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The rare silver gum, *Eucalyptus cordata*, is leaving its trace in the organellar gene pool of *Eucalyptus globulus*

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Abstract

The process of genetic assimilation of rare species by hybridizing congeners has been documented in a number of plant genera. This raises the possibility that some of the genetic diversity found in phylogeographical studies of widespread species has been acquired through hybridization with species that are now rare or extinct. In this fine-scale phylogeographical analysis, we show that a rare eucalypt species is leaving its trace in the chloroplast genome of a more abundant congener. The heart-leafed silver gum, *Eucalyptus cordata*, is a rare endemic of south-eastern Tasmania. Its populations are scattered amidst populations of more abundant related species, including the Tasmanian blue gum, *Eucalyptus globulus*. Using 339 samples from across the full range of both species, we compared chloroplast (cp) DNA haplotype phylogeography in *E. globulus* and *E. cordata*. The genealogy and distribution of chloroplast haplotypes suggest that *E. globulus* has acquired cpDNA from *E. cordata* in at least four different mixed populations. Shared haplotypes are highest in *E. globulus* sampled within 2 km of known *E. cordata* populations and drop to zero at a distance of 25 km from the nearest known *E. cordata* population. Localized haplotype sharing occurs in the absence of obvious hybrid zones or locally shared nuclear ribosomal DNA sequences. Given that the future loss of *E. cordata* from some mixed populations is likely, these findings indicate that phylogeographical analyses of organellar DNA should consider the possibility of introgression, even from species that have been eliminated from the sites of interest.

Keywords: chloroplast, eucalypt genetics, *Eucalyptus*, introgression, phylogeography, hybrid

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Introduction

Among plant species that hybridize readily, the gradual replacement of one species by another at mixed or parapatric populations may be favoured not only by competition, but also by repeated (often unilateral) hybridization. In extreme cases, this may lead to the genetic assimilation of one species by another. Examples can be found in *Helianthus* (Carney *et al*. 2000), *Cercocarpus* (Rieseberg & Gerber 1995), *Argyranthemum* (Levin *et al*. 1996) and *Spartina* (Anttila *et al*. 1998). These observations raise the possibility that some of the genetic diversity currently found in phylogeographical studies of widespread species may have been acquired through hybridization with species that have now been partially or completely eliminated from the sites sampled. This possibility applies in particular to cytoplasmic markers such as chloroplast (cp) DNA, which are generally exchanged between species far more readily than nuclear markers (Rieseberg & Soltis 1991).

Analyses of cpDNA in the Australian forest genus *Eucalyptus* (Steane *et al*. 1998; McKinnon *et al*. 1999; McKinnon *et al*. 2001a) have shown that, like species of *Quercus* (Whittemore & Schaal 1991; Ferris *et al*. 1993; Petit *et al*. 1997; Belahbib *et al*. 2001), *Pinus* (Matos & Schaal 2000) and *Alnus* (King & Ferris 2000), different eucalypt species tend to share matching cp haplotypes within geographical regions. On the island of Tasmania, a complex pattern of haplotype sharing has been demonstrated among 17 species of *Eucalyptus* subgenus *Symphyomyrtus* (McKinnon *et al*. 2001a). While this finding suggests a history of gene flow among species, the actual contribution of any individual species to the chloroplast genome of any other awaits clarification. The situation is complicated by the need to distinguish between alternative causes of haplotype sharing, such as: (1) convergent mutation; (2) shared retention of ancestral polymorphisms

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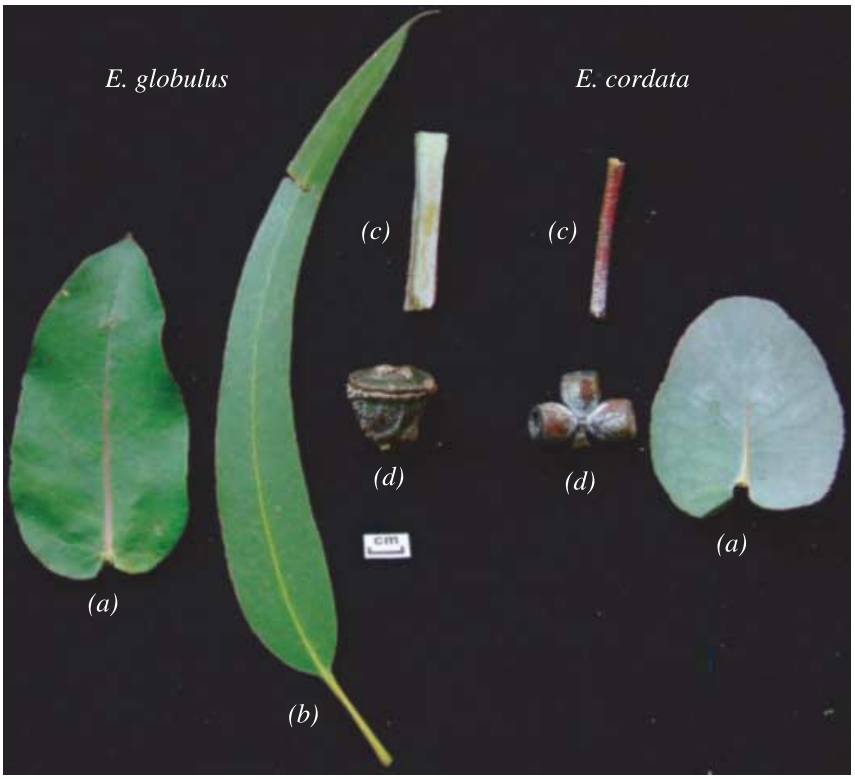

Fig. 1 Foliage, fruit and stems of *Eucalyptus globulus* and *Eucalyptus cordata*. (a) Juvenile foliage (retained by mature *E. cordata*); (b) adult foliage; (c) stem; (d) fruit.

due to incomplete lineage sorting; and (3) transfer of DNA between species (introgression). The last two processes are notoriously difficult to disentangle, but the problem may be approached by a careful consideration of both haplotype genealogy and geographical distribution (Matos & Schaal 2000; Schaal & Leverich 2001). In this fine-scale analysis, we use a phylogeographical approach to show that patterns of cpDNA diversity in a common eucalypt species are consistent with repeated introgression involving a rare species. Our findings underscore the fact that rare or vanishing species may potentially constitute an important contributing factor to genetic diversity in common extant species.

