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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.

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 possess 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 officinal 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 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, Mianyang 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 fixing solutions containing chloroform, PVP, and 2-hydroxy-1-ethanethiol (but without liquid nitrogen), and then ground into tiny pieces by silica (SiO_2) 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.

Table 1
Sources of RAPD 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	Mianyang, Sichuan	009
LS	*A. sinensis* (Oliv.) Diels	Linzhi, Tibet	010

Table 2
Sequences of RAPD primers.

Primer	Sequence 5′-3′
SBS-A1	CAGGCCCTTC
SBS-A3	AGTCAGCCAC
SBS-I1	ACCTGGACAC
SBS-I3	CCGCCTAGTC
SBS-I15	TCATCCGAGG
SBS-M8	TCTGTTCCCC
SBS-M18	CACCATCCGT
SBS-N6	GAGACGCACA
SBS-N19	GTCCGTACTG
SBS-Q16	AAGCGACCTG
SBS-A2	TGCCGAGCTG
SBS-A14	CAATCGCCGT
SBS-I2	GGAGGAGAGG
SBS-I14	TGACGGCGGT
SBS-M6	CTGGGCAACT
SBS-M13	GGTGGTCAAG
SBS-N2	ACCAGGGGCA
SBS-N14	TCGTGCGGGT
SBS-N20	GGTGCTCCGT
SBS-Q20	TCGCCCAGTC

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 ddH_2O. 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.

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 ddH_2O. 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 U.V. 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 group 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.

Table 3
Sequences of ISSR primers.

Primer	Sequence 5′-3′
SBS-A1	CAGGCCCTTC
SBS-A3	AGTCAGCCAC
SBS-I1	ACCTGGACAC
SBS-I3	CCGCCTAGTC
SBS-I15	TCATCCGAGG
SBS-M8	TCTGTTCCCC
SBS-M18	CACCATCCGT
SBS-N6	GAGACGCACA
SBS-N19	GTCCGTACTG
SBS-Q16	AAGCGACCTG
SBS-A2	TGCCGAGCTG
SBS-A14	CAATCGCCGT
SBS-I2	GGAGGAGAGG
SBS-I14	TGACGGCGGT
SBS-M6	CTGGGCAACT
SBS-M13	GGTGGTCAAG
SBS-N2	ACCAGGGGCA
SBS-N14	TCGTGCGGGT
SBS-N20	GGTGCTCCGT
SBS-Q20	TCGCCCAGTC

R = (A, G), Y = (C, T).

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. sinensis*) was minimum (0.41), while between samples 6 and 8 (*A. sinensis* 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 showed 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

