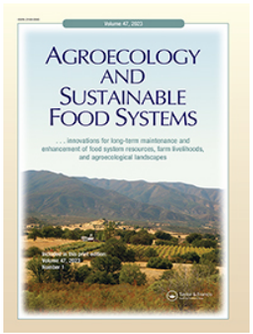

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A data mining approach gives insights of causes related to the ongoing transgene presence in Mexican native maize populations

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ABSTRACT

Mexico is the center of origin of maize and one of the phenomena that imperil maize conservation is transgene presence. Although previous studies have documented transgene presence in Mexican landraces, to date there is no countrywide transgene monitoring protocol, nor systematic analyses assessing which factors could be related with transgene presence and dispersal. In this work, we propose a geographically representative sampling protocol and present empirical data from three sampled states: Mexico City, Oaxaca and Chiapas. To further investigate which environmental and social variables could be associated with transgene presence, we carried out a data mining approach. To assess transgene presence in collected maize samples, we used Real-Time PCR, finding that transgenes were widely distributed across sampled localities: 33% of the localities in Chiapas, 25% in Mexico City and 11% in Oaxaca. The data mining approach allowed us to identify state-specific spatial associations in Chiapas and Oaxaca. In Chiapas, a higher probability of transgene presence appeared related to the coexistence of industrialized maize agriculture, while in Oaxaca it was related with seed exchange. We discuss the importance of implementing a national biomonitoring protocol to increase our understanding of the sources that enable transgene presence and dispersal.

KEYWORDS

Transgenes; maize; center of origin and diversification; biomonitoring; spatial data mining

Introduction

Mesoamerica is the center of origin and domestication of 15% of the crops commonly sowed in the world (Khoury et al. 2016), including maize. From an agri-biological perspective, Mexico is one of the most important genetic

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reservoirs of maize, whose 59 native races and thousands of varieties have been adapted to very different climatic conditions and agronomic practices (Ruiz-Corral, Aguilar, and de Sánchez 2001; Ruiz-Corral et al. 2008, 2013; Wellhausen et al. 1951). These races account for approximately 50% of the world's genetic variability for this crop, according to microsatellite data (Vigouroux et al. 2008). Such variability could help contend with the negative impacts of environmental changes which could imperil future maize production in Mexico (Ureta et al. 2020) and elsewhere (Tigchelaar et al. 2018), through the implementation of both autochthonous and professional breeding efforts (Caldu-Primo et al. 2017; Fernández, Wise, and Garvey 2012; Hufford et al. 2012; Vigouroux et al. 2008). Consequently, the preservation of native maize varieties at their center of origin and diversification is strategic for food security at the national and international level. Preserving maize diversity in Mexico will enable millions of people to keep their livelihood, as well as preserve their diet (Tuxill et al. 2010), traditions and rituals (Brush and Perales 2007; Perales, Benz, and Brush 2005; Tuxill et al. 2010), which in turn have been linked with ethnolinguistic diversity (Brush and Perales 2007; Perales and Golicher 2014; Ureta et al. 2013). To conserve such biocultural diversity, which is subject to a dynamic *in situ* evolutionary process mostly in the hands of small-scale farmers and peasants that sow native maize in diverse landscapes, there is a need to support them and mitigate possible risks (Bellon et al. 2018; Bellon and Van Etten 2014).

Currently, *in situ* maize conservation faces many challenges (Bellon et al. 2018; Brush et al. 2015; Dyer et al. 2014, 2015; Rojas-Barrera et al. 2019). One of them is the ongoing presence of transgenes and their potential introgression into native maize populations (Agapito-Tenfen et al. 2017; Dyer et al. 2009; Ellstrand 2003; Piñeyro-Nelson et al. 2009; Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007; Vazquez-Barrios et al. 2021; Warwick et al. 2008), which could alter endogenous genes, potentially affecting plant characteristics such as seed quality and fitness (Andow and Zwahlen 2006).

While it is very difficult to establish the impacts of recombinant DNA or proteins from transgenic crops on human health, toxicological feeding studies performed in animal models such as rodents, pigs and bovines have shown negative physiological effects (Chowdhury et al. 2003; Kılıç et al. 2008; Lutz et al. 2005; Mesnage et al. 2015; Onose et al. 2008; Séralini, Cellier, and de Vendomois 2007; Séralini et al. 2014; Walsh et al. 2011). Thus, there is growing concern on the potential effects of transgenic DNA and recombinant proteins on human tissues (Mendoza-Almanza et al. 2020; Nawaz et al. 2019). Finally, recent evidence confirms that Bt proteins produced by transgenic crops expressing *Cry* genes have potential allergenic properties (Santos-Vigil et al. 2018; Then and Bauer-Panskus 2017).

Currently, commercial planting of transgenic maize in Mexico has been hindered due to social resistance by diverse groups (grassroots organizations,

individual and small-scale organized peasants, environmental organizations, scientists, and citizens) who have put forth public media campaigns as well as several legal procedures over the past two decades that have translated into a prolonged and almost continuous *de facto* moratorium to open field planting of transgenic maize cultivation at a commercial scale (Hernández-López 2019). Despite these efforts, the presence of transgenic maize has been detected in native maize populations in different regions of the country since the year 2000 (Quist and Chapela 2001); only four years after the large-scale introduction of transgenic maize in the United States (James and Krattiger 1996). After this first detection event, several academic studies have followed, most of them detecting transgene presence (Agapito-Tenfen et al. 2017; Dyer et al. 2009; Ortiz-García et al. 2005; Piñeyro-Nelson et al. 2009; Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007); while only three publicly communicated transgene monitoring efforts have been executed by relevant governmental agencies (INECC 2015, 2017, 2018). Nonetheless, all these biomonitoring efforts are difficult to compare, as each used different sampling protocols, assumptions, and scopes (ranging from a few localities within a state, to several localities across different states). Consequently, it has been hard to infer which phenomena could favor changes in transgene frequency and distribution across space and time. Still, these studies are valuable efforts that allowed scholars and decision takers to have an insight on transgene presence in native maize populations. The overall frequencies of transgenes in native maize populations across the country remain unknown and, given the number of maize plants sowed each year across the country and the dynamic nature of maize agriculture, such frequencies may never be completely established. Thus, there is a need for a sampling strategy that allows comparability and geostatistical analyzes to be performed, as a means to identify possible sources of transgene dispersal and potential processes of transgene introgression. These tools are indispensable instruments to make informed biosafety decisions and to update public policies.

