Genetic diversity characterization of maize populations using molecular markers

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Highlights

- *A wide genetic diversity was found in populations of maize races grown in the state of Chiapas, Mexico, in terms of total number of alleles, alleles per locus and percentage of polymorphic loci.*
- *More than a third were private alleles present in low frequency.*
- *The molecular information analyzed together for racial classification provides a strong foundation to understand the diversification and evolutionary relationships that exist between maize varieties in the state of Chiapas, Mexico.*

Abstract

Seventy-three maize populations were characterized to estimate the genetic distribution and structure of 8 maize races from the state of Chiapas, in addition to a population of the Balsas race of teosinte (*Zea mays* ssp. *parviglumis* Iltis & Doebley). A total of 31 microsatellite *loci* were evaluated in 25 individuals from each population, estimating their genetic diversity and Wright F statistics. The populations were grouped based on principal component and cluster analyses. A total of 787 alleles were counted, with an average of 25.4 alleles per *locus* and 91.8% polymorphic *loci*. Likewise, in the studied populations, 294 exclusive alleles were detected with low frequency, representing 37% of the total alleles. The populations from Zapalote Grande and Tepecintle races were the most differentiated, forming separate, better-defined groups, while the populations from Comiteco, Otolón, and Negro de Chimaltenango races tended to group, showing a relatively scattered allocation within the races. The F_{ST} statistic (differentiation index) was 0.197, indicating that 80.3% of the genetic variation was found among individuals within the accessions, which suggests that, under the current status of Chiapas maize populations, it would be more efficient to apply intra-population recurrent selection than hybridization breeding approaches.

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Introduction

Maize (*Zea mays* L.) is the most important crop with relevance in terms of land cutivated, both nationally and internationally. Mexico has broad maize diversity, with each type of maize already adapted to different local environmental conditions and agricultural systems. Mexico is the center of origin, domestication, and diversification of modern maize races, which are the result of the convergence and combination of natural and cultural processes, including the uses that have been given to this plant species through time that have led to a wide genetic diversity expressed through the different maize populations that are grouped into races (Muñoz *et al*., 2009).

The state of Chiapas represents one of the areas with the highest maize diversity in Mexico (Orozco-Ramírez *et al*., 2017), encompassing the Chiapas-Oaxaca-Guatemala region (Kato Yamakake *et al*., 2009), where maize is the most important cultivated species. In the particular case of maize populations in the state of Chiapas, genetic diversity has been the subject of little scientific attention. The studies made so far have focused on race classification (Doebley *et al*., 1985; Sánchez *et al*., 2000). In other cases (Perales *et al*., 2005; Barrera-Guzmán *et al*., 2020), they have been limited to small samples, or have referred to maize historical and cultural processes (Keleman *et al*., 2009). However,

these studies offer a limited vision of the existing diversity within each race, and little has been done with the current tools to obtain sufficient knowledge to value this richness.

Considering the above-mentioned remarks, molecular markers of simple repeated sequences (SSR) of DNA, commonly known as microsatellites, represent a technology that has proven its reliability in producing genomic fingerprints as well as in providing the description and systematization of the diversity between and within maize populations, overcoming the drawbacks presented by traditional methodologies (Prasanna *et al*., 2010). Therefore, the aim of this research was to use microsatellite markers to assess the genetic diversity, population structure, and relationships among eight maize races from the state of Chiapas, Mexico.

Materials and Methods

Genetic material

Seventy-three representative accessions of 8 maize races from the state of Chiapas, Mexico were analyzed: 19 of Zapalote Grande, 16 of Tepecintle, 13 of Comiteco, 11 of Olotón, 5 of Tehua, 4 of Motozinteco, 3 of Negrito, and 1 of Negro de Chimaltenango, in addition to a teosinte population of the Balsas race (*Zea mays* ssp. *parviglumis* Iltis & Doebley). The sample seeds were provided by the germplasm banks of the International Maize and Wheat Improvement Center and by the Mexican National Institute for Forestry, Agriculture, and Livestock Research, both located in Texcoco, Mexico.

