# Molecular markers in herbal drug technology

**Kalpana Joshi*, Preeti Chavan, Dnyaneshwar Warude and Bhushan Patwardhan**

Bioprospecting Laboratory, Interdisciplinary School of Health Sciences, University of Pune, Pune 411 007, India

**Herbal drug technology is used for converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. The use of chromatographic techniques and marker compounds to standardize botanical preparations has limitations because of their variable sources and chemical complexity. DNA-based molecular markers have utility in the fields like taxonomy, physiology, embryology, genetics, etc. DNA-based techniques have been widely used for authentication of plant species of medicinal importance. Pharmacognosy mainly addresses quality-related issues using routine botanical and organoleptic parameters of crude drugs, and chemoprofiling-assisted characterization with chromatographic and spectroscopic techniques. The new pharmacognosy includes all the aspects of drug development and discovery, where biotechnology-driven applications play an important role. Current focus on chemotype-driven fingerprinting and related techniques requires integration with genotype-driven molecular techniques so that an optimal characterization of botanical materials is possible. This review provides a brief account of various DNA-based technologies that are useful in genotyping and quick identification of botanicals with suitable examples.**

## Current trends in herbal medicine

USE of indigenous drugs from plant origin forms a major part of complementary and alternative medicine/traditional medicine (CAM/TM). The world market for herbal medicine, including herbal products and raw materials has been estimated to have an annual growth rate between 5 and 15%. Total global herbal drug market is estimated as US $62 billion and is expected to grow[1] to US $5 trillion by the year 2050. India has a great wealth of traditional knowledge and wisdom. Ayurveda contributes Rs 3500 crores (US $813 million) annually to the internal market. The Indian medicinal plants-based industry is growing at the rate of 7–15% annually. The value of medicinal plants-related trade in India is estimated at Rs 5000 crores per annum. Global trend leading to increased demands of medicinal plants for pharmaceuticals, phytochemicals, nutraceuticals, cosmetics and other products is an opportunity sector for Indian trade and commerce[2]. Scientifically validated and technologically standardized herbal medicines may be derived using a safe path of reverse pharmacology approach based on traditional knowledge database[3]. This may play a vital role in drug discovery, development and therapeutics, in addition to dealing with a typical Western bias against ayurveda[4]. Herbal drug technology includes all the steps that are involved in converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge will remain important. Herbal medicinal products may vary in composition and properties, unlike conventional pharmaceutical products, which are usually prepared from synthetic, chemically pure materials by means of reproducible manufacturing techniques and procedures. 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[5,6].

## Methods of identification, limitations and emerging techniques

Most of the regulatory guidelines and pharmacopoeias suggest macroscopic and microscopic evaluation and chemical profiling of the botanical materials for quality control and standardization[7–9]. Macroscopic identity of botanical materials is based on parameters like shape, size, colour, texture, surface characteristics, fracture characteristics, odour, taste and such organoleptic properties that are compared to a standard reference material. Microscopy involves comparative microscopic inspection of broken as well as powdered, crude, botanical materials. However, these parameters are judged subjectively and substitutes or adulterants may closely resemble the genuine material. Chemical profiling establishes a characteristic chemical pattern for a plant material, its fractions or extracts. Thin-layer chromatography (TLC) and high performance thin-layer chromatography (HPTLC) are routinely used as valuable tools for qualitative determination of small amounts of impurities. In addition, many analytical techniques such as volumetric analysis, gravimetric determinations, gas chromatography, column chromatography, high performance liquid chromatography and spectrophotometric methods are also frequently used for quality control and standardization[9].

*For correspondence. (e-mail: joshikalpana@hotmail.com)

The use of chromatographic techniques and marker compounds to standardize botanical preparations has limitations because of their variable sources and chemical complexity. Variability in the flavours, aroma and physical characteristics of wine and coffee from year to year and region to region, provide a good analogy. Many factors may affect the ultimate chemical profile of any herb. Intrinsic factors such as genetics and extrinsic factors such as cultivation, harvesting, drying and storage conditions are a few examples[10]. Routine chemotaxonomic studies provide only a qualitative account of secondary metabolites. For quantitative studies, use of specific markers that can be easily analysed to distinguish between varieties, remains a preferred option. Such metabolites being used as markers may or may not be therapeutically active, but should ideally be neutral to environmental effects and management practices[11].

In order to ensure efficacy, selection of the correct chemotype of the plant is necessary. Even when there are many known chemotypes of a plant species, selection of the right chemotype to which clinical effects are attributed is difficult. For example, *Withania somnifera* is reported to have three chemotypes depending upon the presence of a class of closely related steroidal lactones like withanolides, withaferin A, etc. The content of withanolides, withaferin A and other biologically active compounds may vary depending upon the environment, genotype, time of collection of plant material, etc. Hence selection of the right chemotype having therapeutic efficacy is important[12].

