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Identification of DNA polymorphisms in *Angelica acutiloba*

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Abstract *Angelica acutiloba* and related species have been known for their analgesic and sedative effects, and are widely used as the key ingredient in Japanese traditional Kampo medicine. Among various varieties, *Angelica acutiloba* var. *acutiloba* has long been grown in the Obuka area of Nara prefecture, Japan, and has been evaluated as an excellent variety. Recent cultivation of this and other varieties of *Angelica* in various regions of East Asia prompted us to identify DNA polymorphisms that distinguish the *acutiloba* variety from others. Random Amplified Polymorphic DNA analysis and sequencing selected genomic regions among *Angelica* plants of different origins identified several nucleotide changes among the varieties tested, which enabled us to distinguish the *acutiloba* variety from others. We also report an improved protocol for genomic DNA extraction from dried root samples of *Angelica*.

Key words: *Angelica acutiloba*, DNA polymorphism, herbal medicine, PCR, RAPD.

Dried roots of several varieties of *Angelica acutiloba* Kitagawa (Tohki) and related species have been widely used as one of the key ingredients in Japanese traditional Kampo medicine. Among various therapeutic properties, this herbal medicine is especially known for its analgesic and sedative effects, and is mainly used to treat women's diseases. Phytochemical studies suggest that *Angelica* roots contain high level of volatile ligustilide and related metabolties, which are believed to be at least partially responsible for their therapeutic effects. *A. acutiloba* Kitagawa var. *acutiloba* Kitagawa (also called 'Yamato' Tohki) was originally grown in the Obuka area of Nara prefecture, Japan and has been awarded a top reputation in the Japanese herb market for many years amongst varieties available in East Asian countries. *A. acutiloba* Kitagawa var. *sugiyamae* Hikino ('Hokkai' Tohki) and other *A. acutiloba* varieties, as well as related *Angelica* species are currently grown in Japan and other East Asian countries including China and Korea. In the Japanese herb market, the dried roots of various *Angelica* species and varieties are used to prescribe herbal Tohki medicines. Because *Angelica* species outcross and because the *sugiyamae* "Hokkai" variety has been cultivated in various regions of Japan, including Nara prefecture (Hikino 1957), it is important to maintain pure varieties with superior and/or preferred traits.

Several varieties of *A. acutiloba* did not show any nucleotide polymorphisms in the intergenic spacer region of 5S rDNA (Mizukami et al. 1997). Although no restriction fragment length polymorphism (RFLP) was observed between the *acutiloba* variety and the *sugiyamae* variety, a small difference in random amplified polymorphic DNA (RAPD) patterns was detected between the two varieties (Watanabe et al. 1998). Watanabe et al. (1998) also observed differences in RAPD patterns among individual plants of the two varieties, indicative of genetic heterogeneity among the *A. acutiloba* populations grown in Japan. It has also been reported that the two varieties had two-nucleotide differences in the spacer region between the *atpF* and *atpA* genes in the plastid genome (Hosokawa et al. 2006).

To facilitate DNA fingerprinting of *Angelica*, we identified genetic polymorphisms among *A. acutiloba* varieties by RAPD technique and sequencing of selected genomic loci. The DNA markers thus developed were used to distinguish the Tohki variety from others.

Materials and Methods

Random Amplified Polymorphic DNA (RAPD) and e-RAPD analysis

Genomic DNA was extracted by cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980) with some modifications. Fresh leaf was frozen with liquid nitrogen, and ground to fine powder with a motar and pestle. The powder

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was transferred to a 1.5 ml tube containing 500 μl of extraction buffer (50 mM Tris pH 8.0, 35 mM sorbitol, 5 mM EDTA pH 8.0, 10% polyethylene glycol 4000, 5% soluble polyvinyl pyrrolidone and 2% β-mercaptoethanol) and homogenized with a Pellet pestle (Sigma, USA). 400 μl of washing buffer (50 mM Tris pH 8.0, 35 mM sorbitol, 25 mM EDTA pH 8.0, 0.4% β-mercaptoethanol) and 1 μl of 10 mg/ml RNaseA were added and mix well. Then 48 μl of 10% sarcosyl, 64 μl of 5 M NaCl and 52 μl of CTAB solution (8.6% CTAB and 0.7 M NaCl) were added and the solution was mixed gently. The tubes were incubated for one hour at 65°C with occasional mixing by inverting the tubes. Protein, phenolics and other possible contaminants were removed by chloroform extraction by adding 300 μl of chloroform and mixed gently with a rotator for 5 min at room temperature. 300 μl of the aqueous phase was transferred to a new tube, and genomic DNA was precipitated by adding 300 μl of isopropanol and 30 μl of 3 M sodium acetate (pH 5.8). After the tubes were centrifuged at 15,000 rpm for 15 min, the pellet was rinsed with 700 μl of 70% ethanol, air dried and resuspended in 50 μl of dH_2O. DNA was quantified by using spectrophotometer Ultrospec 3000 (GE Healthcare, USA), and the degree of degradation was visually analyzed by agarose gel electrophoresis. We named this CTAB method as 'standard CTAB protocol' for convenience.

