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Usefulness of three DNA-PCR techniques to differentiate Jalapeño pepper varieties

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The cultivation of pepper (*Capsicum annuum*) is one of the oldest in America. However, out of the 25 species described only 5 have been domesticated. The varieties registered and legally protected must comply with International Union for the Protection of New Varieties of Plants (UPOV) guidelines. Nevertheless, simple sequence repeat the morphological descriptors used for this purpose are sometimes subjective and they are altered by the environment conditions. Molecular genetic markers have been used as alternative to reduce these limitations. The aim of this study was to compare the effectiveness of three techniques based on PCR to discriminate varieties of jalapeño pepper with little phenotypic differences and with very similar shape of the fruit. The techniques used were random amplified polymorphic DNA-DNA amplification fingerprinting (RAPD-DAF), inter simple sequence repeat (ISSR) and simple sequen repeat (SSR) through capillary and conventional electrophoresis. Results indicated that the values of genetic variation in the studied jalapeno varieties are different depending on the technique used, which is due to the capacity of each technique to sample the genome.

Keywords: DNA fingerprints, *Capsicum annuum*, jalapeno variety, molecular discrimination, electrophoresis, Mexico

Introduction

Within the *Capsicum* genus, pepper is one of the oldest crops of America. Baral and Bosland[1] indicated that 25 species have been identified, but only five have been domesticated[2-3]. The species *C. annuum*, *C. frutescens* and *C. chinense* are genetically compatible and capable of genetic exchange. Pepper belongs to *C. annuum*, and according to Food and Agriculture Organisation Statitical Database (FAOSTAT)[4], the main producing countries are China, Mexico, Turkey, Indonesia, USA and Spain. Mexico ranked second with an output of 2,379,736 ton. Pepper consumption and cultivation has increased because it is rich in vitamins, antioxidants, β-carotene, flavonoids, anticancer and antimicrobial agents. Also, it presents very variable contents of pigments, flavors, volatile oils, carotenoids, oleoresins and insectiside alkaloids[5], and its importance also lies in the foreign exchange generated. Its cultivation has been carried out mainly during the last decades and new varieties have transcended in the protection of the breeder's rights. Its success is due to having market demands covered, regarding the nutritional quality of the fruit forms, capsaicin content, ornamental potential, adaptation and resistance to pests and diseases, among others[6]. Registered and protected varieties meet the requirements of distinctiveness, uniformity and stability (DUS) proposed by the International Union for the Protection of New Varieties of Plants (UPOV), based on morphological and physiological assessment of descriptors; however, sometimes they are subjective, as many characters are multigenic and usually altered by the environment. For the other hand, pepper breeding programs has been based on a restricted genetic background; as consequence, the morphological differences are rare. Some limitations in varietal descriptions are enhanced through the use of molecular genetic markers (MGM)[7,8], which quickly estimate the degree of homogeneity or genetic differences; this allows the breeder to know precisely the potential of elite parents for improvement. The success of MGM is because individuals or groups of individuals are differentiated or identified using few experiments. This is because genetic information is not altered by the growth stage of the plant, the season, geographical location or agronomic practices.

The molecular techniques MAAP (multiple arbitrary amplicon profiling) such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism

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(AFLP) and DNA amplification fingerprinting (DAF)[9] or inter simple sequence repeats (ISSR)[10,11] explored the whole genome; but others like simple sequence repeats (SSR) differentiate individuals at specific loci[12]. The UPOV recommended SSR technique for varieties discrimination due principally to their highly reproducibility; however, it shares with the others techniques the capacity of mapping genomes from different species, quantifying genetic diversity, and performing marker-assisted selection, genetic fingerprint and varietal differentiation[13]. A major limitation of SSR technique is the time and cost for design specific primers and the for knowledge of the DNA sequence. Moreover, although SSR are present in all eukaryotes, their location and polymorphic level vary among them[13], so it is unlikely that the primers of one species function with a different species; hence the importance of the other techniques that detect anonymous markers. Many SSR markers have been developed for the *Capsicum* genus, to map the pepper genome[7,14-20]; however, their implementation depends on the purpose of the research. This work was developed in order to compare the efficiency of three PCR-based techniques to differentiate varieties of jalapeño pepper, whose contrast in phenotype and shape of the product is minimal.

Materials and Methods

Nine varieties of jalapeño pepper (*C. annuum*) (Tajín, Ixtapa, Villano, Séminis 2290, Grande, Chimenea, Caloro, Chapala and Aquiles) were studied (Fig. 1). The seeds were established in greenhouse conditions and a mixtures of leaves from ten seedlings were used to obtain the DNA with applying the CTAB method[21]. The DNA quantity was estimated by spectrophotometry (ND-1000 Thermo Scientific, USA), and the DNA quality was determined in 1% agarose gels.