Here, we propose a sampling protocol for transgene detection in native maize varieties that is geographically representative and takes into account the proportion of rainfed area in each state, as well as where native maize races have been sowed. With our sampling results, we carried out a spatial data mining analysis to identify possible environmental and social variables that could be favoring transgene presence and dispersal. Transgene presence was monitored in Mexican native maize varieties independently to which race the variety belonged to – native maize varieties (landraces) were considered as such given morphological characteristics as well as when peasants identified them this way. As study cases, we sampled the states of Chiapas, Oaxaca, and Mexico City. We selected two different states where transgenes had been previously detected, such as Mexico City and Oaxaca (Agapito-Tenfen et al. 2017; Dyer et al. 2009; Piñeyro-Nelson et al. 2009; Quist and Chapela 2001;

Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007) in order to identify if there was an ongoing presence of transgenes and if so, their spatial distribution. We also tested our sampling proposal in a previously unsampled state: Chiapas, which is only second to Oaxaca in native maize diversity.

The sampling proposal presented here can be the basis for future nationwide or regionwide biomonitoring efforts. The objectives in the present study were: 1) to develop a stratified sampling protocol with geographic representativeness for the spatial detection of transgenes in native maize, a characteristic that former studies have lacked, as they had been focused on specific localities, 2) to apply this sampling protocol in three states (Chiapas, Oaxaca, and Mexico City) that were taken as study cases, 3) to determine whether transgenes are widely distributed in space and at what frequencies, and 4) to determine potential spatial associations with diverse environmental and social variables to give insight about possible sources of transgenes.

Methods

Sampling protocol for biomonitoring transgene presence in native maize

To accomplish the first two objectives, we based the spatial distribution of our sampling design on the rainfed annual crop spatial representation included in the Land Use/Land Cover (LULC) nationwide cartography provided by the Mexican National Institute for Statistics and Geographic Information (INEGI 2019). Indeed, most of the native maize varietal diversity in Mexico is associated with rainfed annual cultivation systems (Ruiz-Corral et al. 2008), although native maize can also be found in irrigated areas. In this work, we assumed native races are predominantly grown in rainfed areas. The LULC cartography derives from the visual interpretation of satellite imagery (Landsat TM5) acquired in the dry and rainy seasons, with the aid of a nationwide network of agricultural sites for validation, which has been updated roughly every four years since 2000 (INEGI 2013). This cartography is the only one available at the national scale (1:250,000 scale; highest resolution available).

We decided to use INEGI's database and not the one provided by the Mexican Agricultural and Fisheries Information Service (SIAP 2018), which is another nationwide database documenting the distribution and yield of maize, because even when it is updated annually for the purpose of agricultural policy planning and the distribution of public subsidies (SADER [Ministry of Agriculture and Rural Development] 2016), this database relies on local reporting by farmers who might overestimate the areas of maize sown under irrigation, resulting in inconsistencies over time in terms of total cultivated land surface. Furthermore, this database is provided at a municipality level.

Since the annual rainfed agriculture layer of the LULC cartography contained not only maize but also many other crops (although maize cultivation is

a major pattern in this layer), we incorporated information on native maize distribution from CONABIO's (the National Commission for the Use and Knowledge of Biodiversity) national maize landrace collections to increase the probability of finding native maize varieties on the field. This collection involves more than 20,000 samples collected throughout the country and we only used the most recent records from 1970 to 2010 (CONABIO 2011).

Given the different level of certainty pertaining to the sowing of native maize in the different localities, we selected the localities to be sampled using a weighted selection strategy: the largest weight was assigned to the CONABIO localities (weight value $w = 3$), for which crop identity is guaranteed, followed by INEGI localities that coincided at the municipality level with a record of native maize presence (CONABIO (Comisión Nacional para el Conocimiento y Uso de la Biodiversidad) 2011) ($w = 2$), and the lowest weight to the INEGI localities that were present in the rainfed area but did not coincide at the municipality level with a record for native maize presence ($w = 1$).

The total number of sampled localities was established based on the number of plants that could be stored and managed in our facilities at the laboratory of E.R. Álvarez-Buylla at the Ecology Institute of the National Autonomous University of Mexico and at the Plant Physiology laboratory at the Autonomous Metropolitan University-Xochimilco (A. Piñeyro-Nelson). For each state, the number of localities to sample was proportional to the fraction of rainfed annual maize crop area that each state contributed to the national total; however, when this formula translated into less than 40 localities for a given state, we increased the sample size to 40 to guarantee minimal statistical power. The localities sampling procedure was repeated 10,000 times and for each sample, we calculated the total weight, and constructed a frequency distribution of total weight. The sample selected for implementation in the field corresponded to the one at the 95% percentile of this distribution. We chose the sample at this percentile instead of those with the highest total weight to avoid implementing an outlier sample (Figure 1, S1).

