academicJournals

Vol. 7(27), pp. 1969-1978, 17 July, 2013
DOI: 10.5897/JMPR12.211
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Plant-specific primers for the amplification of the nrDNA ITS region in fungus-associated *Pulsatilla* species

Yan-Lin Sun[1], Wan-Geun Park[2], Hyun-Kyung Oh[3] and Soon-Kwan Hong[4,5]*

[1]School of Life Sciences, Ludong University, Yantai, Shandong, 264-025, China.
[2]Department of Forest Resources, Kangwon National University, Chuncheon, 200-701, Korea.
[3]National Institute of Biological Resources, Environmental Research Complex, Gyoungseo-dong, Seo-gu, Incheon, 404-708, Korea.
[4]Department of Bio-Health Technology, Kangwon National University, Chuncheon, 200-701, Korea.
[5]Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, 200-701, Korea.

Accepted 3 June, 2013

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA is the most frequently used marker for distinguishing plant species. Previously published polymerase chain reaction (PCR) primers available for amplifying the ITS region from environmental plant samples provide a high degrees of success while maintaining a broad range of compatibility difficult to discriminate against fungal DNA. Thus, the plant-specific ITS primer design is required to achieve the preferential amplification of plant DNA with discrimination against fungal DNA from environmental plant samples. In this study, two primers, ITS1-F2 and ITS4-R, were newly designed and the specificity of these primers were tested against 14 accessions of *Pulsatilla tongkangensis* and 2 accessions of *Pulsatilla cernua*. Our results showed that ITS4-R, when paired with either a universal primer ITS5 or the newly designed primer ITS1-F2, efficiently amplified DNA from environmental *P. cernua* samples and discriminated against parasitic fungal DNAs, while another newly designed primer ITS1-F2, when paired with either a universal primer ITS4 or ITS4-R, could not preferentially amplify plant DNA or discriminate against fungal DNA. For *P. tongkangensis*, the dismatch of ITS5 primer resulted in noneffective or wrong amplification when paired with either ITS4-R or ITS4, while ITS1-F2, when paired with ITS4-R and ITS4, efficiently amplified plant DNA and both plant and fungal DNAs, respectively. This result suggested that the combination with ITS1-F2 and ITS4-R were intended to be specific to *Pulsatilla* species. This study will be particularly useful for detection and analysis of plant sequences from environmental or fungus-associated materials.

Key words: *Pulsatilla*, nrDNA ITS, plant-specific primer design, fungus-associated plant discrimination, molecular identification.

INTRODUCTION

The internal transcribed spacer (ITS) region contains two variable non-coding regions that are nested within the ribosomal DNA (rDNA) repeat between the highly conserved small subunit, 5.8S, and large subunit rRNA genes (Baldwin et al., 1995). Several features make it the most frequently utilized and convenient target region for molecular identification of plants, fungi, and animals (Álvarez and Wendel, 2003; Kress et al., 2005). The

*Corresponding author. E-mail: soonkwan@kangwon.ac.kr. Tel: 82-33-250-6476. Fax: 82-33-250-6470

advantages of using the ITS region for identification of plant and fungus species are that (a) the entire ITS region is generally between 600 and 800 bp, that makes efficient amplification and sequencing easy; (b) the primers for amplifying the ITS region shared high universality, e.g. the earliest ITS primers, ITS1, ITS4, and ITS5 have gained a wide acceptance for work with fungal and plant ITS region (White et al., 1990); (c) the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute, or highly degraded DNA samples; (d) the ITS region has high species discrimination, that has been much demonstrated especially when used as a region for the barcoding and authentication of herbal medicinal materials (Álvarez and Wendel, 2003; Kress et al., 2005; Karehed et al., 2008). However, due to a very broad range of primer universality, currently available primers could readily amplify the ITS region from both plant and fungal DNAs. This disadvantage affects further progress of the ITS region in species phylogenetic studies.

In our laboratory work, this situation of false-positive PCR amplification has reached an unignorable level, e.g. using universal primers could amplify the ITS regions from not only plant DNA but also fungal DNA in some fungus-associated plant species, including *Dracocephalum argunense*, *Scutellaria baicalensis*, and *Toona sinensis* (data not published). In many natural situations, fungal contamination of plant materials may be inevitable, thus specific or preferential amplification of plant DNA would be desirable. *Pulsatilla cernua* (Thunb., Ranunculaceae) is a perennial herb distributed in Northeastern China, Korea, and Japan (Huang et al., 2002). It is a common early spring flower in China, but is an endangered plant in Japan, because of a rapid decline of populations in the last two decades (Naito and Nakagoshi, 1994). The dried roots of *P. cernua* have been widely used as traditional Chinese medicine (TCM) for thousands of years in China, mainly for the treatment of amoebic and bacterial dysentery (Xu et al., 2010). *P. cernua* is known for containing a large number of compounds, including triterpenoidal saponins, phytosterone and anthocyanins (Xu et al., 2011). Modern pharmacological studies demonstrated that *P. cernua* extracts had a number of effects, such as hypoglycaemic, antitumour, cognition enhancing, neuroprotective, cytotoxic and antiendotoxin activities (Sparg et al., 2004; Han et al., 2007; Seo et al., 2010). Another *Pulsatilla* species, *Pulsatilla tongkangensis* Y.N. Lee & T.C. Lee (Ranunculaceae), a perennial herb was discovered naturally growing on the cliffs of Tongkang, Kangwon province, Korea and endemic to Korea, thus was announced as a new *Pulsatilla* spp., *P. tongkangensis* (Lee, 2000; Lee et al., 2010;Kim et al., 2010). From the morphological characters, *P. tongkangensis* has a shorter length of stem, and flower stalk, compared to other *Pulsatilla* spp. As known, the flower stalk of most of *Pulsatilla* spp. bends during their anthesis, like *P. cernua* (Huang et al., 2002), however, the movement of the floral stalk during anthesis could not been found in *P. tongkangensis*. In Korea, there are six main *Pulsatilla* spp. naturally growing in the well-drained gravel areas or sandy soils or even cavity of calcified rocks, including *P. cernua*, *Pulsatilla davurica*, *Pulsatilla koreana*, *Pulsatilla nivalis*, *Pulsatilla chinensis*, and *P. tongkangensis* (Lee et al., 2004). Because of the sharing of similar morphological characteristics, traditional identification methods have not been able to achieve clear discrimination and effective use of the *Pulsatilla* spp., molecular identification based on analyses of DNA sequence diversity becomes more reliable alternative means.

