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MOLECULAR COMPARISON OF WILD AND COMMERCIAL CHILIES FROM TAMAULIPAS AND TABASCO, MEXICO

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Abstract

The genetic differences among two groups of varieties of chili (*Capsicum* spp.) from different geographical origins (one each from the states of Tabasco and Tamaulipas in the Mexican republic) were studied using AFLP (Amplified Fragment Length Polymorphism) molecular markers. Four Tabasco chili varieties were studied; two cultivars (Jalapeño, Habanero) that belong to *Caspicum annuum* L. and *Capsicum chinense* Jacq., respectively and two wild varieties (Amashito, Ojo de cangrejo), that belong to *Capsicum annuum* var. *glabriusculum.* Five Tamaulipas chili varieties were studied: the Mirador, Piquín huasteco and Ozulamero varieties, which belong to C*apsicum annuum* L, and the Pico paloma and Chilpaya tabasco varieties, which belong to *Capsicum frutescens.* For the analysis of genetic differentiation, four AFLP molecular markers were used, which amplified 150 to 209 bands in the two variety groups. The AMOVA (Analysis of molecular variance) results indicated that the maximum variation was found between the regions (37.0%) and within the populations of each region (37.0%). The estimated variety differentiation was high, F_{ST}=0.373, which indicates that 62.70% from the variation is found within the varieties, and the high values of F_{IS}=0.409 and F_{IT}= 0.629 lead to the assumption that the varieties possess a large number of homozygotes and a substantial loss of heterozygotes. The cluster analysis separated the evaluated varieties by geographical region and by kind; for Tabasco, the wild Amashito and the Ojo de cangrejo were grouped, and the cultivated varieties formed their own group. In the Tamaulipas varieties, this grouping tendency was not observed.

Key words: *Capsicum annuum, Capsicum chinense*, Analysis of molecular variance, Varietal comparison

Introduction

The Solanaceae family, which includes the genus *Capsicum* is very important in Mexico and worldwide; demand for chilies is very high due in part to their nutritional content, and this leads to a large cultivated surface area and great economic benefits. Due to high demand and cultivation, worldwide green chili and dry chili production has increased, reaching 31.2 million tons in 2009 according to the (Anon., 2009). Chilies have many diverse uses; the most important of these are their uses as spices, as a vegetable and in the extraction of colorants (Djian-Caporalino *et al*., 2006).

Nevertheless, according to Kochieva & Ryzhova (2003), relatively few molecular and genetic studies of chili peppers have been performed, compared with other types of crops. Several studies, including McLeod*et al*., (1983), Loaiza-Figueroa *et al*., (1989), Paran *et al*., (1998), Rodríguez *et al*., (1999), and Hernández-Verdugo *et al*., (2001), have analyzed C*apsicums* using isoenzymes and Random Amplification of Polymorphic DNA (RAPD) molecular markers. The results obtained by Loaiza-Figueroa *et al*., (1989) showed that wild and domesticated chili species maintain low levels of genetic variance and that most of this variance is present among different populations. The studies performed by Paran *et al*., (1998) and Rodríguez *et al*., (1999) used molecular markers based on the Random Amplification of Polymorphic DNA (RAPDs) fragments and indicated that the C*apsicum annuum* genome possesses low molecular polymorphism levels. However, these studies were performed using pepper samples obtained from different germplasm banks from Mexico and other countries; this could lead to an underestimation of the genetic variation existing in pepper populations grown under natural conditions. In a study performed by Rodríguez *et al*., (1999), it was discovered that wild and domesticated *C. annuum* species formed a single molecular level group.

Another molecular technique used in pepper studies is the AFLP (Amplified Fragment Length Polymorphism) technique, described by Vos *et al*., (1995), which is considered one of the quickest and least expensive techniques. The AFLP technique is based on the selective amplification of restriction fragments from total genomic DNA (gDNA) using PCR (Polymerase Chain Reaction). The AFLPs can produce varied complexity patterns, depending on the restriction enzymes and the fragment length used in the PCR. According to Paran *et al*., (1998), the AFLP markers segregate in a "Mendelian" way, just like RFLPs. However, the RFLP approach does not detect as many polymorphic loci as the AFLP technique. Barrios *et al*., (2004) reported the genetic diversity of the C*apsicum annuum-chinense-frutescens* complex in Cuba using AFLP markers; this analysis corroborated the taxonomic descriptions performed in a morphological study and was conducted *in situ*.

