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Inter- and intraspecific differentiation of *Capsicum annuum* and *Capsicum pubescens* using ISSR and SSR markers

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ABSTRACT

Mexico is the second largest producer of *Capsicum* sp. fruit, the main consumer worldwide of *Capsicum*, and the country with the highest genetic diversity of *Capsicum*. Polymorphism was evaluated in two Serrano and two Jalapeño cultivars of *Capsicum annuum* and one cultivar of *Capsicum pubescens*. Cultivar differentiation was performed using molecular characterization with ISSR and SSR markers. Using eight ISSR anchored primers, a total of 38 bands were obtained. Band number varied from 15 to 23 by primer and fragment size from 150 to 6000 bp. Two markers provided polymorphic data. Mean values were 0.77 for PIC, 0.74 for MI and 16.08 for *Rp*. The number of alleles per marker identified using SSR markers in both species ranged from 1 to 10. Average PIC values for the SSR were 0.5. Both techniques were useful in distinguishing the two tested *Capsicum* species. Based on PCA and cluster analysis, both techniques efficiently allowed differentiation of the varieties of *C. annuum* from the *C. pubescens*, and among varieties of *C. annuum*, except one variety of Serrano that was grouped with the Jalapeno ones. SSRs used in this study, originally designed for *C. annuum*, resulted in amplification in *C. pubescens*, which demonstrated their usefulness for this species as well because these markers include genes that preserve the same coding regions.

1. Introduction

Chili pepper (*Capsicum annuum*) belongs to the *Solanaceae* family, and *C. annuum* var. Glabriusculum is considered to be the wild ancestor of cultured *C. annuum*. The taxonomic structure of this genus was based upon the information from numerical taxonomy, cross-fertility, cytogenetics, biochemistry, geography and ethnobotany (Nicolaï et al., 2013). This genus grows preferentially on plains and originated in Central and South America (Ahmed, 2013) and includes approximately 25 species, five of which are cultivated to a greater or lesser degree worldwide (Patel et al., 2011). The domesticated species include *C. annuum*, *Capsicum baccatum*, *Capsicum chinense*, *Capsicum frutescens* and *Capsicum pubescens* (Shirasawa et al., 2013).

In Mexico, the wild species show significant genetic changes when converted from wild to cultured populations, thus losing their relative genetic diversity (Nicolaï et al., 2013). Preserving, managing and improving chili pepper species and varieties require evaluation of their degree of variation based on genetic characteristics.

In *Capsicum*, RFLPs have been used to map the gene responsible for the red and yellow colors CCS (capsanthin–capsorubin synthase), and through induced mutation, it has been observed that its deletion increases the intensity of yellow color in the fruit (Lefebvre et al., 1998). AFLPs have been used to map the gene that determines the pungency (locus C locus of chromosome 2) in parental and F2 populations developed by *C. frutescens* (pungent) × *C. annuum* var. *grossum* (not pungent) (Blum et al., 2003); in addition, they were used to locate the P5 and SRH-SNAP-9 markers related to resistance to *Phytophthora* (Kim et al., 2008). Furthermore, four RAPDs specific for three hybrid varieties and 11 RAPDs have been identified as useful in determining the purity of seeds in hybrid varieties that are useful for routine quality control of seeds (Ilbi, 2003) strains. By using RAPDs, it was determined that separation between wild and domesticated populations of *C. annuum* in

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Table 1
Locations and conditions used for the development of *Capsicum* spp. evaluated in this study.

Specie	Code	Planting conditions	Location	Coordinates	Date of planting-harvesting
C. annuum	JN[a,x]	Open field	Ixcuintla, Nayarit	Lat.: 21°48′47.47″N Long.: 105°12′15.58″W	October 2011–February 2012
	SQ[b,x]		El Marques, Querétaro	Lat.: 20°35′0.95″N Long.:	March–June 2012
	JQ[a,x]			100°22′22.62″W	
	SG[b,x]		Celaya, Guanajuato	Lat.: 20°30′53.31″N Long.:	
	JG[a,x]			100°48′53.55″W	
	SE[b,x]	Greenhouse	Chapingo, Estado de	Lat.: 19°25′10″N Long.:	
	JE[a,x]		México	99°7′60″W	
	SS[c,x]	Open field	Guasave, Sinaloa	Lat.: 25°34′0.12″N Long.: 108°28′2.3″W	October 2011–February 2012
	JS[d,x]		Sinaloa de Leyva, Sinaloa	Lat.: 25°49′21.67″N Long.: 108°13′18.01″W	
C. pubescens	ME[e,l]	Greenhouse	Chapingo, Estado de México	Lat.: 19°25′10″N Long.: 99°7′60″W	May–November 2011
	MV[e,l]		Villa Guerrero, Estado de México	Lat.: 18°57′47.18″N Long.: 99°38′12.91″W	April–September 2012
	MP[e,l]		Chignahuapan, Puebla	Lat.: 19°49′60″N Long.: 98°1′60″W	

The combination of the letters denotes the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

northern Mexico occurred, which suggests that artificial selection produces genetic changes associated with domestication (Oyama et al., 2006). Additionally, with RAPDs, it has been possible to distinguish between pungent and non-pungent fruits, while AFLPs was efficient in detecting variability (Paran et al., 1998).

By using COSII, 299 orthologous markers between pepper and tomato were mapped, thus building the first complete genetic map comprising 12 linkage groups (Wu et al., 2009). In the recent publication on the full genome of chili (Kim et al., 2014), 17,397 sets of orthologous genes were identified, comparing chili and tomato genomes, and based on these genes, the speciation time was estimated (19.1 billion years); moreover, all orthologous genes of the route of the capsaicinoids were identified by homology, microsynteny and previous reports. Regarding CAPS markers, these have been developed for recessive alleles of viral resistance used for improving chili (*Capsicum*), for instance to identify the locus Pvr4 as a tool for pyramiding potyvirus resistance genes (Caranta et al., 1999).

SSRs are classified into genomic SSRs and EST-SSRs; the former are designed from the complete genome and EST-SSRs from the transcription of mRNA sequences. In general, EST-SSRs show greater transferability between species/genera because the coding regions of genes are more suitable to be preserved among related species/genus (Shirasawa et al., 2013).