In this study, we have successfully amplified the ITS region from *P. cernua* using universal primers, but with poor plant-specificity. The ITS region could not be effectively amplified using universal primers from natural *P. tongkangensis* materials. The reason of inefficient amplification of plant ITS region was the come down to the severe fungal contamination and super high primer universality; the reason of unsuccessfully amplification was believed to owe to the mismatch of the ITS primers with *P. tongkangensis* DNA sequence. To achieve the effective amplification of ITS region from *P. tongkangensis*, we have designed and tested one new primer set (ITS1-F2/ITS4-R) that in combination specifically amplify the ITS region of plants from fungus-contaminated plant materials. This plant-specificity of newly designed primer set was also suitably applied to the ITS region amplification from *P. cernua*. In combination with universal primers, the newly designed primers could also achieve the preferential ITS amplification from plants. Our focus on plant-specific ITS primers could help more accurately, rapidly amplification of the ITS region from plant materials, even some fungus-associated plant materials. In addition, yet little is known, the ITS region sequences of the new species, *P. tongkangensis*, that furthermore makes against the understanding of the phylogeny between *P. tongkangensis* and other *Pulsatilla* spp. The ITS region sequences of *P. tongkangensis* presented here is the first time to successfully amplify and sequence. Thus, this study also provides more sequence sources to compare to further understand the phylogenetic relationships of the genus *Pulsatilla*.

MATERIALS AND METHODS

Plant

P. cernua (Thunb.) is commonly found around Korea. Two *P. cernua* accessions were collected from the campus of Kangwon National University (KNU), Chuncheon, Korea, and identified by plant systematic expert, Prof. Wan-Geun Park of Department of Forest Resources, Kangwon National University. *P. tongkangensis* Y.N. Lee & T.C. Lee plants were conserved by Prof. Wan-Geun Park, and cultured in the green house of Department of Forest Resources, Kangwon National University. There are fourteen accessions of *P. tongkangensis* in all. Their specimen vouchers and NCBI GenBank accession numbers of the ITS region are listed in Table 1.

Table 1. Specimen vouchers and NCBI accession numbers of plant and fungus materials investigated in this study.

Ecotype	Specimen voucher	NCBI accession number	Abbreviation
Pulsatilla cernua	KNU Haknai-1	JN811070	HN1
	KNU Haknai-2	JN811071	HN2
Pulsatilla tongkangensis	Park h1-1	JN811072	PH1
	Park h4-1	JN811073	PH2
	Park h5-1	JN811074	PH3
	Park h6-1	JN811075	PH4
	Park h7-1	JN811076	PH5
	Park h9-1	JN811077	PH6
	Park h1-2	JN811078	PH7
	Park h4-2	JN811079	PH8
	Park h5-2	JN811080	PH9
	Park h6-2	JN811081	PH10
	Park h7-2	JN811082	PH11
	Park h9-2	JN811083	PH12
	Park h3-1	JN853785	PH13
	Park h8-1	JN853786	PH14
Pulsatilla cernua parasitic fungus	Haknai01	JN811084	FHN1
	Haknai02	JN811085	FHN2
	Haknai03	JN811086	FHN3
Pulsatilla tongkangensis parasitic fungus	*Tongkangensis*01	JN811087	FPH1
	*Tongkangensis*04	JN811088	FPH2
	*Tongkangensis*06	JN811089	FPH3

DNA extraction and PCR amplification

DNA extractions were performed by using the modified cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). PCR was performed using a Gene Amp 9700 PCR system (Applied Biosystems Inc. ABI, Warrington, Cheshire, UK) in 20 to 25 µl volumes with the following reaction components: 1 µl template DNA (~1 to 100 ng), 10 × Ex Taq Buffer (TaKaRa, TAKARA BIO INC., Japan), 200 µmol/l each dNTP, 0.1 µmol/l each primer, and 0.1 µl TaKaRa Ex Taq (5 units/µl, TaKaRa, TAKARA BIO INC., Japan). PCR amplification conditions followed an initial denaturation stem of 95°C for 5 min, 35 amplification cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and a final extension step at 72°C for 1.5 min. After the 35 cycles were completed, the samples were incubated for an additional 7 min at 72°C.

Two universal ITS primers, ITS5 and ITS4 (White et al., 1990), were used to combine with new designed ITS primers, ITS1-F2 and ITS4-R, for more accurately and preferentially amplification from fungus-contaminated plant materials. The accurate location of the newly designed ITS primer is as shown in Figure 1. The amplification products were checked by electrophoresis through 1.0% agarose gel, and then purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) or Gel Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions.

Purified PCR products were then sequenced at MACROGENE advancing through genomics (Korea, http:// dna.macrogen.com/kor/).

Plant-specific ITS primer design

Two plant-specific primers for the ITS region, ITS1-F2 and ITS4-R were designed, located in the 3' end of the 18S rDNA and the 5' end of the 28S rRNA gene, respectively (Figure 1). The sequences used for comparison were obtained from NCBI GenBank database, with Accession Numbers of GU732648 ~ GU732651, JF422893, HQ829829, HQ829821, HQ735289, FJ639908, JF422890 ~ JF422892 for different *Pulsatilla* spp. and GU732647, HQ898650, AB120213, AM267279 for different fungus species parasitic in *Pulsatilla* spp.

The 3' end of 18S rDNA sequences shared relatively high homology between *Pulsatilla* spp. and parasitic fungus species, while the 5' end of 28S rDNA sequences showed some informative nucleotide sites, easy to design plant-specific ITS primer.