Due to the importance of chili peppers in Mexico, the present research was intended to describe the possible genetic diversity distribution pattern and the relationships between and among two types of chilies from two different geographic areas in Mexico using AFLP markers.

Materials and Methods

Plant material: In this study, nine varieties of pepper were used. Four varieties were from the state of Tabasco: Jalapeño, Amashito, Ojo de cangrejo and Habanero. The Jalapeño and Habanero varieties are cultivated types and they belong to the types *Caspicum annuum* L. and *Capsicum chinense* Jacq., whereas the Amashito and Ojo de cangrejo are wild and are classified as *Capsicum annuum* var. *glabriusculum.* From the state of Tamaulipas, five varieties were used: Mirador, Piquín huasteco, Pico paloma, Ozulamero and Chilpaya tabasco; Mirador, Piquín huasteco, and Ozulamero are C*apsicum annuum* L., and Pico paloma and Chilpaya tabasco belong to C*aspicum frutescens* L. The Piquín huasteco and Chilpaya tabasco varieties are wild, the Mirador variety is semi-wild, and the Pico paloma and Ozulamero varieties are cultivated in home backyards.

gDNA extraction and AFLP amplification.: Genomic DNA (gDNA) was extracted from the young leaves of five plants of each variety; from these leaves, three tissue samples of 0.5 g each were taken and macerated in liquid nitrogen (-196º) (Dellaporta *et al.,* 1983). The concentration of the extracted gDNA was measured in a Jenway 6305® UV/vis spectrophotometer at 260 nm absorbance. The quality of gDNA was determined by electrophoresis; 5 µg of DNA was run in a 1 % (p v^{-1}) agarose gel with a TAE buffer (40 mM Triacetate, pH 7.6; 1 mM Na2.EDTA) for two hours at 80 V, and then dyed with ethidium bromide (0.5 mg mL^{-1}).

AFLP molecular markers were used to analyze and compare the varieties in this study. Four combinations of molecular markers (MM) or primers were used (Table 1). The amplified AFLP fragments were separated by electrophoresis through a 6.5% polyacrylamide gel (Vos *et al*., 1995); for this the IRDye™ Fluorescent AFLP® Kit for Large Plant Genome Analysis (LI-COR®, Lincoln, NE) commercial kit was used. To separate the amplified fragments, an IR^2 semi-automatic sequencing system was made (model 4200-029; *LI-COR®*). The bands were identified visually, generating a binary matrix of the presence (1) or absence (0) of a band produced by each primer set.

Table 1.Oligonucleotide sequences used for AFLP analysis in chili.

Adapter	EcoRI	5’- CTCGTAGACTGCGTACC - 3’ 3’- CTGACGCATGGTTAA -5’
	MseI	5’- GACGATGAGTCCTGAG -3’ 3’- TACTCAGGACTCAT -5’
Pre-selective amplification	EcoRI	5 ‘ – AGACTGCGTACCAATTC/A – 3’ + A
	MseI	5'- GACGATGAGTCCTGAGTAA/A -3' + C
Selective amplification	EcoRI	5’- AGACTGCGTACCAATTC -3’ + AAG
	MseI	5'-GACGATGAGTCCTGAGTAA -3' + CAG
	EcoRI	5’- AGACTGCGTACCAATTC -3’ + ACG
	MseI	5’-GACGATGAGTCCTGAGTAA-3’+CAG
	EcoRI	5’- AGACTGCGTACCAATTC -3’ + AAG
	MseI	5’-GACGATGAGTCCTGAGTAA-3’ + CAA
	EcoRI	5’- AGACTGCGTACCAATTC -3’ + ACG
	MseI	5’-GACGATGAGTCCTGAGTAA-3’ + CAA
	EcoRI	5’- AGACTGCGTACCAATTC -3’ + ACT
	MseI	5’-GACGATGAGTCCTGAGTAA-3’ + CTG
	EcoRI	5’- AGACTGCGTACCAATTC -3’ + AGG
	MseI	5’-GACGATGAGTCCTGAGTAA-3’ + CTG

Data management and statistical analysis: We registered the different alleles detected in each pepper variety for every *locus* so that every present band corresponds to an allele, and its absence corresponds to an alternate allele. The polymorphism detected in each variety was estimated by the evaluated markers. The AFLP fragment data were subjected to an Analysis of molecular variance (AMOVA) using GenAlEx6.4 Software (Peakall & Smouse, 2006) to determine the genetic relationships among the pepper varieties.

