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Parallel evolution of dwarf ecotypes in the forest tree *Eucalyptus globulus*

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Summary

• Three small populations of a dwarf ecotype of the forest tree *Eucalyptus globulus* are found on exposed granite headlands in south-eastern Australia. These populations are separated by at least 100 km.

• Here, we used 12 nuclear microsatellites and a chloroplast DNA marker to investigate the genetic affinities of the dwarf populations to one another and to their nearest populations of tall *E. globulus*. Cape Tourville was studied in greater detail to assess the processes enabling the maintenance of distinct ecotypes in close geographical proximity.

• The three dwarf populations were not related to one another and were more closely related to adjacent tall trees than to one another. At Cape Tourville the dwarf and tall ecotypes were significantly differentiated in microsatellites and in chloroplast DNA. The dwarf and tall populations differed in flowering time and no evidence of pollen dispersal from the more extensive tall to the dwarf population was found.

• The three dwarf populations have evolved in parallel from the local tall ecotypes. This study shows that small marginal populations of eucalypts are capable of developing reproductive isolation from nearby larger populations through differences in flowering time and/or minor spatial separation, making parapatric speciation possible.

Key words: ecological speciation, ecotypic variation, eucalypt genetics, *Eucalyptus globulus*, flowering time, gene flow, parallel evolution, parapatric speciation.

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Introduction

The study of genetic differentiation along environmental gradients is fundamental to understanding adaptive evolution (Endler, 1977; Storfer, 1999). Adaptation, the process of genetic change through which a population increases its fitness to its environment, is thought to be driven by natural selection and countered by gene flow among populations. Local adaptation occurs where selective pressures are strong enough to counter gene flow or where gene flow is limited (Endler, 1986; Linhart & Grant, 1996). A consequence of local adaptation is the development within species of ecotypes associated with particular habitats (Turesson, 1922; Volis *et al.*, 2002). Although ecotypes are often assumed to represent single-origin variants that have dispersed into suitable habitats, an accumulating body of evidence suggests that ecotypes can be polyphyletic. Recent molecular data are consistent with the parallel evolution of matching ecotypes at different sites within species of both animals (Rolán-Alvarez *et al.*, 2004; Natoli *et al.*, 2006; Ostbye *et al.*, 2006; Panova *et al.*, 2006) and plants (Brochmann *et al.*, 2000; Levin, 2001; Hogbin & Crisp, 2003). Most polyploid plant species appear to have multiple origins (Soltis & Soltis, 1999), and the same is likely to apply to ecotypes or races that differ in their ploidy.

Similarly, at least one diploid hybrid species has been reported to have multiple origins (*Argyranthemum dundingii*; Brochmann *et al.*, 2000). However, little attention has been given to the possibility of multiple origins of species or ecotypes in the absence of hybridization or gross chromosomal shifts such as a change in ploidy. Such cases of parallel evolution are believed possible when matching selective pressures in different parts of a species' range act on similar progenitor populations (Levin, 2001). This concept has been extended to include the proposal that some diploid plant species may have arisen recurrently in several places or times from a single species (Levin, 2001). However, the connection between local adaptation and the presence and evolution of reproductive isolation remains poorly understood (Schluter, 2001; Coyne & Orr, 2004).

This study investigates the evolutionary origin and population dynamics of a dwarf ecotype of the Australian species, *Eucalyptus globulus*. *Eucalyptus globulus* is a forest and woodland tree, typically growing 15–60 m tall (Curtis & Morris, 1975). It is often a dominant of coastal forests in south-eastern Australia (Williams & Potts, 1996), specifically Victoria and the islands of Tasmania (Fig. 1). The species is genetically diverse with distinct geographic races showing broad-scale differences in numerous quantitative traits (Dutkowski & Potts, 1999), many of which have adaptive significance such as frost tolerance (Tibbits *et al.*, 2006) and drought tolerance (Dutkowski, 1995; Toro *et al.*, 1998). Comparison of racial differentiation based on molecular (F_{ST}) and quantitative (Q_{ST}) data suggests that some of the quantitative traits used to define races in *E. globulus* have been influenced by natural selection, resulting in cases of both phenotypic divergence of parapatric races and phenotypic convergence of allopatric races (Steane *et al.*, 2006). There is also evidence that significant genetic differentiation can occur in *E. globulus* over very short distances (< 10 m) owing to limited seed dispersal (Skabo *et al.*, 1998; Jones *et al.*, 2007). All *Eucalyptus* species including *E. globulus* are believed to have the same chromosome number ($2n = 22$), since there are no confirmed cases of polyploidy in the genus (Potts & Wiltshire, 1997).

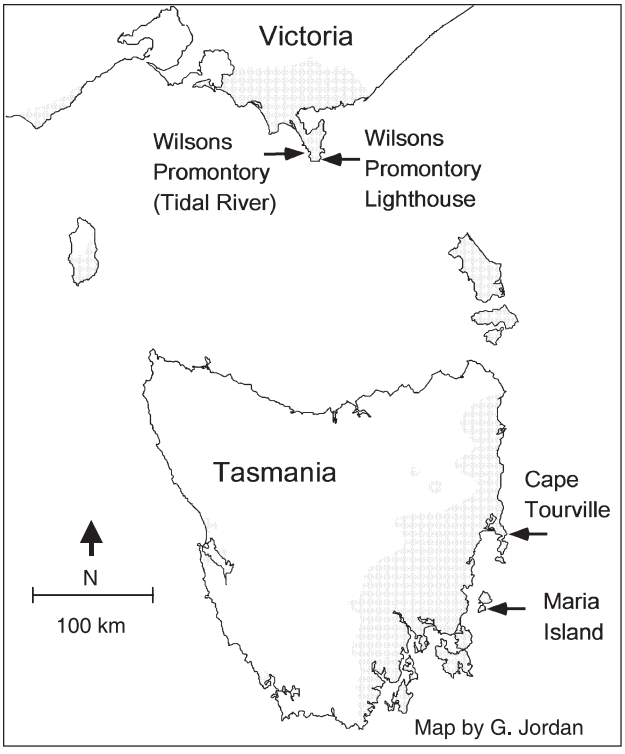

Fig. 1 Map of south-eastern Australia showing the natural distribution of *Eucalyptus globulus* (shaded area) and location of sampling sites.

