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Full Length Research Paper

Authentication of *Caulis clematidis armandii (Chuanmutong)* and differentiation of its common adulterants using RAPD and SCAR markers

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***Caulis clematidis armandii* ("Chuanmutong" in Chinese) has been used as an important traditional Chinese herb for a long time. As recorded, the origins of *Caulis clematidis armandii* are the dried stems of *Clematis armandii* Franch. and *C. montana* Buch. -Ham. But due to the similarity in morphology, it is difficult to distinguish *C. armandii* and *C. montana* from other *Clematis* species. As a result, some *Clematis* species were mistakenly used as *Caulis clematidis armandii*, and caused potentially dangerous consequences. To overcome the limitation of the traditional morphological methods, an efficient molecular method was developed to better distinguish *Caulis clematidis armandii* from its common adulterants. Two random amplified polymorphic DNA (RAPD) fragments specific for *C. armandii* and *C. montana* were sequenced and primers were designed for sequence characterized amplified region (SCAR) markers. The two obtained SCAR markers were specific to both *C. armandii* and *C. montana*, but were absent from other *Clematis* species. The protocol developed in this study can be further applied to rapid authentication of *Caulis clematidis armandii* and its common adulterants.**

Key words: *Athentication, Caulis clematidis armandii, Clematis armandii, Clematis Montana,* RAPD, SCAR.

INTRODUCTION

Traditional Chinese herbs have been used for medicinal purposes for several thousands years. The Chinese herb *Caulis clematidis armandii* ("Chuanmutong" in Chinese) is distributed in southwest China, especially in Sichuan province. In China, medicinal plants *Clematis armandii* Franch and *C. montana* Buch-Ham are classified as *Caulis Clematidis Armandii* and are stated as the official species in Chinese Pharmacopoeia, 2005 (Chinese Pharmacopoeia Committee, 2005). They contain saponins and flavanone glycoside, and are effective in relieving rheumatism, clearing heat, promoting diuresis, and enhancing antibacterial host defense (Guo et al., 2007).

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Abbreviation: RAPD, Random amplified polymorphic DNA; **SCAR,** sequence characterized amplified regions.

Interest in Caulis aristolochiae manshuriensis ("Guanmutong" in Chinese) is increasing due to the aristolochic acid nephropathy, which is induced by aristolochic acid, the predominant toxic contaminant of Chinese herb Caulis aristolochiae manshuriensis (Cosyns, 2003). Thus, many countries have banned the use of Caulis aristolochiae manshuriensis (U. S. Food and Drug Administration, 2000; Martena et al., 2007). In china, the national center for ADR monitoring also published warnings in its Chinese Adverse Drug Reaction information Bulletin (National Center for ADR Monitoring, PRC, 2002). *Caulis clematidis armandii*, with no detectable level of aristolochic acid is used to replace Caulis aristolochiae manshuriensis in 32 Chinese traditional patent medicines (Ministry of Public Health, PRC, 2002).

Based on commercial investigation, *C. peterae* Hand-Mazz, *C. argentilucida* W.T Wang, *C. apiifolia* DC, *C. urophylla* M. Y. Fang, *C. finetiana* Levl et Vant, *C. peterae* var.*trichocarpa* W.T Wang, *C. lasiandra* Maxum. and *C. akebiodes* (Maxim) Hort. ex Veitch were sometimes used inappropriately as Caulis Clematidis Armandii (Guo et al.,

2007). The adulterants of *Caulis clematidis armandii* may have different pharmacological effects. However, it is particularly difficult to distinguish *C. armandii* and *C. montana* from other *Clematis* species by traditional morphological approaches, especially for commercial products in tablet forms.

DNA methods for identification of medicinal plants have been developed, and have been shown as a good complementary method to morphological analysis. PCR based methods including randomly amplified polymorphic DNA (RAPD) can be effectively used for the authentication of the medicinal plant material. Further, development of more specific, sensitive and reproducible markers like RAPD based sequence characterized amplified region (SCAR) can increase industrial application of the molecular techniques (Paran and Michelmore, 1993).

