Available online: www.notulaebotanicae.ro
Print ISSN 0255-965X; Electronic 1842-4309
Not Bot Horti Agrobo, 2014, 42(1):59-65

Genetic Diversity and Relationships in Local Varieties of Eggplant from Different Cultivar Groups as Assessed by Genomic SSR Markers

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Abstract

Spain is a secondary center of diversity for eggplant (*Solanum melongena*). Spanish landraces of eggplant are normally classified in four cultivar groups: Round, Listada de Gandía, Semi-Long, and Long. We have used 19 genomic SSRs for the molecular characterization of 30 eggplant accessions corresponding to the four cultivar groups. Sixteen SSRs of which 15 were polymorphic could be amplified and 65 polymorphic alleles, with a range of two to 11 alleles/locus, were detected. The polymorphism information content (*PIC*) of SSR markers ranged from 0.07 to 0.77, with an average value of *PIC*=0.50. The mean observed heterozygosity (H_o) presented a very low value H_o=0.01, while the mean expected heterozygosity (H_e) had a value of H_e=0.57. Multivariate cluster analyses revealed that a considerable diversity exists within each of the cultivar groups. Listada de Gandía and Long cultivar groups were clearly separated from each other in different branches of phenogram. The principal coordinates analysis (PCoA) confirmed that each of the cultivar groups is genetically diverse and, with the exception of the Round group, they plot in different areas of the PCoA graph. Overall, the results indicate that Spanish eggplant landraces present a high degree of homozygosis, considerable intra-cultivar group diversity, and a certain degree of genetic differentiation. This information is of interest for selection and breeding of eggplant as well as for germplasm conservation.

Keywords: forage, breeding, cultivar groups, fruit shape, landraces, multivariate analysis, *Solanum melongena*

Introduction

Eggplant (*Solanum melongena* L.) was domesticated in Southeast Asia (Meyer *et al.* 2012). From there it spread to other tropical and subtropical areas of the world, where selection and other microevolutive forces led to the development of a wide array of locally adapted landraces of eggplant and to the emergence of secondary centers of diversity (Cericola *et al.*, 2013; Hurtado *et al.*, 2012; Prohens *et al.*, 2005; Tümbilen *et al.*, 2011). Differentiation between eggplants of Southeast Asia on one side and those of the Middle East, Africa and Mediterranean region of Europe on the other have led to the recognition of two major groups of eggplant cultivar groups denominated, respectively, Oriental and Occidental eggplants (Cericola *et al.*, 2013; Vilanova *et al.*, 2012).

The Mediterranean region of Spain is considered as a secondary center of diversity (Hurtado *et al.*, 2012; Prohens *et al.*, 2005) in which four cultivar groups are normally recognized: Round, Listada de Gandía, Semi-Long, and Long (Hurtado *et al.*, 2013; Muñoz-Falcón *et al.*, 2011; Prohens *et al.*, 2005). These four cultivar groups are distinguished mostly by the fruit length/width ratio (i.e., the Round, Semi-Long, and Long cultivar groups) (Cericola *et al.*, 2013; Hurtado *et al.*, 2013; Prohens *et al.*, 2005; Tümbilen *et al.*, 2011), and also for the presence of purple stripes on a white background combined with a fruit shape intermediate between the Round and Semi-Long types in the case of Listada de Gandía (Hurtado *et al.*, 2013; Muñoz-Falcón *et al.*, 2011).

Development of new eggplant varieties addressing old and new breeding objectives (Barchi *et al.*, 2012; Lebeau *et al.*, 2013; Sunseri *et al.*, 2003) requires of genetic diversity. Normally development of new commercial varieties, is based on intra-varietal group crossings, except in the case of the Semi-Long type, which can also be obtained by crossings between Round and Long types. Increasing the genetic base of new eggplant cultivars can be achieved through the incorporation of local landraces in the commercial breeding programmes (Muñoz-Falcón *et al.*, 2009a). Therefore, the study of genetic diversity and relationships of collections of local varieties provides information of relevance for the breeding programmes.

