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Article

Genetic variation of *Pinus pinceana* Gordon evidence of conectivity in fragmented populations

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Abstract:

Pinus pinceana is a Mexican pine species which belongs to the Cembroides subsection. It occurs in a fragmented manner in arid mountainous areas of the Eastern *Sierra Madre* (*SMO*). It is currently included in the Mexican Official Standard NOM-059-SEMARNAT-2010 with the Special Protection status. In order to assess the genetic diversity among its populations, the amount of genetic flow, as well as the genetic distance between them, 180 samples obtained from the northernmost locations were analyzed using the random amplification of polymorphic DNA (RAPD) technique. The genetic diversity in the populations was high, with a polymorphism percentage of 94.7 % and a Shannon diversity index of 48 %. The total variation between the populations was 14.8 % (P=0.001). Most of the variation was determined within the populations (85.18 %) as being high and having a low differentiation (Gst = 0.15). This suggests that, although the distribution of the species across the *SMO* is fragmented and restricted, the interpopulational genetic flow has been sufficient to declare that *P. pinceana* is not in a vortex of genetic extintion. The genetic distance shows the formation of two groups: one with the populations belonging to the state of *Coahulia*, and another with those of *Nuevo León* and *Zacatecas*: this indicates a larger genetic flow between them than within relation to those of *Coahuila* and, probably, the influence of a physographic barrier in the Eastern *Sierra Madre*.

Kew words: Genetic distance, genetic extinction, genetic flow, *Pinus pinceana* Gordon, RAPD, Eastern *Sierra Madre*, genetic variation.

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Introduction

Pinus pinceana Gordon (1858) is an endemic species of Mexico that provides an excellent opportunity to study the effects of fragmentation and the genetic patterns of variation between its populations. It is distributed on a stretch of over 750 km from north to south (Favela and Thomas, 2013) and a surface area of more than 20 000 km^2; however, due to its discontinuous and dispersed distribution, the surface area it covers is actually less than 2 000 km^2. It occurs in three main areas —the northern (*Coahuila*, *Zacatecas*, *Nuevo León* States), the central (*San Luis Potosí* State) and the southern (*Hidalgo* and *Querétaro* States)— separated by mountains and large arid stretches. *P. pinceana* occurs not only scattered among the communities but also in a fragmented manner, isolated by geographic barriers, which supposedly renders the genetic exchange or the connectivity between its populations difficult, especially in the northern area (*Coahuila*), where its isolation is accentuated by large arid stretches, its northernmost populations are localized and its distribution stops.

Genetic isolation favors differentiation between populations due to genetic drift, as shown by studies on the genetic diversity of the species using isoenzymes (Ledig *et al.*, 2001; Molina-Freaner *et al.*, 2001) and chloroplast DNA microsatellites. The environmental dissimilarity between regions suggests the possibility that natural selection has caused differences between populations with characteristics of adaptive importance to specific factors of environmental stress (Ledig *et al.,* 2001).

Pinus pinceana forests are included in the list of endangered natural habitats that demand specific conservation actions, classified as "LRnt" in the IUCN Red List, which means that they are at low risk (IUCN, 2001). Furthermore, they are subject to protection according to the *Norma Oficial Mexicana NOM-059-SEMARNAT-2010* (Mexican Official Standard NOM-059-SEMARNAT-2010) (DOF, 2010), partly because their populations are distributed in a fragmented manner, in small stands, and

perhaps due to the lack of studies on their genetic variability. In addition, according to several authors it is vulnerable, because in some of the visited areas there is little natural regeneration (Perry 1991; Villarreal *et al*., 2009). Also, it is important for the economy of the inhabitants of nearby communities, since these obtain income from the sale of the edible seeds (white pine nuts) and because they use it as a source of fire wood and fuel.

