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#### ARTICLE

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# Maize genetic diversity in traditionally cultivated polycultures in an isolated rural community in Mexico: implications for management and sustainability

Karla Y. Leyva-Madrigal (D<sup>a,e\*</sup>, P. A. Báez-Astorga (D<sup>a\*</sup>, S. Negrete-Yankelevich (D<sup>b</sup>, A. Núñez-de la Mora (D<sup>c</sup>, G. Amescua-Villela (D<sup>d</sup> and I. E. Maldonado-Mendoza (D<sup>a</sup>

<sup>a</sup>Departamento de Biotecnología Agrícola, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Sinaloa, Instituto Politécnico Nacional, Guasave, México; <sup>b</sup>Red de Ecología Funcional, Instituto de Ecología, Xalapa, Veracruz, México; <sup>c</sup>Instituto de Investigaciones Psicológicas, Universidad Veracruzana, Xalapa, Veracruz, México; <sup>d</sup>Centro de Investigaciones Gestálticas (CESIGUE), Xalapa, Veracruz, México; eDepartamento de Ciencias Naturales y Exactas, Universidad Autónoma de Occidente, Los Mochis, Sinaloa

#### ABSTRACT

Background: Maize in Mexico exhibits great genetic diversity, maintained by traditional practices of indigenous and non-indigenous communities, the same practices that have led to crop diversification over centuries. As one of the main staple crops worldwide, safeguarding the genetic diversity of maize is paramount to food security.

Aims: This study evaluated the genetic diversity and population structure of traditionally cultured maize landraces in a rural seasonal agricultural community in Veracruz, Mexico, in order to learn how traditional practices shape these landraces, and propose strategies for their preservation.

**Methods:** We analysed 118 individual maize samples belonging to five morphotypes (white, yellow, black, red and mottled) with eight microsatellite markers.

**Results**: We encountered high genetic diversity, according to expected heterozygosity  $(H_e = 0.61)$ . However, inbreeding coefficient and gene flow values suggested the existence of assortative mating, which causes low genetic differentiation. Population structure analysis identified three genetic pools, independent of grain colour. These findings suggest that all morphotypes belong to the same population, which is sub-structured due to assortative mating and gene flow related to local agronomic management.

**Conclusions:** Current management practices in this community could lead to genetic erosion. In order to preserve diversity, wider regional seed exchange and selection for morphological diversity could be implemented.

# Introduction

As the origin of maize diversification, Mexico has generated great genetic diversity of maize through farming and environmental selection (Perales and Golicher 2014). Maize cultivation in Mexico is carried out under two very contrasting systems; while in the north-west there is intensive and highly mechanised agriculture, in the centre and south of the country there are communities that manage the crop traditionally, cultivating maize landraces called criollos. The latter have been traditionally cultured along with other crops such as beans and pumpkin in a polyculture called a milpa (González-Jácome and Reves-Montes 2014). The milpa, in addition to producing food, is part of an *in-situ* conservation system, which relies on keeping the cultivated species in their natural habitats (Zizumbo-Villareal and Colunga-GarcíaMarin 2008; Serratos-Hernández 2009).

About 80% of the total area of maize sown in Mexico is produced through subsistence farming, while the remaining 20% is produced through mechanised agriculture (Bellon et al. 2009). Although maize yields obtained under the traditional system are lower (1 to 3 Mg ha<sup>-1</sup>), 68% of the national production is harvested in this way (FIRA 2016).

Unlike many hybrid varieties, maize landraces are an important source of phytochemical compounds such as dietary fibre, phenolic compounds (flavonoids and anthocyanins), carotenoids, xanthophylls and vitamins that contribute to human health (Bacchetti et al. 2013; Serna-Saldívar et al. 2013; da Silva Messias et al. 2014; Doria et al. 2015; Hwang et al. 2016). The diversity and quantity of these compounds are intimately linked to the genetic diversity of these landraces. Given the importance of traditional systems as the main

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CONTACT I. E. Maldonado-Mendoza 🖾 ignacioemaldonado@yahoo.com.mx

The present affiliation for Karla Y. Leyva-Madrigal is Departamento de Ciencias Naturales y Exactas, Universidad Autónoma de Occidente, Los Mochis, Sinaloa. \*These authors contributed equally to this work.

Supplemental data for this article can be accessed here.

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producers of this staple crop in the country, practices such as selection and seed exchange with other communities are fundamental for the preservation of diversity, genetic heritage, conservation of maize, and ultimately food security (Thrupp 2000; Dyer and Taylor 2008; Bellon et al. 2009; Bracco et al. 2009; Prasanna 2012).

Initial studies of maize diversity in Mexico used morphological and ecological traits of the crop and identified 64 races (59 native and five introduced), close to one-third (29%) of all maize races (220) identified in Latin America (CONABIO 2012). More recently, the advent of molecular techniques has allowed genetic diversity to be studied using molecular markers such as microsatellites (SSR). This technique has been used due to their codominant nature, high degree of polymorphism, low cost and reproducibility between laboratories (Virdi and Sachdeva 2005; Prasanna 2012; Bedoya-Salazar 2013).