Sequence editing and alignment

For editing and assembly of the complementary strands of the sequencing results, the software program DNAMAN version 6.0 (Lynnon Biosoft Corporation, USA, www.lynon.com) was used. Analogue of our sequences and nucleotide sequence comparisons were detected with Basic Local Alignment Search Tool (BLAST) network services against databases (http://www.ncbi.nlm.nih.gov/). To detect single nucleotide polymorphisms, the multiple sequence alignments of ITS region (ITS1, 5.8S rRNA gene and ITS2) of all the16 *Pulsatilla* spp. and 6 parasitic fungus species were respectively performed also using DNAMAN version 6.0 software.

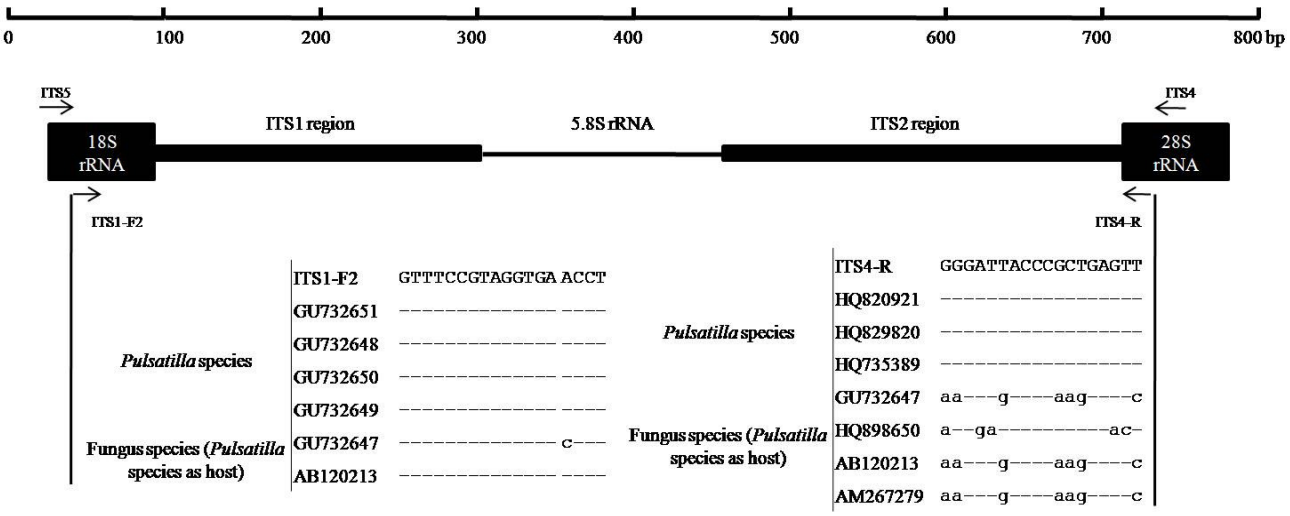

Figure 1. Accurate location of the newly designed ITS primers, ITS1-F2 and ITS4-R. ITS1-F2 and ITS4-R are targeted in the 3' end of 18S rRNA and 5' end of 28S rRNA gene, respectively. All primer sequences are listed in the standard 5' to 3' direction.

Phylogenetic analysis

The phylogenetic relationships among the *Pulsatilla* spp. was estimated after the construction of a phylogram based on multiple sequence alignment of ribosomal DNA ITS sequence with the DNAMAN version 6.0 software (Lynnon Biosoft Corporation, USA, www.lynon.com). The relevant region lengths (ITS1, 5.8S rRNA gene and ITS2) of each sequence were calculated. Genetic distance (GD) was obtained with the help of MEGA software and mean GD of the intraspecific distance was calculated by the sum of individual GD divided by the number of samples.

RESULTS AND DISCUSSION

Using ITS region as a DNA marker began with discriminating some closely-related fungus species and understanding their phylogenetics (White et al., 1990). As the development of plant systematic and identification, the discrimination through molecular identification approach has gradually replaced the traditional plant discrimination. And the ITS region, due to its high primer universality, has become the single most frequently utilized DNA region in plants, fungi, and even animals (Hershkovitz et al., 1999). However, high primer universality also has a disadvantage that these primers simplify a wide range of plant targets and work well to analyze DNA isolated from individual organisms, but do not exclude effectively the fungus sequences in mixed, phytosphere DNA extracts of fungus-associated plants. To resolve this problem, the plant-specific primers should be subsequently designed for analyses of plant ITS sequences (Zhang et al., 1997; Cullings and Vogler, 1998). However, due to the nucleotide variations occurring among not only different plant species but also different fungus species, newly designed primers able to be specific to plants were intended to be specific for certain plant species and specific-associated fungus species.

Plant-specific ITS primer design

In this study, the *Pulsatilla* spp. specific primers of ITS region were newly designed, named by ITS1-F2 and ITS4-R as forward and reverse primer, respectively. Due to relatively high conservation of the 18S and 28S rRNA region, it makes it convenient to design universal primer for the amplification of the ITS region, but also hard to select to amplify certain organisms from potential other species-contaminated materials. When comparing all existing *Pulsatilla* spp. and fungus species parasitic in *Pulsatilla* spp., the accurate sequence alignment was too hard to compare the matches between plant and fungal DNA sequence. Thus, respective sequence alignment of plant and fungi could only be performed in this study. As mismatches at the 3' end of primer are often most critical for efficient amplification (Sommer and Tautz, 1989; Kwok et al., 1990), we tried to find mismatches between plant and fungus sequences at the 3' end of the primer. However, the 18S rRNA shares very highly conserved sequences among the *Pulsatilla* spp. and their parasitic fungus species, that the newly designed ITS1-F2 primer might match both plant and fungal DNAs well (Figure 1). For the reverse primer, it is easier to find some sequence part that matches plant DNA but not fungal DNA, because the fungal ITS sequences among the 5' end of the 28S rRNA gene were variable to the ITS sequences of plants (Figure 1). Therefore, the newly designed primer, ITS4-R was very specific and sensitive to *Pulsatilla* spp. Besides *Pulsatilla* spp., the high plant-specificity of the ITS4-R primer was also investigated and tested on other plant species with potential fungal contamination in our laboratory work, e.g. *Scutellaria baicalensis* Georgi had