Atypical, reproductively mature 'dwarf' populations of *E. globulus*, growing to an average height of less than 4 m and often having multiple stems, are known from three locations on exposed granite headlands (see Fig. 1): Maria Island (Brown & Bayly Stark, 1979) and Cape Tourville (Chalmers, 1992) in eastern Tasmania, and Wilson's Promontory, the southern tip of Victoria (Jordan *et al.*, 2000). These populations would be classified as 'peripheral' in both an ecological and geographic sense (Fig. 1). At Cape Tourville the species changes from a dwarf form on the cliff-top to a forest tree over a distance of 300 m. It has been suggested that the dwarf habit of *E. globulus* at these locations is related to exposure to salt and high winds or water stress (Brown & Bayly Stark, 1979; Chalmers, 1992; Jordan *et al.*, 2000).

Previous work by Chalmers (1992), comparing the dwarf *E. globulus* trees at Cape Tourville with normal-height trees from a second site 500 m inland and a third site a further kilometre inland, demonstrated clinal phenotypic variation in a number of characters. Near the coast, trees became shorter, were more precocious in vegetative phase change and flowering, and showed an increase in drought tolerance and leaf stomatal density. In a common garden experiment using open-pollinated seed from each of Chalmers' three sites, Jordan *et al.* (2000) found a genetic basis to the clinal variation in plant height, habit and age of vegetative phase change. Jordan *et al.* (2000) suggested that the dwarf trees at this site evolved from local tall trees. However, this hypothesis has not been tested against the alternative hypothesis that separate dwarf populations have a common origin. Furthermore, there is no information on whether the dwarf ecotype is maintained by selection in the face of gene flow from adjacent taller trees as suggested by Jordan *et al.* (2000), and as found in sunflowers (Sambatti & Rice, 2006), or whether differentiation has been favoured by the presence of barriers to gene flow.

The present study used both nuclear microsatellites and maternally inherited chloroplast DNA markers (McKinnon *et al.*, 2001) to investigate the genetic affinities of the three dwarf populations of *E. globulus* to one another and to their nearest populations of tall *E. globulus*. The Cape Tourville location was studied in greater detail to assess the processes

enabling the maintenance of distinct *E. globulus* ecotypes in close geographical proximity. We measured flowering time in dwarf and tall Cape Tourville trees and allele frequencies at three microsatellite loci in open-pollinated progeny of dwarf trees to investigate the extent of pollen-mediated gene flow into the dwarf ecotype.

Materials and Methods

Tissue sampling

For the main study, leaf tissue for DNA extraction was collected from 210 mature *E. globulus* (sensu Brooker, 2000; formerly known as *E. globulus* ssp. *globulus*, Kirkpatrick, 1975) trees in natural populations at three locations in eastern Australia (Fig. 1). At Cape Tourville, in eastern Tasmania, samples were taken from 45 dwarf trees (average height ± SD = 3.8 ± 0.8 m) near the lighthouse, 45 tall trees (T1, 16.7 ± 4.9 m) found close to the dwarf ecotype and another 20 tall (T2, 17.2 ± 5.4 m) trees from a more or less parallel transect a further 600 m inland (Fig. 2). The dwarf ecotype is continuous with the tall one only at its northern and southern extremities and is otherwise separated from T1 by up to 300 m of coastal shrubbery. The two tall tree populations (T1 and T2) are in continuous forest. On South Maria Island, in eastern Tasmania, leaf samples were collected from 20 trees of the dwarf (4.9 ± 1.5 m) ecotype on the headland to the east of Haunted Bay and 16 tall (17.9 ± 5.7 m) trees across North and South Maria Island. On Wilson's Promontory, in southern Victoria, leaf samples were taken from 32 dwarf trees (3.9 ± 1.1 m) growing near the lighthouse and 32 tall (26.6 ± 6.5 m) trees growing at Tidal River. Although there are a few scattered tall trees within 5–10 km of the lighthouse, Tidal River, 13 km to the north, is the nearest population of tall trees. To avoid sampling related trees (Skabo et al., 1998, Jones et al., 2007) all tall trees were sampled at least 50 m apart and dwarf trees were usually 10 m apart (at least two tree heights apart), although at Cape Tourville the dwarf trees sampled were usually 25 m apart in order to sample from throughout the distribution of the ecotype.