Previous large-scale studies (national, continental and intercontinental) have characterised a considerable number of Mexican maize landraces (Matsuoka et al. 2002; Pressoir and Berthaud 2004; Reif et al. 2006; González-Castro et al. 2013). However, a micro scale level approach is crucial to understanding how environmental and social conditions and agronomic practices within local communities affect the genetic diversity and population structure of maize landraces.

Ocotepec, is a rural community in Veracruz, eastern Mexico with a series of characteristics that makes it ideal to be used as a model study site for maize genetic diversity using a micro scale level approach. These characteristics include (1) the very limited seed exchange with other communities, a situation that renders Ocotepec an isolated community in terms of gene flow (Louette 1997) and (2) maize management is based on a colour-based classification of the seeds (morphotypes) which is a common practice in farming communities in Mexico (Louette et al. 1997; Herrera-Cabrera et al. 2002; Dyer and Taylor 2008) and other countries in Latin America (van Etten and de Bruin 2007). This classification follows differences in cultivation and culinary preferences of the different communities, where 'special' varieties are sown for certain traditional dishes (Dyer and Taylor 2008; Fernández-Suárez et al. 2013). Previous studies conducted with different maize landraces from Los Tuxtlas, Veracruz, Mexico have suggested functional differences between differently coloured morphotypes at the level of mycorrhizal dependency for P uptake

(Sangabriel-Conde et al. 2014) and interactions with different arbuscular mycorrhiza fungi (AMF) assemblages (Sangabriel Conde et al. 2015).

In the present study we quantified molecular genetic diversity within and among selected accessions of maize landraces from this study site to address the hypotheses that (H1) farmers' traditional practices (seed selection, mixed sowing and genetic isolation resulting from limited or no seed exchange) are suitable for the conservation of genetic and morphological diversity of local maize landraces; and (H2) that the practice of classification of the grains based on colour into five morphotypes (black, white, yellow, red and mottled) reflects genetically structured maize populations.

# Materials and methods

### Plant material and seed sampling

For more than 100 years, farmers in Ocotepec have been cultivating five maize morphotypes (white [W], black [B], yellow [Y], red [R] and mottled [M]) in *milpa* system and/or monoculture (Figure 1). They refer to these types of maize by their grain colour and not by name of races as in other communities. An initial morphological classification to define the races present in this region, led us to conclude that these belonged to the 'Conico' group. All morphotypes belong to the Conico race with influences of other races; Arrocillo (black and yellow morphotypes), Chalqueño (black and red morphotypes) and Coscomatepec (white morphotype). In this region, the typical area sown by each farmer is less than 2 ha and divided into two to four plots that are mainly distributed close to the township, with few distant plots (Figure 2(a,b)). The seeds used reflect the previous year's harvest, which is collected from November to January and stored in the farmers' household in a storage deck called tapanco, a platform made above the kitchen/bedroom or 30-40 cm above the floor (Figure 2(c)), where cobs are separated based on their colour, but the harvests from different plots are mixed. The stored maize is used for family consumption and as seed for the next crop cycle. Some farmers select their seed immediately after harvest, while most make this selection three days before sowing in March. The number of selected cobs by morphotype rely on the area to be sown, but generally only 15 to 50 cobs are selected. Kernels of the tip and base of each ear are discarded and only kernels in the



Figure 1. Maize (*Zea mays*) morphotypes managed in a *milpa* system by farmers in Ocotepec, Veracruz, Mexico. (a) *Milpa* systems are formed by maize (chevron icon), beans (arrow) and pumpkin (triangle). Symbols point to the leaves, flowers and/or fruits of the crops; (b) view of a *milpa* plot; (c) close-up view of a pumpkin flower and fruit; (d) close-up view of bean pods; corn morphotypes of different grain colour: (e) yellow; (f) white; (g) black; (h) red; and(i) mottled.



**Figure 2.** *Milpa* fields sampled and the storage decks used by farmers in Ocotepec, Veracruz, Mexico. Geographical location of the sampled maize storage decks (circles) and the plots (rectangles) of three farmers in Ocotepec, Veracruz, Mexico (a); Close-up of the town central area (b) marked with a dashed square in (a); and storage deck below a kitchen ceiling (c).

middle region are used as seeds. Farmers used this seed batch is used to sow all their plots. To: Farmers used this seed batch to sow all their plots. A less common practice (ca. 25% of the farmers) is to select three cobs from each harvested plot and sow them in the same plot the following year.

Following harvest, storage and selection, sampling was carried out in the tapancos of three randomly selected farmers with three plots each in 2014–2015 (Figure 2(a,b)). Depending on colour availability in each household, two cobs were chosen from household 1: red and black; four from household 2: mottled, white, yellow and black; and six from household 3: three from the white morphotype, one each of black, yellow and red. Ten grains per ear were randomly chosen composing a maternal half-sib family. Grains were grouped by colour to obtain a total of 118 individual seeds, representing five morphotypes/ 12 maternal half-sibs families; yellow (18 individuals; 2 maternal half-sib families), white (40; 4 maternal half-sib families), red (20; 2 maternal half-sib families), mottled (10; 1 maternal halfsib family) and black (30; 3 maternal half-sib families).

