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Random amplified polymorphic DNA and amplified fragment length polymorphism assessment of genetic variation in Nicaraguan populations of *Pinus oocarpa*

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Abstract

***Pinus oocarpa* is the most widely distributed pine species of Mexico and Central America. The natural populations of Nicaragua have been affected by extensive human activities. As a consequence, their size has been reduced, and there is a serious threat to the development of mature woodland. Knowledge of population structures and the genetic diversity of the species is required for the design of sustainable use and conservation strategies. Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers were used to assess the genetic variation among 10 populations from three geographical regions of Nicaragua. Both markers revealed high levels of diversity in these populations. G_{ST} values and analyses of molecular variance (AMOVA) found that most variation was within populations but there is still a significant differentiation between populations indicating that the populations sampled cannot be considered a single panmictic unit. The partitions created by AMOVA also showed that there was little differentiation between populations of different regions, although cluster analyses based on RAPDs and AFLPs indicated a closer relationship among most of the populations from a same geographical region. Management of *P. oocarpa* in Nicaragua should be aimed to maintain the high degree of genetic variation within individual populations that is still observed even in some of these highly degraded populations.**

Keywords: AFLPs, conservation, genetic diversity, *Pinus oocarpa*, population structure, RAPDs

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Introduction

Pinus oocarpa Schiede ex Sclechtendal ssp. *oocarpa* is the most widely distributed pine species of Mexico and Central America. Nicaragua lies at its southern limit (Barnes & Styles 1983), and in this country it is the most common pine, making up some 90% of the pine-forested area (Greaves 1979). It occurs as discrete populations located in mountain areas at altitudes of 600–1300 m in the Northern, Western and Central regions. These populations are suffering degradation as a consequence of the overexploitation of timber and through forest fires. Further, the frequency of deliberate fires has greatly increased as people have attempted to create more arable and grazing land. These phenomena have altered the size and genetic structure of the natural populations of the species, and pose a serious threat to the development of mature woodland (Farjon & Styles 1997).

Knowledge of the structure and pattern of genetic variation in this species is important to the development of appropriate strategies for the *in situ* conservation of natural woods and the regeneration of partially logged forests. This information can also serve as a baseline for determining whether genetic diversity is lost through sampling or conservation involving *ex situ* propagation — something thought possible in other species (Hamrick & Godt 1996). Studies on the genetic variability of *P. oocarpa* have been limited to a few inherited, quantitative traits. Considerable variation has been reported in trials with pines from different parts of Central America, in which open-pollinated families were planted in a range of tropical and subtropical

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environments (Moura *et al*. 1998). These trials were performed by the Central America and Mexico Coniferous Resources Cooperative (CAMCORE) international testing program (Dvorak & Donahue 1992), and have confirmed the economic value of *P. oocarpa* as a forest plantation species, its excellent wood quality (a great advantage over other tropical pines), and the ease with which it can be vegetatively propagated (Moura *et al*. 1998).

Biochemical and molecular markers have proven powerful tools in the assessment of genetic variation, both within and between plant populations. Allozymes have been widely used for quantifying population structure in pines (reviewed by Hamrick & Godt 1989, 1996; Parker & Hamrick 1996). However, their use can be limited either by the number and kind of detectable loci, or the lack of variable loci. Several studies have also questioned the neutrality of isozyme loci, suggesting that both balancing (Karl & Avise 1992) and diversifying (Le Corre *et al*. 1997) selection may operate on some enzymes.

Polymerase chain reaction (PCR)-based fingerprinting techniques provide a more representative sample of the genome than do allozymes, plus a virtually unlimited number of anonymous markers. Moreover, many of the polymorphism revealed by these techniques is likely neutral, although it has been suggested that a proportion of variation at molecular marker loci could be adaptive rather than neutral (Pérez de la Vega 1996). Of the available techniques, random amplified polymorphic DNA (RAPD) (Williams *et al*. 1990) has been widely used in population genetic studies of a large number of plant species, including pines (Szmidt *et al*. 1996; Bucci *et al*. 1997; Thomas *et al*. 1999; Wu *et al*. 1999). One of the major drawbacks of RAPDs is their sensitivity to reaction conditions, which require careful optimization (Weising *et al*. 1995). The use of the amplified fragment length polymorphism (AFLP) technique (Vos *et al*. 1995) circumvents some of the limitations of earlier fingerprinting methods. It is specific, generates a high multiplex ratio, is highly reproducible (Powell *et al*. 1996) and has been used successfully in plant population genetic studies (Muluvi *et al*. 1999; Palacios *et al*. 1999; Russell *et al*. 1999; Schmidt & Jenssen 2000). For both techniques it has been well established that most of comigrating fragments among individuals of the same species are homologous (Waugh *et al*. 1997; Hurme & Savolainen 1999; Wu *et al*. 1999). Nevertheless, both RAPD and AFLP share a potential limitation: their dominant nature precludes direct estimations of allele frequency and can bias calculations of genetic diversity and population differentiation (Lynch & Milligan 1994; Isabel *et al*. 1995, 1999). However, the use of appropriate statistical methods has allowed improved evaluation of intraspecific diversity. The analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992), which is not influenced by the dominance problem, has become an important tool for investigating the partitioning of dominant marker variation (Huff *et al*. 1993; Steward & Excoffier 1996; Bartish *et al*. 1999; Bussell 1999; references therein). AMOVA has been shown to give the most accurate estimate of population differentiation when conifer genotypic and phenotypic RAPD data are compared (Isabel *et al*. 1995, 1999). Diversity estimates can also be obtained based on phenotype frequency using Nei's unbiased statistics (Nei 1978). Although less informative than the corresponding gene diversity parameters, a Hardy–Weinberg equilibrium should not be assumed since unpredictable biases depending on the null-phenotype frequency might be introduced.

