American Journal of Plant Sciences, 2013, 4, 269-273
http://dx.doi.org/10.4236/ajps.2013.42035 Published Online February 2013 (http://www.scirp.org/journal/ajps)

Authentication and Genetic Origin of Medicinal *Angelica acutiloba* Using Random Amplified Polymorphic DNA Analysis

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Received December 18th, 2012; revised January 15th, 2013; accepted January 22nd, 2013

ABSTRACT

Some *Angelica* species are used for medicinal purposes. In particular, the roots of *Angelica acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*, known as "Toki" and "Hokkai Toki", respectively, are used as important medicinal materials in traditional Japanese medicine. However, since these varieties have recently outcrossed with each other, it is difficult to determine whether the Japanese *Angelica* Root material used as a crude drug is the "pure" variety. In this study, we developed an efficient method to authenticate *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* from each other and from other *Angelica* species/varieties. The random amplified polymorphic DNA (RAPD) method efficiently discriminated each *Angelica* variety. *A. acutiloba* var. *sugiyamae* was identified via a characteristic fragment amplified by the decamer primer OPD-15. This fragment showed polymorphisms among *Angelica* species/varieties. The unique fragment derived from *A. acutiloba* var. *sugiyamae* was also found in one strain of *A. acutiloba* var. *acutiloba*, implying that this strain arose from outcrossing between *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*. This RAPD marker technique will be useful for practical and accurate authentication among *A. acutiloba* var. *acutiloba*, *A. acutiloba* var. *sugiyamae*, and their adulterants.

Keywords: *Angelica acutiloba*; Intraspecific Variation; Kampo Medicine; Random Amplified Polymorphic DNA (RAPD)

1. Introduction

The Japanese indigenous species *Angelica acutiloba* Kitagawa var. *acutiloba* Kitagawa (Toki) or *A. acutiloba* Kitagawa var. *sugiyamae* Hikino (Hokkai Toki) are listed in the Japanese Pharmacopoeia (JP), 16th edition [1], and are important medicinal materials for traditional Japanese medicine. The cultivar *A. acutiloba* var. *acutiloba*, which is generally considered to be higher quality than the "Hokkai Toki", was originally grown in Nara Prefecture, Japan. The difference in the quality of the two cultivars has been described in two Japanese historical manuscripts [2,3]. To this day, the quality of *A. acutiloba* var. *acutiloba* is still regarded as being higher than that of other cultivars, and this is reflected in the market price of the root. Since *A. acutiloba* var. *sugiyamae* "Hokkai Toki" has been cultivated in various regions of Japan including Nara Prefecture since the 1950s [4], several *Angelica* strains are thought to be outcrossed [5]. Furthermore, *A. acutiloba* Kitagawa var. *iwatensis* Hikino (Miyama Toki), which is genetically very close to 'Toki', and *A. stenoloba* ("Hosoba Toki" or "Tokachi Toki") grow wild in Japan [6]. Because these species and varieties are closely related to *A. acutiloba*, they readily outcross with *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* [7]. The root of *A. acutiloba* var. *iwatensis* is now not used as a crude drug listed in JP 16th edition, although the morphological features of the roots are very similar among related species of *A. acutiloba* var. *acutiloba* and it is difficult to reliably distinguish them from each other. A previous molecular survey of chloroplast DNA and the internal transcribed spacer of nuclear ribosomal DNA showed some nucleotide polymorphisms that could be used to distinguish between *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* [8]. Although genomic DNA polymorphisms revealed differences among *Angelica* varieties, an efficient and simple discrimination method that can identify varieties of *A. acutiloba* is needed.

The random amplified polymorphic DNA (RAPD) method can be used to study the genetic variability of species, subspecies, varieties, and natural populations [9-12]. The advantage of RAPD analysis is that it is simple, fast [13], and it does not require genomic informa-

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tion [14]. A previous RAPD analysis showed some polymorphism for discriminating *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*, but the polymerphism of *A. acutiloba* var. *sugiyamae* were quite similar to those of *A. stenoloba* [15]. Therefore, clear discrimination marker for *A. acutiloba* varieties and related species was needed. In this study, we report the authentication and genetic variation among *A. acutiloba* varieties and related species as determined using RAPD markers.

2. Materials and Methods

2.1. Plant Materials

The plant materials included nine strains of *Angelica acutiloba* var. *acutiloba*, three of *A. acutiloba* var. *sugiyamae*, and two of *A. acutiloba* var. *iwatensis* from cultivars grown in different locations or native populations. Cultivated strains of the related species *A. stenoloba* ("Hosoba Toki"), *A. stenoloba* f. *lanceolata* ("Tokachi Toki"), and *A. shikokiana* ("Inu Toki") were also collected from various locations in Japan (**Table 1**). All strains/varieties were cultivated in a greenhouse before use in experiments. Each collected plant was identified by its morphological characters.

