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ORIGINAL PAPER

Comparative effectiveness of sugar beet microsatellite markers isolated from genomic libraries and GenBank ESTs to map the sugar beet genome

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Abstract Sugar beet (*Beta vulgaris*) is an important root crop for sucrose production. A study was conducted to find a new abundant source of microsatellite (SSR) markers in order to develop marker assistance for breeding. Different sources of existing microsatellites were used and new ones were developed to compare their efficiency to reveal diversity in mapping population and mapping coverage. Forty-one microsatellite markers were isolated from a *B. vulgaris* ssp *maritima* genomic library and 201 SSRs were extracted from a *B. vulgaris* ssp *vulgaris* library. Data mining was applied on GenBank *B. vulgaris* expressed sequence tags (ESTs), 803 EST-SSRs were identified over 19,709 ESTs. Characteristics, polymorphism and cross-species transferability of these microsatellites were compared. Based on these markers, a high density genetic map was constructed using 92 F_2 individuals from a cross between a sugar and a table beet. The map contains 284 markers, spans over 555 cM and covers the nine chromosomes of the species with an average markers density of one marker every 2.2 cM. A set of markers for assignation to the nine chromosomes of sugar beet is provided.

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Introduction

Sugar beet (*Beta vulgaris L.*) is a crop of primary economic importance in Europe. Due to its importance as a major source for sucrose production, molecular tools supporting sugar beet breeding have been extensively developed. However, a relatively limited amount of data have been made available for public, and most of the genetic maps rely on anonymous restricted use markers (RFLP: Nilsson et al. 1997, SSR: Rae et al. 2000) or on poorly reproducible and transferable markers like RAPD (Uphoff and Wricke 1995; Barzen et al. 1995) and AFLP (Schondelmaier et al. 1996; McGrath et al. 2007). RFLP, AFLP and RAPD markers have nevertheless allowed the assignation of important characters to the nine chromosomes of *B. vulgaris*. Male sterility and beet mosaic virus resistance (Friesen et al. 2006) genes were assigned to chromosome I, annuality (Boudry et al. 1994; El-Mezawy et al. 2002) and root and hypocotyl color genes (Butterfass 1968; Barzen et al. 1992) to chromosome II, rhizomania resistance (Barzen et al. 1997; Pelsy and Merdinoglu 1996; Scholten et al. 1997) and X restorer locus of the

CMS (Wagner et al. 1992; Hagihara et al. 2005) to chromosome III and monogermy (Barzen et al. 1992) and the Z restorer locus (Roundy and Theurer 1974) to chromosome IV. The identification of QTL for sucrose content, yield and quality (Weber et al. 1999; 2000; Schneider et al. 2002) and *Cercospora* leaf spot resistance (Nilsson et al. 1999; Schäfer-Pregl et al. 1999) was also achieved with these markers.

Up to now, SSR markers have been widely used for population genetics in beets (e.g., crop-wild gene flow; Arnaud et al. 2003; Viard et al. 2004; Andersen et al. 2005) but only poorly applied to sugar beet genetics although these codominant markers are particularly suitable for assistance to breeding by characterizing heterozygous states. Moreover, SSRs are both reproducible and easily transferable from one map to another. The development of SSR markers from DNA libraries is labor-extensive and characterized by low yields (Zane et al. 2002). In beet, several genomic DNA libraries have been constructed but only a limited number of SSR markers have been developed (Mörchen et al. 1996; Rae et al. 2000; Cureton et al. 2002; Viard et al. 2002; Richards et al. 2004). An alternative strategy to find SSRs exploits the increased number of EST sequences available in public databases. SSRs have indeed been reported to be more frequent in transcribed regions than in genomic DNA as a whole (Morgante et al. 2002; Fujimori et al. 2003). Using this strategy, SSRs have been successfully identified in monocots like rice, maize, barley, wheat, durum wheat, rye, sorghum, tall fescue (Morgante et al. 2002; Kantety et al. 2002; Hackauf and Wehling 2002; Saha et al. 2004), and dicot species like *Arabidopsis* sp., cotton, grape, tomato, potato, apricot, melon (Areshchenkova and Ganal 2002; Milbourne et al. 1998; Decroocq et al. 2003; Monforte 2003; Qureshi et al. 2004; Cardle et al. 2000). Up to 28,000 sequences of sugar beet ESTs are available in the NCBI GenBank database and the potential of EST data-mining in this species has been shown for mapping 75 functional gene homologs on chromosomes (Schneider et al. 1999).

In this study, we have used two ways to identify SSRs in beets. First, new microsatellites were isolated from a previously described *B. vulgaris* ssp *maritima* genomic library (Viard et al. 2002) and from a *B. vulgaris* ssp *vulgaris* genomic library. Additionally, a data mining approach was conducted on 19,709 public *B. vulgaris* ESTs from the GenBank database to obtain further markers. All the microsatellites obtained with these two strategies were combined to construct a genetic map, and compared for their level of polymorphism, repeat structure and distribution on the genetic map. Finally, the cross-species transferability of these microsatellites was investigated.

Materials and methods

Plant material

In order to maximize the expected polymorphism between the two parents, the mapping population was constructed from an intra-specific cross between a sugar and a table beet rather than between two sugar beets. The female parent was an heterozygous sugar beet with a green hypocotyl and a white root, and was self sterile and monogerm (rr, yy, sfsf, mm). The male parent was an heterozygous table beet with red hypocotyl and root and was multigerm (R-, Y-, Sf-, M-). An F_1 plant with a red hypocotyl (used to enable the selection of a hybrid F1 plant), was selfed to produce a mapping population of 192 F_2 plants.

To test the mendelian segregation, 141 individuals were genotyped with 75 markers. Then, in order to reduce genotyping time, the remaining markers were tested on 92 randomly sampled individuals. One marker was genotyped on 192 individuals.

Hypocotyl and root colors were evaluated on all 192 young plants potted and grown in a greenhouse. These phenotypic markers were scored as dominant [red versus green (R/r) and red versus white (Y/y), respectively].

