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# Genetic variability and structure of natural and domesticated populations of Caribbean pine (*Pinus caribaea* Morelet)

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**Abstract** Isozyme analysis of seed samples derived from natural and managed populations of the tropical pine *Pinus caribaea* vars 'bahamensis' and 'caribaea' was used to assess population genetic structure in its native range and to detect changes occurring during early domestication of the species. Baseline data from natural populations of the two varieties showed that populations sampled as seed are characterized by high gene diversity (mean $H_e = 0.26$) and a low level of inbreeding ( mean $F_{is} = 0.15$). A UPGMA tree of genetic relatedness among populations indicates that the two varieties represent distinct evolutionary units. Within each variety there is significant differentiation among populations, and this is greater for the more fragmented populations of var 'bahamensis' ($F_{st} = 0.08$) than for var 'caribaea' ($F_{st} = 0.02$). Seed from a seed orchard population of var 'caribaea' established within its natural range showed no change in genetic diversity but did show a reduced inbreeding coefficient ($F_{is} = 0.09$) compared with its progenitor populations, suggesting a decrease in selfing and/or biparental inbreeding. A bulked seed sample from an exotic plantation of var 'bahamensis' in Australia displayed a large increase in the inbreeding coefficient ($F_{is} = 0.324$) compared with that found in natural populations, possibly due to elevated self-fertilization. Finally, a bulked seed sample from an exotic plantation population of var 'caribaea' from China showed enhanced genetic diversity, an increase in the inbreeding coefficient and more linkage disequilibrium than its presumed progenitor populations. It was also genetically divergent from them. RFLP analysis of chloroplast DNA variation in the Chinese sample suggested that seeds of the related taxa *P. elliottii* and *P. taeda*, or seeds derived from hybridization with these taxa growing in the seed production area, had been included in the seed crop during harvesting. We conclude that monitoring of appropriate genetic markers may be an effective means of identifying potentially deleterious genetic changes occurring during forest tree domestication.

**Key words** *Pinus caribaea* · Isozymes · cpDNA · Genetic structure · Domestication

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## Introduction

Many forest tree species are in an early and active phase of the crop domestication process (Simmonds 1985). This involves sampling and movement of wild germplasm and the establishment of managed plantation populations both within and outside the natural range of the species. In the course of early domestication substantial unintentional genetic change may occur as a consequence of the sampling process, natural selection and alterations in reproductive behaviour. This may generate significant genetic differences between seed samples harvested from wild stands and those derived from domesticated populations (Savolainen and Karkkainen, 1992).

A proportion of these genetic changes can be detected by monitoring differences in selectively neutral genetic markers scored at the seed stage of the life cycle in native and domesticated populations (Savolainen and Yazdani 1991). Examples of such changes include reduction in genetic marker diversity, indicating a

