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Comparative analysis of polymorphism and chromosomal location of tomato microsatellite markers isolated from different sources

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Abstract Previously isolated tomato (*Lycopersicon esculentum*) microsatellite markers were mainly clustered in the centromeric heterochromatin and not located in euchromatic regions. To achieve a more-uniform distribution of microsatellite markers for genome mapping purposes, a set of tomato microsatellite markers containing dinucleotide simple sequence repeats were developed by screening genomic libraries enriched for single-copy sequences, and screening the tomato EST database. The tomato microsatellites isolated in these ways were characterized by combinations of different types of repeated motifs and they were polymorphic in a set of *L. esculentum* varieties detecting up to four alleles. A total of 20 markers were placed on the genetic map of tomato. Interestingly, all markers isolated from genomic libraries enriched for single-copy sequences by *Pst*I-pre-digestion mapped into the centromeric regions. The majority of markers derived from EST sequences contained predominantly AT microsatellites and were located in euchromatic regions.

Keywords Microsatellites · Genetic mapping · *Lycopersicon esculentum* · Genetic variability

Introduction

Microsatellites, or simple sequence repeats (SSRs), are a class of repetitive sequences which are interspersed and abundant in eukaryotic genomes. Variation in the number of repeats results in length polymorphism at given loci between different individuals. Such polymorphisms can be detected by a pair of locus-specific primers flanking a microsatellite repeat and PCR amplification of the target region (Litt and Luty 1989; Weber and May 1989). Their high level of polymorphism, frequency, and random distribution in the genome make microsatellites useful genetic markers for mapping, and for population and evolutionary studies, especially in organisms with low levels of DNA variation.

The generation of PCR primers for microsatellite markers remains a relatively complex technique, because it requires the isolation of unique flanking sequences specific to each microsatellite locus by the construction and screening of different types of genomic libraries. Increasing amounts of genomic sequences in sequence databases facilitate SSR marker development. Large-scale isolation of functional microsatellite markers has been undertaken for different plant species. For example, more than 120 microsatellites isolated from rice genomic libraries and sequence databases were integrated into the RFLP map and showed an even distribution throughout the rice chromosomes (McCouch et al. 1997). A total of 279 microsatellite-containing loci amplified by 230 primer pairs were placed onto the wheat genetic linkage map. The markers were mapped to all three A, B and D genomes of wheat and were randomly distributed along linkage groups with some clustering in several centromeric regions (Röder et al. 1998). Forty SSRs have been isolated from barley genomic libraries and database sequences, and mapped to seven barley chromosomes (Becker and Heun 1995; Liu et al. 1996). This has been followed by the development of 568 new barley SSRs from enriched small-insert genomic libraries and from new gene sequences in the public databases. Using these markers, a second-generation SSR-based linkage map of the barley genome has been constructed (Ramsay et al. 2000). Recently, an integrated genetic linkage map of the soybean genome consisting of 606 SSR, 689 RFLP, 79 RAPD, 11 AFLP, 10 isozyme, and 26 morphological loci has been published (Cregan et al. 1999). Ninety eight highly informative potato microsatellite markers were isolated from the EMBL sequence database, cDNA and selectively enriched small-insert genomic libraries, and 65 markers were localized at 89 genetic loci across

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the chromosomes giving reasonable genome coverage (Milbourne et al. 1998). Nearly 1000 microsatellites have been mapped in the maize genome and found to be distributed on all ten chromosomes (http://www.agron.missouri.edu/ssr.html).

Dinucleotide simple sequence repeats which are usually randomly distributed in plant genomes dominate among microsatellite markers detecting a high level of polymorphism. Tri- and tetra-nucleotide microsatellites are occasionally used for marker development but their distribution throughout the genomes is different from that of dinucleotide repeats. Trinucleotide repeats have been found to be frequent in coding regions (Wang et al. 1994; Smulders et al. 1997), while tetranucleotide repeats appear to be clustered in the regions of suppressed recombination in the genome, such as centromeres.

