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Development and Characterization of New Microsatellite Markers for *Perilla frutescens* (L.) Britton

Kyu Jin Sa[1], Su Eun Lim[1], Ik-Young Choi[2], Kyong-Cheul Park[2], Ju Kyong Lee[1*]

[1]Department of Applied Plant Sciences, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon, Korea
[2]Department of Agriculture and Life Industry, Kangwon National University, Chuncheon, Korea
Email: *jukyonglee@kangwon.ac.kr

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Abstract

Based on RNA sequences using transcriptome analysis, 37 new simple sequence repeat (SSR) primer sets were developed for *Perilla* species. These new SSR markers were applied to analyze the genetic diversity among 15 accessions of *Perilla* species. A total of 182 alleles were confirmed in 37 loci, with an average of 4.9 alleles per locus and from 2 to 9 alleles per locus. The MAF (major allele frequency) per locus varied from 0.200 to 0.733, with an average of 0.463. The gene diversity (GD) ranged from 0.391 to 0.853, with an average of 0.670. The average polymorphic information content (PIC) was 0.624, ranging from 0.315 to 0.838. The new SSR markers of *Perilla* species reported in this study may provide potential markers to analyze the genetic diversity and genetic relationships of *Perilla* species. In addition, new *Perilla* SSR markers developed from transcriptome analysis can be useful for the identification of cultivars, conservation of *Perilla* germplasm resources, and genetic mapping and designating of important genes/QTLs for future *Perilla* crop breeding programs.

Keywords

Perilla frutescens, Oil Crop, Vegetable Crop, Genetic Diversity, Microsatellites, RNA-Seq

1. Introduction

Perilla frutescens (L.) Britton is widely cultivated in East Asia. There are two varieties based on the uses and morphology, *P. frutescens* var. *frutescens* and var. *crispa*. Generally, var. *frutescens* is used as an oil crop (ren in Chinese, dlggae in Korean and egoma in Japanese), whereas var. *crispa* is used as a vegetable crop or Chinese medicine (zisu in Chinese, cha-jo-ki in Korean and shiso in Japanese) [1] [2] [3]. Today, *P. frutescens* var. *frutescens* and var. *crispa* are extensively cultivated and used in Korea and Japan [2] [3] [4]. Var. *frutescens* is used as both a leafy vegetable and an oil crop in Korea. In contrast, var. *crispa* is used for vegetables or pickles, using the leaves in Japan, and is also used for Chinese medicine in China [2] [3] [5]. In East Asia, the wild species of these two varieties of *Perilla* crop are unknown but weedy plants of two cultivated types of *P. frutescens* have been identified [1] [2] [3]. In East Asia, the weedy plants naturally grow in wastelands, roadsides, around farming fields or farmhouses [2] [3] [5] [6]. The two cultivated types of var. *frutescens* and *crispa* in East Asia have several distinguishing morphological characters including the seed size, stem and leaf color, and plant fragrance. The weedy type of var. *frutescens* has a same stem and leaf color and fragrance as cultivated var. *frutescens* but its seeds are smaller and harder than those of cultivated var. *frutescens* [2] [7]. The weedy type of var. *crispa* is occasionally recognized and used as cultivated var. *crispa* by farmers because of their morphological similarities [2] [3].

Information on the genetic diversity and genetic relationships among *Perilla* crop and their weedy types is very important for successful *Perilla* crop breeding programs, the use of the germplasm resources and conservation. In previous studies, RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), and SSR (simple sequence repeat) analyses showed that the two weedy types of *Perilla* crop were each grouped with the two cultivated types of var. *frutescens* and var. *crispa* [1] [5] [8] [9] [10]. Although these studies were performed to distinguish cultivated types of var. *frutescens* and *crispa* and their weedy types at the DNA level, they did not present a clear classification for these two *Perilla* varieties and their weedy types. Among many DNA molecular markers, SSRs are very abundant in eukaryotic genomes and show a highly variable number of repeats among individuals in a given population [11]. SSRs are often selected for genetic studies such as genetic diversity and relationship analyses because they have advantages such as high reproducibility, polymorphisms, abundance, and codominance in plant genomes [11] [12]. Our previous study successfully isolated SSRs for *Perilla* crop [13] [14] [15] and analyzed *Perilla* accessions collected from various regions [9] [14] [16] [17] [18]. However, the number of SSR markers for clear classification is still lacking.