Several molecular studies (Cericola *et al.*, 2013; Prohens *et al.*, 2005; Tümbilen *et al.*, 2011) have shown that eggplant cultivar groups are genetically diverse. In the case of

Spanish varieties, Prohens *et al*. (2005) studied the AFLP diversity in a collection of eggplants from the Round, Listada de Gandía, Semi-Long, and Long types and found considerable intra-group diversity and genetic differentiation among groups. However, SSRs have proved as more powerful than AFLPs to study the relationships amongst closely related eggplant materials (Muñoz-Falcón *et al*., 2009b). Furthermore, unlike AFLPs, which are dominant, SSRs are co-dominant, which allows determining the levels of observed heterozygosity. In particular, in eggplant, genomic SSRs have proved as much more informative than EST-SSRs (Muñoz-Falcón *et al*., 2011). Therefore, in order to obtain information of interest for eggplant breeding and germplasm conservation, we studied the diversity, heterozygosity and relationships of 30 eggplant varieties from the Region of Valencia, situated in the Mediterranean region of Spain using genomic SSR markers.

Materials and methods

Plant material and DNA extraction

A total of 30 accessions corresponding to four cultivar groups (Round, Listada de Gandía, Semi-Long, and Long) were used (Tab. 1). All the accessions used, except the commercial selection Listada Clemente (Semillas Clemente, Vitoria, Spain), and the breeding line LF3-24 (INRA, France) are local landraces from the region of Valencia (Spain). A number of these accessions have been recently characterized by fruit shape (Hurtado *et al*., 2013), and phenolics content (Plazas *et al*., 2013).

For each accession, genomic DNA was extracted from 75 mg of young leaf tissue using the CTAB method (Doyle and Doyle, 1987). DNA concentration was quantified, after electrophoresis on a 1.0% agarose gel, using a Nanodrop ND-1000 (Nanodrop Technologies, Wilminton, Delaware, USA) spectrophotometer. Samples were adjusted to a DNA concentration of 20 ng/µl. The quality of DNA was evaluated through the 260/280 nm and 260/230 nm absorbance ratios (Sambrook *et al*., 1989).

Molecular characterization

Nineteen genomic highly polymorphic SSR markers developed by Vilanova *et al*. (2012) were used to screen the 30 eggplant accessions (Tab. 2). SSRs were tested following the M13-tail PCR method of Schuelke *et al*. (2000) to facilitate the incorporation of a dye label during the PCR. An M13-tailed forward primer was used in combination with a standard M13 primer dye-labeled with FAM, NED, PET, or VIC fluorophores at its 5'-end.

PCR amplifications were performed in a total volume of 12 µl with 20 ng of DNA, 1.5 mM $MgCl_2$, 0.05 µM of forward primer, 0.25 µM of reverse primer, 0.2 µM of fluorescent M-13 primer, 0.2 mM dNTPs, and 0.04 units of Taq DNA polymerase. Amplifications were carried out in an Eppendorf Mastercycler ep gradient S (Eppendorf AG, Hamburg, Germany) thermocycler. Two different protocols for amplification (T or TD) were used depending on the marker (Tab. 2). The T protocol consisted in an initial step at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 1 min at the appropriated annealing temperature (Tab. 2), 72 °C for 2 min, and final 10 min extension at 72 °C. The TD protocol consisted in an initial step at 94 °C for 2 min, 7 cycles of 94 °C for 15 s, 55-48 °C (beginning with 55 °C in the first cycle and reducing 1 °C in each of the subsequent cycles) for 30 s, 72°C for 45 s, and final 10 min extension at 72 °C; subsequently, other 28 cycles are performed with the following conditions: 94 °C for 15 s, 48 °C for 30 s, 72°C for 45 s, and final 10 min extension at 72 °C.

PCR products were separated on an ABI Prism 3100 Avant (Applied Biosystems, Foster City, California, USA) genetic analyzer using GeneScan 3.7 (Applied Biosystems)

Tab. 1. Accessions used in the present work, fruit weight and fruit length/width ratio (mean±SE), grouped according to their varietal group

