POPULATION LEVEL ANALYSIS TO IDENTIFY SPECIES DIAGNOSTIC RAPD MARKERS FOR CLASSIFICATION OF CENTRAL AMERICAN AND MEXICAN PINES

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ABSTRACT

The practical recognition and definition of taxa is the starting point for much ecological and conservation work. In this paper, we address the utility of molecular markers as an aid to the identification of pine taxa in Central America and Mexico, where morphological data has often been inadequate to delineate species. The diagnostic capability of a sample of phylogenetically informative Randomly Amplified Polymorphic DNA (RAPD) markers identified by FURMAN *et al.* (1997) using a DNA pooling method was investigated for six pine taxa from Central America and Mexico. Six to seven open-pollinated families from six to eight populations represented each taxon (approximately 45 mother trees per taxon). RAPD polymorphisms at the level of populations and individuals were analyzed to describe the relationships of *Pinus caribaea*, *P. greggii*, *P. oocarpa*, *P. patula*, *P. pringlei* and *P. tecunumanii*. Molecular marker variation is generally concordant with the morphology-based taxonomy of Central American and Mexican pines, and that these molecular markers can be used as a reliable aid to the identification of these closely related taxa. Diagnostic markers, which are phylogenetically informative should be useful for discrimination of species, studies of hybridization and introgression and for resolving taxonomic ambiguity.

Keywords: *Pinus patula*, *Pinus greggii*, *Pinus pringlei*, *Pinus caribaea* var. *hondurensis*, *Pinus tecunumanii*, *Pinus oocarpa*.

INTRODUCTION

The taxonomic identity and evolutionary relationships of several of the Central American and Mexican closed-cone taxa of *Pinus* (subsection *Oocarpae*) have been a subject of debate for at least twenty years (STYLES 1976, 1985, EGUILUZ & PERRY 1983, SQUILLACE & PERRY 1993). These taxa include *Pinus greggii* Engelm., *P. jaliscana* Pérez de la Rosa, *P. oocarpa* Schiede ex Schlechtendal, *P. patula* Schiede ex Schlechtendal & Chamisso, *P. pringlei* Shaw and *P. tecunumanii* Eguiluz & J.P. Perry. Many of these Central American and Mexican pines are highly productive in forest plantations in the tropics and subtropics (BARNES & STYLES 1983). The majority of these pines have shown good adaptability in southern and eastern African and many South American countries and could become the most widely planted pines in the tropics and subtropics (BARNES & STYLES 1983, EGUILUZ 1984). A sixth taxon, *Pinus caribaea* var. *hondurensis* (Sénéclauze) W. H. Barrett & Golfari, a member of subsection *Australes*, is also of great economic interest because of its fast growth and wide site adaptation (DIETERS & NIKLES 1997). The established taxonomy of these pines has emphasized morphological differences in bark, needle, cone and seed characteristics (*e.g.*, SHAW 1909, MARTÍNEZ 1948, MIROV 1967, PERRY 1991, FARJON & STYLES 1997). However, these characters vary considerably within species, even within limited geographical areas (SALAZAR 1983, EGUILUZ 1984, MCCARTER & BIRKS 1985). The high degree of variation of many morphological characters within taxon is such that they have not always been effective for discriminating among taxa (MCCARTER & BIRKS 1985). Interspecific crosses of many of the closed-coned taxa produce viable offspring (*e.g.*, FIELDING 1960, CRITCHFIELD 1967). *Pinus caribaea* has the potential to hybridize with both *Pinus tecunumanii* and *P. oocarpa* in natural stands (STYLES *et al.* 1982, FERNANDEZ DE LA REGUERA *et al.* 1988, SQUILLA-

CE & PERRY 1993). Upon occasion, field identification of these taxa is difficult (MCCARTER & BIRKS 1985). This problem is most pronounced with closely related taxa, especially where hybridization and introgression are suspected.

Previously, methods to distinguish the above mentioned closely related taxa have used quantitative morphological characters and discriminant analysis. For example, MCCARTER & BIRKS (1985) conducted linear and canonical variable analyses of fifteen needle and cone morphological characters to discriminate between *P. tecunumanii* and *P. oocarpa*. A similar study was conducted by DVORAK and RAYMOND (1991) to discriminate between *P. oocarpa*, *P. patula* and *P. tecunumanii* and by DONAHUE *et al.* (1996b) to discriminate between northern and southern populations of *P. greggii*. Squillace & Perry (1993) used monoterpenes to discriminate between *P. caribaea*, *P. oocarpa*, *P. patula* and *P. tecunumanii*. For other forest trees, allozymes and other molecular markers have been used to delineate closely related taxa and to study hybridization and introgression (*e.g.*, CONKLE & WESTFALL 1984, BOBOLA *et al.* 1992, 1996), but the general application of these approaches has been limited.

Closely related taxa have relatively few molecular differences that unambiguously diagnose lineages. The proportion of genetic polymorphisms that unambiguously diagnose phylogenetically distinct lineages should increase with time since divergence (*e.g.*, DAVIS 1995). Alleles that differentiate groups of populations are most likely to have arisen due to differential transmission through speciation events (*e.g.*, genetic drift, natural selection, cessation of gene flow, *etc.*; see MAYR 1977, GRANT 1981, HARTL & CLARK 1997), or through lineage sorting where descendant populations and taxa are likely to contain random, and often nonexclusive, subsets of the marker alleles present in an ancestral population (*e.g.*, FLOYD 2002; JOHANNESEN & VEITH 2001; MAYER & SOLTIS 1999; PAGE & CHARLESTON 1997).