In the present study, we designed two reproducible SCAR markers based on the RAPD-generated PCR products, to efficiently distinguish *C. armandii* and *C. montana* from other *Clematis* species, and set up an unequivocal authentication strategy for *Caulis clematidis armandii*.

MATERIALS AND METHODS

Plant materials

Fresh leaf samples of official species (*C. armandii* and *C. montana*) and eight adulterants were collected from different parts of Sichuan province, China: *C. amandii* from Emei Mount (EM1.1), Qingcheng Mount (QCM2.1), Lixian (LX3.1); *C. montana* from Emei Mount (EM1.2), Jiuzhaigou (JZG4.1), Wolong (WL5.1); *C. peterae* from Lixian (LX3.2), Qingcheng Mount (QCM2.2); *C. argentilucida* from Chengdu (CD6.1) , Lixian (LX3.3); *C. apiifolia* from Emei Mount (EM1.3), Pingwu (PW7.1); *C. urophylla* from Emei Mount (EM1.4), Pingwu (PW7.2); *C. finetiana* from Qingchuan (QC8.1), Emei Mount (EM1.5), Leibo (LB9.1); *C. peterae* var.*trichocarpa* from Chengdu (CD6.2), Qingcheng Mount (QCM2.3); *C. lasiandra* from Emei Mount (EM1.6), Pingwu (PW7.3); *C. akebiodes* from Wolong (WL5.2) , Langzhong (LZ10.1), Chengdu (CD6.3).

They were further identified by Professor De Guang Wan, Chengdu University of Traditional Chinese Medicine, and then were deposited in Research Institute of Medicinal Plant, Chengdu University of Traditional Chinese medicine, China. Additionally, in order to test if the designed primers could amplify the SCAR markers, market samples were purchased from Hehuachi Herbal Materials Market, the biggest Herbal Materials Market in southwest China: *C. amandii* (Coll. No. 050101, 060602, 070601); *C. montana* (Coll. No. 040602, 060706, 070602); *C. peterae* (Coll. No. 040501, 050102); *C. argentilucida* (Coll. No. 050103, 060707, 070603); *C. apiifolia* (Coll. No. 040502, 060708); *C. urophylla* (Coll. No. 050104, 060709, 070604); *C. finetiana* (Coll. No. 040603, 050105); *C. peterae* var.*trichocarpa* (Coll. No. 040604, 060710, 070605); *C. lasiandra* (Coll. No. 060603, 070701); *C. akebiodes* (Coll. No. 050106, 060604, 070702).

DNA isolation and RAPD amplification

Fresh leaf tissues (0.2 g) or stem phloem of market samples (1 g) were ground to powder in liquid nitrogen. Genomic DNA was extracted from the powder with a Plant Tissue Kit (Qiagen, USA). In our study, dried market samples were scraped with a scalpel to remove overlying tissues, and then were cleaned by ddH_2O. After ultraviolet radiation treatment for 1 h, genomic DNA was extracted from dried samples.

RAPD assays were performed in a final volume of 20 µl containing 1×*Taq* DNA polymerase buffer, 2.5 mM $MgCl_2$, 150 mM dNTPs, 1 U of Taq DNA polymerase, 0.5 mM primer (RAPD primers SBSA, SBSB, SBSC, SBSI, SBSK, SbSBIO, China), 50 ng template DNA. PCR was performed as follows: one cycle of 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 36°C, followed by 1.5 min at 72°C; and one cycle of 10 min at 72°C. PCR products were separated with electrophoresis in 1.5% (w/v) agarose gel and detected with ethidium bromide.

Cloning and sequencing the RAPD markers

Two RAPD fragments specific to both *C. armandii* and *C. montana* were amplified by the random primer SBSI18 and SBSC13, respectively. Then they were excised from 1.5% (w/v) agarose gel and were purified using Gel Extraction Kit (EZNA, USA). The purified PCR fragment was ligated into PMD-18-T vector (Takara, Japan). The presence of the cloned fragment was confirmed by enzyme digestion of the plasmid DNA and sequencing was conducted using an ABI3730 automated DNA sequencer (Applied Biosystems, USA).

