Development of SSR Markers in Carnation (*Dianthus caryophyllus*)

Tetsuya Kimura^{1*}, Masafumi Yagi², Chikako Nishitani³, Takashi Onozaki², Yoshiyuki Ban¹
and Toshiya Yamamoto³

¹National Center for Seeds and Seedlings, Tsukuba 305-0852, Japan

²National Institute of Floricultural Science, Tsukuba 305-8519, Japan

³National Institute of Fruit Tree Science, Tsukuba 305-8605, Japan

Thirteen SSR (simple sequence repeat) markers were successfully developed in carnation using an enriched genomic library method. All 13 primer pairs designed could successfully amplify target fragments of the expected size for the original variety ‘Francesco’ or ‘Barbara’. Thirty-nine of 41 carnation varieties, except for mutant varieties, were successfully distinguishable using 32 putative alleles and 15 polymorphic bands produced from 13 SSR markers. ‘U Conn Sim’ and ‘White Sim’, which are bud mutants originated from ‘William Sim’, showed an identical genotype to ‘William Sim’. Three (CB026a, CB057a, and CB060a) of 13 SSR markers were thought to be derived from multi-locus because the 3 SSR markers produced more than 3 discrete amplified fragments. The number of alleles per locus ranged from 1 (CB020a and CB041a) to 9 (CB003a), with an average value of 3.2. The values of the observed heterozygosity (H_O) and the expected heterozygosity (H_E) ranged from 0 to 0.59 (mean 0.30) and 0 to 0.68 (mean 0.34), respectively. The power of discrimination (PD) ranged from 0 (CB020a and CB041a) to 0.93 (CB026a) (mean 0.49). The parentage of 2 carnation varieties from intraspecific crosses was analyzed using 10 SSR markers, and the hybridity of the progenic varieties was confirmed. SSR markers were very effective for variety discrimination and determination of carnation parentage.

Key Words: *Dianthus caryophyllus*, genetic diversity, genetic relatedness, parentage test, simple sequence repeat.

Introduction

The genus *Dianthus* consists of more than 300 species and includes many important ornamental species, such as carnation (*Dianthus caryophyllus* L.). Carnation is one of the most important cut flower crops in the world. Many *Dianthus* species are also used for garden and potted plants, and are grown and distributed in temperate climate regions around the world (Mii et al., 1990). More than 30,000 varieties have been listed for *Dianthus* in the International Dianthus Register in England (Gatt et al., 1998). In Japan, about 1,200 varieties are listed in the new variety registration system under the seeds and seedlings law (<http://www.hinsyu.maff.go.jp/>, October 14, 2008). Many carnation varieties have been bred as they are one of the leading commodities in the ornamental industry (Jensen and Malter, 1995).

Recently, molecular markers have been developed and used to identify several characteristics controlling flower longevity (De Beneditti et al., 2003, 2005), resistance

to disease (Onozaki et al., 1999, 2004; Scovel et al., 2001; Yagi et al., 2006), and double flowers (Scovel et al., 1998) in carnation breeding programs. Molecular markers have been also utilized to confirm interspecific hybrids (Nimura et al., 2003, 2006) and variety identification (Smulders et al., 2000, 2003).

In humans and animals, SSR markers of a single locus have been used in parentage analysis and criminal investigations (Glowatzki-Mullis et al., 1996; Hughes, 1992). SSR markers have several advantages over other molecular markers in that they provide a more reliable method for DNA fingerprinting because of their co-dominant inheritance and large number of alleles per locus; therefore, SSR markers can be utilized for variety identification, parentage analysis and genetic relatedness. SSR markers are expected to be used in DNA profiling techniques in order to protect the rights of the breeders of a new plant variety (<http://www.hinsyu.maff.go.jp/>, October 14, 2008).

In previous studies, we established genomic libraries enriched for di-nucleotide repeat motifs and developed SSR markers for pear, peach, and rose (Kimura et al., 2006; Yamamoto et al., 2002a, 2002b, 2002c). The

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* Corresponding author (E-mail: tetsuki@affrc.go.jp).

developed SSR markers were successfully utilized to construct genetic linkage maps, genetic identification, and parentage analysis in pear (Kimura et al., 2002, 2003, 2006; Yamamoto et al., 2002b, 2006).

