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Identification of *Atractylodes* plants in Chinese herbs and formulations by random amplified polymorphic DNA

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KEY WORDS Chinese herbal drugs; *Atractylodes*; DNA

ABSTRACT

AIM: An efficient, precise, and sensitive method for identifying *Atractylodes* plants has been established and will contribute significantly to quality control and scientific analysis in Chinese traditional medicine. **METHODS**: Twenty primers were applied for setting up the RAPD (randomly amplified polymorphic DNA) markers of *Atractylodes* plants, *Atractylodes lancea* DC (*A lancea* DC), *Atractylodes japonica* Koidz (*A japonica* K), and *Atractylodes ovata* DC (*A ovata* DC). The primer OPF03, OPF05, and OPF14 could discriminate them successfully. The results were also able to apply on the Chinese formulations with *Atractylodes* purchased from local markets. **RESULTS**: RAPD was used to investigate phylogenetic relationships among and within closely related species. RAPD analysis reflects heritable changes in the nucleotides sequence in both the coding and noncoding regions, because it is conducted directly from the DNA level. This work first conducted RAPD analysis of *Atractylodes* plants to establish their RAPD makers. **CONCLUSION**: The RAPD markers could be applied extensively in the Chinese herbal formulations.

INTRODUCTION

Atractylodes, Compositae, are perennial herbs distributed in East Asia[1]. The dried rhizomes of the plants are generally used as main ingredients in Chinese formulations for treatment of gastrointestinal diseases. Three species are frequently used in Chinese traditional medicine: *Atractylodes lancea* DC (*A lancea* DC), *Astractylodes japonica* Koidz (*A japonica* Koidz), and *Atractylodes ovata* DC (*A ovata* DC). The somatic chromosome of the three *Atractylodes* plants has been reported to be $2n = 24$[2]. The major essential oils of *Atractylodes* are sequiterpenes. A *japonica* Koidz, called "Tsang-Chu (Cangzhu)" (TC) in Chinese, contains large quantities of atractylon as well as atractylodin[3,4]. Meanwhile, *A lancea* DC and *A ovata* DC called "Pai-Chu (Baizhu)" (PC) in Chinese, contain hinesol and β-eudesomol[5,6].

A lancea DC and *A ovata* DC grow wildly in the lower reaches of the Yangtze River (Yangzi River) in China[7]. Meanwhile, *A ovata* DC is cultivated in Henan, south of the Yellow River, where it is called "Chian-Ping-Shang-Teng (Jiangping Shangdeng)" (CPST) and in Hunan, a province at south of the Yangtze River, where it is known as "Yi-Teng (Yi-deng)" (YT). Their geological isolation makes the two *A ovata* DC somewhat different in morphology, although they are of the same species.

Chinese herbs have been used over five thousand years, and prepared in many ways, including powders, pastes, pills, and so on. Chinese herbs have been classified and identified using morphological and chemical methods[8], but the results have generally been ambiguous, especially in the case of herbal formulas, which typically contain numerous individual herbs. Because the raw material tissues were difficultly preserved intact enough to control the quality of the herbal samples. Thus, DNA testing of carefully preserved samples of the Chinese medicine was the best way of overcoming the difficulties caused by complicated sources and a lack of

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reliable and precise identification methods.

Genetic markers are very valuable in practical breeding programs and genetic research[9]. RAPD (Randomly amplified polymorphic DNA) or AP-PCR (arbitrarily primed polymerase chain reaction)[10,11] was used to investigate phylogenetic relationships among and within closely related species[12-17]. RAPD analysis reflects heritable changes in the nucleotides sequence in both the coding and noncoding regions, because it is conducted directly from the DNA level.

The *Atractylodes* plants sold in local markets in Taiwan are imported from difference sources. Thus significant quality variations exist and can influence the effects of the medicine. This work first conducted RAPD analysis of *Atractylodes* plants to establish their RAPD makers. The results were successfully applied to identify the origins of *Atractylodes* plants from the Chinese formulations, Pingwei San and Wuling San.

