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ORIGINAL PAPER

**Sheh May Tam · Corinne Mhiri · Aat Vogelaar
Marcel Kerkveld · Stephen R. Pearce
Marie-Angèle Grandbastien**

Comparative analyses of genetic diversities within tomato and pepper collections detected by retrotransposon-based SSAP, AFLP and SSR

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Abstract The retrotransposon-based sequence-specific amplification polymorphism (SSAP) marker system was used to assess the genetic diversities of collections of tomato and pepper industrial lines. The utility of SSAP markers was compared to that of amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers. On the basis of our results, SSAP is most informative of the three systems for studying genetic diversity in tomato and pepper, with a significant correlation of genetic relationships between different SSAP datasets and between SSAP, AFLP and SSR markers. SSAP showed about four- to ninefold more diversity than AFLP and had the highest number of polymorphic bands per assay ratio and the highest marker index. For tomato, SSAP is more suitable for inferring overall genetic variation and relationships, while SSR has the ability to detect specific genetic relationships. All three marker results for pepper showed general agreement with pepper types. Additionally, retrotransposon sequences isolated from one species can be used in related Solanaceae genera. These results suggest that different marker systems are suited for studying genetic diversity in different contexts depending on the group studied, where discordance between different marker systems can be very informative for understanding genetic relationships within the study group.

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S. M. Tam · C. Mhiri · M.-A. Grandbastien (✉)
Laboratoire de Biologie Cellulaire,
Institut Jean-Pierre Bourgin,
INRA-Centre de Versailles,
78026 Versailles, France
E-mail: gbastien@versailles.inra.fr
Tel.: +33-1-30-83-3024
Fax: +33-1-30-83-3099

A. Vogelaar · M. Kerkveld
Rijk Zwaan Breeding, P.O. Box 40,
2678 ZG De Lier, The Netherlands

S. R. Pearce
School of Life Sciences, University of Sussex,
Falmer, Brighton, BN1 9QG, UK

Introduction

Tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.) are two economically important crop plants from the Solanaceae family. Both tomato and pepper are grown on a large scale commercially as fresh and processed vegetable crops, with pepper also being a leading condiment. One of the primary needs of the crop industry is the estimation of genetic diversity between cultivated accessions for identification and breeding purposes. To this end, various marker techniques have been successfully applied, either individually or in combination, to study the genetic diversity of various plant species.

The genetic diversity of tomato has been investigated in several studies using isozymes, restriction fragment length polymorphisms (RFLPs), oligonucleotide fingerprinting and simple sequence repeats (SSRs). With the exception of SSRs, limited information was obtained due to a lack of variability that was ascribed to the self-pollinating nature of modern tomato cultivars combined with their narrow genetic base (Alvarez et al. 2001 and references therein). Studies on the genetic diversity of pepper have involved the use of morphological markers and molecular markers such as RFLPs, amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNA (RAPDs) (for review, see Lefebvre 2005). Lefebvre et al. (2001) reported that isozyme variability was very low in *C. annuum*, while RFLP, RAPD and AFLP studies detected higher polymorphisms between small- and large-fruited cultivars but low variability, especially within the large-fruited inbred lines.

A novel group of molecular marker techniques based on insertion polymorphisms generated by the activity of retrotransposons has recently been reported to work efficiently in certain plant species (for review, see Schulman et al. 2004). Retrotransposons are mobile genetic elements that amplify by reverse transcription of an RNA intermediate. The Ty1-copia retrotransposons represent the best-studied retrotransposon group in plants (Kumar and Bennetzen 1999). In particular, the sequence-specific amplification polymorphism (SSAP) technique (Waugh et al. 1997) provides a retrotransposon-based marker system that combines the high resolution of AFLP with the specificity of an oligonucleotide primer anchored on the terminal sequences of a retrotransposon, usually in the long terminal repeat (LTR). The usefulness of SSAP for studying genetic diversity, mapping populations and species relationships has repeatedly been reported, such as for barley (Waugh et al. 1997; Gribbon et al. 1999; Leigh et al. 2003; Schulman et al. 2004), pea (Ellis et al. 1998; Pearce et al. 2000), wheat (Gribbon et al. 1999), oat (Yu and Wise 2000) and alfalfa (Porceddu et al. 2002). Inserted copies of retrotransposons, as opposed to DNA transposons, do not excise, thereby resulting in stable insertions in the genome. In addition, various retrotransposon families in a genome may vary with respect to their insertion activity, allowing the matching of the family used for marker generation to the phylogenetic depth required (Pearce et al. 2000; Leigh et al. 2003; Schulman et al. 2004).

Ty1-copia retrotransposons were first detected in the Solanaceae following the isolation of the Tnt1A element of tobacco (Grandbastien et al. 1989). Tnt1-related elements have been detected in tomato, potato and petunia, and the Tnt1-like Retrolyc1 element has been characterized in tomato and several related wild species (Costa et al. 1999; Araujo et al. 2001). In addition to members of the Tnt1 superfamily, the tomato genome contains other Ty1-copia retrotransposons [Rogers and Pauls 2000; Budiman et al. 2000; The Institute for Genome Research, Rockville, Md. (http://www.tigr.org/tdb/e2k1/plant.repeats/index.shtml)].

The primary objective of the study reported here was to assess the usefulness of SSAP as a marker system for analysing the genetic diversities of tomato and pepper collections. For both collections, we evaluated insertion patterns generated by several retrotransposon families. We then compared the effectiveness of the SSAP marker system with that of other dominant and co-dominant marker systems, namely AFLP and SSR.

Materials and methods

Plant material

The tomato (*Solanum lycopersicum*) collection comprised 34 homozygous lines (designated TOR1 to TOR36, excluding TOR7 and TOR9) distinguished by fruit morphology (Table 1). The pepper (*Capsicum annuum*) collection consisted of 35 homozygous lines (designated PAR4 to PAR39, with the exception of PAR24) that had been classified on the basis of pepper type (Table 1). Nuclear DNA was extracted from fresh young leaves using the CTAB method (Bernatzky and Tanksley 1986).

Ty1-copia elements used for SSAP

For SSAP, primers were derived from two known retrotransposons, the Tnt1 tobacco element (X13777) and the ToRTL1 tomato element (U68072). Two additional retrotransposon sequences, T135 and T265, were isolated from tomato following the protocol of Pearce et al. (1999), with the omission of the motif 2 PCR primer (ADI/MF/LTK). PCR products were subcloned directly into TOPO TA vector (Invitrogen, Paisley, UK) prior to sequencing. The nucleotide sequence data of the T135 and T265 sequences have been submitted to the DDBJ/EMBL/Genbank databases under the accession numbers AY746975 (T135) and AY746976 (T265).