Pinus pinceana has been studied before, using molecular techniques such as isoenzymes (Ledig *et al*., 2001; Ramírez-Herrera 2007). However, the number and distribution of the included localities was not enough to reaffirm the cited haplotypes. Therefore, the present work uses RAPD markers to evaluate the variation of the northern populations, including the northernmost and particularly those that occur in the Eastern *Sierra Madre*. Besides, a recently registered population in the state of *Nuevo León* is considered for the first time. Analyzing exclusively the northern part of the distribution of the species will provide the opportunity to know whether or not there is a population structure, as well as to assess the northernmost and the most isolated populations.

Nuclear RAPD markers have been used to analyze the genetic variation of *Pinus* species and populations (Newton *et al*., 2002; Kurt *et al*., 2011; Cipriano *et al*., 2013; Kovacevic *et al*., 2013; Zhang *et al*., 2013) and to rebuild the phylogenetic relationship of certain taxa (Favela, 2004; Castro-Félix, 2008). The RAPD technique does not require previous knowledge of the genome to be used; furthermore, these markers offer the advantage of being randomly multilocus and generating a large number of polymorphic markers due to the amplification or lack of amplification of the DNA sequence scattered throughout the genome (Williams *et al.*, 1990; Vos *et al.*, 1995), for which more accurate information is obtained than what can be inferred from a single gene (Koopman, 2005). However, the results of RAPD can be deceiving, due to the presence of pollutants (fungi) —a crucial factor for measuring the reproductibility of data.

Other potential drawbacks are the lack of homology of the comigrating fragments and their dominant nature (absence/presence of bands). However, a comprehensive search of literature proves the overall consistency of the data obtained with RAPD with those obtained using microsatellites across a wide range of taxonomic groups (Nerendrula and Nkongolo, 2012; Zhang *et al*., 2013; Cichorz *et al*., 2014; Tomar *et al*., 2014); this shows that they are acceptable for the analysis of the genetic variability of populations.

The objectives of the present study were to: a) document how the genetic diversity, differentiation and distance of RAPD are distributed among the northern *Pinus pinceana* populations, including the population recently discovered in the state of *Nuevo León*; b) compare the data on genetic diversity previously recorded for other *P. pinceana* populations and with other molecular markers, in order to analyze the behavior of the results of the population, and c) infer whether or not there is connectivity between the populations.

Materials and Methods

Biological material

Six populations representing the distribution of *P. pinceana* in northern Mexico (Table 1), specifically those located in the states of *Zacatecas* and *Coahuila* and the population recently registered for *Nuevo León* (Figure 1) (Favela, 2009). 30 individuals separated from one another by a distance of 10 m were selected at random; 10 g of ripe leaves were collected manually from each and stored in plastic bags with 10 g of silica gel.

