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Development and characterization of genomic simple sequence repeat markers in eggplant and their application to the study of diversity and relationships in a collection of different cultivar types and origins

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Abstract The eggplant (*Solanum melongena* L.) genome is the least investigated among the economically most important solanaceous crops. Extensive use of molecular markers will improve eggplant germplasm enhancement and breeding. Microsatellites, or simple sequence repeats, have proved to be very useful for eggplant germplasm management and breeding, but there is limited availability of these polymorphic, codominant, and highly repeatable markers in eggplant. We developed a genomic DNA library enriched with AG/CT, which allowed the identification of 55 new genomic microsatellites. Variation parameters of microsatellite loci analyzed showed high average values. The potential of these markers for fingerprinting was assessed in a collection of 24 accessions, of which 22 correspond to *S. melongena* from different types (landraces, heirlooms, modern F1 hybrids, and obsolete cultivars) and origins, and two to each of the cultivated relatives *S. aethiopicum* and *S. macrocarpon*. The multivariate (cluster and PCoA) analyses clearly differentiated four main clusters: (a) two outgroups formed by *S. aethiopicum* and *S. macrocarpon* accessions, (b) *S. melongena* accessions derived mostly from the Mediterranean basin, Central Europe, Africa, and America ('occidental' eggplants), and (c) *S. melongena* accessions derived mostly from Eastern and Southeastern Asia ('oriental' eggplants). However, no apparent association pattern was found for accessions of the different types. Observed heterozygosity (H_o) values were low, although hybrid cultivars had higher values ($H_o = 0.12$) than non-hybrid materials ($H_o = 0.02$). The new set of eggplant microsatellite markers has proved highly informative and useful for studying the diversity, relationships, and genetic characteristics of an eggplant collection. These markers will be useful for germplasm management and breeding in eggplant.

Keywords Eggplant · Microsatellites · Simple sequence repeat · *Solanum melongena* · Genetic diversity

Introduction

Eggplant (*Solanum melongena* L., Solanaceae) is widely grown in many temperate and tropical regions of the world. The major goals of ongoing breeding programs include yield, resistance to biotic and abiotic stress, fruit quality, postharvest quality, nutritional value, and local market preferences (Daunay 2008). The efficient use of the genetic resources available is critical in order to obtain new improved cultivars that

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satisfy these new requirements. A recent example comes from the application of modern tools to characterize and use exotic germplasm, including wild and cultivated relatives, for the improvement of antioxidant phenolics content in eggplant (Stommel and Whitaker 2003; Prohens et al. 2007; Mennella et al. 2010).

A wide diversity of local eggplant varieties, adapted to different environmental conditions (mostly open field), uses, and preferences, many of which are stored in germplasm collections (Daunay 2008), coexist with modern cultivars (mostly F1 hybrids) specifically adapted to greenhouse cultivation (Muñoz-Falcón et al. 2008a, 2009a). Although eggplant production is increasingly based on modern hybrid cultivars, selections of some locally adapted materials have attained prominence in the last decades and are marketed as heirloom varieties (Daunay 2008; Muñoz-Falcón et al. 2008b, 2009b). Although many differences exist among local varieties from specific regions of the world, two large groups of eggplant varieties are generally considered by breeders: "occidental" or "Western" eggplants (from the Middle East, Africa, Europe, and America) and "oriental" or "Asian" eggplants (from Eastern and Southeastern Asia) (Chadha 1993; Hallard 1996; Daunay and Janick 2007; Daunay 2008; Bohme et al. 2008).

The availability of molecular tools for the fingerprinting and study of diversity and relationships of germplasm and breeding material is essential for adopting effective plant breeding strategies (Collard and Mackill 2008; Xu and Crouch 2008). Molecular markers have also been shown to be good tools for the indirect selection of qualitative and quantitative traits, pedigree analysis, determination of the degree of heterozygosis, establishment of genetic maps, or development of introgression lines (Staub et al. 1996; Dekkers and Hospital 2002). Unfortunately, the eggplant genome is the least investigated among the economically most important cultivated Solanaceae, and attempts to use markers developed in other related crops (e.g. tomato, pepper, potato) have shown important limitations, and in some cases have proven inefficient (Nunome et al. 2009; Frary et al. 2005). For this reason, the use of molecular markers in eggplant breeding has been limited compared to other relevant crops of the same family (Barone et al. 2009; Jo et al. 2010; Danan et al. 2011).

Several studies of genetic diversity in eggplant have been carried out using random amplified polymorphic DNA (RAPD) (Karihaloo et al. 1995; Nunome et al. 2001; Koundal et al. 2006; Singh et al. 2006), amplified fragment length polymorphisms (AFLP) (Mace et al. 1999; Nunome et al. 2001; Furini and Wunder 2004; Prohens et al. 2005; Koundal et al. 2006; Muñoz-Falcón et al. 2008b, 2009b), simple sequence repeat (SSR) (Nunome et al. 2003a, b; Behera et al. 2006; Stàgel et al. 2008; Nunome et al. 2009; Muñoz-Falcón et al. 2009b, 2011), and inter simple sequence repeat (ISSR) (Isshiki et al. 2008) markers. These studies show a relatively low frequency of polymorphism among eggplant cultivars, which is probably caused by the genetic bottleneck associated with its domestication in the Indo-Burma region, an area outside the natural range of its wild ancestor *S. incanum* L. (Lester and Hasan 1991; Weese and Bohs 2010). Therefore, the development of new molecular markers of interest for the management of genetic resources and breeding is a priority.

Microsatellites, or SSRs, are one of the best available marker choices for eggplant genetic studies and breeding due to their high level of polymorphism, high reproducibility, multiallelic nature, codominant inheritance, locus specificity, abundance, and random distribution throughout the genome (Powell et al. 1996; Varshney et al. 2005; Kalia et al. 2011). A number of microsatellite markers are publicly available in eggplant, either genomic microsatellites from SSR-enriched genomic libraries, or genic (expressed sequence tag; EST) microsatellites from in-silico analysis of EST databases (Nunome et al. 2003a, b; Stàgel et al. 2008). The most recent work reported the identification of 1,120 SSRs, of which only 620 were polymorphic (Nunome et al. 2009). Despite this, more markers are needed due to the low frequency of polymorphism found among eggplant cultivars.

