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Diversity and Genetic Structure Inferred with Microsatellites in Natural Populations of *Pseudotsuga menziesii* (Mirb.) Franco (Pinaceae) in the Central Region of Mexico

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Abstract: The amount and structure of the genetic diversity in Mexican populations of *Pseudotsuga menziesii* (Mirb.) Franco, is almost unknown, since most genetic studies have been carried out on populations from Canada and the United States. Here, we applied a set of 12 microsatellite markers to 12 populations (234 trees) from the central region of Mexico in order to determine values of genetic diversity and differentiation. Seventy-three different alleles were identified: an average number of alleles per locus (N_a) of 6.083, effective number of alleles (N_e) of 2.039, observed heterozygosity (H_o) of 0.229, and expected heterozygosity (H_t) of 0.417. Genetic differentiation was high: the coefficient of differentiation (θ) was 0.270, while the coefficient of structure (Φ_{st}) was 0.278. Bayesian analysis identified two genetic groups in central Mexico. The PCoA and the dendrogram were in concordance with the two genetic groups. The results of the analysis of molecular variance (AMOVA) indicate that genetic variation exists mainly within populations (72.149%). Therefore, conservation efforts should focus on as many individuals within populations as possible, to maintain this variation.

Keywords: genetic diversity; microsatellites; fragmentation; conservation

1. Introduction

Pseudotsuga Carriere (Pinaceae) is distributed naturally in western North America and Southeast Asia [1]. *Pseudotsuga menziesii* (Mirb.) Franco, is the most important species of this genus, appreciated for its high quality wood that is easy to work with and has physical properties suitable for use in structures [2]. It is one of the main lumber species in North America, and has been introduced to many parts of the world as an exotic species. Today, there are plantations in southern Germany [3], New Zealand, Argentina, and Chile [4–6].

In Mexico, this species is distributed in the northern region, although isolated stands can also be found in the central part of the country where its distribution is restricted to shaded moist sites, on the sides of ravines and canyons, or protected valleys [7,8]. It forms mixed forests, mainly with species of *Quercus*, *Pinus*, and *Abies religiosa* (Kunth) Schltdl. & Cham., where *P. menziesii* is present as a secondary species [8]. According to the Official Mexican Standard 059 (NOM-059-SEMARNAT), *P. menziesii* is “subjected to special protection (Pr),” meaning that the species is threatened by factors that negatively affect its viability, and that there is the need to promote its recovery and conservation [9]. Moreover, due to anthropogenic factors such as change of use of land, it is included in the red list of threatened species of the International Union for Conservation of Nature (IUCN) [10].

The discontinuous, restricted distribution of populations leads to a high degree of inbreeding, causing the appearance of similar individuals and reducing genetic variation. Consequently, when the individuals inherit identical alleles, they are more likely recessive alleles. This can cause expression of deleterious mutations and undesirable effects in yield [11]. Populations of the central part of Mexico show the lowest fertility and seedling recruiting rates because of inbreeding depression, very likely due to being the smallest and most isolated populations in the country [12–15]. Reduction of genetic diversity can increase the risk of extinction in natural populations by decreasing individual aptitude and their possibility of adapting to changing environmental conditions [16].

Genetic diversity has an important role to play in the conservation and management of genetic resources, especially in the face of changes that may come about. It is predicted that climate change will cause summers to be hotter and drier, and winters to be warmer and more humid [17,18]. Projections under the climate change model HadCM3 (Hadley Centre Coupled Model, version 3) predict a reduction in the current suitable area for the species by the year 2050, whereas by 2080 they could be on the verge of extinction. The model suggests that the main variable decreasing the suitable area would be the reduction in precipitation, rather than the increase in temperature [19]. The species is found in a variety of environmental conditions, suggesting that it must maintain high genetic diversity in order to cope with this environmental heterogeneity [19]. Therefore, it is necessary to establish conservation strategies that maintain this diversity. Mexican populations are an important source of germplasm for managing the species in the eventuality of climate change.

Application of molecular techniques in the analysis of genetic diversity can contribute to the conservation of endangered species, since it is now possible to analyze a large number of polymorphic loci and individuals at the same time [20]. The use of molecular markers allows the estimation of genetic diversity parameters which can be used to inform conservation strategies [21]. Microsatellite markers or SSR (simple sequence repeats) are very useful due to their abundant distribution in the genome and their codominant inheritance [22,23], and they are often derived from non-coding regions [24].

