An investigation into the evolutionary relationships of the North Island alpine *Ranunculus*

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Abstract

Ranunculus nivicola is a member of the alpine *Ranunculus* (Section *Pseudadonis*), a group of 18 species that occupy distinct ecological habitats within mountainous environments of New Zealand and Australia. There are currently three species of alpine *Ranunculus* found in the North Island of New Zealand. Of those three species *R. nivicola* is the only species endemic to the North Island. With a ploidy of 2n = 96, *R. nivicola* is the exception to the hexaploid state (2n = 48) of all other members of the alpine *Ranunculus*. It has been hypothesised that the elevated chromosome number in *R. nivicola* could be explained by a hybridisation event between the other two North Island species, *R. insignis* and *R. verticillatus*, with subsequent polyploidisation.

The aim of the present study was to investigate the evolutionary history and habitat differences of the North Island species of *Ranunculus* through phylogenetic analyses of sequence data and the use of LENZ (Land Environments of New Zealand) data layers with geographic information systems. Nuclear ITS (ITS), chloroplast J_{SA} and trnL - trnF sequences were determined and analysed using median networks and maximum likelihood tree building. Positional data (from GPS grid points and herbarium records) for populations of each of the three species was used to query the LENZ database and extract information on environmental envelopes.

The phylogenetic analyses indicate that *R. verticillatus* and *R. insignis* have different population histories, with range expansion occurring at different periods from the South to the North Island. Multiple events of dispersal between the South and the North Island could be inferred for R. *verticillatus*. However, only a single event of range expansion has occurred for *R. insignis*. A paucity of sequence variation among widely sampled accessions of *R. nivicola* may point to a recent (late Pleistocene-Holocene) origin for this allopolyploid. Interestingly, while *R. nivicola* nuclear ITS sequence is very similar to an extant genotype of *R. verticillatus*, its chloroplast sequence is intermediate between haplotypes shared by *R. insignis* and *R. enysii*. There is no evidence for recurrent allopolyploid formation.

Analyses of environmental data revealed that there were significant differences between the three species. *R. insignis* occupied the widest range of environments, with the

environmental envelopes of *R. verticillatus* and *R. nivicola* occurring as subsets of the *R. insignis* envelope. It was also found that *R. insignis* could potentially occur in areas currently occupied exclusively by *R. nivicola.*

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List of Abbreviations

cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
dNTP	Dinucleotidetriphosphate
GIS	Geographic Information System
GPS	Geographic Positioning System
Indel	Insertion or Deletion
J_{SA}	Junction of the chloroplast short single copy region and inverted repeat A
LENZ	Land Environments of New Zealand
ML	Maximum Likelihood
mtDNA	Mitochondrial DNA
Mya	Million years ago
ITS	Internal Transcribed Spacer
NJ	Neighbor Joining
nDNA	Nuclear DNA
nrDNA	Nuclear ribosomal DNA
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
RNA	Ribonucleic Acid
Subsp.	Subspecies
trnL – trnF	trn L (UAA) 5'exon – trn F (GAA) intergenic spacer region

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Chapter 1 General Introduction

1.1. Introduction to the Themes of the Thesis

1.1.1 Overview

This thesis investigates the role of allopolyploidy in angiosperm evolution through molecular systematics and Geographic Information Systems (GIS) studies on the New Zealand alpine *Ranunculus* (Fisher 1965). Range expansion of taxa into North Island habitats of New Zealand has been inferred based on phylogenetic analyses of chloroplast and nuclear DNA sequences. Habitat differences for diploid and allopolyploid species were characterised using the LENZ (Land Environments of New Zealand) database and ArcView GIS software. Hypotheses are proposed to explain range expansion and extant species distributions.

1.1.2 Role of Polyploidy in Angiosperm Evolution

Polyploidy is regarded as a fundamentally important phenomenon in the evolution of angiosperms (Stebbins, 1971; Grant, 1981; Baack and Staton, 2005). Many studies suggest a significant number of angiosperm species have undergone a polyploidisation event in either their recent evolutionary past (neopolyploids) or in their deep evolutionary past (paleopolyploids) (Soltis and Soltis, 1993; Masterson, 1994; Leitch and Bennett, 1997; Ramsey and Schemske, 1998; Widmer and Baltisberger, 1999; Rieseberg, 2001; Schranz and Osborn, 2004; Soltis *et al.*, 2004b; Adams and Wendel, 2005; Soltis, 2005). It is estimated that 30-80% of angiosperms are either descended from, or are themselves polyploid (Muntzing, 1936; Stebbins, 1950; Grant, 1981; Masterson, 1994). Furthermore, recent data suggest that even species with relatively small genomes, such as *Arabidopsis* (n = 5), have been involved in at least one polyploid event (Simillion *et al.*, 2002; Blanc *et al.*, 2003; Adams and Wendel, 2005). It appears that polyploidy is ongoing and new polyploid taxa have been observed in the last hundred years in several genera (*e.g. Tragopogon* (Ownbey, 1950); *Spartina* (Thompson, 1991), *Senecio* (Lowe and Abbott, 1996) and *Cardamine* (Urbanska *et al.*, 1997)).

Polyploidy is defined as the possession of two or more genomes per cell (Grant, 1981; Ramsey and Schemske, 1998; Soltis and Soltis, 2000). A polyploid species can arise as the result of genome doubling within one species (autopolyploidy) or following interspecific hybridisation (allopolyploidy) (Soltis and Soltis, 2000; Ramsey and Schemske, 2002). Allopolyploids are considered to be more prevalent in nature than autopolyploids, although Soltis and Soltis (2000) suggest that further examination will find that the numbers of autopolyploids are underestimated.

Chromosome doubling has been considered to be maladaptive by some (Levin, 1983) due to factors such as minority cytotype exclusion (Levin, 1975; Rausch and Morgan, 2005), epigenetic instability and the disruption of mitosis and meiosis (Whitton, 2004; Comai, 2005). These features and others have prompted some to downplay the role of polyploidy as a progressive evolutionary state (Dobzhansky, 1950; Wagner, 1970; Mable, 2004). An alternative view is that doubling of chromosome number in allopolyploids is not maladaptive, as it prevents the problems associated with the bringing together of divergent genomes *i.e.* inviability and sterility (Wendel, 2000). Doubling of chromosomes creates a homologue which restores pairing and fertility in chromosomally divergent species (Dobzhansky, 1933). It is thought that genome inflation via polyploidy is temporary and that over time there are rounds of removal of redundant genetic material. Thus over time a species undergoes cycles of expansion via polyploid doubling and contraction via differential gene loss (Adams and Wendel, 2005).

Polyploidy is in effect an instantaneous speciation event with the resulting polyploid usually resistant to introgression with its parental species due to differences in ploidy level (Ramsey and Schemske, 1998; Gross *et al.*, 2003). However, if a newly formed polyploid is to be successful in establishing itself, it needs to be sufficiently different in character from its parental species so as not to be in direct competition with them (Welch and Rieseberg, 2002). Polyploids are often reported to have broader ecological tolerances than their parental species, and this property can facilitate their expansion into habitats not occupied by either parent (Levin, 1983; Schwarzbach *et al.*, 2001). Furthermore, polyploids are purported to be better colonizers than diploids (Soltis and Soltis, 2000). Polyploidisation can also have the effect of changing a plant's morphology into more robust forms (Pal and Khoshoo, 1973) as well as influencing its phenology

(Petit *et al.*, 1997) and physiology (Chen and Tang, 1945). The rearrangement of genes that results from genome doubling can bring about changes that result in the formation of novel phenotypes or the expression of transgressive traits (Adams and Wendel, 2005).

There is significant evidence for the natural occurrence of polyploidy; a question that then arises is whether polyploid formation between two species occurs once or is recurrent. Increasingly, the evidence points towards polyploidy being recurrent in nature (Soltis and Soltis, 1999). Soltis and Soltis (1999) go as far to say that recurrent origins of polyploidy are the rule rather than the exception, and in a study by the same authors (Soltis and Soltis, 1993) they cite 30 examples of recurrent formation from angiosperms, ferns and bryophytes. The mixing of polyploids formed from different populations creates the potential for abundant genetic diversity. The massive potential for genetic variability not only at the individual level but at the population level could be a reason why polyploids are so successful at colonising sites compared to their diploid progenitors.

1.1.3 Polyploidy and the Evolution of the New Zealand Alpine Flora

The high morphological diversity of the New Zealand alpine flora appears to be anomalous given the recent development of the New Zealand mountain ranges (Raven, 1973; Winkworth, 2000; Winkworth *et al.*, 2005). It has been proposed that this diversity is the product of rapid species radiations that occurred in relative isolation (Winkworth *et al.*, 2002b). The role of polyploidy in these radiations is uncertain. Polyploidy has been reported for 63% of New Zealand plant species (Hair, 1966), including species from most of the major angiosperm families present in New Zealand (Table 1.1). However, many plant groups in the New Zealand angiosperm flora have also radiated without forming polyploidy series. The species of New Zealand alpine *Ranunculus* are perhaps one of the best examples of an alpine polyploid group.

One the first proposed allopolyploid species in the New Zealand flora was *Ranunculus nivicola* (Fisher, 1965). It was found that *R. nivicola* has 2n = 96 chromosomes, whilst the nearest known relatives have 2n = 48 chromosomes (Fisher, 1965). It was hypothesised by Fisher (1965) that *R. nivicola* is an allopolyploid of *R. insignis* and *R. verticillatus*. There

are molecular data supporting this hypothesis (Lockhart *et al.*, 2001); however, it remains to be thoroughly tested.

Family	**Example genus**
Asteliaceae	*Astelia* (Wheeler, 1966)
Asteraceae	*Anaphalioides* (Glenny, 1997)
Cyperaceae	*Uncinia* (Hair, 1966)
Ericaceae	*Epacris* (Hair, 1966)
Fabaceae	*Carmichaelia* (Wagstaff *et al.*, 1999)
Iridaceae	*Libertia* (Blanchon *et al.*, 2000)
Lobeliaceae	*Pratia* (Murray *et al.*, 1992)
Onagraceae	*Fuchsia* (Raven, 1979)
Plantaginaceae	*Hebe* (Hair, 1966)
Poaceae	*Poa* (Hair, 1966)
Ranunculaceae	*Ranunculus* (Fisher, 1965)
Rhamnaceae	*Pomaderris* (Hair, 1963)
Rubiaceae	*Coprosma* (Hair, 1966)
Verbenaceae	*Vitex* (Barrell *et al.*, 1997)
Violaceae	*Melicytus* (Molloy and Clarkson, 1996)

Table 1.1: Incidence of polyploidy in the angiosperm flora of New Zealand.

1.1.4 Taxonomy and Systematics of Family Ranunculaceae Juss.

The Ranunculaceae is a large family with a worldwide distribution described as containing between 50-66 genera and approximately 2000 species (Webb *et al.*, 1988; Ziman and Keener, 1989; Hoot, 1991; Johansson and Jansen, 1993). Members of this family, which includes the genus *Ranunculus*, are found predominantly in temperate to arctic/sub-Antarctic climates (Johansson and Jansen, 1993). Outside of montane regions, they are uncommon in tropical and subtropical zones (Ziman and Keener, 1989).

The Ranunculaceae is believed to have originated in the montane temperate floras of the Northern Hemisphere during the Cretaceous (Ziman and Keener, 1989). The family is often considered a "basal" herbaceous eudicot family as members exhibit a number of proposed ancestral traits (Stebbins, 1938; Jury, 1993; APG, 2003), such as numerous and spirally arranged floral parts, apocarpy, imperfect carpel closure and follicles (Hoot, 1991). Yet, it is very successful in the contemporary setting and demonstrates characters considered to be derived, such as finely dissected leaves, achenes, racemose inflorescences and zygomorphic flowers (Hoot, 1991).

On a worldwide scale the distribution of Ranunculaceae can be divided into six floristic kingdoms, with eight subkingdoms, 34 regions and 150 provinces (Ziman and Keener, 1989). Of relevance to this study is the Holoantarctic kingdom which covers South America from latitude 30° south and includes the Neozeylandic region that encompasses New Zealand and its outlying islands.

1.1.5 Genus *Ranunculus* L.

Ranunculus is the largest genus of Ranunculaceae and was described by Linnaeus in the 18th century under the name 'buttercup', which is now the common name of this genus (Rendel, 1987). The genus contains approximately 600 species (Tamara, 1995) and is characteristically morphologically diverse with leaf forms ranging from large peltate leaves to small, highly dissected ones. Parallel evolution appears to be prevalent, with distantly related species often showing convergent leaf morphology (Fisher, 1965).

Polyploidy and hybridisation appear to have been important phenomena in the evolution of this genus (Gregory, 1941; Fisher, 1965; Hörandl *et al.*, 2005).

There is a high degree of ecological diversity within the genus *Ranunculus*, with members found in a variety of terrestrial and aquatic environments, from lowland to alpine zones (Johansson, 1998; Hörandl *et al.*, 2005). *Ranunculus* species have a variety of sexual reproductive strategies such as protogyny and agamospermy, and asexual vegetative reproductive structures such as stolons and bulbs (Fisher, 1965; Hörandl *et al.*, 2005). A high degree of out-crossing is maintained via pollination from generalist insects (Fisher, 1965; Jury, 1993). Species are considered taxonomically difficult to categorise due to high levels of intraspecific morphological diversity, and frequent hybridisation (Rendel and Murray, 1989).

1.1.6 Infrageneric Taxonomy of the New Zealand *Ranunculus*

In the Neozeylandic region there are 38 species of *Ranunculus* divided into three sections. Section *Chrysanthe* (Spach) Benson has 16 species endemic to New Zealand. Section *Epirotes* (Prantel) Benson has 6 species endemic to New Zealand and section *Pseudadonis* F. Muell. has 16 species endemic to New Zealand and 2 species in Australia (Fisher, 1965; Lockhart *et al.*, 2001).

Section *Chrysanthe* contains species found in a range of habitats from sub-alpine to lowland (Rendel and Murray, 1989). Previously, species of section *Epirotes* were divided into two ecologically and genetically distinct groups: those that occupied high-altitude or alpine environments, and lowland plants of wet or damp environments (Rendel, 1987). Tamura (1995) transferred the high altitude and alpine species into section *Pseudadonis*, which is the focus of the current study (Table 1.2).

Ranunculus subgenus *Ranunculus* Peterm.
Members of section *Pseudadonis* F. Muell.
R. anemoneus F. Muell.
R. buchananii Hook. f.
R. crithmifolius subsp. *crithmifolius* Fisher
R. crithmifolius subsp. *paucifolius* (T. Kirk) Fisher.
R. enysii T. Kirk.
R. godleyanus Hook. f.
R. gracilipes Hook. f.
R. grahamii Petrie
R. gunnianus Hook.
R. haastii subsp. *haastii* Fisher
R *haastii* subsp. *pilferus* Fisher
R. insignis Hook. f.
R. lyallii Hook. f.
R. nivicola Hook.
R. pachyrrhizus Hook. f.
R. pinguis Hook. f.
R. scrithalis P.J Garnock Jones
R. sericophyllus Hook. f.
R. verticillatus T. Kirk
R. viridis H.D. Wilson & P.J Garnock Jones

Table 1.2: *Ranunculus* species from section *Pseudadonis* included in this study.

1.1.7 Origin of the New Zealand Alpine *Ranunculus*

The most widely held hypothesis regarding the origin of the New Zealand alpine *Ranunculus* (section *Pseudadonis*) is that they are a derivative of a South American ancestor (Fisher, 1965; Wardle, 1978; Santisuk, 1979; Ziman and Keener, 1989). Alternatively, an ancestor of the New Zealand species may have dispersed via the New Guinea highlands down through the Australian Alps and then to New Zealand (Raven, 1973). It is also

possible that they arose from a lowland relative (Fisher, 1965). The likely candidate for this is *R. acaulis* Banks & Sol. as this species has a wide distribution (Fisher, 1965). However, that hypothesis is not supported by analysis of nuclear and chloroplast DNA sequences (Lockhart *et al.*, 2001).

The prevalence of species in the South Island led Fisher (1965) to conclude that the group originated there, and subsequently dispersed to the mountainous areas of the North, Stewart and sub-Antarctic islands, and into the Australian Alps. There is some molecular support (Lockhart *et al.*, 2001) for dispersal from New Zealand to Australia, with the Australian species *R. gunnianus* and *R. anemoneus* grouping closely with New Zealand species (Lockhart *et al.*, 2001). Such transoceanic dispersal events would have had to occur against the prevailing westerly circumpolar winds. These winds, which still occur today, are thought to have existed since the Tertiary period (Stewart and Neall, 1984), and are a major discussion topic in studies of south-western Pacific biogeography (Raven, 1973; Wagstaff *et al.*, 1999; von Hagen and Kadereit, 2001; Winkworth *et al.*, 2002a; Winkworth *et al.*, 2002b; Munoz *et al.*, 2004).

1.1.8 Habitat Origins: A Result of Tectonic and Climatic Upheaval

New Zealand arrived at its current location *c* 60 million years ago (mya), after splitting off from the super-continent of Gondwana approximately 80 mya and rafting across the Tasman basin (Cooper and Millener, 1993; Cooper and Cooper, 1995). New Zealand is an area of particularly active geological processes, largely due to the fact that it straddles the margins of the Pacific and Indo-Australian continental plates (Fleming, 1979). This marginal position led to an intense period of mountain building in the Southern Alps of the South Island during the last 5-6 million years (Fleming, 1979; Batt *et al.*, 2000; Chamberlain and Poage, 2000). New Zealand has undergone a series of climatic fluctuations; most notably, *c* 18 glacial-interglacial cycles that occurred during the Pleistocene (Cooper and Millener, 1993). The lowering of the sea level with glacial maxima increased the amount of coastal land and joined the three main islands (Te Punga, 1953; Fleming, 1962).

1.1.9 Environmental Specialisation in the New Zealand Alpine *Ranunculus*

Distinctive alpine habitats have been recognised for the alpine species of *Ranunculus* section *Pseudadonis* (Table 1.3). Fisher (1965) identified five of these general environments, which range from rock ledges and cervices among outcrops in the zone of permanent snow to slowly draining seepage zones alongside streams (Table 1.3). These habitats reflect the environmental specialisation of some species. An exception concerns *R. insignis* which is one more environmentally variable species of alpine *Ranunculus*. Fisher (1965) described this species has being restricted to sheltered situations, *i.e.*, protected from direct sunlight or wind. However, it can be observed that there is a gradient in ecological preferences from the North Island where it occurs in damp shady sheltered areas such as stream sides and gorges, to the South Island where *R. insignis* is found in more open tussock grassland or shrub land. While there is going to be variation from these general descriptions it provides a convenient starting point to distinguish between the habitats of the alpine *Ranunculus*.

