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Review Article

Molecular characterization of medicinal and aromatic plants by 5S rRNA NTS and PCR RFLP- A mini review

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The use of plants and plant products for medicine is being practiced from time immemorial. The advent of herbal drug technology has led to the production of medicines and pharmaceutical products from herbs. Secondary metabolites from plants are important economically as drugs, fragrances, pigments, food additives and pesticides. In order to protect consumers from adulteration and for conservation of the plants; authentication and identification of medicinal plants is essential. Traditionally, morphological, phytochemical and analytical techniques are used. In recent times molecular marker based methods are used widely. With the advent of PCR based techniques minute quantities of DNA in order of nanograms are sufficient for molecular analysis. These techniques are more advantageous than the classical morphology based or chemical and analytical methods. Recently the non-transcribed sequence (NTS) region of 5S rRNA has been employed for studying intra specific discrimination. In this short review an attempt has been made to study the use of 5S -rRNA NTS region for molecular fingerprinting in plants.

Identification, detection and quantification of herbal and aromatic plants is generally based on environmental factors such as morphological, chemical, anatomical and developmental factors or by methods of sample storage [Cai *et al*, 1999]. According to environmental conditions, the same genotype may express different chemical patterns or vice-versa [Rubiolo *et al,* 2009]. Chemotypes are referred to as phenotypical expression of a genotype, even though same genotype might give rise to different phenotypes. When plant mixtures are present it is very difficult to identify a particular plant. Plant identification is usually achieved by quantification of active compounds, when toxic or carcinogenic plants are present in powder form. This requires gas chromate-graphic (GC) – or liquid chromatographic (LC)–mass spectra determinations which takes up time and is very cumbersome, whereas DNA based molecular methods is relatively fast and quantification is easier and these molecular methods are effective in genotypic discrimination [Luciano *et al,* 2007; Rubiolo *et al,* 2007]. Since genetic method requires genotype instead of phenotype, DNA-based experiments have become widely employed techniques for rapid identification of herbal medicines. Using PCR approaches, nanogram quantities of DNA are required to amplify and yield sufficient amounts of template DNA for molecular genetic analysis [Bertea *et al*, 2005]

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Recently, 5S rRNA gene spacer region has been used for the phylogenetic relationships of some higher plants. The 5S-rRNA region is present in all ribosomes, except in the mitochondria of few species [Brown *et al*, 1997]. 5S-rRNA undergoes transcription from hundreds to thousands of genes in all eukaryotes. These sequences are tandem repeats of alternative arrays of sequences coding 5S-rRNA and non-transcribed spacers (NTSs) located separately from the 18S-26S rRNA gene clusters [Park *et al*, 2000]. The two gene clusters can be localized on the same chromosome in a linked state [Scoles *et al,* 1988] or on different chromosomes independently in the genome. [Lee *et al*, 1999; Park *et al,* 2000]. The NTS region shows a high variation in base composition and length which has been speculated as it is not under the same selection procedure as the 120bp 5S- rRNA gene. The specific function of 5S-rRNA as a component of the large ribosomal subunit in eukaryotes may be attributed due to its high level of conservation. This 5S-rRNA can be considered as a good model for organization and evolution [Cox *et al*, 1992]. On the basis of these analysis and assumptions, variation in the NTS region has been used in a number of plant species for studying intraspecific variation, mapping 5S-rDNA arrays, genome evolution and phylogenetic reconstruction [Negi *et al,*2002; Baker *et al*,2000 and Trontin *et al,*1999] .

RFLP and PCR amplification

Polymerase chain reaction (PCR), due to its property of amplifying the gene and generating large amount of required DNA is widely used in molecular biology and marker based applications [Bachmann *et al*, 1994]. The property of PCR to perform gene specific amplification has had an effect on research similar to that of the discovery of restriction enzymes and Southern hybridization.

