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Identification of *Angelica acutiloba* and Related Species by Analysis of Inter- and Intra-Specific Sequence Variations in Chloroplast and Nuclear DNA Sequences

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ABSTRACT

Japanese Angelica Root prepared from *Angelica acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*, known in Japan as "Toki" and "Hokkai Toki", is an important crude drug used in Kampo medicine (traditional Japanese medicine). However, since these *Angelica* varieties have recently outcrossed with each other, it is unclear whether Japanese Angelica Root sold for use in Kampo medicine is a pure variety. Here, we describe DNA sequence polymorphisms that can be used to distinguish between *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*. In our analyses, differences in the *trn*K region of chloroplast DNA distinguished among some *A. acutiloba* varieties and related species, but not between *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *iwatensis*. One geographical strain of *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* showed identical sequences in three regions of chloroplast DNA, but differences in the internal transcribed spacer region of nuclear ribosomal DNA. One strain of *A. acutiloba* var. *iwatensis* and *A. acutiloba* var. *sugiyamae* had identical sequences in all of the chloroplast and nuclear ribosomal DNA regions examined. These findings show that *A. acutiloba* var. *acutiloba* has hybridized with *A. acutiloba* var. *sugiyamae* and that the "Hokkai Toki" variety resulted from outcrossing with *A. acutiloba* var. *iwatensis*. Molecular authentication based on analyses of chloroplast and nuclear ribosomal DNA sequences of *A. acutiloba* and related species is an efficient method to authenticate Japanese Angelica Root at the variety level. Therefore, these analyses can determine whether a product is derived from *A. acutiloba* var. *acutiloba* or *A. acutiloba* var. *sugiyamae*.

Keywords: *Angelica acutiloba*; Chloroplast DNA; ITS; Japanese Angelica Root; Kampo Medicine; Sequence Variation

1. Introduction

The Japanese indigenous species of *Angelica acutiloba* Kitagawa var. *acutiloba* Kitagawa (Toki) or *A. acutiloba* Kitagawa var. *sugiyamae* Hikino (Hokkai Toki) are listed in the Japanese Pharmacopoeia, 16th Edition [1], and are precious crude drugs in Kampo medicine (traditional Japanese medicine). The *A. acutiloba* cultivar (also called "Yamato Toki" or "Ohbuka Toki"), is of higher quality than the "Hokkai Toki" cultivar, and was originnally grown in Nara prefecture, Japan. The high quality of this cultivar is described in historical Japanese literature [2,3]. To this day, its quality is still regarded as being higher than that of other cultivars, and this is reflected in the market price of the root. Since the 1950s, the "Hokkai Toki" variety of *A. acutiloba* var. *sugiyamae* has been cultivated in various regions of Japan, including Nara prefecture [4]. *Angelica* species are thought to be outcrossed [5]. There are wild-growing species that that are closely related to *A. acutiloba*, including *A. acutiloba* Kitagawa var. *iwatensis* Hikino (Miyama Toki), which is genetically very close to "Yamato Toki", and *A. stenoloba* ("Hosoba Toki" or "Tokachi Toki") [6]. These species readily outcross with *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* [7].

Previous molecular studies on *A. acutiloba* did not show any nucleotide polymorphisms in the intergenic spacer region of 5S rDNA [8], while *A. acutiloba* var. *iwatensis* collected from different geographical locations showed genetic polymorphisms in a random amplified polymorphism (RAPD) analysis [9]. There were small differences in RAPD patterns between *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* [10], and there were differences among individual plants of these varieties, indicating genetic diversity among *A. acutiloba* varieties. The three varieties of *A. acutiloba* var. *acutiloba*, *A. acutiloba* var. *sugiyamae*, and *A. acutiloba* var. *iwat-*

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ensis had two-nucleotide differences in the spacer region between the *atp*F and *atp*A genes in the plastid genome [11]. Although there are genomic DNA polymorphisms among *Angelica* varieties, these varieties are genetically heterogeneous because of the high rate of cross-pollination [12].