For *P. menziesii*, there are SSR markers available that have been used in various different studies demonstrating their applicability to estimating genetic diversity and structure, pollen introgression, evolutionary history, etc. [6,25–27]. However, most of these studies have focused on native populations of Canada and the United States, where populations are big and show a continuous distribution [27]. In contrast, for Mexico, genetic diversity studies are scarce, and natural populations are fragmented and show a discontinuous distribution. Additionally, Mexican populations of *P. menziesii* have been found to be genetically different from those present in Canada and the United States [19,28,29]. Therefore, because of the characteristics of Mexican populations, we expect diversity and genetic values to be different from those reported for Canada and the United States populations. Here, we investigate the diversity and genetic structure of *P. menziesii* populations in the central region of Mexico using microsatellite markers (first report). The findings will contribute to the sustainable management and conservation of *P. menziesii* germplasm in Mexico.

2. Materials and Methods

2.1. Collection of Plant Material

Samples were collected from 12 *P. menziesii* populations located in the central area of Mexico (states of Tlaxcala, Puebla, Querétaro, and Veracruz (Figure 1, Table 1). Ventura et al. [8] identified 29 populations in this region, covering an area of approximately of 682 ha (area size ranging from 78 to 0.5 ha). For each population, 20 trees were sampled when possible. Adult and young *P. menziessi* trees (mean value of 1.37 m, ranging from 0.12 to 1.12 m at breast height) were sampled along linear transects, considering a minimum distance of 100 m to reduce the probability of sampling from family clusters [30]. Because of the area or the density of the populations (very few scattered trees), in some cases it was not possible to sample the target number (populations Axopilco and Barranca). Needles were identified and conserved fresh until their reception in the laboratory, where they were frozen at -80°C until lyophilization.

2.2. DNA Extraction

The plant material was lyophilized and pulverized mechanically. Genomic DNA was isolated following the CTAB (Cetyl Tri-Metyl Ammonium Bromide) [31] method with modifications, using 50 mg of sample and 1 mL of extraction buffer (100 mM Tris-HCl, 700 mM NaCl, 50 mM EDTA (ethylenediaminetetra-acetic acid), 1 % CTAB, 140 mM 2-mercapto-ethanol and 1% PVP (polivinylpyrrolidone)). The DNA was precipitated by adding chilled absolute ethanol. The DNA pellet was resuspended in 200 μL buffer TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)).

2.3. Quantification of Nucleic Acids

Concentration and purity of the DNA samples were determined by spectrophotometry (NanoDrop, Thermo Scientific, Wilmington, DE, USA) at wavelengths of 260/280 nm. Integrity of the DNA was evaluated in 1% agarose gels, and the migration pattern of the bands was compared with uncut lambda marker (PROMEGA, Madison, WI, USA).

2.4. SSR Analysis

Forty-two pairs of SSR primers were initially screened for polymorphism, and twelve primers were selected for the molecular analysis [25,26] (Table 2). PCR reactions were performed at a final volume of 10 μL . Each reaction included 50 ng of DNA, 0.8 X RedTaq[®] ReadyMix[™] (SIGMA-ALDRICH, St. Louis, MO, USA), 0.5 μM of each primer (SIGMA-ALDRICH, St. Louis, MO, USA), and molecular biology grade water. The PCR amplifications were carried out on a thermocycler Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 40 s at 95°C for denaturation, 40 s for alignment at the adequate temperature of each primer (Table 2), 2 min at 72°C for extension, and a final step of 20 min at 72°C .

PCR products were separated in 8% non-denaturing polyacrylamide gels in 1X TBE (45 mM Tris-Borate, 1mM EDTA) buffer solution at 250V for 2 h 30 min. After electrophoresis, the PCR products were visualized with silver staining [32]. Gel images were captured with the KODAK Gel Logic 100 System (Kodak, Rochester, NY, USA). Fragment sizes were determined with the software GelQuest (SequentiX-Digital DNA Processing, Germany, www.sequentix.de), with which the matrix of codominant data was constructed.