Habitat	Species
High altitude snowfield	*R. buchananii*
	R. grahamii
Snowline fringe	*R. godleyanus*
	R. sericophyllus
	R. pachyrrhizus
Stony debris	*R. crithmifolius* subsp. *crithmifolius*
	R. haastii
Sheltered situations	*R. lyallii*
	R. pinguis
	R. insignis
	R. enysii
	R. nivicola
	R. pinguis
Slowly draining	*R. gracilipes*
	R. verticillatus

Table 1.3: Characteristic habitats of the alpine *Ranunculus* (section *Pseudadonis*) as identified by Fisher (1965).

1.1.10 Morphological Variation of the New Zealand Alpine *Ranunculus*

Morphologically, the New Zealand species follow the familial trend of varying widely in form and leaf shape. For example, consider *R. lyallii* and *R. gracilipes*: *R. lyallii* has large, entire peltate leaves up to 40cm in diameter and scapes up to 1.0m in height, whereas the leaves of *R. gracilipes* may be only 3cm long with bipinnasect divisions, and the entire plant rarely exceeding 10cm in height.

It was proposed by Fisher (1965) that the morphological variation within section *Pseudadonis* fell into eight categories ranging from uniform morphology to variation

recognised at the level of subspecies. At the simplest level are species such as *R. lyallii* which maintain a uniform morphology across their entire range. More complex is the morphological variation observed in *R. verticillatus* and *R. insignis.*

The vegetative morphology of *R. verticillatus* varies widely across its range and within any one population it is possible to observe the entire range of forms. This species is considered to represent a multiform species under Fisher's (1965) system. *R. insignis* also varies significantly in morphology across its range, grading from broadly reniform leaves (10 – 23 cm wide) to lanceolate forms (2.5 to 10cm wide) (Fisher, 1965). However, unlike *R. verticillatus* where the variation has no geographic structure, in *R. insignis* the variation is clinal and regional forms grade continuously through the species' entire population.

1.1.11 Breeding Groups in the New Zealand Alpine *Ranunculus*

In his study of the New Zealand alpine *Ranunculus* (section *Pseudadonis*), Fisher (1965) found that species could be divided into two breeding groups. These groups could be distinguished on the basis of petal number and hair type (Table 1.4). It was hypothesised that these two groups (many-petalled *i.e.* 10-16 petals with silky hair and few-petalled *i.e.* 5-6, petals with coarse hair) represented an ancestral split in the lineage. In the many-petalled, silky-haired group a further division could be made based on flower colour (Table 1.4). The predominant flower colour in the section *Pseudadonis* is yellow, and it is thought that the two white-flowered species (*R. lyallii* and *R. buchananii*) represent a more recent split in the lineage (Fisher, 1965).

Group	Species	Flower colour
Many-petalled, silky hair	*R. lyallii*	White
	R. buchananii	White
	R. haastii	Yellow
	R. grahamii	Yellow
	R. sericophyllus	Yellow
	R. pachyrrhizus	Yellow
	R. verticillatus	Yellow
	R. pinguis	Yellow
	R. nivicola	Yellow
Few-petalled, coarse hair	*R. insignis*	Yellow
	R. godleyanus	Yellow
	R. crithmifolius subsp. *crithmifolius*	Yellow
	R. enysii	Yellow
	R. gracilipes	Yellow

Table 1.4: Morphological division of the New Zealand alpine *Ranunculus* (section *Pseudadonis*), as identified by Fisher (1965).

A recent study by Lockhart *et al.* (2001) of the alpine *Ranunculus* of New Zealand and Australia, using DNA marker sequencing of the nuclear Internal Transcribed Spacer (ITS) region, found that section *Pseudadonis* can be divided into 4 genetically distinct groups (Table 1.5). Group I is comprised of alpine species from New Zealand and Australia. The species of group II are found in alpine and sub-Antarctic environments of New Zealand and Australia. The group III species are all found in alpine areas of New Zealand. *R. scrithalis*, the solitary group IV species, is a scree specialist and is both ecologically and genetically distinct from the other members of section *Pseudadonis* (Lockhart *et al.*, 2001).

Group	Species	Distribution
I	*R. lyallii*	South Island, New Zealand
	R. haastii	South Island, New Zealand
	R. buchananii	South Island, New Zealand
	R. nivicola (ITS)*	North Island, New Zealand
	R. verticillatus	North and South Islands, New Zealand
	R. grahamii	South Island, New Zealand
	R. anemoneus	Mt Kosciusko, Australia
II	*R. sericophyllus*	South Island, New Zealand
	R. pachyrrhizus	South Island, New Zealand
	R. viridis	Stewart Island, New Zealand
	R. pinguis	Campbell and Auckland Islands, New Zealand
	R. gunnianus	Australian Alps, Australia
III	*R .insignis*	North and South Islands, New Zealand
	R. nivicola (J_{SA})*	North Island, New Zealand
	R. godleyanus	South Island, New Zealand
	R. crithmifolius subsp. *crithmifolius*	South Island, New Zealand
	R. crithmifolius subsp. *paucifolius*	South Island, New Zealand
	R. enysii	South Island, New Zealand
	R. gracilipes	South Island, New Zealand
IV	*R. scrithalis*	South Island, New Zealand

Table 1.5: Phylogenetic divisions identified by J_{SA} and ITS, and geographic locations of the members of section *Pseudadonis*. *It is noted that there is conflict in the placement of *R. nivicola*.

These findings of Lockhart *et al.* (2001) were largely congruent with the findings of Fisher (1965). Fisher's many-petalled line is contained in groups I and II and the few-petalled lineage corresponds to group III. The group IV species of *R. scrithalis* was described by Garnock-Jones (1987) and thus was not discussed in Fisher's 1965 monograph. Lockhart *et al.*, (2001) estimated that the primary divergence between group III and groups I and II occurred approximately 5 mya, which coincides with the onset of Tertiary mountain building in New Zealand (Batt *et al.*, 2000).

Lockhart *et al.* (2001) also characterised the same taxa for the chloroplast encoded marker J_{SA}, which is found at the junction of the inverted repeat A (IR_A) and small single-copy region (SSC) of the chloroplast genome. This region was identified as a fast-evolving marker via Amplified Fragment Length Polymorphism (AFLP) studies of the alpine *Ranunculus* (Lockhart and McLenachan, 1997). Phylogenetic analysis of J_{SA} also suggested the major groupings identified with ITS, with the notable exceptions of *R. verticillatus* and *R. nivicola*. In the J_{SA} phylogeny, *R. verticillatus* was sister to the group I species, rather than being a distinct member of the clade as observed in the ITS phylogeny. In the chloroplast phylogeny, *R. nivicola* was associated with group III rather than group I, as was the case in the ITS phylogeny (Table 1.5). The shift of *R. nivicola* from group I, where it is associated with *R. verticillatus*, to group III (in association with *R. insignis*) is consistent with the hypothesis that this species is the progeny of *R. insignis* and *R. verticillatus*.

1.2 Dispersal and Polyploidy in the North Island *Ranunculus*

1.2.1 Introduction to the Study

Currently there are three species of alpine *Ranunculus* in the North Island, namely *R. insignis*, *R. verticillatus* and *R. nivicola* (Fig. 1.1). It has been hypothesised that during the Pleistocene *R. insignis* and *R. verticillatus*, which are thought to have originated in the South Island, expanded their range across Cook Strait and into the North Island (Fisher, 1965; Lockhart *et al.*, 2001). This most likely occurred during glacial periods of the Pleistocene when lower sea levels meant the islands were connected (Te Punga, 1953; Fleming, 1962; Fisher, 1965; Lockhart *et al.*, 2001). Fisher (1965) hypothesised that *R. nivicola* formed in the North Island as an allopolyploid between *R. verticillatus* and *R. insignis*. In support of this inference Fisher (1965) noted that *R. nivicola* exhibits phenotypic traits intermediate between that of its putative parents (Fig. 1.2).

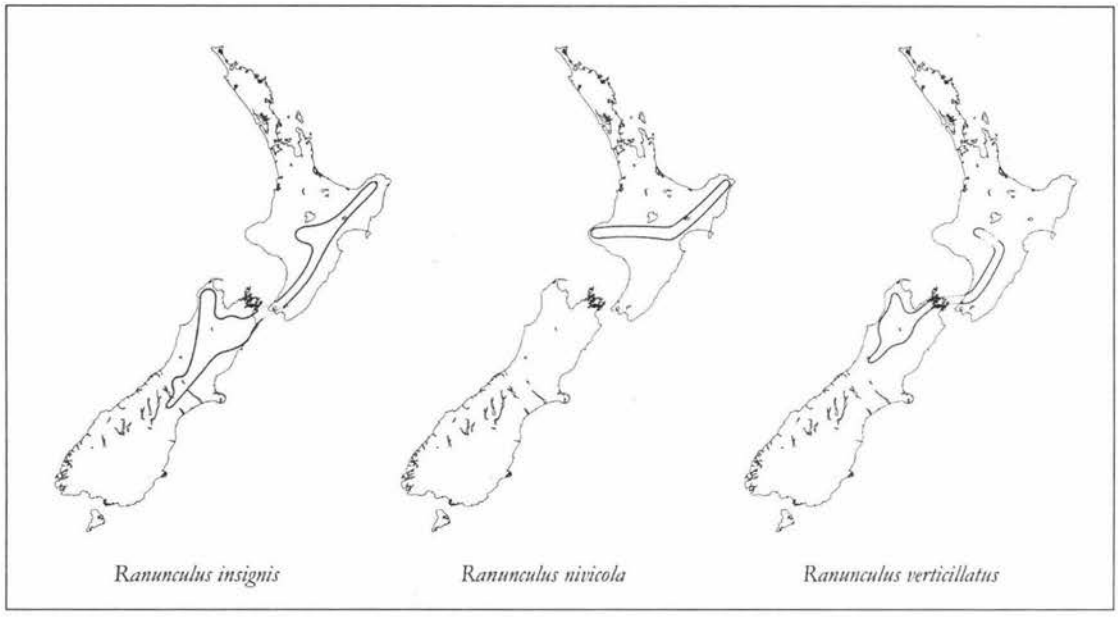

Figure 1.1: The current distributions of *R. insignis*, *R. nivicola* and *R. verticillatus* from Fisher (1965).

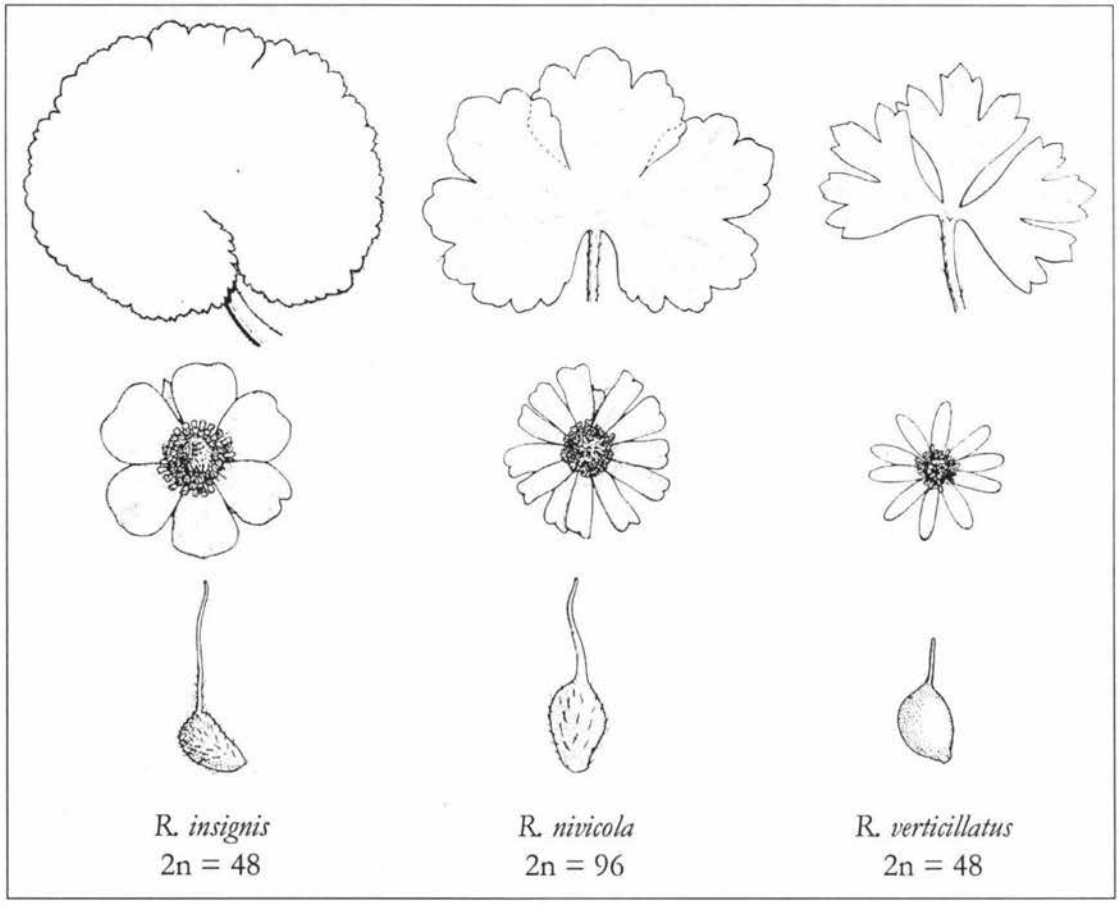

Figure 1.2: Leaf and floral morphology of *R. insignis*, *R. nivicola* and *R. verticillatus*. (Modified from Fisher (1965)).

The principal question that has motivated the present study is whether the formation of the allopolyploid *R. nivicola* might have facilitated range expansion of New Zealand alpine *Ranunculus* into habitats unsuitable for either of the parent species. The present study makes a contribution towards answering this general question by describing phylogenetic relationships of all three species and by analysing ecological diversity with ArcGIS.

1.2.2 Hypotheses and Aims

Hypothesis 1:

Range expansion of *R. insignis* and *R. verticillatus* into the North Island involved rare dispersal events in the evolutionary history of New Zealand alpine *Ranunculus.*

Aim:

Using phylogenetic analyses of J_{SA} and ITS sequences, and more extensive taxon sampling than that used by Lockhart *et al.* (2001), reconstruct the evolutionary relationships among individuals from North and South Island populations of *R. insignis* and *R. verticillatus* and infer the frequency of seed dispersal events between the two islands.

Hypothesis 2:

In the North Island, a single allopolyploidisation event between *R. insignis* and *R. verticillatus* gave rise to *R. nivicola.*

Aim:

With improved taxon sampling compared to that used by Lockhart *et al.*, (2001), perform phylogenetic analyses of J_{SA} and ITS sequences to investigate (a) whether the origin of *R. nivicola* can be identified with respect to North Island populations of *R. insignis* and *R. verticillatus,* and (b) whether or not *R. nivicola* has formed recurrently.

Hypothesis 3:

The polyploid *Ranunculus nivicola* extends the geographic range of alpine *Ranunculus* in the North Island into areas that would not be otherwise occupied by this genus.

Aim:

Use LENZ and ArcGIS techniques to describe the habitat differences of the three North Island species, and infer whether *R. nivicola* occupies habitat that could not be occupied by *R. insignis* or *R. verticillatus.*

Chapter 2 Phylogenetic Investigation of the North Island *Ranunculus*

2.1 Introduction

2.1.1 The Use of Molecular Markers for Phylogenetic Analysis

Molecular markers in combination with tools for phylogenetic analysis are a powerful method for examining phylogenetic relationships at all taxonomic levels, and the field of phylogenetics has become the mainstay for the study of evolutionary histories. Of fundamental importance to a phylogenetic study is the use of genetic regions with the appropriate resolution for the question posed. An important factor in selecting a region for phylogenetic analysis is its potential variability. Under neutral theory, it would be predicted that coding regions will accumulate fewer changes relative to non-coding regions, as they are under strong selective constraint (Kimura and Ohta, 1974) and will be applicable to resolving deep branch topologies. In contrast, non-coding regions are under less constraint and will develop more variation and are therefore appropriate for the determination of relationships of more closely related species in a phylogenetic tree (Kimura and Ohta, 1974; Wolfe and Randle, 2004). Exceptions to these general rules do occur, and this will be considered later.

The most commonly used regions in the study of plant phylogenetics and the focus of this current study have been nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) (Small *et al.*, 2004). Unlike in animals, plant mitochondrial DNA (mtDNA) typically shows a low rate of nucleotide sequence evolution (however see Cho *et al.* (2004) and Parkinson *et al.* (2005)), and often undergoes structural rearrangements (Palmer and Herbon, 1988); for these reasons, mtDNA is not widely used in plant phylogenetic studies (Wolfe *et al.*, 1987; Muse, 2000; Small *et al.*, 2004). An additional source of information that is now receiving some attention is nuclear DNA (nDNA) other than nrDNA, through the use of low copy nuclear genes (Bailey and Doyle, 1999; Sang, 2002; Small *et al.*, 2004). However, the use of these genes for phylogenetic purposes is limited by a lack of universal primers and confounded by the complex evolutionary

dynamics of nuclear gene families (Clegg *et al.*, 1997) and is therefore not yet widely utilised.

2.1.2 Gene Trees versus Species Trees

The use of single gene regions for phylogenetic analysis creates gene trees which may not reflect the full phylogenetic history of a species due to factors such as introgression, hybridisation, lineage sorting, extinction and gene duplication (Pamilo and Nei, 1988; Doyle, 1992; Maddison, 1997; Nichols, 2001; Degan and Rosenberg, 2006). It is important then to use multiple independent markers from different genomes to reconstruct species trees to account for these differences, and to allow for varying rates of sequence evolution in genomes, genes and gene regions.

When multiple independent regions are analysed together there will be one of two possible outcomes with respect to the phylogenetic signal; either they will show a consensus or they will show incongruence. Incongruence among evolutionary trees reconstructed from nrDNA and cpDNA has been widely used to infer events of hybridisation (discussed in McBreen and Lockhart (2006)). This situation occurs because as a general rule, plant nrDNA is biparentally inherited but cpDNA is maternally inherited. A species of hybrid origin will usually have both parental types of a biparentally inherited marker however; concerted evolution (discussed further in Section 2.1.3) can lead to there being only parental type present in the hybrid genome. Thus the placement of a hybrid in a phylogenetic tree may be incongruent as it can have the nrDNA type of parental species A and the cpDNA of parental species B. However, it is also possible that the two independent markers are derived from the same parent, and because both phylogenies will show this, the hybrid origin will effectively be masked. Therefore additional evidence from other sources such as additional markers, genetic studies, crossing studies, and morphology is necessary.