One example of the interest in SSR markers over other molecular markers available in eggplant comes from the works of Muñoz-Falcón et al. (2009b, 2011), who have found that SSR markers are more informative than AFLPs for studying the relationships among closely related materials of the local *Almagro* and *Listada de Gandía* heirlooms, and have allowed the detection of SSR alleles specific and universal to the different selections of these heirlooms. Similarly, Demir et al. (2010) also found that a few selected SSRs discriminated a set of 19 Turkish eggplant genotypes better than RAPD markers.

The aim of this study was to develop a new set of SSR markers from an eggplant enriched genomic

library and to evaluate its utility for the study of diversity and relationships in a set of eggplant materials representing different types and origins. Two related cultivated species from the secondary genepool of eggplant, the scarlet eggplant (*S. aethiopicum*) and the gboma eggplant (*S. macrocarpon*) (Schippers 2000; Daunay 2008), were also included as outgroups and to test the transferability of the new SSR markers to *S. melongena* relatives.

Materials and methods

Plant material and DNA extraction

Twenty-four accessions, of which 22 correspond to *S. melongena* and two to each of the related species *S. aethiopicum* and *S. macrocarpom*, were used for SSRs characterization (Table 1). The *S. melongena* accessions were chosen so that they represented different origins and morphological and molecular characteristics as assessed in previous works (Muñoz-Falcón et al. 2008b, 2009b), as well as different types, including landraces (local varieties traditionally grown in a restricted area), heirlooms (local varieties that have acquired a reputation and have wide diffusion), F1 cultivars, and two obsolete (non-hybrid) cultivars (Table 1). The plant material used in this study is either part of the germplasm collection of the Instituto de Conservacion y Mejora de la Agrodiversidad Valenciana or was obtained from seed companies.

Genomic DNA was extracted from fresh leaves of the eggplant accessions according to the CTAB method procedure (Doyle and Doyle 1987). The quality of DNA was checked on 1% agarose gels and the DNA concentrations estimated using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilminton, DE, USA).

Development of the enriched SSR library

Development of the enriched genomic library was carried out using the commercial F1 hybrid Mulata. Linker was prepared by mixing equal volumes of the oligonucleotides AdapE-forward (5′-CTCGTAGAC TGCGTACC-3′) and AdapE-Reverse (5′-AATTG GTACGCAGTCTAC-3′) to a final concentration of 5 μM. The mix was denatured at 94°C for 5 min, and subsequently incubated at room temperature for 20 min.

Ten micrograms of DNA were completely digested with 60 U of *Eco*RI. A ligation reaction was performed in a total volume of 200 μl using 27 μl of digested DNA, 4 U of ligase T4 and 20 μl of the supplier buffer.

In order to select the fragments containing $(CT)_n$ microsatellite sequences, the PCR product was hybridized to a biotinylated $(GA)_9$ oligonucleotide. Streptavidin-coated paramagnetic particles (Streptavidin MagneSphere Paramagnetic Particles, Promega, Sydney, Australia) were added to recover fragments potentially containing microsatellite sequences. The mix was washed several times and finally eluted with double-distilled water. An aliquot of the elute was PCR-amplified in a volume of 100 μl containing 1 μM AdapE-forward primer, 0.2 mM dNTPs, 1 mM $MgCl_2$, 1× PCR buffer, 1 U of *Taq* polymerase (Roche), in a thermal cycler following the profile: 1 cycle for 5 min at 94°C, 28 cycles of 20 s at 94°C, 20 s at 60°C, and 1 min at 72°C. Finally, the PCR amplified products were column-purified (High Pure PCR Product Purification Kit, Roche Diagnostics GmbH, Mannheim, Germany) and used for the DNA library construction.

The $(CT)_n$ enriched sequences were cloned on pTZ57R/T vector (InsTAclone cloning kit, Fermentas, New York, USA) transformed through thermic shock into *E. coli* competent cells (strain DH5α) and plated onto selective Luria–Bertoni (LB) agar plates (50 μg/ml ampicillin). Recombinant colonies were picked up from the plates, transferred individually onto 96-well plates containing 100 μl of LB, and incubated for 12 h at 37°C.

Colony PCRs were performed using M13 primer, electrophoresed in 1.5% agarose gels, transferred to a nylon membrane (Nylon Membranes Positively Charged, Roche, Mannheim, Germany) and hybridized with a digoxigenin (Dig)-labelled $(AG)_{15}$ oligonucleotide probe. Positive clones were sequenced using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences obtained were vector-trimmed and unique clones were selected. In order to detect the repeat motifs, sequences were analyzed with Websat software (Martins et al. 2009; http://wsmartins.net/websat). Primers complementary to microsatellite flanking regions were designed using

