AFLP polymorphism in restored provenances of *Ceiba aesculifolia* within an urban heat island

Polimorfismo AFLP en sitios de muestreo restaurados de *Ceiba aesculifolia* bajo la influencia de una isla de calor urbano

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Abstract. To quantify the effect on genetic diversity of restoring tree populations in an area under the influence of an urban heat island, we evaluated the genetic diversity of 72 *Ceiba aesculifolia* individuals, from a restoration experiment established from 2170 to 2260 m a.s.l. Reintroduced individuals were compared with the provenance from which the seeds were obtained, and two external provenances, for a total of 123 individuals. Samples were analyzed with three AFLP primer combinations. Polymorphisms of 38.4 to 62.5% were obtained. Genetic diversity estimated with the Simpson index ranged from 0.14 to 0.2. The provenances in the restoration site had higher diversity than the provenance that originated the seeds. Groups formed with provenances with similar genetic diversity (no statistical differences) also shared similar Bayesian mixture proportions. Having higher genetic diversity in restored provenances than in the provenance that originated the seeds can be favorable as the conditions in the site change in the future due to the expected increase in the urban heat island effect as the nearby city expands.

Keywords: Genetic diversity; AFLP; Restoration ecology; *Ceiba aesculifolia*; Protected Natural Area.

Resumen. Para cuantificar el efecto en la diversidad genética de restaurar poblaciones arbóreas en un área bajo la influencia de una isla de calor urbano, evaluamos la diversidad genética de 72 individuos de *Ceiba aesculifolia* en un área de restauración establecida entre los 2170 a los 2260 m s.n.m. Los individuos reintroducidos se compararon con la procedencia de la que se obtuvieron las semillas y dos procedencias externas, alcanzando un total de 123 individuos. Las muestras se analizaron con tres combinaciones de oligonucleótidos para generar AFLP. Se obtuvieron valores de polimorfismo entre 38,4 y 62,5%. La diversidad genética, evaluada con el índice de Simpson, varió entre 0,14 y 0,20. Las procedencias del sitio restaurado tuvieron diversidad mayor que la procedencia de la que se obtuvieron las semillas. Los grupos obtenidos de procedencias con diversidad genética similar (no estadísticamente significativas), también compartieron proporciones similares de las mezclas Bayesianas. Contar con una mayor diversidad genética en las procedencias del sitio de restauración que en la procedencia que originó las semillas puede ser favorable en la medida que las condiciones del sitio cambian en el futuro, en consecuencia del incremento en los efectos de la isla de calor urbano conforme aumenta la ciudad cercana al sitio.

Palabras clave: Diversidad genética; AFLP; Restauración ecológica; *Ceiba aesculifolia*; Areas naturales protegidas.

INTRODUCTION

Determination of genetic population parameters associated with plant introduction in the context of ecological restoration projects constitutes one of the goals of restoration genetics. It is necessary to evaluate the long-term potential of a plantation, considering its high genetic diversity to increase the probability of persistence and thus, to maintain its evolutionary potential (McKay, 2005; Broadhurst et al., 2008; Falk et al., 2009). Lesica and Allendorf (1999) have argued that it is preferable to establish individuals and populations of high genetic diversity when disturbance levels and proposed restoration areas are large. They proposed that hybrid genotypes of various sources in highly perturbed sites would have a greater chance of survival, as opposed to local genotypes that are non-adapted to such severe perturbations. Hufford and Mazer (2003) have warned of the dangers of establishing non-local genotypes, initiating exogamy that does not improve fitness, since they are non-adapted and could cause further damage. For this reason, Sinclair et al. (2009) and Bedolla-García et al. (2011) recommend seed collection from the sites of greatest genetic diversity located as close as possible to the restoration site. This suggests that the problem remains unsolved, and that the effects of the reintroduction of individual plants as part of a restoration effort on various genetic parameters merits further research.

