Genetic diversity and structure of morphologically characterized populations *Pinus ayacahuite* and *Pinus strobiformis* through the analysis of neutral nuclear markers

Análisis de diversidad y estructura genética con marcadores nucleares neutros de poblaciones de Pinus ayacahuite y Pinus strobiformis morfológicamente caracterizadas

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Abstract

Diversity and genetic structure of *Pinus ayacahuite* and *Pinus strobiformis* was assessed by random amplified polymorphic DNA (RAPD) variation. Eleven populations distributed along a latitudinal gradient in Mexico were morphologically identified as P. strobiformis, P. ayacahuite var. veitchii or P. ayacahuite var. ayacahuite. A total of 69 primers were screened, and 10, that amplified 51 intense and reproducible fragments, were chosen for the genetic analysis. None of the 51 fragments were unique to a population. Genotypic diversity (Hj) ranged from 0.222 to 0.287 among populations. The total gene diversity (HT) of *P. ayacahuite* and *P. strobiformis* was 0.276 and 0.318, respectively. F_{ST} values showed most genetic variation to be within populations. Differentiation among wingless populations of P. strobiformis was almost double (F_{ST}=0.179) than that of winged seed populations of P. ayacahuite (FST=0.080). The AMOVA analysis confirmed the-se results. The analysis with STRUCTURE showed three genetic groups and the population of Ce-rro el Potosi as clearly differentiated. This po-pulation and seed dispersal mechanisms other than dissemination by Clark's nutcracker might explain the high differentiation found in P. strobi-formis. Management of *P. ayacahuite* and *P. stro-biformis* in Mexico should be aimed to maintain morphologically and genetically well-differen-tiated populations.

Keywords: Genetic variation; Genetic structure; RAPD; Pinus ayacahuite; Pinus strobiformis

Resumen

La diversidad y la estructura genética de *Pinus ayacahuite* y *Pinus strobiformis* se determinaron con polimorfismos de DNA amplificados al azar (RAPDs). Once poblaciones distribuidas a lo largo de un gradiente latitudinal en México fueron identificadas morfológicamente como P. strobiformis, P. ayacahuite var. veitchii o P. ayacahuite var. ayacahuite. Se tamizaron un total de 69 cebadores, y sólo 10 que amplificaron 51 fragmentos intensos y reproducibles se seleccionaron para el análisis genético. Ninguno de los 51 fragmentos fueron exclusivo de una población. La diversidad genotípica (Hj) varió de 0.222 a

0.287 entre las poblaciones. La diversidad genética total (HT) de P. *ayacahuite* y P. s*trobiformis* fue de 0.276 y 0.318, respectivamente. Los valores de FST mostraron que la mayoría de la variación genética se encuentra dentro de las poblaciones. La diferenciación entre poblaciones de P. strobiformis con semillas sin alas fue casi el doble (F_{ST} = 0.179) que la de las poblaciones de P. ayacahuite de semillas con alas (F_{ST} = 0.080). El análisis de AMOVA confirmó estos resultados. El análisis con el programa STRUCTURE mostró tres grupos genéticos y la población de Cerro el Potosí como diferenciada. Los mecanismos de dispersión de la semilla podrían explicar la alta diferenciación encontrada en P. strobiformis. El manejo de *P. ayacahuite* y *P. strobiformis* en México debe orientarse a mantener poblacio-nes bien diferenciadas desde el punto de vista morfológico y genéticoPalabras clave: variación genetica, estructura genetica, RAPD, *Pinus ayacahuite; Pinus strobiformis.*

Introduction

Pinus ayacahuite Ehrenberg ex Schlechtendal and *Pinus strobiformis* Engelman are two important components of forest ecosystems in Mexico. They are a valuable source of timber as well as the tallest-growing and most regular shaped representatives of the Mexican white pines (Farjon & Styles,1997). *Pinus ayacahuite, P. strobiformis* and *Pinus flexilis* James comprise a complex of closely related species (Critchfield, 1986; Farjon & Styles, 1997; Syring et al 2007). They grow on well drained soils as discrete populations in mountainous areas at altitudes ranging from 2000 to 3600 m. *Pinus strobiformis* occurs naturally in the north of the Mexican Transvolcanic Belt to southwestern Colorado and northern Arizona. The distribution of *P. ayacahuite* ranges from the Mexican Transvolcanic Belt to northern Honduras (Farjon & Styles 1997). *Pinus flexilis* is restricted to the Rocky Mountains and the Basin and Range region of western North America north to southern British Columbia and Alberta (Jorgensen et al 2002).

White pines are traditionally classified within subgenus *Strobus*, Section Strobus, Subsection *Strobi* (Steinhoff & Andresen 1971; Farjon & Styles 1997). Re-cently, they were included in the new section "Quin-quefoliae Duhamel" (Gernandt *et al* 2005; Syring et al 2007). Three varieties of P. *ayacahuite* have been recognized; *P. ayacahuite* Ehrenb. ex Schltdl. var. *ayacahuite,* restricted to the southern part of its distribution range, P. *ayacahuite* var. veitchii (Roezl) G. R. Shaw, found mostly in the northern region of the Mexican Transvolcanic Belt, and P. *ayacahuite* var. brachyptera G. R. Shaw, which is synonymous with P. *strobiformis*, distributed from northern Mexico northward (Perry, 1991; Farjon & Styles 1997).

