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R C Schmidtling [a] & V Hipkins [b]
[a] USDA Forest Service, SRS, Southern Institute of Forest Genetics, 23332 Hwy 67, Saucier, MS, 39574, USA E-mail:
[b] USDA Forest Service, NFGEL Lab., Placerville, CA, USA
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R C Schmidtling[1] and V Hipkins[2]

[1] *USDA Forest Service, SRS, Southern Institute of Forest Genetics, 23332 Hwy 67, Saucier, MS 39574, USA. e-mail: SCHMIDTL@datasync.com.*
[2] *USDA Forest Service, NFGEL Lab., Placerville, CA, USA*

ABSTRACT

Allozymes in bud tissue and monoterpene contents in xylem oleoresin of slash pine (*Pinus elliottii*) were analyzed from populations across the natural distribution, as well as those from other species in the AUSTRALES pines. Allozyme diversity measures of slash pine were similar to those found in other southern pines. The two slash pine varieties, the slower-growing south Florida variety (var. *densa*) and the more commercial "typical" variety (var. *elliottii*), were not separated in the cluster analysis of allozymes. Variation was continuous from south to north in Florida in slash pine, with no distinct transition between the two varieties. The monoterpene data also showed continuous variation between the two slash pine varieties. Expected heterozygosity declined from south to north, supporting the hypothesis that slash pine resided in a Pleistocene refugium in south Florida or the Caribbean, migrating northward at the close of the ice age. Allozyme frequencies as well as monoterpene compositions of slash pine and its AUSTRALES relatives showed a very close relationship between slash pine and Bahamian Caribbean pine (*P. caribaea* Morelet var *bahamensis*).

KEYWORDS: *Pinus caribaea,* AUSTRALES, allozymes, monoterpenes

INTRODUCTION

Slash pine (*Pinus elliottii* Engelm.) is one of the most commercially valuable pines in the United States. It is commonly planted inside and outside its natural range in the Southeastern US (Boyer and South 1984), as well as in exotic plantings in Asia (Pan 1989), South America (Picchi and Barrett 1967), Africa and Australia (Mullin *et al.* 1978).

Taxonomically, slash pine has obvious affinities with other caribbean hard pines, and before 1952 (Little and Dorman) slash pine and *P. caribaea* Morelet were not considered separate species. Slash pine is now divided into two varieties, the "typical" variety (var. *elliottii* Little and Dorman) and the south Florida variety (var. *densa* Little and Dorman). The transition between the two varieties was mapped in the central Florida peninsula by Little and Dorman (1952)(**Figure 1**), although Squillace (1966) and Nikles (1966) found no distinct transition between the varieties and considered the variation to be continuous in the peninsula.

There is some evidence for gene exchange between *P. caribaea* var. *bahamensis* (Griseb.) B. and G. and slash pine in southern Florida, based on analysis of cortical monoterpenes (Squillace *et al.* 1977) as well as chloroplast DNA (Nelson *et al.* 1994, Wagner *et al.* 1992). Recently, Dvorak *et al.* (2000) has shown a very close relationship between *P. caribaea* and slash pine using RAPD's.

The purpose of the present study is to further evaluate the patterns of variation in populations from throughout the slash pine range and the nearby tropical and temperate relatives in the subsection AUSTRALES using allozymes and monoterpenes.

MATERIALS AND METHODS

Plant materials

Dormant buds were collected from 17 geographic sources of slash pine across the natural range (**Figure 1 and Table 1**). Eleven of the sources were of secondary origin, located in provenance test plantings in south Mississippi (Snyder *et al.* 1967, Doudrick *et al.* 1996). Collection specifications were the same for the recent, *in situ* collections as well as the existing provenance tests: Collections were made from trees in natural stands separated from each other by 100 ft (30 m) or more. One of the collections (LA Land race) was from a seed orchard in central Louisiana, from grafted ramets of trees selected in plantations made during the 1930's. Slash pine does not occur naturally west of the Mississippi, but is widely planted there. Collections were also made of longleaf pine (*P. palustris* Mill.) in Wakula County FL, from loblolly pine (*P. taeda* L.) in southwest Mississippi, and from Caribbean pine (*P. caribaea* Morelet) on Grand Bahama Island. Buds were collected from approximately 30 trees from each of the populations.