The present study explored this issue by evaluating the genetic diversity of surviving individuals of *Ceiba aesculifolia* (Kunth) Britten & Baker f. in a restoration project within the Protected Natural Area of Cerro del Punhuato (PNA-CP) in Morelia, Michoacán, Mexico. *Ceiba aesculifolia* is classified in the *Bombacaceae,* a tree that can reach 15 m in height and has a geographical distribution from central Mexico to Honduras (Gibbs & Semir, 2003). Populations of *C. aesculifolia* can be found in Michoacán up to 1900 m.a.s.l., however isolated trees or very small populations reach 2200 m.a.s.l. (Carranza & Blanco-García, 2000). The high altitude distribution range for the species in tropical dry forests has been heavily reduced particularly within the region known as the Bajío, including the vicinity of Morelia where most *C. aesculifolia* remnant populations have few individuals (Rzedowski & Calderón 1987).

Niche shifts for plant species have been proposed as a response to the effects of urban heat islands (Gomez et al., 1998; Jenerette et al., 2007). In urban areas, the increased cover of dark surfaces from buildings and streets (Zhao et al., 2006), alter the heat balance and thus increase temperatures. In fact, temperature is the most affected climatic variable altered by urban development (Pigeon et al., 2007). Temperature increase within the city and its surroundings to rural areas can be as high as 8 °C. In comparison, thus, the term "urban heat island" coined by Manley (1958) is now widely accepted.

Valle-Díaz et al. (2009) explored the urban heat island effect of the city of Morelia in terms of the survival of 320 individuals of *C. aesculifolia*. Plantations on the western slope of the PNA-CP included 80 individuals at each of four sites across an altitudinal gradient (2170, 2200, 2230 and 2260 m.a.s.l.). In the study by Valle-Diaz et al. (2009), *C. aesculifolia* plants were propagated in a greenhouse from seeds collected from the nearest remnant population, just outside the protected area. The remnant population has 15 reproductive individuals. After one year of planting they reported the highest survival (100%) at 2230 m a.s.l. and the lowest (25%) at 2170 m.a.s.l. This implied an altitudinal shift for the optimal establishment of *C. aesculifolia* seedlings. This result contrasts with the 2000 m.a.s.l. altitudinal upper limit of distribution for the Bajío. This finding suggested that, under conditions similar to those found at the study site, assisted migration (Guariguata et al., 2008) across the whole altitudinal gradient should be considered as part of a restoration effort.

Although survival and growth have been considered good approximations to fitness in trees (Ouedraogo et al., 2011), quantifying the genetic composition of the established populations is important both in terms of ecological restoration and for a better understanding of assisted migration as a measure of adaptation to climate change (McLachlan et al., 2007; Aubin et al., 2011; Winder et al., 2011). Genetic analysis with AFLP (Vos et al., 1995) was therefore carried out to: (1) determine the genetic diversity of groups of *C. aesculifolia* individuals established at four altitudinal clines (Valle-Díaz et al., 2009), as well as the progenitor population and two populations external to the PNA-CP; (2) determine the presence of distinct genotypes for the different groups of individuals. The hypothesis is that the altitudinal clines with fewer survivors show a decrease in diversity as opposed to the higher survival clines. Additionally, the altitudinal levels presenting higher survival will have a genetic diversity similar to, or lower than, the progenitor population. It is also expected that, as a result of a small population size and differential selection pressure at the altitudinal clines, populations external to the PNA-CP would be more diverse than both the progenitor population and those used in the restoration effort.

MATERIALS AND METHODS

Location and collection of plant material. The leaves of 129 *Ceiba aesculifolia* individuals were collected from seven collection sites, considering each one of these sites as a provenance; for all provenances, all individuals were sampled with the exception of the provenance from which the seeds for the restoration effort were collected (Valle-Díaz et al., 2009). For this provenance only the reproductive individuals were sampled. The reasoning for this selectivity was that various individuals at this site were irrelevant to the study. This was because they never produced seeds since their monitoring began in 2005. Four provenances located at 2170, 2200, 2230 and