Recently, species diagnostic markers based on Random Amplified Polymorphic DNA (RAPD) (*e.g.*, WILLIAMS *et al.* 1990) have been developed for studies of hybridization and introgression (*e.g.*, FAURE *et al.* 2002; GUADAGNUOLO *et al.* 2001; CARAWAY *et al.* 2001, CHRUNGU *et al.* 1999; PERRON *et al.* 1995; KHASA & DANCIK 1996). In a previous study, FURMAN *et al.* (1997) used a DNA pooling strategy to detect genetic marker differences among eight groups of Central American and Mexican pines, representing six taxa, and identified a large number of phylogenetically informative RAPD markers. The DNA pools were comprised of samples from morphologically well-characterized individuals representative of different taxa or geographic groups within taxa. Analysis using these markers yielded a statistically robust phylogenetic tree, which provided systematic insights on the relationships among the pine taxa. In the study reported here, the diagnostic capability of a sample of phylogenetically informative RAPD markers identified by FURMAN *et al.* (1997) was utilized for eight groups of individuals and populations representing these six taxa from Central America and Mexico. We show that molecular marker variation is generally concordant with the morphology-based taxonomy of Central American and Mexican pines, and can help identify these closely related taxa. Diagnostic markers that are phylogenetically informative should be useful for discrimination of species, studies of hybridization and introgression, and for resolving taxonomic ambiguity.

MATERIALS AND METHODS

Plant material

Seeds collected from trees representing five closed-cone (serotinous) pine taxa (*Pinus greggii, P. oocarpa, P. patula, P. pringlei* and *P. tecunumanii*) and one open-cone (non-serotinous) taxon (*Pinus caribaea* var. *hondurensis*) were obtained from the International Tree Conservation and Domestication Program (CAMCORE), North Carolina State University. Populations of *Pinus greggii* were divided into two subgroups, based on location. The northern subgroup represents populations from northern (N) Mexico (latitude 25° N) and the southern subgroup represents populations from central (C) Mexico (latitude 21° N). The distance between these sets of populations is approximately 400 kilometers. Similarly, populations of *Pinus tecunumanii* were separated into two subgroups, based on altitude. Seed samples obtained from trees above 1500 meters altitude were included in the high (H) elevation subgroup and those collected below 1500 meters were included as low (L) elevation *P. tecunumanii*.

For each of the 8 species' groups, one filled seed was randomly taken from each of six to seven open-pollinated families per provenance. Six to eight provenances represented each group for a total of 45 mother trees per taxonomic entry or 366 individuals across the entire study (Table 1). Seeds were nicked and germinated in 1% hydrogen peroxide for five days. Seed coats were removed and embryos excised, leaving the haploid megagametophyte tissue to be

Table 1. Provenances of Central American and Mexican pines used in population study. Six families were used for all species except *P. pringlei*, which used 7.

Species	Provenance	Designation	Species	Provenance	Designation
P. caribaea	Poptun, Guatemala	CAR1	*P. patula*	Potrero de Monroy, Mexico	PAT1
	La Breá, Honduras	CAR2		Ejido el Rosario, Mexico	PAT2
	Limón, Honduras	CAR3		Corralitla, Mexico	PAT3
	El Pinal (Tikal), Guatemala	CAR3		Santa Maria Papalo, Mexico	PAT4
	Lanquin, Guatemala	CAR3		Zacualtipan, Mexico	PAT5
	Ejido Caobas, Mexico	CAR3		Llano Las Carmonas, Mexico	PAT6
	Alamikamba, Nicaragua	CAR3		Tlacota, Mexico	PAT7
	Isla de Guanaja, Honduras	CAR3		Cumbre de Muridores, Mexico	
P. greggii	El Madraño, Mexico	GREC1	*P. pringlei*	Santa Maria Lachixio, Mexico	PRIN1
	Laguna Atezca, Mexico	GREC2		Santo Domingo Yosonama,	PRIN2
	Laguna Seca, Mexico	GREC3		Mexico	PRIN3
	Valle Verde, Mexico	GREC4		El Guajolote, Mexico	PRIN4
	San Joaquin, Mexico	GREC5		Tlahuitoltepec, Mexico	PRIN5
	Jalamelco, Mexico	GREC6		Sola de Vege, Mexico	PRIN6
	Carrizal Chico, Mexico	GREC7		Acaten, Mexico	
P. greggii	Las Placetas, Mexico	GREN1	*P. tecunumanii*	San Vicente, Guatemala	TECH1
	Cañon Los Lirios, Mexico	GREN2		Chanal, Mexico	TECH2
	Jamé, Mexico	GREN3		Las Trancas, Honduras	TECH3
	Ojo de Agua, Mexico	GREN4		Napite, Chiapas, Mexico	TECH4
	La Tapona, Mexico	GREN5		Finca La Piedad, Guatemala	TECH5
	Loma El Oregano, Mexico	GREN6		El Pinalón, Guatemala	TECH6
	Santa Anita, Mexico	GREN7		Rio Chicquito, El Salvador	TECH7
				Montecristo, El Salvador	TECH8
P. oocarpa	Siguatepeque, Honduras	OOC1	*P. tecunumanii*	San Esteban, Honduras	TECL1
	San Luis Jilotepeque, Guatemala	OOC2		Culmi, Honduras	TECL2
				Gualaco, Honduras	TECL3
	San Lorenzo, Guatemala	OOC3		La Esperanza, Honduras	TECL4
	San José La Arada, Guatemala	OOC4		Yucul, Nicaragua	TECL5
	Las Minas, Guatemala	OOC5		San Rafael del Norte, Nicaragua	TECL6
	San Jeronimo, Guatemala	OOC6		Apante, Nicaragua	TECL7
	El Castaño, Guatemala	OOC7		Las Camelias, Nicaragua	TECL8
	La Lagunilla, Guatemala	OOC8			