The root of *A. acutiloba* var. *iwatensis* is not used as a crude drug for Kampo medicine in Japan [1], although the morphological features are very similar among related varieties of *A. acutiloba*, making them difficult to distinguish from one another. Therefore, a simple method to discriminate among these varieties is required. *A. acutiloba* var. *acutiloba* is also cultivated in China [13]. The Chinese product prepared from the root of *A. acutiloba* var. *acutiloba* is called "Nisshiki Toki" and resembles the Japanese style of "Yamato Toki". This product has been imported into Japan. It is now difficult to find the original varieties of *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*. Therefore, it is important to be able to distinguish among species and to determine the geographic origin of *Angelica* varieties. A reliable identification method will be useful to maintain pure varieties with superior traits for use in Kampo medicine.

In this study, we investigated various DNA sequences to provide useful information for authentication of *A. acutiloba* varieties and related species. The DNA sequences were the *atp*F-*atp*A, *rpl*16-*rpl*14, and *trn*K regions of chloroplast DNA (cpDNA), and the internal transcribed spacer (ITS) of nuclear ribosomal DNA, consisting of five regions; 18S, ITS1, 5.8S, ITS2, and 26S.

2. Materials and Methods

2.1. Plant Materials

As shown in **Table 1**, we collected nine strains of *Angelica acutiloba* var. *acutiloba*, three of *A. acutiloba* var. *sugiyamae*, and two of *A. acutiloba* var. *iwatensis* from cultivars of different location or native populations. We also collected the cultivated strains of related species *A. stenoloba* ("Hosoba Toki"), *A. stenoloba* f. *lanceolata* ("Tokachi Toki"), and *A. shikokiana* ("Inu Toki"). Each collected plant was identified by morphological characters. Three individuals of each strain/variety were cultivated in a greenhouse before use in experiments.

Table 1. Plant materials used in this study and summary of haplotype data for *Angelica acutiloba* and related species.

Sample code	Species	Japanese name	Locality	Origin	DDBJ/GenBank/EBI Data Bank accession number			
					*atp*F-*atp*A	*rpl*16-*rpl*14	*trn*K	*ITS*
TG-1[a]	*A. acutiloba* var. *acutiloba*	Toki	Gumma	Cultivar	AB697525	AB697547	AB697569	AB697591
TG-2	*A. acutiloba* var. *acutiloba*	Toki	Gumma	Cultivar	AB697526	AB697548	AB697570	AB697592
TK	*A. acutiloba* var. *acutiloba*	Toki	Kyoto	Cultivar	AB697527	AB697549	AB697571	AB697593
TY	*A. acutiloba* var. *acutiloba*	Toki	Yamagata	Cultivar	AB697528	AB697550	AB697572	AB697594
TH	*A. acutiloba* var. *acutiloba*	Toki	Hokkaido	Cultivar	AB697529	AB697551	AB697573	AB697595
TC	*A. acutiloba* var. *acutiloba*	Toki	China	Cultivar	AB697530	AB697552	AB697574	AB697596
YH	*A. acutiloba* var. *acutiloba*	Toki	Hokkaido	Cultivar	AB697531	AB697553	AB697575	AB697597
YM	*A. acutiloba* var. *acutiloba*	Toki	Miyagi	Cultivar	AB697532	AB697554	AB697576	AB697598
OT-1	*A. acutiloba* var. *acutiloba*	Toki	Toyama	Cultivar	AB697533	AB697555	AB697577	AB697599
OT-2	*A. acutiloba* var. *acutiloba*	Toki	Toyama	Cultivar	AB697534	AB697556	AB697578	AB697600
OC-1	*A. acutiloba* var. *acutiloba*	Toki	Chiba	Cultivar	AB697535	AB697557	AB697579	AB697601
OC-2	*A. acutiloba* var. *acutiloba*	Toki	Chiba	Cultivar	AB697536	AB697558	AB697580	AB697602
HA	*A. acutiloba* var. *sugiyamae*	Hokkai Toki	Hokkaido	Cultivar	AB697537	AB697559	AB697581	AB697603
HB	*A. acutiloba* var. *sugiyamae*	Hokkai Toki	Hokkaido	Cultivar	AB697538	AB697560	AB697582	AB697604
HC	*A. acutiloba* var. *sugiyamae*	Hokkai Toki	Chiba	Cultivar	AB697539	AB697561	AB697583	AB697605
MM	*A. acutiloba* var. *iwatensis*	Miyama Toki	Miyagi	Cultivar	AB697540	AB697562	AB697584	AB697606
MY	*A. acutiloba* var. *iwatensis*	Miyama Toki	Yamagata	Native population	AB697541	AB697563	AB697585	AB697607
LH	*A. stenoloba*	Hosoba Toki	Saitama	Cultivar	AB697542	AB697564	AB697586	AB697608
LT	*A. stenoloba* f. *lanceolata*	Tokachi Toki	Saitama	Cultivar	AB697543	AB697565	AB697587	AB697609
IM-1	*A. shikokiana*	Inu Toki	Miyazaki	Cultivar	AB697544	AB697566	AB697588	AB697610
IM-2	*A. shikokiana*	Inu Toki	Miyazaki	Cultivar	AB697545	AB697567	AB697589	AB697611
BB	*Peucedanum japonicum*	Botanbofu	Chiba	Native population	AB697546	AB697568	AB697590	AB697612