2.1.3 Nuclear Ribosomal Markers

The nuclear genome of a plant cell contains from several hundred to tens of thousands of copies of nrDNA (Ingle *et al.*, 1975; Bendich and Rogers, 1987), which exist in long tandem arrays (Takaiwa *et al.*, 1985; Jorgensen and Cluster, 1988). In plants rDNA

contains the three coding regions (5' 18S, 5.8S, 26S 3') with transcribed non-coding spacers located between (Internal Transcribed Spacer, ITS) and outside (External Transcribed Spacer, ETS) the coding regions (Takaiwa *et al.*, 1985; Jorgensen and Cluster, 1988). The region separating the transcription units of adjacent rDNA repeats is commonly referred to as the intergenic spacer (IGS) (Bendich and Rogers, 1987; Jorgensen and Cluster, 1988).

Ribosomal DNA markers have been a significant source of phylogenetic information over the past two decades (Grechko, 2002). The structure of the three coding regions is highly conserved, and has been used in the study of deep divergences (Mishler *et al.*, 1994; Kuzoff *et al.*, 1998; Soltis *et al.*, 1999; Soltis *et al.*, 2000). The ITS and ETS regions evolve at a faster rate than the coding regions (Appels and Dvorák, 1982) and are therefore suited for the study of recent divergence (Hillis and Dixon, 1991; Baldwin *et al.*, 1995).

The ITS region is the located between the 18S and 26S regions, encompassing the small 5.8S coding region, and is commonly used for inter-generic and inter-specific studies (Baldwin, 1992). This marker is popular because it is biparentally inherited, universal across plants and fungi, present in high numbers and thus easily isolated, and has limited intra-genomic variability, and inter-genomic variability. It is also thought to have low functional constraints, potentially allowing it to evolve neutrally (Arnheim *et al.*, 1980; Baldwin, 1992; Baldwin *et al.*, 1995; Álvarez and Wendel, 2003).

Whilst there are many advantages, there are also several caveats in using this region that need to be considered. ITS is highly repetitive and may be polymorphic due to incomplete concerted evolution (Arnheim *et al.*, 1980). Concerted evolution is a process whereby different copies of the region do not accumulate mutations independently, and instead become homogenised to the same sequence type. It is hypothesised that this process occurs by high frequency unequal crossing over or gene conversion (Smith, 1976; Arnheim *et al.*, 1980; Zimmer *et al.*, 1980). The homogenising effect maybe advantageous in that it can lead to low intra-genomic variation. Unfortunately, concerted evolution may not be uniform across repeats or taxa (Small *et al.*, 2004), and as a result ITS sequences may not be homogeneous. In automated sequencing, these differences can sometimes be identified as polymorphic bases. However, in some instances, they may remain

undetected, with only the nucleotide with the strongest signal being read for that base position (Small *et al.*, 2004).

Experimental studies have shown that polymerase chain reaction (PCR) may preferentially amplify a particular ITS copy over others due to differences in primer affinity or variable copy numbers of the different sequences (Small *et al.*, 2004). Also, closely related taxa may have had ancestral copies of two or more ITS types that have subsequently been differentially lost. In such cases, the gene tree divergences will tend to be deeper than the actual species divergences, thus giving a phylogenetically misleading signal.

While there are conditions to the use of ITS in phylogenetic analyse it is used in this current study because the overall utility of this marker is suitable for this type of work, and it allows this study to be compatible with previous phylogenetic studies of New Zealand alpine *Ranunculus*.

2.1.4 Chloroplast Markers

The ultra-structure and gene content of the chloroplast genome within angiosperms shows little variation (however, see Palmer and Thompson (1982)), which has made it a popular choice for phylogenetic studies (Palmer and Olmstead, 1994). The typical angiosperm chloroplast genome is comprised of four parts; there are two identical segments that form an inverted repeat separating the rest into a large and a small single copy region (Palmer *et al.*, 1988). This structural stability has facilitated the design of 'universal' PCR primers, and most importantly, it allows for the expectation that specific DNA sequences isolated from different species are orthologous (Small *et al.*, 2004).

The most common rearrangements in the chloroplast are micro-structural changes in the form of insertions and deletions (indels). Smaller indels are thought to occur by processes such as slipped-strand mispairing (Levinson and Gutman, 1987), and the addition and subtraction of short sections of repeat sequence often associated with long stretches of a single nucleotide (Kelchner, 2000). Larger indels are associated with the formation of hairpins and stem-loop structures in secondary DNA (Kelchner, 2000;

Ingvarsson *et al.*, 2003). Indels are often observed in non-coding stretches of the chloroplast, and have been phylogenetically problematic (see Section 2.1.5 for the use indel information in phylogenetic reconstruction).

Features that make cpDNA particularly applicable to phylogenetic reconstruction are that it is haploid, contains coding and non-coding sequences, typically is uni-parentally inherited, and inter-molecular recombination is rare (Doyle and Doyle, 1990; McCauley, 1995; Small *et al.*, 2004). The haploid state means that intra-individual (allelic) variation is absent, and reduces the degree of intraspecific and inter-population variation as the effective population (N_e) size is smaller than that of a diploid genome (1/4 in dioecious plants, and 1/2 in monoecious plants) (Birky *et al.*, 1983). The smaller effective population size of a haploid genome means that coalescence times, and time to fixation of cpDNA haplotypes within a population are relatively short as compared to a diploid genome (Small *et al.*, 2004). Also, there are multiple copies of the chloroplast genome per chloroplast and this, when coupled with the presence of multiple chloroplasts per leaf cell, means that cpDNA is present in relatively high copy number within a genomic DNA extraction. The high copy number means that the chloroplast is readily accessible for PCR amplification.

A central assumption in phylogenetic analyses is that the terminal taxa on a tree have arisen by a bifurcation rather than reticulation. Thus, as a uniparental haploid molecule, the cpDNA phylogeny should by definition be bifurcating. However, hybrid species formation and introgression are well documented in plant evolution (Arnold, 1992; Rieseberg, 1997; Rieseberg and Carney, 1998). In these situations, uniparental inheritance becomes limiting, as the mixed origin of species is not revealed on a bifurcating tree (see Section 2.1.2). Concerns for the use of cpDNA in phylogenetic reconstruction arise when hybridisation and recombination occur, however, the evidence for chloroplast recombination has been weak and controversial (reviewed in Howe (1986)). Also, exceptions to uniparental inheritance have been noted (Corriveau and Coleman, 1988) and furthermore, the common view that chloroplast genomes are simple and stable has been challenged by a review about instances of heteroplasmy in the chloroplast genome, and the transfer of segments of chloroplast DNA to the mitochondrial or nuclear genomes (Wolfe and Randle, 2004). However, it is likely that that these instances and exceptions are rare, and cpDNA is, in general applicable, to phylogenetic reconstruction.

In order to study recent divergences, numerous primers for chloroplast introns and intergenic spacers have been developed (see Kelchner (2000) for a review). It has been observed that the non-coding sequences of the chloroplast often do not evolve in a neutral fashion and both introns and intergenic spacers can embody a considerable degree of structure, similar to that observed in rDNA (Kelchner, 2000). This can mean a region that is particularly variable and informative in one genus may show little or no variation in others (Shaw *et al.*, 2005). Within the alpine *Ranunculus* (Section *Pseudadonis*), Lockhart and McLenachan (1997) identified the J_{SA} region as being fast evolving (see Section 1.1.11). The J_{SA} is a putative coding region and contains a stretch of variable sequence flanked by two conserved regions. While it is a coding region, the variation in J_{SA} has been found to be suitable for distinguishing intra- and interspecific relationships (Lockhart *et al.*, 2001) and is used in this study together with the commonly used trnL – trnF region of Taberlet (1994).

2.1.5 Reconstruction of Phylogenetic Trees

In interspecific studies, models that describe evolutionary relationships typically assume an underlying bifurcating tree. Phylogenetic relationships are commonly displayed as a leaf-labelled tree, which can be either rooted or unrooted (Holder and Lewis, 2003; Huson and Bryant, 2006). There are two general categories of tree reconstruction, *i.e.* local clustering methods and global optimality methods. Clustering methods have the advantage of being computationally faster than global optimality methods, as they construct partial solutions rather than evaluating overall tree space to find the best overall fit (Holder and Lewis, 2003). One clustering method commonly applied is Neighbor Joining (NJ) (Saitou and Nei, 1987).

Global optimality methods start by defining an optimality criterion that is used to evaluate and compare alternate trees. A commonly used optimality method is maximum-likelihood (ML). This method evaluates the probability of the observed data given a specified substitution model and evolutionary tree. The log-likelihood score for all possible trees is calculated at every sequence position and the overall score of a tree is the

sum of this value for all positions. The tree or trees with the highest likelihood are chosen as the optimal solution (Felsenstein, 1981).

In practice, for any realistically-sized data set it is not possible to assess the score of every possible tree. Instead, heuristic methods are used to search tree-space for locally optimal solutions (Whelan *et al.*, 2001; Swofford, 2002). Topological stability of a tree can be assessed by the application of non-parametric bootstrapping. Bootstrapping gives a measure of confidence in branches by repeated re-sampling of a data set to determine how often relationships separated by branches are recovered (Felsenstein, 1985). Bootstrap values are not a measure of accuracy of the phylogeny, but rather, indicate the possible influence of stochastic error among the topology recovered. In the present study, maximum likelihood reconstruction with bootstrap resampling is used to reconstruct the evolutionary history of New Zealand alpine *Ranunculus*.

In intraspecific studies, haplotype networks are often used to display phylogenetic relationships. Commonly used methods include the Templeton Crandall Sing (TCS) network construction method of Templeton *et al.* (1992) as implemented by Clement *et al.* (2000), and the median network method (Bandelt, 1995; Bandelt *et al.*, 1999; Huber *et al.*, 2001). Median networks give a unique representation of relationships where adjacent nodes are always separated by one substitution difference. They are used to analyse sites in the data that comprise no more than two character states. The construction principle relies on using the median operation and an expansion algorithm for building a splits graph (Bandelt, 1995; Huber *et al.*, 2001; McBreen and Lockhart, 2006). The limitations of median networks are that the median operation can infer artificial ancestral nodes and the network can develop a high degree of complexity.

Indels can provide interesting phylogenetic information, yet there is some debate over the most appropriate fashion in which to analyse indel data (see Young and Healy (2003) for a review). An approach used in this current study is to consider an indel as a single event and recode it as a new character with two character states (insertion and gap). This two character state data can be then analysed using a median network. The ease of interpretation of the two character analysis and its ready application to the study of indels were considered suitable for the visualisation of relationships among intraspecific accessions of North Island *Ranunculus,* and are thus used in this study.

2.2 Materials and Methods

2.2.1 Plant Collection Information

The plant material used for this study was primarily collected from natural populations by the author or a collaborator. Some samples were available as dried specimens from previous studies. In all cases the minimum amount of material was sampled to reduce the impact on the population or sample. Samples collected in the field were stored in self-indicating silica gel to desiccate them and prevent microbial degradation and contamination (Chase and Hills, 1991). Voucher specimens will be deposited in the MPN herbarium upon completion of this study

2.2.2 Plant Genomic DNA Extraction

2.2.2.1 CTAB method

Two DNA extraction protocols were used. The first protocol was modified from Doyle and Doyle (1990). 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). A small quantity of each sample was used (approximately 200 mg dry weight) in the DNA extraction process. The extractions were performed in batches of 4-8 samples.

The lower portion of the microcentrifuge tube was placed into liquid nitrogen for approximately 1 minute 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 (Volac). 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 made for each sample.

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 µL 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]). The solution 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 then allowed to cool to below 60°C for approximately 5 minutes. Five hundred µL 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 x 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 are left in 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.

Five hundred µL iso-propanol (BDH) was added to the collected aqueous solution, 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 tube using a wide bore pipette tip. Approximately 500 µL 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 precipitate. If this did occur, it became very difficult to clean the DNA any further or to later re-suspend the DNA.

Washes were repeated with 500 µL 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 16,060g for 1 minute. The supernatant was discarded and the pellet allowed to air dry. Each pellet was re-suspended in 50 µL Milli-Q water. Re-suspended extracted DNA was run on agarose gels (refer to section 2.2.3) to determine the quantity and quality of the DNA. This modified CTAB method typically gave DNA suitable for PCR at concentrations of 10-20 ng/µL.

2.2.2.2 Qiagen DNeasy extraction method

The second DNA extraction protocol employed was the DNeasy plant mini-kit (Qiagen). Samples were extracted as per the kit instructions, including the optional centrifugation step. A small aliquot of eluate was then electrophoresed on an agarose gel (refer to section 2.2.3) to determine the success and concentration of the extraction. Typically, 10-20 ng/µL of DNA would be recovered from 100 mg wet or 20 mg dry leaf tissue. 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).

2.2.3 Quantification of DNA by 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 a 5 µL aliquot of extracted DNA was mixed with 1 µL 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 gel. A size marker, 1 Kb Plus DNA Ladder (Invitrogen, see appendix B) was included on the gels, as well as a High DNA

Mass Ladder (Invitrogen, see appendix B) to quantify the amount of DNA present. Samples were electrophoresed at 5.5 V/cm^{-1} in 1× TAE buffer. Following electrophoresis, the gel was stained with ethidium bromide solution (1.5 mg/L^{-1}) for 20 minutes. Gels were destained in Milli-Q water when background fluorescence was high. DNA on stained gels was 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 a 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 extracted DNA was used to calculate the approximate DNA concentration of the extraction.

2.2.4 Amplification of DNA Markers by PCR

Each PCR reaction contained 1× PCR buffer (1.5 mM $MgCl_2$ [Roche]), 250 μM of each 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. The PCR was routinely supplemented with 1 M betaine solution (Sigma-Aldich), 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 H_2O in place of the DNA template, was always run with each reaction set. The PCR thermocycling protocols used for the amplification of the specific DNA loci are detailed in Table 2.1. The J_{SA} primers used are those of Lockhart *et al.* (2001). The universal ITS4 primer (White *et al.*, 1990) was used in association with an ITS5 (White *et al.*, 1990) primer modified for higher plants, ITS5Hp (Hershkovitz and Zimmer, 1996). The trnL - trnF chloroplast was amplified with primers from Taberlet *et al.* (1991). PCR primers were supplied lyophilised (from Invitrogen) and were diluted to a stock concentration of 1 mM (1 nmol μL^{-1}) with Milli-Q H_2O and were stored at −80°C. Stock solutions were typically diluted 100× to a working solution of 10 μM (10 pmol μL^{-1}) with Milli-Q H_2O and were stored at −20°C.

DNA locus	primer pair	Thermocycling protocol
Chloroplast		
Junction of the inverted repeat A and small single-copy region (J_{SA})	5'primer RERN 3'primer 151A	Initial denature 2 minutes at 94°C then 35 cycles: 1 minute at 94°C 1 minute at 55°C 1 minute at 72°C final extension 5 minutes at 72°C
trn L (UAA) 5 'exon – trn F (GAA) intergenic spacer region	5'primer TabF 3'primer TabC	Initial denature 3 minutes at 94°C then 35 cycles: 30 seconds at 94°C 1 minute at 50°C 1 minute at 72°C final extension 5 minutes at 72°C
Nuclear		
Internal Transcribed Spacer (ITS) region	5'pimer ITS4 3'primer ITS5Hp	Initial denature 2 minutes at 94°C then 35 cycles: 30 seconds at 94°C 30 seconds at 48°C 1 minute at 72°C final extension 5 minutes at 72°C

Table 2.1: Oligonucleotide primers and thermocycling conditions used in the PCR amplification of DNA marker loci (Primer sequences are included in appendix C).

Following thermocycling, a 5 μL aliquot of each amplification was electrophoresed on a 1% (w/v) LE agarose gel as described previously (refer to section 2.2.3) 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.

PCR products usually exhibited only one discrete band on the agarose gel. In this case, an enzymatic cleanup procedure was carried out on the PCR product. Two µl of Shrimp Alkaline Phosphatase (SAP) (1 U/µL [USB]) and 1 µL of Exonuclease III (ExoIII) (10 U/µL [USB]) were added to the PCR product. The PCR product was incubated at 37°C for 30 minutes, then 80°C for 15 minutes. SAP removes the phosphate group from unincorporated dNTPs and ExoIII digests unincorporated single stranded primers which prevents participation of these molecules in the sequencing reaction. The final step of 15 minutes at 80°C denatures both SAP and ExoIII so that they do not interfere with the subsequent 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 manufacturer's instructions. Purified products were quantified on 1% agarose gels (Section 2.3.3).

2.2.5 Sequencing of PCR Products

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 (ng) added to the reaction was calculated by dividing the length of the DNA fragment in base pairs by 20. The mass of DNA in ng was converted to a volume amount (µL) based on the quantification of the DNA as described in Section 2.3.3.

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

Sequence products were purified by CleanSeq dye-terminator removal (Agencourt) following the manufacturer's protocol. Sequence reactions were analysed using the capillary separation service offered by the Palmerston North Allan Wilson Centre Genome Service (AWCGS) using an ABI3730 capillary sequencer (Applied Biosystems). Capillary separation was carried out in accordance with the manufacturer's instructions.

2.2.6 Alignment of DNA Sequences

Sequence files were imported individually into Sequencher 4.1 (Gene Codes), and the complementary strands were aligned. These alignments were inspected visually and edited where necessary. Bases that were ambiguous in both the forward and reverse directions were included in the final alignment as an ambiguous state. Consensus sequences were obtained for each individual and then included in a multiple alignment of consensus sequences for all individuals.

2.2.7 Phylogenetic Tree Building Methods

Aligned DNA sequences for the ITS, chloroplast J_{SA} and trnL - trnF markers used in this study were analysed using median networks and maximum likelihood. Heuristic searches were performed in PAUP* 4b10 (Swofford, 2002) using the Tree Bisection-Reconnection (TBR) swapping algorithm. The optimal model of sequence evolution used was selected from 56 models by using Modeltest 3.7 (Posada and Crandall, 1998) with the Akaike Information Criterion (AIC) (Akaike, 1974). The stability of the topology of the recovered trees was tested by using 100 bootstrap replicates using the 50% majority rule consensus tree (Felsenstein, 1985).

