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A COALESCENT APPROACH TO CHLOROPLAST GENOME RELATIONSHIPS WITHIN AND BETWEEN POPULATIONS OF *PINUS DEVONIANA* IN MEXICO

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ABSTRACT

Pinus devoniana, a member of the *Pinus montezumae* complex (*Ponderosae*) of Mexico and Central America occurs as scattered populations from the western states of Jalisco and Nayarit to Chiapas in the east. Populations within regions, i.e., western or eastern Mexico, are separated by from 75 to 300 km, while the eastern and western populations are more than 700 km apart. Levels of gene flow between populations within regions and between eastern and western Mexico are assessed using the paternally inherited chloroplast genome as a marker. Twenty-five polymorphic restriction chloroplast sites were found in the 175 individuals surveyed; eight restriction sites define the regional identity of populations. The chloroplast phylogeny and relationships within and between populations and regions are discussed in the context of the coalescent.

Key words: chloroplast, coalescent, evolution, haplotypes, phylogeny, *Pinus*.

INTRODUCTION

Isolation of populations by distance and subsequent genetic changes in those isolated populations are the materials of speciation. Consequently, isolated populations have fascinated students of evolution at least since Darwin. Sewall Wright and others since him have developed models and the theoretical framework in which to place isolated populations and the effects of genetic drift, random mutation, and selection on them. Recently interest has focused on the genealogical history of populations beginning with a migration or isolation event and the subsequent differentiation of the populations from one another. Beginning in the early 1980's with the seminal papers of Kingman (1982a, 1982b) a body of work has accumulated regarding the genealogy of genes. A gene tree representing the genealogy of a gene based on nucleotide differences is a way of visualizing the evolutionary history of the gene. If homologous genes are sampled from a number of populations of a species then the genealogy of the gene can be inferred (Takahata 1989), and give insight into the history of the populations. The gene tree shows the most parsimonious relationship between variants that are detected in the sampled gene. This process of looking backward in time to infer phylogenies of genes, termed the coalescent, has been recently reviewed in Ewens (1990) and Hudson (1990).

Since the early 1980s Ewens et al. (1981), Donnelly and Tavaré (1986, 1995), Golding (1987), Templeton et al. (1987, 1993), Excoffier and Langaney (1989), Crandall and Templeton (1993), and Griffiths and Ta-

varé (1998), among others have developed methodology, insights, and statistical methods that aid in the translation of molecular data into evolutionary insights. These methods allow interpretations of data beyond simple parsimony. For example, the θ of Ewens et al. (1981) gives an estimation of genetic diversity within groups based on restriction site data. Templeton et al. (1987, 1993) and Crandall and Templeton (1993) have shown that for very low levels of θ an increase in sample size does not return the investment of time with much additional information; these authors have also devised methods for estimating cladograms and assigning probabilities to those cladograms. Crandall and Templeton (1993) have suggested interpretations for the relative evolutionary age of genotypic classes based on the frequencies of those classes, and the most probable relationship of various frequency classes to one another. These methods can greatly enhance the amount of information gleaned from data sets. Some of these methods are applied to the chloroplast haplotype data presented here.

This paper examines evolutionary relationships among populations that are isolated from one another, but isolated to different degrees. Using chloroplast haplotypes I examine similarities and differences among isolated populations of *Pinus devoniana* Lindley. Most work with isolated populations has focused not on long-lived perennials like *P. devoniana*, but instead on relatively short-lived plants or animals, organisms in which dramatic evolutionary changes due to isolation and drift might be observed. Some classic cases of the results of isolation in plants are 1) the

study by Epling and Dobzhansky (1942) on the effects of isolation and drift on a desert annual, *Linanthus parryae* (A. Gray) E. Green; 2) the classic study of ecotypic differentiation on *Potentilla glandulosa* Lindley by Clausen et al. (1940); and 3) observations of the result of repeated isolation events on Hawaiian tarweeds (*Dubautia*).

The population structure of long lived woody perennials has rarely lent itself to easy detection of intraspecific changes in the genome due to the results of isolation. Long-lived woody perennial populations are known to show little between population genetic differentiation. Most of the variability in woody perennials is found within each population and this variability is shared between populations (Hiebert and Hamrick 1983; Hamrick 1987; Stebbins 1950; Yeh and Layton 1979). Many long-lived perennials have ranges that cover large geographic areas and are wind pollinated. In wind pollinated species, long-distance gene flow prevents differentiation over fairly large areas (Jain and Bradshaw 1966; Silen 1962).

Pinus devoniana, is a member of the *P. montezumae* Lamb. complex of Mexico and Central America (sub-section *Ponderosae*) (Little and Critchfield 1969). *Pinus devoniana* occurs at relatively low elevations, from 1300 to 2400 m (but occasionally to 3000 m) (Mirov 1967). The species is generally considered to be endemic to Mexico, ranging from the western states of Nayarit and Jalisco, north to southern Zacatecas and San Louis Potosí, and south and east to Oaxaca and Chiapas, however, according to some authors (Price et al. 1998) the range extends into Guatemala. Although the geographic range of the species is large, it does not occur as extensive populations. Instead, *P. devoniana* occurs as relatively small scattered populations mixed with oaks in western Mexico, or mixed with other species of pines in eastern Mexico.

The chloroplast genome is paternally inherited through wind borne-pollen in all conifers studied to date (Neale et al. 1986, 1989; Neale and Sederoff 1989; Stein et al. 1989; Szmidi et al. 1987; Wagner et al. 1987), thus long-distance dispersal of the plastid genome is possible. But, the approximately 700 km between western and eastern Mexico populations of *P. devoniana* almost certainly precludes any direct transfer of the chloroplast genome across the range of this species.

The unrecombining nature along with the slow mutation rate, found in some early studies (Banks and Birkey 1985), of chloroplast DNA has led to its widespread use as a taxonomic tool. Chloroplast DNA has been used to identify taxonomic relationships, usually above the species level (Jansen and Palmer 1988; Lavin et al. 1990; Sytsma and Gottlieb 1986; French and Kessel 1989). Chloroplast DNA has also been used to characterize species using few individuals (Hantula et

al. 1989). More recently studies using chloroplast DNA have found intraspecific variability, and this variability has been used to assess partitioning of chloroplast variability within species (Neale et al. 1986; Soltis et al. 1989; Soltis and Soltis 1989).

