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ALLOZYMATIC DIVERSITY IN A NATURAL POPULATION OF *PINUS HARTWEGII* LINDL. IN VERACRUZ, MEXICO

SUMMARY

Understanding the degrees and geographic distributions of genetic variation between tree species is important to planning their management and conservation. The genetic variation of the population of *Pinus hartwegii* Lindl., from the National Park "Cofre de Perote" in Veracruz, Mexico, was assessed at two sites of different altitudes (3,500 and 4,000 m.a.s.l.) to aid in the development of a conservation program for this important forest resource. Ten trees were randomly selected at each site, and at least 20 cones were collected per tree. 25 genetic markers were detected from germinated seeds. The average number of alleles per locus was 1.56, with most alleles found at both altitude sites. The expected heterozygosity along the altitude gradient ranged from 0.235 to 0.260. Based on this data, it is recommended to establish a Forest Genetic Resource Conservation Unit (FGRCU) at 4,000 m with a minimum viable population size (Ne) of 7,645 individuals, and another at 3,500 m with a Ne of 8,563 trees.

Keywords: Allozyme; Alleles; Altitudinal gradient; Polymorphism

INTRODUCTION

Pinus hartwegii Lindl. Is a species confined to the highest mountains of Mexico and Central America, located between 3000 and 4000 m.a.s.l. Its natural distribution is discontinuous in high mountain sites from Nuevo León in northwestern Mexico (approx. 25° LN) to sites near the border between Guatemala and El Salvador (approx. 14° LN) (Perry, 1991). The importance of *P. hartwegii* is not only focused on the production of wood or wood products, it also great ecological importance because it is one of the most tolerant species to low temperatures in Mexico (Viveros-Viveros *et al*. 2007), fulfilling protective functions for other associated resources and providing services such as carbon sequestration, water retention, and buffering the effects of pollution (Musálem

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and Solís 2000). This species constitutes the altitudinal limit of the arboreal vegetation of the high mountains and volcanoes located in important national parks in Mexico, such as the Colima Volcano, Pico de Tancítaro, Nevado de Toluca, Ajusco, Popocatépetl, Iztaccihuatl, and Malinche (Vera-Vilchis and Rodríguez-Trejo 2007). In Veracruz, Mexico, it is found in fragmented populations on the Nauhcampatépetl (Cofre de Perote) and Citlaltépetl (Pico de Orizaba) (Iglesias *et al.* 2012).

The unique and significant elevation distribution of *P. hartwegii* makes it highly susceptible to global warming. In Mexico, it is projected that the average annual temperature will increase by 1.5°C, 2.3°C, and 3.7°C by 2030, 2060, and 2090, respectively, accompanied by a decrease in annual precipitation by 6.7%, 9.0%, and 18.2%, respectively (Sáenz-Romero *et al.* 2010). These changes are expected to lead to a reduction in the habitat of *P. hartwegii* by up to 42% (Arriaga and Gómez 2004). Research by Alfaro-Ramírez *et al*. (2020) suggests a potential loss of distribution area ranging from 29 to 70% in the next 50 years, while García-Amorena *et al.* (2021) anticipate a reduction ranging from 6 to 23% by 2050 and from 6 to 44% by 2070. Furthermore, the recent increase in temperature has resulted in a 10.6% reduction in the species' relative growth (Ricker *et al*. 2007).

The analysis of cones and seeds is a useful tool for understanding the reproductive status of conifers (Owens *et al*. 2005). This is because reproductive characteristics are important for understanding genetic processes in a population and for monitoring the viability of populations (Rajora *et al*. 2000). Due to the low viability of seeds and the high proportion of empty seeds, the reproduction rate of the "Cofre de Perote" population has experienced a reduction (Iglesias-Andreu *et al*. 2006). Given this, it is crucial to know the distribution of the species as well as the patterns of genetic variation within its populations. This species is better adapted to elevations of 3800 m.a.s.l. on Mount Tlaloc and 3900 m.a.s.l. on Nevado de Toluca, based on reproductive indicators. Both populations have shown comparable levels of inbreeding depression associated with seed production across altitudes. These reproductive indicators were affected by environmental factors in the Monte Tláloc and Nevado de Toluca populations, as well as in each of the *P. hartwegii* populations at different altitudes (Andrade-Gómez *et al*. 2021).

