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Research Article

Genetic diversity among cultivated and wild *Panax ginseng* populations revealed by high-resolution microsatellite markers

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ABSTRACT

Background: Ginseng (*Panax ginseng* Meyer) is one of the world's most valuable medicinal plants with numerous pharmacological effects. Ginseng has been cultivated from wild mountain ginseng collections for a few hundred years. However, the genetic diversity of cultivated and wild ginseng populations is not fully understood.
Methods: We developed 92 polymorphic microsatellite markers based on whole-genome sequence data. We selected five markers that represent clear allele diversity for each of their corresponding loci to elucidate genetic diversity. These markers were applied to 147 individual plants, including cultivars, breeding lines, and wild populations in Korea and neighboring countries.
Results: Most of the 92 markers displayed multiple-band patterns, resulting from genome duplication, which causes confusion in interpretation of their target locus. The five high-resolution markers revealed 3 to 8 alleles from each single locus. The proportion of heterozygosity (H_e) ranged from 0.027 to 0.190, with an average of 0.132, which is notably lower than that of previous studies. Polymorphism information content of the markers ranged from 0.199 to 0.701, with an average of 0.454. There was no statistically significant difference in genetic diversity between cultivated and wild ginseng groups, and they showed intermingled positioning in the phylogenetic relationship.
Conclusion: Ginseng has a relatively high level of genetic diversity, and cultivated and wild groups have similar levels of genetic diversity. Collectively, our data demonstrate that current breeding populations have abundant genetic diversity for breeding of elite ginseng cultivars.
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1. Introduction

Ginseng (Korean ginseng or Asian ginseng, *Panax ginseng* Meyer), which belongs to the Araliaceae family consisting of about 1,500 species, has been an important medicinal plant in Northeastern Asia for centuries [1,2]. This perennial plant is well known for its remarkable pharmacological effects including improving the cardiovascular [3] and immune systems [4], prevention of Alzheimer disease [5], and functioning as an anticancer agent [6]. Because of its multifunctional therapeutic effects, ginseng has been cultivated continuously as a high-value crop for hundreds of years and has recently attracted attention in international markets [7]. The therapeutic effects have led many research studies to study the efficacy of its medicinal compounds, but fundamental breeding and genetic studies lag behind this efficacy research. Increased interest in ginseng genomics has recently led to reports of the karyotype [8,9], genome structure and characteristics [10,11], transcriptome profile [12], and evolutionary model [13].

Ginseng grows slowly (~four years/generation) and yields few seeds (~40 seeds/plant), which makes hybridization breeding difficult [14]. It is also sensitive to environmental factors such as light and soil moisture, so maintaining individual plants requires

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Table 1
Summary of 147 *P. ginseng* germplasms used in this study.

Serial numbers	Germplasm type	Number of germplasms	Remarks
1–14	Korean cultivar	14	Registered in Korea Seed & Variety Service
15-118	Breeding line	104	Bred and maintained in the Rural Development Administration, Korea
119-121	Japanese cultivar	3	Collected from Japan (cv. Mimaki)
122-144	Korean wild collection	23	Collected from various areas in Korea
145-147	Russian wild collection	3	Collected from Vladivostok, Russia

considerable effort. In addition, ginseng has a large genome size, estimated to be 3.6 Gbp [15], and complex genome structure, which resulted from recent allotetraploidization and subsequent rapid amplification of diverse repetitive sequences such as retrotransposons [10,16]. Owing to these genome complexities, development of DNA markers for ginseng is difficult. Most simple sequence repeat (SSR) markers used in previous studies showed multiband patterns, which cause confusion in the interpretation of the target locus [17,18]. These problems result in a need for more accurate and useful marker development in ginseng breeding research.

Crop improvement is the objective of plant breeding research, and diversity studies using various genetic resources support exploration of potential genetic resources. Genetic diversity of germplasms can be investigated by numerous techniques, including analysis of morphological and agricultural traits, isozymes, biochemical characteristics, composition of metabolites, and allele analysis using DNA markers. Of those techniques, DNA markers are the most reliable in distinguishing the variation of alleles because they are not influenced by environmental and physiological factors [19]. Specifically, microsatellites, also called SSRs, are distributed in most eukaryotic genomes and have multiallelic forms that provide useful polymorphism information for a locus [20]. Moreover, because of their high reproducibility and codominant properties, SSRs have been broadly applied to diverse studies including phylogenetic analysis [21], authentication of cultivars and related species [18], construction of genetic maps [22], and analysis of genetic diversity [23].