DNA was extracted with Dneasy 96 plant kit (Qiagen) from 12–16 mg of dried leaf.

Beta vulgaris ssp *maritima* microsatellites isolated from genomic libraries

Twenty-three microsatellites originating from different *B. vulgaris* ssp *maritima* libraries were genotyped [Mörchen et al. 1996 (Bvm); Cureton et al. 2002 (BMB); Viard et al. 2002 (Bv); Richards et al. 2004 (SB)]. The 6 BMB-microsatellites came from an enriched genomic library, while the others, 4-Bvm, 5-Bv and 8-SB, came from standard genomic libraries.

Screening of Viard's genomic library (Library A) was continued by sequencing 246 additional positive clones on a Li-Cor automated DNA sequencer Long Reader 4200 (Viard et al. 2002). Homologies between the sequences were identified with the local Blast function of BioEdit 5.0.9. Subsequent microsatellites were prefixed Bv according to those that were previously isolated from the same library.

Beta vulgaris ssp *vulgaris* microsatellites isolated from genomic libraries

Five genomic libraries were developed from a sugar beet resistant to BNYVV (Beet Necrotic Yellow Vein Virus) by AgroGene S.A. (Library B). Microsatellites with $(CA)_n$, $(TC)_n$, $(TTC)_n$, $(ATT)_n$ motifs were screened and 393 sequences were delivered. Homologies between the different

sequences were identified with the local Blast function of BioEdit 5.0.9.

The SSR markers were numbered from FDSB5000 to FDSB5239 with a few exceptions. Ten markers (FDSB1001, 1002, 1005, 1007, 1008, 1011, 1023, 1026, 1027 and 1033) were transferred to the AFLP map recently developed by McGrath et al. (2007) to allow assignation to chromosomes.

Isolation of microsatellites from *Beta vulgaris* ESTs from GenBank database

On June 2003, 19,709 sugar beet ESTs from GenBank were screened for the presence of microsatellites by using the microsatellite search tool MISA (Thiel et al. 2003). The parameter for specifying the minimum number of repeats was set to 10 and 6 for mononucleotide and dinucleotide motifs, whereas it was set to 5 for longer microsatellites with motif lengths of 3–6. An output was generated, summarizing type and position of the microsatellites combined with the outputs of Primer3, such as primers sequences, Tm, and expected product size.

Redundancies among the EST-SSRs were identified and eliminated by screening against the non-redundant unigenes in the Sputnik sugar beet database (Rudd et al. 2003) and TIGR Beet Gene Index (http://www.tigr.org/tdb/tgi/plant.shtml). The non redundant EST-SSRs were designated from FDSB500 to FDSB1483.

The microsatellite markers are available for academic research application with Materials Transfer Agreement. Applications should be sent to P. Devaux.

Determination of protein coding regions in *Beta vulgaris* ESTs

All open reading frames (ORFs) were searched and examined to detect potential protein coding regions (CDS) with a 3-phase Markov chain of order 5 (word length 6) previously trained on known coding sequences (Mielordt 2005). In order to estimate the reliability of the prediction, each EST sequence was shuffled 100 times for both strands and scored again. A *P*-value was assigned depending on how many times the predicted coding ORF has a higher score than a randomly generated one.

Microsatellite genotyping

For all the microsatellites identified, primers were developed with Primer3 (Rozen and Skaletsky 2000) for an amplicon size between 120 and 280 bases for analysis by an automated DNA sequencer Li-Cor. One of the two primers was tailed with M13 forward sequence 5′CACGACGTT GTAAAACGAC3′.

SSR loci were amplified in 15 µl reactions containing: 16 ng DNA template, 1× PCR buffer (Biolabs), 0.1 mM of each dNTP, 0.2 mg/ml of Bovine Serum Albumin, 0.5 pmol of each primer, 0.5 U of Taq DNA polymerase and 1.5 pmol of sequence M13 end-labeled with InfraRedDye-800. Thermocycling was performed in a MJ Research thermocyler using 40 cycles of 94°C for 30 s, 50°C, 53°C or 55°C for 40 s, 72°C for 50 s and a final extension of 15 min. PCR products were analyzed on an automated DNA sequencer (model 4200™, Li-Cor) using an 8% Long ranger (acrylamide) gel. Scoring was performed by visual identification.

Cross-species transferability of microsatellites

Twenty EST-SSRs and 20 genomic-SSRs were used to examine the transferability of microsatellites between different species and sub species of the Amaranthaceae family. Thirty-one accessions representing ten species and two subfamilies (Hohmann et al. 2006) were genotyped with genomic and EST based microsatellites (Table 1). Both the

Table 1 Identification and origin of individuals tested for transferability of SSR according to the revised classification of Hohmann et al. 2006

Subfamily	Tribe	Section	Species	Subspecies	Individuals
Chenopodiodeae			*Spinacia oleracea*		5
			Chenopodium quinoa		5
Betoideae	Hablitzieae		*Patellifolia patellaris*		2
			Patellifolia procumbens		2
			Patellifolia webbiana		2
	Beteae	Beta	*Beta vulgaris*	*vulgaris*	2
			Beta vulgaris	*maritima*	5
			Beta vulgaris	*adanensis*	2
			Beta patula		1
			Beta macrocarpa 2x		1
			Beta macrocarpa 4x		1
		Corollinae	*Beta lomatogona*		2
			Beta corolliflora		1

quality of the amplification and the existence of a polymorphism (number of polymorphic loci) were investigated.

Linkage analysis and map construction

Segregation distortion to the Mendelian ratio (3:1 or 1:2:1) was tested for each marker with a Chi-square test. Those showing a distorted ratio were excluded from initial map construction. Linkage analyses were performed with the software JoinMap® 3.0 (Van Ooijen and Voorrips 2001) using the Kosambi function (Kosambi 1944) to calculate map distance.