DNA extraction and polymerase chain reaction amplification

Total DNA isolation was performed from a sample of 100 mg of mesocotyl, coleoptile, and leaf tissue from 25 5-day-old seedlings from each accession, for a total of 1825 individuals, using a commercial kit (ChargeSwitch® gDNA plant kit, Invitrogen, Waltham, MA, USA) with magnetic beads and automated equipment (KingFisher® Flex Thermo Scientific, Whaltham, MA, USA). To quantify the DNA concentration, an ultra-low volume spectrophotometer was used (Nanodrop 2000, Thermo Scientific, Whaltham, MA, USA), taking into account absorbance readings at 260 nm and a ratio of 260/280 nm to verify the DNA quality. The amplification of the microsatellite fragments was done by polymerase chain reaction (PCR) using primers labeled with fluorescent molecules 6-FAM, HEX, or ROX in the 5' extreme to be identified during capillary electrophoresis. The reaction mixture was a multiplex PCR (several primers simultaneously) in a final volume of 25 µL. The components of each reaction were 4 µL of buffer 0.8X, 1.2 mL of $MgCl_2$ at 1.2 mM; 0.4 µL of dNTPs at 0.16 mM; 1 µL of each primer at 4 pmol; 0.2 µL of *Taq* DNA polymerase (1 U total); 2.5 µL of DNA diluted at 10 ng μL^{-1} and 14.7 µL of water HPLC grade. A thermal cycler (Gene AMP® PCR System 9700, Foster City, CA, USA) was used for the amplification of the microsatellite sequences with the following program: an initial denaturation period of 4 minutes at 95°C, followed by 25 cycles of 1 minute at 95°C of denaturation, 2 minutes at 55°C of alignment, 2 minutes at 72°C of extension; and a final extension of 60 minutes at 72°C. A total of 31 *loci* of microsatellites were evaluated (Table 1), distributed throughout the maize genome. Information on the specific primers can be found at the Maize Genetics and Genomics Database (http://www.maizegdb.org/ssr.php#).

Identification of DNA fragments

The PCR products were processed in a DNA sequencer (Genetic Analyzer ABI 3130®, Applied Biosystems, Foster City, CA, USA) using GeneScan 500 LIZ as the internal marker standard. The program GeneMapper® V. 4.0 was used (Applied Biosystems, Foster City, CA, USA) to interpret the output electropherograms and to determine the size of the fragments required to create a database with the allelic information of each marker per individual.

Statistical analysis

The program POPGENE 1.31 (http://www.ualberta.ca/~fyeh/popgene.pdf) was used to determine diversity parameters, such as the total number of alleles, number of alleles per *locus*, number of exclusive alleles, proportion of polymorphic *loci*, and expected heterozygosity. To determine the genetic structure of the populations, F statistics developed by Wright (1965) were used, which represent the magnitude of the non-random association of alleles in an individual, describing the degree of inbreeding in a hierarchical way within the populations (F_{IS}), between the subpopulations (F_{ST}) and within the total population (F_{IT}).

Principal component analysis was performed with selected alleles based on a correlation matrix using the SAS V.9.0. program (SAS Institute, 2002). A selection of the alleles was carried out to reduce random variation between accessions, arising from the presence of alleles with very low frequency in the cluster analysis, which contribute little to the definition of the genetic structure. To avoid problems of distancing between accessions and the corresponding interpretation, which occurs when low-frecuency or exclusive alleles are involved in one-way analysis of variance with the allelic frequencies as dependent variables, and those with a significance of $p \leq 0.05$ between accessions and allele frequency above than 20%. A phylogenetic analysis was made between populations by the neighbor-joining method (Saitou and Nei, 1987) with the selected alleles, using modified Roger's genetic distance matrix with NTSYSpc V.2.21 program (Rohlf, 2009).

Results and Discussion

Genetic diversity

A total of 787 alleles were found in the 1825 individuals from 73 populations that represented eight maize races and one teosinte race, with an average of 25.39 alleles per *locus* (Table 2). Regarding these parameters, Rocandio-Rodríguez *et al*. (2014) and Herrera-Saucedo *et al*. (2019) reported lower values of 20.52 and 18.38 alleles per *locus* on average, respectively, in maize accessions from the High Valleys in Central and Northeastern Mexico. This could be due to the fact that maize populations in the current study intrinsically harbor more diversity, probably associated with the cultural richness of the different ethnic groups living in the state of Chiapas (Santillán-Fernández *et al*., 2021). By the same token, unique or exclusive alleles were found with a frequency of <0.05, corresponding to 37% out of the total number of alleles found, which also reflects the genetic richness of the populations. These alleles were found in all the assessed markers (Table 2), showing greater presence in the Zapalote Grande race. A similar case referring to the magnitude of occurrence of this type of allele was previously reported by González *et al*. (2013), who found alleles with <0.05 frequency, representing 50% of the alleles found in maize populations from the Mexican tropics. By

