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Characterization of the genetic structure and diversity of maize (*Zea mays* L) landrace populations from Mexico

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Abstract

Maize (*Zea mays* L) is a globally important crop. In Mexico, its center of origin and diversity, it forms part of the culture and staple diet of present-day towns. This condition has allowed the development of a large number of maize landraces. In southern Mexico, in the states of Yucatán, Quintana Roo, Campeche and Chiapas, little attention has been given to the diversity potential that landraces represent in order to make best use of them. This work therefore characterized the genetic structure and diversity of 16 maize landrace populations from the southern region of Mexico using ISSR markers. A total of 69 loci were generated with 100% polymorphism. The analysis revealed the formation of two groups with geographical and genetic origins in the Yucatán Peninsula and the state of Chiapas. Wide genetic diversity was found for the entire sample of assessed populations (He = 0.40 and I = 0.54), as well as in the groups formed (*He* = 0.36, *I* = 0.52 and *He* = 0.35, *I* = 0.50 respectively). At the population level within each group, the populations that formed the Yucatán Peninsula group had greater mean diversity values than in the Chiapas group (*He* = 0.33, *I* = 0.36 and *He* = 0.32, *I* = 0.35 respectively). Based on this information, it is recommended to develop in-situ conservation programs and to increase the existing ex-situ collections in the region that allow us to enhance the germplasm of the maize landraces of Mexico in breeding programs.

Keywords: *Zea mays* L, genetic diversity, landraces, molecular markers

Introduction

Mexico is considered the center of origin, domestication and diversity of maize (*Zea mays* L), a condition that has allowed for the development of a wide variety of landraces. This diversity is largely due to the domestication and selection process performed by indigenous farmers, who are the principal heirs, custodians and breeders of native germplasm (Matsuoka et al, 2002; Fernández et al, 2013).

In southern Mexico, the area composed of the Yucatán Peninsula (Yucatán, Quintana Roo and Campeche) and the state of Chiapas has very particular physiographic, vegetation and agro-ecological characteristics, resulting in wide environmental diversity and ecological niches (Mijangos, 2010). This environmental diversity, in conjunction with the selection process by indigenous farmers, has led to wide morphological and genetic diversity of landraces (González et al, 2013).

In recent decades, a decreasing trend of planting maize landraces in southern Mexico has been reported (Perales and Hernández, 2005; Arias et al, 2007). Maize landrace populations therefore show signs of genetic erosion and a reduction in in-situ conservation, causing the loss of new, exotic and favorable traits with agronomic potential (Arias et al, 2007; Martínez et al, 2008). Fenzi et al (2015) mention that, despite the introduction of improved maize throughout Mexico, farmers in the Yucatán Peninsula still preserve maize landraces, highlighting the importance that these genotypes represent in traditional agriculture. Despite their importance, few studies have been conducted to determine the genetic diversity possessed by these genotypes. Several authors have studied the diversity of maize landrace populations based on morphological traits (Burgos et al, 2000; Camacho and Chávez, 2004; Martínez et al, 2006; Arias et al, 2007). However, for a robust analysis of diversity, combined geographical, morphological and molecular data must be taken into account (Castiñeiras et al, 2007; González et al, 2013).

In this regard, molecular markers are increasingly being used in the genetic analysis of plants. In addition to being highly polymorphic, they are more stable and less influenced by environmental factors (Azofeifa-Delgado, 2006).

Among the molecular techniques used to study genetic diversity in different crops, markers known as

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inter simple sequence repeats (ISSR) offer one of the simplest and most widely used techniques (Carvalho et al, 2002; Amaral Júnior et al, 2011; Idris et al, 2012; Lenka et al, 2015). This technique involves the amplification of a DNA segment present in an amplifiable distance between two identical repeat microsatellite regions in opposite directions (Vijayan, 2005). In addition, ISSR markers are dominant, making them useful for studies of genetic diversity, phylogeny, genetic coding, genomic mapping and evolutionary biology (Lenka et al, 2015). Based on the above, the aim of this work was to characterize the structure and genetic diversity of maize landrace populations in Mexico using ISSR markers.

