ORIGINAL ARTICLE

Chloroplast DNA diversity associated with protected slopes and valleys for hybridizing *Eucalyptus* species on isolated ranges in south-eastern Australia

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ABSTRACT

Aim To relate genetic diversity to topographic features and to investigate genetic interactions between *Eucalyptus* species in a local centre of endemism and diversity in south-eastern Australia.

Location Grampian Ranges, Victoria, Australia.

Methods We documented chloroplast DNA (cpDNA) variation for a group of endemic *Eucalyptus* species (*E. serraensis, E. verrucata* and *E. victoriana*) that dominate rocky, high-elevation ridgelines of the Grampian Ranges and for one closely-related, widespread species (*E. baxteri*) occupying flanking slopes and valleys. We documented genetic patterns across the landscape using cpDNA microsatellites, and related them to topographic features (exposed west-facing versus protected east-facing slopes and valleys). We also determined the extent of local haplotype sharing between populations of endemic species and neighbouring *E. baxteri* downslope with cpDNA microsatellites, and haplotype sharing between the endemic group and more distantly related species (*E. obliqua*, *E. pauciflora* and *E. willisii*) with sequences of the J_{LA}+ chloroplast region.

Results We detected 26 cpDNA microsatellite haplotypes in a relatively small area of *c.* 20 km × 50 km. Populations of *E. baxteri* on east-facing slopes and valleys had greater cpDNA microsatellite diversity than *E. baxteri* and endemic species on exposed west-facing slopes. Endemic species frequently shared chloroplast haplotypes with *E. baxteri* downslope. Sharing of J_{LA}+ haplotypes with species outside the endemic group was mostly restricted to *E. victoriana*, which had cpDNA more similar to the species from other sections of *Eucalyptus* (*E. obliqua*, *E. willisii* and *E. pauciflora*).

Main conclusions Intensive sampling of related species on small isolated mountain ranges allowed us to relate genetic diversity to fine-scale habitats and to document extensive local haplotype sharing between species. This study contributes to a general understanding of the environmental conditions that enable plant population persistence by linking concentrations of genetic diversity to particular habitats.

Keywords
Australian biogeography, chloroplast microsatellites, endemism, *Eucalyptus*, Grampians National Park, introgression, landscape genetics, phylogeography, south-eastern Australia.

INTRODUCTION

Areas where species have persisted for extended periods are important repositories of genetic diversity. Genetic signatures of persistence and recolonization associated with Pleistocene glaciations in the Northern Hemisphere have been identified based on a large body of literature documenting patterns of intraspecific genetic diversity (Beheregaray, 2008). Similar

patterns of genetic diversity have been found in Australia (e.g. Hugall *et al.*, 2002; McKinnon *et al.*, 2004; Byrne, 2007; Nevill *et al.*, 2010), yet postulated regions of long-term species persistence tend to be more geographically restricted and dispersed across the continent than in Northern Hemisphere systems (Byrne, 2008). In regions that were not glaciated in the Pleistocene, but where the distribution of vegetation in glacial periods was affected by colder and drier conditions, complex topography coincides with endemism, genetic diversity, and postulated refugia (Médail & Diadema, 2009). Mountainous regions have unique and stable microclimates relative to the regional climate, and certain microclimates, providing suitable habitat for many organisms during climate fluctuations (Sublette Mosblech *et al.*, 2011; Keppel *et al.*, 2012), for example providing mesic microclimates in the context of a drying regional climate (Byrne, 2008). Matching phylogeographic evidence with sites that have relatively stable climates may help to identify locations where species may persist during future climate change (Dobrowski, 2010). Identifying sites conducive to species persistence should be a conservation priority, both in Australia (Steffen *et al.*, 2009) and globally (Keppel *et al.*, 2012).

In this study, we document patterns of genetic diversity and relationships for *Eucalyptus* species that dominate mid-to-high elevations in the Grampians, an isolated group of mountain ranges in south-eastern Australia. The Grampians region is a local centre of endemism (Crisp *et al.*, 2001) and a putative refugium during Miocene marine incursions and Pleistocene climate shifts (Marginson & Ladiges, 1988). The Grampians are likely to have provided an important gene pool for recolonization of the interior arid region to the north-west of the study area, which was particularly unsuitable for woody plant growth during arid periods (Byrne, 2008). For example, Marginson & Ladiges (1988) suggested that *E. arenacea* colonized Pleistocene-deposited sand sheets in the north-west arid region of Victoria from a source in the Grampians. Patterns of cpDNA variation indicate that the Grampians probably provided habitat for the mesic forest species *Tasmannia lanceolata* during the height of Pleistocene aridity (Worth *et al.*, 2010). Here, we further investigate patterns of genetic diversity within the Grampians at a fine scale to ask whether genetic diversity is distributed evenly across the Grampians landscape or is concentrated in particular habitats. We explicitly consider the effects of landscape position, particularly the effects of west-facing versus east-facing slopes and valleys.

The stringybark eucalypts (*Eucalyptus* subg. *Eucalyptus* ser. *Pachyphloius* Blakely *sensu* Brooker, 2000) include a closely related group of three narrowly endemic species, *E. serraensis* Ladiges & Whiffin, *E. verrucata* Ladiges & Whiffin, and *E. victoriana* Ladiges & Whiffin, which grow only on rocky sandstone outcrops in the Grampian Ranges. These endemic species are related to a more widespread species, *E. baxteri* (Benth.) Maiden & Blakely ex J.M. Black, which occurs on mid-elevation slopes and valleys with sandy soils in the Grampians. Compared with *E. baxteri*, the endemic species have thicker, tougher leaves and larger and generally more robust fruits. These traits are likely to represent adaptations to the harsh conditions on exposed rocky outcrops (Pollock *et al.*, 2011). The endemic species are distributed allopatrically, with *E. serraensis* in the north, *E. victoriana* in the west and *E. verrucata* in the south (Fig. 1), but all three are parapatric with populations of *E. baxteri*. This group has long been of interest because of its variable and extreme morphology. Previous research on morphology (Marginson, 1984; Marginson & Ladiges, 1988; Whiffin & Ladiges, 1992), volatile leaf oils (Whiffin & Ladiges, 1992) and a progeny trial (Marginson, 1984) has confirmed the wide range of morphologies present in the endemic species relative to the widespread *E. baxteri*. This study adds chloroplast DNA (cpDNA) data to further understand the gene flow and taxonomic identity of the species.

