Novel and highly informative *Capsicum* SSR markers and their cross-species transferability

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ABSTRACT. This study was undertaken primarily to develop new simple sequence repeat (SSR) markers for *Capsicum*. As part of this project aimed at broadening the use of molecular tools in *Capsicum* breeding, two genomic libraries enriched for AG/TC repeat sequences were constructed for *Capsicum annuum*. A total of 475 DNA clones were sequenced from both libraries and 144 SSR markers were tested on cultivated and wild species of *Capsicum*. Forty-five SSR markers were randomly selected to genotype a panel of 48 accessions of the *Capsicum* germplasm bank. The number of alleles per locus ranged from 2 to 11, with an average of 6 alleles. The polymorphism information content was on average 0.60, ranging from 0.20 to 0.83. The cross-species transferability to seven cultivated and wild *Capsicum* species was

tested with a set of 91 SSR markers. We found that a high proportion of the loci produced amplicons in all species tested. *C. frutescens* had the highest number of transferable markers, whereas the wild species had the lowest. Our results indicate that the new markers can be readily used in genetic analyses of *Capsicum*.

Key words: Microsatellites; Pepper; Sweet-pepper; SSR markers; Cross-species transferability; *Capsicum annuum*

INTRODUCTION

Capsicum, a genus of the family Solanaceae, contains several species that are grown for food consumption, as well as for medicinal, ornamental or even security purposes. Their fruit, commonly known as pepper, is the most important spice in the world. The top pepper producing areas are South and Central America, India, Hungary, Spain, Italy, and the Southwestern United States (Prince et al., 1993). The cultivated *Capsicum* species include *C. annuum*, *C. frutescens*, *C. baccatum*, *C. pubescens*, and *C. chinense*. From the agricultural perspective, the most important cultivated species is *C. annuum*, which originated in Mexico (Paran et al., 1998).

Genetic variability within a taxon is of great importance for plant breeding. In *Capsicum*, variability and relatedness have long been estimated using polymorphism of morphological traits (Prince et al., 1992). More recently, the detection of DNA polymorphisms, as assessed by molecular markers, have become the standard procedure to study genetic variation. *Capsicum* genetic variation has been studied using different types of molecular markers, including isozyme variants (McLeod et al., 1979), restriction fragment length polymorphisms (Prince et al., 1992), random amplified polymorphic DNA (Paran et al., 1998), amplified fragment length polymorphism (Bradeen and Simon, 1998), inter-simple sequence repeat markers (Dias et al., 2013), as well as microsatellites. Simple sequence repeats (SSRs), or microsatellites, are useful and powerful molecular markers with manifold applications. The potential use of these molecular markers is well documented for *Capsicum* and they have been extensively used for genetic mapping, assessment of overall genetic variation, population and evolutionary genetic studies, and cultivar identification.

Several reports describe the development of microsatellite markers for use in genetic studies of *Capsicum* (Sanwen et al., 2000; Lee et al., 2004; Minamiyama et al., 2006; Yi et al., 2006; Nagy et al., 2007; Portis et al., 2007; Kong et al., 2012; Sugita et al., 2013). However, it can be noted that the number of publicly available molecular markers based on polymerase chain reaction (PCR) for peppers, including SSR markers, is still limited. Most of the SSRs included in published *Capsicum* genetic maps are proprietary markers (e.g., Syngenta, DNA Landmarks, and Seminis - Kong et al., 2012; and VegMarks - http://vegmarks.nivot.affrc.go.jp/, Sugita et al., 2013). Recently, Nicolaï et al. (2012) sequenced the *C. annuum* cv. 'Yolo Wonder' transcriptome using Roche 454 pyrosequencing. They identified 11,849 single nucleotide polymorphisms and 853 putative SSRs (Nicolaï et al., 2012). However, to date, to our knowledge, there are no reports of genotyping tests using these markers. In addition, Cheng et al. (2016) described a large set of unique microsatellite primer pairs derived from *in silico* evaluation of DNA sequences of six different pepper genomes (nuclei, mitochondria,

and chloroplasts). They tested a random set of 160 primer pairs of which only 65 exhibited polymorphisms among the 21 pepper genotypes analyzed. Hence, there is a need for additional publicly available microsatellite markers that are tested and validated for extensive genotyping application in *Capsicum*.

It should also be considered that the majority of the marker development studies have restricted the DNA analysis of new markers to *C. annuum* and some of the cultivated pepper species. Very little is therefore known about application of such markers in experiments with wild relatives of *Capsicum*. Marker-assisted selection could be used to unveil the potential use of wild species as donors of agronomic important traits to cultivated species, such as disease resistance or abiotic stress tolerance. Cross-species SSR marker transferability tests seem, therefore, to be justifiable in pepper species not only for breeding purposes, but also to stimulate the domestication of species with market potential.

In the present study, we described the development of new microsatellite markers for use in genetic analysis of *Capsicum* using DNA libraries enriched for AG/TC repeat sequences. The level of polymorphism and the number of alleles at the identified SSR loci were assessed in different accessions from a germplasm collection. In addition, the extent of primer amplification across a broad range of species was examined to determine the potential utility of the SSR markers in other cultivated and wild *Capsicum* species.

