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The use of semi-automated fluorescent microsatellite analysis for tomato cultivar identification

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Abstract The objectives of this study were to evaluate the usefulness of a fluorescent-analysis method for genotyping PCR-based tomato microsatellite markers (or STMSs) and to establish the value of these markers to generate unique DNA profiles of tomato cultivars. The analyses were performed using forward primers labelled with a fluorochrom and using an ALF express DNA sequencer. In general, analysis of the tomato STMSs revealed distinct allelic peaks. PCR artefacts like stuttering and differential amplification were observed for several tomato STMS markers, but in most cases these artefacts did not seriously hamper allele designation. Comparison of fluorescent and silver-stained allelic profiles revealed a similar distribution of alleles among the test cultivars. Sixteen tomato cultivars were DNA-typed for 20 selected STMS markers using the fluorescent approach. Length polymorphism among the PCR products was detected with 18 of these markers, yielding gene diversity values from 0.06 to 0.74. The number of alleles per microsatellite locus ranged from 2 to 8. As few as four STMSs were sufficient to differentiate between all 16 cultivars, indicating that these markers are especially suitable for a species like tomato which has low levels of variation as detected by other types of markers.

Key words Microsatellites · *Lycopersicon esculentum* · STMS · Cultivar identification · Fluorescent detection

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Introduction

The low genetic diversity of modern tomato cultivars is reflected by a low level of polymorphism for proteins, isoenzymes, and several types of DNA (Miller and Tanksley 1990; Van der Beek et al. 1992; Rus-Kortekaas et al. 1994). An exception is the relatively high level of polymorphism with microsatellite DNA, as demonstrated by Vosman et al. (1992). Microsatellites are DNA sequences consisting of arrays of a basic repeat unit of 2–8 base pairs. These repeats are highly polymorphic, even among closely related cultivars, due to variation in the number of repeat units (see Brown et al. 1996).

Two approaches have been reported for measuring the polymorphism of tomato microsatellites. In the first approach, i.e. multilocus analysis, microsatellite polymorphism was studied by Southern hybridization of restriction enzyme-digested DNA with labelled microsatellite probes. In this way, it was possible to distinguish 11–27 tomato cultivars (Vosman et al. 1992; Philips et al. 1994; Kaemmer et al. 1995). Although the fingerprints thus obtained contained numerous well-reproducible polymorphisms, the amount of work involved makes this approach time consuming. In addition the multilocus approach yielded dominant markers (Arens et al. 1995 a). In the second approach, single-locus PCR analysis, the microsatellites were studied as sequence-tagged microsatellite-site markers (STMSs) by PCR amplification of individual loci and analysis of the PCR products on denaturing sequencing gels. With this approach it has been demonstrated that tomato STMS markers are polymorphic both between species and cultivars (Philips et al. 1994; Arens et al. 1995 b; Broun and Tanksley 1996; Smulders et al. 1997). In general, the number of different alleles per locus was low, mostly 2–4 with a maximum of eight alleles (Smulders et al. 1997).

Different methods have been described to detect PCR-STMS products that were subjected to denaturing

polyacrylamide electrophoresis, including silver staining, isotopic labelling and automated fluorescence. In the tomato studies, STMS detection was carried out using radioactivity (Philips et al. 1994; Broun and Tanksley 1996) or a silver staining method (Arens et al. 1995 b; Smulders et al. 1997). During recent years, it has been shown that PCR-based microsatellite analysis with fluorescent primers and automated fluorescent DNA sequencers can be useful in high-resolution genomic analyses (see Frégeau and Fourney 1993; Schwengel et al. 1994; Gill et al. 1995; Botta et al. 1995; Diwan and Gregan 1997; Pfeiffer et al. 1997).

The present study was undertaken to establish the usefulness of fluorescently labelled STMSs as molecular markers in tomato and to investigate their level of polymorphism in a test set of 16 tomato cultivars. In a previous study, polymorphism was investigated for loci extracted from the EMBL database with only seven cultivars (Smulders et al. 1997). Because of the low level of polymorphism of these loci among the seven test cultivars, and therefore their restricted informativeness, a number of additional loci isolated from a DNA library (Smulders et al., in preparation) were included in the present study.

