

ABSTRACT

CLARK, CATHERINE MARGARET. Genetic diversity and phylogeography in a Tasmanian rainforest conifer (*Lagarostrobos franklinii* (Hook f.) Quinn Podocarpaceae. (Under the direction of Dr. Ronald Sederoff and Dr. Thomas Wentworth).

Genetic variation in Huon pine (*Lagarostrobos franklinii*), a Tasmanian rainforest conifer, was estimated using several marker systems and two spatial scales. Genealogy based methods were used to infer population history of eight Huon pine stands based on chloroplast DNA variation. Chloroplast nucleotide diversity (π) was low (0.00093 ± 0.00006) in a multilocus haplotype generated by three universal chloroplast primers (*trnS-trnT*, *trnD-trnT*, *psbC-trnS*). Five haplotypes were identified; two were widely distributed but the most frequently occurring haplotype was found only in trees in the western portion of the range. Genetic differentiation among populations was significant and showed a high degree of structure ($G_{ST} = 0.26077$ $p \leq 0.00001$). Pairwise comparisons between populations revealed significant structure between the southeastern and northwestern watersheds and significant isolation by distance ($p \leq 0.0220$).

Nucleotide variation was also assessed in segments of three nuclear genes, *4Cl* (4-coumarate: coenzyme A ligase), *ITS2* (intergenic spacer region of ribosomal DNA) and *G3pdh* (glyceraldehyde 3-phosphate dehydrogenase). A total of 1,154 base pairs were sequenced from 79 individuals (158 alleles) representing seven geographic locations. Estimates of nucleotide diversity ($\pi = 0.00089 \pm 0.00007$) and theta (θ) (0.00061 ± 0.00031) were low for the combined loci and similar to chloroplast estimates. There was a higher level of variation at the *4Cl* locus ($\pi = 0.00167 \pm 0.00014$) associated with recombination. Nucleotide diversity for nuclear loci was highest in the subalpine Mt. Read population,

previously described as a putative clonal stand. Population differentiation ($F_{ST} = 0.0130$) was lower than estimated in chloroplast DNA, or in a previous allozyme investigation ($F_{ST} = 0.095$).

Multilocus genotypes based on RAPD markers (random amplified polymorphic DNA) were generated to further investigate genetic diversity in the Huon pine stand at Mt. Read. DNA was analyzed from 63 trees from Mt. Read and genetic diversity compared to 33 Huon pine samples from a wide geographic range. Twelve random decanucleotide primers amplified a total of 35 alleles. Only three of the alleles (8.6%) from the Mt. Read population were polymorphic in contrast to 18 (51.4%) polymorphic alleles in the reference population. Gene diversity at Mt. Read (0.0316 ± 0.1061) was six-fold lower than that found in the reference sample (0.1973 ± 0.2112). Only four unique DNA fingerprints were revealed at Mt. Read and these were spatially clustered in the stand. Each of the 33 isolates in the wide geographic sample exhibited a unique genotype.

The three marker systems of this study, along with a previous allozyme survey, are concordant in indications of a low level of diversity in a Southern Hemisphere conifer. The low level of nucleotide diversity, star-like phylogeny and haplotype distribution of chloroplast DNA suggest that Huon pine has experienced a series of population bottlenecks and colonization events from refugial areas. This is congruent with paleoecological data that suggest that there were major refugial areas on the western coast of Tasmania in addition to small, isolated refugia in other portions of the current range. The low level of variation found in nuclear loci is also compatible with a history of demographic events related to Pleistocene and Holocene environmental variability and long-term range reduction. The limited genetic

variation in the Mt. Read population may be related to minimal gene flow into this stand due to geographic isolation, and extensive vegetative reproduction in a harsh environment.

**GENETIC DIVERSITY AND PHYLOGEOGRAPHY IN A TASMANIAN
RAINFOREST CONIFER (*LAGAROSTROBOS FRANKLINII* (HOOK F.) QUINN
PODOCARPACEAE**

By

CATHERINE M. CLARK

**A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy**


FORESTRY AND BOTANY

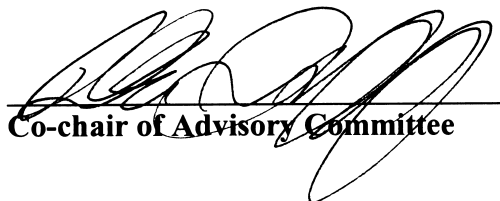
Raleigh

2006

APPROVED BY:









Co-chair of Advisory Committee

For my mother, Jo Graham Clark Herlinger,

and for Ernie Retzel and Ken Garrard

BIOGRAPHY

Catherine M. Clark was born in Pasadena, Texas and grew up and attended school in Houston, Texas. After graduating from Daeflower Free School, she left Houston on a bicycle and ended up in the Santa Cruz Mountains of California. After several cross-country moves, Catherine moved to Boone, North Carolina in 1978 to attend Appalachian State University and received a B.S. in 1984 from the Biology Naturalist program. After completing course work at Appalachian State University, she took an extended trip to Mexico. Some of the experiences of this trip led to a decision to pursue an education in the health care field. After graduating from Wake Technical College with an Associate Degree in Nursing in 1985 she worked as a Registered Nurse for ten years in gerontology, student health, chemical dependency treatment and for the American Red Cross. Catherine then decided to return to studying plant biology and returned to school at North Carolina State University. She received a Master's degree in Botany from North Carolina State University in 1998, based on the study of chloroplast microsatellite variation in North American *Abies*. She then continued her graduate education in a Ph.D. program, co-majoring in Botany and Forestry at North Carolina State University.

ACKNOWLEDGEMENTS

It is a great privilege to pursue an extended educational program. The support, guidance and friendship of family, friends and professional colleagues have made it possible for me to complete this degree. I would like to first thank the members of my committee for their examples of scholarship, education and leadership: Dr. Ron Sederoff, Dr. Tom Wentworth, Dr. Henry Amerson and Dr. Bruce Weir.

A number of sources of financial support and recognition have made my graduate program possible including; an EPA STAR Graduate Fellowship, Department of Forestry Fellowship, Preparing the Professoriate Fellowship, Namkoong Family Graduate Fellowship, The American Chestnut Foundation and a National Science Foundation Small Grant for Exploratory Research.

Members of the Forest Biotechnology Group provided technical assistance with molecular markers. In particular, I am grateful to Dr. Henry Amerson, Saul Garcia, Dr. Arthur Johnson, Dr. David Remington, Dr. Alexander Myburg, and Dr. Ying-Hsuan Sun. Jerri Johnson, secretary for the Forest Biotechnology Group and Sue Vitello, Graduate Program secretary for the Botany Department, provided administrative assistance. Thanks to Dr. Ignazio Carbone of the NCSU Plant Pathology Department who provided assistance with data analysis.

The people and agencies of Tasmania were very gracious in facilitating my field collections. Mick Brown of Forestry Tasmania provided assistance with field collections. Tasmanian Parks and Wildlife provided information on location of Huon pine populations and facilitated the issuance of sample permits. Dr. Greg Jordan of the Plant Biology

Department, University of Tasmania assisted with information on the paleohistory of Huon pine and in finding a field assistant for sample collection. Penny Brodrib was a wonderful, resourceful and knowledgeable field assistant. Georgina Davies was my initial contact in Tasmania and provided names of several contacts that were vital to the success of the field collections. Dr. Alison Shapcott shared her knowledge of her previous population genetic survey of Huon pine.

Dr. Rosanne D'Arrigo of the Tree Ring Laboratory at Lamont-Doherty Earth Observatory first approached members of the Forest Biotechnology Group about working with Huon pine. She and Dr. Ed Cook, also of the Tree Ring Laboratory, collaborated on a subsequent grant proposal involving this species and I am grateful for their assistance with the project. I am also grateful to Dr. Remy Petit and Dr. Antoine Kremer of INRA for hosting me in their lab at Pierroton, France to study DNA extraction from wood specimens.

Finally, I owe very special thanks to both Ernie Retzel and Ken Garrard, two people that have had an extraordinary impact on my life. They provided exceptional support that made it possible for me to complete this program.

TABLE OF CONTENTS

LIST OF TABLES	ix
LIST OF FIGURES	xi
PREFACE.....	1
Literature Cited.....	9
CHAPTER 1: CHLOROPLAST DNA PHYLOGEOGRAPHY IN A TASMANIAN ENDEMIC CONIFER (<i>LAGAROSTROBOS FRANKLINII</i> (HOOK F.) QUINN) PODOCARPACEAE	20
Abstract	21
Introduction	23
Study species.....	26
Materials and Methods.....	29
Sample collection	29
DNA extraction, PCR amplification and sequencing.....	29
Analyses of genetic diversity and sequence neutrality.....	31
Analyses of population structure and gene flow	32
Nested clade analysis.....	35
Results	37
Nucleotide and haplotype diversity	37
Genetic differentiation and migration.....	38
Nested clade analysis.....	39
Discussion.....	41
Genetic diversity and population bottleneck in Huon pine.....	41
Genetic structure and migration	44
Nested clade analysis.....	48
Proposed population history of Huon pine	51
Summary	55
Literature Cited.....	57
Tables and Figures	76

TABLE OF CONTENTS (cont.)

CHAPTER 2: LOW LEVEL OF NUCLEOTIDE DIVERSITY AT THREE NUCLEAR LOCI IN HUON PINE, A TASMANIAN RAINFOREST CONIFER (<i>LAGAROSTROBOS FRANKLINII</i>)	88
Abstract	89
Introduction	90
Study species	92
Materials and Methods	95
Field collection	95
DNA extraction, PCR amplification and sequencing	95
Analyses of genetic diversity and sequence neutrality	99
Analyses of population structure	100
Results	102
Genetic diversity and sequence neutrality	102
Population structure	104
Discussion	106
Nuclear DNA variation	106
Population structure	109
Conclusions and summary	113
Literature Cited	116
Tables and Figures	131
CHAPTER 3: RAPD MARKERS REVEAL CLONAL DIVERSITY WITHIN A HIGH ELEVATION STAND OF HUON PINE (<i>LAGAROSTROBOS FRANKLINII</i>) AT MT. READ, TASMANIA	141
Abstract	142
Introduction	143
Materials and Methods	146
Study species and field collections	146
DNA extraction and RAPD marker analysis	147
Data analysis	149
Results	152
RAPD banding and polymorphism	152
Genotypes	153
Discussion	155
Diversity in Huon pine	155
Clonal variation at Mt. Read	156
Gender expression at Mt. Read	159
Mutation and marker diversity	160
Summary	164
Literature Cited	166

TABLE OF CONTENTS (cont.)

CHAPTER 3: RAPD MARKERS REVEAL CLONAL DIVERSITY WITHIN A HIGH ELEVATION STAND OF HUON PINE (<i>LAGAROSTROBOS FRANKLINII</i>) AT MT. READ, TASMANIA Tables and Figures	180
APPENDICES	188
APPENDIX A. Unique alleles for 271 base pairs of chloroplast <i>trnD-trnT</i> sequence in Phylip format.....	189
APPENDIX B. Unique alleles for 330 base pairs of chloroplast <i>trnS-trnT</i> sequence in Phylip format.....	190
APPENDIX C. Unique alleles for 291 base pairs of chloroplast <i>psbC-trnS</i> sequence in Phylip format.....	191
APPENDIX D. Unique allele for 291 base pairs of <i>G3pdh</i> sequence in Phylip format.....	192
APPENDIX E. Unique alleles for 289 base pairs of <i>ITS2</i> sequence in Phylip format.....	193
APPENDIX F. Unique alleles for 574 base pairs of <i>4CL</i> sequence in Phylip format.....	194
APPENDIX G. Huon pine RAPD data file for RAPDPLOT.....	197
APPENDIX H. Identification of trees from the Mt. Read population tagged for climatological or growth studies	200

LIST OF TABLES

CHAPTER 1: CHLOROPLAST DNA PHYLOGEOGRAPHY IN A TASMANIAN ENDEMIC CONIFER (*LAGAROSTROBOS FRANKLINII* (HOOK F.) QUINN) PODOCARPACEAE

Table 1.1. Sample size, dbh range and watershed for Huon pine populations.....	76
Table 1.2. Segregating nucleotide sites and haplotype configuration in chloroplast DNA sequence from three loci in Huon pine	77
Table 1.3. Estimates of population genetic diversity and sequence neutrality based on chloroplast DNA variation in intergenic spacer regions from <i>trnD-trnT</i> , <i>trnS-trnT</i> and <i>psbC-trnS</i> from 96 Huon pine samples.....	78
Table 1.4. Chloroplast haplotype frequency from eight Huon pine populations (96 samples) based on sequence from <i>trnS-trnT</i> , <i>trnD-trnT</i> and <i>psbC-trnS</i>	79
Table 1.5. Pairwise comparisons of values of G_{ST} (above diagonal) and Nm (below diagonal) between Huon pine populations.....	80
Table 1.6. Coalescent estimates of migration (M), theta (θ) and divergence time (T) between eastern and western watershed populations.....	81
Table 1.7. Results of the nested clade analysis for Huon pine haplotypes	82
Table 1.8. Examples of chloroplast nucleotide diversity in woody plant species	83

CHAPTER 2: LOW LEVEL OF NUCLEOTIDE DIVERSITY AT THREE NUCLEAR LOCI IN HUON PINE, A TASMANIAN RAINFOREST CONIFER (*LAGAROSTROBOS FRANKLINII*)

Table 2.1. Sample size, dbh range and watershed for Huon pine populations.....	131
Table 2.2. Summary of polymorphic sites and haplotype configurations at the <i>4Cl</i> and <i>ITS2</i> loci	132
Table 2.3. Estimates of DNA sequence and haplotype diversity at the <i>4Cl</i> and <i>ITS2</i> loci ...	133
Table 2.4. Tests for departure from neutral evolution at the <i>4Cl</i> locus	135
Table 2.5. Haplotype frequencies at the <i>4Cl</i> and <i>ITS2</i> loci.....	135
Table 2.6. Hardy-Weinberg exact tests by the Markov chain method for the <i>4Cl</i> locus.....	136
Table 2.7. Genotypic differentiation between all pairwise populations. For each comparison χ^2 value is above the diagonal and p -value below the diagonal	137
Table 2.8. Pairwise comparisons of values of F_{ST} (above diagonal) and Nm (below diagonal) between Huon pine populations.....	138
Table 2.9. Estimates of nucleotide diversity in plant and animal species.....	139

LIST OF TABLES (cont.)

CHAPTER 3: RAPD MARKERS REVEAL CLONAL DIVERSITY WITHIN A HIGH ELEVATION STAND OF HUON PINE (*LAGAROSTROBOS FRANKLINII*) AT MT. READ, TASMANIA

Table 3.1. Mt. Read and Reference population samples.....	180
Table 3.2. RAPD primers and loci scored.....	181
Table 3.3. Allele frequencies and gene diversity in polymorphic RAPD loci	182
Table 3.4. Genotype frequencies at Mt. Read	183
Table 3.5. Genotype frequency by transect at Mt. Read.....	184
Table 3.6. Similarity indices between genotypes at Mt. Read	185

LIST OF FIGURES

CHAPTER 1: CHLOROPLAST DNA PHYLOGEOGRAPHY IN A TASMANIAN ENDEMIC CONIFER (*LAGAROSTROBOS FRANKLINII* (HOOK F.) QUINN) PODOCARPACEAE

- Figure 1.1. Huon pine sampling areas in Tasmania, Australia..... 84
Figure 1.2. Geographic distribution of chloroplast haplotypes in Huon pine 85
Figure 1.3. Posterior probability distributions of migration (M) and time since divergence (T) between watersheds..... 86
Figure 1.4. Nested clade diagram based on the 95% most probable connections between haplotypes calculated by TCS version 1.18 (Clement *et al.* 2000)..... 87

CHAPTER 2: LOW LEVEL OF NUCLEOTIDE DIVERSITY AT THREE NUCLEAR LOCI IN HUON PINE, A TASMANIAN RAINFOREST CONIFER (*LAGAROSTROBOS FRANKLINII*)

- Figure 2.1. Huon pine sampling areas in Tasmania, Australia..... 140

CHAPTER 3: RAPD MARKERS REVEAL CLONAL DIVERSITY WITHIN A HIGH ELEVATION STAND OF HUON PINE (*LAGAROSTROBOS FRANKLINII*) AT MT. READ, TASMANIA

- Figure 3.1. Locations of populations sampled..... 186
Figure 3.2. Tree of Mt. Read genotypes and genetic distance measured by the similarity index 187

PREFACE

Introduction

A major goal of population genetic studies of forest trees is to quantify the amount and pattern of genetic variation in both domesticated and native, uncultivated tree species. Inference of the evolutionary forces shaping genetic diversity is an important component for delineation of priorities for conservation of seed resources and unique populations, and for breeding programs. Over 7,300 tree species, or nine per cent of the tree flora of the world, are considered endangered or threatened with extinction (Oldfield *et al.* 1998). This number includes over half of the world's conifer species. Huon pine (*Lagarostrobos franklinii* Hook f.) Quinn, a Tasmanian endemic conifer, is the subject of the following three chapters, and is considered a vulnerable species by The World Conservation Union (Oldfield *et al.* 1998). Fossil floras from the Early Cenozoic (65 mya) show the presence of diverse and widespread rainforest vegetation in mainland Australia and Tasmania (Carpenter *et al.* 1994). During the Oligocene (34 mya) (million years ago) the range of many conifers, including Huon pine, greatly diminished (Carpenter *et al.* 1994; Jordan 1995; 1997). The previously widespread temperate Australian rainforest became restricted to western Tasmania and the eastern mainland during the Late Cenozoic (2 mya). The decline in rainforest vegetation has been attributed to climatic trends of decreasing temperature and precipitation along with increased seasonality and variability of climate (Kershaw 1988). Huon pine populations have also been subject to extensive exploitation since the beginning of European settlement 200 years ago (Gibson 1986; Gibson and Brown 1991) and populations have been lost due to logging, inundation by hydroelectric construction, mining, land clearing and fire (Peterson 1990).

Genetic consequences of climate change

Current predictions for changes in temperature, precipitation and CO₂ levels associated with global climate variation (Karl and Trenberth 2003) must be considered when assessing the potential for adaptation and continued existence in a particular environment. CO₂ has increased approximately 30 per cent since pre-industrial times and half of the increase has occurred in the last 40 years (<http://cdiac.esd.ornl.gov/trends/co2/sio-mlo.htm>). Measurements from ice cores show that CO₂ levels are higher than they have been in the last 650,000 years through six glacial intervals (Siegenthaler *et al.* 2005). Examination of Northern hemisphere temperature records for the last 1200 years show anomalous warmth from the mid 20th century (Osborn and Briffa 2006). It is clear that environmental conditions are changing at a relatively rapid rate, but the direction or the intensity of these changes is uncertain at present. Long-lived tree species can also contribute to our understanding of climate change. The Huon pine population at Mt. Read, one of the sites surveyed in this study, has been used extensively in dendrochronological analyses that infer climate history of Tasmania for the last four millennia (Cook *et al.* 1991; Cook *et al.* 1992; Cook *et al.* 1996; Cook *et al.* 2000). Tree ring analyses for this period show that there has also been anomalous warming in Tasmania and surrounding areas since 1965.

Consequences of vast scale climate change are unknown for forest tree species, but both the degree and the rate of change are of concern for forest tree populations. The maintenance of genetic diversity will have implications for continued evolutionary and reproductive success in a changing environment. Populations that occur at the limits of growth would very likely be affected by a rapid alteration of selective forces. Adaptation

could be constrained by the extended generation times in many long-lived tree species. Adaptive responses could also be hindered by genetic correlations among traits not conducive to selection pressures (Etterson and Shaw 2001). Tree species may be buffered to some extent to withstand genetic loss during range fluctuations as diversity is largely found within rather than among populations (Hamrick 2004). Genetic diversity in trees may also be preserved by habitat heterogeneity in refugial areas provided by topography and microhabitat differentiation. The landscape of western Tasmania provides substantial habitat heterogeneity related to topography that could provide suitable microclimate for Huon pine persistence in refugia. Long life spans in many tree species, such as Huon pine, could also alleviate effects of genetic drift and loss of diversity as there could be many opportunities for infrequent gene flow, particularly with the wind borne pollen of many conifer species (Hamrick 2004).

Genetic impacts of Pleistocene climate change are largely unresolved in forest tree species (Davis and Shaw 2001). Evidence from the fossil record documents contractions and expansions as well as range shifts in tree species in conjunction with cyclical climate change (Huntley and Webb 1989; Ibrahim *et al.* 1996; Davis and Shaw 2001). Range shifts are expected to be a major response for current climate change although contemporary migration is likely to be limited in many cases by an increasingly fragmented landscape and loss of habitat (Groom and Schumaker 1993). Many species moved out of refugial areas at the beginning of the Holocene 10,000 years ago (Thompson 1988; Markgraf 1995; McGlone 1997). For extremely long-lived organisms, there may have been few generations since that time, and it is unlikely that they have reached a state of equilibrium gene flow (Schaal and Olsen 2000). The gene pool of these organisms could reflect both historical migration and

shared ancestral polymorphisms in addition to contemporary gene flow (Hewitt 1996). In addition to range shifts, there is also a genetic response to environmental heterogeneity as selection intensity and direction may be altered, and there is an adaptive response to environmental variation (Davis 2001; Davis and Shaw 2001). The two different mechanisms suggested above have different implications on the genetic structure of populations. In the first case, historical migration patterns should reflect changes in climate conditions, although range shifts in a sessile organism can lead to loss of diversity, with poor or limited dispersal capability and availability of suitable habitat for colonization. In the second case, localized genetic variation could be reduced during periods of climate change due to more intense selection during those periods. By examining tree populations in the context of historical climate changes of the Pleistocene, we may be able to infer some of the genetic consequences for present populations and gain insight into the processes shaping genetic variation in tree species.

Molecular markers and forest trees

Molecular marker studies have been used for over 40 years to estimate genetic diversity in tree species. Different molecular marker systems are subject to different limitations on diversity relating to mutation, functional constraints and selection and may estimate different levels or patterns of genetic diversity in the same species (Echt *et al.* 1998; Esselman *et al.* 1999; Kjølner *et al.* 2004). Isozyme studies have been used for over 30 years to assess genetic variation in woody plant species (Hamrick and Godt 1990; Hamrick *et al.* 1992; Hamrick and Godt 1996). An extensive analysis of the available allozyme literature summarized from many studies showed that long-lived woody plant species exhibit

characteristic high levels of allozyme variation that are correlated with mating system, life span and mode of dispersal (Hamrick *et al.* 1992; Hamrick and Godt 1996). Allozyme studies may have limited resolution due to the relatively small number of loci available, or to low levels of diversity. In recent years, development of other marker systems based on PCR - amplification (Mullis 1990) have provided greater resolution as a larger number of loci and alleles can potentially be generated. Many studies of genetic variation in woody plant taxa are based on arbitrary random sequence primers such as RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) (Hsiao and Rieseberg 1994; Tuskan *et al.* 1996; Pornon *et al.* 2000; Suyama *et al.* 2000; Moriguchi *et al.* 2001). RAPD markers were used in this study (Chapter 3) to evaluate the genetic diversity of a putative clonal stand of Huon pine at Mt. Read. These markers are particularly useful in species with limited preexisting sequence information or low genetic variability, but they are dominant markers and do not provide information on the historical relationship among individuals. Genealogically ordered DNA sequence based markers can often retain phylogenetic information between individuals or populations and provide insight into the historical processes shaping genetic variation. Organelle DNA from both chloroplasts and mitochondria have also been used to infer genetic diversity, population structure and post-Pleistocene history for a number of plant and animal species (Bermingham and Avise 1986; Avise 1994; Byrne and Moran 1994; Bernatchez and Wilson 1998; Huang *et al.* 2001; Huang *et al.* 2002; Hwang *et al.* 2003). In many of these studies, data have been analyzed in the context of extensive fossil databases to infer refugial areas and postglacial migration (Demesure *et al.* 1996; Dumolin-Lapegue *et al.* 1997; Petit *et al.* 1997; Ferris *et al.* 1998; King and Ferris 1998). Genealogically ordered chloroplast DNA sequence was used to

examine genetic diversity and infer population history of eight widespread Huon pine populations (Chapter 1). To date, there are few studies of DNA nucleotide variation of nuclear loci in woody plants (Dvornyk *et al.* 2002; García-Gil *et al.* 2003; Järvinen *et al.* 2003; Kado *et al.* 2003; Brown *et al.* 2004) or undomesticated species (Olsen 2002; Caicedo and Schaal 2004). In Chapter 2, nuclear DNA sequence variation from three loci was analyzed from seven Huon pine populations to examine the amount and geographic pattern of genetic diversity.

Genetic diversity in Huon pine

The three marker systems used in this study of Huon pine provide slightly different information and conclusions about genetic diversity in Huon pine. They are, however, concordant along with a previous allozyme survey in indications of a low level of diversity in a Southern Hemisphere conifer. The results suggest that Huon pine has experienced a series of population bottlenecks and colonization events from refugial areas. This is congruent with paleoecological data that suggest that there were major refugial areas on the western coast of Tasmania in addition to small, isolated refugia in other portions of the current range. Although we do not yet understand the connection between genetic diversity at the molecular level and the ecological level, molecular evolutionary genetics attempts to interpret nucleotide changes in the context of ecology and geography (Purugganan and Gibson 2003). Information on the genes and variation that are important to selection and adaptation is being developed in model organisms such as *Arabidopsis thaliana* (Hanfstingl *et al.* 1994; Miyashita *et al.* 1996; Kawabe and Miyashita 1999), *Zea mays* spp. *mays* (Tenailon *et al.* 2001), *Drosophila melanogaster* (Wang *et al.* 2002) and *Homo sapiens* (Cargill *et al.* 1999).

Patterns of genetic diversity are also being examined in quantitative variation by mapping of QTL (quantitative trait loci) (Doebley *et al.* 1997) and association studies (Templeton 1995) although some types of molecular marker may be poor predictors of differentiation in quantitative traits (Karhu *et al.* 1996). In forest tree species that can live over 2,000 years, such as Huon pine (*Lagarostrobos franklinii* (Hook f.) Quinn) (Francey *et al.* 1984), we can expect to find differences in the manifestation of evolutionary forces on genetic diversity relative to short-lived model systems. Further development of techniques and methods of analysis will improve the resolution of these studies in native populations. Recent technological advances in DNA sequencing technology that make it possible to sequence 25 million base pairs in a four hour period (Margulies *et al.* 2005) could lead to sequencing of entire genomes of both native and model organisms in increasingly short time periods.

LITERATURE CITED

- Avise, J. C. (1994) *Molecular Markers, Natural History and Evolution* Chapman and Hall, New York.
- Bermingham, E., Avise J. (1986) Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* **113**, 939-965.
- Bernatchez, L., Wilson C. C. (1998) Comparative phylogeography of nearctic and palearctic fishes. *Molecular Ecology* **7**, 431-452.
- Brown, G. R., Gill G. P., Kuntz R. J., Langley C. H., Neale D. B. (2004) Nucleotide diversity and linkage disequilibrium in loblolly pine. *Proceedings of the National Academy of Science U S A* **101**, 155255-115260.
- Byrne, M., Moran G. F. (1994) Population divergence in the chloroplast genome of *Eucalyptus nitens*. *Heredity* **73**, 18-28.
- Caicedo, A. L., Schaal B. A. (2004) Population structure and phylogeography of *Solanum pimpinellifolium* inferred from a nuclear gene. *Molecular Ecology* **13**, 1871-1882.
- Cargill, M., Altshuler D., Ireland J., Sklar P., Ardlie K., Patil N., Lane C., Lim E., Kalyanaraman N., Nemesh J., Ziaugra L., Friedland L., Rolfe A., Warrington J., Lipshutz R., Daley G., Lander E. (1999) Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nature Genetics* **22**, 231-238.
- Carpenter, R. J., Hill R. S., Jordan G. J. (1994) Cenozoic vegetation in Tasmania: macrofossil evidence. In: *History of Australian Vegetation. Cretaceous to Recent* (ed. Hill RS), pp. 276-298. Cambridge University Press, Cambridge.

- Cook, E. R., Bird T., Peterson M., Barbetti M., Buckley B., D'Arrigo R., Francey R. (1992) Climatic change over the last millennium in Tasmania reconstructed from tree rings. *The Holocene* **2**, 205-217.
- Cook, E. R., Bird T. D., Peterson M., Barbetti M., Buckley B. M., D'Arrigo R. D., Francey R. J., Tans P. (1991) Climatic change in Tasmania inferred from a 1089 - year tree - ring chronology of Huon pine. *Science* **253**, 1266-1268.
- Cook, E. R., Buckley B. M., D'Arrigo R. D., Peterson M. J. (2000) Warm-season temperatures since 1600 BC reconstructed from Tasmanian tree rings and their relationship to large-scale sea surface temperature anomalies. *Climate Dynamics* **16**, 79-91.
- Cook, E. R., Francey R. J., Buckley B. M., D'Arrigo R. D. (1996) Recent increases in Tasmanian Huon pine ring widths from a sub-alpine stand: natural climate variability, CO² fertilisation, or greenhouse warming? *Papers and Proceedings of the Royal Society of Tasmania* **130**, 65-72.
- Davis, M. B. (2001) Past and future forest response to rapid climate change. In: *Global Biogeochemical Cycles in the Climate System* (eds. Schulze E-D, Heimann M, Harrison S, *et al.*), pp. 167-175. Academic Press, San Diego.
- Davis, M. B., Shaw R. G. (2001) Range shifts and adaptive responses to Quaternary climate change. *Science* **292**, 673-679.
- Demesure, B., Comps B., Petit R. J. (1996) Chloroplast DNA phylogeography of the common Beech (*Fagus sylvatica* L.) in Europe. *Evolution* **50**, 2515-2520.

- Doebley, J., Stec A., Hubbard L. (1997) The evolution of apical dominance in maize. *Nature* **386**, 485-488.
- Dumolin-Lapegue, S., Demesure B., Fineschi S., LeCorre V., Petit R. J. (1997) Phylogeographic structure of white oaks throughout the European continent. *Genetics* **146**, 1475-1487.
- Dvornyk, V., Sirviö A., Mikkonen M., Savolainen O. (2002) Low nucleotide diversity at the *pal1* locus in the widely distributed *Pinus sylvestris*. *Molecular Biology and Evolution* **19**, 179-188.
- Echt, C. S., DeVerno L. L., Anzidei M., Vendramin G. G. (1998) Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Molecular Ecology* **7**, 307-316.
- Esselman, E. J., Jianqiang L., Crawford D. J., Windus J. L., Wolfe A. D. (1999) Clonal diversity in the rare *Calamagrostis porteri* ssp. *insperata* (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. *Molecular Ecology* **8**, 443-451.
- Ferris, C., King R. A., Vainola R., Hewitt G. M. (1998) Chloroplast DNA recognizes three refugial sources of European oaks and suggests independent eastern and western immigrations to Finland. *Heredity* **80**, 584-593.

- Francey, R. J., Barbetti M., Bird T. D., Beardsmore D., Coupland W., Dolezal G. D., Farquhar G. D., Flynn R. G., P.J. F., Gifford R. M., Goodman H. S., Kunda B., McPhail S., Nanson G., Pearman G. I., Richards N. G., Shankey T. D., Temple R. B., Weir B. (1984) Isotopes in tree rings. C.S.I.R.O. Division of Atmospheric Technical Paper, Australia.
- García-Gil, M. R., Mikkonen M., Savolainen O. (2003) Nucleotide diversity at two phytochrome loci along a latitudinal cline in *Pinus sylvestris*. *Molecular Ecology* **12**, 1195-1206.
- Gibson, N. (1986) Huon pine conservation and management, p. 91. Tasmanian Forestry Commission, Hobart, Tasmania.
- Gibson, N., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 2. Population structure and spatial pattern. *Australian Journal Of Ecology* **16**, 223 - 229.
- Groom, M. J., Schumaker N. (1993) Evaluating Landscape Change: Patterns of Worldwide Deforestation and Local Fragmentation. In: *Biotic Interactions and Global Change* (eds. Kareiva PM, Kingsolver JG, Huey RB), pp. 24-44. Sinauer, Sunderland.
- Hamrick, J. L. (2004) Response of forest trees to global environmental changes. *Forest Ecology And Management* **197**, 323-335.
- Hamrick, J. L., Godt M. J., Sherman - Broyles S. L. (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**, 95-124.

- Hamrick, J. L., Godt M. J. W. (1990) Allozyme diversity in plant species. In: *Plant population genetics, breeding, and genetic resources* (eds. Brown AHD, Clegg MT, Kahler AL, Weir BS), pp. 43-82. Sinauer, Sunderland.
- Hamrick, J. L., Godt M. J. W. (1996) Conservation Genetics of Endemic Plant Species. In: *Conservation Genetics: Case Histories from Nature* (eds. Avise JC, Hamrick JL), pp. 281-304. Chapman and Hall, New York.
- Hanfstingl, U., Berry A., Kellogg E. A., Costa III J. T., Rudinger W., Ausubel F. M. (1994) Haplotype divergence coupled with lack of diversity at the *Arabidopsis thaliana* alcohol dehydrogenase locus: role for both balancing and directional selection? *Genetics* **138**, 811-828.
- Hewitt, G. M. (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**, 247-276.
- Hsiao, J.-Y., Rieseberg L. H. (1994) Population genetic structure of *Yushania niitakayamensis* (Bambusoideae, Poaceae) in Taiwan. *Molecular Ecology* **3**, 201-208.
- Huang, S., Chiang Y. C., Schaal B. A., Chou C. H., Chiang T. Y. (2001) Organelle DNA phylogeography of *Cycas taitungensis*, a relict species in Taiwan. *Molecular Ecology* **10**, 2669-2681.
- Huang, S. S. F., Hwang S.-Y., Lin T.-P. (2002) Spatial pattern of chloroplast DNA variation of *Cyclobalanopsis glauca* in Taiwan and East Asia. *Molecular Ecology* **11**, 2349-2358.

- Huntley, B., Webb T. (1989) Migration: species' response to climatic variations caused by changes in the Earth's orbit. *Journal of Biogeography* **16**, 5-19.
- Hwang, S.-Y., Lin T.-P., Ma C.-S., Lin C.-L., Chung J.-D., Yang J.-C. (2003) Postglacial population growth of *Cunninghamia konishii* (Cupressaceae) inferred from phylogeographical and mismatch analysis of chloroplast DNA variation. *Molecular Ecology* **12**, 2689-2695.
- Ibrahim, K. M., Nichols R. A., Hewitt G. M. (1996) Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity* **77**, 282-291.
- Järvinen, P., Lemmetyinen J., Savolainen O., Sapanen T. (2003) DNA sequence variation in *BPMADS2* gene in two populations of *Betula pendula*. *Molecular Ecology* **12**, 369-384.
- Jordan, G. J. (1995) Extinct conifers and conifer diversity in the Early Pleistocene of western Tasmania. *Review of Palaeobotany and Palynology* **84**, 375-387.
- Jordan, G. J. (1997) Evidence of Pleistocene plant extinctions and diversity from Regatta Point, western Tasmania, Australia. *Botanical Journal of the Linnean Society* **123**, 45-71.
- Kado, T., Yoshimaru H., Tsumura Y., Tachida H. (2003) DNA Variation in a Conifer, *Cryptomeria japonica* (Cupressaceae *sensu lato*). *Genetics* **164**, 1547-1559.

- Karhu, A., Hurme P., Karjalainen M., Karvonen P., Kärkkäinen K., Neale D., Savolainen O. (1996) Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? *Theoretical and Applied Genetics* **93**, 215-221.
- Karl, T. R., Trenberth K. E. (2003) Modern global climate change. *Science* **302**, 1719-1723.
- Kawabe, A., Miyashita N. T. (1999) DNA variation in the basic chitinase locus (*ChiB*) region of the wild plant *Arabidopsis thaliana*. *Genetics* **153**, 1445-1453.
- Kershaw, A. P. (1988) Australasia. In: *Vegetation History* (eds. Huntley B, Webb TI), pp. 238 - 306. Kluwer Academic Publishers, Dordrecht.
- King, R., Ferris C. (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. *Molecular Ecology* **7**, 1151-1161.
- Kjølnner, S., Såstad S. M., Taberlet P., Brochmann C. (2004) Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: clonal diversity in *Saxifraga cernua*. *Molecular Ecology* **13**, 81-86.