MATERIAL AND METHODS

Plant material and DNA extraction

Total genomic DNA was extracted from expanded leaves of a single *Capsicum annuum* plant (CNPH 4109) and used to construct the genomic DNA libraries. For the SSR marker characterization, 48 accessions of *C. annuum* from different countries (Table 1) were selected from Embrapa's germplasm collection. For the marker cross-species transferability evaluation, two plants from each of *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and one plant from each of the wild *Capsicum* species (*C. recurvatum*, *C. flexuosum*, *C. campylopodium*, and *Capsicum* sp accession LBB1551) were used for amplicon detection. In all experiments, total genomic DNA extraction followed the standard CTAB procedure (Doyle and Doyle, 1987).

SSR-enriched genomic library construction and screening

Initially, two enriched genomic libraries from a single individual plant of *C. annuum* were constructed with *Tsp509* and *Mse* digested DNA, following the protocols described by Brondani et al. (1998) and Buso et al. (2006). Fragments between 280 and 600 bp were recovered by DEAE-cellulose membrane via electrophoresis. After purification, around 30-µg fragments were recovered and ligated to adaptors with *Tsp509* and *Mse* ends. Fragments containing SSR sequences were selected by hybridization with biotinylated oligonucleotides complementary to the repetitive sequence AG/CT, and recovered by magnetic beads linked to streptavidin. The complex DNA-magnetic beads were amplified by PCR through the utilization of primers complementary to the adaptors. After purification, the fragments were cloned in the plasmid vector pGEM-T and then transformed into *Escherichia coli* strain XL1-Blue.

Table 1. *Capsicum annuum* accessions used to characterize the microsatellite loci.

Accession code	Accession name	Origin
181	Pimentão Italiano	Local variety - SP - Brazil
184	PI 123469	India
187	PI 201234	Mexico
191	Agronômico 10G	Commercial cultivar - Asgrow - Brazil
192	Magda	Commercial cultivar - Agroflora - Brazil
193	Yolowonder	Commercial cultivar - Ferry Morse - USA
196	Cascadura Ykeda	Commercial cultivar - Agroflora - Brazil
199	Ambato	Argentina
200	Vyuco	Argentina
291	Golden Calwonder	USA
295	Keystone resist. Giant 3	USA
296	California Wonder 300	Commercial cultivar - Asgrow - Brazil
332	Margareth	Local variety - Brazil
337	São Carlos	Local variety - Brazil
577	Early Calwonder	USA
580	Lito	Netherlands
582	Latino	Netherlands
641	Paradicsomalaku	uncertain
642	Javitott Cecei	uncertain
649	Plant C-1	uncertain
678	PI 140363	Iran
680	PI 159266	USA
689	PI 169110	Turkey
693	PI 169134	Turkey
703	PI 183441	India
707	PI 194909	Ethiopia
716	PI 241646	Peru
753	CNPH	Brazil
736	PI 357587	Yugoslavia
751	Amador F1	Brazil
754	Salmoai	Senegal
756	UFRJ-A	Brazil
764	UFRJ-B	Brazil
823	Linea 10	Spain
1379	XVR 3-25	USA
1387	Mallorca	Uncertain
1405	Yugoslavia Doce	Brazil
1716	Antibois	France
2171	NCMA (306)	USA
2275	Cascadura Ikeda	Commercial cultivar - Agroceres - Brazil
2282	PM3 F1	Commercial cultivar - Agroceres - Brazil
2474	Bel Rubi	Brazil
2519	Marconi	France
2678	Chili Ancho	USA
2691	Pimiento	USA
2732	Hibrido Fortuna	Brazil
2764	Hot Poken F2	Germany
Pimentão 3	CNPH	Brazil

Hybridization screening for microsatellites, sequencing of positive clones, and primer design

Aliquots from the enriched library were plated for screening on agar plates

containing ampicillin and tetracycline. Recombinant colonies with SSR were identified by hybridization with oligonucleotide probes poly dAdG/dCdT labeled with digoxigenin-11-ddUTP using a DIG oligonucleotide 3'-end labeling kit (Boehringer Mannheim, Indianapolis, IN, USA) following the manufacturer instructions. After high stringency washing, the membranes were processed for chemiluminescent detection and exposed to X-ray film for 2-3 h at 37°C.

Positive clones were grown overnight in liquid LB-ampicillin medium. Plasmid DNA was extracted by alkaline lysis (Sambrook et al., 1989), PCR-amplified, and purified, after which inserts were sequenced on an Applied Biosystems 3700 (Perkin-Elmer, San Jose, CA, USA) DNA sequencer. Primer pairs complementary to sequences flanking the repeat element were designed using the Primer 3 software (Untergasser et al., 2012) set to the following stringency criteria: a primer annealing temperature (Ta) range from 52° to 58°C; a primer pair Ta variance of less than 3°C; GC content ranging from 40 to 60%; and absence of complementarity between primers.

Primer screening and PCR conditions

Microsatellite amplification was performed in a 13-μL reaction mix containing 0.3 μM each primer, 1 U Taq DNA polymerase, 0.2 mM each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, DMSO 50%, and 7.5 ng template DNA. Amplification was performed with the cycling conditions: 96°C for 2 min; 29 cycles of 94°C for 1 min, 54°-72°C (based on each primer pair Ta) for 1 min, 72°C for 1 min; and 72°C for 7 min. The Ta for each primer pair was optimized to produce clear and robust DNA band amplification. Each primer pair was screened for product polymorphism on 3% agarose gels stained with ethidium bromide and size compared to a 1-kb and 100-bp DNA ladder.