Pine chloroplast variability seems to range from quite low to rather high. No chloroplast variation was detected in *P. torreyana* Carrière ($n = 24$), although this species is unique in its lack of variability in general (Waters and Schaal 1991); nor was any chloroplast variation found in a sample of 30 *Pinus taeda* L. (Ali et al. 1991). Only two chloroplast variants were found in *P. monticola* Douglas ($n > 64$) (White 1990), and two in *Pinus densata* Mast. ($n = 20$) (Wang and Szmidi 1990) although this last example of chloroplast variation was determined to be the result of ancient hybridization. Thirteen length variants were found in 371 individuals of *P. contorta* Dougl. Ex Loud. and *P. banksiana* Lamb. in regions of allopatry and in a hybrid zone between the two species (Wagner et al. 1987). However, at least two other species in the *P. montezumae* complex, *P. hartwegii* Lindl. and *P. montezumae*, show a great deal of chloroplast variation (Matos 1992). Matos (1992) found 51 distinct chloroplast haplotypes (restriction sites only) in 350 individuals.

This study addresses the question: what happens at the chloroplast genome level when populations of wind-pollinated, long-lived perennials are isolated from one another by distances of from approximately 75 to 700 km? Do the trees "see" these distances as different, and if so, how much different with regard to chloroplast genome flow? Additionally, given the methods of the coalescent, what can be said about the history of the chloroplast genome of *Pinus devoniana*?

MATERIALS AND METHODS

Collection of Specimens

One hundred seventy-five specimens of *Pinus devoniana* were collected during two field trips to Mexico (see Fig. 1 and Table 1 for collection localities and specific site information). Both pressed voucher specimens (MO and MEXU) and fresh branch tip material were collected for all individuals; all voucher specimens include cones, if available. Branch tip material was kept on wet ice until it was frozen in liquid N₂ prior to DNA extraction.

Description of the Collection Sites

Throughout this paper, collection localities will often be described as sets of collections or sets of localities. This terminology refers to the set of four collections in the western states of Jalisco and Nayarit and to the set of three collections from the eastern states of Oaxaca and Chiapas (Fig. 1).

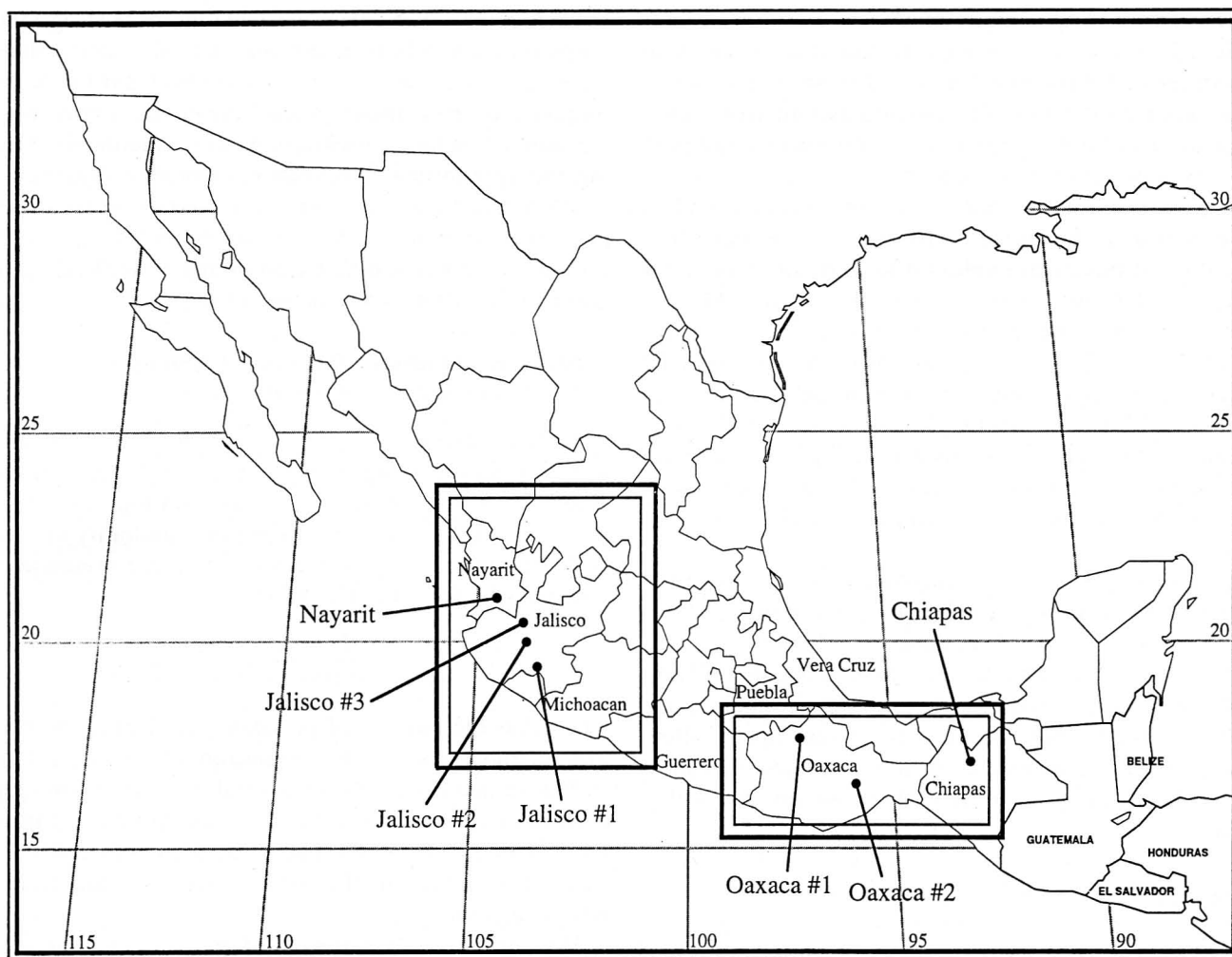


Fig. 1. Collection localities in western and eastern Mexico.