In our previous study, we obtained 15,991 SSR loci from transcriptome sequencing by RNA-seq in one cultivated type (PF98095) of *P. frutescens* var. *frutescens* [19]. In this study, we successfully developed SSR primers from *Perilla* species, and these novel additional SSR markers can be used to analyze the genetic diversity and genetic relationships and to perform QTL mapping among two cultivated types of *Perilla* crop and their weedy types.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

This study used 15 accessions including the cultivated type of var. *frutescens*, cultivated type of var. *crispa* and weedy type of var. *frutescens* to evaluate polymorphisms and identify new SSR markers. 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).

2.2. SSR Marker Development

To construct the transcriptome reference set in a previous study [19], *de novo* assembly of the PF98095 RNA-seq data was performed using Trinity software. The raw reads from NGS sequencing with a Phred quality score of at least 20 and read length of at least 50 bp of HiSeq 2000 data were filtered before assembly. A Perl script MISA tool (https://pgrc.ipk-gatersleben.de/misa) was used to search microsatellite sites in the assembled transcriptome sequences of PF98095. The SSRs with di-, tri-, and tetra-nucleotide repeat units were identified. Based on the SSR flanking sequences, PRIMER 3 software was employed to design the primer pairs. We searched all unigenes in the cultivated type of var. *frutescens* (PF98095) and detected 15,991 SSR loci. In this study, we selected 200 SSR primer sets based on the 80 di-, 60 tri- and 60 tetra-nucleotide types and the number of repeat units.

2.3. SSR Analysis and Data Analysis

SSR amplifications were conducted in a total volume of 20 μl consisting of 20 ng genomic DNA, 1 × PCR buffer, 0.5 μM of forward and reverse primers, 0.2 mM dNTPs, and 1 unit of *Taq* polymerase (Biotools, Madrid, Spain). The PCR profile consisted of an initial denaturation at 95˚C for 3 minutes followed by 36 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds, and 72˚C for 1 minute 30 seconds with a final extension step of 5 minutes at 72˚C. After PCR analysis, the PCR products were resolved on a QIAxcel Screengel system (QIAGEN) with the 0M700 method according to the manufacturer's protocol. The number of alleles, allele frequency, major allele frequency (MAF), gene diversity (GD), and polymorphic information content (PIC) for new SSR markers were calculated using PowerMarker 3.25 [20].

3. Results and Discussion

Among the selected 200 SSR primer pairs, 37 SSR primer pairs had good amplification and polymorphisms among 15 *Perilla* accessions (**Table 1**). However, the remaining 163 SSR primer pairs exhibited a monomorphic band (53) or ambiguous band pattern (33) and poor or no amplification (77) in the *Perilla* accessions. The new 37 SSR primer pairs were used to measure the genetic diversity index, such as the number of alleles, MAF, GD, and PIC among 15 *Perilla* accessions, including two cultivated types of *Perilla* crop and their weedy types in East Asia. 182 alleles were detected in the 15 *Perilla* accessions, with an average of 4.9 alleles per locus, ranging from 60 to 250 bp. The number of alleles per locus ranged from 2 (KNUPE-45 and KNUPE-57) to 9 (KNUPE-42). The MAF per locus varied

Table 1. Characteristics of the 37 SSR loci, including the primer sequence, repeat motif, annealing temperature, allele size range, and genetic diversity index among 15 *Perilla* accessions.