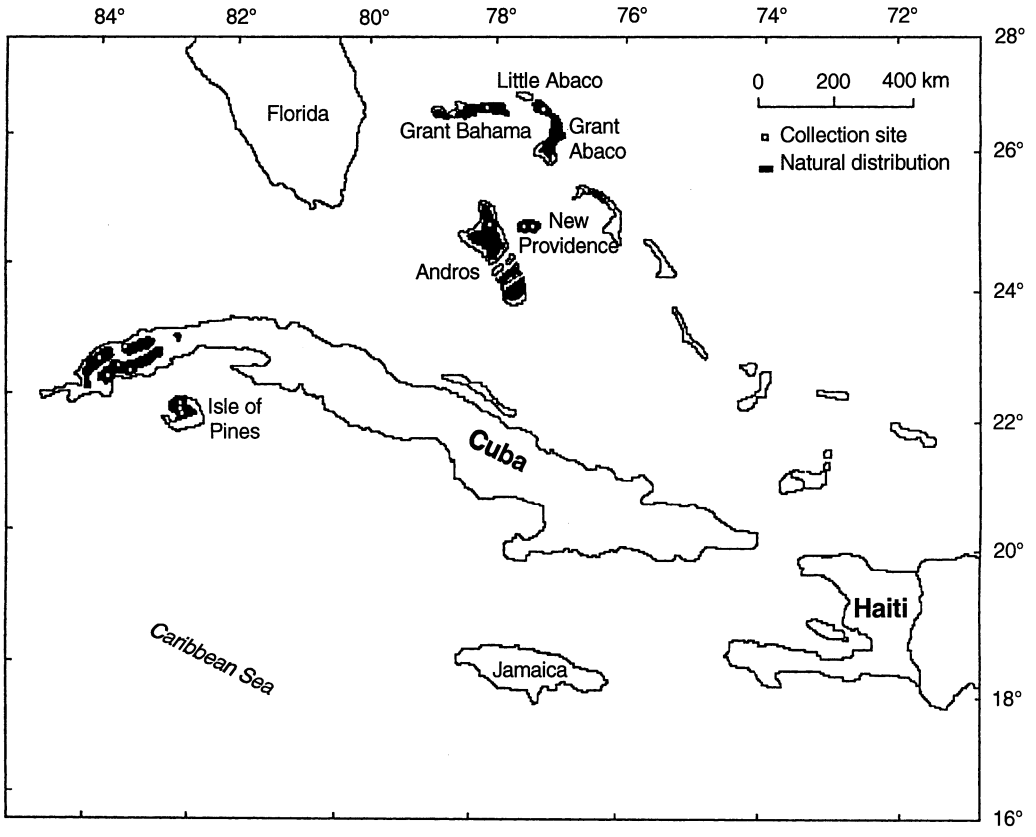


**Fig. 1** Natural distribution of *Pinus caribaea* var 'caribaea' (Cuba and Isle of Pines, now Isla de la Juventud) and var 'bahamensis' (Bahamas). Also shown are locations of natural populations sampled in this study

general reduction in genetic variability of the managed populations, and changes in the single locus and multilocus structure of seed from managed populations, signifying alterations in the breeding system of the species (Ennos 1996).

Certain of these genetic alterations (reduction in diversity, increased inbreeding) are highly undesirable and may compromise the long-term success of genetic improvement programmes which utilize seed from the domesticated populations (Namkoong et al. 1988). It is therefore important during early domestication to monitor the form and extent of unintentional changes in the genetic structure of forest tree populations in order to recognize and avoid future genetic problems.

Caribbean pine, *Pinus caribaea* Morelet, is an important and widely planted tropical conifer (Lamb 1973). For the last 70 years it has been used extensively as an industrial plantation species both within its natural range and as an exotic in tropical and subtropical areas throughout the world. Taxonomically the species has been split into three varieties which occur in different geographic regions (Vidakovic 1991). In the study presented here we were concerned with the genetic structure and genetic changes in two of these, var 'caribaea' Morelet and var 'bahamensis' Barr. et Golf. Natural populations of var 'caribaea' are restricted in distribution to the island of Cuba and the adjacent Isla de la Juventud. Populations of var 'bahamensis' occur to the north on the scattered islands of the Bahamas (Fig. 1).

In recent years native germplasm of *P. caribaea* has been used to establish various types of domesticated populations from which seed is collected for further planting. Some populations have been managed for seed production in situ. Selected genotypes of var 'caribaea' have been used to establish a seed orchard within its native range in Cuba to provide seed for commercial planting (Brodie 1994). In addition, seed collections from both varieties have been used to found plantations in exotic locations, notably Australia and China, These in turn are used as seed sources for further planting. In China at least, the genetic base for these introductions is believed to be narrow (Zheng 1997).

The first objective of this research was to employ isozyme analysis of seed samples to describe the genetic variability and population structure of native populations of *P. caribaea* var 'caribaea' and var 'bahamensis'. The extent of genetic differences between these two varieties was analysed to establish whether they represent different 'evolutionary units' as implied by their varietal status (Moritz 1995).