Margulies, M., Egholm M., Altman W. E., Attiya S., Bader J. S., Bemben L. A., Berka J., Braverman M. S., Chen Y.-J., Chen Z., Dewell S. B., Du L., Fierro J. M., Gomes X. V., Godwin B. C., He W., Helgesen S., Ho C. H., Irzyk G. P., Jando S. C., Alenquer M. L., Jarvie T. P., Jirage K. B., Kim J.-B., Knight J. R., Lanza J. R., Leamon J. H., Lefkowitz S. M., Lei M., Li J., Lohman K. L., Lu H., Makhijani V. B., McDade K. E., McKenna M. P., Myers E. W., Nickerson E., Nobile J. R., Plant R., Puc B. P., Ronan M. T., Roth G. T., Sarkis G. J., Simons J. F., Simpson J. W., Srinivasan M., Tartaro K. R., Tomasz A., Vogt K. A., Volkmer G. A., Wang S. H., Wang Y., Weiner M. P., Yu P., Begley R. F., Rothberg J. M. (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376-380.

Markgraf, V., McGlone M., Hope G. (1995) Neogene paleoenvironmental and paleoclimatic change in southern temperate ecosystems - a southern perspective. *Trends In Ecology & Evolution* **10**, 143 - 147.

McGlone, M. S. (1997) The response of New Zealand forest diversity to Quaternary climates. In: *Past and Future Rapid Environmental Changes: The Spatial and Evolutionary Responses of Terrestrial Biota* (eds. Huntley B, Cramer W, Morgan AV, Prentice HC, Allen JRM), pp. 73-80. Springer-Verlag, Berlin.

Miyashita, N., Innan H., Terauchi R. (1996) Intra- and interspecific variation in the alcohol dehydrogenase locus region of wild plants *Arabis gemmifera* and *Arabidopsis thaliana*. *Molecular Biology and Evolution* **13**, 433-436.

- Moriguchi, Y., Matsumoto A., Saito M., Tsumura Y., Taira H. (2001) DNA analysis of clonal structure of an old growth, isolated forest of *Cryptomeria japonica* in a snowy region. *Canadian Journal of Forest Research* **31**, 377-383.
- Mullis, K. B. (1990) Target amplification for DNA analysis by the polymerase chain reaction. *Annales de Biologie Clinique* **48**, 579-582.
- Oldfield, S., Lusty C., MacKinven A. (1998) The World List of Threatened Trees. World Conservation Press, Cambridge.
- Olsen, K. M. (2002) Population history of *Manihot esculenta* (Euphorbiaceae) inferred from nuclear DNA sequences. *Molecular Ecology* **11**, 901-911.
- Osborn, T. J., Briffa K. R. (2006) The spatial extent of 20th-century warmth in the context of the past 1200 years. *Science* **311**, 841-844.
- Peterson, M. J. (1990) Distribution and conservation of Huon pine, p. 50. Forestry Commission Tasmania, Tasmania.
- Petit, R. J., Pineau E., Demesure B., Bacilieri R., Ducouso A. (1997) Chloroplast DNA footprints of postglacial recolonization by oaks. *Proceedings of the National Academy of Sciences* **94**, 9996-10001.
- Pornon, A., Escaravage N., Thomas P., Taberlet P. (2000) Dynamics of genotypic structure in clonal *Rhododendron ferrugineum* (Ericaceae) populations. *Molecular Ecology* **9**, 1099-1111.
- Purugganan, M., Gibson G. (2003) Merging Ecology, Molecular Evolution, and Functional Genetics. *Molecular Ecology* **12**, 1109-1112.

- Schaal, B. A., Olsen K. M. (2000) Gene genealogies and population variation in plants. *Proceedings of the National Academy of Sciences* **97**, 7024-7029.
- Siegenthaler, U., Stocker T. F., Monnin E., Luthi D., Schwander J., Stauffer B., Raynaud D., Barnola J. M., Fischer H., Masson-Delmotte V., Jouzel J., Tibayrenc M. (2005) Stable carbon cycle-climate relationship during the late Pleistocene. *Science* **310**, 1313-1317.
- Suyama, Y., Obayashi K., Hayashi I. (2000) Clonal structure in a dwarf bamboo (*Sasa senanensis*) population inferred from amplified fragment length polymorphism (AFLP) fingerprints. *Molecular Ecology* **9**, 901-906.
- Templeton, A. R. (1995) A cladistic-analysis of phenotypic associations with haplotypes inferred from restriction-endonuclease mapping or DNA-sequencing. 5. Analysis of case-control sampling designs-Alzheimer's disease and the apoprotein-E locus. *Genetics* **140**, 403-409.
- Tenaillon, M. I., Sawkins M. C., Long A. D., Gaut R. L., Doebley J. F., Gaut B. S. (2001) Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.). *Proceedings of the National Academy of Sciences U S A* **98**, 9161-9166.
- Thompson, R. S. (1988) Western North America. In: *Vegetation History* (eds. Huntley B, Webb TI). Kluwer Scientific, Dordrecht, The Netherlands.

Tuskan, G. A., Francis K. E., Russ S. L., Romme W. H., Turner M. G. (1996) RAPD markers reveal diversity within and among clonal and seedling stands of aspen in Yellowstone National Park. *Canadian Journal Forest Research* **26**, 2088-2098.

Wang, W., Thornton K., Berry A., Long M. (2002) Nucleotide variation along the *Drosophila melanogaster* fourth chromosome. *Science* **295**, 134-137.

CHAPTER 1

**CHLOROPLAST DNA PHYLOGEOGRAPHY IN A TASMANIAN ENDEMIC
CONIFER (*LAGAROSTROBOS FRANKLINII* (HOOK F.) QUINN)
PODOCARPACEAE**

ABSTRACT

Genealogy based methods were used to estimate genetic diversity and phylogeographic history for a Tasmanian endemic conifer, Huon pine (*Lagarostrobos franklinii* (Hook f.) Quinn). DNA from 96 trees collected from eight populations was sequenced using three universal chloroplast primers (*trnS-trnT*, *trnD-trnT*, *psbC-trnS*). There were three segregating sites, one transition and two transversions, in 892 base pairs of sequence. Overall chloroplast nucleotide diversity was low ($\pi = 0.00093 \pm 0.00006$) and varied in stands from 0.0 to 0.00115. Of the five haplotypes identified, two were widely distributed and found in seven of the eight sampled populations. The most frequently occurring haplotype was found only in trees in the western portion of the range. There was significant genetic differentiation among populations overall ($G_{ST} 0.26077$ $p \leq 0.00001$), and significant isolation by distance ($p \leq 0.0220$), so the null hypothesis of no geographic association of haplotypes was rejected. A previous allozyme survey found a much lower level of population differentiation ($F_{ST} = 0.095$). Pairwise comparisons of differentiation between individual populations showed significant structure among populations in the southeastern and northwestern watersheds. Estimates of migration between populations obtained using both parametric and nonparametric methods indicated levels of gene flow consistent with an isolation by distance model. Nested clade analysis indicated that Huon pine genetic diversity has been shaped by historical range expansion and long distance colonization possibly followed by subsequent fragmentation. The low level of nucleotide diversity, star-like phylogeny and haplotype distribution suggest that Huon pine has

experienced a series of population bottlenecks and colonization events from refugial areas. This is congruent with paleoecological data that suggest major refugial areas on the western coast in addition to small, isolated refugia in other portions of the current range.

INTRODUCTION

Paleoecological records illustrate sequential range or habitat shifts of many plant and animal species in both the Northern and Southern Hemispheres in association with glacial and interglacial intervals of the Pleistocene epoch (Kershaw 1988; Delcourt and Delcourt 1991; Pielou 1991; Markgraf *et al.* 1995; Comes and Kadereit 1998; Premoli *et al.* 2003). As a result of contraction and expansion of ranges and effects of Pleistocene environmental variability, we expect cyclical climate change to have a significant effect on the genetic architecture of species (Davis and Shaw 2001). Recent common ancestry, population bottlenecks, and recolonization from refugial areas could be a greater determinant of genetic structure than contemporary gene flow, or stochastic processes such as genetic drift (Hewitt 1996; Comes and Kadereit 1998). Long lived organisms with long generation times, such as woody plant species, may have experienced relatively few generations since the Last Glacial Maximum 18,000 years B. P. (before present) (Bond *et al.* 1993), and it is unlikely that an equilibrium state has been reached (Schaal and Olsen 2000). Population genetic methods based on assumptions of equilibrium gene flow may not provide an accurate approximation of the mechanisms resulting in current population structure and variation.

Although species response differs according to intrinsic and extrinsic factors, repeated range shifts, fragmentation, change in selection pressures and population bottlenecks should be reflected in the species gene pool (Hewitt 2000). Recently developed methods to analyze genealogical data allow inference of the evolutionary processes producing geographic patterns of genetic variation (Hudson 1990; Crandall and Templeton

1993; Avise 1994). This information can be interpreted to provide insights into locations of glacial refugia, and patterns of postglacial colonization (Bermingham and Avise 1986; Freeman *et al.* 2001; Olsen 2002). Genealogically ordered DNA sequence variation can retain the phylogenetic relationships among individuals and populations so that temporal relationships, as well as spatial and frequency distribution can be considered (Hudson *et al.* 1992b; Templeton 1998). Precision is enhanced over that of F_{ST} based estimators (Wright 1951) and in some cases, it is possible to distinguish between population differentiation related to historical migration (Hudson *et al.* 1992b) versus isolation or recombination (Nielsen and Wakeley 2001). Coalescent theory provides a coherent statistical framework to examine migration as both a historical and a contemporary process (Kingman 1982a, b; Hudson 1983, Fu and Li 1999 Review). Integration of parameters for nonequilibrium populations, such as asymmetrical gene flow and unequal population size, further enhance the accuracy of coalescent models in estimating migration (Beerli and Felsenstein 1999; Bahlo and Griffiths 2000; Beerli and Felsenstein 2001; Nielsen and Wakeley 2001). The power of these analyses is increased by a hierarchical implementation that verifies *a priori* assumptions of the coalescent model for sequence neutrality and no recombination (Carbone and Kohn 2001; Carbone *et al.* 2004), and could be particularly valuable in obtaining information from loci with low resolution.

Chloroplast DNA (cpDNA) has been used to infer genetic diversity, population structure and post-Pleistocene history for a number of woody plant species including *Eucalyptus nitens* (Byrne and Moran 1994), *Melaleuca alternifolia* (Butcher *et al.* 1995), *Cycas taitungensis* (Huang *et al.* 2001), *Cyclobalanopsis glauca* (Huang *et al.* 2002) and

Cunninghamia konishii (Hwang *et al.* 2003). In a number of these studies, data have been analyzed in the context of extensive fossil databases to infer refugial areas and postglacial migration, particularly in western European species (e.g. *Quercus* sp. (Dumolin-Lapegue *et al.* 1997; Petit *et al.* 1997; Ferris *et al.* 1998); *Alnus glutinosa* (King and Ferris 1998) and *Fagus sylvatica* (Demesure *et al.* 1996) (Reviewed by Taberlet *et al.* 1998). Chloroplast genomes offer several fundamental advantages for use in evaluating population history within a temporal, nonequilibrium relationship (Schaal *et al.* 1991). The high level of conservation of sequence and gene arrangement in the chloroplast (Palmer and Stein 1986) increase the probability that current genetic diversity originated prior to Pleistocene glacial cycles, so that the variation in populations is due more to haplotype sorting than new mutation (Hewitt 2001). The historical structure is also maintained by non-Mendelian inheritance, non-reticulating lineage and rare instances of recombination (Birky *et al.* 1989). Effective population size is decreased in effectively haploid, uniparentally inherited genomes, and they are as a result more sensitive to genetic drift and stochastic change (Birky 1995). Although the relatively low mutation rate in chloroplast sequence can lead to difficulty in finding high resolution loci, universal PCR primers have been designed to amplify chloroplast intergenic spacers for assessment of genetic diversity when there is limited preexisting sequence data (Demesure *et al.* 1995). The intergenic regions are assumed to be selectively neutral, so they are an appropriate indicator of gene flow and migration (Schaal *et al.* 1998).

In this investigation, genealogically ordered DNA nucleotide variation from three chloroplast loci was used to test the null hypothesis of no geographic association of

haplotypes in a long-lived Australian conifer from a portion of the range. Nucleotide and haplotype variation were used to estimate genetic diversity, population structure and migration history. Using both parametric and nonparametric methods, we inferred the phylogeographic history of Huon pine populations. Molecular chloroplast data and evidence from the paleoecological record were evaluated to assess concordance between genealogy and geography. Organelle variation from this study was compared to that found in a previous allozyme survey.

Study system

Huon pine (*Lagarostrobos franklinii* (Hook f.) Quinn) is a Tasmanian endemic rainforest conifer. Pollen records suggest that Huon pine, or its ancestor, may have existed for more than 100 million years as a component of the Gondwanan flora, and further suggest that it was much more widespread during the late Cretaceous and early Tertiary (Playford and Dettman 1979). In the Southern Hemisphere, fossil floras from the Early Cenozoic show the presence of diverse and widespread rainforest vegetation in mainland Australia and Tasmania (Carpenter *et al.* 1994). Previously widespread rainforest became restricted to western Tasmania and the eastern mainland during the Late Cenozoic. The decline in rainforest vegetation has been attributed to climatic trends of decreasing temperature and precipitation along with increased seasonality and variability of climate (Kershaw 1988). The range of Huon pine has greatly diminished along with that of other conifers since the Oligocene (Carpenter *et al.* 1994; Jordan 1995; 1997). The pollen and fossil record indicate that Huon pine has been present in Tasmania since at least the early Pleistocene (Macphail *et al.* 1993). A temperate rainforest tree, Huon pine is currently distributed largely in protected areas of

southwestern Tasmania in eight watershed systems (Peterson 1990; Gibson and Brown 1991). Distribution is naturally fragmented, as populations are often restricted to narrow gallery forests along river systems, but it is also sometimes found on moist slopes, in association with sclerophyllous vegetation, or in large non-riverine stands (Gibson 1986; Gibson *et al.* 1991). Huon pine is sensitive to moisture requirements and distribution is limited primarily to areas with precipitation totals greater than 1000 mm per year (Gibson *et al.* 1991). The area of living Huon pine was estimated in 1990 at approximately 10,500 hectares (Peterson 1990). Extensive logging since European settlement approximately 200 years ago has substantially reduced populations in some areas. Other populations have also been lost due to inundation following hydroelectric construction, mining, land clearing and fire (Gibson 1986; Gibson and Brown 1991). Huon pine is currently classified by the IUCN as a vulnerable species (Oldfield *et al.* 1998). Estimates are that most of the cool-temperate rainforests in Tasmania are less than 12,000 years old and that Huon pine survived glacial periods in west coast refugia and in a few small, isolated stands (Colhoun *et al.* 1996).

Huon pine is one of the longest-lived species known and individuals can attain ages greater than 2,000 years (Francey *et al.* 1984). They are slow growing, with a typical diameter increase of 0.3 to 2 mm per year and reach a maximum diameter of two meters. Canopy height can reach 25 to 30 meters, but Huon pine is also found as shrubby or alpine forms less than two meters tall (Gibson 1986). The trees are seasonally responsive and highly decay resistant. Subfossil wood has been used to develop tree ring chronologies extending over 3,600 years B. P. to infer climatic history for the Tasman Sea area (Cook *et al.* 1991; 1996; 2000).

Relatively little is known of gene flow or mode of organelle inheritance in Huon pine. Large quantities of seeds can be released during mast years that occur every five to seven years (Shapcott 1991a). Lateral dispersal of seeds by wind is extremely limited (Shapcott 1991a); however, seeds remain buoyant and viable in water for extended periods and water systems may provide an important means of dispersal (Shapcott *et al.* 1995; Shapcott 1997). Even along river courses, dispersal and establishment of Huon pine is quite slow (Gibson 1986). Green rosellas (*Platycercus caledonicus* Gmelin), a type of parakeet, have been observed feeding on the seed and could be both seed predators and dispersal agents (Gibson 1986; Shapcott 1991b). Huon pine is predominantly dioecious, and as a result, it is largely an obligate outcrosser (Quinn 1982). Although it is an outcrossing species, a previous allozyme survey covering the range of the species found populations were highly inbred and genetically similar (Shapcott 1991b; 1997). Extensive vegetative propagation has also been noted (Millington *et al.* 1979) and Shapcott (1997) proposed that Huon pine forms extensive clonal stands at some sites (e.g. Mt Read, Tasmania).

METHODS AND MATERIALS

Sample collection

Leaf tissue was collected from 333 trees in eight populations in Tasmania, Australia during May 2001 (Figure 1.1). These populations were located in four of the eight watersheds in which Huon pine is found. Size class structure, previously characterized by Shapcott *et al.* (1995), varied within stands. Samples were collected from all size classes present at each site (e.g. seedling, juvenile, mature). Population scale analyses in this study were based on a sample of 96 individuals selected from the total number of samples to represent each population and all size classes present in the stand (Table 1.1).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from 35 to 50 mg of leaf tissue homogenized in a Bio101 FastPrep™ FP120 (Carlsbad, CA, USA) using a Qiagen (Valencia, CA, USA) DNeasy® Plant Minikit® with minor modifications of the Qiagen protocol. DNA was quantified on ethidium bromide stained gels visualized with UV light. Thirty-four chloroplast primer pairs were each screened with three DNA samples and the majority of these either failed to amplify or produced multiple banding patterns. Only those producing bright, unambiguous PCR amplification were selected for further screening to evaluate the quality of sequence data generated using the same PCR primers. Three universal chloroplast primer pairs, *trnS-trnT*, *trnD-trnT*, *psbC-trnS* (Demesure *et al.* 1995) generated high quality sequence and were selected for screening with the population samples.

PCR reactions contained ~20 ng template DNA, 10X PCR buffer (Qiagen, Valencia, CA, USA), MgCl₂ 0.5 mM, 200 μM each dNTP, 400 μM each forward and reverse primer, 0.8 units *Taq* polymerase (Qiagen, Valencia, CA, USA), and double distilled water (ddH₂O) for a total volume of 20 μL. Thermal cycling was carried out on a MJ Research, Inc. PTC-100 (Watertown, MA, USA). Parameters consisted of an initial denaturation step of 2 minutes at 95° C, followed by 35 cycles of 95° C for 15 seconds, annealing at 52° C for 30 seconds and extension at 72° C for 2 minutes, followed by a final extension at 72° C for 10 minutes. PCR products were run on ethidium bromide stained agarose gels (1%) in 1X TBE buffer. Single bands were excised and cleaned with a QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA, USA) to prepare DNA template for sequencing reactions. Sequencing reactions consisted of ~50 to 60 ng template DNA, 2 μL Big Dye[™] version 2.0 or 3.1 Ready Reaction Mix (PE Biosystems, Foster City, CA, USA), 5 pmol primer, 2.5X buffer (40 mM Tris, pH 9.0, MgCl₂ 15 μM), and ddH₂O for a total volume of 20 μL. Cycling parameters were 95° C for 30 seconds, 50° C for 10 seconds and 60° C for 4 minutes for a total of 35 cycles. Unincorporated dyedeoxy terminators were removed by centrifugation at 900 rcf for 5 minutes in Multiscreen[™] 96 well filtration plates (Millipore, Bedford, MA, USA: Sephadex[™] G-50-50, Sigma Chemical Co., St. Louis, MO, USA). Reactions were dried in a vacuum manifold (Savant SpeedVac[®] AES2010, Holbrook, NY, USA) and then resuspended in 1.7 μL loading dye (5:1 deionized formamide, 25 mM EDTA, 50 mg/mL Blue Dextran). Membrane combs (The Gel Company, San Francisco, CA, USA) were used to load 0.6 μL on a 0.2 mm, 36 cm 5% Gene-PAGE PLUS 6M urea polyacrylamide gel (Amresco, Solon, OH,

USA). Samples were electrophoresed in 1X TBE buffer on an ABI Prism[®] 377 DNA automated sequencing instrument (PE Biosystems, Foster City, CA, USA). Sequencing parameters were 1800 volts, 50 mA, 150 watts, 48° C, laser power 40.0 mW with a run time of 6 hours for a total of 7072 scans. Quality of all DNA sequence files was evaluated with MacPhred version 0.990722.g (Ewing and Green 1998; Ewing *et al.* 1998) and by visual inspection. Sequencing reactions were repeated for all sequences with ambiguities. Trace files with phred quality scores were input into Sequencher version 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) for alignment and editing and exported in Nexus format. A query sequence for each allele from each locus was submitted to the NCBI BLAST server for nucleotide homology search (Altschul *et al.* 1990). Sequences for *trnS-trnT* did not show a high level of homology with any sequence in the GenBank database. The BLAST algorithm for *psbC-trnS* showed strong similarity to the coding sequence for the *psbC* gene. A ClustalW alignment showed that the variable site in *psbC-trnS* was nonsynonymous and led to an amino acid change (alanine to serine). Portions of *trnD-trnT* show homology with *rps4* coding sequence. Unique sequences from each locus were deposited in GenBank (accession numbers DQ092738-DQ092743).

Analyses of genetic diversity and sequence neutrality

Estimates of inter- and intrapopulation genetic diversity and sequence neutrality were calculated using DnaSP version 4.0 (Rozas *et al.* 2003). Nucleotide diversity was calculated both separately and jointly for the three chloroplast loci. All other diversity calculations consider the three sites as a single multilocus haplotype. Levels of genetic diversity were quantified by estimates of nucleotide diversity, π (π), the average number of nucleotide

differences per site between two sequences (Nei 1987 equation 10.5 or 10.6); haplotype diversity (*hd*) (Nei 1987 equations 8.4 and 8.12 replacing 2n by n); average number of nucleotide differences (*k*) (Tajima 1983); number of segregating sites (*s*); and theta (θ) calculated from *s* (Watterson 1975), where $\theta = 4N_e\mu$ for a diploid organism, N_e is the effective population size and μ the mutation rate per site per generation (Nei 1987 equation 10.3; Tajima 1993, equation 3). Tajima's D (Tajima 1989) and Fu and Li's D* and F* (Fu and Li 1993) were calculated to provide *a priori* inference on sequence neutrality.

Analyses of population structure and gene flow

Approximation of the evolutionary history of populations was facilitated by use of the SNAP Workbench (Suite of Nucleotide Analysis Programs) (Price and Carbone 2005) (<http://www.cals.ncsu.edu/plantpath/faculty/carbone/home.html>), a Java program designed to coordinate a series of population genetic programs in a single interface. DNA sequence files in Nexus format were input into SNAP Workbench and converted to a Phylip alignment format using PAUP version 4.0b10 (Swofford 2000) and the three loci combined in a single file using SNAP Combine (Aylor and Carbone 2003). Sequences were collapsed into unique haplotypes with geographic phenotype using SNAP Map (Aylor and Carbone 2003) and SITES version 1.1 (Hey and Wakeley 1997). Nucleotide substitutions were categorized as transitions or transversions and as phylogenetically informative or uninformative. A site compatibility matrix was generated with SNAP Clade (Markwordt *et al.* 2003). Compatibility matrices were graphically illustrated using SNAP Matrix (Markwordt *et al.* 2003) to show a pattern of compatibility or incompatibility between two or more sites. A single sample from Newall Creek that was the only representative of Haplotype E was

identified as a possible recombinant and removed from further analysis to meet assumptions of the coalescent for no recombination. The three samples from Darwin Crater Track were also removed as the small population size made it difficult to interpret genetic structure estimates. Values for population subdivision, gene flow and all subsequent analyses were calculated with the remaining 92 samples from seven geographical regions. SNAP MAP was used to generate a Seqtomatrix (Hudson *et al.* 1992a) file and then converted to a distance matrix. The Seqtomatrix distance matrix was then used in Permtest, a nonparametric permutation method based on Monte Carlo simulations to assess statistical significance of hierarchical genetic differentiation between populations and regions (K_{ST}) (Hudson *et al.* 1992a). These measures provided the necessary inference regarding population panmixia or subdivision required to proceed with coalescent analyses.

Gene flow and genetic structure between populations was assessed by haplotype based measures (G_{ST} and N_m) (Nei 1973 equation 9). The haplotype based measure was selected as it has been shown to be more powerful when diversity is relatively low (Hudson 2000). Estimates of population structure were based on assumptions of the island model of population structure (Wright 1951) and an infinite sites model (Kimura 1969). The statistical significance of the relationship between the genetic distance matrix and the geographical distance matrix was evaluated by a Mantel test and implemented by Isolation by Distance Web Services (IBDWS) (Jensen *et al.* 2005) (<http://phage.sdsu.edu/~jensen/>). Statistical significance and confidence intervals for the Mantel test were generated using a randomization procedure. Great Circle Distances for the test were generated with

<http://www.wcrl.ars.usda.gov/cec/java/lat-long.htm>, and were based on latitude and longitude measures from populations sampled by Shapcott (1991b).

Based on evidence of geographic subdivision among populations, and validation of assumptions for molecular neutrality and no recombination in the data, two coalescent models were used to estimate migration between geographic regions. MDIV was used to test for evidence of equilibrium gene flow versus shared ancestral polymorphism in pairwise comparisons between subdivided populations (Nielsen and Wakeley 2001). Estimates were based on an infinite sites model without recombination. MDIV implements likelihood and Bayesian analyses using Markov chain Monte Carlo (MCMC) simulations to estimate migration (M), time since divergence (T), and theta (θ). Multiple iterations of the MDIV analysis between each of the individual populations failed to show sufficient convergence in results. This could be related to the low resolution of the sequence. Based on the low values for G_{ST} and high Nm for populations within watersheds, individual stands were grouped by watershed for MDIV analyses. Additionally, to increase the power of estimates, the King and Gordon populations were considered a single unit based on very high estimations of migration and low genetic structure between these two areas. Ten independent runs showed convergence in values among the Huon (Riveaux and Condominium Creek), Pieman (Stanley River and Mt. Read) and Gordon (Gordon River, Teepookana and Newall Creek) populations. With evidence of equilibrium gene flow between the regions indicated by MDIV, MIGRATE (<http://evolution.genetics.washington.edu/lamarc/migrate.html>) was used to estimate equilibrium migration rates (Beerli and Felsenstein 1999; 2001). MIGRATE is a maximum likelihood method based on coalescence and also uses a MCMC approach. Unlike

F_{ST} based estimators, MIGRATE can model symmetrical or asymmetrical gene flow with equal or unequal population sizes. Ten independent runs were performed and showed sufficient convergence in results using populations grouped by watershed as above.

Nested clade analysis

The null hypothesis of no association between haplotype distribution and geography was tested using nested clade analysis (Templeton 1998). The haplotype network with 95 per cent most plausible connections between 1-step mutations was constructed according to the guidelines in Templeton *et al.* (1992), and using TCS version 1.18 (Clement *et al.* 2000). The cladogram was constructed by joining the haplotypes (0-step clades) separated by a single mutation. The 0-step haplotypes were grouped into a network from the tips to the interior haplotype according to the procedure in Templeton *et al.* (1987). The 0-step haplotypes (A, B, C, D) were nested in a single 1-step clade. Each geographical location was then treated as a categorical variable and a permutational contingency test evaluated the randomness of geographical distribution of haplotypes. Pairwise geographic distances (kilometers) and allele frequency information for nested clades were analyzed by GeoDis version 2.2 (Posada *et al.* 2000). This program uses a Monte Carlo method that randomizes the contingency table at each step and calculates the statistical significance of the observed distribution of clade distance, nested clade distance and interior-tip distance. The clade distance (D_c) measures the geographical range of a clade or haplotype (0-step clade) as the average distance an individual haplotype is from the geographical center point of all individuals of that particular clade. The nested clade distance (D_n) is a measure of the geographical distribution of a specific clade relative to clades in the same higher level nesting category. It is calculated as

the average distance of a clade individual from all individuals of the next higher level nesting clade in which it is contained (Templeton 1998). Results of the nested clade analysis were interpreted using the inference key of Templeton (2004).

RESULTS

Nucleotide and haplotype diversity

There were three polymorphic sites in a total of 892 base pairs of DNA sequence obtained from three chloroplast loci (Table 1.2). There were two transversions and one transition, but no insertions or deletions and no evidence of heteroplasmy. Polymorphic sites in *trnD-trnT* and *trnS-trnT* were phylogenetically informative while the *psbC-trnS* site was uninformative. Both the *trnD-trnT* and *trnS-trnT* sequence had similar levels of nucleotide diversity. Sequence from *psbC-trnS* had much lower levels of diversity, perhaps related to functional constraints on mutation in coding sequence. Seven of the eight populations were polymorphic for the combined loci, but mean nucleotide diversity was low ($\pi = 0.00093 \pm 0.00006$) (Table 1.3). Nucleotide diversity ($\pi = 0.00115 \pm 0.00018$) and average pairwise difference ($k = 1.026$) were highest in the two Huon watershed populations, and lowest at the Mt. Read stand, where there was a single haplotype. The remaining five populations had slightly lower values for π and k . Values for θ were highest at Teepookana, which also had the highest number of segregating sites (3) in the population. Several measures were applied to the chloroplast sequence to test for evidence of selection. There was no indication of deviation from the neutral model in individual or total population measurements of Tajima's D ($p \geq 0.10$), Fu and Li's D^* ($p \geq 0.10$), and Fu and Li's F^* ($p \geq 0.10$).

There were five multilocus haplotypes and an overall haplotype diversity of $0.666 (\pm 0.027)$ (Table 1.4). Three haplotypes were abundant; one occurred at low frequency, and there was one singleton identified as a potential recombinant. Teepookana had the highest

level of haplotype diversity, but levels were quite similar at Darwin Crater Track, Gordon River and Stanley River stands. Lower levels of haplotype diversity were found in the Riveaux, Condominium and Newall Creek populations. Haplotype A was the most abundant overall, but was found only in the western populations, and it was the single haplotype found at Mt. Read (Figure 1.2). Haplotypes B and C were the most widely distributed geographically and the only haplotypes found in the Huon watershed populations. The low frequency haplotypes, D and E, were found only in the western watershed populations.

Genetic differentiation and migration

Geographic subdivision among all stands occurred at a moderate level but there was statistically significant geographic subdivision among populations ($K_{ST} = 0.136632$ $p \leq 0.0001$). Total G_{ST} was 0.26077, so approximately 26 per cent of the total variation was found among populations, and 74 per cent was found within populations (Table 1.5). In pairwise population comparisons, differentiation was also significant or highly significant between the Huon watershed populations, Riveaux and Condominium Creek, and Stanley River, Mt. Read, Teepookana and Newall Creek in all cases except between Condominium Creek and Teepookana. Values for G_{ST} were greatest between populations found at the greatest geographical distances in this study, Riveaux Creek and the two Pieman watershed sites. The Mantel test for matrix correlation of genetic distance and geographic distance indicated significant evidence to support an isolation by distance model ($r^2 = 0.3244$ $p \leq 0.0220$ from 1000 randomizations). There was a very low level of differentiation and high levels of migration between the Gordon and King watershed populations. Differentiation was also low between the Gordon River and Pieman stands.

Overall gene flow estimated by DnaSP was $Nm = 1.42$; however, there was a great deal of variation in migration estimates between individual populations (Table 1.5). Pairwise population estimates ranged from $Nm < 1$ between Mt. Read and Huon watershed sites to $Nm = 174$ between Teepookana and the Gordon River site. Posterior probability distributions from MDIV also showed evidence for high levels of gene flow between the combined Gordon and King populations, and the Pieman watershed populations ($M > 5$). As was the case with DnaSP estimates, MDIV showed minimal levels of migration between Huon and Pieman watersheds ($M = 0.2$). MDIV estimates of migration between the Huon and Gordon ($M = 0.50$) watersheds were lower than both DnaSP and MIGRATE estimates and also exhibited a high level of asymmetrical migration from Huon to Gordon watershed populations (Figure 1.3 and Table 1.6). It is possible that this result represents similarity from shared ancestral polymorphism rather than an indication of high levels of gene flow between these watersheds. MIGRATE estimates of migration between watersheds were otherwise similar to those from DnaSP and MDIV. Theta values obtained from MDIV analyses for all pairwise comparisons were similar ($\theta = 0.3$). Estimates from MIGRATE showed similar values for theta. Posterior probability distributions for T (time since divergence) showed essentially no divergence between the Gordon and Pieman stands, and the highest value for divergence time between Huon and Pieman stands ($T = 0.75$).

Nested clade analysis

The nested clade diagram is identical in configuration to the chloroplast haplotype network, and produces a star-like phylogeny that shows the genealogical relationship among haplotypes (Figure 1.4). Haplotype A was the inferred interior based on frequency, and

Haplotypes B, C and D the tips of the 0-step clades. Haplotype E was not included in the analysis as it was a putative recombinant. Geographic distribution of haplotypes is represented in Figure 1.2. Analysis by GeoDis rejected the null hypothesis of no association between geographic location and haplotype distribution ($\chi^2 = 62.3253$ $p \leq 0.0000$). There were no significantly large values for Dc , but values were significantly small for Clade A, Clade C, and the interior to tip comparison (Table 1.7). There were significantly small values for Dn in Clade A and the interior versus tip comparison, and significantly large values for Dn in Clades B and C. Interpretation of these results according to the inference key (Templeton 2004) suggests that populations have been subject to range expansion and long distance colonization with possible subsequent fragmentation.

DISCUSSION

Genetic diversity and population bottleneck in Huon pine

The level of chloroplast DNA diversity in Huon pine is consistent with the hypothesis of a series of population bottlenecks in this species. In addition to long-term widespread range contractions through the Miocene, Huon pine has been subject to range shifts through Pleistocene glacial intervals. Based on low allozyme diversity consisting of both a low level of polymorphism and minimal allelic richness, Shapcott also suggested that Huon pine had likely experienced population bottlenecks (Shapcott 1991b; 1997). The level of chloroplast DNA nucleotide diversity in Huon pine is low compared to many plant species, but is similar to levels of variation in other woody plant taxa that have undergone bottleneck events, such as *Cunninghamia konishii* and *C. lanceolata* (Hwang *et al.* 2003), or *Cyclobalanopsis glauca* (Huang *et al.* 2002) (Table 1.8). Although there was little variation in the range of nucleotide diversity, haplotype diversity values exhibited greater differences. In addition to the low nucleotide diversity, allelic richness is quite limited in Huon pine. Populations in both this study and the allozyme survey differed primarily in allelic frequency rather than alleles. There were other similarities between these two studies. Among the stands surveyed in this study, higher allozyme diversity, as well as the highest value for nucleotide diversity, occurred in the Riveaux and Condominium Creek populations. Although nucleotide diversity was higher in these populations, Riveaux and Condominium Creek both exhibited lower levels of chloroplast haplotype diversity. Mt. Read had similarly low genetic diversity in both studies, with no diversity detected in this study, and minimal diversity found in the allozyme

survey. The two studies differed however in diversity levels of the Gordon, King and Pieman populations. Teepookana had a low level of observed allozyme heterozygosity, but had the highest level of chloroplast haplotype diversity. In comparisons with the seven populations of this study, allozyme heterozygosity for Gordon River, Newall Creek and Stanley River stands were intermediate, although these stands all had relatively high chloroplast haplotype diversity.

Similarly low genetic diversity has been found in other long-lived tree species affected by periods of climate change. Low levels of genetic diversity in *Dacrydium cupressinum*, a New Zealand podocarp, were attributed to bottleneck effects from past range reductions (Hawkins and Sweet 1989; Billington 1991). Fragmented populations of *Fitzroya cupressoides*, a South American podocarp, show evidence of having undergone major range contractions and repeated bottlenecks during glacial intervals (Allnutt *et al.* 1999; Premoli *et al.* 2000; 2003). Based on a star-like phylogeny, low nucleotide diversity and the fossil pollen record, *Cunninghamia konishii*, a Taiwanese endemic gymnosperm, is thought to have undergone population bottlenecks in multiple refugial areas during glacial intervals (Lu *et al.* 2001; Hwang *et al.* 2003). *Pinus torreyana*, a species now restricted to two populations, exhibited no polymorphism at 59 isozyme loci (Ledig and Conkle 1983). Three western North American conifers (*Pinus radiata*, *Pinus muricata*, *Pinus attenuata*), with limited or fragmented distributions, exhibited low levels of chloroplast DNA variation in the *trnD* - *trnT* intergenic spacer region (Hong *et al.* 1993). A widespread species, *Pinus resinosa*, has been subjected to severe bottlenecks during glacial intervals and shows very little intraspecific diversity in RAPD (random amplified polymorphic DNA) or allozyme markers

(Fowler and Morris 1977; Mosseler *et al.* 1992). An extreme example of low diversity in a relictual species is that of *Wollemia nobilis*, a highly endangered Australian conifer with approximately 100 remaining trees (Peakall *et al.* 2003). Hundreds of loci from allozyme, AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) markers revealed no polymorphism in this species. Chloroplast DNA diversity in other Australian species is typically higher than that found in Huon pine. *Lambertia orbifolia* (Byrne *et al.* 1999), *Eucalyptus nitens* (Byrne and Moran 1994), *Melaleuca alternifolia* (Butcher *et al.* 1995) and *Melaleuca linariifolia* (Butcher *et al.* 1995) all exhibit higher levels of chloroplast nucleotide diversity. Chloroplast sequence in *Eucalyptus globulus* produced a hypervariable region yielding over 100 haplotypes in 270 individuals (Freeman *et al.* 2001). Although many species subjected to range contraction exhibit low levels of genetic diversity, not all relict species have low levels of diversity. *Cycas taitungensis*, a Taiwanese gymnosperm with two remaining populations, exhibited relatively high levels of chloroplast nucleotide diversity that was largely attributed to intramolecular recombination (Huang *et al.* 2001).

