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Microsatellite genotyping of carnation varieties

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Abstract A set of 11 sequence-tagged microsatellite markers for carnation (*Dianthus caryophyllus*) was developed using a DNA library enriched for microsatellites. Supplemented with three markers derived from sequence database entries, these were used to genotype carnation varieties using a semi-automated fluorescence-based approach. In a set of 82 cultivars, the markers amplified 4–16 alleles each. The effective number of alleles varied from 1.9 to 6.0. For the eight best scorable markers, heterozygosity was between 0.51 and 0.99. The markers were able to distinguish all cultivars with a unique combination of alleles, except for sport mutants, which were readily grouped together with the original cultivar. In addition, one group of three and one group of six cultivars each had the same combination of 'allelic peaks'. The cluster of three varieties concerned original cultivars and their mutants. The cluster of six consisted of four mutants from the same cultivar and two other varieties.

Keywords Variety · Identification · Distinction · Sequence-tagged microsatellite site · Simple sequence repeat

Introduction

Variety distinction and identification in agricultural and horticultural crops is generally carried out using morphological and physiological markers. However, many of the morphological descriptors used are multi-genic, quantitative or continuous characters, the expression of which may be altered by environmental factors, thereby making it necessary to use greenhouses or extensive field trials. Furthermore, the number of registered varieties increases over time, and because of this it is impossible to check efficiently each newly submitted variety against all old varieties. DNA markers have many advantages for plant variety identification over the more traditionally used morphological markers because of their independence from environmental influences, high level of polymorphism and greater potential for automation. The sequence tagged microsatellite site (STMS) approach has proven to be particularly useful for variety identification and testing in several crops (Plaschke et al. 1995; Corbett et al. 2001; Luce et al. 2001; Bredemeijer et al. 2002; Röder et al. 2002).

In carnation, only a few STMS markers could be developed from EMBL database sequences. They produced banding patterns in 26 *Dianthus* species, including *Dianthus caryophyllus* (Smulders et al. 2000). The STMS markers developed in that study were analysed on denaturing polyacrylamide gel electrophoresis (PAGE) gels in combination with silver staining for visualisation of the bands. The markers seemed promising for the measurement of genetic diversity in natural populations of *Dianthus* species and the identification of carnation varieties. However, the number of markers was too limited for routine use.

To increase the number of STMS loci for carnation, we isolated microsatellite loci using an enrichment procedure; we subsequently designed primers and tested the markers on a set of varieties that were granted Plant Breeders' Rights in The Netherlands during the period 1995–2000 and represent a cross section of modern carnation varieties. In addition, we evaluated the utility of the semi-automated fluorescence-based approach for sizing carnation microsatellite polymerase chain reaction (PCR) products using an ALF-express DNA sequencer.

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Materials and methods

A set of 82 carnation (*Dianthus caryophyllus* L.) varieties was used, of which 70 were granted Plant Breeders' Rights in The

Netherlands during the period 1995–2000 and represent a cross section of modern carnation varieties. Young leaves were frozen immediately and stored at –80 °C until DNA extraction according to Bernatzky and Tanksley (1986) with some modifications as described by Vosman et al. (1992). Microsatellite-enriched libraries were produced by a selective hybridisation procedure (Karagyozov et al. 1993; modified by Van de Wiel et al. 1999) on sonicated genomic DNA of *D. caryophyllus* cv. Danseur. The procedure involved the hybridisation towards immobilised synthetic oligonucleotides complementary to the microsatellite motif. Several di-, tri- and tetranucleotide repeats were tested. The enrichment procedure resulted in 12% of the fragments containing a microsatellite, which is an enrichment of 25–625-fold. Of these sequences, an average of 35% could be used to develop a STMS marker.

Primers for amplification of microsatellites in genomic DNA were designed with the program PRIMER 0.5 (Whitehead Institute for Biomedical Research Cambridge, Mass.). STMS analysis was carried out using the primer pairs listed in Table 1. The genomic sequences of these markers have been deposited in the EMBL genebank (for accession numbers see Table 1).