Pérez de la Rosa (1993) examined the geographical variation of 17 morphological charac-ters among Mexican populations of P. *ayacahuite* and P. *strobiformis* and found a pattern of cline variation in cone morphology and seed wing len-gth from north to south. Cones of P. strobiformis have irregular shapes and variable sizes, and they are usually smaller than those of P. *ayacahuite.* The seeds of P. strobiformis are large and may be wingless or have a vestigial wing (<10 mm), while the typical seeds of P. ayacahuite are relatively smaller with a wing size up to twice the seed's size. A large seed with a wing size intermediate between that of P. ayacahuite and P. *strobiformis* has been described for P. *ayacahuite* var. veitchii (Farjon & Styles 1997).

Seed and cone traits are associated with seed dispersal mechanisms (Samano &Tomback 2003). Pines dispersed by animals have relatively large and wingless seeds; apparently the wingless condition facilitates seed disper-sal by small mammals and birds (Tomback & Linhart 1990; Tomback et al 2005, Tomback *et al* 2011a). In contrast, pines dispersed by wind have small to medium-sized seeds and relatively large wing. Animals exclusively disperse about 25 out of 110 recognized pine species; the rest of the species are dispersed by wind or by both animals and wind (Vander Wall, 2008). Clark's nutcracker (*Nucifraga columbiana*) is an important seed disperser for populations of P. *strobiformis* located at limit of the northern range of its distribution. Southern populations of P. *strobiformis* have different seed dispersal biology, recent information indicates that core

populations south of the dependable range of Clark's nutcracker have larger seeds and may be dispersed by small mammals and jays (Tomback *et al* 2011b).

The genetic diversity of P. *ayacahuite* and P. *strobiformis* has been studied at both lo-cal and wide range of geographical scale. Hernández-González (1990) and Ledig (1998) have studied the levels of genetic variation in populations of P. *ayacahuite* and a few of P. *strobiformis* using allozymes. Chloroplast microsatellite markers have also been used to examine the genetic diversity in P. ayacahuite and P. strobiformis (Ortíz-Medrano et al 2008; Moreno-Letelier & Piñe-ro 2009) However, as suggested by Bower *et al* (2011) the current published population genetic data are not sufficient to compare the genetic diversity and population structure of the P. *ayacahuite-P. strobiformis* complex. Moreover, there is a poor understanding regarding the influence of seed morphology and dispersal mode on genetic diversity and structure of this pine complex. The use of molecular nuclear markers might offer a useful alternative for the analysis of genetic diversity of these pines, but until now, their effecti-veness has not been evaluated.

The purposes of the present study are a) to assess genetic diversity and population structure of populations of P. *ayacahuite* and P. *strobifor-mis* sampled in a latitudinal gradient through the analysis of a nuclear marker, and b) to investigate whether differences in seed morphology are rela-ted to genetic differentiation among populations.

Materials and methods

Field sampling

Plant material was collected from 11 populations of the P. *ayacahuite*-P. *strobiformis* complex during a period of four years (2001-2004), a total of 288 samples were gathered. The number of sampled trees per population varied from 20 to 31, and selected trees were separated by a minimum of 50 m within population.

Table 1
Main characteristics and exact location of the *Pinus ayacahuite- P. strobiformis* complex populations in this study.

Taxon	Population (Collector voucher[a])	n	Region	Latitude (°N)	Longitude (°W)	Altitude (masl)	Mean annual precipitation (mm)	Mean annual Temperature (°C)	Soil type	Forest coverage (%)
P. strobiformis	1 Cananea (1881)	24	North	31°03'18	110°22'59	2470	500	15	Phaeozem, Leptosol, Regosol, Planosol, Luvisol, Cambisol, Vertisol, Fluvisol	55.21
	2 Bocoyna (1882)	23	North	27°43'36	107°40'19	2470	683	15	Regosol, Luvisol, Leptosol, Phaeozem, Umbrisol, Vertisol, Durisol	78.14
	3 Cerro el Potosí (1891)	25	North	24°53'13	100°13'26	3116	500	15	Litic leptosol	79.29
	4 Pueblo Nuevo (1889)	23	North	23°37'32	105°50'14	2224	1400	17	Leptosol, Luvisol, Regosol, Umbrisol, Cambisol, Phaeozem, Fluvisol	99.26
P. ayacahuite										
var. *veitchii*	5 Cuale (1888)	26	North	20°22'41	105°02'37	2530	1840	16	Litosol	99.27
var. *ayacahuite*	6 Real del Monte (1860)	25	Central	20°09'13	98°38'07	2630	1000	16	Andosol, Luvisol	58.24
var. *veitchii*	7 Las Palmas (1646)	25	Central	19°40'	102°25'	2260	800	14	Regosol	63.41
var. *ayacahuite*	8 Xico (1867)	23	Central	19°31'08	97°05'42	2930	1600	13	Andosol, Luvisol	79.97
var. *veitchii*	9 San Rafael (1858)	30	Central	19°12'38	98°44'10	2721	1500	12	Luvisol	94.02
var. *ayacahuite*	10 Ixtlán (1871)	24	South	17°24'37	96°30'43	2770	2350	18	Luvisol, Acrisol, Cambisol, Fluvisol	77.85
var. *ayacahuite*	11 Rancho Nuevo (1874)	23	South	16°40'13	92°33'15	2390	1250	18	Alisol, Luvisol, Leptosol, Gleysol	87.75

[a]Pérez de la Rosa and Vargas (IBUG)

The populations selected were distributed along a latitudinal gradient representative of the distributional range of the pine complex in Mexico.

Figure. 1. Map illustrating the positions of Pinus strobiformis and Pinus ayacahuite populations sampled. (For details of sample locations, see Table 1).