Transferability analysis

To test the cross-species transferability of the SSR loci, we used two plants of each of the cultivated species and one plant of each of the *Capsicum* wild species. There were no additional attempts to optimize PCR conditions in cross-species transferability tests.

SSR locus characterization

The primer pairs showing high-quality PCR-amplified products at the given annealing temperature were used for estimates of allele sizes and for genotype determination. PCR products were separated on 4% denaturing polyacrylamide gels stained with silver nitrate (Bassam et al., 1991) and sized by comparison to a 10-bp DNA ladder. Forty-five primer pairs were used on a set of 48 accessions of the *Capsicum* collection. The number of alleles and allelic diversity were surveyed at each locus. To evaluate the informativeness of markers, the polymorphic information content (PIC), a statistic that incorporates both the number of alleles and the frequency of detection of alleles among the genotypes utilized, was calculated following Botstein et al. (1980).

RESULTS

SSR development

A total of 1000 recombinant colonies of *C. annuum*-enriched genomic library were screened with an AG/TC probe and 475 clones were detected that were positive for microsatellite-containing regions. The DNA sequencing indicated that 259 (55%) clones contained useful sequences for primer design, showing an adequate number of high-quality nucleotides flanking the repeat sequence. Three different categories of repeats were evident in the sequenced clones (Table 1): a) 217 perfect repeats, with no interruptions, ranging from 9 to 44 dinucleotide repeat units; b) 24 compound repeats, where two or more repeats were adjacent to each other; c) 18 imperfect repeats, with interruption in the repeat sequence. Since the library was enriched for (AG) repeats, this was by far the most common repeated motif present in almost all the microsatellite sequences. Nevertheless, (CA) tandems were also observed associated to (GA) repeats in compound sequences.

SSR locus characterization

A total of 144 SSR markers were tested with the DNA used to develop the two DNA genomic libraries. As many as 118 markers amplified very clean and easily interpretable PCR products and did not require further adjustment of the PCR conditions. However, 26 markers showed some non-specific amplification of secondary bands, did not amplify any product, or resulted in bands that could not be interpreted. For this last group of markers, further optimization of the reaction conditions was attempted that will be reported elsewhere.

The PIC for a selected sample of 45 SSR markers was estimated using a set of 48 genotypes of *C. annuum*. Among the 45 loci tested, the number of alleles per locus ranged from 2 to 11, with an average of 6 alleles per locus (Table 2). The PIC value was on average 0.60, ranging from 0.20 to 0.83.

The number of perfect repeats correlated weakly with the PIC values ($r^2 = 0.15$). For instance, primer CaBR64 had 42 repeats and a PIC of 0.81, primer CaBR61 had 29 repeats and the highest PIC value of 0.83, whereas CaBR40 had 23 repeats and a PIC of 0.81.

Transferability

Ninety-one SSR markers were tested on four cultivated and four wild *Capsicum* species (Table 3). The cultivated species with the highest number of markers showing amplicon products was *C. frutescens* (85), followed by *C. chinense* (84) and *C. baccatum* (79). Two markers amplified PCR products only in *C. annuum*. The wild species with the highest number of markers showing amplicon products were *C. recurvatum* and *Capsicum* sp LBB1551, both with 63 markers, followed by *C. campylopodium* (55 markers) and *C. flexuosum* (45 markers). PCR products were obtained for 73 SSR markers (80%) in all cultivated species and 41 markers in all wild species. Twenty-three markers did not show PCR products in any of the wild *Capsicum* species tested.

Table 2. Primer sequences, repeat motif, fragment size, annealing temperature (Ta), total number of alleles (N_A), and polymorphic information content (PIC) for a new set of *Capsicum annuum* SSR markers.

