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Development of RAPD markers for authentication of *Piper nigrum* (L.)

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Abstract

In this study, the RAPD (Random Amplified Polymorphic DNA) technique was employed for authentication of *Piper nigrum* (black pepper) from its adulterant *Carica papaya*. Eight decamer oligonucleotide primers were screened in the RAPD analysis for identification of genuine and adulterant samples using the DNA isolated from the dried seed of samples. Out of eight primers used, five gave species-specific reproducible unique amplicons, which could clearly distinguish genuine as well as adulterant samples having similar morphology. RAPD could thus, help to serve as a complementary tool for quality control of genuine samples sold in the local markets.

Keywords: Adulterant, *Carica papaya*, Herbal medicine, PCR

Introduction

Herbal medicine has been enjoying renaissance among the customers throughout the world. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. Hence, standardization of herbals is a current need. *Piper* species are of high economic and medicinal importance (Nadkarni and Nadkarni, 1954) and several species of *Piper* are ingredients in the formulation of Ayurveda-Chinese-Unani and Compo-System of medicine. *Piper nigrum* (black pepper) (Piperaceae) is one of the

most popular spices in the world, and is also employed in the folk medical protocols of many countries. The fruits of *P. nigrum*, is an important as spice have been widely used since time immemorial in household spices as flavoring agents, and also in various traditional systems of medicine and has also been used in the treatment of cholera and dyspepsia, as well as a variety of gastric ailments and arthritic disorders (Jung and Shin, 1998). Alkamides are particularly interesting, due to their various biological activities, including insecticidal (Kiuchi *et al.,* 1988; Park *et al.,* 2002), anti-bacterial (Reddy *et al.,* 2004), anti-inflammatory (Mujumdar *et al.,* 1990) and antioxidant activity (Kapoor *et al.,* 2009). According to Ayurvedic system of medicine, *P. nigrum* fruits are anthelmintic, antiasthmatic and used to treat pain, piles, insomnia, and epilepsy (Anonymous*,* 2001). Studies on this drug have revealed anticonvulsant (Hu and Davies, 1997) and bioavailability-enhancing properties (Atal *et al.,* 1980; Zutshi *et al.,* 1985; Bano *et al.,* 1987). The chemistry of *P. nigrum* fruits has been extensively investigated and reveals a number of piperidine and pyrrolidine amides. Pharmacological and clinical studies have revealed that piperine has CNS depressant, antipyretic, analgesic, anti-inflammatory (Lee *et al.,* 1984), antioxidant (Khajuria *et al.,* 1997), and hepatoprotective (Koul and Kapil, 1993) activities. Recently, Park *et al.* (2002) and Siddiqui *et al.* (2002, 2003, 2004) performed studies on controlling *Aedes aegypti* larvae by isolating insecticidal constituents from *P. nigrum* fruits.

The fruits contain 1.0–2.5% volatile oil, 5–9% alkaloids, of which the major ones are piperine, chavicine, piperidine, and piperetine, and a resin (Evans, 1997). The terpenes, steroids, lignans, flavones, and alkaloids/alkamides have been identified as the primary constituents of the peppers (Navickene *et al.,* 2000; Parmar *et al.,* 1997). Most of the pharmacological properties of *P. nigrum* fruits are attributed to a piperidine alkaloid, piperine, which is present in the fruits in amounts of 1.7–7.4% (Anonymous, 1998; Epstein, 1993). Piperine has also been shown to enhance the bioavailability of several drugs, for example sulfadiazine, tetracycline, streptomycin (Hu and Davies, 1997), rifampicin, pyrazinamide, isoniazid, ethambutol (Zutshi *et al.,* 1985) and phenytoin (Bano *et al.,* 1987). Due to its diverse pharmacological properties, piperine is important as a biomarker for standardization of fruit of *P. nigrum* and *P. longum* and of polyherbal formulations containing these raw materials.

