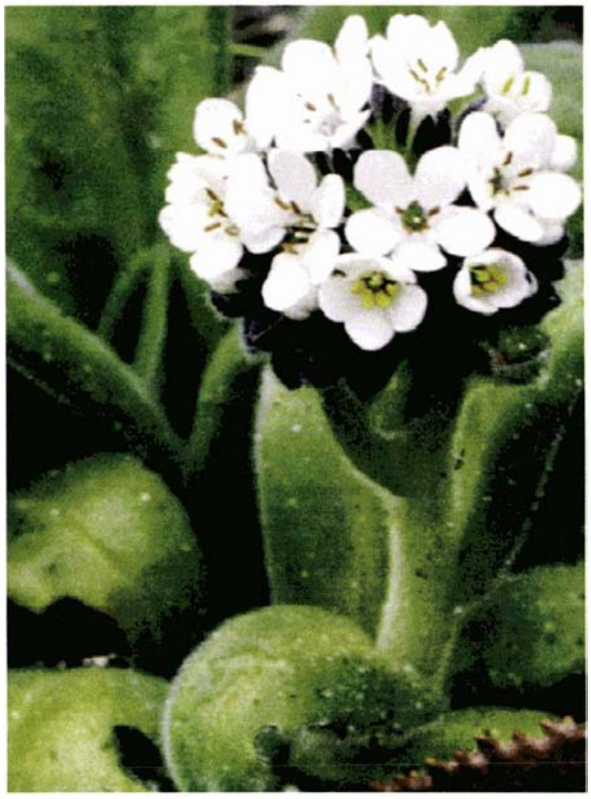

Myosotis rakiura

"With regard to general problems of biogeography, the biota of New Zealand has been, perhaps, the most important of any in the world. It has figured prominently in all discussions of austral biogeography, and all notable authorities have felt obliged to explain its history: explain New Zealand and the world falls into place around it."

Gareth Nelson (1975)

Evolution of the New Zealand Alpine Flora:

Origins, Diversification and Dispersal

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

Doctor of Philosophy
in
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Richard Charles Winkworth

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ABSTRACT

This thesis describes molecular systematic studies that test hypotheses concerning the age and origins of the New Zealand alpine flora. Analyses of nuclear and chloroplast DNA markers for two plant groups that have radiated extensively in the alpine zone of New Zealand – the genus *Myosotis* (Boraginaceae) and the Australasian apioid Umbelliferae – are reported. The molecular results suggest that the diversification of these groups in New Zealand has occurred since the late Tertiary. This finding corroborates recent molecular systematic studies on other New Zealand plant groups. Taken together, these studies suggest that New Zealand's modem floristic diversity was greatly influenced by dramatic global climate change during the late Tertiary and Quaternary. These results are also consistent with those reported for plant groups overseas. In these, recent diversification has occurred with Quaternary climatic fluctuations (Comes & Kadereit, 1998) and colonisation of insular environments (Crawford & Stuessy, 1997)

The molecular analyses also suggest that since the late Tertiary, *Myosotis* and the Australasian Apioideae have been involved in transoceanic dispersal events both to and from New Zealand. However, while most other molecular studies have provided evidence for the importance of circum-polar westerly winds, the present data suggests that, for *Myosotis* and with less confidence the apioid genera, some dispersal events have been in a westerly direction. Since this finding was made, late Tertiary and Quaternary westward dispersal has also been inferred for other New Zealand alpine plants. These observations suggest that passive eastward wind dispersal can not explain the distributions of all southern Pacific plant groups.

The present study, as well as other recent studies, has highlighted the need to develop molecular tools and analytical approaches for describing the potentially complex evolutionary relationships between taxa that have originated since the late Tertiary. Here, the amplified fragment length polymorphism (AFLP) method was investigated as a means of identifying fast evolving genome regions in New Zealand *Myosotis.* Preliminary analyses suggest that this is a useful approach for locating highly variable molecular markers. However, like other rapidly evolving regions (e.g. Buckler *et al.*, 1997) some of the derived markers were multiple copy and polymorphic at different loci within a single genome. This feature of fast evolving genome regions is problematical since bifurcating evolutionary analysis models will poorly represent such complex data. For this reason split-decomposition was investigated as an alternative method for data representation. This approach was found to have both advantages and limitations for studying late Tertiary or Quaternary radiations.

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Thanks to my family for providing motivation and support over the last four years. A special note to my Dad, in answer to your favourite question I can now say, "yes, it's finally finished".

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CHAPTER 1: INTRODUCTION

The nature of the physical environment in the alpine zone of New Zealand, both historically and as it exists today, is expected to have strongly influenced the composition and evolution of the flora which it supports (Wardle, 1986). This chapter provides an overview of the geological and climatic history of New Zealand and discusses the current physical environment of the alpine zone. The varied habitats of the contemporary New Zealand mountains support a diverse alpine flora – various aspects of this flora are also described in this chapter.

Having developed this background, hypotheses concerned with the origins and diversification of the current alpine flora are discussed. Finally, a brief outline of the study is presented.

1.1 GEOLOGICAL AND CLIMATIC CHANGE IN NEW ZEALAND

In this section an introduction to New Zealand's geological and climatic history is given. Evidence suggests that habitats, which today support a diverse alpine flora, are unlikely to have existed in New Zealand before the Pliocene. Figure 1.1 illustrates the major changes to the geography of New Zealand during Cenozoic time.

1.1.1 The Cretaceous and early Tertiary

The ancestral New Zealand landmass was uplifted along the eastern edge of Gondwana during the late Jurassic and early Cretaceous. Initially mountainous, steady chemical and mechanical weathering throughout the Cretaceous Period resulted in a significant lowering of the topology and progressive inundation of marginal areas by the ocean (Stevens, 1985). In the late Mesozoic tectonic activity began to separate New Zealand from the Gondwanan supercontinent (Weissel & Hayes, 1977; Ollier, 1986). Geological evidence suggests that by 85 MYA (million years ago) open ocean existed between New Zealand and Australia, although sea floor spreading continued into the early Paleocene when the current separation of the landmasses was reached (Cooper & Millener, 1993).

In the late Paleocene significant sea floor spreading between Antarctica and Australia began to propel New Zealand northward (Ollier, 1986). These tectonic movements have been correlated with indicators that suggest climatic warming, culminating in subtropical climates during the early Eocene. However, the pollen record and marine fossils indicate that climates had cooled markedly by the late

Eocene (Suggate *et al.*, 1978; McGlone, 1985; Pocknall, 1989). Erosion continued to reduce the ancestral New Zealand landmass in the early Tertiary – by the Eocene most of the land had become peneplanated and the geographic outline changed rapidly (Fleming, 1979; Cooper & Cooper, 1995).

Non-flowering plants such as ferns, fern-allies, cycads and podocarps dominated Early Cretaceous vegetation assemblages. Angiosperm pollen first appeared in New Zealand approximately 100 MYA and by the latest Mesozoic, formed an important component of New Zealand palynofloras (Fleming, 1979; Mildenhall, 1980). Early Tertiary assemblages continued to be dominated by cool temperate Podocarpaceae, with *Casuarina*, Proteaceae and *Nothofagus fusca* type pollens remaining subordinate. However, by the Eocene angiosperms dominated palynofloras (Mildenhall, 1980).

1.1.2 The middle Tertiary

Two characteristic Southern Hemisphere phenomena became established and strengthened during the middle Tertiary. The separation of Antarctica from Australia and New Zealand led to the establishment of circum-Antarctic marine currents during Oligocene time. At first these deep-water marine movements were weak, but as the distance between landmasses progressively increased this current system became a prominent feature of the Southern Ocean. Associated with these marine currents was the development of the West Wind Drift. Unimpeded by landmasses these strong, prevailing westerly winds of high southern latitudes had a dramatic influence on Southern Hemisphere weather patterns (Edwards, 1975; Kennet, 1977; Stevens, 1985). Both these circum-polar systems are thought to have had an important impact on dispersal in the Southern Hemisphere and ensured that New Zealand received colonists from diverse sources, despite the disruption of Cretaceous and early Tertiary land dispersal routes (Close *et al.*, 1978; Fleming, 1979).

New Zealand appears to have been reduced to a narrow, scattered, string of islands during the Oligocene (Figure 1.1). Although the exact extent and geographical distribution of land is uncertain, particle sediment analysis suggests that the remaining land had limited topographical relief (Stevens, 1985; Cooper & Cooper, 1995). The cool climate established in the late Eocene continued during the Oligocene although high rainfall and humidity are suggested to have supported vegetation assemblages of high diversity (Mildenhall, 1980; Burns & Nelson, 1981).

The activation of the Pacific-Australian plate boundary approximately 25 MYA was the beginning of an important and relatively unstable period in the tectonic history of New Zealand (Cooper *et al.*, 1987; Kamp, 1992). Geological evidence suggests that during the Miocene a narrow, elongated land of rugged, although not yet mountainous, relief was uplifted (Stevens, 1985; Cooper & Cooper, 1995). Pollen cores from deep sea drilling and the marine fossil record indicates that New

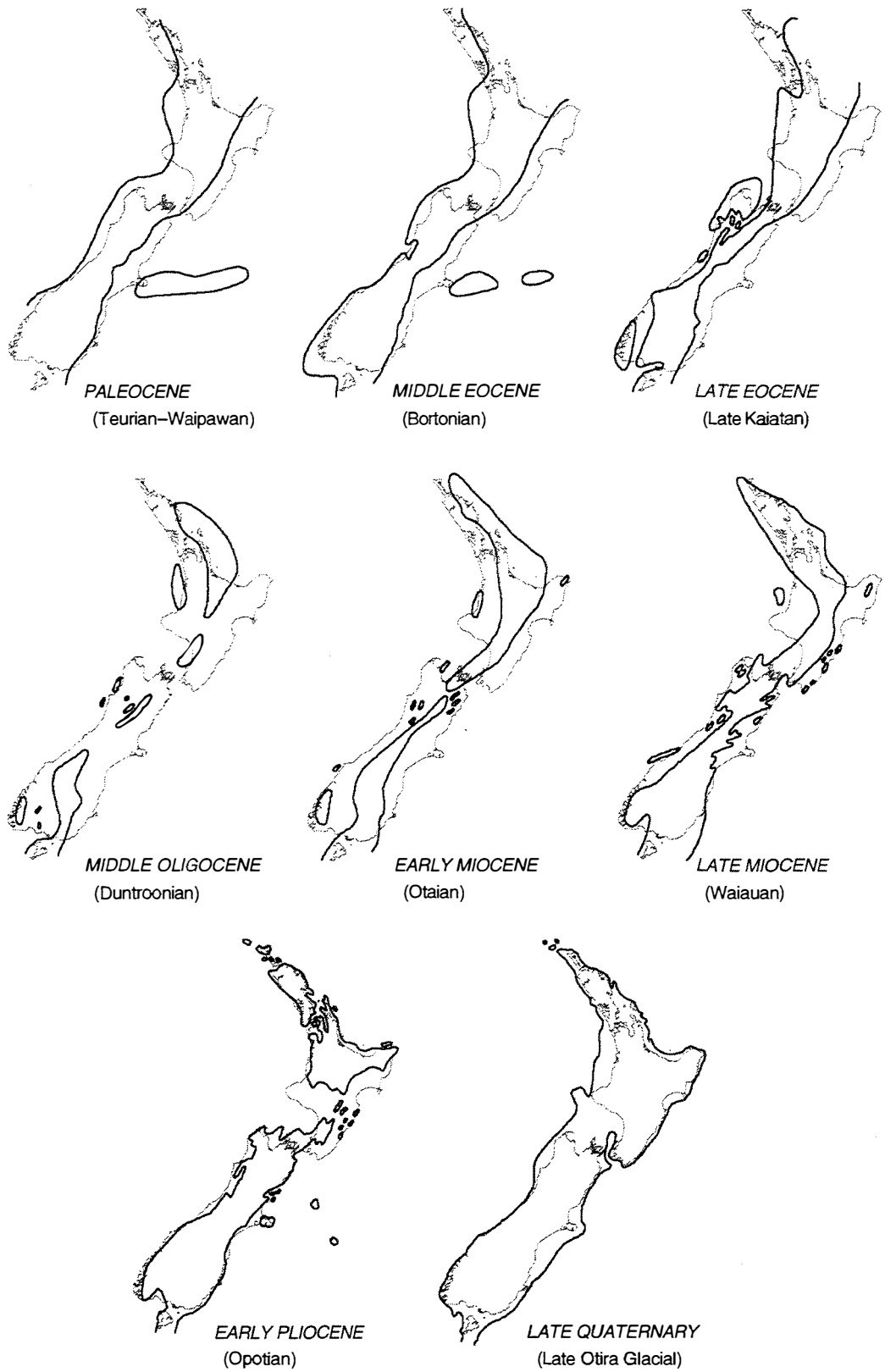


***Figure 1.1**: The changing geographic outline of New Zealand during the Tertiary and Quaternary Periods (modified from Suggate* et al., *1978).*

Zealand experienced warm and possibly subtropical conditions for most of the Miocene (Fleming, 1979). During the late Miocene climates again deteriorated, possibly in response to a northward shift in the boundary between cool polar and warm tropical waters (Pocknall, 1989).

Nothofagus dominated Oligocene palynofloras although several other groups are prominent (Table 1.1). During the late Oligocene and Miocene, regionally distinct pollen assemblages developed – in the north *Nothofagus brassii* pollen dominates but is replaced by *fusca* type in the south (Mildenhall, 1980).

1.1.3 The Pliocene and Pleistocene

Geological evidence indicates that tectonic movements, which were initiated in the Miocene, intensified during the Pliocene (Ollier, 1986). Fission track data and sedimentation rates suggest that approximately 5 MYA, changing interactions between the Pacific and Australian tectonic plates led to the rapid elevation of New Zealand's axial mountain ranges in the late Pliocene and early Pleistocene (Cox & Findlay, 1995; Batt *et al.*, 2000). Marine transgressions and active volcanism also influenced the geography of New Zealand during this time (Fleming, 1979; Stevens, 1985). Climates were generally cool temperate during the Pliocene, although regional

TABLE 1.1
General patterns in the composition of mid Tertiary to Quaternary floras of New Zealand

Geologic Period	Common elements	First appearance of taxa	References
Oligocene (37-23 MYA)	Arecaceae, Podocarpaceae, Myrtaceae, *Casuarina*, *Nothofagus brassii* and *fusca* groups	Asteraceae, Juncaceae, Restionaceae, *Weinmannia*, *Laurelia*, *Myrsine*, *Fuchsia*, *Elaeocarpus*, *Pseudowintera*, *Rhalopstylis*, *Myriophyllum*, *Typha*, *Coprosma*, *Pteridium*	Fleming (1979) Mildenhall (1980) Pocknall (1989)
Miocene (23-5 MYA)	Myrtaceae, Podocarpaceae, Arecaceae, Cyatheaceae, *Nothofagus brassii* and *fusca* groups, *Eucalyptus*, *Casuarina*	*Cordyline*, *Muehlenbeckia*, *Ripogonum*, *Hydrocotyle*, *Dactylanthus*, *Pittosporum*, *Libertia*, *Alectryon*, *Melicytus*, *Macropiper*, *Phormium*, *Griselinia*	Fleming (1979) Mildenhall (1980) Mildenhall & Pocknall (1984) Pocknall & Mildenhall (1984)
Pliocene (5-2 MYA)	Podocarpaceae, Myrtaceae, *Nothofagus brassii* and *fusca* groups, *Dacrydium* – later herbaceous taxa dominate some areas	*Walhenbergia*, *Sarcocornia*, *Quintinia*, *Coriaria*, *Rubus*, *Carmichaelia*, *Gentiana*, *Hebe*, *Epilobium*, *Grammitis*	Wardle (1963, 1978) Raven (1973) Mildenhall (1980)
Quaternary (2 MYA to present)	Various associations reflecting climatic change – Beech-podocarp forest, shrublands etc.	Euphorbiaceae, *Gaultheria* *Parsonia*, *Myosotis*, *Toronia*, *Acacia*, *Acaena*, *Epacris*	Mildenhall (1980)

differences in altitude, rainfall and temperature became increasingly pronounced (Mildenhall, 1980). Pliocene vegetation assemblages were typically podocarp-hardwood forests dominated by cool temperate taxa. Although, in the South Island grass and shrubland associations developed during the latest Tertiary (Table 1.1; Mildenhall, 1980; McGlone, 1985)

In New Zealand, glacial climates became established approximately 2.5 MYA, and glacio-eustatic sea-level changes indicate repeated glacial interglacial cycles (Beu & Edwards, 1984; Suggate, 1990). While the record of glaciation during the late Pliocene and early Pleistocene is incomplete, marine sediment cores and oxygen isotope data provide a more comprehensive record of recent events and suggest that nine glaciations have occurred during the last 0.7 MY (million years). In terrestrial deposits only the last four of these, covering the past 0.35 MY, can be recognised due to subsequent tectonic and erosional processes (Suggate, 1990). During the late Pleistocene climates appear to have changed rapidly and dramatically – alternating between cool, dry climates during glacial maxima and the warm, moist interstadials and interglacials (Hornibrook, 1992; Markgraf *et al.*, 1995). Analysis of marine sediment cores suggests that during the last glacial maximum, circum-polar westerly winds were stronger and extended further north than at present (Thiede, 1979; Stewart & Neall, 1984). These strong, cold winds are likely to have had an important influence on the New Zealand climate during glacial periods.

The repeated glacial cycles have significantly effected South Island landforms. Many of the contemporary features result directly from glacial modelling or modification of glacial constructions. Although the influence of glaciation on the North Island mountains was only minor, processes associated with cold climates (e.g. periglacial mass movement) strongly affected southern North Island landscapes (Pillans *et al.*, 1992). Pleistocene environmental change also had profound effects on the New Zealand vegetation (Raven, 1973). During stadials, forest habitats were mostly confined to northern New Zealand, although in the south small, refugial pockets may have survived in favourable locations (McGlone, 1985; Wardle, 1988). The pollen record suggests that in areas deforested during glacial maxima, extensive grass and shrub dominated assemblages developed and it is suggested that close to glaciated locations, large areas probably remained bare (McGlone, 1985, Markgraf *et al.*, 1995).

1.1.4 The post-glacial period

In New Zealand glaciers began to rapidly retreat approximately 14 000 years ago, although several halts and minor glacial re-advances have occurred since that time. Following the glacial retreat, erosion and depositional processes dramatically altered many New Zealand landforms (Suggate, 1990; Pillans *et al.*, 1992). The onset of post-glacial climates was associated with rising sea-levels, such that by approximately 9 000 years ago New Zealand was separated into the three main

islands. However, current sea-levels were not reached until more recently (Pillans *et al.*, 1992).

During the early post-glacial forest types which had been confined to northern New Zealand rapidly spread through the North Island so that by 12 000 years ago most areas appear to have been reforested. However, fossil pollen profiles suggest that forest vegetation did not become widely established in the South Island until 10 000 years ago (McGlone, 1985; Pillans *et al.*, 1992). Early post-glacial forests were predominantly podocarp-hardwood, reflecting the warm, moist climatic optimum that followed deglaciation. Cooler, drier conditions developed around 7 000 years ago and in these climates, *Nothofagus* populations expanded dramatically (McGlone, 1985).

1.2 THE CONTEMPORARY ALPINE ZONE

Variation in the physical environment of the New Zealand mountains is the result of interactions between ongoing tectonic processes, variation in the underlying geology, past glacial processes and the predominance of westerly weather patterns (Whitehouse & Pearce, 1992). This section discusses present day environmental conditions in the New Zealand mountains.

1.2.1 The New Zealand mountains

Mountains dominate the South Island landscape, extending in an unbroken chain from the inland Kaikoura Range in Marlborourgh to the mountains of Fiordland (Figure 1.2; Whitehouse & Pearce, 1992). The South Island mountains are divided into distinct regions, each with a typical geomorphology and composition. Most of the eastern Southern Alps consist of greywacke and argillite rock types that underwent complex faulting and folding processes during their uplift. These rocks are very prone to frost shattering, giving rise to the extensive scree and talus slopes characteristic of many peaks in this region (Holloway, 1982; Whitehouse & Pearce, 1992). The western mountains of the Main Divide, are formed by harder schist. Uplifted in a complex orogeny, these high mountains were significantly modified by extensive, repeated glaciations and heavy post-glacial erosion (Holloway, 1982). The rounded and undulating mountains of central and north Otago are also composed of schist but, unlike the alpine schist mountains, these have been formed by folding and tilting of crustal sections. The peaks of these block mountains are relatively subdued and their slopes have not been affected by glacial erosion (Holloway, 1982; Whitehouse & Pearce, 1992). The steep-sided mountains of Fiordland and Stewart Island consist predominantly of erosion resistant granite, gneiss, and related rock types. A wide variety of ancient and erosion resistant rock types – granite, gneiss, schists,

***Figure 1.2**: Alpine areas of New Zealand (from Fisher, 1965)*

limestone and marble – also form the mountains of north Westland and north-west Nelson. These steep, rugged mountains rise rapidly from the coastal lowlands and at higher elevations extensive scree slopes are common (Holloway, 1982).

In the North Island the relief is comparatively subdued. The main axial ranges are formed by an extension of the greywacke formation important in the eastern South Island mountains. In the north of the island, several mountain chains form a large, rugged upland area but this narrows in the south where a single range divides the country (Holloway, 1982; Whitehouse & Pearce, 1992). The most distinctive North Island mountains are the geologically young volcanic peaks, three on the Central Plateau and one to the west. These mountains are composed of various volcanic materials, such as ash and scoria – their present form determined by past volcanic eruptions and erosional processes (Holloway, 1982)

1.2.2 Alpine climates

The climatic conditions of the New Zealand mountains, and also those of the lowlands, primarily reflect the isolated oceanic situation and the north to south

distribution of strong topographic relief that intercepts the prevailing westerly weather patterns. As a result of this interaction there is a strong west to east rainfall gradient, especially in the South Island where most precipitation falls on the western mountains and eastern areas are in rainshadow. (Holloway, 1982; Whitehouse & Pearce, 1992). Snowfall in the New Zealand mountains generally occurs between May and October. At high elevation in the South Island extensive, permanent ice and snowfields occur. At lower elevations snowfall may remain for only a few days or for several months, especially in areas of southern aspect or during winter. In comparison, the North Island mountains have little permanent snow or ice (Holloway, 1982; Whitehouse & Pearce, 1992).

Temperature is an important factor in alpine areas, and in the New Zealand alpine zone it is strongly affected by the oceanic situation. Typically, temperatures fluctuate around freezing point throughout the year, rarely climbing above 20°C or falling below – 20°C. However, significant changes in temperature can occur rapidly as the result of changes in wind direction (Holloway, 1982; Whitehouse & Pearce, 1992). Comparisons with mountain regions of continental landmasses indicate that although summer temperatures are similar, winter maxima are much warmer in New Zealand – possibly reflecting the maritime influence. Such comparisons also reveal that the frost-free and growing periods in the New Zealand alpine zone are shorter than for Northern Hemisphere mountains (Holloway, 1982).

1.3 THE NEW ZEALAND ALPINE FLORA

The alpine flora encompasses those plants that grow within the alpine zone – generally considered to be the area between the upper limit of tree growth and the lower limit of permanent snow (Wardle, 1986). This section provides an overview of some characteristic features of the New Zealand alpine flora.

1.3.1 Diversity and endemism

In New Zealand approximately 600 angiosperm species – representing about 30% of the vascular plant flora – occur in alpine areas (Mark & Adams, 1995). Although Southern Hemisphere mountain floras are generally recognised as exhibiting high levels of endemism, the New Zealand mountains have a particularly high number of endemic species which perhaps reflecting the greater isolation from possible source areas (Smith, 1986). Approximately 93% of New Zealand's alpine plant species are endemic to New Zealand and this makes a significant contribution to the overall level of species endemism in New Zealand. At higher taxonomic levels endemism is surprising low with few genera being endemic to New Zealand (Mark & Adams, 1995)

Consistent with the greater extent of alpine habitats in the South Island, most of the alpine plant diversity is found in these mountains. In particular the mountains of the Nelson-Marlborough and Otago-Southland have high diversity, these areas supporting many locally endemic species, or plants with disjunct distributions (Wardle, 1963; Burrows, 1965; McGlone, 1985). By comparison the North Island mountains support an alpine flora of smaller size and lower diversity. Several factors may have limited the possibilities for the evolution of a diverse alpine flora in the North Island. These include (a) the younger age and smaller extent of mountain habitats; (b) post-glacial climatic warmth which may have further reduced the extent of North Island alpine habitats; (c) the relative isolation and instability of habitats on the volcanic peaks (McGlone, 1985; Wardle, 1988).

1.3.2 Alpine plant communities

Alpine plant communities can change dramatically over short distances reflecting the importance of microclimates in the alpine zone. In alpine areas of New Zealand a number of vegetation types and niche habitats are recognised and these are broadly separated into three zones – low alpine, high alpine and nival (Wardle, 1986).

The low alpine zone extends for approximately 300 m above the climatic treeline and is characterised by various, usually well-developed soils that support several vegetation types (Table 1.2). In the New Zealand mountains low alpine communities are typically dominated by tussock (*Chionochloa*) species – an unusual feature for alpine vegetation in areas outside of the tropics. Above the low alpine communities the high alpine belt extends to the permanent snowline. The vegetation is generally low growing and strongly influenced by soil type, exposure and snow cover (Table 1.2; Wardle, 1986; Mark & Adams, 1995)

The upper limit of the alpine zone is defined by the permanent summer snowline, above this extends the nival zone. On slopes too steep or exposed for permanent snow to accumulate and where melt water is available, this habitat supports a few specialised vascular plants (Wardle, 1986; Dawson, 1988; Mark & Adams, 1995).

1.3.3 Specialised forms in the alpine flora

Several specialised growth forms and putative morphological adaptations are generally characteristic of New Zealand alpine plants. In New Zealand cushion and mat forming plants occur in the high alpine zone (e.g. *Raoulia* and *Hectorella*) and in bog habitats (e.g. *Donatia* and *Oreobolus*). This form has been suggested to be a response to consistently low temperatures (Wardle, 1985). The scree slopes of the eastern South Island support several specialised taxa, including *Ranunculus haastii*

TABLE 1.2
Plant communities of the New Zealand alpine zone (following Dawson, 1988; Mark & Adams, 1995)

Vegetation belt	Community type	Description
Low alpine zone	Mixed snow tussock- shrub	mixture of tall tussocks, large herbs (e.g. *Aciphylla*, *Celmisia* and *Ranunculus*) and shrubs; shrubs are usually a combination of alpine and subalpine forms.
	Mixed snow tussock- herbfield	in wetter areas, an extension of the mixed tussock-shrub communities; composition is largely soil dependent but dominated by tussocks and larger herbs.
	Herbfields	similar to the mixed tussock-herbfield, but dominated by *Aciphylla*, *Anisotome*, *Astelia*, *Celmisia* and *Ranunculus*; tussocks are much rarer.
	Bogs	formed in areas where the water table is close to the surface; species composition dependent on altitude, acidity, slope and height of water table; generally dominated by cushion taxa.
High alpine zone	Fellfields	often develop in exposed areas with little soil; characterised by dwarf shrubs, cushion and mat plants, including "vegetable sheep" (*Raoulia* and *Haastia* sp.)
	Snowbanks	sheltered, moist sites that support various species, although communities usually representative of fellfields at higher elevations
	Screes	may span a wide altitudinal range and support a sparse but highly specialised flora that can survive the extreme conditions in this habitat
	Cushionfields	extremely dwarfed vegetation of the central Otago plateau mountains; maintained by the high winds, cold temperatures and frequent freeze-thaw cycles

and *Leptinella atrata*. These plants have features considered to be adaptations to scree habitats, of particular importance are those which reduce mechanical damage caused by movements of the mobile surface (Fisher, 1952). Many New Zealand alpine plants are characterised by large, rigid leaves with glossy adaxial and densely tormentose abaxial surfaces. These features have been alternatively linked with xeromorphy, nutrient deficiency or thermal balance (Wardle, 1985, 1986).

Further, although mountain and island floras generally contain a significant proportion of white-flowered plants, this feature is particularly apparent in the New Zealand alpine flora (Wardle, 1978; Godley, 1979; Lloyd, 1985). This characteristic is well illustrated in genera where related species outside of New Zealand have predominantly brightly coloured flowers, for example *Myosotis* and *Gentiana* (Wardle, 1978; Godley, 1979; Webb & Kelly, 1993). The evolution or maintenance of white-flowered forms has been attributed to the paucity of specialised pollinating insects in New Zealand or linked to a syndrome of characters associated with autogamy (Godley, 1979; Raven & Raven, 1976; Mark & Adams, 1995). It appears that the trend toward white flowers in New Zealand can not be explained by a single

selective mechanism, rather the biology of individual taxa has influenced flower colour (Wardle, 1978; Godley, 1979). A further point of interest has been the origin of coloured flowers in several genera on the subantarctic islands that are represented by white-flowered species on mainland New Zealand. Hypotheses for the origins of these coloured subantarctic species have suggested that these taxa (a) represent ancestral and non-adaptive forms (Wardle, 1978); (b) evolved recently following dispersal from remote sources (Lloyd, 1985). Currently the origin of these forms and the selective regime that has created or maintains them is unknown.

1.4 HYPOTHESES CONCERNING THE ORIGINS OF THE ALPINE FLORA

The apparent contradiction between the recent development of mountains and the diversity of the alpine flora has led to debate over the origins of these plant lineages in New Zealand (Dawson, 1971). This section outlines several hypotheses that have been proposed to explain the origins of the New Zealand mountain flora.

1.4.1 A long history in New Zealand

Cockayne (1928) proposed that the core of the current mountain flora was descended from groups that had inhabited alpine habitats formed during the initial uplift of New Zealand. He suggested that wide environmental tolerances allowed these taxa to survive the unfavourable Cretaceous and Tertiary environments in open habitats where forest vegetation had failed to establish. These taxa later radiated into the alpine niches formed during the Pleistocene (Cockayne, 1928). Despite the capacity of some alpine groups to adapt to lowland and coastal habitats this hypothesis remains doubtful. Given the rapid erosion of New Zealand's early Cretaceous mountains and the evolutionary history of herbaceous flowering plants it seems unlikely that an angiosperm flora, from which the current alpine flora could be derived, would have been present in these earlier alpine habitats (Fleming, 1962; Dawson, 1988).

The hypothesis that the modem alpine flora evolved from cool temperature ancestors that survived the Cretaceous and early Tertiary in the general New Zealand region was further developed by Wardle (1963, 1968, 1978). Wardle (1963) proposed that during the Tertiary, a southern extension to New Zealand supported a cool adapted flora that later gave rise to the modern alpine flora. However, there is no geological evidence to suggest that Tertiary New Zealand extended further to the south than at present. Although, if even small islands were present south of New Zealand these may have maintained a cool temperate flora from which some contemporary alpine plants may be derived (Fleming, 1962; Dawson, 1988). In the two later papers, Wardle (1968, 1978) suggested that despite warm climates and

lowered relief, diverse habitats capable of supporting cool temperate assemblages had existed in Tertiary New Zealand. This perspective was based on the presence of small, taxonomically isolated lineages – such as *Hectorella*, *Haastia* and *Rostkovia* – in the New Zealand alpine flora. Such groups were not expected to have arrived recently, based on their fossil record or present distributions, or to have rapidly changed environmental preferences. The observation that a number of these groups are restricted to apparently older soil types was interpreted as indicating that these taxa formed an ancient element (Wardle, 1968). Also of importance was the occurrence of alpine taxa on infertile, moist soils well below the subalpine zone. Wardle (1968) pointed to the palynological record as indicating similar habitats in Tertiary New Zealand and suggested that a cool temperate flora may have evolved in such environments.

Several authors have noted that lineages long present in the lowland forest flora of New Zealand have contributed to the modem alpine flora. They propose that alpine forms arose in response to changing environments following the onset of mountain building and climate change in the late Pliocene (Smith, 1986; Dawson, 1988). Several predominantly lowland forest genera – such as *Coprosma*, *Hebe*, *Myrsine* and *Pittosporum* – have subalpine or alpine derivatives, some having evolved highly modified morphologies. That taxa apparently derived from lowland ancestors form only a small component of the New Zealand alpine flora has been variously attributed to the young age of the mountains, the ecology of forest habitats and the nature of glacial cycles in New Zealand (Smith, 1986; Markgraf *et al.*, 1995)

1.4.2 Recent dispersal from southern origins

An alternative perspective to those of Cockayne and Wardle suggests that Cretaceous and early Tertiary environments prohibited the survival of cold temperate taxa in New Zealand. Instead, such a flora was suggested to have been restricted to the significantly cooler climate of Antarctica, the current New Zealand alpine flora being derived from elements that dispersed from Antarctica during the Pleistocene (Fleming, 1962). High alpine genera – such as *Donatia*, *Haastia*, and *Hectorella* – would appear to be candidates for such an origin as it seems unlikely that the refugia envisaged by Wardle (1968) could have supported these taxa given their current environmental requirements for cold, high alpine climates (Dawson, 1988).

This hypothesis was questioned by Wardle (1968), who concluded that the absence of certain "key" groups from the subantarctic islands – suggested to be stepping stones for dispersal from Antarctic to mainland New Zealand – indicated that these taxa had been present in New Zealand during the Tertiary. Alternatively, Llyod (1985) has suggested that these islands may have been completely glaciated during the Pleistocene. This perspective suggests that plant groups now represented on

the subantarctic island are the result of late Quaternary dispersal events, rather than being relicts of an earlier Antarctic cool temperate flora.

1.4.3 Recent immigration of northern ancestors

While accepting that plant groups with "obscure phyletic relationships" may have origins in Tertiary New Zealand, Raven (1973) proposed that the ancestors of many New Zealand alpine groups dispersed recently from cool climate areas in the Northern Hemisphere. This hypothesis suggests that the alpine areas of south-east Asia and New Guinea – also uplifted during late Tertiary and Pleistocene – served as stepping stones for the dispersal of pre-adapted alpine lineages to Australia. Having reached the Australian mountains, the dispersal of these groups to the New Zealand alpine zone was mediated by West Wind Drift (Raven, 1973; Raven & Raven, 1976). The fossil evidence for the Tertiary radiation of sympetalous angiosperm groups, and the apparent difficulty of dispersal against the westerly winds, were interpreted as being consistent with this explanation of New Zealand alpine biodiversity (Raven, 1973).

Several authors have supported the suggestion that dispersal has been important for the formation of the New Zealand alpine flora. The significance of Northern Hemisphere groups in the mountain flora has been dicussed by Dawson (1963) – taxa with North Temperate affinities forming the largest component in this analysis. More recently, Pole (1994) and Macphail (1997) have argued for the importance of Australia as a source for the New Zealand flora. However, their view is more extreme. They assume that the erosion of the ancestral landmass during the Cretaceous and early Tertiary resulted in the complete inundation of New Zealand for at least part of the Oligocene. Consequently, all extant New Zealand plant lineages are suggested to have dispersed from Australia since the renewed uplift of land began in Miocene time. This conclusion follows from observations that in New Zealand there is a poor correlation between extant plant species and those from the fossil record of the early Tertiary. Pole (1994) and Macphail (1997) argue that, if the current flora is truly of ancient origin, one must expect extant or morphologically similar forms to be present in the Tertiary fossil record of New Zealand.

1.5 OUTLINE OF THE THESIS

The focus of this investigation has been to test hypotheses on the origins and diversification of New Zealand alpine plants. Molecular phylogenetic data have been determined for two large, predominantly alpine groups – the genus *Myosotis* and the Australasian apioid Umbelliferae. These groups were selected primarily because, as will be discussed, the specific hypotheses about their origins are quite different.

Additional factors also favoured the use of these two groups (a) they have radiated widely in the New Zealand alpine zone; (b) they both have representatives found outside of New Zealand. These features allow tests of diversification pattern and dispersal versus vicariance to be formulated. For *Myosotis* and the austral Apioideae both chloroplast and nuclear loci were characterised by DNA sequencing – as differing modes of inheritance are expected for these two genomes this approach may provide insights into complex patterns of relationship. The internal transcribed spacers (ITS) regions of the nuclear ribosomal subunit were sequenced in both plant groups. This marker has been used at various taxonomic levels, from family to population, and is currently the only nuclear marker widely used at lower taxonomic levels in plant molecular systematics (Baldwin *et al.*, 1995; Soltis & Soltis, 1998). Four chloroplast markers were characterised in this investigation – the *matK* gene, *ndhF* gene, *psbA-trnK* intergenic spacer and the novel JSAD locus. These regions were selected as previous studies suggested that these markers might be phylogenetically useful at the species level (Lockhart *et al.*, in press). Two additional investigations were performed for *Myosotis*. Firstly, a small morphological data set was compiled to investigate the relative levels of morphological diversity in the Australasian and Northern Hemisphere groups. Secondly, a preliminary attempt was made to locate fast evolving regions of the *Myosotis* genome that may be useful for studying the relationships between the New Zealand taxa.

A number of phylogenetic analyses were performed on the DNA sequence data for *Myosotis* and the austral Apioideae – these included both standard (e.g. maximum parsimony and quartet puzzling) and novel (e.g. split-decomposition) analysis methods. This approach was expected to provide a more rigorous method of evaluating the evolutionary relationships between taxa with differing levels of genetic divergence and complexity. The results from this work, along with those from other recent molecular investigations on alpine groups, are presented as a contribution towards understanding the evolution of the New Zealand alpine flora.

