MOLECULAR ECOLOGY

Molecular Ecology (2010) **19**, 2949–2963 doi: 10.1111/j.1365-294X.2010.04725.x

Chloroplast evidence for geographic stasis of the Australian bird-dispersed shrub *Tasmannia lanceolata* (Winteraceae)

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Abstract

Few chloroplast-based genetic studies have been undertaken for plants of mesic temperate forests in the southern hemisphere and fossil-based models have provided evidence of vegetation history only at the broadest scales in this region. This study investigates the chloroplast DNA phylogeography of *Tasmannia lanceolata* (Winteraceae), a fleshy-fruited, bird-dispersed shrub that is widespread in the mountains of southeastern Australia and Tasmania. Thirty haplotypes were identified after sequencing 3206 bp of chloroplast DNA in each of 244 individuals collected across the species' range. These haplotypes showed unexpectedly strong phylogeographic structuring, including a phylogeographic break within a continuous part of the species' range, with the distribution of four major clades mostly not overlapping, and geographic structuring of haplotypes within these clades. This strong geographic patterning of chloroplast DNA provided evidence for the survival of *T. lanceolata* in multiple putative wet forest refugia as well as evidence for additional wet forest species refugia in southeastern Australia. In western Tasmania lower haplotype diversity below the LGM tree line compared to above the LGM tree line suggests that glacial refugia at high altitudes may have been important for *T. lanceolata*. The level of geographic structuring in *T. lanceolata* is similar to gravity dispersed southern hemisphere plants such as *Nothofagus* and *Eucalyptus*. Behavioural traits of the birds transporting seed may have had a strong bearing on the limited transport of *T. lanceolata* seed, although factors limiting establishment, possibly including selection, may also have been important.

Keywords: chloroplast DNA, fleshy fruit, glacial refugia, phylogeographic break, widespread, Winteraceae

Received 3 November 2009; revision received 14 May 2010; accepted 17 May 2010

Introduction

Although not subjected to climatic changes as severe as those at similar latitudes in the northern hemisphere, mid- to high-latitude forests of the southern hemisphere changed in both extent and composition during the Pleistocene (2.5 million years to 12 000 years ago). In temperate parts of Australia, New Zealand and South America, fossil pollen evidence suggests that areas presently covered by mesic forests were dominated by steppe and grassland during the Last Glacial Maximum (LGM; ~19–17 000 years ago), and probably also during earlier glacial periods (Markgraf *et al*. 1995). Recapture of this landscape by forest in the early Holocene has been attributed by several authors to the ability of forest species to recover rapidly by expansion over short distances from multiple 'micro-refugia' (Macphail & Colhoun 1985; McGlone 1985).

However, this model of postglacial recovery for southern hemisphere mesic forests remains largely untested because of the relatively small number of phylogeographic studies in the region (Beheregaray 2008). In southeastern Australia, mesic forests occur in

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a zone of high rainfall (>800 mm) on the island of Tasmania and in the mountain ranges of Victoria and New South Wales (NSW). These forests are mostly dominated by eucalypts and include both open-canopied forests, and pockets of closed-canopied forests in areas of lower fire frequency, especially where rainfall is higher. During the LGM, the climate of southeastern Australia was probably at its most unfavourable for forest growth at any time during the last two million years (Hope 1994). The LGM climate has been described as 'glacial arid' (Macphail 1979), with Tasmania being 6–8 °C colder (Colhoun 1996; Mackintosh *et al.* 2006) and the mountains of the southeast Australian mainland being 8–10 °C colder than the present in their warmest month (Galloway 1965; Barrows *et al.* 2004). The climatic tree line was 1500 m lower than at present in parts of the mainland (Singh & Geissler 1985) and near the present sea level in Tasmania. The climate was also considerably drier than at present, with annual rainfall being perhaps 50% of current levels (Colhoun 2000). As a consequence, the vegetation of most parts of southeastern Australia during the LGM is believed to have been almost treeless (Hope 1994; Hill 2004). Alpine vegetation expanded in mountainous areas of the mainland (Ladd 1979b; McKenzie 1997) and extended to low altitudes in western Tasmania (Colhoun & Van De Geer 1986; Gibson *et al.* 1987). Nevertheless, areas of dry woodlands dominated by *Eucalyptus* and Casuarinaceae (Williams *et al.* 2006), and restricted patches of rainforest are thought to have been present.

Investigations of chloroplast DNA (cpDNA) variation across the ranges of plant species can complement the fossil record by providing independent evidence for the location of glacial refugia. In addition, because cpDNA is dispersed by the seed and not pollen in most flowering plants (Mogensen 1996), the study of cpDNA variation may also provide new insights into the spatial extent of migration for individual species (McCauley 1995; Cain *et al.* 2000). Recent studies of the distribution of chloroplast DNA variation have shed light on how some major southeastern Australian mesic forest tree species responded to past climate change. Chloroplast phylogeographies identified strong structuring of chloroplast variation in the dominant cool temperate rainforest tree, *Nothofagus cunninghamii* (Worth *et al.* 2009), and in *Eucalyptus regnans* (Nevill *et al.* 2009), a tall mesic forest species that co-occurs with rainforest species in areas with infrequent but catastrophic fires. The cpDNA evidence from *N. cunninghamii* supported fossil data indicating the LGM survival of this species in western Tasmania (Van Der Geer *et al.* 1994; Colhoun 2000) and the central highlands of Victoria (McKenzie 1997), and provided strong genetic evidence for LGM survival in eastern Tasmania (Worth *et al.* 2009), a region with only mid-late Holocene fossil pollen records of the species. Importantly, the genetic evidence of *N. cunninghamii* and *E. regnans* supports the hypothesis of Macphail & Colhoun (1985) that the recovery of mesic forest species from LGM climates involved limited migration during the Holocene from multiple glacial refugia.

However, *N. cunninghamii* and *E. regnans* have gravity dispersed seed (Cremer 1966; Howard 1973) and the response of forest species with differing dispersal traits has not been investigated in southeastern Australia. In particular, little is known about the phylogeography of southern hemisphere fleshy fruited plants. As these plants are typically dispersed by vertebrates, especially birds (Snow 1970; Van Der Pijl 1982), they are considered to have a strong potential for efficient seed dispersal (e.g. Ellner & Shmida 1981; Snow & Snow 1988; Fineschi *et al.* 2005). Phylogeographic studies of fleshy-fruited plants have mostly shown low levels of geographic structuring of chloroplast DNA, consistent with expectations of frequent effective dispersal of their seed (Raspe *et al.* 2000; Oddou-Muratorio *et al.* 2001; Aguinagalde *et al.* 2005). However, contrasting results were reported for a few fleshy-fruited species in Europe (Grivet & Petit 2002; Rendell & Ennos 2003; Fér *et al.* 2007). Phylogeographic structuring within *Hedera* sp. in Europe, for example, was attributed to life-history traits restricting the species' capacity to invade established populations (Grivet & Petit 2002). In another study, high seed mobility was inferred for central and northern European populations of *Frangula alnus*, but strong phylogeographic structuring in the southern extreme of the species' range was explained by the absence of migratory birds in this region (Hampe *et al.* 2003).

