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Genetic diversity and population structure analysis in *Perilla frutescens* from Northern areas of China based on simple sequence repeats

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ABSTRACT. In this study, 21 simple sequence repeat (SSR) markers were used to evaluate the genetic diversity and population structure among 77 *Perilla* accessions from high-latitude and middle-latitude areas of China. Ninety-five alleles were identified with an average of 4.52 alleles per locus. The average polymorphic information content (PIC) and genetic diversity values were 0.346 and 0.372, respectively. The level of genetic diversity and PIC value for cultivated accessions of *Perilla frutescens* var. *frutescens* from middle-latitude areas were higher than accessions from high-latitude areas. Based on the dendrogram of unweighted pair group method with arithmetic mean (UPGMA), all accessions were classified into four major groups with a genetic similarity of 46%. All accessions of the cultivated var. *frutescens* were discriminated from the cultivated *P. frutescens* var. *crispa*. Furthermore, most accessions of the cultivated var. *frutescens* collected in high-latitude and middle-latitude areas were distinguished depending on their geographical location. However, the geographical locations of several accessions of the cultivated var. *frutescens* have no relation with

their positions in the UPGMA dendrogram and population structure. This result implies that the diffusion of accessions of the cultivated *Perilla* crop in the northern areas of China might be through multiple routes. On the population structure analysis, 77 *Perilla* accessions were divided into Group I, Group II, and an admixed group based on a membership probability threshold of 0.8. Finally, the findings in this study can provide useful theoretical knowledge for further study on the population structure and genetic diversity of *Perilla* and benefit for *Perilla* crop breeding and germplasm conservation.

Key words: *Perilla frutescens*; Genetic similarity; SSR marker; UPGMA; Geographical location; Polymorphic information content

INTRODUCTION

Perilla frutescens (L.) Britt. is a self-fertilizing crop in the family of mint, Lamiaceae. Depending on their morphological characteristics and availability, this species is divided into two cultivated types. One is *P. frutescens* var. *frutescens* that is a kind of oil crop. It is called by different names in East Asia countries, such as Ren in China, Dlggae in Korea, and Egoma in Japan. The other is *P. frutescens* var. *crispa*, a Chinese medicine or vegetable crop that is called Zisu in Chinese, Cha-jo-ki in Korean, and Shiso in Japanese (Lee and Ohnishi, 2003). *P. frutescens* var. *frutescens* is also used as a vegetable only in Korea, and its seeds are traditionally used in the same way that sesame seeds are used as a seasoning in China, Korea, and Japan from old times. On the other hand, *P. frutescens* var. *crispa* is used as Chinese medicine in East Asia from old times and is used as a spicy vegetable or pickle crop in Japan. Thus, these two cultivated types of *Perilla* crop have been important in East Asia from ancient times (Lee and Ohnishi, 2003; Nitta et al., 2003).

Since *Perilla* crop has been extensively cultivated and used in East Asia from old times, the original birth place of *Perilla* crop has generally been considered as East Asia (Makino et al., 1961; Li, 1969; Nitta, 2001) although the wild ancestor of cultivated types of *Perilla* crop has not yet been identified in East Asia. In East Asia, the cultivated var. *frutescens* is currently the most grown and is used as both an oil crop and a leafy vegetable in Korea. However, the cultivated var. *crispa* is not currently being cultivated because of the decreased use of Chinese medicine, although it is occasionally found as a relict form in Korea (Lee and Ohnishi, 2001; Lee et al., 2002). Conversely, the cultivated var. *crispa* is extensively cultivated and used in Japan, while the cultivated var. *frutescens* is rarely cultivated in Japan. On the other hand, the cultivated var. *frutescens* is grown in the northern area of China. Likewise, the cultivated var. *crispa* is not currently being cultivated in China. Because there have been very few efforts to improve *Perilla* crop through breeding programs, many genotypes of *Perilla* crop still occur as landraces in farmers' fields in East Asia (Lee et al., 2002). A landrace may be defined as active population(s) of a cultivated crop that has a distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted, and associated with traditional farming systems (Harlan, 1975; Villa et al., 2006). Landraces may evolve through natural hybridization with closely wild or weedy relatives, and farmers play a critical role in selecting and adapting new materials using traditional skills and genetic resources (Zeven, 1998).

Previously, various molecular markers have provided useful information regarding genetic diversity and genetic relationships in many crops (Senior et al., 1998; Nitta and Ohnishi, 1999; Prasad et al., 2000; Lee et al., 2002; Hamza et al., 2004; Xia et al., 2005; Lee and Kim, 2007; Sa et al., 2013, 2015; Park et al., 2002, 2015). Among them, random amplified polymorphic DNA or RAPD (Nitta and Ohnishi, 1999), amplified fragment length polymorphism or AFLP (Lee et al., 2002; Lee and Ohnishi, 2003), and simple sequence repeats or SSR (Lee and Kim, 2007; Park et al., 2008; Sa et al., 2013, 2015) markers have been applied in the analysis of genetic diversity and relationships among cultivated and weedy types of *Perilla* crop in East Asia and other countries. Particularly, SSR markers are highly reproducible, polymorphic, generally codominant, and abundant in plant genomes (Powell et al., 1996; Park et al., 2009). As a result of these better features, SSRs have been used to establish genetic diversity and genetic relationships in many crop species (da Cunha et al., 2014; Liu et al., 2014; Yook et al., 2014).