[a], -1, and -2 are the same strain but different individuals.

2.2. Total DNA Extraction and Amplifications

We extracted total DNA from approximately 200 mg young leaf tissue using the modified CTAB method [14]. The extracted DNA was used as the template for polymerase chain reaction (PCR) amplifications. The *atp*F-*atp*A, *rpl*16-*rpl*14, and *trn*K regions, and the entire ITS region containing intergenic spacer regions were amplified with the following primers: atpF1 (TTACGAGGAGCTCTAGAACTCTGAATAGTTGTT TG) and atpR1 (GCCATTACTTCATCAAGACCGTG AATACGAGCAATGCC) for the *atp*F-*atp*A region desinged by Hosokawa *et al*. [11]; PSIDF1 (AAAGATCTAGATTTCGTAAACAACATAGAGGAAGAA) and PSIDR1 (ATCTGCAGCATTTAAAAGGGTCTGAGGT TGAATCAT) for the *rpl*16-*rpl*14 region desinged by Ohta *et al*. [15]; trnKF1 (TGGGTTGCTAACTCAATG G) and trnKR1 (AACTAGTCGGATGGAGTAG) for the *trn*K region desinged by Zhu *et al*. [16]; and ITSF1 (TCCACTGAACCTTATCATTTAG) and ITSR1 (CCA TGCTTAAACTCAGCGGGT) for the ITS region desinged in this study. Each amplification was carried in a reaction mixture containing ~50 ng total DNA, 1×ExTaq buffer, 0.2 mM each deoxynucleotide triphosphate, 1 mM each primer, and 2.5 U ExTaq (TaKaRa, Japan). The amplification conditions were as follows: initial denaturation for 5 min at 95˚C, followed by 30 cycles of 95˚C for 45 s, 60˚C for 1 min, and 72˚C for 2 min, and a final extension at 72˚C for 5 min. The PCR products were purified using the ChargeSwitch-Pro PCR Clean-up kit (Invitrogen, Carlsbad, CA, USA). The purified PCR products were sequenced and analyzed using an ABI PRISM Dye Terminator Cycle Sequencing FS Core kit (Applied Biosystems, USA) and an ABI 3100 DNA Sequencer (Applied Biosystems). The sequences obtained in this study have been registered in DDBJ/GenBank/EBI Data Bank under the accession numbers shown in **Table 1**.

2.3. Sequence Alignments and Phylogenetic Analyses

The nucleotide sequences were aligned using BioEdit software (version 6.0.8.0) [17]. Insertion-deletions (indels) were removed from all data set prior to phylogenetic analysis. Neighbor-joining (NJ) analyses were performed using MEGA 5 [18] by calculating genetic distance based on Kimura's two-parameter model [19]. One thousand bootstrap replications were performed for each tree to evaluate the reliability of the topology.