2.2. Total DNA Extraction

Total DNA was extracted from young leaf tissue (approx. 200 mg) using a modified CTAB method [16]. The DNA concentration was determined by absorbance at 260 nm.

2.3. RAPD Analysis

We used 80 decamer (10-mer) primers (OPA, OPB, OPC, and OPD; Operon Technologies, USA, **Table 2**) in the RAPD analysis. Each 30-μ l PCR mixture contained 200 ng DNA, 1 × ExTaq buffer, 0.2 mM each deoxynucleotide triphosphate, 1 mM each primer, and 2.5 U ExTaq (TaKaRa, Japan). Amplification was performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc.), as follows: initial denaturation for 5 min at 95˚C, followed by 42 cycles of 95˚C for 30 s, 40˚C for 1 min, and 72˚C for 2 min, and a final extension at 72˚C for 10 min. RAPD fragments were separated electrophoretically in 1% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. To optimize the RAPD analysis, we trialed PCR mixtures containing 20, 40, 120, 200, or 400 ng total DNA, and 1, 2.5, or 5 units ExTaq polymerase. To optimize the cycling conditions, we trialed 35, 38, 42, or 45 cycles, and annealing temperatures of 35˚C, 38˚C, 40˚C, 45˚C, or 48˚C.

3. Results and Discussion

In a preliminary study, we used DNA prepared from three varieties of *A. acutiloba* (Toki, Hokkai Toki, and Miyama Toki) as templates, and screened 80 10-mer random primers (OPA, OPB, OPC, and OPD series) to investigate whether they detected polymorphisms. Of the 80 10-mers, 47 did not detect polymorphisms, 29 did not amplify all DNA templates isolated from the three varieties, and 4 gave highly reproducible polymorphic RAPD amplification patterns. From the four primers that de tected polymorphisms, we selected three for further analysis of plant materials as they produced the most distinct and reproducible polymorphisms.

Next, we optimized the PCR conditions for the RAPD analysis, since the reaction conditions can affect the pattern of PCR products [17]. The optimized conditions were as follows: 200 ng DNA, 2.5 U ExTaq polymerase, 42 cycles, and 40˚C annealing temperature (data not shown).

We obtained reproducible amplified DNA fragments for 14 varieties of *A. acutiloba* and 2 species of *A.*

Table 1. Plant materials used in this study.

Sample code	Species	Japanese name	Locality	Origin
TG	*A. acutiloba* var. *acutiloba*	Toki	Gumma	Cultivar
TK	*A. acutiloba* var. *acutiloba*	Toki	Kyoto	Cultivar
TY	*A. acutiloba* var. *acutiloba*	Toki	Yamagata	Cultivar
TH	*A. acutiloba* var. *acutiloba*	Toki	Hokkaido	Cultivar
TC	*A. acutiloba* var. *acutiloba*	Toki	China	Cultivar
YH	*A. acutiloba* var. *acutiloba*	Toki	Hokkaido	Cultivar
YM	*A. acutiloba* var. *acutiloba*	Toki	Miyagi	Cultivar
OT	*A. acutiloba* var. *acutiloba*	Toki	Toyama	Cultivar
OC	*A. acutiloba* var. *acutiloba*	Toki	Chiba	Cultivar
HA	*A. acutiloba* var. *sugiyamae*	Hokkai Toki	Hokkaido	Cultivar
HB	*A. acutiloba* var. *sugiyamae*	Hokkai Toki	Hokkaido	Cultivar
HC	*A. acutiloba* var. *sugiyamae*	Hokkai Toki	Chiba	Cultivar
MM	*A. acutiloba* var. *iwatensis*	Miyama Toki	Miyagi	Cultivar
MY	*A. acutiloba* var. *iwatensis*	Miyama Toki	Yamagata	Native population
LH	*A. stenoloba*	Hosoba Toki	Saitama	Cultivar
LT	*A. stenoloba* f. *lanceolata*	Tokachi Toki	Saitama	Cultivar
IM	*A. shikokiana*	Inu Toki	Miyazaki	Cultivar