Table 1. Key to collection numbers and localities. *Pinus devoniana*.

Collection numbers	Locality and site description	Elevation
#1368–1392	<i>Jalisco #1</i> , at the base of Nevado de Colima. <i>P. devoniana</i> is one of three pines at this elevation. The collection is from the middle of a pasture, and continues along a ravine.	1650 m to 1690 m
#1393–1419	<i>Jalisco #2</i> , just before the first major ridge west of Ayutla on a gravel road to Cuautha. No other species of pine here, mixed pine/oaks woodland. Individuals are generally small, all are less than 15 m, many less than 10 m.	1700 m
#1420–1444	<i>Jalisco #3</i> , in the first creek draw after turning north toward Ameca at LaHuerta on a gravel road. No other pine species, mixed pine/oak woodland on a low hillside.	1420 m
#1446–1470	<i>Nayarit</i> , between 0.1–0.7 km from the intersection of Hwy. 5 and Hwy. 15 on a dirt road. The habitat is open pasture land and ravines. <i>Pinus devoniana</i> is the only pine mixed with oaks.	1130 m
#1618–1642	<i>Chiapas</i> , just off of Hwy. 199, 15.2 km north of the turn off to Chanal. <i>Pinus devoniana</i> is one of three species of pines in this extensive pine forest. Individuals to approx. 20 m tall.	2000 m
#1643–1667	<i>Oaxaca #1</i> , just off Hwy. 135, 16.5 km north of Hwy. 190. This is a small, seemingly isolated population of in a mixed pine forest with scattered oaks; the nearest observed population near Monte Alban. Trees to approx. 20 m tall.	2020 m
#1668–1692	<i>Oaxaca #2</i> , 7.2 km from El Cameron on the gravel road to San Carlos Yaultepec. Trees are located on a dry granite hillside. No other pines in the area, other vegetation consists of grasses and a few scattered trees. Trees to approx. 15 m tall.	1200 m

One hundred specimens of *P. devoniana* were collected from the western edge of the species range in the states of Jalisco and Nayarit. The second set of *P. devoniana* collections, 75 individuals, are from three populations at the eastern edge of the species range in the states of Oaxaca and Chiapas.

Pinus devoniana is one of the few pines found at relatively low elevations in western and central Mexico, thus, it has been exploited to a greater extent for timber and firewood in western and central Mexico than in eastern Mexico where the pine forests are extensive. Consequently, populations in the west may be much more fragmented now than in the relatively recent past. *Pinus devoniana* occurs as scattered populations in the relatively xeric states of Jalisco and Nayarit in western Mexico, occasionally as pure stands but, more frequently it is found in mixed stands with oaks.

In eastern Mexico, *P. devoniana* occurs as small scattered populations in the extensive mixed pine forest that covers much of southeastern Mexico. One collection site, Oaxaca #2 deviates from this general habit of *P. devoniana* in eastern Mexico. This collection site is on a large granite outcrop in a wide, open valley. Other species of pines are found on the near hillsides, but only *P. devoniana* was observed on the valley floor.

DNA Isolation and Analysis

Branch tip material was ground in liquid nitrogen to a fine powder either in a mortar and pestle cooled with liquid nitrogen or an electric coffee grinder cooled with dry ice. The pine powder was stored at -80°C prior to DNA extraction.

Total cellular DNA was extracted from frozen powdered material by a modified CTAB procedure (Murray and Thompson 1980) that yielded 50–70 μg of DNA per approximately 2 g of pine powder.

All DNA samples were digested with *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II, *Sac*I, *Sma*I, and *Xho*I. Additionally, for the purpose of mapping the chloroplast genome, some DNA samples were digested with *Kpn*I and *Pst*I. These additional digests aided in aligning the *P. devoniana* chloroplast map with the previously published restriction map of *P. radiata* D. Don (Strauss et al. 1988).

The digested pine DNAs were separated on horizontal 0.8% agarose gels with a 1X TAE buffer system overnight and DNA was double blotted on to nylon membranes following the general procedure described in Learn and Schaal (1987).

Heterologous petunia chloroplast (*Pst*I cp clones) probes (from J. D. Palmer, Palmer et al. 1983) were hexamer-labeled (Feinberg and Vogelstein 1983) with either $[\alpha\text{-}^{32}\text{P}]\text{-dCTP}$ or $[\alpha\text{-}^{32}\text{P}]\text{-dATP}$, or both. After

hybridization, filters were washed and autoradiographs prepared according to Learn and Schaal (1987), filters were re-washed and sequentially hybridized to 15 petunia cp probes, these probes cover the entire pine chloroplast genome. Restriction sites were mapped using the appropriate combination of double digests.

DNA size fragments were estimated using computer programs written by A. Templeton and J. Learn, J. Lawrence, and H. Chou, based on the FORTRAN program of Schaffer and Sederoff (1981).

Estimation of Genetic Diversity, Population Subdivision, and Phylogeny Reconstruction

Genetic diversity within populations and species was estimated using the formula of Ewens et al. (1981), $\hat{\theta} = 4N\mu$. $\hat{\theta}$ corrects for the fact that restriction site polymorphisms do not represent randomly chosen DNA fragments but are conditional on the presence or absence of each site. The formula is

$$\hat{\theta} = \frac{(k_4 + k_5 + k_6)}{\{(8m_4 + 10m_5 + 12m_6)\ln(n)\}},$$

where k is the number of polymorphic sites for restriction endonucleases with recognition sites that are 4, 5, or 6 base pairs long, m is the total number of sites for each restriction enzyme, and n is the number of individuals (genes or haplotypes) in the sample. All restriction enzymes in this study have six base recognition sequences.

