PART 1- LITERATURE REVIEW

Cryptic Glacial Refugia

PART 2- EXPERIMENTAL THESIS

Chloroplast DNA variation in *Nothofagus cunninghamii* (Hook.) Oerst.

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Submitted in partial requirement for the degree of Bachelor of Science with Honours.

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any degree in any university and that to the best of my knowledge and belief, the thesis contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text.

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ABSTRACT

Nothofagus cunninghamii (Hook.) Oerst. is the dominant component of most cool temperate rainforest in southeastern Australia. *N. cunninghamii* has a long history in the region and has endured through a period of great climatic changes, including the glacial-interglacial cycles. Currently the possible roles of glacial refugia, postglacial migration and long distance dispersal of seed are not well understood to explain the patchy distribution of the species, especially for regions such as northeastern Tasmania where no fossil record of the species is available. The PCR-RFLP technique was employed to determine the pattern of variability of chloroplast DNA using 134 samples across the species range. Four mutations were detected defining five haplotypes, displaying strong geographic structure. A relatively high level of variation detected in a sample size of 72 individuals compared to an initial small sample size of eight demonstrates the importance of using large sample sizes in PCR-RFLP studies for the purpose of finding polymorphic enzyme/fragment combinations. The occurrence of a common and ancestral haplotype across much of the species range makes it impossible to determine the possible roles of migration and fragmentation in regions where this haplotype is the only one present. However, derived haplotypes restricted to the central highlands of Victoria suggest glacial survival, supporting fossil evidence. A haplotype almost restricted to the northeast of Tasmania also suggests glacial survival, but this assertion is not yet definitive since the haplotype was also found in western Tasmania. In addition, two haplotypes restricted to western Tasmania suggest another possible separate refugia in this region. The presence of two haplotypes in southern Victoria may be indicative of a post-glacial migration event across Bass Strait. Further sampling and the detection of greater variation in the chloroplast genome of *N. cunninghamii* are required to make stronger biogeographical conclusions.

CONTENTS

Part 1- Literature Review

Chapter 1- Cryptic glacial refugia……1

1.1 Introduction……1

1.2 How can cryptic refugia be identified……4

1.3 What does the evidence say about cryptic refugia……7

1.4 Conclusions……15

Part 2- Experimental Thesis

Chapter 2- Background……18

2.1 Introduction……..18

2.2 Explaining the current distribution of *N. cunninghamii*: glacial refugia, migration or long distance dispersal?……..20

2.3 Chloroplast DNA……..23

Chapter 3- Materials and Methods……28

31. Sampling Strategy……28

3.2 Molecular Methods……30

Chapter 4- Results……35

4.1 Polymerase Chain Reaction……35

4.2 Restriction Digestion……35

4.3 Genetic relationships and descriptions and description of haplotypes……38

4.4 Distribution of haplotypes……45

4.5 DNA Sequencing……...47

Chapter 5- Discussion……49

5.1 Low variation of chloroplast DNA……49

5.2 Biogeographical inferences from the distribution of hapolytes in *N. cunninghamii*……52

5.3 Future Research……...57

Appendix 1……59

Appendix 2……63

Acknowledgements……..64

References……65

PART 1:

LITERATURE REVIEW

Cryptic Glacial Refugia

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CHAPTER 1

Cryptic glacial refugia

1.1 Introduction

The extensive climatic fluctuations of the Quaternary (the glacial-interglacial cycles), involved large, and sometimes very rapid changes in temperature, precipitation and CO_2 concentration (Davis and Shaw 2001). Changing climates posed problems for the survival of species, and caused major changes in the world's vegetation (Huntley and Birks 1983). This work reviews the intense current debate on where species survived during these extreme conditions of the Quaternary ice ages. Consideration is made of both plants and animals, but the focus is on the survival of plants, especially forest trees, which have received most research attention. The Quaternary refers to the last 1.6 million years (Comes and Kadereit 1998). The last full glacial cycle began at the end of the last interglacial (135 thousand years ago) when the world climate proceeded to glacial conditions. The glacial lasted approximately a hundred and seventeen thousand years ago, giving way to the present interglacial approximately 14000 years ago (Hewitt 1996).

1.1.2 What are refugia?

According to niche theory, species respond to environmental change in four ways: 1) remaining in place by tolerating the changing conditions; 2) adaptation, allowing shift in habitat; 3) migration to suitable areas, sometimes involving long distance dispersal; and 4) extinction from an area resulting in range contraction (Jackson and Overpeck 2000). While many environmental changes influence plant survival, the most intensely studied have been the response to the glacial-interglacial cycles (Bennet *et al.* 1991). Quaternary fossil evidence has indicated extensive shifts in the distribution of species in response to these climatic fluctuations (Willis *et al.* 2000; Brewer *et al.* 2002). At the onset of the Last Glacial (Hewitt 1996) cold adapted species tolerated the conditions and expanded their ranges from steppe and tundra (Frenzel 1968; Ferris *et al.* 1999). The

warm adapted species of temperate forests, unable to tolerate conditions, experienced local extinction. However, for widespread species, not all of their range was equally affected, and they were able to survive where conditions were suitable (Stewart and Lister 2001; Hewitt 1996). In the literature these areas are called refugia, with glacial refugia used to describe an area where a species survived throughout the entire last glacial period (Ravazzi 2002).

Fragmentation of species ranges during glaciations is commonly used to explain current biogeographical patterns, such as the distribution of endemic (Kirkpatrick and Fowler 1998) and disjunct plant populations (McGlone 1985; Schonswetter *et al.* 2003). This hypothesis relies on the species not having recovered their former range, but presumably being in the process of doing so (McGlone 1985). The second explanation for disjunctions involves an origin via long distance dispersal (McGlone 1985). However, until recently palaeoecologists have not given equal consideration to this hypothesis (Pielou 1991) in part due to a lack of understanding of the mechanisms underlying long distance transport. However, Jordan (2001) has demonstrated that observable traits of species can be used to estimate their probability of long distance dispersal.

The history of glacial refugia for forest species can be considered as the continuous presence of tree populations with continual cyclic pulses of outward range expansion and contraction (Grivet and Petit 2003; Hewitt 1996). The rate of expansion of forest trees depends on their dispersal ability (Grivet and Petit 2003), time before reproduction after establishment (Austerlitz *et al.* 2000), and competition with other tree species (Comes and Kadereit 1998). Unlike expansion, contraction of a species' range does not involve migration in response to climate change. Instead temperate trees are considered to die *in situ* in response to severe climatic conditions, with spread backwards impeded by the inability to colonize climax forest (Ferris *et al.* 1999; Tzedakis *et al.* 2003). For members of species complexes (e.g *Eucalyptus*, Griffin et al. 1988; *Quercus*, Matyas and Sperisen 2001) hybridization, followed by introgression, could be an important mechanism of dispersal (Potts and Reid 1988). In the vacant postglacial landscapes, pollen dispersed ahead of a species range may hybridize with an interfertile species in the landscape. Continued backcrossing of hybrids by pollen flow and

eventually complete introgressive replacement, may enable the former species to increase their range faster than through seed dispersal alone (Potts and Reid 1988).

Northern Hemisphere forest expansions from refugia are considered to have been rapid, involving long distance dispersal of seed (Hewitt 1996), and covering vast areas on a continental scale (Willis 1996). High genetic diversity of forest trees in southern Europe, for example, relative to northern areas has led to the hypothesis that colonization involved a series of bottlenecks (Hewitt 1996). Firstly, migration may have happened only from the northern fringes of refugia (Taberlet *et al.* 1998). Long distant dispersants from this fringe established a distance away from the main wave of expansion, which then were the progenitors of colonization of surrounding areas (Ferris and King 1998). The southern populations in refugia may have been prevented from contributing due to being blocked by the northern expanding populations (Vogel *et al.*1999). This process would lead to only a subset of the overall genetic diversity in refugia colonizing areas left vacant by the retreating glaciers (Grivet and Petit 2002; Ferris *et al.* 1999), while the majority of diversity remains *in situ* in refugial areas (Vogel *et al.* 1999).

The intensity of glaciations has differed significantly between hemispheres, with the northern hemisphere experiencing massive ice sheet coverage and an extensive periglacial conditions at middle and high latitudes. In the mid latitudes of the southern hemisphere glaciation was not as extensive during the ice ages (Hewitt 1996), with less continuous ice cover (Premoli *et al.* 2002).

1.1.3 What are cryptic refugia?

In Europe, the hypothesis that temperate trees survived the ice ages in southern refugia has become the dominant paradigm (Stewart and Lister 2001). However, sharp increases in tree pollen abundance in early postglacial northern Europe require rapid estimations of tree spread (up to1500m/year) to fit the hypothesis (Huntley and Birks 1983), which has led to questioning of the southern refugia hypothesis (e.g, Palme and Vendramin 2002). In addition, fossils of vertebrates and plant material from full glacial deposits in central and northern Europe, and some disjunct plant populations (e.g *Pinus*

sylvestris; Cruzan and Templeton 2000), have led to the 'cryptic' refugia hypothesis (Stewart and Lister 2001). It proposes that to explain these apparent anomalies temperate plants may, in fact, have been able to survive the aridity and cold of the full glacial *in situ*, protected in favourable microclimates (Stewart and Lister 2001). These areas are called 'cryptic' glacial refugia, as they are unexpected because of: 1) our understanding of the tolerance and/or adaptive range of species (e.g. McVean 1964); 2) models of glacial climatic conditions (e.g. temperature, aridity and ice coverage, e.g. Kullmann 2002); 3) predictions of biogeographical histories of species from current distribution patterns, and 4) available pollen evidence. In northern Europe, for example, small isolated populations of trees protected by favourable microclimates could have been the origin of temperate tree pollen found in northern and central Europe during the last glacial, which is usually explained as originating via long distance dispersal from southern glacial refugia, or as having been reworked from interglacial deposits (Willis *et al.* 2000; Stewart and Lister 2001; Carrion *et al.* 2003). The concept of cryptic glacial refugia suggests a more complex role of glacial refugia in shaping the world's biota (Stewart and Lister 2001), and may be relevant to southern hemisphere biogeography.

1.2 How can cryptic refugia be identified?

1.2.1 Pollen

Fossil pollen provides the main record of the changes in plant distribution and abundance during the Quaternary (Huntley and Birks 1983). The identification of cryptic refugia requires pollen analytical techniques that are able to determine the nature of the local vegetation in an area. For species with very low production or with non-wind dispersed pollen, local presence can be inferred with confidence. However, for wind-pollinated species with high dispersal and production of pollen, it is difficult to discern between the local presence of a species or pollen produced in, or even outside, the regional area (Willis *et al.* 2000). Pollen sequences from very small sedimentary basins, such as forest hollows, which receive pollen from a reduced area (Brewer *et al.* 2002; Rejmaek 1999) may be used to infer local presence more successfully (see Parshall 2002;

Schauffler 2002). In addition, very small populations (e.g, low density, Bennett 1985) may be undetectable through palynological studies (Birks 1989). Therefore, although fossil pollen can be useful to identify local presence of some plant species, its usefulness is limited for wind-pollinated species, species that reproduce vegetatively, or species with a small number of individuals.

1.2.2 Macrofossils

Macrofossils could provide the most direct evidence for the identification and characterisation of cryptic refugia (Stewart and Lister 2001). This is due to the fact that macrofossils can often be identified to species level, and are rarely transported long distances (e.g Field *et al.* 2000, but see Tallantire 2002). Sites with fossil of autochthonous origins, e.g, logs in peat bogs, (Kullmann 2002); fossil middens of woodrats or sticknest rats (Lyford *et al.* 2003); charcoal (Willis *et al.* 2000); and tree stumps preserved in growth position (Rowell *et al.* 2001) are particularly useful as they enable precise reconstruction of past vegetation but unfortunately such sites are rare (Rowell *et al.* 2001). The accuracy of radiocarbon dating methods used is of crucial importance in fossil studies, in order to distinguish between true cryptic refugia (i.e survival through the entire glacial period) and isolated populations that were established by long distance dispersal long before the main expansion from distant refugia (Lyford *et al.* 2003).

1.2.3 Molecular techniques

Current patterns of neutral intra-specific genetic variation can reflect the fragmentation of species distributions during the full glacial, and postglacial migration (King and Ferris 1998). A number of genetic studies have claimed to have located refugia based on region of highest genetic diversity (Hewitt 1996), or the location of a distinctive haplotype (e.g King and Ferris 1998). Modern populations derived from cryptic refugia may have distinctive genetic differences compared to populations derived from other refugia (Stewart and Lister 2001). Although a range of molecular techniques has been developed, organelle DNA may be the most useful tool (Ennos *et al.* 1999).

Firstly, their haploid status, halving effective population size (Ennos *et al.* 1999), increases susceptibility to genetic drift making genetic differentiation between separate glacial refugia and their colonizing descendants more likely (Petit *et al.* 1997). Furthermore, chloroplasts are maternally inherited in most angiosperms (Fineschi *et al.* 2000), as are mitochondria in conifers (Burban and Petit 2003). This and the generally low mutation rate of the organelle genome means that there is greater retention of historical genetic structure (Comes and Kadereit 1998), than biparentally inherited nuclear markers (Fineschi *et al.* 2000; Palme and Vendramin 2002). Patterns of genetic differentiation within species are the basis for interpretations of biogeographical history, but patterns may arise as result of vicariance events (e.g isolation of a species into separate refugia) or through dispersal. A number of studies have incorporated the use of molecular evolutionary clocks (e.g Ferris *et al.* 1999) to determine the time of divergence between differentiated populations (Martin and Dowd 1988). Comparison of the time of divergence with knowledge of geological or climate history (e.g the ice ages) are then correlated together (see Martin and Dowd 1988). However, the rate of molecular evolution is not known for the majority of species or for specific regions within organellular genomes (McKinnon *et al.* 2001), so its utility may be limited. Problems in the use of plant organelle DNA markers to identify possible cryptic refugia involve the intensity of sampling. The number of samples that can be incorporated into organelle studies is restricted by cost (Freeman *et al.* 2000), therefore reducing the chance of finding rare haplotypes. Also, in a cpDNA study of *Pinus resinosa* the region of the greatest diversity was considered to not be associated with a refugium but a result of admixture of haplotypes from at least two separate refugia and loss of haplotype diversity through genetic drift in shrinking populations in the southern part of its range (Walter and Epperson 2001). Therefore, it is crucial in organellular DNA studies that all lines of evidence are used when interpreting biogeographical histories from patterns of genetic variation, including the phylogenetic relationship among organellular DNA variants (haplotypes) (Grivet and Petit 2002), as well as independent geomorphological, paleoecological and pedological evidence (King and Ferris 1998; Walter and Epperson 2001).

1.3 What does the evidence say about cryptic refugia?

In this section fossil and molecular evidence are discussed primarily in reference to temperate trees of Europe, and in particular, their survival in cryptic refugia during the height of the last glacial (approximately 23,000-18,000 years ago). However, evidence for other examples of cryptic refugia are given for regions outside Europe, and in different plant groups (e.g alpine plants).

1.3.1 Fossil evidence

There is abundant pollen evidence that, in Europe and North America, temperate trees survived the ice ages in southern glacial refugia well south of the icesheets (Huntley and Birks 1993; Soltis *et al.* 1997). The pollen evidence has suggested that glacial refugia occurred in the mountains of the Mediterranean peninsulas (Iberia, Italy, and the Balkans) (Huntley and Birks 1983; Bennett *et al.* 1991; Denefle *et al.* 2000), the coast of the Black Sea (Tarasov 2000), and in California, Mexico and Florida in North America (Hewitt 1996; Davis 1976; Soltis *et al.* 1997). Tzedakis *et al.* (2003) considered that there is no pollen evidence indicating postglacial colonization of temperate trees from central and northern Europe.

What fossil evidence does support survival of temperate trees in northern refugia? So far, most evidence is from macrofossils (Hewitt *et al.* 2001). In Europe, full glacial charcoal macrofossils have been found as far north as Moldova, Romania, the Danube basin and north and west of Hungary, all indicating the full glacial presence of ecosystems similar to boreal and sub-alpine forests (Carcaillet and Vernet 2001). In Austria and Czechia, *Taxus*, *Fagus* and *Ulmus* have been identified that are between 45,000 and 25,000 years ago (Damblon 1997). In southwestern Europe, charcoal of conifers, evergreen and deciduous trees have been identified in rock shelters dated between 34,000 and 13,000 years old (Carcaillet and Vernet 2001), while charcoal of the temperate species, the yew (*Taxus baccata*) and Scot's pine (*Pinus sylvestris*) have been

dated as occurring 18,000 years ago in Slovakia (Stewart and Lister 2001). Macrofossils recovered from Paleolithic sites in northern Spain, dated between 17,000BP and 15,000BP (uncalibrated), have revealed diverse assemblages of temperate and Mediterranean species suggesting a full glacial tree refugium (Carcaillet and Vernet 2001). Carcaillet and Vernet (2001) consider that while these records indicate the presence of tree species during the full glacial in central Europe, the type of vegetation, whether forest, woodland, or treed steppe-tundra with prostrated individuals, cannot be determined. However, Willis *et al.* (2001) noted that these records may be unreliable as many of the macrofossil charcoal assemblages have poor chronological control.