Accession[a]	Code	Fruit weight	Fruit length / width ratio
Round			
B-31	B31	225±17	1.09±0.05
B-32	B32	346±33	1.63±0.05
V-S-9	VS9	233±18	0.83±0.02
V-S-13	VS13	180±12	0.97±0.03
Listada de Gandía			
07-A25-01	07A	330±26	2.31±0.08
IVIA-025	I025	330±16	1.70±0.06
IVIA-347	I347	300±26	1.35±0.06
Listada Clemente	LC	209±23	2.47±0.07
V-S-1	VS1	239±32	1.59±0.07
V-S-2	VS2	278±15	1.60±0.05
V-S-7	VS7	346±20	1.47±0.06
V-S-8	VS8	288±24	2.36±0.10
V-S-10	VS10	169±12	1.56±0.07
V-S-11	VS11	182±9	1.87±0.05
V-S-15	VS15	228±12	1.86±0.03
Semi-long			
B-33	B33	171±8	2.93±0.07
B-36	B36	176±16	2.99±0.12
V-S-14	VS14	250±16	2.88±0.09
V-S-16	VS16	290±19	2.61±0.35
V-S-17	VS17	228±20	2.99±0.12
V-S-18	VS18	168±14	3.47±0.11
Long			
B-35	B35	218±15	3.98±0.22
LF3-24	LF3	181±13	3.99±0.12
V-S-3	VS3	164±12	3.92±0.13
V-S-4	VS4	206±18	3.84±0.14
V-S-5	VS5	232±19	4.43±0.13
V-S-6	VS6	223±13	4.02±0.13
V-S-12	VS12	200±12	5.36±0.11
V-S-19	VS19	252±15	4.52±0.11
V-S-21	VS21	301±24	3.88±0.23

[a]All accessions are local landraces of the Region of Valencia (Spain), except Listada Clemente, which is a commercial selection (Semillas Clemente, Vitoria, Spain) of the Listada de Gandía type, and LF3-24, which is a breeding line from INRA (France).

Tab. 2. SSR markers used in the present study along with their repeat motif, amplification protocol, annealing temperature, and linkage group in which they map (Vilanova *et al*., 2010, 2012)

SSR locus	Repeat motif	Protocol[a]	Annealing temperature	Linkage group
CSM4	$(GA)_{15}$	TD	55-48	8
CSM7	$(CT)_{10}$	TD	55-48	Unknown
CSM12	$(AG)_{12}$	T	51	6
CSM27	$(GA)_{23}$	T	51	3
CSM29	$(AG)_{17}$	TD	55-48	12
CSM30	$(CT)_{20}$	T	51	9
CSM31	$(AG)_{28}$	T	51	1
CSM32	$(AG)_{23}$	T	51	4
CSM36	$(GA)_{27}$	T	51	9
CSM40	$(CT)_{45}$	T	51	4
CSM43	$(AG)_{14}$	TD	55-48	1
CSM44	$(AG)_{14}$	T	51	3
CSM45	$(AG)_{16}$	TD	55-48	5
CSM52	$(TC)_{12}$	T	50	Unknown
CSM54	$(GA)_{19}$	T	51	9
CSM57	$(CT)_{8}$	T	51	Unknown
CSM62	$(GA)_{27}$	T	51	Unknown
CSM74	$(GA)_{26}$	TD	55-48	12
CSM78	$(CT)_{19}$	TD	55-48	10

[a]See text for technical details of the amplification protocols

software. SSR alleles were precisely sized using GeneScan 500 Liz (Applied Biosystems) molecular size standards with Genotyper 3.7 software (Applied Biosystems).

Data analysis

For each SSR locus, the number of polymorphic alleles (N_a), frequency of the predominant allele (f), and effective number of alleles (N_e) was determined using the PowerMaker software (Liu and Muse, 2005).

The polymorphism information content (*PIC*) was calculated as $PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=i+1}^{n} 2 p_i^2 p_i^2$, where n is the total number of alleles detected, p_i is the frequency of the ith allele, and p_j is the frequency of the jth allele (Botstein *et al*., 1980). Also, the observed heterozygosity (H_o), expected heterozygosity (H_e), calculated as $He = 1 - \sum_{i=1}^{n} p_i^2$, were determined. Nei and Li (1979) genetic similarities were calculated and a neighbor-joining phenogram was built using genetic distances with the PowerMaker software (Liu and Muse, 2005) and plotted using TreeView software (Page, 1996). Genetic distances were also used to graphically represent genetic relationships among accessions by principal coordinates analysis (PCoA) using GenAlEx 6.5 software (Peakall and Smouse, 2012).

Results and discussion

SSR characterization and diversity

The 19 SSR markers could be amplified, but for three of them (CSM36, CSM44, and CSM78) the PCR products could not be successfully resolved. The 16 remaining SSR markers were polymorphic with the exception of CSM32, for which only one allele was detected. A total of 65 alleles were detected for the 15 polymorphic SSR loci, with an average number of alleles/locus (N_a) of 4.33 and a range between two (CSM52) and 11 (CSM31) alleles (Tab. 3).