To achieve objectives 2 and 3, we carried out field work for maize sample collection, targeting the localities randomly selected by our algorithm. During field work, we faced several setbacks, the most important ones for sampling purposes entailed cases where the proposed locality could not be reached, or maize ears were not found, or when we did not find maize fields at all. In those cases, we explored the vicinity of the proposed locality, sampling as close as possible to it. We thus were able to sample 151 localities in Chiapas (with a mean of 3 samples per locality), 258 localities in Oaxaca (with a mean of 2.3 samples per locality) and 20 localities in Mexico City (with a mean of 12 samples in each locality). Each sample represents about 10 ears or 1 kg of seeds. The plots from which samples were collected were not measured, as samples were collected in farmer's houses/storage. We georeferenced each locality using a GPS. The intention was to sample the same amount of maize

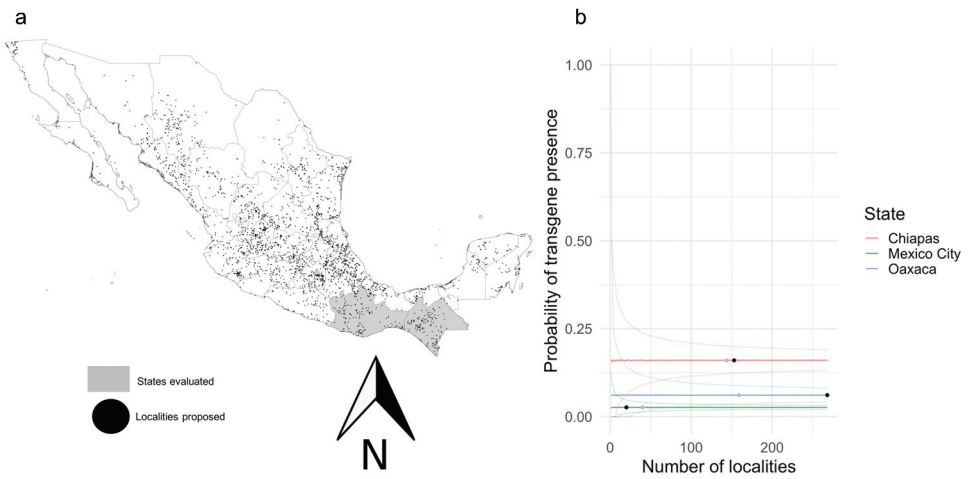

Figure 1. Nationwide sampling proposal and estimation of uncertainty on the probability of detecting transgenic maize samples in three sampled states. Panel A: Map of **Mexico** where black dots represent the proposed number of localities to be sampled. States shaded in gray are the three states sampled: Mexico City, Oaxaca and Chiapas. Panel B: for the three states sampled, we calculated the probability of positive transgene detection given the number of localities sampled per state. Lines represent the estimated mean transgene presence probability obtained with different sample sizes in Chiapas (red), Mexico City (green) and Oaxaca (blue). Confidence bands were obtained through bootstrap. Gray dots: Proposed number of localities to be sampled per state. Black dots: Number of localities sampled.

per locality but it was not always possible. Collected samples were taken to the laboratory and maize ears were given a unique identifier and were visually inspected to assess fungal or insect pest presence. Infested samples were discarded and remaining samples were treated with aluminum phosphide tablets to avoid future infestation. Approximately 100 seeds per ear or 100 g of maize seeds were ground in an industrial blender, which was cleaned between samples to avoid cross contamination; three flour aliquots per sample were used for DNA extraction. Remaining maize samples and flour were stored at room temperature for future use.

DNA extraction, monitoring, and detection of transgenic sequences in maize samples

Flour aliquots of 100 mg were used for DNA extraction following the method described by Doyle and Doyle (1987). DNA concentration was quantified by assessing UV absorption at 260 nm; purity was assessed evaluating the absorption ratio at A_{260nm}/A_{280nm} using a NanoDrop Lite UV-Vis Spectrophotometer (Thermo Scientific). Extracted DNA was stored at −20°C until further use. Recombinant sequences in DNA maize samples were detected using Taq-Man Real-Time PCR. In brief, the 35S promoter from the Cauliflower Mosaic Virus (35S CaMV) and the Nopaline synthase

(T-NOS) genes were detected and assigned as transgenic markers. This strategy would help us cover over 80% of commercial maize events, which have one or both transgenic sequences. If these sequences were identified as positive in the samples, these were further tested for maize-specific transgenic events (i.e. NK603, TC1507, and MON810). Certified reference materials were used as positive controls. The certified reference materials consist of genetically modified maize flour developed by the Institute of Reference Materials and Methods (IRMM, Geel, Belgium). The certified materials employed were ERM-BF415 for NK603 (MON-ØØ6Ø3-6); ERM-418 for TC1507 (DAS-Ø15Ø7-1) and ERM-BF413 for MON810 (MON-ØØ81Ø-6) (Arleo et al. 2020; Carvajal et al. 2017; González-Ortega et al. 2017; Querci, Jermini, and Van den Eede 2006).

Once we determined the number of localities with transgene presence, we could evaluate the uncertainty associated with the number of localities proposed and sampled per state (Figure 2). To reach objective 3, we evaluated the spatial distribution of transgene presence, relative to the spatial distribution of sampled localities. This evaluation was performed by considering presence/absence as a binary marked point pattern process, testing the existence of random labeling through the L-function (Fletcher and Fortin 2018). We also calculated the percentage of localities with transgenic samples for each state.