Material and methods

Plant DNA

Sixteen *Lycopersicon esculentum* cultivars (see Table 2) were obtained from the tomato collection of the Centre of Genetic Resources (CGN, part of CPRO-DLO, The Netherlands). Nuclear DNA was extracted from frozen leaves of three individuals essentially as described by Bernatzky and Tanksley (1986) with some slight modifications (Vosman et al. 1992).

PCR conditions

Twenty seven tomato microsatellite loci, representing various repeat classes, were used for PCR amplification as described by Smulders et al. (1997). The amplification conditions for each locus are listed in Table 1. The forward primers, labelled at the 5′ end with a fluorescent label [Indodicarbocyanine(Cy5) phosphoramidite], were purchased from Pharmacia. Each 25-µl amplification reaction contained: 10 ng of genomic DNA, 0.2 µM of fluorescently labelled forward primer (Pharmacia) and unlabelled reverse primer (Isogen, The Netherlands), 100 µM of deoxyribonucleotides, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.05% (v/v) polyoxyethylene ether (W1), and 0.5 U of *Taq* DNA polymerase (Life Technologies). 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. When appropriate, the annealing temperature was lowered to 50°C, and the number of cycles increased to 35 as indicated in Table 1.

T4 DNA polymerase treatment

The electrophoretograms of the tomato STMS loci after standard PCR were often complicated because of the generation of two peaks per allele differing in size by one base (n and $n + 1$ peak). Most likely this is caused by the ability of *Taq* DNA polymerase to add an extra base to the 3′ end of the amplified fragments (Sprecher et al. 1996). As the degree of extra base addition depended on the locus, and sometimes also on the cultivar (data not shown), allele designation was not always reliable and manual editing was required to correct the selection of allelic peaks. In order to eliminate the problem of extra base addition to the fragments by the *Taq* polymerase, a T4 DNA polymerase treatment was carried out as recommended by Ginot et al. (1996). For this purpose, a 10-µl PCR reaction was mixed with 0.4 U of T4 DNA polymerase (Gibco) and subsequently incubated for 30 min at 37°C. Occasionally, the T4 DNA polymerase treatment generated non-specific, relatively broad peaks that may interfere with the allelic peaks. Loci exhibiting this phenomenon were not selected for identification purposes.

Detection of STMSs

Fluorescent amplification products were resolved on denaturing polyacrylamide gels and analysed on an ALF express DNA sequencer. For this, 4 µl of each T4-treated amplification reaction was mixed with 8 µl of loading buffer, containing Cy5 sizers according to the instructions of Pharmacia. After denaturation at 90°C for 4 min followed by quenching on ice, 4 µl samples were loaded onto a standard sequencing gel (Ready mix from Pharmacia; 6% polyacrylamide, 7 M urea, 0.6 × TBE). Gels were run for 5–6 h at constant power (25 W) at 55°C. Fragment sizes were determined automatically using Pharmacia Fragment Manager (FM) software. Size estimates were rounded up or down because the FM software provides product size as a fraction of a nucleotide. The allele sizes were transferred to an excell spreadsheet for storage and utilization in further analyses.

Results

Separation and detection of STMS alleles

The initial objective of this study was to evaluate the utility of the semi-automated fluorescence-based approach for sizing tomato microsatellite PCR products using an ALF express DNA sequencer. For this purpose we chose 27 tomato microsatellite loci representing various repeat classes and different qualities with respect to the scorability of alleles on silver-stained gels (Smulders et al. 1997).

In general, fluorescence-based analysis of the tomato microsatellite loci revealed a single main peak in homozygotes and two distinct different-sized allelic peaks in heterozygotes (Fig. 1 A). However, in a number of cases additional peaks were observed that may hamper allele designation. Some primer pairs detected an additional locus that was monomorphic; the PCR products were generally smaller in size than those from loci corresponding to the 'expected loci' (Fig. 1 B) similar to those reported for wheat (Bryan et al. 1997). Many primer pairs produced smaller, or larger, less intense products or stutter peaks in addition to the major allelic peak(s) (Fig. 1 A–D). Moreover, some samples showed signs of differential amplification, i.e. differences in peak areas between alleles of heterozygote samples (Fig. 1 C). A number of the 27 loci investigated could not be used because of the problems