Information about percentage of forest cover was obtained from the Land Use/Land cover map from INEGI (2011; scale 1:1000000) using a buffer of 5 km surrounding the populations' patch collection point.

DNA extraction and PCR-RAPD

Total genomic DNA was extracted following the CTAB method with minor modifications (Palomera et al 2008). DNA quality and concentration were determined visually on agarose gels and by UV spectrophotometry. DNA samples were diluted in MilliQ water to a final concentration of 20 ng/µL.

PCR- RAPD reactions were performed according to Williams et al (1990), with minor modifications (Castro-Félix et al 2008). The reaction component concentrations and conditions were optimized for representative samples of P. *ayacahuite* and P. *strobiformis* to give reproducible markers. A total of 69 oligonucleotides primers (series OPA, OPB, OPBB and primers OPC-1 to OPC- 9) were assayed during this study, and ten were selected because of their clear RAPD profile. Each PCR-RAPD reaction proceeded in a 25 µL reaction mix that contained 0.75 U of Taq polimerase (Applied Biosystems, Inc., Calif., USA), 0.2 mM of each dNTP, 0.2 µM of primer (Ope-ron Tech., Calif., USA), 40 ng of genomic DNA, 2.5 mM final concentration of $MgCl_2$ for, OPA-11;
3.5 mM final concentration of $MgCl_2$ for, OPB-19, OPC-03, OPC-06, and 4.5 mM final

concentration of $MgCl_2$ for primers OPA-04, OPA-08, OPB-04, OPB-10, OPBB-07 and OPBB-2. Amplifications were carried out in a MJ Research PTC-0100 Thermal Cycler. Optimal amplification conditions for P. *ayacahuite* and P. *strobiformis* samples consisted of 3 min at 94 ºC, followed by 45 cycles of 1 min at 94 ºC, 2 min at 36 ºC and 2 min at 72 ºC, with a last step of 10 min at 72 ºC. A negative control, without tem-plate DNA, was included in each round of re-actions. Fragments generated by amplification were separated by size on a 1.8% agarose gel run with 1X TBE buffer at 100 V for 4 h, stained with ethidium bromide, and visualized under UV light. Gels were photographed with a Ko-dak DC40 digital camera. The reproducibility of RAPD fragments for each selected primer was tested for the total number of individual sam-ples from one population of P. *ayacahuite*.

Data analysis

Genetic variation

Analysis was restricted to intense and reproducible RAPD fragments. Amplified fragments were scored in each DNA sample, and visualized with the help of the EDAS Kodak photo documentation system (version 3.02). DNA fragment sizes were estimated using a 100 bp DNA size marker. Each fragment was treated as an independent locus with two alleles. Fragments were scored according to their presence (1) or absence (0) to create binary data matrices. The data matrices were analysed with POPGEN version 1.21 (Yeh *et al* 1997), and the proportion of polymorphic loci (P), expected heterozygosity (HE), and Shannon diversity index (I), were estimated for each taxon. A RAPD marker was determined to be polymorphic when the frequen-cy of the most common allele was less than 0.95. Additionally, allele frequencies were estimated with AFLP-surv version 1.0 (Vekemans *et al* 2002) assuming Hardy–Weinberg equilibrium, a Bayesian analysis with a non-uniform prior distribution of allele frequencies was used.

Genetic differentiation and population structure

We used the AFLPsurv program to estimate the Wright's pairwise statistic between populations within each taxon (P. *strobiformis, P. ayacahuite var. ayacahuite* and P. ayacahuite var. veitchii); the observed value of FST was tested, for genetic differentiation, against an ad-hoc distribution obtained after 1000 random permutations. Furthermore, we conducted a non-parametric analysis of molecu-lar variance (AMOVA) with ARLEQUIN version 3.01 (Excoffier et al 1992). Molecular variance, based on pairwise distance between RAPDs phenotypes, was partitioned among and within populations in both the ayacahuite and the strobiformis groups. The variance distribution among regions (northern, central and southern), populations within regions, and within populations was also analysed. We tested variance components against a null model generated after 1000 random permutations. Nei genetic distance (pairwise FST) was estimated with the Tools for population genetic analysis (TFPGA) program (Miller, 1997). Geographic distances between pairs of populations were obtained using the 'Point Distance' tool in ArcInfo version 10 (ESRI, 2012), and taking the center of the popu-lation's patch as the centroid. We used a Mantel test to determine whether there was an associa-tion between genetic distances (pairwise FST) and geographic distances using the TFPGA program (Miller, 1997), the signifiicance of the test was also determined using the program with 1000 permutations. The program Structure v2.3.4 (Pritchard et al 2000) was used to determine the most likely number of genetic groups, or clusters (K). Struc-ture estimates a posterior likelihood value (Pr (X/K)) for each run. Each run consisted of 10,000 burn-in steps and 100,000 collecting Marcov Chain Monte Carlo (MCMC) repetitions. The pa-rameters were set as default (i.e., the Admixtu-re Model without prior population information), and ten runs were performed for each value of K ranging from 2 to 12. The rate of change in the log probability of data between successive K va-lues (ΔK) was used to determinate which value of K was most likely for the data (Evanno et al 2005; Earl *et al* 2012). One set of runs included all locations, and in a second set, data from Cerro el Potosi were excluded.

Results

Markers profile

Of the 69 RAPD primers screened, ten produced clear RAPD patterns and yielded a total of 89 bands. Only 51 intense and reproducible bands were analysed, which represent an average of 5 bands per primers.

