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ORIGINAL PAPER

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Construction of a watermelon BAC library and identification of SSRs anchored to melon or *Arabidopsis* genomes

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Abstract A bacterial artificial chromosome (BAC) library was constructed for watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*) with an average insert-size of 106 kb, providing 21 haploid genome equivalents. The library was used to identify BAC clones that are anchored to probes evenly distributed on the genomes of melon or *Arabidopsis*. Twenty eight probes (representing 66% of the tested probes) from melon and 30 probes (65%) from *Arabidopsis* identified positive BAC clones. Two methods were implemented to identify SSRs from the positively hybridizing BAC clones. First, analysis of BAC end sequences revealed 37 SSRs. For the second method, pooled DNA of BACs identified by the melon probes was used to develop a shotgun library. The library was then screened with synthetic SSR oligonucleotides by hybridization. Sequence analysis of positively hybridizing shotgun clones revealed 142 different SSRs. Thirty eight SSRs were characterized using three watermelon cultivars, five plant introduction (PI) accessions of *C. lanatus* var *lanatus* and four PIs of *C. lanatus* var *citroides*. Of these, 36 (95%) were found to be polymorphic with up to six alleles per marker. Polymorphism information content values for polymorphic markers varied between 0.22 and 0.79 with an average of 0.53. The methods described herein will be valuable for the construction of a watermelon linkage map with SSRs evenly distributed on its genome that is anchored to the genomes of melon and *Arabidopsis*.

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Introduction

Molecular genetic linkage maps are useful tools for plant breeding, gene cloning and genomic analyses and have been developed for many crop species (Chen and Tanksley 2004; Feltus et al. 2004; Joobeur et al. 2004). However, in many instances they contain relatively large gaps between markers and/or reduced genome coverage (Cregan et al. 1999; Liebhard et al. 2003; Song et al. 2005). Such deficiencies can be largely overcome by creating comparative linkage maps which facilitate the identification of markers in targeted regions and increase genome coverage (Choi et al. 2004; Dirlewanger et al. 2004; Huang et al. 2005; Ku et al. 2000; Yogeeswaran et al. 2005).

In addition to the genome coverage, the types of markers used for linkage map construction may determine the extent of its applicability (Joobeur et al. 1998; Liebhard et al. 2003). Compared to Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, restriction fragment length polymorphism (RFLP) and simple sequence

repeat (SSR) markers can be easily transferred between populations that are segregating for different traits. RFLPs and SSRs have the advantage of being codominant and reliable markers. SSRs are preferred markers for plant breeding since they are PCR-based. SSRs are also hypervariable, multiallelic and ubiquitous in plant genomes (Danin-Poleg et al. 2001; Gonzalo et al. 2005; Mba et al. 2001; Vigouroux et al. 2005).

Bacterial artificial chromosome (BAC) libraries are valuable resource for numerous applications in plant genomics, including linkage map construction and elaboration of a physical framework for whole genome sequencing (Budiman et al. 2000; Lorenzen et al. 2005; Nam et al. 2005; Sasaki et al. 2005). BAC libraries have been also employed for the targeted identification of molecular markers, map based cloning and microsynteny analysis (Cregan et al. 1999; Morales et al. 2005; Morishige et al. 2002).

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*) is a member of the Cucurbitaceae family that includes several other economically important species such as cucumber (*Cucumis sativus* va *r. sativus* L.), melon (*Cucumis melo* L.), squash (*Cucurbita pepo*), and pumpkin (*C. maxima*). Compared to melon, watermelon linkage map resources are less advanced. Hashizume et al. (2003) published a linkage map containing 11 linkage groups, the same as the watermelon haploid number. However, most of these markers were RAPDs that are difficult to transfer between populations. Additional linkage maps for watermelon provide only partial genome coverage and contain mainly RAPD markers (Levi et al. 2002, 2001). Melon on other hand, has relatively dense linkage maps, which include characterized genes and agronomically important traits (Brotman et al. 2005; Joobeur et al. 2004; Noguera et al. 2005; Périn et al. 2002; Silberstein et al. 2003) as well as mapped QTLs (Monforte et al. 2004; Perchepied et al. 2005; Périn et al. 2002). Furthermore, expressed sequence tags (ESTs) sequences are being determined and *Agrobacterium*—mediated transformation is available for functional studies (Galperin et al. 2003). Therefore, the development of a comparative map between melon and watermelon will greatly advance the development of genetic studies for watermelon.

The goal of the current research was to identify SSRs that are anchored to melon or *Arabidopsis* in order to initiate the construction of a comparative map between these species. We constructed a BAC library for watermelon with ~21 genome complements. Probes evenly distributed on the melon or *Arabidopsis* genomes were used to screen the watermelon BAC library. Two methods were implemented to identify SSRs from positive BACs. For the first method BAC end sequences were analyzed for the presence of SSRs. For the second method small insert shotgun clones derived from pooled BAC DNA of positive clones were screened with synthetic SSR oligonucleotide repeats. The presence of SSRs was confirmed by sequencing. Thirty eight SSRs were assessed for polymorphism using four watermelon cultivars and eight PIs.

