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Kenji KONDO[a], Mao SHIBA[a], Yuichi YOTSUYANAGI[b], Naoyuki NISHIMURA[b], Takuro MARUYAMA[c] and Yukihiro GODA[c]: **Discrimination between *Atractylodes* Rhizome (Byaku-jutsu) and *Atractylodes lancea* Rhizome (So-jutsu) by the PCR-RFLP Analysis of ITS Region on nrDNA**

nrDNA, ITS 領域の PCR-RFLP 分析による生薬白朮と蒼朮の鑑別（近藤健児[a]，司馬真央[a]，四柳雄一[b]，西村直行[b]，丸山卓郎[c]，合田幸広[c]）

Summary: The purity test for *Atractylodes* rhizome by the molecular biological method based on ARMS was established in the general information on the first supplement of the Japanese Pharmacopoeia 15th ed., to stop commingling of *Atractylodes lancea* rhizome with *Atractylodes* rhizome. The ARMS requires rigorous experimental conditions. Therefore, we established a new, simple, quick and stable method based on PCR-RFLP for discrimination between *Atactylodes* rhizome and *Atractylodes lancea* rhizome.

Dried rhizomes of *Atractylodes macrocephala* Koidz. (= *A. ovata* DC., *Asteraceae*) and *A. japonica* Koidz. are used as the crude drug called *Atractylodes* rhizome (Byaku-jutsu in Japanese), and *A. lancea* DC. and *A. chinensis* Koidz. called *Atractylodes lancea* rhizome (So-jutsu in Japanese) in the Japanese Pharmacopoeia, 15th ed. (JP XV, The Ministry of Health 2006). Discrimination between *Atractylodes* rhizome and *Atractylodes lancea* rhizome using molecular markers had been reported (Mizukami et al. 2000, Cheng et al. 1997).

In the general information on the first supplement of the JP XV (The Ministry of Health 2007), the purity test for *Atractylodes* rhizome by molecular biological method was established to stop commingling of *Atractylodes lancea* rhizome with *Atractylodes* rhizome. This purity test based on the amplification refractory mutation system (ARMS) detects the differences of nucleotide sequences on internal transcribed spacer of nuclear ribosomal DNA (ITS) among the medicinal *Atractylodes* species (Guo et al. 2006). The ARMS requires rigorous experimental condition comparatively because the ARMS detects the nucleotide substitution by existence or nonexistence of PCR products using the specific mismatch primer. Therefore, we tried to discriminate between *Atractylodes* rhizome and *Atractylodes lancea* rhizome by PCR-Restriction fragment length polymorphism (PCR-RFLP) as a simple, quick and stable method.

## Materials and Methods

Dried rhizomes of *Atractylodes macrocephala* (THS 83160-1), *A. japonica* (THS 83160-22), *A. lancea* (THS 83160-18) and *A. chinensis* (THS 82810-1) identified by their ITS sequences were prepared. THS means Tsumura Herbarium specimen. The method of sequencing of ITS region and the species identification by the ITS sequence followed Shiba et al. (2006). Additionally, the hybrid between *A. lancea* and *A. chinensis* (THS 82811-2) was added as a material because many natural hybrids are distributed in China (Shiba et al. 2006).

### Target site for PCR-RFLP analysis

Based on the ITS sequences among the medicinal *Atractylodes* species (Shiba et al. 2006), two nucleotide sites were selected as species specific nucleotide substitutions that were digestible by restriction enzymes. *Atractlodes lancea* only has nucleotide ‘G’ at the 75th site which can be cut by the restriction enzyme *FauI*

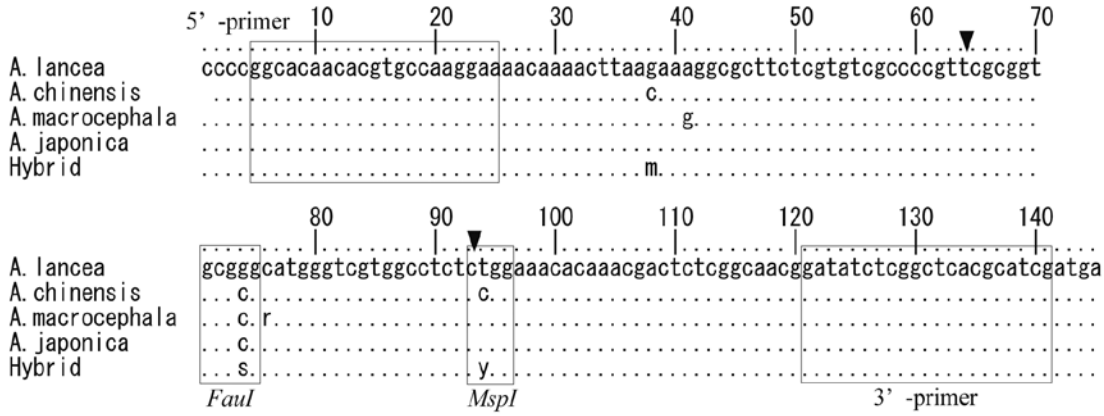


Fig. 1. Amplified DNA region and recognition sites by *FauI* and *MspI* on the ITS sequences in the *Atractylodes* species. The triangle means cut sites by *FauI* and *MspI*. Hybrid is a derivative between *A. lancea* and *A. chinensis*.

