# The major Australian cool temperate rainforest tree *Nothofagus cunninghamii* withstood Pleistocene glacial aridity within multiple regions: evidence from the chloroplast

James R. P. Worth, Gregory J. Jordan, Gay E. McKinnon and René E. Vaillancourt

School of Plant Science, University of Tasmania, Hobart 7001, Tasmania, Australia

Author for correspondence:
*James R. P. Worth*
*Tel: +61 3 6226 2603*
*Email: jworth@utas.edu.au*

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## Summary

• Glacial aridity of the Pleistocene was inhospitable for the cool temperate rainforest tree *Nothofagus cunninghamii* over most of its current range in southeastern Australia, particularly in eastern Tasmania. A chloroplast DNA phylogeographic study was undertaken to investigate whether this species was likely to have survived *in situ* or conforms to a dispersal model of postglacial recovery.

• Twenty-three chloroplast haplotypes were identified by PCR-RFLP and direct sequencing of 2164 base pairs from 213 *N. cunninghamii* individuals collected in a range-wide survey. Fine-scale haplotype distribution was investigated using PCR-RFLP in eastern Tasmania.

• Deep chloroplast divergence occurred in *N. cunninghamii*. The single haplotype of the sister species, *N. moorei*, was nested among *N. cunninghamii* haplotypes. The distribution of *N. cunninghamii* haplotypes supports: multiple glacial refugia in coastal and inland western Tasmania, the centre of haplotype diversity; glacial survival in the central highlands of Victoria, corroborating pollen data; and the long-term occupation of eastern Tasmania because of the presence of a unique deeply diverged chloroplast lineage.

• *Nothofagus cunninghamii* withstood glacial aridity within multiple regions in apparently nonequable climates. This finding contributes to a growing understanding of how the resilience of temperate species during glacial periods has shaped modern biota.

## Introduction

There is a continuing debate on how plants and animals have survived through past major climatic changes, enabling the assembly of temperate communities during the current interglacial. Many authors, particularly using fossil pollen data from the northern hemisphere, have proposed that temperate forest species migrated very rapidly from a few distinct refugia located in regions that escaped the major climatic changes of the glaciations (Huntley & Birks, 1983; Hewitt, 1996). In this model, current temperate tree populations outside known glacial refugia are thought to have been established during the postglacial period via migration over thousands of kilometres (Jackson & Overpeck, 2000), and across both land and sea barriers (Davis *et al.*, 1986; Webb, 1987; Bennett, 1995). More recently, macrofossil and phylogeographic evidence has been used to argue for more complex histories involving expansion from multiple refugia (Stewart & Lister, 2001; McLachlan *et al.*, 2005; Petit *et al.*, 2008). This has resulted in the identification of refugia in locations unexpectedly close to regions where glacial climates had major effects on the environment. The locations of these refugia often conflict with present knowledge of the tolerance range and/or adaptive abilities of species; models of glacial climatic conditions (e.g. temperature, aridity, permafrost and ice cover); interpretations of pollen evidence; and predictions of biogeographical histories of species from current distribution patterns. Such refugia are sometimes referred to as 'cryptic' (Stewart & Lister, 2001; Provan & Bennett, 2008). The southern hemisphere provides opportunities to better

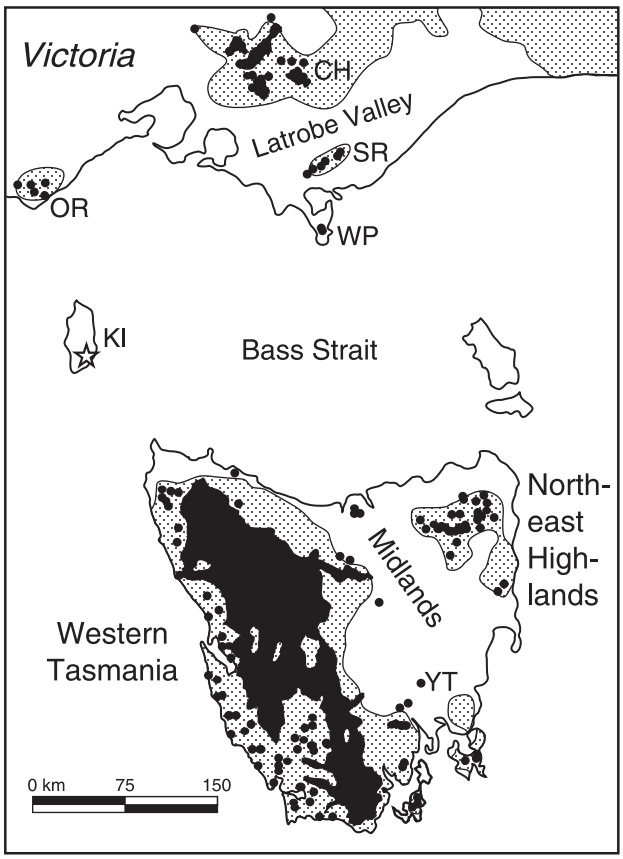


**Fig. 1** Distribution of *Nothofagus cunninghamii* cool temperate rainforest (black areas) in southeastern Australia. Grey stippled areas indicate regions in Tasmania and Victoria receiving over 50 mm of precipitation during the driest month. Geographical names mentioned in text: Victorian central highlands (CH), Strzelecki Ranges (SR), Otway Ranges (OR), Wilsons Promontory (WP), King Island (KI), Yarlington Tier (YT). Some major dry land and sea barriers are also shown. The star on KI indicates the location of a macrofossil site of *N. cunninghamii* dated at *c.* 110 000 yr before present (G. J. Jordan, unpublished).

understand how important large-scale postglacial migrations and/or expansions from multiple glacial refugia may have been in shaping the current forests of the temperate zone.

Temperate rainforests of the southern hemisphere occur from latitudes 28°S to 55°S in South America, New Zealand and Australia. In each of these regions, palaeoecologists have proposed that these forests recovered from glacial climates by expansion from multiple refugia without extensive range shifts (Macphail & Colhoun, 1985; McGlone, 1985; Markgraf *et al.*, 1995). In southeastern Australia, most areas of cool temperate rainforest, as defined by Webb (1959), are dominated by *Nothofagus cunninghamii* (Nothofagaceae). These rainforests have a widespread but discontinuous distribution in the wettest and most fire-protected regions (Hill *et al.*, 1988) and are surrounded by more extensive sclerophyll forests.

Many authors have argued that arid conditions during Pleistocene glaciations would have made almost all of southeastern Australia inhospitable for cool temperate rainforest (Hope, 1994; Hill, 2004). The available pollen evidence in southeastern Australia from the Last Glacial Maximum (LGM) 18 000 yr ago indicates a more or less treeless landscape dominated by glacial steppe vegetation probably to present sea level. Pollen evidence identifies rainforest tree LGM survival in only two places: the western half of Tasmania, where the coastal plains exposed by depressed LGM sea levels may have provided suitable habitat (Kiernan *et al.*, 1983; Colhoun, 2000) and the central highlands of Victoria (McKenzie, 1997).