In this study, RAPDs and AFLPs were used to assess the genetic diversity and population structure of natural populations of *P. oocarpa* ssp. *oocarpa* from Nicaragua. This contributes towards the definition of genetically distinct units needed for conservation purposes. A second objective was to compare RAPD and AFLP markers with respect to the estimates of genetic variation they provide.

Materials and methods

Plant material

Seed samples were collected from 10 natural populations of *Pinus oocarpa* growing in the three regions of the species' natural range in Nicaragua (Fig. 1, Table 1). Nine populations (all except that of Rodeo Grande) were sampled by taking seeds from mature trees separated by a distance of 100 m. The number of trees sampled from each population varied from 13 to 23 (Table 1). The population from Rodeo Grande was analysed from a bulked seed collection (accession number 01899/92) stored at the Danida Forest Seed Centre (Denmark). One seed from each sampled tree, and 19 seeds from the bulked seedlot, were germinated, grown for two months, and the needles collected for DNA extraction.

DNA extraction

Total genomic DNA was extracted following the procedure described by Scott & Playford (1996) with minor modifications. Briefly, DNA was prepared by grinding tissue in 4 mL of extraction buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, 0·35 M sorbitol, 0.1% BSA and 10% polyethylenglycol) at room temperature. The homogenate was filtered, centrifuged and incubated for 15 min at room temperature in 400 µL of wash buffer (50 mM Tris-HCl, pH 8.0, 25 mM EDTA and 0.35 M sorbitol) and 100 µL of 10% sarcosyl. One milliliter of prewarmed CTAB buffer (0.5 M CTAB, 1 M Tris-HCl, pH 8.0, 0.5 M EDTA, 5 M NaCl) was then added and the mixture incubated for 30 min at 55 °C followed by centrifugation. The supernatant was transferred to a new tube, extracted with

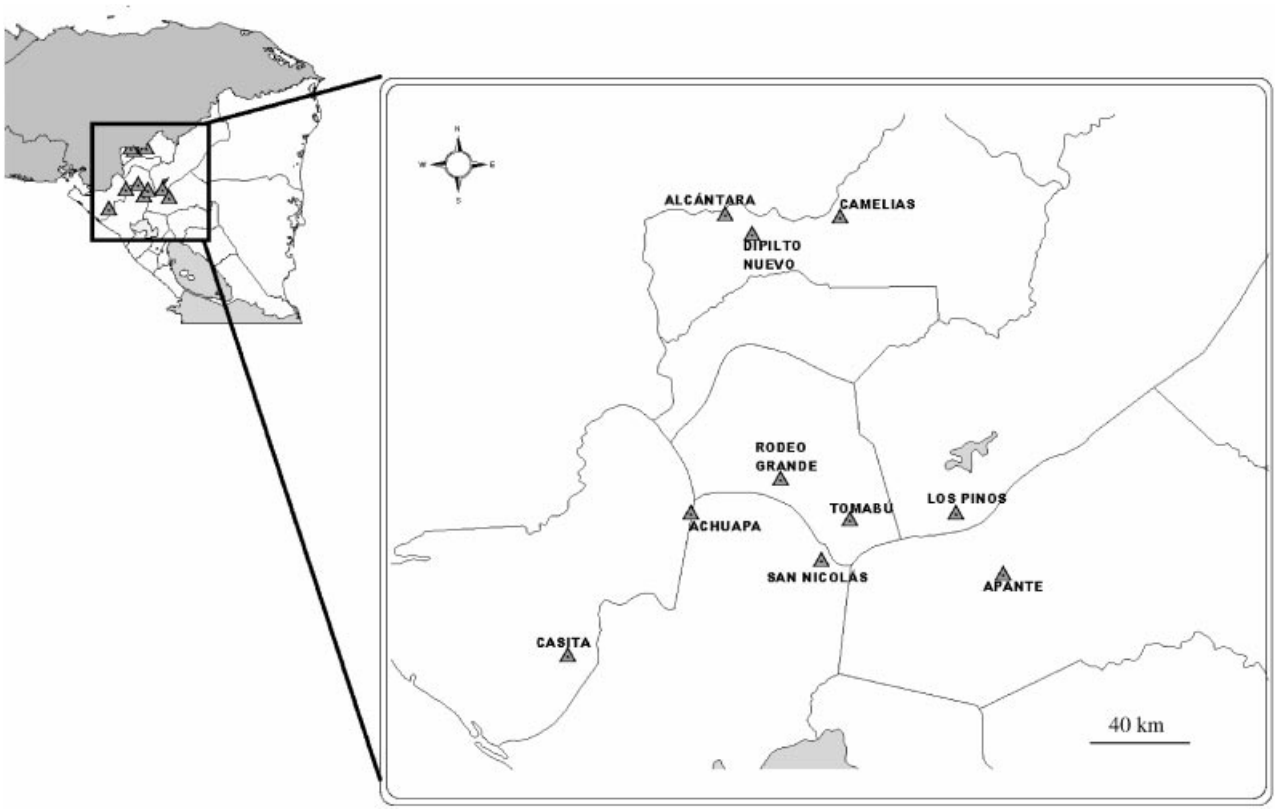

Fig. 1 Location of the 10 *Pinus oocarpa* populations sampled from the North, West and Centre of Nicaragua.

