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Identification of medicinal plants using PCR-RFLP in *Dasamula* - an Ayurvedic drug

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Abstract

Dasamula, an herbal drug formulation consisting of roots of 10 plants, is used widely for the treatment of various diseases but no reliable procedure is available to identify the presence of different prescribed plant material in the drug. Adulteration is rampant in the process of drug preparation. Molecular taxonomy is a reliable and easier way to identify any dried and processed medicinal plants and its adulterants from medicine by using DNA markers. Six out of ten plants of *Dasamula* i.e. *Desmodium giganicum, Aegle marmelos, Solanum xanthocarpum, Solanum indicum, Tribulus terresteris, Oroxylum indicum* plants have been studied to establish their identification using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. Internal transcribed spacer (ITS) region of these plants were PCR amplified by using primers ITS1 (F) and ITS4(R). The size of ITS region varied from 650 bp to 850 bp. RFLP was carried out by restriction digestion of amplified ITS region using *BamHI, HindIII, MspI, MboI, EcoRI, EcoRV, HinfI, AluI*. RFLP showed species-specific variation in the number and size of digested DNA fragments. MspI and EcoRV proved to be the best enzymes as it could generate unique pattern for all the six plants. Variations in the ITS region among the species can be used for conclusive identification of these plant species.

Keywords: DNA molecular markers, ITS-RFLP, *Dasamula* herbal drug formulation, Restriction enzymes, Adulteration, Authentication.

INTRODUCTION

Herbal drug technology is widely used as an alternative medicine/traditional medicine where quality control and standardization by authentic scientific methods is important. One such ayurvedic medicine "*Dasamula*" consisting of roots of ten different plants is used for various ailments in the form of decoction, oil or powder in combination with plant parts. It is a favorite remedy in diarrhea and dysentery, obstinate fever & asthma in different forms.

The roots of ten plants constituting "*Dasamula*" (*Dasa* means "ten" and *Mula* means "roots") are namely *Aegle marmelos, Oroxylum indicum, Gemelina arborea, Alpinia galangal, Premna intrigrifolia, Desmodium giganiticum, Uraria picta, Solanum indicum, Solanum xanthocarpum* and *Tribulus terresteris*. Since roots of these plants form the active constituents of this herbal formulation, it is a general practice that roots are being dug out indiscriminately along with other plants and may get mixed leading to rampant adulteration in the process of preparation of drug.

In addition to this there is no authentic procedure available to identify the presence of different prescribed plant materials in the drug. Mostly the roots are purchased from the open market for preparation of the medicine. Thus, the quality of the product is always in question, since the adulteration may make the product unfit for human consumption.

Correct identification and quality assurance of the starting material is, therefore, an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy [1-3] and the root cause lies mainly with the starting material where the adulteration takes place. These drugs are frequently found to be adulterated with other drugs. Adulteration in herbal drugs may be intentional for monetary gains or unintentional due to lack of awareness and knowledge

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about the authentic plants, confusion in vernacular names between indigenous systems of medicine and local dialect, similarity in morphological and aromatic features of the different plant sources, non-availability of the authentic plants, careless collection and other unknown reasons [4]. Thereby this requires scientific methods for their authentication and standardization.

One of the most reliable and easy ways of authentic identification of the constituent medicinal plants in ayurvedic drugs is molecular marker based identification. Molecular Genetic Marker (MGM) is used to establish the species identification and also to co-relate with the chemical constituents of the plants. MGM may be defined as those derived from direct analysis from genetic polymorphism in the DNA sequences. MGMs are more accurate and preferred over morphological traits, biochemical and chemical markers as MGMs reveal extensive polymorphism at DNA level and are suitable for discriminating closely related genotypes [3]. DNA as molecular markers have several advantages over typical phenotypic markers and are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental factors [5]. DNA can be extracted from fresh or dried organic tissue [6] of the botanical material hence the physical form of the sample for assessment does not restrict detection. DNA-based techniques have been especially useful for authentication of those medicinal plant species, which are frequently substituted or adulterated with other species that are morphological and/or phytochemically indistinguishable [7-10]. A number of DNA-fingerprinting methods have been developed over past few years which include Random Amplification Polymorphic DNA (RAPD) [11], Amplified Fragment Length Polymorphism (AFLP) [12, 13] PCR-RFLP. RAPD technique has been used to determine the components of a Chinese herbal preparation, yu-ping-feng san in which the presence of three herbs have been detected using single RAPD primer [14]. RAPD and PCR-RFLP have been used for authentication of P. ginseng among ginseng populations [15]. Authentication of medicinal Dendrobium sps by ITS of rDNA has been successfully done [16]. DNA sequence analysis and PCR-RFLP were successfully applied to differentiate four medicinal Codonopsis species from related adulterants [17]. ITS-RFLP has also been used for authentication of *Fritillaria pallidiflora* [18], Atractylodes species [19] and others from their adulterants [20-22]. Internal Transcribed Spacers are sequences located in the eukaryotes between the 18S and 5.8S rRNA coding region (ITS1) and between the 5.8S and the 28S coding regions (ITS2). Studies of restriction site variation in the rDNA have shown that while coding regions are conserved, the spacer regions are variable [23] and hence can be used as markers to establish identity of morphologically allied species. The main objective of this study was to establish molecular markers for ten plants, of which six plants was taken for the present study, using RFLP of the Internal Transcribed Spacer (ITS) region, which can be used for authenticated identification and control on the adulteration.