The second objective was to detect and quantify differences in genetic variability and structure between

seed samples derived directly from native stands of *P. caribaea* and those collected from domesticated and managed populations. Domesticated populations included a seed stand and a seed orchard of *P. caribaea* var 'caribaea' found within its native range and exotic plantations of var 'bahamensis' and var 'caribaea' in Australia and China, respectively. From these comparisons inferences were made about the scale and nature of genetic changes, detectable by means of genetic markers, that had occurred during early domestication. Additional analysis of chloroplast DNA markers was used to clarify the origin of the seed sample derived from the exotic plantation in China.

## Materials and methods

### Plant material

Analysis of isozyme variation was conducted on open-pollinated seed sampled randomly from at least 10 maternal trees in each of the native and managed populations under study. Seed of var 'bahamensis' was derived from collections made in the 1970s by the Oxford Forestry Institute for the purpose of establishing international provenance trials (Baylis and Barnes 1989). Five natural populations and one exotic plantation population from Australia were analysed (Table 1).

For var 'caribaea', open-pollinated seed collections were made from four native forests on Cuba and one on the adjacent island of Isla de la Juventud in summer 1994 (Brodie 1994). One of the populations in Cuba (Marbajita) is managed in situ for seed production. Samples were also analysed from the crop of a seed orchard established from 109 plus trees and sited in Mala Aguas, Cuba. The majority of the plus trees in this orchard (68) were from the Marbajita population on Cuba. In addition, a seed sample of *P. caribaea* var 'caribaea' was supplied by the Chinese Academy of Forestry from an exotic plantation in Zhanjiang (Table 1). This is one of the major seed-producing areas for *P. caribaea* in China. The plantations in China are believed to be based on a limited introduction of germplasm made in the 1960 s (Zheng 1997).

### Isozyme analysis

From 35 to 52 seeds were electrophoretically assayed from each sampled population of var 'bahamensis'. At least 50 seeds were scored from each population of var 'caribaea'. Seeds were germinated at room temperature until radicles were 5 mm in length. Diploid embryo tissue was excised, and enzyme extracts were made from this tissue in 0.2 *M* sodium phosphate extraction buffer containing 1 mg/ml dithiothreitol.

Extracts were absorbed onto filter paper wicks and run on horizontal starch gels (11%) for 5 h. Gels were sliced and stained for five enzyme systems coding for eight polymorphic loci using the methods of Cheliak and Pitel (1984). The enzymes scored were AAT (aspartate aminotransferase, E.C.2.6.1.1; 3 loci), IDH (isocitrate dehydrogenase, E.C.1.1.1.42; 1 locus), MDH (malate dehydrogenase, E.C.1.1.1.37; 1 locus), 6PGD (6-phosphogluconate dehydrogenase, E.C.1.1.1.44; 2 loci) and PGM (phosphoglucomutase, E.C.5.4.4.2; 1 locus). Inheritance of isozyme variation was deduced from a separate analysis of segregation in open-pollinated families (Zheng 1997).

### Restriction fragment length polymorphism (RFLP) analysis of chloroplast (cp)DNA

Following seed germination, DNA was extracted from embryos using the CTAB method (Murray and Thompson 1980). Three

**Table 1** Details of populations of *Pinus caribaea* var 'caribaea' and var 'bahamensis' used in study. The number of maternal trees from which seed was sampled are given

| Site name | Site code | Latitude (N) | Longitude (W) | Altitude (m) | Number of trees |
|---|---|---|---|---|---|
| var caribaea | | | | | |
| Los Palacios | PAL 0301-0315 | 22°36′ | 83°14′ | 20–30 | 15 |
| La jagua | JAG 0601-0618 | 22°43′ | 83°38′ | 200–280 | 18 |
| Juan Manuel | MAN 0701-0714 | 22°38′ | 83°33′ | 70–150 | 14 |
| Marbajita[a] | MBJ 2101-2117 | 22°49′ | 83°28′ | 50–70 | 17 |
| Isla de la Juventud | IDJ 2001-2016 | 21°25′<br>21°46′<br>21°43′ | 83°00′<br>83°02′<br>83°55′ | 50–100 | 16 |
| Malas Aguas[b] | SOR 1001-1117 | 22°41′ | 83°53′ | 50 | 43 |
| Zhanjiang[c] | CHN | 21° | 100°(E) | – | – |
| var bahamensis | | | | | |
| San Andros | SA 69/88/1-10 | 24°54′ | 78°01′ | 5 | 10 |
| Adelaide | AD 77/88/1-10 | 25°00′ | 72°26′ | 10 | 10 |
| E. New Providence | ENP 78/88/1-10 | 25°01′ | 77°24′ | 5 | 10 |
| Little Abaco | LA 39/77/1-10 | 26°46′ | 77°30′ | – | – |
| High Rock | HR 07/78/1-10 | 26°30′ | 78°20′ | – | – |
| Byfield[c] | BA Australia | –[d] | – | – | – |