Using the genomic DNA samples extracted from the two *Angelica* ANR and SH1 as templates, PCR reactions were carried out with twenty four decamer primers named Upstream primers in a final volume of 20 μl and 0.25 U of ExTaq DNA polymerase (Takara, Japan) with the PCR cycle as follows; 95°C for 5 min for initial denaturing, then 40 cycles of 95°C for 30 s, 40°C for 2 min and 72°C for 2 min, followed by 5 min of additional extension at 72°C. The primer sets were originally packaged in the Fluorescence Differential Display Kit (Takara, Japan).

In order to achieve higher specificity and reproducibility in identifying polymorphic DNA, we carried out e-RAPD analysis by adding one nucleotide at the 3′ end of the candidate primers as described previously (Tanaka and Taniguchi, 2002). The PCR reactions were conducted as in the case of RAPD analysis. The candidate DNA fragment was subcloned into pGEM-T vector (Promega, USA), and the plasmid was propagated and purified as described elsewhere. The insert of the plasmid was sequenced using T7 promoter primer and SP6 promoter primer by ABI-PRISM 3100-Avant Genetic analyzer (Applied Biosystems, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The chromatograms of the sequence data were analyzed by Finch TV 1.4.0 software (Geospiza, USA).

PCR reactions using gene-specific primers

For PCR reactions to amplify the DNA fragment with the approximate size of 560 bp using specific primers AN_F1 (5′-GATCAATCGCCACTGATTAATTAA-3′) and AN_R1 (5′-GATCAATCGCTATAAAAAGTATC-3′) (RAPD1 primer set), 0.25 U of ExTaq DNA polymerase (Takara, Japan) was used together with under the following PCR cycle; 94°C for 2 min, then 35 cycles of 94°C for 20 s, 56°C for 20 s and 72°C for 30 s, followed by 5 min of additional extension at 72°C.

In order to clone the putative *Angelica LOX*, PCR reactions were carried out using gene-specific primers *LOX*_F1 (5′-GAGCCTTGGTGGCCTAAAAT-3′) and *LOX*_R1 (5′-CTGG-TCCGACTCTGTTCCTC-3′) in a reaction volume of 20 μl with 10 ng template DNA, 0.5 μM of each primer, 0.25 mM dNTPs with 0.25 U of ExTaq DNA polymerase and the buffer system containing 2 mM Mg^{2+} that is included in the ExTaq DNA polymerase package. The PCR cycles described as follows: 94°C for 2 min, then 5 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 30 s, then 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, followed by 5 min of additional extension at 72°C. The spacer region between *atpF* and *atpA* was amplified as described previously (Hosokoawa et al. 2006).

For the detection of the 8-bp insertion within the genome region that was amplified by using RAPD1 primer set, nested-PCR reactions were performed using AS_F2 (5′-AACAAAC-CATGAAGGAAGGG-3′) and AS_R2 (5′-TAGCTCCGATGC-GCATTAAATGC-3′) primers (RAPD2 primer set), since the amplification efficiency of the RAPD2 primer set was not high enough to conduct PCR reactions directly using genomic DNA as templates. Briefly, the first PCR products obtained using the RAPD1 primer set were diluted to a 100-fold and used as templates for the second PCR reactions as follows; 94°C for 2 min, then 35 cycles of 94°C for 20 s, 56°C for 20 s and 72°C for 30 s, followed by 5 min of additional extension at 72°C. The PCR products were separated on 8% polyacrylamide gel electrophoresis, and the DNA fragments were stained with ethidium bromide as described elsewhere. The amplified fragments were sequenced for verification.

The modified protocol for genomic DNA extraction from dried root samples of Angelica.