The heart-leafed silver gum, *Eucalyptus cordata* (subgenus *Symphyomyrtus*, section *Maidenaria*, subsection *Euryota*, series *Orbiculares*; *sensu* Brooker 2000) is a rare endemic eucalypt of south-eastern Tasmania, easily recognized by its persistent, glaucous juvenile foliage (Maiden 1919; Fig. 1). It grows as a stunted mallee shrub in dry near-coastal woodland between altitudes of 100–400 m, but reaches a tree habit on wetter inland sites from 400 to 680 m altitude (Williams & Potts 1996). Its distribution, which falls within an area of 6000 km^2, coincides closely with a modelled former glacial refuge (Kirkpatrick & Fowler 1998) and appears to be relictual (Potts 1988). About 37 scattered populations are known, most of which are sympatric with at least one other species from the same section (Potts 1989). Populations range in size from a single tree to over 5000 trees and are separated from their nearest neighbours by an average of 9.2 km (maximum 36.5 km; Potts 1989). This distance constitutes a significant barrier to both seed and pollen-mediated gene flow in most eucalypt species studied (Potts & Wiltshire 1997), and the majority of *E. cordata* populations are probably completely or almost completely genetically isolated from one another at present.

The Tasmanian blue gum, *Eucalyptus globulus* Labill. (subsection *Euryota*, series *Globulares*; *sensu* Brooker 2000; formerly designated *Eucalyptus globulus* ssp. *globulus*, Kirkpatrick 1974) is a common and economically important forest tree of eastern Tasmania, the Bass Strait islands and south-eastern continental Australia. It is the only eucalypt of series *Globulares* in Tasmania; 11 other species of *Globulares* (including *Eucalyptus maidenii*, *Eucalyptus bicostata*, and *Eucalyptus pseudoglobulus*; formerly designated as subspecies of *E. globulus*, Kirkpatrick 1974) are found in continental Australia. *Eucalyptus globulus* is predominantly a lowland coastal or near-coastal species occurring from sea level to 650 m, and colonizes a range of substrates from fertile wet gullies to infertile dry dune sands and sea cliffs (Williams & Potts 1996). Its range in Tasmania encompasses that of *E. cordata* and the two species are sympatric or parapatric at some locations. *Eucalyptus globulus* and *E. cordata* are readily distinguishable on a number of features including leaf and capsule morphology (Fig. 1). Natural hybrids between them are not common, but have been detected in mixed populations, and both an artificial F_2 and putative natural backcrosses have been reported (Potts 1989).

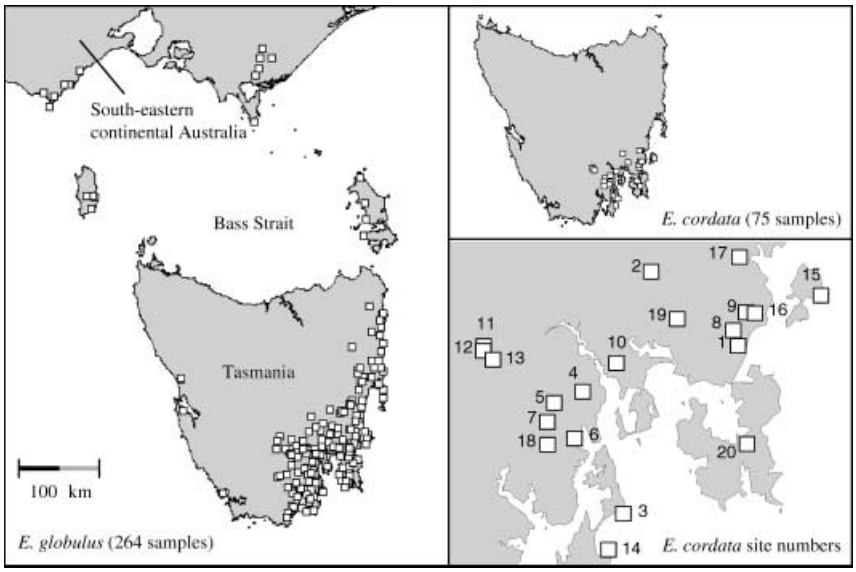

Fig. 2 Sites at which *Eucalyptus globulus* and *Eucalyptus cordata* were sampled for chloroplast (cp) DNA analysis.

Previous phylogeographical analyses (Jackson *et al*. 1999; Freeman *et al*. 2001) have shown that eastern Tasmanian populations of *E. globulus* have a diverse mixture of cpDNA lineages, including one clade (S) not found in mainland *E. globulus*, *E. maidenii*, *E. bicostata*, or *E. pseudoglobulus*. Haplotypes of this clade are found in several other species from section *Maidenaria* in south-eastern Tasmania, but are absent from the same species in central and northern Tasmania (McKinnon *et al*. 2001a). They are dominant only in *E. cordata* and a second rare, related endemic of south-eastern Tasmania, *Eucalyptus morrisbyi* (series *Orbiculares*). The observation that *E. globulus* and *E. cordata* are interfertile raises the possibility that some S clade haplotypes in south-eastern Tasmanian *E. globulus* may have been acquired through past introgressive hybridization with *E. cordata*. Most other species appear less probable as candidate S haplotype donors, either because they do not hybridize naturally with *E. globulus* (e.g. *E. morrisbyi*, *Eucalyptus gunnii*, *Eucalyptus rodwayi*, *Eucalyptus dalrympleana*; Williams & Potts 1996) or because they more commonly have other haplotypes in this region (e.g. *Eucalyptus ovata*; McKinnon *et al*. 2001a). This study aimed to investigate more fully the cpDNA phylogeography of *E. cordata* and to determine whether there was supporting evidence for significant cpDNA exchange between this species and *E. globulus*. A highly variable region of the cp genome, capable of fine discrimination (as used in Freeman *et al*. 2001), was sequenced for allopatric and sympatric populations of *E. cordata* and *E. globulus* across their full geographical ranges. A third, potentially hybridizing species *Eucalyptus viminalis* was present at many mixed *E. cordata*/*E. globulus* populations and was sampled for additional information. To test for introgression of nuclear genes, sequence data from the internal transcribed spacer (ITS) region of nuclear ribosomal (nr) DNA were obtained for selected samples of *E. cordata* and *E. globulus*.