Materials and Methods

Plant material and DNA extraction

Sixteen maize landrace populations from four geographic areas in southern Mexico (Yucatán, Campeche, Quintana Roo and Chiapas) where the landraces are still cultivated were used (Figure 1; Supplementary Table 1). Three populations were obtained from the maize base collection of the Centro de Investigación Científica de Yucatán (CICY) kept at the Parque Científico y Tecnológico de Yucatán, Mexico. Three populations were gathered from a seed fair in the state of Yucatán and 10 populations were collected from farmers in the Yucatán Peninsula and the state of Chiapas. Fifteen individuals per population were germinated using the between-paper method described in Rao et al (2006). Total DNA was extracted from young leaves from a total of 240 individuals using the DNeasy® Plant Mini Kit (QIAGEN) following the supplier's indications. DNA quality was verified by electrophoresis in 1% agarose gel stained with 4 µl of ethidium bromide in 0.5x TBE buffer solution.

PCR amplification and DNA electrophoresis

Six ISSR primers reported in previous studies (Carvalho et al, 2002; Amaral Júnior et al, 2011; Idris et al, 2012) that have shown good amplification results and high levels of polymorphism were selected.

PCR was performed in a final volume of 20 µl containing 1 µl of DNA (10 ng), 2.0 µl 10x buffer, 0.8 µl $MgCl_2$ (50 mM), 0.4 µL dNTPs (10 mM), 0.8 µl primer (10 mM) (Invitrogen) and 0.2 µl Taq DNA polymerase (5 U μl^{-1}) (Invitrogen) suspended in 14.8 µl of ultra-pure water. DNA amplification was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City CA) programmed for 4 min denaturation at 94ºC, followed by 35 cycles of 2 min at 94°C, 1.5 min annealing with temperatures ranging from 41 to 51.5°C depending on the primer used, 1 min at 72ºC and final extension for 7 min at 72°C.

Genetic structure analysis

To assess the genetic structure of the populations, an individual assignment test was performed using STRUCTURE v. 2.3.4 software (Pritchard et al, 2000). Individuals were assigned to a number of groups (K) using a clustering model based on a Bayesian approach by means of an adjustment of the Hardy-Weinberg (HW) equilibrium and linkage disequilibrium. The admixed ancestry model with correlated allele frequencies was used. For each K value (values from one to nine were tested), a total of 10 independent simulations were run. Each simulation consisted of a burn-in period of 10,000 followed by 100,000 Markov chain Monte Carlo (MCMC) steps. The optimal K value was chosen according to the ΔK statistic proposed by Evanno et al (2005) using STRUCTURE HARVESTER (Earl and VonHoldt, 2012). The optimal K value inferred was used to obtain the ancestry graph with STRUCTURE software by conducting a last run using a burn-in period of 10,000 followed by 100,000 MCMC steps. The data generated by STRUCTURE were then used to divide the entire sample into subgroups (called Yucatán Peninsula and Chiapas), for which several diversity and genetic structure indices were measured. Given the dominant nature of ISSR markers, genetic differentiation was calculated by Wright's differentiation index (F_{ST}) with AFLP-Surv 1.0 software (Vekemans, 2002). To calculate the allele frequency, the Lynch and Milligan (1994) square-root model was used, assuming that the genotypic proportions remain in Hardy-Weinberg equilibrium. To give additional support to Wright's F statistic, an analysis of molecular variance (AMOVA) was performed with GenAlEx v.6.5 software (Peakall and Smouse, 2012).

