Microsatellite and morphological analysis of *Eucalyptus globulus* populations

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Abstract: *Eucalyptus globulus* Labill. is native to southeastern Australia and is the most important temperate hardwood plantation species in the world. It consists of four subspecies that are morphologically and geographically distinct but that are linked by morphologically and geographically intermediate populations. The Jeeralang provenance, an intermediate population from southeastern Victoria, is an important source of seed for plantations and genetic material for breeding programs because of its superior growth and wood density. To determine the genetic affinities of this provenance, 154 trees from three subspecies and the Jeeralang provenance were sampled. Analysis of 12 morphological characters verified that the Jeeralang provenance is intermediate between subspecies *bicostata* (Maiden, Blakely, & J. Simm.) Kirkpatr., *globulus* and *pseudoglobulus* (Naudin ex Maiden) Kirkpatr., with individuals having closest affinities to either ssp. *globulus* or ssp. *bicostata*. However, eight microsatellite loci showed that the Jeeralang provenance has greater affinities to Victorian ssp. *globulus* to which it is geographically closest. In contrast, Tasmanian and Victorian ssp. *globulus* are morphologically similar yet appear to be distinct at the molecular level. This study emphasizes the importance of using traits that are unlikely to be influenced by selection when determining the origin and affinities of populations.

Résumé : *Eucalyptus globulus* Labill. est une espèce indigène du sud-est de l'Australie. C'est la plus importante espèce feuillue en plantation au monde. L'espèce est constituée de quatre sous-espèces qui sont morphologiquement et géographiquement distinctes. Cependant, ces sous-espèces sont liées entre elles par des populations morphologiquement et géographiquement intermédiaires. La provenance Jeeralang, une population intermédiaire de la région sud-est de Victoria, représente une source importante de semences pour l'établissement de plantations, en plus de fournir du matériel génétique pour les programmes d'amélioration, en raison de sa supériorité pour la croissance et la densité du bois. Afin de déterminer les affinités génétiques de cette provenance, 154 arbres représentatifs de trois sous-espèces et de la provenance Jeeralang ont été échantillonnés. La caractérisation de 12 caractères morphologiques a permis de confirmer que la provenance Jeeralang était intermédiaire entre sous-espèces *bicostata* (Maiden, Blakely & J. Simm.) Kirkpatr., *globulus* et *pseudoglobulus* (Naudin ex Maiden) Kirkpatr. Les individus de cette provenance avaient plus d'affinités avec ssp. *globulus* et ssp. *bicostata*. Cependant, l'analyse de huit loci de microsatellites a révélé que la provenance Jeeralang avait le plus d'affinités avec la sous-espèce *globulus* de la région de Victoria, dont elle est géographiquement la plus rapprochée. À l'opposé, les individus de la sous-espèce *globulus* des régions de Tasmanie et de Victoria étaient morphologiquement similaires même s'ils étaient distincts à l'échelle moléculaire. Cette étude souligne l'importance d'utiliser des caractères peu ou pas influencés par la sélection naturelle lorsqu'on désire déterminer l'origine des populations et leurs affinités.

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Introduction

Eucalyptus globulus Labill. (blue gum) is the most important pulpwood species in temperate regions of the world, with a plantation area estimated at 1.7×10^6 ha by 1995 (Tibbitts et al. 1997). Breeding programs, aimed at improving

growth and wood quality, are being undertaken in Argentina, Australia, Chile, China, Portugal, and Spain (Tibbitts et al. 1997). *Eucalyptus globulus* has a widespread natural distribution in southeastern Australia, and across this range there is significant spatial genetic variation, especially in reproductive characters. On the basis of this morphological differentiation, Kirkpatrick (1975) amalgamated *E. globulus* and its related taxa into a single species complex with four subspecies: ssp. *bicostata* (Maiden, Blakely, & J. Simm.) Kirkpatr., ssp. *globulus*, ssp. *maidenii* (F. Muell.) Kirkpatr., and ssp. *pseudoglobulus* (Naudin ex Maiden) Kirkpatr. (these will be hereafter referred only by their subspecific names, and *E. globulus* will refer to the species complex). This treatment of the species complex was supported by the analysis of Jordan et al. (1993), who combined the morphological data of Kirkpatrick (1975) with measurements from collections of new populations. The core areas of these four subspecies are both geographically (Fig. 1) and morphologically distinct. Subspecies *maidenii* has the smallest capsules