Table 2. Nucleotide sequences of random primers used in this study.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')	Primer	Sequence (5'-3')	Primer	sequence (5'-3')
OPA-01	CAGGCCCTTC	OPB-01	GTTTCGCTCC	OPC-01	TTCGAGCCAG	OPD-01	ACCGCGAAGG
OPA-02	TGCCGAGCTG	OPB-02	TGATCCCTGG	OPC-02	GTGAGGCGTC	OPD-02	GGACCCAACC
OPA-03	AGTCAGCCAC	OPB-03	CATCCCCCTG	OPC-03	GGGGGTCTTT	OPD-03	GTCGCCGTCA
OPA-04	AATCGGGCTG	OPB-04	GGACTGGAGT	OPC-04	CCGCATCTAC	OPD-04	TCTGGTGAGG
OPA-05	AGGGGTCTTG	OPB-05	TGCGCCCTTC	OPC-05	GATGACCGCC	OPD-05	TGAGCGGACA
OPA-06	GGTCCCTGAC	OPB-06	TGCTCTGCCC	OPC-06	GAACGGACTC	OPD-06	ACCTGAACGG
OPA-07	GAAACGGGTG	OPB-07	GGTGACGCAG	OPC-07	GTCCCGACGA	OPD-07	TTGGCACGGG
OPA-08	GTGACGTAGG	OPB-08	GTCCACACGG	OPC-08	TGGACCGGTG	OPD-08	GTGTGCCCCA
OPA-09	GGGTAACGCC	OPB-09	TGGGGGACTC	OPC-09	CTCACCGTCC	OPD-09	CTCTGGAGAC
OPA-10	GTGATCGCAG	OPB-10	CTGCTGGGAC	OPC-10	TGTCTGGGTG	OPD-10	GGTCTACACC
OPA-11	CAATCGCCGT	OPB-11	GTAGACCCGT	OPC-11	AAAGTCTGGG	OPD-11	AGCGCCATTG
OPA-12	TCGGCGATAG	OPB-12	CCTTGACGCA	OPC-12	TGTCATCCCC	OPD-12	CACCGTATCC
OPA-13	CAGCACCCAC	OPB-13	TTCCCCCGCT	OPC-13	AAGCCTCGTC	OPD-13	GGGGTGACGA
OPA-14	TCTGTGCTGG	OPB-14	TCCGCTCTGG	OPC-14	TGCGTGCTTG	OPD-14	CTTCCCCAAG
OPA-15	TTCCGAACCC	OPB-15	GGAGGGTGTT	OPC-15	GACGGATCAG	OPD-15	CATCCGTGCT
OPA-16	AGCCAGCGAA	OPB-16	TTTGCCCGGA	OPC-16	CACACTCCAG	OPD-16	AGGGCGTAAG
OPA-17	GACCGCTTGT	OPB-17	AGGGAACGAG	OPC-17	TTCCCCCCAG	OPD-17	TTTCCCACGG
OPA-18	AGGTGACCGT	OPB-18	CCACAGCAGT	OPC-18	TGAGTGGGTG	OPD-18	GAGAGCCAAC
OPA-19	CAAACGTCGG	OPB-19	ACCCCCGAAG	OPC-19	GTTGCCAGCC	OPD-19	CTGGGGACTT
OPA-20	GTTGCGATCC	OPB-20	GGACCCTTAC	OPC-20	ACTTCGCCAC	OPD-20	ACCCGGTCAC

stenoloba and *A. shikokiana*, using three primers (OPA-16, OPD-3, and OPD-15). *A. acutiloba* varieties and *A. stenoloba* had many bands that differed from those of *A. shikokiana* (**Figure 1**). All of the *A. acutiloba* var. *acutiloba* strains showed the same RAPD profile using the OPD-3 primer, except for *A. acutiloba* var. *acutiloba* collected from Yamagata. The 1.6-kbp band observed in the fragment profile of *A. acutiloba* var. *acutiloba*, *A. acutiloba* var. *iwatensis* collected from Miyagi, and *A. stenoloba* after amplification with the OPA-16 primer was absent from the profiles of *A*. *acutiloba* var. *sugiyamae*, *A*. *acutiloba* var. *iwatensis* collected from Yamagata, and *A. stenoloba* var. *lanceolata*. The fragment pattern of *A. acutiloba* var. *iwatensis* collected from Miyagi differed from that of *A*. *acutiloba* var. *iwatensis* collected from Yamagata. Therefore, *A*. *acutiloba* var. *iwatensis* showed geographic variations in its genetic polymerphisms, similar to the findings of a previous study [8]. In addition, the profiles of *A. acutiloba* var. *iwatensis* collected from Miyagi were quite similar to that of *A. acutiloba* var. *acutiloba* after amplification with the OPA-16 primer. This implied that *A. acutiloba* var. *acutiloba* prescribed as a crude drug is closely related to a geographical strain of *A. acutiloba* var. *iwatensis*. Another *A. acutiloba* var. *iwatensis* collected from Yamagata showed generally the same fragment pattern as that of *A. acutiloba* var. *sugiyamae* after amplification with the OPD-15 primer, but the patterns differed after amplification with the OPA-16 primer. These results indicated that there was geographical variation among varieties of *A. acutiloba* var. *iwatensis*, and the genetic background of one of the varieties of *A. acutiloba* var. *iwatensis* was very similar to that of *A. acutiloba* var. *sugiyamae*. Among three strains of *A. acutiloba* var. *sugiyamae,* the slight differences in their fragment patterns after amplification with the OPD-3 primer might reflect indigenous polymorphisms that occurred independently during long-term maintenance of these traditional strains.

