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Effects of native forest regeneration practices on genetic diversity in *Eucalyptus consideniana*

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Abstract Impacts of forest harvesting and regeneration practices on genetic diversity in the Australian native forest species *Eucalyptus consideniana* Maiden (yertchuk) were examined using 29 Mendelian DNA markers (18 RFLPs and 11 microsatellites). Two replicate logging coupes were studied from each of the two most commonly employed silvicultural treatments: clear felling with aerial re-sowing and the seed tree system. For each coupe, genetic diversity measures were compared between a sample of the sapling regeneration and a corresponding control sample from bordering unharvested trees. When calculations were performed over all 29 loci, significant reductions of allelic richness (A_R), effective number of alleles (A_E) and/or expected heterozygosity (H_E) were detected on one or both of seed tree coupes, but on neither of the clear falls. When calculations were performed over the 11 microsatellites alone, all three measures, A_R, A_E and H_E, were significantly reduced on both of the seed replicates but on neither of the two clear falls. In contrast, when the RFLPs were examined separately, there were no significant reductions of diversity on either of the two seed tree coupes or on the two clear falls. These results suggest that genetic erosion is more likely under the seed tree system than under clear-felling with aerial re-sowing and that there is greater statistical power to detect it with microsatellites than with RFLPs. A Monte Carlo simulation to test the statistical significance of the number of apparently lost or gained alleles showed that significant losses of alleles above specified threshold frequencies occurred only in the two seed tree replicates. Three of the four control and regeneration population pairs were significantly differentiated, as indicated by exact tests or by pairwise F_{ST} estimates. Comparisons of CONTML dendrograms, constructed for the regeneration populations only versus the control populations only, indicated that genetic drift was significantly promoted under forest management. No significant decreases in observed heterozygosity, or increases in the panmictic index (*f*), were observed in any of the comparisons suggesting that inbreeding was not promoted by a single rotation of forest management.

Keywords Genetic diversity · Silviculture · RFLPs · Microsatellites · *Eucalyptus consideniana*

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Introduction

Genetic diversity within species is thought to be critical to their long term survival in changing environments (Newman and Pilson 1997; Westemeier et al. 1998; Booy et al. 2000). It has been internationally recognised that sustainable forest management should not lead to a long term decline in the genetic diversity present in forest dwelling species (Montréal Process Working Group 1999). Trees are keystone species in forest ecosystems, whose genetic diversity should not be adversely impacted by commercial forestry harvesting and regeneration practices (Young et al. 2000). With regard to the regeneration of tree species in harvested native forests in Australia, there is a policy mandate to conserve both local species composition and local gene pools (Commonwealth of Australia 1992). The degree to which the latter goal, the conservation of local gene pools within harvested tree species, is achieved by regeneration practices is largely unknown. In light of the large numbers of tree species harvested on a world wide scale, it is surprising that the impacts of forest management practices on the genetic diversity of regeneration have been studied in only a very limited number of species (e.g., Knowles 1985; Neale 1985; Gömöry 1992; Stoehr

and El-Kassaby 1997; Adams et al. 1998; Gillies et al. 1999; Rajora 1999; Thomas et al. 1999; El-Kassaby 2000; Macdonald et al. 2001; Perry and Bousquet 2001; Lee et al. 2002; Glaubitz et al. 2003). The majority of these studies have been in northern hemisphere gymnosperms, with only two in tropical species (Gillies et al. 1999; Lee et al. 2002), and only a single study to-date within the species-rich and commercially important genus *Eucalyptus* (Glaubitz et al. 2003).

In Australia large areas of native eucalypt forest are harvested for sawlogs and/or woodchips, and then regenerated either by the aerial application of tree seed to clear falls or via the seed tree system. Seed for aerial re-sowing of clear falls is generally collected from only a small number of trees. Alternatively, under the seed tree system, only a limited number of seed trees of each species are left behind on a logging coupe (i.e. a cut block or harvested area). Hence, there is a potential for bottlenecks to occur under forest management, resulting in loss of genetic diversity over the whole genome. Depending on their severity, the occurrence of such bottlenecks should be diagnosable using genetic markers.

We recently completed a large-scale study in the Australian native forest species *Eucalyptus sieberi*, in which statistically significant changes in diversity measures were not detected over a single rotation (Glaubitz et al. 2003). We attributed the genetic resilience of this species to its local abundance (most common tree species locally) along with favourable characteristics of its reproductive biology, which have led to it being promoted by silviculture (Lutze 1998). Since less abundant species may be more genetically sensitive (Boyle 2000), it is of interest whether rare or minor eucalypt species on the same study site share the genetic stability of *E. sieberi*. *Eucalyptus consideniana* Maiden (yertchuk), the closest relative of *E. sieberi* (Boland et al. 1984), provides a good contrast in that it is a minor species in the logged portions of the site whose relative abundance has fallen dramatically in the regeneration. The proportional representation of *E. consideniana* has fallen from 13% prior to logging to just 5% in the regeneration (on clear falls and seed tree coupes) at 3 years post-harvest (Lutze 1998), and is predicted to fall even further by 10 years post-harvest (Mark Lutze, Victoria Department of Natural Resources and Environment, personal communication). *E. consideniana* further contrasts with *E. sieberi* in terms of its commercial value; while *E. sieberi* is commercially the most important species in this forest type (the lowland sclerophyll forests of the East Gippsland region of the state of Victoria), *E. consideniana* has little commercial value due to its poor form (Boland et al. 1984). Hence, the temptation may exist for forest managers to increase the economic value of the regrowth forests by reducing the proportional representation of *E. consideniana*, possibly putting this species at a disadvantage.