Materials and methods

Sampling

Sampling of *E. cordata* (75 individuals from 20 populations; Fig. 2) covered the species' natural geographical range. Where possible, five widely spaced trees were sampled from each population; five populations were represented by single samples. At 11 mixed populations, three samples each of *E. globulus* and/or *E. viminalis* (including its sister taxa, *E. dalrympleana* and *E. rubida*, with which it intergrades) were also collected. Previously analysed samples of *E. globulus* (excluding former subspecies now raised to specific status) were incorporated from the dataset of Freeman *et al*. (2001) to give high-density coverage of the full range of the species (in total 264 samples from 157 different populations; Fig. 2). Additional samples of Tasmanian *E. viminalis*/*dalrympleana*/*rubida* were also incorporated to give a total of 58 samples from 38 populations. Individuals were selected on the basis that they were morphologically representative of their species. Trees were tagged, their geographical co-ordinates were recorded and voucher specimens were collected for storage at the School of Plant Science, University of Tasmania. Leaf tissue was collected and stored in airtight bags at 4 °C, or frozen under liquid nitrogen and stored at –70 °C, until used for DNA extraction.

DNA extraction, polymerase chain reaction (PCR) and sequencing

DNA purification followed the cetyltrimethylammonium bromide (CTAB) method of Doyle & Doyle (1990), with addition of polyvinylpyrollidone (2%) to the CTAB isolation buffer. The following minor modifications were made: (1) replacement of β-mercaptoethanol (2% v/v) with dithiothreitol

Table 1 Mutations defining the 19 haplotypes found in *Eucalyptus cordata*. The common (presumably ancestral) state in *E. cordata* is indicated. For character descriptions see Appendix S1

	Character													
	31	35.1	40	40.1	44	45	46	53	62	62.2	72.1	84	91.1–91.2	91.4–91.6
Base position	231	290	297–305	306	324	325–350	351	379	448–470	462	511	648–658	749–827 (minisatellite)	828–914 (minisatellite)
Common state	T	A	T_8	A	T	$(A_5)T(A_{13})$	0	C	0	?	G	A_{10}	1 copy	1 copy
Haplotype														
JS41													2	
JS43														
JS47						$(A_5)T(A_{14})$		A				A_{11}	0	
JS48	G						G	A				A_{11}	0	
JS53				T					1	T			2	
JS54									1	G			2	
JS58						$(A_5)T(A_{12})$								
JS71						$(A_5)T(A_{14})$					T			
JS73									1	T			2	
JS77						A_{18}								
JS80						$T(A_{13})$							2	
JS83						A_{19}								
JS88			T_9			A_{22}								
JS89				T									2	
JS95		T			0	A_{20}							2	
JS96						$(A_5)T(A_{10})$								
JS99														3
JS100												A_{11}		
JS101												A_{11}	2	

(7 mM) and (2) incubation of CTAB extracts at 55 °C instead of 60 °C. The J_{LA+} region of the chloroplast genome, comprising the *rpl2-trnH* intergenic spacer, the *trnH* gene, and the *trnH-psbA* spacer (approximately 630 bp in total) was amplified and sequenced following the methods of Freeman *et al*. (2001). Sequences were lodged with GenBank (accession numbers AY620868–AY620886 for *E. cordata* haplotypes; AY620887–AY620895 for *E. viminalis*/*dalrympleana* haplotypes; and AY620896–AY620902 for *E. globulus* haplotypes). The 5.8S nr DNA and flanking internal transcribed spacer (ITS)-1 and ITS-2 (637 bp in total) were amplified according to Steane *et al*. (1999) and the amplified products were sequenced without cloning using the primers ITS-4 and ITS-5 (White *et al*. 1990). Sequences were lodged with GenBank (accession numbers AY615654–AY615668 for *E. cordata* ITS haplotypes; AY615669–AY615680 for *E. globulus* ITS haplotypes). ITS sequences from additional species were taken from the dataset of Steane *et al*. (1999, 2002).

Phylogenetic analysis of chloroplast haplotypes

Sequences of the J_{LA+} region for *E. cordata* and *E. globulus* were aligned manually and scored for 28 base substitutions, 10 1-bp indels, 13 larger indels, five polyA/T tracts and three minisatellites (Appendix S1; available online). Indels were scored as presence/absence, variable-length polyA/T tracts were scored as single multistate characters and minisatellite repeats were scored as stepwise mutations (one mutation per repeat). Haplotypes were classed as identical only if they matched at every sequence character. Haplotypes were named according to Freeman *et al*. (2001), except that the prefix 'J' was omitted; new haplotypes were named by clade (Freeman *et al*. 2001) and numbered in order of discovery. All variable characters were used to construct the haplotype network for *E. cordata*. The character matrix (Table 1) was imported into the program PAUP version 3.1.1 (Swofford 1991), and a matrix of distances among haplotypes was computed. The distance matrix was imported into the program TCS 1.13 (Clement *et al*. 2000) and the intraspecific haplotype network was generated based on the principle of statistical parsimony using the 95% limit. The network was nested according to the rules of Templeton (Templeton *et al*. 1987; Templeton & Sing 1993). The haplotype network for *E. globulus* was constructed using the method described for *E. cordata*. However, owing to the greater variability and phylogenetic depth in *E. globulus*, it was necessary to collapse the large number of haplotypes by excluding four rapidly evolving polyA/T regions from the analysis (characters 14, 36–46, 53–54, and 84; Appendix S1). These regions

were either too variable to be phylogenetically informative in *E. globulus* (characters 14, 36–46 and 53–54) or were clearly homoplasious (character 84). The resulting *E. globulus* network is therefore a simplified representation of variability in that species. The character matrix for *E. globulus* is available online (Appendix S2).