Hudson's (1989) estimate of the variance of $\hat{\theta}$ is calculated with the formula

$$\widehat{\text{Var}}(\hat{\theta}) = \frac{\hat{\theta}}{k} + \frac{\hat{\theta}^2}{\{ \ln(n) \}} \sum_{i=1}^{n-1} \frac{1}{i^2}.$$

Wright's F_{st} (1969) was estimated using haplotype frequency data (BIOSYS, Swofford 1989). N_m (Wright 1951), the number of migrants per generation between the eastern and western populations, corrected for haplotype data was calculated by geographic region using the equation

$$F_{st} = \frac{1}{N_m + 1}.$$

Local N_m values, within the set of four western populations and the set of three eastern populations, were estimated using the method of Slatkin and Maddison (1989). This method depends on knowing the phylogeny of the nonrecombining segments of DNA sampled, and the geographic location of each sample is considered a multistate character. The Slatkin and Maddison (1989) method gives a minimum N_m that is consistent with the phylogeny and distribution of the segment of DNA of interest. The phylogeny of genes and geographic location indicates the past number of migration events, s , necessary for the current distri-

bution; s , along with n , the number of haplotypes, was used to estimate Nm from table 2 in Slatkin and Madison (1989).

Relationships between *P. devoniana* haplotypes were visualized by two methods. First, cladograms were generated by entering restriction site gains and losses, that is presence/absence, data into PAUP (Swofford 1991). The data were analyzed both weighted (1.3:1, 2:1) and unweighted, with and without an outgroup (*Pinus ponderosa* Laws), and unrooted and mid-point rooted. Weighting restriction site gains more than restriction site losses reflects the greater probability of a loss. This is based on the fact that a mutational event that results in a change in one of the recognition nucleotides rendering it unrecognizable by the enzyme is more probable than that a mutational event will occur in a "near" recognition sequence, i.e., the other five (in the case of 6-base recognizing enzymes) and will be properly positioned, making it a site gain. Since the actual ratio of gains to losses and vice versa events is not known with much certainty for any particular organism or stretch of DNA, a variety of biologically realistic weighting schemes was used with these data. If all trees agree regardless of weighting—within biologically reasonable limits—the believability of the topography of the tree is increased substantially.

Heuristic searches were carried out using random stepwise addition with 10 and 100 replications. Cladograms were also generated that included both site and length variation. Here, site gains to losses were weighted 1.3:1, while insertions and deletions were weighted 1:1. Because PAUP designates haplotypes as different from each other if one haplotype is missing data present for the other, the addition of length variation was used only to clarify relationships between restriction site haplotypes that differed only because of missing data.

Distance and data matrices were used to construct more informative cladograms showing the exact restriction site mutational steps between haplotypes. Information from the data matrix, when referenced back to the chloroplast DNA maps for site changes, allowed the identification of specific restriction sites involved in defining haplotypes. Since *P. devoniana* haplotypes were specific to eastern or western Mexico, the most parsimonious connections were made between haplotypes within the regions and between these two geographic regions of Mexico. Specific restriction site changes were identified, and intermediate haplotypes assumed. This process gives a step-by-step connection between eastern and western haplotypes and assumes that nature takes the most parsimonious path from unique haplotype to unique haplotype.

After cladograms for the eastern and western Mexico *P. devoniana* chloroplast haplotypes were constructed, the probability that the one-step, 2-step, etc.

connections are statistically significant was calculated using the method of Templeton et al. (1993). This method of assigning cladogram uncertainty uses information from both the restriction site differences between two haplotypes and from the restriction sites shared between two haplotypes. The Templeton et al. (1993) method allows the calculation of the probability that two haplotypes differ by j restriction sites and share m restriction sites using the equation

$$\hat{P}_j = \prod_{i=1}^j (1 - \hat{q}_i),$$

where q_i , the oldest polymorphic restriction site (the index mutation) is estimated through a Bayesian analysis. The program for estimating P_j was provided by A. Templeton. The parameters entered are: j , m , b (a transition-transversion bias—no bias was assumed here), r (the length of the restriction endonuclease recognition sequence), and u (the H of Hudson [1989]). Because *P. devoniana* haplotypes tend to differ from one another at several restriction sites resulting, in some cases, in $P < 0.95$ using the above equation, parsimony+1 was also calculated. The equation

$$\sum_{i=0}^y \sum_I \prod_{k=1}^i q_{j(k)} \prod_{k=i+1}^x (1 - q_{j(k)}),$$

where I refers to the set of all permutations of the x age ranks, employs the same parameters, j , m , b , r , and u , as above. This equation (Templeton et al. 1993) calculates the probability that haplotypes are connected by a parsimonious, or by at most one additional mutation, relationship.

RESULTS

Chloroplast Genome

Pinus devoniana is one of four species of pines in the closely related *P. montezumae* complex. The chloroplast genome of *P. devoniana* is compared here with that of *P. hartwegii* and *P. montezumae*, other members of the complex for which chloroplast genome data are available (Matos 1992). The *P. devoniana* chloroplast genome, like its close relatives *P. hartwegii* and *P. montezumae*, is 127 kb long if all the longest length variants are included in the map. The eight restriction enzymes used in this study characterized 0.73% of the chloroplast genome. The chloroplast genome of *P. devoniana* was cut at 155 restriction sites, 25 of which were polymorphic. Of these polymorphic restriction sites, 13 are unique to *P. devoniana* compared to *P. hartwegii*, while 12 of the polymorphic restriction sites found in *P. devoniana* are also found in *P. hartwegii*. *Pinus devoniana* consistently differs from *P. hartwegii* at a minimum of five specific restriction site gains, one *Sma*I site and four *Xho*I sites.

Table 2. Key to restriction site haplotypes of *Pinus devoniana*. Haplotypes are listed by locality. The designations Jalisco #1, Jalisco #3, etc. without specific individual numbers following them indicates that this haplotype class includes all individuals collected at this locality other than those given their own haplotype number.