Genetic relationships among 16 maize landrace populations from Mexico

To assess genetic relationships among populations, a dendrogram was constructed using UPGMA algorithm linkage and Nei's genetic distance modified by Lynch and Milligan (1994). The tree topology was assessed by bootstrap analysis with 1,000 replicates using CONSENSUS software from the PHYLIP package (Felsenstein et al, 2005). The tree was displayed and edited with MEGA 6 software (Tamura et al, 2013).

Genetic diversity analysis

Genetic diversity was quantified on two levels: 1) for the entire sample; and 2) between the two groups

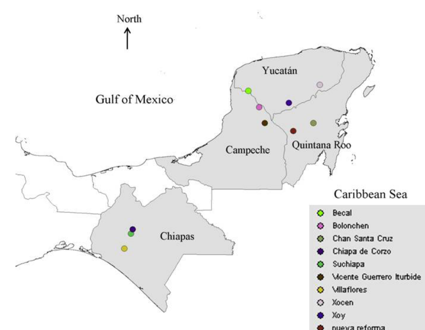

Figure 1 - Locations of origin of the 16 maize landrace populations from Mexico.

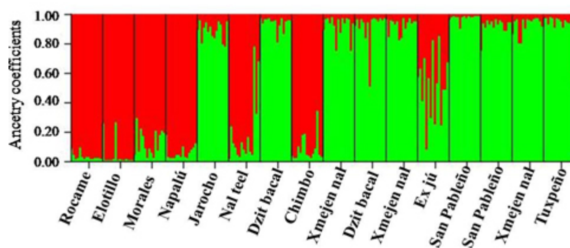

Figure 2 - Inferred population structure for K = 2 groups colored red and green. Each individual is represented by a thin vertical line, divided into two colored segments that represent the individual estimated membership to each group.

identified with STRUCTURE software, called Yucatán Peninsula and Chiapas. To determine whether there were statistically significant differences, Student's t-test was performed on values of genetic diversity between the groups formed with a significance level of $P < 0.05$ using InfoStat software (Di Rienzo et al, 2011). Given the dominant nature of ISSR markers, genetic diversity was calculated by the Shannon-Weaver index (I). In addition, the expected heterozygosity (*He)* was calculated as a measure of comparison with other studies that mostly use co-dominant markers. For calculation of the diversity indices [number of polymorphic loci (*P*), percentage of polymorphic loci (*%P*) and expected heterozygosity (*He*)], the Lynch and Milligan (1994) square-root model was followed, assuming that populations remain in Hardy-Weinberg equilibrium. All the above parameters were calculated with AFLP-Surv 1.0 software (Vekemans, 2002). The Shannon-Weaver diversity index (I) was calculated with PopGen version 1.31 software (Yeh et al, 1999).

Results

Genetic structure of 16 maize landrace populations from Mexico

The ΔK statistic method of Evanno et al (2005) (Supplementary Figure 1) suggested that the entire sample was composed of two genetically distinct groups (K = 2). The bar plot (Figure 2) shows the distribution of the populations in the two groups found (group one colored red and group two colored green respectively) and the grouping of populations was observed to be consistent with the geographical origin of the populations. Group one (red), was composed mostly of populations from the state of Chiapas, Mexico, named the Chiapas group. Group two (green) mostly included populations from the Yucatán Peninsula, and was therefore called the Yucatán Peninsula group.

Focusing on percentages of membership of the 16 populations within each of the observed groups (Chiapas and Yucatán Peninsula), the Ex jú population was the only one that did not show a clear trend, with a higher percentage of membership (51%) to the Yucatán Peninsula group than to the Chiapas group (49%). The genetic differentiation values (Table 1) were high for the entire sample (*Fst* = 0.18). Nevertheless, they were higher at the group level (*Fst* = 0.22 and *Fst* = 0.24 for the Chiapas and Yucatán Peninsula groups respectively), supporting the formation of the two groups observed with STRUCTURE. At the entire sample level, 33% of the variation was explained by differences within populations (*Hs*) and 27% by differences within the groups (Table 1). These results agree with the analysis of molecular variance (AMOVA), which indicated that 66% of the total variance was distributed within populations, while 29% was distributed among the populations and 5% between the observed groups.