Genotypification

The molecular techniques applied in this study were RAPD-DAF, ISSR and SSR. The amplified product was analyzed on acrylamide gels and/or capillary electrophoresis using an ABI3130 sequencer. For RAPD-DAF, 20 primers of the C Roth series (Carl Roth GmbH Co) were evaluated; for the ISSR technique, nine anchored primers were used: A(GACA)$_3$GACGC, (GACA)$_4$GT, AC(GACA)$_4$, (CA)$_8$RT, (AC)$_8$YG, DBDA(CA)$_7$, (GA)$_8$YC, (TCC)$_5$RY, VDV(CT); and 24 pair primers, previously. reported by Lee *et al*[7], were used for the SSR analysis. After a preliminary trial, only the informative primers were selected. (Tables 1 and 2). To develop the RAPD-DAF technique, total DNA was first digested with the enzyme

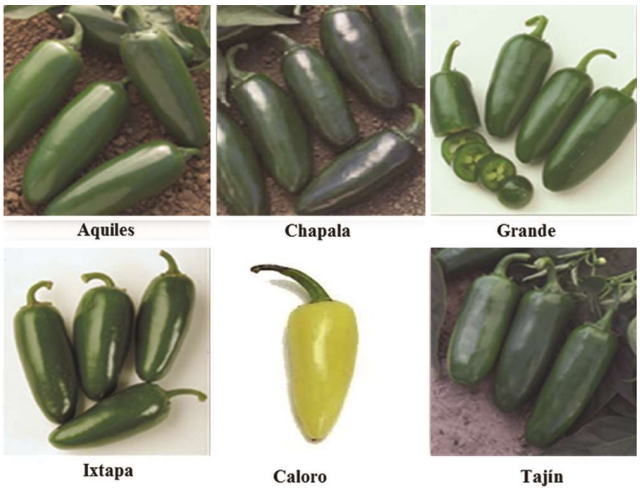

Fig. 1 — Jalapeño pepper varieties studied.

Table 1 — RAPD-DAF and ISSR primers used for analysis, sequence, annealing temperature (T_m), number of amplified fragments, number of polymorphic fragments and percentage of polymorphisms detected in varieties of jalapeño pepper (*Capsicum annum* L)

Primer	Sequence 5′- 3′	T_m (°C)	Total number of fragments	Total number of polymorphic fragments	Percentage of polymorphisms
RAPD-DAF					
C-08	TGG ACC GGT G	40	15	13	86.6
C-09	CTC ACC GTC C	40	6	4	66.6
C-14	TGC GTT CTT G	40	17	16	94.1
Total			38	33	82.4
ISSR					
1	A(GACA)$_3$ GACGC	54	43	43	100
2	(GACA)$_4$ GT	54	20	18	90
3	AC(GACA)$_4$	54	28	26	92.8
4	(AC)$_8$ YG	60	31	23	74.1
5	(GA)$_8$ YG	60	27	22	81.4
Total			149	132	87.7

Y: residue substituted C (cytosine) and T (thymine)

*Eco*R1; then the digested products were used as template DNA in PCR reactions. This method becomes DNA molecule and facilitates the recognition of the PCR primers, increasing the reproducibility of DNA profiles. PCR reaction mixtures for RAPD-DAF and ISSR were of 25 µL and contained water, 200 µM dNTPs, 1X *Taq* buffer, 2.5 mM $MgCl_2$, 20 pM of primer, 1.5U *Taq* DNA polymerase and 80 ng of DNA. For conventional SSR (acrylamide gels separation), the reaction mixture for PCR was 25 µL with 10 pM of each primer, 0.2 mM dNTPs, 1X *Taq* buffer, 3 mM $MgCl_2$, 1U *Taq* DNA polymerase and 40 ng of template DNA.