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Fig. 1. The distribution of the *Eucalyptus globulus* subspecies and their intergrades in southeastern Australia (modified from Jordan et al. 1993) and the locations of sites sampled in this study. The number of trees sampled at each locality (cluster of symbols) is given. Within the two large circles are small intergrade populations.

of the complex with up to seven fruit per umbel, while *globulus* has large, solitary capsules. Subspecies *bicostata* and *pseudoglobulus* are both three fruited, with *pseudoglobulus* having smaller fruit, longer pedicels, and fewer ribs on the capsules than *bicostata*. While the core areas of these subspecies are distinct, they are linked by extensive zones of clinal intergradation, characterized by intermediate capsule morphology.

The forest industry has shown most interest in *globulus*, and in the early years of domestication, King Island was the favoured provenance because of its fast growth in field trials (Volker and Orme 1988). However, there has subsequently been a shift in breeding objectives towards a combination of higher wood density together with faster overall growth rate (Borralho et al. 1993). This change has resulted in increasing interest in the intergrade populations, especially those in

the Strzelecki Ranges in Victoria, because of their high wood density and relatively fast growth (noted by Dutkowski and Potts 1999). The Jeeralang provenance is the main area within the Strzelecki Ranges from which seed for pulpwood plantations has been sampled in recent years. However, with the rapid expansion of plantation forestry, demand for seed from Jeeralang is increasing, and seed collectors need to know how far outside the Jeeralang core they can extend their sampling to obtain the superior genotypes. Problems have also arisen in the labelling of Jeeralang seed, as the provenance has been difficult to assign to one subspecies. On average, the Jeeralang provenance is morphologically intermediate between subspecies *bicostata*, *globulus*, and *pseudoglobulus* (Jordan et al. 1993) with a diverse range of phenotypes present, and as such, its genetic affinities and origin are as yet unresolved. Several hypotheses have been

put forward (see Kirkpatrick 1975; Jordan et al. 1993; Nesbitt et al. 1995), but these are inconclusive as they are based on morphological traits (which may be influenced by selection) or have limited sample size.

Microsatellites, also known as simple sequence repeats (SSRs), are a class of molecular markers that do not appear to have any real function in the nuclear genome and are, therefore, probably selectively neutral (Jarne and Lagoda 1996), unlike morphological characters that may be subject to selection. Microsatellites have high levels of polymorphism (Glaubitz and Moran 2000) and are, therefore, extremely useful for fine-scale genetic analyses. As microsatellites are a relatively recent discovery, there have been only a few eucalypt population studies using them (Byrne et al. 1996; Brondani et al. 1998; Steane et al. 2001; McGowen et al. 2001).

This study focuses on the Jeeralang provenance and uses morphology and molecular markers to elucidate its relationship with *bicostata*, *globulus*, and *pseudoglobulus*. Most of the morphological traits used by Kirkpatrick (1975) and Jordan et al. (1993) are measured to verify the intermediacy of the Jeeralang provenance relative to the other three subspecies. Eight of the microsatellite loci developed by Steane et al. (2001) are used to determine the genetic relationships between these groups, and characterize their levels of diversity and inbreeding.