# **DNA** extraction

Seeds were disinfected by hydrothermal treatment (Leyva-Madrigal et al. 2015) and germinated on Luria Bertani agar (LB, Sigma Aldrich, USA, Cat. No. L3022). Germinated seeds were then transferred to 500 mL polyurethane recipients containing vermiculite and allowed to grow for 14 days under greenhouse conditions. Seventy milligrams of leaf tissue were collected from each individual plantlet and were immediately frozen in liquid nitrogen and subsequently ground in a mixer mill (Tissue Lyser II, Qiagen). Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quality and purity of genomic DNA were estimated by measuring the 260/280 nm ratio in a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) and by electrophoresis in a 1% agarose gel. DNA was diluted to a final concentration of 10 ng  $\mu$ L<sup>-1</sup> for use in the polymerase chain reaction.

# Microsatellite genotyping

Initially, 21 SSR markers were selected, covering all 10 chromosomes and according to a PIC value > 0.7 reported in previous studies (Register et al. 2001; Sharma et al. 2010; Pineda-Hidalgo et al. 2013; Wasala and Prasanna 2013) and tested in our morphotypes. Twelve SSR were polymorphic, however as four of them presented difficulties for amplification in some individuals they were discarded and eight were used for further analyses.

PCR was performed in a volume of 25 µL containing 10-20 ng DNA template, 1.5 mM MgCl<sub>2</sub>, 0.5 mM of each dNTP, 0.4 µM of forward and reverse primers, and 1 U of Taq DNA (Invitrogen, polymerase Brazil, Cat. No. 11615-050). The programme used was 5 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, 30 sec annealing at the respective Tm (Table 1), 20 sec extension at 72°C, and a final extension at 72°C for 5 min. A touchdown PCR (TD-PCR) was developed for microsatellite phi083. Similar PCR conditions were used except that in the first 20 cycles annealing was set at 63°C with 0.3°C decrements per cycle, followed by 15 cycles at an annealing temperature of 56°C. PCR products were analysed by capillary electrophoresis in a QIAxcel system using the QIAxcel DNA High resolution kit (Qiagen). Allele sizing was

Table 1. Characteristics of the eight microsatellite loci used in this study of Zea mays landraces, Ocotepec, Veracruz, Mexico.

Locus	RSª	CLp	Forward	Reverse	OAR(pb) <sup>c</sup>	Tm <sup>d</sup>
phi96100 <sup>2</sup>	ACCT	2.01	AGGAGGACCCCAACTCCTG	TTGCACGAGCCATCGTAT	268–296	62.5
phi083 <sup>4</sup>	AGCT	2.04	CAAACATCAGCCAGAGACAAGGAC	ATTCATCGACGCGTCACAGTCTACT	128–148	56
phi127 <sup>4</sup>	AGAC	2.08	ATATGCATTGCCTGGAACTGGAAGGA	AATTCAAACACGCCTCCCGAGTGT	98-126	65
phi072 <sup>3</sup>	AAAC	4.00	ACCGTGCATGATTAATTTCTCCAGCCTT	GACAGCGCGCAAATGGATTGAACT	132–160	66.9
phi031 <sup>4</sup>	GTAC	6.04	GCAACAGGTTACATGAGCTGACGA	CCAGCGTGCTGTTCCAGTAGTT	185-201	58.7
umc1545 <sup>4</sup>	AAGA	7	GAAAACTGCATCAACAACAAGCTG	ATTGGTTGGTTCTTGCTTCCATTA	49-85	56.4
phi015 <sup>2</sup>	AAAC	8.08	GCAACGTACCGTACCTTTCCGA	ACGCTGCATTCAATTACCGGGAAG	81-105	65
phi065 <sup>4</sup>	CACTT	9.03	AGGGACAAATACGTGGAGACACAG	CGATCTGCACAAAGTGGAGTAGTC	128–153	61.6

SSR References: <sup>1</sup>Register et al. (2001); <sup>2</sup>Sharma et al. (2010); <sup>3</sup>Pineda-Hidalgo et al. (2013); <sup>4</sup>Wasala and Prasanna (2013).

<sup>a</sup>Repeat sequence.

<sup>b</sup>Chromosome location (Bin).

<sup>c</sup>Observed allelic range in base pairs.

<sup>d</sup>Annealing temperature used for PCR.

determined using ScreenGel software (QIAGEN, v1.0.2.0; Ambion Inc., Austin, TX).

#### **Genetic diversity**

A genotype accumulation curve was plotted with the *genotype\_curve* function in the R package 'POPPR' (Kamvar et al. 2014) using 1,000 resampling to assess the power of the microsatellite markers to discriminate between individuals of the dataset and to ensure that the observed diversity did not increase with the addition of another marker.

Allele number ( $N_a$ ), effective number of alleles ( $N_e$ ), observed ( $H_o$ ) and expected heterozygosity ( $H_e$ ), private alleles (PA), Shannon's information index (I) and endogamy index ( $F_{IS}$ ) were calculated in GENALEX 6.5 (Peakall and Smouse 2012). Allelic richness corrected for differences in sample size was calculated using Fstat 2.9.3.2 (Goudet 1995) according to El Mousadik and Petit (1996):

$$\widehat{ar}_{(n)} = \left\lfloor \sum_{i=l}^{k} 1 - \frac{\left(2N - N_{i}\right)}{\left(2N - N_{i}\right)} \right\rfloor$$

where Ni represents the number of occurrences of the *i*th allele among the 2N sampled genes.