Another difficulty encountered in the selection of the correct plant material is to establish the identity of certain species that may be known by different binomial botanical names in different regions. For example, Shankhapushpi, which is an important 'medhya rasayan' in ayurveda is equated with any one of the following plants depending upon the region in India: *Canscora decussata*, *Evolvulus alsinoides* and *Clitoria ternata*[13]. Certain rare and expensive medicinal plant species are often adulterated or substituted by morphologically similar, easily available or less expensive species. For example, *Swertia chirata* is frequently adulterated or substituted by the cheaper *Andrographis paniculata*[14].

In view of these limitations there is need for a new approach that can complement or, in certain situations, serve as an alternative. Some of the newly emerging techniques for ensuring correct botanical identity and quality include Herboprint™, which in addition to chemoprofile also considers ayurvedic properties[15], and capillary electrophoresis which is a faster, precise and sensitive method and has recently been used to ascertain the botanical identity and quality of *Ephedrae herba*[16], *Coptidis rhizoma*[17], *Ginseng radix*[18] and *Paeoniae radix*[19].

Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites and other macromolecules such as nucleic acids. Secondary metabolites as markers have been extensively used in quality control and standardization of botanical drugs. Here we focus only on DNA markers, which may have several advantages over typical phenotype markers. DNA markers 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[20]. DNA can be extracted from fresh or dried[21,22] organic tissue of the botanical material; hence the physical form of the sample for assessment does not restrict detection. Various DNA-based methods for species characterization and adulteration detection in medicinal plants; agricultural crops and genetically modified (GM) foods have been published.

## Types of DNA markers used in plant genome analysis

Various types of DNA-based molecular techniques[11,23,24] are utilized to evaluate DNA polymorphism. These are hybridization-based methods, polymerase chain reaction (PCR)-based methods and sequencing-based methods.

### *Hybridization-based methods*

Hybridization-based methods include restriction fragment length polymorphism (RFLP)[24] and variable number tandem repeats[25]. Labelled probes such as random genomic clones, cDNA clones, probes for microsatellite[26] and minisatellite[27] sequences are hybridized to filters containing DNA, which has been digested with restriction enzymes. Polymorphisms are detected by presence or absence of bands upon hybridization.

### *PCR-based methods*

PCR-based markers involve *in vitro* amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide primers and the thermostable DNA polymerase enzyme. PCR-based techniques where random primers are used, include random amplified polymorphic DNA (RAPD)[28,29], arbitrarily primed PCR (AP–PCR)[30] and DNA amplification fingerprinting (DAF)[31,32]. Inter simple sequence repeats (ISSRs)[33] polymorphism is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type. Polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats. A recent approach known as amplified fragment length polymorphism (AFLP)[34,35] is a technique that is based on the detection of genomic restriction fragments by PCR amplification.

Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor-homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity[36].

### *Sequencing-based markers*

DNA sequencing can also be used as a definitive means for identifying species. Variations due to transversion, insertion or deletion can be assessed directly and information on a defined locus can be obtained. Genetic variation occurs extensively at the single nucleotide level. Direct sequencing can efficiently identify such single nucleotide polymorphisms that usually depend on how closely related are the organisms being compared. Other sequencing-based strategies include analysis of the variable internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). The ITS region of 18s–26s rDNA has proved to be a useful sequence for phylogenetic studies in many angiosperm families. The level of ITS sequence variation suitable for phylogenetic analysis is found at various taxonomic levels within families, depending on the linkage. A number of researchers have also sequenced other regions of DNA such as trnK of chloroplast and spacer region of 5s rDNA as diagnostic tools for authentication purpose.

## Applications of molecular markers in herbal drug technology

DNA-based molecular markers have proved their utility in fields like taxonomy, physiology, embryology, genetics, etc. As the science of plant genetics progressed, researchers have tried to explore these molecular marker techniques for their applications in commercially important plants such as food crops, horticultural plants, etc. and recently in pharmacognostic characterization of herbal medicine.