considering the percentage of polymorphic *loci* as the race average, we obtained a level of 91.8%, demonstrating that maize races grown in Chiapas possess broad genetic diversity. The race with the lowest percentage of polymorphic *loci* was Tehua, with 74.8%, whereas the race with the highest percentage was Motozinteco, with 95.1%. In a research study conducted by Sánchez *et al*. (2000), using 21 enzymatic systems in 209 accessions, representing 59 maize races in Mexico, the percentaje of polymorphic loci ranged between 48 and 80%, which is lower than the percentages obtained in this study.

Table 1. *Loci* of the microsatellites used in the analysis of maize populations.

Locus	Bin number	Repetitive unit Repetitiva	Fragment size (pb)
phi127	2.07	GTGC	113-132
phi051	7.06	AGG	131-143
phi115	8.03	ATAC	291-308
phi015	8.08	TTTG	73-109
phi033	9.02	CCT	234-266
phi053	3.05	ATGT	170-214
phi072	4.01	CAAA	127-164
phi093	4.08	CTAG	275-290
phi024	5.01	CCT	354-373
phi085	5.06	GCGTT	231-265
phi034	7.02	CCT	121-159
phi121	8.04	CCG	93-104
phi056	1.01	GCC	236-259
phi064	1.11	ATCC	65-115
phi050	10.03	AAGC	79-93
phi96100	2.01	ACCT	232-299
phi101249	Unknown	AGAT	111-160
phi109188	5.03	AAAG	145-175
phi029	3.04	AG-AGGG	144-176
phi073	3.05	AGC	184-200
phi96342	10.02	ATCC	230-251
phi109275	1.03	AGCT	119-149
phi427913	1.01	ACG	118-145
phi265454	1.11	AGG	216-242
phi402893	2.XX	AGC	203-247
phi346482	1.XX	AGG	114-152
phi308090	4.04-4.05	AGC	185-226
phi330507	5.02-5.06	CCG	131-151
phi213398	4.01-4.04	ACC	285-312
phi339017	1.03	AGG	139-166
phi159819	6.00-6.08	CCG	121-146

Table 2. Parameters of genetic diversity from maize populations in the State of Chiapas, Mexico.

Race	Accessions	Alleles	Alleles per *locus*	Exclusive alleles	Polymorphic *loci*, %	Expected heterozygosity
Balsas (Teosinte)	1	166	5.35	15	100.00	0.644
Comiteco	13	406	13.06	45	93.05	0.709
Motozinteco	4	262	8.45	9	95.14	0.669
Negrito	3	240	7.81	5	92.47	0.704
Negro de Chimaltenango	1	133	4.62	2	93.55	0.551
Olotón	11	374	12.06	25	92.38	0.718
Tehua	5	269	8.48	9	74.84	0.679
Tepecintle	16	474	13.32	79	93.75	0.722
Zapalote Grande	19	495	14.03	105	91.00	0.697
Total	73	787	-	294	-	
Average	-	-	25.39	-	91.80	0.677

This difference might arise from the involvement of a larger number of populations per race, resulting in the expression of higher genetic variation, apart from the fact that isoenzymatic markers, by nature, are less polymorphic than microsatellites (Azofeifa-Delgado, 2006).

The expected heterozygosity was expressed with an overall average value of 0.68. The highest values were found in the Olotón and Tepecintle races, with 0.72. The Negro de Chimaltenango race had a value of 0.55, which is the lowest among all races. Pineda-Hidalgo *et al*. (2013) reported similar values of 0.72 after assessing 28 populations from the state of Sinaloa with 20 microsatellite markers. Furthermore, Rocandio-Rodríguez *et al*. (2014) obtained a value of 0.71 after analyzing maize populations from Mexico's High Central Valleys. The values obtained in this study show that there is higher genetic diversity in maize populations from Chiapas than in maize populations from other Mexican regions.