CHAPTER 2: MATERIALS AND METHODS

2.1 PLANT MATERIAL AND COLLECTION

The plant material used for the investigations in this thesis was collected directly from natural populations, either by the author or a collaborator, or were from existing herbarium specimens when it was not possible to make field collections. In all cases a minimal amount of material was collected so as to reduce the impact on the individual or population being sampled or to reduce damage to the herbarium specimen.

2.1.1 Sources and collection of *Myosotis* and outgroup taxa tissue samples

For the *Myosotis* species and associated outgroup taxa (*Borago*, *Echium*, *Myosotidium*, *Plagiobothrys* and *Symphytum*) which were collected in the field, tissue samples were usually taken from 2-3 individuals within the population being examined. Usually 3-5 leaves were removed from each individual although, less frequently, inflorescences were also collected for DNA extraction. The tissue sample for each individual was transferred to a small re-sealable plastic bag or vial which contained approximately 15 g of self-indicating silica gel (6-8 mesh, BDH) to rapidly dry the tissue, and in this way preserve the DNA (Chase & Hills, 1991). If the taxon had large or leathery leaves, the leaf sample was gently torn or cut into smaller pieces. Slower dehydration of plant tissue, over a period of greater than 24 hours, usually resulted in degradation of the DNA and hence less effective extraction. A herbarium voucher specimen was also collected where appropriate. Taxa collected in the field and used in these investigations are listed in Appendix I.

It was not possible to make field collections for a large number of *Myosotis* species found outside of New Zealand. However, numerous herbarium samples for taxa that occur overseas were kindly provided by Professor Dr. Jurke Grau (Staatsherbarium München [MSB]) making it possible to greatly broaden the geographic scope of this project. The tissue samples usually consisted of a few leaves either loose or associated with a short stem – where possible an inflorescence was also included. Tissue for three additional overseas taxa, *Myosotis vestergrenii* (Missouri Botanical Garden Herbarium [MO]), *M. australis* (New Guinea; Manaaki Whenua, Landcare Research Herbarium [CHR]) and *M. exarrhena* (Australian National Botanical Garden Herbarium [CBG]) was also collected from herbaria. Taxa sampled in this way and included in this work are also listed in Appendix I.

2.1.2 Sources and collection of *Aciphylla*, *Anisotome*, and *Gingidia* tissue samples

Plant samples of *Aciphylla*, *Anisotome*, and *Gingidia* collected in the field were preserved as described in the previous section. Tissue samples for two Australian taxa, *Aciphylla glacialis* and *A. simplicifolia* were provided by Tristan Armstrong (Australian National University, Canberra) and *Anisotome procumbens* was collected by Gregory Jordan (University of Tasmania, Hobart). To increase the range of taxa, material was also collected from herbarium specimens. The Landcare Research Herbarium supplied tissue for a number of New Zealand species of *Aciphylla*, *Anisotome* and *Gingidia*. A list of taxa sampled for this study is presented in Appendix II.

2.2 NUCLEIC ACID EXTRACTION

All tissue samples were processed in an identical fashion – using a protocol modified from Doyle & Doyle (1990) – whether collected directly from the field or obtained from herbaria. Prior to processing the dried plant samples, it was necessary to prepare a tool for grinding the tissue. A glass pasteur pipette (Volac) was heated in a bunsen flame, close to where the pipette narrowed, until the two sections separated. The thicker section of the pipette was again passed through the flame to create a small bulb at the end that could be used to grind the frozen tissue sample in a 1.6 mL microcentrifuge tube. One tool was prepared for each sample to be processed.

A small amount (100-200 mg) of dried tissue was transferred, using clean tweezers, to a fresh 1.6 mL microcentrifuge tube (Axygen Scientific). The tube was then suspended in liquid nitrogen to rapidly freeze the dried tissue fragments. This was usually accomplished by submerging the lower portion of the microcentrifuge tube in the liquid nitrogen whilst holding the cap with forceps. Once the contents were frozen, 1-2 minutes, the tube was removed from the liquid nitrogen and the tissue sample crushed using the glass tool. In order to thoroughly disrupt the plant material it was usually necessary to dislodge the sample from the bottom of the tube, by gentle agitation, and refreeze the sample before continuing to grind. A 600 µL aliquot 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]) was added and mixed with the finely ground tissue using the glass tool and gentle agitation. The tube was then placed in a heating block at 65°C, and incubated for approximately 30 minutes, with occasional inversion and gentle agitation. The half-hour incubation period appeared to

optimise nucleic acid yield while minimising the effects of chemical reduction and degradation on the DNA.

Following incubation, the tube was allowed to cool for approximately 5 minutes, so that the temperature of the solution dropped below 65°C. A 600 µL aliquot of chloroform was then added to the extraction mixture. Addition of chloroform without the cooling step resulted in the chloroform boiling and a large reduction in nucleic acid yield. The two solutions were mixed by repeated inversion and then left to stand for 2-3 minutes to allow separation of the aqueous and organic phases. The tube was then briefly centrifuged at 5000 × g, to completely separate the phases and compact the interface. After centrifugation a wide bore pipette was used to transfer the upper aqueous phase of the extraction to a clean 1.6 mL microcentrifuge tube. It was important to use a wide bore tip, or a narrow bore tip which had the lower 5 mm removed, at this and all later stages of the extraction protocol, so as to minimise possible mechanical shearing of the DNA due to pipetting. To the collected aqueous solution was added 600 µL isopropanol; the two solutions were mixed by gentle inversion and the tube incubated on ice for 10-15 minutes. At this point the nucleic acids appeared either as a brownish-white, mucus-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 the lower two thirds of the solution, including any precipitates, was transferred to a clean 1.6 mL tube using a wide bore pipette tip. Approximately 600 µL of 80% ethanol was added to the microcentrifuge tube. The tube was then very gently rocked to mix. It was important at this stage not to agitate the tube too vigorously, as the DNA was prone to collapsing on itself due to the pigment proteins still associated with it. If this did occur, it became very difficult to clean the DNA any further or to later resuspend the DNA.

The appearance of a precipitate at this stage dictated the procedure that was used to further clean the DNA. If the DNA had remained as one or a few large aggregates then these were collected using a wide bore pipette tip and transferred to a clean 1.6 mL microcentrifuge tube which contained 600 µL 80% ethanol. The tube was then gently inverted to thoroughly wash the DNA. The precipitated DNA was transferred to a clean tube containing fresh 80% ethanol a further two times. With each wash it was possible to agitate the tube with increasing vigour as progressively more contaminants were removed from the DNA. After three or four ethanol washes the DNA was usually white or only slightly coloured and appeared more or less fluffy in texture. The tube was then briefly centrifuged to pellet the DNA, the supernatant discarded and the pellet allowed to air dry or dried, under vacuum, using a Speed Vac Concentrator (Savant). Each pellet was finally resuspended in 20-50 µL 10:1 Tris EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

Alternatively, if the DNA had fragmented into a large number of small particles it was necessary to briefly centrifuge the tube at 3000-4000 × g in order to collect as

much of the DNA as possible. The centrifugation was repeated if the DNA fragments had not adhered to the walls of the tubes, although it was very important not to centrifuge the tubes too strongly at this stage. If large amounts of pigment were associated with the DNA pellets these were subsequently hard to remove. Following centrifugation the supernatant was discarded and a 600 µL aliquot of 80% ethanol was added to the collected DNA. The tubes were gently flicked and inverted to dislodge the DNA from the walls of the tubes. The centrifugation and washing procedure was repeated a further three or four times depending on the amount of pigment associated with the pelleted DNA following the initial spin. After the final ethanol wash the supernatant was discarded. The pellets were then either allowed to air dry or were dried, under vacuum, using a Speed Vac Concentrator. Again the DNA pellet was finally resuspended in 20-50 µL 10:1 TE buffer.

Once completely resuspended, a 5 µL aliquot of each solution was electrophoresed as described in Section 2.3.

2.3 AGAROSE GEL ELECTROPHORESIS

Aliquots of each sample, either extracted DNA or amplification products, were combined with 1-2 µL of 10× loading buffer (27.5% [w/v] Ficoll Ty 400 [Pharmarcia], 0.44% [w/v] bromophenol blue [Serva] and 0.44% [w/v] xylene cyanol [Sigma]) and the mixture loaded onto a 1% (w/v) Seakem LE agarose (FMC BioProducts)/1× Tris acetate EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.0]) gel. Either the 1Kb or 1Kb plus DNA ladder (both from Gibco BRL) were routinely included on electrophoretic gels as size standards. Samples were electrophoresed at approximately 3-5 V/cm in 1× TAE buffer. Following electrophoresis the DNA samples were visualised by ethidium bromide fluorescence on a UV transilluminator (wavelength 302 nm, UVP Incorporated) and a digital photograph of the illuminated gel taken using a video camera (Panasonic) and ImagePC software (Scion).

2.4 GENOMIC DNA PURIFICATION FOR THE POLYMERASE CHAIN REACTION

For a number of DNA samples isolated from herbarium specimens an additional purification step was required in order to amplify the target loci using the polymerase chain reaction (PCR). This involved the separation of high molecular weight DNA from possible contaminants in the extracted solution by gel electrophoresis. Aliquots of 10-15 µL extracted DNA solution were mixed with 1-2 µL 10× loading buffer and loaded on to a 1.5% (w/v) NuSieve GTG agarose (FMC BioProducts)/1× TAE buffer gel. In

order to avoid possible contamination between DNA samples, either during loading or at later stages, samples were loaded only into every second lane. A lane was also left unused between the 1Kb plus DNA Ladder, loaded as a size standard, and the adjacent sample lanes. Samples were then electrophoresed at 3-5 V/cm in 1× TAE buffer. Following electrophoresis, the DNA was visualised by ethidium bromide fluorescence on a UV transilluminator. To ensure that the gel did not come in contact with any DNA products on the surface of the transilluminator it was first covered with a clean layer of clear plastic film. The high molecular weight DNA band for each sample was excised using a sterile scalpel blade and each gel slice transferred to a clean 1.6 mL microcentrifuge tube. To each tube was added a volume of Milli-Q H_2O equal to the weight of the agarose slice in milligrams. The tubes were then placed at 65°C for 3-5 minutes to melt the agarose. DNA samples purified in this way were stored at 4°C until use.

2.5 AMPLIFICATION OF DNA MARKERS BY PCR

2.5.1 Double-stranded PCR amplifications of DNA marker loci using Q-solution

Multiple sample amplifications of marker loci were regularly carried out in 0.2 mL reaction tubes (Sorenson BioScience) using PCR. Each reaction mixture contained 1× Q solution (Qiagen), 1× PCR buffer (Tris-HCl, KCl, $[NH_4]_2SO_4$, 1.5 mM $MgCl_2$, [pH 8.7 at 20°C]; Qiagen), 250 µmol of each deoxy-nucleosidetriphosphate (dNTP; Boehringer Mannheim), 10 pmol 3' primer, 10 pmol 5' primer, and 1 U of *Taq* DNA polymerase (5 U/µL; Qiagen) in a final amplification volume of 20 µL including template. The combinations of oligonucleotide primers used for PCR amplification of specific marker loci are detailed in Table 2.1 and primer sequences are provided in Appendix III. To minimise the possibility of cross-contamination between reactions when preparing multiple amplifications the DNA template was always added last. When extracted DNA solutions provided the template for PCR, 10-100 ng of total cellular DNA was routinely used. Typically this involved using a 1 µL aliquot of the DNA solution diluted ten-fold in Milli-Q H_2O. If the DNA had been gel purified it was necessary to melt the diluted agarose slice, at 65°C, prior to addition to PCR reactions. A 4 µL aliquot of melted agarose was used as template. In addition to the sample tubes a negative control, containing 1 µL Milli-Q H_2O (i.e. no DNA), was always amplified with each reaction set. Thermocycling was carried out in either a MJ Research PTC-200 DNA engine or MJ Research PTC-150 Minicycler both of which had heated lid attachments that prevented evaporation of the reaction mixture. The PCR programs used for the amplification of the specific DNA loci are detailed in Table 2.1. All the thermocycling programs included an initial hold for 2 minutes at 94°C to

TABLE 2.1
Oligonucleotide primers and thermocycling conditions used in the PCR amplification of DNA marker loci

DNA locus	Oligonucleotide primer pair	Thermocycling conditions
3' region of the *matK* locus (chloroplast)	3' primer tmK3R 5' primer tKMY2F	initial denature 2 minutes at 94°C then 35 cycles : 1 minute at 94°C 1 minute at 50°C 2 minutes at 72°C final extension 5 minutes at 72°C indefinite hold at 4°C
Noncoding *psbA-trnK* intergenic spacer (chloroplast)	3' primer PSBAR 5' primer tmK3F	initial denature 2 minutes at 94°C then 35 cycles : 1 minute at 94°C 1 minute at 50°C 1 minute at 72°C final extension 5 minutes at 72°C indefinite hold at 4°C
JSAD locus (chloroplast)	3' primer AFLP151 or 151A 5' primer JSAD3F	initial denature 2 minutes at 94°C then 35 cycles : 1 minute at 94°C 1 minute at 45°C 3 minutes at 72°C final extension 5 minutes at 72°C indefinite hold at 4°C
Internal Transcribed Spacer (ITS) locus (nuclear)	3' primer ITS5 5' primer ITS4	initial denature 2 minutes at 94°C then 35 cycles : 1 minute at 94°C 1 minute at 48°C 1 minute at 72°C final extension 5 minutes at 72°C indefinite hold at 4°C
5' region of *ndhF* locus (chloroplast)	3' primer ND972F or ND1318F 5' primer ND2110R	as for the 3' region of the *matK* locus

completely denature the double stranded DNA templates and a final 5 minute extension time at 72°C to ensure that the reaction products were fully extended. The ramping rate, the speed at which the thermocycler moves between temperatures, was the default value for the PCR machines (approximately 1-2°C/second) except during amplification of the JSAD locus when this was limited to 1°C/second.

Following thermocycling, a 2 µL aliquot of each amplification was electrophoresed on a 1% (w/v) LE agarose/TAE gel as described in Section 2.3 to determine the efficacy, by comparison to the DNA ladder, of individual reactions. Amplifications which produced a large amount of the target fragment were purified and sequenced (see Sections 2.6 and 2.8). However if the target locus had amplified only weakly, as determined by gel electrophoresis, it was necessary to attempt to amplify the target locus using one of a number of approaches as described Sections 2.5.2 and 2.5.3.

2.5.2 Semi-nested PCR amplifications of chloroplast *matK*, JSAD and *ndhF* loci

When the chloroplast *matK*, JSAD or *ndhF* fragments amplified only weakly it was necessary to use a semi-nested PCR approach to amplify the target region. Semi-nested PCR uses one of the original amplification primers and a second primer, with a binding site within the target fragment, to amplify a portion of the desired locus. To amplify the full target fragment using a nested approach it was necessary to prepare a number of reaction sets, each with a different combination of PCR primers. Reaction mixtures were prepared, as described in Section 2.5.1, the combinations of oligonucleotide primers used in these amplifications are outlined in Table 2.2. Primer sequences are presented in Appendix III. When the extracted DNA solution had been used for the initial PCR amplification of either the *matK* or *ndhF* locus a 1 µL aliquot of a ten fold dilution, in Milli-Q H_2O, of first round reaction was added as template to the nested amplifications. However for second round amplifications of the JSAD region, a 1 µL aliquot of the first round reaction product was used undiluted. A 1 µL aliquot of neat first round product was also used in subsequent rounds if the initial DNA extraction had been gel purified. Two control reactions were prepared with second round amplifications. The first a simple water blank, contained 1 µL Milli-Q H_2O. A re-amplification of 1 µL first round negative, either a ten fold dilution or undiluted depending on the samples, was performed as a second control. Use of double control amplifications provided confirmation that both rounds of PCR were free from contamination. The reaction mixtures were then thermocycled as described in Table 2.2. Following thermocycling, the efficacy of the amplifications was determined, as previously, by electrophoresing a 2 µL aliquot of each reaction on a 1% (w/v) LE agarose/TAE gel.

2.5.3 Double-stranded DNA amplification of the nuclear ITS locus using Enhancer solution

For a number of DNA samples extracted from herbarium samples it was necessary to use Enhancer solution in order to amplify the ITS locus. As before, PCR was carried out in 0.2 mL reaction tubes with amplification volumes of 20 µL. Cocktails contained 2× Enhancer solution (Gibco BRL), 1× PCR buffer (Qiagen), 50 mM $MgCl_2$, (Qiagen), 250 µmol of each dNTP (Boehringer Mannheim), 10 pmol primer ITS4, 10 pmol primer ITS5, and 1 U of *Taq* DNA polymerase (Qiagen) per reaction. To the reaction mixtures was added 10-100 ng of the appropriate genomic DNA and a negative control was also included with each reaction set. Thermocycling was as described for the nuclear ITS locus in Table 2.1. A 2 µL aliquot of each PCR amplification was electrophoresed on a 1% (w/v) LE agarose/TAE gel as described in Section 2.3 to determine the efficacy, by comparison to the DNA marker, of the PCR.

TABLE 2.2
Oligonucleotide primers and thermocycling conditions used in the nested PCR amplification of DNA marker loci

DNA locus	Oligonucleotide primer pairs	Thermocycling conditions
3' region of *matK* locus (chloroplast)	3' primer trnK3R 5' primer tKMY1F or tKMY1FB AND 3' primer trnK3AR 5' primer tKMY2F	initial denature 2 minutes at 94°C then 35 cycles : 1 minute at 94°C 1 minute at 50°C 2 minutes at 72°C final extension 5 minutes at 72°C indefinite hold at 4°C
5' region of *ndhF* locus (chloroplast)	3' primer ND1318F 5' primer ND1762R AND 3' primer ND1656F 5' primer ND2110R	as for 3' region of *matK* locus
JSAD locus (chloroplast)	3' primer 151A 5' primer REANA AND 3' primer 151B 5' primer REMYB AND 3' primer 151C 5' primer JSAD3F	as for 3' region of *matK* locus

2.6 PURIFICATION AND QUANTIFICATION OF PCR AMPLIFIED FRAGMENTS FOR AUTOMATIC SEQUENCING OR CLONING

Routinely PCR amplified products from single amplifications were purified using the QIAquick PCR Purification Kit (Qiagen) or alternatively the CONCERT Rapid PCR Purification System (Gibco BRL) both of which utilise spin columns to remove unincorporated primers, dNTPs and other reagents from the amplified fragments. The manufacturers protocols were followed although minor modifications were made to the elution procedures, as outlined. When using the QIAquick PCR Purification Kit the final elution was carried out using 30 µL Milli-Q H_20 and the column assembly was stood for 2-3 minutes before centrifuging for 2 minutes at 10000 × g. A 30 µL aliquot of heated Milli-Q H_20, as described in the manufacturers protocol, was used to elute amplified fragments when using the CONCERT Rapid PCR Purification System. The cartridge/collection tube assembly was again allowed to stand for 2-3 minutes before centrifugation at 10000 × g for 3 minutes. Occasionally it was necessary to pool several amplifications so that the concentration of PCR product after purification would be high enough for DNA sequencing. In these cases, an appropriate volume of the binding solution was added to each reaction tube and the contents of the tubes then loaded into a single spin column before proceeding as above.

Following purification, a 2 µL aliquot of each cleaned amplification product was quantified by agarose gel electrophoresis as described in Section 2.3. Included on electrophoretic gels was an aliquot of Low DNA Mass Ladder (Gibco BRL). The concentration of the purified PCR fragments was estimated by comparison to the Low DNA Mass Ladder.

2.7 CLONING OF PCR PRODUCTS

PCR amplified fragments were cloned in several circumstances. When direct sequencing of purified nuclear ITS PCR products indicated that a taxon may have multiple forms of this locus, cloning of products allowed the forms to be characterised separately. Cloning of Amplified Fragment Length Polymorphism (AFLP) derived bands was performed for similar reasons. Amplification of the excised AFLP bands may have resulted in more than one product of a similar size being generated. If this occurred direct sequencing would be impossible, but cloning enabled single fragments to be examined. The *matK* locus of several taxa initially proved difficult to sequence presumably due to a lack of primer specificity. Cloning these PCR products provided a simple means to sequence them.

2.7.1 Blunt-end ligation of purified PCR products into the pGEM-T vector or pGEM-T Easy vector

The PCR products used for cloning were purified by spin column as described in Section 2.6 or Section 2.12.3. Ligation reactions were carried out in 1× T4 DNA ligase buffer (30 mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 1 mM ATP; Promega), 1 U T4 DNA ligase (Promega), 50 ng pGEM-T or pGEM-T Easy vector (both from Promega) and 20-40 ng purified PCR product in a total volume of 10 µL. The amount of PCR amplified fragment added was optimised for each product, as described in the manufacturer's protocol, as this depends on fragment length. Prepared reaction tubes were agitated and then briefly centrifuged to collect the contents at the bottom of the tubes. Ligations were incubated overnight at 4°C and were left at that temperature until use later the following day.

2.7.2 Preparation of selective agar plates

Growth of host bacteria under a selective regime identifies groups of cells that have been transformed with a plasmid containing an insert. The technique used an antibiotic, Ampicillin, to select for those cells which were maintaining the vector and a blue/white colour difference to distinguish those that contained a plasmid with an insert. For colour selection host cells are grown on media containing 5-bromo-4-chloro-

3-indolyl β-D-galactopyranoside (X-gal) and isopropylthiogalactoside (IPTG). The enzyme β-galactosidase, which is encoded by the vector and induced by IPTG, metabolises X-gal thereby staining bacterial colonies blue. Activity of this enzyme is destroyed by insertion of a PCR fragment, resulting in a white colony.

Luria-Bertani agar (1.5% [w/v] bacteriological agar, 1% [w/v] tryptone, 1% [w/v] NaCl, 0.5% [w/v] yeast extract) was prepared and autoclaved for 20 minutes at 103 Kpa. After sterilisation the medium was allowed to cool before being dispensed in approximately 30 mL aliquots to petri dishes. Agar plates prepared in this way were either used immediately or stored for up to 3 months at 4°C. If previously stored plates were to be used, these were stood on the bench for approximately an hour to allow them to reach room temperature. Immediately prior to use each plate was supplemented with Ampicillin to 100 µg/mL (Sigma), 0.3% (w/v) X-gal (Sigma) and 6 mM IPTG (Sigma). The three solutions were aliquoted on to each plate and then distributed over the agar surface using a glass spreader. The plates were then left at room temperature for approximately 30 minutes before use.

2.7.3 Transformation of ligated vector and insert into E. coli cells and plating out of cells

Either MAX efficiency DH5∝™ or subcloning efficiency DH5∝™ competent cells (both from Gibco BRL) were used as hosts for the pGEM-T vectors. In general the manufacturers protocol was followed; however several modifications were made, as outlined below.

The stock of competent cells, which was stored at -80°C, was thawed on ice prior to use. A 30 µL aliquot of competent cells was added to each of the ligation reactions, the solution gently agitated to mix, and the tube stood on ice for approximately 30 minutes. Following incubation the cells were heat shocked according to manufacturer's protocols (i.e. MAX efficiency DH5∝™ competent cells 45 seconds at 42°C and subcloning efficiency DH5∝™ competent cells 20 seconds at 37°C). Immediately after heat shock treatment the tubes were stood on ice for 2 minutes. A 260 µL aliquot of sterile Luria-Bertani (LB) medium (1% [w/v] tryptone, 1% [w/v] NaCl, 0.5% [w/v] yeast extract) was added to each tube. The tubes were then agitated at 225 rpm and 37°C for 1 hour. After incubation the contents of each tube was spread on to individual LB agar/Ampicillin/X-gal/IPTG plates prepared as described in Section 2.7.2. The plates were stood at room temperature for approximately 15 minutes to allow the surface to dry slightly before being inverted and incubated overnight at 37°C.

2.7.4 Isolation and culturing of transformant colonies

The following morning, plates were checked for white colonies and then placed at 4°C for several hours to promote further development of the colour reaction. Discrete white colonies were transferred to individual, sterile 15 mL screw top plastic tube (Nalgen NUNC International) which contained 4 mL LB medium and Ampicillin to 100 µg/mL, using a sterilised wire loop. Routinely 2-6 white colonies were cultured per agar plate. The culture tubes were then incubated overnight at 37°C with agitation.

2.7.5 Extraction of plasmid DNA from *E. coli* cell culture by the Rapid Boil technique

Plasmid DNA was routinely extracted using the Rapid Boil technique (Holmes & Quigley, 1981) with some modifications as follows. Using sterile technique, 1.5 mL of cell culture was transferred from the culture tubes to 1.6 mL microcentrifuge tubes. The microcentrifuge tubes were then centrifuged at 13000 × g and room temperature for 2 minutes, after which the supernatant was discarded. This process was repeated and, after discarding the supernatant, the cell pellets were resuspended in 400 µL HQ STET buffer (100 mM NaCl, 10 mM Tris- HCl [pH 8.0], 1 mM EDTA, 5% [v/v] Triton X-100 [Sigma]). A 30 µL aliquot of lysozyme (10 mg/mL in TE [pH 8.0]; Boehringer Mannheim) was added to each tube, the solutions mixed by agitation, and the tubes placed in a boiling water bath for 40 seconds. After boiling the tubes were centrifuged at 13000 × g and room temperature for 10 minutes. The cell debris, which appeared as a white mucus-like pellet, was removed from each tube using a clean sterile toothpick. The DNA was then precipitated by the addition of 50 µL 3M NaOAc (pH 5.2) and 500 µL isopropanol; the solutions were mixed by gentle inversion and allowed to stand at room temperature for 5 minutes. Tubes were again centrifuged at 13000 × g and room temperature for 10 minutes. After centrifugation the supernatant was decanted from each tube, taking care to retain the pelleted DNA. A 500 µL aliquot of 70% ethanol was added and the tubes inverted repeatedly to wash the DNA pellet. The tubes were then centrifuged as before but for only 3 minutes, after which the supernatants were again discarded. The pelleted DNA was either allowed to air dry or was dried, under vacuum, using a Speed Vac Concentrator. When dry each DNA sample was resuspended in 100-200 µL TE buffer.

Plasmid DNA isolated in this way was not suitable for direct DNA sequencing and it was necessary to perform an RNase digestion and phenol/chloroform extraction. To each DNA solution 5 µL ribonuclease A (2 mg/mL in Milli-Q H_2O, DNase free; Sigma) was added, the solutions mixed by inversion and the tubes incubated at 37°C for 10 minutes. Following incubation an equal volume of Tris-buffered phenol (pH 8.0) was aliquoted to each tube and mixed vigorously

by inversion. The tubes were then centrifuged at 13000 × g and room temperature for 5 minutes to separate the aqueous and organic phases and to compact the precipitated proteins at the interface. After centrifugation the aqueous phases were transferred to clean 1.6 mL microcentrifuge tubes, taking care to avoid the white precipitate at the interface. A half volume each of Tris-buffered phenol and chloroform was added to the tubes, the solutions were then mixed and centrifuged as before. A further solvent extraction was performed as described – however one volume of chloroform only was used. After the final chloroform extraction the aqueous layers were again transferred to clean 1.6 mL tubes. The DNA was precipitated by the addition of 1/10 volume 3M NaOAc (pH 5.2) and 2.5 volumes ice cold absolute ethanol. The solutions were mixed by gentle agitation and then incubated at –20°C for 2 hours. After incubation the tubes were centrifuged at 13000 × g and room temperature for 10 minutes to sediment the precipitated DNA. The supernatant was carefully discarded and a 500 µL aliquot of 70% ethanol was added to each tube. The tubes were inverted repeatedly to wash the DNA pellet and then centrifuged at 13000 × g and room temperature for 3 minutes. A second ethanol wash was performed, after which the ethanol solution was discarded and the pellets allowed to air dry or dried under vacuum. The dried DNA pellets were finally resuspended in 50 µL 10:1 TE and stored at 4°C until use. For long term storage, extracted DNA solutions were transferred to -80°C.

2.7.6 Characterisation of cloned inserts by restriction enzyme digestion

To confirm that PCR amplified fragments of the appropriate size had been successfully cloned, the plasmid DNA was cut with restriction enzymes to excise the insert. The digestion procedure was dependent on the vector used.

If the PCR fragments were originally ligated into the pGEM-T vector these were excised using a two-step digestion process. The first digestion, with *Apa* I, was carried out in 1.6 mL microcentrifuge tubes using a total volume of 25 µL. Reaction mixtures contained 1× SuRE/Cut™ buffer A (66 mM potassium-acetate, 33 mM Tris-acetate, 10 mM magnesium-acetate and 0.5 mM DTT; Boeringher Mannheim), 0.5 U *Apa* I enzyme (10 U/µL, Boeringher Mannheim), and 200-300 ng plasmid DNA. The reaction tubes were gently agitated, briefly centrifuged to collect the contents at the bottom of the tubes and then incubated at 30°C for 30 minutes. A 5 µL aliqout of *Pst* I enzyme reaction cocktail was then added. This mixture contained 1× SuRE/Cut™ buffer A, 0.6 M NaCl, 0.5 U *Pst* I enzyme (10 U/µL, Boeringher Mannheim). The tubes were again agitated and centrifuged before a further 30 minute incubation at 37°C. After incubation the reactions were placed on ice until electrophoresed.

When the pGEM-T Easy vector system was used excision of the cloned fragments required only a single digestion. Enzymatic reactions were performed in a reaction volume of 10 µL which contained 1× NEBuffer for *Eco* RI (50mM NaCl,

100 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$ and 0.025% [v/v] Triton X-100 [pH 7.9 at 25°C]; New England Biolabs), 10 U *Eco* RI (10 U/µL, New England Biolabs), and 200-300 ng plasmid DNA. Reaction tubes were gently agitated, briefly centrifuged to collect the contents at the bottom of the tube and then incubated at 37°C for 30 minutes. After incubation the reaction tubes were placed on ice until electrophoresis.

An aliquot of each digestion reaction equal to half the total reaction volume was combined with 1-2 µL 10× loading buffer and the mixture loaded onto a 1% (w/v) Seakem LE agarose/1× TAE gel. Sample electrophoresis, detection and photography were as previous described in Section 2.3. The size of cloned PCR fragments was determined by comparison to the DNA ladder. Clones that contained PCR fragments of the appropriate size were sequenced as described in Sections 2.8.

2.7.7 Characterisation of cloned inserts by PCR

As an alternative to the methods outlined in Section 2.7.4, 2.7.5, and 2.7.6, a PCR based approach was also used to screen transformant colonies. A region of the plasmid containing the insertion site was amplified directly from bacterial cells using specific PCR primers.

Reaction cocktails were prepared as described in Section 2.5.1 and contained 10 pmol M13 forward and 10 pmol M13 reverse primers (Promega). Clean 10 µL pipette tips were used to transfer cells from transformant colonies to reaction tubes. A negative control was also included with each reaction set. Thermocycling consisted of an initial hold at 94°C for 3 minutes to ensure cell lysis and to denature the double stranded DNA templates; followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 1 minute at 72°C. A final extension period of 72°C for 5 minutes was also incorporated. After thermocycling the reactions were held at 4°C. A 1-2 µL aliquot of each PCR amplification was electrophoresed on a 1% (w/v) LE agarose/TAE gel as described in Section 2.3 to determine the efficacy, by comparison to the DNA marker, of the PCR. Amplification products of the appropriate size (i.e. those identified as containing the required insert) were purified and sequenced as described in Sections 2.6 and 2.8 respectively.

2.8 DIRECT AUTOMATIC SEQUENCING OF DNA TEMPLATES

2.8.1 Automatic sequencing reactions

Purified PCR fragments and cloned amplification products were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). In this chemistry each dideoxy nucleoside is labelled with a different and specific fluorescent dye. Thus, when a dideoxy nucleoside is incorporated into an

extending fragment, the chain is simultaneously broken and labelled with a specific dye.

Automatic sequencing reactions were typically carried out using 25-45 ng of purified PCR product or 100–200 ng plasmid DNA in a total volume of 10 µL. For each reaction a 4 µL aliquot of Terminator Ready Reaction Mix (Perkin-Elmer) was dispensed to a labelled 0.2 mL reaction tube. To this was added 1.6 pmol of the appropriate primer, an appropriate volume of template and if necessary Milli-Q H_2O to bring the volume up to 10 µL. For automatic sequencing reactions, oligonucleotide primers were always used freshly diluted to a concentration of 1 pmol/µL from the 10 pM/µL working stock. Reactions were then thermocycled in a MJ Research PTC-200 DNA Engine or PTC-150 Minicycler using a program which consisted of 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. Ramping between temperature steps was limited to 1°C/second throughout the program. At the completion of thermocycling the reactions were held at 4°C.

Extension products were subsequently purified by ethanol precipitation. For each reaction 1 µL 3M NaOAc (pH 5.2) was added to a labelled 1.6 mL microcentrifuge tube. The entire 10 µL reaction volume was added, followed by a 25 µL aliquot of ice-cold absolute ethanol. The solutions were mixed by gentle agitation and the tubes incubated on ice for approximately 5 minutes before being centrifuged for 15 minutes at 13000 × g and 4°C. After centrifugation, the supernatant was carefully removed from each tube using an automatic pipette. A 700 µL aliquot of ice-cold 80% ethanol was added to each tube, which was then repeatedly inverted to rinse the pellet. The tubes were then centrifuged as before for 15 minutes. The ethanol was decanted and the tubes placed at 37°C for approximately 30 minutes. Dried extension products were either resuspended and electrophoresed immediately or stored at -20°C until required.

2.8.2 Electrophoresis of automatic sequencing reactions

Sequencing reaction products were electrophoresed at the Massey University Sequence Analysis Facility. The products were analysed using an Applied Biosystems 373A DNA Sequencing System, an electrophorectic detection system that automates the separation of labelled sequencing products and base determination. Extension products were loaded onto a single lane of a polyacrylamide gel and electrophoresed. An argon laser was used to excite the labelled fragments as they migrated past a fixed point on the gel. The resulting fluorescent emissions were collected and converted into DNA sequence information.

2.9 PHYLOGENETIC ANALYSIS OF DNA SEQUENCES

Phylogenetic analysis, typically describes the inference of evolutionary relationships between taxa or sequences. The process by which relationships are inferred from sequences can be characterised by several components: the data alignment, any required correction for unobserved changes under a substitution model, the tree selection criteria and the evaluation of the recovered tree or trees (Penny *et al.*, 1992). This section briefly reviews these components and describes analytical methods used in this investigation.

2.9.1 The data alignment

Phylogenetic analysis of sequence data is dependent upon positional homology between the sequences – the nucleotides at a given position in the DNA sequence should trace their ancestry to a single position that occurred in a common ancestor of those sequences. To create a sequence alignment that satisfies the requirement for positional homology it may be necessary to insert gaps of one or more nucleotides into certain sequences, these gaps corresponding to insertions or deletions (indels) (Morrison & Ellis, 1997). With increasing taxon sampling ensuring positional homology between sequences of different length may become very difficult.

The simultaneous alignment of multiple sequences is a non-trivial problem and it is not feasible to make such alignments when there are more than ten taxa. One solution is to break the sequence length into many small parts and align each section independently and simultaneously – an approach called "divide and conquer" (Stoye, 1998; http://bibiserv.TechFak.Uni-Beilefeld.DE/dca). Another approach, which again reduces the complexity of the alignment problem, is to use progressive alignment procedures that sequentially align pairs of DNA sequences. In such methods, the order in which sequences are aligned is based on some estimate of the amount of genetic divergence between the sequences. With the most similar sequences aligned first and more divergent ones added later. Whilst this approach allows multiple alignments to be constructed in polynomial time – that is, the time to complete the alignment increases linearly with the number of taxa – the alignment order is known to bias the outcome of subsequent evolutionary tree building. This issue has been shown to be particularly important when evolutionary distances between sequences are large and when indels are common (Lake, 1991; Lockhart *et al.*, 1996b). However, for closely related sequences the extent of alignment ambiguity is expected to be small and so this approach has been adopted in the present study. Irrespective of the alignment method used, comparative studies (Morrison & Ellis, 1997; Hickson *et al.*, 2000) have shown that it is usually necessary to visually inspect and question

the resulting alignment – this is important since current analytical methods infer positional homology based on similarity and not structural homology.

2.9.2 Tree reconstruction methods

Various methods have been developed for the reconstruction of evolutionary relationships, although these generally fall into two distinct categories – "algorithmic" or "optimality" methods (Swofford *et al.*, 1996). Outlined here are the methods used in the present study.

ALGORITHMIC METHODS. Algorithmic approaches to phylogenetic inference define a series of procedures, the algorithm, that lead to the clustering of taxa. Examples include UPGMA and neighbor-joining. Since these approaches do not evaluate alternative trees, they are computationally faster than methods which seek to find some overall best fit of the data to an evolutionary model (Swofford *et al.*, 1996).

Neighbor-joining (NJ) is a form of cluster analysis for distance data. However, unlike other clustering methods neighbor-joining is not constrained by a requirement for lineages to have evolved at equal rates and so this approach does not require a molecular clock. However, for the method to be consistent – that is, given enough data it will converge to the correct tree – this approach requires the data to be "additive", a condition that will only be met if the sequences have been appropriately corrected for unobserved changes. This method first produces an initial data matrix, which is normalised by adjusting the divergence between pairs of nodes by their average distance from all others. The tree is progressively assembled – first the two closest nodes are joined and this common point added to the data matrix replacing the terminal nodes and branches in the data matrix. This process is repeated until only two nodes and a single branch remain in the matrix (Saitou & Nei, 1987; Swofford *et al.*, 1996; Page & Holmes, 1998).