A recent study by Willis *et al.* (2000) identified macroscopic charcoal particles from 31 full glacial sedimentary sequences in Hungary. While boreal trees *Picea*, *Larix*, and *Pinus* dominated, at one site, the temperate tree *Carpinus betulus* was present. This finding was considered by Willis *et al.* (2000) to be inconsistent with regional glacial climatic conditions predicted for this time. Independent fossil mollusk evidence from the same site supported this anomalous finding, with temperate species dominating the mollusk assemblage. Willis *et al.* (2000) interpreted these findings as indicating that there were "oases" in central Europe where temperate flora and fauna could survive *in situ* through the full glacial conditions. These microenvironments could be the product of the increased humidity and protection from wind afforded by the surrounding coniferous trees (Willis *et al.* 2000). Analogous communities of conifers with small pockets of temperate trees are found at the southern edge of modern boreal forests (Willis *et al.* 2000). Carcaillet and Vernet (2001) questioned the occurrence of *Carpinus betulus* this far north, suggesting that the wood anatomy of *C. betulus* is very hard to distinguish from that of *C. orientalis*, a drought resistant shrub (Grivet and Petit 2003).

Further north in the Swedish Scandes, Kullmann (2002) found stems and roots of the boreal trees *Betula pubescens*, *Picea abies* and *Pinus sylvestris* preserved *in situ*; radiocarbon dates indicating occurrence between 14,000- 5300 years ago. Kullman (2002) considered that the occurrence of trees at this elevation soon after the last glacial maximum (about 18,000 years ago) may have an origin in unglaciated areas such as the nearby coast of Norway. The current successful growth and reproduction of tree species at the study site, with a mean summer temperature of only 5ºC (about 5ºC cooler than is

generally considered to be required for tree species), suggests that, when climatic reconstructions are considered, stunted trees could have survived in refugia on the coast of Norway during the last glacial maximum, enabled by the relative temperate conditions of the ocean (Stewart and Lister 2001). A long distant dispersal origin, however, could explain the early arrival of trees in the Swedish Scandes. In the same region fossil leaves and fruits of oaks (*Quercus*), elm (*Ulmus*), hazel (*Corylis*) and alder (*Alnus*) dated at between 8500-8000 years before present indicate the early arrival of temperate trees soon after deglaciation (Stewart and Lister 2001). A Norwegian coastal refugium is supported by the findings of fossil Red Squirrel (*Sciurus vulgaris*) in cave deposits close to the Norwegian Coast, a species with a close association with both *P. abies* and *P. sylvestris* (Kullmann 2002).

Overall, the macrofossil evidence suggests that a number of temperate trees may have survived through the glacial in some regions of northern and central Europe, with strong evidence for survival of hornbeam (*Carpinus betulus*) at least as far north as Hungary (Willis 1996). Further fossil discoveries are needed to better understand the glacial history of temperate trees in Europe, such as the location and species composition of cryptic refugia and their northern limit.

The South Island of New Zealand was heavily affected by the ice ages. Ice cover during the glacial maximum is estimated to have been extensive along the Southern Alps, while pollen diagrams indicate widespread non-forest vegetation below the ice (McGlone 1985). Where tree species survived during this time is unclear. Well dispersed *Nothofagus* pollen has been found in full glacial sequences but is unlikely to represent local vegetation. However, reafforestation appears to have taken only about 500 years at the end of the glacial. This is extremely rapid considering the low migration capacity of *Nothofagus* (McGlone 1985). McGlone (1985) proposed that forest must have occurred in scattered and isolated favourable microclimates, probably on the lowered coastline and coastal ranges (McGlone 1985).

1.3.2 Molecular evidence

1.3.2.1 Southern refugia vs. cryptic refugia hypothesis

Tzedakis *et al.* (2003) considered that there is no clear evidence for temperate cryptic refugia in north and central Europe, after numerous studies of temperate trees and other plants (e.g, Grivet and Petit 2003; Besnard *et al.* 2002; Cronberg 2000; Matyas and Sperisen 2001; Trewick *et al.* 2002; Tyler 2000; Mohanty *et al.* 2000; Grivet and Petit 2002; Dumolin-Lapaque *et al.* 1997; Demesure *et al.* 1996). However, Stewart (2003) disagrees, suggesting that the pattern of genetic variation in the polyploid rockfern (*Asplenium ceterach*) (Trewick *et al.* 2002) and sedge (*Carex digita*) (Tyler 2000) indicate that they survived in temperate northern cryptic refugia. High cpDNA haplotype diversity in diploid populations of the rockfern, which have lower colonizing abilities than tetraploids, suggest a northern refugium in the Pannonian-Balkan region, Romania, (Trewick *et al.* 2002). Glacial refugia including south-eastern European Russia and central Europe are indicated by allozymes in the sedge (*Carex digitata*; Tyler 2002). However, Tzedakis *et al.* (2003) consider that the there is no clear association of these plants with temperate forest, and, therefore, that they may be of limited value in determining the existence of temperate tree refugia. Trewick *et al.* (2002) suggest that a distinct cpDNA haplotype in *Fagus sylvicata* (Demesure *et al.* 1996) in Hungary indicates survival in a refugium close to the periglacial zone in that area. However, the distinct haplotype differs from the most widespread haplotype by only one mutation (Demesure *et al.* 1996), therefore a postglacial origin of the haplotype cannot be ruled out.

1.3.2.2 Scottish populations of *Pinus sylvestris*

Scot's pine, *Pinus sylvestris*, is widely distributed in western Europe, extending from Norway to the mountains of southern Spain (Sinclair *et al.* 1999). A highly disjunct population occurs in the northwest of Scotland (Sinclair *et al.* 1999), the origin of which has been the subject of great interest and investigation. Birks (1989), using isochrone pollen mapping techniques, demonstrated that two separate postglacial expansions in abundance of *P. sylvestris* pollen occurred, one in northwestern Scotland between 8500 and 7900 years ago and independently in southern England, approximately 9500 years ago. Considering the predictions of the severity of the climate in Scotland and the extent of glaciation (Birks 1989; Taberlet *et al.* 1998) during the Last Glacial, it has generally been considered that *P. sylvestris* could not have survived (Godwin 1975) and must have dispersed there during the postglacial (Birks 1989). High nuclear genetic diversity in the Scottish populations, despite considerable range contraction by natural and human causes (Kinloch *et al.* 1986) and evidence from mitochondrial DNA (Sinclair *et al.* 1999) has provided strong evidence for a contrary origin in a cryptic refugium (Sinclair *et al.* 1999; Cruzan and Templeton 2000; Stewart and Lister 2001). This finding is consistent with the recognition as a separate taxonomic variety of the Scottish populations (Kinloch *et al.* 1986; Birks 1989).

1.3.2.3 Alpine plants of the European Alps

Whether alpine plants survived in the ice-covered European Alps during the last glaciation (Gugerli and Holderegger 2001), has been the subject of strong debate (Stehlik *et al.* 2001), which has not been helped by a lack of alpine plant fossils (Stehlik 2000). Two hypotheses have been proposed: 1) total extinction within glaciated areas, followed by colonisation of vacant alpine areas from peripheral refugia during deglaciation (*tabula rasa* hypothesis; Schonswetter *et al.* 2002), and 2) long term *in situ* survival within glaciated regions on ice-free mountain peaks above the ice shield (nunatak hypothesis; Stehlik 2000). Molecular techniques have provided the opportunity to better answer such questions (Gugerli and Holderegger 2001). Amplified fragment length polymorphism (AFLP) studies have identified considerable genetic differences between peripheral

populations and Central Alp populations in the subalpine perennial herb *Erinus alpinus* (Stehlik *et al.* 2002a) and the alpine herb *Rumex nivalis* (Stehlik 2002). In addition, the distribution of two divergent groups of cpDNA haplotypes identified in the perennial cushion plant *Eritrichium nanum* indicate survival of the species on separate nunataks in the west and east central Alps during the ice ages (Stehlik *et al.* 2002b). *In situ* survival could be a common response of alpine plants to the Quaternary glaciations (Stehlik *et al.* 2002b). However, an AFLP study of the high alpine plant *Phyteuma globulariifolium* does not indicate survival in the glaciated Central Alps, but rather survival in peripheral ice-free areas (Schonswetter *et al.* 2002), which may reflect the stochastic nature of plant survival in these areas.

1.3.2.4 Cryptic refugia of *Fagus grandifolia* and *Acer rubrum*

In North America, Clark (2002) surveyed the distribution of cpDNA variation in two eastern North American tree species, American beech (*Fagus grandifolia*) and red maple (*Acer rubrum*) with a resultant pattern suggesting that in both species populations survived in cryptic refugia at higher latitudes than expected from pollen evidence, close to the Laurentide Ice Sheet.

1.3.2.5 Eastern Tasmanian forest glacial refugia

During the Quaternary, Tasmania was subject to at least five glaciations (Colhoun *et al.* 1996) each associated with significant contraction of the forest vegetation (Macphail 1979) and dominance of alpine and herbland communities (McKinnon *et al.* submitted). In western Tasmania fossil evidence indicates coastal refugia for rainforest and (eucalypt) woodland during the last glacial (Colhoun 2000). In eastern and southeastern Tasmania, which was considerably more arid than the west of Tasmania during the glacials (Macphail 1979; Jackson 1999), there is currently no fossil evidence indicating where forest refugia may have survived (Kirkpatrick and Fowler 1998). However, the current high level of endemism of eucalypt species in southeastern Tasmania has led to proposals that glacial refugia may have occurred in this region (McKinnon *et al.* 2001). Models of the distribution of forest vegetation in Tasmania during the Last Glacial Maximum (Kirkpatrick and Fowler 1998) support the presence of

southeast refugia, although concerns have been expressed about the validity of this model (e.g, McKinnon *et al.* submitted). This is because Kirkpatrick and Fowler (1998) based their climate assumption on the dominant rainforest species *Nothofagus cunninghamii* having survived the last glacial through to present in eastern Tasmania, which would suggest that rainfall was similar to present (McKinnon *et al.* submitted). Dispersal by seed from rainforest refugia in western Tasmania was considered unlikely due to the limited dispersal ability of *N. cunninghamii*. In fact, some evidence suggests that eastern Tasmania was considerably more arid during the last glacial than at present (e.g Bowden 1983) and, therefore, may have been more inhospitable for forest vegetation than Kirkpatrick and Fowler (1998) predict (McKinnon *et al.* submitted). A cpDNA study across 17 hybridizing eucalypt species, including the southeastern endemics, throughout Tasmania (McKinnon *et al.* 2001) has provided some important additional information. A widespread haplotype group (Jc), also found in continental Australia, was found in nearly all species across Tasmania, but was uncommon in the southeast. A second haplotype group (Js) had a restricted distribution in the Storm Bay region of southeastern Tasmania. The distribution of the Js haplotype fits closely with the limits of a refugium proposed by Kirkpatrick and Fowler (1998). McKinnon *et al.* (2001) proposed a glacial refugia hypothesis to explain this pattern: during the glaciations an isolated forest refugia survived in the southeast, where the Js mutation arose and has since experienced little postglacial migration since. The Jc haplotype may have migrated relatively recently from northern forest refugia in the postglacial period through central Tasmania which would have been treeless at the time. The low abundance of the Jc haplotype in the south and the restriction of those widespread eucalypt species polymorphic for the Js and Jc haplotype in the southeast, suggest that the Jc haplotype has only recently made contact with the Js haplotype. A separate origin of the two haplotypes is also supported by the estimated divergence time between the two Js and Jc haplotypes at between 0.8 to 3 million years. These examples demonstrate the importance of genetic studies, especially where fossil evidence is not available (Premoli et al 2002).

1.3.3 Cryptic populations

The fossil and molecular studies described above have been interpreted as evidence for species tolerating the full glacial conditions in favourable microclimates. In the modern environment situations analogous to these past refugia are not uncommon. Populations, or even individuals, have been found surviving in areas that are unexpected, outside the estimated tolerance range of the species. Microenvironments are created by a combination of factors related to topography, geology and climate (Danin 1999). There are many known examples of this phenomenon of which a small number will be discussed here.

In central Sahara, a total of 233 individuals of the conifer, *Cupressus dupreziana* survive in ravines and wadis, where (although highly variable) average annual rainfall amounts to 30 mm (Abdoun and Beddiaf 2002). In the deserts and steppe of Jordan, the Sinai and Israel, mesophytic plant species occur in the crevices of rocks and around permanent springs (oases) (Danin 1999*).* For example, *Pistacia atlantica,* a tree normally found occurring in open forests in the moist areas of Israel, is found in the Negev Highlands (100 mm rainfall per annum) in the water accumulation zone of a large smooth faced limestone outcrop. The population size of some species in these desert refugia can be very low, with for example, only three individuals of the tree species *Arbatus andrachne* surviving near springs in south-western Jordan (Danin 1999). It survives in an area with less than 200 mm of annual rainfall whereas the closest trees live with 500-600 mm of annual rainfall in northern Jordan and the Judean mountains (Danin 1999). Danin (1999) regards *P. atlantica*, and most other mesophytic trees and shrubs found in the region, to be relicts of moister periods.

The conifer *Picea mariana,* is found beyond the latitudinal tree line in Canada where it survives by layering, with no sexual reproduction (Laberge *et al.* 2000). In this case survival is related to the reproductive plasticity of the species under the harsh conditions (Mejias *et al.* 2002), rather than micro-environmental factors, involving an increased allocation of resources to vegetative reproduction, rather than sexual reproduction (Laberge *et al.* 2000).

In monsoonal Arnhemland, Northern Territory, Australia, taxa considered to be relictual from cooler periods, including the insect *Aphroteniella* and the conifer

Podocarpus, occur isolated in a deep sandstone gorge where the vegetation itself and sheltered aspect provide a cool and humid microclimate even during the dry season (Cranston 1998).

In Tasmania, Australia, isolated stands of cool temperate rainforest dominated by *Nothofagus cunninghamii* can be found in regions outside the predicted range of the species (Harle *et al.* 1993). An isolated stand at Yarlington Tier, southeastern Tasmania, occurs in a relatively dry region with the lowest rainfall known in the range of the species. A high groundwater table, protection from fire and frequent cloud cover are factors that allow the species to survive in a largely inhospitable area (Harle *et al.* 1993).

1.4. Conclusions

The available evidence suggests that during the last glaciation, and presumably other glacials, plant species were able to tolerate conditions by surviving in cryptic refugia, in many parts of the world.

In the Northern Hemisphere, cryptic refugia could explain some important observations of the pollen record (Birks 1989). One such problem has been the fast rates of dispersal estimated during the postglacial (Cain *et al.* 1998). As mentioned earlier, species are estimated to have migrated rapidly, typically at 50-500m/yr, although sometimes as much as 1500m/year in hazel (*Corylis avellana*) and up to 2000m/yr in alder (*Alnus glutinosa*)(Birks 1989; Hewitt 1999). This rapid plant dispersal is unexplainable by current mean seed-dispersal distances of tree species, so has been explained through infrequent long-distance dispersal events (Clark *et al.* 1998; Cain *et al.* 1998; Jackson and Overpeck 2000). The large expanses of uncolonised regions in the northern hemisphere exposed after the retreat of the glaciers is considered to have promoted these exceptionally high rates of migration (Ferris *et al.* 1999). However, migration rates generated from models of dispersal incorporating long distance dispersal components have predicted smaller spread rates than estimated (Ronce 2001). Also, in North America, the rapid plant migration cannot be explained by plant dispersal by Native Americans (MacDougall 2003). Recolonization of the postglacial environment from the southern refugia and supplemented by refugia further north may provide a better

explanation (Ronce 2001). This has important implications, as the ability of species to migrate rapidly in response to climate change, has been considered important for the long-term maintenance of biomes in the face of future anthropogenic climate change (Clark *et al.* 1998; Nathan *et al.* 2002). For example, after molecular evidence suggested that two temperate North American trees may have survived further north near the icesheets than previously understood estimations of the migration rate were lowered from 1000m a year to <100m a year (McLachlan unpublished). This lower estimation of dispersal rate is more consistent with knowledge of life history and dispersal ability of the species (Ronce 2001).