Western Mexico Collections	
Haplotype 52.	Jalisco #1 Jalisco #3 Nayarit
Haplotype 53.	Jalisco #1 1371
Haplotype 54.	Jalisco #1 1381
Haplotype 55.	Jalisco #1 1383
Haplotype 56.	Jalisco #2
Haplotype 57.	Jalisco #3 1421 Nayarit 1446, 1458
Haplotype 58.	Jalisco #3 1428 Nayarit 1452
Eastern Mexico Collections	
Haplotype 59.	Chiapas Oaxaca #1 Oaxaca #2
Haplotype 60.	Chiapas 1619–20
Haplotype 61.	Chiapas 1628
Haplotype 62.	Oaxaca #1 1661
Haplotype 63.	Oaxaca #2 1686

These apparently species-specific restriction sites are detected by the 23 kb petunia probe in the case of the *Sma*I site, and by the 21 kb, 11.7 kb, 9 kb, and 1 kb petunia probes in the case of the *Xho*I restriction sites. When compared with the *P. hartwegii* and *P. montezumae* chloroplast genomes, the *P. devoniana* chloroplast genome has three species-specific deletions totaling 6.9 kb and has four species specific insertions totaling 6.7 kb. The 25 chloroplast restriction site polymorphisms found in the 175 *P. devoniana* individuals included in this study resulted in 12 distinct haplotypes listed by locality in Table 2.

The polymorphic restriction sites are segregated geographically. The western set of populations had 15 polymorphic restriction sites, while the eastern Mexico localities had ten. Eight restriction sites appear to define the regional identity of individuals, that is, eastern and western populations of *P. devoniana* consistently differ at all eight restriction sites. With regard to their chloroplast haplotypes, individuals of *P. devoniana* tend to differ from each other at a number of restriction sites. This distribution of restriction site changes in *P. devoniana* differs from that found in *P. hartwegii* and *P. montezumae*, where 38 polymorphic restriction sites resulted in 51 different chloroplast haplotypes (Matos 1992).

Haplotype frequencies are given in Table 3 by collection site. Haplotype frequencies are also given in Fig. 3, the phenogram shows the set of three or four collection sites in eastern or western Mexico, respectively. Haplotypes [52] and [57] are noted with asterisks in Table 3, and Fig. 3 and 4. These two haplotypes were indistinguishable on the basis of their restriction site profiles due to missing data, however, if length variation is taken into account, haplotypes [52] and [57] are resolved as distinct haplotypes. The combined haplotype frequency haplotypes [52] and [57] are given at the bottom of the Table 3. Remarkably few haplotypes account for almost all individuals in both eastern and western Mexico. Haplotype [59] occurred in 70 of the 75 individuals collected in the eastern states of Oaxaca and Chiapas. Three of the four remaining haplotypes occurred in only one individual each; haplotype [60] was found in two individuals at the Chiapas collection locality. Two haplotypes, haplotypes [52] and [56], account for 92 of the 100 individuals collected in western Mexico. Haplotype [56], differs from haplotype [52] by one *Dra*I site difference and

Table 3. Frequency of *Pinus devoniana* restriction site haplotypes by collection locality.

Haplotype	Western Mexico				Eastern Mexico		
	Jalisco #1	Jalisco #2	Jalisco #3	Nayarit	Chiapas	Oaxaca #1	Oaxaca #2
52.*	0.88	0	0.92	0.88	0	0	0
53.	0.04	0	0	0	0	0	0
54.	0.04	0	0	0	0	0	0
55.	0.04	0	0	0	0	0	0
56.	0	1.00	0	0	0	0	0
57.*	0	0	0.04	0.08	0	0	0
58.	0	0	0.04	0.04	0	0	0
59.	0	0	0	0	0.88	0.96	0.96
60.	0	0	0	0	0.08	0	0
61.	0	0	0	0	0.04	0	0
62.	0	0	0	0	0	0.04	0
63.	0	0	0	0	0	0	0.04
*52 + 57	0.88	0	0.96	0.96	0	0	0
n=	25	25	25	25	25	25	25

* Due to missing data, these haplotypes could not distinguished on the basis of restriction sites; they are different from one another if insertions-deletions are considered.

Table 4. Genetic variation among geographically isolated populations of *Pinus devoniana*.

Collection locality	<i>n</i>	<i>k</i>	$\hat{\theta}$	Vâr $\hat{\theta}$
Jalisco and Nayarit	100	15	0.0015177	1.081774×10^{-4}
Oaxaca and Chiapas	75	8	0.0009962	1.003750×10^{-4}

n, the number of individuals in the population; *k*, the number of polymorphic restriction sites per sample; $\hat{\theta}$, Ewens et al. (1981) measure of nucleotide diversity using restriction site data; Vâr $\hat{\theta}$, Hudson's (1989) estimate of the variance of $\hat{\theta}$.

was the only haplotype found in the 25 individuals collected at Jalisco #2. The remaining five haplotypes found in western Mexico are represented by single individuals.

Nucleotide Diversity

Levels of nucleotide diversity, $\hat{\theta}$, for *P. devoniana* are low within regions of Mexico, especially in eastern Mexico where $\hat{\theta}$ is only a little more than half the value for western populations (Table 4). The small values of $\hat{\theta}$ in the western populations of *P. devoniana* are equal to the lowest values of $\hat{\theta}$ found in the closely related *P. hartwegii* and *P. montezumae*. The eastern Mexico population values of $\hat{\theta}$ are much lower than those found in other populations of *P. montezumae* complex pines for which $\hat{\theta}$ has been estimated (Matos 1992).

Measures of Population Subdivision and Migration

F_{st} , Wright's (1969) popular measure of population subdivision is the ratio of between-population variance to the total variance expected at complete fixation. F_{st} can vary from a value of 1, if populations are fixed for different alleles, to zero, when populations have identical allele frequencies. Restriction site haplotype data can be considered to be one, very well sampled, allele. F_{st} for the combination of all populations of *P. devoniana* is: $F_{st} = 0.792$. Given this F_{st} , N_m , the estimated number of migrants per generation, is calculated to be 0.263, well below the threshold level of 1, indicating that migration is nonexistent or minimal.