Table 1 Sampled populations of *Pinus oocarpa*, number of plants sampled at each site, region and exact location

Population	No. of plants	Region	Latitude (°N)	Longitude (°E)	Altitude (m)
Achuapa (ACH)	18	Central	86°39′	13°02′	600
Alcántara (ALC)	22	Northern	86°32′	13°44′	1180
Apante (APA)	17	Central	85°53′	12°53′	900
Camelias (CAM)	13	Northern	86°17′	13°46′	970
Casita (CAS	20	Western	86°57′	12°41′	1140
Dipilto Nuevo (DPN)	23	Northern	86°30′	13°43′	1100
Pinos (PIN)	22	Central	86°00′	13°02′	1380
Rodeo Grande (RGR)	18	Central	86°26′	13°07′	1200
San Nicolás (SNI)	22	Central	86°20′	12°55′	1250
Tomabú (TOM)	20	Central	86°16′	13°01′	820

chloroform:isoamylalcohol (24:1) and again centrifuged. The supernatant from this procedure was incubated for 15 min at 37 °C with RNase (10 mg/mL), and the DNA ethanol-precipitated and resuspended in TE.

RAPD procedure

RAPD reactions were carried out essentially as described by Williams *et al*. (1990) in 25 μL total volumes containing 25 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of primer, 100 μM dNTPs and 1 U of *Taq* polymerase (Sigma). Amplification was initiated by denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 36 °C for 3 min and 72 °C for 2 min, and a final extension step of 5 min at 72 °C. Amplification products were separated by electrophoresis in 1.5% agarose gels. Gels were stained with 0.5 mg/mL ethidium bromide for 30 min and then photographed with a digital camera under UV light.

A total of 90 primers from the University of British Columbia (UBC, Vancouver, Canada) and Operon Technologies, Inc. (USA) were screened using three individuals randomly chosen from the 195 surveyed. Twelve primers that gave high intensity, easily scorable and reproducible

bands were selected. These were four UBC primers (BC-223, BC-228, BC-411 and BC-610), and eight Operon primers (OPE-14, OPE-18, OPM-13, OPR-07, OPS-08, OPS-18, OPT-04 and OPT-12).

AFLP procedure

The AFLP procedure was that described by Vos *et al*. (1995) with minor modifications. Three hundred and fifty ng of DNA was restricted with either *Eco*RI and *Mse*I or *Pst*I and *Mse*I (3.5 U of each) for 3 h at 37 °C in 50 μL of the buffer described by the above authors. Adaptors were ligated to the fragments obtained by adding 10 μL of a ligation mixture containing 5 μM *Mse*I adaptor and 0.5 μM *Eco*RI or *Pst*I adaptor. Samples were incubated for 3 h at 37 °C. Preselective amplification was performed using primers with a single selective nucleotide (A). Sequences of adaptors and primers were those described by Vos *et al*. (1995) for the *Eco*RI and *Mse*I ends, and those described by Powell *et al*. (1996) for the *Pst*I end.

Selective amplification was performed using pairs of primers with three selective nucleotides. In order to choose the primer combinations revealing clear, reproducible polymorphisms, 30 *Eco*RI–*Mse*I and 10 *Pst*I–*Mse*I primer combinations were screened in six individuals from different populations. Four *Pst*I–*Mse*I primer combinations were selected for use with all individuals: P-ACC/M-ATC; P-ACC/M-ACC; P-ACC/M-AAC; and P-ACT/M-AGA. Either *Eco*RI or *Pst*I primers were end-labelled with [γ^{33}P]-ATP. The following PCR conditions were used: 13 cycles each of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 60 s. The annealing temperature was reduced by 0.7 °C in each of these cycles to reach a final temperature of 56 °C. In these conditions, the reaction was continued for 30 cycles, followed by a final extension step at 72 °C for 10 min Amplified DNA samples were mixed with 95% formamide loading buffer, heated at 94 °C for 5 min, and quickly cooled on ice. An amount of 3.5 μL of each sample was loaded on a 5% polyacrylamide gel containing 7.5 M urea in TBE, and run at 110 W for 2 h. After electrophoresis, gels were dried for 2 h and exposed to an autoradiographic film for 2 days.

Data analysis

RAPD and AFLP bands were scored visually as either present or absent in each DNA sample. Analysis was restricted to polymorphic bands, i.e. reliable scored bands with a frequency of less than 95% for the most common phenotype. Each data file was investigated for nonrandom associations between individual pairs of bands using the correlation test of the SIMINT program (NTSYS software) (Rohlf 1993).

Based on phenotype frequency, diversity values for each marker were calculated for each population using Nei's unbiased distances (Nei 1978):

$$h_i = \frac{n\,(1 - \Sigma_i p_i^2)}{n - 1}$$

where p_i is the frequency of the presence or absence of a band in the population, and n the number of individuals analysed. Diversity values for each primer or primer combination were calculated as:

$$H_j = \frac{\Sigma h_i}{r}$$

where r is the number of markers revealed by each primer or primer combination. Diversity values for each single population (H) were calculated as the mean h_i value over all markers. Similarly, diversity values were also calculated for the whole sample (H_T), taking into account the total number of individuals analysed.

Total phenotypic diversity was partitioned into intra- and interpopulational components. Intrapopulational diversity was calculated for each primer or primer combination as the weighted (by sample number) average of the H_j values. Intrapopulational diversity was also calculated on a per marker basis from the H-values. The component of diversity within populations is H_{POP}/H_T and the component between populations (G_{ST}) is $(H_T - H_{POP})/H_T$ (Nei 1977). The overall G_{ST} value was calculated from the average per marker values.