Table 1 Eggplant accessions used in this study, cultivar type and origin

Accession name	Code	Accession type	Origin
S. melongena			
Prosperosa	PRO	Heirloom	Italy (Tomato Growers Seeds, USA)
Listada de Gandía	LdG	Heirloom	Valencia, Spain
Rami	RAM	Heirloom	Egypt
C-S-23	CS23	Landrace	Gavá, Barcelona, Spain
PI491260	PI491260	Landrace	Tsakoniki, Greece
Almagro	ALM	Heirloom	Almagro, Ciudad Real, Spain
AFR-S-1	AFRS1	Landrace	El Kelaa, Morocco
Nadia	NAD	F1 hybrid	Italy (Reimer Seeds, USA)
Manjri Gota	MAN	Heirloom	India (Reimer Seeds, USA)
Mulata	MUL	F1 hybrid	Ramiro Arnedo S.A., Spain
SUD-S-5	SS5	Landrace	La Habana, Cuba
Florida High Bush	FHB	Obsolete cultivar	USA (Tomato Growers Seeds, USA)
INRA11 Dourga	INRA11	Obsolete cultivar	INRA, France
B-S-5	BS5	Landrace	Palma de Mallorca, Baleares, Spain
RNL019	RNL19	Landrace	Klouekanme, Benin
BBS-189	BBS189	Landrace	Adzopé, Abidjan, Ivory Coast
Fairy Tale Hybrid	FTH	F1 hybrid	Tomato Growers Seeds, USA
Kermit	KER	F1 hybrid	Thailand (Asian Vegetable Seeds, USA)
ASI-S-1	ASIS1	Landrace	Beijing, China
Long White Angel	LWA	F1 hybrid	China (Asian Vegetables Seeds)
Thai Long Green	TLG	F1 hybrid	Thailand (Asian Vegetable Seeds, USA)
Ping Tung Long	PTL	Heirloom	Taiwan (Evergreen Seeds, USA)
S. aethiopicum			
BBS-157	BBS157	Landrace	Boudoukou, Abidjan, Ivory Coast
S. macrocarpon			
BBS-178	BBS178	Landrace	Abengourou, Abidjan, Ivory Coast

the program Primer3 (Rozen and Skaletsky 2000; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Microsatellites were named with a three-letter code followed by a number. The first letter is a C (for COMAV; Instituto de Conservación y Mejora de la Agrodiversidad Valenciana), the second and the third are SM (for *S. melongena*). Since our microsatellites were developed from a genomic library, we consider not to BLAST them against the available EST-SSR datasets because a few or no hits were expected.

Microsatellite characterization

Microsatellites were amplified following the M13-tail method described by Schuelke (2000) to facilitate the incorporation of a dye label during PCR. Amplifications were performed in a total volume of 12 µl with 10 ng DNA, 1 mM $MgCl_2$, 0.05 µM of forward primer, 0.25 µM of reverse primer, 0.2 µM fluorescent-labelled M-13 primer, 0.2 mM dNTPs, and 1 U of *Taq* polymerase in 1× PCR buffer. Conditions of the PCR amplification were as follows: 1 cycle for 2 min at 94°C, 35 cycles of 15 s at 94°C, 30 s at the appropriated annealing temperature (Table 2), 45 s at 72°C, followed by 10 min extension at 72°C. Microsatellite alleles were resolved on an ABI Prism 3100 DNA sequencer (Applied Biosystems) using GeneScan 3.7 software and precisely sized using GeneScan 500 LIZ molecular size standards with GenoTyper 3.7 software (Applied Biosystems).

Data analysis

Marker analysis was performed using the matrix of allele size and the program PowerMarker (Liu and

Muse 2005). The following parameters were calculated: number of alleles per locus (A), polymorphic information content (PIC) values calculated as PIC $= 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$ (where n is the total number of alleles detected, p_i the frequency of the ith allele, and p_j the frequency of the jth allele) (Botstein et al. 1980), observed heterozygosity (H_o), expected heterozygosity (H_e), calculated as $H_e = 1 - \sum_{i=1}^{n} p_i^2$ (where p_i is the frequency of the ith allele) (Nei 1973), and fixation index (F_{is}), calculated as $F_{is} = 1 - (H_o/H_e)$(Wright 1965).

In order to evaluate the potential of the SSR markers obtained for diversity studies, a similarity matrix was constructed scoring the amplified fragments as present (1) or absent (0) in each microsatellite loci. Dice's similarity values (Dice 1945) were calculated for 1,000 bootstrapped data matrices using Phyltools 1.32 software (Buntjer 1997). Subsequently, a consensus phenetic tree based on the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm (Sneath and Sokal 1973) was built with the Phylip 3.62 package (Felsenstein 1989). Dice's similarity values (Dice 1945) were used to graphically represent genetic relationships among accessions by principal coordinate analysis (PCoA) (Gower 1966) using the NTSYS 2.0 software package (Rohlf 1993).

Results

Development of microsatellite markers

A total of 896 colonies showed insert by colony PCR, and 182 of them strongly hybridized with the $(GA)_{15}$ Dig-labelled probe. One hundred and sixty-nine clones gave readable sequences, of which 112 were unique sequences. We found a total of 73 microsatellites, of which 67 contained at least one $(AG)_n/(CT)_n$ repeat, while different motifs were found in the remaining six SSRs. Of these, most (57) were simple microsatellites, with the number of repeats ranging from 5 to 45, five had interrupted repeats, and five had compound repeats. On the other hand, six sequences showed different microsatellite motifs (three for AT, and one for each of TTG, TAT and AAAT). Thirty-two out of 39 sequences without microsatellites had a rich AG content. Primers were designed for 73 microsatellite flanking sequences. Amplification was successful for 55 of them (75.3%), which are described in Table 2. The remaining 18 microsatellites gave complex patterns or no amplification.

SSR polymorphism

All the microsatellites developed (55) amplified discrete bands in the set of eggplant accessions studied as well as in the accessions of the related species *S. aethiopicum* and *S. macrocarpon*. When we took into account only *S. melongena* accessions, 41 of the microsatellites (74.5%) were polymorphic, but this number increased to 47 (85.5%) when the two accessions of the related species *S. aethiopicum* and *S. macrocarpon* were included. Most SSRs with a low number of CT/AG repeats ($n \leq 11$) were monomorphic in the materials analyzed. Three of the primers (CSM15, CSM33, and CSM71) had banding patterns corresponding to the presence of two loci. Since the difference in size was great enough to distinguish them unambiguously in all cases, they were not discarded from the study and were tagged with an A or B letter to discriminate them (e.g., CSM15A, and CSM15B).

