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Identification of dried rhizomes of *Coptis* species using random amplified polymorphic DNA

Kur-Ta Cheng[1], Hsien-Chang Chang[2], Ching-Hua Su[3], Feng-Lin Hsu[4,5]

[1]*Graduate Institute of Pharmacognosy Science, Taipei Medical College, Taipei, Taiwan, Republic of China*

[2]*National Laboratories of Foods and Drugs, Department of Health Executive Yuan, Taipei, Taiwan, Republic of China*

[3]*Center of Biotechnology, Taipei Medical College, Taipei, Taiwan, Republic of China*

[4]*School of Pharmacy, Taipei Medical College, No. 250, Wu-Hsing Street, Taipei, Taiwan, Republic of China*

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Abstract. The Random amplified polymorphic DNA (RAPD) technique was adopted in the study to identify eight kinds of Coptidis Rhizomes and one kind of Picrorhizae Rhizoma, a pharmaceutical replacement for the former in some provinces of China. The DNA isolated from the dried rhizome of the samples were used as templates in polymerase chain reactions with twenty random decamer primers. Four of the primers, OPT-09, OPT-15, OPT-18, and OPT-20, revealed reproducibly distinct RAPD profiles. The primers possessed different abilities to detect variations in the *Coptis* species. RAPD analysis provides an efficient approach to identifying the sources of Coptidis Rhizoma medicines on the market.

Keywords: Coptidis; *Coptis*; Fingerprinting; Identification; RAPD.

Introduction

The genus *Coptis* (Ranunculaceae) comprises approximately sixteen species in the world. Of these, six species are found in mainland China and only one, *Coptis quinquefolia,* in Taiwan (Huang, 1996). Three species of *Coptis* originating from China. *C. chinensis, C. deltoidea*, and *C. teetoides* are usually found in the Taiwan herbal market. They are cultivated principally in Sichuan and Yunnan provinces. The rhizome of the plant is used for medicine. The active constituents of the Coptidis Rhizome are known to be the protoberberine alkaloids. It is a Chinese herbal drug, the properties of which include dispelling heat, drying dampness, removing toxins, and purging fever (Hsu et al., 1985).

In 1990, a new DNA fingerprint technique, called RAPD (random amplified polymorphic DNA), was developed, in which DNA fragments are amplified by a polymerase chain reaction (PCR) using a single arbitrary primer (Welsh and McClelland, 1990; Williams et al., 1990), resulting in genetic polymorphisms observed between different genomes when amplified products are separated on agarose gel by electrophoresis. The advantages of the technique are its simplicity and rapidity, its ability to detect extensive polymorphisms, and the fact that only small quantity of DNA is needed. The RAPD technique has been used to identify medicinal plants, for instance, tea (*Camellia sinensis*) (Wachira et al., 1995) and yam (*Dioscorea bulbifera*) (Ramser et al., 1996). In these studies, phylogenetic relationships have also been assessed. RAPD markers which are specific to a taxonomic level or link to a particular trait also facilitate the identification and the breeding of plants. Two kinds of spruce, white spruce [*Picea glauca* (Moench) and Voss] and Engelmann spruce (*Picea engelmannii* Parry), were easily differentiated using two species-specific RAPD markers which were generated from two random primers (Khasa and Dancik, 1996). In our laboratory, we obtained two RAPD markers generated from one primer to differentiate the expensive medicinal plants *Anoectochilus formosanus* and *A. koshunensis* and their hybrids (unpublished information).

Dried Chinese herbs have also been analyzed by RAPD. The fingerprints of the dried roots of *P. ginseng, P. quinquefolius*, *P. notoginseng*, and their adulterants were generated for identification purpose (Shaw and But, 1995). Several herbal drugs on the market still cannot be identified based on their morphological or histological characteristics. In the present study, RAPD analysis was employed to discriminate eight kinds of Coptidis Rhizomes (huanglien) from one another and from their replacement, Picrorhizae Rhizoma (Huhuanglien). The molecular identification of Chinese herbal medicines is essential because their products are usually sold in powder form and cannot be identified according to morphological or histological characteristics. RAPD, requiring only limited DNA, provides an accurate way to differentiate the materials. We hope that the technique can be an alternative tool with ap-

[5]Corresponding author.

plications to the quality control of Chinese herbal medicines.

