Efficiency of improved RAPD and ISSR markers in assessing genetic diversity and relationships in *Angelica sinensis* (Oliv.) Diels varieties of China

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**Abstract**

*Background:* *Angelica sinensis* is a well-known traditional Chinese medicinal plant. We aimed to assess the genetic diversity and relationships in *A. sinensis* cultivars collected from different locations of China and also some other *Angelica* species.

**Results:** We employed an improved random amplified polymorphic DNA (RAPD) technique for the amplification of DNA materials from ten *Angelica* cultivars, and the results were verified by inter-simple sequence repeat (ISSR) analysis. Twenty six RAPD primers were used for RAPD, and the amplified bands were found highly polymorphic (96%). Each primer amplified 8–14 bands with an average of 10.25. The cluster dendrogram showed that the similarity coefficients ranged from 0.41 to 0.92. The similarity coefficients were higher among different cultivars of *A. sinensis*, and lower among different species. Twenty ISSR primers were used for the amplification, and each primer generated 6–10 bands with an average of 7.2 bands per primer. The cluster dendrogram showed that the similarity coefficients ranged from 0.35 to 0.89.

**Conclusions:** This study genetically characterized the *Angelica* species, which might have a significant contribution to the genetic and ecological conservation of this important medicinal plant. Also, this study indicates that the improved RAPD and ISSR analyses are important and potent molecular tools for the study of genetic diversity and authentication of organisms.

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1. Introduction

The herb *Angelica sinensis* (Oliv.) Diels, also *A. sinensis*, from Apiaceae family, has a long history of usage in Traditional Chinese Medicine (TCM). This plant is indigenous to China, and locally known as ‘Dang gui’ (当归). *A. sinensis* is often called ‘female ginseng’, as its major usage is in female health related diseases [1,2,3]. In addition to effect in ameliorating female reproductive complications, *A. sinensis* also possesses a number of other medicinal or health beneficial activities [4,5]. For example, different extracts or specific active ingredients from *A. sinensis* show potent anticancer activities [6,7]. Extracted natural products from *A. sinensis* have been proved successful also against cardiovascular complications, hepatic diseases, inflammation, etc. [4,8,9,10]. Although the traditional medicinal formula of *A. sinensis* is available in many countries, the plant is indigenous to China only. It is suspected that it may face extinction threat in future. Molecular studies and genetic characterization of this plant might be a useful tool for the ecological and genetic conservation of this valuable medicinal plant.

*A. sinensis* is mainly cultivated in Sichuan, Gansu, Hubei and Yunnan of P.R. China. The cultivars native to Min county of Gansu are famous for their official components and good qualities. Due to the slightly different morphological characteristics of *Angelica* in their dried roots, several other species are usually considered to be “Dang gui” by error. The species of *Angelica acutiloba* and *Levisticum officinale* are the main adulterants or substitutes for *A. sinensis* in China. *L. officinale* is native to southwest Asia and Europe, and was introduced into China in 1957 [11]. More comprehensive molecular systematic studies show that *L. officinale* and *A. sinensis* are sister taxa. *A. acutiloba* native to Japan and Korea, is distributed in the northeast of China. The root is used as a substitute for the crude drug of *A. sinensis*; however, the substitutes and adulterants have little medicinal importance. Inevitably, the confusion may compromise the genuine resources and therapeutic effect of this TCM, and even imperil the safety of consumers.

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In the era of modern biotechnology, several molecular techniques have been developed for the genetic studies and characterizations of different organisms, among which random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP), and simple sequence repeat (SSR) analysis are well established and widely used [12,13,14,15]. RAPD is a more reliable molecular technique for the genetic characterization of organisms, especially of plants. However, this technique was reported to have some limitations like poorer reproducibility and lower production. We have developed an improved method of RAPD analysis by prolonging the RAMP time, and hence name RAMP-PCR instead of traditional RAPD-PCR [16].

There are few reports on the genetic characterization of A. sinensis, among which most are very preliminary works and are more than a decade long old [17,18]. In this study, we have analyzed ten samples of Angelica cultivars and adulterants by using improved RAPD analysis and then verified the results by using ISSR analysis. The detailed genetic characterization and distance analysis among the samples might have significant roles in the genetic and ecological conservation of Angelica species.