Table 3 shows the parameters of variability studied for the polymorphic SSRs. When considering only *S. melongena* accessions, the number of SSR alleles detected was 203, ranging from two to 15 per locus, with an average of 4.7 alleles per locus. The allelic frequencies (p) varied from 0.02 to 0.95, with a mean value of 0.21. One hundred and forty alleles (70.0%) were considered rare (i.e., $p \leq 0.10$) and 2 (1.0%) were almost fixed ($p \geq 0.90$) (data not shown). The mean expected heterozygosity (H_e) was 0.52, and ranged from 0.86 in CSM36 to 0.09 in CSM13 and CSM33a. Mean observed heterozygosity (H_o) was 0.06, and ranged from 0.00 for 16 markers, for which no heterozygous individuals were found, to 0.24 for CSM36 marker. The H_o value for F1 hybrids was 0.12, while for the non-hybrid materials it was 0.02. Wright's fixation index (F_{is}) had an average value of 0.88, ranging from 0.41 to 1.00. F_{is} for F1 hybrids was 0.64, while for the rest of non-hybrid materials it was 0.96.

The most informative marker (CSM36; PIC = 0.85) was able to distinguish the highest number of *S. melongena* accessions, whereas the least informative markers (CSM13 and CSM33A; PIC = 0.08) were only able to distinguish one accession (ASI-S-1) from the rest. Taking into account all loci across the *S. melongena* accessions, the average PIC was 0.47.

Table 2 Characteristics of the 55 genomic microsatellite primers developed from *S. melongena* Mulata

Marker name	Repeat	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Temperature (°C)	Predicted size (bp)
CSM4	$(GA)_{15}$	GCGTACCAATTCTAACCACAAG	GTAATCCGCTTCCCATTTCTC	59.3	213
CSM7	$(CT)_{10}$	CGACGATCACCTTGATAACG	CCTAAATGCAGAGTTTCCAAAG	58.6	201
CSM9	$(CT)_{7}$	TGCGTACCAATTCGACATTCT	GCATTTGCTAGGAATTTACCG	59.6	207
CSM12	$(AG)_{12}$	CAATGGTATGTCTCCACTCGTC	AAGCTAAACATGAGATGCCGAT	59.8	210
CSM13	$(CT)_{8}$	TCTCCTCTCTTCATCTTTCTCG	AAGACTGGGAGAGGGACCAG	59.3	189
CSM15	$(CT)_{8}$	TCGGTCCTTTTGTTAAGCATC	GATATGAGTGTCGAGAGACCCC	59.6	292
CSM16	$(AG)_{15}$	ACGTGCCATTTCAAACTTGG	TCCTTTTCTTGAGCTGAATTTG	59.8	224
CSM18	$(CT)_{9}$	AAATTGTCAAACCTCGACACC	TAAGCAGTGGAAAACAAAACCC	59.7	112
CSM19	$(TG)_{16}(AG)_{10}$	CACTGATGCCAGTAATTGTGC	TTGACCTGCTCCAAAGTTCC	59.7	160
CSM20	$(GT)_{16}(GA)_{10}$	TTAGTGCCAGCAAAAATTGG	TTTTAAGCTTTAGCGCTCTCC	58.5	212
CSM21	$(AG)_{11}$	ATTTGACAACTGCCACATCG	ACCATGGGAAAGCGTATGAG	59.8	245
CSM23	$(AAG)_{7}$	TTTCCACTCAACATAGGCTTTTAG	CTTCCCCTCTCCCAAGAAAG	59.6	151
CSM25	$(AG)_{5}$	TCCACCAGCGTTAACCTCAG	TATCTTTGTGCGGGCTTTTC	60.7	188
CSM26	$(TTG)_{5}$	CCCAGAAAAGGCTCATTGTTAG	GTCGAGGCAATCCAAATTACTC	60.0	230
CSM27	$(GA)_{23}$	TGTTTGGAGGTGAGGGAAAG	TCCAACTCACCGGAAAAATC	60.0	206
CSM29	$(AG)_{17}$	GGATGAAATGAAGGCTTAGGG	GCCATCCTCATCTTTGATGG	60.2	236
CSM30	$(CT)_{20}$	CACTGTTCCTGGTTGCTGTG	TTTAGCTTTAGCCCATCTACCG	60.1	172
CSM31	$(AG)_{28}$	CAACCGATATGCTCAGATGC	GCCCTATGGTCATGTTTTGC	59.8	259
CSM32	$(AG)_{23}$	TCGAAAGTACAGCGGAGAAAG	GGGGGTTTGATTTTCATTTTC	59.6	248
CSM33	$(CT)_{11}$	CTCCTCTTGGTGGAGCTCAG	TTTAGAGGGCGTTTGGATTG	60.1	231
CSM34	$(CT)_{14}$	TCAAATTTCTCCTCCCCAAG	GTTCCTATTCGCCACGTCAC	59.8	221
CSM35	$(CT)_{10}$	GGACCACAAGGACTGTCACC	GAGTTCCCTGCATACCTTGG	60.0	215
CSM36	$(GA)_{27}$	CCTCAATGGCAGTAGGTCAGA	GTTCTTTGAGCCTCCAGTGC	60.1	344
CSM40	$(CT)_{45}$	AGGGCAAGTGCTGATAAACG	TCACGAAATGATGCCTCTTC	59.5	299
CSM41	$(CT)_{12}$	AACCTTGAGGGGCATTCAG	GTCACGGCTTGGAACAGAAG	60.4	205
CSM42	$(TA)_{5}$	ACGCCCATGAGGTTCTAGTG	CATGCATTAGTGACTTGAAAGTTG	59.5	177
CSM43	$(AG)_{14}$	ATTTTAACCCCGGGAAAATG	ACCGCTTCTAGGTTTTGCAC	59.6	250
CSM44	$(AG)_{14}$	CGTCGTTGTAACCCATCATC	TTGCCAAATTCCTTGTGTTC	58.7	249
CSM45	$(AG)_{16}$	TTGAAAAGGGCAATTTCGTC	ATTCTCCTCTGAAACCCTTGC	59.9	190
CSM46	$(AG)_{9}$	CCAATTCTGCAGGTTTTCTTC	ATTGGCCAAATGAAACTTCC	58.9	224
CSM47	$(AG)_{9}$	CAACATTCTCAGCAAGCATAGC	GCAACTCCTGAAGACGGAAG	60.0	171