Smulders et al. (2000, 2003) reported that the 8 simple sequence repeat (SSR) markers developed could be used to evaluate the genetic diversity of *Dianthus* species and identify carnation varieties. However, the number of SSR markers is still limited for efficient variety identification and confirmation of hybrids in carnation; therefore, it is necessary to increase the number of SSR markers for genetic studies.

In this study, we isolated and developed polymorphic SSRs from enriched genomic libraries of carnation varieties. Genetic identification of 41 standard- and spray-type varieties was performed using established SSR markers. The hybridity of 2 varieties derived from 2 combinations of intraspecific crosses was examined using 10 SSR markers.

Materials and Methods

Plant materials and DNA extraction

Genomic DNAs used for SSR isolation were extracted for a standard-type variety 'Francesco' and a spray-type variety 'Barbara' using a Genomic-tip20/G column and the G2 Buffer method (Yamamoto et al., 2006).

Forty-one carnation (*Dianthus caryophyllus* L.) varieties, were used including 23 standard-type and 18 spray-type varieties (Table 1). Plant materials were obtained from the National Center for Seeds and Seedlings (NCSS, Tsukuba, Japan) and the National Institute of Floricultural Science (NIFS, Tsukuba, Japan). 'Miracle Rouge', '85-11' and their parent varieties were used for hybrid confirmation.

Genomic DNAs were isolated from fresh young leaves using the DNeasy Plant Mini Kit (Qiagen, Japan) according to the manufacturer's instructions. One hundred milligrams of fresh leaf tissue was homogenized in liquid nitrogen. The tissue powder obtained was suspended in 400 µL AP1 lysis buffer, 4 µL RNase A solution (100 mg·mL⁻¹), 8 µL 2-mercaptoethanol and 100 mg polyclar AT, and then incubated at 65°C for 10 minutes. One hundred and thirty microliters of AP2 buffer was added to the solution and put on ice for 5 minutes. After centrifugation at 15,000 rpm for 10 min, the solution was applied to the QIAshredder spin column. After centrifugation, the flow-through fraction was recovered. AP3 buffer was added to the suspension and then added to the DNeasy Plant Mini Prep Column. After washing with AW buffer, genomic DNA was eluted with 50 µL of sterilized Milli-Q water.

SSR isolation

Enriched genomic libraries were constructed from 'Francesco' and 'Barbara'. Genomic DNAs were digested with *Eco* RI and *Mse* I, and then ligated into adaptors using the AFLP Analysis System I (Invitrogen,

USA) at twice the scale of the manufacturer's instructions. Pre-PCR was conducted using *Eco*-primer (5'-GACTGCGTACCAATTC-3') and *Mse*-primer (5'-GATGAGTCCTGAGTAA-3'). Amplification was performed in a 200 µL solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each of dNTPs, 10 pmoles of *Eco*-primer and 60 pmoles of *Mse*-primer, adaptor-ligated DNA, and 10 units of Taq DNA polymerase (Invitrogen). Amplification was performed with 5–10 cycles at 90°C for 30 s, 56°C for 60 s, and 72°C for 60 s, for denaturing, annealing and primer extension, respectively. PCR products were hybridized with 100 pmoles of 3'-biotin-labelled (AG)₁₅ oligonucleotides after denaturation. The sample obtained was mixed thoroughly with 100 µg of Dynabeads M-280 Streptavidin (Dyna, Norway) in STEX buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.1% Triton X-100, pH 8.0) for 30 min.

The conjugates were collected with a magnet and washed with STEX buffer 3 times. The washed conjugates were suspended in 1/10 TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). After boiling for 3 min., the target DNAs were recovered from the supernatant solution. PCR was conducted using the enriched DNAs as templates under the same conditions as for pre-PCR except that 25 cycles were adopted. The PCR products were ligated into the pCR2.1 vector and transformed into competent *Escherichia coli* cells (Original TA Cloning Kit, Invitrogen). Colony blot hybridization was performed to select the plasmids containing repeat sequences. Recombinants were transferred onto nylon membranes (Hybond N⁺, Amersham, UK) and then hybridized with 5'-biotin-(AG)₁₅ oligonucleotides as a probe. Positive clones were detected using the BrightStar BioDetect kit (Ambion, USA), according to the manufacturer's instructions. Positive plasmids were sequenced using a PRISM 377 DNA sequencer (PE-Applied Biosystems, USA) after cycle sequence reaction with BigDye Terminator Cycle Sequencing Kit (PE-Applied Biosystems). SSR primer sets were designed using the software OLIGO ver. 6.0 (Molecular Biology Insights, USA).