used for DNA purification. Megagametophytes were stored at –80 °C.

DNA extraction and DNA fragment amplification

Total genomic DNA was isolated from megagameto-phyte tissue using the Pure Gene Isolation Kit (Gentra Systems, Inc., Minneapolis, MN). Each DNA pellet was resuspended in 50 µl of rehydration solution provided in the kit. DNA concentration and size were monitored on a 0.8% agarose gel by comparison to lambda DNA standards. DNA preparations were then diluted to 1ng/µl concentration with sterile distilled water.

DNA amplification for RAPD marker analysis was based on WILLIAMS *et al.* (1990). Decamer DNA primers were obtained from Operon Technologies, Inc., Alameda, CA. Each amplification reaction contained 1.5 µl of 10X reaction buffer (100 mM Tris HCl, pH 8.8; 500 mM KCl; 1% Triton-X100; 25mM $MgCl_2$); 100 µM each of dATP, dCTP, dGTP, dTTP; 0.2 µM primer; 5 ng of genomic DNA template and 0.95 units of Taq DNA polymerase, in a total volume of 15 µl. Amplification was carried out in 96-well plates using an MJ Research PTC-100 thermal controller. The thermal program parameters were: 41 cycles of 1 min at 92 °C, 1 min at 35 °C and 2 min at 72 °C. A total of 50 primers was used to assay DNA amplification. Approximately 12 individual seed parents representing each of the 8 groups were analyzed on 96-well plates, for a total of 4 plates per primer assayed. The reactions using the 8 groups as templates were loaded next to each other on gels to allow compari-

sons. Two controls were included for each plate; one contained all components except primer and the second contained all components except DNA template.

Amplification products (RAPD fragments) were analyzed by horizontal gel electrophoresis in 2% agarose TBE gels and detected by ethidium bromide staining (SAMBROOK 1989). Gels were videographed over UV light with an Eagle Eye Video Imager (Stratagene). RAPD fragments were scored for presence or absence across the samples analyzed. Fragment sizes in base pairs were estimated by comparison to a mixture of fragments of known size (1KB ladder, Life Technologies) using the software SEQUAID II (Rhoads & Roufa 1990).

Data compilation and analysis

Over 18,000 RAPD reactions for the 366 DNA samples were amplified using 50 decamer primers. RAPD fragments polymorphic among the DNA samples were scored as discrete characters (1 = present, 0 = absent). Any plates in which the control reactions produced bands were repeated and, if bands persisted, were discarded from the study. Only RAPD markers of high amplification intensity were analyzed.

Cluster analysis was used to identify natural groupings based on molecular markers. Unweighted pair-group method using an arithmetic average (UPGMA) (Sneath & Sokal 1973) was carried out using the computer program POPGENE v1.1 (YEH *et al.* 1996). UPGMA defines the inter-cluster distance as the average of all pairwise distances for members of two clusters (WEIR 1996). A dendrogram was created from the results of the UPGMA analyses.

Population frequencies of the 135 markers that were generated by the 50 primers (see results) were calculated for each group to identify diagnostic markers for the classification of individuals. In this study, a marker is considered to be species diagnostic if it has a frequency greater than or equal to 90 percent in one group and a frequency less than or equal to 10 percent in another group. A second data set comprised of 366 individuals scored for these diagnostic RAPD markers was constructed.

Nonparametric discriminant analysis (PROC DISCRIM, method=npar, k-nearest-neighbor; SAS, ver-sion 6.11) was used to classify individuals into groups based on the species diagnostic marker data. This analysis was completed on untransformed data. A discriminant function was then determined for this set of diagnostic markers. Verification of the discriminant function was accomplished by random resampling of individuals. Individuals were randomly placed into one of two separate data sets. Approximately one half of the individuals representing each of the 8 groups was placed into each data set. One data set was used to derive a discriminant function and this function was then applied to the second data set. A total of 100 random iterations were completed. The average rate of correct reclassification was thus calculated for 100 replicate pairs of samples drawn from 366 individuals. For each replicate pair, a discriminant function was derived from one sample of approximately 158 individuals representing the 8 groups and its classification rate was tested on the second sample of the remaining individuals. Although the same data set was used