Variation in allozyme composition has been widely used since 1960 to investigate patterns of genetic variation in conifers due to its simple Mendelian inheritance and co-dominant expression (Hamrick and Godt 1996). The use of haploid (n) tissue from the megagametophyte of conifer seeds has been used to assess genetics and facilitate genetic studies. Through the use of allozyme, genetic variation has been studied in several conifer species, e.g. in *Pinus cembra* L. and *P. sibirica* Du Tour (Politov *et al*. 2008), *Pinus sylvestris* L. (Bilgen and Kaya 2007; Przybylski *et al.* 2020), and *Pinus nigra* J.F. Arnold (Scaltsoyiannes *et al*. 2009). Knowledge of the genetic variation in species or populations allows the establishment of better management or conservation strategies (Przybylski *et al.* 2020).

Although this species is important, especially in the *P. hartwegii* population of the "Cofre de Perote" in Mexico, little is known about the effects of altitude on the genetic variation levels of this species. Moderate genetic differentiation was found along the altitudinal gradient in the *P. hartwegii* population from Michoacán State in the “Pico de Tancítaro” National Park of Mexico, according to a study conducted there. For this reason, this study was performed to evaluate the genetic variation of the *P. hartwegii* population at "Cofre de Perote" in two locations along an altitudinal gradient, to devise an efficient management and conservation strategy.

MATERIAL AND METHODS

The *P. hartwegii* population of the “Cofre de Perote” National Park in Veracruz, Mexico, is located at 19°15' N latitude and 97º W longitude, in the central-western part of the Veracruz state (Fig. 1). In this rugged and mountainous area, two sites were located along an altitudinal gradient, one at 4000 m.a.s.l. (Location 1) and the other at 3,500 m.a.s.l (Location 2).

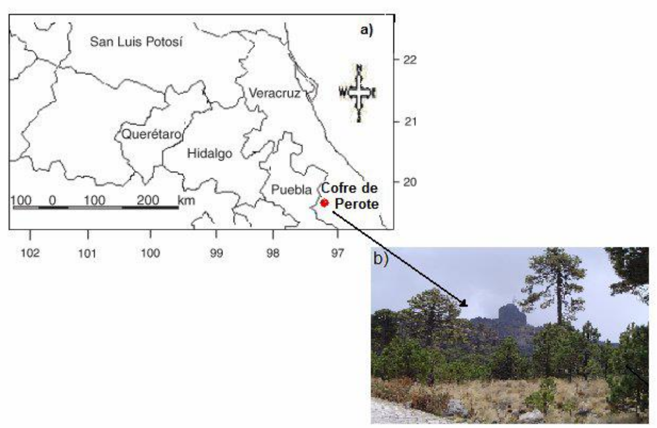

Fig.1. a) Location of *P. hartwegii* population in “Cofre de Perote”, Veracruz, Mexico. b) *P. hartwegii* forest.

The geographic area under study is characterized by rocky but moderately deep soils, poor organic matter composition, and an average annual temperature of 12 °C (SMN, 1984). Bulk seed lots were used for the population study; each seed lot was collected from 10 trees per altitudinal site. In 2008, at least 20 cones were gathered from each tree's central crown. At room temperature, the seeds were germinated in Petri dishes filled with moist agrolite. The megagametophyte of each seed was removed for enzymatic examination when the roots were three to five millimeters long.

200 µL of 0.2 M phosphate extraction buffer, pH 7.5, was used to cold homogenize the samples (Hodgkiss, 1998). After centrifuging the samples for ten minutes at 14,000 rpm, the supernatants were subjected to electrophoresis. Isoenzymes in polyacrylamide gels were examined using a discontinuous vertical electrophoresis system (Ornstein, 1964).

For the electrophoretic separations, separation and compaction polyacrylamide gels at 12.5% and 5%, respectively, and Tris-Glycine buffer pH 8.3, were used. These were carried out at 160 volts for 5 hours. The staining of nine isoenzyme systems followed conventional protocols (Vallejos, 1983) with slight modifications. Still, only three of them (Esterase, EST: E.C. 3.1.1.2, aspartate aminotransferase, GOT: E.C. 2.6.1.1, and Acid phosphatase, ACP: E.C. 3.1.3.2) showed good resolution and repeatability. Different loci encoding the same allozyme were designated based on their relative mobility. Intraspecific enzyme mobility was verified by comparing the different isomorphs in the same gel.