Analysis of haplotype sharing between species

To distinguish clearly between introgression and lineage sorting, haplotype sharing between *E. cordata* and *E. globulus* was considered in terms of both the geographical distributions of shared haplotypes and their positions (i.e. tip or interior) in the haplotype networks for each species. Significant associations between *E. cordata* haplotypes and geography were tested using the program GEODIS 2.0 (Posada *et al*. 2000) with the 2003 inference key. To determine whether the presence of matching haplotypes in *E. globulus* was correlated with geographical proximity to *E. cordata*, the distance was calculated between every sampled *E. globulus* individual and every known population of *E. cordata* (including unsampled populations). *E. globulus* individuals were divided into roughly equal-sized groups (around 40 per group) according to their distance from the closest *E. cordata* population and contingency χ^2 testing with the Yates correction was used to determine whether shared haplotypes were distributed randomly among groups.

Results

Phylogeographical analysis of E. cordata

Sequence analysis of the J_{LA+} region in 75 individuals of *E. cordata* covering its full geographical range (Fig. 2) identified 14 variable sequence characters defining 19 haplotypes (Tables 1 and 2; Fig. 3). Relative to other Tasmanian species of section *Maidenaria*, *E. cordata* has a shallow cpDNA phylogeny, lacking haplotypes from the most common *Maidenaria* clade (C; McKinnon *et al*. 2001a). Based on the number of mutational connections, the analysis identified S43 as the probable ancestral haplotype in *E. cordata*. In agreement with coalescent theory, which predicts that interior, ancestral haplotypes will be present at higher frequency than tip haplotypes, S43 was the commonest and most widespread haplotype in *E. cordata*. All other haplotypes were confined to single populations, except S47, which was found in the two easternmost populations (sites 15 and 16; Table 2 and Fig. 2). Nine of the 15 populations with replicate samples were fixed for single haplotypes.

Statistical testing for association of cpDNA variation in *E. cordata* with geography (nested clade analysis) required the resolution of some ambiguities in the haplotype network. The four localized haplotypes S58, S77, S83 and S96 differed from S43 and from each other at a hypervariable polyA/T tract that was scored as a single multistate character (character 45; Table 1). This character enabled fine discrimination among haplotypes, but was of little value for determining the phylogenetic relationship among haplotypes. Based on coalescent theory, the probable derivation of haplotypes S58, S77, S83 and S96 is from the widespread S43, rather than from one another, but other relationships are also possible. For the purpose of testing associations between haplotypes and geography, these haplotypes were all grouped into the same one-step clade with S43 (Templeton & Sing 1993). Two other potential links in the network connected S89 to S53, and S100 to S101. In both cases the putatively linked haplotypes were well separated geographically and the links, which were judged to arise from parallel mutations in characters 40.1 and 84, respectively (Table 1), were broken.

Nested clade analysis found significant geographical structuring of cpDNA variation within the *E. cordata* cladogram as a whole, as well as within clades 1-2, 1-8, 2-1, and 2-2. The chief finding of importance to this study was that Dc (within-clade dispersal) values were significantly low ($P < 0.05$) for haplotypes S41, S58, S77, S80, S83 and S96, and clades 1-1, 1-4 (haplotype S99), 2-1 and 2-3. In other words, these haplotypes and clades had significantly restricted geographical ranges within *E. cordata*. The majority of tip haplotypes and tip clades with restricted distributions fell within the range of their respective interior haplotypes or clades, and the main phylogeographical inference drawn for *E. cordata* using the GeoDis inference key was therefore restricted gene flow with isolation by distance.

Haplotype sharing between E. cordata *and* E. globulus

Comparison of cpDNA sequences from *E. cordata* (75 samples from 20 populations) and *E. globulus* (264 samples from 157 populations) taking every variable sequence character into account identified six shared haplotypes: S41, S43, S47, S77, S83 and S99. Figures 3 and 4 show the positions of shared haplotypes in the *E. cordata* and *E. globulus* haplotype networks. Owing to the greater phylogenetic depth in *E. globulus*, the network for this species has been condensed by excluding four rapidly mutating polyA/T regions; haplotypes S77 and S83 are shown collapsed into S43. The major haplotype clade in *E. globulus*, previously identified by Freeman *et al*. (2001), is the C clade, which is common throughout the species' range. The S clade is localized to eastern Tasmania, but shows high haplotypic diversity. In both species, S haplotypes are centred on the shared haplotypes S43 and S41. Haplotypes S77 and S83 are two of many highly localized polyA derivatives of S43 in both species. Haplotype S47 and related haplotypes from the same site (S48 in *E. cordata* and S72 in *E. globulus*, site 16) form tip clades in both species and S99 is a tip haplotype in both species.

Table 2 *Eucalyptus cordata* populations sampled for cpDNA analysis. Samples of *Eucalyptus globulus* and *Eucalyptus viminalis/dalrympleana/rubida* from the same sites are also shown; an additional 232 samples of *E. globulus* and 28 samples of *E. viminalis* were collected from 146 and 25 other sites, respectively (not shown)