Materials and methods

Bacterial artificial chromosome library construction

A BAC library for watermelon was constructed using the high fruit-quality inbred line 97103. High-molecular weight DNA isolation and library construction was realized as described by Luo et al. (2003). Nuclear DNA embedded in an agarose plug was partially digested using the *Hin*dIII restriction enzyme and then subjected to two rounds of size selection by pulsed-field gel electrophoresis. The size-selected digested DNA was ligated into *Hin*dIII—digested and dephosphorylated Copy-Control™ pCC1BAC™ cloning vector (Epicentre, Madison, WI, USA). The ligation reaction was desalted using 0.1 M glucose/1% agarose cone on ice for 1.5 h before transformation into electrocompetent TransforMax EPI300 cells (Epicentre). Using a Q-bot (Genetix Ltd., Christchurch, Dorset, U.K.), recombinant colonies were picked from plates containing IPTG, Xgal and chloramphenicol and arrayed into 384-well plates containing freezing media. To determine the average insert-size for the library, plasmid DNA was isolated from 114 random BAC clones, and subjected to digestion with the restriction enzyme *Not*I. The insert size was determined after analyzing the digestion product by pulsed-field gel electrophoresis (Luo et al. 2001).

In order to screen the watermelon BAC library by hybridization, the recombinant colonies were gridded at high density using a Genetix Q-bot on positively charged nylon filters Hybond-N+ (Amersham, USA) according to the method described by Luo et al. (2001).

Screening the watermelon BAC library with melon and *Arabidopsis* probes

A total of 42 melon cDNA probes (MC probes) distributed on its genome were selected for screening the watermelon BAC library (Oliver et al. 2001). Based on previously published Southern blot analysis, the selected probes are present in single or low copy number, thus they are useful for comparative analysis. The *Arabidopsis* probes were obtained by PCR amplification of ~800 bp fragments using primers designed from genes of the conserved ortholog set (COS) identified by Fulton et al. (2002). A list of the primers used to amplify the COS probes is indicated in Table 1. For this analysis, 46 COS probes were selected based on two criteria; (1) evenly distributed on the *Arabidopsis* genome and (2) the corresponding COS markers were mapped on the tomato genome. The BAC library screening was done according to the method described by Budiman et al. (2000). The hybridization temperatures were 65°C for melon probes and 50°C for *Arabidopsis* probes.

Table 1 List of primers used to amplify the *Arabidopsis* COS probes

COS probe[a]	Forward primer	Reverse primer
T0055	CCTGATTTGGTGAAATGGGT	CAAGAGCGAGACGAGGAAGT
T0256	AGCTGCCAATTTCTTGAGGA	ATTTGCTTCCCAAGCTCAGA
T0266	CAACTTCACAGCATCATGGG	TTGAGTTTAACGGAGTCGGG
T0276	TTTGAGAATGCTTGTGGTCG	TTCAAATTCTCCTCGACGCT
T0506	CTTTTGCCACAGCATAAGCA	ATACCCTTTGTTCACCAGCG
T0585	ACAAGGGGAAGCTGGAGACT	GATGCAAAGAAATGCTGCAA
T0620	ATTGAAAATCGAGTGGGCAG	CCCTTCCTGGATCTTTGTGA
T0632	CAGACCTCGAGCTCAATTCC	TTCTCGGAAACTGCAAATCC
T0687	CACGTGGCGATAGTCTACGA	TGGTCTCTGCTTCACCACTG
T0688	TGGACTGGCAAGTGTTGGTA	ACCAAACCGAGGACTGCATA
T0761	TTGTGTGCAGGGATTAACCA	CCGACCTGCAAAAGAATAGG
T0766	AAGGTTTGTGCAGTTGGGAG	CCACGACATGTTTGACCAAG
T0774	ACCTAATACGACGACGGTGG	TCTTTCAAATCGCCAGAACC
T0800	AACAAGCCCTACATGGAACG	TTGACCCCACGTCTAAAACC
T0834	TCGCTCCTCAGGTTCAATTT	TCCGCCGTTGATTTAATCTC
T0883	GATTTCGACTATGGTGCGCT	TCGGTGCCTACAGAGGAGAT
T1066	TTAGGCAAAAGAAGCTGGGA	CTGTCTTCCTCTGCCTCACC
T1110	AGCTGAAGAAGCGGACATTC	GGTCGTGCTGTAAGCTCCTC
T1119	TTATGCCTTCCAGAGAGCGT	AACGCAAAGCCTGTATGGTT
T1151	TCCATCGTTTCACCAATCAA	CAAATCCCCATAAGGCTGAA
T1152	GACTCAATCAAGCGGCTAGG	ACACCTTCACACTCGGAACC
T1179	TCCTTCTCCTCTCCCTCTCC	TCCCTGGCTTGAGAAGCTAA
T1185	GGTCTCTCTGTCGTTCGGAG	TCTCCGGTCAGATTATTGCC
T1227	TTCTCTCGTCCCCACGTATC	TTTTGGGCTAAGTGAAACGC
T1238	TATTGAATCAGACCCTCCCG	ATTGAGAACAACCACCTCGC
T1260	AAGGTGTACGACGAAATGCC	ATGCCTCGTATTGATCAGCC
T1291	ATTTCCAGCATTTTTCACCG	CATTGCCACCAAAAGTGATG
T1327	TCTCATTCCCTTCCTTGTGC	TTTCGAAGAAGCGGATCTGT
T1349	TAGGATTGCGATTAAAGCCG	ATCGAGCACCACTCTTTGCT
T1359	GGGCTTGAGTATGACCTGGA	GCGGAAATTGATAGATCCGA
T1401	GAGCTATGCACTTGCGTGAA	TGTTGCCCTGATAAGGTTCC
T1413	TGAAGCGATTCGTGAAGAGA	ACCGGTCAGTGTCAAAAAGG
T1430	GAAGCCTCAGTTTTGGGATG	TACCAAAGGGCTTGGTTTCA
T1449	GCAGAAGGGAGAGTTCGAGA	AGCCCAAGGTAGCAAAGACA
T1462	AAACAAGGCGGCTTAAATCA	TGGATAGAGAATGCCTTGGG
T1471	TAGGAAACAGGTTGGATGCC	TGCTTTGCTTCATGTCCATC
T1480	GATAAGCGGTTTGGGAAACA	GGCCACTGATGTAACGGTCT
T1493	TTGGTTTCTAAATGCGGCTT	CCATTGCTGGCATGTACAAC
T1510	AAAAGAACTTCCTTCGCCGT	CACGGTAATGTTTCAACCCC
T1564	AGGCTGACCATATGCTTGCT	TCCCCAAGCTCCATAATCTG
T1601	GAGTCGCATTGGAAGGAGAG	CCGAATGTATCGGCTCCTAA
T1662	TGCTAGTGGAGCAACACCTG	TTCAAGGCCAACTCCATTTC
T1736	CGATTCGCTAGGAATTACGC	CCTCGAGTTGGTTTCTTGGA
T1784	GCTCATCTTTTCCCCAATGA	ATGCCTAAACTTCCCGGACT
T1789	CGCCTCGTTTCTTAAAGTCG	CACAGGAAACACCGTTAGCA
T1794	GCTACTGGCTGAGGTTTTGG	TGCTTCTCGTGCCTATCCTT