Materials and methods

Plant material

Leaf tissue (for DNA extraction) and a herbarium specimen, including buds and capsules, were collected from 154 adult trees in natural stands in southeastern Victoria and eastern Tasmania (Fig. 1). In most cases, trees sampled were at least 100 m apart to avoid sampling closely related trees (see Skabo et al. 1998). Twenty-eight trees from the Jeeralang provenance were sampled, as were 20–37 trees each of “core” ssp. *bicostata*, *globulus*, and *pseudoglobulus* (as defined by Kirkpatrick 1975) from several Victorian localities occurring as close as possible to the Jeeralang provenance, as well as 41 trees from Tasmanian ssp. *globulus* (Fig. 1). For the morphological and molecular analyses, five groups were defined as populations: core *bicostata*, Tasmanian core *globulus*, Victorian core *globulus*, core *pseudoglobulus*, and core Jeeralang.

Morphometric methods

For each Victorian tree sampled, 12 capsule, inflorescence, and leaf characters were measured, following the character descriptions of Kirkpatrick (1975) and Jordan et al. (1993) (see caption in Fig. 2). Five average-sized umbels with all parts intact were selected from the youngest capsule age-class, and the middle capsule of each umbel (where applicable) was measured. Inflorescence data were collected from up to 70 umbels per tree. Three representative leaves from each tree were photocopied, and the dimensions were measured.

Analyses were undertaken on the means of the five capsule and three leaf replicates from each tree. The variables were transformed as in Jordan et al. (1993) to optimize population normality and remove the correlation between popu-

lation means and variances. Multivariate analyses of the three subspecies (*bicostata*, *globulus*, and *pseudoglobulus*) were undertaken using canonical variate analysis (CVA; Phillips et al. 1973), an ordination technique that maximizes differences between defined groups relative to within-group variation. The Jeeralang and other intergrade individuals were placed within the multivariate discriminant space defined by the three subspecies. The subspecies core to which each Jeeralang individual was morphologically closest was calculated using the classification criterion based on a linear function derived from the pooled within-group covariance matrix (SAS Institute Inc. 1996), potentially dividing the Jeeralangs into three morphs: Jeeralang b (*bicostata*-like), Jeeralang g (*globulus*-like), and Jeeralang p (*pseudoglobulus*-like). As the three-group analysis may force Jeeralang to fall intermediate between the cores, a separate analysis was undertaken, treating Jeeralang as a fourth defined group. Both the three-group and four-group CVAs were performed using all 12 morphological variables. For all multivariate analyses, the vectors indicating the direction of variation in each character were constructed from the standardized canonical coefficients. The lengths of the vectors were proportional to the ANOVA *F* values, calculated between subspecies groups for each trait. CVA and ANOVA were undertaken using the PROC DISCRIM and PROC GLM procedures, respectively, in the statistical package SAS Institute Inc. (1996).

Molecular methods

Total genomic DNA was extracted following the protocol of Doyle and Doyle (1990), using an incubation temperature of 55°C. Following extraction, DNA concentration and purity were estimated using agarose gel electrophoresis with ethidium bromide staining, comparing with a standard molecular weight marker (λ *HindIII*). Where necessary, DNA was cleaned by re-precipitation in the presence of high salt concentration (2.0 M NaCl). The eight “EMCRC” microsatellite primer pairs used in this study were designed by Steane et al. (2001). The forward primers were synthesized with a fluorescent label (either HEX or TET) attached to the 5' end to allow detection of polymerase chain reaction (PCR) products. For each DNA sample, PCR amplification of each of the eight EMCRC microsatellite loci was performed, with PCR reaction mixture (25 μ L final volume) containing 1 $\times$ *Taq* polymerase reaction buffer (Fisher Biotech; 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/mL gelatin); 100 μ g/mL BSA (bovine serum albumin); 2 mM MgCl₂; 120 μ M each of dATP, dCTP, dGTP, and dTTP; 5 nmol of each primer; 2.2 units *Taq* DNA polymerase; and 40 ng genomic DNA. The PCR amplification was performed in a PTC-100 programmable thermal controller (MJ Research, Inc.) using the annealing temperatures given by Steane et al. (2001). The touchdown PCR cycling program used for each locus varied according to annealing temperature, while all other temperatures and times remained constant. For an annealing temperature of 55°C, the program was as follows: 94°C for 2 min; 10 cycles of 94°C for 1 min, 57°C for 55 s, and 72°C for 50 s; 10 cycles of 94°C for 1 min, 55°C for 55 s, and 72°C for 50 s; 10 cycles of 94°C for 30 s, 53°C for 55 s, and 72°C for 50 s; and a final 5-min extension at 72°C.