### *Genetic variation/genotyping*

It has been well documented that geographical conditions affect the active constituents of the medicinal plant and hence their activity profiles[37]. Many researchers have studied geographical variation at the genetic level. Estimates of genetic diversity are also important in designing crop improvement programmes for management of germplasm and evolving conservation strategies. RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana*[38], neem[39], *Juniperus communis* L.[40], *Codonopsis pilosula*[41], *Allium schoenoprasum* L.[42], *Andrographis paniculata*[43] collected from different geographical regions. Similarly, different accessions of *Cannabis sativa*[44] have been discriminated using ISSR markers and those of *Arabidopsis thaliana* L. Heynh.[45] have been differentiated using cleaved amplified polymorphic sequence and ISSR markers. Inter- and intra-species variation has also been studied using DNA-based molecular markers. Interspecies variation has been studied using RFLP and RAPD in different genera such as *Glycerrhiza*[46], *Echinacea*[47], *Curcuma*[48] and *Arabidopsis*[49]. RAPD and RFLP have also been applied for characterization of *Epimedium*[50] species at the genetic level. Members of three different species of *Scutellaria*[51], Chinese medicinal plants and three subspecies of *Melissa officinalis*[52] have been discriminated using RAPD. Varietal characterization of Kenaf (*Hibiscus cannabinus* L.)[53] has been done with the help of agronomical and RAPD data. Varietal identification and genetic purity test in pepper and *Capsicum annuum* were carried out using RAPD markers[54]. RFLP technique was used for interspecific genetic variation within the genus *Capsicum* and also for DNA fingerprinting of pepper cultivars[55]. RAPD has served as a tool for the detection of variability in Jojoba (*Simmondsia chinensis* L. Schneider)[56], *Vitis vinifera* L.[57] and tea (*Camellia sinesis*)[58]. An attempt has been made to understand population structure of *Podophyllum peltatum* to establish commercial level propagation of useful secondary metabolites using molecular markers[59]. Also, high genetic diversity has been shown in *Podophyllum hexandrum* species from Himachal Pradesh, India[60]. Genetic variation and relationships among and within *Withania* species[61], and genetic relationships among papaya and its wild relatives (*Caricaceae*)[62] have been revealed using AFLP markers. Genetic variation within *Brassica campestris* cultivars has been studied using AFLP and RAPD markers[63].

Phylogenetic relationship has been studied among citrus and its relatives using SSR markers[64]. RAPD has been used to construct genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla*[65]. RAPD markers have been developed for genetic mapping of Pacific yew (*Taxus bravifolia* Nutt.)[66]. An attempt has been made to develop a physical AFLP map of the complex *Arabidopsis* genome by combining gel-based AFLP analysis with *in silico* restriction fragment analysis using the published genome sequence[67].

### *Authentication of medicinal plants*

DNA-based techniques have been widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable.

Dried fruit samples of *Lycium barbarum* were differentiated from its related species using RAPD markers[68]. The

RAPD technique has also been used for determining the components of a Chinese herbal prescription, yu-ping-feng san. In this study the presence of three herbs (*Astragalus membanaceus* (Fisch.) Bge., *Ledebouriella seseloides* Wolff and *Atractylodes macrocephala* Koidz) in the formulation have been detected using a single RAPD primer[69].

Three RAPD primers have been identified that could successfully discriminate between three species of *Atractylodes*, from Chinese formulation purchased from local markets[70]. In another study, three random primers were used to reveal the genetic variability of *Astragalus* medicine materials sold in Taiwan market. SSCP analysis was also conducted on PCR products from the ITS-1 region of rDNA in order to differentiate the two *Astragalus* species[71]. Primers have been designed for hybridization with the hypervariable ends of microsatellite loci that could reveal DNA-polymorphism among five *Eucalyptus* species[72]. DAF has been used to identify the Chinese traditional medicine, *Magnoliae officinalis*, its counterfeits and substitutes[73]. An RAPD primer that is selective for an elite strain Aizu K-111 of *Panax ginseng*, including its cultured tissues has been identified[74]. RAPD and PCR–RFLP analysis have been used for authentication of *P. ginseng* among ginseng populations[75]. Some researchers have used a new approach called Direct Amplification of Length Polymorphism (DALP) for authentication of *Panax ginseng* and *Panax quinquefolius*[76]. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of rDNA has been done successfully[77]. A DNA microarray for detecting processed medicinal *Dendrobium* species (*Herba dendrobii*) was constructed by incorporating the ITS1-5.8s- ITS2 sequences of *Dendrobium* species on a glass slide. The established microarray could detect the presence of *D. nobile* in a Chinese medicinal formulation containing nine herbal components[78]. Molecular authentication of *Atractylodes*-derived crude drugs (Jutsu) was done with the help of PCR–RFLP and direct sequencing of chloroplast trnK. Two regions (Region1 and Region2) inside the chloroplast trnK were selected as molecular markers for identification and discrimination of *Atractylodes* rhizome (Byaku-jutsu) and *Atractylodes* Lancea rhizome (So-jutsu). Based on polymorphism in the restiction site for *Hinf*1 in Region1 fragment (260 bp), it was possible to discriminate between the two species. By direct sequencing of Region 2 (436 bp) and comparison of the nucleotide sequence datasets, we could not only discriminate Byaku-jutsu and So-jutsu, but also identify the original plant species of each crude drug specimen[79].