This study investigates the chloroplast phylogeography of a bird dispersed, fleshy fruited shrub *Tasmannia lanceolata*, a widespread species that transgresses a number of major dryland barriers in continental Australia and the island of Tasmania (Fig. 1). We also examine whether bird dispersal in *T. lanceolata* has resulted in a different history for this species than for the gravity dispersed mesic forest taxa, that is, one that may have involved extensive tracking of climate (McGlone 1996) and dispersal between populations, including movements across dryland barriers.

Materials and methods

The study species

Tasmannia lanceolata (Poiret) A. C. Smith (Winteraceae), mountain pepper, is a dioecious (Curtis & Morris 1993), insect pollinated (Peter McQuillan, personal communication) shrub or small tree occasionally reaching heights

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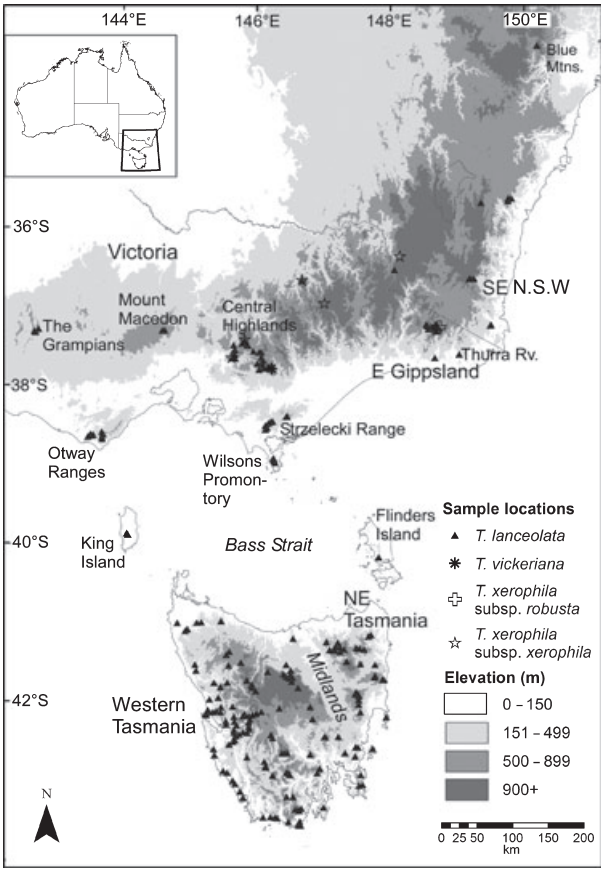


Fig. 1 Location of all 244 *Tasmannia lanceolata* samples included in this study indicated by dark triangles (in some cases representing two to three individual plants). Some areas of unsuitable habitat mentioned in text are labelled in italics. Collection locations of three *Tasmannia* taxa that have overlapping distributions with *T. lanceolata* are also indicated, *T. xerophila* subsp. *xerophila* (five samples), *T. xerophila* subsp. *robusta* (three samples) and *T. vickeriana* (two samples).

of 10 m (Floyd 1989). The dried fruit is increasingly becoming utilized as a pepper substitute. *T. lanceolata* is one of eight species of *Tasmannia* in Australia (Guymer 2007) and is distributed across ~ 10° of latitude within the mountainous regions of southeastern Australia as well as the island of Tasmania (Figs 1, 5 and 6), where it is the only representative of the genus. *T. lanceolata* is restricted to locations where annual rainfall exceeds ~ 930 mm (Sniderman 2007) and occurs in a wide range of vegetation types, including wet *Eucalyptus* forest, near the margins or within cool temperate rainforests, along creek lines in dry *Eucalyptus* forest, and, in Tasmania, in alpine shrublands (Vink 1970). It is common as an early successional species in disturbed forest and grassland (Read & Hill 1983; Read 1989) but also colonizes canopy gaps in mature forests (Ellis 1985). Apart from some differences in depth of stomatal encryption observed by Barnes *et al.* (2000) and clines in leaf size along altitudinal gradients (Casey 1991), *T. lanceolata* is markedly uniform in morphology.

T. lanceolata possesses small berry-like fruits (3–7 mm in diameter, 0.17–0.25 g in weight, French 1991; Borzak 2003), each containing 4–18 shiny and irregular shaped seeds (Guymer 2007). The Green Rosella (*Platycercus caledonicus*; Psittacidae) is considered the main bird disperser of *T. lanceolata* at low altitudes in Tasmania (Read & Hill 1983) and often regurgitates intact seed of *T. lanceolata* (Read & Hill 1983). In the same region, the omnivorous Black Currawong (*Strepera fuliginosa*; Cracticidae) also consumes *T. lanceolata* and regurgitates intact seed (Cash 1998; Borzak 2003). *Platycercus* and *Strepera* species are also common throughout the mainland range of *T. lanceolata* (Blakers *et al.* 1984), though their role in dispersal has not been confirmed. Ants also disperse the fruit of *T. lanceolata* (Howard 1974).

Sampling

Leaves were sampled from 244 *T. lanceolata* individuals spanning the distributional range of the species (Fig. 1; see Table S1, Supporting information for a list of all *T. lanceolata* sample locations). Two hundred and thirty of these individuals were separated by more than 400 m, while fourteen individuals at least 10 m apart were sampled from some small, isolated populations.

Tasmannia is a diverse genus of approximately 50 species (Guymer 2007). Hybridization appears to be important in the evolution of the genus and has been reported between *Tasmannia* species in Australia and in Papua New Guinea (Vink 1970; Sampson *et al.* 1988). Historical hybridization can lead to sharing of haplotypes among species (McKinnon *et al.* 2001), which can complicate the interpretation of phylogeographic patterns (Alvarez & Wendel 2006). We examined the potential for chloroplast exchange between *T. lanceolata* and other species via hybridization by sampling all other southeastern Australian species and subspecies of *Tasmannia*. Thus, multiple samples of taxa that sometimes co-occur with *T. lanceolata*: *T. xerophila* subsp. *xerophila* (five samples), *T. xerophila* subsp. *robusta* (three samples) and *T. vickeriana* (two samples) were collected (Fig. 1), as well as one sample each of *T. glaucifolia*, *T. purpurascens* and *T. stipitata*, and three samples of *T. insipida*. For collection information of these taxa, see Table S2, Supporting information. Three other species of Winteraceae were used as outgroups: one sample of the New Zealand species *Pseudowintera colorata* (cultivated at the University of Tasmania), one sample of the South American species *Drimys winteri* (grown in a private collection by Ken Gillanders, southern Tasmania) and *D. granadensis* provided by a complete cpDNA sequence in GenBank (Cai *et al.* 2006).