Fig. 2. Plots of individual canonical variables from analysis using 12 morphological variables. The multivariate space is defined by the three subspecies cores (for *globulus*, only Victorian samples are used in the analysis), with values for Jeeralang individuals superimposed in this space. The proportion of variation explained by each axis is shown. The vector diagram indicates the direction of the eight main variables in the space defined by CV1 and CV2, with length proportional to the F value. C/U, number of capsules per umbel; LWP, length to widest point of leaf; peduncle, peduncle length; pedi, pedicel length; disc, disc height; CD, capsule diameter; ribs, number of ribs on capsule; LL, lamina length excluding petiole. The broken line indicates the division of the Jeeralang group into *bicostata*-like (Jeeralang b) and *globulus*-like (Jeeralang g) morphs.

The PCR products were combined with two volumes of formamide B-blue loading buffer (formamide with 0.1 mg/mL bromophenol blue sodium salt) and denatured at 95°C for 4 min before electrophoretic separation on 15-cm 4% denaturing acrylamide gels at 40°C, using a Gel-Scan 2000 real-time acrylamide gel system (Corbett Research). Alleles were sized by comparison with the Gene Scan 350 (TAMRA) size standard, using the computer software package Gene Profiler™ (Scanalytics, Inc.). Repeats of individuals were included (both within and among gels) to assess the repeatability of the size analysis. Where stutter bands were observed, the darkest, and usually largest, band was considered the true allele. When only one allele was observed, homozygosity was assumed (ignoring possible null alleles). Molecular weights determined for the same fragment within a gel, and for the same individual between gels, usually varied within 1 base pair (bp) of the bin size. Fragments falling within these size ranges were grouped into single-allele bin sizes (data not shown).

The genotypes at each locus, for each individual, were analysed using the software program GDA (Lewis and Zaykin 2001). The genotype data and allele frequencies were used to calculate the following measures of diversity, averaged for each population and each locus: the number of alleles per locus (A), the percentage of polymorphic loci (P), observed heterozygosity (H_o), and Hardy–Weinberg ex-

pected heterozygosity (H_e). The inbreeding coefficients F_{IS} (inbreeding in individuals relative to their subspecies, equivalent to Wright's fixation index, F), F_{ST} (inbreeding in subspecies relative to the total *E. globulus* complex), and F_{IT} (inbreeding in individuals relative to the total complex) were also calculated. A pairwise F_{ST} matrix was used to delineate relationships among groups within *E. globulus*, with the 95% confidence interval (CI) on F_{ST} values derived from 1000 bootstraps.

To summarize the genetic distances between *bicostata*, *globulus* (Tasmania and Victoria), Jeeralang, and *pseudoglobulus*, a two-dimensional ordination of the groups was derived using global nonmetric multidimensional scaling (GNMDS, Minchin 1987). Analysis was performed using 10 randomized starts in one, two, three, and four dimensions, and in this case, the two-dimensional solution was the best summarization of the genetic distance matrix. The analysis was undertaken using the package DECODA and associated NMDS program (Minchin 1987).

The probability of each Jeeralang individual being from the four predefined populations (*bicostata*, Tasmanian *globulus*, Victorian *globulus*, and *pseudoglobulus*) was determined using "structure" (Pritchard et al. 2000). The average q (estimated membership in each population) for each Jeeralang individual was derived from 1000 Markov chain Monte Carlo (MCMC) repetitions, following a burnin of 1000.

Table 1. Overall genetic parameters for each of the eight microsatellite loci used in this study.