Materials and Methods

Plant Materials

Eight samples of Coptidis Rhizomes and one sample of Picrorhizae Rhizoma used in the study were provided by National Laboratories of the Food and Drug Department of the Health Executive Yuan. The nine samples contain: 1. *Coptis chinensis* Franch (Ranunculaceae); 2. *C. deltoidea* C. Y. Cheng et Hsiao; 3. *C. teetoides* C. Y. Cheng; 4. *C. omeiensis* (Chen) C. Y. Cheng; 5. *C. quinquefolia* Miquel; 6. *C. japonica* Makino var. *dissecta* Nakai; 7. *C. trifolia* (L.) Salisb; 8. *C. teeta* Wall; 9. *Picrorhiza scrophulariaeflora* Pennell (Scrophulariaceae).

DNA Extraction

Plant tissue was washed in 70% ethanol for 5 min and in sterile deionized water for 1 min, using sonication to avoid surface contamination. After being air dried, the sample was ground into powder with mortar and pestle. DNA were extracted from various dried rhizomes using a modified CTAB (cetyltrimethylammonium bromide) procedure (Rogers and Bendich, 1985). Approximately 0.1 g of the powdered rhizome was added to 1.2 ml of 2X CTAB extraction buffer [2% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 0.2% 2-mercaptoethanol]. The suspension was incubated in a water bath at 90°C for 30 min with occasional shaking, then cooled to room temperature and extracted with one vol. aqueous phenol/chloroform/isoamyl alcohol (25:24:1) twice. After centrifugation at 12,000 *g* for 10 min, 0.1 vol. 65°C 10% CTAB buffer [10% CTAB; 0.7 M NaCl] was added to the upper aqueous. The mixture was extracted by phenol/chloroform/isoamyl alcohol extraction once more. The two phases were separated by centrifugation, and 1 vol. CTAB ppt buffer [1% CTAB; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0] was added to the aqueous phase. The mixture was centrifuged at 12,000 *g* for 20 min. The pellet was washed with 70% ethanol twice, then dissolved in 450 µl sterilized 1/10X TE. The solution was used for RAPD reaction.

RAPD Reaction

Twenty decamer oligonucleotide primers (kit T) for RAPD analysis were purchased from Operon Technologies Inc. (Alameda, Calif., U.S.A.). One µl of DNA sample (= 0.22 mg powdered rhizome) was amplified in 25 µl of a PCR mixture consisting of 1X PCR buffer (Boehringer Mannheim, Germany), 0.2 mM dNTP, 0.8 µM primer, and 0.625 U T*aq* polymerase (Boehringer Mannheim). For DNA amplification, a Perkin Elmer Cetus 2400 DNA thermocycler was programmed for 45 cycles of 20 sec at 94°C, 40 sec at 45°C, and 50 sec at 72°C. The RAPD fragments were separated electrophoretically on 1.5% agarose gel in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) and stained with ethidium bromide.

Results and Discussion

The RAPD technique has frequently been used for detection of genetic variability in plants. The advantages of the approach are: the limited amount of DNA required, procedure simplicity, and the absence of any need for isotopes or prior genetic information. These characteristics are especially advantageous for the identification of Chinese herbal medicines because little DNA exists in the dried materials, and sequence data are difficult to obtain. To elevate the quality of extracted DNA from Coptidis Rhizome, a high concentration of CTAB was used to remove a large amount of protein, polysaccharide, and alkaloids from the material. It has also been reported that the GTC (guanidine isothiocyanate) method can efficiently extract genomic DNA for RAPD reaction from the fresh and dried root of the *Panax* species (Ozeki et al., 1996). In the present study, the concentration of template DNA was too low to determine by spectrophotometer, so we tested a series of RAPD reactions with various amounts of DNA using one selected primer which generated consistent patterns among the samples used. The appropriate dilution of the DNA solution was therefore determined. The fingerprints of RAPD by varying amounts of DNA showed the presence or absence of some products, which resulted in a decrease of fidelity (Munthali et al., 1992). Extracted DNA quality is considered the major factor affecting the reproducibility of RAPD (Micheli et al., 1994). DNA contamination and wound DNA both will easily introduce variable RAPD patterns.