There has been particular controversy about whether two regions of southeastern Australia contained glacial refugia: the highlands of northeast Tasmania and southern Victoria (Fig. 1). Depauperate cool temperate rainforest communities are reasonably extensive in these regions (Busby, 1984) but they receive < 50% of the precipitation of the wetter parts of western and southern Tasmania (Nunez, 1978) where the most geographically extensive cool temperate rainforests exist. The available geomorphological and pollen-based evidence indicates extensive glacial aridity during the LGM in northeast Tasmania and southern Victoria (Galloway, 1965; Bowler, 1982; Colhoun, 2002), with the Australian desert thought to have extended to within 100 km of current cool temperate rainforest populations (Bowden, 1983; Hill & Bowler, 1995). In northeast Tasmania, palaeoclimatic modelling can reconstruct conditions favourable for LGM survival of cool temperate rainforest only by invoking eastern Tasmanian climates with similar rainfall to the present (Kirkpatrick & Fowler, 1998), contrary to the evidence for much higher aridity. Even this modelling could only identify refugia in the wettest part of eastern Tasmania, Blue Tier. An alternative explanation involving dispersal rather than glacial refugia for the occurrence of rainforest in northeast Tasmania must invoke Holocene dispersal across > 150 km from the nearest documented refugia in western Tasmania, a scenario that has been considered unlikely because of the low dispersal capacity of many rainforest species (Dodson & Ono, 1997; Kirkpatrick & Fowler, 1998), particularly *N. cunninghamii*. Herein lies the conundrum: the areas where cool temperate rainforest currently occur can only have arisen from *in situ* glacial refugia if our understanding of glacial climates is wrong, and/or the ecological tolerance range of species during past climatic changes was greater than would be predicted from their modern distribution. However, if rainforest did not survive in multiple regions, extensive range shifts must be invoked (Jordan, 2003). Therefore, the cool temperate rainforest system of southeastern Australia provides an opportunity to test the relative roles of multiple refugial survival and postglacial dispersal.

This study aims to address this conundrum by investigating the chloroplast DNA phylogeography of the dominant

cool temperate rainforest tree in southeastern Australia, *N. cunninghamii*. The current distribution of chloroplast DNA (cpDNA) haplotype variation across a species' range can provide independent evidence for the history of genetic exchange by seed and isolation of populations (Schaal *et al.*, 1998). Isolated populations may differentiate over time and, through genetic drift, form distinct genetic lineages. Dispersal can result in the territorial expansion of genetic lineages (Avise, 1994) and the sharing of lineages between populations. Chloroplast DNA phylogeographic studies have been used to investigate the location of glacial refugia and migration histories of temperate forest mostly in the northern hemisphere (Soltis *et al.*, 1997; Okaura & Harada, 2002; Petit *et al.*, 2003). Although range-wide cpDNA phylogeographic studies have been completed in some southeastern Australian sclerophyll forest *Eucalyptus* species (Byrne & Moran, 1994; Freeman *et al.*, 2001), this study is the first cpDNA phylogeography of a widespread cool temperate rainforest species in Australia. This study assesses the contributions of multiple glacial refugia to postglacial recovery of *N. cunninghamii*. Specifically we address whether glacial survival occurred outside putative refugia in coastal western Tasmania and the central highlands of Victoria.

## Materials and Methods

### Study species

*Nothofagus cunninghamii* (Hook.) Oerst, or myrtle beech, is a long-lived, evergreen, monoecious, wind-pollinated tree reaching 50 m in height (Curtis, 1967), but at the altitudinal maximum of its range may be reduced to a compact shrub under 50 cm. The species is remarkably uniform in morphology across its range apart from variation in leaf size, which is strongly correlated with summer temperatures (Jordan & Hill, 1994). Seed is gravity-dispersed, generally one tree height from the mother tree (Howard, 1973; Hickey *et al.*, 1982; Tabor *et al.*, 2007), but may be dispersed downstream in water courses (Howard, 1973). The species' distribution is broken by some major dry land and sea barriers (Howard & Ashton, 1973; Fig. 1). Across its range, this species is confined to cool, humid climates where rainfall exceeds *c.* 1000 mm $yr^{-1}$ with at least 50 mm rainfall during the driest month (Jackson, 1965; Busby, 1986; Lindenmayer *et al.*, 2000). The small populations outside this climatic range (e.g. Yarlington Tier; Fig. 1) are all special topographic sites with precipitation supplemented by ground water and/or cloud stripping (Harle *et al.*, 1993). *Nothofagus cunninghamii* does not currently co-occur with any other species of its subgenus, *Lophozonia*. Its sister species, *N. moorei*, is restricted to mountain ranges of northern New South Wales and southern Queensland (Busby, 1986), *c.* 780 km north of the northernmost population of *N. cunninghamii*. The western Tasmanian endemic, *N. gunnii* (subgenus *Fuscospora*), sometimes co-occurs with *N. cunninghamii* but the two species do not hybridize (Hill & Read, 1991).

### Sampling

Fresh leaves were collected from 342 adult trees (327 stands) of *N. cunninghamii* in natural populations, including nearly all known parts of the species' distribution, apart from some remote parts of western Tasmania. Only one tree was sampled from most stands, but up to three widely spaced individuals were sampled at some locations, including some very isolated populations (see Supporting Information, Tables S1 and S2, for list of sample locations). Latitude, longitude and altitude information were recorded for each sample collected. Tree form was noted. Five individuals of *N. moorei* were also sampled from the northern (Lamington National Park, Springbrook National Park and Bar Mountain) and southern extremes of this species' range (two samples from Barrington Tops National Park *c.* 490 km south of the northern populations of this species). For use as outgroups, leaf samples of the New Zealand endemic *N. menziesii* and the Chilean species *N. glauca* were obtained from the Royal Tasmanian Botanical Gardens, Hobart, Tasmania. Phylogenies based on morphology, nuclear DNA and chloroplast DNA each indicate that *N. moorei* is the sister of *N. cunninghamii*, that *N. menziesii* is sister of this clade and that the resulting clade is sister to a small clade containing *N. glauca* Manos (1997). These samples were analysed in two groups: a range-wide survey whereby a haplotype phylogeny was created from chloroplast sequence and PCR-RFLP data using 213 *N. cunninghamii* samples from across the distribution of the species and all outgroups; and a fine-scale study of haplotype distribution that used three PCR-RFLP characters and screened 149 samples from the northeast highlands of Tasmania (including 20 samples used in the previous study). For this study, individual trees were sampled a minimum of *c.* 1 km apart.

### Molecular methods

Total genomic DNA was extracted from 1 g of adult leaves, following the CTAB protocol of Doyle & Doyle (1990). DNA concentration and purity were assessed using agarose gel electrophoresis with ethidium bromide staining and comparison with a standard molecular weight marker (Lambda *Hind*III). DNA concentration was standardized at 5 ng $\mu l^{-1}$.

