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MICROSATELLITE MARKERS FOR IDENTIFICATION AND REGISTRATION OF ROSE VARIETIES

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Microsatellite markers for identification and registration of rose varieties

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Introduction

Roses are divided into three groups, botanical species, old garden roses and the modern roses. Over 25,000 varieties of modern roses have been described (Cairns, 2000). The first hybrid tea rose was introduced in 1867 and since then more than 10,000 hybrid tea have entered the market. At the Centre for Variety Research, the Netherlands approx. 80 candidate varieties are examined each year for granting of Plant Breeders' Rights (PBR). Variety registration and protection in roses is based on morphological characteristics described in the UPOV guidelines. Considering the large number of varieties in 'common knowledge', it can easily be seen that it is impossible to take them all into account when examining candidates for PBR. Especially side-by-side comparison of applicants with older varieties is difficult or even impossible as many varieties are not easily available and maintaining large reference collections is far to expensive. Therefore, alternative approaches, such as the construction of electronic photo- and molecular databases, are currently being evaluated.

Several first-generation molecular marker techniques have been used for genotyping roses and extensive literature is available (Rajapakse et al.. 1992, Torres et al. 1993, Vainstein & Ben-Meir 1994, Ballard et al. 1995). These marker systems have some major drawbacks for variety identification. They lack high levels of polymorphisms, are difficult to reproduce, are time consuming or provide complex patterns undesirable for database building (Vosman 1998). In contrast, microsatellites are highly polymorphic and have the advantage of providing a codominant marker system based on PCR technology. In a sequenced tagged microsatellite site (STMS) approach they provide simple banding patterns, especially suitable for automated and objective analysis and therefore easy to store in a database. New varieties or new markers can easily be added to an existing database (Vosman et al. 2000). This STMS approach was successfully demonstrated in a collaborative study for the construction of databases for tomato and wheat (Vosman et al. 2001, Bredemeijer et al. in prep.)

In this paper we present the results of our study on the use of microsatellite markers from *Rosa hybrida* for the characterisation of hybrid tea and rootstock varieties as well as the construction of a molecular database. Varieties of rootstocks were included since identification based on morphological characteristics is often unsatisfactory because of the small differences between the varieties within a species. Possible future applications of these markers in variety registration are discussed.

Material and Methods

Plant material
Material used in this study consisted of 46 Hybrid Tea (*R.hybrida*) and 26 rootstock varieties belonging to *R. canina* (13), *R. indica* (8), *R. chinensis* (1), *R. rubiginosa* (1), *R. rubrifolia glauca* (1), hybrids (2) and 4 hiprose varieties beloning to *R. carolina* . Young leaves of a single individual were used for DNA analysis.

Microsatellite isolation, marker development and detection of polymorphism
Microsatellites were isolated from enriched small-insert genomic libraries essentially as described by Karagyozov et al. (1993) and modified by Van der Wiel et al. (1999). Microsatellite-containing clones were sequenced and primer pairs were designed using the program PCR Plan. For the characterization of STMS primers the PCR amplification products were separated on a 6% acrylamide gel and visualized with silver staining according to Promega Silver sequence DNA sequencing system as described by Wiel et al.(1999). Fluorescent amplification products were detected using an ALFexpress DNA sequencer (Pharmacia) as described by Bredemeijer et al. (1998).

Creating the STMS database
STMS markers with high quality patterns were selected for the creation of the database. A reference allele system as previously described for tomato (Bredemeijer et al. 2000) was used. The alleles of each candidate were scored and scores transferred to a numeric database in an Excel spreadsheet for storage and further analyses.

Data analysis
Co-dominant scoring of the markers in heterozygote samples to assess the complete genotype of a variety was complicated by the polyploid nature of roses. Although differences were found in the amounts of product for different alleles, it turned out to be very difficult to use these differences to estimate whether a particular allele was present one, two or three times, and in this way deduce the actual genotype of a variety. Therefore only the allele composition was scored and recorded in a presence/absence matrix. We refer to this as the allelic phenotype (Becher et al. 2000). The allelic phenotypes for 76 varieties were determined for 23 STMS loci. A similarity matrix was calculated and the varieties were clustered using the unweighted pair group method using arithmetic averages (UPGMA) module of NTSYS, version 2.1.