2260 m.a.s.l. in the PNA-CP (labeled with an E, followed by the altitude at which they are located: E2170, E2200, E2230 and E2260). These provenances comprise the surviving trees from the experiment of Valle-Díaz et al. (2009). The 15 reproductive individuals from the "progenitor" provenance (2020 to 2060 m.a.s.l.) were distant by ca. 400 m from the restoration experiment. Two sites external to the PNA were used for comparative purposes; "Cuitzeo" and "Tzitzio" at 22.7 and 24.8 kms from the restoration experiment, respectively (Fig. 1, Table 1).
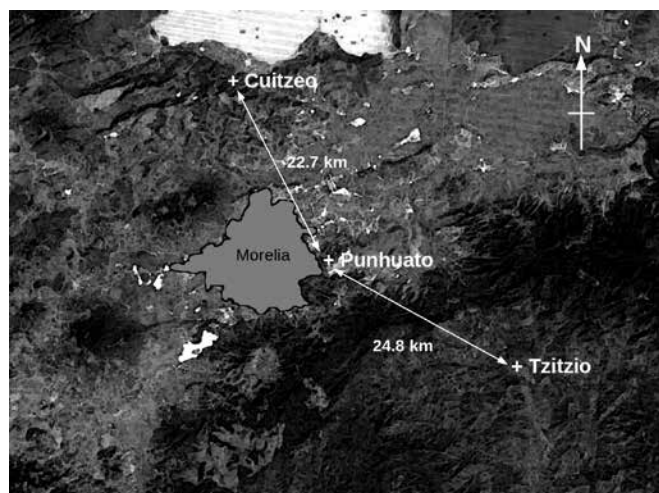
Fig. 1. Location of the provenances and distance between the three geographical locations, including the Punhuato protected area. The progenitor and restoration provenances (E1970, E2200, E2230 and E2260) where within 400 m from each other in the Punhuato.

Fig. 1. Localización de las procedencias y distancia entre las localidades geográficas incluyendo el área protegida del Punhuato. La procedencia de los progenitores así como las del sitio de restauración (E1970, E2200, E2230 y E2260) se encuentran a 400 metros de distancia en el Punhuato.

DNA extraction and amplification of AFLP. DNA extraction was carried out using the MINI-PREP protocol of Lefort and Douglas (1999). The amplified fragment length polymorphisms (AFLP) (Vos et al., 1995) were obtained using the AFLP ligation and preselective amplification kit for regular plant genomes (N/P 402004) from Applied Biosystems®. The manufacturers' directions were followed. Selective amplification was conducted with three primer pair combinations: *Mse*I CAT- *Eco*RI ACC, *Mse*I CTA-EcoRI ACC and *Mse*I CTC-*Eco*RI ACT. Amplification products were sent to Macrogen Inc. of Korea for band detection and size calculation. The resulting electropherograms were analyzed with Peak Scanner v1.0 (Applied Biosystems®), eliminating bands with intensities of < 100 nm. The remaining bands were then analyzed using the RawGeno R CRAN package (Arrigo et al., 2009), where bands of < 50 bp and > 500 bp were eliminated and a binary matrix (0 for absence of band and 1 for presence of band) was exported for further analysis.

Statistical analysis. The comparison of genetic diversity among provenances was obtained through the Rarefaction by the bootstraping method in RaBot proposed by Scotti et al. (2013); two R scripts were provided by the authors. The first script was used for filtering the markers in genotypic disequilibrium to further analyze only the independent markets complying with RaBot requirements. The second script for executing RaBoT for AFLP "phenotypes" codified for presence/absence data of AFLP fragments within the R platform (R Development Core Team 2014). RaBoT calculates the genetic diversity for each genetic marker with Simpson's Index for two populations at a time. In this method, as explained by Scotti et al. (2013), the observed diversity in the smaller population is computed for each genetic marker. In the larger population subsamples are randomly drawn with replacement with sample sizes equal to the size of the small population. Genetic diversity (H) is computed for each subsample and

Table 1. Collection sites and number of individuals collected per provenance.
Tabla 1. Sitios de colecta y número de individuos muestreados por procedencia.