Previous studies have documented widespread incongruence between maternally inherited chloroplast DNA markers and species-level taxonomy between closely related, co-occurring *Eucalyptus* species (McKinnon *et al.*, 1999, 2001, 2004), and we therefore expect some level of gene sharing between stringybark eucalypts in the Grampians. We are particularly interested in how three closely related species with morphologically variable populations exist in the same small region despite the likelihood that they are reproductively compatible. We quantify the taxonomic and spatial extent of chloroplast gene sharing between: (1) populations of the three endemic high-elevation species (*E. serraensis*, *E. verrucata* and *E. victoriana*) and paired locations of *E. baxteri* downslope on individual peaks; (2) this stringybark group and species from other taxonomic sections within *Eucalyptus* subg. *Eucalyptus* (*E. obliqua* L'Hér., *E. willisii* subsp. *falciformis* Newnham, Ladiges & Whiffin, and *E. pauciflora* subsp. *parvifructa* Rule).

MATERIALS AND METHODS

Study site

The Grampian Ranges (Grampians National Park) mark the south-western extent of the Great Dividing Range in Victoria, Australia (Fig. 1). The climate is relatively mild with hot summers and cool, wet winters averaging 600 mm year^{-1} rainfall. The ranges consist of Devonian sandstone that has been uplifted to a cuesta formation, with steep environmental gradients. Three main ranges run north–south with valleys in between, providing a range of habitats. Species composition changes across environmental gradients over short distances, especially in relation to geology and soil type (Enright *et al.*, 1994).

Sample collection

Existing taxonomic descriptions for the three stringybark species endemic to the Grampians are based on collections from a limited number of localities because the difficult terrain prevents easy access to many populations. We used aerial photography to identify additional populations for explora-

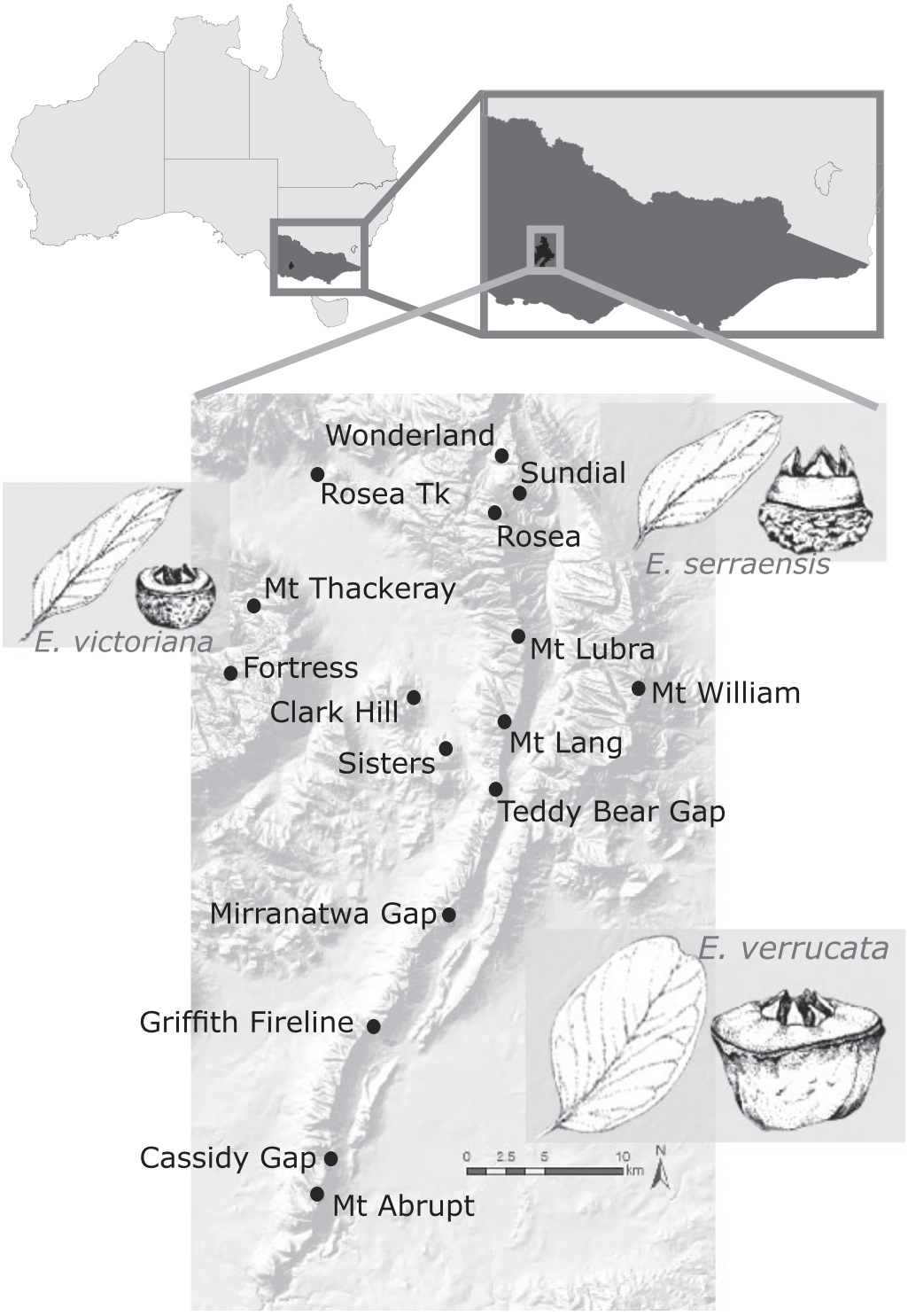

Figure 1 Map of the Grampians National Park (black) located in western Victoria (grey), south-eastern Australia (37°15′ S, 142°28′ E), showing sampling locations (black dots). Fruits and leaf shapes are illustrated for each of the endemic species (drawings modified from Ladiges & Whiffin, 1993). *Eucalyptus serraensis* occupies the northern half of the Serra Range from Teddy Bear Gap to Wonderland; *E. verrucata* extends from Mirranatwa Gap to Mount Abrupt; *E. victoriana* is found on the two highest peaks in the Victoria Range: Mount Thackeray and The Fortress; *E. baxteri* (not shown) is widespread and surrounds populations of endemic species (see Fig. 3).

tion. Populations of *E. serraensis* and *E. verrucata* were distinguished from other eucalypts in aerial photographs by their shorter stature and more horizontal leaf orientation. Populations that were not described in previous studies but were morphologically similar to existing descriptions are indicated in Table 1. Some stands had morphologies intermediate

Table 1 List of species, locations (latitude, longitude and elevation range), number of individuals included in cpDNA microsatellite analysis (*n*), observed cpDNA microsatellite haplotypes, and observed J_{LA}+ sequence haplotypes at each site for *Eucalyptus* species in the Grampians National Park, Victoria, Australia. Haplotypes are followed by the number of individuals in parentheses. Missing data are indicated with dashes. Letters in parentheses in the species column indicate the aspect of *E. baxteri* sites (E = east-facing; W = west-facing). * sites not sampled by Whiffin & Ladiges (1992), Ladiges & Whiffin (1993) and Marginson (1984). See Appendix S3 for a Google Earth file with study site locations.