Identification of genetic variation is an essential ability for the long-term success of breeding programs and maximizes the use of germplasm resources (Rao, 2004). China has a long history of the cultivation of *Perilla* crop in East Asia, but there has been little research on the genetic diversity of accessions of *Perilla* crop. Therefore, the objective of this study was to establish the genetic diversity of *Perilla* crop from the northern areas of China based on SSR markers. The findings in this study can play an important role in the protection and conservation of accessions of *Perilla* species in China.

MATERIAL AND METHODS

Plant materials and DNA extraction

Seventy-seven accessions of *P. frutescens* were collected in different provinces of China in the year 2015. The accession numbers and localities of *Perilla* crop are shown in Table 1 and Figure 1. Seventy-seven accessions included 54 accessions of the cultivated type of the var. *frutescens* from high-latitude areas (northeastern regions of China) and 23 accessions of the cultivated type of the var. *frutescens* from middle-latitude areas (Northwest and North China). Only 3 accessions of the cultivated type of the var. *crispa* were collected in this study. The subset of each collection was deposited in the National Agrobiodiversity Center, Rural Development and Administration, Jeonju, Republic of Korea, for permanent seed preservation. These *Perilla* seeds collected were germinated on Petri dishes. The germinated seeds were sown in a nursery in May of 2016. After seedlings have come out, they were transplanted into the farm of Kangwon National University. Total DNA was extracted from the leaf tissues of a representative individual plant for each accession following the Plant DNAzol Reagent protocol (GibcoBRL Inc., Grand Island, NY, USA).

SSR analysis and silver staining

SSR amplifications were conducted in a total volume of 20 μL consisting of 20 ng genomic DNA, 1X PCR buffer, 0.5 μM forward and reverse primers, 0.2 mM dNTPs, and 1 U Taq polymerase (Biotools, Spain). The PCR profile consisted of initial denaturation at 95°C for 3 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min 30 s, with a final extension step of 5 min at 72°C. After PCR, 5 μL the final products were mixed

Table 1. *Perilla* accessions from different regions of China used for SSR analysis.

Code No.	Accession No.	City and Province	Country	Type
1	CH1	Harbin, Hei Longjiang	CHN	Cultivated type of var. *frutescens*
2	CH3	Harbin, Hei Longjiang	CHN	Cultivated type of var. *frutescens*
3	CH35	Hailin, Hei Longjiang	CHN	Cultivated type of var. *frutescens*
4	CH38	Jiamusi, Hei Longjiang	CHN	Cultivated type of var. *frutescens*
5	CH46	Heihe, Hei Longjiang	CHN	Cultivated type of var. *frutescens*
6	CH56	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
7	CH58	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
8	CH60	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
9	CH62	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
10	CH63	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
11	CH64	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
12	CH65	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
13	CH66	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
14	CH67	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
15	CH68	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
16	CH69	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
17	CH70	Helong, Jilin	CHN	Cultivated type of var. *frutescens*
18	CH71	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
19	CH72	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
20	CH73	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
21	CH75	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
22	CH78	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
23	CH79	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
24	CH45	Yanji, Jilin	CHN	Cultivated type of var. *frutescens*
25	CH80	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
26	CH81	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
27	CH82	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
28	CH83	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
29	CH85	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
30	CH86	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
31	CH87	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
32	CH88	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
33	CH16	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
34	CH17	Longjing, Jilin	CHN	Cultivated type of var. *frutescens*
35	CH20	Longjing, Jilin	CHN	Cultivated type of var. frutescens
36	CH22	Longjing, Jilin	CHN	Cultivated type of var. frutescens
37	CH23	Longjing, Jilin	CHN	Cultivated type of var. frutescens
38	CH13	Changchun, Jilin	CHN	Cultivated type of var. frutescens
39	CH24	Baishan, Jilin	CHN	Cultivated type of var. frutescen
40	CH32	Changbai, Jilin	CHN	Cultivated type of var. *frutescens*
41	CH33	Tonghua, Jilin	CHN	Cultivated type of var. *frutescens*
42	CH43	Tonghua, Jilin	CHN	Cultivated type of var. *frutescens*
43	CH14	Siping, Jilin	CHN	Cultivated type of var. *frutescens*
44	CH42	Jilin, Jilin	CHN	Cultivated type of var. *frutescens*
45	CH27	Jilin, Jilin	CHN	Cultivated type of var. *frutescens*
46	CH28	Jilin, Jilin	CHN	Cultivated type of var. *frutescens*
47	CH4	Shenyang, Liaoning	CHN	Cultivated type of var. *frutescens*
48	CH5	Shenyang, Liaoning	CHN	Cultivated type of var. *frutescens*
49	CH7	Liaoyang, Liaoning	CHN	Cultivated type of var. *frutescens*
50	CH8	Liaoyang, Liaoning	CHN	Cultivated type of var. *frutescens*
51	CH11	Tianjin	CHN	Cultivated type of var. *frutescens*
52	CH34	Zhengzhou, Henan	CHN	Cultivated type of var. *frutescens*
53	CH37	Tianshui, Gansu	CHN	Cultivated type of var. *frutescens*
54	CH30	Tianshui, Gansu	CHN	Cultivated type of var. *frutescens*
55	CH39	Tianshui, Gansu	CHN	Cultivated type of var. *frutescens*
56	CH49	Pingliang, Gansu	CHN	Cultivated type of var. *frutescens*
57	CH55	Longnan, Gansu	CHN	Cultivated type of var. *frutescens*
58	CH40	Longnan, Gansu	CHN	Cultivated type of var. *frutescens*
59	CH29	Longnan, Gansu	CHN	Cultivated type of var. *frutescens*
60	CH51	Qingyang, Gansu	CHN	Cultivated type of var. *frutescens*
61	CH41	Haozhou, Anhui	CHN	Cultivated type of var. *frutescens*
62	CH44	Haozhou, Anhui	CHN	Cultivated type of var. *frutescens*
63	CH50	Suqian, Jiangsu	CHN	Cultivated type of var. *frutescens*
64	CH31	Huai'an, Jiangsu	CHN	Cultivated type of var. *frutescens*
65	CH26	Jilin, Jilin	CHN	Cultivated type of var. *frutescens*
66	CH12	Changchun, Jilin	CHN	Cultivated type of var. *frutescens*
67	CH6	Shenyang, Liaoning	CHN	Cultivated type of var. *frutescens*
68	CH10	Anguo, Hebei	CHN	Cultivated type of var. *frutescens*
69	CH48	Tianshui, Gansu	CHN	Cultivated type of var. *frutescens*
70	CH9	Cangzhou, Hebei	CHN	Cultivated type of var. *frutescens*
71	CH25	Weifang, Shandong	CHN	Cultivated type of var. *frutescens*
72	CH36	Zhengzhou, Henan	CHN	Cultivated type of var. *frutescens*
73	CH47	Baoding, Hebei	CHN	Cultivated type of var. *frutescens*
74	CH52	Yantai, Shandong	CHN	Cultivated type of var. *crispa*
75	CH2	Harbin, Hei Longjiang	CHN	Cultivated type of var. *crispa*
76	CH54	Zhaoyuan, Shandong	CHN	Cultivated type of var. *crispa*
77	CH53	Zhaoyuan, Shandong	CHN	Cultivated type of var. *crispa*