The frequency of the predominant allele (f_p) ranged between 0.36 (CSM31) and 0.97 (CSM74), although with the exception of the latter SSR locus (CSM74) in all cases the f_p values have been below 0.65 (Tab. 3). The effective number of alleles (N_e) ranged between 1.07 (CSM74) and 4.85 (CSM31), and with the exception of CSM74 and

Tab. 3. SSR polymorphic markers, number of alleles per locus (N_a), frequency of the predominant allele (f), number of effective alleles per locus (N_e), polymorphism information content (*PIC*), observed heterozygosity (H_o), and expected heterozygosity (H_e) in the studied collection of 30 eggplant accessions

SSR locus	N_a	f	N_e	*PIC*	H_o	H_e
CSM4	6	0.43	3.03	0.61	0.04	0.67
CSM7	3	0.55	2.21	0.46	0.00	0.55
CSM12	3	0.50	2.14	0.42	0.00	0.53
CSM27	4	0.57	2.47	0.54	0.00	0.59
CSM29	3	0.56	2.31	0.49	0.00	0.57
CSM30	3	0.54	2.12	0.42	0.00	0.53
CSM31	11	0.36	4.85	0.77	0.00	0.79
CSM40	6	0.39	3.32	0.65	0.00	0.70
CSM43	3	0.59	2.04	0.41	0.00	0.51
CSM45	3	0.52	2.14	0.42	0.00	0.53
CSM52	2	0.58	1.95	0.37	0.00	0.49
CSM54	5	0.37	3.74	0.69	0.00	0.73
CSM57	5	0.63	2.a25	0.52	0.04	0.55
CSM62	6	0.38	3.38	0.65	0.00	0.70
CSM74	2	0.97	1.07	0.06	0.00	0.06
Mean	4.33	0.53	2.60	0.50	0.01	0.57

CSM52, which are the only polymorphic markers with N_a=2 (and therefore N_e<2), all loci had N_e>2 (Tab. 3).

The average value for the *PIC* value of the SSR markers tested was of 0.50, but the *PIC* value of individual SSR markers ranged between 0.07 (CSM74) and 0.77 (CSM31) (Tab. 3). The mean value for the observed heterozygosity (H_o) was very low (H_o=0.01), corresponding to H_o=0.00 values for 13 out of the 15 polymorphic SSR loci and to low values (H_o=0.04) for the two remaining loci (CSM4 and CSM57). Conversely, the mean value for the expected heterozygosity (H_e) was much higher (H_e=0.57), with values for individual SSR loci ranging from 0.06 (CSM74) to 0.79 (CSM31).

Multivariate analyses

The cluster analysis performed showed that the four cultivar groups present a considerable diversity (Fig. 1). However, a clear separation of the Long and Listada de Gandía accessions in different basal branches of the phenogram was observed (Fig. 1). All the Listada de Gandía accessions, except accession VS11, are clustered together in one of the sub-branches of the phenogram. Also, all Long accessions, except B35, are clustered together in another sub-branch in which there are also a Round (B32) and a Semi-Long (B33) accessions. Semi-Long, and particularly, the Round accessions, do not present this pattern of clustering, and are scattered in different branches of the phenogram.

When considering the principal coordinates analysis (PCoA), the first and second principal coordinates account, respectively, for 47 % and 13 % of the total variation. The representation of accessions in the PCoA graph shows that the cultivar groups are diverse, although each of them plot in different parts of the graph (Fig. 2).

In this respect, all Listada de Gandía accessions present positive values of the first coordinate and either low positive or negative values of the second coordinate and plot together, not being intermingled with other cultivar groups. Only the odd VS11 accession plots closer to a Round accession (VS9) than to the other Listada de Gandía accessions. All Long accessions present negative values for the first coordinate and either low positive or negative values for the second coordinate (Fig. 2).