Purelink Plant DNA extraction kit (Invitrogen, USA) was used to obtain high quality genomic DNA samples from dried root samples of *Angelica* plants, basically according to the manufacturer's protocol, but with additional procedures as described below. A dried root material, 50 mg, was ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a 1.5-ml tube containing 250 μl of the buffer R2 and briefly vortexed. The remaining DNA purification steps were done following the manufacturer's protocol until the genomic DNA was eluted with 149 μl of dH_2O and the eluate was mixed with 28 μl of 5 M NaCl and 23 μl of 8.6% CTAB, and extracted three times with 200 μl of chloroform. DNA was precipitated by adding equal volume of isopropanol and 20 μl of 3 M sodium acetate (pH 5.8) at −20°C for 30 min, and centrifuged at 15,000 rpm for 15 min. The pellet was washed with 500 μl of 70% EtOH, dried and resuspended in 50 μl of dH_2O. DNA was quantified by using spectrophotometer Ultrospec 3000 (GE Healthcare, USA), and the degree of degradation was visually analyzed by agarose gel electrophoresis. The PCR reaction was performed using as described above. For comparison, genomic DNA was also extracted either by 'standard' CTAB method or using DNAzol (Invitrogen, USA) with additional steps of general chloroform extraction or phenol extraction followed by chloroform extraction by adding equal volume of either equilibrated phenol (pH 7.9) (Sigma, USA) or chloroform, respectively.

Sequence analysis

To achieve higher throughput, the amplified DNA fragments from various dried root samples of *Angelica* were either directly purified using QIAquick PCR Purification Kit (Qiagen, Germany) or extracted from the agarose gel by QIAquick Gel Extraction Kit (Qiagen, Germany) for direct sequencing. Both strand of the DNA fragments were sequenced for verification. The DNA sequences in FASTA format were aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) and processed with BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html), which are available on-line. Sequence similarity search was conducted using BLAST program (Altschul et al. 1990; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

Results and discussion

Identification of a candidate DNA marker by RAPD analysis

We used fresh leaf of *Angelica* which have been cultivated and carefully maintained for years in the Obuka area of Nara prefecture, Japan, as the reference plants (ANR) for *Angelica acutiloba* var. *acutiloba*, and 'Hokkai' Tohki which was grown in Hokkaido prefecture (SH1) (Table 1) to amplify genomic DNA for RAPD analysis. The symbol ANR and SH1 stand for the *A. acutiloba* variety grown in Nara as the reference line, and the *A. acutiloba* var. *sugiyamae* grown in Hokkaido prefecture, respectively. Since a reliable reference line was not available to us for the *A. sugiyamae* variety, we used *Angelica* plants grown in several different places as 'Hokkai' Tohki. All the genomic DNA samples were extracted from dried roots unless otherwise mentioned. The root samples used in this study were shown in Table 1.

RAPD analysis identified several candidate primers that may distinguish ANR from SH1; primer No. 15 (5′-GATCAATCGC-3′) produced band patterns which were clearly different between ANR and SH1. Although multiple PCR products were amplified, we focused on

Table 1. *Angelica* samples used in this study.

Sample code	Internal code	Species and suspected variety	Cultivation site
ANR	YNaA_07_1	*Angelica acutiloba* var. *acutiloba*	Obuka, Nara prefecture, Japan
SH1	Hokkai Tohki	*Angelica acutiloba* var. *sugiyamae*	Hokkaido prefecture, Japan
AN1	YNA	*Angelica acutiloba* var. *acutiloba*	Nana prefecture, Japan
AN2	YNC	*Angelica acutiloba* var. *acutiloba*	Nana prefecture, Japan
AC1	YCD	*Angelica acutiloba* var. *acutiloba*	Southern part of China
AH1	YHC	*Angelica acutiloba* var. *acutiloba*	Hokkaido prefecture, Japan
SH2	HHE	*Angelica acutiloba* var. *sugiyamae*	Hokkaido prefecture, Japan
SK1	HCo_7	*Angelica acutiloba* var. *sugiyamae*	Chungchong-namdo, Republic of Krea
AS1	KanT_Xi_sho_070702_1	*Angelica sinensis*	Kansu, China
AS2	KanT_Ch5_Xi_070702	*Angelica sinensis*	Kansu, China

ANR is the reference sample for *Angelica acutiloba* var. *acutiloba*. The first and second alphabets of the sample code represent the species and the cultivation sites, respectively, whereas the third alphabet denotes the sample number except for ANR, which is the reference variety. The first alphabet; A, *Angelica acutiloba* Kitagawa var. *acutiloba* Kitagawa; S, *Angelica acutiloba* Kitagawa var. *sugiyamae* Hikino. The second alphabet; N, Nara prefecture; H, Hokkaido prefecture; C, China; K, Korea.