Genetic relationships among 16 maize landrace populations from Mexico

The UPGMA dendrogram (Figure 3) showed a similar topology to the one observed in the STRUCTURE analysis: two main groups were formed (here called A and B), mainly composed of populations from the Yucatán Peninsula (group A) and central Chiapas (group B). Within group A, two subgroups (A1 and A2) were formed. The A1 subgroup was essentially composed of populations from the transition zone from Campeche-Chiapas and the A2 subgroup was formed by populations from the southeast Yucatán Peninsula. The second group (B) was essentially formed by populations from central Chiapas.

With some exceptions, in general the topology observed in the STRUCTURE and UPGMA results agree with each other. Within the exceptions, it is important to indicate the incorporation of the Elotillo population in the Yucatán Peninsula group, when in the STRUCTURE analysis it belongs to the Chiapas group. Despite the Nal tel population originating in the Yucatán Peninsula, both analyses placed it in group B, composed of populations from Chiapas. Conversely, despite the Jarocho population having its origin in the central zone of Chiapas, both analyses located it in group A, composed of populations from the Yucatán Peninsula. Finally, the Xmejen nal and Dzit bacal populations, isolated to both groups, have their origin in a seed bank.

Genetic diversity of 16 maize landrace populations from Mexico

A total of 69 loci were generated with a range of 200 to 2,000 bp and 100% polymorphism (Supplementary Table 2). Primers $(AG)_8T$ and $(GA)_8T$ generated the greatest number of loci with 17 and 16 respectively. Both genetic diversity indices evaluated (*He* and *I*) were high for the entire sample (0.40 and 0.54 respectively). At the group level, the percentage

Table 1 - Genetic structure of maize landraces from Mexico based on six ISSR primers.

Level	*n*	*Hs* (SE)	*Fst* (SE)
Entire sample	16	0.33 (0.003)	0.18 (0.08)
Chiapas	6	0.27 (0.008)	0.22 (0.146)
Yucatán Peninsula	10	0.27 (0.006)	0.24 (0.072)

n - population number, *Hs* - heterozygosity within population, *Fst* - genetic differentiation, *SE* - standard error

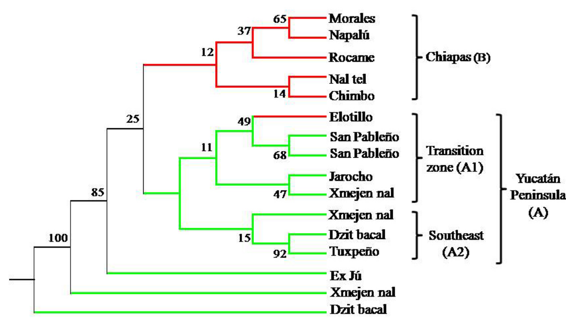

Figure 3 - UPGMA dendrogram based on Nei's genetic distance (1972) modified by Lynch and Milligan (1994). Populations are colored according to cluster analysis by structure in Figure 2.

of polymorphic loci (%*P*), expected heterozygosity (He) and Shannon index (I) estimators for the Yucatán Peninsula group had higher values compared to the Chiapas group (98.6%, 0.36, and 0.52, respectively; Table 2). Nevertheless, no statistically significant differences were found for the diversity values (*He* and *I*).

The percentage of polymorphic loci (%*P*), expected heterozygosity (*He*) and Shannon index (*I*) were calculated at the population level within the groups formed by STRUCTURE software. Within the Chiapas group, the highest values of expected heterozygosity (*He*) and the Shannon index (*I*) were found in the Rocame population (*He* = 0.34, *I* = 0.39). The Morales population had the highest percentage of polymorphism and high levels of genetic diversity (%*P* = 88.4, *He* = 0.33, and *I* = 0.38). On the other hand, the Elotillo population had the lowest values of genetic diversity evaluated (%*P* = 66.7%, *He* = 0.30, and *I* = 0.27; Table 3).

