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Effect of selective logging on the genetic differentiation of *Juglans pyriformis* Liebm. populations

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Abstract: *Juglans pyriformis* Liebm. (*Juglandaceae*) is a threatened and endemic tree that grows in the cloud forest of Mexico. Natural populations of this species have been reduced due to, among others, changes in land use, overexploitation, and logging, with probable effects on its genetic diversity and structure. To determine the levels of variation and genetic structure of two populations with different silvicultural regimes, six inter-simple sequence repeat (ISSR) primers were used to amplify DNA from 35 individuals from a high-logging population and 32 from a low-logging population. The results show a higher polymorphism in the low-logging population (81.5%) compared to the high-logging population (77.4%). The genetic differentiation coefficient (*PhiPT*) values (0.109), genetic distance (0.134) and STRUCTURE analysis (*Fst* = 0.2271, *P* = 0.04) show significant genetic differentiation between populations. Rare, private, and monomorphic bands were detected in both populations. These results confirm the trend of reduced genetic variation due to logging.

Keywords: cedar walnut; genetic diversity; molecular marker; population genetics

Mexican Montane Cloud Forests (MCF) are of great importance due to the extraordinary biodiversity they harbour, composed of approximately 2 500 to 3 000 vascular plant species with 30% endemism (Rzedowski 1996). However, they are among the most threatened ecosystems at the national level and are currently highly fragmented. The main threats these forests are facing are climate change, illegal logging, and conversion to agricultural crops, pastures, and urban expansion. Overall, 26% of these forests have been reduced, equivalent to a 2% annual loss, which especially affects endemic species (Challenger 2003).

Juglans pyriformis Liebmann (*Juglandaceae*), commonly known as cedar walnut, is an endemic species of the Mexican cloud forests. The distribution of its natural populations is discontinuous and geographically restricted to the states of Hidalgo, Oaxaca, and Veracruz (Manning 1960; Narave-Flores 1983; Luna-Vega et al. 2006). Nowadays, many of these populations are found in isolated hard-to-access places, reduced because of deforestation, fragmentation, and destruction of their habitat from changes in land use and the intense exploitation of their wood, which is highly

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appreciated for its excellent quality (Narave-Flores 1983; Challenger 2003; Luna-Vega et al. 2006). Thus, it is presently listed as a threatened species by NOM-059-ECOL-2010 [Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) 2010].

In this regard and based on the criteria of the International Union for the Conservation of Nature's (IUCN) Red List categories for agricultural and horticultural plants, Hammer and Khoshbakht (2005) include *J. pyriformis* among taxa whose habitats have been reduced so drastically to a critical level that they are considered in immediate danger of extinction. Several studies show a restricted distribution with a low density of this species in the state of Veracruz, Mexico (Narave-Flores 1983; López-Gómez et al. 2008). In Veracruz, cedar walnut occurs only in the central area, from the municipality Misantla to Orizaba, forming discrete populations separated by geographical barriers at altitudes ranging from 1 200 m a.s.l. to 1 600 m a.s.l. (Narave-Flores 1983). In the Coatepec-Huatusco region, Veracruz, a density of 2 individuals·ha^{-1} is reported in coffee polyculture and 3 individuals·ha^{-1} in cloud forest fragments (López-Gómez et al. 2008). There are no established crops, populations are fragmented and geographically isolated, the seeds have been indiscriminately extracted and the populations have been under forestry management and harvesting of healthy and mature trees (Servicio Forestal Oriente 2000; Acosta-Hernández et al. 2011a).

The study, carried out on populations in Veracruz, reports morphological and dasometric differences within and between populations of *J. pyriformis* in San José Buenavista (SJB) and Coacoatzintla (COA), two localities with different forest management strategies. These were associated with a natural geographic discontinuity of this species and mainly due to the harvesting carried out in SJB (Acosta-Hernández et al. 2011a). Therefore, it was assumed that a process of genetic differentiation between the two populations and the loss of genetic variability could begin.

