Shared phylogeographic patterns and widespread chloroplast haplotype sharing in *Eucalyptus* species with different ecological tolerances

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Abstract We examined the phylogeography of three south-east Australian trees (*Eucalyptus delegatensis, Eucalyptus obliqua*, and *Eucalyptus regnans*) with different tolerances, in terms of cold, drought, fire and soil to explore whether species with different ecologies share major phylogeographic patterns. A second aim of this study was to examine geographic patterns of chloroplast DNA (cpDNA) haplotype sharing among the three study species. Trees of *E. delegatensis* (n=120), *E. obliqua* (n=265) and *E. regnans* (n= 270) were genotyped with five cpDNA microsatellite markers. The species shared major phylogeographic disjunctions, and common patterns at proposed glacial refugia (generally high haplotype diversity) and areas thought to have been treeless during the Last Glacial Maximum (LGM) (low diversity). Inter-specific sharing of haplotypes was extensive, and fixation of shared, regional haplotypes was more frequent in areas postulated as having been treeless at the LGM. Despite ecological differences, chloroplast microsatellite data suggest the three species have responded to past climatic changes in a similar way, by persisting in multiple, generally common refugia. We suggest that the natural ability of eucalypt species to hybridise with others with quite different or broader ecological tolerances may provide an "insurance policy" for response to rapidly changing abiotic conditions.

Keywords climate change, comparative phylogeography, *Eucalyptus*, hybridisation, resilience, southern hemisphere.

Introduction

Much of the existing temperate zone forest has developed since the Last Glacial Maximum (LGM) (Davis 1976) and the current distributions of species and the assemblages we now observe are the result of the individual responses of the

species involved. While increasing numbers of species histories have been inferred from geographic patterns of genetic markers (reviewed in Beheregaray 2008), we know relatively little (compared to the northern hemisphere) about the extent to which species that occur together now in the temperate forests of the southern hemisphere have had parallel responses to past climate change. The temperate, tall *Eucalyptus*-dominated forests of south-eastern Australia provide a unique system in which to test hypotheses about species response to climate change as there are numerous morphologically and ecologically distinct species, many with sympatric distributions.

In far south-eastern Australia, tall eucalypt forests are largely restricted to high rainfall coastal and montane regions (Boland et al. 2006). During colder, more arid, glacial periods, species of these forest types are thought to have survived either *in situ* in suitable micro sites or in coastal refugia and then colonised the landscape when conditions became more favourable. Evidence from the fossil pollen record (McKenzie 2002; McKenzie and Kershaw 2000, 2004; McKenzie 1997; McKenzie and Kershaw 1997; Kershaw et al. 1991; Kershaw et al. 2007; Macphail and Jackson 1978; Sigleo and Colhoun 1981; Fletcher and Thomas 2007), combined with climate modelling of species distributions (Kirkpatrick and Fowler 1998) and a limited number of studies using molecular markers (Freeman et al. 2001; Jackson et al. 1999; McKinnon et al. 2004a; McKinnon et al. 2001; Pollock et al. 2012; Steane et al. 1998; Nevill et al. 2010; Worth et al. 2009; Worth et al. 2010; Worth et al. 2011), has allowed the identification of putative refugia and treeless areas during glacial maxima for forest species in south-eastern Australia. On the mainland, multiple refugia for tree species are thought to have existed in the Otway Ranges, the south Central Highlands, South Gippsland and East Gippsland regions of the mainland state of Victoria. On the island of Tasmania, refugia were likely in the west, south-east, east and north-east. Areas thought to have been

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treeless during the LGM include the Central Highlands of Victoria and the central regions of Tasmania. However, it is possible that tree species persisted in these regions during the LGM in disagreement with current knowledge of their environmental tolerances or at low densities and thus not detected in the fossil pollen record.

Tall eucalypt forests are seen as vulnerable to anthropogenic climate change, particularly to increasing temperature, decreasing rainfall and altered fire regime (Hughes 2003). Within these forests there are three main commercially important species in subgenus *Eucalyptus*: alpine ash, *Eucalyptus delegatensis* R.T.Baker; messmate, *Eucalyptus obliqua* L'Hér; and mountain ash, *Eucalyptus regnans* F.Muell. These species are members of the informal 'Ash' group of eucalypts, considered to fall into two clades, viz. the "green ashes" (including *E. obliqua* and *E. regnans*), and the "blue" ashes (including *E. delegatensis*) (Ladiges et al. 1989). They occur in adjacent stands in many regions, often as an altitudinal replacement series, but have distinct ecological tolerances, especially with respect to cold, drought, soil fertility and fire (see Methods). It is not known to what extent responses to climate change during the LGM were shared between these three "Ash" species and, in particular, whether they contracted to similarly located refugia and colonised similar areas. For example, during the LGM *E. regnans* may have been restricted to a narrower distribution than either *E. delegatensis* or *E. obliqua* due to its inferior cold and drought tolerance or more restricted range of ecotypes or phenotypic plasticity. A comparative phylogeographic study of these species will thus provide additional information from the southern-hemisphere on the effect of contrasting ecological traits on the genetic structure of temperate forests.

This study will also add to our understanding of the sharing between eucalypt species of maternally inherited chloroplast markers. Comparative phylogeographic

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studies strongly suggest that chloroplast DNA (cpDNA) is shared extensively between eucalypt species in south-eastern Australia, supporting the proposition that reticulate evolution has played an important role in the evolution of eucalypts. These studies have focused mostly on the island of Tasmania and on species from the largest subgenus, *Symphyomyrtus* (McKinnon et al. 1999; McKinnon et al. 2001; Steane et al. 1998; Jackson et al. 1999; McKinnon et al. 2004b; McKinnon et al. 2004a). In Tasmania, particular chloroplast types are associated with hypothesised refugia and colonised areas and, an individual's chloroplast type is more strongly influenced by geographical location than its taxonomic classification. However, it is not known whether particular species always share haplotypes, or whether sharing is more common at certain locations i.e. in areas postulated as treeless during the LGM.

In this study we used cpDNA microsatellite markers (Steane et al. 2005) to examine geographic patterns of chloroplast diversity and structure in *E. delegatensis* and *E. obliqua*. The results were compared to cpDNA data previously obtained for *E. regnans* (Nevill et al. 2010) and *E. obliqua* (Bloomfield et al. 2011) in order to: 1) explore whether montane eucalypt species with different ecological tolerances share phylogeographic patterns; 2) infer historical patterns of hybridisation and introgression between species; and 3) to provide data on patterns of genetic variation to assist in the formulation of gene pool management guidelines for three of the most commercially important tall forest species.

Methods

Study species

The three species in this study have different ecological tolerances. *Eucalyptus delegatensis* is a subalpine species and is restricted to latitudes between 35˚ and 43˚ S, and between 850 m and 1500 m in elevation on the mainland and 160-1200 m. A subspecies occurs in Tasmania (*Eucalyptus delegatensis subsp. tasmaniensis)*, which has thick bark over the entire trunk and the larger limbs. In the mainland subspecies the rough bark extends only part-way up the trunk. *Eucalyptus delegatensis* can grow over 90 m tall, is the most cold tolerant of the three, is fire-sensitive, and moderately drought tolerant (Boland et al. 2006). *Eucalyptus obliqua* is highly morphologically variable and one of the most widely distributed eucalypt species, with ecotypes occurring in situations from coastal heath to a tall over-storey above cool-temperate rainforest. It is found between the latitudes of 28˚ and 43.5˚ S, from sea level to 1200 m and grows to 90 m in height (Boland et al. 2006). *Eucalyptus obliqua* is the least fire sensitive of the study species and occurs across a much wider ecological range and has variable cold and drought tolerance, depending on provenance (Brown et al. 1976). *Eucalyptus regnans*, grows to heights in excess of 100 m, and is discontinuously distributed between 37.2˚ and 43.3˚ S on and south of the Great Dividing Range in Victoria between 200 and 1100 m and in Tasmania between sea level and 700 m. The species is often found in small, disjunct stands in gullies and on sheltered slopes and occurs only on sites with high rainfall and deep fertile soils (Ashton 1958; Boland et al. 2006). The species is the most drought and fire sensitive of the three study species and like *E. obliqua*, has variable cold tolerance depending on provenance . These three species are predominantly out crossing, with pollination by a range of non-specific insects and vertebrates and seed dispersal by gravity over relatively small distances, generally within two tree heights (Boland et al. 2006).