3. Results and Discussion

The intergenic spacer sequences of *atp*F-*atp*A in *A. acutiloba* varieties were 48 - 50 base pairs (bp) in length and contained two polymorphic sites (**Table 2**). The *atp*F-*atp*A sequences were identical among all strains of *A. acutiloba* var. *sugiyamae*, *A. acutiloba* var. *acutiloba* from Yamagata, *A. acutiloba* var. *iwatensis* from Yamagata, *A. stenoloba*, *A. stenoloba* f. *lanceolata*, and one individual of *A. shikokiana*. There were intraspecific variations in *atp*F-*atp*A sequences in three strains of *A. acutiloba* var. *acutiloba* (TG, OT, and OC) and *A. shikokiana*. Two individuals of each of the TG (TG-1 and TG-2), OT (OT-1 and OT-2), and OC (OC-1 and OC-2) strains of *A. acutiloba* var. *acutiloba* contained indels of the T-nucleotide at 31 bp. There was also an indel of the T-nucleotide at 30 bp between two individals of *A. shikokiana*. Therefore, there were intraspecific variations in this region of the chloroplast genome both among and within strains of *A. acutiloba* var. *acutiloba* and *A. shikokiana*. It was previously reported that some varieties of *A. acutiloba* var. *acutiloba* (such as "Toki", "Yamato Toki" and "Ohbuka Toki") could be distinguished from other varieties (such as *A. acutiloba* var. *sugiyamae* and *A. acutiloba* var. *iwatensis*) by differences in the intergenic spacer sequences between the *atp*F and *atp*A genes [11]. In this study, however, the *atp*F-*atp*A region could not be used to authenticate *A. acutiloba* varieties and related species. The *atp*F-*atp*A sequence of *Peucedanum japonicum* (AB697546 in GenBank), which is a genus related to *Angelica* in the Umbelliferae, contained a 3-bp deletion between nucleotide positions 29 and 31, compared with the *atp*F-*atp*A sequences in *Angelica* species determined here. Therefore, this region may be useful to distinguish *Angelica* from related genera *Peucedanum*, but cannot be used to distinguish *Angelica* species from each other.

In three varieties of *A. acutiloba*, the sequences of the *rpl*16-*rpl*14 spacer region consisted of 463 bp that were identical to AB199891 in GenBank, which is the *rpl*16-*rpl*14 region in *A. acutiloba* var. *acutiloba*. Although there were no variations in this region between *A. acutiloba* and *A. stenoloba*, *A. stenoloba* f. *lanceolata* contained a T-nucleotide insertion at nucleotide position 449 (**Table 2**). In *A. shikokiana*, there was a 31-bp insertion between nucleotides 365 and 396 in this region. *A. shikokiana* differs morphologically from *A. acutiloba* and *A. stenoloba* varieties, based on phenotypic traits. Therefore, the genetic background of *A. shikokiana* likely differs from that of the other species.

The *trn*K sequence was identical among *A. acutiloba* var. *acutiloba* from Gumma, Kyoto, Hokkaido, Miyagi, Toyama, and Chiba, and *A. acutiloba* var. *iwatensis* from Miyagi (**Table 2**). The *trn*K sequence in the Chinese *A. acutiloba* var. *acutiloba* had one base substitution at nucleotide position 2238. Thus, the Chinese *A. acutiloba* var. *acutiloba* could be distinguished from the others based on this sequence difference. In *A. acutiloba* var.

Table 2. Nucleotide sequence variation and the classification of cpDNA and ITS in Angelica acutiloba and related species[a].