Marker	Repeat motif	Primer pair sequences (5'-3')	Fragment size (bp)	Ta (°C)	N_A	PIC
CaBR20	$(AG)_{27}$	CCgTAAAgAAATCAAACCAC gCATgCACACATAAACACTC	108-122	56	4	0.40
CaBR23	$(TC)_{31}$	ggCTCCTAggTATgCACCAg AATgTgATgCACAgTgCACC	162-174	56	7	0.77
CaBR24	$(TC)_{11}$	gTTATCTCCTTTTCCCAATC AAATgTTAggAACTCACCAg	80-90	56	2	0.30
CaBR25	$(TC)_{17}$	gCCTCTCTCTTATATTTCAg gTTTAATAggATgACAgAgC	117-129	56	5	0.57
CaBR30	$(CT)_{28}$	CCCTTTgAAACCTgATCTTg gATggACTTgATgAgATTgC	108-144	56	4	0.56
CaBR36	$(CT)_{22}$	gggTAAAggTACTTAgTAgA gTTgTATTgCTTTAgCTCAg	112-136	56	6	0.57
CaBR38	$(CT)_{12}$	ATCCggCggAgCTTCATAAC gCCgATCgATATATggATgC	94-98	56	3	0.45
CaBR39	$(CT)_{13}$	CATCCATATATCgATCggCT TTTCgACCATgTTCAgATCC	89-95	56	4	0.43
CaBR40	$(CT)_{23}$	TCAgACACCAAgCCATCAA gCAAgCTAATggCATggTA	124-142	56	9	0.81
CaBR49	$(AG)_{21}$	CTATCTTCgCATATAggCAg AATCTCTgTggCTgACTCAA	146-154	56	4	0.37
CaBR53	$(TC)_{25}$	CgACCTTCAggACAgATCAT CTggTAACCTAgAAAggCAg	220-260	56	5	0.65
CaBR58	$(TC)_{14}$	CggAgAAgAACTAgACgATT CTTgACAAACATCCACCACT	153-157	56	3	0.45
CaBR59	$(TC)_{17}$	CCAgggTACTTTgTCTTTCC ggAgCCTCggCACATAAA	116-128	56	3	0.37
CaBR61	$(AG)_{29}$	gAAgAgTCCTACTCAATCTA CTATATggTTTCACTCCTTC	84-134	56	11	0.83
CaBR63	$(CT)_{21}$	gAgTgCCTATCgATgTCTTT AgCTATCTAATTgCACCAAg	146-172	56	9	0.60
CaBR64	$(AG)_{42}$	AgAgTgTCCCATgCATAC gCTTATTCCAAAggTCTC	114-156	56	9	0.81
CaBR65	$(TC)_{24}$	gAgggTTTgTCATCTTATTg CTgAgACAgAAATTCTTgCT	100-136	56	4	0.64
CaBR67	$(GA)_{25}$	CCgAgAAAATgCACACAA TgACATACTCTTCCTACAgCTA	90-114	56	6	0.62
CaBR68	$(TC)_{21}$	TAATAgAAgCCCgCCCCT ATgCATgAgACTgTTgTTATggTA	114-132	56	5	0.65
CaBR73	$(AG)_{25}$	gCAAggAgAgAATCAAggTT ggAggTCAACACTTggATTAg	318-320	56	2	0.50
CaBR75	$(TC)_{26}$	CAACACTAAgTggTCATTCg CTgAgACAgAAATTCTTgCT	133-171	56	6	0.65
CaBR76	$(AG)_{24}$	gCTAgTCACgTCAATCTgTT CATTCTCTTCTTCTCAAACg	116-140	56	7	0.73
CaBR77	$(AG)_{24}$	gCATggTACTTCTTAgCATT gACACCAAgCCATCAATTAT	108-122	56	6	0.74
CaBR79	$(TC)_{26}$	CACTgggTATgTTgTTgTAA CCgTAAAgAAATCATACCAC	120-138	56	4	0.45
CaBR80	$(TC)_{20}(TG)_7$	CTCAAgTgTgCCAggTgATT gAgAgACAggAAgAgACgTACA	106-132	56	4	0.63
CaBR81	$(TC)_{13}(CA)_4$ $(CG)_6(TC)_{11}$ $(CA)_5(CG)_5$	gCAACTTCTCCgAgACAATC TTTCTAgTgCATgggACgTA	178-182	56	3	0.52
CaBR82	$(AG)_{37}$	gCACATgCACgTACAACC ggggTAAAAggCATTgTg	172-190	56	8	0.78
CaBR85	$(TC)_{25}$	CTTCACTTTgCTCACCCTAC TTACTggTTCAAgAgggAAA	116-154	56	10	0.72
CaBR88	$(AG)_{22}$	AATggATgTTCCCTTgCTTT CAACTgATCAACCATTCCgT	148-164	56	7	0.59
CaBR90	$(AG)_{17}$	AAggAggAACAAgAACAACC ATTggCAAgCACATgTAACT	120-132	56	4	0.32
CaBR93	$(AG)_{25}$	gCAATATgTTgAgTAgCTgT ACCCATACAAATCATCCAC	160-182	56	6	0.79
CaBR97	$(AG)_{38}$	gTgTgTCTgTgTgCATgAgC gCCTTCAgCTgTggTTATg	130-170	56	6	0.77

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Table 2. Continued.

Marker	Repeat motif	Primer pair sequences (5'-3')	Fragment size (bp)	Ta (°C)	N_A	PIC
CaBR98	$(AG)_{20}$	CgAgCTAATggCATggTACT TCAgACACCAAgCCATCAAT	132-146	56	7	0.76
CaBR100	$(AG)_{17}$	ATggCATggTACTTCTTAgCA CCAAgCCATCAATTATgCAA	138-152	56	7	0.71
CaBR106	$(AG)_{16}$	CgAggAAgCATTTAATgAgA CCTAgTCAATTggATAgTggg	168-182	56	6	0.78
CaBR107	$(AG)_{13}$	AAgCTAATggCATggTACTTCT CCAAgCCATCAATTATgCAA	144-154	56	6	0.73
CaBR112	$(TC)_{19}TA$ $(TG)_{12}$	CCTCCATTCTTCTATTgTACAgg TTgCACCCgCAgATTAgTT	136-142	56	4	0.69
CaBR113	$(GT)_{13}(GA)_{26}$	CAgAACTTAgCAgAggACCAg ATgTTCTgAgTCCACgATgC	138-190	56	5	0.48
CaBR115	$(AG)_{20}$	CATgAgACTgTTgTTATgg TgTCCAgTTTATgTTAgTTA	142-160	56	5	0.65
CaBR116	$(AG)_{22}$	gCATggTACTTCTTAgCATT gACACCAAgCCATCAATTA	118-130	56	7	0.74
CaBR124	$(TC)_{23}$	CgACCTTCAggACAgATCAT ggCAgAACATACTgAgACAgAA	156-192	56	5	0.63
CaBR136	$(AG)_{16}$	GTggACTAACAgACTCAACg CTgATgATgCAgAACATgAT	160-166	56	3	0.64
CaBR137	$(TC)_{18}$	CgTCCTTTATTTCTTgCTT AAAgAgAgAgATAgAAAggg	64-82	56	6	0.69
CaBR138	$(TC)_{27}$	ATCCCATTCTCATTTCTAgg gCAAgCAgTTTTTAgTgAgA	156-158	56	2	0.20
CaBR144	$(GT)_5(GA)_{21}$	TAgAAACACAAAATgTCCCC CCATTgACAAggACAATTCT	116-132	56	6	0.75

Table 3. Marker denomination, repeat motif, annealing temperature (Ta), primer sequence, and cross-species amplification for 91 microsatellite loci tested with DNA from four cultivated *Capsicum* species (*C. annuum*, *C. baccatum*, *C. chinense*, and *C. frutescens*) and four wild *Capsicum* species (*Capsicum* sp accession LBB1551, *C. recurvatum*, *C. flexuosum*, and *C. campylopodium*).

