

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Evolutionary relationships of the Castle Hill buttercup
(*Ranunculus crithmifolius* subspecies *paucifolius*).

A thesis presented in partial fulfilment of the requirements for the degree
of

Master of Science
in
Plant Biology

at Massey University, Palmerston North,
New Zealand.

Morore Morgan Piripi

2005

Abstract

The Castle Hill buttercup (*Ranunculus crithmifolius* subsp. *paucifolius*) is a rare plant found only in a small area of limestone gravel at Castle Hill. Known as Kura Tawhiti in Maori, the region is renowned for an abundance of rare and endangered plants and has historically been an important area of Maori activity. The Castle Hill buttercup has a long conservation history, starting in 1948 and continuing to the present day. Recently the population of *Ranunculus crithmifolius* subsp. *paucifolius* has again declined to the point where further conservation effort is needed.

Lockhart *et al.* (2001) found that the Castle Hill buttercup showed ambiguous phylogenetic results when chloroplast and nuclear DNA markers were sequenced. It was theorised that the Castle Hill buttercup was a product of one or more events of diploid hybridisation, which would account for these ambiguous phylogenetic results. The aims of this study were to investigate the Castle Hill buttercup and its closest relatives using phylogenetic methods. Data was gathered from nuclear ribosomal ITS and chloroplast *JSA* DNA marker sequencing and the multi-locus fingerprinting (MLF) methods ISSR and AFLP.

No evidence was found in this study to support the hypothesis that the Castle Hill buttercup is a diploid hybrid, but both MLF techniques showed a level of genetic distinctiveness between *R. crithmifolius* subsp. *paucifolius* and its sister subspecies *R. crithmifolius* subsp. *crithmifolius*. Other alpine *Ranunculus* taxa studied showed genetic groupings related to geography. Most notably, the species *R. enysii* was divided into two separate genetic groups, one in the Waimakariri basin area, and one located in the southern South Island. This southern group was itself divided into two genetically distinct groups, located in the east and west of the southern South Island.

Comparison of the different data gathering methods used in this study showed that MLF has a higher phylogenetic resolution than DNA marker sequencing and was able to determine genetic differences between individual accessions. AFLP was found to be superior to ISSR for use in New Zealand alpine *Ranunculus* due to greater consistency between duplicate reactions.

Acknowledgements

Firstly I would like to thank my supervisor Pete Lockhart for starting this whole thing off, without your patience and enthusiasm this would never have happened.

I am also extremely grateful for the use of equipment of the Allan Wilson Centre for Molecular Ecology and Evolution and for financial assistance from the Allan Wilson Centre, the New Zealand Marsden Fund, the Manaaki Whenua Landcare Research bursary and the Manaaki Tauiira Trust.

Huge thanks go to Trish McLenachan and Andrew Clarke for countless manuscript revisions and lab assistance, that must have been so incredibly dull... Dave Havell's beard is a little greyer on account of the massive amount of work he put into organising herbarium vouchers, keeping track of samples and scrambling down slopes that give goats the willies. Big thanks also to Wim Hordijk, Sandra Martin, Hannah Riden and all the people who clambered up half the mountains in the South Island looking for plants the same colour as rocks.

I am indebted to many people for offering help with all sorts of problems, in particular Richard for suffering cryogenic burns in the line of duty, Gillian for squeaky-clean emergency PCR, Mike for boldly going where field ecologists fear to tread and Simon for receiving innumerable stab wounds. I am also obliged to the denizens of the Boffin Lounge and the PLEB and Farside labs for help and encouragement. I must also thank my parents for their constant support.

I would also like to thank the staff at DOC Arthur's Pass and Rachel Puentener of Te Runanga o Ngai Tahu for help with collection permits. I am also grateful to Gnome Hannah-Brown of DOC Christchurch and Joan Vurdman of Ngai Tuahuriri for allowing me to present my research proposal and for a valuable history lesson.

Lastly, I need to thank Sue for putting up with never seeing her husband, and for providing constant encouragement, love and cups of tea.

List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
cpDNA	Chloroplast DNA
cpJ _{SA}	Chloroplast J _{SA}
DNA	Deoxyribonucleic Acid
dNTP	Dinucleotidetriphosphate
DOC	Department Of Conservation
InDel	Insertion or Deletion
ISSR	Inter-Simple Sequence Repeat
ITS	Internal Transcribed Spacer
IUCN	International Union for the Conservation of Nature and natural resources
J _{SA}	Junction of the chloroplast Short Single Copy region and Inverted repeat A
MLF	Multi-Locus Fingerprinting
nITS	Nuclear ITS
NJ	Neighbor Joining
NNET	Neighbor-Net
nrDNA	Nuclear Ribosomal DNA
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction

Table of Contents

1	Introduction	1
1.1	The Castle Hill buttercup	1
1.2	Reasons behind conservation	2
1.2.1	Justification of conservation	2
1.2.2	Conservation categories	2
1.3	A New Zealand example - the Castle Hill buttercup	6
1.3.1	Conservation history	6
1.3.2	Conservation value of the Castle Hill buttercup	7
1.4	The Alpine Ranunculi of New Zealand	8
1.4.1	Distribution	8
1.4.2	Morphology.....	9
1.4.3	Habitat	9
1.5	Taxonomic uncertainty of the Castle Hill buttercup	10
1.5.1	Taxonomic history	10
1.5.2	Research history	11
1.5.3	Molecular findings	11
1.6	Geological history of New Zealand alpine buttercups	12
1.6.1	Glacial refugia.....	12
1.6.2	Disjunct distributions	13
1.6.3	Biogeographic research.....	13
1.7	Hybridisation	14
1.7.1	Hybridisation in New Zealand	14
1.7.2	Hybridisation of New Zealand alpine buttercups	15
1.8	Conservation genetics of the Castle Hill Buttercup	15
1.9	Focus of this research.....	16
1.9.1	The context of this project.....	16
1.9.2	Plant molecular markers.....	17
1.9.3	Problems with commonly used markers	17
1.9.4	Multilocus DNA fingerprinting.....	18
1.9.5	Phylogenetic methods	19
1.10	Hypotheses	20
2	Materials and Methods.....	21
2.1	Sampling	21
2.2	DNA extraction.....	23
2.2.1	CTAB extraction method	23
2.2.2	Qiagen DNeasy extraction method	24
2.3	Agarose gel electrophoresis	25
2.4	PCR amplification of marker regions for direct sequencing	27
2.5	Cleanup of PCR for direct sequencing.....	27
2.6	DNA sequencing protocol.....	28
2.7	ISSR PCR protocol	29
2.8	AFLP PCR protocol.....	29
2.8.1	Restriction endonuclease digestion of DNA	29
2.8.2	Preparation of oligonucleotide linkers	32

2.8.3	Ligation of linkers to restriction fragments.....	32
2.8.4	Pre-selective amplification of ligation products	32
2.8.5	Selective amplification of pre-amp PCR products.....	33
2.9	PAGE of AFLP and ISSR PCR products	33
2.9.1	Preparation of plates for PAGE.....	33
2.9.2	Preparation of polyacrylamide gel matrix.....	34
2.9.3	Loading and electrophoresis of the polyacrylamide gels.....	35
2.9.4	Silver staining protocol of polyacrylamide gels.....	36
2.10	Scoring of ISSR and AFLP gels	39
2.11	Sequence editing	39
2.12	Phylogenetic protocol.....	39
3	Results.....	41
3.1	Results pertaining to Hypothesis I.....	41
3.1.1	DNA sequence data.....	41
3.1.2	Analyses of nITS data	41
3.1.3	Analyses of J _{SA} data	44
3.1.4	Fingerprint data	47
3.1.5	ISSR profiles	47
3.1.6	AFLP profile	50
3.2	Results pertaining to hypothesis II.....	54
3.2.1	Analyses of nITS data	54
3.2.2	Analyses of J _{SA} data	55
3.2.3	Analyses of ISSR profiles	55
3.2.4	Analyses of AFLP profiles.....	56
3.3	Results pertaining to hypothesis III	56
3.3.1	Analyses of nITS data	56
3.3.2	Analyses of J _{SA} data	57
3.3.3	Analyses of ISSR profiles	57
3.3.4	Analyses of AFLP profiles.....	57
4	Discussion.....	58
4.1	Discussion of cpJ_{SA} and nITS DNA sequence results.	58
4.2	Discussion of Multilocus Fingerprint results.....	61
4.3	Discussion of methods.....	63
4.3.1	DNA sequence analysis vs. Multi-locus fingerprinting	63
4.3.2	Subjective factors in data	63
4.3.3	AFLP vs. ISSR.....	63
4.4	Discussion of hypotheses.....	65
4.4.1	Hypothesis I: That there is genetic distinctiveness between regions in the Group III New Zealand alpine <i>Ranunculus</i>	65
4.4.2	Hypothesis II: That the Castle Hill buttercup (<i>R. c. paucifolius</i>) is genetically distinct from <i>R. c. crithmifolius</i>	65
4.4.3	Hypothesis III: That the Castle Hill buttercup is of diploid hybrid origin. 66	
4.4.4	Genetic variation within <i>R. c. paucifolius</i>	67
4.5	Conservation and this study.....	67
5	References	69

6	Appendices	78
6.1	Appendix A: Collection information of <i>Ranunculus</i> samples used in analyses.	78
6.2	Appendix B: Invitrogen DNA ladder standards	80
6.3	Appendix C: Oligonucleotides used in DNA sequencing, AFLP and ISSR	81

List of Figures

Figure 1.1-	IUCN threatened species Red List categories (IUCN, 2001).....	3
Figure 1.2–	Conservation classification system for New Zealand plants (after de Lange and Norton 1998)	4
Figure 1.3 -	Conservation classification system for New Zealand (Molloy <i>et al.</i> 2002)	5
Figure 1.4 -	Photograph of Lance McCaskill reserve, Kura Tawhiti.....	7
Figure 2.1 -	South Island map showing locations of accessions used in this study.....	22
Figure 2.2 -	Example of an agarose gel containing size and mass ladders and J _{SA} PCR amplification products.....	26
Figure 2.3 -	Diagram of the AFLP reaction (modified from (Clarke, 2001)).....	31
Figure 2.4 -	ISSR primer #835 silver stained polyacrylamide gel.....	38
Figure 3.1 -	Neighbor –Joining graph derived from nITS DNA sequences	42
Figure 3.2 -	Neighbor-Net graph derived from nITS DNA sequences	43
Figure 3.3 -	Neighbor-Joining graph derived from J _{SA} DNA sequences	45
Figure 3.4 -	Neighbour-Net Graph derived from J _{SA} DNA sequences	46
Figure 3.5 -	Neighbor-Joining graph derived from ISSR MLF data.....	48
Figure 3.6 -	Neighbor-Net graph derived from ISSR MLF data.....	49
Figure 3.7 -	Neighbor-Joining graph derived from AFLP MLF data	51
Figure 3.8 -	Neighbor-Net graph derived from AFLP MLF data	52
Figure 3.9 -	Leaf outlines of <i>R. enysii</i> taken from Fisher (1965) p42. (A) Leaves from Lake Lyndon, Waimakariri Basin. (B) Leaves from Mt. Cleughearn, Fiordland. (C) Leaves from the Rock and Pillar Range.	53
Figure 3.10 -	Herbarium voucher photographs of southern <i>R. enysii</i> analysed in this study (see appendix A for full specimen details). (A) Specimen <i>enysii</i> _M5 from Mount Lyndon, Waimakariri Basin. (B) Specimens <i>enysii</i> _M12 and <i>enysii</i> _M13 from Borland Burn, Fiordland. (C) Specimen <i>enysii</i> _M14 from the Rock and Pillar Range, Otago.	54
Figure 4.1 -	Regional groupings of <i>R. enysii</i> suggested from DNA marker sequences. (1) Waimakariri basin. (2) Borland Burn, Fiordland. (3) Nevis River valley. (4) Umbrella Mountains. (5) Rock and Pillar Range.....	59

List of Tables

Table 1.1 - A comparison of several systems of species classification for conservation	4
Table 1.2 - Distributions of New Zealand alpine <i>Ranunculus</i>	9

1 Introduction

1.1 The Castle Hill buttercup

The Castle Hill buttercup (*Ranunculus crithmifolius* subspecies *paucifolius*) is one of New Zealand's rarest plants. Endemic to the Kura Tawhiti region, the plant exists only as a consequence of cultivation and management since the 1950s, and has since become an iconic example of a rare species brought back from the brink of extinction by extensive conservation effort. The Kura Tawhiti/Castle Hill area (43.223922° S 171.717081° E) is located in the Broken River basin northwest of Christchurch. Kura Tawhiti is an important site for local Maori as it is considered a Ngai Tahu topuni site. This name recognises and is symbolic of the Ngai Tahu custom of Rangatira (Chiefs) placing their cloaks over an area as a symbol of their power and authority over the region. The area was also a historically important stopover for the local Ngai Tuahuriri iwi when travelling towards the East coast for fishing expeditions (Joan Vurdman, pers. comm., 2003).

In the present study, direct DNA sequencing and DNA fingerprinting have been used to investigate the evolutionary origin of the Castle Hill Buttercup and to determine its relationship to other alpine buttercups of the Kura Tawhiti region. This study was motivated by recent observations suggesting that the population of Castle Hill buttercups has once again declined, and that the genetic distinctiveness of this species is unclear (Lockhart *et al.*, 2001). An important aim of the present study has been to investigate the extent of hybridisation amongst species closely related to the Castle Hill buttercup, and to determine whether or not *R. crithmifolius* subsp. *paucifolius* is a diploid hybrid species. Although diploid hybridisation (interspecific hybridisation without a change in ploidy level) has been speculated as being important in evolution of the New Zealand flora (Rattenbury, 1962), genetic evidence for this is lacking. Thus it is hoped that findings from the present study will provide some insight into the general question of whether or not hybridisation is important for explaining extant

alpine plant biodiversity in New Zealand. Answers to this question will enable us to make informed decisions concerning conservation of our native taonga.

1.2 Reasons behind conservation

1.2.1 Justification of conservation

There are at least three main reasons for the conservation of species. Crozier (1997) identifies these as being (a) moral – the assumption that all species have a right to exist, (b) aesthetic – the belief that species have a natural beauty and should be preserved, and (c) utilitarian – the belief that human lives are enriched by the presence of other species, or that we can derive some product or benefit from them. Regardless of the justification used, the common theme of conservation in New Zealand and overseas is that of preserving biodiversity, defined in the United Nations (Secretariat of the Convention on Biological Diversity, 1992) as “...the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems”. Unfortunately without information of phylogenetic distinctiveness to elucidate processes that explain biodiversity, it is problematic to assess and evaluate the threatened status of animal and plant species. When considering conservation issues, phylogenetic distinctiveness is relevant to both category (b) and (c) above (Crozier, 1997).

1.2.2 Conservation categories

The most commonly used classifications for evaluating threat status are perhaps the Red List categories list (IUCN, 2001) of the International Union for the Conservation of Nature and natural resources (IUCN), more commonly known as the World Conservation Union. These categories (Fig. 1.1) use an organism’s distribution or habitat range, its occurrence within this habitat, population size and rate of population decline to assess and categorise endangered species. However, these criteria are

problematic to implement in New Zealand as they do not take into account the relatively small size of the country, the short time period of many recent species declines and the large number of taxa with naturally restricted ranges and/or small population sizes (Molloy *et al.*, 2002).

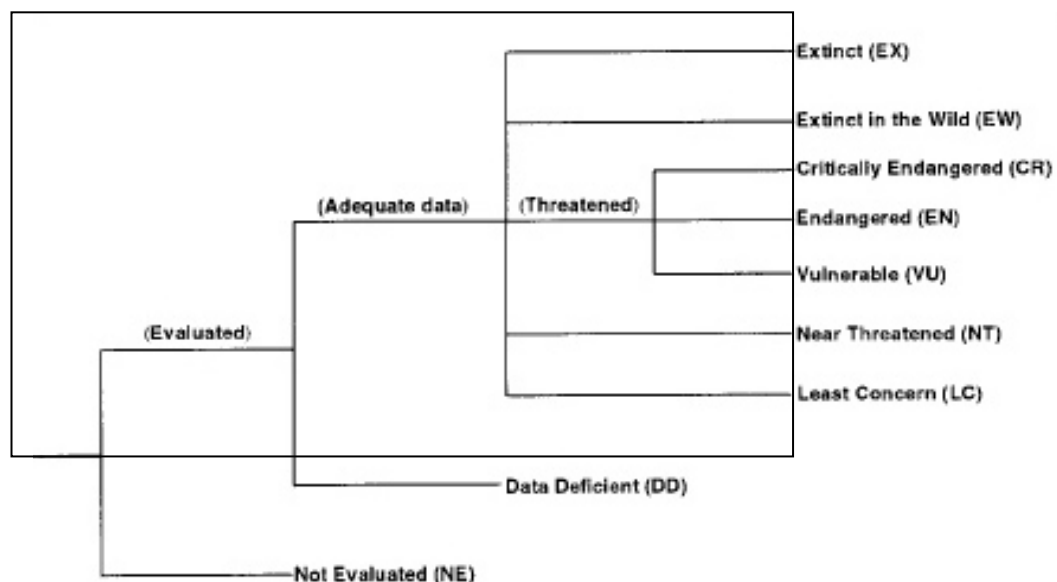


Figure 1.1- IUCN threatened species Red List categories (IUCN, 2001)

In 1999 at a species threat classification workshop, the New Zealand Department of Conservation (DOC) evaluated the suitability of IUCN criteria for use in New Zealand (Fig. 1.1) along with de Lange and Norton's (1998) classification system for rare plants (Fig. 1.2). A comparison of the classification categories in these two systems is presented in Table 1.1. A subset of the DOC species priority criteria was also evaluated (Molloy and Davis, 1994). This system assigned scores to species based on the taxonomic distinctiveness, status, vulnerability, value to humans and threats facing the species. A higher score under this ranking means that a species should have higher priority for conservation.

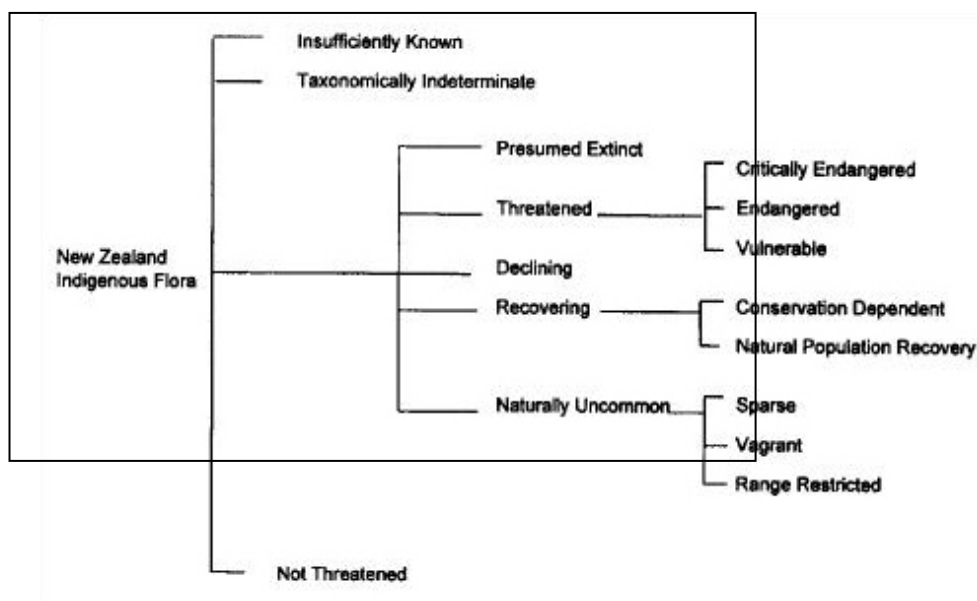


Figure 1.2– Conservation classification system for New Zealand plants (after de Lange and Norton 1998)

IUCN v3.1 (2001)	DeLange and Norton, 1998	Molloy <i>et al.</i> , 2002
Critically endangered	Critically endangered	Nationally Critical
Endangered	Endangered	Nationally endangered
Vulnerable	Vulnerable	Nationally vulnerable
Vulnerable	Declining/Naturally uncommon/Recovering	Serious decline
Near Threatened	Declining/Naturally uncommon/Recovering	Gradual decline
Least concern	Declining/Naturally uncommon/Recovering	Range Restricted

Table 1.1 - A comparison of several systems of species classification for conservation

The workshop concluded at this time that none of the schemes evaluated were ideal for the New Zealand situation, and that a new classification system should be made by combining elements from all three of these methods. As a result, a new classification system for use in New Zealand was proposed (Molloy *et al.*, 2002) (Fig. 1.3). A comparison of the classification categories in this system with the systems previously mentioned can be seen in Table 1.1. This scheme attempts to take into account New Zealand's relatively small land size, the rapid decline of many of our native species,

the restricted distributions and population sizes of many of our taxa and features of the New Zealand environment that make it problematic to implement the IUCN criteria (Molloy *et al.*, 2002).

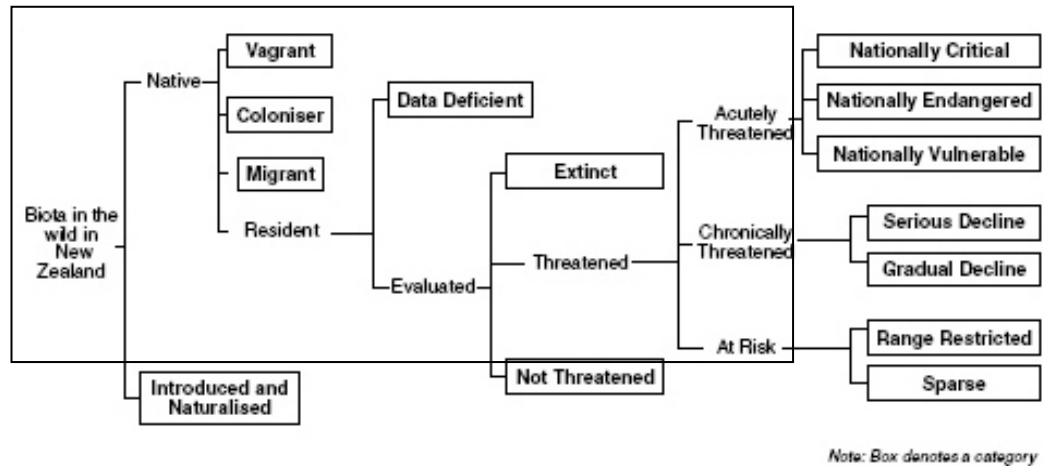


Figure 1.3 - Conservation classification system for New Zealand (Molloy *et al.* 2002)

Like the IUCN red species list, the Molloy *et al.* (2002) proposal makes no attempt to prioritise taxa for conservation purposes, but rather concentrates on providing an indication of threat levels. Ideally, all threatened and endangered taxa would receive conservation management. Unfortunately this is impractical due to financial and logistical constraints, so some system of allocating appropriate levels of conservation priority is required. A system for deciding priorities should take into account existing threat levels to the taxa, as well as perceived or extrapolated risk. Other factors such as cultural values also need to be taken into consideration, as often conservation priority is not solely based on threat factors (Mace and Lande, 1991). Taxa that are not regarded as highly threatened may nevertheless have high conservation priority because of these additional factors. Clearly, these issues are complex and thus priorities were not specified as part of the Molloy *et al.* (2002) proposal. The aim instead was to provide for a “New Zealand Threat Classification System focused at the national level, which would provide a more sensitive classification [than IUCN criteria] for taxa occurring in naturally restricted distributions and in small numbers due to New Zealand’s island and mountainous geography”.