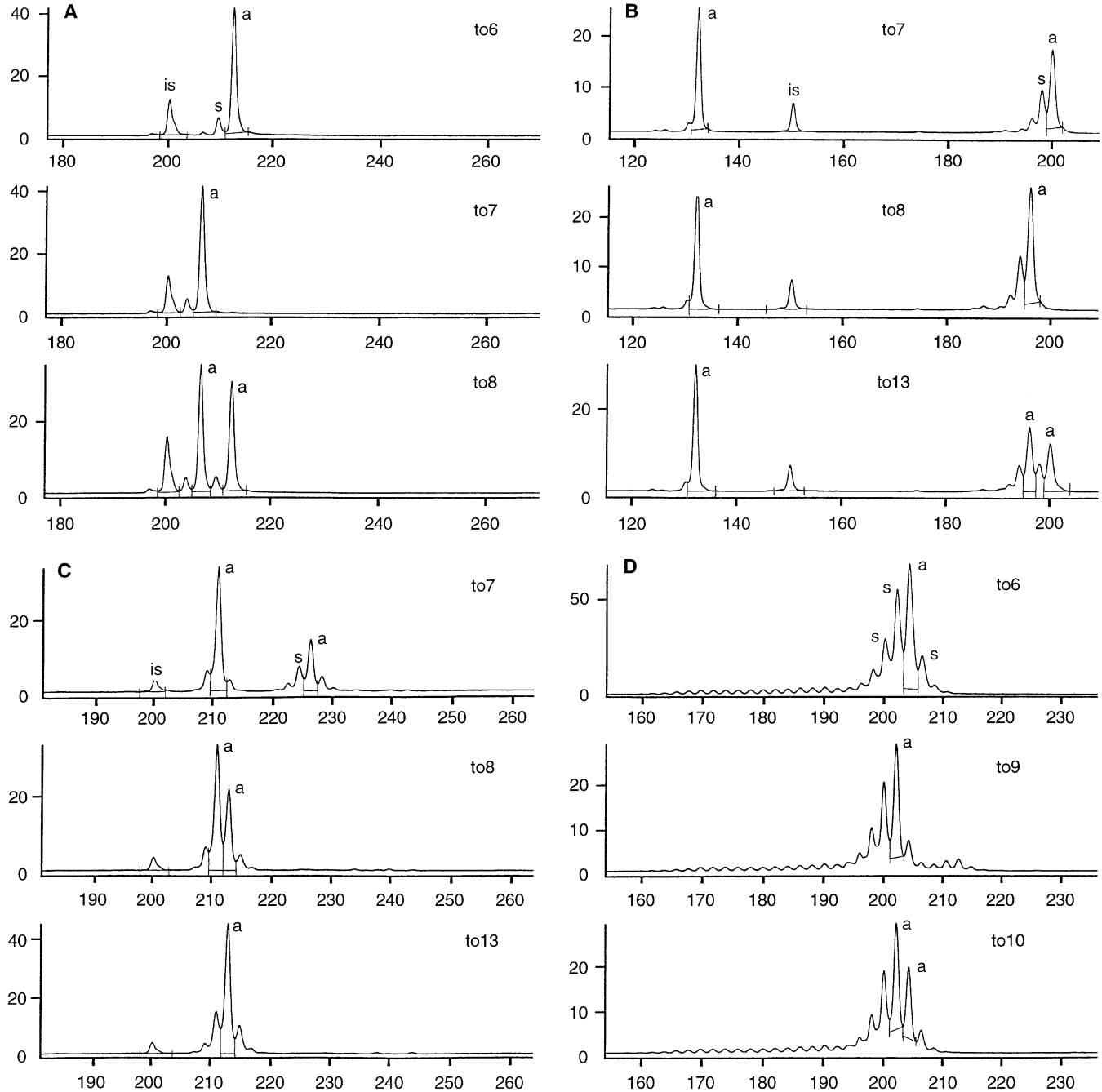

Fig. 1A–D Typical electrophoretograms of four tomato STMS markers differing in stutter characteristics and the amplification of alleles in heterozygotes. **A** LEE6 (low stutter), **B** LEB102A (additional monomorphic locus), **C** LEMDDNa (differential amplification), **D** LEEFIAa (high stutter). Each panel shows the relative fluorescence intensity (Y-axis) and the size of the fragments in base-pairs (X-axis). *a* = allelic peak, *s* = stutter peak, *is* = internal sizer peak

of interpretation caused by stuttering and differential amplification, especially when stutter peaks from one heterozygous allele overlapped the other. Additionally, in the case of mononucleotide repeats allele designation was problematical as adjacent size types were difficult to distinguish. Nevertheless, allele designation was possible for 20 of the 27 loci investigated, including loci that gave ladders of bands with the silver staining procedure (Smulders et al. 1997).