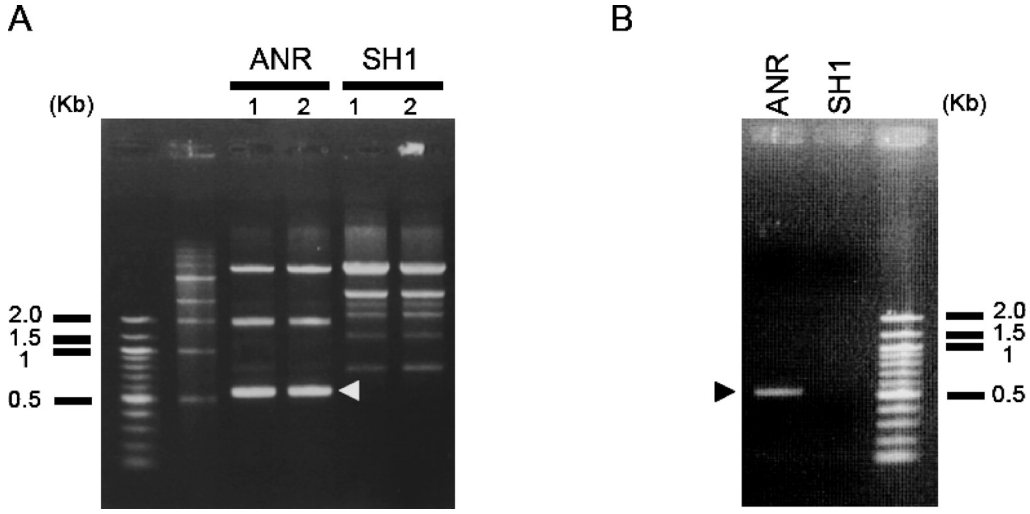

Figure 1. RAPD analysis of *Angelica acutiloba* varieties; (A) RAPD analysis using a decamer primer yielded DNA fragment (arrowhead) specific to the *A. acutiloba* variety (ANR). A *sugiyamae* variety is indicated by SH1. Two independent DNA extractions and PCR amplifications were performed as indicated by 1 and 2. (B) Specific primers, Toki_t15_4yamato_fw1 and Toki_t15_4yamato_rv1, amplified single DNA fragment. PCR reactions were performed using two gene-specific primers AN_F1 and AN_R1, which are the derivatives of the decamer No. 15.

the PCR product with an approximate size of 560 bp, assuming that a small fragment would be amplified more efficiently than large fragments (Figure 1A). We then conducted e-RAPD (Tanaka and Taniguchi 2002) to achieve higher specificity and reproducibility. When thymine or cytosine was added to the 3′ end of the primer No. 15, the 560 bp band was amplified but other bands became less complex (data not shown). After the 560 bp band was cloned into pGEM-T vector (Promega, USA), the insert DNA was sequenced. The result showed that the cloned *Angelica* DNA sequence did not exhibit significant similarity to any DNA sequences deposited in the database. Based on the DNA sequence information, we then designed specific PCR primers AS_F1 (5′-GATCAATCGCCACTGATTAATTAA-3′) and AS_R1 (5′-GATCAATCGCTATAAAAAGTATC-3′), and conducted PCR analysis. This RAPD1 primer set amplified a 560 bp fragment from ANR but not from SH1 (Figure 1B). The amplified DNA fragment was sequenced for verification.

Modified protocol for genomic DNA extraction from dried root samples

Since the dried root samples, but not fresh leaf materials, are generally available for DNA polymorphic analysis, it is essential to establish an efficient and reliable DNA extraction protocol from dried roots of *Angelica*. The quality of the genomic DNA from ANR obtained by various extraction protocols was analyzed by conducting PCR reaction using the RAPD1 primer set the same PCR condition. The results showed that the modified protocol that we developed using PureLink Plant successfully yielded high quality genomic DNA which could be used as templates for PCR analysis (Figure 2, lane 7). On the other hand, six other protocols based either on general