Table 2 continued

Marker name	Repeat	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Temperature (°C)	Predicted size (bp)
CSM48	$(GA)_{13}n(AT)_6n(AG)_5$	GAGACTGGCTGTTTATGGTGTG	TTTTCTAATTGGACCAGAGACTTC	58.8	250
CSM49	$(AG)_{10}$	GGTGTGGTGTTAGGGAAACG	GCATCCTTCTTTGCCATCAG	60.5	219
CSM50	$(TAT)_4$	CAAGAAATGATGTGCCTTGG	GTGGGATGAAATGCAACAAG	59.1	185
CSM52	$(TC)_{12}$	CTTGGGTCACAAAAGGTTCC	TCACCGAAAAAGATCCAACC	59.7	235
CSM53	$(AG)_7$	CGGCGACAAAATTAGTGAGC	ACCCACGGATACTCCTTCTC	59.4	250
CSM54	$(GA)_{19}$	ATGTGCCTCCATTCTGCAAG	TGGGTGGGATGCTGAGTAAG	61.1	227
CSM55	$(AG)_{10}$	TCAAGCACGGTTGATACTGG	CATGGGTGCAGCAAGAAAC	60.0	208
CSM57	$(CT)_8$	TTTTGGTCCCAAACTTTTCC	ACCTTCAAGCTTCACGATTTAG	58.3	208
CSM58	$(GA)_9AA(GA)_5$	TTGTCCTCTTTCAGCCTTCC	CCCAATGCATGCCACTTAC	59.7	212
CSM59	$(CT)_{10}$	TTCATTCTCGTCTGCTGTGG	GATCATGTGTTTTCAACATCTCC	59.2	164
CSM60	$(GAA)_3(GA)_5$	TTGTTGGTCGTCTGGTTTTG	CCACCATGAACCCATTTTTC	59.8	236
CSM62	$(GA)_{27}$	TAAACACGCGATGAGAGAGG	TGGAAGAATATCCATTAACATTCAAG	59.4	234
CSM63	$(CT)_{10}$	CAGCCATGGACCACATTTTAC	ACATGCCACTCATGTTGGTG	60.3	150
CSM65	$(TC)_8$	CAACCCCCAAATCCCTAAAT	GAGGAAGAGAAGCGGTGGTC	60.6	157
CSM67	$(AT)_5$	GCGTACCAATTCAAGACAAGG	AATGTTAGAACCCGCATCTCAT	59.7	335
CSM68	$(AT)_5$	GATCCATGTCACTCAAATTCCC	AGTTCGCAAGGTCTCTTTACTGA	60.1	355
CSM69	$(GAGAAA)_7$	ACATATTGAGGGGCGGAATC	GGAACTATTGCAAGATGAAGCA	60.2	160
CSM71	$(CT)_{16}$	CACCAATACTTCGCCATCCT	TCACGAGCGTTAAGACAACAA	59.7	186
CSM73	$(CT)_{22}$	TTCAACATAGCCTGGACCATT	AATGCAGGGTTTGGACTTCA	60.0	209
CSM74	$(GA)_{26}$	ATAAGGCCATTTGCTGGTGA	AAAGTTTCCCATGTTTCACG	59.0	187
CSM75	$(AG)_{11}$	TGCGTACCAATTCTTAGCTTCTC	GGGATACGTGTGGGATTCG	60.5	250
CSM76	$(GA)_5C(AG)_8$	TCTGTCAACATAATTGTATACATGCAG	TGGAAAAATAATATAACTAGGCAAACA	59.1	239
CSM77	$(TC)_{16}$	CCAAGTTGCTTGGTAGCTCA	TCAAACCGTTCCTCTGCTCT	59.5	208
CSM78	$(CT)_{19}$	AGGGAGGAGCTCTCGTGTG	CAATAACGTAGCTTAATTACTCCCAAG	60.2	288

For each marker, the repeat motif, forward and reverse primers, annealing temperature, and predicted size in Mulata are presented

Table 3 Characteristics of the polymorphic microsatellites in the materials studied, grouped by taking into account only *S. melongena* accessions, or all the materials (*S. melongena* plus the two *S. aethiopicum* and *S. macrocarpon* accessions)