Locus	<i>n</i>	<i>A</i>	<i>H_o</i>	<i>H_e</i>	<i>F_{IS}</i>	<i>F_{IT}</i>	<i>F_{ST}</i>
EMCRC1a	150	24	0.46	0.92	0.47	0.51	0.09
EMCRC2	152	20	0.71	0.90	0.19	0.22	0.04
EMCRC3	152	18	0.70	0.86	0.14	0.19	0.06
EMCRC5	144	30	0.65	0.86	0.21	0.26	0.06
EMCRC7	134	18	0.57	0.82	0.20	0.32	0.15
EMCRC10	141	16	0.42	0.88	0.51	0.53	0.03
EMCRC11	152	18	0.78	0.90	0.09	0.14	0.05
EMCRC12	149	21	0.67	0.81	0.06	0.20	0.15
Mean		20.6	0.62	0.87	0.24	0.30	0.08 (0.05–0.11)

Note: Inbreeding coefficients (F_{IS} , F_{IT} , F_{ST}) were based on the five populations listed in Table 2. The 95% confidence interval on the mean F_{ST} value, derived from 1000 bootstraps, is given in parentheses. *n*, number of individual trees (varies among loci, as clear amplification products were not obtained for all individuals); *A*, observed number of alleles per locus (mean *A* across loci is A_s , the average number of alleles per locus for the species complex); H_o , observed heterozygosity; H_e , expected heterozygosity (mean H_e across loci is H_T , the total expected heterozygosity for the species complex).

Results

Morphological relationships within *Eucalyptus globulus*

The number of capsules per umbel, pedicel length, and capsule diameter were the most important characters in differentiating the three subspecies cores (*bicostata*, *globulus*, and *pseudoglobulus*; Fig. 2). When placed in the multivariate space defined by three-group CVA, the Jeeralang individuals fell intermediate between the subspecies cores, tending more towards *globulus* and *bicostata* than *pseudoglobulus* on the basis of morphological characters. Some Jeeralang individuals fell within the *bicostata* and *globulus* subspecies cores. The Jeeralang provenance is morphologically variable, and for each Jeeralang individual, the subspecies core to which it was morphologically closest was calculated, potentially dividing the Jeeralangs into three morphs. Fifteen individuals were most similar to *globulus* (Jeeralang g), 10 were closest to *bicostata* (Jeeralang b), and no Jeeralang individuals had closer affinities to *pseudoglobulus* than to either of the other subspecies cores. When the Jeeralang provenance was treated as a distinct group (four-group CVA), the group also fell effectively intermediate between the three subspecies cores, deviating only slightly on the third axis, which represented a small amount of the variance (5%; data not shown).

Microsatellite variation within *Eucalyptus globulus*

All eight loci used were highly polymorphic with 16–30 alleles per locus ($A_s = 20.6$; Table 1). Each population had up to 12.4 alleles per locus ($A_p = 11.3$; Table 2). The high level of polymorphism was also reflected in the mean and observed heterozygosity per population ($H_o = 0.62$, $H_e = 0.82$; Table 2) and the total expected heterozygosity for the species complex ($H_T = 0.87$; Table 1).

The observed heterozygosity was less than the expected within population heterozygosity for all loci, as reflected in the positive F_{IS} for all loci (Table 1). The positive mean F_{IS} of 0.238 (Table 1) indicates an overall excess of homozygosity within populations compared with that expected under random mating. Some loci (e.g., EMCRC1a, EMCRC7, and EMCRC12) had higher F_{ST} values than other loci (Table 1), indicating that these loci were more informative in differentiating populations. No single population ap-

Table 2. Genetic diversity parameters for each population in this study.

Population	<i>n</i>	<i>A</i>	<i>H_o</i>	<i>H_e</i>	<i>F</i>
<i>bicostata</i>	28	11.9	0.63	0.87	0.28
<i>globulus</i> (Tas.)	36.5	12.4	0.71	0.83	0.15
<i>globulus</i> (Vic.)	36.4	11.0	0.57	0.77	0.26
Jeeralang	26.9	9.9	0.61	0.75	0.20
<i>pseudoglobulus</i>	19	11.1	0.57	0.87	0.35
Mean		11.3	0.62	0.82	0.25

Note: See Table 1 for definitions of variables. *F*, Wright's fixation index.

peared to be much more diverse than any other (Table 2, H_e values). All populations had a higher level of expected diversity than observed, and therefore, each had a positive *F* value (Table 2).