Most of the ginseng cultivars registered in Korea have been bred by selection of individual plants from arable fields of mixed local landrace population [24]. Although the registered cultivars have a generally uniform phenotype, relatively higher heterogeneity is observed in ginseng compared with other annual crops because it takes so long to develop pure inbred lines by self-pollination. For example, it takes at least ~32 years to develop eight generations of a single-seed descent self-pollinated progeny. Consequently, ginseng germplasms are expected to be highly diverse and heterogeneous. Although several studies have been performed to analyze genetic diversity among accessions, only small populations were used [17,25].

In this study, a total of 92 polymorphic microsatellite markers were developed based on ginseng whole-genome sequence data. Five markers with high-resolution allele diversity for a single locus were selected and used to investigate the genetic diversity of 147 ginseng genetic resources including cultivated and wild populations. The relationship between cultivated and wild groups was elucidated in more detail than previous studies from this comprehensive analysis of diversity in various ginseng resources. These results will provide valuable understanding about the genetic diversity and relationships between ginseng populations. The SSR markers developed here will be useful for further research including analysis of the population structure, genetic map construction, seed purity testing for specific cultivars [26], and marker-assisted selection for breeding.

2. Materials and methods

2.1. Plant material and DNA isolation

A total of 147 ginseng individual plants were used for analysis of genetic diversity, including various cultivars, breeding lines, and wild collections (Table 1; Supplementary Table 1). Among these resources, 14 cultivars and 104 breeding lines of ginseng were provided from the Rural Development Administration (Eumseong, Korea), and three "Mimaki" cultivars originating in Japan were collected from the research field of Seoul National University (Suwon, Korea). Twenty-three wild collections were collected from Hwacheon, Hamyang, and Yeongjongdo in Korea, and the remaining three wild resources were collected from Vladivostok, Russia.

The fresh leaves were frozen in liquid nitrogen and ground using a mortar and pestle. Genomic DNA was isolated through modified cetyltrimethylammonium bromide methods [27]. The quantity and quality of the extracted DNA was measured using a NanoDrop ND-1000 (Thermo Scientific Inc., Wilmington, DE, USA). For polymerase chain reaction (PCR) analysis, the DNA concentration of each sample was adjusted to 10 ng/μL.

2.2. Microsatellite primer design

Scaffold sequences generated from *P. ginseng* cv. Chunpoong (CP) as a part of the ginseng genome project were used for development of microsatellites [15]. Assembled sequences of longer than 200 Kbp were selected, and SSR motifs inside these long sequences were explored using the Pear script Microsatellite identification tool (http://pgrc.ipk-gatersleben.de/misa/misa.html) with default parameters. Primer pairs of 23 to 28 nucleotides were designed in the regions flanking the SSR motifs using the Primer3 version 0.4.0 web program (http://bioinfo.ut.ee/primer3-0.4.0/). The PCR product size was adjusted to be between 150 and 500 bp.

2.3. PCR amplification and electrophoresis

PCR amplification was carried out in a 25-μL volume containing 1U *Taq* polymerase, 1 × reaction buffer, 0.2 mM dNTPs, and 0.2 × Enhance solution (Inclone Biotech, Yongin, Korea), 20 ng genomic DNA, and 10 pmole of each primer. The following thermal cycle conditions were used: 10 min at 94°C; 35 cycles of 20 s at 94°C, 20 s at 54°C, and 20 s at 72°C; and 7 min at 72°C for final extension. The PCR products were separated using 1% agarose gel to confirm amplification. The polymorphism of microsatellites and genotype of ginseng genetic resources was identified by electrophoresis for 2 hours using 3% agarose and 12% nondenaturing polyacrylamide gels. The gels were stained with ethidium bromide and destained for 15 min with distilled water. The products were visualized under UV light. In the case of the gws1070 marker, amplified fragments were separated on a Fragment Analyzer™ 96 platform that has an automated capillary electrophoresis system using the dsDNA 905 Reagent Kit (Advanced Analytical Technologies Inc., Ankeny, IA, USA), and the

Table 2
Five high-resolution SSR markers used for diversity analysis.