1.3 A New Zealand example - the Castle Hill buttercup

1.3.1 Conservation history

The Castle Hill Buttercup is a good example of this type of conservation problem. Sheep and rabbit grazing in the area in the 1940s left a population of only 32 plants (McCaskill, *c.* 1982). What was to become the reserve area of 6.4 hectares was fenced in March 1948. An immediate improvement could be seen the next year, with over one hundred new seedlings and young plants counted (McCaskill, *c.* 1982). A programme involving weeding, pest control and careful seed collection and planting succeeded in stabilising the population at over 400 plants by 1978 (McCaskill, *c.* 1982). A census count was performed by Havell, Hordijk and Piripi in 2003, in which 89 clumps were counted throughout the reserve. This recent observation shows that the population has once again fallen to dangerously low numbers. A photograph of the reserve area taken from the northern cliff face is shown in Figure 1.4.



Figure 1.4 - Photograph of Lance McCaskill reserve, Kura Tawhiti

1.3.2 Conservation value of the Castle Hill buttercup

It is clear that continuing human intervention is needed to ensure its survival, but should scarce conservation resources be spared to do so? The cultural value of the Castle Hill buttercup is clear as it is only found in a small area within the Kura Tawhiti region, and this in itself may be sufficient to give it high conservation priority. However, of relevance are also its degree of morphological and ecological distinctiveness in relation to other endemic New Zealand alpine *Ranunculus*. The phylogenetic distinctiveness of this species is also relevant when determining its conservation value as it is applicable under the aesthetic (b) and utilitarian (c) criteria discussed by Crozier (1997). The phylogenetic distinctiveness of the Castle Hill buttercup is also the major subject of this thesis.

1.4 The Alpine Ranunculi of New Zealand

1.4.1 Distribution

The genus *Ranunculus* is common and cosmopolitan, with 300-500 species worldwide. Species with alpine distributions are found in most temperate world regions including North and South America, Europe, Asia and Australia. New Zealand has 16 species of alpine *Ranunculus*, all within section Epirotes. Two of these, *R. crithmifolius* and *R. haastii*, each have two subspecies. These taxa all form part of a monophyletic group that originated in New Zealand during the Late Tertiary Period and began to diversify with the onset of Pliocene mountain building (Lockhart *et al.*, 2001). A recent study by Lockhart *et al.* (2001) using DNA marker sequencing found that the New Zealand alpine Ranunculi are separated into 4 genetically distinct groups. Group I consists of *R. lyalli*, *R. buchananii*, *R. haastii*, *R. nivicola*, *R. verticillatus* and *R. grahamii*, group II consists of *R. sericophyllus*, *R. pachyrrhizus*, *R. viridis* and *R. pinguis*. The focus of this study, group III, contains *R. insignis*, *R. godleyanus*, *R. crithmifolius*, *R. enysii* and *R. gracilipes*, while group IV is restricted to a single species, *R. scrithalis*.

Lockhart *et al.*'s (2001) DNA sequencing studies have also established that two species of Australian alpine buttercup, *R. anemoneus* and *R. gunnianus* belong to groups I and II respectively, and dispersed to Australia from New Zealand during the Pleistocene. Of the New Zealand alpine Ranunculi, *R. insignis* and *R. verticillatus* are found in both main islands. The species found only in the South Island are *R. sericophyllus*, *R. pachyrrhizus*, *R. scrithalis*, *R. lyalli*, *R. buchananii*, *R. haastii*, *R. grahamii*, *R. godleyanus*, *R. crithmifolius*, *R. enysii* and *R. gracilipes*, while *R. nivicola* is found exclusively in the North Island. *R. viridis* is found in Stewart Island, while *R. pinguis* is found only on the sub-Antarctic Auckland Island and Campbell Island. This distribution is also shown in Table 1.2

Species	Locations found within New Zealand
<i>R. insignis</i>	North and South Islands

<i>R. verticillatus</i>	North and South Islands
<i>R. nivicola</i>	North Island only
<i>R. buchananii</i>	South Island only
<i>R. crithmifolius</i>	South Island only
<i>R. enysii</i>	South Island only
<i>R. gracilipes</i>	South Island only
<i>R. grahamii</i>	South Island only
<i>R. godleyanus</i>	South Island only
<i>R. haastii</i>	South Island only
<i>R. lyalli</i>	South Island only
<i>R. pachyrrhizus</i>	South Island only
<i>R. sericophyllus</i>	South Island only
<i>R. viridis</i>	Stewart Island
<i>R. pinguis</i>	sub-Antarctic Islands

Table 1.2 - Distributions of New Zealand alpine *Ranunculus*

1.4.2 Morphology

Morphologically, the New Zealand species vary widely in form and leaf shape. *R. lyalli* has large, entire peltate leaves up to 40cm in diameter and scapes up to 1.0m in height. In comparison, the leaves of *R. gracilipes* may only be 3cm long with bipinnasect divisions, the entire plant rarely exceeding 10cm in height. Morphology can also show extreme variation intraspecifically as well as between species; *R. enysii* is a good example of this. The least dissected specimens (formerly known as *R. berggrenii*) from the Carrick Range near Cromwell have leaves that are approximately 2cm in length, shallowly trilobate and almost orbicular in overall shape. The most divided leaves are palmate, with up to five ternately lobed leaflets and are found in Canterbury and Fiordland. Intraspecific variation can be correlated with the geographical distribution of the species (e.g. *R. enysii* and *R. insignis*), but this is not always the case (e.g. *R. verticillatus*).

1.4.3 Habitat

The New Zealand alpine Ranunculi are also found in a wide variety of habitats (Dave Havell, pers. comm., 2004; (Fisher, 1965). *R. lyalli* and the North Island form of *R.*

insignis occur in damp shady areas such as stream sides and gorges, while South Island *R. insignis* is often found in tussock grassland or shrubland. *R. grahamii* is usually found in snowfields, while *R. buchananii* is found in high altitude scree or on wet cliffs. *R. godleyanus* is found at the snowline fringe, typically above 2000m, often near snowmelt channels or temporary tarns. *R. sericophyllus* and *R. pachyrrhizus* occur in similar habitats to *R. godleyanus* but *R. pachyrrhizus* is found in the block schist mountains east of Mount Aspiring in the Central Otago mountain zone, while *R. sericophyllus* grows throughout the central Southern Alps in suitable habitats. *R. haastii* is found on coarse scree slopes, while *R. crithmifolius* grows in finer screes with high proportions of gravel, or in compacted scree. *R. crithmifolius* subsp. *paucifolius* is found only in the fine limestone debris at Castle Hill. Two other buttercups highly localised in their distribution are *R. scrithalis*, found on fine clay screes in the Eyre Mountains and *R. viridis* which is confined to the summits of granite outcrops in the Tin Range of Stewart Island. *R. pinguis* grows only on Auckland Island and Campbell Island on open stony ground and cliff ledges.

1.5 Taxonomic uncertainty of the Castle Hill buttercup

1.5.1 Taxonomic history

Although ecologically distinct, the morphological distinctiveness of the Castle Hill buttercup has been unclear, and taxonomic revisions have led to numerous taxonomic reassignments. Most recently, citing a number of morphological similarities, such as glaucous leaf surfaces with brown epidermal pitting, versus a relatively small number of differences, Fisher (1965) combined the three plants known as *R. crithmifolius*, *R. chordorhizos* and *R. paucifolius* into one species. That is, *R. crithmifolius* and *R. chordorhizos* were subsumed into *R. crithmifolius* subsp. *crithmifolius* whilst *R. paucifolius* was relegated to subspecies status: *R. crithmifolius* subsp. *paucifolius*. This classification recognises that the Castle Hill buttercup is morphologically similar

to *R. crithmifolius* subsp. *crithmifolius*, but that there is also considerable difference in shape of the leaves, which are much less dissected, with wider segments, than *R. crithmifolius* subsp. *crithmifolius*.

1.5.2 Research history

The botanical monograph “The Alpine Ranunculi of New Zealand” by F. J. Fisher (1965) provides the most recent overview of biological diversity for the group including studies on morphological diversity and breeding relationships. Included in this monograph were chromosome counts of all alpine *Ranunculus* species; except for *R. nivicola* with 96 chromosomes, the entire group is regarded as ancient hexaploids of the *Ranunculus* base number of eight (Fisher, 1965). Understanding of taxonomic relationships within the group has been further advanced by a recently published study on genetic diversity; “Phylogeny, dispersal and radiation of New Zealand alpine buttercups: molecular evidence under split decomposition”, a paper by Lockhart *et al.* (2001).

1.5.3 Molecular findings

The study by Lockhart *et al.* (2001) characterised a small number of accessions for all eighteen recognised taxa (species and subspecies) of New Zealand alpine buttercups and two Australian species through phylogenetic analysis of nuclear Internal Transcribed Spacer (ITS) and chloroplast (*J_{SA}*) DNA sequences. The authors found that the alpine Ranunculi of New Zealand consist of four phylogenetic groups, and that divergence of these groups began approximately 5 million years ago. This is an estimate that coincides with the onset of the late Tertiary orogeny in New Zealand (Batt *et al.*, 2000), suggesting that the first novel species of alpine buttercups in New Zealand may have evolved in response to the creation of new habitats and niches. These genetic studies indicate that the phylogenetic groups I and II correspond closely with Fisher’s (1965) “many petals, silky hair” group, while group III which includes the Castle Hill buttercup is equivalent with Fisher’s “few petals, coarse hair” breeding group. In their analyses Lockhart *et al.* (2001) also found evidence to suggest that the

species *R. sericophyllus* and *R. lyalli* were paraphyletic. In the study of Lockhart *et al.* taxon sampling was insufficient to draw conclusions about paraphyly of group III species. However, the preliminary chloroplast DNA results for this group were surprising, suggesting that the Castle Hill buttercup was genetically distinct from the other subspecies of *R. crithmifolius*. An important specific aim of this thesis has therefore been to examine in more detail the genetic diversity of species in group III: *R. insignis*, *R. godleyanus*, *R. enysii*, *R. crithmifolius* and *R. gracilipes*.

1.6 Geological history of New Zealand alpine buttercups

1.6.1 Glacial refugia

An interesting finding in the studies of Lockhart *et al.* (2001) was that the species *R. lyalli* and *R. sericophyllus* were found to be paraphyletic in analyses of two independent molecular markers. The authors hypothesised that this phenomenon may indicate regional speciation from distinct Pleistocene glacial refugia in the central South Island and the southern South Island. The last New Zealand glacial maximum ended approximately 10,000 years ago and is termed the Otiran glaciation (Gage and Suggate, 1958). The glacial advances during this period covered extensive areas near Kumara in north Westland and in the Waimakariri Basin on the eastern side of the Southern Alps. *Ranunculus crithmifolius* subsp. *paucifolius* is found only in the McCaskill reserve in the Kura Tawhiti area – a known hotspot for rare New Zealand plants, including a forget-me-not (*Myosotis colensoi*), two whipcord koromiko, (*Hebe cupressoides* and *H. armstrongii*) and a tussock (*Carex inopinata*). The Castle Hill region is thought to have been glaciated in the last glaciation period (Burrows and Moar, 1996; Gage, 1958, 1977; Gage and Suggate, 1958). The abundance of endemic plants in the region, or those with restricted distributions centred around this locale, suggests that Castle Hill may have been a glacial refugium where these plants survived. Alternatively, novel species may have evolved in the area after the glaciers retreated, creating new habitats available for colonisation.

1.6.2 Disjunct distributions

The existence of South Island glacial refugia may explain observations of north/south species disjunctions in the South Island. First discussed by Willet (1950), the most well known example of a species disjunction in the South Island is the “beech gap”, so called because there is little or no *Nothofagus* beech forest between the Taramakau and Paringa rivers on the west coast of the South Island, despite an apparently suitable habitat. North-south disjunct distributions have also been noted for many other taxa (Heads, 1998) including *Celmisia traversii* (Wardle, 1963) and *Drapetes laxus* (Burrows, 1965). Some have at times argued that these species distributions are geologically old and possibly due to events that occurred in the Oligocene or Miocene epochs (Cooper and Cooper, 1995; Heads, 1998; Heads and Craw, 2004; McGlone, 1985), others have suggested that such disjunctions arose during the Pleistocene (McGlone *et al.*, 2001; Trewick and Wallis, 2001; Wallis and Trewick, 2001). Willet (1950) for example suggested that the heavy glaciation of the South Island during the late Pleistocene caused the unusual South Island distribution of *Nothofagus* species by causing local extinction. Wardle (1963) similarly proposed that the high numbers of plants endemic to Southland/Otago and to the Nelson/Marlborough districts of the South Island is a result of plants surviving Pleistocene extinction events in these non-glaciated areas throughout the Otiran glaciation.

1.6.3 Biogeographic research

To date, relatively few attempts have been made to test hypotheses that explain disjunct distributions of New Zealand native plant species. Nevertheless, a general consensus from the study of genetic diversity of sequence data for many plant groups (Stoekler, 2001; Wagstaff and Garnock-Jones, 2000; Winkworth *et al.*, 2002) suggest that events of the Pleistocene may be a more appropriate explanation for observed species distribution patterns than earlier geological events. On a global scale, in recent years findings from both DNA and palynological studies emphasise the importance of Pleistocene climate change for understanding plant species distributions (Comes and Kadereit, 1998).

One of the few recent attempts to test hypotheses of the importance of glacial refugia in the South Island of New Zealand is the work by Heenan and Mitchell (2003). These authors applied phylogenetic techniques to morphological and ITS DNA sequence data in eight species of *Pachycladon* as well as one undescribed species. They considered the potential alpine habitat available during the last glacial maximum for *Pachycladon* species and concluded that *P. fastigiata* was likely to have been eradicated from the high Southern Alps by Pleistocene glacial activity. In contrast they argued that *P. enysii* may well have survived the Otiran in “nunataks”, ice-free mountain regions that protruded above the glacial ice sheet. Similar inferences for *in situ* survival of species have been suggested in the Northern Hemisphere (Schonswetter *et al.*, 2003; Schonswetter *et al.*, 2004; Stehlik *et al.*, 2002; Stehlik *et al.*, 2001) where large scale glaciations once covered much of the European Central Alps.

1.7 Hybridisation

1.7.1 Hybridisation in New Zealand

Glacial refugia may act passively and allow species to survive *in situ*, however it has been argued that they may also act as species pumps to promote species diversification (Willis and Whittaker, 2000), possibly through hybridisation-differentiation cycles (Ehrendorfer, 1959). Indeed hybrid speciation has been suggested as playing a significant role in the evolution of the New Zealand flora (Rattenbury, 1962). However, its frequency of occurrence and true evolutionary significance in the New Zealand flora remains to be tested.

1.7.2 Hybridisation of New Zealand alpine buttercups

At present there are only limited molecular data available on the Castle Hill Buttercup that might indicate its closest genetic relatives and origins. Nevertheless, analyses of nuclear ITS (nITS) sequences suggest a close phylogenetic relationship with *R. crithmifolius* subsp. *crithmifolius*, whilst analyses of chloroplast DNA (cpDNA) sequences have suggested closer relationships with *R. insignis* and *R. enysii* (Lockhart *et al.*, 2001). This discrepancy of phylogeny may be explained if the species is hybrid in origin; different genetic lineages may be evident because cpDNA is maternally inherited in *Ranunculus* (Corriveau and Coleman, 1988), while nuclear DNA is biparental.

Several other naturally occurring alpine Ranunculi are thought to be hybrids, and Fisher (1965) compiled an extensive list of putative natural and experimental hybrids. *R. crithmifolius* subsp. *crithmifolius* has been suggested to hybridise with *R. insignis* in the field and will produce fertile hybrids with this species under cultivation. At the time of his study (pre 1965) Fisher mentioned that *R. insignis* and *R. crithmifolius* subsp. *paucifolius* were often seen flowering at the same time at Castle Hill, an observation that can still be made today.

1.8 Conservation genetics of the Castle Hill Buttercup

Most conservation genetic studies that seek to help evaluate threatened species status and determine conservation priority involve characterisation of the degree of genetic distinctiveness at neutral gene loci. Interpretation of the data from these loci may not be straightforward in interspecific studies because plants such as *Ranunculus crithmifolius* subsp. *paucifolius* and its relatives are products of alpine species radiations, phenomena in which hybridisation may play a significant role (Ehrendorfer, 1959; Stebbins, 1959). Sensible management of alpine plant species requires an understanding of the radiation events, the underlying genetic processes and the effect of these processes on speciation. The present study of *Ranunculus crithmifolius* subsp. *paucifolius* illustrates the potential and problems of genetic data when used for conservation purposes.

1.9 Focus of this research

1.9.1 The context of this project

This study attempts to contribute to a better understanding of alpine plant biodiversity in New Zealand through specific studies made on alpine *Ranunculus*. These studies have involved DNA sequence determinations and phylogenetic analyses of the nITS regions and the chloroplast J_{SA} (cp J_{SA}) region from closely related species belonging to the “group III” New Zealand alpine buttercups (*R. crithmifolius*, *R. enysii*, *R. insignis* and *R. gracilipes*) recognised by Lockhart *et al.* (2001). Both molecular markers were implemented successfully in the earlier work of Lockhart *et al.* (2001), and although they have some limitations (as will be discussed), additional taxon sampling with these markers has allowed the testing of specific hypotheses that arose from this earlier work.

Additionally, Amplified Fragment Length Polymorphism (AFLP) and Inter – Simple Sequence Repeat (ISSR) fingerprint profiles were used to provide finer resolution analysis of the population of *Ranunculus crithmifolius* subsp. *paucifolius*, as these methods provide a relatively fast means of analysing many genetic loci at once. ISSR (Ziętkiewicz *et al.*, 1994) is a potentially rapid and powerful technique now being widely adopted by plant biologists for studying hybrid species (Garcia-Maroto *et al.*, 2003; Wolfe *et al.*, 1998). ISSR was also successfully used by Smissen *et al.* (Smissen *et al.*, 2003) to genetically identify populations of the New Zealand alpine genus *Raoulia*. Analyses of AFLP (Vos *et al.*, 1995) profiles are considered more robust than ISSR methods (Archak *et al.*, 2003; McGregor *et al.*, 2000) and provide resolution at intraspecific levels on a finer scale than nITS sequences (Wolfe *et al.*, 1998). These studies were implemented with the aim of providing a measure of the genetic diversity of the remaining Castle Hill buttercup plants, and to find the extent and significance of diploid hybridisation in the Castle Hill buttercup population.

1.9.2 Plant molecular markers

Nuclear ITS and chloroplast markers have made significant contributions to the field of plant phylogenetic reconstruction. Together they can provide much phylogenetic information, and the nITS region has also been used to verify the occurrence of hybridisation in plant studies (Andreasen and Baldwin, 2003). nITS ribosomal DNA sequences are one of the mainstays of plant molecular phylogenetics (Álvarez and Wendel, 2003). Since its introduction the nITS region has been analysed in numerous recent phylogenetic studies (Álvarez and Wendel, 2003), due in large part to the advantages of simple experimental protocols and perceived low mutation rates coupled with high amounts of information (Baldwin *et al.*, 1995).

Chloroplast DNA data have also been used extensively in phylogenetic studies (Olmstead and Palmer, 1994). The major advantages of studying cpDNA are its genetic simplicity and its stability. The presence of multiple chloroplasts per cell, and multiple genomes in each chloroplast make experimental work simple and have helped make it the most widely used source of genetic data for plant phylogenetic studies (Álvarez and Wendel, 2003).

1.9.3 Problems with commonly used markers

However, the scientific community has become reliant on the tools of cpDNA and nuclear ribosomal DNA (nrDNA), often to the exclusion of other tools that may be better suited to the task at hand (Álvarez and Wendel, 2003). The highly repetitive nature of nrDNA markers such as ITS gives it properties that may make it less suitable for phylogenetic studies than other genes in some circumstances. There are many copies of nrDNA in plant genomes and it has been recognised for some time that these are subject to “concerted evolution” (Arnheim *et al.*, 1980; Fuertes Aguilar *et al.*, 1999). That is, the different sequences tend to homogenise towards the same sequence by gene conversion or high-frequency crossing over. In most studies, PCR is used to amplify a single consensus sequence that is assumed to be representative of all the different sequences. Unfortunately, concerted evolution is not uniform across repeats or taxa (Small *et al.*, 2004), so nrDNA sequences may not be homogeneous.

In automated sequencing these differences can be read as polymorphic bases which may subsequently remain undetected, be ignored or the strongest peak read as the actual base at that position. In experimental work, differences across copies can cause PCR to preferentially amplify a particular sequence over others due to differences in primer affinity or variable copy numbers of the different sequences. The multi-copy nature of the nITS marker also means that paralogous sequences are possible; i.e. sequence divergence that occurs soon after gene duplication, leading to two different sequences descended from a common ancestor in the same species.

Chloroplast DNA also has caveats which should be taken into consideration before it is used in phylogenetic studies. It is generally assumed that cpDNA is non-recombining, but some evidence has been shown to the contrary (Marshall *et al.*, 2001). Another generally accepted view is that inheritance is uniparental, but exceptions have been noted e.g. *Geranium* and *Pisum* (Corriveau and Coleman, 1988; Wolfe and Randle, 2004). Evidence has also arisen to challenge the common view that chloroplast genomes are simple and stable. Wolfe and Randle (2004) reviewed instances of heteroplasmy in the chloroplast genome and the transfer of segments of chloroplast DNA to the mitochondrial or nuclear genomes.

In hybrid studies, bifurcating trees from cpDNA will identify a hybrid species as belonging to the clade of one parent, without revealing its mixed origin. If an independent nuclear marker such as ITS was inherited from the other parent and was included in the same study the two phylogenies will show different closest relatives, thus identifying the parentage (e.g. the hybrid has the ITS of species A and the cpDNA of species B). However this trait can be equally disadvantageous if the two markers are derived from the same parent, as both phylogenies will show this, effectively masking a hybrid origin.

1.9.4 Multilocus DNA fingerprinting

Some of the problems associated with using single-locus DNA sequences can be resolved by using multilocus DNA fingerprints (MLF). Amplifying and analysing many genetic loci at once means fingerprints have much finer resolution than single locus analyses. This is especially useful in intraspecific studies as individuals can be

distinguished by their fingerprint profiles. This aids in the discovery and classification of hybrid species. One of the disadvantages of multilocus fingerprinting when compared with DNA marker sequencing is that there is no way of determining whether bands of the same size are truly homologous unless fingerprint bands are isolated and sequenced. Additionally, no information is known about the nature of the loci; they are essentially anonymous. This means that uninformative or unsuitable loci are given the same weight as all others. However, problems of doubtful homogeneity and anonymity are overcome by the large number of loci that can be amplified and analysed at once, greatly improving the tree-building properties of the data.