We also made use of monoterpene analysis of oleoresin samples collected by Nikles (1966)[1] and Coyne[2]. Samples for both data sets were collected using glass vials inserted into holes drilled into the sapwood at breast-height (Coyne 1965). Collections were made in 26 different populations of eight taxa and hybrids (**Table 2**). Monoterpenes were assayed using gas chromatography, following procedures and conditions that were nearly identical for both sample sets (Nikles 1966, Coyne and Keith 1972).

Enzyme electrophoresis

Isozyme band patterns were investigated using dormant vegetative bud tissue as the enzyme source material from individual tree collections. Scales were removed from terminal buds and a small portion (8 mm^3) of meristem was dissected and submerged in three drops of modified Cheliak and Pitel (1984) extraction buffer (where mercaptoethanol was removed). Samples were frozen at -7°C until electrophoresis.

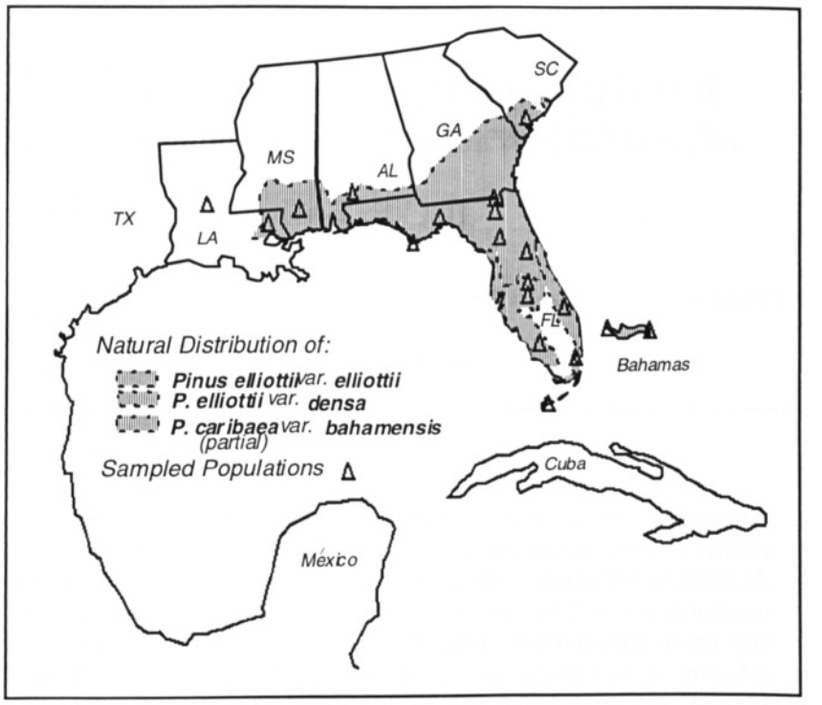

Figure 1. *Map of the southeastern United States and adjacent Caribbean showing the natural distribution of slash pine (adapted from Critchfield and Little 1966) and sample points for allozyme analysis.*

Table 1. *Population origins, number of trees sampled per population, mean number of alleles per locus (N_a), percent loci polymorphic (P_l), and expected heterozygosity (H_e) at 22 loci.*