Molecular marker technology based on DNA polymorphism has shown rapid development and advancement in field of molecular genetics [Semagan *et al*, 2006]. The ability of DNA markers to identify species even when morphological identification is not possible proves to be extremely advantageous. DNA polymorphisms can occur anywhere in the genome, including coding and non-coding, single-copy or repetitive DNA. Restriction digestion can also be used to detect sequence polymorphisms of PCR amplified products [Harada *et al*, 1994]. The digested or amplified DNA products are run on an agarose / acrylamide gel. The bands on gels are visualized under UV after staining with ethidium bromide / silver staining. These bands serve as DNA markers [Bachmann *et al*, 1994].

Molecular fingerprinting of medicinal and aromatic plants by 5S-rRNA gene

5S-rRNA-NTS gene RFLP analysis has given successful results from several medicinal and aromatic plants. Successful comparison of NTS region has been obtained at both inter-specific and intra-specific levels. Few examples from gymnosperms and angiosperms are considered below.

In certain essential oil producing plants such as *Picea glauca* and *Pseudotsuga menziesii,* the NTS region shows variation not only in size (from 101 bp in *P. glauca* to 880 bp in *P.menziesii*) but also in the number of different size classes [Liu *et al*, 2003]. In the conifer *Pinus radiata*, sequencing of the gene gave a repeat unit of 524 bp after cloning and characterization [Gorman *et al*, 1992]. A molecular analysis of 5S-rDNA of white spruce, *P. glauca*, revealed the presence of two classes of repeating units, one of 221 bp, corresponding to the PCR amplification product, and another of approximately 600 bp [Brown *et al*, 1997]. Eucalyptus from family *Myrtaceae* is cultivated world over for several of its medicinal, aromatic and

biological properties. Australia is unique in having a single genus of this plant [Batish *et al*, 2008; Boland *et al*, 1993]. In two *Angophora* species and nineteen Eucalyptus species; the variability of the non-coding intergenic region was due to a 50 bp repeating element, which has undergone duplication and modification in certain taxa. On the basis of this it was identified that species of Eucalyptus were more similar to that of *Angophora* than to non-blood-wood species of Eucalyptus [Udovicic *et al*, 1995].

In Douglas fir *(Pseudotsuga spp*.), a medicinal plant having antifungal activity plants known to produce an essential oil characterized by monoterpenes [Tesevic *et al,* 1998]. Sequencing and Southern hybridization of the 5S-rRNA gene revealed repeat units of 888 and 871 bp in length, the latter with a 17 bp deletion in the NTS. At the 5'end of the coding region, immediately upstream a 35bp region of NTS showed high similarity to other conifers but not to other published plant 5S-rDNA sequences [Amarasinghe *et al*, 1998].

In the silver fir (*Abies alba Mill*.), another essential oil producing plant, characterized by terpenoids [Duquesnoy *et al*, 2007], DNA sequences of around 550 and 700bp were detected after PCR amplification and Southern blotting using a hybridization probe. It resulted in the amplification of a 75bp sequence of short unit class in the middle of spacer region which was revealed by sequence analysis of the spacers [Besendorfer *et al*, 2005]. In *L. decidua* and *L. kaempferi* of the genus *Larix,* using homologous probe hybridization and selective amplification of gene and spacer region; sequence analysis revealed divergent size unit classes of around 650 and 870 bp [Timeryanov *et al,* 1994; Trontin *et al*, 1999].

Analysis of the 700 bp 5S-rRNA gene spacer region of *Acorus graminues* and three types of *Acorus calamus* revealed the variation in the presence of β-asarone and led to classification of two chemotypes of *Acorus calamus*. In Chemotype A β-asarone is a major constituent and chemotype B is characterized mainly by sesquiterpenoids [Sugimoto *et al*, 1999]. 5S–rRNA gene repeats from the family *Solanaceae,* tobacco (*Nicotiana rustica*) and tomato (*Lycopersicon esculentum*), were isolated and sequenced. Differences were observed in *Lycopersicon* species [Smith *et al*, 1996]. In tobacco two gene repeats were observed for 5S rRNA. One was 430bp long which is more abundantly formed and the other is 521bp long. In tomato it was found out to be 355bp. Due to unequal crossover, non-significant, repeating sequence elements that are non-conserved have been observed [Venkateshwarlu *et al,* 1991]. Capsicum, another important plant from the family *Solanaceae*; after sequence analysis, revealed that 5S- rRNA gene varied in size from 278-300bp and the coding region was highly conserved. Further studies revealed that while *C. chinense, C. frutescens and C. annuum* formed one lineage*, C. baccatum* was revealed to be a species intermediate between the former three species and *C.pubescens*. [Park *et al,* 2000].