Table 2

Total number of bands amplified per polymorphic primer, number and percentage of polymorphic bands.

Primer	5'-3'	Bands	Size range	Polymorphic
OPA-04		7	450-1500	4
OPA-08		5	550-1100	5
OPA-11		6	650-1150	4
OPB-04		6	500-1200	2
OPB-10		5	700-1500	2
OPB-19		3	650-1400	2
OPBB-02		5	650-900	3
OPBB-07		4	650-1000	2
OPC-03		4	1000-1500	2
OPC-06		6	900-2200	3

The number of bands scored per primer varied from two (OPB-19) to seven (OPA-04) in the size ran-ge of 450 to 2200 bp. None of the 51 bands were unique to a particular population. Based on allelic frequencies, 29 loci were polymorphic in at least one population.

Genetic variation

Gene frequencies revealed percentages of polymorphic loci from 41% for Las Palmas to 61% for Cuale.

Table 3

Estimates of genetic diversity (% P, H_j, I) within populations of *Pinus strobiformis*, *Pinus ayacahuite* var. *ayacahuite* and *P. ayacahuite* var. *veitchii*.

Taxon	Population	% P	H_j (SE)	I (SD)
P. strobiformis				
	Cananea	51	0.279 (0.022)	0.300 (0.311)
	Bocoyna	49	0.260 (0.022)	0.280 (0.306)
	Cerro el Potosí	59	0.239 (0.022)	0.308 (0.279)
	Pueblo Nuevo	55	0.276 (0.023)	0.322 (0.309)
P. ayacahuite var. *veitchii*				
	Cuale	61	0.287 (0.022)	0.346 (0.296)
	Las Palmas	41	0.222 (0.023)	0.234 (0.300)
	San Rafael	45	0.227 (0.023)	0.255 (0.298)
var. *ayacahuite*				
	Real del Monte	53	0.255 (0.023)	0.293 (0.296)
	Xico	55	0.270 (0.021)	0.304 (0.293)
	Ixtlán	59	0.254 (0.022)	0.315 (0.289)
	Rancho Nuevo	57	0.265 (0.023)	0.308 (0.290)

Expected heterozygosity levels (Hj) ranged from 0.222 to 0.287 among the eleven populations. The population of Cuale exhibited the highest heterozygosity estimation whereas Las Palmas and San Rafael showed the lowest levels.

Table 4

Estimates total gene diversity (Ht), within population gene diversity (Hs) and differentiation index (F_{ST}) for *P. strobiformis*, *P. ayacahuite* and all populations. p= significance level.

Taxon	n	Ht	Hs	F_{ST}	P
P. strobiformis	4	0.318	0.264	0.172	<0.05
P. ayacahuite	7	0.276	0.254	0.080	<0.05
var. *veitchii*	3	0.265	0.245	0.075	<0.05
var. *ayacahuite*	4	0.274	0.261	0.049	<0.05
All populations	11	0.293	0.258	0.121	<0.005

The Shannon diversity Index ranged from 0.346 for Cuale to 0.234 for Las Palmas. The genetic varia-tion analysis at the species and variety level showed higher values of total heterozygosity (HT), and mean population diversity index (HS) for P. *strobiformis* than those for P. *ayacahuite*; genetic diver-sity parameters were higher in var. *ayacahuite* than in var. veitchii.

Genetic differentiation and population structure

The estimation of FST for all 11 populations was 0.121, which suggested that about 12% of the total RAPD diversity was due to differences among the populations (Table 4). The differentiation index of P.*strobiformis* was greater than that of P. *ayacahuite*, but considerably lower (0.097) when data from Cerro el Potosi was excluded from the P. strobiformis group. Within P. ayacahuite, the var. ayacahui-te had a lower FST index than the var. veitchii. These results were in broad agreement with those of AMOVA. The analysis of all populations revealed that 81.38% of the total variance is attributable to differences within populations and 17.34% to differences among populations.

Table 5

Analysis of molecular variance (AMOVA) conducted at the regional level and within and among populations of *Pinus ayacahuite* and *Pinus strobiformis*. P= significance level.

Source of variation	d.f.	Sum of squares	Variance	% total variance	P
Regions					
Among regions	2	53.72	0.06	1.28	<0.05
Within regions	8	180	0.81	17.34	<0.001
Within populations	242	920.99	3.80	81.38	<0.001
P. strobiformis					
Among populations	3	101.27	1.37	25.61	<0.001
Within populations	83	330.84	3.98	74.39	<0.001
P. ayacahuite					
Among populations	6	97.68	0.53	12.51	<0.001
Within populations	159	590.14	3.71	89.49	<0.001

When the analysis was restricted to P. *strobifor-mis* or P. *ayacahuite* populations, partitioning of variation within and between populations showed that most of the total variation existed within populations. However, higher popula-tion differentiation was detected in P. *strobifor-mis* (25.61%) when compared to P. *ayacahuite* (12.51%). Moreover, AMOVA estimations within regions and among regions revealed that only a small proportion of the total variation detected by RAPDs (1.28%) was attributed to differences among regions. The Mantel test results indica-ted that geographical distance was not correla-ted with genetic distance either when using the complete dataset (r=0.1892, p=0.18) or when using the P. strobiformis data (r=0.6302, p=0.08). The correlation between genetic and geogra-phic distance was positive and significant (r= 0.6160, p<0.05) when the P. *ayacahuite* data were considered separately.