For these reasons we chose to examine in this study the impacts of native forest harvesting and regeneration practices on genetic diversity in *E. consideniana*, utilising the same field site as in our *E. sieberi* study (Glaubitz et al. 2003). Two replicate coupes were examined from each of the two most commonly used silvicultural treatments, the seed tree system and clear-felling with aerial re-sowing. We report herein the results from comparisons of genetic diversity measures over 29 Mendelian loci (11 microsatellites and 18 RFLPs) between samples of regeneration on these coupes and corresponding control samples from bordering unharvested trees.

Materials and methods

Field site and sampling strategy

The Cabbage Tree Creek Silvicultural Systems Project (SSP) of the Victoria Department of Natural Resources and Environment (DNRE), situated near Orbost, Victoria (latitude and longitude: 37 41′S 148 44′E), was used as the field site for this study. It lies within a lowland sclerophyll forest dominated by *E. sieberi, Eucalyptus globoidea*, and *Eucalyptus baxteri*. *E. consideniana* is one of six additional, less common, eucalypt species. The distribution of these six minor and locally rare species is dependent on the microsite; *E. consideniana* tends to occur on drier, more nutrient poor soils. The Victoria DNRE have examined a broad assortment of silvicultural treatments on this 400 ha site, which prior to their study had not previously been harvested (Lutze 1998). For the present genetic diversity study we have examined only those that are in most common operational use in this forest type: the seed tree system and clear felling with aerial re-sowing. Two replicate logging coupes were selected for each of these silvicultural treatments (referred to as ST1 and ST2 for the seed tree system and CF1 and CF2 for the clear felling system). CF2 was harvested in 1989 while ST1, ST2 and CF1 were harvested in 1990.

From the *E. consideniana* regeneration on each of the four selected logging coupes, 50 saplings were sampled in the year 2000. Comparative unharvested controls for each of these regeneration samples were composed of 50 mature trees sampled from the unlogged forest bordering the corresponding logging coupe. In the case of the two seed tree treatments (ST1 and ST2), all residual *E. consideniana* seed trees were also sampled and included with the corresponding control population (four seed trees in each case). In total, 408 trees were sampled. From each sample DNA was extracted from approximately 5 g of leaf material following the protocol of Glaubitz et al. (2001).

RFLP and microsatellite markers

Genotypes were obtained for 394 of the 408 sampled trees at 29 Mendelian loci: 18 single-copy nuclear RFLPs and 11 microsatellites. Twenty three of these 29 marker loci have been placed on either a *Eucalyptus globulus* genetic linkage map (Thamarus et al. 2002) or a *Eucalyptus nitens* map (Byrne et al. 1995). RFLP methodology followed that of Byrne et al. (1994). For all 18 RFLP loci, *Eco*RV was the sole restriction enzyme used to digest the DNA samples. The 18 probes were selected from a wider array of available eucalypt single-copy nuclear RFLP probes (Byrne et al. 1998; Thamarus et al. 2002) based upon their level of polymorphism and ease of genetic interpretation in a preliminary screen of *Eco*RV-digested DNA samples from 24 mature *E. consideniana* trees (54 candidate probes were screened in total). Three of the 18 selected RFLP probes were from known genes, 11 were from an *E. globulus* cDNA library (Byrne et al. 1994) and the remaining four were from an *E. nitens* genomic library (Byrne et al. 1994). The three known-gene probes were 4-coumarate Coenzyme A ligase and caffeoyl Coenzyme A O-methyltransferase (4CL and CCoAOMT; Thamarus et al. 2002) and a cellulose synthase homologue (ECS3; Simon Southerton, personal communication). All three were from *E. globulus*. The 11 cDNA probes were c030,

Table 1 PCR conditions[a] specific to each of the 11 microsatellites

Microsat	Batch	Label[b]	T_A	[$MgCl_2$]	[primer]	[enhancer]
Eg089	1	D3	55 °C	2.0 mM	0.2 μM	–
Eg115	1	D4	55 °C	2.0 mM	0.2 μM	–
En010	1	D2	55 °C	2.0 mM	0.2 μM	1X
En016	1	D3	55 °C	2.0 mM	0.2 μM	–
Es115	1	D4	55 °C	2.0 mM	0.2 μM	–
Es255	1	D2	55 °C	2.0 mM	0.4 μM	–
Eg098	2	D2	55 °C	4.0 mM	0.4 μM	–
Es054	2	D3	65 °C	4.0 mM	0.2 μM	–
Es140	2	D3	55 °C	1.5 mM	0.2 μM	–
Es157	2	D2	55 °C	1.5 mM	0.2 μM	–
Es211	2	D4	55 °C	2.0 mM	0.2 μM	–

[a] T_A, annealing temperature; enhancer, PCRx Enhancer (Life Technologies)
[b] Label, the WellRED fluorescent dye (Beckman Coulter) that the forward primer is labelled with

c092, c113, c116, c135, c136, c137, c288, c299, c395 and c451 (Byrne et al. 1998). The four genomic DNA probes were g174, g183, g250 and g409 (Byrne et al. 1998).