Six CAPS markers, MP001, MP043, MP079, MP110, MP175, MP096 (Schneider et al. 2002), corresponding to the RFLP markers of Barzen et al. (1992, 1995), were genotyped to assign the linkage groups to the chromosomes. Ten SSRs from Library B were also shared with McGrath's population (2007) to complete the assignation. The chromosomes were named according to the Butterfass trisomics series nomenclature (Butterfass 1964; Schondelmaier and Jung 1997).

Results

Microsatellite isolation

Among the 246 clones positive for the presence of a SSR in Library A (Viard et al. 2002), 132 clones with a SSR were sequenced. After removal of redundant sequences, primers could be designed for 41 out of the 71 remaining clones (Table 2). Among the 393 positive sequences from Library B, 201 different amplifiable microsatellites were obtained and corresponding primer pair sets were developed.

In total, 528 clones from all the genomic libraries were sequenced to identify the SSRs. On observation, 66.1% of the sequences from Library A and 41.7 % from Library B were found unsuitable for designing primers due to absence of SSR or because of too short sequences. Both libraries considered, 30.8% of the sequences did not contain an SSR and flanking sequences were too short to design primers for 15% of the sequences that did contain an SSR. A high level of redundancy was also observed for 77% of the Library A sequences and 79% of the Library B sequences. In the Library A, one $(CT)_n$ clone was present in 53 copies. Moreover, a satellite sequence (Santoni and Bervillé 1992) corresponding to a centromeric associated repeat unit (Schmidt and Heslop-Harrison 1996) that has been previously reported in another *B. vulgaris* ssp *maritima* library (Mörchen et al. 1996) was found in 17 $(CA)_n$ clones. For the Library B, up to 36 copies of a same $(CT)_n$ sequence were found and, all sequences considered, 152 redundant clones corresponded to 27 unique sequences. Altogether, over the two genomic libraries, 253 non-redundant sequences with an SSR were found and could be used to design primers (Table 3).

Out of 19,709 ESTs available in the GenBank database, 880 (4%) contained at least one SSR. Once redundant sequences were discarded, 779 different ESTs with SSR were obtained (Table 3). Most of the EST-SSR sequences were unique or they belonged to contigs of less than 6 EST members, although some clusters of ESTs contained up to 45 ESTs with SSRs. There was no redundancy between the sequences from libraries A or B and EST-SSRs. For EST-SSRs, 105 microsatellites (16.5%) showed an amplicon 4–6 times larger as compared to the length of the SSR regions contained in the EST sequences. The unexpected large size of these amplicons prevented their use on the automated Li-Cor sequencer.

Microsatellite characteristics

The trinucleotide repeats were more abundant in ESTs (47.5%) than in genomic DNA libraries (35.2%). An opposite result was found for dinucleotide repeats (33% vs 64.4%; Table 3). Even though trinucleotide repeats were more common than dinucleotide repeats, the most common microsatellite repeat type in ESTs was $(CT)_n$ (28%) followed by $(AAC)_n$ (11%), $(TGA)_n$ (11%) and $(AGA)_n$ (11%). The repeat types $(GCG)_n$, $(GC)_n$ and $(C)_n$ were the less frequent type (0.6, 0.2, 0.4% respectively).

Among 812 non redundant ESTs containing an SSR, 552 (68%) contained full ORFs or fragments with a *P*-value of 0.05 or lower. Of all sequences considered, i.e., 557,968 nucleotides, 201,079 nucleotides (36%) are in coding regions and 356,889 nucleotides (63.9%) are in other regions (UTRs and others sequences). Triple repeats were found enriched in coding regions (57%) and mono repeats depleted in coding regions (12%) (Table 4). The CDS with triple repeats led to peptides with 5–7 repeats of a single amino acid. The repeats concerned all the amino acids but cysteine, tyrosine and tryptophan. Asparagine, serine and glycine repeats were the most frequent (respectively 15, 12 and 9.5%) and alanine and isoleucine repeats the less frequent (2.5%).

Polymorphism in the mapping population

All of the 731 EST-SSRs and 242 genomic-SSRs were amplified on parents and on the F_1 parent of the mapping population to check for amplification and polymorphism.

For most repeat motifs, 40–60% of the microsatellites were polymorphic. Microsatellites with a $(GGT)_n$, $(CTG)_n$ and $(AAG)_n$ core sequence were less polymorphic than the majority of microsatellites whereas microsatellites with a $(CT)_n$ or $(ATT)_n$ core sequence were more polymorphic than the majority.

Table 2 *Beta maritima* microsatellites isolated from Viard's library (2002)

