

Difference of ITS sequences of Akebia plants growing in various parts of Japan

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Difference of ITS sequences of *Akebia* plants growing various parts of Japan

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Abstract The stem of *Akebia* plant, “Mokutsu”, is a crude diuretic and antiphlogistic drug. Japanese products prepared from wild *Akebia* plants covers most of the Mokutsu market. Two *Akebia* plants, *Akebia quinata* Decaisne (Aq) and *A. trifoliata* Koidzumi (At) of Lardizabalaceae, are standardized as “Mokutsu” in Japanese pharmacopoeia. These 2 *Akebia* plants along with *A. × pentaphylla* Makino (Ap), which is considered a hybrid with the morphology of Aq and At, can be distinguished by DNA sequence analysis of internal transcribed spacers 1 and 2 (ITS) of nuclear ribosome DNA. Here, we reported the result of molecular genetic analysis of *Akebia* plants in various wild habitats in Japan. We found that each of three *Akebia* plants could be distinguished by their locality at five points of nucleotide sequence at 91, 128, 133, 134 and 221 in ITS. Plants with a comparable habitat had similar nucleotide sequences at these five points. We also found Aq with ITS and nucleotide deletion at position 86 that was distributed only around Awajishima in Shikoku (A), Harimanada (B) and Kinki (C), including the chief production center of *Akebia Caulis*. The results of these ITS sequences enabled discrimination of the locality of *Akebia Caulis*.

Keywords *Akebiae Caulis*, ITS-1, ITS-2, *Akebia quinata*, *Akebia × pentaphylla*, *Akebia trifoliata*

Introduction

The stem of *Akebia quinata* Decaisne (Aq) and *A. trifoliata* Koidzumi (At) of Lardizabalaceae, is prescribed as the Kampo crude drug “Akebia Caulis”, “Mokutsu” in Japanese, in the modern Japanese pharmacopoeia [1]. “Mokutsu” is effective as a diuretic and an antiphlogistic, and the Japanese products cover most of the Mokutsu market [1, 2]. *Akebia* plants were morphologically identified by the shapes and number of the leaves, and also the size and color of the flowers. *A. quinata* usually has 5 oval-shaped leaflets and white or pale purple male and female flowers. *A. trifoliata* has 3 wavy-edged leaflets and deep purple male and female flowers [3, 4], but there is another *Akebia* plant, *A. × pentaphylla* Makino (Ap), which is considered a hybrid of *A. quinata* and *A. trifoliata*. The typical morphology of *A. × pentaphylla* has 5 leaflets with slightly wavy edges and pale purple male and female flowers [3, 4], although sometimes individuals have boundary morphologies of *A. quinata* and *A. × pentaphylla* as well as *A. trifoliata* and *A. × pentaphylla*. It is difficult to distinguish the species from crude drugs prepared from these *Akebia* plants. For proper usage of the crude drugs, well-defined methods for specifying plants species are necessary. DNA analysis is a practical way to identify medicinal plants as well as crude drugs [5, 6, 7]. Previously, we found that these 3 *Akebia* plants can be distinguished by DNA sequence analysis of internal transcribed spacers 1 and 2 (ITS) [8]. Here, we report the results of molecular genetic analysis of

Akebia plants in various wild habitats in Japan. We focus on the genetic distinction of *Akebia* plants in the habitat.

Materials and Methods

Plant and crude drug materials

Akebia specimens were collected in various parts of Japan (Fig. 1). The plants were identified by the color and morphology of flowers as well as the shape and number of the leaves. Representative specimen and crude drug numbers and information on the collection sites are given in Table 1.

Isolation of total DNA

From the dried leaves of collected specimens, about 50–70 mg were frozen in liquid nitrogen and ground into fine powder. Total DNA isolation from the powder was performed using a DNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's protocol with minor modifications.

PCR amplification

Amplification of the ITS region was performed by polymerase chain reaction (PCR) using 100–120 ng of total DNA as a template in 25 μ l of reaction mixture containing 2.5 μ l of 10 \times PCR buffer for KOD-Plus, 0.2 mM of each dNTP, 1.0 mM of MgSO₄, 0.5 units of KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and 0.4 μ M of each primer Akebi-f (GCT CCT ACC GAT TGA ATG GT) and Ake-26SR (GTA AGT TTC TTC TCC TCC GC). Amplification was carried out under the following conditions: hot start at 94°C for 2 min, 30 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 68°C for 45 sec, and a final extension at 68°C for 5 min. Three microliters of the PCR product were analyzed by agarose gel electrophoresis and then the remaining product was purified using a QIA quick PCR Purification Kit (QIAGEN).

Sequencing procedure

The purified PCR product was subjected to direct sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primers, Akebi-f and Ake-26SR, were used to prime the sequencing reactions of the ITS region. DNA sequences were aligned using DNASIS (version 3.0)

software (Hitachi, Tokyo, Japan).