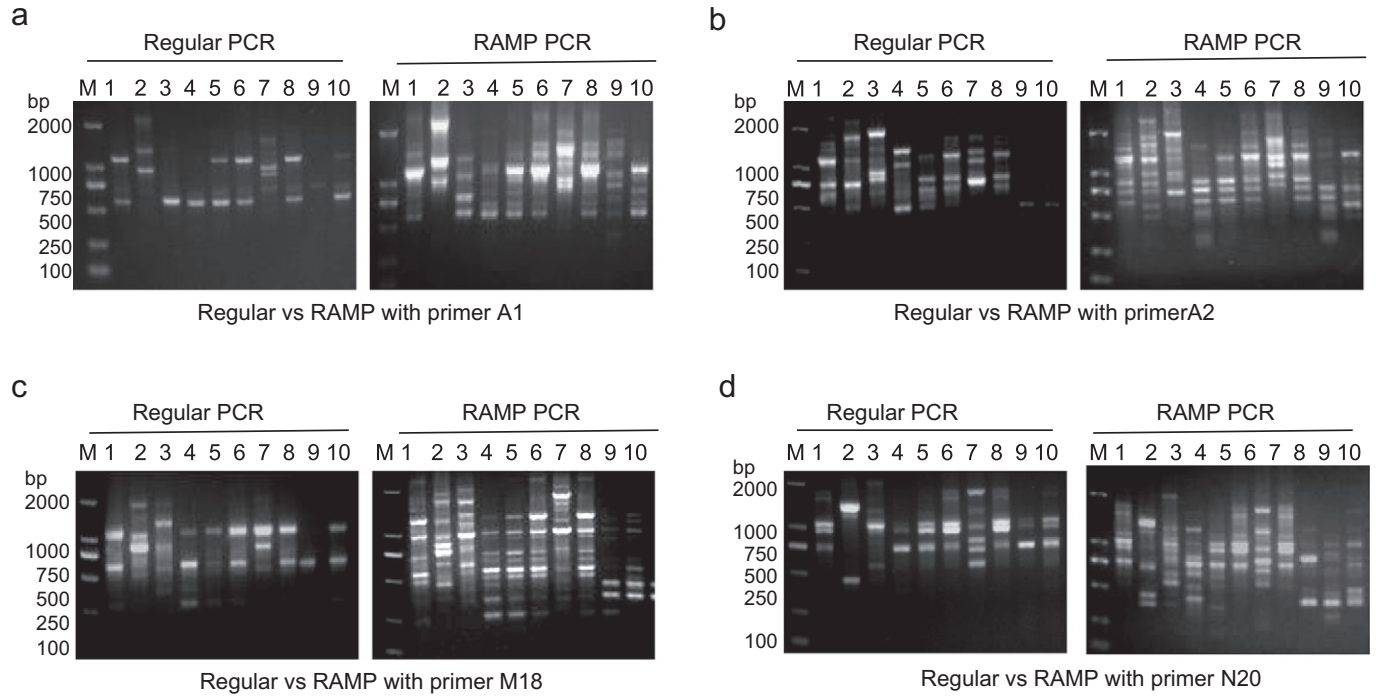


Fig. 2. Comparison between regular RAPD amplification with a 100% RAMP rate and improved RAPD amplification with a 5% RAMP rate. Lanes 1–10 represent different samples of *Angelica* cultivars listed in Table 1. (a) The comparison result by primer SBS-A1. (b) The comparison result by primer SBS-A2. (c) The comparison result by primer SBS-M18. (d) The comparison result by primer SBS-N20. Left panels indicate the results of regular RAPD amplification (regular PCR); right panels indicate the results of improved RAPD amplification (RAMP PCR). Lane "M" represents DL2000 DNA marker with indicated molecular weight size (bp).

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

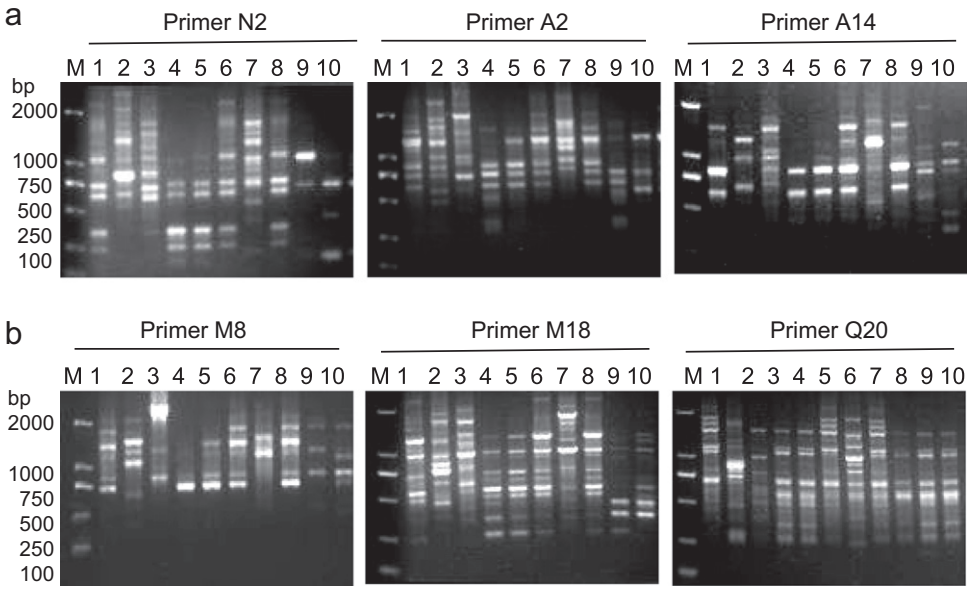
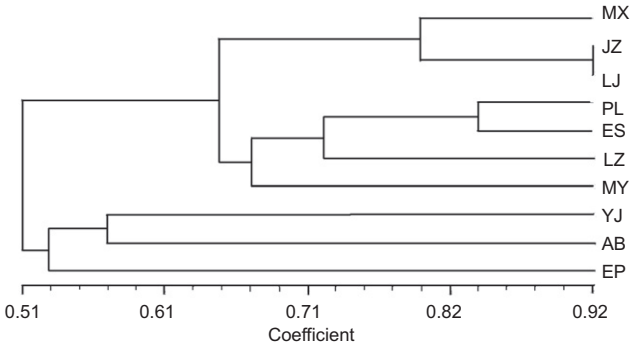