[a] Managed as a seed stand
[b] Seed orchard
[c] Exotic plantation
[d] Data not available

**Table 2** Estimates of mean number of alleles per locus ($n_a$), percentage polymorphic loci (P%), unbiased gene diversity ($H_e$) (Nei 1978) and inbreeding coefficient within populations ($F_{is}$) for natural and managed populations of *P. caribaea* vars 'caribaea' and 'bahamensis'. Also given is the value of $F_{st}$ among the natural populations within each variety. (Standard errors are given in brackets)

| Population | $n_a$ | P(%) | $H_e$ | $F_{is}$ | $F_{st}$ |
|---|---|---|---|---|---|
| var caribaea | | | | | |
| PAL | 2.3 (0.3) | 62.5 | 0.274 (0.094) | 0.139** | |
| JAG | 2.1 (0.4) | 50.0 | 0.250 (0.096) | 0.107 | |
| MAN | 2.3 (0.4) | 75.0 | 0.286 (0.086) | 0.173** | |
| MBJ | 2.3 (0.4) | 62.5 | 0.297 (0.097) | 0.156** | |
| IDJ | 2.3 (0.4) | 50.0 | 0.253 (0.101) | 0.167** | |
| Mean | 2.26 | 60 | 0.272 | 0.148 | 0.020 (0.008)** |
| SOR | 2.3 (0.3) | 62.5 | 0.274 (0.091) | 0.09 | |
| China | 2.4 (0.2) | 87.5 | 0.350 (0.065) | 0.221** | |
| var bahamensis | | | | | |
| SA | 2.1 (0.4) | 50.0 | 0.225 (0.086) | 0.218** | |
| AD | 1.9 (0.3) | 62.5 | 0.261 (0.094) | 0.180** | |
| ENP | 2.4 (0.3) | 50.0 | 0.227 (0.091) | 0.107 | |
| LA | 2.3 (0.3) | 62.5 | 0.243 (0.086) | 0.014 | |
| HR | 2.5 (0.3) | 62.5 | 0.249 (0.080) | 0.139** | |
| Mean | 2.24 | 57.5 | 0.241 | 0.164 | 0.078 (0.005) ** |
| BA | 2.4 (0.3) | 62.5 | 0.248 (0.093) | 0.324** | |

** Differences from F = 0 are significant at $P < 0.01$

bulked embryo samples of var 'caribaea' from Cuba (SOR Table 1), var 'hondurensis' (supplied by Oxford Forestry Institute) and putative var 'caribaea' from China (CHN Table 1) were extracted. Two single embryo extracts were made from var 'caribaea' from Cuba (SOR Table 1) and three from putative var 'caribaea' from China (CHN Table 1). Two-microgram aliquots of DNA from each sample were digested to completion with *Bam*HI, separated on 1% agarose gels in TAE buffer for 16 h at 0.5 V/cm, then transferred to Hybond-N membrane (Amersham) and linked to the membrane by baking at 80°C for 2 h. A cloned fragment (pPCB28) from the chloroplast genome of *Pinus contorta* Dougl. (Lidholm and Gustafsson 1991) was radioactively labelled with $\alpha[^{32}P]$-dCTP (Amersham) using 'Ready to Go Labelling Beads' (Pharmacia Biotech) following the manufacturer's instructions. Southern hybridization was conducted overnight at 65°C in a Hybaid mini-10 hybridization oven (Sambrook et al. 1989). After washing at 65°C hybridized blots were exposed to X-ray film in the presence of an intensifying screen for 24 h. The sizes of the restriction fragments were determined by comparison with a 1-kb ladder.