Among all molecular markers, ISSRs and SSRs were found to be highly polymorphic and informative (Sestili et al., 2008); they were also complementary because ISSRs is dominant and SSR is codominant. A number of studies have used microsatellites (SSR) in *C. annuum* to characterize and generate a molecular genetic map of SSR loci (Lee et al., 2004; Minamiyama et al., 2006; Portis et al., 2007); to study genetic diversity (Aguilar-Melendez et al., 2009; Contreras-Toledo et al., 2011; Hanáček et al., 2009; Rai et al., 2013); or to design SSR primers that are transferable between *Capsicum* species (Ince et al., 2009, 2010). In *C. annuum*, ISSR have been used to analyze genetic polymorphism (Ahmed, 2013; Ruanet et al., 2005), to generate molecular profiles and determine genetic variability (Kumar et al., 2001; Thul et al., 2012) and to conduct molecular studies of changes in fruit shape induced by grafts (Tsaballa et al., 2013). The main goal of the present study was to use ISSR and SSR markers to differentiate between *C. annuum* and *C. pubescens* species and among Jalapeño and Serrano varieties of *C. annuum* species.

2. Materials and methods

2.1. Plant material

Four hybrid *C. annuum* varieties were used: Jalapeño var. Hulk and Serrano var. RSS-C36 (Agroseeds Chicuate S. de R.L); and Jalapeño var. Ahutlán and Serrano var. San Tomás (Seminis Vegetable Seeds, Inc., St. Louis, Missouri, USA) (Table 1).

One *C. pubescens* variety was used: Manzano var. Grajales ST (Universidad Autónoma de Chapingo, Estado de México, Mexico). *C. annuum* seeds were planted directly in open fields in all regions except in Chapingo, where they were started in greenhouses (35–40 °C). *C. pubescens* seeds were cultivated under greenhouse conditions (18–25 °C) in all considered regions. Seeds were germinated and then transplanted to field conditions at 50-cm intervals. Plants grown under greenhouse conditions were transplanted to black polyethylene bags (40 cm wide; 45 cm high); each bag contained 50% red volcanic (tezontle) gravel in the lower part and 25% Growing Mix and 25% fine red volcanic (tezontle) sand in the upper part.

2.2. Marker development

Extraction of DNA and molecular analysis were performed using young leaves. These were first decontaminated by soaking in ddH_2O, 30% NaClO and 10% NaClO for 5 min each, followed by 70% ethanol and finally sterile ddH_2O for 5 min. The sterilized leaves were then stored at −20 °C until use (Troconis-Torres et al., 2012).

2.2.1. DNA extraction

Genomic DNA was extracted using a Plant DNAzol® kit (Cat no. 10978-021, Invitrogen, Life Technologies Corp., Carlsbad, California) following manufacturer's instructions. The DNA was quantified at 260 nm and its purity measured at a 260/280 nm absorbance ratio. All measurements were conducted with a Nanodrop™ 1000 V3.7 spectrophotometer (Thermo Fisher Scientific, Silverside Road, Bancroft Building, Suite 100, Wilmington, Delaware 19810, U.S.A.), although DNA quantity was also estimated in 1% agarose gels.

2.2.2. ISSR analysis

Eight anchored ISSR primers synthesized by IDT (Integrated DNA Technologies, Coralville, Iowa, USA) were used. These anchored primers have an extended portion of bases in the 5′ or 3′ end of their sequence, to increase the specificity of the amplicon (Table 2). Total volume for the PCR reactions was 25 μL, containing Taq DNA Polymerase buffer with added 25 mM $MgCl_2$ 1×, 200 μM dNTPs, 1.5 U GoTaq DNA Polymerase (PROMEGA), 60 ng genomic DNA and 20 pM primer. Amplification was performed with a Geneamp PCR system 9700 thermocycler (Applied Biosystems, 5791 Van Allen Way, PO Box 6482, Carlsbad, California, USA) following a thermocycle of one cycle at 94 °C for 1 min; 35 cycles [94 °C for 30 s; 59 °C for 30 s; 72 °C for 1.5 min]; and a final extension of 72 °C for 5 min. Amplification products were separated in 5% polyacrylamide gels (Sambrook et al., 1989), using a 1 kb molecular

Table 2
Characteristics of the ISSR primers used for genotyping analysis of *Capsicum* spp.

Primer	Short sequence	Extended sequence (5′–3′) R = (A,G); Y = (C,T)	Number of bases
Iso_1	$AC(GACA)_4$	ACGACAGACAGACAGACA	18
Iso_2	$(AC)_8$ YG	ACACACACACACACACYG	19
Iso_3	$(AGAC)_4$ GC	AGACAGACAGACAGACGC	18
Iso_6	$(GA)_8$ YC	GAGAGAGAGAGAGAGAY	19
T	$(GAGA)_4$ YC	GAGAGAGAGAGAGAGAYC	19
U	$(CA)_8$ RT	CACACACACACACACART	19
W	$(GACA)_4$ GT	GACAGACAGACAGACAGT	18
X	$(TCC)_5$ RY	TCCTCCTCCTCCTCCRY	19

weight marker (O'GeneRuler, Thermo Scientific) as reference. Gels were stained with 0.2% $AgNO_3$ following a conventional protocol (Valadez-Moctezuma and Kahl, 2000), and photographed with a visible light transilluminator. Both the PCR reactions and gel separations were performed in triplicate to confirm reproducibility.

2.2.3. SSR analysis

Using multiple PCR, 24 microsatellite loci were amplified (Lee et al., 2004). Primers were marked with 6-FAM or HEX florescent labels (Applied Biosystems) on the 5′ terminal to allow for their detection in a Genetic Analyzer 3130® capillary sequencer (Applied Biosystems). In a previous study and to streamline the SSR analysis by using a sequencer, groups of the same SSR primers used in this study were evaluated and found as capable of amplifying their respective marker more efficiently in the same reaction mixture of PCR (Contreras-Toledo et al., 2011). However, some markers were analyzed individually, as they have different T_M conditions (alignment temperature). Data were recorded for locus name, repeated unit, sequence, product size, T_M, total alleles and polymorphic information content (PIC) (Table 5). Multiple PCR amplification was performed using a 25 μL reaction mixture containing 0.2 mM nucleotides, 4 mM $MgCl_2$, 1.6× buffer, 40 ng DNA, 1 U Taq DNA polymerase and 15 pmol of each primer. The thermocycle was one cycle at 94 °C for 4 min; 35 cycles [94 °C for 60 s; 65 °C for 1 min; 72 °C for 2 min] and a final extension at 72 °C for 12 min. The PCR products were analyzed with the GeneMapper® V. 4.0 program (Applied Biosystems).