PCR was carried out in a total reaction volume of 25 µl containing 10 ng of genomic DNA, 0.2 µ*M* of fluorescently labelled forward primer (Pharmacia, Woerden, The Netherlands) and unlabelled reverse primer (Isogen, Maarssen, The Netherlands), 100 µ*M* of deoxyribonucleotides, 50 m*M* KCl, 20 m*M* Tris-HCl (pH 8.4), 1.5 m*M* $MgCl_2$, 0.05% (v/v) polyoxyethylene ether (W1) and 0.5 U of *Taq* DNA polymerase (Life Technologies, Breda, The Netherlands). Amplifications were performed in microtiter plates using a Hybaid Omni Gene thermal cycler. Basically, the amplification conditions were: one cycle of 94 °C for 3 min; 30 cycles of 55 °C for 45 s, 72 °C for 1 min 45 s and 94 °C for 45 s. After the final cycle, one cycle of 55 °C for 45 s and 72 °C for 3 min was added (Bredemeijer et al. 1998). PCR products were denatured by the addition of 8 µl of loading buffer containing Cy5 sizers (Pharmacia) to 4 µl PCR reaction and heating to 90 °C for 4 min followed by quenching on ice. Samples (4 µl) were loaded onto short sequencing gels (Gibco, Gaithersburg, Md.; 6% polyacrylamide, 7 *M* urea, 1.0 × TBE) and separated at 15 W constant power on an ALFexpress DNA sequencer (Pharmacia).

Fragment sizes were determined automatically using Fragment Manager (FM) software (Pharmacia). The criteria for allelic peak selection were as described by Esselink et al. (2002) with one addition: in the case of dinucleotide repeats, intermediate peaks between two allelic peaks that differed by 2 bp were scored as the largest allele.

Results and discussion

In general, fluorescence-based analysis of the carnation microsatellite markers revealed one to four (depending on ploidy level and heterozygosity) clear, well-separated peaks. The markers amplified 4–16 different alleles per locus (Table 1). In diploid cultivars, no more than two alleles would be expected. Nevertheless, some primer pairs (DCA221, DCD224, MS-DCAMCRBSY) generated more than two PCR products in some of the diploid cultivars, suggesting that not all STMS markers are single-locus markers (Fig. 1B, D; four fragments amplified in diploid cv. Obelix). The multiple fragments generated by the MS-DCAMCRBSY primers are possibly a consequence of the fact that it is a member of a multigene family (Smulders et al. 2000).

Although polymorphism, number of effective alleles and distribution across the genome are important criteria for choosing STMS markers, scorability of a marker may be even more important. Some of the 11 markers investigated could not be scored reliably when applying the general peak selection criteria that are currently in use in our laboratory for crops like tomato, wheat and rose (Bredemeijer et al. 1998; Esselink et al. 2002). The problematical primer pairs generated strong stuttering and poor resolution of large fragments containing TG repeats (Fig. 1B) or a relatively high number of low peaks (Fig. 1D) that were near or below the selection thresholds used to distinguish peaks from background (borderline cases). In several cases ‘allelic’ peaks were observed that differed 1 bp or less in size (Fig. 1A). Such differences in size may be caused by microheterogeneity (i.e. alleles differing slightly in size among cultivars, possibly due to point mutations which would change the base pair composition among alleles of the same length) and extra base addition [n/n+1 forms due to differential addition of an extra A by the DNA polymerase (Brownstein et al. 1996)]. Because the intra-gel variation was around 1 bp, these peaks were scored as one allele. It is obvious that specific peak selection criteria have to be applied, in addition to the existing ones, to allow semi-automated genotyping. The lack of amplification with a number of samples (Table 1), which occurred in replicate experiments, may be due to the presence of null alleles or a result of sensitivity of these loci for DNA quality (Smulders et al. 2000). A marker-dependent decrease in PCR quality of DNA was also observed in tomato (data not published).

The degree of polymorphism for the best eight primer pairs (quality 1 and 2 in Table 1) was tested using a set of 82 varieties of which three cultivars occurred in duplo. These STMS markers amplified 4–16 alleles (on average, 7.8) per marker. Observed heterozygosity varied between 0.51 and 0.99. As a measure of the information content, the number of effective alleles (A_e) was calculated for each marker. The A_e values for the eight best markers ranged from 1.9 to 6.5 (Table 1). The higher the A_e value, the higher the discriminatory power of a locus.