Sample code	*atp*F-*atp*A[b]		*rpl*16-*rpl*14			*trn*K																*ITS*																							
	30	31	365 -396[c]	448	449	463	918	1134	1841	1893	2021	2125	2193	2208	2238	2262	2255	2368	2433	93	98	110	119	161	167	168	173	279	460	545	547	568	590	592	605	640									
TG-1, TK, YM, OT-2, OC-2	T	T	-	T	-	T	A	C	T	T	A	G	C	T	T	C	A	-	C	C	T	G	C	C	G	C	G	C	C	G	C	A	T	C	T	C									
TG-2, TH, YH, OT-1, OC-1	T	-	-	T	-	T	A	C	T	T	A	G	C	T	T	C	A	-	C	C	T	G	C	C	G	C	G	C	C	G	C	A	T	C	T	C									
TY	-	-	-	T	-	T	A	C	T	T	A	G	A	T	T	C	A	T	C	C	T	G	C	C	G	C	G	T	C	G	C	A	T	C	T	C									
TC	T	T	-	T	-	T	A	C	T	T	A	G	C	T	C	C	A	-	C	C	T	G	C	C	G	C	G	C	C	G	C	A	T	T	T	C									
HA, HB, HC, MY	-	-	-	T	-	T	A	C	T	T	A	G	A	T	T	C	A	T	C	C	T	G	C	C	G	C	G	C	C	G	C	A	T	C	T	C									
MM	T	T	-	T	-	T	A	C	T	T	A	G	C	T	T	C	A	-	C	C	T	G	G	C	G	C	G	C	C	G	C	A	T	C	T	C									
LH	-	-	-	T	-	A	A	C	T	T	A	G	C	T	T	C	A	T	C	C	T	G	C	C	A	C	G	C	C	G	C	A	T	C	T	C									
LT	-	-	-	T	T	A	A	C	T	T	A	G	C	T	T	C	A	T	C	C	T	G	C	C	G	C	G	C	C	G	C	A	T	C	T	C									
IM-1	T	-	+	-	-	T	G	A	C	G	G	T	C	C	T	A	C	T	A	T	A	A	C	T	G	T	T	C	T	A	T	G	C	C	A	T									
IM-2	-	-	+	-	-	T	G	A	C	G	G	T	C	C	T	A	C	T	A	T	A	A	C	T	G	T	T	C	T	A	T	G	C	C	A	T									

[a] Number indicates position of the variable site in intragenic region based on the sequence of TG; [b] Dash indicates deletion; [c] Plus between 365 and 396 shows insertion of 31 bp specific to TG.

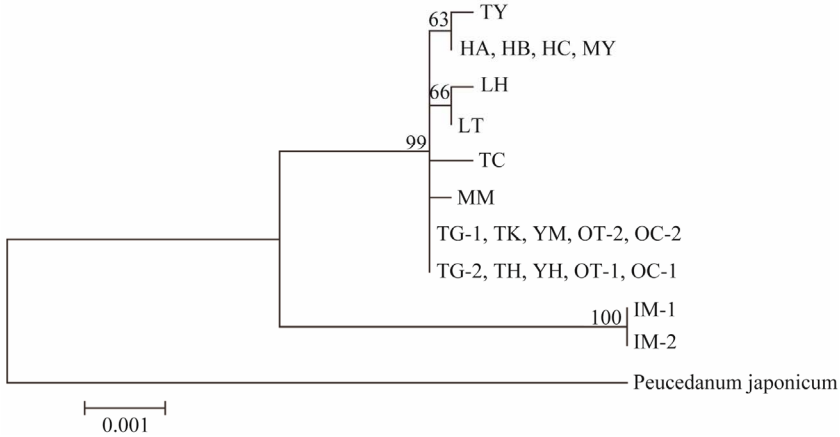

Figure 1. Neighbor-joining tree based on combined analysis of cpDNA (*atp*F-*atp*A, *rpl*16-*rpl*14, *trn*K) and ITS sequences. Tree was outgroup-rooted using sequence data from *Peucedanum japonicum*. Numbers beside internal branches indicate Bootstrap values (>50%) calculated from 1000 replicates.

acutiloba from Yamagata, three strains of *A. acutiloba* var. *sugiyamae*, and *A. acutiloba* var. *iwatensis* from Yamagata, the *trn*K sequences had an A instead of a C at nucleotide position 2193, and often contained a T-nucleotide insertion at nucleotide position 2368. Two varieties of *A. stenoloba* and the "Hokkai Toki" variety also had the T-nucleotide insertion at nucleotide position 2368, and the latter had a T-to-A substitution at nucleotide position 463. The *trn*K sequence in *A. shikokiana* had 10 base substitutions compared with that in *A. acutiloba*.