2.2.4. ISSR data analysis

Each ISSR band was treated as an independent locus and visually codified as having polymorphics absent ("0") or present ("1") for each sample; only consistent bands were used in the analysis. Differences in band intensity between different sample profiles were not considered. The resulting molecular data were used to generate a cluster analysis. Genetic similarity between accessions was calculated using the DICE similarity coefficient (Dice, 1945) with the NTSYSpc ver. 2.2 software (Applied Biostatistics, Inc.). Clustering was conducted using the UPGMA (Unweighted Pair Group Method using Arithmetic average) algorithm. The PIC, Marker Index (MI) and resolution power (*Rp*) of each primer were calculated because they are widely used in studies with molecular markers and allow for comparison of the information provided by the latter, such as polymorphic content and their capability of distinguishing between genotypes (*Rp* y MI). The PIC value was calculated with the formula $PIC_i = 1 - \Sigma\,(P_{ij})^2$ (Qiu et al., 2013), where P_{ij} is the *i*th band shown by the *j*th primer added for all of the bands shown by the primers. The MI of each ISSR primer was calculated as MI = PIC × ηβ, where PIC is the mean PIC value, η is the number of bands and β is the proportion of polymorphism. The *Rp* value was calculated with the formula $Rp = \Sigma I_b$, where I_b represents band informativeness as calculated with the formula $I_b = 1 - (2 \times |0.5 - p|)$, where *p* is the proportion of bands containing band I (Prevost and Wilkinson, 1999). A principal components analysis (PCA) was also run to locate the markers to which the Manzano, Jalapeño and Serrano cultivar groups were attributed. This was run based on the correlations matrix generated from the allele frequencies using the Statgraphics Centurion XVI ver. 16.1.11 program (StatpointTechnologies, 2009).

2.2.5. SSR data analysis

Using the POPGENE program (Yeh et al., 1999), the number of alleles per locus for each individual was counted to generate population allele frequencies and the proportion of polymorphic loci. In addition, a PCA was run based on the correlations generated from the allele frequencies, using Statgraphics Centurion XVI ver. 16.1.11 (StatpointTechnologies, 2009). As a complement, a grouping analysis was performed with the NTSYSpc® V.2.1 program (Rohlf, 2004), using the Rogers genetic distance modified by Wright (1978) and the UPGMA cluster method. The PIC was calculated for each SSR locus based on its allele frequency, using the formula $PIC_i = 1 - \sum P_{ij}^2$.

3. Results

3.1. ISSR analysis

Of the eight evaluated ISSR primers, Iso_1 and Iso_2 were selected because they showed amplification product sizes ranging from 150 to 6000 bp; the primers Iso_3, Iso_6, T, U and W showed lower molecular weights products. The results obtained with these primers, such as annealing temperature (TM), total bands (TB), polymorphic bands (PB), range of the amplification product (bp), percentage of polymorphism (%*P*), polymorphic information content (PIC), marker index (MI) and resolving power (*Rp*) are shown in Table 3. Primer Iso_1 amplified 23 total bands, and product size ranged from 150 to 5000 bp. Of these, 21 were polymorphic bands with 91.3% polymorphism; other values were 0.68 (PIC), 0.62 (MI) and 11.67 (*Rp*). Primer Iso_2 amplified 15 total bands, and product size ranged from 150 to 5000 bp, all of which were 100% polymorphic, with a product size range of 150 to 6000 bp; other values were 0.86 (PIC), 0.86 (MI) and 20.50 (*Rp*). Together, these two primers amplified 38 polymorphic bands in the 12 tested samples. Taken together, the band number for the two primers ranged from 15 to 23 and fragment size from 150 to 6000 bp. Mean values were 0.77 for PIC, 0.74 for MI and 16.08 for *Rp*. Of the two, Iso_2 was more informative than Iso_1 in terms of polymorphism.

Primer Iso_2 provided visible monomorphic bands at 500, 750 and 1000 bp (Fig. 1). We can observe in the Serrano samples bands approximately 5000 bp that are not present in the samples of Jalapeño.

Similarity coefficients were calculated using the DICE coefficient based on the Iso_1 and Iso_2 markers (Fig. 2, Table 4). Coefficients for *C. pubescens* ranged from 0.63 to 0.79, with proximity between $MV^{e,l}$ and $MP^{e,l}$ (0.79) and lesser genetic similarity between $MV^{e,l}$ and $ME^{e,l}$ (0.63). Values for *C. annuum* ranged from 0.44 to 0.94, with greater similarity between $SG^{b,x}$ and $SQ^{b,x}$ (0.94) and less between $JS^{d,x}$ and $JE^{a,x}$ (0.44). Among the Serrano and Jalapeño varieties, similarity values ranged from 0.50 to 0.83, with more similarity

Table 3
Polymorphic information of *Capsicum* spp., with primers ISO_1 and ISO_2.

Primer	T_M (°C)	TB	PB	Product size (bp)	%P	PIC	MI	Rp
Iso_1	59	23	21	150–5000	91.3	0.68	0.62	11.67
Iso_2	59	15	15	150–6000	100	0.86	0.86	20.50
Average	–	38	36	150–6000	95.65	0.77	0.74	16.08

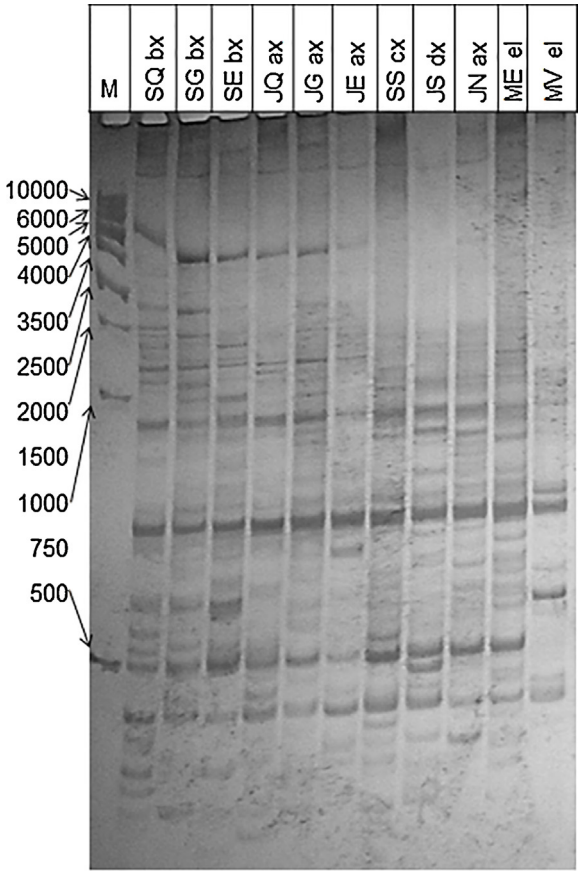

Fig. 1. ISSR profiles detected with Iso_2 primer, sequence $(AC)_8$ YG in *Capsicum* spp. Lane M corresponds to 1 kb molecular weight marker. The combination of the letters denotes the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

between $JG^{a,x}$ and $SG^{b,x}$ (0.83) and less between $SG^{b,x}$ and $SQ^{b,x}$, and $JS^{d,x}$ (0.50). The similarity index ranges for *C. pubescens* (0.63–0.79), *C. annuum* (0.44–0.94) and Jalapeño–Serrano (0.50–0.83) indicated overall high similarity and low diversity among the tested cultivars.