A. acutiloba var. *acutiloba* and *A. acutiloba* var. *sugiyamae* were discriminated by polymorphic bands generated by the OPA-16 primer. The fragment pattern of *A. acutiloba* var. *sugiyamae* after amplification with the OPD-15 primer contained a characteristic 1.1-kbp band. This was not found in *A. acutiloba* var. *acutiloba* strains, except for the strain collected from Yamagata. Therefore, this fragment could be useful to clarify whether the Japanese Angelica Root material is derived from *A. acutiloba* var. *acutiloba* or *A. acutiloba* var. *sugiyamae*. Although the same fragment was found in the Yamagata strain of *A. acutiloba* var. *acutiloba* using the OPD-15 primer, this strain showed a similar fragment pattern to that of other *A. acutiloba* var. *acutiloba* strains after am-

plification with the OPD-3 primer. Since previous studies have shown that the sequences of three regions in the chloroplast genome of *A. acutiloba* var. *acutiloba* from Yamagata were identical to those of *A. acutiloba* var. *sugiyamae* [8], these results demonstrated that the Yamagata strain was an interspecific hybrid derived from outcrossing between *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*.

Authentic identification of *A. acutiloba* varieties is necessary for customers using these materials as a crude drug for traditional Japanese medicine to ensure the quality of the Japanese Angelica Root material. The traditional methods to authenticate "Toki", "Hokkai Toki" and their adulterants are mainly based on morphological characters of the plants. In general, the differences in morphological characters and certain active compounds are subtle and ambiguous [18]. The RAPD analysis of *A. acutiloba* and related species represents an efficient method to authenticate Japanese Angelica Root, and confirm whether it is derived from "Toki" or "Hokkai Toki". The molecular authentication of these regions is a highly sensitive and stable method. The authentication results were reliable and were not affected by the physical form or physiological conditions of the plant samples. Therefore, the RAPD marker method described in this study could be used for practical and accurate authentication of *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*.

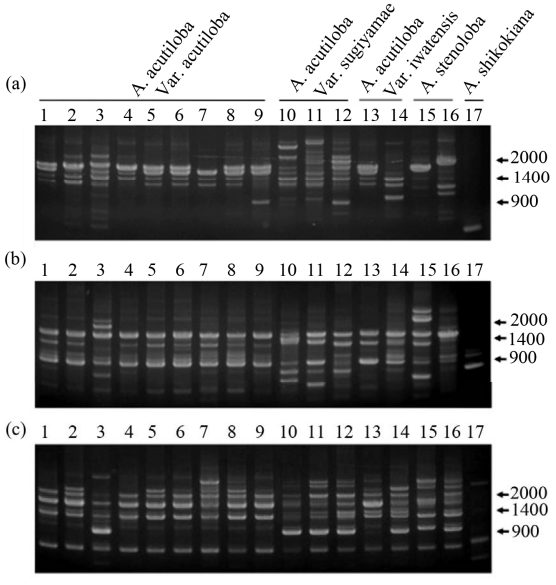

Figure 1. RAPD profiles generated by OPA-16 (a), OPD-3 (b) and OPD-15 (c) primers. Lanes 1 - 9: *A. acutiloba* var. *acutiloba* (TG, TK, TY, TH, TC, YB, YM, OT, OC; refer to Table 1 for codes), lanes 10 - 12: *A. acutiloba* var. *sugiyamae* (HA, HB, HC), lanes 13 and 14: *A. acutiloba* var. *iwatensis* (MM, MY), lane 15: *A. stenoloba* (LH), lane 16: *A. stenoloba* f. *lanceolata* (LT), lane 17: *A. shikokiana* (IM).

The Miyagi strain of *A. acutiloba* var. *iwatensis* showed a similar genetic background to *A. acutiloba* var. *acutiloba* and the Yamagata strain of *A. acutiloba* var. *iwatensis* showed a similar genetic background to *A. acutiloba* var. *sugiyamae*. These findings suggested that *A. acutiloba* var. *iwatensis* was a historical parent of both cultivars of *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*. These RAPD analyses can be used to elucidate the origins of both *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*. Since *A. acutiloba* var. *iwatensis* grows wild in Japan [6], further studies are required to clarify the history of outcrossing among these closely related species and varieties.

4. Acknowledgements

We are grateful to Prof. K. Koike, Faculty of Pharmaceutical Sciences, Toho University, Mr. H. Hayasaka, Graduate School of Pharmaceutical Science, Tohoku University, Mr. M. Murakami, Toyama Prefectural Institute for Pharmaceutical Research, and Dr. T. Shibata, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, for supplying *A. acutiloba* plants. We thank Assoc. Prof. K. Hotta, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, for providing *A. acutiloba* var. *sugiyamae* plants.

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