Marker name	Repeat motif		Primer sequence (5′ -> 3′)	Melting temperature (°C)	Product size (bp)		Target genome sequence position[1]
gws218	$(CCTTTTT)_n$	F	TCAAATAATCATATCACACCCATCA	60.3	130 - 160	S	Pg_scaffold0784
		R	ACCAAAATAAAGATTAGCGACAATG	59.8		P	259,244 - 259,376
gws450	$(CCACAA)_n$	F	TGGTAATAGTTGAGACAAAATTGCAT	60.2	190 - 250	S	Pg_scaffold1503
		R	GGTTTGTTCATTGTATATGCTCCTG	61.0		P	193,908 – 194,098
gws454	$(ATAG)_n$	F	AAAGGAATACAAGAAAGAGGGAGAA	60.0	100 - 130	S	Pg_scaffold3767
		R	TAAAGAATTTGGATCCACCTACAAA	60.1		P	30,965 - 31,069
gws936	$(AGGCAGA)_n$	F	AGAGTAGCAGACTAGCAGTGGAGAG	59.9	160 - 190	S	Pg_scaffold2001
		R	TGTTCTTTTAGGCATTCGGTATGTA	61.0		P	458,078 – 458,257
gws1070	$(GAAGCAT)_n$	F	TTCCAAACATAAAAGAAAACTGACC	59.8	230 - 290	S	Pg_scaffold1638
		R	GTCTTGAAAACTTACCGAATTGAAA	59.9		P	69,551 – 69,790

F, forward; P, position; R, reverse; S, scaffold name; SSR, simple sequence repeat.
[1] Target genome sequence position was estimated based on the published genome assembly sequence of *P. ginseng* cv. Chunpoong [15].

genotype of each samples was confirmed using PROSize™ 2.0 version 1.3.1 software included in the analyzer.

2.4. Microsatellite selection and analysis of genotyping data

Two main cultivars, cv. CP and cv. Yunpoong, were used in prescreening all primers for polymorphism. Among the confirmed polymorphic microsatellites, five high-resolution markers were selected for genetic diversity analysis of the 147 individuals (Table 2). The genotype of each locus was determined by precise manual verification of several repetitions (Supplementary Table 1). The number of alleles and genotypes, major allele frequency (MAF), gene diversity (GD), proportion of heterozygosity (H_e), polymorphism information content (PIC), and Nei's genetic distance [28] of each microsatellite locus were calculated using a PowerMarker version 3.25 program [29]. Phylogenetic analysis between ginseng germplasms was carried out based on Nei's genetic distance and the unweighted pair group method with arithmetic mean using the MEGA 7.0 program [30].

3. Results and discussion

3.1. Development of microsatellites in polyploid ginseng

A ginseng reference genome assembly was recently published, composed of 3.0 Gbp from 9,845 scaffolds using the CP cultivar [15]. This genome assembly was used in this work to identify SSR motifs from the scaffold sequences. Polymorphic SSR markers were identified by screening for polymorphisms between CP and Yunpoong cultivars. Out of a total of 1,215 primer pairs containing various SSR motifs, only 92 SSRs were verified to show polymorphism between the two cultivars (Supplementary Table 2). The polymorphism rate was estimated at 7.57%, lower than that of other plants [31,32] but similar to previous research on ginseng [33].