Locus	*S. melongena*					All materials				
	No of alleles	H_e	H_o	PIC	F_{is}	No of alleles	H_e	H_o	PIC	F_{is}
CSM12	5	0.68	0.09	0.62	0.87	5	0.68	0.09	0.62	0.87
CSM13	2	0.09	0.00	0.08	1.00	3	0.16	0.00	0.16	1.00
CSM15A	3	0.52	0.00	0.42	1.00	3	0.52	0.00	0.42	1.00
CSM15B	4	0.33	0.00	0.31	1.00	4	0.33	0.00	0.31	1.00
CSM16	3	0.49	0.00	0.41	1.00	5	0.57	0.00	0.51	1.00
CSM19	3	0.54	0.19	0.45	0.65	4	0.61	0.17	0.54	0.71
CSM20	3	0.45	0.10	0.37	0.78	5	0.54	0.09	0.48	0.83
CSM21	3	0.49	0.05	0.43	0.91	5	0.57	0.04	0.52	0.93
CSM23	–	–	–	–	–	2	0.09	0.00	0.08	1.00
CSM26	3	0.53	0.00	0.43	1.00	4	0.57	0.00	0.48	1.00
CSM27	9	0.74	0.10	0.71	0.87	10	0.76	0.09	0.74	0.88
CSM29	6	0.79	0.10	0.76	0.88	6	0.79	0.10	0.76	0.88
CSM30	5	0.67	0.14	0.63	0.80	5	0.67	0.14	0.63	0.80
CSM31	10	0.83	0.14	0.82	0.84	12	0.86	0.13	0.85	0.85
CSM32	8	0.75	0.05	0.71	0.94	11	0.78	0.08	0.76	0.89
CSM33A	2	0.09	0.00	0.08	1.00	3	0.16	0.00	0.16	1.00
CSM33B	2	0.20	0.05	0.18	0.77	5	0.33	0.08	0.31	0.74
CSM34	3	0.24	0.00	0.23	1.00	3	0.24	0.00	0.23	1.00
CSM35	–	–	–	–	–	2	0.08	0.00	0.08	1.00
CSM36	15	0.86	0.24	0.85	0.72	17	0.88	0.22	0.87	0.75
CSM4	10	0.83	0.14	0.82	0.83	12	0.86	0.13	0.84	0.85
CSM40	6	0.71	0.09	0.67	0.87	6	0.71	0.09	0.67	0.87
CSM41	3	0.28	0.05	0.26	0.84	4	0.39	0.04	0.36	0.89
CSM43	5	0.63	0.00	0.59	1.00	7	0.69	0.00	0.66	1.00
CSM44	9	0.78	0.18	0.75	0.77	10	0.79	0.17	0.77	0.78
CSM45	6	0.76	0.00	0.72	1.00	7	0.78	0.00	0.74	1.00
CSM46	2	0.43	0.09	0.34	0.79	4	0.52	0.08	0.45	0.84
CSM47	3	0.22	0.05	0.21	0.78	4	0.29	0.05	0.28	0.84
CSM48	3	0.55	0.05	0.47	0.92	4	0.59	0.04	0.52	0.93
CSM52	6	0.51	0.05	0.49	0.91	8	0.59	0.04	0.57	0.93
CSM53	–	–	–	–	–	2	0.09	0.00	0.08	1.00
CSM54	7	0.78	0.05	0.75	0.94	9	0.81	0.05	0.79	0.94
CSM55	2	0.28	0.00	0.24	1.00	3	0.41	0.00	0.37	1.00
CSM57	5	0.66	0.10	0.60	0.85	5	0.66	0.10	0.60	0.85
CSM58	3	0.31	0.18	0.28	0.41	3	0.31	0.18	0.28	0.41
CSM60	–	–	–	–	–	2	0.08	0.00	0.08	1.00
CSM62	5	0.64	0.05	0.58	0.93	5	0.64	0.05	0.58	0.93
CSM63	–	–	–	–	–	3	0.16	0.00	0.15	1.00
CSM65	2	0.19	0.00	0.17	1.00	3	0.33	0.00	0.30	1.00
CSM67	2	0.36	0.00	0.30	1.00	6	0.47	0.09	0.42	0.81
CSM69	2	0.50	0.09	0.37	0.82	4	0.57	0.08	0.48	0.85
CSM7	6	0.53	0.18	0.50	0.66	8	0.60	0.17	0.58	0.72

Table 3 continued

Locus	*S. melongena*					All materials				
	No of alleles	H_e	H_o	PIC	F_{is}	No of alleles	H_e	H_o	PIC	F_{is}
CSM71A	–	–	–	–	–	3	0.13	0.05	0.13	0.65
CSM71B	4	0.58	0.09	0.49	0.84	4	0.58	0.09	0.49	0.84
CSM73	4	0.68	0.00	0.62	1.00	5	0.71	0.00	0.66	1.00
CSM74	6	0.47	0.00	0.45	1.00	8	0.56	0.00	0.54	1.00
CSM75	–	–	–	–	–	3	0.20	0.00	0.19	1.00
CSM76	3	0.25	0.00	0.24	1.00	5	0.37	0.00	0.36	1.00
CSM77	2	0.43	0.00	0.34	1.00	4	0.52	0.00	0.45	1.00
CSM78	8	0.72	0.10	0.68	0.87	8	0.72	0.10	0.68	0.87
Mean	4.72	0.52	0.06	0.47	0.88	5.46	0.51	0.06	0.47	0.90

Number of alleles detected, expected heterozyosity (H_e), observed heterozygosity (H_o), polymorphic information content (PIC) and Wright's fixation index (F_{is}) are presented

When we considered all the accessions (i.e., *S. melongena* plus the relatives *S. aethiopicum* and *S. macrocarpon*), the number of alleles detected increased to 273, with a mean of 5.46 alleles per locus. Markers CSM63, CSM23, CSM71A, CSM75, CSM53, CSM60 and CSM35, which were monomorphic in *S. melongena*, had one or two different alleles in these *S. melongena* relatives. When these two accessions were added to the calculation of variation parameters, mean expected ($H_e = 0.51$) and observed ($H_o = 0.06$) heterozygosity showed very slight variation. A small increase in Wright's fixation index (F_{is}) was also detected (0.90), while the average PIC remained unchanged (0.47).

Multivariate analysis

SSR-based genetic distance values between accessions ranged from 0.26 between AFR-S-1 and PI491260 to 0.61 between Listada de Gandia and Thai Long Green. The phenogram obtained by UPGMA cluster analysis clearly distinguished three main clusters (Fig. 1). The outgroup clusters, supported by a 100% bootstrap value, includes and differentiates the two *S. aethiopicum* and *S. macrocarpon* outgroup accessions. The third major cluster grouped 16 *S. melongena* accessions of different types derived from the Mediterranean basin, Central Europe, Africa, and America as well as the Indian Manjri Gota heirloom. Given that the origin of 15 accessions out of these 16 accessions can be traced back to Europe, Africa and America, we labelled this group "occidental". The fourth cluster was composed of five *S. melongena* accessions derived from Eastern and Southeastern Asia, and also includes the Fairy Tale Hybrid originating from America (Tomato Growers Seeds, USA). We labelled this cluster "oriental". Within the "occidental" cluster, robust nodes (with a bootstrap value $\geq$ 50%) were found connecting Florida High Bush and SUD-S-5 (85%), Listada de Gandia and Rami (70.4%), PI-491260, Almagro, and AFR-S-1 (62.6%), and B-S-5 and INRA11 Dourga (58.6%).