Principal components analysis (PCA) of the binary matrix of 38 bands for *Capsicum* spp. primers Iso_1 and Iso_2 showed the first two components explained 47.84% of the variability in the genotypes. Weights for the markers (PC1) and component 2 (PC2) indicated the Serrano cultivars ($SE^{b,x}$, $SG^{b,x}$, $SQ^{b,x}$) to be associated with markers Iso2_m, Iso1_f, Iso1_b, Iso2_k, Iso2_l, Iso2_i and Iso2_p (Fig. 3). The Manzano cultivars ($Mp^{e,l}$ and $MV^{e,l}$) were associated with Iso1_m and Iso1_f, while the Jalapeños ($JG^{a,x}$, $JE^{a,x}$ and $JQ^{a,x}$) associated with Iso1_u, Iso2_b and Iso2_a. Marker Iso1_n, associated with both Jalapeños ($JS^{d,x}$, $JN^{a,x}$) and Serranos ($SS^{c,x}$), was situated in the same component.

3.2. SSR analysis

Twenty-four SSR markers were evaluated to identify the genetic profile of twelve *Capsicum* spp. cultivars. These markers produced polymorphic and monomorphic alleles; the results presented (Table 5) are according to the amplification profiles of different markers; they include the following:

(1) AF039662 produced two alleles (A and B) with 112–122 bp. These two alleles were designated as A and B. Allele A (112) was observed in nine cultivars ($JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$) and allele B (122) in all twelve cultivars ($ME^{e,l}$, $MV^{e,l}$, $MP^{e,l}$, $JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$). The PIC value was 0.50.
(2) CM0005 also produced two alleles (A and B) with 159–162 bp. Allele A (159) was present in nine cultivars ($JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$) and allele B (162) in three ($ME^{e,l}$, $MV^{e,l}$ and $MP^{e,l}$). The PIC value was 0.50.
(3) CAN130829 produced two alleles (A and B) with 184–192 bp. Allele A (184) was observed in nine cultivars ($JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$) and allele B in three cultivars ($ME^{e,l}$, $MV^{e,l}$ and $MP^{e,l}$). The PIC value was 0.50.
(4) HpmS1_6 produced two alleles (A and B) with 183–184 bp. Allele A was present in two cultivars ($ME^{e,l}$, $MV^{e,l}$) and allele B in nine ($JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$). The PIC value was 0.66.
(5) HpmS 2_13 produced two alleles (A and B) with 232–253 bp. Allele A (232) was observed in all twelve cultivars ($ME^{e,l}$, $MV^{e,l}$, $MP^{e,l}$, $JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$) and allele B (253) in nine ($JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$). The PIC value was 0.71.
(6) CAN010950 produced two alleles (A and B) with 259–276 bp. Allele A (259) was present in nine cultivars ($JQ^{a,x}$, $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JS^{d,x}$ and $JN^{a,x}$) and allele B (276) in three ($ME^{e,l}$, $MV^{e,l}$ and $MP^{e,l}$). The PIC value was 0.54.
(7) HpmS1_172 produced ten alleles (A–J) with 321–345 bp, which were designated as A, B, C. D, E, F, G, H, I and J. All ten alleles were present in no more than four cultivars: A (321) was present in $ME^{e,l}$, $MV^{e,l}$ and $MP^{e,l}$; B (323) was present only in $MV^{e,l}$; C (324) was present only in $MP^{e,l}$; D (329) was present only in $ME^{e,l}$; E (335) was present in $JG^{a,x}$ and $SS^{c,x}$; F (339) was present only in $SG^{b,x}$; G (340) was present in $JQ^{a,x}$, $JE^{a,x}$, $SQ^{b,x}$ and $JN^{a,x}$; H (343) was only present in $JQ^{a,x}$; I (344) was present in $JE^{a,x}$, $JG^{a,x}$, $SQ^{b,x}$, $SG^{b,x}$; and J (345) was present in $JQ^{a,x}$ and $SS^{c,x}$. The PIC value was 0.91.

The 24 SSR markers produced a total of 118 alleles. The amplified fragments varied in size from 100 bp (HpmS 1_214) to 344 bp (HpmS 1_172). All 118 fragments resulted in polymorphic profiles among the twelve cultivars. The mean PIC value was 0.77; the highest was 0.91 (HpmS1_172) and the lowest was 0.50 (AF069362, CM0005 and CAN130829).

The genetic distances were calculated based on the 24 SSR markers and using the Rogers coefficient modified by Wright. For *C. pubescens*, the range was from 0.32 to 0.40 (Table 6), with a short distance between $MV^{e,l}$ and $MP^{e,l}$ (0.32) and less similarity between $ME^{e,l}$ and $MP^{e,l}$ (0.40). The genetic distance values within *C. annuum* were within a range of 0.40 to 0.59, with $SQ^{b,x}$ and $JQ^{a,x}$ being close (0.40) and $SS^{c,x}$ and $JE^{a,x}$ more distant (0.59). The range values for the Serrano cultivars were 0.41 to 0.53, with more similarity between $SQ^{b,x}$ and $SG^{b,x}$ (0.41) and less between $SS^{c,x}$ and $SG^{b,x}$ (0.53).