PowerMarker V3.25 software (Liu and Muse 2005) was used to estimate the percentage of the most frequent allele (%MFA) and the polymorphic information content (PIC), the latter following the equation:

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2 \left[ \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} p_i^2 p_j^2 \right]$$

where *pi* is the frequency of the *i*th allele, and *n* is the number of alleles (Botstein et al. 1980).

#### **Genetic structure**

Deviations from Hardy-Weinberg Equilibrium (HWE) were evaluated for each microsatellite locus in each morphotype, with a significance of 0.05 and 1000 permutations in POPPR. Genetic differentiation among maize morphotypes was evaluated over all loci by calculating  $F_{ST}$  (infinite allele model: IAM) (Weir and Cockerham 1984) and  $R_{ST}$  (stepwise mutation model: SMM) (Slatkin 1995) pairwise estimate in Arlequin 3.5.2.2 (Excoffier and Lischer 2010) to enable a critical comparison between both statistical strategies and obtain all possible information on the population structure,

since each set of microsatellites can fit to one or the other mutation model, depending on the factors acting on the population (e.g. migration, mutation). Additionally, pairwise estimate of migration, expressed as number of migrants (Nm; Wright 1951) was calculated over all loci with GENALEX 6.5 using 999 permutations for significance testing.

We used three different approaches to evaluate/ analyse population structure: a model-based method (Bayesian clustering), and two methods based on genetic similarities (Dendrogram and PCoA). A Bayesian cluster analysis was made using STRUCTURE v. 2.3.4 (Pritchard et al. 2000) software. STRUCTURE runs were based on 500,000 iterations, after a burn-in length of 100,000, assumed correlated allele frequencies, and an admixture model. We tested from 1 to 5 groups (K), and 15 replicates for each K value were made in order to examine the convergence. The  $\Delta K$  ad hoc method described by Evanno et al. (2005) and implemented in the online tool Structure Harvester (Earl and vonHoldt 2012) was used to estimate the most likely K; (2) a distance dendrogram was constructed according to the neighbourjoining method (NJ), using Provesti's and Bruvo's genetic distances on the POPPR package; (3) to carry out the principal coordinate analysis (PCoA) a genetic distance matrix was first calculated using the Codom-genotypic option (Smouse and Peakall 1999) for multiallelic codominant data and the PCoA was drawn using the distance-standardised method as implemented in GENALEX 6.5. The population structure inferred by STRUCTURE was evaluated through an analysis of molecular variance (AMOVA) using Arlequin 3.5.2.2. Distance between alleles was computed as number of different alleles (FST; Weir and Cockerham 1984) and sums of squared differences in repeat numbers (R<sub>ST</sub>; Slatkin 1995). Statistical significance of the variance was tested using 10,000 permutations. Genetic differentiation among groups was assessed by comparing average numbers of pairwise differences between groups (PiXY); average number of pairwise differences within groups (PiX and PiY); and the corrected average pairwise difference (PiXY - (PiX + PiY)/2) using Arlequin 3.5.2.2.

# Results

## **Genetic diversity**

Twelve microsatellite loci were genotyped in all morphotypes, but only eight (phi96100, phi083,

phi127, phi072, phi031, umc1545, phi015 and phi065) were used for further analysis due to high percentage of missing data in microsatellites phi053, phi079, phi006 and phi059. The genotype accumulation curve (Figure S1) showed a linear increase in the number of multi-locus genotypes as the number of loci increased. After four loci sampled, variance and the number of new genotypes detected decreased drastically and the curve reached a plateau with seven loci sampled. This confirms the effectiveness of the eight microsatellite markers to discriminate between 100% of the individuals analysed, indicating that the genetic variability was adequately sampled.

The genetic diversity analysis revealed a total of 57 alleles, with the number of alleles per locus ranging from 5 to 11 (Table 2). Shannon's information index ranged from 0.78 (phi127) to 1.90 (phi072), with a mean of 1.27. For all loci, except phi031,  $H_o$  was lower than  $H_e$ , and  $F_{IS}$  values ( $F_{IS} > 0$ ) suggest an excess of homozygotes (Table 2). PIC values across loci ranged from 0.36 (phi127) to 0.78 (phi072) (Table 2). Excepting phi031 and phi083,

 Table 2. Summary statistics for each microsatellite loci of Zea mays landraces, Ocotepec, Veracruz, Mexico.