1.9.5 Phylogenetic methods

Studies in this thesis have also involved phylogeographic analyses that seek to test for the existence of regionally specific biodiversity patterns. A feature is the use of the phylogenetic network analysis method Neighbor-Net (Bryant and Moulton, 2004) to help visualise species phylogenies, in contrast to “gene tree phylogenies”. Standard phylogenetic method using neighbor-joining phylogenies are also used in this study for investigating the nature of the Castle Hill buttercup.

1.10 Hypotheses

The experiments described in subsequent chapters were designed to test the following specific hypotheses:

(I) that there is genetic distinctiveness between regions among the group III (Lockhart *et al.*, 2001) New Zealand alpine *Ranunculus*.

This group contains species which are closely related to the Castle Hill buttercup as evidenced in both molecular (Lockhart *et al.*, 2001) and classical breeding studies (Fisher, 1965), and may shed some light onto the origins of the Castle Hill buttercup

(II) that the Castle Hill buttercup is genetically distinct from its closest relatives in the group III (Lockhart *et al.*, 2001) New Zealand alpine *Ranunculus*.

The results of this hypothesis are relevant to the evaluation of the conservation status of the Castle Hill buttercup, and to the management of the McCaskill Reserve and the Kura Tawhiti area.

(III) that the Castle Hill buttercup has a diploid hybrid origin.

This issue is also relevant to the evaluation of the conservation status of the Castle Hill buttercup and will help us to understand the evolutionary relationship between *Ranunculus crithmifolius* subsp. *paucifolius* and its closest genetic relatives.

Although not accepted taxonomic nomenclature, in the interests of brevity and comprehensibility in this thesis the abbreviations *R. c. paucifolius* and *R. c. crithmifolius* have been used to refer to *Ranunculus crithmifolius* subsp. *paucifolius* and *Ranunculus crithmifolius* subsp. *crithmifolius* respectively at many points.

2 Materials and Methods.

2.1 Sampling

Samples used in this study were collected from South Island locations in November 2002 by Peter Lockhart, David Havell, Hannah Riden, Sandra Martin, and Morore Piripi. Additional samples were collected from the Richmond Range, Kura Tawhiti, Ben Ohau and Dansey's Pass in December 2003 by David Havell, Wim Hordijk, Richard Carter and Morore Piripi. Herbarium samples were used for some locations which were not available from recent collections (See appendix A for details). Some DNA sequence information was previously published by Lockhart *et al.* (2001). Figure 2.1 shows the locations of all accessions used in this study. Collected samples were stored on self-indicating silica gel crystals to dessicate samples and prevent microbial degradation and contamination (Chase and Hills, 1991). Some samples were stored fresh at -80°C .

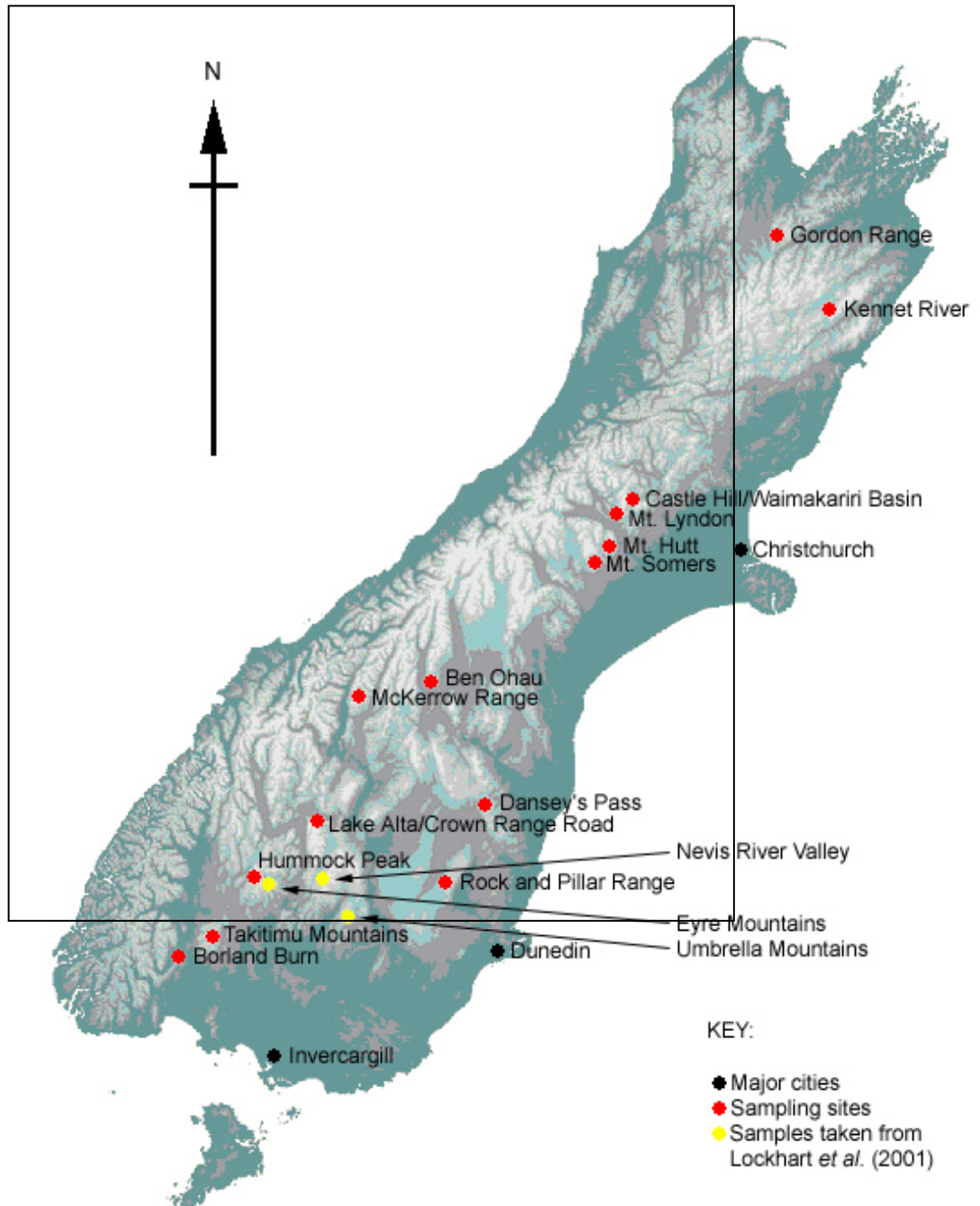


Figure 2.1 - South Island map showing locations of accessions used in this study

2.2 DNA extraction

2.2.1 CTAB extraction method

Two DNA extraction protocols were used. The first protocol was modified from that used by Doyle and Doyle (1990). Small quantities of each sample were used (approximately 200 mg dry weight) in each extraction process. Each batch of extractions contained 4-8 samples. The extraction protocol for each sample was identical, the portion of dry tissue to be used in each extraction was cut off using a sterile scalpel blade and transferred to a 1.7 mL microcentrifuge tube (Axygen Scientific). The lower portion of the tube was placed into liquid nitrogen for approximately one minute (min) to freeze the tissue sample. The frozen sample was then crushed using a glass crushing tool. This tool was made prior to extraction from a glass Pasteur pipette. The Pasteur pipette was heated in a Bunsen burner flame, close to where the pipette starts to narrow. As the glass melted the two sections of the Pasteur pipette were pulled apart. The thin section was discarded and the melted tip of the thicker section was then melted further in the Bunsen flame whilst turning it, to create a glass bulb on the end. This bulb was a good size to crush the frozen sample in the 1.7 mL microcentrifuge tube. To avoid contamination a new crushing tool was created for each sample to be used in the DNA extraction.

Typically samples were refrozen and crushed two or three times to create a fine powder in the base of the microcentrifuge tube. This powder was suspended in 500 μ L of cetyl trimethyl ammonium bromide (CTAB) extraction buffer (2% [w/v] CTAB, 1% [w/v] polyvinyl-pyrrolidone [PVP], 1.4 M sodium chloride [NaCl], 100 mM Tris-HCl [pH 8] and 20 mM ethylenediaminetetra-acetic acid [EDTA]). This was mixed gently using the crushing tool to dislodge the powdered plant sample from the sides and base of the tube. This mixture was then incubated in a 60°C heating block for 10 minutes. The tube was allowed to cool for approximately five minutes to below 60°C, after which 500 μ L of chloroform (BDH) was added to the test tube and mixed vigorously by inverting the test tube several times. This mixture was left to sit at room temperature for 2 minutes and then centrifuged at $16,060 \times g$ for 1 minute to separate the two liquid phases. The DNA and CTAB remain in the upper aqueous

phase, while the chloroform and cellular components form the lower organic phase. Polysaccharide precipitates and cellular debris aggregate at the interface. The upper aqueous phase containing the DNA was carefully removed and transferred to a fresh 1.7 mL microcentrifuge tube. A pipette tip which had the lower 5 mm removed was used for this transfer. Removing the end of the pipette tip is important at this stage as it avoids mechanical shearing of the DNA, which may otherwise occur with a smaller bore.

To the collected aqueous solution, 500 μ L iso-propanol (BDH) was added and the two solutions were mixed by gentle inversion. At this point the nucleic acids appeared either as a brownish-white, cotton-wool like precipitate or if there had been any degradation, a narrow band of flocculent formed which floated at approximately half the depth of the solution. In both cases any of these visible precipitates were transferred to a clean 1.7 mL microcentrifuge tube using a wide bore pipette tip. Approximately 500 μ L of 80% (v/v) ethanol was added to the microcentrifuge tube. The tube was then very gently inverted to mix. It was important at this stage not to agitate the tube too vigorously, as the DNA was prone to collapsing on itself trapping contaminants within the DNA. If this did occur, it became very difficult to clean the DNA any further or to later resuspend the DNA.

Washes were repeated with 500 μ L 80% (v/v) ethanol up to 4 times until the DNA was only slightly discoloured and had a cotton-wool like appearance. The DNA in 80% (v/v) ethanol was centrifuged to pellet the DNA at $16,060 \times g$ for 1 min. The supernatant was discarded and the pellet allowed to air dry. Each pellet was resuspended in 50 μ L Milli-Q water. Resuspended extracted DNA was run on agarose gels (refer to section 2.3) to determine if the DNA extraction had been successful. This modified CTAB method typically gave DNA suitable for Polymerase Chain Reaction (PCR) at concentrations of 10-20 ng/ μ L.

2.2.2 Qiagen DNeasy extraction method

The second DNA extraction protocol employed the Qiagen DNeasy plant mini-kit (Qiagen). DNA extracted by this method was used for samples intended to be run as

Inter-Sequence Simple Repeat (ISSR) or Amplified Fragment Length Polymorphism (AFLP) fingerprints as the PCR reactions for these techniques gave clearer bands when column extracted DNA was used. Samples were extracted as per the kit instructions, including the optional extra centrifugation step. A small aliquot of eluate was then electrophoresed on an agarose gel (see section 2.3) to determine the success and DNA concentration of the extraction. Typically, from 100 mg wet or 20 mg dry leaf tissue, 1000-2000 ng of DNA would be recovered. This amount would vary depending on the age of the sample and the effectiveness of the silica gel: much less DNA was obtained from old (3-6 years) or herbarium samples and those with pink silica gel, indicating that the gel had reached its water absorption capacity.

2.3 Agarose gel electrophoresis

Agarose gels were made by adding LE agarose powder (Roche) to 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) to a concentration of 1% (w/v). This mixture was heated in a microwave until all agarose had dissolved and was cooled to below 55°C before it was poured into a gel tray. Molten agarose was stored in an incubator at 60°C ready for use.

For agarose DNA electrophoresis, 5 µL aliquots of the DNA extraction were mixed with 1 µL of 10× loading buffer (27.5% [w/v] Ficoll Ty 400 [Pharmacia], 0.44% [w/v] bromophenol blue [Serva electrophoresis] and 0.44% [w/v] xylene cyanol [Sigma]), and 4 µL of Milli-Q water and then loaded into a 1% agarose/1× TAE buffer gel. A size marker, 1Kb Plus DNA Ladder (Invitrogen, see appendix B) was included on the gels, as well as a High DNA Mass Ladder or Low DNA Mass Ladder (Invitrogen, see appendix B) depending on the expected size of the product, to quantify the amount of DNA present. Ladders were used at half the recommended concentration. Samples were electrophoresed at 5.5 V.cm⁻¹ in 1× TAE buffer. Following electrophoresis the DNA samples were stained with ethidium bromide solution (1.5 mg.L⁻¹) for 20 min. Gels were destained in Milli-Q water when background fluorescence was high. Stained gels were visualised by ethidium bromide fluorescence on a UV transilluminator (wavelength 302 nm, Bio-Rad) and a digital

photograph of the illuminated gel was taken using the supplied video camera and Bio-Rad Quantity One software (4.4.0). The band in the High DNA Mass Ladder (which is of known mass) that most closely matched the fluorescence of the extraction was used to estimate the DNA concentration of the extraction. A typical example of agarose gel electrophoresis can be seen at Figure 2.2.

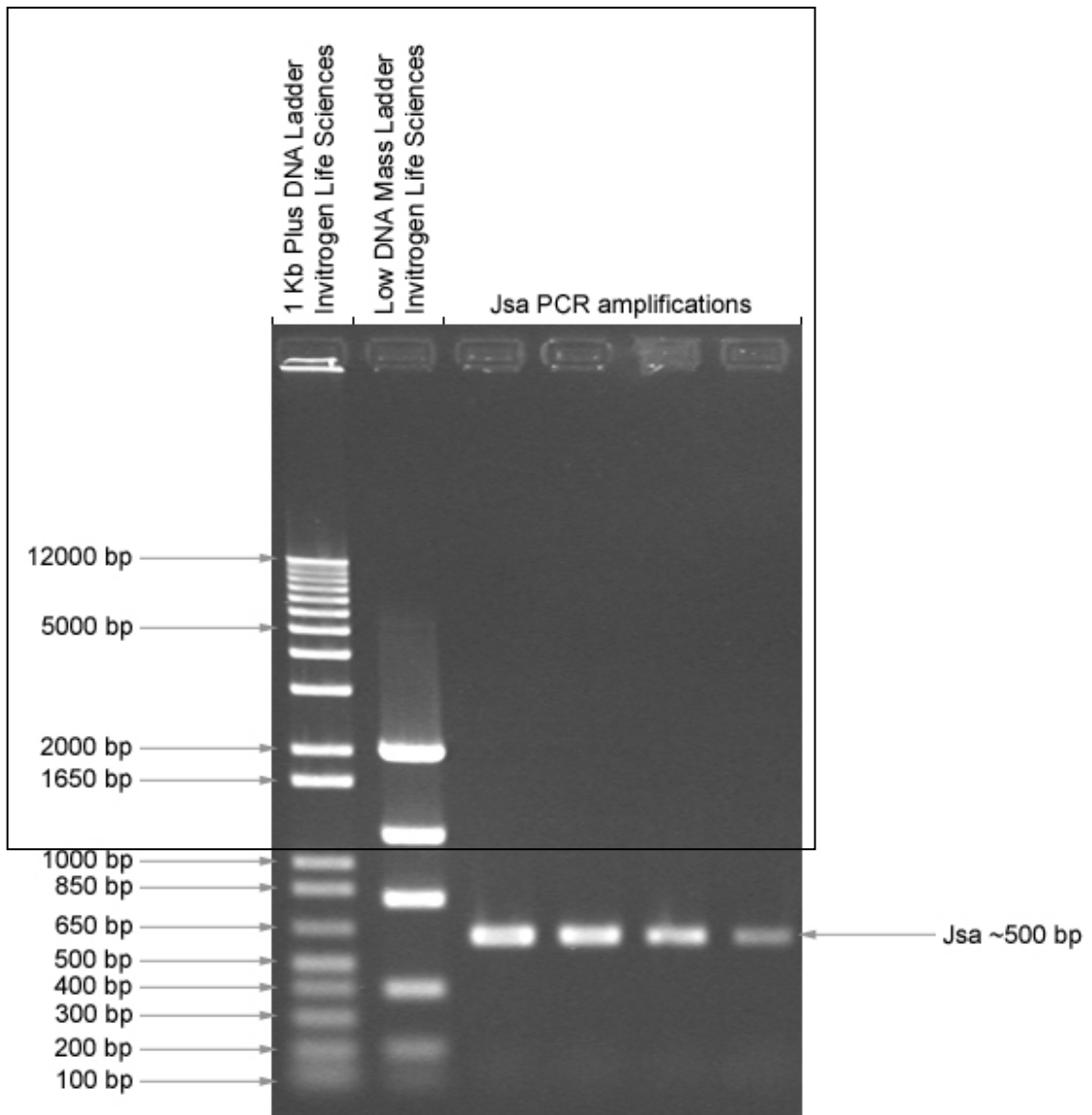


Figure 2.2 - Example of an agarose gel containing size and mass ladders and J_{SA} PCR amplification products

2.4 PCR amplification of marker regions for direct sequencing

PCRs contained 1× PCR buffer (containing 1.5 mM MgCl₂ [Roche]), 250 μM of each dinucleotidetriphosphate (dNTP), 10 pmol each of a forward and reverse primer, 1 U *Taq* DNA polymerase (Roche) and 1 μL 10-100ng DNA template in a total volume of 20 μL. PCRs were routinely supplemented by 1M betaine solution (Sigma) which is known to enhance PCR of difficult templates (Frackman *et al.*, 1998).

To minimise the possibility of cross-contamination between reactions when preparing multiple amplifications the DNA template was always added last. One negative control, containing 1 μL Milli-Q H₂O in place of the DNA template, was always run with each reaction set.

ITS and J_{SA} primers used were those used by Lockhart *et al.* (2001) (see appendix C for primer sequences). The thermocycling protocol for ITS amplification was as follows: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 48°C for 30 sec, 72°C for 1 min, followed by 1 cycle of 72°C for 5 min. For J_{SA} amplification the denaturing step (94°C 30 sec) was changed to 94°C for 1 min and the primer annealing step (48°C for 30 sec) was changed to 55°C for 1 min.

Following thermocycling, a 5 μL aliquot of each amplification was electrophoresed on a 1% (w/v) agarose gel as described previously (section 2.3) to determine the success of individual reactions. Reactions were electrophoresed with the 1 Kb Plus DNA Ladder as a size standard and the Low DNA Mass Ladder (Invitrogen) as a quantity standard.

2.5 Cleanup of PCR for direct sequencing

Usually, PCR products exhibited only one discrete band on the agarose gel. In this case an enzymatic cleanup procedure was carried out on the PCR product. 2 μL of Shrimp Alkaline Phosphatase (SAP) (1U/μL [USB]) and 1 μL of Exonuclease I (ExoI) (10U/μL [USB]) were added to the PCR product. The PCR product was then

incubated at 37°C for 30min. then 80°C for 15 minutes. SAP removes the phosphate group from unincorporated dNTPs and ExoI removes unincorporated single stranded primers which prevents participation of these molecules in the sequencing reaction. The incubation at 80°C denatures both SAP and ExoI so that they do not degrade the components of the sequencing reaction.

In cases where two or more bands were observed on the initial gel, the remaining PCR product (15 µL) was run on a 1% (w/v) agarose electrophoresis gel (refer to section 2.3). The band corresponding to the size of the fragment of interest was cut from the agarose gel using a sterile scalpel blade and the DNA extracted using the Perfectprep PCR purification kit (Eppendorf) according to the manufacturers instructions.

2.6 DNA sequencing protocol

Dideoxy sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions contained 2 µL of ready reaction premix, 1× BigDye Terminator v3.1 Sequencing Buffer, 3.2 pmol of primer for sequencing in the desired direction and the appropriate amount of PCR product template in a total volume of 20 µL. The amount of PCR product added to the reaction was calculated by dividing the length of the DNA fragment in base pairs by 20 to give the amount of DNA in ng. The mass of DNA in ng was converted to a volume amount (µL) based on the quantification of the DNA as described in section 2.4 above.

The sequencing reaction thermocycling protocol was as follows: 27 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. All temperature ramping speeds were set to 1°C/sec.

Sequence reactions were analysed using the capillary separation service offered by the Palmerston North Allan Wilson Centre Genome Service using an ABI3730 capillary sequencer. Capillary separation was carried out in accordance with the manufacturers instructions.

2.7 ISSR PCR protocol

ISSR primers from the University of British Columbia primer set #9 were used in this study. Preliminary screening of the primers was performed by Dr. Kim McBreen. Primers that showed strong amplification (#811, #835 and #844 (for sequences see appendix C)) were selected for use with *Ranunculus* spp. in this study.

ISSR PCR reactions contained 1× PCR buffer, 30 nmol of supplementary MgCl₂, 1× betaine, 250 μM of each dNTP, 1 U *Taq* DNA polymerase, 20 pmol of ISSR primer and 1 μL (200-500 pg) DNA template in a total volume of 20 μL.

The ISSR PCR reaction thermocycling protocol was as follows: 94°C for 2 min, then 35 cycles of 94°C for 1 min, 51.5°C for 1 min, 72°C for 1 min, followed by 72°C for 5 min. Amplified DNA fragments were stored at 4°C for no more than 24 hours before being subjected to Polyacrylamide Gel Electrophoresis (PAGE).

2.8 AFLP PCR protocol

For a diagrammatical explanation of the AFLP method, see Fig. 2.3.

2.8.1 Restriction endonuclease digestion of DNA

AFLP digests contained 1× reaction buffer (50 mM potassium acetate [KOAc] [Sigma], 10 mM magnesium acetate [MgOAc] [Sigma] and 10 mM Tris-HCl [pH 7.5]), 10 U of *Eco*RI (Roche), 10 U of *Mse*I (New England Biolabs), and 250ng of genomic DNA in a total volume of 25 μL.

Digestion reactions were incubated at 37°C for 2 hours. 5 μL of the digested DNA template was then electrophoresed on an agarose gel (see section 2.3), along with 2 μL of an undigested DNA template as a control. Staining, rinsing and photography of the agarose gels were carried out as in section 2.3. A properly digested sample appeared as a smear down the length of the gel and complete absence of high

molecular weight DNA. After a complete digest had been confirmed, the reactions were incubated at 70°C for 5 min to denature the restriction enzymes. Restriction digests were stored at -20°C.

