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Original Articles

# Identification of Angelica Species by Pyrosequencing

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**Objective :** Angelica species are some of the most medicinally important materials in Oriental medicine. This study was performed to determine if Angelica species could be identified by genetic analysis and to verify Pyrosequencing analyses, which were used to assess genetic variation.

**Methods :** The DNAs of *Angelica acutiloba, Angelica gigas* and *Angelica sinensis* were extracted. We have investigated the typing of single-base variations of Angelica species in DNA by using Pyrosequencing.

**Results :** *Angelica gigas* showed a different pattern compared with *Angelica acutiloba* and *Angelica sinensis*. The peak of Angelica gigas was very weak in the second C nucleotide base compared with that of the others. The peak of *Angelica acutiloba* was present in the fourth C nucleotide base compared with that of the others. From these results we verified that our Angelica species-specific sequencing primer was well designed.

**Conclusion :** Pyrosequencing analysis might be able to provide the identification of the Angelica species.

***Key Words:*** *Angelica acutiloba, Angelica gigas, Angelica sinensis*, Pyrosequencing, Identification

## Introduction

Angelica species (*Angelica acutiloba, Angelica gigas* and *Angelica sinensis*) are some of the most medicinally important genera in oriental medicine and have been used in traditional Korean, Chinese and Japanese medicines for a long time[1]. For centuries, Angelica species have been used traditionally for invigorating blood circulation[2,3]. The herbal supplement market has been growing rapidly in the past decades[4,5]. Angelica species are regarded as important medicines in Korea, and China. Between these species, *Angelica gigas* is known to tonify the blood more effectively than *Angelica acutiloba* and *Angelica sinensis*. So in the Korean market, the price of *Angelica gigas* is higher than that of other Angelica species.

Many commercial Angelica species products are extremely difficult to identify in the form of slice, powder, or extract. The identification via analysing chemical profiles is also very difficult for many variables such as the soil condition, climate, and nutritional factors.

This study was performed to determine if Angelica

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species could be identified by genetic analysis of Pyrosequencing method, which was used to assess genetic variation.

## Materials and Methods

### 1. Samples and Purification of DNA

*Angelica acutiloba*, *Angelica gigas* and *Angelica sinensis* samples were obtained from the Herbal Garden of Kyung Hee University, Korea. 3 g of Angelica species plant material in the form of whole root was firstly minced with a sterile scalpel and pulverized to a powder using a sterilized mortar and pestle. A DNA isolation kit (DNeasy, No. 69104) (Qiagen Inc., Valen cia, CA) was used as described in the manufacturer' s instructions with slight modifications. 300 mg of the powdered sample was used in the purification procedure. Before sample elution, the columns were dried at 37℃ for 5 minutes to evaporate residual ethanol. Samples were eluted in a total volume of 200 ul of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

### 2. Preparation for Pyrosequencing and Genotyping

The extracted DNA was amplified by polymerase chain reaction (PCR). The internal transcribed spacer regions (ITS) of each Angelica species was amplified using 25 ng of DNA, 5 pmol of each primer; forward was 5' - AAGGATCATTGTCGAATCCT -3' and reve rse was 5' - ACGGACGTACAATTCAGTTTTAA -3' . The PCR amplification was performed by using 0.5 unit Taq polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The 30 ul of PCR reaction mixtures were 10 mM Tris-HCl, pH 9.0, 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1% Triton-X 100, 0.01 % [v/v] stabilizer, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 0.1 M of each oligonucleotide primer. The PCR steps were denaturation of 5 minutes at 95℃, 30 cycles of 30 seconds at 95℃, 30 seconds at 60℃, and 30 seconds at 72℃ with a PCR System (Astec, Fukuoka, Japan). The reverse primer was biotinylated toallow the preparation of single-stranded DNA. The quality of PCR products was controlled by 1.5% of agarose gel electrophoresis.