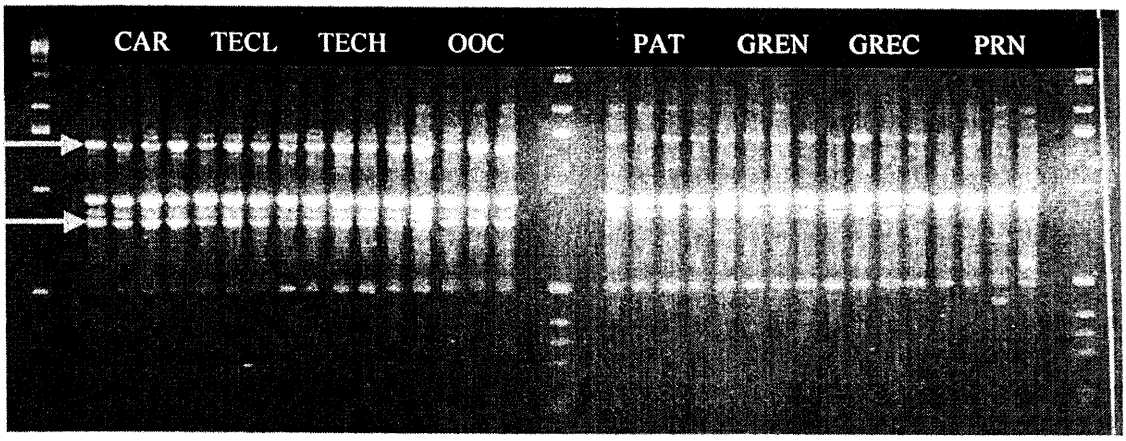

Figure 1. Subset of population survey of RAPD markers for primer AH14. Arrows on the left show markers that distinguish taxa: AH14-1425 (top) and AH14-792. Lanes 1, 20 and 39 are the 1KB ladder (Life Technologies). Lanes 2, 19, 21 and 38 were left empty. Each remaining lane used template DNA from one individual of a given species. Four individuals per species group were used. Lanes 3–6 contain RAPD fragments for *P. caribaea*, lanes 7–10 for *P. tecunumanii* (low elevation), lanes 11–14 for *P. tecunumanii* (high elevation), lanes 15–18 for *P. oocarpa*, lanes 22–25 for *P. patula*, lanes 26–29 for *P. greggii* North, lanes 30–33 for *P. greggii* Central and lanes 34–37 for *P. pringlei*.

both to define and evaluate the discriminant function, those individuals being tested were different from those used to define the discriminant function (*e.g.*, CHMIELEWSKI 1995).

RESULTS

RAPD marker analysis

RAPD analysis was used to detect genetic marker differences among individuals representing 6 taxa. The DNA samples were scored for the presence/absence of RAPD markers using 50 decamer primers (Figure 1). The 50 primers yielded 135 easily scored polymorphisms. Of these RAPD markers, 72 were chosen for their ability to discriminate between the 8 groups in a previous study (Furman *et al.* 1997). The remaining 63 markers were chosen to provide a representative sample of marker variation among individuals. The data set used for the cluster analyses consisted of 366 individuals scored for 135 polymorphic RAPD markers.

Cluster analyses (UPGMA)

A principal purpose of this study was to analyze the natural variation of individuals and populations of the six pine taxa listed in *Plant Material* and to ascertain if the described variation fell into well-defined groups. UPGMA analysis was used to evaluate the RAPD marker variation and defined two main subgroups which were well diverged (Figure 2). Subgroup 1 included populations of *P. patula*, *P. pringlei* and the two geographically distinct samples of *P. greggii*. The variation for these three taxa fell into four well-defined and well-separated groups, including a clear separation between the northern and southern populations of *P. greggii*. Subgroup 2 included populations of *P. caribaea*, *P. oocarpa* and the two *P. tecunumanii* samples defined by altitude. Two distinct clusters within this second subgroup resulted in a clear and well-defined separation between *P. caribaea* populations, representing