We estimated the genetic diversity patterns H_T, H_S, D_{ST} and G_{ST} (Nei, 1973; Culley *et al*., 2002). We also estimated the genetic distances among every population or variety pairs as described by Nei (1972) and calculated the following Wright F-statistics: F_{IS} (measures of variation of heterozygosity within the varieties with respect to the expected result based on the allelic frequencies under random mating), F_{ST} (reduction of expected heterozygosity under random mating in a hierarchical level in relation with a superior level, attributable to the variety differentiation in the genetic groups), and the F_{IT} (overall inbreeding coefficient).

The estimation of the genetic distances was made with the Dice (Nei & Li, 1979) method, which is estimated as $\frac{2a}{(2a+b+c)}$, where a= the total number of common bands for the subjects i and j (1,1), b= the total number of bands that are present in subject i, but not in subject j (1,0), and c= the total number of bands that are present in subject j, but not in subject i (0,1). This method considers the absence of a band to have a minor biological importance, so this coefficient has a full meaning in the DNA similarity function. The dice method is the only one with a biological relevance because it expresses the probability that a band that is present in a subject is in another subject too; it is interpreted as the relationship between the matching band number between two subjects and the total band number (subject’s bands average). With the data obtained from the total bands in each variety, a genetic distance matrix was elaborated, which was used later to build a dendrogram with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in order to compare the Tabasco varieties to those from Tamaulipas. The dendrogram was generated with the programs Freetree and Treeview (Page, 1996). The cophenetic correlation coefficient (r), which compared the original matrix and the similarity one (Nuñez, 2011), was calculated using the NTSYSpc 2.02 software (Rohlf, 1998).

Results and Discussion

On average, a greater number of bands (Table 2) were found in the varieties from Tamaulipas (194.4 bands) than in those from Tabasco (155.5 bands). Regarding the common band number (data not shown), Tamaulipas’ varieties generally presented a higher common band average than the Tabasco ones, with the exception of the “Chilpaya tabasco” variety from Tamaulipas, which had the lowest common band number. The number of bands found in our research is similar to the one reported by Kochieva & Ryzhova (2003), but greater than that reported by Paran *et al*., (1998) and by Votava *et al*., (2002) using RAPDs in *Capsicum annuum* var. *glabriusculum* populations. On average, the studied loci were more polymorphic in the Tamaulipas varieties than the Tabasco ones (Table 2). Among the nine varieties, the Ojo de cangrejo from Tabasco presented the highest percentage of polymorphic loci (41.84%), followed by the Mirador (45.74%) and Pico paloma (41.49%) samples from Tamaulipas.

Table 2. Total band number, estimated statistics and polymorphism levels detected in chili varieties from Tabasco and Tamaulipas.

Code	Population	Band Num.	H_S	H_T	D_{ST}	G_{ST}	LP (%)
Tabasco wild samples							
2	Amashito	154	0.14	0.46	0.32	0.70	30.50
3	Ojo de cangrejo	150	0.19	0.39	0.20	0.51	41.84
Tabasco cultivated samples							
1	Jalapeño	156	0.08	0.51	0.48	0.86	17.73
4	Habanero	162	0.08	0.52	0.44	0.85	18.44
	Average	155.5	0.12	0.47	0.39	0.73	27.13
Tamaulipas wild samples							
6	Piquín huasteco	202	0.11	0.66	0.55	0.83	24.11
9	Chilpaya tabasco	171	0.12	0.53	0.41	0.77	25.89
Tamaulipas semi-wild samples							
5	Mirador	187	0.20	0.54	0.34	0.63	45.74
Tamaulipas backyard cultivated samples							
7	Pico paloma	203	0.18	0.60	0.42	0.70	41.49
8	Ozulamero	209	0.15	0.66	0.51	0.77	33.69
	Average	194.4	0.15	0.60	0.45	0.74	34.18