Figure 1. Geographic location of natural populations of *Pseudotsuga menziesii* (Mirb.) Franco, where plant material was collected. Left map represents the most northern surveyed populations in the states of Veracruz and Querétaro. Right map shows populations located in the states of Tlaxcala and Puebla. Names of populations are as follows: (1) La Barranca, (2) Carbonero Jacales, (3) Tlaxco, (4) Cruz de León, (5) Emiliano Zapata, (6) Tlalmotolo, (7) La Rosa, (8) Villareal, (9) Cuatemoxla, (10) La Caldera, (11) Axopilco, (12) Apizaquito. The color of the dots indicates the genetic group assigned by the STRUCTURE analysis.

Table 1. Geographic data of the locations of the *Pseudotsuga menziesii* populations analyzed in this study.

Population ¹	State	Number of Individuals	Altitude (m.a.s.l.) ²	Longitude W	Latitude N
La Barranca	Querétaro	16	2187	99°38'44"	21°08'59"
Carbonero Jacales	Veracruz	20	2589	98°28'15"	20°25'16"
Tlaxco	Tlaxcala	20	2698	98°05'42"	19°37'21"
Cruz de León	Puebla	20	2825	97°52'19"	19°34'52"
Emiliano Zapata	Tlaxcala	20	2884	97°55'00"	19°33'31"
Tlalmotolo	Puebla	20	2739	97°44'18"	19°33'05"
La Rosa	Tlaxcala	20	2863	97°54'32"	19°31'23"
Villareal	Tlaxcala	20	2976	97°53'53"	19°31'11"
Cuatxmola	Puebla	20	3125	97°49'46"	19°30'01"
La Caldera	Puebla	20	2866	97°52'15"	19°29'58"
Axopilco	Tlaxcala	18	2880	97°55'37"	19°28'10"
Apizaquito	Puebla	20	3100	97°18'41"	19°12'11"

¹ Populations are sorted based on LN values. ² m.a.s.l.= meters above sea level.

Table 2. Characteristics of 12 pairs of microsatellite markers used for the analysis of genetic diversity and structure of 12 *Pseudotsuga menziesii* (Mirb.) Franco populations.

Locus	Sequence (3' to 5')	Observed Size (bp)	Tm (°C)
PmOSU_3H4	F: TTTGCCGTCACATTTT ATTG R: GCATCTTTCAGGCATAGTCT	198–202	55
BCPsmAG27	F: ACGGGGAGGGAGGGTAAC R: CCTTCCTCTCCACTTTCAACC	120–124	55
PmOSU_3B9	F: TGTGTAAAAATGTCTAATCC R: ACTACTATTCGAGGTTTCT	152–164	50
PmOSU_4E9	F: GTTGGTTGTGTATATTCAGTTT R: GCCTCTTCTTGGTTTGGT	116–122	55
PmOSU_3G9	F: ATTCTTTTGAGACCTACTT R: CTTCAAAAATTCCTACAACA	150–158	50
PmOSU_2B6	F: TTGTTGGGTATAATTTTCA R: TAATAAAATAGCTCTAACCC	150–180	47
PmOSU_2D6	F: GGAAAATATACATCTCACGAC R: AAGCATGCGTACTAGGTG	164–176	55
PmOSU_2D9	F: TCGATTTACGCTTTTTTCTC R: TGTTTATCCCCAGTCTCAAG	162–176	55
BCPsmAG20	F: CATAGAGAGGGGCATATCAA R: ACCCCCGAACCGTTACTAC	126–146	55
PmOSU_2G12	F: CAAGGACTCATATGGGAAA R: AACATCAGTAATAACCTTTT	240–254	50
PmOSU_2D4	F: TTATTGCACATGAGTATTATGA R: CAGATGTTGTTTTTATACCAC	150–190	50
PmOSU_783	F: GAGCTGATGCCTTGAAGACT R: CAAGTCAGTTCACAATTCCT	250–270	55

Source: BCPsm markers [25] and PmOSU markers [26].

2.5. Data Analysis

POPGENE 1.31 [33] was used to estimate the following genetic diversity parameters at population level: percentage of polymorphic loci (%P), average number of alleles per locus (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and number of migrants (N_m). The number of private alleles (N^P), defined as alleles that are found only in a single population among a broader group of populations [34], was estimated using GenAlex 6.5 [35]. Principal coordinate analysis (PCoA) was performed at the population level to display graphically their dispersion. It was calculated using the genetic distance between pairs of accessions [35].