Site		Latitude (°)	Longitude (°)	Species	Haplotypes (number of samples)
1	Bream Ck	−42.79672	147.84009	*E. cordata*	**S43 (3)**
				E. globulus	**S43 (3)**
				E. viminalis	**S43 (2)**
2	Brown Mt	−42.59779	147.52171	*E. cordata*	**S41 (5)**, S101
				*E. globulus**	S81 (2), S94
				E. vim/dal	**S41 (2)**, S98
3	Cape Queen Elizabeth	−43.25377	147.42502	*E. cordata*	S43 (6)
				E. globulus	S76 (3)
4	Chimney Pot Hill	−42.92374	147.2757	*E. cordata*	S96 (3), S95 (2)
				E. globulus	S43 (3)
				E. viminalis	S90 (2), S93
5	Combes Hill	−42.95456	147.17163	*E. cordata*	S43
6	Electrona	−43.05078	147.24558	*E. cordata*	S43 (6)
7	Herringback foot	−43.00593	147.14601	*E. cordata*	**S43 (5)**
				E. globulus	**S43 (3)**
				E. vim/rub	**S43 (2)**
8	Hospital Creek	−42.75542	147.82242	*E. cordata*	S58 (3)
				E. globulus	S97 (3)
				E. viminalis	S91 (2), S92
9	Jacob Hill	−42.7061	147.86817	*E. cordata*	S43
10	Meehan Range	−42.84558	147.39749	*E. cordata*	**S83 (5)**
				E. globulus	**S83 (2)**, S41
11	Moogara site 1	−42.80067	146.9144	*E. cordata*	S80 (5)
				E. globulus	CC56 (2)
				E. dal	S69 (3)
12	Moogara site 2	−42.81328	146.91316	*E. cordata*	S43 (5)
13	Mt Lloyd	−42.83761	146.94861	*E. cordata*	**S99 (3)**, **S100 (2)**
				E. globulus	CC56 (2), **S99**
				E. vim/dal	**S100 (3)**
14	Penguin Island	−43.3512	147.37141	*E. cordata*	S43
15	Maria Island	−42.65726	148.13835	*E. cordata*	S47
16	Ponybottom Ck	−42.70801	147.89995	*E. cordata*	**S47 (2)**, S48
				E. globulus	**S47 (2)**, S72
				E. viminalis	CC53 (3)
17	Prosser River	−42.55626	147.84173	*E. cordata*	**S77 (5)**
				E. globulus	**S77 (2)**, S78
				E. viminalis	S38 (2), S79
18	Snug Plains	−43.06716	147.14738	*E. cordata*	S43 (2), S88 (2), S89
19	Square Mt	−42.72519	147.61804	*E. cordata*	S53, S54, S73
20	Taranna	−43.06209	147.87686	*E. cordata*	S71

Cases of local haplotype sharing within mixed populations are shown in bold. *, The sampled species was slightly separated from *E. cordata* (1–2 km away); *vim*, *viminalis*; *dal*, *dalrympleana*; *rub*, *rubida*.

Geographical distribution of shared haplotypes

The four shared tip or putative tip haplotypes (S47, S77, S83 and S99) were significantly geographically restricted or belonged to geographically restricted clades in *E. cordata*. S47 was found only at sites 15 and 16, while S77, S83 and S99 were found only at sites 17, 10 and 13, respectively (Fig. 2, Table 2). These four haplotypes showed a matching restricted distribution in *E. globulus*, being found only in mixed *E. globulus*/*E. cordata* populations at sites 16, 17, 10 and 13, respectively. Haplotype S72, a derivative of S47 found in *E. globulus*, was also restricted to site 16.

Haplotype S43 was the common ancestral haplotype in *E. cordata* and was also the most common S clade haplotype

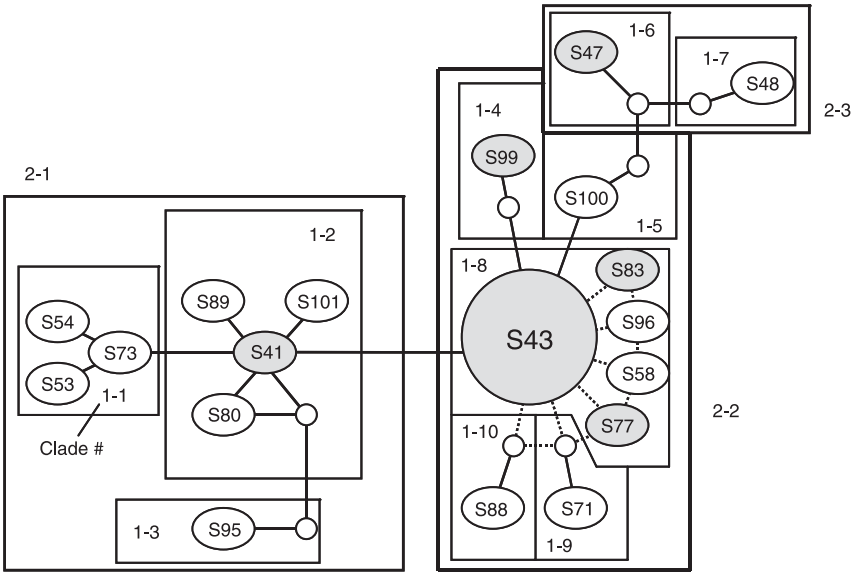

Fig. 3 Chloroplast (cp) DNA haplotype network for *Eucalyptus cordata*. Haplotypes shared with *Eucalyptus globulus* are tinted grey. Lines connecting haplotypes represent single character state changes; empty nodes depict missing haplotypes. The putative ancestral haplotype is shown larger. The precise phylogenetic relationship among the haplotypes connected by the dotted lines is ambiguous (see text).