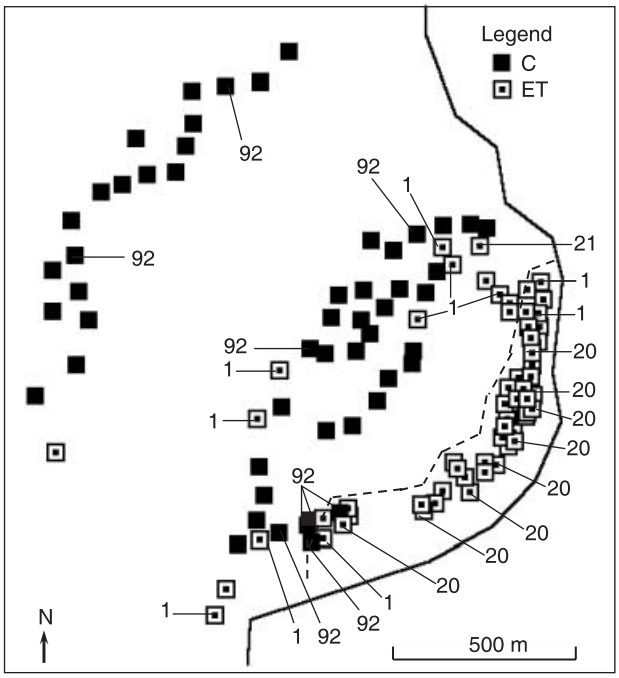

Fig. 2 Map of Cape Tourville (south-eastern Australia) showing location of sampled *Eucalyptus globulus* trees and distribution of their chloroplast DNA haplotypes. The coastline is shown as a thick black line and each symbol represents a sampled tree. The cliff-top dwarf population on Cape Tourville is delimited by a broken line and further inland are the T1 and T2 tall populations. Except at its northern and southern extremities the dwarf population is not continuous with the tall (T1) population as there is a gap with no *E. globulus* trees (area with no sample between the dwarf and T1). There is a continuous forest containing *E. globulus* between T1 and T2 and beyond T2. Individuals with haplotypes ET1, ET20, ET21 or C92 are indicated.

Molecular methods

Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method of Doyle & Doyle (1990) with initial incubation at 55°C. DNA was cleaned using Prep-a-gene DNA purification kit (Bio-Rad, Hercules, CA, USA) as required. Polymerase chain reaction (PCR) amplification was carried out using eight of the EMCRC microsatellite primer pairs designed for *E. globulus* by Steane *et al.* (2001) (EMCRC1a, 2, 3, 5, 6, 7, 10 and 11) and four of the EMBRA microsatellite primer pairs of Brondani *et al.* (1998) designed for *E. grandis* (EMBRA10, 11, 12 and 17). Amplification of PCR products followed Jones *et al.* (2002) using the annealing temperatures given by Steane *et al.* (2001) and Brondani *et al.* (1998). All PCR reactions were carried out using a PTC-100 thermocycler (MJ Research Inc., Watertown, MA, USA). Allele sizes were estimated by either of the following methods: (1) PCR products were separated using a Gel-Scan (2000) real-time acrylamide gel system (Corbett Research, Sydney, Australia); fragments were sized by comparison with either a TAMRA-labelled 60–500 bp ladder (Invitrogen, Mt Waverley, Victoria, Australia) or a CXR-labelled 60–400 bp ladder (Promega, Madison, WI, USA); allele sizes were estimated using the Gene Profiler (Scanalytics Inc., Fairfax, VA, USA) computer software package and visual inspection as in Jones *et al.* (2002); (2) PCR products were separated using a Beckman Coulter CEQ 8000 Genetic Analyses System; CEQ DNA standard 400 size markers were included in each lane, following Jones *et al.* (2007), and alleles were identified using the Beckman Coulter CEQ 8000 fragment analysis software. Duplicate samples were run within and between gels and across machines (i.e. Gel-Scan 2000 and Beckman Coulter CEQ 8000) to assess

the reliability of allele size estimates. Where stutter bands were observed, the band with the highest peak was considered the true allele. Homozygosity was assumed when only one allele was observed. Alleles were binned in 2-base increments (e.g. fragments in the range 201.1–203.0 were binned as 202; data not shown). The full set of 12 microsatellites was assayed on 20 trees from each population at Cape Tourville and the dwarf at Maria Island, 16 tall trees at Maria Island and 32 trees at each of the Wilson's Promontory populations.

To determine the chloroplast DNA (cpDNA) haplotype clade of the 210 trees, the hypervariable J_{LA} region, located at the junction of the large single copy and inverted repeat A, was amplified according to Vaillancourt & Jackson (2000). The PCR products were sized using agarose gel electrophoresis, following McKinnon *et al.* (2001), using control samples of the C, S and ET clades as size standards. The PCR products differed in size according to their haplotype clade (McKinnon *et al.*, 2001). To verify the membership of size variants to their haplotype clade, the longer and more informative J_{LA+} region was amplified and sequenced in 39 trees (29 from Cape Tourville, four dwarf and two tall from Wilson's Promontory, and four from Maria Island) following Freeman *et al.* (2001). J_{LA+} contains J_{LA} and both consist mainly of intergenic spacer (Steane, 2005). The DNA sequences were aligned with those in the chloroplast DNA database of Freeman *et al.* (2001), identical haplotypes receiving the same name while new ones were numbered in sequence. All DNA sequences were deposited in GenBank.

Flowering survey at Cape Tourville

The sampled trees at Cape Tourville that had flower buds were monitored at 4-weekly intervals from the start of the flowering season. As a number of the trees in the T1 and T2 populations had no buds, extra trees (3 and 5, respectively) within these were included in the flowering survey. Hence, flowering was monitored in 14, 16 and 18 trees in the dwarf, T1 and T2 populations, respectively. The numbers of buds that were unopened, flowering and had finished flowering were recorded for each tree on each visit. The number of buds per tree averaged 196 and ranged from 11 to 644 across all trees. Flowering in over half the trees of each population spanned at least 50 d and one tree spanned more than 100 d. The mean flowering time of each tree was calculated based on the percentage of buds in flower at each assessment (day 1 was 1 January, 2001). These data were then used to test the significance of the difference between populations in their mean number of days to flowering using PROC GLM of SAS (Version 9.1; AS, Cary, NC, USA). The general flowering pattern of each population was graphed by averaging the percentage of open flowers per tree over all trees in each population for each assessment date. An isolation index (I) between populations (X and Y) was calculated using these data following Antonovics (2006), where:

$$I = 1 - \left(\frac{B}{1 - B} \right)$$

and the frequency of between population mating was calculated as

$$B = \sum_{i=1}^{n} \frac{X_i Y_i}{X_i + Y_i}$$

where X_i and Y_i were the mean percentage of open flowers per tree in each population at date i, standardized by the population total summed across n assessment dates. This isolation index ranges from 0 to 1, where 0 signifies panmixis and 1 total isolation.