Amplification was performed on an Applied Biosystem 2700 thermocycler; RAPD-DAF thermocycling conditions were: 94°C, 4 min; 35 cycles (94°C 1 min; 40°C, 40 s; 72°C, 1.5 min) and 72°C, 5 min for final extension. The ISSR program was 94°C, 4 min; 35 cycles (94°C, 1 min; 54°C/60°C, 40 s; 72°C, 2 min) and final extension cycle of 72°C for 10 min. SSR amplification was obtained by initial denaturation for 4 minutes at 94°C; 30 cycles (94°C,1 min; 53°C to 69°C, 1 min; 72°C, 2 min); the final extension was 12 min at 72°C. The molecular markers 1 kb DNA ladder (Promega, USA), 100 bp (Promega, USA) and 1 kb (Fermentas, USA) were used to estimate molecular weight of the amplified bands. The voltage applied was 220 V for 1.5 h in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA pH 8) using dual MGV-216-33 vertical electrophoresis gel system (CBS, USA). Silver nitrate solution ($AgNO_3$) 0.2% was used to stain DNA fragments[23]. The electrophoresis were repeated at least three times. The profiles in all cases were always reproducible. The SSR markers analyzed with capillary electrophoresis generally read once and the molecular weight of the loci was determined directly in the sequencer machine.

Statistical Analysis

Genomic relationships between the nine pepper varieties estimated with the random techniques (RAPD-DAF and ISSR) were established and visualized in clusters applying taxonomy NTSYS pc 2.2 software (Applied Biostatics, Inc.). The procedure was the following: Dice similarity coefficient, hierarchical algorithm unweighted pair group method with arithmetic averages (UPGMA) and SAHN (Sequential, Agglomerative, Hieratical and Nested) cluster analysis. For SSR markers, allele frequencies were estimated, genetic distances were calculated applying Nei 72 coefficient and then the dendrogram was generated using NTSYS pc 2.2 software.

Results and Discussion

Out of the initial markers analyzed in the present study, 3 RAPD-DAF, 5 ISSR, 6 SSR markers

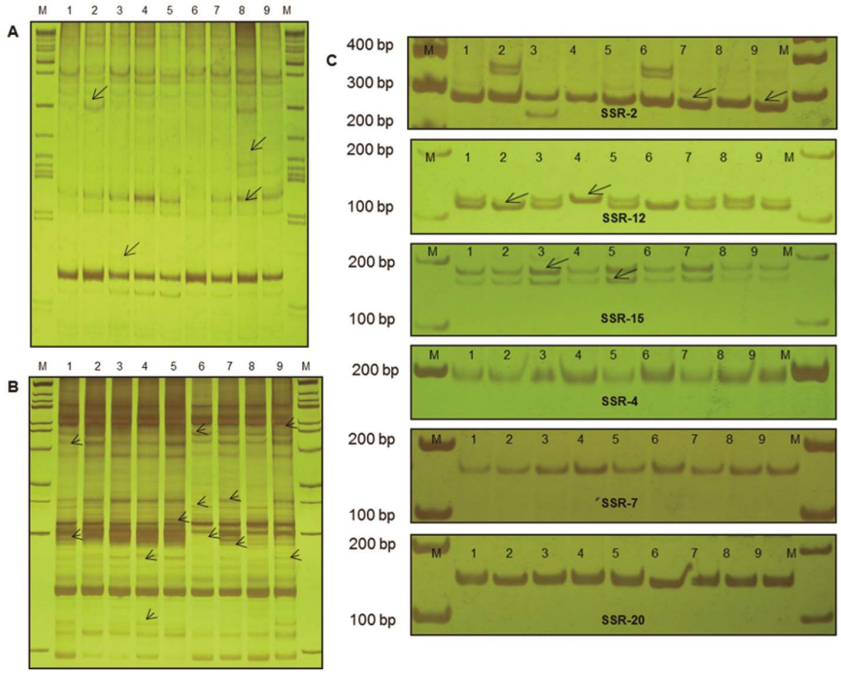

Fig. 2 — DNA profiles of 9 varieties of pepper Jalapeño as shown by three different techniques. Lane 1-9: Tajín, Ixtapa, Caloro, Villano, Séminis 2290, Grande, Chimenea, Chapala and Aquiles: A. DAF-RAPD profiles obtained with the primer C-14. M: 1 kb molecular marker (Promega), 2B. ISSR profiles obtained with primer (AC: 8 YG. M: 1 kb molecular marker (Fermentas), C. SSR markers. The SSR-2, 12 and 15 were polymorphic (indicated with arrows); and SSR-4, 7 and 20 only detected one allele. M: 100 bp molecular marker (Promega).

Table 2 — SSR locus, repeated unit, annealing temperature (T_M) and alleles into varieties of jalapeño pepper (*Capsicum annum* L.)