The *Allium* family has over 500 members. The 5S-rRNA gene revealed an efficient probe sequence with a 320 bp NTS, flanking partial 5S coding sequences in *Allium fistulosum* [Lee *et al*, 1999]. *A. cepa* and *A. schoenoprasum* possess 5S rRNA genes of different sizes. But they possess a common 75bp sequence [Shibata *et al*, 2002].

The plants belonging to the *Linum* genome are known for high quantity of essential fatty acids which are used for medicinal purposes [Krist *et al*, 2003]. Five distinct groups of the genome were considered in which the intra specific variations were studied and groups 3-5 were highly divergent. Intraspecific sequence variations among five distinct groups of 5S-rRNA genes indicate that 5S-ribosomal genes most closely resemble other angiosperm 5S

genes, while groups 3–5 are highly divergent. Sequence variation was higher in the spacer region than in the transcribed region for all pairwise comparisons. [Schneeberger *et al*, 1992]

Sequences of 5S-rRNA gene spacer were used to identify *Epimedium brevicornu, E. sagittatum, E. wushanense, E. pubescens* and *E. koreanum.* These species are listed as source plants of Chinese medicine 'Ying Yang Huo' in the Chinese Pharmacopoeia. In the *Epimedium* species the neighbor – joining method was used in the sequence analysis. In the 5S-rRNA gene spacer of *E. pubescens, E. wushanense* and *E. brevicornu* a position specific nucleotide was found. The 5S-rRNA gene spacer of *E. koreanum* showed a 19bp deletion and it was most divergent from the other four endemic Chinese species of *Epimedium* [Akhlaghi *et al*, 2007].

The genus *Brassica* is known for its medicinal properties. In *Brassica campestris* the 5S-rDNA repeat unit is 495 bp in length and consists of a highly conserved 119 bp coding region and a variable non-coding spacer region. In *Brassica nigra,* at the 5S-rRNA level the homology to other representatives of the same family was 97–100%. In the *Eruca sativa* genome two families of the 5S-rRNA co-exist. The 0.5 kb-size family consists of the 5S-rRNA genes (S4) that have coding regions similar to those of other reported plant 5S-rDNA sequences, whereas the 1 kb-size family consists of 5S-rRNA gene variants (S1) that exist as 1 kb *BamHI* tandem repeats [Singh *et al*, 1994].

Summary of study on few plants where PCR-RFLP technique was used.

The 5S-rRNA spacer region of both diploid (β-asarone-free) and triploid (β-asarone rich) *Acorus calamus* were amplified by PCR, by designing primers for the NTS region. The amplified products were set up for restriction digestion using *EcoR1*. The restriction digestion profile showed to be different for both the cyto-types. Further phyto-chemical analysis and cytotype studies were performed to confirm the results [Bertea *et al,* 2006].

In the *Asteraceae* family 5S-rRNA spacers helped in correct identification of wild *Saussurea lappa* which is a highly endangered plant [Werkhoff *et al*, 1991]. After sequencing it was found that similarity existed between ITS-1, ITS-2 and 5S-rRNA intergenic spacers among *S. lappa* and related species. But the intraspecific variation was quiet low. It was also found out that there are few unique changes in the sequences which can be used as differentiation markers [Chen *et al*, 2004]. In the genus *Fritillaria* there are around 25 species and varieties which carry the name *Beimu* in commercial markets. After DNA extraction, PCR amplification was done by designing primers to the conserved coding region. By comparing the sequences, it was found that diversity was found in the spacer region. Rapid identification was done later by using *EcoRI* [Cai *et al,* 1999].