OPTIMALITY CRITERIA. These methods have two steps – the definition of an optimality criterion that is used to evaluate and compare alternative trees, and an algorithm for calculating the value of this criterion for each tree and searching amongst trees. Here the algorithm is a tool, allowing alternative phylogenies to be evaluated and ranked according to the specific criterion. However, although optimality methods offer advantages over algorithmic approaches they are computationally slower as they need to track much more information in evaluating support for phylogenetic trees (Swofford *et al.*, 1996). This problem is illustrated in Figure 2.1.

Searching for the optimal tree or groups of trees can be performed using either "exact" or "heuristic" methods. There are two exact searching strategies – "exhaustive" and "branch and bound" – and although these methods guarantee to

discover all optimal trees this may not be computationally feasible. For small numbers of taxa, typically less than ten, an exhaustive search can evaluate all possible trees. If the data set contains less than twenty taxa a "branch and bound" approach can be used. Rather than evaluating all possible trees this method progressively eliminates areas of the search space that can only contain suboptimal solutions. That is, if the value of the optimality criterion for a particular search path exceeds that of the current best tree then further exploration of this region is unnecessary – the addition of more taxa to the tree cannot possibly reduce the value of the optimality criterion. For data sets containing more than twenty taxa, heuristic approaches are employed to find the optimal tree. These generally operate by a "hill climbing" method – an initial tree is produced which undergoes progressive rearrangements in an attempt to improve the tree, rearrangements stop when no further improvements are possible under the defined criterion. Heuristic approaches cannot guarantee to find the optimal tree, but in simulation studies are known to perform well in recovering model evolutionary trees (Swofford *et al.*, 1996).

ALGORITHMIC METHODS

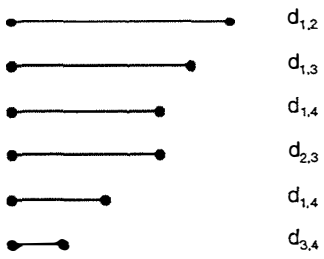
For distance methods the number of possible "paths" is given by:

$$\frac{n(n-1)}{2}$$

Where n = the number of taxa. So for,

4 taxa	6 paths
5 taxa	10 paths
6 taxa	15 paths

Examples of paths in a four taxon situation.



OPTIMALITY METHODS

Taxa	Possible patterns
A	1 1 0 0 0 1 1 1
B	1 0 1 0 0 1 0 0
C	1 0 0 1 0 0 1 0
D	1 0 0 0 1 0 0 1

For parsimony methods and two character state data the number of patterns to evaluate is given by:

$$\frac{2^n-(2n+2)}{2}$$

Where n = the number of taxa. So for,

4 taxa	3 patterns
5 taxa	10 patterns
6 taxa	25 patterns

For maximum likelihood, with two character states, the number of patterns to evaluate is given by:

$$2^{2n-5}$$

So for,

4 taxa	8 patterns
5 taxa	64 patterns
6 taxa	128 patterns

***Figure 2.1**: A comparison of the amounts of information evaluated by different phylogenetic methods.*

Maximum parsimony methods attempt to minimise the number of changes or mutations required to explain the observed data on a given tree (or trees, if there is more than one solution of equal length). The optimal solution is that which minimises the number of hypothesised events of parallelism or convergence that are required to fit a bifurcating evolutionary model onto that data. Although parsimony does not assume an explicit model of evolution, it does require certain assumptions before phylogenetic interpretation can be made. The process of sequence substitution needs to be simple and there should be few multiple changes in the data – if not then parsimony may become inconsistent (Swofford *et al.*, 1996; Steel *et al.*, 2000).

Maximum likelihood (ML) evaluates the probability of the observed data given a specified substitution model and evolutionary tree. That is, maximum likelihood attempts to identify the tree, or phlyogenetic hypothesis, which is most likely to have given rise to the data (Felsenstein, 1981; Swofford *et al.*, 1996). Most implementations require "stationary" substitution models that assume a uniform process across the underlying phylogeny. Sequence positions are expected to evolve identically in process – although perhaps at different rates – and independently (the "iid" assumption). The log-likelihood score for all possible trees is calculated at every sequence position, and the overall score for any tree is the sum of this value for all positions. The tree, or trees, with the best overall score are chosen as the optimal solution. Maximum likelihood methods perform well in simulations, although, like parsimony and other methods of phylogeny reconstruction they may also become inconsistent given the complexities of biological data (e.g. Lockhart *et al.*, 1996a; Steel *et al.*, 2000).

QUARTET METHODS. These methods evaluate all possible groups of four taxa, called "quartets", and the most well supported of these are then used to construct a tree or network structure. This approach to tree building may have elements of both algorithmic and optimality criterion methods. In "split-decomposition" algorithms work in polynomial time to both evaluate support for quartets and build graphs. In "quartet puzzling" optimality criteria are used to evaluate support for quartets and subsequently join the best quartets into a phylogenetic tree.

Split-decomposition provides a useful approach for examining data structure as it evaluates whether the data actually fit a "treelike", bifurcating evolutionary model. For this reason split-decomposition is expected to be helpful for testing biogeographic hypotheses in recently speciated plant groups (e.g. Winkworth *et al.*, 1999; Lockhart *et al.*, in press). This approach consists of three distinct steps. Initially, all quartets are evaluated, using either parsimony, distance or maximum likelihood, to identify the two most strongly supported unrooted trees. Typically, support for the possible unrooted trees is determined by a distance calculation, and is called the "isolation index". The two most strongly supported trees from every quartet (see Figure 2.2A),

each representing an internal "split" in the data, are said to be "weakly compatible" and are used to derive a "split system" which defines the relationships between all taxa in the data set. As some quartets in the split system will identify the same split (see Figure 2.2B), it is necessary to choose the isolation index value that will be used to define the branch length for that split. As currently implemented, a conservative approach is taken – the minimum isolation index value is used to represent a given split. The split system also contains splits that represent external branches in the final tree, the standard implementation also selecting the smallest isolation index value for such splits. However, this approach has been shown to over-represent external branch length. Therefore in the present study edge lengths were optimised using a least-squares function. Finally, the split system is displayed as a "splits-graph". This graphical representation will be treelike if the data has few incompatibilities, however, if there are numerous conflicts in the data, possibly as the result of convergence or reticulate evolution, the splits-graph will be a "boxlike" network (see Figure 2.2C). To aid in the interpretation of graphs, a fit statistic is also defined – this is simply the sum of splits in the graph divided by the sum of splits in the split system. Values range from 0% to 100% and will have a lower value if the branch lengths are a poor approximation of distances in the original distance matrix (Swofford *et al.*, 1996; Huson, 1998; Lockhart *et al.*, in press)

Quartet puzzling identifies, using a criterion such as maximum likelihood or parsimony, the best supported of the three possible unrooted trees for each quartet of taxa. A global heuristic search, which is suggested to work well in finding optimal trees, is then used in 1000 "puzzling steps" to determine the extent of compatible relationships in a final tree. The quartet puzzle scores for edges in such trees have an advantage over bootstrapping when the data shows relatively few substitutions. In quartet puzzling an edge will receive a high puzzle value – suggesting strong support for that edge – when it is compatible with the remaining data, even if there is only one nucleotide position which suggests the relationship. However, under the same conditions bootstrapping is likely to place low support on the same edge. As there are few patterns supporting the arrangement resampling the data set is unlikely to recover this site in a large number of replicates.

2.9.3 Evaluating the recovered tree(s)

In evolutionary tree building there are no tests for consistency, there are only tests for "convergence" (Penny *et al.*, 1992). Such tests indicate whether the topology of the reconstructed tree, made using a certain method, is expected to be stable, given more data of the type already analysed. Examples of such measures include "bootstrapping", "jackknifing" and tests of tree length difference.

Statistical resampling techniques, such as "bootstrapping" and "jackknifing", can be implemented to estimate the stability of an evolutionary tree by resampling the

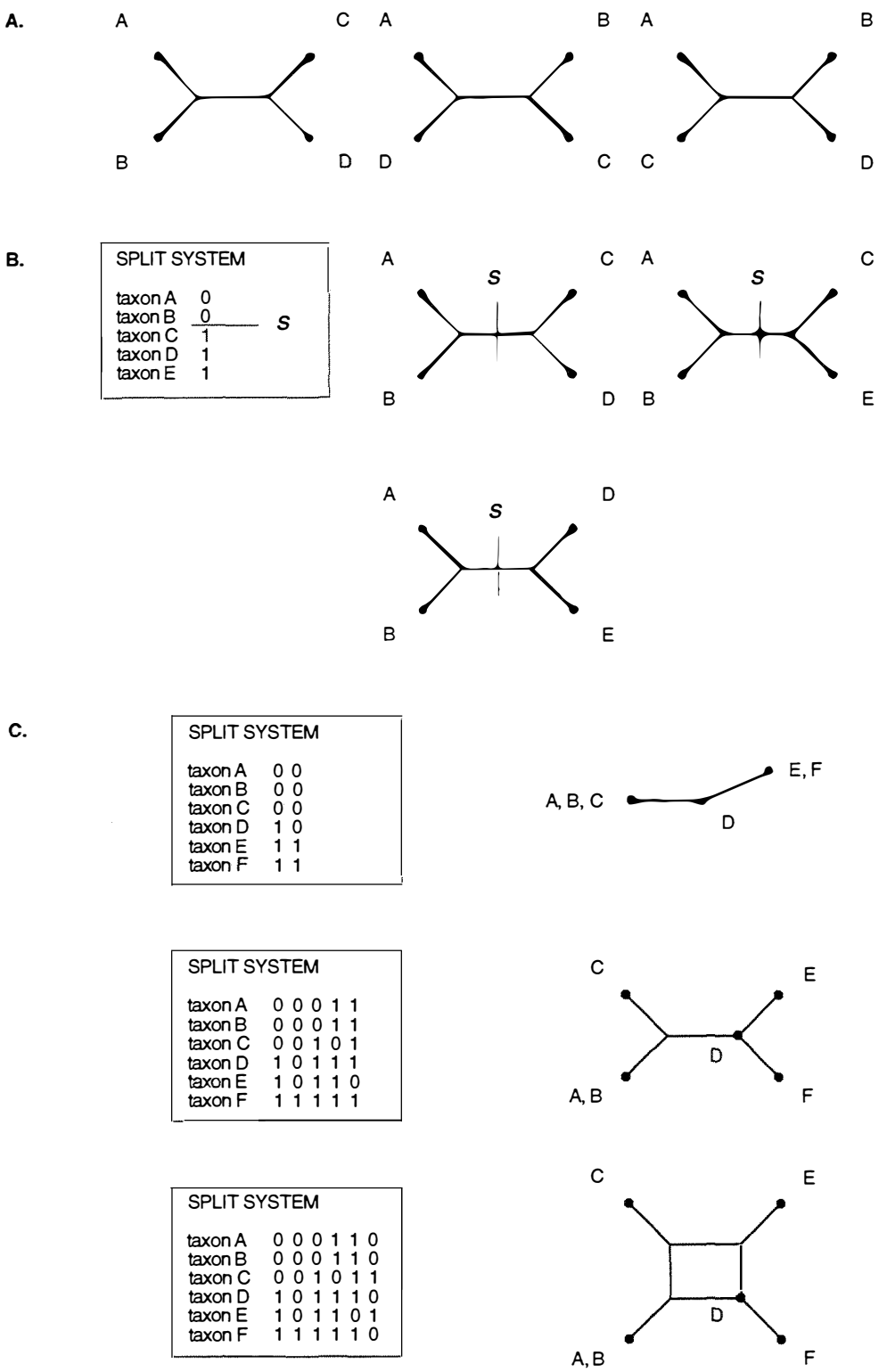


***Figure 2.2**: Split decomposition. **A**. Three possible unrooted trees for a single quartet, a fourth "star tree" with no internal edge is also possible. **B**. Examples of compatible splits. All trees consistent with split "S" in the split system. **C**. Examples of split systems and reconstructed splitsgraphs. Each of the split systems has been used to construct the corresponding splits-graph, however note that only the splits are represented, not the value of the isolation index for each split (modified from Lockhart* et al., *in press).

original data set (Swofford *et al.*, 1996). In general, bootstrapping can be defined as "sampling with replacement" – for evaluating phylogenetic trees, this involves randomly resampling the original data set, with replacement, to create multiple new data sets with the same number of characters as the original. A tree is produced from each of the replicate data sets, the properties of which are recorded and used to determine a consensus tree. On to the internal edges of the consensus tree are placed values that indicate the proportion of times a given edge was recovered from tree building on the replicate data sets. Bootstrapping does not establish that a given phylogeny is correct. Rather this approach provides a measure of the relative support in the data for a tree given a specified tree building procedure. High bootstrap values indicate that relatively large numbers of uncontradicted patterns support the arrangement and suggest that the addition of further data, with a similar structure, should not markedly alter the choice of optimal tree shape for the given tree building method. There are two possible explanations for low bootstrap values – either there are relatively few informative patterns, although these may be uncontradicted, which support the relationship or that similar numbers of conflicting pattern types are present in the data (Efron, 1982; Swofford *et al.*, 1996).

2.9.4 Molecular clock analysis

The suggestion that genes evolve at some constant rate, therefore allowing the estimation of divergence times by comparing molecular sequences – a "molecular clock" – has been controversial. It is clear that a universal molecular clock is unlikely; rate heterogeneity between gene regions and widely divergent lineages has been well documented. Differing rates of molecular divergence within genomes has been associated with differing selective constraints, while between lineage variation has been attributed to a number of lineage specific factors – such as generation time, metabolic rate and the number of germ-line DNA replications. Although a generalised molecular clock is unlikely, the existence of "local" molecular clocks has been advocated. That is, between closely related taxa with similar life histories and molecular processes, a consistent rate may be expected at an individual locus. (Hillis *et al.*, 1996; Page & Holmes, 1998)

One method available to test for the applicability of a molecular clock is the "likelihood ratio test". In this approach maximum likelihood trees are inferred for a data set both with and without the assumption of a molecular clock. The overall likelihood scores for the two trees are used to derive a measure of similarity, which is distributed as χ^2 with (n-2) degrees of freedom, where n is the number of sequences (Hillis *et al.*, 1996; Page & Holmes, 1998). Although the test is sensitive, it may be subject to violations of the evolutionary model specified. A more general test is the relative rates test described by Steel *et al.* (1996). This examines triplets of sequences and tests whether the distance between sequences on either side of a node are statistically

equivalent (see Figure 2.3). This procedure was used to investigate the substitution properties of the ITS region in the present work.

2.10 DNA FINGERPRINTING USING THE AFLP SYSTEM

The AFLP technology is a novel DNA fingerprinting technique that combines restriction fragment analyses and PCR amplification. The approach is illustrated in Figure 2.4.

2.10.1 Restriction enzyme digestion of genomic DNA

Genomic DNA was digested in a reaction mixture containing 50 mM potassium acetate (KOAc, Sigma), 10 mM magnesium acetate (MgOAc, Sigma), 10 mM Tris-HCl (pH 7.5), 4 U *Mse* I (New England Biolabs), 1 U *Eco* RI (New England Biolabs) and 150 –200 ng genomic DNA in a total volume of 25 µL. The reactions were incubated at 37°C for 3 hours. To ensure complete digestion the tubes were agitated and then briefly centifuged at intervals during the incubation. After incubation the tubes were heated at 70°C for a further 15 minutes to denature the restriction enzymes. Digestions were either used immediately or stored at –20°C until use. The efficacy of genomic DNA digestion was determined by gel electrophoresis: 5 µL aliquots of each digestion reaction were electrophoresed on 1% (w/v) Seakem LE agarose/1× TAE gel as previous described in Section 2.3. Typically, a smear of low molecular weight DNA, between 100 base pairs (bp) and 1.5 kilobase pairs (KB) was observed.

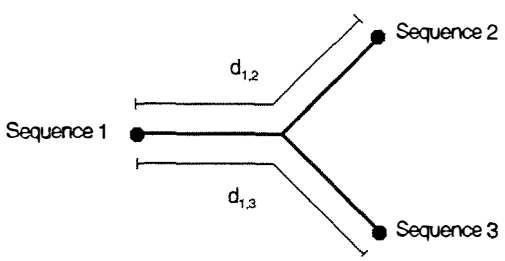


***Figure 2.3**: Path length comparison for the relative rates test of Steel* et al. *(1996). This procedure tests whether $d_{1,2}$ and $d_{1,3}$ are statistically equivalent, an assumption that is required for the existence of clock-like change between the three sequences.*

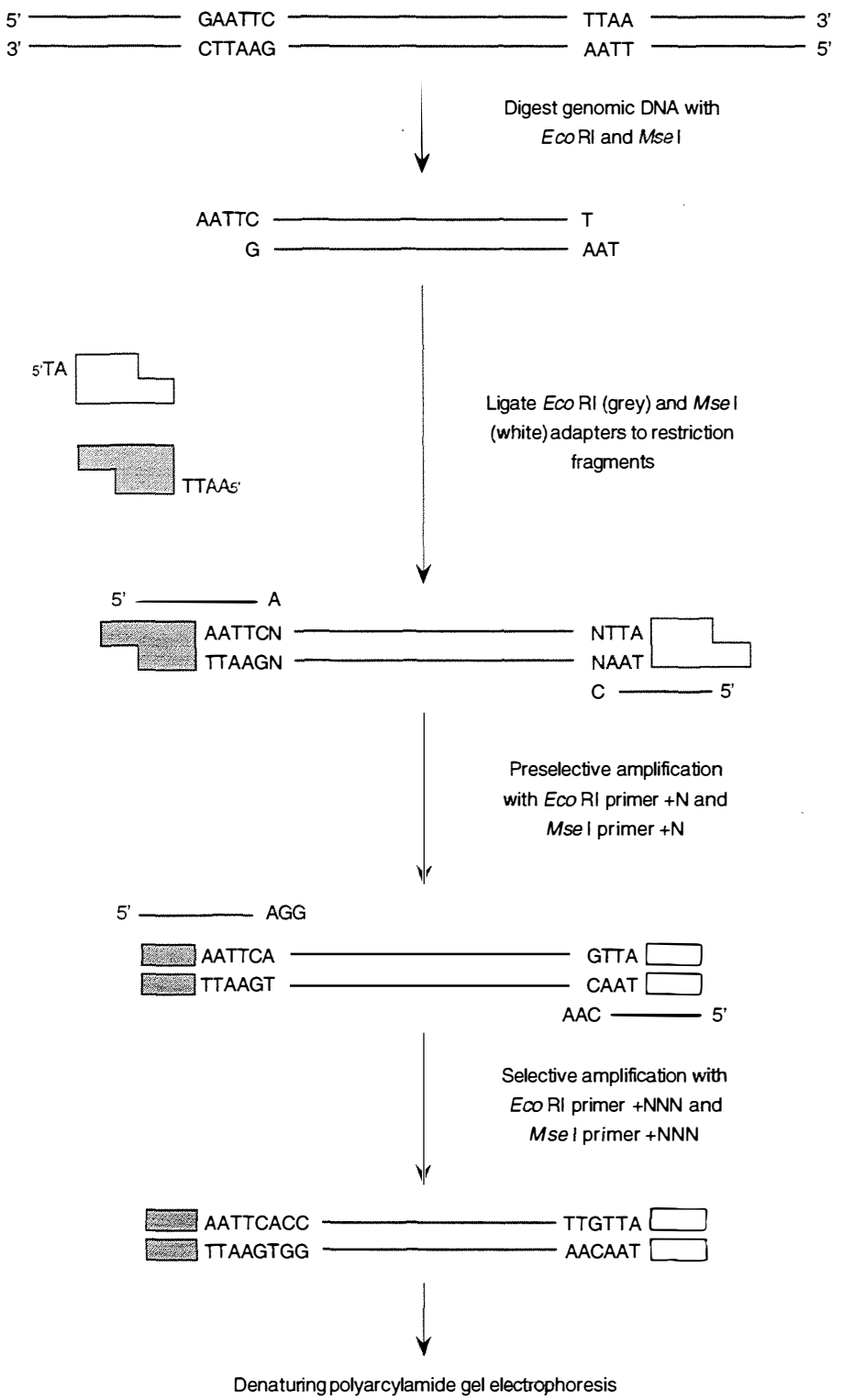


***Figure 2.4**: Example of the AFLP procedure using one primer pair (modified from the AFLP™ Analysis System I instruction manual [Gibco BRL])*

2.10.2 Preparation of oligonucleotide adapters

A pair of double-stranded adapters, which provided targets for the pre-selective and selective amplification primers, were prepared by annealing two single-stranded oligonucleotides (see Figure 2.4). A reaction cocktail was prepared for each type of adapter. The *Eco* RI adapter cocktail contained 45% (v/v) 10:1 TE buffer, 5 pmol *Eco* RI adapter I and 5 pmol *Eco* RI adapter II in a total volume of 100 µL. The *Mse* I adapter mixture consisted of 45% (v/v) 10:1 TE buffer, 50 pmol *Mse* I adapter I, and 50 pmol *Mse* I adapter II again in a total volume of 100 µL. Sequences of the oligonucleotides used to prepare AFLP adapters are given in Appendix IV. Both cocktails were incubated at 94°C for 4 minutes before being allowed to cool slowly, on the benchtop, to room temperature.

2.10.3 Addition of adapter sequences to genomic DNA fragments

Adapters were ligated to the digested genomic DNA fragments in a reaction volume of 20 µL containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM adenosine-5'-triphosphate (ATP), 25 mg/mL bovine serum albumin, 50 pmol *Mse* I adapter, 5 pmol *Eco* RI adapter, 1 Weiss U T4 DNA ligase (New England Biolabs) and 60-80 ng of digested genomic DNA from Section 2.10.1. The tubes were gently agitated to mix and then briefly centrifuged to collect the contents at the bottom of the tube. Ligation reactions were incubated at 4°C overnight; the following day reactions were either used immediately or transferred to –20°C for storage until use.

2.10.4 Pre-selective PCR amplifications

All pre-selective amplifications were performed using oligonucleotide primers that had a single base overhang – an adenosine on the *Eco* RI primer and a cytosine on the *Mse* I primer (see Figure 2.4). Reactions were carried out in 1× Q solution (Qiagen), 1× PCR buffer (Qiagen), 250 µmol of each dNTP (Boehringer Mannheim), 10 pmol *Eco* RI+A pre-selective primer, 10 pmol *Mse* I+C pre-selective primer, and 1 U of *Taq* DNA polymerase (Qiagen) in a total volume of 20 µL including template. Primer sequences are given in Appendix IV. The template, 1 µL 1:5 dilution of ligation product, was added to the reactions last. Thermocycling was performed in a MJ Research PTC-200 DNA engine using 20 cycles of 30 seconds at 94°C, 1 minute at 56°C and 1 minute at 72°C. The ramping speed for the program was limited to 1°C/second; after completion of thermocycling, the reactions were held at 4 °C.

Agarose gel electrophoresis of half the reaction volume was routinely used to determine the efficacy of the amplifications. Sample electrophoresis and detection of

amplified fragments was as previously described in Section 2.3. Typically, a smear was observed between 100 bp and 1 KB in size.

2.10.5 Selective PCR amplifications

The primers used for selective amplifications had a three base overhang, which included the single adenosine or cytosine of the pre-selective primers (see Figure 2.4). Selective amplifications were carried out in a total volume of 20 µL, including template, and contained 1× PCR buffer (Qiagen), 50 mM $MgCl_2$, 250 µmol of each dNTP (Boehringer Mannheim), 10 pmol *Eco* RI+ANN selective primer, 10 pmol *Mse* I+ANN selective primer, 1 U of *Taq* DNA polymerase (Qiagen) and 1 µL of undiluted pre-selective amplification product. The combinations of selective primers used are detailed in Table 2.3; primer sequences are given in Appendix IV. Thermocycling was again performed in a MJ Research PTC-200 DNA engine. The PCR program consisted of an initial hold at 94°C for 2 minutes, 6 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 1 minute at 72°C, 6 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C and 24 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 1 minute at 72°C. A final extension period of 72°C for 5 minutes was also incorporated. At the completion of thermocycling the reactions were held at 4°C.

Agarose gel electrophoresis was used to determine the efficacy of the amplifications prior to acrylamide gel electrophoresis. An 8 µL aliquot of each selective PCR amplification was loaded onto a 2% (w/v) Seakem LE agarose/TAE gel. Sample electrophoresis and the determination of the amplification efficiency was as described previously in Section 2.3. Typically, a series of discrete bands, of between 100 b p and 1 Kb in size, were observed.

2.11 PREPARATION, ELECTROPHORESIS AND SILVER-STAINING OF DENATURING POLYACRYLAMIDE GELS

2.11.1 Preparation of polyacrylamide gels

Prior to pouring the polyacrylamide gel, the glass plates were scrubbed with detergent under hot running water and then repeatedly rinsed to remove any soapy residue. After washing, the plates were dried with a clean paper towel and one side of each cleaned with ethanol. Approximately 2 mL of 80% ethanol was pipetted onto the surface of each plate, which was then wiped with a clean paper towel. This procedure was repeated twice more for each plate. The cleaned face of the shorter glass plate was then treated with a Bind-Silane solution consisting of 2 mL 95% ethanol, 0.5% (v/v) glacial acetic acid and 0.05% (v/v) Bind-Silane (Pharmarcia). The

TABLE 2.3
Combinations of selective amplification primers used for AFLP analysis

Eco RI primer	*Mse* I primer
Eco RI - AGG	*Mse* I - CTG
Eco RI - ACC	*Mse* I - CTC, *Mse* I - CTG
Eco RI - ACG	*Mse* I - CAC, *Mse* I - CTC, *Mse* I - CTG
Eco RI - AAT	*Mse* I - CAT, *Mse* I - CTA
Eco RI - ATA	*Mse* I - CAT, *Mse* I - CTG

solution was applied to the glass plate using a Kimwipe (Kimberley-Clark) and allowed to dry for 4-5 minutes. Excess Bind-Silane was removed by cleaning the plate three times with 2 mL of 95% ethanol and wiping with a paper towel after each application. A small amount of RAIN-X®, 2-3 mL, was applied to the cleaned side of the longer glass plate with a paper towel according to the manufacturers directions. Before use, the plate was washed with 5-10 mL Milli-Q H_2O and polished with a Kimwipe. Once dry, the plates were assembled with a pair of 0.4 mm spacers (Gibco BRL) in position, to form a glass sandwich. The assembly was then clamped together using a S2 casting boot (Gibco BRL). It was important during this procedure not to allow the treated faces of the glass plates to come into contact, as the resulting interaction caused the gel to bind to both surfaces.

A 5% denaturing polyacrylamide gel solution was prepared which contained 8 M urea, 10% (v/v) Long Ranger gel solution (FMC BioProducts), and 1× Tris borate EDTA (TBE) buffer (90 mM Tris-borate and 1 mM EDTA [pH 8.0]) in a total volume of 70 mL. The solution was then filtered through 2 pieces of Whatman 1 filter paper. Before pouring the gel, 350 µL of 10% (w/v) ammonium persulphate (APS, Sigma) and 35 µL NNN'N'-tetramethylethylenediamine (TEMED, BDH) were added and the solution mixed with gentle agitation. Using a 50 mL syringe the gel solution was slowly dispensed into the glass sandwich taking particular care not to introduce air bubbles. Once the gel was poured, a pair of sharktooth combs (5.7 mm point-to-point spacing; Gibco BRL) were inserted, in reverse orientation, to form the sample well. The combs were clamped into position with several large bulldog clips and the assembly covered loosely with plastic film. To allow complete polymerisation the gel was left at room temperature for at least 1 hour and routinely it was left overnight.

Once polymerised, and immediately prior to use, the casting boot and bulldog clips were removed and the outside of the glass plates cleaned. The gel sandwich was then placed into the S2 electrophoresis apparatus (Gibco BRL), and each of the buffer tanks filled with approximately 600 mL of 1× TBE buffer. After removing the combs, a 25 mL syringe filled with 1× TBE was used to flush any acrylamide fragments, excess urea or air bubbles from the sample well. The gel was then pre-run at 35-40 W for 30 minutes.

2.11.2 Preparation of a DNA size standard for electrophoresis on polyacrylamide gels

The DNA size standard electrophoresed with AFLP reactions was the 100 bp DNA Ladder (Gibco BRL). This was prepared in volumes of 100 µL by diluting 1 µL 100 bp DNA Ladder (1 µg/µL) in 99 µL of formamide loading dye (98% [v/v] formamide, 10 mM EDTA, 0.05% [w/v] bromophenol blue and 0.05% [w/v] xylene cyanol). The solution was prepared in a 0.2 mL reaction tube and denatured at 94°C for 4 minutes in the PCR machine, then rapidly cooled to 4°C. After initial use, the ladder was stored at –20°C; denaturation was not repeated prior to subsequent applications.

2.11.3 Preparation of samples for electrophoresis on polyacrylamide gels

While the polyacrylamide gel was pre-running, the samples were prepared for electrophoresis. Typically 4.5 µL of each selective amplification or 2 µL of a PCR amplified novel AFLP marker (see Section 2.12) was combined with 1-1.5 µL of formamide loading dye in clean 0.2 mL reaction tubes. The samples were denatured as described above for the DNA ladder and then placed on ice until loaded.

2.11.4 Sample loading and electrophoresis

After pre-running the polyacrylamide gel the sample well was flushed out using a 1× TBE filled syringe. The pair of sharktooth combs were inserted into the sample well, taking care not to trap any loose polyacryamide fragments and ensuring that the points of the teeth were resting on the gel surface rather than piercing it. Before dispensing a sample onto the gel, the well into which it was to be loaded was again flushed. Using a long, flat 0.37 mm gel tip (SSI) 5-6 µL of the buffer was removed from the well and dispersed into the upper buffer tank. This process was repeated three times in each well. A 2-5 µL aliquot of denatured sample, either selective amplification or novel AFLP derived marker, was immediately loaded into the clean well using the long, flat 0.37 mm gel tip. Two 4 µL loadings of the 100 bp DNA Ladder were usually made with each reaction set, one either side of the samples. Samples were usually loaded side by side, although gaps of one or a few wells were routinely left between sets of reactions for ease of interpretation. If it was intended to isolate polymorphic bands from the gel, a single well gap was left between sample loadings to reduce the likelihood of cross-contamination during loading, electrophoresis or later excision of bands.

If selective amplifications were being electrophoresed, the gel was typically run at 35-40 W until the bromophenol blue dye front was 3-6 cm from the bottom of the polyacryamide gel. However for novel AFLP markers, electrophoresis was generally

longer to allow separation of the similarly sized bands. If the dye fronts were tending to "frown" (i.e. the inner samples were running slower than samples closer to the edge of the gel) the wattage was reduced to 30 W to minimise the effect.

2.11.5 Detection of DNA fragments by silver staining of polyacrylamide gels

Prior to electrophoresing the polyacrylamide gel 4 L Milli-Q H_2O and the developing solution, 4 L 280 mM sodium carbonate (Na_2CO_3 anhydrous, AnalaR, BDH), were placed at –20°C until use. Immediately prior to use, the chilled sodium carbonate solution was made to 0.16% (v/v) formaldehyde (37%, Sigma) and 50 µM sodium thiosulphate ($Na_2S_2O_3$, BDH).

Following electrophoresis the gel sandwich was removed from the apparatus and the glass plates immediately separated by inserting a scalpel blade between them. The short glass plate, to which the acrylamide gel adhered, was transferred to a developing tray containing 4 L 10% (v/v) acetic acid solution. This was agitated on a mechanical shaker for 2 hours to remove the urea from the gel. After the wash, 2 L of the acetic acid solution was retained and stored at –20°C for later fixing of the gel. The gel was transferred to a second developing tray that contained 2 L Milli-Q H_2O and agitated for 2-3 minutes. This water rinse step was repeated twice more, each time transferring the gel to a developing tray containing clean Milli-Q H_2O. After the final water rinse, the gel was placed in 3 L of stain solution consisting of 6 mM silver nitrate ($AgNO_3$, BDH) and 0.15% (v/v) formaldehyde and gently agitated for 1 hour.

After staining, the gel was quickly rinsed, for approximately 5 seconds, in 2-3 L of chilled Milli-Q H_2O, and immediately transferred to a developing tray containing half the chilled developing solution. The tray was vigorously agitated by hand to disperse the brown precipitate that formed; when the precipitate was no longer apparent, the tray was agitated mechanically. After approximately 5 minutes DNA bands became visible, at which point the gel was transferred to a second tray containing the remaining chilled developing solution. Agitation was continued until the bands were clearly discernible, taking care not to over-develop the gel which resulted in a dark background. The developing reaction was then stopped by the addition of the chilled acetic acid solution. To achieve the best results, the gel was usually removed from the developing solution, the acetic acid added and the two solutions mixed vigorously by hand before the gel was retumed to the tray. The gel was mechanically agitated for a further 2-5 minutes until bubbles were no longer being generated. A 2 minute rinse in Milli-Q H_2O was then used to remove the acetic acid from the gel. If polymorphic bands were to be excised from the gel this was done immediately. The gel was then allowed to air dry overnight at room temperature.

2.12 ISOLATION, CHARACTERISATION AND USE OF NOVEL DNA MARKER SYSTEMS IDENTIFIED USING THE AFLP SYSTEM

2.12.1 Isolation of polymorphic bands from silver stained polyacrylamide gels

Polymorphic loci of interest were identified on polyacrylamide gels soon after completion of silver staining. These were usually DNA bands between 250 bp and 1 kb in size that occurred in two or more, but not all, of the samples that had been electrophoresed. A representative of each identified band size was excised from the gel using a clean sterile scalpel blade and transferred to a clean labelled 1.6 mL microcentrifuge tube. A 20 µL aliquot of Milli-Q H_2O was then added to each tube. The tubes were stored overnight at 4°C to allow diffusion of the DNA from the polyacrylamide matrix, for long term storage gel slices were placed at -80°C.

2.12.2 PCR amplification of polymorphic bands isolated from silver stained polyacrylamide gels

Re-amplification of polymorphic bands isolated from AFLP profiles was regularly carried out using PCR. Each reaction mixture contained 1× Q solution (Qiagen), 1× PCR buffer (Qiagen), 250 µmol of each dNTP (Boehringer Mannheim), 10 pmol *Mse* I+C pre-selective primer, 10 pmol *Eco* RI+A pre-selective primer, and 1 U of *Taq* DNA polymerase (Qiagen) in a final amplification volume of 20 µL. For these amplifications it was important to use a primer solution freshly diluted from the 1 nM/µL stock solution as older dilutions proved less effective. A 1 µL aliquot of the AFLP band eluate was used as template. Thermocycling consisted of an initial hold at 94°C for 2 minutes followed by 35 cycles of 1 minute at 94°C, 1 minute at 50°C and 2 minutes at 72°C. A final 5 minute extension time at 72°C was used as before. At the completion of the program reactions were held at 4°C.

A 2 µL aliquot of each amplification was electrophoresed on a 1% (w/v) Seakem LE agarose/TAE gel as described in Section 2.3 to determine the efficacy of the PCR.

2.12.3 Purification of PCR amplified polymorphic bands

The amplified products from single reactions were purified using the QIAquick Gel Extraction Kit (Qiagen) which separates target fragments from unincorporated primers, dNTPs and other reagents on an agarose gel before amplification products are removed from the agarose using a spin column. In this way it was possible to isolate fragments of a specific size which were to be cloned.

The total amplification volume was electrophoresed on a 1% (w/v) Seakem LE agarose/1× TAE buffer gel as described in Section 2.3. To avoid possible contamination, either during loading or at later stages, samples were loaded into every second well. A well was also left unused between the 1Kb plus DNA Ladder, loaded as a size standard, and the adjacent sample lanes. After electrophoresis the amplification products were visualised by ethidium bromide fluorescence on a UV transilluminator which was first covered with a layer of clear plastic film. DNA bands of the appropriate size were carefully excised from sample lanes using sterile scalpel blades and each transferred to a clean 1.6 mL microcentrifuge tube. The manufacturers protocol for the isolation of the PCR products from the gel slices was followed, although minor modifications were made, as outlined. The two optional steps, addition of isopropanol to the agarose/solubilisation buffer mixture and rinsing of the spin column with solubilisation buffer were both used. Aliquots of 30 µL Milli-Q H_20 were used to elute amplification products from the spin columns which were stood for 2-3 minutes before centrifuging for 2 minutes at 10000 × g. After purification, a 2 µL aliquot of each cleaned PCR product was quantified by agarose gel electrophoresis as described in Section 2.3. Included on electrophoretic gels was an aliquot of Low DNA Mass Ladder and either the 1Kb or 1Kb plus DNA ladder. The concentration of the purified fragments was then assessed by comparison to the Low DNA Mass Ladder.

2.12.4 Cloning of re-amplified polymorphic AFLP bands

The cloning of the re-amplified polymorphic AFLP bands was carried out as described in Section 2.7.

2.12.5 Characterisation of cloned polymorphic AFLP bands

Sequencing was carried out as described in Section 2.8. Once determined, the sequences were screened against the EMBL/GENBANK databases using the BLAST program (website: http://www.ncbi.nlm.nih.gov/blastest/blast.cgi).

2.12.6 Primer design

Primers were designed internally at both the 3' and 5' ends of cloned fragments. Care was taken to match melting temperatures between primers and to avoid strings containing repetitive sequences or consecutive guanine or cytosine nucleotides.

