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Construction of EST-SSR Databases for Effective Cultivar Identification and Their Applicability to Complement for Lettuce (*Lactuca sativa* L.) Distinctness Test

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Abstract

The objectives of this study were to construct a database of expressed sequence tag (EST)-simple sequence repeat (SSR) markers to identify lettuce cultivars. A set of 370 EST-SSR primer pairs were applied for fingerprinting the lettuce cultivars. Fifty-eight EST-SSR markers showed hyper-variability and were able to differentiate 92 cultivars. A total of 176 polymorphic amplified fragments were obtained by the 58 markers, and two to eight SSR alleles were detected for each l˚Cus with an average of three alleles per locus. Average polymorphism information content (PIC) was 0.425, ranging from 0.022 to 0.743. Cluster analysis was based on Jaccard's distance coefficients using the method of unweighted pair group. In this method we used arithmetical averages (UPGMA) algorithm categorized 4 major groups, which were in accordance to morphological traits. The eight cultivars of three groups with 100% genetic similarity through SSR analysis were investigated by phenotypic traits. These cultivars including these pairs are very similar in 27 morphological characteristics. Therefore, these EST-SSR markers could be used to select similar cultivars through management of reference collection to complement distinctiveness test of lettuce cultivars.

Keywords

Expressed Sequence Tags, Simple Sequence Repeats, Cultivar Identification, Distinctness Test

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1. Introduction

Lettuce (*Lactuca sativa* L., Asteraceae) is one of the most widely cultivated vegetables [1]. Sixty-three lettuce cultivars obtained Plant Variety Protection (PVP) at the Korea Seed & Variety Service. This is the fourth highest number among vegetable crops in Korea (http://www.seed.go.kr). A new plant variety requires novelty, denomination, distinctness, uniformity, and stability (DUS) to get registered for plant breeder rights based on the International Union for the Protection of New Varieties of Plants (UPOV) regulations. The morphological characters are used for the DUS test. It is necessary to conduct two growing cycles at the fields or greenhouse for growth trials. Moreover, morphological characters are quantitatively inherited and their expression is affected by environmental factors. Thus, a rapid and robust "DNA marker technique" has been used to identify cultivars for the DUS test [2]-[4].

DNA markers have many advantages to identify cultivars due to their independence from environmental influences. The UPOV suggests that simple sequence repeat (SSR) markers are suitable for a DNA profiling database due to their multi-allelic nature, reproducibility, high polymorphism, easy automation, and co-dominant inheritance [5]. SSRs are present in both coding and noncoding regions [6]. Expressed sequence tags (ESTs)-SSRs have advantages over genomic SSRs representing noncoding regions because they are present in coding regions and the expressed sequence data can be easily retrieved from the public databases [7]. Moreover, EST-SSRs may enhance the applicability of DNA markers by expressing the variation in transcribed genes [8]. However, the development of SSR markers in lettuce has been very limited because it is costly, and labor intensive. There have been above 300 SSR markers described in the literature [9]-[11]. In addition, there are no reports on applicability for the DUS test using molecular marker in commercial lettuce cultivar. Therefore, we constructed EST-SSR profiles databases to effectively identify 92 cultivars widely cultivated in Korea and investigated the possibility of utilization in the distinctiveness tests by comparing morphological characterization of lettuce cultivars with limited variability through SSR analysis.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

92 commercial lettuce cultivars were used in this study (**Table 1**). DNA was extracted from the 20 seeds of each cultivar. DNA isolation was carried out using NucleoSpin® Plant II (Macherey-Nagel Cat. 740 770.250) based on the manufacturer's protocol. DNA concentration was adjusted to 20 ng using Nanodrop (Thermo Scientific, Rockford, IL, USA) and then used for polymerase chain reaction (PCR) analysis.

2.2. Screening of the Lettuce EST Database and Primer Design

A total of 81,330 ESTs were downloaded from NCBI (to July 1, 2011) and assembled with CAP3 [12]. A web tool called microsatellite identification (MISA) (http://pgrc.ipk-gatersleben.de/misa/) was used to search for SSRs in the unigenes with a minimum of six repeats units for a di-nucleotide, five repeats units for a tri-nucleotide, five repeats units for a tetra-nucleotide, four repeats units for a penta-nucleotide and hexa-nucleotide. SSR primers were designed with the Primer 3 program [13] and were used to generate PCR products of 100 - 300 bp at annealing temperatures (T_a) of 50˚C - 60˚C. Primers were synthesized by Bioneer Company (Daejeon, Korea).

2.3. PCR and Electrophoresis

The 370 SSR primers were used to identify lettuce cultivars. PCR amplifications were performed in 25 μL volume containing 20 ng template DNA, 10 pmol·μL^{-1} forward and reverse primers, 2.5 mM dNTP mixture, 10× PCR buffer solution, and 1 unit *Taq* polymerase (Genet Bio, Seoul, Korea). All primer combinations consisted of a 4 min initial denaturation at 94˚C followed by 40 PCR cycles of 30 s at 94˚C, 30 s at 55˚C, 45 s at 72˚C, and a final 10 min extension at 72˚C on T professional™ thermocycler (Biometra, Göttingen, Germany). To select informative SSR markers, seven cultivars ("Gangpungjeokchima", "Numberone", "Icered", "Eboniblack", "Topgreen", "Chirivel", and "Ace") among the 92 cultivars were first screened to select polymorphic SSR markers. The PCR products of the seven cultivars were separated on denatured 6% polyacrylamide gels and then silver stained with the DNA Silver Staining Kit (Promega Cat. Q4132, Madison, WI, USA) according to the manufacturer's instructions. From the genotyping result of the seven lettuce cultivars, SSR primer pairs with

Table 1. The 92 cultivars used to construct the DNA profile database using EST-SSR markers.