Collection site	Altitude (m. a.s.l.)	North	West	No. of individuals
E2170	2170	19° 41' 59"	101° 07' 49"	7
E2200	2200	19° 41' 59"	101° 07' 49"	21
E2230	2230	19° 41' 59"	101° 07' 49"	30
E2260	2260	19° 41' 59"	101° 07' 49"	14
Established	2170 to 2260	19° 41' 59"	101° 07' 49"	72
Progenitor	2020 to 2060	19° 42' 60"	101° 08' 21.49"	15
Cuitzeo	2000	19° 53' 56.05"	100° 07' 40.20"	16
Tzitzio	1600	19° 34' 48.47"	100° 55' 27.61"	20
			Total	123

for each marker, and the median genetic diversity is obtained. If there is no real difference in diversity between the small and the large population, beyond the effect of population sampling stochasticity, then the genetic diversity value of the small population should be drawn, for each locus, from the same distribution as the random subsamples from the large population and the same locus. As a consequence, individual marker ΔH values should have the same probability of being positive or negative, and at the genome level, there should be equal numbers of positive and negative ΔH values. Departures from this expectation were tested by a Chi-square test and were taken as suggestive of real, genome-wide differences in diversity. RaBot allows to statistically test genome-wide differences in diversity from a large number of loci and from populations of different sizes.

In addition, Bayesian cluster analysis was carried out as implemented in Structure V 2.3 (Pritchard et al., 2000). This allowed characterizing genetically homogenous individuals within a provenance. Utilizing the admixture model without prior provenance information, ten separate runs were conducted to determine the K values of verosimilarity (from 1 to 10). The method of Evanno et al. (2005) was utilized to select the optimum value of selected clusters or genetic groups present in the sample (K). Runs were performed with a burn-in of 10000 generations, and data collected from 100000 additional Monte-Carlo steps.

RESULTS

A total of 445 loci were obtained with three AFLP combinations for 123 individuals. The highest percentage of polymorphic loci obtained was for the Tzitzio provenance (56.6%) and the lowest for Cuitzeo (38%; Table 2). The mean diversity of genetic markers for each provenance calculated as the Simpson index ranged from 0.1482 for the Cuitzeo provenance to 0.1999 for the provenance at 2260 m.a.s.l. in the restoration area.

Table 2. Intra-population genetic structure in seven *C. aesculifolia* provenances.
Tabla 2. La estructura genética intrapoblacional de las siete procedencias de *C. aesculifolia*.

Provenance	No. of individuals	No. of loci	Polymorphic loci percentage	No. of polymorphic loci
E2170	7	445	278	62.5%
E2200	21	445	231	51.9%
E2230	30	445	227	51.0
E2260	14	445	247	55.5%
Progenitor	15	445	194	43.6%
Cuitzeo	16	445	171	38.4%
Tzitzio	20	445	252	56.6%

Table 3. Results of RaBoT tests between Ceiba aesculifolia provenances. For each cell, proportion of markers for which diversity is lower in the smaller population, χ^2 and P-value.
Tabla 3. Resultados de la prueba de RaBot entre las procedencias de Ceiba aesculifolia. Para cada celda, la proporción de marcadores para los que la diversidad es menor en la población pequeña, χ^2 y el valor de P.

Provenance	E2170	E2260	Progenitor	Cuitzeo	Tzitzio	E2200
E2260	0.52					
	$\chi^2 = 0.356$	*				
	$P = 0.550$					
Progenitor	0.35	0.39				
	$\chi^2 = 20.43$	$\chi^2 = 15.01$	*			
	$P < 0.0001$	$P = 0.0001$				
Cuitzeo	0.35	0.34	0.43			
	$\chi^2 = 22.39$	$\chi^2 = 31.39$	$\chi^2 = 1.237$	*		
	$P < 0.0001$	$P < 0.0001$	$P = 0.266$			
Tzitzio	0.48	0.46	0.60	0.62		
	$\chi^2 = 0.336$	$\chi^2 = 2.49$	$\chi^2 = 11.64$	$\chi^2 = 17.83$	*	
	$P = 0.562$	$P = 0.114$	$P = 0.0006$	$P < 0.0001$		
E2200	0.44	0.41	0.60	0.61	0.48	
	$\chi^2 = 4.74$	$\chi^2 = 9.45$	$\chi^2 = 11.21$	$\chi^2 = 14.22$	$\chi^2 = 0.318$	*
	$P = 0.029$	$P = 0.002$	$P = 0.0008$	$P = 0.0002$	$P = 0.572$	
E2230	0.38	0.33	0.59	0.64	0.49	0.53
	$\chi^2 = 12.64$	$\chi^2 = 31.01$	$\chi^2 = 9.12$	$\chi^2 = 21.11$	$\chi^2 = 0.109$	$\chi^2 = 1.014$
	$P = 0.0004$	$P < 0.0001$	$P = 0.0025$	$P < 0.0001$	$P = 0.741$	$P = 0.314$