[a] COS probes names are according to Sol Genomics Network (http://www.sgn.cornell.edu/cgi-bin/search/markers/cos_list.pl)

Shotgun library construction from pooled BAC DNA

Positive BAC clones identified by the melon probes were fingerprinted and 1–2 BAC clones from each contig were selected. The BAC DNA of all selected clones was mixed in equal amounts and the pooled DNA was used to construct a shotgun library. BAC DNA was isolated using standard alkaline lysis procedure with modification according to the method described by Joobeur et al. (2004). A total of 4 μg (100 ng/μl) of DNA was sheared using a speed code of 4 for 20 cycles with GeneMachine Hydroshear (Genomic Solutions, Ann Arbor, MI, USA). DNA fragments of 1.0–1.5 kb were agarose-gel purified and used to construct the shotgun library. The resulting library was arrayed and then gridded on filters as described by Luo et al. (2001).

Screening the shotgun library for the presence of SSRs

The shotgun library was screened by hybridization for the presence of SSRs according to the method described by Lowe et al. (2004). Two pools of synthetic SSR oligonucleotides were used as probes; the first pool (Probe I) contained: $(TA)_{15}$, $(GT)_{15}$, $(GTT)_{15}$, $(TTAA)_{10}$, and the second pool (Probe II) contained $(AAT)_{10}$, $(AAAT)_{10}$, $(GA)_{15}$ and $(GAA)_{10}$. These SSR oligonucleotides were selected for screening because they are relatively frequent in melon. Each oligonucleotide was individually end labeled using (γ-^{32}P)-ATP (MP Biomedicals, Irvine, CA, USA) and T4 polynucleotide kinase (Promega, Madison, WI, USA). Clones identified as containing putative SSRs, were then sequenced from both ends. Plasmids and BAC ends were sequenced as previously described by Joobeur et al. (2004).

Sequence analysis and SSR characterization

First, vector sequence was eliminated. Only sequences with PHRED (Ewing and Green 1998) quality value >20 were kept. The sequences were then aligned using CAP3 (Huang and Madan 1999) and searched for the presence of SSRs. BAC ends and shotgun sequences were analyzed separately.

The software Sputnik (http://www.espressosoftware.com/pages/sputnik.jsp) was used for SSR identification. For this work, we defined SSRs as being mononucleotide repeats >15 bp, dinucleotide repeats >14 bp, trinucleotide repeats >15 bp, tetranucleotide repeats >16 bp and pentanucleotide repeats >20 bp (Cardle et al. 2000). SSRs were divided into two classes depending on the repeat size (Temnykh et al. 2001). Class I contains SSRs with repeat size ≥20 bp and class II consisted of SSRs with size between 15 and 20 bp.

Using the default setting, primers complementary to sequences flanking selected SSRs were designed with the program Primer 3 (Rozen and Skaletsky 2000). The range size of the PCR product was selected between 100 and 400 bp. In order to characterize selected putative SSRs, the corresponding primers were used to analyze eight watermelon US plant introduction (PI) accessions (including five PIs of the *C. lanatus* var lanatus and three PIs of *C. lanatus* var *citroides*) and four watermelon cultivars (Table 2). PCR amplification and SSR analysis was realized with ABI 377 (PE Biosystems, Foster City, CA, USA) according to the method described by Joobeur et al. (2004). The polymorphism information content (PIC) value for each polymorphic SSR was calculated as described by Katzir et al. (1996).