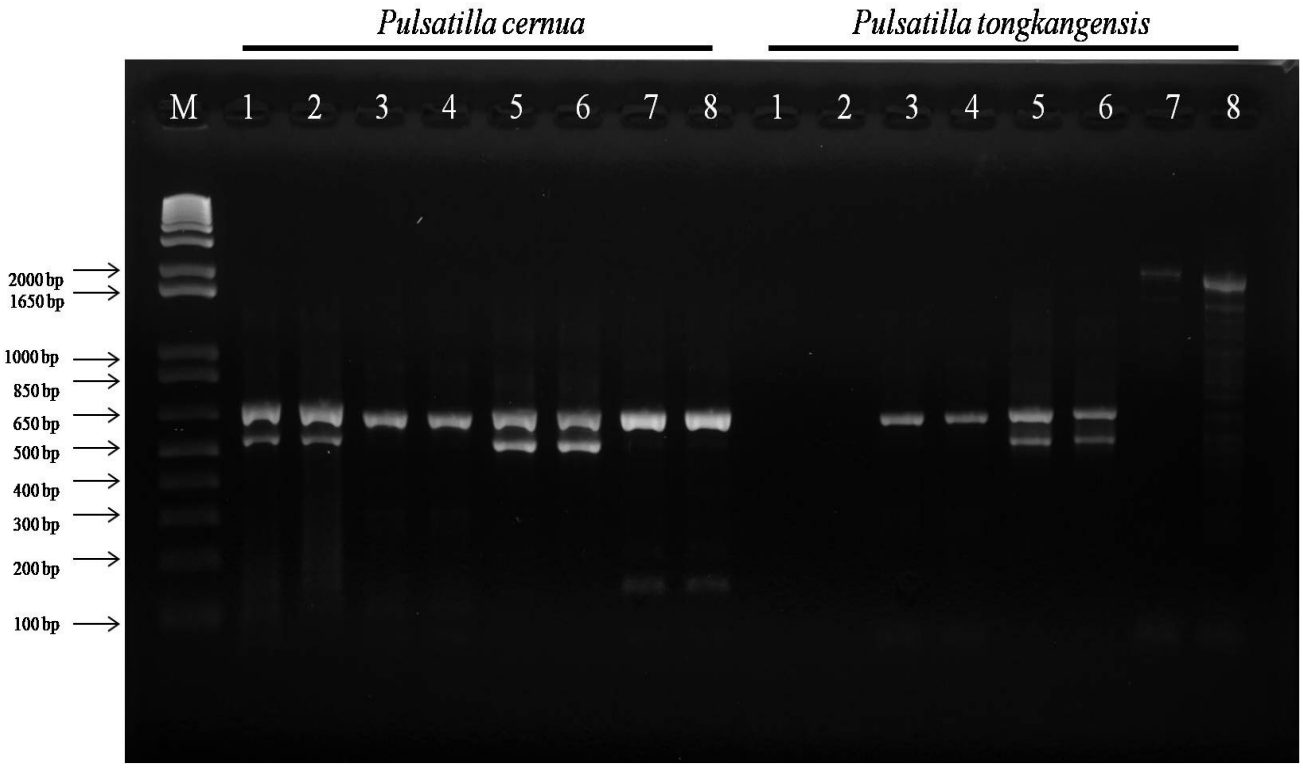

Figure 2. Efficient and preferentially amplification of the ITS region of *P. cernua* and *P. tongkangensis*. Lane 1, 2: PCR products using ITS4 and ITS5 primers; Lane 3, 4: PCR products using ITS1-F2 and ITS4-R primers; Lane 5, 6: PCR products using ITS1-F2 and ITS4 primers; Lane 7, 8: PCR products using ITS5 and ITS4-R primers. The odd and even lanes means one independent plant organism of relevant *Pulsatilla* species respectively.

shown high plant-specificity of the ITS4-R/ITS1-F2 primer set (data not published).

PCR amplification

The amplification ability of four primer pairs including ITS4/ITS5, ITS1-F2/ITS4-R, ITS1-F2/ITS4, and ITS5/ITS4-R, were tested in this study (Figure 2). The amplification results were very different from both *P. cernua* and *P. tongkangensis*, and primer pairs. The *P. cernua* DNAs could amplify efficiently with all of the primer pairs, but using ITS4/ITS5 and ITS1-F2/ ITS4 primer pairs produced two bands. After purifying and sequencing these both PCR products, it was known that the bigger size product was plant ITS region and the smaller size product was the fungal ITS region. Using ITS1-F2/ITS4-R and ITS5/ITS4-R primer pairs, it could preferentially amplify a single clear band from the fungus-contaminated *P. cernua* DNAs. Compared with the size of PCR product, it was trended to be plant ITS region, and the suggestion was strongly supported by our sequencing results. It was said that in combination of ITS4-R primer, the plant DNA isolated from natural *P. cernua* materials was preferentially amplified to the apparent, exclusion of fungal DNA (Figure 2). This result suggested that ITS4-R was specific to *P. cernua* DNA but not fungal DNA, while other three primers including ITS1-F2, ITS4, and ITS5 were not specific neither to *P. cernua* DNA nor fungal DNA. For the new species, *P. tongkangensis*, the universal primer set, ITS4/ITS5 did not work when used to amplify the ITS region, resulting in either no product or wrong amplified product (Figure 2). This result indicated that both or at less one of both universal primers mismatched the *P. tongkangensis* ITS region. So, the occurrence of this result absolutely puzzled us at the beginning, because there was no any sequence sources of *P. tongkangensis* published in GenBank database of NCBI, that limits the free comparison of DNA sequences of *P. tongkangensis* with those from other plant species. Luckily, the newly designed primers, ITS1-F2 and ITS4-R with the original design intention of *Pulsatilla* spp. specific ITS primers, could amplify the ITS region from fungus-contaminated *P. tongkangensis* materials, and the amplification ability was not low. Using both newly designed primers, ITS1-F2 and ITS4-R, not only effectively amplified the ITS region from *P. tongkangensis*, but showed very high plant-specification. However, when combined with one universal primer, ITS4 or ITS5, the amplification ability was largely decreased: in combination with the universal primer ITS4, the reactions could successfully amplify, but the plant-specification was

Table 2. Comparisons of nucleotide variable sites occurring between *P. cernua* and *P. tongkangensis* investigated in this study.