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Molecular methods

Total genomic DNA was extracted from 0.25 g of adult leaves using Qiagen DNeasy Plant Mini Kit. DNA quantity and quality were assessed by agarose gel electrophoresis with ethidium bromide staining and comparison with a standard molecular weight marker (Lambda *Hin*dIII). Six regions of chloroplast DNA were amplified using PCR for all samples: the *pet*N–*psb*M and *psb*M–*trn*D intergenic spacers using primers *pet*N1–*psb*M2R and *psb*M2–*trn*D (Lee & Wen 2004); the *trn*L intron and the *trn*L–*trn*F intergenic spacer using primers c–d and e–f (Taberlet *et al.* 1991); and the *trn*K intron (including part of the *mat*K gene) using primers K1-*mat*K1 and *mat*K6-K2 (Demesure *et al.* 1995; Grivet & Petit 2002). All PCR reactions were performed in a total volume of 25 μL containing 2.5 mM $MgCl_2$; 100 μg/mL of Bovine Serum Albumin; 80 μM each of dATP, dCTP, dGTP and dTTP; 5 pM of each primer; 1x 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 approximately 10 ng of genomic DNA (1–2 μL of gDNA). PCR amplifications were performed using a MJ Research PTC-225 Thermal Cycler. PCR conditions were as follows: for *pet*N1–*psb*M2R and *psb*M2–*trn*D reactions, an initial 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min at 51 °C and extension for 2 min at 72 °C, and a final extension step for 10 min at 72 °C; for c–d and e–f, an initial 1 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C and extension for 45 s at 72 °C, and a final extension step for 7 min at 72 °C; for K1-*mat*K1 and *mat*K6-K2, an initial 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 51 °C and extension for 1.5 min at 72 °C, and a final extension step for 10 min at 72 °C.

PCR products were purified using the Qia-Quick PCR purification kit. DNA sequencing was performed in one direction using the forward primers *pet*N1, c, K1 and *mat*K6 and the reverse primers *trn*D and f. A large deletion of 447 bp was found that distinguished *T. lanceolata* *psb*M–*trn*D sequences from those of the other *Tasmannia* taxa and outgroup samples. This indel could be used as a barcode to discriminate *T. lanceolata* from other Australian *Tasmannia* species. To obtain longer read length with these other taxa an internal forward primer (5′ ACTCGGGATCTAATCCCATAGAGA 3′) was used. Sequencing reactions were performed in a MJ Research PTC-225 Thermal Cycler using ABI Prism Bigdye Terminator v 3.0 Cycle Sequencing Kits with AmpliTaq DNA polymerase and run on a 3730*xl* DNA Analyzer. Sequences were aligned using Sequencher 4.5, and checked by eye for incorrect base calls, base pair (bp) substitutions and DNA insertions or deletions (indels). DNA polymorphisms detected in only one sample, including those found in the outgroup samples and the single samples of *T. stipitata* and *T. purpurascens* (except for *T. glaucifolia*), were validated by repeating the PCR and sequencing reaction. Gaps were positioned to maximize conformity to known indel types such as simple and inverted repeats of adjacent sequences (Crayn *et al.* 2006) and to minimize the number of inferred indel events (Graham *et al.* 2000). Two indels that overlapped were scored as a multistate character. Characters that occurred only in outgroup samples and *Tasmannia* taxa samples other than *T. lanceolata*, within the 447 bp section of sequence that was not present in the *psb*M–*trn*D intergenic spacer sequences of *T. lanceolata*, were excluded from the data set.

Phylogenetic estimation and spatial analyses

Maximum parsimony (MP) analysis of cpDNA haplotypes was undertaken using PAUP* version 4.0b10 (Swofford 2000). All characters, including indels, were treated as unordered and of equal weight. The MP analysis used a heuristic search with 1000 replicates of stepwise, random branch swapping addition sequence followed by tree-bisection-reconnection (TBR). Branch support was assessed by bootstrap analysis (Felsenstein 1985) with 100 bootstrap replicates using the same search parameters as those in the parsimony analysis except that 10 heuristic search replicates were undertaken instead of 1000 and 'max trees' was set to 1000. *Pseudowintera colorata*, *D. winteri* and *D. granadensis* were used as outgroups. In addition, a median-joining network with equal weighting of all characters was constructed for all *T. lanceolata* haplotypes using Network 4.5.0.2 (Bandelt *et al.* 1999).

A 3-bp length inversion with perfect flanking inverted repeats (IR's) of 14 bp and $\Delta G = -14.3$ kcal/mol (calculated using OligoAnalyzer 3.1 http://test.idtdna.com/analyzer/Applications/OligoAnalyzer/) within the *psb*M–*trn*D intergenic spacer was excluded from all analyses. Short inversions associated with long IR's show high hairpin stability (Kelchner & Wendel 1996; Catalano *et al.* 2009) and are recognized as being highly homoplastic (Kelchner & Wendel 1996; Sang *et al.* 1997; Graham *et al.* 2000). The inversion identified in this study was inferred as having shifted states at least eight times among the samples (data not shown). Neutrality was tested for each of the fragments separately using Tajima's *D* (Tajima 1989) and Fu and Li's *D** and *F** statistics (Fu & Li 1993) undertaken with DnaSP 4.90.1 (Rozas *et al.* 2003) using all 244 *T. lanceolata* samples.

The spatial structuring of *T. lanceolata* haplotypes, clades and subclades was investigated using a number of methods. Firstly, the single nearest geographic

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neighbour for each sample was determined by a specially written macro in SAS 9.1. This program also performed a permutation test (Manly 1991) with 10 000 randomized repeats testing whether the nearest neighbour of each sample was more often of the same haplotype, subclade and clade than expected by chance (a proxy of spatial structure). In order to do this analysis samples that occurred less than 400 m apart (mostly affecting samples from isolated populations) were excluded. Using the same data set, the program SGS version 1.0 d (Degen *et al.* 2001b) was used to identify the number of subclades/clades and haplotypes in common between pairs of samples at 20 spatial distance classes, with 1000 permutations. Clark and Evans' index (R) (Ripley 1981) was calculated using the same program. A clumped and aggregated distribution is indicated by a values of index (R) < 1, an index value of 1 indicates a random distribution and a regular distribution is described by values (R) > 1 (Degen *et al.* 2001a).

Haplotype diversity was compared between samples in three regions (eastern Tasmania, mainland southeastern Australia and western Tasmania) through rarefaction analysis (Simberloff 1979) following the procedure described in (Worth *et al.* 2009). Western Tasmania was defined as being west of a line passing through the northern and southern midlands of the island. Rarefaction analysis was also undertaken by altitude in western Tasmania where low altitude was defined as below and equal to 150 m above sea level (m asl), the approximate estimated tree line during the Last Glacial Maximum (Colhoun 1985; Gibson *et al.* 1987). High altitude was defined as above and equal to 500 m asl, which divided the remaining samples approximately evenly.