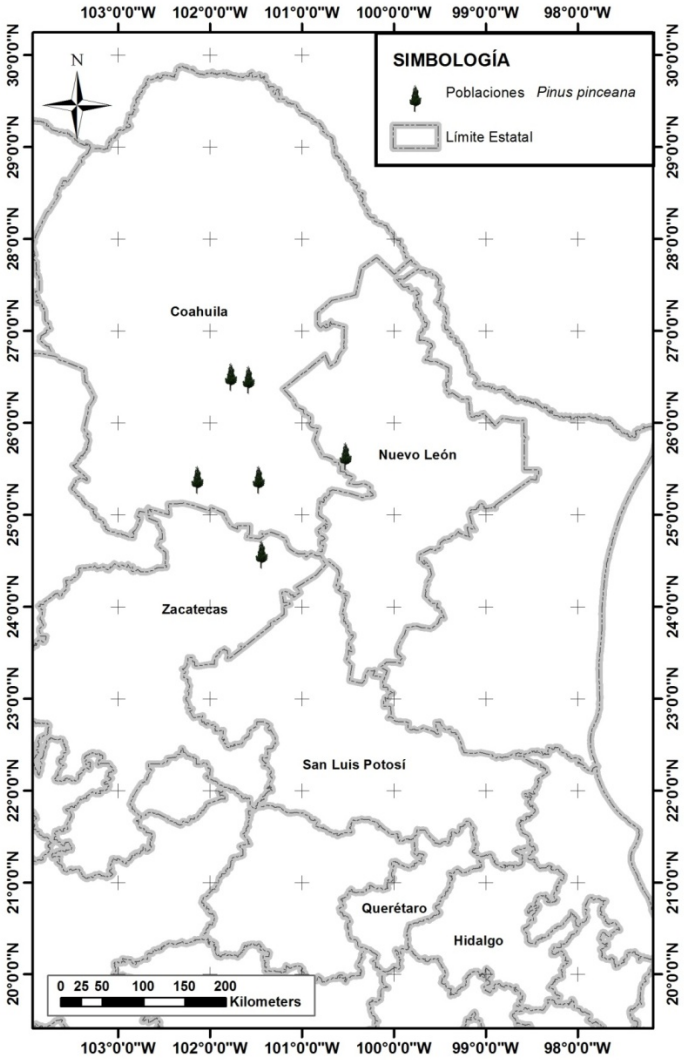


Figure 1. Geographic distribution of the sampled *Pinus pinceana* Gordon populations of the Eastern *Sierra Madre*.

Table 1. List of sampled *Pinus pinceana* Gordon populations of the Eastern *Sierra Madre*.

	Location	Coordinates		Altitude
		N	W	(m)
1	*La Noria*, *Cuatro Ciénegas*, *Coahuila*	25°23'08''	101°10'03''	2 050
2	*La Palmosa*, *Cuatro Ciénegas*, *Coahuila*	25°17'29''	101°10'03''	2 340
3	*La Casita*, *Parras*, *Coahuila*	25°15'08''	101°34'08''	2 200
4	*El Jaralito*, *Gral. Cepeda*, *Coahuila*	25°21'42''	101°27'76''	2 500
5	*Cañón del Moroso*, *Santa Catarina*, *Nuevo León*	25°37'46''	100°31'34''	1 500
6	*Las Lajas*, *Concepción del Oro*, *Zacatecas*	24°33'35''	101°26'25''	2 400

DNA extraction

The DNA was extracted from 1 g of dry leaves of the 30 individuals collected in each population, following the method of Doyle and Doyle (1990), and ammonium acetate was added to remove the excess carbohydrates (Hollingsworth *et al.*, 1999).

RAPD analysis

In order to select the oligos to be used in all the samples 15 random sequence decanucleotides of the OPA, OPB, OPC, OPG and OPP operon primers for RAPD (Operon RAPD kits technologies, Alameda). For this purpose, 16 of the 160 existing individuals were selected, and finally six with clear, reproductible shift patterns were chosen for the corresponding analyses.

The PCR reactions were prepared in a final volume of 25 μL of a mixture of the following reagents: 20 ng of genomic DNA in a 1X buffer (Invitrogen 10X)

with 2.5 mM $MgCl_2$, 0.2 nM dNTP´s, 0.50 M primer, 2 % formamide, and 1 Unit of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). A control tube without genomic DNA having all the components of the reaction was included in order to rule out contamination.

PCR conditions

The DNA was amplified using a programmable thermal cycler (Thermo Electron Px2 Thermal Cycler PCR PCYL220 Issue 1, HBPX2110). The reaction conditions for RAPD utilized were: 94 °C (2 min) for the initial separation of the DNA chains; 2 touchdown cycles were subsequently added, in which the reaction alignment temperature decreased by 2 °C in each cycle. These were followed by 41 cycles of 93 °C (30 sec), 35 °C (1 min), 72 °C (2 min) and 72 °C (5 min) for the final stretch.