No.	Cultivars	Horticultural type	No.	Cultivars	Horticultural type
1	Joara	Leaf (red)	47	Asiaoraettajeokchima	Leaf (red)
2	Hotred	Leaf (red)	48	Jingangjeokchungmyeon	Leaf (red)
3	Daepungjeokchima	Leaf (red)	49	Chamjinhanjeokchungmyeon	Leaf (red)
4	Hongssam	Leaf (red)	50	Pungbuheukchungmyeon	Leaf (red)
5	Myungpumtojongjeokchukmyeon	Leaf (red)	51	Evergreen	Leaf (green)
6	Jeoksamgakchae	Leaf (red)	52	Ongreen	Leaf (green)
7	Onpungjeokchima	Leaf (red)	53	Cheongpungchima	Leaf (green)
8	Operajeokchukmyeon	Leaf (red)	54	Cheongpungyeoreumchima	Leaf (green)
9	Mipungpochabjeokchukmyeon	Leaf (red)	55	Yeoreumgaeryangdambae	Leaf (green)
10	Hongpungchima	Leaf (red)	56	Greencheongchima	Leaf (green)
11	Jangpungyeoreumchima	Leaf (red)	57	Sanggreen	Leaf (green)
12	Taepungyeoreumjeokchukmyeon	Leaf (red)	58	Topgreen	Leaf (green)
13	Hapungjeokpogi	Leaf (red)	59	Jeilsangchae	Leaf (green)
14	Jinsunhongjeokchukmyeon	Leaf (red)	60	Jeilcheongchukmyeon	Leaf (green)
15	Asiajinppalchima	Leaf (red)	61	Garyangdambaesangchu	Leaf (green)
16	Jeoksamgakchu	Leaf (red)	62	Hanbiccheongchima	Leaf (green)
17	Sunmangjeokchukmyeon	Leaf (red)	63	Greenglace	Leaf (green)
18	Chamjon	Leaf (red)	64	Scanstar	Leaf (green)
19	Yeonsanhongjeokchukmyeon	Leaf (red)	65	Snowgreen	Leaf (green)
20	Honghwajeokchukmyeon	Leaf (red)	66	Hanbatcheongchima	Leaf (green)
21	Dabaljeokchukmyeon	Leaf (red)	67	Sambokmeokchima	Leaf (black)
22	Yeoreumchammakjeokchima	Leaf (red)	68	Meokchima	Leaf (black)
23	Mujeokyeoreumjeokchima	Leaf (red)	69	Heukssammeokchima	Leaf (black)
24	Manhongpochab	Leaf (red)	70	Sunjoheukchima	Leaf (black)
25	Jinpungjeokchukmyeon	Leaf (red)	71	Mujeokyeoreumheukchima	Leaf (black)
26	Hartjeokchima	Leaf (red)	72	Meokdoli	Leaf (black)
27	Redstar	Leaf (red)	73	Eboniblack	Leaf (black)
28	Manchuredstar	Leaf (red)	74	Heukssamchima	Leaf (black)
29	Ttuksumcheongchukmyeon	Leaf (red)	75	Peoseutgyeolgu	Crisphead
30	Yeolgangjeokchima	Leaf (red)	76	Beseutgyeolgu	Crisphead
31	Rosequeenjeokchukmyeon	Leaf (red)	77	Wintergreen	Crisphead
32	Rubella	Leaf (red)	78	Buttikkeu	Crisphead
33	Rubella2ho	Leaf (red)	79	Eurake	Crisphead
34	Danpungjeokchima	Leaf (red)	80	Pungseong	Crisphead
35	Gangpungjeokchima	Leaf (red)	81	Adam	Crisphead
36	Numberone	Leaf (red)	82	Sensation	Crisphead
37	Icered	Leaf (red)	83	Chirivel	Crisphead
38	Jeokdan	Leaf (red)	84	Yeoreumgohyangdambae	Romaine
39	Sunpungpochabjeokchukmyeon	Leaf (red)	85	Cheonpung	Romaine
40	Ace	Leaf (red)	86	Sanggeurangssam	Romaine
41	Misunjeokchukmyeon	Leaf (red)	87	Sijeoseugreen	Romaine
42	Hwahongjeokchukmyeon	Leaf (red)	88	Mansang	Romaine
43	Redsunjeokchukmyeon	Leaf (red)	89	Cheonsang	Romaine
44	Pochabijeokchukmyeon	Leaf (red)	90	Starromaine	Romaine
45	Jeokchima	Leaf (red)	91	Sunnyredbutter	Butterhead
46	Sunhongjeokchukmyeon	Leaf (red)	92	Sunredbutter	Butterhead

highly reproducible and clear band patterns were respectively labeled at the end site of the forward primer with FAM, VIC, NED, and PET dye and then PCR amplification was performed. The PCR products (4 µL) were separated on 2% agarose gels and then 1 - 3 µL was mixed with 200 µL water depending on the intensity of the PCR products. 1.5 µL aliquot of diluted PCR product, 10 µL of deionized formamide and 0.25 µL of size marker (LIZ500 size standard) were mixed and denatured for 5 min at 94˚C. The PCR products of 94 cultivars were analyzed by capillary electrophoresis (Genetic Analyzer 3130XL, Applied Biosystems, Foster City, CA, USA) using the manufacturer's instructions. The allele determination for the SSR markers was evaluated with the GeneMapper 3.7 software program (Applied Biosystems).

2.4. Data Analysis

Peaks were scored with regard to the presence and absence of peaks for genotypes. Scores of "1" and "0" designated the presence and absence of peaks for each SSR marker allele, respectively. Polymorphism information content (PIC) was calculated via the formula established by [14]:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the j^{th} allele for the i^{th} locus summed across all alleles for the locus. The cluster analysis was based on Jaccard's similarity coefficient using the unweighted pair group method using arithmetical averages [15] method in the NTSYSpc 2.10b [16] software package. Mantel test was employed for the correlation between genetic and morphological distances [17].