Marker	Repeat motif	Ta (°C)	Primer sequence	*C. annuum*	*C. baccatum*	*C. chinense*	*C. frutescens*	LBB1551	*C. recurvatum*	*C. flexuosum*	*C. campylopodium*
CaBR2	$(CT)_5T_8$	60	ACTACCgCCATCTTCCAC ATTCgAgTgTTggAAgTTAC	X	X	X	X	X	X	X	X
CaBR3	$(TC)_{18}$	54	CTAgTATCCTTACTTTCTCTCTC AgCCACgACAATCAATAg	X	X	X	X	X	X	X	X
CaBR4	$(AG)_{14}$	56	CATgATgTTAagAgAgAAgA ATCTCATCTCCTTTCTCTTT	X	X	X	X	X	X	X	X
CaBR5	$(AT)_8T_8$	54	TgTgTTgACTggTATgTTCC ACACAgTCATAAATgggTTC	X	X	X	X	X	X	X	X
CaBR10	$(AG)_{27}$	54	AAACACgAgAgAgAgAgA CgAgTgTggTgAATATgA	X	–	X	X	X	X		X
CaBR11	$(CT)_{12}$	56	gTTgTTATCTCCTTTTCCCA AAATgTTAggAACTCACCAg	X	X	X	X	X	X	X	X
CaBR13	$(CT)_{12}$	60	CAgACAATgCTCCTTCTC CAATCTTCCCAACTgCTC	X	X	X	X	X	X	X	X
CaBR15	$(CT)_{10}$	50	gCTCTTATCAATgAACCA AAgAAgAggAAgAAgACC	X	X	X	X				X
CaBR17	$(CT)_{11}$	52	CTCATTTCAAATCTCAAg CTgAATTCCAATTATgTg	X	X	X	X				
CaBR20	$(AG)_{27}$	56	CCgTAAAgAAATCAAACCAC gCATgCACACATAAACACTC	X	X	X	X	X	X		
CaBR22	$(TC)_7$	56	ATCCCTgTTgCTCTgATCTA CCAACAgATTgAAgCTgATA	X	X	X	X	X	X	X	
CaBR23	$(TC)_{31}$	56	ggCTCCTAggTATgCACCAg AATgTgATgCACAgTgCACC	X	X	X	X	X	X	X	X
CaBR24	$(TC)_{11}$	56	gTTATCTCCTTTTCCCAATC AAATgTTAggAACTCACCAg	X	X	X	X	X	X		X
CaBR25	$(TC)_{17}$	56	gCCTCTCTCTTATATTTCAg gTTTAATAggATgACAgAgC	X	X	X	X				X
CaBR27	$(CA)_{12}(CT)_{17}AT$ $CG(CT)_9$	60	gCAgAggACCAgTTAgCATA TgTTCTgAgTCCACgATgCT	X	X	X	X				
CaBR28	$(CT)_{11}(TA)_6$	56	ATgATgACCTTgTTgAgCAg TTgTATgTgTgTgCgTgT	X	X	X					
CaBR30	$(CT)_{28}$	56	CCCTTTgAAACCTgATCTTg gATggACTTgATgAgATTgC	X			X	X	X		
CaBR31	$(CT)_{20}$	56	CAACCTTACAAATCATCCA TTATTgAgAgggAgAggACA	X	X	X	X	X	X	X	X
CaBR34	$(GA)_{21}$	54	gggTTAAgCAATATgTTgAg CggTCTgTCATTACATCAAA	X	X	X	X	X	X		
CaBR35	$(CT)_{22}$	50	TTgTCCAgTTTATgTTAg TggTAATATTggTTCTTg	X	X	X	X				
CaBR36	$(TC)_{22}$	56	gggTAAAggTACTTAgTAgA gTTgTATTgCTTTAgCTCAg	X	X	X	X	X	X	X	X
CaBR37	$(CT)_{16}$	56	gAAACCATTgAAgCTCCC TCggCTCTATCTACAACg	X	X		X				
CaBR38	$(CT)_{12}$	56	ATCCggCggAgCTTCATAAC gCCgATCgATATATggATgC	X	X	X	X	X			X
CaBR39	$(CT)_{13}$	56	CATCCATATATCgATCggCT TTTCgACCATgTTCAgATCC	X	X	X	X	X	X	X	X
CaBR43	$(TC)_{14}$	58	AgCTTAACCATAACCAACCA ggTgCATgACATTTgAgTTA	X	X	X	X				
CaBR49	$(GA)_{21}$	56	CTATCTTCgCATATAggCAg AATCTCTgTggCTgACTCAA	X	X	X	X				

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Table 3. Continued.

