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POPULATION STRUCTURE ANALYSIS OF HABANERO CHILI (*CAPSICUM CHINENSE* JACQ.) WITH AFLP

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Abstract

The structure of four Habanero Chili pepper populations (*C. chinense* Jacq.), was estimated with AFLP (Amplified fragment length polymorphisms) markers. Three of the four evaluated populations were selected in Tabasco and one from Campeche, each population consisted of 15 individual plants. The six oligonucleotides combinations used at this research showed products of 34 to 422 pb, and produced 1722 total bands. Combination E-AAG/M-CAG amplified the largest bands number (329) and E-ACA/M-CTG detected the minor bands number (190). The populations of Mucuychacán, Campeche, presented the largest polimorphism (1371 polimorphic bands), with mean of 228.5 bands polimorphic per oligonucleotide, the polimorphic bands minor number was observed at Cucuyulapa, Tabasco with 1216. The AMOVA (Variance Molecular Analysis) showed a genetic differentiation index of F_{ST}= 0.13, the variability explained among populations were minor (14.14%), that within populations (85.86%). The populations structure was determined with a value of deltaK=3. The cluster analysis grouped the plants of Santa Cruz Tlacotalpa as an independent population, and some plants of Mucuychacán, Cucuyulapa and Ranchería El Habanero populations were grouped at the cluster I.

Key words: *C. chinense* populations, AFLPs, Polymorphism, Molecular marker, AMOVA.

Introduction

According to MacNeish (1964), the chili (*Capsicum* spp.) was known to the human civilization of the Western Hemisphere from the year 7500 b.c. The chili was domesticated by the natives of Mesoamerica and South America between the years 5200 and 3400 b.C., placing the chili as the crop with greater antiquity in being sown in America (Long-Solís, 1998). During domestication process of *Capsicum* genus, number of species were generated viz. *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* R. & P. (Anon., 1983). Of these, *C. annuum* is the one of greater economic and agricultural importance (Paran *et al*., 1998). Together with squash plant (*Cucurbita pepo* L.), maize (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.), chili, until today is one of the primary foods at diet of the Neotropical civilization of Mesoamerica (Perry *et al*., 2007). In Mexico, the pre-Columbian cultures contributed a lot in the chili domestication, and were generated lot of variability of cultivated forms that today are mantained in the country. Thanks to the diversity of agro-ecological environmental and the human participation, who generated a wide range of forms in colors, aromas, flavors and sizes that constituted a valuable contribution of Mexico to world gastronomy. Habanero chili (*C. chinense* Jacq.) is native of South America and its diversity center is the Amazonas basin, extending to Bolivia. A lot of people believe that Habanero chili was introduced from Cuba to Mexico per Yucatan state, until today *C. chinense* is grown in the three states that form the Yucatan Peninsula (Campeche, Quintana Roo and Yucatan), and also in Tabasco and Chiapas states (Eshbaugh, 1993). Habanero chili as a crop contribute to the economy of the national and international markets of the Caribbean islands (Moses *et al*., 2013).

A number of studies have been conducted on the population structure of *Capsicum* spp., by Taranto *et al*., (2016), Xiao-min *et al*., (2016); Hill *et al*., (2013); Se-Jong *et al*., (2012); Pacheco-Olvera *et al*., (2012), and of *C. chinense* by Baba *et al*., (2015); González-Pérez *et al*., (2014); Moses *et al*., (2013).

The objective of this research was to estimate the genetic structure of four populations of *C. chinense* Jacq, to determinate the possible selecction effects applaied by the chili growers at the states of Tabasco and Campeche, Mexico.

Materials and Methods

From the four populations of chili that were studied, three were obtained from Tabasco and one from Campeche (Table 1). In each locality, seeds were individualy collected from each one of the 15 plants that formed each population. The 60 collections or individual plants belong to *C. chinense* Jacq.

Table 1. Origin of *Capsicum chinense* Jacq. populations.

Name	Location and municipality collection	State
RH1 a RH15	Ranchería El Habanero, Cárdenas	Tabasco
MC1 a MC15	Mucuychacán, Campeche	Campeche
SCTP1 a SCTP15	Santa Cruz, Tacotalpa	Tabasco
CC1 a CC15	Cucuyulapa, Cunduacán	Tabasco

Table 2. Primers characteristics used to determine genetic structure of four Habanero chili (*C. chinense* Jacq.) populations form Tabasco and Campeche, Mexico.