MATERIALS AND METHODS

Material: The Six authenticated plants namely *Aegle marmelos, Oroxylum indicum, Desmodium giganiticum, Solanum indicum, Solanum xanthocarpum* and *Tribulus terresteris* collected from the nursery of department of Pharmacy, Panjab University were used as subject for the study.

DNA extraction: The leaves of all the subjects were cleaned and processed immediately for DNA extraction using modified CTAB method [24] or dried and preserved at -80○C for future use.

ITS Amplification: The ITS region comprising of ITS1-5.8S rRNA gene-ITS2 of isolated DNA from these plants was amplified using primer sets as ITS1F (5'-AAG TCG TAA CAA GGT TTC CGT AG-3') & ITSR (5'-TCC TCC GCT TAT TGA TAT GC-3') (GENEX, India.). Amplification reaction of 50µl volume containing 1x Taq polymerase assay buffer (75mM Tris HCl pH 8.8, 20mM (NH4)2SO4, 2.0 mM MgCl2 and 0.01% Tween-20), 0.2 mM of each dNTPs (MBI Fermentas), 0.5µM of each forward and reverse

primer, 1.25U of Taq polymerase (MBI Fermentas) and 100ng of DNA was performed using Eppendorf Master Cycler. The amplification was carried out using the following program: an initial denaturation at 95˚C for 5 min, then 30 cycles of denaturation at 95˚C for 30 sec, annealing at 52˚C for 30 sec, extension at 72˚C for 1 min and a final extension at 72 ˚C for 7 min.

Amplified products were resolved in 1.5% Agarose gel at 75 V, 1x TAE buffer for 1 hr. A 100 bp DNA ladder (Fermentas, MBI) served as standard molecular weight marker. The gel were stained with ethidium bromide (0.5µg/ml) and documented under gel documentation system (Ultra Lum gel imager, USA).

ITS- RFLP

The restriction enzyme digestion was carried out using 1 µg of amplified PCR product digested by 10 units of restriction enzyme for 16 hrs. The following enzymes were used: ApaI, AluI, Bam HI, EcoRI, EcoRV, HinfI, Hind III, MboI, MspI, and after digestion each enzyme was inactivated by incubation at the specific temperature as per the specifications of the manufacturer (Fermentas, MBI). The restriction fragments were size separated by electrophoresis on 2% Agarose gel at 60 V for 2hr. A 100 bp DNA ladder (Fermentas, MBI) served as standard molecular weight marker. The staining and documentation was same as in ITS amplification.

Table 1: ITS Restriction fragment length polymorphisms of the plants used in this study

	Fragment length(s) (bp) after restriction digestion with									
Plants	**ITS[a]**	**EcoRI**	**EcoRV**	**HinfI**	**MspI**	**HindIII**	**MboI**	**AluI**	**ApaI**	**BamHI**
Desmodium giganticum	750	750	450+300	350+280 +110*	520+140*	750	400+310*	650+50 +50	750	690+60
Aegle marmelos	730	730	440+290	400+350	680+50	730	730	650+50*	730	730
Solanum indicum	680	680	410+330*	350+290*	280+200* +6bands<50	550+ 120*	600+70*	680	480+ 100 +100	680
S. xanthocarpum	850	850	600+250	330+260 80+80+ 50+50	360+250 200+110	850	850	850	850	850
Tribulus terresteris	770	770	50+320	360+250 +160	380+280 +100	770	500+190 +80	770	690+ 80	770
Oroxylum indicum	780	780	450+320*	780	250+200 200+130	780	380+310 +50	780	700+ 80	780

a) ITS1-5.8S-ITSs amplified product size.