Primers orientated towards the element's 5′ end were defined in the LTRs of each retrotransposon (Table 2). The Tnt1 primer used in tomato and pepper was the OL16 primer from tobacco, which successfully amplified Tnt1-related Retrolyc1 sequences from various tomato species (Araujo et al. 2001). In addition, the ToRTL1- and T265-derived primers were used in tomato, and the T135-derived primer was used in pepper.

SSAP method

The SSAP technique described in Waugh et al. (1997) was adopted with modifications. Genomic DNA (final concentration of 500 ng) was digested with 5 U of

Table 1 Tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) lines grouped by fruit characteristics

	Lines
Tomato collection (fruit morphology)	
Round	TOR1, 15, 19, 22, 23, 24, 25, 26, 27, 28, 32, 33, 36
Intermediate	TOR2, 4, 5, 8, 12, 13, 30, 35
Flesh	TOR3, 6, 10, 11, 14, 16, 17, 18, 20, 21, 29, 31
Cherry	TOR34
Pepper collection (pepper type)	
Bell pepper	
Blocky NL/Spain	PAR4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22
Dolma	PAR23, 32
Lamuyo	PAR29, 30, 31, 35, 36
Conical pepper	
Dulce Italiano	PAR25, 26, 27, 28
Sivri	PAR33, 34
Charliston	PAR37, 38, 39

Table 2 SSAP adaptors and primer sequences

Type	Primer/adaptor name	Sequence (5′ → 3′)
SSAP retrotransposon primers (position in LTR sequence)[a]	Tnt1-OL16 (+ 317, +339)ToRTL1 (+97, +117)T135.1 (+55, +75)T265.2 (+43, + 63)	TTCCCACCTCACTACAATATCGC CCCTGGGTTTGTTTCATCTGC TGGTCAAAAATGGGTTGGTCC GGTACTCCCTAACAGGCTTGG
SSAP adaptors	double-stranded *Eco*RI adaptordouble stranded *Csp*6I adaptor	CTCAGGCTCGTAGACTGCGATCC AATTGGATCGCAG CTGGACGATGAGTCCTGAGA TATCTCAGGACT
SSAP adaptor primers (restriction site)	E00 (*Eco*RI)C00 (*Csp*6I)	GTAGACTGCGATCCAATTC GACGATGAGTCCTGAGATAC

[a]Retrotransposon primers are in reverse complement to the long terminal repeat (LTR) sequences

restriction enzymes *Eco*RI (Invitrogen) or *Csp*6I (MBI Fermentas, Souffelweyersheim, France) in 1× final concentration buffers supplied by the manufacturers in a final volume of 25 µl and the mix incubated at 37°C for 3 h. Into the digested DNA was added 25 µl of ligation mix, which consisted of the corresponding adaptor (Table 2; 1 p *M*, Sigma Genosys, Haverhill, UK), 0.2 m *M* ATP (Invitrogen), 1 U T4 DNA ligase, 1× final concentration T4 ligase buffer (Invitrogen) and sterilized deionized water to reach the final volume. The mix was incubated at 37°C for a further 3 h, after which the DNA was stored at −20°C until used. Primer-labelling PCR was carried out in 25-µl reactions containing the following: 2 µl template DNA, 0.3 µl *Taq* polymerase (Invitrogen), 2.5 µl 10× PCR buffer (Invitrogen), 1 µl 50 ng/µl [^{33}P]-labelled retrotransposon primer (Perkin Elmer, Paris, France; Table 2), 1 µl 50 ng/µl E00 or C00 primer (Table 2), 0.75 µl 50 m *M* $MgCl_2$, 1.2 µl 5 m *M* dNTP (Invitrogen) and sterilized deionized water to reach the final volume. PCR were performed following the protocol of Melayah et al. (2001). Each species was assayed using three primer sets: tomato— Tnt1-C00, ToRTL1-E00 and T265-E00; pepper—Tnt1-E00, Tnt1-C00 and T135-E00. Amplified products were separated on 6% denaturing polyacrylamide gels. After drying, the gels were exposed to Kodak Biomax films (Eastman Kodak, Rochester).

AFLP method

The AFLP technique (Vos et al. 1995) was performed according to Van Eck et al. (1995) with minor modifications. Following the ligation of the *Eco*RI (adaptor E) and *Mse*I (adaptor M) adaptors to the restricted DNA, the selection of biotinylated ligation products using streptavidin-coated magnetic beads was replaced by a 20-fold dilution of the ligation mixture. Selective amplification was achieved by adding one or more additional nucleotides onto the PCR primers. Pre-amplification consisted of a stringent touchdown temperature profile with the primers having one additional 3′ nucleotide, and the subsequent PCR amplification procedure used fluorescently end-labelled (FAM, JOE, TAMRA; Perkin Elmer) E + 3 primers in combination with M + 3 primers. Nine primer combinations were used (tomato: E35-M47, E35-M48, E35-M49, E35-M50, E35-M60, E35-M61, E35-M62, E41-M50, E38-M61; pepper: E41-M50, E35-M48, E35-M49, E35-M61, E38-M59, E38-M50, E38-M61, E40-M50, E35-M59) . Samples were loaded on a 5% polyacrylamide gel and analysed with an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, Calif.). A GS-500 ROX-labelled size standard (Perkin Elmer) was loaded in each lane to facilitate the automatic analysis of the gel and the sizing of the fragments. The computer programme GENESCAN 2.0.2 was used for data analysis and to generate sample files. The data were further processed using GENOTYPER 2.0 (Perkin Elmer) for the analysis of GENESCAN files generated by the ABI 377 DNA Sequencer.

SSR method

Two sets of 16 (for tomato) and 13 (for pepper) proprietary SSR primers (Rijk Zwaan Breeding, De Lier, The Netherlands) were used. Amplification reactions were performed in a total volume of 25 µl, which contained 10 ng genomic DNA, 0.32 μM of each primer [of which one primer was fluorescently end-labelled (FAM, JOE, TAMRA; Perkin Elmer)], 100 μM deoxyribonucleotides, 50 m *M* KCl, 20 m *M* Tris-HCl (pH 8.4), 1.5 m *M* $MgCl_2$ and 1 U *Taq* DNA polymerase (Roche, Indianapolis, Ind.). The reactions were performed in 96-well plates in a Perkin Elmer 9700 thermocycler with the following cycling conditions: one cycle of 94°C for 5 min; 35 cycles of 55°C for 1 min, 72°C for 2 min and 94°C for 1 min. After the final cycle, 1 cycle of 55°C for 1 min and 72°C for 7 min was added. The PCR amplification products were verified by 5% polyacrylamide gel electrophoresis (PAGE), and the data generated were processed as described for the AFLP method.