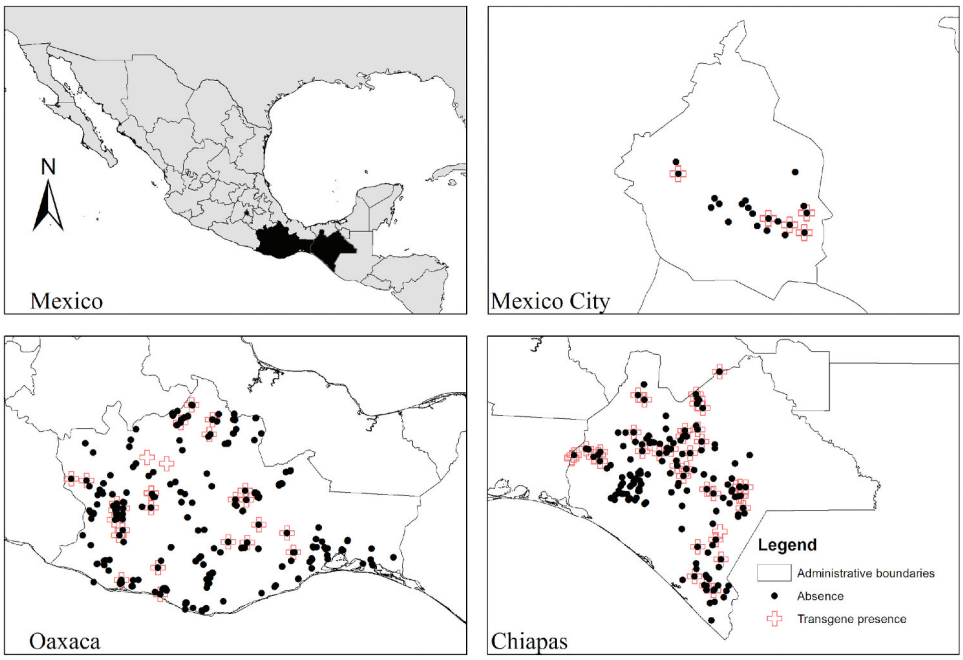

Figure 2. Localities sampled and transgene presence in three Mexican States. Map of Mexico with the three states sampled shaded in black. For each state, black dots and red crosses represent the localities where samples were collected. Black dots: transgene absence; red crosses: transgene presence.

Transgene spatial associations with social and environmental variables

Finally, objective 4 was to identify the spatial associations of the maize samples positive for transgenes with environmental and social variables in order to gain a better understanding of specific factors that could be related with a higher probability of transgene presence. To reach this goal we used a data mining approach proposed by Stephens et al. (2009) and previously implemented for maize data by Ureta et al. (2013). This method is based on Bayesian probability and allows obtaining the probability of presence under a socio-environmental profile. Stephens and collaborators proposed two metrics: epsilon (ε) and score (S). ε values allow finding spatial associations between the factor of interest and other variables. In this particular case, we aimed to analyze transgene presence across the sampled geographic area and 61 environmental and social variables (e.g., temperature, precipitation, vegetation type, population density, indigenous population density, area covered with rainfed fields, area covered with irrigated fields, among others). We divided variables' values into deciles for the analysis, but for some variables the number of categories had to be reduced in order to avoid categories to overlap (Table S5).

ε is the probability of presence of a factor (in this case, transgenes in a maize sample) given a variable (i.e., a specific temperature range) that takes into account the sample size of the studied variable and helps to identify if there is a significant spatial relation or not. To incorporate the sample size, Stephens and colleagues (2009) proposed the following equation that was used in the present study:

$$\varepsilon\left(\frac{B_i}{I_j}\right) = N_{Ij}\left(P\left(B_i/I_j\right) - P(B_i)\right)/\left(N_{Ij}P(B_i)(1 - P(B_i))\right)^{1/2} \quad (1)$$

where N_{Ij} is the number of cells in which a specific variable I_j is distributed, $P(B_i|I_j)$ the probability of finding a transgene when variable I_j is present and $P(B_i)$ the probability of finding transgenes. ε works as a hypothesis test that assumes a normal distribution and uses $\varepsilon(B_i|I_j) = 2$ as a critical value. The number 2 corresponds to two standard deviations in a 95% confidence interval.

As mentioned above, the other important metric to be calculated is S. This metric allows constructing probability maps of transgene presence given the presence of a specific profile of variables (in this case, environmental and social variables) through a Bayesian approximation. The equation to calculate the score values is the following:

$$S\left(\frac{B_i}{I'}\right) = \sum_{k=1}^{N} S\left(\frac{B_i}{I_k}\right) = \sum_{k=1}^{N} \ln(P\left(\frac{I_k}{B_i}\right)/P\left(\frac{I_k}{A_i}\right)); \quad (2)$$

where B_i is the number of localities in which transgenes are present, A_i the number of localities in which transgenes are not present, I_k the number of localities in which a studied variable is present and I' a variables profile. Thus, $S(B_i|I')$ represents a measure of the probability of finding the distribution variable B_i when the variables profile is I', while $P(I_k/B_i)$ is the probability of finding variable B_i when variable I_k is present and $P(I_k/A_i)$ is the probability of not finding B_i when factor I_k is present.

Results

A sampling protocol for transgene detection in native maize and its implementation in Chiapas, Mexico city, and Oaxaca

Based on the rainfed area putatively sowed with native maize, we generated a sampling proposal composed of 2556 localities covering the entire country. Additionally, we decided to set the minimum locality number per state to 40 as a means to have a more robust sample size that would allow for robust statistical analyses as we empirically tested for Mexico City (see sections below). Consequently, the sample size was adjusted in 14 out of 32 states, thus accruing to 2802 localities (Table 1). Given that localities were randomly chosen as our focus was on generating a representative sample size per state in each run, specific localities can differ among runs but overall geographic representativity remains constant (see Supplementary Material Fig S1). The R code used to generate them is available in our supplementary material (Script S1). In order to test our sampling proposal in the fields, we applied it in three Mexican states. Two with the highest number of native maize landraces – Oaxaca and Chiapas – and another of importance for local food security (Mexico City). Adjustments in the number and selected localities were needed in all three states due to three main factors: inaccessibility of selected localities, lack of maize cultivation in the selected locality and incorporation of expert knowledge by local colleagues. The last two cases attest to a need for a more precise and updated geographic data set than the one that was publicly available to construct our algorithm, as we encountered several localities where maize had not been cultivated in the recent past (10–15 years). This is not surprising, as one of the two input databases used in our algorithm had data spanning from 1970 until 2010 (CONABIO (Comisión Nacional para el Conocimiento y Uso de la Biodiversidad) 2011). During field work, knowledge from local maize experts allowed us to recognize important groups of localities where native maize varieties are cultivated that were not considered in the federal census and thus, we adjusted the number of localities to be sampled. This was especially relevant for Oaxaca, but also for Chiapas. The final number of localities sampled per state was very similar to the original number proposed by our biomonitoring protocol in the state of Chiapas (151