In general, higher levels of genetic diversity are associated with long-lived species with long generation times (Hamrick *et al.* 1992). Older trees and their genotypes remain in, and continue to contribute to the population gene pool along with new colonists. However, species with restricted ranges, or island endemics such as Huon pine, often have lower levels of allozyme polymorphism and heterozygosity, and reduced allelic richness compared to widespread species (Hamrick and Godt 1990). This is largely attributed to the greater effects of genetic drift in small populations. Repeated fragmentation or constraint to refugia during glacial intervals could lead to the loss of low frequency alleles through the effects of

inbreeding and genetic drift (Nei *et al.* 1975). In diploid loci, even though average heterozygosity can remain high, allelic richness may be reduced. Both the intensity of the bottleneck and the population growth rate after the bottleneck affect the average heterozygosity level after a bottleneck event (Nei *et al.* 1975). Effects on organelle sequence could be even more pronounced related to the lower effective population size.

Genetic structure and migration

Forest trees, in particular outbreeding, wind pollinated conifers with widespread distribution, typically maintain a relatively high level of genetic diversity within populations, and a relatively low level of differentiation among populations compared to many plant species (Hamrick *et al.* 1992). The relatively long life span, tall stature and greater potential for dispersal could contribute to the lower amount of genetic structure of nuclear genes in woody plant species (Hamrick 2004). In contrast to the low level of allozyme differentiation among populations ($F_{ST} = 0.095$) (Shapcott 1997), the multilocus chloroplast haplotype shows significant genetic structure ($G_{ST} 0.26077$ $p \leq 0.0000$) in Huon pine populations. Population differentiation measured in organelle sequence is typically higher than that found for nuclear genes (Petit *et al.* 1993a), and could reflect the lower effective population size and increased effects of genetic drift and stochasticity. Populations may become increasingly differentiated as new mutations arise (Hudson 1990). The level of differentiation found in the Huon pine allozyme survey was comparable to that found in other wind pollinated species, but somewhat higher than typically found in other gymnosperms, or long lived species (e.g. *Sequoiadendron giganteum* (Fins and Libby 1982) and *Pinus longaeva* (Lee *et al.* 2002)).

Although mode of organelle inheritance and dispersal is important in determining population structure of uniparentally inherited loci, the mode of organelle inheritance is unknown for Huon pine. The level of genetic structure exhibited in chloroplast haplotypes could be indicative of either a relatively low level of gene flow via pollen and seeds if cpDNA is paternally inherited in Huon pine, or a high level of dispersal by seed if cpDNA is maternally inherited. Species with wind dispersed pollen and paternal cpDNA inheritance often display lower G_{ST} values (e.g. *Pinus resinosa* 0.121 (Echt *et al.* 1998); *Pinus radiata* 0.162 (Moran *et al.* 1988); *Pinus rigida* 0.039 (Guries and Ledig 1982)). Larger values for G_{ST} are often found if cpDNA is maternally inherited, particularly if the seeds are dispersed by animals (e. g. *Quercus robur* 0.92 (Petit *et al.* 1993b); *Fagus sylvatica* 0.83 (Demesure *et al.* 1996)); *Eucalyptus nitens* 0.62 (Byrne and Moran 1994). In the gymnosperms, paternal plastid inheritance is seen in many, but not all, conifers studied (Mogensen 1996), including members of the Taxodiaceae (Neale *et al.* 1989) and Pinaceae (Neale *et al.* 1986; Neale and Sederoff 1989). Maternal cpDNA inheritance has been identified in at least one member of the Cupressaceae, *Cunninghamia konishii* (Lu *et al.* 2001). Biparental transmission of cpDNA has been found in many species. An examination of 88 angiosperm families found biparental transmission of cpDNA in 27 per cent of the families (Harris and Ingram 1991).

Many of the values for G_{ST} and Nm in this study are consistent with either a low level of gene flow between stands, or long-term separation. The high level of genetic structure could also be related to post glacial dispersal that resulted in a loss of alleles, or founding by individuals with few alleles. Although the overall value for G_{ST} is high, there is variation in levels of differentiation between individual stands, and stands grouped by catchment. The

highest level of differentiation and lowest value for migration between stands is seen in comparisons of the Huon and Pieman watershed populations. Gene flow estimates based on G_{ST} are consistent with coalescent-based estimates in indicating minimal levels of migration between the Huon and Pieman watershed populations. The posterior probability estimates generated by MDIV indicate the highest value for time since divergence between these watersheds of the three pairwise comparisons. The Huon and Pieman watersheds represent the greatest geographical distance in this study, so it is not unreasonable to assume that gene flow would be severely restricted between these sites. An isolation by distance model is supported by the significant value for the Mantel test. Genetic distance measures based on allozyme diversity were also higher between the more isolated sites including Mt. Read, Pieman River and King River to other catchments (Shapcott 1997).

Comparisons of migration between the Pieman and Gordon watershed populations also show a fairly high level of agreement in results between parametric and nonparametric methods. All measures indicate a high level of migration between catchments that could also be related to shared ancestral polymorphism. The posterior probability for time since divergence indicates that these areas are essentially not diverged. Results of MIGRATE and MDIV analyses differ only in estimates of migration between the Huon and Gordon watershed populations. MIGRATE analysis shows a fairly high level of asymmetrical gene flow from the Huon into the Gordon watershed, and a low level of migration from the Gordon into the Huon watershed. The MDIV value indicates a minimal level of migration between these two areas. Allozyme diversity indicated that the Gordon and Huon catchments were not differentiated when grouped by watershed (Shapcott 1997). The headwaters for the

Gordon and Huon watersheds arise in the same geographic area, so it is unclear whether this result indicates a relictual link, or is evidence of contemporary gene flow. With past population fragmentation with no current gene flow, populations could still show similarity based on shared ancestry and imply there was current gene flow (Templeton 1998). The lack of resolution for the Gordon and Huon catchments could be related to the low variability of the chloroplast haplotypes in this study.

Limited gene flow leading to isolation by distance is consistent with the distribution of Huon pine, which has a naturally fragmented distribution. Huon pine populations are primarily limited to narrow bands along river systems. In comparisons of *Pinus* species with continuous versus discontinuous ranges, those with disjunct ranges typically have a higher G_{ST} (mean 0.181) than those with continuous ranges (mean 0.053) (Hamrick and Godt 1996). The *Pinus* species with higher G_{ST} values also exhibited more variability in levels of population diversity. There are three known non-riverine stands greater than 200 hectares, but these stands, which include Teepookana, account for approximately 40 per cent of known Huon pine habitat (Gibson *et al.* 1991). Huon pine seed exhibit poor lateral seed dispersal, but can remain buoyant and viable after extended periods in water (Shapcott 1991a). Dispersal down waterways via either seeds or vegetative material is rapid (Gibson and Brown 1991); however, the spread away from rivers and streams is quite slow. Poor dispersal ability could affect gene flow between populations (Hamrick *et al.* 1992), and with limited gene flow between populations, the dominant process shaping genetic structure will become genetic drift (Hamrick 2004).

Nested clade analysis

The low level of nucleotide diversity in Huon pine limits the resolution of the nested clade analysis; however, the results suggest that the nonrandom distribution of haplotypes indicates a history of range expansion and long distance colonization. The analysis also suggests possible subsequent fragmentation of populations. Although the star phylogeny produced in this study lacks depth, and is characterized by minimally branching haplotypes that coalesce rapidly, it is still possible to discover significant evolutionary associations (Templeton 1999 review). There are no missing intermediate haplotypes in this data set, so it appears that this study provides a good representation of the available chloroplast variation from the populations investigated.

Under a scenario of range expansion, only a subset of the variation present in ancestral or refugial areas moves to new regions, so tip clades are found in the expanded region and are more geographically widespread than interior clades (Cann *et al.* 1987). As a result, the tip clades could have significantly large Dcs or Dns , and tip to interior distances are reversed. In addition, the distances for interior versus tip clades can be reversed from that expected with restricted gene flow (Templeton 1998). Under restricted gene flow, simulations and theory show that the geographical range of a haplotype is strongly correlated with the age of the haplotype, and the oldest haplotypes are the most widespread (Neigel and Avise 1993). With range expansion, some older haplotypes from source populations will be confined to the pre-expansion area, and as a result will have significantly small Dcs and Dns . The younger haplotypes found in the expanded regions will have significantly large Dcs and Dns (Templeton 1998).

The distribution of Huon pine is concentrated primarily in the drainage systems surrounding Macquarie Harbour in the central west coast area of Tasmania (Gibson *et al.* 1991). Populations from areas surrounding Macquarie Harbour have the highest levels of allelic richness and haplotype diversity in this study. This area appears to be the center of chloroplast haplotype diversity for this species. The overall frequency and distribution of Haplotype A in western areas of the range near the putative center of diversity makes it the most likely ancestral haplotype based on evidence that the most abundant haplotype is the root and younger haplotypes the tips (Templeton *et al.* 1995). The conclusion of range expansion in this study is based on the distribution of Haplotypes B and C, and the significant values for clade and nested clade distances. These two haplotypes appear at low frequency in the west coast areas with the highest levels of haplotype diversity, and also occur at higher frequencies in the Huon watershed. Haplotype A, the putative ancestral haplotype, was not detected in the Huon watershed. These results could indicate that there was a spread from west coast populations to eastern areas with long distance colonization. The Huon watershed populations in the southeast contain a subset of the diversity found near the west coast. The geographic pattern of chloroplast haplotype distribution illustrated in Figure 1.4 is consistent with the hypothesis of range expansion and long distance colonization.

If there is recent shared ancestry, as is the case with range expansion, there could be a high level of genotypic similarity between populations that imply a high level of gene flow (Templeton 1998). With population expansion, F_{ST} or G_{ST} or other traditional measures of gene flow could imply extensive gene flow and might not be biologically relevant

(Templeton 1998). If the populations in the Huon watershed resulted from long distance colonization, we would expect lower haplotype diversity related to founder effects. Allelic richness and haplotype diversity both decrease in the southeastern portions of the range although nucleotide diversity is higher in both Riveaux and Condominium Creek populations. A similar pattern of high heterozygosity, but low allelic diversity was seen outside refugial areas in European Beech (*Fagus sylvatica*) (Comps *et al.* 2001).

Based on measures of population differentiation in this study, it is unlikely that there is contemporary gene flow between populations at the most extreme ends of the geographical range. The largest geographic distance is approximately 200 kilometers between Riveaux Creek and Stanley River stands, and 120 kilometers between Gordon River mouth and Condominium Creek. In a species, such as Huon pine, with limited dispersal of seeds except along waterways, it is more likely that the pattern of chloroplast distribution in this study is related to founder effects from long distance dispersal. In Eastern North America and Western Europe, trees migrated to newly opened landscape after the retreat of glaciers at rates of 10 to 100 km per century (Davis 1981; Delcourt and Delcourt 1987). Fossil pollen assemblages show disjunct colonies up to 40 kilometers from the leading edge of the range of American Beech (*Fagus grandifolia*), an animal dispersed species. In Eastern Hemlock (*Tsuga canadensis*), a wind dispersed species, jump distances of 80 to 100 kilometers have been documented (Davis *et al.* 1986). Long distance dispersal from refugial areas could lead to dominance of these genotypes and decreased diversity in the areas colonized (Hewitt 1996). The pattern of colonization can also be important in determining structure. In several European species colonized by long distance seed dispersal, very large areas are found with

similar genotypes and a high level of genetic structure. This pattern has been particularly noticeable in species with maternal inheritance of cpDNA such as *Quercus robur* (Petit *et al.* 1997) and *Fagus sylvatica* (Demesure *et al.* 1996). Repetition of long distance dispersal over time and large distances could lead to a loss of both allelic richness and genetic diversity.

There are a number of examples in tree species that reflect contraction to refugia and subsequent migration. With few exceptions, a common finding in these studies is higher levels of diversity in refugial areas and decreasing diversity in more recently colonized areas. Diversity levels were lower outside refugial areas in *Quercus* sp. (Dumolin-Lapegue *et al.* 1997), *Alnus glutinosa* (King and Ferris 1998), *Eucalyptus loxophleba* (Byrne and Hines 2004) and *Fagus sylvatica* (Demesure *et al.* 1996). In the Pacific Northwest of North America, genetic variation in plant populations is often reduced in northern populations, and northern and southern populations are genetically differentiated (Soltis *et al.* 1997). It is, however, not always the case that diversity is highest in refugial areas. In 22 widespread European tree and shrub species, populations with the highest levels of diversity were found north of refugial areas (Petit *et al.* 2003). This was attributed to admixture of divergent lineages colonizing from separate refugia. The highest level of diversity in this study was found in an area that had been under glacial ice during the last glacial interval.

Proposed population history of Huon pine

The amplitude of climate change in temperate parts of the Southern Hemisphere has been smaller than that of many temperate areas of the Northern Hemisphere, perhaps because the larger ocean areas in relation to land mass moderate temperature fluctuations (Markgraf *et al.* 1995). Climate change appears to have been accompanied by retraction and expansion

of species from refugial areas (Markgraf *et al.* 1995) without the continent wide migrations of the Northern Hemisphere (Huntley and Webb 1989; Huntley 1993). Although Tasmania has an ocean moderated climate, there has still been sufficient climate change to lead to the extinction of a number of woody plant species in the last 18 million years (Macphail *et al.* 1993; Jordan 1997). The mountains of Western Tasmania have been glaciated during at least five separate periods of the Pleistocene (Colhoun 1985; Kiernan *et al.* 2001). Evidence for more extensive ice formation in Tasmania is found for middle and earlier Pleistocene glaciations (Colhoun *et al.* 1996).

During the Last Glacial Maximum approximately 18,000 years B. P., icecaps and outlet glaciers were present throughout the West Coast Range and Central Plateau of Tasmania (Colhoun *et al.* 1996). Temperatures during this period were 5° C to 6.5° C cooler than at present and average precipitation was reduced (Colhoun 1985; Colhoun and van de Geer 1987). Grasslands and sclerophyllous vegetation expanded at this time and rainforest taxa were largely confined to the western coast and isolated mountains (Markgraf *et al.* 1995). The ice had largely disappeared by 10,000 years B. P., but development of ice had been greater in the western, more humid areas of Tasmania (Colhoun *et al.* 1996) that largely coincide with the current range of Huon pine (Macphail 1979). There was a rise in temperature and associated rise in precipitation between 11,500 and 9,500 years B. P., associated with Holocene warming that led to an expansion of trees across Tasmania. Postglacial climates in southern Tasmania were characterized by a strong gradient in precipitation from the west to the east (Macphail 1979). The lack of moisture, primarily in the east, delayed the expansion of forest in eastern Tasmania until after 9,500 years B. P. The

Lower Gordon River valley pollen record, spanning most of the last 8,000 years, indicates that rainforest species were well established in the west by this time and have remained dominant through the Holocene (Harle *et al.* 1999). The high elevation, alpine stand of Huon pine at Mt. Read has been present for at least 11,000 years, based on dating of subfossil wood (Francey *et al.* 1984). The Stanley River population has also been present for over 10,000 years (Barbetti *et al.* 1993). Between 5,000 and 8,000 years B. P., climates in southern Tasmania were increasingly humid and perhaps warmer (Macphail 1979). With a more suitable environment, by 7,800 years B. P. rainforests were found beyond their current range limits. The climate has since become increasingly unstable and frosts and drought have become more common (Macphail 1979).

Paleoecological records from all last glacial assemblages from western Tasmania include a small component of rainforest trees that include Huon pine pollen (Colhoun and van de Geer 1987). A Late Pleistocene deposit from the southwestern Tasmanian coast at least 38,000 years old contains both macrofossil and pollen identified as Huon pine (Jordan *et al.* 1991). Pollen assemblages from a central western Tasmania site 25,000 to 40,000 years old contain a small quantity of temperate rainforest pollen including that of Huon pine. The small quantities found in the pollen record are consistent with isolated stands of rainforest trees during glacial intervals (Colhoun and van de Geer 1987). Vegetation modeling based on palynological and ecological information suggests that Tasmanian rainforest vegetation survived the last glacial interval near west coast valleys and in very small refugial areas in the east (Kirkpatrick and Fowler 1998). Based on the combined evidence, it has been

suggested that there were widespread small stands of rainforest trees during glacial intervals (Macphail 1979; Colhoun 1985; Colhoun and van de Geer 1986).

The landscape of western Tasmania provides substantial habitat heterogeneity related to topography that could have provided microclimate suitable for Huon pine persistence. The current distribution occurs in an altitudinal range of 0 to 1000 meters (Gibson *et al.* 1991). Habitat heterogeneity in refugial areas could also act to preserve diversity by providing suitable microclimate conditions. During glacial intervals, trees may have persisted on west coast coastal plains and river valleys, but some Huon pine could have persisted in a shrubby alpine form. In southern Tasmania, primarily along the Huon and Picton Rivers, Huon pine occurs as a low growing component of a scrub community in areas with rainfall totals that are smaller than the predicted limits for rainforest cover (Gibson *et al.* 1991). Marginal areas such as this are likely to disappear as climate conditions become drier during glacial intervals, particularly as there are strong west to east precipitation gradients in Tasmania.

The oldest known macrofossils in Tasmania were recorded from Early Pleistocene sediments (between 0.78 and 1.64 million years old) (Macphail *et al.* 1993). Although pollen attributed to Huon pine is common, macrofossils are rare. It has been suggested, due to the lack of macrofossils, that Huon pine could have been uncommon in the distant past and could be a relatively recently evolved species (Hill 1990). Another possibility is that the lack of pollen evidence could have been produced by a larger group of podocarp species that have since become extinct. This may in time be clarified with further finds of macrofossil evidence. Evidence from the fossil record suggests that Huon pine persisted in multiple refugial areas and subsequently expanded its range. The current restricted distribution of

Huon pine populations could be associated more with slow dispersal of the species rather than a narrow niche. Huon pine is still expanding its range and currently occupies only a portion of its potential range (Read and Hill 1988; Gibson *et al.* 1991).

Summary

It is difficult to draw inference on population history based on a single gene genealogy without a paleoecological record (Lascoux *et al.* 2003). There is stochastic variability in evolution at a single locus (Ewens 1983) and variation might not be sufficient to resolve population history. This study demonstrates the potential of cpDNA, together with available paleoecological information, to provide insight into the genetic structure and post-Pleistocene history of a Southern Hemisphere conifer. A hierarchical approach that validates the assumptions of analyses has been used to maximize the resolution of a low definition locus. Measurements were made using both parametric and nonparametric methods in an attempt to define contemporary and historic evolutionary processes shaping genetic variation and population structure in Huon pine. The nature of this analysis is inferential and alternative explanations cannot be excluded. However, given these caveats, aspects of the molecular data are compatible with the hypothesis of population bottlenecks from both long term and Pleistocene glacial range contraction. In the most recent post-glacial period, the history is consistent with range expansion and long distance colonization. Pollen records suggest that there were multiple refugial origins for current Huon pine populations, a pattern found in other Southern Hemisphere species. Macrofossil and pollen databases provide some estimates of past species distribution, but this record is often incomplete or inconclusive because of poor conditions for deposition and preservation (Petit *et al.* 1997).

Paleoecological information is limited in Huon pine, so it is difficult to infer routes of recolonization, or identify specific small refugial areas at present. The history of post-glacial migration could be further obscured by multiple refugial origins. Further sampling of Huon pine populations and evaluation of genetic diversity with both nuclear and organelle loci could further resolve refugial areas and post-Pleistocene migration. The low level of chloroplast nucleotide diversity is similar to that in other relict species and suggestive of a history of population bottlenecks.

The most significant findings of this study can be summarized as follows:

1. Overall, there is significant population structure in Huon pine exhibited in chloroplast DNA. Genetic structure is significant between the populations in the Huon watershed and many of the northwestern populations. This structure fits an isolation by distance model and is supported by a significant Mantel test between genetic distance and geographic location.
2. The pattern of haplotype distribution suggests a history of range expansion and long distance colonization from western refugial areas to southeastern populations.
3. The populations surrounding Macquarie Harbor appear to be the center of chloroplast haplotype diversity for Huon pine. This is consistent with information from the fossil record that suggests that the west coast was the principal refugial area during glacial intervals.

LITERATURE CITED

- Allnutt, T. R., Newton A. C., Lara A., Premoli A., Armesto J. J., Vergara R., Gardner M. (1999) Genetic variation in *Fitzroya cupressoides* (alerce), a threatened South American conifer. *Molecular Ecology* **8**, 975-987.
- Altschul, S. F., Gish W., Miller W., Meyers E. W., Lipman D. J. (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**, 403-410.
- Awise, J. C. (1994) *Molecular Markers, Natural History and Evolution* Chapman and Hall, New York.
- Aylor, D., Carbone I. (2003) SNAP Combine and Map. Department of Plant Pathology, North Carolina State University, Raleigh, NC, USA.
<http://www.cals.ncsu.edu/plantpath/faculty/carbone/home.html>
- Bahlo, M., Griffiths R. C. (2000) Inference from gene trees in a subdivided population. *Theoretical and Population Biology* **57**, 79-95.
- Barbetti, M., Bird T., Dolezal G., Taylor G., Francey R. J., Cook E., Peterson M. (1993) Radiocarbon variations from Tasmanian conifers: first results from Late Pleistocene and Holocene logs. *Radiocarbon* **34**, 806-817.
- Berli, P., Felsenstein J. (1999) Maximum likelihood estimations of migration rates and effective population numbers in two populations using a coalescent approach. *Genetics* **152**, 763-773.

- Beerli, P., Felsenstein J. (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proceedings of the National Academy of Sciences U S A* **98**, 4563-4568.
- Bermingham, E., Avise J. (1986) Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* **113**, 939-965.
- Billington, H. L. (1991) Effect of population size on genetic variation in a dioecious conifer. *Conservation Biology* **5**, 115-119.
- Birky, C. W. (1995) Uniparental inheritance of chloroplast and mitochondrial genes: mechanisms and evolution. *Proceedings of the National Academy of Science* **92**, 11331-11338.
- Birky, C. W. J., Fuerst P., Maruyama T. (1989) Organelle gene diversity under migration, mutation and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics* **121**, 613-627.
- Bond, G., Broecker W., Johnsen S., McManus J., Labeyrie L., Jouzel J., Bonani G. (1993) Correlations between climate records from North Atlantic sediments and Greenland ice. *Nature* **365**, 143-147.
- Butcher, P. A., Byrne M., Moran G. F. (1995) Variation within and among the chloroplast genomes of *Melaleuca alternifolia* and *M. linariifolia* (Myrtaceae). *Plant Systematics and Evolution* **194**, 69-81.
- Byrne, M., Hines B. (2004) Phylogeographical analysis of cpDNA variation in *Eucalyptus loxophleba* (Myrtaceae). *Australian Journal Of Botany* **52**, 459-470.

- Byrne, M., Macdonald B., Coates D. (1999) Divergence in the chloroplast genome and nuclear rDNA of the rare Western Australian plant *Lambertia orbifolia* Gardner (Proteaceae). *Molecular Ecology* **8**, 1789-1796.
- Byrne, M., Moran G. F. (1994) Population divergence in the chloroplast genome of *Eucalyptus nitens*. *Heredity* **73**, 18-28.
- Cann, R. L., Stoneking M., Wilson A. C. (1987) Mitochondrial DNA and human evolution. *Nature* **325**, 31-36.
- Carbone, I., Kohn L. M. (2001) A microbial population - species interface: nested cladistic and coalescent inference with multilocus data. *Molecular Ecology* **10**, 947-964.
- Carbone, I., Liu Y.-C., Hillman B. I., Milgroom M. G. (2004) Recombination and migration of *Cryphonectria hypovirus* 1 as inferred from gene genealogies and the coalescent. *Genetics* **166**, 1611-1629.
- Carpenter, R. J., Hill R. S., Jordan G. J. (1994) Cenozoic vegetation in Tasmania: macrofossil evidence. In: *History of Australian Vegetation. Cretaceous to Recent* (ed. Hill RS), pp. 276-298. Cambridge University Press, Cambridge.
- Clement, M., Posada D., Crandall K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1660.
- Colhoun, E. A. (1985) Glaciations of the West Coast Range, Tasmania. *Quaternary Research* **24**, 39-59.
- Colhoun, E. A., Hannan D., Kiernan K. (1996) Late Wisconsin glaciation of Tasmania. *Papers and Proceedings of the Royal Society of Tasmania* **130**, 33-45.

- Colhoun, E. A., van de Geer G. (1986) Holocene to Middle Last Glaciation vegetation history at Tullabardine Dam, Western Tasmania. *Proceedings Of The Royal Society Of London* **B229**, 177-207.
- Colhoun, E. A., van de Geer G. (1987) Vegetation history and climate before the maximum of the last glaciation at Crotty, Western Tasmania. *Papers and Proceedings of the Royal Society of Tasmania* **121**, 69-74.
- Comes, H. P., Kadereit J. W. (1998) The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**, 432-438.
- Comps, B., Gomary D., Letouzey J., Thiebout B., Petit R. J. (2001) Diverging trends between heterozygosity and allelic richness during postglacial colonization in European beech. *Genetics* **157**, 389-397.
- Cook, E. R., Bird, T. D., Peterson, M., Barbetti, M., Buckley, B. M., D'Arrigo, R. D., Francey, R.J., Tans, P. (1991) Climatic change in Tasmania inferred from a 1089 - year tree - ring chronology of Huon pine. *Science* **253**, 1266-1268.
- Cook, E. R., Francey, R. J., Buckley, B. M., D'Arrigo, R. D. (1996) Recent increases in Tasmanian Huon pine ring widths from a sub-alpine stand: natural climate variability, CO² fertilisation, or greenhouse warming? *Papers and Proceedings of the Royal Society of Tasmania* **130**, 65-72.

- Cook, E. R., Buckley B. M., D'Arrigo R. D., Peterson M. J. (2000) Warm-season temperatures since 1600 BC reconstructed from Tasmanian tree rings and their relationship to large-scale sea surface temperature anomalies. *Climate Dynamics* **16**, 79-91.
- Crandall, K., Templeton A. (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics* **134**, 959-969.
- Davis, M. B. (1981) Quaternary history and the stability of forest communities. In: *Forest Succession: Concepts and Application* (eds. West DC, Shugart HH, Botkin DB), pp. 132-153. Springer-Verlag, New York.
- Davis, M. B., Shaw R. G. (2001) Range shifts and adaptive responses to Quaternary climate change. *Science* **292**, 673-679.
- Davis, M. B., Woods K. D., Webb S. L., Futyama R. P. (1986) Dispersal versus climate; expansion of *Fagus* and *Tsuga* into the Upper Great Lakes region. *Vegetatio* **67**, 93-103.
- Delcourt, H. R., Delcourt P. A. (1991) *Quaternary Ecology. A paleoecological perspective* Chapman & Hall, London.
- Delcourt, P. A., Delcourt H. R. (1987) *Long - term forest dynamics of the temperate zone* Springer - Verlag, New York.

- Demesure, B., Comps B., Petit R. J. (1996) Chloroplast DNA phylogeography of the common Beech (*Fagus sylvatica* L.) in Europe. *Evolution* **50**, 2515-2520.
- Demesure, B., Sodzi N., Petit R. J. (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology* **4**, 129-131.
- Dumolin-Lapegue, S., Demesure B., Fineschi S., LeCorre V., Petit R. J. (1997) Phylogeographic structure of white oaks throughout the European continent. *Genetics* **146**, 1475-1487.
- Echt, C. S., DeVerno L. L., Anzidei M., Vendramin G. G. (1998) Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Molecular Ecology* **7**, 307-316.
- Ewens, W. J. (1983) The role of models in the analysis of molecular genetic data, with particular reference to restriction fragment data. In: *Statistical Analysis of DNA Sequence Data* (ed. Weir B), pp. 45-73. Marcel Dekker, New York.
- Ewing, B., Green P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* **8**, 186-194.
- Ewing, B., Hillier L., Wendl M. C., Green P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8**, 175-185.
- Ferris, C., King R. A., Vainola R., Hewitt G. M. (1998) Chloroplast DNA recognizes three refugial sources of European oaks and suggests independent eastern and western immigrations to Finland. *Heredity* **80**, 584-593.

- Fins, L., Libby W. J. (1982) Population variation in *Sequoiadendron*: seed and seedling studies, vegetative propagation, and isozyme variation. *Silvae Genetica* **31**, 102-110.
- Fowler, D. P., Morris R. W. (1977) Genetic diversity in red pine: evidence for low genic heterozygosity. *Canadian Journal Forest Research* **7**, 343-347.
- Francey, R. J., Barbetti M., Bird T. D., Beardsmore D., Coupland W., Dolezal G. D., Farquhar G. D., Flynn R. G., P.J. F., Gifford R. M., Goodman H. S., Kunda B., McPhail S., Nanson G., Pearman G. I., Richards N. G., Shankey T. D., Temple R. B., Weir B. (1984) Isotopes in tree rings. C.S.I.R.O. Division of Atmospheric Technical Paper, Australia.
- Freeman, J. S., Jackson H. D., Steane D. A., McKinnon G. E., Dutkowski G. W., Potts B. M., Vaillancourt R. E. (2001) Chloroplast DNA phylogeography of *Eucalyptus globulus*. *Australian Journal Of Botany* **49**, 585-596.
- Fu, Y.-X., Li W.-H. (1993) Statistical tests of neutrality of mutations. *Genetics* **133**, 693-709.
- Fu, Y.-X., Li W.-H. (1999) Coalescing into the 21st Century: An Overview and Prospects of Coalescent Theory. *Theoretical Population Biology* **56**.
- Gibson, N. (1986) Huon pine conservation and management, p. 91. Tasmanian Forestry Commission Report number 86/3, Hobart, Tasmania.
- Gibson, N., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 2. Population structure and spatial pattern. *Australian Journal Of Ecology* **16**, 223-229.

- Gibson, N., Davies J., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 1. Distribution, floristics and environmental correlates. *Australian Journal Of Ecology* **16**, 215-222.
- Guries, R. P., Ledig F. T. (1982) Genetic diversity and population structure in pitch pine (*Pinus rigida* Mill.). *Evolution* **36**, 387-402.
- Hamrick, J. L. (2004) Response of forest trees to global environmental changes. *Forest Ecology And Management* **197**, 323-335.
- Hamrick, J. L., Godt M. (1996) Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London B* **351**, 1291-1298.
- Hamrick, J. L., Godt M. J., Sherman - Broyles S. L. (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**, 95-124.
- Hamrick, J. L., Godt M. J. W. (1990) Allozyme diversity in plant species. In: *Plant population genetics, breeding, and genetic resources* (eds. Brown AHD, Clegg MT, Kahler AL, Weir BS), pp. 43-82. Sinauer, Sunderland.
- Harle, K. J., Hodgson D. A., Tyler P. A. (1999) Palynological evidence for Holocene paleoenvironments from the lower Gordan River valley, in the World Heritage Area of southwest Tasmania. *The Holocene* **9**, 149-162.
- Harris, S. A., Ingram R. (1991) Chloroplast DNA and biosystematics: The effects of intraspecific diversity and plastid transmission. *Taxon* **40**, 393-412.

- Hawkins, B. J., Sweet G. B. (1989) Genetic variation in Rimu: An investigation using isozyme analysis. *New Zealand Journal Of Botany* **27**, 83-90.
- Hewitt, G. M. (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**, 247-276.
- Hewitt, G. M. (2000) The genetic legacy of the Quaternary ice ages. *Nature* **405**, 907-913.
- Hewitt, G. M. (2001) Speciation, hybrid zones and phylogeography - or seeing genes in space and time. *Molecular Ecology* **10**, 537-549.
- Hey, J., Wakeley J. (1997) A coalescent estimator of the population recombination rate. *Genetics* **145**, 833-846.
- Hill, R. S. (1990) The fossil history of Tasmania's rainforest tree species. *Tasforests* **1**, 5-12.
- Hill, R. S., Macphail M. K. (1985) A Fossil Flora from Rafted Plio-Pleistocene Mudstones at Regatta Point, Tasmania. *Australian Journal of Botany* **33**, 497-517.
- Hong, Y.-P., Hipkins V. D., Strauss S. H. (1993) Chloroplast DNA diversity among trees, populations and species in the California closed-cone pines (*Pinus radiata*, *Pinus muricata* and *Pinus attenuata*). *Genetics* **135**, 1187-1196.
- Huang, S., Chiang Y. C., Schaal B. A., Chou C. H., Chiang T. Y. (2001) Organelle DNA phylogeography of *Cycas taitungensis*, a relict species in Taiwan. *Molecular Ecology* **10**, 2669-2681.
- Huang, S. S. F., Hwang S.-Y., Lin T.-P. (2002) Spatial pattern of chloroplast DNA variation of *Cyclobalanopsis glauca* in Taiwan and East Asia. *Molecular Ecology* **11**, 2349-2358.

- Hudson, R. (1983) Testing the constant - rate neutral allele model with protein sequence data. *Evolution* **37**, 203-217.
- Hudson, R. (1990) Gene genealogies and the coalescent process. *Oxford Surveys in Evolutionary Biology* **7**, 1-44.
- Hudson, R., Boos D., Kaplan N. (1992a) A statistical test for detecting geographic subdivision. *Molecular Biology and Evolution* **9**, 138-151.
- Hudson, R. R. (2000) A new statistic for detecting genetic differentiation. *Genetics* **155**, 2011-2014.
- Hudson, R. R., Slatkin M., Maddison W. P. (1992b) Estimation of levels of gene flow from DNA sequence data. *Genetics* **132**, 583-589.
- Huntley, B. (1993) Species-richness in North-temperate zone forests. *Journal of Biogeography* **20**, 163-180.
- Huntley, B., Webb T. (1989) Migration: species' response to climatic variations caused by changes in the Earth's orbit. *Journal of Biogeography* **16**, 5-19.
- Hwang, S.-Y., Lin T.-P., Ma C.-S., Lin C.-L., Chung J.-D., Yang J.-C. (2003) Postglacial population growth of *Cunninghamia konishii* (Cupressaceae) inferred from phylogeographical and mismatch analysis of chloroplast DNA variation. *Molecular Ecology* **12**, 2689-2695.
- Jensen, J., Bohonak A. J., Kelley S. T. (2005) Isolation by distance, web service. *BMC Genetics* **6**, 13.

- Jordan, G. J. (1995) Extinct conifers and conifer diversity in the Early Pleistocene of western Tasmania. *Review of Palaeobotany and Palynology* **84**, 375-387.
- Jordan, G. J. (1997) Evidence of Pleistocene plant extinctions and diversity from Regatta Point, western Tasmania, Australia. *Botanical Journal of the Linnean Society* **123**, 45-71.
- Jordan, G. J., Carpenter R. J., Hill R. S. (1991) Late Pleistocene vegetation and climate near Melaleuca Inlet, south-western Tasmania. *Australian Journal Of Botany* **39**, 315-333.
- Kershaw, A. P. (1988) Australasia. In: *Vegetation History* (eds. Huntley B, Webb TI), pp. 238 - 306. Kluwer Academic Publishers, Dordrecht.
- Kiernan, K., Lauritzen, S.E., Duhig, N. (2001) Glaciation and cave sediment aggradation around the margins of the Mt Field Plateau, Tasmania. *Australian Journal of Earth Science* **48**, 251-263.
- Kimura, M. (1969) The number of heterozygous nucleotide sites maintained in a finite population due to steady flux of mutations. *Genetics* **61**, 893-903.
- King, R., Ferris C. (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. *Molecular Ecology* **7**, 1151-1161.
- Kingman, J. F. C. (1982a) The coalescent. *Stochastic Processes and their Applications* **13**, 235-248.
- Kingman, J. F. C. (1982b) On the genealogy of large populations. *Journal of Applied Probability* **19A**, 27-43.

- Kirkpatrick, J. B., Fowler M. (1998) Locating likely glacial forest refugia in Tasmania using palynological and ecological information to test alternative climatic models. *Biological Conservation* **85**, 171-182.
- Lascoux, M., Palmé A. E., Cheddadi R., Latta R. G. (2003) Impact of Ice Ages on the genetic structure of trees and shrubs. *Philosophical Transactions Of The Royal Society Of London Series B Biological Sciences* **359**, 197-207.
- Ledig, F. T., Conkle M. T. (1983) Gene diversity and genetic structure in a narrow endemic, Torrey pine (*Pinus torreyana* Parry ex. Carr). *Evolution* **37**, 79-85.
- Lee, S.-W., Ledig F. T., Johnson D. R. (2002) Genetic variation at allozyme and RAPD markers in *Pinus longaeva* (Pinaceae) of the White Mountains, California. *American Journal of Botany* **89**, 566-577.
- Lu, S.-Y., Peng C.-I., Cheng Y.-P., Hong K.-H., Chiang T.-Y. (2001) Chloroplast DNA phylogeography of *Cunninghamia konishii* (Cupressaceae), an endemic conifer of Taiwan. *Genome* **44**, 797-807.
- Macphail, M. K. (1979) Vegetation and climates in Southern Tasmania since the last glaciation. *Quaternary Research* **11**, 306-341.
- Macphail, M. K., Jordan G. J., Hill R. S. (1993) Key periods in the evolution of the Cenozoic flora and vegetation of Tasmania I. the Early-Middle Pleistocene. *Australian Journal Of Botany* **41**, 673-707.