Sample abbreviation	Nucleotide sequence (bp)[1]																													
	ITS1 region									ITS2 region																				
	2	3	5	8	9	11	38	70	74	418	472	537	580	588	589	593	594	595	599	604	605	609	610	613	614	620	621	624	625	
HN1	.	C	.	.	G	C	G	C	A	C	T	A	.	.	.	.	T	C	.	.	A	.	C	.	.	A	G	T	.	
HN2	T	.	A	G	-	T	-	-	-	-	-	-	.	.	.	.	-	-	.	A	-	.	-	.	.	-	-	-	.	
PH1	T	.	G	G	C	.	.	T	G	T	C	G	.	.	.	G	-	-	G	.	.	.	-							
PH2	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G					
PH3	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G					
PH4	T	.	.	G	C	.	-	-	G	T	C	G	.	C	.	G	-	-	G	.	-	.	-							
PH5	.	-	.	G	C	.	-	Y	G	T	C	G	.	.	.	.	-	-	.	.	-	T	.	.	G					
PH6	.	-	.	G	C	.	-	T	G	T	C	G	G	C	C	.	-	-	.	.	-	.	-							
PH7	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G	.	.	-	.	
PH8	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	C	G	T	.	-	.	-							
PH9	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	C	G	.	.	-	T	
PH10	.	-	.	G	C	.	-	-	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G	.	.	-	.	
PH11	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G	.	.	-	.	
PH12	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G	.	.	-	.	
PH13	.	-	.	G	C	.	-	-	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	.	G	.	.	.	.	
PH14	.	-	.	G	C	.	-	T	G	T	C	G	.	.	.	.	-	-	.	.	-	.	-	G	G	.	.	.	.	

[1]The number means the nucleotide site number in the complete ITS1 and ITS2 region of *Pulsatilla* species. Y is the combination of T and C; . means nucleotide site indel; - means nucleotide site identical to that of the top line; non-filled position means the completion of nucleotide sequence.

low; in combination with the universal primer ITS5, the reactions could not produce any accurate amplification from neither plants nor fungi. These results suggested that ITS1-F2 might well match the ITS regions of both *P. tongkangensis* and parasitic fungi but not ITS4-R, and the universal primer ITS4 could match *P. tongkangensis* DNA, but not another universal primer ITS5. Due to the primer location of ITS1-F2 existing in the downstream of that of ITS5, the accurate, relevant primer binding site of ITS5 in *P. tongkangensis* DNA sequence could not be checked in the present work, but the hypothesis that universal primer ITS5 did not match *P. tongkangensis* would be proved using an upstream primer of ITS5 in the future.

Single nucleotide polymorphisms between *P. cernua* and *P. tongkangensis*

To further understand the phylogeny between *P. cernua* and *P. tongkangensis*, the single nucleotide polymorphisms were analyzed in this study. In the total ITS region, there were 29 nucleotide substitutions or indels obtained in all, among which 9 nucleotide variable sites were located in the ITS1 region and 20 sites in the ITS2 region (Table 2). The single nucleotide polymorphisms occurred not only between *P. cernua* and *P. tongkangensis*, but among accessions of same species. Among all the variable nucleotide sites, five ones at position 9, 74, 418, 472, and 537 bp were species-specific, occurring completely between *P. cernua* and *P. tongkangensis* but not among accessions of each species (Table 2). To understand whether these variable sites were *P. tongkangensis*-specific, single nucleotide polymorphism were further analyzed combined with other *Pulsatilla* spp. at these nucleotide sites (Table 3). The detailed result was presented in the following part of phylogenetic relationship among the *Pulsatilla* spp.

Decently, accumulating evidence suggests that nucleotide variations might occur among ITS

Table 3. Comparisons of four nucleotide variable sites occurring only between *P. cernua* and *P. tongkangensis* in this study, with other existing *Pulsatilla* species.

Species	Nucleotide sequence (bp)[1]				References
	74	418	472	537	
P. cernua	A	C	T	A	This study and HQ829820
P. tongkangensis	G	T	C	G	This study
P. violacea	G	C	C	G	JF422892, JF422893, JF422894
P. sp.	G	C	C	G	GU732651
P. chinensis	G	C	C	G	GU732650
P. cernua × *P. dahurica*	R	C	Y	G	HQ829821
P. dahurica	G	C	C	A	GU732648
P. dahurica	G	C	C	G	HQ735289
P. halleri	.	C	C	G	FJ639908
P. rubra	G	C	C	G	JF422891
P. turczaninovii	G	C	C	G	GU743649
P. albana	G	C	C	G	JF422890

[1]The number means the nucleotide site number in the complete ITS1 and ITS2 region of *Pulsatilla* species. R is the combination of A and G; Y is the combination of T and C; . means nucleotide site indel.