### *Detection of adulteration/substitution*

RAPD technique was adopted to identify eight types of dried *Coptis* rhizomes and one type of *Picrorrhiza* rhizome, a substitute for the former in the Chinese herbal market[80]. *P. ginseng* is often substituted by *P. quinquefolius* (American ginseng). Sequence characterized amplified region (SCAR), AP–PCR, RAPD and RFLP have been successfully applied for differentiation of these plants and to detect substitution by other closely related species[81–83]. Characterization of *Echinacea* species and detection of possible adulterations have been done using RAPD technique[84]. DNA fingerprinting and polymorphism in the Chinese drug 'Ku-Di-Dan' (herba elephantopi) and its substitutes were studied using AP–PCR and RAPD. The results were used for authentication of 'Ku-Di-Dan' and its substitutes[85]. DNA fingerprinting of *Taraxacum mongolicum* (herba taraxaci) and its adulterants of six species of Compositae was demonstrated using AP–PCR and RAPD[86]. Bulb of *Fritillaria cirrhosa,* an official drug of *Chinese Pharmacopoeia* (1995), is commonly used as an antitussive and expectorant. It has often been adulterated with similar bulbs of other related species. Specific DNA-based primers have been designed for authentication of *F. cirrhosa* at the genomic level[87].

A molecular marker that is specific to medicinal rhubarb-based on chloroplast trnL/trnF sequence which is absent in its adulterants has been identified[88]. DNA sequence analysis of rDNA ITS and PCR–RFLP were explored for their application in differentiating four medicinal *Codonopsis* species from their related adulterants, *Campanumoea javania* and *Platycodon grandiflorus*. The technique allowed effective and reliable differentiation of *Codonopsis* from the adulterants[39].

### *Marker assisted selection of desirable chemotypes*

Along with authentication of species identity, prediction of the concentration of active phytochemicals may be required for quality control in the use of plant materials for pharmaceutical purposes. Identification of DNA markers that can correlate DNA fingerprinting data with quantity of selected phytochemical markers associated with that particular plant, would have extensive applications in quality control of raw materials. AFLP analysis has been found to be useful in predicting phytochemical markers in cultivated *Echinacea purpurea*[89] germplasm and some related wild species. RAPD fingerprint has been developed to support the chemotypic differences in oil quality of three different genotypes of *Pelargonium graveolens*[90] and flavonoid composition of *Aconitum*[91] species. DNA profiling has been used to detect the phylogenetic relationship among *Acorus calamus* chemotypes differing in their essential-oil composition[92]. *Artemisia annua*, a source of antimalarial compound artemisinin, shows variation in artemisinin content all over India. These chemotype variants of *A. annua* L. have been characterized using RAPD markers. This study also revealed existence of high levels of genetic variation in the Indian population despite geographical isolation and opens out a possibility of further

genetic improvement for superior artemisinin content. An attempt has also been made to study variation in essential-oil components and interspecific variations using RAPD technique[93]. Morphological, chemical and genetic differences in twelve basil (*Ocimum gratissimum* L.) accessions were studied to determine whether volatile oil and flavonoids can be used as taxonomical markers and to examine the relation between RAPDs and these chemical markers[94].

### *Medicinal plant breeding*

ISSR–PCR has been found to be an efficient and reliable technique for the identification of zygotic plantlets in citrus interploid crosses[95]. Molecular markers have been used as a tool to verify sexual and apomictic offspring of intraspecific crosses in *Hypericum perforatum*, a well-known antihelminthic and diuretic[96]. An attempt has been made towards marker-assisted selection of fertile clones of garlic with the help of RAPD markers[97]. RAPD markers have been successively used for selection of micropropogated plants of *Piper longum* for conservation[98].

### *Applications in foods and nutraceuticals*

DNA-based molecular markers have been used extensively for a wide range of applications in food crops and horticultural plants[6,10,99]. These applications include study of genetic variation, cultivar identification, genotyping, cross-breeding studies, identification of disease-resistant genes, identification of quantitative-trait loci, diversity analysis of exotic germplasms, sex identification of dioeceous plants, phylogenetic analysis, etc. Recently, the application of DNA-based molecular markers is being explored in the field of nutraceuticals.

According to the new European Council legislation[100], the labelling of food or food ingredients produced from, or containing licensed genetically modified organisms must indicate the inclusion of these ingredients where they are present at or above a level of 1%. In compliance with the labelling regulation for GM foods, several countries in Europe such as Germany and Switzerland, have extensively developed PCR methods for both identification and quantification purposes.

In response to reports of unlicensed GM ingredients in food in the international market, the Food Safety Authority of Ireland has completed a survey to determine the levels of GM maize ingredients in tortilla chips and taco shells on sale in Ireland, using the PCR technique[101]. Where sufficient GM DNA was present in the sample, quantitative analysis was undertaken using real-time PCR.

Primers specific for inserted genes in Roundup Ready™ soybean have been found to be suitable for detection and discrimination of GM soybean from non-GM products[102]. In another study, Roundup Ready soybeans, Bt 176 maize and Cecropin D capsicum have been successfully discriminated from non-GM products using primers specific for inserted genes and crop endogenous genes[103].