Number of SSR	Locus	Repeating unit	Fragment size (according to Lee *et al*, 2004)	T_M (°C)	Fragment size (pb) and frequency Jalapeno studied	Number of alleles
1	*AF039662*	T_{26IMP}	114	53	112 (8), 122 (8)	2
2	*AF244121**	$(TTG)_{5IMP}(AT)_{6IMP}(GT)_{3IMP}$	238	54	223 (4), 239 (1), 243 (2), 244 (10), 246 (1)	5
3	*CAN010950*	$(TA)_9$	254	56	262 (18)	1
4	*CAN130829**	T_{16}	184	64	185 (18)	1
5	*CM005*	$(CCA)_8$	160	56	157 (2), 160 (14)	2
6	*HpmsCaSIG19*	$(CT)_6(AT)_8(GTAT)_5$	218	54	221 (1), 223 (5), 225 (4), 230 (6)	4
7	*HpmsAT2-14**	$(AAT)_{16IMP}$	174	65	176 (18)	1
8	*Hpms1-1*	$(CA)_{12}(TA)_4$	283	56	260 (7), 270 (2), 276 (5)	3
9	*Hpms1-5*	$(AT)_{11}(GT)_{17}$	311	62.5	296 (8), 309 (8)	2
10	*Hpms1-6*	$(AT)_2(GT)_4(AT)_8(GT)_{13}$	197	59.5	191 (1), 197 (15)	2
11	*Hpms1-62*	$(TG)_{23}(AG)_9$	186	54	188 (2), 190 (16)	2
12	*Hpms1-106**	$(AAAAAT)_4$	159	69	158 (10), 163(1), 164 (7)	3
13	*Hpms1-111*	$(AAT)_{11}$	159	56	148 (6), 152 (9), 158 (3)	3
14	*Hpms1-143*	$(AG)_{12}$	221	53	224 (11), 229 (7)	2
15	*Hpms1-148**	$(GA)_{14}$	197	65	184 (9), 193 (9)	2
16	*Hpms1-168*	$(TA)_{17}(GA)_{12}$	208	62.5	170 (2), 171 (4), 172 (1), 178 (9), 179 (2)	5
17	*Hpms1-172*	$(GA)_{15}$	344	62.5	340 (8), 341 (1), 344 (7)	3
18	*Hpms1-173*	$(GA)_{16}(TG)_2$	163	53	157 (5), 163 (4), 165 (8), 167 (1)	4
19	*Hpms1-214*	$(GTTT)_2(TTG)_9$	100	59.5	95 (1), 100 (3), 101 (14)	3
20	*Hpms1-274**	$(GTT)_7$	174	65	176 (9), 179 (5)	2
21	*Hpms2-2*	$(GT)_9$	146	53	148 (14), 151 (4)	2
22	*Hpms2-13*	$(AC)_{12}(AT)_4$	259	56	234 (10), 240 (2), 255 (2)	3
23	*Hpms2-21*	$(AT)_{11}(AC)_9(ATAC)_{10}$	295	64	287 (6), 288(1), 23 293 (1), 295 (2)	4
24	*Hpms2-24*	$(CT)_{17}(CA)_5A_{21}$	205	56	185 (15), 202 (3)	2

* SSR also separated in acrylamide gels

generated reproducible, clear and distinct amplified products (Table 2, Fig. 2). For SSR markers analyzed capillarity, 24 pair primers were used. It is particularly worth noting that the RAPD-DAF technique produced reproducible fingerprints as an advantage of the combination of both techniques (RAPD and DAF). Similar results was reported by Jiménez-Galindo *et al*[22], who used a similar strategy. RAPD-DAF technique provided fewer amount of amplified fingerprints compared to ISSR, technique as expected (Table 1; Fig. 2A and B)[24-25]; however, the rate of polymorphism was similar in both techniques.

The SSR markers generated several alleles (1-5), depending on the primer, and in some cases new alleles were detected from those reported by Lee *et al*[7], indicating the presence of the genetic richness in the pepper crop and allelic variation in the jalapeño varieties used in this research (Table 2). Allelic values obtained from SSR markers, conventionally separated in polyacrylamide gels and with capillary electrophoresis, were very similar. The data in capillary electrophoresis gives the exact weight of the microsatellite fragment size. However, for the SSR-20 primer, the size difference between the two alleles was of 3 bases, this difference was difficult to estimate in the polyacrylamide gel. Thereby, we can be more sure of the number of alleles and their exact weight using capillary electrophoresis.

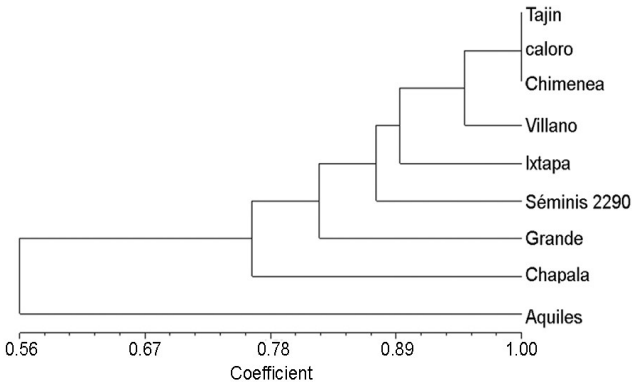

Fig. 3 — Dendrogram generated with data from RAPD-DAF technique in Jalapeño pepper varieties.