To obtain more information on genetic diversity among varieties and individuals of *Angelica*, especially among *A. acutiloba* varieties, we determined the sequences of the ITS region, including the 18S, ITS1, 5.8S, ITS2, and 26S regions. There were no variations in this region among individuals of *A. acutiloba* var. *acutiloba* (except for the strains from Yamagata and China), and all *A. acutiloba* var. *sugiyamae*. There was one unique substitution at position 279 in the 5.8S region of *A. acutiloba* var. *acutiloba* from Yamagata, and a C-to-T substitution at position 592 in the ITS2 region of Chinese strain. Also, there was a one-nucleotide substitution at position 119 in the ITS1 region in *A. acutiloba* var. *iwatensis* from Miyagi and at position 167 in the ITS1 region in *A. stenoloba*. There were 13 nucleotide substitutions in the ITS sequence of *A. shikokiana*, compared with this region in the other varieties. The ITS region and the cpDNA sequence were identical between one strain of *A. acutiloba* var. *iwatensis* and all strains of *A. acutiloba* var. *sugiyamae*. These results imply that the 'Hokkai Toki' is derived from *A. acutiloba* var. *iwatensis*. In a previous study, *A. acutiloba* var. *iwatensis* from different geographical locations showed genetic polymorphisms in a RAPD analysis [9]. Because of that result and the differences in sequences of both the *trn*K and ITS regions, further analysis should be conducted using other *A. acutiloba* var. *iwatensis* varieties.

We constructed a neighbor-joining tree based on Kimura's two-parameter model [19] for the 10 haplotypes (**Table 2**) based on the combined analysis of cpDNA (*atp*F-*atp*A, *rpl*16-*rpl*14 and *trn*K) and ITS sequences (**Figure 1**). The monophyly of *A. shikokiana* and other species was supported by a bootstrap value of 100. This result indicates that *A. acutiloba* is more closely related to two varieties of *A. stenoloba* than to *A. shikokiana*.

The traditional methods for authenticating crude drugs and adulterants are based on the morphological characters of the plants. In general, differences among morphological characters and compounds are subtle and ambiguous [20]. Based on sequence analyses of the *trn*K and ITS regions in *A. acutiloba*, we have proposed a method to authenticate the origin of Japanese Angelica Root; that is, whether it is derived from *A. acutiloba* var. *acutiloba* or *A. acutiloba* var. *sugiyamae*. The molecular authentication of these regions is a highly sensitive and stable method. The authentication results are reliable and are not affected by the physical form or physiological conditions of the plant samples. Therefore, the method based on the combined cpDNA and ITS sequences by the addition of conventional methods is useful for practical and accurate authentication of *A. acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae*. This method will be useful in raw material production processes and for quality control of "Toki" produced for use in Kampo medicine in Japan.

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REFERENCES

[1] Hirokawa Publishing Co., "The Japanese Pharmacopoeia," 16th Edition, Tokyo, 2011.

[2] E. Kaibara, "Chapter Galenicals Toki," *Yamato Honzo*, Vol. 6, 1709.

[3] R. Terashima, "Chapter Herbs Toki," *Wakansansazue*, Vol. 93, 1713.

[4] H. Hikino, "Bibliographical Research and History of Productions of Toki or Tang Kuei 2," *Yakugaku Kenkyu*, Vol. 29, 1957, pp. 1059-1078.

[5] Ministry of Health and Welfare, Pharmaceutical Affairs Bureau, "Yakuyou-Shokubutsu Saibai to Hinshitsu-Hyoka Part 1," *Yakuji Nippo*, Tokyo, 1992, pp. 41-50.

[6] T. Gotoh, T. Tanaka, T. Kawamura and Y. Noro, "Phytosociological Studies of the Communities on *Angelica acutiloba* and *A. acutiloba* var. *iwatensis* (1) Gifu Prefecture and Its Surroundings," *Natural Medicines*, Vol. 49, 1995, pp. 255-260.

[7] K. Hatano, I. Nishioka and S. Iwasa, "Cytogenetical Studies of Umbelliferous Plants. I. 1) The Karyotype and Cross-Compatibility on the Original Plants of Japanese Toki," *Shoyakugaku Zasshi*, Vol. 28, 1974, pp. 51-64.