Table 1. Proposed number of samples per state for a mid- and long-term biomonitoring protocol in Mexico.

Mexican state	Rainfed area (10^3 ha)	Recommended number of localities	Corrected number of localities	Number of sampled localities
Veracruz	2242.455	236	236	
Zacatecas	1841.451	194	194	
Jalisco	1774.975	187	187	
Oaxaca	**1507.948**	**159**	**159**	**258**
Tamaulipas	1469.863	155	155	
Chiapas	**1370.472**	**144**	**144**	**151**
Puebla	1351.604	142	142	
Chihuahua	1248.284	131	131	
Michoacán	1186.279	125	125	
San Luis Potosí	1190.332	125	125	
Durango	1025.361	108	108	
Sinaloa	1000.574	105	105	
Guanajuato	914.698	96	96	
Guerrero	909.047	96	96	
Estado de México	826.832	87	87	
Hidalgo	781.129	82	82	
Nuevo León	547.918	58	58	
Tabasco	397.495	42	42	
Nayarit	359.553	38	40	
Coahuila	325.296	34	40	
Querétaro	306.563	32	40	
Tlaxcala	284.144	30	40	
Sonora	255.135	27	40	
Yucatán	248.169	26	40	
Campeche	200.596	21	40	
Morelos	168.988	18	40	
Baja California	150.237	16	40	
Aguascalientes	132.155	14	40	
Quintana Roo	130.734	14	40	
Colima	95.496	10	40	
Mexico City	**34.394**	**4**	**40**	**20**
Baja California Sur	0.842	0	40	
Nationwide Total	24279.019	2556	2802	429

Rainfed area: agricultural area where irrigation is not employed and native maize varieties are more commonly grown. *Recommended number of localities*: the number of localities recommended to be sampled given the rainfed area in each state. *Corrected number of localities*: correction of the recommended number of localities to guarantee minimal statistical power. *Number of sampled localities*: number of localities sampled in the three states reported here.

final; 144 original) while in both Oaxaca (258 final; 159 original) and Mexico City (20 final; 4 original), it was higher (Figure 1). Although 40 localities were the minimum number of suggested localities per state for statistical purposes, in Mexico City it was only possible to sample 20 localities. Still, this number is 5 times higher than the number of localities suggested by our sampling algorithm given the amount of rainfed area present in this state (see Table 1). When evaluating our biomonitoring proposal (see Figure 1), we corroborated that 20 localities do allow for correctly estimating the probability of finding transgenes on the field in Mexico City. Also, it has been proposed for other predictive models in correlative analysis, that 20 localities are a good

number to creative predictive models (Pearson et al. 2007). Thus, we decided to continue with our data mining method in all three states sampled.

Unfortunately, the predictive power of the set of variables analyzed was not strong enough to project a transgene-presence probability map given a socio-environmental profile in the states evaluated (S; see Supplementary Material, Fig. S2), but it was possible to find significant spatial associations with different environmental and social variables (ε).

Frequency of detected transgenes and spatial associations

Purified DNA from all maize samples collected was analyzed using Q-PCR to assess the presence of transgenic markers NOS-T and P35S; positive samples for either or both markers were further analyzed for specific recombinant events NK603, TC1507, and MON810. We found transgenes in native maize populations in all three states evaluated and widely distributed across geographic space (Figure 2). While previous studies had detected transgenes in Oaxaca and Mexico City (Agapito-Tenfen et al. 2017; Piñeyro-Nelson et al. 2009; Quist and Chapela 2001; Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007), this is the first study that reports transgene sequences in native maize samples from the state of Chiapas, which was the state with the highest percentage of samples positive for transgenes. In Chiapas, we found transgenic maize samples in 33% of the localities surveyed; in Mexico City, 25% of localities had at least one sample positive for transgenes, while in Oaxaca positive samples for transgenes were distributed in 11% of the surveyed localities (Figure 2). The number of maize samples that yielded positive results for transgenes differs from the above estimates, as on average, three farmers were surveyed per locality (3 farmers per locality in Chiapas, 2.3 in Oaxaca and 12 in Mexico City). Furthermore, many farmers had more than one native maize variety. Thus, in terms of positive maize samples per state, the percentages resulted as follows: 12.6% (58/461 samples) for Chiapas; 2.3% (6/258 samples) for Mexico City and 7% (41/626 samples) for the state of Oaxaca. The transgenic marker that was more frequently amplified was NOS-T, the CaMV 35S promoter in second place, while the identification of specific maize transgenic events was low (for further details see report by Álvarez-Buylla 2018).

We also demonstrated that in Chiapas, transgenic samples are spatially aggregated, in Oaxaca they are marginally aggregated and in Mexico City the number of localities surveyed (20) did not allow us to establish a spatial pattern different from random (Figure 3). For Oaxaca, while the distribution was quite different from the random model, it was still within the confidence band of this model. This result is also in accordance with the finding of no significant spatial associations of transgenic samples for Mexico City, but significant associations were found in Chiapas and Oaxaca (Table 1).