Data analysis

All distinct bands were scored as present (1) or absent (0). Binary SSAP, AFLP and SSR data have been

submitted to the GERMINATE database (http://bioinf.scri.sari.ac.uk/cgi-bin/germinate/germinate.cgi). Genetic distance was used as a measurement of relatedness among samples, and the resultant binary data analysed using PAUP*4.10 (Swofford 2002). Pairwise distances were computed using the MEAN CHARACTER DIFFERENCE option in PAUP*4.10. The microsatellite genetic distance D_{AS} is identical to the pairwise differences calculated using mean character difference in PAUP*4.10 (Fisher et al. 2002).

The neighbor-joining (NJ) (Saitou and Nei 1987) algorithm in PAUP* clustered pairwise distances using the MINIMUM EVOLUTION option, and clade support was estimated by 1,000 NJ bootstrap replications. Mantel's test (Liedloff 1999) was used to assess the strength of correlation between two observed pairwise distance matrices and the test statistic tested for significance against 1,000 random permutations. The ratio of $\sqrt{(\text{mean sampling variance})}$ and mean genetic distance was used to estimate precision following Lefebvre et al. (2001).

Genetic variation of the two collections were studied using POPGENE ver.1.31 (Yeh and Boyle 1997), which allowed the analyses of dominant (SSAP/AFLP) and co-dominant (SSR) diploid data. For SSAP and AFLP, the data obtained include the observed (n_a) and expected (n_e) number of alleles (Kimura and Crow 1964), percentage polymorphism, the polymorphic index content (PIC) simplified from Anderson et al. (1993) and the evaluation of non-neutral sites by the Ewens-Watterson neutrality test (Manly 1985). The PIC was determined over all loci as $\text{PIC} = 1 - \Sigma p_I^2$ where p_I is the frequency of the Ith allele. For dominant marker systems, only the presence and absence of alleles are represented. For SSR, the same parameters were obtained with the addition of the fixation index (F_{IS}; Wright 1978) and the two-locus linkage disequilibrium (LD) (Burrows' composite index and chi-square test for significance; Weir 1979).

In addition, for SSAP data, the programme DNASP - ver. 3.99 (Rozas et al. 2003) was used to perform Tajima's neutrality test (Tajima 1989) and analyse percentage of LD between pairs of informative sites for individual datasets. The chi-square test was used to detect significant linkage disequilibrium and the Bonferroni procedure to correct for multiple tests was applied (Weir 1996). The marker index (MI), a product of PIC/expected heterozygosity, the proportion of polymorphic loci and the total number of loci detected per primer set (Powell et al. 1996; Porceddu et al. 2002) were calculated and used to compare the usefulness of SSAP in relation to the other marker systems.

Results

Genetic variation of tomato and pepper collections assessed by SSAP

SSAPs were performed using two known retrotransposon families, Tnt1 (Grandbastien et al. 1989) and ToRTL1 (Daraselia et al. 1996). Two newly characterized retrotransposon sequences, namely T135 and T265 isolated from tomato following Pearce et al. (1999), were also used. As different retrotransposons can produce variations in the quality of SSAP patterns (i.e. band intensity versus background intensity, scorability and abundance of bands; Leigh et al. 2003), we selected for each species retrotransposon primers that provided the best SSAP profiles, namely Tnt1, ToRTL1 and T265 for tomato, and Tnt1 and T135 for pepper. Figure 1 shows examples of SSAP gels obtained with different primer combinations.

For tomato, analyses of the three primer sets yielded a total of 138 scored bands of which 79 (57.3%) were polymorphic (Table 3). Genetic variation values estimated using the three retrotransposon datasets were fairly similar, with Tnt1-C00 showing most variation; for example, 11–13% higher polymorphism than T265-E00 and ToRTL1-E00. Unique bands (specific to only one line) were found in three different lines (one to three bands). Mantel's tests revealed significant correlations ($P < 0.01$) between the three primer sets (high between ToRTL1-E00 and T265-E00, $r = 0.75$; lower between each of these two and Tnt1-C00, $r = 0.18$ and 0.16, respectively). The three datasets were combined for subsequent inference of genetic relationships.

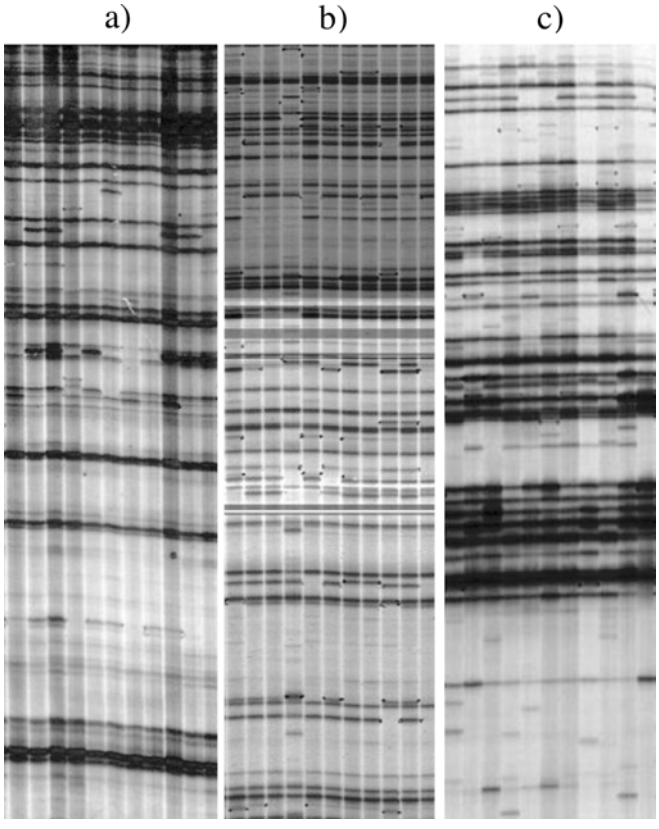

Fig. 1 Comparison of SSAP insertion profiles of three different retrotransposon primer sets on 11 tomato lines: **a** ToRTL1-E00, **b** T265-E00, **c** Tnt1-C00

Table 3 Genetic variation values and neutrality tests for different tomato and pepper SSAP datasets