2.3 Results

2.3.1 Collection and Sequence Data

Accessions of *Ranunculus insignis*, *R. verticillatus* and *R. nivicola* were collected following the distribution maps of Fisher (1965) (Fig. 1.1). The geographic regions sampled for *R. insignis*, *R. verticillatus* and *R. nivicola* are shown in Figure 2.1.

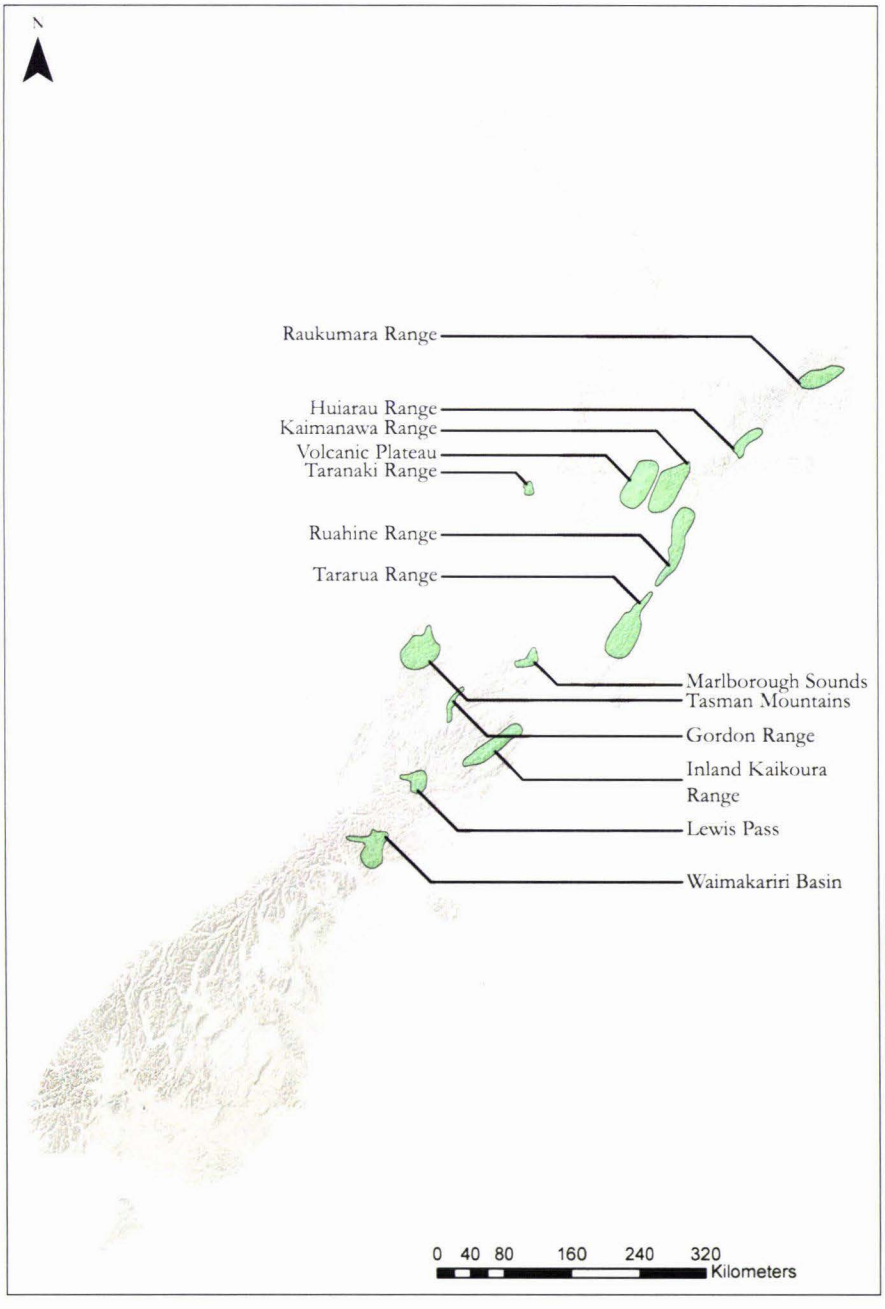

Figure 2.1: Location map showing regions sampled for *R. insignis*, *R. verticillatus* and *R. nivicola*.

Sequence data were obtained for the ITS marker from 28 accessions of *R. insignis*, 23 of *R. verticillatus* and 10 of *R. nivicola*. For the J_{SA} region sequence data was obtained from 31 accessions of *R. insignis*, 19 accessions of *R. verticillatus* and 12 accessions of *R. nivicola*. The trnL - trnF region was sequenced for a subset of J_{SA} accessions (7 for *R. insignis*, 9 for *R. verticillatus* and 4 for *R. nivicola*). Some characteristics of all three datasets are shown in Table 2.2.

No significant base composition bias was observed for the three regions. Consistent with findings in other genera (Boudraa and Pascale, 1987; Oliver *et al.*, 1989; Howe *et al.*, 2002), the nuclear marker had a higher GC content than the chloroplast markers (Table 2.2). In the J_{SA} sequence alignment there were two indel events (Table 2.2). These indel events were simple direct repeats of the Golenberg *et al.* (1993) IB type, and were only observed in accessions of *R. verticillatus*.

A full list of accessions and voucher numbers are included in the appendix. Sequence data will be submitted to GenBank at the completion of the study.

Region	Length	Variable sites	Parsimony Informative sites	Indels	Mean GC Content
Nuclear					
ITS	584	25 (4.28%)	19 (3.25%)	0	51.5%
Chloroplast					
J_{SA}	513	26 (5.07%)	20 (3.90%)	2	36.4%
trnL - trnF	956	15 (1.57%)	13 (1.36%)	0	35.1%

Table 2.2: Nuclear and chloroplast alignment statistics for the study species.

2.3.2 Sequence Diversity in the North Island *Ranunculus*

Intraspecific variation was characterised for the three North Island *Ranunculus* species and visualized using median networks. Intraspecific variation was not assessed for the trnL – trnF marker, as this marker was only sequenced for a subset of the total sequences used.

R. nivicola

No sequence variation was observed among the 12 accessions for *R. nivicola* for J_{SA} nor the 10 accessions sequenced for ITS. This contrasted with the findings for *R. insignis* and *R. verticillatus*.

R. insignis

Seven distinct chloroplast J_{SA} haplotypes were observed in 31 accessions of *R. insignis*, three of which are represented by multiple accessions (haplotypes 1, 2, and 3). The median network relating these sequences is shown in Figure 2.2. Based on a phylogenetic analysis of these sequences with those of other New Zealand alpine *Ranunculus* (Fig. 2.6), the most likely root position is on the internal node (haplotype 2) which is represented by numerous *R. insignis* accessions from the Waimakariri basin (Fig. 2.1). Interestingly, this analysis (Fig. 2.6) also indicated that haplotype 2 is also shared by some accessions of *R. enysii*. All the North Island and most northern South Island accessions are represented by haplotype 1. Haplotype 3 is comprised of *R. insignis* accessions from Mount Hutt (geographically south of the Waimakariri Basin area); this haplotype is shared with accessions of *R. crithmifolius* subsp. *crithmifolius* (Fig. 2.6). Accessions from Foggy Peak, Porters Pass, and Castle Hill are separate haplotypes that are geographically within the Waimakariri Basin area, and are most closely linked to the Waimakariri haplotype (2). The accession from Travers Peak (Lewis Pass area) also forms a separate haplotype; this accession is geographically within the area covered by the northern haplotype (1) and is most closely related to these accessions.

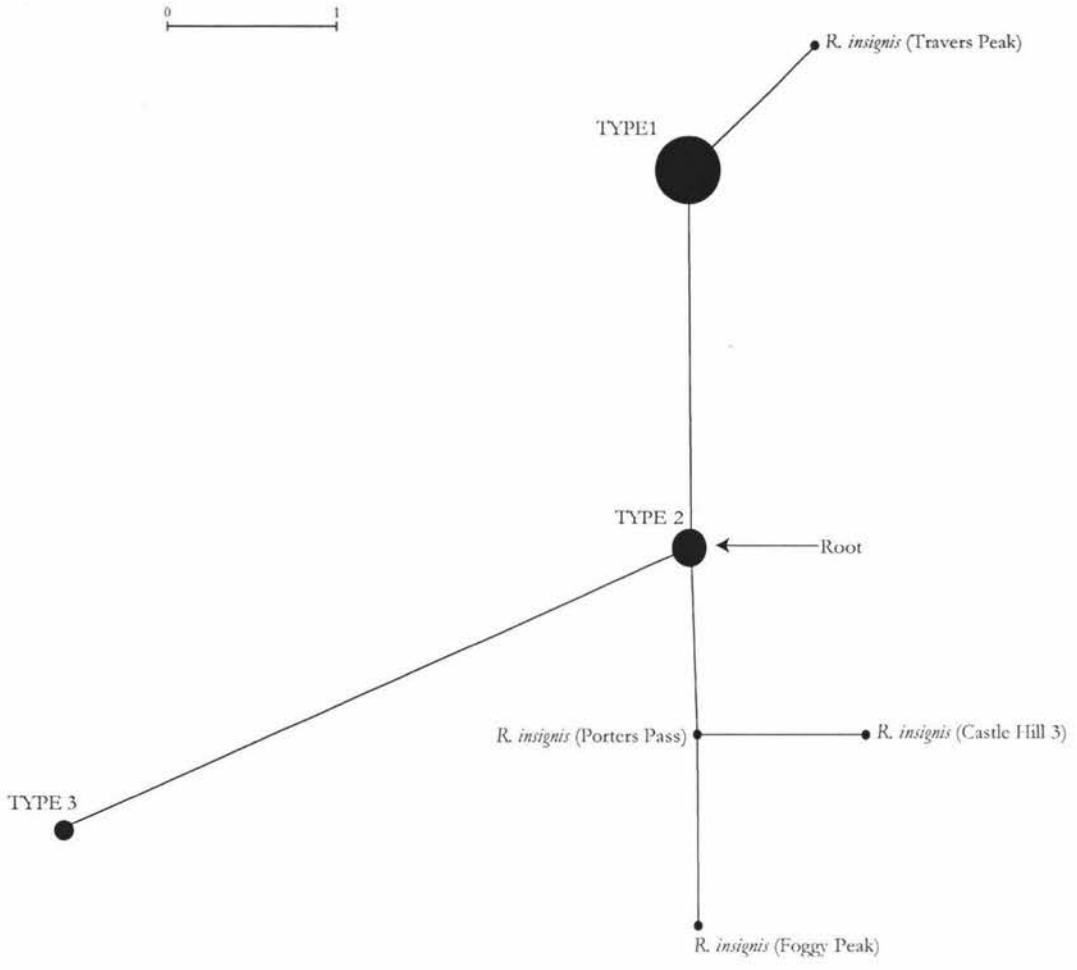

Figure 2.2: Median network of the J_{SA} intraspecific variation in *R. insignis*.

In the ITS region, there are numerous (15) polymorphic sites in the 28 accessions of *R. insignis* indicating multiple alleles in some individuals. If these sites are removed from analysis then all ITS sequences, with the exception of the Mount Arthur (Tasman Mountains) accession, are identical.

R. verticillatus

The J_{SA} sequence for *R. verticillatus* contained two regions where there were direct repeats (Table 2.3). Excluding these regions from analyses resulted in 10 haplotypes among the 19 accessions of *R. verticillatus* (data not shown). When the repeats were recoded to create three new splits then 11 haplotypes could be inferred. The only difference between the two datasets (gaps removed vs. gaps recoded and included) was that types 1 and 2 from the recoded analysis form a single haplotype in the analysis with the gap information removed. The median network relating the 11 haplotypes of the recoded sequences is

shown in Figure 2.3. The most likely root position, as indicated by phylogenetic analyses with other NZ alpine *Ranunculus* is the type 2 node (Fig 2.5).

J_{SA} Indels	
No indel	GAAGAACAGGAAGAA----------GAGGAAGAGGCAACCG
A1	GAAGAACAGGAAGAACAGGAAGAA---------GAGGAAGAGGCAACCG
A2	GAAGAACAGGAAGAACAGGAAGAACAGGAAGAAGAGGAAGAGGCAACCG
Recoded (r)	
No indel A	AA
rA1	TA
rA2	TT
No indel B	AAAAAGAAAAAG------GAAAA
B1	AAAAAGAAAAAGGAAAAGGAAAA
Recoded (r)	
No indel B	A
rB1	TG

Table 2.3: Indels observed in J_{SA} sequences of *R. verticillatus*.

While there is some geographic association of haplotypes, multiple events of seed dispersal are needed to explain the observed geographic distribution. If the graph is rooted in the purposed position, then the origin could be either in the North or South Island. From Figure 2.4 it can be seen that this analysis would infer a minimum of 3 and maximum of 8 dispersal events.

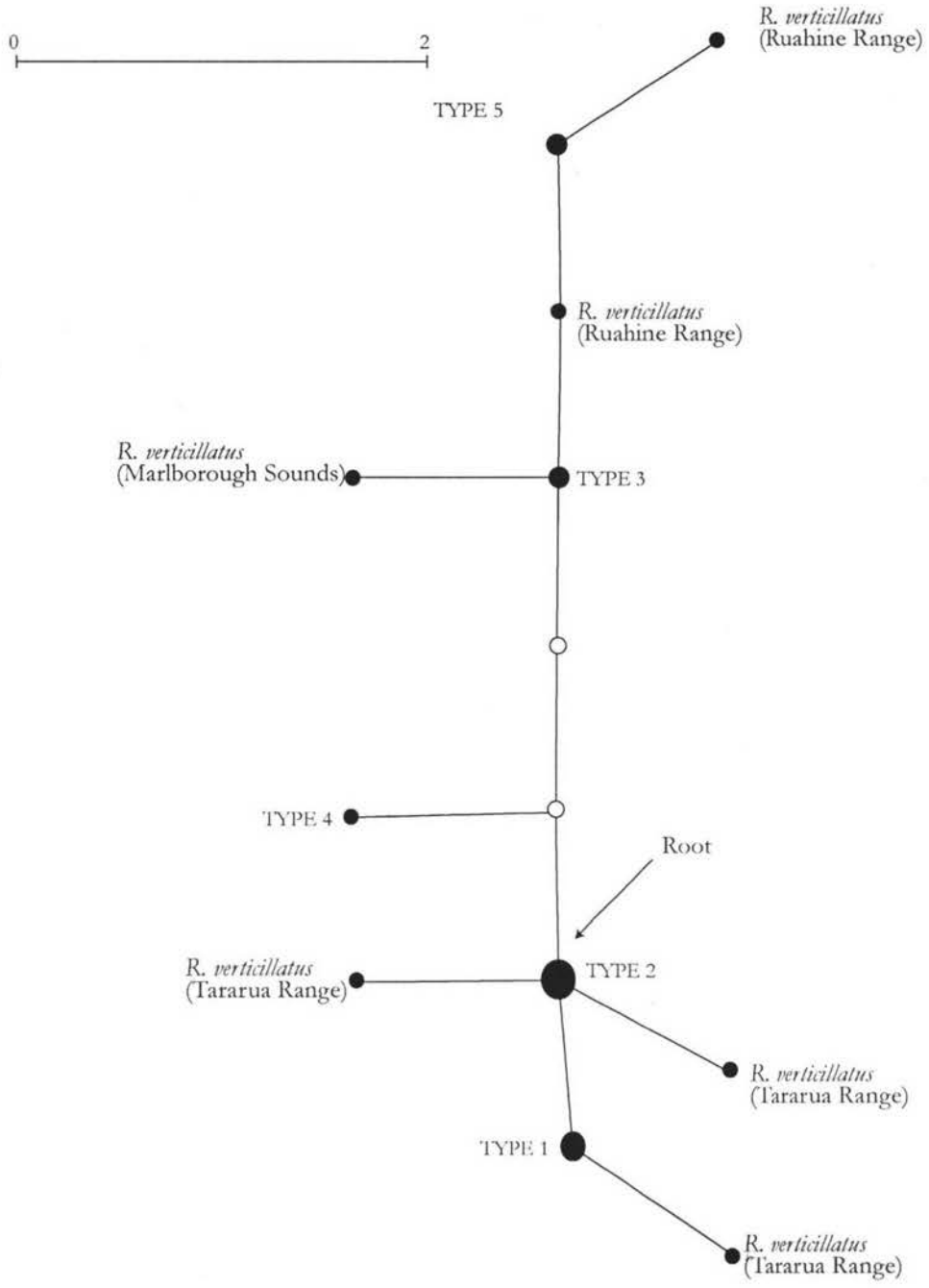

Figure 2.3: Median network of intraspecific variation in *R. verticillatus* for the J_{SA} region, with gaps recoded.

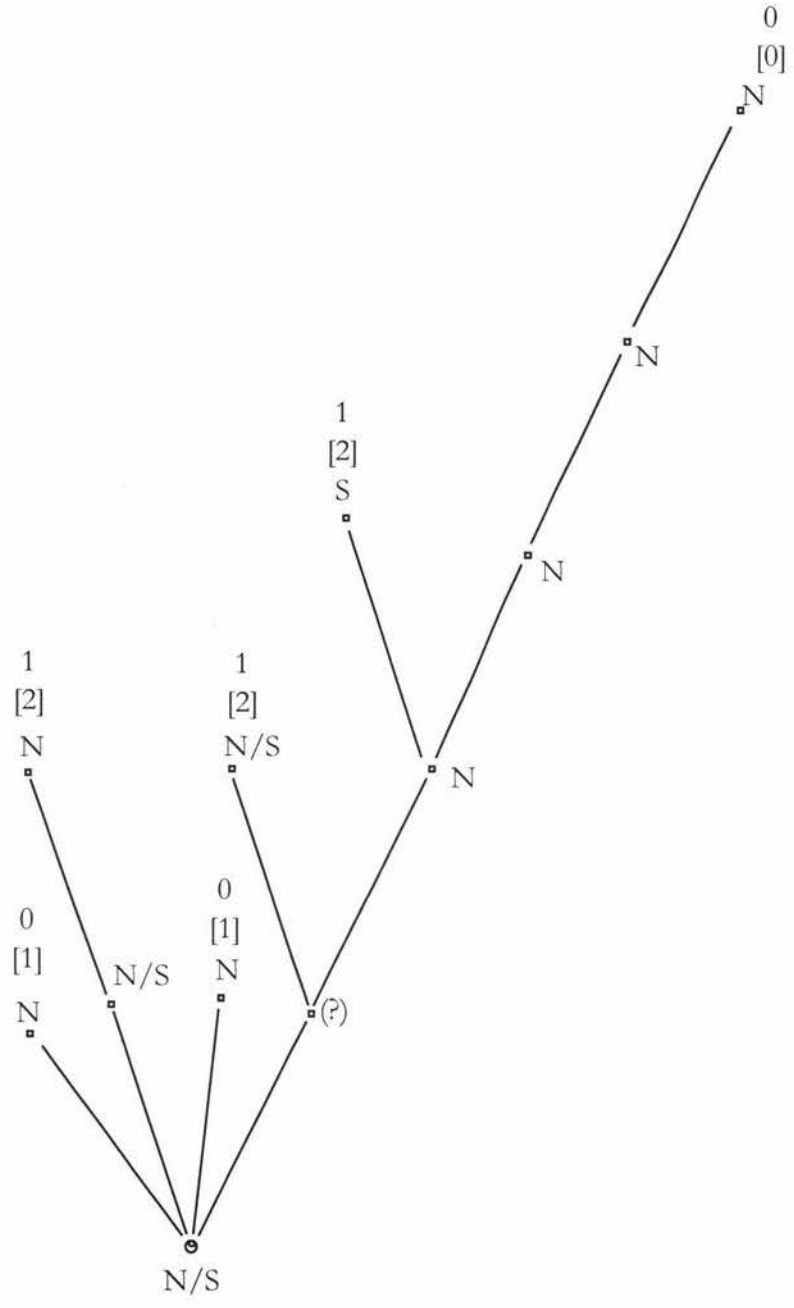

Figure 2.4: Rooted median joining graph of J_{SA} data for *R. verticillatus* depicting the geographic origin of the nodes. The number in brackets is the maximum number of dispersal events needed to explain the geographic relationship. The number above is the minimum estimate.