2.12.7 PCR amplification and characterisation of AFLP derived marker loci

Reaction cocktails were prepared as described in Section 2.5.3 and contained 10 pM forward primer and 10 pM reverse primer for a given AFLP derived marker. Initial attempts at amplification used thermocycling conditions as described for the ITS locus in Table 2.1, although an annealing temperature 4°C below the melting temperature of the primers, as determined by the GC method, was incorporated. If necessary the thermocycling conditions for each primer pair were further optimised in later amplifications. A 2 µL aliquot of each reaction was electrophoresed on a 2% (w/v) LE agarose/TAE gel as described in Section 2.3 to determine the efficacy, by comparison to the DNA marker, of the PCR. Electrophoresing amplification products on agarose gels of high concentration permitted the identification of situations where primers amplified products of multiple sizes. If PCR products appeared as single bands in all samples, these fragments were purified and then further characterised by DNA sequencing (see Sections 2.6 and 2.8). Where multiple amplification products were apparent these samples were electrophoresed on polyacrylamide gels, as described in Section 2.11, to allow accurate comparison of band sizes.

CHAPTER 3: THE EVOLUTION AND BIOGEOGRAPHY OF THE GENUS *MYOSOTIS* L. (BORAGINACEAE)

This chapter reports analyses DNA sequences and morphological diversity in the genus *Myosotis*. The origins and evolution of the genus are discussed.

3.1 INTRODUCTION

3.1.1 The family Boraginaceae (A. L. de Jussieu)

The Boraginaceae is a large family consisting of more than 100 genera and 2000-2500 species distributed throughout temperate, subtropical and tropical regions. The family is most diverse in the Mediterranean region, but comparatively poorly represented in colder zones (Al-Shehbaz, 1991; Heywood, 1993). More than half the species of the Boraginaceae are assigned to one of ten large genera – examples include *Cordia*, *Cynoglossum, Heliotropium* and *Myosotis*. A significant proportion of the remaining genera are small, each containing less than five species (Al-Shehbaz, 1991).

Members of the Boraginaceae are typically annual to perennial herbs, shrubs or trees, with stems, leaves and inflorescences that are covered with rough hairs. Leaves are usually simple, exstipulate, often containing cystoliths and arranged alternately on terete stems. The Boraginaceae have characteristic inflorescences consisting of one or more scorpiod or helicoid cymes that uncoil progressively as the flowers open (Al-Shehbaz, 1991; Heywood, 1993). Flowers are blue, pink, yellow, or white in colour and are typically actinomorphic, although irregular flowers occur in *Echium* and some related genera. The perianth is pentamerous – the sepals may be free or fused at the base and the sympetalous corolla is generally salverform or campanulate. In many, five "floral scales" or formices occur opposite the corolla lobes. These scales, which are formed by invaginations of the corolla, generally constrict the throat of the corolla tube and may act as nectar guides (Al-Shehbaz, 1991; Heywood, 1993). The five epipetalous stamens of the androecium alternate with the corolla lobes. Each stamen consists of a slender filament and a bilocular anther that dehisces by longitudinal slits. A variety of pollen morphologies occur in the Boraginaceae (Al-Shehbaz, 1991). The superior ovary consists of two fused carpels, although the formation of false septa often results in four locules. Each locule contains a single ovule – various types have been reported, anatropous, unitegmic, erect or sub-

horizontal and either tenuinucellar or psuedocrassinucellar. A single, simple, gynobasic or terminal style is common in the Boraginaceae, although in some genera the apex may be divided into two or four lobes (Heywood, 1993; Mabberley, 1993). Predominantly, flowers are insect pollinated, although both bird and bat pollination have been observed. Self-fertilisation has been reported in some members of the family as have several outbreeding systems including self-incompatibility, separate female flowers, and heterostyly (Al-Shehbaz, 1991; Heywood, 1993). Within the Boraginaceae the fruits are generally a schizocarp of four one-seeded nutlets, more rarely a drupe, berry or loculicidal capsule. Seeds have little or no endosperm and contain a straight or curved embryo (Willis, 1973; Al-Shehbaz, 1991; Heywood, 1993). Several seed dispersal mechanisms are reported in the family – the fruiting calyxes or nutlets may be adapted for wind or water dispersal, several groups have adaptations for external or internal transport by birds or mammals (Al-Shehbaz, 1991).

The Boraginaceae are a taxonomically controversial family – there has been much debate over intrafamilial relationships, familial boundaries and wider affiliations within the angiosperms. In the past, authors have variously treated the ordinal placement of the Boraginaceae and have suggested affiliations with several families (e.g. Thorne, 1976; Dahlgren, 1980; Cronquist, 1981). Currently available molecular and morphological data suggests a close relationship with the Hydrophyllaceae and several recent classification systems have placed both these families within the Polemoniales (Al-Shehbaz, 1991; Chase *et al.*, 1993). Subfamilial classification is based mainly on features of the style and fruit, while tribal and generic boundaries within the Boraginaceae largely rely on nutlet attachment. However, the reliance on characteristics of the fruit for intrafamilial classification has been suggested as leading to the recognition of unnatural groups (Al-Shehbaz, 1991).

3.1.2 The genus Myosotis

DISTRIBUTION AND MORPHOLOGY. The genus *Myosotis* consists of approximately 100 species distributed predominantly in the temperate zones of both hemispheres, although a few taxa occur in alpine regions of the tropics. *Myosotis* has two centres of diversity – one in western Eurasia, where approximately 60 taxa occur, and the other in New Zealand, with approximately 35 endemic species formally described and several undescribed taxa (Al-Shehbaz, 1991). Outside these two regions of diversity, the genus is poorly represented. A few Eurasian taxa have ranges that extend into North America and Africa but fewer than ten species are restricted to areas outside these centres of diversity and are found in North America, South America, Africa, New Guinea and Australia (Al-Shehbaz, 1991).

This morphologically well defined genus has numerous features characteristic of the Boraginaceae. Members of the genus are annual to perennial herbs of various habits – cushion form to sprawling or erect. Plants are usually softly pubescent with entire, alternate leaves, the lower ones usually petiolate but becoming sessile in the upper parts of the plant (Webb *et al.*, 1988; Al-Shehbaz, 1991). The inflorescences of *Myosotis* are either bracteate or ebracteate and are characteristically a determinant, scorpiod or raceme-like cyme. Rarely – as in the New Zealand endemic *M. uniflora* – the inflorescences are solitary. Calyxes are pubescent, usually strongly divided and generally persistent, often enlarging and enclosing the nutlets as the fruit develops. The sympetalous corolla is blue, purple, brown, yellow or white in colour and is salverform, campanulate, or funnelform in shape. *Myosotis* is distinguished by the contorted arrangement of the corolla in bud, the remainder of the subfamily Boraginoideae – to which *Myosotis* is generally assigned – have imbricate aestivation. The epipetalous stamens may be included or exserted well beyond the corolla and as a consequence anthers are either similar in length to the filaments or shorter. Anthers generally have apical appendages (Grau & Schwab, 1982). The glaborous, four-locular ovary supports a slender style that may be included or exserted. The fruits are four small, glossy, brown or black nutlets that are ovoid to ellipsoid in shape and usually have a distinct rim (Moore, 1961; Webb *et al.*, 1988; Al-Shehbaz, 1991). Dispersal has not been well studied in *Myosotis*, although several observations have been reported. Mammals are suggested to disperse nutlets – large mammals, such as deer, internally as the result of eating the whole plant and smaller species externally, the hooked trichomes of the calyxes clinging to fur (Ridley, 1930). Birds have been observed eating *Myosotis* nutlets, although it is unknown if these remain viable, and those species which have an elaiosome are suggested to be ant dispersed (Bresinsky, 1963; Steyermark, 1963).

TAXONOMIC HISTORY. Although morphological features clearly define the genus, species relationships within *Myosotis* remain poorly understood. *Myosotis* has been considered taxonomically complex, with little consensus on the limits, rank and infrageneric classification (Al-Shehbaz, 1991). An early treatment by de Candolle (1846) relied primarily on the nature of the corolla scales and anther exsertion to distinguish five sections. Table 3.1 describes the characters upon which the sections of de Candolle (1846) are based. Five Southern Hemisphere taxa were included within this treatment, which has been used by numerous authors – including Gürke (1897) – since it was first proposed. A later revision of the genus (Stroh, 1941), which considered many new species, rejected the sections *Strophiostoma* Endl. and *Phyllocephalum* Boiss., but retained the remaining three. Approximately 30 Australasian taxa were included in this treatment and were divided between sections *Exarrhena* and *Myosotis*. Both Moore (1961) and Grau & Liens (1968) have

TABLE 3.1
Outline of de Candolle's (1846) infrageneric classification of *Myosotis* (following Grau & Schwab, 1982)

Section	Morphological characteristics
Myosotis DC.	corolla tube with scales, though generally small; anthers longer than filaments and not exserted beyond corolla tube; seeds without caruncula
Exarrhena DC.	corolla tube with scales; anthers shorter than filaments and exceeding corolla tube; seeds without caruncula
Gymnomyosotis DC.	corolla tube without scales; anthers shorter than filaments and exceeding corolla tube; seeds without caruncula
Strophiostoma Endl.	corolla tube with scales; anthers included; areole of seed with prominent caruncula
Phyllocephalum Boiss.	flowers grouped closely together in dischasium inflorescence; areole of seed with prominent caruncula

questioned the usefulness of this classification. The problem is particularly well illustrated in New Zealand – several species pairs, which are indistinguishable on the basis of vegetative characteristics, are nevertheless assigned to different sections due to differences in the degree of anther exsertion. Moore (1961), despite her concerns, produced the current taxonomic treatment of New Zealand *Myosotis* based on this arrangement. Grau & Liens (1968) have similar objections; they cite a European example where the taxon has been assigned to section Myosotis despite the occurrence of exserted anthers.

Grau and co-authors (Grau & Liens, 1968; Grau & Schwab, 1982), using a number of morphological characters, reassessed the infrageneric taxonomy of *Myosotis*. Based on a study of pollen morphology, Grau & Liens (1968) suggested a primary division between Northern and Southern Hemisphere representatives. However, they also noted that a third, smaller group of taxa related to the Northern Hemisphere annual species, *M. discolor*, were intermediate. In addition to expanding the analysis of pollen morphology, a more recent study investigated several microcharacters of the stigma, corolla scales and anthers (Grau & Schwab, 1982). The more extensive sampling of this later investigation confirmed the presence of three distinct pollen classes within the genus and supported the conclusions drawn by Grau & Liens (1968) on the distribution of these pollen types. In all Northern Hemisphere taxa except the "*discolor* group" the pollen was found to be highly uniform. In contrast, Grau & Schwab (1982) indicated that variability in pollen morphology of the Australasian taxa may be taxonomically useful for further dividing this southern group. Analysis of stigma morphology and the floral scales was consistent with the division of the genus based on pollen morphology. Both these floral structures indicated similarity between the Australasian and *discolor* group species and distinctiveness from the remainder of the genus. Several subgeneric groupings were also apparent based on features of the sterile anther apex. Although these groups did not correspond to the division of the genus indicated by pollen,

corolla scale and stigma characters they were compatible with this partition. It was suggested that anther characters may also prove taxonomically useful, especially within the small pollen group of Northern Hemisphere taxa (Grau & Schwab, 1982). Based on their observations Grau & Schwab (1982) proposed a new infrageneric classification which divided the genus into two sections – *Myosotis* and *Exarrhena* – although named for two of de Candolle's sections, these were redefined as described in Table 3.2.

BIOGEOGRAPHY OF THE AUSTRALASIAN *MYOSOTIS*. Grau & Leins (1968) identified two major groups within *Myosotis* based on differences in pollen size and shape – a Northern Hemisphere group with small pollen grains and a predominantly Southern Hemisphere group with larger grains. Further, these authors noted the uniformity of the pollen in the Northern Hemisphere taxa with small grains whereas pollen of the southern group was polymorphic with respect to surface appearance. Although suggesting that further study was required, these authors proposed that the pollen diversity in Australasia pointed to these taxa as ancestral and therefore that Northern Hemisphere *Myosotis* had been derived from southern ancestors.

Consistent with this interpretation, Wardle (1963, 1968) proposed that much of the New Zealand alpine flora, including *Myosotis*, arose through diversification of taxa present on southern ocean lands following the separation of Gondwana. This hypothesis assumes the survival of a cool adapted flora in the general New Zealand region through the warm Tertiary Period. Later, this hypothesis asserts, this element diversified in response to the onset of climate change and mountain building in the late Pliocene and Pleistocene. A similar hypothesis, proposed by Fleming (1962), suggests that Antarctica acted as a Tertiary refuge for cool temperate taxa, which subsequently colonised the developing alpine zone of New Zealand. Apparently based on the assumption that the ancestral flower colour in *Myosotis* was blue, Wardle (1978) suggested that this genus may have dispersed to New Zealand from Antarctica via the subantarctic islands, where the two endemic species are blue flowered.

Alternatively, Raven (1973), Pole (1994) and Macphail (1997) have proposed a north to south route of long distance dispersal to explain founding lineages of alpine groups, including *Myosotis*, in New Zealand. In contrast to the hypotheses of Fleming and Wardle, they suggest evolutionary origins are very recent. Although acknowledging the apparent ancestral status of Australasian *Myosotis*, Raven (1973) suggested that during the Pliocene and Pleistocene the ancestors of New Zealand *Myosotis*, as well as those of other New Zealand alpine groups, dispersed from the Northern Hemisphere. Dispersal was suggested to have been restricted to the recent geological past – only in the late Pliocene and Pleistocene was dispersal facilitated by the formation of alpine habitats in Asia, New Guinea and Australia which

TABLE 3.2
Outline of the infrageneric classification of *Myosotis* proposed by Grau & Schwab (1982)

Infrageneric division	Morphological characteristics	Distribution and content
Section *Myosotis*	pollen grains small, constricted at equator and with fine perforations at poles; stigmas two lobed with papillae only slightly differentiated; corolla scales with long papillae	all Eurasian, African and North American species except those belonging to the *discolor* group
Section *Exarrhena* – austral group	large pollen grains, surface conspicuously sculptured; simple stigmas with large club shaped papillae; corolla scales with short papillae	all Australasian and South American taxa
Section *Exarrhena* – *discolor* group	large pollen grains, but lacking surface sculpturing or perforations found in other groups; form of corolla scales and stigmas as in the austral group	a small group of taxa distributed in Eurasia, but with one species endemic to east Africa

could have acted as "stepping-stones" (Raven, 1973; Smith, 1986). Further, although the West Wind Drift was established in the middle Tertiary, the strengthening of this meteorological pattern during Quaternary glacial periods may have increased the opportunities for dispersal westward across the Tasman Sea (Stewart & Neall, 1984). The suggestion that the ancestors of many New Zealand alpine plant groups were dispersed to New Zealand is also consistent with the palynological record. Many groups in the New Zealand alpine flora, including *Myosotis*, only appear in the fossil record during the latest Tertiary and Quaternary (Fleming, 1979; Mildenhall, 1980)

These different hypotheses on the origins of austral *Myosotis* make specific predictions about the distribution of genetic diversity within the genus. If, as suggested by Wardle (1963, 1968) and others, *Myosotis* has had a long history in the Southern Hemisphere – and assuming no widespread extinctions in this region – then it could be expected that the austral taxa would be a genetically diverse group. Indeed, Grau & Leins (1968) proposal that *Myosotis* arose in the Southern Hemisphere suggests that the austral taxa may be genetically more diverse than their Northern Hemisphere relatives. In contrast Raven's (1973) hypothesis predicts that between the austral *Myosotis,* genetic diversity would be low compared to that between Northern Hemisphere taxa.

The conflicting ideas about the origins of *Myosotis* in New Zealand also have implications for the dispersal of the genus between Southern Hemisphere lands, especially with respect to dispersal between New Zealand and Australia. Raven's (1973) hypothesis proposed that *Myosotis* first became established in Australia, then colonised the alpine zone of New Zealand. Subsequent dispersal either from New Zealand or directly from Australia would have established the subantarctic island

populations. This perspective emphasises the importance of the West Wind Drift. However, Wardle (1978) suggests other weather patterns may facilitate trans-Tasman dispersal in a westward direction and that such a direction of dispersal may be a better explanation of some trans-Tasman alpine plant distributions. Again these possibilities make specific predictions about the structure of inferred phylogenetic graphs. Raven's (1973) hypothesis predicts that in a rooted phylogeny the Australian representatives would be basal to the New Zealand taxa. In contrast, Wardle's (1978) hypothesis suggests that the Australian species would be nested within a radiation of New Zealand taxa.

3.2 MATERIALS AND METHODS

3.2.1 Collection of DNA sequences

Tissue samples for this investigation were either collected in the field, and preserved in silica gel, or obtained from existing herbarium specimens. DNA was extracted from tissue samples with a CTAB protocol, modified from Doyle and Doyle (1990). The DNA marker regions were then amplified from genomic DNA using either a single amplification or a semi-nested PCR approach. Purified amplification products were either sequenced directly or, when necessary, cloned prior to sequencing. When determining the sequence of marker loci, both DNA strands were characterised. DNA sequences from opposing strands were aligned to each other in MT Navigator (Perkin Elmer) and ambiguities checked against the chromatographs. Detailed descriptions of these procedures are given in Chapter 2

3.2.2 Phylogenetic analysis of DNA sequences

Preliminary alignments of DNA sequences were obtained using the progressive multiple alignment procedure, ClustalX (Thompson *et al.*, 1994). These alignments were then inspected visually and corrected where necessary. The occurrence of insertion/deletion (indel) events in DNA sequences required the introduction of gaps into alignments. A conservative approach was taken toward indels – gapped sequence positions were excluded from analyses, as were any variable positions immediately flanking inferred indels. Similarly, all ambiguous sequence positions were removed from analyses. For molecular clock tests, which were carried out on the nuclear ITS data set, only sequence positions within the ITS1 and ITS2 regions were considered. The data alignments are provided as NEXUS format in Disc Appendix I.

Phylogenetic analyses were performed using various methods as implemented in the programs PAUP*4.0b3a (Swofford, 1998), PHYLIP 3.573c (Felsenstein, 1995) and SplitsTree3.0 (Huson, 1998).

3.2.3 Morphological analysis

To confirm earlier suggestions that Southern Hemisphere *Myosotis* display greater morphological diversity than the Eurasian lineages, a small morphological character set was compiled. The data was gathered from a number of publications, including several regional floras, and personal observations (R. Winkworth & A. Robertson). The morphological data set and the list of publications from which the information was collected are provided in Appendix VI. The morphological data was re-coded, as described in Appendix VI, for Principle Component Analysis (PCA) in the statistical package SPSS (1997).

3.3 DATA ANALYSIS

3.3.1 Aligned sequence data

The high degree of similarity between the DNA sequences characterised for each molecular marker resulted in aligned data matrices that contained little alignment ambiguity. Statistics from the aligned data matrices for *Myosotis* sequences are presented in Table 3.3. These are the first DNA sequences reported for *Myosotis*, and for two of the loci – *matK* gene and *trnK-psbA* intergenic spacer – they are the first reported for members of the Boraginaceae. DNA sequences for the nuclear ITS-1 region (Böhle *et al.*, 1996) and chloroplast *ndhF* gene (Ferguson, 1998) have been previously reported from the Boraginaceae.

Similarity searches of the GENBANK nucleotide database confirmed the identity of the DNA sequences determined in this thesis. Database searches using the complete ITS region (including ITS-1, 5.8s and ITS-2) from *Myosotis* show high similarity to other angiosperm ITS sequences in regions corresponding to the highly conserved ribosomal genes, but less similarity to other reported ITS-1 and ITS-2 regions. This finding is consistent with the suggestion that ITS-1 and ITS-2 sequences are unlikely to retain positional homology in comparisons at higher taxonomic levels (Baldwin *et al.*, 1995). When ITS-1 sequences were used in database searches the corresponding DNA sequences for *Echium* (Böhle *et al.*, 1996) were recovered. Further the ITS-1 sequence determined for *Echium vulgare* in this thesis matches, with very high similarity (approximately 99%), the sequence previously reported by these authors.

The alignments for the *matK* locus and *trnK-psbA* spacer regions showed very high similarity to previously reported chloroplast genome sequences. The first 763 nucleotides of the *matK* alignment corresponded to the 3' region of the *matK* gene from *Nicotiana tabacum* – nucleotide positions 2126-2879 in the complete chloroplast

TABLE 3.3
Statistics from the aligned data matrices of *Myosotis* DNA sequences

	nuclear ITS region (ITS-1/5.8S/ITS-2)	Chloroplast		
		3' region of *matK*	5' region of *ndhF*	*trnK-psbA* intergenic spacer
Number of taxa sequenced	34	34	14	14
Sequence length range (bp)	646-649	882-923	725-731	223-250
Aligned length (bp)	659	925	731	250
Number of indels	11	11	1	6
Number of excluded sites	49	58	6	28
Number of constant sites	511	801	687	211
Number of varied sites	99	66	36	11
Number of parsimony informative sites	68	34	19	4
%GC content range (all included sites)	55.6-58.9	32.4-33.4	27.1-28.1	32.9-33.3
%GC content mean (all included sites)	57.2	33.0	27.4	32.9
%GC content range (varied sites only)	42.4-58.6	51.5-65.7	44.4-63.4	36.4-54.5
%GC content mean (varied sites only)	52.5	63.3	49.4	45.5

genome sequence for *N. tabacum*. In the intergenic spacer alignment for *Myosotis*, nucleotides 1-11 corresponded to the first eleven positions in the *trnK* gene of *N. tabacum* and positions 131-250 matched the 5' end of the *psbA* gene for the same species. In both these alignments the remaining sequence positions showed little similarity or obvious positional homology with available chloroplast genome sequences. This observation is consistent with the expectation that these regions were non-coding. DNA sequences for the *ndhF* gene have previously been reported for seven taxa of the Boraginaceae (Ferguson, 1998). The sequence alignment reported here corresponds, with high similarity, to the 5' region (bases 1350-2777) of the *ndhF* gene in *Cryptantha flavoculata* (Boraginaceae).

3.3.2 Phylogenetic gene trees

Aligned DNA sequences for the nuclear ITS and chloroplast *matK* regions characterised in the present study were analysed under two evolutionary tree building approaches – maximum parsimony and quartet puzzling with maximum likelihood as the chosen optimality criterion. Parsimony analyses were performed in PAUP*4.0b3a (Swofford, 1998) using the heuristic search option and the "tree-bisection-reconnection" (TBR) swapping algorithm with accelerated transformation

(ACCTRAN) optimisation in effect. A single most parsimonious tree of 75 steps was recovered in analyses of the *matK* data set; this tree had a consistency index (CI) of 0.933 and retention index (RI) of 0.956. Analyses of ITS sequences produced three equally parsimonious trees of 194 steps with CI of 0.691 and RI of 0.855, that differing only in the relationships inferred between *Myosotis alpestris*, *M. lithospermifolia*, *M. semiamplexicaulis*. Figure 3.1 presents bootstrap consensus trees using parsimony (50% majority rule with groups compatible with this tree retained) of ITS and *matK* data sets. Quartet puzzle analyses were also performed in PAUP*4.0b3a (Swofford, 1998). For these analyses the transition/transversion ratio and proportion of invariable sites for the ML criterion were first estimated on a neighbor-joining tree. These trees are presented in Figure 3.2

The topology of trees produced from chloroplast and nuclear markers were largely compatible with each other. Placement of only two sequences, those from *M. persoonii* and *M. abysinnica*, differ markedly between evolutionary trees derived from the nuclear and chloroplast markers (Figures 3.1 and 3.2). Further, although indels were removed from analyses, all but one was compatible with the phylogenetic reconstructions based on nucleotide substitution. A single, 1 bp indel in the ITS region – shared by the *M. sylvatica* lineage and the North American species, *M. macrosperma* and *M. verna* (sequence position 432) – was not compatible with the evolutionary trees reported.

The gene trees presented here indicate five distinct groups within *Myosotis* – four with taxa largely restricted to the Northern Hemisphere (two species in South Africa being exceptions) and one containing both Eurasian representatives and all Southern Hemisphere species. These groups are described in Table 3.4. Although these groups are distinct from each other, relationships between them are not resolved as bifurcating. In these analyses, the main lineages are generally separated from each other by polytomies (e.g. Figures 3.1 and 3.2). Within the *M. exarrhena*

TABLE 3.4
Major groupings of *Myosotis* sequences indicated by gene trees derived from chloroplast and nuclear DNA markers

Sequence group	Species
M. alpestris group	*M. alpestris*, *M. lithospermifolia*, *M. propinqua*, *M. semiamplexicaulis*
M. sylvatica group	*M. afropalustris* (*matK* sequence only), *M. arvensis*, *M. decumbens* subsp. *decumbens*, *M. sylvatica*, *M. vestergrenii*
M. laxa group	*M. debilis*, *M. laxa* var. *caespitosa*, *M. rehsteneri*
M. refracta group	*M. refracta*
M. exarrhena group	*M. abyssinica*, *M. albiflora*, *M. albo-sericea*, *M. australis* (three accessions), *M. brockiei* (nITS sequence only), *M. cadmea*, *M. capitata*, *M. congesta*, *M. discolor*, *M. exarrhena*, *M. goyenii*, *M. incrassata*, *M. macrantha*, *M. macrosperma*, *M. matthewsii*, *M. persoonii*, *M. rakiura*, *M. ruscinonensis*, *M. stricta* (*matK* sequence only), *M. verna*

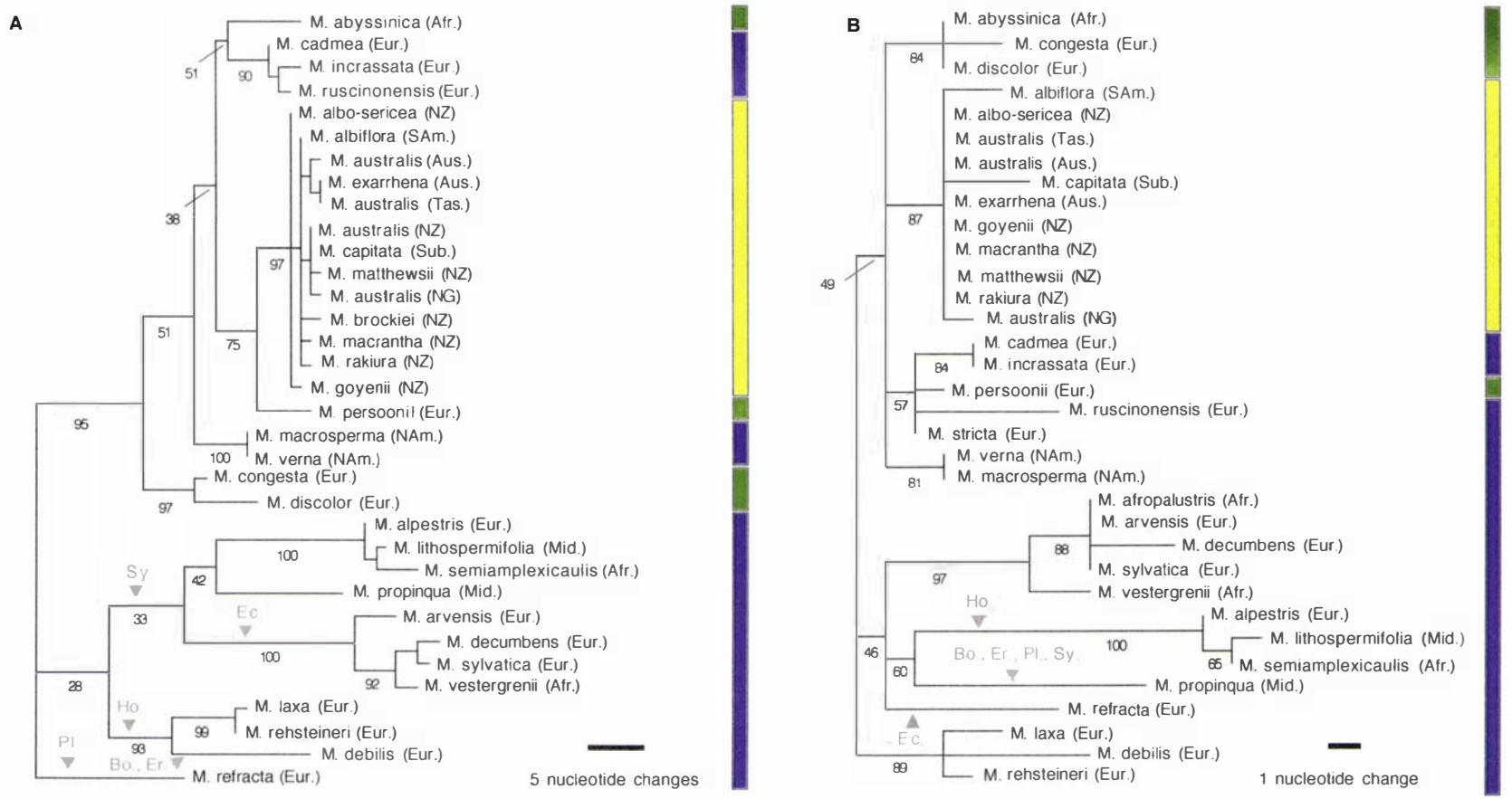


Figure 3.1 : Majority rule bootstrap consensus trees using parsimony, edge lengths estimated under ACCTRAN (using PAUP 4.0b3a). **A**. Data set for 34 Myosotis taxa from the ITS region of the nrDNA (610 nucleotides). **B**. Data set for 34 Myosotis taxa from the 3' region of the chloroplast matK gene locus (867 nucleotides). Subgeneric groups of Grau & Schwab (1982) are indicated – yellow, Section Exarrhena austral group; green, Section Exarrhena discolor group; blue, Section Myosotis. Preferred outgroup rooting positions are marked by grey arrows, outgroups are denoted by two letter code – Bo., Borago officinalis; Ec., Echium vulgare; Er., Eritrichium nanum; Ho., Myosotidium hortensia; Pl., Plagiobothrys albiflorum; Sy., Symphytum × uplandicum. Generalised distributions for taxa are indicated by abbreviations: Afr. – Africa; Aus. – Australian mainland; Eur. – Europe; Mid. – Middle East; NAm – North America; NZ – New Zealand; SAm. – South America; Sub. – subantarctic islands; Tas – Tasmania.. Bootstrap values (250 replicates) for internal edges are given.

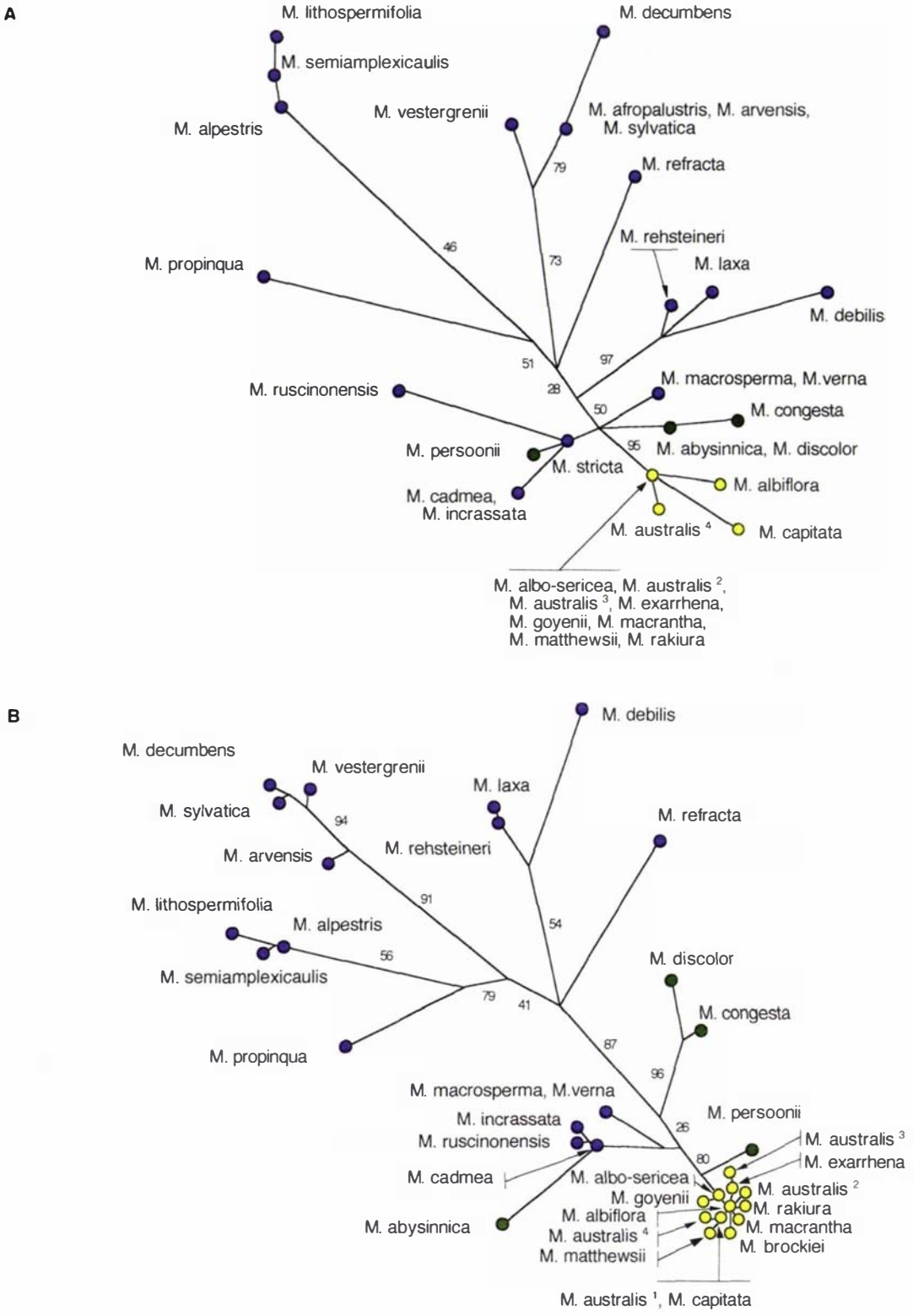


Figure 3.2: Quartet Puzzle trees with edge lengths estimated using maximum likelihood (using PAUP 4.0b3a). **A**. Data set for 34 Myosotis taxa from the 3' region of the chloroplast matK gene locus (867 nucleotides). Puzzle support values for internal edges are given. **B**. Data set for 34 Myosotis taxa from the ITS region of the nrDNA (610 nucleotides). Puzzle support values for internal edges are given Distribution of M. australis accessions – [1]New Zealand; [2]Mt. Kozciuscko, Australia; [3]Tasmania, Australia; [4]New Guinea. Subgeneric groups are denoted as for Figure 3.1.

group there is also limited bifurcating phylogenetic structure. Many relationships are represented as polytomies and many branches are short with low support values (e.g. Figures 3.1, 3.2).

3.3.3 Relative genetic diversity and outgroup rooting

The branch lengths in gene trees derived from *matK* and ITS sequences (Figures 3.1 and 3.2) indicate that the greatest genetic diversity occurs amongst the Northern Hemisphere taxa of *Myosotis* – an observation consistent with a Northern Hemisphere origin for the genus. To test this inference, ITS and *matK* sequences were determined for six potential outgroups from other genera of the Boraginaceae and these were used to root the *Myosotis* gene trees. For each outgroup taxon a series of trees were constructed – using the ITS and *matK* data sets independently – in which the placement of the selected outgroup onto the *Myosotis* tree was varied. The optimal root position for each outgroup was identified using a maximum likelihood criteria (for ITS ti/tv= 1.7725, p_{inv}= 0.8052; for *matK* ti/tv= 1.4121, p_{inv}= 0.6381) and evaluated for significance under the Kishino-Hasegawa sites test (in PAUP*4.0b3a). In these tests, hypotheses proposing a Northern Hemisphere origin for the genus (i.e. the outgroup joined a branch within the Eurasian lineages) were always favoured over those suggesting *Myosotis* arose in the Southern Hemisphere. However, gene trees indicating a Southern Hemisphere origin were only significantly worse in 63% of the tests at the $P< 0.10$ level (data not shown). The optimal rooting position for each outgroup is shown in Figure 3.1.

Combining DNA sequence data from different gene loci has been shown to improve phylogenetic resolution in some situations (Cunningham, 1997). This approach was used here to further investigate the suggested Northern Hemisphere origin of *Myosotis*. In addition to the nuclear ITS and chloroplast *matK* sequences, two other chloroplast markers, the 5' region of *ndhF* and the *trnK-psbA* intergenic spacer, were also characterised for a representative sample of Southern and Northern Hemisphere *Myosotis* taxa and two outgroups. Before combining the sequence data for these four loci, the partition homogeneity test (Michevich & Farris, 1981) as implemented in PAUP*4.0b3a, was used to test whether the partition of data sets was random. The *P* values for all pairwise comparisons between loci and a simultaneous test of all loci together are presented in Table 3.5. Only one of these comparisons (*matK* vs. *psbA* for the data set including *Echium* sequences) suggested possible incongruence between these data sets at a $P= 0.05$ threshold. However, both Sullivan (1996) and Cunningham (1997) have indicated that this threshold may be too conservative for the partition homogeneity test. Therefore, the four loci were combined for further phylogenetic analysis. Using this combined data set the optimal root position was again tested for significance under the Kishino-

TABLE 3.5
***P* values from partition homogeneity test with 1000 replicates for various partitions in the combined data set**

Partitions compared	*P* values for taxa sets investigated		
	Myosotis only	*Myosotis* + *Echium*	*Myosotis* + *Plagiobothrys*
Pairwise			
ITS vs. *matK*	0.881	0.446	0.843
ITS vs. *ndhF*	0.917	0.838	0.491
ITS vs. *psbA*	1.000	1.000	0.761
matK vs. *ndhF*	1.000	1.000	0.368
matK vs. *psbA*	1.000	0.054	0.517
ndhF vs. *psbA*	1.000	0.606	1.000
Simultaneous for all loci	0.985	0.7160	0.593

Hasegawa sites test (ti/tv= 1.4149 and p_{inv}= 0.0 and 0.8637 were used). As in tests that used the ITS or *matK* sequence data separately, the optimal placements for the outgroups were always within the exclusively Northern Hemisphere groups whenusing the combined data set. More importantly, in tests of the combined data, root positions suggesting a Southern Hemisphere origin for the genus were always significantly worse at the $P< 0.05$ level and in all but one case these hypotheses were rejected at the $P< 0.01$ level (data not shown).

