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GENETIC VARIATIONS AND RELATIONSHIPS OF CULTIVATED AND WEEDY TYPES OF *PERILLA* SPECIES IN KOREA AND JAPAN USING MULTI DNA MARKERS

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Abstract

The genus *Perilla*, known as an oil crop or a Chinese medicine, vegetable crop, is widely cultivated in East Asia. It occurs in two distinct varieties, var. *frutescens* and var. *crispa*, and their genetic relationship is still obscure. To understand the genetic diversity and relationships of the cultivated and weedy types of *Perilla* crops in Korea, Japan and China, we evaluated the genetic variation of 20 accessions by 3 rDNA markers. Among these three markers, the nuclear ribosomal DNA (nrDNA) internal transcribed spacers (ITS) region of *Perilla* crops showed less sequence variations than the 5S and 18S genes. There were abundant variable nucleotide sites appearing in the 5S and 18S genes. Especially in the 18S gene, the variable nucleotide sites showed specificity between some *Perilla* type and other varieties. JPN1 showed 6 special variable nucleotide sites differing from other varieties, resulting in the farthest phylogenetic relationship in the 18S tree. CHI15 shared 8 special variable sites, also showing far phylogenetic relationship with other varieties. According to the sequence analysis result, the cultivated types of Korean var. *frutescens* showed relatively more genetic diversity than those of Japanese var. *frutescens*, while Korean var. *crispa* showed lower genetic diversity than those of Japanese var. *crispa*. However, the intra- or inter-variety genetic distance did not have significant difference. This work provided more sequence resources of *Perilla* crops and evidences to evaluate the genetic variation and relationships. Our result would help molecular type identification, functional plant breeding and trait improvement of *Perilla* crops.

Key words: *Perilla*; Genetic diversity; Phylogenetic relationship; Molecular identification; Species discrimination

Introduction

Perilla frutescens (Labiatae) Britton, as the exclusive species of the genus *Perilla*, is a self-fertilizing crop that is widely cultivated in East Asia and introduced in Europe, Russia and USA as an oilseed crop (Nitta *et al*., 2003). *P. frutescens* L. occurs in two distinct varieties based on its morphological characters and special uses: *P. frutescens* (L.) Britton var. *frutescens*, an oil crop, and *P. frutescens* (L.) Britton var. *crispa* (Thunb.) W. Deane, a Chinese medicine or spicy vegetable crop (Honda *et al*., 1994, 1996; Nitta & Ohnishi, 1999; Lee & Ohnishi, 2001).

Because *Perilla* is propagated through seeds, there are both forms in folk, the cultivated and weedy forms (Pandey & Bhatt, 2008). The cultivated varieties are generally grown by direct sowing or raised in nursery beds for transplanting annually. The weedy varieties usually occur in farmer's fields, as dynamic populations of a cultivated plant. Because they should evolve through natural hybridization with closely related wild or weedy relative, adapt natural and local environment and traditional farming systems, the weedy varieties often show genetically diverse (Harlan, 1975; Villa *et al*., 2006). Previous studies about genetic diversity between cultivated and weedy forms reported that weedy forms were genetically intermediate between the two cultivated crops (Welsh & McClelland, 1990; Williams *et al*., 1990). The reason was explained by that the weedy forms might result from crossing between the two crops, escaped crops, or ancestor of the crops (Nitta & Ohnishi, 1999). To improve the physiological trains of *Perilla* crops through breeding programs, the study about genetic diversity and relationships among cultivated and weedy types is always in process.

Recently, PCR-based molecular identification methods have become routine, efficient means for accurate authentication among plant species (Joshi *et al*., 2004; Sultan *et al*., 2013; Turi *et al*., 2012; Shinwari *et al*., 2011; Pervaiz *et al*., 2010; Rabbani *et al*., 2010). DNA-based molecular markers share very abundant, useful information, which we could use to study genetic diversity and relationships between the *Perilla* cultivated and weedy varieties. On the way of understanding the genetic diversity and relationships of *Perilla* varieties, many scientists have done much attempt. Nitta & Ohnishi (1999) and Nitta *et al*. (2003) have identified weedy *P. frutescens* types using random amplified polymorphic DNA (RAPD) and grouped them into two cultivated types. Lee & Ohnishi (2003) analyzed the genetic diversity and relationships of the cultivated *Perilla* species with their weedy forms using amplified fragment length polymorphism (AFLP) markers. Kwon *et al*. (2005) designed simple sequence repeat (SSR) markers for *Perilla* species, and Park *et al*. (2008) analyze the accessions from East Asian countries using SSR markers. The morpho-biochemical and

molecular markers are used to screen elite genotypes (Jan *et al*., 2017, Shinwari *et al*., 2014; Jan *et al*., 2016; Shinwari *et al*., 2013; Mahmood *et al*., 2010). However, little information was found using ribosomal DNA (rDNA) markers to analyze genetic diversity and relationships between the cultivated and weedy varieties. In the present study, we evaluated the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA), 5S and 18S ribosomal DNA (rDNA) genes to analyze the genetic diversity and relationships among 20 accessions consisting of cultivated and weedy varieties from Korea, Japan and China.

ITS region of nrDNA, comprising the ITS1 intergenic spacer, 5.8S rRNA, and the ITS2 intergenic spacers, is one of the most commonly used regions for species identification according to the Barcode of Life data systems (http://www.boldsystems.org) and NCBI GenBank (http://www.ncbi.nlm.nih.gov) database (Coleman, 2003). The 5S rRNA genes, which are present in multiple copies in the eukaryotic genome, are also the most widely used gene family for the determination of phylogenetic relations. These genes contain the coding region and a non-transcribed spacer region; the coding region of 5S rDNA genes is highly conserved and commonly 120 bp in length, whereas the non-transcribed spacer (NTS) region is of different size in different species depending on the coding region and exhibits high variation (Campell *et al*., 1992; Sastri *et al*., 1992). Another rDNA gene, 18S is also widely used for species discrimination because of high frequency of nucleotide variation (Soltis *et al*., 2000; Bleidorn *et al*., 2003).