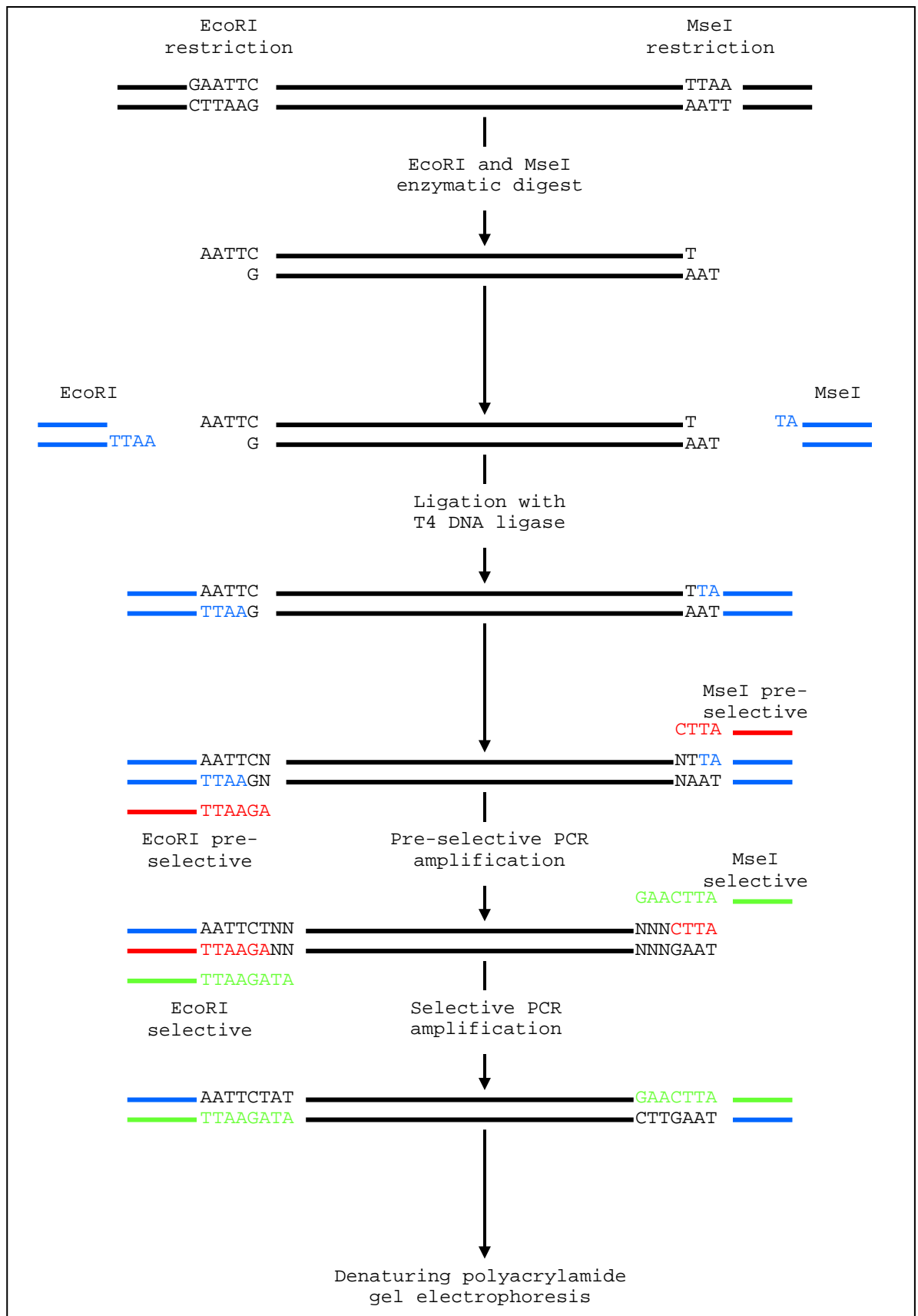


Figure 2.3 - Diagram of the AFLP reaction (modified from (Clarke, 2001))

2.8.2 Preparation of oligonucleotide linkers

Double stranded oligonucleotide linkers were prepared by annealing two single stranded oligonucleotides (for sequences see appendix C). The *EcoRI* annealing reaction contained 45% (v/v) 10:1 TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]), 5 pmol *EcoRI* linker 1 and 5 pmol *EcoRI* linker 2. The *MseI* annealing reaction contained 45% (v/v) 10:1 TE buffer, 50 pmol *MseI* linker 1 and 50 pmol *MseI* linker 2. These reactions were both performed in a final volume of 100 μ L. The reactions were heated to 95°C for 4 min and then allowed to cool slowly on the benchtop to room temperature as slow cooling favours perfect annealing of the linkers. The *EcoRI* and *MseI* linkers were stored at -20°C for later use.

2.8.3 Ligation of linkers to restriction fragments

Ligation reactions contained 1 \times Ligation Reaction buffer (Roche), 5pmol of double-stranded *EcoRI* linker and 50pmol of double-stranded *MseI* linker, 1 Weiss U of T4 DNA ligase (Roche), and 5 μ L of DNA digest in a total volume of 20 μ L. The ligation reactions were incubated at 37°C for 3 hours. Ligation reactions were stored at -80°C for further use.

2.8.4 Pre-selective amplification of ligation products

Pre-selective amplification (pre-amp) reactions contained 1 \times PCR buffer, 1M betaine 250 μ M of each dNTP, 10 pmol of *EcoRI*+A pre-amp primer and 10 pmol *MseI*+C pre-amp primer, 1 U *Taq* DNA polymerase (Roche) and 1 μ L ligation template were in a total volume of 20 μ L.

The AFLP pre-amp reaction thermocycling protocol was as follows: 20 cycles of 94°C for 30 sec, 56°C for 1 min, 72°C for 1 min. All temperature ramping speeds were set to 1°C/sec.

2.8.5 Selective amplification of pre-amp PCR products

Selective amplification primers were chosen based on previous work (Andrew Clarke, pers. comm.). Selective amplification reactions of pre-amp PCR products contained 1× PCR buffer, 50 mM supplementary MgCl₂, 250 μM of each dNTP, 10 pmol *MseI*+CAAG selective primer and 10 pmol *EcoRI*+ATA selective primer, 1 U *Taq* DNA polymerase, and 1 μL of pre-amp PCR product in a total volume of 20 μL (for sequences see appendix C).

A modified touchdown PCR AFLP selective amplification thermocycling protocol was used as follows: 94°C for 2 min, 12 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min. 24 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min., followed by 72°C for 5 min.

Selective amplification PCR products were run out using PAGE.

2.9 PAGE of AFLP and ISSR PCR products

AFLP and ISSR polyacrylamide gels were prepared electrophoresed and stained using the method of Andrew Griffith (pers. comm.). This method is an adaptation of the Vos *et al.* (1995) and Gibco (*c.* 1998) recommended methods for AFLP.

2.9.1 Preparation of plates for PAGE

To prepare an acrylamide gel for pouring, the long and short glass plates were first cleaned thoroughly. This was done by scrubbing the plate with detergent under running water. The plate was then dried with a paper towel, and cleaned with 70% (v/v) ethanol which was wiped off with another paper towel. This process was repeated twice more, so that each plate was washed three times. After the final wash, the plates were cleaned once more with ethanol, and wiped dry with Kimwipes (Kimberly-Clark) to remove any lint from the cleaned surfaces.

Only one side of each plate was cleaned, so it was essential to note which side it was, as this side would be facing the gel itself. It was important to ensure that nothing touched or dirtied the cleaned surface, as this would affect the pouring and subsequent running and staining of the gel.

The cleaned side of the long plate was treated with Windshield Rain Repellent (Prestone) solution according to the manufacturer's instructions. The Rain Repellent was allowed to dry, and the plate was then cleaned with 3 mL of 99.7-100% (v/v) ethanol. Both Rain Repellent and ethanol were applied with Kimwipes.

The cleaned side of the short plate was treated with a solution of Bind-Silane (Pharmacia). This solution was prepared as follows: 2 mL 99.7-100% (v/v) ethanol, 0.5% (v/v) glacial acetic acid (AnalaR BDH) and 0.05% (v/v) Bind-Silane. The solution was prepared in a 2 mL microtube (Gibco BRL) and spread evenly over the plate using a Kimwipe. The Bind-Silane solution was allowed to dry, then excess Bind-Silane was removed from the plate by three successive washes of 3 mL 99.7-100% (v/v) ethanol applied with a Kimwipe.

The plates were then assembled clean sides together, in a glass sandwich separated by 0.4mm plastic spacers (Gibco BRL). When doing this, it was important to make sure that the surfaces of the plates did not touch, as this would lead to the gel adhering to both plates rather than the short plate alone. The glass sandwich was then firmly seated in an S2 casting boot (Gibco BRL).

2.9.2 Preparation of polyacrylamide gel matrix

70 mL of 5% (w/v) denaturing polyacrylamide gel solution was prepared as follows: 8M urea (USB), 10% (v/v) of 50% (w/v) 19:1 acrylamide (BDH):bis-acrylamide (BDH) solution and 1× Tris Borate EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The solution was then filtered through two Whatman No. 1 filter circles. Immediately prior to pouring the gel, 350 μ L of 10% (w/v) ammonium persulphate (APS [Bio-Rad]) solution and 35 μ L of N,N,N',N'-

tetramethylethylenediamine (TEMED [BDH]) were added to the filtrate and mixed thoroughly. The solution was then immediately drawn up into a needleless 50 mL syringe. The acrylamide solution was dispensed from the syringe into the glass sandwich through the hole in the base of the casting boot, taking care that no bubbles were formed in the process. Once the gel had been poured, two 5.7 mm (point-to-point spacing) shark-tooth combs (Gibco BRL) were inserted approximately 6 mm into the top of the gel in reverse orientation to form the gel wells. Great care was taken to ensure the combs were inserted perfectly horizontally and even, as this would affect the straightness of the gel run. Once the combs were satisfactorily placed, they were held in place by three large bulldog clips. The top end of the gel was sealed with plastic cling film to prevent the gel dehydrating, and the boot/gel assembly was laid at a 5° angle using the legs of the casting boot as support, and left overnight to allow the acrylamide solution to polymerise completely.

2.9.3 Loading and electrophoresis of the polyacrylamide gels

Once the gel was completely polymerised, the cling film, casting boot, bulldog clips and combs were removed from the gel sandwich. The outside surfaces of the glass plates were then cleaned with ethanol to remove any dried buffer or polyacrylamide. The gel sandwich was loaded into the S2 electrophoresis rig (Gibco BRL), and the buffer tanks were filled with 1× TBE buffer. A 2 mL disposable plastic pipette was used to flush out the sample well to remove crystals of urea, polyacrylamide fragments and bubbles. The gel was then pre-run for 30 minutes at 70W.

A 100 bp DNA Ladder (Invitrogen, see appendix B) was used as a size standard on acrylamide gels. The ladder was prepared as follows: 198 µL formamide loading dye (98% [v/v] formamide (BDH), 10 mM EDTA, 0.05% [w/v] bromophenol blue and 0.05% [w/v] xylene cyanol) and 2 µL 100 bp DNA Ladder (1µg/ µL). The solution was prepared in a 200 µL PCR microtube and then irreversibly denatured by heating to 94°C for 5 minutes in a thermocycler. The formamide ladder solution was stored at -20°C for further use.

Both ISSR and AFLP amplification fragments were prepared by adding 15 μ L formamide loading dye to the entire 20 μ L PCR reaction, and irreversibly denaturing at 94°C for 5 minutes in a thermocycler.

All ISSR and AFLP reactions were performed in duplicate and loaded side-by-side to ensure consistency of the bands. Where possible, duplicate reactions were performed from independent DNA extractions. Where there was insufficient material for two DNA extractions, a duplicate reaction\series of reactions would be performed on the same DNA from the beginning of the reaction process.

The two shark-tooth combs were inserted into the gel so that the teeth were resting on the gel surface. The gels were loaded from the left side, the first two lanes of a gel were always loaded with 7 μ L of denatured 100 bp DNA Ladder to allow easy orientation of developed gels. 7 μ l of denatured PCR product was loaded in each well, interspaced at regular intervals with a lane of 100 bp DNA Ladder. The final lane of a gel was always loaded with 100 bp DNA Ladder.

Acrylamide gels were run at 70W until the xylene cyanol dye front had just run off the end of the gel. This usually took about three hours.

2.9.4 Silver staining protocol of polyacrylamide gels

Milli-Q water was used at all stages of this protocol. At the start of electrophoresis, 4 L of fresh developing solution (280 mM anhydrous Na₂CO₃ [AnalaR BDH] in H₂O) and 2 L of Milli-Q H₂O were chilled to ~5°C by placing the solutions at -20°C until required.

When electrophoresis had finished, the gel sandwich was removed from the gel rig, and the plastic spacers removed. The glass plates were then pried apart, and the short plate to which the gel was attached was placed in a large tray containing 4 L of 10% (v/v) acetic acid. The tray was agitated on a mechanical shaker for 1 hour, this removed urea from the gel. The gel was then removed from the acid and placed in another plastic tray. 2 L of the acetic acid was retained and cooled to ~5°C by placing

the solution at -20°C until required. Acetic acid was removed from the gel by agitating it in 2 L of water for approximately 4 minutes. The water was then poured off and the rinsing repeated twice more with a fresh 2 L of water used for each rinse.

After rinsing, the gel was transferred to another tray containing 3 L of staining solution (6 mM AgNO_3 [BDH] and 0.15% [v/v] formaldehyde [37% (v/v) BDH] in H_2O) and agitated for 1 hour. The 4 L of pre-chilled developing solution was then made up to 0.16% (v/v) formaldehyde and 50 μM sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$ [BDH]).

The gel was removed from the stain and placed in a tray containing the 2 L of pre-chilled H_2O for not more than five seconds to quickly cool the gel and to remove excess stain. The gel was transferred to another tray containing 2 L of the developing solution and immediately agitated by hand to disperse the brown precipitate formed. The chilled H_2O was retained. The gel was then agitated by mechanical shaker until the first DNA bands became visible. At this point, the gel was transferred to another tray containing the remaining 2 L of developing solution and developed until the bands reached the desired intensity without allowing the background to become too dark. To stop the developing reaction and fix the gel, the chilled 2 L of 10% acetic acid reserved from the previous step was added to the developer. The mixing of the acid and carbonate solutions caused the formation of bubbles. When the bubbling ceased, the gel was properly fixed and was transferred to the reserved chilled H_2O without agitation to remove excess acetic acid. Developed gels were stored upright overnight to dry before any analysis was undertaken. An example of a developed polyacrylamide gel can be seen at Figure 2.4.

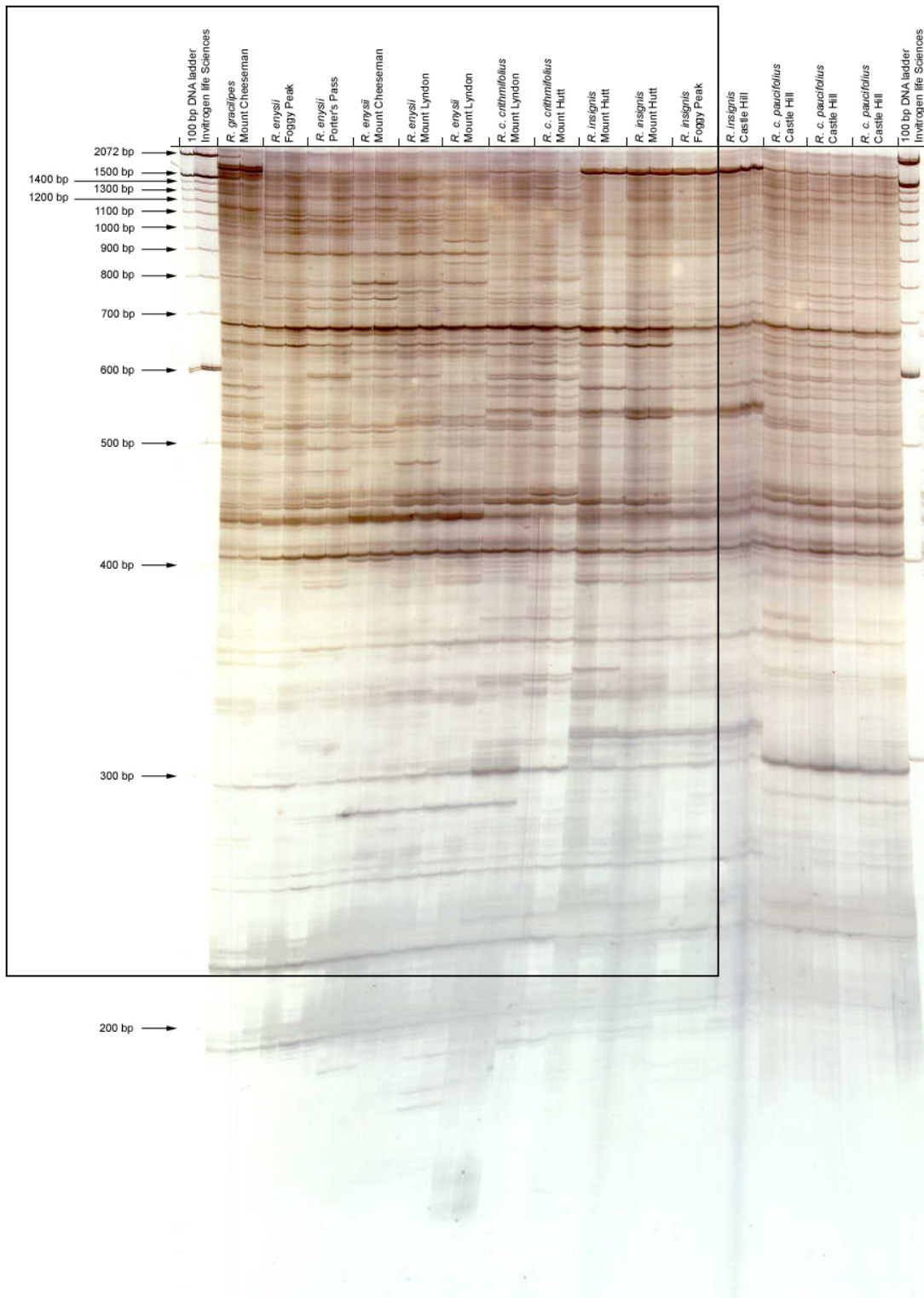


Figure 2.4 - ISSR primer #835 silver stained polyacrylamide gel

2.10 Scoring of ISSR and AFLP gels

AFLP and ISSR gels were scored blind by three people to avoid investigative bias. If a band was present in **both** replicates of a reaction, it was deemed to be a true amplified fragment, if a band appeared in only one replicate, it was ignored as a spurious result. Bands in different taxa that had migrated the same distance were deemed to be homologous. The presence or absence of a band at a particular locus could be recorded as binary data for each sample, and this was used for all further analyses.

2.11 Sequence editing

DNA sequences were manually edited using the programs MacClade (Sinauer Associates) and MtNavigator (Applied Biosystems), sequence alignment was performed manually, no ambiguous alignments were found.

2.12 Phylogenetic protocol

Neighbor-Joining (NJ) (Saitou and Nei, 1987) is a heuristic method of constructing a bifurcating phylogeny, that is extremely popular for use in molecular phylogenetic studies such as this one due to its statistical consistency and computational efficiency with large numbers of taxa. NJ is a distance-based method, it uses dissimilarities rather than site patterns (e.g. those found in DNA or amino acid sequence alignments) to reconstruct relationships. The NJ method uses a greedy algorithm to progressively cluster taxa in pairwise fashion and calculates the branch lengths as sequences are added to the growing clusters. It differs from the popular Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method in that the order that taxa are clustered depends not only on the difference between individual taxa, but also on the net divergence of each taxon from all other taxa. The NJ branch length calculation is also more rigorous than that of UPGMA and does not assume a molecular clock. However, if the best explanation for the evolution of a data set is not a tip

labelled bifurcating model, then the NJ method is at a disadvantage as it exclusively produces tip labelled bifurcating graphs.

Neighbor-Net (NNET) (Bryant and Moulton, 2004) is a relatively new method which in some respects is similar to Neighbor Joining. With this method, taxa are progressively clustered based on a criterion similar to the S_{ij} matrix used in Neighbor Joining. However, unlike Neighbor Joining, taxa are often paired with more than one other taxon during clustering. If the data are not tree-like, the algorithm will produce a planar graph (a graph wherein all the relationships can be visualised in a plane) that contains reticulations. The branch lengths in the graph are determined by a least squares optimisation criterion after construction of the relationships. NNET has improved performance over the split decomposition method (Bandelt and Dress, 1992; Huson, 1998) for studies of interspecific phylogeny since their splits graphs show more resolution than split decomposition splits graphs when sequence/fingerprint data exhibits higher levels of phylogenetic signal incompatibility.

Neighbor-Net and Neighbor-Joining phylogenies were constructed using a beta version of SplitsTree4 (Huson and Bryant, in preparation). For DNA sequence data, missing and ambiguous bases were excluded from the analysis. Default program settings were used for all phylogenies.

3 Results

3.1 Results pertaining to Hypothesis I

Hypothesis I: that there is genetic distinctiveness between regions among the group III (Lockhart et al., 2001) New Zealand alpine buttercups.

3.1.1 DNA sequence data

To construct phylogenetic graphs from edited DNA sequence ambiguous data was removed from the analyses. This approach reduces phylogenetic noise, but can also remove informative bases, as will be discussed in later sections.

3.1.2 Analyses of nITS data

Neighbor Joining (NJ) and NeighborNet (NNET) analyses of nITS data (602 base pairs, 30 substitutions) produce similar results (Figs. 3.1 and 3.2), and show that *R. enysii* is separated into two strongly supported groups, with southern accessions (M11, M12, M13, P2 and P3) being genetically distinct from accessions collected from further north (M1-10). *R. c. crithmifolius* also shows variation between regions. Accessions from Ben Ohau and the Eyre Mountains group separately from the main *R. crithmifolius* group, as does the accession from the Gordon Range. In contrast to *R. crithmifolius* and *R. enysii*, accessions of *R. gracilipes* are genetically very similar. The sequences from widely sampled *R. insignis* populations are also homogeneous, and identical to the sequences from many accessions of *R. gracilipes*.