Population differentiation

All loci had alleles unevenly distributed across the sampled range of *E. globulus* as indicated by the positive F_{ST} values for each locus (Table 1). For example, the 279-bp allele at locus EMCRC7 occurred at a high frequency in Jeeralang and Victorian *globulus* (frequency 0.72 and 0.64, respectively; data not shown); however, it was rare in *bicostata* and *pseudoglobulus* (0.09 and 0.08, respectively; data not shown). The overall F_{ST} (degree of population differentiation) for this study was 0.08 with a 95% confidence interval of 0.05–0.11 (Table 1).

Relationships between the groups within the species complex were represented by pairwise F_{ST} values and Nei's (1972) genetic distance (Table 3). The affinities of these populations based on Nei's (1972) genetic distances were summarized in a two-dimensional GNMDS ordination (Fig. 3). The Jeeralang provenance was significantly closer to Victorian *globulus* than any other group ($F_{ST} = 0.01$, $D = 0.10$, Table 3), while all other groups were similarly differentiated from the Jeeralang provenance (i.e., with overlapping 95% CIs on F_{ST} values; Table 3). The *bicostata* and *pseudoglobulus* populations also grouped quite closely ($F_{ST} = 0.04$, $D = 0.54$; Table 3), although the F_{ST} value was not significantly smaller than the F_{ST} between *bicostata* or *pseudoglobulus* and each of the remaining *E. globulus*

Table 3. Pairwise percentage F_{ST} values (above diagonal) and Nei's 1972 genetic distance (D , below diagonal) for populations of *Eucalyptus globulus*.

	<i>bicostata</i>	Tasmanian <i>globulus</i>	Victorian <i>globulus</i>	Jeeralang	<i>pseudoglobulus</i>
<i>bicostata</i>	—	0.08 (0.05–0.11)	0.10 (0.04–0.18)	0.10 (0.04–0.18)	0.04 (0.03–0.06)
Tasmanian <i>globulus</i>	0.81	—	0.08 (0.05–0.12)	0.09 (0.05–0.13)	0.08 (0.05–0.11)
Victorian <i>globulus</i>	0.78	0.51	—	0.01 (0.005–0.02)	0.08 (0.03–0.14)
Jeeralang	0.73	0.53	0.10	—	0.08 (0.04–0.15)
<i>pseudoglobulus</i>	0.54	0.86	0.58	0.60	—

Note: The 95% confidence interval on F_{ST} values, derived from 1000 bootstraps, is given in parentheses.

Fig. 3. Two-dimensional GNMDS ordination of Nei's (1972) genetic distances between populations of *Eucalyptus globulus* (from Table 3).

groups. Tasmanian *globulus* was not significantly closer to Victorian *globulus* than any other group (i.e., all CIs for Tasmanian *globulus* vs. other groups were overlapping; Table 3). The ordination shows that both the Jeeralang and Victorian *globulus* were genetically intermediate between three differentiated populations: *bicostata*, Tasmanian *globulus*, and *pseudoglobulus* (Fig. 3). The affinities of the Jeeralang provenance to Victorian *globulus* was confirmed by the analysis with "structure" (Pritchard et al. 2000). The mean probability of each Jeeralang individual being a member of the Victorian *globulus* population was 0.81. Twenty-three of 28 Jeeralang individuals were assigned to the Victorian *globulus* population, two individuals were admixtures of Victorian and Tasmanian *globulus*, one appeared to be an admixture of *bicostata* and Victorian *globulus*, one was an admixture of *pseudoglobulus* and Victorian *globulus*, while one had closest affinities to *bicostata*.