Our objective was to explore the level of genetic diversity and relationships between the cultivated *P. frutescens* and their weedy varieties using these three rDNA markers. This work would provide more evidences for further understanding of the genetic diversity and relationships of *Perilla* species, and help to improve variety conservation and breeding programs.

Materials and Methods

Plant materials: Twenty *Perilla* accessions including 10 *P. frutescens* var. *frutescens* and 10 var. *crispa*, were collected from main Asian habitats, Korea, Japan and China. Among these accessions, there were 15 cultivated types and 5 weedy types in all. The cultivated types were sown annually at the local, while the weedy types were all landraces, adapting and combined with naturally environment and traditional farming system. Fresh leaf tissues of these 20 *Perilla* accessions were sampled and immediately stored in liquid nitrogen condition for DNAs extraction. The specimen vouchers and other detailed information about collection regions and countries were shown in Table 1.

DNA extraction, PCR amplification and sequencing: DNA extractions were performed by using the modified cetyltrimethylammonium bromide (CTAB) method described by Doyle & Doyle (1987). The ITS1-5.8S-ITS2 region was amplified using universal primers ITS1 and ITS4 (White *et al*., 1990) in 20 µl PCR reaction. The rDNA 5S gene was amplified using universal primers 5SF (forward primer) and 5SR (reversed primer, Hizume, 1993). And the rDNA 18S gene was amplified using universal primers 18SF (forward primer) and 18SR (reversed primer, Sogin, 1990). The reaction components for effective PCR amplification are 1 µl of template DNA (~1-100 ng), 10 µl 2 × PCR Dye Master Mix (containing 2 × Taq DNA polymerase, 2 × PCR buffer, 2 × dNTP, and moderate loading dye, QIAGEN, Korea), and 0.1 µmol l^{-1} of each primer (including forward primer and reversed primer). PCR amplification was conducted using this set of primers with the following program: 35 cycles of denaturation at 95℃ for 1 min, annealing 54-57 ℃ for 1 min, and a final extension step at 72℃ for 1 min. The amplification products were checked by electrophoresis through 1.0% agarose gel, and then purified before DNA sequence analysis using a QIA quick PCR Purification Kit (QIAGEN, Korea) or Gel Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at BGI in Beijing, China (http://www.genomics.cn/index).

Sequence editing and alignment: For editing and assembly of the complementary strands, the software program DNAMAN version 6.0 (Lynnon Biosoft Corporation, USA, http://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/). The multiple sequence alignments of ITS1-5.8S-ITS2 region, 5S and 18S genes were also performed using DNAMAN version 6.0 software, to detect single nucleotide polymorphisms.

Phylogenetic analysis: We assessed intraspecial genetic divergences by using pair wise distance calculations (Meyer & Paulay, 2005). Jaccard coefficients used to represent identity among the ecotypes were calculated by similarity coefficient [Sj = a/(a+u)]. In the total ITS region, ITS1 and ITS2 region, '1' was used for base variation and '0' was used for no variation; 'a' represents the number of the same bases and 'u' represents the number of different bases between two accessions. The phylogenetic relationship among 20 *Perilla* accessions was estimated after the construction of a phylogram based on multiple sequence alignment of various DNA sequences with the DNAMAN version 6.0 software. Based on the typical variable nucleotide sites in the total ITS region sequence, the phylogenetic relationship among our materials was estimated again. Genetic distance (GD) was obtained with the help of MEGA software and mean GD of the intravariety and intervariety distance was calculated by sum of individual GD divide by number of samples.

Statistical analysis: Statistically significant differences between the means were determined by based on two-way analysis of variance (ANOVA) using Duncan's multiple-range test (Duncan, 1955). A P value of less than 0.05 was considered significant.

Table 1. Specimen voucher and collection geographical region information of *Perilla frutescens* materials investigated in this study.

Sample No.	Specimen voucher	Genotype	Type	Collection region	Collection country	NCBI Accession Number		
						nrDNA ITS	rDNA 18S	rDNA 5S
1.	KOR1	*Perilla frutescens* var. *frutescens*	Cultivated	Miryang-shi, Kyongsangnam-do	South Korea	KF012852		
2.	KOR2	*Perilla frutescens* var. *frutescens*	Cultivated	Hapchon-gun, Kyongsangnam-do	South Korea	KF012853	KR921727	KT225341
3.	KOR3	*Perilla frutescens* var. *frutescens*	Cultivated	Yang-yang-gun, Kangwon-do	South Korea	KF012854	KR921728	KT225342
4.	KOR4	*Perilla frutescens* var. *frutescens*	Cultivated	Yonchon-gun, Kyonggi-do	South Korea	KF012855		KT225343
5.	KOR5	*Perilla frutescens* var. *frutescens*	Cultivated	Chongson-gun, Kangwon-do	South Korea	KF012856	KR921729	KT225344
6.	JPN1	*Perilla frutescens* var. *frutescens*	Cultivated	Kawanishi-gun, Hokkai-do	Japan	KF012857	KR921730	KT225345
7.	JPN2	*Perilla frutescens* var. *frutescens*	Cultivated	Sannohe-gun, Aomori-ken	Japan	KF012858	KR921731	KT225346
8.	JPN3	*Perilla frutescens* var. *frutescens*	Cultivated	Waga-gun, Iwate-ken	Japan	KF012859	KR921732	KT225347
9.	JPN4	*Perilla frutescens* var. *frutescens*	Cultivated	Minamiaizu-gun, Fukushima-ken	Japan	KF012860	KR921733	KT225348
10.	JPN5	*Perilla frutescens* var. *frutescens*	Cultivated	Chichibu-gun, Saitama-ken	Japan	KF012861	KR921734	KT225349
11.	JPN6	*Perilla frutescens* var. *crispa*	Cultivated	Utsunomiya-shi, Tochigi-ken	Japan	KF012862	KR921735	KT225350
12.	JPN7	*Perilla frutescens* var. *crispa*	Cultivated	Kurita-gun, Shiga-ken	Japan	KF012863		KT225351
13.	JPN8	*Perilla frutescens* var. *crispa*	Cultivated	Yonezawa-shi, Yamagata-ken	Japan	KF012864	KR921736	
14.	JPN9	*Perilla frutescens* var. *crispa*	Cultivated	Higashishirakawa-gun, Fukushima-ken	Japan	KF012865	KR921737	KT225352
15.	JPN10	*Perilla frutescens* var. *crispa*	Cultivated	Kaminoyama-shi, Yamagata-ken	Japan	KF012866		KT225353
16.	KOR12	*Perilla frutescens* var. *crispa*	Wild	Inje-gun, Kangwon-do	South Korea	KF012867	KR921738	KT225354
17.	KOR18	*Perilla frutescens* var. *crispa*	Wild	Pochon-gun, Kyonggi-do	South Korea	KF012868	KR921739	KT225355
18.	KOR19	*Perilla frutescens* var. *crispa*	Wild	Hongchon-gun, Kangwon-do	South Korea	KF012869	KR921740	KT225356
19.	KOR20	*Perilla frutescens* var. *crispa*	Wild	Andong-shi, Kyongsangbuk-do	South Korea	KF012870	KR921741	KT225357
20.	CHI15	*Perilla frutescens* var. *crispa*	Wild	Yichang, Hubei	China	KF012871	KR921742	KT225358