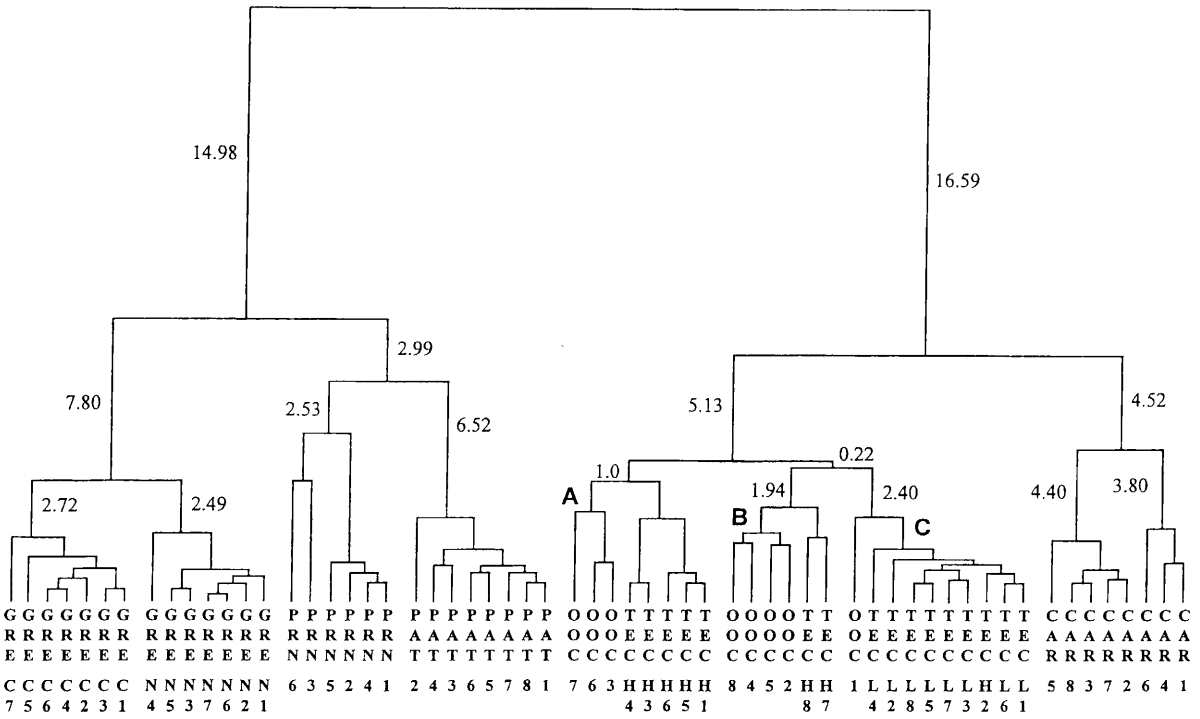

Figure 2. Results of UPGMA clustering analysis for 6 species (8 groups) from RAPD marker population data (numbers after species designation identify provenance). Seventeen diagnostic marker differences distinguished the two main subgroups. Dendogram based on Nei"s (1978) genetic distance. Also shown are 3 subclusters of *P. oocarpa*/*P. tecunumanii* denoted "A", "B" and "C". Subcluster A contains *P. oocarpa* populations San Lorenzo, San Jeronimo, and El Castaño from Guatemala and *P. tecunumanii* H populations San Vicente, Finca La Piedad, and El Pinalon from Guatemala; Las Trancas from Honduras and Napite from Mexico. Subcluster B contains *P. oocarpa* populations San Luis Jilotepeque, San Joséé La Arada, Las Minas, and La Lagunilla from Guatemala and *P. tecunumanii* H populations Rio Chicquito and Montecristo from El Salvador. Subcluster C contains *P. oocarpa* population Siguatepeque from Honduras, *P. tecunumanii* H population Chanal from Mexico, and all populations of *P. tecunumanii* L.

one cluster, and a second cluster comprising populations of *P. tecunumanii* and *P. oocarpa*. Within this cluster, three distinct subclusters were found; each was defined by a number of distinct marker differences (Figure 2).

One subcluster (subcluster A) consisted of a mixture of *P. oocarpa* provenances and high elevation *P. tecunumanii*. The *P. oocarpa* provenances included San Lorenzo, San Jeronimo and El Castaño from Guatemala; high elevation *P. tecunumanii* provenances included San Vicente, Finca La Piedad and El Pinalón from Guatemala, Las Trancas from Honduras and Napite from Mexico. A second subcluster (subcluster B) was also mixed, and consisted of *P. oocarpa* provenances of San Luis Jilotepeque, San Jose La Arada, Las Minas and La Lagunilla from southeastern Guatemala and high elevation *P. tecunumanii* provenances of Rio Chicquito and Montecristo from El Salvador. A third subcluster (subcluster C) placed one provenance of high elevation *P. tecunumanii* (Chanal, Mexico) and one provenance of *P. oocarpa* (Siguatepeque, Honduras) together with all low elevation *P. tecunumanii* provenances. These three *P. tecunumanii*/*P. oocarpa* subclusters are geographically separated, following a north to south progression (Figure 3).

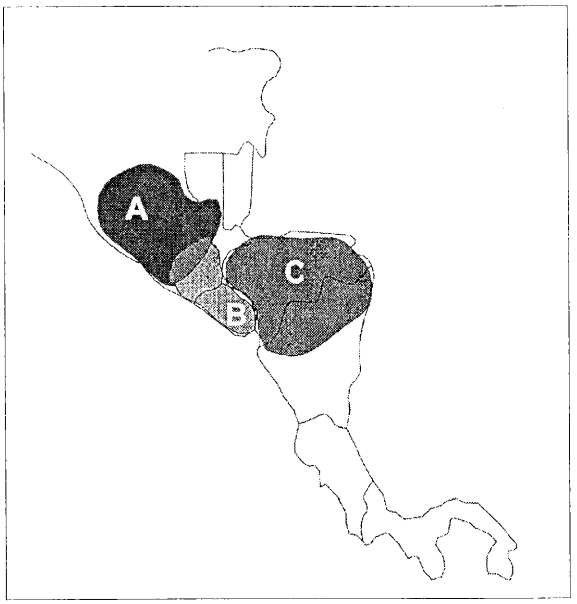

Figure 3. Geographical location of *P. oocarpa* / *P. tecunumanii* subclusters resulting from UPGMA clustering analysis.