Genetic structure of the population

The values of the F_{IS} genetic differentiation coefficient for the assessed populations showed the highest values for Negro de Chimaltenango and Balsas (teosinte) races (with 0.425 and 0.314 respectively), indicating a major deficiency in heterozygotes. The Motozinteco race had the lowest value (0.156). In general, these values show that populations deviate from Hardy-Weinberg equilibrium, since most *loci* showed an excess of heterozygotes. The loss of homozygosity became more evident in the populations of previously mentioned races with high values of F_{IS} (Table 3); which might be the result of non-random mating by successive increases of small samples in germplasm banks, consistent with the findings of other studies, such as the research work conducted by Reif *et al*. (2005) and by Rocandio-Rodríguez *et al*. (2014).

The amplitude of values in the genetic differentiation coefficient F_{ST} ranged from 0.178 in the Motozinteco race to 0.434 in the Tehua race, with an overall average of 0.197 for all the races. The latter value was higher than the value reported by Sánchez *et al*. (2000) in the same races assessed in this study and the results of Pressoir and Berthaud (2004) in their trials with landrace populations from Oaxaca, Mexico. In this study, the average value of 0.197 for the F_{ST} coefficient suggests a high level of genetic differentiation, where 80.3% of the genetic variation was found in individuals within the accessions and only 19.7% was found between accessions.

The high values of F_{IS} and F_{ST} in this study can be explained by the fact that the populations came from germplasm banks, where conservation and regeneration are done *ex situ*, in a single environment. Under such conditions, recombination occurs among a small number of individuals within the populations, leading to a greater possibility of genetic drift and subsequent inbreeding (Richads *et al*., 2010). According to this information and based on the genetic structure of the assessed populations and their current status, it is more efficient to apply intra-population recurrent selection than applying hybridization breeding approaches.

Marker-based similarity relationships among races from the state of Chiapas, Mexico

A principal component analysis based on the frequencies of the 203 selected alleles was performed. The first 16 components contributed 54.7% to the total variation. The first principal component contributed 6.5% to the total variation, while the second one contributed 5.5% (Table 4). Alleles *phi308090-M*, *phi015J*, *phi050-F*, *phi101249-D*, *phi050-G*, *phi101249-G*, *phi127-C*, *phi101249-C*, *phi159819-B*, *phi064-O*, *phi339017-H*, *phi96100-N* and *phi265454-D* were the greatest contributors to the definition of variability in principal component 1, whereas alleles *phi159819-F*, *phi050-G*, *phi056-L*, *phi072-a*, *phi072-A*, *phi015-V*, *phi121-C*, *phi072-M*, *phi96342-I*, *phi159819-H* and *phi213398-I* contributed to the definition of variability to a greater extent in component 2.

The scatter plot of populations constructed with components 1 and 2 (Figure 1) shows that populations from Zapalote Grande, Tehua and Motozinteco races had the greatest differentiation, forming better defined groups, while populations from Comiteco, Olotón and Negro de Chimaltenango races tended to form relatively scattered groups, probably as a result of the consistent gene flow among them, since at least the first 2 populations are cultivated in temperate and semi-warm neighboring areas, where seed exchange is a common practice among farmers.

Some populations from Olotón, Comiteco and Tehua races were not included in any of the corresponding groups, as was expected; instead, the populations were scattered through Quadrants I, II and III. In the study conducted by Reif *et al*. (2006), these races showed closeness, probably because they share common genetic traits as well as a degree of similarity in geographic origin. Although it is important to mention that Reif and collaborators used only one accession from each race. The Olotón and Negrito races are grown in high altitude regions, while the Comiteco race is distributed in areas of transition from temperate to semi-warm climates. Doebley *et al*. (1985) found that the Comiteco, Olotón, Tehua, Tepecintle and Zapalote Grande races were not segregated in well-defined complexes but formed a con-

Table 3. Wright F statistics estimated from 31 *loci* of microsatellites, using populations of 8 maize races from the state of Chiapas, Mexico and 1 teosinte race.