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At locations where the three species co-occur, *E. delegatensis* is generally found at the highest elevations, and *E. regnans* and *E. obliqua* at lower elevations (Ashton 1958; Boland 2006). In drier areas *E. regnans* may not occur or *E. delegatensis* may be absent in areas with lower topography. *Eucalyptus regnans* and *E. delegatensis* typically occur in single species stands, commonly even-aged because adult trees are usually killed by even low intensity fire and regenerate primarily from seed (Ashton 1958; Boland 2006). *Eucalyptus obliqua* in contrast often occurs with a variable mix of other *Eucalyptus* species and, because it has a greater capacity for vegetative survival by re-sprouting after fire, these stands are typically multi-aged. Weak reproductive barriers exist between many eucalypt species (Griffin et al. 1988), and hybridisation between *E. obliqua* and *E. regnans* is common, with morphologically intermediate individuals found at most locations where they grow in adjacent stands (Ashton 1958; Ashton 1981). Hybridisation between *E. delegatensis* and either *E. obliqua* and *E. regnans* also occurs (Griffin et al. 1988) , but appears less common. Hybridisation between the three study species and many other species in subgenus *Eucalyptus* not included in the study has also been recorded (Griffin et al. 1988). The three taxa are, nonetheless, "good species" in a morphological sense, being distinguished by key characters of bark, leaves, inflorescences and fruits. They are placed across two sections and three series in the classification of Brooker (2000); phylogenetic analyses based on morphology, nuclear ribosomal DNA sequences, and genome-wide markers (Ladiges et al. 1989; Ladiges et al. 1992; Steane et al. 2011; Ladiges et al. 2010) support the placement of *E. delegatensis* in a separate clade ("blue ashes") to the two other species, and morphological analyses suggest that *E. obliqua* and *E. regnans* are not each others' closest relatives (Ladiges et al. 1989).

Sampling

The sampled populations are located between latitudes 37˚ and 43˚ S on the south-eastern tip of mainland Australia and the large island of Tasmania (see Table 1 and Fig. 2 for details). Samples were collected from 120 *E. delegatensis* and 155 *E. obliqua* trees at 12 and 16 locations, respectively. The sampling strategy was designed to complement previous studies on *E. obliqua* (Bloomfield et al 2011) and *E. regnans* (Nevill et al. 2010). We collected samples from locations that represent a subset of the ranges of *E. delegatensis* and *E. obliqua*, however, the range of *E. regnans* was comprehensively sampled. Samples were collected from *E. delegatensis* and *E. obliqua* trees estimated to be at least 70 years of age (regenerated prior to the silvicultural movement of seed between geographic regions), wherever they occurred near an *E. regnans* sampling location in the earlier study. Where possible, ten individuals of each species were sampled per location and sample trees were at least two tree heights apart to avoid sampling closely related individuals. However, at a number of locations, fewer than ten individuals were sampled due to the small number of suitable individuals. *Eucalyptus delegatensis*, *E. obliqua* and *E. regnans* co-occurred at 12 locations, with *E. obliqua* and *E. regnans* co-occurring at a further 15 locations.

Chloroplast microsatellite analysis

Genomic DNA was extracted from cambial scrapings using a protocol adapted from Tibbits et al. (2006), with modifications described by Nevill et al. (2010). We used the five chloroplast microsatellite loci (EMCRC60cp, EMCRC67cp, EMCRC74cp, EMCRC86cp and EMCRC90cp; (Steane et al. 2005) and the same methods of amplification, capillary separation and allele sizing as a previous study of chloroplast variation in *Eucalyptus regnans* (Nevill et al. 2010). DNA standards from that

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study were included to ensure consistency in fragment sizing. Wherever allele scores were ambiguous, samples were re-amplified. If allele scoring remained ambiguous after re-amplification, the samples were excluded from the study. Chloroplast microsatellites can be affected by homoplasy and mutation models for loci are not generally clear. However, they were used in this study rather than, for example, chloroplast sequence because of the need to integrate our data with those previously collected (Bloomfield et al. 2011; Nevill et al. 2010).

Genetic data analysis

In this paper a haplotype was considered to be a distinct combination of size variants at the five cpSSRs. The number of haplotypes, number of unique haplotypes (the number of haplotypes found only in that species at that location) and haplotypes shared was calculated for each species at each location. The extent of introgression (i.e. genetic similarity) between species was calculated using introgression ratios (IG) from Belahbib et al. (2001) which are based on intra- and interspecific identities from Dumolin-Lape`gue et al. (1999). Introgression ratios for each species pair in each region (Mainland and Tasmania) and for both regions combined were quantified using data from populations where two of the study species co occur. 95% confidence intervals for IG values were calculated by means of simple percentile bootstrap with 1000 iterations, resampling populations. Code was written in SAS version 9.3 and is available on request.

For each species, mean within-population genetic diversity, total genetic diversity, and population differentiation were calculated treating alleles as unordered (h_S, h_T and G_{ST}) and ordered (v_S, v_T, and R_{ST}), where the inferred number of mutational steps between alleles is considered to carry additional information, following the

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method of Pons and Petit (1996) using PERMUT (available at

www.pierroton.inra.fr/genetics/labo/Software/PermutCpSSR/). The differentiation

parameters G_{ST} and R_{ST} were compared for each species to test for phylogeographical

structure. If R_{ST} is significantly higher than G_{ST} this indicates the presence of

phylogeographic structure (Pons and Petit 1996). Separate estimates are calculated for

the mainland, Tasmanian and all populations. Differences between species in mean

within-population genetic diversity, total genetic diversity and population differentiation

were tested using t tests. A rarefaction estimate of haplotype richness for each species at

each location for the minimum sample size of five was produced using ADZE (available

at http://rosenberglab.stanford.edu/adze.html) (Szpeich et al. 2008). These data were

then analysed by Kruskall-Wallis procedure (using the NPAR1WAY procedure in SAS

9.3) to test for differences between species overall, regions overall, and between species

within each region. The null hypothesis tested in each case is that the median within-

population haplotype richness is the same in all groups.

A statistical parsimony network of haplotypes was constructed using Network

4.2.0.1 (available at http://www.fluxus-engineering.com/sharepub.htm#a10). The

median joining network algorithm was used to construct the shortest, least complex

network (Bandelt et al. 1999). The distance between haplotypes is the sum of nucleotide

differences over the five chloroplast microsatellite loci. In order to examine the

geographic structure of haplotype diversity a haplotype frequency map was produced

for each species.

A linear regression model was used to investigate the dependence of the number of

haplotypes, number of unique haplotypes and haplotype sharing on population distance

from the coast or with elevation.

Results

Haplotype variation and relationships

The five cpDNA microsatellite loci genotyped in 120 individuals of *E. delegatensis* (12 locations), 265 individuals of *E. obliqua* (27 locations), and 270 individuals of *E. regnans* (27 locations) were all polymorphic and produced a total of 30 different size variants and, when combined, 40 different haplotypes (Table 2; haplotype numbers follow the format of Nevill et al. 2010). This study revealed 13 new haplotypes in addition to the 31 found in the extensive study on *E. regnans*. This study of cpSSRs showed a total of 10 haplotypes in *E. delegatensis*, 22 in *E. obliqua*, ,and 27 in *E. regnans*.

The relationships between haplotypes are shown in Fig. 3. Haplotypes were connected to between one and five others and generally separated by one or two nucleotides, however, a number of haplotypes were separated by greater than three nucleotides from the next closest haplotype (H9, H11 and H40). The longest internal branch in the network separates two major clades (clade 1 and clade 2), corresponding generally to the geographic origin of the individuals. All Tasmanian haplotypes, regardless of species, were located within clade 1. Victorian haplotypes were divided between clades 1 and clades 2. All accessions of *E. obliqua* from the Otway Ranges populations of Upper Ford R (Site 16) and Carisbrook (Site 15), Kallista (Site 2) and Mt Disappointment (Site 4) in the south Central Highlands and nine individuals of *E. regnans* from Upper Ford River (Site 16) and one from Carisbrook (Site 15) were located in clade 1 (i.e. all individuals with H1). All individuals from other regions of Victoria, regardless of species, fell within clade 2. A subgroup of clade 2 contains a

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group of haplotypes, present in all three species, that occurs predominantly in East Gippsland (Sites 8-11).