The data obtained from electrophoretic variation at different altitudinal sites was used to establish a matrix. Various population genetic parameters such as allele richness (A), percentage of polymorphic loci with a 99% polymorphism criterion, and expected heterozygosity (He) were calculated for each altitude level using TFPGA software (Miller, 1997). A cluster analysis was conducted based on genetic distances between the study sites using the Nei method (Nei, 1972) and the unweighted pair group method with arithmetic means (UPGMA). The effective size of the viable population was determined using the expected heterozygosity value and a regression formula based on a mutation rate of 1×10-5, as per data from Millar and Libby (1991) for coniferous species, and the equation of Crow and Kimura (1972). The regression formula is Ne=-984.58+ (36723 He).

RESULTS AND DISCUSSION

Twenty-five loci were identified for the three isozyme systems analyzed. Esterase isoenzymes had a greater number of polymorphic loci (Table 1).

Table 1. Allozyme composition in a *P. hartwegii* population from "Cofre de Perote", Veracruz, Mexico

Isoenzyme System	***Loci***		
	Polymorphic	**Monomorphic**	**Total**
ACP	2	1	3
EST	12	0	12
GOT	9	1	10
Total	22	3	25

ACP: Acid Phosphatase (EC. 3.1.3.2); EST: Esterase (EC. 3.1.1.1); GOT: aspartate aminotransferase (EC. 2.6.1.1)

A substantial isoenzyme variation was found between the altitudinal sites evaluated. The altitudinal site of 4,000 m.a.s.l. showed the presence of a smaller number of polymorphic loci, while the altitudinal site of 3,500 m.a.s.l. was the one that showed the greatest polymorphism. This could be due to a differential genetic variation along the altitudinal transect examined (Table 2).

High values of genetic diversity were observed because of relatively high values of the number of polymorphic loci, the mean number of alleles per locus, and the heterozygosity observed in both sites. However, some differences were observed between them (Table 2).

Table 2. Isoenzyme variation and genetic diversity estimation of two altitudinal locations from “Cofre de Perote”, Veracruz, Mexico

Locations	Isoenzyme systems						Percentage *Loci* Polymorphic (P)	Expected heterocigocity (He)
	ACP		EST		GOT			
	P	M	P	M	P	M		
Location 1	2	1	9	3	4	6	56	0.235
Location 2	1	2	10	2	6	4	68	0.260
Population	-	-	-	-	-	-	62	0.250

ACP: Acid Phosphatase (EC. 3.1.3.2); EST: Esterase (EC. 3.1.1.1); GOT: aspartate aminotransferase (EC. 2.6.1.1); P: Polymorphic loci; M: Monomorphic loci: Location 1: 4000 m.a.s.l; Location 2: 3500 m.a.s.l.

The average number of alleles per locus (A) was 1.56. Some alleles varied in frequency, but it was found that both sites shared 56% of the allele variants detected (Fig. 2).

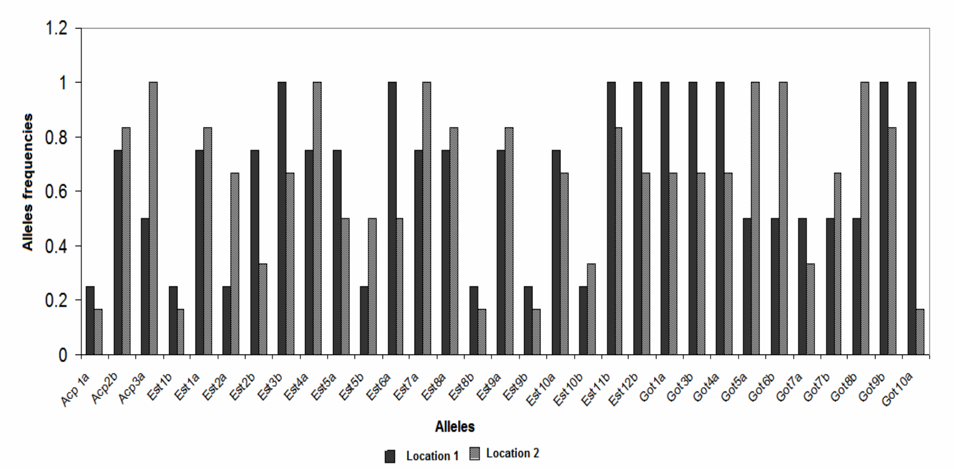

Fig. 2. Allele frequency variation for loci showed in two altitudinal locations of *P. harwegii* from “Cofre de Perote”, Veracruz, Mexico