Blank spacers in the table above mean no submitted sequences

Results and Discussion

PCR amplification of DNA markers: To amplify the ITS1-5.8S-ITS2 region of *P. frutescens*, the universal ITS primer set, ITS1 and ITS4 (White *et al*., 1990) were used in this study. The nrDNA ITS regions were successfully amplified from all 20 *Perilla* materials, with about 670 bp and submitted in NCBI Gen Bank database (with accession numbers of KF012852-KF012871). The analogue of the PCR products was detected through BLAST on NCBI server (http://www.ncbi.nlm.nih.gov/). The analogue suggested that our sequencing results were 99% similar to *P. frutescens* ITS1-5.8S-ITS2 region sequence (NCBI GenBank accession number: DQ667246), 99% similar to *P. frutescens* var. *acuta* ITS1-5.8S-ITS2 region sequence (NCBI GenBank accession number: AB685326), and 99% similar to *P. frutescens* var. *auriculatodentata* ITS1-5.8S-ITS2 region sequence (NCBI GenBank accession number: KC011251).

Using universal primer set of the rDNA 5S genes (Hizume, 1993), this region was not easy to successfully amplify. Vague bands or multi bands were often obtained in the electrophoresis result. By DNA purification and PCR condition optimization, the rDNA 5S genes were efficiently amplified from 18 *Perilla* materials, with about 500 bp and submitted in NCBI GenBank database (with accession numbers of KT225341-KT225358). The analogue of the PCR products was detected through BLAST on NCBI server (http://www.ncbi.nlm.nih.gov/). The analogue suggested that our sequencing results were 85% similar to *P. frutescens* var. *frutescens* 5S rRNA gene sequence (NCBI GenBank accession number: EF6730411), 79% similar to *P. frutescens* var. *auriculatodentata* 5S rRNA gene sequence (NCBI GenBank accession number: EF673040), and 78% similar to *P. frutescens* var. *crispa* 5S rRNA gene sequence (NCBI GenBank accession number: EF673043).

The rDNA 18S gene was amplified using universal primer set (Sogin, 1990), and the complete sequences were assembled using DNAMAN software version 6.0. There were 16 rDNA 18S gene sequences amplified from our *Perilla* materials, with about 1800 bp and submitted in NCBI GenBank database (with accession numbers of KR921727- KR921742). The analogue of the PCR products was detected through BLAST on NCBI server (http://www.ncbi.nlm.nih.gov/). The analogue suggested that our sequencing results were 99% similar to *Schizonepeta tenuifolia* 18S rRNA gene sequence (NCBI GenBank accession number: JN802671), 99% similar to Lamium purpureum 18S rRNA gene sequence (NCBI GenBank accession number: HQ384694), and 99% similar to *Paulownia tomentsa* 18S rRNA gene sequence (NCBI GenBank accession number: AJ236039). Except there was no existing 18S rRNA gene sequence in NCBI GenBank database, our sequences were all identical with existing *Perilla* sequences. Our 18S rDNA sequences were identical with existing 18S rDNA gene sequences from other related plant species. These results forcefully proved the validity of our amplification.

Sequence analysis of DNA markers: Compared to the rDNA 5S and 18S gene sequences, the nrDNA ITS region sequences were little variable, showing high similarity among cultivated and weedy *Perilla* varieties investigated in this study. The rDNA 5S gene sequences showed the highest nucleotide variation among these three DNA markers, with 76.39% similarity rate among all investigated *Perilla* accessions. Compared to the coding region, the non-transcribed spacer region appeared more prominent variation that was also supported by previous study (Persson, 2000). In addition, the rDNA 18S gene sequence also showed high sequence variation, however, because of long length, the variation rate was not very high.

There were some special variable nucleotide sites occurring between some variety and other varieties, that might become the primary reason of grouping in the phylogenetic tree. There were 6 variable nucleotide sites occurring between JPN1 and other varieties, located at 28 bp, 31 bp, 33 bp, 35 bp, 41 bp, and 911 bp (Table 2). Among these 6 sites, 4 were caused by nucleotide substitution, while 33 bp and 911 bp belonged to nucleotide indels. Among 5 Japanese var. *frutescens* cultivated types investigated in this study, JPN1 and JPN3, still shared 7 particular variable nucleotide sites, located at 943-945 bp, 947 bp, 965 bp, 966 bp, and 1011 bp (Table 3). And no nucleotide changes were found in JPN2, JPN4, and JPN5 18S gene sequences, that suggested that there was also genetic divergence appearing in cultivated types. The sole var. *crispa* Chinese weedy variety (CHI15) had 8 particular variable nucleotide sites, located at 43 bp, 44 bp, 47 bp, 48 bp, 51 bp, 52 bp, 57 bp, and 127 bp (Table 4). The variation pattern was mostly caused by nucleotide substitution, but only one site (44 bp) was caused by nucleotide indel. In addition, KOR5, a Korean var. *frutescens* cultivated variety, showed seven particular variable nucleotide sites, located at 777 bp, 790 bp, 795 bp, 804 bp, 810 bp, 895 bp, and 1049 bp (Table 5), among which 810 bp was nucleotide indel site, and others were nucleotide substitution. At 822 bp, KOR5, KOR12, and KOR18 showed a 'T' nucleotide addition, however, they were even not part of the same variety and cultivation type (Table 1). This result suggested that KOR5 might have much germplasm exchange with var. *crispa* type in natural environment.