- Markgraf, V., McGlone M., Hope G. (1995) Neogene paleoenvironmental and paleoclimatic change in southern temperate ecosystems - a southern perspective. *Trends In Ecology & Evolution* **10**, 143 - 147.
- Markwordt, J., Doshi R., Carbone I. (2003) SNAP Clade and matrix. <http://www.cals.ncsu.edu/plantpath/faculty/carbone/home.html>
- Millington, R. J., Jones R., Brown D., Vernon B. (1979) Huon pine - endangered? Environmental Studies Occasional Paper No. 9. University of Tasmania, Hobart.
- Mogensen, H. L. (1996) The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany* **83**, 383-404.
- Moran, G. F., Bell J. C., Eldridge K. G. (1988) The genetic structure and the conservation of the five natural populations of *Pinus radiata*. *Canadian Journal of Forest Research* **18**, 506-514.
- Mosseler, A., Egger K. N., Hughes G. A. (1992) Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markers. *Canadian Journal of Forest Research* **22**, 1332-1337.
- Neale, D. B., Marshall K. A., Sederoff R. R. (1989) Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. *Proceedings of the National Academy of Sciences U S A* **86**, 9347-9349.
- Neale, D. B., Sederoff R. R. (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theoretical and Applied Genetics* **77**, 212-216.

- Neale, D. B., Wheeler N. C., Allard R. W. (1986) Paternal inheritance of chloroplast DNA in Douglas fir. *Canadian Journal of Forest Research* **16**, 1152-1154.
- Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences, USA* **70**, 3321-3323.
- Nei, M. (1987) *Molecular Evolutionary Genetics* Columbia University Press, New York.
- Nei, M., Maruyama T., Chakraborty M. (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**, 1-10.
- Neigel, J. E., Avise J. C. (1993) Application of a random-walk model to geographic distributions of animal mitochondrial DNA variation. *Genetics* **135**, 1209-1220.
- Nielsen, R., Wakeley J. (2001) Distinguishing migration from isolation: A Markov chain Monte Carlo approach. *Genetics* **158**, 885-896.
- Oldfield, S., Lusty C., MacKinven A. (1998) *The World List of Threatened Trees*. World Conservation Press, Cambridge.
- Olsen, K. M. (2002) Population history of *Manihot esculenta* (Euphorbiaceae) inferred from nuclear DNA sequences. *Molecular Ecology* **11**, 901-911.
- Palmer, J. D., Stein D. B. (1986) Conservation of chloroplast genome structure among vascular plants. *Current Genetics* **10**, 823-833.
- Peakall, R., Ebert D., Scott L., Meagher P., Offord C. (2003) Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). *Molecular Ecology* **12**, 2331-2343.

- Peterson, M. J. (1990) Distribution and conservation of Huon pine, p. 50. Forestry Commission Tasmania, Tasmania.
- Petit, R. J., Aguinagalde I., de Beaulieu J. L., Bittkau C., Brewer S., Cheddadi R., Ennos R., Fineschi S., Grivet D., Lascoux M., Mohanty A., Muller-Starck G., Demesure-Musch B., Palme A., Martin J. P., Rendell S., Vendramin G. G. (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* **300**, 1563-1565.
- Petit, R. J., Kremer A., Wagner D. B. (1993a) Finite island model for organelle and nuclear genes in plants. *Heredity* **71**, 630-641.
- Petit, R. J., Kremer A., Wagner D. B. (1993b) Geographic structure of chloroplast DNA polymorphisms in European oaks. *Theoretical and Applied Genetics* **87**, 122-128.
- Petit, R. J., Pineau E., Demesure B., Bacilieri R., Ducouso A. (1997) Chloroplast DNA footprints of postglacial recolonization by oaks. *Proceedings of the National Academy of Sciences* **94**, 9996-10001.
- Pielou, E. C. (1991) *After the Ice Age* University of Chicago Press, Chicago.
- Playford, G., Dettmann M. (1979) Pollen of *Dacrydium franklinii* Hook. f. and comparable early Tertiary microfossils. *Pollen et Spores* **20**, 513-534.
- Posada, D., Crandall K. A., Templeton A. R. (2000) GeoDis: A program for the Cladistic Nested Analysis of the Geographical Distribution of Genetic Haplotypes. *Molecular Ecology* **9**, 487-488.

- Premoli, A. C., Kitzberger T., Veblen T. T. (2000) Isozyme variation and recent biogeographical history of the long - lived conifer *Fitzroya cupressoides*. *Journal of Biogeography* **27**, 251-260.
- Premoli, A. C., Vergara R., Souto C. P., Lara A., Newton A. C. (2003) Lowland valleys shelter the ancient conifer *Fitzroya cupressoides* in the Central Depression of southern Chile. *Journal of the Royal Society of New Zealand* **33**, 623-631.
- Price, E. W., Carbone I. (2005) SNAP: workbench management tool for evolutionary population genetic analysis. *Bioinformatics* **21**, 402-404.
- Quinn, C. J. (1982) Taxonomy of *Dacrydium* Sol. ex Lamb. amend de Lamb. (Podocarpaceae). *Australian Journal Of Botany* **30**, 311-320.
- Read, J., Hill R. S. (1988) Comparative responses to temperature of the major canopy species of Tasmanian cool temperate rainforest and their ecological significance. I. Foliar frost resistance. *Australian Journal Of Botany* **36**, 131-143.
- Rozas, J., Sanchez-DelBarrio J. C., Messeguer X., Rozas R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496-2497.
- Schaal, B. A., Hayworth D. A., Olsen K. M., Rauscher J. T., Smith W. A. (1998) Phylogeographic studies in plants: problems and prospects. *Molecular Ecology* **7**, 465-474.

- Schaal, B. A., Leverich W. J., Rogstad S. H. (1991) A comparison of methods for assessing genetic variation in plant conservation biology. In: *Genetics and conservation of rare plants* (eds. Falk DA, Holsinger KE), pp. 123-134. Oxford University Press, New York.
- Schaal, B. A., Olsen K. M. (2000) Gene genealogies and population variation in plants. *Proceedings of the National Academy of Sciences* **97**, 7024-7029.
- Shapcott, A. (1991a) Dispersal and establishment of Huon pine (*Lagarostrobos franklinii*). *Papers and Proceedings of the Royal Society of Tasmania* **125**, 17-26.
- Shapcott, A. (1991b) Studies in the population biology and genetic variation of Huon pine (*Lagarostrobos franklinii*), p. 84. National Rainforest Conservation Program and Department of Parks, Wildlife and Heritage, Hobart, Hobart, Tasmania.
- Shapcott, A. (1997) Population genetics of the long-lived Huon pine *Lagarostrobos franklinii*: An endemic Tasmanian temperate rainforest tree. *Biological Conservation* **80**, 169-179.
- Shapcott, A., Brown M. J., Kirkpatrick J. B., Reid J. B. (1995) Stand structure, reproductive activity and sex expression in Huon pine (*Lagarostrobos franklinii* (Hook L.) Quinn). *Journal of Biogeography* **22**, 1035-1045.
- Soltis, D. E., Gitzendanner M. A., Strenge D. D., Soltis P. S. (1997) Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant Systematics and Evolution* **206**, 353-373.

- Swofford, D. L. (2000) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, MA.
- Taberlet, P., Fumagalli L., Wust-Saucy A. G., Cosson J. F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7**, 453-464.
- Tajima, F. (1983) Evolutionary relationships of DNA sequences in finite populations. *Genetics* **105**, 437-460.
- Tajima, F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphisms. *Genetics* **123**, 585-595.
- Tajima, F. (1993) Measurement of DNA polymorphism. In: *Mechanisms of Molecular Evolution* (eds. Takahata N, Clark AG). Sinauer Associates, Sunderland, MA.
- Templeton, A. R. (1998) Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7**, 381-397.
- Templeton, A. R. (1999) Uses of evolutionary theory in the human genome project. *Annual Rev. Ecol. Syst.* **30**, 23-49.
- Templeton, A. R. (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology* **13**, 789-810.
- Templeton, A. R., Boerwinkle E., Sing C. F. (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics* **117**, 343-351.

- Templeton, A. R., Crandall K. A., Sing C. F. (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619-633.
- Templeton, A. R., Routman E., Phillips C. A. (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* **140**, 767-782.
- Watterson, G. (1975) On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology* **7**, 188-193.
- Wright, S. (1951) The genetical structure of populations. *Annals of Eugenics* **15**, 323-354.

Table 1.1. Sample size, dbh range and watershed for Huon pine populations.

Location	Abbr.	<i>n</i>	dbh range	Watershed
Riveaux Creek	RC	13	16.5 cm–130 cm	Huon
Condominium Creek	CC	13	< 1 cm–3 cm	Huon
Teepookana	TK	14	6.5 cm–136 cm	King
Newall Creek	NC	13	6 cm–70 cm	King
Darwin Crater Track	DC	3	40 cm	King
Gordon River	GR	13	10 cm–178 cm	Gordon
Stanley River	SR	13	< 1cm–102 cm	Pieman
Mt. Read	MR	14	2 cm–80 cm	Pieman

Abbr., abbreviation of population name; *n*, sample size; dbh, diameter at breast height (1.4 meters)

Table 1.2. Segregating nucleotide sites and haplotype configuration in chloroplast DNA sequence from three loci in Huon pine.

Primer pair ¹	<i>trnS-trnT</i>	<i>trnD-trnT</i>	<i>psbC-trnS</i>
Sequence length (bp)	330 bp	271 bp	291 bp
Site number	1	2	3
Position in locus	6	208	96
Site type	v	t	v
Character type	i	i	u
Consensus	A	C	G
Haplotypes (f)			
A (45)	A	C	G
B (25)	A	T	G
C (22)	C	C	G
D (3)	A	C	T
E (1)*	C	T	G

¹Demesure *et al.* 1995

bp, base pairs; v, transversion; t, transition; i, phylogenetically informative;

u, phylogenetically uninformative; (f), number of occurrences of haplotype in 96 samples;

*Haplotype E was an inferred recombinant haplotype and was excluded from coalescent analyses to meet assumptions of the model

Table 1.3. Estimates of population genetic diversity and sequence neutrality based on chloroplast DNA variation in intergenic spacer regions from *trnD-trnT*, *trnS-trnT* and *psbC-trnS* from 96 Huon pine samples.

Population	<i>n</i>	<i>k</i>	<i>s</i>	θ (<i>SD</i>)	π (<i>SD</i>)	Fu and Li's D*	Fu and Li's F*	Tajima's D
Total	96	0.829	3	0.00065 (0.00040)	0.00093 (0.00006)	0.83028 ns	0.94629 ns	0.75655 ns
Riveaux Creek	13	1.026	2	0.00072 (0.00055)	0.00115 (0.00018)	0.95275 ns	1.28188 ns	1.66129 ns
Condominium Creek	13	1.026	2	0.00072 (0.00055)	0.00115 (0.00018)	0.95275 ns	1.28188 ns	1.66129 ns
Teepookana	14	0.78	3	0.00106 (0.00069)	0.00087(0.00018)	-1.03687 ns	-1.03012 ns	-0.52939 ns
Newall Creek	13	0.667	2	0.00072 (0.00055)	0.00075 (0.00024)	0.95275 ns	0.83322 ns	0.09664 ns
Darwin Crater Track	3	0.667	1	0.00075 (0.00075)	0.00075 (0.00035)	na ¹	na ¹	na ¹
Gordon River	13	0.744	2	0.00072 (0.00055)	0.00083 (0.00018)	0.95275 ns	0.92936 ns	0.43193 ns
Stanley River	13	0.744	2	0.00072 (0.00055)	0.00083 (0.00018)	0.95275 ns	0.92936 ns	0.43193 ns
Mt. Read	14	0	0	0	0.0000	na ²	na ²	na ²

n, the number of nucleotide sequences; *k*, average number of pair-wise differences between the *i*th and the *j*th DNA sequences (Tajima 1983, equation A3); *s*, segregating sites (Watterson 1975); θ , an estimation of the mean population mutation rate per site from *s*, segregating nucleotide sites and *n*, sample size (Watterson 1975); π , nucleotide diversity, the average number of nucleotide differences per site between two sequences (Nei 1987, equations 10.5 or 10.6); Fu and Li's D* (1993); Fu and Li's F* (1993); Tajima's D (Tajima 1989)
 ns, not significant ($p > 0.1$); * $0.05 < p < 0.1$; ** $0.01 < p < 0.05$; *** $0.001 < p < 0.01$; **** $p < 0.001$
 na¹ insufficient number of sequences for neutrality estimation; na² no variation found in population
SD, standard deviation

Table 1.4. Chloroplast haplotype frequency from eight Huon pine populations (96 samples) based on sequence from *trnS-trnT*, *trnD-trnT* and *psbC-trnS*.

Population	Haplotypes					<i>hd</i> (<i>SD</i>)
	A	B	C	D	E	
Total frequency	0.46875	0.26042	0.22917	0.03125	0.0104	0.666 (0.027)
Riveaux Creek	0	0.3846	0.6154	0	0	0.51282 (0.082)
Condominium Creek	0	0.6154	0.3846	0	0	0.51282 (0.082)
Teepookana	0.5	0.3571	0.0714	0.0714	0	0.65934 (0.090)
Newall Creek	0.6923	0.0769	0.1538	0	0.0769	0.52564 (0.153)
Darwin Crater Track	0.3333	0	0.6667	0	0	0.66667 (0.314)
Gordon River	0.5385	0.1538	0.3077	0	0	0.64103 (0.097)
Stanley River	0.5385	0.3077	0	0.1538	0	0.64103 (0.097)
Mt. Read	1	0	0	0	0	0

hd, haplotype diversity (Nei 1987, equations 8.4 and 8.12 but replacing 2n by n);
SD, standard deviation

Table 1.5. Pairwise comparisons of values of G_{ST} (above diagonal) and Nm (below diagonal) between Huon pine populations (RC, Riveaux Creek; CC, Condominium Creek; TK, Teepookana; NC, Newall Creek; GR, Gordon River; MR, Mt. Read; SR, Stanley River).

Total $G_{ST} = 0.26077^{**}$ $Nm = 1.42$							
	RC	CC	TK	NC	GR	MR	SR
RC	—	0.01328	0.16479*	0.28925*	0.13140	0.60060**	0.20892**
CC	37.14	—	0.12387	0.29926**	0.15401	0.60060**	0.16844**
TK	3.54	3.54	—	0.02959	0.00285	0.20530	-0.03186
NC	1.23	1.17	16.40	—	-0.00747	0.06933	0.02710
GR	3.31	2.75	174.68	-67.39	—	0.18580	0.01664
MR	0.33	0.33	1.94	6.71	2.19	—	0.18580
SR	1.89	2.47	-16.19	17.95	29.55	2.19	—

G_{ST} and Nm (Nei 1973) equation 9 and calculated as equations 5 and 6 in Hudson *et al.* (1992b) by DnaSP; Statistical significance of population differentiation determined with Hudson's K_{ST} (1000 Monte Carlo simulations) (Hudson *et al.* 1992a); * $p \leq 0.05$, ** $p \leq 0.01$.

Table 1.6. Coalescent estimates of migration (M), theta (θ) and divergence time (T) between eastern and western watershed populations.

Watershed	MIGRATE				MDIV			
	$2N_e m$ estimates x = recipient				θ^a	θ^b	M	T
1. Huon	—	0.1	0.1	0.000232 (0.2069)	0.3 (0.00034)	Huon-Pieman	0.2	0.75
2. Gordon	4	—	2	0.000820 (0.7314)	0.3 (0.00034)	Huon-Gordon	0.5	0.4
3. Pieman	0.2	1	—	0.000375 (0.3345)	0.3 (0.00034)	Gordon-Pieman	>5	N.D.

Estimates of migration are based on 92 Huon pine samples grouped in populations as follows: Huon (Condominium and Riveaux Creek), Gordon (Gordon River, Teepookana and Newall Creek), and Pieman (Stanley River and Mt. Read).

$2N_e m$ is the effective number of migrants exchanged between two populations each generation, where N_e is the effective population size, and m is the migration rate per generation. Estimates of $2N_e m$ were generated with 10 independent simulations in MIGRATE to ensure convergence of values. An example of how to read the migration matrix: For the Gordon watershed population, 3, x indicates that migration from population 3 (Pieman) into x = (Gordon) is $2N_e m = 2$.

θ^a is the coalescent based, per site estimate of $\theta = 2N_e \mu$ (Beerli & Felsenstein 1999). The per locus population estimate is shown in parentheses.

θ^b is the coalescent per locus estimate from MDIV (the per site estimate is shown in parentheses). M is the scaled migration rate $2N_e m$, and T is the scaled divergence time $(T/2N_e)$.

N.D. equals populations not diverged. Graphical representations of M and T from MDIV analyses are shown in Figure 2.

Table 1.7. Results of the nested clade analysis for Huon pine haplotypes.

1-Step Clade			
Location	Haplotype	D_c	D_n
Int.	A	54 ^S	82 ^S
Tip	B	99	98 ^L
Tip	C	76 ^S	97 ^L
Tip	D	90	93
	I-T	-35 ^S	-15 ^S
Chain of Inference		1-2-11-12-13	RE - LD

The nested clade arrangement is shown in Figure 3. Chain of inference indicates the inference key from Templeton (2004) and the numbers indicate the steps taken in the key to reach a conclusion based on biological inference. Following the numbers are the conclusions based on the inference key, where RE is range expansion and LD is long distance colonization. The presence of two or more symbols indicates that the data does not contain enough information to select a single alternative. Superscript S or L indicates significantly small or large values respectively ($p \leq 0.05$) for D_c , D_n or I-T.

Table 1.8. Examples of chloroplast nucleotide diversity in woody plant species.

Species	π
<i>Cunninghamia konishii</i> (Hwang <i>et al.</i> 2003)	0.00190
<i>Cunninghamia lanceolata</i> (Hwang <i>et al.</i> 2003)	0.00176
<i>Cycas taitungensis</i> (Huang <i>et al.</i> 2001)	0.01268
<i>Cyclobalanopsis glauca</i> (Huang <i>et al.</i> 2002)	0.00065
<i>Eucalyptus loxophleba</i> (Byrne & Hines 2004)	0.08800
<i>Eucalyptus nitens</i> (Byrne & Moran 1994)	0.08400
<i>Lagarostrobos franklinii</i> (this study)	0.00093
<i>Lambertia orbifolia</i> (Byrne <i>et al.</i> 1999)	0.07200
<i>Melaleuca alternifolia</i> (Butcher <i>et al.</i> 1995)	0.01700
<i>Melaleuca linariifolia</i> (Butcher <i>et al.</i> 1995)	0.15000

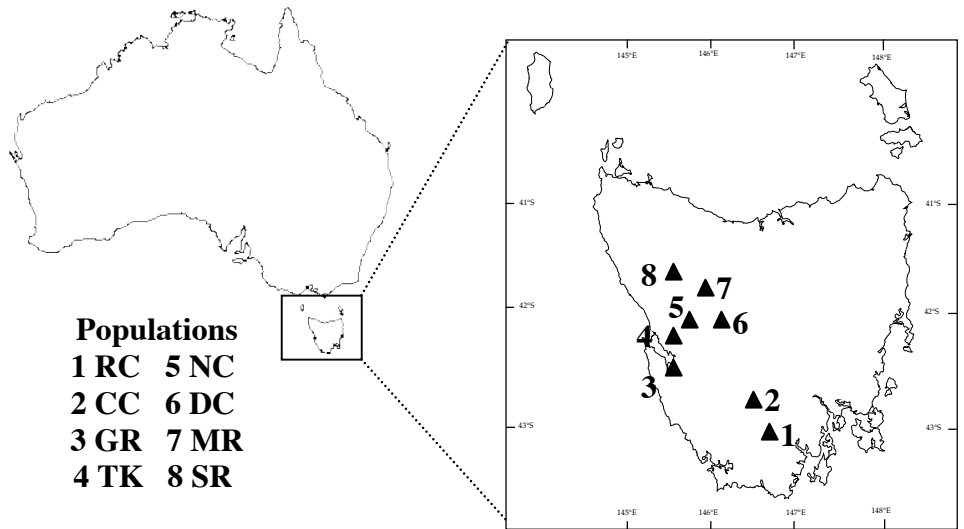


Figure 1.1. Huon pine sampling areas in Tasmania, Australia. Triangles (▲) represent sampling areas. (RC, Riveaux Creek; CC, Condominium Creek; GR, Gordon River; TK, Teepookana; NC, Newall Creek; DC, Darwin Crater Track; MR, Mt. Read; SR, Stanley River)

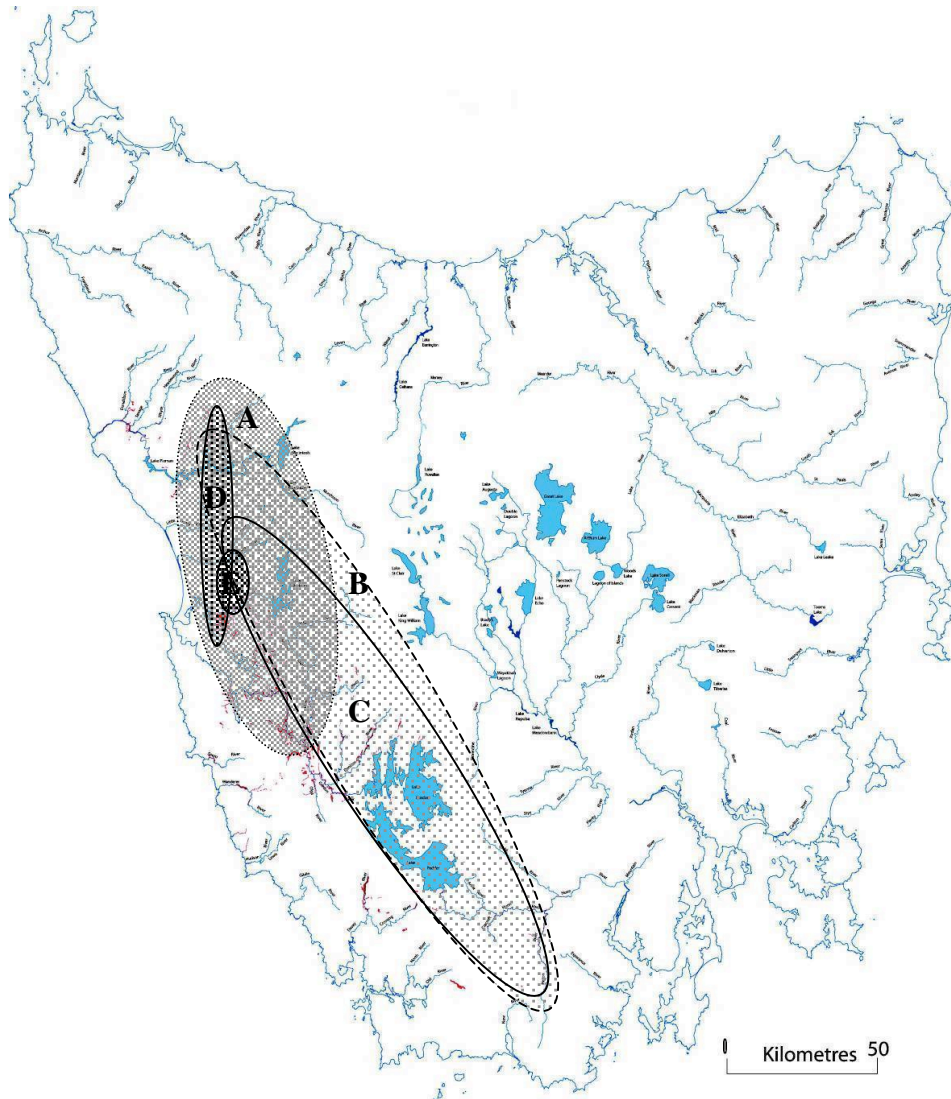


Figure 1.2. Geographic distribution of chloroplast haplotypes in Huon pine. Letters (A, B, C, D, E) represent Haplotypes. Dashed line indicates Haplotype B.

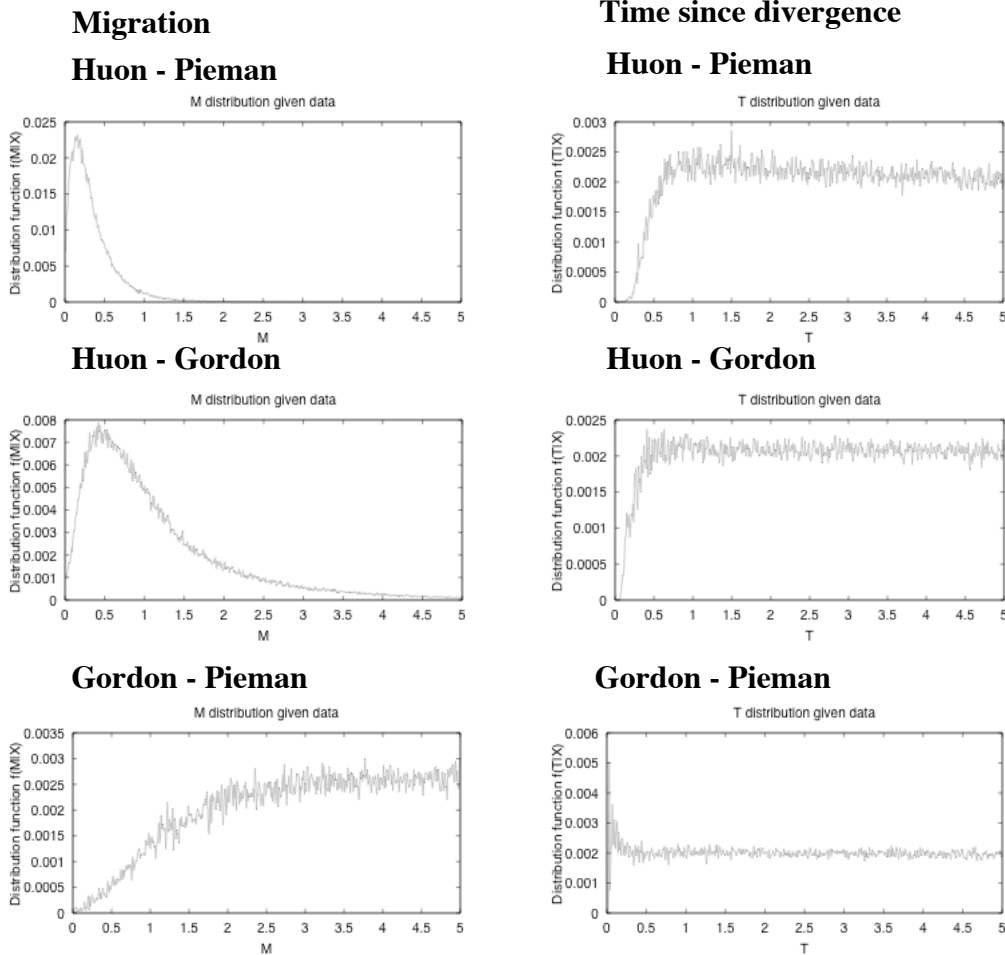


Figure 1.3. Posterior probability distributions of migration (M) and time since divergence (T) between watersheds: Huon (Riveaux and Condominium Creek), Gordon (Gordon River, Teepookana and Newall Creek) and Pieman (Stanley River and Mt. Read) estimated using MDIV. An initial 500,000 steps in the chain were used to simulate a sufficient number of genealogies before approximating the posterior distribution (Nielsen & Wakeley 2001). Posterior probability distribution estimations were based on a chain length of 2,000,000, Max $M = 5$, $T = 5$. Ten independent replicates with different starting random number seeds and the same assumptions and parameters were similar in all cases. MDIV results were plotted with gnuplot version 3.7 (<http://www.gnuplot.info/>).

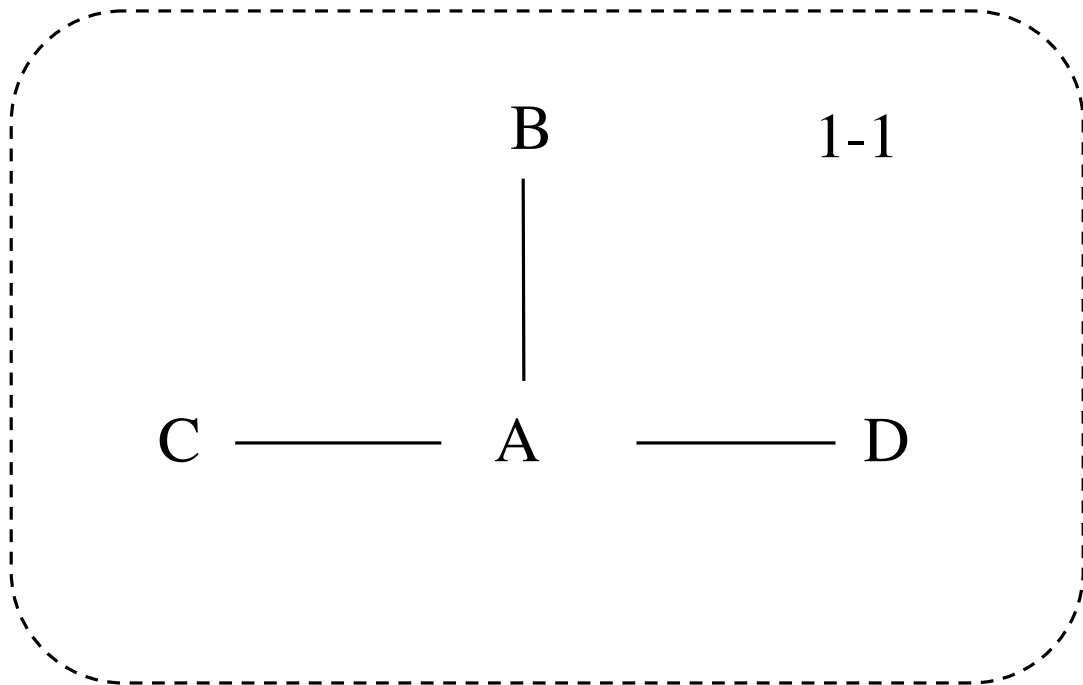


Figure 1.4. Nested clade diagram based on the 95% most probable connections between haplotypes calculated by TCS version 1.18 (Clement *et al.* 2000). Each line represents a single mutation and letters (A, B, C, D) represent haplotypes.

CHAPTER 2

**LOW LEVEL OF NUCLEOTIDE DIVERSITY AT THREE NUCLEAR LOCI IN
HUON PINE, A TASMANIAN RAINFOREST CONIFER
(*LAGAROSTROBOS FRANKLINII*)**

ABSTRACT

DNA nucleotide variation in a Tasmanian rainforest conifer (*Lagarostrobos franklinii*) was investigated in segments of three genes, *4Cl* (4-coumarate: coenzyme A ligase), *ITS2* (intergenic spacer region of ribosomal DNA) and *G3pdh* (glyceraldehyde 3-phosphate dehydrogenase). A total of 1,154 base pairs were sequenced from 79 individuals (158 alleles) representing seven geographic locations. Estimates of nucleotide diversity (π) (0.00089 ± 0.00007) and theta (θ) (0.00061 ± 0.00031) were low for the combined loci. Although total nucleotide diversity was low, there was a higher level of variation at the *4Cl* locus ($\pi = 0.00167 \pm 0.00014$). Polymorphism occurred at a frequency of less than five percent at the *ITS2* locus and was absent at the *G3pdh* locus. The highest level of nucleotide diversity was found in the high elevation Mt. Read population, previously described as a putative clonal stand composed entirely of male trees. There was no indication of significant nonrandom union of either gametes or haplotypes. Although there was no evidence for departure from neutral molecular evolution at the *4Cl* locus, the lack of variation at *G3pdh* and extremely low variation at *ITS2* could be evidence of selective forces restraining diversity at these sites. Both nucleotide diversity and population differentiation ($F_{ST} = 0.0130$) were lower than previous estimates in chloroplast DNA from the same individuals ($\pi = 0.00093$, $G_{ST} = 0.2607$) (Chapter 1). Population differentiation was also lower than that based on allozyme variation ($F_{ST} = 0.095$) (Shapcott 1991b). The very low level of variation found in nuclear loci is compatible with a history of demographic events such as population bottlenecks, and range contraction and expansion related to Pleistocene environmental variability.

INTRODUCTION

One of the fundamental aims of population genetics is to quantify genetic diversity and the evolutionary forces influencing patterns of variation. Single nucleotide polymorphisms (SNP's) are an important tool for estimating population genetic diversity and providing inference on evolutionary processes. Improvements in technology to generate DNA sequence have made it possible to study nucleotide variation in a variety of plant and animal species. Extensive DNA SNP surveys are available for model organisms, or economically important species such as *Arabidopsis thaliana* (Hanfstingl *et al.* 1994; Miyashita *et al.* 1996; Kawabe and Miyashita 1999), *Zea mays* ssp. *mays* (Tenaillon *et al.* 2001), *Drosophila melanogaster* (Wang *et al.* 2002) and *Homo sapiens* (Cargill *et al.* 1999). Large-scale nucleotide surveys that encompass multiple loci reveal common patterns apparent in genetic variation (Tenaillon *et al.* 2001). One common finding is that genetic diversity varies across species, and there are wide differences in variation found in some comparisons. For example, genetic variation in *Pinus taeda* (Brown *et al.* 2004) is approximately five-fold higher than that found in *Homo sapiens* (Cargill *et al.* 1999). Levels of diversity also vary within species among loci related to differences in mutation rates, selective forces and recombination (Cargill *et al.* 1999, Tenaillon *et al.* 2001, Brown *et al.* 2004). The distribution of SNP polymorphisms can also reflect population history and demography through signatures of migration, genetic drift, and contraction and expansion of population size and range (Templeton 1998; Olsen 2002; Carbone *et al.* 2004). A fourth common observation is the correlation of the level of nucleotide variation with the

recombination rate (Begun and Aquadro 1992). The variability in DNA sequence diversity across species and loci illuminates the potential for wide variability of interpretations of population history based on a single locus as sequence is subject to stochastic variability, or different functional constraints (Ewens 1983).

Examination of nuclear DNA diversity at multiple loci in tree species could provide important information for the conservation and management of genetic diversity with changing global climate (Karl and Trenberth 2003). Most multilocus SNP studies have examined short-lived animal and plant species. For extremely long-lived organisms, there may have been relatively few generations since the last Pleistocene glacial maximum approximately 18,000 years BP (before present) (Webb and Bartlein 1992), and it is unlikely that they have reached a state of equilibrium gene flow (Schaal and Olsen 2000). The gene pool of these organisms could reflect both historical migration, and shared ancestral polymorphisms, in addition to contemporary gene flow (Hewitt 1996). To date, there are few studies of nucleotide variation of nuclear loci in woody plants (Dvornyk *et al.* 2002; García-Gil *et al.* 2003; Järvinen *et al.* 2003; Kado *et al.* 2003; Brown *et al.* 2004), or undomesticated species (Olsen 2002; Caicedo and Schaal 2004). There are two published studies examining nuclear variation in tree species at three or more loci (Kado *et al.* 2003; Brown *et al.* 2004). Long-lived woody plant species show characteristic levels of allozyme variation that are correlated with mating system, life span and mode of dispersal (Hamrick *et al.* 1992; Hamrick and Godt 1996). In forest tree species that can live over 2,000 years, such as Huon pine (*Lagarostrobos franklinii* (Hook f.) Quinn) (Francey *et al.* 1984), we can expect to find

differences in the manifestation of evolutionary forces on genetic diversity compared to short-lived model systems.