3.3.4 Morphological diversity

In the principal component analysis of morphological characters (Figure 3.3) the intrageneric groups of Grau & Schwab (1982) fall into three non-overlapping regions of the sample space. The comparatively limited area occupied by the data points representing the two Northern Hemisphere groups suggests limited morphological diversity within each of these groupings. In contrast, high levels of morphological variation are suggested between the Southern Hemisphere taxa due to the wider distribution of the data points within the sample space.

3.3.5 Age of the Australasian lineage in the Southern Hemisphere

The maximum length of time *Myosotis* has been in the Southern Hemisphere was investigated by calculating the oldest possible age for a Southern Hemisphere ancestor (Figure 3.4). These analyses were made on a subset of the nuclear ITS data set that included the austral species and the closest Northern Hemisphere relatives to this lineage. The age estimates were determined using a number of different rate calibrations.

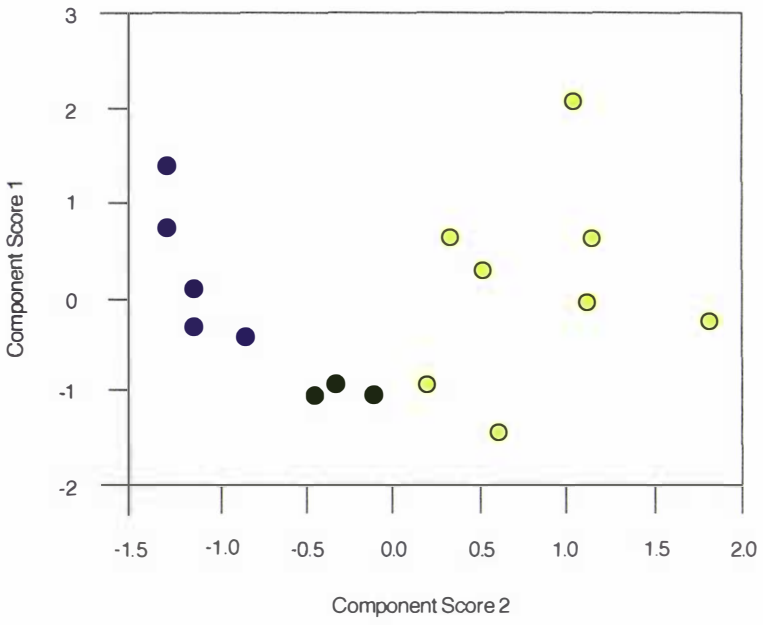


Figure 3.3: Scattergraph for a Principal Components Analysis (PCA) involving 17 morphological characters that could be scored for 16 representative taxa. Data points are colour coded by subgeneric group as denoted for Figure 3.1. Data taken from Grau & Leins, 1968; Grau & Schwab, 1982; various regional floras not cited and from authors personal observations.

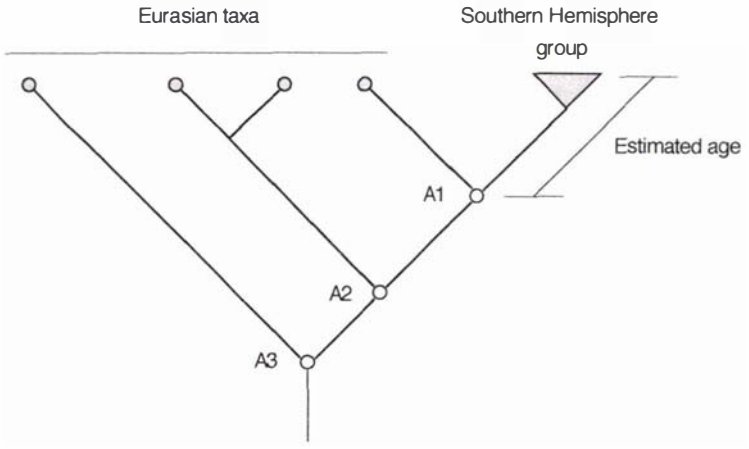


Figure 3.4: Estimating the possible age of the Southern Hemisphere ancestor. Grey shading indicates extant taxa (circles) or groups of taxa (triangle) and open circles represent inferred ancestral taxa. Ancestor A1 could have had either a Eurasian or Southern Hemisphere distribution but taxa A2 and A3 are expected to have been Eurasian. The estimate of the maximum possible age for the austral ancestor assumes the most parsimonious suggestion – a Eurasian distribution for A1.

The first calibration was derived from the earliest fossil record for *Myosotis* in New Zealand – pollen of Quaternary age (Mildenhall, 1980). A maximum likelihood tree (ti/tv= 2.9961, p_{inv}= 0.0) was constructed for the data set and from this sequence divergences were estimated using independent paths of taxa which crossed the root position indicated in Figure 3.5. When these sequence divergences were calibrated with a date of 2 MY, a conservative estimate for the age of the austral radiation based on the first pollen record, the ITS sequence evolution rate was calculated to be 7.20×10^{-4}-1.81×10^{-3} substitutions/site/MY. Given these evolutionary rates, the point estimates indicate that the earliest possible Southern Hemisphere ancestor existed only 11.1-5.6 MYA (Table 3.6).

Further calibrations for ITS sequence evolution were based on the results of Sang *et al.* (1994) for *Dendroseris* (Asteraceae), a genus endemic to the Juan Fernandez Islands. In their investigation, an evolutionary rate of 3.94×10^{-3} substitutions/site/MY was calculated using all pairwise comparisons of taxa in the *Dendroseris* radiation. Since this rate was calculated using non-independent paths of taxa, a second estimate was calculated based on distances for independent paths in the data of Sang *et al.* (1994). This was done as described for the *Myosotis* rate calculation, using the age of the Juan Fernandez Islands (4 MY; Sang *et al.*, 1994) as a limit for the possible age of the *Dendroseris* radiation. For this calculation *D. micrantha* (two accessions) and *D. pruinata* were excluded as these did not consistently conform to the assumptions of a molecular clock under the relative rate test (Test_Clock function) of SplitsTree3.0 (Huson, 1998). This second procedure estimated the rate of ITS sequence evolution for *Dendroseris* to be 3.03×10^{-3}-5.58×10^{-3} substitutions/site/MY. Using these calibrations, the maximum likelihood point estimate (with ti/tv= 2.9961 and p_{inv}= 0.0) for the possible age of the austral *Myosotis* lineage was calculated to be less than 5.0 MY (Table 3.6).

TABLE 3.6

Age estimates for the origin and diversification of the austral lineage of *Myosotis*

Age estimate for	Divergence in ML tree	Estimated ages (MY)		
		Sang *et al.* (1994) calibration	Re-estimated *Dendroseris* rate	Pollen based calibration
Inferred austral ancestor to the last common Northern Hemisphere ancestor	0.00659	1.7	1.2-2.2	3.6-9.1
Radiation of the austral species (from estimate of greatest divergence)	0.00868	2.2	1.6-2.9	N/A
Overall age of the austral group	0.01522	3.9	2.7-5.0	5.6-11.1

Variance on these estimates was tested under the Kishino-Hasegawa sites test (in PHYLIP 3.573c). In this, optimal bifurcating phylogenies were constructed using the closest Northern Hemisphere relatives to the austral group (as in Figure 3.5) and a single Southern Hemisphere species – either *M. exarrhena* or *M. albo-sericea*, as these represented the extremes of genetic diversification from an inferred austral ancestor. The resulting trees were constrained for topology and with respect to the length of the branch joining the representative austral taxon to the inferred Northern Hemisphere ancestor. A series of trees were then compared in which the age of the southern lineage was varied from 0.6 to 100 MY. In trees using *M. albo-sericea* the likelihood scores for reconstructions were significantly worse ($P< 0.10$) for hypotheses that suggested the austral lineage was older than 29 MY (assuming the conservative pollen calibration) and 6.9 MY (assuming a conservative *Dendroseris* ITS calibration). For estimates using *M. exarrhena* these values were 41.1 MY and 9.8 MY respectively.

3.3.6 Dispersal in the Southern Hemisphere

The direction of dispersal events in the Southern Hemisphere indicated by the nuclear ITS gene tree (Figure 3.5) was further investigated by testing how robustly the closest Northern Hemisphere relative joined different branches of the Southern Hemisphere phylogeny. Competing hypotheses for placement of *M. persoonii* onto a quartet puzzle tree reconstructed for the austral species from ITS sequences were evaluated under maximum likelihood criteria (ti/tv= 2.9961 and p_{inv}= 0.0 and 0.7) using the Kishino-Hasegawa sites test (in PAUP*4.0b3a).

Maximum likelihood estimation equally favoured three root positions. These optimal placements were all effectively equivalent to the reconstruction shown in Figure 3.5, as the *M. persoonii* sequence joined either the terminal branches of *M. albo-sericea* or *M. goyenii*, or the branch joining these two sequences to the remainder of the tree (data not shown). Only two of the possible outgroup placements tested under the Kishino-Hasegawa sites test were significantly worse at the $P< 0.10$ level. These were terminal branches leading to the Australian taxa, *M. exarrhena* and *M. australis* (Tasmanian accession). The remaining hypotheses had $P< 0.32$ (data not shown). Although not significantly better under the Kishino-Hasegawa sites test, the optimal outgroup placements indicate New Zealand as the most likely centre for the origin of the extant Southern Hemisphere taxa. Quartet puzzle values also favour this conclusion (93% support; Figure 3.5). Placements of the Northern Hemisphere sequence onto branches leading to other taxa from other Southern Hemisphere lands have less than 1% support. These results provide strong evidence for New Zealand as a centre for Southern Hemisphere dispersal of *Myosotis* and these results are discussed further in Section 3.4.6.

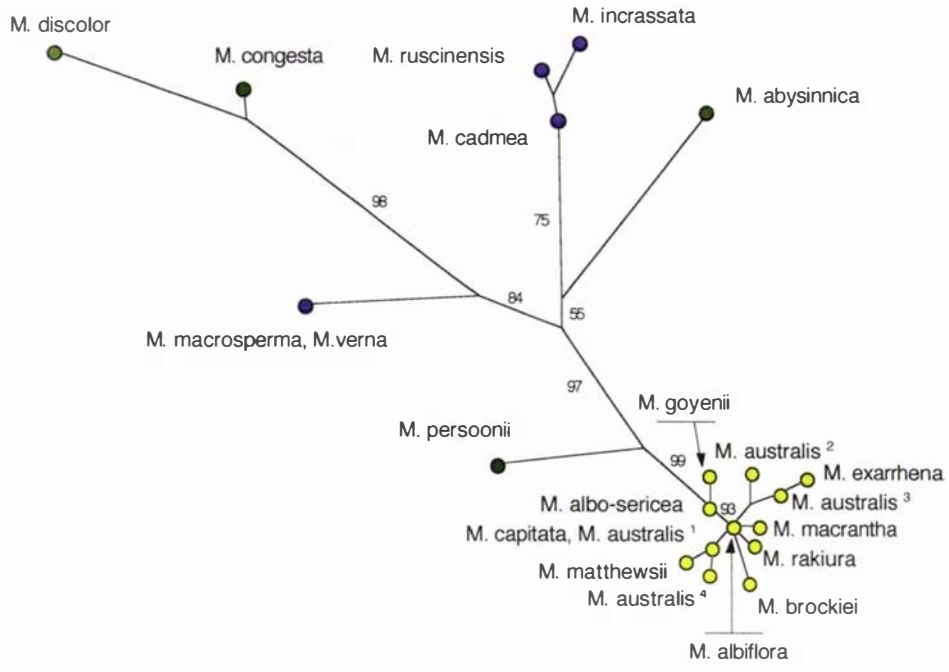


Figure 3.5: Quartet-puzzle tree with edge lengths estimated using maximum-likelihood (PAUP 4.0b3a), made using a data set of 634 nucleotides from the ITS region of the nrDNA for 22 Myosotis taxa. Puzzle support values for internal edges are given. Subgeneric groups denoted as for Figure 3.1 and distribution of M. australis accessions denoted as for Figure 3.2.

3.3.7 Dispersal in the Northern Hemisphere

Within the predominantly Northern Hemisphere Myosotis lineages, limited genetic diversification is observed between taxa that are geographically isolated. Examples of this pattern of genetic diversity include M. afropalustris, M. abyssinica, M. semiamplexicaulis, M. vestergrenii, M. macrosperma and M. verna. The inference that long distance dispersal explains the distribution of these Northern Hemisphere taxa is discussed in Section 3.4.7.

3.4 DISCUSSION

3.4.1 Aligned sequence data

The DNA sequences determined for Myosotis contain low levels of phylogenetic signal. However, the aligned sequence data also display very little ambiguous alignment or homoplasy. The gene trees constructed from these data suggest a number of important points concerning the biogeography and evolution of Myosotis.

3.4.2 The similarity between gene trees: phylogeny?

The high degree of similarity between gene trees derived from nuclear and chloroplast sequences indicates that these different loci reflect a common evolutionary history and phylogeny for *Myosotis*. In these trees, the lack of bifurcating structure is due to a low number of sequence positions that support conflicting patterns of relationship between lineages. These competing signals possibly reflect periods of rapid diversification during the evolution of the genus.

The placement of two taxa differ in trees derived from the chloroplast and nuclear DNA. In these, the incongruent placement of *M. abysinnica* and *M. persoonii* in trees derived from these genetic markers is possibly explained by homoplasy (sequencing error is not an explanation as the samples were independently sequenced twice in both directions). For example, only one nucleotide substitution (at position 791, a thymine to cystosine) is required to move *M. persoonii* in to the ancestral polytomy, an arrangement compatible with the ITS topology. An alternative explanation might be that "chloroplast capture" has caused the alternative placement of these taxa in gene trees from different markers. Chloroplast capture involves the introgression of a chloroplast genome from one taxon into a population, or populations, of another through hybridisation (Schaal *et al.*, 1998). In the case of *M. persoonii* it would require pollen from to have repeatedly fertilised individuals in a population within the *Myosotis stricta* chloroplast group. As a result plants morphologically recognisable as *M. persoonii*, and expected to have a substantial proportion of the *M. persoonii* nuclear genome, contain the chloroplast genome of a different species. It may be possible to distinguish between these possibilities by characterising chloroplast and nuclear markers for a wider sample of *M. abysinnica* and *M. persoonii* populations.

3.4.3 Relative genetic diversity and outgroup rooting

Phylogenetic analyses of ITS and *matK* DNA sequences indicate more diversity between Northern Hemisphere taxa than between Southern Hemisphere taxa. This finding is also supported by analyses of two further chloroplast markers, the 5' region of the *ndhF* gene and the *trnK-psbA* intergenic spacer (Winkworth *et al.*, 1999). This pattern of genetic diversity suggests a Northern Hemisphere origin for the genus – an interpretation supported by tests of outgroup placement. In particular, analyses using a combined data set indicate that hypotheses suggesting a Southern Hemisphere origin are always significantly worse than those supporting a Northern Hemisphere origin. The use of combined data sets, especially when these suggest differing evolutionary histories, has been controversial. Some authors have suggested that such data should not be combined, while others favour joining data sets, at least under certain conditions (Cunningham, 1997). In this case the results of

the partition homogeneity test and the similarity of gene trees derived from ITS and *matK* sequences suggests that the evolutionary histories of the chloroplast and nuclear genomes have been similar.

Although tests of outgroup root position on *Myosotis* phylogenies strongly suggest the genus arose in the Northern Hemisphere, an optimal root placement within a Northern Hemisphere lineage was not identified with confidence. Therefore, the identity of the ancestral *Myosotis* lineage remains uncertain.

3.4.4 Morphological diversity

Principal component analysis of morphological characters indicates that greater morphological diversity exists between Southern Hemisphere taxa than is apparent for the Northern Hemisphere groups. This result is consistent with the earlier findings of Grau and co-workers (Grau & Liens, 1968; Grau & Schwab, 1982) that suggest greater morphological diversity between the Australasian taxa. Interestingly, the pattern of morphological diversity is in direct contrast to the genetic results. This pattern of low genetic and high morphological diversity suggests that the extensive morphological differentiation of the austral *Myosotis* species has occurred only recently. In New Zealand this may be correlated with striking climatic fluctuations and geological change in the late Tertiary and Quaternary (Wardle, 1968; Raven, 1973). The finding of recent morphological diversification in the New Zealand alpine flora is discussed further in Chapter 6.

In the Northern Hemisphere, the major lineages of *Myosotis* appear to have arisen shortly after the origin of the genus. However, the branching pattern within lineages indicates that many of the contemporary species have arisen in recent evolutionary time. This finding suggests that global environmental change during late Tertiary and Quaternary may also have been important for establishing the modern species diversity of Eurasian *Myosotis*.

3.4.5 Age of the Australasian lineage in the Southern Hemisphere

Based on the relative diversity of pollen and certain floral characters between the extant Northern and Southern Hemisphere species, Grau & Schwab (1982) have suggested that the Eurasian lineages arose from taxa that had an ancient presence in the Southern Hemisphere. This hypothesis is not supported by the molecular data presented here. Given the estimated age of the Australasian lineage it is suggested that the Southern Hemisphere austral group has arisen through dispersal rather than vicariance. The poor resolution of relationships between the austral lineage and the remainder of the genus makes it difficult to identify the closest Northern Hemisphere relatives to the Australasian species. However, in analyses of the nuclear ITS region the *discolor* group species, *Myosotis persoonii*, was the closest Eurasian relative –

this is consistent with the suggestion of Grau & Schwab (1982) that the *discolor* group are closely related to the Australasian taxa.

The pattern of low genetic diversity within the Australasian lineage suggests that it is the result of a single introduction to the Southern Hemisphere and that it has recently undergone a period of diversification. Further, the branching patterns in gene trees (Figures 3.2, 3.5) indicate that this Southern Hemisphere diversification has primarily been the result of speciation in New Zealand.

3.4.6 Dispersal in the Southern Hemisphere

The recent radiation of the Australasian taxa has been characterised by events of transoceanic dispersal between Southern Hemisphere lands. Phylogenetic analyses of ITS sequences provide compelling evidence that during the late Tertiary and Quaternary, New Zealand was a source for transoceanic dispersal in many directions. Successful dispersal events occurred south-west to the subantarctic islands, west to Australia, north-west to New Guinea and south-east towards South America (Figure 3.5). The importance of these results for hypotheses on dispersal in the Southern Hemisphere is discussed further in Chapter 6.

3.4.7 Dispersal in the Northern Hemisphere

The molecular results reported here also support the importance of long distance dispersal from Eurasian sources for the establishment of *Myosotis* in Africa and North America. Both the nuclear and chloroplast markers indicate that the South African taxon, *M. semiamplexicaulis*, has arisen by the dispersal from the *M. alpestris* lineage. Two further African species – *M. afropalustris* (South Africa) and *M. vestergrenii* (northeastern Africa) – are derived from the *M. sylvatica* lineage. Due to the poor resolution of the relationships between Section *Exarrhena* and the remainder of the genus further investigation is needed to resolve the ancestry of the African endemic *Myosotis abyssinica*, although it too may have arisen by dispersal of a Eurasian ancestor.

Two taxa endemic to North America are represented in these analyses. The genetic evidence suggests that the white-flowered species, *M. macrosperma* and *M. verna*, are derived from a Eurasian ancestor by a single dispersal event. This would appear to be consistent with the morphological similarity of these two species, which have previously been treated as conspecific (e.g. Steyermark, 1963; Smith, 1978, 1988).

3.4.8 Implications of molecular analyses for the intrageneric classification of *Myosotis*

The issue of monophyly in taxonomy is well debated and it is widely accepted that only monophyletic groups should be formally recognised in taxonomic classification (Donoghue & Cantino, 1988). While the molecular results reported here support certain aspects of the intrageneric classification proposed by Grau & Schwab (1982), in particular the close relationship between the *discolor* and austral groups of Sect. *Exarrhena*, they do not provide evidence for the monophyly of either of the currently recognized sections. These molecular results indicate the need for a revised intrageneric classification – perhaps with consideration of the genetic lineages identified in the present analyses.

3.4.9 Taxon sampling

Insufficient or inappropriate taxon sampling may bias the conclusions drawn from systematic studies. While the taxon sampling for this study was not complete – approximately one third of the recognised *Myosotis* species were included – it does cover much of the known morphological variation and geographical distribution of the genus. Two factors suggest that additional sampling of known taxa is unlikely to significantly effect the inferences on the origins and diversification of austral *Myosotis*. Firstly, the study includes most of the austral taxa that occur outside of New Zealand, one subantarctic island species is the exception. Secondly this investigation has sampled much of the variation in morphology and distribution found in New Zealand *Myosotis*. However, increased sampling of Northern Hemisphere species may well be worthwhile – such studies may identify additional genetic groupings and may also clarify the relationships between the Northern Hemisphere lineages identified in this thesis.

3.4.10 Other recent studies

Recent molecular systematic studies on other New Zealand alpine plant groups suggest that these lineages also have recent origins in New Zealand, most likely arriving via dispersal and subsequently undergoing rapid morphological radiations. Examples include *Hebe* (Wagstaff & Garnock-Jones, 1998), *Gingidia* (Mitchell *et al.*, 1998), the Gnaphalieae (Breitwieser *et al.*, 1999), and *Ranunculus* (Lockhart *et al.*, in press). Dispersal of one or a few disseminules and subsequent rapid morphological radiation of the colonising lineage has also been reported for elements of oceanic island floras (e.g. Baldwin, 1992; Sang *et al.*, 1994; Sang *et al.*, 1995b; Böhle *et al.*, 1996; Kim *et al.*, 1996). The apparent importance of rapid morphological diversification in some plant groups suggests important issues conceming the study of recent plant radiations – these are discussed in Chapter 5.

CHAPTER 4: DIVERSIFICATION OF THE AUSTRALASIAN APIOID UMBELLIFERAE (JUSS.)

This chapter reports the analysis of DNA sequences from the nuclear ITS and novel chloroplast marker, JSAD, for the Australasian apioid Umbelliferae. The study has been motivated by the intention to examine hypotheses of relationship within the Australasian apioid genera and, more generally, to further test hypotheses on the origins of the New Zealand alpine flora.

4.1 INTRODUCTION

4.1.1 The Umbelliferae

The Umbelliferae, or Apiaceae Lindl., is a morphologically well defined group of angiosperms. This large family of 300-455 genera and more than 3000 species is cosmopolitan in distribution, though more common in temperate upland regions and comparatively rare in the tropics (Heywood, 1993; Downie & Katz-Downie, 1996; Downie *et al.*, 1998).

MORPHOLOGY OF THE APIACEAE. Members of the Apiaceae are predominantly annual to perennial herbs; more rarely woody growth results in a tree or shrub habit. Creeping and cushion habits also occur. Leaves are arranged alternately and are usually dissected, although simple leaves occur in certain genera (e.g. *Hydrocotyle*). The family is commonly recognised by several features of flower, seed morphology and the characteristic chemistry of many species (Heywood, 1993; Downie *et al.*, 1998; see Table 4.1).

Perhaps the most recognisable feature of the Umbelliferae are the characteristic umbellate inflorescences – complex structures in which the pedicels of individual flowers arise from a single point on the main floral axis, or peduncle. Compound inflorescences are common in the Apiaceae. In these complex structures groups of flowers form small umbels, termed "umbellets" or "umbellules", which are organised in an umbellate arrangement. Typically, all the flowers in an umbel are carried at the same level due to a progressive increase in pedicel length toward the periphery of the inflorescence (Willis, 1973; Heywood, 1993). The visual impact of an inflorescence may be increased in a number of ways – increasing the number or size of inflorescences; grouping the flowers of the inflorescence closely together;

TABLE 4.1
Characteristic features of the Apiaceae (from Heywood, 1993; Downie *et al.*, 1998)

Structure	Characteristic form
Inflorescence	usually a simple or compound umbel - although possibly highly modified or reduced to a single flower (e.g. *Hydrocotyle*); bracts commonly subtend the umbel rays and, in compound umbels, the umbellets; varying in number and size.
Flowers	flowers very uniform; perianth consisting of a highly reduced calyx and five petals; five free stamens; an inferior ovary with two locules each containing a single pendulous, anatropous ovule; two styles supported by a stylopodium.
Fruits	specialised; two one-seeded mericarps suspended from a bifurcating central carpohore.

modification of the marginal flowers of umbels, much like ray florets in the Asteraceae; or the bracts that subtend umbels, and umbellets in compound structures, may be enlarged or coloured (Heywood, 1993; Mabberley, 1993). Although floral structure is uniform in the Apiaceae, there is a wide diversity of reproductive biologies (Bell, 1971). Flowering is highly organised, within an inflorescence the flowers open sequentially, beginning at the outer whorl and progressing to the centre. Often the stamens mature before the female parts become receptive, this is termed protandrous – although in a few genera stigmas mature first (protogyny) (Bell, 1971). In the Apiaceae the degree of sexual differentiation varies, individual flowers may be male or female (e.g. *Sanicula*), or an entire inflorescence or individual (e.g. as in the dioecious genus *Gingidia*) may be unisexual. In some taxa, perfect and unisexual flowers occur in the same umbel, the relative proportions of each flower type being dependent on the position of the umbel in the flowering sequence (Heywood, 1993). The majority of species are self fertile and are probably fertilised geitonogamously – that is, a flower may receive pollen from a flower within the same inflorescence. A wide variety of insects are known to pollinate the Umbelliferae including Lepidoptera, Hymenoptera (including unspecialised forms), and Diptera (Bell, 1971; Heywood, 1993).

In the Umbeliferae the basic fruit type is a dry schizocarp, consisting of two one-seeded mericarps, that usually contain an oily endosperm and a small embryo. The outer surface of the mericarp usually has five primary ridges, with four vallecular ridges between these. Between species mericarps vary greatly in the presence of surface appendages, the distribution of oil and resin canals and the presence of calcium oxalate crystals in the fruit wall. The appendages, which may consist of tubercles (swellings), wings, hairs, spines, or hooks, have been related to seed dispersal mechanisms. The wide variation in the type, colour and size of the surface modifications has been relied on for taxonomic characters (Heywood, 1993; Lee & Downie, 1999, Downie *et al.*, 2000b).

TAXONOMIC RELATIONSHIPS WITHIN THE APIACEAE. The Apiaceae is recognised as a natural group based on the shared presence of umbellate inflorescences, specialised fruits and floral morphology. However, despite a general economic importance and nearly four centuries of study, the relationships within the Apiaceae remain poorly understood (Plunkett *et al.*, 1996; Katz-Downie *et al.*, 1999). The most widely accepted classification of the family was proposed more that a century ago. Drude (1897-1898) divided the Umbelliferae into three subfamilies – Apioideae, Hydrocotyloideae and Saniculoideae – and twelve tribes based largely on features of the mericarp (Downie & Katz-Downie, 1996; Plunkett *et al.*, 1996). An outline of Drude's system is given in Table 4.2. Recent molecular (e.g. Plunkett *et al.*, 1996; Downie *et al.*, 1998; Downie *et al.*, 2000 b, c), morphological and anatomical studies (Judd *et al.*, 1994) suggest that the Apioideae and Saniculoideae are monophyletic sister groups. However, many authors have expressed dissatisfaction with Drude's classification system, especially in respect of tribal circumscriptions and the failure of the system to reflect evolutionary relationships (Theobold, 1971; Heywood, 1971; Shneyer *et al.*, 1995). Several other classification schemes have been proposed for the family but none have gained widespread acceptance, largely due to similar reliance on one or a few characters (Katz-Downie *et al.*, 1999, Downie *et al.*, 2000c).

The subfamily Apioideae is the largest, most economically important and taxonomically complex of the three subfamilies within Apiaceae. The shared presence of several features (e.g. compound umbels, specialised fruits, terminal style arising

TABLE 4.2
Outline of Drude's (1897-1898) classification of the Umbelliferae (modified from Heywood, 1993)

Subfamily	Characteristics	Tribes and main genera
Hydrocotyloideae	fruit with a woody endocarp, lacking free carpophore; no secretory vittae or restricted to primary ribs; stipules present.	Hydrocotyloideae; *Hydrocotyle* Mulineae; *Azorella*
Saniculoideae	fruit with soft parenchymatous endocarp; ring-like disc surrounds base of style; secretory canals various.	Saniculeae; *Eryngium*, *Astrantia*, *Sanicula* Lagoecieae; *Lagoecia*, *Petagnia*
Apioideae	fruit with soft endocarp, possibly hardened by woody subepidermal layers; style on apex of disc; stipules absent	Echinophoreae; *Echinophora* Scandiceae; *Scandix*, *Chaerophyllum*, *Anthriscus*, *Myrrhis* Coriandreae; *Coriandrum* Smymieae; *Smymium*, *Conium*, *Cachrys*, *Scaligeria* Apieae (Ammieae); *Bupleurum*, *Seseli*, *Pimpinella*, *Apium*, *Ligusticum* Peucedaneae; *Angelica*, *Ferula*, *Heracleum*, *Pastinaca* Laserpiteae; *Laserpitum*, *Thapsia* *Dauceae*; *Daucus*, *Torilis*, *Caucalis*

from a stylopodium, and the absence of stipules) indicates that the subfamily is a natural group, however taxonomic relationships within the Apioideae remain uncertain (Heywood, 1993; Downie & Katz-Downie, 1996). It has been suggested that as many of the tribes and subtribes are erected solely on minor details of mericarp morphology they do not reflect evolutionary history, and are maintained for convenience (Downie & Katz-Downie, 1996). Further it is apparent that many New World taxa, described since the scheme was originally published, have been forced into Eurasian groups (Dawson & Webb, 1978). A number of authors have attempted to improve the classification of the Apioideae. These schemes, which have used characters such as pollen and seedling morphology (e.g. Cerceau-Larrival, 1962) or placed importance on fruit characters other than those used by Drude (e.g. Koso-Poljansky, 1916), are not widely accepted. Recently, information from DNA-DNA hybridisation and serology have been added to the large body of data on anatomy, morphology and chemistry. A growing number of molecular phylogenetic studies, which have used both chloroplast and nuclear DNA sequence markers, have begun to investigate the relationships within the subfamily (e.g. Downie & Katz-Downie, 1996; Downie *et al.*, 2000a, b, c). These studies have offered little support for the either the classification of Drude (1897-1898) or other proposed schemes (Downie *et al.*, 1998).

4.1.2 The Australasian apioid Umbelliferae

The subfamily Apioideae is represented in New Zealand by 95 species in nine genera. New Zealand members of the group are predominantly plants of montane habitats, 82% having distributions centred on upland areas. Five of the nine genera – *Aciphylla* J. R. et G. Forst., *Anisotome* Hook. f., *Gingidia* Dawson, *Lignocarpa* Dawson and *Scandia* Dawson – have restricted distributions, being either endemic to New Zealand or extending only to Australia (Dawson,1971; Dawson & Webb, 1978).

MORPHOLOGY AND DISTRIBUTION. The five predominantly New Zealand genera of the subfamily Apioideae are morphologically diverse – each genus is distinctive, and morphological differentiation within the larger genera is extensive. In general, these genera can be divided into two broad groups on the basis of reproductive biology and morphology (Table 4.3). The differences in the architecture and arrangement of within the southern apioid genera are illustrated in Figure 4.1.

The genus *Aciphylla* consists of around 40 species, including two Australian endemics, and has a highly distinctive morphology. Many species are spinescent and characterised by narrow, elongate inflorescences with numerous reduced compound umbels. However, the genus is currently recognised on foliar characters. The leaves are apetiolate and unifacial – the leaf adaxial surface is reduced to a

TABLE 4.3
Broad groupings of the Australasian genera of the subfamily Apioideae (following Dawson, 1971)

Genera	Common morphological features
Gingidia, Lignocarpa, Scandia	gynodioecous; leaflet teeth not modified as bristles or spines; staminodes highly reduced in female flowers; minute oil tubes on mericarp ribs
Aciphylla, Anisotome	dioecous; usually leaflet teeth are modified as bristles or spines; in female flowers the staminodes are differentiated into anther and filament; oil tubes are usually prominent on the mericarp ribs

narrow groove on the upper face of the secondarily flattened lamina. This definition of the genus results in the inclusion of several taxa that lack the distinctive spinescent habit and the narrow inflorescence (Dawson, 1971). Several morphological groups have been identified within *Aciphylla*. The New Zealand species can be divided into two groups based on patterns of leaf dissection and the relative length of the "stipules". Each of these groups can then be further subdivided based on inflorescence form – broad, open or narrow, elongate (see Figure 4.1).

Anisotome is more typically apioid in morphology, although a number of high alpine species have a mat or cushion habit. The genus is characterised by soft, bifacial, pinnately compound leaves, and broad inflorescences. Within the New Zealand species two groups can be recognised. The first consists of three taxa – *A. lanuginosa*, *A. imbricata* var. *imbricata* and *A. imbricata* var. *prostrata* – which occur at higher altitudes in the southern South Island. The group is characterised by very reduced petioles, generally sessile inflorescences, and broad, short fruits with obscure ribs. The other group consists of taxa with well defined petioles, inflorescences that are never sessile and narrow mericarps with conspicuous ribs. Within this group a further division can be recognised. Six small taxa occur in alpine areas of Stewart Island and the South Island, of which one – *A. aromatica* – extends into the North Island mountains. Four larger taxa form a second group. These are endemic to the coastal areas of the southern South Island, Stewart Island and the subantarctic islands (Dawson, 1971). *Anisotome* is represented in Australia by one endemic species, *A. procumbens* (Webb, 1986).

A rosette, herbaceous habit and axillary inflorescences (see Figure 4.1) distinguish *Gingidia*. This genus is represented by eight species, five endemic to New Zealand, two restricted to Australia and one shared between these two lands. Again two groups can be recognised in New Zealand – three species with winged lateral mericarp ribs (e.g. *G. montanum*, *G. trifoliolatum*) and two unwinged species (e.g. *G. flabellatum*) (Dawson, 1971).

Scandia consists of two species and is distinguished by a caulescent semi-climbing habit, terminal inflorescences, and winged lateral ribs on the mericarps. One species – *S. geniculata* – is restricted to coastal or lowland areas of the eastern South

Island and southern North Island, and is characterised by weak stems and small simple leaves. The second species, *S. rosaefolia*, has two forms, one with broad leaflets and occurring rocky coastal sites in Northland, the other with generally narrower leaves restricted to coastal or lowland areas of the north-eastern North Island (Dawson, 1971).

The ditypic genus, *Lignocarpa* is distinctive within the Australasian apioid Umbelliferae. Plants are very fleshy and have a rosette habit, inflorescences are axillary and bear female flowers that have only vestigial petals and staminodes. In addition, the mericarp wall is heavily lignified, an unusual feature in the Apioideae, which is suggested to be a modification to the steep scree habitats in which this genus grows (Dawson, 1971).

TAXONOMIC HISTORY OF THE AUSTRALASIAN APIOIDEAE. The taxonomic history of the predominantly New Zealand apioid genera has been problematic. This has largely been attributed to the attempts of earlier workers to place the New Zealand taxa within a European framework. The difficulties are apparent from the contradictory placement of the three largest genera – *Aciphylla*, *Anisotome* and *Gingidia* – within

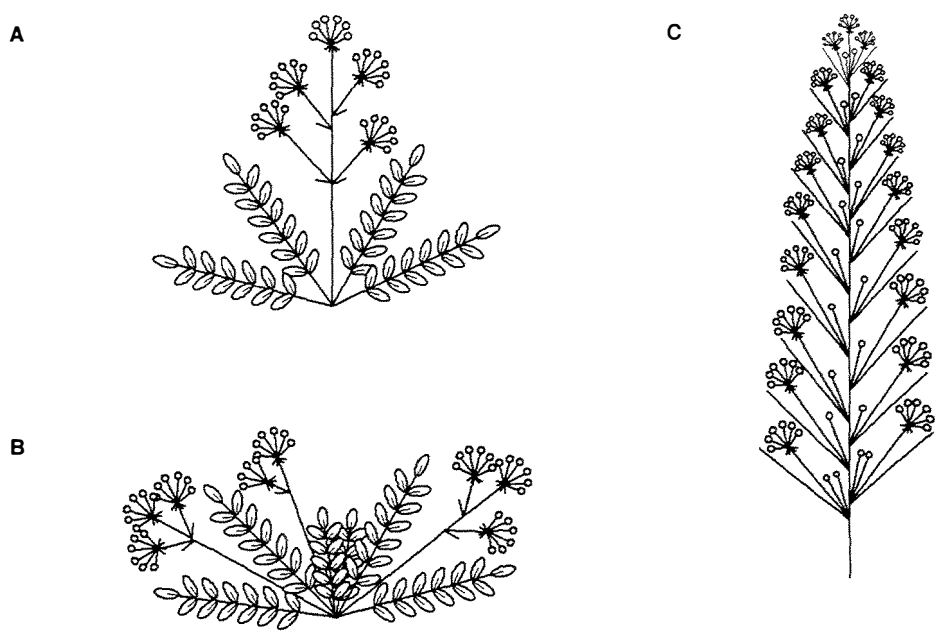


***Figure 4.1**: Inflorescence types in the New Zealand apioid Umbelliferae (each open circle represents a simple umbel). **A**. Broad terminal inflorescence as in* Anisotome *and some members of* Aciphylla *(note that* Scandia *also has terminal inflorescences however these are produced from caulescent stems rather than a rosette as shown). **B**. Axillary inflorescences as in* Gingidia *and* Lignocarpa. *__C__. Narrow, terminal inflorescence as in most* Aciphylla *species (modified from Dawson, 1971)*

various Northern Hemisphere taxa (Dawson & Webb, 1978; Mitchell *et al.*, 1998). Table 4.4 summarises the taxonomic history of the Australasian apioid genera.