Phylogenetic relationship based on DNA marker sequences: According to the nrDNA ITS region sequences, an ITS phylogenetic tree of all *Perilla* varieties were constructed, with two groups forming: KOR2, JPN1, JPN3, and JPN4 forming one group, and other varieties forming the other group (data not shown). The cultivated or weedy varieties in each group shared very high similarity rate with each other. All var. *crispa*, whether cultivated or weedy types were divided into one group, nevertheless all var. *frutescens* types were separated into two groups, that suggested that var. *frutescens* had more genetic divergence than var. *crispa*. And the cultivated and weedy types of var. *crispa* were divided into one group, irrespective of collection region.

Table 2. Special variable nucleotide sites in the rDNA 18S gene sequences, mostly appearing between JPN1 and other varieties.

Sample No.	Specimen voucher	Variable nucleotide sites								
		28bp	30bp	31bp	33bp	34bp	35bp	36bp	41bp	911bp
1.	KOR2	G	C	T	T	G	A	A	T	A
2.	KOR4	G	C	T	T	G	A	A	T	A
3.	KOR5	G	C	T	T	G	A	-	T	A
4.	JPN1	A	C	A	-	G	T	A	G	-
5.	JPN2	G	A	T	T	G	A	A	T	A
6.	JPN3	G	A	T	T	G	A	A	T	A
7.	JPN4	G	A	T	T	G	A	A	T	A
8.	JPN5	G	A	T	T	G	A	A	T	A
9.	JPN6	G	A	T	T	G	A	A	T	A
10.	JPN8	G	A	T	T	G	A	A	T	A
11.	JPN9	G	A	T	T	G	A	A	T	A
12.	KOR12	G	A	T	T	G	A	A	T	A
13.	KOR18	G	C	T	G	T	A	A	T	A
14.	KOR19	G	A	T	T	G	A	A	T	A
15.	KOR20	G	A	T	T	G	A	A	T	A
16.	CHI15	G	G	T	T	G	A	A	T	A

- Means nucleotide indel.

Table 3. Special variable nucleotide sites in the rDNA 18S gene sequences, mostly appearing between JPN1 and JPN3, and other varieties.

Sample No.	Specimen voucher	Variable nucleotide sites				
		943-945bp	947bp	965bp	966bp	1011bp
1.	KOR2	CAT	T	-	G	-
2.	KOR4	CAT	-	T	G	-
3.	KOR5	CAT	-	-	G	-
4.	JPN1	TCA	T	G	A	A
5.	JPN2	CAT	-	-	G	-
6.	JPN3	TCA	T	G	A	A
7.	JPN4	CAT	-	-	G	-
8.	JPN5	CAT	-	-	G	-
9.	JPN6	CAT	-	-	G	-
10.	JPN8	CAT	-	-	G	-
11.	JPN9	CAT	-	-	G	-
12.	KOR12	CAT	-	-	G	-
13.	KOR18	CAT	-	-	G	-
14.	KOR19	CAT	-	-	G	-
15.	KOR20	CAT	-	-	G	-
16.	CHI15	CAT	-	-	G	-

- Means nucleotide indel.

Table 4. Special variable nucleotide sites in the rDNA 18S gene sequences, mostly appearing between CHI15 and other varieties.

Sample No.	Specimen voucher	Variable nucleotide sites							
		43bp	44bp	47bp	48bp	51bp	52bp	57bp	127bp
1.	KOR2	A	A	A	A	A	G	T	G
2.	KOR4	A	A	A	A	A	G	T	G
3.	KOR5	A	A	A	A	A	G	T	G
4.	JPN1	A	A	A	A	A	G	T	G
5.	JPN2	A	A	A	A	A	G	T	G
6.	JPN3	A	A	A	A	A	G	T	G
7.	JPN4	A	A	A	A	A	G	T	G
8.	JPN5	A	A	A	A	A	G	T	G
9.	JPN6	A	A	A	A	A	G	T	G
10.	JPN8	A	A	A	A	A	G	T	G
11.	JPN9	A	A	A	A	A	G	T	G
12.	KOR12	A	A	A	A	A	G	T	G
13.	KOR18	A	A	A	A	A	G	T	G
14.	KOR19	A	A	A	A	A	G	T	G
15.	KOR20	A	A	A	A	A	G	T	G
16.	CHI15	G	-	C	C	C	C	G	C

- Means nucleotide indel.

Table 5. Special variable nucleotide sites in the rDNA 18S gene sequences, mostly appearing between KOR5 and other varieties.

Sample No.	Specimen voucher	Variable nucleotide sites							
		777bp	790bp	795bp	804bp	810bp	822bp	895bp	1049bp
1.	KOR2	G	T	G	T	T	-	-	-
2.	KOR4	G	T	G	T	T	-	-	-
3.	KOR5	A	C	T	C	-	T	G	G
4.	JPN1	G	T	G	T	T	-	-	-
5.	JPN2	G	T	G	T	T	-	-	-
6.	JPN3	G	T	G	T	T	-	-	-
7.	JPN4	G	T	G	T	T	-	-	-
8.	JPN5	G	T	G	T	T	-	-	-
9.	JPN6	G	T	G	T	T	-	-	-
10.	JPN8	G	T	G	T	T	-	-	-
11.	JPN9	G	T	G	T	T	-	-	-
12.	KOR12	G	T	G	T	T	T	-	-
13.	KOR18	G	T	G	T	T	T	-	-
14.	KOR19	G	T	G	T	T	-	-	-
15.	KOR20	G	T	G	T	T	-	-	-
16.	CHI15	G	T	G	T	T	-	-	-

- Means nucleotide indel.