Study species

Huon pine is a Tasmanian endemic rainforest conifer currently distributed largely in protected areas of southwestern Tasmania in eight watershed systems (Peterson 1990; Gibson and Brown 1991). Distribution is naturally fragmented, as populations are often restricted to narrow gallery forests along river systems, but stands are also found on moist slopes, in association with sclerophyllous vegetation, or in large non-riverine stands (Gibson 1986; Gibson *et al.* 1991). Huon pine was part of a diverse and widespread rainforest in mainland Australia and Tasmania from the Early Cenozoic era 65 mya (million years ago) (Carpenter *et al.* 1994). The previously widespread range of Huon pine has greatly diminished along with that of other conifers since the Oligocene 34 mya (Carpenter *et al.* 1994; Jordan 1995; 1997), and trees are currently restricted to humid areas of western Tasmania. Fossil pollen assemblages that include Huon pine pollen suggest that, in addition to long term range reduction, the species has been subject to contraction and expansion related to Pleistocene glacial intervals. During glacial intervals, populations were most likely found in west coast refugia in addition to small, isolated stands of rainforest (Macphail 1979; Colhoun 1985; Colhoun and van de Geer 1986; Macphail *et al.* 1993; Macphail *et al.* 1994). Vegetation modeling based on palynological and ecological information also suggests that Tasmanian rainforest vegetation survived the last glacial interval near west coast valleys and in very small refugial areas in the east (Kirkpatrick and Fowler 1998). Estimates are that most of the cool, temperate rainforests in Tasmania are less than 12,000 years old and that Huon pine

survived glacial periods in west coast refugia and in a few small, isolated stands (Colhoun *et al.* 1996). Huon pine populations have also been subject to extensive reductions from logging since European settlement approximately 200 years ago, and it is currently classified by the IUCN (The World Conservation Union) as a vulnerable species (Oldfield *et al.* 1998). Populations have been lost due to inundation following hydroelectric construction, mining, land clearing and fire (Gibson 1986; Gibson and Brown 1991). Isozyme (Shapcott 1991b; 1997) and chloroplast DNA (Chapter 1) investigations of Huon pine show limited diversity that is perhaps indicative of a history of population bottlenecks. Chloroplast DNA also exhibited significant population structure and a geographic pattern of distribution that suggested a history of range expansion and long distance colonization.

Relatively little is known of Huon pine reproductive biology or mode of organelle inheritance. Lateral dispersal of seeds by wind is extremely limited (Shapcott 1991a); however, seeds remain buoyant and viable in water for extended periods, and it is thought that water systems provide an important means of dispersal (Shapcott *et al.* 1995; Shapcott 1997). Even along river courses, dispersal and establishment of Huon pine is quite slow (Gibson 1986). Green rosellas (*Platycercus caledonicus* Gmelin), a type of parakeet, have been observed feeding on the seed, and could be both seed predators and dispersal agents (Gibson 1986; Shapcott 1991b). Huon pine is predominantly dioecious so it is largely an obligate outcrossing species (Quinn 1982).

In this study, DNA sequence variation was analyzed at three nuclear loci to assess patterns of genetic diversity within and among seven populations of Huon pine. Two of the principal estimators of the population mutation rate per nucleotide site, $\theta = 4N_e\mu$, are

Watterson's theta (θ), based on the number of segregating sites, where N_e is the effective population size, μ is the mutation rate per generation (Watterson 1975), and π (π) is the average nucleotide pairwise difference between two individuals in a population (Nei 1987). Estimates for θ are not dependent on allele frequency, whereas those for π are based on both the number of polymorphic sites and allele frequencies. Differences in the two measures could indicate a history of selection, or effects on other demographic parameters. Diversity at nuclear loci was compared to that found in previous allozyme and chloroplast nucleotide surveys. Variation was examined across loci to compare patterns of selection, linkage disequilibrium and recombination.

METHODS AND MATERIALS

Field collection

Leaf samples were collected from eight geographic locations in Tasmania during May of 2001. A total of 333 samples were obtained from four of the eight watershed systems in which Huon pine is found (Figure 2.1). Samples were collected to represent all size classes present in stands (e.g. seedling, juvenile, mature). Size class structure of populations was previously characterized (Shapcott *et al.* 1995). A sample of 96 individuals, previously used in a survey of chloroplast DNA diversity (Chapter 1), was selected from the total to represent all geographic locations, and the size class structure of each stand. In some cases, DNA template from these samples was difficult to amplify or produced poor quality sequence after repeated attempts. These individuals were removed from the sample of 96 at each locus. Estimates of genetic diversity and population structure in this study were ultimately based on a sample of 79 individuals from seven geographic locations (Table 2.1).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from 35 to 50 mg of leaf tissue homogenized in a Bio101 FastPrep™ FP120 (Carlsbad, CA, USA) using a Qiagen (Valencia, CA, USA) DNeasy® Plant Minikit® with minor modifications of the Qiagen protocol. DNA was quantified on ethidium bromide stained gels visualized with UV light relative to standards. At the time this study was undertaken, there were two nuclear DNA sequences available in GenBank for Huon pine, *ITS2* and 18S rDNA. Two of the primers for this study were designed based on

existing sequences in GenBank, previously published primers designed for universal amplification (Strand *et al.* 1997), and degenerate primers based on multiple alignments from GenBank sequences of other species and developed with CODEHOP software (Rose *et al.* 1998). Genes that were targeted included *G3pdh* (glyceraldehyde 3-phosphate dehydrogenase), *4Cl* (4-coumarate: coenzyme A ligase), *Needly* (ortholog of *Floricaula / Leafy* genes), CCoAOMT (caffeoyl-CoA/5-hydroxyferuoyl-CoA 3-O-methyltransferase), COMT (caffeate 3-O-methyltransferase), *ITS2* (internal transcribed spacer of the nuclear ribosomal DNA) *PtaAGP6* (arabinogalactan protein in *Pinus taeda*), *Cad* (cinnamyl alcohol dehydrogenase), *Chs* (chalcone synthase) (Strand *et al.* 1997) *Adh* (alcohol dehydrogenase) (Strand *et al.* 1997) and *Tpi* (triose phosphate isomerase) (Strand *et al.* 1997). Many of these primer pair combinations produced multiple bands or otherwise poor PCR amplification in Huon pine. Only those producing bright, unambiguous PCR amplification were selected for further screening to evaluate the quality of sequence data generated using the same forward and reverse PCR primers.

Amplicons from *G3pdh*, *4Cl* and *Cad* were selected for cloning, so PCR products were run on ethidium bromide stained agarose gels (1%) in 1X TBE buffer and single bands excised and cleaned with a QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA, USA). This template was used for cloning with the TOPO TA Cloning[®] Kit and protocol (Invitrogen, Carlsbad, CA, USA). DNA template produced from cloning was sequenced with M13 forward and reverse primers. Huon pine specific primers were developed based on sequence generated with the M13 primers using Primer 3 software (Rozen and Skaletsky 2000). These primers were then screened with three to six DNA samples for evaluation of

sequence quality. The *Cad* fragment produced very poor quality sequence after several attempts and was not considered for further use. Primer pairs selected for screening with the population sample were *4Cl*-f 5' AACTGGGTCGTGCTTCATTC 3' and *4Cl*-r 5' GGCGAGAATCCCAACATTTA 3', *G3pdh*-f 5' GACACGATTCATCCTCGGT 3', and *G3pdh*-r 5' GGTTTAAACGAATTCGCCCT 3'. Primer sequence for the *ITS2* region, *ITS2*-f 5' GCATCGATGAAGAACGTAGC 3' and *ITS2*-r 5' CACGCTTCTCCAGACTACA 3', was obtained from a previous study (Sinclair *et al.* 2002). PCR primers used to generate fragments were also used as sequencing primers. The *4Cl* locus was sequenced in both directions, and the *G3pdh* and *ITS2* sites were each sequenced with the forward primer. Approximately ten percent of *G3pdh* and *ITS2* alleles were also sequenced with the reverse primer to verify trace file accuracy.

PCR reactions contained ~20 ng template DNA, 10X PCR buffer (Qiagen, Valencia, CA, USA), MgCl₂ 0.5 mM, 200 μM each dNTP, 400 μM each forward and reverse primer, 0.8 units *Taq* polymerase (Qiagen, Valencia, CA, USA), and double distilled water (ddH₂O) for a total volume of 20 μL. Thermal cycling was carried out on a MJ Research, Inc. PTC-100 cycler (Watertown, MA, USA). Parameters consisted of an initial denaturation step of 2 minutes at 95° C, followed by 35 cycles of 95° C for 15 seconds, annealing at 52° to 54° C for 30 seconds and extension at 72° C for one to two minutes depending on product size, followed by a final extension at 72° C for 10 minutes. PCR products were run on ethidium bromide stained agarose gels (1%) in 1X TBE buffer. Single bands were excised and cleaned with a QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA, USA) to prepare DNA template for sequencing reactions. Sequencing reactions consisted of ~50 to 60 ng template DNA, 2

μL Big Dye™ version 2.0 or 3.1 Ready Reaction Mix (PE Biosystems, Foster City, CA, USA), 5 pmol primer, 2.5X buffer (40 mM Tris, pH 9.0, MgCl_2 15 μM), and ddH₂O for a total volume of 20 μL . Cycling parameters were 95° C for 30 seconds, 50° C for 10 seconds and 60° C for 4 minutes for a total of 35 cycles. Unincorporated dideoxy terminators were removed by centrifugation at 900 rcf for 5 minutes in Multiscreen™ 96 well filtration plates (Millipore, Bedford, MA, USA) with Sephadex™ G-50-50 (Sigma Chemical Co., St. Louis, MO, USA). Reactions were dried in a vacuum manifold (Savant SpeedVac® AES2010, Holbrook, NY, USA) and then resuspended in 1.7 μL loading dye (5:1 deionized formamide, 25 mM EDTA, 50 mg/mL Blue Dextran). Membrane combs (The Gel Company, San Francisco, CA, USA) were used to load 0.6 μL on a 0.2 mm, 36 cm 5% Gene-PAGE PLUS 6M urea polyacrylamide gel (Amresco, Solon, OH, USA). Samples were electrophoresed in 1X TBE buffer on an ABI Prism® 377 DNA automated sequencing instrument (PE Biosystems, Foster City, CA, USA). Sequencing parameters were 1800 volts, 50 mA, 150 watts, 48° C, laser power 40.0 mW with a run time of 6 hours for a total of 7072 scans.

DNA sequence file quality was evaluated with MacPhred version 0.990722.g (Ewing and Green 1998; Ewing *et al.* 1998) and by visual inspection. Sequencing reactions were repeated for all sequences with ambiguities and for approximately ten percent of all samples to verify accuracy in base calls. Base call probability scores were greater than phred 30 for over 90 percent of samples. A phred score of 30 indicates a probability of one in one thousand that a base call is in error, or 99.9 percent accuracy. Heterozygotes appeared with a double peak in the polymorphic positions in chromatograms, and no more than two peaks appeared at any site. Two of the polymorphic sites at the 4*Cl* locus occurred in an area of

overlap of forward and reverse sequence, and chromatograms with double peaks for heterozygotes were the same in every instance. The identity of the two haplotypes at any locus was inferred through the haplotype subtraction method of Clark (1990). Trace files with phred quality scores were put into Sequencher version 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) for alignment and editing and exported in Nexus format. A query sequence from each locus was submitted to the NCBI BLAST server for nucleotide homology search (Altschul *et al.* 1990). Open reading frames (ORFs) were identified with BLAST homology and subsequent CLUSTALW (Thompson *et al.* 1994) alignments with several other angiosperm or gymnosperm sequences to confirm identity. The partial sequences from each locus are called by putative locus names. Intron and exon boundaries were determined through comparison with GenBank sequences and the presence of the preserved GT...AG intron boundaries. Sequences for unique haplotypes were deposited in GenBank (accession numbers DQ272225-DQ272235).

Analyses of genetic diversity and sequence neutrality

Estimates of inter- and intrapopulation genetic diversity and sequence neutrality were calculated using DnaSP version 4.0 (Rozas *et al.* 2003). Nucleotide diversity (π) and theta (θ) were calculated both separately and jointly for the three loci. All other diversity calculations consider the loci separately. Levels of genetic diversity were quantified by estimates of nucleotide diversity, π (π), the average number of nucleotide differences per site between two sequences (Nei 1987 equation 10.5 or 10.6); haplotype diversity (hd) (Nei 1987 equations 8.4 and 8.12 replacing $2n$ by n); average number of nucleotide differences (k) (Tajima 1983); number of segregating sites (s) and theta (θ) calculated from s (Watterson

1975), where $\theta = 4N_e\mu$ for a diploid organism, N_e is the effective population size and μ the mutation rate per site per generation (Nei 1987 equation 10.3; Tajima 1993, equation 3). Under the neutral theory of molecular evolution and the infinite sites model, θ and π both estimate the parameter $\theta = 4N_e\mu$. Tajima's D (Tajima 1989) tests the neutral prediction that θ and π are equivalent. A significant negative D value indicates that there is an excess of rare polymorphisms, and a positive D indicates that there is an excess of intermediate frequency alleles. Fu and Li's D* and F* (Fu and Li 1993) were calculated to provide additional *a priori* inference on sequence neutrality.

The minimum number of recombination events at the 4 *Cl* locus was estimated using the four-gamete test (Hudson and Kaplan 1985). Statistical significance of gametic association was established by the χ^2 method. Hudson's method (1987) was used to estimate the recombination parameter $C = 4Nr$, where N is the population size and r the recombination rate per gene, based on the variance in the number of pairwise differences, and the minimum number of recombination events (R_M). Linkage disequilibrium and significance of the association between polymorphic sites was assessed by Fisher's exact tests. Linkage disequilibrium was measured at informative sites at the 4*Cl* locus as the correlation coefficient R (Hill and Robertson 1968). The *ITS2* and *G3pdh* loci were not assessed as polymorphism occurred at a frequency of less than five percent.

Analyses of population structure

Genotypic data for 4*Cl* alleles was examined for departure from Hardy-Weinberg equilibrium. Exact tests were used to test the null hypothesis that the union of gametes is random (Weir 1996; Guo and Thompson 1992) using Genepop (Raymond and Rousset

1995b). Unbiased p -values for each population were estimated through a Markov chain method and globally by Fisher's method (Raymond and Roussett 1995a). Population differentiation both globally and between all pairwise combinations was assessed using Fisher's exact test (Goudet *et al.* 1996). F_{ST} was evaluated using the method of Hudson *et al.* (1992). The statistical significance of the relationship between the genetic distance matrix and the geographical distance matrix was evaluated by a Mantel test implemented by Isolation by Distance Web Services (IBDWS) (Jensen *et al.* 2005). Statistical significance and confidence intervals were generated using a randomization procedure with 1000 iterations. Great Circle Distances for IBDWS were based on latitude and longitude measures from populations sampled by Shapcott (1991b).

RESULTS

Genetic diversity and sequence neutrality

Seventy-nine individuals were examined at portions of three genes: *4Cl*, *G3pdh* and *ITS2*. A total of 1,154 base pairs were aligned for the three loci, and 158 alleles examined for a total of 182,332 base pairs examined. There were four segregating sites, each with two variants, and no insertions or deletions (Table 2.2). Three of the polymorphic sites were found at the *4Cl* locus, one in the *ITS2* sequence, and no variation was detected at the *G3pdh* locus. All of the variable sites were silent (introns and synonymous variation), although one site occurred in an exon region of *4Cl*. Sequence at *4Cl* consisted of one partial and one complete exon, and an intron. The majority of the *G3pdh* locus was coding sequence. The *ITS2* sequence consisted of the complete second intergenic spacer region, and a segment of 28S ribosomal RNA.

Values for nucleotide variation are summarized in Table 2.3 (s , k , π , θ , hd). Values for both π (0.00089 ± 0.0007) and θ (0.00061 ± 0.00031) were low for the three loci combined. These values indicate that approximately six to eight nucleotide sites are polymorphic per 10,000 base pairs examined in this study. When the loci were assessed individually, the highest levels of diversity were found in the *4Cl* sequence ($\pi = 0.00167 \pm 0.00014$). The Mt. Read population had the highest level of nucleotide diversity at this locus, and Condominium Creek had the lowest. This high level of diversity was an unexpected result as the Mt. Read stand has previously been identified as having minimal allozyme variation (Shapcott 1997). All of the trees assessed at the Mt. Read site were heterozygotes.

The *ITS2* locus had a very low level of variation overall with a single polymorphic site ($\pi = 0.00025 \pm 0.00010$). Variation at the *ITS2* locus was found in only four out of the seven populations. Three tests for evidence of selection in the regions examined were applied to the *4Cl* locus. There was no indication of significant deviation from the expectation of neutrality by Fu and Li's F^* and D^* (Fu and Li 1993) or Tajima's D (Tajima 1989). Values for tests for selection for each population at the *4Cl* locus are summarized in Table 2.4.

There were eight unique sequence haplotypes at the *4Cl* locus, two from *ITS2*, and a single haplotype from the *G3pdh* locus (Table 2.5). For the three loci combined, there were eight to eleven haplotypes, depending on gametic phase. Total haplotype diversity was moderately high at the *4Cl* locus ($hd = 0.595$) (Table 2.3) and quite low at *ITS2* ($hd = 0.074$). One of the two haplotypes at the *ITS2* locus occurred at very high frequency ($f = 0.9620$). The most common *4Cl* haplotype occurred at a frequency of over 60 percent. Together with the second most common haplotype at the *4Cl* locus, this accounted for almost 75 percent of the variation. The two most common *4Cl* haplotypes were also the only ones found in all seven geographic locations. The remaining six *4Cl* haplotypes occurred at very low frequency and none were found in all stands. The lowest level of haplotype diversity at the *4Cl* locus was found at Condominium Creek ($hd = 0.394$), and the highest at Riveaux Creek ($hd = 0.736$), populations separated by only 25 kilometers. The Condominium Creek stand was the smallest population examined in this study, and also appeared to have the youngest population with no tree larger than three centimeters diameter at breast height. Haplotype diversity was also high at the Mt. Read population ($hd = 0.716$). Five of the eight haplotypes occur with a frequency less than or equal to five percent. All populations except

Condominium Creek contain at least one of these low frequency haplotypes. Exact tests for Hardy–Weinberg equilibrium at the *4Cl* locus could not reject the null hypothesis of random union of gametes in individual populations or in the total sample (Table 2.6).

There were significant levels of linkage disequilibrium between polymorphic sites in the *4 CL* locus. All pairwise comparisons between the three segregating sites exhibit highly significant levels of nonrandom association ($p \leq 0.0001$ Fisher's exact test). Recombination was detected between *4Cl* positions 86 and 322, and between positions 322 and 348. An estimate of the minimum number of recombination events within each locus (R_M) was two. Estimates of $C (= 4Nr)$ per gene were 10.4, and between adjacent sites 0.0181.

Population structure

Tests for the distribution of *4Cl* genotypes across populations could not reject the null hypothesis that they are distributed equally across populations, except between Teepookana and Gordon River ($p = 0.0486$) (Table 2.7). Comparisons of genotypic differentiation between Gordon River and three other populations, Riveaux Creek, Condominium Creek and Mt. Read, although not significant at the 0.05 level, had a p -value of less than 0.1. Genotypic variation for total populations was nonsignificant (p -value of the log-likelihood G-based exact test 0.2051 ± 0.0190). The lack of differentiation between most populations indicates that genotypes are distributed in a fairly homogeneous and undifferentiated manner across the *4Cl* locus. Total F_{ST} for all populations at the *4Cl* locus is low ($F_{ST} = 0.0130$), and also indicates that populations have a very low level of genetic differentiation. In pairwise comparisons of F_{ST} (Table 2.8), there was no evidence of significant genetic structure by K_{ST} analysis. All populations exhibit very low levels of genetic structure with values ranging

from -0.0037 to 0.0896 . There was no evidence to support an isolation by distance model ($Z = 33.1854$, $r = 0.2272$, $p \geq 0.1640$ from 1000 randomizations).

DISCUSSION

Nuclear DNA variation

This study adds to the growing database of nucleotide variation in woody plant species by examining genetic diversity at three nuclear loci in Huon pine. Estimates of DNA nucleotide diversity in Huon pine were quite low overall for the combined loci ($\pi = 0.00088 \pm 0.0007$) and were consistent with a hypothesis of a series of population bottlenecks. Extensive vegetative propagation has also been noted in Huon pine in some populations, including Mt. Read (Millington *et al.* 1979; Shapcott 1997), and could contribute to the low level of diversity. Previous surveys of diversity in Huon pine at both nuclear (Shapcott 1991b; 1997) and chloroplast loci (Chapter 1) also attributed a low level of genetic variation to a history of range shifts and subsequent population bottlenecks. The level of nucleotide diversity found in this study was very similar, to that found previously in chloroplast DNA (Chapter 1). Low levels of genetic diversity attributed to range contractions and bottleneck effects are seen in a number of long-lived forest tree species. *Dacrydium cupressinum* (Hawkins and Sweet 1989; Billington 1991), *Fitzroya cupressoides* (Allnutt *et al.* 1999; Premoli *et al.* 2000; 2003) and *Wollemia nobilis* (Peakall *et al.* 2003), three Southern Hemisphere conifers, show evidence of historical range contractions and bottlenecks during Pleistocene glacial intervals. Two Northern Hemisphere conifers, *Pinus torreyana* (Ledig and Conkle 1983) and *Pinus resinosa* (Fowler and Morris 1977; Mosseler *et al.* 1992), show similar patterns of low diversity attributed to population bottlenecks. In general, higher levels of genetic diversity are associated with long-lived species with long generation times

(Hamrick *et al.* 1992). In an extensive analysis of the available allozyme literature, outbreeding woody plants had among the highest estimates of heterozygosity in plants (Hamrick and Godt 1996). Island endemics however, such as Huon pine, or species with otherwise restricted ranges, often have lower levels of allozyme polymorphism and heterozygosity and reduced allelic richness compared to widespread species (Hamrick and Godt 1990).

The average values for both π (0.00088) and θ (0.00060) in Huon pine are low compared to estimates from other species (Table 2.9). The multilocus average of nucleotide diversity in Huon pine is four to seven-fold lower than that found in either *Cryptomeria japonica* ($\pi = 0.00383$) (Kado *et al.* 2003), or *Pinus taeda* ($\pi = 0.00640$) (Brown *et al.* 2004). Similarly to the average diversity from 19 loci examined in *Pinus taeda* (Brown *et al.* 2004), variation in Huon pine was lower than that found in *Zea mays* (Tenailon *et al.* 2001), *Arabidopsis thaliana* (Shepard and Purugganan 2003) and *Drosophila* sp. (Moriyama and Powell 1996), but was greater than that found in humans (Cargill *et al.* 1999). In single locus comparisons of woody plant species, overall diversity in Huon pine was similar to that seen at the *Pal1* locus in *Pinus taeda* (Harkins 2000), and two phytochrome loci (*PHYP* and *PHYA*) in *Pinus sylvestris* (García-Gil *et al.* 2003). Nucleotide diversity in Huon pine was lower than that found at the *Pal1* locus in *Pinus sylvestris* (Dvornyk *et al.* 2002), and *BpMADS2* from *Betula pendula* (Järvinen *et al.* 2003). Diversity in Huon pine is also lower than that found in single locus estimates from a number of annual plant species such as *Arabidopsis thaliana* (*Chi*) (Kuittinen and Aguadé 2000), *Hordeum vulgare* (*Adh*)

(Cummings and Clegg 1998), *Leavenworthia stylosa* (*PgiC*) (Filatov and Charlesworth 1999), and *Solanum pimpinellifolium* (*Vac*) (Caicedo and Schaal 2004).

The range in genetic diversity across loci in Huon pine ($\pi = 0$ to 0.00160) illustrates one of the inherent difficulties in relying on estimates from a single locus to infer population history. Similar patterns of variation in the range of nucleotide diversity have been found in other plant and animal species. In two other woody plant species, *Cryptomeria japonica* (Kado *et al.* 2003) and *Pinus taeda* (Brown *et al.* 2004), there was a wide range in levels of nucleotide diversity across loci. Total variation of silent and nonsynonymous sites in *Pinus taeda* varied across a 10-fold range. In *Drosophila* sp. there was a five-fold difference (Moriyama and Powell 1996), and in maize there was a 16-fold difference in genetic diversity of loci surveyed on chromosome one (Tenaillon *et al.* 2001).

Lack of variation at the *G3pdh* locus in Huon pine may indicate that this locus is subject to strong selective forces, or has perhaps undergone a recent selective sweep (Kaplan *et al.* 1989). In contrast to the lack of variation at the *G3pdh* locus in Huon pine, *Manihot esculenta* had an average nucleotide diversity of 0.0049 at the *G3pdh* locus (Olsen 2002). Polymorphism at the *ITS2* locus in Huon pine occurred at a frequency of less than five percent. The *ITS* regions are most often used in phylogenetic studies between species because mutation rates and coalescence times are often suitable for evaluating relationships between species and genera (Baldwin *et al.* 1995). However, intraspecific polymorphism in *ITS* regions has been demonstrated in taxa such as *Zea* sp. (Buckler and Holtsford 1996), *Calycadenia* sp. (Baldwin 1993), *Mimulus guttatus* (Ritland *et al.* 1993) and *Clematis fremontii* (Learn and Schaal 1987). Rates of mutation in *ITS* regions appear to be correlated

with life form, and it appears these regions evolve more slowly in woody plants from ancient lineages than in herbaceous species of more recent origin (Baldwin *et al.* 1995).

Variation in Huon pine was highest at the *4Cl* locus, and was associated with a low level of intragenic recombination and significant levels of linkage disequilibrium. Correlation of recombination rates with levels of genetic diversity has been noted in multi-locus studies of maize (Tenaillon *et al.* 2001) and *Drosophila* (Begun and Aquadro 1992). The association of increased diversity could be related to enhanced effects of background selection and hitchhiking with recombination (Kaplan *et al.* 1989; Begun and Aquadro 1992). Estimates for recombination rates ($C = 4Nr$) may not be very reliable when sample sizes are small, or values for θ are small, as in Huon pine, as variances of these estimates could be very large (Wall 2000). Single locus estimates are also subject to stochasticity, as are measures of nucleotide diversity. The value for C in Huon pine at the *4Cl* locus was 10.4 per gene, or 0.01810 per base pair. This value is midrange for values seen in *Cryptomeria japonica* (0.0003 to 0.0335) (Kado *et al.* 2003). The Huon pine recombination rate is an order of magnitude higher than the average in *Pinus taeda* (0.00175), although diversity at the *4Cl* locus in that species was almost seven-fold higher ($\theta = 0.00594$) (Brown *et al.* 2004) than that found in Huon pine ($\theta = 0.00089$). Although variation at the *4Cl* locus in Huon pine was low, tests for evidence of selection could not reject the null hypothesis of neutral molecular evolution.

Population structure

The low observed value for overall F_{ST} (0.0130) in Huon pine is consistent with findings for other outbreeding, wind pollinated conifers (Hamrick *et al.* 1992). Forest trees

with widespread distribution typically maintain a relatively high level of genetic diversity within populations, and a relatively low level of differentiation among populations compared to many plant species (Hamrick *et al.* 1992). The relatively long life span, tall stature and greater potential for dispersal could contribute to a decrease in genetic structure of nuclear genes in woody plant species (Hamrick 2004). The low levels of genetic divergence observed in conifers (~ 0.05) imply high levels of migration among populations, and low levels of genetic drift (Hamrick *et al.* 1992). A low level of differentiation could be also be related to recent shared ancestry that results in a high level of genotypic similarity between populations. Nuclear DNA differentiation in this study is lower than that found for allozymes ($F_{ST} = 0.095$) (Shapcott 1991b; 1997), or chloroplast DNA ($G_{ST} = 0.26077$) (Chapter 1) thus indicating that populations are largely homogeneous. The single significant pairwise population comparison of genetic structure was found between Gordon River and Teepookana stands, an unexpected result since chloroplast differentiation between these two populations was extremely low. The F_{ST} value in Huon pine was also lower than typically found in other wind pollinated species or other long lived gymnosperms (e.g. *Sequoiadendron giganteum* (Fins and Libby 1982) and *Pinus longaeva* (Lee *et al.* 2002)).

In contrast to the low level of nuclear differentiation among populations, the multilocus chloroplast haplotypes previously examined showed significant genetic structure in Huon pine populations. Huon pine nuclear DNA sequence exhibited no indication of an isolation by distance pattern, although cpDNA showed marked differences between northwestern and southern populations. Population differentiation measured in organelle sequence is typically higher than that found for nuclear genes (Petit *et al.* 1993a), and could

reflect the lower effective population size and increased effects of genetic drift and stochasticity. In species with maternally inherited cpDNA, the difference in population structure between nuclear and chloroplast sequence is often quite pronounced. A review of primary data from population studies of organelle DNA variation from 183 species found that the level of population differentiation was largely impacted by the mode of genome inheritance (Petit *et al.* 2005). Consistently higher levels of differentiation are found in maternally inherited DNA than either paternally or biparentally inherited DNA (Petit *et al.* 2005). This pattern of cytonuclear differentiation is found in tree species from Africa (El Mousadik and Petit 1996), Australia (Moran 1992; Byrne and Moran 1994), Europe (Comps *et al.* 1990; Petit *et al.* 1993b; Zanetto *et al.* 1994; Demesure *et al.* 1996) and North America (Latta *et al.* 2001). Population differentiation is typically lower in species with wind dispersed pollen and paternally inherited chloroplasts (e.g. *Pinus resinosa* 0.121 (Echt *et al.* 1998); *Pinus radiata* 0.162 (Moran *et al.* 1988); *Pinus rigida* 0.039 (Guries and Ledig 1982)). In gymnosperms, differentiation is highest in maternally inherited mitochondrial DNA ($G_{ST} = 0.764$) with much smaller averages for both paternally inherited chloroplast DNA ($G_{ST} = 0.165$), and nuclear DNA ($G_{ST} = 0.116$) (Petit *et al.* 2005).

In comparisons of long-lived tree species and annual plants, there is often a much more pronounced level of genetic structure between nuclear and organellar genes in annual plants (Austerlitz *et al.* 2000). Demographic modeling and computer simulation show that the pronounced founder effects seen in annual plant species are dramatically decreased, even if there is limited pollen flow, when the long generation times of trees are considered (Austerlitz *et al.* 2000). Reproduction in long-lived species occurs over longer time periods,

and delayed reproduction with an extended juvenile phase could lead to greater opportunities for diverse genotypes to become established (Hamrick 2004). There are no published estimates of generation time in Huon pine, but observations have been made that reproductive structures do not in general appear on trees smaller in stature than 1.5 meters (Shapcott 1991a). With the very slow growth rates of Huon pine, approximately 0.3 to 2 mm in diameter per year (Francey *et al.* 1984), generation time could be 50 to 100 years or more. Older trees and their genotypes remain in and can contribute to the population gene pool along with new variation, so a number of different age classes are often present. Delayed reproduction may also lead to opportunities for subsequent colonization of open habitat by different genotypes. A higher number of founders, and founders from more than one year could become established and result in a higher level of diversity (Austerlitz *et al.* 2000). In annual plants, reproduction in the second and subsequent years can be dominated by the initial colonists, thus resulting in a lower level of diversity (Austerlitz *et al.* 1997).

Modeling indicates that founder events during recolonization after climate change may lead to a high level of genetic structure among populations, and low within population diversity (Austerlitz *et al.* 1997). Although modeling predicts a high level of structure related to founder effects, many tree species exhibit a pattern of high intra-population diversity and low among population differentiation (Hamrick *et al.* 1992). High levels of migration would also be required to explain the lack of nuclear differentiation (Ennos 1994) but these hypotheses are largely based on assumptions of equilibrium gene flow. Life history traits in addition to gene flow are required to explain the low genetic differentiation of nuclear loci exhibited by woody plant species (Austerlitz *et al.* 2000).

Conclusions and summary

The current distribution of Huon pine is concentrated primarily in the drainage systems surrounding Macquarie Harbour in the central west coast area of Tasmania (Gibson *et al.* 1991). Populations from areas surrounding Macquarie Harbour on the west coast have the highest levels of allelic richness and haplotype diversity in chloroplast DNA, and appear to be the center of chloroplast haplotype diversity for this species. Diversity in nuclear DNA sequence appears to be more homogeneous in distribution, with no evidence of significant non-random distribution. The lowest levels of diversity in nuclear DNA were found in the smallest populations. There is otherwise no apparent founder effect noted in the eastern populations as there was with chloroplast DNA.

The low level of nucleotide diversity in nuclear DNA of Huon pine could be related to a history of long-term range reduction, and contraction and expansion to refugia during periods of environmental instability. Repeated fragmentation, or constraint to refugia during glacial intervals could also lead to the loss of low frequency alleles through the effects of inbreeding and genetic drift (Nei *et al.* 1975). Both the intensity of the bottleneck, and the population growth rate after the bottleneck affect the average heterozygosity level after a bottleneck event (Nei *et al.* 1975). Huon pine has a naturally fragmented distribution, as populations are primarily limited to narrow bands along river systems. It is unlikely that there is a high rate of gene flow between populations as terrestrial seed dispersal is poor. Seeds can, however, remain buoyant in water for extended periods (Shapcott 1991b). Dispersal down waterways can be rapid (Gibson and Brown 1991), but the spread away from rivers and streams is quite slow. Huon pine is thought to be still expanding its range after the Last

Glacial Maximum, and currently occupies only a portion of its potential range (Read and Hill 1988; Gibson *et al.* 1991).

This study demonstrates the potential of nuclear DNA from multiple loci to provide insight into the pattern of genetic diversity of a Southern Hemisphere conifer. The low levels of nucleotide diversity found in this study are similar to levels of diversity observed in chloroplast DNA in Huon pine, and are consistent with a history of population bottlenecks following long-term range reduction and habitat shifts during the Pleistocene. The variation in levels of nucleotide diversity among the three loci in Huon pine is similar to patterns found in larger studies of other species, and illustrates one of the inherent problems in relying on a single locus to infer population history. The lack of variation at the *G3pdh* locus, and low level of variation at the *ITS2* locus could indicate selective forces constraining diversity at these sites, or perhaps fixation of beneficial alleles. A higher level of genetic variation at the *4Cl* locus was associated with significant linkage disequilibrium and recombination. The highest level of nucleotide diversity in this study was found at the high elevation Mt. Read site. Although this stand may have a high level of vegetative reproduction, this study showed that there were at least five genotypes present at Mt. Read. The lowest levels of diversity were found in the populations with the smallest area and size class distributions, Condominium Creek and Newall Creek. The low level of genetic structure in Huon pine for nuclear loci is consistent with levels found in other long-lived conifers, but is quite different from the significant structure found for chloroplast DNA (Chapter 1). Nuclear and organellar structure differ also in that there is no evidence to support an isolation by distance model in nuclear DNA, as was found in chloroplast DNA. Further sampling of Huon pine populations,

and evaluation of genetic diversity with both nuclear and organelle loci could provide greater resolution of historical and contemporary migration in Huon pine.

LITERATURE CITED

- Allnutt, T. R., Newton A. C., Lara A., Premoli A., Armesto J. J., Vergara R., Gardner M. (1999) Genetic variation in *Fitzroya cupressoides* (alerce), a threatened South American conifer. *Molecular Ecology* **8**, 975-987.
- Altschul, S. F., Gish W., Miller W., Meyers E. W., Lipman D. J. (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**, 403-410.
- Austerlitz, F., Jung-Miller B., Godelle B., Gouyon P.-H. (1997) Evolution of coalescence times, genetic diversity and structure during colonization. *Theoretical Population Biology* **51**, 148-164.
- Austerlitz, F., Mariette S., Machon N., Gouyon P.-H., Godelle B. (2000) Effects of colonization processes on genetic diversity: differences between annual plants and tree species. *Genetics* **154**, 1309-1321.
- Baldwin, B. G. (1993) Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: Chromosomal and morphological evolution reexamined. *American Journal of Botany* **80**, 222-238.
- Baldwin, B. G., Sanderson M.J., Porter J. M., Wojciechowski M. F., Campbell C. S., Donoghue M. J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**, 247-277.
- Begun, D. J., Aquadro C. F. (1992) Levels of naturally occurring DNA polymorphism correlate with recombination rates in *D. melanogaster*. *Nature* **356**, 519-520.

- Billington, H. L. (1991) Effect of population size on genetic variation in a dioecious conifer. *Conservation Biology* **5**, 115-119.
- Brown, G. R., Gill G. P., Kuntz R. J., Langley C. H., Neale D. B. (2004) Nucleotide diversity and linkage disequilibrium in loblolly pine. *Proceedings of the National Academy of Sciences U S A* **101**, 15255-15260.
- Buckler, E. S., Holtsford T. P. (1996) *Zea* systematics: ribosomal ITS evidence. *Molecular Biology and Evolution* **13**, 612-622.
- Byrne, M., Moran G. F. (1994) Population divergence in the chloroplast genome of *Eucalyptus nitens*. *Heredity* **73**, 18-28.
- Caicedo, A. L., Schaal B. A. (2004) Population structure and phylogeography of *Solanum pimpinellifolium* inferred from a nuclear gene. *Molecular Ecology* **13**, 1871-1882.
- Carbone, I., Liu Y.-C., Hillman B. I., Milgroom M. G. (2004) Recombination and migration of *Cryphonectria hypovirus 1* as inferred from gene genealogies and the coalescent. *Genetics* **166**, 1611-1629.
- Cargill, M., Altshuler D., Ireland J., Sklar P., Ardlie K., Patil N., Lane C., Lim E., Kalyanaraman N., Nemesh J., Ziaugra L., Friedland L., Rolfe A., Warrington J., Lipshutz R., Daley G., Lander E. (1999) Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nature Genetics* **22**, 231-238.
- Carpenter, R. J., Hill R. S., Jordan G. J. (1994) Cenozoic vegetation in Tasmania: macrofossil evidence. In: *History of Australian Vegetation. Cretaceous to Recent* (ed. Hill RS), pp. 276-298. Cambridge University Press, Cambridge.