In addition to the unstable taxonomic placement of these genera within the family, their generic boundaries have been subject to change. Originally reinstated because of distinctive morphology (e.g. spinescent habit, narrow inflorescence and ribbed mericarps) *Aciphylla* has been variously redefined by later authors. Oliver (1956) differentiated *Aciphylla* and *Anisotome* based on the presence or absence of stipules at the sheath apex. This resulted in the inclusion of several species with soft leaves and broad inflorescences within the genus. Alternatively, Dawson (1968) has distinguished *Aciphylla* by apetiolate, unifacial and secondarily flattened leaves. This author interpreted the "stipules" as the first leaflet pair, and in taxa where these leaf segments were absent, suggested that the joint associated with the leaflet was still apparent. The reinstatement of *Gingidium* by Dawson (1961) was based on several features that distinguish the genus from both *Anisotome* and *Angelica*, to which it had been previously assigned. Based on these characters Dawson (1961) transferred two species of *Anisotome* – *A. deltoidea* and *A. filifolia* – to *Gingidium*. Later revisions, which included a consideration of several new characters, resulted in the exclusion of several taxa from *Gingidium* – the species previously transferred from *Anisotome* were returned and several species were segregated into the new genera, *Scandia* and *Lignocarpa* (Dawson, 1967a, b). Following a change in the generic name, two Australian species of *Seseli*, were transferred to *Gingidia* (Dawson, 1974, 1976).

Generally, the Northern Hemisphere representatives of subfamily Apioideae are divided among many numerically small genera that are distinguished by details of mericarp structure. Dawson & Webb (1978) proposed that adopting such an approach in New Zealand would lead to the recognition of several new genera, which they asserted was undesirable, instead suggesting that the grouping of the New Zealand species into five genera was satisfactory. While these authors noted that the distinction between *Anisotome* and *Aciphylla* was well justified, they suggested the close relationship between *Scandia* and *Gingidia* may warrant their combination in a single genus. The position of *Lignocarpa* was considered to be unclear (Dawson & Webb, 1978).

TAXONOMIC RELATIONSHIPS OF THE AUSTRALASIAN GENERA WITHIN THE APIOIDEAE.

The taxonomic relationships of the Australasian apioid Umbelliferae have been described as obscure (Webb, 1986). Theobold (1971) has placed *Aciphylla*, *Anisotome* and *Lignocarpa* within the large tribe Ammieae, and the remaining two genera in Peucedaneae, apparently based on the presence of broadly winged mericarps in these groups. However, the characters shared by this group of taxa – long-lived, glaborous, and sexually dimorphic perennials – suggest that they form a natural group of closely related species (Webb & Druce, 1984; Webb, 1986). The

TABLE 4.4
Taxonomic history of the Australasian apioid Umbelliferae (following Dawson & Webb, 1978)

Genus	Notes on taxonomic history
Aciphylla	erected in 1775 from a specimen of *Aciphylla squarrosa*; later transferred to the Northern Hemisphere genus *Laserpitum* (as *L. Aciphylla*) then to *Ligusticum* (as *L. Aciphylla*); reinstated the original name reinstated in 1844based on distinctive morphology; generic limits have been variously altered since reinstatement (see text)
Anisotome	established in 1844 with two species and distinguished by soft leaves and broad inflorescences; subsequently included within *Ligusticum* and, later, *Aciphylla* ; authors followed one of these two treatments until *Anisotome* reinstated in 1911based on relative numbers of mericarp oil glands; generic limits altered since reinstatement (see text)
Gingidia	first described from the type specimen, *Gingidium montanum*, but transferred to *Ligusticum* (as *L. Gingidium*) in 1786; later *G montanum* was transferred to *Anisotome* and then to the Northern Hemisphere genus *Angelica*; subsequently identified species were also included in *Angelica*; *Gingidium* was reinstated in 1961, but later the generic limits were revised (see text); name changed to *Gingidia* as when *Gingidium* J. R. et G. Forst. was found to be invalid
Scandia	segregated from *Gingidium* in 1967; distinguished by caulescent semi-climbing habit and terminal inflorescences
Lignocarpa	based on fleshy texture, rudimentary petals in the female flowers, and woody endocarp in the mericarps this genus was segregated from *Gingidium*.

occurrence of natural intergeneric hybrids (Webb & Druce, 1984) and recent molecular data (Mitchell *et al.*, 1998) support this perspective. Interpretation of mericarp morphology has been suggested as placing the New Zealand apioid Umbelliferae within the tribe Apiaeae (= Ammieae). The leaf form typical of this tribe is found in some species of *Aciphylla* and *Anisotome*. However, other features characteristic of this tribe, such as biennial habit and andromonoecious breeding systems, do not occur in the New Zealand genera (Webb, 1986).

Recent molecular studies with limited taxon sampling have suggested that the current tribal and, in some cases, generic level taxonomy of the Apioideae does not reflect evolutionary relationships. Investigations using the ITS region of the nuclear ribosomal DNA regions have found that the New Zealand apioid genera are related to three Northern Hemisphere taxa – the genera *Lecokia*, *Ligusticum* and *Smyrnium* – forming a clade well separated within the subfamily (Downie *et al.*, 1998; Plunkett & Downie, 1999). Similar results have also been reported for chloroplast markers. However, in these studies *Ligusticum* does not feature in the clade and its placement on the phylogenies does not suggest a close relationship to the New Zealand apioid genera (Downie *et al.*, 1998)

BIOGEOGRAPHY. As previously stated, two general hypotheses have been proposed to explain the origins of the New Zealand alpine flora. One suggests a long history for these lineages within the general New Zealand area (Cockayne, 1928; Fleming, 1962; Wardle, 1963, 1968, 1978), the other proposes recent dispersal of

alpine groups from the north (Raven, 1973; Pole, 1994; Macphail, 1997). These alternative hypotheses for the origin of the austral apioid genera make specific predictions about the levels of genetic diversity within this group and their relationships to outgroup taxa. If these genera have a long history in the Southern Hemisphere, and assuming no lineage specific extinction, it may be expected that the austral Apioideae would be genetically diverse and highly diverged from outgroup taxa. In contrast if, as Raven (1973) suggests, the austral taxa are recent additions to the Southern Hemisphere then the Australasian Apioideae would be expected to be closely related to Northern Hemisphere relatives and there to be little genetic diversity within the group. Indeed, recent phylogenetic analyses of molecular data within the Apioideae suggest a northern origin for the Australiasian genera. These data suggest a sister group relationship between the southern lineage and the Northern Hemisphere genera *Lecokia*, *Smyrnium* and possibly *Ligusticum* (Downie *et al.*, 1998; Plunkett & Downie, 1999). Although the clade to which the Australasian apioid genera belong is relatively basal, Plunkett & Downie (1999) have proposed that these southern genera are recent arrivals in the region. These authors interpret the proposed origin of the Apioideae in North Africa and the close relationship between the Northern Hemisphere and Southern Hemisphere taxa as suggesting recent long distance dispersal to Australasia with subsequent radiation of the southern group.

Dawson (1971) investigated the diversification of the New Zealand apioid Umbelliferae, and has favoured separate origins for the gyndioecious (*Gingidia*, *Lignocarpa* and *Scandia*) and dioecious groups (*Aciphylla* and *Anisotome*). This author pointed out that *Gingidia*, *Lignocarpa* and *Scandia* are capable of surviving in warmer climates and proposed that these three genera may have originated in northern New Zealand. *Scandia*, because of its shrubby habit, is suggested to represent the most ancestral group. The remaining two genera, *Aciphylla* and *Anisotome,* are proposed to have had a cool southern origin. Dawson's (1971) hypothesis was that the reduction in land area during the relatively cool Oligocene resulted in the separation of the common ancestor of *Aciphylla* and *Anisotome* into two isolated populations. An island in the general region of the modern subantarctic islands and the southern South Island supported the origin of *Anisotome*. In contrast, the ancestors of *Aciphylla* are thought to have originated on land isolated closer to the modern Chatham Islands. However, Dawson (1971) does not exclude other possible interpretations of the available data. Other possible hypotheses of origin include, (a) evolution on Antarctica and subsequent dispersal to New Zealand; (b) a mainland New Zealand origin with later colonisation of the Chatham and subantarctic islands, both of which had been previously suggested (e.g. Wardle, 1963, 1968; Fleming, 1962). The latter hypothesis was considered unlikely by Dawson (1971) given the unique occurrence of *Anisotome* on the subantarctic islands and *Aciphylla* only on the Chatham Islands.

A New Zealand origin of this group implies that related Australian taxa are derived, and some authors (e.g. Wardle, 1978) have suggested that the extant Australia species arrived there recently by transoceanic dispersal. However, the significant morphological differences between the New Zealand and Australia species led Dawson (1971) to suggest that these taxa are part of an ancient Gondwanan element in the floras of New Zealand and Australia, separated through vicariance. A further, and opposing view, is that the New Zealand apioid Umbelliferae are derived from Northern Hemisphere groups that arrived in Australasia since the early Pliocene (Raven, 1973; Webb, 1986). In reaching this conclusion Webb (1986) examined characters, including many also considered by Dawson, from both New Zealand and Australian members of the three, shared genera – *Aciphylla*, *Anisotome* and *Gingidia*. This analysis suggested that in each genus, the New Zealand species were more derived than the Australian representatives. Webb (1986) concluded that these genera arose in Australia, from some unidentified ancestor, subsequently dispersing to New Zealand where the diversity of available alpine habitat allowed extensive differentiation of form. The two endemic New Zealand genera, *Scandia* and *Lignocarpa*, were suggested to have evolved following arrival of the group in New Zealand. Subsequent east-west dispersal to Australia was also proposed – the shared species, *Gingidia montanum*, was suggested to have recently colonised Australia.

The different hypotheses for the diversification of the austral Apioideae make specific predictions about the structure of inferred phylogenetic graphs. Dawson's (1971) hypothesis predicts that in a rooted phylogeny the Australian and New Zealand representatives would form two distinct groups. Further, within the New Zealand clade three groups would be expected – an *Anisotome* lineage with subantarctic island and southern New Zealand taxa basal, an *Aciphylla* lineage in which Chatham Island taxa were basal and a lineage containing *Gingidia*, *Lignocarpa* and *Scandia*. In contrast the hypotheses of Wardle (1978) and Webb (1986) do not predict an initial split between the Australian and New Zealand taxa as suggested by Dawson (1971). Rather these two proposals suggest that the genera shared between Australia and New Zealand (i.e. *Aciphylla*, *Anisotome* and *Gingidia*) form monophyletic groups. Webb's (1986) hypothesis predicts within these genera the Australian species are be ancestral, whereas Wardle (1978) suggests that the Australian taxa would be nested within the New Zealand radiation.

4.2 MATERIALS AND METHODS

4.2.1 Data collection from *Aciphylla*, *Anisotome* and *Gingidia*

For this investigation DNA was extracted using a modified CTAB protocol (Doyle & Doyle, 1990) from tissue samples preserved in silica gel, or obtained from existing herbarium specimens. Amplification of DNA marker regions was either by a single amplification or a nested PCR approach. Marker loci were characterised from both DNA strands. These sequences were aligned to each other in MT Navigator (Perkin Elmer) and chromatographs used to resolve ambiguities. A detailed description of these procedures is given in Chapter 2.

4.2.2 Additional DNA sequences

Additional DNA sequences for the nuclear ITS locus were included in this study. Several sequences from Mitchell *et al.* (1998) were used to increase ingroup sampling. Sequences (ITS1 and ITS2 regions only) for three outgroup taxa – *Lecokia cretica*, *Ligusticum scoticum* and *Smymium olusatrum* – selected from a phylogeny for the subfamily Apioideae (Downie *et al.*, 1998) were also included. GENBANK (http://www.ncbi.nlm.nih.gov) accession numbers and journal references for these sequences are provided in Appendix II

4.2.3 Phylogenetic analysis

The progressive multiple alignment procedure, ClustalX (Thompson *et al.*, 1994), was used to obtain preliminary alignments which were then checked by eye. For phylogenetic analyses all ambiguous sequence positions were removed, as were gapped positions and any variable sites that immediately flanked inferred indels. In analyses of the ITS region that involved the outgroup taxa and for molecular clock analyses, the 5.8S gene was excluded. Data alignments are provided as NEXUS format in Disc Appendix I.

Phylogenetic analyses were performed using various methods as implemented in the programs PAUP*4.0b3a (Swofford, 1998), PHYLIP 3.573c (Felsenstein, 1995) and SplitsTree3.0 (Huson, 1998).

4.3 DATA ANALYSIS

4.3.1 Aligned sequence data

The aligned data matrices contained little alignment ambiguity due to the high degree of similarity between the DNA sequences. Statistics from the aligned data matrices for the Australasian apioid genera are presented in Table 4.5. DNA sequences from *Aciphylla*, *Anisotome* and *Gingidia* have previously been reported for the ITS1/5.8S/ITS2 locus (Mitchell *et al.*, 1998). Further, there is a large number of ITS1 and ITS2 sequences reported of other members of the subfamily Apioideae (e.g. Downie *et al.*, 1998; Downie *et al.*, 2000a). The JSAD region is a novel chloroplast marker that has recently been shown to provide phylogenetic resolution within the genus *Ranunculus* (Lockhart *et al.*, in press). The sequences presented here for the JSAD marker are the first reported for taxa outside that genus.

Similarity searches of the GENBANK nucleotide sequence database confirmed the identity of the DNA sequences determined in this thesis. The ITS alignment presented here shows very high similarity (< 95%) to previously published sequences for this marker from members of the Australasian apioid genera (e.g.

TABLE 4.5
Statistics from the aligned data matrices of DNA sequences from the Australasian apioid genera *Aciphylla*, *Anisotome* and *Gingidia*

	Nuclear ITS region (ITS-1/5.8S/ITS-2)	Chloroplast JSAD region
Number of taxa sequenced	30	24
Sequence length range (bp)	616-617	1562-1586
Aligned length (bp)	620	1586
Number of indels	4	3
Number of excluded sites	30	26
Number of constant sites	533	1526
Number of varied sites	57	33
Number of parsimony informative sites	36	15
% GC content range (all included sites)	54.1-55.4	34.2-34.7
%GC content mean (all included sites)	54.9	34.5
%GC content range (varied sites only)	55.0-75.0	53.3-80.0
%GC content mean (varied sites only)	67.6	65.0

Mitchell *et al.*, 1998). Database searches also recovered, with lower similarity values, DNA sequences for the ITS1 and ITS2 regions of other genera of the family Apiaceae. The JSAD alignment matched the corresponding regions in previously reported chloroplast genome sequences. Similarity was confined to short segments of the alignment that were separated by regions of low similarity and little obvious positional homology. This pattern is consistent with earlier observations that DNA sequences for the JSAD region vary considerably between different angiosperm taxa.

4.3.2 Phylogenetic gene trees

The evolutionary tree building method of maximum parsimony was used to analyse the aligned data matrices for the nuclear ITS marker. Parsimony analyses were performed in PAUP*4.0b3a (Swofford, 1998) using the heuristic search option and TBR swapping algorithm with ACCTRAN optimisation in effect. Parsimony analyses of ITS sequences produced 24 equally parsimonious trees of 146 steps with CI of 0.822 and RI of 0.857. As no sequences from the outgroup taxa were available for the JSAD region, parsimony analyses – which were used primarily to represent the relationships between the ingroup and the outgroup taxa – were not performed. Figure 4.2 presents a bootstrap consensus tree using parsimony (50% majority rule with groups compatible with this tree retained) from the ITS data set. Quartet puzzle analyses, with a maximum likelihood optimality criterion, were also performed in PAUP*4.0b3a (Swofford, 1998) and used to investigate both the ITS and JSAD data sets. For these analyses the transition/transversion ratio and proportion of invariable sites for the ML criterion were first estimated on a neighbor-joining tree. These trees are presented in Figure 4.3

Phylogenetic gene trees reconstructed from ITS and JSAD regions showed very little similarity to each other. In many cases the trees showed conflicting patterns of relationship between sequences. The rooted ITS gene tree (Figure 4.2) had two well resolved groups of sequences. The first consisted of DNA sequences from *Anisotome* and *Gingidia*. A second, larger group contained sequences from all three genera investigated. Within this group, sequences from *Aciphylla* and *Anisotome* were not resolved from each other as distinct lineages. The chloroplast gene tree (Figure 4.3B) does not support the division of the Australasian Apioideae into the groupings suggested by the nuclear marker. Phylogenetic inference from this marker contradicted the ITS trees (Figure 4.2 and 4.3A) in suggesting that *Gingidia* sequences formed a monophyletic lineage. Contradictory patterns of relationship are also evident between sequences from *Aciphylla* and *Anisotome*. Although gene trees derived from nuclear and chloroplast markers do not suggest a common phylogeny, these trees are consistent in that neither supports these two genera as monophyletic groups.

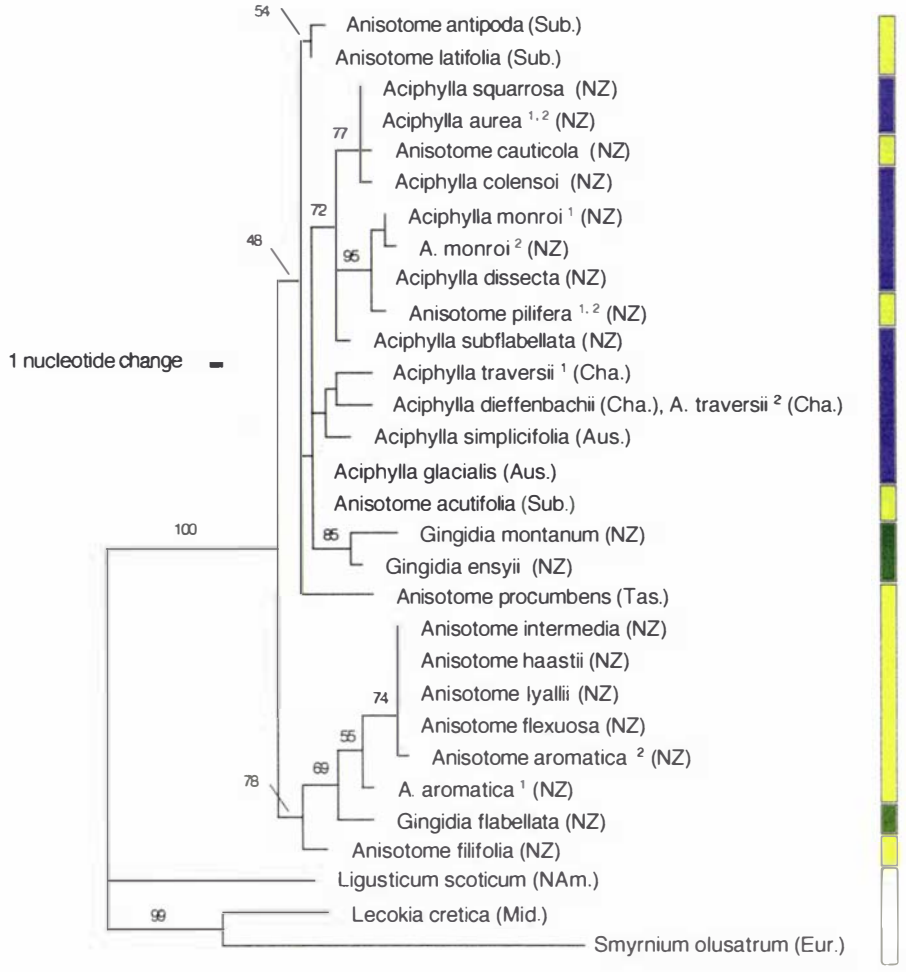


Figure 4.2 : Parsimony majority rule bootstrap consensus tree of ITS1 and ITS2 regions of the nrDNA (374 nucleotides) for 30 Apioideae, edge lengths estimated under ACCTRAN (using PAUP 4.0b3a). Genera are denoted – blue, Aciphylla; yellow, Anisotome; green, Gingidia. Three outgroup taxa Lecokia cretica, Ligusticum scoticum and Smyrnium olusatrum are indicated in white. Five taxa are represented by two accessions: Aciphylla aurea – [1]Mt. Cook, [2]Waipara, Canterbury; Aciphylla monroi – [1]Porters Pass, Canterbury, [2]Craigeburn, Canterbury (Mitchell et al., 1998); Aciphylla traversii – [1]cultivated (B. Rance), [2]cultivated (Otari Native Botanic Garden); Anisotome aromatica – [1]Mt. Hikurangi, East Cape, [2]cultivated (Mitchell et al., 1998); Anisotome pilifera – [1]cultivated (B. Rance), [2]Havelock River, Canterbury. Generalised distributions for taxa are indicated by abbreviations: Aus. – Australian mainland; Cha. – Chatham Islands.; Eur. – Europe; Mid. – Middle East; NAm – North America; NZ – New Zealand; Sub. – subantarctic islands; Tas – Tasmania. Bootstrap values (250 replicates) for internal edges are given.

4.3.3 The level of genetic diversity

The rooted gene tree (Figure 4.2) for the ITS1 and ITS2 regions indicates that sequences from Aciphylla, Anisotome and Gingidia form a single closely related group of taxa. Within this group there is little phylogenetic structure, many relationships between sequences are not resolved as bifurcating and many of the branches are short with relatively low support values (Figure 4.3). Although the patterns of relationship suggested by phylogenetic analysis of the chloroplast marker differ

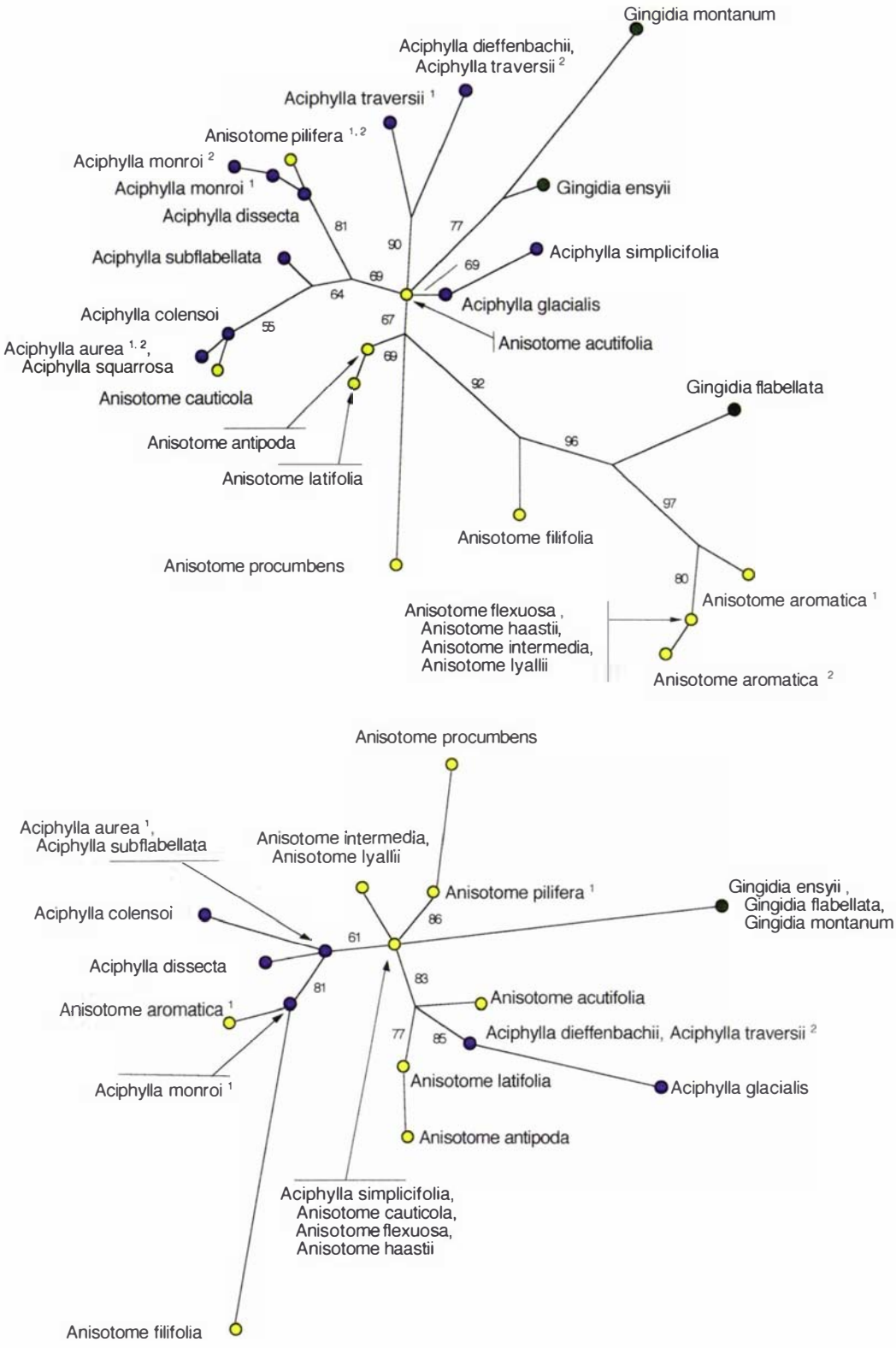


Figure 4.3: Quartet Puzzle trees with edge lengths estimated using maximum likelihood (using PAUP 4.0b3a) for Aciphylla, Anisotome and Gingidia. **A**. Dataset for 30 austral apioid taxa from the ITS region of the nrDNA (410 nucleotides). Puzzle support values for internal edges are given. **B**. Data set for 24 Australasian Apioideae from the chloroplast locus, JSAD (1586 nucleotides). Puzzle support values for internal edges are given. Genera and distribution of accessions are denoted as for Figure 4.2.

markedly from those supported by the ITS region, gene trees derived from the JSAD regions also indicate limited genetic diversity and close relationships between sequences.

4.3.4 Age of the Australasian lineage in the Southern Hemisphere

Nuclear ITS sequences which met the assumptions of a molecular clock, tested using the Test_Clock function of SplitsTree3.0 (Huson, 1998), were used to estimate a maximum limit for the age of the Southern Hemisphere lineage. These estimates were calculated using different calibrations for the rate of sequence evolution in the ITS region. The first calibration was based on the earliest appearance of *Gingidia* pollen in New Zealand during the upper Miocene (Mildenhall, 1980) and calculated as described in Section 3.3.5. Further calibrations were based on the results of Sang *et al.* (1994) for *Dendroseris* - calculation of these has also been described in Section 3.3.5. Given maximum likelihood estimates (ti/tv= 1.9844, p_{inv}= 0.6702) of sequence divergence, it is suggested that the best point estimate for the oldest age of a Southern Hemisphere ancestor is 68 MY (assuming the pollen calibration) and 23.5 MY (based on the *Dendroseris* rate calibrations) (Table 4.6). Further, using the sequence evolution rate derived from the results of Sang *et al.* (1994) the diversification of the Australasian taxa is estimated to be not more than 8.8 MY old.

The Kishino-Hasegawa sites test was used to investigate the variance on estimates for the maximum possible age of the Southern Hemisphere lineage. In this, optimal maximum likelihood trees were constructed using the three outgroup taxa and a single taxon from the southern lineage – either *Anisotome aromatica* (Mitchell *et al.*, 1998 accession) or *Anisotome latifolia* which represent the extremes of sequence divergence in the group. These trees were constrained for topology and branch length to the austral taxon. A series of trees were then compared in which the age of the southern lineage was varied. The likelihood scores for trees using *A. aromatica* were significantly worse ($P< 0.10$) for hypotheses that suggested the Australasian lineage was older than 156.8 MY (based on the conservative pollen calibration) and 40.4 MY (assuming the conservative *Dendroseris* calibration). In trees using *A. latifolia*, all alternative hypotheses were significantly worse at $P= 0.10$.

4.3.5 Dispersal in the Southern Hemisphere

The direction of dispersal events in the Southern Hemisphere indicated by the nuclear ITS gene tree (Figure 4.2) was further investigated by testing alternative positions for the branch joining a Northern Hemisphere relative to the Southern Hemisphere phylogeny. Competing hypotheses for the placement of each outgroup onto a quartet puzzle tree for the austral species were evaluated under maximum

TABLE 4.6
Age estimates for the origin and diversification of the austral lineage of the Apioideae

Age estimate for	Divergence in ML tree	Estimated ages (MY)		
		Sang *et al.* (1994) calibration	Re-estimated *Dendroseris* rate	Pollen based calibration
Inferred austral ancestor to the last common Northern Hemisphere ancestor	0.04443	11.3	8.0-14.7	19.5-57
Radiation of the austral species (from estimate of greatest divergence)	0.02679	6.8	4.8-8.8	N/A
Overall age of the austral group	0.07122	18.1	12.8-23.5	30.5-68

likelihood criteria (ti/tv= 1.9844, p_{inv}= 0.6702) using the Kishino-Hasegawa sites test (in PAUP*4.0b3a).

For all three outgroups maximum likelihood estimation favoured the root position illustrated in Figure 4.2. However, in this analysis many alternative root positions were not rejected as significantly worse at the $P< 0.1$ level. Further, some placements – including hypotheses suggesting an Australian or subantarctic island origin for the group – were only marginally worse than the preferred root position (*P* values ranged from 0.1018-0.9343; data not shown).

4.4 DISCUSSION

4.4.1 Aligned Sequence data

Only low levels of phylogenetic signal were observed between the DNA sequences determined for Australasian apioid Umbelliferae. These data also showed very little homoplasy or alignment ambiguity. Several important points concerning the evolutionary history of this group are suggested by the molecular data.

4.4.2 Phylogenetic gene trees

Discordance between the topologies of gene trees derived from nuclear and chloroplast sequences has often been explained by introgressive hybridisation (Rieseberg, 1991; Rieseberg & Soltis, 1991). Hybridisation in the Australasian apioid Umbelliferae is also suggested by occurrence of different sequence forms and length variants – the latter were identified in one accession of *Aciphylla monroi* and one of *Anisotome cauticola* (this data was excluded when reconstructing phylogenies) – in DNA sequences from the ITS region. A process termed "concerted evolution" usually

maintains the integrity of the nrDNA copies (Baldwin *et al.*, 1995). However, it has been shown that following hybridisation the mechanism of concerted evolution may breakdown allowing more than one sequence type to occur within a hybrid individual (e.g. Sang *et al.*, 1995). The suggestion of hybridisation from observations on the molecular data is consistent with reports of hybridisation based on morphological comparisons – Webb & Druce (1984) list 44 interspecific and intergeneric hybrids. Taken together these observations suggest that hybridisation may have had an important role in the evolutionary history of the Australasian Apioideae. The importance of hybridisation for the diversification of New Zealand alpine plant groups is discussed further in Chapter 6.

In these gene trees, the lack of bifurcating structure is due of low numbers of conflicting signals that support contradictory relationships between lineages. This result is consistent with rapid diversification of this group.

4.4.3 The level of genetic diversity and outgroup rooting

The phylogenetic resolution and branching pattern in evolutionary trees for both the chloroplast and nuclear markers suggest that *Aciphylla*, *Anisotome* and *Gingidia* form a closely related group of species. The phylogenetic reconstructions of Mitchell *et al.* (1998) would suggest that the two remaining genera of the southern Apioideae (*Lignocarpa* and *Scandia*), which were not considered in this study, have also arisen within this complex. Further, these genetic results are consistent with the findings of Webb and Druce (1984) and Webb (1986), who proposed that these five genera were closely related based on general features of morphology, reproductive biology and life history.

In the present analyses, sequences from the genera *Lecokia*, *Ligusticum* and *Smyrnium* were used to root ITS gene trees as recent molecular studies have identified them as the closest relatives to the austral genera (Downie *et al.*, 1998; Plunkett & Downie, 1999). However, these analyses still have limited sampling – for example Downie *et al.* (1998) report sequences for 119 species in 83 genera, the Apioideae consists of 250-400 genera and possibly 3000 species. Therefore, although a Northern Hemisphere origin seems likely, the closest relatives of the austral genera may not yet have been identified. If the outgroups in these analyses are not the closest relatives to the Australasian lineage and closer relatives can be found, then their analysis may help improve estimates for the age of the austral group.

4.4.4 Age of the Australasian lineage in the Southern Hemisphere

Dawson (1971) has suggested that the austral genera of the Apioideae have an ancient presence in the Southern Hemisphere, and possibly represent a

Gondwanan relict. In general the molecular data do not support this hypothesis, instead suggesting that dispersal explains the presence of the Australasian lineage in the Southern Hemisphere. This conclusion arises from age estimates for the austal lineage based on the calibration of Sang *et al.* (1994), the sequence evolution rate re-calculated for *Dendroseris* and less conservative pollen calibrations. Age estimates derived from the most conservative pollen calibrations cannot exclude the possibility of a vicariant Gondwanan distribution. However, as the Apiaceae do not appear in the any pollen record until the early Eocene (Muller, 1981), it is suggested that the family is unlikely to have occurred in the Southern Hemisphere at the time of Gondwana.

The pattern of low genetic diversity within the austral lineage suggests that it is the result of a single introduction to the Southern Hemisphere and that diversification of the group has occurred only relatively recently. The branching patterns in gene trees (Figure 4.2) indicate that diversification has primarily occurred in New Zealand. The recent radiation of plant groups in New Zealand may be correlated with environmental change during the late Tertiary and Quaternary. Patterns of recent diversification in the New Zealand alpine flora are discussed further in Chapter 6.

4.4.5 Dispersal in the Southern Hemisphere

The diversification of the Australasian apioid Umbelliferae during the late Tertiary and Quaternary has been characterised by successful long distance dispersal events. Phylogenetic analyses of nuclear ITS sequences suggest that New Zealand was a source for transoceanic to the subantarctic islands, the Chatham Islands and Australia. In this last case the molecular data provide some evidence that successful dispersal has occurred more than once – *Anisotome procumbens* may represent a separate dispersal to that which established *Aciphylla simplicifolia* and *A. glacialis*. The results from the chloroplast marker also suggest the occurrence of dispersal between Southern Hemisphere lands. However, without evidence of for the root position, the direction of dispersal cannot be determined. These results are discussed further, in the context of Southern Hemisphere dispersal, in Chapter 6.

4.4.6 Implications of molecular analyses on the generic level taxonomy of the Australasian Apioideae

The Australasian apioid genera are a conspicuous and morphologically diverse group. The two largest genera are currently diagnosed by leaf characters (Dawson, 1968), although they are more generally divided by gross habit – *Aciphylla* characteristically being hard, spineose plants whereas *Anisotome* are generally soft herbs. However, the phylogenetic analyses presented here do not support the

current circumscription of *Aciphylla*, *Anisotome* and *Gingidia* based on morphological characteristics. These analyses indicate that the current taxonomy does not reflect the evolutionary history of the group and suggest that the morphological syndromes on which the treatment is based may have arisen (or been lost) independently a number of times.

Further genetic study will be required to produce a taxonomic revision that reflects the evolutionary relationships within the Australasian apioid Umbelliferae. In order to develop an understanding of diversification in this group it is expected that extensive population sampling and highly variable markers from both the chloroplast and nuclear genomes will be required.

4.4.7 Taxon sampling

Taxon sampling can bias the inferences drawn from phylogenetic analyses. In this study the sampling of the austral apioid Umbelliferae was not complete, only about one half of the recognised species were included. However, given that the sampling of this group includes representatives of all taxa that occur outside of New Zealand, it is unlikely that further sampling of known taxa would alter the inferences on the origins and diversification of austral Apioideae. This is not to say that further sampling would not be worthwhile. Indeed such efforts may be important in two respects (a) may provide further insights into the role of hybridisation in the diversification of this group; (b) if more appropriate outgroups could be sampled it may improve estimates of the lineage's age.

4.4.8 Other recent studies

Recent molecular systematic studies have suggested that several New Zealand alpine plant groups may have undergone rapid morphological radiations since the late Tertiary (e.g. Mitchell *et al.*, 1998; Wagstaff & Garnock-Jones, 1998; Breitwieser *et al.*, 1999; Lockhart *et al.*, in press). Similar patterns of rapid morphological diversification have also been reported for elements of oceanic island floras (e.g. Baldwin, 1992; Sang *et al.*, 1994; Sang *et al.*, 1995b; Böhle *et al.*, 1996; Kim *et al.*, 1996). In some cases (e.g. Baldwin 1997; Lockhart *et al.*, in press) hybridisation has been implicated in the diversification of these groups – a finding that is consistent with the suggested importance of hybridisation in plant speciation (Ehrendorfer, 1958; Stebbins, 1984; Arnold, 1997). However, while these studies suggest the importance of hybridisation further investigation is required to fully document the nature of diversification. These observations on recent diversification of plant groups leads suggests several issues concerning the study of recent plant radiations, these are discussed in Chapter 5.