2. Materials and methods

2.1. Experimental materials and reagents

The RAPD primers (SBS Genetech Corporation, Table 1), ISSR primers (UBC Primer Set #9, Table 2), 2 × PCR Taq MasterMix (TianGen Biotech Co., Ltd, Beijing, China) and DNA Markers (Takara Biotechnology Co. Ltd, Dalian, China) were reported previously [12]. Other reagents were analytical grade reagents as described at our previous experiments [16,19].

2.2. Collection of Angelica samples

A total of ten Angelica cultivars, including seven of A. sinensis from Minxian and Pingliang of Gansu, Miyanyang and Jiuzhaigou of Sichuan, Lijiang of Yunnan, Linzhi of Tibet and Enshi of Hubei, and A. acutiloba (Sieb. et Zucc.) Kitag. from Yanji of Jilin, A. acutiloba from Aba of Sichuan, and L officinale Koch from Changchun of Jilin, were collected from different geographic locations of China in this study (Fig. 1) (Table 1). The fresh leaf materials were sampled in fields and identified by Prof. Pixian Shui. The plants are deposited in the medicinal botanical nursery of Luzhou Medical College.

2.3. Extraction of DNA

Total genomic DNA was extracted from silica-gel-dried leaves and commercially crude materials using a modified CTAB (Cetyl trimethylammonium bromide) method described previously [12,20,21, 22]. Angelica plant materials were first fixed in solutions containing chloroform, PVP, and 2-hydroxy-1-ethanethiol (but without liquid nitrogen), and then ground into tiny pieces by silica (SiO₂) for the extraction of DNA. DNA quality was checked by a 0.8% agarose gel electrophoresis and spectrophotometry [19]. The final concentration of all DNA samples was adjusted to 10 ng/μL for PCR, and stored at -20°C till use.

2.4. RAPD-PCR

Twenty six SBS primers, purchased from SBS Genetech Corporation (Beijing, China) were initially evaluated for polymorphism by improved RAPD analysis, among which twenty four primers amplified DNA well with polymorphic profiles for data analysis (Table 2). Contents of the PCR system (10 μL) were prepared as follows: 1 μL of primers (2.5 μmol/L), 1 μL (10 ng) of Angelica species DNA template, 5 μL of 2 × PCR Taq Mastermix and 3 μL of ddH₂O. The PCR condition was as follows: Initial denaturation at 95°C for 90 s, followed by 40 cycles of 40 s at 94°C, 60 s at 36°C, 90 s at 72°C, and final extension of 5 min at 72°C. PCR of each accession was executed in a machine of “Applied Biosystems Veriti® 96-Well Thermal Cycler” (Life Technology, USA). The RAMP rate from annealing to extension was adjusted from 2.5°C/s (100% ramp rate) to 0.125°C/s (5% ramp rate) for Angelica cultivars using our previously established ramp PCR conditions [12], to compare the resolution and production of the two methods in the present study. All the PCRs were repeated three times for each of the 10 samples.

| Sample | Species | Source | No. |
|--------|---------|--------|-----|
| MX     | A. sinensis (Oliv.) Diels | Minxian, Ganshu | 001 |
| YJ     | A. acutiloba (Sieb. & Zucc.) Kitag. | Yanji, Jilin | 002 |
| AB     | A. acutiloba (Sieb. & Zucc.) Kitag. | Aba, Sichuan | 003 |
| PL     | A. sinensis (Oliv.) Diels | Pingliang, Gansu | 004 |
| HB     | A. sinensis (Oliv.) Diels | Enshi, Hubei | 005 |
| JZ     | A. sinensis (Oliv.) Diels | Jiuzhaigou, Sichuan | 006 |
| EP     | L officinale Koch | Changchun, Jilin | 007 |
| LJ     | A. sinensis (Oliv.) Diels | Lijiang, Yunnan | 008 |
| MY     | A. sinensis (Oliv.) Diels | Enshi, Sichuan | 009 |
| LS     | A. sinensis (Oliv.) Diels | Linzhi, Tibet | 010 |