Sixteen regions of cpDNA were amplified using universal primers (*trnD–trnT*, *trnS–trnf*$_M$, *trnK–trnQ*, *rpoC1–trnC*, *trnV–rbcL*, *rpl23–psbA3*, *atpH–atpI*, *atpI–rpoC2*, *rpoC2*-f–*rpoC2*-r, *orf184–petA*, *petA*-f–*psbE*-r, *clpp–psbB*, *psbB–petB*, *petB–petD*, *trnH–trnK* and *trnK–trnK*) (Demesure *et al.*, 1995; Dumolin-Lapégue *et al.*, 1997b; Grivet *et al.*, 2001). All PCR reactions were performed in a total volume of 25 µl containing 2.5 mM $MgCl_2$; 100 µg $ml^{-1}$ bovine serum albumin; 80 µM

each of dATP, dCTP, dGTP and dTTP; 5 pM of each primer; 1 × PCR buffer (67 mM Tris-HCl, 16.6 mM $(NH_4)_2$ $SO_4$, 0.5% Triton X-100 and 5 μg of gelatin); two units of *Taq* DNA polymerase; and *c*. 10 ng of genomic DNA. PCR amplification was performed by a MJ Research PTC-225 Tetrad thermocycler (GMI, Inc., Ramsey, MN, USA) as follows: an initial melt of 4 min at 94°C; 30 cycles of 45 s at 92°C, 45 s at annealing temperature (see Table S3 for details of annealing temperatures of each fragment), 4 min at 72°C; and a final extension for 10 min at 72°C. PCR products were digested with a range of restriction enzymes in a total reaction volume of 20 μl containing 5–10 μl of PCR product. The products of the restriction digests were size-fractionated in a 2.2% agarose gel in TBE at 100 V for 90 min. Restriction fragment length polymorphisms (RFLPs) were identified visually by comparing restriction fragment patterns between samples.

Screening of eight *N. cunninghamii* samples (representing all major parts of the species range) and 150 fragment/restriction endonuclease combinations (using *Taq*I, *Hinf*I, *Alu*I, *Dpn*II, *Hae*III, *Hinp1*I, *Rsa*I, *Dde*I, *Msp*I, *Hph*I, *Nco*I, *Ssp*I, *Ase*I, *Sty*I, *Nci*I, *Dra*I, *Cla*I, *BstU*I, *EcoR*V) revealed one RFLP. Screening of 48 or 72 samples and 16 fragment/restriction endonuclease combinations detected two additional RFLPs. All samples for the range-wide survey were screened for the three fragment/restriction endonuclease combinations that detected polymorphisms.

Owing to the paucity of cpDNA variation detected using PCR/RFLP, cpDNA fragments of all samples for the range-wide survey were sequenced. These were partial sequences of the intergenic spacer regions *petN1–psbM2*R, *psbM2–trnD* (Lee & Wen, 2004), *trnS–trnf*$_M$ (Demesure *et al*., 1995), and *trnL–trnF* (Taberlet *et al*., 1991) and the intron between *rps16*/1 F–*rps16*/1 R (Nishizawa & Watano, 2000). PCR conditions were as follows: *petN1–psbM2*R and *psbM2–trnD*, denaturation for 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C; *trnS–trnf*$_M$, denaturation for 4 min at 94°C, followed by 30 cycles of 45 s at 92°C, 45 s at 62°C, and 4 min at 72°C; *trnL–trnF*, denaturation for 1 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C and 45 s at 72°C; *rsp*16/1 F–*rsp*16/1 R, denaturation for 3 min at 95°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. All had a final extension for 10 min at 72°C, except *trnL–trnF*, which had a final extension of 7 min at 72°C. Before sequencing, PCR products were purified using the Qia-Quick PCR purification kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia). Sequencing reactions were performed using a Beckman Coulter Quick Start Kit following a modified protocol using 0.64 μl of 5 μM primer, and 6 μl of purified PCR product in a final volume of 10 μl. Sequence reactions were analysed using a Beckman Coulter CEQ 2000 automated sequencer (Beckman Coulter, Inc., Fullerton,CA, USA). Polymorphisms detected in only one sequencing reaction were checked by repeating the PCR and the sequencing reaction. In all cases where unexpected haplotype distributions were found (e.g. *N. moorei*), sequences and PCR-RFLP analyses were repeated, and samples rechecked.

For the fine-scale study of northeast Tasmanian haplotypes, restriction endonucleases that would enable the quick and easy identification of individuals carrying either the C1 or NE1 haplotypes (see later) were identified using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php). The restriction endonuclease *Hae*III cut the *trnL–trnF* fragment of C1 individuals once, and zero times in NE1 individuals as a result of a 17 base pair (bp) deletion. The endonuclease *Hpy*188III was found to distinguish the *psbM2–trnD* fragment of individuals carrying NE1 haplotype as a result of an extra restriction site in this haplotype. For this analysis a new internal reverse primer was developed (5′ ... CCGGGACTCGTCTTTATCATACTTC ... 3′) that amplified a cpDNA fragment *c*. 540 bp in length compared with the original *c*. 1200 bp fragment. This allowed better separation of the polymorphic fragments that differed by 77 bp in length between C1 and NE1 haplotypes. All 149 samples were screened with these two new endonuclease/fragment combinations and the previously identified *atpI–rpoC2* fragment/*Taq*I combination.

## Phylogenetic relationships of haplotypes

Evolutionary relationships between haplotypes (including *N. glauca*, *N. menziesii* and *N. moorei*) were investigated by maximum parsimony analysis undertaken using the program PAUP* version 4.0b10 (Swofford, 2000). PCR-RFLP polymorphisms, single nucleotide polymorphisms and insertions/deletions were scored as binary characters, except for two indel variants (characters 1 and 2; Table 1), which were scored as a multi-state character with three states. In addition, parallel variation was seen in two adjacent base pairs (a doublet). This was treated as a single character (character 20, Table 1). Parsimony analysis was undertaken using a heuristic search with 1000 replicates of stepwise, random branch swapping addition sequence followed by tree-bisection-reconnection (TBR). As a result of significant levels of homoplasy of some characters (in particular, characters 19 and 20; Table 1), all characters were reweighted iteratively by the maximum value of their rescaled consistency index (Farris, 1969). A strict consensus of all the shortest trees found using this procedure was constructed. Branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 heuristic search pseudo-replicates using the same search parameters as those in the parsimony analysis. *Nothofagus glauca* and *N. menziesii* were used as outgroups in all searches based on the topologies identified by (Manos, 1997).

## Spatial clustering and regional haplotype diversity

Within the 213 samples collected across the range of *N. cunninghamii*, the spatial structuring of haplotypes was

**Table 1** *Nothofagus cunninghamii* haplotypes (and the single *N. moorei* haplotype) with cpDNA sequence and PCR/RFLP characters shown in comparison to the *N. cunninghamii* C1 haplotype