Results

Watermelon BAC library construction

We constructed a BAC library for watermelon using DNA of the high fruit-quality inbred line 97103. The library was developed using the *Hin*dIII restriction enzyme and contains 92,160 clones arrayed into 240–384-microtiter plates. Analysis of 114 random clones showed that 98% contained an insert with an average size of 106 kb (Fig. 1). The same analysis showed that 76% of the clones have an insert size greater than 100 kb (Fig. 2). Based on an estimated watermelon genome size of ~430 Mbp (Arumuganathan and Earle 1991) the library contains ~21 haploid genome equivalents. The library is represented by a set of 5 filters each containing 18,432 BAC independent clones; thus each filter represents ~4 × haploid genome equivalents.

Table 2 Origin and subspecies of the plant material used for SSR characterization

PI accession/ cultivar name	Subspecies and origin
PI 379249	*Citrullus lanatus* var. *lanatus*; Yugoslavia
PI 470249	*C. lanatus* var. *lanatus*; Indonesia
PI 542115	*C. lanatus* var. *lanatus*; Botswana
PI 543211	*C. lanatus* var. *lanatus*; Bolivia
PI 612459	*C. lanatus* var. *lanatus*; South Korea
PI 296341	*C. lanatus* var. *citroides*
PI 189225	*C. lanatus* var. *citroides*
PI 271769	*C. lanatus* var. *citroides*
Dixielee	*C. lanatus* var. *lanatus*; USA
90-4304	*C. lanatus* var. *lanatus*; USA
New Hampshire Midget	*C. lanatus* var. *lanatus*; USA
97103	*C. lanatus* var. *lanatus*; USA

BAC library screening with melon and *Arabidopsis* probes

Probes evenly distributed on the melon and *Arabidopsis* genomes were used to screen the watermelon BAC library. A total of 46 *Arabidopsis* and 42 melon probes were individually hybridized to a single filter of the BAC library. After hybridization 66% (28 probes) and 65% (30 probes) of the melon and *Arabidopsis* probes identified positive clones, respectively. Probes with positive hybridization identified between 1 and 21 BAC clones with an average of 4.8 (Table 3) roughly equivalent to the estimated genome coverage represented by one filter of the library. DNA fingerprinting analysis with the FPC program of 290 BACs revealed that clones identified by each probe belong to a single contig. The BAC ends from positive BACs were sequenced to identify possible SSRs.

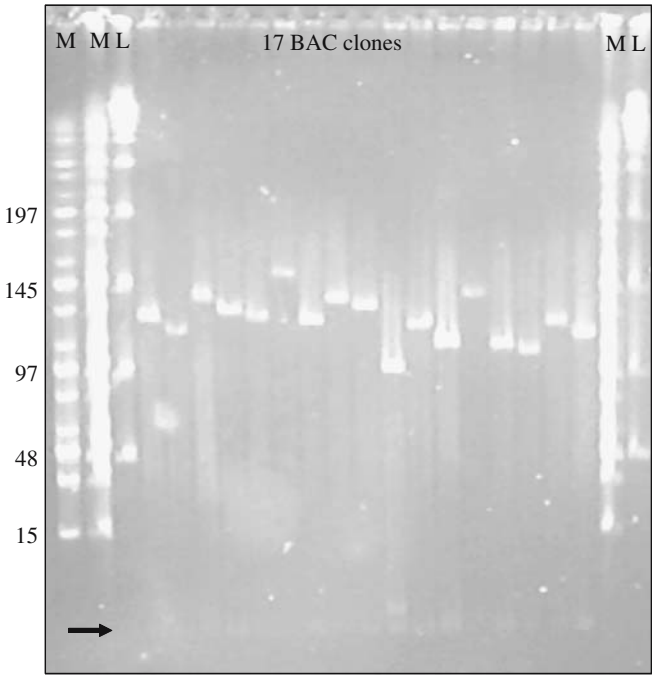

Fig. 1 Insert size estimation for the watermelon BAC library. Extracted BAC DNA was digested with *Not*I and separated using pulsed-field gel electrophoresis. Markers used to estimate the insert size were the Midrage I (*M*) and lambda ladder (*L*), from New England Biolabs (Ipswich, MA, USA). Values on the left are in kilobytes. The 7.5-kb common band (*arrow*) corresponds to the cloning vector

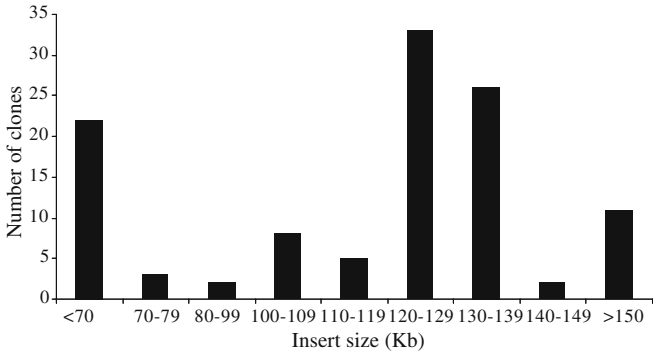

Fig. 2 Insert-size distribution of 114 random BAC clones. The insert-size was estimated after analyzing the *Not*I-digestion products by pulsed-field gel electrophoresis

SSR frequency in the BAC ends

A total of 290 BAC ends were successfully sequenced. After analysis with CAP3, 199 unique sequences covering 120,775 bp were identified. To identify repetitive sequences, the obtained sequences were searched against the non redundant protein database using BLASTX (Altschul et al. 1990). A total of 20 sequences (10%) presented similarity to transposon-like sequences.