Locus	Primer F	Primer R	Amplification T°C	Repeat	Allele size (pb)
BvAT1	TTAGCAACAATTGGAGGGTT	TCTCCTCAAAATTCCATCCA	45	(AT)6	158
BvAT2	CTCATATCGATTCGGTTCAGA	TTATGAACACACCCACAGCAA	48	(TA)9	181
BvATCT1	TCAATGAATTCAGCTTCTGAGC	AGAGGAAGAGGAGTTTGTGTGG	48	(TA)14(GA)11	200
BvATT1	GTGCACCATTGTTCTCCTT	CTCAAATTTATCAGTAGTATC	50	(ATT)7	208
BvATT2	CGGCAACCAATCAATCTAGG	AGGGTTTCGGGTCATGCTAT	50	(ATT)7i	151
BvATT3	TTTCTTCCTCCAATTTCTGACTG	TCTTGGATTATTTGACGGAAA	40	(ATT)6i	229
BvATT4	GCCCTGTTTTTAAGAGCCTTT	ACGGGTTGGGGTTTTATTTC	48	(ATT)13i	249
BvATT5	TCAGTTCAGTTCAGCTCCATTC	TGAATTCGATTTTCTAAAGGGGTA	40	(ATT)34i	245
BvATT6	CCGAAATTAACACAACCGACT	GGCACGTTATCAGGAGATGG	55	(TTA)5i	214
BvATT7	GTGTCAAGATTCTAAGTGAGAACG	TTGGAGAATATCGGCCAAAG	55	(ATT)24i	226
BvCA2	CCTTGCTAGTTGCTGCTGTG	GCATATGTACAAGAGAGCCGTTT	55	(CA)6	198
BvCA4	AAACCATCCCATGTTTGGAG	GGATACCAAATACAAAGTACCTGC	50	(GT)9i	151
BvCA5	GAGTCTCGAGCATTCTGGATAAA	GATGAATACAGGCCCCAGAA	55	(CA)7	190
BvCT1	CGTACGAGCTCGAATTTTAT	TGAACACAATGTACCTGATGA	40	(CT)12	183
BvCT2	CTACTGCATTCAGCTCCTCC	CCAGTTCTGAGGAGAATCCA	40	(CT)11	189
BvCT3	CCTTTCAAATATAATGCACTGAA	GAAACCAGAGAGACGCGA	52	(CT)14	248
BvCT5	GATCATCAAGAGAATTAAATATAT	GACCTTGATGCAGGAGCTT	50	(CT)25i	158
BvCT6	TGAAACGTGAATGGTGAGGA	CTCCCCCAATCTCGGAAC	50	(CT)7	111
BvCT7	CCACGGAACTTACCCGTTTT	TAGACGGGAGAATGCGATGT	54	(CT)11	143
BvCT8	GCTGTTTCCTGTGTGTAATATTGTT	CTGCAGAGATATTCAGCTCCA	40	(CT)6(CTT)6	155
BvCT9	TCACATGGGTCCCAATTTTT	GCCTTTGCTATTTCCCATGC	55	(CT)8i	143
BvCT10	TCCCCACTTTGAATGATTGAG	CCCAACTGGCAACTGAAATC	48	(CT)8i	201
BvCT11	GACATCGCCTTGACTTCCTT	TCGTGCTGAGCCTGATTTTA	50	(CT)10	200
BvCT12	TACCGCATTTGTGGCAAGTA	GGTACTGGAACCTGGGAAT	54	(CT)22	227
BvCT13	CCGTTTTCAAAGGGTTTTTG	GGGAAGAGAAGAGAGAGATTAGGG	55	(CT)18	187
BvCT14	TAAATGTCGAACGCTGACCA	TCCTGAAGCAGGCATATTGA	55	(CT)6i	213
BvCTGT1	CGTGGCTTGACTGAAAGTCTC	GGGCAAAACAGTCCTCAAAA	50	(CT)7(GT)10i	201
BvCTT1	AGATCTGGATCTGCCCCTTT	AAGCAGAAAAGATGTGACAAAAGA	48	(TTC)6i	185
BvGAA2	TGGCAGGGTCACTTATGACT	GGTTGCTCAACCCATACATC	40	(GAA)4	171
BvGAA3	TTCCCTCTTCCAAAGAAAGGT	TCAAGGACATGTTCAAGGTGTT	50	(GAA)5i	155
BvGGC2	GGTGCTCATCCAGCCTAATC	GGGCAACCGACCATATTCTA	48	(GGC)4i	137
BvGTGTT1	GGTTGGTGCACGAAGTGAC	GCCTAGAAGGTGGGAACTCA	48	(CA)4(CAA)3	224
BvGTT2	AAAAACCCACCCTCGTTCTT	TCTGCACTGAAATCGCTGTT	50	(GTT)7	156
BvGTT3	ACTTGCCATTCCACTCCACT	GGTGTCTCCAATTGTTTGCTT	50	(TGT)4i	176
BvGTT4	TGGGGTAAAACTTCCCACAA	ACCTGGAAATTTGAGCCACA	55	(CTT)3(GTT)6	170
BvGTT5	GCCAACAGGAGAACACATCA	TTTCCATACGCTTTGCCATC	55	(CAA)6i	209
BvGTT6	GAAATTAGGCGACTACTTGCAG	GGGCACAAAAACACACCTCT	48	(GTT)8i	171
BvGTT7	TTAAGACCCAACTTTCGTTGA	TGTAAATTCTTCTCTAATTCCCAT	48	(CAA)6i	199
BvGTT8	TTTTCTGCCCTTGTTTGACA	TCTTCCCCTAACAATCCAAATG	50	(GTT)6i	124
BvGTT9	GCCAATCGGCATAATAGGAG	GATCACTCTCAACCGCC	55	(GTT)6	151
BvTAC1	GGGAGCTCTCTGCCTTTTG	CATGACCATTACCATTACTCTCCA	50	(TAC)5	167

i imperfect repeat

Most of the amplified SSRs were monolocus and were scored in a codominant way. Only 18 (7.1%) of the genomic SSRs from Library B and 166 (22.6%) EST-SSRs were multilocus or gave multiple banding patterns. Among the multilocus SSRs from Library B, nine showed two loci and the others revealed complex patterns. For the markers with a complex pattern, one or several unambiguous and clearly segregating bands were scored in a dominant way. Among

Table 3 Comparative numbers of microsatellite markers identified from genomic libraries and ESTs

Repeat type	Genomic libraries			ESTs		
	SSRs	Repeats		SSRs	Repeats	
		Maximal	Average		Maximal	Average
Mono	1 (0.04%)	17	17	126 (16.1%)	31	16
Di	163 (64.4%)	82	24	257 (33%)	22	7
Tri	89 (35.2%)	49	17	370 (47.5%)	16	6
Tetra	–			9 (1.2%)	10	6
Penta	–			2 (0.2%)	5	5
Hexa	–			15 (2%)	14	6
Total	253			779		

For each core sequence (repeat type), the number of loci (SSR) and their length (repeats) are indicated

– not applicable

Table 4 Distribution of microsatellites with different repeat types in the coding regions of ESTs

SSR type	Mono	Di	Tri
SSR total	126	257	370
SSR in CDS	15 (12%)	92 (36%)	212 (57%)
bp Total	1618	3686	6474
bp in CDS	175 (11%)	1386 (38%)	3705 (57%)
Average length	13	14	17
Average length in CDS	12	15	17
Average length in other regions	13	14	18

the markers mapped, 244 (83.3%) SSRs were codominant and 49 (16.7%) dominant.