Independently of the number of localities sampled, the aggregation in the distribution of transgenes is related with the number of localities with transgene-positive samples. Thus, even when in Oaxaca we sampled more localities (258), the number of localities with positive samples was lower than in Chiapas (41 v. 58), and consequently it was harder to evaluate aggregation and spatial associations. The same reasoning applies to Mexico City, in which the number of samples positive for transgene presence was just 6.

In Chiapas, spatial associations were found in irrigated and "wet low mid-altitude" areas (Hartkamp 2001), with temperatures between 23°C and 27°C and precipitation between 33 and 43 mm in the coldest quarter of the year.

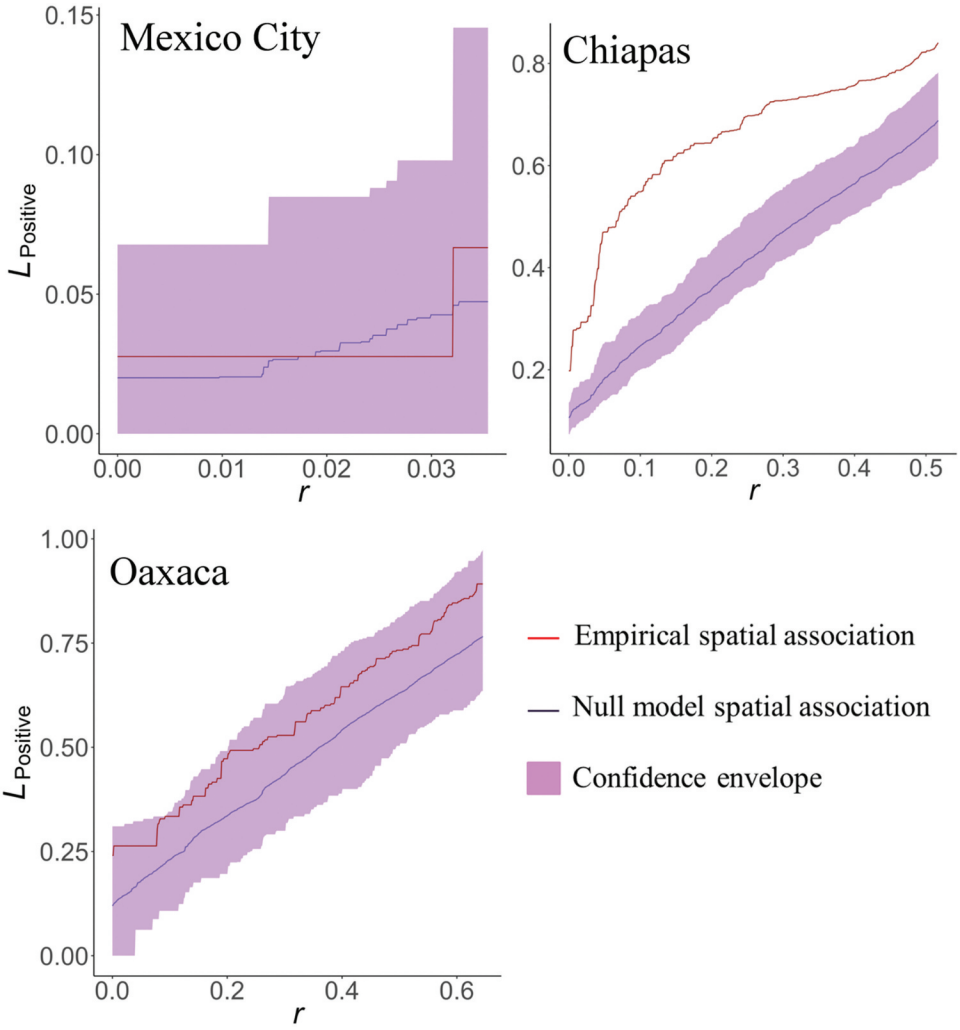

Figure 3. Analysis of the spatial association of the presence of transgenic maize samples in three Mexican states. Values of the empirical L-function (red line), a measure of spatial association, at different influence radii (r). Values above the confidence band (mauve) of the null model (purple line) correspond to a spatial aggregation of the detected transgenes.

Transgene presence in Chiapas was also associated with very small slopes and strong maize consumption (Supplementary Material Table 2). In Oaxaca, the spatial association with transgene presence was determined in localities where indigenous population was high, while localities with transgene presence had different altitudes and slopes. In contrast with Chiapas, the calculated associations in Oaxaca were related with higher temperatures, higher precipitation and proximity to waterbodies. In this state, we also found an association between transgene presence and distance to railways (Supplementary Material Table 2). Finally, even when the proposed data mining approach might help in projecting potential distributions for the subject of interest, in this case the predictive power (obtained through score values) of transgene presence was very low and we were not able to geographically extrapolate our results.

Discussion

The need for a nationwide biomonitoring strategy for mid and long term assessment of transgene presence

In this study, we used an efficient stratified sampling model designed with geographic representativeness to decrease the uncertainty in detecting transgenes in native maize samples. This model can be replicated in future surveys in any state within Mexico and if applied consistently, it will allow for the analysis of changes in transgene frequencies across space and time. Previous scientific efforts conducted in Oaxaca, Mexico City and other parts of the country had detected transgene presence in particular localities only (Agapito-Tenfen et al. 2017; Piñeyro-Nelson et al. 2009; Quist and Chapela 2001; Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007); some studies focused on particular regions within a state (Agapito-Tenfen et al. 2017; Piñeyro-Nelson et al. 2009; Quist and Chapela 2001; Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007); while, to our knowledge, the only study that considered a nationwide representation – surveying several localities in 14 states as a means to represent the different kinds of agricultural practices in the country – had as a central motivation the understanding of the socio-economic dynamics of Mexican farmers, rather than to produce a geographic representation of the different regions where native maize varieties were being sowed at the time (Dyer et al. 2009). A relevant finding of our study is that as far as we know, this is the first report documenting transgene presence in maize varieties from the state of Chiapas, which is only second to Oaxaca in the number of native maize varieties sowed (CONABIO (Comisión Nacional para el Conocimiento y Uso de la Biodiversidad) 2011) and at the highest rate (Figure 2). The high proportion of transgenic maize found in Chiapas, however, could be an overestimation of the actual proportion because