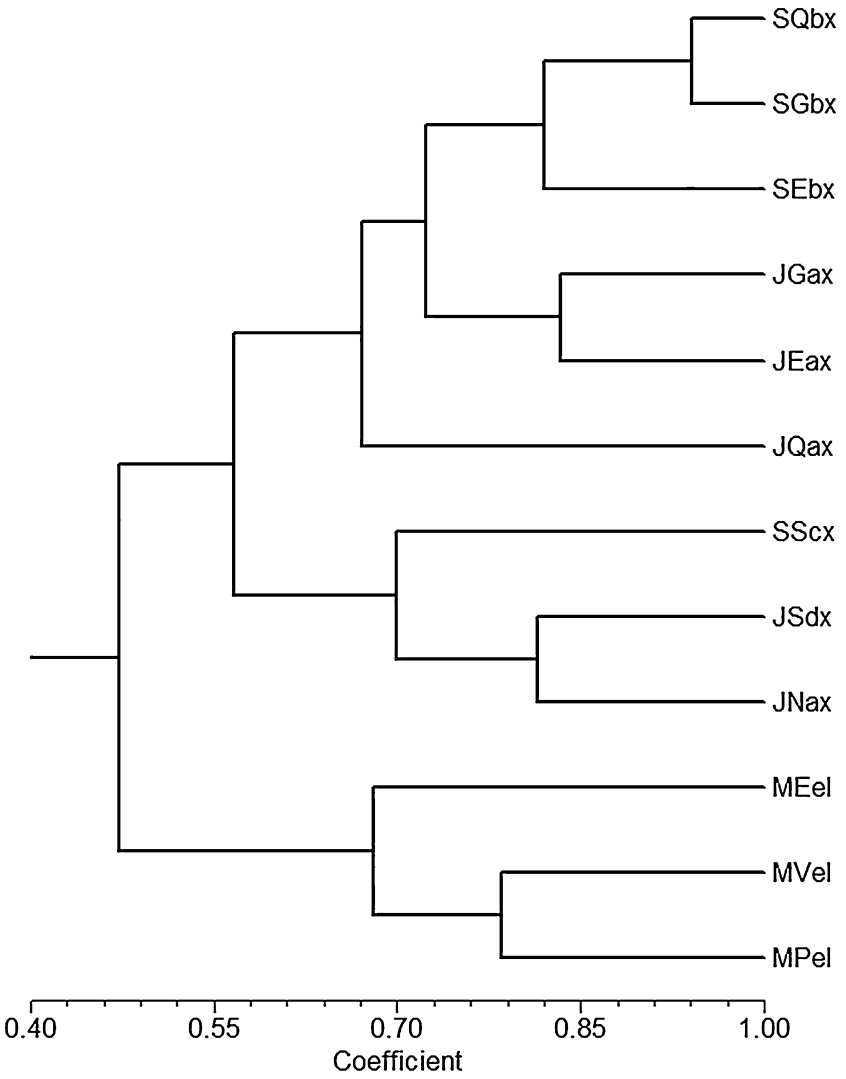

Fig. 2. Dendrograms obtained with primers Iso_1 (AC(GACA)$_4$) and ISO_2 ((AC)$_8$ YG). using the DICE coefficient for clustering of species. The combination of the letters denote the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

Table 4
Similarity matrix for leaf samples of *Capsicum* spp., based on ISSR markers.

	SQ^{b,x}	SG^{b,x}	SE^{b,x}	SS^{c,x}	JS^{d,x}	JN^{a,x}	JQ^{a,x}	JG^{a,x}	JE^{a,x}	ME^{e,l}	MV^{e,l}	MP^{e,l}
SQ^{b,x}	1											
SG^{b,x}	0.94	1										
SE^{b,x}	0.82	0.82	1									
SS^{c,x}	0.57	0.64	0.48	1								
JS^{d,x}	0.5	0.5	0.54	0.62	1							
JN^{a,x}	0.62	0.62	0.53	0.78	0.81	1						
JQ^{a,x}	0.69	0.69	0.65	0.54	0.6	0.52	1					
JG^{a,x}	0.76	0.83	0.65	0.7	0.52	0.58	0.67	1				
JE^{a,x}	0.76	0.76	0.59	0.7	0.44	0.58	0.67	0.83	1			
ME^{e,l}	0.54	0.59	0.57	0.57	0.51	0.5	0.46	0.5	0.44	1		
MV^{e,l}	0.36	0.43	0.3	0.64	0.38	0.43	0.31	0.43	0.35	0.63	1	
MP^{e,l}	0.41	0.47	0.46	0.64	0.56	0.62	0.44	0.41	0.41	0.73	0.79	1

The combination of letters denote the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

The values for the Jalapeños were 0.37 to 0.57, showing a close genetic relationship between JN^{a,x} and JS^{d,x}, and distance between JS^{d,x} with JQ^{a,x} and JE^{a,x}.

The PCA for the allele frequencies of the 118 alleles detected for the 24 SSR loci used in the tested *Capsicum* spp. showed the three primary components accounted for 53.03% of total marker variability (Fig. 4). Principal component 1 (PC1) explained 33.72% of data variability and component 3 (PC3) explained 8.77%. The PC1 separated the Manzano from the Serrano and Jalapeño cultivars, and its positive axis was influenced by 17 alleles (AF244121 A, HpmS 1_148 E, AF039662 A, CM0005 A, CAN130829 A, HpmS 1_5 C, HpmS 1_168 F, HpmS 2_24 C, HpmS 1_111 A, CAN010950 A,

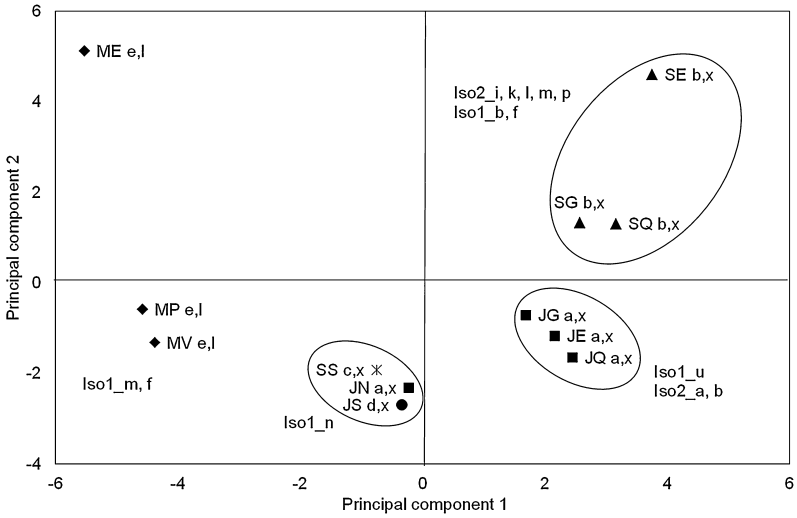

Fig. 3. Principal component analysis based on the two first principal components derived from 38 bands amplified by two primers ISSR among 12 pepper landraces. The combination of the letters denote the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

Table 5
Characteristics of SSR primer* evaluated in *Capsicum* spp.