Most cultivars could be discriminated from each other based on the information obtained from the eight best markers. Average genetic similarity among pairs of cultivars was 0.49. In addition to the three pairs of duplicate samples only two other pairs, one group of three and one group of six cultivars had the same combination of alleles. One pair and the cluster of three varieties concerned original cultivars and their mutants. The latter was not entirely unexpected since mutants may result from a single point mutation only. It is extremely unlikely that such a mutation would involve a microsatellite marker used in this study. The cluster of six consisted of four mutants from the same cultivar and two other cultivars. The presence of the two other cultivars in this cluster cannot be explained straightforward, but it could simply be due to sample swapping.

In summary, we have developed a set of highly discriminatory microsatellite markers that can be used for genotyping carnation varieties and grouping mutants with the original variety, in a semi-automated, medium-

Table 1 Characterisation of 14 polymorphic microsatellite markers in *Dianthus caryophyllus*

Marker	Accession number of the genomic sequence	Repeat[a]	Primer sequences (forward, reverse)	PCR conditions T_m (°C) cycles	Product Sizes	Number of alleles in 82 varieties	A_e[b]	H_o[c]	Quality of patterns[d]	Remarks[e]
DCA221	AJ490808	$(CT)_{35-1}$	5′-CAACTGGTATTGAGAAGTGTTG 5′-AACCTTGAAATGGATTTGG	55-30	116–156	10	6.0	0.95	2	C, E
DCB109	AJ490809	$(GT)_{24-1}$	5′-ATAATTCACTTAACGGAAGGC 5′-AATTAAGGTCCACTACATCCC	55-30	91–123	6	3.1	0.83	2	D
DCB131	AJ490810	$(GT)_{30}$	5′-AGAGCCCCTCCGATG 5′-AGGTCTGTCCGTACGGTAC	55-30	190–244	4			3	G
DCB134	AJ490811	$(GT)_{23-1}$	5′-AAGAAGCATGCAATCATCTT 5′-CATTACAATCATATCACCCGT	55-35	131–201	7			3	A, G
DCB135	AJ490812	$(T)_{27-2}(GT)_{28}$	5′-TTATGCGGTTTGCTGC 5′-AGCGCAATTCAGGACTAAT	55-35	128–215	9			3	G
DCB140	AJ490813	$(GT)_{39}$	5′-TTCTCCTTCACTTGACTACGA 5′-TCCAAACTGATATTCCCATTA	55-30	110–179	8			3	G
DCD010	AJ490814	$(CTT)_{24-2}$	5′-GCATTCGTTTTTCCTTCTACT 5′-AACAACGTTCAGACAACCTAA	55-30	135–219	16	3.7	0.65	1	A(18), B
DCD105	AJ490815	$(CTT)_{27}$	5′-CCCTATCATACTTCTTAGCTGC 5′-TAACATATCGAAGAAACGATTG	55-30	193–246	6			4	A(8)
DCD224	AJ490816	$(CTT)_{10}$	5′-CGTCACAAGCTCTAAATCTTT 5′-AACCAAAAACCCTTCTAACAC	55-30	123–184	8	3.8	0.99	1	C, D
DCE218	AJ490817	$(T)_{67-11}$	5′-TTTCATAGGAGACTAACATAAATCC 5′-GGGTGAAATTTAGGTAGAAA	55-30	127–176	7	2.4	0.51	1	A(33)
DCF115	AJ490818	$(A)_{56-10}$	5′-TTTACGAACAAACGATCATTT 5′-CCTAATCAACAACAAGTTTCTATG	55-30	144–162	5	1.9	0.69	1	B, F
MS-DCAMCRBSY	Z18952	$(CAA)_{17}$	Smulders et al. 2000	55-35	92–141	9			4	C
MS-DINCARACC	M60619	$(CAA)_{17}$	Smulders et al. 2000	55-35	190–227	6	2.9	0.84	1	C
MS-DINMADSBOX	L40805	$(TA)_7$	Smulders et al. 2000	55-30	128–136	4	2.5	0.71	1	B, D

[a] A minus sign indicates deviation from the perfect repeat; e.g. (T)76-11 is a (T)76 repeat with an 11-bp difference from the repeat unit