Geographic structure and levels of haplotype diversity

For the three species, within-population cpSSR diversity (h_S v_S) was low relative to total diversity (h_T v_T) and there was strong population differentiation (Table 3). Levels of diversity were not significantly different between the three species in all pair-wise comparisons (P values all > 0.05). However, *E. delegatensis* had fewer haplotypes in all pair-wise comparisons, and *E. obliqua* always had lower numbers of haplotypes than *E. regnans*. High haplotype diversity was found at several locations across the study. For example, at Quarry Creek (Site 9 Fig. 2) in East Gippsland five haplotypes were found in a total of 30 sampled individuals of *E. delegatensis* (H10 and H39), *E. obliqua* (H10) and *E. regnans* (H10, H11, H12 and H13). Similarly, high haplotype diversity was found at the South Gippsland location of Mirboo East (Site 13 Fig. 2), with five haplotypes found in a total of 20 sampled individuals of *E. obliqua* (H2, H3, H4 and H35) and *E. regnans* (H2, H3, H4 and H5). We found no significant differences for median within-population haplotype richness in all pairwise comparisons between populations or regions (Table 4).

The haplotype distribution was highly geographically structured and similar in the three species (Fig. 2). The pattern previously found in *E. regnans* (Nevill et al. 2010) was also found in *E. delegatensis* and *E. obliqua* i.e. fixation or near fixation of certain haplotypes in some regions whereas other regions show high relative haplotype diversity. For example, at high elevation sites in the Central Highlands of Victoria (sites 5, 6 and 7), only a single haplotype H4, was found in all sampled individuals of the three species (90 individuals). Similarly, in the central region of Tasmania (sites

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24 and 25), there is near fixation of haplotype H20 (58 of 60 individuals) in all three species. In contrast, in the East Gippsland region (sites 8, 9, 10 and 11) 15 haplotypes were found in 110 individuals of the three species (*E. delegatensis*, 6 haplotypes across 30 individuals; *E. obliqua*, 8 haplotypes across 40 individuals; *E. regnans*, 10 haplotypes among 40 individuals). For *E. delegatensis*, *E. obliqua* and *E. regnans* both overall and at a state level R_{ST} was not significantly higher than G_{ST} (P values all > 0.05).

Haplotype sharing

In general, haplotype sharing was common (14 out of 40 haplotypes) (Tables 1 and 2 and Figs 2, 3 and 4), with four common haplotypes shared among all three species, and 10 shared between *E. obliqua* and *E. regnans*. Sharing of haplotypes between all three species was particularly prevalent in the Central Highlands of Victoria (H4 at sites 5, 6 and 7) and central Tasmania (H20 sites 24 and 25). Overall, 35% of haplotypes detected were shared between at least two species with sharing occurring at 20 of the 27 included locations. Introgression ratios (IGs) were positive for all species comparisons indicating local sharing of haplotypes (Table 5). Introgression ratios ranged from 0.42 (*E. obliqua* and *E. regnans* in Tasmania) to 0.79 (*E. delegatensis* and *E. regnans* in Tasmania) and there were no consistent geographic or species pair patterns.

   Even though sharing of haplotypes between species was common, most haplotypes (26 out of 40) were confined to one species. Haplotypes detected in only one species were observed at many locations and in most regions of the study, but were particularly common in East Gippsland (10 of 26 unique species haplotypes). Overall, six haplotypes were confined to *E. delegatensis*, seven haplotypes were confined to *E. obliqua*, and thirteen to *E. regnans* (Tables 1 and 2). There were many locations

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where one of the species was not found to share any haplotypes with the other species (Figs 2 and 4). For example, at Ben Nevis (Site 20) in north-east Tasmania, all *E. delegatensis* and *E. obliqua* individuals were fixed for H24 whilst two different haplotypes were found in *E. regnans* (H20, H21), but not H24. Similarly, at Toolangi (Site 3) and Mt Disappointment (Site 4) in the Central Highlands of Victoria, *E. obliqua* contained haplotypes H40 and H1 respectively whilst *E. regnans* was fixed for H4.

We found no relationship between the number of haplotypes, number of unique haplotypes or introgression ratio and either population distance from the coast or elevation.

Discussion

Congruence of phylogeographic patterns

The cpSSR data suggest that, despite differing ecological tolerances, *E. delegatensis*, *E. obliqua* and *E. regnans* have responded to past climatic changes in a similar way. The species share phylogeographic patterns, suggesting a common history of large-scale colonisation or severe bottleneck events (i.e. Central Highlands of Victoria), and persistence in many localised refugia during glacial maxima. This study contributes to a growing understanding of the resilience of temperate tree species to climatic change in south-eastern Australia (e.g. Worth et al. 2009; Nevill et al. 2010; Worth et al. 2010) and supports the hypothesis of Macphail and Colhoun (1985) that mesic forest species in southeastern Australia persisted in multiple refugia and migrated short distances following the LGM; this in contrast to the large-scale, long distance colonisation events, from a small number of refugia that characterise many (but not all) boreal and northern hemisphere temperate zone tree species (Jackson and Overpeck 2000).

Localities were well differentiated from one another based on G_{ST} and R_{ST} values, however, R_{ST} was not significantly greater than G_{ST} indicating that there was little statistical support for phylogeographic structure. This suggests that across the study area 'related' (via stepwise mutation) haplotypes were not more likely than 'unrelated' haplotypes to be found together at a locality. This is surprising as there appears to be quite a clear phylogeographic pattern and could possibly be the result of phylogenetically distinct variants sharing the same size cpSSR variant (homoplasy) in the data.

In general, 'diversity hotspots' at both a population (e.g., Mirboo East (Site 13) and Quarry Creek (Site 9)), and regional scale (e.g., South Gippsland and East Gippsland) were common to the three species (Fig. 2). Finding this pattern consistently in the three species suggests that these 'hotspots' are possibly: 1) at or close to sites of long term persistence (refugia) for one or more species, which has allowed maintenance, over time, of genetic diversity; 2) suture zones (Remington 1968; Hewitt 1996) where distinct maternal lineages have converged (e.g. *Quercus robur* southern Finland Ferris et al. 1998); or 3) a combination of these factors. That diversity hotspots were associated with regions identified *a priori* as glacial refugia suggests that this at least partly accounts for observed diversity.

In line with northern hemisphere studies (e.g. Heuertz et al. 2006; Maliouchenko et al. 2007) it might have been expected that species with different ecological requirements could show evidence of distinctive phylogeographic histories. In particular, *E. delegatensis* (most cold tolerant) and *E. obliqua* (most drought tolerant and by far the most widespread of the three) were expected to have maintained larger effective population sizes in colder and drier climates and thus exhibit higher levels of polymorphism and lower fixation rates during the LGM than *E. regnans* (with

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very narrow tolerances and currently restricted to sites with high rainfall, deep soils and infrequent frosts). This was not found to be the case, as median within population allelic richness was not significantly different amongst the three groups (between species overall, regions overall and species within regions)(Table 4). It is possible that the climatic niche of *E. regnans* was broader during previous glacial maxima and the species was able to maintain similar effective population sizes to the other study species. In particular, genetic diversity may have been supplemented through gene flow from other species (e.g. possibly *E. delegatensis* and *E. obliqua* and others not included in this study) with greater tolerances to cold and drought. Hybridisation is generally seen as a negative for the conservation of biodiversity (Muhlfeld et al. 2009), particularly if one of the species involved has been moved deliberately or accidentally via anthropogenic actions or changes to biotic and/or abiotic conditions have allowed a species to colonise a new region (Allendorf and Luikart 2007). However, hybridisation can facilitate adaptation and increase evolutionary potential by introducing genetic variation (Hoffmann and Sgrò 2011). The natural ability of eucalypt species to hybridise with others with quite different or broader ecological tolerances may provide an "insurance policy" for response to rapidly changing abiotic conditions where a species does not have the necessary range of alleles for it to rapidly adapt, but may acquire beneficial alleles by introgression with other species.