In addition, cryptic refugia could explain lack of pollen evidence indicating the initial colonization by pioneer species (e.g pine and birch) followed by late successionary species such as oak, ash and beech suggested by north-European forest succession models (Willis 1996). Instead there appears to have been an immediate increase of diverse mixed deciduous woodland (Willis 1996).

The roles of cryptic refugia in shaping the biota appear to have been different between the Northern and Southern Hemisphere. In Europe, for example, cryptic refugia of temperate trees may have supplemented the survival and recolonisation of the European biota (Stewart and Lister 2001). In the Southern Hemisphere where land area is relatively small compared to the Northern Hemisphere (Hewitt 1996) it follows that a high proportion of warm adapted species ranges were affected by glacial climate, and therefore species must have survived *in situ* in favourable microclimates. In Australia, Chile and New Zealand there is good evidence for this occurring (McKinnon *et al.* submitted; McGlone 1985; Markgraf *et al.* 1995). The present relatively simple flora of cool temperate rainforest in Tasmania reflects the forced contraction during glacials of species into small favourable microenvironments. This favoured survival of species with a wide tolerance range that are now dominant components of the rainforest vegetation, while the fossil record indicates the extinction of a number of conifers and angiosperms with warm temperate affinities during the Quaternary (Macphail *et al.* 1993). In Europe, the number of extinctions has probably been less with fossil evidence suggesting that despite the glacial periods the same suite of temperate flora expanded into northern Europe during warm interglacials over the past 1.8 million years (Willis 1996).

In conclusion survival of species in cryptic refugia has had important implications for the current biota in both the Northern and Southern Hemispheres. The evidence for cryptic refugia contributes to current understanding of the tolerance ability of plant species and the climatic conditions during the glacial-interglacial cycles of the Quaternary (e.g the extent of ice cover, Stewart and Lister 2001). The best evidence for cryptic refugia in the future may come from macrofossils, which unlike molecular techniques, are able to indicate the existence, location and duration of refugia most accurately (Stewart and Lister 2001).

PART 2-

EXPERIMENTAL THESIS

Chloroplast DNA variation in *Nothofagus cunninghamii* (Hook.) Oerst.

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Submitted in partial requirement for the degree of
Bachelor of Science with Honours.

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CHAPTER 2

Background

2.1 Introduction

The last 10 million years has seen marked changes in the world vegetation (Traverse 1982), due to a general cooling trend, increased seasonality, decreased rainfall and large, rapid climatic fluctuations at high and middle latitudes (Macphail *et al.* 1993). The vegetation of much of the temperate world, including southeastern Australia, has acquired much of its character during this period (Macphail *et al.* 1993). In particular, the temperate rainforest component of the Australian vegetation has suffered severe contraction in its range and a reduction in species diversity (Martin 1990; Carpenter *et al.* 1994). Vegetation with temperate rainforest affinities was far more widespread in Australia during the Tertiary (Kemp 1978), the extent of which is well demonstrated by the discovery of fossils of the current Southern Hemisphere temperate rainforest genus *Nothofagus* in late Eocene (55-34 million years ago) sediments from southwestern Western Australia (Hill *et al.* 1996). Some time in the Pleistocene, temperate rainforest became confined to southeastern Australia and to some isolated areas of the east coast of Australia (Read and Brown 1996). As a result, temperate rainforest is now a relatively minor component of the Australian vegetation.

Nothofagus cunninghamii (Hook.) Oerst. is the dominant component of most cool temperate rainforest in southeastern Australia (Howard and Ashton 1973). Currently *N. cunninghamii* is restricted to the wetter regions of Victoria and the island of Tasmania (Howard and Ashton 1973; Lindenmayer *et al.* 2000). Its distribution is characterized by some major disjunctions, with Bass Strait and the dry regions of the Tasmanian Midlands and central Victoria constituting the most significant barriers to dispersal (Jones *et al.* in press). In addition, some minor disjunctions occur within regions, for example, in eastern Tasmania (Fig 2.1).

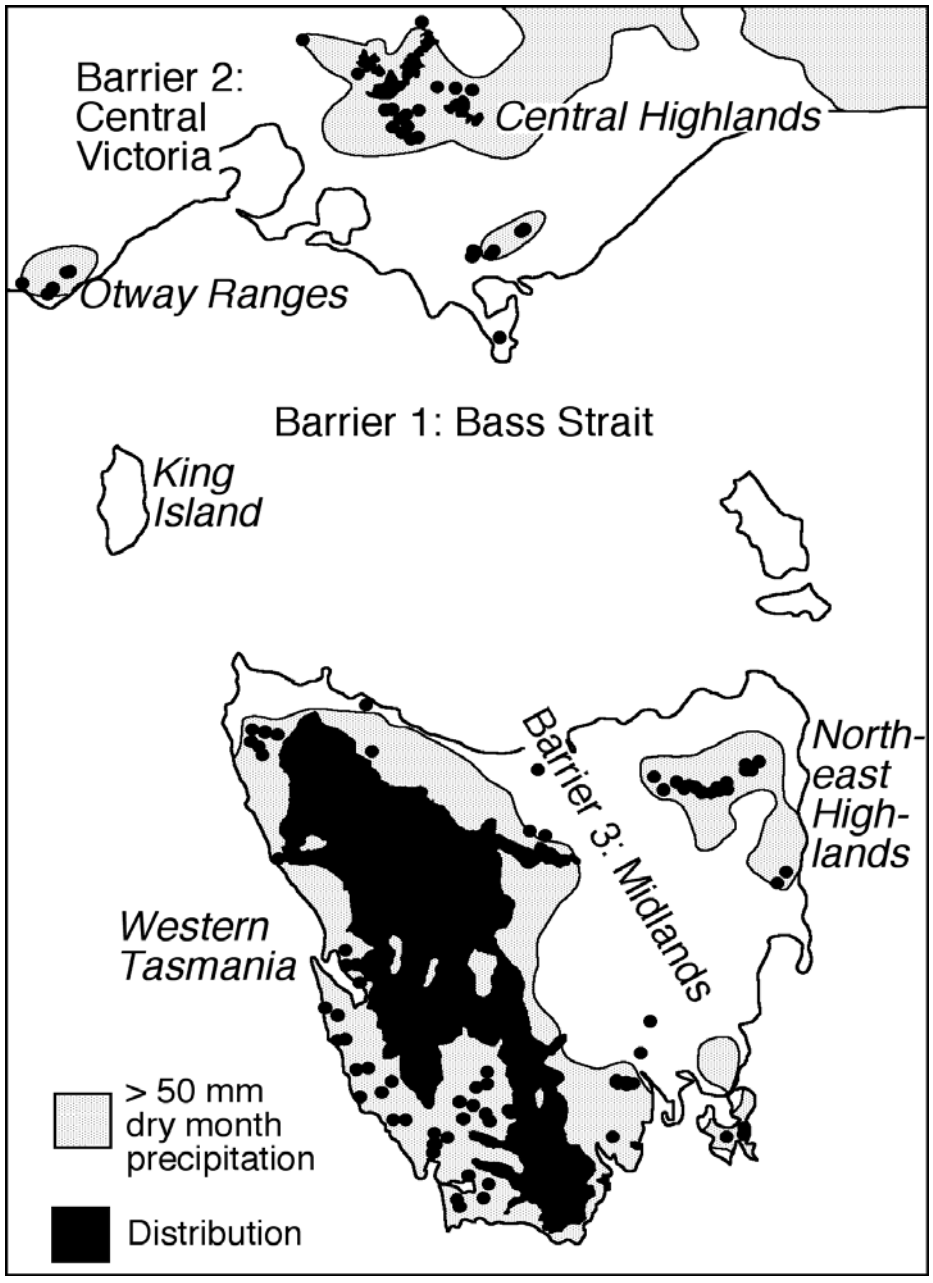

Fig 2.1 The current distribution of *Nothofagus cunninghamii* (black) and the species predicted range based on the average rainfall of the driest month (grey; Busby 1986). The three major barriers to gene flow; 1) Bass Strait, 2) the dry regions of Central Victoria, and 3) the Tasmanian Midlands, are shown. Information on the broad distribution of *Nothofagus cunninghamii* was obtained from Howard and Ashton (1973) and personal observation, and information on some small and isolated stands, particularly in eastern Tasmania, was obtained from Harle *et al.* (1993), Neyland and Brown (1993), respectively.

The species has a long history in Australia, with available fossil information suggesting that *N. cunninghamii* evolved from a clade of species in the subgenus *Lophozonia* that has been present in Australia for 55 million years, with the modern species recognizable in the fossil record for at least the last 1 million years (Jordan 1999). The current distribution of *N. cunninghamii* is no doubt the result of complex interactions of the species with both past and present environments, along with stochastic events (e.g. glacial survival). For example, Busby (1986) considered that the present absence of the species from the highlands of northeastern Victoria, despite suitable climatic conditions, is due to the severe effects of the last ice age and the species slow recovery due to poor dispersal. The ecological versatility of *N. cunninghamii* (Hill *et al.* 1996) is considered to have been an important factor in its survival through the climatic changes of the last 10 million years, particularly the glacial-interglacial cycles (Read and Brown 1996). The tolerance of this species to a wide range of conditions is demonstrated by the species large altitudinal range. *N. cunninghamii* occurring from sea level to over 1500m (Howard and Ashton 1973), where it ranges from a 50m tall tree at low altitudes (e.g. Parsons *et al.* 1975) to a compact shrub under 50cm in height at the extremes of its altitudinal range (Reid and Brown 1996).

2.2 Explaining the current distribution of *N. cunninghamii*: glacial refugia, migration or long distance dispersal?

Considering the severe climatic changes that have characterised the past 10 million years a number of important questions exist concerning the history of *N. cunninghamii*. One of the most significant of these is where the species survived over this period and the nature of its migration across the landscape. The low seed dispersal ability (Hickey *et al.* 1982) of *N. cunninghamii* has led to the hypothesis that the current disjunct distribution of the species is the result of fragmentation from a more widespread distribution in the past due to an increase in unsuitable habitat (e.g. Macphail and Colhoun 1985; Harle *et al.* 1993). This scenario infers that the species survived through at least five glacial periods (Colhoun *et al.* 1996) in glacial refugia

within each major region of its distribution. However, during glacial periods many parts of the current range of *N. cunninghamii* would have been highly stressful places for rainforest species. Glacial climates, particularly in Tasmania, were severe relative to current interglacial conditions. For example, the climate during the Last Glacial, which is the best understood, is estimated to have had temperatures approximately 6ºC colder than present (Colhoun *et al.* 1996), and a likely 50% reduction in rainfall (Colhoun 2000). These conditions allowed the development of ice caps on upland areas of Tasmania and extensive periglacial conditions in both Tasmania and the southeast of mainland Australia (Davies 1974; McKenzie 1997).

The available fossil record is limited in its ability to tell us whether *N. cunninghamii* survived in glacial refugia in each region that it now occupies. The species is known to have survived continuously for at least 350,000 years in western Tasmania (Colhoun and van der Geer 1998; Colhoun 2000) and is also known to have been present before the last glacial in the Central Highlands of Victoria, surviving through the Last Glacial Maximum (McKenzie 1997). While some fossil record exists for other regions of the species distribution, for example Wilsons Promontory and the Otway Ranges, these records extend only 5000 years and 7000 years before present, respectively (Hope 1974; McKenzie and Kershaw 1997). These records start 9000 and 7000 years after the end of the Last Glacial Maximum and are, therefore, uninformative as to glacial survival in these areas. Currently no fossil evidence of *N. cunninghamii* is available for eastern Tasmania.

Other explanations of the cause of the disjunctions that do not require the survival through the glacial cycles must be also be considered. Observations in the European and North American fossil record suggests that forest trees with large seeds and generally low seed dispersal abilities (e.g. oaks) were capable of rapid migrations in response to climatic changes, over large areas (Reid's paradox; Clark *et al.* 1998). For example, migration rates are estimated to have been 50-500m/year, and sometimes as much as 1500m/year in hazel (*Corylis avellana*; Birks 1989). This rapid migration is considered by Ferris *et al.* (1999) to have been promoted by a lack of competition in the vacant landscapes exposed by the retreat of glaciers in Europe. During glacial periods in Tasmania and the Central Highlands of Victoria, periglacial

conditions were widespread creating a nearly treeless environment (Macphail 1979; McKenzie 1997; Colhoun 2000). Rapid migration across current barriers during wetter periods (or with more even rainfall) could explain some of the large disjunctions or minor disjunctions within regions. Interchange between Victoria and Tasmania of *N. cunninghamii* could have occurred in the past across the relatively shallow Bass Strait (mostly <100m deep), a land bridge that has been repeatedly exposed and submerged by changes in sea level since at least the Early Miocene (17 million years ago; Baillie 1989). A time lag between the amelioration of climate after glacial periods (end of Last Glacial 14000 years ago) and the closure of the land bridge across Bass Strait (6400 years ago; Belperio 2002) by the rise of sea level, could have allowed migration between Tasmania and Victoria by *N. cunninghamii* if the climate was suitably wet. This possibility is supported by macrofossils of the species on King Island thirty eight thousand years old (Hill *et al.* 1996). Macphail (1979) and McKenzie (1997) have observed periods of expansion of *N. cunninghamii* in the fossil record beyond present limits due to periods of wetter conditions in both Tasmania (8000 and 5000 years ago; Macphail 1979) and Victoria (7000-4000 years ago; McKenzie 1997), repectively. These periods, and similar periods before it, could explain some major or minor disjunctions within the species distribution. Evidence exists for the rapid postglacial migration of other *Nothofagus* species, for example New Zealand *Nothofagus* species (see McGlone 1985).

The third possible origin of disjunctions in the range of *N. cunninghamii* is via long distance dispersal of seed. There is a general insistence in the literature that *Nothofagus* is incapable of long distance seed dispersal (Hill *et al.* 1996). Indeed, this seems to be supported by the seed possessing only small wings and being heavier than normal wind dispersed seed (McGlone *et al.* 1996), and experimental data suggesting average dispersal is only one or two canopy heights away from the mother tree (Hickey *et al.* 1982). A number of lines of evidence suggest that the potential dispersal distance of *Nothofagus* seed could be greater. For example, stands of *Nothofagus* in New Zealand isolated up to 25km from the nearest populations are considered to have their origin via rare long distance dispersal of seed (Burrows 1977; Wardle 1980; Haase 1990). Evidence for even greater dispersal of seed in *Nothofagus* is considered in the origin of the genus in New Zealand. The *Nothofagus* subgeneras *Lophozonia* and *Brassospora*

appear in the New Zealand fossil record well after the severing of dry land routes between Australia and New Zealand, approximately 25 million years ago for *Lophozonia* and forty-three million years ago for *Brassospora* (Macphail *et al.* 1996). These subgenera appeared first in Australia before New Zealand (Hill *et al.* 1996). The unlikelihood that these subgenera were present in New Zealand earlier but did not contribute to the pollen record while doing so in Australia and South America has led to the suggestion that at some stage *Nothofagus* has crossed the 2000km Tasman Sea from Australia to New Zealand (Pole 1994; Macphail 1997). This assertion is supported by fossil evidence suggesting an origin via long distance seed dispersal for other New Zealand taxa (Mildenhall 1980). While the mechanisms underlying long distance transport in *Nothofagus* are not understood, McGlone *et al.* (1996) consider storm- force winds or perhaps seed stuck in birds plumage or feet could be possible vectors. Overall, the possibility that rare long distance seed dispersal has had a role in the distribution of *Nothofagus* species suggests that a similar role of long distance dispersal in the distribution of *N. cunninghamii* is possible.

2.3 Chloroplast DNA

While the fossil record has been a major contributor to the understanding of the past history of plant species, phylogeography is increasingly becoming an important and independent source of information. Phylogeography is the study of the relationship between the phylogeny of current intra-and/ or interspecific genetic variation and its geographical distribution (Dumolin-Lapegue *et al.* 1997). The tool of choice in plant phylogeography is chloroplast DNA (Avise 1998). This is due to the maternal inheritance of the genome in most angiosperms and, therefore, the distribution of chloroplast DNA variability is likely to reflect the history of genetic exchange by seed or isolation across a species range (Schaal *et al.* 1998). Isolated populations differentiate by mutations and through genetic drift form distinct genetic lineages over time. Migration events result in the expansion of the distribution of genetic lineages (Avise 1994). The haploid status of the chloroplast genome makes it particularly susceptible to genetic drift, a process that occurs during both range fragmentation and migration, and increases

structuring of variation (Ennos *et al.* 1999). This is due to cpDNA having half the effective population size of that found in the nuclear genome. Furthermore, because of the constant rate of evolution of the cpDNA genome, the depth of common ancestry of populations can be inferred by the their degree of genetic similarity (Schaal *et al.* 1998). The roles of fragmentation and migration (the species spatio-temporal dynamics) in a species past history may be inferred by examining the phylogeny of chloroplast DNA variation and overlaying this over a geographical framework (Ennos *et al.* 1999).