CHAPTER 5: PHYLOGENETIC ANALYSIS OF LATE TERTIARY AND QUATERNARY PLANT SPECIES RADIATIONS

Analyses of the molecular data presented in this thesis, and the findings of recent molecular investigations of other plant groups, indicate that explanations of modern plant biodiversity require an understanding of late Tertiary and Quaternary diversification processes. However, the phylogenetic study of recently evolved species complexes is problematical. One limitation is that commonly used tree building methods often poorly represent the complex patterns of relationship displayed by fast-evolving genes in recently radiated plant groups. A second limitation is the current lack of molecular markers displaying levels of genetic variation appropriate for studying relationships between closely related taxa. This chapter describes observations and results from preliminary studies addressing these issues.

5.1 OBSERVATIONS ON RECENT SPECIES RADIATIONS

Molecular studies indicate that for numerous plant groups, the late Tertiary and Quaternary have been periods of intense speciation and a time when plant distributions changed rapidly (Comes & Kadereit, 1998; Winkworth *et al.*, 1999). Generally, these investigations have concluded that speciation has been associated with Quaternary climatic fluctuations (e.g. Crawford *et al.*, 1985; Hungerer & Kadereit, 1998) or the colonisation of insular environments (e.g. Baldwin, 1992; Böhle *et al.*, 1996). Despite the dramatic morphological and ecological diversity that characterises some recent radiations, these same lineages display little genetic differentiation – probably reflecting the relatively short period of time in which these species complexes have evolved. In gene trees, the phylogenetic relationships between recently speciated groups are often represented as polytomies and many of the branches are short with low support values (e.g. Baldwin, 1992; Böhle *et al.*, 1996; this thesis). Although taxa are often distinct, the lack of genetic differentiation suggests – and in some cases field observations confirm – that hybridisation, introgression and polyploidy may be occurring between diverged lineages (Schaal *et al.,* 1998). The observation that recombination of genetic material from differentiated parental taxa and differential transfer of cytoplasmic and nuclear genomes may be associated with reticulate evolution suggests that such processes are likely to result in complex patterns of inheritance.

Reticulate evolution may also effect the evolutionary process at certain genetic loci. For example, the internally transcribed spacer regions of the nuclear ribosomal DNA are commonly used in studies of closely related plant species because these loci exhibit rapid, concerted evolution. However, observations suggest that following hybrid formation the mechanism of gene conversion may be disrupted (Baldwin *et al.*, 1995). In this case the presence of heteroplasmic nucleotide positions may reflect the failure of concerted evolution to homogenise differentiated parental ITS repeats following hybridisation (Sang *et al.*, 1995a). A hybrid origin may also be indicated by DNA sequence patterns that suggest reticulate relationships. Such patterns may arise when differentiated parental loci are recombined in hybrid genomes through partial gene conversion and crossover events (Buckler *et al.*, 1997; Aguilar *et al.*, 1999; Huber *et al.*, in press).

5.2 RECONSTRUCTING PHYLOGENY IN RECENT SPECIES RADIATIONS

5.2.1 Are bifurcating methods useful for studying recent radiations?

That reticulate evolution is suggested to be a feature of many recently radiated plant groups indicates that patterns of phylogenetic relationship and marker evolution in these groups are likely to be complex. Since bifurcating tree building approaches – such as parsimony and maximum likelihood – cannot directly reconstruct reticulate evolution, phylogenetic patterns resulting from hybridisation or polyploidisation will be misrepresented on bifurcating trees (Baldwin *et al.*, 1995).

Another potential problem for using certain bifurcating evolutionary models to study recently radiated species complexes is that sequences of extant taxa may not have diverged from those of recent common ancestors. The occurrence of putatively ancestral sequences in extant lineages is likely due to the short evolutionary period since these groups diversified – either the ancestral taxa are extant or contemporary species have not accumulated nucleotide substitutions that would differentiate them from extinct ancestors. Consequently, phylogenetic methods that force taxa onto the tips of bifurcating trees (such as neighbor-joining and parsimony) are expected to be of limited usefulness for representing these evolutionary relationships.

5.2.2 Networks: an alternative method

Bifurcating phylogenetic tree representations indicate only compatible signals in the data, and as a result significant phylogenetic information may be lost. Network methods are a promising alternative to bifurcating approaches as these procedures can represent more of the information contained in DNA sequences (see Figure 5.1). Two network methods receiving recent attention are split-decomposition (Lockhart *et*

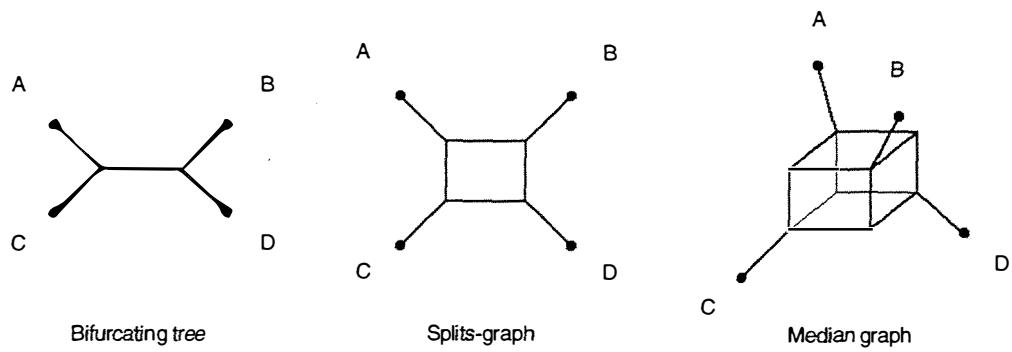


***Figure 5.1**: A comparison of the information that can be represented by different types of tree building procedure. In the bifurcating tree shown only the relationship (AC – BD) can be represented. In the splits-graph sequence patterns that suggest (AC – BD) + (AB – CD) can both be visualised. The median graph provides the most complex representation as (AC – BD) + (AB – CD) + (AD – CB) can be visualised simultaneously.*

al., in press), which represents the strongest weakly compatible signals in the data (see Section 2.9.2 for background) and median networks that can potentially display all of the phylogenetic information contained in sequence data (Huber *et al.*, in press).

This section presents some observations and results from recently radiated plant groups that were investigated using split-decomposition, as implemented in SplitsTree3.1 (Huson, 1998). These analyses highlight the potential and limitations of split-decomposition for studying recent plant diversification.

5.2.3 Desirable properties of split-decomposition representation

Several properties of split-decomposition make this method useful for reconstructing phylogenetic relationships in recently diversified plant groups. Obvious advantages of this approach for studying closely related species complexes are (a) this method does not assume a bifurcating evolutionary process; (b) that conflicting phylogenetic signals can be represented; (c) that ancestral haplotypes are not forced away from internal nodes in reconstructed trees. Using split-decomposition, the information in the sequence data can be more fully visualised. Thus, it may be possible to differentiate between data that is treelike and bifurcating, and that which is characterised by more complex relationships, Importantly, exploring the data structure in this way may allow more appropriate inferences of evolutionary history to be made.

Splits-graph representations for the diversification of the two New Zealand plant groups studied in this thesis and two well studied oceanic island groups, the Hawaiian silversword alliance (Baldwin & Robichaux, 1995; Baldwin & Sanderson, 1998) and *Dendroseris* from the Juan Fernandez Islands (Sang *et al.*, 1995b) are presented in Figures 5.2-5.5. The data sets used for analyses are given in Disc

Appendix I. In three of these groups – *Dendroseris* is the exception – there is evidence that some of the extant taxa have ancestral haplotypes. Further, there is also evidence of incompatibilities – patterns suggesting contradictory relationships – in three of the examples. These conflicting patterns may indicate that reticulate evolution has been a feature of diversification in the silversword alliance, *Dendroseris* and the Australasian Apioideae.

5.2.4 Edge length estimation in split-decomposition representation

One limitation to implementing spit-decomposition in the context of testing biogeographic and phylogeographic hypotheses concerns the calculation of edge lengths in splits-graphs. The original implementation of split-decomposition was intended to be conservative. However, in some situations (e.g. when using large data sets and when the associated fit statistic is low) this approach suffers a systematic bias. This appears to occur because for successively more internal edges in a reconstructed tree more possibilities exist for calculating the isolation indices (Figure 5.6). Therefore, with finite sequence length the probability of obtaining an artificially low isolation index value is higher for more internal edges. This bias generally results in the length of internal edges being under-estimated in comparison to external branches – in the worst case, internal edges may be collapsed to a polytomy.

A least-squares criterion (Moulton, Bryant & Huson, unpublished) was investigated and found to greatly improve estimates of internal edge length. The difference that a least-squares optimisation can make to estimates of internal branches is illustrated in Figures 5.7 and 5.8. In these examples up to a four-fold difference in the estimates of branch length are observed – these edge lengths becoming more similar to those obtained under maximum likelihood tree building. While promising, this approach is also limited in that the least-squares estimates of edge length are made on a topology determined by the original and more conservative implementation. Consequently, if an internal edge is collapsed to zero length by the initial procedure the length cannot be re-estimated by the least-squares approach and the edge length will remain zero (i.e. a polytomy). One possible solution is to construct splits-graphs from overlapping subsets of closely related taxa within a larger data set, using a compatibility analysis to join these small trees together. (e.g. Disk-Covering Method, Huson *et al.*, 1998, 1999). Such an approach is suggested to be more efficient and accurate than "naive" split-decomposition (Huson *et al.*, 1998). Currently a problem for this approach is that it is unclear what procedure could be used to join the sub-trees.

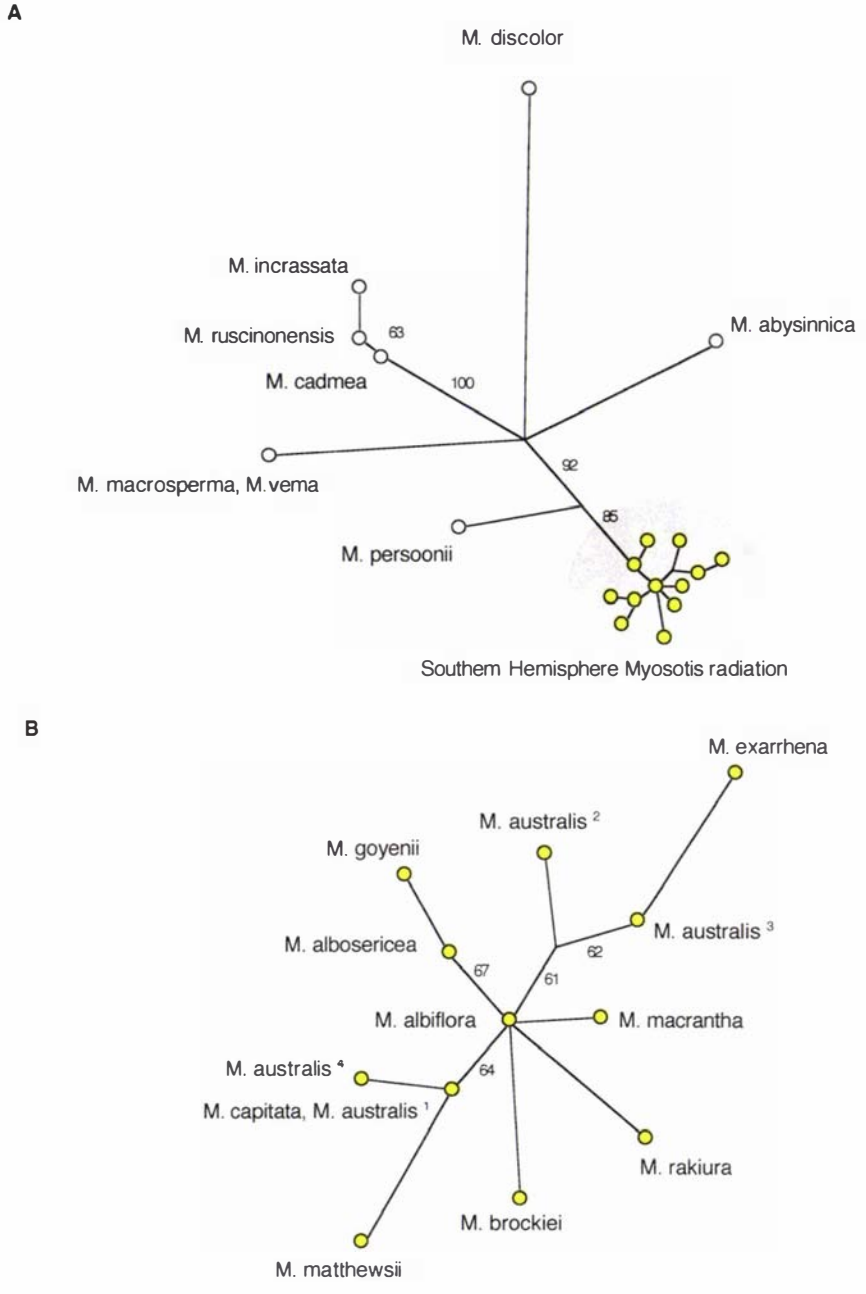


Figure 5.2: Splits-graphs (using Huson, 1998; SplitsTree 3.1) made using nuclear ITS data sets from representatives of the Southern Hemisphere radiation of Myosotis and the most closely related Northern Hemisphere taxa. **A**. Relationship of the austral Myosotis group to Northern Hemisphere species of Section Exarrhena discolor group and Section Myosotis. The graph was reconstructed from a data set of 462 nucleotides (Fit statistic = 74.9). **B**. Relationships within the austral Myosotis group reconstructed from a data set of 467 nucleotides (Fit statistic = 100). The distribution of M. australis accessions – [1]New Zealand; [2]Mt. Kozciuscko, Australia; [3]Tasmania, Australia; [4]New Guinea.

Figure 5.3 (Following page): Splitsgraphs (using Huson, 1998; SplitsTree 3.1) made using nuclear ITS data sets from representatives of the Australasian apioid Umbelliferae and outgroup genera. The three Australasian apioid genera are indicated – blue, Aciphylla; yellow, Anisotome; green, Gingidia. **A**. Relationship of the austral genera to the most closely related Northern Hemisphere genera reconstructed from a data set of 376 nucleotides (Fit statistic = 62.0). **B**. Relationships within the Australasian Apioideae reconstructed from a data set of 410

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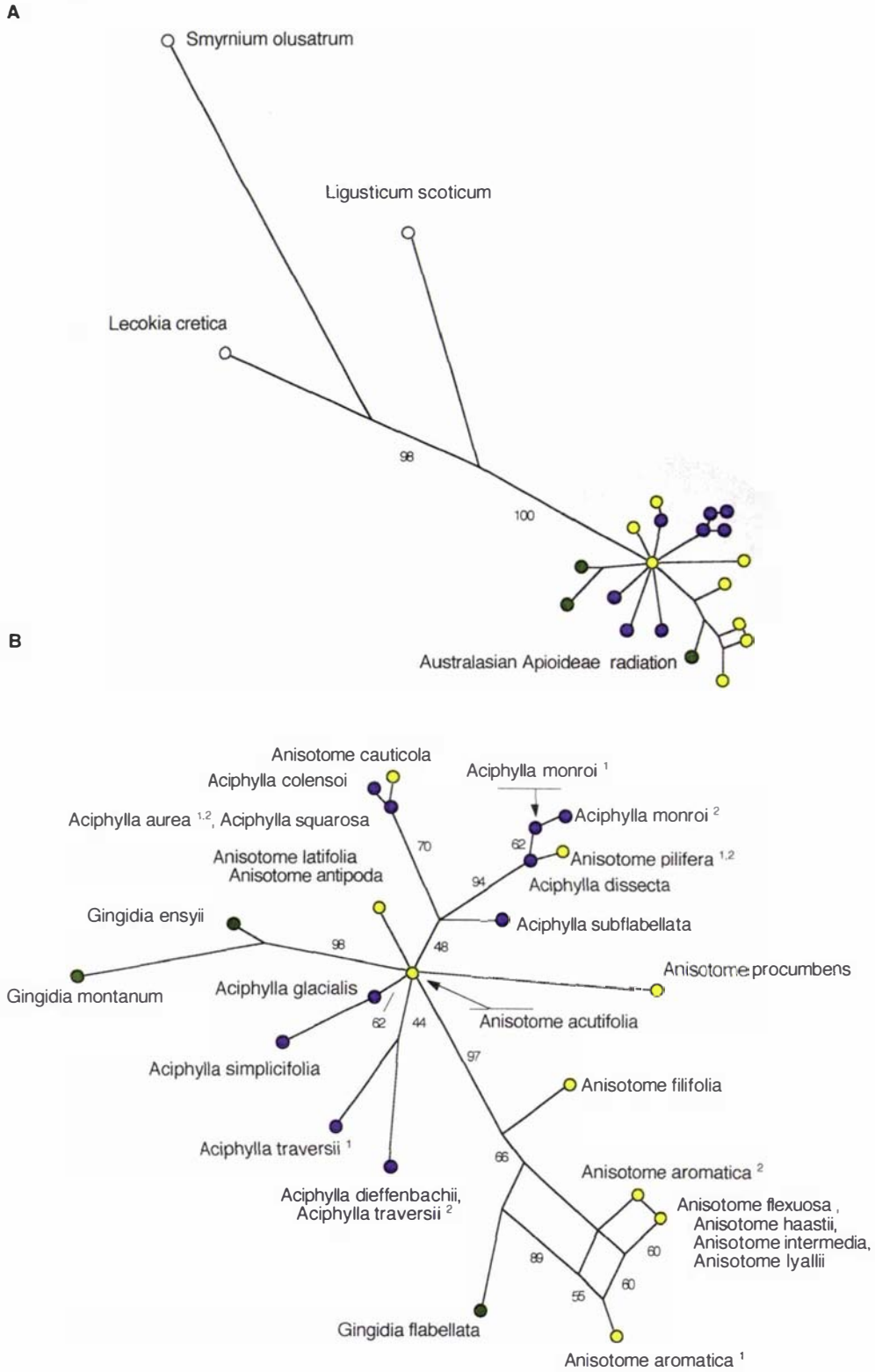


Figure 5.3: (continued from previous page) nucleotides (Fit statistic = 70.1). Five taxa are represented by two accessions, Aciphylla aurea - [1]Mt. Cook, [2]Waipara, Canterbury; Aciphylla monroi - [1]Porters Pass, Canterbury, [2]Craigeburn, Canterbury (Mitchell et al., 1998); Aciphylla traversii - [1]cultivated (B. Rance), [2]cultivated (Otari Native Botanic Garden); Anisotome aromatica - [1]Mt. Hikurangi, East Cape, [2]cultivated (Mitchell et al., 1998); Anisotome pilifera - [1]cultivated (B. Rance), [2]Havelock River, Canterbury. Bootstrap values (250 replicates) for internal edges are given. Bootstrap values (250 replicates) for internal edges are given.

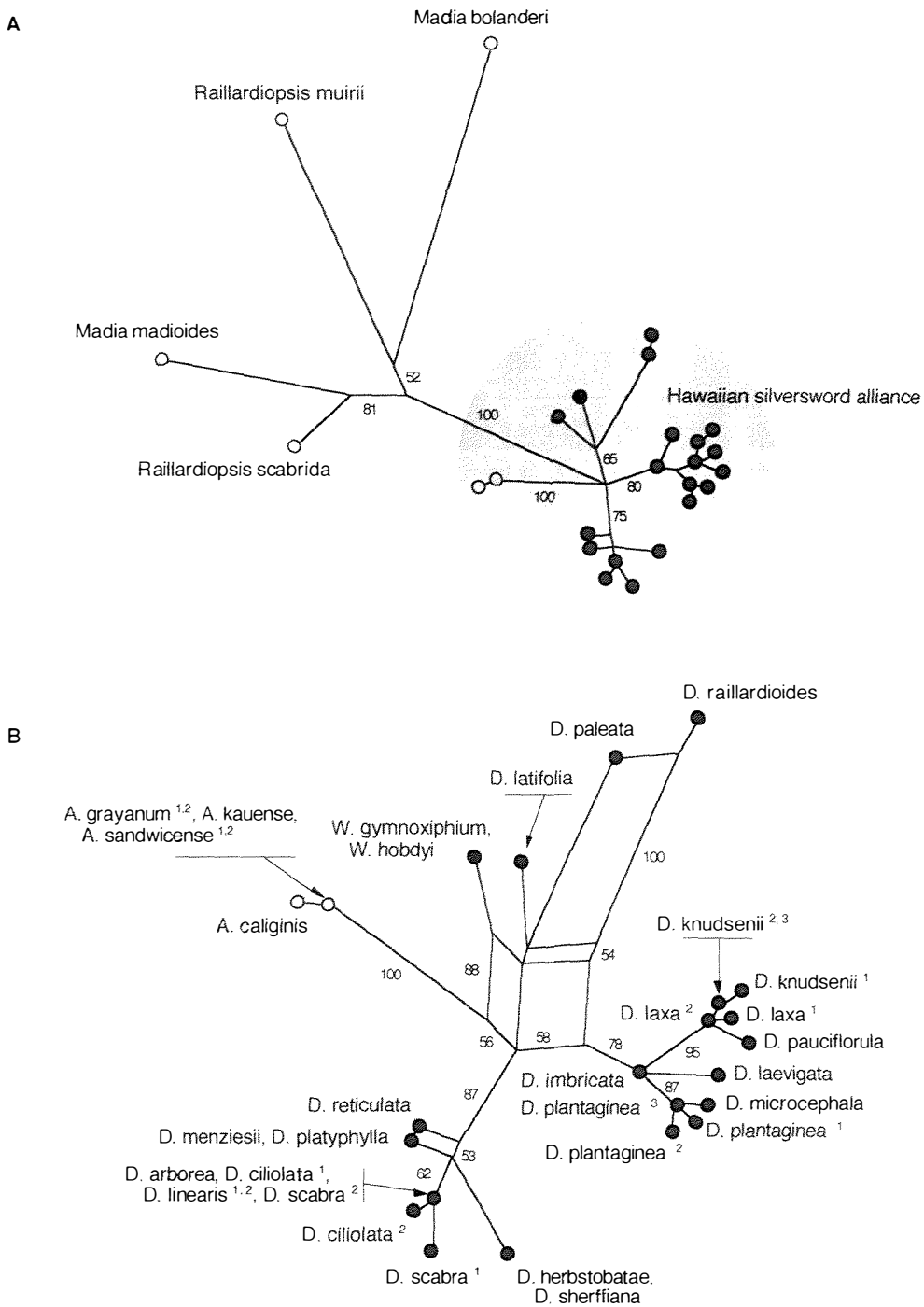


Figure 5.4: Splits-graphs (using Huson, 1998; SplitsTree 3.1) made using nuclear ITS data sets from representatives of the Hawaiian silversword alliance and outgroup genera. The three silversword genera are indicated – yellow, Argyroxiphium; blue, Dubautia; green, Wilkesia. **A**. Relationship of the silverswords to closely related species in the genera Madia and Raillardiopsis reconstructed from a data set of 583 nucleotides (Fit statistic = 74.3). **B**. Relationships within the silversword alliance reconstructed from a data set of 597 nucleotides (Fit statistic = 75.2). Eight taxa are represented by multiple accessions: A. grayanum – [1]East Maui, [2]West Maui; A. sandwicense – [1]subsp. sandwicense, [2]subsp. macrocephalum; D. ciliolata – [1]subsp. glutinosa, [2]subsp. ciliolata; D. knudsenii – [1]subsp. knudsenii, [2]subsp. filiformis, [3]subsp. nagatae; D. laxa – [1]subsp. hirsuta, [2]subsp. laxa; D. linearis – [1]subsp. linearis, [2]subsp. hillebrandii; D. plantaginea – [1]subsp. plantaginea, [2]Kaua'i , [3]subsp. humilis; D. scabra – [1]subsp. leiophylla, [2]subsp. scabra. Edge lengths in the graphs are optimised using a least-squares function (see Section 5.2.4). Bootstrap values (250 replicates) are given

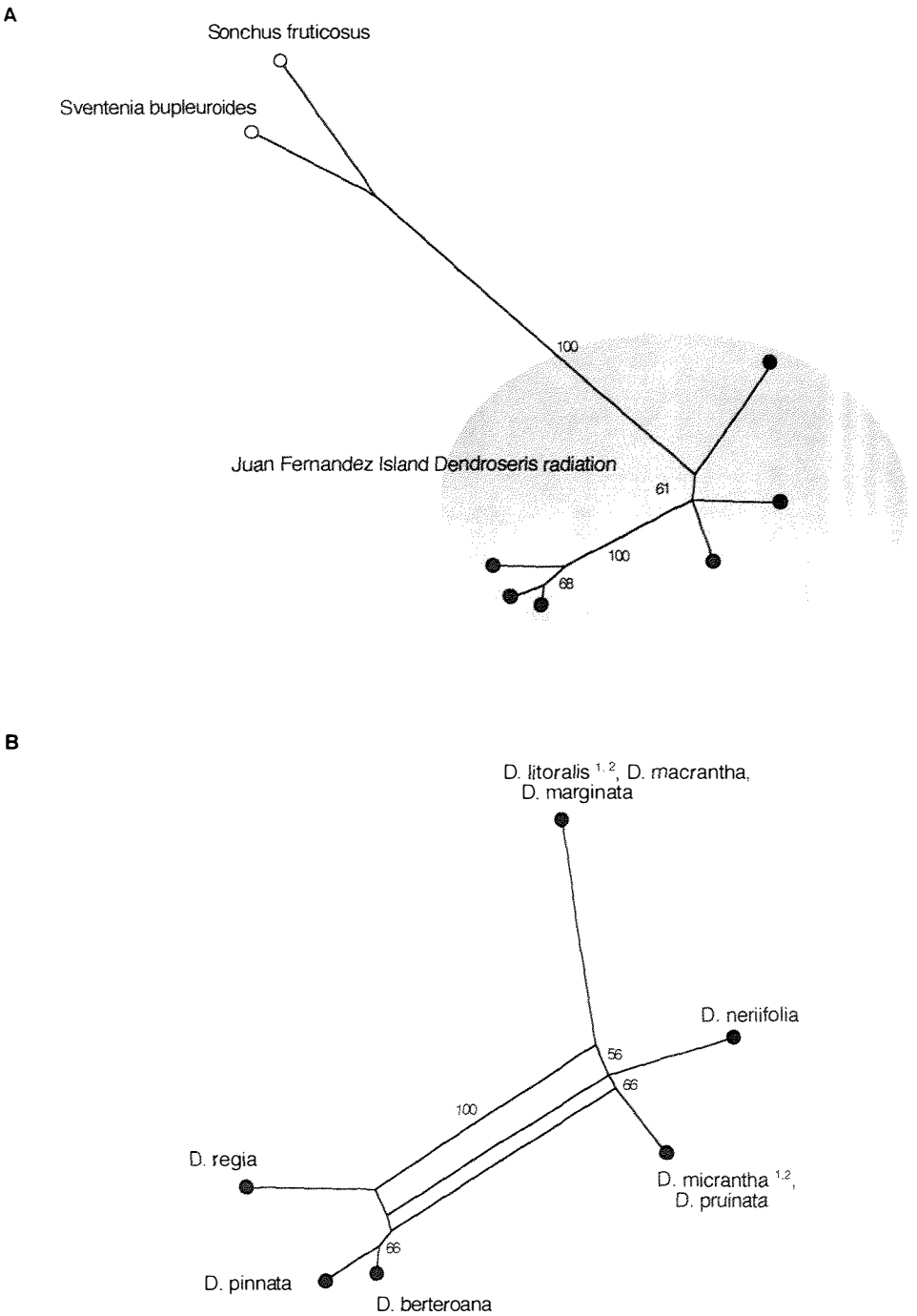
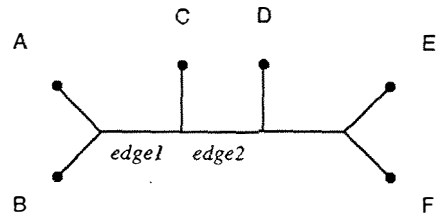


Figure 5.5: Splits-graphs (using Huson, 1998; SplitsTree 3.1) made using nuclear ITS data sets from representatives of the Juan Fernandez Island endemic genus Dendroseris and outgroup genera. **A**. Relationship of Dendroseris to species of the closely related genera Sonchus and Sventenia reconstructed from a data set of 463 nucleotides (Fit statistic = 87.4). **B**. Relationships within Dendroseris reconstructed from a data set of 470 nucleotides (Fit statistic = 100). Two taxa are represented by multiple accessions: D. litoralis – [1]cultivated, [2]Puerto Francés; D. micrantha – [1]Cerro Alto, [2]Puerto Francés. Edge lengths in the graphs are optimised using a *least-squares* function (see Section 5.2.4). Bootstrap values (250 replicates) are given.



$$edge1 = \min\left(\frac{d_{ac}+d_{bd}-d_{ab}-d_{cd}}{2};\frac{d_{ac}+d_{be}-d_{ab}-d_{ce}}{2};\frac{d_{ac}+d_{bf}-d_{ab}-d_{cf}}{2};\frac{d_{ad}+d_{bc}-d_{ab}-d_{cd}}{2};\right.$$
$$\frac{d_{ad}+d_{be}-d_{ab}-d_{de}}{2};\frac{d_{ad}+d_{bf}-d_{ab}-d_{df}}{2};\frac{d_{ae}+d_{bc}-d_{ab}-d_{ce}}{2};\frac{d_{ae}+d_{bd}-d_{ab}-d_{de}}{2};$$
$$\left.\frac{d_{ae}+d_{bf}-d_{ab}-d_{ef}}{2};\frac{d_{af}+d_{bc}-d_{ab}-d_{cf}}{2};\frac{d_{af}+d_{bd}-d_{ab}-d_{df}}{2};\frac{d_{af}+d_{be}-d_{ab}-d_{ef}}{2}\right)$$

$$edge2 = \min\left(\frac{d_{ad}+d_{be}-d_{ab}-d_{de}}{2};\frac{d_{ad}+d_{bf}-d_{ab}-d_{df}}{2};\frac{d_{ae}+d_{bd}-d_{ab}-d_{de}}{2};\frac{d_{ae}+d_{bf}-d_{ab}-d_{ef}}{2};\right.$$
$$\frac{d_{af}+d_{bd}-d_{ab}-d_{df}}{2};\frac{d_{af}+d_{be}-d_{ab}-d_{ef}}{2};\frac{d_{ad}+d_{ce}-d_{ac}-d_{de}}{2};\frac{d_{ad}+d_{cf}-d_{ac}-d_{df}}{2};$$
$$\frac{d_{ae}+d_{cd}-d_{ac}-d_{de}}{2};\frac{d_{ae}+d_{cf}-d_{ab}-d_{cf}}{2};\frac{d_{af}+d_{cd}-d_{ac}-d_{df}}{2};\frac{d_{af}+d_{ce}-d_{ac}-d_{ef}}{2};$$
$$\frac{d_{bd}+d_{ce}-d_{bc}-d_{de}}{2};\frac{d_{bd}+d_{cf}-d_{bc}-d_{df}}{2};\frac{d_{be}+d_{cd}-d_{bc}-d_{de}}{2};\frac{d_{be}+d_{cf}-d_{bc}-d_{ef}}{2};$$
$$\left.\frac{d_{bf}+d_{cd}-d_{bc}-d_{df}}{2};\frac{d_{bf}+d_{ce}-d_{bc}-d_{ef}}{2}\right)$$

Figure 5.6: *An example of the calculation of isolation indices for internal edges of a splits-graph. The more internal an edge is, the greater the number of quartets that need to be considered.*

5.2.5 Heteroplasmic sites

Currently information from heteroplasmic nucleotide positions, which may indicate that a taxon has hybrid origins (Sang *et al.*, 1995a; Aguilar *et al.*, 1999), cannot be represented in tree building analyses. Using bifurcating methods, one approach to this problem has been to derive a phylogenetic framework for non-hybrid species and then superimpose recognised hybrid taxa onto this tree (e.g. Sang *et al.*, 1995a). However, this requires that hybrids be identified prior to tree building and consequently the approach risks that the analyses may become subjective. While split-decomposition has the potential to represent data patterns that indicate reticulate evolution, at present split-decomposition also cannot represent the information at heteroplasmic sites. In the examples presented in Figures 5.2-5.5 a total of 56 heteroplasmic sites were removed from the analyses prior to constructing the splits-graphs. Clearly then the relationships within these groups are likely to be more complex than is represented visually in these graphs.

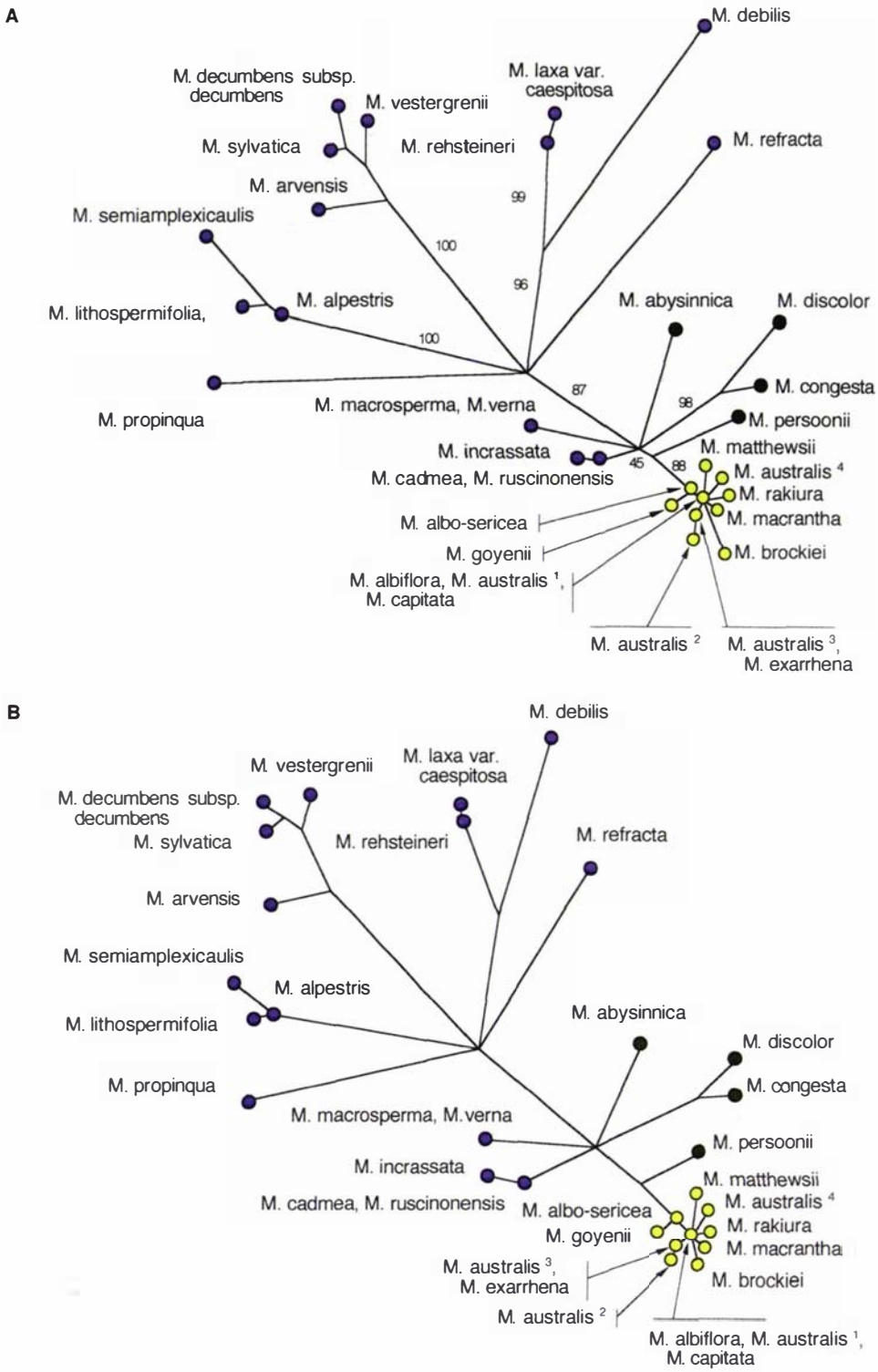


Figure 5.7: Splits-graphs (using Huson, 1998; SplitsTree 3.1) made using a data set of 610 nucleotides from the ITS region of the nuclear ribosomal DNA for 34 Myosotis taxa. **A**. Edge lengths estimated by the standard implementation of SplitsTree (i.e. the minimum value of the isolation index for a given split). Bootstrap values (250 replicates) are given for most internal edges. Fit statistic = 55.4. **B**. Edge lengths optimised using a least-squares function as implemented in SplitsTree 3.1. Subgeneric groups of Grau & Schwab (1982) are indicated – yellow, Section Exarrhena austral group; green, Section Exarrhena discolor group; blue, Section Myosotis. Distribution of M. australis accessions – [1]New Zealand; [2]Mt. Kozciuscko, Australia; [3]Tasmania, Australia; [4]New Guinea. Bootstrap values (250 replicates) for internal edges are given.