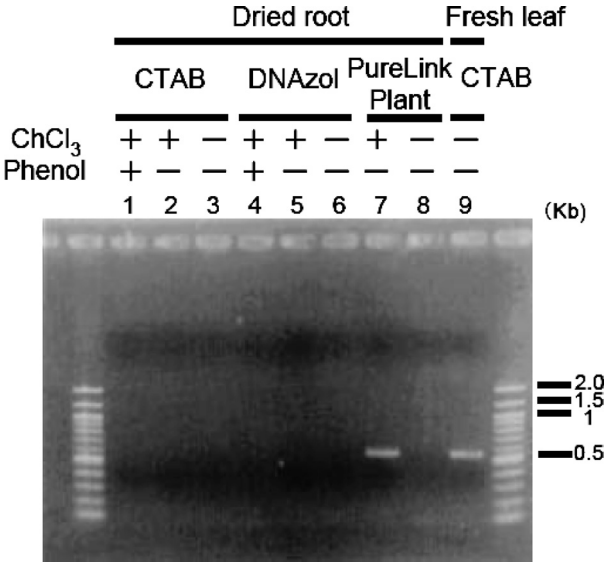

Figure 2. Development of a genomic DNA extraction protocol suitable for dried *Angelica* roots; Genomic DNA was extracted from dried root of ANR by various extraction procedures. Lane1-3, genomic DNA samples were extracted by CTAB-based protocol; lane 4-6, genomic DNA samples were extracted using DNAzol; lane 7 and 8, genomic DNA samples were extracted using PureLink Plant. Genomic DNA extracted by a 'standard' CTAB method from fresh leaf (lane 9) was used as a positive control for PCR reaction. ChCl3, chloroform extraction; phenol, phenol extraction.

Figure 3. An alignment of the DNA sequences amplified by using the RAPD1 primer set. Identical nucleotides among eight *Angelica* plants were boxed in black. AS1 and AS2 were not aligned simply because DNA fragments were not amplified from these two samples by using RAPD1 primer set. The heterogenous DNA sequence of the 8-bp insertion (GTCTGAGA) was indicated as bold letters with shaded background.

Table 2. Heterogeneity of DNA sequences among *Angelica* plants.

Sample code	RAPD1				*atpA–F*	
	Heterozygosity	8-pb insertion	Single nucleotide polymorphism		Single nucleotide polymorphism	
			Position 51	Position 347	Position 33	Position 34
ANR	Homo	−	A	T	T	T
AN2	Homo	−	A	T	T	T
SK1	Homo	+	G	C	T	T
AN1	Hetero	+/−	A	T	T	T
AC1	Hetero	+/−	G	C+T	T	T
AH1	Hetero	+/−	G	C+T	T	T
SH1	Hetero	+/−	G	C+T	T	T
SH2	Hetero	+/−	A	T	*	*
AS1		no amplification			T	*
AS2		no amplification			T	*

A genetic locus was amplified by PCR primer sets RAPD1 and *atpA–F*. The symbol +/− indicates that the PCR reactions using the RAPD1 primer set amplified the DNA fragments either with or without the 8-bp insertion, which was verified by DNA sequencing. No DNA fragments were amplified with the RAPD1 primer set when genomic DNA from AS1 and AS2 varieties were used. The asterisks indicate the absence of the corresponding nucleotides.