Results

Chloroplast variation

A total of 3206 bp of aligned sequence (equal to 2% of the *Drimys granadensis* chloroplast genome; Cai *et al.* 2006) was obtained for all *T. lanceolata* samples. Overall, 157 single base pair nucleotide or indel polymorphisms were observed, including 35 polymorphisms among samples of *T. lanceolata*. Within *T. lanceolata* this variation defined 30 haplotypes (Table S3, Supporting information). The polymorphisms in *T. lanceolata* included 27 single nucleotide polymorphisms (23 transitions and four transversions) and eight indels. The indels varied in length from 1 to 8 bp. Five were exact repeats of the preceding DNA sequence. Partial aligned sequence lengths obtained for *T. lanceolata* were as follows: the *pet*N1–*psb*M intergenic spacer, 331 bp; the *psb*M–*trn*D intergenic spacer, 677–694 bp; *trn*L intron, 491–496 bp; the *trn*L–*trn*F intergenic spacer, 368 bp; and for the *trn*K intron, K1–*mat*K1, 598–606 bp and *mat*K6-K2, 700–711 bp, respectively. All neutrality tests implemented, Tajima's D and Fu and Li's D^* and F^* statistics, were nonsignificant ($P > 0.05$), indicating there was no evidence that the cpDNA regions studied in *T. lanceolata* were under selection. All variant sequences were deposited in GenBank (Table S4, Supporting information).

Phylogenetic relationships of Tasmannia

Parsimony analysis of the full cpDNA data set yielded 151 most parsimonious trees. All haplotypes within *Tasmannia* taxa formed a strongly supported clade (bootstrap percentage, BP = 100%; Fig. 2) defined by 53

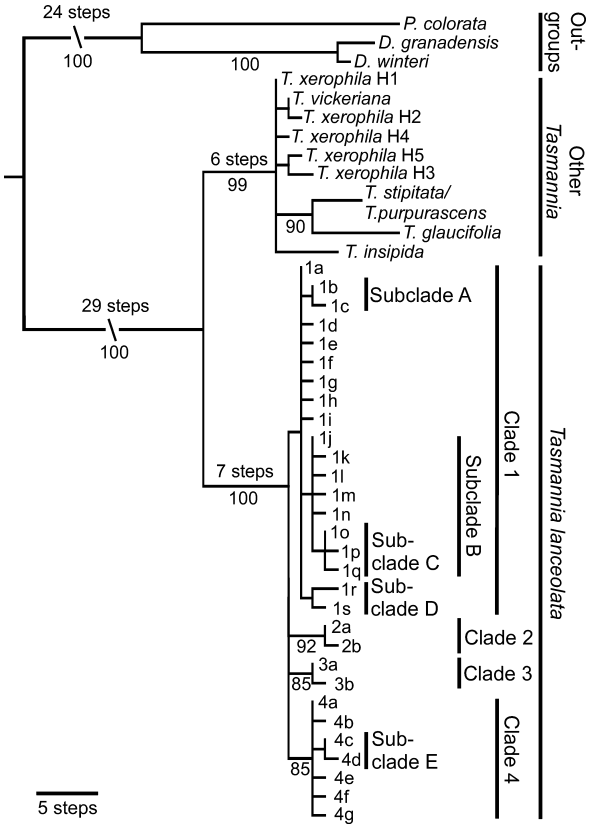


Fig. 2 The inferred phylogenetic relationships among *Tasmannia lanceolata* chloroplast haplotypes and their relationship to other Australian *Tasmannia* species and outgroups constructed via maximum parsimony analysis of 100 informative and 57 uninformative chloroplast characters. This tree is identical to the strict consensus of 151 most parsimonious trees (L = 167, CI = 0.964, RI = 0.986, RC = 0.963). Each haplotype is represented by a number–letter combination. Haplotype clades and subclades within *T. lanceolata* are indicated with numbers and letters (A–E). Bootstrap values above 50% are shown below for branches of length greater than one (Zander 2004). For samples of *T. xerophila* taxa the different haplotypes observed have been given names H1–H5.

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Table 1 Nearest neighbour analysis of Tasmanian and mainland Australian samples of *Tasmannia lanceolata* chloroplast DNA variation. The number of observed individuals (*n*) with a nearest neighbour of the same clade (clades 1, 2, 3 or 4), subclade (A–E) and haplotype are shown with probabilities

	Tasmania (n = 155)			Mainland (n = 75)		
Nearest neighbour	Observed	Expected	P	Observed	Expected	P
Of same clade	136 (88%)	79.8 (52%)	<0.001	69 (100%)	65.1 (94%)	<0.001
Of same subclade	129 (84%)	77.9 (51%)	<0.001	65 (94%)	23.9 (35%)	<0.001
Of same haplotype	106 (69%)	57.1 (37%)	<0.001	53 (77%)	16.5 (24%)	<0.001

differences from a clade containing *P. colorata*, *D. winteri* and *D. granadensis* (Fig. 2). The *Tasmannia* clade was split into two strongly supported clades (Fig. 2), one consisting of all 30 *T. lanceolata* haplotypes (BP = 100%) and the other containing all other *Tasmannia* taxa (BP = 99%). These clades differed by a total of 13 synapomorphies (Fig. 2). The monophyly of *T. lanceolata* suggests that hybridization with other living species of *Tasmannia* has not played a significant role in its evolution and phylogeography. In contrast, the mainland taxa, *Tasmannia xerophila* subsp. *xerophila*, *T. xerophila* subsp. *robusta* and *T. vickeriana* were not monophyletic at the chloroplast level. The 30 haplotypes within *T. lanceolata* were divided into four clades, three of which (clades 2, 3 and 4) had high bootstrap support (Fig. 2). The largest clade (clade 1) was supported by only one nucleotide substitution (Table 1) and contained four subclades (subclades A, B, C and D). Clade 4 contained an additional subclade (subclade E) supported by 1 bp substitution. The haplotype network (Fig. 3) is consistent with the relationships of *T. lanceolata* haplotypes inferred from parsimony analysis, but suggests that haplotype 1a may be ancestral within *T. lanceolata*.

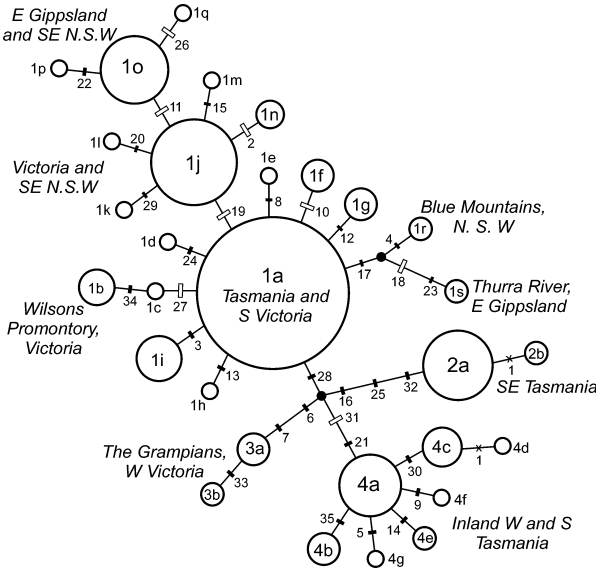


Fig. 3 Median-joining network of all *Tasmannia lanceolata* haplotypes. Each haplotype is represented by a circle and a number–letter combination as in Fig. 2. The size of each circle is proportional to the frequency of the haplotype. Lines connecting each haplotype are proportional to the number of character differences between them. Non-homoplasious single nucleotide base changes are indicated by solid bars, homoplasious single nucleotide base change by crosses, and indels (all non-homoplasious) by open bars. Small filled circles represent inferred haplotypes that were not found.