of a possible sampling bias: we could obtain only a small sample of maize planted in the North-Eastern part of the state, comprising the Lacandon rainforest and surrounding territories (Figure 2), where it is documented that indigenous communities cultivate their own maize varieties (Contreras-Cortés et al. 2013). The Frailesca region is highly productive in maize, but still our sampled localities from the Frailesca region could be overrepresented in the total sample with respect to localities in the region of the Lacandon rainforest, with the possible effect of artificially boosting transgene frequency detected in Chiapas. Our data also corroborate transgene presence in two previously sampled states (Mexico City and Oaxaca). Given that transgenes were widely distributed in the maize populations of the three states evaluated, we hypothesize that the presence of transgenes is not due to isolated events. Our results suggest that 20 years after the first report of adventitious transgene presence in maize varieties in Mexico (Quist and Chapela 2001), this phenomenon is either ongoing or has left a molecular fingerprint in some native maize populations in regions that still have high diversity of this staple crop.

In Mexico, the Law on Biosecurity of Genetically Modified Organisms (LBOGM, 2005) was created to regulate at the federal level the experimental release, confined use, import, and export of genetically modified organisms. Despite the existence of this Law and the fact that from 1998 onwards a *de facto* moratorium to open field planting of transgenic maize varieties in the country has been almost continuous, between 2009 and 2013 experimental and pilot-sized plantings of transgenic maize were legally allowed in some Northern states of the country (Sandoval 2017). These plantings may have contributed to the transgenes we detected, as several transgenic escapes have been previously documented for various genetically modified crops (Price and Cotter 2014; Bauer-Panskus, Hamberger, and Then 2013 respectively).

Even with the consistent evidence of transgene presence in native maize populations across time (Agapito-Tenfen et al. 2017; Dyer et al. 2009; Piñeyro-Nelson et al. 2009; Quist and Chapela 2001; Rendón-Aguilar et al. 2019; Serratos-Hernández et al. 2007), public governmental initiatives oriented to detect and identify transgenic sequences in maize have been haphazard (INECC (Instituto Nacional de Ecología y Cambio Climático) 2015; Mercer and Wainwright 2008). Furthermore, these results are not comparable through time. With our results, it is possible to put forth specific biosecurity actions. For example, given the significant spatial associations between the presence of transgenic maize and the proximity to railways, specific efforts geared toward the contention of train freight spills and monitoring of maize grown close by could be put in place.

Since Mexico is the center of origin and diversification of many staple crops, the potential risks associated with the presence of transgenes in these cultivars and their wild relatives, as well as the biosecurity policies and actions enforced by the Mexican government have been analyzed and discussed from several

perspectives (i.e. agronomical, ecological, sociological) and many authors coincide that biosecurity governance in Mexico has likely overlooked the presence of transgenic plants due to inadequate sampling and suboptimal molecular detection approaches (Bellon 2010; Bonneuil, Foyer, and Wynne 2014; Hernández-López 2020).

The importance of Mexican native maize varieties as living genetic reservoirs has been variously acknowledged by multiple scholars (Bellon et al. 2018; Hufford et al. 2012; Kato Yamakake et al. 2009; Rojas-Barrera et al. 2019; Vigouroux et al. 2008; Wellhausen et al. 1951). In this context, transgene presence and diffusion across maize varieties in Mexico posits additional potential risks that can become synergistic with other phenomena that affect the *in situ* conservation of native maize varieties. Despite this situation, there is a general consensus regarding the need for the implementation of measures and incentives to strengthen the biocultural environment that enables the dynamic *in situ* conservation of native maize varieties, which can serve a twofold purpose of strengthening local food security and reducing the risk of genetic erosion (Bellon et al. 2021).

The sampling proposal presented here, if adapted and implemented at a national scale and at regular time periods (i.e., every 4 generations or 4 years), could serve a twofold objective: 1) to provide insight of transgene flow and potential introgression into native maize varieties and – if adequate genetic markers are implemented – to evaluate changes in genetic diversity and structure that can hint to processes of genetic erosion in this crop (Rojas-Barrera et al. 2019), and 2) to understand how farmer seed management dynamics, including seed utilization, acquisition, and sharing strategies, can hinder or stimulate transgene flow, through a written survey applied to participating producers (Brush et al. 2015; Dyer and López-Feldman 2013; Dyer et al. 2009). This data would enable the federal authorities to redesign regulations and biosecurity strategies, and would set a basis to decide if additional regional or locality-specific sampling efforts should be undertaken in order to understand, contain, or manage recombinant sequences present in native maize seed lots, taking into account farmer's adaptation strategies to climate change and their responses to transgene presence in their seed lots (Mercer, Perales, and Wainwright 2012). In this sense, active involvement from the communities where transgenes are detected would be crucial for a bottom-up process to decide biosecurity measures. Such measures would also benefit from more detailed region-specific studies that determine if cultural practices favor an increase or decrease of transgene dispersal throughout a community (e.g. Dyer, López-Feldman, and Niedz 2013; Dyer et al. 2009). In this context, questions pertaining practices such as the sowing of imported grain that is genetically modified, presence of trace amounts of transgenic seed mixed with isogenic hybrid seeds and the potential introduction through undeclared commercial practices or by migrant farm workers of

transgenic seed should be addressed, as has been suggested previously by several scholars (Álvarez-Buylla and Piñeyro 2014; Agapito-Tenfen and Wickson 2018; Dyer et al. 2009).