Location	Species	Coordinates	Elevation (m a.s.l.)	*n*	cpDNA microsat haplotype	J_{LA}+ haplotype
Victoria Range						
Fortress (FT)*	*E. victoriana*	37.309° S, 142.304° E	855–912	6	B (1), C (5)	II (1)
	E. baxteri (E)	37.323° S, 142.315° E	850–900	3	M (2), T(1)	VII (2)
Mount Thackeray (TH)	*E. victoriana*	37.277° S, 142.345° E	955–975	5	A (5)	II (1)
	E. victoriana/ *E. baxteri* (E)	37.293° S, 142.330° E	858–864	2	H (2)	VII (1)
	E. baxteri (E)	37.243° S, 142.346° E	573–578	5	E (3), G (1), M (1)	VII (2)
Northern Serra Range						
Mount Lang (LA)*	*E. serraensis*	37.320° S, 142.501° E	867–903	9	W (9)	VII (1)
	E. baxteri (W)	37.325° S, 142.481° E	287–331	8	V (3), W (5)	VII (1)
Mount Lubra (LU)*	*E. serraensis*	37.258° S, 142.515° E	940–955	8	M (7), N (1)	VII (2)
	E. baxteri (W)	37.245° S, 142.475° E	306–325	5	N (5)	VII (1)
Valley–Lubra Park (VL)*	*E. baxteri* (E)	37.259° S, 142.553° E	420–431	5	M (1), N (2), O (1), U (1)	VII (3)
Mount Rosea	*E. serraensis*	37.197° S, 142.506° E	1000–1006	2	–	VII (2)
Valley–Rosea Track (VR)*	*E. baxteri* (W)	37.192° S, 142.421° E	298–311	5	O (5)	VII (1)
Valley–Clark Hill (VC)*	*E. baxteri* (E)	37.303° S, 142.441° E	258–279	5	B (1), R (2), S (2)	II (1), VII (1)
Valley–Sisters (VS)*	*E. baxteri* (W)	37.332° S, 142.469° E	268–273	6	R (6)	VII (2)
Sundial (SU)	*E. serraensis*	37.164° S, 142.517° E	719–735	4	F (4)	VII (2)
	E. baxteri (W)	37.177° S, 142.479° E	686–695	5	O (5)	VII (1)
Teddy Bear Gap (TB)	*E. serraensis*	37.356° S, 142.497° E	517–540	4	W (4)	VII (1)
	E. baxteri (W)	37.340° S, 142.488° E	324–352	8	W (8)	VII (2)
Wonderland (WD)	*E. serraensis*	37.161° S, 142.515° E	657–685	10	M (9), N (1)	VIII (2)
	E. baxteri (W)	37.152° S, 142.446° E	431–448	4	O (4)	VII (1)
Mount William Range						
Mount William (WL)	*E. serraensis/E. baxteri*	37.295° S, 142.603° E	1108–1148	8	L (5), M (3)	VII (2)
	E. baxteri (W)	37.288° S, 142.592° E	846–964	5	M (2), O (3)	VII (1)
Southern Serra Range						
Cassidy Gap (CG)*	*E. verrucata/E. baxteri*	37.572° S, 142.365° E	500–540	10	K (10)	VII (1)
	E. baxteri (E)	37.571° S, 142.362° E	345–442	5	J (2), X (3)	VII (2)
Griffith Fireline (GF)*	*E. verrucata*	37.491° S, 142.400° E	537–548	10	L (10)	VII (1)
	E. baxteri (E)	37.501° S, 142.412° E	273–283	5	P (2), Q (2), R (1)	VII (3)
Mirranatwa Gap (MG)	*E. verrucata*	37.428° S, 142.460° E	484–525	10	D (10)	VII (3)
	E. baxteri (E)	37.433° S, 142.460° E	293–305	3	I (3)	VII (2)
Mount Abrupt (AB)	*E. verrucata* – large-fruited	37.593° S, 142.357° E	806–815	4	Z (4)	VII (1)
	E. verrucata	37.593° S, 142.357° E	785–806	16	Y (16)	VII (1)
	E. baxteri (E)	37.589° S, 142.359° E	430–451	6	J (2), X (4)	VII (2)
Additional species						
Serra Valley						
Teddy Bear Gap Rd	*E. obliqua*	37.347° S, 142.508° E	335	–	–	II (1)
Stockyard Track	*E. willisii*	37.328° S, 142.518° E	370	–	–	II (1)
Mount William Range						
Yarram Park Rd	*E. obliqua*	37.380° S, 142.547° E	400–405	–	–	II (1)
Mount William	*E. pauciflora*	37.296° S, 142.604° E	1131	–	–	I (1)
Mount William	*E. pauciflora*	37.295° S, 142.603° E	1148	–	–	I (1)
Victoria Valley						
Greens Creek	*E. obliqua*	37.138° S, 142.509° E	320–330	–	–	II (1)
Serra Track Clark Hill	*E. obliqua*	37.294° S, 142.435° E	260–265	–	–	II (1)
Moora Track	*E. obliqua*	37.260° S, 142.457° E	240	–	–	II (1)

between one of the endemic species and *E. baxteri* and are labelled accordingly (e.g. *E. verrucata*/*E. baxteri*, Table 1).

Leaves were collected from a total of 189 individuals. These included: 15–20 individuals from each of 12 locations spanning the distributions of *E. serraensis*, *E. verrucata* and *E. victoriana*; 5–10 individuals from each of 13 locations at which *E. baxteri* bordered populations of these endemics; and three *E. baxteri* populations in the valley between mountain ranges. We aimed to sample the endemic species at 15–20 m intervals and *E. baxteri* at 40–60 m intervals along elevational transects. Given the height and seed weight of the species, this spacing reduced the probability that individuals were closely related (Cremer, 1977; Jones *et al.*, 2007). Leaves were dried and stored in silica gel. A GPS point was recorded for each location and for most individuals within populations. Fruits, a voucher specimen, and ecological and environmental data were collected at each location. Vouchers are held at the University of Melbourne Herbarium (MELU).

DNA extraction

Genomic DNA was extracted from leaf material according to a cetyltrimethyl ammonium bromide (CTAB) procedure. Approximately 12 mg of silica-dried leaf tissue from each individual was disrupted either by grinding tissue frozen in liquid nitrogen or using a mixer-mill (model MM300; Retsch, Haan, Germany). Immediately following tissue disruption, 800 μL of extraction buffer was added and samples were initially incubated for at least one hour. We followed Tibbits *et al.* (2006) from the extraction step onwards.