MATERIALS AND METHODS

Materials The Chinese herbal formulations, Pingwei San and Wuling San, were purchased from local herb markets in Taiwan. The *Atractylodes* plants were identified based on their appearance and pharmacognostic histological anatomy. Four species samples of the *Atractylodes* plants, *A lancea* DC, *A japonica* Koidz, and *A ovata* DC, including CPST and YT, were obtained.

DNA isolation High molecular weight genomic DNAs were isolated by following a slightly modified version of the procedure described previously[18-21]. In a chilled mortar, the samples were fixed in liquid nitrogen, ground into a fine powder, and collected using the lid of a sterile eppendorf tube. Four hundred microliters of extraction buffer (urea 6.5 mol/L, NaCl 350 mmol/L, Tris HCl 50 mmol/L, pH 8.0, edetic acid 20 mmol/L, 2 % sarcosin, 5 % phenol reagent) preincubated at 65 ℃ was added. Following vigorous mixing and the addition of 100 μL 20 % SDS (sodium dodecyl sulfate), the mixture was placed in tubes and kept at 65 ℃ for 15 min. An equal volume of isoamyl alcohol-chloroform was then added and shaken gently until it emulsified. The tubes were centrifuged at 12 000 × *g* for 15 min, producing a clear supernatant fraction with some floating residues. Cold isopropanol was added for precipitating genomic DNA. The precipitate was dried and treated with DNase and free RNase at 37 ℃ for 10 min. Then, the samples were extracted using the phenol/chloroform method. The DNA pellet was vacuum dried and dissolved in an appropriate volume of TE buffers (Tris HCl 10 mmol/L, pH 6.5, edetic acid 1 mmol/L). A set of standard DNA stock solution was prepared for PCR at a fixed concentration of 10 mg/L and stored at −20 ℃.

RAPD reaction PCR reaction mixture (50 μL final volume) contained approximately 20 ng genomic DNA, 4 mmol/L each of dNTPs (Promega), 1DZB Buffer (Finnzymes), 1 unit of DyNaZyme (Finnzymes), and 20 pmol of random primer (OPERON technologies kit F; OPF). The samples were subjected to enzymatic amplification in a MJ Research Inc. Programmable Thermal Controller 45 times, according to the following thermal cycle: 1 min at 92 ℃, 3 min at 35 ℃, and 2 min at 72 ℃. After the final cycle, samples were incubated for a further 12 min at 72 ℃ then held at 4 ℃ prior to analysis. Fragments generated by amplification were separated according to size on 1.5 % agarose gels run in 1XTBE (Tris HCl 89 mmol/L, pH 8.3, boric acid 89 mmol/L, edetic acid 5 mmol/L) with ethidium bromide and visualized under illumination with ultraviolet light (312 nm).

RESULTS

RAPD analysis of genomic DNA from *Atractylodes* plants The genomic DNAs extracted from *Atractylodes* species were analyzed using RAPD with 20 random decamer oligonucleotide primers. Primer 5 (OPF05), 5′ CCGAATTCCC 3′, displayed a distinct and unique band of RAPD markers, in about 450 bp (Fig 1). The result indicated OPF05 could be used to detect *Atractylodes* species because the marker is conserved. Additionally, the PCR product of TC, *A japonica* Koidz, from primer 3 (OPF03), 5′ CCTGATCACC 3′, can only see the fingerprints of 200 bp on agarose analysis (Fig 2). Meanwhile, the other products derived from PC, *A lancea* DC and *A ovata* DC, including CPST and YT, can see not only the fingerprints of 200 bp but also the fingerprints of 400 bp. The RAPD markers generated from OPF03 can be distinguished between TC and PC (Fig 2). Different band patterns were clearly revealed after amplification by using primer 14 (OPF14), 5′ TGCTGCAGGT3′. The PCR products of A lancea DC were 200, 300 and 400 bp, and that of A ovata DC (CPST and YT) were 150, 250, and 350 bp, the bands of PCR from *A japonica* K were 100 and 150 bp (Fig 3).