Also, all Semi-Long accessions plot in the same area of

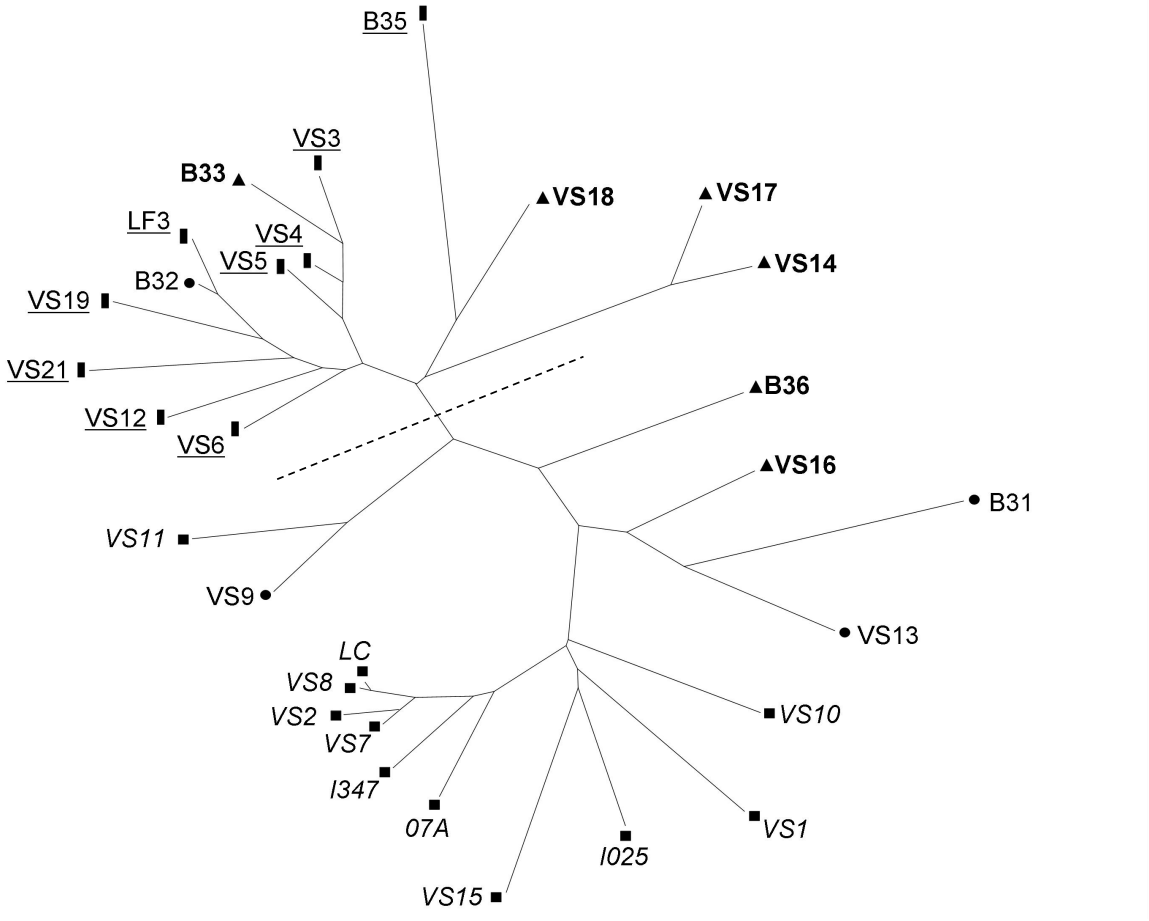

Fig. 1. Unrooted neighbor-joining phenogram of 30 eggplant accessions based on 15 polymorphic SSR markers. Phenetic relationships were derived from genetic distances (Nei and Li, 1979). The different groups of accessions are represented by different symbols and font types: Round (● and normal font); Listada de Gandía (▪ and italics font); Semi-long (▲ and bold font); Long (▌ and underlined font). The dashed line separates the phenogram branches that contain all Long accessions (above) and all the Listada de Gandía accessions