Amplification and screening of chloroplast microsatellites

We initially screened eight individuals of *E. serraensis* and *E. verrucata* for amplification and polymorphisms with 10 chloroplast DNA microsatellite primer pairs developed by Steane *et al.* (2005): EMCRC59cp, EMCRC60cp, EMCRC62cp, EMCRC65cp, EMCRC67cp, EMCRC74cp, EMCRC84cp, EMCRC85cp, EMCRC86cp and EMCRC90cp. All primer pairs amplified a product of the expected size range and four were polymorphic (EMCRC59cp, EMCRC60cp, EMCRC67cp and EMCRC86cp). Using these four primers, 189 further samples were assayed.

Polymerase chain reaction (PCR) amplification of microsatellites was completed in a Mastercycler 5330 thermocycler (Eppendorf, North Ryde, NSW, Australia) using a final reaction mix of 1× NH_4 PCR buffer (Bioline), 3 mM $MgCl_2$ (Bioline), 400 μM of each dNTP (Invitrogen, Carlsbad, CA, USA), 0.1 μM of each forward and reverse primer, 0.04% bovine serum albumin (Sigma), 0.05 U *Taq* DNA polymerase (Bioline), and 1 μL DNA per 10 μL PCR mix. Forward PCR primers were fluorescently labelled with 6-FAM or HEX dyes. PCR reaction conditions were: initial denaturation for 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, annealing temperature of 55 °C for 30 s, and an extension of 72 °C for 1 min. PCR products were initially checked for amplification success using gel electrophoresis (2% agarose, 0.5× TBE buffer). We repeated PCR amplification for a randomly chosen 10% of the samples to check for contamination. Successfully amplified products were assayed by capillary electrophoresis using an ABI 3730 DNA analyser (Applied Biosystems, Ann Arbor, MI, USA) and allele sizes were scored automatically using GeneMapper 4.0 (Applied Biosystems) and/or manually using PeakScanner 1.0 software (Applied Biosystems). Discrepancies that occurred between automated and manual scoring resulted from alleles being outside the expected size range defined in the automated scoring. In those cases, we used the manually interpreted results.

Sequencing of the J_{LA}+ region

The J_{LA}+ region of cpDNA was sequenced for a subset of 50 samples that included at least one individual from each cpDNA microsatellite haplotype, each site, and each taxon within each site. The J_{LA}+ region was also sequenced for eight individuals from each of three additional co-occuring species (*E. obliqua*, *E. willisii* and *E. pauciflora*). We used the forward primer *euro_rpl2* (GCGTCCTGTAGTAAGAGGAG) from Payn *et al.* (2007) and the reverse primer *eucpsbA* (GGAGCAATAAC CAACACTCTTG) from Freeman *et al.* (2001). For these reactions, PCR reaction mixes were as defined above and PCR conditions were: initial denaturation of 94 °C for 1 min followed by 34 cycles of 94 °C for 20 s, 64 °C for 30 s, and 72 °C for 30 s. PCR products were purified and sequenced in both directions using the initial amplification primers, ABI BigDye terminators (Applied Biosystems), and an ABI 3730XL DNA Analyzer by Macrogen (Seoul, Korea). Contiguous sequences were assembled and edited in Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA), and aligned in BioEdit 7.0.9.0 (Hall, 1999) using ClustalW (Thompson *et al.*, 1994) with 1000 bootstraps of a neighbour-joining algorithm. PCR sequence lengths were *c.* 780 bp. We aligned 600 bp and final edits were made manually. Sequences are deposited in GenBank (accession numbers JX187448–JX187505).

Analysis of J_{LA}+ region

Unique haplotypes were distinguished by base substitutions, excluding insertions and deletions. Relationships among haplotypes were determined using the median-joining method in Network 4.5 (Bandelt *et al.*, 1999), which allows multistate sequence data. We coded the haplotype network with species identity and plotted haplotypes onto a hillshade surface derived from a 20 m digital elevation model (produced by the Department of Sustainability and Environment, Victoria) in ArcGIS 9.3.1 (ESRI, Redlands, CA, USA).

Analysis of cpDNA microsatellites

Haplotype network

For analysis, unique haplotypes were assigned to each distinct combination of allele sizes across all loci. A haplotype network

was produced using the reduced median option in NETWORK 4.5. This method produces a network linking all the most parsimonious pathways among haplotypes and retaining closed loops to allow homoplasy (parallel mutations or reversals) to be identified and examined (Bandelt *et al.*, 1995). The most likely sequence of mutations was identified for each reticulation based on the tenets of coalescent theory that a rare haplotype is more likely to evolve from a common haplotype and that haplotypes are more likely to be connected to other haplotypes located nearby (Crandall & Templeton, 1993). We coded the haplotype network with region and species identity. The haplotypes were overlain onto a hillshade surface derived from a 20 m digital elevation model in ARCGIS 9.3.1 to visualize potential historical seed migration patterns.

Genetic variation between geographic regions and species

We determined the extent of genetic differentiation in two ways: (1) between and within species; and (2) between and within geographic regions (northern Serra Range/Mount William Range, southern Serra Range and Victoria Range) with two AMOVAs based on ϕ_{PT} distances (similar to F_{ST} for diploid data) for all pairwise groups. Statistical significance was determined by comparison to 999 random permutations of the data.

Local genetic similarity between paired sampling locations

The extent of introgression (i.e. genetic similarity) between species was quantified for all collecting sites with a pair containing one of *E. victoriana*, *E. verrucata* or *E. serraensis*, and the closely related stringybark species *E. baxteri*. We used introgression ratios (IG) from Belahbib *et al.* (2001), which are based on intra- and interspecific identities from Dumolin-Lapègue *et al.* (1999). Local introgression was estimated with the IGR parameter from Palme *et al.* (2004). Specifically, we asked if individuals from a focal endemic population were more or less related to other endemic populations elsewhere than to the adjacent *E. baxteri* downslope. We correlated IGR to log-geographic distance with a Mantel test.

Geographic structure

The effect of geographical distance on genetic distance was determined by Mantel tests for correlations between dissimilarity matrices, using the package VEGAN 2.0-2 (Oksanen *et al.*, 2011) in R 2.14.1 (R Development Core Team, 2011). We correlated Nei's linear genetic distance with log geographic distance (in km) with a Mantel test for all individuals, disregarding taxonomic status. A partial Mantel test, which assigns a partial correlation that is conditional upon a third matrix of species identity, was used to determine the extent that species identity influences the isolation-by-distance relationship. Pearson's *r* correlation statistic was used to determine the strength of correlation between matrices. Significance was determined by comparing the frequency distribution of the correlation coefficient from 999 random permutations of the data matrices to that of the original data.

Genetic diversity

We calculated how haplotype diversity varied between three groups: (1) all endemic species; (2) *E. baxteri* collected from east-facing slopes and valleys, which generally have deeper soil and are sheltered from wind and sunlight; and (3) *E. baxteri* collected from exposed west-facing slopes. We accounted for different numbers of individuals at collection locations by rarefaction. We determined the mean number of haploptypes found for five individuals at each location using the 'rarefy' function in the package VEGAN 2.0-2.