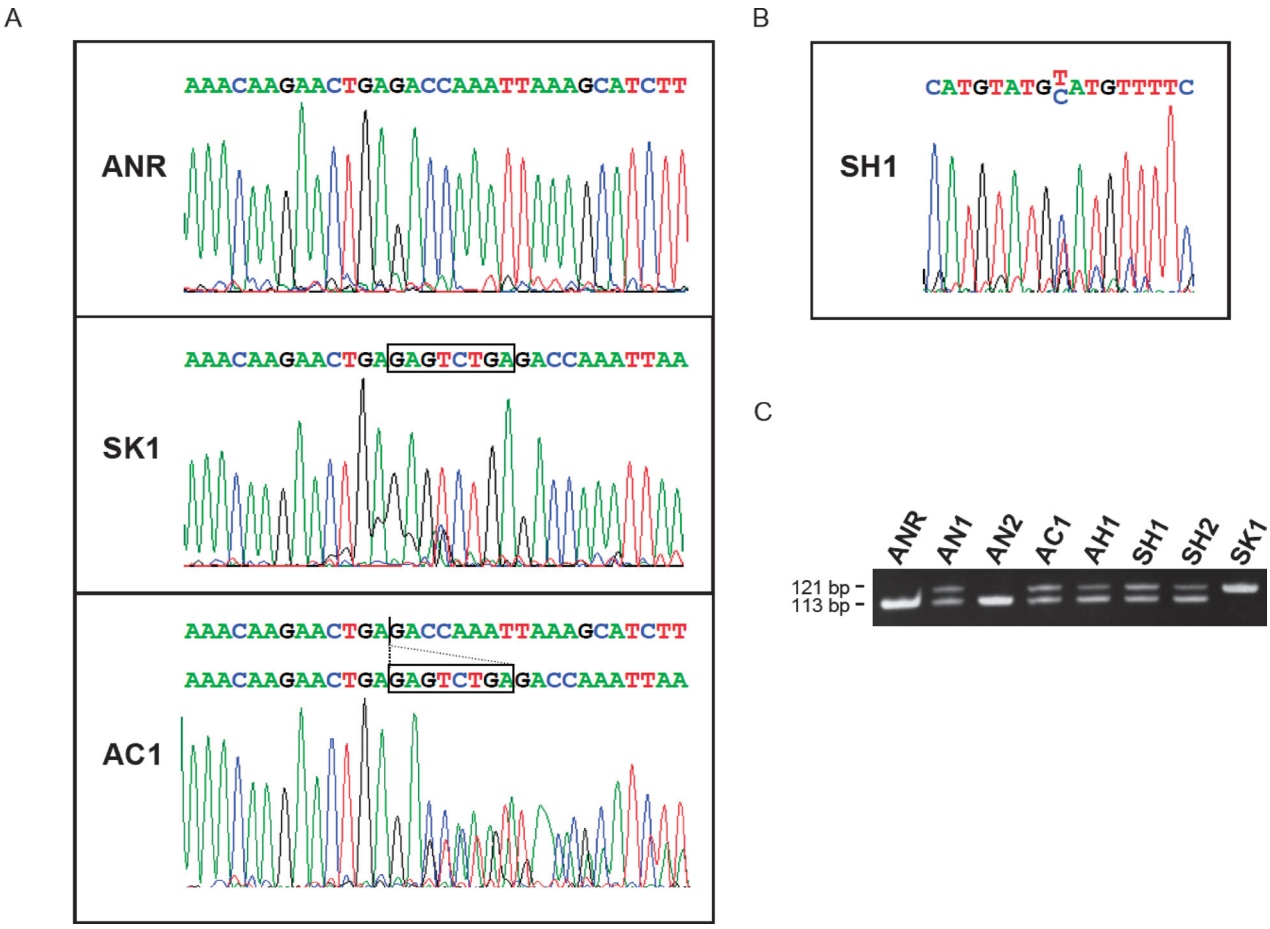

Figure 4. Genetic heterozygocity found *Angelica* plants used in this study. (A) Chromatograms from DNA sequencing results of ANR, SK1 and AC1 near the 8-bp insertion site. The DNA fragments were amplified from genomic DNA samples derived from respective *Angelica* plants by using RAPD1 primer set, and were sequenced by using AN_F1. The chromatogram of ANR is shown as an example of the variety which is homozygous at this locus without the 8-bp insertion. When genomic DNA samples from AN1, AH1 and SH2 were used, virtually the same results were obtained as in the case of AC1. Only the area near the 8-bp insertion was shown. The 8-bp insertion is boxed. (B) Heterozygocity of SH1 at Position 347. Note that the chromatographic peaks of thymine and cytosine overlap. Chromatograms from DNA sequencing of AN1 and AC1 showed virtually identical results to that of SH1. (C) PCR analysis of the 8-bp insertion. The PCR products obtained by using RAPD1 primer set were diluted to a 100-fold and used as templates for the second PCR reaction using RAPD2 primer set. The PCR products were separated by 8% polyacrylamide gel electrophoresis and stained with ethidium bromide.

CTAB method or DNAzol in combination with phenol extraction and chloroform extraction failed to purify genomic DNA compatible with PCR reactions (Figure 2, lane 1–6). Therefore, our modified protocol effectively removed possible inhibitory contaminants for PCR amplification. The DNA extraction protocol described here will be a useful tool for DNA finger printing of *Angelica* and possibly of other herbal samples.

DNA polymorphisms among A. acutiloba

The RAPD1 primer set was used to analyze ten different *Angelica* dried roots available in the market (Table 1). Unexpectedly, purified genomic DNA did not give clear-cut amplification or non-amplification from dried root samples, possibly because of varied extent of degradation of genomic DNA (data not shown). Moreover, this putatively *acutiloba* variety-specific primer set amplified a DNA fragment of the same size when genomic DNA samples from dried roots from the *sugiyamae* variety was used. We then compared the nucleotide sequences of the ~0.5 kb fragments after direct sequencing. The DNA sequences obtained from SK1, AC1, AH1 and SH1 contained two single nucleotide changes (A to G at position 51 and T to C at position 347) compared to those from the reference ANR and an additional plant of putative *acutiloba* variety (AN2) (Figure 3 and Table 2). Moreover, there is an insertion of the 8-bp sequence (GAGTCTGA) in SK1. Notably, AH1 and AC1 were labeled as the *acutiloba* varieties, but have distinct DNA polymorphisms characteristic to the *sugiyamae* variety.