## DNA markers as new pharmacognostic tool

Traditionally, pharmacognosy mainly addressed quality-related issues using routine botanical and organoleptic parameters of crude drugs. Pharmacognosy became more interdisciplinary because of subsequent advances in analytical chemistry. These developments added emphasis on chemoprofiling-assisted characterization with chromatographic and spectroscopic techniques. The new pharmacognosy includes all aspects of drug development and discovery, where biotechnology-driven applications will play an important role.

Extensive research on DNA-based molecular markers is in progress in many research institutes all over the world. This technique remains important in plant genome research with its applications in pharmacognostic identification and analysis. Chinese researchers have applied DNA markers extensively for characterization of botanicals from the Chinese materia medica. These markers have shown remarkable utility in quality control of commercially important botanicals like Ginseng, Echinacea, Atractylodes. In India several agricultural universities and research institutes are actively involved in exploring DNA-based techniques in genotyping of medicinal plants. Although considerable progress has been made in DNA marker technology, applications of these techniques for characterizing semi-processed and processed botanical formulations to ensure the desirable quality remain underutilized.

Although DNA analysis is currently considered to be cutting-edge technology, it has certain limitations due to which its use has been limited to academia. In order to establish a marker for identification of a particular species, DNA analysis of closely related species and/or varieties and common botanical contaminants and adulterants is necessary, which is a costly and time-consuming process. Isolation of good-quality DNA suitable for analysis from semi-processed or processed botanicals is also a challenge. Another important issue is that DNA fingerprint will remain the same irrespective of the plant part used, while the phytochemical content will vary with the plant part used, physiology and environment. DNA fingerprinting ensures presence of the correct genotype but does not reveal the contents of the active principle or chemical constituents. Hence DNA analysis and pharmacognostic techniques for chemoprofiling such as TLC, HPTLC, etc. will have to be used hand in hand rather than in isolation. Identification of quantitative-trait loci[34] that are closely linked to a biologically active phytochemical will prove to be useful. Several attempts have been made in recent

years, to correlate DNA markers with qualitative and quantitative variations in phytochemical composition among closely related species[87–92]. Proper integration of molecular techniques and analytical tools will lead to the development of a comprehensive system of botanical characterization that can be conveniently applied at the industry level for quality control of botanicals.

Ayurvedic classification of medicinal plant is based on basic principles and therapeutic characters that may have a genetic basis. We have undertaken an exploratory study on the use of molecular markers for quick identification of botanical materials in crude, semi-processed and processed herbal formulations. Our strategy involves identification of species-specific marker after screening a number of species and/or varieties of the medicinal plant using random oligonucleotide primers, followed by cloning and subsequently converting it to SCAR markers for better specificity and reproducibility. Also, application of RAPD markers has been explored for standardization of botanical formulations containing ayurvedic medicines like *Emblica officinalis*[104] and *Tinospora cordifolia*[105].