Multivariate PCoA analysis was performed in order to complement the information obtained with the cluster analysis. The first and the second coordinates accounted for 14.0 and 9.4% of the total variance respectively. The PCoA graph (Electronic Supplementary Material Fig. 1) shows a clear separation between the "occidental" group accessions, positioned at the right-hand side of the graph, and "oriental" accessions, which are plotted in the left-hand part. The commercial accession Fairy Tale Hybrid, included in the "oriental" group, plots closer to the African accession RNL-19, which forms part of the "occidental" group, rather than to other "oriental" accessions. Within the "occidental" group a differentiation is observed according to the second coordinate, so that eight accessions plot together in the upper part of the graph (high positive values of the second coordinate), another six plot in the lower part (negative values), and two others plot in between (with values close to 0 for this second coordinate) (Electronic Supplementary Material Fig. 1). No association between these three subgroups within the "occidental"

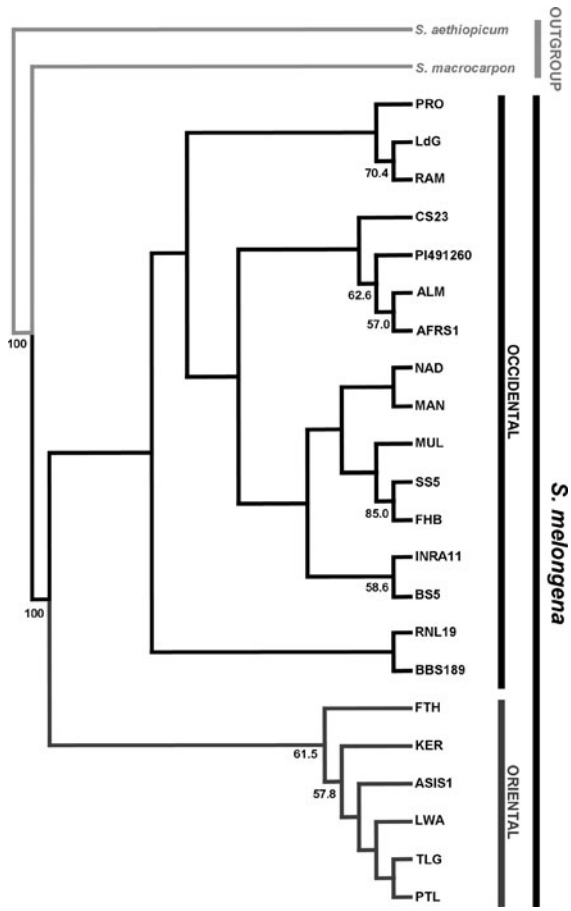

Fig. 1 Hierarchical clustering analysis (UPGMA algorithm with bootstrap supporting values, 1,000 replicates) of the 24 eggplant accessions (22 of *S. melongena*, one of *S. aethiopicum*, and one of *S. macrocarpon*) based on Dice genetic distances calculated with 47 polymorphic SSRs. Only bootstrap values over 50% are shown. Accession codes are reported in Table 1

group with origin or cultivar characteristics was apparent, although the two commercial F1 hybrids (Mulata and Nadia) plotted in the first subgroup.

Discussion

Genomic libraries enriched for specific dinucleotide repeats are useful for identifying highly polymorphic SSR markers (Chakraborty et al. 1997; Schug et al. 1998; Kalia et al. 2011) Using a genomic library enriched for AG/CT repeats, we were able to design 55 new genomic microsatellites that gave a successful amplification. This is an important addition to the publicly available genomic SSR markers in eggplant. Forty-one of the new SSRs proved to be polymorphic in a set of accessions of *S. melongena* (74.5%), and 47 (85.5%) when the related species *S. aethiopicum* and *S. macrocarpon* were considered. Studies carried out in 25 plant microsatellite libraries reported that, on average, 82.3% of loci producing PCR products are polymorphic (Squirrel et al. 2003), which is a similar value to the one obtained with our set of SSR markers and materials. This is a significant fact, given that an important genetic bottleneck is thought to have taken place during eggplant domestication and, in consequence, cultivated eggplant has a narrow genetic base (Lester and Hasan 1991; Furini and Wunder 2004; Weese and Bohs 2010). It is also remarkable that the polymorphism found by us is higher than that obtained in eggplant with genomic SSRs by Nunome et al. (2003a; 69.5%), Nunome et al. (2003b; 13.7%), and Nunome et al. (2009; 56.7%), and with EST-SSRs by Stàgel et al. (2008; 28.2%), and Nunome et al. (2009; 30.3%). The higher polymorphism of the genomic SSRs, already observed in a study on striped eggplants (Muñoz-Falcón et al. 2011), is to be expected, as these markers are mostly associated with non-coding regions, while EST-SSRs derive from expressed regions of the genome (Kalia et al. 2011).

The high average values for the number of alleles detected per locus (4.7), the expected heterozygosity (0.52) and the PIC values (0.47) obtained for *S. melongena* in this study indicate that the SSR markers developed can be of great utility for germplasm management and breeding programmes in eggplant. In general, the values estimated for the variation parameters obtained by us were also higher than those observed in former studies in eggplant. In this respect, Nunome et al. (2003a) obtained a mean of 3.1 alleles per locus and a H_e of 0.38 when evaluating 11 *S. melongena* lines by means of 16 polymorphic dinucleotide genomic microsatellites. The same authors (Nunome et al. 2003b) evaluated the same 11 accessions using trinucleotide genomic microsatellites and observed that the number of alleles per locus (2.1) and H_e (0.31) were even lower. This agrees with previous studies which suggested higher mutation rates in dinucleotide than in trinucleotides repeats (Chakraborty et al. 1997; Schug et al. 1998) and may also explain why our SSRs (mostly dinucleotide genomic SSRs) get relatively high values of variation