H_S= within-populations gene diversity estimates, H_T= total gene diversity estimates, D_{ST}= among populations gene diversity estimates, G_{ST}= relative magnitude of gene differentiation among populations, LP= polymorphism level

In relation to the percentage of polymorphic loci, our results also outperform the 16.5% and 22.0% polymorphism found in peppers by Kochieva & Ryzhova (2003) and Paran *et al*., (1998), respectively. The high polymorphism detected in the present investigation agrees with the results in black cohosh from Nyree *et al*., (2002) and those in hops from Shaun & Henning (2005); it is possible that this is due to the diversity of the *C. annuum* var. *glabriusculum*, *C. frutescens* and *C. Chinense* Jacq material analyzed in this study.

In Table 2, it can also be observed that the Jalapeño and Habanero varieties, both from Tabasco, presented the lowest average diversity (H_S=0.08 for each). The varieties with the highest diversity value were Mirador from Tamaulipas (H_S=0.20) and Ojo de cangrejo from Tabasco (H_S=0.19). The total genetic diversity (H_T) varied from 0.32 to 0.52 in the samples from Tabasco and from 0.53 to 0.66 in the samples from Tamaulipas. Most of the populations evaluated showed considerable haplotype levels in their source region, as demonstrated by the resulting genetic differentiation coefficient (G_{ST}) values (Table 2). The average G_{ST} values of 0.73 and 0.74 for each variety group indicate that 73.0 and 74.0% of the total genetic diversity detected was explained by the differences within the populations. The diversity within the varieties (D_{ST}) from Tamaulipas was slightly higher than that of the ones from Tabasco (Table 2). The estimated genetic diversity values (H_T) within the C*apsicum* varieties in our work (Table 2) are higher than the values reported on the same genus by McLeod *et al*., (1983), Loaiza-Figueroa *et al*., (1989), and Paran *et al*., (1998); these prior studies estimated diversity using cultivars that were obtained from genebanks in the US, Mexico, and Europe, and obtained similar diversity values to the ones found in the domesticated and wild populations form the Mexican northwest by Hernández *et al*., (2006). The diversity values are also greater than the H_T values found by Coulibaly *et al*., (2002) in wild cowpea annuals cultivated from different geographic regions in Africa. The discrepancy between the results obtained in this and prior studies is most likely due either to the germplasm used in each investigation, or to the use of different species, in the case of the cowpea study.

Finding average H_T values that are higher in the cultivated and sub-cultivated populations than the ones that are wild and semi-wild from Tabasco and Tamaulipas. This can be attributed to the fact that such populations belong to the *C. annuum*, *C. chinense* and *C. frutescens* varieties. The previous facts can be corroborated with the D_{ST} (genetic diversity among populations) values, which were higher for the cultivated populations than the wild ones. These results coincide with the ones reported by Hernández *et al*., (2006).

The G_{ST} (genetic differentiation coefficient) values found in this research surpassed the 0.17 reported by Hernández *et al*., (2006), and almost equaled the 0.32 that was reported in the "poblano" pepper by Contreras *et al*., (2011). This discrepancy and coincidence in the G_{ST} values may be due to the existing diversity in the evaluated populations in each study, or due to the number of species the populations belong to; this was the case in the study performed byLoaiza-Figueroa *et al*., (1989), who found values of G_{ST}=0.91 (domesticated) and G_{ST}=0.90 (wild) by analyzing five populations of domesticated pepper species and their closest wild relatives.

Table 3. Analysis of molecular variance (AMOVA) based on 282 AFLP markers in C*apsicum*.

Source of variation	d.f.	Sum of squares	Mean squares	Variance component	Variation (%)
Among regions	1	482.35	482.35	29.44	37
Among populations	7	629.13	89.88	20.23	26
Within populations	18	525.33	29.19	29.19	37
Total	26	1636.82		78.85	100

F_{IS} = 0.409**, F_{IT} = 0.629**, F_{ST} = 0.373**

Table 4. Genetic distances (GD) among chili varieties estimated with the Dice coefficient.