1. Report of the Inter Regional Workshop on Intellectual Property Rights in the Context of Traditional Medicine, Bankok, Thailand, December, 2000.
2. Singh, J., Singh, A. K. and Khanuja, S. P. S., *Pharma Bio World*, 2003, **1**, 59–66.
3. Vaidya, A. D. B., Vaidya, R. A. and Nagaral, S. I., *J. Assoc. Physicians India*, 2001, **49**, 534–537.
4. Patwardhan, B., Chopra, A. and Vaidya, A. D. B., *Curr. Sci.*, 2003, **84**, 1165–1166.
5. Straus, S. E., *N. Engl. J. Med.*, 2002, **347**, 1997–1998.
6. De Smet PAGM, *N. Engl. J. Med.*, 2002, **347**, 2046–2056.
7. *Indian Herbal Pharmacopoeia*, Indian Drug Manufacturers' Association, Mumbai, 2002.
8. *British Herbal Pharmacopoeia*, British Herbal Medicine Association, 1996.
9. *Quality Control Methods for Medicinal Plant Materials*, WHO, Geneva, 1998.
10. An exploration of current issues in botanical quality: A discussion paper, Natural Health Products Directorate, Health Products and Food Branch, Canada, February, 2002.
11. Joshi, S. P., Ranjekar, P. K. and Gupta, V. S., *Curr. Sci.*, 1999, **77**, 230–240.
12. Bhutani, K. K., *East. Pharm.*, 2000, 21–26.
13. Rao, E. V., *East. Pharm.*, 2000, 35–38.
14. Sri Bhava Misra, *Bhavaprakash* (eds Misra, B. and Vaisya, R.), Chaukhambha Sanskrit Sansthan, Varanasi, 1999, vol. 1, pp. 73–75.
15. Vijaya Kumar, D. and Raghavan, K. V., Novel Chromatographic fingerprinting method for standardization of single medicines and formulations, Indian Institute of Chemical Technology, Hyderabad. WO 0246739-EP2 0000991 991-263397CSIR G01N30-88.
16. Liu, Y. M., Sheu, S. J., Chiou, S. H., Chang, S. H. and Chen, Y. P., *Planta Med.*, 1993, **59**, 376–378.
17. Liu, Y. M., Sheu, S. J., Chiou, S. H., Chang, S. H. and Chen, Y. P., *Phytochem. Anal.*, 1994, **5**, 256–260.
18. Chuang, W. C., Wu, S. K., Sheu, S. J., Chiou, S. H., Chang, H. C. and Chen, Y. P., *Planta Med.*, 1995, **61**, 459–465.
19. Chuang, W. C., Wu, S. K., Sheu, S. J., Chiou, S. H., Chang, H. C. and Chen, Y. P., *Planta Med.*, 1996, **62**, 459–465.
20. Chan, K., *Chemosphere*, 2003, **52**, 1361–1371.
21. Warude, D., Chavan, P., Kalpana, J. and Patwardhan, B., *Plant Mol. Biol. Rep.*, 2003, **21**, 1.
22. Singh, M., Bandana and Ahuja, P. S., *Plant Mol. Biol. Rep.*, 1999, **17**, 171.
23. Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A., *Mol. Breed.*, 1996, **2**, 225–238.
24. Botstein, B., White, R. L., Skolnick, M. and Davis, R. W., *Am. J. Hum. Genet.*, 1980, **32**, 314–331.
25. Nakamura, Y., Julier, C., Wolff, R., Holm, T., O'Connell, P., Lepert, M. and White, R., *Nucleic Acids Res.*, 1987, **15**, 2537–2547.
26. Litt, M. and Luty, J. A., *Am. J. Hum. Genet.*, 1989, **44**, 397–401.
27. Jeffrey, A. J., Wilson, V. and Thein, S. L., *Nature*, 1985, **314**, 67–73.
28. Williams, J. G. K., Kubelik, A. R., Livak, J., Rafalski, J. A. and Tingey, S. V., *Nucleic Acids Res.*, 1990, **18**, 6531–6535.
29. Welsh, J. and McClelland, M., *Nucleic Acids Res.*, 1990, **18**, 7213–7218.
30. Welsh, J. and McClelland, M., *Nucleic Acids Res.*, 1991, **19**, 861–866.
31. Caetano-Anolles, G., Bassam, B. J. and Gresshof, P. M., *Bio/technology*, 1991, **9**, 553–557.
32. Caetano-Anolles, G. and Bassam, B. J., *Appl. Biochem. Biotechnol.*, 1993, **42**, 189–200.
33. Zietkiewicz, E, Rafalski, A. and Labuda, D., Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, 1994, **20**, 176–183.
34. Zabeau, M., *European Patent Application*, Publication No. 0534858A1, 1993.
35. Vos, P. *et al.*, *Nucleic Acids Res.*, 1995, **23**, 4407–4414.
36. Kumar, L. S., *Biotechnol. Adv.*, 1999, **17**, 143–182.
37. Oleszek, W., Stochmal, A., Karolewski, P., Simonet, A. M., Macias, F. A. and Tava, A., *Biochem. Syst. Ecol.*, 2002, **30**, 1011–1022.
38. Shasany, A. K., Kukreja, A. K., Saikia, D., Darokar, M. P., Khanuja, S. P. S. and Kumar, S., *PGR Newsl.*, 1999, **121**, 27–31.
39. Farooqui, N., Ranade, S. A. and Sane, P. V., *Biochem. Mol. Biol. Int.*, 1998, **45**, 931–939.
40. Adams, R. P., Pandeyb, R. N., Leverenzc, J. W., Digdardd, N., Hoeghe, K. and Thorfinnssonf, T., *Sci. Hortic.*, 2002, **96**, 303–312.
41. Fu, R. Z., Wang, J., Zhang, Y. B., Wang, Z. T., But, P. P., Li, N. and Shaw, P. C., *Planta Med.*, 1999, **65**, 648–650.
42. Friesen, N. and Blattner, F. R., *Planta Med.*, 1999, **65**, 157–160.
43. Padmesh, P., Sabu, K. K., Seeni, S. and Pushpangadan, P., *Curr. Sci.*, 1999, **76**, 833–835.
44. Kojoma, M., Iida, O., Makino, Y., Sekita, S. and Satake, M., *Planta Med.*, 2002, **68**, 60–63.
45. Barth, S., Melchinger, A. E. and Lubberstedt, T., *Mol. Ecol.*, 2002, **11**, 495–505.
46. Yamazaki, M., Sato, A., Shimomura, K., Saito, K. and Murakoshi, I., *Biol. Pharm. Bull.*, 1994, **17**, 529–1531.
47. Kapteyn, J., Goldsbrough, B. and Simon, E., *Theor. Appl. Genet.*, 2002, **105**, 369–376.
48. Chen, Y., Bai, S., Cheng, K., Zhang, S. and Nian, L., *Zhongguo Zhong Yao Za Zhi*, 1999, **24**, 131–133.
49. Lind-Hallden, C., Hallden, C. and Sall, T., *Hereditas*, 2002, **136**, 45–50.
50. Nakai, R., Shoyama, Y. and Shiraishi, S., *Biol. Pharm. Bull.*, 1996, **19**, 67–70.
51. Hosokawa, K., Minami, M., Kawahara, K., Nakamura, I. and Shibata, T., *Planta Med.*, 2000, **66**, 270–272.
52. Wolf, H. T., Berg, T. V. D., Czygan, F. C., Mosandl, A., Winckler, T., Zundorf, I. and Dingermann, T., *Planta Med.*, 1999, **65**, 83–85.
53. Cheng, Z., Lu, B. R., Baldwin, B. S., Sameshima, K. and Chen, J. K., *Hereditas*, 2002, **136**, 231–239.
54. Hulya, I., *Sci. Hortic.*, 2003, **97**, 211–218.