Race	F_{IS}	F_{IT}	F_{ST}
Balsas	0.314	0.314	-
Comiteco	0.188	0.385	0.243
Motozinteco	0.156	0.306	0.178
Negrito	0.183	0.372	0.231
Negro de Chimaltenango	0.425	0.425	-
Olotón	0.230	0.412	0.235
Tehua	0.219	0.558	0.434
Tepecintle	0.211	0.376	0.210
Zapalote Grande	0.186	0.385	0.244
Average	0.235	0.392	0.197

F_{IS}, inbreeding within the populations; F_{IT}, global inbreeding coefficient; F_{ST}, inbreeding between populations.

tinuum. In this regard, González *et al*. (2013) mentioned that the Zapalote Grande race is losing genetic variability because it has the lowest percentage of interracial variation (1.06%). The distribution of populations from the Tepecintle race extends through Quadrants I, III and IV, showing greater dispersion than the Zapalote Grande race, probably due to a higher gene flow with other populations. Therefore, in the current stage and at the molecular level, these races do not have a well-defined genetic identity but rather a diffuse identity. It should be noted that Ortega Corona *et al*. (2011) believed that both races were endangered.

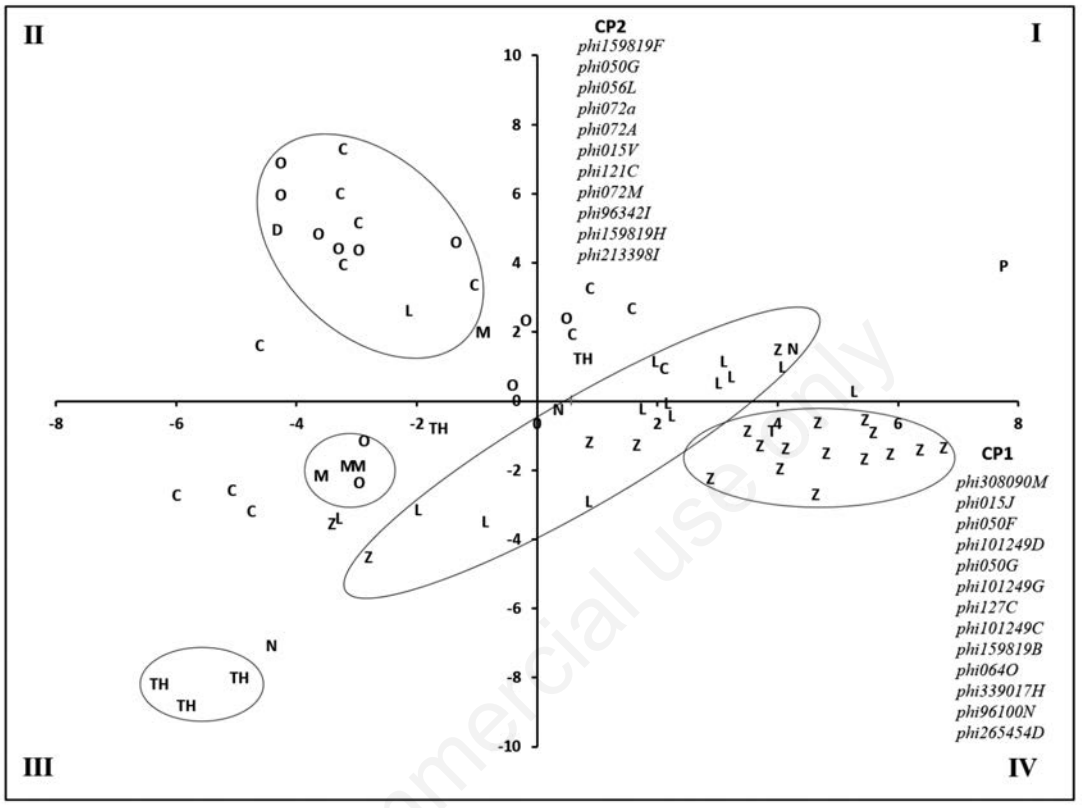

Figure 1. Scatter plot of 73 maize populations from the state of Chiapas based on the first two principal components produced by the frequency of 203 alleles (P: *Parviglumis*, C: Comiteco, M: Motozinteco, N: Negrito, D: Negro de Chimaltenango, O: Olotón, TH: Tehua, L: Tepecintle, Z: Zapalote Grande). The chart shows the most influencing alleles in every principal component.

Table 4. Eigenvalues and explained variance of the first 16 components produced with 31 *loci* from microsatellites.