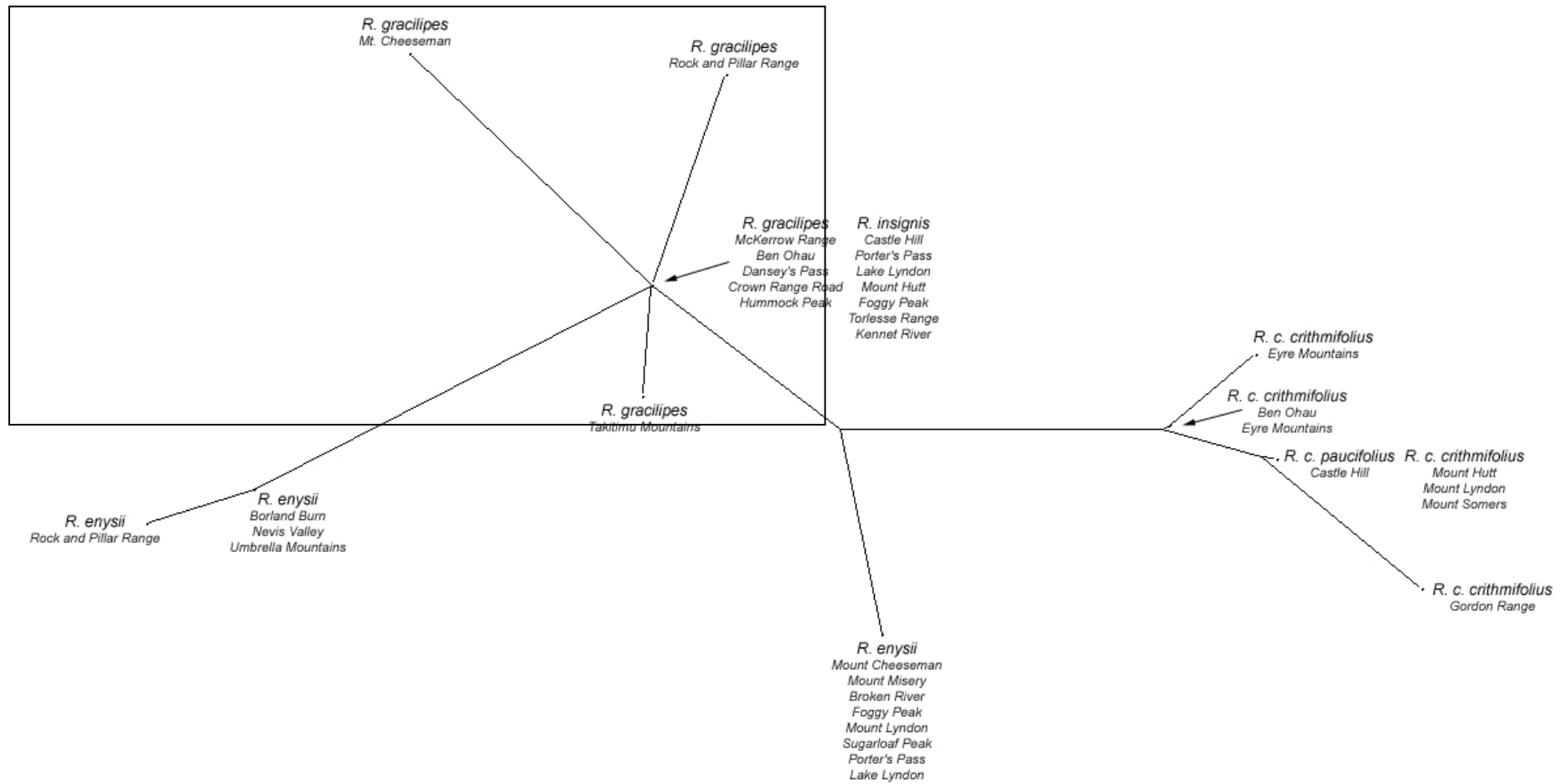


Figure 3.1 - Neighbor -Joining graph derived from nITS DNA sequences

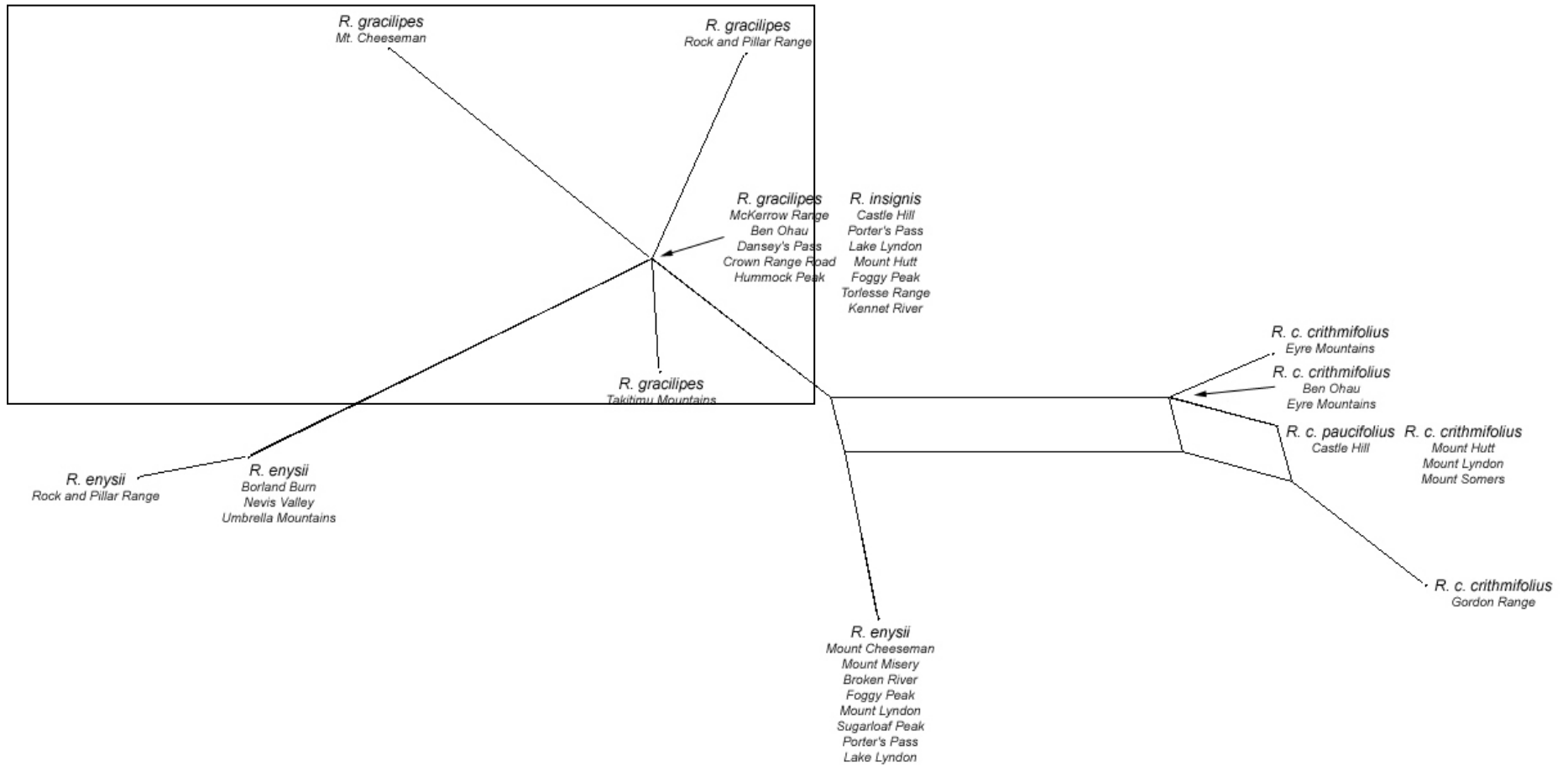


Figure 3.2 - Neighbor-Net graph derived from nITS DNA sequences

3.1.3 Analyses of J_{SA} data

The NJ and NNET graphs constructed from the cpJ_{SA} data (490 base pairs, 19 substitutions) are structurally identical, *i.e.* there are no reticulations in the NNET graph, indicating that there are no site incompatibilities in the J_{SA} data. In contrast to the results from analyses of nITS data, analyses of J_{SA} sequences (Figs. 3.3 and 3.4) indicate genetic variation within regions within *R. insignis*. Two accessions of *R. insignis* from Mount Hutt (M4 and M12) are identical to two accessions of *R. c. crithmifolius* (M2 and M6), while the remainder of the *R. insignis* (M1-3, M5-11, M13 and M14) accessions are closely related to the northern group of *R. enysii* (M1-8 and M10). The northern *R. enysii* group is genetically distinct from the more southern accessions (M11-14, P2, P3). The southern group of *R. enysii* is itself divided into two regionally distinct groups, an eastern group from the Rock and Pillar Range and Umbrella Mountains (M11, M14 and P3) and a western group from Fiordland (M12 and M13). One accession of *R. enysii* (P2) clustered with accessions of *R. gracilipes* from the Rock and Pillar Ranges.

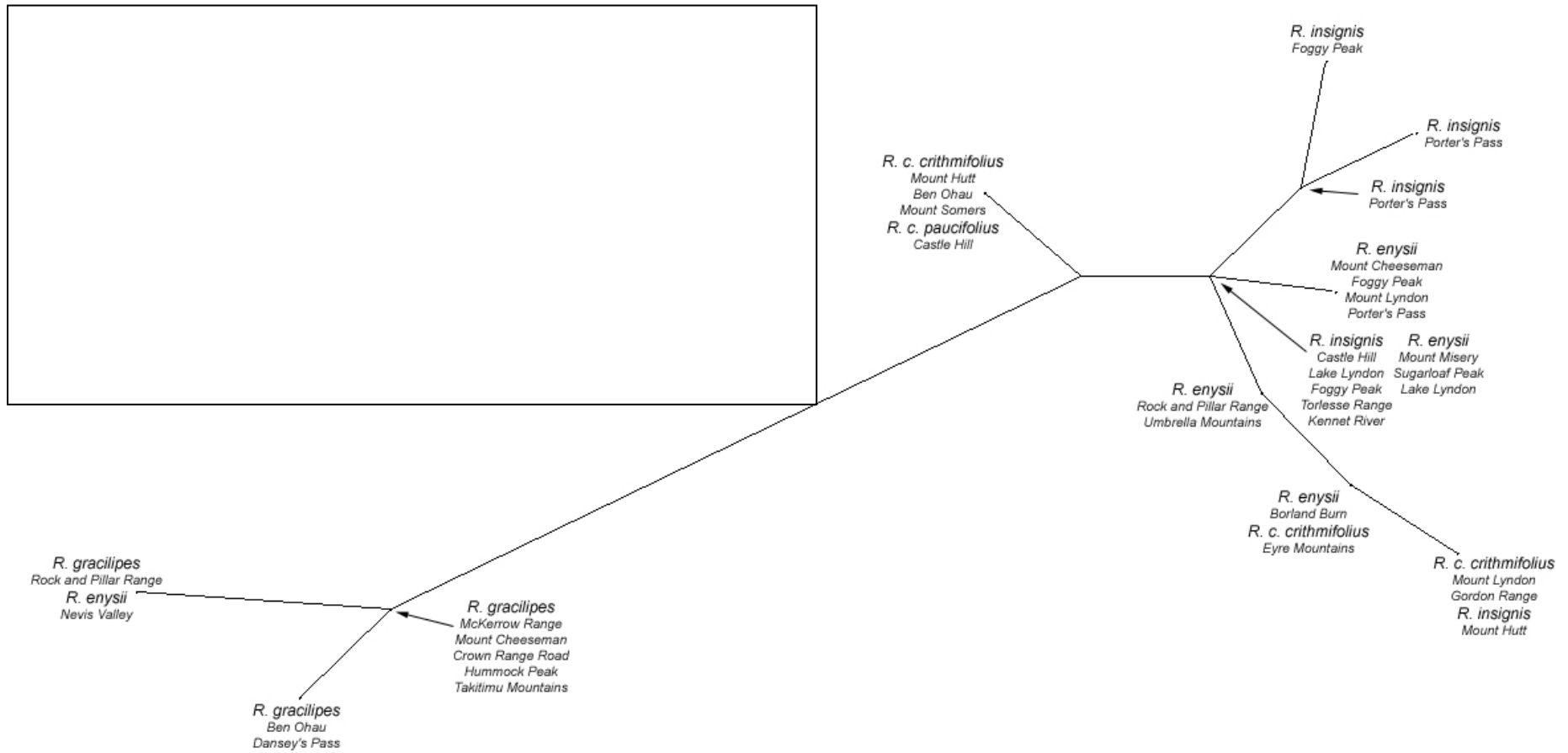


Figure 3.3 - Neighbor-Joining graph derived from J_{SA} DNA sequences

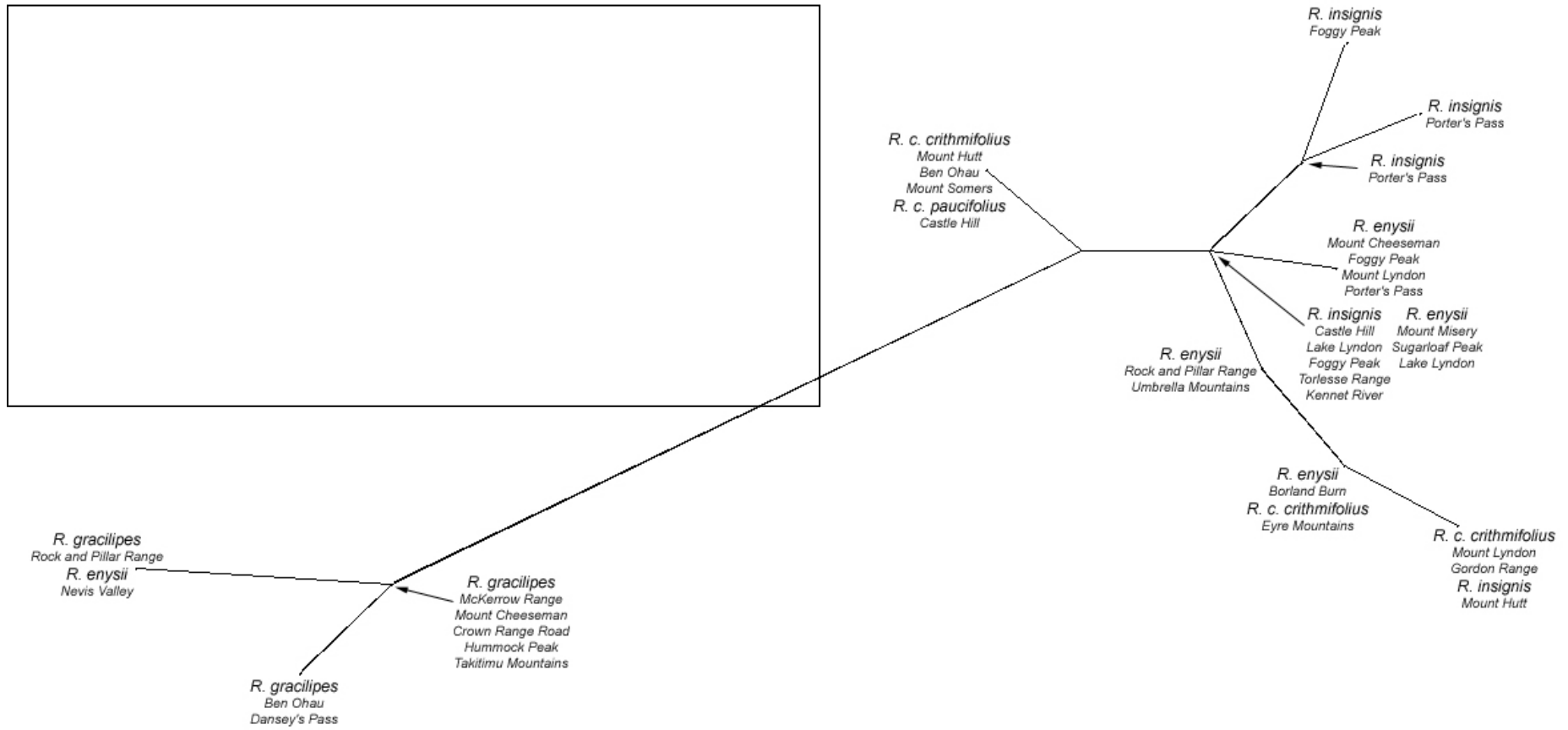


Figure 3.4 - Neighbour-Net Graph derived from JSA DNA sequences

3.1.4 Fingerprint data

Interpretation of fingerprint profiles is more subjective than sequence analyses. For this reason profiles were scored blind and independently by three individuals. The issue of subjectivity and its impact on inferences is later discussed. The observations emphasised here concern features of phylogenetic graph structure common to analyses of data from all individual scorers.

3.1.5 ISSR profiles

NNET and NJ graphs derived from ISSR profiles (Figs. 3.5 and 3.6) showed that individual accessions of the same taxa grouped together in phylogenetic reconstructions. No consistent regional groupings were shown between independent scorings, *i.e.* in some scorings (e.g. with *R. insignis*) the profiles showed genetic distinctiveness between regions, but in other scorings, these regional groupings were not seen. In other cases, (e.g. as with *R. enysii*) regional groupings were not preserved.

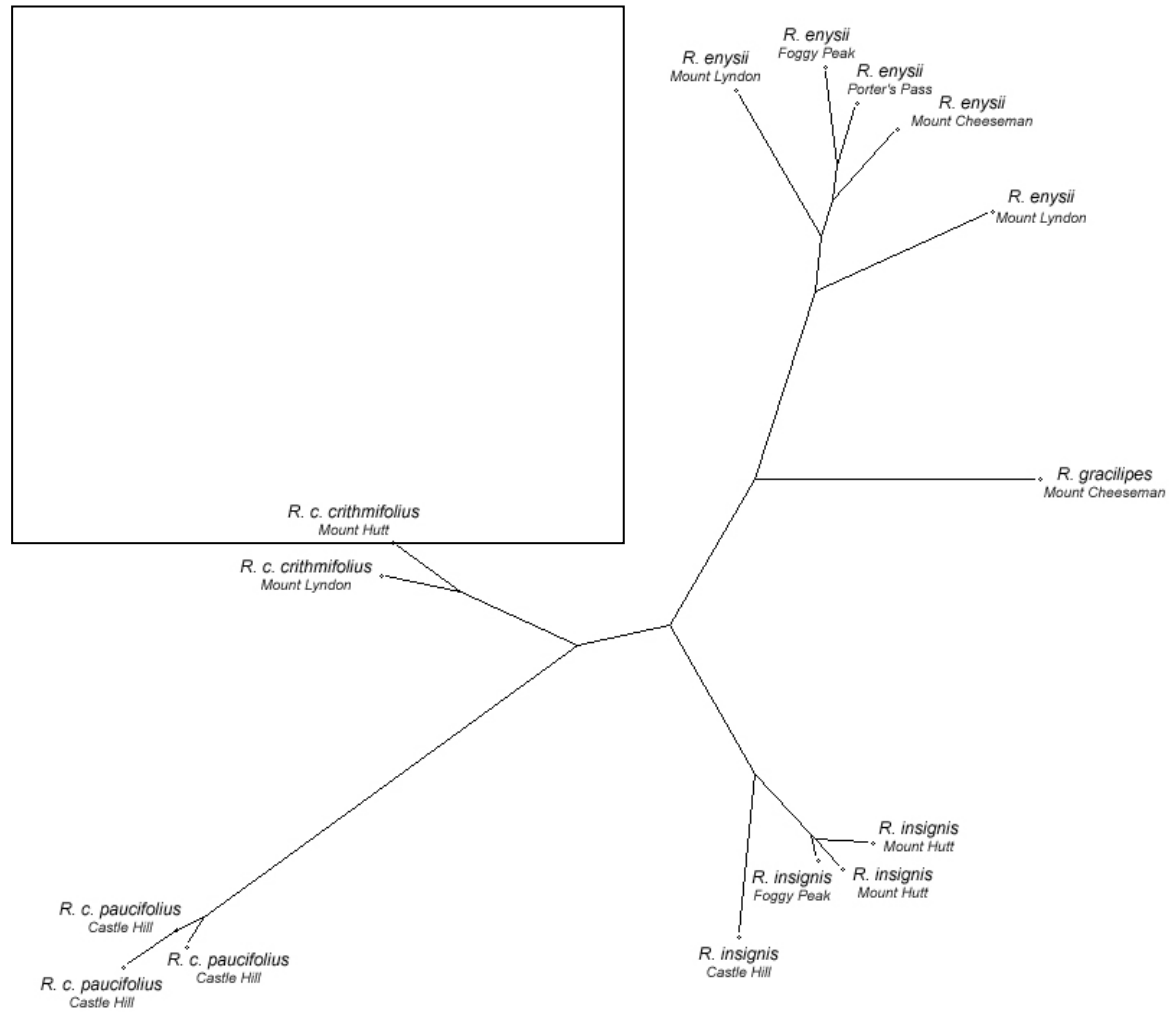


Figure 3.5 - Neighbor-Joining graph derived from ISSR MLF data

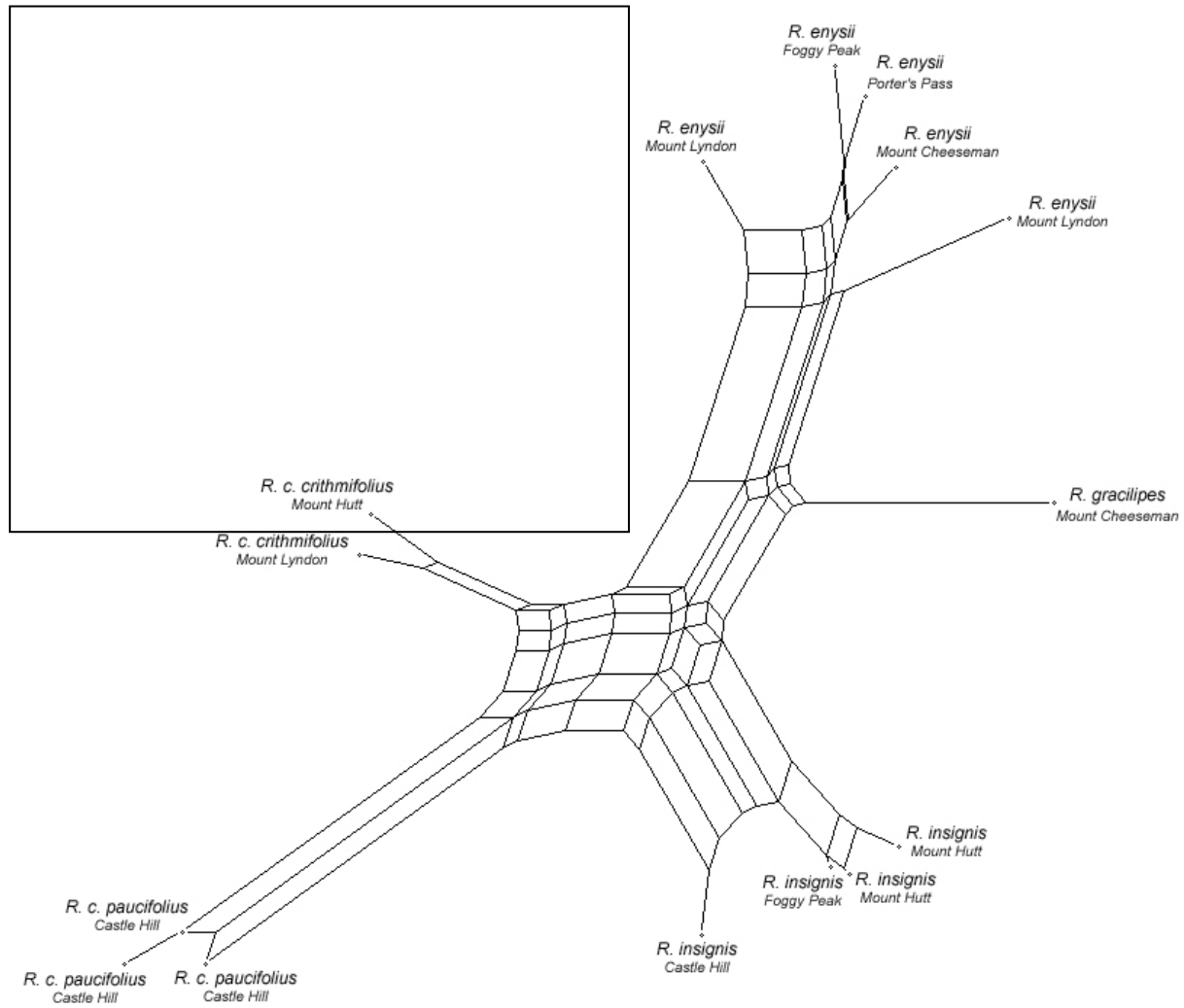


Figure 3.6 - Neighbor-Net graph derived from ISSR MFL data

3.1.6 AFLP profile

NNET and NJ graphs derived from the AFLP data from different scorings are incongruent in displaying inferred relationships between taxonomically distinct and geographically disjunct taxa. However some features were common to all scorings. Of particular note is that graphs derived from three independent scorings (e.g. Figs. 3.7 and 3.8) suggest that *R. enysii* is paraphyletic. Whilst *R. enysii* from Fiordland (enysii_M12 and enysii_M13) group together, they are genetically distinct from the other *R. enysii* accessions. This is also evident with *R. enysii* accessions from the Rock and Pillar range (enysii_M11 and enysii_M14), and corroborates similar findings from cpJ_{SA} and nITS DNA sequence analyses.

