCR trials the annealing temperature 36 °C has been used which was determined with gradient PCR. DNA denaturation is a critical step in DNA amplification reactions. For most DNA amplification reactions incubation time for DNA denaturation is 1 min at 94 °C.

In this investigation, 6 RAPD primers produced 24 polymorphic bands that unambiguously discriminatedCassia spsincluding *C. angustifolia*,*C. Acutifolia* and *C. Tora* respectively. This RAPD marker exhibited 57.14% polymorphism among these species with 6 decamer primers. Our results indicated the presence of wide genetic variability among different *Cassia* species. Variations in the DNA sequences lead to the polymorphism and greater polymorphism is indicative of greater genetic diversity. All four species of *Cassia* were differentiated from each other based on unique band obtained in PCR amplification. *C. angustifolia* had unique bands; 1, 1, 0, 0, 0 and 1 with primers OPC-3, 4, 17, 18, 19 and 20, respectively.

These bands were reproducible when PCR reaction was repeated thrice. Among the different primers utilized, OPC-3 and OPC-4 produced maximum number of polymorphic bands and may be used in future for the identification of these *Cassia* species. The cluster analysis clearly showed the genetic divergence among these species. These four species are clustered into two groups: First group had *C. angustifolia* and *C. acutifolia* which had high similarity (72.73%) to each other as compared to the second group which had *C. tora*. However, the *C. angustifolia* and *C. acutifolia* have morphological similarities in leaves to a great extent and in dried state it is very difficult to differentiate to each other, but in RAPD analysis both species showed more genetic divergence.

The advantages of this technique are its rapidity, simplicity and avoidance of any need such as genetic information about the plant prior to the commencement of the experiment. These characters are especially advantageous for the identification of any herbal drugs because of little DNA existing in the dried material. So, our study revealed that RAPD markers can be used for the identification of commercial *Cassia* species and could be a useful tool to supplement the distinctness, uniformity and stability analysis for

plant samples to maintain their identity for the protection in the future. Our study clearly indicated that RAPD markers could be used effectively to authenticate genuine as well as their respective adulterant samples sold in the local herbal markets.

References:

1. Acharya et al., 2005L. Acharya, A.K. Mukherjee, P.C. Panda, P. Das: Molecular characterization of five medicinally important species of *Typhonium* (Araceae) through random amplified polymorphic DNA (RAPD)
2. Genetic diversity and molecular authentication of wild populations of *Dendrobiumofficinale* by RAPDeCAM, 40 (2005), pp. 1028-1032.
3. S.C. Ferreira-Machado, M.P. Rodrigues, A.P.M. Nunes, F.J.S. Dantas, J.C.P. De Mattos, C.R. Silva, E.G. Moura, R.J.A.C. Bezerra , A. Caldeira-de-Araujo
4. Genotoxic potentiality of aqueous extract prepared from *Chrysobalanusicaco* L. leaves
5. Toxicol.Lett., 151 (2004), pp. 481-487 S. Irshad, J. Singh, P. Kakkar, S. Mehrotra
6. Molecular characterization of *Desmodium* species – an important ingredient of 'Dashmoola' by RAPD analysisFitoterapia, 80 (2009),pp. 115-118S.A. Jordan, D. G. Cunningham, R.J. Marles
7. Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion Afr. J. Biotech. 6 (2007),S. Khan, K.J. Mirza, M. Tayaab, M.Z. Abdin
8. RAPD profile for authentication of medicinal plant *Glycyrrhizaglabra* Linn MAPSB, 3 (1) (2009), pp. 48-51 S. Khan, K.J. Mirza, M.Z. Abdin
9. Development of RAPD markers for authentication of medicinal plant *CuscutaReflexa* EurAsian J.BioSci., 4 (1) (2010), pp. 1-7 S. Khan, K.J. Mirza, F. Anwar, M.Z. Abdin
10. Development of RAPD Markers for authentication of *Rutagraveolens* (L) and its adulterant MAPSB, 5 (1) (2011), pp. 58-61 U. Kiran, S. Khan, K.J. Mirza, M. Ram, M.Z. Ab din
11. PCR based detection of adulteration in the market samples of turmeric powder Food Biotech., 18 (2004), pp. 299-306
12. C.R. Silva, M.R. Monteiro, H.M. Rocha, A.F. Ri beiro, A. Caldeir.
13. Assessment of anti-mutagenic and genotoxic potential of senna (*Cassia angustifolia* Vahl.) aqueous extract using in vitro assaysToxicol. Vitro, 22 (1) (2008), P.H.A. Sneath, R.R. Sokal
14. Authentication of the family Polygonaceae in Chinese pharmacopoeia by DNA barcoding techniqueJ.Ethnopharmacol., 124 (2009), pp. 434-439W.L. Stern, M.W. Morris, W.S. Judd
15. Genome-based approaches to the authentication of medicinal plantsPlanta Med., 74 (2008), pp. 603-623 G.E. Trease, W.C. Evans
16. Identification of *Dendrobium* species by a candidate DNA barcode sequence: the chloroplast psb A-trnHintergenicregionPlanta Med., 75 (2009), pp. 667-669; Y. Zhang, P.P. But et al

***

V. E.Vijaya Sekhar
Lecturer in Botany A. C. College, Guntur, A.P.
Email: ezravsekhar @gmail.com
T. Sujatha
Lecturer in Chemistry, Sri Sarathi Institute of Engineering & Technology,
Nuzividu, Krishna Dist, A.P.