2.5. Morphological Analysis

Morphological characters were analyzed according to the DUS characteristics of the lettuce test guideline prescribed by the Korea Seed & Variety Service (http://www.seed.go.kr). 29 were used among 39 morphological characters of lettuce for the DUS test.

3. Results and Discussion

3.1. Development of EST-SSR Markers

With 81,330 lettuce ESTs from the NCBI database, 41,609 singletons and 8452 contigs were identified by CAP3 software. In total, 4229 SSR loci from singletons [11] and 807 SSR loci from contigs were identified using the MISA program. Of 807 SSR loci, the highest proportion was trinucleotides (455, 56.4%), dinucleotides (266, 33%), hexanucleotides (42, 5.2%), pentanucleotides (31, 3.8%), and tetranucleotides (13, 1.6%). These results support the previous studies that trinucleotide repeats occurred in higher frequency than different repeat motif in lettuce [7] [18].

3.2. EST-SSR Polymorphisms in Lettuce

The 348 SSR primers developed previously [9]-[11] and 22 SSR primers among 710 primers designed in this study were used to screen in seven cultivars. Of the 370 SSR primers, 82 showed polymorphisms among tested cultivars (**Table 2**). 58 of the 82 polymorphic markers were selected on the basis of easy scoring, high reproducibility and peak quality among cultivars. Thereafter, 92 cultivars were examined using 58 SSRs by an automated DNA sequencing system (**Figure 1**). The PIC, number, and size of the alleles for the 92 cultivars were analyzed using the 58 EST-SSR markers (**Table 3**). A total of 176 alleles were obtained ranging from 2 - 8 alleles with an average of 3.03 alleles. The polymorphism information content (PIC) values, which are reflective of allele diversity and frequency among the varieties, were not uniformly high for the tested SSR l˚Ci. The average PIC value was 0.425 and it ranged from 0.022 (SML-052) to 0.743 (KSL-26). The variation at SSR loci in 92 cultivars are summarized in **Table 3**.

Several reports have described PIC value of SSR analysis in lettuce. This result was higher than 0.32 reported by [10] but lower than 0.55 of [19] and 0.56 of [20]. This may be mainly due to genetic background of tested cultivars and chromosome loci linked to SSR marker. Generally, the level of polymorphism for the EST-SSR

Table 2. SSR markers screened to identify lettuce cultivars and polymorphism of the SSR markers.

Number of screened markers	Type of SSR makers	Polymorphism (%) of amplified SSR markers	SSR marker source
20	EST-SSR of lettuce (*L. sativa* L.)	1/20 (5%)	Jeuken *et al*. (2008)
61	EST-SSR of lettuce (*L. sativa* L.)	30/61 (49.2%)	Simko (2009)
267	EST-SSR of lettuce (*L. sativa* L.) singletons	47/267 (17.6%)	Hong *et al*. (2013)
22	EST-SSR of lettuce (*L. sativa* L.) contigs	4/22 (18.2%)	Hong *et al*. (2013) and in this study
Total 370		82/370 (22.2%)	

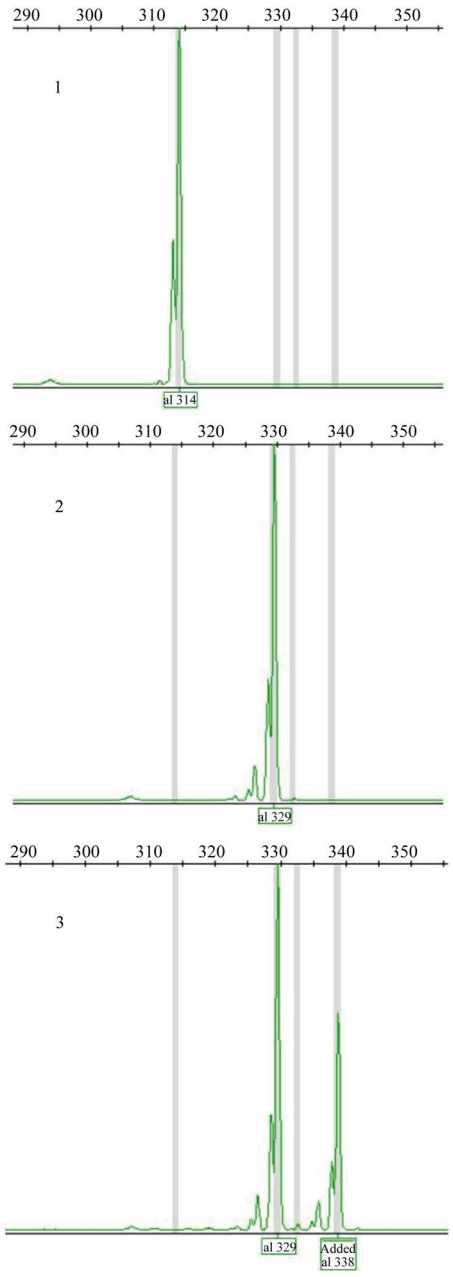

Figure 1. Amplified SSR fragments of 3 lettuce cultivars at SML-022 locus. The PCR products were separated using a Genetic Analyzer 3130XL (Applied Biosystems, USA) and detected using GeneMapper software (Applied Biosystems, USA). Lane 1: "Joara", Lane 2: "Daepungjeokchima", Lane 3: "Peoseutgyeolgu".

Table 3. Description of 58 polymorphic EST-SSR markers selected to construct a DNA profiling database for lettuce.

