Study on genetic polymorphism of genus *Acorus* and its application for standardization of Acori Graminei Rhizoma

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Introduction

Acori Graminei Rhizoma is originated from the rhizome of *Acorus gramineus* Solander and has been used as analgesic, sedative, stomachic and anthelmintic drugs in Japan. While the rhizome of *A. calamus* Linn. also has been used as a stomachic in Asian countries, but its demand is few in Japan. Although Acori Graminei Rhizoma is imported from China, the botanical source of this drug mainly used in China is not *A. gramineus* but *A. tatarinowii* Shott [1]. In China, there are four *Acorus* species including above 3 species [2]. The rhizome of *Acorus* species has been reported to have sedative, anticonvulsant, enhancing memory, lowering blood lipids, regulating heart rate, stomachic, antispasmodic, antimicrobial, anticancer, antiepileptic activities besides toxicity [1]. For the safe and efficient uses of Acori Graminei Rhizoma or Acori Tatarinowii Rhizoma, the standardization is necessary in China and Japan. Until now a lot of chemical studies on the rhizomes of *A. calamus* were performed to demonstrate several variations on composition [3-5]. However, comparative study between the rhizomes of *A. gramineus* and *A. tatarinowii* is few because the correct identification of two drugs as well as plants is very difficult. In order to find out molecular marker for identification and further to clarify their chemical composition, the study on genetic polymorphism of genus *Acorus* is carried out. Until now, we have investigate the ITS sequences of 41 plant specimens belonging to the 3 *Acorus* species which were collected from Anhui, Zhejiang and Chongqing in China and 17 crude drug samples from markets of Japan and China. The results indicated significant genetic polymorphism in ITS sequences of *A. tatarinowii* and *A. gramineus* [6]. Eight types of ITS sequences (types T1~T8) were found in *A. tatarinowii* and three types (G1~G3) in *A. gramineus*.

In the present study, we collected plant specimens from Guangxi, China and crude drug samples from Japan, China and Korea, subsequently analyzed their ITS sequences and incorporated the results to the previous achievement, in order to obtain more evidences for clarification of genetic variations for standardize Acorus Rhizoma.

Materials and Methods

Plant and crude drug samples

Fifteen plant specimens which were identified as *A. gramineus* by ourselves were collected from Guangxi Province, China (Table 1, Fig. 1), and six crude drug samples collected from markets of China Japan and Korea (Table 2). Vouchers are deposited at the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan.

Table 1 Plant materials of *A. gramineus* and their types of ITS sequences.

Code No.	Location of Collection	Date of collection	**Type of ITS sequence**
SL1	Shanglin, Guangxi, China	2012.10.	**N3**
SL2	Shanglin, Guangxi, China	2012.10.	**N1**
L1	Longan, Guangxi, China	2012.10.	**N4**
L2	Longan, Guangxi, China	2012.10.	**N4**
L3	Longan, Guangxi, China	2012.10.	**N5**
L4	Longan, Guangxi, China	2012.10.	**N1**
L5	Longan, Guangxi, China	2012.10.	**N3**
L6	Longan, Guangxi, China	2012.10.	**N5**
L7	Longan, Guangxi, China	2012.10.	**N1**
L8	Longan, Guangxi, China	2012.10.	**N5**
L9	Longan, Guangxi, China	2012.10.	**N4**
L10	Longan, Guangxi, China	2012.10.	**N1**
L11	Longan, Guangxi, China	2012.10.	**N1**
L12	Longan, Guangxi, China	2012.10.	**N1**
L13	Longan, Guangxi, China	2012.10.	**N2**

Fig. 1 Plant Samples from Shanglin (left) and Longan (right), Guangxi, China

Table 2 Crude drug materials and their types of ITS sequences, as well as identification results

No.	Name	TMPW No.	Produced area	Market	Type of ITS sequence	Identity
1	セキショウブ	26407	Anhui, China	Tochimoto Tengaido, Osaka, Japan	**T3**	*A. tatarinowii*
2	石菖蒲	26537	Sichuan, China	TCM market, Chongqing, China	**T8**	*A. tatarinowii*
3	石菖蒲	26600	Anhui, China	Huangshan, Anhui, China	**C1**	*A. calamus*
4	石菖蒲	26617	Jiangxi, China	Qizhou, Zhejiang, China	**T1**	*A. tatarinowii*
5	石菖蒲	27715	Guangxi, China	Nanning, Guangxi, China	**G3**	*A. gramineus*
6	石菖蒲	26926	Jeju, Korea (Wild)	Seoul, Korea	**T1**	*A. tatarinowii*

Genomic DNA Extraction

Total DNA of dry leaves (40-80 mg) was extracted using DNeasy Plant Mini Kit (QIAGEN, Germany), and crude drugs (120-200 mg) using Blood & Cell Culture DNA Mini Kit (25) (QIAGEN, Germany). DNA quality and quantity were determined by electrophoresis on 1% agarose gel stained by UltraPower DNA/RNA Safedye (Gellex International ltd., Japan).