There was no significant difference between the *bicostata*-like (Jeeralang b) and the *globulus*-like (Jeeralang g) morphs within the Jeeralang provenance ($F_{ST} = -0.01$, 95% CI = -0.03 to 0.01 ; data not shown). Similarly, there was no significant difference between Jeeralang b and Victorian *globulus* ($F_{ST} = -0.006$, 95% CI = -0.02 to 0.003 ; data not shown) and Jeeralang g and Victorian *globulus* ($F_{ST} = 0.01$, 95% CI = 0 to 0.02 ; data not shown). However, both Jeeralang b and Jeeralang g morphs were significantly differentiated from *bicostata* ($F_{ST} = 0.10$ in both cases, with lower CI boundaries greater than zero; data not shown).

Discussion

Microsatellite diversity in *Eucalyptus globulus*

This study (using 154 individuals) is the largest microsatellite study in *Eucalyptus* to date. All of the microsatellite loci used had high levels of polymorphism. The levels of diversity estimated here for the whole *E. globulus* complex are comparable with microsatellite studies in many other forest trees, such as *Eucalyptus nitens* (Dean & Maiden) Maiden (Byrne et al. 1996), *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. (Streiff et al. 1998), *Melaleuca alternifolia* (Maiden and Betcher) Cheel (Rossetto et al. 1999), *Camellia japonica* L. (Ueno et al. 2000), and *Caryocar brasiliense* Camb. (Collevatti et al. 1999) but higher than those found in the endangered tropical tree *Swietenia humilis* Zucc., probably the result of forest fragmentation of the latter (White et al. 1999).

In this study, the observed heterozygosity is lower than expected (Table 2), indicating a marked deviation from Hardy-Weinberg expectations. This contrasts with isozyme studies that show that Wright's fixation index is usually negative in adult *Eucalyptus* populations because of selection against homozygosity through the life-span (Potts and Wiltshire 1997). These studies usually sample a much smaller population unit than in our case. Therefore, the excess homozygosity detected in this study is most likely due to the Wahlund effect (temporal or spatial substructuring of populations into gene pools differing in their allele frequencies), as most "populations" in this study covered a wide geographic range and consisted of many, possibly genetically differentiated "subpopulations".

Genetic relationships between the subspecies cores

The core samples of each taxon were well differentiated, both morphologically and at the molecular level. Capsule morphology characters (in particular number of capsules per umbel, pedicel length, and capsule diameter) were most influential in differentiating the subspecies, as shown previously by Kirkpatrick (1975) and Jordan et al. (1993). Microsatellite loci were also useful for differentiating populations, especially the loci with high F_{ST} values (e.g., EMCRC1a, EMCRC7, and EMCRC12). Although Tasmanian and Victorian *globulus* are morphologically similar (Kirkpatrick 1975; Jordan et al. 1993), the two groups appear to be distinct at the molecular level. The distinction between Tasmanian and Victorian *globulus* has also been shown using RAPD markers (Nesbitt et al. 1995) and quantitative traits (Dutkowski and Potts 1999). While capsule morphology is a key taxonomic trait, in this case, capsule morphology gives a biased impression of the differences between Tasmanian and Victo-

rian *globulus*. It appears that genetic drift between these geographically separate populations has occurred at the molecular level, while similarity in selection pressure may have resulted in large capsules occurring in both areas. Large capsules may have a selective advantage in dry, coastal, more fire-prone areas (such as eastern Tasmania and the coastal plains of southern Victoria), as a seed insulation mechanism during fire (Bradstock et al. 1994; House 1997). The larger flowers of *globulus* may also be a mechanism to avoid pollen swamping from smaller flowered species when invading new habitats (i.e., reproductive character displacement; Howard 1993). Subspecies *globulus* cannot act as the maternal parent in crosses with smaller flowered species, as the pollen tubes of smaller flowered species cannot grow down the full length of the *globulus* style (Gore et al. 1990).