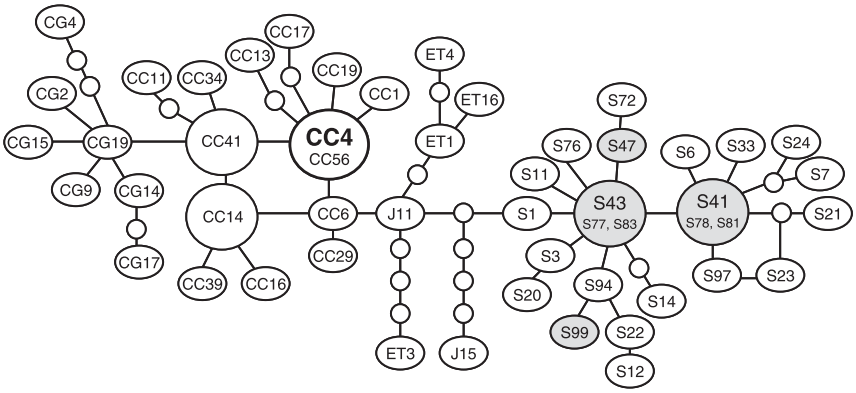

Fig. 4 Condensed haplotype network for *Eucalyptus globulus*, after excluding rapidly evolving polyA/T regions. Four minor loops have been broken. Lines connecting haplotypes represent single character state changes; empty nodes depict missing haplotypes; and large nodes depict haplotypes found in more than 10 *E. globulus* populations (after collapsing haplotypes). The commonest haplotype is CC4. Haplotypes shared with *Eucalyptus cordata* are tinted grey.

found in *E. globulus*. This haplotype was found in allopatric as well as sympatric populations of both species. Again, however, the full geographical distribution of this haplotype in *E. globulus* closely matched its distribution in *E. cordata*, with two main geographical clusters around sites 1 and 7. In total, this haplotype was found in 11 populations of *E. globulus*, only three of which also contained *E. cordata*. The remaining populations were 1.9, 2.3, 3.2, 5.4, 8.4, 8.5, 11 and 14 km from the nearest known population of *E. cordata*. The mean distance between populations of *E. globulus* with S43 and the nearest known population of *E. cordata* with S43 was 7.8 km (maximum 14 km).

The only shared haplotype that showed a nonmatching distribution in the two species was S41. This haplotype was significantly restricted to a single site in *E. cordata*, despite its interior position in the haplotype network. Its distribution in *E. globulus* was wider (six populations). Three of these were within 2 km of *E. cordata* populations that had closely related haplotypes (S43 and S83), but the other three were 15, 17 and 24 km from any known population of *E. cordata*.

To test whether the presence of *E. cordata* haplotypes in *E. globulus* was significantly correlated with proximity to *E. cordata*, the entire *E. globulus* dataset was divided into seven roughly equal-sized groups according to distance from the nearest *E. cordata* population (0–2 km, 2–10 km, 10–20 km, 20–50 km, 50–100 km, 100–250 km and 250–550 km). The frequency of shared haplotypes for the different distance groups is shown in Fig. 5. Shared haplotypes peaked in the 0–2 km distance group and dropped to zero by 25 km. Contingency χ^2 testing showed that the proportion of shared haplotypes in *E. globulus* sampled 0–2 km from *E. cordata* was significantly higher than expected under random distribution of shared haplotypes in *E. globulus* within both the total region of haplotype sharing (up to 50 km from *E. cordata*; $P < 0.001$) and the main region of haplotype sharing (up to 20 km from *E. cordata*; $P < 0.05$).

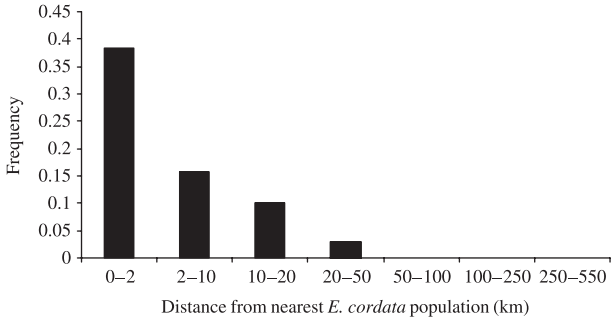

Fig. 5 Frequency of all *E. cordata* haplotypes in *E. globulus* as a function of distance from the nearest known population of *E. cordata*.

Haplotypes found in sympatric E. viminalis

Nine of the sampled *E. cordata* populations were sympatric with populations of *E. viminalis* or its intergrading sister species, *E. dalrympleana* and *E. rubida*. All three species have been observed to hybridize with *E. cordata*, and both *E. viminalis* and *E. rubida* have been observed to hybridize with *E. globulus* (Williams & Potts 1996), raising the possibility that haplotype sharing between *E. globulus* and *E. cordata* could be mediated by hybridization of both species with *E. viminalis* or its sister taxa. To investigate this possibility, samples of *E. viminalis/dalrympleana/rubida* were analysed whenever sympatric with *E. cordata* (Table 2) and at 29 additional Tasmanian populations (not shown). Local haplotype sharing between *E. globulus* and *E. cordata* was apparently not dependent on *E. viminalis*, since it occurred both in the absence of *E. viminalis* (e.g. site 10) and when *E. viminalis* was present but had different haplotypes (e.g. sites 16 and 17). Local haplotype sharing between *E. viminalis/dalrympleana* and *E. cordata* also occurred independently of *E. globulus* (e.g. site 2). However, at sites 1 and 7 both *E. globulus* and *E. viminalis* shared haplotype S43 with *E. cordata*. This haplotype was not found at any other sampled populations of *E. viminalis/dalrympleana/rubida*.

ITS sequences

To determine whether local cpDNA sharing between *E. cordata* and *E. globulus* was accompanied by sharing of nuclear DNA markers, sequences from the ITS region of nr DNA were obtained for 15 samples of *E. globulus* and 19 of *E. cordata* at sites showing shared cp haplotypes, and compared with sequences from additional sites and related species (Table 3). Species of section *Maidenaria* show little ITS sequence divergence (Steane *et al.* 1999), and no fixed differences were found between *E. globulus* and *E. cordata*. However, at most sites the local ITS sequences of *E. cordata* differed from those of sympatric *E. globulus*, despite cpDNA sharing. For example, mutations found in *E. globulus* at base pairs 68, 306, 447 and 452 were not detected in any sympatric *E. cordata*. Mutations found at base pairs 84, 129, 140, 183, 433 and 479 in *E. cordata* were not found in sympatric *E. globulus*. Thus, although both *E. globulus* and *E. cordata* showed ITS sequence heterogeneity, both within populations and within individuals, this could not be readily attributed to exchange of nuclear DNA between the two species.