The 11 microsatellite markers came from three different eucalypt species: six from *E. sieberi* (Es054, Es115, Es140, Es157, Es211 and Es255; Glaubitz et al. 2001), three from *E. globulus* (Eg089, Eg098 and Eg115) and two from *E. nitens* (En010, En016; Byrne et al. 1996). Primer sequences for all are available at http://www.ffp.csiro.au/tigr/molecular/index.html. The annealing temperatures, magnesium chloride concentrations and primer concentrations used for each microsatellite marker are given in Table 1. For En010 only, PCRx Enhancer (Life Technologies) was included at 1 × concentration. Other than these specific parameters, the PCR reaction and cycling conditions were the same as those given in Glaubitz et al. (2001). Separation and detection of the microsatellite alleles was carried out on a CEQ™ 2000 DNA Analysis System (Beckman Coulter) in two batches of five or six microsatellites each, a single primer from each microsatellite being labelled on its 5′end with one of the dyes D2, D3 or D4 (Table 1).

Data analysis and hypothesis testing

To facilitate hypothesis testing, software was written in the computer programming language C++ to calculate the following genetic diversity measures for each sampled population: allelic richness (= average number of alleles, A_R), effective number of alleles (A_E) (Kimura and Crow 1964), observed heterozygosity (H_O), unbiased expected heterozygosity (H_E) and the panmictic index or inbreeding coefficient (*f*), the latter calculated according to Weir and Cockerham (1984). The accuracy of the measures calculated by our program was confirmed by comparison with the output of the programs GDA (Lewis and Zaykin 2000) and POPGENE (Yeh et al. 1997). GDA was also used to test whether the estimates of *f* for each population, and of F_{ST} for each control/regeneration population pair, significantly deviated from zero, by bootstrapping over loci 10,000 times in all cases. Exact tests for genotypic differentiation for each pair-wise comparison were performed at each locus according to Goudet et al. (1996) using GENEPOP (version 3.3; Raymond and Rousset 1995a). GENEPOP calculated global *p*-values across loci using Fisher's method (Raymond and Rousset 1995b).

As estimates of genetic diversity measures may not be normally distributed (Archie 1985; Weir 1996), we adapted our C++ computer program to perform hypothesis testing in relation to apparent reductions in diversity measures using a randomisation, or permutation, approach (Manly 1997). Sampled trees were randomly shuffled between corresponding control/regeneration population pairs 100,000 times (preserving the original sample sizes) and the difference in each diversity measures (A_R, A_E, H_O, H_E and *f*) calculated for each shuffle. In this manner, we generated for each diversity measure the probability distribution of the difference that would be observed under the null hypothesis that the two populations were genetically identical. Since we are concerned with whether harvesting and regeneration practices result in a *reduction* of genetic diversity (or an *increase* in inbreeding, as measured by *f*), we performed one-tailed tests. The one-tailed probability was computed as the proportion of the differences obtained by randomisation that were greater than or equal to the original difference obtained from the unshuffled data.

For each control/regeneration population pair, the statistical significance of the numbers of 'apparently lost' and 'apparently gained' alleles (i.e. those alleles appearing in the sample from one of the populations but not in the sample from the other) were determined via Monte Carlo simulation. Each simulation calculated the probabilities, under the null hypothesis of no allele frequency differences between the two populations in question, that the observed numbers of apparently lost or gained alleles were the result of sampling error, assuming that both populations were products of random mating. For each population pair, one million pairs of random samples were drawn from the pooled, multilocus allele-frequency distribution, preserving the original sample sizes at each locus within each population (thereby accounting for the effect of missing data). The numbers of 'apparently lost' and 'apparently gained' alleles were tallied for each random sample, and the resulting distributions were used to calculate the probabilities of our observed outcomes.

The CONTML program from the computer package PHYLIP (Felsenstein 1993) was used to construct a maximum-likelihood dendrogram describing the relationships between the eight populations under a model of pure genetic drift (Felsenstein 1981). To test the reliability of the obtained topology, 10,000 bootstrap re-samples of loci were performed using SEQBOOT in PHYLIP. In addition, separate dendrograms were constructed for each generation: one for the four control populations separately (CPO, for "control populations only") and one for the four regeneration populations separately (RPO, for "regeneration populations only"). To determine the statistical significance of the difference between the total spans of the CPO and RPO dendrograms, identical sets of 10,000 bootstrap re-samples over loci were obtained for both, by using the same random seed in SEQBOOT. The bootstrap distribution of the 10,000 *differences* in total span was then recovered from the two resulting 'treefiles' (produced by CONTML), using a homemade C++ computer program. The proportion of this distribution that fell on or below zero reflects the probability that the longer dendrogram was actually of equal or smaller length than the other, and was thus used to determine the statistical significance of the difference in total span.

Results

Population genetic measures

Comparisons of four genetic diversity measures (A_R, A_E, H_O and H_E) between all four of the control/regeneration

Table 2 Genetic diversity measures[a] for *E. consideniana* in four harvested coupes and their corresponding controls (based on 18 RFLPs and 11 microsatellites)

Population	n	A_R	A_E	H_O	H_E
CF1					
control	48	7.62 (221)	3.34	0.586	0.600
regeneration	46	7.55 (219)	3.32	0.608	0.616
difference[b]		–0.07 (–0.9%)	–0.03 (–0.8%)	+0.022 (+3.7%)	+0.016 (+2.5%)
probability[c]		0.532	0.436	0.857	0.910
CF2					
control	50	7.97 (231)	3.36	0.608	0.615
regeneration	48	7.69 (223)	3.19	0.595	0.604
difference[b]		–0.28 (–3.5%)	–0.17 (–5.0%)	–0.013 (–2.1%)	–0.011 (–1.7%)
probability[c]		0.249	0.071	0.244	0.133
ST1					
control	54	7.69 (223)	3.41	0.585	0.602
regeneration	48	7.14 (207)	3.04	0.603	0.582
difference[b]		–0.55 (–7.2%)	–0.37* (–10.9%)	+0.018 (+3.0%)	–0.020* (–3.3%)
probability[c]		0.177	**0.005**	0.871	**0.039**
ST2					
control	52	7.97 (231)	3.58	0.603	0.620
regeneration	48	7.21 (209)	3.39	0.610	0.606
difference[b]		–0.76* (–9.5%)	–0.19* (–5.5%)	+0.007 (+1.2%)	–0.014 (–2.2%)
probability[c]		**0.010**	**0.048**	0.660	0.096
Overall	394	11.24 (326)	3.55	0.599	0.612