	Sample				
Populations Sampled1	Lat °	N size	N_a	P_l	H_e
Pinus elliottii var. *densa (P.e.d.)*					
11 Big Pine Key, FL	24.6	35	2.18(0.24)2	63.6	0.197(0.039)
12 Everglades, FL	25.4	31	1.86(0.19)	59.1	0.170(0.041)
2 Collier Co. FL	26.1	30	2.05(0.19)	68.2	0.177(0.040)
21 Okeechobee, FL	27.0	31	2.09(0.20)	68.2	0.189(0.041)
3 Highlands Co., FL	27.6	32	2.14(0.22)	68.2	0.194(0.042)
P.e.d. - P.e.e. Transition					
205 Polk Co.,FL	28.1	30	2.05(0.21)	68.2	0.176(0.043)
Pinus elliottii var. *elliottii (P.e.e.)*					
4 Disney, FL	28.4	35	2.09(0.22)	63.6	0.187(0.044)
5 Marion Co., FL	29.2	35	2.00(0.20)	59.1	0.170(0.041)
6 Baker Co., FL	30.2	35	2.14(0.23)	63.6	0.165(0.037)
203 Baker Co., FL	30.2	29	2.05(0.21)	63.6	0.158(0.035)
61 Wakula Co., FL	30.2	23	1.95(0.18)	63.6	0.169(0.037)
62 Cape San Blas, FL	29.7	31	1.95(0.19)	63.6	0.170(0.039)
207 Monroe Co., AL	31.1	38	2.09(0.20)	68.2	0.174(0.042)
209 Harrison Co., MS	30.5	30	2.00(0.21)	63.6	0.147(0.038)
211 St Tammany, LA	30.6	30	1.77(0.16)	59.1	0.173(0.042)
201 Colleton Co., SC	32.3	30	2.14(0.20)	72.7	0.175(0.038)
70 LA Land Race	-	31	2.23(0.25)	63.6	0.194(0.041)
Pinus elliottii Means	2.04	64.8	0.173		
Pinus caribaea var. *bahamensis (P.c.b.)*					
East Grand Bahama	26.6	32	2.14(0.21)	72.7	0.210(0.047)
West Grand Bahama	26.6	32	2.18(0.21)	72.7	0.233(0.047)
P. palustris	30.2	30	1.68(0.20)	45.5	0.107(0.039)
P. taeda	31.2	32	2.18(0.21)	72.7	0.196(0.047)

[1] *Populations with single digit I.D. are from a 4-year-old provenance test located in south Mississippi (Doudrick* et al. *1996), those with double digit I.D. were collected* in situ, *and those with triple digit I.D. are from a 45-year-old provenance test in south Mississippi (Snyder* et al. *1967).*

On the morning of the electrophoretic run, extracts were prepared by thawing samples, macerating the bud tissue with a Dremel MultiPro drill press, and absorbing the slurry onto 3mm wide paper wicks. Wicks were inserted into 11% starch gels (Sigma Chemical Co.) that accommodated 30 samples. The preparation and running of the gels are modifications of Adams *et al.* (1990) and Conkle *et al.* (1982). A total of 662 trees were genotyped at 22 isozyme loci using three buffer systems. Buffer system 'LB' (gel and tray buffer 'A' of Adams *et al.*(1990)) was used to resolve enzyme systems leucine aminopeptidase (LAP), phosphoglucomutase (PGM), shikimic acid dehydrogenase (SKD), and diaphorase (DIA). Buffer system 'SB' (a modification of gel and tray buffer 'B' of Adams *et al.* (1990), where the electrode buffer was pH 8.0), was used to resolve enzyme systems triosephosphate isomerase (TPI), catalase (CAT), and glutamic oxaloacetate transaminase (GOT). Buffer system 'MC8' (a modification of gel and tray buffer 'C' of Adams *et al.* (1990), where the stock solution was adjusted to pH 8.0), was used to resolve isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), fluorescent esterase (FEST), and malate dehydrogenase (MDH). Running conditions and stain recipes follow Adams *et al.* (1990) and Conkle *et al.* (1982). After the dye marker migrated 8 cm, gels were cut horizontally into four to seven slices, stained and scored.