Marker	Repeat motif	Ta (°C)	Primer sequence	*C. annuum*	*C. baccatum*	*C. chinense*	*C. frutescens*	LBB1551	*C. recurvatum*	*C. flexuosum*	*C. campylopodium*
CaBR50	$(TATG)_{10}(TC TA)_2(TC)_{18}$	58	AACgCACTTAgCATATgTC ggCAgCTTgTAAgTAgTgAA	X	X		X	X	X	X	
CaBR52	$(GT)_{14}(AG)_{14}TA GC(GA)_{10}$	54	TAgCAgAggACCAgTTAgCA ATgTTCTgAgTCCACgATgC	X	X	X	X	X	X	X	X
CaBR53	$(TC)_{25}$	56	CgACCTTCAggACAgATCAT CTggTAACCTAgAAAggCAg	X	X	X	X	X	X	X	X
CaBR54	$(AG)_{25}$	56	gCAgAACATACTgAgACAgA CTAAgTggTCATTCgAAgAg	X	X	X	X	X	X	X	X
CaBR55	$(GT)_7(GA)_{23}$	56	CAgCgCTAAACAgAAggAA CTCTCTAAACACAAACggCT	X	X	X	X	X	X		X
CaBR58	$(TC)_{14}$	56	CggAgAAgAACTAgACgATT CTTgACAAACATCCACCACT	X	X	X	X	X	X	X	X
CaBR59	$(TC)_{17}$	56	CCAgggTACTTTgTCTTTCC ggAgCCTCggCACATAAA	X	X	X	X	X	X	X	X
CaBR61	$(AG)_{29}$	56	gAAgAgTCCTACTCAATCTA CTATATggTTTCACTCCTTC	X							
CaBR63	$(CT)_{21}$	56	gAgTgCCTATCgATgTCTTT AgCTATCTAATTgCACCAAg	X	X	X					
CaBR64	$(AG)_{42}$	56	AgAgTgTCCCATgCATAC gCTTATTCCAAAggTCTC	X	X	X	X	X	X		X
CaBR65	$(TC)_{24}$	56	gAgggTTTgTCATCTTATTg CTgAgACAgAAATTCTTgCT	X	X	X	X	X	X	X	X
CaBR66	$(TC)_{18}$	54	CAACCCATACAAATCATCCA AgggTTgAgCAATTCATAgA	X	X	X	X	X	X	X	X
CaBR67	$(GA)_{25}$	56	CCgAgAAAATgCACACAA TgACATACTCTTCCTACgCTA	X	X	X	X	X	X	X	X
CaBR68	$(TC)_{21}$	56	TAATAgAAgCCCgCCCCT ATgCATgAgACTgTTgTTATggTA	X	X	X	X	X	X	X	X
CaBR73	$(AG)_{25}$	56	gCAAggAgAgAATCAAggTT ggAggTCAACACTTggATTAg	X	X	X	X	X	X		X
CaBR76	$(AG)_{24}$	56	gCTAgTCACgTCAATCTgTT CATTCTCTTCTTCTCAAACg	X	X	X	X	X	X		
CaBR77	$(AG)_{24}$	56	gCATggTACTTCTTAgCATT gACACCAAgCCATCAATTAT	X	X	X	X	X	X	X	X
CaBR78	$(AG)_{13}$	52	TTACAgAgAgCgATggAAgC CTTTCTTTCTCCCgCgTT	X		X	X			X	
CaBR79	$(TC)_{26}$	56	CACTgggTATgTTgTTgTAA CCgTAAAgAAATCATACCAC	X	X	X	X				
CaBR80	$(TC)_{20}(TG)_7$	56	CTCAAgTgTgCCAggTgATT gAgAgACAggAAgAgAcgTACA	X	X	X	X	X	X	X	X
CaBR81	$(TC)_{13}(CA)_4$ $(CG)_4(TC)_{11}$ $(CA)_{15}(CG)_5$	56	gCAACTTCTCCgAgACAATC TTTCTAgTgCATggACgTA	X	X	X	X	X	X	X	X
CaBR82	$(AG)_{37}$	56	gCACATgCACgTACAACC ggggTAAAAggCATTgTg	X	X	X	X	X	X	X	X
CaBR83	$(TC)_{21}$	58	CAAATATgAAgTTCTCgCTC ggTTgAgCAATATgTTgAgT	X	X	X	X	X	X	X	X
CaBR85	$(TC)_{25}$	56	CTTCACTTTgCTCACCCTAC TTACTggTTCAAgAgggAAA	X	X						
CaBR86	$(CT)_{15}$	48	TCCTTTTCTCTCAATTCT TCgAggATACAgTCTCAA	X	X	X	X				
CaBR88	$(AG)_{22}$	56	AATggATgTTCCCTTgCTTT CAACTgATCAACCATTCCgT	X	X	X	X	X	X	X	X
CaBR89	$(AG)_{17}$	62	ggTTCATgCTCAgTAgATTg ggAACTgAgAATgATTACCAC	X	X	X	X	X	X	X	X
CaBR90	$(AG)_{17}$	56	AAggAggAACAAgAACAACC ATTggCAAgCACATgTAACT	X	X	X	X	X	X		
CaBR91	$(TC)_{23}$	50	TgTCCAgTTTATgTTAgTTA ggTTCTTgATTTCATATAgT	X	X	X	X	X	X	X	X
CaBR92	$(AG)_{22}$	48	TggTACTTCTTAgCATTgAg CgATgACATTAACgTACACA	X	X	X	X	X	X		
CaBR93	$(AG)_{25}$	56	gCAATATgTTgAgTAgCTgT ACCCATACAAATCATCCAC	X	X	X	X	X	X	X	X
CaBR95	$(AG)_{20}$	56	CgCATATAggCAgATCAAAT AATCTCTgTggCTgACTCAA	X	X	X	X	X	X	X	X
CaBR97	$(AG)_{38}$	56	gTgTgTCTgTgTgCATgAgC gCCTTCAgCTgTggTTATg	X	X	X	X				
CaBR100	$(AG)_{17}$	56	ATggCATggTACTTCTTAgCA CCAAgCCATCAATTATgCAA	X	X	X	X	X	X	X	X
CaBR101	$(AG)_{17}$	56	TgATTAgggCgTAgggTTTA TATTCCTCTTCACCTCCgCT	X	X	X	X	X	X		X
CaBR102	$(AG)_{19}$	60	ggCAAgCTAATggCATggTA CCAAgCCATCAATTATgCAA	X	X	X	X	X	X	X	X
CaBR106	$(AG)_{16}$	56	CgAggAAgCATTTAATgAgA CCTAgTCAATTggATAgTggg	X		X	X				
CaBR107	$(AG)_{13}$	56	AAgCTAATggCATggTACTTCT CCAAgCCATCAATTATgCAA	X	X	X	X	X	X	X	X
CaBR108	$(AG)_{24}$	58	ACTTTTggTTgggAgAAACT AACCCACACCTATTTCCTCT	X	X	X	X	X	X	X	X
CaBR110	$(TC)_{21}$	56	gTCCAgTTTATgTTAgTTATTATgg TTCTTgATTTCATATAg	X	X	X	X		X		
CaBR112	$(TC)_{19}TA(TG)_{12}$	56	CCTCCATTCTTCTATTgTACAgg TTgCACCCgCAgATTAgTT	X							
CaBR113	$(GT)_{13}(GA)_{26}$	56	CAgAACTTAgCAgAggACCAg ATgTTCTgAgTCCACgATgC	X	X	X	X				
CaBR115	$(AG)_{20}$	56	CATgAgACTgTTgTTATgg TgTCCAgTTTATgTTAgTTA	X	X		X	X	X		X
CaBR116	$(GA)_{22}$	56	gCATggTACTTCTTAgCATT gACACCAAgCCATCAATTA	X	X	X	X	X	X	X	X
CaBR118	$(AG)_{21}$	56	CAAggAgAgAATCAAggT TTATAATCCCTCTCTTTgTg	X	X	X	X				
CaBR123	$(TC)_{28}$	58	TCAAATCTCAAgTTCTTgCT TgggCAgTATgTTgAgTAgT	X	X	X	X	X	X	X	X
CaBR124	$(TC)_{23}$	56	CgACCTTCAggACAgATCAT ggCAgAACATACTgAgACAgAA	X	X	X	X	X	X	X	X
CaBR125	$(TC)_{20}$	56	gATggACTTCATTATgATgC AAgAAggTgCATgTgTAgAA	X	X	X	X				
CaBR127	$(TC)_{22}$	56	CgCTACgTTAgTATgTTAT CCACgACAATCAATAgAA	X		X	X				
CaBR129	$(GA)_{23}$	60	gCAAACTCATAATAgAAgC AgATACTTCTCTgTTCTCCT	X		X	X				
CaBR130	$(AG)_{44}$	58	TgCATACgCTggTgTgTC TTCAgCTgTggTTATggg	X	X	X	X	X	X	X	X
CaBR135	$(TC)_{22}$	60	AATCTCAAgTTCTTgCACCT ggTTgAgCAATATgTTgAgT	X	X	X	X	X	X	X	X
CaBR136	$(GA)_{16}$	56	GTggACTAACAgACTCAACg	X		X	X				X