Figure 3.9 shows leaf outlines taken from Fisher (1965) of *R. enysii* leaves from the Waimakariri area, Fiordland and the Rock and Pillar Range, while Figure 3.10 shows photographs of herbarium specimens analysed in this study. Comparison of these figures illustrates some of the regional morphological variation in *R. enysii* and precludes any obvious misidentification of the Borland and Rock and Pillar Range *R. enysii* samples used in this study, as they clearly fall within the recorded morphological variation of this species.

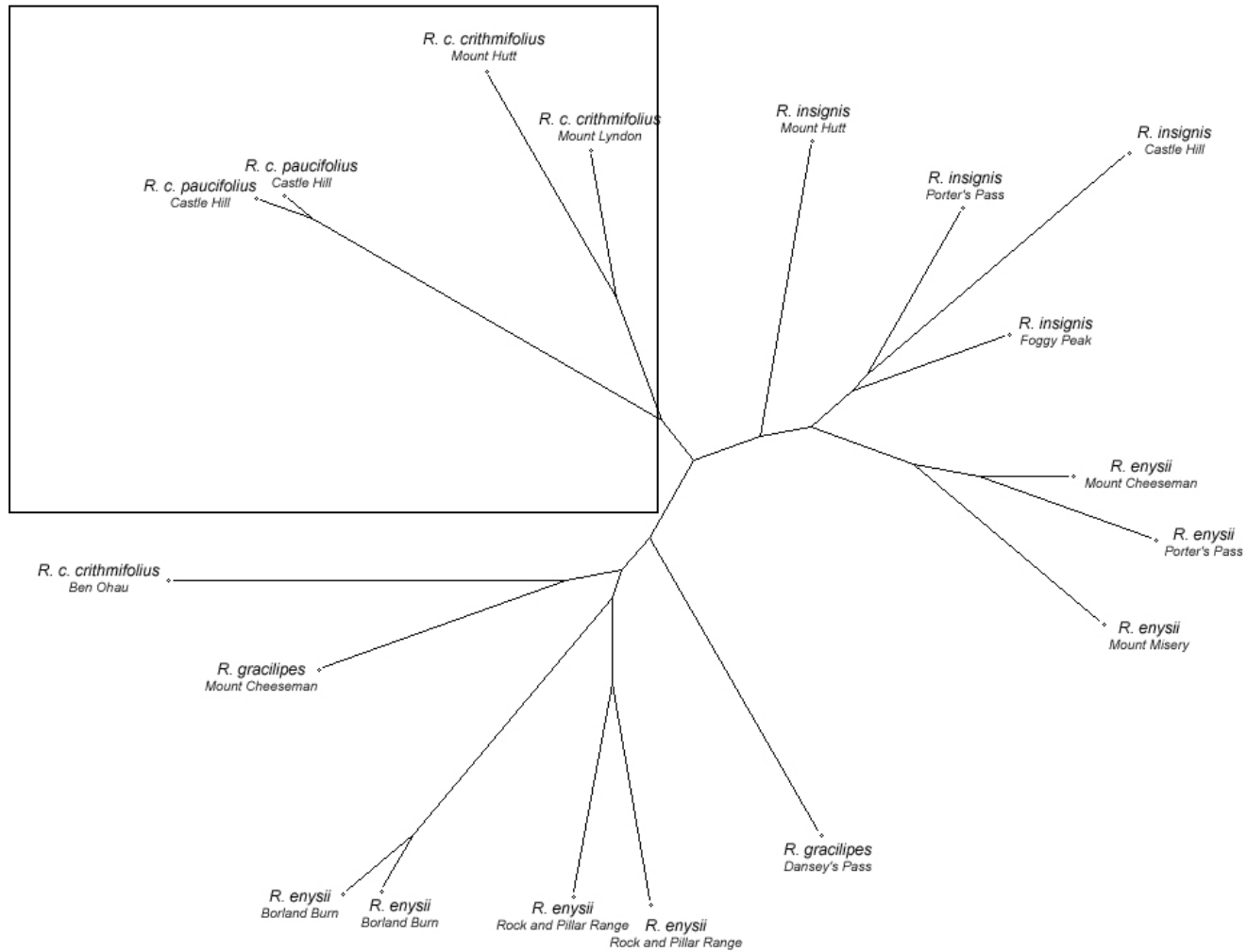


Figure 3.7 - Neighbor-Joining graph derived from AFLP MLF data

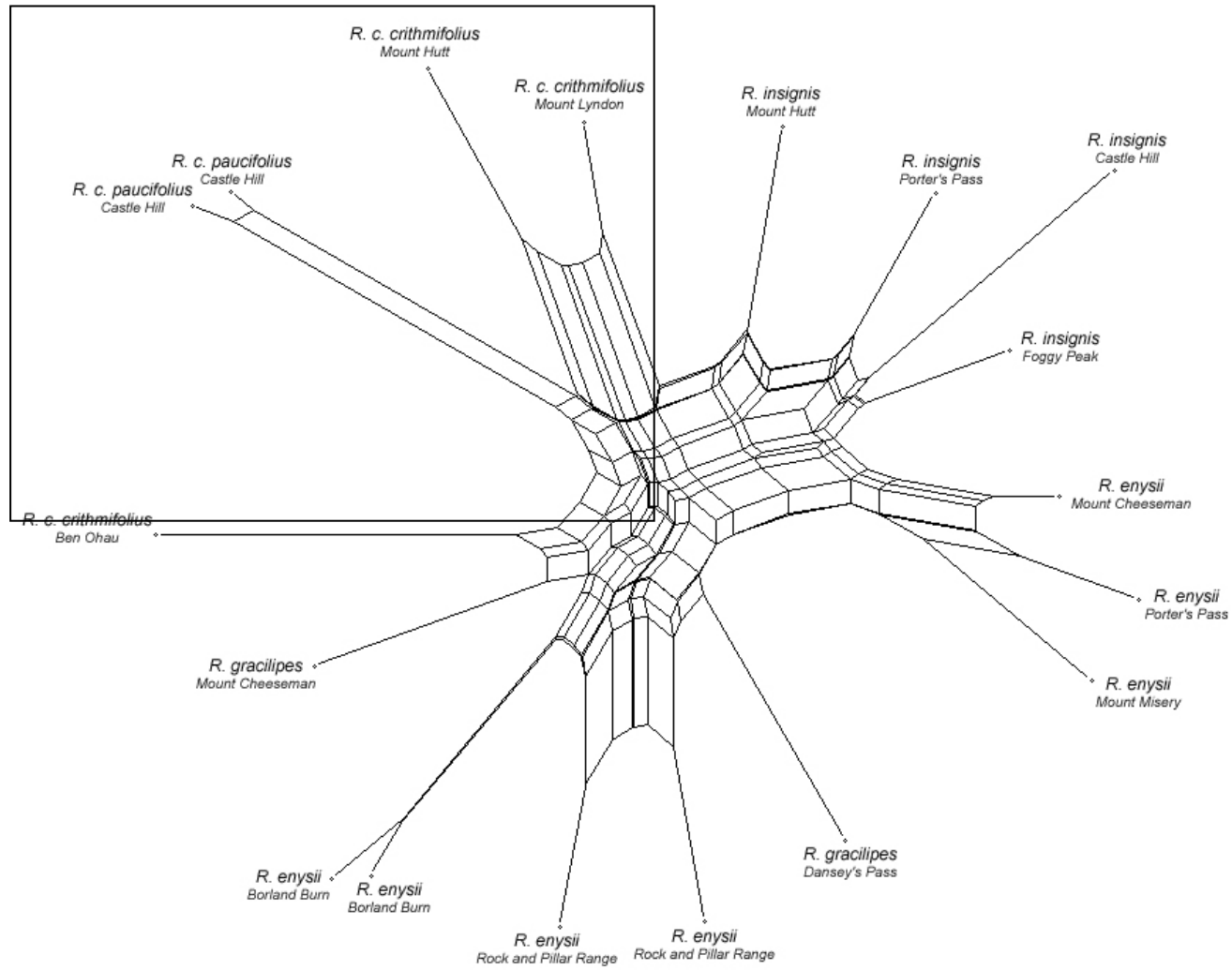


Figure 3.8 - Neighbor-Net graph derived from AFLP MLF data

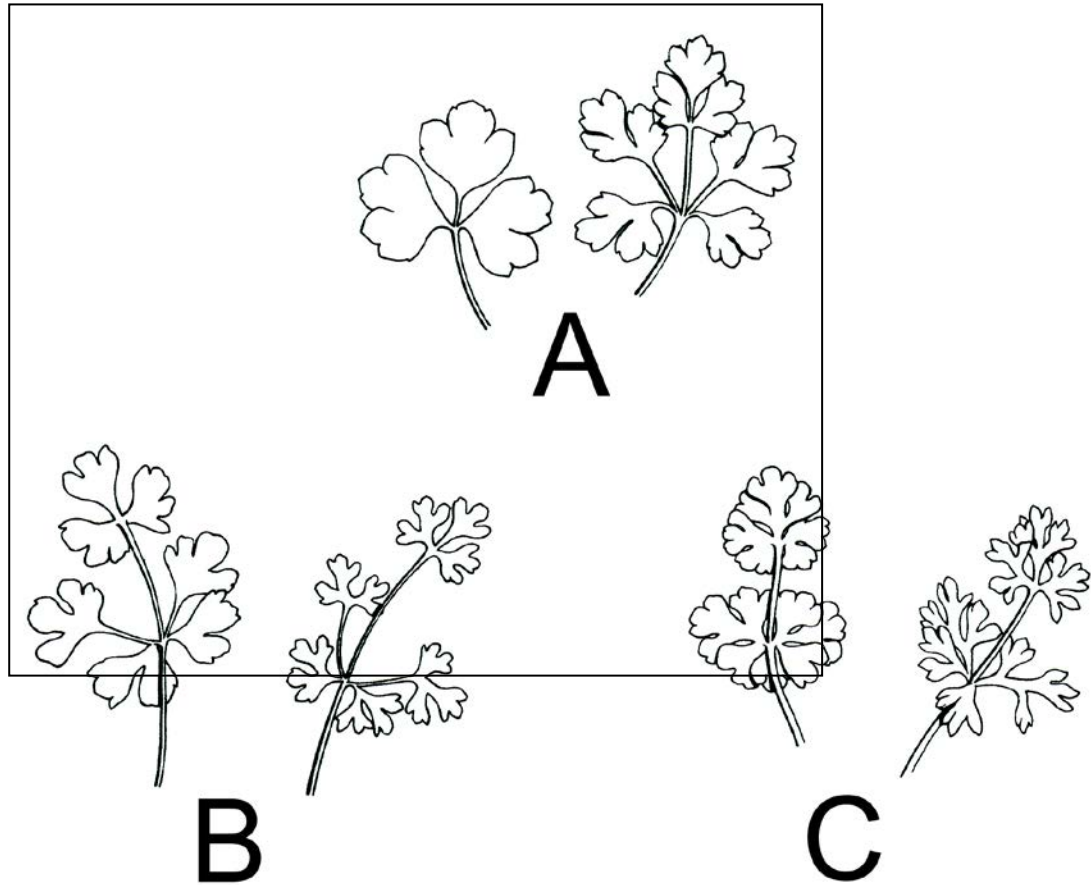


Figure 3.9 - Leaf outlines of *R. enysii* taken from Fisher (1965) p42. (A) Leaves from Lake Lyndon, Waimakariri Basin. (B) Leaves from Mt. Cleughearn, Fiordland. (C) Leaves from the Rock and Pillar Range.

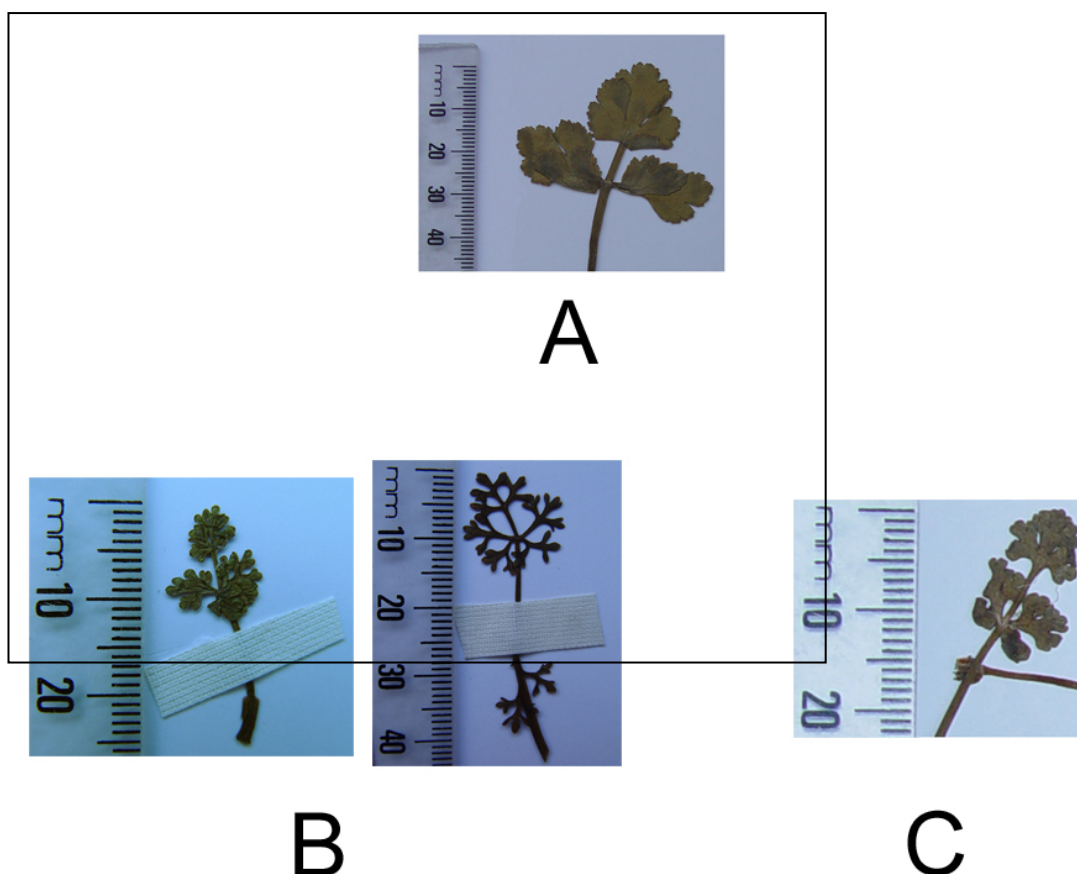


Figure 3.10 - Herbarium voucher photographs of southern *R. ensyii* analysed in this study (see appendix A for full specimen details). (A) Specimen *ensyii*_M5 from Mount Lyndon, Waimakariri Basin. (B) Specimens *ensyii*_M12 and *ensyii*_M13 from Borland Burn, Fiordland. (C) Specimen *ensyii*_M14 from the Rock and Pillar Range, Otago.

3.2 Results pertaining to hypothesis II

Hypothesis II: that the Castle Hill buttercup is genetically distinct from its closest relatives in the group III (Lockhart et al., 2001) New Zealand alpine Ranunculus.

3.2.1 Analyses of nITS data

nITS sequences showed inter and intraspecific variation amongst only some group III (Lockhart *et al.*, 2001) *Ranunculus* species (Figs. 3.1, 3.2). Accessions of *R. insignis* were genetically very similar to each other and to the widely dispersed *R. gracilipes*. In contrast, southern (M11, M12, M13, P2 and P3) accessions of *R. ensyii* were observed to be genetically distinct from more northern accessions (M1-10). Three

accessions of *R. c. paucifolius* were found to be identical in sequence. This genotype was also shared by some accessions of *R. c. crithmifolius* (nos. M1, M2 and M5). In both the NNET and NJ graphs, all *R. crithmifolius* taxa grouped together, to the exclusion of all the other species.

3.2.2 Analyses of J_{SA} data

A number of observations can be made from analysis of the J_{SA} data. The three accessions of *R. c. paucifolius* are identical to each other and to the majority (nos. M1, M3, M4 and M5) of the *R. c. crithmifolius* accessions. Two accessions of *R. c. crithmifolius* (nos. M2 and M6) showed very distinct genotypes from the remainder of the *R. crithmifolius* accessions and were identical to two accessions of *R. insignis* (M4 and M12). In contrast to the nITS sequences, accessions of *R. insignis* showed some genetic variation, although no phylogeographic pattern was apparent. *R. enysii* again demonstrated genetic distinctiveness between northern and southern accessions. Two accessions of *R. c. crithmifolius* (P1 and P2) were genetically identical to accessions of *R. enysii* from Borland Burn. All accessions of *R. gracilipes* were genetically similar and distinct from the other species. A characteristic 12 base InDel separated *R. gracilipes* from the other taxa. One accession of *R. enysii* also shared this feature, grouping with *R. gracilipes*.

3.2.3 Analyses of ISSR profiles

Figures 3.5 and 3.6 show NNET and NJ graphs obtained from analysis of ISSR presence/absence data derived from two profiles obtained using UBC primer 835 and 844 (for primer sequences see appendix C). Different scorings are incongruent in displaying inferred relationships between taxonomically distinct and geographically disjunct taxa. However some features were common to all scorings and are discussed here. In these networks, *R. c. paucifolius* always groups with *R. c. crithmifolius*. However, unlike the findings from direct marker sequencing studies, the Castle Hill buttercups are genetically distinct from *R. c. crithmifolius*, although only a subset of the available *R. c. crithmifolius* accessions were genotyped in this fashion.

Phylogenetic analysis of ISSR profiles also indicates that the other species conform to the established taxonomy, unlike the findings from direct sequencing. That is, all *R. insignis* accessions group together and all *R. enysii* accessions group together.

3.2.4 Analyses of AFLP profiles

Further analyses were carried out on an extended taxon data set using AFLP. The data to construct the NNET and NJ graphs were presence/absence data derived from the primer combination *EcoRI*+ATA and *MseI*+CAAG (for primer sequences see appendix C). As with the ISSR profiles, AFLP profiles were scored by three independent observers. A consistent feature of the phylogenetic graphs (Figs. 3.7, 3.8) was that *R. c. paucifolius* were genetically distinct from other taxa in the networks. However, taxonomic groups were not found to be coherent as was observed in the ISSR profiles. The genetic distinctiveness observed between Fiordland, Otago and northern *R. enysii* samples in direct sequencing analyses was also observed in the AFLP NNET graphs.

3.3 Results pertaining to hypothesis III

Hypothesis III: that the Castle Hill buttercup has a diploid hybrid origin.

3.3.1 Analyses of nITS data

Both NJ and NNET graphs of the nITS data (Figs. 3.1, 3.2) show that *R. c. paucifolius* is identical to three accessions of *R. c. crithmifolius* (nos. M1, M2 and M5) and closely related to the other accessions. The *R. crithmifolius* taxa are grouped together, excluding the other species.

3.3.2 Analyses of J_{SA} data

As with nITS, the cpJ_{SA} data (Figs. 3.3, 3.4) shows that *R. c. paucifolius* is genetically identical to some accessions of *R. c. crithmifolius* (M1, M3, M4 and M5), although two *R. c. crithmifolius* accessions (M2 and M6) do not group with the rest of the *R. crithmifolius* taxa, instead grouping with two *R. insignis* accessions from Mount Hutt. Two more *R. c. crithmifolius* accessions (P1 and P2) group with accessions of *R. enysii* from Borland Burn.

3.3.3 Analyses of ISSR profiles

In the NJ and NNET graphs derived from ISSR data (Figs. 3.5, 3.6), *R. c. paucifolius* groups most closely with *R. c. crithmifolius*, although the distances involved vary between individual scorers.

In these networks, *R. c. paucifolius* is adjacent to *R. c. crithmifolius* in all three scorings. In one scoring, the *R. crithmifolius* taxa are split from all the other taxa, while in the other two networks there are small splits between *R. c. paucifolius* and *R. c. crithmifolius*.

3.3.4 Analyses of AFLP profiles

In NJ and NNET analyses of AFLP profiles (Figs. 3.7, 3.8), accessions of *R. c. paucifolius* consistently groups together throughout the individual codings and is genetically distinct from *R. c. crithmifolius*. The placement of the *R. c. crithmifolius* accessions varies between the individual scorings of the profile.

These analyses provide no evidence that the Castle Hill buttercup was formed by the process of diploid hybridisation. This finding is later discussed in light of other observations reported in this chapter that may implicate hybridisation as a common process involving other group III (Lockhart *et al.*, 2001) *Ranunculus* species.

4 Discussion

4.1 Discussion of cpJ_{SA} and nITS DNA sequence results.

The study by Lockhart *et al.* (2001) which this work continues, found that phylogenies assembled by using nuclear ITS data clustered the subspecies *R. c. paucifolius* and *R. c. crithmifolius*, whereas cpJ_{SA} DNA phylogenies were inconclusive with their placement of *R. c. paucifolius*. In this study, both nITS and cpJ_{SA} analysis show that *R. c. paucifolius* is identical in sequence to a number of accessions of *R. c. crithmifolius*. This study also found that the *R. c. paucifolius* chloroplast type is present within the diversity of *R. c. crithmifolius*. These differences are attributed to the increase in the number of accessions sampled. Thus there is no evidence apparent from DNA sequence analysis that *R. c. paucifolius* is genetically distinct from *R. c. crithmifolius*. Equally, the DNA sequence analysis shows no evidence that *R. c. paucifolius* is a diploid hybrid species. Since it has been suggested (Wolfe *et al.*, 1998) that conventional molecular markers may not be sufficiently sensitive to detect diploid hybrid speciation among closely related taxa, this provided motivation to perform the MLF studies discussed in section 4.2.

Evidence for regional genetic variation of *R. enysii* is found in both the nITS and J_{SA} results. nITS analysis indicates the presence of three genetically distinct groups of *R. enysii*, formed by accessions taken from three geographically distant locations. The first genetic group is from the well sampled Waimakariri river basin area and will be referred to as the ‘Waimakariri Group’. The second group is from the Rock and Pillar Range approximately 95km north-northwest of Dunedin and will be referred to as the ‘North Otago Group’. The third group was sampled from the Borland Burn basin in the Hunter mountains of Eastern Fiordland, the Nevis River valley, and the Umbrella Mountains and is termed the ‘East Fiordland Group’. These last two groups will be collectively referred to as the ‘Southern Group’.

These three phylogenetic groups are also detected by analysis of the cpJ_{SA} DNA region, but not all of the accessions are consistent with nITS in their phylogenetic

placement. Two accessions which grouped with the East Fiordland Group under nITS results (ensyii_P2 and P3) exhibit different placement in cpJ_{SA} analysis. The accession of *R. ensyii* from the Umbrella Mountains is identical to the North Otago Group in cpJ_{SA} analysis, while the accession of *R. ensyii* from the Nevis River Valley groups with *R. gracilipes* from the Rock and Pillar Ranges. The variability shown by these accessions might be attributed to hybrid speciation, particularly as ensyii_P2 possesses the distinctive *R. gracilipes* cpJ_{SA} type. Fig. 4.1 shows the groupings of *R. ensyii* as indicated by nITS and cpJ_{SA} marker sequence.