Analysis with the Sputnik program revealed 37 SSRs, of which 12 SSRs were from class I (repeat size >20 bp). The overall SSR frequency was one SSR every 3.3 kb, compared to one every 10 kb for class I SSRs. These results also indicated that one SSR was found every 7.8 BAC ends and class I SSRs were found every 24 BAC ends.

Shotgun library construction and screening for SSRs by hybridization

DNA from 1 to 2 randomly selected BAC clones (total of 41 BACs) identified by each melon probe was isolated, pooled and used to construct a shotgun library. For 18 melon probes we selected two BAC clones and for 5 probes we chose one BAC clone. The total physical region covered by the selected BAC clones was estimated to range between ~2,400 and 4,300 kb, assuming an average insert size of 100 kb and complete to minimal overlap of BACs. The shotgun library contained a total of 12,288 recombinant clones with an average insert size of 1 kb. Thus, the expected shotgun library coverage of the region was between 2 and 3.5 fold.

After screening the shotgun library with synthetic SSR oligonucleotide probes, 439 positive clones were identified; 68 with probe I (a mixture of synthetic SSRs: $(TA)_{15}$, $(GT)_{15}$, $(GTT)_{15}$, and $(TTAA)_{10}$) and 371 with probe II [$(AAT)_{10}$, $(AAAT)_{10}$, $(GA)_{10}$ and $(GAA)_{10}$]. A total of 384 positive clones (all the clones identified by probe I and 316 of the clones identified by probe II) were subjected to sequencing from both ends.

Table 3 Melon and *Arabidopsis* probes cross hybridizing with watermelon BAC clones

Probes [a]	N. BAC[b]	MLG[a]	Probe[c]	N. BAC[b]	A CH[d]
MC04	11	G4	T0055	7	2
MC21	21	G12	T0256	2	5
MC33	8	G4	T0266	4	4
MC92	4	G7	T0506	1	2
MC120	2	G6	T0585	6	1
MC124	1	G2	T0761	4	1
MC216	3	G6	T0766	3	4
MC224	2	G12	T0774	1	3
MC226	1	G12	T0800	4	2
MC231	1	G5	T0883	16	1
MC235	5	G2	T1066	9	2
MC244	1	G2	T1110	5	4
MC253	9	G3	T1119	10	5
MC256	7	G4	T1152	5	3
MC264	9	G5	T1185	3	1
MC276	5	G4	T1227	11	5
MC279	1	G6	T1238	5	4
MC290	2	G12	T1291	6	5
MC295	1	G8	T1327	3	1
MC326	2	G5	T1349	3	3
MC337	3	G5	T1359	2	1
MC340	6	G8	T1401	3	1
MC356	3	G1	T1449	6	5
MC373	5	G3	T1471	5	2
MC375	1	G5	T1480	8	5
MC376	11	G8	T1493	6	3
MC387	1	G3	T1510	3	2
MC388	3	G5	T1601	4	1
			T1662	4	4
			T1784	5	5

[a] Melon linkage groups and probes nomenclature is according to Oliver et al. (2001)
[b] Number of positive BAC clones identified after screening one filter of the watermelon library
[c] COS probe nomenclature is as indicated in the Sol Genomics Network (http://www.sgn.cornell.edu/cgi-bin/search/markers/cos_list.pl)
[d] *Arabidopsis* chromosome number

Simple sequence repeat frequency in the selected shotgun clones

For the selected shotgun clones, a total of 647 high-quality sequences were obtained. Analysis with CAP3 revealed 78 contigs with sizes ranging between 125 and 4,598 bp and an average of 1,255 bp. All contigs but two presented 2–16 sequence reads. Two contigs (with 4,197 and 4,598 bp) presented exceptional large number of reads (105 and 113). One hundred and thirteen singlets were also identified, with a size ranging between 178 and 737 bp with an average of 521 bp. The total sequences derived from the selected clones covered 156,306 bp. BLASTX search revealed seven sequences (three contigs and four singlets) with similarity to transposon-like sequences, representing a frequency of one sequence every ~22 kb. For comparison purposes, we sequenced 52 random clones from one end, resulting in a total of 34,981 bp. BLASTX analysis showed the presence of six sequences with similarity to transposon-like sequences representing one sequence every 6 kb. These results indicate that the selected clones were not enriched for repetitive sequences.