Phylogeographic structuring of T. lanceolata cpDNA

The chloroplast variation observed within *T. lanceolata* displayed statistically significant phylogeographic structuring. The analyses of nearest geographic neighbours showed that individuals were much more likely to be geographically nearest to individuals of the same haplotype (159 of 230) or the same clade (205 of 230) than to individuals of different haplotypes or clades ($P << 0.001$). Strong spatial structuring occurred within Tasmania and on the mainland (Table 1). In addition, the number of haplotypes and subclades/clades in common within 20 spatial distance classes differed strongly from random spatial distribution (Fig. 4). Positive spatial structure was observed up to a distance of 90–120 km for the analysis based on haplotypes and 210–240 km for the analysis based on subclades/clades. Negative spatial structure was observed at a distance >300 km for both the haplotype and subclade/clade analyses. Strong spatial structuring of clades/subclades was also demonstrated by the presence of a very low Clark and Evans' index (R) value of 0.26.

Haplotype diversity was similar between mainland southeastern Australia and western Tasmania and lowest in eastern Tasmania (Table 2). In western Tasmania, where a total of 14 haplotypes were observed, haplotype diversity was not significantly different (P = 0.212; randomization test) between mid (151–499 m asl) and high (≥500 m asl) altitudes with 8.0 and 8.7 haplotypes, respectively (Table 3). Haplotypes diversity at mid and high altitudes differed significantly (P < 0.001) from the

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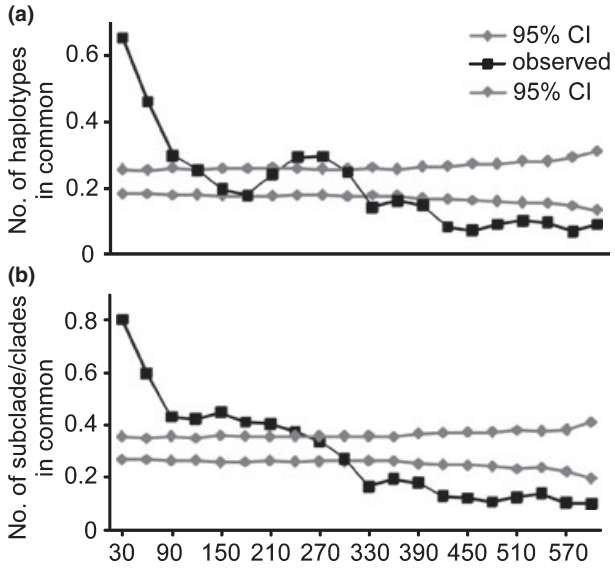


Fig. 4 Distogram depicting the mean number of *Tasmannia lanceolata* haplotypes (a) and subclade/clades (b) in common between all pairs of individuals within 20 spatial distance classes plotted against spatial distance classes (km), together with the 95% confidence limits.

Table 2 Rarefied haplotype diversity in *Tasmannia lanceolata* within eastern Tasmania, mainland southeastern Australia (value in brackets was calculated excluding samples from the Grampians and Blue Mountains) and western Tasmania. Values are the mean number of haplotypes when randomly subsampled to 43 samples, ± standard errors

Region	Rarefied haplotype diversity
Eastern Tasmania	3.0 ± 0.0
Mainland	11.7 ± 1.5 (10.0 ± 1.3)
Western Tasmania	9.6 ± 1.3

Table 3 Rarefied haplotype diversity in *Tasmannia lanceolata* within three altitudinal zones in western Tasmania. Values are the mean number of haplotypes when randomly subsampled to 33 samples, ±standard errors. The haplotypes observed within each altitudinal zone and their frequency is also shown, with haplotypes observed in only one zone shown in bold

Altitudinal zone in western Tasmania	Rarefied haplotype diversity	Haplotypes observed (frequency)
≤150 m asl	3.8 ± 0.44	1a(35), **1d**, 1g (3), 4a (2)
151–499 m asl	8 ± 0.0	1a (23), 1f, 1g, 1i, 4c (3), 4e (2), **4f**, **4g**
≥500 m asl	8.7 ± 0.51	1a (5), **1e**, 1f (3), 1i (7), 2a (2), 4a (13), **4b** (4), 4c (3), **4d**

mean of 3.8 haplotypes observed at low altitudes (≤150 m asl).

A significant feature of the chloroplast phylogeography of *T. lanceolata* was the restricted distribution of most clades, subclades and individual haplotypes (Figs 5 and 6). Populations in three regions harboured endemic clades: clade 4 (7 haplotypes and 12.3% of all *T. lanceolata* samples) in inland western and southern Tasmania; clade 2 (2 haplotypes and 8.6% of all samples) in the predominantly dry (under ~700 mm) central eastern Tasmania; and clade 3 (2 haplotypes and 2.5% of all samples) in the Grampians, Victoria (Fig. 5). The Grampians is an isolated massif, ~210 km from the nearest occurrence of the species at Mount Macedon and the Otway Ranges, with two small populations of *T. lanceolata* (Fig. 1).

Haplotypes of clade 1 (76% of all samples) were extensively distributed in Tasmania and included all haplotypes of *T. lanceolata* observed on mainland Australia, excluding the Grampians populations. Haplotype 1a (41.4% of all samples) was frequent, and often the sole haplotype, in parts of Tasmania and the Bass Strait islands, and extended to the southernmost parts of mainland Australia (Fig. 5). Strong phylogeographic structuring of haplotypes from clade 1 is evident with four subclades (A, B, C and D) occurring in distinct geographical locations where haplotype 1a is rare or absent (Fig. 6).

In addition, the lack of geographic overlap of clades and subclades was marked and was characteristic of not only disjunct populations (e.g. Wilsons Promontory and the Grampians), but also occurred within more or less continuous populations with no obvious barriers to seed flow (Figs 5 and 6). The only regions where overlap was observed were: (i) in western Tasmanian, at the northern and southern edges of the range of the distribution of clade 4, where haplotypes of both clade 1 and 4 were observed and (ii) in the Strzelecki Ranges of southern Victoria where haplotype 1a of clade 1 was in close proximity to haplotypes of subclade B (Figs 5 and 6). Four haplotypes trangressed major areas of unsuitable habitat; haplotype 1a, which spanned Bass Strait and occurred in both northeastern and western Tasmania across the dry Tasmanian Midlands, 1j and 1o in Victoria and southeast NSW, and 2a in eastern Tasmania on both sides of the Midlands.