- Clark, A. (1990) Inference of haplotypes from PCR-amplified samples of diploid populations. *Molecular Biology and Evolution* **7**, 111-122.
- Colhoun, E. A. (1985) Glaciations of the West Coast Range, Tasmania. *Quaternary Research* **24**, 39-59.
- Colhoun, E. A., Hannan D., Kiernan K. (1996) Late Wisconsin glaciation of Tasmania. *Papers and Proceedings of the Royal Society of Tasmania* **130**, 33-45.
- Colhoun, E. A., van de Geer G. (1986) Holocene to Middle Last Glaciation vegetation history at Tullabardine Dam, Western Tasmania. *Proceedings Of The Royal Society Of London* **B229**, 177-207.
- Comps, B., Thiébaud B., Paule L., Merzeau D., Letouzey J. (1990) Allozyme variability in beechwoods (*Fagus sylvatica* L.) over central Europe: spatial distribution among and within populations. *Heredity* **65**, 407-417.
- Cummings, M. P., Clegg M. T. (1998) Nucleotide sequence diversity at the alcohol dehydrogenase 1 locus in wild barley (*Hordeum vulgare* ssp. *spontaneum*): An evaluation of the background selection hypothesis. *Proceedings of the National Academy of Sciences USA* **95**, 5637-5642.
- Demesure, B., Comps B., Petit R. J. (1996) Chloroplast DNA phylogeography of the common Beech (*Fagus sylvatica* L.) in Europe. *Evolution* **50**, 2515-2520.
- Dvornyk, V., Sirviö A., Mikkonen M., Savolainen O. (2002) Low nucleotide diversity at the *pal1* locus in the widely distributed *Pinus sylvestris*. *Molecular Biology and Evolution* **19**, 179-188.

- Echt, C. S., DeVerno L. L., Anzidei M., Vendramin G. G. (1998) Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Molecular Ecology* **7**, 307-316.
- El Mousadik, A., Petit R. J. (1996) Chloroplast DNA phylogeography of the argan tree of Morocco. *Molecular Ecology* **5**, 547-555.
- Ennos, R. A. (1994) Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* **72**, 250-259.
- Ewens, W. J. (1983) The role of models in the analysis of molecular genetic data, with particular reference to restriction fragment data. In: *Statistical Analysis of DNA Sequence Data* (ed. Weir B), pp. 45-73. Marcel Dekker, New York.
- Ewing, B., Green P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* **8**, 186-194.
- Ewing, B., Hillier L., Wendl M. C., Green P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8**, 175-185.
- Filatov, D. A., Charlesworth D. (1999) DNA polymorphism, haplotype structure and balancing selection in the *Leavenworthia* PgiC locus. *Genetics* **153**, 1423-1434.
- Fins, L., Libby W. J. (1982) Population variation in *Sequoiadendron*: seed and seedling studies, vegetative propagation, and isozyme variation. *Silvae Genetica* **31**, 102 - 110.
- Fowler, D. P., Morris R. W. (1977) Genetic diversity in red pine: evidence for low genic heterozygosity. *Canadian Journal Forest Research* **7**, 343-347.

- Francey, R. J., Barbetti M., Bird T. D., Beardsmore D., Coupland W., Dolezal G. D., Farquhar G. D., Flynn R. G., Fraser P.J., Gifford R. M., Goodman H. S., Kunda B., McPhail S., Nanson G., Pearman G. I., Richards N. G., Shankey T. D., Temple R. B., Weir B. (1984) Isotopes in tree rings. C.S.I.R.O. Division of Atmospheric Technical Paper, Australia.
- Fu, Y.-X., Li W.-H. (1993) Statistical tests of neutrality of mutations. *Genetics* **133**, 693-709.
- García-Gil, M. R., Mikkonen M., Savolainen O. (2003) Nucleotide diversity at two phytochrome loci along a latitudinal cline in *Pinus sylvestris*. *Molecular Ecology* **12**, 1195-1206.
- Gibson, N. (1986) Huon pine conservation and management, p. 91. Report No. 86/3. Tasmanian Forestry Commission, Hobart, Tasmania.
- Gibson, N., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 2. Population structure and spatial pattern. *Australian Journal Of Ecology* **16**, 223-229.
- Gibson, N., Davies J., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 1. Distribution, floristics and environmental correlates. *Australian Journal Of Ecology* **16**, 215-222.
- Goudet, J., Raymond M., De Meeüs T., Rousset F. (1996) Testing differentiation in diploid populations. *Genetics* **144**, 1933-1940.
- Guo, S. W., Thompson E. A. (1992) Performing the exact test of Hardy-Weinberg proportions for multiple alleles. *Biometrics* **48**, 361-372.

- Guries, R. P., Ledig F. T. (1982) Genetic diversity and population structure in pitch pine (*Pinus rigida* Mill.). *Evolution* **36**, 387-402.
- Hamrick, J. L. (2004) Response of forest trees to global environmental changes. *Forest Ecology And Management* **197**, 323-335.
- Hamrick, J. L., Godt M. (1996) Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London B* **351**, 1291-1298.
- Hamrick, J. L., Godt M. J., Sherman - Broyles S. L. (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**, 95-124.
- Hamrick, J. L., Godt M. J. (1990) Allozyme diversity in plant species. In: *Plant population genetics, breeding, and genetic resources* (eds. Brown AH, Clegg MT, Kahler AL, Weir BS), pp. 43-82. Sinauer, Sunderland.
- Hanfstingl, U., Berry A., Kellogg E. A., Costa III J. T., Rudinger W., Ausubel F. M. (1994) Haplotype divergence coupled with lack of diversity at the *Arabidopsis thaliana* alcohol dehydrogenase locus: role for both balancing and directional selection? *Genetics* **138**, 811-828.
- Harkins, D. M. (2000) Nucleotide diversity for two genes in the lignin biosynthesis pathway of loblolly pine (*Pinus taeda* L.) M.S., North Carolina State University.
- Hawkins, B. J., Sweet G. B. (1989) Genetic variation in Rimu: An investigation using isozyme analysis. *New Zealand Journal Of Botany* **27**, 83-90.

- Hewitt, G. M. (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**, 247-276.
- Hill, W. G., Robertson A. (1968) Linkage disequilibrium in finite populations. *Theoretical and Applied Genetics* **38**, 226-231.
- Hudson, R., Boos D., Kaplan N. (1992a) A statistical test for detecting geographic subdivision. *Molecular Biology and Evolution* **9**, 138-151.
- Hudson, R., Slatkin M., Maddison W. (1992b) Estimation of levels of gene flow from sequence data. *Genetics* **132**, 583-589.
- Hudson, R. R. (1987) Estimating the recombination parameter of a finite population model without selection. *Genetical Research* **50**, 245-250.
- Hudson, R. R., Kaplan N. L. (1985) Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics* **111**, 147-164.
- Järvinen, P., Lemmetyinen J., Savolainen O., Sapanen T. (2003) DNA sequence variation in *BPMADS2* gene in two populations of *Betula pendula*. *Molecular Ecology* **12**, 369-384.
- Jensen, J., Bohonak A. J., Kelley S. T. (2005) Isolation by distance, web service. *BMC Genetics* **6**, 13.
- Jordan, G. J. (1995) Extinct conifers and conifer diversity in the Early Pleistocene of western Tasmania. *Review of Palaeobotany and Palynology* **84**, 375-387.

- Jordan, G. J. (1997) Evidence of Pleistocene plant extinctions and diversity from Regatta Point, western Tasmania, Australia. *Botanical Journal of the Linnean Society* **123**, 45-71.
- Kado, T., Yoshimaru H., Tsumura Y., Tachida H. (2003) DNA Variation in a Conifer, *Cryptomeria japonica* (Cupressaceae *sensu lato*). *Genetics* **164**, 1547-1559.
- Kaplan, N. L., Hudson R. R., Langley C. H. (1989) "Hitchhiking effect" revisited. *Genetics* **123**, 887-899.
- Karl, T. R., Trenberth K. E. (2003) Modern global climate change. *Science* **302**, 1719-1723.
- Kawabe, A., Miyashita N. T. (1999) DNA variation in the basic chitinase locus (*ChiB*) region of the wild plant *Arabidopsis thaliana*. *Genetics* **153**, 1445-1453.
- Kirkpatrick, J. B., Fowler M. (1998) Locating likely glacial forest refugia in Tasmania using palynological and ecological information to test alternative climatic models. *Biological Conservation* **85**, 171-182.
- Kremer, A., Kleinschmit J., Cottrell J., Cundall E. P., Deans J. D., Ducousso A., König A. O., Lowe A. J., Munro R. C., Petit R. J., Stephan B. R. (2002) Is there a correlation between chloroplastic and nuclear divergence, or what are the roles of history and selection on genetic diversity in European oaks? *Forest Ecology And Management* **156**, 75-87.
- Kuittinen, H., Aguade M. (2000) Nucleotide variation at the CHALCONE ISOMERASE locus in *Arabidopsis thaliana*. *Genetics* **155**, 863-872.

- Latta, R. G., Linhart Y. B., Mitton J. B. (2001) Cytonuclear disequilibrium and genetic drift in a natural population of Ponderosa pine. *Genetics* **158**, 843-850.
- Learn, G. H. J., Schaal B. A. (1987) Population subdivision for ribosomal DNA repeat variants in *Clematis fremontii*. *Evolution* **41**, 433-438.
- Ledig, F. T., Conkle M. T. (1983) Gene diversity and genetic structure in a narrow endemic, Torrey pine (*Pinus torreyana* Parry ex. Carr). *Evolution* **37**, 79-85.
- Lee, S.-W., Ledig F. T., Johnson D. R. (2002) Genetic variation at allozyme and RAPD markers in *Pinus longaeva* (Pinaceae) of the White Mountains, California. *American Journal of Botany* **89**, 566-577.
- Macphail, M. K. (1979) Vegetation and climates in Southern Tasmania since the last glaciation. *Quaternary Research* **11**, 306-341.
- Macphail, M. K., Alley N., Truswell E. M., Sluiter I. R. (1994) Early Tertiary vegetation: evidence from spores and pollen. In: *History of Australian Vegetation. Cretaceous to Recent* (ed. Hill RS), pp. 189-261. Cambridge University Press, Cambridge.
- Macphail, M. K., Jordan G. J., Hill R. S. (1993) Key periods in the evolution of the Cenozoic flora and vegetation of Tasmania I. the Early-Middle Pleistocene. *Australian Journal Of Botany* **41**, 673-707.
- Millington, R. J., Jones R., Brown D., Vernon B. (1979) Huon pine - endangered? Environmental Studies Occasional Paper No. 9. University of Tasmania, Hobart.

- Miyashita, N., Innan H., Terauchi R. (1996) Intra- and interspecific variation in the alcohol dehydrogenase locus region of wild plants *Arabis gemmifera* and *Arabidopsis thaliana*. *Molecular Biology and Evolution* **13**, 433-436.
- Moran, G. F. (1992) Patterns of genetic diversity in an Australian tree species. *New Forests* **6**, 49-66.
- Moran, G. F., Bell J. C., Eldridge K. G. (1988) The genetic structure and the conservation of the five natural populations of *Pinus radiata*. *Canadian Journal of Forest Research* **18**, 506-514.
- Moriyama, E. N., Powell J. R. (1996) Intraspecific nuclear DNA variation in *Drosophila*. *Molecular Biology and Evolution* **13**, 261-277.
- Mosseler, A., Egger K. N., Hughes G. A. (1992) Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markers. *Canadian Journal of Forest Research* **22**, 1332-1337.
- Nei, M. (1987) *Molecular Evolutionary Genetics* Columbia University Press, New York.
- Nei, M., Maruyama T., Chakraborty M. (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**, 1-10.
- Oldfield, S., Lusty C., MacKinven A. (1998) *The World List of Threatened Trees*. World Conservation Press, Cambridge.
- Olsen, K. M. (2002) Population history of *Manihot esculenta* (Euphorbiaceae) inferred from nuclear DNA sequences. *Molecular Ecology* **11**, 901-911.

- Peakall, R., Ebert D., Scott L., Meagher P., Offord C. (2003) Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). *Molecular Ecology* **12**, 2331-2343.
- Peterson, M. J. (1990) Distribution and conservation of Huon pine, p. 50. Forestry Commission Tasmania, Tasmania.
- Petit, R. J., Duminil J., Fineschi S., Hampe A., Salvini D., Vendramin G. G. (2005) Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molecular Ecology*, 689-701.
- Petit, R. J., Kremer A., Wagner D. B. (1993a) Finite island model for organelle and nuclear genes in plants. *Heredity* **71**, 630-641.
- Petit, R. J., Kremer A., Wagner D. B. (1993b) Geographic structure of chloroplast DNA polymorphisms in European oaks. *Theoretical and Applied Genetics* **87**, 122-128.
- Premoli, A. C., Kitzberger T., Veblen T. T. (2000) Isozyme variation and recent biogeographical history of the long - lived conifer *Fitzroya cupressoides*. *Journal of Biogeography* **27**, 251-260.
- Premoli, A. C., Vergara R., Souto C. P., Lara A., Newton A. C. (2003) Lowland valleys shelter the ancient conifer *Fitzroya cupressoides* in the Central Depression of southern Chile. *Journal of the Royal Society of New Zealand* **33**, 623-631.
- Quinn, C. J. (1982) Taxonomy of *Dacrydium* Sol. ex Lamb. amend de Lamb. (Podocarpaceae). *Australian Journal Of Botany* **30**, 311-320.

- Raymond, M., Rousset F. (1995a) An exact test for population differentiation. *Evolution* **49**, 1280-1283.
- Raymond, M., Rousset F. (1995b) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**, 248-249.
- Read, J., Hill R. S. (1988) Comparative responses to temperature of the major canopy species of Tasmanian cool temperate rainforest and their ecological significance. I. Foliar frost resistance. *Australian Journal Of Botany* **36**, 131-143.
- Ritland, C. E., Ritland K., Straus N. A. (1993) Variation in the ribosomal internal transcribed spacers (ITS1 and ITS2) among eight taxa of the *Mimulus guttatus* species complex. *Molecular Biology and Evolution* **10**, 1273-1288.
- Rose, T. M., Schultz E. R., Henikoff J. G., Pietrokovski S., McCallum C. M., Henikoff S. (1998) Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly-related sequences. *Nucleic Acids Research* **26**, 1628-1635.
- Rozas, J., Sanchez-DelBarrio J. C., Messeguer X., Rozas R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496-2497.
- Rozen, S., Skaletsky H. (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds. Krawetz S., Misener S), pp. 365-386. Humana Press, Totowa, NJ.
- Schaal, B. A., Olsen K. M. (2000) Gene genealogies and population variation in plants. *Proceedings of the National Academy of Sciences USA* **97**, 7024-7029.

- Shapcott, A. (1991a) Dispersal and establishment of Huon pine (*Lagarostrobos franklinii*). *Papers and Proceedings of the Royal Society of Tasmania* **125**, 17-26.
- Shapcott, A. (1991b) Studies in the population biology and genetic variation of Huon pine (*Lagarostrobos franklinii*), p. 84. National Rainforest Conservation Program and Department of Parks, Wildlife and Heritage, Hobart, Hobart, Tasmania.
- Shapcott, A. (1997) Population genetics of the long-lived Huon pine *Lagarostrobos franklinii*: An endemic Tasmanian temperate rainforest tree. *Biological Conservation* **80**, 169-179.
- Shapcott, A., Brown M. J., Kirkpatrick J. B., Reid J. B. (1995) Stand structure, reproductive activity and sex expression in Huon pine (*Lagarostrobos franklinii* (Hook L.) Quinn.). *Journal of Biogeography* **22**, 1035-1045.
- Shepard, K. A., Purugganan M. D. (2003) Molecular Population Genetics of the Arabidopsis CLAVATA2 Region: The Genomic Scale of Variation and Selection in a Selfing Species. *Genetics* **163**, 1083-1095.
- Sinclair, W. T., Mill R. R., Gardner M. F., Woltz P., Jaffré T., Preston J., Hollingsworth M. L., Ponge A., Möller M. (2002) Evolutionary relationships of the New Caledonian heterotrophic conifer, *Parasitaxus usta* (Podocarpaceae), inferred from chloroplast *trnL-F* intron/spacer and nuclear rDNA ITS2 sequences. *Plant Systematics and Evolution* **233**, 79-104.
- Strand, A. E., Leebens-Mack J., Milligan B. G. (1997) Nuclear DNA-based markers for plant evolutionary biology. *Molecular Ecology* **6**, 113-118.

- Tajima, F. (1983) Evolutionary relationships of DNA sequences in finite populations. *Genetics* **105**, 437-460.
- Tajima, F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphisms. *Genetics* **123**, 585-595.
- Tajima, F. (1993) Measurement of DNA polymorphism. In: *Mechanisms of Molecular Evolution* (eds. Takahata N, Clark AG). Sinauer Associates, Sunderland, MA.
- Templeton, A. R. (1998) Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7**, 381-397.
- Tenaillon, M. I., Sawkins M. C., Long A. D., Gaut R. L., Doebley J. F., Gaut B. S. (2001) Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.). *Proceedings of the National Academy of Sciences U S A* **98**, 9161-9166.
- Thompson, J. D., Higgins D. G., Gibson T. J. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.
- Wall, J. D. (2000) A comparison of estimates of the population recombination rate. *Molecular Biology and Evolution* **17**, 156-163.
- Wang, W., Thornton K., Berry A., Long M. (2002) Nucleotide variation along the *Drosophila melanogaster* fourth chromosome. *Science* **295**, 134-137.

- Watterson, G. (1975) On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology* **7**, 188-193.
- Webb, T., Bartlein P. J. (1992) Global changes during the last 3 million years: climatic controls and biotic response. *Annual Review of Ecology and Systematics* **23**, 141-173.
- Weir, B. S. (1996) *Genetic Data Analysis II* Sinauer, Sunderland, Massachusetts.
- Zanetto, A., Roussel G., Kremer A. (1994) Geographic variation of inter-specific differentiation between *Quercus robur* L., *Quercus petraea* (Matt.) Liebl. I. Monolocus patterns of variation. *Forest Genetics* **1**, 111-123.

Table 2.1. Sample size, dbh range and watershed for Huon pine populations.

Location	Abbr.	<i>n</i>	dbh range	Watershed
		79		
Riveaux Creek	RC	11	16.5 cm–130 cm	Huon
Condominium Creek	CC	11	< 1 cm–3 cm	Huon
Teepookana	TK	13	6.5 cm–136 cm	King
Newall Creek	NC	12	6 cm–70 cm	King
Stanley River	SR	12	< 1cm–102 cm	Pieman
Mt. Read	MR	10	2 cm–80 cm	Pieman
Gordon River	GR	10	10 cm–178 cm	Gordon

Abbr., abbreviation of population name; *n*, sample size; dbh, diameter at breast height (1.4 meters)

Table 2.2. Summary of polymorphic sites and haplotype configurations at the *4Cl* and *ITS2* loci.

Locus	<i>4Cl</i>			<i>ITS2</i>	
Position in locus	86	322	348		139
Consensus	T	C	G		A
Site type	t	v	v		t
Character type	i	i	i		u
Haplotypes (f)					
C1 (97)	.	.	C	I1 (152)	A
C2 (21)	C	.	.	I2 (6)	G
C3 (8)	C	A	.		
C4 (5)	C	A	C		
C5 (14)	C	.	C		
C6 (6)	.	A	C		
C7 (2)	.	A	.		
C8 (5)	.	.	.		

bp, base pairs; v, transversion; t, transition;

i, phylogenetically informative; u, phylogenetically uninformative; (f), number of occurrences of haplotype.

The *G3pdh* locus produced a single haplotype so is not shown.

Table 2.3. Estimates of DNA sequence and haplotype diversity at the *4Cl* and *ITS2* loci.

Population	<i>s</i>	<i>k</i>	π (<i>SD</i>)	θ (<i>SD</i>)	<i>hd</i> (<i>SD</i>)
Total for 3 combined loci	4	1.033	0.00089 (0.0007)	0.00061 (0.00031)	na
<i>4cl</i>					
Total populations	3	0.959	0.00167 (0.0004)	0.00093 (0.00054)	0.595 (0.042)
Riveaux Creek	3	1.251	0.00218 (0.00032)	0.00142 (0.00083)	0.736 (0.090)
Condominium Creek	2	0.615	0.00107 (0.00034)	0.00096 (0.00068)	0.394 (0.119)
Teepookana	3	1.049	0.00183 (0.00036)	0.00137 (0.00079)	0.646 (0.094)
Newall Creek	3	0.764	0.00133 (0.00028)	0.00140 (0.00081)	0.591 (0.104)
Stanley River	3	0.920	0.00160 (0.00035)	0.00140 (0.00081)	0.594 (0.105)
Mt. Read	3	1.305	0.00227 (0.00031)	0.00147 (0.00085)	0.716 (0.086)
Gordon River	3	0.763	0.00133 (0.00036)	0.00147 (0.00085)	0.500 (0.122)
<i>ITS2</i>					
Total populations	1	0.074	0.00025 (0.00010)	0.00061 (0.00061)	0.074 (0.028)
Riveaux Creek	1	0.091	0.00031 (0.00028)	0.00095 (0.00095)	0.091 (0.081)
Condominium Creek	0	0	0	0	0
Teepookana	1	0.077	0.00027 (0.00024)	0.00091 (0.00091)	0.077 (0.070)
Newall Creek	1	0.159	0.00055 (0.00033)	0.00093 (0.00093)	0.159 (0.094)
Stanley River	1	0.159	0.00055 (0.00033)	0.00061 (0.00061)	0.159 (0.094)
Mt. Read	0	0	0	0	0
Gordon River	0	0	0	0	0

s, segregating sites (Watterson 1975); *k*, average number of pair-wise differences between the *i*th and the *j*th DNA sequences (Tajima 1983, equation A3); π , nucleotide diversity, the average number of nucleotide differences per site between two sequences (Nei 1987, equations 10.5 or 10.6); θ , an estimation of the mean population mutation rate per site from *s*, segregating nucleotide sites and *n*, sample size (Watterson 1975); (*SD*), standard deviation; *hd*, haplotype diversity (Nei 1987, equations 8.4 and 8.12)

Table 2.4. Tests for departure from neutral evolution at the *4Cl* locus.

Population	Fu and Li's D*	Fu and Li's F*	Tajima's D
Total populations	0.79012 ns	1.13812 ns	1.33281 ns
Riveaux Creek	0.99199 ns	1.25596 ns	1.34556 ns
Condominium Creek	0.85062 ns	0.79650 ns	0.27286 ns
Teepookana	0.96844 ns	1.07096 ns	0.82111 ns
Newall Creek	-0.18894 ns	-0.19630 ns	-0.12171 ns
Stanley River	0.97946 ns	0.93198 ns	0.36608 ns
Mt. Read	1.00649 ns	1.29760 ns	1.45182 ns
Gordon River	-0.12425 ns	-0.18574 ns	0.26042 ns

Fu and Li's D* (1993); Fu and Li's F* (1993); Tajima's D (Tajima 1989)
 ns, not significant ($p > 0.05$)

Table 2.5. Haplotype frequencies at the *4Cl* and *ITS2* loci.

Population	RC	CC	TK	NC	SR	MR	GR	Total (f)
<i>4Cl</i>								
C1	0.5000	0.7727	0.5769	0.6250	0.6250	0.5000	0.7000	0.6139
C2	0.0909	0.0909	0.1538	0.1667	0.1667	0.1500	0.1000	0.1329
C3	0.0909	0	0.0769	0	0.0417	0.1500	0	0.0506
C4	0	0	0	0	0.0417	0.0500	0.1500	0.0316
C5	0.1364	0.1364	0.0385	0.0833	0.0833	0.1500	0	0.0886
C6	0.0455	0	0.1154	0.0417	0.0417	0	0	0.0379
C7	0.0455	0	0.0385	0	0	0	0	0.0127
C8	0.0909	0	0	0.0833	0	0	0.05	0.0316
<i>ITS2</i>								
I1	0.0455	0	0.0385	0.0833	0.0833	0	0	0.0380
I2	0.9445	1	0.9615	0.9617	0.9167	1	1	0.9620

(f) = total frequency

(RC, Riveaux Creek; CC, Condominium Creek; GR, Gordon River TK, Teepookana; NC, Newall Creek; MR, Mt. Read; SR, Stanley River)

Table 2.6. Hardy-Weinberg exact tests by the Markov chain method for the 4*Cl* locus.

Population	<i>p</i> -value (SE)
Riveaux Creek	0.3206 (0.0163)
Condominium Creek	0.4395 (0.0056)
Teepookana	0.7088 (0.0138)
Newall Creek	0.1501 (0.0091)
Stanley River	0.1084 (0.0111)
Mt. Read	0.1640 (0.0074)
Gordon River	0.1607 (0.0072)
All populations (Fisher's method)	$\chi^2 = 20.1171$ df = 14

Hardy Weinberg exact tests (Guo and Thompson 1992; Weir 1996); 1000 iterations of Markov chain
df = degrees of freedom

Table 2.7. Genotypic differentiation between all pairwise populations. For each comparison χ^2 value is above the diagonal and p -value below the diagonal.

	RC	CC	TK	NC	SR	MR	GR
RC	—	2.919	0.975	0.734	0.869	0.840	5.018
CC	0.23234	—	3.946	1.689	0.741	4.114	5.422
TK	0.61422	0.13903	—	1.932	0.401	2.568	6.048
NC	0.69290	0.42983	0.38057	—	0.475	4.119	3.054
SR	0.64766	0.69051	0.81814	0.78843	—	0.477	1.971
MR	0.65701	0.12781	0.27697	0.12750	0.78783	—	5.273
GR	0.08134	0.06648	0.0486*	0.21722	0.37332	0.07162	—

Df (degrees of freedom) = 2

p -value of the probability test (Fisher's exact test) using the method of Raymond and Rousset (1995) * $p \leq 0.05$

(RC, Riveaux Creek; CC, Condominium Creek; TK, Teepookana; NC, Newall Creek; GR, Gordon River; MR, Mt. Read; SR, Stanley River)

Table 2.8. Pairwise comparisons of values of F_{ST} (above diagonal) and Nm (below diagonal) between Huon pine populations.

Total $F_{ST} = 0.0130$ $Nm = 18.93$							
	CC	RC	TK	NC	GR	MR	SR
CC	—	0.05142	0.02482	-0.04032	-0.00375	0.08966	-0.00807
RC	4.61	—	0.00017	0.01824	0.05313	-0.01883	0.01165
TK	9.82	1488.76	—	0.00021	-0.02439	0.01896	-0.02415
NC	-6.45	13.45	1196.26	—	-0.01281	0.05202	-0.02367
GR	-66.90	4.45	-10.50	-19.77	—	0.06828	-0.03060
MR	2.54	-13.52	12.94	4.56	3.41	—	0.01087
SR	-31.25	21.21	-10.60	-10.81	-8.40	22.76	—

(RC, Riveaux Creek; CC, Condominium Creek; TK, Teepookana; NC, Newall Creek; GR, Gordon River; MR, Mt. Read; SR, Stanley River)

F_{ST} and Nm (Hudson) equation 9 and calculated as equations 5 and 6 in Hudson *et al.* (1992b) by DnaSP; Statistical significance of population differentiation determined with Hudson's K_{ST} (1000 Monte Carlo simulations) (Hudson *et al.* 1992a)

Table 2.9. Estimates of nucleotide diversity in plant and animal species.

Species	Locus	Nucleotide diversity	Reference
<i>Lagarostrobos franklinii</i>	3 loci	0.00088 (π)	This study
<i>Pinus taeda</i>	19 loci	0.00658 (θ) S	(Brown <i>et al.</i> 2004)
<i>Cryptomeria japonica</i>	7 loci	0.00383 (π) S	Kado <i>et al.</i> 2003
<i>Pinus sylvestris</i>	<i>Pal1</i>	0.00490 (π) S	(Dvornyk <i>et al.</i> 2002)
<i>Pinus taeda</i>	<i>Cad</i> and <i>Pal1</i>	0.0069 (π) <i>Cad</i> 0.0007 (π) <i>Pal</i>	(Harkins 2000)
<i>Pinus sylvestris</i>	<i>PHYP</i> and <i>PHYA</i>	0.0024 (π) S	(Garcia-Gil <i>et al.</i> 2003)
<i>Betula pendula</i>	<i>BpMADS2</i>	0.0043 (π) S	(Järvinen <i>et al.</i> 2003)
<i>Arabidopsis thaliana</i>	11 loci	0.0063 - 0.0579 (π)	(Shepard & Purugganan 2003)
<i>Arabidopsis thaliana</i>	<i>Chi</i>	0.004 (π)	(Kuittinen & Aguadé 2000)
<i>Zea mays</i> ssp. <i>mays</i>	21 loci	0.0096 (θ)	(Tenaillon <i>et al.</i> 2001)
<i>Manihot esculenta</i>	<i>G3pdh</i>	0.0049 (π)	(Olsen 2002)
<i>Hordeum vulgare</i>	<i>Adh1</i> <i>Adh3</i>	0.00182 (π) 0.0219 (π)	(Cummings & Clegg 1998)
<i>Leavenworthia stylosa</i>	<i>PgiC</i>	0.051 (π)	(Filatov & Charlesworth 1999)
<i>Solanum pimpinellifolium</i>	<i>Vac</i>	0.0064 (π)	(Caicedo & Schaal 2004)
<i>Drosophila</i> sp.	41 loci	0.0040 (π)	(Moriyama & Powell 1996)
<i>Homo sapiens</i>	106 loci	0.0005 (π)	(Cargill <i>et al.</i> 1999)

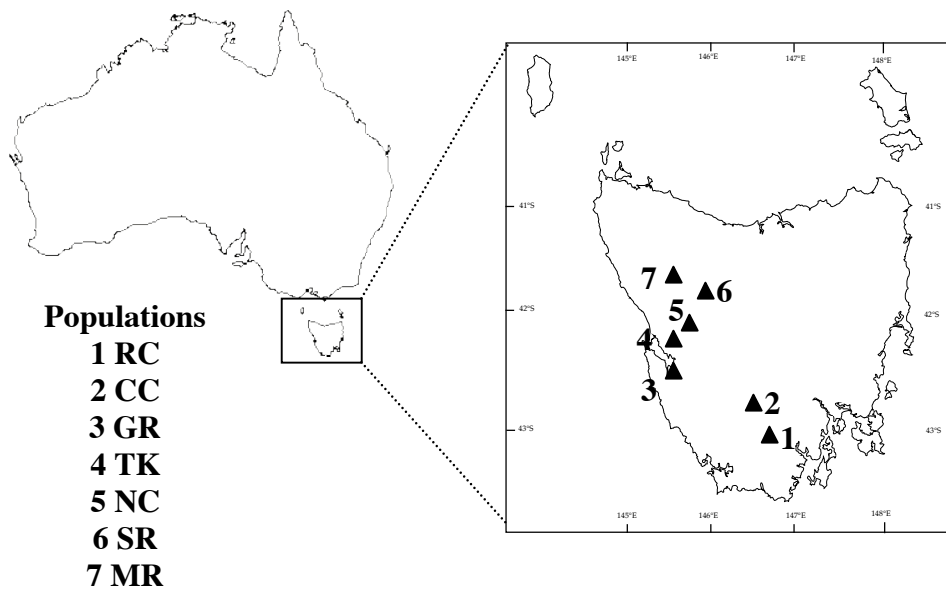


Figure 2.1. Huon pine sampling areas in Tasmania, Australia. Triangles (▲) represent sampling areas. (RC, Riveaux Creek; CC, Condominium Creek; GR, Gordon River; TK, Teepookana; NC, Newall Creek; MR, Mt. Read; SR, Stanley River)

CHAPTER 3

RAPD MARKERS REVEAL CLONAL DIVERSITY WITHIN A HIGH ELEVATION STAND OF HUON PINE (*LAGAROSTROBOS FRANKLINII*) AT MT. READ, TASMANIA

ABSTRACT

Multilocus genotypes based on RAPD markers (random amplified polymorphic DNA) were generated to investigate genetic diversity in a putative clonal population of Huon pine (*Lagarostrobos franklinii*) at Mt. Read, Tasmania. This population may reproduce entirely by vegetative reproduction as only male trees have been observed in the stand. Sixty-three DNA samples were analyzed from Mt. Read, and their genetic diversity compared to 33 reference DNA samples from a wide geographic area. Twelve random decanucleotide primers amplified a total of 35 alleles. Only three of the alleles (8.6%) from the Mt. Read population were polymorphic, in contrast to 18 (51.4%) polymorphic alleles in the reference population. Gene diversity (Nei 1973) at Mt. Read (0.0316 ± 0.1061) was six-fold lower than that found in the reference sample (0.1973 ± 0.2112). Four unique DNA fingerprints were generated in the 63 samples from Mt. Read and were not found in the other populations. The 33 samples from the reference population each had a unique genotype. The average similarity index for the Mt. Read population was 0.9738. The spatial distribution of the Mt. Read genotypes is consistent with multiple ramets of genets, as two genotypes were found only in the upper half and two found only in the lower half of the stand. Previous investigations of genetic diversity show minimal or no variation with allozymes and chloroplast loci, but a higher level of diversity in nuclear DNA sequence. The low level of genetic variation in the Mt. Read population could be related to limited gene flow due to geographic isolation, and extensive vegetative reproduction in a harsh environment.

INTRODUCTION

A disjunct population of Huon pine (*Lagarostrobos franklinii* (Hook f.) Quinn), a Southern Hemisphere podocarp, forms a sexually and genetically distinct population (Shapcott 1991b) at Mt. Read, Tasmania, Australia. Although Huon pine is predominately dioecious (Quinn 1982), extant trees at this site produce only pollen cones (Shapcott 1991b; 1997). Given the presence of only male trees, extensive vegetative reproduction may be the only means by which this stand has propagated and survived on Mt. Read (Millington *et al.* 1979; Shapcott 1991b). Accurate assessment of genetic diversity and relatedness is essential for the evaluation of long-term evolutionary potential of forest tree populations. Previous surveys of diversity in Huon pine with allozymes (Shapcott 1991b; 1997), and with chloroplast (Chapter 1) and nuclear DNA sequences (Chapter 2) discovered low levels of diversity consistent with a long-term history of range reduction and Pleistocene habitat shifts (Carpenter *et al.* 1994; Jordan 1995; 1997). Results from Mt. Read exhibit minimal allozyme variation and no chloroplast variation. Nuclear DNA sequence revealed a relatively high level of variation at the *4Cl* locus (4-coumarate coenzyme: A ligase) at the Mt. Read site that was associated with a high level of recombination (Chapter 2).

Delineation of genetically distinct individuals and assessment of levels of genetic diversity are complicated in species that reproduce both sexually and asexually. In plant species that reproduce vegetatively, unique genotypes (genets) may be comprised of multiple morphological units (ramets) (Harper 1977). Although individual ramets, or clones, can be identified through examination of root systems (Cook 1983; Moriguchi *et al.* 2001), this method is impractical in large organisms. In many species, the extent of genetic variation and

spatial distribution of genets has been assayed by a variety of molecular markers including RAPD (random amplified polymorphic DNA) (Smith *et al.* 1992; Hsiao and Rieseberg 1994; Tuskan *et al.* 1996), AFLP (amplified fragment length polymorphism (Pornon *et al.* 2000; Suyama *et al.* 2000; Moriguchi *et al.* 2001), allozymes (Parker and Hamrick 1992, Parks and Werth 1993), microsatellites (Zhou *et al.* 2003) and ISSR (intersimple sequence repeats) (Tani *et al.* 1998). Each type of genetic marker may have inherent bias in location, extent of variation, and selection. In *Pinus pumila*, a comparison of methods to determine closely related genotypes showed that PCR-based markers provided greater resolution when comparing isozyme, RAPD and ISSR (Tani *et al.* 1998). This was primarily attributed to the ability to generate larger numbers of polymorphic alleles.