with 10 µL electrophoresis loading buffer (98% formamide, 0.02% BPH, 0.02% Xylene C, and 5 mM NaOH). After denaturing and quick cooling, 2 µL of each sample was loaded onto 6% denaturing (7.5 M urea) acrylamide-bisacrylamide gel (19:1) in 1X TBE buffer and

then electrophoresed at 1800 V and 60 W for 130 min. The separated fragments were then visualized using a silver staining kit (Promega, Madison, WI, USA).

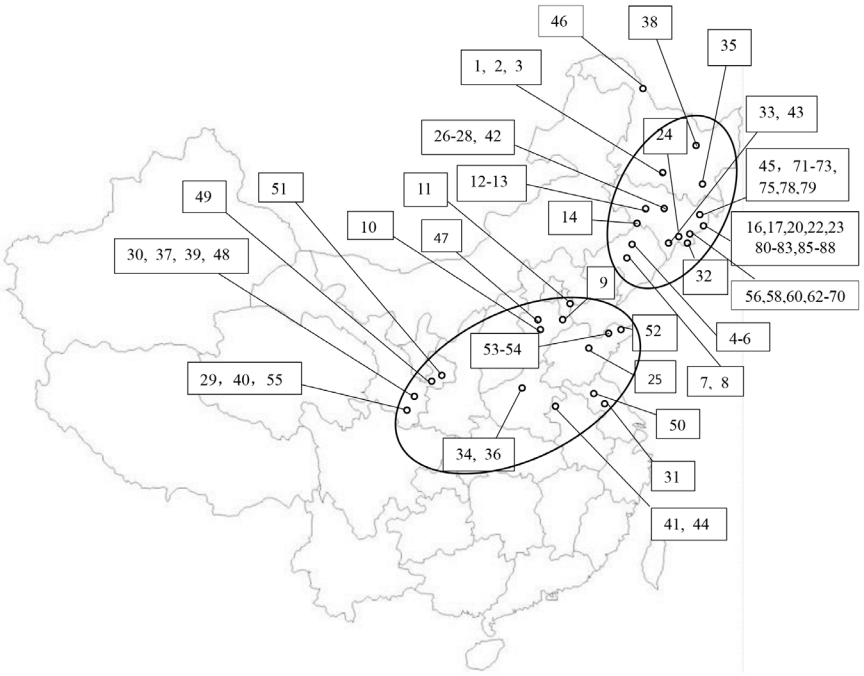

Figure 1. Collecting sites of accessions of cultivated types of *Perilla* crop were evaluated in this study. *Perilla* accessions collected from high-latitude area and middle-latitude area were respectively shown in the upper and lower oval circles.

Data analysis

Fragments amplified using the SSR primers were scored as presence (1) or absence (0). Power Marker version 3.25 (Liu and Muse, 2005) was applied to obtain the information on the number of alleles, allele frequency, major allele frequency (MAF), gene diversity (GD), and polymorphic information content (PIC). Genetic similarities were calculated for each pair of lines using the Dice similarity index (Dice, 1945). To illustrate the genetic relationship of samples, the similarity matrix was then used to construct an unweighted pair group method with arithmetic mean (UPGMA) dendrogram by the application of SAHN-Clustering from NTSYS-pc.V.2.1 (Rohlf, 1998). The STRUCTURE 2.2 software (Pritchard and Wen, 2003) was used for the investigation of a population structure of 77 accessions of *Perilla* crop. To identify the number of clusters (K), the software was run using admixture model with correlated allele frequencies following Hardy-Weinberg equilibrium. Five independent runs were performed for each simulated value of K = 1 to 10, with a burn-in period of 100,000 runs followed by 100,000 Monte Carlo Markov Chain (MCMC) replications. The real number of population was detected by plotting Ln(PD) derived ΔK, an *ad hoc* quantity against each K (Evanno et al., 2005). The result was computed and visualized using the Structure Harvester software (http://taylor0.biology.ucla.edu/struct_harvest/).