However, 44% of the alleles detected were specific to one site or another. Based on these results, a Nei (1978) genetic identity of 0.877 and a genetic distance (Nei 1972) of 0.131 were estimated (Fig. 3).

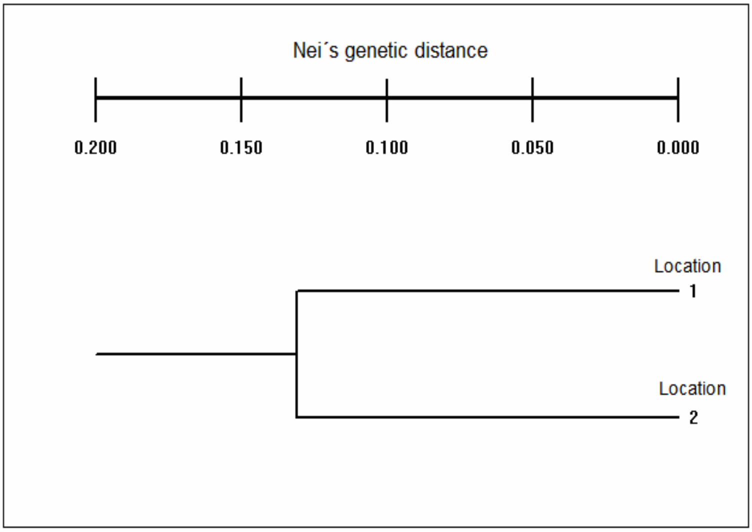

Fig. 3. UPGMA Dendrogram for the two altitudinal locations of *P. hartwegii* based on the Nei method (Nei 1972)

The close genetic distance observed shows that the average difference between these sites does not exceed 1% of the structural genes. The subpopulation located at the highest altitude presented six alleles: ACP_3-b, EST_4-b, EST_7-b, GOT_5-b, GOT_6-a, and GOT_8-a, which varied in frequencies between 0.25 and 0.5. In comparison, the subpopulation located at a lower altitude presented nine alleles: EST_3-a, EST_6-b, EST_{11}-a, EST_{12}-a, GOT_1-b, GOT_3-a, GOT_4-b, GOT_9-a, and GOT_{10}-b, with frequencies that ranged between 0.167 and 0.833. Finally, it was found that the minimum population size (Ne) estimated for the lowest altitude site was 8,563 individuals, while for the highest altitude site, it was 7,645 individuals.

Conifers constitute one of the groups of species with the greatest genetic variability (Ledig, 1998), and the population of *P. hartwegii* from “Cofre de Perote”, Veracruz, is no exception. The average number of alleles per locus (A) detected in this population was like that found when analyzing nuclear DNA, mitochondrial DNA, and chloroplast DNA (1.7, 1.5, and 1.6, respectively) of this species in the protection area of flora and fauna of “Nevado de Toluca” (Heredia-Bobadilla *et al*. 2019).

The average value of polymorphic loci (62%) detected in this population was higher than that found in “Pico de Tancítaro”, located in Michoacán, Mexico (58.3%) (Viveros-Viveros *et al*., 2010) and that of mitochondrial DNA (33.3%) detected in the “Nevado de Toluca” flora and fauna protection area (Heredia-Bobadilla *et al*., 2019). This value can be considered high, like that detected in other species of the genus, such as *Pinus nigra* (74%; Turna *et al*. 2006), *P. sylvestris* (76.5%; Korshikov *et al*. 2005), and *P. hartwegii*, in which its variation in nuclear DNA was evaluated (78.9%) (Heredia-Bobadilla *et al*. 2019).