Continued on next page

Table 3. Continued.

Marker	Repeat motif	Ta (°C)	Primer sequence	*C. annuum*	*C. baccatum*	*C. chinense*	*C. frutescens*	LBB1551	*C. recurvatum*	*C. flexuosum*	*C. campylopodium*
CaBR136	$(GA)_{16}$	56	GTggACTAACAgACTCAACg CTgATgATgCAgAACATgAT	X		X	X				X
CaBR137	$(TC)_{18}$	56	CgTCCTTTATTTCTTgCTT AAAgAgAgAgATAgAAggg	X		X	X				
CaBR138	$(TC)_{27}$	56	ATCCCATTCTCATTTCTAgg gCAAgCAgTTTTTAgTgAgA	X		X	X				
CaBR139	$(GA)_7C_4(GA)_{19}$	56	gAAggATTACACAATATgTT TTTCAAATCTgAAgTTCTCT	X		X		X	X		
CaBR140	$(GA)_{12}$	58	gTgTgTCTgTgTgCATgAgC TTCAgCTgTggTTATgggA	X	X	X	X				
CaBR141	$(GA)_{13}$	56	TTTCgACCATgTTCAgATCC CATCCATATATCgATCggCT	X	X	X	X	X	X	X	X
CaBR142	$(GA)_{12}$	58	gCCgATCgATATATggATgC TTTgTgCCgCATCACTCC	X	X	X	X	X	X		X
CaBR143	$(GA)_{21}$	56	TATgCCCACCTCTAAAgAAA AACCATCTgTTCATCTCCAT	X	X	X	X	X	X	X	
CaBR144	$(GT)_5(GA)_{21}$	56	TAgAAACACAAAATgTCCCC CCATTgACAAggACAATTCT	X	X	X	X	X	X		X
CaBR145	$(CT)_{24}$	58	CATTCTCTTCTTCTCAAACg CACgTCAATCTgTTgTgAAA	X	X	X	X	X	X	X	X
CaBR147	$(AG)_{22}$	58	gAAgggTTgAgCAATATA CTTAAgTTCTCgCTCTTC	X	X	X	X	X	X		X
CaBR149	$(CT)_4T_4(CT)_{21}$	52	CAAACCATACAAATCATCTAAT TTgTATTATTgAgAgggA	X	X	X	X	X	X	X	X
CaBR150	$(AG)_{14}$	52	CTTCTTAgCATTgAgAgTTC AATATCAgACACCAAgCC	X	X	X	X	X	X		