Principal component	Eigenvalue	Explained variance, %	Cumulative variance, %
1	13.37	6.58	6.58
2	11.35	5.59	12.17
3	9.79	4.82	17.00
4	8.37	4.12	21.12
5	7.69	3.79	24.90
6	7.41	3.65	28.55
7	6.86	3.38	31.93
8	6.23	3.07	35.00
9	5.82	2.87	37.87
10	5.59	2.75	40.62
11	5.17	2.55	43.17
12	5.13	2.53	45.69
13	5.00	2.46	48.16
14	4.79	2.36	50.52
15	4.38	2.16	52.68
16	4.25	2.10	54.77

The previous groupings are similar to the groups shown in the phylogram of Figure 2, produced by the frequency of the 203 selected alleles, showing consistency in the associations. Populations BSSM445 and BSSM446 from the Olotón race are the closest to the teosinte from the Balsas race (*Zea mays* ssp. *parviglumis* Iltis & Doebley), which was considered an external group, suggesting that this race, and in fact races from high areas, as had already been mentioned by Vigouroux *et al*. (2008), have a genetic relationship relatively close to the immediate predecessor of cultivated maize.

A total of 6 groups were defined in general from the set of populations represented in the phylogram of Figure 2. Group 1 contains 2 subgroups: subgroup 1a mainly comprises populations of the temperate zone from the Olotón (2), Comiteco (3) and Motozinteco (3) races, while subgroup 1b included populations from the Tehua race. Group 1 concentrated the races from the temperate region mostly, with very few races from the semi-warm zone, such as the Comiteco and Tehua races.

Group 2 comprises 4 well-defined accessions from the Olotón race. However, they showed a different genetic relationship that separated them from the accessions initially represented in Subgroup 1a, where there are also populations from the Comiteco and Motozinteco races. This typical association was also mentioned by Ortega *et al.* (1991). Furthermore, Perales *et al*. (2005) reported a very narrow genetic distance (<0.02) between populations from the Olotón and Comiteco races, leading to implied similarities.

Group 3 contains most populations from Olotón race (5 populations), indicating that this race has relatively scattered genetic variability and tends to join other populations from the Comiteco and Negro de Chimaltenango races. This has been reported by Sánchez *et al*. (2000), who classified these races as being within the group of late maturity.

Group 4 mainly consists of maize populations of the semi-warm region Tehua race, although there are also some populations from the Tepecintle and Zapalote Grande races of warm regions, considered races of intermediate maturity adapted to semi-warm areas (Sánchez *et al*., 2000). Group 5 was mainly represented by 5 populations from the Comiteco race, including 3 populations from the Tepecintle race and 2 populations from the Zapalote Grande