The distribution of cpDNA haplotypes has been used to infer the biogeographical history, especially the response to the ice ages, of numerous plant taxa on all continents. The glacial history of temperate European trees has received much research attention. Continental scale studies of cpDNA variation of the hornbeam (*Carpinus betulus*), olive (*Olea europeae*), hazel (*Corylis avellana*), common ivy (*Hedera* spp.), black alder (*Alnus glutinosa*) and common beech (*Fagus sylvicata*) (Demesure *et al.* 1996; Besnard et al 2002; Grivet and Petit 2002; Palme and Vendramin 2002; Grivet and Petit 2003) show strong structuring of cpDNA variation. This was used to infer the locations of refugia in southern European peninsulas and northern postglacial colonization routes. Other studies have found weak structuring of cpDNA variation (e.g. Raspe *et al.* 2000; Mohanty *et al.* 2001; Rendell and Ennos 2002) in species that are characterized by efficient seed dispersal mechanisms (e.g. wind or animal dispersed) (Hampe *et al.* 2003). At a regional scale, examination of the distribution of cpDNA variation has provided strong evidence that some alpine plants (e.g. *Eritrichium nanum*) were able to survive the heavy glaciation of the Alps separately on ice-free nunataks and in peripheral refugia (Stehlik 2001).

For studies of cpDNA phylogeography to be informative an appropriate level of intraspecific chloroplast variation must be detected. Developing molecular techniques to detect intraspecific variation has not been without its difficulties partly as a result of the low average rate of evolution of chloroplast DNA, but also the practicalities of the techniques themselves (Wolfe *et al.* 1987), resulting in a low probability of detecting intraspecific variation. Most early studies of cpDNA variation used it as a marker to examine plant systematics and evolution above the species level (Soltis *et al.* 1992). The earliest studies of within species variation relied on the labor intensive technique of

isolating pure chloroplast DNA followed by restriction digest (e.g. Bank and Birky 1985; Dally and Second 1989; Sahuquillo and Lumaret 1999). The requirement to isolate pure cpDNA, and not the use of restriction enzymes, severely limited the number of samples analysed. Restriction enzymes cleave (restrict) DNA at specific nucleotide sequences (recognition sites) and generate fragments of the original product. Variation in the nucleotide sequence between samples are detected by the differences in fragment size as a result of the creation or loss of restriction sites by mutation of single nucleotides (Parker *et al.* 1998) or through insertion or deletion mutations (indels) (Curtis and Clegg 1984). This size variation is known as "restriction fragment length polymorphism" (RFLP) (Parker *et al.* 1998). The invention of Southern hybridization (Southern 1975) brought about great improvement in the analysis of cpDNA. This technique allowed larger number of samples to be analysed but is still considered time consuming (e.g. Vaillancourt and Weeden 1992) compared to polymerase chain reaction (PCR) based techniques (Avise 1994). The use of PCR in combination with RFLP to detect variation has improved the ability to detect variation (Mohanty *et al.* 2000). The PCR-RFLP technique involves amplification of specific universal target sequences of cpDNA followed by screening of polymorphism through digestion with restriction enzymes (Wang and Szmidt 2001). The PCR based technique has a number of major advantages as it allows a greater resolution of fragments, is more rapid (Dumolin- Lapegue *et al.* 1997) and allows the ability to isolate specific areas of the genome that generally have faster rates of evolution (Wolfe *et al.* 1987; Ogihara *et al.* 1992). A number of universal cpDNA markers have been developed, where pairs of primers have been placed in conserved sequences flanking more variable regions (Taberlet *et al.* 1991; Demesure *et al.* 1995; Dumolin–Lapegue *et al.* 1997), including lengths of non-coding sequence that have a higher frequency of mutations (Taberlet *et al.* 1991). While the PCR-RFLP approach has been shown to be efficient in the detection of sequence variation, the amount of variation detected depends largely on the number of restriction enzymes used in a study and the number of different regions that are successfully amplified using PCR. Intraspecific cpDNA site variation has been reported in many species (reviewed by Soltis *et al.* 1992). The direct sequencing regions of interest provides the most detailed analysis

of DNA differentiation (Parker *et al.* 1998), although this technique is more time consuming than PCR-RFLP analysis.

Chloroplast DNA phylogeographic studies in southeastern Australia are so far limited to the genus *Eucalyptus*, the dominant of non-rainforest forest vegetation (e.g. Byrne *et al.* 1994; Jackson *et al.* 1999; Freeman *et al.* 2001; McKinnon *et al.* 2001). To date no study into the distribution of cpDNA variation has been completed in cool temperate rainforest plants in Australia. Currently, the only studies of cool temperate rainforest plants using molecular techniques consist of two rangewide isozyme studies of the Huon Pine (*Lagarostrobus franklinii*), a Tasmanian endemic, and Sassafras (*Atherosperma moschatum)* (Shapcott 1993; Shapcott 1997) and an allozyme study investigating the clonality of the extremely restricted *Lomatia tasmanica* (Lynch *et al.* 1998). In addition, few genetic studies have been undertaken in the genus *Nothofagus* (e.g. Premoli 1997; Marchelli *et al.* 1998; Marchelli and Gallo 2001), despite the genus being "...the key to southern hemisphere biogeography" (van Steenis 1972). Understanding the history of *N. cunninghamii* in terms of locations of glacial refugia and migration routes is of particular importance, as it would provide invaluable insights into past climate in southeastern Australia, particularly the severity of the glacial cycles (McKenzie and Kershaw 1997). This is made possible by an excellent understanding of the bioclimatic profile of the species derived from climatic parameters correlated with the distribution of extant populations (Busby 1986), together with a considerable body of work on the species ecology and ecophysiology (Howard 1973a; Howard 1973b; Howard 1973c; McKenzie and Kershaw 1997; Hovenden and Brodribb 2000; Hovenden and Schimanski 2000; Hovenden 2001). In addition, understanding the biogeographical history of *N. cunninghamii* may be relevant to the understanding of other cool temperate rainforest species.

In summary, this study aims to 1) identify variation in the chloroplast genome across the range of *N. cunninghamii* and 2) determine the possible roles of glacial refugia, migration and/or long distance dispersal in explaining the current distribution of *N. cunninghamii*.

CHAPTER 3

Materials and Methods

3.1 Sampling Strategy

Leaf tissue was collected from 170 adult trees in natural stands from across the latitudinal and altitudinal range of *Nothofagus cunninghamii* (Appendix 1). This includes samples from all regions of the species distribution (Fig. 3.1). The sampling strategy aimed to sample as extensively in each region as possible, with an objective to sample each individual tree at least 10km from the nearest individual. Most known small isolated stands were also sampled. Multiple samples were collected with a minimum distance of approximately 50m between trees sampled at the Dazzler Ranges, Lookout Hill and Blue Tier, as these populations were considered as potentially significant (e.g. putative glacial refugia, or remnant populations). Parts of the southwest, central and northwestern Tasmania were not sampled in this study due to inaccessibility.

From each tree a herbarium specimen and a sample of foliage with young expanded leaves for DNA extraction were collected. The UTM and altitude was recorded for each tree sampled using a GPS. In addition samples of *N. moorei* and *N. menziesii* were obtained from the University of Tasmania (collected by G. J. Jordan) and the Tasmanian Royal Botanic Gardens, respectively.

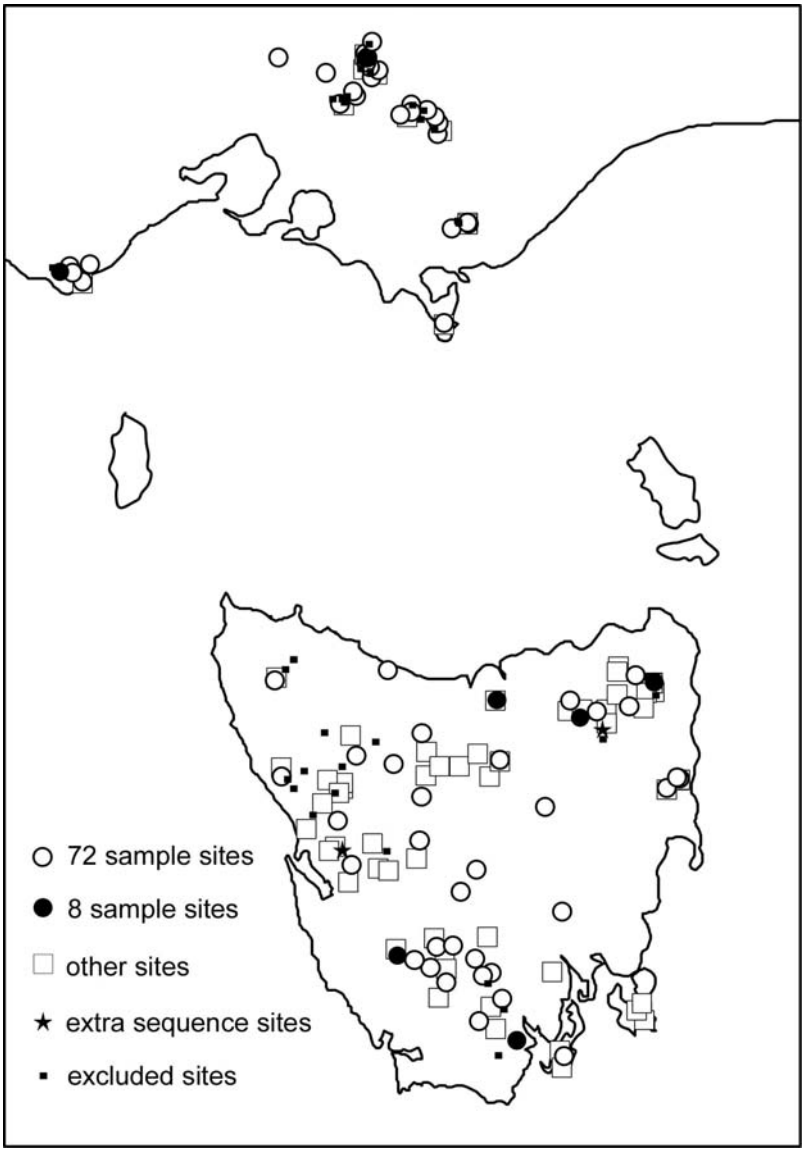

Fig 3.1 The 170 sites sampled for *N. cunninghamii* across its range. Open circles represent samples that were used in the 72 sample screening; filled circles represent samples used in the initial eight sample screening size; open squares represent other sites not used in the former analyses; stars represent those samples sequenced for the S_fM locus in addition to the sample size of eight; small filled squares represent those samples with missing data.

3.2 Molecular Methods

3.2.1 *DNA Extraction*

Total genomic DNA was extracted following the CTAB protocol of Doyle and Doyle (1990), with the following modifications: using 1g of adult leaf tissue and 5ml of CTAB buffer with 4% polyvinylpyrrolidone and 35µl of 1M DTT and incubating for 30 min at 55°C. All samples were cleaned by re-precipitation in the presence of high salt concentration (2.0M NaCl). DNA concentration and purity of all samples was assessed using agarose gel electrophoresis with ethidium bromide staining, comparing with a standard molecular weight marker (Lamda *Hind* III). DNA concentration of samples was standardized at 5ng per micro-litre.

3.2.2 *Polymerase Chain Reaction*

PCR was used to isolate different regions of the chloroplast genome. In order to maximize the number of cpDNA regions isolated in *N. cunninghamii*, an initial experimental phase was undertaken involving the testing of thirty-three universal chloroplast DNA primer pairs (described in Demesure *et al.* (1995); Dumolin-Lapegue *et al.* (1997); Sang *et al.* (1997); Grivet *et al.* (2001); Huang *et al.* (2002)) (Table 3.1).

Each cpDNA primer pair was initially tested using the annealing temperature described by Demesure *et al.* (1995), Dumolin- Lapegue *et al.* (1997), Sang *et al.* (1997), Grivet *et al.* (2001), Huang *et al.* (2002). The reaction mixture (25µl final volume) contained 2.5 mM $MgCl_2$; 100µg/mL BSA (Bovine Serum Albumin); 80µM each of dATP, dCTP, dGTP and dTTP; 5 picomoles of each primer; 1 x PCR buffer (67 mM Tris-HCI (pH 8.8), 16.6 mM $(NH_4)_2 SO_4$, 0.5% Triton X-100 and 5µg of Gelatin); 2 units of *Taq* polymerase (Promega; Madison, Wisconsin); and 10 ng of genomic DNA. PCR amplification was performed in a Corbett Research thermocycler as follows: an initial 4min at 94°C to denature DNA; 30 cycles of 45s at 92°C; 45s at annealing temperature (see Table 3.2); 4 min at 72°C; and a final extension of 10 min at 72°C.

Table 3.1 Forward and reverse primer pairs for the 33 primer pairs used in this study.

Name	Primer forward (5'-3')	Primer reverse (3'-5')	Reference
HK	trnH	trnK	a
K_1K_2	trnK	trnK	a
CD	trnC	trnD	a
DT	trnD	trnT	a
Sf_M	trnS	$trnf_M$	a
AS	psaA	trnS	a
K2Q	trnK2	trnQr	b
QR	trnQ	trnRr	b
C_1C	rpoC1	trnCr	b
TC	trnT	psbCr	b
f_MA	$trnf_M$	psaA	b
FV	trnF	trnVr	b
VL	trnV	rbcLr	b
AH	psbA	trnH	c
23A3	rpl23	psbA3'	d
QS	trnQ	trnS	d
SR	trnS	trnR	d
HI	atpH	atpI	d
IC2	atpI	rpoC2	d
C2C2	rpoC2-f	rpoC2-r	d
CS	trnS	orf184	d
L106	rbcLf	orf106	d
OA	orf184	petA	d
AE	petAf	psbEr	d
18p	rps18	clpp	d
pB	clpp	psbB	d
B1B2	psbB	psbB	d
B2B3	psbB	petB	d
BD	petB	petD	d
16l2	rpl16	rpl2	d
TL	trnT	trnL	e
VM	trnV	trnM	e
GP	petG	trnP	e

a, Demesure *et al.* (1995); b, Dumolin-Lapegue *et al.* (1997); c, Sang *et al.* (1997); d, Grivet *et al.* (2001); e, Huang *et al.* (2002)

Table 3.2 Amplification conditions and the approximate size (bp) of the PCR product for each primer pair that successfully amplified *N. cunninghamii* cpDNA.

Name	Annealing Temperature (^oC)	Polymerase	Approximate Size (bp) [a] in *N. cunninghamii*
HK	62	normal *Taq*	1800
K_1K_2	55	normal *Taq*	2300
DT	54	normal *Taq*	1000
Sf_M	62	normal *Taq*	1200
AS	57	normal *Taq*	2500/500[b]
K2Q	57	normal *Taq*	1400
QR	61	normal *Taq*	2800/1800 [b]
C_1C	50	Advantage™ *Taq*	3200
VL	57	Advantage™ *Taq*	3000
AH	54	normal *Taq*	550
23A3	48	Advantage™ *Taq*	3200
HI	50	normal *Taq*	1200
IC2	58	normal *Taq*	2000
C2C2	58	normal *Taq*	2700
OA	52	normal *Taq*	2600
AE	42	Advantage™ *Taq*	1900
pB	51	normal *Taq*	2500
B2B3	52	Advantage™ *Taq*	2500
BD	55	normal *Taq*	1800

[a] bp equals number of nucleotide bases pairs in PCR product

[b] PCR amplification produced double banded products

A different thermocycle was used for the AH locus with an initial 5min at 94°C to denature DNA; 30 cycles of 60s at 94°C, 60s at 46°C, 1 min at 72°C and a final extension of 5 min at 72°C. The quality of PCR products was assessed by electrophoresis on agarose gels (1%) stained with ethidium bromide.