Using the procedure set out in Slatkin and Maddison (1989), N_m values for the four populations in western Mexico and the three populations in eastern Mexico were estimated. In order to calculate N_m by their method, cladograms were estimated using parsimony and N_m was calculated following their protocol. Figure 2 gives *s*, the minimum number of migration events to account for the distribution of haplotypes, for each set of populations. The minimum number of migration events are shown in Fig. 2 at the nodes and tips of the cladogram where haplotypes are found at more than one locality. *n*, the number of alleles, or in this case haplotypes, from each population in either

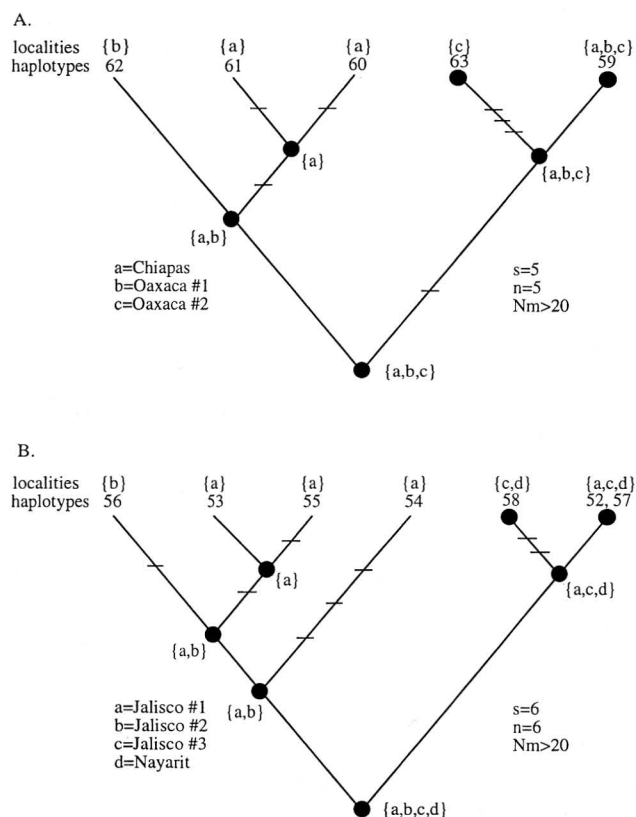


Fig. 2. Cladograms for *Pinus devoniana* populations from: A. eastern Mexico, and B. western Mexico. The numbers at the tips are the restriction site haplotype numbers and the letters are the collection sites. Bars across the lines of descent indicate mutations.

eastern (Fig. 2A) or western (Fig. 2B) Mexico is counted from the top of the cladograms. N_m was estimated from table 1 in Slatkin and Maddison (1989). In both cases, $N_m > 20$, indicating either that there is substantial amounts of gene flow between sets of populations, or that they have not been isolated from one another long enough to differentiate much with regard to chloroplast haplotypes.

Graphical Displays of Haplotype Relationships

Restriction site gains versus restriction site losses were weighted 1:1, 1.3:1, and 2:1. For restriction site gain weights above 1:1, the resultant cladograms were topographically identical for from 10 or 100 stepwise additions of the data. Weighting gains:losses 1:1 changed the topography slightly for western Mexico haplotypes. The 1:1 weighting scheme resulted in 26 minimum length trees. It is generally agreed that weighting restriction site gains equal to losses is less realistic than giving higher weight for restriction site gains.

The following results are for the 1.3:1 weighting scheme, although the reasoning would be the same for any of the above. One shortest cladogram was produced, the next shortest trees were 3-steps longer and

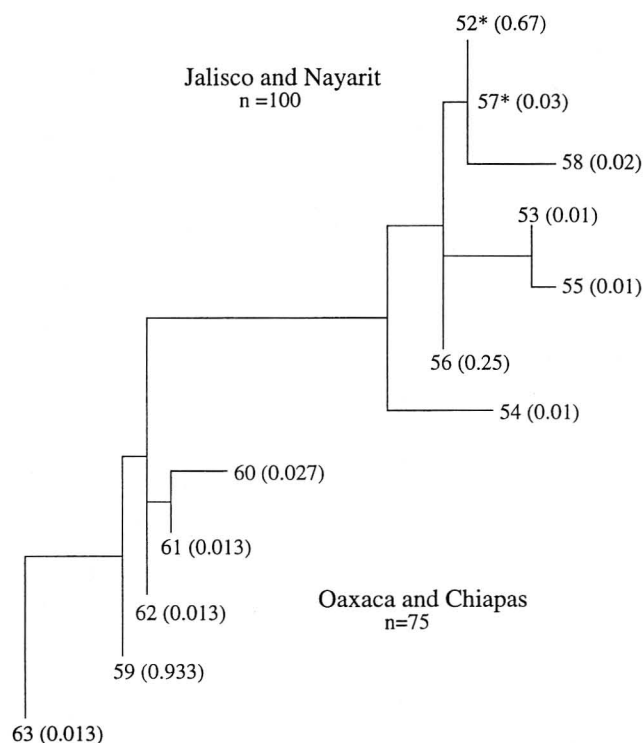


Fig. 3. Unrooted maximum parsimony phylogram of *P. devoniana* restriction site haplotypes and the total frequency of each haplotype in western Mexico (Jalisco and Nayarit) or eastern Mexico (Oaxaca and Chiapas). One shortest tree of 340 steps, was generated from 10 or 100 replications of random stepwise addition of the data. Restriction site gains: losses are weighted 1.3:1. Essentially identical trees were generated by a weighting scheme of 2:1 and 1:1. See Table 2 for a key to collection sites and haplotypes.