Non-random distribution of tetranucleotide repeats has been demonstrated by the genetic mapping of the long stretches of GATA and GACA motifs in tomato (Arens et al. 1995) which have been clustered around centromeres. Interestingly, this clustering of microsatellite markers was not limited to tetranucleotide repeats in tomato. Microsatellite markers based on various types of dinucleotide microsatellite repeats isolated from small-insert genomic libraries revealed a predominantly centromeric location after genetic mapping (Broun and Tanksley 1996; Areshchenkova and Ganal 1999), which was unusual compared to other plant species. In order to elucidate whether the centromeric location is a peculiar feature of tomato microsatellites or a drawback of the isolation procedures used, we have investigated different sources of microsatellite sequences from the tomato genome for their level of polymorphism, repeat structure and chromosomal location.

Materials and methods

Plant material and DNA isolation

The *Lycopersicon esculentum* variety VFNT cherry was used for the construction of small-insert libraries and as a reference stock. The test set of tomato cultivars and the tomato mapping population have been described previously (Tanksley et al. 1992; Areshchenkova and Ganal 1999). Total genomic DNA was isolated according to Bernatzky and Tanksley (1986) from leaf tissue.

Construction and screening of plasmid libraries enriched for single-copy sequences

Total DNA from the tomato cultivar VFNT cherry was pre-digested with the restriction enzyme *Pst*I. Restriction fragments in the size range between 2 and 7 kb were purified from a 1% agarose gel using the Geneclean kit (Dianova). The size-selected DNA fragments were further digested with *Sau*3A, *Bam*HI, or *Acs*I. After digestion with *Sau*3AI or *Bam*HI, DNA fragments were precipitated with ethanol and ligated into the pUC18 *Bam*HI/BAP vector, and after digestion with *Acs*I into the pUC18 *Eco*RI/BAP vector (Amersham Pharmacia). *Escherichia coli* XL10-Gold ultracompetent cells (Stratagene) were transformed according to the manufacturer's protocol and plated onto 22×22 cm plates. Using the automated colony picking system BioPick (BioRobotics Ltd.), single colonies were transfered into 384-well microtiter plates. High-density colony filters for hybridization were prepared using the robotic gridding system BioGrid (BioRobotics Ltd.). High-density filters were hybridized with $(GA)_n$, $(GT)_n$, $(TGA)_n$, $(GTA)_n$ and $(GATA)_{10}$, oligonucleotide probes at 65°C and washed to a stringency of 0.5×SSC, 0.1%SDS. Single positive clones were used to isolate plasmid DNA according to the plasmid mini protocol (Qiagen).

Search of the EST database for microsatellites

Computer searches were performed using the TIGR (The Institute for Genome Research) Tomato Gene Index (LGI, release version 1.2). The LGI (http://www.tigr.org/tdb/lgi/index.html) was searched for a minimum number of ten repeating units for all types of dinucleotide, trinucleotide and GATA repeats using the WU-BLAST 2.0 search program.

Primer designing and PCR conditions

Oligonucleotide primer pairs were designed from unique sequences flanking the microsatellite motifs using the Primer 0.5 program (provided by E. Lander, Whitehead Institute, USA). Primers were selected to be approximately 20-bp long and to have a G+C content of 45–50%. One primer was labelled with fluorescein for fragment analysis on an A.L.F sequencer. PCR reactions on tomato DNA were performed as described previously (Areshchenkova and Ganal 1999).

Allele detection and genetic mapping

In order to determine the polymorphism level of the isolated microsatellite markers, genomic DNA from the test cultivars was used as a template for PCR amplification with each primer pair. Fragment analysis was carried out on an A.L.F. DNA sequencer (Pharmacia) using short gel cassettes. Denaturing 6% polyacrylamide gels, 0.35-mm thick, were prepared using SequaGel XR solutions (Biozym). Gels were run in 1×TBE buffer under 600 V, 40 mA and 50 W with 2 mW laser power and a sampling interval of 0.84 s. Fragment sizes were determined using the computer program Fragment manager 1.2 (Pharmacia) and internal size standards. Linkage analysis was performed using the MAPMAKER software (Lander et al. 1987).