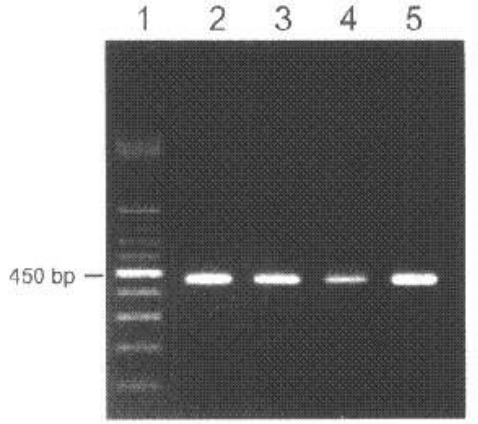

Fig 1. The RAPD fingerprints of *Atractylodes* species generated with OPF05 primer. Lane 1: DNA marker; Lane 2: *A ovata* DC (CPST); Lane 3: *A ovata* DC (YT); Lane 4: *A lancea* DC; and Lane 5: *A japonica* K.

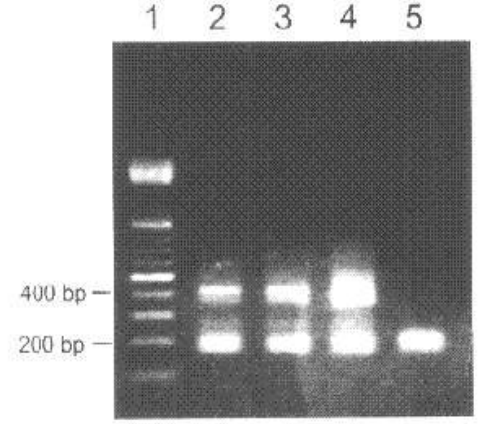

Fig 2. The RAPD fingerprints of *Atractylodes* species generated with OPF03 primer. Lane 1: DNA marker; Lane 2: *A ovata* DC (CPST); Lane 3: *A ovata* DC (YT); Lane 4: *A lancea* DC; and Lane 5: *A japonica* K.

RAPD analysis of *Atractylodes* plants in Chinese herbal formulations RAPD markers were applied to identify the *Atractylodes* species in Chinese formulations sold in local markets. Two formulas, Pingwei San and Wuling San, were selected because they contained *Atractylodes* plants. First, OPF05 was used to identify the *Atractylodes* plants in the Chinese formulas. The RAPD markers of the formulas with OPF05 were 450 bp conserved from among the *Atractylodes* plants (Fig 4). OPF03 and OPF14 were applied in sequence on the

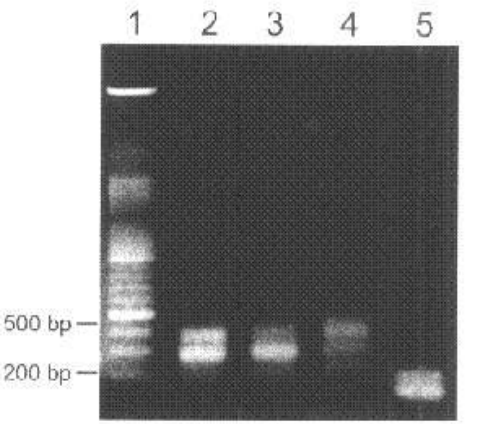

Fig 3. The RAPD fingerprints of *Atractylodes* species generated with OPF14 primer. Lane 1: DNA marker; Lane 2: *A ovata* DC (CPST); Lane 3: *A ovata* DC (YT); Lane 4: *A lancea* DC; and Lane 5: *A japonica* K.

PCR for classifying the species of the *Atractylodes* plants. In the RAPD analysis with OPF03, matching RAPD patterns (200 bp of PCR product) indicated that the *Atractylodes* plant of the Pingwei San was *A japonica* Koidz. Similarly, YT was found to be one of the components of Wuling San (Fig 5). The results demonstrated that the RAPD markers could be applied extensively in the Chinese herbal formulations.