RAPD markers (Welsh and McClelland 1990; Williams *et al.* 1990) provide a highly efficient method for generating arbitrarily primed PCR fragments. A single random primer can often generate a large number of loci and detect multiple polymorphic alleles. This technique has been used in a wide variety of studies including assessment of genetic diversity and population structure (Russell *et al.* 1993), genotyping individuals (Hsiao and Rieseberg 1994), estimation of spatial distribution of genets (Tani *et al.* 1998) and construction of genomic maps (Rieseberg *et al.* 1993). Since the primers are arbitrary random sequences, they are not likely to have bias for coding or noncoding regions, and may be more representative of the genome than some other methods (Lynch and Milligan 1994). In species with large genomes, such as conifers (Wakamiya *et al.* 1993; Hizume *et al.* 2001), RAPD markers most likely represent non-coding sequences (Lynch and Milligan 1994), although

organelle DNA has been reported in some studies of RAPD diversity (Lorenz *et al.* 1994; Aagard *et al.* 1995; 1998).

There are also limitations intrinsic to using RAPD markers. RAPD loci are dominant, so only the recessive homozygote can be reliably scored. Since the dominant/null heterozygote cannot be distinguished from the dominant/dominant homozygote, estimates of genetic diversity may be biased. The recessive homozygote, or null/null can be distinguished, so allele frequencies and gene diversity can be estimated under some assumptions including Hardy Weinberg equilibrium (Lynch and Milligan 1994). Reported problems with amplification of RAPD markers, such as the appearance of intermittent bands, reaction failures (Ellsworth *et al.* 1993), or difficulty with reproducibility (Yu and Pauls 1992; Jones *et al.* 1997) can be addressed by meticulous adherence to protocol, and selection of robust primers.

The primary objective of this study was to develop an estimate of the extent of genotypic diversity present in RAPD markers in the Huon pine population at Mt. Read. The level of diversity comprising the Mt. Read stand was compared to estimates based on a wide geographic sample of Huon pine. Results from the RAPD variation were also compared to results in previous investigations of genetic variation at Mt. Read with allozyme loci, and chloroplast and nuclear DNA sequences.

MATERIALS AND METHODS

Study species and field collections

Huon pine is a Tasmanian endemic conifer found in eight watershed systems of southwestern Tasmania (Peterson 1990; Gibson and Brown 1991). Distribution of Huon pine is naturally fragmented as populations are largely restricted to narrow gallery forests along river systems, although the species is also sometimes found on moist slopes, in association with sclerophyllous vegetation, or in large non-riverine stands (Gibson 1986; Gibson *et al.* 1991). Huon pine has also been heavily exploited since European settlement and is currently classified as a vulnerable species by the IUCN (The World Conservation Union) (Oldfield *et al.* 1998).

The Mt. Read population of Huon pine forms a subalpine, rainforest community at 950 to 1000 meters in a species rarely found above 500 meters (Peterson 1990). Conditions in this high elevation population are harsh, but pollen cores from a cirque lake below the population demonstrate the presence of this stand since at least the late glacial period 14,000 to 10,000 years B.P. (before present) (Anker *et al.* 2001). The Mt. Read Huon pine population has been used extensively in dendrochronological analyses to infer the last 4,000 years of climate history for Tasmania, and the Tasman Sea area (Cook *et al.* 1991; 1992; 2000). Huon pine at elevations above 700 meters exhibit a strong growth response to seasonal temperatures (Buckley *et al.* 1997). Tree ring analyses for this period show that there has been anomalous warming in Tasmania and surrounding areas since 1965. Many of

the extant trees in the Mt. Read population are over 1000 years old (Cook *et al.* 1996) in a species that can live as long as 2,000 years (Francey *et al.* 1984).

Huon pine is the dominant vegetation in the stand found on the southern slope of Mt. Read above Lake Johnston. The growth of trees at this site differs from that observed at seven other Huon pine populations in that the orientation of branches is largely horizontal, and there is obvious layering. Trees were growing in obvious clumps at this site, and samples were collected when possible at least five meters from the nearest cluster. Where multiple stems were present, samples were collected from the largest stem in a cluster. Collections were made in five transects across the slope beginning at the top of the stand and ending at the base near Lake Johnston. Sampling in the middle of the stand occurred at approximately 10 to 15 meter intervals. Two of the transects were completed on cleared areas marked by grid coordinates (Transect 3 2400S 575E and Transect 4 L65600N 378340E).

A total of 63 foliage samples were collected at Mt. Read and all of these were included in this analysis (Figure 3.1 and Table 3.1). Nineteen of the 63 trees sampled at the Mt. Read population were tagged for climatological or growth studies (Appendix H). An additional collection of 33 DNA samples representing seven geographically widespread populations previously assayed for nucleotide diversity at multiple chloroplast and nuclear loci (Chapter 1; 2) were included in this study to provide a baseline reference for RAPD marker diversity in Huon pine.

DNA extraction and RAPD marker analysis

Total genomic DNA was extracted from 35 to 50 mg of leaf tissue homogenized in a Bio101 FastPrep™ FP120 (Carlsbad, CA, USA) using a Qiagen (Valencia, CA, USA) DNeasy®

Plant Minikit[®] with minor modifications of the Qiagen protocol. DNA template was quantified on ethidium bromide stained gels visualized with UV light and diluted to 1 ng per μL .

Four DNA templates were screened with 120 random sequence decanucleotide primers. Twelve primers were selected for this survey based on amplification of clear, unambiguous bands. Primer names were based on Operon Technologies, Inc., system of alphanumeric designations. RAPD bands were identified by the primer name followed by the size in base pairs of the fragment. Reactions were carried out in a total volume of 15 μl in 96 well Falcon microtitre plates (Becton Dickinson and Co., Oxnard, CA, USA) and consisted of 5 ng template DNA, 15 μg BSA (non-acetylated bovine serum albumin) (New England Biolabs, Inc., Beverly, MA, USA), 1.5 μL of 10 X PCR Buffer (containing 100 mM Tris-HCl, 500 mM KCl, 1% Triton-X 100, final pH 9.0), 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.5 mM MgCl_2 , primer 30 ng (Sigma-Genosys, The Woodlands, TX, USA or MWG Biotech, High Point, NC, USA), *Taq* polymerase 0.8 U, (Qiagen, Valencia, CA, USA or Roche, Indianapolis, IN, USA) and 0.94 μL of ddH₂O. Reactions were covered by 50 μL of mineral oil (Bristol – Meyers Squibb, NY, NY, USA) prior to thermal cycling. PCR amplification was carried out in a 96 well plate thermal cycler (MJ Research PT-100 thermal cycler (Bio - Rad Laboratories, Waltham, MA, USA) with parameters slightly modified from Williams *et al.* (1990). Reactions consisted of 41 cycles of denaturation at 92° C x 1 minute; 35° C annealing for 1 minute; and extension at 72° C for 2 minutes. Prior to gel electrophoresis 2 μL loading dye (0.25% bromophenol blue, 40% sucrose and 0.002% ethidium bromide) was incorporated into the reaction mixture via centrifugation. Fragments

were separated by gel analysis on 1.5% agarose gels in 1X TBE buffer and electrophoresed 5 hours at approximately 150V in a 20 cm x 40 cm horizontal rig (Model A3, Owl Scientific, Cambridge, MA, USA). PCR amplification products were visualized and recorded with an Eagle Eye TM video imaging system (Stratagen, La Jolla, CA, USA). Size of fragments was determined by comparison with either a 100 base pair or 1Kb Plus ladder (Invitrogen, Carlsbad, CA, USA). In order to verify reproducibility, all PCR amplification was repeated one or more times using the same DNA template and reaction parameters on a separate agarose gel. Only bands that appeared on both runs with a frequency greater than five percent, and with intense, unambiguous amplification were included in this analysis. There was negligible difference between runs and this consisted primarily of differences in intensity of amplification, although in a few cases there were bands present on one gel that were not present in another. These bands were not scored or considered in data analysis.

Data analysis

The following assumptions were made regarding the RAPD bands: each marker corresponds to only one amplified product (i.e. there is no comigration of fragments with the same molecular weight) and each locus can have only two states, present or absent. Mendelian inheritance was assumed when polymorphic bands were repeatable and found in multiple individuals. RAPD bands were scored as band present (1), band absent (2), or (0) for missing data because of failed or ambiguous amplification. A binary matrix was created based on the phenotype of presence or absence for each band scored. When a band was present, the assumption was made that the individual was either a dominant homozygote or

heterozygote. The null allele is assumed to be due to a mutation at the primer binding site and when an individual was assessed as null, which indicated a recessive homozygote.

Genetic diversity within the Mt. Read population and the reference sample was quantified in three ways. The percentages of within-population polymorphic loci, allele frequencies and Nei's gene diversity, or heterozygosity (Nei 1973) were estimated using POPGENE version 1.31 (Yeh and Boyle 1997). Allele frequency and gene diversity were estimated assuming Hardy – Weinberg equilibrium and independence of nuclear loci. Under Hardy Weinberg equilibrium, allele frequencies are denoted by p and $q = 1 - p$ so in a segregating population the expected frequencies are $P_{AA} = p^2$, $P_{BB} = q^2$ and $P_{AB} = 2pq$. RAPD markers are dominant, so only the recessive homozygote can be directly estimated (q^2), and frequencies of dominant homozygotes and heterozygotes inferred. Although in Shapcott's (1997) allozyme survey only 8 out of 32 populations conformed to Hardy Weinberg expectations at the *Idh* locus, the Mt. Read population, due to low diversity in the stand, was in Hardy Weinberg equilibrium.

The binary matrix was used as an input file for the software program RAPDPLOT version 3.0 (Black 1995) to estimate the similarity index between each pairwise combination in both the Mt. Read, and the reference population based on the number of shared fragments (Lynch 1990). The similarity index provides an estimation of similarity, or the fraction of bands shared by individuals x and y (Nei and Li 1985), based on the formula $(S) S_{xy} = \frac{2n_{xy}}{n_x + n_y}$ where n_{xy} is the number of bands shared by individuals x and y ; n_x is total bands in isolate x ; and n_y the total bands in isolate y . Average similarity values were also calculated for the Mt. Read stand, the reference sample, and each of the five transects from Mt. Read. Statistical

significance was evaluated by a t-test. A similarity matrix for Mt. Read genotypes was used as an infile for the Phylip 3.5c (Felsenstein 1989) Neighbor program for cluster analysis. A phenogram for the Mt. Read genotypes was plotted using the Phylip Drawtree program.

Unique genotypes were identified based on total marker configuration, and isolates were assigned identity based on genotype. The total number of genotypes from the Mt. Read stand was compared to that found in the reference sample. Genotypic diversity in the Mt. Read population was also characterized by a measure of clonal diversity (Ellstrand and Roose 1987) (G), a measure of the number of clones detected divided by the sample size (N).

RESULTS

RAPD banding and polymorphism

Twelve RAPD primers amplified unambiguous alleles at 35 loci in Huon pine DNA samples (Table 3.2). The amplified bands were stable and produced predictable banding patterns across multiple reactions. The number of bands per primer ranged from one to six, with an average of approximately three per primer. There were a total of 18 polymorphic bands (51.4%) in both the total 96 samples, and the 33 reference samples. Variation was low in the Mt. Read population, where only 29 of the total 35 bands appeared, and three of the RAPD loci (8.6%) were polymorphic. Seventeen bands were monomorphic in all populations. Four bands that were polymorphic in the reference sample were absent in the Mt. Read stand. An additional two monomorphic bands were also not present in the Mt. Read stand but present in the reference sample. The three polymorphic bands in the Mt. Read stand were also polymorphic in the reference population.

Allele frequencies and gene diversity are shown for polymorphic alleles that occurred with a frequency of at least five per cent in the Mt. Read, reference, and total samples in Table 3.3. As seen in Table 3.3, the three polymorphic bands at Mt. Read were more frequently absent, or in the homozygous recessive state, than present. Gene diversity for the total sample of 96 was 0.1153 (\pm 0.1383). When considered separately, the reference population value for gene diversity was higher (0.1973 \pm 0.2112), and the value for Mt. Read was much lower (0.0316 \pm 0.1061).

Genotypes

The RAPD analysis produced a total of 37 genotypes. There were four unique multilocus genotypes from 63 samples at Mt. Read (Table 3.4). Based on the formula $P = [S^2 + (1 - S)^2]^{n/s}$ (Weising *et al.* 1995), where P is the probability that all markers are shared between a pairwise comparison, S is the average similarity index and n is the average number of markers per sample, the probability of two isolates from the Mt. Read population sharing all bands by chance was 6.68×10^{-26} , or virtually zero. The G value for Mt. Read was 0.06 (4/63). Each of the 33 samples in the wide geographic sample had a unique genotype. None of the Mt. Read genotypes were found outside of the population, and none of the reference sample genotypes were found at Mt. Read. Samples that exhibited the same alleles at all 29 loci were assumed to be members of the same genet. The most common genotype at Mt. Read, Genotype 1, was found only in the upper half of the stand, and the second most common, Genotype 4, only in the bottom half of the stand (Table 3.5). Both of these common genotypes were found over twice as often as the other two genotypes. The phylogenetic relationship among the Mt. Read genotypes measured by the distance in similarity indices is shown in Figure 3.2. Similarity index values ranged from 1 to 0.9455 at the Mt. Read stand, indicating that pairwise comparisons share approximately 95 to 100 per cent of bands. The average value was 0.9738 ± 0.0204 , which indicates that within the stand approximately 97 per cent of markers are the same. Similarity index values between genotypes are shown in Table 3.6. Average similarity values were compared between transects at Mt. Read and values ranged from 0.9455 to 0.9825 with no significant difference between transects. The

range of similarity values for the reference population was 0.9831 to 0.8148, with a mean value of 0.9021 ± 0.0396 .

DISCUSSION

Diversity in Huon pine

This study examined the utility of RAPD markers for characterizing intraspecific diversity in a putative clonal stand of Huon pine. Reproducibility and the robust nature of the markers selected was demonstrated. Results indicate a very low level of diversity within the Huon pine population at Mt. Read ($h = 0.0316$) compared to the wide geographic sample ($h = 0.1973$), with a six-fold difference between the two. Several estimates from other tree species assayed with RAPD markers show diversity levels similar to, or higher than that of the wide geographic sample. In three pine species (*Pinus attenuata*, *P. muricata*, *P. radiata*) the pooled estimate of heterozygosity was 0.15 (Wu *et al.* 1998). Aspen seedlings (*Populus tremuloides*) have a weighted average heterozygosity ranging from 0.18 to 0.24 (Stevens *et al.* 1999). In Pawpaw (*Asimina triloba*) $h = 0.25$ (Huang *et al.* 2000), and in Sugar Maple (*Acer saccharum*) $h = 0.288$ to 0.300 (Gunter *et al.* 2000). Long-lived forest tree species typically possess high levels of genetic variation when compared to herbaceous species; however, endemic species typically have only 30 percent of the variation found in widespread species (Hamrick *et al.* 1992). Based on Huon pine's endemism, and history of long-term range reduction and habitat shifts through the Pleistocene (Carpenter *et al.* 1994; Jordan 1995; 1997), a low level of genetic diversity related to probable population bottlenecks is a reasonable expectation for this species.

Small population size and isolation at Mt. Read make this stand vulnerable to loss of genetic diversity through genetic drift and inbreeding (Hartl and Clark 1989; Barrett and

Kohn 1991; Ellstrand and Elam 1993). Geographic isolation of this stand could also limit gene flow and have a major impact on diversity. The Mt. Read Huon pine population is approximately 25 kilometers or more from any other Huon pine stand. Dispersal and establishment of Huon pine is quite slow even in favorable environments (Gibson 1986), but the altitude of Mt. Read represents an extreme environment for this species. Lateral dispersal of seeds by wind is extremely limited as seeds have no adaptations to enhance wind-borne spread (Shapcott 1991a). Water systems are thought to be the primary mode of dispersal in Huon pine, as seeds remain buoyant and viable in water for extended periods (Shapcott *et al.* 1995; Shapcott 1997). One possibility for long distance gene flow in Huon pine by routes other than along waterways may be animal dispersal by Green rosellas (*Platycercus caledonicus* Gmelin), a type of parakeet. These birds have been observed feeding on the seed and could be both seed predators and long range dispersal agents (Gibson 1986; Shapcott 1991b).

Clonal variation at Mt. Read

Low levels of genetic diversity at Mt. Read may also result from extensive vegetative reproduction in this population. Huon pine has a demonstrated capacity to reproduce asexually through layering, production of suckers, coppicing and growth from cuttings (Millington *et al.* 1979). RAPD markers showed that there were at least four genets comprising the Mt. Read stand, with distribution of ramets spatially clustered. The unlikely probability of observing multiple copies of each of the four genotypes ($P = 6.68 \times 10^{-26}$) leads to the conclusion that reproduction is largely, or entirely vegetative. The similarity index computes a number based on shared bands between zero and one. A value of 1 indicates a

genetically identical morphological unit, or clone. The average similarity index between individuals at the Mt. Read stand was 0.9738, but there were many values indicating identical genotypes or ramets of one genet. *Calamagrostis porteri*, a highly clonal plant, displayed similarity indices comparable to Mt. Read Huon pine with values of 0.935 to 1.000 (Esselman *et al.* 1999). The mean value for Mt. Read is higher than that observed between either parents and offspring (0.90), or siblings (0.93) in *Cryptomeria japonica* (Moriguchi *et al.* 2001). In *Rhododendron ferrugineum*, a clonal Ericaceae, similarity values were high (0.82, 0.79 and 0.84) in two studies at different locations (Escaravage *et al.* 1998; Pornon *et al.* 2000) indicating a high degree of relatedness between genets (Weising *et al.* 1995; Busemeyer *et al.* 1997). In aspen, seedling stands displayed lower levels of similarity (0.755) than mature populations (0.941) that appear to be of largely clonal origin (Tuskan *et al.* 1996).

The Mt. Read population of Huon pine appears to be dominated by a few large clones that could be very old, considering the age of the stand and individual trees within the stand. Asexual populations can be quite large and long-lived, for example in the fungus *Armillaria bulbosa* (Smith *et al.* 1992), which covers at least 15 hectares, and is estimated to be at least 1,500 years old. *Cryptomeria japonica* forms several high altitude stands with a single detected genotype, while lower elevation stands exhibit much higher diversity (Taira *et al.* 1997; Moriguchi *et al.* 2001). In a review of studies of clonality in plants, 25 out of 27 species revealed multiple clones both within and among populations (Ellstrand and Roose 1987). The average G value (Genotypes divided by number sampled) for the clonal plants was 0.17, but ranged from 0.002 to 1.00 (all have the same genotype) (Ellstrand and Roose

1987). Clonal diversity in the Mt. Read trees is much lower than typically found in clonal populations ($G = 0.0635$) (Ellstrand and Roose 1987) indicating a high ratio of ramets to genets. The putative clonal nature of the Mt. Read trees means that there may have been limited gene flow into this stand in historical time, further impacting genetic diversity. Even if seeds continue to arrive, clonal populations typically exhibit low recruitment from seeds (Eriksson 1989; Pornon *et al.* 1997). Growth of ramets and intraspecific competition between clones leads to the potential for a few clones to persist and control the site (Soane and Watkinson 1979).

Although counterintuitive, asexually reproducing species in general have the same level of genetic diversity as species that reproduce sexually (Ellstrand and Roose 1987; Hamrick and Godt 1989). Tree species that reproduce both sexually and asexually typically have higher levels of diversity (Hamrick *et al.* 1992), perhaps related to long lifespan and decreased susceptibility to stochastic events. In long-lived species, the extended juvenile period provides the opportunity for recruitment and establishment of multiple genotypes in newly available habitat. If clones persist at a site for hundreds or thousands of years, even with minimal recruitment genetic diversity can be high. The low level of diversity at Mt. Read could also be related to intense selective forces in a harsh environment. The high altitude stand is unusual in a species usually found in lowland temperate rainforests. The genets at Mt. Read could be the product of adaptation to microsite variability and diversifying selection in a harsh environment (Antonovics and Ellstrand 1984; Ellstrand and Roose 1987). There are many instances of plant adaptation to local conditions documented in the literature (Antonovics *et al.* 1971; Brady *et al.* 2005). Vegetative reproduction could be

an advantage for plants with reproductive barriers such as inbreeding, or seedling survival under harsh conditions, and could provide an advantage in rapid colonization of a new habitat at a lower reproductive cost (Silander *et al.* 1985). Limited genetic diversity provides limited potential for shuffling of genetic material and adaptation to altered environmental conditions.

Gender expression at Mt. Read

Genetic variation at Mt. Read is also affected by the absence of female cone production. Although no female plants have been observed at Mt. Read in recent times, there is the possibility that at some time in the past 10,000 years there has been sexual reproduction of Huon pine at Mt. Read. The Tasmanian climate was warmer and precipitation totals higher during the early Holocene 6,000 to 8,000 years B.P. (Macphail 1979; Markgraf *et al.* 1995). Conditions during the Holocene warming may have been more favorable for development of female trees. There is a possibility that the Mt. Read Huon pine produce only male cones (Shapcott *et al.* 1995; Shapcott 1997) as a response to stress. There appears to be a strong tendency in dioecious plants to have a higher ratio of male plants under stressful or harsh conditions (Freeman *et al.* 1976; Lloyd and Bawa 1984). Conifers also exhibit a strong tendency to form a higher proportion of male cones in stressful environments (Floyd 1983; Whitham & Mopper 1985). Gender ratios in several tree species were shown to change with the successional status of a site (Falinski 1980). Male trees were more prevalent in earlier successional stages. Female trees predominated in the oldest stages of vegetation development, and equal ratios were found on mid-successional communities.

Plant taxa can also in some instances alter their gender expression in response to environmental variables (Freeman *et al.* 1980). In a study of constancy of gender expression in Red Maple (*Acer rubrum*), bisexual trees were variable in gender expression through time (Primack and McCall 1986). Although predominately dioecious, four percent of Huon pine greater than one meter in height were monoecious, or bisexual (Shapcott *et al.* 1995). Although the overall ratio of male to female trees was not significantly different, ten out of twenty-five populations examined for gender expression were found to have significant differences in the ratio of males to females (Shapcott *et al.* 1995). The gender ratio in stands did not appear to be associated with climate, disturbance regime, or the structure and density of the stand (Shapcott *et al.* 1995). Climate was, however, associated with floristic variation in species composition of Huon pine communities (Gibson *et al.* 1991), and there was a significant correlation between proportion of female trees and sites with similar composition of species (Shapcott *et al.* 1995). Shapcott (1993) also found that populations with higher proportions of female trees were more genetically similar, and suggested that gender differences may have been influenced by selection related to ecological differences more than by environmental differences that induce differences in gender expression (Shapcott *et al.* 1995).

Mutation and marker diversity

Genetic variation is the fundamental basis for evolutionary change and is affected by mutation and selection as well as migration, drift and mating systems (Hartl and Clark 1997). The evolutionary histories of allozyme, chloroplast and nuclear DNA sequence, and RAPD markers are affected by differences in mutation and selection. As a result, they could yield

different results in Huon pine, yet all contribute to the understanding of diversity in this species. RAPD primers are based on arbitrary random sequences that in theory should represent coding and noncoding regions equally (Lynch and Milligan 1994), and experience fewer constraints on mutation in noncoding regions (Begun and Aquadro 1993; Baruffi *et al.* 1995). Allozyme loci are protein coding regions, and as such could be subject to sampling bias from strong selective forces. Chloroplast sequence is often highly conserved compared to nuclear or mitochondrial DNA (Wolfe *et al.* 1987). Chloroplast sequence is also subject to increased effects of genetic drift and stochasticity due to lower effective population size (Petit *et al.* 1993). A wide range in levels of nucleotide diversity across loci is observed in large-scale studies in both plant and animal species. A 10-fold range in diversity was shown in *Pinus taeda* (Brown *et al.* 2004), and a five-fold range in *Drosophila* sp. (Moriyama and Powell 1996). Genes on a single chromosome in maize exhibited a 16-fold difference in genetic diversity (Tenailon *et al.* 2001). Absence of variation at nuclear loci may indicate strong selective forces or a recent selective sweep (Kaplan *et al.* 1989). The estimate produced by this study probably underestimates the true extent of diversity in this population due to limited variation. Ellstrand and Roose (1987) found there were significant correlations between the number of genotypes discovered and the number of characters scored. PCR based markers could provide a more complete picture of the variation present in the Mt. Read population of Huon pine with the application of more loci.

The level of genetic variation at RAPD loci in this study is higher than that shown with allozymes (Shapcott 1991b; 1997), based on measures of heterozygosity. Overall heterozygosity for allozymes in Huon pine was $h = 0.043$ and at Mt. Read $h = .011$, a third to

a quarter of the level shown in RAPD markers. There are relatively few loci available for isozyme studies, so resolution in patterns of clonal diversity is often low compared to PCR-based markers. Allozymes at Mt. Read showed that 90 per cent of individuals were the same (Shapcott 1991b). Low allozyme diversity in Huon pine consisted of both a low level of polymorphism and minimal allelic richness (Shapcott 1991b). The lowest level of resolution at Mt. Read was shown with chloroplast nucleotide diversity (Chapter 1). Nucleotide diversity, or π (π), the average number of nucleotide differences per site between two sequences (Nei 1987) was assayed in eight Huon pine populations. Diversity was low compared to other plant taxa ($\pi = 0.00093$), but there was no diversity observed in 14 samples from Mt. Read, where a single haplotype was found.

Although nucleotide diversity at three nuclear loci in seven populations estimated a similar low level of diversity ($\pi = 0.00089$) (Chapter 2), nuclear variation at Mt. Read for the *4Cl* locus was the highest reported in seven populations ($\pi = 0.0027$). Variation at the *4Cl* locus was associated with a low level of intragenic recombination, and significant levels of linkage disequilibrium. Two other nuclear loci showed no variation at Mt. Read. Diversity at the *4Cl* locus delineated five haplotypes at the Mt. Read stand compared to the four haplotypes delineated by RAPD analysis. The 14 trees used in both the chloroplast and nuclear diversity surveys were included in the RAPD assay at Mt. Read, so these known isolates have been sampled by three different methods. Correlation between haplotypes for *4Cl* and RAPD is negligible ($r = 0.1704$).

Although not included in calculation of diversity for this study, the appearance of a number of bands with frequencies of less than five per cent, suggests the occurrence of

somatic mutation in Huon pine. Many of the low frequency bands appeared in only one or two DNA samples, but were completely repeatable. This phenomenon has been reported in other studies of plant species that reproduce vegetatively (Schaal and Learn 1988; Capossela *et al.* 1992; Hsiao and Rieseberg 1994; Tuskan *et al.* 1996; Tani *et al.* 1998; Moriguchi *et al.* 2001). For example, differences were detected in offspring from obligate asexually reproducing *Taraxacum officinale* (King and Schaal 1990). Other studies report no evidence of somatic mutation (Smith *et al.* 1992; Cloutier *et al.* 2003). It is possible, especially considering the great age of the trees at Mt. Read, that this low frequency RAPD variation in Huon pine is due to somatic mutation. Somatic mutations increase diversity both within individuals and within populations (Whitham and Slobodchikoff 1981; Silander 1985), especially in long-lived species (Antolin and Strobeck 1985; Ledig 1986). Somatic mutation should be a more prominent process in long-lived tree species as mutations can accumulate through time (Antolin and Strobeck 1985). Long-lived plants may also have higher rates of mutation per generation than annual plant species. Mutation rates in mangrove trees are 25 times higher than in short lived buckwheat or barley (Kiekowski and Godfrey 1989). One hypothesis to explain higher levels of somatic mutation in trees is that it provides an evolutionary advantage in long-lived plants exposed to many pathogens or predators over a lifetime (Whitham *et al.* 1984). The meristem system of plants is also conducive to the development of somatic mutations (Kiekowski and Godfrey 1989). As meristematic tissue gives rise to reproductive as well as somatic tissue, mutations in meristematic tissue can be incorporated into the germ line and be entirely heritable (Whitham and Slobodchikoff 1981). Mutations that occur in somatic tissues could be confined to one or a few individuals (King and Schaal 1990).

Many studies have reported differences in the number of clones detected using different marker systems. The assumption is usually made that the most accurate estimate comes from the marker with the highest resolution (Esselman *et al.* 1999). This is not a testable hypothesis unless the number of genets is known objectively. In a study of the herbaceous species *Saxifraga cernua*, the difference between the number of clones detected with RAPD and AFLP markers (Kjølner *et al.* 2004) was explained by sampling error and stochastic variation in assortment of alleles associated with recombination. In Red pine (*Pinus resinosa*), surveys showed little to no allozyme variation or RAPD variation (Fowler and Morris 1977; Mosseler *et al.* 1992), but diversity was present in chloroplast microsatellites (Echt *et al.* 1998). A perennial grass, *Calamagrostis porteri*, exhibited similar disparity in the number of genets resolved using different molecular markers (Esselman *et al.* 1999), with allozymes revealing the fewest. There are also examples of studies that show concordance in results with PCR-based markers and allozymes. Resolution of genets and ramets was similar with different molecular markers in both *Malacothamnus fasciculatus* var. *nesioticus* (Swenson *et al.* 1995) and *Haloragodendron lucasii* (Sydes and Peakall 1998).

Summary

This study provides important information for long-term conservation efforts of the distinct Huon pine population at Mt. Read, Tasmania. RAPD markers reveal exceptionally low diversity at Mt. Read in comparison to a wide geographic sample of Huon pine. Only four genotypes were found at Mt. Read, and each of these genotypes was present in multiple individuals. The genotypes were spatially clustered, with two of them found only in the upper portion, and two genotypes only in the lower portion of the population. Recruitment to this

population of Huon pine appears to be low, either related to isolation and limited gene flow, or to clonal spread of initial colonists with subsequent low recruitment. Further resolution of the genetic diversity at this stand will require the application of many more markers such as RAPD, or AFLP as genetic diversity in the Mt. Read stand is low.

LITERATURE CITED

- Aagaard, J. E., Krutovskii K. V., Strauss S. H. (1998) RAPD markers of mitochondrial origin exhibit lower population diversity and higher differentiation than RAPDs of nuclear origin in Douglas-fir. *Molecular Ecology* **7**, 801-812.
- Aagaard, J. E., Vollmer S. S., Sorensen F. C., Strauss S. H. (1995) Mitochondrial DNA products among RAPD profiles are frequent and strongly differentiated between races of Douglas fir. *Molecular Ecology* **4**, 441-447.
- Anker, S. A., Colhoun E. A., Barton C. E., Peterson M., Barbetti M. (2001) Holocene vegetation and paleoclimatic and paleomagnetic history from Lake Johnston, Tasmania. *Quaternary Research* **56**, 264-274.
- Antolin, M. F., Strobeck C. (1985) The population genetics of somatic mutation in plants. *The American Naturalist* **126**, 52-62.
- Antonovics, J., Bradshaw A. D., Turner R. G. (1971) Heavy metal tolerance in plants. *Advances in Ecological Research* **7**, 1-85.
- Antonovics, J., Ellstrand N. C. (1984) Experimental studies of the evolutionary significance of sexual reproduction I. a test of the frequency-dependent selection hypothesis. *Evolution* **38**, 103-115.
- Barrett, S. C., Kohn J. R. (1991) Genetic and evolutionary consequences of small population size in plants: implications for conservation. In: *Genetics and Conservation of Rare Plants* (eds. Falk DA, Holsinger KE), pp. 3-30. Oxford University Press, Oxford.

- Baruffi, L., Damiani G., Guglielmino C. R., Bandi C., Malacrida A. R., Gasperi G. (1995) Polymorphism within and between populations of *Ceratitidis capitata*: comparison between RAPD and multilocus enzyme electrophoresis data. *Heredity* **74**, 425-437.
- Begun, D. J., Aquadro C. F. (1993) African and North American populations of *Drosophila melanogaster* are very different at the DNA level. *Nature* **365**, 548-549.
- Black, W. C. (1995) Statistical analysis of arbitrarily primed PCR patterns in molecular taxonomic studies. In: *Molecular Biology, Species Diagnostics Protocols: PCR and Other Nucleic Acid Methods* (ed. Clapp CL), pp. 39-55. Humana Press, Totowa, N. J.
- Brady, K. U., Kruckeberg A. R., Bradshaw Jr. H. D. (2005) Evolutionary ecology of plant adaptation to serpentine soils. *Annual Review of Ecology and Systematics* **36**, 243-266.
- Brown, G. R., Gill G. P., Kuntz R. J., Langley C. H., Neale D. B. (2004) Nucleotide diversity and linkage disequilibrium in loblolly pine. *Proceedings of the National Academy of Sciences U S A* **101**, 15255-15260.
- Buckley, B. M., Cook E. R., Peterson M. J., Barbetti M. (1997) A changing temperature response with elevation for *Lagarostrobos franklinii* in Tasmania, Australia. *Climatic Change* **36**, 477-498.
- Busemeyer, D. T., Pelikan S., Kennedy R., Rogstad H. (1997) Genetic diversity of Phillipine *Rubus molucannus* L. (Rosaceae) populations examined with VNTR DNA probes. *Journal of Tropical Ecology* **14**, 867-884.

- Capossela, M., Silander Jr. J., Jansen R., Bergen B., Talbot D. (1992) Nuclear ribosomal DNA variation among ramets and genets of white clover. *Evolution* **46**, 1240-1247.
- Carpenter, R. J., Hill R. S., Jordan G. J. (1994) Cenozoic vegetation in Tasmania: macrofossil evidence. In: *History of Australian Vegetation. Cretaceous to Recent* (ed. Hill RS), pp. 276-298. Cambridge University Press, Cambridge.
- Cloutier, D., Rioux D., Beaulieu J., Schoen D. (2003) Somatic stability of microsatellite loci in Eastern white pine, *Pinus strobus* L. *Heredity* **90**, 247-252.
- Cook, E. R., Bird T., Peterson M., Barbetti M., Buckley B., D'Arrigo R., Francey R. (1992) Climatic change over the last millenium in Tasmania reconstructed from tree rings. *The Holocene* **2**, 205-217.
- Cook, E. R., Bird T. D., Peterson M., Barbetti M., Buckley B. M., D'Arrigo R. D., Francey R. J., Tans P. (1991) Climatic change in Tasmania inferred from a 1089 - year tree - ring chronology of Huon pine. *Science* **253**, 1266-1268.
- Cook, E. R., Buckley B. M., D'Arrigo R. D., Peterson M. J. (2000) Warm-season temperatures since 1600 BC reconstructed from Tasmanian tree rings and their relationship to large-scale sea surface temperature anomalies. *Climate Dynamics* **16**, 79-91.
- Cook, E. R., Francey R. J., Buckley B. M., D'Arrigo, R. D. (1996) Recent increases in Tasmanian Huon pine ring widths from a sub-alpine stand: natural climate variability, CO² fertilisation, or greenhouse warming? *Papers and Proceedings of the Royal Society of Tasmania*, **130**, 65-72.

- Cook, R. E. (1983) Clonal plant populations. *American Scientist* **71**, 244-253.
- Echt, C. S., DeVerno L. L., Anzidei M., Vendramin G. G. (1998) Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Molecular Ecology* **7**, 307-316.
- Ellstrand, N. C., Elam D. R. (1993) Population genetic consequences of small population-size - implications for plant conservation. *Annual Review Of Ecology And Systematics* **24**, 217-242.
- Ellstrand, N. C., Roose M. L. (1987) Patterns of genotypic diversity in clonal plant species. *American Journal of Botany* **74**, 123-131.
- Ellsworth, D. L., Rittenhouse K. D., Honeycutt R. L. (1993) Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14**, 214-217.
- Eriksson, O. (1989) Seedling dynamics and life histories in clonal plants. *Oikos* **55**, 231-238.
- Escaravage, N., Questiau S., Pornon A., Doche B., Taberlet P. (1998) Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology* **7**, 975-982.
- Esselman, E. J., Jianqiang L., Crawford D. J., Windus J. L., Wolfe A. D. (1999) Clonal diversity in the rare *Calamagrostis porteri* ssp. *insperata* (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. *Molecular Ecology* **8**, 443-451.
- Falinski, J. B. (1980) Vegetation dynamics and sex structure of the populations of pioneer dioecious woody plants. *Vegetatio* **43**, 23-38.

- Felsenstein, J. (1989) PHYLIP (Phylogeny Inference Package) version 3.2, *Cladistics*, 5, 164-166.
- Floyd, M. E. (1983) Dioecy in five *Pinus edulis* populations in the Southwestern United States. *American Midland Naturalist* **110**, 405-411.
- Fowler, D. P., Morris R. W. (1977) Genetic diversity in red pine: evidence for low genic heterozygosity. *Canadian Journal of Forest Research* **7**, 343-347.
- Francey, R. J., Barbetti M., Bird T. D., Beardsmore D., Coupland W., Dolezal G. D., Farquhar G. D., Flynn R. G., Fraser P.J., Gifford R. M., Goodman H. S., Kunda B., McPhail S., Nanson G., Pearman G. I., Richards N. G., Shankey T. D., Temple R. B., Weir B. (1984) Isotopes in tree rings. C.S.I.R.O. Division of Atmospheric Technical Paper No. 4, Australia.
- Freeman, D. C., Harper K. T., Charnov E. L. (1980) Sex change in plants: Old and new observations and new hypotheses. *Oecologia* **47**, 222-232.
- Freeman, D. C., Klikoff L. G., Harper K. T. (1976) Differential resource utilization by the sexes of dioecious plants. *Science* **193**, 597-599.
- Gibson, N. (1986) Huon pine conservation and management, p. 91. Tasmanian Forestry Commission, Hobart, Tasmania.
- Gibson, N., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 2. Population structure and spatial pattern. *Australian Journal Of Ecology* **16**, 223-229.