DISCUSSION

The enrichment procedure used in this study proved to be effective and enabled the development of *Capsicum* DNA libraries enriched for microsatellite sequences. Across these libraries, approximately 50% of the sequenced clones contained short repeats. Brondani et al. (1998) used the same enrichment procedure and found similar results for eucalyptus. Of the 259 different microsatellite containing useful sequences for primer design, 83.8% were composed of perfect repeats, 9.3% of compound, and 6.9% of imperfect repeats. A high percentage of markers based on perfect repeats is desirable, especially for population studies, where allele calling of compound and imperfect repeat markers is complex and challenging.

The number of alleles per marker ranged from 2 to 11, with a mean of 6 alleles. This value is higher than that found in similar studies on self-pollinating plants (Hokanson et al., 1998) and in another study with peppers (Cheng et al., 2016), but similar to results obtained in *Phaseolus vulgaris* (Buso et al., 2006). The degree of polymorphism detected by the SSRs was measured using the PIC value. The PIC values obtained for the 45 *Capsicum* SSR markers among 48 genotypes averaged 0.60. By comparison, gene diversity values and/or PIC values obtained for other autogamous species were found to be 0.43 for tomato (Panaud et al., 1996), 0.69 for rice (Bredemeijer et al., 1998), 0.63 for bean (Buso et al., 2006), 0.77 for *Capsicum* (Ibarra-Torres et al., 2015), and varying from 0.05 to 0.64 in *Capsicum* by Cheng et al. (2016). The information content of a marker can be determined by taking into account its ability to differentiate between individuals. The larger the number of alleles detected by a marker, the greater its potential utility appears to be. According to the criterion suggested by Botstein et al. (1980), a PIC value above 0.5 is considered high and indicates a high usefulness of the marker. Therefore, the set of *Capsicum* microsatellites reported here should be useful for germplasm characterization of both cultivated *Capsicum* as well as its wild relatives and might contribute to the understanding of the genetic relationships among *Capsicum* accessions, wild taxa, or both.

The characterization of the *Capsicum* germplasm, as reported for many other "orphan species", i.e., plant species for which we have little genetic and/or genomic knowledge, is usually limited by budget constraints. Thus, unequivocal identification of each accession is essential to avoid duplicate samples, which increases germplasm maintenance costs. The set

of microsatellites described here, combined with appropriate allele-sizing algorithms and multiplex, semi-automated genotyping systems, could be used to characterize *Capsicum* germplasm collections. This would expedite the process of selecting representative germplasm sets for conservation, characterization, or breeding. In addition, because SSR markers are codominant, polymorphic, and stable, they should be very useful for genomic mapping and gene or QTL tagging, if a large number of SSRs is developed.

The ability to use the same SSR primers in different plant species depends on the extent to which the primer sites flanking SSRs are conserved between related taxa. The cross-species transferability of SSR markers in *Capsicum* was found to be high, with high percentages of loci producing amplicons in all tested species. This indicates that a high level of sequence conservation exists within the primer regions of these *Capsicum* species. The high transferability rate of the SSR loci indicates that these markers are powerful tools for synteny analysis in *Capsicum*. Furthermore, our study identified novel candidate SSR markers for *C. flexuosum*, *C. recurvatum*, and *C. campylopodium*, which had not been investigated in previous *Capsicum* SSR development studies.

The evolutionary conservation of these loci allows for successful cross-species amplification using the same primers and amplification conditions. This facilitates their potential use in phylogeny studies, investigations into levels of genetic variation, and other population parameters such as gene flow in the different species of the *Capsicum* genus. Recently, Ibarra-Torres et al. (2015) used SSR markers originally developed for *C. annuum* to analyze *C. pubescens* genotypes showing high genetic homology among both species. Ince et al. (2010) also reported that microsatellite primer pairs amplified genomic targets of *C. annuum*, *C. baccatum*, *C. chacoense*, *C. chinense*, *C. frutescens*, and *C. pubescens*, also indicating species transferability within *Capsicum*. In beans, Yu et al. (2000) identified SSR primers from *Vigna aconitifolia*, a distantly related species, which amplified *Phaseolus vulgaris* DNA. Ashkenazi et al. (2001) identified SSR markers obtained from tomato capable of producing PCR products from the potato genome.

In conclusion, we report the development of a new set of SSR markers for *Capsicum* that is immediately available for use by the scientific community. The possible applications of these markers range from cultivar identification, analysis of genetic diversity to trait mapping and marker-assisted selection.

Conflicts of interest

The authors declare no conflict of interest.

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