The seed of *Carica papaya* is less or more similar to the seed of *Piper nigrum* and easily adulterated in the local markets. Similarly, several herbal drugs on the market still cannot be identified or authenticated based on their morphological or histological characteristics. Use of a wrong herb may be ineffective or it may worsen the condition and may even cause death. In Belgium, the use of a traditional Chinese medicine (TCM) contaminated with plants from the *Aristolochia* species resulted in an epidemic of subacute intestinal nephropathy. Many of the affected patients required kidney transplantation. When 19 kidneys and urethras removed from 10 patients were examined histologically, neoplasms were detected in 40% (Cosyns *et al.,* 1999). Ideally, authentication should be done from the harvesting of the plant material to the final product. Morphological as well as biochemical markers used in the authentication of herbal drugs have many limitations due to the impact of environmental conditions. DNA-based molecular markers however, are important tools in quality assurance and preservation of germplasm of medicinal plant species. Our major objective therefore, was to develop a DNA-based molecular tool for

accurate identification of *P. nigrum* in a local market to differentiate and authenticate it from *C. papaya.*

Piper was chemically reviewed by many workers (Parmar *et al.,* 1997; Sengupta and Ray, 1987) but, very little work on their adulteration has been carried out (Madan *et al.,* 1996; Paradkar *et al.,* 2001). The mature berries of *P. nigrum* are black in colour. The Mature fruits of papaya contain numerous grey-black spherical seeds 5 mm in diameter (Villegas, 1997). The antifertility properties of papaya, particularly of the seeds, have been the subject of significant evaluation using animal models, especially in India where there is interest in the development of a safe and effective oral male contraceptive (Lohiya *et al.,* 1999). A complete loss of fertility has been reported in male rabbits, rats and monkeys fed an extract of papaya seeds (Lohiya *et al.*, 1999; Pathak *et al*., 2000; Lohiya *et al*., 2002) suggesting that ingestion of papaya seeds may adversely affect the fertility of human males or other male mammals. A number of early studies, largely conducted in India, suggested that unripe papaya fruit, latex extracts or papaya seeds have deleterious effects on pregnancy in laboratory animals (Schmidt 1995). The contraceptive efficacy and toxicological screening of the two principal compounds, MCP I and ECP I, isolated from the seeds of *Carica papaya*, in male albino rats at the standardized dose regimen, at 50 mg/kg b.w./day, for a period of 360 days and up to 90 days of treatment withdrawal have been reported (Lohiya *et al*., 2002).

Materials and methods

A genuine sample of *Piper nigrum* (L.) was provided by Central Council for Research in Unani Medicine (CCRUM), Hyderabad. The samples for authentication were purchased from local markets of Khari Baoli, Delhi, India. The material was identified at the National Institute of Science Communication and Information (NISCAIR) by Dr. H. B. Singh and voucher (NISCAIR/RHMD/consult/-2007-08/937/121) was deposited in Herbarium.

Reagents and chemicals

The stock solution concentration were: CTAB 3% (w/v), 1MTris–Cl (pH 8), 0.5M EDTA (pH 8), 5M NaCl, absolute ethanol (AR grade), chloroform-IAA (24:1 [v/v]), polyvinylpyrrolidone (PVP) (40 000 mol wt) (Sigma), β -mercaptoethanol. All the chemicals used in the experiments were of analytical grade. The extraction buffer consisted of CTAB 3% (w/v), 100 mM Tris–Cl (pH 8), 25 mM EDTA (pH 8), and 2M NaCl respectively. The polyvinylpyrrolidone and β-mercaptoethanol were added freshly prepared.