Genetic relationships among the nine pepper varieties were visualized in the Figures 3, 4 and 5.

The RAPD-DAF technique (Fig. 3) separated the Aquiles variety from others at genetic similarity (Dice coefficient) of 0.56. The varieties Tajín, Caloro and Chimenea were grouped in the same cluster without any genetic differentiation when applying RAPD-DAF techniques. Villano, Ixtapa, Séminis 2290, Grande and Chapala varieties are clearly different with minimum genetic similarity ranged from 0.77 to 0.96. The ISSR technique also separated the Aquiles variety (Fig. 4), indicating scarce genetic relationship to the rest. Two main groups were labelled: the first one grouped the varieties Tajín, Ixtapa, Caloro, Villano, Séminis 2290, and Chimenea, of which Caloro and Séminis 2290 varieties are the most similar; the second group included the varieties Grande and Chapala. The genetic distance shown between pepper varieties in this study is very low in both dendrograms (Figs. 3 & 4); similar results were shown by Yang *et al*[26] using similar techniques. Oyama *et al*[27] and Hernández *et al*[28] indicated the genetic distance already, of 0.20 separated populations of domesticated Serrano, Jalapeño and Morrón pepper varieties.

The grouping resulted from the SSR markers (Fig. 5) showed some similarity with the information obtained from the random markers (Figs. 3 & 4). In particular, the Aquiles variety is still maintained distanced from the other varieties. This technique formed two groups, the first one made up of Grande and Ixtapa varieties. The second group consists of the Caloro, Villano, Chapala, Séminis 2290, Chimenea and Tajín varieties; it is emphasized that the varieties Chimenea and Tajín are very similar, while the rest do differentiate. The utility of molecular genetic markers is relative depending on the study purpose. In this work, the three techniques (and two modes of separation for SSR markers) provided different degrees of information; but in all cases, the varieties Tajín, Chimenea and Caloro were maintained in the same group, indicating that these varieties are similar genetically. SSR marker are preferred to varietal differentiation for legal purposes due to their reproducibility and quality of information; however, this technique should only be determined by capillary electrophoresis, as reported by Lee *et al*[7] and Contreras *et al*[29], for pepper varieties.

In conclusion, the selected primers for RAPD-DAF and ISSR techniques amplified sufficient DNA markers to differentiate eight varieties of Jalapeño peppers used in the study. The RAPD-DAF and ISSR techniques provided greater amounts of DNA fingerprinting, compared with SSR, and allowed to differentiate the varieties of Jalapeño peppers at a minimum distance. The SSR markers separated on conventional acrylamide gels limit allelic exact allocation values, while the SSR markers in capillary electrophoresis separate the alleles present in the different varieties with precise molecular weights.

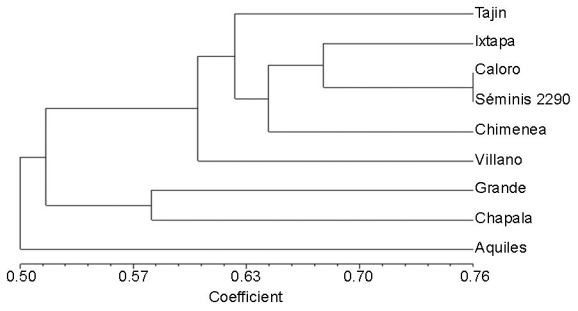

Fig. 4 — Dendrogram obtained with data from ISSR technique in Jalapeño pepper varieties.

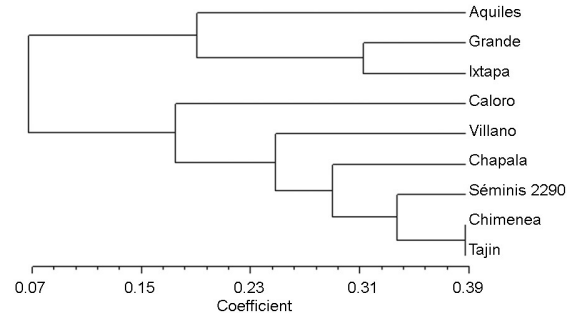

Fig. 5 — Dendrogram obtained with data from SSR in Jalapeno pepper varieties.

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