A Mantel test [36] was applied to the dataset to determine the relationships between the genetic and geographic distances among *P. menziessi* populations from all the surveyed locations in the central part of Mexico. The overall population subdivision was estimated using Wright's F statistics (F_{IT} , F_{IS} and F_{ST}) [37] using POPGENE 1.31 [33]. Additionally, the coefficient of genetic structure (Φ_{st}) [37] was calculated by the analysis of molecular variance (AMOVA), using 10,000 permutations to test the significance of the hierarchical population analysis using ARLEQUIN v. 3.5 [38].

Graphical representation based on genetic distances [39] was constructed using the methodology unweighted pair group method using arithmetic average (UPGMA) algorithm [40], implemented in TFPGA v. 1.3 [41].

A test of genetic structure was done by Bayesian analysis of individual assignment, assuming a model in which between one and 12 populations could be grouped. The program was executed using 250,000 iterations of MCMC (Monte Carlo Markov Chains), 50,000 replicates, and 10 repetitions for each K , using the software STRUCTURE 2.3.4 [42].

3. Results

3.1. Genetic Diversity

We analyzed 234 individuals from 12 populations of *P. menziessi* from the central region of Mexico. A total of 73 different alleles were detected, using 12 SSR markers. Allele number ranged from three (PmOSU_3H4, BCPsmAG27 and PmOSU_3B9) to ten (PmOSU_783), with an average of 6.08 alleles per locus (Table 3). Among these alleles, 22 alleles were private. Locus PmOSU_2D6 had the most private alleles, whereas loci PmOSU_2B6, BCPsmAG20 and PmOSU_2D4, had only one.

Table 3. Average estimates of parameters fixation indexes, gene flow, and private alleles in the 12 pairs of microsatellites analyzed.

Locus	N_a	F_{IS}	F_{IT}	F_{ST}	N_m	N^P
PmOSU_3H4	3	1.000	1.000	0.075	3.067	0
BCPsmAG27	3	−0.599	−0.318	0.176	1.404	0
PmOSU_3B9	3	0.764	0.780	0.070	3.325	2
PmOSU_4E9	4	0.450	0.559	0.197	0.958	0
PmOSU_3G9	5	0.077	0.754	0.733	0.089	3
PmOSU_2B6	6	0.701	0.853	0.507	0.246	1
PmOSU_2D6	7	−0.047	−0.016	0.029	8.677	6
PmOSU_2D9	7	−0.295	−0.185	0.084	2.575	2
BCPsmAG20	8	0.554	0.636	0.183	1.098	1
PmOSU_2G12	8	0.227	0.359	0.170	1.346	2
PmOSU_2D4	9	0.944	0.964	0.358	0.527	1
PmOSU_783	10	0.028	0.082	0.054	4.654	4
Mean	6.08	0.234	0.452	0.285	0.647	

N_a = Average number of alleles per locus, F_{IS} = Coefficient of inbreeding within populations, F_{IT} = Coefficient of total inbreeding, F_{ST} = Coefficient of differentiation, N_m = Number of migrants, N^P = number of private alleles.

At the population level, overall percentage of polymorphism was 100%, and it fluctuated between 58.33% (Apizaquito) to 100% (La Rosa). The average number of alleles (N_a) per population was 2.677. The population of Cruz de León had a lower N_a value (2.167), while the population of Carbonero Jacales had a higher value (3.166). The effective number of alleles (A_e) ranged from 1.416 (Tlaxco) to 2.067 (Carbonero Jacales), with a mean value of 2.039. The mean values of observed heterozygosity (H_o) and expected heterozygosity (H_e) were 0.230 and 0.302 respectively. H_o values varied from 0.162 for population of Tlaxco to 0.304 for Tlamotolo. The total expected heterozygosity (H_t) was 0.417 and varied by populations, where the population of Tlaxco had the lowest values (0.226), and the population of Carbonero Jacales the highest (0.383). The population of Tlaxco, showed the lowest

values for A_e , H_o , H_e , and N^P , whereas the population of Carbonero Jacales showed the highest values for the same variables (Table 4).

Table 4. Genetic diversity parameters in the 12 natural populations of *Pseudotsuga menziesii* (Mirb.) Franco, included in this study.