The number of genetic groups for all the eight locations resulted in K=3 with a K=90.84. This analysis recognized the genetic distinctness of Cerro el Potosí, which was almost completely constituted by a single genetic group found in low proportion in Rancho Nuevo. The rest of the populations appeared to have heterogeneous proportions from the other two genetic groups, probably highlighting groups of individuals that are genealogically related. Individuals from Pueblo Nuevo and Cuale show similar admixtu-red proportions. The number of genetic groups when data from Cerro el Potosi were excluded resulted in K=2 K=12.06. Patterns of popula-tion structure were similar to those observed above.

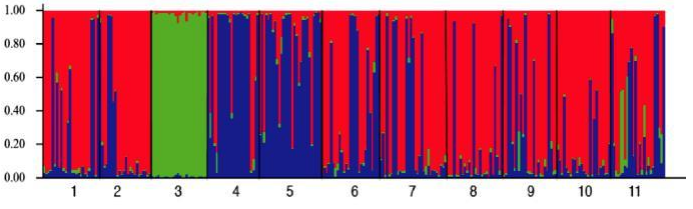

Figure. 2. STRUCTURE graph for all 288 sampled plants showing three genetic components, the rate of change in the log probability of data between successive K values (ΔK=90.84) was used to determinate which value of K was most likely for the data. (Numbers refer to sample locations as indicated in Table.

Discussion

Genetic variation

Pines are among the group of organisms with the highest genetic diversity (Piñero *et al* 2008). Approximately 50 species have been stu-died mainly using allozyme markers, and the ave-rage expected heterozygosity (H_E) for the genus is 0.198, for P. *strobiformis* (two populations) and P. *ayacahuite* (fourteen populations), allozymes exhibit similar diversity levels (H_E=0.154) (Ledig, 1998). In our study, RAPD markers detected high levels of genetic variation for both taxa, these results are similar to the average obtained with RAPDs in other pines, such as: P. *tabulaeformis* (H_E=0.285), P. *oocarpa* (H_E=0.358), P. *halepensis* (H_E=0.319), P. *brutia* (H_E=0.34) and P. s*ylvestris* (H_E=0.37) (Cui *et al* 2008; Díaz et al 2001; Gómez et al 2001; Kandedmir et al 2004; Szmidt et al1996).

Total and intrapopulation variation parameters were slightly higher for P. strobiformis than in P. *ayacahuite*. The only published heterozygo-sity estimate for each taxon was based on two populations of P. *strobiformis* from Durango (H_E=0.106), two populations of the veitchii variety from central Mexico (H_E=0.281) and eight popu-lations of the ayacahuite variety from southern Mexico, Guatemala, and Honduras (H_E=0.102) (Hernández-González, 1990). Moreno-Letelier & Piñero (2009) reported high genetic diversity for P. strobiformis (H_E=0.856) especially in western Mexico, whereas eastern populations were less variable and more genetically similar to P. *aya-cahuite* of central Mexico (H_E=0.557), but their results are not directly comparable with those from other pines, because they used chloroplast microsatellites.

Overall, in Mexico the populations of P. *strobiformis* are less disturbed than those of P. ayacahuite. Farmers and loggers use P. *ayaca-huite* for low grade construction timber, furniture and wood carving, so the species has been hea-vily exploited for a long period of time (Farjon & Styles 1997). Furthermore, the lowest indices of genetic diversity observed in P. *ayacahuite* var. veitchii from Las Palmas and San Rafael might be explained by reforestation practices for which

only seeds from a small number of different parent trees are used. Harvesting for timber and pressure from urban development, identified as the main threats for this taxon (Bower et al 2011), are not regulated. The IUCN assess both taxa as "least concern", but considering the degree of representation of these taxa in the protected areas of Mexico of 7.5% for P. *ayacahuite* var. *ayacahuite*, 6.3% for var. veitchii and 9.5% for P. *strobiformis*, (Aguirre et al 2010) coupled with the above mentioned threats and taxonomic controversy, current status becomes a very serious issue for most of the populations of the complex.

Genetic differentiation and structure

Pinus *strobiformis* has a significant level of gene-tic differentiation among populations (Table 4). Moreover in the ayacahuite group, differentiation among populations of the veitchii variety is higher than among populations of the *ayacahuite* variety. Gene flow among populations is determined by geographical distances and the mechanism of seed dispersal. The significant positive correlation between geographical and genetic distances within P. a*yacahuite* suggests that geographically separated populations have lower gene flow. Isolation by distance was not detected in P. *strobiformis.*

The AMOVA analysis reveals that only a small percentage of the total variance is explained by differences among regions and most of the genetic variance is due to differences within populations. A greater genetic variance is observed among populations of P. *strobiformis* than among populations of P. *ayacahuite*. Our results contrast with findings of Ledig (1998) as the allozyme data revealed a higher differentiation in P. *ayacahuite* (F_{ST}=0.222) relative to P. strobiformis (F_{ST}= 0.047). The STRUCTURE graph (Figure 2) shows three genetic components shared between individuals from the sampled populations; this result reflects the close relationship between P. ayacahuite and P. *strobiformis*. The population of Cerro el Potosi is the most differentiated, showing mainly one genetic component, which can be found in a very small proportion in the rest of the populations. This result is in agreement with Farjon & Styles (1997) who pointed out the unusual cone morphology of Cerro el Potosi and with Frankis (2009) who described it as a new species of white pine (Pinus stylesii Frankis ex Businský). Individuals from Cuale and Pueblo Nuevo show the same proportion of the genetic components so we can infer that there is genetic flow among these locations, this result highlights the extend of the controversy between taxonomists, who have considered individuals from Cuale and Pue-blo Nuevo either as different species, or the same species (Farjon & Styles (1997); Looney & Waring, 2013; Moreno-Letelier & Piñero 2009). Finally, it is a surprise to find that all the rest of the popu-lations have very similar proportions of the three genetic components suggesting they constitute a single panmictic unit. Additionally, to understand the speciation process and the phylogenetic re-lationships in the *Pinus flexilis*, P. *strobiformis* and P. ayacahuite species complex, Moreno-Le-telier et al (2018) evaluated genetic and ecolo-gical differentiation using multilocus sequence data (cytoplasmic and housekeeping nuclear genes) and ecological niche model comparisons. Their sequence data results show a clear diffe-rentiation of P. *ayacahuite.* However, ecological niche differences and candidate genes for drou-ght tolerance show a strong differentiation in P. *flexilis.*