Previous studies of walnut genetic diversity between cultivars and natural populations from different geographic locations were carried out using different markers like isozymes (Ninot, Aletà 2003), random amplification of polymorphic DNA (RAPD) (Ferrazini et al. 2007; Shah et al. 2019), microsatellites (SSR) (Pollegioni et al. 2006a, 2009; Ross-Davis et al. 2008; Bernard et al. 2018), amplified fragment length polymorphism (AFLP) (Ali et al. 2016), and inter-simple sequence repeat (ISSR) (Christopoulos et al. 2010; Pollegioni et al. 2003, 2006b; Ghanbari et al. 2019). Among these markers, inter-simple sequence repeat (ISSR) markers are superior compared to other molecular markers (RAPD and AFLP) due to their speed of amplification, high reproducibility of the bands, and detection of high levels of polymorphism. Their use does not require prior knowledge of sequences in the genome to pre-design the sequence of the primer (Zietkiewicz et al. 1994).

Considering the advantages of ISSR and the fact that there are no genetic studies on *J. pyriformis*, the main objective of this study was to assess the genetic variability within and between the San José Buenavista (high logging strategy) and Coacoatzintla (low logging strategy) populations of cedar walnut through ISSR markers, to determine the effect of forestry management on the genetic diversity of these populations.

MATERIAL AND METHODS

The study of the intra- and interpopulation genetic variability of *J. pyriformis* was carried out in two natural populations in central Veracruz. The first population is in the congregation of San José Buenavista (SJB) in the Altotonga municipality at latitude 19°48'N and longitude 97°03'W, at an altitude of 1 600 m a.s.l., on 32.36 ha (Acosta-Hernández et al. 2011b). The second one is in the Coacoatzintla locality (COA), Coacoatzintla municipality, at 19°39'N and 96°59'W, at an altitude of 1 487 m a.s.l., on approximately 73 ha (Acosta-Hernández et al. 2011a). Between the two populations, there are many geographic barriers (Figure 1).

Sampling strategy. In both populations, we established circular sampling sites of 400 m^2 in size with *J. pyriformis* trees. In SJB, 35 sampling sites were established in areas of cloud forest remnants and grasslands, and 32 sites in COA in forest remnants, grasslands, and backyards. To avoid the effect of co-distribution, distances between sites were at least 50 m. Fresh, healthy leaves were collected from one tree at each site, obtaining 35 samples from the SJB population and 32 from the COA population. The collected leaf samples were stored at –20 °C until DNA isolation.

DNA isolation. Genomic DNA was extracted three times from 40–45 mg of leaf tissue using the CTAB method of Stewart and Via (1993). The

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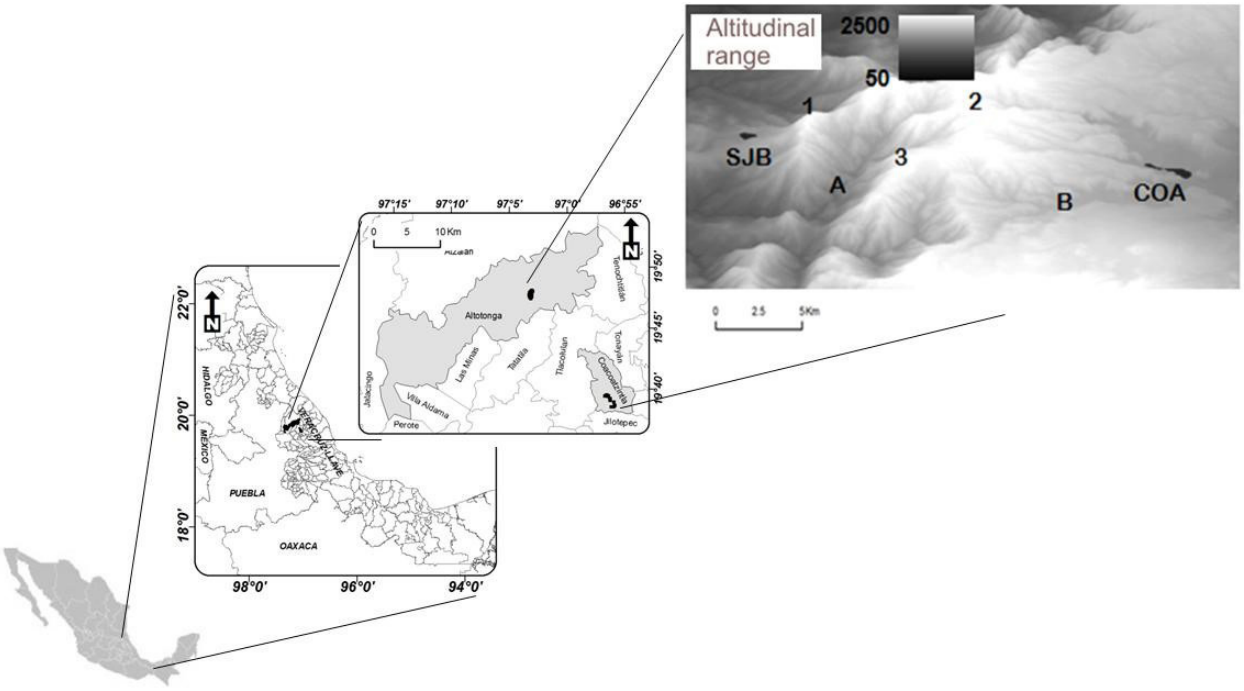