S. No.	1	2	3	4	5	6	7	8	9
1.		0.587	0.517	0.609	0.437	0.496	0.461	0.496	0.455
2.			0.660	0.576	0.393	0.456	0.404	0.460	0.441
3.				0.552	0.339	0.385	0.362	0.401	0.367
4.					0.456	0.526	0.485	0.520	0.466
5.						0.696	0.645	0.582	0.536
6.							0.761	0.749	0.659
7.								0.717	0.631
8.									0.653
9.									

Cophenetic correlation coefficient r = 0.92

In the AMOVA performed with AFLP bands, a very large differentiation in the allelic frequencies among the populations from both regions (F_{ST}=0.373) could be observed. Because of this, it can be inferred that the heterozygote deficit was due to a major non-random mating effect within the populations (F_{IS}=0.409). In other words, the AMOVA results indicate that 37.0% of the total genetic variation was distributed between regions and 26.0% was distributed between the populations from each region (Table 3). These values (F_{ST} and F_{IS}) were statistically significant ($p \leq 0.001$), indicating a clear difference not only between the regions but also between the populations in each region. The high values of F_{IS} (average heterozygote deficiency or excess in each population) and F_{IT}=0.629 (average heterozygote deficiency or excess in each population group) in the evaluated populations lead to the assumption that the populations possess a large number of homozygotes and a substantial loss of heterozygotes. The differences found in the three main AMOVA variation sources (Table 3), indicate clear differences between regions, between populations, and within the populations. The variation values between regions and between populations were moderate (37.00% and 26.00%). The F_{ST}=0.373 value indicates that the differentiation in the allelic frequencies between populations from each region is high, which means that either there has not been random mating between the populations (Anon., 2004) or there is genetic isolation among the populations. The F_{ST} found in our investigation is more than three times greater than the one reported by Contreras *et al*., (2011) in different types of "ancho" pepper from Puebla (Poblano, Loco, and Miahuateco, among others). The high F_{ST} value found in our research could have been observed because *Capsicum annuum, C. frutescens* and *C. chinense* populations were included, and among these there were wild, semi-wild and domesticated samples. The discrepancy between these results could have arisen because the prior authors used germplasm of the *C. annuum* kind, while we used *C. annuum*, *C. frutescens* and *C. chinense* Jaq. The F_{IS} and F_{IT} values found in our study could be the result of the non-random mating that has occurred naturally within the populations (Anon., 2004). Nevertheless, *Capsicum* is considered an autogamous plant, depending on the natural conditions (temperature and relative humidity, mainly), and has a certain degree of natural mating or allogamy.

The genetic distances matrix (Table 4) was used to build a dendrogram (Fig. 1). The similarity values between the evaluated populations fell into a range of 0.339 to 0.761. The resulting dendrogram showed two well-defined groups, one containing the populations from Tabasco and another with the Tamaulipas varieties. Within each group, subgroups were formed. In the Tabasco group, one subgroup is formed by the wild Amashito and Ojo de cangrejo varieties, and another subgroup is formed by the Jalapeño hybrid and the Habanero variety. In the varieties from the state of Tamaulipas, even though there were subgroups formed, there was no similar separation between varieties; this means that the wild varieties did not group with other wild samples, and similarly, the domesticated varieties did not form a subgroup either. When comparing the matrix obtained using the Dice method and the cophenetic matrix, we found a correlation value of r=0.92 (Table 3); according to Anon., (2010) and Nuñez (2011), the grouping of individuals sampled according to the established classification by it is very good.

Regarding the genetic distances found between the populations from Tamaulipas and Tabasco, it is supposed that they do not have a phylogenetic relationship because they did not group together in the dendrogram, which raises the possibly that the genome of each population is not conserved. Shitthiwong *et al*., (2005) reported similar grouping results and genetic distance values by classifying ten accessions of *C. annuum* from Thailand. However, our results disagree with those of Rodríguez *et al*., (1999), who found in 100 *Capsicum annuum* accessions that the wild and domesticated populations grouped together. According to Kochieva & Ryzhova(2003), the C*apsicum* cultivar genome is highly conserved, as the genetic distances were very small (0.021 to 0.072).

