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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