[b] A_e (number of effective alleles) is estimated as the reciprocal of $\sum Pi^2$, where Pi is the frequency of the 1st allele in the 82 varieties examined. For each variety, regardless of putative ploidy level, the presence of each allele was scored once, except when plants were homozygous, in which case the allele was scored twice

[c] Calculated excluding cultivars with more than two alleles for that locus

[d] Quality 1, No or weak stutter peaks, well scorable; 2, stutter peaks present, but product still scorable; 3, scoring of large fragments not reliable; 4, scoring difficult, reproducibility bad (after Smulders et al. 1997)

[e] A, No amplification in some cultivars (between brackets, the number of cultivars that gave no amplification); B, differential amplification of alleles; C, possibly multilocus marker (more than two products); D, microheterogeneity (i.e., alleles differ slightly in size among cultivars); E, peaks around or below the selection thresholds that are used to distinguish peaks from background (borderline cases); F, presence of low broad artefact peaks (ignored for assigning alleles); G, presence of large fragments (ignored for assigning alleles)

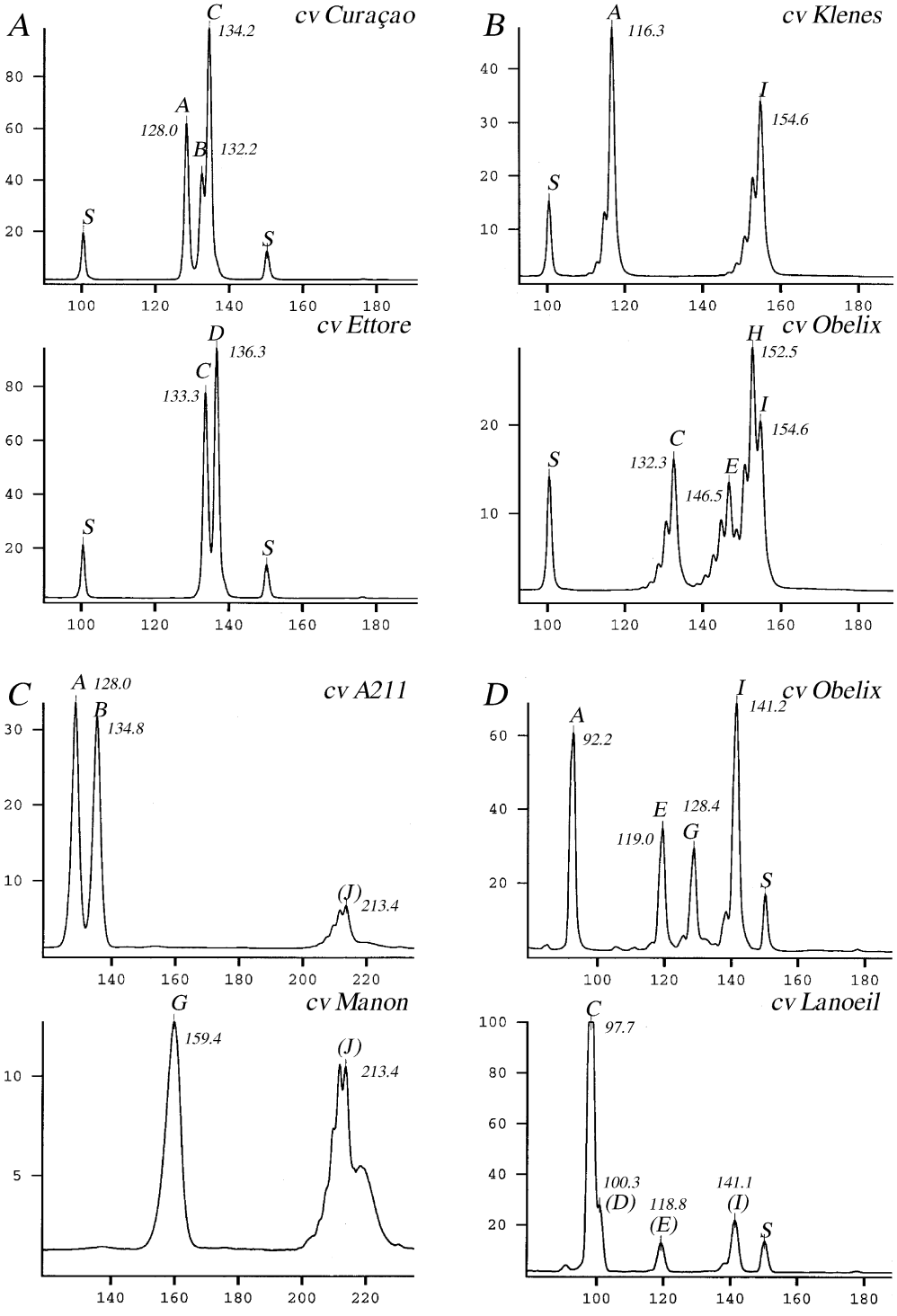

Fig. 1A–D Typical electropho-retograms of four carnation STMS markers differing in scorability of the allelic peaks. **A** MS-DINMADSBOX (quality 1), **B** DCA221 (quality 2), **C** DCB135 (quality 3), **D** MS-DCAMCRBSY (quality 4). Each panel shows the relative fluorescence intensity (*Y-axis*) and the size of the fragments in base-pairs (*X-axis*). In the electropherograms, *A*, *B*, *C*, *D*, *E*, etc. are allele codes used for database construction. Peaks with the allele code *in parenthesis* were not designated as alleles using the standard set of criteria (Bredemeijer et al. 1998; Esselink et al. 2002). *S* Internal sizer peak

throughput procedure. These markers may be used for breeding and population studies in several *Dianthus* species as well as for tracing infringements on Plant Breeders' Rights and quality control purposes.

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References

Bernatzky R, Tanksley SD (1986) Genetics of actin-related sequences in tomato. Theor Appl Genet 72:314–321