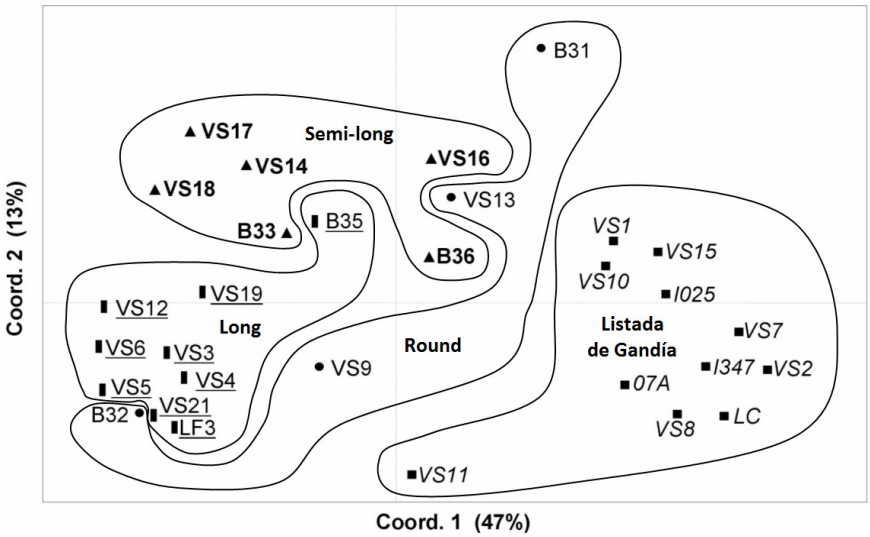

Fig. 2. Relationships between the 30 eggplant accessions based on 15 polymorphic SSRs according to the first and second principal coordinates obtained from a principal coordinates analysis (PCoA) (47% and 13% of the total variation accounted by the first and second coordinates, respectively). The different groups of accessions are represented by different symbols and font types: Round (● and normal font); Listada de Gandía (▪ and italics font); Semi-long (▲ and bold font); long (▮ and underlined font)

the PCoA graph, with either low positive or negative values for the first coordinate and positive values for the second coordinate. Conversely to the other cultivar groups, the four Round accessions are scattered in different parts of the PCoA graph, with two accessions presenting positive values for both the first and second coordinates and situated close to Semi-Long accessions, and two accessions with negative values for both coordinates, with one accession (B32) close to Long accessions and the other (VS9) close to the odd VS11 Listada de Gandía accession (Fig. 2).

Discussion

Genomic SSRs that previously proved to be highly polymorphic in eggplant (Vilanova *et al.*, 2012) have been found to be of great value for evaluating the genetic diversity and relationships in a collection of eggplants from different cultivar groups. The screening of this collection has revealed a high degree of polymorphism for the SSR markers tested, confirming that the Mediterranean region is a secondary center of diversity for eggplant (Cericola *et al.*, 2013; Hurtado *et al.*, 2012; Prohens *et al.*, 2005). Muñoz-Falcón *et al.* (2011) have shown that in eggplant genomic SSR markers are more polymorphic than EST-SSRs. Our results seem to confirm these results and that the genomic SSRs developed from an enriched genomic library by Vilanova *et al.* (2012) are particularly useful for the study of relationships in germplasm collections.

The same SSRs used by us were tested by Vilanova *et al.* (2012) in a collection of 22 *S. melongena* from different origins, including Occidental and Oriental types of eggplant. For the 15 polymorphic loci used in our study these authors found an average number of alleles per locus (6.47) higher than ours (4.33). Hurtado *et al.* (2012) in a study of 52 accessions from China, Spain, and Sri Lanka shared seven of the SSR markers that have been polymorphic in our study and also found a higher average number of alleles per locus (8.86) than we found for these seven SSR markers in our study (4.43). Also, Cericola *et al.* (2013) in a wide study of 238 eggplant materials from different origins shared four SSRs with our study. For these four SSRs, Cericola *et al.* (2013) found on average 7 alleles/locus, while these same alleles in our collection presented a mean of 5.5 alleles/locus.

The higher diversity found by Vilanova *et al.* (2012), Hurtado *et al.* (2012), and Cericola *et al.* (2013) probably is a consequence of the fact that our collection comes from a single geographic area (Region of Valencia, in the Mediterranean coast of Spain). In fact, when considering only the 14 Spanish accessions used in the Hurtado *et al.* (2012) study the number of alleles per locus (3.86) is similar to ours (4.43). Amazingly, for the CSM32 loci, which has been monomorphic in our collection, Hurtado *et al.* (2012) and Vilanova *et al.* (2012) found 12 and 8 alleles, respectively, indicating that selection or genetic drift in the materials of the collection we have evaluated have led to fixation of one specific allele.