Analysis of the selected clone sequences revealed 142 SSRs with a frequency of one SSR every 1,101 bp. Therefore one SSR was found every 4.5 high-quality sequences. Class I SSRs (total of 53 SSRs) were estimated to occur every 2.95 kb and every 8 high-quality sequences. In order to compare the SSR frequency in the selected and random shotgun clones, we searched the 52 random sequences for the presence of SSRs. Nine SSRs were found in 34,980 bp, representing one SSR every 3.8 kb. This was similar to the frequency observed in the BAC ends (one SSR every 3.3 kb). Thus, the SSR frequency was ~3.5 fold higher in selected than in random clones. A total of four class I SSRs were identified presenting a frequency of one SSR every 8.7 kb. Class I SSR frequency was ~3 fold higher in selected than in random clones. An increase of the SSR frequency was observed for all the SSR motifs used in the oligonucleotide probes, except for AAC and AATT repeats (Table 4).

Characterization of selected SSRs

Primers were designed for 46 selected SSRs derived from the shotgun library and were used to analyze the watermelon plant material. A total of 38 primer pairs (82%) produced PCR products (Table 2). The sequence of these 38 primer pairs, the expected size and number of alleles identified for the corresponding SSRs are indicated in Table 5. All the primers but one (MCPI-23) produced a fragment with the expected size using DNA from the watermelon cultivar 97103. One primer pair (MCPI-07) produced other bands in addition to the expected locus product. Of the 38 SSRs that produced a PCR product, 36 were polymorphic. The number of alleles identified by each primer pair ranged from 2 to 6 for all the plant material and between 2 and 4 for the *lanatus* type. The polymorphism information content value ranged between 0.22 and 0.79 (Table 5).

Table 4 Simple sequence repeat type frequency in the analyzed sequences

SSR group	SSR type[a]	SSRs frequency [b]		
		BAC ends	Random shotgun clones	Selected shotgun clones
Probe I	AAC	−(0)	−(0)	156 (1)
	AATT	60 (2)	17 (2)	39 (4)
	AC	−(0)	−(0)	13 (12)
	AT	30 (4)	35 (1)	9.8 (16)
Probe II	AAAT	120 (1)	−(0)	22 (7)
	AAG	−(0)	35 (1)	8.6 (18)
	AAT	20 (6)	35 (1)	9.7 (16)
	AG	60 (2)	35 (1)	9.7 (16)
Subtotal		8 (15)	5.8 (6)	1.7 (90)
Other		5.5 (22)	11.6 (3)	3 (52)
Total		3 (37)	3.8 (9)	1 (142)

[a] SSR types were divided into three groups; used in probe I, used in probe II and not used in either probe (other)
[b] SSR frequency was calculated by dividing the total sequence length by the number of identified SSRs. The total sequence length for BAC ends, random shotgun clones and selected shotgun clones is 120, 35 and 156 kb, respectively. The parentheses indicate the number of SSRs for each type. (−) Indicates an undefined value

Discussion

Watermelon BAC library screening

We constructed a BAC library for watermelon with an estimated coverage of 21X haploid genome complements. The library was arrayed at high density on five filters, each representing ~4 × haploid genome complements. Forty two melon and 46 *Arabidopsis* probes were used individually to screen one filter of the library. A total of 58 probes detected positive clones with an average of 4.8 clones per probes, providing an experimental estimation of the coverage for a single filter.

In this study we found that 65% of *Arabidopsis* probes hybridized to watermelon BAC clones. Oliver et al. (2001) found that 57% of the *Arabidopsis* probes hybridized to melon DNA in genomic southerns. This rate was not significantly different from our observed rate ($P=0.74$). The fact that melon and watermelon are closely related species likely explains these similar observations. Dominguez et al. (2003) found a cross-hybridization rate varying between 10% for *Helianthus annuus* and 60% for *Solanum tuberosum* using *Arabidopsis* ESTs with high sequence conservation to rice. The cross hybridization rate we observed between the Arabidopsis COS probe and watermelon was in the same range.

Melon is more closely related to watermelon than *Arabidopsis*; melon and watermelon are from the same family of the Rosid I clade, however, *Arabidopsis* belongs to the Rosid II clade (Chase et al. 1993). Thus we expected a higher cross hybridization rate for melon probes than for *Arabidopsis* probes. The similarity of the cross hybridization rates observed for melon (66%) and *Arabidopsis* (65%) probes may be explained as follows. In our experiments the hybridization temperature was less stringent (50°C) for the *Arabidopsis* probes than for melon probes (65°C) and unlike for melon the *Arabidopsis* probes were selected to be highly conserved across taxa.

Using the same annealing temperature we used for the melon probes, Oliver et al. (2001) found that all nine cDNA cucumber probes hybridized to melon genomic DNA. Silberstein et al. (1999) also showed that more than 84% of cucumber cDNA probes detected fragments in melon genomic DNA, with reduced hybridization temperature (55°C). The lower rate of cross hybridization between melon and watermelon we observed compared to results reported for cucumber and melon may reflect taxonomic relationships between these species. Cucumber and melon are in the same genus, however, melon and watermelon belong to different tribes (Andres 2004). Fukao et al. (2004) reported cross hybridization rates of around 66% for species from different tribes of the Poaceae family, similar to our findings.