Statistical analysis

Allozyme data provided several estimates of genetic variation using BIOSYS I (Swofford and Selander 1989): Mean number of alleles per loci (N_a), percent loci polymorphic (P_l, 100% criterion), and expected heterozygosity (H_e). Diploid genotypes were also transformed for multivariate analysis using the technique of Smouse and Williams (1982). For each allele at a locus minus one, the value of 0.5 was assigned when the allele was present and 0 when the allele was absent. The score when the allele at the locus is in the homozygous state would be 0.5 + 0.5 = 1.0, when it is in the heterozygous state, 0.5 + 0.0 = 0.5. For individuals without the allele the score would be 0. This is equivalent to a measure of the amount of an allele in each individual. Data sets with more than ten alleles can be assumed to have a normal distribution (Smouse and Williams 1982). Transformed allozyme data as well as monoterpene data were analyzed using SAS (1990) multivariate analysis of variance and canonical discriminant analysis.

RESULTS AND DISCUSSION

Allozyme analysis

Overall, 20 of the 22 allozyme loci were polymorphic in slash pine (91%), with an average of 3.0 alleles per

Table 2. *Monoterpene composition of stem-gum samples from slash pine and related taxa.*

Taxa2	Source3	Component1							
		n	a-Pin	Camph	b-pin	D_3-car	Myrc	Lim	b-phel
					%				
Pee - MS	2	10	46.5	0.36	34.37	0.01	0.27	0.01	18.5
Pee - MS	2	7	58.2	0.67	34.24	0.36	0.79	0.01	4.7
Pee - FL	1	14	60.7	0.79	21.91	0.01	2.36	1.05	13.2
Pee \ *Ped*	2	13	30.1	0.43	5.79	0.09	0.56	0.01	63.0
Ped - 13	1	13	37.4	1.05	2.90	0.30	2.95	1.46	54.2
Ped - 14	1	8	51.2	1.16	4.74	0.23	2.88	1.39	38.4
Ped - 16	1	16	44.1	1.06	5.49	0.16	2.87	1.32	45.1
Ped - 18	1	12	36.8	1.08	3.53	0.42	2.79	1.18	54.3
Pcb - 1	1	15	30.4	1.01	2.55	2.67	3.04	1.44	58.9
Pcb - SA1	1	10	40.9	0.69	5.36	3.78	2.66	1.14	45.5
Pcb - 3	1	15	39.0	1.00	3.97	3.48	3.29	2.05	47.3
Pcb - 5	1	17	32.7	0.81	2.10	8.29	3.21	1.38	51.5
Pcb - 9	1	15	41.0	1.11	3.79	10.65	3.16	1.53	38.6
Pcb - 11	1	15	26.0	0.77	1.83	9.41	3.17	0.98	57.8
Pcb - SA11	1	12	38.1	0.68	3.91	0.30	3.08	1.43	52.5
Pcb - 17	1	15	36.3	1.45	2.94	0.59	2.87	1.70	54.2
Pcb - 20	1	16	28.1	0.84	2.31	14.64	3.26	1.79	49.0
Pcb - 21	1	16	33.3	0.97	2.56	0.49	2.70	1.26	58.7
Pcc - 1	1	9	62.1	0.72	4.91	2.50	2.01	0.50	27.2
Pcc - 2	1	12	64.0	1.41	5.55	0.21	2.76	0.41	25.7
Pcc	2	11	58.9	0.68	5.56	3.77	3.82	3.55	23.7
Pch - BH	1	12	68.1	0.48	10.81	0.04	2.75	1.08	16.8
Pch - H	1	11	64.4	0.69	6.49	0.05	3.05	1.19	24.1
Pch - N	1	11	65.0	0.72	8.02	0.06	3.40	1.15	26.7
P cubensis	1	10	89.1	0.73	3.63	0.04	2.42	1.01	3.0
Pp X Pee	2	23	54.3	0.17	41.29	0.61	0.01	0.01	3.6

1 Alpha pinene, camphene, beta pinene, delta-3-carene, myrcene, limonene, and beta phellandrine, respectively.

2 Pee = Pinus elliottii *var.* elliottii, Ped = P. elliottii *var.* densa, Pcb = P. caribaea *var.* bahamensis, Pcc = P. caribaea *var.* caribaea, Pch = P. caribaea *var.* hondurensis, Pp X Pee = Pinus palustris X P. elliotti *var.* elliottii *F1 hybrid.*