DNA extraction and purification

DNA was isolated from dried seed using a modified CTAB method (cetyl trimethyl ammonium bromide (Khan *et al.,* 2007). The fine powder was transferred to the microcentrifuge tube containing freshly prepared 600 µl of extraction buffer (100 mM Tris buffer pH 8, 25 mM EDTA, 2 M NaCl, 3% CTAB and 3% polyvinyl pyrrolidone). The suspension was gently mixed and incubated at 65ºC 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 at12000 rpm/min for 10 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 ºC for 30 min. The nucleic acid was collected by centrifuging at 1000 rpm/min 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 (10 mM tris buffer pH 8, 1 mM EDTA) at room temperature. The contaminating RNA was eliminated by treating the sample with RNase A (10 mg/ml) for 30 min at 37 ºC. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

RAPD reaction

The RAPD reaction was performed according to the method developed by (McClelland *et al*., 1995). The reactions were carried out in 25 µl volume in a tube using eight random decanucleotide primers, OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, and OPC-8 (Operon Technologies Inc., USA). Each reaction tube contained 30 ng template DNA, 1.5 mM $MgCl_2$, 300 µM of dNTPs, 2.5 µl1x*Taq* DNA polymerase buffer, 25 pM decanucleotide primer and 1.5 units of *Taq* DNA polymerase (Genei, India). Amplification was performed in a DNA thermal cycler (Techne Thermal cycler, England) using the following conditions: 95 ºC for 3 min; 36 cycles at 94 ºC for 1 min, 35.6 ºC for 30 s and 72 ºC for 1 min; final extension at 72 ºC for 2 min. PCR products were resolved on 1.2% agarose gel in 1xTAE buffer. The DNA was stained with 0.5 mg/mL ethidium bromide, visualized and photographed under a UV transilluminator. A sample without template DNA was included as a negative control in each experiment to check contamination. Electrophoretic profile was visualized under UV radiation and photographed with UV transilluminator, U.K. The sizes of DNA fragments were estimated by comparison with standard Ladder 1kb and 100 bp (Banglore Genei, India).

Results and discussion

Piper nigrum was chosen to test the reliability of quality control using RAPD technique. The dried seed of *P. nigrum* is more or less similar in morphology with those of *C. papaya*. The RAPD technique carried out in replicate on both plants for reproducibility of this technique using 32 decamer primers. Of the 8 primers used, 5 generated reproducible, unique, monomorphic and polymorphic fragments in PCR amplification using the genomic DNA extracted from seed. The genuine and adulterant samples were discriminated by the presence or absence of unique bands in RAPD profile. The total number of unique bands specific to genuine as well as adulterant samples with different primers is summarized in Figs 1-2 and Table 1.

According to WHO general guidelines for methodologies on research and evaluation of traditional medicines, first step in assuring quality, safety and efficacy of traditional medicines is correct identification. RAPD markers obtained through primer screening, specific to *P. nigrum* and *C. papaya* were reproducible and clearly differentiated both species of different genera. The most of the primers gave unique bands in both species. The primers OPC-1, OPC-4, OPC-6, OPC-7 and OPC-8 clearly discriminated *P. nigrum* as well as *C. papaya* by the presence and absence of unique bands (Fig 1, 2).

Table 1. The unique bands specific to *Piper nigrum* and *Carica papaya* samples with 8 decamer oligonucleotides primers obtained in PCR amplification.

Species	Fig. 1 Size of unique bands (bp)				Fig. 2 Size of unique bands (bp)			
	OPC-1	OPC-2	OPC-3	OPC-4	OPC-5	OPC-6	OPC-7	OPC-8
Piper nigrum	600	0	0	1100, 680	0	1600, 900	1000	800, 700, 650, 600
Carica Papaya	0	0	0	0	0	800	250	0
Total no of unique bands/primer	1	0	0	2	0	3	2	4