Study of progeny of dwarf trees at Cape Tourville

At the end of the flowering season 16–20 capsules were collected from across the top of the canopy of the 14 dwarf trees at Cape Tourville that had been monitored for flowering. The seeds collected from these capsules would be from previous flowering seasons. Seeds from each tree were grown in a glasshouse. DNA was extracted from leaf tissue of 12 progeny of each tree using approximately 1 cm^2 of leaf tissue placed in a tube with a tungsten carbide bead and 250 µl of homogenization buffer (0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 M ethylenediaminetetraacetic acid (EDTA), 2 mM polyvinylpyrrolidone (PVP)-40, 14 mM dithiothreitol (DTT)). The tissue was then homogenised by shaking at a rate of 30 oscillations s^{-1} for 2 min using a 300 MM mixer mill (Retsch, Haan, Germany). Immediately following homogenization 250 µl of 2× CTAB buffer was added (4% CTAB, 0.1 M Tris-HCl, 1.4 M NaCl). The extraction protocol then followed Doyle & Doyle (1990) but with 55°C incubations. The 168 progeny were genotyped using the microsatellites EMCRC5, EMCRC10 and EMBRA12 with PCR conditions as explained above.

Data analysis

Genetic diversity analysis was carried out using the software package GDA (Lewis & Zaykin, 2001). Analyses used 160 trees genotyped with 12 microsatellite loci, with each Cape Tourville population and the dwarf population at Maria Island represented by 20 trees, the population of tall trees at Maria Island represented by 16 trees, and each population at Wilson's Promontory represented by 32 trees. Genotype data were used to calculate the following genetic diversity measures, averaged for each population and each locus: number of alleles per locus (A), observed heterozygosity (H_o), Hardy–Weinberg expected heterozygosity (H_e), and the F-statistics F_{IS} and F_{ST}. Relationships between sites were quantified using pairwise F_{ST} and Nei, 1972) genetic distance (D). For all F-statistics, 99% confidence intervals were determined using

2000 bootstraps across loci. The degree of genetic differentiation between the populations was summarized using UPGMA clustering of Nei's (1972) genetic distance. Allelic richness was calculated for each population using FSTAT version 2.9.3.2 (Goudet, 1995). Tests of significance in levels of allelic richness and expected heterozygosity between groups of samples were performed using FSTAT. Two different tests were carried out, one in which all the dwarf populations were compared with the tall populations and one in which the Victorian populations were compared with the Tasmanian populations.

Differences in the frequency of the most common allele at each locus were tested across the three populations of Cape Tourville using a χ^2 contingency test with a Bonferroni adjustment (α/number of loci) applied to allow for multiple loci being tested (Snedecor & Cochran, 1980). Based on the outcome of this analysis, a further 50 trees (25 dwarf and 25 T1) from Cape Tourville were sampled and genotyped at three loci (EMCRC5, EMCRC10 and EMBRA12) together with the 168 open-pollinated progenies. We used χ^2 contingency tests to compare allele frequencies between the three populations of mature trees (dwarf, T1 and T2) with those of the dwarf progeny at Cape Tourville. The minimum outcrossing rate of the dwarf population was calculated using these three loci through identification of progenies possessing nonmaternal alleles.

Table 1 Overall genetic diversity parameters for each of the 12 microsatellite loci used in the *Eucalyptus globulus* study

Locus	*n*	Allele size range	A	H_o	H_e	F_{IS}	F_{ST}
EMCRC 1a	154	150–208	16	0.55	0.85	0.29	0.11
EMCRC 2	149	155–185	15	0.77	0.82	0.02	0.06
EMCRC 3	156	119–145	9	0.56	0.72	0.05	0.20
EMCRC 5	151	180–254	21	0.63	0.85	0.14	0.16
EMCRC 6	154	150–202	19	0.68	0.79	0.07	0.09
EMCRC 7	155	248–312	17	0.68	0.87	0.09	0.15
EMCRC 10	152	311–343	14	0.70	0.90	0.15	0.09
EMCRC 11	149	223–253	14	0.71	0.86	0.05	0.14
EMBRA 10	155	116–154	18	0.69	0.91	0.12	0.15
EMBRA 11	154	100–162	19	0.80	0.81	–0.06	0.08
EMBRA 12	157	107–151	17	0.76	0.87	0.06	0.09
EMBRA 17	156	120–164	19	0.83	0.87	–0.02	0.07
Mean	153		16.5	0.70	0.84	0.08	0.12

n, Number of trees sampled (varies between loci because clear amplification products were not obtained for all loci); A, observed number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity. F_{IS} and F_{ST} are fixation indices determined using the seven populations in Table 2, where F_{IS} = degree of inbreeding in each individual relative to its local population and F_{ST} = inbreeding in local populations relative to the total sample. The allele size range in base pairs is also given.