sequences even in one organism, because this kind of organisms are hybrids, either diploid or polyploids, of disparate parents (Sang et al., 1995; Buckler et al., 1997). For both *Pulsatilla* spp., some nucleotide variations showed accession-specific, that might express the hybridization result. Although there was no report indicating that *P. tongkangensis* could be hybridized with *P. cernua*, a hybridization behavior between *P. tongkangensis* and *P. koreana*, and was suggested based on randomly amplified polymorphic DNA (Lee et al., 2010). Due to initially growing in a very isolated area in Tongkang, Korea, *P. tongkangensis* was recorded as a new species and identified based on leaf morphology, the orientation of flower buds before anthesis, and flower colors (Lee, 2000; Lee and Kim, 2006). However, *P. cernua*, as one of the mostly common *Pulsatilla* spp. in Korea like *P. koreana*, made natural hybridization possible between these two species. The mostly apparent variable site typical of hybridization was position 70 bp, that both *P. cernua* accessions HN1 and HN2 showed C at this site, while most *P. tongkangensis* accessions showed nucleotide substitution with T, excluding accession PH4, PH5, PH10, and PH13 (Table 2). In particular, PH5 showed combination with T and C at position 70 bp, further suggesting that *P. tongkangensis* could be hybridized with other *Pulsatilla* spp. In addition, position 2 and 3 bp might also represent the hybrid result, that *P. cernua* accession HN1 shared nucleotide indel at position 2 bp with most *P. tongkangensis* accession exclusion of PH1 and PH4, and HN2 shared the same nucleotide sequence with PH1 and PH4 at position 2 bp; on the contrary, HN2 shared nucleotide indel with PH1 and PH4 at position 3 bp, and HN1 showed the same nucleotide sequence as other *P. tongkangensis* accessions (Table 2).

Besides with *P. cernua* and *P. koeana*, *P. tongkangensis* might be capable to hybridize with other *Pulsatilla* spp., such as *P. davurica*, *P. nivalis*, and *P. chinensis* growing in the overlapping geographic areas in Korea. The occurrence probability of hybridization made the understanding of accession-specific variable nucleotide sites more easily, e.g. position 580 and 589 bp in PH6 showed one nucleotide insertion, position 594 and 599 bp in PH8 showed one nucleotide substitution, and position 609 bp in PH5 had one nucleotide insertion (Table 2).

Phylogenetic relationship among the *Pulsatilla* spp.

Despite the fact that the ITS region sequence was available in many *Pulsatilla* spp. including *Pulsatilla violacea*, *P. cernua*, *P. chinensis*, *Pulsatilla dahurica*, *Pulsatilla halleri*, *Pulsatilla rabra*, *Pulsatilla turczaninovii*, and *Pulsatilla albana*, no ITS sequence source but any other sequence sources of *P. tongkangensis* were found in NCBI GenBank database until were submitted in the early October in 2011. In general, sequences from samples difficult to amplify and sequence were scarcely few. For *P. tongkangensis*, besides this reason, its habitat endemic to Korea also led to the scarcity of sequence sources. According to the DNA alignment result, there were 59 nucleotide variable sites occurring in all among the 571 bp common length of the ITS region of *Pulsatilla* spp. (data not shown). Not like assumed, nucleotide variations did not mainly limit between *P. tongkangensis* and other *Pulsatilla* spp., on the contrary, some *Pulsatilla* spp. showed identical nucleotide sequences to *P. tongkangensis* but not to the common species, *P. cernua*. At the four *P. tongkangensis*-specific variable sites, this

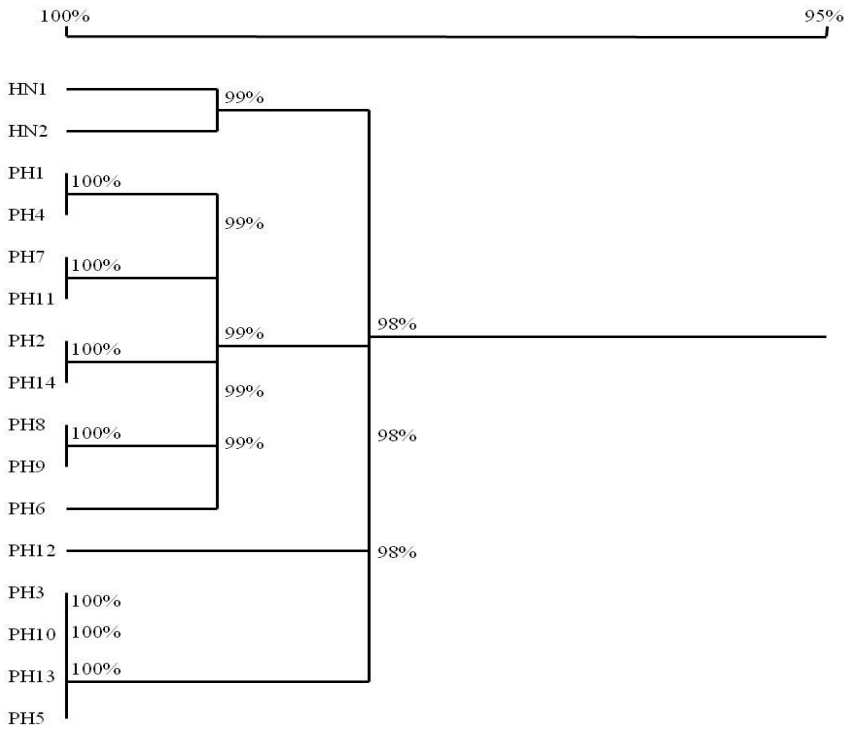

Figure 3. Phylogenetic tree constructed using the ITS region sequences of two *P. cernua* accessions and fourteen *P. tongkangensis* accessions.

Table 4. Genetic distance (GD) and homology rate evaluated by pairwise percentage similarity of the ITS region of species of *Pulsatilla* species.