Many of the 92 polymorphic markers showed complex multiple-band patterns, which makes it difficult to identify genotypes when applied to large germplasm collections (Supplementary Table 2). Consequently, we selected five high-resolution markers that distinguish genotypes for their corresponding single locus. The

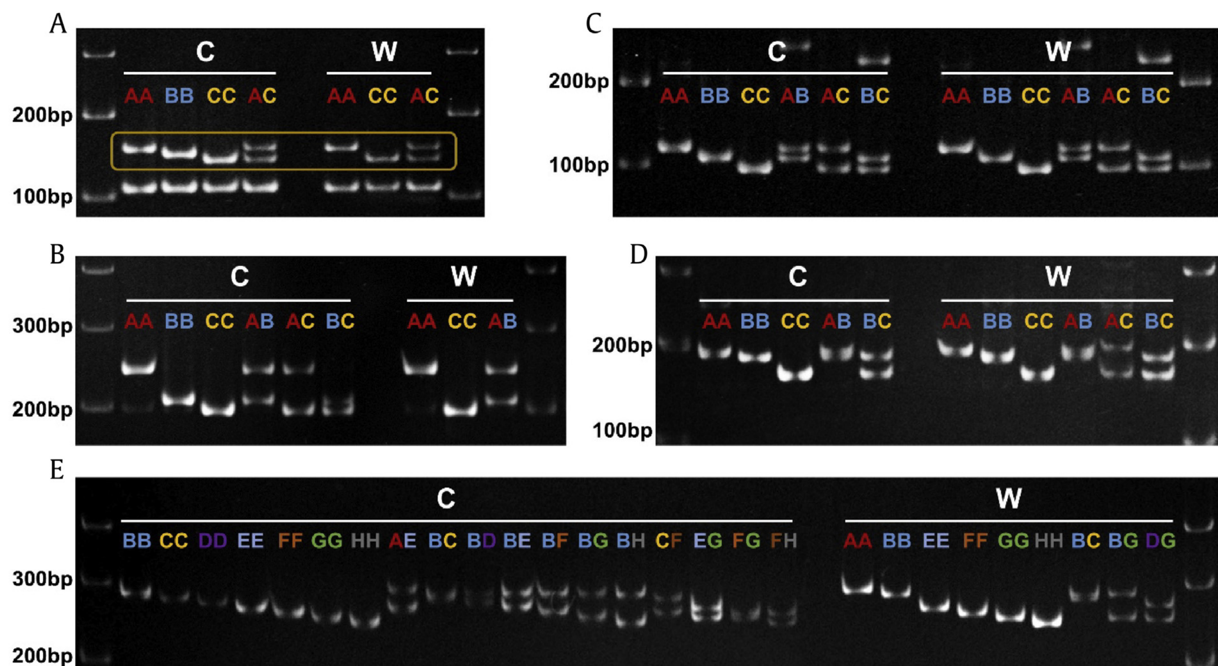

Fig. 1. Representative genotypes in each locus between the cultivated and wild ginseng groups. (A) gws218, (B) gws450, (C) gws454, (D) gws936, and (E) gws1070. The characters at the top of bands represent the genotype determined for each sample. The brown box around the upper bands in (A) gws218 represents the target locus.
C, cultivated ginseng group; W, wild ginseng group.