Table 5 Simple sequence repeat markers used to analyze the watermelon panel

SSR name	SSR motif	Primer sequence (5′–3′)	Allele no.	Expected size (bp)	PIC
MCPI-03	(TG)8	GCATAAACCACCTGTGAGTGG ATGGCTTTGCGTTTCATTTC	2	218	0.44
MCPI-04	(AC)36	AGCAAATGCATGGGGAAAAC TGTTGAATGGAGGCTTTGAG	4	237	0.71
MCPI-05	(TA)9N36(GT)8	ATTTCTGGCCCCAGTGTAAG GAACAACGCAACCACGTATG	5	188	0.61
MCPI-07	(AAG)9	GGTTATGGCCATCTCTCTGC GAGAGTGGGCGTAAGGTGAG	3	249	0.57
MCPI-09	(AAT)11	TCAATTCCAATCATCCATCC TAATGGCCGGACTTTATGC	3	208	0.49
MCPI-10	(AAAT)8	GATGATTTGTTTGTTCTGATCTTTG AAACCATCACTGAGAACAAAAGG	2	291	0.44
MCPI-11	(AG)20	GAGCAGGGGAGAAGGAAAAC CCAGTAGCTTTTTCCGATGC	5	241	0.68
MCPI-12	(AAG)7N69(AT)26	GGAGTAGTGGTGGAGACATGG TCCTTTCTCTTTCGCAAACTTC	4	246	0.66
MCPI-13	(AG)25	TTCCTGTTTCATGATTCTCCAC TCAGAATGGAGCCATTAACTTG	6	211	0.69
MCPI-14	(AAT)15	TCAAATCCAACCAAATATTGC GAGAAGGAAACATCACCAACG	5	240	0.75
MCPI-15	(AAT)14	GCAAAATGCAACTGTTTATCG CCATTATGATTTCAATCAATCTCC	3	241	0.43
MCPI-16	(AG)11(AAG)5	TGCTCAATCCACCCTTTCTC AAAAACAGCAACTCTCCCATC	3	246	0.57
MCPI-17	(CA)10N14(AAGG)4	CAGAAATTTTGAATAACGCCAAC TGACTGCATTAGGGTAGAAACG	2	218	0.44
MCPI-18	A21(AG)10	CCGAAGCAAGATGGTTTTC AAACCGATATGCCTGTCTGC	2	244	0.44
MCPI-20	(AAG)5N191(AAG)4	GATCTCCTCAAAGCCTTACCG CGGATCCGATAATCTGCTG	3	273	0.49
MCPI-21	(AG)11	AAAGTTTTCATGCCAACGTATC TCAGCCAATATGGTCAAATAGC	4	193	0.67
MCPI-23	(AAT)13	CCACCGACTTGCTTTTCTTC TGTCACCATTTGAACCAAGG	2	174[(a)]	0.46
MCPI-24	(AAT)10	GAACTTTCAAATTTACAACAACAAAC CAATTTAATCCCTTCCATGC	2	250	0.50
MCPI-25	(AG)13	TACCTCAGTCGACGCTGTTG GATTTGTGTGGAACCCAAGC	2	180	0.44
MCPI-26	(AAT)12	CAGAGGAACGAGAGGGAGTG GGGGAGCCCATATTTTAACC	5	223	0.63
MCPI-27	(AAT)13(AAAAT)4	GGGAAATTAGCCCTTTTGTTG AATGGATGGGATCGTGCTAC	3	184	0.56
MCPI-28	(AAG)9	AATGTTAAGCAGTAAGCACATGG ACACCGGAGAAGGTGAATTG	4	285	0.63
MCPI-29	(AG)13	CACAATCAAGGAAGGTTCAGC TGAGCAAGCCAACAGAAGTG	3	233	0.54
MCPI-30	(AAG)10	GCTTTGAAGTTTGTTTAATTTTAGTCC CGCCTCACGCTCTCTCTAAC	3	266	0.64
MCpI-31	(AAG)6A22	TAACCGTCACCAACCCATTC TCCAAAATTGGTCGGATTTG	2	252	0.44
MCPI-32	(AAG)5(ATC)8	AAGGCTGCAGAGACCATGAC AATGATGAAGAACGGGCAAG	4	264	0.54
MCPI-33	(AG)8N173(TA)8	CGTCATTTGAGAGCATTGGA TCCAATTTTGTTTAGTGACATAGAGTGC	6	271	0.79
MCPI-34	(AG)11	CCAAATTGGACCAGAACCAC AAGCCGTCAGTCTCGGTTAG	2	300	0.50
MCPI-37	(AAT)9	AATCTTCCCCATGCCAAAAC GACTTCCAAACCCTCCCTTC	4	166	0.51
MCPI-39	(TA)8N27(AAG)5	AGGCCCAAAACCTAACTTGC CTTTTGCCCTCGCTCTTTC	2	290	0.47
MCPI-40	(AAT)9	AAAAATTTGAAAATTAGGTGAGGAG TTTTGACTAGGTGTACACTACCTTTG	2	285	0.22
MCPI-41	(AAT)7	AGGTGGTATGTCGCTCATCC GTGGGAGATGTGTGAGCTTG	2	182	0.44
MCpI-42	(AAG)11	TGCTTAAACCTCCGTTCTGG ATTTTCTTCAGCTGCGTTCC	3	127	0.49

Table 5 (Contd.)