No.	Primer name	EST/Contig ID	Repeat motif	Primer sequence (5'-3')		Ta (˚C)	Product sizes	No. of alleles	PIC
1	SML-001	QGC15N13	(CATGAT)6	F: CCATGGATCCTGTGTGAAGA	R: CACCATGTTCCACTTCCACTT	55	176 - 198	4	0.581
2	SML-002	QGH4c05	(TTC)17	F: GTGATTGCATGCCAAATGAA	R: TTAGTAGCCCGCATGCTTTT	55	204 - 225	4	0.310
3	SML-003	QGA14A20	(GTTTT)5	F: CGGGCTGGTTTTGATTTTTA	R: TGTCAAATCGTCACGTGGTT	55	114 - 119	2	0.418
4	SML-007	QGG10L02	(TCACCA)19	F: ACACTTGCCGATTCCTTCAC	R: ACCCGTGTTGAAAATGGAGA	55	184 - 202	3	0.519
5	SML-013	Cntg-4755	(GAA)14…(CTG)5	F: TCCCATGATGGAGAGACTCA	R: CCCAAAAGGGAATAGCAACC	55	265 - 276	3	0.434
6	SML-015	Cntg-1438	(TGTTA)16	F: TTGAGGAGGGCATTTACGTC	R: GAGGCGTATCTCCAAGGTGT	55	254 - 269	3	0.471
7	SML-019	Cntg-1238	(ATATG)5	F: AAGGAGGAAAGTATGGTGAGGA	R: TGAAATGAAGCAACACACGA	55	163 - 168	2	0.375
8	SML-020	Cntg-419	(AATG)6	F: GTGGTCGTGATGATGCTTTG	R: TGCAATCCCTCTTTTCTTCAA	55	223 - 227	2	0.141
9	SML-038	Cntg-4846	(CCA)4	F: ACCTCCTCCGTCACCAAGT	R: TCGCAAATTTTCTTGCCTTT	55	189 - 192	2	0.291
10	SML-039	Cntg-5632	(CCCCTT)2	F: ATTACCCCTGGCCTTATGCT	R: TCGTATCTTGGCTGCTCCAT	55	229 - 235	2	0.444
11	SML-042	Cntg-6454	(CGGA)2 (AGGA)3 AAG (A)11* (GAAAGA)2	F: CATGAAGTGTTTTGGGGTGA	R: GGCCTTTCATTTCTTCCTCA	55	189 - 193	3	0.435
12	SML-043	Cntg-649	(T)14	F: TTCTTTCGCCCATCTGAAAC	R: AAACAGGGGGCTAACGATCT	55	192 - 198	3	0.468
13	SML-045	Cntg-7478	(AAG)9	F: ACAAAACCGTTTCACCCAAA	R: AGCCCTGTCCTCTTCAGGAT	55	220 - 226	3	0.375
14	SML-048	Cntg-7935	(T)12	F: TTTGAGGCAGGTGTTAGCTG	R: GAACAAAGTTACAACGAGAGGAAA	55	114 - 123	3	0.337
15	SML-051	QGC13L24	(TAA)8	F: CTCAAGGCGGCATCTACTTC	R: GACCCCTTGCTTGTTTCAGA	55	237 - 240	2	0.315
16	SML-052	Cntg-2202	(CAT)5	F: CTGTAGCCGGGAATTGAAGT	R: TGCCCCTAAACAAGACCTACA	55	219 - 228	2	0.022
17	SML-054	Cntg-3649	(TA)3	F: CCATGCCATGCAGTATACCTT	R: AAAAATGACTGCATACTTTGTGAA	55	269 - 271	3	0.529
18	SML-055	Cntg-3666	(TGA)15…(ATG)9	F: CTGCGTGTTTTAAGCCGTTT	R: TCCATAATAATATAATCGCACCAA	55	224 - 239	2	0.378
19	SML-056	QGE4h11	(TTA)14	F: GCCTAGTCCAATTGCTTTGC	R: CAGCTTAACATACTTTTGTTCATTCA	55	183 - 189	2	0.350
20	SML-057	QGB14L18	(GAA)14…(CTG)5	F: TCCCATGATGGAGAGACTCA	R: CCCAAAAGGGAATAGCAACC	55	266 - 278	3	0.437
21	SML-059	Cntg-4454	(TCT)12	F: GATAAAGGCTGGACCGATGA	R: GTTTGGTTTGGTTTGGCAAG	55	177 - 208	3	0.536
22	SML-061	Cntg-3414	(AACA)6	F: GAGTGCTGAGAAAGCCCAAG	R: TAAGCTGCTCTTCCCTCCTG	55	203 - 207	2	0.402
23	SML-021	Cntg-1077	(TA)8	F: TTGGGAGAATTTTCATTTCCA	R: AGTCATCTTTTTCACCCCACA	55	172 - 180	4	0.521
24	SML-022	Cntg-1211	(ATC)13	F: GGGCCTCAAATCCTCTCTG	R: TGTTCTTCCCCTCTTTGGAA	55	314 - 338	4	0.511
25	SML-026	Cntg-2481	(GAA)11	F: GGGTTCTCATTGGCTGACAT	R: TGTCTTCCAACCAAAACATACA	55	172 - 198	3	0.500
26	SML-028	Cntg-2789	(A)15	F: TGATCCAGGCTCTCCAGAAT	R: CACGACCATGAATGATAAGTGC	55	170 - 181	3	0.511
27	SML-032	Cntg-3460	(T)4	F: TTGGATTTGGGGTGATGAAT	R: GCATAGTAATTTGACATTTTGGCATA	55	215 - 216	2	0.465
28	SML-036	Cntg-4454	(TCT)12… (CCAAA)4	F: CTGCTGCTGTTTTGCTCTTG	R: CCTGAGGTGAGGTTGTCAAT	55	214 - 217	2	0.137
29	SML-037	Cntg-4499	(AAC)3	F: TTTTTCCCGATCTTTGCATC	R: AGCGAATCTTTGCTTTTTCG	55	211 - 214	2	0.282