Results

Isolation of microsatellites from genomic libraries enriched for single-copy sequences

Tomato libraries in plasmid vectors were constructed by cloning small inserts of total VFNT cherry genomic DNA pre-digested with the methylation-sensitive restriction enzyme *Pst*I. *Pst*I does not cleave at its recognition sequence of 5′-CTGCAG-3′ if the 5′cytosine is methylated. The 5′CNG3′ is the most-common site for methylation in plant genomes and the C-T-G site is methylated in approximately 70% of all cases (Gruenbaum et al. 1981). Most repetitive sequences are completely methylated at this site; therefore, this enzyme cleaves preferentially in single- or low-copy DNA. Thus, cloning with restriction enzymes like *Pst*I yields libraries enriched for single-copy sequences and reduces the number of microsatellites derived from regions of highly repeated DNA. Selected 2–7-kb fractions of *Pst*I-restriction DNA frag-

Table 1 Repeat structure, primer sequences of tomato microsatellite markers and the number of alleles in detected in twelve *L. esculentum* lines and *L. pennellii* LA716

Microsatellite marker	Repeated sequence	T_m, °C	Fragment size, bp	Primer pair, 5′–3′	Number of alleles
Markers isolated from genomic libraries enriched for single-copy sequences					
TMS52	$(AC)_{14}(AT)_{18}$	55	152	TTCTATCTCATTTGGCTTCTTC TTACCTTGAGAATGGCCTTG	3
TMS54	$(TA)_{14}(CA)_{15}$	60	226	TTGGTCTAGAACGATGAGCA GCCATGCATCACTGAATGAC	2
TMS56	$(CT)_{19}$	55	120	GATCTCAAAGGATGAACAATAC TCATTAGGAGATTCTTTGTATCA	4
TMS57	$(GT)_{13}(AT)_5$	60	260	ACATGACCGGTTGACGACTA AAATTGTCCACATGGTGGGT	1
TMS58	$(TA)_{15}(TG)_{17}$	60	225	CATTTGTTGTATGGCATCGC CAGTGACCTCTCGCACAAAA	2
TMS59	$(AT)_5(GT)_{11}(AT)_{11}$	55	100	TGAACGGGCCTTCTGTTATC ATCATCATTATAGTTCTTAAGTGAT	2
TMS60	$(AT)_{13}(CA)_{23}(AT)_{22}$	60	242	ATGCAGTTCCAAGCATCATT TTGCCACATTAATGTTGAAGT	2
TMS63	$(AT)_4(GT)_{18}(AT)_9$	60	150	GCAGGTACGCACGCATATAT GCTCCGTCAGGAATTCTCTC	3
TMS65	$(TA)_{25}(GA)_{20}$	60	308	AGCTTCATCCATTACGCCAC GTGCATCTGGCGTACCTACC	4
TMS66	$(GT)_{19}(AT)_4$	60	201	GGGTTAATAAAGCAATGTAGCG CTCTTCATTAAAGTTGCCGC	2
Microsatellite markers generated from EST sequences					
TC1843	$(CAG)_8(AAT)_{12}$	60	567	ATGGAGTTTCAGGACCACTT AGGATGATTCAATATATCCGC	3
TC1107	$(AT)_{11}$	58	97	TCCATCTCTCTCTAGACCTTTCT TTCTTAAATCCTCTCACTCA	2
TC948	$(TA)_{38}$	60	150	TTTTCGCGTTAAGAGATGTT CCGCCATACTGATACGATAG	2
TC11	$(AG)_{11}$	60	91	TCAACACAGAGAAAATAGGCA CAGCTTGCTCAGCCAGC	3
TC461	$(TAT)_{15}$	60	185	GGCTGCCTCGGACAATG TTATTGCCACGTAGTCATGA	3
EST245053	$T_{12}(GT)_6(GA)_6$	58	228	CCATTTAAATGACCCTATGCT AATCAAAAAGAATCTAAGCCCT	2
EST253712	$(AT)_{14}$	55	140	GAAATGAAGCTCTGACATCAAA TCATTGCTTGCATATGTTCATG	4
EST258529	$(TA)_{17}$	50	127	AACACCCTTTATTCAGATTCC GCATAAAAATGTTAAAGGGG	2
EST248494	$(TA)_{13}T_{20}$	60	211	CTGAAACGAGACAGAGGAAG AGCTGAGTACGTCTCCCATG	2
EST259379	$(TA)_{20}$	55	151	TTGGTCTCCCTCTTTATGCC GGCTTCATTGATGAACCCAT	3
EST268259	$T_{11}(GT)_4(AT)_4(GT)_8$	55	127	GCTGCTCCTATTGGTTACCA TCTCCTTATTTGGATTGGCC	2