Bredemeijer GMM, Arens P, Wouters D, Visser D, Vosman B (1998) The use of semi-automated fluorescent microsatellite analysis for tomato cultivar identification. Theor Appl Genet 97:584–590

Bredemeijer GMM, Cooke RJ, Ganal MW, Peeters R, Isaac P, Noordijk Y, Rendell S, Jackson J, Röder MS, Wendehake K,

Dijcks M, Amelaine M, Wickaert V, Bertrand L, Vosman B (2002) Construction and testing of a microsatellite database containing more than 500 tomato varieties. Theor Appl Genet 105:1019–1026

Brownstein ML, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques 20:1004–1010

Corbett G, Lee D, Donini P, Cooke RJ (2001) Identification of potato varieties by DNA profiling. Acta Hortic 546:387–390

Esselink D, Smulders MJM, Vosman B (2002) Identification of cut-rose (*Rosa hybrida*) and rootstock varieties using robust Sequence Tagged Microsatellite markers. Theor Appl Genet (in press)

Karagyozov L, Kalcheva ID, Chapman M (1993) Construction of random small-insert genomic libraries highly enriched for simple sequence repeats. Nucleic Acids Res 21:3911–3912

Luce C, Noyer JL, Tharreau D, Ahmadi N, Feyt H (2001) The use of microsatellite markers to examine the diversity of the genetic resources of rice (*Oryza sativa*) adapted to European conditions. Acta Hortic 546:221–235

Plaschke J, Ganal MW, Röder MS (1995) Detection of genetic diversity in closely related bread wheat using microsatellite markers. Theor Appl Genet 91:1001–1007

Röder MS, Wendehake K, Korzun V, Bredemeijer G, Isaac P, Cooke RJ, Vosman B, Ganal MW (2002) Construction and analysis of a microsatellite-based database for European wheat cultivars. Theor Appl Genet (in press)

Smulders MJM, Bredemeijer G, Rus-Kortekaas W, Arens P, Vosman B (1997) Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. Theor Appl Genet 94:264–272

Smulders MJM, Rus-Kortekaas W, Vosman B (2000) Microsatellite markers useful throughout the genus *Dianthus*. Genome 43:208–210

Van de Wiel C, Arens P, Vosman B (1999) Microsatellite retrieval in lettuce (*Lactuca sativa* L.). Genome 42:139–149

Vosman B, Arens P, Rus-Kortekaas W, Smulders MJM (1992) Identification of highly polymorphic DNA regions in tomato. Theor Appl Genet 85:239–244