					%			
Microsatellite	Na <sup>a</sup>	Ne <sup>b</sup>	Ho <sup>c</sup>	He <sup>d</sup>	MFA <sup>e</sup>	PIC <sup>f</sup>	la	FIS
phi96100	8	4.44	0.61	0.77	31	0.74	1.60	0.21
phi065	5	1.80	0.19	0.45	72	0.41	0.87	0.58
phi015	7	3.05	0.50	0.67	41	0.61	1.31	0.26
umc1545	8	2.53	0.42	0.60	60	0.58	1.32	0.30
phi031	5	2.86	0.69	0.65	52	0.60	1.22	-0.07
phi072	11	5.05	0.69	0.80	33	0.78	1.90	0.13
phi083	7	2.30	0.53	0.57	61	0.52	1.14	0.07
phi127	6	1.66	0.28	0.40	76	0.36	0.78	0.30
Average	7.13	2.65	0.49	0.61	53	0.58	1.27	0.22

<sup>a</sup>Number of alleles.

<sup>b</sup>Efective number of alleles.

<sup>c</sup>Observed heterozygosity.

<sup>d</sup>Expected heterozygosity.

<sup>e</sup>Percentage of the most frequent allele.

<sup>f</sup>Polymorphic information content. <sup>g</sup>Shannon's information index.

<sup>h</sup>Endogamy index.

Table 3. Summary statistics of the genetic diversity within *Zea* mays morphotypes, Ocotepec, Veracruz, Mexico.

Morph	Naª	Ne <sup>b</sup>	Rsc	Pa <sup>d</sup>	Ho <sup>e</sup>	He <sup>f</sup>	la	F <sub>IS</sub> <sup>h</sup>
Y	4.50	2.59	3.99	5	0.42	0.57	1.09	0.27
W	5.75	2.88	4.37	7	0.48	0.60	1.20	0.19
R	4.50	2.81	4.04	0	0.54	0.61	1.15	0.11
М	3.50	2.29	3.5	0	0.50	0.51	0.92	0.02
В	5.13	2.72	4.15	4	0.50	0.59	1.16	0.15
Total	4.68	2.65	4.01	3.2	0.49	0.58	1.10	0.15

<sup>a</sup>Number of alleles.

<sup>b</sup>Effective number of alleles.

<sup>c</sup>Allelic richness

<sup>d</sup>Private alleles

<sup>e</sup>Observed heterozygosity.

<sup>f</sup>Expected heterozygosity. <sup>g</sup>Shannon's information index.

<sup>h</sup>Endogamy index.

all the loci showed deviations from HWE (data not shown).

All loci were polymorphic in all morphotypes (Table 3). The number of alleles per locus within morphotypes ranged from 3.5 (M) to 5.75 (W), while the Ne ranged from 2.29 to 2.88. The high discrepancy between N<sub>a</sub> and N<sub>e</sub>, as well as the % MFA denote the predominance of a few alleles and the low frequencies of the others (Figures S2 and S3). Morphotypes W, B and R were the most diverse, according to the values obtained for I and  $H_e$  (Table 3).  $H_o$  was lower than  $H_e$  in all morphotypes, and F<sub>IS</sub> values suggest an excess of homozygotes. Deviations from HWE were observed in all morphotypes (Figure S4). The R morphotype registered the highest number of loci with deviations from panmixia (5; phi96100, phi065, umc1545, phi031 and phi127). Private alleles were only registered in morphotypes Y (5), W (7) and B (4), most of them with low frequency.

#### **Population structure**

Low levels of differentiation were observed as shown by pairwise comparisons of F<sub>ST</sub> among morphotypes (average  $F_{ST}$  was 0.032) (Table 4). The highest F<sub>ST</sub> value resulted from the comparison between yellow and red morphotypes (0.066, P < 0.001) and the lowest between white and black morphotypes (0.024, P < 0.01) (Table 4). Pairwise value of R<sub>ST</sub> between yellow and red morphotypes was higher than F<sub>ST</sub> and significantly different from zero ( $R_{ST} = 0.170, P < 0.001$ ), suggesting a contribution of the SMM to differentiation in these morphotypes (Table S1). R<sub>ST</sub> values calculated for comparison between red - mottled, red - black and mottled - black morphotypes were lower than  $F_{ST}$ . In general, the yellow morphotype showed the highest and significant pairwise values for F<sub>ST</sub> and  $R_{ST}$  (Table S1). The estimation of gene flow through the number of migrants showed a high exchange between morphotypes, mainly between the black and white morphotypes (Nm = 12.191) (Table 4).

**Table 4.** Pairwise comparison of  $F_{ST}$  (below the diagonal) and Nm (above the diagonal) between *Zea mays* of Ocotepec, Veracruz, Mexico.

Morph	Y	W	R	М	В
Y		9.014	3.904	5.711	5.282
W	0.032**		9.962	8.237	12.191
R	0.066***	0.029**		5.021	10.617
Μ	0.050**	0.037*	0.053**		8.193
В	0.050***	0.024***	0.027**	0.036*	

\*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

Bayesian analysis suggested the existence of genetic structure. Three independent runs were highly consistent and LnP(D) values reached a plateau at K = 3 (Figure S5). The latter was confirmed following the methods used by Evanno et al. (2005) (Figure S5). In general, population structure did not agree with the initial colour classification of the seeds. We observed high levels of admixture between morphotypes (Figure 3). At K = 2 our data set was divided into two groups (group 1, red; 2, green) consisting of individuals of all morphotypes (Figure 3(a,b)). At K = 3 the membership coefficients of the individuals in group 2 (green) remained high (0.745-0.984), while the original red group split in two, red and blue, both with high membership coefficients (0.774-0.973 and 0.-749-0.978, respectively) with some admixed individuals (Figure 3(c,d); Table S2). At K = 4 the membership coefficients decrease drastically increasing the number of admixed individuals (Figure 3(e,f)). Only group 2 remained as in K = 2and K = 3 with slight variation in membership values.