Interestingly, AN1, AC1, AH1 SH1 and SH2 showed genetic heterogeneity in this genome region that is amplified by using the RAPD1 primer set. When the PCR-amplified fragments from AN1, AC1, AH1 SH1 and SH2 were directly sequenced from either end, a single chromatogram was obtained just before the 8-bp insertion but two distinct chromatograms overlapped after the insertion (Figure 4A and Table 2). Heterozygocity of AC1, AH1 and SH1 was also evident from the two sequence reads (cytosine and thymine) at Position 347, where homozygous plants with the 8-bp insertion had cytosine and homozygous plants lacking the insertion had thymine (Figure 4B). These findings also corroborate with the results that two distinct DNA fragments (121 bp and 113 bp) were amplified by PCR when genomic DNA samples were used from AN1, AC1, AH1, SH1 or SH2, while single DNA fragments were obtained from ANR, AN2 and SK1 (Figure 4C). These results imply that the five *Angelica* plants (AN1, AC1, AH1, SH1 and SH2) are heterozygous at the site of the 8-bp insertion, which possibly arose by cross pollination between a plant with the 8-bp insertion and another plant

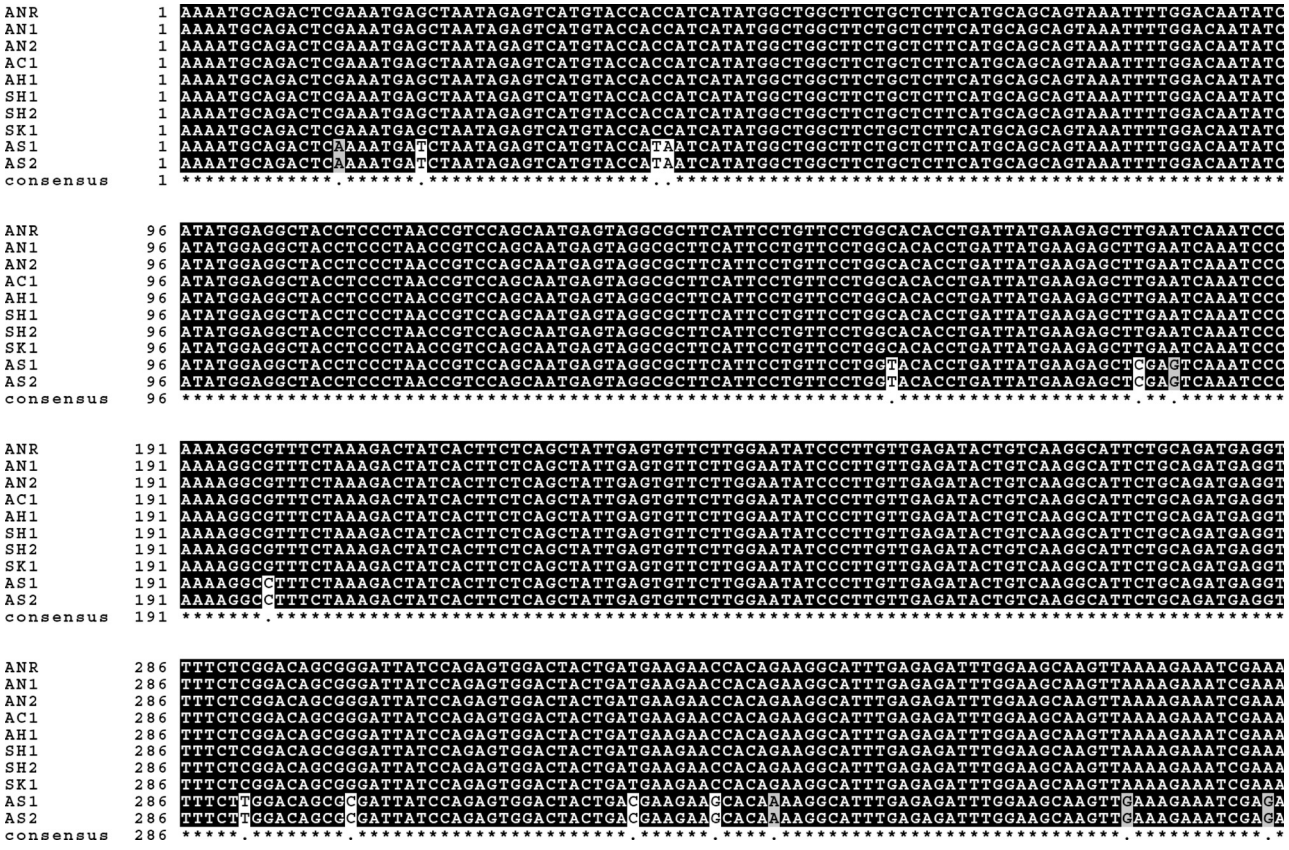

Figure 5. An alignment of the DNA sequences of the putative *Angelica lipoxygenase* gene fragments. Identical nucleotides among eight *Angelica* plants were boxed in black.

without the insertion.

DNA polymorphisms between A. acutiloba *and* A. sinensis

Since the RAPD1 primer set did not amplify DNA fragments from *A. sinensis* Diels, which is also marketed as the Kan Tohki, we tried to amplify polymorphic loci from *A. acutiloba* and *A. sinensis* by PCR. We first amplified the spacer region between the plastidic *atpF* and *atpA* genes, as reported by Hosokawa et al. (2006). The nucleotide positions of 33 and 34 were deleted in the SH2 plant, but these positions were thymines in ANR and other plants putatively classified either as the *acutiloba* variety or the *sugiyamae* variety (Table 2). In *A. sinensis*, the thymine at Position 34 was missing.

We next used a publicly available EST database of celery (Vilaine et al. 2003; Divol et al. 2005), which is in the same Apiaceae family. We chose the putative celery lipoxygenase (*LOX*; AY607693) and designed a primer set for *LOX*, based on the putative celery *LOX*. The obtained DNA fragments of 0.5 kb were directly sequenced and analyzed to show that all the tested plant samples of *A. acutiloba* showed an identical DNA sequence, but *A. sinensis* samples contained multiple nucleotide changes (Figure 5), thereby clearly distinguishing these two *Angelica* species.