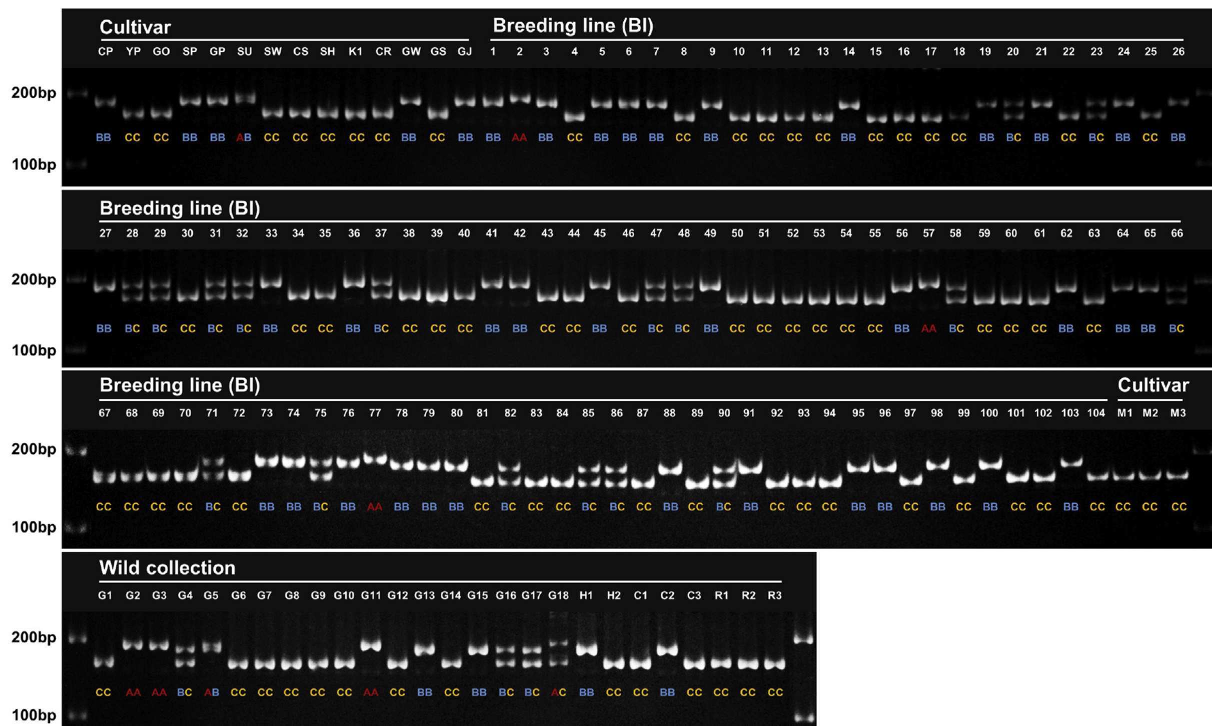

Fig. 2. Intraspecies diversity among 147 ginseng germplasms identified by the gws936 marker. Each sample is represented by the abbreviation defined in Supplementary Table 1. The label at the bottom of the bands indicates the genotypes of each sample.

representative genotypes of each locus are shown in Fig. 1. Among the selected markers, gws218 showed additional bands around 100 bp, common to all samples (Fig. 1A). This phenomenon was frequently observed in previous studies on ginseng SSR markers [17,18,33,34] and did not disappear after altering PCR conditions and extending primer lengths. This characteristic confounds the interpretation of allelic diversity for a marker as correct genotyping can be confused by coamplification of other duplicated chromosomal regions. Actually, previous works on ginseng have reported too many alleles in a locus and high H_e values for SSR markers [25,35]. Because of the complex duplicated genome structure of ginseng, it is necessary to use high-resolution markers that can clearly distinguish single target locus, as shown in this study.

3.2. Allele diversity among 147 ginseng germplasms

The genotypes of 147 ginseng germplasms were surveyed for the loci of the five SSR primers (Fig. 2). Statistical analysis of each locus was performed using the determined genotype data of each individual to evaluate the intraspecific diversity (Table 3A; Fig. 3). All five markers showed clear polymorphic amplification products that provided unambiguous genotyping data for each individual. A total of twenty alleles were identified, and the number of alleles per locus ranged from three to eight. The four loci, gws218, gws450, gws454, and gws936, had three alleles, whereas gws1070 had eight different alleles among the 147 germplasms. The MAF at each locus ranged from 43.5% for gws1070 to 87.8% for gws218. On average, 62.4% of the germplasms contained a common major allele. The GD of each locus broadly ranged from 0.218 to 0.734, with an average of 0.508. The H_e of germplasms ranged from 2.7 % to 19.0 %, with an average of 13.2%. The PIC value, which indicates the usefulness of a marker, ranged from 0.199 to 0.701, with an average of 0.454.

Ginseng has abundant genetic diversity at polymorphic loci. All five loci had more than three alleles, and most of the possible heterozygous genotypes were observed. The MAF for the five loci suggests that there is no predominant allele, but there is relatively even allele distribution within the population. Moreover, GD values were higher than 0.5 in the three loci, gws454, gws936, and gws1070. Although ginseng had low frequency of SSR polymorphism compared with other plants, the polymorphic loci had an abundance of intraspecies allele diversity [17,18]. The genetic diversity may have originated from ecological characteristics of

Table 3
Characteristics of the five high-resolution SSR loci among ginseng germplasms.