Polymorphism of STMSs

In order to determine the levels of variability of the STMSs selected on the basis of scorability, genomic DNA from 16 tomato cultivars was used. These cultivars represent a wide spectrum of tomato differing in

Table 1 Characteristics of the STMS markers used to generate DNA profiles of 16 tomato cultivars by amplification with fluorescence-tagged forward primers, resolution on polyacrylamide gels and analysis on a fluorescent DNA fragment analyzer

Locus	Repeat	PCR conditions Ta (°C), cycles	Internal Cy5 standards	Product size (bp)	Number of alleles	Diversity index[d]
ATTa[a]	(TTA)5CT(ATT)8ATC … complex repeat	50–30	150–250	222	1	0
JACKP1[a]	(GATA)*n*(GACA)*n* … complex repeat	55–30	250–300	364–381	3	0.44
LE20592 (D)[b]	(TAT)15-1(TGT)4	55–30	100–200	162–168	3	0.44
LE21085 (D)	(TA)2(TAT)9-1	50–35	50–200	103–116	2	0.34
LEB102 A (A)	(TA)6(CA)33(TA)4	55–30	150–250	194–200	3	0.56
LEB136 (A)	(AT)5(GT)34-4(TA)6-1	55–30	200–300	238–244	3	0.36
LEB181 (A)[c]	(CTAT)5(CT)14-2(AT)9-1	50–30	150–250	297–299	2	0.50
LECHI3 (D)	(TA)6-1(GA)4	55–30	100–200	126	1	0
LED1 A(A)	(TCT)21TCCTTCC(TCT)6	50–35	100–200	168–174	3	0.56
LED4 (A)	(TCT)32-1	50–30	150–250	189–192	2	0.17
LED10 (A)	(TCT)29-2	55–30	200–300	205–306	3	0.40
LEE6 (A)	(GTT)28-3	55–30	200–300	206–212	2	0.50
LEE11 (A)	(CAA)36	50–35	50–200	138–193	8	0.74
LEE102 (A)	(GTT)88 imperfect	55–30	150–250	278–301	3	0.28
LEEF1Aa (D)	(TA)8(ATA)9	50–30	100–250	198–213	7	0.67
LELE25 (D)	(TA)11	50–35	150–250	219–223	3	0.55
LELEUZIP (D)	(AGG)6-1TT(GAT)7	55–30	100–200	101–104	2	0.12
LEMDDNa (D)	(TA)9	55–30	200–300	211–226	3	0.44
LESATTAGA (D)[c]	(TA)11(GA)11	50–30	150–250	173–211	4	0.61
LEWIPIG (D)	(CT)4(AT)4	55–30	200–300	247–254	2	0.06

[a] Broun and Tanksley 1996; Phillips et al. 1994
[b] (D) = STMS extracted from database (Smulders et al. 1997); (A) = STMS isolated by Smulders et al. (in preparation)
[c] PCR products not treated with T4 and separated on Gibco acrylamide
[d] Diversity index was calculated using the formula $D = 1 - \Sigma Pi^2$ where Pi is the frequency of the I^{th} allele in the 16 cultivars examined

the type of fruits formed and in their resistance (see Table 1 in Rus-Kortekaas et al. 1994). Half of the selected cultivars were inbred cultivars, whereas the other cultivars were F_1 hybrids. Eighteen of the 20 microsatellites showed polymorphism among the 16 test-set cultivars. The number of alleles detected per polymorphic locus ranged from two to eight (Table 1). In total, 58 alleles were found, 15 of which were unique. The number of unique alleles per cultivar varied from zero to three for 15 of the 16 cultivars; cv Mirabell (to2), however, had five unique alleles. It appeared that closely related cultivars showed more similarity in STMS fingerprint patterns than cultivars of more remote types. Thus, the two closely related cvs San Marzano Lampadone and San Marzano differed by only two loci (LEEF1Aa and LEE11), both shown in Table 2, while the more remote cvs Moneymaker and Mirabell differed by seven loci (data not shown). All the F_1 cultivars were heterozygous for three or more loci. Of the eight inbred cultivars, six (Moneymaker, Pipo, San Marzano Lampadone, San Marzano, Marmande and Mirabell) were homozygous for all loci while two cultivars (UC 82B and Roma VF) were heterozygous for one or two of the polymorphic loci (data shown for four loci in Table 2) suggesting heterogeneity.