Dendrograms based on Bruvo's and Provesti's genetic distances between individuals (Figures S6 and S7), strongly supported the existence of three genetic groups (bootstrap 100%). Cluster I in Bruvo's dendrogram (Figure S6) mainly grouped individuals assigned to the blue group (Figure 3 (b)) by STRUCTURE, while cluster II was composed of individuals assigned to the red group (Figure 3(b)). Cluster III grouped individuals of the three STRUCTURE groups; however, most of the individuals assigned to the green group (Figure 3(b)) were grouped in this cluster. Provesti's dendrogram was slightly different from Bruvo's. Clusters I and II were mainly composed by individuals assigned to the red group in STRUCTURE, while cluster III was subdivided into two groups: a, individuals of the blue group in STRUCTURE and *b*, a mix of individuals of the three populations predominating those assigned to the green group. In both cases, clustering was somewhat different from the one identified in STRUCTURE, however the same number of genetic groups (K = 3) and high levels of admixture between groups remained. The principal coordinate analysis (PCoA) also suggested the existence of three genetic groups, which are made up of individuals belonging to all morphotypes (Figure 4). The grouping was very similar to that obtained in STRUCTURE.

The AMOVA carried out among the groups established by STRUCTURE showed that more than 70% of the genetic variation is within individuals ( $F_{ST} = 0.265$ , P < 0.05;  $R_{ST} = 0.188$ , P < 0.05) (Table 5). Values of both fixation indices were similar; this is expected in cases where gene flow is high and differentiation among groups low, as observed in this study. Pairwise differences between groups showed a very similar intra- and inter-group variation (Table 6). Group 1 (red) and 2 (green) recorded the highest intra-group variation in the two distance methods employed (Table 6, numbers in the diagonal). Inter-group differences were



**Figure 3.** Population structure of *Zea mays* landraces when K = 2 (a, b), K = 3 (c, d) and K = 4 (e, f) sorted by morphotype (a, c, e) and by the membership coefficient (Q-value; b, d, f). Different colours represent different genetic groups. Maize morphotypes are separated by a vertical black line. Each individual is represented by a thin vertical bar. Numbers on the y-axis represent the membership coefficient of each maize individual to a genetic group. Shaded bars in (b) and (d) represent admixed individuals with low Q-values for each genetic group (< 0.70).



**Figure 4.** Principal coordinate analysis (PCoA) plot of *Zea mays* morphotypes from Ocotepec, Veracruz, Mexico, based on eight microsatellite markers. The scatter plot shows the first and second principal coordinates that best explain the variability in the population. The red (group 1), green (group 2) and blue (group 3) circles represent the suggested groups by the STRUCTURE software. Symbols represent the maize morphotype as follows: open square: white; closed square: black; yellow diamonds: yellow; red triangles: red and closed circles: mottled. The plot coordinates explain 31% of the total variance.

Table 5. Analysis of molecular variance (AMOVA) of three genetic groups identified by STRUCTURE in *Zea mays*, Ocotepec, Veracruz, Mexico.

		IAM <sup>a</sup>				SMM <sup>b</sup>				
Source of variation	d.f.	Sum of squares	% var.	F <sub>ST</sub>	P-value	d.f.	Sum of squares	% var.	R <sub>ST</sub>	P-value
Among groups	2	60.070	17.48	0.109*	0.000	2	176.868	4.95	0.146	0.000
Among individuals within groups	89	213.468	9.06	0.175*	0.000	89	2102.953	13.92	0.049*	0.000
Within individuals	92	177.000	73.46	0.265*	0.000	92	1618.500	81.13	0.188*	0.000

<sup>a</sup>Infinite allele model; <sup>b</sup> Stepwise mutation model; \*, P < 0.05.

**Table 6.** Pairwise population differentiation for genetic groups identified by STRUCTURE in *Zea mays*, Ocotepec, Veracruz, Mexico. Above diagonal: Average number of pairwise differences between groups (PiXY). Diagonal elements: Average number of pairwise differences within groups (PiX). Below diagonal: Corrected average number of pairwise difference (PiXY-(PiX+PiY)/2). Distances were based on the number of different alleles ( $F_{ST}$ ) and sum of squared size difference ( $R_{ST}$ ).

	F <sub>ST</sub>			R <sub>st</sub>			
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	
Group 1	4.809	5.918	4.642	47.450	49.685	40.939	
Group 2	1.096**	4.835	5.515	1.691*	48.537	41.600	
Group 3	0.510**	1.370**	3.454	2.403**	2.521**	29.621	

Corrected average pairwise differences that are statistically different, \* P < 0.05; \*\* P < 0.001. Group 1 correspond to the red population, group 2 to the green population and group 3 to the blue population, all defined by STRUCTURE software.

significant between all groups based on both indexes ( $F_{ST}$  and  $R_{ST}$ ) (Table 6). A detailed review of allele frequencies among the three genetic groups (sub-populations) showed variations and the presence of private alleles in all groups (Table S3).