A correlation among seed dispersal mechanisms and genetic differentiation at the population le-vel has been reported in tree species (Hamrick et al 1992); a higher intraspecific differentiation has been observed when seeds are ingested by animals or dispersed by gravity in relation to seed dispersion by wind. Similarly to our results in P. *strobiformis*, Delgado (2002) found a high population differentiation in five pine species that have large and wingless seeds. Therefore seed dispersal mechanisms other than dispersion by Clark's nutcracker might be another factor that explains the relatively higher differentiation observed in P. strobiformis. Tomback et al (2011b) mentioned that P. *strobiformis* has different seed dispersal biology in areas where nutcrackers are neither residents nor reliable dispersers. In populations from southeastern Arizona, some seed dispersal may occur by Steller's jays (*Cyanocitta stelleri*)

and dispersal of fallen seeds by nocturnal rodents is probably the most important mechanism. For a peripheral population of the closely related limber pine (*Pinus flexilis*), seed dispersal may occur primarily by nocturnal rodents (Tomback et al 2005), which has been proposed to be the cause of the reduced genetic diversity and subs-tructure previously reported in the same popula-tion (Schuster & Mitton 2000), the reason could be that small mammals have limited movements compared to nutcrackers in the main range of limber pine, so we suggest small mammals are potentially the most important seed dispersers for P. *strobiformis* in Mexico. The spatial gene-tic structure analyze with AFLP fingerprints of P. strobiformis from Durango showed a very weak spatial distance – genetic distance relationship, probably caused by a strong seed interchange, likely prompted by birds, such as the Mexican Jay, Aphelocoma wollweberi (Quiñones-Pérez et al 2014), and due to the continuous and broad geographic distribution of this tree species (Looney & Waring 2013).

For P. *ayacahuite* it could be the other way around, since this species has a small seed with a large wing and the populations show low levels of population differentiation, wind should play the most impor-tant role in dispersing this seeds species.

Unfortunately, the study of seed dispersal mechanism in this species complex in Mexico has not gone beyond the anecdotal or initial observation state, we can solely infer which it might be by comparing the genetic diversity and differentiation statistics with those of other pine species. We can also define it based on the morphological traits exhibited by the seed, but it is clear that a detail analysis should be carried out. We can point out that the levels of genetic differentiation and diversity observed are similar to those reported for species with wind or animal primary seed dispersal mechanisms.

Conservation decisions should be based on quantitative genetic variation as well as on patterns of variation of molecular markers, which are generally assumed to be selectively neutral. Our study using RAPD markers did not reflect significant genetic differences among P. *strobiformis*, P. *ayacahuite* and the varieties. Morphological differences among these taxa might be a result of large distribution and fragmentation of the populations as well as the high diversity of habitats. Moreover, besides the observed morphological differentiation, recent work (Aguirre-Gutierrez et al 2015) has also detected significant ecological niche differentiations between these species, showing their similarity but rejecting the equivalency of their ecological niches. Thus, we consider it is important to conserve populations that represent these ecological and genetic variations. The levels of genetic diversity within and among populations of P. *ayacahuite* and P. *strobiformis* should be used to define appropriate units for conservation and for germplasm conservation. Particularly, the high degree of differentiation recorded between populations of P. *strobiformis* suggests that populations throughout the natural range of the taxa should be incorporated into conservation plans. Genetic material from populations of P. *ayacahuite* var. veitchii with the highest levels of genetic variation should be used to promote regeneration of the more genetically impoverished populations.

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References

Aguirre-Gutiérrez, J. & J.F. Duivenvoorden. 2010. Can we expect to protect threatened species in protected areas? A case study in the genus Pinus in Mexico. Revista Mexicana de Biodiversidad 81(3): 875-882. [(Printed) ISSN: 1870-3453; (Online) ISSN:2007-

8706]

Aguirre-Gutiérrez, J., H. Serna-Chaves, A.R. Villalo-bos-Arámbula, J.A. Pérez de la Rosa & N. Raes. 2015. Similar but not equivalent: Ecological niche comparison between closely-related Mexican white pines. Diversity and Distributions 21(3): 245-257. [(Online) ISSN: 1472-4642]

Bower, A.D., S.C., McLane, A., Eckert, S., Jorgensen, A., Schoettle & S. Aitken. 2011. Conservation Gene-tics of High Elevation Five-Needle White Pines. pp. 98–117. In: Keane, R.E., Tomback, D.F., Murray, M.P., Smith, C.M. (Eds.), The Future of High-Elevation, Five-Needle White Pines in Western North America: Proceedings of the High Five Symposium. Proceedings RMRS-P-63. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station. 376 pp. Online at http://www.fs.fed.us/rm/pubs/rmrs_p063.html