Primer pairs that did not produce a PCR product, or PCR products that were of poor quality (e.g. low yield or more than one PCR product), were further tested under a range of annealing temperatures. Each primer pair was tested, using the

eight-sample size, at 12 different annealing temperatures in 2-4°C intervals above and below the given annealing temperature. Those primer pairs that subsequently remained unsuccessful after manipulation of annealing temperature were tested using the BD Advantage™ 2 PCR Kit (BD Biosciences Clontech). This kit is particularly useful for the amplification of large DNA fragments. The PCR reaction mixture (20µl final volume) using BD Advantage™ 2 PCR Kit contained: 1 x BD Advantage™ 2 PCR Buffer (40mM Tricine-KOH (pH 8.7), 15mM KOAc, 3.5mM Mg $(OAc)_2$, 3.75µg/mL, 0.005% Tween 20, 0.005% Nonidet-P40); 26 µM each of dATP, dCTP, dGTP and dTTP; 0.5µl of BD Advantage™ 2 Polymerase Mix; 5 picomoles of each primer; and 10 ng of genomic DNA.

3.2.3 *Detecting variation in the cpDNA of* N. cunninghamii- *PCR/RFLP*

Eight individuals (Fig 3.1) were amplified with 16 cpDNA primer pairs. These eight samples were selected to be representative of the major disjunctions in the geographic range of *N. cunninghamii* and, because of marked altitude difference within species, incorporated both high and low altitude forms.

Fourteen PCR fragments (DT, Sf_M, K2Q, C_1C, VL, 23A3, HI, IC2, C2C2, OA, AE, pB, B2B3, BD) were digested with eight restriction enzymes. Two fragments, HK and K_1K_2, were digested with 19 restriction enzymes. The specific recognition site and the specific reaction conditions for each enzyme (as recommended by New England Biolabs, Beverly MA) are described in Appendix 2. An aliquot of 5µl of PCR product was digested in a restriction digestion reaction (20µl final volume) containing 100µg/mL BSA (Bovine Serum Albumin), 1 x enzyme buffer (New England Bioproducts) and 5 U of enzyme. The digestion step was undertaken at 65°C for 3 hours for *Taq*I and overnight at 60°C for *Bst*UI, and at 37°C overnight for all other enzymes. The total 20µl reaction volume was loaded onto a 2% agarose gel and restriction fragments were separated by electrophoresis using Tris Borate EDTA buffer (1x) at 100V for 90 min. The gels were stained with ethidium bromide and photographed under UV light. RFLPs were identified visually by comparing restriction fragment patterns between samples. The size of the

restriction fragments was assessed by comparison with a 100bp molecular weight marker (Promega; Madison, Wisconsin).

Because of a lack of variation detected between the eight samples, seventy-two samples were amplified for five loci (C_1C, IC2, C2C2, OA and pB). The 72 samples chosen included multiple samples from each major region of the distribution of *N. cunninghamii*, and samples from all small isolated populations sampled (Fig 3.1). The five PCR fragments were digested with two restriction enzymes *Taq*I and *Hinf*I under the same conditions as described above.

Those fragment/enzyme combinations that produced variation between samples either using the eight or 72 sample screening size were screened for all 170 samples of *N. cunninghamii* to determine the geographic distribution of variants. Samples of *N. moorei* and *N. menziesii* were also screened with variable fragment/ enzyme combinations to provide information on the eventual status of restriction sites in *N. cunninghamii*.

3.2.4 DNA Sequencing

The nucleotide sequence of the AH and Sf_M loci were determined by direct sequencing of the PCR products. The same eight samples as used above, and an additional two samples (sample number 35 from western Tasmania and sample number 12 from the northeast highlands of Tasmania) were used for the S_fM locus (Fig. 3.1). PCR products were purified using a QIAGEN Quick PCR purification kit (QIAGEN Pty Ltd Vic, Australia). PCR products were sequenced in both forward and reverse directions. Sequence reactions were performed using the Beckman Coulter protocol as described in Poke *et al.* (2003). Products were sequenced on a Beckman Coulter CEQ 2000 automated sequencer. Sequence data was analysed using Sequencher software (Gene Codes Corporation MI, USA). Sequences were manually checked for incorrect base calls, heterozygous sites, polymorphisms, insertions/deletions (indels), and length variations (microsatellite like variation in numbers of a particular base occurring in a region between different samples). Sequences were compared to others in Genbank using the computer software program BLAST (http://www.ncbi.nlm.nih.gov).

CHAPTER 4

Results

4.1 Polymerase Chain Reaction

Nineteen primer pairs produced an amplification product in *N. cunninghamii*. Five loci were amplified using the BD Advantage™ 2 PCR Kit. A total of 34,850bp of single banded amplified product of the cpDNA genome of *N. cunninghamii* was isolated. Two primer pairs produced double-banded products under all conditions and were not used further. The approximate sizes of each primer pair product are described in Table 3.2. All loci were situated in the large single copy region, except 23A3, which amplified a region in the inverted repeat.

4.2 Restriction Digestions

The number of fragments visualized for each primer pair/ enzyme combination is given in Table 4.1 and 4.2. A total of 488 restriction fragments were observed in the eight samples screened, but only one variable character was observed. This was at the K_1K_2 locus using *Taq*I. No restriction site mutations were observed in a total of 339 restriction sites assayed, representing a total of 1389bp or nearly 1% of a 150,000bp chloroplast genome (the average size in most species; Curtis and Clegg 1984). The restriction enzymes *Taq*I and *Hinf*I generated the most fragments across 14 loci on average at 4.38 and 4.71 respectively (Table 4.2).

Table 4.1 The number of fragments observed after digestion of 14 primer pairs with eight restriction enzymes of *N. cunninghamii*. The average number of fragments for each restriction enzyme is shown. No variation between samples was observed for these primer pair/ enzyme combinations. – denotes missing data.

	Restriction Enzyme							
Locus	*Taq*I	*Hinf*I	*Alu*I	*Dpn*II	*Hae*III	*Hinp*1I	*Rsa*I	*Dde*I
DT	3	3	2	4	3	1	2	2
Sf_M	3	4	3	4	3	1	2	2
K2Q	-	4	3	2	2	2	3	2
C_1C	7	6	6	7	2	3	4	6
VL	7	6	6	4	3	-	2	4
23A3	3	8	5	5	3	3	4	4
HI	2	4	3	3	2	1	1	3
IC2	6	5	4	3	3	1	4	4
C2C2	5	6	3	4	4	3	7	6
OA	7	6	5	6	3	2	4	6
AE	4	4	1	2	3	3	3	2
pB	3	2	4	4	5	3	6	3
B2B3	4	5	6	-	4	1	4	2
BD	3	3	3	5	2	3	2	4
Average	4.38	4.71	3.86	4.08	3.00	2.08	3.43	3.57

Table 4.2 The number of fragments observed after digestion of the cpDNA fragments HK and K_1K_2 using 19 restriction enzymes across the eight samples of *N. cunninghamii*. One mutation was observed at the K_1K_2 locus using *Taq*I.

	Locus	
Restriction Enzyme	HK	K_1K_2
*Taq*I	1	8
*Hinf*I	2	2
*Alu*I	6	1
*Dpn*II	4	3
*Hae*III	2	1
*Hinp*1I	2	1
*Rsa*I	2	3
*Dde*I	2	1
*Msp*I	2	3
*Hph*I	1	1
*Nco*I	1	1
*Ssp*I	2	3
*Ase*I	3	1
*Sty*I	5	1
*Nci*I	3	3
*Dra*I	2	4
*Cla*I	1	2
*BstU*I	4	4
*EcoR*V	1	2

Three mutations were revealed in the survey of 72 samples after restriction digestion of the C2C2, IC2 and OA locus with *Taq*I. No variation was detected using the other loci/ enzyme combinations. The screening of all the *N. cunninghamii* individuals (n=170), and samples of *N. moorei* and *N. menziesii*, was conducted using the variable locus/ enzyme combinations K_1K_2/ *Taq*I, IC2/ *Taq*I, OA/ *Taq*I and C2C2/ *Taq*I. No new mutations were identified within *N. cunninghamii* with this expanded screening size. Three mutations, two at the OA locus using *Taq*I and one at the K_1K_2 locus with *Taq*I, were identified between *N. cunninghamii* and *N. menziesii*.

4.3 Genetic relationships and description of haplotypes

Overall four mutations were identified in *N. cunninghamii* using restriction digestion with the enzyme *Taq*I of the eight and seventy-two sample sizes. These mutations defined five haplotypes in *N. cunninghamii*. A further three mutations were identified between *N. cunninghamii* and *N. menziesii*. No mutational differences were found between *N. moorei* and haplotype 1 of *N. cunninghamii*. A simple haplotype network was constructed to represent the relationship between haplotypes (Fig. 4.5). Haplotype 1 occupies the central position in the network. All other haplotypes could be explained as being derived from it, and all differ from haplotype 1 by only one mutation. The mutational changes between haplotype 1 are described in Table 4.3. Mutation 1 occurs in *N. menziesii* at the K_1K_2 and is the result of the loss of a restriction site (Fig. 4.1). Mutation 2 occurs in *N. cunninghamii* at the K_1K_2 locus and is the result of either a 150bp deletion or the creation of a new restriction site resulting in a 150bp fragment that could not be observed because it migrated with other fragments of similar size (Fig 4.1). Mutation 3 in *N. cunnghamii* is caused by the loss of a restriction site in the IC2 locus (see Fig 4.2). Mutation 4 at the OA locus in *N. cunninghamii* is the result of a mutation at a restriction site causing the failure of cutting by the restriction enzyme (Fig 4.3). Mutation 5 in *N. menziesii* at the OA locus is the result of a 170bp deletion or the creation of a restriction site (Fig 4.3). Mutation 6, also in *N. menziesii* at the same locus, is probably the result of the loss of a restriction site, although the 20bp fragment cannot be visualized being too small (Fig 4.3). Mutation 7 occurs in *N. cunninghamii* at the C2C2 locus and is the result of the loss of a restriction site (Fig. 4.4).

Table 4.3 Restriction fragment length changes in haplotypes 2 to 5 in *N. cunninghamii* and in *N. menziesii,* compared to the fragment size of haplotype 1 of *N. cunninghamii* and *N. moorei.*

Mutation	Enzyme	Fragment	Change	Haplotype
1	*Taq*I	K_1K_2- 1	610 + 50 → 660	*N. menziesii*
2	"	K_1K_2- 2	550 → 400 + X	2
3	"	IC2 –1	870 +140 → 1010	3
4	"	OA- 1	620 + 410 → 1030	4
5	"	OA- 4	410 → 220 + X	*N. menziesii*
6	"	OA- 7	120 + 20 → 140	*N. menziesii*
7	"	C2C2- 1	530 + 280 → 810	5

Arrows indicate the direction of change as inferred from condition in *N. moorei* and haplotype 1 in *N. cunninghamii.* X = fragment not visualized because of either small size, or superimposed bands.

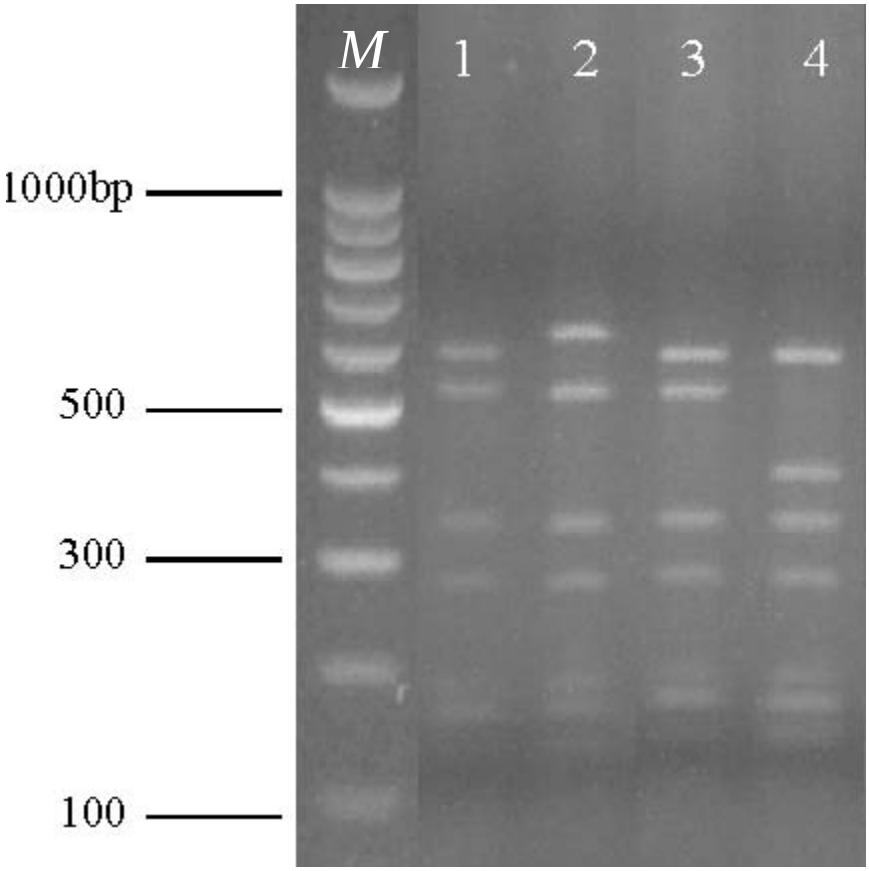

Fig 4.1. Restriction fragments produced by the digestion of K_1K_2 PCR product with *Taq*I for *N. moorei* (lane 1), *N. menziesii* (defined by mutation one) (lane 2) and for *N. cunninghamii* haplotype 1 (lane 3) and haplotype 2 (defined by mutation 2) (lane 4), after electrophoresis in 2% agarose gels. Lane M= Molecular weight marker (100bp ladder; Promega; Madison, Wisconsin). The size in base pairs is given on the left margin of the image.

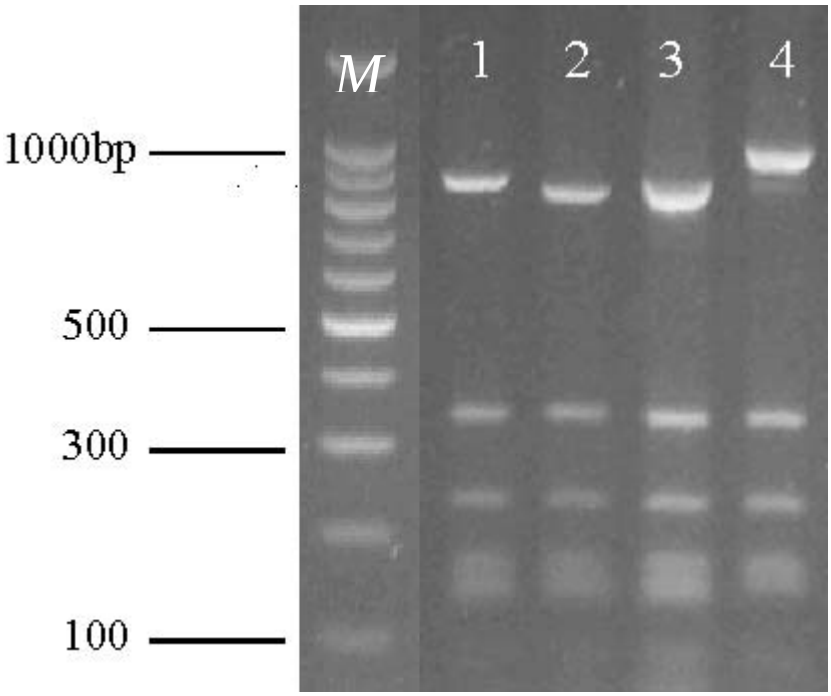

Fig 4.2. Restriction fragments produced by the digestion of IC2 PCR product with *Taq*I for *N. moorei* (lane 1), *N. menziesii* (lane 2) and for *N. cunninghamii* haplotype 1 (lane 3) and haplotype 3 (defined by mutation three) (lane 4), followed by electrophoresis in 2% agarose gels. M= Molecular weight marker (100bp ladder; Promega; Madison, Wisconsin). The size in base pairs is given on the left margin of the image.