SSR name	SSR motif	Primer sequence (5′–3′)	Allele no.	Expected size (bp)	PIC
MCPI-44	(AAG)9	ATTCAAAACGCAAGGGTCAG ATCAGGGGTACCACCTCCTC	2	158	0.44
MCPI-46	A19(TA)16	CAAACAAAAACTTAGGAACTAGATTG TTAGCCATGAGGCGTGTACC	4	201	0.45
MCPI-47	(AAAT)5	TTGCCATTGAAATTTTGAGAAG TCAAATTTTGTTTCTTGGAAATG	2	249	0.44

[a] The observed PCR fragment size differed from expected

Identification of SSR markers anchored to melon and *Arabidopsis* genomes

The primary motivation for this work was the identification of watermelon SSRs that can be used to anchor melon and *Arabidopsis* genomes to watermelon. To achieve this objective, we selected probes that are distributed on the melon or the *Arabidopsis* genome to derive markers for watermelon. We expect that these probes, particularly those of melon, would result in markers that are more uniformly distributed on the watermelon genome than randomly developed markers and thus will facilitate the construction of a linkage map that represents the whole genome of watermelon. To derive markers from the selected probes, we first used them to screen the watermelon BAC library. Hybridizing BAC clones were then used to identify SSR markers using two methods.

For the first method, we searched the BAC end sequences for SSRs. Class I SSRs (that are more likely to be polymorphic) were found to occur every 10 kb. This frequency was similar to the result of sequencing of random clones from the shotgun library (frequency was one class I SSR every 8.7 kb). van Leeuwen et al. (2003) previously reported a frequency of one class I SSR every 4.0 kb in a single analyzed melon BAC clone. This discrepancy likely reflects the non random distribution of SSRs in plant genomes (Temnykh et al. 2001). Analysis of genomic DNA of *Arabidopsis*, rice, soybean, maize and wheat, revealed that the frequency of SSRs varied between 2.2 (*Arabidopsis*) to 5.5 (maize) (Morgante et al. 2002). However, the authors used less stringency criteria to define an SSR; the minimum SSR size was 12 bp compared to 14 bp in our work.

In the second method, we constructed a shotgun library from pooled DNA of selected BAC clones identified by melon probes. The shotgun library was then screened for the presence of SSRs. Cregan et al. (1999) used a similar method for the targeted identification of SSR markers in soybean; however in the current work we used sheared rather than enzyme-digested BAC DNA. We hypothesized that shearing would result in more random clones than DNA digestion with restriction enzymes. Song et al. (2005) compared both types of libraries from wheat genomic DNA and found a lower level of redundancy using sheared DNA, which was reflected in a higher frequency of unique sequences that are derived from a single clone. In our work 67% of unique sequences (out of 191) were each derived from a single clone. Cregan et al. (1999) found a lower frequency of unique sequences (14% of a total of 35) derived from a single clone. The difference in efficiency may be attributed to the contrast between the methods used (shearing versus restriction enzyme digestion) and/or the difference in the number of SSRs present in the starting DNA used for subcloning. Based on the work of van Leeuwen et al. (2003) for melon, additional SSRs are expected to be present in the BAC clones used for the shotgun library. Indeed, 90 SSRs of the type used in screening the shotgun library were identified (Table 3). Thus, based on an estimated size (2,400–4,300 kb) of the physical region covered by the BAC clones used for the shotgun library construction, the estimated frequency is one SSR every 27–48 Kb. However, van Leeuwen et al. (2003) found the same SSR type to occur every 1.2 kb. These results indicate further screening and sequencing more clones of the shotgun library is likely to result in the identification of additional SSRs.

Compared to BAC end sequencing the utilization of shotgun library has two main advantages; (1) BAC end sequencing is usually more challenging than regular plasmid sequencing. (2) Our results indicated that the number of sequences needed to identify a class I SSR is ~3 fold higher for the BAC end sequencing method than the shotgun library method. The shotgun method resulted in the identification of informative SSR markers; Analysis of 12 watermelon genotypes with 38 SSRs revealed a high level of polymorphism; 94% of the markers detected polymorphism and the PIC value for the polymorphic markers had an average of 0.53. The PIC value is a measure of the polymorphism level detected by a particular marker and is dependent on the number of alleles detected and their distribution in the population tested. Similar trends of polymorphism have been reported in melon. Using 13 melon genotypes, Danin-Poleg et al. (2001), found 75% of 40 SSR markers were polymorphic and the PIC value for the polymorphic markers was 0.52.

In the work reported here, we implemented an approach to isolate watermelon SSR markers that are anchored to melon or *Arabidopsis* probes. A BAC library for watermelon was constructed and screened with melon and *Arabidopsis* probes that are evenly distributed on their genomes. Positive BAC clones were then used to isolate SSRs. A total of 38 SSRs were used to analyze 12 watermelon genotypes and revealed a high level of

polymorphism. Markers identified using this targeted approach will likely have better coverage of the watermelon genome and thus will be more suited for quantitative trait loci identification and bulk segregant analysis in populations segregating for important traits. It is expected that the application of the reported approach will result in the construction of comparative maps between watermelon, melon and *Arabidopsis* and in the transfer of valuable genetic and genomic information between these species.

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