RaBot tests (Table 3) indicated that the provenances with the lowers diversity values, Cuitzeo (0.1482) and the Progenitor (0.1632) did not differ between them, but differed from all the other provenances. The provenances at the lowest 2170 m.a.s.l. (0.1834) and highest altitudes 2260 m.a.s.l. (0.1999) and Tzitzio did not differ among themselves, but differed from the four remaining provenances. Provenances at intermediate elevations 2200 (0.1744) and 2230 m.a.s.l. (0.1742) did not differ between them but from all the others.

Bayesian cluster analysis indicated that the optimum number of genetic populations was K = 3 with an average LP (D) value of -16081.68. These three genotype units (Ks1, Ks2 and Ks3) were present in different Bayesian admixture proportions in the seven provenances (Fig. 2). The provenances with the largest genotypic proportion of Ks1 (Blue) were E2200 (44.97%) and E2230 (50.25%); Ks2 (Orange) in the progenitor (46.07%) and Cuitzeo (77.35%), and finally Ks3 (Yellow) in E2170 (57.45%), E2260 (371.72%) and Tzitzio (60.29%) (Table 4).

Table 4. Percentage for each Ks cluster found in each population. Values greater than 40% are shown in bold text and the colors assigned to each cluster in the figures in parenthesis.
Tabla 4. Porcentaje de cada grupo Ks encontrado en cada población. Los valores mayores del 40% se muestran en negritas y el color asignado a cada grupo en las figuras en paréntesis.

Populations	Ks1 (Blue)	Ks2 (orange)	Ks3 (yellow)
E2170	31.13	11.42	**57.45**
E2200	**44.97**	33.26	21.78
E2230	**50.25**	18.98	30.77
E2260	15.69	12.59	**71.72**
Progenitor	35.39	**46.07**	18.55
Cuitzeo	6.15	**77.35**	16.50
Tzitzio	6.32	33.39	**60.29**

DISCUSSION

Differences in the genetic diversity highlights Cuitzeo (0.15) as the least diverse provenance, and the provenance at 2260 m.a.s.l. (0.20) as the highest; the difference amongst these two of 0.25% indicates that higher diversity in restored provenances can be achieved. The difference between the progenitor provenance and the provenances in the PNA-CP varied between 6 and 18%. Diversity values of the restoration site were always higher than the diversity of the progenitor provenance.