Figure 1. Location of *Juglans piryformis* populations

SJB – San José Buenavista; COA – Coacoatzintla; Hills 1 – La Concordia; 2 – La Nopalera; 3 – El Cuervo; A – Barranca Bedolla; B – Barranca El Tecolote

DNA was diluted to 50 ng·μL^{-1} in sterile double distilled water, stored at –20 °C and used for PCR amplification. Isolated DNA and PCR products were confirmed by gel electrophoresis on 0.9% and 1.8% agarose, respectively, in 0.5× TBE buffer (40 mM Tris-HCl, 20 mM boric acid, 1 mM EDTA, pH 8.0) with a horizontal electrophoresis system (CONSORT®; iLife Biotech, India) at 100 V for 60 min. Gels were stained with ethidium bromide (1μg·mL^{-1}) and then visualized with a long-wave UV light source (CONSORT SPTF12®, iLife Biotech, India) and photographically documented using a camera (Canon PowerShot® G6, Canon, Japan). A 100 bp DNA ladder (Invitrogen, Thermo Fisher Scientific, Mexico) was used to determine the molecular weight of the amplified products. To assess the presence of nonspecific amplification fragments, each sample was amplified three times; in all cases, blanks containing all PCR components except DNA were included. The concentration of the extracted DNA was determined using a spectrophotometer (Perkin Elmer, USA) at 260 nm and 280 nm.

ISSR analysis. To establish adequate resolution and reproducibility of the ISSR technique, two assays were performed with DNA obtained from ten individuals from each population of *J. pyriformis* included in this study. Six ISSR primers (834, 841, 856, 888, 890, and 891) (Promega, USA) from the University of British Columbia, Canada (UBC) were used for the amplification (Table 1). PCR reactions were performed in a 25 μL volume containing: 50 ng template DNA, 1× PCR buffer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 1.25 U Taq DNA polymerase (Promega, USA), 0.2 mM dATP, dGTP, dCTP and dTTP, primers (Promega, USA), according to Zietkiewicz et al. (1994).

Table 1. UBC (University of British Columbia) primers and their 5'–3' sequences used for Inter Simple Sequence Repeat (ISSR) analysis

ISSR Primers (UBC)	Sequences (5'–3')
834	AGAGAGAGAGAGAGAGYT
841	GAGAGAGAGAGAGAGAYC
856	ACACACACACACACACYA
888	BDBCACACACACACACA
890	VHVGTGTGTGTGTGTG
891	HVHTGTGTGTGTGTGT

Single-letter abbreviations for mixed-base positions: Y – C or T; V – A, C or G; H – A, C or T; B – C, G or T; D – A, G or T

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Genetic analysis. The amplified bands were coded with values of 1 = presence and 0 = absence. Unclear and non-reproducible bands were not included in the analysis. The average number of amplified fragments per primer (*N*) and the percentage of polymorphism at 95% (Hedrick 2000) were calculated. Polymorphism values were compared by a chi-squared test with prior transformation of the data ($x = \sqrt{\text{ArcSin}(\%)}$). Because of the dominant nature of the ISSR marker, total heterozygosity was determined by the method of Lynch and Milligan (1994) using the TFPGA 1.3 package (Tools for Population Genetic Analysis) (Miller 1997). To determine differences in heterozygosity between populations, the Wilcoxon nonparametric test was applied using STATISTICA (Version 8 for Windows; 2007).