Furthermore, data and insights attained through both genetic analyses of transgene presence throughout time and space, as well as a detailed understanding of seed management practices could strengthen previous theoretical proposals that have modeled the potential distribution and fate of introduced transgenes into maize (meta) populations in Mexico (Piñeyro-Nelson et al. 2009; Van Heerwaarden et al. 2012). Thus, transgene detection and identification efforts undertaken with a consistent and representative sampling protocol, together with theoretical approaches, can allow for a better understanding and thus protection of the *in situ* dynamics of local maize selection and conservation in different parts of the country (Bellon et al. 2018).

Analysis of transgene presence in context: insights from a data mining approach

Our data mining approach helped us obtain significant spatial associations with some environmental and social variables and enabled us to assess which distinct variables were important in each of the two states analyzed (Chiapas and Oaxaca). Nevertheless, we did not reach enough predictive power to project in the geography where transgenes can potentially be found given a socio environmental profile (Supplementary Material Fig. S2). A very interesting result is that in Chiapas transgene presence is not randomly distributed, suggesting that there are social and environmental factors that are related to transgene presence, and probably dispersal. The type of variables associated with transgene presence in Chiapas relate more with the existence of irrigated fields, such as the "area dominated by irrigation" (Supplementary Material Table 2). This result gives us some insight about the role that industrialized maize agriculture in Chiapas could be playing in transgene introduction. Chiapas' industrialized agriculture induces farmers to buy hybrid seeds yearly to transnational seed companies; most of them are the same that commercialize transgenic seeds in other countries (Trueba Carranza 2012). The internal policies concerning the enforcement of biosecurity and segregation measures in seed companies are unknown to us, but given our results and evidence in other countries like Brazil (Fernandes et al. 2022), it is possible to hypothesize that transgene presence in native maize populations in Chiapas might be an outcome of gene flow from improved hybrid seed lots used in irrigated fields that could in turn have some transgenic seed in them. A recent survey that analyzed the presence of transgenes in some hybrid seed samples collected in various parts of the country found some seed lots with transgenes (Trejo-Pastor et al. 2021); even when this study lacked a representative sample of all the hybrid seed varieties that are sold in Mexico, it gives some support to our

hypothesis. The fact that transgene presence in native maize populations might be related to transnational seed companies is one of the reasons why biosecurity policies should be strengthened and the national capacity for producing our own seeds needs to be recovered. Hybrid seeds coming from transnational companies not only represent a risk in terms of transgene contamination, but they are also being sold at a very high cost, reducing farmer's net income significantly (Ureta et al. 2020).

Even when in Oaxaca the localities sampled exceeded the localities recommended by our geographic representative sampling proposal, the predictive power was not enough to project potential transgene presence. This result might be associated with the enormous complexity of the system under study. Still, as in Chiapas, transgenes are also spatially aggregated and significant spatial associations between social and environmental variables were found. Associations between transgene presence and social and environmental factors in this state were more related with the presence of indigenous populations and a dominance of rainfed fields, i.e., with small-scale rainfed agriculture. There was also a significant spatial association with the proximity to railways, probably hinting at the possibility that imported grain used as seed could be a source of transgenes. However, the fact that transgenes were detected across several environments in this state could mean that they have been present for several years and have thus been unknowingly incorporated into the seed exchange dynamics that are common among peasants in Oaxaca. These dynamics and practices need to be better understood to increase our understanding on the role they could be playing in transgene dispersal. Consequently, we strongly recommend evaluating the influence of small-scale agriculture dynamics on transgene presence, as was previously documented by Dyer and colleagues (2009).

In Mexico City, samples positive for transgene presence were not aggregated, but this result could be related with the relatively low number of positive records registered. A higher number of localities are needed to potentially increase the number of positive localities and consequently, enable for potential spatial associations and increase the predictive power of our model. However, we could corroborate the presence of transgenes in Mexico City, as previously documented by Serratos-Hernández and collaborators (2007), suggesting that over a decade after this work was published, no effective measures have been implemented despite this state being established as a "transgene-free zone" since 2009 (GODF 2009), a measure that was further written into the local political constitution when this entity became a state in 2017 (CPCDMX 2017).

Conclusions

To our knowledge, this study is the first attempt to standardize a sampling protocol aimed to detect and identify transgenic sequences in native maize populations, encompassing the entire geographic area where they can

currently be found in Mexico. It is also the first analysis focused on providing insight about the potential sources of transgene introduction and dispersion, through a geographic data mining approach. We believe that the information generated in this work can be helpful for the national biosecurity authorities. Furthermore, our approach, if adapted to other geographies and agronomic contexts, could be useful for other countries that are centers of origin and diversity of specific crop species, where their importance warrants the implementation of sustained seed sampling schemes and transgene monitoring. We would strongly recommend for future studies to follow a similar sampling proposal as the one presented here in order to make results across time and geography comparable. We are aware of the difficulties of following a strict sampling protocol during field work, but it is still important to strive at standardizing sampling efforts.

Highlights

- A geographic-based nationwide sampling protocol was proposed for maize in Mexico; this approach could be adapted for transgene biomonitoring in other crops and other countries.
- We applied this sampling protocol in three states, finding that transgenes are widely distributed across the analyzed states: Chiapas (33% of localities), Mexico City (25%), and Oaxaca (11%).
- This is the first time that transgenes are detected in Chiapas, which is one of the most agrodiverse areas of Mesoamerica.

A data mining approach suggests that transgenes in Chiapas are spatially associated with irrigated agriculture, while in Oaxaca they seem to be related with seed exchange dynamics.

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