Within the Yucatán Peninsula group (Table 3), the Xmejen nal population from Nueva Reforma village and the Jarocho population had the highest values of genetic diversity (*He* = 0.34, *I* = 0.40 and *He* = 0.34, *I* = 0.39, respectively). The population with the highest percentage of polymorphism was Dzit bacal, belonging to Xocen village. The lowest values of genetic diversity were found in San Pableño (%*P* = 81.2, *He* = 0.30, and *I* = 0.31).

Discussion

Molecular characterization of maize landraces from Mexico

ISSR markers have been successfully used in maize diversity analysis by different authors (Carvalho et al, 2002; Amaral Júnior et al, 2011; Idris et al, 2012). In this study, the six ISSR primers selected generated a total of 69 loci with 100% polymorphism. Compared to Carvalho et al (2002), who evaluated the diversity of Brazilian maize landraces, in our study primers $GATA_4$ and $GACA_4$ generated a higher percentage of polymorphism. Using the primers $(GA)_8T$ and $(AG)_8YT$, a total of 16 and 15 polymorphic loci were generated, respectively. These numbers were greater than those reported by Amaral Júnior et al (2011) with popcorn populations in Brazil. Likewise, Idris et al (2012) studied the variation in maize populations of Sudan and found fewer loci and a lower percentage of polymorphism with primers $(AG)_8T$ and $(GA)_8T$, but a greater number of loci and a higher percentage of polymorphism than we reported here with the primer $GATA_4$. These differences found with the same primer could mainly be due to the fact that Mexico is the center of origin and domestication of maize. Also, our study evaluated maize landrace populations that are genetically more diverse.

Table 2 - Genetic diversity observed at different levels of maize populations from Mexico.

Level	*n*	*P*	%*P*	*He*	*I* (ES)
Entire sample	16	69	100	0.40	0.54 (0.15)
Chiapas	6	66	95.7	0.35^{ns}	$0.50 (0.20)^{ns}$
Yucatán Peninsula	10	68	98.6	0.36^{ns}	$0.52 (0.16)^{ns}$

n - population number, P - polymorphic loci number, %P - percentage of polymorphism, He - expected heterozygosity, I - Shannon index, SE - standard error, ns - not significant

Population structure of maize landraces from Mexico

The analysis based on the STRUCTURE model identified two genetically differentiated groups congruent with their geographical origin. The dendrogram obtained with the UPGMA algorithm, despite some exceptions, corroborated the grouping pattern detected by the STRUCTURE analysis (Figures 2 and 3). Among the exceptions in the UPGMA are the Ex jú, Xmejen nal and Dzit bacal populations, which originated in the Yucatán Peninsula. In the first case, the non-inclusion of the Ex jú population within one of the groups observed could be due to the high degree of crossing, as revealed by the individual assignment analysis (Figure 2). In the following two cases (Xmen nal and Dzit bacal populations), the result could be due to the origin of the seeds used. In this study, both populations come from a gene bank where regeneration processes are rare. In this regard, Rocandio-Rodriguez et al (2014) mention that the regeneration process in gene banks occurs outside the original environment, causing genetic isolation.