Process	Enzime	Primer sequence
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' + ACA
	MseI	5'-GACGATGAGTCCTGAGTAA-3' + CTG
	EcoRI	5'-AGACTGCGTACCAATTC-3' + AAC
	MseI	5'-GACGATGAGTCCTGAGTAA-3' + CTG

Seed of each one of the populations was grown in polystyrene traysin in the greenhouse of the Center for Genomic Biotechnology of the National Polytechnic Institute (CBG-IPN) in Reynosa, Tamaulipas. Peat-moss and pearlite (50:50), were used as substrate. After 30 days of germination (dag), young leaves of five plants per collection, from which the genomic DNA (gDNA) was extracted using the protocol of Dellaporta *et al*., (1983). The collections samples were analyzed using six AFLP combinations (Amplified fragment length polymorphisms) as specified by Vos *et al*., (1995) in his protocol, using the commercial kit IRDyeTM Fluorescent AFLP® Kit for Large Plant Genome Analysis (LI-COR®) with adapters and primers (Table 2), corresponding to restriction sites for MseI and EcoRI enzymes, according to the manufacturer's instructions (LI-COR® Biosciences, Lincoln, NE, USA). All amplification reactions were performed in an i-Cycler Standard 3. The amplified fragments separation was carried out in a semi-automatic IR2 sequencing system (model 4200-029, LI-COR®, Lincoln, NE, USA) by means of polyacrylamide gel electrophoresis.

The amplicons were identified visually, with them, a binary matrix of presence (1) and absence (0) was generated for each of the primer's used in this investigation. The percentage of polymorphism was estimated from the data obtained from the intra and interpopulation genetic diversity patterns. The analysis of molecular variance (AMOVA) was performed with the statistical package GenAlEx version 6.502 (Peakall & Smouse, 2006), and in this, the variance within and between populations was estimated. With the statistical package DARwin versión 6.0.14 (Perrier and Jacquemoud-Collet, 2006), the similarity analysis of habanero chili populations was carried out. Finally, the population genetic structure was estimated through a Bayesian inference analysis with STRUCTURE 2.3.4 software (Pritchard *et al*., 2010), calculations were made for one, two, three and four populations with the mixed model parameters and correlated allele frequencies. The computation was made with 30 iterations for each population, with pre-run data consisting of 5000 'burn-in period' followed by 50,000 Monte Carlo Markov chains (MCMC), similar to the proposed by Sung-Chur (2013) to obtain the optimum delta K value that represents the number of differentiated populations of *C. chinense*. The results obtained were analyzed with the software Structure Harvester version v0.6.94 (Earl & VonHoldt, 2012), with the information obtained, a final analysis was calculated for optimal deltaK, the parameters considered were mixed model and correlated allele frequencies followed by 500,000 'burn-in period' and 750,000 MCMC.

Table 3. Polymorphism obtained by combinations of pairs of AFLP primers in habanero chili pepper (*C. chinense* Jacq.) from Tabasco and Campeche, Mexico.

Primers combination	Amplified	Bands polymorphic	Unique	Polymorphism (%)
E-AAG/M-CAA	291	291	20	100
E-AAG/M-CAG	329	329	16	100
E-ACA/M-CTG	190	190	14	100
E-ACG/M-CAG	320	320	10	100
E-ACG/M-CAA	276	276	50	100
E-AAC/M-CTG	316	316	44	100
Total	**1722**	**1722**	**153**	**100**

Table 4. Summary of the polymorphism between populations of *C. chinense* Jacq., from Tabasco and Campeche, Mexico.