Haplotype sharing

Interspecific sharing of cpDNA haplotypes was extensive and geographical location was more important than species classification in determining an individual's cpDNA haplotype. This finding is consistent with studies of eucalypts (Steane et al. 1998; Jackson et al. 1999; McKinnon et al. 1999, 2001, Pollock et al. 2013), other southern hemisphere plants (e.g. Acosta and Premoli 2010) as well as European and North

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American studies of *Quercus* (e.g. Petit et al. 1997), *Fraxinus* (e.g. Heuertz et al. 2006) and *Betula* spp. (e.g. Palme, 2004). The main causes of interspecific sharing of cpDNA haplotypes are convergent evolution, retention of ancestral polymorphism (incomplete lineage sorting) and hybridisation and introgression (Palme et al. 2004). Convergent evolution is unlikely in this case as mutation events are rare and 35% of haplotypes detected were shared between at least two species. Ancestral polymorphism is possible but unlikely given the degree of divergence between haplotypes and the strong geographic structuring of haplotype sharing (Meudt and Bayly 2008; McKinnon et al. 2001). Accordingly, hybridisation is the most parsimonious explanation for chloroplast sharing among the three study species, and geographic coincidence of shared haplotypes in multiple species in multiple locations has been found in many studies of euclaypts (McKinnon et al. 2004a; McKinnon et al. 2001; Jackson et al. 1999; Steane et al. 1998; McKinnon et al. 2004b; Pollock et al. 2012). Sharing of haplotypes is so pervasive (at 20 of 27 sites) that introgression has presumably occurred on a range of time scales (sharing of both internal and tip haplotypes indicates both historical and recent introgression events) and in a range of situations, e.g. both at sites considered glacial refugia (i.e. Otway Ranges and the south Central Highlands on the mainland and on the island of Tasmania, the west, south-east, east and north-east) and those colonised since the LGM (i.e. central highlands of the mainland and central Tasmania).

Of particular note is that our study shows complete or near complete fixation of regional haplotypes, across the three species, in areas believed to have been treeless during the LGM, i.e. in the central highlands of Victoria (sites 5–7) and central Tasmania (site 24–25; Fig. 4). In contrast, in adjacent, putatively refugial areas there are many haplotypes, with species sometimes sharing haplotypes (e.g. Murdunna, Site 27) and at other times not (e.g. Kallista (Site 2) and Powelltown (Site 1)) (Figs 2 and

4). Such a pattern of haplotype sharing could be explained by: (1) the presence of many haplotypes in past refugia, some shared between species and some not, but due to chance events only one haplotype which was shared between all species was able to establish in the colonised region; (2) the selective advantage, under current or past conditions, of a specific haplotype in certain regions; or (3) introgression between species that is an integral part of the colonisation process. The first of these hypotheses is not well supported. Chance success and persistence of a single haplotype across all three species in recolonised areas also seems unlikely when we have seen that often haplotypes are not shared between species in refugia and it appears to have happened in the two separate recolonised regions identified in this study (H4 in Victoria and H20 in Tasmania). Secondly, a selective advantage of one haplotype across a broad region seems unlikely given that the species occupy a range of environments at a local scale (e.g. *E. obliqua* on lower, drier sites; *E. delegatensis* on colder, higher elevation sites). However it is possible that the shared haplotypes might historically have conferred a selective advantage, in what were broadly drier and colder conditions during the LGM. Finally, it has been previously proposed that introgression between eucalypt species occurred either in glacial refugia, where previously separated species were reduced to small population sizes and pushed into sympatry, or during the colonisation of the landscape (McKinnon et al. 2004a) but the relative importance of these two scenarios is unclear. However, there is increasing evidence to suggest that in many genera, hybridisation and introgression are frequently associated with species range expansions (e.g. Potts and Reid 1988; Petit et al. 2004; Currat et al. 2008) with maternally inherited genetic markers tending to be more commonly transferred to invading species than are bi-parentally inherited markers (Arnold 1997; Wu and Campbell 2005). A limitation of this study is that only chloroplast markers have been used. As the chloroplast

does not recombine or does so only rarely, these markers are inherited as one unit, essentially a single locus. Further studies using neutral nuclear markers might help unravel the effective population sizes of each of the species in refugia and colonised areas and enable us to examine in greater detail the nature of introgression between the three species.

Introgression ratios

Introgression ratios were similar but generally higher than those found between *E. serraensis*, *E. verrucata*, *E. victoriana* and *E. baxteri*, which were also studied with cpSSRs (Pollock et al. 2013). Introgression ratios (IG) differed between geographic regions (Tasmania and Mainland) and species pairs and there were no consistent patterns. For example, the *E. obliqua*/*E. regnans* species pair had the highest IG in Tasmania and the lowest on the mainland (Table 5). The relatively low IG between *E. obliqua* and *E. regnans* on the mainland was surprising given hybridisation between *E. obliqua* and *E. regnans* is common, with morphologically intermediate individuals found at most locations where they grow in adjacent stands (Ashton 1958; Ashton1981).

Conservation implications

In south-eastern Australia, areas of tall eucalypt forest clear-felled for timber harvesting are regenerated using the direct sowing of seed, and to a far lesser extent, the seed tree system (Flint and Fagg 2007). Current regulatory requirements specify the maintenance of the species composition and local genotypes of those species that existed prior to harvesting operations (Commonwealth of Australia 1992). Seed production in eucalypts is strongly influenced by rainfall, and drier than average conditions in many regions over the last three decades have resulted in a

reduction in mass flowering events and seed crops, hence there is a temptation for forest managers to use seed collected from other locations. If climate change predictions are correct, sourcing of seed on site is likely to be challenging in an increasingly dry environment, not only because of poor seed production but also because changes in fire regimes associated with climate change may cause landscape-wide loss of mature obligate seeder forests (Bowman et al. 2014).

Our results are compatible with current strategies for reforestation that suggest: (1) seed should be obtained from the immediate area or currently defined seed zones; and (2) that the genetic distinctiveness of outlier populations be a conservation priority. This would preserve existing patterns of inter-specific chloroplast types and diversity, which are particularly apparent in some regions. Similar recommendations were made from a more extensive study of genetic variation in *E. obliqua* in Tasmania where a similar geographic pattern on chloroplast variation but only weak nuclear microsatellite structure were found. Bloomfield et al. (2011) suggested that seed transfer zones defined by Forestry Tasmania were suitable for maintaining historical patterns of genetic diversity in *E. obliqua* and that when transfer between seed zones was required, priority could be given to transfers within the geographic zones defined by the cpDNA.

Further support for the use of local genotypes is provided by the finding of high but variable levels of chloroplast sharing between species in our study (also variable in terms of species involved). The high level of chloroplast sharing between species within a locality and its obvious importance in the evolution of eucalypts supports the current conservative management objectives such as preservation of the original species composition (all eucalypt species present at a site prior to harvesting) that potentially maintain the existing evolutionary processes that maintain the collective adaptive potential of eucalypt species at a site.

Acknowledgements

Data Archiving Statement

Chloroplast microsatellite data has been submitted to the TreeGenes Database. Accession numbers are: *E. delegatensis*, TGDR 024; *E. obliqua*, TGDR 026; *E. regnans*, TGDR 027. Each accession number is presented with its allelic composition for the five loci used in our study.

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Table 1 Geographic location, population name and species sampled (*Eucalyptus delegatensis*, *Eucalyptus obliqua* and *Eucalyptus regnans*) for 27 sites used in this study. n is the sample size. n/hap is the number of different haplotypes found in individuals sampled at 1) that location and 2) that region. n/unq is the number of haplotypes found only in 1) that population and 2) that region. S, haplotypes shared between species at a location.