RESULTS

SSR polymorphism and genetic variation among *Perilla* accessions from different regions of China

The genetic variability at each SSR locus was measured regarding the number of alleles, MAF, GD, and PIC (Table 2). A total of 21 SSR loci showed polymorphism, producing a total of 95 alleles among the 77 accessions of *Perilla* crop. The number of alleles per locus varied widely from 2 at GBPF201, KWPE32, and KWPE56 to 11 at GBPF204, with an average of 4.52 alleles. The MAF per locus varied from 0.442 (GBPF204) to 0.948 (GBPF201, GBPF91), with an average of 0.746. The GD of each locus ranged from 0.098 at GBPF201 to 0.741 at GBPF204, with an average of 0.372 (Table 2). The PIC values ranged from 0.094 at GBPF201 to 0.714 at GBPF204, with an average of 0.346 (Table 2).

Table 2. Characteristics of the 21 SSR loci including primer sequence, repeat motif, allele size range, allele numbers, major allele frequency, gene diversity, and PIC among 77 accessions of *Perilla* crop from different regions of China.

SSR loci	Primer sequence	Repeat motif	Allele size (bp)	No. of alleles	MAF	GD	PIC
GBPF201	F-AGACTCGTTTCACAATTTCTCC R-CATTCCCACCTCATGTTACG	$(GA)_8(GT)$ $(GA)_5$	150-155	2	0.948	0.098	0.094
GBPF179	F-TGAATCATCCCAAACGAGAT R-TCGCTTCTCTCTCATGGATT	$(TGA)_5$	170-182	5	0.623	0.565	0.528
GBPF172	F-ATCGGTCTTTGAAATCACCA R-TGAAATTTCTTGCCGTTACC	$(GA)_{11}$	260-276	3	0.636	0.517	0.452
GBPF135	F-CTTCTGAGGCCAACATTGAG R-AGGGCTCGGTTGAATCTTAC	$(CT)_{20}$	190-216	5	0.909	0.171	0.168
GBPF75	F-CATAGTTCATGGCTTCCACC R-CCTGAGCACAGAAACAGATCA	$(CT)_{12}$	147-153	3	0.922	0.146	0.141
GBPF70	F-CCCTCCAAATCAATATTCCA R-TAGCTGCCATACGAACATGA	$(ATTTG)_3,(AC)_5$	215-240	4	0.922	0.148	0.144
KWPE25	F-ACATTTAAGAGAGAGAGCAAG R-ACGAACGGGCTTCAATCTT	$[(GT)_8(GA)_{14}]$	213-220	3	0.896	0.191	0.182
KWPE26	F-GAGGCAATGCTGGTACTTC R-GAACGGGCTTCAATCTTC	$[(AG)_6(AG)_7(GA)_{13}]$	235-245	3	0.909	0.169	0.162
KWPE32	F-AGAACAACATTGTAGCTCGG R-ACGACCAACCAGTAGATGAT	$(CCT)_4$	148-154	2	0.883	0.206	0.185
KWPE39	F-AGAACAACATTGTAGCTCGG R-GACGAACCAGCAAACGAC	$(CCT)_4$	330-400	4	0.545	0.598	0.533
KWPE48	F-CACCCCATCTTTTTGGAT R-AGCAGGATGGTGGTGGTC	$(GA)_9$	215-226	3	0.896	0.192	0.183
KWPE57	F-ATCACATCTCTCTCTTTCTGGA R-CCAGTCACTCCATCATCTCTA	$(CT)_{16}$	155-181	8	0.468	0.721	0.692
KWPE56	F-AAGCAGTGGACTGATTGTTT R-ACAAAATCCAATTACTTTCTGC	$(TG)_9$	105-107	2	0.506	0.500	0.375
KWPE53	F-ACTCACCAGAAGAGAAGAAGA R-GCCACTGACCTGTTAATATCTG	$(CT)_{16}$	194-224	6	0.667	0.512	0.482
KWPE58	F-AGAGAGTTACCTGCGATTTTC R-CTTCAATATTCGGCCATCTT	$(TG)_9(AG)_{12}$	163-175	5	0.870	0.238	0.232
KWPE19	F-CAACCCTTCACGATCACTAT R-AAATAACGGCCGATTCTAC	$(ACG)_7$	250-271	6	0.688	0.498	0.471
KWPE29	F-AAGACAAGGAGGAAGATGC R-ATAGGTGTTCGCTCTCCTGTG	$(GAA)_5$	210-245	7	0.532	0.664	0.632
GBPF111	F-ATCATGGATGAATCGCACTT R-CATTCTCCAAATGTTACTCTATTT	$(ACACA)_8$	161-200	6	0.857	0.259	0.250
GBPF155	F-TTTGTGACAATACGCACCAC R-CCAATTGCTCAATGCTCTCT	$(GAA)_{10}$	281-305	4	0.597	0.587	0.545
GBPF204	F-TCGAAAAATTGCAGATCACC R-TTGTCTTTTGCCTCTTTTGC	$(AG)_{17}$	130-144	11	0.442	0.741	0.714
GBPF91	F-CCACTCAAATCCGCTTCTAA R-AATGTTGGTTGCGTTTCATT	$(AG)_9$	221-230	3	0.948	0.100	0.096
Average				4.52	0.746	0.372	0.346

MAF: major allele frequency; GD: genetic diversity; PIC: polymorphic information content.