Fig. 1. The localities of samples of Angelica cultivars from different regions in China. Spots in dark blue indicate cities and lines in light blue indicate the Yellow River (up) and the Yangtze River (down).
2.5. ISSR amplification

From the initial screening of thirty-five ISSR primers from UBC Primer Set #9, twenty primers were selected and amplified DNA well with polymorphic bands (Table 3). ISSR amplifications were performed in 10 μL reactions consisting 1 μL of 2.5 μmol/L primers, 1 μL of DNA template of Angelica samples, 5 μL of 2 × PCR Taq Mastermix, and 3 μL of ddH₂O. The PCR condition was as follows: Initial denaturation at 95°C for 90 s, followed by 35 cycles of 40 s at 94°C, 30 s at 50°C, 90 s at 72°C, and final extension of 5 min at 72°C [12]. PCR of each accession was executed in the above mentioned “Applied Biosystems Veriti® 96-Well Thermal Cycler”.

2.6. Agarose gel electrophoresis

Agarose gel electrophoresis was described previously [12]. The amplified PCR products were separated by electrophoresis on a 1.8% agarose gel in 1 × TAE buffer. Gels were visualized by 0.5 μg/mL ethidium bromide staining, and the images were documented using the ChemiDoc XR (Bio-Rad, USA) under UV. lights. Unambiguous and reproducible bands in successive amplifications were selected for scoring.

2.7. Data analysis

Clear bands in the gel profiles were recorded as present (1) and absent (0). The similarity matrix (SM) and the similarity index (SI) were calculated by using SM coefficient. The dendrogram based on unweighted pair method with arithmetic mean algorithm (UPGMA) was generated by the use of the SAHN module in a NTSYS pc 2.1 package [23].

3. Results

3.1. Comparison between regular and improved RAPD amplification technique for DNA analysis in Angelica cultivars

To increase the RAPD amplification efficiency and get more specific bands, PCR was first performed to amplify the DNA materials by adjusting RAMP time from annealing to extension with a ramp rate for 5% (0.125°C/s) and 100% (2.5°C/s) from ten Angelica cultivar samples in Table 1 with RAPD primers SBS-A1, SBS-A2, SBS-M18 and SBS-N20, respectively. Specifically, in primer SBS-A1, each sample amplified 1–5 bands in regular PCR (Fig. 2a, left panel), whereas the PCR products and DNA bands were clearly increased (3–7 bands) in RAMP PCR (where RAMP rate was adjusted from 100 to 5%) (Fig. 2a, right panel); In primer SBS-A2, each sample amplified 2–5 bands in regular PCR (Fig. 2b, left panel), the PCR products and DNA bands were clearly increased (3–9 bands) in RAMP PCR with a 5% RAMP rate (Fig. 2b, right panel); In primer SBS-M18, each sample amplified 1–4 bands in regular PCR (Fig. 2c, left panel), whereas the PCR products and DNA bands were clearly increased (4–9 bands) in RAMP PCR with a 5% RAMP rate (Fig. 2c, right panel); In primer SBS-N20, each sample amplified 2–6 bands in regular PCR (Fig. 2d, left panel), whereas the PCR products and DNA bands were clearly increased (3–7 bands) in RAMP PCR with a 5% RAMP rate (Fig. 2d, right panel). In addition, the signal intensities of the PCR bands at the corresponding bands in the left panels of Fig. 2 were stronger than that in the left panels, which demonstrates that the amount of production was higher by improved RAPD PCR than that by regular RAPD PCR. Thus, the improved RAPD condition was very useful in the genetic analysis of Angelica cultivars by prolonging ramp time from annealing to extension, where the production, resolution, and reproducibility of RAPD were significantly improved. Therefore, this improved condition was chosen to complete amplification assays with other primers for ten DNA samples of Angelica cultivars.