The polymorphism of the EST-SSRs (47.8%) was lower as compared to the polymorphism of the genomic SSRs (61.5%) from the genomic Library B. The *B. vulgaris* ssp *maritima* SSR from genomic Library A showed a level of polymorphism (45.2%) similar to the EST-SSRs. Half of the EST-SSR markers [dinucleotide repeats (56.4%), trinucleotide repeats (48%)] were polymorphic whatever the position of the SSR in the CDS or in non coding regions in ESTs was.

Cross-species transferability

Transferability of EST-SSRs to the different species of Beteae tribe was high and ranged from 100% for *B. vulgaris* subspecies and *B. patula* to 65% for *B. corolliflora* (Table 5). On the contrary, species from Hablitzieae tribe and Chenopodiodeae subfamily only amplified for 20–15% of the EST-SSRs. Genomic-SSRs had a significantly lower transferability than EST-SSRs on both *B. vulgaris adanensis* (70%) and *B. patula* (65%) species (Chi-square test, $P < 0{,}01$). The transferability of genomic-SSRs to the selected species of Beteae tribe ranged from 100 to 40% and from 15 to 10% for the other species: the difference to the EST-SSRs was, however, not significant (Chi-square test, $P < 0.05$). The transferability of both kinds of microsatellites was high within the same tribe but low outside of Beteae. Most of the EST-SSRs and genomic SSRs amplified genomic regions of the expected size in the majority of the species of the Beteae tribe, but not in distantly related species.

All species considered, the genomic-SSRs and EST-SSRs showed similar levels of polymorphism on the 31 accessions of the Amaranthaceae family for both the number of alleles (1–8 alleles versus 3–11 alleles) and PIC values (PIC = 0.59 vs PIC = 0.68).

Table 5 Transferability of genomic SSRs and EST-SSRs on 31 accessions of the Amaranthaceae family

	Chenopodiodeae		Betoideae										
			Hablitzieae			Beteae						Corollinae	
	S	Q	Pat	Proc	Web	B	Vm	Va	Pa	Ma^2	Ma^4	Lo	Co
% Amplification								**	**				
Genomic-SSR	10	15	15	15	15	95	100	70	65	70	80	65	40
EST-SSR	20	15	15	20	15	100	100	100	100	95	95	80	65
% Polymorphism													
Genomic-SSR	0	0	0	0	33	63	85	50	–	–	–	31	–
EST-SSR	25	66	66	75	33	75	95	20	–	–	–	56	–

– not applicable

**Significant at $P < 0.01$

Linkage analysis

The genotyping of the parents of the mapping population revealed that the two parents were heterozygous. Indeed, 14% of the SSRs showed two alleles for the red beet parent and 20% for the sugar beet parent. Due to this heterozygosity, the corresponding markers were not always informative on the F_2 mapping progeny since the same allele was inherited from the two parents in the F_1. Then, 282 polymorphic markers (SSRs and CAPS) were amplified and mapped on the 92 individuals of the progeny. Genetic segregation in the F_2 population was analysed for a total of 282 molecular loci and two morphological loci. Forty-nine markers (14%) showing a skewed segregation were excluded from initial map construction. Since their further introduction in the pool of data did not disturb the constitution of the linkage groups initially obtained, they were, therefore, incorporated in the map. Two hundred and eighty-four loci were organized in nine linkage groups at a minimum LOD score of seven and two loci remained unassigned.

The linkage groups were assigned to the nine chromosomes of the species by one to five anchoring markers each (Table 6; Fig. 1) common with maps of McGrath et al. (2007) and Schneider et al. (2002). The anchoring markers were all SSR markers, apart from two morphological characters and three CAPS. No discrepancy was found on assignation among the anchoring markers common with the McGrath map. The hypocotyl color gene allowed the assignation to chromosome II (Butterfass 1968; Barzen et al. 1995; MacGrath et al. 2007) and the male sterility gene the assignation to chromosome I (Friesen et al. 2006, MacGrath et al. 2007). The male sterility character was not segregating in this mapping population but it was attributed to the corresponding linkage group in a sugar beet × sugar beet population (unpublished results). Three CAPS markers have allowed assignation to chromosomes I, IV, VI and one EST-SSR, FDSB 957, corresponding to the gluthatione reductase gene (gr), have allowed the assignation to chromosome III (Schneider et al. 2002). One difference was obtained for the CAPS MP43 that mapped on the chromosome IV rather than on the chromosome III as expected