Discussion

Origin of cp haplotype sharing

The overall pattern of cpDNA sharing between *E. globulus* and *E. cordata* appears most consistent with multiple instances of local introgression in different mixed populations. Among-species sharing of haplotypes caused by incomplete lineage sorting preferentially involves ancestral (interior) haplotypes (Schaal & Leverich 2001), while sharing of recently derived (tip) haplotypes is more likely to arise through introgression. Shared tip haplotypes could be generated by the occurrence of parallel mutations, particularly in rapidly evolving sequence regions, but this would be unlikely to produce geographical concordance of tip haplotypes across species. In this study, four putative tip haplotypes (S47, S77, S83 and S99) have matching, highly localized distributions in *E. cordata* and *E. globulus*. No shared tip haplotypes have nonmatching or scattered geographical distributions, as might be expected under parallel mutation. The frequency of all shared haplotypes is highest in *E. globulus* individuals sampled within 2 km of *E. cordata* populations (Fig. 5) and drops off rapidly to almost zero by 15 km, indicating a strong correlation between haplotype sharing and species proximity.

In addition to sharing tip haplotypes localized to sympatric populations, *E. cordata* and *E. globulus* share two more widespread haplotypes interior to the cpDNA networks of both species (S41 and S43). Sharing of interior haplotypes is more ambiguous than sharing of tip haplotypes, as it may arise through a variety of processes, including incomplete lineage sorting, recent introgression, and/or ancient introgression. A potential role for lineage sorting in the sharing of these haplotypes is supported by the facts that (1) both haplotypes also occur in *E. viminalis*; (2) both haplotypes have many unique derivatives in *E. globulus* and *E. cordata*; and (3) occurrences of haplotype S41 in *E. globulus* and *E. cordata* show relatively poor geographical correlation. Conversely, a potential role for introgression of haplotype S43 is supported by its tightly linked distribution in all three species. Occurrences of S43 in *E. viminalis* are limited to two sites where *E. cordata* and *E. globulus* also have S43, while occurrences of S43 in *E. globulus* closely surround occurrences of S43 in *E. cordata* to a maximum distance of 14 km. Given the evidence for introgression of tip haplotypes at four different sites, it is credible that S43 has also been exchanged recurrently between *E. cordata* and *E. globulus* over

Table 3 Comparison of internal transcribed spacer (ITS) sequences from *Eucalyptus cordata*, *Eucalyptus globulus*, and related eucalypt taxa of section *Maidenaria*

Site	Species	Samples	Haplotypes	bp 68 (C)	84 (T)	129 (C)	140 (G)	183 (A)	230* (T)	249 (0)	306 (G)	433 (A)	447 (C)	452 (0)	479 (G)	562 (T)	603 (G)
1	*E. cordata*	3	S43		C + T	C + T	G + T		C + T			A + G			C + G		A + G
	E. globulus	3	S43						C + T							C + T	
10	*E. cordata*	5	S83					G	C	T						C + T	
	E. globulus	2	S83	C + T					C + T		G + T		C + T	A + 0			A + G
13	*E. cordata*	3	S99, S100				G + T	G + A	C						C + G	C + T	
	E. globulus	3	S99, CC56	C + T					C + T					A			
16	*E. cordata*	3	S47, S48				T		C						C + G		
	E. globulus	3	S47, S72						C + T		G + T		C + T	A + 0		C + T	
17	*E. cordata*	5	S77		C + T	C + T	G + T		C + T			A + C + G			C + G		A + G
	E. globulus	3	S77, S78	C + T					C + T				C + T	A + 0		C + T	
7	*E. cordata*	1	S43						C								
11	*E. cordata*	1	S43						C								
Vic	*E. globulus*	1															
NSW	*E. maidenii*	1											T				
NSW	*E. bicostata*	1															A
Vic	*E. pseudoglobulus*	1															
NSW	*E. corticosa*	1															
Qld	*E. scoparia*	1															

The common nucleotide is indicated following the base pair number (bp); base heterogeneity indicates the presence of within-individual or within-population heterogeneity. Autapomorphies are not shown. *Eucalyptus cordata* is from series *Orbiculares*; *E. globulus*, *Eucalyptus maidenii*, *Eucalyptus bicostata* and *Eucalyptus pseudoglobulus* are from series *Globulares*; *Eucalyptus corticosa* is from series *Acaciiformes* and *Eucalyptus scoparia* is from series *Microcarpae*. Abbreviations: Vic, Victoria; NSW, New South Wales; Qld, Queensland; 0, missing base. *This base is often polymorphic for C and T in species of section *Maidenaria* (G. E. McKinnon, unpublished).

time, producing a matching range in both species. Since S43 is ancestral in *E. cordata*, both recent and ancient transfer of this haplotype to *E. globulus* could have occurred. Limited expansion of *E. globulus* carrying S43, and/or loss of *E. cordata* from some sites through natural extinction or landclearing, could then have led to the presence of S43 in some allopatric populations of *E. globulus*.

The nature of introgression

Several lines of evidence suggest that shared haplotypes were acquired by *E. globulus* from *E. cordata* and not vice versa. First, *E. cordata* appears to be completely fixed for S clade haplotypes, while *E. globulus* has a mixture of C and S haplotypes in south-eastern Tasmania and predominantly C haplotypes elsewhere in its range. The absence of C haplotypes in *E. cordata* suggests that introgression is not bidirectional. Second, *E. cordata* populations form a series of small islands surrounded by a larger 'sea' of *E. globulus*. Pollen flow from *E. globulus* to *E. cordata* is likely to far exceed pollen flow in the opposite direction, making *E. cordata* the probable maternal parent (and thus the probable chloroplast donor; Byrne *et al*. 1993; McKinnon *et al*. 2001b) to any F_1 hybrids. For the same reason, subsequent backcrossing of hybrids is likely to favour *E. globulus* as the pollen parent. The observed pattern of small populations of *E. cordata* surrounded by *E. globulus* populations with both matching and unique haplotypes is consistent with invasion of *E. globulus* by both seed and pollen dispersal. Lastly, crossing experiments suggest that natural hybrids between the two species are more likely to have *E. cordata* as the maternal parent. Although successful artificial crosses in both directions have been reported, as well as a vigorous F_2 (Potts 1989), pollination experiments have shown a barrier to fertilization of *E. globulus* females by *E. cordata* and several other species from the same section (including *E. viminalis*; Potts & Savva 1989). As interspecific crosses in the reverse direction often show greater success, this barrier may be due to the long style of *E. globulus* preventing the pollen tubes of smaller-flowered species from reaching the ovary (Gore *et al*. 1990).