To compare the genetic diversity of the cultivated var. *frutescens* between high-latitude (53 accessions) and middle-latitude (20 accessions) regions in northern China, we also measured the number of alleles, MAF, GD, and PIC among the 73 accessions of the cultivated var. *frutescens* (Table 3). The average number of alleles was 3.52 and 3.19 alleles for accessions of the cultivated var. *frutescens* from high-latitude and middle-latitude areas in northern China, respectively. The average GD was 0.266 and 0.410 for accessions of the cultivated var. *frutescens* from high-latitude and middle-latitude areas in northern China, respectively. Finally, the average PIC values were 0.246 and 0.373 for accessions of the cultivated var. *frutescens* from high-latitude and middle-latitude areas in northern China, respectively (Table 3). These results show that accessions of the cultivated var. *frutescens* from middle-latitude area exhibited higher GD and PIC values than those of the cultivated var. *frutescens* from high-latitude areas.

Table 3. Number of alleles, genetic diversity, and polymorphic information content obtained from each SSR locus in accessions of the cultivated var. *frutescens* collected from high-latitude and middle-latitude areas of China.

Markers	Accessions of cultivated var. *frutescens* (N = 53, high-latitude areas)			Accessions of cultivated var. *frutescens* (N = 20, middle-latitude areas)		
	No. of alleles	GD	PIC	No. of alleles	GD	PIC
GBPF201	2	0.073	0.070	2	0.180	0.164
GBPF179	5	0.479	0.446	4	0.570	0.492
GBPF172	2	0.282	0.242	3	0.595	0.528
GBPF135	2	0.037	0.036	3	0.265	0.247
KWPE25	2	0.073	0.070	2	0.255	0.222
KWPE26	2	0.037	0.036	2	0.255	0.222
KWPE32	2	0.229	0.203	2	0.180	0.164
KWPE39	4	0.589	0.539	3	0.515	0.424
KWPE48	3	0.206	0.195	2	0.180	0.164
KWPE57	6	0.559	0.534	8	0.800	0.777
KWPE56	2	0.478	0.364	2	0.420	0.332
KWPE70	2	0.073	0.070	2	0.095	0.090
KWPE53	5	0.384	0.362	5	0.665	0.604
KWPE58	3	0.142	0.136	3	0.265	0.247
KWPE19	5	0.329	0.312	5	0.745	0.708
GBPF75	1	0.000	0.000	2	0.180	0.164
KWPE29	7	0.538	0.515	4	0.720	0.666
GBPF91	2	0.037	0.036	1	0.000	0.000
GBPF204	9	0.634	0.603	5	0.765	0.729
GBPF111	4	0.109	0.107	3	0.340	0.314
GBPF155	4	0.300	0.286	4	0.625	0.578
Mean	3.52	0.266	0.246	3.19	0.410	0.373

GD: genetic diversity; PIC: polymorphic information content.

Genetic relationships among accessions of *Perilla* crop from different regions of China

The phylogenetic tree was constructed using UPGMA and revealed that 77 *Perilla* accessions were clustered into four major groups with a genetic similarity of 46% (Figure 2). Group I contained 69 accessions of the cultivated var. *frutescens*. Group II contained 2 accessions of the cultivated var. *frutescens* and 1 accession of the cultivated var. *crispa* (CH53). Group III contained only 2 accessions of the cultivated var. *frutescens*. Group IV contained only 3 accessions of the cultivated var. *crispa*. Besides, accessions in Group I were further subdivided into four sub-clusters with a genetic similarity of 60%. The first sub-cluster contained 4 accessions of the cultivated var. *frutescens* from high-latitude areas and 1 accession of the cultivated var. *frutescens* from middle-latitude areas. The second sub-cluster contained

56 accessions of the cultivated var. *frutescens* from high-latitude (47 accessions) and middle-latitude (9 accessions) areas. The third sub-cluster contained 5 accessions of the cultivated var. *frutescens* from middle-latitude areas and 1 accession of the cultivated var. *frutescens* from high-latitude areas. The fourth sub-cluster contained 2 accessions of the cultivated var. *frutescens* from high-latitude and middle-latitude areas, respectively.