Continued

No.	Primer name	EST/Contig ID	Repeat motif	Primer sequence (5'-3')		Ta (˚C)	Product size	No. of alleles	PIC
30	KSL-1	CLSS10849	(CAA)10	F: CACCACTCCATTTCATCCCA	R: GCTCATTCCCAAACCCAGAT	55	162 - 171	3	0.197
31	KSL-37	CLSM1424	(AGA)15	F: TCTCTTGCTCCAATACCCGA	R: GTATCGGGCTCATGTCCCTT	55	125 - 155	6	0.666
32	KSL-51	CLSZ1624	(ATG)10	F: CCCCTACCACCACCAAAGTC	R: TACCAAATGACATGCACCCC	55	184 - 205	4	0.461
33	KSL-271	CLSS8197	(ATG)12	F: ACAAAGGCAAGATTGGGTCA	R: GCGGATATGCAGCCATAACA	55	238 - 250	3	0.505
34	KSL-316	QGC8F02	(AAT)11	F: CGCAGCCTTCAAAACTACCA	R: AGCAACTGAAATCCAACCCC	55	272 - 281	2	0.461
35	KSL-317	QGC8E09	(ATG)11	F: TGTGGATCTGAATGGGCATC	R: TGCAAGAATGTTGGCTTCCT	55	248 - 251	2	0.496
36	KSL-357	CLSS10992	(TGA)14	F: GCAGCAACAAGAAACCCAAA	R: GCCCCAACATCATCATCATC	55	255 - 283	2	0.356
37	KSL-87	CLSY5704	(CT)17	F: GCGGGATCGATACTTACCCT	R: ATCATCGACGGGCTTTTCTT	55	256 - 272	7	0.614
38	KSL-173	CLSM444	(CT)14	F: ATAGTCACGACTCACGCCCA	R: CCATTTTCCTCTTTCTGCGA	55	155 - 165	4	0.608
39	KSL-245	CLSM513	(AG)16	F: CTTCACCTCCGGAATCCTGT	R: GAGGCACGACTGCCATTTAG	55	269 - 283	4	0.138
40	KSLC-4	A35	(GGT)5	F: TGGGGGAACATCACAAACAC	R: ACTTCCGACCACCAATAGGG	55	196 - 199	2	0.328
41	KSLC-30	B14	(CA)6	F: GGGGCCTCTATCCACTCTCA	R: AAAGTCCAGCCATCTCTGCC	55	162 - 164	2	0.437
42	KSLC-322	L146	(TTATA)4	F: CTCCTCCGGGAAACTATGGA	R: TCTCCAACACAACACCCACC	55	290 - 300	3	0.349
43	KSLC-443	R439	(GAAGAG)4	F: GACGATGACGACGTGGAAAG	R: CCTCCAGCTGCAAACCAGTA	55	262 - 271	2	0.063
44	KSL-7	CLSS10499	(TCT)12	F: TGCTCAATCTCGAGCTTATCCT	R: ATGTGCCCACAAGGAAGACA	55	378 - 396	3	0.505
45	KSL-26	CLSM14994	(TC)16	F: GGGCTTTCTCTCCTTTCCTTT	R: AATTTGGATCCTGTCGAGGG	55	300 - 319	8	0.743
46	KSL-32	CLSM14764	(CT)14	F: CGGGGAGCATTTAGTGTGTG	R: AATTTGGGGTCCGATTTGAG	55	210 - 218	5	0.610
47	KSL-43	CLSM1373	(ATC)9(TTC)8	F: GACGCAAACCTTTACCAGCA	R: TCATTCCATTCCATTGGGTG	55	269 - 281	3	0.501
48	KSL-44	CLSM13555	(TCACCA)4tcat (CATCAC)5catcg (CCATCA)5	F: CGATTCCTTCACCTCCACCT	R: CTGTCAATCGTGCCAGTTCC	55	229 - 246	3	0.510
49	KSL-75	CLSY7906	(TC)6tt(TC)6	F: AGAGGCTTTCTACGCCAACC	R: TGAGGAGGGGGAAGTTCATC	55	205 - 207	2	0.444
50	KSL-83	CLSY6646	(AG)6tgtgt(GA) 7aa(GT)6	F: GCGGAGCTTCTTTCTCACCT	R: GGAAAGAAAACGCATTTCGAG	55	249 - 287	2	0.315
51	KSL-92	CLSY517	(CT)20	F: GGTCTCTTTCCTCTGCCCTG	R: TCGCGTTCTGAAGTAGCCAT	55	188 - 196	5	0.735
52	KSL-97	CLSY4815	(CT)11	F: CGCAGAAAAGGGATCAGACA	R: TCAGAGACACTGCAAAAGGGA	55	223 - 232	3	0.616
53	KSL-102	CLSY4549	(TGA)5tgt(TGA)6	F: AAGCCCCAATGACGAATCTC	R: TGTCAATAGCAATGGCGAAA	55	313 - 320	3	0.121
54	KSL-115	CLSM11208	(CT)11	F: CATTGCACTCCGTCATCTCC	R: GGGTTGATTCCGAAAGTTCC	55	210 - 212	2	0.477
55	KSL-119	CLSM10279	(TC)16	F: TTCGACTCGTCTTCGACGC	R: CGATGTCACACCACCCATCT	55	271 - 279	4	0.642
56	KSL-123	CLSL2393	(ATC)13	F: ATTGTAACTTCTGCGGGCCT	R: GCCTCACATGTTCTTCCCCT	55	336 - 360	4	0.511
57	KSL-137	CLSZ3622	(TGA)9caatgatgaaa aagatgatgcagag-gaggcagatgat-gatgctggagatgaa-gatttctcaggagaa-gaaaggggga-gag(GAT)6gaagaag accctagtgaa-gatcctaagg-caaatggtaatcaagac-gac(GAT)7gacgacg ac(GAT)6	F: TTCTCTGAGCTTCACAAGAGGG	R: TCATCACCATCATCATTTCCC	55	281 - 298	4	0.666
58	KSL-152	CLSM12854	(GAC)5gatgagc (AAG)8	F: CGAGGTCGAAGAAGAGGAGG	R: TCCCATTAGGATCTCTGCCC	55	268 - 284	2	0.063
	Total							176	24.63
	Mean							3.03	0.425+

markers was lower than that for the genomic SSR markers. The reason might be that EST-SSR markers are based on the translated region [21]. However, the set of 58 EST-SSR markers selected in this study might be very informative for identifying 92 commercial lettuce cultivars in Korea.