Polymerase Chain Reaction (PCR) Amplification

Primers for amplification of ITS regions are listed in Table 3 and their locations are indicated in Fig. 2. The PCR was performed in a 25 μL reaction mixture, containing 0.4 mM of each dNTP, 0.4 mM of each primer, 1×PCR buffer and 0.5 U KOD-Plus-Neo (Toyobo Life Science, Japan) and 1 μL of template DNA. The thermal cycle conditions for amplification were as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 50 s, and 68 °C for 60 s, and final extension at 68 °C for 20 min. 2 μL of resulting PCR-amplicon was detected by 1.0% agarose gel electrophoresis and the remained part was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, U.S.A.). The amplification strategy was showed in Fig. 2.

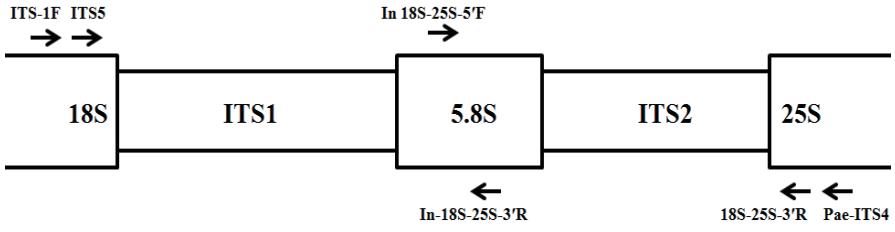

Fig. 2 Location of primers used in PCR amplification and sequencing of ITS region

Table 3 Sequences of primers used in this study

Primer name	Primer sequences (5′→3′)	Length (bp)
Forward primer		
ITS-1F	TCCACTGAACCTTATCATTTAG	22
In 18S-25S-5′F	TCTCGCATC GATGAAGAACG	20
ITS5	GGAAGGAGAAGTCGTAACAAGG	22
Reverse primer		
In-18S-25S-3′R	GACTCGATGGTTCACGGGATTCT	23
18S-25S-3′R	CCATGCTTAAACTCAGCGGGT	21
ITS4	TCCTCCGCTTATTGATATGC	20

Sequence Determination

The purified PCR products served as template, sequencing reaction were performed using the Big Dye Terminator V3.1 (Applied Biosystems, U.S.A.) with the same primers as in PCR amplification and several inside primers. The thermal cycling condition was 96 °C for 1 min, 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. After further purification using Big Dye XTerminator™ Purification Kit (Applied Biosystems, U.S.A.), the supernatants were injected to and analyzed on an automated DNA sequencer (ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems, U.S.A.). The obtained sequences were edited and aligned by the AutoAssemble program (Version 1.3.0, Applied Biosystems, U.S.A.), and adjusted manually where necessary.

The boundaries of ITS1, 5.8S and ITS2 regions were assigned by comparison with a reference sequence in Genbank FJ874939.

Results and Discussion

The nucleotide sequences of rDNA ITS region of fifteen plant samples from Guangxi, China, and six crude drug samples collected from markets of China, Japan, and Korea were determined and compared. Amplification products (~800 bp fragment) of the whole ITS region (ITS1-5.8S-ITS2) were successfully obtained by using the total DNA from plant specimens as template. However, it is difficult to amplify the whole ITS region in the case of crude drug samples, due to severe degradation of the extracted total DNA. Therefore, we separately amplified the ITS1-5.8S(5'-end) and 5.8S(3'-end)-ITS2 regions for the crude drug samples, subsequently decided their sequences and finally assembled them as one piece for comparison.