Castro-Félix, P., J.A., Pérez de la Rosa, G., Vargas, S., Velásquez, A., Santerre, F. López-Dellamary & A.R. Villalobos-Arámbula. 2008. Genetic Relationships among Mexican White Pines (Pinus, Pinaceae) based on RAPD Markers. Biochemical Systematics and Ecology 36(7): 523-530. [ISSN: 0305-1978]

Critchfield, W.B. 1986. Hybridization and classification of the white pines (Pinus section Strobus). Taxon 35(4): 647–656. [(Online) ISSN: 1996-8175]

Cui, L., C. Baofeng & W Mengben. 2008. Population Genetic Structure of Pinus tabulaeformis in Shanxi Plateau, China.Russian Journal of Ecology 39(1): 34-40. [(Printed) ISSN: 1067-4136; (Online) ISSN: 1608-3334]

Delgado, P., A., Cuenca, A.E., Escalante, G. Molina-Freaner & D. Piñero, D. 2002. Comparative genetic structure in pines: evolutionary and conservation consequences. Revista Chilena de Historia Natural 75 (1): 27-37. [(Printed) ISSN: 0716-078X

Díaz, V., L.M Muñiz, & E. Ferrer. 2001. Random amplified polymorphic DNA and amplified fragment length polymorphism assessment of genetic variation in Nicaraguan populations of Pinus oocarpa. Molecular Ecology 10(11): 2593-2603. [(Online) ISSN: 1365-294X]

Earl, D.A. & M von Holdt-Bridgett. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evan-no method. Conservation Genetics Resources 4(2): 359-361. [(Printed) ISSN: 1877-7252 ; (Online) ISSN: 1877-7260]

ESRI, 2012. Redlands. CA, USA: ESRI Inc. http://www.esri.com

Evanno, G., S. Regnaut & J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 14 (8): 2611-2620. [(Online) ISSN: 1365-294X]

Excoffier, L., P.E. Smousse & J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA data. Genetics 131(2): 479-491. [(Online) ISSN: 1943-2631]

Farjon, A. & S B.T Styles. 1997. Flora Neotropica. Pinus (Pinaceae). New York Botanical Garden. The Bronx, New York, USA. 291 pp. [(ISBN: 0893274119]

Frankis, M., 2009. The High Altitude White Pines of Mexico and the Adjacent SW USA. International Dendrology Society Yearbook 2008, pp. 63–72.

Gernandt, D.S., G., Geada, S., Ortiz & A. Liston. 2005. Phylogeny and classification of Pinus. Taxon 54(1): 29-42. [(Online) ISSN: 1996-8175]

Gómez, A., R. Alía & M.A. Bueno. 2001. Genetic diversity of Pinus halepensis Mill. populations detected by RAPD loci. Annals of Forest Science 58(8): 869-875. [(Printed) ISSN: 1286-4560; (Online) ISSN: 1297-966X]

Hamrick, J. L., M.J.W. Godt & S.L. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. New Forest 6(4): 95-124

Hernández-González, J.J., 1990. Variación genética isoenzimática en Pinus ayacahuite y sus dos variedades de México y Centroamérica. MSc Thesis, Universidad Autónoma Chapingo, Chapingo, México.

INEGI, 2011.Uso del Suelo y Vegetación. Instituto Nacional de Estadística y Geografía. México. <http://www.inegi.org.mx/geo/contenidos/recnat/usosuelo/inf_e1m.aspx>.

Jorgensen, S., J.L. Hamrick & P.V. Wells. 2002. Regional patterns of genetic diversity in Pinus flexilis (Pinaceae) reveal complex species history. American Journal of Botany 89(5): 792–800. [(Online) ISSN: 1537-2197]

Kandedmir, G.E., I. Kandemir & Z. Kaya. 2004. Genetic Va-riation in Turkish Red Pine (Pinus brutia Ten.) Seed Stands as Determined by RAPD Markers. Silvae Ge-netica 53(4): 169-175.

Ledig, F.T., 1998. Genetic variation in Pinus. In: Richardson, D.M. (Ed.), Ecology and Biogeography of Pinus. Cambridge University Press, Cambridge, pp. 251-280. ISBN: 521 55176 5.

Looney, C.E. & K.M. Waring. 2013. Pinus strobiformis (southwestern white pine) stand dynamics, regeneration, and disturbance ecology: A review. Forest Ecology and Management 287: 90–102. [(Printed) ISSN: 0378-1127]

Miller, M.P., 1997. Tools for population genetic analyses (TFPGA) 1.3: a windows program for the analysis of allozyme and molecular population genetic data. Computer Software Distributed by the Author. Northern Arizona University.

Moreno-Letelier, A. & D. Piñero. 2009. Phylogeographic structure of Pinus strobiformis Engelm. across the

Chihuahuan Desert filter-barrier. Journal of Biogeography 36(1): 121-131. [Online ISSN: 1365-2699]

Moreno-Letelier, A., A. Ortiz-Medrano, D. Piñero, & T.G. Ba-rraclough, 2018. Genetic and ecological divergence in Pinus flexilis-Pinus strobiformis-Pinus ayacahuite species complex: Clues to understanding speciation in pines. In: Schoettle, Anna W.; Sniezko, Richard A.; Klie-junas, John T., eds. Proceedings of the IUFRO joint con-ference: Genetics of five-needle pines, rusts of forest trees, and Strobusphere; 2014 June 15-20; Fort Collins, CO. Proc. RMRS-P-76. Fort Collins, CO: U.S. Depart-ment of Agriculture, Forest Service, Rocky Mountain Research Station. p. 8.