Results

Microsatellite affinities

The 12 nuclear microsatellite loci were polymorphic, with 9–21 alleles observed per locus (mean 16.5; Table 1). The number of alleles per locus was smallest in the dwarf population from Wilson's Promontory, despite this population having one of the largest sample sizes (Table 2). There was no significant difference in allelic richness between dwarf and tall populations overall; however, the difference between Tasmanian and Victorian populations was significant ($P = 0.02$). At all loci (Table 1) and in all populations (except the tall trees at Wilson's Promontory; Table 2) observed heterozygosity was lower than expected heterozygosity, with differences of up to 0.30 (EMCRC1a; Table 1). The overall lower levels of observed compared with expected heterozygosity within populations are reflected in the mean positive F_{IS} values (Table 1). These values were positive for most loci (mean = 0.08) indicating that there is a higher level of homozygosity than would be expected under random mating. All populations had positive *F*-values (Table 2) except for the tall population from Wilson's Promontory, which indicates that there is either inbreeding or spatial substructuring within most populations. There was no obvious difference in expected heterozygosity between populations in Tasmania (Table 2). The dwarf population at Wilson's Promontory had the lowest expected heterozygosity. However, there was no significant difference in expected heterozygosity between dwarf and tall populations, or between Tasmanian and Victorian populations.

The most closely related populations were the two populations of tall trees at Cape Tourville, T1 and T2, which had nonsignificant differentiation ($F_{ST} = 0.02$; Table 3). The dwarf Cape Tourville population was genetically closest to the adjacent T1 population ($F_{ST} = 0.03$) while the Maria Island dwarf population was more genetically similar to its adjacent tall population ($F_{ST} = 0.03$) than to any other populations (Table 3). The two dwarf populations in Tasmania were the most genetically differentiated populations within Tasmania ($F_{ST} = 0.10$). The population of tall trees at Wilson's Promontory was more similar to the Tasmanian populations than to the nearby population of dwarf trees ($F_{ST} = 0.18$). The dwarf population at Wilson's Promontory was highly differentiated from all others. These relationships are summarized in a UPGMA dendrogram (Fig. 3).

Chloroplast DNA differentiation

Analysis of the J_{LA} region of chloroplast DNA found that all trees belonged to clades or groups of haplotypes previously identified in a range wide study of *E. globulus* (Freeman *et al.*, 2001). There was pronounced differentiation between tall and dwarf populations at Cape Tourville. The dwarf Cape Tourville population was almost fixed (96%) for J_{LA} chloroplast haplotypes within the size range corresponding to

Table 2 Genetic diversity parameters for each population of *Eucalyptus globulus* used in this study

Location	Population	*n*	A	Ar	H_o	H_e	*F*
Cape Tourville	Dwarf	19.8	8.8	7.9	0.73	0.81	0.09
	Tall T1	19.4	9.7	8.7	0.71	0.83	0.14
	Tall T2	19.8	9.6	8.6	0.74	0.82	0.10
Maria Island	Dwarf	19.9	8.3	7.5	0.69	0.79	0.12
	Tall	15.6	8.6	8.3	0.67	0.80	0.17
Wilson's Promontory	Dwarf	29.5	5.9	5.0	0.58	0.62	0.07
	Tall	29.5	8.4	6.6	0.76	0.73	–0.05
Mean		21.9	8.5	7.5	0.70	0.77	0.09

n, Mean number of trees sampled per locus in each population; A, mean number of observed alleles per locus; Ar, average allelic richness per locus standardized to a population size of 14; H_o, observed heterozygosity; H_e, expected heterozygosity; *F*, Wright's Fixation Index.

Table 3 Pairwise F_{ST} values (above diagonal) and Nei's (1972) genetic distance (below diagonal) for *Eucalyptus globulus* populations in this study

Population	Cape Tourville dwarf	Cape Tourville tall T1	Cape Tourville tall T2	Maria Island dwarf	Maria Island tall	Wilson's Promontory dwarf	Wilson's Promontory tall
Cape Tourville dwarf		0.03*	0.05*	0.10*	0.08*	0.20*	0.12*
Cape Tourville tall T1	0.28		0.02	0.06*	0.05*	0.21*	0.11*
Cape Tourville tall T2	0.36	0.23		0.05*	0.03*	0.17*	0.09*
Maria Island dwarf	0.71	0.44	0.36		0.03*	0.21*	0.12*
Maria Island tall	0.60	0.42	0.29	0.25		0.17*	0.08*
Wilson's Promontory dwarf	0.97	1.18	0.69	0.96	0.68		0.18*
Wilson's Promontory tall	0.66	0.61	0.45	0.60	0.42	0.60	

*, F_{ST} was significantly different from 0 at $P < 0.01$.

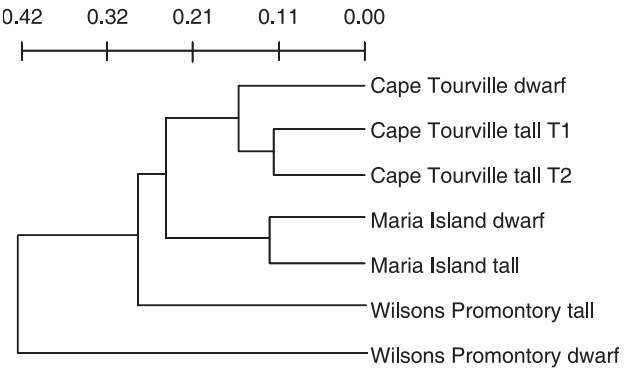

Fig. 3 An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram based on Nei's (1972) genetic distance (*D*) between the *Eucalyptus globulus* populations used in this study. All pairwise comparisons of populations showed that they were significantly different from one another (F_{ST}, Table 3), except for the two tall populations (T1 and T2) at Cape Tourville.