| Haplotype | *rps16* intron *petN1-psbM2*R spacer | | | | | | | *psbM2-trnD* spacer | | | | | | | | | | *trnS-trnfM* spacer | | | | | | *trnL-trnF* spacer | | | | | | | | | | | PCR-RFLP polymorphisms | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Character | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| C1 | . | . | C | C | . | . | . | . | T | T | G | A | T | A | A | C | C | T | T | TC | . | T | A | A | . | T | . | . | A | G | G | A | . | G | 0 | 0 | 0 | 0 |
| WT1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | |
| WT2 | I | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | |
| WT3 | | | | | | | | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | 1 | | |
| WT17 | | | | | | | | | | | T | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | |
| WT18 | | | | | | | | | | | | | | | | | | | G | GA | | | C | | | | | | | | | | | | | 1 | | |
| V1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | |
| V2 | | | | | | | | | | | | | | | | | | | | GA | | | | | | | | | | | | | | | 1 | | | |
| V3 | | | | | | | | | | | | | | C | | | | | | GA | | | | | | | | | | | | | | | 1 | | | |
| WT4 | | | | T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WT5 | | | | | | | | | | | | | | | | | | | | GA | | | | | | | | | | | | | | | | | | |
| WT6 | | | | | D | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WT7 | | | | | D | | | | | | | | | | | | | | | | | | | C | | | | | | | | | | | | | | |
| WT9 | | | | | | | | | | | | | | | | | | | | | | | | | | | I | | | | | | | | | | |
| WT10 | | | | | | | | | | | | | | | | | | | G | | | | | | | | | | | | | | | | | | |
| WT8 | | | | | | | | | | | | | | | | | | | | | I | | | | | | | | | | T | | | | | | |
| WT11 | | | | | | | | | | | | | | | | | | | G | GA | | | | | | | | | | T | T | C | | | | | | |
| WT12 | | | | | | I | | | | | | | | | | | | | G | | | | | | | | | | | T | T | C | | | | | | |
| WT13 | | I | T | | | | I | D | | | | | | | C | | | | | GA | | | | | | C | | | | | T | | | A | | | | |
| WT14 | | | | | | | | D | | | | C | | | | A | | | G | | | | | | I | C | | | | | T | | | | | | | |
| *N. moorei* | | | | | | | | D | | G | | C | | | | A | A | | G | | | | | | | C | | | G | | T | | | | | | | 1 |
| WT15 | | | | | | | | D | | | | | | | | A | A | | G | | | G | | | | C | | | | | T | C | | | | | | |
| WT16 | | | | | | | | D | C | | | | | | | A | A | | G | | | | | C | | C | I | | | | T | | | | | | | |
| NE1 | | | | | | | | D | | | | | G | | | A | A | | | GA | | | | | | C | | | | | T | | D | | | | 1 | |
| *N. glauca* | | | | | D | | | D | | | | | | | | A | A | | G | GA | | | | C | | C | | | | | T | | | | 1 | | | |
| *N. menziesii* | | | | | | | | D | | | | | | | | A | A | | | GA | | | | | | C | | | | | T | | | | | | | |

The characters unique to *N. glauca* and *N. menziesii* are not shown. Indels are shown as insertions (I) or deletions (D) as determined from the C1 haplotype. Order of haplotypes follows their order in the strict consensus maximum parsimony (MP) tree.

investigated. The single nearest geographic neighbour for each sample was determined using a specially written macro in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). This program also performed a permutation test (Manly, 1997) with 10 000 randomized repeats testing whether the nearest neighbour of each sample was more often of the same haplotype than expected by chance (a proxy of spatial structure). This was carried out across all samples and within each region (Victoria, eastern Tasmania and western Tasmania). This procedure was also applied to the 149-sample set from northeast Tasmania in the fine-scale study.

Haplotype diversity was also compared across these regions through rarefaction analysis (Simberloff, 1979). The three regions were randomly subsampled to the size of the least sampled region ($N = 26$) 10 000 times. Differences in haplotype richness were then tested using a permutation test (Manly, 1997) with 10 000 randomized repeats, programmed in SAS 9.1 (SAS Institute Inc.). Similar analyses were performed comparing low-, medium- and high-altitude samples in western Tasmania, rarefying each to 43 samples. Low altitude was defined as < 150 m above sea level (m asl), the estimated treeline during the Last Glacial Maximum (Colhoun, 1985; Gibson *et al.*, 1987). High altitude was defined as > 500 m asl, which divided the remaining samples approximately evenly and is far above any plausible estimate of LGM tree lines given that all palaeoclimatic estimates show LGM temperatures at least 5°C lower than present (Galloway, 1965; Colhoun, 1985, 2000).

## Results

### cpDNA variation and phylogenetic relationships

A total of 35 polymorphisms defining 23 different haplotypes (Table 1) were found within the 213 samples of *N. cunninghamii*. Three PCR-RFLP polymorphisms were identified after restriction digest of three fragments (*trnK–trnK*, *atpI–rpoC*2 and *orf*184–*petA*), all using the restriction endonuclease *Taq*I (Table 2). Twenty single nucleotide polymorphisms (six transitions and 14 transversions), one doublet sequence polymorphism and 11 indels from one to 23 bp in length were identified from a total aligned sequence length of

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**Table 2** Molecular weights in base pairs (bp) of the polymorphic fragments obtained using the variable fragment/restriction endonuclease combinations found in *Nothofagus cunninghamii* and the five *N. moorei* samples

| Polymorphic fragments | Character number | Primary state[a] → Variant (bp) | Haplotype/species |
|---|---|---|---|
| *trnK–trnK*-(*Taq*I)2[b] | 35 | 540 → 420 | V1, V2, V3, *N. glauca* |
| *orf*184–*petA*-(*Taq*I)1 | 36 | 620 → 1030 | WT1, WT2, WT3, WT17, WT18 |
| *atpI–rpoC2*-(*Taq*I)1 | 37 | 870 → 1010 | NE1 |
| *atpI–rpoC2*-(*Taq*I)3 | 38 | 250 → 330 | *N. moorei* |

[a]Major pattern determined from the C1 haplotype.
[b]Polymorphic fragment number (largest to smallest) after size fractionation.

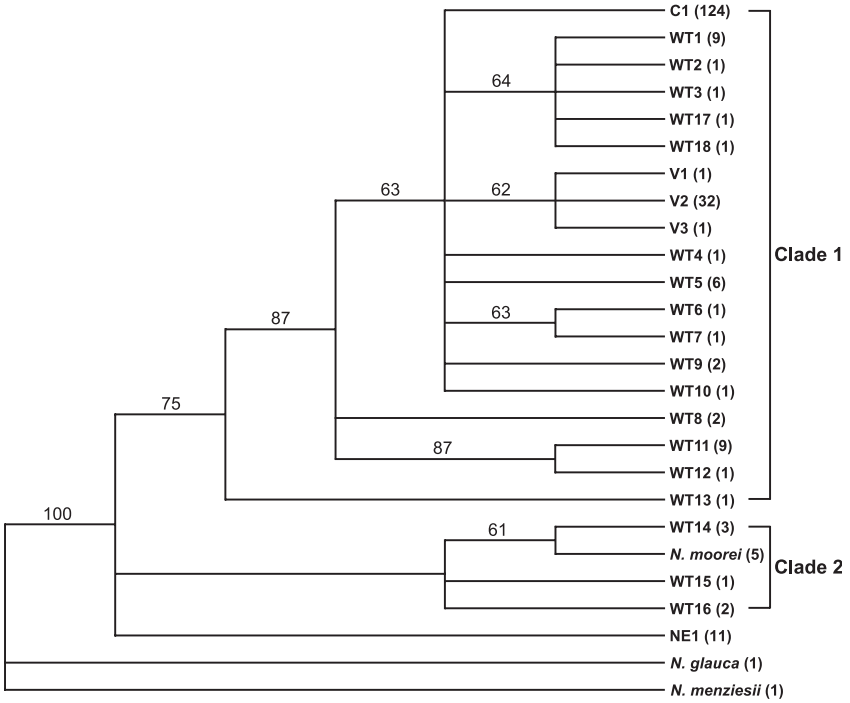


**Fig. 2** Inferred phylogenetic relationships of all haplotypes found in *Nothofagus cunninghamii* and *N. moorei* based on maximum parsimony (MP) analysis, with iterative reweighting of all characters. This MP strict consensus was based on 412 most parsimonious trees (L = 70.34, CI = 0.97, RI = 0.96, RC = 0.93). Bootstrap values above 60% are shown above branches. Haplotype names indicate the region in which they were found (WT, western Tasmania; V, Victoria; NE, northeast Tasmania), except for the most widespread haplotype C1. The number of occurrences for each haplotype is shown in brackets after the haplotype name.