Discussion

The cpDNA variation in *T. lanceolata* shows a remarkably strong phylogeographic structure, with most clades, subclades and haplotypes geographically restricted. Hypothetically, the observed cpDNA structure could have arisen recently through rapid evolution of the

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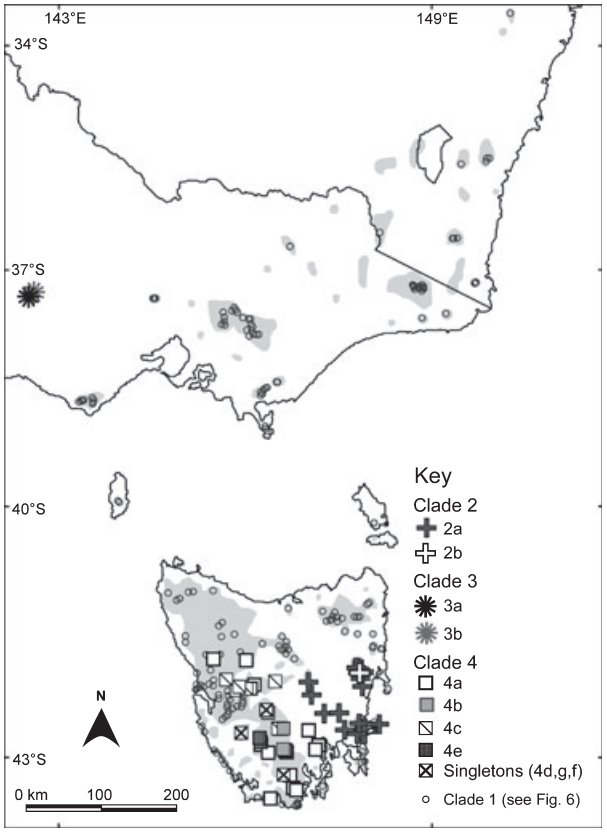


Fig. 5 Distribution of *Tasmannia lanceolata* haplotypes from clades 2, 3 and 4 overlying the natural distribution of the species (grey). For reference, each sample location where haplotypes of clade 1 were observed is shown as a small open grey circle. Each haplotype is indicated except for singletons (haplotypes observed only once), which share the same symbol. Information on the distribution of *T. lanceolata* was obtained from the Natural Values Atlas (Department of Primary Industries and Water, Tasmanian Government), Australia's Virtual Herbarium (http://www.ersa.edu.au/avh/), Floyd (1989, 1990), Raleigh *et al.* (1994) and personal observations.

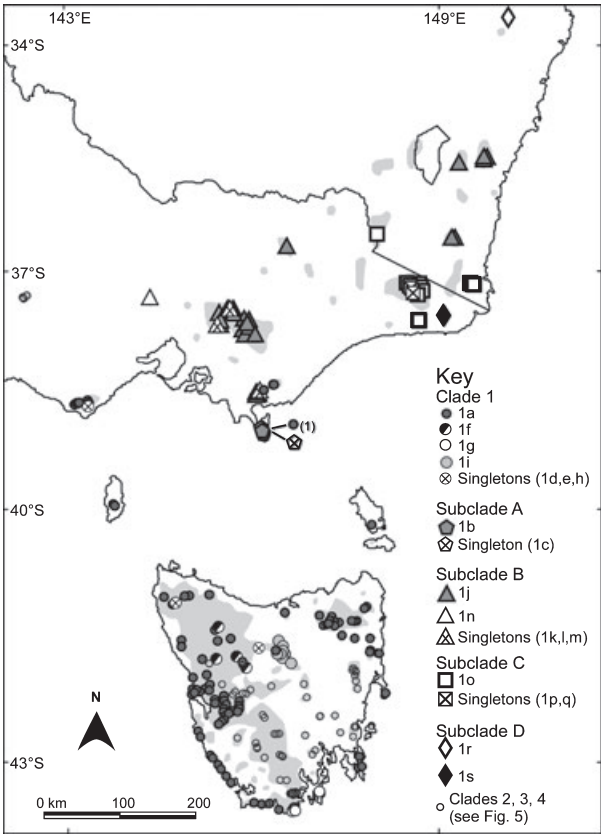


Fig. 6 Distribution of *Tasmannia lanceolata* haplotypes from clade 1 overlying the natural distribution of the species (grey). For reference, each sample location bearing haplotypes of other clades is displayed as a small open grey circle. Each haplotype is distinguished except for singletons (haplotypes observed only once), which share the same symbol.

chloroplast. However, this is very unlikely considering evidence for slow chloroplast evolution (Wolfe *et al.* 1987; Clegg *et al.* 1994), especially in Winteraceae (Marquínez *et al.* 2009), and the significant number of mutations observed within the species (a maximum of 8 between the most diverged haplotypes; Fig. 4). The strong 'patchiness' of haplotype clades or subclades is most likely the consequence of the existence of isolated refugia within or close to those patches through the Last Glacial Maximum (LGM) and possibly multiple glacial periods of the Pleistocene. The large number of geographically restricted clades and subclades suggests the former presence of multiple refugia for *T. lanceolata*. In addition, the strong phylogeographic structure of *T. lanceolata* provides compelling evidence that Holocene dispersal was limited for this species.

In western Tasmania, an endemic clade (clade 4) and five endemic clade 1 haplotypes provides further evidence for the importance of this part of southeastern Australia as a reserve of genetic diversity of mesic forest species (Worth *et al.* 2009) and supports the LGM fossil pollen record of *T. lanceolata* in the region (see Fig. S1, Supporting information). Western Tasmania has similar haplotype diversity to mainland Australia, despite being geographically smaller. In western Tasmania, this diversity is concentrated above the LGM climatic tree line with five haplotypes found only at mid (151–499 m asl) to high altitudes (≥500 m asl) versus one haplotype restricted to low altitudes below the LGM tree line (≤150 m asl). The potential role of high altitude refugia for mesic woody species is supported by the presence of multiple high altitude endemic haplotypes in *N. cunninghamii* (Worth *et al.* 2009). Apart from the possibility that LGM populations of *T. lanceolata* that survived below the LGM tree line have subsequently experienced extensive extinction in western

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Tasmania, the higher haplotype diversity at mid to high altitudes suggests that glacial refugia above the LGM tree line may have been important for *T. lanceolata* in this region.

This study provides unexpected evidence for long-term survival of wet climate species in two regions: central eastern Tasmania and the Grampians of western Victoria. Each of these areas harbours an endemic *T. lanceolata* clade. In central eastern Tasmania *T. lanceolata* and other wet climate species are uncommon and restricted to small and scattered populations. In addition, no cool temperate rainforest or wet *Eucalyptus* forest refugia were inferred in this area at the height of the LGM using palaeoclimatic reconstruction (Kirkpatrick & Fowler 1998). Similarly, the Grampians are isolated from other mesic regions, climatically very marginal for wet climate species, and it is likely that LGM climates in the area were much drier than at present (Crowley & Kershaw 1994). However, there is other evidence suggesting that these regions may have provided glacial refugia for a range of woody species. Chloroplast DNA evidence suggests the long-term survival of *Eucalyptus* subgenus *Symphomyrtus* (a clade of trees or shrubs now ranging from very wet to very dry climates) in central eastern Tasmania (Freeman *et al.* 2001; McKinnon *et al.* 2001). Furthermore, both central eastern Tasmania and the Grampians are centres of local endemism of plant species (Kirkpatrick & Brown 1984; Crisp *et al.* 2001). It would be of interest to examine the level of genetic divergence of other mesic forest species that occur in these regions, for example, *Dicksonia antarctica*, *Notelaea ligustrina* (Jeanes 1999), *Pomaderris apetala* (Walsh 1999) and *Prostanthera lasianthos* (Conn 1999).