[a] n, number of trees sampled; A_R, allelic richness (total no. alleles in parentheses); A_E, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

[b] Negative difference indicates a loss of diversity (percentage difference in parentheses). Significant reductions indicated with an asterisk ($\alpha = 0.05$)

[c] One-tailed probability for an apparent reduction in diversity under the null hypothesis. Based on 100,000 random shuffles of individuals between the control and regeneration populations, using the difference in the diversity measure as the test statistic. Values less than 0.05 shown in bold

population pairs and calculated over all 29 loci are shown in Table 2. Also shown in Table 2 are the one-tail probabilities of the observed 'reductions' occurring under the null hypothesis. From these it can be seen that significant reductions ($\alpha = 0.05$) in diversity measures were observed only in the seed tree regeneration: A_E and H_E were significantly reduced in ST1 ($p = 0.005$ and 0.039 respectively) and A_R and A_E were significantly reduced in ST2 ($p = 0.010$ and 0.048 respectively). Significant losses of diversity were not detected on the clear falls. No significant reductions in H_O were observed in any of the populations, as H_O appeared to increase in the regeneration in all cases except for CF2. The three measures other than H_O (A_R, A_E and H_E) were consistently reduced to a greater extent in the regeneration on the seed tree coupes than in the clear falls.

The genetic diversity measures A_R, A_E and H_E are reported separately for the RFLPs and the microsatellites in Table 3 (since no significant reductions in H_O were observed, this statistic was omitted from the table). In the overall sample of 394 trees A_R, A_E and H_E over the 18 RFLP loci were 9.2, 2.1 and 0.50 respectively while they were 14.6, 5.9 and 0.79 across the 11 microsatellites. Similar total numbers of alleles were uncovered by the two sets of markers: 165 alleles at the 18 RFLPs, and 161 alleles across the 11 microsatellites. When the RFLP loci were examined separately, there were no significant reductions of A_R, A_E or H_E in any of the regeneration populations. However, separate examination of the microsatellites showed significant reductions of all three measures in both the ST1 and ST2 regeneration, but not in the regeneration on the two clear falls (CF1 or CF2). Furthermore, the *p*-values from the significant tests in Table 3 are all much smaller than their counterparts in Table 2. These results indicate that losses of genetic diversity are more likely to occur under the seed tree system than under clear felling with aerial re-sowing, and that there is far greater statistical power to detect such losses with microsatellite loci than with RFLPs.

Comparisons and changes of the panmictic index (*f*) over each of the population pairs and based upon all 29 loci are shown in Table 4. No significant increases in *f* were observed. In fact, in three of the four comparisons *f* was lower in the regeneration, suggesting a *reduction* of inbreeding. Performance of a two-tailed test showed a significant reduction of *f* in the ST1 regeneration relative to its control ($p = 0.005$). However, in most cases *f* was quite close to zero (indicating random mating genotypic proportions). Only the estimate of *f* for the ST1 control (0.029) was significantly different from zero (Table 4). Similar results were obtained when RFLPs and microsatellites were examined separately.

Pair-wise F_{ST} values based upon all 29 loci (Table 5) significantly differed from zero in three out of the four control/regeneration pairs, the exception being CF2. The results were in qualitative agreement with those from exact tests for differentiation (Table 5), which also indicated significant differentiation in the same three pairs. However, the exact tests appear to be far more

Table 3 Genetic diversity measures[a] reported for RFLPs and microsatellites separately

Population	RFLPs (18 loci)			Microsatellites (11 loci)		
	A_R	A_E	H_E	A_R	A_E	H_E
CF1						
control	5.67	2.07	0.492	10.82	5.43	0.778
regeneration	5.94	2.22	0.521	10.18	5.12	0.771
difference[b]	+0.28	+0.15	+0.029	–0.64	–0.31	–0.007
% change	+4.9%	+7.1%	+5.9%	–5.9%	–5.6%	–0.9%
probability[c]	0.878	0.974	0.963	0.225	0.173	0.299
CF2						
control	6.00	2.14	0.513	11.18	5.36	0.783
regeneration	6.00	2.07	0.498	10.45	5.03	0.779
difference[b]	0.00	–0.07	–0.015	–0.73	–0.34	–0.003
% change	0.0%	–3.1%	–3.0%	–6.5%	–6.3%	–0.4%
probability[c]	0.602	0.153	0.145	0.131	0.122	0.380
ST1						
control	5.61	1.98	0.483	11.09	5.74	0.797
regeneration	5.39	2.02	0.477	10.00	4.70	0.754
difference[b]	–0.22	+0.04	–0.006	–1.09*	–1.05**	–0.043**
% change	–4.0%	+2.1%	–1.2%	–9.8%	–18.2%	–5.4%
probability[c]	0.467	0.763	0.343	**0.049**	**0.002**	**0.001**
ST2						
control	6.00	2.14	0.509	11.18	5.95	0.802
regeneration	5.61	2.18	0.504	9.82	5.37	0.774
difference[b]	–0.39	+0.04	–0.005	–1.36**	–0.58**	–0.028**
% change	–6.5%	+1.6%	–1.0%	–12.2%	–9.7%	–3.5%
probability[c]	0.177	0.691	0.364	**0.005**	**0.017**	**0.007**
Overall[d]	9.17	2.10	0.503	14.64	5.91	0.789