The genetic differentiation coefficient (*PhiPT*) was calculated in accordance with the AMOVA method with the GenAlEx program (Peakall, Smouse 2006). To determine whether the estimates of *PhiPT* for each locus differed significantly from zero, we calculated the statistics of chi-squared (χ^2):

$$\chi^2 = (2N) \times PhiPT \times (k - 1) \text{ with } (k - 1) \times (s - 1) \times df \quad (1)$$

where:

N – size of the sample;
PhiPT – genetic differentiation coefficient;
k – mean number of alleles;
s – number of populations (Octavio-Aguilar et al. 2009)
df – degrees of freedom.

In addition, using the MVSP software (Version 3.2; 1985–2010) (Miller 1997), the genetic distance was calculated according to Nei and Li (1979) for dominant markers and a UPGMA analysis was performed for Nei and Li's similarity of individuals. A comparison of *Fst* calculated with the software STRUCTURE (Version 2.3.4, 2012) was made using 50 000 repeats and 50 000 burns in periods for K2, K3 and K4 groups, considering the origin of individuals in the analysis (Pritchard et al. 2000; Falush et al. 2003).

RESULTS AND DISCUSSION

A total of 67 DNA samples were analysed from both populations of *J. pyriformis*. The average number of amplified fragments was *N* = 55.3, of which *N* = 29.7 ± 4.5 corresponded to SJB and *N* = 25.6 ± 4.7 to COA. The six primers under study amplified 106 polymorphic fragments ranging from 150 bp to 1 400 bp. The proportion of polymorphic loci (%*P*) and the heterozygosity (*H*) are shown in Table 2.

The χ^2 test revealed significant differences between the populations ($\chi^2 = 21.81$, $P = 0.0006$) in the percentages of polymorphism transformed by each ISSR; in contrast, the Wilcoxon test showed no significant differences ($z = 1.58$, $P = 0.11$) in the heterozygosity values between the two populations. These results evidence that the COA population is indeed more polymorphic (81.13%) than SJB (76.41%), but not different in their degree of heterozygosity, although the COA population showed slightly higher heterozygosity ($H = 0.335 \pm 0.17$) than SJB ($H = 0.301 \pm 0.18$) (Table 2).

In SJB, the six primers revealed the presence of 22 unique bands, half of them obtained with primer UBC 890, and 3 rare bands with a frequency of ≤ 0.05. One of these bands was obtained with primer UBC 856 and the other two with primer UBC 888. In COA, all six primers detected 16 unique bands and 3 rare bands. One of the rare bands was obtained with primer UBC 890 and the other two with primer UBC 888 (Figure 2).

The detected variation evidenced the existence of significant genetic differentiation between both populations ($\chi^2 = 14.59$, $df = 1$, $P = 0.0001$), which was confirmed by the values obtained for *PhiPT* (0.109) and the Nei and Li distance (0.134). The STRUCTURE analysis showed consistent differentiation when 92% of individuals in SJB and 89% of individuals in COA were correctly assigned. This consistency in sample assignment and explained variance decreased as the number of means increased ($k = 3$ and $k = 4$) (Figure 3).

The obtained results confirm that the six ISSR primers tested were useful for identifying the level of genetic variability in *J. pyriformis*, and this information is important due to the lack of previous knowledge of this aspect in these populations. Our results are consistent with similar studies conducted with ISSR markers in *Juglans* species. The range in base pairs (150 bp to 1 400 bp) is within the limits reported for the same ISSR primers in *J. regia* (246 bp to 1 968 bp) by Pollegioni et al. (2006b) and in *J. nigra* and *J. regia* (307–2 440 bp) by Pollegioni et al. (2006a).