To investigate whether differences existed in STMS patterns between individual plants, DNA isolated from seedlings of nine plants of the true-breeding cv Moneymaker and the hybrid cv Calypso was used for PCR-amplification of the two most discriminative microsatellites, i.e. LEE11 and LEEF1Aa. No differences in STMS profiles among the individual plants of each cultivar were found (data not shown).

The polymorphism of the microsatellite markers observed in this study provides a system for the identification of tomato cultivars. Complete differentiation between the 16 cultivars could be achieved by genotyping as few as four loci (e.g. the loci shown in Table 2). DNA typing of more of the loci will certainly allow many more tomato cultivars to be separated. An aid for selecting additional loci is the information content of a locus, which can be determined by taking into account its ability to differentiate between cultivars. As a measure of information content we used the gene diversity (D). The values of gene diversity associated with each of the tomato STMS markers were rather low, ranging from 0.06 to 0.74 when calculated based upon the set of 16 cultivars (Table 1).

Discussion

The initial objective of this study was to evaluate the potential of semi-automated fluorescent PCR-product analysis of tomato microsatellites. Three important

Table 2 Identification of tomato cultivars using microsatellites

Cultivar	Cultivar code[a]		LEEF1Aa alleles[b]	LEE11 alleles	LE21085 alleles	LELE25 alleles
Pipo	to3	–	198/–	170/–	116/–	223/–
Evita	to13	F_1	198/208	167/170	116/–	223/–
Mirabell	to2	–	200/–	170/–	103/–	219/–
Moneymaker	to1	–	198/–	170/–	103/–	223/–
Liberto	to12	F_1	198/–	170/177	103/–	223/–
Blizzard	to14	F_1	198/–	141/170	103/–	223/–
UC82B	RKO6	–	206/–	141/193	116/–	221/–
San Marzano Lampadone	to4	–	213/–	138/–	103/–	221/–
San Marzano	to5	–	202/–	164/–	103/–	221/–
Marmande	to6	–	204/–	141/–	103/–	221/–
Carma	to10	F_1	202/204	138/174	103/116	221/–
Roma VF	to9	–	202/–	141/167	103/–	221/–
Trend	to11	F_1	198/–	167/170	103/–	221/–
Calypso	to15	F_1	198/–	170/–	103/–	221/223
Dombito	to7	F_1	198/–	138/170	103/–	221/223
Vision	to8	F_1	198/–	141/170	103/–	221/223

[a] Codes for cultivars used in figures and text (see Rus–Kortekaas et al. 1994)
[b] The length of the alleles is given in base pairs

phenomena that are known to affect the discrete generation of STMSs and thus hamper allele sizing were observed, i.e. extra base addition, stuttering and the differential amplification of alleles.

The extra base addition is not necessarily a real problem in allele designation and sizing. The use of a T4 DNA polymerase treatment after PCR, as recommended by Ginot et al. (1996), appeared to generate distinct peaks representing the true allele (*n* form). An alternative would be the use of modified primers (Brownstein et al. 1996) or *Pfu* DNA polymerase during PCR because this enzyme does not exhibit terminal transferase activity (see Sanchez et al. 1996). In a preliminary experiment using *Pfu* DNA polymerase similar results were obtained for three of the four loci analysed (LED1A, LEEFIAa, LEMDDNa). However, in the case of LE21085 products of unexpected size were generated.

A second PCR artefact that was observed for several tomato STMSs is known as repeat slippage giving rise to extra amplification products that are one or more repeat units larger or smaller than the authentic alleles (Sprecher et al. 1996). In a number of heterozygous cultivars the artefact peaks and true allelic peaks overlapped, sometimes causing problematical interpretation.

Nevertheless, the fluorescent approach offered a greater clarity of interpretation as compared to the silver-stain approach. In the first place because only one DNA strand is detected with the fluorescent analysis method and in the second place because it is possible to use fluorescent band intensity as an objective measure.