ments were further digested with one of the following enzymes *Sau3A*I, *BamH*I or *Acs*I and inserted into the pUC18 plasmid.

Approximately 40000 clones derived predominantly from the *PstI/Sau3A* and PstI/*Acs*I libraries were analysed by colony hybridization. The number of clones hybridizing to poly(GT) and poly(GA) oligonucleotides with respect to the total number of analysed clones was less than 0.1%. This ratio was four-times lower than the percentage obtained during Lambda library screening (Areshchenkova and Ganal 1999). The determination of the insert sizes of these libraries revealed an average size of 250 base pairs compared to the 1-kb average insert size of previously used Lambda libraries. Increasing the average insert size of the tomato clones to a size comparable to the Lambda libraries was performed by a second size selection before ligation. The average insert size of 750 bp increased the number of positive clones to 0.25%.

A total of 27 positive recombinant clones were sequenced from the plasmid libraries of tomato, of which 22 contained dinucleotide repeats. Five clones failed to show microsatellites, primarily due to large insert sizes that prevented full sequencing of the inserts. All isolated GT repeats and the majority of GA microsatellites were associated with AT repeats. The average number of

Table 2 Comparative analysis of polymorphisms detected with microsatellite markers in 12 *L.esculentum* lines and *L.pennellii* LA716

Microsatellite markers generated from:	Phage libraries	Plasmid libraries	EST sequences
Total number of markers	25	10	11
Average number of alleles in 12 *L.esculentum* lines and *L.pennellii* LA716	3.0	2.5	2.55

GT repeats was 18, and GA motifs were on-average 22-times repeated. Thus, microsatellites isolated from the plasmid libraries of tomato showed the same repeat length and complex structure as SSRs isolated previously from the Lambda libraries. No microsatellites were isolated by hybridizations with trinucleotide and GATA probes. Five (23%) of the isolated microsatellites were located near the restriction sites. For 16 microsatellites primer sets could be designed. Out of 16 synthesized primer pairs ten (62.5%) yielded functional markers (Table 1).

Generation of microsatellite markers from EST sequences

LGI release version 1.2 contained more than 27000 tomato EST sequences. In order to identify microsatellites from expressed parts of the tomato genome, LGI was searched with all possible di- and tri-nucleotide repeats and also GATA simple-sequence repeats using BLAST similarity searching. A total of 250 SSR-containing sequences were identified, among them only 55 (0.2% of total number) contained microsatellites with more than ten repeated motifs. AT dinucleotide repeats were by far the most-frequent class of microsatellites followed by GA repeats. AAT repeats were the most-abundant among trinucleotide repeats followed by the complementary ATT motif which were repeated on average seven times. No tetranucleotide microsatellites were found except for one TTAA and one TTTA repeat, which were repeated five times each.