DNA Preparation for pyrosequencing was performed according to manufacturer' s standard protocol (Pyrosequencing AB, Uppsala, Sweden)[6]. The streptavidin sepharose beads (Streptavidin Sepharose HP, Amersham Pharmacia Biotech, Uppsala, Sweden) were immobilized to PCR products. The sequencing primer of Angelica species was 5' - GCAAAA TGACCCGCT -3' and it was designed so that the terminal residue hybridized to the base immediately adjacent to the mutation site from Pyrosequencing AB (http://www.pyrosequencing.com). By incubation at room temperature for 10 minutes, 20 ul of biotinylated PCR products were immobilized onto streptavidin-coated sepharose beads, the immobilized PCR products were transferred to a Millipore 96-well filter plate (Millipore, Bedford, MA, USA). Vacuum was used to eliminate the different solutions and reagents to obtain pure, single-stranded DNA while the beads remained in the wells)[7]. In 55 ul of 4 M acetic acid containing 0.35 uM of sequencing primer the beads with the immobilized template were resuspended. Then the 45 ul of suspension was transferred to a PSQ 96 plate (Pyrosequencing AB, Uppsala, Sweden)[8].

By using PSQ 96 Sample Prep Thermoplate (Pyrosequencing AB, Uppsala, Sweden) the PSQ 96 plate containing the samples was heated at 90℃ for 5 minutes for sequencing primer annealing, and moved to room temperature for 10 minutes. Then the PSQ 96 Plate was placed into the process chamber of the PSQ 96 instrument (Pyrosequencing AB, Uppsala, Sweden)[9]. The enzymes, substrates, and nucleotides were dispensed from a reagent cassette into the wells by

using the PSQ 96 SNP Reagent Kit (Pyrosequencing AB, Uppsala, Sweden), The light that was generated when a nucleotide is incorporated into a growing DNA strain. From this process the polymorphism of Angelica species was genotyped automatically.

## Results and Discussion

There are several types of DNA sequence variation, including single base pair difference - Single Nucleotide Polymorphisms (SNP), differences in the copy number of repeated sequences, and insertions and deletions. The first is the most frequent. The method for identifying the origin of a product is very important. So characteriza tion and scoring of genetic variations is increasingly necessary to correlate phenotype and genotype differe nces.

We have investigated the possibility of typing single-base variations in the Angelica species' DNA by using a recently developed sequencing technique, called Pyrosequencing. To determine if Angelica species could be identified by Pyrosequencing method, we performed a Pyrosequencing analysis of *Angelica acutiloba*, *Angelica gigas* and *Angelica sinensis* samples cultivated in Herbal Garden of Kyunghee University, Korea. The Pyrosequencing results of *Angelica species* showed different patterns. *Angelica gigas* showed a different pattern compared with Angelica acutiloba and Angelica sinensis. The peak of *Angelica gigas* was very week in the second C nucleotide base compared with that of the others. The peak of *Angelica acutiloba* was present in the fourth C nucleotide base compared with that of the others.

We designed the Angelica species specific sequencing primer to identify *Angelica acutiloba*, *Angelica gigas* and *Angelica sinensis*. In sequence *Angelica acutiloba* has a CGTC nucleotide base, *Angelica gigas* has a CGTT nucleotide base and *Angelica sinensis* has a TGTA nucleotide base (Fig. 1-3). From these results we verified that our Angelica species -specific sequencing primer was well designed.