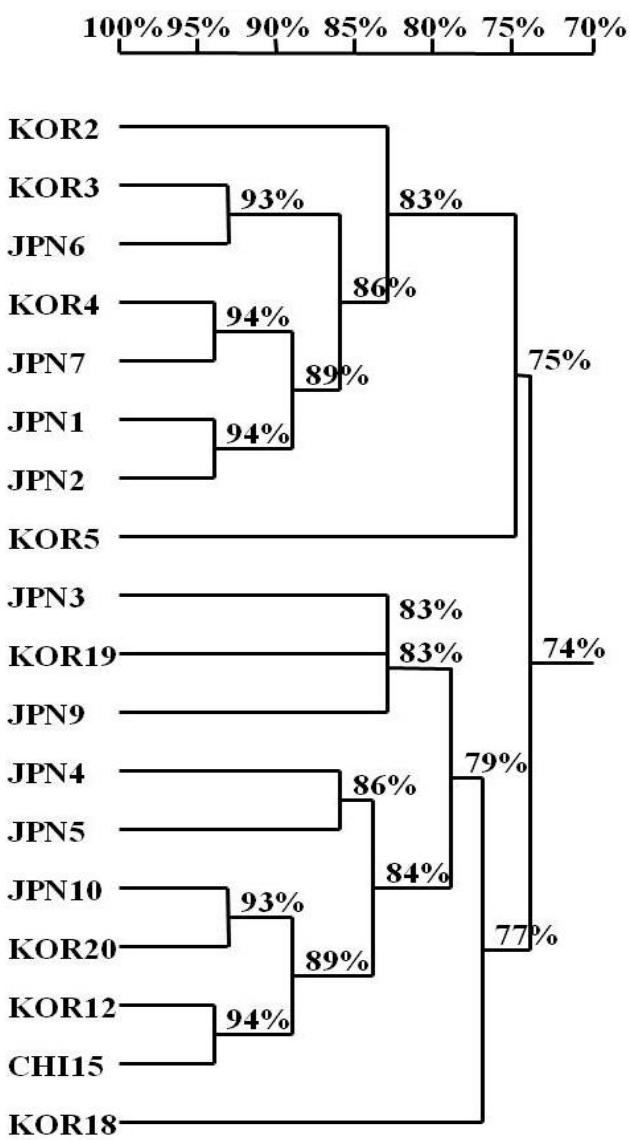

Fig. 1. Phylogenetic tree based on the rDNA 5S gene sequences of *Perilla* varieties.

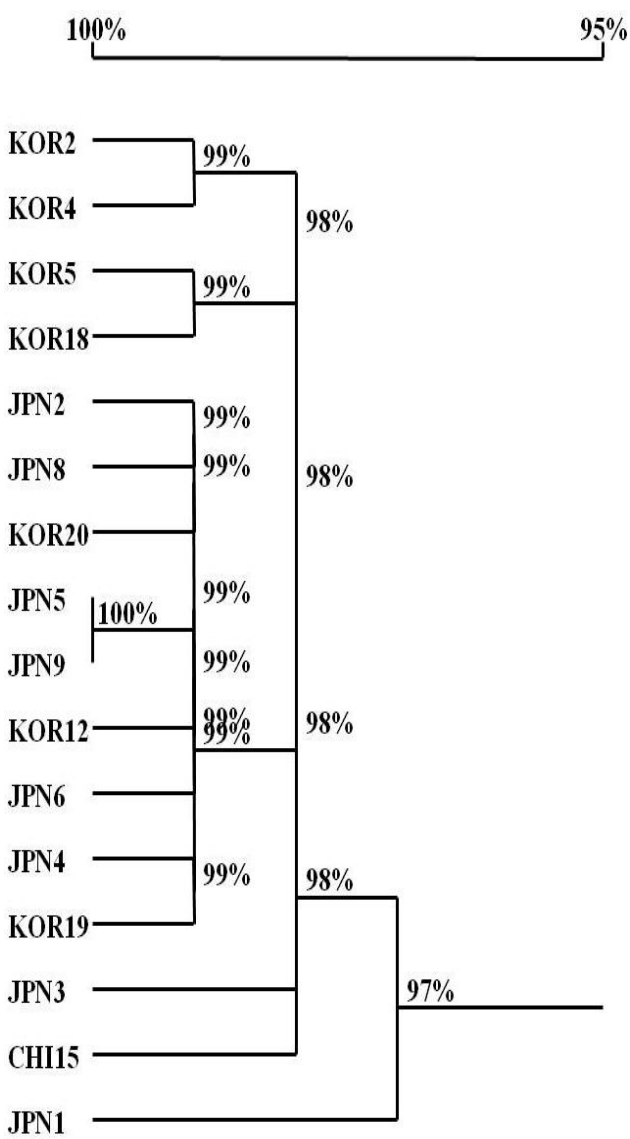

Fig. 2. Phylogenetic tree based on the rDNA 18S gene sequences of *Perilla* varieties.