For the majority of microsatellites in EST sequences, primers could not be designed because the repeats were located in non-translated regions at the ends of ESTs. Only 20 completely sequenced uninterrupted dinucleotide microsatellites could be used for primer design. The majority of those sequences contained the AT dinucleotide or other AT-rich trinucleotide repeats. Eleven (55%) primer pairs amplified a single fragment of the expected size (Table 1). The remaining 45% of microsatellite markers contained predominantly AT repeats and resulted in the amplification of multiple fragments. The AT microsatellite markers amplified multiple fragments more often than GA or GT microsatellites.

Polymorphism of microsatellites in *L.esculentum* varieties

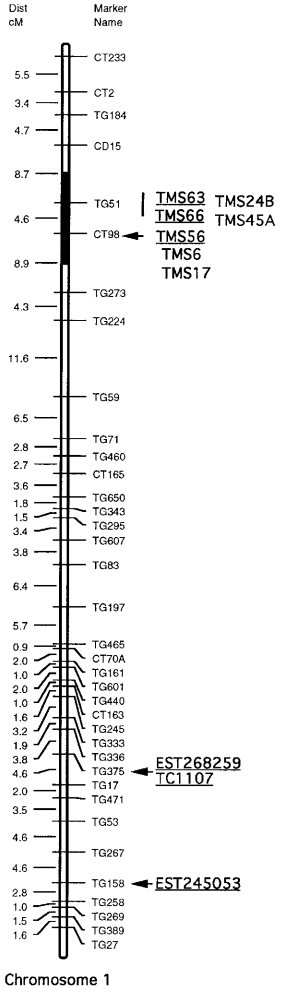
The microsatellite markers which amplified fragments in the expected size range were used to study allelic diversity in the cultivated tomato and *Lycopersicon pennellii*. For the 21 analyzed microsatellites, a total of 53 alleles could be identified in the test set of tomato lines ranging from one allele to a maximum of four different alleles. In most cases, the *L. pennellii* accession LA716 had either a unique or a null allele (Table 1) in accordance with previous results (Areshchenkova and Ganal 1999). The 10 markers generated from plasmid libraries detected on-average 2.5 alleles in the investigated *L. esculentum* accessions and *L. pennellii* LA716. The microsatellite markers that were generated from the EST sequences detected an average of 2.55 alleles compared to an average of 3 alleles for genomic microsatellite markers isolated from Lambda phages (Table 2).

Genetic mapping of tomato microsatellites

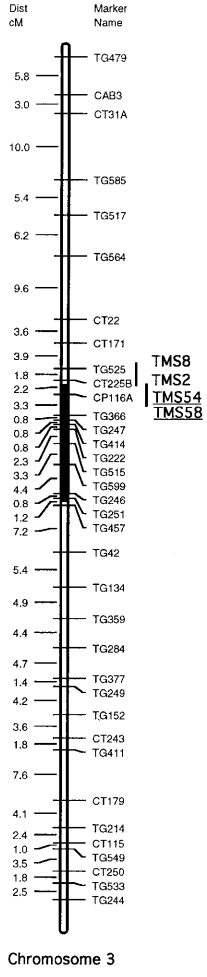
Genetic mapping of the isolated microsatellite markers was performed using an F2 population derived from the *L.esculentum* TA55×*L.pennellii* LA716 interspecific cross. This segregating population was used for the construction of a saturated RFLP map of tomato (Tanksley et al. 1992). When surveying the markers for polymorphism between the two parents, it was noted that only half of the microsatellite markers yielded defined fragments in *L. pennellii* after PCR amplification. Absence of the amplification product in *L. pennellii* was scored as a null allele. The map position of each microsatellite marker was determined on the existing high-density linkage map.