	Tomato collection (34 lines)				Pepper collection (35 lines)			
	Retrotransposon primer set			Combined data[a]	Retrotransposon primer set			Combined data
	ToRTL1-E00	T265-E00	Tnt1-C00		Tnt1-E00	Tnt1-C00	T135-E00	
Total number of bands	46	91	36	–	50	51	38	–
Number of bands scored	38	65	35	138	47	50	24	121
Number of polymorphic bands	20	36	23	79	31	48	13	92
Polymorphic bands (%)	52.63	55.38	65.71	57.25	65.96	96.0	54.17	76.03
$n_a - n_e$	0.229	0.254	0.376	0.278	0.372	0.440	0.235	0.373
Polymorphic index content (PIC)[b]	0.172	0.176	0.177	0.175	0.174	0.302	0.184	0.229
Tajima's D neutrality test	1.400	1.003	−0.396	–	0.7220	−0.098	1.077	–
Significance under intermediate recombination	Significant $P < 0.05$	Significant $P < 0.05$	Not significant $P > 0.10$	–	Not significant $P > 0.10$	Not significant $P > 0.10$	Not significant $P > 0.10$	–
Number (%) of non-neutral sites[c]	5/38 (13.2%)	3/65 (4.6%)	1/35 (2.9%)	9/123 (7.3%)	3/47 (6.4%)	3/50 (6%)	2/24 (8.3%)	8/121 (6.6%)
Sites at lower frequency	3 (7.89%)	2 (3.08%)	1 (2.9%)	6 (4.88%)	2 (4.26%)	3 (6%)	2 (8.3%)	7 (5.79%)
Sites at higher frequency	2 (5.26%)	1 (1.54%)	0	3 (2.44%)	1 (2.13%)	0	0	1 (0.83%)
Significant linkage disequilibrium (%)[d]	9.47	1.75	1.98	–	2.58	1.68	5.13	

[a] Data obtained from three retrotransposons were combined together and re-analysed
[b] Anderson et al. (1993)
[c] According to Ewens-Watterson neutrality test (Manly 1985)
[d] According to χ^2 test and corrected by Bonferroni procedure (Weir 1996)

For pepper, 121 bands were scored from the three primer sets, of which 92 (76.03%) were polymorphic (Table 3). Similar genetic variation values were detected between the Tnt1-E00 and T135-E00 datasets, but markedly different ones were observed between Tnt1-E00 and Tnt1-C00, with the latter reflecting a much higher level of variation. Unique bands were found in five lines (one band). As Mantel's tests again revealed significant correlations ($P < 0.01$) between the three primer sets (Tnt1-E00/C00, $r = 0.36$; Tnt1-E00/T135-E00, $r = 0.51$ and Tnt1-C00/T135-E00, $r = 0.32$), the three datasets were combined. The Tnt1-E00 and Tnt1-C00 datasets, although obtained from the same retrotransposon primer, were considered to be non-overlapping datasets because of the very low possibility that a given insertion may be located close to both an *Eco*RI and a *Csp*6I restriction site, especially since the two restriction sites have a very different GC content and are likely to be present in different compartments of the genome. Molecular characterization of nine Tnt1-E00 and 16 Tnt1-C00 polymorphic bands from the SSAP gels indicated that only one band corresponded to the same insertion (unpublished data) . Therefore, combining the datasets was not expected to significantly bias genetic distance calculations.

Tajima's neutrality tests carried out under no recombination showed that all retrotransposon datasets did not significantly deviate from neutral expectation. *D* values for different retrotransposon datasets ranged from −0.396 (Tnt1) to 1.4 (ToRTL1) in tomato and from −0.098 (Tnt1) to 0.7220 (T135) in pepper. When the neutrality tests were performed under the more probable assumption of intermediate recombination, the insertion frequencies of ToRTL1 and T265 in tomato were found to deviate slightly from neutrality ($P < 0.05$) (Table 3). Surprisingly, both *D* values were positive, which indicates balancing selection. No change detected from the pepper datasets suggested that globally, the effect of genetic drift is more important than selection. The Ewens-Watterson tests for both tomato and pepper showed that most of the retrotransposon insertion frequencies were neutral, with only a total of nine insertions for tomato (7.3%) and eight insertions for pepper (6.6%) deviating from the expectations of the neutral infinite alleles model (Table 3). While fairly constant in individual pepper datasets (6–8.3%), the proportion of non-neutral insertions was substantially diverse in tomato, ranging from 2.9% for Tnt1 up to 13.2% for ToRTL1. For all elements, most non-neutral insertion frequencies consisted of insertions at lower frequencies. For SSAP, estimates of linkage disequilibria were low and fairly similar for pepper (1.68–5.13%) and more diverse for tomato (1.75–9.47%).

Genetic variation of tomato and pepper collections assessed by AFLP

The nine primer sets yielded 845 bands in tomato, of which 123 (14.56%) were polymorphic, and 1,432 bands

in pepper, of which 115 (8.031%) were polymorphic (Table 4). For tomato, the average number of bands generated per primer set was 94 (range: 62–130), with an average of 14 polymorphic bands per primer set (range: 9–21). For pepper, a higher average number of bands were generated per primer set—159 (range: 115–211)—with an average of 13 polymorphic bands per primer set (range: 8–19). The majority of the polymorphic AFLP loci were neutral, with a much lower level of non-neutral loci in pepper (0.49%) than in tomato (2.01%) (Table 4).

Genetic variation of tomato and pepper collections assessed by SSR

A total of 16 and 13 primer sets were used to analyse the tomato and pepper collections, respectively. The microsatellite loci were all polymorphic with two to three alleles and two to four alleles observed at each locus for tomato and pepper, respectively. In total, 39 different alleles were scored for tomato and 31 different alleles were scored for pepper (Table 4). For tomato, four loci had at least one allele unique to an accession and for pepper, one locus had at least one allele unique to an accession.

Genetic variation values for both collections were fairly similar (Table 4). The mean values of the H_e estimates for tomato and pepper indicate a considerable amount of genetic variation for inbreeding species, with the H_e value for tomato (0.3929) being slightly higher than that for pepper (0.3536). However, differences were seen in F_{IS} values, and in tomato, four loci exhibited heterozygote excess with the F_{IS} values of the remaining loci ranging from 0.8652 to 1.0. The average inbreeding coefficient for tomato, 0.6920, was considerably lowered by these four loci. Pepper exhibited a much higher average inbreeding coefficient, 0.9740, with most of its loci (11 of 13) showing F_{IS} values of 1.0. F_{IS} values ranged from 0.8111 to 1.0, indicating a higher amount of inbreeding in the pepper collections than in the tomato collections. For tomato, the percentage of loci under selection was 18.75% (three loci) and the percentage of linkage disequilibrium was 5.26%. For pepper, no locus was under selection, and the percentage of linkage disequilibrium detected was lower, 1.72%.

Comparison between the three marker systems in tomato and pepper

For tomato and pepper, a wide variation was observed for the average number of loci recovered per primer set for each marker system. However, for both plants, SSR recovered the lowest ratio and AFLP the highest ratio of loci (Table 5). Genetic variation represented by percentage polymorphism (Tables 3, 4) and PIC/H_e (Table 5) was the lowest when calculated from the AFLP dataset and the highest when calculated from the SSR dataset for both tomato and pepper. Therefore, there was no association between percentage of polymorphic loci and total number of scored loci. The highest ratios of number of polymorphic loci per primer set were observed for SSAP, being 1.93-fold and 2.4-fold higher than for AFLP and 10.79-fold and 12.86-fold higher than for SSR for tomato and pepper, respectively (Table 5). The highest marker index was calculated for SSAP, being sevenfold and 12-fold higher than AFLP and SSR, respectively, in tomato, and 21-fold and 20-fold higher than AFLP and SSR, respectively, in pepper.