Detection and processing of PCR products with RAPD

The amplifications were replicated for each primer and for each population according to the standardized protocol. The DNA samples of the population were inserted in the agarose gels. The amplified products were separated in an agarose gel electrophoresis at 1.8 % in a TBE 1x buffer, with a constant difference of potential of 135 volts during 2 hours and 30 minutes. The gels were dyed during 15 minutes in a SB 1X buffer with ethidium bromide, and finally, the banding patterns were observed under UV light and photographed on a transilluminator (MultiDoc-It™ Imaging System, UVP®, USA).

Data analysis

With the banding pattern obtained through the separation during the electrophoresis of the various amplified fragments, a presence-absence matrix was built and usually recorded as present bands (1), consisting of all the dominant homozygotes (AA) and heterozygotes (Aa), and as absent bands (0), consisting of recessive homozygotes (aa). Binary matrices were developed for each primer and population; these were then used to estimate the genetic variability and structure of *P*. *pinceana* populations.

The binary matrix was analyzed using the POPGENE v32 software (Yeh *et al*., 1997) to measure the genetic variability. The genetic variation values for each population were: the Shannon diversity index (I) (Lewontin, 1972) and the polymorphic loci percentage (P). The statistical analysis of Nei's genetic diversity (1973) was used to calculate the genetic differentiation between populations (Gst).

The genetic distance (Jaccard) was calculated based on the binary matrix and was subsequently subjected to a Principal Coordinates (PCO) analysis using the PAST software, version 3 (Hammer *et al.* 2001), which produces a visual representation of the genetic relationship within and between the populations. Furthermore, a dendrogram was built using the UPGMA (Unweighted Pair Group Method with Arithmetic averages) method based on the genetic distance between pairs of populations. The bootstrap analysis was carried out with 1 000 repetitions. The FAMD (Fingerprint Analysis with Missing Data) software, version 1.25, was used for both analyses (Schlüter and Harris, 2006).

The analysis of molecular variance (AMOVA) was carried out for the structure of the population in order to evaluate the variation between and within the populations using the Harlequin software (Schneider *et al*., 2000). The levels of significance for AMOVA are computed by means of non-parametric permutations of the set of data with 100 permutations. The Arlequin software generates a statistic-ϕ (Excoffier *et*

al., 1992) analogous to Wright's FST (Wright, 1951). This method has been used with RAPD data (Favela, 2004, 2010).

Results

Amplification using RAPD markers

Six of the first 15 assayed markers yielded reproductible band patterns that are easily resolved; these were used with 180 sampled individuals. The six primers generated a total of 76 bands whose sizes ranged between 1 500 and 250 pb. A range of 10 to 16 bands per primer was obtained.

Data analysis

Genetic diversity. The analysis of the genetic diversity of the populations carried out with POPGENE revealed that the population of *Cuatrocienégas*, *Coahuila*, exhibits a larger genetic diversity, as shown in Table 2, with high I values (I= 0.44 and I = 0.43). The lowest value corresponded to the population of *Las Lajas*, *Zacatecas* (I= 0.37).

The average polymorphic loci (P) of the populations ranged between 77.32 and 84.21 %. The population of *La Noria Cuatrocienégas*, *Coahuila*, exhibited the greatest polymorphism, and the one with the least polymorphism was that of *Las Lajas*, *Zacatecas*. (Table 2).

It should be noted that the populations with the highest diversity values are those *La Noria* and *La Palmosa*, in *Cuatrociénegas*, *Coahuila*; both are located on the northernmost strip of the distribution interval of *P. pinceana*.

Table 2. Genetic diversity estimators for each one of the six *Pinus pinceana* Gordon populations in Mexico, based on RAPD.

Population	N	Shannon index (I)	Polymorphic loci percentage (P)
La Noria	30	0.44	84.21
La Palmosa	30	0.43	77.63
La Casita	30	0.39	78.95
El Jaralito	30	0.41	78.95
Cañón del Moroso	30	0.41	81.58
Las Lajas	30	0.37	76.32
Total	180	0.48	94.74

N = Number of individuals used in the analysis of each population.

