Genetic Diversity Within and Between Natural Populations of *Eucalyptus occidentalis* (Myrtaceae)

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(Received 23rd May 2003)

Summary

Eucalyptus occidentalis is endemic to the south-west of Australia, occurring in small isolated populations in wet depressions and along drainage lines. The level of genetic diversity and pattern of structuring within and between populations was investigated using nuclear RFLP analysis of 10 populations. The level of genetic diversity was moderate and similar in the populations from the main range but lower in the outlier populations from the eastern end of the range. There was no evidence of inbreeding within the populations. The level of population differentiation was low but significantly different from zero, and the populations from the eastern end of the range showed higher levels of differentiation from each other and from the populations in the main range. The pattern of genetic diversity in *E. occidentalis* indicates historical connectivity between populations and the species was probably more abundant with a wider distribution in the past, except for the eastern end of the range where the species distribution appears to have always been fragmented. The pattern of population differentiation is similar to the pattern of differentiation in quantitative traits that has been observed in provenance trials. Sampling strategies for breeding programs should focus on the main range of the species.

Key words: genetic diversity, differentiation, RFLP, *Eucalyptus*.

Introduction

Eucalyptus occidentalis Endl., a species endemic to south-western Australia, has been extensively planted in many regions around the world due to its adaptability to dry temperate climates and saline soils. The species has been successfully introduced into southern Europe, North Africa and the Middle East, and has also performed well in California, Mexico and Chile (Chippendale, 1983), and the drier regions of south-eastern Australia (Marcar *et al.,* 1995). Within its natural range in the southern wheatbelt and subcoastal areas of Western Australia *E. occidentalis* is commonly found in wet, clayey depressions (Brooker and Kleinig, 1990), drainage lines and around the borders of freshwater and brackish lagoons. In these environments the ground surface around the trees may be inundated for several months during winter and spring and the species is adapted to soil salinity and seasonal waterlogging. The species also occurs in sandy duplex soils on alluvial flood plains and at the base of granite rock outcrops, particularly in the northern part of its range. While it is typically planted in lower parts of the landscape on heavy soils, *E. occidentalis* has proved to be adaptable to a wide range of soil types in exotic plantings, including clays, well drained loess soils and sands (Jacobs, 1981; Zohar and Moreshet, 1987). It displays excellent survival and moderate growth rates in arid, semi-arid and sub-humid winter rainfall climates.

Eucalyptus occidentalis has been utilised for its wood products, in such activities as building poles, pilings, posts and heavy construction. The heartwood is pale, hard, somewhat straight grained and durable in damp soils. It has potential for pulpwood production, with Kraft pulp yields of 50% from irrigated plantations in South Australia (Clark and Rawlins, 1999) but has low pulp tearing resistance due to short fibre length. Non-wood products of *E. occidentalis* include the flowers, which have value for honey production, and the bark is reported to have high levels of tannin (Jacobs, 1981). *Eucalyptus occidentalis* is planted for environmental services such as soil conservation and erosion control in hilly areas of Calabria and Sicily (Jacobs, 1981), and amenity, shade and shelterbelt purposes in Mediterranean countries (Harwood, 2000).

In Australia there is renewed interest in utilisation of *E. occidentalis* (Harwood, 2000) due to increasing salinisation of landscapes resulting from rising water tables bring naturally accumulated salt deposits to the soil surface (George *et al.,* 1997). Tolerance of salinity and water logging make it an ideal species for planting on salt-affected lands with rising water tables, although salinities over 15 ds m^{-1} Ece are detrimental to its growth (Marcar *et al.,* 1995). In these environments it can improve water usage, reduce water tables (Biddiscombe *et al.,* 1989; Pepper and Craig, 1986), enhance ecosystem function and also provide a commercial return from otherwise unproductive sites.

