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

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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 J_{SA} 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.

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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

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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 (43.223922º S 171.717081º 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).

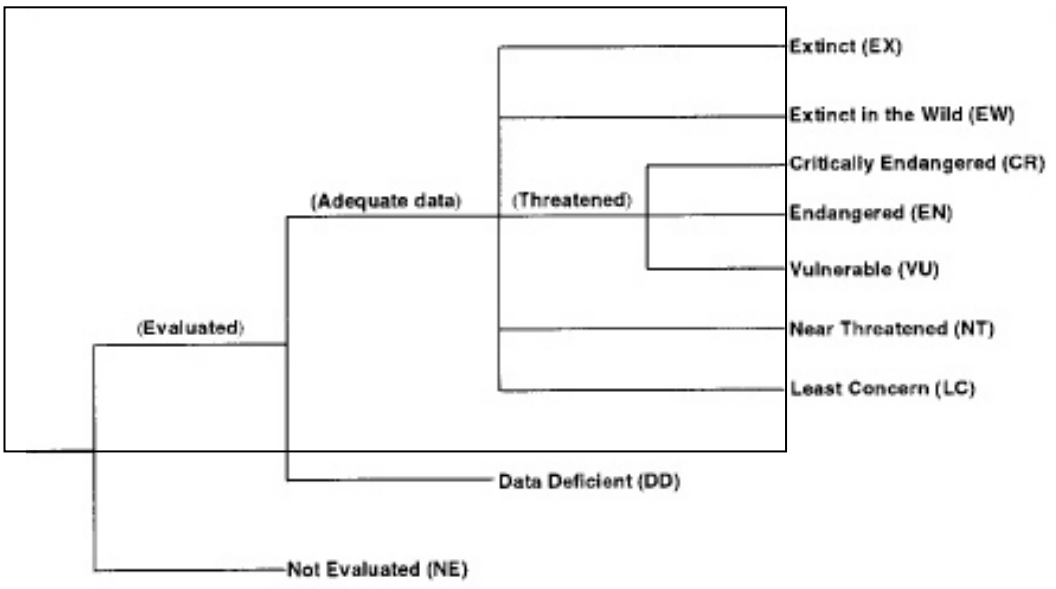

Figure 1.1- IUCN threatened species Red List categories (IUCN, 2001)

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.

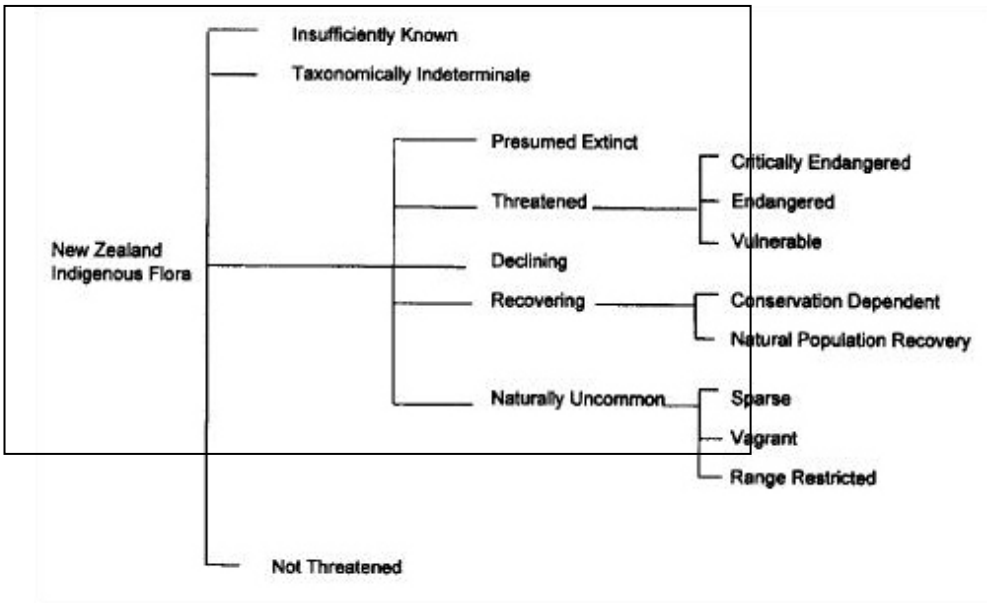

Figure 1.2– Conservation classification system for New Zealand plants (after de Lange and Norton 1998)

IUCN v3.1 (2001)	DeLange and Norton, 1998	Molloy *et al.*, 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).

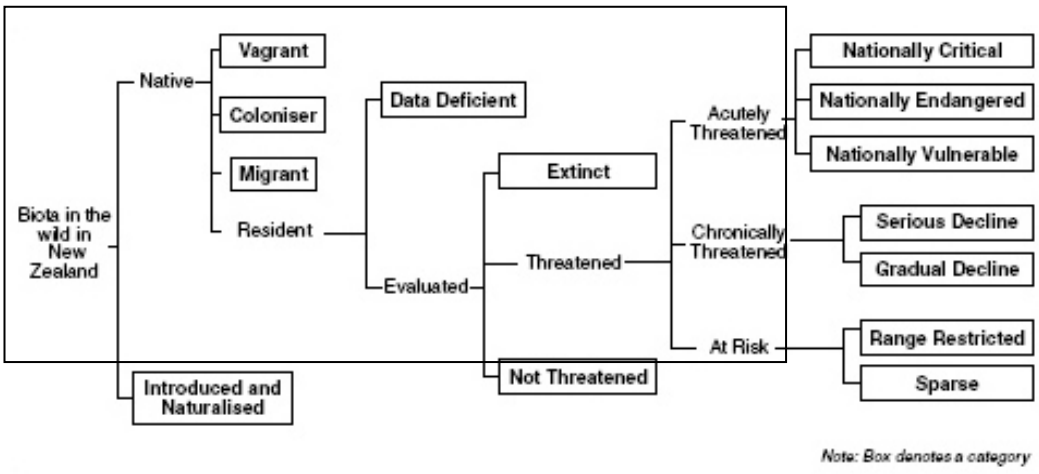

Figure 1.3 - Conservation classification system for New Zealand (Molloy *et al.* 2002)

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
R. insignis	North and South Islands

R. verticillatus	North and South Islands
R. nivicola	North Island only
R. buchananii	South Island only
R. crithmifolius	South Island only
R. enysii	South Island only
R. gracilipes	South Island only
R. grahamii	South Island only
R. godleyanus	South Island only
R. haastii	South Island only
R. lyalli	South Island only
R. pachyrrhizus	South Island only
R. sericophyllus	South Island only
R. viridis	Stewart Island
R. pinguis	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. lyallii* 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. pachyrrihzus* occur in similar habitats to *R. godleyanus* but *R. pachyrrihzus* 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 (Stoeckler, 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 pres ent 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} (cpJ_{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.