All microsatellite markers could be mapped except TMS57 which was monomorphic. The mapping results are shown in Fig. 1. The 22 markers isolated previously from tomato phage libraries (Areshchenkova and Ganal 1999) did not map in a random fashion onto the tomato chromosomes. Independent of the type and number of repeated motifs, the map position of all microsatellite markers was at, or close to, the position of the presumed centromere of the respective chromosome. Interestingly, the mapping of the nine microsatellites isolated from the *Pst*I pre-digested tomato plasmid libraries revealed that

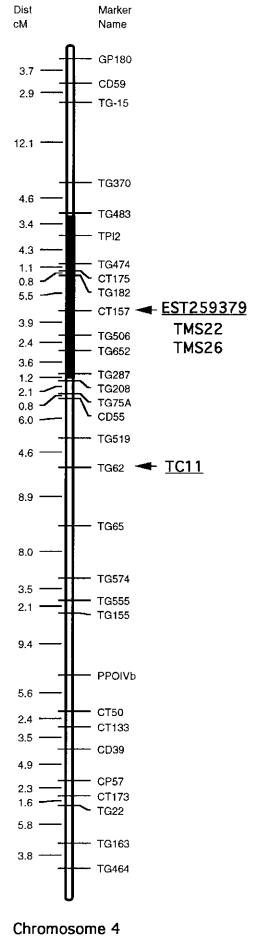
Fig. 1 Map position of tomato microsatellite markers. The map position of 20 microsatellite markers (underlined) generated from the *Pst*I-pre-digested genomic DNA and the ESTs was integrated into the existing framework of the tomato high-density genetic map and are *underlined*. Only those chromosomes are shown onto which these new markers could be mapped. Previously mapped TMS markers are not underlined and are indicated for comparison. The most-likely position of the centromere is indicated by a *black bar* in the chromosome picture ▶