ITS sequences from 23 accessions of *R. verticillatus* also contained polymorphic sites (9); when these were removed from the analysis, 4 haplotypes could be inferred. The median network describing the relationship between these is shown in Figure 2.5. There is little geographic structure in the median joining graph. Haplotype 1 is comprised of accessions

from both the North and South Islands. Under analysis with other members of the alpine *Ranunculus,* haplotype 2, which is comprised of North Island accessions, is the most likely root position for this species. This result is particularly interesting as this *R. verticillatus* haplotype is shared with *R. nivicola.*

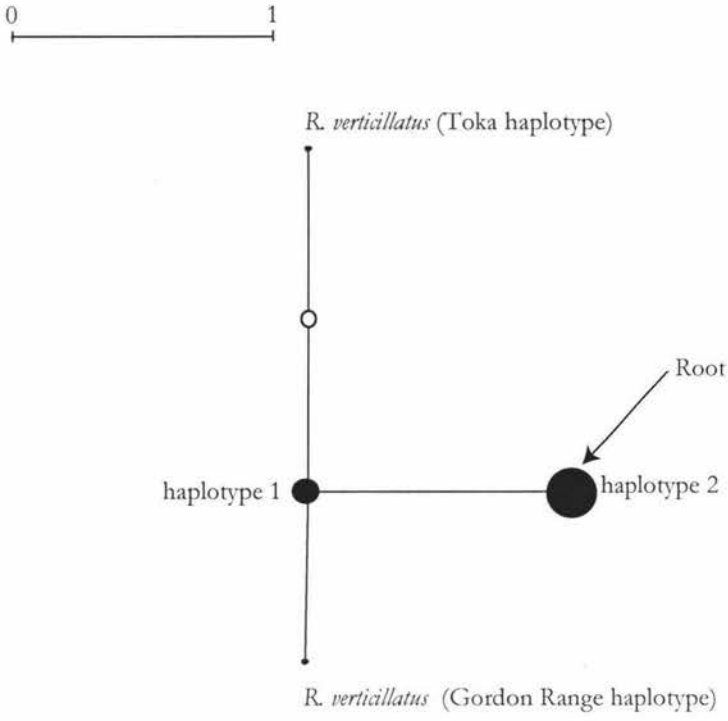

Figure 2.5: Median joining graph of the *R. verticillatus* ITS sequences.

2.3.3 Maximum Likelihood Analyses of the Alpine *Ranunculus* Phylogeny

To investigate the hypothesis that *R. verticillatus* and *R. insignis* are the parental species of *R. nivicola*, and to examine the phylogenetic relationships of these species within the overall alpine *Ranunculus* (section *Pseudadonis*) phylogeny, Maximum Likelihood (ML) analyses were performed on an expanded dataset. The expanded dataset contained representatives of all 18 species of alpine *Ranunculus* from the North, South, Stewart, Campbell and Auckland Islands, and the Australian species of section *Pseudadonis*. The additional *Ranunculus* sequences were available from Lockhart *et al.* (2001) and Piripi (2005) (See appendices for the list of accession numbers).

The ML analysis was conducted on the haplotypes using the median joining networks described in Section 2.3.2. For the ITS dataset this analysis gave a set of 2 haplotypes of *R. insignis*, 4 of *R. verticillatus*, and 1 of *R. nivicola*. For the J_{SA} there were 7 haplotypes of *R. insignis*, 10 of *R. verticillatus* and 1 of *R. nivicola*.

An additional chloroplast maker, the trnL - trnF region, was included in this analysis. A subset of the North Island *Ranunculus* accessions used in the previous analyses were sequenced for this marker in association with a subset of the species used by Lockhart *et al.* (2001) and Piripi (2005).

The ML analysis and bootstrapping were performed in PAUP* 4.0b10 (Swofford, 2002) as described previously (Section 2.2.7). The optimal model of sequence evolution was estimated for the three alignments by Modeltest 3.7 (Posada and Crandall, 1998) using the Akaike Information Criteria AIC (Akaike, 1974) (Table 2.4).

Region	Model of Sequence evolution	Optimal tree Score	Proportion of invariable sites	Gamma Shape parameter
Nuclear				
ITS	SYM+I+G (Zharkikh, 1994)	-lnL = 1929.7211	0.5525	0.9682
Chloroplast				
J_{SA}	K81uf+G (Kimura, 1981)	-lnL = 1374.3839	NA	0.1030
trnL-F	TVM+I (Posada and Crandall, 1998)	-lnL = 1572.4631	0.8939	NA

Table 2.4: The estimated models of sequence evolution for the three markers used in this study.

ML analysis of ITS sequences recovered the four lineages identified by Lockhart *et al.* (2001) (I, II, III, IV), however the cpDNA indicted that *R. verticillatus* was distinct from the rest of the group I species. Base of this result dividing group I into two subgroups, IA and IB, is warranted (Figs. 2.7 and 2.8). The important observation relevant to the hypothesised relationship between the North Island *Ranunculus* is that for ITS, *R. nivicola* and *R. verticillatus* are sister species, whilst in the chloroplast analyses, *R. nivicola* and *R. insignis* both occurred in the same group but their relationship to one another was unresolved. In contrast to the well-supported clade comprising all accessions of *R. verticillatus*, *R. insignis* was not found as a monophyletic clade in any analysis.

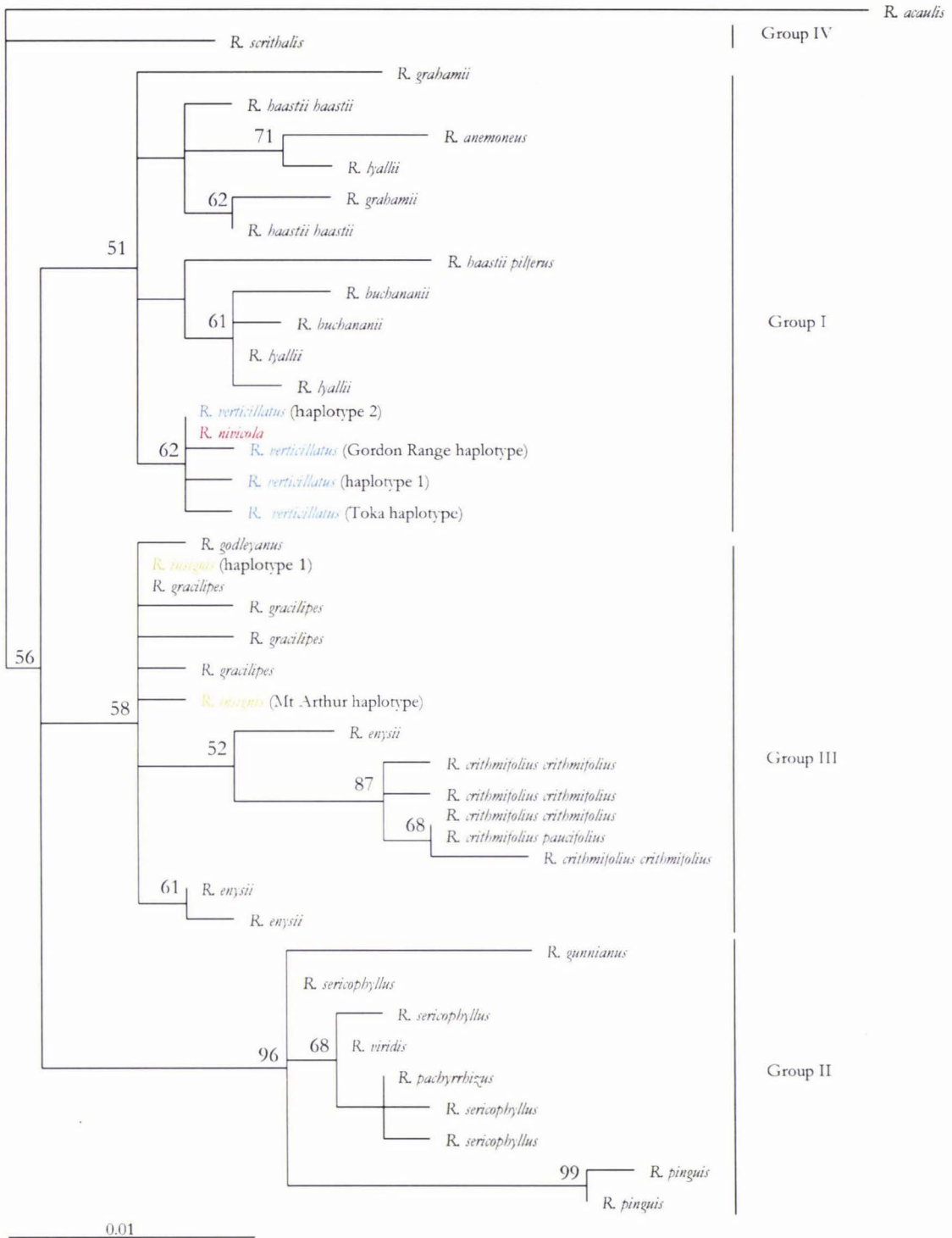

Figure 2.6: Maximum-likelihood tree based on ITS sequences of alpine *Ranunculus*. Bootstrap values with greater than 50% support are shown above their respective branches. Major clades are labelled I, II, III, IV, and correspond to those labels used by Lockhart *et al.* (2001).

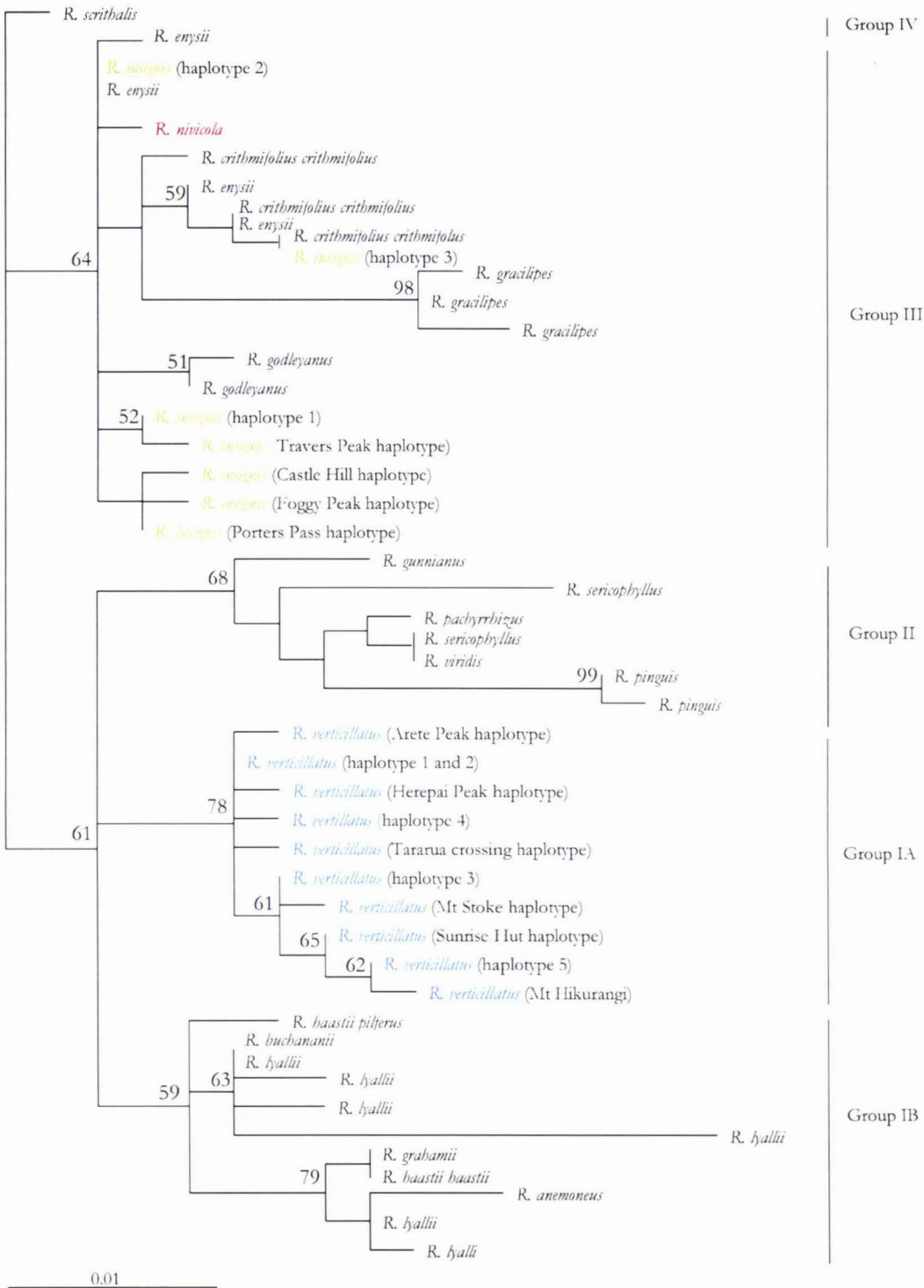

Figure 2.7: Maximum-likelihood tree based on J_{SA} sequences of alpine *Ranunculus*. Bootstrap values with greater than 50% support are shown above their respective branches. Major clades are labelled IA, IB, II, III, and IV.

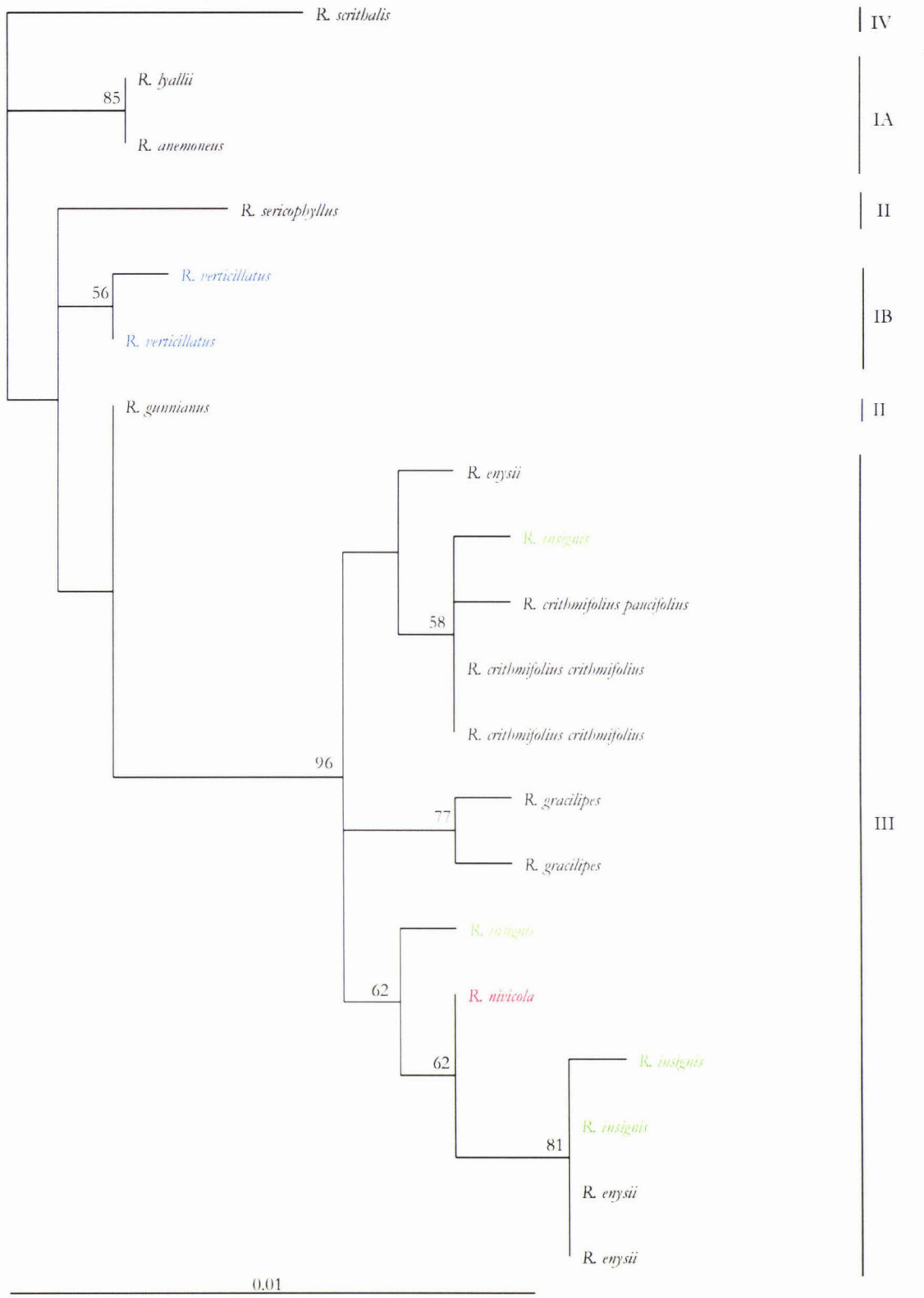

Figure 2.8: Maximum-likelihood tree based on trnL - trnF sequences of alpine *Ranunculus*. Bootstrap values with greater than 50% support are shown above their respective branches. Major clades are labelled IA, IB, II, III and IV.