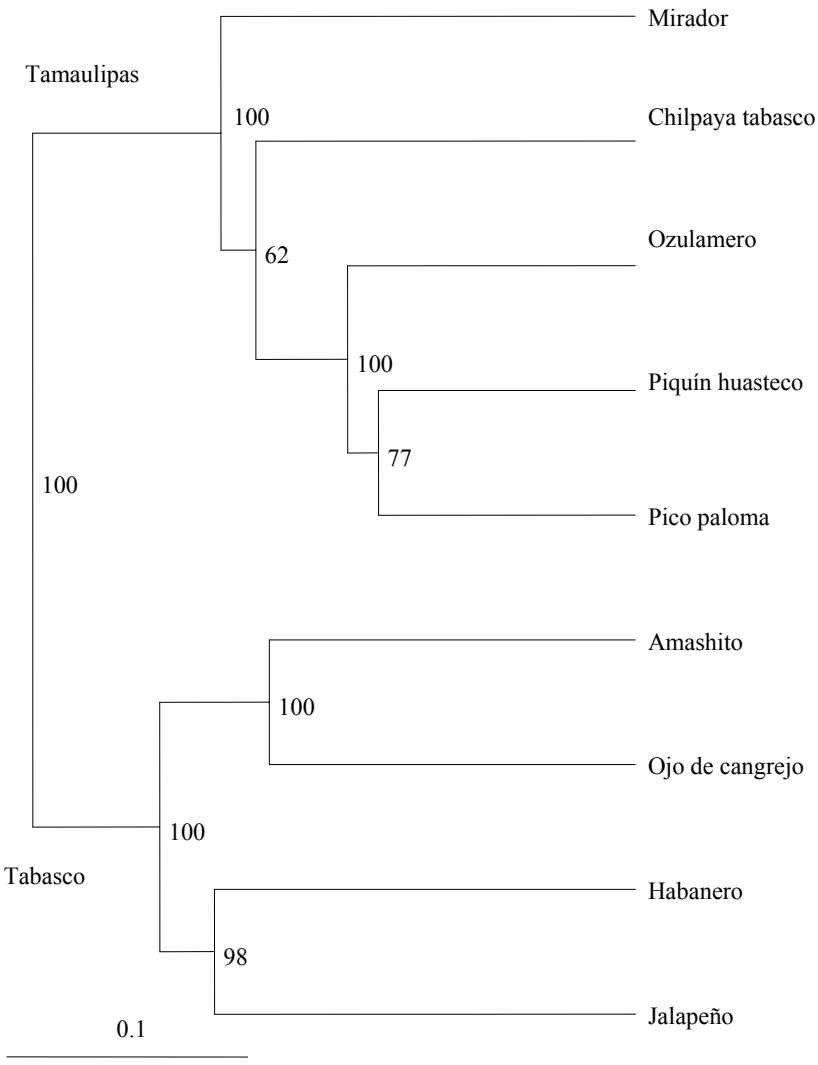

Fig. 1. Genetic distance dendrogram based on AFLP markers among 9 chili varieties from two states (Tabasco and Tamaulipas). Numbers on branches correspond to bootstrap values (1000 replications).

Conclusions

The results of our investigation showed that a high level of genetic diversity among chili peppers exists and that the populations from the state of Tabasco are genetically well differentiated from the ones from the state of Tamaulipas. This was corroborated by the dendrogram, where it could be observed that the populations from each region formed separate groups and that varieties formed well-defined subgroups within each regional group.

According to the AMOVA result, it was determined that 37% of the variance is found between regions and that 26% is found between populations of each region. The data also showed that there is a close relationship between the wild Amashito and "Ojo de cangrejo" populations from Tabasco, but similar close relationships were not observed in the Tamaulipas populations. Based on the genetic distance values found and the grouping pattern in the populations, it can be inferred that these populations can be used in genetic improvement programs; such programs could take advantage of heterotic effects in desirable characteristics of economic importance, such as fruit size and the yield.

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