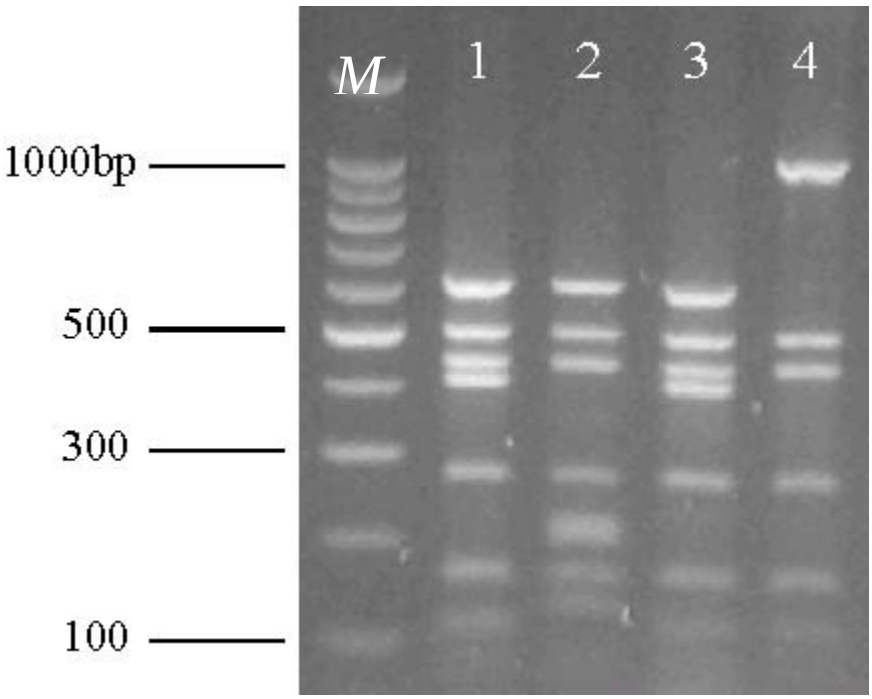

Fig 4.3. Restriction fragments produced by the digestion of OA PCR product with *Taq*I for *N. moorei* (lane 1), *N. menziesii* (defined by mutation five and six) (lane 2) and for *N. cunninghamii* haplotype 1 (lane 3) and haplotype 4 (lane 4), after electrophoresis in 2% agarose gels. M= Molecular weight marker (100bp ladder; Promega; Madison, Wisconsin). The size in base pairs is given on the left margin of the image.

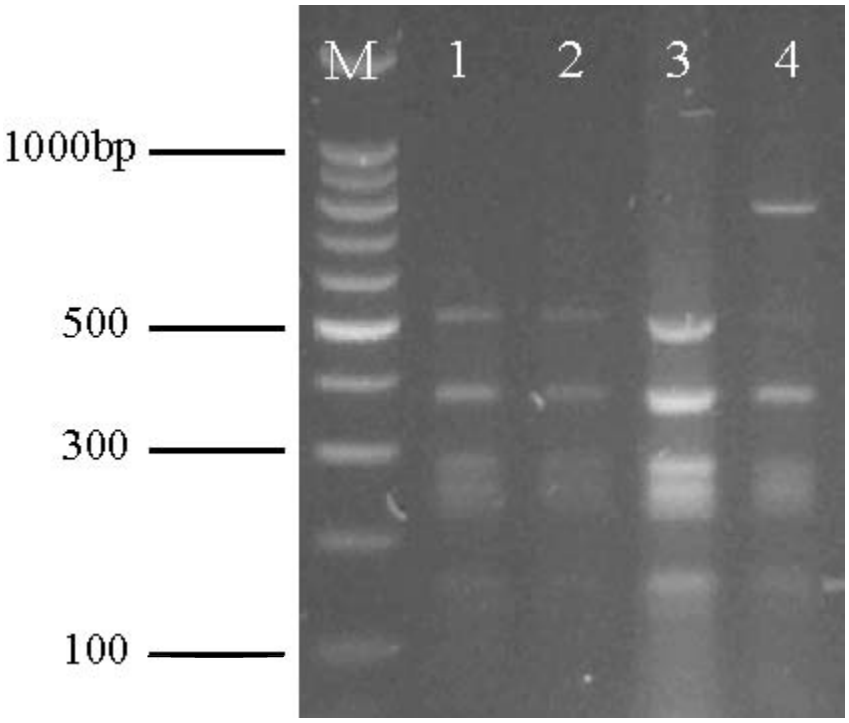

Fig 4.4. Restriction fragments produced by the digestion of C2C2 PCR product with *Taq*I for *N. moorei* (lane 1). *N. menziesii* (lane 2) and for *N. cunninghamii* haplotype 1 (lane 3) and haplotype 5 (defined by mutation seven) (lane 4), after electrophoresis in 2% agarose gels. Lane M= Molecular weight marker (100bp ladder; Promega; Madison, Wisconsin). The size in base pairs is given on the left margin of the image.

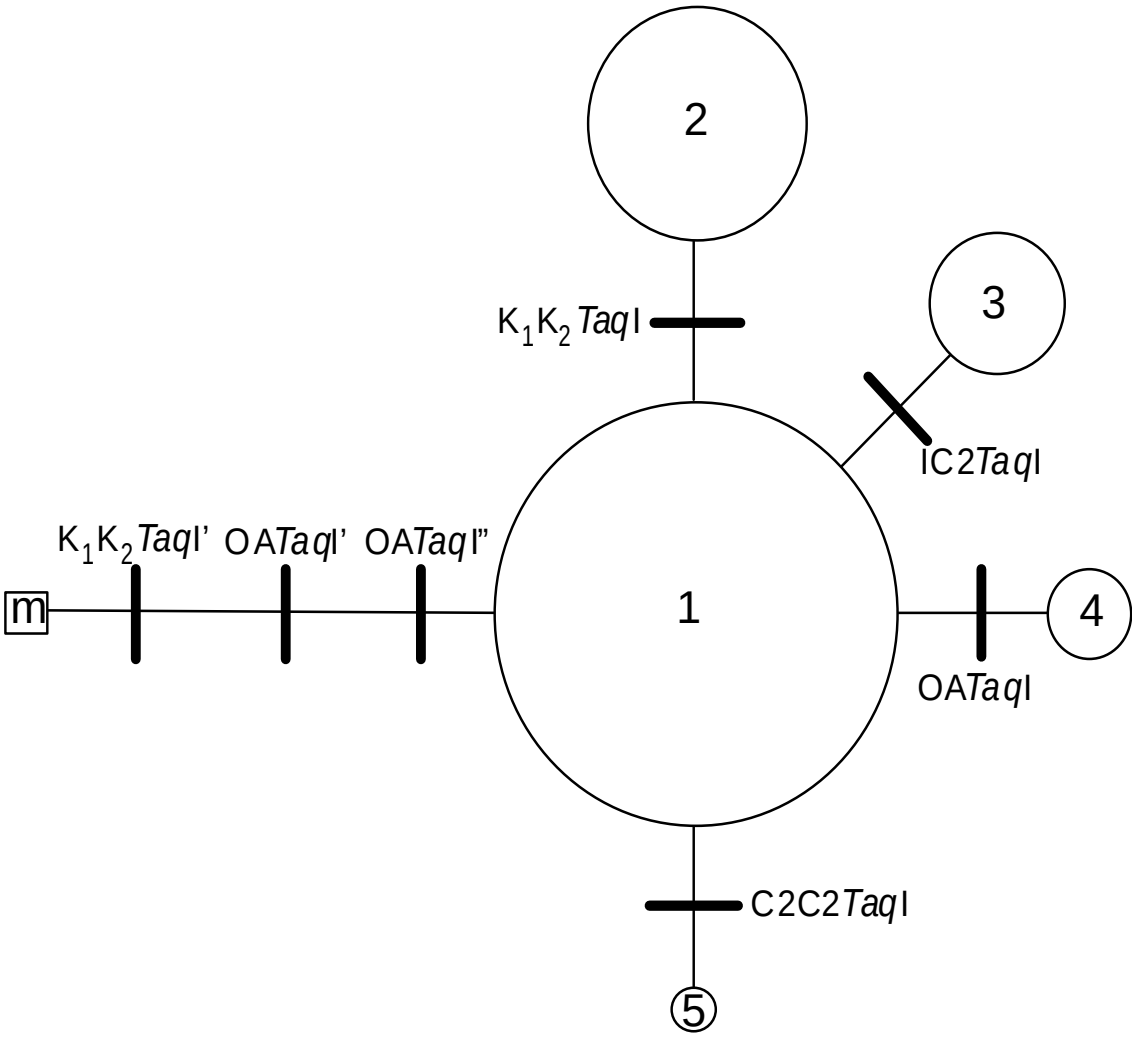

Fig 4.5 Network of the 5 haplotypes identified in *N. cunninghamii*. The area of the circles is proportional to the haplotype frequencies. Intersecting lines indicate a single mutational step difference from the common haplotype, with loci enzyme combinations identifying mutation shown. *N. moorei* is identical to haplotype 1 for all loci/enzyme combinations surveyed. m = *N. menziesii*.. Numbers refers to the haplotype number.

4.4 Distribution of Haplotypes

Of the 170 individuals screened the haplotype of 36 samples could not be determined because of missing data at one or more of the polymorphic loci/ enzyme combinations (Appendix 1). Therefore the discussion of distribution of haplotypes refers only to those 134 samples with no missing data.

The distribution of the five haplotypes in *N. cunninghamii* shows strong geographic structure (Fig 4.6). Haplotype 1 is the most common (detected in 90 samples) and widespread across Tasmania and all regions of Victoria, except the Central Highlands (Fig. 4.6). Haplotype 2 (29 samples) is found in all samples from the Victorian Central Highlands and is present with Haplotype 1 in the Strezlecki Ranges. Haplotype 3 is found in nine samples from the northeast highlands of Tasmania, and in one individual from the Great Western Tiers. Haplotype 4 was detected in four samples in western and central Tasmania, and Haplotype 5 was found in only one individual from Lake St Clair. All three areas with multiple samples collected (Dazzler Ranges, Blue Tier and Lookout Hill) were fixed for the same haplotype. In addition, no association was found between haplotype and altitude of origin. For example, high altitude forms of *N. cunninghamii* on Mt Sprent and in the Sentinel Range possessed different haplotypes (haplotype 1 and haplotype 4, respectively).

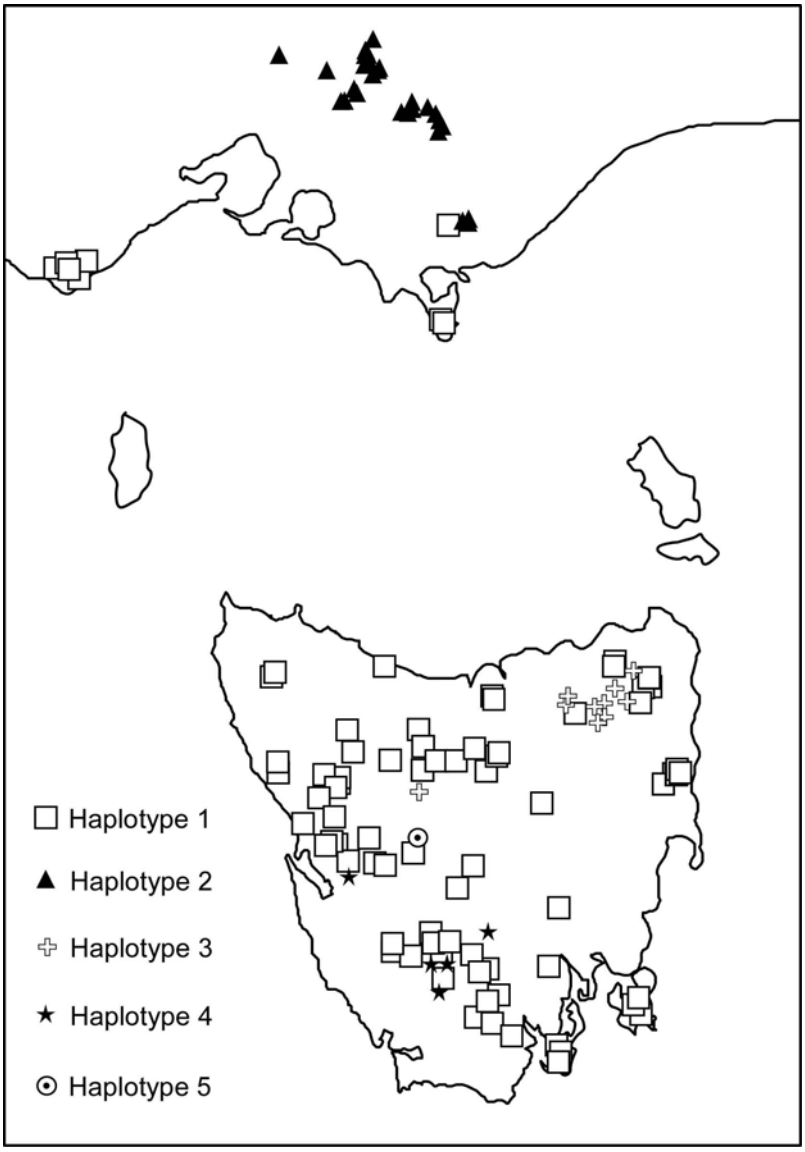

Fig 4.6 The distribution of the five haplotypes identified in *N. cunninghamii* by PCR/RFLP screening.

4.5 DNA Sequencing

A total of 441bp of the AH locus was successfully sequenced. No variation was detected between the 8 samples. 1200bp of the S_fM locus was successfully sequenced. A mutation at a poly-T region (bp 442; Table 4.5) and a small indel (bp 780-781; Table 4.5) were identified. These two mutations defined three variants whose distribution is shown in Fig. 4.7. Variant A was shared between widespread samples in western Tasmania, northeastern Tasmania, and the Victorian Central Highlands and is most likely the ancestral form of these three variants. Three samples, two from northeastern Tasmania and one from the Dazzler Ranges shared variant B. Variant C was found in four samples from western, northwestern and southern Tasmania and the Otway Ranges (Fig. 4.7; Table 4.5).

Table 4.5 Summary of the DNA sequence variation observed between samples and their base position in the S_fM locus. The PCR/RFLP haplotype of each individual is shown for comparison.

	Base Position			
Variants	780, 781	442	Samples	PCR/RFLP Haplotype
A	G A	$(T)_{10}$	24	1
"	"	"	102	3
"	"	"	12	2
B	T C	$(T)_{10}$	9	1
"	"	"	141	?1
"	"	"	154	1
C	T C	$(T)_{11}$	35	1
"	"	"	79	1
"	"	"	166	?1
"	"	"	72	1

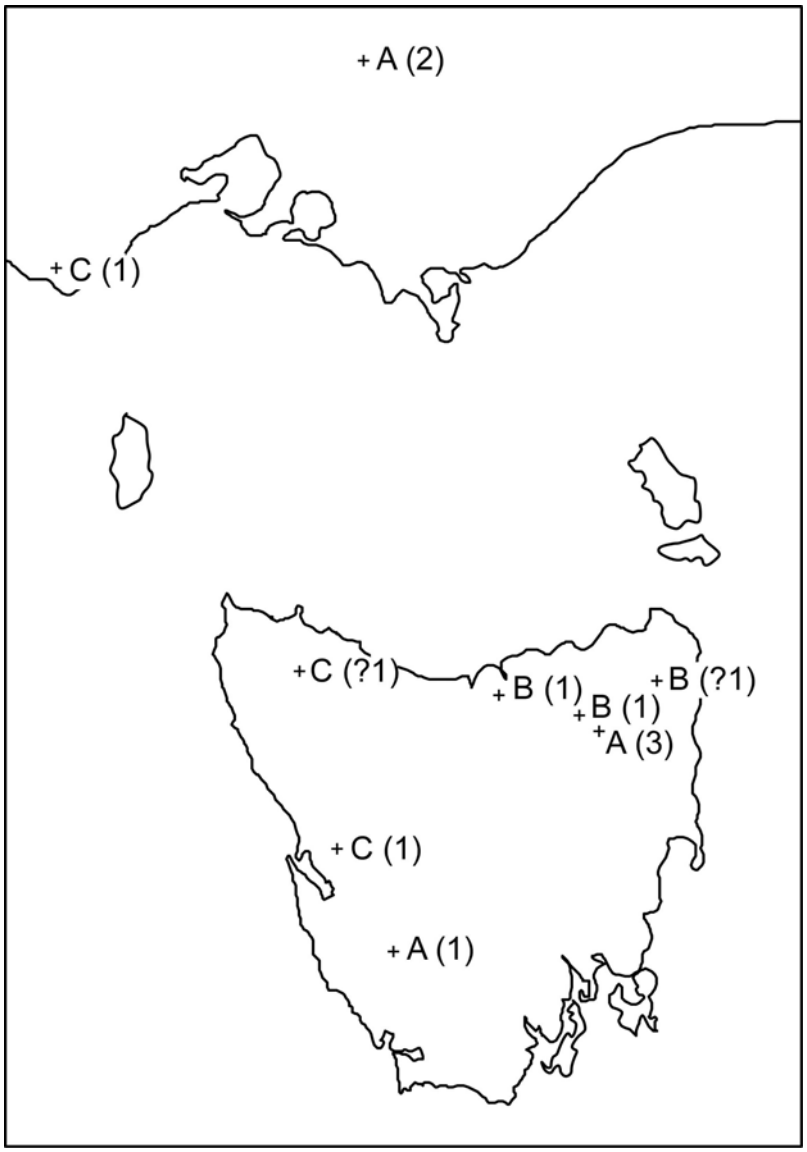

Fig 4.7. The three variants defined by sequence data and their distribution. The PCR/RFLP haplotype is indicated for each sample in parenthesis.