Fig. 3. The representative results of banding files in *Angelica* cultivars obtained by improved RAPD amplification (5% RAMP rate). (a) Primers for SBS-N2, SBS-A2 and SBS-A14. (b) Primers for SBS-M8, SBS-M18 and SBS-Q20. Lanes 1–10 represent different samples listed in Table 1. Lane "M" represents DL2000 DNA marker.

a



b

Sample	1	2	3	4	5	6	7	8	9	10
1	1									
2	0.46	1								
3	0.54	0.57	1							
4	0.62	0.43	0.55	1						
5	0.68	0.45	0.53	0.83	1					
6	0.79	0.41	0.50	0.63	0.74	1				
7	0.54	0.51	0.54	0.48	0.50	0.53	1			
8	0.80	0.42	0.52	0.66	0.77	0.92	0.58	1		
9	0.59	0.47	0.66	0.67	0.63	0.56	0.55	0.62	1	
10	0.65	0.42	0.63	0.74	0.72	0.61	0.50	0.65	0.72	1

Fig. 4. Dendrogram of ten *Angelica* cultivar samples by improved RAPD. (a) Dendrogram of *Angelica* cultivars based on improved RAPD PCR amplification files. Bar on the bottom indicates similarity index based on S.M. coefficients. (b) Genetic distance dendrogram for *Angelica* cultivars by improved RAPD.

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

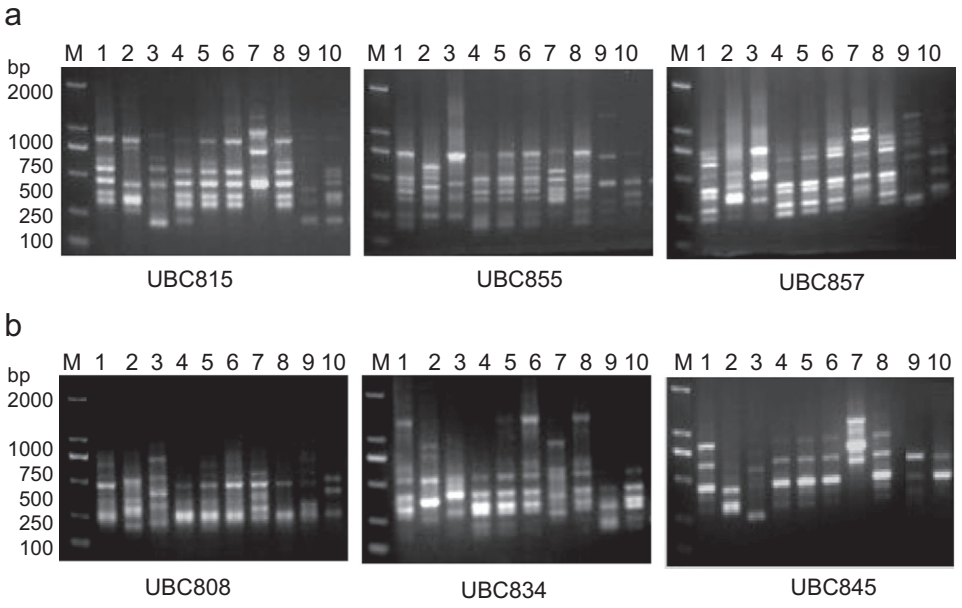
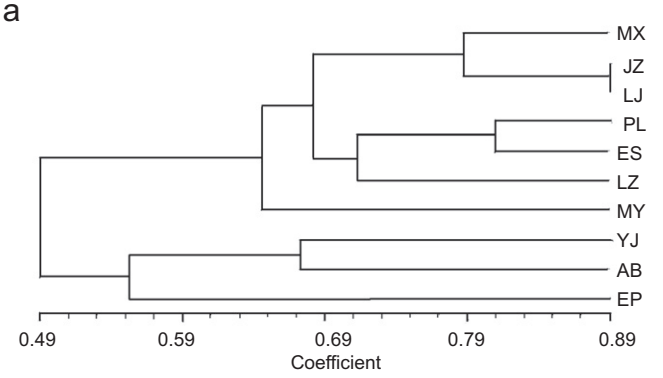