RESULTS

The relatively conserved J_{LA}+ sequence region revealed cpDNA relationships between the target group (*E. serraensis*, *E. verrucata*, *E. victoriana* and *E. baxteri*) and more distantly related species from different sections of *Eucalyptus* (*E. obliqua*, *E. willisii* and *E. pauciflora*). CpDNA microsatellites proved useful for documenting fine-scale patterns within the target group.

J_{LA}+ region

We detected seven polymorphic sites and four unique haplotypes for 58 individuals (see Appendix S1 in Supporting Information). Haplotype sharing between the target group and other species was limited to *E. victoriana* and one *E. baxteri* individual (Fig. 2a), which both shared J_{LA}+ haplotype II with *E. obliqua* and *E. willisii*. The *E. victoriana* populations and *E. baxteri* individual that shared haplotype II were geographically restricted to the Victoria Range in the western portion of the study site (Fig. 2a). A relatively large disjunction (four transversions and one transition) occurred between haplotype II and haplotype VII. Haplotype VII was found in all samples of *E. baxteri* except for one individual from Clark Hill, all *E. verrucata* individuals, and most *E. serraensis* individuals (Fig. 2a, see Appendix S2). The remaining two *E. serraensis* individuals from Wonderland had haplotype VIII (Fig. 2a).

CpDNA microsatellites

The cpDNA microsatellites distinguished 26 unique multilocus haplotypes (Fig. 2b, see Appendix S2). Common haplotypes tended to be located internally on the haplotype network (with the exception of haplotype W) and centrally located within the study region (Figs 2b & 3a). Rare haplotypes tended to be found on the periphery of the haplotype network but were distributed throughout the study area (Figs 2b & 3a). A morphologically distinct, large-fruited population of *E. verrucata* at Mount Abrupt contained four private alleles at locus EMCRC86cp, and the *E. baxteri* population at Mount Lang contained four private alleles for EMCRC67cp.

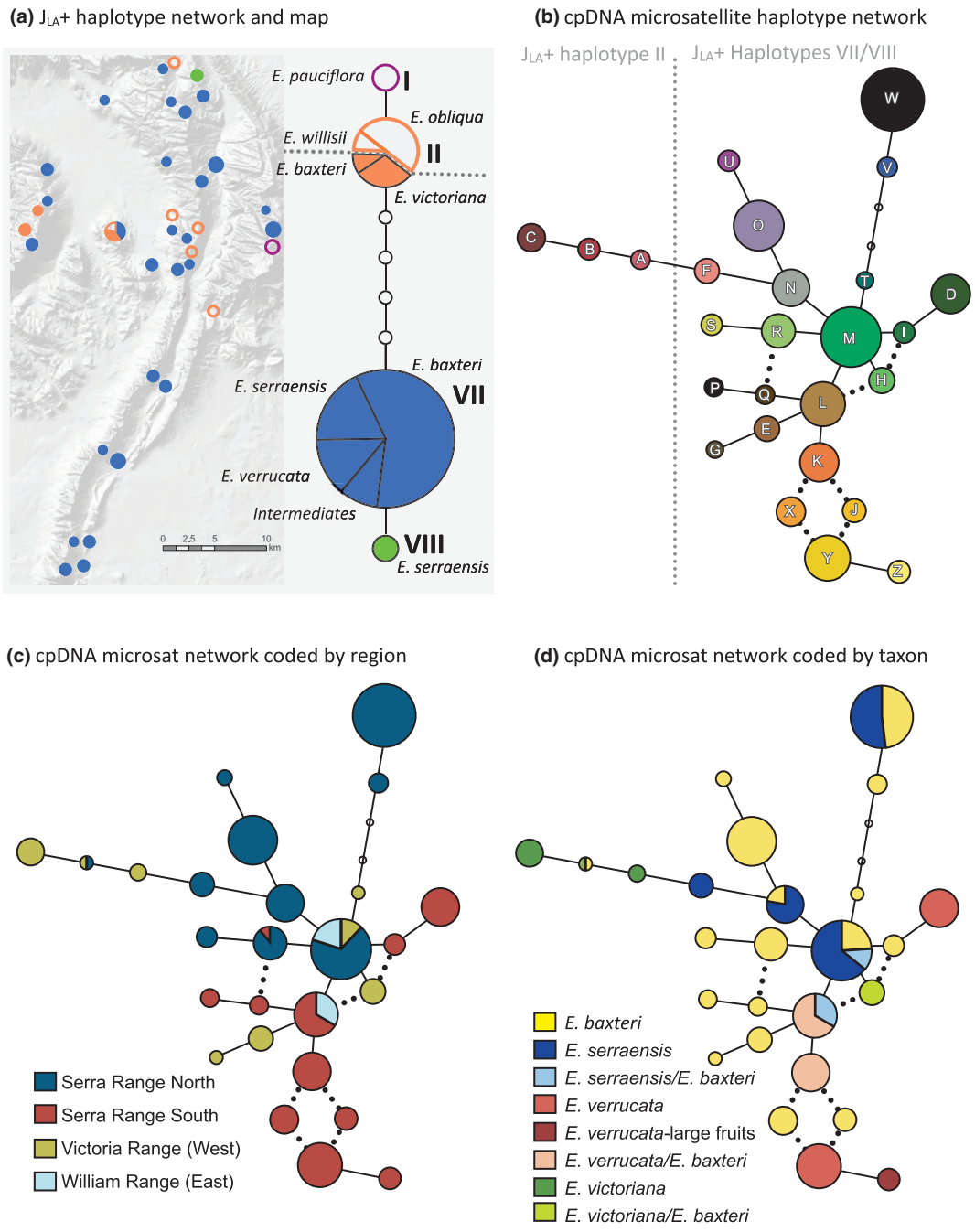

Figure 2 (a) Haplotype network and map of J_{LA}+ sequence data for *Eucalyptus* species from the Grampians National Park, Victoria, Australia. The sizes of the circles represent the number of individuals with each haplotype. Connecting lines are a single mutation. Hollow nodes on the network indicate positions not represented by individuals. Hollow circles on the map denote samples of *E. willisii*, *E. obliqua* and *E. pauciflora*; paired samples of endemic species and *E. baxteri* are arranged as shown in Fig. 3. (b) Haplotype network based on cpDNA microsatellite data for *Eucalyptus baxteri*, *E. serraensis*, *E. verrucata* and *E. victoriana*. Letters represent unique multilocus haplotypes. Closed loops were retained with less likely mutation steps shown with dotted lines. (c, d) cpDNA microsatellite haplotype network colour-coded by geographic region (c; as defined in Table 1) and taxon identity (d).