3 Original source of the data: 1 - Nikles (1966), 2 - Coyne (1974) unpublished data.

locus. On an individual population basis, an average of 64.8% of the loci in the slash pine populations had more than one allele (**Table 1**). The average slash pine population had 2.04 alleles per locus and an expected heterozygosity of 0.173. The LA "land race", a collection made in a seed orchard in central Louisiana, had slightly higher than expected allozyme diversity, similar to seed orchard samples in loblolly (Schmidtling *et al.* 1999), and longleaf pines (Schmidtling and Hipkins 1998).

Although the loblolly and longleaf pines are represented by only single populations, measures of genetic variability are comparable to those previously published (Schmidtling *et al.* 1999, Schmidtling and Hipkins 1998) (**Table 1**). Loblolly pine averaged slightly higher than slash pine in allozyme variability, with 2.18 alleles per locus, 72.7% polymorphic loci, and an expected heterozygosity of 0.196. Longleaf pine averaged lower in allozyme variability, with 1.68 alleles per locus, 45.5% polymorphic loci and an expected heterozygosity of 0.107. Allozyme variability in the Caribbean pine was higher than that found in slash pine and more comparable to loblolly pine, with 2.16 alleles per locus, 72.7% loci polymorphic, and an expected heterozygosity of 0.221.

In examining the spatial variation in the frequency of alleles for our data, we found only continuous variation across the range of slash pine. The dividing line between the south Florida variety (*densa*) and the "typical" northern variety (*elliottii*) is not distinct and there is a transition zone where intermediate types occur (Squillace 1966). The presence of this transition zone has been ascribed to hybridization between the two varieties (Mergen 1954). If hybridization were occurring in the transition zone, one might expect greater variability in sources in this zone than in areas north or south of the zone. Squillace (1966) did not find greater variability in this transition zone in morphological traits, rather he found the greatest variability in the south.

Similarly, there was no greater variability in allozymes in the transition zone (**Table 1**). Population #205 in our study was collected as a "typical" (var. *elliottii)* source from the transition area. Growth and morphology of the resultant seedlings, however, indicated that the source was more closely related to the *densa* variety (Snyder *et al.* 1967). Source #205 is not any more variable than the adjacent sources (**Table 1**). There does appear to be a decrease in allozyme variability from south to north, however.

In a range-wide study of longleaf pine (Schmidtling and Hipkins 1998) a distinct decrease in allozyme variability was found from west to east. This was interpreted as evidence for a single refugium for longleaf pine in south Texas or north Mexico during the last Pleistocene ice age maximum (ca. 14,000 years before present). The loss in adaptively neutral allozyme alleles resulted from stochastic processes occurring during the rapid migration north and east at the close of the Pleistocene.

Expected heterozygosity is probably the best measure of variability in allozymes. If we plot expected heterozygosity versus distance along the proposed migration route for slash pine at the close of the Pleistocene (Schmidtling 2000), we see a decrease in heterozygosity with distance from the refugium (**Figure 2**). This is consistent with the proposed Pleistocene refugium in south Florida / Bahamas, and a migration