All the estimates of correlation coefficients among pairwise genetic distance matrices generated by the different marker systems calculated using Mantel's test were highly significant (1,000 permutations, $P < 0.01$). For tomato, SSAP and AFLP showed the highest correlation ($r = 0.7362$), whereas lower correlations were detected between SSAP and SSR ($r = 0.5037$) and AFLP and SSR ($r = 0.5018$). Overall, correlations between pairwise genetic distances were lower between the marker systems in pepper. SSAP and SSR had the highest correlation ($r = 0.4486$), followed by SSAP with AFLP ($r = 0.3674$) and AFLP and SSR ($r = 0.2682$).

Table 4 Genetic variation values and neutrality test for AFLP and SSR tomato and pepper datasets

	Tomato collection (34 lines)		Pepper collection (35 lines)	
	AFLP	SSR	AFLP	SSR
Number of primer sets	9	16	9	13
Number of bands scored	845	39	1,432	31
Number of polymorphic bands	123	39	115	31
Polymorphism (%)	14.56	100	8.031	100
$n_a - n_e$	0.0665	0.66	0.0357	0.751
PIC (AFLP) or expected heterozygosity H_e (SSR)[a]	0.0461	0.3929	0.0263	0.3536
Number (%) of non-neutral sites (AFLP)/loci (SSR)[b]	17/845 (2.01%)	3 (18.75%)	7/1,432 (0.49%)	0
Sites/loci at higher frequency	5 (0.59%)	3 (18.75%)	3 (0.21%)	0
Sites/loci at lower frequency	11 (1.30%)	0	3 (0.21%)	0
Sites/loci at equal frequency	1 (0.12%)	0	1 (0.07%)	0

[a] Anderson et al. (1993) or Nei (1973)
[b] According to Ewens-Watterson neutrality test (Manly 1985)

Table 5 Comparison of informativeness and genetic distances obtained with the SSAP, AFLP and SSR marker systems

	Tomato collection (34 lines)			Pepper collection (35 lines)		
	SSAP	AFLP	SSR	SSAP	AFLP	SSR
Number of primer sets	3	9	16	3	9	13
Number of loci per primer set	46	93.89	2.44	40.33	159.11	2.385
Number of polymorphic loci per primer set	26.33	13.67	2.44	30.67	12.78	2.385
Observed number of alleles per locus (n_a)	1.573	1.146	2.438	1.76	1.08	2.385
Effective number of alleles per locus (n_e)	1.294	1.079	1.778	1.388	1.045	1.634
PIC (SSAP, AFLP) or H_e(SSR)[a]	0.175	0.0461	0.3929	0.229	0.0263	0.3536
Marker index (MI)[b]	4.61	0.630	0.393	7.022	0.336	0.354
Mean genetic distance	0.168	0.042	0.311	0.204	0.0213	0.306
Minimum genetic distance	0.036 (TOR10/17)	0.006 (TOR16/17)	0 (TOR10/11)	0.0579 (PAR5/7; 7/9; 8/9; 11/12; 29/36)	0.00281 (PAR7/16)	0 (PAR5/6; 7/9; 13/16; 19/22; 30/35)
Maximum genetic distance	0.326 (TOR14/34)	0.086 (TOR4/17)	0.641 (TOR4/15; 31/34)	0.397 (PAR22/34)	0.0517 (PAR8/29)	0.645 (PAR34/10,11,12, 15,18; 17/37)
Mean sampling variance	9.865×10^{-4}	4.680×10^{-5}	5.082×10^{-3}	1.298×10^{-3}	1.446×10^{-5}	6.247×10^{-3}
Estimated precision	0.187	0.163	0.229	0.177	0.179	0.258

[a]Anderson et al. (1993) or Nei (1973)
[b]Powell et al. (1996)

Genetic relationships within the tomato and pepper collections

Genetic distances and corresponding mean sampling variances calculated from the three different marker systems for tomato and pepper are shown in Table 5. SSAP and AFLP, but not SSR, were able to uniquely fingerprint each of the tomato and pepper lines. For tomato, the estimation of genetic distances was more precise using AFLP, followed by SSAP and, lastly, by SSR. In contrast, for pepper, genetic distances were calculated more precisely using SSAP, followed by AFLP, and, lastly, by SSR.

All of the NJ trees show well-resolved relationships among the tomato and pepper lines (Figs. 2, 3), except for the slightly lowered resolution seen on the SSR NJ tree for pepper. For tomato, the SSR NJ tree (Fig. 2) shows the highest range of genetic distance between pairs of lines (0–0.641) (Table 5). The highest number of bootstrap-supported groups was obtained from AFLP, followed by SSAP and SSR. The SSR NJ tree shows the clearest delimitation with the least misplaced individuals on the basis of fruit morphology. Two main clusters are easily distinguishable, with the first consisting of round with dispersed intermediate lines (C1) and the second consisting of two sub-clusters comprising flesh-type lines (C2). The SSAP and AFLP NJ trees have similar clustering patterns, which also differentiate between round and flesh fruit types, with the latter type comprising two sub-clusters, and the intermediate-type lines scattered among the previous two types. A notable difference displayed by the SSAP and AFLP NJ trees is in the placement of one of the flesh-type sub-clusters, being genetically more similar to the round-type cluster than to the other flesh-type sub-clusters. With all of the marker systems, the unique cherry-type line (TOR34) was genetically isolated and positioned closer to the round-type lines.

For pepper (Fig. 3), the SSR NJ tree again shows the highest range of genetic distances (0–0.645) (Table 5). Both SSAP and AFLP datasets showed the highest number of bootstrap-supported groups followed by SSR. Genetic relationships among pepper lines inferred from the three marker systems generally agreed with each other and with pepper types, mainly on the basis of fruit characteristics. The NJ trees clearly separate the genetically distant Sivri (pungent) and Charliston (sweet) conical pepper types from the Blocky and Dolma sweet bell/large-fruited pepper types with relatively limited genetic distances. Shifty positions are found for the Dulce Italiano (sweet conical) and Lamuyo (sweet blocky) types. The Dolma type PAR32 line, however, clusters with conical Sivri and Charliston pepper types with all three marker systems.