Note: * Restriction fragments less than 50 bp were not considered.

RESULTS AND DISCUSSION

High quality DNA was extracted from all six plants. The yield of the DNA varies from 50ng to 1µg. The ITS regions of six subjects were successfully amplified and ranged between 680 to 850 bp (Fig.1) (Table.1). The PCR products were digested with nine different restriction enzymes of which EcoR1 had no site in any of the six ITS products. Result of restriction digestion of PCR products with different enzymes are summarized in Table 1 and shown in Figure 2.

In the ITS region restriction digestion shows specific patterns for a species and any variation in the pattern within a species, can be detected as polymorphism.

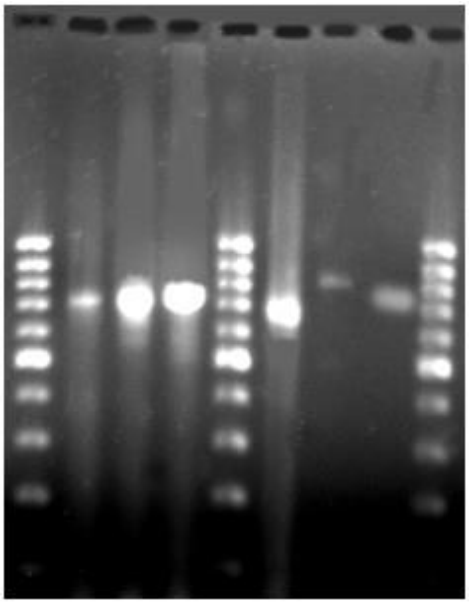

Figure 1: PCR-Amplified ITS product of six plants: Lane 2: *Aegle marmelos*, Lane 3: *Desmodium giganticum*, Lane 4: *Tribulus terrestris*, Lane 6: *Solanum indicum*, Lane 7: *Solanum xanthocarpum*, Lane 8: *Oroxylum indicum*, Lanes 1,5 9: 100bp marker.

The present study has shown that the restriction digestion of PCR amplified rDNA ITS region with nine different restriction endonucleases could establish markers for the six plants. BamHI and HindIII had restriction site only in the ITS of *Desmodium giganticum* and *Solanum indicum* respectively with different size fragments in each and hence could be used as markers for these two plants. The inability of HindIII and EcoRI to cut the ITS region of most of the plants may be attributed to the methylation [25], since HindIII will not cleave DNA if the cytosine residue in the recognition sequence AAGCTT is methylated. Alternatively it might not have site in this region. EcoRV and MspI produced restriction patterns in the ITS of all the six plants, however EcoRV produced similar fragments for *D. giganticum, T. terrestris* and *O. indicum* and is not suitable for generating markers for these three plants. Markers generated by MspI can be very well applied for all the six plants to determine their botanical origin and distinguish them from their allied species. HinfI generated patterns can be used for all the plants except *O. indicum* where it does not have any restriction site. MboI and ApaI produced restriction pattern for three plants each, all different from each other (Table1) and can be used for generating marker. AluI could not generate patterns of much utility to be used for identifying these plants. EcoRI could not establish any marker for the six plants as it probably did not have site in the ITS region.

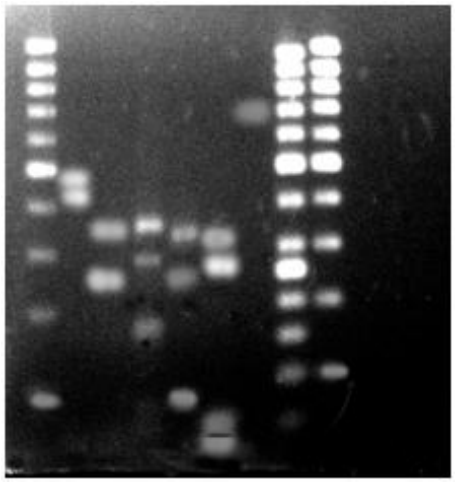

Figure 2: Restriction digestion of PCR-Amplified ITS product of six plants with HinfI: Lane 2: *Aegle marmelos*, Lane 3: *Solanum indicum*, Lane 4: *Tribulus terrestris*, Lane 5: *Desmodium giganticum*, Lane 6: *Solanum xanthocarpum*, Lane 7: *Oroxylum indicum*, Lanes 1, 9: 100bp marker, Lane 8: 50 bp marker.