Table 6 Markers allowing assignation to chromosomes

Chromosome	Assignation markers	Primers R	Primers F	Markers type	Common with:
I	MP175	Available upon request on the corresponding article		CAPS	(3)
	A male sterility			Morpho	(4, 5)
II	Y root color			Morpho	(1, 2, 5)
	FDSB1300	AATTTAAACGCGAGAGCAGC	TCAGCTTCTGGGCTTTTTGT	SSR	(5)BQ584037
III	FDSB1027	CAGGCATGAGTAGCATGAACTAAAG	GCTGGATGCTGACAACTATGAAAC	SSR	(5)
	FDSB957	TCAATCCATCTCTATTCTCTCCG	GTCATGGTTGGTCGATCCTT	SSR	(3) marker *gr*
IV	FDSB1002	GAAAACGGAGTTCAGTCAGGGA	CCTTAAACCTAAAAACGCCAGC	SSR	(5)
	MP79	Available upon request on the corresponding article		CAPS	(3)
	FDSB1023	TCTCTCTCCCCCTAAAAGTTCA	GTAGCTAGTTCAGCAATCTTCGC	SSR	(5)
	SB06	AAATTTTCGCCACCACTGTC	ACCAAAGATCGAGCGAAGAA	SSR	(5)
	SB07	TGTGGATGCGCTTTCTTTTC	ACTCCACCCATCCACATCAT	SSR	(5)
V	SB04	ACCGATCACCAATTCACCAT	GTTTTGTTTTGGGCGAAATG	SSR	(5)
	SB15	CACCCAGCCTATCTCTCGAC	GTGGTGGGCAGTTTTAGGAA	SSR	(5)
VI	MP110	Available upon request on the corresponding article		CAPS	(3)
	BvGTT1	CAAAAGCTCCCTAGGCTT	ACTAGCTCGCAGAGTAATCG	SSR	(5)
	FDSB568	TTCTGGGGATGATTTCTTCG	CCGGGACAGAGAGAACAGAG	SSR	(5)BQ591966
VII	FDSB1011	CAACTTATTTAAGCCTTTTAGTGC	GATCCATTTATTTCGTGTTGA	SSR	(5)
	FDSB502	GCAAAAACCCAAAACCCTTT	TTTCTCTCTCCTCCTCTTCCTC	SSR	(6) USDA07
	FDSB990	TCTCACCTGAAATCCGAACC	CCATCCGTAACTCGGTGACT	SSR	(6) USDA13
	FDSB1250	TTCACCGCCTGAATCTTTTC	CGACGAAGAATCGGGTAAAA	SSR	(5, 6) USDA5
VIII	FDSB1007	ATTAGAATAGCATCAATTGTGG	CCTTATAGTTGGAATTGAGAAA	SSR	(5)
IX	FDSB1001	ACTTCAACCACTATCACAAAGTGAG	ATCTTATGCTGCCATGACCA	SSR	(5)
	FDSB1427	TTGAAGGCTCACCTCAAACAAA	CTGTTGCTGTTGCTGTTGCT	SSR	(6) USDA10
	FDSB1033	GCTGAGATGATGTTTGTTAGGGC	TTCAAATCGCCATCTCCCAG	SSR	(5)

(1) Butterfass 1968, (2) Barzen et al. 1995, (3) Schneider et al. 2002, (4) Friesen et al. 2006, (5) McGrath et al. 2007, (6) SES Vanderhave reported by McGrath et al. 2007

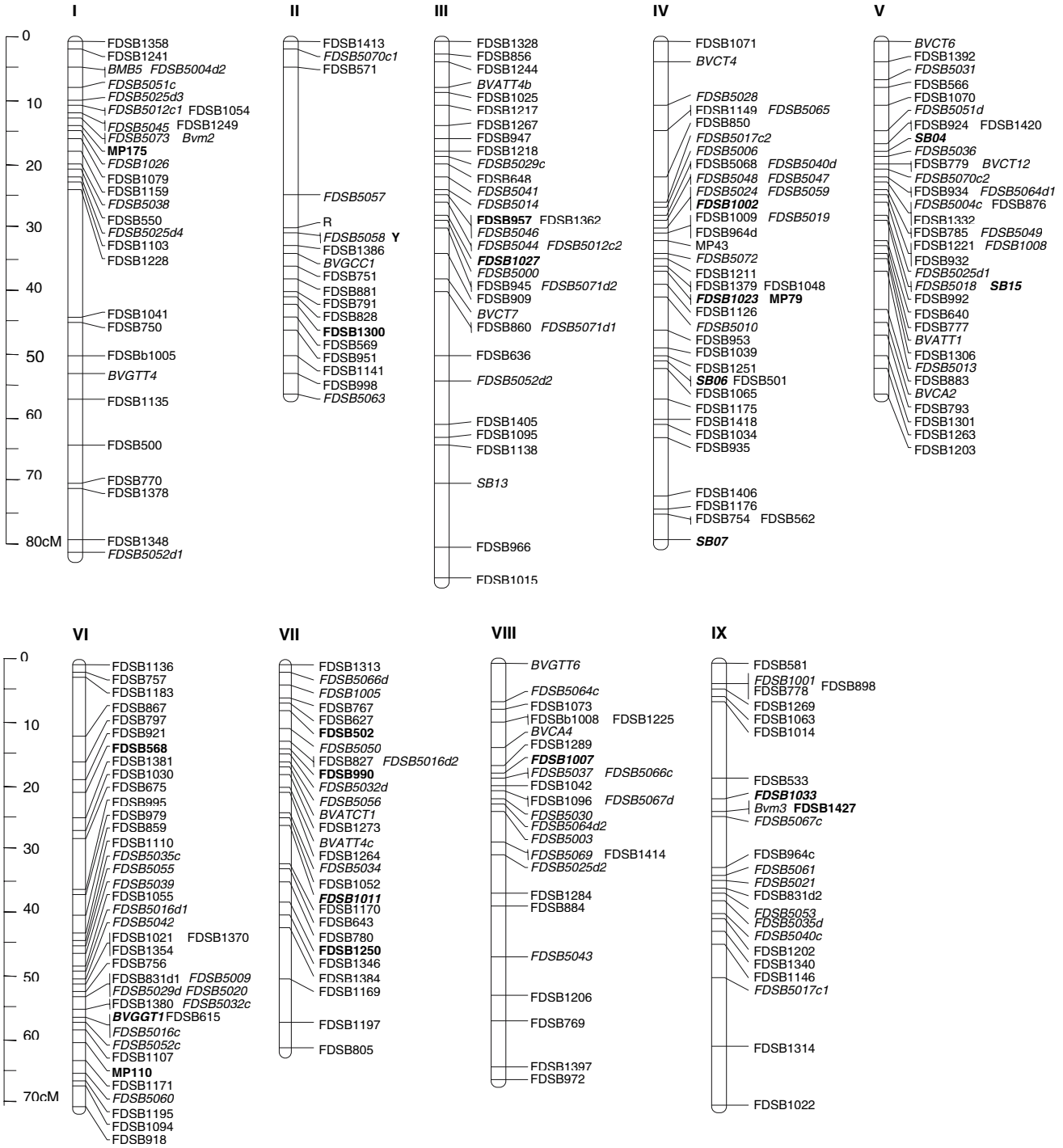

Fig. 1 Genetic map of the nine chromosomes of sugar beet × red beet progeny. Genomic SSRs are in *italic* and EST-SSRs in *plain text*; markers that have allowed assignation of linkage groups to chromosomes are in *bold face*

(Schneider et al. 2002). This marker showed several segregating fragments that could explain the discrepancy in chromosomal location if the fragments scored in both maps were not the same. Almost all the distances and orders between the markers common to our map and the one of McGrath were conserved. The two exceptions were the position of FDSB1023 on chromosome IV and the distance between SB04 and SB15 on chromosome V. This distance was shorter on our map than in McGrath's, despite a larger overall size of the linkage group V.