While the simplest interpretation is that *E. globulus* has acquired cpDNA haplotypes through direct hybridization with *E. cordata*, the potential role of additional hybridizing species must be considered. *Eucalyptus viminalis*, which has been reported to hybridize with both *E. globulus* and *E. cordata* (Williams & Potts 1996), was present at several mixed populations and shared haplotype S43 with both species at sites 1 and 7. In theory, this species might have acted as chloroplast donor to both *E. globulus* and *E. cordata* at these sites, or as an intermediary in hybridization between the two species. The former possibility seems unlikely given that S43 is the common ancestral haplotype in *E. cordata* but is found only at these two sites in *E. viminalis*, while the latter possibility is not supported by cpDNA evidence from other sites containing all three species. *Eucalyptus ovata*, *E. johnstonii* and *E. barberi* are thought to be involved in hybridization with *E. cordata* at sites 1, 7 and 16, respectively (Potts 1989) and are also capable of hybridizing with *E. globulus* (Williams & Potts 1996), raising the possibility of more complex interactions. However, at other sites these species are absent, and direct gene flow between *E. cordata* and *E. globulus* remains the most obvious explanation for haplotype sharing.

Site disturbance by human activity is apparently not necessary for gene flow between *E. globulus* and *E. cordata*, since three of the sites demonstrating localized introgression (sites 10, 16 and 17) are in natural forest. Potts (1989) noted that hybridization between *E. cordata* and other species is a natural phenomenon and not associated with site disturbance. However, the two sites at which *E. cordata*, *E. globulus* and *E. viminalis* all share haplotype S43 have suffered some disturbance through clearing and/or road-building in the past 200 years, which may have influenced natural patterns of hybridization. Site disturbance has been shown to promote hybridization in diverse genera such as *Iris* (Anderson 1949), *Rorippa* (Bleeka & Hurka 2001), and *Banksia* (Lamont *et al*. 2003).

Despite evidence for local cpDNA transfer, there is no evidence for local transfer of nuclear ITS sequences between *E. globulus* and *E. cordata*. The ITS sequences in both species are heterogeneous within individuals, and within and among populations, but polymorphisms are not shared among sympatric *E. globulus* and *E. cordata*. The finding of cytoplasmic marker exchange, combined with little or no detectable nuclear DNA exchange, is quite common in plant genera (reviewed by Rieseberg & Soltis 1991; examples include *Quercus*, *Populus* and *Salix*). Reasons for the discrepancy include selective barriers preventing the introgression of blocks of the nuclear genome (Rieseberg *et al*. 1995; Martinsen *et al*. 2001). Backcrossing of interspecific hybrids to one parental species is also expected to dilute the nuclear genomic contribution of the other parent in later generations. In the case of the ribosomal RNA genes, this process may be accelerated by concerted evolution. Aguilar *et al*. (1999) found that, in artificial *Armeria* hybrids, ITS sequences were already homogenized by the F_2 generation and showed a bias favouring one parent; in backcrosses, sequences were rapidly homogenized in favour of the recurrent parent.

Implications for phylogeographical analysis

Extinction by hybridization (assimilation) is recognized as a risk for rare, insular species surrounded by more abundant hybridizing congeners (Levin *et al*. 1996). This raises the possibility that vanishing species may leave their traces in the organellar genomes of hybridizing species that have assimilated them. Field observations indicate that hybridization with co-occurring species such as *E. globulus*, *E. viminalis*, *E. ovata/brookeriana* and *E. johnstonii* is one of several

factors (also including habitat disturbance, competition, and in-breeding) threatening the long-term survival of *E. cordata* (Potts 1989). The present study has found that cpDNA phylogeography in *E. globulus* is consistent with acquisition of *E. cordata* haplotypes at several mixed populations. In the likely event that *E. cordata* is eventually eliminated from some of these populations (e.g. site 17, which is currently in poor condition following bush fires), the origin of this acquired cpDNA diversity will be obscured. Based on the distribution of S43, which occurs in *E. globulus* up to 14 km from any known *E. cordata* population, it is possible that the cpDNA of *E. globulus* already contains the signature of some lost *E. cordata* populations and that the origins of other acquired haplotypes have already been obscured.

These findings show that interpretation of cpDNA phylogeography needs to take into consideration not only the possibility of introgression between extant sympatric species, but also the possibility of introgression involving species that have been locally or globally eliminated. Given the close relationship between local environment and species dominance in *Eucalyptus*, many eucalypt species must have undergone redistribution and/or extinction during climate changes of the Pleistocene, and their potential contribution to cpDNA diversity in extant species presents a major challenge for phylogeographical analysis. The same principle applies to other genera in which the risk of local species extinctions is combined with weak reproductive barriers between species.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2364/MEC2364sm.htm

Appendix S1. Variable characters from the J_{LA+} region of cpDNA used to define haplotypes in *Eucalyptus globulus* and *Eucalyptus cordata*. Characters that were variable in *E. cordata* are highlighted in grey. Other characters were variable only in *E. globulus*. Indels were scored as absence/presence; polyA/T characters were scored as single multistate characters; minisatellite repeats were scored as stepwise mutations (one mutation per repeat). Character ID refers to a larger-scale eucalypt chloroplast database (available from the authors on request).

Appendix S2. Data matrix used to construct the *Eucalyptus globulus* cpDNA haplotype network. Owing to the greater phylogenetic depth in this species, haplotypes were collapsed for network construction by excluding four of the hypervariable regions used for haplotype definition.

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