There has been no significant breeding in *E. occidentalis* although provenance trials have been planted in several countries. These trials show significant differences between provenances for growth and stem form, and the generally consistent performance of some provenances suggests good prospects for improvement of *E. occidentalis* through selection and breeding (Harwood, 2001). The variable performance in provenance trials suggests that there may be genetic differentiation between populations and structuring of genetic diversity within the species, although the high degree of adaptability to a range of site conditions may indicate a lack of significant local adaptation to particular environments. The geographical restriction of the species to small isolated populations also suggests that population differentiation may be high. Estimates of genetic diversity and structuring within species are widely used as tools to assess genetic resources in order to capture a broad base of genetic diversity and guide activity within breeding programs (Moran *et al.,* 2000) and this knowledge will be useful for the *E. occidentalis* breeding program. Co-dominant markers provide greatest power in population genetic analyses, and for eucalypts both microsatellite and RFLP markers have been developed. Eucalypt microsatellite markers show good conservation across species (Byrne *et. al.,* 1996; Brondani *et al.,* 1998; Steane *et al.,* 2001), although in practice the number of loci that are useful for population genetic analysis in any particular species can be limited. In contrast, RFLP markers are readily transferable across species and have been shown to be highly variable in eucalypts (Byrne, 1999; Hines and Byrne, 2001; Butcher *et al.,* 2002). Therefore, nuclear RFLP markers were used to investigate genetic diversity and structuring within *E. occidentalis*.

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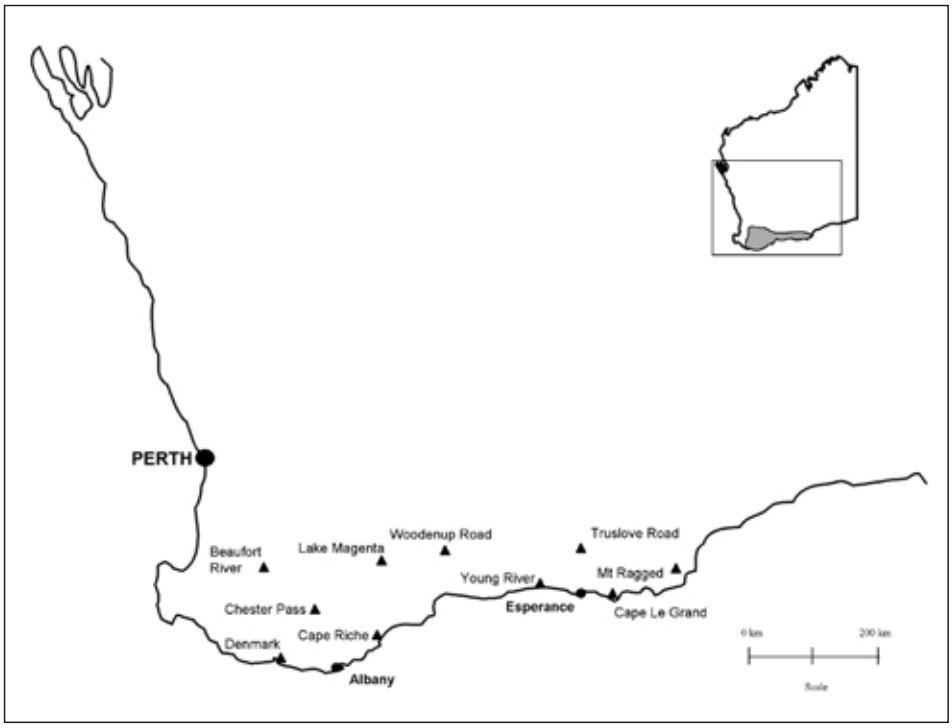

Figure 1. – Distribution and location of sampled populations of *Eucalyptus occidentalis.*

Materials and Methods

Plant material

Leaf samples were collected from ten individuals from each of ten populations of *E. occidentalis* from throughout its geographic range (*Figure 1*). Samples were also collected from one population of *E. kessellii* for use as an outgroup. Extraction of DNA from the leaves was carried out as in BYRNE *et al.* (1998). DNA of all individuals was digested with either *Bgl*II or *Eco*RV, Southern blotted and hybridised with 30 RFLP probes (c092, c113, c115, c116, c135, c136, c170, c238, c299, c333, c395, c411, c451, g059, g067, g086, g095, g099, g142, g154, g174, g183, g195, g233, g243, g250, g256, g261, g425, g474; BYRNE *et al.,* 1995). Restriction digestion and hybridisation were described in BYRNE and MORAN (1994), and probe plasmids were amplified through PCR then labelled with ^{32}P by the random priming method (FEINBERG and VOGELSTEIN, 1983).