AMOVA (Excoffier *et al*. 1992) was used to estimate variance components for both RAPD and AFLP phenotypes, partitioning the variation into intrapopulational, interpopulational, intraregional and interregional values. Two matrices containing Euclidean distances between all pairs of phenotypes were used as input distance matrices (Huff *et al*. 1993). Variance components were tested statistically by nonparametric permutational procedures using 1000 permutations. Two of the three geographical regions sampled were represented by more than one population. AMOVAS were also performed for populations within each of these two regions (Northern and Central). Genetic differentiation (Φ_{ST}) between pairs of the *P. oocarpa* populations, and levels of significance, were also calculated. All analyses were performed using ARLEQUIN 1.1 software (Schneider *et al*. 1997).

Mean genetic differentiation values (Φ_{ST}) obtained with the two marker types were compared using the χ^2 test, as proposed by Allendorf & Seeb (2000) for F_{ST}.

The Mantel test was used to estimate the possible association between different distance matrices: geographical distances, Φ_{ST} based on RAPD, and Φ_{ST} based on AFLP, using the MXCOMP program (NTSYS software). Significance was determined using 1000 permutations.

Each pairwise matrix was used to construct a dendrogram by the neighbour-joining method (Saitou & Nei 1987) using the PHYLIP package (Felsenstein 1993). Cophenetic value matrices were produced from the tree matrices using

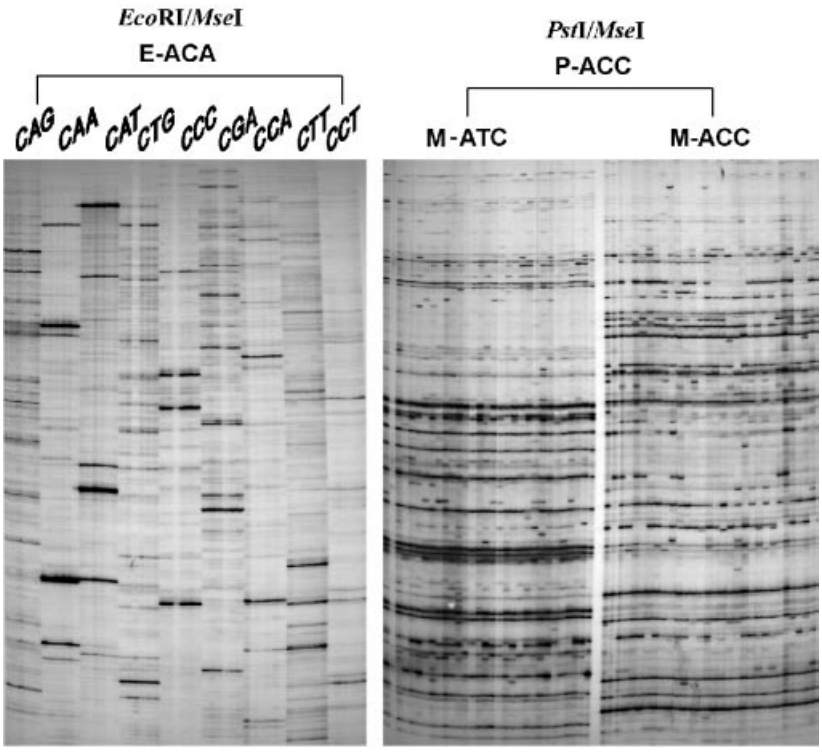

Fig. 2 AFLPs generated from genomic DNA of six *Pinus oocarpa* individuals using different *Eco*RI–*Mse*I primer combinations (left), and of individuals from Dipilto Nuevo and Casita populations using two *Pst*I–*Mse*I primer combinations [P-ACC/M-ATC and P-ACC/M-ACC, respectively (right)].

the COPH program (NTSYS software). They were then employed to check the goodness of fit of cluster analyses by comparing them to the Φ_{ST} matrices using a Mantel test.

Results

The RAPD profile

Twelve of the 90 primers tested showed banding patterns with very high reproducibility and clear band resolution. These 12 primers produced a total of 131 distinct bands ranging from 300 to 2000 bp. The number of scored bands per primer ranged from six for OPE-18, to 16 for OPR-07, with a mean number of 10.9 per primer.

Seventy-two of the 131 bands were polymorphic across the whole sample. None showed correlation with any other. Therefore, each of the 72 polymorphic bands were considered independent characters. The percentage of polymorphic markers ranged from 93.33% for primer OPG-10, to 20% for primer BC-228. Populations were similarly polymorphic for the whole set of bands scored. Thus, the percentage of polymorphism ranged from 51.91% for Camelias and Dipilto Nuevo, to 48.85% for Apante and Pinos with a mean of 50.53%. No band was fixed exclusively in a single population, and most of the 72 polymorphic bands were also polymorphic in each single population. When only these 72 bands were analysed, the mean percentage of polymorphism per population was 82.78%.

AFLP profiles

All 30 of the AFLP primer combinations *Eco*RI–*Mse*I tested produced highly complex profiles of more than 150 bands per lane. Many bands were faint, and overlapping was frequent. The complexity of the profiles was clearly reduced when *Pst*I–*Mse*I primer combinations were used. All 10 combinations tested produced around 100 well separated, sharp bands per gel lane. Examples of the profiles obtained with both kinds of primer combination are shown in Fig. 2.

The four primer combinations selected produced a total of 392 bands. The number of scored bands ranged from 105 for P-ACT/M-AGA, to 90 for P-ACC/M-AAC, with a mean of 98. A total of 162 bands were polymorphic across the entire sample. Four bands amplified with P-ACC/M-AAC correlated completely. Three were therefore excluded from further analysis because they might have represented the same locus. The percentage of polymorphic markers ranged from 43.77% for P-ACC/M-AAC, to 32.25% for P-ACC/M-ACC.