Genetic differentiation

The total coefficient of genetic differentiation (Gst) between the populations was 0.15. This value describes how the variation is distributed between the studied populations and ranges between 0 to 1; the value closest to 0 means that the populations share more genes, and the closer the value is to 1, the further the populations are from each other and the less genetic material they share.

Genetic structure of *P. pinceana* populations

The AMOVA indicates that ≈85.18 % of the total variation is registered within the populations (P< 0.001). Significantly, 14. 82 % of the remaining variation is found between the six *P. pinceana* populations (P< 0.001) (Table 3). A FST value of 0.1482, equivalent to the GST value cited above (0.15), was observed.

Table 3. AMOVA based on the analysis of RAPD markers in six *Pinus pinceana* Gordon populations.

Source of variation	g.l.	Sum of squares	Components of the variance	Percentage of variation (%)	P
Between populations	5	319.470	1.89	14.82	p<0.001
Within populations	162	1 763.25	10.88	85.18	p<0.001
Total	167	2 082.72	12.77		
F_{ST} = 0.1481					

[a]= Levels of significance based on 1 000 interactions.

Genetic distance

Jaccard's distance was utilized to carry out an analysis of principal coordinates, which is a graphic representation of the genetic relationship between the six analyzed *P. pinceana* populations. The first two principal components of the distance for the RAPD describe 4.64 and 4.24 % of the variation, respectively. The samples of the individuals of each population were represented as a continuous dispersion, with the populations of *La Noria*, *La Palmosa*, *La Casita* and *El Jaralito* in the state of *Coahuila*. The populations of *Cañón del Moroso*, *Nuevo León* and *Las Lajas de*

Zacatecas appear clearly separate from the rest of the populations in a distinct group (Figure 2).

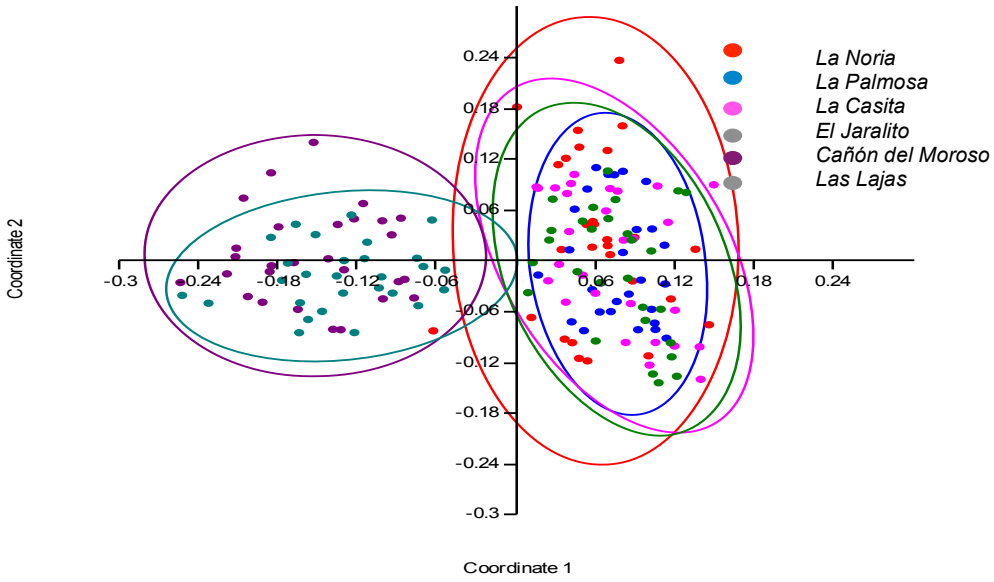
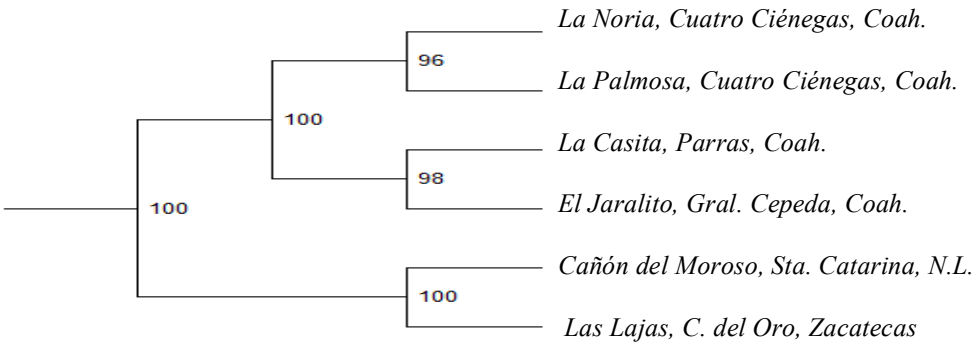