In addition to this evidence for otherwise unexpected refugia, the cpDNA phylogeographic evidence supports the long-term survival of this species in other regions lacking LGM fossil records for any mesic forest species, including *T. lanceolata* (see Fig. S2, Supporting information). Thus, endemic chloroplast variants occur in Wilsons Promontory (subclade A), the east Gippsland region (subclade C and haplotype 1s) and the Blue Mountains (haplotype 1r). Both Wilsons Promontory and east Gippsland also contained endemic chloroplast haplotypes of the mesic eucalypt, *E. regnans* (Nevill *et al.* 2009) and the former has previously been postulated as containing glacial refugia for forest species (Ladd 1979a, 1991; Hope 1994).

The origin of the distribution of the four haplotypes that transgress significant areas of uninhabitable landscape (1a, 1j, 1o and 2a) is ambiguous but does not contradict evidence for extreme geographic stasis within this species. Haplotype 1a occurs widely in Tasmania and southern Victoria, 1j occurs widely in eastern Victoria and southeastern NSW, 1o occurs in small isolated patches across either side the New South Wales/Victorian border and 2a occurs scattered in small pockets in central eastern Tasmania. In each case, it is possible that they may have been involved in long distance dispersal (pre or post LGM) but each of these haplotypes is the ancestral form for the clade or subclade that occurs across their respective regions. The presence of these haplotypes could also therefore be the result of persistence since prior to the differentiation of haplotypes within each clade. The correlation between the geographical extent and relative ages of the ancestral haplotypes (haplotype 1a is older and more widespread than 1j, which is in turn older and more widespread than 1o) is consistent with the latter explanation. Furthermore, arguments for recent dispersal of these haplotypes are difficult to reconcile with the apparent lack of long distance dispersal in derived haplotypes of these clades/subclades, unless there has been strong selection against invading haplotypes (as discussed below).

Notwithstanding the previous arguments, the almost complete restriction of haplotype 1a to coastal areas, combined with its disjunct distribution spanning Bass Strait, requires some explanation. Some clues may be gained from the remarkably similar sharing of ancestral haplotypes between Tasmania and southern Victoria (most notably between the Otway Ranges and Tasmania) in other tree species, *E. globulus* (Freeman *et al.* 2001) and *N. cunninghamii* (Worth *et al.* 2009), a chloroplast pattern that could be the result of a common movement of forest species during favourable past climates across Bass Strait. The ancestral status of the haplotypes in all three cases means that any movement may predate the last exposure of the Bassian plain during the LGM. In *T. lanceolata*, although the inferred ancestral haplotype 1a is most extensively distributed in Tasmania there is no reason to infer a centre of origin of the species in Tasmania since the current distribution of haplotype 1a could have been impacted by extinction. There is extensive evidence for Pleistocene extinction of wet forest species in Tasmania (Jordan 1997), and the impact of Pleistocene climates on mainland Australia on such species may have been even greater (Hope 1994). In western Tasmania, the rarity of haplotype 1a from high altitude areas may involve association with lowland-adapted genotypes.

Explaining evidence for limited dispersal in a fleshy fruited plant

The strong phylogeographic structure of *T. lanceolata* provides evidence that bird dispersal of *T. lanceolata* fruit/seed has not resulted in effective dispersal frequent enough to erase the genetic signals of glacial

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survival of this species. This contrasts with the weak chloroplast patterning generally observed in fleshy fruited plants in Europe (Raspe *et al.* 2000; Oddou-Muratorio *et al.* 2001; Aguinagalde *et al.* 2005) and is more similar to chloroplast phylogeographies of gravity dispersed mesic forest trees investigated in southeastern Australia so far, including *N. cunninghamii* (Worth *et al.* 2009) and *E. regnans* (Nevill *et al.* 2009), as well as South American *Nothofagus* (Marchelli & Gallo 2006; Azpilicueta *et al.* 2009; Acosta & Premoli 2010). The limited Holocene (and pre-Holocene) dispersal of *T. lanceolata* is probably due to limited transport of seed and other ecological or environmental factors that limit successful establishment. Factors pertaining to seed set and seed viability can be discounted to explain the strong phylogeographic structuring. For example, fruit production, which is positively correlated with visitation of birds (Howe & Estabrook 1977; Takahashi & Kamitani 2004), and seed viability are unlikely to be limiting because *T. lanceolata* commonly produces large seed crops and seedlings of this species are abundant in suitable sites (J. Worth & G. Jordan, personal observations).

Close examination of the relevant dispersal agents reveals that the transport of fruit of *T. lanceolata* may be restricted. Unlike many birds of central and northern Europe (Snow 1970), few terrestrial temperate Australian birds are migratory (French 1992). This apparently applies to both known avian dispersers of *T. lanceolata*, *Platycercus caledonicus* and *Strepera fuliginosa* (Ratkowsky & Ratkowsky 1978), although exploratory movements may be quite large (hundreds of kilometres) in some Australian species (Wimbush 1969). However, the observation that one species of currawong, *S. graculina*, regurgitates most seed five to fifteen minutes after fruit consumption (Bass 1990) suggests that this species is unlikely to transport seed great distances. There is no reason to suspect markedly different behaviour in *P. caledonicus* or *S. fuliginosa*. Alternative vectors for *Tasmannia lanceolata* seed appear unlikely to transport seeds far. Individual movements of the known mammalian consumers of *T. lanceolata* fruit, the brushtail possum (*Trichosurus vulpecula*) and the red-bellied wallaby (*Thylogale billardierii*), are unlikely to exceed 2 km (Clout & Efford 1984; le Mar 2002). For many fleshy-fruited species, a large proportion of the fruit fall to the ground (Rey & Alcantara 2000; Guan *et al.* 2006), and may then be transported by ants (Howard 1974) or water (Hampe & Arroyo 2002). Ant dispersal is generally limited to a few metres (Andersen 1988) while water transport does not provide any mechanism to cross catchments. Although the leaves of *T. lanceolata* are thought to have been an occasional food source for the Tasmanian Aborigines (Cane *et al.* 1979), there is no reason to suspect that Aborigines deliberately transported viable seed of this species for long distances.

Several factors may decrease the probability of successful establishment of *T. lanceolata* after long distance transport of seed. One such factor is the dioecy of the species, because successful establishment of a new population of such species depends on either multiple dispersal events to the same sites or long distance pollination. Some molecular evidence supports this as a process limiting effective dispersal (Schaefer & Renner 2009). However, regurgitants of *T. lanceolata* fruit typically contain multiple seed, potentially reducing the biogeographic significance of dioecy in this species (Baker & Cox 1984).