Marker frequencies for groups

The frequency of each marker was calculated for each of the 8 groups. In this study, a marker is defined as species diagnostic if it has a frequency of greater or equal to 90 percent in one group and a frequency of less than or equal to 10 percent in another group. Of these 135 RAPD markers, 72 were previously identified as able to differentiate taxa from the analysis of pooled DNA samples (FURMAN *et al*, 1997). Forty of the 72 markers chosen to differentiate taxa from the DNA pooling strategy were verified from population data (56%). Twenty additional species diagnostic markers were identified from the population data (32% of a representative set). Thus, a total of 60 markers was found to be species diagnostic for at least two of the groups. These molecular marker differences are the result of an initial screening to detect marker differences among groups, and therefore may not be representative of genome wide divergence. Marker frequencies for a random sample of 20 species diagnostic RAPD markers are presented in Table 2. The remaining set of 75 markers was selected at random, and therefore should represent a substantial and unbiased sample of genome wide variation.

Discriminant analysis

The second objective of this study was to devise an efficient means of classifying individuals into groups using molecular markers. Nonparametric discriminant analysis of the data was carried out to assess the potential for the species diagnostic marker data to be used to correctly classify individuals. The discriminant criterion is determined by a measure of generalized squared distance (FISHER 1936). Nonparametric discriminant methods are based on group-specific probability densities that do not make assumptions about the distributions of the data (SAS 1991). Evaluation of the resulting discriminant function was accomplished by random resampling of individuals. Individuals of the original data set were randomly placed into two separate data sets. One data set was used to derive a discriminant function that was applied to the second data set. The average rate of correct reclassification was very high for all groups: *P. caribaea* (98 %), *P. greggii* C (95 %), *P. greggii* N (100 %), *P. patula* (96 %), and *P. pringlei* (100 %), *P. oocarpa* (72 %), *P. tecunumanii* H (86 %) and *P. tecunumanii* L (86 %) (Table 3). A discriminant function for the 60 species diagnostic RAPD markers can be used to predict group assignments for individual trees and to identify individuals, which may be the result of hybridization or introgression events. A list of the 135 RAPD markers and 60 species diagnostic RAPD markers can be found in FURMAN (1997 – pages 71 & 72).

Table 2. Within group frequency of a subset of 20 species diagnostic RAPD markers.

Primer marker (bp)	CAR	TECL	TECH	OOC	PAT	GREN	GREC	PRIN
B8 (2808)	0	0	0	0	0.98	0.90	1	0.67
C19 (648)	0	0	0	0	1	1	1	1
E2 (1076)	0.17	0.04	0	0.02	1	1	1	1
I14 (3173)	0.94	0.96	0.73	0.96	0.02	0	0	0
L15 (1100)	0	0	0	0	0.98	1	1	1
L15 (610)	1	1	0.92	0.85	0	0	0	0
S11 (637)	1	1	1	0.96	0.04	0.02	0	0.40
T13 (1733)	1	1	1	1	0	0	0	0
V10 (1800)	1	1	1	0.92	0	0	0	0.45
V10 (1450)	0	0	0	0	0.90	1	0.07	0.98
V10 (1200)	1	1	1	1	0	0	0	0.40
W6 (3227)	0.96	1	0.96	0.94	0	0	0	0
AH14 (1425)	1	1	1	1	0	0	0	0
AK4 (414)	0	0	0	0	1	1	1	1
AQ1 (1156)	1	1	1	1	0	0	0	0
AS6 (3573)	0.98	0.94	0.96	0.96	0	0	0	0
AV13 (2149)	0	0	0	0	0.96	1	1	0.90
AX8 (2137)	0	0	0	0	1	1	0.98	1
AX8 (1112)	0	0	0	0	1	1	1	0.95
AX8 (451)	1	1	1	0.98	0	0	0	0.10

Table 3. Cross validation of species groups for 100 random iterations of nonparametric discrimination analysis of 60 species diagnostic RAPD markers.

	CAR	OOC	TECH	TECL	PAT	PRIN	GREC	GREN
CAR	**98**	0	0	2.00	0	0	0	0
OOC	0	**72.15**	19.29	8.57	0	0	0	0
TECH	0	4.76	**86.31**	8.92	0	0	0	0
TECL	0	7.88	6.25	**85.86**	0	0	0	0
PAT	0	0	0	0	**95.83**	0	0	4.17
PRIN	0	0	0	0	0	**100**	0	0
GREC	0	0	0	0	0	0	**94.74**	5.26
GREN	0	0	0	0	0	0	0	**100**

DISCUSSION

The biological basis for taxonomic classification is genetic differentiation resulting from evolutionary divergence. The analysis of molecular marker variation is a powerful tool to obtain insights into the distribution and evolution of species and populations. Phylogenetic processes are likely to be reflected in the variation of molecular markers that differentiate taxa (*e.g.*, OLMSTEAD 1995, VRBA 1995). In this study, RAPD markers were chosen for their ability to differentiate taxa defined by morphological differences. We would expect to find molecular markers that do indeed differentiate groups defined by morphology and that the cluster analysis would mirror the organization of taxa if enough species diagnostic markers were found. Many of the markers used in this study were previously identified as phylogenetically informative from a study of marker differences among pooled DNA samples (FURMAN *et al.* 1997). If species divergence occurred long ago, a large number of markers should be found which distinguish species. Such marker differences establish well-diverged groups in a cluster analysis. Conversely, recently diverged groups would be expected to have relatively few marker differences that distinguish them. Such differentiation can provide evidence for a phylogen-

etic definition of a group of species (DAVIS & NIXON 1992).