All of the 41 markers showing a skewed segregation ($P < 0.001$) mapped on chromosome V. The eight remaining markers with a skewed segregation ($P < 0.05$) were dispersed on chromosomes I, II, IV, VI and IX. All the

distorted markers that mapped on chromosome V were characterized by a lack of the sugar beet (female parent) allele and an excess of the red beet (male) allele. For the marker BvCA2, it was even necessary to genotype 191 individuals to find a few individuals with the female allele.

The final map (Fig. 1) spanned 555 cM with linkage groups size varying between 54.6 and 84.4 cM. The nine chromosomes seemed well covered with the exception of two gaps on chromosomes II and IX that remain difficult to fill. The EST-SSR markers have mapped uniformly on the nine chromosomes whereas the genomic SSRs from Library B showed a high level of clustering.

Discussion

To develop the available resource of sugar beet SSR markers, two ways to identify SSR were explored. Microsatellites were isolated from genomic libraries and about 19,000 sugar beet EST were systematically searched for SSR.

Although the genomic libraries have allowed the obtention of 253 new SSR, several restrictions have reduced the efficiency of genomic library screening. One of the most important is redundancy between the SSRs isolated. This redundancy between the sequences of a same library might have been lowered if genomic libraries could have been enriched for single copy sequences. Indeed, only 53% of the clones were redundant with an enriched genomic library of rye-grass (Hirata et al. 2006) as compared to 79% for genomic Library B. However, such an enriched genomic library, probably would not have decreased the number of clones unsuitable to design primers, since the level of unsuitable clones reached 74.3 and 46.5% for rye-grass and strawberry enriched libraries respectively (Hirata et al. 2006; Monfort et al. 2006), which is similar to the level of clones unsuitable to design primers from Libraries A and B. There was only one homology between the sequences of libraries A and B, suggesting a partial coverage of the genome during library construction. There was no redundancy between the sequences from libraries A or B and EST-SSRs, meaning that the regions of the genome targeted during the genomic library construction were mainly non coding regions, and supporting the common assumption that genomic SSRs are neutral markers. For 16.5% of the EST-SSRs, the introns have introduced an unexpected size variability of some amplified fragments. These results are in agreement with previous studies in plants since 21.3% and 22.5% of the SSRs amplification gave an amplicon larger than expected on bread wheat (Zhang et al. 2005) and barley (Thiel et al. 2003) respectively.

However, despite this loss of usable SSRs, our results confirmed the efficiency of data mining on public EST libraries as an easily-accessible source of SSRs. Indeed, the number of usable microsatellites was significantly higher in ESTs (779) than in the two genomic DNA libraries (A and B) (253). Thus, in species for which a high number of EST sequences are available, data mining is an efficient alternative to genomic library construction (costly and time-consuming) to identify SSRs markers.

The characterization of SSRs from both origins did not point out basic differences with those previously isolated in other species. The trinucleotide repeats were more abundant in sugar beet ESTs than in genomic DNA libraries and an opposite distribution was found for dinucleotide repeats. Similar results were described for *Arabidopsis* and barley for which the number of trinucleotide repeats was doubled in coding regions (Morgante et al. 2002; Thiel et al. 2003). In sugar beet ESTs, $(CT)_n$ is the most prevalent dimeric motif as in ESTs and GenBank data for most species studied except tomato (Areshchenkova and Ganal 2002) and loblolly pine (Liewlaksaneeyanawin et al. 2004) for which (AT)*n* is the most frequent. Among the trimeric repeats, $(CCG)_n$ is the most frequent for rice, barley, maize and sorghum (Temnykh et al. 2001; Kantety et al. 2002; Thiel et al. 2003) but it is not prevalent on wheat, *Arabidospsis* (Cardle et al. 2000), tomato (Areshchenkova and Ganal 2002), cotton (Qureshi et al. 2004) as in sugar beet (this study).

The distribution of sugar beet microsatellites with different repeat type in the CDS region support the theory to find triple repeats enriched in coding regions (57%) and mono repeats depleted in coding regions (12%). Similarly, 65.4 and 64% of the SSRs were trinucleotide repeats in exons of *Arabidopsis* and rice, respectively. (Lawson and Zhang 2006). This pattern is likely due to negative selection against frameshift mutations in coding regions that disrupt the protein (Metzgar et al. 2000). Surprisingly, the SSRs are distributed between the CDS region and the rest of the ESTs approximately with the respective occurrence of these regions. The repeats concerned all the amino acids but cysteine, tyrosine and tryptophan. Asparagine, serine and glycine repeats were the most frequent (respectively, 15, 12 and 9.5%). Similarly for *Arabidopsis*, the three more frequent types of amino acid repeats were serine (27.5%), proline (11.9%) and glycine (11.8%), cysteine and tyrosine were the rarest and tryptophan was missing (Lawson and Zhang 2006).