Individual populations showed a similar level of polymorphism, ranging from 35.97% for Casita, to 39.54% for Tomabú, with an average of 37.86%. This value was lower than that found in the RAPD analysis. However, when taking into account the 159 polymorphic bands across the entire sample, the mean percentage of polymorphism per population was 93.33%. This indicates that most of the polymorphic bands studied were also polymorphic in each

Table 2 Estimates of diversity (H_j) within each of 10 populations of *Pinus oocarpa* based on 12 RAPD primers and mean diversity (H) across markers

Primer	ACH	ALC	APA	CAM	CAS	DPN	PIN	RGR	SNI	TOM
OPE-14	0.368	0.221	0.113	0.359	0.195	0.190	0.348	0.346	0.378	0.441
OPE-18	0.425	0.000	0.243	0.269	0.199	0.087	0.087	0.105	0.082	0.253
OPG-10	0.381	0.390	0.357	0.458	0.346	0.409	0.409	0.421	0.360	0.389
OPM-13	0.370	0.467	0.289	0.410	0.412	0.446	0.285	0.380	0.274	0.303
OPR-07	0.399	0.338	0.428	0.359	0.377	0.462	0.352	0.416	0.397	0.432
OPS-08	0.160	0.331	0.059	0.077	0.095	0.178	0.123	0.235	0.260	0.271
OPS-18	0.461	0.382	0.262	0.251	0.258	0.340	0.299	0.310	0.246	0.357
OPS-04	0.168	0.427	0.245	0.308	0.368	0.324	0.000	0.277	0.082	0.000
OPT-12	0.296	0.411	0.462	0.303	0.419	0.403	0.248	0.467	0.305	0.336
BC-223	0.425	0.334	0.340	0.333	0.381	0.313	0.299	0.334	0.425	0.397
BC-228	0.229	0.485	0.309	0.372	0.289	0.482	0.169	0.307	0.383	0.389
BC-411	0.462	0.366	0.450	0.442	0.396	0.393	0.397	0.377	0.408	0.394
All markers	0.381	0.363	0.350	0.370	0.351	0.374	0.317	0.367	0.343	0.362

Table 3 Estimates of diversity (H_j) within each of 10 populations of *Pinus oocarpa* based on four AFLP primer combinations and mean diversity (H) across markers

Primer combination	ACH	ALC	APA	CAM	CAS	DPN	PIN	RGR	SNI	TOM
P-ACC/M-ATC	0.347	0.371	0.274	0.346	0.298	0.381	0.320	0.370	0.330	0.375
P-ACC/M-ACC	0.293	0.321	0.343	0.318	0.334	0.316	0.305	0.310	0.325	0.341
P-ACC/M-AAC	0.284	0.347	0.349	0.328	0.282	0.301	0.318	0.282	0.323	0.344
P-ACT/M-AGA	0.356	0.410	0.396	0.380	0.371	0.376	0.337	0.380	0.364	0.417
All markers	0.323	0.367	0.342	0.346	0.322	0.347	0.323	0.338	0.338	0.373

population. As for RAPD profiles, no band was found to be unique to a single population.

Diversity analysis

Genetic diversity measurements (H_j) within populations were calculated for each primer or primer combination and mean values (H) were averaged across markers. Tables 2 and 3 show the results based on RAPD and AFLP data, respectively.

RAPD primers varied in their power to detect diversity within populations. Some primers, such as BC-411, OPR-07 or OPG-10, revealed high diversity levels in all populations, whereas others detected very variable diversity levels. For example, OPE-18 detected a level of 0.425 in Achuapa and 0.0 in Alcántara. Averaged over all markers, Pinos displayed the lowest level of intrapopulational diversity (H = 0.317), and Achuapa the highest (H = 0.381). The mean diversity values across the 10 populations was 0.358.

AFLP primer combinations, however, yielded more homogeneous diversity estimates. Small differences were found both among populations evaluated with a primer combination, and in individual populations evaluated with different primer combinations. With respect to the former, the greatest differences among diversities were found with P-ACC/M-ATC, ranging from H_j = 0.274 in Apante, to H_j = 0.381 in Dipilto Nuevo. With respect to the latter, the greatest differences were found in Apante, which showed an H_j of 0.395 with P-ACT/M-AGA, and an H_j of 0.274 with P-ACC/M-ATC. Averaged over all markers, Casita showed the lowest diversity value (H = 0.322) and Tomabú the highest (H = 0.373). The mean diversity values across the 10 populations was 0.342, very similar to the found with RAPDs.

Partition of the total diversity by population

Averaged values for intrapopulational diversity (H_{POP}), and diversity values in the whole sample (H_T), were used to calculate the level of diversity within and between populations. Results obtained for the RAPD and the AFLP analyses are shown in Tables 4 and 5, respectively.