	Population	Latitude S	Longitude E	*E. delegatensis* n	n/hap	n/unq	*E. obliqua* n	n/hap	n/unq	*E. regnans* n	n/hap	n/unq	S
1	Powelltown	37.78	145.82	-	-	-	10	2	1	10	2	1	0 of 4
2	Kallista	37.92	145.38	-	-	-	10	1	0	10	2	0	0 of 3
3	Toolangi	37.52	145.52	-	-	-	10	1	1	10	1	0	0 of 2
4	Mt Disappointment	37.42	145.20	-	-	-	10	1	0	10	1	0	0 of 2
5	Rubicon	37.33	145.93	10	1	0	10	1	0	10	1	0	1 of 1
6	Mt Useful	37.68	146.53	10	1	0	10	1	0	10	1	0	1 of 1
7	Mt Erica	37.92	146.37	10	1	0	10	1	0	10	1	0	1 of 1
8	Dargo	37.46	147.25	10	2	1	10	2	1	10	2	1	1 of 4
9	Quarry Creek	37.43	147.65	10	2	1	10	1	0	10	4	3	1 of 5
10	Buchan	37.23	148.05	10	2	2	10	3	1	10	2	0	2 of 3
11	Yalmy River	37.35	148.45	-	-	-	10	3	0	10	2	0	2 of 3
12	Wilsons Promontory	39.10	146.37	-	-	-	10	1	1	10	1	1	0 of 2
13	Mirboo East	38.52	146.38	-	-	-	10	4	1	10	4	1	3 of 5
14	Traralgon Creek	38.43	146.52	-	-	-	5	1	0	10	1	1	0 of 2
15	Carisbrook	38.60	143.75	-	-	-	10	1	0	10	2	0	1 of 2
16	Upper Ford R	38.65	143.48	-	-	-	10	1	0	10	2	0	1 of 2
	Mainland			60	6	4	155	15	6	160	17	8	
17	Smithton	41.02	145.37	-	-	-	10	2	0	10	1	0	1 of 2
18	Beaconsfield	41.30	146.75	-	-	-	10	2	0	10	1	0	1 of 2
19	Lisle	41.25	147.37	-	-	-	10	1	0	10	2	1	0 of 3
20	Ben Nevis	41.42	147.55	10	1	0	10	1	0	10	3	1	1 of 4
21	Goulds Country	41.22	148.15	-	-	-	10	2	0	10	2	0	1 of 3
22	Levendale	42.52	147.53	10	2	1	10	1	0	10	3	1	1 of 4
23	Swan Port	42.40	147.85	10	1	0	10	1	0	10	1	0	1 of 2
24	Florentine Valley	42.48	146.45	10	1	0	10	1	0	10	1	0	1 of 1
25	Styx	42.82	146.60	10	1	0	10	1	0	10	2	0	1 of 2
26	Strathblane	43.37	146.97	-	-	-	10	2	1	10	2	1	1 of 3
27	Murdunna	42.97	147.95	10	1	0	10	2	0	10	2	0	2 of 3
	Tasmania			60	4	1	100	7	1	110	10	4	
	All samples			12	10	5	265	22	17	270	27	12	

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Table 2 Characteristics of 40 haplotypes detected with five chloroplast microsatellite markers in *Eucalyptus delegatensis* (del), *Eucalyptus obliqua* (obl) and *Eucalyptus regnans* (reg) and species which harboured each haplotype. Allele sizes are in base pairs (bp)

Haplotypes	Species	Allele size (bp) per primer				
		EMCRC60cp	EMCRC67cp	EMCRC74cp	EMCRC86cp	EMCRC90cp
H1	obl, reg	194	233	127	147	229
H2	obl, reg	195	235	135	145	230
H3	obl, reg	195	235	135	145	229
H4	del, obl, reg	194	235	135	145	230
H5	reg	194	234	134	144	229
H6	reg	198	236	135	145	230
H7	reg	196	235	135	145	230
H8	reg	196	236	135	145	230
H9	reg	190	235	135	145	230
H10	dele, obl, reg	197	235	135	145	229
H11	reg	194	235	135	144	219
H12	reg	194	235	137	144	231
H13	reg	196	241	135	144	231
H14	obl, reg	194	235	135	145	229
H15	obl, reg	196	241	135	145	230
H16	obl, reg	196	235	135	143	229
H17	obl, reg	196	235	135	145	229
H19	obl, reg	195	234	127	147	231
H20	dele, obl, reg	194	234	127	147	231
H21	reg	194	234	127	148	231
H22	reg	195	232	127	147	231
H23	reg	195	231	127	147	231
H24	dele, obl, reg	195	234	127	148	231
H25	reg	195	235	127	148	231
H26	obl, reg	193	234	127	147	231
H28	reg	194	235	127	147	231
H30	obl, reg	196	234	127	148	231
H32	obl	195	236	135	145	230
H33	obl	194	235	137	146	230
H34	del	195	235	137	146	230
H35	obl	197	236	135	145	230
H36	obl	196	235	138	148	230
H37	del	195	235	135	145	219
H38	del	197	235	138	147	230
H39	del	197	235	135	146	230
H40	obl	194	235	141	145	230
H41	del	195	234	127	148	230
H42	obl	194	233	127	147	231
H43	del	195	234	127	149	231
H44	obl	196	234	135	145	230

Table 3 Chloroplast microsatellite marker haplotype diversity (within population diversity h_s, v_s and total diversity h_T, v_T) and differentiation (G_{ST}, R_{ST}) statistics in three species of *Eucalyptus* and by regions. M, mainland populations; T, Tasmanian populations; O, overall. n, number of individuals; n/hap is the total number of different haplotypes found in each species; n/hap sh is the number of haplotypes shared between species. Standard deviations over populations are given in parentheses. NC, not calculable.

Species	n	n/hap	n/hap sh	h_S	h_T	G_{ST}	v_S	v_T	R_{ST}
E. delegatensis									
sympatric with *E. obliqua* (M)	60	6	2	0.18 (0.09)	0.64 (0.12)	0.72 (0.06)	0.27 (0.25)	0.63 (0.43)	0.58 (NC)
sympatric with *E. regnans* (M)	60	6	2	0.18 (0.09)	0.64 (0.12)	0.72 (0.06)	0.27 (0.25)	0.63 (0.43)	0.58 (NC)
sympatric with *E. obliqua* (T)	60	4	2	0.06 (0.06)	0.81 (0.03)	0.93 (0.07)	0.02 (0.02)	0.82 (0.18)	0.97 (0.03)
sympatric with *E. regnans* (T)	60	4	2	0.06 (0.06)	0.81 (0.03)	0.93 (0.07)	0.02 (0.02)	0.82 (0.18)	0.97 (0.03)
sympatric with *E. obliqua* (O)	120	10	4	0.12 (0.06)	0.88 (0.05)	0.86 (0.06)	0.09 (0.09)	0.88 (0.20)	0.89 (0.07)
sympatric with *E. regnans* (O)	120	10	4	0.12 (0.06)	0.88 (0.05)	0.86 (0.06)	0.09 (0.09)	0.88 (0.20)	0.89 (0.07)
E. obliqua									
sympatric with *E. delegatensis* (M)	60	7	2	0.19 (0.12)	0.68 (0.19)	0.72 (0.14)	0.22 (0.16)	0.68 (0.30)	0.68 (0.18)
sympatric with *E. regnans* (M)	155	15	8	0.16 (0.07)	0.89 (0.19)	0.82 (0.07)	0.29 (0.13)	0.89 (0.27)	0.67 (0.16)
sympatric with *E. delegatensis* (T)	60	5	2	0.20 (0.13)	0.80 (0.10)	0.75 (0.14)	0.38 (0.26)	0.77 (0.33)	0.52 (0.12)
sympatric with *E. regnans* (T)	100	7	6	0.22 (0.08)	0.74 (0.07)	0.70 (0.09)	0.38 (0.23)	0.72 (0.36)	0.48 (0.10)
sympatric with *E. delegatensis* (O)	120	12	4	0.19 (0.08)	0.88 (0.05)	0.88 (0.05)	0.25 (0.13)	0.88 (0.18)	0.71 (0.14)
sympatric with *E. regnans* (O)	265	22	14	0.19 (0.05)	0.92 (0.02)	0.80 (0.05)	0.17 (0.07)	0.92 (0.13)	0.82 (0.08)
E. regnans									
sympatric with *E. delegatensis* (M)	60	10	2	0.27 (0.13)	0.68 (0.21)	0.60 (0.07)	0.43 (0.39)	0.65 (0.45)	0.33 (NC)
sympatric with *E. obliqua* (M)	160	17	8	0.20 (0.06)	0.82 (0.08)	0.76 (0.07)	0.27 (0.12)	0.82 (0.33)	0.67 (0.13)
sympatric with *E. delegatensis* (T)	60	6	2	0.22 (0.09)	0.77 (0.11)	0.72 (0.08)	0.43 (0.32)	0.73 (0.26)	0.41 (0.26)
sympatric with *E. obliqua* (T)	110	10	6	0.23 (0.06)	0.76 (0.10)	0.70 (0.06)	0.47 (0.21)	0.74 (0.21)	0.36 (0.13)
sympatric with *E. delegatensis* (O)	120	16	4	0.24 (0.07)	0.87 (0.06)	0.72 (0.07)	0.13 (0.10)	0.88 (0.16)	0.85 (0.08)
sympatric with *E. obliqua* (O)	270	27	14	0.21 (0.05)	0.90 (0.03)	0.77 (0.05)	0.09 (0.04)	0.91 (0.06)	0.90 (0.04)

Table 4 Within population chloroplast haplotype richness in three species of *Eucalyptus* and by regions, using rarefaction estimates at a common sample size of 5.