When all the DNA polymorphisms found in three different loci of *Angelica* genome (namely *LOX*, the *atpA*-F spacer, and the DNA fragment amplified using the RAPD1 primer set) are combined, one *Angelica* sample which was grown in Nara prefecture (AN2) showed the identical polymorphisms as the authentic *acutiloba* (Yamato Tohki) variety (ANR) but differed from other *Angelica* samples at several polymorphic loci. We also found molecular evidence for extensive hybridization between different varieties of *A. acutiloba* (Figure 4, Table 2). These data suggest that *Angelica* plants grown in the wide area of Japan may be genetically heterogenous, probably due to the high rate of cross pollination. The data also implied that it is important to maintain the elite *A. acutiloba* varieties by preventing cultivation of different varieties in the same field, and to distinguish them by molecular markers. Development of lager sets of DNA markers is underway for more accurate and reliable tests to judge *A. acutiloba* varieties.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J. Mol Biol* 215: 403–410

Divol F, Vilaine F, Thibivilliers S, Amselem J, Palauqui JC, Kusiak C, Dinant S (2005) Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens.* *Plant Mol Biol* 57: 517–540

Hikino H (1957) Bibliographical research and history of productions of "Toki" or "Tang kuei" 2. *Yakugaku Kenkyu* 29: 1059–1078 (in Japanese)

Hosokawa K, Hishida A, Nakamura I, Shibata T (2006) 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 Med* 72: 570–571

Mizukami H, Hao BS, Tanaka T (1997) Nucleotide sequence of 5S-rDNA intergenic spacer region in *Angelica acutiloba*. *Natural Medicines* 51: 376–378

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res* 19: 4321–4326

Tanaka J, Taniguchi F (2002) Emphasized-RAPD (e-RAPD): a simple and efficient technique to make RAPD bands clearer *Breeding Sci* 52: 225–229

Vilaine F, Palauqui JC, Amselem J, Kusiak C, Lemoine R, Dinant S (2003) Towards deciphering phloem: a transcriptome analysis of the phloem *Apium graveolens.* *Plant J* 36: 67–81

Watanabe A, Araki S, Kobari S, Sudo H, Tsuchida T, Uno T, Kosaka N, Shimomura K, Yamazaki M, Saito K (1998) In vitro propagation, restriction fragment length polymorphism, and random amplified polymorphic DNA analyses of *Angelica plants.* *Plant Cell Rep* 18: 187–192