Geographically structured cpDNA variation

Microsatellite variation was congruent with that of the J_{LA}+ sequences in that *E. victoriana* and one *E. baxteri* individual from Clark Hill (left arm in Fig. 2b) were differentiated from the remaining members of the stringybark group. Microsatellite variation corresponded more closely to geography (Fig. 2c) than species identity (Fig. 2d). Quantitative evidence for the role of geography in structuring microsatellite haplotypes included: (1) regional differences explained more genetic variation than species identity with AMOVA (55% and 26%, respectively; Table 2); and (2) Mantel tests showed a positive

Table 2 Partitioning of molecular variance (AMOVA) for cpDNA microsatellite data of *Eucalyptus* species in the Grampians National Park, Victoria, Australia, with degrees of freedom (d.f.), sum of squares (SS) and the percentage of variance attributed to the following groupings: (a) between and within taxa (morphologically intermediate populations excluded) and (b) between and within the three regions (northern Serra Range/Mount William Range, southern Serra Range and Victoria Range).

		cpDNA		
	Groups	d.f.	SS	%
(a)	Between species	3	224	26
	Within species	172	876	74
(b)	Between regions	2	386	55
	Within regions	186	758	45

relationship between geographic distance and genetic distance ($r = 0.37$, $P < 0.001$), with a slightly weaker relationship between genetic and geographic distance when accounting for species identity ($r = 0.35$, $P < 0.001$), implying an overall isolation-by-distance relationship mostly unaffected by species identity.

Local differentiation from E. baxteri *varies between endemic species*

We further investigated the influence of taxonomy on local genetic structure by quantifying the genetic similarity between each endemic species and *E. baxteri* only for the sites in which both were present. This method reduced the potential of obscuring local genetic signal by averaging over species and regions.

Introgression ratios (IG) ranged from 0.2 for *E. victoriana* to 0.6 for *E. serraensis*, indicating that *E. victoriana* is relatively distinct from *E. baxteri*, while *E. serraensis* shared the most haplotypes with *E. baxteri* from the same geographic region (Table 3). Only one *E. baxteri* individual from Clark Hill, in the valley between the Victoria and Serra ranges, shared a J_{LA}+ haplotype and microsatellite haplotype with *E. victoriana* (and this J_{LA}+ haplotype was also shared with *E. obliqua* and *E. willisii*). The Mount Thackeray population, with morphology intermediate between *E. victoriana* and *E. baxteri*, had cpDNA more similar to *E. baxteri* than *E. victoriana* (Table 1).

On average, populations of *E. serraensis* and *E. verrucata* had more similar cpDNA to *E. baxteri* downslope than to other neighbouring conspecific populations (Table 3). The most convincing case of local interspecific cpDNA similarity was in the Mount Lang–Teddy Bear Gap region (Figs 2b & 3b), where *E. serraensis* and *E. baxteri* shared the highly differentiated haplotype W (Fig. 2b). Also striking were populations of *E. verrucata* with unique cpDNA haplotypes that were more similar to those of *E. baxteri* downslope than to neighbouring populations of *E. verrucata*, suggesting historical, geographically localized exchange of genes. This

Table 3 Introgression ratio (IG) of *Eucalyptus* species in the Grampians National Park, Victoria, Australia, based on cpDNA microsatellite variation, and geographic parameters with standard errors for each geographic region. IG values based on interspecific identities and intraspecific identities (from Dumolin-Lapègue *et al.*, 1999). IG is the ratio of interspecific : intraspecific identities summed over each region (see Belahbib *et al.*, 2001, for calculation). IG values range from 0 (complete genetic differentiation between taxa) to 1 (no species effect on genetic variation). IGR is the IG among all conspecific endemic populations (*E. verrucata*, *E. serraensis* or *E. victoriana*) divided by the IG between the endemic population and the *E. baxteri* population from that same site. IGR values > 1 indicate individuals from focal populations are more related to individuals from conspecific populations than to the *E. baxteri* at that site. IGR values < 1 indicate endemic populations are more related to *E. baxteri* than to other endemic populations.

Range	IG	IGR
Serra North (*E. serraensis*)	0.58	0.65 ± 0.13
Serra South (*E. verrucata*)	0.49	0.85 ± 0.05
Victoria Range (*E. victoriana*)	0.20	1.12 ± 0.10

was seen (Figs 2b & 3b) at Mirranatwa Gap (where *E. verrucata* had the unique haplotype D, related to haplotype I in adjoining *E. baxteri*) and at Mount Abrupt (where *E. verrucata* had unique haplotypes Y and Z, related to haplotypes X and/or J in adjoining *E. baxteri*). The haplotypes of these populations of *E. verrucata* were many steps apart on the haplotype network, with haplotypes of *E. baxteri* interposed between them (Fig. 2b).

CpDNA patterns vary with landscape configuration and aspect

One part of the haplotype network (haplotypes K, X, J, Y, Z in Fig. 2b) broadly corresponds with geographic position for sampling locations in the southern Serra Range. Haplotypes from neighbouring populations of *E. verrucata* and *E. baxteri* are one or two steps apart and differ in a stepwise manner along the southern Serra Range (Fig. 3c).

Eucalyptus baxteri populations sampled from the east-facing slopes and valleys had more diverse cpDNA microsatellite haplotype assemblages than both *E. baxteri* sampled from west-facing slopes and endemic species on ridges (Fig. 4). The two most diverse populations were *E. baxteri* at Clark Hill and *E. baxteri* east of Mount Lubra (Fig. 3b), which had three and four cpDNA microsatellite haplotypes, respectively, in the five individuals sampled. The Clark Hill and Mount Lubra sites are both centrally located between ranges (Fig. 3a,b). Conversely, *E. baxteri* on western slopes in the northern Serra Range showed the least diversity with one relatively widespread fixed microsatellite haplotype O (purple in Fig. 3a). *Eucalyptus verrucata* had consistently low within-population genetic diversity, with each population containing one microsatellite haplotype (Fig. 3c).