2164 bp per sample. A total of 39 other polymorphisms were identified when adding samples of *N. moorei*, *N. menziesii* and *N. glauca* (data not shown). Two single nucleotide polymorphisms and one RFLP polymorphism were unique to the five *N. moorei* samples, which were all of the same haplotype (Fig. 3). All unique sequences were deposited in Genbank (see Table S4).

Parsimony analysis with iterative reweighting of the full cpDNA dataset yielded 412 most parsimonious trees based on 20 informative characters (Figs 2, 3). Haplotypes of *N. cunninghamii* and *N. moorei* formed a monophyletic group. Within *N. cunninghamii*, 19 haplotypes formed a large, partially resolved clade (clade 1) with good support (bootstrap percentage, BP = 75%; Fig. 2). Within clade 1, the most common and widespread haplotype (C1), occurred in a subclade with a BP of 63% (subclade A; Fig. 3). Subclade A also included 11 uncommon haplotypes found only in western Tasmania (WT1–WT7, WT9, WT10, WT17 and WT18), and a clade (62% BP support) of three haplotypes found only in the central highlands of Victoria (V1–V3). The remaining haplotypes within clade 1, haplotypes WT8, WT11, WT12 and WT13, fell outside subclade A (Fig. 3). An unsupported sister clade to clade 1 (clade 2; Fig. 2) contained three additional western Tasmanian haplotypes. Surprisingly, the single *N. moorei* haplotype was nested within this clade (Fig. 2), with *N. moorei* differing from *N. cunninghamii* haplotype WT14 by five characters. A single haplotype unique to northeast Tasmania (NE1) fell outside both clades 1 and 2.

## Distribution, spatial clustering and regional haplotype diversity

The C1 haplotype was the most frequent (58.2% of samples) and widespread of all haplotypes observed in *N. cunninghamii*. This haplotype occurred across the entire range of the species

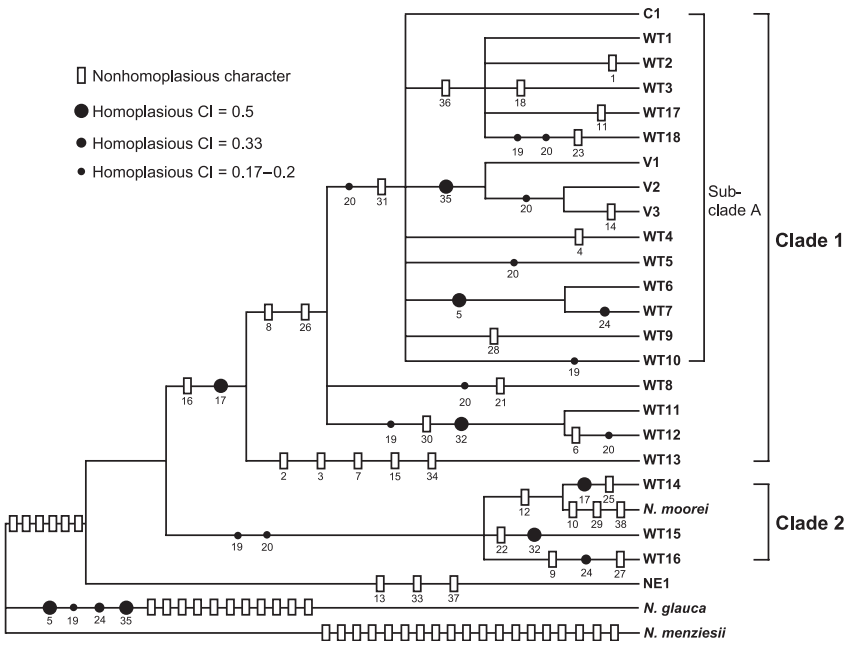


**Fig. 3** One of 412 most parsimonious trees with iterative reweighting. Numbers below branches indicate the *Nothofagus cunninghamii* and *N. moorei* character numbers given in Table 1. Open rectangles represent nonhomoplasious characters, including PCR-RFLP, single nucleotide polymorphisms and indels. Black circles represent homoplasious characters, with size of circles indicating consistency index (see key).

**Table 3** Rarefied haplotype diversity within regions

| | | Probability[b] | |
|---|---|---|---|
| Region | Rarefied haplotype diversity ($N = 26$)[a] | NE Tasmania | Victoria |
| Northeast Tasmania | 2.0 ± 0.0 | | |
| Victoria | 2.9 ± 0.7 | 0.161 | |
| Western Tasmania | 7.1 ± 1.6 | 0.001 | 0.004 |

[a]Values are the mean number of haplotypes when randomly subsampled to 26 samples, ± SE. [b]Probability of pairwise comparisons of diversity between regions.

except the Victorian central highlands, and was almost the only haplotype found in large areas of northwest Tasmania, and populations in the Otway Ranges, Wilsons Promontory, and geographically peripheral populations in Tasmania (Fig. 4a). Haplotype V2 was the second most frequent haplotype (15.0%) and occurred across the central highlands of Victoria. Haplotypes C1 and V2 occurred together in a contact zone of the two haplotypes in the Strzelecki Range (Fig. 4a). Haplotype NE1 (5.1%) was observed only in the northeast highlands of Tasmania (Fig. 4b). The remaining 18 haplotypes were all restricted to western and southern Tasmania and, apart from WT1 (4.2%), WT5 (2.8%) and WT11 (4.2%), were rare (< 1.3% of all samples; Fig. 4b–d). Five of the rare haplotypes were observed only at high altitudes in inland regions of western and southern Tasmania (WT2 at 900 m asl, WT3 at 805 m asl, WT6 at 681 m asl, WT9 at 610 m asl and WT12 at 960 m asl). Seven (WT4, WT7, WT10, WT13, WT15, WT17 and WT18) were found only at low altitudes (all below 80 m asl) near the coast or inland rivers. High- and low-altitude haplotypes were scattered throughout the phylogenetic tree.