Results

Isolation and characterisation of microsatellite markers
A detailed description of the microsatellite isolation process in rose will be published elsewhere (Esselink et al., in prep.). Thirty-four markers were selected on the basis of pattern quality, using the standards described by Smulders et al. (1997).
For these primerpairs, all with quality 1 and 2, fluorescent forward primers suitable for analysis on the ALFexpress where synthesized. A set of 10 varieties was used for pattern quality assessment. Although promising in the first selection with PAGE/silverstaining, 11 primerpairs were discarded for various reasons. The remaining markers were used for fingerprinting the set of 76 varieties.

Marker characteristics
For the evaluation of the markers 26 rootstocks, 46 Hybrid Tea and 4 hiprose varieties were analysed in duplicate with 23 well scorable STMS markers. After the first analysis a set of varieties representing all the alleles were included during following electrophoretic runs and were used for allele designation. Although the use of a sophisticated DNA sequencer in combination with internal sizers allows accurate fragment sizing, nevertheless small differences in fragment sizes of the same alleles were observed between different electrophoretic runs. The advantage of using an 'allelic ladder' is that genotyping of varieties becomes independent of experimental variation, that allele assignment is based on fragments with comparable sequence, and that new alleles can be identified. For a given microsatellite locus between two and nine reference varieties were necessary to produce all alleles for the

ladder; when appropriate, different DNA samples (varieties) were pooled to decrease the number of reference lanes on the gel.

Table 1 shows some characteristics of the markers used in this study.

Moderate stutter bands were solely found among dinucleotide repeats, low stutter bands among both dinucleotide and trinucleotide repeats and two trinucleotide markers showed no stutter bands at all. The observed stutter characteristics did not interfere with the allele assignment for any of the STMS markers.
For a number of STMS markers allele sizes differ one or multiple repeat units from each other, i.e separated by steps of 2 bp for dinucleotide repeats or 3 bp for trinucleotide repeats. However, sometimes this relation was not obvious for all alleles. This suggests that other types of sequence variation may also be involved in allelic diversity (Table 1). It complicated the allele designation of a single allele of some loci (e.g. RhAB26, RhP518).

Another important characteristic of the STMS markers observed is the occurrence of differences in band intensity in heterozygous varieties. This was a generally observed phenomenon among the STMS markers tested. Although differences exist between the selected loci, most alleles could be easily identified since the relative peak area of the less intense band was usually well over 20% of the main peak. This difference in amplification is partly due to differences in the number of copies of the alleles present, but may also depend on differences in amplification efficiency of alleles.

Variety identification using STMS markers
Five to 18 alleles were generated by each STMS marker, and a total of 254 alleles was amplified (Table 2). In the 26 rootstock and 4 hiprose varieties, 232 alleles were amplified. In the 46 Hybrid Tea varieties, 119 alleles were detected. In total 135 unique rootstock and 22 unique Hybrid Tea alleles were detected, 97 alleles were present in both groups of varieties. The 23 markers unequivocally identified all varieties with an unique phenotype, except for one group of 8, one group of 4 and three groups of 2 varieties. In all these cases, the varieties within these groups were known duplicates or mutants (sports) from initial varieties. Taking this into account, a complete differentiation between the varieties of rootstocks has been achieved. A unique phenotype for all varieties was already obtained by fingerprinting with as few as 2 microsatellites (e.g RhAB40 together with RhEO506 or RhP507 with RhD201). For Hybrid Tea varieties, all except two varieties were distinguished by RhEO506 with RhD201. Addition of the data of e.g. RhP517 differentiated between the remaining two varieties. Several other combinations of 2 or 3 STMS loci were also capable of identifying all the varieties tested. The high discriminating power of the loci suggests that a selection of the most robust STMS markers could differentiate any two varieties within rootstocks or Hybrid Teas.

Figure 1 shows the UPGMA tree obtained with the marker data. In this tree the rootstocks are clearly distinct from the Hybrid tea varieties and the branches for all varieties are very long, indicating a large genetic distance between the varieties. From this figure the identical genotypes mentioned before can also be readily identified.

Discussion

Variety identification
In many crops several thousands of varieties are registered. To be able to identify them to a very large extent by a unique fingerprint it is necessary that the markers used are highly discriminative. Using all data presented in this study, the 76 rose varieties were divided into

63 genotypes. Fifty eight varieties showed a unique genotype. Besides that there were three groups of two varieties, one group of 4 and one of 8 varieties that showed group specific genotypes (Fig.1). These observations were in line with the groupings based on morphological characteristics. The group with 8 varieties consisted of rootstock varieties that were found to be not distinct in their morphological characteristics. The group of four consisted of one original variety and 3 color mutants derived from this variety. The three groups of two varieties consisted of 1) a duplicate sample, 2) an original variety and one mutant and 3) morphological identical varieties. In another study, it was shown that with only four microsatellite markers described here, more than 99% of the 250 varieties under study could be distinguished (Van Hoof et al. 2000).