- Gibson, N., Davies J., Brown M. (1991) The ecology of *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae) in Tasmania. 1. Distribution, floristics and environmental correlates. *Australian Journal Of Ecology* **16**, 215-222.
- Gunter, L. E., Tuskan G. A., Gunderson C. A., Norby R. J. (2000) Genetic variation and spatial structure in sugar maple (*Acer saccharum* Marsh.) and implications for predicted global-scale environmental change. *Global Change Biology* **6**, 335-344.
- Hamrick, J. L., Godt M. J. (1989) Allozyme diversity in plant species. In: *Plant Population Genetics, Breeding and Genetic Variation in Plants* (eds. Brown AH, Clegg MT, Kahler AL, Weir BS). Sinauer Associates Inc., Sunderland, MA.
- Hamrick, J. L., Godt M. J., Sherman - Broyles S. L. (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**, 95-124.
- Harper, J. L. (1977) *Population Biology of Plants* Academic Press, London.
- Hartl, D. L., Clark A. G. (1997) *Principles of Population Genetics* Sinauer, Sunderland, MA.
- Hizume, M., Kondo T., Shibata F., Ishizuka R. (2001) Flow cytometric determination of genome size in the Taxodiaceae, Cupressaceae *sensu stricto* and Sciadopityaceae. *Cytologia* **66**, 307-311.
- Hsiao, J.-Y., Rieseberg L. H. (1994) Population genetic structure of *Yushania niitakayamensis* (Bambusoideae, Poaceae) in Taiwan. *Molecular Ecology* **3**, 201-208.
- Huang, H., Layne D. R., Kubisiak T. L. (2000) RAPD inheritance and diversity in Pawpaw (*Asimina triloba*). *Journal of the American Society of Horticultural Science* **125**, 454-459.

- Jones, C., Edwards K., Castaglione S., Winfield M., Sala F., van de Wiel C., Bredemeijer G., Vosman B., Matthes M., Daly A., Brettschneider R., Bettini P., Buiatti M., Maestri E., Malcevski A., Marmioli N., Aert R., Volckaert G., Rueda J., Linacero R., Vazquez A., Karp A. (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding* **3**, 381-390.
- Jordan, G. J. (1995) Extinct conifers and conifer diversity in the Early Pleistocene of western Tasmania. *Review of Palaeobotany and Palynology* **84**, 375-387.
- Jordan, G. J. (1997) Evidence of Pleistocene plant extinctions and diversity from Regatta Point, western Tasmania, Australia. *Botanical Journal of the Linnean Society* **123**, 45-71.
- Kaplan, N. L., Hudson R. R., Langley C. H. (1989) "Hitchhiking effect" revisited. *Genetics* **123**, 887-899.
- Kiekowski, E. J., Godfrey P. J. (1989) Ageing and mutation in plants. *Nature* **340**, 389-391.
- King, L. M., Schaal B. A. (1990) Genotypic variation within asexual lineages of *Taraxacum officinale*. *Proceedings of the National Academy of Sciences U S A* **87**, 998-1002.
- Kjølnner, S., Sæstad S. M., Taberlet P., Brochmann C. (2004) Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: clonal diversity in *Saxifraga cernua*. *Molecular Ecology* **13**, 81-86.

- Ledig, F. T. (1986) Heterozygosity, heterosis and fitness in outbreeding plants. In: *Conservation Biology: the Science of Scarcity and Diversity* (ed. Soulé ME), pp. 77-90. Sinauer Associates, Inc., New York.
- Lloyd, D. G., Bawa K. S. (1984) Modification of the gender of seed plants in varying conditions. *Evolutionary Biology* **17**, 255-338.
- Lorenz, M., Weihe A., Borner T. (1994) DNA fragments of organellar origin in random amplified polymorphic DNA (RAPD) patterns of sugar beet (*Beta vulgaris* L.). *Theoretical and Applied Genetics* **88**, 775-779.
- Lynch, M. (1990) The similarity index and DNA fingerprinting. *Molecular Biology and Evolution* **7**, 478-484.
- Lynch, M., Milligan B. G. (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology* **3**, 91-99.
- Macphail, M. K. (1979) Vegetation and climates in Southern Tasmania since the last glaciation. *Quaternary Research* **11**, 306-341.
- Markgraf, V., McGlone M., Hope G. (1995) Neogene paleoenvironmental and paleoclimatic change in southern temperate ecosystems - a southern perspective. *Trends In Ecology and Evolution* **10**, 143-147.
- Millington, R. J., Jones R., Brown D., Vernon B. (1979) Huon pine - endangered? Environmental Studies Occasional Paper No. 9, University of Tasmania, Hobart.

- Moriguchi, Y., Matsumoto A., Saito M., Tsumura Y., Taira H. (2001) DNA analysis of clonal structure of an old growth, isolated forest of *Cryptomeria japonica* in a snowy region. *Canadian Journal of Forest Research* **31**, 377-383.
- Moriyama, E. N., Powell J. R. (1996) Intraspecific nuclear DNA variation in *Drosophila*. *Molecular Biology and Evolution* **13**, 261-277.
- Mosseler, A., Egger K. N., Hughes G. A. (1992) Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markers. *Canadian Journal of Forest Research* **22**, 1332-1337.
- Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA* **70**, 3321-3323.
- Nei, M. (1987) *Molecular Evolutionary Genetics*, Columbia University Press, NY.
- Nei, M., Li W. H. (1985) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA* **76**, 5269-5273.
- Oldfield, S., Lusty C., MacKinven A. (1998) *The World List of Threatened Trees*. World Conservation Press, Cambridge.
- Parker, K. C., Hamrick J. L. (1992) Genetic diversity and clonal structure in a columnar cactus, *Lophocereus schottii*. *American Journal of Botany* **79**, 86-96.
- Parks, J. C., Werth C. R. (1993) A study of spatial features of clones in a population of bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). *American Journal of Botany* **80**, 537-544.

- Peterson, M. J. (1990) Distribution and conservation of Huon pine, p. 50. Forestry Commission Tasmania, Tasmania.
- Petit, R. J., Kremer A., Wagner D. B. (1993) Finite island model for organelle and nuclear genes in plants. *Heredity* **71**, 630-641.
- Pornon, A., Escaravage N., Thomas P., Taberlet P. (2000) Dynamics of genotypic structure in clonal *Rhododendron ferrugineum* (Ericaceae) populations. *Molecular Ecology* **9**, 1099-1111.
- Pornon, A., Escaravage N., Till-Bottraud I., Doche B. (1997) Variation of reproductive traits in *Rhododendron ferrugineum* L. (Ericaceae) populations along a successional gradient. *Plant Ecology* **130**, 1-11.
- Primack, R. B., McCall C. (1986) Gender variation in a red maple population (*Acer rubrum*; Aceraceae): a seven year study of a 'Polygamodioecious' species. *American Journal of Botany* **73**, 1239-1248.
- Quinn, C. J. (1982) Taxonomy of *Dacrydium* Sol. ex Lamb. amend de Lamb. (Podocarpaceae). *Australian Journal Of Botany* **30**, 311-320.
- Rieseberg, L. H., Choi H., Chan R., Spore C. D. (1993) Genomic map of a diploid hybrid species. *Heredity* **70**, 285-293.
- Russell, J. R., Hosein J., Johnson E., Waugh R., Powell W. (1993) Genetic differentiation of cocoa (*Theobroma cacao* L.) populations revealed by RAPD analysis. *Molecular Ecology* **2**, 89-97.

- Schaal, B. A., Learn G. H. (1988) Ribosomal DNA variation within and among plant populations. *Annals of the Missouri Botanical Garden* **75**, 1207-1216.
- Shapcott, A. (1991a) Dispersal and establishment of Huon pine (*Lagarostrobos franklinii*). *Papers and Proceedings of the Royal Society of Tasmania* **125**, 17-26.
- Shapcott, A. (1991b) Studies in the population biology and genetic variation of Huon pine (*Lagarostrobos franklinii*), p. 84. National Rainforest Conservation Program and Department of Parks, Wildlife and Heritage, Hobart, Hobart, Tasmania.
- Shapcott, A. (1993) The population genetics of two temperate rainforest trees, *Lagarostrobos franklinii* (Hook f.) Quinn (Huon pine), and *Atherosperma Moschatum* Labill. (Sassafras) Ph.D., University of Tasmania.
- Shapcott, A. (1997) Population genetics of the long-lived Huon pine *Lagarostrobos franklinii*: An endemic Tasmanian temperate rainforest tree. *Biological Conservation* **80**, 169-179.
- Shapcott, A., Brown M. J., Kirkpatrick J. B., Reid J. B. (1995) Stand structure, reproductive activity and sex expression in Huon pine (*Lagarostrobos franklinii* (Hook L.) Quinn.). *Journal of Biogeography* **22**, 1035-1045.
- Silander, J. (1985) Microevolution in clonal plants. In: *Population biology and evolution of clonal organisms* (eds. Jackson J, Buss L, Cook R), pp. 107-152. Yale University Press, New Haven, CT.
- Smith, M., Bruhn J., Anderson J. (1992) The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. *Nature* **356**, 428-431.

- Soane, I. D., Watkinson A. R. (1979) Clonal variation in populations of *Ranunculus repens*. *New Phytologist* **82**, 557-573.
- Stevens, M. T., Turner M. G., Tuskan G. A., Romme W. H., Gunter L. E., Waller D. M. (1999) Genetic variation in postfire aspen seedlings in Yellowstone National Park. *Molecular Ecology* **8**, 1769-1780.
- Suyama, Y., Obayashi K., Hayashi I. (2000) Clonal structure in a dwarf bamboo (*Sasa senanensis*) population inferred from amplified fragment length polymorphism (AFLP) fingerprints. *Molecular Ecology* **9**, 901-906.
- Swensen, S. M., Allen G. J., Howe M., Elisens W. J., Junak S. A., Rieseberg L. H. (1995) Genetic analysis of the endangered island endemic *Malacothamnus fasciculatus* (Nutt.) Greene var. *nesioticus* (Rob.) Kear (Malvaceae). *Conservation Biology* **9**, 404-415.
- Sydes, M. A., Peakall R. (1998) Extensive clonality in the endangered shrub *Haloragodendron lucasii* (Haloragaceae) revealed by allozymes and RAPDs. *Molecular Ecology* **7**, 87-93.
- Taira, H., Tsumura Y., Tomaru N., Ohba K. (1997) Regeneration system and genetic diversity of *Cryptomeria japonica* growing at different altitudes. *Canadian Journal of Forest Research* **27**, 447-452.
- Tani, N., Tomaru N., Tsumura Y., Araki M., Ohba K. (1998) Genetic structure within a Japanese stone pine (*Pinus pumila* Regel) population on Mt. Aino-Dake in central Honsyu, Japan. *Journal of Plant Research* **111**, 7-15.

- Tenaillon, M. I., Sawkins M. C., Long A. D., Gaut R. L., Doebley J. F., Gaut B. S. (2001) Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.). *Proceedings of the National Academy of Science U S A* **98**, 9161-9166.
- Tuskan, G. A., Francis K. E., Russ S. L., Romme W. H., Turner M. G. (1996) RAPD markers reveal diversity within and among clonal and seedling stands of aspen in Yellowstone National Park. *Canadian Journal Forest Research* **26**, 2088-2098.
- Wakamiya, I., Newton R. J., Johnston J. S., Price H. J. (1993) Genome size and environmental factors in the genus *Pinus*. *American Journal of Botany* **80**, 1235-1241.
- Weising, K., Nybom H., Wolff K., Meyer W. (1995) *DNA Fingerprinting in Plants and Fungi* CRC Press, Boca Raton, Florida.
- Welsh, J., McClelland M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213-7218.
- Whitham, T. A., Slobodchikoff C. N. (1981) Evolution by individuals, plant/herbivore interactions, and mosaics of variability: the adaptive significance of somatic mutations in plants. *Oecologia* **49**, 287-292.
- Whitham, T. G., Mopper S. (1985) Chronic herbivory: Impacts on architecture and sex expression of pinyon pine. *Science* **228**, 1089-1091.

- Whitham, T. G., Williams A. G., Robinson A. M. (1984) The Variation Principle: Individual Plants as Temporal and Spatial Mosaics of Resistance to Rapidly Evolving Pests. In: *A New Ecology* (eds. Price PW, Slobodchikoff CN, Gaud WS), pp. 16-51. Wiley - Interscience, New York.
- Williams, J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A., Tingey S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.
- Wolfe, K., Li W.-H., Sharp P. M. (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences USA* **84**, 9054-9058.
- Wu, J. Y., Krutovskii K. V., Strauss S. H. (1998) Abundant mitochondrial genome diversity, population differentiation and convergent evolution in pines. *Genetics* **150**, 1605-1614.
- Yeh, F. C., Boyle T. J. (1997) Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**, 157.
- Yu, K., Pauls K. P. (1992) Optimization of the PCR program for RAPD analysis. *Nucleic Acids Research* **20**, 2606.
- Zhou, Z., Miwa M., Nara K., Wu B., Nakaya H., Lian C., Miyashita N., Oishi R., Maruta E. (2003) Patch establishment and development of a clonal plant, *Polygonum cuspidatum*, on Mount Fuji. *Molecular Ecology* **12**, 1361-1373.

Table 3.1. Mt. Read and Reference population samples.

Population	Abbreviation	N	Dbh range	Watershed
Mt. Read	MR	63	2 cm–80 cm	Pieman
Riveaux Creek	RC	4	16.5 cm–130 cm	Huon
Condominium Creek	CC	3	< 1 cm–3 cm	Huon
Teepookana	TK	9	6.5 cm–136 cm	King
Newall Creek	NC	5	6 cm–70 cm	King
Stanley River	SR	6	< 1cm–102 cm	Pieman
Gordon River	GR	6	10 cm–178 cm	Gordon

N = number of samples; dbh = diameter at breast height

Samples from Riveaux Creek, Condominium Creek, Teepookana, Newall Creek, Stanley River and Gordon River comprise the reference population.

Table 3.2. RAPD primers and loci scored.

Primer	Sequence	Loci scored*
A5	AGG GGT CTT G	520, 820, 1075, 1800, 1900, 2000
A9	GGG TAA CGC C	375, 430, 680
B7	GGT GAC GCA G	550
B12	CCT TGA CGC A	890, 1200
B20	GGA CCC TTA C	330, 480, 1400
C4	CCG CAT CTA C	790
D11	AGC GCC ATT G	475, 790, 1800
D20	ACC CGG TCA C	650, 780, 890, 1000
E18	GGA CTG CAG A	550, 800, 975
J7	CCT CTC GAC A	590, 630
K4	CCG CCC AAA C	640, 650, 750, 1000, 1300
T7	GGC AGG CTG T	440, 1400

* = molecular weight in base pairs of fragments scored

Table 3.3. Allele frequencies and gene diversity in polymorphic RAPD loci.

Population	Mt. Read (n=63)			Reference (n=33)			Total (n=96)		
	Absent	Present	<i>h</i>	Absent	Present	<i>h</i>	Absent	Present	<i>h</i>
A5_520	0	1	0	0.2462	0.7538	0.3712	0.0846	0.9154	0.1549
A5_1075	0.7127	0.2873	0.4095	0.3892	0.6108	0.4755	0.6015	0.3985	0.4794
A5_1800	0	1	0	0.6124	0.3876	0.4747	0.2063	0.7937	0.3274
A5_1900	0	1	0	0.3482	0.6518	0.4539	0.1197	0.8803	0.2107
A5_2000	1	0	0	0.5590	0.4410	0.4930	0.8515	0.1485	0.2530
A9_375	0	1	0	0.3482	0.6518	0.4539	0.1197	0.8803	0.2107
A9_430	1	0	0	0.8528	0.1472	0.2611	0.9494	0.0506	0.0961
A9_680	0	1	0	0.1741	0.8259	0.2875	0.0598	0.9402	0.1125
B12_890	0.8262	0.1738	0.2872	0.9211	0.0789	0.1453	0.8588	0.1412	0.2425
B20_1400	0	1	0	0.3482	0.6518	0.4539	0.1197	0.8803	0.2107
C4_790	0	1	0	0.6276	0.3724	0.4674	0.8720	0.1280	0.2232
D11_790	1	0	0	0.6276	0.3724	0.4674	0.8720	0.1280	0.2232
D20_1000	0	1	0	0.2540	0.7460	0.3790	0.0838	0.9162	0.1535
E18_550	0	1	0	0.8528	0.1472	0.2511	0.9494	0.0506	0.0961
E18_975	0	1	0	0.6513	0.3487	0.4542	0.2239	0.7761	0.3475
J7_590	0	1	0	0.3892	0.6108	0.4755	0.1338	0.8662	0.2318
J7_630	0.7127	0.2873	0.4095	0.7177	0.2823	0.4052	0.7144	0.2856	0.4080
K4_650	1	0	0	0.9211	0.0789	0.1453	0.9729	0.0271	0.0528
Total			0.0316			0.1973			0.1153

h = Nei's gene diversity (1973)

Only polymorphic alleles are shown although estimates of diversity included all loci.

Table 3.4. Genotype frequencies at Mt. Read.

Genotype	Frequency
1	0.365
2	0.127
3	0.175
4	0.333

Table 3.5. Genotype frequencies in each transect at Mt. Read.

Transect	N	Genotype 1	Genotype 2	Genotype 3	Genotype 4
1	5	0.80	0.20	0	0
2	11	0.73	0.27	0	0
3	15	0.73	0.27	0	0
4	29	0	0	0.38	0.62
5	3	0	0	0	1.0

Transect 1 was located at the upper elevation of the Mt. Read Huon pine population. Transects 2 through 5 were found at successively lower elevations through the stand. N = sample size

Table 3.6. Similarity indices between genotypes at Mt. Read.

Genotype 1			
Genotype 2	0.9825		
Genotype 3	0.9456	0.9643	
Genotype 4	0.9630	0.9455	0.9811

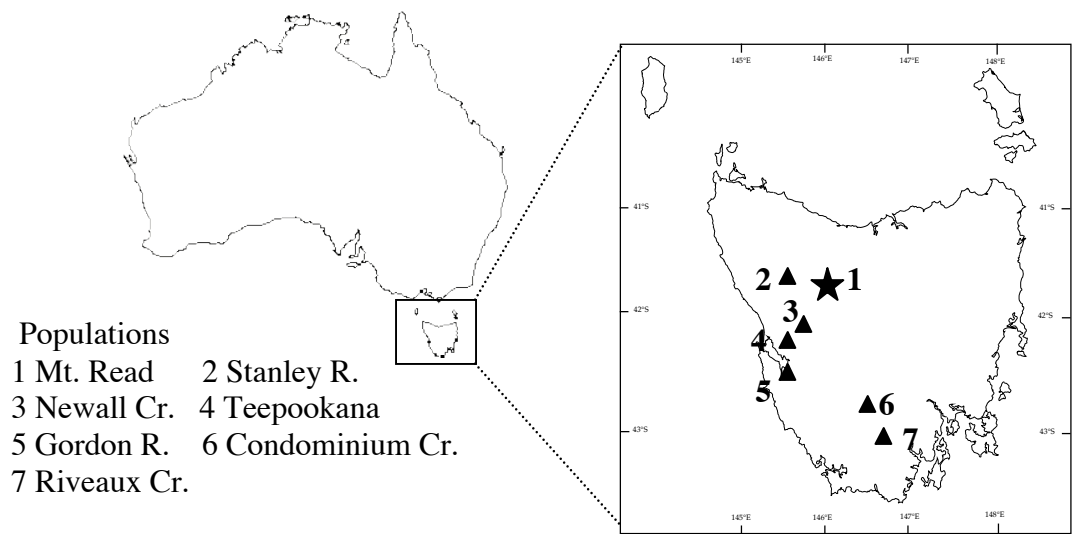


Figure 3.1. Locations of populations sampled.

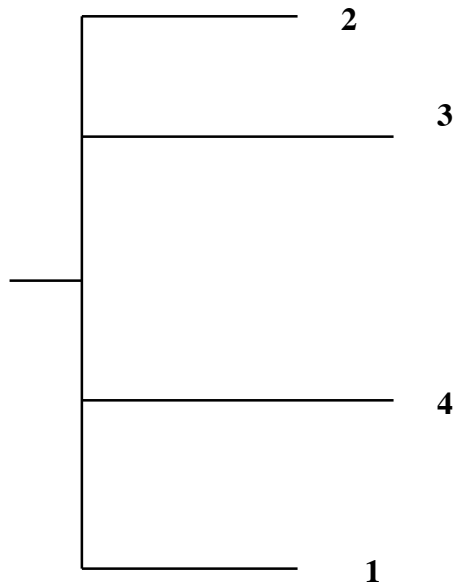


Figure 3.2. Tree of Mt. Read genotypes and genetic distance measured by the similarity index. Numbers indicate genotypes.

APPENDICES

APPENDIX A. Unique alleles for 271 base pairs of chloroplast *trnD* - *trnT* sequence in Philip format. Numbers indicate isolate designations.

208

CAAAATGGATCCCTAGATCAATAAATCCGCATATGGATAGATATTTATCCGCTTT
ATTGTTCCAGGGCCAATGATGTTCCAATCATGTCACTTATTTATAATCAAATATA
TTCAAACAATAATTTGTTTGAGCTATTCGTTCTAGTCATAAAAGGGTGACATCTG
TACCCACAATTGTCAGGGAGGAACTATATGTTCCATTATTTTTCAGCCTATGAG
ATGAGAAGGGTAATCTTATTAATAATTTTTTTGGCAGGATTACCCTTCACCC

222

CAAAATGGATCCCTAGATCAATAAATCCGCATATGGATAGATATTTATCCGCTTT
ATTGTTCCAGGGCCAATGATGTTCCAATCATGTCACTTATTTATAATCAAATATA
TTCAAACAATAATTTGTTTGAGCTATTCGTTCTAGTCATAAAAGGGTGACATCTG
TACCCACAATTGTCAGGGAGGAACTATATGTTCCATTATTTCTCAGCCTATGAG
ATGAGAAGGGTAATCTTATTAATAATTTTTTTGGCAGGATTACCCTTCACCC

APPENDIX B. Unique alleles for 330 base pairs of chloroplast *trnS-trnT* sequence in Phylip format. Numbers indicate isolate designations.

71

AATAAAAAAATTCTAATGTAACCTACAGAAGAAATCTGAAATATAGATTTAACG
GAACAATTCTATCTATTTGCCTGCTCTGGGAGAGCAGAGAGAGAAGGGATAAGG
GTGTAATTGCCAGATATCCAGGAATCCTTCATTTTCATTCTCAATTTAGGCTCGA
CGGGAAAATACTCTATGACCAACAATTCATTAATGTCCAAACCGATGCTTATAT
TATCGATTATTTGATTA ACTACTCCTTTATATTGTGACGAATAAAGAGTCAAATA
AATGGTACTTTATGCTTCATTTCATACTTTTGAAATCGTAAAGAGAGATTTTTTT

51

AATAACAAAATTCTAATGTAACCTACAGAAGAAATCTGAAATATAGATTTAACG
GAACAATTCTATCTATTTGCCTGCTCTGGGAGAGCAGAGAGAGAAGGGATAAGG
GTGTAATTGCCAGATATCCAGGAATCCTTCATTTTCATTCTCAATTTAGGCTCGA
CGGGAAAATACTCTATGACCAACAATTCATTAATGTCCAAACCGATGCTTATAT
TATCGATTATTTGATTA ACTACTCCTTTATATTGTGACGAATAAAGAGTCAAATA
AATGGTACTTTATGCTTCATTTCATACTTTTGAAATCGTAAAGAGAGATTTTTTT

APPENDIX C. Unique alleles for 291 base pairs of chloroplast *psbC-trnS* sequence in Phylip format. Numbers indicate isolate designations.

214

GACTTATCAATTTGTCGGGTAAATTA ACTCGGGGCTCATGTGGCTCATGCCGGATT
AATTGTATTCTGGGCCGGAGCAATGAACCTATTTGAAGTGGCTCATT TTTGTACCG
GAAAAACCTATGTATGAACAAGGATTGATTTTACTTCCCCATCTGGCTACTCTAG
GATGGGGAGTCGGTCCTGGTGGGGAAATTGTGGACACTTTCCCCTACTTTGTATC
TGGAGTACTTCACTTAATTTCTTCTGCTGTTTTAGGTTTTGGTGGTATTTATCATG
CACTCGTCGGACCCG

78

GACTTATCAATTTGTCGGGTAAATTA ACTCGGGGCTCATGTGGCTCATGCCGGATT
AATTGTATTCTGGGCCGGAGCAATGAACCTATTTGAAGTGTCTCATT TTTGTACCG
GAAAAACCTATGTATGAACAAGGATTGATTTTACTTCCCCATCTGGCTACTCTAG
GATGGGGAGTCGGTCCTGGTGGGGAAATTGTGGACACTTTCCCCTACTTTGTATC
TGGAGTACTTCACTTAATTTCTTCTGCTGTTTTAGGTTTTGGTGGTATTTATCATG
CACTCGTCGGACCCG

APPENDIX D. Unique allele for 291 base pairs of *G3pdh* sequence in Phylip format.

```
TGTCAGCGTGTTGTCCGGGGGCGTAGCAGCGCACTTGTCATCCGACTCACCCCTG
TATGGGCCCTCGGCGGCATCCTTGATGGTCTTCTTGATCTCCTCGTAGCTCGCAG
CCTTCTCAATGCGGCAAGTCAAGTCGACCACGGATACGTTCTTGGTGGGGACGC
GCATCGACATGCCGGTGAGCTTGCCATTCAGCGAAGGGATGACCTTGCCGACGG
CCTTTGCGGCGCCAGTAGAGCTGGGGATGATGTTCTCTGCTGCAGCACGGCCTCC
GCGCCAATCCTTGTTGGA
```

APPENDIX E. Unique alleles for 289 base pairs of *ITS2* sequence in Phylip format.
Numbers designate isolates.

7

GCGGGCGGGAAGAGCGGACATGGCCGTCCGTGCCCTCCCGTGGTGCGGTTCGGCT
GAAAAATGCACGCTGGGCCGTCGCCCCCGGCCCGACGAGCGGTGGCTCCGTTG
CGAGTCGGCGTTTGTTCGGGCTCGGGGAACGTGCGGTCCGGTACCGAAGAAGTTT
GCACACCTTCCGCGTCCCCAGGTCAGGCGAGACCACCCGCTGAGCTTAAGCATA
TCAATAAGCGGAGGAAAAGAACTTACGAGGATTCCCCTAGTAACGGCGAGCGA
ACCGGGAACAGCCCAGCAT

111

GCGGGCGGGAAGAGCGGACATGGCCGTCCGTGCCCTCCCGTGGTGCGGTTCGGCT
GAAAAATGCACGCTGGGCCGTCGCCCCCGGCCCGACGAGCGGTGGCTCCGTTG
CGAGTCGGCGTTTGTTCGGGCTCGGGGAACGTGCGGTCCGGTATCGAAGAAGTTT
GCACACCTTCCGCGTCCCCAGGTCAGGCGAGACCACCCGCTGAGCTTAAGCATA
TCAATAAGCGGAGGAAAAGAACTTACGAGGATTCCCCTAGTAACGGCGAGCGA
ACCGGGAACAGCCCAGCAT

APPENDIX F. Unique alleles for 574 base pairs of *4CL* sequence in Phylip format. Numbers designate isolates.

155

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTTATCATGCAAAAATTCAACATCACGAC
GTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCCCTTCGTGCCACCC
ATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCCA
TCCGGATGATCACGGCGGGCGGCAGCCTCTCGGAGAGGATCTCGAAGACGCCC
TCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCCCCCCAAAA
AATTCAGTTTTGTTTTGTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTGG
GATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCCGGGCTAGCA
AGGAATCTTGGGTTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGGT
ACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTCT
TTACCACACAACCAAGCAGGTGAA

77

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTTATCATGCAAAAATTCAACATCACGAC
GTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCCCTTCGTGCCACCC
ATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCCA
TCCGGATGATCACGGCGGGCGGCAGCCTCTCGGAGAGGATCTCGAAGACGCCC
TCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCACCCAAAA
AAATTCAGTTTTGTTTTGTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTG
GGATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCCGGGCTAGC
AAGGAATCTTGGGTTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGG
TACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTC
TTTACCACACAACCAAGCAGGTGAA

APPENDIX F. Unique alleles for 574 base pairs of *4CL* sequence in Phylip format (cont'd).

14

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTTATCATGCAAAAATTCAACATCACGAC
GTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCTTCGTGCCACCC
ATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCCA
TCCGGATGATCACGGCGGGCGGCGCCTCTCGGAGAGGATCTCGAAGACGCC
TCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCACCCCAAAA
AAATTCAGTTTTGTTTTCTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTG
GGATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCGGGCTAGC
AAGGAATCTTGGGTTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGG
TACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTC
TTTACCACACAACCAAGCAGGTGAA

94

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTCATCATGCAAAAATTCAACATCACGA
CGTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCTTCGTGCCACC
CATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCC
ATCCGGATGATCACGGCGGGCGGCGCCTCTCGGAGAGGATCTCGAAGACGCC
CTCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCCCCCCAAA
AAATTCAGTTTTGTTTTCTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTG
GGATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCGGGCTAGC
AAGGAATCTTGGGTTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGG
TACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTC
TTTACCACACAACCAAGCAGGTGAA

304

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTCATCATGCAAAAATTCAACATCACGA
CGTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCTTCGTGCCACC
CATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCC
ATCCGGATGATCACGGCGGGCGGCGCCTCTCGGAGAGGATCTCGAAGACGCC
CTCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCACCCCAAAA
AAATTCAGTTTTGTTTTCTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTG
GGATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCGGGCTAGC
AAGGAATCTTGGGTTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGG
TACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTC
TTTACCACACAACCAAGCAGGTGAA

APPENDIX F. Unique alleles for 574 base pairs of *4CL* sequence in Phylip format (cont'd).

111

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTCATCATGCAAAAATTCAACATCACGA
CGTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCCCTTCGTGCCACC
CATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCC
ATCCGGATGATCACGGCGGGCGGCGCGCCTCTCGGAGAGGATCTCGAAGACGCC
CTCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCACCCCAAAA
AAATTCAGTTTTGTTTTGTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTG
GGATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCCGGGCTAGC
AAGGAATCTTGGGTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGG
TACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTC
TTACCACACAACCAAGCAGGTGAA

297

TGATTTTGTGCGTGTTGCCCTCTTTCATATCTTGTCCCTCAATTCGGTGCTGCTCT
GCGCGCTCCGCGTCGGCGCCGCCATCCTTATCATGCAAAAATTCAACATCACGAC
GTTGCTGGAGCTGATCGAGAAATACAAAGTCACCGTTGCCCCCTTCGTGCCACCC
ATTGTTTTGGAGATCGCCAAAACCCCATCGTTGCCAACTACGACGTGTCCTCCA
TCCGGATGATCACGGCGGGCGGCGCGCCTCTCGGAGAGGATCTCGAAGACGCC
TCCGAGCCCGCTTCCCGTCCGCCAAATTCGGCCAGGTAAACTCCCCCCCCAAAA
AATTCAGTTTTGTTTTCTTTTCTAATCGAATTTTTTGTCTGTGTATTGAATTTGTG
GATCTTCATTGAATTTAGGGTTACGGCATGACGGAGGCGGGGCCCGGGCTAGCA
AGGAATCTTGGGTTCGCGAAGGAGCCTTCCCTGTGAAATCAGGGTCCGGCGGT
ACCGTTGTCCGCAACGCCAGATGAAGATTATCGACACGGAGACCGGAGCCTCT
TTACCACACAACCAAGCAGGTGAA

APPENDIX G. Huon pine RAPD data file for RAPDPLOT.

NUMBER OF INDIVIDUALS: 96

TITLE: MT. READ RAPDS

NUMBER OF FRAGMENTS: 35

(35(1X,A8))

A5-520-- A5-820-- A5-1075- A5-1800- A5-1900- A5-2000- A9-375-- A9-430-- A9-680--
B7-550-- B12-QQQ- B12-1200 B20-330- B20-480- B20-1400 C4-790-- D11-475- D11-
790- D11-1800 D20-650- D20-780- D20-890- D20-1000 E18-550- E18-800- E18-975- J7-
590-- J7-630-- K4-640-- K4-650-- K4-750-- K4-1000- K4-1300- T7-440-- T7-1400-

(A3,1X,35A1)

217 11111212112111121211111211111211111
225 11111212112111121211111211111211111
218 11111212111111121211111211111211111
226 11111212111111121211111211111211111
219 11111212112111121211111211111211111
227 11111212111111121211111211111211111
220 11111212112111121211111211111211111
228 11111212112111121211111211111211111
221 11111212112111121211111211111211111
229 11111212112111121211111211111211111
222 11111212112111121211111211111211111
230 11111212111111121211111211111211111
223 11111212112111121211111211111211111
231 11111212112111121211111211111211111
224 11111212112111121211111211111211111
232 11111212112111121211111211111211111
233 11111212112111121211111211111211111
241 11111212112111121211111211111211111
234 11111212112111121211111211111211111
242 11111212111111121211111211111211111
235 11111212112111121211111211111211111
243 11111212112111121211111211111211111
236 11111212111111121211111211111211111
244 11111212112111121211111211111211111
237 11111212112111121211111211111211111
245 11111212112111121211111211111211111
238 11111212111111121211111211111211111
246 11111212112111121211111211111211111
239 11111212112111121211111211111211111
247 11211212112111121211111211121211111
240 11111212111111121211111211111211111
248 11211212112111121211111211121211111
249 11211212111111121211111211121211111
258 11211212112111121211111211121211111

APPENDIX G: Huon pine RAPD data file for RAPDPLOT. (cont'd)

250 11211212112111121211111211121211111
259 11211212112111121211111211121211111
251 11211212112111121211111211121211111
260 11211212111111121211111211121211111
253 11211212112111121211111211121211111
261 11211212111111121211111211121211111
254 11211212112111121211111211121211111
262 11211212112111121211111211121211111
255 11211212112111121211111211121211111
263 11211212111111121211111211121211111
256 11211212111111121211111211121211111
264 11211212111111121211111211121211111
257 11211212112111121211111211121211111
265 11211212112111121211111211121211111
266 11211212111111121211111211121211111
274 11211212111111121211111211121211111
267 11211212112111121211111211121211111
275 11211212111111121211111211121211111
268 11211212111111121211111211121211111
276 11211212112111121211111211121211111
269 11211212111111121211111211121211111
277 11211212112111121211111211121211111
270 11211212112111121211111211121211111
278 11211212112111121211111211121211111
271 11211212112111121211111211121211111
279 11211212112111121211111211121211111
272 11211212111111121211111211121211111
216 11111212112111121211111211111211111
273 11211212112111121211111211121211111
62 1111112112111111111111111120211111
74 111111211211111111111112111211111
315 211111121121111111111112111211211111
76 1121111211111111111111121112111211111
322 11111221112111211111111211211211111
92 1111111112111121211111211121211111
332 11211212112111211111111211111111111
103 11111212212111121211111212121211111
41 11121121112111121211111111121211111
112 11211112112111121211111211121211111
46 111211111211112121111121222111111
189 111212121121111111111111121211111
50 1120201211111111111110211121211111
193 21111212112111221211111211111211111

APPENDIX G: Huon pine RAPD data file for RAPDPLOT. (cont'd)

128 11121212112111111111111212111211111
201 11111122111111121211111211111211111
138 1111121111211111111111111211121211111
154 11111212112111121211111212121111111
297 11111112112111121211111211121211111
174 11121111112111111111111111111211111
116 1112111211211111111111111111121211111
180 11122112112111221211111211111211111
117 11111212111111111111111211121211111
282 11122112112111111111111212121211111
123 11111111121111111111111111121211111
285 11111112112111121211111212111211111
1 11121112112111121211111212111211111
291 11221112112111111111111112211111111
7 111211211121111111111110212111111111
294 11111112111111111111111212121211111
11 1111121111211111111111111212111211111
295 11111112112111111111111212121211111
27 11122112112111121211111211211211111

APPENDIX H. Identification of trees from the Mt. Read population tagged for climatological or growth studies.

Tree sample #	Tag identification
218	2
219	4
220	5
221	8
222	10
223	9
224	MRLH 4
226	12
251	34
253	33
255	35
257	36
260	37
261	38
262	39
263	40
267	MRLH 1
274	MRLH 2
277	18