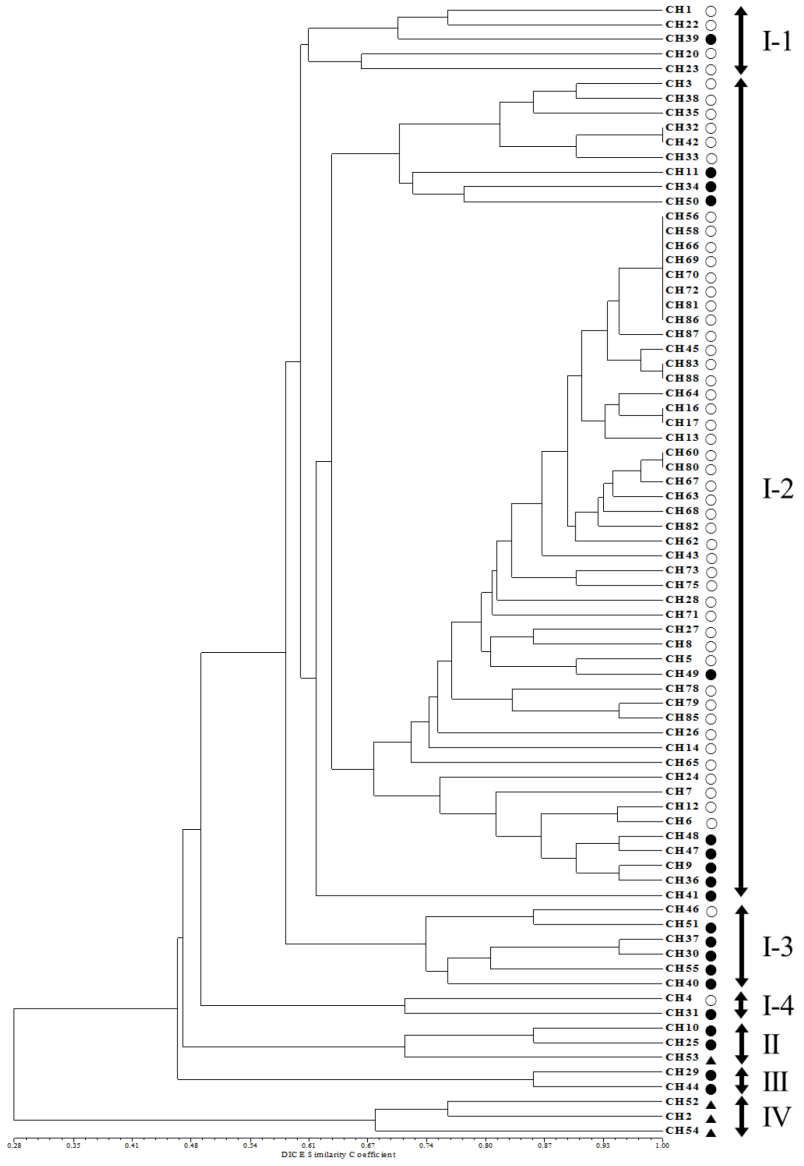

Figure 2. UPGMA dendrogram based on the SSR markers. The accessions of *Perilla* crop from high-latitude and middle-latitude areas of China are shown in Table 1. Open circle: cultivated var. *frutescens* from high-latitude area, filled circle: cultivated var. *frutescens* from middle -latitude area, filled triangle: cultivated var. *crispa*.

In our analysis, all accessions of the cultivated var. *frutescens* were discriminated from the cultivated var. *crispa*, except for one accession (CH53), which belonged to the group of the cultivated var. *frutescens*. Furthermore, in the case of the cultivated var. *frutescens*, most accessions of the cultivated var. *frutescens* collected in high-latitude and middle-latitude areas were distinguished depending on their geographical location, except for several accessions.

Population structure

To understand the genetic structure among the 77 *Perilla* accessions from high-latitude and middle-latitude areas in northern China, we used a model-based approach in the STRUCTURE software to subdivide each accession into their corresponding subgroups. We ran STRUCTURE for fixed K values ranging from one to ten and performed five runs for each K. This study applied the *ad hoc* measure ΔK using the method developed by Evanno et al. (2005) to overcome the difficulty in interpreting the real K values. The highest value of ΔK for the 77 *Perilla* accessions was for K = 2 (Figure 3).

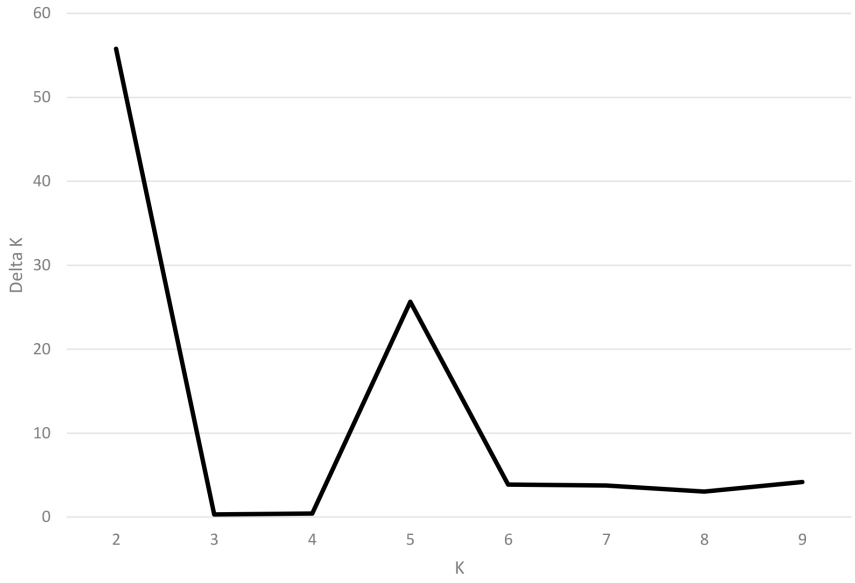

Figure 3. Magnitude of Δ*K* as a function of *K*. The peak value of Δ*K* was at *K* = 2, suggesting two genetic clusters in the *Perilla* accessions from high-latitude and middle-latitude areas of China.