PCR amplification of SSRs

Thirteen SSR primer pairs originating from enriched SSR clones were used for PCR amplification (Table 2). PCR amplification was performed in a 20 µL solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each of dNTPs, 10 pmoles of each forward primer labeled with fluorescent chemicals (Fam, Ned or Vic) and unlabelled reverse primer, 10 ng of genomic DNA, and 0.5 unit of Taq DNA polymerase (Invitrogen). Amplification was performed with 35 cycles at 94°C for 1 min, 55–60°C for 1 min and 72°C for 2 min, for denaturation, annealing, and primer extension, respectively. PCR products were separated and detected using a PRISM 3100 DNA

Table 1. Plant materials used in this study.

No.	Variety name	Flower type	Source ^z
1	Aicardi	standard	NCSS
2	Brelag	standard	NCSS
3	Design	standard	NCSS
4	Empire	standard	NCSS
5	Felix	standard	NCSS
6	Francesco	standard	NCSS
7	Keipacks	standard	NCSS
8	Kobuchi Santo	standard	NCSS
9	Laville Dorina	standard	NCSS
10	Miracle Rouge	standard	NIFS, hybrid of 63-41 and 62-48
11	Novelpink	standard	NCSS
12	Opera	standard	NCSS
13	Riccardo	standard	NCSS
14	Saleya	standard	NCSS
15	Sorriso	standard	NCSS
16	U conn Sim	standard	NIFS, bud mutant from William Sim
17	White Sim	standard	NIFS, bud mutant from William Sim
18	William Sim	standard	NIFS
19	62-48	standard	NIFS, male parent of Miracle Rouge
20	63-7	standard	NIFS, male parent of 85-11
21	63-35	standard	NIFS, female parent of 85-11
22	63-41	standard	NIFS, female parent of Miracle Rouge
23	85-11	standard	NIFS, hybrid of 63-35 and 63-7
24	Alicetta	spray	NCSS
25	Alpine Rose	spray	NCSS
26	Barbara	spray	NCSS
27	Breime	spray	NCSS
28	Breto	spray	NCSS
29	Bridal White	spray	NCSS
30	CFPC Rose Malea	spray	NCSS
31	Hilfines	spray	NCSS
32	HY Bis	spray	NCSS
33	Kleoya	spray	NCSS
34	Koreno	spray	NCSS
35	Lilac	spray	NCSS
36	Mystic Green	spray	NCSS
37	Ronia	spray	NCSS
38	Rose Clavel	spray	NCSS
39	Stadisoc	spray	NCSS
40	Super Gold	spray	NIFS
41	Vega	spray	NCSS

^z Abbreviations of varieties sources are as follows. NCSS: National Center for Seeds and Seedlings, NIFS: National Institute of Floricultural Science.

sequencer (Applied Biosystems). The size of the amplified products was calculated based on internal standard DNA (HD400ROX, Applied Biosystems) using GeneScan software (Applied Biosystems).

Statistical analysis

The heterozygosity and power of discrimination of carnations were estimated for SSR loci. The observed heterozygosity (H_O) and the expected heterozygosity (H_E) were calculated using CERVUS ver. 2.0 software

(Marshall et al., 1998). H_O was calculated as the number of heterozygous genotypes at a given locus divided by the total number of genotypes scored at that locus. H_E was calculated using an unbiased formula from allele frequencies assuming Hardy-Weinberg equilibrium (equation 8.4, Nei, 1987). The power of discrimination (PD) was calculated based on the frequency of each genotype according to the formula: $PD = 1 - \sum p_i^2$ where p_i is the frequency of the i^{th} genotype of each SSR locus (Kloosterman et al., 1993). A phenogram of 41 varieties

Table 2. Characteristics of 13 microsatellite markers derived from an enriched genomic library of carnation.