Statistics	Ranchería El Habanero	Mucuychacan	Santa cruz	Cucuyulapa	Total	$\bar{X}$	Sd
Gene's copies	15	15	15	15	60	15.00	0.00
Useful *loci*	1722	1722	1722	1722	1722	1722	0.00
polymorphs *loci*	1292	1371	1241	1216	1719	1280	68.415

$\bar{X}$= Mean, Sd= Standard deviation, Sum of squares of frequencies = 0.0667, Genetic diversity (Standard index) = 1.0000 +/- 0.0243

Results and Discussion

The AFLP primers combinations used at this investigation (ACA/CTG, AAC/CTG, AAG/CAG, ACG/CAG, AAG/CAA and ACG/CAA), amplified products from 34 to 422 bp and generated 1722 polymorphic bands. Baba *et al*., (2015), with seven AFLP primers combinations found bands with a size of 60-502 bp in accessions of Habanero chili pepper. In our research E-AAG/M-CAG combination detected a greater number of bands (329), and E-ACA/M-CTG showed lower number of bands (190), the average polymorphism found was 287 bands (Table 3). The polimorphic bands number detected in our investigation was greater that 6.5 mean that was reported by Paran *et al*., (1998); them used 34 populations (mostly commercials varieties) of *C. annuum*, the mentioned researchers conclued of their results that the populations evaluated showed high genetic similitarity. Aktas *et al*., (2009), reported 49.8% of average bands for each AFLP primers used. On the other hand, our results differed with that reported by De Freitas (2007), who found low variability in Habanero accessions pepper´s chili´s of Brazil, this might be that De Freitas employed two pairs of oligonucleotids AFLP EcoRI/MseI +3/+3 with different sequence (AGG/CAC y ACG/CAA), these oligos showed litte informative efficiency, since they generated only 137 polymorphic bands ranging between 50 and 490 bp. González-Pérez *et al*., (2014) found with SSR in *C. chinense* 125 allels and 41.2 unique allels. Baba *et al*., (2015); reported fewer bands (302) than those visualized in our research. Dhaliwal *et al*., (2014) in *C. annuum* found an average of 2.78 alleles per locus and reported maximum 4 alleles per pair of initiators, and concluded that the AFLP detected greater polymorphism than the SSR markers. Albrecht *et al*., (2012) in their study (where accessions of *C. baccatum*, *C. annuum*, *C. chinense* and *C. frutescens* were evaluated), of the five accessions of *C. chinense* included in their investigation, found only five bands and concluded that *C. chinense* and *C. frutescens* species were related. Lijun & Xuexiao (2012), used ISSR markers type to estimate the population structure in accessions of C*apsicum* spp., the ISSR´s used per them amplified 135 bands of which 102 were polymorphic, the band size was 100 to 200 bp, these results showed that the AFLP´s reproduced higher number of bands and were more polymorphic in pepper chili than other types of markers Ibarra-Torres *et al*., (2015) utilized ISSR and SSR in Capsicum *annuum* and Capsicum *pubescens* and reported 38 bands with weights between 150 to 6000 bp.

The analysis at an intrapopulation level for 15 genotypes and haplotypes with 1722 useful loci, different at the haplotype level indicated that *C. chinense* from Mucuychacan, Campeche, showed the highest polymorphism value (1371 polymorphic sites), followed by the populations of Tabasco. The total polymorphism of the populations evaluated was 99.8% (Table 4), Islam *et al*., (2016), in *C. annuum*, found 61% polymorphism, a value lower than that found in our research. On the other hand in lotus (Nelumbo), Fu (2011), using seven AFLP primers and reported the bands number ranging from 75 to 300 bp and 28.1% polymorphism, Aktas *et al*., (2009) found 26% polymorphism and 12.5 polymorphic bands per primer, according to these results it could be established that the effectiveness of molecular markers, in this case AFLP, could be depend to a great extent on the species in which they are applied. The lowest molecular diversity index was presented by the population of Cucuyulapa and the highest one by Mucuychacán, Campeche, with respect to the other two populations of Habanero chili pepper from Tabasco.