parameters. Stàgel et al. (2008), when considering 11 polymorphic EST-SSRs in 38 *S. melongena* accessions, found a rate of 3.1 alleles per locus and an average PIC value of 0.38. Usually, genomic microsatellites tend to be more polymorphic than EST-SSRs (Kalia et al. 2011), but in this case, the results are similar to those obtained by Nunome et al. (2003a) using genomic microsatellites. This may be explained by the fact that Stàgel et al. (2008) used a higher number of accessions from a broader range of origins than Nunome et al. (2003a, b). Nunome et al. (2009) also evaluated genomic and EST-SSRs in eight eggplant accessions, obtaining a mean of 2.2 and 1.4 alleles per locus respectively. The PIC values in the Nunome et al. (2009) study were also low, being 0.27 for the genomic SSRs and 0.13 for the EST-SSRs. Demir et al. (2010), using five eggplant SSRs developed by Nunome et al. (2009) and selected for their high PIC, found an average of 4.8 alleles per locus using 20 Turkish accessions, which is similar to the average value found by us. However, if we just consider our five SSR markers with the highest PICs, the average number of alleles per locus in our study would have been 10.0, which is also a greater value than that obtained by Demir et al. (2010). The fact that we have used a wide diversity of materials, with different types and different origins, from four continents, may also have contributed to the high values of the variation parameters. However, if we exclude the clearly distinct "oriental" accessions from the analyses, the average values of number of alleles per locus and PICs of the 15 "occidental" accessions are still high (3.2 and 0.32 respectively), suggesting that the methodology used is useful for developing highly polymorphic SSR markers.

The results of this study also show a low level of observed heterozygosity (H_o) in *S. melongena*. The mean F_{is} was close to 1 (0.88) indicating an evident deficiency of heterozygotes. Similar results were reported by Nunome et al. (2003a, b) and Muñoz-Falcón et al. (2009a, b) in different *S. melongena* materials. This suggests a high level of inbreeding, probably due to the mostly autogamous nature of eggplant (Quagliotti 1979; Pessarakli and Dris 2004). A low level of observed heterozygosity is detected even in commercial hybrids ($H_o = 0.12$). This provides evidence that the present breeding programme methods use a narrow elite genepool for the development of new hybrid cultivars, resulting in an overall reduction of the heterozygosity of the hybrids (Muñoz-Falcón et al. 2009a). Given that heterosis for yield traits has been detected in eggplant when crossing genetically distant parents (Sidhu et al. 2004; Rodríguez-Burruezo et al. 2008), the results obtained suggest that introduction of new germplasm in eggplant breeding programmes could be useful for increasing the heterozygosity and heterosis of hybrids.

The evaluation of the SSR markers developed as potential tools for fingerprinting has been demonstrated, as all the accessions used have had a unique SSR fingerprint. In fact, SSRs have proved very useful for studying variation among closely related materials of eggplant (Muñoz-Falcón et al. 2009b, 2011). Their usefulness for establishing relationships among the materials has been studied by means of UPGMA clustering and PCoA analysis. The cluster analysis clearly differentiates four groups. The outgroups include the scarlet (*S. aethiopicum*) and gboma (*S. macrocarpon*) eggplants clusters, which are mainly cultivated in Africa. The third and fourth clusters include, respectively, what we have called "occidental" and "oriental" accessions. In general, eggplants from Europe, Africa, Middle East and America are morphologically different from Asian eggplants (Chadha 1993; Hallard 1996; Daunay and Janick 2007). This is evidence that a genetic differentiation between "occidental" and "oriental" eggplants has occurred, which may have important implications for conservation of genetic resources and breeding. In this respect, it remains to be studied whether hybrids between the two types of eggplant present heterosis for yield and potential commercial interest. However, although "occidental" and "oriental" accessions were clearly separated, the relationship among the accessions belonging to each group is in general unclear, and the subclusters formed appear to show no association based on the origin or type of material.

The fact that Manjri Gota, an Indian heirloom, groups with the "occidental" accessions, derived from local Indian germplasm, is not a surprise. In former studies (Muñoz-Falcón et al. 2008b), the Manjri Gota accession we have used was found to be morphologically and molecularly similar to Mediterranean accessions. *S. melongena* was domesticated in the Indo-Burma center of origin (Lester and Hasan 1991; Weese and Bohs 2010), from where it was introduced into the Middle East, Africa, and Europe (Prohens et al. 2005; Daunay 2008).This may lead us to

speculate that, among others, materials genetically similar to Manjri Gota were brought from India into western regions of the Old World and through the action of microevolutive forces gave rise to the materials of eggplant typical of the Middle East, Africa, and Europe. In any case, further research should be done to investigate the reason for the clustering of Manjri Gota with "occidental" eggplants. Also, the clustering of Fairy Tale Hybrid, which is an F1 hybrid with small and elongated fruits and is morphologically similar to other "oriental" eggplants (Muñoz-Falcón et al. 2009a) suggests that it might have had materials derived from Asian eggplants in its parentage. Further molecular work may help to clarify this issue.

Prior studies have suggested that Asian varieties show wider morphological and genetic diversity than Western types (Lester and Hasan 1991; Weese and Bohs 2010). Here we have found that the diversity measured as H_e (Nei 1973) of the "occidental" and "oriental" groups established by us was similar (0.48 and 0.43 for "occidental" and "oriental" groups, respectively). This is probably due to the fact that the diversity of Asian eggplants was much more underrepresented in the "oriental" group than the diversity of Western types was in the "occidental" group. In this respect, most of the "oriental" accessions evaluated are commercial hybrids and no heirlooms typical of this region are represented (Lester and Hasan 1991; Daunay 2008). A wider diversity would probably have been found if more Asian landraces and heirlooms had been available and included in this study.

The complete level of transferability of microsatellites to the related scarlet (*S. aethiopicum*) and gboma (*S. macrocarpon*) eggplants is of great relevance for the breeding of these neglected crops, in which few genetic improvement efforts have been undertaken up to now (Lester and Thitai 1989; Schippers 2000; Seck 2000). The availability of these SSR markers will help in the conservation of genetic resources, as well as in studying the diversity, establishing relationships, and breeding of both African eggplant crops. It will also facilitate the construction of interspecific genetic linkage maps, and will help to accelerate the introgression of useful genes of eggplant relatives into the more economically important *S. melongena*.

In conclusion, the 55 newly developed eggplant microsatellite markers developed using the enriched genomic library strategy have proved highly informative and useful for studying the diversity and relationships of a set of eggplant materials, and represent a significant improvement in the available eggplant genomic resources. This new set of molecular tools as well as the information derived from its application to a collection of eggplant materials will be useful for germplasm management and breeding research in eggplant.

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