3.3. Genetic Relationships among the Lettuce Cultivars

A dendrogram based on the similarity coefficients of the 92 cultivars was constructed. The dendrogram scale varied from 0.29 to 1.00 (**Figure 2**) and were clustered into four major groups at a similarity level of 0.40. Cluster I consisted of cultivars with red leaf lettuce, which was split into three clusters with a mean similarity of 0.47.

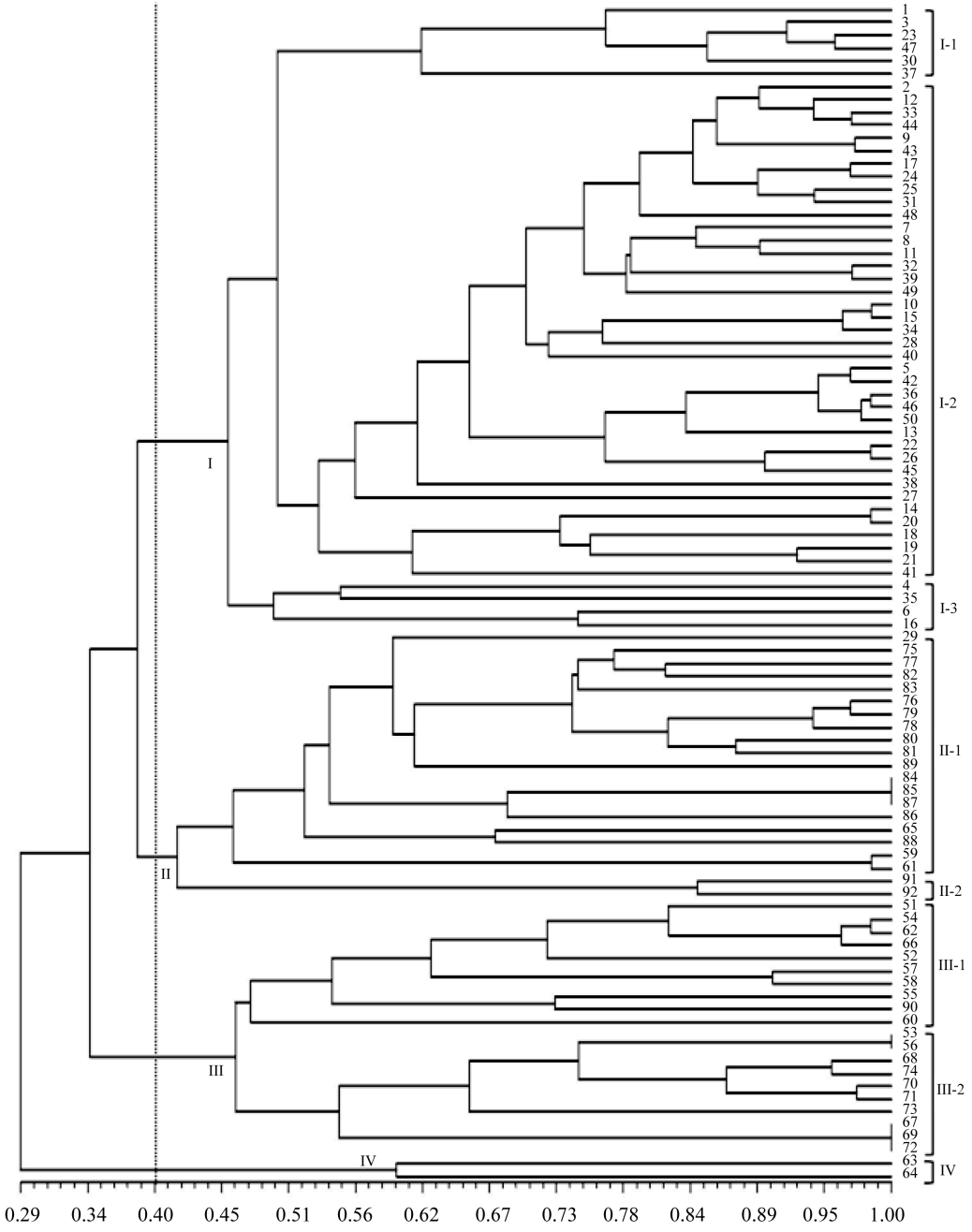

Figure 2. Phenetic dendrogram of 92 lettuce cultivars based on SSR markers. The scale at the bottom is Jaccard's coefficient of similarity. The numbers (1 - 92) at right refer to the list of cultivars in **Table 1**.