The analysis of molecular variance (AMOVA), showed significance $p \leq 0.05$ (Table 5) with variance between and within populations of 14.14 and 85.86.0%. Similar results (15.26 and 84.74%) were reported by Nimmakayala *et al*., (2014), in their study of linkage disequilibrium and population structure analysis with SSR in *C. annuum*, Islam *et al*., (2016), while working with 171 collections of *C. annuum*, found percentages of variance explained between and within accessions of 45 and 54%. The percentage of variance between populations reported by Islam *et al*., (2016) exceeded that was reported in our work, but the percentage of variation within populations was lower than that found in our investigations. On the other hand, Albrecht *et al*., (2012) reported 25 and 75% for variation between accessions and within accessions. The discrepancy between the percentages of variation between and within populations reported in both investigations, may be due to the fact that they evaluated accessions of *C. annuum*, *C. frutescens* and *C. chinense*, whereas in our investigation only *C. chinense* was evaluated. The value of F_{ST} was 0.13 indicate that the genotypes of each population showed moderate genetic differentiation. Almost similar to the F_{ST} of 0.1526 was reported by Nimmakayala *et al*., (2014).

Table 5. Analysis of molecular variance from populatios of *C. chinense* Jacq., from Tabasco and Campeche, Mexico.

Source	d.f.	Sum of squares	Variance components	Variation (%)
Between populations	3	2271.334	35.9270	14.14
Wihtin populations	56	12219.467	218.2048	85.86
Total	59	14490.800	254.1318	100

AFLP genetic analysis formed a cladogram (Fig. 1) with five groups: the first included 10 plants, seven were of Mucuychacán (MC), two from Rancheria El Habanero (RH) and one from Santa Cruz Tacotalpa (SCTP). The second group consisted of the largest number of plants (16), of these 15 were of Cucuyulapa, and one of Santa Cruz Tacotalpa (SCTP), the third group had 8 habaneros from Mucuychacán with three from Ranchería El Habanero, the fourth group conglomerated 10 plants of Ranchería El Habanero, and the fifth group included 13 individuals from Santa Cruz Tacotalpa (SCTP).

The population structure of Habanero chili´s from Tabasco contrasts with the one reported for Brazilian habanero peppers by De Freitas (2007), pointed out the formation of major groups of these chili peppers and high genetic variability among them. This difference could be explained as (De Freitas, 2007) two AFLP oligonucleotides with different sequential structure. Too, it could have contributed to this difference in the results found by De Freitas (2007), the method of grouping employed (Neighbor-joining) that is different from the one used in the present work (UPGMA). Dhaliwal *et al*., (2014) found that their populations were grouped into nine clusters and that oligonucleotides AFLP detected greater polymorphism than SSRs, hence more clusters were formed.

On the other hand, Nimmakayala *et al*., (2014), reported with SSR that the evaluated cultivars of *C. annuum* formed five clusters, and Taranto *et al*., (2016) found that variants of C*apsicum* spp. were grouped according to geographical origin and the characteristics of the fruit. These differences in the evaluated germplasm might be due to the geographical origin of each germplasm. Rana *et al*., (2014) when using RAPD and ISSR, found on average 42 to 44% polymorphism, and variants of *C. annuum* formed defined groups except the genotypes ACC-2 and Mahog, with which they suggested that by crossing they would form a segregating population with maximum genetical diversity.

The phylogenetic tree obtained from AFLP data showed clustering of habanero accessions based on their geographical origin (Fu, 2011, found similar results in Lotus), and degree of diversity, where the Campeche accessions were more diverse and with greater genetic dissimilarity with respect to those of Tabasco. In addition, the AFLP primers were discriminant within the same species when separating plants according to their collection of origin. It is possible that the similarity showed between plants of the Cucuyulapa and Mucuychacan poputlations is due the exchange seeds carried out by the sowers of habanero pepper in these localities. Also among the Habanero chilli peppers of Tabasco high variability and polymorphism was observed, which reflected the absence of better defined genetic groups, which might be due to the creole nature of this crop, or certain selective management by the farmers of the area, the gene flow between germplasm and / or the possible genetic recombination between them (Fig. 2).