the Eastern Tasmanian group (ET; Fig. 2; Table 4). By contrast, most trees in the T1 population (71%) had haplotypes within the size range of the central (C) clade, while the remaining 29% had haplotypes within the size range of the ET clade. The T2 population, approximately 1 km from the dwarf population, was virtually fixed (95%) for C haplotypes (Fig. 2). Sequencing the more informative J_{LA+} region of chloroplast DNA confirmed the assignment of samples to clades and distinguished three different ET haplotypes but only one C haplotype (C92) at Cape Tourville (Table 4). The most common ET haplotype in the tall T1 population was ET1, which was found previously in *E. globulus* by Freeman *et al.* (2001) in four nearby locations on the east coast of Tasmania. The most common haplotype in the dwarf population was ET20, which was restricted to this population (i.e. not found in other populations here or by Freeman *et al.*, 2001) and differed from ET1 by one extra nucleotide in a microsatellite region. The third ET haplotype, ET21, was a single occurrence within the T1 population and differed from ET1 by a 20 bp insertion. The dwarf trees shared haplotype ET1 with the tall trees at the northern and southern extremes of the dwarf population and C92 at the southern extreme of the dwarf population (Fig. 2), which is consistent with localized seed dispersal.

On Wilson's Promontory, all samples possessed a J_{LA} fragment of the same size (Table 4). Upon sequencing the J_{LA+} fragment it was found that the four dwarf tree samples had

Table 4 Frequency of each chloroplast size variant (corresponding to group or clade C, S and ET) in each population of *Eucalyptus globulus* as determined by sizing the J_{LA} region of cpDNA

		Frequency of each cpDNA size variant			Frequency of each cpDNA haplotype sequenced							
Location	Population	C	S	ET	ET1	ET20	ET21	C92	S43	Cc34	Cg13	Cg33
Cape Tourville	Dwarf	2		43	3	8		2				
	Tall T1	32		13	8		1	5				
	Tall T2	19		1				2				
Maria Island	Dwarf		20						2			
	Tall	2	14						1	1		
Wilson's Promontory	Dwarf	32									4	
	Tall	32										2
GENBANK No.					AY829640	AY829641	AY829642	EF428977	AY620869	EF434409	EF434408	EF428976

To verify the membership of size variants to their haplotype clade, the longer and more informative J_{LA+} region was sequenced in some of the trees.

haplotype Cg13 while the two tall trees sequenced had haplotype Cg33. Those two haplotypes differed by a small indel but belonged to the same group of haplotypes (Cg), which is nearly endemic to south-eastern Victoria (Freeman *et al.*, 2001; Freeman *et al.*, in press). On Maria Island, no chloroplast differentiation was detected between the dwarf and tall populations (Table 4); two tall trees had C haplotypes and the remainder of the trees on the island had haplotypes from a third clade (southern; S), which is common only in southern Tasmania (Freeman *et al.*, 2001).

Pollen mediated gene flow at Cape Tourville

There were significant differences between Cape Tourville populations in the frequency of the most common allele at two (EMCRC5 and EMCRC10) out of the 12 loci. In addition, the dwarf population at Cape Tourville possessed a relatively common allele for EMBRA12 (allele 109), which was not found in any other population in the first analysis before sample size was increased. This was the only private allele with a frequency greater than 0.2. Allele frequencies at these three microsatellite loci were determined for 168 progeny from 14 dwarf trees in order to study gene flow by pollen dispersal from the tall to the dwarf population. The minimum outcrossing rate over all trees was 93%. Null alleles were rare and were identified only in the progeny of two dwarf trees at the EMCRC5 locus and a third dwarf tree at the EMCRC10 locus. In order to increase our precision in estimating allele frequencies at these three loci in parental populations, we genotyped all available Cape Tourville trees, giving total sample sizes of 45, 45 and 20 for dwarf, T1 and T2 populations, respectively (Fig. 4). With the larger sample sizes, the frequencies of the most common alleles of EMCRC5 and 10 changed little, but allele 109 of EMBRA12 was no longer confined to the dwarf population, with two of the 45

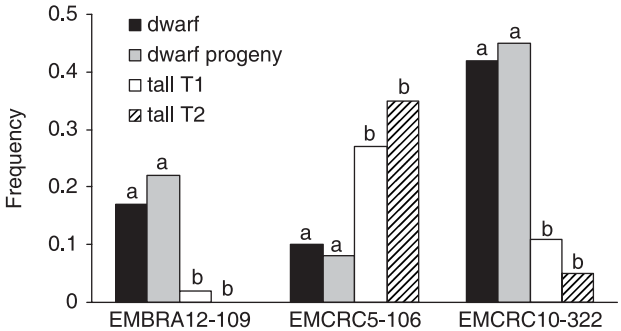

Fig. 4 Frequency of allele 109 for locus EMBRA12 and the most common allele for loci EMCRC5 and EMCRC10 in *Eucalyptus globulus* at Cape Tourville (south-eastern Australia) shown for the dwarf (number of individuals $n = 45$), dwarf progenies ($n = 168$), tall T1 ($n = 45$) and tall T2 ($n = 20$) populations. Populations at each locus with different letters (a, b) are significantly different ($P < 0.05$). Allele EMBRA12-109 was not found in the T2 trees.

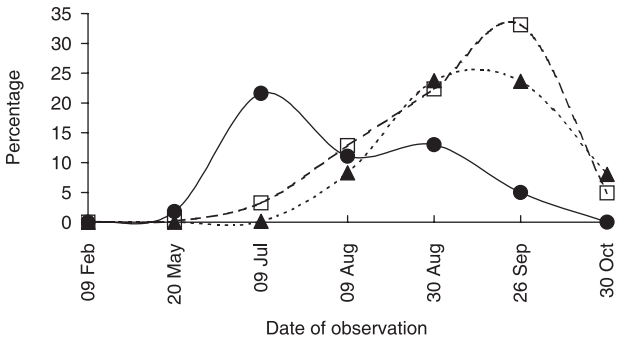

Fig. 5 Flowering time of the three populations (dwarf, circles; tall T1, squares; tall T2, triangles) of *Eucalyptus globulus* at Cape Tourville (south-eastern Australia) during 2001. Mean percentage of open flowers per tree is shown for each population. This was determined by calculating the percentage of buds in flower on each tree at each visit and averaging the result across each site.