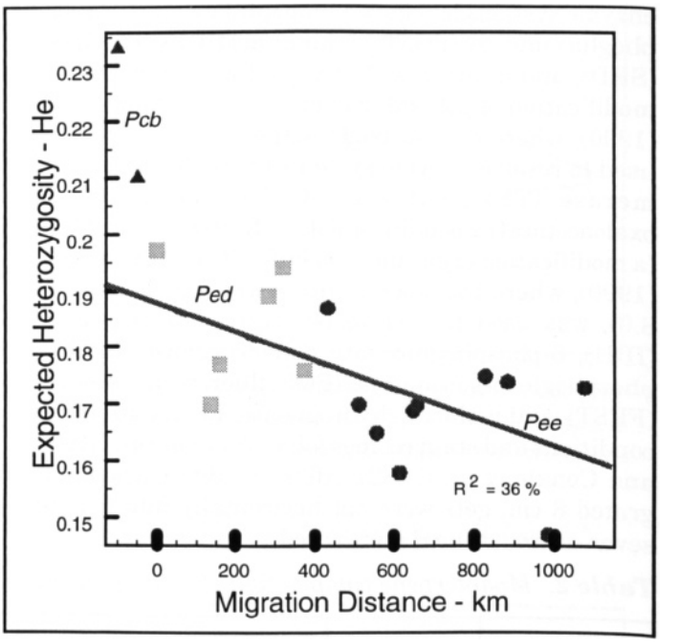

Figure 2. *Plot of expected heterozygosity H_e for slash pine allozyme data versus migration distance from a putative Pleistocene refugium near Key West, FL. H_e is also shown for the Bahamian Caribbean pine (*Pcb*), but is not included in the regression. Data for the LA land race is also not included.*

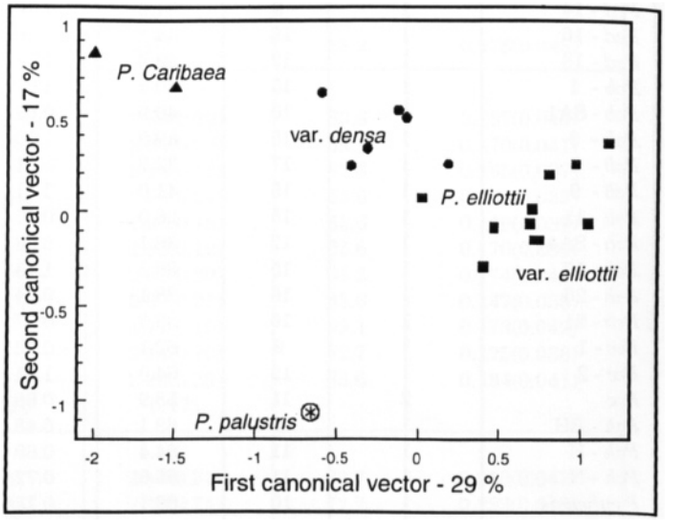

Figure 3. *Plot of first and second canonical vectors (representing 46% of the total variation) from the multivariate analysis of transformed allozyme data. Loblolly pine is not shown, as it is widely separated from the data shown.*

northward after the retreat of the ice (Schmidtling 2000). There is no discernable increase in heterozygosity at the transition between the varieties.

Continuous variation is also evident in a plot of the first two canonical vectors from the canonical analysis of the transformed allozyme data (**Figure 3**). It appears that the continuum includes the Caribbean pine from Grand Bahama(*Pcb*), although *Pcb* is separated from *Ped* by some distance. Longleaf pine is well separated from *Pcb, Ped,* and *Pee*. Loblolly is not shown because it is completely off the chart. These results are in accord with those of Dvorak *et al.* (2000).

Monoterpene analysis

The most obvious difference among taxa in monoterpene composition of stem oleoresin is in the proportion of beta pinene and beta phellandrine (**Table 2**). The northern variety of slash pine (*Pee*) has relatively low levels of beta phellandrine and high levels of beta pinene. The only obvious difference between slash pine and caribbean pine is in the proportion of delta-3-carene, which is very low in both races of slash pine, but can be as high as 14% in *Pcb*. Samples from *Pinus cubensis* contain almost 90% alpha pinene with only small amounts of other constituents. The longleaf pine X slash pine hybrid essentially had only alpha and beta pinene, more similar to pure longleaf pine (Franklin and Snyder 1971) than to slash pine.

A plot of the first two canonical vectors from the monoterpene analysis shows a much closer relationship between the south Florida slash pine (*Ped*) and the Bahamian Caribbean pine (*Pcb*) (**Figure 4**) than the canonical analysis of the allozyme data (**Figure 3**). Both analyses show a rather distant relationship between longleaf pine and the other taxa, in accord with Dvorak *et al.* (2000).