[a] Abbreviations follow those in Table 2
[b] Negative difference indicates a loss of diversity. Significant reductions indicated with asterisks (*$p < 0.05$; **$p < 0.01$)
[c] One-tailed probability for an apparent reduction in diversity under the null hypothesis. Based on 100,000 random shuffles of individuals between the control and regeneration populations, using the difference as the test statistic. Values less than 0.05 shown in bold
[d] Calculated based on the entire study sample of 394 trees

Table 4 Differences in the inbreeding coefficient, *f*, between four harvested coupes and their corresponding controls

Item	CF1	CF2	ST1	ST2
f in control[a]	0.024	0.012	*0.029*	0.029
f in regeneration[a]	0.012	0.015	–0.035	–0.006
Change[b]	–0.012	+0.003	–0.064	–0.035
Probability[c]	0.663	0.446	0.998	0.934

[a] Single population *f* estimates that significantly differ from zero are shown in italics (10,000 bootstraps across loci; $\alpha = 0.05$)
[b] Negative change indicates a reduction in inbreeding in the regeneration
[c] One-tailed probability of an apparent increase in *f* under the null hypothesis of no change (based upon the differences in *f* from 100,000 randomisations)

sensitive to allele frequency differences, yielding very small *p*-values relative to the F_{ST} results.

Apparent loss or gain of alleles

In total, 326 alleles were detected in this study. The majority of these were rare alleles: 224 alleles were present at a frequency of less than 5% in the overall sample of 394 trees, with 35 present in only a single copy (frequency of 0.13%). Differences in allelic composition between all pairs of control and regeneration samples were entirely due to the presence or absence of such rare alleles (<5% in overall frequency). The frequency distributions of alleles detected in the controls but absent in their corresponding regeneration populations (="apparently lost alleles") are shown in Fig. 1. In these histograms, allele frequencies are given in "copy number" units (referring to the number of copies of the allele in the control sample), where a copy number of 1 corresponds to an allele frequency of between [1/(54 × 2)=] 0.93% and [1/(48 × 2) =] 1.04% depending on the relevant control population. The total number of apparently lost (or gained) alleles for each population pair are given in Table 6. We estimated the probabilities that these outcomes could have occurred by chance, under the null hypothesis of no allele frequency differences between each population pair, via Monte Carlo computer simulation (as described in the Materials and methods, under the assumption of random mating). The results of the simulations are shown in Table 6. The total number of apparently lost alleles was highly significant in all four cases. However, the total number of apparently gained alleles was significantly greater than null hypothesis expectations only in CF1 and ST1. The strongest contrast

Table 5 Tests for differentiation between each control and regeneration population pair

Type	CF1	CF2	ST1	ST2
F_{ST} (control vs regen)	0.008*	0.002	0.020*	0.007*
95% C.I.[a]	0.014	0.006	0.028	0.014
	0.003	–0.001	0.012	0.001
Exact test (genotypic)				
χ^2 (over all 29 loci)	114.3	73.46	Infinity	110.5
probability (df = 58)	0.00001	0.0830	Zero	0.00004

* Significantly different from zero based upon 10,000 bootstrap re-samples of loci (α = 0.05)
[a] C.I., confidence interval

Table 6 Apparently lost or gained alleles in each control and regeneration population pair

Item	CF1	CF2	ST1	ST2
Total no. apparently lost alleles	43*	37*	45*	42*
(probability[a])	(0.000)	(0.004)	(0.000)	(0.000)
Total no. apparently gained alleles	41*	29	29*	20
(probability[a])	(0.000)	(0.105)	(0.019)	(0.488)
Specific outcomes:				
Copy no. threshold in control (T1)	4 copies	3 copies	5 copies	4 copies
No. apparently lost alleles $\geq$ T1	3	4	3*	6*
(probability[a])	(0.219)	(0.551)	(0.039)	(0.009)
Copy no. threshold in control (T2)	6 copies	4 copies	10 copies	5 copies
No. apparently lost alleles $\geq$ T2	1	1	1*	1
(probability[a])	(0.243)	(0.807)	(0.017)	(0.538)

[a] Calculated via Monte Carlo simulation – see text for details
* Significant at α = 0.05

between the number of apparently lost and gained alleles occurred in ST2, where there were 22 more alleles 'lost' than 'gained'.

The simulations also estimated the probabilities of specific outcomes relating to the histograms in Fig. 1. Here we estimated the probability (under the null hypothesis) that an equal or greater number of alleles at or above a particular copy number threshold in the control sample would have been absent from the regeneration sample. Two copy number thresholds (T1 and T2 in Table 6) were set for each population pair, based upon the corresponding histogram in Fig. 1. When such thresholds were employed, significant losses above the given thresholds were observed only in the seed tree replicates (Table 6). For example, the apparent loss from the ST1 regeneration of an allele present in ten copies in the ST1 control was statistically significant (p = 0.017). Further, the apparent loss from the ST2 regeneration of six alleles present in four or more copies in the ST2 control was also significant (p = 0.009).