Across the whole species, the single nearest geographic neighbour analysis detected significant spatial clustering of haplotypes ($P < 0.001$). This may be because significant structuring was evident in Victoria ($P < 0.001$) and in northeast Tasmania ($P < 0.01$). Within western Tasmania the nearest neighbour analysis did not indicate significant clustering ($P > 0.05$). This may be partly due to the presence of C1 haplotype throughout this region, because there was significant clustering ($P = 0.04$) of samples excluding the C1 haplotype. Haplotype diversity was significantly higher in western Tasmania (over twice as high for sample size rarefied to 26 samples) than either northeast Tasmania or Victoria (Table 3). The diversities of the latter two regions were not significantly different. In western Tasmania, a significantly higher ($P < 0.001$; randomization test) number of haplotypes occurred at low altitudes (12 haplotypes from 43 samples < 150 m asl) and at high altitudes (12 haplotypes

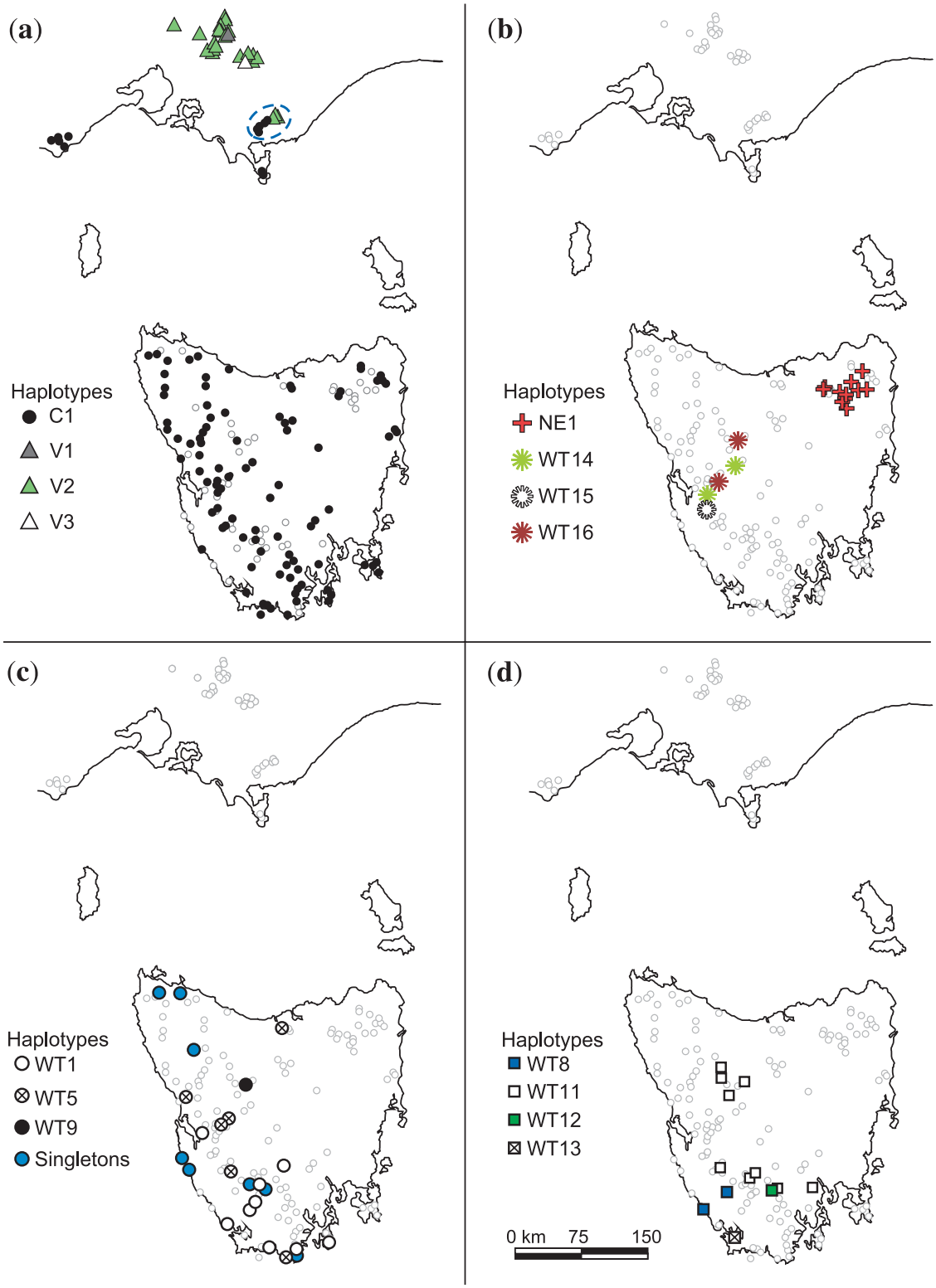


**Fig. 4** Distribution of haplotypes observed in *Nothofagus cunninghamii*. For reference, each of the sample locations is displayed on each map as a small open grey circle underlying the haplotypes shown. (a) Distribution of C1 and the minor subclade V1–V3 haplotypes and an inferred contact zone of the C1 and V2 haplotypes in the Strzelecki Range in Victoria (broken circle). (b) Distribution of clade 2 haplotypes in *N. cunninghamii* (WT14, WT15 and WT16) and the NE1 haplotype. (c) Distribution of haplotypes within subclade A of clade 1, excluding the C1 and V1–V3 haplotypes. Singletons are haplotypes that were observed once. (d) Distribution of haplotypes within clade 1 outside the large subclade A (WT8, WT11, WT12 and WT13).

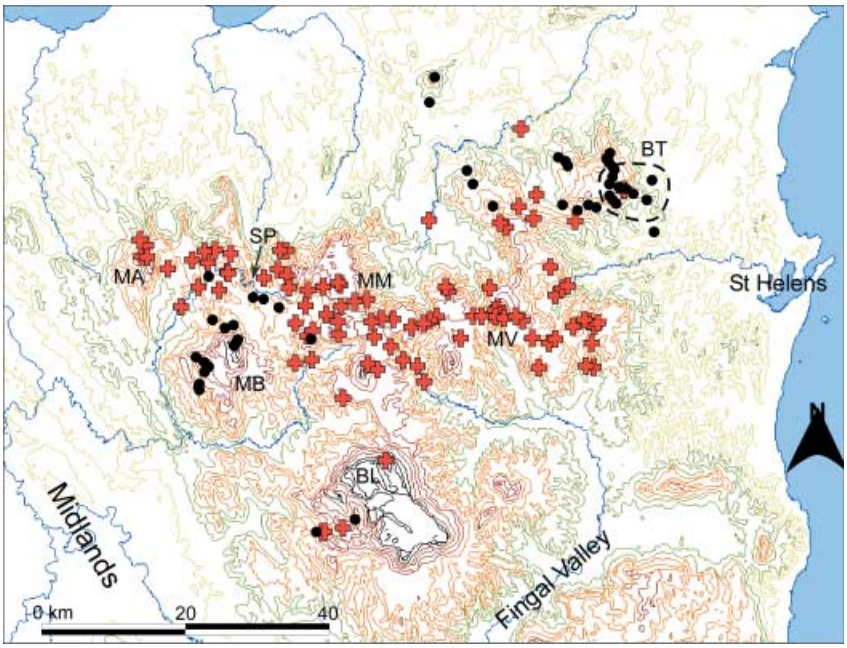


**Fig. 5** Distribution of the NE1 (crosses) and C1 (circles) haplotypes in 149 *Nothofagus cunninghamii* individuals covering almost the entire species range in the northeast highlands of Tasmania. Features mentioned in the text are Blue Tier (BT), Ben Lomond (BL), Mt Barrow (MB), Mt Arthur (MA), Mt Maurice (MM), Mt Victoria (MV), and the St Patricks River (SP). The black broken circle surrounds the purported location of the Blue Tier glacial refugium from Kirkpatrick & Fowler (1998).

from 43 samples > 500 m asl) than at intermediate altitudes (five haplotypes from 48 samples, or when rarefied to 43 samples, 4.9 haplotypes).