The populations analyzed were structured in three groups K = 3, this value is similar to that reported by Taranto *et al*., (2016), and found that delta K = 3, grouped the accessions in three clusters too. On the other hand, González-Pérez *et al*., (2014); and Lee *et al*., (2016), found values of delta K = 6 and delta K = 10, which could be explained by the different variants of the chili germomplam that were used in their research. In our work, the first group (cluster I), included plants of Ranchería El Habanero (13), Cucuyulapa (8) and the total (15) of Mucuychacán, Campeche, with value of F_{ST} = 0.2580, the second cluster grouped the 15 individuals of Santa Cruz Tacotalpa with value of F_{ST} = 0.2433 and the third group or cluster includes two individuals of Rancheria El Habanero plus seven of Cucuyulapa with value of F_{ST} = 0.5017. Similar results were also obtained by Nicolaï *et al*., (2013); Hill *et al*., (2013), although they included in their work different varieties of chili, among them the 'Campana' and small spicy ones, as well as cultivars of *C. chinense*, *C. frutescens* and *C. baccatum*, and *C. chacoense*, *C. galapagoense*, *C. pubescens*, *C. baccatum*, *C. praetermissum* (Taranto *et al*., 2016). Lee *et al*., (2016) reported different population structure and greater number of clusters (10), which could be explained because they included different variants of *Capsicum* namely of *C. cardenesi*, *C. chacoense y C. galapoense*.

Using four AFLP, De Freitas (2007) characterized morphologically and molecularly some accessions of *C. chinense* Jacq., from Brazil, and reported that the morphological descriptors (61 different) were adequate to characterize the genetic diversity of this species and indicated that the characteristics of fruit color and place of origin of the collection or accesion showed less association with the dissimilarity as opposed to the shape of the fruit. However, two AFLP´s markers were efficient to characterize chili habanero germplasm. Although, no relationship was found between the genetic similarity and the popular names or the origin places of the samples, dissimilarity was found between 2 and 47.9% with an average of 22.3%, which showed that there was genetic variability in *C. chinense*.