In this study of six pine taxa, RAPD polymorphisms at the level of populations and individuals were analyzed to describe the relationships of *Pinus caribaea*, *P. greggii*, *P. oocarpa*, *P. patula*, *P. pringlei* and *P. tecunumanii*. The resulting lineages and clusters defined by the genetic marker variation were generally congruent with the established morphology-based taxonomy. Analysis of the natural variation of the individuals and populations resulted in well-defined and well-separated groups. Similarly, the discriminant analysis showed that the markers provide a useful set of characters to classify most individuals into taxon and subtaxon groups. The analyses confirm previous work (FURMAN *et al.* 1997) that showed these six taxa divided into two major and distinct subgroups. *Pinus patula*, *P. pringlei* and *P. greggii* belonged to one major subgroup and *P. caribaea*, *P. oocarpa* and *P. tecunumanii* belonged to the second distinct subgroup. These main subgroups were distinguished by 17 diagnostic markers (Figure 2), and indicate that these major lineages had diverged prior to speciation events that gave rise to the current taxa.

Within these major subgroups, the cluster analysis clearly separated the northern and central Mexican populations of *P. greggii*. These geographically defined groups were also defined by four diagnostic marker differences. The level of differentiation between these two groups was roughly the same magnitude as the differentiation between species for other pairs of these taxa. Common garden studies and studies in natural stands have shown significant differences between populations of *P. greggii* from northern and central Mexico in height growth (DVORAK *et al.* 1996), monoterpene chemistry (DONAHUE *et al.* 1996), leaf, cone and seed morphology (DONAHUE & LOPEZ-UPTON, 1996), and allozyme variation (HERRERA *et al.* 1997). Thus, the marker data, combined with terpene differences and common garden studies, provide support for the recognition of the northern and central provenances of *P. greggii* as separate units for breeding and conservation. The two groups have now been identified as separate varieties (DONAHUE & LOPEZ-UPTON 1999). The level of divergence of lineages that justifies naming of species remains a matter of taxonomic judgement. In other groups of organisms, morphologically indistinguishable cryptic species have been diagnosed using other characteristics, including molecular markers (*e.g.*, BEEBE *et al.* 2002; HUNG *et al.* 1999; BAKER *et al.* 1995; WILKERSON *et al.* 1995). Further study of the morphological differentiation between these two groups is warranted to determine how these groups should be classified.

The taxonomy and marker lineages were not congruent for *Pinus tecunumanii* and *P. oocarpa* (Figure 2). None of the 135 markers identified in this study definitively discriminated between these two taxa. The lack of molecular marker differences could indicate that: (1) the groups are too closely related and we have reached the limit of resolution of the morphological taxonomy to define groups, or (2) the groups are becoming indistinguishable as a result of hybridization and introgression (or convergence of lineages). The fact that all markers are found, some at low frequency, in both of these taxa could indicate that fixation has not yet occurred, and that these taxa are only recently diverging and therefore have not yet accumulated a large amount of differentiation at the DNA sequence level. Convergence of lineages may also be an explanation for the lack of marker differences between populations of high elevation *P. tecunumanii* and *P. oocarpa* within a given geographic region. For example, most of the populations of subcluster A (see Figure 2) are from the Sierra de Las Minas range of eastern Guatemala. The *P. oocarpa* provenances of San Lorenzo, San Jeronimo and El Castaño all occur sympatrically, or nearly sympatrically, with *P. tecunumanii*. Within these populations, trees are found that intergrade between these two taxa (DVORAK, unpublished data). Similarly, the high elevation provenances of San Vicente, Finca La Piedad and El Pinalón all occur in close proximity to *P. oocarpa* stands. Although it is possible that gene flow is occurring between sympatric populations resulting in extensive introgression within a given geographic area, it is equally likely that both groups (taxa) inherited these markers from a common ancestor. This marker study was designed to provide diagnostic markers to use in studies of introgression. The fact that these types of markers were not found for these taxa does not establish introgression, and further studies will be necessary to test the hypothesis of convergent lineages.

The clustering resulting from the UPGMA analysis identified three distinct subclusters, each consisting of provenances of *P. tecunumanii* and *P. oocarpa*, defined by distinct marker differences (Figure 2). Subcluster A and subcluster B were comprised of both high elevation *P. tecunumanii* and *P. oocarpa* individuals, while subcluster C was comprised of all of the low elevation *P. tecunumanii* individuals and only a small number of individuals from *P. oocarpa* and high elevation *P. tecunumanii*. A discriminant analysis showed that marker data provided a substantially higher frequency of correct

Table 4. Cross validation of species groups for 100 random iterations of nonparametric discriminant analysis of 60 species diagnostic RAPD markers: TEC/OOC subclusters.