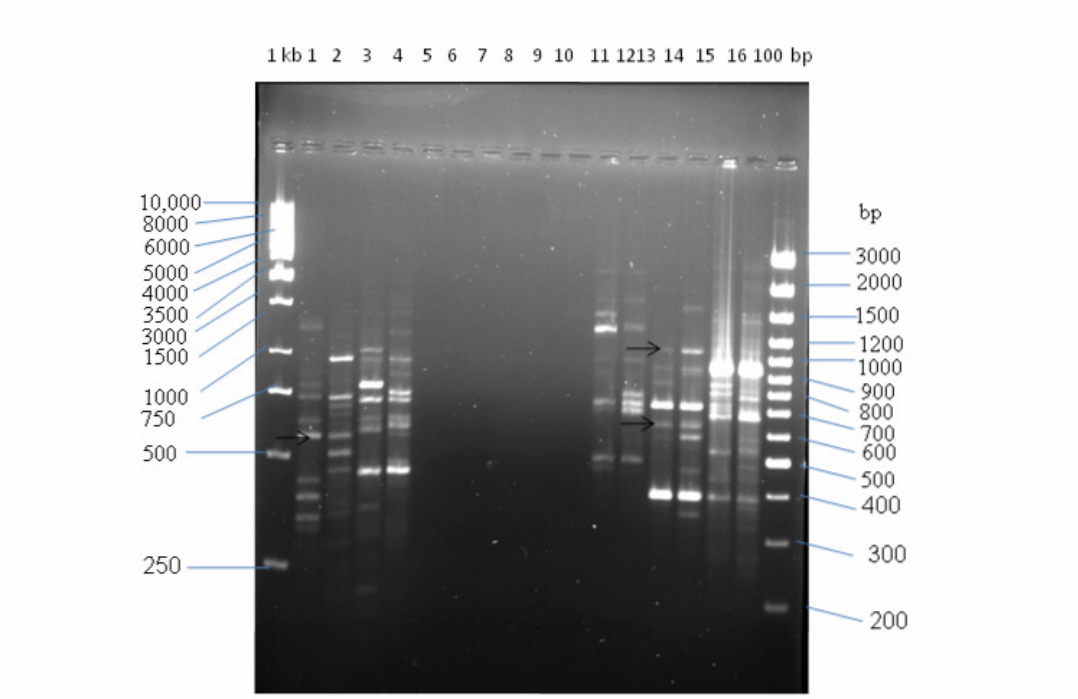

Figure 1. RAPD analysis carried out with primer: OPC-1(lanes-1-4), OPC-2(lanes-5-8), OPC-3(lanes-9-12), OPC-4(lanes-13-16) on genomic DNA isolated from seed of *Piper nigrum* (lanes-1,2,5,6, 9,10, 13,14) and *Carica papaya* (lanes-3,4,7,8,11,12,15,16). 1kb and 100 bp are DNA ladder.