The SSR NJ tree shows a clear distinction between the pepper fruit types. One cluster (labelled L) consists of all of the Srivi and Charliston types, except for PAR38, that are clearly separated from the rest, being closest to the isolated Dolma line PAR32. Dulce Italiano

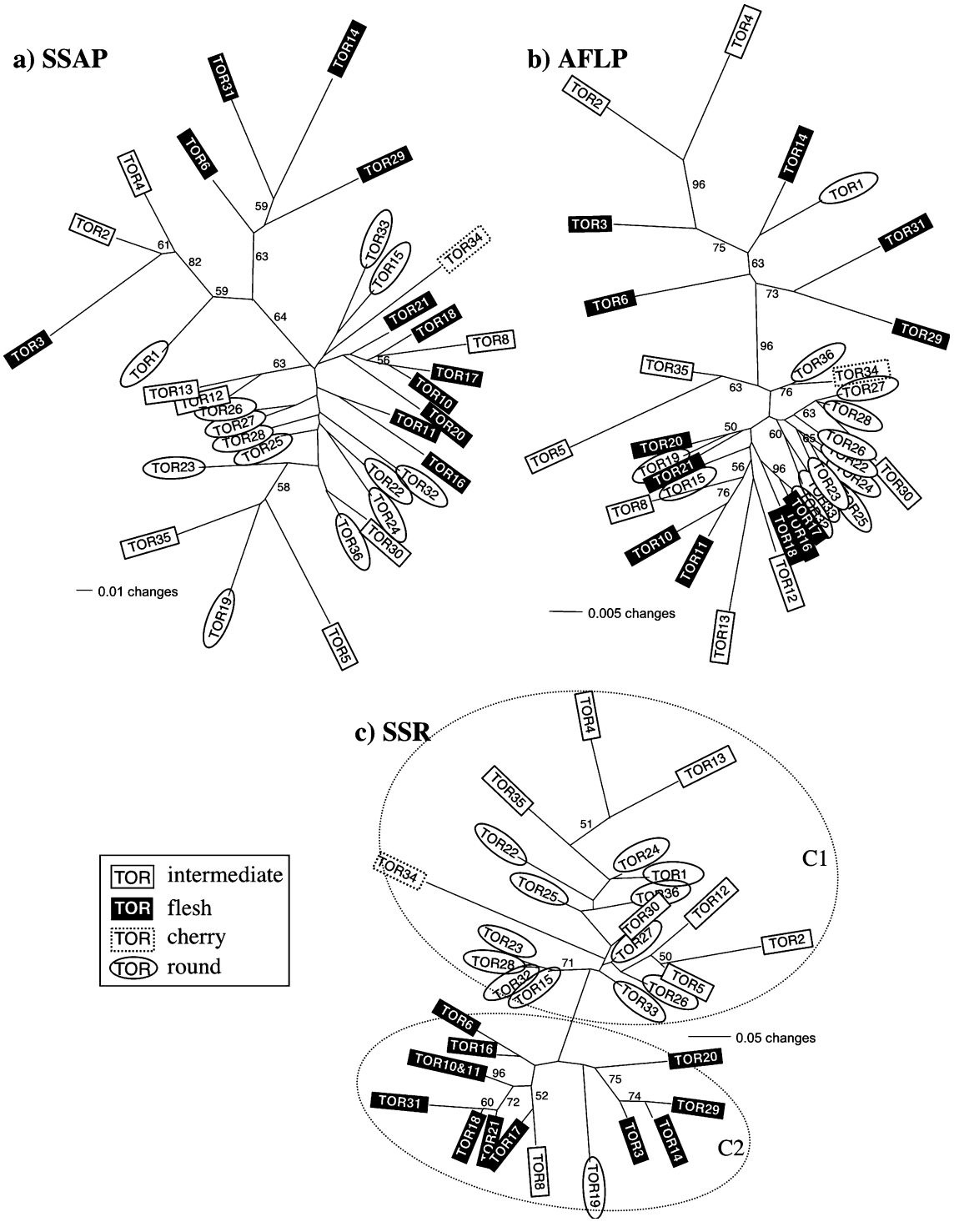

Fig. 2 Genetic relationships among tomato lines depicted by Neighbor Joining (NJ) trees based on three different marker systems: **a** combined SSAP obtained from the three retrotransposon datasets, **b** AFLP, **c** SSR. Lines are classified according to fruit morphology. Genetic distances are indicated below each tree, numbers next to the branches indicate levels of bootstrap support (only levels above 50% are indicated)

types, however, are interspersed among the bell pepper lines. The SSAP tree shows the clearest separation of a cluster (labelled S) consisting of all Blocky and Dolma sweet bell types, except for PAR32, from a second group comprising two sub-clusters, the first sub-cluster being the Lamuyo and Dulce Italiano types, which are closely related to the second sub-cluster, the Sivri and Charliston types. Here too, however, some conical Dulce