SSR marker	Forward and reverse primer sequences (5'-3')	Motif	Ta ^z (°C)	PCR ^y product size (bp)	No. of alleles (No. of polymorphic bands)	Single/multi locus	H _O ^x	H _E ^x	PD ^w
CF003a	F: CAGTTGAAGAAGTTTGAATGAATCGC R: TTCTCTCTCTAAACCCCCCAA	(GAA) ₅ GTT(AG) ₉	55	127	3	single	0.59	0.57	0.73
CB003a	F: TCTGCTTGATCTAATGGTGGACA R: ATGGCTTTCTCTAGGCTTTGGG	(TA) ₂₀ (AG) ₁₄	55	187	9	single	0.49	0.68	0.83
CB004a	F: GAITGGGTGGGGCGTGA R: CCATCCTCCTCCTTTCTATCTTCCCT	(AG) ₁₂	55	157	2	single	0.02	0.02	0.05
CB011a	F: GGATTGTTGGACTAGGATGA R: AGGACATGGATGATTTACAGTG	(AG) ₁₅	55	206	3	single	0.49	0.49	0.69
CB016a	F: CCTGGTGTGATAATGAATACG R: AATAGCCTCCTCTCCTCTCCTC	(AG) ₁₀	55	185	4	single	0.46	0.49	0.67
CB018a	F: AGTGTCTAAGAGTCTAAGTTGTG R: GTAATACAATGGCATAACTTCA	(AGAGAGT) ₆	55	110	4	single	0.49	0.52	0.71
CB020a	F: GTGTGTGAAAATGGAAAAAGTGTG R: CCGTGTATAAGAACCATGAAAGCA	(GT) ₆ (GA) ₈	55	134	1	single	0	0	0
CB026a	F: CCGAGTGGAAAGAGAGAGAG R: GGAGGGAGTAGAAAATAGTGTG	(AG) ₃₁	55	175	(8)	multi	—	—	0.93
CB027a	F: TGTATGCAAAATTAGGCGAGAGAG R: AAAATCTGGAAAAGGACTTGGC	(GA) ₇ (AG) ₁₁	55	172	2	single	0	0.05	0.05
CB041a	F: CCCCCGCATGAGCATAC R: ACGAAGTAAATTTACATAATTTTATCAG	(TA) ₈ (GA) ₇	55	124	1	single	0	0	0
CB047a	F: AGGCTAAAATGCTGTGCTCGT R: GATAAACTTTTCTCTCTCTCATCTCTC	(AT) ₁₄ (AG) ₃ T(GA) ₁₅	55	149	3	single	0.41	0.57	0.74
CB057a	F: GTTATCCGCCATTGTGCT R: AAAAACATAAATCCAAATACCTCC	(AG) ₁₄	55	123	(4)	multi	—	—	0.76
CB060a	F: CCCTTTCAAAACCCTAAATAAACT R: AACTCTATGTTATATCCGGACCT	(AT) ₈ (AT) ₈ (AG) ₇	55	171	(3)	multi	—	—	0.26

^z Ta denotes annealing temperature.^y The PCR product size refers to the sequenced clone from 'Francesco' or 'Barbara'.^x H_O and H_E denote observed and expected heterozygosities, respectively.^w PD denotes the power of discrimination.

was constructed using UPGMA (unweighted pair-group method using arithmetic averages) based on Nei's genetic identity (Nei and Li, 1979). The program NTSYS-pc, ver. 2.01 was used to construct the phenogram (Rohlf, 1998). Parentage was analyzed based on the inheritance of one allele at a single SSR locus transmitted from the parent to the offspring.

Results and Discussion

SSR marker development

A total of 141 positive clones were screened from 615 colonies with genomic libraries enriched for (AG)/(TC) of carnation varieties 'Francesco' and 'Barbara' by blot hybridization. After sequencing these positive clones, 62 independent sequences containing 7 or more repeat motifs were obtained with ca. 13.0 repeats on average. The average insert size of the clones obtained was ca. 381 bp, ranging from 101 bp to 997 bp. Only one set of sequences showed duplication. Of 62 sequences, 48 contained complete (AG)/(TC) repeats and the others had interrupted repeats or combined motifs of (AG)/(TC) and other units. For example, the CB018a SSR sequence contained 6 repeats of complete (AGAGAGT)/(TCTCTCA) (Table 2). Six sequences showed 2 repeat regions apart from each.