S/N	Species	1	2	3	4	5	6	7	8	9	10
1	*P. halleri*	-	97.80	99.10	98.00	98.65	99.50	99.50	99.20	98.90	97.80
2	*P. albana*	0.022	-	97.50	97.00	97.05	97.70	97.70	97.80	97.70	99.10
3	*P. cernua*	0.009	0.025	-	97.60	98.40	98.90	99.00	99.25	98.60	97.50
4	*P. chinensis*	0.020	0.030	0.024	-	98.40	97.90	97.90	96.80	98.40	97.00
5	*P. dahurica*	0.013	0.030	0.016	0.016	-	98.00	98.55	98.05	98.70	97.05
6	*P. rubra*	0.005	0.023	0.011	0.021	0.02	-	99.60	99.20	98.90	97.70
7	*P. sp.*	0.005	0.023	0.01	0.021	0.0145	0.004	-	98.20	99.00	97.70
8	*P. tongkangensis*	0.008	0.022	0.0075	0.032	0.0195	0.008	0.018	-	97.90	97.80
9	*P. turczaninovii*	0.011	0.023	0.014	0.016	0.013	0.011	0.01	0.021	-	97.70
10	*P. violacea*	0.022	0.009	0.025	0.03	0.0295	0.023	0.023	0.022	0.023	-

GD was presented below the axlewire; homology rate was presented above the axlewire.

situation was also found, e.g. *P. violacea*, *P. chinensis*, *P. dahurica*, *P. rubra*, *P. turczaninovii*, and *P. albana* shared the same nucleotide sequence at site 74, 472, and 537 bp (Table 2). One hybrid species by *P. cernua* and *P. dahurica* (HQ829821) showed clear homogenization to that of either parental species due to the concerted evolution in these four nucleotide variable sites, with site 74 and 472 bp from both parental species and site 537 bp from *P. dahurica* species. Although there was one *P. dahurica* sequence showing that site 537 bp is identical to *P. cernua* but not the *P. cernua* × *P. dahurica* hybrid species; the ITS region of this hybrid species showed high homogenization level. The new species, *P. tongkangensis* had only one specific variation at site 418 bp.

In the phylogenetic tree constructed by all the ITS region sequences, both *P. cernua* accessions formed a monophyletic group, sharing 99% of homology with each other, while *P. tongkangensis* accessions showed three one- or multi-level groups, parallel to the *P. cernua* monophyletic group (Figure 3). In the phylogenetic tree constructed by the ITS region of all the *Pulsatilla* spp., no

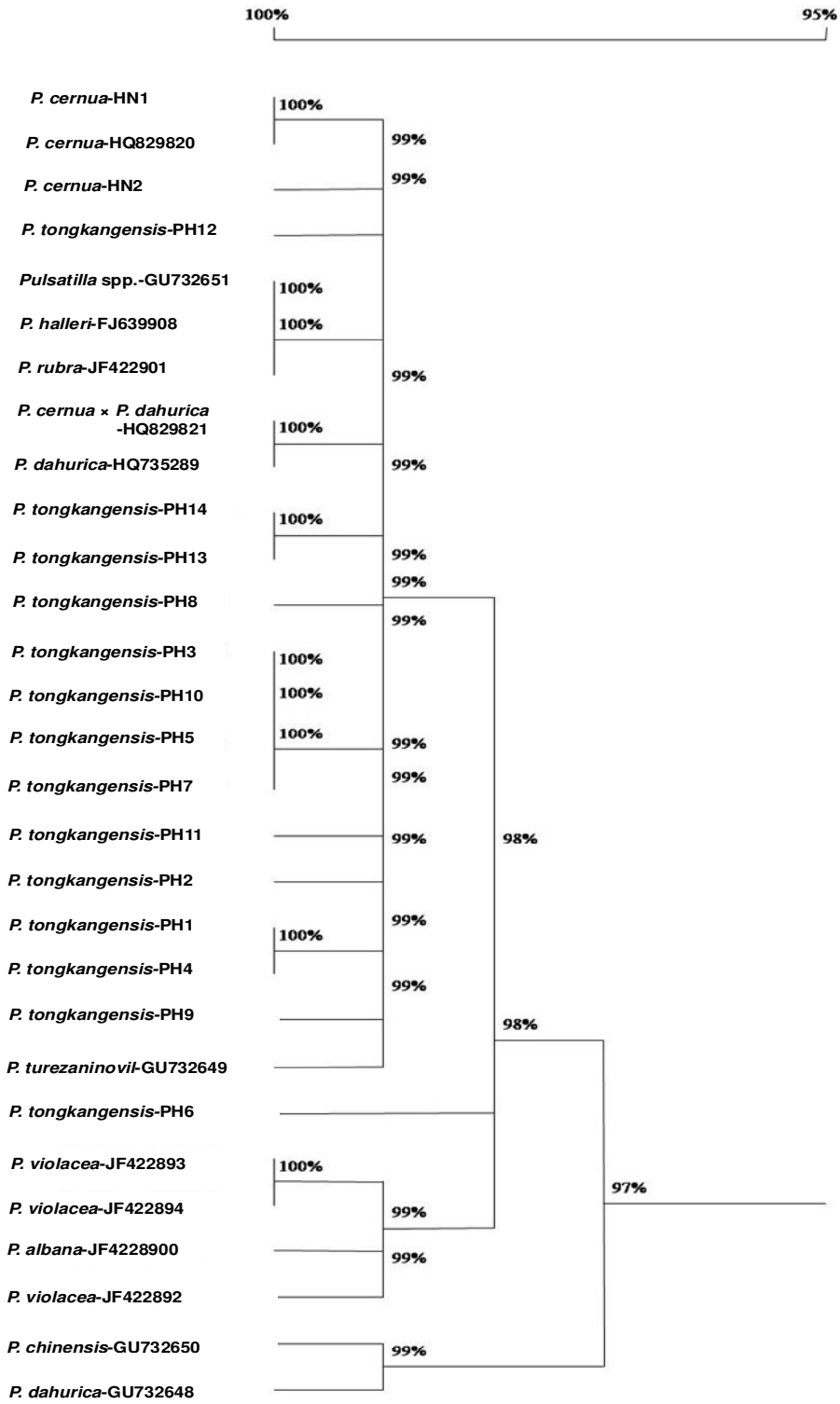

Figure 4. Phylogenetic tree constructed by all available ITS sequence sources of *Pulsatilla* species in NCBI GenBank database and in this study.

clear monophyly of certain species was found. *P. cernua* species showed the highest identity rate of 99.30% among three different accessions, while *P. dahurica* species showed the lowest one of 98.10%. Between both *Pulsatilla* spp., *P. chinensis* and *P. tongkangensis* were found to have the highest dissimilarity (3.2%), followed by *P. chinensis* and *P. albana* (3.0%), while *P. rubra* and *Pulsatilla* spp. were found to have the highest similarity

(99.60%), followed by *P. rubra* and *P. halleri* (99.50%, Table 4).