(recognition site: GCGGG, cut site: between the 64th and 65th), and *Atractylodes chinensis* only has nucleotide 'C' at the 94th site which can be cut by *MspI* (recognition site: CCGG, cut site: between the 93th and 94th, Fig. 1). Primers were designed to amplify the 137 bases of DNA fragment containing the two nucleotide sites. 5′-primer: 5′-GGC ACA ACA CGT GCC AAG GAA AA-3′, 3′-primer: 5′-CGA TGC GTG AGC CGA GAT ATC C-3′.

**DNA extraction and amplification**

For DNA extraction and amplification, Ampdirect® Plus enzyme kit (Shimadzu Biotech, 241-08890-92) were applied. Ampdirect® Plus enzyme kit with Ampdirect® Plus and Nova Taq™ Hot Start DNA Polymerase allow simple and quick DNA extraction, and stable DNA amplification from crude drugs. Shredded crude drug material (20 mg) within SNET buffer (400 μL) incubated at 55˚C for overnight (about 16–18 hr). The SNET buffer contains Tris-HCl pH 8.0 (20 mM), EDTA (5 mM), NaCl (400 mM), SDS (0.3 %) and Protainase K (200 μg/mL). Following the incubation, DNA polymerase was inactivated at 95˚C for 5 min. After precipitating solids by centrifuge, the supernatant was used as template DNA solution. Amount of DNA within the template DNA solution is difficult to estimate accurately by $OD_{260\ nm}$ observation because the solution contains many foreign substances.

The PCR mixture contained 2× Ampdirect® Plus with $MgCl_2$ and dNTP (10 μL), the above-described 5′- and 3′-primers (0.5 μM), Nova Taq™ Hot Start DNA Polymerase (0.5 units), the template DNA solution (0.5 μL), and added D.W. to 20 μL of total mixture volume. The PCR mixture was prepared under cool conditions.

DNA amplification by PCR was carried out under the following conditions: precycling denaturation at 95˚C for 10 min, 40 cycles of denaturation at 95˚C for 30 sec, annealing at 65˚C for 15 sec, and elongation at 72˚C for 7 min; and stored at 4˚C.

**Digestion with the restriction enzyme**

For the digestion of the PCR products by *FauI* (New England BioLabs Inc.), a 15 μL reaction containing 3 μL template DNA, one unit of enzyme and 1× SEBuffer B was incubated at 55˚C for 2 hr. For *MspI* (New England BioLabs Inc.), a 15 μL reaction containing 3 μL template DNA, 20 units of enzyme and 1× NEBuffer B was incubated at 37˚C for 2 hr. After the incubation, the enzymes were inactivated at 72˚C for 10 min. The digested DNA fragments were detected by electrophoresis using E-gel® 4% agarose (Invitrogen).

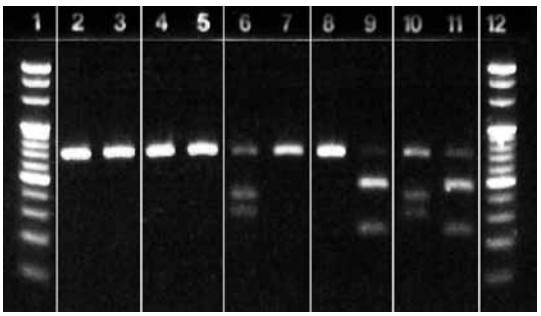


Fig. 2. Electrophoretic pattern in the *Atractylodes* species. 1, 12. DNA ladder (20 bp). 2, 3. *A. macrocephala* (*FauI*, *MspI*). 4, 5. *A. japonica* (*FauI*, *MspI*). 6, 7. *A. lancea* (*FauI*, *MspI*). 8, 9. *A. chinesis* (*FauI*, *MspI*). 10, 11. Hybrids between *A. lancea* and *A. chinensis* (*FauI*, *MspI*).