Marker name	Number of alleles	Number of genotypes	MAF	GD	H_e	PIC
147 ginseng germplasms						
gws218	3	4	0.878	0.218	0.027	0.199
gws450	3	6	0.697	0.462	0.122	0.410
gws454	3	6	0.534	0.597	0.163	0.526
gws936	3	6	0.575	0.527	0.156	0.432
gws1070	8	20	0.435	0.734	0.190	0.701
Mean	4	8.4	0.624	0.508	0.132	0.454
121 cultivated individuals only						
gws218	3	4	0.880	0.214	0.025	0.197
gws450	3	6	0.669	0.491	0.140	0.436
gws454	3	6	0.550	0.593	0.165	0.524
gws936	3	5	0.566	0.515	0.149	0.409
gws1070	8	18	0.467	0.711	0.198	0.677
Mean	4	7.8	0.626	0.505	0.136	0.449
26 wild individuals only						
gws218	2	3	0.865	0.233	0.038	0.206
gws450	3	3	0.827	0.292	0.038	0.259
gws454	3	6	0.462	0.582	0.154	0.491
gws936	3	6	0.615	0.544	0.192	0.484
gws1070	8	9	0.288	0.802	0.154	0.774
Mean	3.8	5.4	0.612	0.491	0.115	0.443

GD, gene diversity; H_e, heterozygosity; MAF, major allele frequency; PIC, polymorphic information content.

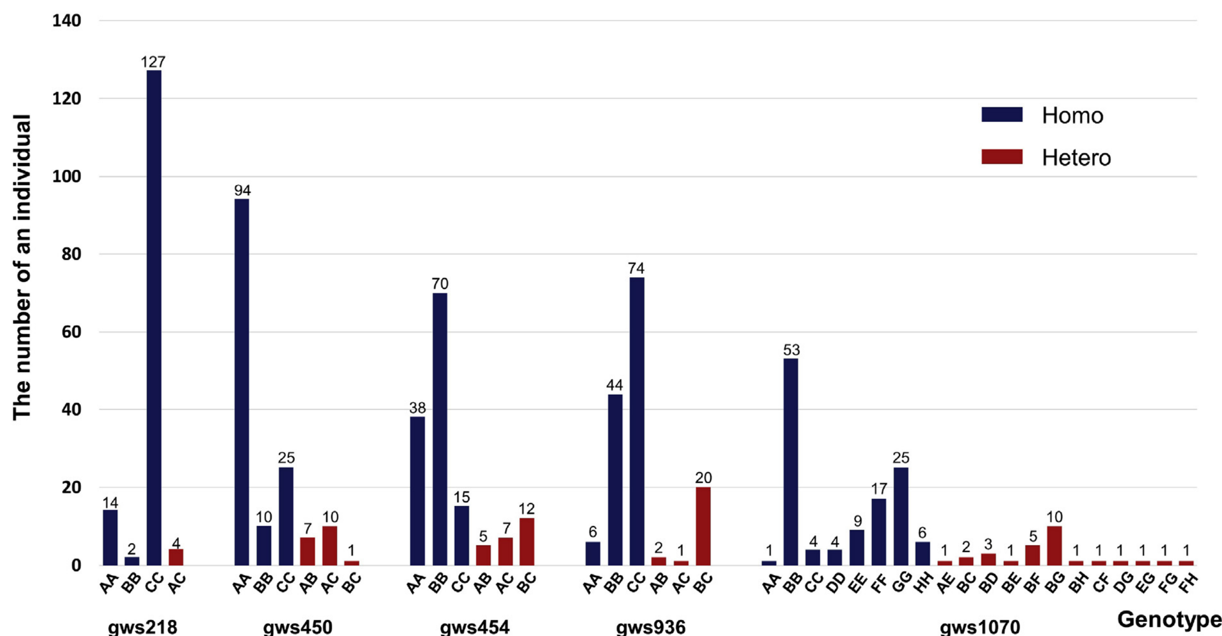

Fig. 3. Genotype distribution of each SSR locus among 147 ginseng germplasms. Unidentified genotypes in each locus were not included in this graph. SSR, simple sequence repeat.

ginseng and its unique cultivation environment. Ginseng grows very slowly (~four years/generation); therefore, homogenization of genotypes by self-pollination takes longer than that in other annual plants. In fact, the registered cultivar showed approximately 10% of off-type alleles in previous research [17].