The expected heterozygosity estimates of 0.25 could be considered high. Few species belonging to the *Pinus* genus have demonstrated expected heterozygosity values exceeding 0.20. Hiebert and Hamrick (1983) estimated values between 0.189 and 0.227 for the expected heterozygosity of 41 *Pinus* species. Some species have values higher than 0.340, such as *P. longaeva* D. K. Bailey. The variation values identified in this study agree with the findings of Delgado *et al.* (1999), who have demonstrated that conifer populations in Mexico and Central America exhibit higher levels of polymorphism and differentiation than other populations situated at higher latitudes.

It has been suggested that the genetic variation in *Pinus* spp., is a consequence of relatively high rates of cross-pollination (Hamrick and Godt, 1989; Hamrick *et al.* 1992) and the effective gene flow associated with long-distance pollen dispersal or homogeneity selection pressure (Wheeler and Guries 1982). At the site located at 3,500 m.a.s.l. greater variation was found, with greater allele richness: 42 total alleles (nine specific), a higher level of polymorphism (68%), greater heterozygosity (0.26), and a lower number of fixed alleles (8). While at the site, located at 4000 m.a.s.l. less variation was found, with 39 total alleles (six specific) and 56% polymorphism, as well as a heterozygosity of 0.235 and a greater number (11) of fixed alleles.

The variations in allele composition detected between sites could be an adaptive response of the population to spatial heterogeneity (Furnier and Adams 1986). Based on the specific and fixed alleles found, some ecological elements that are primarily related to topography and altitude may constitute the structural elements of an allele pattern. Another possibility is that bottlenecks that arise during range expansion cause genetic variation to decline during forest movements (Newton *et al.* 1999). In forest species, Ohsawa and Ide (2008) have described similar cases in which populations at higher altitudes have comparatively less variation.

It is interesting to note that this population, like others of the same species, faces problems of fragmentation and isolation. The results obtained in this work indicate that it is convenient to apply permissive sampling strategies to preserve the allele richness existing at different altitudinal levels. Conservation programs should include both, representative genes and specific genes, to preserve maximum allele richness. These results provide information for the first time on the genetic variability of *P. hartwegii* from the “Cofre de Perote”, despite the relatively small number of loci and altitudinal gradients considered in this study. To conserve a representative sample of current genetic diversity, it is recommended that at least one forest genetic resources' conservation unit (FGRCU) be established at 4,000 m with a minimum viable population size (Ne) of 7,645 individuals, and another at 3,500 m with a Ne of 8,563 trees.

However, if the current global warming trend continues (Sáenz-Romero *et al.* 2010), an assisted migration to higher altitude areas in other mountains would be necessary. Seeds would have to be collected from current populations, the plants reproduced in a nursery, and new ex-situ conservation plantations established at a site about 400 m above sea level higher than the seed collection site. This would allow the populations to readjust to the climate to which they are adapted. According to estimates for this species based on the Canadian General Circulation Model and the A2 model emission scenario (Sáenz-Romero *et al.* 2010), the climate in 2030 will be about 400 m higher than today. Higher elevation populations such as the “Cofre de Perote” would no longer have a place to migrate, as its peak is 4,282 m above sea level. Therefore, migration to other high mountains or volcanoes of the Mexican volcanic axis, such as “Iztaccihuatl, Popocatepetl, or Citlaltepetl” (Pico de Orizaba), should be encouraged. It can be argued that local populations of *P. hartwegii* on these volcanoes may be suffering from inbreeding depression due to gene flow from foreign plantations. However, it should be considered that local populations would also progressively lose their adaptation to climate change, which would undoubtedly represent a greater risk in the long term. It is hoped that future studies that include a greater number of molecular and population markers will provide a better understanding of the levels and patterns of the genetic structure of this important and valuable forest resource.

CONCLUSIONS

The population of *P. hartwegii* in the “Cofre de Perote” in Veracruz, Mexico, was found to have a high level of genetic diversity. The lower altitude site has higher genetic diversity and allelic richness than the higher altitude site, due to the pattern of distribution of genetic diversity observed. Based on this data, it is advisable to establish a Forest Genetic Resource Conservation Unit (FGRCU) at altitudes of 3,500 m and 4,000 m.

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