

Development and Characterization of New Microsatellite Loci from Lemon (*Citrus limon*)

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ABSTRACT

Microsatellite loci are highly informative genetic markers useful for population genetic studies like linkage mapping and parentage determination. This study reports the identification and characterization of seven polymorphic microsatellite loci in *Citrus*. The loci were isolated from two libraries constructed from genomic DNA non-enriched for TC and AC and enriched for AC repeats. The 7 microsatellites included four 'perfect' dinucleotide repeats and three 'imperfect' repeats. These markers yielded 4 to 9 alleles per locus (mean 6.14) in a survey of 32 *Citrus* cultivars. Average observed heterozygosity ranged from 43 to 72% and expected heterozygosity ranged from 57 to 78%. The levels of polymorphism found in this study suggest that these microsatellite loci can become an important tool for genetic studies in *Citrus*.

Key Words: *Citrus*; SSR; Polymorphism; Genomic library; Molecular markers

INTRODUCTION

Citrus is one of the most important fruit crops in the world. Genetic improvement of *Citrus* species through conventional breeding methods has been hampered by the long juvenile period, high heterozygosity, large plant size and nucellar embryony (Gmitter *et al.*, 1992). Genetic markers, which are stable, detectable in all tissues and independent of environmental conditions or production practices, have emerged as efficient and powerful tools in *Citrus* in a wide range of applications including cultivar identification (Fang & Roose, 1997; Filho *et al.*, 1998; Novelli *et al.*, 2000; Bernet *et al.*, 2004), phylogenetics studies (Pang *et al.*, 2003), zygotic and nucellar seedlings identification (Ruiz *et al.*, 2000; Oliveira *et al.*, 2002) and the construction of linkage maps (Kijas *et al.*, 1997; Sanker & Moore, 2001) for marker assisted breeding and map-based cloning of genes. Of the many molecular techniques available to researchers, simple sequence repeats (SSRs) or microsatellites are becoming increasingly widespread because it is co-dominant, highly polymorphic, frequently and evenly distributed throughout the genome and regarded to be the most reliable marker.

A major limitation of SSRs is the time and cost required to isolate and characterize each locus when pre-existing DNA sequence is not available (Kijas *et al.*, 1994; Ahamad *et al.*, 2003). In *Citrus*, enough sequence information is not available in database to design primers for microsatellite analysis. Hence, their development in plant species is still limited to a small number of laboratories. But if microsatellite markers are developed, their use will be effective and convenient in various analyses (Suwabe *et al.*, 2002). In the present work, we

report the development of 7 microsatellite primers using non-enrichment and enrichment protocol and their polymorphism in 32 cultivars of *Citrus*.

MATERIALS AND METHODS

Microsatellite analyses were performed on 32 *Citrus* cultivars representing different species. The plant materials were collected from Citrus Research Institute of Iran. Genomic DNA was extracted from young leaves according to the modified method of Murray and Thompson (1980).

We used a hybridization selection method (Kijas *et al.*, 1994; Ahmad *et al.*, 2003) with some modifications to create a non-enriched TC and AC repeats library and an enriched AC library. To construct a non-enriched library, approximately 16 µg of genomic DNA from lemon (*C. limon*) was fully digested with the restriction enzyme *TaqI* (Promega) and electrophoresed in 1.5% low melting point (1 mp) agarose in TAE buffer (10 mm Tris-HCl, pH 8.0, 0.5 mm Na-acetate, 0.5 mm EDTA). DNA fragments between 300 to 800 bp were selected and purified from the 1 mp agarose using Qiaquick DNA Purification Columns (Qiagen). Plasmid vector pBluescript SK (+) (Stratagene) was digested with *AccI*-cut and dephosphorylated. About 100 ng size-fractionated genomic DNA were ligated with 300 ng of prepared vector using T4 DNA ligase (Promega). The product forms the primary ligation library and used to transform competent XL 1-Blue cells. Recombinant colonies were transferred to Hybond N⁺ membrane discs (Amersham) using 0.4 M NaOH capillary southern blotting as described by the manufacture. Membranes were then neutralized by washing in 2X SSC (1X SSC = 150 mm NaCl, 15 mm sodium citrate) for 5 min. The library was

screened for presence of repeats by hybridization using [³²P] end-labelled (AG)₁₀ and (TG)₁₀ probes, to select colonies containing similar sequences. Positive plaques were isolated and amplified by PCR using T7 and SK primers. PCR products were then transferred to membrane as previously described. In order to find the correct positive, membranes were again hybridized with (GA)₁₀ and (TG)₁₀ probes and positive clones were sequenced.