Of the twenty primers screened, four, OPT-09: 5'CACCCCTGAG3', OPT-15: 5'GGATGCCACT3', OPT-18: 5'GATGCCAGAC3', and OPT-20: 5'GACCAATGCC3', showing distinct and polymorphic fingerprints were selected to differentiate these Coptidis Rhizomes (Figure 1). For decreasing RAPD products, we elevated the annealing temperature to 45°C. In the OPT-9 reaction (Figure 1A), only one or two fragments were produced for each sample. None of them showed the same pattern for each other except *C. deltoidea* and *C. teeta*. A 350 bp product produced from a blank reaction was proved not homologous with the products from *C. teetoides* and *C. japonica* in Southern hybridization (data not shown). In the OPT-15 reaction (Figure 1B), all sample patterns were different from each other except *C. quinquefolia, C. japonica* var. *dissecta*, and *C. teeta* while *P. scrophulariaeflora* revealed a significant deviation in the RAPD fingerprint from the *Coptis* samples. The products from the OPT-18 reaction (Figure 1C) were fewer than from the OPT-15 reaction. The RAPD results between repetitive reactions sometimes changed in the strength of the products, while the patterns were still reproducible. In RAPD analysis, the reaction conditions should be stringently controlled to obtain high reproducibility. The reproducibility of RAPD is affected by DNA quality, primer concentration, different thermal cyclers, and the brand of DNA polymerase used (Meunier and Grimont, 1993; MacPherson et al., 1993; Ellsworth et al., 1993). In the OPT-20 reaction (Figure 1D), a 670 bp marker was present