Group	Locus	Repeat	Primer (forward/reverse)	Size (pb)	T_M (°C)	Total alleles	PIC
1	HpmS 1-62	$(TG)_{23}$ $(AG)_9$	6-FAM-catgaggtctcgcatgatttcac/ggagaaggaccatgtactgcagag	188–210	55	4	0.83
	AF244121	$(TTG)_5$† $(AT)_6$† $(GT)_3$†	6-FAM-tacctcctcgccaatccttctg/ttgaaagttctttccatgacaacc	222–246	50	6	0.84
	HpmS 1_148	$(GA)_{14}$	HEX-ggcggagaagaactagacgattagc/tcacccaatccacatagacg	183–194	55	6	0.84
	HpmS CASIG19	$(CT)_6$ $(AT)_8$ $(GTAT)_5$	HEX-catgaatttcgtcttgaaggtccc/aagggtgtatcgtacgcagcctta	222–254	55	4	0.77
2	HpmS 1_1	$(CA)_{12}$ $(TA)_4$	6-FAM-tcaacccaatattaaggtcacttcc/ccaggcggggattgtagatg	260–276	50	7	0.87
	HpmS 1_274	$(GTT)_7$	HEX-tcccagacccctcgtgatag/tcctgctccttccacaactg	170–180	55	5	0.8
	AF039662	T_{26}†	6-FAM-ccccctcgtctctctttattt/ttgcaaatcttttgtcaattttt	112–122	50	2	0.5
	CM0005	$(CCA)_8$	6-FAM-catgaccaccatgaggata/gatagccacgagcatagtatt	159–162	50	2	0.5
3	HpmS 1_5	$(AT)_{11}$ $(GT)_{17}$	6-FAM-ccaaacgaaccgatgaacactc/gacaatgttgaaaaaggtggaagac	284–316	55	4	0.79
	HpmS 1_172	$(GA)_{15}$	HEX-gggtttgcatgatctaagcatttt/cgctggaatgcattgtcaaaga	321–345	55	10	0.91
	HpmS 1_168	$(TA)_{17}$ $(GA)_{12}$	HEX-gccccgatcaatgaatttcaac/tgatttttgggtggagagaaaacc	167–177	55	6	0.83
	CAN130829	T_{16}	6-FAM-gctaattacttgctccgttttg/aatgggggagtttgttttgg	184–192	55	2	0.5
4	HpmS 2_24	$(CT)_{17}$ $(CA)_5$ A_{21}	6-FAM-tcgtattggcttgtgatttaccg/ttgaatcgaatacccgcaggag	183–204	55	6	0.87
	HpmS 2_21	$(AT)_{11}$ $(AC)_9$ $(ATAC)_{10}$	HEX-tttttcaattgatgcatgaccgata/catgtcattttgtcattgatttgg	286–304	50	4	0.87
	HpmS 1_111	$(AAT)_{11}$	HEX-aagcttatccctttcaaatataa/atatctcacgtattgcggattctt	151–184	50	8	0.88
7	HpmS 1_214	$(GTTT)_2$ $(TTG)_9$	6-FAM-tgcgagtaccgagttctttctag/ggcagtcctgggacaactcg	88–100	50	7	0.85
y	HpmS AT_214	$(AAT)_{16}$†	6-FAM-tttagggtttccaactcttcttcc/ctaaccccaccaagcaaaacac	175–187	50	6	0.83
9	HpmS 2_2	$(GT)_9$	6-FAM-gcaaggatgcttagttgggtgtc/tcccaaaattaccttgcagcac	146–156	55	6	0.83
	HpmS1_6	$(AT)_2$ $(GT)_4$ $(AT)_8$ $(GT)_{13}$	HEX-tccataacttcacccatgagtatga/gcaacacccacattcccttctc	183–184	55	2	0.66
	HpmS 1_106	$(AAAAAT)_4$	HEX-tccaaactacaagcctgcctaacc/ttttgcattattgagtcccacagc	151–163	55	3	0.69
8	HpmS 2_13	$(AC)_{12}$ $(AT)_4$	HEX-tcacctcataagggcttatcaatc/tccttaaccttacgaaaccttgg	232–253	50	2	0.7
y	CAN010950	$(TA)_9$	6-FAM-gattttggtggcagaagaattgg/tgcactttcgaagcaaacaaacc	259–276	55	2	0.54
10	HpmS 1_173	$(GA)_{16}$ $(TG)_2$	HEX-tgctgggaaagatctcaaaagg/atcaaggaagcaaaccaatgc	152–172	50	7	0.86
	HpmS 1_143	$(AG)_{12}$	6-FAM-aatgctgagctggcaaggaaag/tgaaggcagtaggtggggagtg	221–228	55	6	0.83

† Imperfect repeat.
* Lee et al. (2004).

Table 6
Genetic distances matrix of *Capsicum* spp., with SSR markers.

	$SQ^{b,x}$	$SG^{b,x}$	$SE^{b,x}$	$SS^{c,x}$	$JS^{d,x}$	$JN^{a,x}$	$JQ^{a,x}$	$JG^{a,x}$	$JE^{a,x}$	$ME^{e,l}$	$MV^{e,l}$	$MP^{e,l}$
$SQ^{b,x}$	0											
$SG^{b,x}$	0.41	0										
$SE^{b,x}$	0.52	0.51	0									
$SS^{c,x}$	0.52	0.53	0.51	0								
$JS^{d,x}$	0.52	0.55	0.49	0.53	0							
$JN^{a,x}$	0.43	0.44	0.52	0.52	0.55	0						
$JQ^{a,x}$	0.40	0.46	0.55	0.56	0.57	0.44	0					
$JG^{a,x}$	0.50	0.51	0.57	0.56	0.55	0.52	0.56	0				
$JE^{a,x}$	0.48	0.43	0.51	0.59	0.57	0.37	0.51	0.47	0			
$ME^{e,l}$	0.80	0.80	0.78	0.84	0.71	0.82	0.81	0.82	0.84	0		
$MV^{e,l}$	0.79	0.80	0.77	0.81	0.68	0.82	0.80	0.79	0.82	0.34	0	
$MP^{e,l}$	0.76	0.77	0.74	0.78	0.67	0.79	0.77	0.76	0.79	0.40	0.32	0

The combination of the letters denote the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

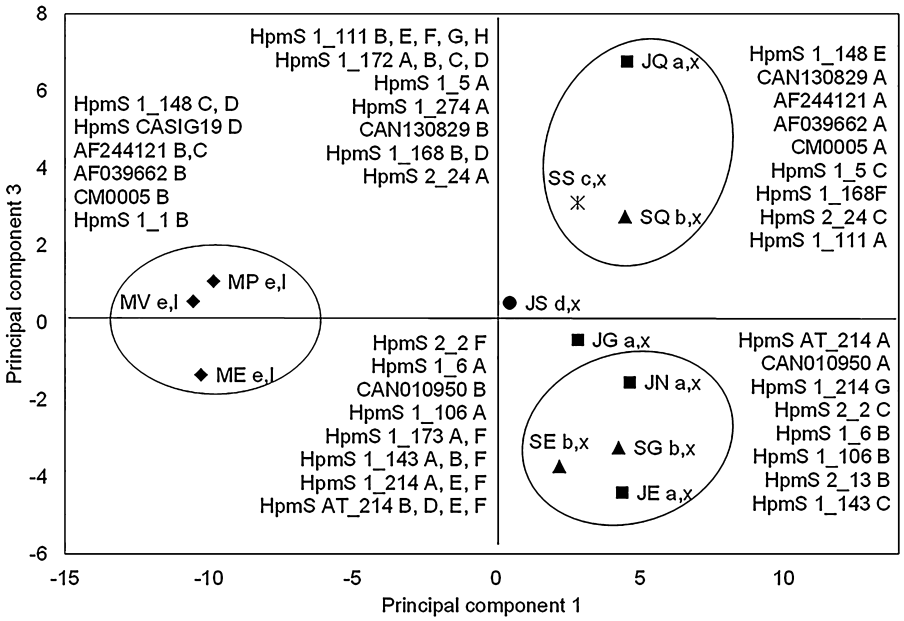

Fig. 4. Principal component analysis based on the three first principal components derived from 118 alleles amplified by 24 loci SSR among 12 pepper landraces. The combination of the letters denote the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