[8] H. Mizukami, B. S. Hao and T. Tanaka, "Nucleotide Sequence of 5S-rDNA Intergenic Spacer Region in *Angelica acutiloba*," *Natural Medicines*, Vol. 51, 1997, pp. 376-378.

[9] M. Kohjyouma, O. Iida, N. Yoshida, Y. Hatakeyama, M. Satake, S. Sekita and H. Kohda, "Random Amplified Polymorphic DNA Analysis of *Angelica acutiloba* and Its Varieties," *Natural Medicines*, Vol. 52, 1998, pp. 130-134.

[10] A. Watanabe, S. Araki, S. Kobari, H. Sudo, T. Tsuchida, T. Uno, N. Kosaka, K. Shimomura, M. Yamazaki and K. Saito, "*In Vitro* Propagation, Restriction Fragment Length Polymorphism, and Random Amplified Polymorphic DNA Analyses of *Angelica* Plants," *Plant Cell Reports*, Vol. 18, No. 3-4, 1998, pp. 187-192. doi:10.1007/s002990050554

[11] K. Hosokawa, A. Hishida, I. Nakamura and T. Shibata, "The Sequences of the Spacer Region between the *atpF* and *atpA* Genes in the Plastid Genome Allows Discrimination among Three Varieties of Medicinal *Angelica*," *Planta Medica*, Vol. 72, No. 6, 2006, pp. 570-571. doi:10.1055/s-2005-916257

[12] J. Murata, H. Oki, K. Kakutani and T. Hashimoto, "Identification of DNA polymorphisms in *Angelica acutiloba*," *Plant Biotechnology*, Vol. 25, No. 2, 2008, pp. 157-163. doi:10.5511/plantbiotechnology.25.157

[13] K. Fukuda, K. Murata, H. Matsuda and T. Tani, "History and the Present of Cultivation and Production of Angelica Root, Yamato-Toki, in Japan," *Yakushigaku Zasshi*, Vol. 44, 2009, pp. 10-17.

[14] M. W. Lassner, P. Peterson and J. I. Yoder, "Simultaneous Amplification of Multiple DNA Fragments by Polymerase Chain Reaction in the Analysis of Transgenic Plants and Their Progeny," *Plant Molecular Biology Reporter*, Vol. 7, No. 2, 1989, pp. 116-128. doi:10.1007/BF02669627

[15] S. Ohta, S. Osumi, T. Katsuki, I. Nakamura, T. Yamamoto and Y. Sato, "Genetic Characterization of Flowering Cherris (*Prunus* subgenus *Cerasus*) Using *rpl*16-*rpl*14 Spacer Sequence of Chloroplast DNA," *Journal of the Japanese Society for Horticultural Science*, Vol. 75, No. 1, 2006, pp. 72-78. doi:10.2503/jjshs.75.72

[16] S. Zhu, H. Fushimi, G. Han, T. Tsuchida, T. Uno, A. Takano and K. Komatsu, "Molecular Identification of 'Chuanxiong' by Nucleotide Sequence and Multiplex Single Base Extension Analysis of Chloroplast *trnK* Gene," *Biological and Pharmceutical Bulletin*, Vol. 30, No. 3, 2007, pp. 527-531. doi:10.1248/bpb.30.527

[17] T. A. Hall, "BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT," *Nucleic Acids Symposium Series*, Vol. 41, 1999, pp. 95-98.

[18] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, "MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods," *Molecular Biology and Evolution*, Vol. 28, 2011, pp. 2731-2739. doi:10.1093/molbev/msr121

[19] M. Kimura, "A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide Sequences," *Journal of Molecular Evolution*, Vol. 16, No. 2, 1980, pp. 111-120. doi:10.1007/BF01731581

[20] Z. Y. Yang, Z. Chao, K. K. Huo, H. Xie, Z. P. Tian and S. L. Pan, "ITS Sequence Analysis Used for Molecular Identification of the *Bupleurum* Species from Northwestern China," *Phytomedicine*, Vol. 14, 2007, pp. 416-423. doi:10.1016/j.phymed.2007.04.009