Clustering bar plots with K = 2 are shown in Figure 4. At K = 2, all 77 *Perilla* accessions were divided into two groups. However, some *Perilla* accessions were admixed within these two groups. There was no clear geographic structure among the 77 *Perilla* accessions from high-latitude and middle-latitude regions in northern China, which was confirmed by UPGMA analysis (Figures 2 and 4). Therefore, we performed an analysis according to the method of Wang et al. (2008) based on a membership probability threshold of 0.8. As a result, the 77 *Perilla* accessions were divided into Group I, Group II, and an admixed group. Group I included 25 accessions (CH56, CH58, CH60, CH62, CH63, CH64, CH66, CH67, CH68,

CH69, CH70, CH72, CH75, CH45, CH80, CH81, CH82, CH83, CH86, CH87, CH88, CH13, CH16, CH17, CH43) of the cultivated var. *frutescens* from high-latitude areas of China. Group II included 16 accessions (CH30, CH34, CH37, CH39, CH46, CH55, CH40, CH29, CH51, CH44, CH31, CH6, CH9, CH10, CH24, CH25) of the cultivated var. *frutescens* and 4 accessions (CH2, CH52, CH53, CH54) of the cultivated var. *crispa*. The admixed group comprised 25 accessions (CH1, CH3, CH35, CH38, CH65, CH71, CH73, CH78, CH79, CH85, CH20, CH22, CH23, CH32, CH33, CH14, CH42, CH26, CH12, CH27, CH28, CH4, CH5, CH7, CH8) of the cultivated var. *frutescens* from high-latitude areas of China and 7 accessions (CH11, CH49, CH41, CH50, CH36, CH47, CH48) of the cultivated var. *frutescens* from middle-latitude areas of China.

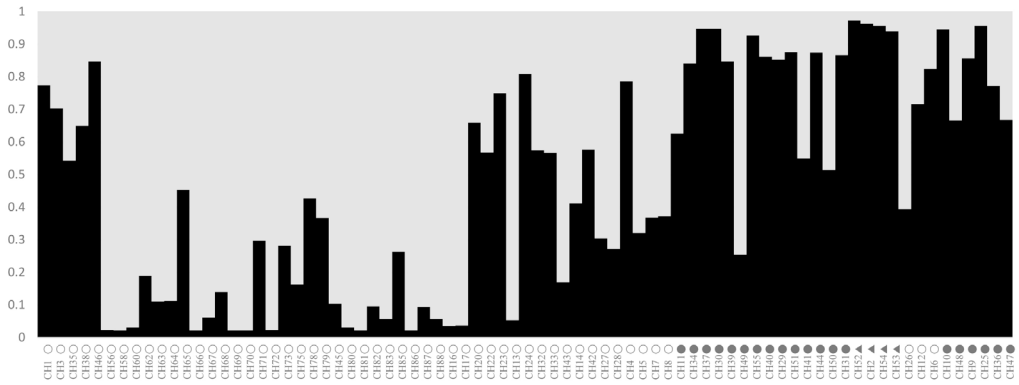

Figure 4. Population structure of 77 accessions of two cultivated types of *Perilla* crop based on 21 SSRs for K = 2. Open circle: cultivated var. *frutescens* from high-latitude area, filled circle: cultivated var. *frutescens* from middle -latitude area, filled triangle: cultivated var. *crispa*.

DISCUSSION

Genetic diversity (or variation) is usually thought of as the amount of genetic variability among individuals of a variety or population of a species (Brown, 1983). It results from the many genetic differences between individuals and may be manifested in differences in DNA polymorphism (e.g., RFLP, RAPD, AFLP, and SSR markers), in biochemical characteristics (e.g., in protein structure or isoenzyme properties), in physiological properties (e.g., abiotic stress resistance or flowering habits), or in morphological characters such as seed color or plant height (Rao and Hodgkin, 2002). Morphological characters or physiological properties were the major methods for germplasm classification or genetic resource evaluation in previous studies. As a consequence of the effects of environmental conditions or other factors on the final results, classification or evaluation accuracy was usually reduced. To obtain an accurate germplasm evaluation, modern biotechnological tools at the DNA-based molecular markers have provided useful information regarding genetic diversity and genetic relationships. Among the various types of DNA molecular markers, SSR markers are useful for analysis in plant genetics, and ecology since the hypervariable nature of SSRs results in the production of high levels of allelic variations, even among very closely related individuals (Powell et al., 1996; Park et al., 2009).

In this study, 21 SSR primer sets were applied for analysis of genetic diversity among *Perilla* accessions collected in high-latitude and middle-latitude areas of China. According to our results, a total of 95 alleles with 21 SSRs were detected segregating in the 77 *Perilla* accessions from different areas of China, which yielded an average of 4.52 alleles per locus. This value appears to be low when compared to the effective number of alleles per SSR locus in the results of previous studies by Lee and Kim (2007) and Sa et al. (2013). They detected a total of 101 alleles with 11 SSRs among 70 *Perilla* accessions from East Asia, with an average allele number of 9.2 per locus (Lee and Kim, 2007), and a total of 165 alleles using 18 SSR markers in 56 *Perilla* accessions from Korea and Japan with an average of 9.2 alleles yielded at each locus (Sa et al., 2013). Furthermore, the average value of genetic diversity in this study is much lower than the values of previous studies by Lee and Kim (2007) and Sa et al. (2013). The reason for the low values of genetic diversity and allele numbers per locus obtained in the current study might be attributed to different materials of *Perilla* accessions when compared with the previous studies by Lee and Kim (2007) and Sa et al. (2013). For example, most materials used in this study are accessions of the cultivated var. *frutescens*, and only 4 accessions of the cultivated var. *crispa* were collected from high-latitude and middle-latitude areas of China. Unfortunately, at the beginning of this study, we did not collect weedy accessions of two cultivated types of *Perilla* crop in these areas. While in the previous studies, they used many accessions of cultivated and weedy types of *Perilla* crop in East Asia (Lee and Kim, 2007; Sa et al., 2013). They reported that the accessions of two weedy types of *Perilla* crop had higher genetic diversity than accessions of two cultivated types of *Perilla* crop (Lee and Ohnishi, 2003; Lee and Kim, 2007; Sa et al., 2013). This result indicated that in domestication through direct and indirect human selections during the evolutionary stage from wild type to cultivated type, some alleles were lost, and the level of polymorphism and genetic diversity were reduced.