Monoterpene contents of oleoresin are quite suitable for studies of geographic variation, since they are highly heritable and little affected by environment (Squillace and Wells 1981). This is evident in **Table 2** and **Figure 4**. Two of the *Pcb* samples were collected from provenance tests in South Africa (SA-1 and SA-11), whereas the other samples were collected *in situ* (Nikles 1966). It is apparent that very little difference exists in the monoterpene composition in samples collected on different continents in different climates. Similarly, the Coyne collections were made in south Mississippi, in provenance tests and natural stands. The *Pcc* sample collected there, as well as the *Pee / Ped* transition sample (the same as source #205 in the allozyme analysis) are comparable to the samples collected *in situ*.

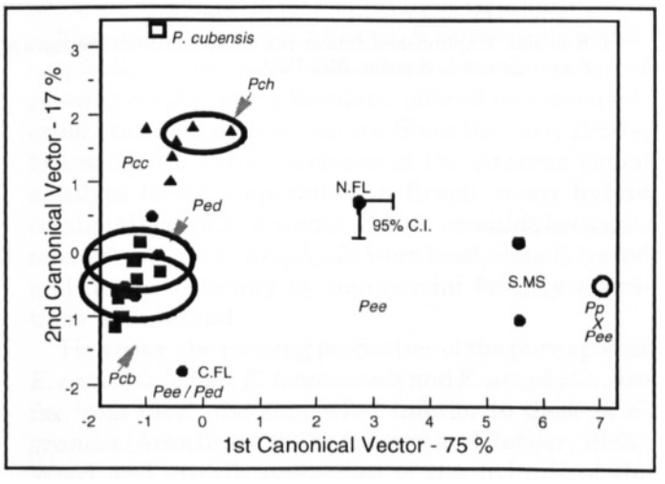

Figure 4. *Plot of first and second canonical vectors (representing 92% of the total variation) from the multivariate analysis of monoterpene data.*

CONCLUSIONS

Both the monoterpene and allozyme analyses suggest a very close relationship between slash pine and Bahamian Caribbean pine. Natural hybrids in adjacent plantations of *Pcc* and *Pee* have been reported (Slee 1971). Seed yields of controlled hybrids between slash pine and Caribbean pine are related to the geographic distance among the taxa. For these hybrids, the order of seed yields were: *Pcb X Pee* > *Pcc X Pee* > *Pch X Pee* (Nikles 1995). There may be some influence of longleaf pine on the evolution of *Pee* as suggested by Nikles (1966), but the evidence presented here is not conclusive. Artificial hybrids among most of the southern pines have been made (Snyder and Squillace 1966), including the slash X longleaf hybrid. Natural hybrids between longleaf and slash pine have been reported in north Florida (Mergen 1958), but the evidence is not very conclusive. A group of putative natural hybrids from the same area were determined to belong to one species or the other, with no intermediates[3].

Recent evidence has shown natural hybridization between *Pch* and *P. tecunumanii*, at the far western end of the distribution of Caribbean pine taxa (Dvorak *et al.* 2000). This suggests a closer relationship between the southern pines and the closed-cone pines than was previously thought, and may indicate a Mexican origin for slash and Caribbean pines.

The data presented here show continuous variation from *Pch* to *Pcc* to *Pcb* to *Ped* to *Pee*, which supports a hypothesized migration route from southern Mexico through the Caribbean islands and up the Florida peninsula. During the most recent Pleistocene ice age, about 14,000 years before present, sea level was around 100 meters lower than currently, exposing large areas of the continental shelf. The Bahamas would have consisted of several large islands, rather than the many small islands. Distances between the mainland and the islands would have been less, facilitating migration after the close of the Pleistocene.

The somewhat arbitrary nature of species determination is shown here. One could certainly justify calling the slash pine / Caribbean pine complex one species with five varieties.

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[1] Original data kindly supplied by Dr Nikles.

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[3] T. Kubisiak. Unpublished data on file at the Southern Institute of Forest Genetics, Saucier, MS, USA.