Since *J. pyriformis* is an endemic species of restricted distribution with isolated populations, it is to be expected that its populations show the lower mean genetic variability compared to related species of wide geographic distribution (Hamrick, Godt 1989). We found some similarity in the values of polymor-

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Table 2. Genetic diversity of two populations of *Juglans pyriformis* obtained by inter-simple sequence repeat (ISSR) markers

ISSR primer (UBC)	No. amplified bands	Range (bp)	San José Buenavista (SJB)			Coacoatzintla (COA)		
			N	%*P*	*H*	*N*	%*P*	*H*
834	17	200–1 250	32	88.23	0.290	30	82.35	0.321
841	21	250–1 400	34	75.00	0.313	30	68.75	0.288
856	16	150–1 300	32	80.95	0.308	22	76.19	0.329
888	14	300–1 100	23	78.57	0.320	20	92.86	0.407
890	21	250–1 100	32	47.61	0.176	24	85.71	0.351
891	17	200–1 400	25	94.12	0.386	28	83.35	0.295
Average ± SD	–	–	29.7 ± 4.5	76.41 ± 16.15	0.301 ± 0.18	25.6 ± 4.7	81.13 ± 8.27	0.335 ± 0.17

N – average number of amplified fragments; %*P* – proportion of polymorphic loci at 95%; *H* – heterozygosity; UBC – University of British Columbia