Dendrogram

Relationships between the eight populations are shown in a continuous character maximum-likelihood (CONTML) dendrogram (Fig. 2). The CONTML method fits a model of pure genetic drift in which rates of evolutionary change (via drift) are allowed to vary (Felsenstein 1981). In this dendrogram, the four regeneration populations all grouped with their respective controls, generally with strong bootstrap support (Fig. 2). ST1 regen lay farthest from its corresponding control while CF1 regen was the second farthest. Hence there was no consistent pattern in which the replicates from one treatment (e.g. the seed tree system) showed more drift between controls and regeneration than those from the other treatment (e.g. the clear fall system). Inclusion of the regeneration populations did, however, lead to apparent expansion of the dendrogram. Branches leading to ST1 regen, CF1 regen and ST2 regen protruded well beyond the rough circle defined by the four controls.

This expansion effect was investigated further by constructing two separate CONTML dendrograms (data not shown), one for the four control populations only (CPO) and one for the four regeneration populations only (RPO). The total span of the RPO dendrogram was 0.031 vs 0.018 for the CPO dendrogram, a 72% increase. Bootstrapping over loci 10,000 times showed that this difference was highly significant (p = 0.0000), indicating that genetic drift has been promoted by regeneration practices.

Discussion

The results of this study indicate that in *E. consideniana*, reductions of genetic diversity are more likely to occur under the seed tree system than under clear felling with aerial re-sowing. This suggests that the effective population size (N_e) was smaller under the seed tree system, which may be explained in part by the larger number of seed sources that contributed to the *E. consideniana* seed lots sown on the clear falls in comparison to the number

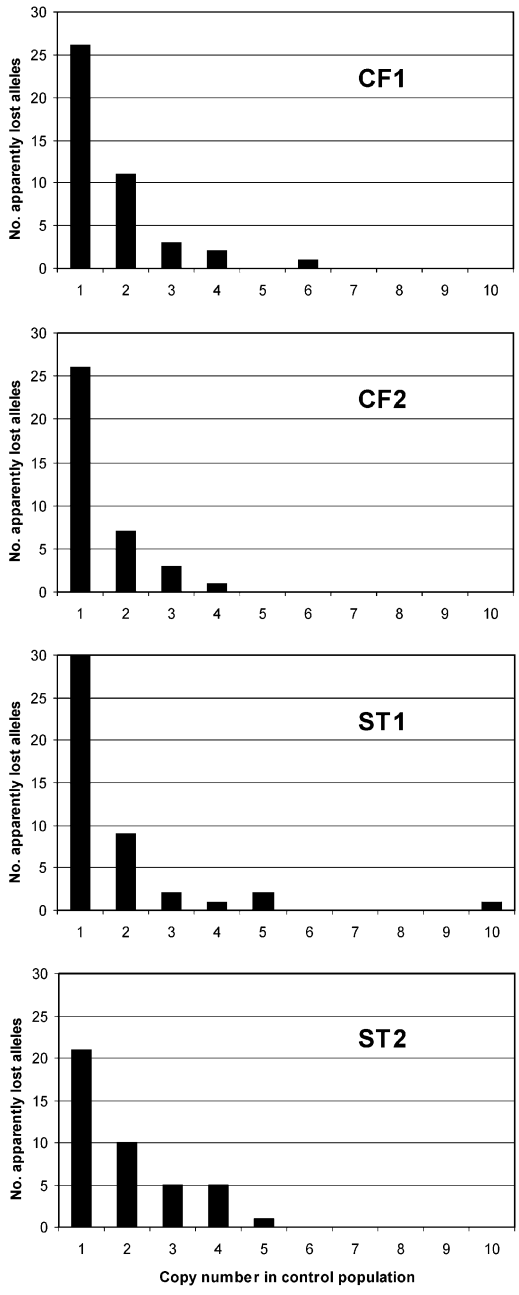

Fig. 1 Frequencies in the control population of apparently lost alleles for the four control vs. regeneration comparisons in *E. consideniana*. A copy number of one corresponds to an allele frequency of approximately 1%

of *E. consideniana* seed trees left behind on the seed tree coupes. The *E. consideniana* seed lots sown on the clear falls (CF1 and CF2) were collected from seven and five trees respectively while each of the seed tree coupes (ST1 and ST2) had only four residual *E. consideniana* seed trees. However, as these differences are not very large, it is likely that there are one or more additional factors at work. One such factor is the *evenness* of the relative contributions of each seed source to the *E. consideniana* sapling population, which can have a strong effect on N_e. It is likely that the relative contributions of the seed sources were more even on the clear falls than on the seed tree coupes, resulting in a larger contrast in N_e between the two silvicultural treatments than that suggested solely by the *number* of seed sources. On the clear falls, relatively even quantities of seed from several half-sib families were mixed and then evenly distributed across each coupe. In contrast, on the seed tree coupes, a number of factors could have led to uneven contributions of the seed trees to the regeneration, including (1) their spatial configuration (on both ST1 and ST2, two of the four seed trees were close to the coupe boundary), (2) poor seed crops on some seed trees, and (3) differential survival of saplings from different seed trees due to microhabitat differences.