Ideally, both STMS alleles generated from a heterozygous sample should yield similar peak heights (Frégeau and Fourney 1993). However, in the present study differential amplification of alleles was observed for some tomato loci causing a lower yield of some alleles. This could be the result of some divergence in the primer sequence which produces an allele with less homology and therefore less PCR product (Lavi et al. 1994). In addition, the effect of mismatches on amplification may vary with primer length, sequence context and reaction conditions (Devos et al. 1995).

A comparison of fluorescent and silver-stained allelic profiles for three loci (LELE25, LELEUZIP and LEMDDNa) revealed a similar distribution of the alleles among the test-set cultivars, but the allele sizes differed by 1–3 bp depending on the STMS marker (data not shown). The size differences of corresponding alleles observed between the two approaches are most likely due to differences in the acrylamide and the size-standards employed. This assumption is supported by the fact that allele sizing with the fluorescent system itself appeared to be dependent on the type of acrylamide and on the sizer combination (data not shown). These effects are probably based on the fact that the mobility of DNA is dependent upon the sequence, as well as size, of the DNA fragment, so that the repeat nature of STMSs may influence the structure of the DNA product and alter the migration upon electrophoresis relative to non-repeat size standards (Frégeau and Fourney 1993; Sprecher et al. 1996). Although the precision of allele size determination with the fluorescent ALF express system used in this study (intragel $\pm$ 0.2 bp; intergel $\pm$ 0.3 bp) was comparable to the variation found in other studies (Diwan and Gregan 1997), the inclusion of allelic ladders will provide additional confidence for inter- and intra-laboratory comparisons that may include altered electrophoretic systems, buffer conditions, or size standards (Sprecher et al. 1996).

The ultimate goal of the study reported here was to investigate the usefulness of STMSs as molecular markers in tomato and to determine their level of polymorphism and information content for use in cultivar identification. For this purpose we have analysed the variation of 20 STMSs in a test set of 16 tomato cultivars (Table 1). Eighteen loci appeared to be polymorphic. The number of alleles detected per polymorphic locus ranged from 2 to 8 which is low in comparison with values for microsatellite loci in other crops such as soybean (Maughan et al. 1995), maize (Taramino and Tingey 1996) and grapevine (Thomas and Scott 1993), but in agreement with previous studies on tomato STMSs (Philips et al. 1994; Broun and Tanksley 1996). The low polymorphic nature of the microsatellite loci was not unexpected because of the narrow genetic base of modern cultivars, combined with the self compatible nature of this species (Miller and Tanksley 1990). As expected, the frequency of heterozygotes among the eight true-breeding cultivars for each of the loci was low. Two of these cultivars were not uniform for one or two loci. For the F_1 hybrid cultivars and for the breeding cultivars that are homozygous for all loci one would expect STMS profiles that are identical among individual plants of a cultivar, as has been observed with the multilocus approach (Vosman et al. 1992). Indeed, we did not detect any difference in the profiles of two STMSs among individual plants of a true-breeding cultivar and a F_1 hybrid.

As a measure of polymorphism information content, gene diversity values were calculated for each of the tomato STMSs based upon the set of 16 cultivars. These values, which can be used as a relative measure of the utility of each STMS, ranged from 0.06 to 0.74 (Table 1) and are low compared to those reported for other crops (Maughan et al. 1995; Brown et al. 1996; Taramino and Tingey 1996). It was possible to distinguish all 16 cultivars with a selection of four STMS markers (Table 2). On the basis of the gene diversity values, the number of unique genotypes (cultivars that can be distinguished) can be estimated as described by Brown et al. (1996). Theoretically, the 18 polymorphic microsatellite loci, having an average diversity of 0.44, will provide more than 34 000 genotypes, assuming that the alleles of one locus are not linked to the alleles of other loci (see Brown et al. 1996). Practically, the number of usable STMSs, and consequently the total discrimination ability, will be lower. In the first place, an STMS marker like LEEFIAa may be not very appropriate for large-scale application because this locus produces difficult-to-score products. Secondly, linkage of a number of the STMS markers used in this study cannot be excluded. GATA- and GACA-containing microsatellites in tomato seem to cluster in the same chromosomal regions (Arens et al. 1995 a). In order to obtain a better choice of microsatellite loci for tomato identification purposes a selection of additional STMSs will be carried out.

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