SSR loci	Forward sequence	Reverse sequence	Repeat motif	T_a	Allele size (bp)	No. of alleles	MAF	GD	PIC
KNUPF-41	CCAAAATCTCCATGTTATTGCT	ACACACATCAGGCTTTCTCTCT	(AT)7	53	115 - 150	7	0.467	0.729	0.704
KNUPF-42	CGAATTCAATAGGGAAAAATGA	AGACTCAAATCATAGGAGTTTACGA	(AT)7	53	140 - 165	9	0.267	0.853	0.838
KNUPF-43	GTCAAATGAAATTCACACATTTTA	GTAAATGGGAATTTTTGAGGAG	(AT)7	51	145 - 155	6	0.467	0.720	0.690
KNUPF-44	ATCTCCACAGATTTCACTCCTG	AATTGATTTCGTTTTACGGAGA	(AT)7	53	155 - 165	6	0.533	0.667	0.637
KNUPF-45	AGACGTTGTGTACAAATTGACG	TCTGCACTCAAATATACAAGGC	(AT)7	52	160 - 165	2	0.733	0.391	0.315
KNUPF-46	AAATTTATTGGCGTGTATCGAG	TTGAATTTGCTGCAGTTGTATC	(TG)9	53	150 - 160	4	0.400	0.720	0.672
KNUPF-47	TCCAAAACCCTGATTCTGTAAC	AATTTGATCCATGGGATCTTC	(TG)9	53	215 - 225	6	0.467	0.693	0.652
KNUPF-48	TGTCCATAAATGTTCAACCAGA	TCACCTATCATTTTCCATTGTG	(AG)26	53	125 - 135	5	0.333	0.738	0.692
KNUPF-49	CTAGGTGTGGGTGATTTTCAAT	AAACTACCTACCACCATTTCCC	(AG)17	53	125 - 135	6	0.267	0.809	0.781
KNUPF-50	TCGTGAATGAGGGTGGTG	GCTGCTATTGGCATTTCTTATG	(CT)17	54	160 - 185	7	0.267	0.827	0.804
KNUPF-51	CCTCCTCTAATACATGTTTCTGC	TGCAGCTTCTGTTATCTTGAAA	(AG)22	52	175 - 185	6	0.533	0.667	0.637
KNUPF-52	AAGACTGCATCTTTCACCAACT	TTTCTTTATACACACATCGGCA	(CT)17	53	205 - 215	4	0.600	0.578	0.531
KNUPF-53	GATTCATCATTCAGCTCTCTCC	ATGACCAATGGATTAAACAAGG	(CT)17	53	205 - 220	7	0.200	0.827	0.803
KNUPF-54	GCCATTTGGAGATGGAATG	ATTTCGAGACAAAAGCAACAAT	(GC)5	53	140 - 150	4	0.400	0.711	0.660
KNUPF-55	TGCTGTTGATGACTTGTATGGT	ATGAGATTTGGCTTCACAGAGT	(AGC)7	53	240 - 250	6	0.333	0.773	0.740
KNUPF-56	CCTATGCATCCTTTCCAAATAA	TACGAGGTTCTGCAAGAAAAAT	(AGC)7	53	240 - 245	4	0.333	0.738	0.690
KNUPF-57	AGCAGCACTCTTCTTCTTGTTC	TCTGCAGAAGTTGTAGTCGATG	(ATC)7	53	170 - 175	2	0.533	0.498	0.374
KNUPF-58	GTATATGTGTGGGAAGGTTGCT	TCAATTTCCTCATCAAATCAAA	(ATG)7	53	215 - 220	3	0.600	0.551	0.485
KNUPF-59	AATCTCGATGCCTAACAACAGT	TTCCTTGTAAATCCAGCTAAGG	(CAG)7	53	140 - 150	4	0.600	0.578	0.531
KNUPF-60	GCAATGGACATCTGTGAGAGTA	AATTGTGGTAATCATAGGGCAG	(CAG)7	53	180 - 185	4	0.667	0.507	0.462
KNUPF-61	GGGATACCCAAATTTCTACCAT	TCATGAAAAATCCAAACATTCA	(CAG)7	53	220 - 225	4	0.600	0.560	0.501
KNUPF-62	CCATCCTTCTTGTTCAACTCAT	AATGTTGATGAGGAGACGTTTT	(CAT)7	53	185 - 190	4	0.467	0.667	0.610
KNUPF-63	AATGTATTTTCGGCAGAGAGAA	CGGAGTTCAGAGCAAAGATTAT	(CGT)7	53	145 - 150	6	0.400	0.711	0.666
KNUPF-64	TTTGAAGGTCTAACAGTGTCTGAA	AACTGAGATTTTGACCAAGCAG	(CTA)7	53	230 - 240	5	0.400	0.720	0.674
KNUPF-65	TAAATCAAGTTGGTAAGCATGG	CAGAAAACTACCTCCATATCGC	(CTA)7	52	240 - 245	3	0.400	0.658	0.584
KNUPF-66	GTCCTTTTGTCAAGAGACTGCT	CCTTCTCCCTTTGAAGAAAAGT	(GCT)7	53	235 - 245	4	0.467	0.613	0.537
KNUPF-67	ATTGATTCTCTATCAACCTGGC	CTCATCATCGGATCAACCTAGT	(GCT)7	53	225 - 240	7	0.467	0.729	0.704
KNUPF-68	GAGTGAAATGCTCGACTTGAT	CAAGTCTCATTCTTTCCAGACC	(GTA)7	52	150 - 155	4	0.467	0.676	0.623
KNUPF-69	TCTTCTCCAAGTCATGTCTTCTT	TGGAGTGGTCGAGAGAAGTAGT	(TCA)7	53	60 - 65	3	0.600	0.560	0.499
KNUPF-70	GAGATCAATAGTGGCAGTGGTT	AAACTAAACCAATGGCGTAGAA	(TCG)7	53	125 - 135	5	0.467	0.693	0.650
KNUPF-71	GAAGAATGCATCAGTAACACGA	ATGCTGGCCAAGTAATAAGAGA	(AGCT)4	53	190 - 200	6	0.333	0.791	0.762
KNUPF-72	TAATTTGAGGGATTCCTTTCCT	CGCCACCCTTACTACTTCATAC	(TCGA)4	53	235 - 245	4	0.400	0.711	0.660
KNUPF-73	CCATTCTTCAATTCGATCAACTA	TCTGCAAATCATCCAGTTAAAA	(CTAG)4	53	135 - 145	3	0.733	0.427	0.388
KNUPF-74	TTGACTGTACCAGAGCATCAAG	GGGTACACTCACAACTCTACCAA	(AAAT)6	53	185 - 200	7	0.467	0.729	0.704
KNUPF-75	CATATTCTCACCACCAAACTCC	GAGAAGAGAAGGAAGCAAACAA	(CTTT)7	53	160 - 180	6	0.400	0.738	0.700
KNUPF-76	AAAGTTTAGACAGCCCAACAAA	CTTAGCGTCAAGAAACAGCAG	(AGGG)6	53	210 - 215	5	0.600	0.587	0.547
KNUPF-77	TTTTTGGTTGCTTTTTCTTGAT	AGCAGATAAAATGTGCTGGATT	(TATG)10	53	155 - 165	4	0.467	0.649	0.586
					Average	4.9	0.463	0.670	0.624