Ortíz-Medrano, A., A. Moreno-Letelier & D. Piñero. 2008. Fragmentación y expansión demográfica en las pobla-ciones mexicanas de Pinus ayacahuite var. ayacahuite. Boletin de La Sociedad Botánica de México. 83: 25-36. [(Printed) ISSN: 0366-2128]

Palomera, V., P. Castro-Félix & A.R.Villalobos-Arámbula. 2008. High Yield of high Quality DNA from Vegetative and Sexual Tissues of Mexican White Pine (Pinus ayacahuite). African Journal of Biotechnology. 7(1): 51-54. [(Online) ISSN: 1684-5315]

Pérez de la Rosa, J.A., 1993. Taxonomía de Pinus strobifor-mis y Pinus ayacahuite. MSc Thesis, Colegio de Pos-graduados, Montecillo, México.

Perry, J.P., 1991. The Pines of Mexico and Central America. Timber Press, Portland Oregon.

Piñero, D., et al., 2008. La diversidad genética como instrumento para la conservación y el aprovechamiento de la biodiversidad: estudios en especies mexicanas, In: Capital Natural de México, Vol. I: Conocimiento Actual de la Biodiversidad. Conabio, México, pp. 437-494.

Pritchard, J.K., M. Stephens & P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155(2): 945-959. [(Online) ISSN: 1943-2631]

Quiñones-Pérez, C. Z., S.L. Simental-Rodríguez, C. Sáe-nz-Romero, J.P. Jaramillo-Correa & C. Wehenkel. 2014. Spatial genetic structure in the very rare and spe-cies-rich Picea chihuahuana tree community (Mexico). Silvae Genetica 63(4): 149-158.

Samano, S. & D.F Tomback. 2003. Cone opening phenology, seed dispersal, and seed predation in southwestern white pine (Pinus strobiformis) in southern Colorado. Ecoscience 10(3): 319-326. [(Printed) ISSN: 1195-6860; (Online) ISSN: 2376-7626]

Schuster, W.S.F & B. Mitton. 2000. Paternity and gene dis-persal in limber pine (Pinus flexilis James). Heredity 84(3): 348-361. [(Printed) ISSN: 0018-067X; (Online) ISSN: 1365-2540]

Steinhoff, R.J & J.W. Andresen. 1971. Geographic variation in Pinus flexilis and Pinus strobiformis and its bearing on their taxonomic status. Silvae Genetica 20:159-167.

Syring, J., K. Farrel, R. Businský, R. Cronn & A. Liston. 2007. Widespread Genealogical Nonmonophyly in Species of Pinus Subgenus Strobus. Systematic Biology 56(2): 163-181. [(Printed) ISSN: 1063-5157; (Online) ISSN: 1076-836X]

Szmidt, A.E., X.R. Wang & M.Z. Lu. 1996. Empirical assess-ment of allozyme and RAPD variation in Pinus syl-vestris (L.) using haploid tissue analysis. Heredity 76: 412–420. [(Printed) ISSN: 0018-067X; (Online) ISSN: 1365-2540]

Tomback, D.F., A.S. Schoettle, K.E. Chevalier & C.A. Jones. 2005. Life on the edge for limberpine: Seed dispersal within a peripheral population. Écoscience 12(4):519-529. [(Printed) ISSN: 1195-6860; (Online) ISSN: 2376-7626]

Tomback, D.F., Achuff, P., Schoettle, A.W., Schwandt, J.W., Mastrogiuseppe, R.J., 2011a. The Magnificent High-Ele-vation Five-Needle White Pines: Ecological Roles and Future Outlook. In: Keane, R.E., Tomback, D.F., Murray, M.P., Smith, C.M. (Eds.), The Future of High-Elevation, Five-Needle White Pines in Western North America: Proceedings of the High Five Symposium. Proceedings RMRS-P-63. Fort Collins, CO: US Department of Agri-culture, Forest Service, Rocky Mountain Research Sta-tion, pp. 2–28.

Tomback, D.F. & Y.B Linhart. 1990. The evolution of bird-dispersed pines. Evolutionary Ecology 4(3):185-219. [(Printed) ISSN: 1063-5157; (Online) ISSN: 1076-836X]

Tomback, D.F., Samano, S., Pruett, E.L., Schoettle, A.W., 2011b. Seed dispersal in limber and southwestern white pine: comparing core and peripheral populations. In: Keane, R.E., Tomback, D.F., Murray, M.P., Smith, C.M. (Eds.), The Future of High-Elevation, Fi-ve-Needle White Pines in Western North America: Proceedings of the High Five Symposium. Procee-dings RMRS-P-63. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station, pp. 69–71.

Vander Wall, S.B., 2008. On the relative contributions of wind vs. animals to seed dispersal of four Sierra Neva-da pines. Ecology 89:1837–1849. [(Online) ISSN: 1939-9170]

Vekemans, X., T. Beawens, M. Lemaire & I. Roldan-Ruiz. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Molecular Ecology 11(1): 139-151. [(Online) ISSN: 1365-294X]

Williams, J., A. Kubelik, K. Livak, J. Rafalski & S. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18(22): 6531-6535. [(Printed) ISSN: 0305-1048; (Online) ISSN: 1362-4962]

Yeh, F.C., R.C. Yang, T.B.J. Boyle, Z.H. Ye & J.X. Mao. 1997. POPGENE, the User-friendly Shareware for Popula-tion Genetic Analysis. Molecular Biology and Biote-chnology Centre, University of Alberta, Canada 10: 295-301.