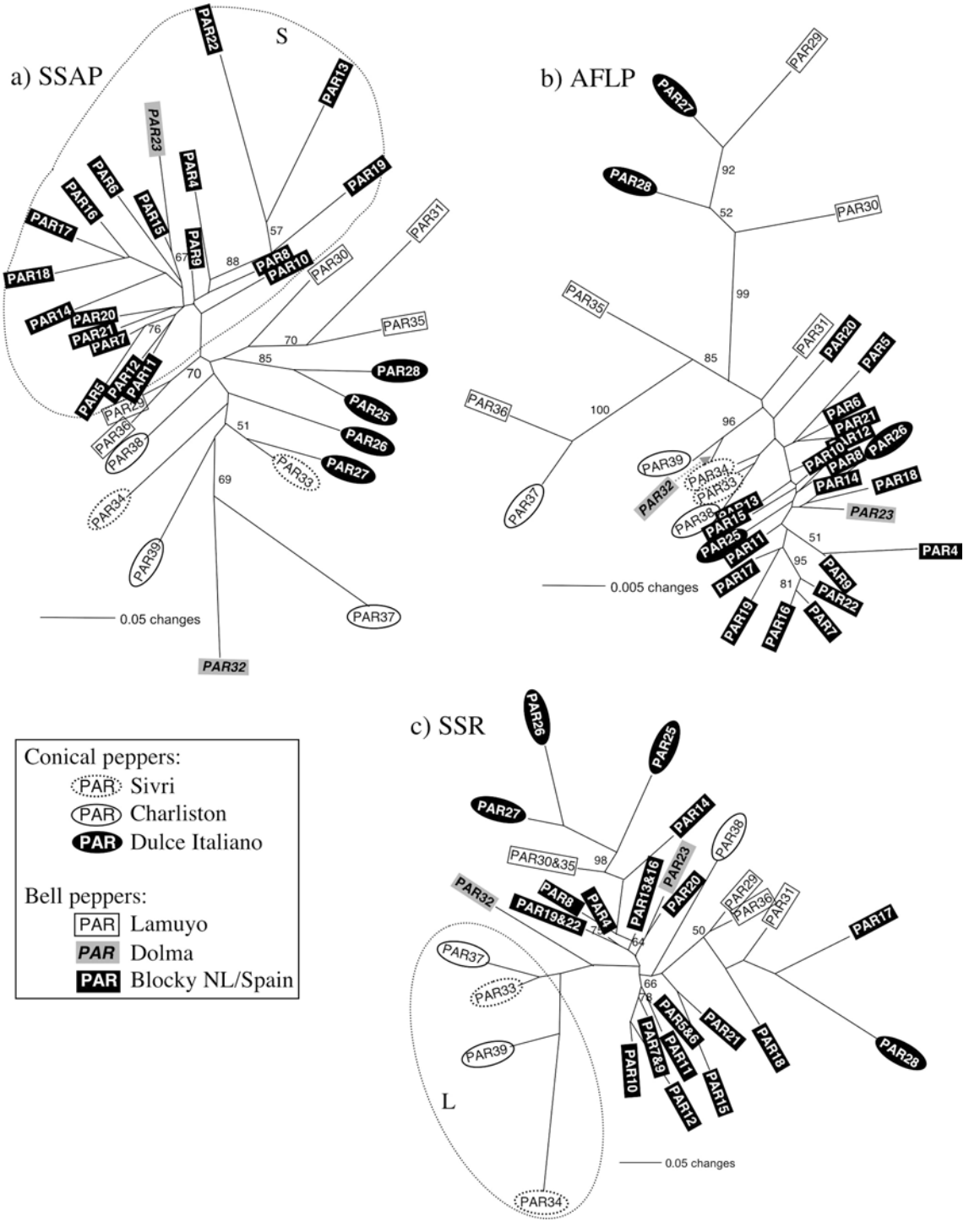

Fig. 3 Genetic relationships among pepper lines depicted by NJ trees based on three different marker systems: **a** combined SSAP obtained from the three retrotransposon datasets, **b** AFLP, **c** SSR. Lines are classified according to pepper type. Genetic distances are indicated below each tree, numbers next to the branches indicate levels of bootstrap support (only levels above 50% are indicated)

Italiano lines are closer to some Lamuyo bell pepper lines. The AFLP tree has a similar clustering pattern in that Blocky and Dolma bell pepper types are clustered and distinct from Sivri and Charliston conical types (together with PAR32). Lamuyo bell peppers are, however, clearly separated from other bell peppers, and Dulce Italiano conical peppers are interspersed with bell peppers.

Discussion

SSAP is an efficient marker system for evaluating genetic variation in tomato and pepper

The major objective of this study was to assess the usefulness of SSAP relative to two other marker systems, AFLP and SSR. SSAP and AFLP analyse a larger number of bands per assay than SSR, which has a significant effect on the cost per assay. SSAP revealed the highest number of polymorphic bands per assay. On the basis of the marker index, SSAP was the most informative system when compared to AFLP and SSR. On the whole, we found that SSAP was an efficient marker system for studying genetic variation in tomato and pepper. This is in agreement with results from previous studies. For example, Waugh et al. (1997) found SSAP to be 25% more polymorphic than AFLP in barley, and Ellis et al. (1998), in *Pisum*, observed that SSAP exhibited a threefold improvement over AFLP in discriminating accessions. In *Medicago sativa* L., SSAP showed the highest assay efficiency index and marker index when compared to SAMPL (Selective amplification of microsatellite polymorphic loci) and AFLP (Porceddu et al. 2002). AFLP offers the possibility to apply the same combination of adaptor primers with various selective nucleotides, which increases the possibility of detecting polymorphism, albeit reducing the number of bands per profile. This is also possible for SSAP markers based on high copy number retrotransposons, such as BARE-1 of barley (Waugh et al. 1997; Leigh et al. 2003) and Tms1 of *Medicago sativa* (Porceddu et al. 2002), both present in thousands of copies. The SSAP patterns we observed with adaptor primers devoid of selective nucleotides suggest that the retrotransposons used in our study are present in low copy number in tomato and pepper, with the possible exception of T265 in tomato. The possibility to increase marker numbers was thus not feasible in our study and we did not test various combinations of adaptor primers. However, it should be pointed out that plant genomes usually contain many different retrotransposon families as well as other types of transposable elements and that their combined use could increase the possibility of detecting polymorphisms.

SSAP is useful for assessing genetic relationships in tomato and pepper

For the purpose of inferring genetic relationships within tomato and pepper industrial collections, the highest mean and range of genetic distances were obtained from the SSR dataset. However, the SSR NJ trees were less resolved and may be the least precise due to its high polymorphism level. The marker system with the most ability to precisely estimate genetic distance was AFLP for tomato and SSAP for pepper. We also observed that AFLP had the highest number of bootstrap-supported groups for tomato, while both AFLP and SSAP had the highest bootstrap-supported groups for pepper.

Differences among marker techniques with respect to grouping lines on the basis of genetic distance have also been shown in earlier investigations. Some investigators reported a good correlation between datasets (dos Santos et al. 1994; Thormann et al. 1994; Lu et al. 1996; Nagaoka and Ogihara 1997), while others reported lower correlations (Beer et al. 1994; Powell et al. 1996; Pejic et al. 1998; Giancola et al. 2002). Rus-Kortekas et al. (1994) and Russell et al. (1997) suggested that higher band sharing would make a technique more suitable for genetic relatedness studies. They attributed the lack of correlation between SSRs and other marker systems to the high levels of polymorphism seen between pairwise comparisons. Powell et al. (1996) reported that SSRs correlated well with AFLPs and RFLPs only at the interspecies level. Conversely, the results from other studies confirmed the usefulness of SSRs for germplasm assessment and evolutionary studies precisely due to its greater resolving power (Yang et al. 1994; Olufowote et al. 1997; Pejic et al. 1998, Giancola et al. 2002).

In our study, the tomato collection can be divided into four distinct fruit morphology groups—round, cherry and two distinct fleshy types. We found a high similarity in the genetic relationship of the groups depicted by SSAP and AFLP, which indicates that the SSAP and AFLP marker systems reflect the same underlying divergences. There is a notable discrepancy in the placement of the two fleshy fruit-type sub-clusters. In using SSR, we did not detect an overly high level of polymorphism as shown by the moderate values of H_e and observed number of alleles per locus. However, there were higher percentages of loci under selection and under linkage disequilibrium. Therefore, we do not believe that the difference in genetic relationships inferred by the AFLP/SSAP trees and the SSR tree, respectively, can be attributed solely to differences in the level of polymorphism detected by each marker system, but rather that they reflect the complexity of the inheritance of the fruit morphology characters.

Previous studies have shown that many loci control the phenotypes of tomato fruit shape and size (quantitative trait loci, QTLs), and these have been mapped (Grandillo et al. 1999; van der Knaap et al. 2002). Dillman et al. (1997) and Burstin and Charcosset (1997) showed that the relationship between molecular and phenotypic distances computed from quantitative traits is clearly not linear but displays a triangular shape. Low marker distances are systematically associated with low phenotypic distances, whereas high marker distances correspond to either low or high phenotypic distances. Consequently, the linear coefficient of correlation between two distances decreases as the number of QTLs involved in the

variation of the traits considered for phenotypic distance increase.