Cluster II consisted of cultivars with Crisphead, Romaine, Green leaf, and Butterhead, which split into two clusters with a mean similarity of 0.43. Cluster II-1 consisted of cultivars with Crisphead, Romaine, Green leaf, and cluster II-2 cultivars with consisted of the Butterhead type. Crisphead was clustered in one group with a similarity level of 0.70. Cluster III consisted of cultivars with green leaf and black leaf at a similarity level of 0.47. Cluster III-1 contained cultivars with green leaf, and cluster III-2 contained black leaf except for "Cheongpungchima" and "Greencheongchima". Cluster IV contained cultivars with the green leaf of "Greenglace" and "Scanstar". Generally, the clustering of 92 lettuce cultivars was mainly categorized into 4 major groups corresponding to morphological traits. However, eight cultivars (Group 1: "Cheongpungchima" and "Greencheongchima", Group 2: "Sambokmeokchima", "Heukssammeokchima", and "Meokdoli", Group 3: "Yeoreumgohyangdambae", "Cheonpung", and "Sijeoseugreen") were not discriminated by the 58 EST-SSR markers. These cultivars may be developed by parents with narrow genetic background, and a limited number of SSR markers were used to identify for lettuce cultivars.

3.4. Correlation between EST-SSR Markers and Morphological Characters

We investigated morphological traits for 3 pairs with 100% genetic similarity. However, cultivars in each pairs were not distinct for the 29 morphological traits under same environmental condition (**Table 4** and **Figure 3**). In

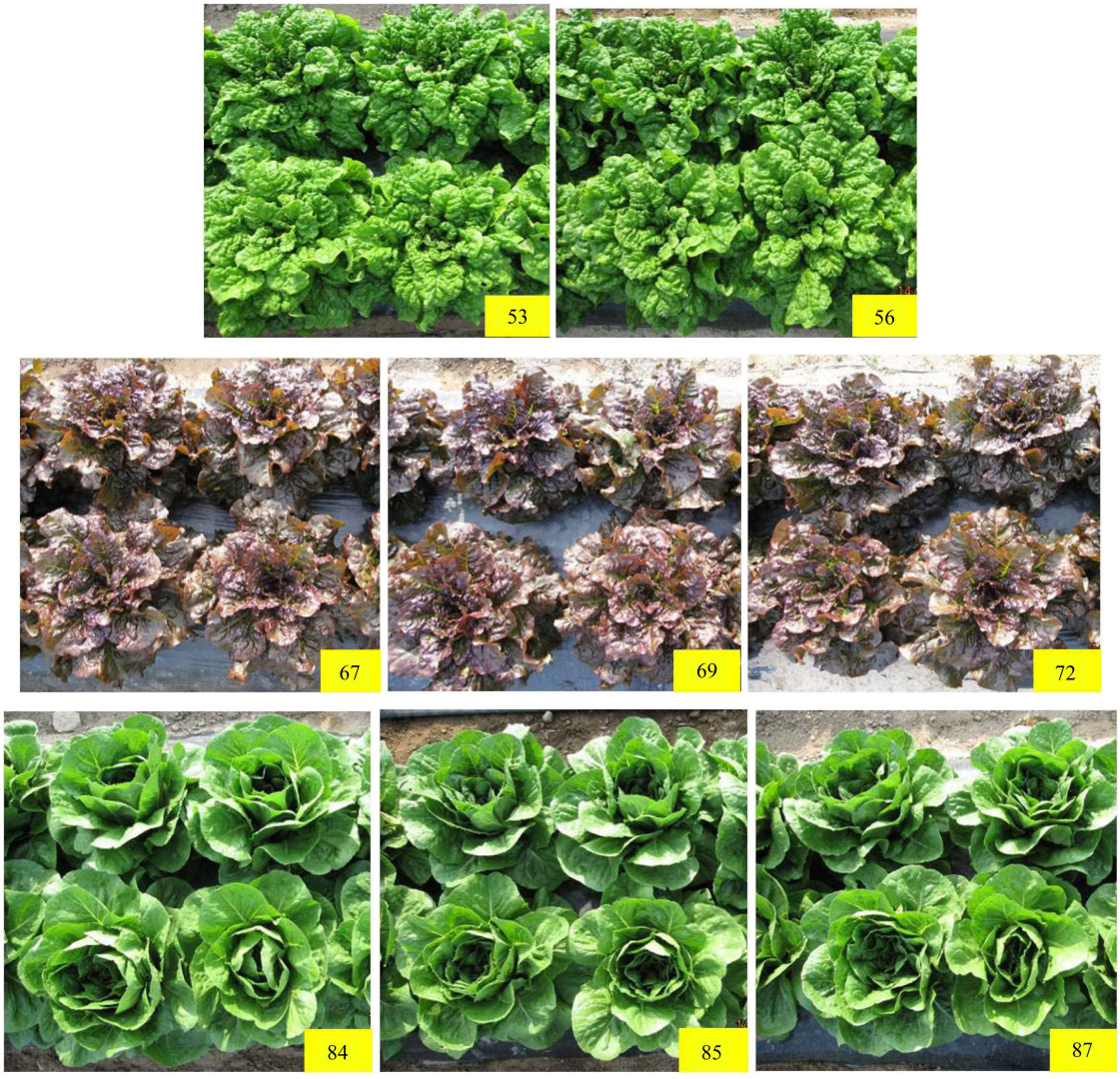

Figure 3. Morphological description of the eight cultivars representing 100% genetic similarity. The numbers in the pictures represent lettuce cultivar numbers in **Table 1**.

Table 4. Some morphological characteristics of the eight cultivars used for the distinctness test.