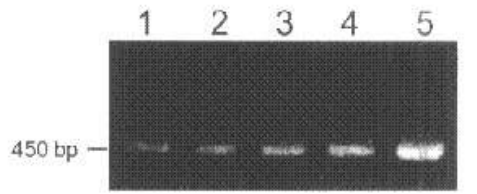

Fig 4. The RAPD fingerprints of *Atractylodes* species generated with OPF05 primer. Lane 1: *A ovata* DC (CPST); Lane 2: *A lancea* DC; Lane 3: *A japonica* K; Lane 4: Pingwei San; and Lane 5: Wuling San.

DISCUSSION

RAPD is a simple and rapid means of establishing the polymorphism between biology without DNA sequencing. Only about a nanogram of DNA is required and the samples can be assayed in large quantities. The primers in this work reflected that genetic polymorphism among *Atractylodes* plants was obtained from the twenty randomly designed decamer primers screened in the RAPD

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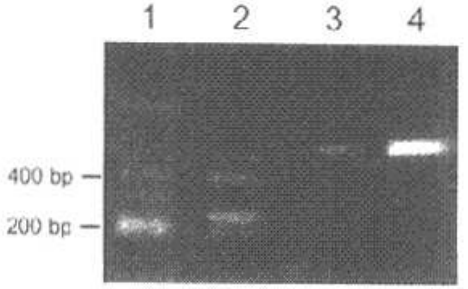

Fig 5. The RAPD fingerprints of *A ovata* DC (YT), *A japonica* K, Pingwei San, and Wuling San generated with OPF03 primer. Lane 1: *A japonica* K; Lane 2: Pingwei San; Lane 3: *A ovata* DC (YT); and Lane 4: Wuling San.

analysis. The RAPD markers from OPR05 are conserved in *Atractylodes* plants. However, those from OPR03 are only conserved in *A lancea* DC and *A ovata* DC. The analytical results indicated that *A lancea* DC was genetically resembled *A ovata* DC. The RAPD markers for differentiating between CPST and YT are close because the samples are from the same species but different sources. The RAPD markers were gathered and analyzed using a SIMQUAL (similarity for qualitative data) routined to generate Jacquard's similarity coefficient. These similarity coefficients were used to construct dendrograms using the unweighted pair-group method with arithmetic averages (UPMGA). The genetic relationships were established (unpublished data) and were found to be the same as the results from the morphological and chemical classification.

The investigation is the first to report RAPD analysis of *Atractylodes* plants in Chinese herbal formulas. *A japonica* K was identified in the *Atractylodes* plant of the Pingwei San and YT was identified in Wuling San by using RAPD markers. According to classical Chinese herbal manuals, Wuling San contains PC, *Poria cocos*, *Polyporus umbellatus*, *Alisma orientalis*, and *Cinnamomum*, while Pingwei San consisted of TC, *Magnolia officinalis*, *Citrus reticulata*, *Ziziphus jujuba*, and *Zingiber officinale*. Thus the results of this study was correspond to the traditional recipes for the herbal formulas studied. Accordingly, an efficient, precise and sensitive method for identifying *Atractylodes* plants has been established, and will contribute significantly to quality control and scientific analysis in Chinese traditional medicine.

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以任意放大多型性 DNA 之方法鉴别中药及复方中的术类

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关键词 中草药；术类；DNA

目的：藉由分子生物学的技术，鉴别中药复方中的单品药材—术类，以提升药材分析的灵敏度，进而促使药材品管升级. **方法**：藉由任意序列的引子，先以单品药材—术类为模板来建立该药材常用的品种，苍术及白术等之 RAPD 标志，以应用于复方之检测. **结果**：在 RAPD 的标志分析中，可得知引子 OPF05 对术类具有保留性，而引子 OPF03 则仅在白术有保留性，因而可以应用于区分苍术与白术. 另外，白术诸种中，可由引子 OPF14 加以鉴别，而同种不同产地的白术，江坪上等与一等则可由引子 OPF13 得到区别. **结论**：中药材的鉴定，传统上以外型及化学方法加以区分，在本论文中，在分子层次上进行鉴定，除了灵敏度增加外，在复方及粉剂中，亦可有良好的鉴别效果.

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