Values for intrapopulational diversity (H_{POP}/H_T) were found to vary with the RAPD primer used. For example, OPT-04 indicated that 61.7% of total diversity was within populations, while OPE-18 estimated this to be 97.9%. However, all the primers detected more variability within, rather than between, populations. The intrapopulational diversity component, averaged over all markers, was

Table 4 Partitioning of diversity within and between populations of *Pinus oocarpa* for 12 RAPD primers

Primer	H_{POP}	H_T	H_{POP}/H_T	G_{ST} $(H_T - H_{POP}/H_T)$
OPE-14	0.294	0.336	0.875	0.125
OPE-18	0.144	0.147	0.979	0.020
OPG-10	0.390	0.423	0.922	0.078
OPM-13	0.363	0.404	0.899	0.101
OPR-07	0.397	0.424	0.936	0.064
OPS-08	0.186	0.277	0.671	0.329
OPS-18	0.319	0.405	0.788	0.212
OPT-04	0.216	0.350	0.617	0.383
OPT-12	0.370	0.420	0.881	0.119
BC-223	0.358	0.377	0.95	0.050
BC-228	0.345	0.369	0.935	0.065
BC-411	0.399	0.425	0.939	0.061
All markers	0.358	0.403	0.888	0.112

Table 5 Partitioning of diversity within and between populations of *Pinus oocarpa* for four AFLP primer combinations

Primer combination	H_{POP}	H_T	H_{POP}/H_T	G_{ST} $(H_T - H_{POP}/H_T)$
P-ACC/M-ATC	0.342	0.375	0.912	0.088
P-ACC/M-ACC	0.321	0.351	0.915	0.085
P-ACC/M-AAC	0.316	0.337	0.938	0.062
P-ACT/M-AGA	0.378	0.407	0.929	0.071
All markers	0.342	0.369	0.927	0.073

estimated to be 88.8%, while that between populations was 11.2%.

In the AFLP analysis, all four primer combinations produced similar estimates of intrapopulational diversity. The average level of diversity within populations was 92.7%, whereas that between populations was 7.3%.

AMOVA analysis

AMOVA estimates within populations, between populations, within regions and between regions (Table 6) showed that most of the total variation existed within populations. The RAPD analysis gave a value of 87.77%, and AFLP gave 91.37%. Both analyses provided evidence of significant genetic structuring. Also, a small but significant proportion of the total variation detected by RAPDs (3.6%) was attributed to differences between regions. AFLPs failed to detect this divergence (0.95%). When the studies were restricted to populations within a particular region, RAPD analysis showed the Northern populations to be as equally differentiated as the Central populations (8.30 vs. 9.20%), whereas with AFLPs, more variation was detected between Central populations (9.27%) than between Northern populations (3.43%).

Mean Φ_{ST} estimates obtained from RAPDs and AFLPs were not significantly different when either the whole set of populations was analysed ($\chi^2 = 12.574$; 9 d.f.; $P > 0.10$) or when only a group of populations was studied ($\chi^2 = 5030$; 5 d.f.; $P > 0.30$, for the Central populations, and $\chi^2 = 4.84$; 2 d.f.; $P > 0.05$, for the Northern populations).

For both RAPDs and AFLPs, all pairwise Φ_{ST} values between populations were significant. This indicates that individuals of each population were more similar to their co-members than to individuals of other populations. Broadly, pairwise Φ_{ST} values revealed a larger separation between populations from different regions than any pair of populations from the same region. The smallest values were found for populations from the same region, i.e. 0.029 between Achuapa and San Nicolás using RAPDs, and 0.031 between Camelias and Dipilto Nuevo using AFLPs. However, geographical distances were not correlated with differentiation values. The Mantel test gave values of $r = 0.239$ for the comparison between matrices of geographical distances and pairwise Φ_{ST} based on RAPDs, and $r = 0.048$ for the comparison between matrices of geographical distances and pairwise Φ_{ST} based on AFLPs. Neither were significant.

Neighbour-joining analysis was performed for each of the two Φ_{ST} data sets (Fig. 3). Although the Mantel test comparing the two matrices showed a poor but significant correlation ($r = 0.395$), both unrooted trees show a remarkably similar topology. There were two clusters, one containing all the Central region populations except for Rodeo Grande. This population, along with Casita and the three Northern populations, grouped into a second cluster. In the first, Achuapa and San Nicolás were closely related, whereas Apante, Pinos and Tomabú appeared in a second subgroup. Within the second cluster, Camelias, Dipilto Nuevo and Casita formed one subcluster, and Rodeo Grande and Alcántara another. Goodness-of-fit analyses suggest both trees to be moderately reliable. The Mantel test comparing Φ_{ST} values matrices and cophenetic matrices gave values of $r = 0.742$ for RAPDs and 0.533 for AFLPs.

Discussion

Both RAPD and AFLP analyses were very effective at detecting genetic variation in the *Pinus oocarpa* genome. None of the 195 individuals sampled were characterized by the same RAPD nor AFLP phenotype. While the majority of primers tested (78 out of 90) provided no reliable fragment patterns, all 10 AFLP primer combinations were useful once the enzyme combination *Pst*I–*Mse*I was selected. As previously reported for other species (Huff *et al*. 1993; Bartish *et al*. 1999), RAPD primers differed substantially in their ability to reveal diversity within *P. oocarpa* populations. This confirms the need for using a large number of RAPD primers in order to prevent

Table 6 Analysis of molecular variance (AMOVA) using either 72 RAPD markers or 159 AFLP markers, conducted between and within the three regions and within all populations, between and within populations from the Northern region and between and within populations from the Central region. The data show the percentage of total variance contributed by each component, the significance (*P*) of the variance components and Φ-statistics

	RAPD			AFLP		
Source of variation	% total variance	Φ-statistics	*P*	% total variance	Φ-statistics	*P*
Between regions	3.60	$\Phi_{CT} = 0.036$ $\Phi_{SC} = 0.086$ $\Phi_{ST} = 0.122$	< 0.01	0.95	$\Phi_{CT} = 0.0095$ $\Phi_{SC} = 0.076$ $\Phi_{ST} = 0.086$	> 0.05
Between populations within regions	8.63		< 0.001	7.58		< 0.001
Within populations	87.77		< 0.001	91.37		< 0.001
Between populations of the Northern region	8.30	$\Phi_{ST} = 0.083$	< 0.001	3.43	$\Phi_{ST} = 0.034$	< 0.001
Within populations of the Northern region	91.70		< 0.001	96.73		< 0.001
Between populations of the Central region	9.20	$\Phi_{ST} = 0.092$	< 0.001	9.27	$\Phi_{ST} = 0.093$	< 0.001
Within populations of the Central region	90.80		< 0.001	90.73		< 0.001