### Isozyme data analysis

For each population genetic diversity was assessed in terms of mean number of alleles per locus, percentage of polymorphic loci (95% criterion) and gene diversity, $H_e$ (expected heterozygosity). Within each population single-locus genetic structure was investigated by testing for deviations from the Hardy-Weinberg (H-W) equilibrium using the exact test available in the GENEPOP package (Raymond and Rousset 1995). The extent and direction of deviation from the H-W equilibrium within each population was quantified by calculating the weighted mean of $F_{is}$ across all loci together with the significance of deviation from zero using the FSTAT package (Weir and Cockerham 1984; Goudet 1995). Multilocus genetic structure was investigated by calculating genotypic linkage disequilibrium between all pairs of loci using GENEPOP.

Within each variety the degree of differentiation among populations was estimated by calculating $F_{st}$ and its standard error over all loci using FSTAT. The genetic relationships among all populations were found by estimating Nei's genetic distance among all pairs of populations (Nei 1978). The matrix of genetic distances was used to construct a UPGMA tree of genetic relatedness among populations using the BIOSYS package (Swofford and Selander 1989).

## Results

### Isozyme analysis

Table 2 summarizes measures of isozyme gene diversity in both the natural and managed populations of *P. caribaea* vars 'caribaea' and 'bahamensis'. Levels of isozyme diversity are high within natural populations. Mean numbers of alleles per locus range from 1.9 to 2.5, percentage of polymorphic loci from 50% to 75% and gene diversity from 0.227 to 0.297. Mean values of diversity for natural populations of the two varieties are very similar.

Gene diversity values for managed populations are very similar to those of their progenitor populations for the seed stand and seed orchard population of var 'caribaea' and the Australian population of var 'bahamensis'. In contrast, there appears to be an elevated level of isozyme variability in the Chinese population of var 'caribaea' ($H_e = 0.35$ compared with a mean value for natural populations of this variety of 0.272).

Tests for departure from H-W equilibrium showed significant deviations for at least one locus for every population, whether natural or managed. All population values of $F_{is}$ are positive, indicating a deficit of heterozygotes (Table 1). For the natural populations mean values of $F_{is}$ are similar for vars 'caribaea' (0.148) and 'bahamensis' (0.164). However $F_{is}$ in the seed orchard sample is lower ($F_{is} = 0.09$), while the deficiencies of heterozygotes in the exotic plantations are substantially higher ($F_{is} = 0.221$ in the Chinese and $F_{is} = 0.327$ in the Australian populations) than in their natural counterparts.

Genotypic linkage disequilibrium was not significant for any pairs of loci in any of the populations of var

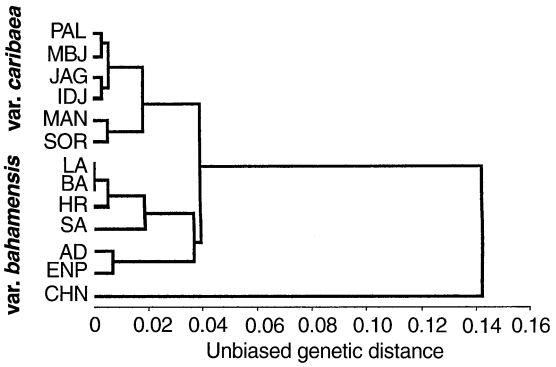


**Fig. 2** UPGMA tree of genetic relatedness of natural and managed populations of *Pinus caribaea* vars 'caribaea' and 'bahamensis' based on matrix of Nei's genetic distances between populations derived from isozyme data. See Table 1 for site codes