Interestingly it was found that among the six plants studied ITS of *Solanum xanthocarpum* does not get cleaved by majority of the enzymes used, except EcoRV, HinfI and MspI despite having the largest ITS region (850bp). This shows that ITS region of *S. xanthocarpum* has the lowest susceptibility to restriction digestion of the endonucleases and therefore a combinational study of ITS along with other genes like rbcl and matK using other enzymes could reveal more information. However, the three enzymes used in our study could still be used sufficiently well as all of them generated very unique patterns in *S. xanthocarpum*. MspI proves to be the best enzyme to establish unique patterns for each of the six plants followed by EcoRV thereby establishing their identity.

ITS has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races) because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA). Variation among individual rDNA repeats can sometimes be observed within the ITS. ITS-RFLP based identification and authentication of medicinal herbs in traditional medicine like Panax species, *Fritillaria pallidiflora, Atractylodes* species was established by various workers [15, 17-19]. The ITS sequences of several medicinal species, including Panax ginseng (Renshen), *Panax quinquefolius* (Xiyangshen), and Codonopsis (Dangshen) species, have been patented and their distinct RFLP pattern was developed to distinguish them [26]. ITS-RFLP based botanical identification of allied species of Dendrobium in a Chinese drug "*Shihu*" has been reported [27].

Most of the work on ITS-RFLP has been carried out for the authentication of plants mainly in Chinese medicines and rarely for the Indian system of medicines especially Ayurveda. Our work is an attempt to identify the authenticated botanical origins of the herbs of an ayurvedic drug "*Dasamula*" constituting the roots of ten plants as to prevent adulteration with their allied species by developing molecular markers using ITS-RFLP which is a simple, reliable and quick procedure for their identification and less expensive than the sequencing and sequence based methods. As ayurvedic drugs are not free from adulteration it is important to have an authenticated molecular identification method along with the available phytochemical methods of identification. Once their identification is established they can be co-related with the quantity and quality of their active phytochemical components and a standard database can constructed. Our strategy involves identification of all the ten plants by establishing molecular markers and further comparing them with their allied species, and if necessary sequencing of the ITS of all the plants. This could be finally being applied at the industry level for quality control of the products.

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REFERENCES

1. De Smet, P.A.G.M., Keller. K., Hansel, R., Chandler, R.F., 1992**.** Adverse effects of herbal drugs. Vol. 1. Springer-Verlag, Heidelberg.
2. Strauss, S. E., 2002. Herbal medicines- What's in the Bottle. New Eng J Med 7: 1997-1998.
3. Joshi, K., Chavan, P., Warude, D., Patwardhan, B., 2004. Molecular markers in herbal drug technology. Current Science, 87 (2): 159-165.
4. Mitra, S.K., Kannan, R., 2000. A Note on Unintentional Adulterations in Ayurvedic Herbs.
5. Chan, K., 2003. Some aspects of toxic contaminants in herbal medicines. Chemosphere. 52:1361-1371.
6. Warude, D., Chavan, P., Kalpana, J., Patwardhan, B., 2003**.** DNA isolation from fresh and dry plant samples with highly acidic tissue extracts. Plant Molecular Biololgy Reports. 21: 1.
7. Cao, L., Li, S.X., Wei, B.Y., Huang, D., Xu, F., Tong, Z.Y., 2010. Optimizing RAPD reaction system and authentic genuineness related genetic background of *Fructus evodia*. Chinese Traditional Herb Drugs. 41(6):975-978.
8. Hussain, M.A., Bedi, Y.S., 2012. Authentication of Picrorhiza kurrooa Royle ex Benth. using DNA fingerprint. International Journal of AgriScience. 2(6):511-521.
9. Padiya, R. H., Patel, E.D., Acharya, Rabindrayan., 2013. Development of RAPD (Random Amplified Polymorphic DNA) Markers for Authentification of *Limnophila heterophylla* (Roxb.) Benth. International Journal of Pharmacy and Bioogical Archives. 4(1):70-74.
10. Suwanchaikasam, P., Phadungchareon, T., Sukhada, S., 2013. Authentication of the Thai medicinal plants sharing the same common name 'Rang Chuet': *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma aff.* amada by combined techniques of TLC,

PCR-RFLP fingerprints and antioxidant activities. Science Asia. 39:124-133.