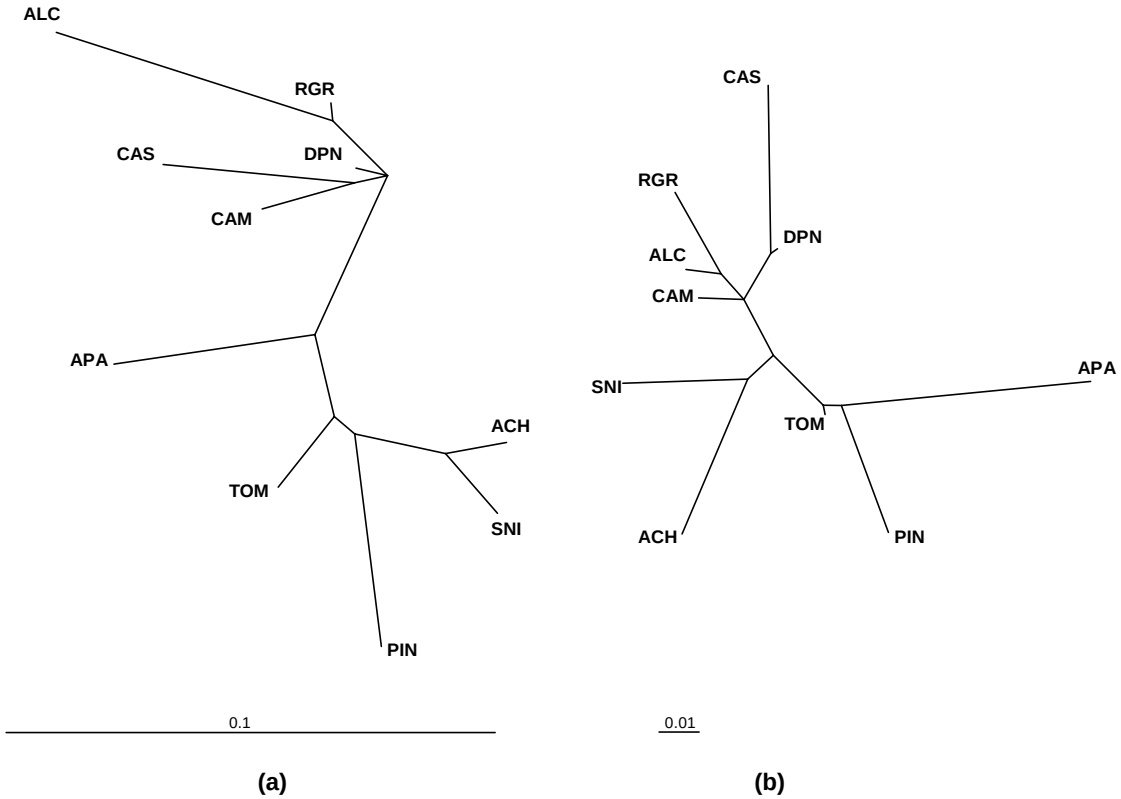

Fig. 3 Neighbour-joining analyses showing genetic relationships of 10 populations of *Pinus oocarpa*: Alcántara (ALC), Camelias (CAM) and Dipilto Nuevo (DPN) from the Northern region; Casita (CAS) from the Western region; Achuapa (ACH), Apante (APA), Pinos (PIN), Rodeo Grande (RGR), San Nicolás (SNI) and Tomabú (TOM) from the Central region. (a) Unrooted tree based on RAPD data. (b) Unrooted tree based on AFLP data.

bias in the estimation of population genetic parameters. In contrast, and in agreement with other authors (Sharma *et al*. 1996; Muluvi *et al*. 1999), selected AFLP primer combinations produced fairly homogeneous estimates of diversity. These results, together with the large number of AFLP markers scored per gel lane, counter the potential drawbacks of the AFLP technique [more time-consuming, more technically demanding and more expensive than RAPDs (Karp & Edwards 1995)].

In the present study, RAPDs and AFLPs showed a similar level of diversity for each single population (Tables 2 and 3). Taking both kinds of markers simultaneously, no population was consistently more or less diverse than any other. Casita, which is the most peripheral and degraded population of those studied, showed similar levels of genetic diversity to the other populations. This agrees with several studies that failed to detect reduced genetic variation for peripheral, compared to Central, populations of forest trees (Tigerstedt 1973; Betancourt *et al*. 1991; Bucci *et al*. 1997). However, in 1998, Hurricane Mitch caused a sudden reduction in the size of Casita, and samples for this study were taken after that event. As a consequence, it is to be expected that the population will experience a loss of genetic diversity over following generations. Based on theoretical considerations, the rate of loss is inversely proportional to effective population size (Nunney & Elam 1994). Therefore, the conservation of this population should be based on maintaining a substantial effective population size that could be promoted by replantation with either local source of seeds or from populations close related to it.