All varieties evaluated here have been in PBR trails and two (nrs 36 and 42) were rejected because they were not homogenous. Rose varieties are propagated vegetatively and differences between individuals can only arise from somatic mutations or changes. The microsatellite analysis most likely would not have detected differences among individual plants of a non-homologenous variety if they are due to somatic mutations.

From the results presented above we can conclude that:

- all seedling varieties can be identified uniquely
- all derived genotypes (mutants and duplicates) are identified by the genotype of the initial variety. They do not show a unique genotype with the markers used.
- The STMS marker are highly discriminative
- STMS fingerprinting is highly reproducible, as identical material comes out identical.

Future use of the markers in variety registration

Variety registration requires that a candidate variety is compared with all existing varieties in common knowledge. For roses this would mean a comparison with more than 10,000 existing varieties of which many are not easily available. To facilitate a comparison with the existing varieties, use should be made of databases containing the molecular profiles of all varieties. To facilitate the construction of such databases, genotyping should be automated as far as possible and the microsatellite markers used in this study are very suitable for this. Criteria for good markers have been identified previously (Vosman et al. 2000). To make the whole procedure even more effective the molecular database should be combined with a searchable photo database.

Proposal for a procedure to implement STMS markers in rose registration

For this the following procedure is suggested. First genotype all existing varieties using seven highly polymorphic microsatellite markers (one per chromosome). When two varieties are identical using these markers, then use a second set of 7 markers. When they are again identical than grow them side by side as they are most likely either sports or very closely related genotypes. Homogeneity and stability testing of the variety should be done in the second phase, during propagation of the material with the breeder or by the inspection services.

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Table 1: Characteristics of the 23 selected microsatellite markers for rose