A similar technique was used for the identification of thujone free chemotypes of *Artemisia umbelliformis*. After the identification of thujone free A. *umbelliformis* by horticultural techniques, the sequence of both the 5S-rRNA genes were studied and gene specific primers were designed for *A.umbelliformis.* By PCR-RFLP method using *RsaI* and *TaqI* restriction enzymes, the two chemotypes were clearly distinguished [Rubiolo *et al*, 2007]. In the *Salvia* species DNA fingerprinting of the 5S-rRNA-NTS was used for the precise identification of *S. divinorum*. After comparing the sequence diversity of the spacer regions, it was found to be greater in *S.divinorum* than in *S.officinalis.* After designing specific primers, PCR-RFLP method was performed using *NdeI* and *TaqI*. An *NdeI* site absent in *S.officinalis* was found in *S.divinorum* NTS region at 428-433bp. For *TaqI*, multiple sites (161–164, 170–173 and 217–220 bp) were found in *S.*

officinalis, whereas a unique site was found in *S. divinorum* (235–238 bp) [Bertea *et al*, 2006].

A similar approach was employed for the identification of *Astragalus* species. *Astragalus* genus which is mainly found in China has around 300 species and varieties and thus making the identification of the plant difficult. After amplification, sequencing and comparison of the 5S-rRNA of around 300 bp restriction mapping showed diversity in the spacer region leading to the genetic identification of *Huangqi* [Ma *et al*, 2000].

Merits and Demerits

This mini review gives brief information regarding the PCR-RFLP of the 5S-rRNA NTS method and its application which has been applied to several taxa belonging to different families and orders. It describes how molecular fingerprinting can be rapidly used for differentiating chemotypes and genotypes. The major advantages of using this method being overcoming taxonomic un-certainties and also in determining the quality/origin of the plants [Gnavi *et al*,2009].

It can also help in forensic investigations and detect adulteration in plant mixtures and subsequent identification of the plant. Because the 5S-rRNA genes are highly conserved, their sequence analysis is used for inferring phylogenetic relationships among deep branches of eukaryotes. The spacer region has a faster rate of divergence in comparison to highly conserved coding region. This proves to be very informative for the phylogenetic study even at inter-specific and inter-generic levels. Generally phytochemical analysis methods such as GC-MS or LC-MS are used for taxonomic and chemical identification but PCR- RFLP and other molecular methods are more accurate and less time consuming when compared to these [Besendorfer *et al*, 2005]

Environmental conditions such as biotic and abiotic stresses and also phenotypic plasticity make taxonomic distinction and identification of plants difficult. This in turn affects gene expression and also formation of product or molecule. In laboratories where phytochemical studies are done, DNA based techniques are not that widely used as these techniques require prerequisites such as designing of specific primers and sequencing. DNA based studies has been limited only to academia as the process and its marker based studies on a specific species involves lots of inter related studies and is quiet expensive. Isolating good quality DNA, which can be used for analysis of processed botanicals, is difficult. The major drawback is that DNA fingerprinting does not reveal the chemical constituents or active principles. Hence, DNA analysis and phytochemical analysis. (e.g: GC–MS, HPLC, LC–MS, etc) are to be used hand-in-hand rather than in isolation.

When considering chemotypes for DNA fingerprinting verification of the samples is necessary, as due to meiotic rearrangements DNA markers will lose their value and their stability needs to be checked. Currently, some controversy exists over the use of DNA barcoding, [Holmes *et al*, 2004] mainly due to the perception that this method would decrease the value of traditional morphology-based taxonomy. However more and more plant DNAs are being sequenced. Species-level discrimination with more than one locus and the need to look for algorithms for barcoding from more than two DNA regions has been stressed by Kress and co-workers [Kress *et al*, 2005].

Conclusion

Progress in the field of plant genetics has led to commercial application of molecular marker techniques. Chemical complexity and variable sources are disadvantageous to chromatographic

techniques. DNA based marker studies have been applied to food crops and horticultural plants and also for authentication of medicinal plants. This review paper has described briefly one of most recent and useful technique for identification and authentication of aromatic plants with the help of 5Sr RNA spacer region. PCR-RFLP and the use of specific markers designed with specific primers for the spacer region of 5S r RNA has proved to be very useful. This tool needs further research for wider application and characterization of medicinal and aromatic plants.

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