To increase the efficiency of microsatellite isolation, an enriched library was developed. For this purpose, a primary ligation library was prepared as described above. Predominantly single-stranded copies of the variable-length inserts ligated into pBluescript SK (+) were obtained by asymmetric PCR using a 10:1 molar excess 0.1 μM of T7 primer and 0.01 μM of SK primer. The asymmetric amplifications create a predominantly single-stranded DNA population. The 20 μL reaction contained 2.0 μL of 10X buffer (Sigma), 1.5 mm MgCl₂, 0.2 mm of each dNTP, 0.5 unit of DNA *Taq* polymerase (Sigma) and 50 ng of primary ligation library. The cycling profile was: 94°C/4 min, 32 cycles/94°C/1 min, 55°C/1 min, 72°C/1 min and a final extension at 72°C/4 min. Thus, in this manner, a single-stranded library was prepared.

A total of 1.0 μg of biotinylated oligoprobe (TG) 10 was attached to 50 μL of streptavidin-coated magnetic beads (Promega) in 100 μL of 5X SSC (1X SSC = 150 mm NaCl, 15 mm sodium citrate) at room temperature for 20 min. Excess un-bound oligo was removed with 3X 100 μL washes in 5X SSC before the beads were resuspended in 35 μL 20X SSC. The predominantly single-stranded product was denatured at 98°C for 10 min before being hybridized to the prob-bead complex at 48°C for 20 min. Beads were repeatedly sedimented by application of a magnetic field and washed 4X for 5 min each at 48°C in 100 μL low-stringency solution (2X SSC) followed by 4X 5 min each at 48°C in 100 μL high-stringency wash solution (1X SSC). Following these washes, the beads were resuspended in 0.15 M NaOH and incubated at room temperature for 20 min to release any bound sequence. The beads were then removed and supernatant was accurately neutralized by addition of 2.2 μL 10X TE (100 mm Tris-HCl, 10 mm EDTA, pH 7.3), 2.7 μL 1.25 M acetic acid. The supernatant was desalted by passage through Qiaquick PCR Purification Kit (Qiagen).

An aliquot from desalted supernatant was amplified exponentially as described earlier. A light smear in the range 300 to 800 bp on 1.5% agarose gel stained with ethidium bromide showed recovery of DNA from the enrichment. The PCR fragments for (TG) n sequences were re-ligated into pGEM T-easy vector using T4 DNA ligase. These recombinants were transferred into competent DH5Alpha E. coli cells. Cloned DNA insert size was determined by transferring white colonies to tube containing 30 μL of H₂O and incubating for 5 min at 95°C. A total of 15 μL of this lysate was used in 25 μL PCR amplification reaction with T7 and SP6 primers. Colonies having sufficient insert size were transferred to LB plus ampicillin medium at 37°C over

night. Plasmid DNA was extracted using Plasmid Mini Prep Kit (Qiagen) and the sequence was obtained on ABI PRISM Genetic Analyser with BigDye Terminator Sequencing Kit (Applied Biosystems).

A total of 27 clones from non-enriched and enriched libraries were sequenced. Eleven complete sequences containing microsatellite arrays were identified. 8 primer pairs were designed using the programme primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and synthesized (Geneworks, Australia).

To test each primer set for successful amplification of target genomic sequence and their ability to create polymorphism, an experiment with populations of *Citrus* was conducted. SSRs were amplified in a total volume of 20 μL containing 50 ng of *Citrus* genomic DNA, 10 mm Tris-HCl pH 8.3, 1.5 mm MgCl₂, 50 mm KCl, 100 nm of each specific primer, 0.2 mm total dNTP, 0.5 U of *Taq* DNA polymerase (Bioline), using the following temperature profile: 94°C/5 min, 32 cycles/94°C/1 min, 52°C/30 sec, 72°C/1 min and a final extension at 72°C/4 min. The PCR were denatured at 95°C for 3 min and resolved in denaturing gel containing 6% "polacrylamide", 4.6 M urea and 1X TBE buffer (45 mm Tris-Boric, 1 mm EDTA pH 8.0). Electrophoresis was conducted at 100 V for 8 - 10 h, after which the gels were stained with silver nitrate. The sizes of the alleles in the loci were determined using a 100 bp DNA standard (GeneRuler).