However, there are other potential seed sources that might have contributed to the regeneration on either the clear falls or the seed tree coupes. These include edge trees, the soil-based seed bank and logging slash. Bordering unharvested trees are unlikely to have contributed much to the sampled regeneration, as saplings near (within approximately 50 m of) the coupe boundaries were not included in the sample, and eucalypt seeds are unlikely to disperse farther than that (Cremer 1977). Moreover, the soil-based seed bank is unlikely to have been a significant factor, as it is ephemeral in most eucalypt species and contributes little to regeneration (e.g. Wang 1997). However, it is feasible that logging slash made a significant contribution to the regeneration on either the clear falls or the seed tree coupes. Grouping of clear fall regeneration populations with their respective controls (with strong bootstrap support) in the CONTML dendrogram (Fig. 2) suggests that this may have been the case. Such grouping would not be expected if the vast majority of the regeneration was derived from the aerially sown seed. This is particularly true for CF1, as the seed sown on that coupe was collected from trees that were all located at least 1.3 km (and at most 2.1 km) distant, and much closer to the other three coupes. It is less surprising, however, that the CF2 control and regeneration populations grouped together as, in this case, the sources of the aerially sown seed were much closer (five trees all within a radius of 0.6 km, the closest being only about 0.2 km distant from CF2). Substantial contribution of seed from logging slash to the regeneration could act as a buffer reducing potential deleterious genetic consequences of management practices. Hence, it would seem that, if seed from logging slash has truly played a role at all, it has made a larger relative contribution to the regeneration on the clear falls than on the seed tree coupes. However, there is no apparent reason why this would have been the case, as on both the clear falls and the seed tree coupes, the seed bed was prepared, after harvest, by burning.

Separate analyses of the RFLPs and microsatellites indicate that the microsatellites employed were more sensitive indicators of genetic loss due to regeneration practices than were the RFLPs. Tests for diversity reductions solely based upon the 11 microsatellite loci strengthen our conclusion that diversity losses occurred

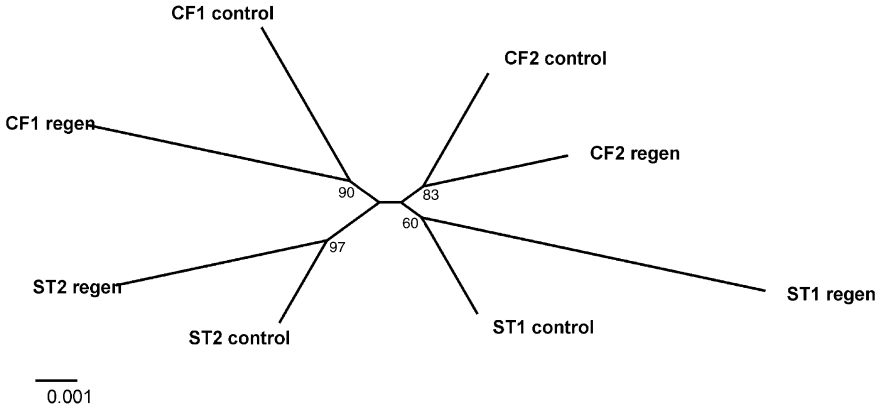

Fig. 2 Continuous character maximum-likelihood (CONTML) dendrogram based upon the allele frequencies in the eight sampled populations ('regen' refers to regeneration). *Numbers* next to the nodes show the percentage of bootstrapped trees (out of 1,000 bootstrap samples over loci) in which the indicated grouping was conserved

under the seed tree system but not under clear felling with aerial re-sowing: in both of the seed tree populations, there were significant reductions of all three diversity measures, A_R, A_E and H_E, with the significant tests all having smaller *p*-values than the corresponding tests based on all 29 loci (Table 3). In contrast, tests based solely upon the 18 RFLPs did not reveal any statistically significant change in these measures. Hence it is clear that greater statistical power for detecting diversity losses was provided by the microsatellites. We attribute this greater power not only to the larger number of alleles that were present per locus in the microsatellites, but also to the greater evenness in their allele frequencies: the ratio of overall A_R to A_E was 2.5 in the microsatellites vs 4.4 in the RFLPs. Since microsatellites are currently far more commonly used and are less labour intensive than Mendelian RFLPs, it is encouraging that they also appear to provide superior statistical power to detect genetic losses.

In regard to potential deleterious genetic impacts of forest management practices, our concern should not only focus on whether levels of diversity have been reduced, but also on whether the genetic makeup of tree populations is being dramatically altered. It is conceivable under the clear fall system – either with aerial sowing or planting – that levels of genetic diversity could be maintained, or even increased, even though the original tree population has been replaced by regeneration that is genetically distinct. Such undesirable genetic alteration is more likely to occur if the regeneration is derived from seed that is not collected locally. For this reason, in studies such as this, analyses of allelic losses (rather than just net change in allelic richness) and of population differentiation should accompany analyses of diversity levels.

The higher the frequency of an apparently lost allele in the control sample, the less likely that its absence from the regeneration sample was simply due to sampling error. Hence our Monte Carlo simulation focussed on the *least rare* of the apparently lost alleles by setting threshold copy numbers based on their observed copy numbers in the control population (Table 6). The simulation results were in concordance with those from the tests for diversity reductions and suggested that significant losses of alleles above threshold frequencies are more likely to occur under the seed tree system than the clear fall system, at least as practiced on the study site. It seems that the use of locally collected seed to re-sow the clear falls mitigated potential allelic losses under this system.