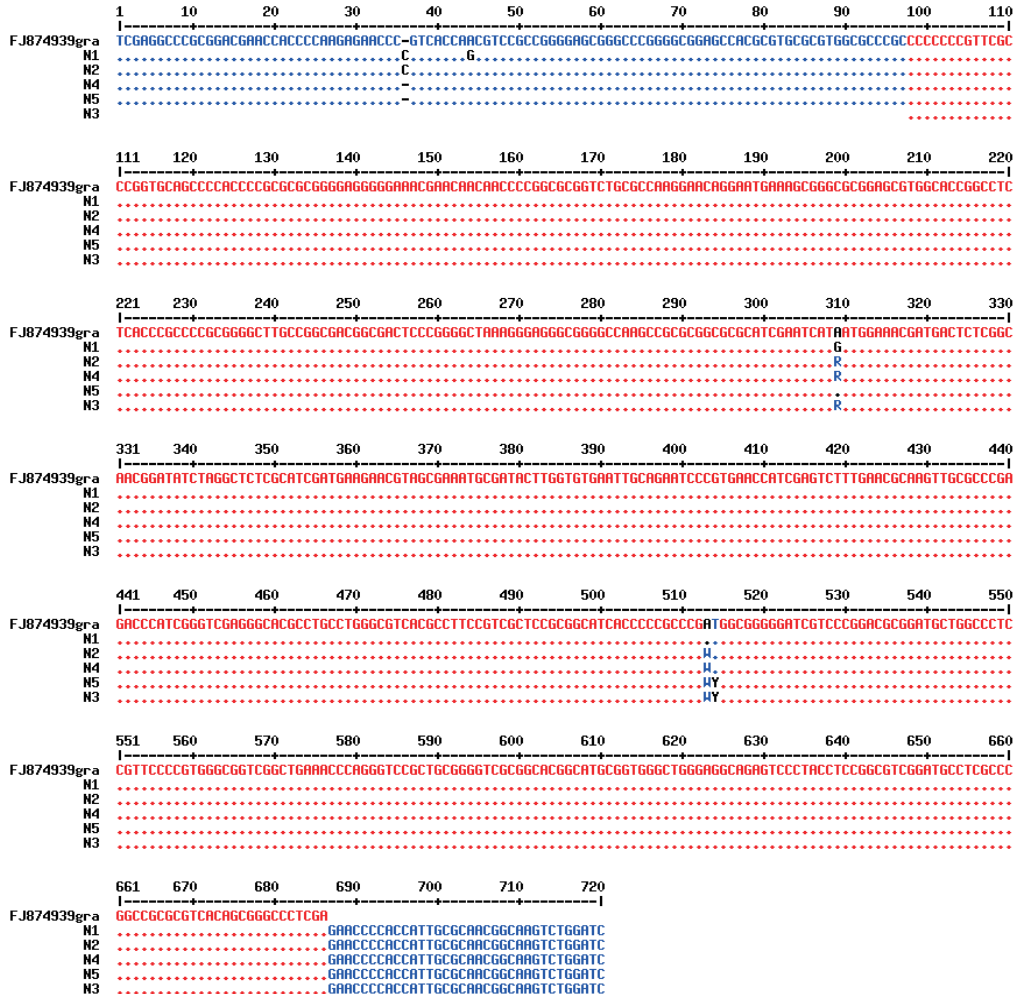

Fig. 3 Comparison of ITS sequences

FJ874939: A reference ITS sequence of *A. gramineus* registered in GenBank. Dot means the same nucleotide as that in the 1st line of the FJ874939gra sequence. R=A & G, W=A & T, Y=C & T.

As for the fifteen plant specimens collected from Guangxi, China, the whole lengths of the ITS region were sequenced except for four samples (SL1, L5, L6 and L7) in which about 100 bp sequence at the 5'-end of ITS1 region were unreadable. Five types of sequences (type N1 ~ N5) were detected and none of them was identical to our previously decided sequences (types G1~G3 and types T1~T8). Though BLAST search in GenBank, these sequences showed high homology to a reference sequence of *A. gramineus* (Accession No. FJ874939) in Genbank. The sequences comparison is shown in Fig. 3. Four sites of nucleotide substitutions at alignment positions 44th, 309th, 513rd and 514th, and one site of indel at positions 36th were observed among them. In addition, additive nucleotides (double peaks in sequencing electrophorogram) were detected at the nucleotide positions 309th, 513rd and 514th in the types N2~N5 sequences, presented as R[Guanine (G) & Adenine (A)], W[A & Thymine (T)] and Y[T & Cytosine (C)], which might infer a hybrid origin of such specimens. Type N1 was a putative pure line sequence.