T1 trees heterozygous for this allele. The two tall populations were not significantly different from one another at these three loci. No significant differences between the dwarf trees and their progeny were found in allele frequencies (Fig. 4). However, the dwarf trees and their progenies were significantly differentiated from both the tall populations. There was no tendency for the allele frequencies in the open-pollinated progenies to deviate towards those of the tall populations (as would be expected with some pollen-mediated gene flow between them); rather, the deviations were in the opposite direction for all three loci (Fig. 4).

There was a statistically significant difference ($F_{2,45} = 12.7$; $P < 0.001$) between the three Cape Tourville tree populations in the mean number of days to flower (Fig. 5). There was no significant difference between the two tall populations ($P > 0.05$) and the significant variation between populations was caused by the early flowering of the dwarf trees (the average number of days ± SE taken for buds to flower from the 1 January: dwarf 217 ± 7.0, T1 253 ± 6.5, T2 263 ± 6.2). The isolation index between the dwarf population and the adjacent tall population T1 was 0.44, that between the dwarf and T2 was 0.56, and both were much higher than the isolation between the two tall populations (I = 0.11). Peak flowering was reached in half of the dwarf trees before flowering started in any trees of the tall populations (data not shown). Peak flowering in the other dwarf trees was less focused (more stretched), although a secondary flowering peak occurred approx. 2 wk earlier than peak flowering in the tall trees (Fig. 5).

Discussion

Origin of the dwarf cliff-top populations in *E. globulus*

Polyploid plant species often appear to have multiple origins (Soltis & Soltis, 1999), and the same is likely to be true for polyploid ecotypes or races. Nevertheless, little attention has been given to the possibility of multiple origins of species or ecotypes in the absence of hybridization or gross chromosomal shifts, as would appear to be the case with the *E. globulus* cliff-top populations. Maritime ecotypes (including dwarf ecotypes) showing adaptations to extremes of salt, wind, light and/or aridity are recognized within numerous plant species (Pegtel, 1972; Ahmad & Wainwright, 1976; KurTa & ChangHung, 1997; Daehler *et al.*, 1999). Only a few studies have explored the possibility that these or other plant ecotypes may have arisen repeatedly as a result of similar selection pressures on progenitor populations in different locations (Levin, 2003). Based on morphological analysis, Brochmann *et al.* (1995) suggested that a coastal, mesophytic ecotype of *Frankenia ericifolia* had evolved from a more xerophytic coastal ecotype independently in three different Cape Verde Islands. A morphological and RAPD analysis of the *Zieria smithii* complex (Hogbin & Crisp, 2003) found close genetic relationships between inland populations of *Z. smithii* and nearby headland populations of an associated ecotype, *Zieria* sp. aff. *smithii*. The authors concluded that this ecotype might have arisen independently on at least 10 different headlands.

The rare dwarf ecotype of *E. globulus* appears to have evolved independently in three disjunct locations in south-eastern Australia. Although isolated from one another by sea and distance, the three locations at which the dwarf ecotypes grow are environmentally very similar: granite cliffs that are highly exposed to wind and salt spray. The three dwarf populations were among the least genetically similar in the study based on microsatellites and each was dominated by chloroplast haplotypes from different groups or clades. Within Tasmania, tall and dwarf populations from Maria Island were most closely related to one another based on microsatellite analysis, and were significantly differentiated from Cape Tourville tall and dwarf populations at three loci: EMCRC1a, EMCRC5 and EMBRA10 (data not shown). Moreover, tall and dwarf trees on Maria Island shared a chloroplast haplotype (S43) not found at Cape Tourville. The dwarf population from Cape Tourville was most closely related to its nearest tall population based on microsatellite data and shared chloroplast haplotypes (ET1, C92), although the most common haplotype in the dwarf population (ET20) was unique to this population. The dwarf ecotype at Wilson's Promontory also had a chloroplast type matching that of the closest tall population of *E. globulus* (haplotype group Cg), although the particular haplotypes were different. The eastern Victorian populations of *E. globulus* are dominated by haplotypes from the Cg group, which is not found in Tasmania and is rare in other locations (Freeman *et al.*, 2001; Freeman *et al.*, in press). The dwarf ecotype at Wilson's Promontory was highly differentiated from all other populations in the microsatellite analysis, consistent with results from a RAPD marker study of populations throughout the range of the species (Nesbitt *et al.*,

1995). This divergence most likely results from inbreeding and drift as a consequence of its small population size and geographic isolation. Its low level of genetic diversity supports this hypothesis. Common environment progeny trials have indicated that the dwarf ecotype of *E. globulus* from Wilson's Promontory is the most extreme of the dwarf populations in its height, growth rate and precocious onset of vegetative and reproductive phase change (Jordan *et al.*, 2000). Its extreme form could be attributable to stronger selection on the exposed headland at Wilson's Promontory combined with lower levels of gene flow from surrounding tall trees than at the other locations.