In our previous studies, SSR markers were developed from pear and rose (Kimura et al., 2006; Yamamoto et al., 2002a), in which 107 and 83 positive clones were screened from 927 and 192 clones, respectively. Eighty-five independent sequences were obtained containing 8 or more repeat motifs in pear, and 55 independent sequences contained 7 or more repeat motifs in rose. The rate of independent sequences containing the repeat motif for the positive clones was 9.2% and 16.1% in pear and rose, respectively. In this study, the rate of independent sequences containing the repeat motif was 10.1% in carnation, which showed a similar value of that of pear. The average number of repeats in independent sequences containing repeat motifs was ca. 20.0 and 16.1 in pear and rose, respectively. The number of ca. 13.0 repeats on average in carnation was less than in pear and rose. The average insert size of ca. 381 bp obtained in carnation was larger than ca. 360 bp in pear and smaller than 489 bp of rose. The number of sequences containing complete (AG)/(TC) repeats in carnation was greater than that of sequences with interrupted repeats or combined motifs, as observed in pear and rose. The rate of sequences containing complete (AG)/(TC) repeats in carnation was 77.4%, which was larger than the 69.4% in pear and 65.5% in rose. SSR markers could be successfully established from enriched genomic libraries of carnation varieties by the same method applied for pear and rose.

Genetic variation evaluated by SSR markers

Thirteen SSR markers were used to evaluate genetic variation in 41 carnation varieties. All 13 primer pairs

designed could successfully amplify the target fragments of the expected size for the original variety 'Francesco' or 'Barbara' (Table 2). Ten SSR markers (CF003a, CB003a, CB004a, CB011a, CB016a, CB018a, CB020a, CB027a, CB041a, and CB047a) produced 1 or 2 discrete amplified fragments for 41 carnation varieties. In particular, the shape of amplified fragments by CB018a SSR was simple and easy for scoring. On the other hand, the 3 SSR markers (CB026a, CB057a, and CB060a) produced 3 or more discrete amplified fragments. Since most carnation varieties are diploid with a chromosome number of $2n=30$ (Gatt et al., 1998), these 3 SSR markers might amplify 2 or more loci.

Standard carnation varieties, 'U Conn Sim', 'White Sim' and 'William Sim', stored at NIFS showed identical peak patterns in all used SSR markers shown as group B in Figure 1. It was reported that both 'U Conn Sim' and 'White Sim' were bud mutants originating from 'William Sim' (Mato et al., 2000), and the current results of SSR analysis accorded with the breeding process. Thirty-nine of 41 carnation varieties except for mutant varieties, were successfully distinguished using 32 putative alleles and 15 polymorphic bands produced from 13 SSR markers (Table 2 and Fig. 1). It was considered that SSR analysis is a very effective tool for variety identification in carnation.

In F_1 varieties of 'Miracle Rouge' and '85-11' bred from intraspecific crosses of *D. caryophyllus* L., 10 of the 13 SSR markers produced 1 or 2 discrete amplified fragments. On the other hand, the other 3 SSR markers (CB026a, CB057a, and CB060a) produced more than 3 discrete amplified fragments, which were not applied for hybridity confirmation. In the hybridity confirmation of 'Miracle Rouge', 4 SSR loci (CF003a, CB003a, CB011a, and CB018a) showed more than 2 putative alleles at all 4 SSR loci. The other 6 SSR loci showed identical SSR genotypes for both parents. The alleles of 128 bp and 128 bp of 'Miracle Rouge' at SSR CF003a were inherited from '63-41' and '62-48', respectively (Table 3). Similarly, the alleles of 190 bp and 203 bp at CB003a were transmitted from '63-41' and '62-48', respectively. For the other two SSR markers, the alleles of 210 bp and 214 bp at CB011a and the alleles of 93 bp and 98 bp at CB018a were also inherited from '63-41' and '62-48', respectively.

In the parentage of '85-11', 5 SSR loci (CF003a, CB003a, CB011a, CB018a, and CB047a) showed more than 2 putative alleles at all 5 SSR loci. SSR alleles of 210 bp and 214 bp of '85-11' at CB011a were inherited from '63-35' and '63-7', respectively (Table 3). Similarly, the SSR alleles of '85-11' were inherited from its parent variety without discrepancy for the other 4 SSRs CF003a, CB003a, CB018a, and CB047a; therefore, parent-offspring relationships of both 'Miracle Rouge' and '85-11' confirmed that the hybrids inherited SSR alleles from their parents without any discrepancy. It is considered that SSR analysis was very useful for

Fig. 1. Phenogram of the 41 genotypes of standard- and spray-type varieties of carnation. The phenogram was produced using the UPGMA method of Nei's genetic identity (Nei and Li, 1979) between varieties.

hybridity confirmation in carnation.