### Northeast highlands haplotype fine-scale study

Of a total of 149 *N. cunninghamii* individuals sampled from the northeast highlands, 52 carried the widespread C1 haplotype and 97 the NE1 haplotype. A high degree of spatial structuring of haplotypes was observed, with two small clusters of the C1 haplotype near the eastern (Blue Tier) and western (Mt Barrow) edges of the region, separated by a large area occupied only by the NE1 haplotype (Fig. 5). The number of individuals with a nearest neighbour bearing the same haplotype was highly nonrandom ($P < 0.001$). The contact zone between the Mt Barrow cluster and the NE1 haplotype appears to coincide with the course of the St Patricks River. The two haplotypes overlapped only at the southern extreme of the range and at Blue Tier. Both NE1 and C1 haplotypes occurred at altitudes ranging from < 200 m asl to > 1200 m asl. Both included large tree and small subalpine shrub forms.

## Discussion

### Antiquity of haplotypes

*Nothofagus cunninghamii* was found to harbour remarkably deep and ancient chloroplast divergence. This is strongly indicated by the nesting of the single *N. moorei* haplotype amongst the chloroplast variation found within *N. cunninghamii*. Fossil evidence implies that the split between *N. moorei* and *N. cunninghamii* occurred at least 0.78 million yr ago (mya) because this is the minimum age of fossils of both *N. cunninghamii* and an extinct sister species (Jordan, 1999; Jordan & Hill, 1999). It is unlikely that *N. moorei* is a more recent derivative of *N. cunninghamii*, because *N. moorei* retains pleisomorphic leaf and reproductive traits, whereas *N. cunninghamii* has a range of apomorphic traits (Jordan & Hill, 1999). Indeed, it is likely that the divergence is much older, with estimated molecular dates ranging from 4.2 to 43.6 mya based on chloroplast DNA and 5.1 to 22.4 mya based on ITS sequence data (Knapp *et al.*, 2005; unpublished dates from the analyses of Cook & Crisp, 2005; M. Crisp pers. comm.).

There are other explanations for the nesting of the single *N. moorei* haplotype within haplotype variation observed in *N. cunninghamii*. One hypothesis is transfer of chloroplast DNA through direct hybridization between *N. cunninghamii* and *N. moorei*. Plant species can capture the maternally inherited chloroplast of other species through repeated backcrossing of interspecific hybrids (as shown in *Quercus* (Dumolin-Lapégue *et al.*, 1997a) and *Eucalyptus* (McKinnon *et al.*, 2004)). However, recent chloroplast capture is unlikely in the present case because *N. moorei* and *N. cunninghamii* are now separated by *c.* 780 km, and *N. moorei* has a single haplotype across a 500 km range. By contrast, ancient chloroplast capture (either with *N. moorei* or using an extinct species as a bridge) is possible. Extinct relatives of *N. cunninghamii* and *N. moorei* are known to have occurred in Tasmania up to the Early Pleistocene (Hill & Read, 1991; Jordan, 1999). The likelihood of ancient hybridization is heightened by the fact that barriers to hybridization appear low within *Nothofagus*

subgenus *Lophozonia*. Thus, spontaneous hybrids occur in cultivation between New Zealand and Chilean species (Wingston, 1979), and natural hybridization between species is common in Chile and Argentina (Donoso & Landrum, 1979; Marchelli & Gallo, 2001, 2004). A final hypothesis is lineage sorting, in which the gene pool of *N. cunninghamii* has retained several disparate ancestral cpDNA lineages whose divergence pre-dated speciation. If correct, this interpretation would indicate that divergence of clades 1 and 2 and haplotype NE1 is likely to be millions of years old.

### Western Tasmania: the major reservoir of chloroplast diversity

Western Tasmania is the stronghold of haplotype diversity in *N. cunninghamii* with 18 endemic haplotypes including three clade 2 haplotypes (WT14, WT15 and WT16) that demonstrate considerable divergence from the commonest haplotype (C1). The rarefaction analyses show clearly that this high diversity is not an artefact of more intensive sampling in this region. The antiquity of haplotypes and high haplotype diversity within western Tasmania are both consistent with a long history of *N. cunninghamii* within the region. Similar interpretations of long-term occupation of an area based on high chloroplast diversity and ancestral variation have been made in a three *Quercus* spp. complex in the western Mediterranean basin (Jiminez *et al.*, 2004) and *Lithocarpus* spp. in southeast Asia (Cannon & Manos, 2003). This is the first genetic evidence that demonstrates the importance of western Tasmania as a reservoir of genetic diversity for cool temperate rainforest species in southeastern Australia, a role that has been postulated based on the restriction of many paleo-endemic (e.g. *Athrotaxis* spp., *Lagarostrobos franklinii*, *N. gunnii*, *Microcachrys tetragona* and *Diselma archeri*) cool temperate rainforest plant species to this region (Colhoun, 1985).

### Molecular evidence for glacial refugia outside regions of expected survival

The complex patchy distribution of haplotypes of *N. cunninghamii* across its range strongly supports the concept of multiple glacial rainforest refugia. The evidence for this comes from many parts of the species' range containing endemic haplotypes combined with evidence of antiquity of the haplotypes. In terms of the location of glacial refugia, the high haplotype diversity at low altitudes in western Tasmania (including seven rare haplotypes observed only below 80 m asl) is remarkably consistent with estimates of the LGM tree line (Colhoun, 1985; Gibson *et al.*, 1987) where survival of cool temperate rainforest species is thought to have occurred (Colhoun, 2000). However, the significant number of haplotypes restricted to high altitudes (a total of five; including three haplotypes observed only above 800 m asl) suggests that *N. cunninghamii* may also have survived well above the estimated LGM tree line, but below the permafrost zone. The high diversity of haplotypes at high altitudes is unlikely to be a result of a 'phalanx' type postglacial expansion, whereby a high diversity of haplotypes can be retained during migration (Hewitt, 1996), because five of the high-altitude haplotypes were not found at low or mid-altitudes. Evidence for high-altitude refugia was unexpected because *N. cunninghamii* currently has a very limited occurrence above the climatic tree line (Macphail, 1975; Harle *et al.*, 1993; Kirkpatrick & Fowler, 1998).

The restriction of haplotype NE1, which fell outside both clades 1 and 2, to the northeast highlands of Tasmania (Fig. 1) is stark evidence for glacial survival of *N. cunninghamii* within this region. Considering that the divergence of this haplotype from other *N. cunninghamii* haplotypes is at least as old as the divergence between *N. cunninghamii* and *N. moorei* haplotypes, *N. cunninghamii* has plausibly survived through multiple glacial/interglacial cycles of the Pleistocene in the northeast of Tasmania. An explanation that does not require glacial survival must involve Holocene dispersal from a population outside the northeast. However this scenario is unlikely since neither the NE1 haplotype nor any related haplotype was observed in 187 samples covering nearly the entire species' range in western Tasmania and Victoria, including all populations close to the northeast highlands. We infer, therefore, that glacial refugia harbouring the NE1 haplotype occurred in the vicinity of some of the major mountain peaks (e.g. Mt Victoria, Mt Maurice and Mt Arthur), or in lowland riparian habitats, within the haplotype's current distribution. This is the first genetic evidence of glacial refugia for cool temperate rainforest species in the northeast highlands. This finding fits well with the occurrence of a distinctive invertebrate fauna that includes some slow-dispersing centipede, millipede, snail and beetle species endemic to the region (Mesibov, 1994, 1997; Munks *et al.*, 2004).