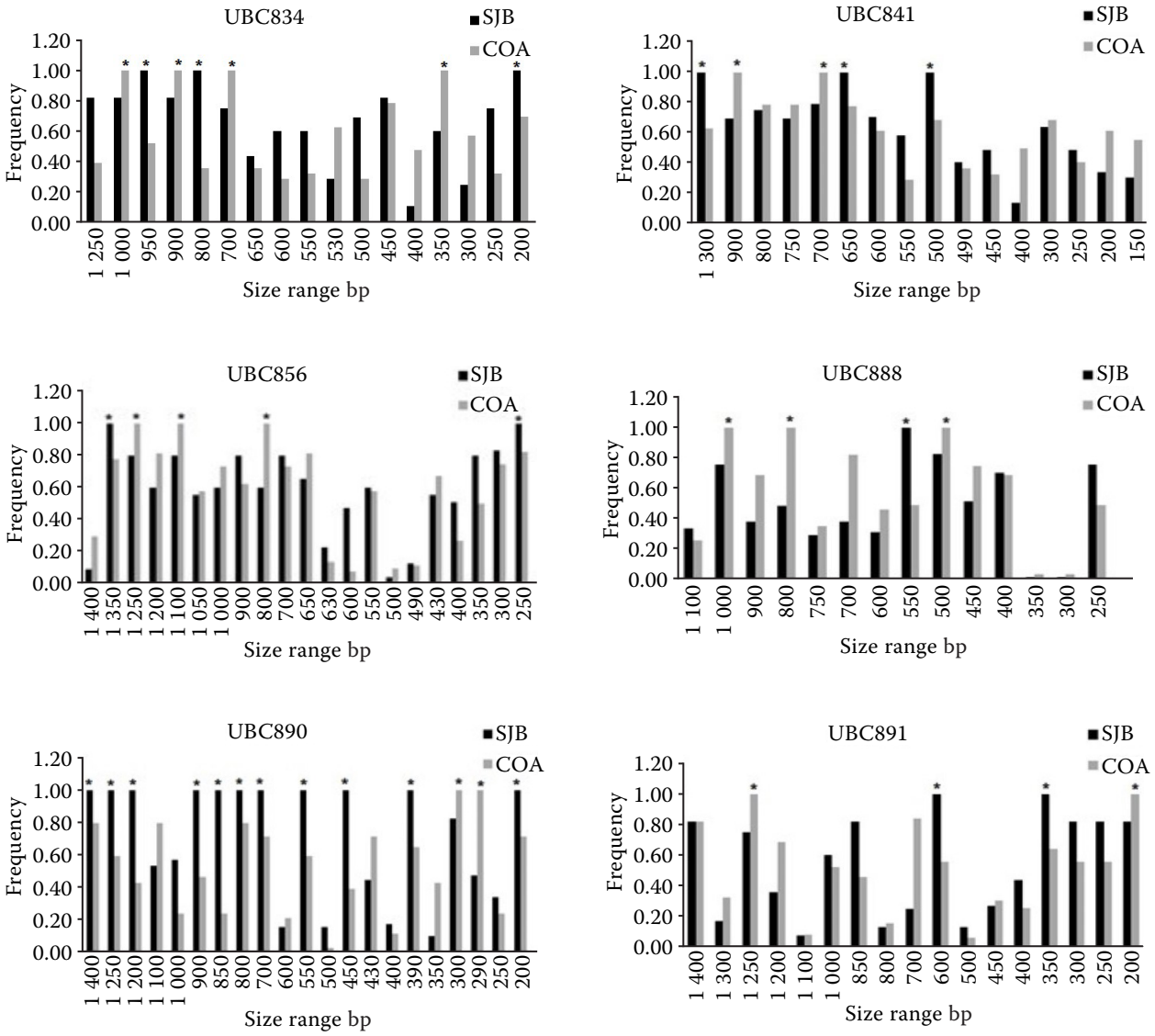

Figure 2. Frequency of alleles of each ISSR primer in *Juglans pyriformis* populations from San José Buenavista (SJB) and Coacoatzintla (COA)

*Unique and monomorphic loci

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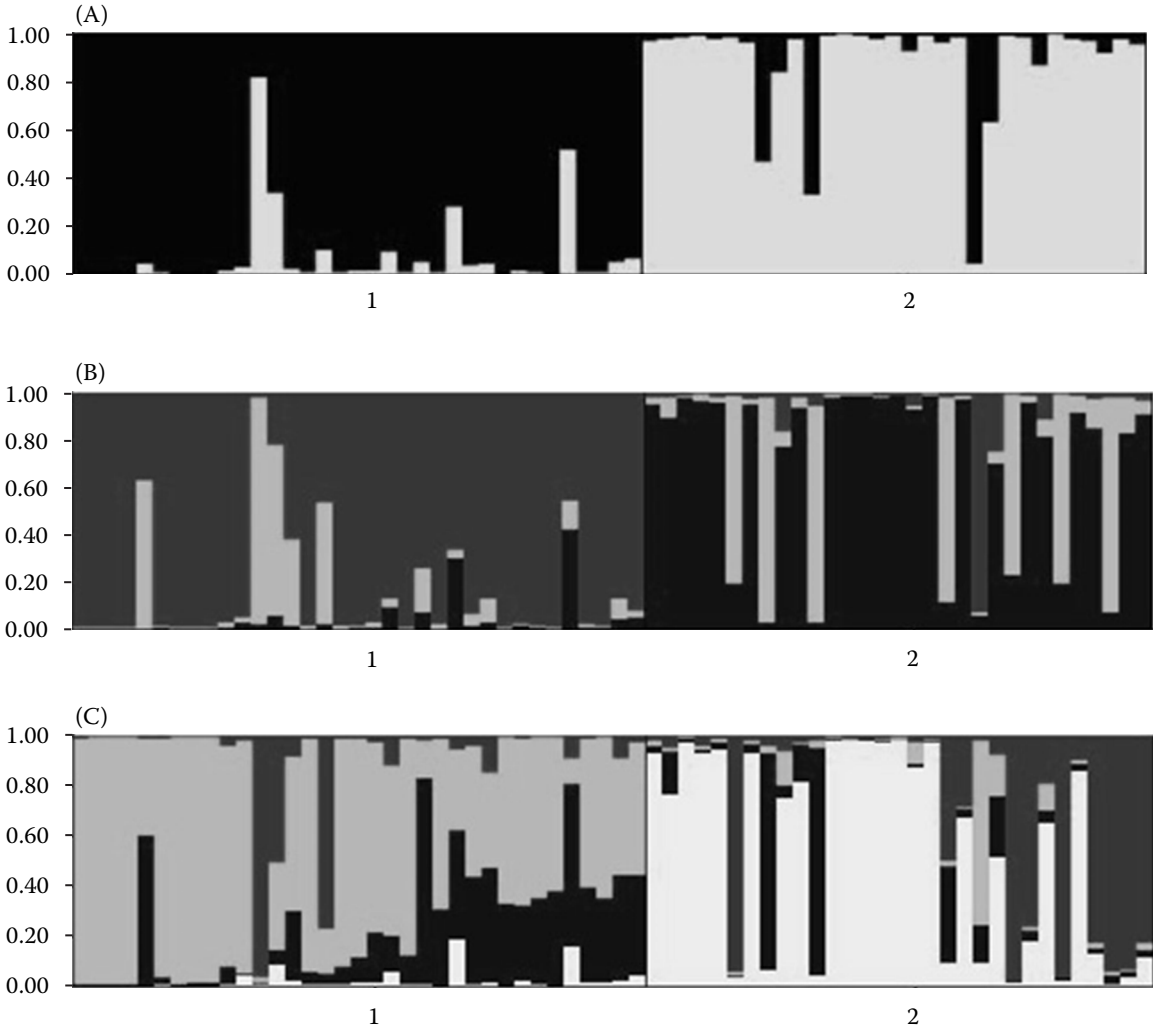