HpmS 1_214 G, HpmS AT_214 A, HpmS 2_2 C, HpmS 1_6 B, HpmS 1_106 B, HpmS 2_13 B and HpmS 1_143 C). These alleles were associated with *C. annuum* cultivars ($JS^{d,x}$, $JN^{a,x}$, $SQ^{b,x}$, $JE^{a,x}$, $SG^{b,x}$, $SE^{b,x}$, $SS^{c,x}$, $JG^{a,x}$ and $JQ^{a,x}$). In its negative axis, PC1 grouped 35 alleles (AF244121 B, C; HpmS 1_148 C, D; HpmS CASIG19 D; AF039662 B; CM0005 B; HpmS 1_1 B; HpmS 1_274 A; CAN130829 B; HpmS 1_168 B; HpmS 1_172 A, B, C, D; HpmS 2_24 A; HpmS 1_111 B, E, F, G, H; HpmS 1_214 A, E, F; HpmS AT_214 B, D, E, F; HpmS 2_2 F; HpmS 1_6 A; HpmS 1_106 A; HpmS 1_173 A, F; HpmS 1_143 A; HpmS 1_143 B, F; and CAN010950 B), all identified in *C. pubescens* cultivars ($MV^{e,l}$, $MP^{e,l}$ and $ME^{e,l}$).

4. Discussion

In the most recent decades, researchers have shown that PCR-based techniques (SSR and ISSR) are effective for studying the relationship or diversity between different species. They have been used primarily to study similarity between different varieties of *Capsicum*. Additionally, molecular markers such as RAPDs have been used to estimate the genetic diversity of *Capsicum* in Mexico, considered as the primary center of origin of this genus (Votava and Bosland, 2001). In the present study, we found that both ISSR and SSR markers were useful in differentiated and characterized samples of two chili pepper species and two cultivars of *C. annuum* (Figs. 2 and 5). Both markers detected polymorphic and monomorphic alleles amplified from *C. annuum* and *C. pubescens*. The genetic distances shown in the SSR analysis identified interspecific differences between *C. annuum* and *C. pubescens* but could not make intraspecific distinctions between the Jalapeño and Serrano varieties of *C. annuum*. Taking as a basis the PCA and the cluster analysis with ISSR and SSR markers, both species were clearly separated. The separation of *C. pubescens* varieties from *C. annuum* is probably due to *C. annuum*, *C. baccatum*, *C. chinense* and *C. frutescens* showing interspecific compatibility to each other, unlike *C. pubescens* (Shirasawa et al., 2013). The cluster analysis with ISSR shows a separation. In the cluster analysis with ISSR within varieties of *C. annuum*, Jalapeno and Serrano peppers were separated with 0.56 similarity coefficient, except for a sample of Serrano chili ($SS^{c,x}$) that was gathered with samples of Jalapeño chili ($JN^{a,x}$ y $JS^{d,x}$). This behavior is related to the genomic similarity between *C. annuum* L. and *C. chinense*, recently reported (Kim et al., 2014), which is based on the sequencing of the entire genome of chili. The PCA helped to locate the ISSR markers corresponding to the Jalapeño, Serrano and Manzano clusters (Fig. 3). These markers may be useful in future studies to design specific ISSR primers for intraspecific differentiation between Jalapeño and Serrano varieties of *C. annuum*. Of note is that the Jalapeño cultivars from the states of Sinaloa (cv. Ahutlan) and Nayarit (cv. Hulk) were grouped by the Iso1_n marker. By using AFLPs in *Capsicum* accessions, loose groupings based on their geographic location, with accessions from southern India near each other (Gaikwad et al., 2001), have been observed. In studies using ISSR primers, an interspecific separation of *C. annuum* varieties with respect to *C. baccatum*, *C. chinense*, *C. eximium*, *C. frutescens* and *C. luteum* (Thul et al., 2012) is observed. In a similar analysis, clusters of varieties of *C. annuum* and *C. frutescens* with *C. baccatum* have been observed, and these clusters are separated from *C. chinense* (Dias et al., 2013), which suggests certain relationships and possible gene exchange among these species. In a study where varieties from different countries were included, clusters of *C. chinense*, *C. frutescens* and *C. annuum* are observed as separated from *C. baccatum* and *C. pubescens* (Lijun and Xuexiao, 2012), which is consistent with the separation between *C. annuum* and *C. pubescens* observed in the present work. Lijun and Xuexiao also demonstrated the efficiency of 15 ISSR primers, among them the sequence $(AC)_8$ YG, included in the present study (first ISO_2); furthermore, the clusters are not associated with the place of origin of varieties, and the authors claim that *C. pubescens* shows a lower genetic polymorphism. Diversity within *C. pubescens* Ruiz & Pavon, *C. eximium* and *C. cardenasii* was estimated, observing that *C. eximium* was distant from *C. pubescens* and closely related to *C. cardenasii* and showing that variation of *C. pubescens* was maximal (Votava and Bosland, 2001). Using RAPD and biochemical analysis, commercial samples of *Capsicum* from Mexico showed a close relationship between the amount and type of carotenoids and genetic profile in addition to separation between *C. annuum* L. and *C. chinense* Jacq. by the first OPE-18 (Troconis-Torres et al., 2012). Through RFLPs, it has been