Designing SCAR primers and their amplification of genomic regions

Based on the sequenced RAPD amplicon two SCAR primer pairs (T1/T2 and G1/G2) that could amplify approximately 141 and 298 bp, respectively, of the genomic sequence from *C. armandii* and *C. montana* were designed. The two SCAR primer pairs were used for PCR amplifications of genomic DNA from the fresh leaf samples and market samples of the ten *Clematis* species. PCR was performed as follows: 94°C for 2 min; 30 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s; and a final extension at 74°C for 5 min. Furthermore, PCR amplification for identification of market samples of *C. armandii* and *C. montana* from the eight adulterants of official species was done.

RESULTS

RAPD analysis

In a preliminary survey, a total of 357 amplified bands were screened against ten *Clematis* species 330 of them (92.4%) were polymorphic and were examined in this study. The average number of polymorphic DNA bands amplified by each primer was 66. We also found that 5 of 100 primers (Table 1), including SBSI18 and SBSC13, demonstrate highly reproducible polymorphic RAPD amplification patterns (Figure 1).

RAPD bands were transformed into a binary character matrix (“1” for the presence and “0” for the absence of a band at a particular position). Genetic relationships among the 10 species were analyzed with the unweighted pair-group method (UPGMA) cluster analysis of the program numerical taxonomy and multivariate analysis system version 2.10e using the Nei's distance parameter (Rohlf, 2000). The dendrogram indicated that the ten *Clematis* species were clustered into two genetic cluster

Table 1. Sequences of 5 selected primers giving highly reproducible polymorphic RAPD amplification patterns. The amplification band numbers of each primer is also listed.

Primer	Sequences (5'-3')	Number of amplified bands
SBSC02	GTG AGG CGT C	62
SBSC05	GAT GAC CGC C	80
SBSC13	AAG CCT CGT C	80
SBSI18	TGC CCA GCC T	56
SBSK18	CCT AGT CGA G	79

Table 2. *C. armandii* and *C. montana* specific SCAR primer pairs designed from their sequenced RAPD amplicon.

RAPD primer	SCAR primer pair	Number of base pairs (bp)	Sequence (5'–3')	G+C content (%)	Annealing temperature
SBSI18	T1	22	GGT GCC ATG AAT TGA TTT GTA G	40.9	55 ℃
	T2	23	TTC TCA CCG TGA TGA CTA AGT GC	47.8	
SBSC13	G1	22	CCG GAA CTT GTT ATT AGA TGC T	40.9	54 ℃
	G2	23	CAT CAT AAG TCA ACT ATG GAG GC	43.5	

groups (Figure 2), one contained *C. montana*, the other contained all the other species. It was also demonstrated that DNA methods have the potential of playing an important role in identifying *Clematis* species with a similar genetic background.

Sequencing of RAPD markers

RAPD primers SBSI18 and SBSC13 consistently amplified a single, strong band of approximately 480 and 1050 bp, respectively, for *C. armandii* and *C. montana*, which was absent in the rest of the 8 *Clematis* species (Figure 1). The two bands were named as SBSI18.478 and SBSC13.1062, respectively, and were considered as putative specific markers, and were cloned and sequenced.

The two sequences are 478 and 1062 bp, respectively, and are given in Figure 3. The sequences have been submitted to GenBank under accession numbers (EU283825 and EU283824, EU283822 and EU283823). Furthermore, homology searches were performed in Genebank's non-redundant database using the BLAST algorithm. BLAST results showed that SBSI18.478 and SBSC13.1062 had no existing homologies of known nucleotide sequences.

Designing SCAR primers and their amplification of genomic regions

On the sequences of the cloned SBSI18.478 and SBSC13.1062, two primer pairs were designed as SCAR primers (more details in Table 2). Using T1/T2 and G1/G2 primer pairs, the expected amplified fragments of 141 bp and 298 bp were obtained from *C. armandii* and *C. montana* respectively. Meanwhile, no amplified fragment was obtained from any other *Clematis* species (data not shown).