## Results and Discussion

The PCR products of the medicinal *Atractylodes* species digested by the restriction enzyme *FauI* and *MspI* were indicated on an electrophoretic profile (Fig. 2). All of the PCR products of *Atractylodes* rhizome (Byaku-jutsu) derived from *A. macrocephala* and *A. japonica* were not digested by *FauI* or *MspI*, and were observed as one band (137 bp) on the electrophoretic profile. In *Atractylodes lancea* rhizome (So-jutsu), the PCR products of *A. lancea* were digested by *FauI* only and were observed as the three bands (137, 77 and 62 bp) on the electrophoretic profile. The PCR products of *A. chinensis* were digested by *MspI* only and were observed as the three bands (137, 91 and 48 bp) on the electrophoretic profile. The PCR products of the hybrid between *A. lancea* and *A. chinensis* were digested by *FauI* and *MspI*, and were observed as three bands.

Digestion of PCR products of *A. lancea* and *A. chinesis* by *FauI* or *MspI* expected that two bands would be observed on the electrophoretic profile. However, in this study, the PCR products of *A. lancea* and *A. chinensis* produced digestion of the three bands on the electrophoretic profile (Fig. 1). According to the expected length of digested band, the shorter two bands were derived from the digestion by *FauI* or *MspI*. The longest bands (137 bp) were derived from the undigested PCR products. These undigested PCR products were not digested under different experimental conditions that the enzyme reaction times were set longer or amount of the enzymes were increased. It has been suggested that the ITS sequences in *A. macrocephala* and *A. japonica* is ancestral phylogenetically from *A. lancea* or *A. chinensis* (Shiba et al. 2006). *Atractylodes lancea* and *A. chinensis* would retain the ancestral minor ITS copy which is indigestible by *FauI* or *MspI*.

Though remaining the undigested PCR products, the three bands derived from the digestion of the PCR products by *FauI* or *MspI* were recognized as *A. lancea*, *A. chinensis* and their hybrid. Thus, the discrimination between *Atractylodes* rhizome derived from *A. macrocephala* and *A. japonica*, and *Atractylodes lancea* rhizome derived from *A. lancea* and *A. chinensis* is possible by the PCR-RFLP analysis using *FauI* and *MspI*. On the electrophoretic profile after the enzyme reaction by *FauI* and *MspI* for the PCR products, one band only was recognized with *Atractylodes* rhizome, 2 or 3 bands are recognized on the reaction of *FauI* or *MspI* with *Atractylodes lancea* rhizome.

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The Ministry of Health, Labor and Welfare. 2007. The Japanese Pharmacopoeia, 15th ed., the first supplement. Tokyo (in Japanese).

第 15 改正日本薬局方第一追補では，生薬白朮に蒼朮が混入するのを防止するために ARMS 法に基づいた分子生物学的手法を用いた純度試験法を参考情報として収載している．この ARMS 法は種特異的な非結合性プライマーを用いた PCR 反応により，その PCR 産物の有無から種特異的塩基置換の違いを検出するため，対象とするサンプルの DNA の状態や PCR などの実験条件が限定的である．そこで PCR-RFLP 法をベースとして，簡便，迅速で安定的に白朮と蒼朮を鑑別する方法を開発した .

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# Hiroyoshi OHASHI[a] and Kazuaki OHASHI[b]: **Three White-Flowered Forms of *Lespedeza* (*Leguminosae*) in Japan**

日本産白花のハギ（マメ科）3 新品種（大橋広好[a]，大橋一晶[b]）

Summary: Three new white-flowered forms of *Lesepdeza* found in Japan are described: *L. pilosa* (Thunb.) Siebold & Zucc. f. *virginea* H. Ohashi from Kagawa Pref., *L. cyrtobotrya* Miq. f. *virginea* H. Ohashi from Ibaraki Pref., and *L. thunbergii* subsp. *thunbergii* f. *miyagialba* H. Ohashi & K. Ohashi from Miyagi Pref.

White-flowered forms of *Lespedeza* have been recognized as a variant from original purplish-flowered forms and hence they have been treated at the rank of form. Only *L. japonica* L. H. Bailey was described as a species, but this is now recognized as a form of *L. thunbergii* (DC.) Nakai. Several white-flowered forms have been known in the subgenus *Macrolespedeza*, probably because the subgenus is characteristic in having purplish larger flowers than another subgenus, *Lespedeza,* having white, yellow or purple smaller flowers. *Lespedeza pilosa* (Thunb.) Siebold & Zucc. f. *virginea* H. Ohashi in this paper is, to our knowledge, the first white-flowered form in the subgenus *Lespedeza* reported so far.

1. *Lespedeza pilosa* (Thunb.) Siebold & Zucc. f. **virginea** H. Ohashi, f. nov.