	CAR	SUB1	SUB2	SUB3	PAT	PRIN	GREC	GREN
CAR	**96.65**	0.06	0.07	3.22	0	0	0	0
SUB1	0	**96.29**	2.44	1.27	0	0	0	0
SUB2	0.13	1.64	**93.89**	4.34	0	0	0	0
SUB3	0.17	0.62	0.21	**99**	0	0	0	0
PAT	0	0	0	0	**97.71**	1.07	0.89	0.33
PRIN	0	0	0	0	3.48	**96.09**	0.28	0.15
GREC	0	0	0	0	0.10	0	**95.84**	4.05
GREN	0	0	0	0	0	0	5.15	**94.85**

reclassification to subclusters than to morphology-defined taxonomic groups (Table 4). Furthermore, the geographic distributions of the populations that comprised these three subclusters (Figure 3) appeared to be geographically separated (parapatric). ELDREDGE (1995) noted that it is possible to have a situation in which one portion of a species is more closely related phylogenetically to a second species than it is to other portions of its own reproductive community (*i.e.*, species). ELDREDGE (1995) further explained that such a situation could arise if a part of a species were to share one or more shared-derived traits (synapomorphies) with the second species and these traits were not found in other populations of the first species.

The geographic distribution of subclusters distinguished by diagnostic markers may also suggest that the individuals assigned to different subclusters could belong to different, independent, non-interbreeding lineages (*e.g.*, TEMPLETON 1989). The interpretation of *P. tecunumanii* and *P. oocarpa* subclusters as lineages raises questions concerning the relationships of individuals classified to these taxa. The *P. tecunumanii* (high elevation) and *P. oocarpa* morphological types were different in appearance, and could have resulted from a polymorphism that segregated in the two lineages (subcluster 1 and 2). Alternatively, the *P. tecunumanii* and *P. oocarpa* types could have diverged independently within subcluster A and subcluster B. The pooling used to identify diagnostic markers combined individuals by morphologically defined taxa (*P. tecunumanii* and *P. oocarpa*). The marker differences resulting in subcluster A and B were probably underrepresented because the composition of the original DNA pools was based on the established *P. tecunumanii* and *P. oocarpa* types. One approach to address this issue further would be the construction of new DNA pools based on the *P. tecunumanii* and *P. oocarpa* types within each subcluster. It may also be of interest to examine, under controlled environmental conditions, the morphological variation of these populations in terms of the groupings suggested by molecular variation. As stated above, the taxa used in this study are defined by needle, bark and cone morphology. Such morphological differences could be due to recent mutation at a few loci and subsequent selection, and may not reflect the true genetic relatedness of groups or individuals. All of the populations used in this study have been planted in field trials across a number of sites by CAMCORE and their assessment might resolve this question.

Phylogenetic analysis provides a conceptual basis for understanding the distribution of diagnostic markers among lineages and an explanation for the results obtained by DNA pooling methods and RAPD markers. For example, the diagnostic markers that distinguish the two major groups in the cluster analysis would almost certainly be identified by comparison of band phenotypes from DNA pools that combined samples from the three taxa within each group. Within each main cluster, *i.e.*, at the level of taxa, great care must be taken to choose the individuals comprising each pooled sample. In our study, 56 % of a sample of the candidate markers identified by the DNA pooling strategy (FURMAN *et al.* 1997) were shown to be species diagnostic by gene frequency estimates for the different taxa. Screening for phylogenetically informative markers appears to be a time effective method for identifying a relatively large number of such diagnostic markers. This method is particularly useful for closely related species, which have not undergone prolonged and well-isolated speciation events.

Our results of the cluster analysis show a clear separation between the two geographically defined groups of *P. greggii*, and, to a great extent, the two elevation groups of *P. tecunumanii*. These results corroborate the morphological and monoterpene differences noted previously (DVORAK & RAYMOND 1991, DONAHUE & LOPEZ-UPTON, 1996, DONAHUE

et al. 1996, DONAHUE & LOPEZ-UPTON 1999). There thus appears to be a correlation between the quantitative trait differences noted in provenance trials and differentiation at the level of molecular markers. This result is in contrast to other studies, which show no noticeable correlation between morphological (quantitative) traits and molecular differentiation (*e.g.*, THOMAS & HUNT 1993, KARHU *et al.* 1996). These studies, however, have concentrated on the sampling of representative genomic variation and have interpreted differences between or within group polymorphism as a measure of differentiation. If quantitative variation is the result of evolutionary adaptation to different environments, molecular markers should differentiate these populations. Analyses based on these molecular marker differences should thus predict patterns of feature diversity that are of interest for conservation and for breeding (Faith 1994). The use of RAPD markers has been expanded to assess the evolutionary history of the Oocarpae and Australes subsections (DVORAK *et al.* 2000a) and to determine conservation strategies for *Pinus maximinoi* (DVORAK *et al.* 2002). In addition, a recent research emphasis at CAMCORE includes the use of interspecific hybridization for enhanced growth and wood properties of *Pinus*.

Diagnostic markers clearly differentiating among groups can be used as discriminant alleles to classify taxonomically ambiguous individuals. The genetic markers identified in this study, therefore, could be useful for assessing hybridization and introgression. While the individuals chosen for this study closely resembled the established morphological types, many individuals in natural populations are difficult to classify. Accessions in conservation and breeding programs could be assayed for diagnostic markers using the discriminant analysis approach. Most of the taxa of *Pinus* could be interpreted as distinct lineages or collections of lineages and diagnosed with molecular markers. This study shows the potential for a molecular marker approach to address "species boundary" questions of practical interest in breeding and conservation programs, uniting both molecular and taxonomic approaches to delineating species, subspecies or populations.

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