Fig. 5. The representative results of different ISSR maker patterns in *Angelica* cultivars obtained by ISSR. (a) Primers for UBC815, UBC855 and UBC857. (b) Primers for UBC808, UBC834 and UBC845. Lanes 1–10 represent different samples listed in Table 1. *Lane* "*M*" represents DL2000 DNA marker.

a



b

Sample	1	2	3	4	5	6	7	8	9	10
1	1									
2	0.36	1								
3	0.53	0.67	1							
4	0.62	0.40	0.48	1						
5	0.70	0.43	0.53	0.80	1					
6	0.78	0.35	0.55	0.64	0.74	1				
7	0.52	0.55	0.56	0.49	0.52	0.53	1			
8	0.78	0.38	0.55	0.70	0.81	0.89	0.58	1		
9	0.63	0.42	0.64	0.64	0.63	0.59	0.51	0.68	1	
10	0.65	0.38	0.61	0.73	0.69	0.60	0.51	0.66	0.70	1

Fig. 6. Dendrogram of ten *Angelica* cultivars by using ISSR primers. (a). Dendrogram of *Angelica* cultivars based on amplification files by ISSR primers. Bar on the bottom indicates similarity index based on S.M. coefficients. (b) Genetic distance dendrogram for *Angelica* cultivars.

genetic characterization of *A. sinensis* or other *Angelica* species by RAPD analysis, but those works were very preliminary, and they did not evaluate the genetic distance analysis clearly. However, an internal transcribed spacer (ITS) region analysis of *Angelica* cultivars showed that they are different from each other genetically and they are easily distinguishable [31].

ISSR analysis is another important molecular technique for the genetic characterization [13,15,26,32]. To verify the improved RAPD analysis result of our study, we employed the ISSR technique for amplifying DNA materials of all ten *Angelica* samples and analyzed the results by cluster dendrogram. Interestingly and expectedly, ISSR analysis evaluated similar trend of genetic characteristics in *Angelica* samples. To our knowledge, this is the first report on the genetic characterization of *A. sinensis* by combining improved RAPD with ISSR analysis, and this characterization could be useful for the preservation of genetic diversity and *Angelica* population.

Twenty varieties of *Angelica* are endemic to the Hengduan Mountains and its adjacent regions, which appear to be both a refugium and a major diversification center for *Angelica* [11,33,34]. The wild resources of *A. sinensis* are distributed in the province of Shaanxi, Sichuan, Gansu and Tibet, which belong to the Hengduan Mountains. However, the expansion of the wild species cultivation was limited because of the smaller size and poorer quality. Southeast of Gansu is the main cultivation area of *A. sinensis*, and stands at 90% of the total production [35]. There are great genetic differences of *A. sinensis* from the cultivation areas in Gansu province such as Pinliang, Minxian, Zhangxian and Weiyuan because of different natural environments and local ecological conditions [36]. In this study, *A. sinensis* from different cultivation areas were clustered into two clades. Samples from Minxian, Jiuzhaigou and Lijiang have close affinity. The sample from Pinliang was close to that from Enshi, which are grouped together with that from Linzhi and Mianyang. The results indicated that the samples from Minxian and Pinliang of Gansu province have great genetic variations. The excellence of cultivated germplasm was introduced and cultivated widely. The germplasm of *A. sinensis* from Jiuzhaigou of Sichuan province and Lijiang of Yunnan province may come from Minxian. The plants cultivated in Enshi of Hubei province might be introduced from Pinliang. The results of cluster analysis based on RAPD and ISSR markers of *A. sinensis* have no consistency with the geographical distributions of *A. sinensis* samples. The distance between samples might be due to plant germplasms from different cultivation areas.