Character code	Character description	Characteristics	Type of char.	Note	Group 1		Group 2			Group 3		
					53	56	67	69	72	84	85	87
1	Seedling: anthocyanin coloration	Absent	QL	1	1	1	9	9	9	1	1	1
		Present		9								
2	Seedling: size of cotyledon	Small	QN	3	5	5	5	5	5	5	5	5
		Medium		5								
		Large		7								
3	Seedling: shape of cotyledon	Narrow elliptic	QN	3	5	5	5	5	5	5	5	5
		Elliptic		5								
		Broad elliptic		7								
4	Leaf: attitude at 10 - 12 leaf stage	Erect	QN	3	5	5	5	5	5	5	5	5
		Semi-erect		5								
		Prostrate		7								
5	Leaf blade: division	Absent	QL	1	1	1	1	1	1	1	1	1
		Present		2								
6	Plant: diameter	Very small	QN	1	7	7	5	5	5	7	7	7
		Small		3								
		Medium		5								
		Large		7								
		Very large		9								
7	Plant: head formation	No head	PQ	1	1	1	1	1	1	2	2	2
		Semi heading		2								
		Joined-up type		3								
		Wrapped-overtype		4								
8	Leaf: thickness	Thin	QN	3	7	7	5	5	5	7	7	7
		Medium		5								
		Thick		7								
9	Leaf: attitude at harvest maturity	Erect	QN	3	5	5	5	5	5	5	5	5
		Semi-erect		5								
		Prostrate		7								
10	Leaf: shape	Narrow elliptic	PQ	1	2	2	2	2	2	2	2	2
		Medium elliptic		2								
		Broad elliptic		3								
		Circular		4								
		Transverse broad obtrullate		5								
		Transverse narrow obtrullate		6								
		Obovate		7								
		Broad obtrullate		8								
		Triangular		9								
11	Leaf: color of outer leaves	Yellowish	PQ	1	2	2	5	5	5	2	2	2
		Green		2								
		Greyish green		3								
		Blueish green		4								
		Reddish		5								
12	Leaf: intensity of color of outer leaves	Very light	QN	1	5	5	7	7	7	5	5	5
		Light		3								
		Medium		5								
		Dark		7								
		Very dark		9								
13	Leaf: anthocyanin coloration	Absent	QL	1	1	1	9	9	9	1	1	1
		Present		9								
14	Leaf: intensity of anthocyanin coloration	Very weak	QN	1	-	-	7	7	7	-	-	-
		Weak		3								
		Medium		5								
		Strong		7								
		Very strong		9								

Continued

Character code	Character description	Characteristics	Type of char.	Note	Group 1		Group 2			Group 3		
					53	56	67	69	72	84	85	87
15	Leaf: distribution of anthocyanin	Localised	QL	1	-	-	2	2	2	-	-	-
		Entire		2								
16	Leaf: kind of anthocyanin distribution	Diffused only	QL	1	-	-	1	1	1	-	-	-
		In spots only		2								
		Diffused and in spots		3								
17	Leaf: glossiness of upper side	Absent or very weak	QN	1	3	3	7	7	7	5	5	5
		Weak		3								
		Medium		5								
		Strong		7								
		Very strong		9								
18	Leaf: surface profile of outer leaves	Concave	QN	3	5	5	5	5	5	5	5	5
		Flat		5								
		Convex		7								
19	Leaf: blistering	Absent or very weak	QN	1	9	9	3	3	3	1	1	1
		Weak		3								
		Medium		5								
		Strong		7								
		Very strong		9								
20	Leaf: size of blisters	Small	QN	3	7	7	5	5	5	3	3	3
		Medium		5								
		Large		7								
21	Leaf blade: degree of undulation of margin	Absent or very weak	QN	1	5	5	5	5	5	1	1	1
		Weak		3								
		Medium		5								
		Strong		7								
		Very strong		9								
22	Leaf blade: presence of incisions of margin on apical part	Absent	QL	1	1	1	1	1	1	1	1	1
		Present		9								
23	Leaf blade: depth of incisions of margin on apical part	Shallow	QN	3	-	-	-	-	-	-	-	-
		Medium		5								
		Deep		7								
24	Leaf blade: degree of incisions on margin on apical part	Sparse	QN	3	-	-	-	-	-	-	-	-
		Medium		5								
		Dense		7								
25	Leaf blade: venation	Not flabellate	QL	1	1	1	1	1	1	1	1	1
		Flabellate		2								
26	Axillary sprouting	Absent or very weak	QN	1	1	1	1	1	1	1	1	1
		Weak		3								
		Medium		5								
		Strong		7								
		Very strong		9								
27	Bitter taste	Absent	QL	1	1	1	1	1	1	1	1	1
		Present		9								
28	Time of harvest maturity	Early	QN	3	5	5	5	5	5	5	5	5
		Medium		5								
		Late		7								
29	Time of beginning of bolting under long day conditions	Very early	QN	1	7	7	7	7	7	7	7	7
		Early		3								
		Medium		5								
		Late		7								
		Very late		9								

QL: qualitative, QN: quantitative, PQ: pseudo-qualitative. The upper numbers refer to the list of cultivars in **Table 1**.

addition, lettuce breeders were in agreement with our results of morphological traits. The correlation between molecular and morphological data for 8 cultivars in 3 pairs was analyzed by the [17] and revealed a good fit between the two data (r = 0.82). These results may be due to using large number of SSR markers derived from EST representing coding regions, and precise genotyping through DNA sequencing system for cultivar identification.

UPOV established the possible use of molecular markers for a DUS test [22]. They suggested four possible application models by technical committee based on research by the working group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT). Option 1 is to use molecular markers that are directly linked to traditional characteristics (gene-specific markers). Option 2 is to calibrate the threshold levels for molecular characteristics against the minimum distance for traditional characteristics. Option 3 is to use the molecular marker characteristics. Option 4 is combining phenotypic and molecular distances to manage a cultivar collection. Of the four options, options 1, 2, and 4 are positive assessment models. Option 3 is not a positive assessment [22]. We tested the morphological characteristics for the cultivars with 100% genetic similarity on the basis of molecular markers. Their correlation was a good fit (r = 0.82). Thus, we conclude that the EST-SSR markers selected in this study will be useful for choosing the most similar cultivars to candidate cultivars, protection of plant breeder's rights, and an alternative choice to conduct a DUS test in lettuce.

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