The homology rate between some different species was even higher than that among different accessions of the same species, suggesting that nucleotide variation among all the *Pulsatilla* spp. was not only geographical origin-related but also species-related. Aligning DNA sequences of all the *Pulsatilla* spp., the identity rate among them reached a relatively high level of 97% (Figure 4).

Conclusion

Fungus contamination always puzzles the ITS amplification from natural plant materials, particularly some medicinal plant species. Two newly designed primers specific for *Pulsatilla* spp. presented here is anticipated to serve as the basis for a wide ranging system to analyze plant materials, particularly in association with fungi. In addition, these new primers sharing high universality provide us more combinations of primer sets for the amplification, and could also supplement some cases that are difficult to successfully amplify using universal primers, such as *P. tongkangensis* investigated in this study. By using multiple primer sets, we expect to maintain a wide range of universality and a high degree of comparability between PCR reactions. And these multiply primer sets are being attempted for plant-specific ITS amplification in other plant species materials in our laboratory. Two newly designed primers were developed with an emphasis on discrimination between plant and fungal sequences and should be particularly useful for studies of plant materials where samples also contain high levels of background fungal DNA.

ACKNOWLEDGEMENTS

This work was supported by Nutraceutical Bio Brain Korea 21 Project Group and New Talent Introduction Project, Ludong University (LY2012008).

REFERENCES

Álvarez I, Wendel JF (2003). Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol., 29:417-434.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowsi MF, Campbell CS, Donoghue MJ (1995). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on Angiosperm Phylogeney. Ann. Mo. Bot. Gard. 82:247-277.

Buckler ES, Ippolito A, Holtsford TP (1997). The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. Genetics, 145:821-832.

Cullings KW, Vogler DR (1998). A 5.8S nulclear ribosomal RNA gene sequence database: applications to ecology and evolution. Mol. Ecol. 7:913-923.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15.

Han CK, Park YH, Jin DQ, Hwang YK, Oh KB, Hand JS (2007). SK-PC-B70M from *Pulsatilla koreana* improves scopolamine-induced impairments of memory consolidation and spatial working memory. Brain Res. 1184:254-259.

Hershkovitz MA, Zimmer EA, Hahn WJ (1999). Ribosomal DNA sequences and angiosperm systematic. In: Hollingsworth PM, Bateman RM, Ornall RJ (eds) Molecular Systematics and Plant Evolution: Taylor & Francis, London pp 268-326.

Huang SQ, Takahashi Y, Dafni A (2002). Why dose the flower stalk of *Pulsatilla cernua* (Ranunculaceae) bend during anthesis? Am. J. Bot. 89:1599-1603.

Karehed J, Groeninckx I, Dessein S, Motley TJ, Bremer B (2008). The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoceae. Mol. Phylogenet. Evol. 40:843-866.

Kim ZS, Jo DG, Jeong JH, Kim YH, Yoo KO, Cheon KS (2010). Genetic diversity and structure of *Pulsatilla tongkangensis* as inferred from ISSR markers. Korean J. Plant Res. 23:360-367.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005). Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. USA. 102:8369-8374.

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda I, Levenson C, Sninsky JJ (1990). Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1model studies. Nucleic Acids Res. 18:999-1005.

Lee AK, Yuan T, Suh JK, Choi DS, Choi IY, Roh MS, Joung YH, Ko JY, Lee JS (2010). *Pulsatilla tongkangensis*, a natural hybrid swarm population hybridized with *P. koreana* based on RAPD and SNPs of chloroplast DNA. Hort. Environ. Biotechnol. 51:409-421.

Lee WK, Choi HW, Bang JW (2004). Karyotype analysis of five species of genus *Pulsatilla*. Korean J. Med. Crop Sci. 12:490-493.

Lee YN (2000). Genus *Pulsatilla* in Korea. Bull. Korea Plant Res. 1:9-15.

Lee YN, Kim YS (2006). I. New taxa of Korean Flora. 1. Two new forms of *Pulsatilla tongkangensis* Y. Lee et T. C. Lee. Bull. Korea Plant Res. 6:2-3.

Naito K, Nakagoshi K (1994). The conservation ecology of *Pulsatilla cernua* (Thumb.) Spreng. (Ranunculaceae), an endangered species in Japan. In: Song Y, Dierschke H, Wang X (eds) Applied vegetation ecology: East China Normal University Press, Shanghai, China, pp. 263-269.

Sang T, Crawford DJ, Stuessy TF (1995). Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. USA. 92:6813-6817.

Seo JS, Yun JH, Baek IS, Leem YH, Kang HW, Cho HK, Lyu YS, Son HJ, Han PL (2010). Oriental medicine Jangwonhwan reduces Abeta(1-42) level and beta-amyloid deposition in the brain of Tg-APPswe/PS1dE9 mouse model of Alzheimer disease. J. Ethnopharmacol. 128:206-212.

Sommer R, Tautz D (1989). Minimal homology requirements for PCR primers. Nucleic Acids Res. 17:6749.

Sparg SG, Light ME, Van Staden J (2004). Biological activities and distribution of plant saponins. J. Ethnopharmacol. 94:219-243.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols-a guide to methods and applications: Academic Press, San Diego, Calif, pp 315-322.

Xu HJ, Shi XW, Ji X, Du YF, Zhu H, Zhang LT (2011). Qualitative and quantitative determination of nine main active constituents in *Pulsatilla cernua* by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. J. Sep. Sci. 34:308-316.

Xu YJ, Bai L, Liu YH, Liu Y, Xu TH, Xie SX, Si YS, Zhou HO, Liu TH, Xu DM (2010). A new triterpenoid saponin from *Pulsatilla cernua*. Molecules. 15:1891-1897.

Zhang W, Wendel JF, Clark LG (1997). Bamboozed again! Inadvertent isolation of fungal rDNA sequences from bamboos (Poaceae: Bambusoideae). Mol. Phylogenet. Evol. 8:205-217.