11. Williams, J. G. K., Kubelic, A. R., Livak, K. J., Rafalski, J. A., Tingley, S.V., 1990. DNA polymorphism applied by arbitary primers are useful as genetic markers. Nucleic Acid Research. 18:6531-6535.
12. Vos, P., Hoger, R., Bleeker, M., Raijans, M., Lee, Van de T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique or DNA Fingerprinting Nucleic Acid Research. 19:86-866.
13. Schimdt, K., Jenson, K., 2000. Genetic structure and AFLP variation of remnant populations in the plant *Pedicularis palustris* (Scrophulariacea) and its relation to population size and reproductive components. American journal of Botany. 87:678-689.
14. Cheng, K. T., Tsav, H. S., Chen, C. F., Chou, T. W. 1998. Determination of the components in a Chinese prescription, yu-ping-feng san, by RAPD analysis. Planta Medica. 64(6): 563-565.
15. Um, J. Y., Chung, H. S., Kim, M. S., Na, H. J., Kwon, H. J., Kim, J. J., Lee, K. M., Lee, S. J., Lim, J. P., Do, K. R., Hwang, W. J., Lyu, Y. S., An, N. H., Kim, H. M., 2001. Molecular authentication of Panax ginseng species by RAPD analysis and PCR-RFLP. Biological and Pharmaceutical Bulletin. 24: 872-875.
16. Lau, D.T., Shaw, P.C., Wang, J., and But, P.P. (2001). Authentication of medicinal Dendrobium species by the internal transcribed spacer of ribosomal DNA, Planta Medica*,* **67**(5):456-60
17. Baum, B. R., Mechanda, S., Livesey, J. F., Binns, S.E., Arnoson, J.T., 2001. Predicting quantitative phytochemical markers in single Echinacea plants or clones from their DNA Fingerinting. Phytochemistry. 56: 543-549.
18. Wang, C. Z., Li, P., Ding, J. Y., Jin, G. Q., Yuan, C. S., 2005. Identification of *Fritillaria pallidiflora* using diagnostic PCR and PCR-RFLP based on nuclear ribosomal DNA internal transcribed spacer sequences. Planta Medica. 71:384-386.
19. Mizukami. H., Okabe, Y., Kohda, H., Hiraoka, N., 2000. Identification of the crude drug atractylodes rhizome (Byaku-jutsu) and atractylodes lancea rhizome (So-jutsu) using chloroplast *TrnK* sequence as a molecular marker. Biol Pharm Bull. 23:589-594.
20. Fernandez, A., Garcia, T., Gonzalez, I., Asensio, L., Rodriguez, M. A., Hernandez, P. E. Martin, R., 2002. Polymerase chain reaction-restriction fragment length polymorphism analysis of a 16S rDNA gene fragment for authentication of four clam species. Journal of Food Protein. 65: 692-695.
21. Kaundun, S. S., Matsumoto, S., 2003. Identification of processed Japanese green tea based on polymorphisms generated by STS-RFLP analysis. Journal of Agriculture and Food Chemistry. 51: 1765-1770.
22. Yang, D. Y., Fushimi, H., Cai, S. Q., Komatsu, K., 2004. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system (ARMS) analyses of medicinally used Rheum species and their application for identification of Rhei Rhizoma. Biological and Pharmaceutical Bulletin. 27: 661-669.
23. Gerbi, S. A., 1985. Evolution of ribosomal DNA in: MacIntyre, R. J., (Ed.) Molecular Evolutionary Genetics Plenum, New York, pp. 419-517.
24. Biswas, K., Biswas, R., 2011.A modified method to isolate genomic DNA from plants without liquid nitrogen, Current Science*,* 100(11):1622-1624*.*
25. Rafalski, J. A., Wiewiorowski, M., Scoll, D., 1983. Organisation of ribosomal DNA in yellow lupine (*Lupeus luteus)* and sequence of the 5S RNA gene. FEBS Letter. 152: 241-246.
26. Shaw, Pang-Chui., Wong, Ka-Lok., Chan, A. Wai-Kit., Wong, Wai-Cheong., But, P. Pui-Hay., 2001. Patent applications for using DNA technologies to authenticate medicinal herbal material. Chinese Medicine. 4:21-32.
27. Zhang, T., Xu, L. S., Wang, Z. T., Zhou, K. Y., Zhang, N., Shi, Y. F., 2005. Molecular identification of medicinal plants: *Dendrobium chrysanthum, Dendrobium fimbriatum* and their morphologically allied species by PCR-RFLP analyses. Yao Xue Xue Bao. 40(8): 728-733.