The presence of abrupt boundaries between the distributions of clades in Tasmania suggests that establishment effects may be significant for *T. lanceolata*. The phylogeographic breaks observed between clade 1 and 4 in western Tasmania, and between clade 2 and clades 1 and 4 in central eastern Tasmania (Figs 5 and 6) could be viewed as being 'snapshots' in time of slowly expanding fronts that harbour the different clades (Neigel & Avise 1986). These phylogeographic breaks could therefore have arisen solely from the lack of effective dispersal (Irwin 2002). An alternative is that competition between *T. lanceolata* individuals reduced the number of effective dispersal events, and therefore slowed the expansion of haplotypes into occupied regions (Hewitt 1996). The development of phylogeographic breaks may also have been impacted by demographic 'inertia'. Populations of *T. lanceolata* in central eastern Tasmania may suffer from demographic 'inertia' as they are widely scattered and separated by dry, and/or fire prone habitat. In this region, low population density, small patch size and patch isolation that characterize these populations may prevent the species exceeding critical thresholds of propagule output (Grime 2002), a process thought to be particularly important in insect-pollinated species (Groom 1998), such as *T. lanceolata*. However, these factors are less likely to be involved in the haplotype boundaries in western Tasmania where *T. lanceolata* populations are generally large and nearly continuous.

Possible selection

Selection for local haplotypes may have contributed to the strong chloroplast patterns observed in *T. lanceolata*, however, all tests for selection acting on the chloroplast sequences were nonsignificant. This, coupled with the near absence of recombination in the chloroplast genome (Ennos *et al.* 1999), suggests that it is unlikely that differential selection for haplotypes has contributed significantly to the observed chloroplast patterns. However, different chloroplast haplotypes may be

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associated with divergent nuclear genotypes (Cruzan & Arnold 1999). Therefore, selection against the nuclear genome of immigrants because of maladaptation may have contributed to the phylogeographic breaks observed within *T. lanceolata*. For example, a phylogeographic break of chloroplast haplotypes within the continuous range of the tropical rainforest tree *Cedrela odorata* in Central America was concomitant with the distribution of two distinct ecotypes of the species (Cavers *et al*. 2003). In *T. lanceolata*, the occurrence of haplotype 1a at mostly low altitudes in western Tasmania is consistent with this distribution being the result of inherited associations of this haplotype with ecotypes adapted to specific ecological characteristics of the habitats of such regions. However, the PCA analysis of the climatic range of haplotypes in Tasmania shows that haplotype 1a has a broad climatic range that almost completely overlaps with haplotypes of clade 2 and clade 4 (see Fig. S2, Supporting information). Furthermore, where strong phylogeographic breaks are evident, the climatic evidence does not provide a convincing case for selection for different ecotypes. In addition, the differences in the climates occupied by haplotypes of clades 2 and 4 could be explained by poor dispersal alone, restricting these clades to separate geographic regions that have contrasting macro-climates associated with a strong rainfall gradient from west to east in Tasmania. Experimental work, including reciprocal field trials, could help resolve the importance of selection in explaining the distribution of haplotypes.

Conclusions

The strong phylogeographic structure in *T. lanceolata* provides evidence that the species has remained within its current range through at least the LGM, and occupied locations that have not previously been reported as glacial refugia for any mesic plant species. The assembly of modern populations has been dominated by the local movements of seed, including during the wet forest expansion of the Holocene, while effective seed flow over long distances (i.e. hundreds of kilometres) has been rare. Considering the probable antiquity of chloroplast variation, the strong phylogeographic structuring of haplotypes may be the consequence of such geographic stasis for multiple glacial-interglacial cycles. The slow effective rate of dispersal of *T. lanceolata* appears to be related to the absence of vectors of long distance dispersal that can distribute seed across the patchy distribution of the species. The two bird species known to consume its fruit have not dispersed it over long enough distances to prevent the development of strong phylogeographic structuring, possibly due to their quick regurgitation of the seed. In addition, factors such as dioecy, and/or selection may be involved in high post-dispersal establishment failure in this species. The implications of this study are twofold. Firstly, it shows that plants with perceived strong dispersal potential may be unable to respond to unfavourable future climatic or landscape change by dispersal and may have to tolerate or adapt to changed conditions or face local or global extinction. Secondly, it provides a strong example supporting the conclusion of Duminil *et al*. (2007) that many factors need to be considered when predicting the potential for effective long-distance dispersal in plants, including vector behaviour and events that effect survival post-dispersal.

Acknowledgments

We would like to thank John and Noela Cross, Jasmine Janes, Anthony Mann, Jon Marsden-Smedley, Simon Whittock, Darren Williams and Dave Woods for sample collection; Ken Gillanders for access to his private collection; and the Department of Sustainability and Environment (Victoria) and the Department of Environment, Climate Change and Water (NSW) for collection permits. We would also like to thank Gregor Sanders for help with construction of Fig. 1. This work was supported by a Discovery grant DP0557260 from the Australian Research Council awarded to René Vaillancourt.

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This study was carried out within the School of Plant Science at the University of Tasmania as part of James Worth's doctoral research on the phylogeography and conservation of Australian cool temperate rainforest plants. J. Worth is mostly interested in utilising molecular tools to better understand processes that shape animal and plant species' distribution and diversity. J. Marthick is interested in plant and animal population genetics and the role of epigenetic markers in cancer genetics. G. Jordan chases the enigma of plant distribution using tools ranging from fossils and plant anatomy to ecology

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and evolutionary reconstruction. G. McKinnon's research encompasses mainly eucalypt population genetics, systematics and hybridization. R. Vaillancourt is a population geneticist specializing in population and conservation genetics of Australian plant species and genomics of *Eucalyptus*.

Supporting information

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

Table S1 *Tasmannia lanceolata* sample information for the range-wide chloroplast study (244 samples). The clade or subclade and haplotype determined for each sample is shown

Table S2 *S*ample information for all *Tasmannia* taxa used as outgroups (16 samples). The haplotype determined for each sample is shown

Table S3 Single base pair nucleotide polymorphisms and insertions/deletions characterising all 30 chloroplast DNA haplotypes observed in *Tasmannia lanceolata*, shown in comparison to the most frequent haplotype 1a. The number of *T. lanceolata* samples with each haplotype is shown in parentheses following the haplotype name. Vertical numbers indicate the bp position of each polymorphism in the complete sequence alignment. The states at variable sites in *T. lanceolata* are also shown for other *Tasmannia* species, *Drimys* and *Pseudowintera*. **T. xerophila* includes *T. xerophila* subsp. *xerophila*, *T. xerophila* subsp. *robusta* and *T. vickeriana* which were identical for the thirty characters variable within *T. lanceolata* but differed for other characters not shown. ** *T. stip./purp.* denotes the single samples of both *T. stipitata* and *T. purpurescens* which were identical for all characters variable within *T. lanceolata* and all other characters not shown

Table S4 Genbank accession numbers for all *Tasmannia lanceolata* haplotypes, variable sequences for other *Tasmannia* species and subspecies and outgroups

Fig. S1 Fossil pollen record of *Tasmannia lanceolata* (Winteraceae) with the current distribution of the species shown in grey. Two macrofossil sites of the species are indicated by arrows. The pollen records of *Tasmannia* on mainland Australia may be other species of the genus not present in Tasmania.

Fig. S2 Estimates of climates for each of the 138 sample sites in Tasmania were made using BIOCLIM (Houlder *et al*. 2003). BIOCLIM created synthetic estimates of 35 climate parameters reflecting annual and seasonal temperature, precipitation, evaporation and moisture availability, from point locations (latitude, longitude and altitude), using a three dimensional spline based on long-term meteorological records. To summarise this large amount of inter-correlated data, principal components analysis was run on all 35 parameters.

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