Figure 2. Graph of principal coordinates of the genetic distance for six *Pinus pinceana* Gordon populations (Jaccard Similarity Coefficient).

The dendrogram shown in Figure 3 was obtained from the genetic distance matrix of pairs using the unweighted pair clustering method with arithmetic average; it revealed that the populations of *Coahuila* are closer and constitute two clades: one consisting of the populations of *Cuatro Cienégas* (*La Noria* and *La Palmosa*), and another, of the populations of *Parras* and *General Cepeda* (*La Casita* and *El Jaralito*). Equally, the populations of *Nuevo León* (*El Moroso* Canyon) and those of *Zacatecas* (*Las Lajas*) are a separate clade, as they are genetically close. The bootstrap values for each population were highly significant and range between 96 and 100.



The *bootstrap* values are shown in the split of each group.

Figure 3. Dendrogram of the groups of the six *Pinus pinceana* Gordon populations, according to the unweighted pair clustering analysis with arithmetic average based on the Jaccard distance estimated with RAPD markers.

Discussion

In the present study, the utilized RAPD markers proved to be a quick, efficient technique for the study of the genetics of *P. pinceana* populations due to their high polymorphism observed in agarose gels. These served as the basis to measure the genetic variation through its components: genetic diversity, differentiation and distance.

Genetic diversity between *Pinus pinceana* populations

Estimated using the Shannon index (I=0.48), the genetic diversity follows a pattern in which the northernmost populations have the highest values: *La Noria* and *La Palmosa* are first (0.44 and 0.43), followed by *El Jaralito* and *Sta. Catarina* (0.41), *La Casita* and *Las Lajas* (0.39 and 0.37); we may infer that the framentation may have triggered the divergence between the populations, some of which generated new, unique alleles as a result of the isolation.

However, in average, the Shannon indices obtained for all the studied populations (0.48) do not differ from that of other pine species (0.45) and of other conifers and brodleaves (0.53) (*Araucaria*, *Fitzorya* and *Cedrela*) (Gillies *et al.*;1997; Allunt *et al.,*1999; Bekessy *et al.,* 2002).

The polymorphic loci percentage is another important indicator for determining the level of genetic variation of an area, as a species with a high value exhibits a high adaptability to the environment and, conversely, a species with a poor adaptability to the environment may be eliminated through natural selection (Zhang *et al.*, 2013); the value for *P. pinceana* was relatively high (79.80 %) in contrast with other pinyon pine species like *P. culminicola* Andresen & Beaman (57.3 %), (Favela, 2010), *P. cembroides* var. *bicolor* Little and *P. johannis* M.-F. Robert*,* with 69.9 and 78.1 %, respectively (Favela, 2004). This shows that each population contains a considerable fraction of the genetic diversity of the species, with values above those documented for endemic species (Hamrick *et al*., 1992; Hamrick y Godt, 1996).