Ten SSR loci, except for CB026a, CB057a, and CB060a, were used to evaluate genetic variation derived from a single locus. The number of alleles per locus ranged from 1 for CB020a and CB041a to 9 for CB003a, with an average value of 3.2 (Table 2). The average value of the observed heterozygosity (H_O) among the 10 loci was 0.30, ranging from 0 (CB020a, CB027a, and CB041a) to 0.59 (CF003a). The average value of the expected heterozygosity (H_E) among the 10 loci was 0.34, ranging from 0 (CB020a, CB027a, and CB041a) to 0.68 (CB003a). The value of the observed heterozygosity (H_O) was relatively lower than that in Smulenders et al. (2003), reflecting that genetically restricted varieties of double flower phenotypes might be used in this study. The power of discrimination (PD) in the 13 SSR markers ranged from 0 (CB020a, CB041a) to 0.93 (CB026a) with a mean value of 0.49.

Carnation varieties are usually identified based on morphological characteristics; however, it has been pointed out that phenotypic traits are often influenced by environmental conditions and human judgments. For

example, some standard- and spray-type varieties do not have easily distinguishable flower characteristics due to phenotypic overlaps caused by environmental conditions, even if standard- and spray-type varieties usually have double and semi-double flower characteristics, respectively (Scovel et al., 1998).

For the genetic relatedness of 41 carnation varieties, analysis based on SSR genotypes revealed that they were mixed among standard- and spray-type varieties (Fig. 1). It was described that the first spray-type variety was a mutant originated from 'William Sim' at 1952 (Hosoya, 1999). As each spray-type variety was derived from the standard type with various genotypes, standard and spray varieties were mixed by UPGMA analysis.

Seven varieties ('Miracle Rouge', '62-48', '63-7', '63-35', '63-41', '85-11', and 'Brelag') were grouped to form cluster group A. Six varieties, except for 'Brelag', were obtained from NIFS (Fig. 1). Two pairs showing parent-offspring relationships were grouped very closely. For example, '62-48' (male parent) vs. 'Miracle Rouge' (offspring) and '63-7' (male parent) vs. '85-11' (offspring) showed high genetic identity, and '63-7',

Table 3. SSR genotypes of 41 carnation varieties obtained in this study.