Figure 3. STRUCTURE analysis (A) K2; (B) K3; (C) K4; the lower variance within groups (variance of ln likelihood for K2 = 907.7, K3 = 1 203.4 and K4 = 2 340.5) and the best allocation comes in two averages

phism percentage obtained in our study (SJB 76.4%; COA 81.13%) to that detected in *J. regia* (83.68%) (Pollegioni et al. 2006b) using the same six ISSR primers. Furthermore, the heterozygosity detected in both populations of *J. pyriformis* (SJB 0.301 ± 0.07, COA 0.335 ± 0.04) was higher than that obtained at 0.060 in *J. regia* (Alfonsín et al. 2004). These results could be explained by the advantages of ISSR markers in terms of the high polymorphism they detect and, as they tend to be longer, having at least 14 di- or trinucleotide repeats, they provide greater reproducibility of bands (Zietkiewicz et al. 1994; Wolfe 2005). In addition, the apparent high genetic diversity of *J. pyriformis* could be reduced since the obtained DNA samples are from adult trees, thus representing vestigial diversity with respect to tree age, which could be considered a characteristic of the species.

The selective forest management carried out in the SJB population constitutes the main difference in the history of the studied populations, so it is possible that the dysgenetic selection carried out to leave only the best-conformed trees, mostly adults of reproductive age (Acosta-Hernández et al. 2011b), could have negatively affected the levels of genetic variability detected in terms of polymorphism percentages (χ^2 = 21.81, P = 0.0006) and heterozygosity observed in the SJB population (Table 2). It is possible that the recent elimination of a large proportion of individuals in a short time (three years), due to forest management (Servicio Forestal Oriente 2000), could explain the presence of more exclusive bands in SJB than in COA. Furthermore, the trend shown by the SJB population towards a reduction in genetic variation was con-

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firmed in this study by the loss and fixation of some bands. The presence of a higher number of unique bands in SJB could also reflect its history. Rare bands may be the first to be lost because of foundation effects, bottlenecks, and other sporadic fluctuations in the population size (Luikart et al. 1998).

The levels of genetic variability obtained in the present study are consistent with the morphological variation reported for both populations (Acosta-Hernández et al. 2011b) and confirm the relationship between the population size and the genetic variation maintained in populations under forest management reported by Putz et al. (2001) and Wickneswari et al. (2004). That is, although the sample size in the morphological study of the SJB was larger than that of the COA, the analysis of the evaluated morphological attributes showed that the COA population has more variations (112 individuals grouped into three clusters) than the SJB (148 individuals grouped into two clusters), which coincides with the levels of genetic diversity found in both populations.