55. Prince, J. P., Lackney, V. K., Angels, C., Blauth, J. R. and Kyle, M. M., *Genome*, 1995, **38**, 224–231.
56. Amarger, V. and Mercier, L., *Biochimie*, 1995, **72**, 931–936.
57. Tessier, C., David, J., This, P., Boursiquot, J. M. and Charrier, A., *Theor. Appl. Genet.*, 1999, **98**, 171–177.
58. Wachira, F. N., Waugh, R., Hackett, C. A. and Powell, W., *Genome*, 1995, **38**, 201–210.
59. Lata, H., Moraes, R. M., Douglas, A. and Scheffler, B. E., *Trends in New Crops and New Uses* (eds Janick, J. and Whipkey, A.), ASHS Press, Alexandria, VA, 2002, pp. 537–539.
60. Singh, B. M., Sharma, K. D., Katoch, M., Guleria, S. and Sharma, T. R., *PGR Newsl.*, 1999, **124**, 57–61.
61. Negi, M. S., Singh, A. and Laksmikumaran, M., *Genome*, 2000, **43**, 975–80.
62. Van, D. B., Breyne, P., Goetghebeur, P., Romijn-Peeters, E., Kyndt, T. and Gheysen, G., *Theor. Appl. Genet.*, 2002, **105**, 289–297.
63. Das, S., Rajagopal, J., Bhatia, S., Srivastava, P. S. and Lakshmikumaran, M., *J. Biosci.*, 1999, **24**, 233–240.
64. Pang, X. M., Hu, C. G. and Deng, X. X., *Yi Chuan Xue Bao*, 2003, **30**, 81–87.
65. Grattapaglia, D. and Sederoff, R., *Genetics*, 1994, **137**, 1121–1137.
66. Gocmen, B., Jermstad, K. D., Neale, D. B. and Kaya, Z., *Can. J. For. Res.*, 1996, **26**, 497–503.
67. Peters, J. L., Constandt, H., Neyt, P., Cnops, G., Zethof, J., Zabeau, M. and Gerats, T., *Plant Physiol.*, 2001, **127**, 1579–1589.
68. Zhang, K. Y., Leung, H. W., Yeung, H. W. and Wong, R. N., *Planta Med.*, 2001, **67**, 379–381.
69. Cheng, K. T., Tsay, H. S., Chen, C. F. and Chou, T. W., *Planta Med.*, 1998, **64**, 563–565.
70. Chen, K. T. *et al.*, *Acta Pharmacol. Sin.*, 2001, **22**, 493–497.
71. Cheng, K. T., Su, B., Chen, C. T. and Lin, C. C., *Am. J. Chin. Med.*, 2000, **28**, 273–278.
72. Matsuda, M., Kojima, E., Izumi, M. and Murakami, K., *Nucleic Acids Symp. Ser.*, 1997, **37**, 169–170.
73. Wang, T., Su, Y., Zhu, J., Li, X., Zeng, O. and Xia, N., *Zhong Yao Cai*, 2001, **24**, 710–715.
74. Yukiko, T. K., Asaka, I. and Ichio, I., *Biol. Pharm. Bull.*, 2001, **24**, 1210–1213.
75. Um, J. Y. *et al.*, *Biol. Pharm. Bull.*, 2001, **24**, 872–875.
76. Ha, W. Y., Yau, F. C., But, P. P., Wang, J. and Shaw, P. C., *Planta Med.*, 2001, **67**, 587–589.
77. Lau, D. T., Shaw, P. C., Wang, J. and Shaw, P. C., *Planta Med.*, 2001, **67**, 456–460.
78. Zhang, Y. B., Wang, Z. T., But, P. P. and Shaw, P. C., *Planta Med.*, 2003, **69**, 1172–1174.
79. Mizukami, H., Okabe, Y., Kohda, H. and Hiraoka, N., *Biol. Pharm. Bull.*, 2000, **23**, 589–594.
80. Cheng, K. T., Chang, H. C., Su, C. H. and Hsu, F. L., *Bot. Bull. Acad. Sin.*, 1997, **38**, 241–244.
81. Shaw, P. C. and But, P. P., *Planta Med.*, 1995, **61**, 466–469.
82. Wang, J., Ha, W. Y., Ngan, F. N., But, P. P. H. and Shaw, P. C., *Planta Med.*, 2001, **67**, 781–783.