3.2. Amplification of DNAs of Angelica cultivars by RAMP RAPD

A total of twenty six primers were used in the improved RAPD analysis for the evaluation of DNA polymorphism, and twenty four primers (Table 1) obtained reproducible polymorphic amplification bands. Fig. 3a and Fig. 3b showed that six representative primers (SBS-N2, SBS-A2, SBSA14, SBS-M8, SBS-M18 and SBS-Q20) generated representative reproducible polymorphic amplification bands by RAMP PCR. From twenty four polymorphic amplification primers, a total of 205 bands were obtained, where each primer had 8–14 amplified bands with an average of 10.25 bands per primer. However, only a total of 126 bands were obtained, with an average of 6.3 bands per primer by regular RAPD PCR in the Angelica DNA samples (data not shown). The approximate range of band size was 150–2500 bp, and 96% of the bands were found polymorphic. These results demonstrate a high level of polymorphism in DNA samples of Angelica species by our improved RAPD technique.

3.3. Genetic distance and cluster analysis of RAMP RAPD

Based on the RAMP-PCR amplification profiles, a cluster dendrogram was obtained (Fig. 4a and Fig. 4b). The dendrogram showed that the similarity coefficients among the ten of Angelica cultivars ranged from 0.41 to 0.92. The similarity coefficient between samples 2 and 6 (A. acutiloba and A. sinesis) was minimum (0.41), while between samples 6 and 8 (A. sinesis from Jiuzhaigou and Lijiang) was maximum (0.92) (Fig. 4b).

3.4. Amplification of Angelica cultivar DNA by ISSR PCR

Twenty ISSR primers were amplified well, which produced 144 clear and reproducible fragments. Each primer had 6–10 amplified bands with an average of 7.2 bands per primer. The approximate range of band size was 200–1800 bp. The representative results are shown in Fig. 5 by ISSR primers UBC815, UBC855, UBC857, UBC808, UBC834 and UBC845. These results provide a clear detection of polymorphism in DNA samples of Angelica cultivars by ISSR amplification.

3.5. Genetic distance and cluster analysis of ISSR PCR

A cluster dendrogram was also obtained based on the ISSR banding profiles, and showed similar results with improved RAPD
in terms of similarity coefficients among ten varieties of Angelica cultivars (Fig. 6a and Fig. 6b). The dendrogram showed that the similarity coefficients among the ten samples ranged from 0.35 to 0.89. The similarity coefficient between samples 6 and 8 (A. sinensis from Jiuzhaigou and Lijiang) was maximum (0.89), and between samples 2 and 6 (A. acutiloba and A. sinensis) was minimum (0.35) (Fig. 6b).

4. Discussion

RAPD is a well-known molecular marker technique, which has been used for years as an important research tool for studying genetic characterization of organisms [11,12,13,24]. Moreover, RAPD has also been successfully used for the study of genetic diversity in newly found or synthetic species of organisms, which are agriculturally and
industrially significant. However, RAPD has been frequently complained for its poorer reproducibility and lower production. Like our previous studies [12,16,25,26,27,28], in this study we have overcome the limitation of traditional RAPD by employing an improved RAPD or RAMP-PCR. The DNA band number amplified by RAMP-PCR was nearly double than that by traditional RAPD-PCR, and also the amplifications were reproducible. Previous studies indicated that the number of bands produced by traditional RAPD amplifies an average of 5 bands per reaction [29]. Our RAMP-PCR amplified DNA band numbers from different Angelica samples were varied from 8 to 14, with an average of 10.25. The amplification bands were found highly polymorphic (96%), which indicates that the species of Angelica genus possess a big genetic distance from each other. The cluster dendrogram showed that the similarity coefficients among the ten of Angelica cultivars ranged from 0.41 to 0.92. The similarity coefficients were higher among different samples of A. sinensis, and lower among different species of Angelica genus. This indicates that the species within Angelica genus are genetically highly variable. This is the first complete RAPD analysis of Angelica cultivars from different regions of China. Some previous studies [17,18,30] attempted to investigate the
The major success of this study is to characterize the Angelica cultivars, especially *A. sinensis* genetically, which might have a significant contribution to the genetic conservation and ecological conservation of this important medicinal plant. Also, we indicate that the improved RAPD analysis is an important and potent molecular tool which can be used for the genetic characterization and authentication of organisms, especially medicinal plants.

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### Conflict of interest

None.

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