T_a: annealing temperature, MAF: major allele frequency, GD: genetic diversity, PIC: polymorphic information content.

from 0.200 (KNUPE-53) to 0.733 (KNUPE-73), with an average of 0.463. The GD ranged from 0.391 (KNUPE-45) to 0.853 (KNUPE-42), with an average of 0.670. The average PIC was 0.624, ranging from 0.315 (KNUPE-45) to 0.838 (KNUPE-42) (Table 1).

The analysis of the three groups of *Perilla* accessions (cultivated and weedy types of var. *frutescens* and cultivated type of var. *crispa*) using the 37 SSR primers showed that the average number of alleles ranged from 2.6 for the weedy type of var. *frutescens* to 3.4 for the cultivated type of var. *crispa*. The average GD were 0.586 and 0.461 for the cultivated and weedy types of var. *frutescens*, respectively, and 0.629 for the cultivated var. *crispa*. The average PIC were 0.524, 0.406 and 0.565 for the cultivated and weedy types of var. *frutescens* and cultivated var. *crispa*, respectively (Table 2). *Perilla* crop is widely distributed and cultivated in Korea, Japan and China. This information is about the genetic diversity of *Perilla* may be useful for the preservation of germplasm resources in East Asia.

Table 2. Estimates of allele number, MAF, gene diversity and PIC of 37 SSR primers among cultivated and weedy types of *Perilla*.