Genetic affinities and origin of the Jeeralang provenance

When placed in the morphological framework defined by the three subspecies, the Jeeralang population falls intermediate between the cores, with morphological affinities closer to *bicostata* and *globulus* than to *pseudoglobulus*. The morphological intermediacy of the Jeeralang population has been shown before (Jordan et al. 1993). However, it was necessary to verify that this relationship held for the trees sampled in this study, so that the patterns of morphological and molecular variation could be compared directly.

At the molecular level, the Jeeralang population has greater affinities to Victorian *globulus* rather than *bicostata* or *pseudoglobulus*. Because more of the Jeeralang individuals had morphological affinities to *globulus* than to *bicostata* in our sample (i.e., 15 vs. 10), we thought that the molecular similarity of the Jeeralang and *globulus* populations may have been due to a greater frequency of *globulus*-like morphs in our Jeeralang sample. When the Jeeralangs were split into their morphs, however, both morphs grouped more closely to *globulus* than to *bicostata*, even though the Jeeralang b morph is morphologically most similar to *bicostata*. There was no significant difference, at the molecular level, between these two groups.

Kirkpatrick (1975) suggested that the intermediate populations within the *E. globulus* complex arose through primary differentiation (divergence within a continuous series of populations resulting from varying selection pressures) rather than secondary intergradation (hybridization and introgression between previously isolated populations). In contrast, Jordan et al. (1993) argued that the pattern of variation in capsule morphology in the complex is best reconciled by secondary intergradation. In this case, it was argued that full differentiation of the *globulus* morph occurred in isolation in Tasmania and the high variability and intermediate morphs in Victoria were due to secondary contact following migration northwards back into the range of ancestral populations. However, if there had been recent contact between differentiated populations then we would expect intermediate microsatellite frequencies, higher variability, and an association between morphological and molecular variation within the intermediate population (Endler 1977; Rieseberg and Wendel 1993). None of these criteria for recent secondary intergradation were observed in our study. Zones of ancient secondary contact or primary differentiation are

difficult to distinguish based on current patterns of variation (Endler 1977). The absence of any Tasmanian cpDNA type in the Victorian *globulus* would argue against seed migration from Tasmania into this area (Freeman et al. 2001), although pollen-mediated invasion of ancestral, intermediate populations on the Victorian coastal plain and resurrection of the *globulus* morphology cannot be dismissed. However, regardless of the origin, our data would strongly argue that selection has played an important role in maintaining the morphological differentiation between the *globulus* phenotypes on the coastal plain and the intermediate phenotypes in the Strzelecki Ranges (such as the Jeeralang provenance). The absence of significant differentiation between morphologically distinctive populations would suggest that high levels of gene flow is currently occurring, or has occurred in the recent past, between these geographically proximal populations.

Practical implications

As demand for *E. globulus* seed increases, seed collectors need to explore gene pools other than the Jeeralang provenance for superior genotypes. This study indicates that Jeeralang has closest genetic affinities to Victorian *globulus*, and therefore, seed collectors should perhaps move further south when the Jeeralang seed is in short supply. Some of these surrounding populations have close quantitative affinities to the Jeeralang provenance (Dutkowski and Potts 1999).

The Victorian ssp. *globulus* appears to be differentiated from the Tasmanian core *globulus* at the molecular level. Most of this differentiated Victorian ssp. *globulus* exists in small, fragmented stands on farmland in South Gippsland and is under immediate threat because of lack of regeneration and potential expansion of plantations with non-native genotypes in the area. As these remnants are unique variants of ssp. *globulus* and are a record of the past distribution of *E. globulus*, they are of high conservation value and require protection. The sampling of these populations in this study was not extensive enough to determine the genetic consequences of fragmentation and small population size (e.g., pollen swamping from nearby abundant species, inbreeding, drift, and bottlenecks; Savolainen and Kuittinen 2000) on these remnant stands. Future molecular studies should address these issues. Additionally, the differentiation between Tasmanian and Victorian ssp. *globulus* allows for the characterization of unique "marker alleles". These may be used to detect genetic pollution of native stands from mainland provenances of *globulus* planted in Tasmania or vice versa.

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