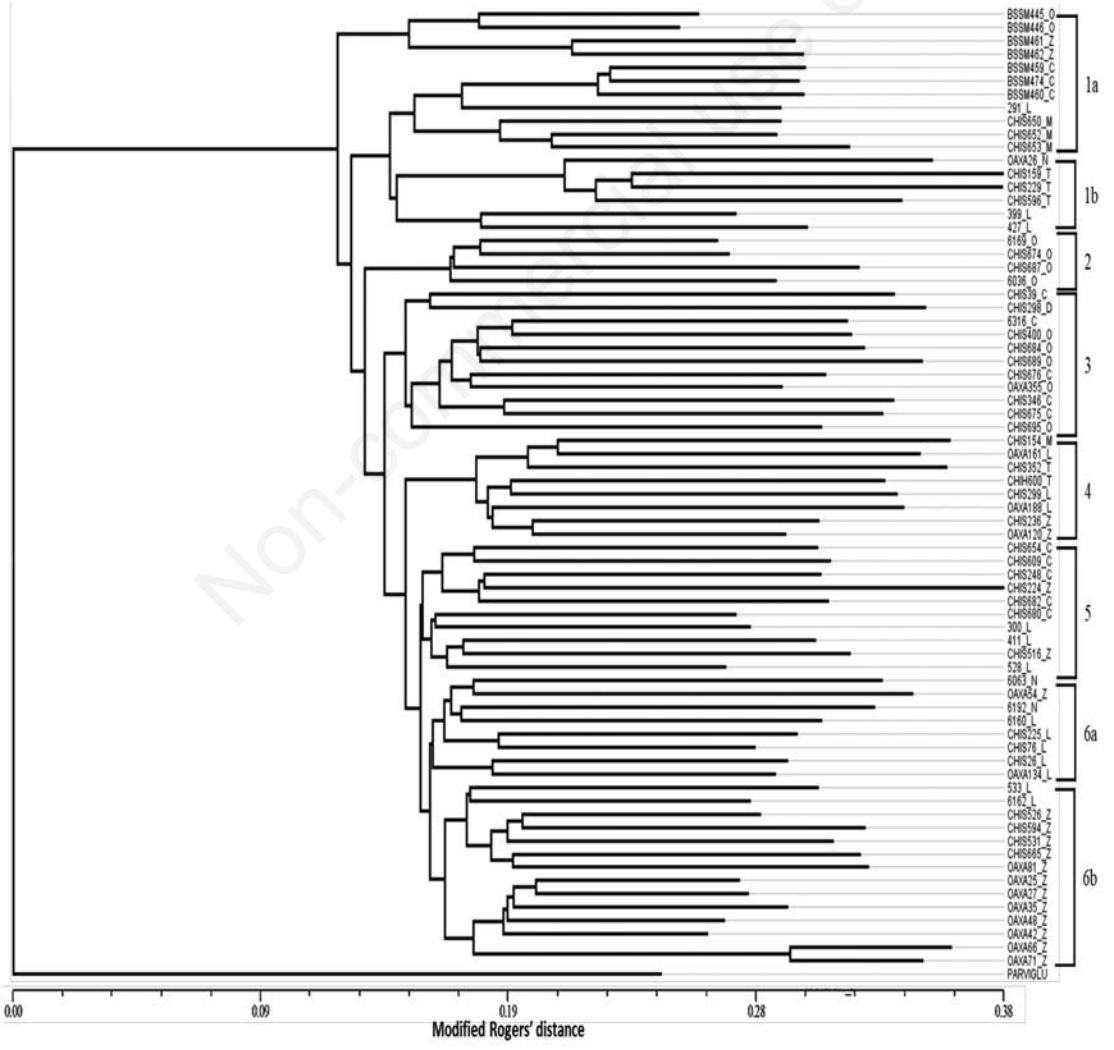

Figure 2. Phylogram of 73 maize populations from the state of Chiapas, Mexico, as determined by the Neighbor-Joining method, using modified Rogers' genetic distances, based on the frequency of the 203 selected alleles.

race. This grouping reflects the interaction between populations from warm and semi-warm regions. In this regard, Brush and Perales (2007) argued that this dynamic is the result of the ethnic diversity impacting maize race distribution beyond its primary habitats due to seed exchange among farmers. On the other hand, Perales and Hernández (2005) reasserted those remarks by mentioning that spatial diversity is greater in some regions of fast transition from temperate to warm climates.

The group with the strongest definition in terms of racial belonging was Group 6, where 2 subgroups were identified: subgroup 6a, which mostly consists of populations from the Tepecintle race, and subgroup 6b, which is formed by populations from the Zapalote Grande race. Both groups are grown in warm regions, which coincides with the grouping proposed by Sánchez *et al*. (2000). The results show that there is genetic variability in the races from the state of Chiapas, and its complexity begins at the molecular level. Some populations joined other races sharing the same origin, which explains the presence of common alleles.

Conclusions

A broad genetic diversity was found in populations of maize races growing in the state of Chiapas, expressed by the parameters describing the total number of alleles, average alleles by *locus*, and percentage of polymorphic *loci*, with more than a third of the exclusive alleles present in low frequency. The population of the Motozinteco race showed the highest percentage of polymorphic *loci*, while Olotón and Tepecintle races had the highest expected heterozygosity. Populations of the Tepecintle and Zapalote Grande races formed well-defined groups, allowing their differentiation as separate groups. Regarding the remaining races in this study, some populations that were classified *a priori* under a certain racial group were located in different groups, indicating higher genetic complexity than previously considered. There is high genetic differentiation in maize races from Chiapas, and the majority of such genetic variation is found in individuals within the same populations, reassuring the importance of using intra-population recurrent selection instead of hybridization breeding approaches as a strategy to benefit from the use of these genetic resources by generating improved open-pollinated varieties.

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