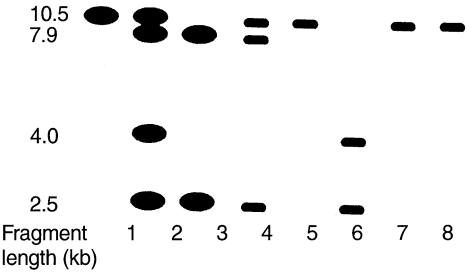


**Fig. 3** Results of cpDNA RFLP analysis of *Pinus caribaea* seed samples. Lanes represent total DNA extracts from bulked or single embryos digested with *Bam*HI and hybridized to the pPCB28 chloroplast probe from *P. contorta*. *Lanes 1, 2* and *3* are bulked seed extracts of var 'caribaea' from Cuba, Chinese sample CHN and var 'hondurensis', respectively, *lanes 4* to *6* are individual embryo extracts from the CHN sample, *lanes 7* and *8* are individual embryo extracts of var 'caribaea' from Cuba

'bahamensis'. In var 'caribaea' a single (but different) pair of loci was in significant linkage disequilibrium in each of 3 populations. However in 1 population, the exotic plantation in China, linkage disequilibrium was significant between four pairs of loci.

Table 2 also gives the values of genetic differentiation, $F_{st}$, among the natural populations within each variety. These values are combined estimates over all loci. $F_{st}$ for both varieties is significantly greater than zero, indicating genetic differentiation among populations. However the $F_{st}$ value for var 'bahamensis' (0.078) is some fourfold greater than for var 'caribaea' (0.02).

Figure 2 shows the UPGMA tree of genetic relatedness among all populations sampled. The natural populations fall clearly into two clusters corresponding exactly to the two varieties 'caribaea' and 'bahamensis'. The seed stand and seed orchard populations fall within the 'caribaea' cluster, the subspecies group from they are derived. Similarly, the Australian plantation population falls within its progenitor 'bahamensis' group. However, the plantation population from China is genetically distant from any other population, natural or exotic (Nei's genetic distance D = 0.14).

## cpDNA analysis

Figure 3 illustrates the RFLP banding patterns obtained for the bulked and individual samples of *P. caribaea* using the *Bam*HI-pPCB28 enzyme-probe combination. This enzyme-probe combination has previously been used to distinguish among the varieties of *P. caribaea* and to distinguish them from related species (Nelson et al. 1994). Both the bulked and individual samples of var. 'caribaea' from Cuba show only a single strong 10.5 kb band as reported by Nelson et al. (1994). The bulked sample from var '*hondurensis*' shows two strong bands of 7.9 and 2.5 kb., which is again as expected from Nelson et al. (1994). However, the bulked sample of putative var 'caribaea' from China (CHN) has bands at 10.5, 7.9, 4.0, and 2.5 kb. Individual samples of CHN have either one band (10.5 kb), two bands (4.0 and 2.5 kb) or three bands (10.5, 7.9 and 2.5 kb.). The second of these banding patterns shown by CHN was previously reported only from the related species *P. elliottii*, and the third banding pattern only from the related species *P. taeda* (Nelson et al. 1994).

## Discussion

One clear result to emerge from this study is the genetic distinctiveness of the two varieties of *P. caribaea* in terms of frequencies of nuclear isozyme markers. Differences between the varieties with respect to vigour and adaptation have previously been demonstrated in field trials (Kemp 1973). Variation in the chloroplast DNA of the two varieties has also been seen within a limited sample of individuals (Nelson et al. 1994). The present results confirm that the two varieties should be regarded as separate 'evolutionary units', rather than differently adapted populations of a single genetic unit (Moritz 1994).

The two varieties of *Pinus caribaea* studied here both possess fragmented distributions, being confined to islands of varying size and degrees of isolation within the Caribbean sea. Despite these fragmented distributions all populations retain large quantities of isozyme variation, equivalent to those found in continuously distributed species of outcrossing coniferous trees (Hamrick et al. 1992). Effective population sizes have presumably remained large enough over long time periods to prevent the erosion of genetic diversity by drift. Nevertheless, the populations have been sufficiently isolated for genetic drift to lead to differentiation between them. This is much more marked in var 'bahamensis', which possesses the more fragmented population structure, as anticipated from theory. Previous analyses of the third variety of *P. caribaea*, var

'hondurensis', which is located on the South and Central American mainland, have also revealed high levels of isozyme variation comparable to those found in the present study (Matheson et al. 1989).