*Haplotypes [52] and [57] are equivalent with regard to restriction sites, they differ from one another if length variation is included.

there were eight of these. The 3-step longer trees differed only slightly, if at all, in topology from the shortest tree. Figure 3, the phylogram of the shortest tree from the 1.3:1 weighting scheme, shows the relationships of chloroplast haplotypes inferred from the restriction site data. The phylogram is shown here rather than the cladogram to convey the somewhat more information shown in branch lengths that are proportional to the number of inferred changes (Swofford 1991). Figure 3 shows the pooled haplotype frequencies for each region, either east (Oaxaca and Chiapas) or west (Jalisco and Nayarit). As discussed above, in the eastern Mexico populations, one haplotype dominates; in the western Mexico populations, one haplotype is at extremely high frequencies at three localities (haplotype [52]) while a fourth locality is fixed for a haplotype ([56]) that differs from the other by a single *DraI* restriction site change (Fig. 4).

Figure 4 shows the stepwise cladogram that most parsimoniously connects *P. devoniana* chloroplast haplotypes. Figure 4 gives specific restriction site changes at each step, each "+" or "-" in this cladogram represents a unique event. For example, between

the eastern and western Mexico sets of haplotypes there is a step designated as *EcoRV*. Each of the four "+" or "-" next to the "EcoRV" represents one restriction site change, they are grouped simply to conserve space. Within the Jalisco and Nayarit set of haplotypes there is a group of four haplotypes, haplotypes [52], [53], [55], and [56] that form an ambiguous box of haplotypes. These haplotypes could not be more clearly resolved using these restriction site data.

Table 5 gives the estimation of the probability of parsimony for 1-step, 2-step, etc. connections between haplotypes shown in Fig. 4 using the equations of Templeton et al. (1993). Haplotype [54] from western Mexico and haplotype [63] from eastern Mexico are distinct from the remainder of their clades. Nonetheless, these two haplotypes can be unambiguously assigned to their respective clades.

DISCUSSION

The *Pinus devoniana* chloroplast genome is remarkably conservative with regard to mutations when compared with two of its closest relatives in the *P. montezumae* complex, *P. hartwegii* and *P. montezumae*. The *P. devoniana* chloroplast genome is also remarkably conservative if compared to angiosperms that have displayed intraspecific chloroplast variability (reviewed in Harris and Ingram 1991). However, much of the angiosperm variability noted in Harris and Ingram (1991) is length polymorphism, and thus is a less conservative character than restriction site polymorphisms.

The two geographic regions of Mexico where large numbers of samples of *P. devoniana* were collected have little, if any, chloroplast gene flow between them. Chloroplast haplotypes are completely differentiated by geographic region. This is not surprising since these sets of collections are at least 700 km apart with few populations of *P. devoniana* between these two edges of its range (Critchfield and Little 1966). Geographic chloroplast differentiation is also not surprising in light of data showing that *P. hartwegii* and *P. montezumae* chloroplast haplotypes are not shared between populations only 125 km apart (Matos 1992). Isolated populations of pines in the *P. montezumae* complex seem to differentiate with regard to their chloroplast haplotypes relatively easily, although no time frame has been proposed for these isolation events.

Low levels of $\hat{\theta}$ (Table 4) are consistent with the slow rate of change and unrecombining nature of the chloroplast genome. Indeed, taking into account the above mentioned distribution within individuals of the restriction site changes, that is that *P. devoniana* individuals tend to have a relatively large number of restriction site polymorphisms that define relatively few haplotypes, the value of $\hat{\theta}$ may be unnaturally high

even at these low levels. It would seem that *P. devoniana* is a paradigm for the much touted conservative nature and lack on intraspecific variability of the chloroplast genome (Banks and Birky 1985; Hantula et al. 1989). Just a few individuals from eastern and western Mexico would have shown almost as much haplotype diversity, that is, very little, as these large samples. Haplotypes [52] and [57] (indistinguishable at the restriction site level) account for 70% of the 100 individuals sampled in the west, while haplotype [59] accounts for 93.3% of all 75 individuals sampled from eastern Mexico.

These data lend support from a natural population for the idea set out in equation 5 of Crandall and Templeton (1993)

$$E(\text{no. mutations} | n \text{ defining events}) = \sum_{\lambda=1}^n \frac{\theta}{\lambda + \theta - 1} \\ = \theta \sum_{j=0}^{n-1} \frac{1}{\theta + j},$$

where a defining event is either a coalescence or a mutation, λ is the number of lineages at a given time, and j is the j th coalescent event. This equation means that for very low values of θ , increasing the number of sampled individuals (n) has little effect on the number of haplotypes found in the sample. Unfortunately, this information is rarely apparent as sampling begins.

Populations of *P. devoniana* within geographic regions, that is, eastern or western Mexico, are for the most part quite similar with regard to their chloroplast haplotypes and are contained in distinct clades (Fig. 3 and 4). There is little differentiation between populations from collection localities in eastern or localities in western Mexico, and calculated migration events, N_m , is high (Fig. 2). These high values of N_m could reflect: 1) that there is current migration of chloroplast haplotypes between localities, or 2) that the chloroplast genome in this species is very stable and remains unchanged or little changed for long periods of time, or 3) that the separate localities have only recently been separated from one another with regard to chloroplast migration.

Although the calculated N_m is high ($N_m > 20$), indicating much migration of the chloroplast genome per generation, the first alternative does not seem to mesh well with one observation from western Mexico. The collection site designated Jalisco #2 does not support the high calculated estimate of N_m . Jalisco #2 is fixed for haplotype [56], but Jalisco #2 is intermediate in distance and in a straight line between all three remaining collection localities in western Mexico (Fig. 1). Haplotype [56] was not found at any of the other three western Mexico localities. If viable pollen is transferred from Jalisco #1 to, for example, the Nayarit collection site which is nearly 250 km to the north