Data analysis

The banding patterns were interpreted according to a Mendelian multi-allelic model. Allelic diversity parameters and homogeneity tests of allele frequency distribution among populations were calculated using POPGENE (YEH *et al.,* 1997). Gene diversity parameters were calculated according to NEI (1973) using FSTAT (GOUDET, 2001). Climatic variables of annual precipition, annual mean temperature, annual mean moisture index and altitude were obtained from BIOCLIM (HOULDER *et al.,* 2000). Association between climatic variables and genetic diversity parameters were tested using a regression analysis. Measures of inbreeding and genetic differentiation were calculated using WEIR and COCKERHAM's (1984) estimates, which are unbiased for small sample size, using FSTAT (GOUDET, 2001). Confidence intervals were estimated by bootstrapping over loci 1000 times. Regression of population differentiation against geographical distance was calculated and tested for significance using a Mantel randomisation test (MANTEL, 1967). A hierarchical cluster analysis was carried out using UPGMA based on unbiased genetic distance measures (NEI, 1978). The significance of nodes in the dendrogram was assessed by bootstrapping with 100 replications.

Results

Twenty-five of the probes produced interpretable fragment patterns that were scored according to an alleles/locus model. The remaining five probes produced multiple banded patterns, resulting from hybridisation to two or more loci, and were not scored. At the species level polymorphism was high and only one locus was monomorphic across all populations. Within single populations up to nine loci were monomorphic. The number of alleles detected at a locus ranged from one to 17, with the maximum number of alleles in any one population being ten. The distribution of alleles showed similar numbers of rare alleles (frequency < 0.1) and common alleles (frequency > 0.5). The proportion of rare alleles in populations ranged from 23–40% with an average of 28%, and the proportion of common alleles ranged from 23–33% with an average of 25%. In most populations the proportion of rare and common alleles were similar or the number of rare alleles was higher than common alleles, except for the two eastern populations in which the proportion of rare alleles was lower than the common alleles. All populations contained low but similar numbers of unique alleles (average 4.8% unique in each population) except for the Mt Ragged population which had over twice the number of unique alleles (10.6%). The unique alleles were generally rare alleles but 31% had frequencies > 0.1. Homogeneity tests of alleles frequencies identified significant ($p < 0.05$) differences across populations in around half of the loci (14 out of 25 loci). Many of these differences involved the Cape Le Grand and Mt Ragged populations and when these populations were removed only nine of the loci showed significant differences in allele frequencies across the remaining populations. Inspection of allele frequencies showed that the Cape Le Grand and Mt Ragged populations had changes in common alleles (frequency > 0.5) at three loci and changes in moderate frequency alleles (> 0.2) at two loci. These populations also had the lowest number of alleles over all loci.

Table 1. – Allelic diversity parameters for populations of *Eucalyptus occidentalis*. *A,* mean number of alleles per locus; *P,* mean number of polymorphic loci (0.99 criterion); H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, fixation index. Standard errors in parentheses.

Population	A	P	H_o	H_e	F_{IS}
Beaufort River	3.1 (0.6)	80	0.344 (0.095)	0.341 (0.094)	-0.069 (0.025)
Denmark	2.9 (0.6)	76	0.336 (0.095)	0.351 (0.094)	0.006 (0.045)
Chester Pass	3.6 (0.7)	88	0.408 (0.093)	0.411 (0.088)	-0.038 (0.046)
Cape Riche	3.0 (0.6)	72	0.392 (0.107)	0.379 (0.102)	-0.095 (0.038)
Lake Magenta	3.2 (0.7)	76	0.368 (0.094)	0.386 (0.095)	-0.028 (0.054)
Woodenup Rd	2.8 (0.5)	76	0.316 (0.088)	0.320 (0.092)	-0.072 (0.034)
Young River	3.1 (0.6)	72	0.320 (0.093)	0.356 (0.099)	0.033 (0.044)
Truslove Rd	3.1 (0.6)	76	0.336 (0.093)	0.340 (0.089)	-0.049 (0.056)
Cape Le Grand	2.6 (0.5)	72	0.308 (0.091)	0.326 (0.090)	-0.008 (0.070)
Mt Ragged	2.6 (0.5)	72	0.292 (0.095)	0.297 (0.092)	-0.040 (0.034)
Mean	3.0 (0.03)	76	0.342 (0.004)	0.351 (0.003)	-0.036 (0.004)