Within natural populations of vars 'caribaea' and 'bahamensis' there appears to be a low but significant level of inbreeding, at least at the seed stage of the life cycle assayed here. Mating system studies indicate that this is caused by a low frequency (5–10%) of self-fertilization (Matheson et al. 1989; Zheng and Ennos 1997). A very low level of linkage disequilibrium was found in these predominantly outcrossing natural populations, as expected from population genetic theory.

Using the data from natural populations as a baseline we can explore the extent of genetic change, detectable from isozyme markers, that has occurred in the three managed populations sampled here. No change in the genetic structure of seed from the managed seed stand is evident. Likewise, changes in the seed sample from the Cuban seed orchard appear to be small. The only trend noted is a slight reduction in the inbreeding coefficient, $F_{is}$. This may indicate a reduction in the rate of selfing and/or biparental inbreeding in this managed population, a result found in other assessments of seed orchards (Moran et al. 1989; Savolainen and Karkkainen 1992) and confirmed by more detailed studies of the mating system of these *P. caribaea* populations (Zheng and Ennos 1997).

Seed from the exotic population of var 'bahamensis' grown in Australia shows essentially the same degree of isozyme diversity as natural populations of this variety and high genetic similarity. Thus, no loss of genetic variation or change in allele frequencies through founder effects is apparent. However, the analysis of $F_{is}$ indicates a greatly elevated level of inbreeding in the seed sample. This may be due to increased levels of self fertilization in the exotic location where difficulties with flowering may restrict the supply of outcross pollen.

The most dramatic difference in genetic structure between seeds from natural and managed populations is displayed by the Chinese sample of var 'caribaea', CHN. This shows increased levels of isozyme diversity compared with those in its putative progenitor populations. In addition, it displays increases in $F_{is}$ and elevated levels of gametic disequilibrium compared with natural populations of var 'caribaea'. Moreover, it is separated from these populations by a very large genetic distance in the UPGMA tree.

Possible explanations for these results are either that the Chinese sample represents a mixture of seed bulked from genetically distinct populations or taxa or that it is the product of hybridization with such populations or taxa. Mixing of populations with different allele frequencies may increase genetic diversity and will generate both an apparent deficit of heterozygotes (Walhund 1928) and significant linkage disequilibrium, as found in the sample.

Analysis of cpDNA from the CHN sample lends weight to these arguments. cpDNA markers found previously only in *P. elliottii* and *P. taeda* are present in the CHN sample. cpDNA in pines is paternally inherited (Neale and Sederoff 1989). The presence of cpDNA markers from *P. elliottii* and *P. taeda* in the CHN sample could therefore be due either to the inclusion of pure seed from these two taxa in the sample or to interspecific hybridization following pollen flow from *P. elliottii* and *P. taeda* into the *P. caribaea* stand. Both species are known to be grown as exotics in the vicinity of the CHN seed stand in the Zhanjiang region of China (Zheng 1997), and hybrids between *P. caribaea* and *P. taeda* have been recorded (Duffield and Righter 1953). This result is of considerable practical importance since these three taxa are adapted to quite different climatic conditions (Nikles 1967). Planting a mixture of seed in any one climatic region is likely to lead to failure of a considerable proportion of the crop through maladaptation.

The results of this study demonstrate that both isozyme and cpDNA genetic markers can be of considerable practical utility in monitoring a defined but limited set of genetic changes that may occur during the process of domestication of tree species. The results obtained from such selectively neutral genetic markers are complementary to traditional provenance and progeny trials which provide data on differences in adaptive genetic variation (Zheng et al. 1994). Isozyme studies have shown that the loss of genetic variation through inadequate sampling does not appear to have taken place in any of the managed populations studied. A similar lack of genetic sampling effects has previously been reported in studies of domestication of indigenous conifers (Szmidt and Muona 1985; Savolainen and Yazdani 1991). However, increases in inbreeding in an exotic location and the contamination of samples by closely related exotic taxa planted in adjacent areas are problems which have been clearly identified. These will need to be addressed if long-term tree improvement programmes with *P. caribaea* in exotic locations are to succeed.

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