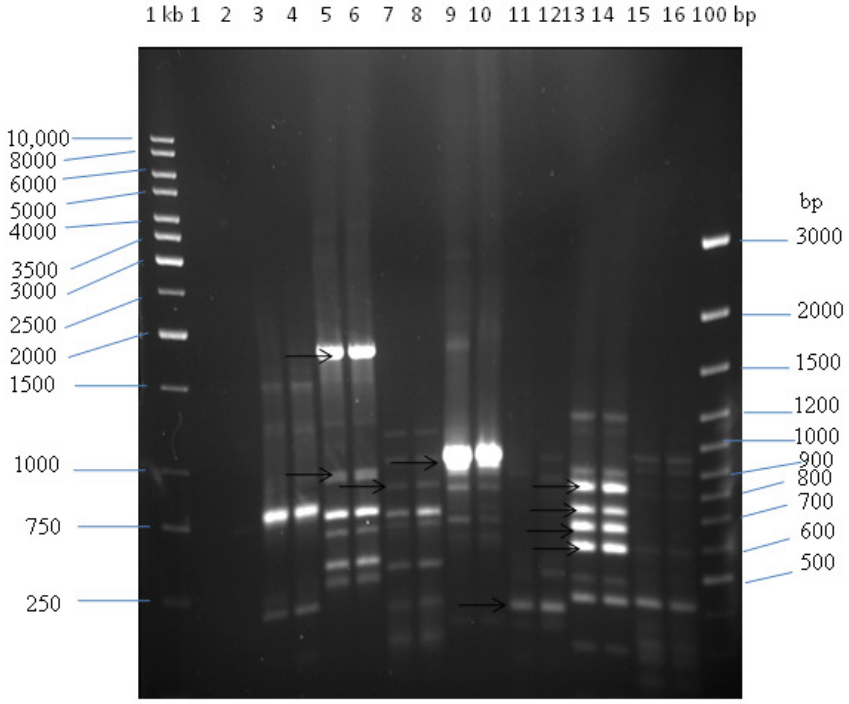

Figure 2. RAPD analysis carried out with primer: OPC-5 (lanes-1-4), OPC-6 (lanes-5-8), OPC-7 (lanes-9-12), OPC-8 (lanes-13-16) on genomic DNA isolated from seed of *Piper nigrum* (lanes-1, 2,5, 6, 9, 10, 13, 14) and *Carica papaya* (lanes-3, 4, 7, 8, 11, 12, 15, 16). 1kb and 100 bp are DNA ladder.

The single primer in our study was found to discriminate the genuine as well as adulterant sample. The RAPD analysis has been widely used for differentiation of a large number of medicinal species from their close relatives or adulterants, including *Panax* species (Shaw and But, 1995), *Coptis* species (Cheng *et al.,* 1997), *Astragalus* species (Cheng *et al.,* 2000), *Lycium barbarum* (Cheng *et al.,* 2000), *Panax ginseng* species (Um *et al.,* 2001), *Echinacea* species (Nieri *et al.,* 2003), turmeric (Sasikumar *et al.,* 2004), *Astragali radix* (Na *et al.,* 2004), *Dendrobium officinale* (Ding *et al.,* 2005), *Typhonium* species (Acharya *et al.,* 2005), *Dendrobium* species and its products (Zhang *et al.,* 2005), *Tinospora cordifolia* (Wild.) Miers ex Hook & Thomas (Rout 2006), *Mimosae tenuiflorae cortex* (Rivera-Arce *et al.,* 2007), *Rahmannia glutinosa* cultivars and varieties (Qi *et al.,* 2008), *Desmodium species* (Irshad *et al.,* 2009) and *Glycirrhiza glabra* (Khan *et al.,* 2009). The RAPD technique has been used for determination of the components in an Ayurvedic herbal prescription, Rasayana Churna to identify three Ayurvedic medicines, dried stem of *Tinospora cordifolia*, dried fruit of *Emblica officinalis* and dried fruit of *Tribulus terestris* (Shinde *et al.,* 2007). The marker with 600 bp is specific to *Tinospora cordifolia*; the marker 500 bp is specific to *Emblica officinalis* and the remaining marker >1000 bp was present in *Tribulus terestris* (Shinde *et al.* 2007). The medicinal species of *Selaginella* and variation of the same species from different habitats was authenticated by RAPD analysis (Li *et al.,* 2007). The advantages of RAPD techniques include their simplicity, rapidity, the low amount of genomic DNA required and the fact that isotopes and prior genetic information are not required (Micheli *et al.,* 1994). Our RAPD marker proved to be extremely reproducible under a wide variation of

amplification conditions; it is clearly visible up to an annealing temperature of 38 ºC (data not shown), and changes in the origin of the primer, the *Taq* polymerase and the thermal cycler used do not affect the result. Accordingly, an efficient, precise and sensitive method for identifying genuine medicinal plant material has been established and will contribute significantly in quality control. In this study, we suggest that RAPD method is convenient for identifying *P. nigrum* and its adulterant *C. papaya* in the local markets. More high quality linked DNA markers; sequenced characterized amplified regions (SCAR) will be developed in further studies, which can provide an alternative tool to monitor the quality of herbal medicines. Significance of present work is that single primer can differentiate genuine as well as adulterant samples.

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