The allelic diversity measures for each population, and mean over all populations, are presented in *Table 1*. The values for the mean number of alleles per locus (*A*), mean number of polymorphic loci (*P*), observed heterogygosity (H_o) and the Hardy-Weinberg expected panmictic heterozygosity (H_e) were moderate to high and were generally similar across populations. The highest allelic diversity occurred in the Chester Pass population in the centre of the range and the lowest diversity occurred in the two populations at the eastern end of the range, Mt Ragged and Cape Le Grand. Although not linear there was a general trend for populations in the west of the distribution to have higher heterozygosity than those in the east. Correlation of genetic diversity parameters and climatic variables showed a trend for decreased observed heterozygosity and polymorphic loci with increasing temperature ($p = 0.183$, $p = 0.039$ respectively), and increasing polymorphic loci with increasing altitude ($p = 0.174$), but only the association between temperature and polymorphic loci was significant. The inbreeding coefficient, F_{IS}, was generally not significantly different from zero and the negative values may reflect bias due to small sample sizes. The mean estimates of total genetic diversity showed moderate levels of diversity ($H_T = 0.373$) with the majority present within populations, although some diversity was maintained between populations ($G_{ST} = 5.9\%$).

Although the majority of variation (94%) occurred within populations, the level of population differentiation at the species level ($\theta = 0.065$) was significantly different from zero based on 95% confidence intervals (*Table 2*). This value was influenced by the differentiation of the two populations at the eastern end of the range, Cape Le Grand and Mt Ragged. The level of population differentiation was lower throughout the main range of the species (average between populations = 0.042). The differentiation between Cape Le Grand and the rest of the populations was 0.114, and Mt Ragged had a differentiation of 0.088 from the rest of the populations. Cape Le Grand and Mt Ragged were not similar to each other and had a population differentiation of 0.177. There was no significant association between population differentiation (θ) and geographic distance across the species as assessed by a Mantel test ($p = 0.3560$).

Table 2. – Measures of inbreeding coefficients, population differentiation and gene diversity for *Eucalyptus occidentalis*. *F,* overall inbreeding coefficient, θ, coancestry coefficient, f, degree of inbreeding within populations, H_T total genetic diversity, G_{ST}, relative degree of gene diversity among populations, 95% confidence intervals given in parentheses.

F	θ	f	H_T	G_{ST}(%)
0.088	0.065	0.025	0.373	5.9
(0.045, 0.132)	(0.050, 0.081)	(-0.014, 0.067)		

Unbiased estimates of genetic distance between all pair-wise comparisons of *E. occidentalis* populations were low (0.0671). The highest distance occurred between the eastern populations at Cape Le Grand and Mt Ragged (0.1032), and the lowest distance (0.0056) occurred between Young River and Truslove Road. An UPGMA analysis of population relationships showed the populations of *E. occidentalis* clustered together and clearly differentiated from the outgroup *E. kessellii*. (*Figure 2*). Within *E. occidentalis* there was little structure, except for the two populations from the eastern end of the range, Mt Ragged and Cape Le Grand, which showed differentiation from each other and from the rest of the populations. None of the nodes had significant bootstrap values.

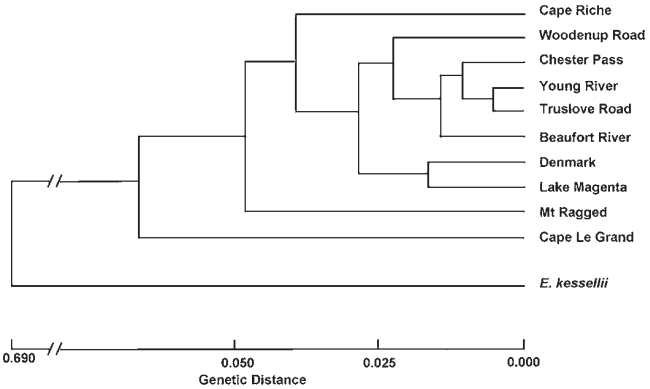

Figure 2. – UPGMA analysis of genetic distance between populations of *Eucalyptus occidentalis* and *E. kessellii*.