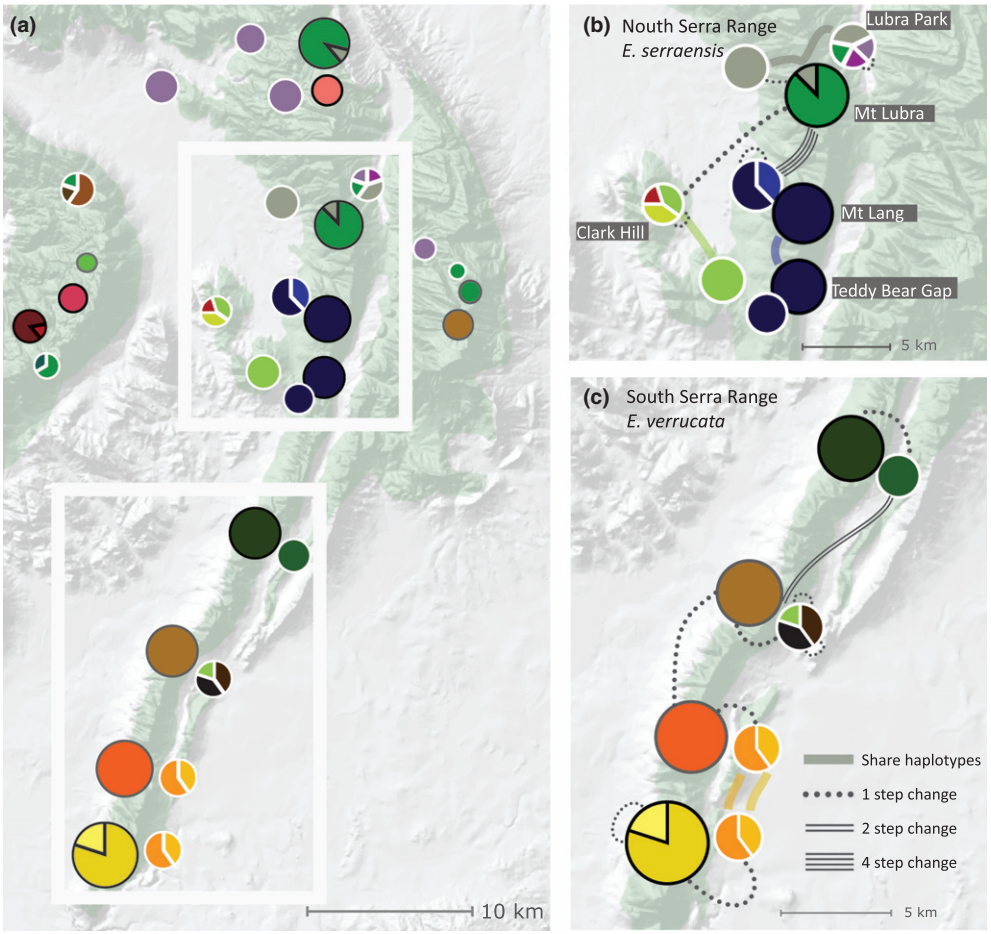

Figure 3 (a) CpDNA microsatellite haplotypes in *Eucalyptus* species and hillshade map derived from a digital elevation model of the Grampians National Park, Victoria; haplotype networks representing the northern Serra Range (b) and southern Serra Range (c). The distribution of *E. baxteri* is shaded green. The sizes of circles represent the number of individuals sampled at that location. Circles for endemic species are outlined in black, intermediate forms (e.g. *E. verrucata*/*E. baxteri*) are outlined in grey, and *E. baxteri* pies are outlined in white. Pie pieces represent the relative number of individuals for locations with > 1 haplotype. Haplotype colours are the same as in Fig. 2a. Line types for (b) and (c) represent the number of mutations separating haplotypes. Solid lines represent haplotype sharing; the line colour corresponds to haplotype identity.

DISCUSSION

The molecular evidence presented here suggests that land forms influence patterns of chloroplast genetic diversity, shape seed migration routes for colonization, and provide climatic conditions for the persistence of restricted haplotypes. Local haplotypes are shared extensively between closely related species along elevation gradients and, in some cases, between relatively distantly related taxa (*Eucalyptus* species from different taxonomic sections).

CpDNA diversity across the landscape

Species distributions in topographically complex regions within temperate Australia are often phylogenetically structured (for a review, see Byrne *et al.*, 2011). The Grampian Ranges contain a diverse assemblage of chloroplast microsatellite haplotypes (n = 26) and geographic structuring within a relatively small area of *c.* 20 km × 50 km. The allelic diversity is comparable to that of *E. regnans* (three identical loci were used in the present study) in putative Pleistocene refugia (Nevill *et al.*, 2010). This is in contrast to low levels of cpDNA diversity seen in eucalypts in areas postulated to have been recolonized following the alleviation of Pleistocene cold periods in south-eastern mainland Australia (Nevill *et al.*, 2010) and Tasmania (McKinnon *et al.*, 2001), which are fixed for one or a small number of haplotypes.

Protected slopes and valleys more genetically diverse than exposed slopes

The uneven distribution of cpDNA haplotype diversity across the landscape may be a historical legacy of species interactions or species responses to past climate fluctuations. The greater genetic diversity of *E. baxteri* in the protected east-facing slopes and valleys could have resulted from introgression with

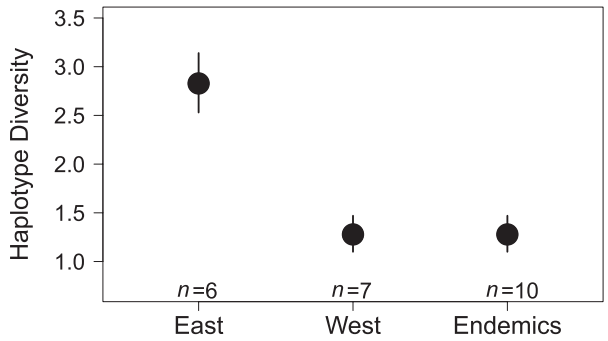

Figure 4 Chloroplast haplotype diversity from microsatellite data for three groups of *Eucalyptus* species from the Grampians National Park, Victoria, Australia: *E. baxteri* collected from sampling locations on sheltered east-facing slopes and valleys (East), *E. baxteri* from exposed west-facing slopes (West), and all endemic species collections (*E. serraensis*, *E. verrucata*, *E. victoriana* and *E. baxteri*/endemic intermediates). Haplotype diversity is the mean number of haplotypes found per five individuals at each sampling location determined by rarefaction. Error bars are standard errors of the mean number of haplotypes per five individuals at each location for each group. Sample sizes (i.e. collection locations) are shown above *x*-axis labels. Locations with fewer than five individuals were excluded from this analysis.

E. obliqua (in the green ash clade; Ladiges *et al.*, 1989), which often co-occurs with *E. baxteri* at mid-elevations. However, *E. obliqua* individuals sampled in this study have a distinct J_{LA}+ haplotype from *E. baxteri* (except one *E. baxteri* individual), and thus introgression is unlikely to be the cause of greater diversity in the protected slopes and lowlands.