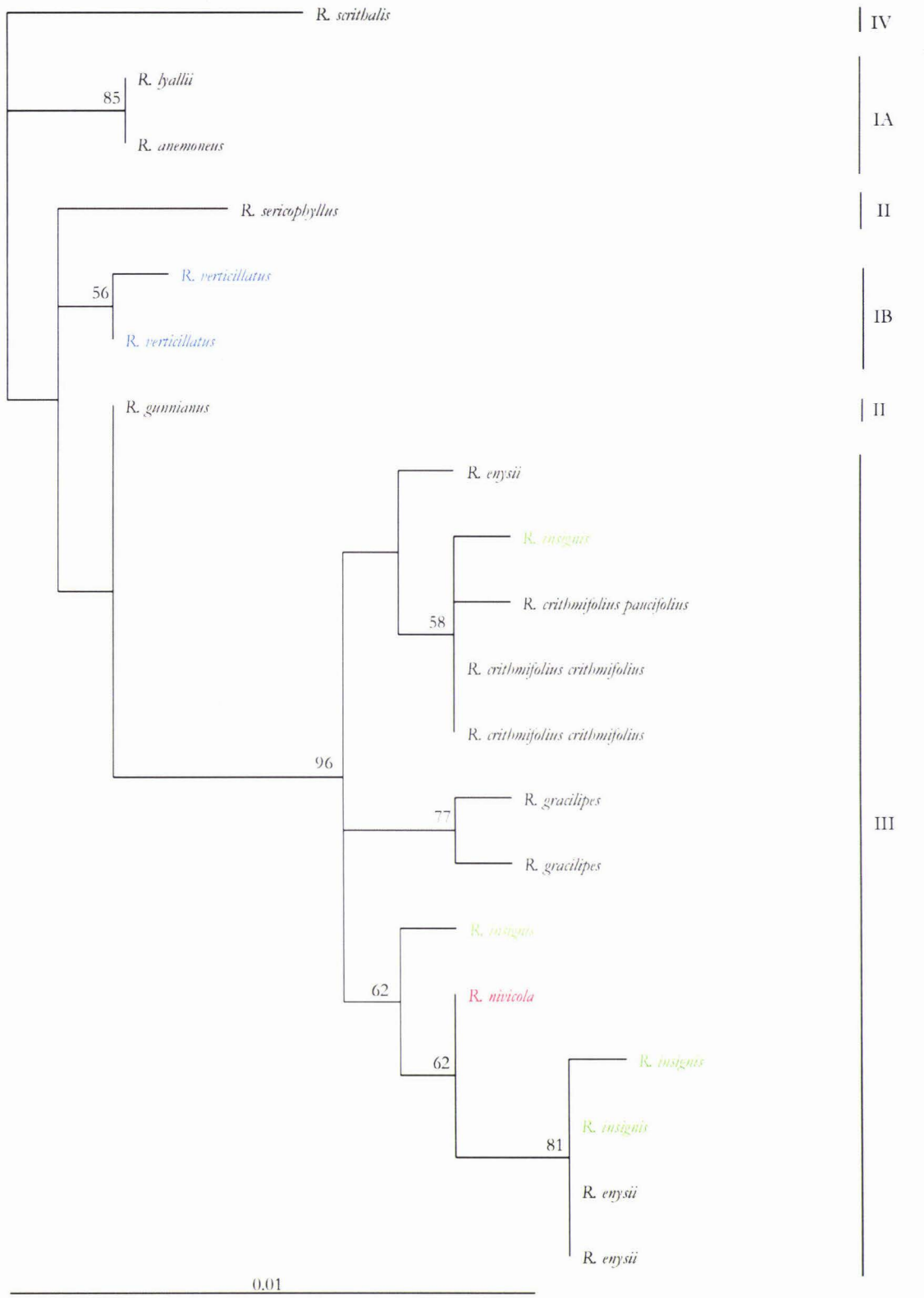

Figure 2.8: Maximum-likelihood tree based on trnL - trnF sequences of alpine *Ranunculus*. Bootstrap values with greater than 50% support are shown above their respective branches. Major clades are labelled IA, IB, II, III and IV.

2.4 Discussion

2.4.1 Range expansion of *R. insignis* and *R. verticillatus* into the North Island involved rare dispersal events in the evolutionary history of New Zealand alpine *Ranunculus*.

As outlined previously, Fisher (1965) hypothesised that alpine *Ranunculus* was able to expand its range into the North Island from the South Island during the last glacial maxima. During this period, the lower sea level would have connected the North and South Islands (Te Punga, 1953; Fleming, 1962), and the depressed forest line and landscape dominated by tundra vegetation may have facilitated this expansion (Fisher 1965). If it is assumed that *Ranunculus* species were only able to cross the Cook Strait into the North Island during the last glacial period, then it is expected that the population history of *R. verticillatus* and *R. insignis*, both currently present in the North Island, would be similar.

However, it was found that the patterns of genetic variation for *R. verticillatus* and *R. insignis* were not similar. The geographic distribution of distinct *R. verticillatus* haplotypes suggests multiple dispersal events between the South and North Islands for this species (Fig. 2.4), and that the period of existence of this species in the North Island has been sufficient to allow genetic diversification of this species in the North Island. In contrast, *R. insignis* from the North Island was found to be genetically uniform for the same molecular markers, suggesting that *R. verticillatus* has been established in the North Island for longer than *R. insignis*. The use of molecular clocks are a source of much debate (Kay *et al.*, 2006) and may be of limited use when sequences show low amounts of sequence variation (Comes and Kadereit, 1998). Nevertheless, if a substitution rate of approximately 0.5% to 2.5% nucleotide divergence per 1 million years is assumed (Koch *et al.*, 2003), then the amount of sequence divergence observed among North Island and South Island *R. insignis* sequences is consistent with this species having radiated into the North Island during the Pleistocene-Holocene period. Lockhart *et al.* (2001) estimated that the primary divergence of the alpine *Ranunculus* occurred approximately 5 mya and the level of genetic variation in *R. verticillatus* would suggest that this species expanded its range into the North Island not long after this divergence. If this inference is correct,

then it draws attention to the observation that *R. verticillatus* has a much more geographically restricted North Island distribution than *R. insignis*. Possible explanations for this might include differences in interacting species dynamics in the North Island and/or availability of suitable North Island habitats. This issue is considered further in Chapter 3.

2.4.1.1 Chloroplast and Nuclear Variation in *R. verticillatus* and *R. insignis*

It was observed that for both *R. verticillatus* and *R. insignis* sequence data from ITS showed less geographic structure than that from cpDNA. Transmission of cpDNA and nrDNA occurs by different processes. cpDNA is generally transmitted via the movement of seeds and pollen, whereas the ITS is transmitted through the movement of seed. Direct estimates of pollen and seed dispersal in numerous taxa indicate that the vast majority of such movement is locally restricted (Schaal *et al.*, 1998). In support of this observation, Armstrong (2004) found that the seeds of Australian alpine *Ranunculus* (Section *Acris* Schur) only travelled a few centimetres from the parental plant. This restricted dispersal in association with the smaller effective population size of the chloroplast genome (Section 2.1.4) means that genetic differentiation among populations is generally more pronounced for cpDNA than nDNA (Palme *et al.*, 2003).

The discovery of two indels in the *R. verticillatus* J_{SA} sequence was unexpected as micro-structural changes are more common in non-coding regions of the chloroplast. The J_{SA} region of the chloroplast is a putative coding region and it was observed that the insertion maintained the base triplet pattern. The position of the J_{SA} on the junction point of the inverted repeat and large single copy chloroplast DNA regions could make it more prone to change (Lockhart *et al.*, 2001).

A low level of variation in the markers used for this study was detected. It has been noted that direct sequencing markers are sometimes insufficiently variable to differentiate between closely related species (Wolfe *et al.*, 1998a). Multilocus fingerprinting techniques, such as Amplified Fragment Length Polymorphism (AFLP) and Inter Simple Sequence Repeat (ISSR) can be used to provide finer resolution. 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 for studying hybrid species (Wolfe *et al.*, 1998b; Garcia-Maroto *et al.*, 2003). ISSR was also successfully used by 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 reproducible than ISSR methods (McGregor *et al.*, 2000; Archak *et al.*, 2003) and may provide resolution at intraspecific levels on a finer scale than ITS (and other) sequences (Wolfe *et al.*, 1998b). 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 individual fragments are isolated and sequenced. Additionally, no information is known about the nature of the loci; they are essentially anonymous. It can mean that uninformative or unsuitable loci are given the same weight as all others. However, problems of doubtful homology 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.

Initial trails of ISSR fingerprinting were successful with differences observed between South and North Island *R. insignis* accessions (Carter *et al.*, unpublished). However, Piripi (2005) found problems with consistency of the ISSR gels. Another approach currently being trailed is fluorescent AFLP.

2.4.1.2 Sequence Diversity as it Relates to Morphological Diversity

R. insignis and *R. verticillatus* exemplify the morphological variability typical of the New Zealand alpine *Ranunculus.* As outlined in Section 1.1.10, these two species exhibit contrasting patterns of morphological variation. In *R. insignis*, variation is clinal with a gradient of forms throughout its geographic range. Fisher (1965) described the morphological variation in *R. verticillatus* as multiform. Within multiform populations, the full range of morphological forms found in the species can be present. If morphology is a reflection of the genetic variation present in a population, then it could be expected that *R. verticillatus* and *R. insignis* would show contrasting patterns of genetic variation. Furthermore, it has been shown that the leaf morphology of *R. verticillatus* can be directly controlled by light exposure (Fisher, 1954).

The accessions collected from the Waimakariri Basin area (Fig. 2.1) were found to encompass the highest sequence diversity in *R. insignis*. Interestingly, the Waimakariri Basin also is where the greatest morphological diversity of *R. insignis* is observed (Allan, 1926) and this coincides with the greatest degree of geographic range overlap with closely related species (*i.e. R. enysii*, *R. gracilipes*, *R. crithmifolius* subsp. *crithmifolius* and *R. crithmifolius* subsp. *paucifolius*). Fisher (1965) hypothesised that there are no strong barriers between species that now comprise the Lockhart *et al.* (2001) group III, so species in this group maybe freely able to hybridise. The phylogenetic analyses support the hypothesis that there is (or was at some point in the past) localised hybridisation between *R. insignis* and the group III species (Figs. 2.6, 2.7, and 2.8).

Fisher (1965) suggested that the morphological variation present in *R. verticillatus* was due it being an 'old' hybrid that represents the fully introgressed form of two species that no longer exist as separate entities. Such a hypothesis might explain why it is phylogenetically distinct from other group I species. Fisher also speculated that the variation in *R. verticillatus* may have arisen via hybridisation with *R. insignis*; however, no sequence data support this hypothesis.

2.4.2 In the North Island a single allopolyploidisation event between *R. insignis* and *R. verticillatus* gave rise to *R. nivicola*.

The phylogenetic results clearly support a hybrid origin of *R. nivicola*, as was suggested by Fisher (1965) and Lockhart *et al.* (2001). In support of an allopolyploid origin of *R. nivicola*, the ITS analysis found *R. nivicola* to be a sister species to *R. verticillatus* in group I, and cpDNA analysis showed its nearest relatives to be to *R. insignis*, *R. enysii*, and other group III species. The lack of genetic variation in *R. nivicola* and *R. insignis* sequences meant that there was no geographic signal in the data and it is not currently possible to determine where *R. nivicola* originated.

2.4.2.1 Concerted Evolution of *R. nivicola* ITS

In the bi-parentally inherited ITS marker, it could be expected that a hybrid would contain sequence information from both parental species (Soltis *et al.*, 2004a). However, in *R. nivicola*, we only found evidence for a single ITS type (that of *R. verticillatus*). Furthermore, no polymorphic bases were found in *R. nivicola* sequences, and this result indicates a single ITS type, although cloning of ITS sequences would be necessary to conclusively demonstrate this. The presence of a single ITS type has been observed in other allopolyploid plants, and this is typically attributed to concerted evolution (Wendel *et al.*, 1995; Franzke and Mummenhoff, 1999; Volkov *et al.*, 1999; Koch *et al.*, 2003; Kotseruba *et al.*, 2003). As covered previously (Section 2.1.3), concerted evolution may effectively remove one of the parental repeats from hybrid genomes, thus homogenising the nrDNA in the hybrid (Wendel *et al.*, 1995). The exact nature of this process is not well understood. It is possible that this process may take many sexual generations, however, genomic change in a newly formed polyploid can be rapid (Song *et al.*, 1995; Feldman *et al.*, 1997; Ozkan *et al.*, 2001) and concerted evolution may occur across a time span of decades (Kovarik *et al.*, 2005).

2.4.2.2 Chloroplast Variation in *R. nivicola*

The cpDNA sequence data do not provide conclusive evidence about the maternal progenitor of *R. nivicola*. A high degree of intraspecific variation was observed amongst the *R. insignis* cpDNA sequences and this variation is such that it does not form a monophyletic group. It could be that this variation is obscuring the relationship between *R. insignis* and *R. nivicola*. Alternatively, it is possible that another group III species, (*i.e.* not *R. insignis*) is the maternal parent of *R. nivicola*. However, given the genetic similarities between *R. nivicola* and *R. insignis* the data available would suggest that *R. insignis* is the maternal progenitor.

2.4.2.3 Single Origin of *R. nivicola*

Many polyploid species are recognised as being of recurrent origin. The presence of distinct geographic races within polyploid species is one of the clearest indicators of recurrent polyploid formation (Soltis and Soltis, 1993; Soltis and Soltis, 1999; Soltis and Soltis, 2000; Soltis *et al.*, 2004a). However, this geographic signal was not observed in analyses of *R. nivicola* sequences. There was no intraspecific genetic variation found in either nrDNA or cpDNA sequences of *R. nivicola*. This finding contrasts greatly with levels of variation found in other closely related species. Based on the findings of this study, it is proposed that *R. nivicola* is of a single origin.

Chapter 3 The Influence of the Environment on the Distribution of the North Island *Ranunculus*

3.1 Introduction

3.1.1 Niches and Predictive Models

The inferences regarding the spatial and environmental correlations between an organism and its surrounding area are based on the premise that a species occupies a definable environment or niche. A niche, as it was conceptualised by Grinnell (1924), described the ultimate distributional unit of a species or subspecies over a geographic area or habitat type. The concept of a niche was also described by Elton (1927), with the inclusion of limitations by climatic and physical barriers and allowing for interaction with other species. Effectively what Grinnell (1924) described is now called a "fundamental niche", and what Elton (1927) described is a species' "realized niche" (Hutchinson, 1957). A fundamental niche represents the total geographic area a species could potentially occupy in the absence of other species. However, a species seldom occupies all of this area; for example, factors such as predation, disease and parasitism may limit a species' distribution, resulting in a realized niche.

A species distribution and potential range can be modelled using the ideas of niche theory. It is not possible to include every niche dimension of a species in a model, as they are numerous and often unknown. However, with currently available digitized map coverages in association with Geographic Information Systems (GIS) it is possible to cover major physical variables that commonly structure a species macro-distribution (Anderson *et al.*, 2002). Such models attempt to define a species fundamental niche, or more accurately, its environmental niche, by combining known localities from distribution records with environmental data to produce an environmental envelope for a species. The model of a species' environmental niche will likely be an over-prediction. In areas predicted as suitable but not currently occupied, factors such as dispersal barriers, predation, local extinction and competition can then be investigated.

3.1.2 Speciation and Geographic Competition

A major source of contention in the study of speciation is whether species arise in isolation (allopatric speciation) or in association (sympatric speciation). There is need for reproductive isolation between species, but also there is the necessity that species are sufficiently different to their parental species such that they are not in direct competition for resources. Hutchinson (1957) suggested that competition with other species was a particularly important influence on a species' realized niche. A species' geographic range may be restricted by closely related species (Miller, 1967; Brown, 1971; Anderson *et al.*, 2002; Anderson and Martínez-Meyer, 2004). This situation occurs because morphologically similar species commonly share ecological requirements that can prevent their coexistence via competition (Tansley, 1917; Gause, 1934; MacArthur, 1972; Anderson *et al.*, 2002).

The geographic partitioning of the environment is of particular interest for the formation of polyploid species. The change in ploidal level can create instantaneous reproductive isolation, so a newly formed polyploid species can exist in sympatry with its parental species. Nevertheless, there is still the need to exploit a niche not occupied by its parental species (Lumaret *et al.*, 1987). It has been suggested that hybrid species often exhibit transgressive traits that allow them to exist in novel habitats, and the high incidence of polyploid species at higher altitudes and in artic environments has led to the notion that polyploid species are better suited to more extreme environments than diploid species (Löve and Löve, 1949; Stebbins, 1984; Brochmann *et al.*, 2004). Although there are no general rules for polyploid establishment, it was proposed by Ehrendorfer (1980) that successful neopolyploid races tended to originate under unstable environmental conditions and the best chance for the origin and establishment of a neopolyploid is in widespread successional to sub-climax communities and areas being settled by invasion floras. If a neopolyploid is established under stable environmental conditions with stable climax vegetation, then the established species will tend to prevail and the neopolyploid will have habitats and areas much narrower than their ancestral species.

3.1.3 Ecological Modelling in New Zealand

To date, ecological/geographical studies of the New Zealand flora have mostly focused on lowland forest species and have seldom been done in conjunction with a phylogenetic hypothesis (Yee and Mitchell, 1991; Leathwick, 1998; Lehmann *et al.*, 2002; Zaniewski *et al.*, 2002; Heenan and Mitchell, 2003; Lloyd *et al.*, 2003; Rutledge *et al.*, 2004). The alpine flora, which is the focus of the current study, has previously received little attention in a phylogenetic context (but see Heenan and Mitchell (2003)). The lowland focus is also observed in the global sense; some reasons for this are the complex topographic and climatic environment of high altitude zones, as well as the fact that there is often poor data availability on these areas (Guisan *et al.*, 1998).