In our analyses of population differentiation, based on either pair-wise F_{ST} or exact tests (Table 5), we found that the control and regeneration population pairs were significantly differentiated for both of the seed tree replicates (ST1 and ST2), but for only one of the clear falls (CF1). The lack of significant differentiation between the control and regeneration populations of CF2 may reflect the relative proximity of its seed sources (noted above). On the other hand, the observed significant differentiation (for CF1, ST1 and ST2) may be due in part to the use in this study of bordering unharvested trees as controls, rather than the ideal situation in which control trees would be sampled from the coupe itself prior to (or at the time of) harvest. Although the exact tests were highly significant (reflecting their high statistical power), the F_{ST} values in question were not very large. In the presence of fine-scale spatial genetic structure, allele frequencies in the bordering controls may not have been perfectly representative of those in the corresponding population of trees present on the coupes prior to harvest. (Note that this same caveat with regard to spatial effects also pertains to the above analyses of apparently lost or gained alleles).

However, the finding that the RPO dendrogram was significantly longer in span than the CPO dendrogram (72% longer; $p = 0.0000$) suggests that the significant differentiation observed was due to more than just fine-scale spatial structure. If such spatial structure was the main cause of the observed differentiation within control/regeneration pairs, then the RPO dendrogram should not be significantly larger than the CPO dendrogram, since both sets of populations are in virtually the same spatial configuration. The fact that the RPO dendrogram was indeed bigger and that this difference was highly significant, suggest that genetic drift has been promoted by harvesting and regeneration practices. The control popu-

lations were themselves the product of genetic drift (in combination with other genetic forces) under 'natural' conditions over several generations; it seems highly unlikely that the continuation of such natural conditions would have resulted in such a large amount of genetic drift over a single generation. Further evidence that genetic drift was promoted by regeneration practices is provided by the fact that the average of the pair-wise F_{ST} values for the four control-regeneration comparisons (0.0091) was more than three times higher than that for all possible control vs control comparisons (0.0028). Since all control vs control comparisons involve larger geographic distances than do those between corresponding control/regeneration pairs, the typical F_{ST} value between the harvested trees and their adjacent controls was likely less than 0.0028.

The results of this study suggest that for minor species, such as *E. consideniana* on the study site, the clear fall system may be superior to the seed tree system in terms of genetic diversity conservation. As a higher degree of control over the genetic composition is afforded via aerial re-sowing, it would be feasible to even *increase* the amount of genetic variation in the regeneration by applying seed collected from a larger number of trees (e.g. 30 or more) over a broader area. However, as we found in our companion study of *E. sieberi* on the same site (Glaubitz et al. 2003), repeated application of the same seedlot to different harvested coupes can lead to genetic homogenisation. Furthermore, use of too few (i.e. less than five) trees as seed sources could lead to genetic erosion. In order to conserve local gene pools, a single seed lot should not be applied too widely, and should be collected from a large number of trees in the area local to where it will be sown.

The results of our companion study (Glaubitz et al. 2003) suggested that *locally common* species, such as *E. sieberi*, are more resilient to potential losses of genetic diversity under the seed tree system than the *locally minor* species studied here. In spite of the apparent negative consequences of the seed tree system to the genetic diversity of minor species such as *E. consideniana*, continued use of this system may be desirable for economic, ecological or political reasons. If that is the case, then it may be possible to ameliorate the potential loss of alleles in minor tree species by supplementing the seed crop with locally collected seed sown by hand.

Other studies of genetic diversity in tree regeneration after logging using genetic markers have yielded varying results. Some have indicated that diversity in regenerated populations has been reduced (Gömöry 1992; Stoehr and El-Kassaby 1997; Adams et al. 1998; Rajora 1999; Macdonald et al. 2001; Lee et al. 2002) while others have found no differences (Knowles 1985; Neale 1985; Thomas et al. 1999; El-Kassaby 2000; Perry and Bousquet 2001). It is difficult to compare results among these studies since they vary in the type of genetic markers used, the number of loci examined, the relative local abundance of the study species, and in the statistical power of their experimental designs. Furthermore, statistical tests were sometimes not performed. Where they were performed the tests used were invariably classical, parametric tests. As the assumptions of these tests may be violated when they are applied to genetic diversity measures, the conclusions drawn may be invalid. In this study, as in our *E. sieberi* study (Glaubitz et al. 2003), we have avoided this pitfall by employing non-parametric, randomisation-based tests.

Most studies of the impacts of forest management practices on genetic diversity, including this one, suffer from the shortcoming that only a single rotation was examined. It may require several rotations before genetic erosion due to forest management becomes obvious. For example, it is possible that reductions in genetic diversity in *E. consideniana* occurred not only under the seed tree system, but also under the clear fall system, but were too small there to be reliably detected over a single rotation, given the level of statistical power inherent in our experimental design. Furthermore, although there was no evidence in this study that a single rotation of forest management resulted in the promotion of inbreeding (as no significant decreases in H_O, or increases in f, were observed in either the clear fall or the seed tree coupes), inbreeding would be far more likely to build up appreciably in subsequent rotations. This is particularly so under the seed tree system, where the future regeneration will be derived from matings within and among a limited number of half sib families. On the other hand, it is possible that short term allelic losses (or promotion of inbreeding) will be compensated for by pollen flow from surrounding unharvested stands. Long term studies over multiple rotations are needed, but are generally impractical in forest trees. Large scale retrospective studies may be possible, in which a large sample of stands that have been subjected to multiple rotations are compared to a similar number of unharvested control stands, provided that a sufficient number of control stands exist. Such large scale studies are currently more feasible with isozyme markers (e.g. McDonald et al. 2001) than with DNA markers. However, the advent of high throughput, highly multiplexed DNA markers (Chicurel 2001) may soon make them feasible at the DNA level in trees, with far more extensive sampling of the genome than can be achieved with the limited number of polymorphic isozymes generally available.

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