L. officinale and *A. acutiloba* are used as adulterants and substitutes of *A. sinensis* in many areas of China. It is difficult to identify them by using the method of morphology, or physical and chemical identification. The phylogenetic tree shows the close relationship among the species of *A. sinensis*, *L. officinale* and *A. acutiloba*, which is consistent with the previous study by using ITS sequences [31]. *A. sinensis* could be accurately distinguished from *L. officinale* and *A. acutiloba* by specific bands and the phylogenetic tree. The genetic distance between different species or cultivars might have grown during the course of evolutionary speciation, and the distance between intra-species samples might have grown due to geographical isolation, which has been indicated in previous studies [12,26,31,32]. A future research study investigating the genetic characterization of *A. sinensis* from different regions of the world, and mapping their genetic distance by RAPD or ISSR would be interesting. Also the development of sequence-characterized amplified region (SCAR) markers by molecular cloning of the RAPD fragments can establish a precise molecular tool for the identification of *A. sinensis*.

5. Concluding remarks

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.

References

[1] Dog TL. Menopause: A review of botanical dietary supplements. Am J Med 2005; 118:98–108. http://dx.doi.org/10.1016/j.amjmed.2005.09.044.
[2] Circosta C, Pasquale RD, Palumbo DR, Samperi S, Occhiuto F. Estrogenic activity of standardized extract of *Angelica sinensis*. Phytother Res 2006;20:665–9. http://dx.doi.org/10.1002/ptr.1928.
[3] Mazaro-Costa R, Andersen ML, Hachul H, Tufik S. Medicinal plants as alternative treatments for female sexual dysfunction: Utopian vision or possible treatment in climacteric women? J Sex Med 2010;7:3695–714. http://dx.doi.org/10.1111/j.1743-6109.2010.01987.x.
[4] Chao WW, Lin BF. Bioactivities of major constituents isolated from *Angelica sinensis* (Danggui). Chin Med 2011;6:29. http://dx.doi.org/10.1186/1749-8546-6-29.
[5] Chen XP, Li W, Xiao XF, Zhang LL, Liu CX. Phytochemical and pharmacological studies on Radix *Angelica sinensis*. Chin J Nat Med 2013;11:577–87. http://dx.doi.org/10.1016/S1875-5364(13), 60067-9.
[6] Lee JG, Hsieh WT, Chen SU, Chiang BH. Hematopoietic and myeloprotective activities of an acidic *Angelica sinensis* polysaccharide on human $CD34^+$ stem cells. J Ethnopharmacol 2012;139:739–45. http://dx.doi.org/10.1016/j.jep.2011.11.049.