On the other hand, in case of the northern areas of China, accessions of the cultivated var. *frutescens* from middle-latitude areas, which had a smaller population size of materials compared to those collected from high-latitude areas, showed much higher genetic diversity and PIC values than those of the cultivated var. *frutescens* from high-latitude areas (Table 3). This result indicated that the cultivated var. *frutescens* from middle-latitude areas might have higher genetic diversity than that of the high-latitude areas. PIC was used to measure genetic diversity (Vaiman et al., 1994). PIC value was applied in the measurement of the efficiency of polymorphic loci. If PIC > 0.5, 0.5 > PIC > 0.25, and PIC < 0.25, locus polymorphisms can be assessed as high, medium, and low, respectively (Xie et al., 2010). In this study, the average value of PIC was 0.346, indicating that 21 SSR loci used in this study showed average level polymorphism for the accessions of *Perilla* crop from the northern areas of China. Meanwhile, the PIC values of 6 SSR loci, such as GBPF179, GBPF155, GBPF204, KWPE39, KWPE57, and KWPE29, showed more than 0.5 (Table 3). The five markers, GBPF155, GBPF204, KWPE39, KWPE57, and KWPE29, also showed the high values of PIC for the accessions of *Perilla* from East Asia and other countries (Sa et al., 2013, 2015; Woo et al., 2016). Therefore, these SSR markers were considered useful and highly informative for the analysis of genetic diversity in *Perilla* accessions from the northern areas of China and might be effectively applied in differentiating the polymorphic rate of a marker at a specific locus.

As shown in Figure 2, 77 accessions of *Perilla* crop were divided into 4 main groups by the analysis of the UPGMA dendrogram. Most accessions of the cultivated var. *crispa*, except one accession (CH53), were obviously separated from the accessions of the cultivated

var. *frutescens*. The result was consistent with the previous study by Sa et al. (2013). They reported that SSR markers are useful for distinguishing between two cultivated types of *Perilla* crop. However, one accession (CH53) of the cultivated var. *crispa* was situated in the group of the cultivated var. *frutescens*. Similar results have also been reported in the previous studies by Lee and Ohnishi (2003) and Sa et al. (2013). Namely, several accessions of var. *frutescens* and var. *crispa* were ambiguously classified on the basis of AFLP and SSR markers (Lee and Ohnishi, 2003; Sa et al., 2013). These results might be thought as a natural crossing that occurs between the two cultivated types of *Perilla* crop, as previously reported by Nitta and Ohnishi (1999) and Lee and Ohnishi (2001). By the result of the UPGMA dendrogram, most accessions of the cultivated var. *frutescens* collected from the middle-latitude area of China were classified from accessions of the cultivated var. *frutescens* from the high-latitude area of China. Based on the analysis of the population structure, Group I only consisted of accessions of the var. *frutescens* from high-latitude areas. Group II contained most *Perilla* accessions from middle-latitude areas except CH6, CH24, and CH46. However, the geographical locations of several accessions of the cultivated var. *frutescens* were not consistent with their positions in the UPGMA dendrogram and population structure. Similar results were also obtained with RAPD (Nitta and Ohnishi, 1999) and AFLP (Lee and Ohnishi, 2003) analysis. This result implies that the diffusion of accessions of the cultivated *Perilla* crop in the northern areas of China might be through multiple routes. Besides, an admixture of accessions of the cultivated *Perilla* crop from different areas of China was observed in Figure 4. The occurrence of this phenomenon may be attributed to the following factors. Firstly, overlapping distribution of *Perilla* accessions grown in the vicinity of the boundary between high-latitude and middle-latitude areas caused an occurrence of gene introgression among accessions in different regions. Second, the topography of areas where these accessions were collected was major in the plain. Gene flow happened through pollens, seeds, and other natural approaches easily in the plain areas, degrading the variation and leading to an unclear structure among cultivated *Perilla* accessions from different regions. Human activities may be the main reason for the ambiguous classification of these accessions. Gene flow can be influenced significantly by human activities (Meng et al., 2015). Seeds of accessions from high-latitude areas may be transferred to northwestern areas of China or other middle latitude areas via human business activities. Probably, the occurrence of this migration or introduction within same regions or between different regions caused these accessions to be categorized ambiguously under the analysis of UPGMA.

The investigations on genetic diversity, population structure, and collection and conservation of germplasm resources have benefited from the utility of SSR markers. The valuable information on the genetic structures and relationships of *Perilla* accessions from northern areas of China have been provided by using the 21 SSR markers in this study. Through the comprehensive analysis of genetic diversity, genetic relationship, and population structure among these Chinese *Perilla* resources, the basic genetic knowledge of Chinese *Perilla* has been obtained and can be used as a reference for genetic diversity studies, germplasm, conservation, and breeding strategies in the future.

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