Discussion

The level of genetic diversity in *E. occidentalis* was moderate compared to other eucalypt species that have been analysed using RFLP analysis. It had lower diversity than *E. kochii* ($H_T = 0.514$; Byrne, 1999), *E. loxophleba* ($H_T = 0.418$; Hines and Byrne, 2001) and *E. camaldulensis* ($H_T = 0.53$; Butcher et al., 2002) which all have more widespread distributions. But it had similar diversity to *E. angustissima* subsp. *angustissima* ($H_T = 0.371$; Elliott and Byrne, 2003) that also occurs in small populations in the subcoastal area of southern Western Australia. The higher diversity in the more widespread species is likely to be related to the allelic distributions that are highly skewed towards rare alleles. In contrast the distribution of alleles in *E. occidentalis* was slightly U-shaped with similar proportions of rare and common alleles. This pattern of allele frequency distribution is similar to that commonly observed in allozyme studies (Chakraborty *et al.,* 1980) where the number of alleles and level of heterozygosity is generally lower than that detected using RFLP markers. The lower diversity in *E. occidentalis* compared to the widespread eucalypt species may also be related to small population size although there is no evidence that this is leading to inbreeding within the populations.

Eucalyptus occidentalis showed moderate levels of population differentiation at the species level but this was influenced by differentiation of the eastern populations as there was little genetic structuring among populations within the main distribution of the species. The level of differentiation between populations of *E. occidentalis* is lower than that detected in *E. angustissima* subsp angustissima (θ = 0.136, ELLIOTT and BYRNE, 2003) which has a similar distribution but a smaller range than *E. occidentalis*, although the higher differentiation of populations at the eastern end of the range is similar to the level of differentiation in *E. angustissima* subsp *angustissima.* A number of factors contributed to the differentiation of the two eastern populations. Both populations showed differences in the frequency of common and moderate frequency alleles at a number of loci, lower number of alleles over all loci and greater proportion of common alleles than rare alleles. In addition the Mt Ragged population had a larger number of unique alleles than any other population. These differences suggest that the populations have a complex history involving the influences of drift, fluctuating population sizes and possible selection.

The low differentiation within the main range of the distribution is unexpected as the small, isolated nature of the populations would be expected to lead to differentiation through the action of drift and possibly inbreeding. However, there was no evidence for the influence of either factor in the genetic diversity of the species as assessed in this study. The influence of drift and inbreeding on genetic diversity and structuring occurs over long time frames particularly in organisms such as trees with long generation times. Therefore the population similarity and random mating in *E. occidentalis* reflect historical conditions rather than recent influences. While south-west Australia did not experience glaciation there was cyclic variation in climatic conditions through the Pleistocene era (HOPPER *et al.,* 1996). During periods of wetter climatic conditions *E. occidentalis* may have been more abundant with larger populations than currently observed. The onset of aridity at the end of the Pleistocene would have resulted in drier conditions through the species distribution leading to population contraction to the wetter sites that characterise the species current ecological niche. Thus the lack of differentiation most likely reflects greater levels of population connectivity in historical times with insufficient time since population contraction for differentiation to develop. The greater differentiation and lower genetic diversity of the eastern outlier populations suggests that they have been isolated over time rather than through recent history or through recent colonisation in this area. Historical isolation of populations in a sporadic distribution would have allowed time for population differentiation to develop. The current distribution of *E. occidentalis* east of Esperance is more fragmented and sporadic than through the main range and this may represent the historical pattern of distribution in this region.