Population diversity values were within the range of those reported in other pine species investigated using RAPDs. Similar mean levels of diversity (0.37) were found in *P. sylvestris* (Szmidt *et al*. 1996), a higher value (0.46) in *P. contorta* (Thomas *et al*. 1999), and lower values (0.16, 0.17 and 0.23) in *P. muricata*, *P. attenuata*, and *P. radiata*, respectively (Wu *et al*. 1999). The present results contrast with the expected mean genetic diversity (0.158), estimated using isozymes, across a large number of *Pinus* species (Hamrick *et al*. 1992). Dominant molecular markers and codominant isozymes provided different information (phenotypic instead of allelic diversity). Also, in isozyme studies, both monomorphic and polymorphic loci are included. Therefore, it is risky to directly compare the obtained population diversity of *P. oocarpa* with that of other *Pinus* species based on isozyme data.

Analyses of RAPDs and AFLPs, using partition of phenotypic diversity estimates and AMOVA, showed population differentiation levels in very good agreement (Tables 4, 5 and 6). Given the large size of *Pinus* genomes, ranging from 19.5 to 26.5 pg (O'Brien *et al*. 1996), RAPDs would be expected to mostly reflect random amplification of noncoding repetitive DNA, which can diverge and change sequence rapidly (Charlesworth *et al*. 1994). As repetitive DNA sequences tend to be more heavily methylated than single copy and coding DNA, it might be anticipated that AFLP markers generated by a DNA methylation-sensitive enzyme, such as *Pst*I, might have been subjected to different evolutionary forces than RAPD markers. However, the similarity between the mean G_{ST} and Φ_{ST} values with both marker types indicates that, as a whole, both RAPDs and AFLPs must be taken as neutral characters in the populations analysed. However, since several RAPD primers revealed high G_{ST} values, i.e. OPS-08, OPS-18 and OPT-04, it cannot be discarded that some markers could be influenced by selection either directly or indirectly if they were closely linked to loci experiencing differing selection pressure (Latta & Mitton 1997).

Our results indicate that the majority of genetic diversity is contained within populations but there is still appreciable differentiation among populations, as indicated by Wright (1978) for F_{ST} values between 0.05 and 0.15. Populations of the Northern region showed lower levels of differentiation, specially in the AFLP-based analysis, than the populations of the Central region. Similar results have also been obtained in analysis with other molecular markers (Díaz 2001). This implies that the populations of *P. oocarpa* cannot be considered a single panmictic unit, although they are closely related (as might be expected given the size of the area sampled). Geographical distances between populations vary from 14 to 261 km. Given this, and taking into account the biological features of the species, i.e. its high outcrossing rate and large pollen dispersion distance, a high effective gene flow is to be expected. This would act as a homogenization factor between nearby populations. Accordingly, RAPD analysis has shown larger values for populational genetic differentiation in other pine species (ranging from 0.45 to 0.179), in agreement with the greater distance between the sampled populations (Bucci *et al*. 1997; Wu *et al*. 1999).

No significant correlations were seen between either RAPD- or AFLP-based differentiation and geographical distance between pairs of populations. However, geographically more distant populations were more dissimilar than populations from the same region. This suggests that, following isolation by distance, the partitioning of genetic variation in *P. oocarpa* has been influenced by the contrasting effects of genetic drift and gene flow. Moreover, in spite of the small regional divergence shown by AMOVA, cluster analysis revealed a good relationship between populations and their geographical location. This was especially evident for Central region populations. Five out of six populations sampled, all except Rodeo Grande, were clustered together. Mantel tests comparing pairwise Φ_{ST} and geographical distance matrices for these five populations gave significant results for both RAPD and AFLP data ($r = 0.705$ and $r = 0.771$, respectively). Because Rodeo Grande was

the only population not directly sampled, but received as a bulk seed sample, it may be that the seeds analysed were a nonrandom sample of the population gene pool. Direct sampling of this population must be performed before any conclusions can be drawn about its apparently discordant pattern of differentiation. A lack of correlation between pairwise Φ_{ST} values and geographical distances was found for the Northern populations. Compared to the Central populations, these are larger, less altered by distribution discontinuities owing to rural development, and all three are relatively near one another. This facilitates periodic gene exchange. The close relationship between Casita and the Northern populations might be explained by either historical relationships, meaning these populations shared a recent common ancestry, or, more likely, by the prevailing wind of the flowering season.

RAPDs and AFLPs detected similar, high levels of genetic diversity and a concordant distribution of variation within and between *P. oocarpa* populations. The fact that much of the variation was found within populations suggests that sampling from a few populations, for either conservation or breeding activities, may capture a large proportion of the variation of the species in Nicaragua. Nevertheless, sampling from a wide range of populations is still advisable as there are significant RAPD and AFLP differences between populations and regions.

Analyses carried out in this study can also be considered a helpful tool in conservation genetics of populations of *P. oocarpa* in Nicaragua. In their present state, Northern populations have a large size. They are located near each other and to populations of Southern Honduras which favour genetic interchanges. These features should be enough by themselves to maintain the observed levels of genetic variation if uncontrolled exploitation is avoided. By contrast, most of the Central populations and Casita are of limited size and more fragmented than Northern populations. They are more vulnerable although they still retain links with neighbouring populations via gene flow, and are thus able to maintain considerably genetic variability. Management in the Central and Western regions should be aimed at preserving these populations and to promote a regeneration of the more degraded ones by using either unselected, local seed or in Casita, using genetic material from its close related Northern populations.

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Verónica Díaz is a final year PhD student working on molecular population genetics of *Pinus oocarpa* under the supervision of Dr Esther Ferrer. Luis Miguel Muñiz is a PhD student working on molecular markers useful for genetic mapping. The main interest of our research is the development of molecular markers for application in problems of plant genetics and the conservation of both cultivated and wild species.