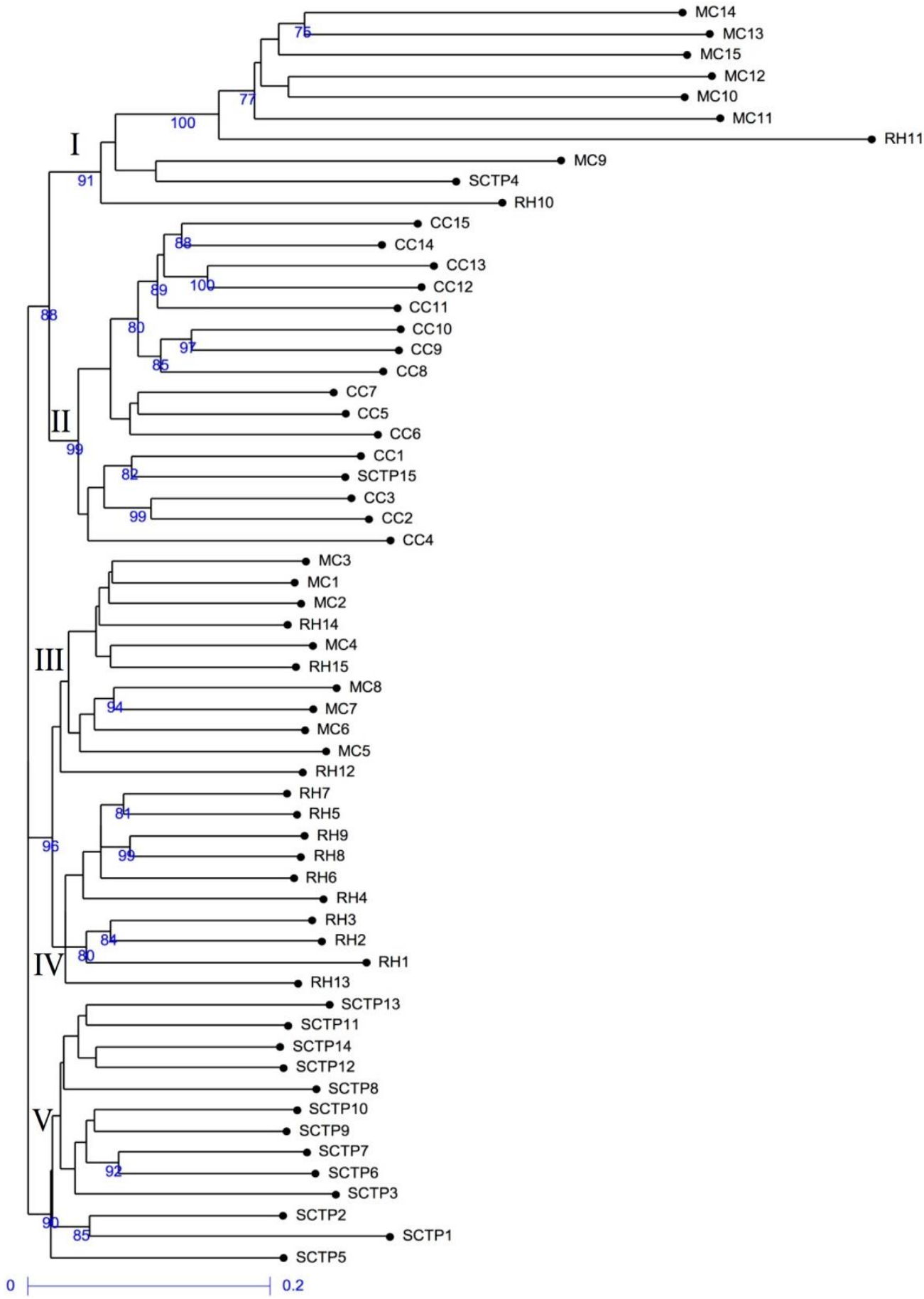

Fig. 1. Dendrogram of 60 Habanero chilli pepper plants (*C. chinense* Jacq.) using the UPGMA method.

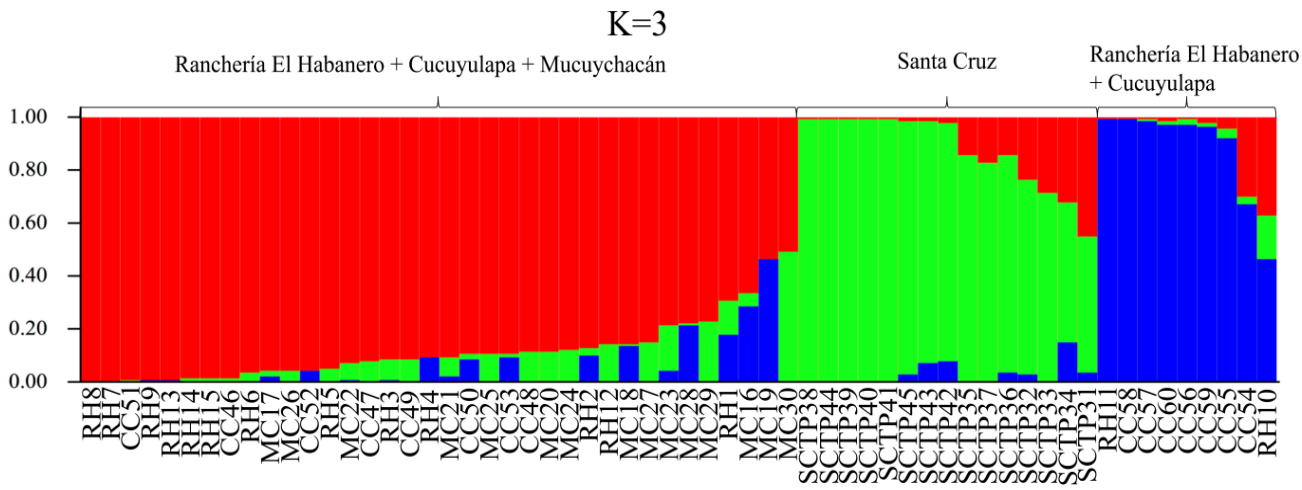

Fig. 2. Population structure determination through AFLP of 60 habanero (*C. chinense* Jacq.) chili pepepr plants.

Conclusions

Differences significantives were found in the genetic diversity patterns of *C. chinense* Jacq. The population of Mucuychacan, Campeche, behaved as the most diverse intra-population and genetically different from the cultivated populations of Habanero chili´s from Tabasco. The AFLP´s used in this study were adequate to differentiate and characterize the chili plants studied. Based on the results found in the present research, it can be established the ocurrence of a possible effect of selection by producers on the populations of habanero cultivated chili in Tabasco and Campeche states.

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