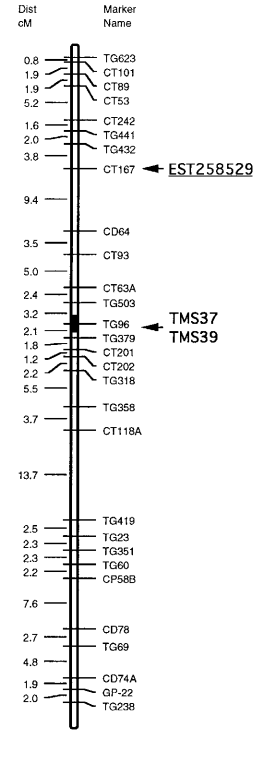
Chromosome 1



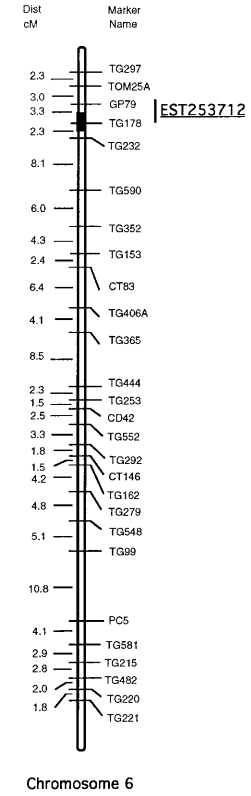
Chromosome 3



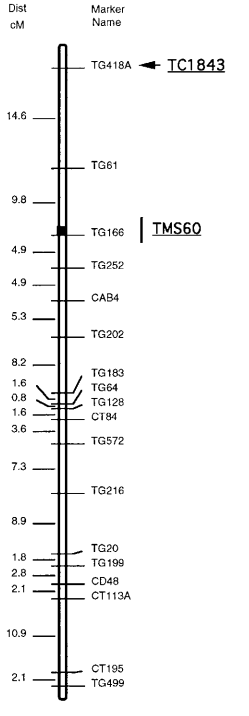
Chromosome 4



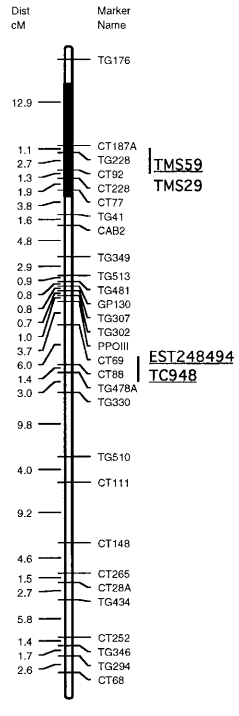
Chromosome 5



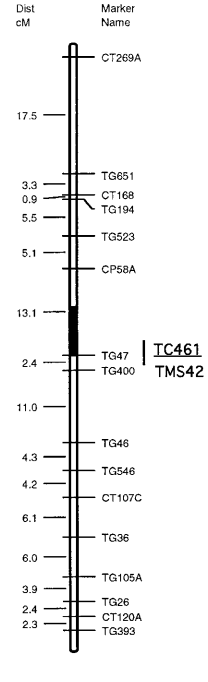
Chromosome 6



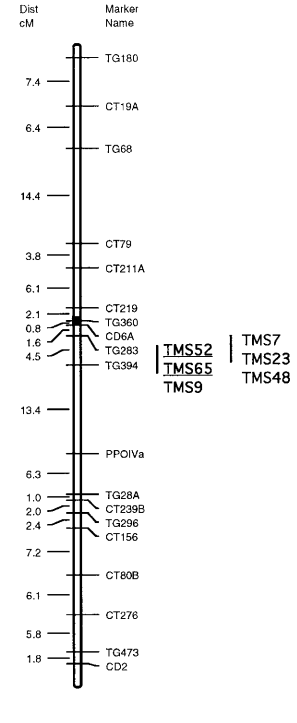
Chromosome 7



Chromosome 8



Chromosome 11



Chromosome 12

these markers were also located in the centromeric heterochromatin.

Compared to the markers isolated from genomic clones, significantly different map positions were observed for most of the microsatellites generated from EST sequences. Eleven markers were mapped onto the genetic map. Three of them (EST253712, EST259379 and TC461) were localized in the centromeric regions of chromosome 6, 4 and 11 respectively, but eight could be placed in regions distant from the centromeres. For example, seven markers isolated from genomic libraries clustered in the centromeric region of chromosome 1, but the three EST markers TC1107, EST268259 and EST245053 mapped to the end of the long arm of this chromosome.

Discussion

Tomato microsatellites directly generated from phage libraries displayed a high level of polymorphism compared to other molecular markers of tomato. However, genetic mapping of a number of such markers revealed that they are predominantly located in the centromere-associated heterochromatin (Areshchenkova and Ganal 1999) and thus have only limited use. This clustering of microsatellite markers in centromeric regions could be due to the fact that a large proportion of the tomato repeated DNA sequences is concentrated in the heterochromatic regions, and many microsatellite markers isolated from genomic libraries are derived from such regions.

To circumvent an eventual bias towards the isolation of microsatellite markers from repetitive DNA we used pre-digestion with the restriction enzyme *Pst*I which has been applied successfully for the enrichment of single- and low-copy DNA sequences during the isolation of probes for RFLP mapping. The microsatellite motifs isolated from the *Pst*I pre-digested DNA fraction were very similar to the motifs of the microsatellite markers isolated from the unselected genomic libraries containing not only the GA and GT but also long stretches of the AT motif which was not selected during the screening of the clones. In the previous study using Lambda phage libraries we found that more than two-thirds of all markers isolated with GA and GT motifs contained composite microsatellite repeats predominantly with AT repeats. With this similarity in structure, the nine functional microsatellite markers isolated from the *Pst*I-enriched DNA fraction mapped to the centromeric regions of tomato chromosomes, suggesting that the pre-selection for low-copy and under-methylated sequences has not improved the distribution of tomato microsatellite markers.

These data suggest that the bias towards the isolation of centromere-associated microsatellites is not caused by the marker isolation or generation procedure as has been observed for AFLPs. Approximately 90% of all AFLP markers generated by *Eco*RI/*Mse*I primer combinations are clustered around the tomato centromeres (Haanstra et al. 1999). This has been attributed to the fact that such primer combinations are unbiased with respect to repeated and methylated DNA and thus are preferentially amplifying repeated and heterochromatic sequences. In this case, the use of primer combinations such as *Pst*I/*Mse*I, which select for unmethylated and low-copy DNA, reduces the bias considerably and creates markers which are more-evenly distributed.