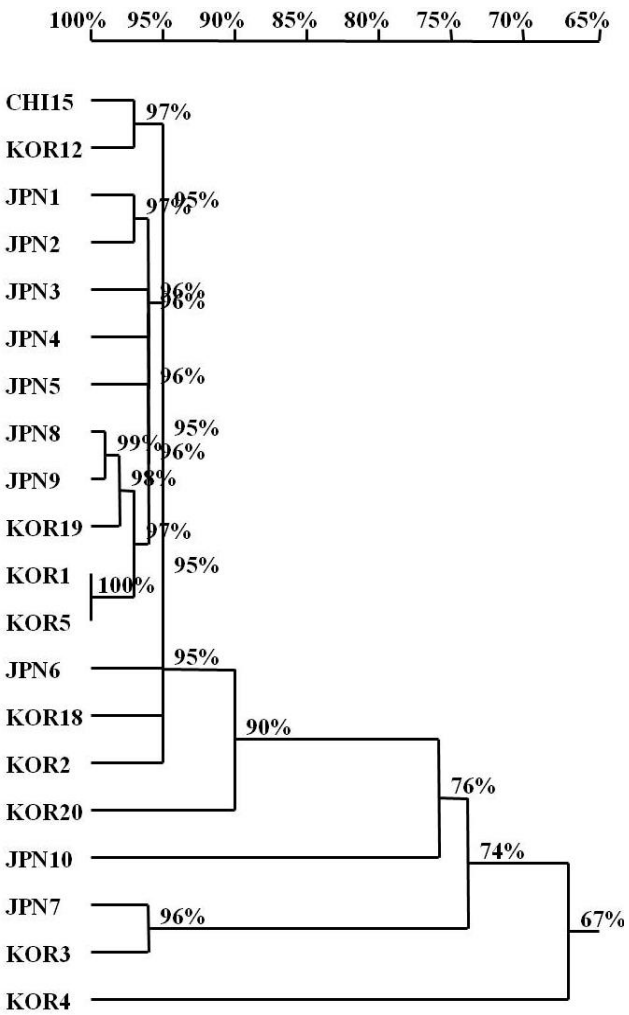

Fig. 3. Phylogenetic tree based on the combined DNA marker sequences of the nrDNA ITS, 18S and 5S rDNA regions.

Seen from the rDNA 5S phylogenetic tree, there were also two groups obtained (Fig. 2). One group was mainly consisted of var. *frutescens*, except JPN3, JPN4, and JPN5 were discriminated. JPN6 and JPN7, var. *crispa* cultivated type, were also included in this group. However, the weedy types of var. *crispa* were divided into the main var. *crispa* group, without exception, that again suggested that var. *crispa* showed less genetic variation than var. *frutescens*. This result was not consentient by the report of Sa *et al*. (2012), in which Sa *et al*. regarded the cultivated type of var. *frutescens* as a more domesticated type showing more genetic variations than the cultivated type of var. *crispa* based on the morphological characteristics. However, Sa *et al*. (2012) could not find concrete evidence between the weedy types of var. *frutescens* and var. *crispa*. The mixing between both varieties suggested that their phylogenetic relationship was so close, and only using rDNA 5S gene sequences might not estimate accurately.

In the 18S phylogenetic tree, JPN1 was the farthest from other varieties, that might be mainly caused by six special variable nucleotide sites appearing in the rDNA 18S gene sequences (Table 2, Fig. 2). The other Japanese *Perilla* species being the same variety with JPN1 showed dispersive distribution in the phylogenetic trees due to certain nucleotide variation (Figs. 1, 2). Among those varieties, JPN3 was the case, showing relatively far phylogenetic relationship from other Japanese var. *frutescens* varieties (Fig. 2). Besides, the Japanese var. *crispa* species also showed dispersive distribution in the phylogenetic trees (Figs. 1, 2), that suggested that inter-variety divergence was relatively obvious among the *P. frutescens* var. *crispa* varieties. This result was supported by the report of Sa *et al*. (2012). CHI15, being Chinese var. *crispa* weedy type, shared the second far phylogenetic relationship in the tree (Fig. 2), that was mainly caused by having eight special variable nucleotide sites between CHI15 and other varieties (Table 4). JPN5 and JPN9, being Japanese var. *frutescens* and var. *crispa* cultivated type, respectively, however, showed very high phylogenetic relationship in the tree (Fig. 2). KOR5 and KOR18, being Korean var. *frutescens* cultivated and var. *crispa* weedy type, respectively, also had close relationship, that might be caused by the nucleotide addition occurring at 822 bp (Fig. 2, Table 5). These results suggested that the mixing degree of both varieties was high, and only to use more samplings and more diversified identification means, we could further discriminate them clearly.

Combined with 3 rDNA marker sequences, we constructed a combined phylogenetic tree (Fig. 3) and calculated the genetic distance between each both varieties (Table 6). In the combined tree, Korean var. *frutescens* varieties showed dispersive distribution, however, Japanese var. *frutescens* varieties showed compact distribution. The genetic distance (GD) of var. *frutescens* strongly testified this conclusion, with mean intravariety GD within Korean accessions significantly higher than that within Japanese accessions (Table 7). This result proved the statement that Korea is the secondary habitat when *Perilla* crops was diffused or transferred from China or Japan and remained (Lee & Ohnishi, 2003), and so the Korean accessions showed abundant genetic diversity. Var. *crispa* varieties, irrespective of geographical region or cultivation type, showed dispersive distribution in the combined tree. The mean intravariety GD result of Japanese accessions also showed significant difference compared to that of var. *frutescens* (Table 7). This result strongly proved that var. *crispa* had high genetic variation than var. *frutescens*. Because the Korean var. *crispa* accessions investigated in this study were weedy types, so we did not use them to make comparison. And in previous studies, cultivated type of var. *frutescens* and of var. *crispa* could be sharply separated according to the AFLP result (Lee & Ohnishi, 2003), however, still occurring ambiguities of intraspecific relationships of the weedy types. Combined with GD of all *Perilla* accessions, the intervariety GD did not show significant difference, except of Korean var. *crispa* weedy accessions (Table 7). This result was directly related with the sampling situation of weedy types, so we considered the weedy types of Korean var. *crispa* were from only one accession. For Japanese accessions, intravariety GD of var. *frutescens* was significantly lower than that of var. *crispa*, but both were all not significantly different with intervariety GD (Table 7). Analyzed all *Perilla* accessions, the intra-variety and inte-rvariety GDs appeared similar level, and had no distinction of geographical region.

Table 6. Genetic distance of 20 *Perilla* accessions investigated in this study.