[7] Huang MH, Lin SZ, Lin PC, Chiou TW, Harn YW, Ho LI, et al. Brain tumor senescence might be mediated by downregulation of S-phase kinase-associated protein 2 via butylidenephthalide leading to decreased cell viability. Tumor Biol Biol 2014;35: 4875–84. http://dx.doi.org/10.1007/s13277-014-1639-0.
[8] Xin YF, Zhou GL, Shen M, Chen YX, Liu SP, Chen GC, et al. *Angelica sinensis*: A novel adjunct to prevent doxorubicin-induced chronic cardiotoxicity. Basic Clin Pharmacol Toxicol 2007;101:421–6. http://dx.doi.org/10.1111/j.1742-7843.2007.00144.x.
[9] Zhang S, He B, Ge J, Li H, Luo X, Zhang H, et al. Extraction, chemical analysis of *Angelica sinensis* polysaccharides and antioxidant activity of the polysaccharides in ischemia–reperfusion rats. Int J Biol Macromol 2010;47:546–50. http://dx.doi.org/10.1016/j.ijbiomac.2010.07.012.
[10] Yeh JC, Cindrova-Davies T, Belleri M, Morbidelli L, Miller N, Cho CW, et al. The natural compound *n*-butylidenephthalide derived from the volatile oil of Radix *Angelica sinensis* inhibits angiogenesis *in vitro* and *in vivo*. Angiogenesis 2011;14:187–97. http://dx.doi.org/10.1007/s10456-011-9202-8.
[11] She ML, Pu FT, Pan ZH, Watson MF, Cannon JFM, Homes-Smith I, et al. Apiaceae. In: Flora of China Editorial Committee, editor. Flora of China, vol. 14. St. Louis, MO: Missouri Botanical Garden Press; 2005. p. 1–205.
[12] Fu J, Yang L, Khan MA, Mei Z. Genetic characterization and authentication of *Lonicera japonica* Thunb. by using improved RAPD analysis. Mol Biol Rep 2013;40:5993–9. http://dx.doi.org/10.1007/s11033-013-2703-3.
[13] Noormohammadi Z, Hasheminejad-Ahangarani FY, Sheidai M, Ghasemzadeh-Baraki AS, Alishah O. Genetic diversity analysis in Opal cotton hybrids based on SSR, ISSR, and RAPD markers. Genet Mol Res 2013;12:256–69. http://dx.doi.org/10.4238/2013.January.30.12.
[14] Shakeel M, Ilyas M, Kazi M. Evaluation of synthetic hexaploid wheats (derivative of durum wheats and *Aegilops tauschii* accessions) for studying genetic diversity using randomly amplified polymorphic DNA (RAPD) markers. Mol Biol Rep 2013;40:21–6. http://dx.doi.org/10.1007/s11033-012-1943-y.
[15] Hadian J, Raeisi S, Nadjafi F, Khadivi-Khub A. DNA typing and genetic relations among populations of *Kelussia odoratissima* using ISSR and SRAP markers. Plant Syst Evol 2014;300:1525–32. http://dx.doi.org/10.1007/s00606-013-0979-3.
[16] Fu JJ, Li LY, Xu X, Wang Z, Tang G, Yin CM, et al. An improved method for increasing the efficiency of the technique of random amplified polymorphic DNA (RAPD). Hereditas 2000;22:251–2.
[17] Xu H, Fabricant DS, Piersen CE, Bolton JL, Pezzuto JM, Fong H, et al. A preliminary RAPD-PCR analysis of *Cimicifuga* species and other botanicals used for women's health. Phytomedicine 2002;9:757–62. http://dx.doi.org/10.1078/094471102321621403.
[18] Zhao KJ, Dong TTX, Tu PF, Song ZH, Lo CK, Tsim KWK. Molecular genetic and chemical assessment of radix *Angelica* (Danggui) in China. J Agric Food Chem 2003;51: 2576–83. http://dx.doi.org/10.1021/jf026178h.
[19] Fu JJ. Short protocols in medical molecular biology. Beijing: China Medical Science Press; 2012.
[20] Yang L, Fu S, Khan MA, Zeng W, Fu J. Molecular cloning and development of RAPD-SCAR markers for *Dimocarpus longan* variety authentication. SpringerPlus 2013;2: 501. http://dx.doi.org/10.1186/2193-1801-2-501.
[21] Yang L, Khan MA, Mei Z, Yang M, Zhang T, Wei C, et al. Development of RAPD-SCAR markers for *Lonicera japonica* Thunb. (Caprifoliaceae) variety authentication by improved RAPD and DNA cloning. Rev Biol Trop 2014;62:1649–57.