Also, despite the Nal tel population having originated in the Yucatán Peninsula, both analyses (STRUCTURE and UPGMA) placed it within group B composed of Chiapas populations. Conversely, despite the Jarocho population having its origin in the central zone of Chiapas, both analyses located it in group A, composed of populations from the Yucatán Peninsula. These changes are strongly supported by the individual assignment analysis (Figure 2). The reason could be related to the exchange of seeds among farmers, since the Nal tel population comes from a collection made at a seed fair. In this regard, Van Etten and de Bruin (2006) and Castiñeiras et al (2007) mention the complexity of seed exchange and supply networks among rural communities. According to

Table 3 - Genetic diversity observed in maize landrace populations within each of the groups formed

Group/population	Village	%*P*	*He* (ES)	*I* (ES)
Chiapas				
Rocame	Chiapas de Corzo	82.6	0.34 (0.02)	0.39 (0.27)
Morales	Villaflores	88.4	0.33 (0.02)	0.38 (0.27)
Nal teel	Xoy	81.2	0.33 (0.02)	0.36 (0.26)
Chimbo	Suchiapa	82.6	0.31 (0.02)	0.36 (0.28)
Napalú	Suchiapa	82.6	0.31 (0.02)	0.34 (0.29)
Elotillo	Villaflores	66.7	0.30 (0.02)	0.27 (0.27)
Mean			0.32 (0.02)	0.35 (0.27)
Yucatán Peninsula				
Xmejen nal	Nueva Reforma	89.9	0.34 (0.02)	0.40 (0.27)
Jarocho	Chiapa de Corzo	89.9	0.34 (0.02)	0.39 (0.26)
Dzit bacal	Xoy	87.0	0.32 (0.02)	0.38 (0.26)
Xmejen nal	Chan Santa Cruz	85.5	0.34 (0.02)	0.37 (0.27)
Tuxpeño	Xoy	81.2	0.32 (0.02)	0.37 (0.28)
Dzit bacal	Xocén	91.3	0.33 (0.02)	0.35 (0.25)
San Pableño	Bécal	87.0	0.33 (0.02)	0.35 (0.28)
Xmejen nal	Bolonchén	87.0	0.33 (0.02)	0.34 (0.29)
Ex Jú	Vicente Guerrero	85.5	0.33 (0.02)	0.34 (0.28)
San Pableño	Vicente Guerrero	81.2	0.30 (0.02)	0.31 (0.28)
Mean			0.33 (0.02)	0.36 (0.27)

%*P* - percentage of polymorphism, *He* - expected heterozygosity, *I* - Shannon index, *SE* - standard error

their preferences, farmers access seeds from other families, communities, villages and even between regions (Castiñeiras et al, 2007). In this regard, voluntarily or not, the seed flow between local populations can easily change the characteristics of a population. Furthermore, it is common to find populations with names that correspond to landraces. However, not all names assigned to local populations really correspond to landraces. Therefore, we must be careful in assuming that the local name is equal to the name of the landrace (Camacho and Chavez, 2004).

The genetic differentiation values (*Fst*) found for the total area and groups (0.18 and 0.24, respectively) indicated that 18 and 24 percent of the total variation was distributed between the populations studied. According the criterion of Snyder et al (1985), who consider *Fst* values ranging between 0.15 and 0.25 to be indicative of high genetic divergence, our results indicated that maize landrace populations in the region have a high level of genetic differentiation, showing that the populations under study are being efficiently conserved by local farmers with a high level of genetic identity of their populations. These results support the results of the STRUCTURE and UPGMA analyses.

Similarly, López et al (2009) observed similar values of genetic differentiation (*Gst* = 0.15) in the Isthmus region. In other cases, higher values are reported, such as Rocandio-Rodriguez et al (2014) for the central region of the high valleys of Mexico, with values of *Gst* = 0.24. Reif et al (2006), using 25 maize accessions from 24 different races and microsatellite markers, found a value of 0.21 for genetic differentiation for the total populations studied. An exceptional case is reported by Pressoir and Berthaud (2004), for the valleys of Oaxaca, with a genetic differentiation value of 0.003 between the villages studied. These differences observed between our results and those reported by the authors mentioned above can be attributed to the type of marker, the number of populations used and the area explored in each study. Overall, the above cases (except for Pressoir and Berthaud, 2004) are categorized by high genetic differentiation and limited gene flow between populations. As such, it has been found that despite the high gene flow and seed exchange that may exist in a region, genetic differentiation of maize is strongly influenced by the selection processes performed by farmers in each production cycle (Pressoir and Berthaud, 2004).