No.	Variety name	SSR genotype ^z										
		CF003a	CB003a	CB004a	CB011a	CB016a	CB018a	CB026a	CB027a	CB047a	CB057a	CB060a
1	Aicardi	128/132	190/190	162/162	210/210	218/218	98/98	181/189	178/178	128/148	126/132	178/195
2	Brelag	128/132	190/203	162/162	210/214	218/218	93/93	152/187	178/178	150/150	126/126	178/195
3	Design	128/128	229/229	162/162	206/210	190/218	93/93	185/189	178/178	128/148	126/132	178/195
4	Empire	128/132	190/231	162/162	206/210	193/218	98/107	181/187/189	178/178	150/150	121/126	178/195
5	Felix	132/132	190/190	162/162	210/210	218/218	93/98	179/189	178/178	148/150	126/126	178/195
6	Francesco	129/132	190/190	162/162	206/206	218/218	98/113	187/189	178/178	128/150	126/126	178/195
7	Keipacks	128/132	190/229	162/162	210/210	193/218	98/98	152/181/189	178/178	128/148	126/126	178/185/195
8	Kobuchi Santo	128/132	229/229	162/162	210/210	190/218	98/98	181/189	178/178	148/150	126/132	178/195
9	Laville Dorina	128/128	190/214	162/162	210/210	218/218	98/98	189/189	178/178	148/150	126/126	178/195
10	Miracle Rouge	128/128	190/203	162/162	210/214	218/218	93/98	152/189	178/178	148/148	113/126	178/195
11	Novelpink	128/132	190/190	162/162	210/214	218/218	98/98	189/189	178/178	128/128	126/132	178/195
12	Opera	128/132	190/231	162/162	206/210	193/218	98/98	181/187/189	178/178	148/150	121/126/132	178/195
13	Riccardo	128/128	190/190	162/162	210/210	218/218	98/98	185/187	178/178	150/150	126/132	178/195
14	Saleya	128/132	190/229	162/162	206/210	193/218	98/98	179/189	178/178	150/150	121/126	178/195
15	Sorriso	128/132	229/229	162/162	210/210	218/222	98/98	187/189	178/178	128/150	126/126	178/195
16	U con Sim	128/129	190/229	162/162	210/210	190/218	93/98	181/187	178/178	148/148	113/121	178/195
17	White sim	128/129	190/229	162/162	210/210	190/218	93/98	181/187	178/178	148/148	113/121	178/195
18	William Sim	128/129	190/229	162/162	210/210	190/218	93/98	181/187	178/178	148/148	113/121	178/195
19	62-48	128/128	190/190	162/162	206/214	218/218	93/98	152/189	178/178	148/148	113/126	178/195
20	63-7	132/132	190/203	162/162	210/214	218/218	93/98	152/187	178/178	148/148	113/126	178/195
21	63-35	128/132	190/203	162/162	210/210	218/218	93/98	148/187	178/178	148/150	113/113	178/195
22	63-41	128/132	190/203	162/162	210/214	218/218	93/93	152/187	178/178	148/148	113/126	178/195
23	85-11	132/132	190/203	162/162	210/214	218/218	93/93	148/187	178/178	148/148	113/126	178/195
24	Alicetta	132/132	190/190	162/162	206/210	190/218	98/98	179/189	193/193	148/148	121/126	178/185/195
25	Alpine Rose	128/132	190/229	162/162	206/210	218/218	98/98	179/179	178/178	148/148	126/126	178/185/195
26	Barbara	128/128	190/190	162/162	206/210	190/218	98/113	181/187	178/178	148/150	126/126	178/195
27	Breime	128/129	190/190	162/162	210/210	218/218	98/98	152/189	178/178	150/150	126/126	178/195
28	Breto	128/128	190/229	162/162	210/210	190/218	93/113	152/179	178/178	148/148	126/126	178/180/195
29	Bridal White	128/132	245/245	162/162	210/210	190/190	93/98	152/179	178/178	148/148	113/126	178/185/195
30	CFC Rose Malea	129/132	229/229	162/162	206/210	190/218	98/98	181/187	178/178	148/148	121/126	178/195
31	Hilfines	128/128	229/229	162/162	206/210	190/218	98/113	168/181	178/178	148/150	126/126	178/195
32	HY Bis	129/132	190/203	162/162	206/210	190/218	98/98	187/187	178/178	148/150	126/132	178/195
33	Kleoya	128/128	190/190	162/162	206/210	190/218	98/113	148/187	178/178	150/150	126/126	178/195
34	Koreno	129/132	229/231	162/162	206/210	190/218	98/98	179/189	178/178	150/150	126/126	178/195
35	Lilac	128/128	229/229	162/162	210/210	218/218	93/98	179/187	178/178	148/150	126/126	178/195
36	Mystic Green	128/132	229/229	162/162	206/206	218/218	93/98	181/181	178/178	150/150	121/126	178/195
37	Ronia	128/128	212/227	162/162	210/210	—/— ^y	98/98	179/179	178/178	150/150	126/126	178/195
38	Rose Clavel	128/128	212/212	162/162	206/206	190/190	98/98	152/189	178/178	148/150	113/126	178/185/195
39	Stadisoc	128/132	—/— ^y	154/162	210/210	190/218	93/113	179/187	178/178	148/148	126/126	178/195
40	Super Gold	128/128	190/205	162/162	210/214	190/218	93/98	152/179	178/178	150/150	126/126	178/195
41	Vega	128/129	190/190	162/162	206/210	190/190	93/98	152/187	178/178	148/150	132/132	178/195

^z The values of SSR genotypes indicate the size of amplified fragment as base pair (bp). All varieties showed identical SSR genotypes of 140/140 at the CB020a and 132/132 at the CB041a.

^y no amplification.

'63-41', and '63-48' were F_1 hybrids derived from the same cross of '945-1' and '945-17' (Onozaki et al., 2006), therefore, it was considered that the results obtained were quite reasonable.

In this study, we could successfully develop 13 SSR carnation markers. These SSR markers will be utilized for genetic identification and hybridity confirmation of intraspecific crosses, and are expected to be put to practical use to protect the rights of breeders as a DNA carnation profiling technique. SSR markers and DNA profiling systems of mat-rush (*Juncus effusus* L.), small beans, sweet cherry and pear are used to protect the rights of breeders (<http://www.hinsyu.maff.go.jp/>, October 14, 2008); however, the number of SSR markers is still limited for DNA profiling in other plant species. Many DNA markers, such as SSR markers, are needed to support the variety registration system under the seeds and seedlings law based on UPOV (International Union for the Protection of New Plant Varieties, Switzerland). Furthermore, it is necessary to develop many more SSR markers for various plants to be put to practical use in DNA profiling.

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