Because of these results, we assume that the centromeric association of tomato microsatellite markers is most likely due to an uneven distribution of the GA and GT arrays in the genome. By in situ hybridization, it has been shown that certain dinucleotide microsatellite motifs are clustered in centromeric regions of sugar beet (Schmidt and Heslop-Harrison 1996). If microsatellite motif-clustering is the problem, one solution would be the generation of microsatellite markers from sequences with motifs other than GA or GT and directly from genes. Microsatellite markers have been generated from the nucleotide sequence database for a number of plants. In potato, more than 50 microsatellite markers have been generated from genes and cDNA clones (Milbourne et al. 1998) and mapped. Smulders et al. (1997) have isolated tomato microsatellite markers directly from the sequence databases but no mapping position of these markers is available until now. As an alternative, we have screened part of the available tomato EST database for microsatellite repeats.

Within more than 27,000 tomato ESTs only 20 could be identified which had microsatellites of more than ten repeating units for which it was possible to design primers. The majority of microsatellites identified in this way harboured the AT motif; the AT motif, in conjunction with T repeats or trinucleotide repeats with the motifs AAT or TAT, defining different classes of microsatellites than the ones isolated from the genomic DNA by screening with the GT and GA motifs. Mapping of the markers generated from ESTs, with approximately two-thirds of them being outside the centromeric regions, shows that AT repeats are expected to be more-randomly distributed throughout the genome. Their distribution in the tomato genome is similar to the distribution of RFLP markers on the high density map of tomato (Tanksley et al. 1992). Nevertheless, these markers reveal with an average of 2.55 alleles in a set of tomato varieties and in *L. pennellii* LA716 a high level of polymorphism. This level of polymorphism is slightly higher than for the microsatellite markers isolated from the *Pst*I-selected DNA (2.5 alleles). In comparison, the unbiased genomic microsatellite markers display on average 3.0 alleles in the same reference set of tomato lines. The allele numbers of microsatellites in tomato varieties are relatively low compared to other plant speices and reflect the narrow germplasm from which current tomato varieties are being developed (Miller and Tanksley 1990). Currently, research is underway to determine the necessary number of tomato microsatellites for the generation of unique microsatellite fingerprints for tomato varieties.

In summary, these results indicate that the GA and GT microsatellites are clustered in the centromeric heterochromatin of tomato, and the isolation of microsatellite markers harbouring these motifs will very likely result in a high level of clustering around the centromeres independently of the isolation procedure. A solution to this problem is the isolation of microsatellite markers from ESTs since the database currently contains nearly 100 000 sequences (http://www.tigr.org/tdb/lgi/index.html). However, the use of ESTs for microsatellite isolation is complicated by two factors. One problem is that the microsatellite repeats are frequently located to close to the cloning site in the 5′ or 3′ untranslated region, eliminating nearly two-thirds of all ESTs for primer design. The other problem is that microsatellite arrays in genes and ESTs have usually less than ten repeating units. For tomato, it has previously been shown by Smulders et al. (1997) that microsatellite markers derived from such short arrays in known genes are significantly less-polymorphic than markers generated from longer arrays. On the other hand, it has to be proven that polymorphisms at such loci are attributed to the short microsatellite arrays themselves rather than to the other DNA rearrangements.

Another alternative would be to generate tomato microsatellite markers from AT repeats. This repeat type is the most prevalent in plant genomes and the high number of AT repeats in the ESTs indicates that they are more-evenly distributed throughout the tomato genome. This is confirmed by the sequence analysis of a contig of more than 150 kilobases in the euchromatin of tomato, where numerous long AT microsatellites could be identified but very little other dinucleotide arrays (K. Ernst and M. Ganal, unpublished results). However, AT microsatellites are more difficult to screen for because of the low annealing temperature for AT sequences, and also primer pairs designed for AT microsatellite markers often amplify multiple fragments.

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