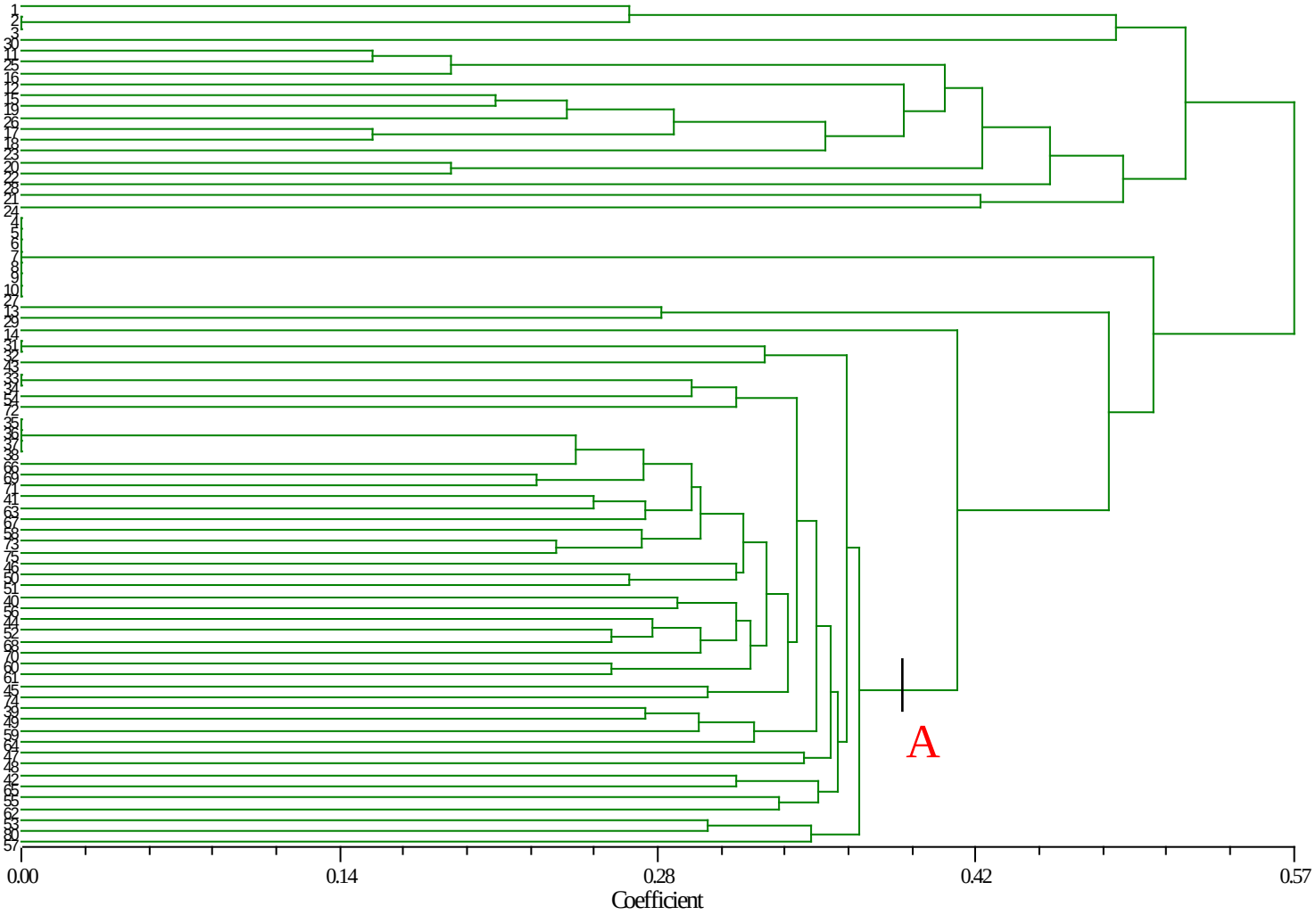
STMS Marker	Repeat sequence	linkage group (Debener et al.,2001)	locus pattern characteristics	
			stutter	scorability
RhAB1	(CT)3(GT)9(GA)10		low/moderate	1
RhAB13	(GA)11-1(GT)8	4	low	3
RhAB15	(GT)19-2(GA)16	2	low/moderate	1
RhAB22	(GT)13(GA)13	6	low/moderate	1
RhAB26	(GT)18-2(GA)17		low	2*
RhAB40	(TC)14(AC)11-1(T)8-2(CAT)2(GAT)4-1	4	moderate	3
RhB19	(GT)10		low	2
RhB303	(GA)11		low/moderate	1
RhBK4	(AT)5(GT)12		low	2*
RhD201	(TCT)33		low	1*
RhD206	(TCT)14	2	low	1
RhD221	(TCT)21-1	4	low	1
RhE2a	(TGT)33-12		no	1
RhE2b	(TGT)20-6		low	1
RhE3	(TGT)21		low	2*
RhEO506	(CAG)6(CAA)18-7(CAG)6	2	low	2
RhI402	(GTG)11-2	3	low	1
RhJ404	(GAG)6	4	low	3
RhO517	(CCG)3(GAG)3-1(GAC)7	1	low	1
RhP507	(TGA)42-11	4	low	1
RhP518	(CAT)4CAATT(CAT)6CAATT(CAT)6	5	low	2*
RhP519	(TGA)11-1		low	2*
RhP524	(AAT)5(GAT)8(GAC)7(AAT)9-2(GAT)9-1		no	3*

* loci showing one or a few alleles that differed around 1 bp and therefor could not be scored accurately. These variants were assigned to the same allele.

Table 2 : Total number of detected alleles and allelic phenotypes for each locus

STMS	30 rootstock varieties		46 Hybrid Tea varieties		Total numbers	
Marker	alleles	Phenotypes	alleles	phenotypes	Alleles	phenotypes
RhAB1	6	8	3	7	7	12
RhAB13	13	9	4	4	13	13
RhAB15	16	17	5	10	17	27
RhAB22	11	13	4	5	12	18
RhAB26	16	16	7	20	17	36
RhAB40	16	18	8	16	16	32
RhB19	8	12	3	8	8	18
RhB303	9	10	6	18	9	27
RhBK4	7	4	4	6	8	9
RhD201	13	19	4	10	14	25
RhD206	17	17	4	5	18	22
RhD221	7	11	5	10	8	17
RhE2a	5	5	4	5	5	8
RhE2b	9	13	6	11	10	21
RhE3	8	6	5	6	9	11
RhEO506	18	15	6	14	18	27
RhI402	6	7	5	7	7	12
RhJ404	7	8	4	7	7	11
RhO517	4	8	4	10	5	14
RhP507	16	17	10	17	18	34
RhP518	6	7	4	5	7	11
RhP519	8	9	5	10	10	18
RhP524	6	8	9	31	11	38
Total no. of alleles	232	257	119	242	254	461
Average no. per marker	10	11	5	11	11	20

Figure 1: Clusteranalysis (UPGMA) of the rose varieties. Numbers 1 – 30 are rootstocks, 31 - 80 are the hybrid tea varieties. Both sets contain reference as well as candidate varieties.



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