Population	N	% P	N_a	A_e	H_o	H_e	N^P
La Barranca	16	183.83	2.750	1.796	0.301	0.371	4
Carbonero Jacales	20	183.33	3.166	2.067	0.238	0.383	5
Tlaxco	20	175.00	2.250	1.416	0.162	0.226	0
Cruz de León	20	166.67	2.167	1.509	0.191	0.257	1
Emiliano Zapata	20	175.00	2.666	1.518	0.200	0.246	3
Tlaxmotolo	20	183.33	2.666	1.752	0.304	0.365	1
La Rosa	20	100.00	3.000	1.809	0.225	0.364	3
Villareal	20	183.33	2.500	1.550	0.231	0.286	2
Cuatexmola	20	183.33	2.916	1.693	0.270	0.320	2
La Caldera	20	166.67	2.750	1.615	0.220	0.267	1
Axopilco	18	175.00	2.833	1.635	0.231	0.287	0
Apizaquito	20	158.33	2.333	1.519	0.187	0.251	0
Mean		177.86	2.667	1.657	0.230	0.302	
* Overall	234	100.00	6.083	2.039	0.229	0.417	

N = Number of individuals; % P = Percentage of polymorphism; N_a = Average number of alleles per locus, A_e = Effective number of alleles, H_o = Observed heterozygosity, H_e = Expected heterozygosity, N^P = Number of private alleles. *To calculate the overall values, we assumed that there was no population structure, and that there was a single panmictic population.

3.2. Genetic Structure

The within population inbreeding coefficient (F_{IS}) varied from -0.599 (BCPsmAG27) to 1.000 (PmOSU_3H4) with a mean of 0.234 , indicating a significant deficiency of heterozygosity (Table 3). The inbreeding coefficient determined for the total population (F_{IT}) per locus ranged from -0.318 (BCPsmAG27) to 1.000 (PmOSU_3H4) with a mean of 0.452 , suggesting a deficiency of heterozygotes in the populations. Meanwhile, the population differentiation coefficient (F_{ST}), which measures the degree of genetic differentiation among populations, ranged from 0.029 (PmOSU_2D6) to 0.733 (PmOSU_3G9) with a mean of 0.285 , indicating a high differentiation among all the populations [43]. Overall, the values of the F statistics obtained in our study suggest considerable inbreeding in the analyzed *P. menziesii* populations. The number of migrants (N_m) per generation inferred from the data of the 12 loci analyzed was 0.62 , which is considered low [44].

The analysis of molecular variance (AMOVA) was carried out considering the 12 populations studied, calculating the molecular variation attributable to differentiation between and within the populations. The highest percentage of variation (%V) was found within the populations (%V = 72.182) and in lower proportion, between populations (%V = 27.818). The coefficient of genetic structure was $\Phi_{st} = 0.278$. The Fisher combined likelihood test or the Raymond and Rousset [45] exactitude test showed that allele frequencies between pairs of populations were significantly different, at a confidence level of 95% ($p < 0.05$) (Table 5). The coefficient of differentiation θ [37], which is analogous to the Wright coefficient of F_{ST} [43], was 0.27 in the 12 Mexican populations of *P. menziesii* of our study.

Distinct genetic groups of accessions were identified with different methods of grouping. Two different groups were formed using PCoA on the first three coordinates, explaining 72.79% (cumulative values) of the total variability (Figure 2). Likewise, the dendrogram generated from genetic distances [39] using the UPGMA method reflected two groups (Figure 3). Populations from Emiliano Zapata, Cruz de León, La Caldera, Cuatexmola, Tlaxmotolo, and La Barranca, clustered in one group (Group I). Populations Apizaquito, La Rosa, Tlaxco, Axopilco, Carbonero Jacales, and Villareal clustered in a different group (Group II).

Table 5. Analysis of molecular variance (AMOVA) in 12 natural populations of *Pseudotsuga menziesii* (Mirb.) Franco.

AMOVA				
	Sum of Squares	Variance Component	Percentage of Variation	Φ_{st}
Between populations	1323.957	0.711	27.860	0.278
Within population	1835.968	1.841	72.149	
Total	1159.925	2.552	100.000	

$p < 0.05$ based significance tests (10,000) permutations.