Genetic diversity of maize landraces in Mexico

The level of genetic polymorphism detected in the region was high, and similar to that found by Rocandio-Rodriguez et al (2014) for 109 maize populations of the central region of the high valleys of Mexico, who reported polymorphism of 92.8%. In South America, Carvalho et al (2002) and Amaral Júnior et al (2011) reported lower percentages of polymorphism (75.8% and 89%, respectively) with ISSR markers than those found in this study. Lenka et al (2015) analyzed 49 maize inbred lines with 12 ISSR markers and reported polymorphism values of 95%. The high values found in our work may be due to the wide genetic variation per se present in landrace populations, as well as the characteristics of the marker used.

The values of expected heterozygosity (*He*) and the Shannon index (*I*) obtained for the study area show wide genetic diversity (*He* = 0.40 and *I* = 0.54). Rocandio-Rodriguez et al (2014) reported an expected heterozygosity of 0.71 with 31 microsatellite primers and 109 maize accessions. Pressoir and Berthaud (2004) reported heterozygosity values of 0.7 in the region of the central valley of Oaxaca based on 11 microsatellite primers and 31 maize populations. Gonzalez et al (2013) used 30 microsatellite primers and 196 accessions from the Mexican Tropics and reported genetic diversity values of 0.57. However, if we consider only the Gulf of Mexico, South Pacific, and Yucatán Peninsula subgroup, genetic diversity was 0.53, which was similar to the Shannon index obtained in our work. López et al (2009) reported lower values than those found in this study, with an expected heterozygosity of 0.22 for 40 populations from the Isthmus of Tehuantepec and 19 isozyme loci. Sanchez et al (2000) evaluated 209 accessions belonging to 59 landraces from Mexico with 37 enzyme loci and reported a total genetic diversity of 0.27.

These differences in genetic diversity values are clearly related to the type of marker used and the mutagenic rate characteristic of each one, but also to sample size, the selected populations and individuals selected to represent each population. Comparisons between diversity analyses of different types of marker should always be taken with caution (Garoia et al, 2007). It is important to note that genetic diversity (*He*) calculated using dominant markers can vary from 0 to 0.5, with the maximum reached when the

frequencies of absence and presence of a band are equal. This consideration clearly indicates that it is not possible to directly compare the absolute values of genetic diversity with different marker data, particularly when comparisons are made between dominant and co-dominant markers (Bitocchi et al, 2015).

At the level of sub-areas, although estimates of diversity, expected heterozygosity (*He*) and the Shannon index (*I*) for the Yucatán Peninsula had higher values than the Chiapas group, statistically significant differences were not observed for P < 0.05. However, at the population level (Table 3) higher diversity values can be observed in the populations that constitute the Yucatán Peninsula group.

The high heterozygosity values found in this study support a wide genetic diversity and variability in the maize landrace populations evaluated for the study area. Based on this information, it is clear that in-situ and ex-situ conservation programs should be developed for the purpose of promoting the use of native maize germplasm in the region, as it is a potential source of genes that can be used in breeding programs.

Conclusion

In general, the topology observed with the STRUCTURE and UPGMA results reveals the formation of two main groups with a defined geographical and genetic origin. The genetic structure was high for the entire sample, and the same was true for both groups observed (Yucatán Peninsula and Chiapas). The analysis of molecular variance (AMOVA) indicated that genetic diversity was distributed mainly within populations and not among populations. Wide genetic diversity was found at the total level (*He* = 0.40 and *I* = 0.54), as well in the groups observed. At the population level within each group, the populations of the Yucatán Peninsula group had greater average diversity values than those of the Chiapas group. It is recommended to develop in-situ conservation programs and to increase existing ex-situ maize landrace collections in the region for assessment and use in breeding programs.

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