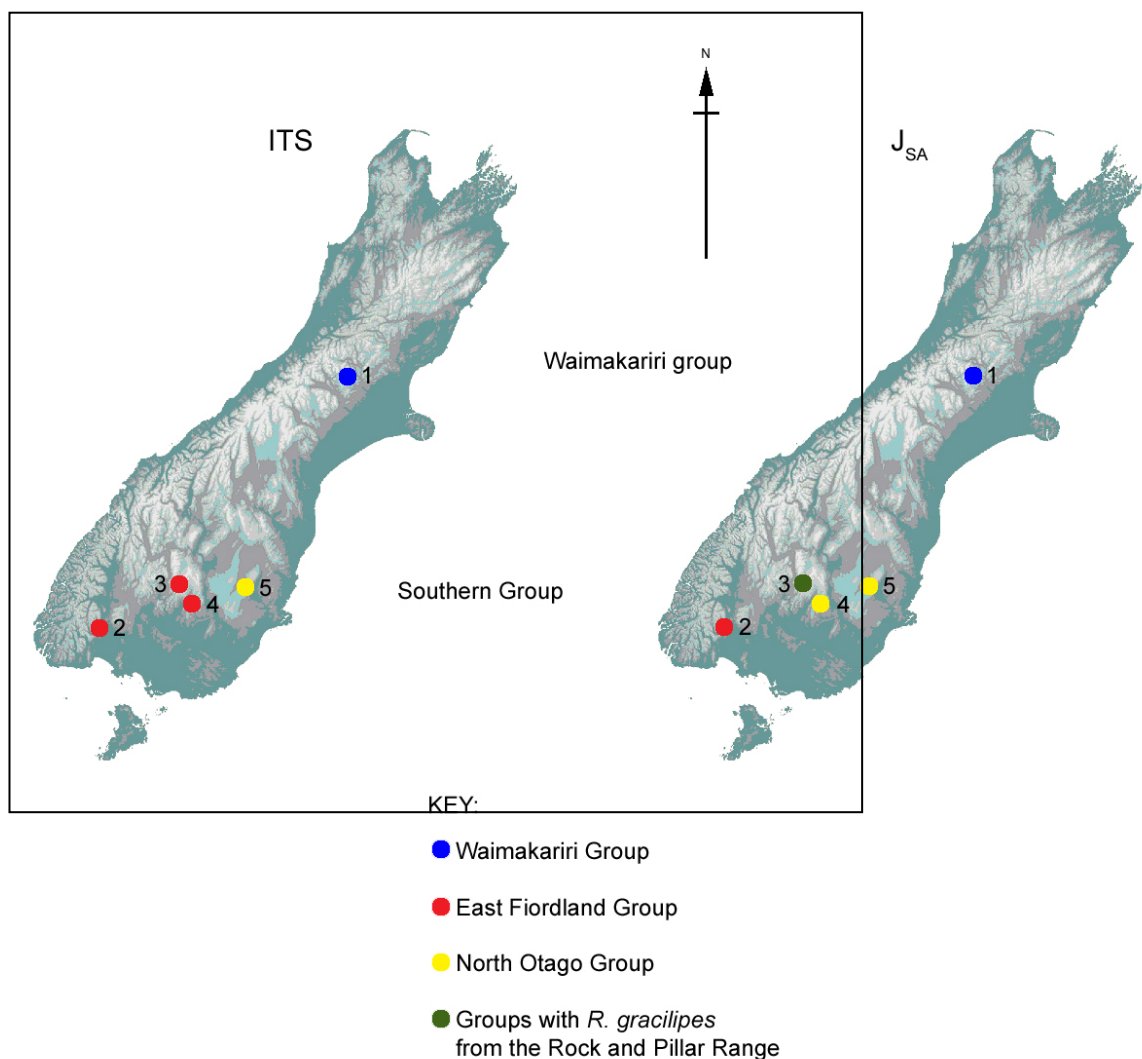


Figure 4.1 - Regional groupings of *R. ensyii* suggested from DNA marker sequences. (1) Waimakariri basin. (2) Borland Burn, Fiordland. (3) Nevis River valley. (4) Umbrella Mountains. (5) Rock and Pillar Range.

In both markers, it is apparent that the three groups are phylogenetically separated along a north/south axis as the two southern groups from North Otago and East Fiordland are more closely related to each other than to the Waimakariri group.

In addition to the regional grouping of *R. enysii*, a regional group consisting of the two accessions of *R. c. crithmifolius* from Ben Ohau is shown by the nITS data. However, this grouping is not supported by the cpJ_{SA} data, and the evidence for this relationship is less convincing. A third regional grouping supported by both the nITS and cpJ_{SA} data consists of the two accessions of *R. c. crithmifolius* from the Eyre Mountains. In nITS analyses, these accessions group with the *R. c. crithmifolius* accessions from Ben Ohau, whereas cpJ_{SA} analyses show them to be most closely related to the East Fiordland group of *R. enysii*.

Regional variation of *R. insignis* was also found with the cpJ_{SA} marker, but not with the nITS data. The accessions of *R. insignis* from Mt. Hutt are distinct from the remainder of the *R. insignis* samples and identical to accessions of *R. c. crithmifolius* from Mt. Lyndon and the Gordon Range. It is possible that the genetic similarity between these two groups is a consequence of convergent evolution, although these changes could also be accounted for by hybrid speciation or introgression. It is noted that both *R. insignis* and *R. c. crithmifolius* are currently found in close proximity on Mt. Hutt.

Transfer of the biparentally inherited nITS locus requires only pollen dispersal. Whereas the chloroplast genome is maternally inherited (Corriveau and Coleman, 1988). For this distribution of the cpJ_{SA} marker to be explained by dispersal, seeds or reproductive plant fragments must have been transported approximately 400km north to or from the Gordon Range near Nelson. If this was the case, it seems likely that this dispersal would have occurred northwards rather than southwards, as the Gordon Range is at the northern extreme of the range of *R. crithmifolius*, and there is no record of this plant being collected in the regions between the Waimakariri basin and the southern Wairau gorge (Fisher, 1965). A better explanation for this distribution could be repeated range expansion and contraction caused by varying climatic conditions.

4.2 Discussion of Multilocus Fingerprint results

Due to the success of Wolfe *et al.* (1998) in confirming the diploid hybrid origin of *Penstemon clevelandii*, ISSR was selected as the initial method to investigate the possible diploid hybrid origin of *R. c. paucifolius*.

Although the relative simplicity of the ISSR method is an advantage over other fingerprinting methods, implementation in this study proved to be problematic. Consistency of duplicate ISSR reactions was very low, resulting in a high proportion of unusable profiles. Two ISSR gels were sufficiently consistent to score, and the ISSR data obtained were derived from them. Only a limited number of accessions were available for analysis at the time these consistent gels were produced, with the result that the range of accessions examined in the ISSR data is limited.

AFLP was investigated as an alternative technique to bypass some of the consistency problems encountered with ISSR. Some recent studies have shown that AFLP can provide more reproducible results than ISSR (Archak *et al.*, 2003; McGregor *et al.*, 2000). Initial results showed that the DNA fragments amplified were indeed more consistent between duplicates than ISSR, but time restrictions prevented the full-scale implementation of the method in this study. One AFLP profile was of sufficiently good quality to be included in the results analysis.

Three individual scorers were used to score each fingerprint profile in this study, with the aim of reducing the subjectivity inherent in the scoring process. The data obtained from the ISSR fingerprint profiles produced extremely consistent phylogenies between individual scorings, whereas the phylogenies obtained from individual scorings of AFLP contained some inconsistencies. However, in most cases these inconsistencies concerned relationships between geographically or taxonomically distinct taxa, suggesting that the method (at least for the primer combination trialled) was at the limits of its resolution. In such a situation an explanation for the variable data obtained from different scorers might be simply that the different scorers may have interpreted the developed acrylamide gel differently. It is likely that further

experimentation with AFLP using other primer combinations would provide a better indication of the nature of this discrepancy.

MLF was shown to give far greater phylogenetic resolution when compared to DNA marker sequencing; both DNA marker phylogenies showed large groups of accessions with identical sequence, whereas both MLF methods confirmed genetic variability between individual accessions, whilst at the same time being robust with respect to duplicate samples.

Like the DNA sequence analysis, networks of ISSR and AFLP data consistently show that *R. c. crithmifolius* is the closest relative of the Castle Hill buttercup. However, unlike DNA sequence data, the MLF results show genetic distinctiveness between the sister subspecies *R. c. paucifolius* and *R. c. crithmifolius*. No evidence is found in the MLF graphs to support the hypothesis that *R. c. paucifolius* is a hybrid species.

The regional genetic distinctiveness seen in the cpJ_{SA} and nITS analyses is also apparent in the fingerprint profiles. The consensus of the AFLP results shows support for the regional genetic distinctiveness of *R. enysii* noticed in the DNA sequence phylogenies; the Waimakariri group of *R. enysii* is separate from the Southern accessions, while the Southern group is divided into the distinct East Fiordland and North Otago groups. This region-specific pattern could not be tested using ISSR, as the accessions involved had not been collected when these profiles were obtained. The grouping of accessions of *R. insignis* and *R. c. crithmifolius* shown in the cpJ_{SA} analysis is not seen in the analysis of AFLP and ISSR fingerprint profiles. Although the accessions of *R. insignis* from Mt. Hutt are adjacent in the phylogenies derived from ISSR profiles, they do not group with the accessions of *R. c. crithmifolius* from Mt. Lyndon and Gordon Range. This is also the case when AFLP data is examined, the accessions involved show no sign of the close genetic relationship displayed in the cpJ_{SA} phylogenies.

4.3 Discussion of methods

4.3.1 DNA sequence analysis vs. Multi-locus fingerprinting

As noted in the introduction, a great deal of research has been done using single-locus DNA markers such as nITS. However, it has been noted that these markers are sometimes insufficiently variable to differentiate between closely related species (Wolfe *et al.*, 1998). This was indeed the case in this study; no difference was found between accessions of *R. c. paucifolius* and *R. c. crithmifolius* in analysis of either nITS or cpJ_{SA} DNA sequence, whereas both forms of MLF were able to distinguish between these closely related accessions. It is concluded that MLF is superior to single locus DNA sequence analysis in the ability to differentiate between closely related species.

4.3.2 Subjective factors in data

Reducing subjective elements in data is an important consideration in studies of this nature. Sequencing error aside, most DNA sequences have elements of subjectivity introduced by alignment (automatic or otherwise) and manual sequence editing (Lee, 2004). The subjective elements introduced by manual editing can be reduced somewhat by the removal of heteroplasmic sites from analysis. Subjectivity in manually scored MLFs can be mitigated somewhat by blind scoring using multiple scorers, but a certain subjective element remains. However as MLF profiles diverge from each other it becomes clear that phylogenetic relationships quickly become difficult to infer.

4.3.3 AFLP vs. ISSR

Different fingerprinting techniques produce different results in different species. This study provided an opportunity to compare the performance of ISSR and AFLP on wild *Ranunculus* species.

The literary consensus on the relative reproducibility and variability of ISSR and AFLP is summarised here; AFLP has greater reproducibility than ISSR, *i.e.* duplicate AFLP reactions have more consistent banding patterns when electrophoresed than ISSR reactions. Whereas ISSR has greater genetic variability, *i.e.* a greater proportion of ISSR bands are polymorphic than those of AFLP reactions performed on the same sample (Saini *et al.*, 2004).

A possible explanation for the relative properties of these techniques may be that AFLP works by exploiting mutations such as Single Nucleotide Polymorphisms (SNPs) and InDels that change the occurrence of restriction enzyme sites, as well as InDels between restriction sites that cause fragment length polymorphisms. Whereas ISSR takes advantage of the variation in microsatellite regions, which are known to be one of the fastest evolving regions of genomes (Futuyma, 1998)

Although microsatellite regions are fast-evolving, ISSR primers are anchored at the 3' end *e.g.* 5'(GA)₈C 3'. Therefore the loci amplified by the PCR do not include the actual microsatellite sequence. However, InDels in the sequences between compatible primers will result in different sizes of amplified PCR products, forming different fingerprint profiles. It is also possible that some regions of a genome are more prone to variability and mutation than others. Thus it might be suggested that the areas of the genome near the hypervariable microsatellite regions will have higher variability than the genomic average. This may lead to the increased variability over AFLP shown by ISSR.

The low reproducibility of ISSR when compared to AFLP may be attributed in part to the nature of PCR. Microsatellite regions are prone to PCR error due to the possibility of a primer misbinding during the annealing stage. This contrasts with AFLP, as ligating linkers to endonuclease restriction sites creates non-repeating synthetic primer sites.

4.4 Discussion of hypotheses

4.4.1 Hypothesis I: That there is genetic distinctiveness between regions in the Group III New Zealand alpine *Ranunculus*.

The experimental work undertaken in this study suggests paraphyly for two species within group III (Lockhart *et al.* 2001) of the New Zealand alpine *Ranunculus*. The strongest evidence for paraphyly is provided for *R. enysii* (based on analysis of two gene loci and MLF data), while a weaker case can be made for paraphyly of *R. c. crithmifolius* (based on analysis of two gene loci). The paraphyly observed may be a consequence of a number of geologically recent events that are theorised to act as so-called “species pumps”, creating environmental conditions which encourage speciation and radiation of organisms. These events include periods of rapid mountain building (in this case, the Kaikoura Orogeny) and the advancement and retreat of glaciers (in this case, the Otiran Glacial Stage (Gage and Suggate, 1958)). In any event, the finding of paraphyly in this study is consistent with results showing paraphyly in groups I and II of the New Zealand alpine *Ranunculus* (Lockhart *et al.*, 2001).

If the paraphyly seen elsewhere in the group III lineage in *R. enysii* is due to the effects of mountain building episodes or other geographical events, then it seems reasonable to assume that these events may have affected the lineages giving rise to the Castle Hill buttercup. It was hypothesised that studying the regional variation that arises from these geological events may illustrate potential complexity in *R. c. paucifolius*.

4.4.2 Hypothesis II: That the Castle Hill buttercup (*R. c. paucifolius*) is genetically distinct from *R. c. crithmifolius*.

The amount of genetic distinctiveness required to define a new species is by no means subject to a set standard (Lee, 2004), despite the desires of some to make it so (Tautz *et al.*, 2003). While it is true that there is always some genetic variation between

individuals, obviously each is not a separate species. The difficulties inherent in striking a balance between “lumping” taxa together and “splitting” them into more and smaller divisions has been discussed for some time (Hey, 2001) and the debate shows no signs of stopping.

The results obtained in this study show that *R. c. paucifolius* is genetically distinct from its sister subspecies *R. c. crithmifolius*. Although DNA marker sequences did not detect any difference between the two subspecies, differences between the two taxa were detected by MLF techniques. The degree of difference shown between the two taxa in the fingerprint analysis is less than the variation shown between other species in the analysis, but greater than that seen between individuals of the same species.

This result suggests that the degree of genetic separation is below the level required to restore *R. c. paucifolius* to full species status, but that there is greater difference between the two taxa than can be accounted for by the variation between individuals. When the clear differences in habitat and the fact that a distinct population of *R. c. paucifolius* has been maintained without being introgressed out of existence are taken into account, the taxonomic rank of *R. c. paucifolius* as a subspecies seems appropriate when this new information is considered. For a new taxonomic reassignment to be considered for *R. c. paucifolius*, further DNA marker sequencing showing a greater difference between *R. c. paucifolius* and *R. c. crithmifolius* would have to be performed, preferably involving at least one single-copy nuclear marker.

4.4.3 Hypothesis III: That the Castle Hill buttercup is of diploid hybrid origin.

The results presented in this study provide no support for the hypothesis that *R. c. paucifolius* is a diploid hybrid species. In analysis of chloroplast and nuclear DNA markers, *R. c. paucifolius* was essentially identical in sequence to *R. c. crithmifolius*, its sister subspecies. AFLP and ISSR DNA fingerprinting did show a degree of genetic difference between the two taxa, but in both cases, *R. c. crithmifolius* was shown to be *R. c. paucifolius*' closest relative.

4.4.4 Genetic variation within *R. c. paucifolius*

Genetic variation was discovered within the population of *R. c. paucifolius* by the application of MLF, finding at least three different genotypes (see Fig. 2.4). This might be considered a surprising result when the history of the Castle Hill region is considered.

It is difficult to determine whether the *R. c. paucifolius* clumps counted are single plants, clumps of many seedlings or vegetative clones of a single individual. As a consequence of this, most recent references have been to “clumps” (McCaskill, *c.* 1982). The historically unsettled nature of the reserve has led to *R. c. paucifolius* being restricted through a recent population bottleneck. Prior to the fencing of the reserve in 1948 there were 32 plants. Weeding and transplantation of seedlings increased the population to 177 clumps containing 400 plants by 1978 (McCaskill, *c.* 1982), but recent observation has shown a restricted population of 89 clumps. Genetic variation in small populations is often expected to be low due to bottlenecks, and some studies support this (Bauert *et al.*, 1998; Landergott *et al.*, 2001). However, some research has shown that this might not necessarily be the case (Friar *et al.*, 2000). A thorough analysis of genetic variation of the population of Castle Hill buttercups would provide valuable information in determining future conservation strategies in the Kura Tawhiti area.

4.5 Conservation and this study.

These results raise questions about future conservation in the Kura Tawhiti area. The population of Castle Hill buttercups has decreased sharply since the last count in 1978, and barring human conservation effort, is likely to continue to decrease. Extinction seems a likely outcome if the situation is ignored.

However, is the Castle Hill buttercup worth preserving? The present study suggests that taxonomically *R. c. paucifolius* should remain a subspecies, as the genetic variation observed was insufficient to justify a taxonomic reassignment. Further

study would be required to resolve this issue. As a subspecies, *R. c. paucifolius* is likely to be assigned lower conservation priority than it would receive if it possessed full species status.

The finding that the Castle Hill buttercup is genetically distinct is also relevant to this question, as it is a valid argument for preservation under criteria (b) and (c) of Crozier (1997). Although taxonomically only a subspecies, the lack of overlap in the range of leaf shapes between *R. c. paucifolius* and *R. c. crithmifolius* combined with the uniqueness of *R. c. paucifolius*' habitat and the molecular evidence shown in this report, all indicate that the Castle Hill buttercup is nevertheless morphologically, ecologically and genetically distinct, and the conservation value of the McCaskill nature reserve would be severely compromised if *R. c. paucifolius* were to become extinct. The scientific value of *R. c. paucifolius* is also important, as it gives the opportunity to study evolution, alpine radiation, and differences in genetics and physiology between closely related taxa. This last prospect is particularly interesting, as there are few examples of New Zealand studies which take a defined ecological habitat into consideration. The study of the Castle Hill Buttercup would provide a valuable opportunity to address this knowledge gap.

R. c. paucifolius is a distinct and unusual component of New Zealand's biodiversity, offering the opportunity for scientific study of hybridisation and species concepts and formation. As the main conservation effort required in the area would only be regular weeding of introduced plants, it would reflect badly on New Zealand specifically, and on people in general if we were to let an endemic taxon perish through simple neglect.

5 References

Álvarez, I. and Wendel, J. F. (2003). "Ribosomal ITS sequences and plant phylogenetic inference." Molecular Phylogenetics and Evolution **29**(3): 417-434.

Andreasen, K. and Baldwin, B. G. (2003). "Nuclear ribosomal DNA sequence polymorphism and hybridization in checker mallows (*Sidalcea*, Malvaceae)." Molecular Phylogenetics and Evolution **29**(3): 563-581.

Archak, S., Gaikwad, A. B., Gautam, D., Rao, E., Swamy, K. R. M. and Karihaloo, J. L. (2003). "Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India." Genome **46**(3): 362-369.

Arnheim, N., Krystal, M., Schmickel, R., Wilson, G., Ryder, O. and Zimmer, E. (1980). "Molecular Evidence for Genetic Exchanges among Ribosomal Genes on Nonhomologous Chromosomes in Man and Apes." Proceedings of the National Academy of Science USA **77**(12, [Part 2: Biological Sciences]): 7323-7327.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. and Donoghue, M. J. (1995). "The ITS Region of Nuclear Ribosomal DNA - a Valuable Source of Evidence on Angiosperm Phylogeny." Annals of the Missouri Botanical Gardens **82**(2): 247-277.

Bandelt, H.-J. and Dress, A. W. M. (1992). "Split decomposition: A new and useful approach to phylogenetic analysis of distance data." Molecular Phylogenetics and Evolution **1**(3): 242-252.

Batt, G. E., Braun, J., Kohn, B. P. and McDougall, I. (2000). "Thermochronological analysis of the dynamics of the Southern Alps, New Zealand." GSA Bulletin **112**(2): 250 - 266.

- Bauert, M. R., Kalin, M., Baltisberger, M. and Edwards, P. J. (1998). "No genetic variation detected within isolated relict populations of *Saxifraga cernua* in the Alps using RAPD markers." Molecular Ecology **7**(11): 1519-1527.
- Bryant, D. and Moulton, V. (2004). "Neighbor-Net: An Agglomerative Method for the Construction of Phylogenetic Networks." Molecular Biology and Evolution **21**(2): 255-265.
- Burrows, C. J. (1965). "Some discontinuous distributions of plants within New Zealand and their ecological significance." Tuatara **13**: 9-29.
- Burrows, C. J. and Moar, N. T. (1996). "A mid Otira Glaciation palaeosol and flora from the Castle Hill Basin, Canterbury, New Zealand." New Zealand Journal of Botany **34**: 539-545.
- Chase, M. W. and Hills, H. H. (1991). "Silica gel: An ideal material for field preservation of leaf samples for DNA studies." Taxon **40**: 215-220.
- Clarke, A. C. (2001). Did Polynesian voyagers introduce the sweet potato (*Ipomoea batatas*) into Oceania from South America? A start on the molecular evidence. IMBS. Palmerston North, Massey University.
- Comes, H. P. and Kadereit, J. W. (1998). "The effect of Quaternary climatic changes on plant distribution and evolution." Trends in Plant Science **3**(11): 432-438.
- Cooper, A. and Cooper, R. A. (1995). "The Oligocene Bottleneck and New Zealand Biota: Genetic Record of a past Environmental Crisis." Proceedings: Biological Sciences **261**(1362): 293-302.
- Corriveau, J. L. and Coleman, A. W. (1988). "Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 Angiosperm species." American Journal of Botany **75**(10): 1443-1458.

Crozier, R. H. (1997). "Preserving the information content of species: Genetic Diversity, Phylogeny, and Conservation Worth." Annual Review of Ecology and Systematics **28**(1): 243-268.

de Lange, P. J. and Norton, D. A. (1998). Revisiting rarity: a botanical perspective on the meanings of rarity and the classification of New Zealand's uncommon plants. Ecosystems, entomology and plants. The Royal Society of New Zealand Miscellaneous Series. **48**: 145-160.

Ehrendorfer, F. (1959). "Differentiation-Hybridization cycles and polyploidy in *Achillea*." Cold Spring Harbour Symposia on Quantitative Biology **24**: 141 - 151.

Fisher, F. J. F. (1965). The Alpine *Ranunculi* of New Zealand. Wellington, R. E. Owen, Government Printer.