CHAPTER 5

Discussion

5.1 Low variation of chloroplast DNA

In total four mutations were detected in the chloroplast DNA (cpDNA) of *N. cunninghamii*. Three of the four mutations are likely the result of point mutations. This result is not consistent with Clegg *et al.* (1994) who consider that insertion/deletion mutations account for much of the genetic variation detected using the PCR/RFLP method. The number of mutations detected in this study is low relative to some other studies (Table 5.1) considering the number of loci/enzyme combinations used (150). There are four main alternative explanations for this low observed diversity in *N. cunninghamii*.

The first alternative is that the PCR-RFLP technique used in this study failed to detect variation that exists in the genome of *N. cunninghamii*. While a large number of restriction enzymes were used in the eight-sample screening size in total only about 1% of the entire genome was analysed for variation. This certainly would have restricted the amount of variation detected. However, other studies have detected higher levels of variation while analysing even less of the genome (Table 5.1). The finding of three mutations in the expanded sample size of 72 suggests that the number of samples used in the initial screening was a limiting factor in the detection of variation and that the variation may in fact be higher than implied by the analysis of eight samples. This highlights the importance of using large sample sizes in PCR-RFLP studies, especially considering that that the ancestral haplotype is sometimes widespread across a species distribution. It is difficult to compare the amount of cpDNA variation detected between different studies due to the differences in life and past histories affecting the chance of cpDNA genetic differentiation in species, and the differences in the number of PCR fragments and enzymes employed in PCR-RFLP studies. However, in general the amount of variation detected has been

low (Weising and Gardner 1999), but highly variable between species. At the extremes no variation has been reported for the alpine plant *Erinus alpinus* (Stehlik *et al.* 2002a), while cpDNA variation has been useful in infering the biogeographical history in other species (e.g Grivet and Petit 2003) (Table 5.1). Therefore the ability to make biogeographical interpretations from PCR-RFLP studies has varied widely between studies.

Table 5.1 Examples of the number of chloroplast DNA mutations detected by other studies using PCR/RFLP technique. **N= *the number of individuals used to screen for variation.***

Species	Distribution	*N*	Loci/ enzyme	Mutations	Reference
Hazel	Europe	248	17	4	Palme and Vendramin (2003)
N. nervosa	S. Andes	22	25	3	Marchelli *et al.* (1998)
Hornbeam	Europe	100	28	5	Grivet and Petit (2003)
Holly	Europe	12	103	2	Rendall and Ennos (2003)
Erinus alpinus	NW Alps	13	72	0	Stehlik *et al.* (2002)
Frangula alnus	Europe	46	36	11	Hampe *et al.* (2003)
Castanea sativa	Europe	181	99	6	Fineschi *et al.* (2000)
Eritrichium nanum	Alps	148	117	17	Stehlik *et al.* (2002)

The second explanation is that the low number of mutations detected is due to the low mutation rate of the chloroplast genome in *N. cunninghamii*. The cpDNA mutation rate of plants is at least four times slower than that of the nuclear genome (Wolfe *et al.* 1987), and is considered to be even slower for long-lived forest trees (Dumolin-Lapegue *et al.* 1997), which would include *N. cunninghamii*.

The third possible explanation for the low variation observed in *N. cunninghamii* is that the whole species has experienced at least one severe bottleneck in the past, as a result of the extinction of the species from most of its range, and a resultant loss of diversity. The ability of *N. cunninghamii* to restore diversity after such an event would be limited considering the longevity of the species, with a

maximum stem lifespan of between 450-500 years (Read 1999), and its vegetative capacity of regeneration (Howard 1973b). For example, if the bottleneck event occurred during the Last Glacial Maximum18000 years ago (Colhoun *et al.* 1996), considering an average lifespan of two centuries, *N. cunninghamii* would have had as few as 90 generations to recover variation. Lifespan is the relevant measure of generation time because the vast majority of reproductive effort in trees results in complete failure (Cannon and Manos 2003). However, the evidence for *N. cunninghamii* having passed through a single bottleneck in the past is not strong. Firstly, the fossil record suggests that the species survived in more than one region during the Last Glacial (McKenzie 1997; Colhoun 2000). In addition, the species was likely to be widespread in western Tasmania during the last glacial with temperature, not rainfall, being the strongest limiting factor (Colhoun 2000). McKenzie (1997) considered that pollen evidence for survival of *N. cunninghamii* through the last glacial in the Central Highlands of Victoria indicated the species survived temperatures that would not be expected based on current knowledge of the species temperature tolerance. Therefore, the species may have been more widespread in western Tasmania than suggested by Colhoun (2000). The survival of *N. cunninghamii* in separate regions would work to increase diversity through genetic drift and isolation, therefore, catastrophic bottlenecks is unlikely to explain the low variation detected in the species.

The fourth alternative to explain the low diversity is natural selection. While universal markers that have been developed have been considered to be selectively neutral, the non-coding component of the amplified products is only a portion of the total length of sequence amplified. The absence of recombination in the chloroplast genome (Birky 1995; Ennos *et al.*1999) means that if a mutation occurred that gave a selective advantage and was finally fixed, then all other parts of the genome would also be fixed, working to reduce chloroplast variation. This is called a ‘selective sweep’ (Maruyama and Birky 1991). The likelihood of this occurring is dependant on the rate at which new advantageous mutations occur (Ennos *et al.* 1999), which is probably low. However, this possibility cannot be disproved.

5.2 Biogeographical inferences from the distribution of haplotypes in *N. cunninghamii*

The possession of haplotype 1 by both *N. cunninghamii* and *N. moorei*, and *N. menziesii* differing by three mutations, suggests a closer relationship between *N. cunninghamii* and *N. moorei* than either species to *N. menziesii.* This relationship is supported by phylogenetic analysis of these species based on vegetative and reproductive morphological data combined with molecular (chloroplast DNA and nuclear DNA data) (Jordan and Hill 1999). In this study outgroup analysis suggests that haplotype 1 is ancestral within *N. cunninghamii*. However, identification of the ancestral haplotype using simple outgroup methods (i.e *N. moorei* and *N. menziesii*) is not definitive. While haplotype 1 remains the most likely candidate due to its presence in sister taxa *N. moorei* (Jordan and Hill 1999), it is possible that all the haplotypes found in *N. cunninghamii* haplotypes were transferred to both species via their common ancestor. In other words *N. cunninghamii* may not be monophyletic with regard to haplotype. However, two other lines of evidence supporting the inference that haplotype 1 is ancestral in *N. cunninghamii* can be invoked: 1) it is the internal haplotype within the haplotype network (Schaal *et al.* 1998), and 2) it is the most abundant and geographically widespread. Under this scenario *N. menziesii* haplotype may have been derived from the ancestral state haplotype 1.

Due to the ancestral status of haplotype 1 where it occurs with no other haplotypes such as the Otway Ranges, Wilsons Promontory, Douglas Apsley and large areas of the western half of Tasmania, it is not possible to make any biogeographical inferences about its distribution. There are two possible opposing interpretations that can be made: 1) first that due to its internal position in the hapotype network, its distribution is the result of fragmentation (Schaal *et al.* 1998), for example between the Otway Ranges and western Tasmania or Wilsons Promontory. Under this interpretation these populations could have remained *in situ* for a very long period of time, throughout the evolutionary history of *N. cunninghamii*. The second interpretation is that the widespread distribution of the ancestral haplotype has arisen as a result of post-glacial migration (Stehlik *et al.* 2002b), or in some regions through a long distance dispersal event. In western

Tasmania the pollen evidence for *N. cunninghamii* supports this second interpretation, with evidence for postglacial recolonization during the last postglacial replacing areas formerly occupies by alpine vegetation and ice caps during the glacial period. However, survival in glacial refugia cannot be ruled out within this region, while no interpretation can be made for the Otway Ranges, Wilsons Promomtory and Douglas Apsley where the fossil record is limited.

Stronger biogeographical interpretations can be made where the ancestral haplotype is found with derived haplotypes. In two major regions of the distribution of *N. cunninghamii* the ancestral haplotype occurs with derived haplotypes, that is haplotype 3 in the northeast highlands of Tasmania, which is almost confined to the region, and with haplotype 4 in western Tasmania, which appears to be endemic to this region. The presence of haplotype 3 in western Tasmania needs to be confirmed, because of this apparent geographic anomaly. In a study of the distribution of cpDNA variation within the genus *Lithocarpus* in southeast Asia, Cannon and Manos (2003) found that the ancestral haplotype was widespread across its range, with a high level of endemism within each major region of its patchy distribution. This pattern was interpreted as each region having experienced little migration and extinction allowing both the accumulation of divergent haplotypes and the persistence of an ancient haplotype within each region (Cannon and Manos 2003). This interpretation was particularly strong with the identification of 76 DNA sequence haplotypes. In comparison, the diversity of derived haplotypes is very low in this study but a similar explanation whereby *N. cunninghamii* has remained for a long period in the northeast highlands and western Tasmania can be inferred. This interpretation is strengthened by the widespread distribution of each haplotype within each region. Haplotype 3 in the northeast highlands covers a geographic range of at least 50km, and haplotype 4 in western Tasmania covers a greater geographic range of almost 100km. The widespread distribution of these haplotypes must have arisen as a result of postglacial expansion into the vacant landscape from one or more refugia within each region of their distribution. An origin via migration is unlikely since these haplotypes are not found, or are rare, in other regions (although fully understanding the distribution of haplotype 3 in western Tasmania may change this interpretation for the northeast

highlands of Tasmania). The widespread distribution of these haplotypes is strong evidence against these haplotypes being derived from recent (i.e current interglacial) mutations, as a mutation (that must originate from only one individual) would take a long time to spread over a wide range into an already occupied landscape due to the low dispersal capacity of *N. cunninghamii* and competition. This widespread distribution therefore supports the hypothesis that these haplotypes have been present in each region for a long period. Although a wider distribution of these haplotypes in the past with subsequent extinction in other regions through genetic drift cannot be ruled out. Survival for a long period by *N. cunninghamii* implies glacial survival in the northeast highlands of Tasmania and Western Tasmania. While the evidence for glacial survival through pollen evidence is extremely strong for western Tasmania (Colhoun 2000) no fossil evidence to date has been found of *N. cunninghamii* in the northeast highlands. The evidence here is consistent with other genetic and non-genetic evidence that is available. Similar to rainforest species in Tasmania, eucalypts, the dominant of non-rainforest vegetation, were also severely restricted in their distribution during the last glacial. McKinnon *et al.* (2001) surveyed the sequence variation of the J_{LA} region of cpDNA (found to be hypervariable by Vaillancourt and Jackson 2000), in 17 species of Tasmanian *Eucalyptus* subgenus *Symphomyrtus*. The near restriction of a shared haplotype clade (J_{et}) to eastern Tasmania was considered to indicate a glacial refugia for eucalypts in this region (McKinnon *et al.* 2001). While eucalypts and *N. cunninghamii* have different ecological tolerances, glacial survival of eucalypts in the region suggests that climate was warm enough to allow survival of tree species, but perhaps as shrubs rather than tree form (Duncan 1990). *N. cunninghamii* may have survived in small populations or even individual trees on steep southfacing slopes supplemented by water runoff and/or in deep gullies, or at high altitudes with water availability supplemented by orographic rainfall. The ability of the species to survive in regions where climate is highly marginal is supported by the presence of some small populations of *N. cunninghamii* today that are able to survive in special topographical conditions (e.g. Yarlington Tier; Harle *et al.* 1993). Eastern Tasmania also has a high level of local

endemic plant species (Kirkpatrick and Brown 1984), which has long been interpreted as evidence for an eastern Tasmania forest glacial refugia.

The presence of two derived haplotypes in western Tasmania could represent evidence for glacial survival of *N. cunninghamii* in one or more refugia. Haplotype 4 is of particular interest. Its distribution corresponds to a number of areas that have been identified as possible forest refugia (Kirkpatrick and Fowler 1998), for example near Bird River. Its widespread distribution could be a result of expansion out of southwestern refugia during the present interglacial. However, further sampling of the southwest of Tasmania is needed to reveal the full biogeographical importance of this haplotype.

Little biogeographical inferences can be made of haplotype 5 since it is restricted to only a single individual. The simplest explanation is that it represents a recent mutation. Further sampling in the proximity of this haplotype will give a greater understanding of this haplotypes biogeographical significance.

Haplotype 2 appears fixed in the Central Highlands of Victoria. The presence of this distinct haplotype, and its wide geographical range of at least 165km (for reasons discussed earlier in this chapter), both support fossil evidence for glacial survival in the region (McKenzie 1997). Currently *N. cunninghamii* is very restricted in it distribution in the region, occurring in only small stands on moist southeast facing slopes or creek gullies (Read and Brown 1996). McKenzie (1997) discovered low but consistent pollen evidence at the height of the last glacial in this region, suggesting survival of *N. cunninghamii* in small patches or even as isolated trees. This low population size would constitute a severe bottleneck and is therefore consistent with the absence of haplotype 1 and the fixation of haplotype 2 in the Central Highlands of Victoria. It would be particularly interesting to find variation within this haplotype, if it exists.

Both haplotypes 1 and 2 are present in the Strzelecki Ranges. *N. cunninghamii* has a very restricted distribution in this area, surviving in only the most protected south facing slopes and creek gullies. While there is no fossil evidence available as to whether the species survived in the region, the presence of extensive relict linear dunes in northern Wilsons Promontory and southeast Gippsland suggest

past periods of aridity, probably during the Last Glacial (Hill and Bowler 1995), in close proximity to the Strzelecki Ranges. The relatively low altitude of the ranges reducing the likely effect of oreographic rainfall and a small area would have provided less opportunity for survival than in the Central Highlands. Therefore, the most likely explanation for the presence of two haplotypes in this region is due to the admixture of haplotypes as a result migration from separate refugia possessing the different haplotypes. A similar pattern of chloroplast DNA variation was observed in *Pinus resinosa*, where high diversity of haplotypes was found in an area that is known was glaciated, also suggesting admixture in this region from separate refugia (Walter and Epperson 2001). These refugia would most likely be in the highlands of Victoria, but the source of haplotype 1 in Wilsons Promontory and the Strzelecki Ranges is unknown. While a source in Victoria (e.g. the Otway Ranges) cannot be ruled out, migration from a refugium in Tasmania is also possible. In fact the close similarity between the Tasmanian and Victorian floras implies a long history of interchange of flora between Victoria and Tasmania across land bridges exposed across Bass Strait during glacial times (Hill *et al.* 1999). This interchange of flora may have been facilitated by a time lapse between the amelioration of conditions after glacials and subsequent rise in sea level (Belperio 2002), which has occurred repeatedly over the last 17 million years (McKinnon *et al.* submitted). Freeman (2001) found strong evidence for recent seed mediated gene flow across Bass Strait by eucalypts based on the sharing of a cpDNA haplotype between western Tasmania and Victoria. A problem exists under this hypothesis as it suggests that individuals bearing haplotype 1 have migrated across the land bridge from Tasmania to Victoria, a far greater distance than haplotype 2 is postulated to have travelled. This apparent greater capacity of migration of haplotype 1 could indicate that individuals bearing this haplotype possessed a selective advantage. The more rapid migration of haplotype 1 may have blocked the southern advance of haplotype 2, unable to establish in an already occupied area. It is possible that haplotype 2 was able to advance further than the Strzelecki Ranges but has since been lost due to genetic drift or was not detected in this study.

5.3 Future research

This research has demonstrated that chloroplast DNA can be used to help understand the past history of *N. cunninghamii*, the dominant of most cool temperate rainforest in Australia. However, more variation in the chloroplast genome would be useful to enable stronger biogeographical conclusions to be made. This study has shown that the PCR/RFLP method when used with large numbers of samples is effective in discovering variation. Further screening of cpDNA universal loci successfully amplified in *N. cunninghamii* in this study using a large sample size could reveal further variation. Restriction enzymes such as *Alu*I, *Dpn*II, *Rsa*I or *Dde*I, which produced a high average number of fragments in the loci screened, provide good potential of detecting further variation using the PCR/RFLP technique.