Markers with PIC values greater than 0.4 are usually considered informative for genetic studies [36]. Four of our five markers had PIC values more than 0.4. In particular, gws1070 is regarded as a "highly informative" marker. These markers could provide helpful information for diversity analysis including construction of a genetic map, quantitative trait mapping, and evolutionary studies.

3.3. *Paralogous targets derived from genome duplication result in overestimation of heterozygosity*

Of the 92 polymorphic markers identified here, 77 microsatellites (83.7 %) showed complex multiple-band patterns that cause confusion in the interpretation of the genotype for the target locus (Supplementary Table 2). In fact, several previous studies have reported that ginseng has a heterozygosity of more than 0.5 [25,35]. However, in this study using single-locus markers, the H_e of the population was determined to be 2.7–19.0%, which is lower than that of previous reports. Most plants showed homozygous genotype distribution for the microsatellite loci included in this study. The markers with multiple bands must be handled carefully because of the possibility of misinterpretation, wherein an independent locus is obscured by the interference of paralog sequences derived from different loci.

A multiple-band pattern is presumed to reflect the genome structure. Recent studies have shown that *P. ginseng* has a complex genome structure, derived from several rounds of whole-genome duplication [8,10]. Because of the recent allotetraploidization, most of the euchromatic regions are two copies with an almost identical sequence [13]. This sequence similarity increases the chance for coamplification of two paralogs that confuse correct genotyping. Therefore, organisms such as ginseng with a complex or polyploid genome structure require more careful approaches to develop markers that have locus-specific amplification and eliminate nontarget, paralogous, noisy regions [37].

3.4. *Genetic relationship between cultivated and wild groups*

Statistical analysis of diversity was carried out on 121 cultivated (Table 3B) and 26 wild ginseng groups (Table 3C). All alleles were identified in both groups, with the exception of the B allele of gws218, which was not identified in any wild groups (Fig. 1). For three loci, gws218, gws454, and gws936, the genotypes were evenly distributed within both groups. Although the cultivated ginseng groups exhibited slightly more variations than the wild groups, there was a similar pattern of variability between the two groups (Table 3). Phylogenetic analysis showed that wild and cultivated groups were intermingled (Fig. 4). These results imply that there is no significant difference in genetic diversity between the wild and cultivated ginseng groups.

Typically, higher genetic variations are identified in wild populations compared with cultivated populations because of the homogenizing effects of domestication [38,39]. However, in ginseng, polymorphic alleles were evenly distributed in the two groups evaluated. This phenomenon could be explained by examining the cultivation process of the plant. In the domestication process, ginseng is cultivated from diverse collections of wild mountain ginseng, and local landrace cultivars are maintained by bulky seed harvest [2]. These methods have maintained the genetic diversity from the wild populations in cultivated ginseng populations.

The preservation of genetic diversity in cultivated populations is important because it allows breeders to ensure genetic resources for crop improvement, adaptable to diverse environments and climate changes [40]. This study found that ginseng had a high level of intraspecific diversity in both groups, which means that the cultivated ginseng populations and breeding collections have sufficient diversity to be developed by further selection of agricultural characteristics, such as in recently developed ginseng cultivars in Korea [24,41].