Figure 2. Graphical dispersion of 12 populations using principal coordinate analysis (PCoA), showing two main groups indicated by dotted circles with colors green (Group I) and red (Group II). Groups were formed in accordance to the UPGMA (unweighted pair group method using arithmetic average) dendrogram. The color of the dots indicate the genetic groups assigned by the STRUCTURE analysis. **Figure 3.** UPGMA dendrogram of 12 natural populations of *Pseudotsuga menziesii* (Mirb.) Franco, constructed from genetic distances [39]. Support values for each branch are indicated in parentheses.

The Mantel test conducted with 1000 permutations revealed a weak positive correlation between genetic distances and geographic distances ($r = 0.24$; $p = 0.102$), indicating that genetic differentiation does not seem to be due to gene flow produced by a model of isolation because of distance.

The Bayesian analysis of assignment of individuals, by comparing likelihoods, showed that the most probable number of genetic groups is $K = 2$ [46] (Figure 4). We assumed a model of intercrossing, and that the populations derive from a common ancestor. Each individual of each population was assigned to one of the two genetic groups. Populations were clustered evenly, six in each group. About 75% of the populations showed strong ancestry values (>0.80 , Table 6). The proportion of color of each of the individuals in each population represents the fraction of ancestry relative to each group (Figure 5).

Figure 4. Delta K values for STRUcTURE analysis of *Pseudotsuga menziesii* (Mirb.) Franco populations, where ΔK , calculated according to Evanno et al. [46] is plotted against the number of modeled gene pools (K).

Table 6. Estimated proportion of ancestry (Q) [42] for the 12 *Pseudotsuga menziesii* (Mirb.) Franco populations analyzed.

Population	Genetic Group	
	I	II
Emiliano Zapata	0.965	0.035
Cruz de León	0.979	0.021
La Caldera	0.978	0.022
Cuatexmola	0.788	0.212
Tlalmotolo	0.107	0.893
Apizaquito	0.022	0.978
La Rosa	0.049	0.951
Axopilco	0.303	0.697
Tlaxco	0.173	0.827
Villareal	0.849	0.151
Carbonero Jacales	0.562	0.438
La Barranca	0.145	0.855

Figure 5. Graphic representation of the genetic structure of the *Pseudotsuga menziesii* (Mirb.) Franco populations analyzed. Each column represents each of the populations. The K groupings are indicated by colors. The numbers on the axis indicate the coefficient of ancestry of the populations.

4. Discussion

4.1. Genetic Diversity

Prior to this study, little information was found in public databases for genetic studies of Mexican populations of *P. menziesii* using molecular markers. Most of the reported studies focused mainly on morphological [47] and phenological characteristics [48], mating patterns [15], and seed viability and dispersion [12,14]. Cruz-Nicolás et al. [49] analyzed Mexican populations from the northern and central regions of Mexico. Using isozyme markers, Cruz-Nicolás et al. determined levels of genetic diversity of $H_t = 0.077$ ($H_e = 0.051$ for the central region). Although this was the first analysis of the levels of genetic diversity in Mexican populations, a direct comparison between isozymes and microsatellites would be inappropriate without taking into account their differing capacity to detect genetic variation [27]. However, their results were the first to report low levels of genetic diversity in these populations and the presence of genetic differentiation ($F_{ST} = 0.298$).

Previously, Amarasinghe and Carlson [25] and Slavov et al. [26] developed microsatellite markers and estimated genetic diversity parameters in *P. menziesii* populations from Canada and the United States, respectively. From these primers, 12 SSR markers were used in the present study to determine the genetic diversity parameters in populations of *P. menziesii* from the central region of Mexico. Polymorphism levels of the selected panel were high (Table 4), with a mean value of 77.86% across the 12 populations of this study. The mean number of alleles ($N_a = 6.08$) and the heterozygosity ($H_t = 0.417$) detected in this study were lower than those reported in other studies in *P. menziesii* using microsatellites ($N_a = 8$, $H_e = 0.673$ [25], $N_a = 23$, $H_e = 0.855$ [26] and $N_a = 24.03$, $H_e = 0.936$ [27]).