	KOR1	KOR2	KOR3	KOR4	KOR5	JPN1	JPN2	JPN3	JPN4	JPN5	JPN6	JPN7	JPN8	JPN9	JPN10	KOR12	KOR18	KOR19	KOR20	CHI15
KOR1	0																			
KOR2	0.002	0																		
KOR3	0.014	0.272	0																	
KOR4	0.014	0.367	0.265	0																
KOR5	0	0.064	0.268	0.374	0															
JPN1	0.009	0.053	0.267	0.37	0.065	0														
JPN2	0.012	0.047	0.269	0.367	0.057	0.028	0													
JPN3	0.011	0.064	0.273	0.362	0.076	0.06	0.049	0												
JPN4	0.015	0.063	0.274	0.367	0.074	0.051	0.042	0.036	0											
JPN5	0.009	0.061	0.264	0.362	0.071	0.046	0.035	0.044	0.036	0										
JPN6	0.009	0.055	0.264	0.36	0.059	0.046	0.041	0.061	0.058	0.05	0									
JPN7	0.011	0.271	0.04	0.266	0.27	0.264	0.267	0.271	0.273	0.267	0.266	0								
JPN8	0.011	0.021	0.267	0.294	0.019	0.022	0.019	0.015	0.018	0.015	0.016	0.268	0							
JPN9	0.006	0.069	0.264	0.361	0.079	0.066	0.057	0.044	0.056	0.052	0.064	0.266	0.011	0						
JPN10	0.014	0.253	0.303	0.254	0.255	0.259	0.254	0.259	0.261	0.25	0.25	0.305	0.255	0.256	0					
KOR12	0.014	0.061	0.271	0.363	0.071	0.05	0.042	0.049	0.044	0.033	0.054	0.27	0.017	0.047	0.255	0				
KOR18	0.011	0.075	0.269	0.362	0.085	0.07	0.061	0.059	0.064	0.055	0.07	0.267	0.017	0.038	0.257	0.049	0			
KOR19	0.009	0.065	0.266	0.359	0.07	0.053	0.045	0.042	0.047	0.04	0.054	0.268	0.014	0.034	0.252	0.044	0.044	0		
KOR20	0.011	0.115	0.264	0.369	0.129	0.122	0.126	0.106	0.114	0.115	0.118	0.265	0.02	0.112	0.251	0.122	0.113	0.12	0	
CHI15	0.015	0.066	0.268	0.363	0.077	0.056	0.051	0.063	0.055	0.045	0.06	0.271	0.022	0.057	0.248	0.028	0.056	0.052	0.124	0

Table 7. Statistical analysis of intravariety and intervariety genetic distance (GD) of var. *frutescens* and var. *crispa*.

Genetic distance of var. *frutescens*								
Korean accessions			Japanese accessions			Entire accessions		
Minium	Maximum	Intravariety	Minium	Maximum	Intravariety	Minium	Maximum	Intravariety
0	0.374	0.164aA	0.028	0.051	0.0427bB	0	0.374	0.1318aA
Genetic distance of var. *crispa*								
Korean accessions			Japanese accessions			Entire accessions		
Minium	Maximum	Intravariety	Minium	Maximum	Intravariety	Minium	Maximum	Intravariety
0.044	0.257	0.0592bB	0.011	0.305	0.1957aA	0.011	0.305	0.1344aAB
Genetic distance of *Perilla frutescens*								
Korean accessions			Japanese accessions			Entire accessions		
Minium	Maximum	Intervariety	Minium	Maximum	Intervariety	Minium	Maximum	Intervariety
0.009	0.369	0.16195aA	0.015	0.273	0.1298abA	0.006	0.369	0.1296aA

The values of intravariety and intervariety GD were mean GD calculated by sum of individual GD divide by number of samples. The same letter followed by the intravariety and intervariety genetic distances means no significantly different at $p<0.05$ according to two-way ANOVA using Duncan's multiple-range test, and the different letter means significant difference. The upper case means statistical result of intra-variety GD, while the lower-case letter means statistical result of inter-variety GD

Conclusion

Three rDNA markers used in this study, though, could not obviously separate the cultivated types of both varieties, still provided more evidences to evaluate genetic relationships of *Perilla* crops. Our result of the investigated accessions will be used to help functional plant breeding and trait improvement in *Perilla* crop.

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References

Bleidorn, C., L. Vogt and T. Bartolomaeus. 2003. New insights into polychaete phylogeny (Annelida) inferred from 18S rDNA sequences. *Mol. Phylogenet. Evol.*, 29: 279-288.

Campell, B.R., Y. Song, T.E. Posch, C.A. Cullis and C.D. Town. 1992. Sequence and organization of 5S ribosomal RNA-encoding genes of Arabidopsis thaliana. *Gene*, 112: 225-228.

Coleman, A.W. 2003. ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends Genet.*, 19: 370-375.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.*, 19: 11-15.

Duncan, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics*, 11: 1-42.

Harlan, J.R. 1975. Our vanishing genetic resources. *Science*, 188: 618-621.

Hizume, M. 1993. Chromosomal localization of 5S rRNA genes in *Vicia faba* and *Crepis capillaries*. *Cytologia*, 58: 417-421.

Honda, G., A. Yuba, T. Kojima and M. Tabata. 1996. Chemotaxonomic and cytogenetic studies on *Perilla frutescens* var. citrodora (*Lemon egoma*). *Nat. Med.*, 48: 185-190.

Honda, G., Y. Koezaka and M. Tabata. 1994. Genetic studies of colour and hardness in *P. frutescens*. *Jpn. J. Breed.*, 40: 469-474.

Jan, S.A., Z.K. Shinwari, M.A. Rabbani, I.A. Niaz and S.H. Shah. 2017. Assessment of quantitative agro-morphological variations among *Brassica rapa* diverse populations. *Pak. J. Bot.*, 49(2): 561-567.

Jan, S.A., Z.K. Shinwari, M.A. Rabbani. 2016. Determining genetic divergence among *Brassica rapa* ecotypes through electrophoretic mobility of total seed proteins. *The J. Anim. Plant Sci.*, 26(6): 1758-1764.