In general, the genetic variation in terms of the various diversity indices exhibits a geographic pattern as higher values are observed in the populations of *Coahuila*, the northernomost of which —*La Noria* and *La Palmosa* in *Cuatro Ciénegas*— have the highest value, followed by those of *La Casita* and *El Jaralito*. These populations are separated from those of *Nuevo León* and *Zacatecas*, probably due to the existence of geographic barriers that have favored their isolation and, as the information flow between populations is determined by the geographic distance. Nevertheless, they

still share alleles, perhaps because they were originally all one, and with the passage of time this original population became fragmented —a fact that favored the onset of the divergence between the populations, which in turn generated new, unique alleles in some of them as it accomplishes to finish the genetic structure of the species as fragmented in three patches, given that in its study includes six populations, each of which marks the fragmentation, allotting two for each patch to the north, center and south of the geographic distribution.

Genetic differentiation between *Pinus pinceana* populations

The statistic (FST) is utilized to estimate the proportion of the genetic variation within and between the populations; it is particularly useful for dominant data such as RAPD (Excoffier, 2001), whose variation ranges between 0 and 1. Values closer to 0 indicate that the allele frequencies are the same in all the populations and that there has been no differentiation; the maximum value possible is 1, when each population is fixed at different alleles (Piñero *et al.*, 2008). The value in the studied populations (0.15) was comparable to the FST (0.152) registered by Ledig *et al.* (2001), but it was lower than those estimated by Molina-Freaner (2001) (0.24) and Ramírez-Herrera (2007) (0.16) for *Pinus pinceana* using isoenzyimes, and for other *Pinus* taxa located in scattered, isolated populations (Hamrick, 2004).

This value indicates that 15 % of the genetic diversity of the species corresponds to the one existing between populations and is very close to the estimate for other pinyon pine, *P*. *rzedowskii* Madrigal & M. Caball. (17 %) (Delgado *et al.*, 1999), an endemic taxon in Mexico also distributed in fragmented, isolated populations. It is also worth mentioning that a FST above 0.10 is regarded as indicating a strong interpopulational genetic differentiation, which is unusual in allogamous species like those of the *Pinus* genus (Hamrick *et al.,* 1992; Ledig, 1998). Only in certain conifers with a restricted natural distribution or with a strong fragmentation of their

populations have similar genetic differentiation values been documented between populations (Ledig, 1998).

Most of the variation in *Pinus pinceana* populations was registered within these (85.18 %). This result is similar to those registered for other pine species (Ledig, 1998; Favela, 2010) and woody plants (Hamrick *et al*., 1992). Furthermore, they are comparable to data obtained from RAPD, in which most of the examined species exibit high levels of interpopulational genetic variation (Xue *et al*., 2006; Favela, 2010), while the degree of genetic variation between populations was 14.82 %. High levels of diversity and differentiation between populations seem to be usual among Mexican conifer taxa (Ledig *et al.*, 2001; Fazekas and Yeh, 2006; Favela, 2010).

Genetic and geographic distances between *Pinus pinceana* populations

Figure 2, which depicts the Jaccard similarity coefficient and is subjected to the PC analysis, evidences a separation between the populations of *Nuevo León* and *Zacatecas* and those of *Coahuila*, indicating that they are genetically the most distant. From this it may be inferred that there is a greater genetic flow between the populations of *Coahuila* (*La Noria*, *La Palmosa*, *La Casita* in *Parras* and *El Jaralito*) than between those of *Nuevo León* (*Cañón del Moroso*) and *Zacatecas* (*Las Lajas*). Previous studies (Ramírez-Herrera, 2007) also show a separation with respect to the populations of *Sierra de Parras* and *Las Norias*, respectively.