A phylogenetic tree based on ITS sequences of the 3 *Acorus* species, including type N1 and

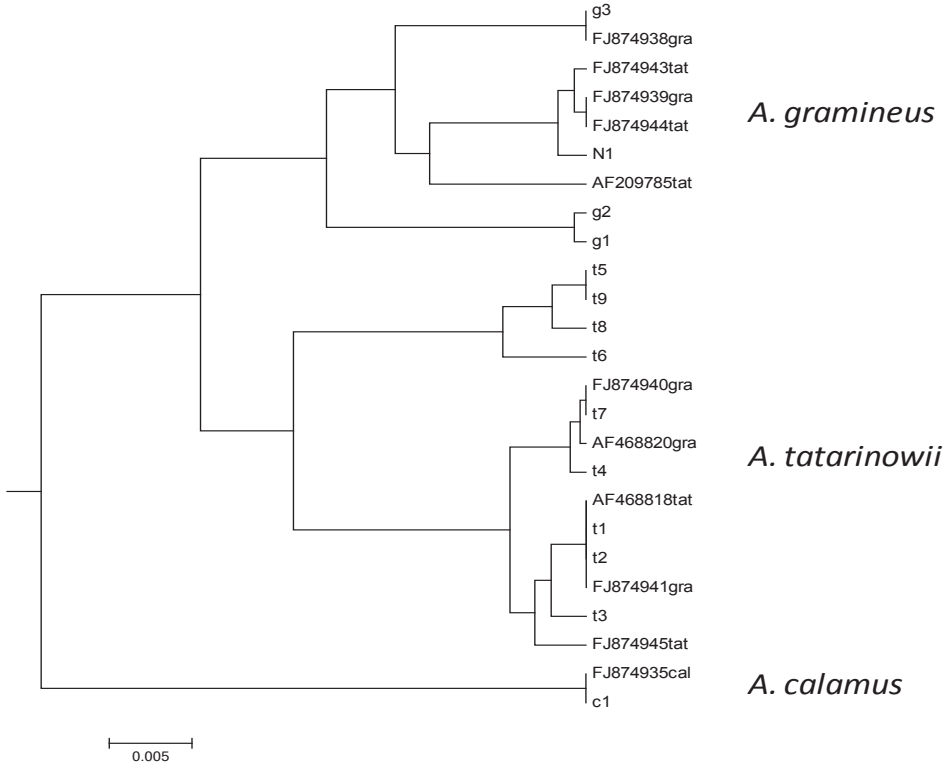

Fig. 4 Phylogenetic tree based on ITS sequences from the three *Acorus* species

(T1~T8: ITS sequences of *Acorus tatarinowii*, G1~G3: ITS sequences of *A. gramineus*, C1: ITS sequences of *A. calamus*. FJxxxxxx and AFxxxxxx: Accession No. of ITS sequences of *Acorus* species registered in GenBank.

our previously determined sequences, was reconstructed by UPGMA method (Fig. 4). Three main clades were formed. *A. calamus*, located at a basic position, showed far relationship from the other two species; *A. tatarinowii* and *A. gramineus* were clustered into two clades. Therefore, we tentatively named them as *A. calamus-, A. tatarinowii-* and *A. gramineus*-clades. Type N1 belonged to the *A. gramineus*-clade, which was consistent with our identification.

Six crude drugs were analyzed and their ITS sequence were compared with our previously obtained data. Sample No. 1 (TMPW No. 26407), which was produced in Anhui Prov., China and available in Japanese market, possessed type T3 sequence. Therefore, it was identified as deriving from *A. tatarinowii*. Four samples (No. 2~5) collected from various locations in China showed types T8, C1, T1 and G3 sequences; thus their botanical origins were identified as *A. tatarinowii*, *A.calamus*, *A. tatarinowii* and *A. gramineus*, respectively. Although these 4 crude drugs were sell as "ShiChangpu (Sekisyoubu)" in markets, their botanical sources are completely different, which must result in unstable and unexpected therapeutic effects. Especially, the sample No. 3, which was collected for local market in Anhui Prov., China, should be Changpu (Syoubu), but not "ShiChangpu (Sekisyoubu)." Sample No. 6 which was collected from Seoul, Korea and was claimed to be from wild source growing in Jeju Island, revealed type T1 sequence. Therefore, it was identified as *A. tatarinowii*.

This study found out several new types of ITS sequences of *A. gramineus*, proving more evidences for clarification of genetic variations of Acorus Rhizoma, and demonstrated the ITS sequence was useful and powerful for authentication of Acorus Rhizoma.

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