The differentiation of chloroplast haplotypes between tall and dwarf trees at Cape Tourville gave additional information regarding the origin of the dwarf ecotype at this location. The bulk of the dwarf population was fixed for a chloroplast haplotype that differed by only one base pair from a haplotype (ET1) found at low frequency in the tall ecotype nearby and in four other tall populations of *E. globulus* in north-eastern Tasmania (dataset of Freeman *et al.*, 2001). We think that the most likely hypothesis to explain the near fixation for this rare haplotype is an ancient founder event by seed, followed by enough pollen dispersal from the nearby tall population to replenish nuclear genetic diversity in the dwarf population, but with little realized seed dispersal between populations since the original founding event. Microsatellite evidence argues against a recent founder event, since the level of microsatellite genetic diversity in the dwarf Cape Tourville ecotype is comparable with that of the tall populations. However, genetic diversity in nuclear DNA is less affected by bottlenecks than chloroplast DNA diversity (Ennos *et al.*, 1999) and past bottlenecks can be difficult to detect using nuclear markers.

Barriers to gene flow between dwarf and tall ecotypes

The present study detected barriers to gene flow between different ecotypes of *E. globulus* at Cape Tourville on a fine geographic scale (*c.* 300 m). CpDNA data suggest that realized seed-mediated gene flow into and out of the dwarf ecotype is extremely limited. This could be attributable to selection against seed immigrants (Clausen *et al.*, 1948; Clausen & Hiesey, 1958; Endler, 1986) and/or limited seed dispersal. Seeds of eucalypt species such as *E. globulus* have no specialized adaptations for dispersal and most seed falls within two canopy heights of a source (Potts & Wiltshire, 1997). Pollen-mediated gene flow into the dwarf ecotype also appears to be limited, contrary to what may be expected for such a small, marginal population in close proximity to a large population of a different ecotype (Kirkpatrick & Barton, 1997; Barbour *et al.*, 2005). Microsatellite analysis at three loci possessing alleles of distinct frequency between populations failed to detect pollen-mediated gene flow into the dwarf population from nearby tall trees. The high outcrossing rate in the dwarf open-pollinated progenies indicates that most progenies were informative concerning their paternal origin. While our total sample size would not be expected to detect rare pollination events, significant pollen swamping of the localized dwarf ecotype by the more extensive tall ecotype is clearly not occurring. In fact, the deviation in frequency of all three microsatellite alleles was never in the direction of the tall forest population.

There are two obvious barriers to gene flow between dwarf and tall trees at the Cape Tourville site. The bulk of the dwarf population is separated from the nearest tall *E. globulus* by a gap of approx. 200–300 m of coastal shrubbery (Fig. 2). This partial spatial discontinuity may deter the movement of the birds and insects that pollinate *E. globulus* (Hingston *et al.* 2004) and may also act as a significant barrier to seed dispersal. Such partial spatial discontinuities are common in eucalypt species because of their specific habitat requirements and may be enough to reduce gene flow and enhance local adaptation. In the present case, this physical barrier cannot be separated from a temporal barrier to pollen-mediated gene flow arising from a significant difference in flowering time between the dwarf and tall ecotypes. Such differences in flowering time between proximal conspecific populations of eucalypts have been reported previously (Potts & Reid, 1985a,b; Savva *et al.*, 1988). In the present case, it is unclear whether differences in flowering time between the tall and dwarf ecotypes are plastic or genetic. However, such differences have previously been found to have a strong genetic component in common environment field trials (Gore & Potts, 1995). If genetic, such differences could simply be another component of adaptation to this cliff-top environment or could have evolved as a means of reducing gene flow. Differences in flowering time have evolved between populations of the grasses *Agrostis tenuis* and *Anthoxanthum odoratum* that are adapted to toxic soils from abandoned mines and nearby populations on uncontaminated soils (McNeilly & Antonovics, 1968; McNeilly & Bradshaw, 1968; McNeilly, 1968); these examples are often cited in support of parapatric speciation. The isolation index between the dwarf and the adjacent tall population of *E. globulus* was 0.44 and was comparable to that found by Antonovics (2006) between populations of *A. odoratum* at a mine boundary ($I = 0.43$). Regardless of the cause, this study shows that effective barriers to gene flow can exist between adjacent eucalypt populations over very short distances, enhancing opportunities for adaptation and parapatric speciation.

The only other molecular study addressing the issue of parallel evolution in *Eucalyptus* is that of McGowen *et al.* (2001). That study examined the possibility that an alpine shrub eucalypt, *Eucalyptus vernicosa*, had evolved from clinally linked lower altitude populations of *Eucalyptus subcrenulata* on three different mountains. In that study, microsatellite analysis supported a single origin for *E. vernicosa* rather than parallel evolution from *E. subcrenulata*. By contrast, the present study confirms the existence of parallel ecotypes within *E. globulus* and raises the prospect that not only

ecotypes but species (Johannesson, 2001), could have evolved in parallel in *Eucalyptus* through differentiation along environmental gradients.

The existence of barriers to gene flow between dwarf and nearest tall populations of *E. globulus* at Cape Tourville and Wilson's Promontory indicate that we may be witnessing the parallel evolution of new species of dwarf eucalypt. The process of ecological speciation involves establishment of populations in marginal habitats, followed by progressive genetic refinement of those populations through natural selection (Levin, 2003). The accumulation of adaptive divergences between neospecies and their progenitor populations is opposed by immigration, and is unlikely where a small outlier population is exposed to constant pollen and seed influx from a larger progenitor population (Levin, 2001). Although speciation and adaptation can occur in the presence of some gene flow (Rieseberg *et al.*, 2004), it is clear that even partial reproductive isolation can help the maintenance of genetic differences as long as there is strong divergent selection. This study shows that a small marginal population, such as the dwarf ecotype at Cape Tourville, is capable of developing reproductive isolation from a nearby larger population through differences in flowering time and/or minor spatial separation. Even if the cause of this reproductive isolation is entirely environmental, if stable through time, it maybe enough, together with selection and adaptation, to maintain genetic differences between populations, thus making parapatric speciation possible.

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