[22] Cheng J, Long Y, Khan MA, Wei C, Fu S, Fu J. Development and significance of RAPD-SCAR markers for the identification of *Litchi chinensis* Sonn. by improved RAPD amplification and molecular cloning. Electron J Biotechnol 2014;18. http://dx.doi.org/10.1016/j.ejbt.2014.11.004.
[23] Rohlf FJ. NTSYS-pc: Numerical taxonomy system ver.2.1. Setauket, New York: Exeter Pub. Ltd; 2002[cited July 6, 2014, Available from Internet: http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html].
[24] Zhang GF, Guan JM, Lai XP, Lin J, Liu JM, Xu HH. RAPD fingerprint construction and genetic similarity of *Mesona chinensis* (Lamiaceae) in China. Genet Mol Res 2012; 11:3649–57. http://dx.doi.org/10.4238/2012.October.4.12.
[25] Mei ZQ, Fu SY, Yu HQ, Yang LQ, Duan CG, Liu XY, et al. Genetic characterization and authentication of *Dimocarpus longan* Lour. Using an improved RAPD technique. Genet Mol Res 2014;13:1447–55. http://dx.doi.org/10.4238/2014.March.6.3.
[26] Mei Z, Yang L, Khan MA, Yang M, Wei C, Yang W, et al. Genotyping of *Ganoderma* species by improved random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analysis. Biochem Syst Ecol 2014; 56:40–8. http://dx.doi.org/10.1016/j.bse.2014.04.012.
[27] Mei Z, Khan MA, Zeng W, Fu J. DNA fingerprints of living fossil *Ginkgo biloba* by using ISSR and improved RAPD analysis. Biochem Syst Ecol 2014;57:332–7. http://dx.doi.org/10.1016/j.bse.2014.09.007.
[28] Long Y, Cheng J, Mei Z, Zhao L, Wei C, Fu S, et al. Genetic analysis of litchi (*Litchi chinensis* Sonn.) in southern China by improved random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR). Mol Biol Rep 2014. http://dx.doi.org/10.1007/s11033-014-3755-8.
[29] Lima ATB, de Souza VAB, Gomes RLF, Lima PSC. Molecular characterization of cajá, *Spondias mombin* (Anacardiaceae), by RAPD markers. Genet Mol Res 2011;10: 2893–904. http://dx.doi.org/10.4238/2011.November.25.1.
[30] Zhou H, Shao M, Ji K, Li X, Li Y, Jin L. RAPD analysis of *Angelicae sinensis* seeds from six different regions in Gansu. J Med Plant 2010;1:12–3.
[31] Feng T, Liu S, He X. Molecular authentication of traditional Chinese medicinal plant *Angelica sinensis* based on internal transcribed spacer of nrDNA. Electron J Biotechnol 2010;13. http://dx.doi.org/10.2225/vol13-issue1-fulltext-13.
[32] Kumla S, Doolgindachbaporn S, Sudmoon R, Sattayasai N. Genetic variation, population structure and identification of yellow catfish, *Mystus nemurus* (C&V) in Thailand using RAPD, ISSR and SCAR marker. Mol Biol Rep 2012;39: 5201–10. http://dx.doi.org/10.1007/s11033-011-1317-x.
[33] Chen WW, He XJ, Zhang XM, Pu JX. Pollen morphology of the genus *Angelica* from southwest China and its systematic evolution analysis. Acta Bot Boreali Occident Sin 2007;27:1364–72.
[34] Feng T, Downie SR, Yu Y, Zhang X, Chen W, He X, et al. Molecular systematics of *Angelica* and allied genera (Apiaceae) from the Hengduan Mountains of China based on nrDNA ITS sequences: Phylogenetic affinities and biogeographic implications. J Plant Res 2009;122:403–14. http://dx.doi.org/10.1007/s10265-009-0238-4.
[35] Zhang SZ, He LP, Han LM, Yang WX, Liu LL. Study on wild *Angelica* resources. Mod Chin Med 2012;4:33–6.
[36] Gao WY, Qin EQ, Xiao XH, Yu HM, Gao GY, Chen SB, et al. Analysis on genuineness of *Angelica sinensis* by RAPD. Chin Tradit Herb Drugs 2001;10:926–9.