SSR loci are highly informative when they present a great number of alleles (N_a), and have low values for the predominant allele frequency (f_p), i.e., with f_p values close to the theoretical minimum of $f_p=1/N_a$ (Botstein *et al.*, 1980; Powell *et al.*, 1996). In our case, some markers have presented a high number of alleles and presented relatively low values for the predominant allele (f_p), resulting in high values for the number of effective alleles (N_e) polymorphic information content (*PIC*), and expected heterozygosity (H_e) confirming that highly relevant information on the diversity and relationships of eggplant can be obtained with a relatively low number of genomic SSR markers (Hurtado *et al.*, 2012; Muñoz-Falcón *et al.*, 2009, 2011).

The low values of observed heterozigosity (H_o) were

expected as eggplant is fundamentally autogamous (Pessarakli and Dris, 2004), and the materials used are non-hybrid. In this respect, Muñoz-Falcón (2009) also found very low values for H_o in eggplant landraces (H_o<0.03), but substantially higher values (H_o=0.38) for commercial F1 hybrids. Cericola *et al*. (2013) also found that most eggplant landraces used in their study had low heterozygosity values, and only 38 out of 238 materials had H_o>0.10. The high level of homozygosity in eggplant landraces shows that pure lines can easily be derived by individual selection from these materials.

The multivariate analysis with SSR markers shows that, as already found by Prohens *et al.* (2005) and Tümbilen *et al.* (2011) with AFLP markers, and by Cericola *et al*. (2013) that considerable genetic diversity exists within each of the cultivar groups studied, which were mostly distinguished by the fruit shape. Also, Muñoz-Falcón *et al.* (2011) using SSR markers also found that one of the groups studied here (Listada de Gandía) was genetically diverse.

The existence of intra-varietal group genetic diversity has important implications for selection and breeding as it indicates that important genetic advances can be obtained with intra-cultivar group selection, and also that hybrids heterotic for yield may be obtained when crossing genetically different accessions of the same cultivar group (Rodríguez-Burruezo *et al.*, 2008).

Here we have found that a certain degree of genetic differentiation exists among the four cultivar groups. This is in agreement with previous reports (Cericola *et al*., 2013; Prohens *et al.*, 2005; Tümbilen *et al*., 2011), in which cultivar groups were found to present a moderate degree of differentiation. However, the Round group accessions were intermingled in the multivariate analyses with accessions of other groups indicating that this cultivar group is genetically highly variable. Wild relatives of eggplant have round, ovoid, or obovoid fruit shape (Knapp *et al.*, 2013), indicating that the fruit shape characteristic of the Round cultivar group (Hurtado *et al.*, 2013) is an ancestral trait probably present in the first domesticated eggplants. As occurred in tomato (Brewer *et al.*, 2007), artificial selection of mutations affecting fruit shape has led to other fruit shapes, like those characteristic of the Listada de Gandía, Semi-Long and Long types, which may have undergone genetic bottlenecks resulting in a lower diversity and higher degree of genetic differentiation. However, further studies should be undertaken to confirm this hypothesis.

In our study, the Long and Listada de Gandía groups are clearly differentiated at the genetic level. In this respect, Muñoz-Falcón *et al.* (2011) and Prohens *et al*. (2005) found that the Listada de Gandía cultivar group was genetically clearly differentiated from the rest of eggplant materials of other types. In our study, only one of the Listada de Gandía accessions used (VS11) seems to be genetically closer to one Round accession (VS9) that to other Listada de Gandía materials. This accession is characterized by an odd shape for the Listada de Gandía type, as it is ovoid instead of having the normal obovoid shape characteristic of the Listada de Gandía materials (Hurtado *et al.*, 2013) and may have been derived from introgression of the striped trait characteristic of Listada de Gandía into a different genetic background.

Conclusions

A reduced number of selected genomic SSR markers have allowed detecting considerable genetic variation in a collection of eggplants including different cultivar types. These markers have also been useful for studying relationships among four cultivar groups differentiated by fruit shape, showing that they are highly homozygous, have considerable intra-varietal group diversity, and present a certain degree of genetic differentiation. In particular the Listada de Gandía and Long varietal groups are clearly differentiated from each other. This information is of interest for the genetic improvement and conservation of genetic resources of eggplant.

Acknowledgements

This research has been partially funded by Ministerio de Economía y Competitividad and FEDER (grant AGL2012-34213) and by Universitat Politècnica de Valencia (grants SP20120681 and PAID-06-11 Nr. 2082).

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