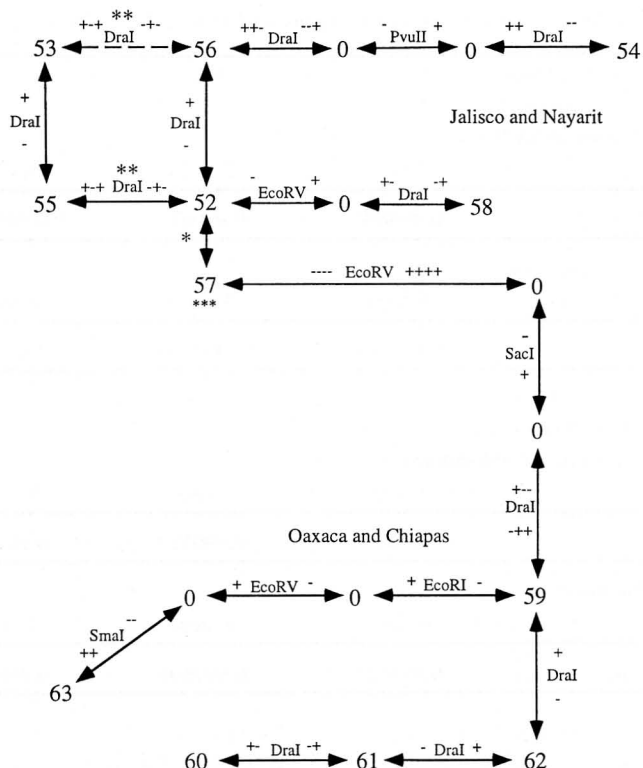


Fig. 4. *Pinus devoniana*, restriction site changes between haplotypes. Specific restriction site gain/loss identified with each haplotype are identified, for example, haplotype [59] has a *DraI* site that has been lost in haplotype [62], this is the only difference between these two haplotypes identified in this study.

*Haplotypes [52] and [57] are equivalent with regard to restriction sites; they are different from each other if length variation is included in the analysis.

**The three *DraI* gains/losses that differentiate haplotype [53] from [56] are the same three *DraI* gains/losses that differentiate haplotype [55] from [52].

***Haplotype [57] shares its unique length variation (that identifies it as different from haplotype [52]) with all eastern haplotypes except haplotype [63].

northwest, or the more proximal Jalisco #3 collection site, it must by-pass the Jalisco #2 collection site, only 75 km away. This seems improbable. A more likely alternative is that the high values of N_m are a reflection of the stability of the chloroplast genome and that similarities between far-flung populations do not necessarily reflect current gene flow. Instead, the similarities more probably reflect a common history and subsequent differentiation within each population. The other alternative is, of course, inadequate sampling. However, sample sizes were moderately high ($n = 25$ per locality, $S_n = 100$ in western Mexico) especially after considering the low value of θ (Table 4).

Donnelly and Tavaré (1986) developed a coalescent model incorporating the age structure of alleles. One outcome of their work was that the expected rank of alleles by age is equal to the rank by their frequency, or

$$E(\text{rank in age of allele } i) = C(n, i/n),$$

Table 5. Probability of Parsimony and Probability of Parsimony + 1.

a. Jalisco and Nayarit. $n = 100$ $H = 0.035485$						
$\hat{\theta} = 0.0015177$						
Probability of Parsimony:						
	1-step	2-steps	3-steps	4-steps	5-steps	6-steps
P =	0.996806	0.990414	0.980863	0.968268	0.95284	0.9349
Parsimony + 1:						
	6-steps	7-steps	8-steps	9-steps	10-steps	11-steps
P =	0.998267	0.996906	0.994958	0.992346	0.98902	0.984954
b. Oaxaca and Chiapas. $n = 75$ $H = 0.0221784$						
$\hat{\theta} = 0.000996199$						
Probability of Parsimony:						
	1-step	2-steps	3-steps	4-steps	5-steps	6-steps
P =	0.996826	0.990573	0.981517	0.970099	0.956835	0.942211
Parsimony + 1:						
	6-steps	7-steps	8-steps	9-steps	10-steps	11-steps
P =	0.998625	0.997691	0.996447	0.994876	0.992972	0.990734

where n_i is the number of individuals of allele (i) in the sample of n individuals, and C is a normalizing constant depending on the number of alleles. Additionally, Golding (1987) observed that older alleles have more mutational connections than younger alleles; Crandall and Templeton (1993) have lent support to these ideas using empirical data from *Drosophila* and added that mutational connections increase with increasing frequency of haplotypes. Another prediction from coalescent theory supported by Crandall and Templeton (1993) is that the number of mutational connections to a haplotype is proportional to the frequency of the haplotype in the population. Lastly, Takahata (1988) predicted that for partially isolated populations the probability that allele i is the oldest is proportional to the number of subpopulations, s , in which it is found, or:

$$P(\text{allele } i \text{ is the oldest}) = Cs_i.$$

The above predictions seem to be well supported by this *Pinus devoniana* chloroplast haplotype data set. In the west, haplotypes [52] and [57] have the highest frequency. Haplotypes [53] and [55] can be connected by identical restriction site mutations to both haplotype [56] and to haplotypes [52] and [57] (Fig. 4). Since haplotypes [52] and [57] are the highest frequency haplotypes in the west and since haplotypes [53] and [55] are found at collection localities with high frequencies of haplotypes [52] and [57] (Table 3), the seemingly equally parsimonious connections are really unequal when the above predictions of the coalescent are taken into account. Thus, the less probable connection is shown with a dashed arrow in Fig. 4.

In light of the work of Donnelly and Tavaré (1986), Takahata (1988), and Templeton et al. (1993), the ancestral chloroplast was more like haplotypes [52] and [57] from western Mexico and haplotype [59] from the eastern Mexico than any of the less frequent haplotypes resolved by these data. Additionally, Fig. 4 supports the propositions that: 1) the high frequency/older haplotypes will have more mutational connections, that is more low frequency/derived haplotypes associated with them, and 2) singletons, haplotypes represented by a single individual, will tend to be at the ends of mutational branches (Golding 1987; Excoffier and Langaney 1989; Crandall and Templeton 1993).

These data strongly suggest that there is no gene flow between eastern and western Mexico populations of *Pinus devoniana* and that this has been the case for an extended period of time. These data also suggest that the sampled localities in eastern and western Mexico are genetically isolated from one another and are; 1) maintaining at low frequencies chloroplast haplotypes that occurred at low frequencies in the founding populations, or 2) slowly producing unique chloroplast haplotypes that are not shared with other populations because of restricted or non-existent gene flow between them.

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