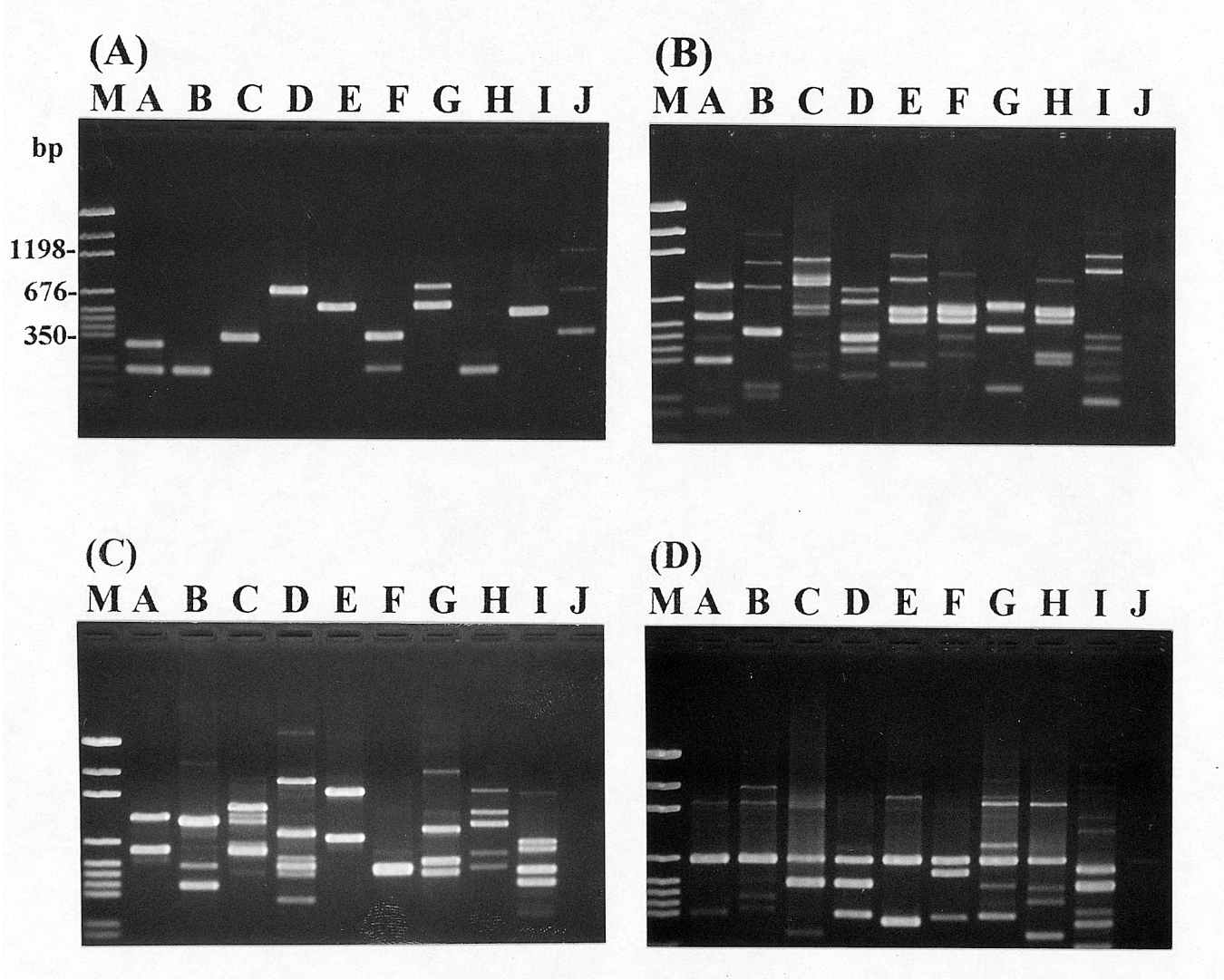

Figure 1. The RAPD fingerprints generated with (A) OPT-09 primer (B) OPT-15 primer. (C) OPT-18 primer (D) OPT-20 primer. M: DNA marker; A: *Coptis chinensis*; B: *C. deltoidea*; C: *C. teetoides*; D: *C. omeiensis*; E: *quinquefolia*; F: *C. japonica* var. *dissecta*; G: *C. trifolia*; H: *C. teeta*; I: *Picrorhiza scrophulariaeflora*; J: blank reaction.

in all *Coptis* species used in the study, but not in *P. scrophulariaeflora.* The marker may be used to distinguish between *Coptis* species and their replacement.

Direct amplification from solid tissue samples without DNA extraction has been reported (Panaccio et al., 1993; Williams and Ronald, 1994). The efficiency of the PCR reaction was dependent on the characteristics of the sample. Low polysaccharide and polyphenolic content in the sample was thought to be suitable for direct PCR. In the study, we failed in the directed amplification with dried material. Few DNA released and high metabolites existing in the dried rhizomes might have been the limiting factors. Thomson and Henry (1995) developed an extracted buffer system, which released the most DNA from the dried leaves of several crops and was successful for directing RAPD reactions. Application of the approach to dried Chinese herbal medicines is underway to simplify the procedure of identification.

The available components content in Chinese herbal medicines varies with the species of plant, so the work of identification is important to the market. The RAPD technique provides a sensitive and fast method which facilitates the identification of a large number of herbs. In the present study, RAPD was employed to differentiate eight *Coptis* species, the fingerprints of which easily showed polymorphisms among the species. The samples purchased from stores were assessed by RAPD using these selected primers. The procedure of DNA extraction was also modified to apply to other kinds of Chinese herbal medicines, such as Folium, Semen, and Fructus in subsequent studies.

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以逢機擴增多型性 DNA 鑑別乾燥根莖之黃連品種

鄭可大[1] 張憲昌[2] 蘇慶華[3] 徐鳳麟[4]

[1]私立臺北醫學院生藥學研究所
[2]行政院衛生署藥物食品檢驗局
[3]私立臺北醫學院生物技術中心
[4]私立臺北醫學院藥學系

本研究以逢機擴增多型性 DNA （RAPD） 分析技術鑑別八個黃連品種及其一代用品－胡黃連。全 DNA 自乾燥的中藥材根莖樣品中抽出，並分別用二十條含十個逢機排列之核酸酸的引子，進行 RAPD 分析。結果有四條引子反應的產物，在各個樣品之間，顯示再現性之不同電泳圖譜。此四條引子分別為 OPT -09、OPT -15、OPT -18 及 OPT -20。不同的引子，在黃連品種的遺傳變異上，具有不同的偵測能力。RAPD 提供快速、有效鑑定品種的方法，篩選反應效果良好的引子，其結果將可作為中藥品質管制的參考。

關鍵詞：黃連；指紋分析；品種鑑定；逢機擴增多型性 DNA 。