# Discussion

Maize has been studied mainly on a wide geographical scale and much is unknown about the genetic

diversity, population structure and the factors affecting both in smaller regions with very particular characteristics as is the case of the community of Ocotepec. The present study describes the genetic diversity and population structure of 12 half-sibling families of five maize morphotypes, based on SSR genotyping. Despite the limited sampling, results reported here provide valuable insights into genetic diversity and population structure of Ocotepec's maize landraces, laying the groundwork for future studies.

All SSR loci analysed were polymorphic and informative, as shown by high PIC values and the genotype accumulation curve. Six out of eight loci recorded high PIC values (≥0.50) reflecting a high allelic variation and a large informativeness of these SSR markers, especially in those with values greater than 0.70 (phi96100 and phi072) (Botstein et al. 1980; Hildebrand et al. 1992). Low PIC values were recorded in loci phi065 and phi127, evidencing an imbalance in the frequency of alleles, as demonstrated by the percentage of the most frequent allele  $(\geq 70\%)$  in these loci. According to the genotype accumulation curve, the set of eight SSR loci proved to be appropriate for sampling the genetic variability of these maize morphotypes as the curve reached a plateau after 4-5 loci, with 90% of the genotypes being detected.

The genetic diversity found in this study is high ( $H_e = 0.61$ ) and consistent with previous reports despite this being a study undertaken at a local scale. Several populations of Mexican maize landraces studied using different sets of microsatellites have reported Nei's gene diversity  $(H_e)$  values ranging between 0.40 and 0.72, with an average of 0.57 (Pressoir and Berthaud 2004; Santacruz-Varela et al. 2004; Reif et al. 2006; González-Castro et al. 2013; Pineda-Hidalgo et al. 2013; Santos et al. 2017). Moderate to high genetic diversity is expected in maize in Latin America, especially in Mexico, as the centre of origin of this cereal and one of the main centres of its diversification (Santacruz-Varela et al. 2004; Bedoya-Salazar 2013).

Population structure analysis by the three explored approaches, showed the existence of three genetic groups (sub-populations) with no relationship to half-sib families, morphotypes or storage decks (farmer) sampled (Figures 3 and 4; Figures S6 and S7). An AMOVA analysis of these three groups defined by STRUCTURE, showed that most of the genetic variation was within individuals (more than 70%). Nevertheless, pairwise differentiation showed significant F<sub>ST</sub> and R<sub>ST</sub> values among all groups. A detailed review of the allele frequencies showed that almost all alleles are evenly distributed among the three genetic groups, except for a few variations and private alleles present in each group (Table S3). Variation in allele frequencies among groups within a population and the slight increase in the proportion of homozygotes [H<sub>e</sub>/H<sub>o</sub> relationship  $(H_e > H_o)$  and  $F_{IS}$  values] found in this study are distinctive of sub-structuring in a population (Wang 2018). This phenomenon is very common

in non-random mating, kin-structured and/or isolated populations as the one reported here (Anderson and Dunham 2008; Pilot et al. 2010; van Heerwaarden et al. 2010; Garnier-Géré and Chikhi 2013; Toosi et al. 2018).

Farmers shape crop diversity and population structure through seed selection and exchange (Dyer and Taylor 2008; Labeyrie et al. 2014). Selected seed lots determine the allelic composition in the new crop cycle and the resulting harvest. Seed selection by Ocotepec farmers is kin-structured as many kernels from a few cobs (ca.15-50, depending on availability and area to be sown) are used in the seed lot conformation that generate half-sib families. This represents a sample with limited genetic variability and of similar genetic composition. Spatial distribution of maize morphotypes within and between plots, flowering overlap of all morphotypes and limited or no seed exchange increase the rate of close relatives mating (positive assortative mating; identical alleles) (Kahler et al. 1989). As a result of these seed selection practices plants in the established crop have a certain degree of kinship (e.g. full-, half-siblings, cousins).

Although we found high genetic diversity  $(H_e = 0.61)$  in Ocotepec landraces, our findings do not support our first hypothesis. We provided evidence that the genetic material in this locality may be prone to genetic erosion due to, among other factors, current management practices. We suggest that Ocotepec farmers reintroduce and/or develop local and culturally-relevant practices to avoid a greater reduction in heterozygosis. Effective practices include the (a) promotion of seed selection directed to increase maize morphological diversity and improve performance, (b) expansion of the geographical range of seed exchange through participation in agricultural fairs and other networking activities, that promote the introduction of native maize races and/or varieties from a wider region that yield well locally (Louette 1997, 2000; Dyer and Taylor 2008; Bellon et al. 2011; Álvarez-Buylla et al. 2011; Aguirre-Gómez and García-Leaños 2012; Engels et al. 2014; Andersen et al. 2018), and (c) plant selection prior to harvesting for the seed batch conformation to improve genetic diversity. Plant selection allows farmers to observe specific characteristics (height, number of cobs, stem thickness, health and other qualitative traits) and to direct specifically their postharvest seed selection. Once desirable plants are

identified, the best cobs (healthy) can be selected, shelled and seeds mixed thoroughly and stored for use in the following year's crop. This comprehensive strategy would contribute to expanding the phenotypic and genetic variability of the local maize by promoting improvements through traditional breeding techniques (Herrera-Cabrera et al. 2002; Carrera-Valtierra et al. 2011; Aguirre-Gómez and García-Leaños 2012).