Higher diversity within the restore populations has also been reported in other studies (Alonso et al., 2014). The opposite has also been found (Cruz Neto et al., 2014); the outcome seems to depend both on the mating system of the species under study and the seed collection procedure. *Ceiba aesculifolia* is a strict self-incompatible species (Quesada et al., 2013) suggesting that
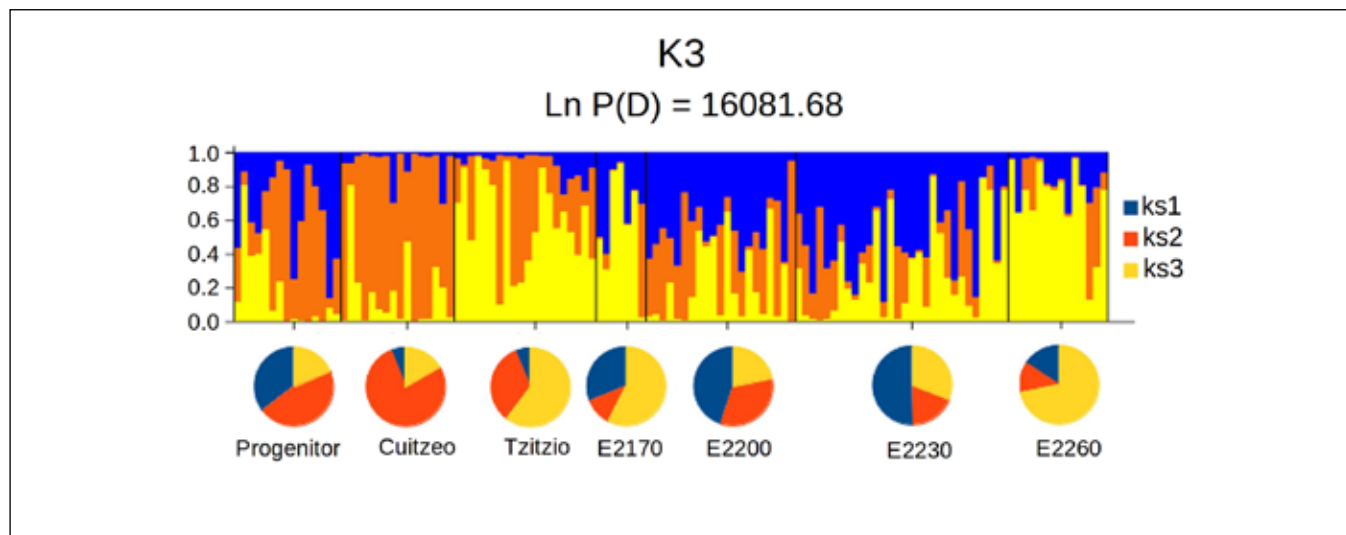
Fig 2. Bar graph produced by Structure V 2.3 for K3. Columns represent each individual. Black lines delimit the provenance and each different genotype combination is denoted by a color. Individuals with two or more colors are the product of a mixture of genotypes. The percentage contribution of each distinct genotype Ks for each provenance is shown below.
Fig 2. Gráfica de barras producida por Structure V 2.3 para K3. Cada individuo está representado por una columna. Las líneas negras delimitan las procedencias y cada combinación diferente de genotipos se muestra en un color. Los individuos que muestran dos o más colores son el producto de una mezcla de genotipos. Debajo se muestra el porcentaje de la contribución de cada genotipo Ks distinto para cada procedencia.

the higher diversity in the restored provenances might be a consequence of this.

The genetic diversity among the natural remnant provenances differed from 9% to 19%. The highest difference is close to those of other species which have suffered severe reduction in population sizes, such as *Adansonia digitata* with a range of 20% (Assogbadjo et al., 2006).

The highest diversity was found in the provenances from the restoration area in the PNA-CP with fewer remaining individuals at E2170 (with seven individuals) and E2260 (with fourteen individuals). This result, while contradictory to our initial hypothesis, has also been reported in *Adansonia digitata* (Malvaceae), where the highest variation of H_E = 0.35 was obtained for a provenance of six individuals, whilst for another 10 provenances the range obtained was H_E = 0.22 to 0.32 f (Assogbadjo et al., 2006). Likewise in *Vochysia ferruginea* (Vochysiaceae), the highest diversity of H_E = 0.32 was found for a provenance of just six individuals, and diversities of H_E = 0.16 to 0.30 for another five provenances with 16 to 20 individuals (Cavers et al., 2005). This pattern has been attributed to species of early or median succession as compensating process, which may be occurring in the *C. aesculifolia* of the PNA-CP.

There is consistency between the results of the genetic diversity tests using RaBot and the Bayesian cluster analysis. The provenances that did not show statistical differences between them also had the highest proportion of each of the Ks; that is, E2200 and E2230 showed the highest proportion in Ks1; Cuitzeo and the Progenitor provenances had the highest proportion in Ks2, and Tzitzio, E2170 and E2260 in Ks3.

The genetic diversity of the restoration site provenances was higher than that for the progenitor provenance. Although the genetic identity between them was high, all provenances shared the three Ks genotypes, albeit in different proportions, indicating a genetic connectivity between the provenances. This type of genetic connectivity is common in plant species such as *C. aesculifolia* with high levels of pollen and seed dispersal (Cavers et al., 2005; Kindt et al., 2009).

It is important to mention that the altitudinal clines where survival was highest were the intermediate altitudes at 2200 and 2230 m.a.s.l. as reported by Valle-Díaz et al. (2009); furthermore, these provenances had similar genetic diversity and the highest proportion of Ks1. Populations possessing high genetic diversity in restored provenances can be favored as the conditions in the site change in the future due to the increment in the urban heat island effect as the nearby city expands.

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