Frackman, S., Kobs, G., Simpson, D. and Storts, D. (1998). "Betaine and DMSO: Enhancing Agents for PCR." Promega Notes **65**: p27.

Friar, E. A., Ladoux, T., Roalson, E. H. and Robichaux, R. H. (2000). "Microsatellite analysis of a population crash and bottleneck in the Mauna Kea silversword, *Argyroxiphium sandwicense* ssp *sandwicense* (Asteraceae), and its implications for reintroduction." Molecular Ecology **9**(12): 2027-2034.

Fuertes Aguilar, J., Rossello, J. A. and Nieto Feliner, G. (1999). "Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae)." Molecular Ecology **8**(8): 1341-1346.

Futuyma, D. J. (1998). Evolutionary Biology. Sunderland, Massachusetts, Sinauer Associates.

Gage, M. (1958). "Late Pleistocene glaciations of the Waimakariri Valley, Canterbury, New Zealand." New Zealand Journal of Geology and Geophysics **5**: 531-544.

- Gage, M. (1977). Glacial geology. Cass: History and natural science in the vicinity of the Cass field station. Burrows, C. J. Christchurch, New Zealand, Department of Botany, University of Canterbury: 67-77.
- Gage, M. and Suggate, R. P. (1958). "Glacial chronology of the New Zealand Pleistocene." Bulletin of the Geological Society of America **69**: 589-598.
- Garcia-Maroto, F., Garrido-Cardenas, J. A., Gomez-Mercado, F., Guil-Guerrero, J. L. and Alonso, D. L. (2003). "Natural hybridisation and phylogenetic position of *Saxifraga trabutiana* (Saxifragaceae) inferred from ISSR markers and ITS sequences." Annales Botanici Fennici **40**(1): 5-13.
- Heads, M. (1998). "Biogeographic disjunction along the Alpine fault, New Zealand." Biological Journal of the Linnean Society **63**(2): 161-176.
- Heads, M. and Craw, R. (2004). "The Alpine Fault biogeographic hypothesis revisited." Cladistics **20**(2): 184-190.
- Heenan, P. B. and Mitchell, A. D. (2003). "Phylogeny, biogeography and adaptive radiation of *Pachycladon* (Brassicaceae) in the mountains of South Island, New Zealand." Journal of Biogeography **30**(11): 1737-1749.
- Hey, J. (2001). "The mind of the species problem." Trends in Ecology and Evolution **16**(7): 326-329.
- Huson, D. (1998). "SplitsTree: analyzing and visualizing evolutionary data." Bioinformatics **14**(1): 68-73.
- Huson, D. and Bryant, D. (in preparation). "Estimating phylogenetic trees and networks using Splitstree4."
- IUCN (2001). IUCN Red List Categories and Criteria: Version 3.1. Gland, Switzerland and Cambridge, UK, IUCN.

- Landergott, U., Holderegger, R., Kozłowski, G. and Schnell, J. J. (2001). "Historical bottlenecks, decrease genetic diversity in natural populations of *Dryopteris cristata*." *Heredity* **87**: 344-355.
- Lee, M. S. Y. (2004). "The molecularisation of taxonomy." *Invertebrate Systematics* **18**(1): 1-6.
- Lockhart, P. J., McLenachan, P. A., Havell, D., Glenny, D., Huson, D. and Jensen, U. (2001). "Phylogeny, radiation, and transoceanic dispersal of New Zealand alpine buttercups: Molecular evidence under split decomposition." *Annals of the Missouri Botanical Gardens* **88**(3): 458-477.
- Mace, G. M. and Lande, R. (1991). "Assessing Extinction Threats toward a Reevaluation of IUCN Threatened Species Categories." *SO - Conservation Biology*. **5**(2). 1991. 148-157.
- Marshall, H. D., Newton, C. and Ritland, K. (2001). "Sequence-Repeat Polymorphisms Exhibit the Signature of Recombination in Lodgepole Pine Chloroplast DNA." *Molecular Biology and Evolution* **18**(11): 2136-2138.
- McCaskill, L. W. (c. 1982). The Castle Hill buttercup (*Ranunculus paucifolius*) A story of preservation. Special publication No. 25, Tussock Grasslands and Mountain Lands Institute (TGMLI), Lincoln College, Canterbury, New Zealand.
- McGlone, M. S. (1985). "Plant biogeography and the late Cenozoic history of New Zealand." *New Zealand Journal of Botany* **23**: 723 - 749.
- McGlone, M. S., Duncan, R. P. and Heenan, P. B. (2001). "Endemism, species selection and the origin and distribution of the vascular plant flora of New Zealand." *Journal of Biogeography* **28**(2): 199-216.
- McGregor, C. E., Lambert, C. A., Greyling, M. M., Louw, J. H. and Warnich, L. (2000). "A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm." *Euphytica* **113**(2): 135-144.

Molloy, J., Bell, B., Clout, M., de Lange, P., Gibbs, G., Given, D., Norton, D., Smith, N. and Stephens, T. (2002). Classifying species according to threat of extinction. A system for New Zealand. Threatened species occasional publication 22: 26.

Molloy, J. and Davis, A. (1994). Setting priorities for the conservation of New Zealand's threatened plants and animals. Wellington, New Zealand, Department of Conservation.

Olmstead, R. G. and Palmer, J. D. (1994). "Chloroplast DNA Systematics: A Review of Methods and Data Analysis." American Journal of Botany **81**(9): 1205-1224.

Rattenbury, J. A. (1962). "Cyclic Hybridization as a Survival Mechanism in the New Zealand Forest Flora." Evolution **16**(3): 348 - 363.

Saini, N., Jain, N., Jain, S. and Jain, R. K. (2004). "Assessment of genetic diversity within and among Basmati and non-Basmati rice varieties using AFLP, ISSR and SSR markers." Euphytica **140**(3): 133-146.

Saitou, N. and Nei, M. (1987). "The Neighbor-Joining Method - a New Method for Reconstructing Phylogenetic Trees." Molecular Biology and Evolution **4**(4): 406-425.

Schonswetter, P., Paun, O., Tribsch, A. and Niklfeld, H. (2003). "Out of the Alps: colonization of Northern Europe by East Alpine populations of the Glacier Buttercup *Ranunculus glacialis* L. (Ranunculaceae)." Molecular Ecology **12**(12): 3373-3381.

Schonswetter, P., Tribsch, A., Stehlik, I. and Niklfeld, H. (2004). "Glacial history of high alpine *Ranunculus glacialis* (Ranunculaceae) in the European Alps in a comparative phylogeographical context." Biological Journal of the Linnean Society **81**(2): 183-195.

Secretariat of the Convention on Biological Diversity, U. N. E. P. (1992). Convention on Biological Diversity. United Nations Conference on Environment and Development (UNCED), Rio de Janeiro, Brazil, United Nations Environment Programme.

- Small, R. L., Cronn, R. C. and Wendel, J. F. (2004). "L. A. S. JOHNSON REVIEW No. 2. Use of nuclear genes for phylogeny reconstruction in plants." Australian Systematic Botany **17**: 145-170.
- Smitsen, R. D., Breitwieser, I., Ward, J. M., McLenachan, P. A. and Lockhart, P. J. (2003). "Use of ISSR profiles and ITS-sequences to study the biogeography of alpine cushion plants in the genus *Raoulia* (Asteraceae)." Plant Systematics and Evolution **239**(1 - 2): 79-94.
- Stebbins, L. G. (1959). "The Role of Hybridization in Evolution." Proceedings of the American Philosophical Society **103**(2): 231-251.
- Stehlik, I., Blattner, F. R., Holderegger, R. and Bachmann, K. (2002). "Nunatak survival of the high Alpine plant *Eritrichium nanum* (L.) Gaudin in the central Alps during the ice ages." Molecular Ecology **11**(10): 2027-2036.
- Stehlik, I., Schneller, J. J. and Bachmann, K. (2001). "Resistance or emigration: response of the high-alpine plant *Eritrichium nanum* (L.) Gaudin to the ice age within the Central Alps." Molecular Ecology **10**(2): 357-370.
- Stoekler, K. (2001). Origins and Evolution of the New Zealand Forest Flora. IMBS. Palmerston North, Massey University: 234.
- Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. and Vogler, A. P. (2003). "A plea for DNA taxonomy." Trends in Ecology and Evolution **18**(2): 70-74.
- Trewick, S. A. and Wallis, G. P. (2001). "Bridging the "beech-gap": New Zealand invertebrate phylogeography implicates pleistocene glaciation and pliocene isolation." Evolution **55**(11): 2170-2180.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). "AFLP: a new technique for DNA fingerprinting." Nucleic Acids Research **23**(21): 4407-4414.

- Wagstaff, S. J. and Garnock-Jones, P. J. (2000). "Patterns of diversification in *Chionohebe* and *Parahebe* (Scrophulariaceae) inferred from ITS sequences." New Zealand Journal of Botany **38**: 389 - 407.
- Wallis, G. P. and Trewick, S. A. (2001). "Finding Fault with Vicariance: A Critique of Heads (1998)." Systematic Biology **50**(4): 602-609.
- Wardle, P. (1963). "Evolution and distribution of the New Zealand flora, as affected by Quaternary climates." New Zealand Journal of Botany **1**(1): 3-17.
- Willet, R. W. (1950). "The New Zealand Pleistocene snow line, climatic conditions, and suggested biological effects." The New Zealand Journal of Science and Technology **32B**(1): 18-48.
- Willis, K. and Whittaker, R. (2000). "The Refugial Debate." Science **287**(5457): 1406-1407.
- Winkworth, R. C., Grau, J., Robertson, A., W. and Lockhart, P. J. (2002). "The origins and evolution of the genus *Myosotis* L. (Boraginaceae)." Molecular Phylogenetics and Evolution **24**: 180 - 193.
- Wolfe, A. D. and Randle, C. P. (2004). "Recombination, heteroplasmy, haplotype polymorphism, and paralogy in plastid genes: Implications for plant molecular systematics." Systematic Botany **29**(4): 1011-1020.
- Wolfe, A. D., Xiang, Q.-Y. and Kephart, S. R. (1998). "Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands." Molecular Ecology **7**(9): 1107-1125.
- Wolfe, A. D., Xiang, Q.-Y. and Kephart, S. R. (1998). "Diploid hybrid speciation in *Penstemon* (Scrophulariaceae)." Proceedings of the National Academy of Science USA **95**(9): 5112-5115.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). "Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification." Genomics **20**: 176-183.

6 Appendices

6.1 Appendix A: Collection information of *Ranunculus* samples used in analyses.

Taxon	Sample	Origin	Region	Latitude	Longitude	Altitude	Voucher
<i>Ranunculus c. crithmifolius</i>	crithmifolius_M1	Mount Hutt	Canterbury	-43.52	171.55	1300m	MPN28946
<i>Ranunculus c. crithmifolius</i>	crithmifolius_M2	Mount Lyndon	Waimakariri	-43.2988	171.657	1400m	MPN28951
<i>Ranunculus c. crithmifolius</i>	crithmifolius_M3	Ben Ohau	South Canterbury	-44.24	169.96	1200m	MPN28947
<i>Ranunculus c. crithmifolius</i>	crithmifolius_M4	Ben Ohau	South Canterbury	-44.23	169.95	1420m	MPN28948
<i>Ranunculus c. crithmifolius</i>	crithmifolius_M5	Mount Somers	Canterbury	-45.625	171.32	960m	MPN28949
<i>Ranunculus c. crithmifolius</i>	crithmifolius_M6	Gordon Range	Marlborough	-41.59	172.97	1250m	MPN28950
<i>Ranunculus c. crithmifolius</i>	crithmifolius_P1	Eyre Mountains	West Otago	-45.37	168.71	1000m	CHR509774
<i>Ranunculus c. crithmifolius</i>	crithmifolius_P2	Eyre Mountains	West Otago	-45.52	168.38	1000m	MPN24673
<i>Ranunculus gracilipes</i>	gracilipes_M1	Rock and Pillar Range	Otago	-45.457	167.993	1300m	MPN28967
<i>Ranunculus gracilipes</i>	gracilipes_M2	Lake Alta	Central Otago	-45.05	168.81	1600m	CHR529048
<i>Ranunculus gracilipes</i>	gracilipes_M3	McKerrow Range	Northwest Otago	-44.2	169.265	1460m	MPN28966
<i>Ranunculus gracilipes</i>	gracilipes_M4	Ben Ohau	South Canterbury	-44.23	169.4	1100m	In processing
<i>Ranunculus gracilipes</i>	gracilipes_M5	Dansey's Pass	East Otago	-44.953	170.373	950m	MPN28965
<i>Ranunculus gracilipes</i>	gracilipes_M6	Mount Cheeseman	Waimakariri	-43.1556	171.665	1660m	MPN28964
<i>Ranunculus gracilipes</i>	gracilipes_M7	Crown Range Road	West Otago	-44.995	168.921	888m	In processing
<i>Ranunculus gracilipes</i>	gracilipes_M8	Hummock Peak	West Otago	-45.03	168.298	1360m	MPN28963
<i>Ranunculus gracilipes</i>	gracilipes_M9	Takitimu Mountains	West Otago	-45.722	168.006	400m	In processing
<i>Ranunculus gracilipes</i>	gracilipes_M10	Rock and Pillar Range	Otago	-45.47	169.993	1310m	MPN28968
<i>Ranunculus insignis</i>	insignis_M1	Castle Hill	Waimakariri	-43.225	171.718	740m	MPN28952
<i>Ranunculus insignis</i>	insignis_M2	Porter's Pass	Waimakariri	-43.296	171.742	900m	MPN28953
<i>Ranunculus insignis</i>	insignis_M3	Lake Lyndon	Waimakariri	-43.29	171.695	1200m	MPN28954
<i>Ranunculus insignis</i>	insignis_M4	Mount Hutt	Canterbury	-43.52	171.55	1300m	MPN28955
<i>Ranunculus insignis</i>	insignis_M5	Foggy Peak	Waimakariri	-43.2886	171.745	1200m	In processing
<i>Ranunculus insignis</i>	insignis_M6	Castle Hill	Waimakariri	-43.225	171.718	780m	In processing
<i>Ranunculus insignis</i>	insignis_M7	Foggy Peak Ridge	Waimakariri	-43.2886	171.745	1200m	In processing
<i>Ranunculus insignis</i>	insignis_M8	Porter's Pass	Waimakariri	-43.296	171.742	780m	MPN28959
<i>Ranunculus insignis</i>	insignis_M9	Torlesse Range	Waimakariri	-43.286	171.81	1200m	MPN28960
<i>Ranunculus insignis</i>	insignis_M10	Castle Hill	Waimakariri	-43.22	171.718	780m	MPN28962

Taxon	Sample	Origin	Region	Latitude	Longitude	Altitude	Voucher
<i>Ranunculus insignis</i>	insignis_M10	Castle Hill	Waimakariri	-43.22	171.718	780m	MPN28962
<i>Ranunculus insignis</i>	insignis_M11	Torlesse Range	Waimakariri	-43.277	171.819	1310m	MPN28961
<i>Ranunculus insignis</i>	insignis_M12	Mount Hutt	Waimakariri	-43.518	171.547	1380m	MPN28958
<i>Ranunculus insignis</i>	insignis_M13	Kennet River	Awatere	-42.019	173.343	900m	MPN28957
<i>Ranunculus insignis</i>	insignis_M14	Castle Hill	Waimakariri	-43.22	171.7186	750m	MPN28956
<i>Ranunculus enysii</i>	enysii_M1	Mount Cheeseman	Waimakariri	-43.1591	171.65787	1620m	MPN28939
<i>Ranunculus enysii</i>	enysii_M2	Mount Misery	Waimakariri	-43.06	171.711	1200m	MPN28941
<i>Ranunculus enysii</i>	enysii_M3	Broken River	Waimakariri	-43.1285	171.69	1350m	MPN28937
<i>Ranunculus enysii</i>	enysii_M4	Foggy Peak	Waimakariri	-43.2886	171.745	1200m	MPN28936
<i>Ranunculus enysii</i>	enysii_M5	Mount Lyndon	Waimakariri	-43.2917	171.6928	1200m	MPN28942
<i>Ranunculus enysii</i>	enysii_M6	Sugarloaf Peak	Waimakariri	-43.0327	171.7763	900m	MPN28932
<i>Ranunculus enysii</i>	enysii_M7	Mount Lyndon	Waimakariri	-43.288	171.679	1200m	MPN28940
<i>Ranunculus enysii</i>	enysii_M8	Porter's Pass	Waimakariri	-43.29	171.745	1000m	MPN28934
<i>Ranunculus enysii</i>	enysii_M9	Mount Cheeseman	Waimakariri	-43.1679	171.6609	1500m	MPN28943
<i>Ranunculus enysii</i>	enysii_M10	Lake Lyndon	Waimakariri	-43.296	171.678	1420m	MPN28935
<i>Ranunculus enysii</i>	enysii_M11	Rock and Pillar Range	Otago	-45.38	170.117	1200m	MPN28945
<i>Ranunculus enysii</i>	enysii_M12	Borland Burn	Fiordland	-45.38	170.11	1200m	MPN28933
<i>Ranunculus enysii</i>	enysii_M13	Borland Burn	Fiordland	-45.757	167.3901	1200m	MPN28944
<i>Ranunculus enysii</i>	enysii_M14	Rock and Pillar Range	Otago	-45.38	170.11	1200m	MPN28938
<i>Ranunculus enysii</i>	enysii_P2	Nevis Valley	West Otago	Not available	Not available	Not available	MPN24610
<i>Ranunculus enysii</i>	enysii_P3	Umbrella Mountains	Central Otago	Not available	Not available	Not available	MPN24611
<i>Ranunculus c. paucifolius</i>	paucifolius_M1	Castle Hill	Waimakariri	-43.2239	171.71714	750m	No Voucher (Endangered)
<i>Ranunculus c. paucifolius</i>	paucifolius_M2	Castle Hill	Waimakariri	-43.2239	171.71714	750m	No Voucher (Endangered)
<i>Ranunculus c. paucifolius</i>	paucifolius_M3	Castle Hill	Waimakariri	-43.2239	171.71714	750m	No Voucher (Endangered)
<i>Ranunculus c. paucifolius</i>	paucifolius_M4	Castle Hill	Waimakariri	-43.2239	171.71714	750m	No Voucher (Endangered)

Table A- Collection information of *Ranunculus* samples used in analyses.

6.2 Appendix B: Invitrogen DNA ladder standards

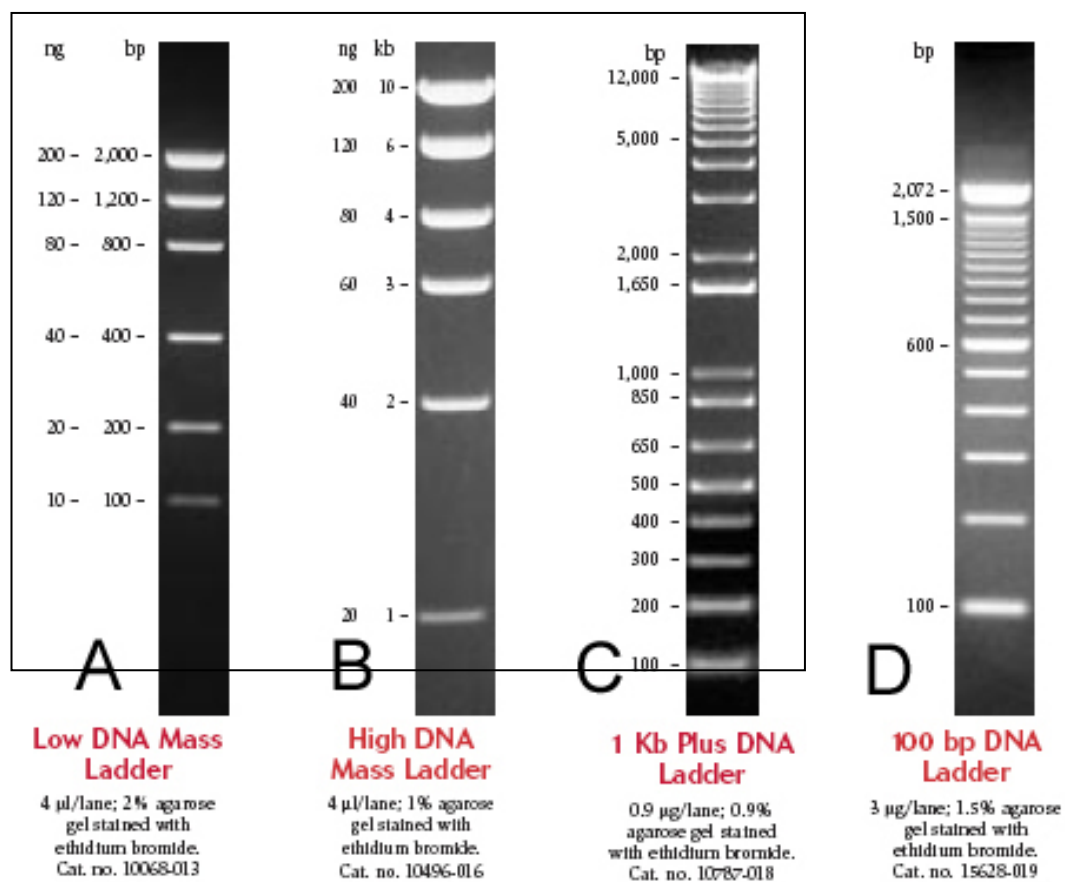


Figure B - Invitrogen DNA ladder standards. (A) Low DNA Mass Ladder. (B) High DNA Mass Ladder. (C) 1 Kb Plus DNA Mass Ladder. (D) 100 bp DNA Mass Ladder.

6.3 Appendix C: Oligonucleotides used in DNA sequencing, AFLP and ISSR

Direct sequencing marker primer sequences		
Locus	Primer Name	Primer Sequence
ITS 1 & 2 rDNA	ITS4	5' TCCTCCGCTTATTGATATGC 3'
	ITS5	5' GGAAGTAAAAGTCGTAACAAGG 3'
Chloroplast J _{SA}	J _{SAF}	5' ATTATYAATGAAGGYAATACWATATATTTTC 3'
	J _{SAR}	5' CAAATTCCAATGACCAAATAGTTGG 3'

AFLP Linker Sequences	
Linker Name	Primer Sequence
MseI linker 1	5' TACTCAGGACTCAT 3'
MseI linker 2	5' GACGATGAGTCCTGAG 3'
EcoRI linker1	5' CTCGTAGACTGCGTACC 3'
EcoRI linker2	5' AATTGGTACGCAGTCTAC 3'

Selective AFLP primers (selective bases are highlighted)	
Primer Name	Primer Sequence
MseI Pre-amp primer	5' GATGAGTCCTGAGTAAC 3'
MseI Selective primer	5' GATGAGTCCTGAGTAACAAG 3'
EcoRI Pre-amp primer	5' GACTGCGTACCAATTC 3'
EcoRI Selective primer	5' GACTGCGTACCAATTCATA 3'

University of British Columbia primer set #9 microsatellite primers	
Primer Name	Primer Sequence
#811	5' GAGAGAGAGAGAGAGAC 3'
#835	5' AGAGAGAGAGAGAGAGYC 3'
#844	5' CTCTCTCTCTCTCTRC 3'

Table C - Oligonucleotides used in DNA sequencing, AFLP and ISSR