Several provenance trials of *E. occidentalis* have been conducted. In Australia, provenance trials involving a small number of mother trees from ten provenances were planted at five sites in South Australia, although only two sites had seed lots from all provenances. Assessment of growth and form showed no significant differences between sites but did show significant differences between provenances due to the poor performance of two provenances from the eastern end of the distribution (Fairlamb J, Bilman P and Harwood C, unpublished). There was little difference between the performance of the remaining provenances from the main distribution but the rankings were consistent and the Grass Patch and Ravensthorpe provenances performed best, although they were only planted at two sites. Unpublished results from provenance trials in Italy also found that Grass Patch and Ravensthorpe were among the fastest growing provenances (cited in HARWOOD, 2001). The poor performance of the provenances from the eastern end of the range is consistent with the reduced genetic diversity and high differentiation from the rest of the populations that was found in this study. The similar performance of the provenances from the main distribution is also consistent with the similar levels of genetic diversity and lack of genetic differentiation identified with molecular markers. The good performance of the Grass Patch and Ravensthorpe provenances does not appear to be correlated with the RFLP genetic diversity. The Grass Patch provenance could not be sampled for this study because it was inaccessible at the time of collection. But the Truslove population in this study is within 15 km of the original Grass Patch collection at Swan Lagoon, and the Woodenup population is 15km from Ravensthorpe. There is no indication of anything particular about the Truslove and Woodenup populations in this study as they both have diversity values and climatic variables in the middle of the range of all populations.

In contrast to other trials a provenance trial at an arid site in Israel involving five provenances found that the Cape Le Grand provenance produced the best height, and a provenance from north of Esperance (in the Grass Patch area) had the poorest height (ZOHAR and MORESHET, 1987). These results are in direct contrast to the results from trials in Australia and Italy, and may be related to the aridity of the site in Israel where any adaptation to aridity in the Cape Le Grand provenance may confer an advantage.

Forest trees generally exhibit low levels of differentiation for molecular markers but higher levels of differentiation for adaptive traits and this discrepancy is attributed to the affects of different evolutionary forces on the loci involved (KREMER *et al.,* 2000). KREMER *et al.* (2000) compared differentiation in quantitative traits and differentiation in molecular markers for three forest tree species and found the differentiation in quantitative traits was consistently higher than the differentiation in molecular markers. However these species all showed low levels of differentiation for molecular markers (F_{ST} = 0.019–0.041; KREMER *et al.,* 2000). Correlation between performance in provenance trials and estimates of genetic differentiation using molecular markers have been observed here in *E. occidentalis*, and have also been detected in the widespread species *E. camaldulensis* (BUTCHER *et al.,* 2002) and in *Picea abies* (COLLIGNON *et al.,* 2002). Therefore while low marker differentiation does not equate to low quantitative variation, detection of differentiation using molecular markers indicates quantitative variation is highly likely.

The genetic diversity identified in *E. occidentalis* suggests that the genetic resources of the species can be captured by sampling a few populations within the main range of the distribution. There is no evidence to suggest that the Grass Patch and Ravensthorpe provenances should have inherently better performance than other provenances from the main range, and more extensive provenance trials would be recommended before concluding that they are the preferred provenances for a breeding program. The differentiation and lower diversity of the eastern populations is consistent with their poor performance in provenance trials and suggests that there would be little benefit in including them in a breeding program for Australian conditions, although their performance on arid sites may be advantageous for planting on harsh sites in other countries. The natural environment of the populations is threatened by increasing salinisation of the landscape and *ex-situ* conser-

vation of the genetic resource will be important to ensure ongoing access to a broad base for the breeding program.

Acknowledgements

We thank W. O'SULLIVAN for making leaf collections. Funding was provided by Natural Heritage Trust grant no. 003071.

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Clonal Propagation of Teak (*Tectona grandis* Linn. f.): Effect of IBA Application and Adventitious Root Regeneration on Vertically Split Cuttings

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(Received 18th June 2003)

Summary

The experiment was conducted on leafy mono-nodal cuttings of 1-year-old seedlings. Each cutting was split vertically into two equal halves. The auxin treatments included 1000 and 2000 ppm IBA. The cuttings were planted under mist for rooting. Even untreated vertically split cuttings rooted profusely and 62.67% rooting was recorded in the controls. However, maximum per cent rooting (88.00%) was observed in 2000 ppm IBA followed by 1000 ppm IBA (80.00%). Furthermore, IBA treatment increased per cent sprouting, mean number of leaves, shoots and their length, mean number of roots and their length; the effectiveness of IBA increased with its increas-