The mean number of alleles observed and expected heterozygosity parameters were compared with other studies that used a similar set of microsatellite markers. Molecular studies on pollination dynamics of *P. menziesii* orchards [50,51] at the locus level (PmOSU_3B9, PmOSU_2D4, PmOSU_2G12, and PmOSU_3G9) displayed higher values ($N_a = 20.2$, $H_o = 0.651$ [50], $N_a = 23$, $H_o = 0.712$, $H_e = 0.916$ [51]) than those detected in the Mexican populations with the same markers ($N_a = 6.3$, $H_o = 0.116$, $H_e = 0.421$). These results are in agreement with previous studies suggesting that Mexican populations from the central region display lower levels of genetic diversity than populations in other regions of North America [25–27,50,51]. Populations of *P. menziesii* in the central region of Mexico are fragmented, and present a discontinuous distribution in the form of small patches or stands that are isolated from one another [1,19]. Paleoclimatic studies suggest that *P. menziesii* in Mexico emerged during the era of glaciation. During this period, the *P. menziesii* populations of northern Mexico

migrated southward, where they found refuge in the mountain systems. In the interglacial events, the populations expanded northward and to the high parts of the mountains, where today's populations formed with a certain level of isolation. These climate changes could have caused bottlenecks that reduced genetic variation and led to interpopulation differentiation, while the populations distributed in the United States and Canada were secondary points of convergence in the interglacial periods, giving rise to greater genetic diversity [19,52].

In the case of small populations, reproductive isolation can lead to genetic drift [53], increasing genetic differentiation among populations, homozygosity, and inbreeding [54]. Low values of genetic diversity have also been reported in small populations of other species with restricted distribution in Mexico, such as *Picea mexicana* Martínez ($H_t = 0.125$) [55], and in different species of *Abies* that have isolated distribution, such as *A. flinckii* Rushforth ($H_e = 0.064$ – 0.158), *A. guatemalensis* Rehder ($H_e = 0.044$ – 0.094), *A. hickeli* Flous & Gausson ($H_e = 0.073$ – 0.135) and *A. religiosa* ($H_e = 0.008$ – 0.235) [52].

Generally, levels of genetic diversity are closely related to the degree of disturbance of the populations [56,57]. Values of genetic diversity obtained for populations of *P. menziesii* for the central region are lower than those reported for populations in Canada and the United States. However, for a better estimate of the genetic diversity of the species in the country, it would be necessary to analyze populations from the northern region, since both regions have been found to be genetically different [47–49].

4.2. Genetic Structure

At the locus level, we detected deficiencies of heterozygotes, as indicated by the values of Wright fixation indexes $F_{is} = 0.234$ and $F_{it} = 0.452$, in all the analyzed populations (Table 3). High levels of homozygosity are characteristic of genetic drift acting on small populations as differentiation continues [53]. According to Wright [43], the genetic differentiation among populations is high when $F_{st} > 0.25$. The results of the present study indicate that genetic variation exists among the populations ($F_{st} = 0.285$). This result is counter to other studies, wherein the authors report no genetic differentiation [27,58]. The value of the coefficient of genetic differentiation, $\theta = 0.27$, analogous to the coefficient F_{st} , was high. Populations with high levels of heterozygosity detected with microsatellite loci can have very low levels of differentiation [59], as in the case of studies reported for other regions in North America, while in our study, the populations analyzed had a deficiency of heterozygotes and high values of differentiation (Table 3).

Populations of *P. menziesii* from the central region of Mexico have been characterized as having a high production of empty seeds, while the viable seeds are smaller in size with respect to populations from the northern region [60]. Moreover, when evaluating germination, higher rates of germination and survival are observed in seeds from the northern region [12]. These characteristic effects of inbreeding (morphological and genetic) observed in the central populations suggest that the process of genetic drift is associated with a high rate of selfing (deficiency of heterozygotes, Table 3).

Gene flow in the analyzed populations was limited, as indicated by the number of migrants (N_m) per generation inferred from the data of the 12 loci analyzed ($N_m = 0.647$, Table 3). In populations of the state of Washington (USA) and British Columbia (Canada), a $N_m = 5.00$ has been reported for this species [58]. For the Pinaceae family, to which *P. menziesii* belongs, values of up to 17.2 migrants of the genus *Pinus* [61] have been reported. This limited gene flow is in agreement with the high levels of endogamy and genetic structure identified in these populations. It is possible that the reduced gene flow in the analyzed population is a consequence of population fragmentation and size, and of local genetic differentiation [8].