SSR loci	No. of alleles			Major Allele Frequency			Gene Diversity			PIC		
	Group1	Group2	Group3	Group1	Group2	Group3	Group1	Group2	Group3	Group1	Group2	Group3
	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)
KNUPF-41	3	4	4	0.600	0.400	0.400	0.560	0.720	0.720	0.499	0.672	0.672
KNUPF-42	4	4	5	0.400	0.400	0.200	0.720	0.720	0.800	0.672	0.672	0.768
KNUPF-43	4	1	4	0.400	1.000	0.400	0.720	0.000	0.720	0.672	0.000	0.672
KNUPF-44	3	2	4	0.600	0.800	0.400	0.560	0.320	0.720	0.499	0.269	0.672
KNUPF-45	1	2	2	1.000	0.800	0.600	0.000	0.320	0.480	0.000	0.269	0.365
KNUPF-46	3	3	3	0.600	0.400	0.400	0.560	0.640	0.640	0.499	0.563	0.563
KNUPF-47	4	2	4	0.400	0.600	0.400	0.720	0.480	0.720	0.672	0.365	0.672
KNUPF-48	4	4	2	0.400	0.400	0.600	0.720	0.720	0.480	0.672	0.672	0.365
KNUPF-49	4	4	3	0.400	0.400	0.400	0.720	0.720	0.640	0.672	0.672	0.563
KNUPF-50	5	3	5	0.200	0.400	0.200	0.800	0.640	0.800	0.768	0.563	0.768
KNUPF-51	3	2	4	0.600	0.800	0.400	0.560	0.320	0.720	0.499	0.269	0.672
KNUPF-52	2	2	4	0.800	0.800	0.400	0.320	0.320	0.720	0.269	0.269	0.672
KNUPF-53	4	5	5	0.400	0.200	0.200	0.720	0.800	0.800	0.672	0.768	0.768
KNUPF-54	3	3	4	0.400	0.600	0.400	0.640	0.560	0.720	0.563	0.499	0.672
KNUPF-55	3	3	3	0.400	0.400	0.600	0.640	0.640	0.560	0.563	0.563	0.499
KNUPF-56	3	3	3	0.600	0.600	0.400	0.560	0.560	0.640	0.499	0.499	0.563
KNUPF-57	2	2	2	0.600	0.800	0.800	0.480	0.320	0.320	0.365	0.269	0.269
KNUPF-58	3	1	3	0.600	1.000	0.600	0.560	0.000	0.560	0.499	0.000	0.499
KNUPF-59	3	3	2	0.600	0.600	0.600	0.560	0.560	0.480	0.499	0.499	0.365
KNUPF-60	2	2	3	0.800	0.800	0.400	0.320	0.320	0.640	0.269	0.269	0.563

Continued

KNUPF-61	3	1	3	0.400	1.000	0.400	0.640	0.000	0.640	0.563	0.000	0.563
KNUPF-62	3	2	2	0.600	0.800	0.600	0.560	0.320	0.480	0.499	0.269	0.365
KNUPF-63	3	2	4	0.600	0.800	0.400	0.560	0.320	0.720	0.499	0.269	0.672
KNUPF-64	2	4	2	0.600	0.400	0.600	0.480	0.720	0.480	0.365	0.672	0.365
KNUPF-65	3	2	3	0.400	0.800	0.400	0.640	0.320	0.640	0.563	0.269	0.563
KNUPF-66	3	2	3	0.400	0.600	0.600	0.640	0.480	0.560	0.563	0.365	0.499
KNUPF-67	3	3	5	0.600	0.600	0.200	0.560	0.560	0.800	0.499	0.499	0.768
KNUPF-68	4	3	3	0.400	0.600	0.600	0.720	0.560	0.560	0.672	0.499	0.499
KNUPF-69	2	2	3	0.600	0.600	0.600	0.480	0.480	0.560	0.365	0.365	0.499
KNUPF-70	3	4	2	0.600	0.400	0.600	0.560	0.720	0.480	0.499	0.672	0.365
KNUPF-71	4	3	4	0.400	0.600	0.400	0.720	0.560	0.720	0.672	0.499	0.672
KNUPF-72	3	3	3	0.600	0.600	0.600	0.560	0.560	0.560	0.499	0.499	0.499
KNUPF-73	3	1	3	0.600	1.000	0.600	0.560	0.000	0.560	0.499	0.000	0.499
KNUPF-74	4	2	4	0.400	0.800	0.400	0.720	0.320	0.720	0.672	0.269	0.672
KNUPF-75	3	3	5	0.400	0.600	0.200	0.640	0.560	0.800	0.563	0.499	0.768
KNUPF-76	3	2	3	0.400	0.800	0.600	0.640	0.320	0.560	0.563	0.269	0.499
KNUPF-77	3	3	3	0.600	0.600	0.600	0.560	0.560	0.560	0.499	0.499	0.499
Average	3.1	2.6	3.4	0.524	0.643	0.465	0.586	0.461	0.629	0.524	0.406	0.565

Group 1: Cultivated var. *frutescens*, Group 2: Weedy var. *frutescens*, Group 3: Cultivated var. *crispa.*

Our study results using new *Perilla* SSR primers validate the proposal that the weedy types of *Perilla* species are the key taxon in understanding the origin of the two cultivated types of var. *frutescens* and var. *crispa.* The new *Perilla* SSR primers described in this study should facilitate confirmation of the genetic diversity and could be used for the identification of cultivars, conservation of *Perilla* germplasm resources, and genetic mapping and designating of important genes/QTLs for *Perilla* crop breeding programs.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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