Sequencing of the S_fM locus using all samples needs to be completed. Although only 10 samples were sequenced at this locus, the distribution of variants suggest some intriguing biogeographical patterns that will contribute to the interpretations of past history made from the present PCR/RFLP haplotypes, with the Otway ranges and Western Tasmania sharing a variant suggesting a close relationship between these regions. The northeast highlands of Tasmania and the isolated population at the Dazzler Ranges share a variant supporting the suggestion in this study that the northeast highland populations are derived from a northeastern refugia. Also, a widespread ancestral variant persists in the central highlands, western Tasmania and northeastern Tasmania.

Chloroplast microsatellites also provide the opportunity to detect further variation. Ten primer pairs have been developed by (Weising and Gardner 1999) that allow the amplification by PCR of microsatellite regions within the chloroplast genome. Microsatellites are a class of repetitive DNA (Powell *et al.* 1995) and consist of tandemly reiterated, short DNA sequnce motifs (Wang *et al.* 1994). They are abundant in chloroplast genomes and found to exhibit length variation (Powell *et al.* 1995) as a result of variable repeat copy number. This variation can be visualized by PCR and electrophoretic separation of amplification products (Weising and Gardner 1999). Eight of the primer pairs developed by (Weising and Gardner 1999) are applicable across a wide range of dicotyledonous angiosperms. Chloroplast

microsatellites have been shown to be useful for the detection of variation in a number of studies and have increased the number of haplotypes identified when used in conjunction with PCR-RFLP technique using universal primers (e.g Palme and Vendramin 2002; Grivet and Petit 2003). However, the use of chloroplast microsatellites in phylogeographical studies may be limited by significant size homoplasy (Doyle *et al.* 1998).

Appendix 1. Localities samples for DNA analysis. Some sample sites share the same locality name. Note Central Highlands refers to the Victorian Central Highlands.

			UTM		Altitude	
N°	Location	Region	easting	northing	(m.a.s.l)	Haplotype
59	Coolangatta Road	Bruny Island	523200	5199350	440	1
60	Cuthberts Road	Bruny Island	525850	5194710	175	1
61	Sheepwash Creek	Bruny Island	524400	5188100	340	1
57	Dove Lake	Central Tasmania	413638	5387965	974	1
64	Frenchmans Cap	Central Tasmania	403500	5319950	1200	1
65	Philps Creek	Central Tasmania	410320	5318500	430	1
129	Dee River	Central Tasmania	468100	5318050	650	1
30	Wayatinah	Central Tasmania	457729	5303483	261	1
31	Lake St Clair	Central Tasmania	430723	5337315	304	5
32	King William Creek	Central Tasmania	428712	5326543	815	1
33	Collingwood River	Central Tasmania	413009	5328090	435	?
34	Raglan Creek	Central Tasmania	399710	5336547	505	1
153	Holwell Gorge	Dazzler Ranges	480492	5431066	304	1
154	Holwell Gorge	Dazzler Ranges	480530	5430720	302	1
130	Apsley River	Douglas-Apsley	593302	5372185	504	1
131	Apsley River	Douglas-Apsley	593301	5372192	525	1
132	Lookout Hill	Douglas-Apsley	601954	5378531	476	1
133	Lookout Hill	Douglas-Apsley	601926	5378531	476	1
134	Lookout Hill	Douglas-Apsley	601877	5378478	474	1
135	Lookout Hill	Douglas-Apsley	601811	5378417	474	1
136	Lookout Hill	Douglas-Apsley	602015	5378573	472	1
137	Douglas River	Douglas-Apsley	600243	5379169	404	1
155	Dasher River	Great Western Tiers	432218	5408269	360	1
156	Mersey River	Great Western Tiers	435272	5397041	310	1
157	Jacksons Creek	Great Western Tiers	431981	5366482	645	3
158	Mersey Forest Road	Great Western Tiers	434844	5381333	452	1
159	Lake Mackenzie Road	Great Western Tiers	443795	5387468	1136	1
160	West Rope Road	Great Western Tiers	456815	5387561	510	1
161	Long Ridge	Great Western Tiers	469030	5395732	263	1
162	Cluan Tier	Great Western Tiers	483455	5390720	722	1
163	Cluan Tier	Great Western Tiers	483577	5390708	732	1
128	Projection Bluff	Great Western Tiers	476800	5380700	1240	1
19	Millers Bluff	Great Western Tiers	513362	5359650	1090	1
4	St Patricks River	North-east Tasmania	528509	5424201	360	3
5	Myrtle Bank Road	North-east Tasmania	529981	5430103	500	3
6	Diddleum Plains	North-east Tasmania	537802	5425156	590	1
7	Ben Ridge Road	North-east Tasmania	553728	5425156	860	3
8	St Patricks River	North-east Tasmania	547436	5422929	640	3
9	Mt Barrow	North-east Tasmania	535375	5419110	1200	1
10	Ben Lomond	North-east Tasmania	555130	5404069	1340	?

11	Telopea Road	North-east Tasmania	554059	5415923	890	3
12	Billybrook Creek	North-east Tasmania	549623	5412146	680	3
138	Ransom River	North-east Tasmania	590065	5433095	105	?

Appendix 1. Cont'd

			UTM		Altitude	
N°	Location	Region	easting	northing	(m.a.s.l)	Haplotype
139	Poimena Road	North-east Tasmania	585047	5436920	579	1
140	Blue Tier	North-east Tasmania	584088	5442602	647	1
141	Blue Tier	North-east Tasmania	583996	5442671	649	?
142	Blue Tier	North-east Tasmania	583944	5442829	648	1
143	Blue Tier	North-east Tasmania	584075	5442906	662	1
144	Blue Tier	North-east Tasmania	584161	5443012	683	?
145	Blue Tier	North-east Tasmania	584179	5442528	644	1
146	Weldborough Pass	North-east Tasmania	580047	5436141	570	1
147	Moorina	North-east Tasmania	572904	5446854	118	3
148	Mt Horror	North-east Tasmania	561549	5453553	670	1
149	Mt Horror Road	North-east Tasmania	560890	5450304	220	1
150	Ringarooma River	North-east Tasmania	560850	5435083	205	3
151	South George River	North-east Tasmania	578194	5426139	261	1
152	Mt Victoria	North-east Tasmania	568616	5426222	700	3
75	Emu River	North-west Tasmania	409850	5450300	70	1
164	Holder Rivulet	North-west Tasmania	351850	5454600	140	?
165	Holder Rivulet	North-west Tasmania	351850	5454600	140	1
166	Milkshake Hills	North-west Tasmania	346150	5448100	150	?
167	Sumac Spur 2	North-west Tasmania	335450	5443490	170	1
168	Sumac Road	North-west Tasmania	336500	5446400	60	1
66	Russell River	South-east Tasmania	477950	5250050	360	1
67	Denison Road	South-east Tasmania	479519	5240285	340	?
68	Huon River	South-east Tasmania	485071	5232628	80	1
69	Mt Picton Track	South-east Tasmania	469871	5217822	485	1
70	Hartz Mountains	South-east Tasmania	480860	5213758	910	1
71	Nevada Peak Track	South-east Tasmania	472270	5247961	960	1
72	Esperance River	South-east Tasmania	493600	5205400	80	1
73	Tahune Airwalk	South-east Tasmania	477687	5228306	70	1
74	Arve River Road	South-east Tasmania	489930	5223210	320	?
105	Styx Valley	South-east Tasmania	467250	5259370	380	1
1	Mt Field	South-east Tasmania	475380	5274700	350	?
3	Moonlight Flats	South-east Tasmania	485530	5189300	560	?
21	Mt Wellington	South-east Tasmania	518062	5251601	1100	1
169	Yarlington Tier	South-east Tasmania	524583	5290342	670	1
58	Mt Fortescue	Tasman Peninsula	578500	5219600	380	1
62	Fortescue Bay Road	Tasman Peninsula	573500	5226000	170	1
63	Waterfall Bay Walk	Tasman Peninsula	577000	5230700	480	1
13	Mt Read	Western Tasmania	378907	5366291	1136	1
14	Lake Gordon	Western Tasmania	447250	5261300	530	?
15	Sentinel Range	Western Tasmania	437800	5253300	900	1
16	Scotts Peak Dam	Western Tasmania	443100	5234500	300	4

17	Frodshams Pass	Western Tasmania	448300	5253500	360	4
18	Condominium Creek	Western Tasmania	448300	5243700	320	4
22	Clear Hill Road	Western Tasmania	440237	5273923	760	1

Appendix 1. Cont'd

			UTM		Altitude	
N°	Location	Region	easting	northing	(m.a.s.l)	Haplotype
23	Clear Hill Road	Western Tasmania	441923	5267060	340	1
24	Mt Sprent	Western Tasmania	415138	5261605	1050	1
25	Gordon River Rd	Western Tasmania	427183	5258371	320	1
26	Timbs Track	Western Tasmania	452603	5267965	520	1
27	Gordon Dam	Western Tasmania	415250	5266750	110	1
28	Snake Gully	Western Tasmania	380167	5372633	165	1
29	Mt Black	Western Tasmania	380285	5376525	661	1
35	Mt Jukes Road	Western Tasmania	378297	5332404	96	1
36	Bird River	Western Tasmania	383787	5310999	93	1
37	Crotty River	Western Tasmania	385971	5321232	200	4
38	near Strahan	Western Tasmania	375225	5334326	332	1
39	near Strahan	Western Tasmania	371079	5331587	246	1
40	Henty River	Western Tasmania	356137	5346089	254	1
41	near Zeehan	Western Tasmania	364274	5351862	159	1
42	Heemskirk River	Western Tasmania	351813	5369218	176	?
43	near Corrina	Western Tasmania	339781	5379666	210	?
44	Pieman River	Western Tasmania	339663	5386800	18	1
45	near Corrina	Western Tasmania	347660	5375320	220	1
46	near Stanley River	Western Tasmania	358693	5380940	262	?
47	Huskinson River	Western Tasmania	370158	5378492	135	?
48	Mt Read high	Western Tasmania	378907	5366291	1136	1
49	Mt Read low	Western Tasmania	377651	5370020	637	?
50	Murchison Highway	Western Tasmania	366670	5363092	216	1
51	Henty Glacial Moraine	Western Tasmania	376841	5350426	396	1
52	Tullah	Western Tasmania	383673	5383675	289	1
53	Bulgobac River	Western Tasmania	389135	5393411	660	?
54	Luina	Western Tasmania	371854	5406056	681	1
55	Mt Pearce	Western Tasmania	385336	5407962	652	?
56	Vale River	Western Tasmania	405508	5400083	887	1
83	Kingslake NP	Central Highlands	337620	5855451	633	?
84	Wirra-Willa	Central Highlands	369069	5845270	617	2
85	Yarra Ranges NP	Central Highlands	377367	5825699	644	2
86	Yarra Ranges NP	Central Highlands	378390	5824876	644	?
87	Mt Donna Buang	Central Highlands	380806	5825131	644	2
88	Mt Donna Buang	Central Highlands	383695	5825654	1257	2
89	Yarra Ranges NP	Central Highlands	385123	5823414	935	?
90	Yarra Ranges NP	Central Highlands	386145	5825624	909	?
91	Yarra Ranges NP	Central Highlands	386921	5827516	680	?
92	Yarra Ranges NP	Central Highlands	388950	5830096	714	?
93	Yarra Ranges NP	Central Highlands	387042	5833000	657	2
94	Cumberland Road	Central Highlands	395779	5845550	964	2

95	Cumberland Road	Central Highlands	399375	5842488	998	?
96	Cumberland Road	Central Highlands	402174	5843727	999	2
97	Cumberland Road	Central Highlands	402937	5845451	983	?

Appendix 1. Cont'd

			UTM		Altitude	
N°	Location	Region	easting	northing	(m.a.s.l)	Haplotype
98	Camberville Road	Central Highlands	403801	5846979	983	2
99	Lady Talbot Road	Central Highlands	393788	5848714	436	2
100	Lady Talbot Road	Central Highlands	398085	5849088	436	2
101	Lady Talbot Road	Central Highlands	395757	5852038	852	2
102	Blue Range Road	Central Highlands	394322	5855453	268	2
103	Blue Range Road	Central Highlands	394623	5858557	268	2
104	Ruoaks Road	Central Highlands	395035	5858308	1074	2
106	Ruoaks Road	Central Highlands	396398	5855419	1134	2
107	Royston River Road	Central Highlands	399540	5865927	222	2
108	Royston River Road	Central Highlands	401243	5861798	240	2
109	Thompson Valley	Central Highlands	445192	5808182	1067	?
110	Mt Erica	Central Highlands	444441	5805361	635	2
111	Mt Erica	Central Highlands	442460	5804836	757	?
112	Thompson Valley	Central Highlands	443097	5812196	840	2
113	Thompson Valley	Central Highlands	440709	5816434	967	2
114	Thompson Valley	Central Highlands	437278	5818088	1069	2
115	Thompson Valley	Central Highlands	435478	5820861	1084	?
116	Thompson Valley	Central Highlands	429830	5821612	1123	2
117	Thompson Valley	Central Highlands	425326	5824305	1037	?
118	Noojee	Central Highlands	425441	5819522	1097	2
119	Noojee	Central Highlands	422639	5817123	1045	2
120	Noojee	Central Highlands	418226	5817584	797	2
2	Mt Baw Baw	Central Highlands	435469	5812423	1300	2
76	Maits Rest	Otway Ranges	208850	5707073	272	?
77	Maits Rest	Otway Ranges	208850	5707073	272	1
78	Melba Gully State Park	Otway Ranges	193034	5713904	368	1
79	Melba Gully State Park	Otway Ranges	192954	5713933	374	?
80	Deppler Creek	Otway Ranges	213706	5718527	379	1
81	Wait-a-While Road	Otway Ranges	200374	5717417	541	1
82	Aire River	Otway Ranges	202283	5713070	187	1
121	Mack Creek	Strzelecki Ranges	463003	5745549	594	?
122	Tarra Bulga NP	Strzelecki Ranges	462453	5747236	668	2
123	Tarra Bulga NP	Strzelecki Ranges	459498	5744876	497	?
124	Tarra Bulga NP	Strzelecki Ranges	460172	5743437	265	?
125	Jeeralang Creek	Strzelecki Ranges	451843	5742223	476	1
TB1	Tarra Bulga NP	Strzelecki Ranges	462179	5746015	650	2
TB2	Tarra Bulga NP	Strzelecki Ranges	462179	5746015	650	2
126	Sealers Cove	Wilsons Promontory	446786	5679802	10	1
127	Sealers Cove	Wilsons Promontory	446786	5679802	10	1

Appendix 2. Recognition sequence, and reaction conditions, of the restriction enzymes used in this study.

Restriction Enzyme	Recognition Sequence	Enzyme Buffer	Incubation Temperature(ºC)
*Taq*I	5'...T^CGA... 3' 3'...AGC^T...5'	U	65
*Hinf*I	5'...G^ANTC…3' 3'…CTNA^G…5'	2	37
*Alu*I	5'...AG^CT...3' 3'...TC^GA...5'	2	37
*Dpn*II	5'…^GATC…3' 3'…CTAG^…5'	U	37
*Hae*III	5'…GG^CC…3' 3'…CC^GG…5'	2	37
*HinP1*I	5'…G^CGC…3' 3'…CGC^G…5'	2	37
*Rsa*I	5'…GT^AC…3' 3'…CA^TG…5'	1	37
*Dde*I	5'…C^TNAG…3' 3'…GANT^C…5'	3	37
*Msp*I	5'…C^CGG…3' 3'…GGC^C…5'	2	37
*Hph*I	5'…GGTGA$(N)_8$^…3' 3'…CCACT$(N)_7$^…5'	4	37
*Nco*I	5'…C^CATGG…3' 3'…GGTAC^C…5'	4	37
*Ssp*I	5'…AAT^ATT…3' 3'…TTA^TAA…5'	U	37
*Ase*I	5'…AT^TAAT…3' 3'…TAAT^TA…5'	3	37
*Sty*I	5'…C^C(AorT)(AorT)GG…3' 5'…GG(TorA)(TorA)C^C…3'	3	37
*Nci*I	5'…CC^(CorG)GG…3' 5'…GG(GorC)^CC…3'	4	37
*Dra*I	5'…TTT^AAA…3' 3'…AAA^TTT…5'	4	37
*Cla*I	5'…AT^CGAT…3' 3'…TAGC^TA…5'	4	37
*Bst*UI	5'…CG^CG…3' 3'…GC^GC…5'	2	60
*Eco*RV	5'…GAT^ATC…3' 3'…CTA^TAG…5'	3	37

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