Another explanation for the uneven distribution of genetic diversity is that *E. baxteri* populations persisted on the deep soils of sheltered east-facing slopes and valleys and retreated (and subsequently recolonized) from exposed west-facing slopes during past climate fluctuations. Tree species survival in mid-latitude ranges requires conditions to remain above a minimum threshold for moisture availability during arid periods and shelter from cold air currents (Tzedakis *et al.*, 2002). The shallow western slopes of the Grampians cuesta formations have skeletal soils and are exposed to strong westerly winds. The average maximum daily wind gust is 65 km h^{-1} on the summit of Mount William compared with 37 km h^{-1} in a nearby open lowland weather station (Bureau of Meteorology Station 089000). Palynological data from the area indicate that the climate was up to 10 °C cooler and drier with stronger winds and less fire in the Last Glacial Maximum than today. Eucalypts were present but restricted to pockets of favourable habitat (D'Costa *et al.*, 1989). The moisture deficit threshold is likely to have been exceeded at times during the Pleistocene, given that exposed western slopes are only marginally suited for tree growth under current conditions, and this could have resulted in *E. baxteri* retreating downslope. If this were the case, then the gradual uninterrupted dip slopes (and intense winds) may have allowed rapid recolonization by one haplotype following the alleviation of arid conditions. Sites exposed to hot northerly and westerly winds have been shown to experience more rapid recent climate change than more protected sites in eastern New South Wales, Australia (Ashcroft *et al.*, 2009). If the climate on the western slopes of the Grampians is changing more rapidly, the exposed slopes may become unsuitable for tree growth in the future, and the sheltered locations may become important gene pools of genetic diversity.

Discordance of cpDNA and taxonomy

Discrepancies between cpDNA and taxonomy have been attributed to interspecific gene flow in eucalypts (McKinnon *et al.*, 2001, 2010) and other large genera such as *Quercus* (Lexer *et al.*, 2006; Lumaret & Jabbour-Zahab, 2009). Gene flow via seeds is generally limited for eucalypts, which lack dispersal appendages (Potts & Reid, 1988), and is especially limited for short-statured species, in which seed dispersal probably only occurs a few metres from the parent (Cremer, 1977). Cases of interspecific gene flow can be inferred over ancestral polymorphism when haplotype sharing occurs near the tips of the haplotype network (Schaal & Leverich, 2001) and in close geographic proximity (Muir & Schlötterer, 2005).

Many cases of haplotype sharing are likely to have resulted from interspecific gene flow in this study. The most likely case of introgression involved haplotype sharing between the target group (all *E. victoriana* and one *E. baxteri* individual on the Victoria Range) and more distantly related species (*E. willisii* and *E. obliqua*) (J_{LA}+ haplotype II, Fig. 2a). *Eucalyptus willisii* (a peppermint) and *E. obliqua* (a green ash; Ladiges *et al.*, 2010) are from different taxonomic sections within *Eucalyptus*. Members of the target group that shared haplotypes with distantly related species were restricted to the western portion of the study area. Interspecific gene flow is likely to have occurred between the stringybarks, the peppermint, and the green ash in this area, given that the species are distantly related and haplotype sharing was restricted to a small area.

The cause of haplotype sharing is more difficult to disentangle in some instances. For example, the sharing of J_{LA}+ haplotype II between *E. victoriana* and one individual of *E. baxteri* could have resulted from ancestral lineage polymorphism or gene flow. *Eucalyptus victoriana* is the least morphologically distinct from *E. baxteri*, to the extent that Brooker (2000) included *E. victoriana* within a broad circumscription of *E. baxteri*. Whiffin & Ladiges (1992) hypothesized, based on volatile leaf oil composition, that *E. victoriana* – restricted to the Victoria Range in the western region of the Grampians – was the most recent of the three endemics to differentiate from *E. baxteri*. The close geographical proximity of haplotype sharing between *E. victoriana* and *E. baxteri* suggests introgression, but the species are not distantly related enough to rule out ancestral polymorphism.

Introgression levels along environmental gradients for *E. verrucata* and *E. serraensis* with *E. baxteri* are comparable with those seen in other large genera, such as *Betula* (Palme *et al.*, 2004) and *Quercus* (Belahbib *et al.*, 2001), in which

species are known to hybridize. The finding that populations of *E. serraensis* and *E. verrucata* shared more cpDNA (according to microsatellites) with downslope *E. baxteri* than with other conspecific populations indicates that gene flow occurs predominantly between neighbouring populations. For example, populations of *E. serraensis* at Mount Lubra and Mount Lang are both morphologically recognizable and typical of the taxon, yet are relatively distantly located on the cpDNA network (Fig. 3b). Each population shares a haplotype with *E. baxteri* downslope, and the shared haplotypes are located at the tips of the haplotype network, providing support for a hypothesis of local introgression.

Another process that could lead to incongruence between variation in cpDNA and morphology is independent origins (parallel evolution) of high elevation morphotypes on different ridges from *E. baxteri*-like ancestors. Such a scenario has been proposed for stunted, coastal morphotypes of *E. globulus*, which were more closely related to the neighbouring tall trees than to other dwarf populations (Foster *et al.*, 2007). It has also been proposed for other plants of extreme environments (e.g. Hogbin & Crisp, 2003). Parallel evolution is also possible in the Grampians stringybarks given the morphological variation between populations and the likelihood that many of the common traits separating the endemics from *E. baxteri* are adaptive. For example, leaves of the endemic species have a low specific leaf area (Pollock *et al.*, 2011), which indicates investment in resource conservation over rapid growth (Reich *et al.*, 1999). Resource conservation would be advantageous on rocky sites where growth is limited by root crowding, where water and nutritional capacity is reduced, and where temperatures are more extreme (Poesen & Lavee, 1994). The larger capsules seen in these populations may offer increased seed protection, especially from high intensity fire (Bradstock *et al.*, 1994), which occurs close to the ground (Van Wagner, 1973).

Parallel evolution is most plausible in the case of *E. verrucata*, because the forms are highly variable with dissimilar cpDNA. For example, *E. verrucata* at Mirranatwa Gap is three mutational steps away from the next most genetically similar population of *E. verrucata* at Griffith Fireline and seven steps away from the population at the type locality of *E. verrucata* on Mount Abrupt. Parallel evolution and introgression could be further distinguished with suitable nuclear markers. If high-elevation populations shared unique nuclear genotypes, then a hypothesis of speciation followed by subsequent introgression with *E. baxteri* would be favoured.

CONCLUSIONS

The intensive sampling scheme we used allowed us to relate genetic diversity to local climates. We detected local pockets of cpDNA diversity on sheltered east-facing slopes and valleys with deeper soils relative to genetically homogenous populations on west-facing slopes with shallower, skeletal soils and intense winds. This study also lends support to the growing body of evidence that interspecific gene flow is prevalent where highly outcrossing, long-lived species with limited dispersal co-occur. Potentially interbreeding species should be included in phylogeographic studies for a comprehensive picture of genetic structure and in order to better inform evolutionary inferences.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 List of haplotypes and polymorphic sites for the J_{LA}+ cpDNA region.

Appendix S2 List of 26 cpDNA unique microsatellite haplotypes for 190 individuals.

Appendix S3 Google Earth (.kmz) file with study site locations.

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BIOSKETCH

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Author contributions: L.J.P. collected the data in the field, carried out lab work, analysed data, and wrote the manuscript; M.J.B. and P.A.V. helped conceptualize the project and plan a sampling strategy; P.G.N. helped with lab work; M.J.B., P.A.V. and P.G.N. helped with data analysis and provided comments on manuscript drafts.

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