At present, Pyrosequencing is performed in an automated microtiter-based Pyrosequencer instrument, which allows simultaneous analysis of samples within 15 minutes. Each round of nucleotide dispensing takes approximately 1 minute and thus offers a rapid way to determine the exact sequence of the genetic variations,

Genetic identification of traditional Chinese herbs

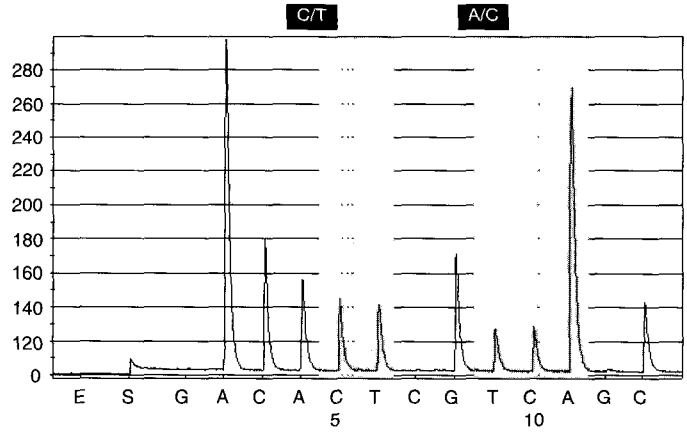


**Fig. 1.** Pyrosequencing of *Angelica acutiloba*

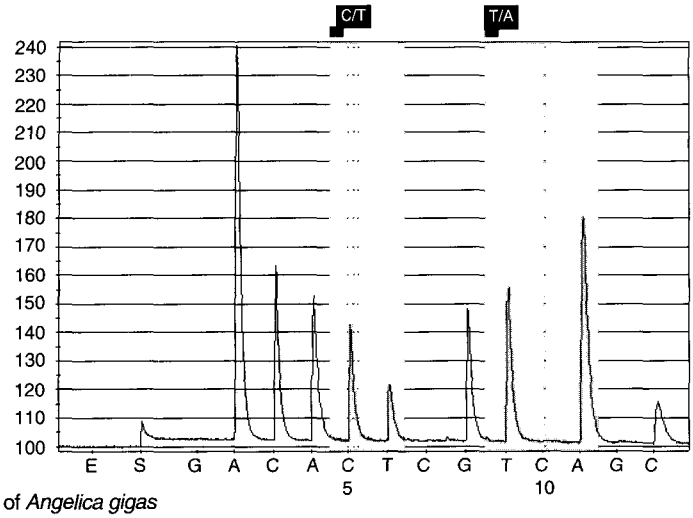


**Fig. 2.** Pyrosequencing of *Angelica gigas*

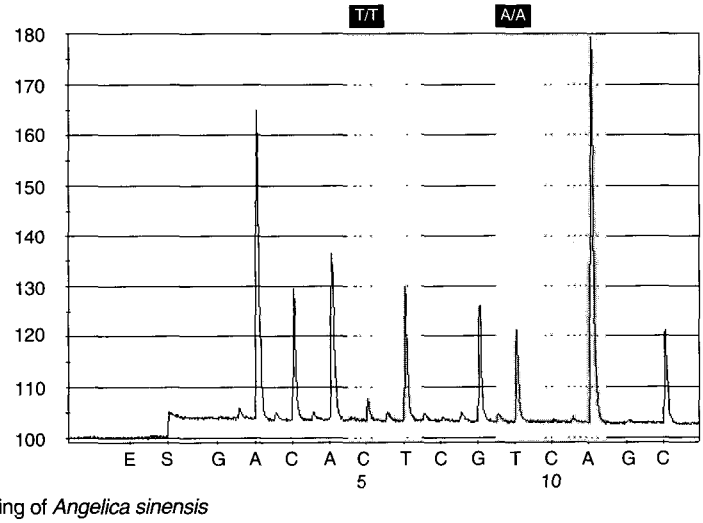


**Fig. 3.** Pyrosequencing of *Angelica sinensis*

should help to ensure the safe use of Chinese herbs especially in case the plant has toxicity. So, the method for identifying the origin is very important.

These results suggest that Pyrosequencing methods are suitable for authentication of the concerned Angelica species. This work shows that typing of genetic variations can efficiently be performed by Pyrosequencing by using an automated system for pattern recognition software. In conclusion, Pyrosequencing analyses might be able to identify the Angelica species. Now Pyrosequencing analyses might be able to provide the identification of Angelica species.

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