The separation of the two flesh-type fruit sub-clusters between the round-type cluster on the AFLP and SSAP trees but their close relationship on the SSR tree can perhaps be explained by the polygenic inheritance of the fruit-shape trait. Burstin and Charcosset (1997) noted that molecular markers that are generally neutral will not have any direct effect on quantitative traits. A strong relationship between marker and morphological distance is expected only if there is linkage disequilibrium. Two lines that are similar at marker loci sharing common alleles at the QTLs will thus be phenotypically close. When the four loci found to be involved in linkage disequilibrium were excluded from analysis, we found that the genetic relationships displayed by SSR were more in concordance with AFLP and SSAP, which is in agreement with the fact that linkage disequilibrium can have a considerable effect on genetic relationships (tree not shown). However, the four loci displaying linkage disequilibria were not permanently removed from SSR analysis, as this resulted in a considerable loss of tree resolution due to the already low number of alleles detected. More importantly, we wish to highlight that different marker systems can reflect different contexts of genetic relationships. Molecular markers have the ability to discriminate between a close similarity as a consequence of different breeding sources and close similarity due to high relatedness (Dillman et al. 1997).

The pepper collection can be divided into two major clusters—a cluster of sweet bell pepper types with lower genetic distances among lines and another cluster of more diverse conical pepper types, pungent and sweet. As with tomato, we did not detect an overly high level of variation from SSR. Our genetic variation results generally agree with those obtained from other studies of pepper (Lefebvre et al. 1993, 2001; Paran et al. 1998). Genetic relationships inferred by the SSAP, AFLP and SSR marker systems are in general agreement with each other, indicating that SSAP also reflects the underlying genetic divergences of the pepper collection. Lefebvre et al. (1993) in their study using RFLPs found that bell peppers grouped together, separately from the small-fruited pepper accessions that included both the pungent and non-pungent accessions, suggesting that the criteria of fruit shape was more determinant than pungency.

Minor differences in tree topologies can be explained by the shifty positions of the groups of Dulce Italiano conical peppers and Lamuyo bell peppers. Dulce Italiano types were grouped with the Blocky bell types on the SSR tree, and the Lamuyo types were grouped with the Sivri and Charliston conical types on the SSAP tree, while most of the Lamuyo and Dulce Italiano lines were interspersed together and basal to the Sivri and Charliston conical types on the AFLP tree. The Lamuyo and Dulce Italiano types thus appear to be intermediate pepper types.

Patterns and the neutrality of retrotransposon insertion polymorphisms in tomato and pepper

The population dynamics of retrotransposons may vary depending on activation and maintenance factors—and thus vary in their informativeness. Reciprocally, SSAP analyses enable indirect comparative observations to be made on the activity of retrotransposons (Ellis et al. 1998; Gribbon et al. 1999; Pearce et al. 2000). The Tnt1 retrotransposon was originally isolated from tobacco (Grandbastien et al. 1989), and related Retrolyc1 elements are present in tomato (Costa et al. 1999; Araujo et al. 2001). We report here the presence of Tnt1 in *Capsicum*, a third Solanaceous genus. A primer designed in the 3′ region of the LTR of the Tnt1-94 tobacco element (OL16) was able to amplify Tnt1 elements in tomato and in pepper, showing very good conservation of this region in some Tnt1-related elements over diverse Solanaceae host genera. Similarly, the T135 retrotransposon sequence, originally isolated from tomato, is also present in pepper. These observations indicate that some of the LTR sequences of retrotransposons isolated from one Solanaceae species can be used in related genera of the same plant family.

The ToRTL1-E00 and T265-E00 datasets in tomato and the Tnt1-E00 and T135-E00 datasets in pepper showed similar levels of genetic diversity values, suggesting that the activity level of these retrotransposon families is fairly similar in the two sets of hosts. Small differences between the observed and expected number of alleles, noted for all elements from tomato and pepper, indicate non-structuring in the collections, so there has been no strong burst of transposition activity restricted to a subset of each of the collections.

Molecular marker studies often assume a strictly neutral model of evolution as the basis for analysing and interpreting results (Ford 2002). In our study, only the insertion site profiles of ToRTL1-E00 and T265-E00 for tomato showed significant departures from neutrality based on Tajima's test (under intermediate recombination) with both datasets showing balancing selection. For the insertion frequencies of individual sites, the Ewen-Watterson's tests showed that the majority of the sites were neutral. This identified genetic drift as the important factor influencing the population dynamics of these retrotransposons in the tomato and pepper lines studied. If most markers are neutral, transposition activity higher than neutral mutation rates will provide more polymorphic sites evolving with neutral insertion frequencies that will influence the D value. In addition, linkage disequilibria between selected insertion sites for ToRTL1 may be partially accountable for the high positive D values.

Conclusion

Our study has established that the SSAP marker system is useful for studying genetic diversity among tomato

and pepper industrial lines. The combination of polymorphism and the large number of bands obtained per assay shows that SSAP is the most informative marker system of the three tested. SSAP showed about four- to ninefold more diversity than AFLP, had the highest ratio of number of polymorphic band per assay and the highest marker index. SSAP requires initial investment in terms of knowledge of sequence information of terminal sequences (LTRs) of retrotransposons, but retrotransposon sequences are now fairly easy to obtain, being abundant in many databases, such as the TIGR Plant Repeat Database (http://www.tigr.org/tdb/e2k1/plant.repeats/index.shtml), and a rapid non-specific isolation strategy of Ty1-copia element LTR sequences is available (Pearce et al. 1999). Furthermore, retrotransposon sequences isolated from one species can be used in other genera of the same plant family. Polymorphic SSAP bands can be extracted and converted into site-specific markers that allow rapid automatized screenings of larger germplasm collections (Flavell et al. 2003).

Estimates of genetic relationships were significantly correlated between different SSAP datasets and between SSAP, AFLP and SSR datasets, thereby demonstrating the reliability of the SSAP technique in both species. For tomato, a higher correlation was found between SSAP and AFLP; this in contrast with lower correlation observed between the former two marker systems and SSR. For pepper, SSAP was more correlated to SSR than AFLP. Our results from tomato show SSAP as being more suitable for inferring overall genetic variation and relationships, which is important in germplasm management. Co-dominant SSR has the ability to detect genetic relationships based on specific traits, most probably caused by its sensitivity to neutrality and/or linkage disequilibrium, and would be more useful for studies of specific sets of inbred lines and breeding material with special characteristics. Our results for pepper show a general agreement between each marker system and with the pepper types. Discordance between different marker systems can be very informative for understanding genetic relationships within the study group.

Overall, knowledge of genetic diversity estimated from different marker systems should provide different levels of information that should cater to the different needs of plant breeding programmes and the management of germplasm resources.

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