On the other hand, it is known that, in mature forests, dysgenetic selection in favour of superior phenotypes changes the genetic structure and negatively affects the phenotypic structure of the population, so that the removal of phenotypically superior trees has a greater impact on the gene pool of subsequent generations (Finkeldey, Ziehe 2004). This is valid for the SJB population, where 37.2% of the individuals with the best characteristics are grouped into a single cluster, in contrast to COA, where 55% of the largest and best shaped trees are grouped into two clusters (Acosta-Hernández et al. 2011a, b). Considering that *J. pyriformis* is monoecious, with anemophilous pollination, we consider that selective extraction in favour of superior phenotypes increases the probability of inferior phenotypes transmitting their genes to subsequent generations and could decrease the potential of the population to adapt to and survive the environmental change (Hawley et al. 2005). The effects of selective forest management studied in two mature *Pinus strobus* forests showed an 80% loss of rare alleles ($P < 0.01$), a 50% reduction in the latent genetic potential (*LP*) of each forest, as well as a 25% reduction in its allelic richness after 75% of the trees had been harvested (Buchert et al. 2002), suggesting that the ability of the gene pool to adapt to changing environmental conditions may have been reduced.

Therefore, if a strategy for the conservation of *J. pyriformis* populations is not established, the current level of genetic variability is at risk due to the set of factors acting between populations and, in the long term, the species could suffer an evolutionary setback (Finkeldey, Ziehe 2004). In particular, the SJB population is at highest risk, as the significant decline in the population size has led to increased genetic drift and subsequently increased levels of inbreeding, which will need to be assessed in future studies using codominant markers.

Finally, the values of *PhiPT* (0.109) and genetic distance (0.134) of Nei and Li (1979) and the lower variance between individuals within two groups shown by the STRUCTURE analysis (907.7) revealed the existence of genetic differentiation between the two populations. However, the STRUCTURE analysis suggests that we are in the presence of two populations with a common origin. It might be possible to explain these results based on the geological history of the Montane Cloud Forest (Mesophyll Mountain Forest, *sensu* Rzedowski 1996) in Mexico and on fossil registers of the floristic components of this forest in Veracruz.

CONCLUSION

According to Rzedowski (1965), the mesophyll forest has existed in Mexico since the Tertiary, and the important proportion of its endemic species could be the result of the fragmented distribution it presents today, although it must have been more continuous during some of the past geological eras. In this regard, Luna-Vega et al. (1999) formulated a preliminary hypothesis on the historical relationships of the cloud forest through the Parsimony Analysis of Endemicity. Based on this, the Mexican MCF could be grouped into five large mesophilic forests (clades), which diverged from a previously continuous forest that was fragmented by major climatic changes. This hypothesis seems to be supported by the findings of Graham (1976) on the presence of fossil pollen of *J. pyriformis* in a lignite formation that presents remnants of the vegetation of 10–12 million years ago, from the upper Miocene, located in Paraje Solo, Veracruz, Mexico. Considering previous studies, we cannot dismiss the possibility that distribution patterns of *J. pyriformis* are associated with the pattern of fragmentation and distribution of the mountain cloud forest (Rzedowski 1965;

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Luna-Vega et al. 1999). Thus, the current populations of *J. pyriformis* in the state of Veracruz constitute a group of fragmented and relict subpopulations that have maintained a genetic flow, which would indicate a common origin. However, the fragmentation and reduction of this ecosystem by anthropogenic activities (Challenger 2003), combined with the isolating effect of geographic barriers (not evaluated), have increased the distance between the populations and probably contributed to insufficient genetic flux among them. Under this argument, we might assume that the genetic and morphological differentiation between populations is recent. The presence of unique and rare bands in both populations also supports the existence of genetic differentiation between the populations under study. We considered the immediate effect following a sharp population decline where rare alleles are lost faster than heterozygosity, creating a transient excess of heterozygosity relative to the allele number (Amos, Hoffman 2010), but ISSR, as all dominant markers, only estimates heterozygosity based on the putative combinations of alleles according to Lynch and Milligan (1994); in this context, rare alleles increase the possible combinations and result in the same transient excess of heterozygosity. According to the *PhiPT* value, Nei's genetic distance and that of the STRUCTURE analysis, the studied populations are different with a common origin. Although this is a preliminary study of genetic diversity in this species, the obtained results provide evidence how these populations could be differentiated by logging strategies.

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