The strong spatial structuring of the C1 and NE1 haplotypes observed in the northeast Tasmanian highlands suggests at least two, and plausibly more, refugia within the northeast. The two C1 haplotype patches near the eastern and western extremes of the distribution of *N. cunninghamii* in the northeast (Mt Barrow and Blue Tier) may best be explained as being derived from postglacial expansion from populations that survived the LGM *in situ* within these two areas. It is unlikely that two long-distance seed dispersal events from western Tasmanian refugia were able to found both these patches during the Holocene. A long-distance seed dispersal explanation is especially unlikely considering these events would have had to occur in a narrow window before individuals harbouring the NE1 haplotype were able to expand into these two areas.

Chloroplast DNA evidence for multiple refugia within northeast Tasmania is hard to reconcile with the strong

evidence that the eastern Tasmanian climate was drier than present during the LGM. The evidence for this comes from widespread occurrence of arid-formed sand dunes in lowland parts (Bowden, 1983; Colhoun, 2002; Duller & Augustinus, 2006) and the confinement of glaciation to one very small part of the southeastern flank of the Ben Lomond plateau (Barrows *et al*., 2002; Fig. 5), despite these mountains being as high as the heavily glaciated mountains of western Tasmania (Derbyshire, 1966; Colhoun & Fitzsimons, 1990). In particular, it is difficult to reconcile arid-formed sand dunes with sufficient growing season rainfall required to sustain *N. cunninghamii*. Even by forcing their palaeoclimatic model to have relatively wet climates in eastern Tasmania during the LGM, Kirkpatrick & Fowler, (1998) could not infer climates suitable for *N. cunninghamii* in the western and central parts of the northeast (i.e. outside of Blue Tier, BT; Fig. 5), areas which our data suggests should have contained refugia.

One possible explanation for survival in such dry environments would be that the past tolerance of the species was greater than would be predicted from its current distribution. This hypothesis is reinforced by the high diversity of haplotypes found at high altitudes in western Tasmania – at altitudes more than 300 m above the estimated last glacial treeline. The ability of *N. cunninghamii* to coppice from basal buds after desiccation of the main stem (Howard, 1973) may have contributed to the persistence of this species through glacial periods, although it appears unlikely that individuals could have survived by this mechanism through the full 8000-yr span of the peak of the last glacial. This would mean that all life stages would have been exposed to full glacial climates.

## The widespread subclade and Victorian populations

Although subclade A contained 86% of the samples in the broad-range survey and occurred across the entire range of *N. cunninghamii*, it is a relatively derived clade (Fig. 3) and at least three, and plausibly five or more, haplotype lineages were present at the time that subclade A evolved. Given that these other lineages are now very restricted in spatial range and mostly uncommon, this would suggest that individuals carrying haplotypes from subclade A expanded to cover the entire range of the species, while other haplotypes present at that time were displaced or failed to expand. Trees carrying haplotypes of subclade A may have had an adaptive advantage over other clades. This expansion is likely to predate the last glacial given the number of derived haplotypes in this clade. Although the diversity of subclade A haplotypes is highest in western Tasmania, which suggests that this region is likely the origin of this clade, the presence of the *N. moorei* haplotype (now geographically located more than 1000 km to the north of Tasmania) nested within the *N. cunninghamii* lineage shows that the ancient history of the subgenus in southeastern Australia is complex.

The phylogeny (Fig. 3) further suggests that the C1 haplotype is likely to be the ancestral haplotype for subclade A because all the other haplotypes within this subclade can most parsimoniously be derived from it. Thus, the widespread distribution of C1 is not evidence for Holocene migration. While Holocene migration may be a possible explanation, the presence of the C1 haplotype in southern Victoria may equally represent an earlier expansion of subclade A. The presence of fossil leaves and fruit of *N. cunninghamii* on King Island in the Last Interglacial (G. J. Jordan, unpublished) would suggest a potential corridor for this expansion (Fig. 1).

The central highland Victorian subclade V1–V3 corroborates the pollen evidence from within this region for *in situ* LGM survival of *N. cunninghamii* (McKenzie, 1997). An alternative scenario whereby these haplotypes arose after Holocene migration into the region is unlikely because of the presence of three related, endemic haplotypes and the complete absence of the C1 haplotype that is elsewhere so widespread.

The haplotype contact zone in the Strzelecki Ranges (Fig. 4a) may represent recent migration of the C1 and/or V2 haplotypes into this region. Haplotype contact zones in *Fagus crenata* in central Japan (Kobashi *et al*., 2006) and *Quercus robur* in southern Finland (Ferris *et al*., 1998) have been interpreted as Holocene events. However, whether the 'islands' of cool temperate rainforest in the Otway Ranges and Wilsons Promontory are derived from glacial refugia contained within these two regions is uncertain, a question that may be addressed by phylogeographic studies of more widespread rainforest species that occur in one or both of the Otway Ranges and Wilsons Promontory, such as *Acmena smithii* (Myrtaceae), *Atherosperma moschatum* (Atherospermataceae), *Elaeocarpus reticulatus* (Elaeocarpaceae), *Hedycarya angustifolia* (Monimiaceae), *Lomatia fraseri* (Proteaceae), *Pittosporum bicolor* (Pittosporaceae) and *Tasmannia lanceolata* (Winteraceae).

## Conclusions

The complex patchy distribution of haplotypes provides strong evidence that most current populations are derived from short-range dispersal probably < 100 km from nearby glacial refugia. This contrasts with the continent-wide postglacial movements apparent in some forest trees in Europe (Demesure *et al*., 1996; Dumolin-Lapégue *et al*., 1997a). Considering the antiquity of haplotypes in *N. cunninghamii*, patterns of genetic variation almost certainly significantly pre-date the LGM, a finding that adds to the increasing literature on the ever deeper phylogeographies present in current populations of forest trees (Lumaret *et al*., 2002; Grivet *et al*., 2006; Hampe & Petit, 2007; Magri *et al*., 2007). This study provides the first genetic evidence of the importance of the topographically diverse western half of Tasmania in providing long-term buffered climates for rainforest species throughout the Pleistocene. Interestingly,

the chloroplast evidence indicates that *N. cunninghamii* was able to survive in regions that were unexpected, based on our knowledge of glacial climates in southeastern Australia. The most extreme case appears to be the strong evidence for multiple refugia of *N. cunninghamii* within the northeast highlands, a finding that adds to the growing body of evidence for temperate tree survival in nonequable climates beyond the understood physiological tolerances of species during the LGM and further demonstrates the important role that mountainous regions have in providing refugia though climatic changes (Hewitt, 2000, 2004).

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** *Nothofagus cunninghamii* sample information for the range-wide chloroplast study (213 samples), including sample information for the five *N. moorei* samples

**Table S2** Sample information for the Tasmanian northeast highlands haplotype fine-scale study (129 samples)

**Table S3** Amplification conditions and the approximate size (bp) of PCR products for each primer pair that successfully amplified *N. cunninghamii* cpDNA

**Table S4** Genbank accession numbers of each unique sequence and the aligned length in base pairs obtained for each of the five fragments sequenced in *N. cunninghamii*

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