The primer pairs, T1/T2 and G1/G2, were further tested for their ability to specifically and efficiently amplify SCAR markers in market samples of Caulis Clematidis Armandii and its eight adulterants (Figure 4). Our results indicated that the two fragments were absent from the eight adulterants, but were obtained from market samples of *C. armandii* and *C. montana*.

DISCUSSION

SCAR is suitable for a precise and rapid identification at the species level and can be used to detect samples by using primers specifically designed on the basis of RAPD markers. This method has been used for identification of traditional herbs and medicinal plants such as genus *Artemisia* (Lee et al., 2006), *Panax* species (Wang et al., 2001), *Phyllanthus* species (Dnyaneshwar et al., 2006). In this study, we successfully distinguished *C. armandii* and *C. montana*, the official species of Caulis Clematidis Armandii from other *Clematis* species.

It is very difficult to obtain high quality DNA from dried herbs. Thus, establishing a reliable DNA fingerprinting for the dried samples of *Clematis* species is very important

for SCAR analysis. Furthermore, polymorphisms between *Clematis* species were efficiently detected using RAPD analysis. As shown in Figure 2, that *C. armandii* and *C. montana* belong to two different genetic cluster groups

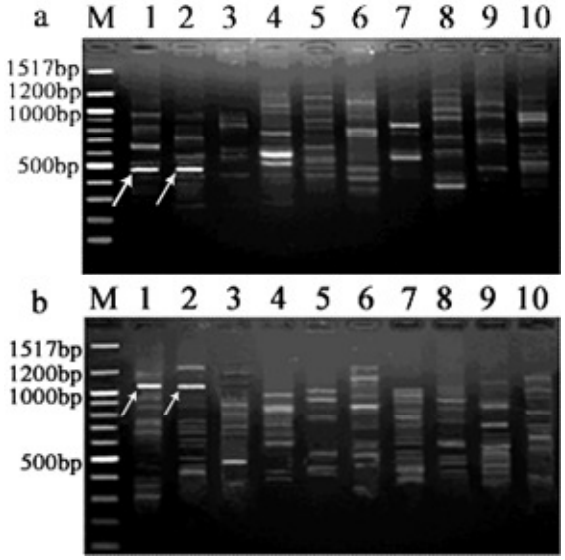

Figure 1. RAPD profiles of *Clematis* species generated by SBSI18 (a) and SBSC13 (b). Arrow indicates polymorphic band in *C. armandii* and *C. montana*. Lane M: 100bp DNA ladder; Lane 1: *C. armandii*; Lane 2: *C. Montana*; Lane 3: *C. peterae*; Lane 4: *C. argentilucida*; Lane 5: *C. apiifolia*; Lane 6: *C. urophylla*; Lanes 7: *C. finetiana*; Lane 8: *C. peterae* var.*trichocarpa*; Lane 9: *C. lasiandra*; Lane 10: *C. akebiodes*.