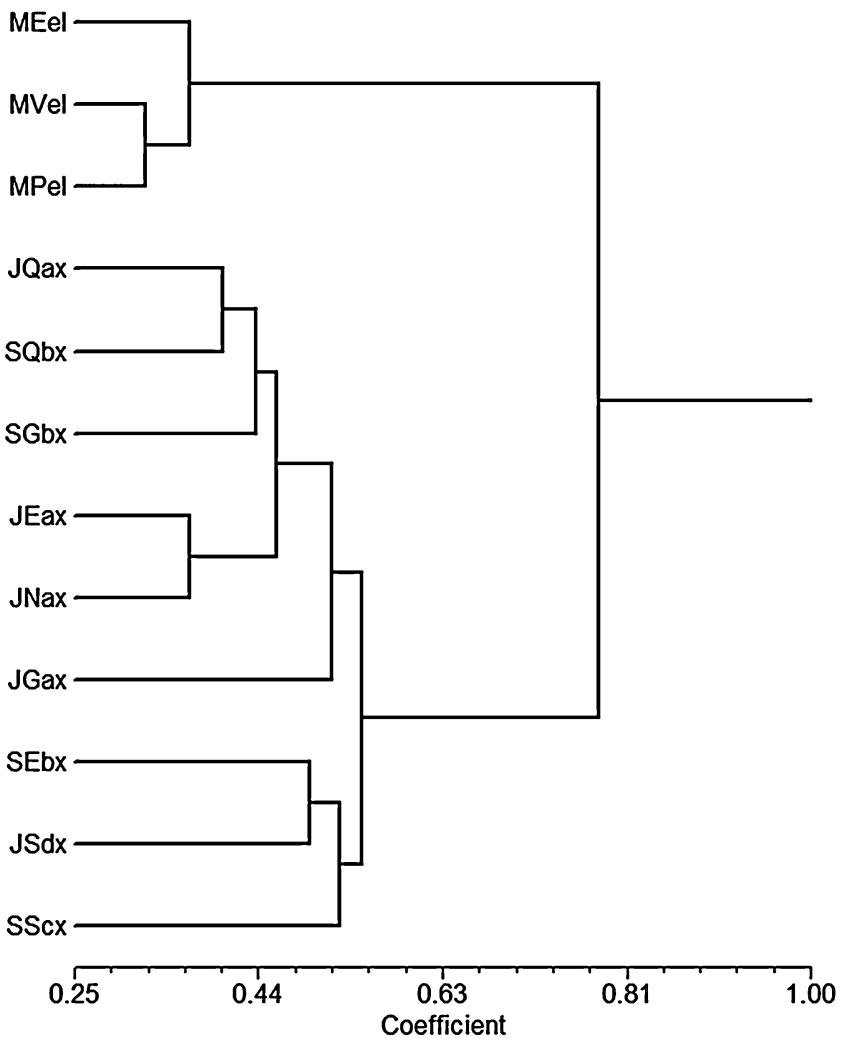

Fig. 5. Dendrograms obtained from the correlation matrix with 24 SSR markers, using. Rogers (modified by Wright) coefficient for clustering of species. The combination of the letters denote the following: type of pepper (J: Jalapeño, S: Serrano, M: Manzano), location (N: Nayarit, Q: Querétaro, G: Guanajuato, E: Estado de Mexico, S. Sinaloa, V: Villa Guerrero, P: Puebla), variety ((a) Hulk, (b) RSS-C36, (c) Santo Tomas, (d) Ahutlán, (e) Grajales ST) and material type (x: hybrid, l: line).

observed that genetic variation was greater among *C. annuum* and *C. baccatum* than between cultivars of *C. annuum* (Lefebvre et al., 1993).

The PCA with the SSR markers located markers that probably correspond to interspecific separation, which coincides with the cluster analysis. The Manzano cultivars were probably grouped by a series of 19 markers. It is important to consider that the SSR markers used here were designed for *C. annuum* (Lee et al., 2004); however, these amplified in *C. pubescens*, which suggests a high genomic homology. This may be due to cross SSRs transferability, by conservation of gene coding regions within related species. Such an effect has been demonstrated in various cultivars including *Capsicum* (Shirasawa et al., 2013), so that the nucleotide sequences in coding regions of *Capsicum* species are substantially preserved. Successful amplification of SSR primer pairs in different species suggests its usefulness in similar studies with other species of *Capsicum* and its possible use for producing DNA species-specific and accession-specific markers, while allowing identification of the genetic relationship among other *Capsicum* species (Ince et al., 2009). On the other hand, in the recent sequencing of the complete genome of chili (Kim et al., 2014), it was observed that *C. annuum* genomically diverges from *C. chinense* at a rate of 1.85%, and this is important because they are two different species. These similarities, in turn, may explain the functionality of SSR primers in different species of chili.

Overall, the ranges of values of genetic distance within *C. pubescens* and *C. annuum* (Serrano and Jalapeño peppers) indicate a high genetic relationship.

The PCA analysis separated both *Capsicum* species according to specific alleles. *C. pubescens* was separated mainly by 39 specific alleles, and the varieties of *C. annuum* were separated by 79 specific alleles. These could be applied in future research as potential markers exclusive of Manzano pepper and interspecific differentiation studies. Interspecific differentiation between *C. annuum* and *C. pubescens* was clear in the cluster analysis (Fig. 5), but no distinction was visible between the Jalapeño and Serrano varieties of *C. annuum*. Interspecific clusters have been observed with SSR between large species of *Capsicum* germplasm (Nicolaï et al., 2013); the groups were independent of the geographical origin of the species; however, they permitted observation of slight differences between wild and cultivated species. In studies with AFLPs, a separation between cultivars and pure lines of *C. annuum* has been observed; however, the level of variation among *C. annuum* samples was quite low (Aktas et al., 2009). It has also been observed that *C. pubescens* and *C. baccatum* have more genetic distance from *C. annuum* and therefore are not crossable (Nam et al., 1997). In a

cluster analysis with SSR markers (Portis et al., 2007), it was observed that *C. annuum* varieties have higher genetic distance with respect to species *C. pubescens*, *C. baccatum*, *C. chacoense*, *C. praetermissum* and *C. eximium*.

5. Conclusion

The present data show that ISSR markers were useful for determining molecular differences based on DNA markers of two *Capsicum* species (*C. pubescens* and *C. annuum*) and two varieties of *C. annuum* species. Additionally, these markers showed interspecific differences between *C. pubescens* and *C. annuum* and intraspecific differences between Serrano and Jalapeño varieties cultivated in three different environments (the states of Querétaro, Guanajuato and Mexico). On the other hand, using SSR markers, interspecific differentiation between *C. annuum* and *C. pubescens* was clear in the cluster analysis, but no distinction was visible between the Jalapeño and Serrano varieties. In addition, SSR markers originally designed for *C. annuum* amplified in *C. pubescens*, which may be due to cross-transferability due to the high genetic homology showed by both species. Molecular markers (alone or combined) are a useful tool for a better understanding of the genetic diversity of *Capsicum*. Studies on desired characteristics in cultured and wild populations of different geographical origins should be extended to provide knowledge that is of interest for phytoimprovement.

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