Joshi, K., P. Chavan, D. Warude and B. Patwardhan. 2004. Molecular markers in herbal drug technology. *Curr. Sci.*, 87: 159-165.

Kwon, S.J., J.K. Lee, N.S. Kim, J.W. Yu, A. Dixit, E.G. Cho and Y.J. Park. 2005. Isolation and characterization of SSR markers in *Perilla frutescens* Britt. *Mol. Eco. Notes*, 5: 454-456.

Lee, J.K. and O. Ohnishi. 2001. Geographical differentiation of morphological characters among *Perilla* crops and their weedy types in East Asia. *Breed. Sci.*, 51: 247-255.

Lee, J.K. and O. Ohnishi. 2003. Genetic relationships among cultivated types of *Perilla frutescens* and their weedy types in East Asia revealed by AFLP markers. *Genet. Resour. Crop Evol.*, 50: 65-74.

Mahmood, T., S. Muhammad and Z.K. Shinwari. 2010. Molecular and morphological characterization of *Caralluma* species. *Pak. J. Bot*., 42(2): 1163-1171.

Meyer, C.P. and G. Paulay. 2005. DNA barcoding: error rates based on comprehensive sampling. *PLoS Biol.*, 3: e422.

Nitta, M. and O. Ohnishi. 1999. Genetic relationships among two *Perilla* crops, shiso and egoma, and the weedy type revealed by RAPD markers. *Gen. Genet. Syst.*, 74: 43-48.

Nitta, M., J.K. Lee and O. Ohnishi. 2003. Asian *Perilla* crops and their weedy forms: their cultivation, utilization and genetic relationships. *Econ. Bot.*, 57: 245-253.

Pandey, A. and K.C. Bhatt. 2008. Diversity distribution and collection of genetic resources of cultivated and weedy type in *Perilla frutescens* (L.) Britton var. *frutescens* and their uses in Indian Himalaya. *Genet. Resour. Crop Ev.*, 55: 883-892.

Park, Y.J., A. Dixit, K.H. Ma, J.K. Lee, M.H. Lee, C.S. Chung, M. Nitta, K. Okuno, T.S. Kim, E.G. Cho and V.R. Rao. 2008. Evaluation of genetic diversity and relationships within an on-farm collection of *Perilla frutescens* (L.) Britt. using microsatellite markers. *Genet. Res. Crop Ecol.*, 55: 523-535.

Persson, C. 2000. Phylogeny of the neotropical Alibertia group (Rubiaceae), with emphasis on the genus *Alibertia*, inferred from ITS and 5S ribosomal DNA sequences. *Am. J. Bot.*, 87: 1018-1028.

Pervaiz, Z.H., M.A. Rabbani, Z.K. Shinwari, M.S. Masood and S.A. Malik. 2010. Assessment of genetic variability in Rice (*Oryza sativa* L.) germplasm from Pakistan using RAPD markers. *Pak. J. Bot*., 42(5): 3369-3376.

Rabbani, M.A., M.S. Masood, Z.K. Shinwari and K.Y. Shinozaki. 2010. Genetic analysis of Basmati and non-Basmati Pakistani Rice (*Oryza sativa* L.) cultivars using microsatellite markers. *Pak. J. Bot*., 42(4): 2551-2564.

Sa, K.J., J.A. Kim and J.K. Lee. 2012. Comparison of seed characteristics between the cultivated and the weedy types of *Perilla* species. *Hortic. Environ. Biotechnol*., 53: 310-315.

Sastri, D.C, K. Hilu, R. Appels, E.S. Lagudah, J. Playford and B.R. Baum 1992. An overview of evolution in plant 5S DNA. *Plant Syst. Evol*., 183: 169-181.

Shinwari, S., F. Akbar, M.A. Rabbani, A.S. Mumtaz and Z.K. Shinwari. 2013. Evaluation of genetic diversity in different genotypes of *Eruca sativa* from Pakistan by SDS-Page analysis. *Pak. J. Bot.*, 45(4): 1235-1240.

Shinwari, Z.K., H. Rehman and M.A. Rabbani. 2014. SDS-Page based genetic divergence in Safflower (*Carthamus tinctorius* L.). *Pak. J. Bot.*, 46(3): 811-815.

Shinwari, Z.K., S. Sultan and T. Mehmood. 2011. Molecular and morphological characterization of selected *Mentha* species. *Pak. J. Bot*., 43(3): 1433-1436.

Sogin, M.L. 1990. Amplification of ribosomal RNA genes for molecular evolution studies. In: *PCR protocols, a guide to methods and applications*. (Eds.): Innis, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White.Academic Press, New York, pp. 307-322.

Soltis, D.E., P.S. Soltis, M.W. Chase, M.E. Mort, D.C. Albach, M. Zanis, V. Savolainen, W.H. Hahn, S.B. Hoot, M.F. Fay, M. Axtell, S.M. Swensen, L.M. Prince, W.J. Kress, K.C. Nixon and J.S. Farris 2000. Angiosperm phylogeny inferred from 18S rDNA, rbcL, and atpB sequences. *Bot. J. Linn. Soc*., 133: 381-461.

Sultan, M., N. Zakir, M.A. Rabbani, Z.K. Shinwari and M.S. Masood. 2013. Genetic diversity of guar (*Cyamopsis tetragonoloba* L.) landraces from Pakistan based on RAPD markers. *Pak. J. Bot.*, 45(3): 865-870.

Turi, N. A., Farhatullah, M.A. Rabbani and Z.K. Shinwari. 2012. Genetic diversity in the locally collected *Brassica* species of Pakistan based on microsatellite markers. *Pak. J. Bot*., 44(3): 1029-1035.

Villa, T.C.C., N.Maxted, M. Scholten and B. Ford Lloyd. 2006. Defining and identifying crop landraces. *Plant Genet. Resour.*, 3: 373-384.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res*., 18: 7213-7218.

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

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.*, 18: 6531-6535.

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