The implementation of these strategies in communities such as Ocotepec is feasible, relatively cost-effective, and increasingly important to preserving local crop diversity and improving the yield and resilience of maize landraces. Moreover, these genetic diversity preservation efforts, must be complemented by *ex situ* conservation and creation of seed banks to represent all local morphotypes as a preventive measure in case of a catastrophic event, whether natural or man-made (ex. excess or lack of rain, extreme cold or warm weather, increased frequency and severity of disease or emergence of new ones).

Our second hypothesis was not supported either since the differentiation by colour did not correlate with the structure of the population. We found, what appears to be a single sub-structured population, whose genetic diversity maintenance depends on the correct representation of each sub-population in the seed lot. An imbalance or the lack of any genetic group(s) can lead to the loss of certain alleles, and the overrepresentation of the alleles of the larger sub-population present in the seed lot, affecting the overall genetic diversity and population structure. This is also true for morphotypes as private alleles were found, so sowing of all morphotypes is important.

The imbalance of genetic groups in our sampling had an effect on our results. As group 2 (green) was slightly under-represented it probably lead to the lack of precision in the estimate of Ne and He and downstream analyses ( $F_{IS}$ ,  $F_{IT}$  and  $F_{ST}$ ) due to miscalculation of allele frequencies (Sánchez-Montes et al. 2017; Wang 2018). As the sub-structure found is cryptic/hidden to the farmers, since grouping did not obey to morphotype or farmer, it would be difficult to ensure a more even sampling of each sub-population. It is therefore suggested that in future studies, a larger number of cobs from the different plots sown by each farmer are included in the sample that represents each farmer's seed lot. Nevertheless, the inclusion of relatives in the sample remains necessary to adequately represent Ocotepec's maize landraces because they are common in the population. Recent studies have proposed new approaches for the calculation of genetic and population estimators in samples with related individuals, since this normally occurs in wild populations (Wang 2018). The difficult task is to determine the proportion that truly represents the population and avoid biased/imprecise parameter estimates. One strategy is to conduct pilot studies to determine the sample size, proportion of relatives and the number of markers needed for each specific species and population (Sánchez-Montes et al. 2017). In this respect, the results of this study can serve as a reference for future maize sampling and analyses in this and other small communities with similar characteristics.

It remains to be determined how generalisable among milpa-growing communities are the genetic signs of inbreeding we found in Ocotepec maize. They might be common, since erosion of traditional agricultural practices, such as seed selection and exchange has been reported as a widespread issue (Perales et al. 2003; Chambers and Brush 2010; Bellon et al. 2011) most likely as a consequence of migration, agricultural abandonment and ageing of the rural population (Bellon and Berthaud 2004). Our findings highlights a critical situation that warrants timely strategic policy-level interventions directed towards supporting local crop diversity. These can be achieved through a series of evidence-based and integrated inter-sectorial programmes aimed at fostering conditions for seed preservation and exchange among isolated communities. Similarly, culturally sensitive and locally relevant knowledge exchange and technical support programmes promoting contextspecific management practices among traditional farmers would contribute greatly to this effort. Traditional farmers play an important role as the ultimate guardians of this emblematic Mesoamerican crop and its genetic wealth on which a large proportion of human population relies for sustenance and cultural identity in the case of Mexico.

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#### Notes on contributors

*Karla Y. Leyva-Madrigal* is interested in the study of the diversity, conservation and improvement of maize, as well as the study of crop diseases.

*P. A. Báez-Astorga* is interested in molecular biology, plant biodiversity conservation and biological control agents to assist crop growth.

*S. Negrete-Yankelevich* is a soil ecologist interested in the consequences of land use practices on soil conservation and food security in tropical areas.

A. Núñez-de la Mora is a biological anthropologist. Her research focuses on the application of evolutionary anthropology to reproductive and health issues combining field and laboratory methodologies.

*G. Amescua-Villela* is a psychotherapist specialising in children, couples and parents.

*I. E. Maldonado-Mendoza* is interested in the molecular ecology of the rhizosphere, the arbuscular mycorrhizal fungi of maize and antagonistic interactions between bacteria and fungi associated with maize.

### ORCID

Karla Y. Leyva-Madrigal D http://orcid.org/0000-0002-3748-821X

P. A. Báez-Astorga (D) http://orcid.org/0000-0001-6193-8228

S. Negrete-Yankelevich (b) http://orcid.org/0000-0002-4429-6662

A. Núñez-de la Mora () http://orcid.org/0000-0002-1609-0771

G. Amescua-Villela (b) http://orcid.org/0000-0002-0984-2883

I. E. Maldonado-Mendoza (D) http://orcid.org/0000-0001-9952-1508

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