Species	Region	Mean haplotype diversity
E. delegatensis	Mainland	1.5
	Tasmania	1.1
	Overall	1.3
E. obliqua	Mainland	1.4
	Tasmania	1.4
	Overall	1.4
E. regnans	Mainland	1.6
	Tasmania	1.5
	Overall	1.5

Table 5 Introgression ratio estimates for each region and for both regions combined with bootstrap 95% percentile confidence intervals based on 10000 bootstrap samples for three species, *Eucalyptus delegatensis* (del), *Eucalyptus obliqua* (obl) and *Eucalyptus regnans*(reg)

Species	Both regions		Tasmania		Mainland	
	IG	CI	IG	CI	IG	CI
del,obl	0.72	(0.66, 0.75)	0.68	(0.64, 0.70)	0.76	(0.65, 0.82)
del,reg	0.64	(0.57, 0.66)	0.50	(0.44, 0.53)	0.80	(0.62, 0.83)
obl,reg	0.54	(0.49, 0.57)	0.70	(0.61, 0.74)	0.43	(0.37, 0.46)

Figure captions

Fig. 1 Location of 10 geographic regions referred to in this study (Otway Ranges, Central Highlands, south Central Highlands, South Gippsland, East Gippsland, north Tasmania, north-east Tasmania, east Tasmania, south-east Tasmania, and central Tasmania). (R) following the name of a region indicates hypothesized refugia, (C) indicates hypothesized re/colonised region and (?) indicates unknown
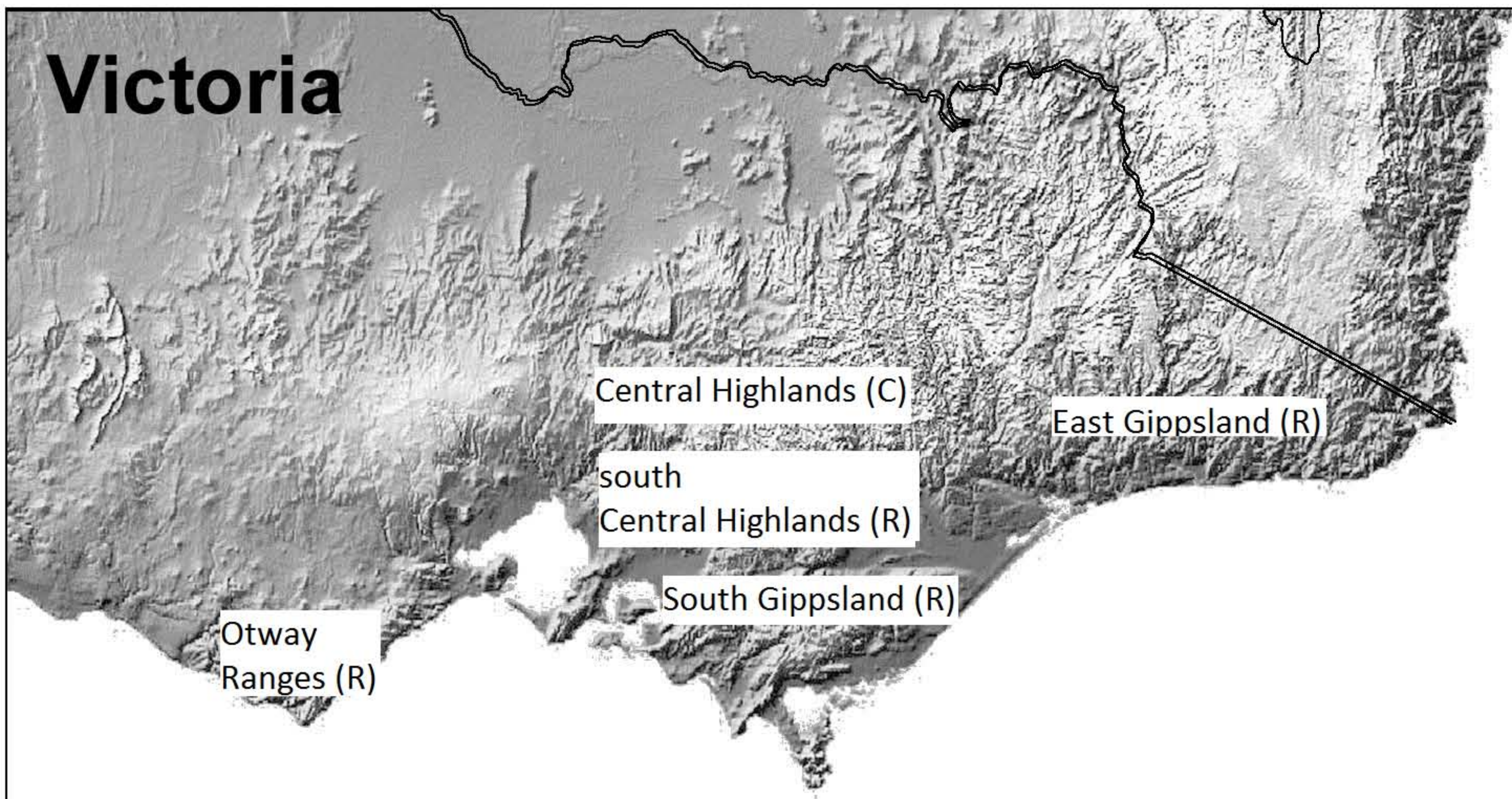
Fig. 2 Geographic distribution and chloroplast haplotype frequency in (a) *Eucalyptus delegatensis*, (b) *Eucalyptus obliqua* and (c) *Eucalyptus regnans*. Population numbers are the same as those used in Table 1. The size of sections of the pie charts corresponds to the number of individuals with that haplotype

Fig. 3 Statistical parsimony network of chloroplast haplotypes found in *Eucalyptus delegatensis*, *Eucalyptus obliqua* and *Eucalyptus regnans* showing the number of nucleotide differences between haplotypes summed over the five chloroplast microsatellite loci. Circle size indicates relative haplotype frequency; haplotypes found in fewer than 10 individuals are represented with a symbol of the same size. Branch length indicates distance between haplotypes. Circle colour indicates species in which haplotypes were found. Latent nodes (unobserved haplotypes), indicated by small, unlabelled red circles, are included to complete the network
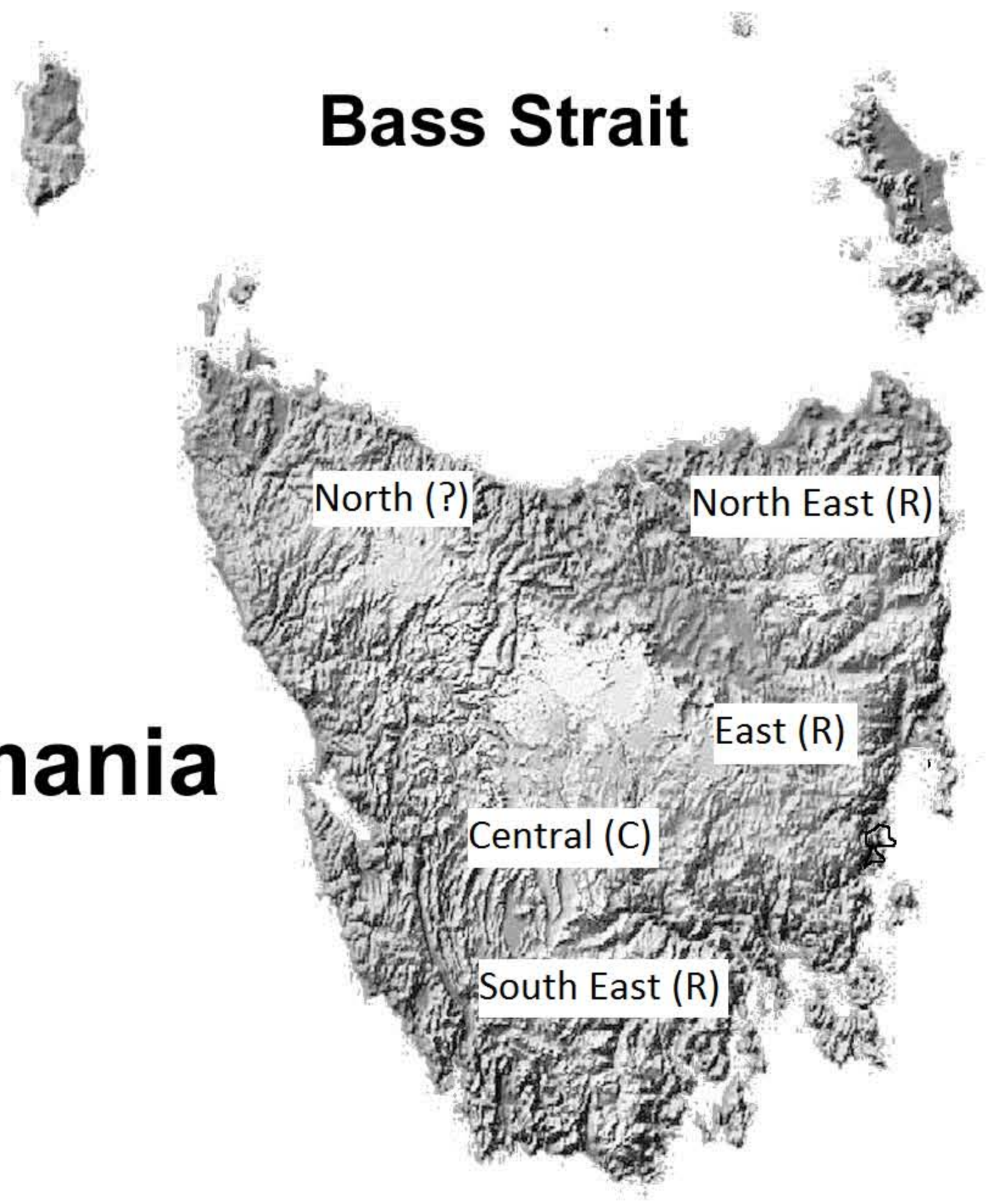
Fig. 4 The geographic pattern of haplotype sharing between *Eucalyptus delegatensis*, *Eucalyptus obliqua* and *Eucalyptus regnans*. A red circle indicates sharing of some but not all haplotypes found at that site; a dark blue circle indicates sharing of no haplotypes