The higher percentage of variation determined with the AMOVA was within the populations (73.05%). At a lower proportion (26.95%) was the variation detected is between populations. This could be due to the biology of the species. In general, long-living populations, panmictic, pollinated by wind, and of late succession have higher levels of variation within populations [62,63]. Additionally, metapopulation structure also has an impact in genetic diversity values. Metapopulation of species

is caused by land fragmentation, creating spatially separated populations, which in some cases can interact. This fragmentation could favor genetic drift, hampering gene flow. As a result, there is a reduction of genetic diversity, especially in cross-pollinated species [64].

The coefficient of genetic structure for the species, derived from the AMOVA, was $\Phi_{st} = 0.278$ ($p < 0.05$), which is similar to the characteristic for long-living perennial plants $\Phi_{st} = 0.25$ [62]. This value suggests the existence of genetic structure, as a consequence of restricted gene flow and evolution, regardless of the populations within the identified genetic groups.

The PCoA, dendrogram, and Bayesian analysis of assignment of individuals (STRUCTURE) were consistent in identifying two genetic groups. However, the results regarding the genetic relatedness of the populations were inconsistent with the expectation that populations geographically closer would be more similar than those that are more distant (Figures 1 and 3). These results are in agreement with the performed Mantel tests, where we found a positive correlation between geographic distance and genetic distance ($r = 0.240$, $p = 0.102$), although this correlation was not significant—a possible reason might be the small sampling range. These results suggest that, although geographic distances do not seem to play a significant role in genetic differentiation, there may exist barriers due to characteristics of the landscape that impede or reduce gene flow among the 12 populations studied.

Knowledge of genetic diversity values and population genetic differentiation is essential to identifying current threats and elucidating mechanisms to protect endangered species [65,66]. The results of this study show that the central populations of *P. menziesii* analyzed still harbor significant levels of genetic diversity, despite the fragmentation status, illegal exploitation, overgrazing, inappropriate cone collecting, pest attack, and climate change [67]. Given these precedents, it is imperative in situ conservation strategies be set up for natural populations with high genetic diversity and private alleles, particularly for populations Carbonero Jacales ($He = 0.383$, $N^P = 5$), La Barranca ($He = 0.371$, $N^P = 4$), Tlalmotolo ($He = 0.365$, $N^P = 1$), La Rosa ($He = 0.364$, $N^P = 3$), and Cuatemoxla ($He = 0.320$, $N^P = 2$). In situ conservation must be complemented with ex situ conservation strategies. There are ongoing efforts by national institutions working on the recollection of cones and seeds from different populations of *P. menziesii* in the country. Collection of vegetative samples should also be considered for in vitro conservation, particularly from those isolated populations and/or those with ancient trees. Populations from La Barranca, Carbonero Jacales, and Apizaquito are isolated from the main cluster of populations (Figure 1), and are at high risk of genetic erosion due to their isolated status. The PCoA distribution also shows these populations are further away from the main clusters (Figure 2). The results of the AMOVA indicate that genetic variation exists mainly within populations. Therefore, conservation efforts should focus on many individuals within populations as possible, to maintain this variation.

5. Conclusions

In the present study, we analyzed 12 populations of *P. menziesii* from the central region of Mexico, using 12 microsatellite markers to determine diversity and genetic differentiation. Different values of genetic diversity were detected among all analyzed populations, although the overall value was lower than other results reported using microsatellite markers in populations from North America. Molecular data revealed heterozygous deficiency in all populations, and high genetic differentiation among populations. These results, along with the small size and restricted distribution of the populations, and location at the border of the natural distribution, indicate genetic drift, increasing genetic differentiation among population, homozygosity, and inbreeding within populations.

Bayesian analysis showed two main genetic groups that were in agreement with the PCoA and the UPGMA dendrogram, and although there was a positive correlation between genetic and geographic distances, this was not significant. Genetic variation exists within populations; thus conservation efforts should focus on as many individuals as possible within populations, in order to capture most of the genetic variants within populations. Based on genetic diversity values and the number of private alleles, in situ conservation strategies should be implemented for populations Carbonero Jacales,

La Barranca, Tlalmotolo, La Rosa, and Cuatexmola. In situ conservation preserves the natural evolution of the species while maintaining its interactions with other organisms and ecological processes. These strategies should also be complemented with ex situ conservation activities.

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