According to Hollingsworth and Ennos (2004), the clustering of individuals of different populations in the same clade in a tree is interpreted as evidence of the genetic flow between populations. This can be equally observed in the tree obtained using the UPGMA method because two clades are formed; the first groups the populations of *Coahuila* and comprises two subclades, one encompassing the

populations of *Cuatro Ciénegas —La Noria* and *La Palmosa—*, and another, those of *Parras* and *Gral. Cepeda*. The populations of *Cañón del Moroso*, *Sta. Catarina*, *Nuevo León* and *Las Lajas*, *Zacatecas*, form a single group. This makes it possible to infer that there is a greater genetic flow between the populations of *Nuevo León* and *Zacatecas*, compared to the populations of *Coahuila*. This is an important datum, for in previous studies (Ledig, 2001; Ramírez-Herrera, 2007) the populations of *Zacatecas* were separated from among the northern population groups, together with those of *Coahuila*.

The variation between the populations may be due to the ecological differences in each of the two groups. The first is constituted by regions with less precipitation (350 a 400 mm) than the regions belonging to the second group —where the precipitation is 400 a 500 mm— and, in general, by the climatic and orographic regions defined as the Eastern *Sierra Madre*.

Various studies on *P. pinceana* record similar data, separating the northern populations (*Coahuila*) from those of central and southern Mexico; now, with the results obtained from the recently described population of *Nuevo León* (Favela *et al*., 2009), the manner in which they are separated from those of *Zacatecas* is evident.

In researches by Ledig *et al*. (2001) it is possible to distinguish the formation of two clades clearly separating the northern from the southern populations, as well as the populations of *Las Lajas* and *Parras, Coahuila*, also included in this study, in subclades. Ramírez-Herrera (2007) has submitted a graph in which two clades are formed: one for the northern populations only, and another with those of the center and the south. In the northern populations we may observe two subclades, one of which separates the population of *Las Norias*, considered in the present study, while the other subclade consists of the populations of *Zacatecas*. Finally, Villarreal *et al*. (2009) conclude that there are two groups of *P. pinceana* pinyon pine forests, based on their floristic and distribution differences: those of the northern

region (*Coahuila*, *Zacatecas* and *San Luis Potosí*) and those of the southern region (*Querétaro* and *Hidalgo*).

Conclusions

The genetic variation in the *Pinus pinceana* populations is high and agrees with previous studies.

The degree of genetic differentiation between the *P. pinceana* populations is low but consistent with the values registered for other conifer species. The present differentiation suggests that, although the *P. pinceana* populations are restricted, isolated and fragmented, the genetic flow between populations, at least today, has not led to the dramatic loss of genetic variation or differentiation between the populations.

The measures of genetic distance between the *Pinus pinceana* populations show that the populations of *Coahuila* have a greater genetic flow between one another and that they are genetically more distant from those of *Nuevo León* and *Zacatecas*. This suggests the existence of two fragments (populations of *Coahuila* and populations of *Nuevo León* and *Zacatecas*) and the influence of a physiographic barrier in that part of the Eastern *Sierra Madre*.

In general, we conclude that the RAPD technique is reliable, even though it has proved controversial, as the results and values obtained through it are not different from those previously obtained with other molecular markers. Therefore, although *Pinus pinceana* has been categorized as a vulnerable species with fragmented populations, the populations hitherto analyzed preserve their genetic connectivity.

In the future, further studies including those biotic and abiotic factors that may be involved in speciation patterns, particularly orographic and geological parameters, will set the guidelines to understand the influence of geographic barriers on the

species. It would also be interesting to study those biotic interactions that may have an impact on the diversification of the species.

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Conflict of de interests

The authors declare no conflict of interests.

Contribution by author

Verónica Aguirre Limón: field and laboratory work and writing of the manuscript; Glafiro Alanís Flores: correction of the manuscript; José Ignacio González Rojas: review of results y and help in statistical analysis; Adriana Flores Suárez: review and discussion of laboratory results and review of the manuscript; Susana Favela Lara: responsible for the original project, review of field and laboratory results, molecular analysis and review of the manuscript.