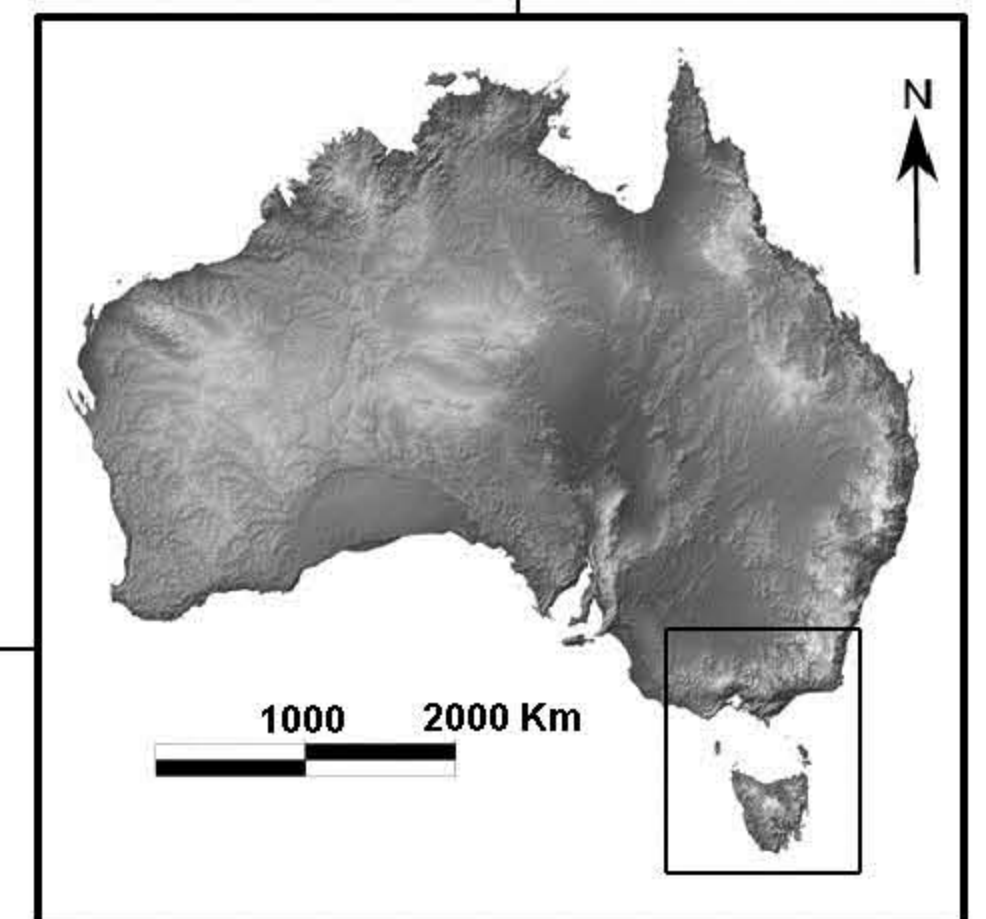at that site; a light blue circle indicates sharing of all haplotypes at that site
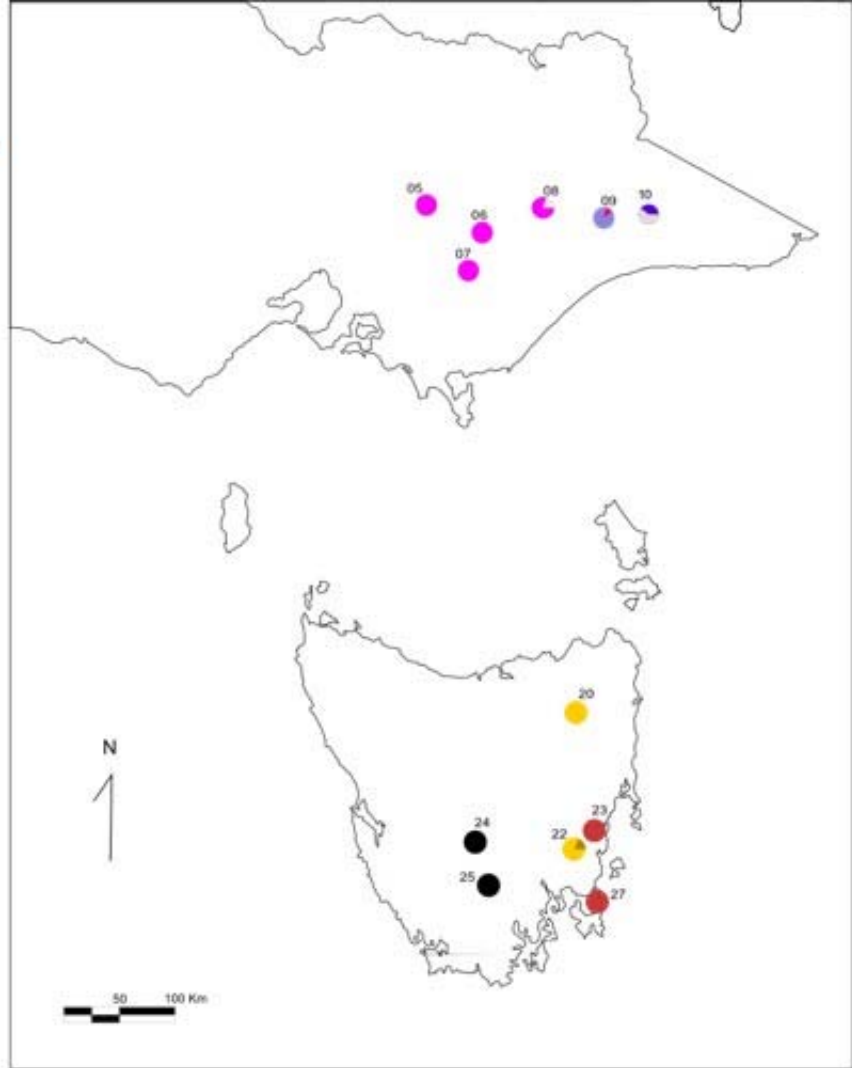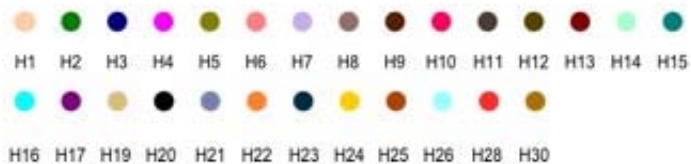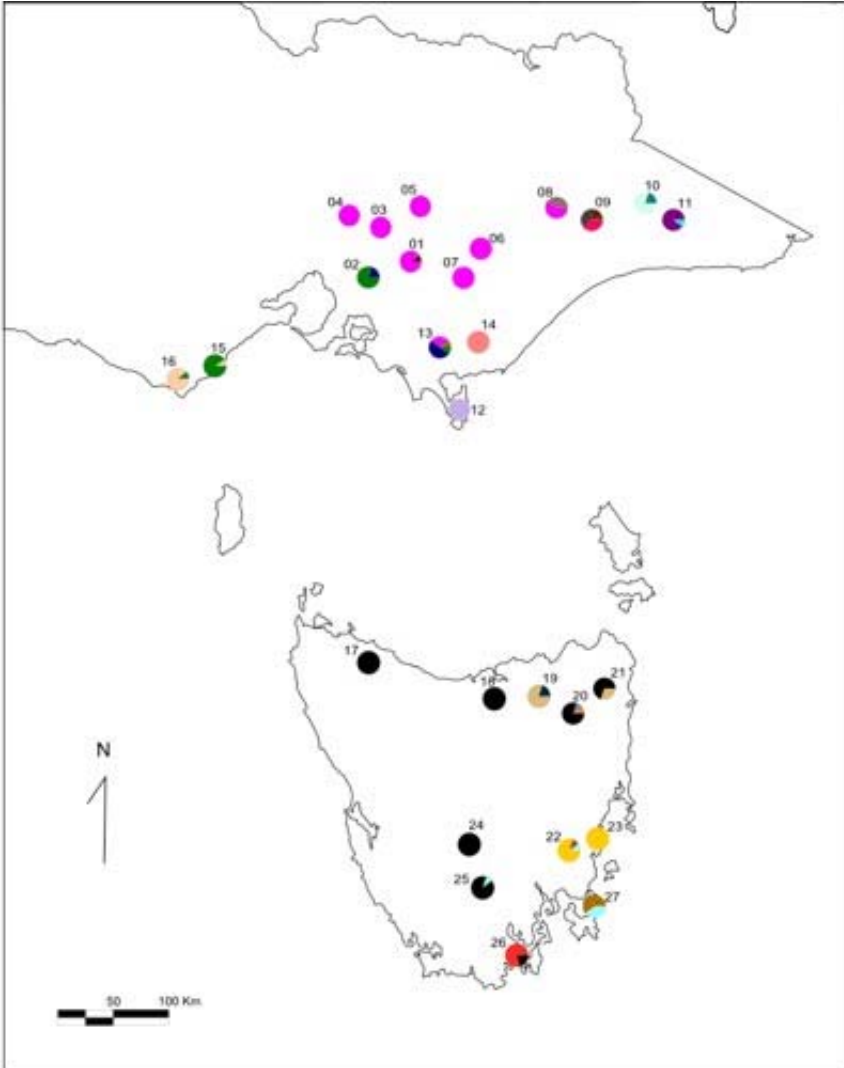
Victoria