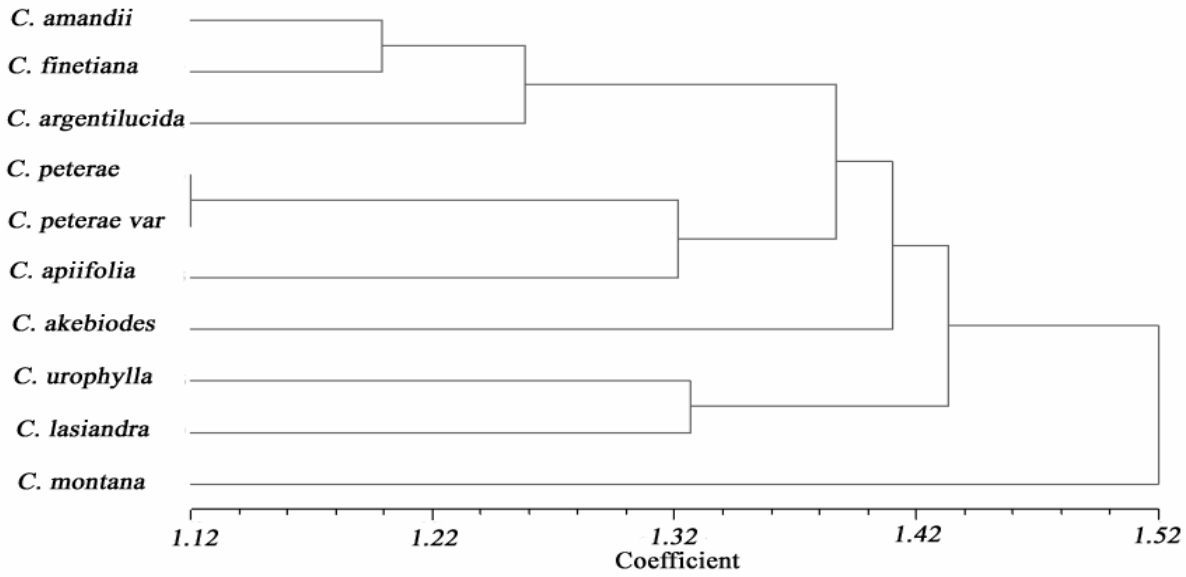

Figure 2. Dendrogram constructed by UPGMA cluster analysis of RAPD data of 10 *Clematis* species.

suggested that a relatively high genetic diversity. But they were both used as Caulis Clematidis Armandii in Chinese Pharmacopoeia. Thus, it is necessary to perform a systematic study of the major components and pharmacological effects for *C. armandii* and *C. montana*.

In summary, the two SCAR primer pairs, T1/T2 and G1/G2, could be used for the authentication of *C. armandii* and *C. montana*, and to differentiate them from other adulterant species traded in the market. This will be useful in quality control of herbal medicines as well as in Selecting *Caulis Clematidis Armandii* samples for research purposes. Moreover, the SCAR method in the present study has potential routine diagnostic procedures for species identification. Converting species-specific RAPD fragments into SCAR markers are easy to identify in any other plants species without prior sequence knowledge, and the approach described here could easily be generalized and applied to other plants.

Figure 3. Nucleotide sequences of polymorphic RAPD amplicon SBSI18.478 (a) and SBSC13.1062 (b) from *C. armandii* and *C. montana*. SBSI18, SBSC13, T1/T2 and G1/G2 primer sequences are highlighted in the two sequences, respectively.

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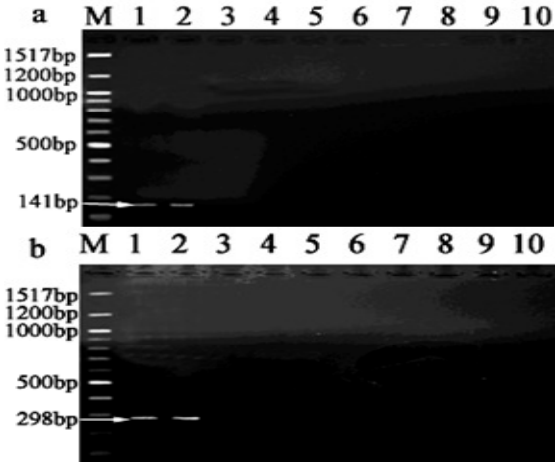

Figure 4. PCR amplification of genomic DNA of market samples (stem phloem) by SCAR primer paires T1/T2 (a) and G1/G2 (b). Amplification can be observed only in *C. armandii* and *C. montana* samples. Lane M: 100bp DNA ladder; Lane 1: *C. armandii*; Lane 2: *C. montana*; Lane 3: *C. peterae*; Lane 4: *C. argentilucida*; Lane 5: *C. apiifolia*; Lane 6: *C. urophylla*; Lanes 7: *C. finetiana*; Lane 8: *C. peterae* var.*trichocarpa*; Lane 9: *C. lasiandra*; Lane 10: *C. akebiodes*.

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