83. Mihalov, J. J., Marderosian, A. D. and Pierce, J. C., *J. Agric. Food Chem.*, 2000, **48**, 3744–3752.
84. Wolf, H. T., Zundorf, I., Winckler, T., Bauer, R. and Dingermann, T., *Planta Med.*, 1999, **65**, 773–774.
85. Cao, H., But, P. P. and Shaw, P. C., *Yao Xue Xue Bao*, 1996, **31**, 543–553.
86. Cao, H., But, P. P. and Shaw, P., *Zhongguo Zhong Yao Za Zhi*, 1997, **22**, 197–200.
87. Li, Y. F., Li, X. Y., Lin, J., Xu, Y., Yan, L., Tang, F. and Chen, F., *Planta Med.*, 2003, **69**, 186–188.
88. Yang, M., Zhang, D., Liu, J. and Zheng, J., *Planta Med.*, 2001, **67**, 784–786.
89. Baum, B. R., Mechanda, S., Livesey, J. F., Binns, S. E. and Arnason, J. T., *Phytochemistry*, 2001, **56**, 543–549.
90. Shasany, A. K., Aruna, V., Darokar, M. P., Kalra, A., Bahl, J. R., Bansal, R. P. and Khanuja, S. P. S., *J. Med. Aromat. Plant Sci.*, 2002, **24**, 729–732.
91. Fico, G., Spada, A., Bracab, A., Agradic, E., Morellib, I. and Tomea, F., *Biochem. Syst. Ecol.*, 2003, **31**, 293–301.
92. Sugimoto, N., Kiuchi, F., Mikage, M., Mori, M., Mizukami, H. and Tsuda, Y., *Biol. Pharm. Bull.*, 1999, **22**, 481–485.
93. Sangwan, R. S., Sangwan, N. S., Jain, D. C., Kumar, S. and Ranade, S. A., *Biochem. Mol. Biol. Int.*, **47**, 935–944.
94. Vieira, R. F., Grayer, R. J., Paton, A. and Simon, J. E., *Biochem. Syst. Ecol.*, 2001, **29**, 287–304.
95. Tusa, N., Abbet, L., Ferrante, S., Lucreti, S. and Scarano, M. T., *Cell. Mol. Biol. Lett.*, 2002, **7**, 703–708.
96. Steck, N., Messmer, M., Schaffner, W. and Bueter, K. B., *Plant Biol.*, 2001, 622–628.
97. Etoh, T. and Hong, C. J., *Acta Hortic.*, 555, II International Symposium on edible Alliaceae.
98. Parani, M., Anand, A. and Parida, A., *Curr. Sci.*, 1997, **73**, 81–83.
99. Sharma, H. C., Crouch, J. H., Sharma, K. K., Seetharama, N. and Hash, C. T., *Plant Sci.*, 2002, **163**, 381–395.
100. http://europa.eu.int/eur-lex/en/lif/dat/2000/en_300R0049.html.
101. http://www.fsai.ie/industry/tortilla_survey.pdf.
102. Lin, H. Y., Chiang, J. and Shih, D. Y. C., *J. Food Drug Anal.*, 2001, **9**, 160–166.
103. Deng, P., Zhao, J., Liu, J. and Fang, S., *Wei Sheng Yan Jiu*, 2002, **31**, 37–40.
104. Warude, D., DNA fingerprinting: A new pharmacognostic tool in drug development, M Pharm dissertation, Faculty of Pharmaceutical Sciences, Bharati Vidyapeeth, Pune, 2003.
105. Patil, M., Chemical and pharmacognostic investigations of *Tinospora cordifoloia*. Ph D thesis, Faculty of Pharmaceutical Sciences, University of Pune, 2003.


ACKNOWLEDGEMENTS. P.C. and K.J. thank Council for Scientific and Industrial Research, Technology and Business Development Division, and (D.W.) the University Grants Commission, New Delhi for financial assistance.

Received 13 October 2003; revised accepted 8 April 2004