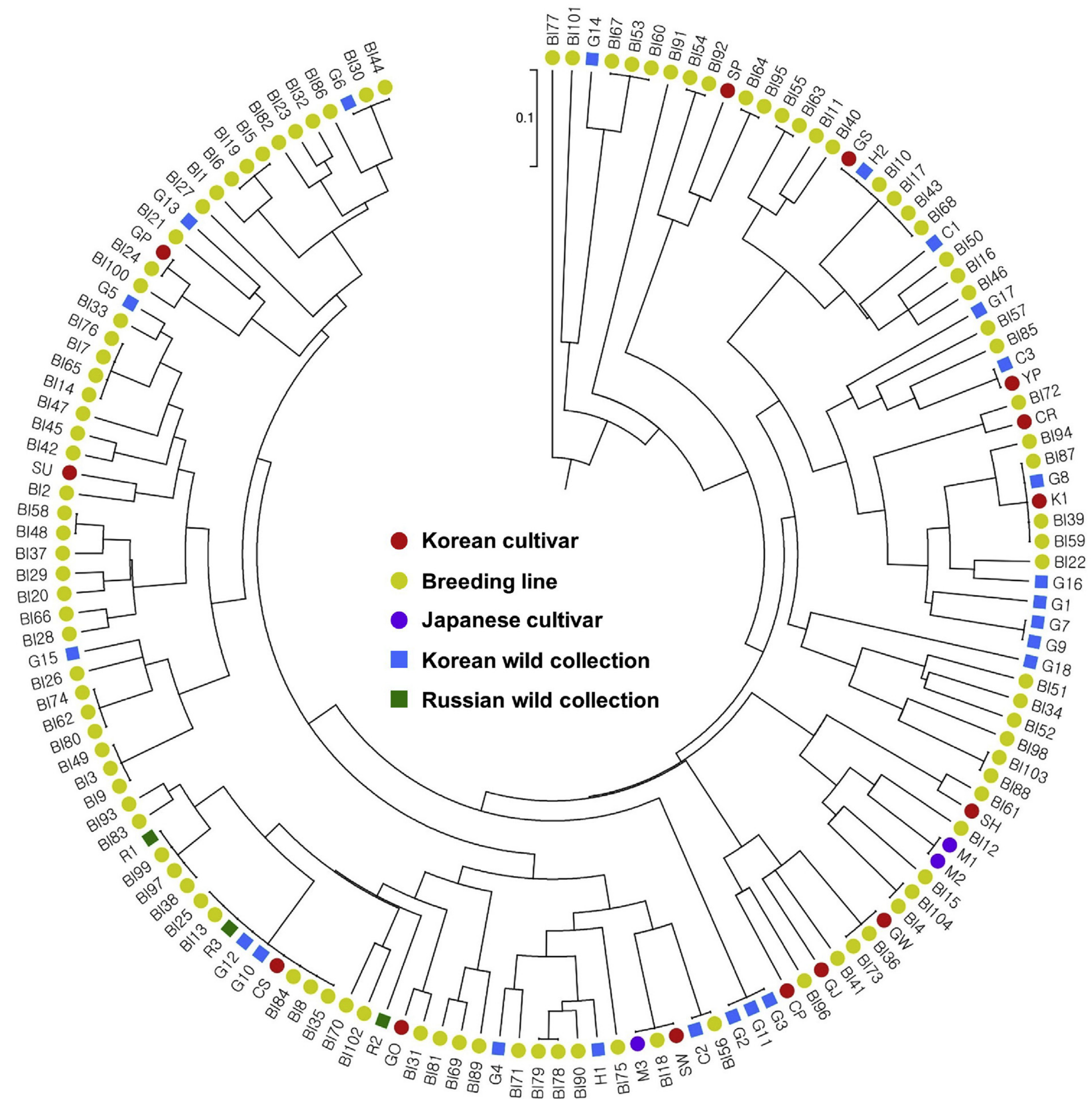

Fig. 4. Genetic relationship among 147 ginseng germplasms including cultivated and wild groups. The phylogenetic tree was constructed using the UPGMA method by five SSR markers. Abbreviations for each sample are described in Supplementary Table 1.
SSR, simple sequence repeat; UPGMA, unweighted pair group method with arithmetic mean.

4. Conclusions

We developed 92 polymorphic SSR markers in ginseng and selected five high-resolution SSR markers that show distinguishable alleles for single target locus. Genetic diversity analysis including 147 ginseng genetic resources showed a high level of polymorphism and diversity in the populations. Previous studies of ginseng variability may have overestimated the H_e value because of duplex bands that resulted from the duplicated genome structure. In addition, statistical and phylogenetic analysis demonstrated that there are no significant differences in genetic diversity between the wild and cultivated ginseng groups. This indicates that abundant genetic variation has been maintained in local landrace and breeding populations. These results extend our understanding of the genetic structure of populations and domestication in ginseng. Furthermore, the markers developed here will provide valuable information for further research including diversity studies, genetic mapping, cultivar authentication, seed purity testing for a cultivar, and marker-assisted selection.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.05.008.

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