Cloning procedure of ITS

The ITS region of total DNA was amplified by PCR using tagged primer set Ake-HD-F (TAA AAA GCT TGC TCC TAC CGA TTG AAT GGT) and ER-Ake-26SR (TTA TGA ATT CGT AAG TTT CTT CTC CTC CGC). After purification using the QIA Quick PCR Purification Kit (QIAGEN), 100–200 ng PCR products were digested with restriction enzymes Hind III and EcoR I (Takara Biotech) in 10 µl reaction mixture containing 10 mM Tris-HCl pH 7.5, 10mM MgCl₂, 1mM dithiothreitol, and 50mM NaCl at 37°C for 1 hr, and then the reaction mixture was heated at 70°C for 15 min. Four microliters of the reaction mixtures were combined with 5ng of the plasmid Bluescript SK (-) digested with the same enzymes and 5 µl of DNA Ligation Kit Ver.2.1 (Takara Biotech). The ligation reaction was performed at 16°C overnight. Competent cells (Competent high DH5α, Toyobo) were transformed with the ligated product as recommended by the manufacturer's protocol. The transformed cells were spread on Luria broth (LB) agar plates containing ampicillin (Amp) (2% tryptone, 1% extract yeast dried, 2% NaCl, 3% agar, and 100 µg/ml ampicillin) and incubated at 37°C overnight. Bacterial colonies were picked up separately, and sub-cultured in liquid LB medium containing ampicillin (100 µg/ml). After overnight incubation, the bacteria were

collected by centrifugation and plasmids were obtained following the standard protocol [9]. DNA was extracted according to the method of alkaline isolation of plasmid DNA. Plasmid DNA was treated with RNase A, and then deposited by polyethylene glycol. The sediment was used as a template when amplifying the ITS region by PCR. The PCR product was purified and then subjected to direct sequencing.

Results and Discussion

Comparison of ITS sequences of Aq, At and Ap collected in various habitats

Samples were collected from various wild habitats as shown in Table 1 and Fig. 1. Intensive field searches were performed in Shikoku (A), Harimanada (B), Kinki (C), Shimane prefecture (D), Kitakyusyu (E) and Ishikawa prefecture (F), collecting and analyzing 61, 54, 52, 26, 23, and 12 specimens, respectively. Previously, we found that 2 *Akebia* plants can be distinguished by DNA sequence analysis of the ITS region. Table 2 shows examples of ITS sequences of specimens morphologically identified as Aq, Ap and At. Among these areas, Shikoku (A) is the chief production centers of *Akebia* Caulis. The ITS region of some specimens morphologically identified as Aq, such as A-3, was subjected to the cloning procedure, since their sequences were unclear due

to overlapping sequences. Sequencing analysis was performed using the clones of plasmids containing the ITS region. We found that these species had overlapping common ITS of Aq and that with nucleotide deletion at position 86. Direct sequencing together with the cloning result showed sequence uniformity within Aq or At specimens, while 19 nucleotide differences were found between these two species. Likewise, those of specimens identified as Ap could not be analyzed by direct sequencing. Using the cloning procedure, Tables 2 show that Ap had both common sequences of Aq and At. The result clearly indicates that Ap is a hybrid of Aq and At. The finding of various DNA types in Ap suggests that the species may show morphological variation. *Akebia* plants exhibiting overlapping sequences were not found in Kitakyusyu (E).

Comparison of ITS sequence of *Akebia* plants in different localities

Table 3 shows distinguishable features of the ITS sequence of *Akebia* plants collected from various habitats. For example, at nucleotide 91, Aq from Harimanada (B) and Kinki (C) were T and At was C, respectively. Both Aq and At of specimens from Shikoku (A) had equal T/C. The Sea of Japan showed almost totally T for Aq and At. Another example at nucleotide 128, G, was found for specimens from all habitats and species, except that At from Ishikawa was T. Furthermore, Aq with overlapping common sequences and deletion at position 86 was found in specimens collected in

Shikoku (A), Harimanada (B) and Kinki (C). Table 4 shows the percent appearance of Aq with nucleotide deletion at position 86 for each prefectures. Aq with nucleotide deletion at position 86 was appeared mainly around Harimanada and Awajishima. We found the specimens identified morphologically as Aq and At had respective common ITS sequences, which were distinguishable from each other [8]. In recent years, the chemical analysis of leaves, stems, and bark of At and Aq revealed that they had distinguishable triterpene profiles [10, 11, 12]. Comparison of triterpene-rich fractions of extract from *Akebia* plants by TLC found that Ap had an intermediate profile to Aq and At [8]. The Aq specimens with a deletion were only found in limited area, which contained the chief production center of *Akebia Caulis*. It is interesting whether the Aq specimens with a deletion have different chemical constituents as well as medical properties from other specimens. Furthermore, the ITS sequence analysis of crude drug suggested that Lot US262208-1, Lot US262208-2 and T068 were originated from Aq of Shikoku (A) while T057 was from Ap. Thus, combined examination of these sequences in ITS made it possible to discriminate the locality of *Akebia Caulis*.

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Legends for Tables and Figures

Table 1 *Akebia* samples used in this study

Table 2 Mutations in ITS 1 and 2 of samples

Table 3 Difference in ITS of *Akebia* plant collected from various habitats

Aq : *A. quinata*, At : *A. trifoliata*

Table 4 Prefecture respective Incidences of *A.quinata* (Aq) with deletion at position 86 in ITS (%)

Fig. 1 Map of collection sites A : Shikoku area, B : Harimanada area, C : Kinki area,

D : Shimane Pref. , E : Kitakyusyu area, F : Ishikawa Pref.

Table 3 Difference in ITS of *Akebia* plants collected from various habitats

Base No. Collecting site	91	128	133	134	221
A: Shikoku area	T&C	G	Aq : G, At : T	Aq : A, At : A>G	Aq : T>C, At : T
B: Harimanada area	Aq : T, At : C	G	Aq : G, At : T	Aq : A, At : A>G	Aq : T>C, At : T
C: Kinki area	Aq : T, At : C	G	Aq : G, At : T	Aq : A, At : A&G	T
D: Shimane Pref.	T>C	G	Aq : G, At : T	Aq : A, At : G>A	T
E: Kitakyusyu area	T>C	G	Aq : G, At : T	Aq : A, At : A>G	T>C
F: Ishikawa Pref.	T>C	Aq : G, At : T	G	A	T
Others	Aq : T, At : C	G	G	A	T>C





		
		
		
		
		
		
		
		
		
		
		
		
		
		
		
		
		
		