3.1.4 The Land Environments of New Zealand Database

With the recent development of the Land Environments of New Zealand (LENZ) database (Manaaki Whenua - Landcare Research) it may be possible to model a species distribution in a complex alpine environment. The LENZ system is an environmental classification database constructed to investigate species-environment relationships. LENZ is divided -into four hierarchical levels (Levels I-IV). Within each level, the landscape is divided into areas of similar environmental conditions. These divisions are based on a measure of environmental distance which is calculated from digitised maps of climatic and physical variables considered most likely to influence the distribution of a species (Table 1.6) (Leathwick *et al.*, 2002).

The levels are structured such that each progressive level contains a larger number of subdivisions, so that Level I is divided into 20 divisions, level II 100, level III 200 and level IV 500. The subdivisions of the levels are subsets of lower levels, *i.e.* division A at level I becomes A1-A7 at level II, A1.1-A7.3 at level III and A1.1a-A7.3b at level IV.

The LENZ database has been used primarily as a tool in conservation management examining threatened and relic floras (*e.g.* Walker *et al.* (2004), Rutledge *et al.* (2004), Walker *et al.* (2005), and Walker and Rogers (2005)). The current study represents the first application of the LENZ database to a phylogenetic problem.

LENZ variables
1) Mean annual temperature (°C)
2) Minimum temperature of the coldest month (°C)
3) Rainfall (mm)
4) Mean annual solar radiation ($MJ/m^2/day$)
5) Winter solar radiation ($MJ/m^2/day$)
6) October vapour pressure Deficit (kPa)
7) Water balance ratio (Calculated from Solar Radiation, Mean Temperature and Rainfall)
8) Soil induration (Soil hardness)
9) Soil water deficit (mm)
10) Soil drainage (Soil description)
11) Soil particle size (mm)
12) Exchangeable calcium in the soil (Mg/100g)
13) Acid soluble phosphorus in the soil (Mg/100g)
14) Soil age
15) Slope (°)
16) Elevation (m)

Table 3.1: The underlying LENZ variables.

3.2 Materials and Methods

3.2.1 Geographic Data Collection

The geographic data used for this study was collected from natural populations in association with sampling for genetic analysis. These data were collected by using a Global Positioning System (GPS) device to record the location of the site. Additional geographic data was available from herbarium (AK, WELT, CHR, and MPN) vouchers. The grid point data were stored as a database in New Zealand Map Grid (NZMG) format (Appendix A).

LENZ level IV data were used for this study as an expanded dataset containing a more full description of the underlying environmental variables was made available by Manaaki Whenua - Landcare Research.

3.2.2 Predicted Species Distribution Method A: LENZ-based classification

As outlined previously in Section 3.1.4, a LENZ layer is divided into areas of similar of environmental characteristics. This is modelled on a computer as a series of information-containing cells, for example:

A1	B1	D1
B1	C1	C1
C1	D1	B1
D1	**A1**	**A1**

The A1 cells in this example represent three areas with similar environmental conditions; if a plant occurs in one those A1 cells, then potentially it could be found in all three of those areas.

To develop a predictive model of a species distribution using the LENZ based classification of the landscape, the database of grid points for a species (Section 3.2.1) was imported into ArcView 3.2 (Environmental Systems Research Institute, ESRI) GIS software, where it was 'joined' with the LENZ data. To join the species grid point data with LENZ data, a script was written using Avenue programming language. This script produced a table that contained the LENZ classification for the sites at which the grid points were found, *e.g.*

Location	EASTING	NORTHING	LENZ Level IV Environment
Mount Hikurangi	2774725	6151925	P8.2a
Mount Arthur	2485270	6000285	P2.1b
Mount Peel	2475665	6007495	P1.1c

ArcMap 9.1 (ESRI) was then used to produce a map which displayed all the cells that are covered by those LENZ divisions to represent the environmental envelope.

3.2.3 Predicted Species Distribution Method B: Extrapolation from environmental parameters

This second method is modified from Rutledge *et al.* (2004). In method A, it was assumed that if a plant was found in one cell it could potentially occur in all cells of that type. It is further possible that a plant could be found in all similar cells, and not exclusively the exact type.

The first step for method B is the same as above. That is the database of grid points for a species imported into ArcView 3.2 (ESRI) and joined with the LENZ data. From the 'joined' data file, the minimum and maximum value of the 15 underlying LENZ variables was determined for each record. The absolute minimum and absolute maximum for each variable was then determined for a species. Using this data, a series of 15 masks were created using ArcMap 9.1 (ESRI). A mask is a map layer which highlights suitable areas for a given variable. These masks were then joined to create an overall mask. The overall mask was an intersection of the 15 masks, and only contained areas that had the support

of all the variables. The overall mask was then overlaid onto a map of New Zealand to display the areas of available habitat.

3.2.4 Analysis of Overlap in Environmental Space

Multivariate analysis of variance (MANOVA) was used to investigate trends in the environmental data. Additionally, density plots were constructed to visualise the differences between the three species. These analyses were performed in R 2.21 (2005) and S-PLUS (2005).

3.3 Results

3.3.1 Geographic Data Collection

A total of 78 GPS points was available for this study (Table 3.2). GPS points were collected from the North and South Islands because *R. insignis* and *R. verticillatus* are found on both islands. Following the guidelines of Stockwell and Peterson (2002), the number of data points for each species was considered to be sufficient for this type of study. The distribution for these three species is well described by Fisher (1965). To limit sampling bias, an even distribution of points throughout the range of the species was collected.

Species	Points from the North Island	Points from the South Island	Total Number of Data Points
R. insignis	25	24	49
R. verticillatus	26	21	47
R. nivicola	27	NA	27

Table 3.2: Total number of GPS Data points for the study species.

3.3.2 Environments Occupied by the North Island *Ranunculus*

R. insignis

For *R. insignis*, the two methods produced complementary results. Both approaches indicated that *R. insignis* could be found more widely than it is currently (Fig. 3.1). In particular, the maps showed that *R. insignis* could be found on Mount Taranaki, Mount Hauhungatahi and in the Kaimanawa Range, which are all areas currently occupied by *R. nivicola* but not *R. insignis*.

In comparison to the ecological diversity observed in other members of the alpine *Ranunculus*, *R. insignis* occupies relatively diverse environments and it is noted that *R. insignis* is found in different environments in the North Island as compared to the South Island (Section 1.1.9). To test this difference between the North and South Islands, the analyses for the two methods were performed again, first excluding all South Island points and then excluding all North Island points. Method A was limited in its predictive ability when the dataset was reduced. Method B was able to recover a better representation of the range of the points removed. However, the removal of the North Island points resulted in an under-prediction in the most northern sites. The removal of the South Island points for method B made only a minor difference, and all known locations of *R. insignis* were recovered (data not shown).

North and South Island data were compared using MANOVA analysis with a Bonferroni correction for multiple comparisons. Four (26%) of the variables (minimum temperature, mean annual temperature, October vapour pressure deficit and acid soluble phosphorus) examined showed significant differences at the 5% level between the North Island and the South Island.

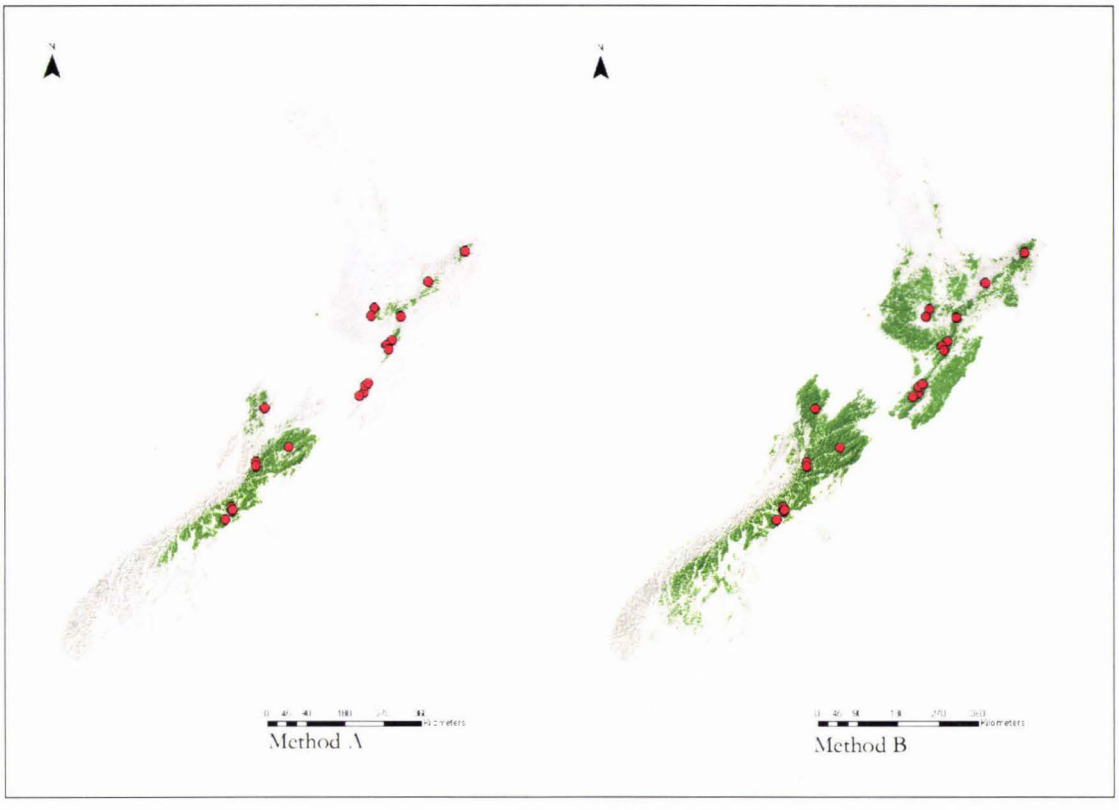

Figure 3.1 Method A and B results for *R. insignis*. The area of green is the predicted coverage, and the red points are the GPS locations used to develop the model.

R. verticillatus

In contrast to the wide distribution predicted for *R. insignis*, *R. verticillatus* had a much more restricted range under both methods (Fig. 3.2). The predictive maps under both methods indicated that *R. verticillatus* is found in most suitable areas in the North Island. The predictions for the South Island range of *R. verticillatus* suggested that it could potentially occupy a more southern range. Following the approach outlined for *R. insignis*, the dataset was rerun using points exclusively from one island. It was found that it was not possible to predict the current distribution based on data from one island alone (data not shown).

When the North Island data were compared to the South Island using the method outlined previously, it was found that they were significantly different for five (33%) of the variables tested (Minimum temperature, water balance, slope, acid soluble phosphorus and annual Rainfall).

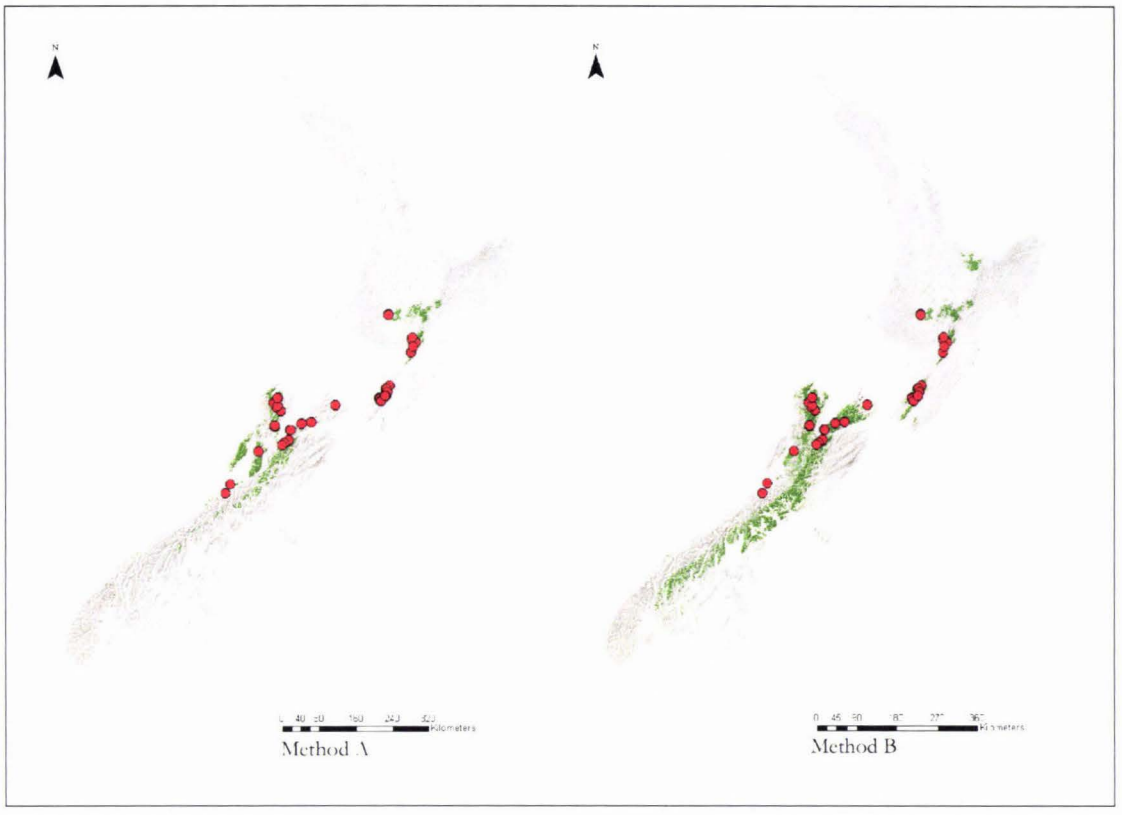

Figure 3.2: Method A and B results for *R. verticillatus*. The area of green is the predicted coverage, and the red points are the points used to develop the model.

R. nivicola

The limited range of *R. nivicola* predicted for method A indicated that *R. nivicola* could be found only in the areas that this species currently occupies. However, method B indicated that *R. nivicola* could exist in the South Island, but not in southern ranges of the North Island. The South Island areas highlighted under method B are very similar to the current range of *R. insignis*, which is a putative parent of the allopolyploid *R. nivicola* (see Chapter 2).

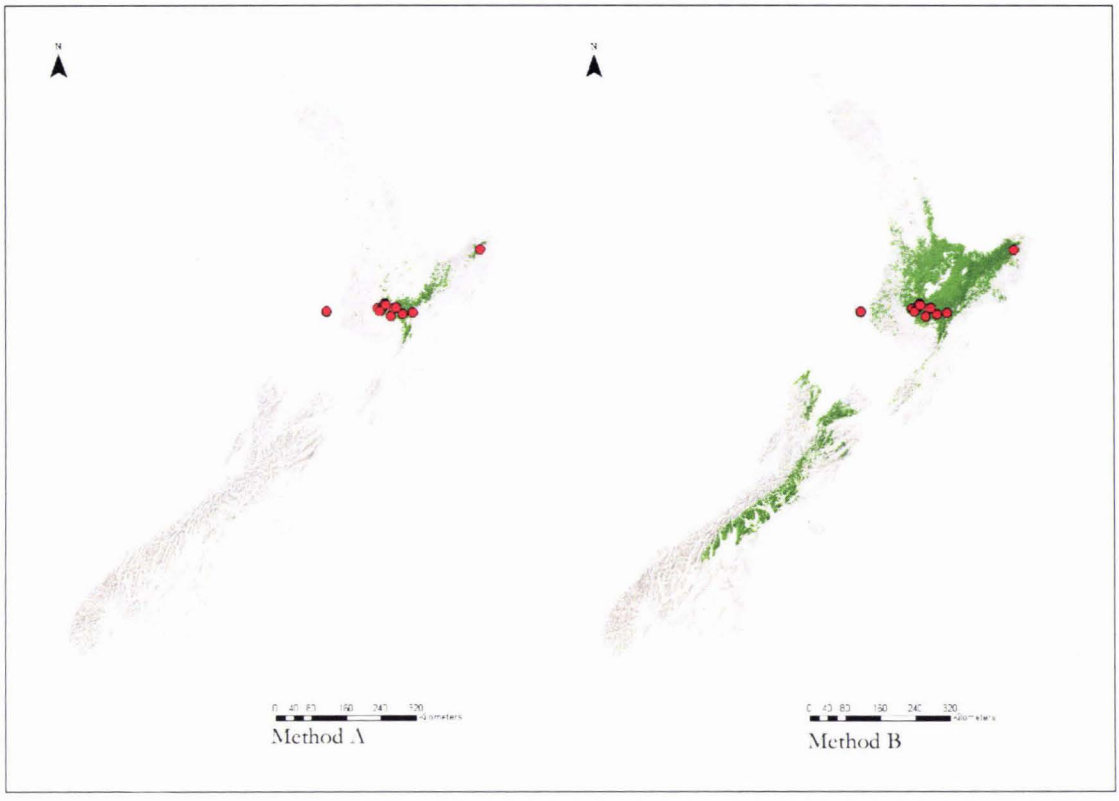

Figure 3.3: Method A and B results for *R. nivicola*. The area of green is the predicted coverage, and the red points are the points used to develop the model.

3.3.3 Comparison of the North Island *Ranunculus* Environments

The North Island environmental data for *R. insignis* and *R. verticillatus* were compared with *R. nivicola* using density plots and MANOVA. Density plots are a measure of the frequency at which a value is found in the data set. Using this approach, *R. nivicola* and *R. insignis* had similarly broad environmental tolerances, however, in the North Island they were significantly different for twelve (80%) of the variables tested. The results indicate that *R. insignis* and *R. nivicola* have the same range of values for mean annual temperature but the peak density is different (Fig. 3.4). This trend was observed for minimum annual temperature, water balance, acid soluble phosphorus, exchangeable calcium and average rainfall (data not shown). The North Island values for *R. verticillatus* were more similar to those of *R. nivicola*; however, they were still significantly different for nine (60%) of the variables tested. Furthermore, relative to *R. insignis*, *R. verticillatus* has a much narrower range for many variables, yet when the North Island data points for the two species were compared, they were not significantly different for any of the variables tested.