Since ESTs are coding sequences of functional genes, the polymorphism at the within-species level was expected to be lower as compared to genomic-SSRs supposed to be mainly derived from non-coding regions. These theoretical expectations were confirmed in durum wheat, 53% versus 25% (Eujayl et al. 2001), in rice, 83.8% versus 54% (Cho et al. 2000) and in our data, 47.8% versus 61.5%. However, the *B. vulgaris* ssp *maritima* microsatellites, although coming from genomic libraries, showed a level of polymorphism

(45.2%) similar to the EST-SSRs. A relationship between the degree of polymorphism and the average number of repeat was reported for barley (Thiel et al. 2003) and loblolly pine (Liewlaksaneeyanawin et al. 2004) and could explain the higher level of polymorphism of the genomic SSRs from Library B. Indeed, the genomic SSRs from Library B had a higher number of repeats on average [$(di)_{24}$, $(tri)_{17}$] when compared to EST-SSRs [$(di)_7$, $(tri)_6$] and *B. vulgaris* ssp *maritima* genomic SSRs [$(di)_{10}$, $(tri)_8$]. Of the EST-SSR markers, the dinucleotide repeats were more polymorphic (56.4%) than the trinucleotide repeats (48%). Such a lower polymorphism of the trimeric EST-SSRs was also previously reported for loblolly pine (Liewlaksaneeyanawin et al. 2004) and rice (Cho et al. 2000). Surprisingly, among the complete ESTs, there was no influence of the position of a SSR in the CDS or in non coding regions on polymorphism. Indeed, half of the EST-SSRs were polymorphic whatever their position in ESTs was. Two hypotheses emerge from this result. First, the corresponding protein region concerned by the extension or shortening of a CDS-SSR should not be involved in the activity of the protein. Second, when the CDS-SSR is a dinucleotide repeat, the variation in the SSR alleles should cover units of at least three repeats (corresponding to two amino acids) not to disrupt the protein. Contrary to the level of polymorphism that is lower in EST-SSRs than in genomic-SSRs, the transferability across species is expected to be enhanced in EST-SSRs. Indeed, as the EST-SSR markers are developed from coding sequences, a higher level of conservation between species of the same genus could be expected, relative to genomic-SSRs. An efficient transferability among species was demonstrated for wheat, barley, apricot, grape or cotton (Eujayl et al. 2001; Decroocq et al. 2003; Sorrells 2000; Guo et al. 2006). Moreover, the level of transferability of EST-SSRs between genus of the same tribe was much greater than for SSRs isolated from genomic libraries for barley and wheat (Holton et al. 2002; Röder et al. 1995; Kantety et al. 2002; Zhang et al. 2005). As for sugar beet, both EST-SSRs and genomic-SSRs showed a similar and high level of transferability across genus of the same tribe pointing out that SSR markers show a great potential for comparative mapping on Beteae tribe. Moreover, for all species considered, in contrast to the difference seen in the mapping population, the genomic-SSRs and EST-SSRs showed similar levels of polymorphism on the accessions of the Amaranthaceae family for both the number of alleles and PIC values. The discrepancy with previous studies might have resulted from less number of microsatellites used in this transferability study.

Both genomic and EST-SSRs developed were used to construct a genetic map of a sugar beet × table beet cross. Twenty-four anchoring markers have shown that this SSR map is congruent with an AFLP one (McGrath et al. 2007). This good correspondence between the two maps had been strengthened by the fact that both maps have been constructed on a F_2 progeny of a cross between a sugar and a table beet. Moreover, the numerous distortions that mapped on chromosome V have been reported elsewhere (Pillen et al. 1992; Schumacher et al. 1997; Weber et al. 1999). Most of them have been attributed to the presence of lethal alleles at the end of the linkage groups (Wagner et al. 1992). The sugar beet × table beet AFLP map also had distortions on chromosomes V and IX. In this AFLP map, the entire chromosomes V and IX favor the female allele contrary to what we have obtained. These distortions were attributed to incompatible gene interactions between sugar and table beets rather than to the segregation of sub-lethal alleles (McGrath et al. 2007).

The final map spanned 555 cM with linkage groups size varying between 54.6 and 84.4 cM. This size was in the range (526–815 cM) of published sugar beet maps (McGrath et al. 2007; Barzen et al. 1995). Only two maps have a smaller average distance between markers than the present one (2.24 cM). A high density RFLP map containing 413 markers had an average distance between markers of 1.5 cM (Halldén et al. 1996) and an AFLP map (McGrath et al. 2007) had an average distance between two markers of 1.61 cM. However, the RFLP map contained a number of large gaps reaching up to 30 cM whereas our map has only four gaps of more than 10 cM: two of 11 cM and two of 19 cM. Although dense, the AFLP map (McGrath et al. 2007) seemed to cover a smaller portion of chromosomes I, III and IX than this SSR map. Indeed, in McGrath's map, the common markers were located nearer the end of the linkage group.

The sugar beet genomic SSRs showed a high level of clustering on the genetic map as did RAPDs, RFLPs (Pillen et al. 1992; Nilsson et al. 1997) and AFLPs (Schondelmaier et al. 1996) and contrary to the EST-SSR markers that have mapped more uniformly. Similarly, in tomato, the genomic SSRs tended to cluster on centromeric regions whereas the EST-SSRs were more well distributed along euchromatic regions (Areshchenkova and Ganal 2002). The clustering on centromeric regions was suspected to result from an uneven distribution of (GT) and (GA) on this species. In sugar beet, it has been suggested that $(CA)_8$ were mainly located around centromeric regions (Schmidt and Heslop-Harisson 1996). In this study, there was no preferential clustering according to the SSR motif, all the different motifs being scattered along the chromosomes (data not shown). The ten $(CA)_n$ SSR markers mapped were scattered along the genetic map although they mapped preferentially on dense regions of different chromosomes as reported for a less saturated microsatellite map (Rae et al. 2000).

In conclusion, by taking benefit from ESTs available in public databases, we have identified a new class of SSRs

for sugar beet genotyping. ESTs are publicly available and EST-SSRs can be identified easily with various software packages. EST-SSR markers were found to be numerous and thus can be used to generate dense linkage maps with a small amount of clustering.

For genome wide isolation approaches, the linkage disequilibrium between a SSR and a gene of interest is fortuitous Gene-targeted strategies are more likely to yield SSRs that are relevant to the goals of marker-assisted breeding, since they provide a route to access potential candidate genes directly. Indeed, mapping expressed genes homologs with known functions can allow to identify the genetic factors that affect important traits if their map position coincides with those of significant QTLs.

Finally, the comparison of the transferability of genomic SSRs and EST-SSRs in beets showed that the later could be valuable tools for diversity studies on related sugar beet species. They exhibit a high number of alleles and are characterized by a more elevated level of polymorphism than standard genomic SSRs.

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