Central Highlands (C)

East Gippsland (R)

south
Central Highlands (R)

South Gippsland (R)

Otway
Ranges (R)

Bass Strait

North (?)

North East (R)

East (R)

Tasmania

Central (C)

South East (R)

N

1000   2000 Km

A

●	●	●	●	●	●	●	●	●	●
H4	H10	H20	H24	H34	H37	H38	H39	H41	H43

H1	H2	H3	H4	H10	H14	H15	H16	H17	H19	H20	H24	H26	H30	H32

H33	H35	H36	H40	H42	H44

| H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H11 | H12 | H13 | H14 | H15 |

| H16 | H17 | H19 | H20 | H21 | H22 | H23 | H24 | H25 | H26 | H28 | H30 |

Legend:
- E. delegatensis (blue)
- E. obliqua (grey)
- E. regnans (green)
- E. obliqua and E. regnans (red)
- All three species (black)

Clade 2

Haplotypes: H39, H6, H44, H35, H7, H2, H10, H13, H15, H8, H32, H3, H17, H16, H4, H14, H11, H5, H9, H20, H28, H26, H12, H40, H33, H34, H38, H37, H36, H22, H42, H23, H1, H21, H19, H30, H24, H43, H41, H25

Clade 1

4 nucleotides
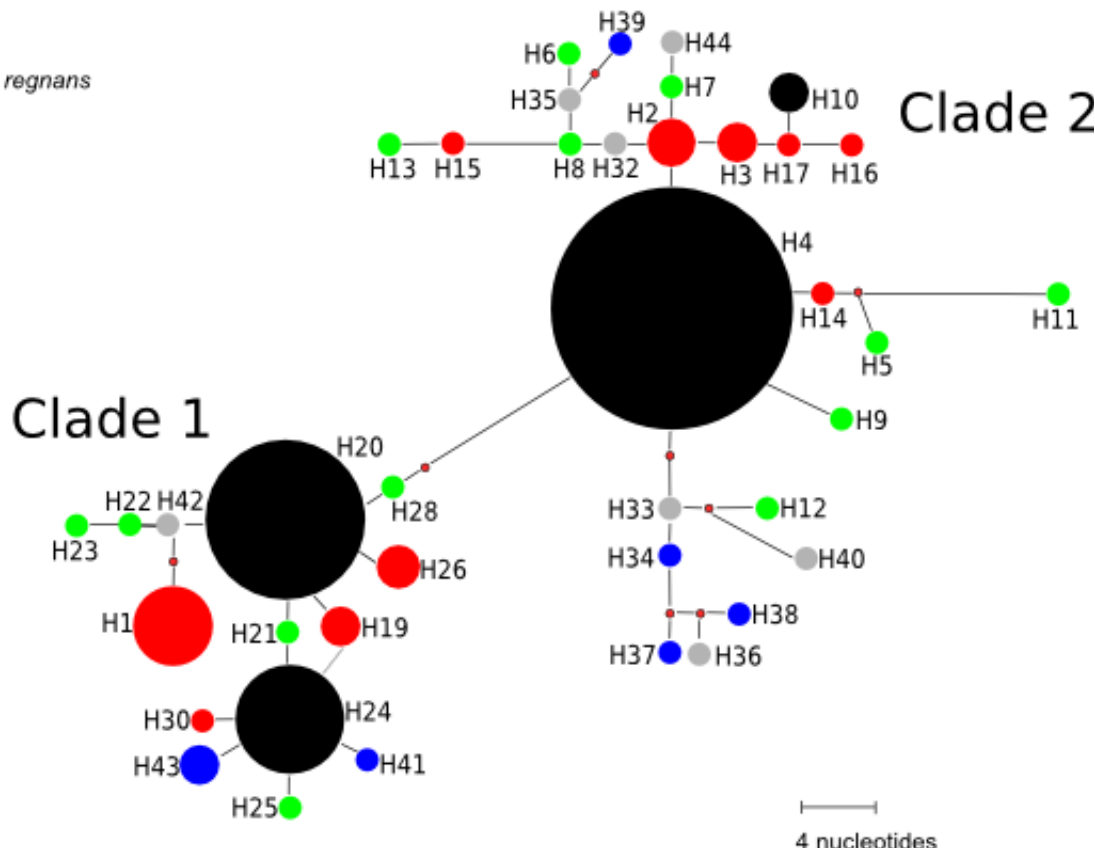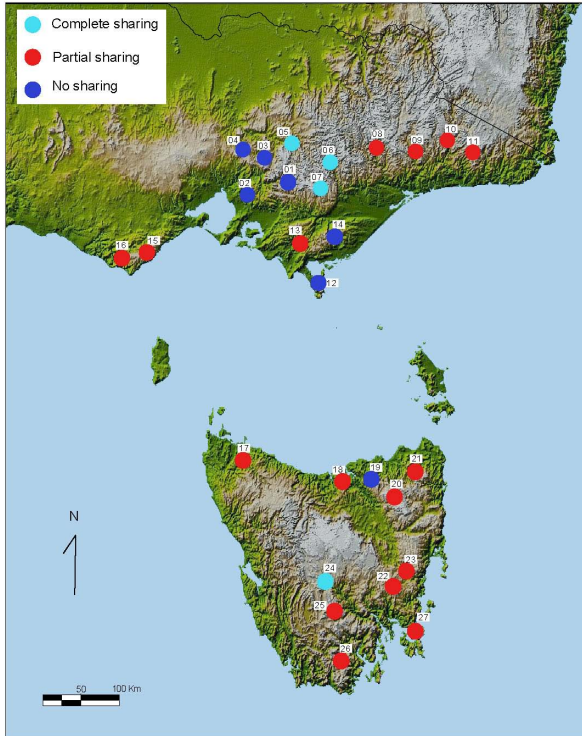
Author/s:
Nevill, PG; Despres, T; Bayly, MJ; Bossinger, G; Ades, PK

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