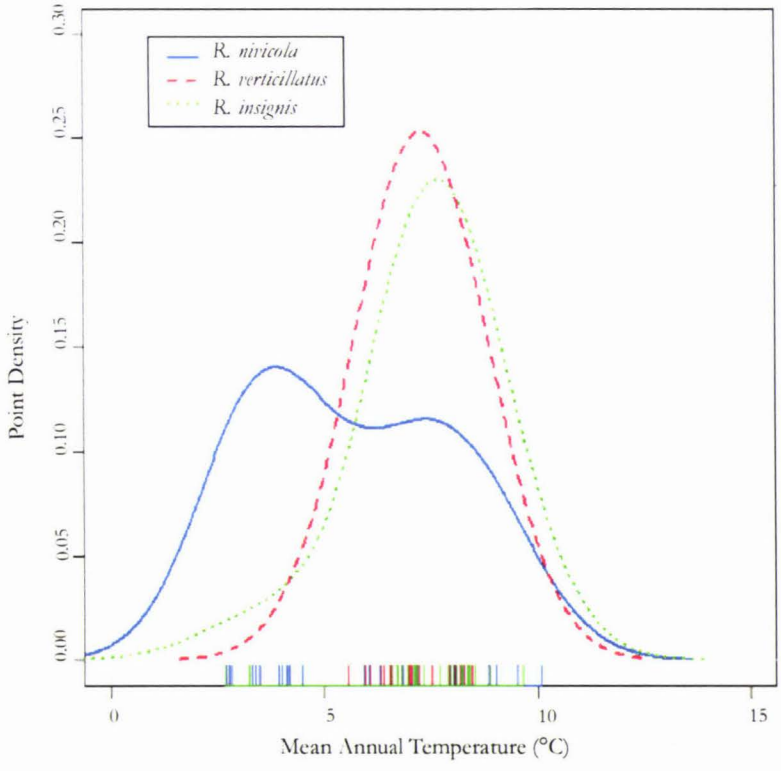

Figure 3.4: Comparison of point density for values of mean annual temperature for the study species.

3.4 Discussion

3.4.1 The polyploid *Ranunculus nivicola* extends the geographic range of alpine *Ranunculus* in the North Island into areas that would not be otherwise occupied by this genus

At the outset of the present study, it was hypothesised that the observed differences in range of the North Island *Ranunculus* were explained by range expansion of the polyploid *R. nivicola* into environments not suited for either of the parental species. This hypothesis predicts that the polyploid will have wider ecological tolerances than its parental species. When the North Island data alone were considered for predicting potential geographic distributions, it was found that *R. insignis* had the widest environmental envelope of the three species. The predicted envelopes of *R. verticillatus* and *R. nivicola* occurred within the range covered by *R. insignis*. However, within that range, the three species showed significant differences for many variables (Fig 3.4).

R. insignis is currently found in a diversity of habitats ranging from riverbank margins of established lowland forest to alpine tussock land. This indicates that this species can potentially occupy a wide environmental envelope. The predicted range of potential habitat for *R. insignis* does at first appear to be an over-estimation, but from personal observation of *R. insignis* in the field it is clear that *R. insignis* is an ecologically diverse species well suited to a range of environments.

The ArcGIS analysis suggests that *R. nivicola* is currently occupies environments that could be otherwise inhabited by *R. insignis*. The phylogenetic analyses (Chapter 2) reveal a low level of genetic diversity in *R. insignis* and *R. nivicola*. For *R. insignis*, this low level of genetic diversity means that it has probably recently expanded its range into the North Island. The low genetic diversity in *R. nivicola* is suggestive of recent formation of this species and also of recent range expansion. The environmental envelope predictions and the observation on genetic diversity that are reported here suggest that the distributions of these two species may not currently be in equilibrium with respect to each other, and that these species are yet to exploit all available habitats. Habitat differences as inferred from the ArcGIS analysis can not explain the absence of *R. insignis* on Mt Taranaki. It is

not clear whether this result reflects a lack of resolution in the LENZ database or an absence of niche differentiation between the two species. More detailed investigation of possible niche differentiation between *R. insignis* and *R. nivicola* could involve studies of microclimate and investigation of physiological tolerances (Lloyd *et al.*, 2003). Preliminary results examining the relative rates of photosynthesis in *R. nivicola* and *R. insignis* (Carter *et al.*, unpublished) have suggested potential differences in photosynthetic rates. Niche competition between these species has not yet been studied, but is clearly an important consideration in understanding extant distributions.

It was observed in the phylogenetic investigation of the North Island *Ranunculus* (Chapter 2) that *R. verticillatus* appeared to have had multiple dispersal events between the North and South Islands (Fig. 2.4). This suggested that the dispersal ability of *R. verticillatus* is not the reason for its limited North Island range. The environmental envelope for this species indicates that it is currently found in most of the available habitat, meaning that suitable habitat is a limiting factor for *R. verticillatus* in the North Island. The phylogenetic analyses reported here suggest that *R. verticillatus* has been in the North Island for a longer period than *R. insignis*. Given the overlap in the environmental envelopes of *R. insignis* and *R. verticillatus*, it is possible that the plastic *R. insignis* may be actively competing with *R. verticillatus* at some sites. Interestingly, Fisher (1965) predicted that *R. verticillatus* should occur in a number of South Island habitats where it was absent. He was uncertain whether *R. verticillatus* was restricted due to unsuitable climate, topography or because of competition. ArcGIS analyses suggests that *R. verticillatus* is not climatically limited in the West Coast of the South Island, favouring an hypothesis that there are other factors limiting the southward expansion of *R. verticillatus*.

Further evaluation of extant distributions will need to consider not only present day conditions, but also environmental disturbances and habitat modification of the recent past. Phylogenetic analyses to reveal population histories of plant communities will be helpful here. Such analyses may also need to consider the effect of many species interactions. For example, hybridisation involving *R. insignis* and other *Ranunculus* species in the South Island (Chapter 2) may be relevant to understanding the diversity of environments in which *R. insignis* is found.

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Appendices

Appendix A: Collection Information of *Ranunculus* Samples used in Analyses

Species	Collection Locality	Easting	Northing	Voucher
Ranunculus acaulis	Mason Bay, Stewart Island, New Zealand	2107590	5346915	MPN24592
Ranunculus anemoneus	Mt Kosciusko, Australia	36° 27' S	148° 16' E	MPN24588
Ranunculus buchananii	Skeleton Lake, South Island, New Zealand	2190800	5520300	CHR50922
	Lake Wapiti, South Island, New Zealand	2072690	5558330	CHR52051
Ranunculus crithmifolius subsp.*crithmifolius*	Ben Ohau 1, South Island, New Zealand	2267220	5658810	MPN28947
	Gordon Range, South Island, New Zealand	2507501	5957625	MPN28950
	Eyre Mountains 1, South Island, New Zealand	2149600	5520700	CHR509774
Ranunculus crithmifolius subsp. *paucifolius*	Castle Hill 1, South Island, New Zealand	2405801	5775324	No voucher (endangered)
Ranunculus enysii	Borland Burn 1, South Island, New Zealand	2283603	5532637	MPN28933
	Rock and Pillar, South Island, New Zealand	2284151	5532656	MPN28945

Species	Collection Locality	Easting	Northing	Voucher
R. enysii	Mount Cheeseman. South Island, New Zealand	2400871	5782448	MPN28939
	Mt Misery, South Island, New Zealand	2405022	5793530	MPN28941
Ranunculus godleyanus	Eric Stream, South Island, New Zealand	2319280	5753690	CHR499398
	Malte Brun, South Island, New Zealand	2290400	5734400	MPN24609
Ranunculus gracilipes	Rock and Pillar 1, South Island, New Zealand	2118518	5516161	MPN28967
	Mt Cheeseman, South Island, New Zealand	2401444	5782853	MPN28964
	Ben Ohau 1, South Island, New Zealand	2222475	5658128	In processing
	Takitimu Mountains, South Island, New Zealand	2121325	5486827	In processing
	Mckerrow Range, South Island, New Zealand	2211549	5660979	MPN28966
Ranunculus grahamii	Aiguilles Rouge, South Island, New Zealand	2291320	5733090	In processing
Ranunculus gunnianus	Mt Kosciusko, Australia	36° 27' S	148° 16' E	MPN24587
Ranunculus haastii subsp. *haastii*	Amuri, South Island, New Zealand	2487400	5862200	CHR532284
	Mt Hutt, South Island, New Zealand	2392600	5743200	CHR518452
	Broken River, South Island, New Zealand	2403650	5785450	In processing

Species	Collection Locality	Easting	Northing	Voucher
Ranunculus haastii subsp. *pilferus*	Eyre Mountains, South Island, New Zealand	2141405	5534640	In processing
Ranunculus insignis	Mt Hutt 1, South Island, New Zealand	2392794	5742219	MPN28955
	Mt Hutt 2, South Island, New Zealand	2392548	5742437	MPN28958
	Castle Hill 1, South Island, New Zealand	2405873	5775211	MPN28952
	Castle Hill 2, South Island, New Zealand	2405873	5775211	In processing
	Castle Hill 3, South Island, New Zealand	2405864	5775766	MPN28962
	Castle Hill 4, South Island, New Zealand	2405913	5775767	MPN28956
	Torlesse Range 1, South Island, New Zealand	2413443	5768545	MPN28960
	Torlesse Range 2, South Island, New Zealand	2414159	5769556	MPN28961
	Lake Lydon, South Island, New Zealand	2404117	5767962	MPN28954
	Porters Pass 1, South Island, New Zealand	2407941	5767354	MPN28953
	Porters Pass 2, South Island, New Zealand	2407941	5767354	MPN28959
	Foggy Peak, South Island, New Zealand	2408172	5768180	In processing
	Ada Pass, South Island, New Zealand	2464967	5878171	In processing
	Travers Peak, South Island, New Zealand	2462145	5867493	In processing
	Kennet River, South Island, New Zealand	2538417	5909925	MPN28957
	Mt Arthur, South Island, New Zealand	2484100	6000125	In processing

Species	Collection Locality	Easting	Northing	Voucher
R. insignis	Mt Holdsworth, North Island, New Zealand	2715000	6036420	In processing
	Herepai Peak, North Island, New Zealand	2722300	6054800	In processing
	Arete Peak, North Island, New Zealand	2715300	6048600	In processing
	Mt Hector, North Island, New Zealand	2701200	6026850	In processing
	Rangiwahai Track, North Island, New Zealand	2767625	6141060	In processing
	Armstrong Saddle, North Island, New Zealand	2781280	6152710	In processing
	South Crater, North Island, New Zealand	2738225	6226800	In processing
	Turoa, North Island, New Zealand	2728020	6208045	In processing
	Mangapohutau, North Island, New Zealand	2866875	6284425	In processing
	Mad-dog Peak, North Island, New Zealand	2800500	6206775	In processing
	Mt Hikurangi, North Island, New Zealand	2954405	6352730	In processing
Ranunculus lyallii	Mt Anglem, South Island, New Zealand	2121250	5373700	In processing
	Mt Tutoko, South Island, New Zealand	2114220	5611740	MPN24601
	Mt Cook, South Island, New Zealand	2275840	5719760	MPN24603
	Franz Joseph, South Island, New Zealand	2279890	5750950	MPN24670
	Hump Ridge, South Island ,New Zealand	2070200	5437400	CHR110835

Species	Collection Locality	Easting	Northing	Voucher
R. lyallii	Mt George, South Island, New Zealand	2071000	5437600	CHR306134A
Ranunculus nivicola	Lower Tama Lake, North Island, New Zealand	2734695	6219215	In processing
	Mt Hauhungatahi, North Island, New Zealand	2720970	6216360	In processing
	Mt Taranaki 1, North Island, New Zealand	2603780	6210935	In processing
	Mt Taranaki 2, North Island, New Zealand	2603980	6211205	In processing
	Mt Taranaki 3, North Island, New Zealand	2603710	6211860	In processing
	Mt Taranaki 4, North Island, New Zealand	2603710	6210560	In processing
	North West Taranaki, North Island, New Zealand	2599670	6210955	In processing
	Poukai West Taranaki, North Island, New Zealand	2597475	6217470	In processing
	Soda Springs 2, North Island, New Zealand	2736385	6226090	In processing
	Soda Springs 3, North Island, New Zealand	2736520	6226120	In processing
	South Crater, North Island, New Zealand	2738040	6226460	In processing
	Umukarikari 1, North Island, New Zealand	2760980	6226000	In processing
	Umukarikari 2, North Island, New Zealand	2761130	6226015	In processing
	Umukarikari 3, North Island, New Zealand	2761150	6226200	In processing
	Umukarikari 4, North Island, New Zealand	2761340	6225735	In processing

Species	Collection Locality	Easting	Northing	Voucher
R. nivicola	Umukarikari 5, North Island, New Zealand	2760950	6225720	In processing
Ranunculus pachyrrhizus	Remarkables, South Island, New Zealand	2181415	5562370	In processing
	Old Man Range, South Island, New Zealand	2376200	5742800	In processing
Ranunculus pinguis	Auckland Island, New Zealand	2019692	4924528	MPN24590
	Campbell Island, New Zealand	2242380	4734942	MPN24591
Ranunculus scrithalis	Eyre Mountains, South Island, New Zealand	2153300	5533300	MPN24600
				In processing
Ranunculus sericophyllus	Pudding Rock, South Island, New Zealand	2277550	5729450	In processing
	Beatham Valley, South Island, New Zealand	2288020	5734580	In processing
	Otira Face, South Island, New Zealand			In processing
	Mt Cook, South Island, New Zealand	2274015	5717080	MPN24596
	Mt Franklin, South Island, New Zealand	2400510	5814365	MPN24597
	Temple Basin 3, South Island, New Zealand	2395040	5810415	In processing
	Rome Ridge, South Island, New Zealand	2389750	5808635	In processing
Ranunculus verticillatus	St Arnaud Range, South Island, New Zealand	2500400	5931300	In processing
	Cobb Valley, South Island, New Zealand	2469400	6015775	In processing

Species	Collection Locality	Easting	Northing	Voucher
Ranunculus verticillatus	Mt Arthur, South Island, New Zealand	2484100	6000125	In processing
	Mt Peel, South Island, New Zealand	2475665	6007495	In processing
	Gordon Range, South Island, New Zealand	2506800	5956400	In processing
	Mt Stoke 1, South Island, New Zealand	2602640	6012430	In processing
	Mt Stoke 2, South Island, New Zealand	2602640	6012430	In processing
	Mt Hector, North Island, New Zealand	2700960	6028330	In processing
	Mt Holdsworth, North Island, New Zealand	2714200	6034890	MPN24607
	Mitre Peak, North Island, New Zealand	2717320	6042490	In processing
	Arete Peak, North Island, New Zealand	2715300	6048600	In processing
	Herepai Peak, North Island, New Zealand			In processing
	Pukamatawai, North Island, New Zealand	2715300	6048600	In processing
	Mt Toka, North Island, New Zealand	2771270	6130850	In processing
	Armstrong Saddle, North Island, New Zealand	2781325	6153615	In processing
	Mt Hikurangi, North Island, New Zealand	2953600	6354400	In processing
	Mt Hauhungatahai, North Island, New Zealand	2722075	6054650	In processing
Ranunculus viridis	Tin Range, Stewart Island, New Zealand	2114230	5333480	No voucher (endangered)

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

Appendix B: Invitrogen DNA Ladder Standards

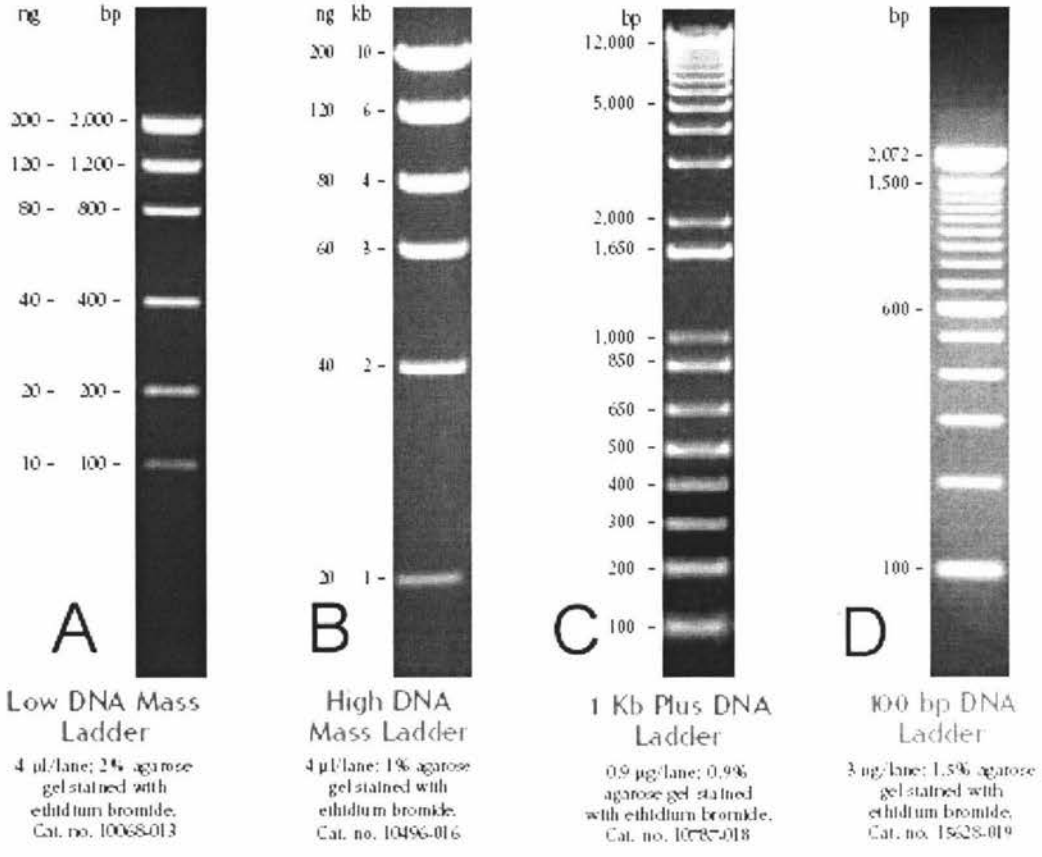

Figure B - Invitrogen DNA ladder standards. (A) Low DNA Mass Ladder. (B) High DNA Mass Ladder. (C) 1 Kb Plus DNA Mass Ladder. (D) 100 bp DNA Mass Ladder.

Appendix C: Oligonucleotides used in DNA sequencing

Direct sequencing marker primer sequences		
Locus	Primer Name	Primer Sequence
Nuclear		
ITS	ITS4	5′ TCCTCCGCTTATTGATATGC 3′
	ITS5hp	5′ GGAAGTAAAAGTCGTAACAAGG 3′
Chloroplast		
J_{SA}	J_{SA}F	5′ ATTATYAATGAAGGYAATACWATATATTTTC 3′
	J_{SA}R	5′ CAAATTCCAATGACCAAATAGTTGG 3′
trn L	trn L c	5′ CGAAATCGGTAGACGCTACG 3′
	trn L f	5′ ATTTGAACTGGTGACACGAG 3′

Table C - Oligonucleotides used in DNA sequencing.