RESULTS AND DISCUSSION

The level of enrichment after two purifications of enriched DNA without post hybridization was 50%. When our results were compared with post hybridization with isotope labeled probes of van der Schoot *et al.* (2000) and chemiluminescent probes of Kijas *et al.* (1994), our enrichment protocol without post hybridization resulted in more microsatellite containing clones. This study has dealt with the successful and development of individual microsatellite from enriched and non-enriched methods. The enrichment method has a number of advantages; one of the most important is the speedy production of highly enriched microsatellite sequence population suitable for cloning and screening.

Among selected colonies from enriched and non-enriched methods, 81% had correct insert. There were 2 identical colonies after sequencing the inserts, alongside 37% of inserts weren't suitable for sequencing. Following screening and sequencing, primer sequences were designed to amplify each repetitive array.

Of the 8 tested primer pairs in 32 cultivars of *Citrus*, one had to be discarded due to nonspecific amplification. The result is a panel of 7 primer sets. Each can specifically amplify a single copy locus containing a microsatellite repeat. Summary data for the 7 microsatellites are presented in Table I.

Table I. Sequences of 7 microsatellite primers developed in *Citrus* and their characterization in 32 cultivars including annealing temperature (T_a), allele size range, number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosities, Proportion of null alleles and Gene Bank accession number

Primer	Sequence (5'-3')	Repeat	T_a °C	Size	N_a	H_O	H_E	Prop. alleles	Null Gene Accession no.	Bank
AMB1	F: TCGGCGTTCTCTCTCTCTCTCT R: TACTTGGACGTCGGGGACTA	(TC) ₇	48	173-199	5	0.431	0.699	0.157	BV209017	
AMB2	F: ACGCATTGACTGCTCTGTG R: AATTTTCAGAGGTGCCGTGAG	(TC) ₇	52	142-162	5	0.583	0.576	-0.004	BV209014	
AMB3	F: AACACACACACTCGCCTCAC R: CAGCCAAATGTGGAGAGACC	(TC) ₈	52	142-178	7	0.701	0.784	0.046	BV209015	
AMB5	F: CCATGCACAAAACTCACAC R: TGGGGGTGTTGAATGGTAAT	(CA) ₉	52	130-168	9	0.720	0.686	-0.020	BV209012	
AMB7	F: GCATGGAAGCTCTCTCCAAA R: TTCACACACGTGCTCGTACA	(CA) ₅ TATA(CA) ₃ CG(CA) ₄	52	174-208	6	0.646	0.677	0.018	BV209011	
AMB8	F: TGAACATATTTGCCCTTGGGA R: TTGTTTTGTGTGCTTGTGAGG	(CA) ₆ A(CA) ₅	52	148-178	7	0.629	0.761	0.074	BV209013	
AMB10	F: TACTGTGGGAAGGGATCTG R: GACTCCTTCCAGCACTTTGC	(A) ₉ G(A) ₅ G(A) ₆	52	161-179	4	0.590	0.582	-0.005	BV209016	
Average					6.14	0.614	0.680			

The 7 microsatellites included four "perfect" dinucleotide repeats and three "imperfect" repeats. The number of alleles per locus (N_a), and observed (H_O) and expected heterozygosities (H_E), were calculated according to Nei (1973). The seven loci are polymorphic, with the number of alleles ranging from 4 to 9 (mean 6.14); H_O ranged from 0.431 to 0.720 and H_E from 0.576 to 0.784 (Table I). The proportion of null alleles for each locus was calculated as $(H_E - H_O) / 1 + H_E$ following Brookfield (1996). The locus AMB1 showed values over 10% in cultivars, suggesting the possibility of null alleles.

This study reveals the abundance of microsatellites in the *Citrus* genome and we isolated more microsatellites from an enriched library than a non-enriched library without post hybridization or use of radioisotopes. Our results indicated conservation of microsatellite loci among chosen *Citrus* cultivars. In crux, these microsatellite loci valuable tools for cultivar identification, germplasm diversity as well as linkage analysis studies of *Citrus*.

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