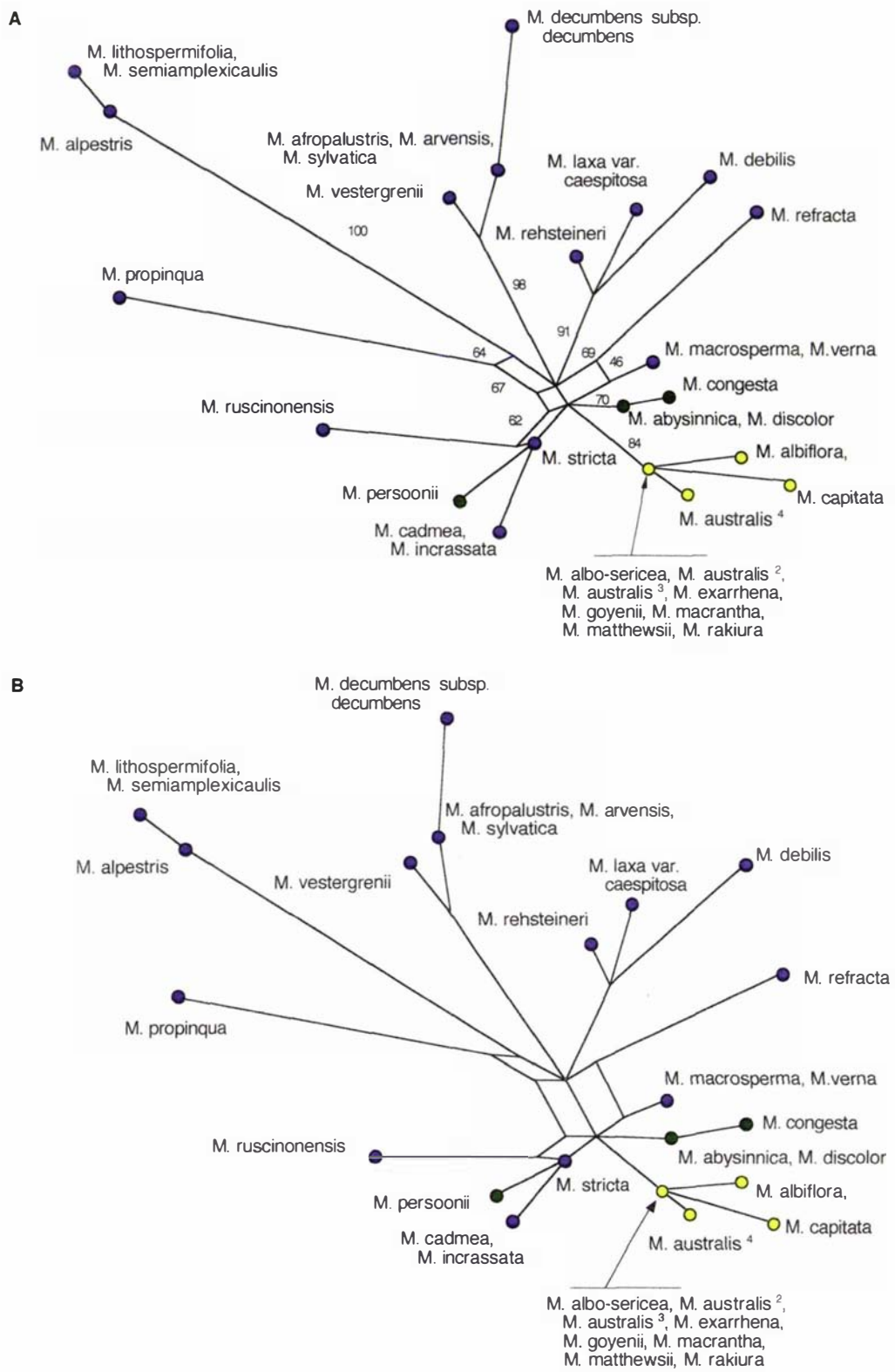


Figure 5.8: Splits-graphs (using Huson, 1998; SplitsTree 3.1) made using a data set of 867 nucleotides from the 3' region of the chloroplast maturase K gene locus for 34 Myosotis taxa. **A**. Edge lengths estimated by the standard implementation of SplitsTree (i.e. the minimum value of the isolation index for a given split). Bootstrap values (250 replicates) are given for most internal edges. Fit statistic = 81.2. **B**. Edge lengths optimised using a least-squares function as implemented in SplitsTree 3.1. Subgeneric groups and distribution of M. australis accessions are denoted as for Figure 5.7.

The development of coding schemes that allow interpretation of heteroplasmic nucleotide positions is required before accurate phylogenetic representations of hybrid data and Quaternary radiations can be made. One possible approach may be to re-code heteroplasmic positions as multiple data columns – each representing one sequence type. However, such schemes also need to consider the problem of over-representing some partitions in subsequent phylogeny reconstruction. This is an important area of future research.

5.3 DEVELOPING NOVEL MOLECULAR MARKERS FOR STUDYING RECENT SPECIES RADIATIONS

Given the potential complexity of evolutionary relationships between recently diverged taxa – hybridisation, introgression and polyploidy may characterise the evolution of such groups – molecular markers are needed that are both highly variable and distributed throughout plant genomes. This section describes observations and preliminary efforts in identifying markers for studying recently evolved plant groups.

5.3.1 Using DNA fingerprint profiles to locate sequence specific markers

Although DNA fingerprinting may provide a powerful assessment of genetic similarity, the interpretation of these data for phylogeny reconstruction is problematical. Much of the difficulty associated with the use of DNA fingerprint profiles in tree building analyses is due to uncertainty in homology assessment. That is, between closely related groups of individuals it may be possible to identify homologous fragments but with more distantly related taxa this is extremely difficult (Whitkus *et al.*, 1994; Mueller & Wolfenbarger, 1999). However, while DNA fingerprint analysis may be of limited use for reconstructing some phylogenies, these approaches provide very effective methods for identifying variable regions of plant genomes (Lou & Boutry, 1995; Lockhart & McLenachan, 1997). The approach that is outlined here utilises the sensitivity and reliability of AFLP to locate polymorphic genome regions from which sequence specific PCR markers – sometimes called "SCAR" or "STS" markers (e.g. Melotto *et al.*, 1996; Shan *et al.*, 1999; Schupp *et al.*, 1999) – can be derived.

Molecular markers of this type have several advantages when studying natural plant populations. Although DNA fingerprinting may provide fine scale resolution of genetic variation, screening large numbers of accessions is labour intensive. In contrast, AFLP derived markers can allow a large number of accessions to be screened far more rapidly. Typically, the effectiveness of AFLP is reduced when DNA quality is poor and this may limit the use of herbarium specimens – an important

source of material for rare or otherwise inaccessible taxa (McLenachan *et al.,* in press). However, as derived PCR markers amplify only one, or a few, genetic loci they are more likely to be useful in such situations.

5.3.2 Conversion of AFLP markers to sequence specific PCR markers

For this preliminary study, AFLP was used to locate regions of phylogenetic variation that would be potentially useful for resolving relationships between members of the Southern Hemisphere *Myosotis* lineage. AFLP profiles for three species – *M. albo-sericea*, *M. arnoldii* and *M. capitata* – were produced as described in Sections 2.10 and 2.11. Polymorphic AFLP markers were isolated from these profiles and used as templates for PCR. Following amplification, PCR fragments were cloned and sequenced. This information was then used to design pairs of specific oligonucleotide primers to target the ends of AFLP fragments. Using these primers the polymorphic regions were amplified from other *Myosotis* species, the PCR products being characterised by gel electrophoresis and DNA sequencing. A detailed description of the procedures used to convert AFLP fragments to sequence specific PCR markers is presented in Section 2.12.

A total of 25 polymorphic AFLP fragments were excised from polyarcylamide gels, of which only three failed to amplify using the *Mse* I and *Eco* RI pre-selective amplification primers. Of the fragments that were successfully re-amplified, six were chosen at random for further investigation – resulting in three markers that had promising levels of variation. The amplification primers for these three markers are given a Appendix V.

5.3.3 Characteristics of AFLP derived markers in Myosotis

MYOAT3 AND MYOGC5. For the derived markers MYOAT3 and MYOGC5, genetic variation was characterised with respect to the number and size of the alleles amplified. Figure 5.9 illustrates the variability observed for these markers in various New Zealand taxa and across a wider geographic selection of species from Section *Exarrhena*. When the different fragment profiles were simply treated as haplotypes, distinct groups could be identified within the austral lineage. Based on MYOAT3 profiles the austral taxa can be divided into eight groups, whereas four haplotype groups can be identified using MYOGC5. Both these markers were also amplified from several Northern Hemisphere taxa – subspecies of *M. sylvatica* and the closely related *M. decumbens* var. *decumbens* – displaying variation in size and number of alleles similar to that observed between Southern Hemisphere species (data not shown). In both of these markers the fragments amplified are presumed to be from homologous regions of the *Myosotis* genome.

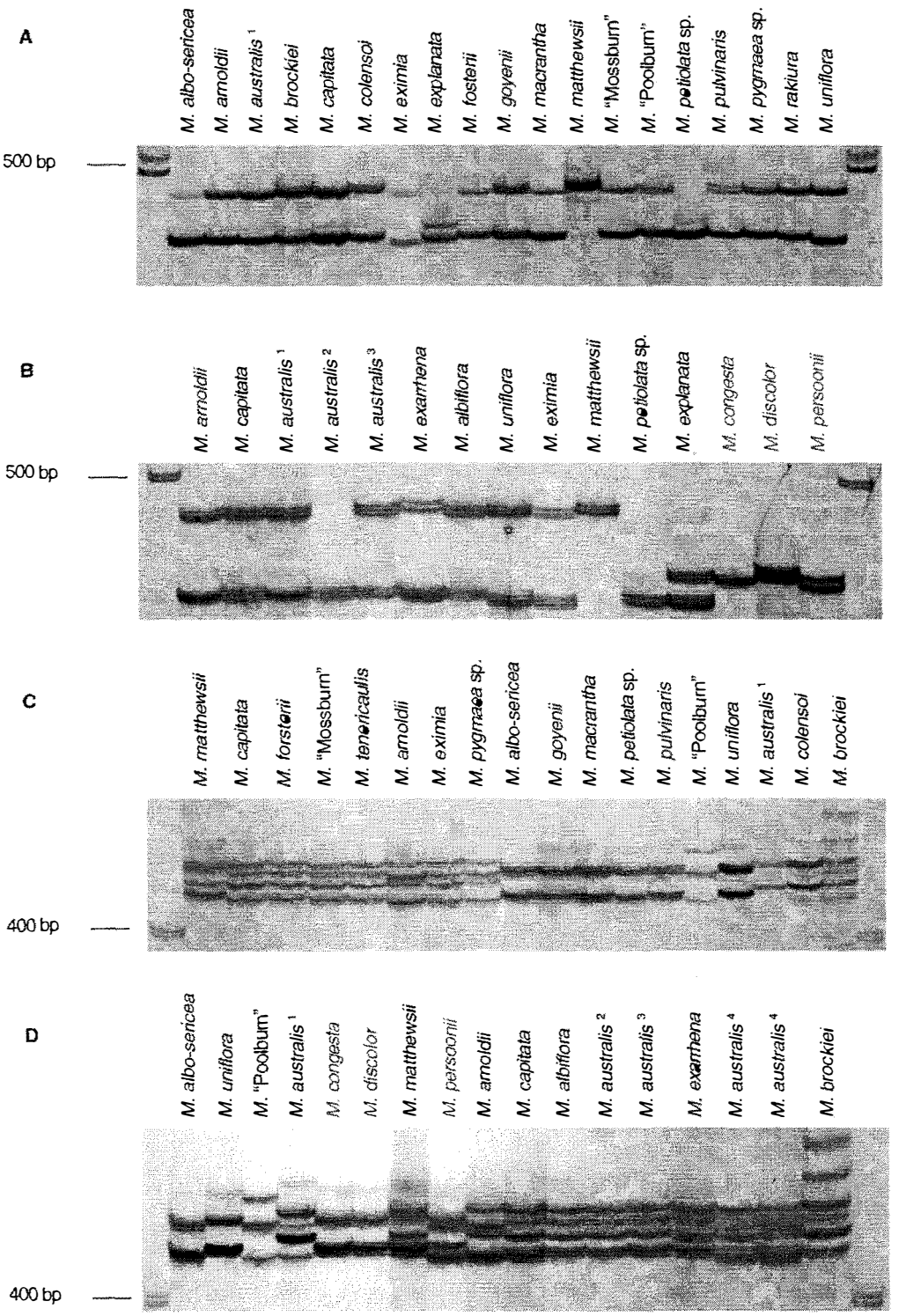


***Figure 5.9**: Electrophoretic profiles of amplified alleles from the AFLP derived markers MYOAT3 and MYOGC5. **A.** Variation in amplified alleles of MYOGC5 for New Zealand* Myosotis *species. **B.** Amplified allele variation in MYOGC5 for* Myosotis *Section Exarrhena. Accessions cover the geographical range of the austral group and extent of variation in New Zealand,* discolor *group taxa (labelled in green) included for comparison. **C.** Amplified allele variation in MYOAT3 are shown for a range of New Zealand* Myosotis *species. **D.** MYOAT3 allele variation for* Myosotis *Section Exarrhena. Accessions selected as in B. Samples were amplified, electrophoresed and visualised as described in Sections 2.11 and 2.12.7. Distribution of* M. australis *accessions are denoted as for Figure 5.7.*

MYOAT6. The single PCR product amplified for the sequence specific marker MYOAT6, was characterised by DNA sequencing in 17 Southern Hemisphere taxa (see Disc Appendix I). Excluding inferred indels, the sequence alignment was 35 nucleotides in length. Although 49 (13.8%) nucleotide positions were variable, most were either uninformative (43 singleton sites) or heteroplasmic in some taxa (9 sites). Only three parsimony informative sites were identified. Two of these sites support a split that partitions three New Zealand sequences (from *Myosotis capitata*, *M. explanata* and *M. macrantha*) away from the remaining sequences. The pattern at the third position is compatible with this split and groups the three Australian sequences (*M. australis* [Mt. Kozciuscko], *M. australis* [Tasmania] and *M. exarrhena*) with one from New Zealand (*M. brockiei*).

Polymorphic regions previously identified from AFLP profiles have been characterised by the presence of short repetitive motifs – both direct and inverted (Lou & Boutry, 1995; Lockhart *et al.*, in press; McLenachan *et al.*, in press). In the MYOAT6 sequences indel patterns were also associated with a repetitive structure. In most sequences a motif of 15 nucleotides was present as a single copy. However, this region was duplicated in *M. macrantha*, and in the Tasmanian accession of *M. australis* the motif was within a 22 bp deletion (relative to the most common allele type).

While the levels of variation observed for these AFLP derived markers are encouraging, the extent to which species and populations are fixed for this variation needs to be investigated before the phylogenetic significance of these patterns can be evaluated. Nevertheless, the levels of variation identified in these preliminary studies suggest that AFLP derived markers may provide tools for investigating population dynamics in New Zealand *Myosotis*.

5.3.4 Potential limitations of this approach

Although the conversion of polymorphic AFLP fragments to sequence specific PCR markers may produce potentially useful tools for studying recently radiated plant groups this approach does have limitations.

One potential limitation of this approach is that the method is labour intensive. If only a few taxa or individuals are to be screened then it may not be efficient to undertake this type of marker development. A further disadvantage is that the markers are likely to be transferable only among similar taxa – that is markers developed for a particular plant group are unlikely to be informative in unrelated genera. However, this may be a general problem when studying relationships in recently radiated groups.

5.3.5 Future development of approaches to locating sequence specific PCR markers for studying recent species radiations

The approach outlined here could be developed in several ways. One possible extension of this method would be to locate larger polymorphic regions. For this, the initial digestion of the genomic DNA could be done using single restriction enzymes with relatively low cutting frequency – possibly enzymes with recognition sites of six or eight nucleotides in length. Due to the greater size of such regions these may provide more informative variation than those described here (e.g. MYOAT3). Using other DNA fingerprinting techniques to identify polymorphic regions would be another possible development of this approach. A technique that may be promising in this respect is inter-simple sequence repeats (ISSR), which has recently been used to characterise patterns of patterns of reticulate evolution in several closely related lineages (e.g. Wolfe *et al.,* 1998; Beismann *et al.*, 1997).

CHAPTER 6: SYNTHESIS OF MOLECULAR STUDIES CONCERNING THE EVOLUTION OF THE NEW ZEALAND ALPINE FLORA

The flora of New Zealand has evolved in an insular environment characterised by a diverse array of habitats and a dynamic geological and climatic prehistory. The apparent contradiction between recent uplift of mountain habitats in New Zealand – historically an area of low geological relief and relatively warm climate – and a diverse alpine flora, has stimulated debate over the origins of New Zealand's alpine plant groups (Dawson 1971). Varying interpretations of character evolution, species relationships and distributions both between Southern Hemisphere lands and within New Zealand itself have added to the controversy (Webb, 1986). This uncertainty suggests that while traditional approaches to investigating the evolutionary history of the modern flora may provide important clues to New Zealand's prehistory, they are unlikely to resolve issues concerning the origins and evolution of the New Zealand alpine flora. However, molecular systematics provides a further technique for the examination of such questions, allowing evolutionary hypotheses concerning the history of particular taxonomic groups to be tested quantitatively (Carlquist, 1996). In this chapter a synthesis of recent molecular studies concerning the evolution of the New Zealand alpine flora is presented.

6.1 THE AGE AND ORIGINS OF THE NEW ZEALAND ALPINE FLORA

6.1.1 The age and origins of New Zealand alpine lineages

HYPOTHESES. The diversity of the New Zealand alpine flora has raised questions about the possible age of these lineages in New Zealand. Competing hypotheses have been extreme in suggesting a recent or ancient presence for groups now well represented in the flora. The scenarios proposed by Cockayne (1928), Fleming (1962, 1963) and Wardle (1963, 1968, 1978) all suggest that at least some modern alpine groups have a long history in the Southern Hemisphere. These authors propose the survival of a cool-adapted element through the warm Tertiary period either in New Zealand, where they inhabited open habitats (Cockayne, 1928; Wardle, 1968), or on cool temperate lands to the south (Wardle, 1963; Fleming, 1963). A long history in New Zealand has also been proposed for elements in the alpine flora that may be derived from lowland forest ancestors. These lowland groups are

suggested to have colonised alpine habitats following the uplift of the mountains during the late Tertiary and Quaternary (Dawson, 1963; Smith, 1986). A contrasting perspective is that most – if not all – of the New Zealand mountain flora arrived by transoceanic dispersal after the Pliocene-Pleistocene uplift of mountains in Australasia (Raven, 1973; Smith, 1986; Pole, 1994; Macphail, 1997). This hypothesis is consistent with the suggestion that strengthening of westerly winds during the Pliocene and Pleistocene provided increased opportunities for dispersal from Australia to New Zealand (Raven, 1973; Stewart & Neall, 1984; Smith, 1986).

MOLECULAR AND FOSSIL DATA. The molecular analyses reported in Chapters 3 and 4, as well as recent studies on other alpine plant groups – including *Hebe* (Wagstaff & Garnock-Jones, 1998), the Gnaphalieae (Breitwieser *et al.*, 1999) and *Ranunculus* (Lockhart *et al.*, in press) – are consistent with the hypothesis that some plant groups are recent additions to the New Zealand biota, most likely arriving during the Pliocene or Pleistocene. This conclusion is corroborated by the observation that many alpine plant genera have appeared in the New Zealand palynological record only since the Pliocene (Fleming, 1979; Mildenhall, 1980). Although transoceanic dispersal seems to provide an explanation for the occurrence of these groups in the New Zealand mountains, the general significance of this phenomenon for the establishment of the alpine flora remains largely unknown. Certainly the current evidence – both molecular and otherwise (see Smith, 1986) – suggests that long distance dispersal events have strongly influenced the composition of the modern New Zealand alpine flora. However, many of the small, taxonomically isolated groups (e.g. *Haastia*, *Hectorella*, and *Phyllachne*) that have been suggested as representing ancient lineages in the mountain flora remain unstudied. It will be interesting to see whether future molecular systematic investigations of these provide evidence for vicariant evolutionary histories.

6.1.2 Could dispersal account for the presence of all extant plant groups in New Zealand?

The argument of Pole (1994) and Macphail (1997) is extreme in suggesting that all extant New Zealand plant lineages – both forest and alpine – have arrived from Australia by long distance dispersal since the Miocene. These authors base their argument on the expectation that extant, or morphologically similar forms, should occur in the pre-Miocene fossil record if the current flora is truly of ancient origin. Since this continuity has not been observed, Pole and Macphail conclude that all plant taxa have arrived in New Zealand recently. However, this conclusion should be treated carefully.

Firstly it should be pointed out that the rapid and profound morphological differentiation observed in many of the genera studied (see Section 6.2.1) cautions against over-interpreting the paleobotanical evidence. A good example is seen in *Myosotis*. In this genus, a greater diversity of vegetative form and pollen type occurs between austral group species than between Northern Hemisphere taxa despite the austral lineage being more recently evolved. Given that dramatic species diversification has apparently been a common phenomenon in New Zealand during the last five million years, it is questionable whether extant taxa would necessarily be recognised in the fossil record. Hence, it is possible that lineages present in New Zealand at the end of the Miocene may have undergone profound morphological changes. If they did, this could make them unrecognisable in the fossil record and also make the expectation of Pole and Macphail unrealistic. This may be particularly so, given that the record is limited by infrequent preservation and low taxonomic resolution (Macphail, 1997).

Pole's (1994) hypothesis that, following the Oligocene marine transgression, the entire New Zealand biota was replaced by dispersed taxa is also questionable when viewed against other data. Geological evidence suggests the occurrence of at least a small amount of emergent land during the Oligocene drowning (Cooper & Cooper, 1995). Even if only a few small, low-lying islands remained at the height of the Oligocene marine transgression it is expected that these would have permitted the survival of at least certain elements of the terrestrial biota. The continuous fossil record provided by Macphail (1997) also suggests a similar conclusion. Further, the occurrence of obviously very ancient austral groups at lower elevations in modern New Zealand is also of importance. Examples of such groups include seed plants like certain members of Araucariaceae, Podocarpaceae, Winteraceae (Suh *et al.*, 1993; Karol *et al.*, 1999), Proteaceae (Hoot & Douglas, 1998), and *Nothofagus* (Manos, 1997), or animals such as *Leiopelma* (frogs), *Sphenodon* (tuatara) and some terrestrial gastropods (Cooper & Millener, 1993). Interpreting these as continuous lineages in the New Zealand region is clearly more parsimonious than invoking events of long distance dispersal (Linder & Crisp, 1995).

The molecular data, when viewed with the fossil and geological evidence, casts doubt on Pole's (1994) hypothesis for the complete disappearance of the terrestrial New Zealand biota during the Oligocene and it's *de novo* formation by long distance arrivals in the Miocene. Certainly some ancient lineages will have suffered extinction, however, the environmental crises that punctuated the Oligocene and later the Pliocene and Pleistocene would have caused the loss of both long-standing inhabitants and recent colonists alike. Although both extinction and dispersal have been of great importance in the evolution of the New Zealand flora it seems unlikely that all pre-Oligocene lineages have been preferentially lost and replaced by recent immigrants.

6.1.3 The importance of New Zealand in Southern Hemisphere dispersal

Various lines of evidence – palynology (Mildenhall, 1980; Pole, 1994), chromosome analyses (Godley, 1967, Sykes & Godley, 1968), morphological and biochemical studies (Godley, 1967; Murray, 1986; Gamock-Jones, 1993; Swenson & Bremer, 1997) – provide support for the suggestion that dispersal has been important for establishing the current distributions of many Australasian and Pacific plant groups. However, the role of New Zealand in Southern Hemisphere dispersal patterns has been controversial, particularly with respect to westward dispersal from New Zealand to Australia. Raven (1973) considered that westward dispersal from New Zealand was highly unlikely due to the prevailing West Wind Drift. In contrast, both Fleming (1976) and Wardle (1978) have argued for at least a limited flow of plant material in an easterly direction. Recently, the increasing number of DNA sequencing studies – summarised in Table 6.1 – have allowed hypotheses concerning dispersal in the Southern Hemisphere to be investigated further.

Phylogenetic analyses of DNA sequences from many plant groups suggest the importance of westerly winds for dispersal in the Southern Hemisphere. However, the patterns of relationship in some groups, including those of the genus *Myosotis* reported in this thesis, cannot be explained by West Wind Drift. The molecular analyses provide compelling evidence that for *Myosotis* transoceanic dispersal from New Zealand has occurred in many directions – including westward to Australia and northwest to New Guinea, in both these cases against the West Wind Drift. Phylogenetic analyses of nuclear ITS sequences also suggest that during the late Tertiary and Quaternary westward long distance dispersal has occurred for the Australasian apioid Umbelliferae, *Hebe, Chionohebe, Parahebe, Ranunculus* and *Gentiana* (see Table 6.1). The finding that dispersal has occurred against the West Wind Drift may not be surprising given that anticyclones in the southern Pacific frequently produce easterly airflow between New Zealand and Australia (Wardle, 1978). Further, the potential for such dispersal events has recently been demonstrated by a manned balloon flight from New Zealand to Australia (Wallington, 2000). For each taxon the precise mechanism of dispersal will have depended on the morphological characteristics of the fruits or seeds. In some, easterly winds may have actively dispersed diseminules to Australia, while for groups that lack any obvious adaptations for wind dispersal (e.g. *Myosotis*) this seems an unlikely mechanism. In plant groups where the opportunities for wind dispersal appear to be limited by seed or fruit characteristics, vectors such as birds may have facilitated dispersal. Indeed bird-mediated dispersal has been favoured as a mechanism for transoceanic dispersal of diseminules in several plant groups (Godley, 1967; Carlquist, 1996; Swenson & Bremer, 1997)

In contrast to earlier suggestions that New Zealand represents an isolated Gondwanan refuge, fossil evidence and recent genetic studies indicate that New

Zealand has received and supplied many immigrants since the break-up of the southern supercontinent. However, the evolutionary significance of these events will have depended on the opportunities for establishment following transoceanic dispersal. Prior to the late Tertiary, opportunities for the establishment of founding populations may not have existed to the same extent as in more recent geological time. The dramatic geological and climatic changes of the last 5 MY are likely to have

TABLE 6.1
Recent molecular studies that have inferred long distance dispersal within the Southern Hemisphere

Plant Family	Tribe/Genus	Direction of dispersal[a]	Reference
Nothofagaceae	*Nothofagus*	Australia → New Zealand	Martin & Dowd (1993)
Asteraceae	Gnaphalieae	Australia → New Zealand Australia → New Guinea	Breitwieser *et al.* (1999).
	Microseris	Australia ↔ New Zealand	Vijverberg *et al.* (1999)
Fabaceae	*Sophora*	New Zealand → Pacific islands New Zealand → south Atlantic islands New Zealand → subantarctic islands	Hurr *et al.* (1999)
	Montigena	Australia → New Zealand	Wagstaff *et al.* (1999)
	Carmichaelia	New Zealand → Lord Howe Island	Wagstaff *et al.* (1999)
Viscaceae	*Korthalsella*	Australia → New Zealand	Molvray *et al.* (1999).
Stylidiaceae	*Oreostylidium*	Australia → New Zealand	Laurent *et al.* (1999)
Corynocarpaceae	*Corynocarpus*	New Guinea → New Zealand	Wagstaff & Dawson (2000)
Myrtaceae	*Metrosideros*	New Zealand → Pacific islands	Wright *et al.* (2000)
Brassicaceae	*Cardamine*	Australia → New Zealand	Mitchell & Heenan (2000)
Ranunculaceae	*Ranunculus*	New Zealand → Australia New Zealand → subantarctic islands	Lockhart *et al.* (in press)
Tetrachondraceae	*Tetrachondra*	South America → New Zealand	Wagstaff *et al.* (in press)
Scrophulariaceae	*Hebe*	New Zealand → South America New Zealand → subantarctic islands	Wagstaff & Garnock-Jones (in press), S. Wagstaff (pers. comm.)
	Parahebe	New Zealand → New Guinea	Wagstaff & Garnock-Jones (in press)
	Chionohebe	New Zealand → Australia	Wagstaff & Garnock-Jones (in press)
Gentianaceae	*Gentianella*	New Zealand → Australia South America → New Zealand	D. Glenny (pers. comm.)
Apiaceae	*Aciphylla*	New Zealand ↔ Australia New Zealand ↔ Chatham Islands	This thesis
	Anisotome	New Zealand ↔ Australia New Zealand ↔ subantarctic islands	This thesis
Boraginaceae	*Myosotis*	New Zealand → New Guinea New Zealand → South America New Zealand → Australia New Zealand → subantarctic islands	This thesis

Note: (*a*) Single-headed arrows indicate the inferred direction of dispersal and double-headed arrows indicate regions are linked by long distance dispersal but that the direction of dispersal was unresolved.

created many available niches – these habitats may well have allowed the establishment of founding populations following transoceanic dispersal. In some cases, groups that have established in New Zealand following long distance dispersal have acted as sources for subsequent dispersal to other Southern Hemisphere lands (e.g. *Myosotis*). Such events have resulted in the establishment of novel species in disjunct Southern Hemisphere locations.

6.2 RAPID MORPHOLOGICAL DIFFERENTIATION IN GENERA OF THE NEW ZEALAND ALPINE FLORA

6.2.1 Recent evolution of plant biodiversity in insular environments

Several botanical researchers have speculated that some genera, well represented in the New Zealand alpine zone, speciated to occupy alpine and sub-alpine habitats newly formed during the Pliocene and Pleistocene (Wardle, 1968; Raven, 1973). A growing number of molecular studies involving speciose genera, which occur predominantly in New Zealand's mountain areas, exhibit patterns of radiation consistent with these suggestions. In these groups, despite large amounts of morphological differentiation and the development of strong ecological preferences, little DNA sequence variation has been observed in commonly assayed marker loci. The low levels of genetic diversity observed within these New Zealand groups, which contain diverse and conspicuous forms, suggests that their morphological diversity has arisen relatively recently. In addition to the examples studied this work, similar patterns of evolution have been described for *Hebe* (Wagstaff & Garnock-Jones, 1998), *Gingidia* (Mitchell *et al.*, 1998), the Gnaphalieae (Breitwieser *et al.*, 1999), *Carmichealia* (Wagstaff *et al.*, 1999), and *Ranunculus* (Lockhart *et al.*, in press). The observation, from the molecular data, that New Zealand alpine plant genera have diversified only recently may explain the high incidence of hybridisation in the New Zealand alpine flora. Morphologically well differentiated and geographically separate taxa are often interfertile – presumably despite morphological differences there has been insufficient time for the development of genetic barriers to hybridisation (Wardle, 1963).

Observations indicating the rapid morphological diversification of plant groups in New Zealand parallel those of plant diversification on other island archipelagos. Examples include *Bidens* (Carr, 1987) and the Silversword alliance (Robichaux *et al.*, 1990; Baldwin, 1992) from the Hawaiian Islands, *Robinsonia* (Sang *et al.*, 1995b) and *Dendroseris* (Sang *et al.*, 1994) from the Juan Fernandez Islands and on the Macaronesian Islands *Echium* (Böhle *et al.*, 1996) and *Sonchus* (Kim *et al.*, 1996). For most of the island plant groups studied the morphological, biochemical, cytological and

genetic evidence supports a hypothesis of recent transoceanic dispersal and establishment of the lineage by a single ancestor – or possibly a small number of genetically very similar individuals – followed by rapid evolution. In many cases the available evidence suggests that diversification of plants in insular habitats has been adaptive and is characterised by elevated speciation rates, increased morphological diversity, and marked differentiation of ecological characteristics (Givnish, 1997).

6.2.2 Evolution of specialised forms in the New Zealand alpine flora

In general, alpine plants have adaptations allowing survival in harsh mountain environments. In New Zealand the great diversity of alpine habitats is likely to have provided opportunities for the evolution of specialised alpine types. Many examples occur, including species with small coriaceous leaves (e.g. *Celmisia brevifolia*, *Ourisia glandulosa*, and *Ranunculus pachyrrhizus*), cushion growth forms (e.g. *Myosotis uniflora, Phyllachne colensoi*, and *Raoulia eximia*) and in scree plants, well developed root systems (e.g. *Anisotome carnosula*, *Lobelia roughii* and *Ranunculus haastii*) (Fisher, 1952; Allan, 1961; Mark & Adams, 1995). Phylogenetic analyses of molecular data for groups containing species with such morphological features are consistent with the evolution of these characters since the onset of mountain building during the Pliocene (e.g. Breitwieser *et al*., 1999; Lockhart *et al*., in press; this thesis).

In the New Zealand biota, an interesting question has been whether the colourful flower displays of some subantarctic island endemics – such as *Myosotis capitata* and *Anisotome latifolia* – are ancestral or more recently derived from less conspicuous relatives found in the alpine areas of mainland New Zealand. Wardle (1978) proposed that in these groups, coloured flowers represented a non-adaptive and primitive state from which the white-flowered mainland forms have evolved. Alternatively, the wide taxonomic distribution of this feature, the relative youth of the groups involved and the apparent recent origin of the subantarctic floras has been interpreted as suggesting the recent evolution of coloured subantarctic island forms (Lloyd, 1985). In the phylogenetic trees reported for *Myosotis* and the Australasian Apioideae, the subantarctic island species occupy relatively derived position within a predominantly New Zealand radiation – a pattern consistent with a recent origin of these species. Similar patterns of recent origin have also been reported for subantarctic island species of *Sophora* (Hurr *et al*., 1999), *Ranunculus* (Lockhart *et al*., in press) and *Hebe* (S. Wagstaff, pers. comm). However, in these groups the subantarctic island forms do not have distinctively coloured flowers.

6.2.3 Plant developmental genetics: A basis for understanding Pliocene and Pleistocene speciations

Recent advances in understanding plant developmental genetics may explain the rapid diversification of plant groups within the New Zealand alpine zone. Evidence from both natural populations and domesticated crops suggests that, in some cases, fundamental changes in plant morphology can arise from relatively small amounts of genetic change (Comes, 1998). A growing number of studies suggest that changes at a few gene loci, each with large and non-additive effects, can lead to dramatic morphological differences between recently evolved plant species (Bradshaw *et al.*, 1995; Bradley et *al.*, 1997; Doebley *et al.*, 1997; Comes, 1998). For example in *Senecio vulgaris* L. (Asteraceae) the features that characterise an ecological shift to a weedy habit (i.e. lack of seed dormancy, early flowering and discoid flowers) are controlled by three single gene loci (Comes, 1998). Similarly genetic evidence suggests that the changes to stem architecture and the morphology of reproductive structures associated with the domestication of maize from it's wild progenitor, teosinte, are under the control of five quantitative trait loci (Doebley *et al.*, 1997). Epigenetic mutations, which are not necessarily accompanied by changes in DNA sequence, may also represent a significant process in the evolution of plant form. Cubas *et al.* (1999) identified a naturally occurring *Linaria vulgaris* mutant where changes to the degree of methylation at a specific locus result in a fundamental change in floral symmetry. Although, these authors note that this mutation type is susceptible to somatic reversion, they suggest that the increased mutation rate and effects on local recombination associated with DNA methylation may result in longer term evolutionary effects (Cubas *et al.*, 1999).

Given that only minor alterations in genetic structure can produce substantial morphological changes, and that such changes may occur over relatively short periods of time, these mechanisms presumably provide a means for plants to adapt rapidly to changing environmental and competitive pressure. Further, if such mechanisms operate, it may not be surprising to find that dramatic morphological evolution of groups in the New Zealand alpine flora has been accompanied by little genetic change.

6.2.4 Have hybridisation and polyploidy had roles in the rapid evolution of the New Zealand alpine flora?

It has been suggested for some time that hybridisation and polyploidy may have important roles in plant speciation (e.g. Ehrendorfer, 1958; Rattenbury, 1962; Stebbins, 1984; Arnold, 1997). Recent studies suggest that these phenomena may

help provide an explanation for the recent patterns of radiation observed in New Zealand and elsewhere.

HYBRIDISATION. The general consequences of hybridisation and introgression in plants have been well debated and it is now widely recognised that hybridisation is an important source of novel, stable evolutionary lineages (Abbott, 1992; Rieseberg & Wendel, 1993; Arnold, 1992, 1997). Indeed, molecular studies on hybridisation have concluded that hybrid formation may facilitate rapid speciation (Rieseberg, 1997; Ungerer *et al*., 1998). Given the frequent occurrence of hybridisation in New Zealand alpine genera – reported for *Ranunculus* (Fisher, 1965), *Leptinella* (Lloyd, 1972), *Epilobium* (Raven, 1972; Raven & Raven, 1976), the Australasian Apioideae (Webb & Druce, 1984), and the Gnaphalieae (Ward, 1997) – it has been suggested that this phenomenon has been of considerable evolutionary importance in the New Zealand mountain flora (Raven, 1972, 1973).

The frequency of hybrids in the New Zealand alpine flora may be explained in terms of the profound changes in climate and geology that occurred during the Pliocene and Pleistocene. Hybrids often occupy sites outside the geographical or ecological range of parental species. This has been attributed to the benefits of fixed heterotic genotypes, reduced mutational load and the increased genetic variation and evolutionary novelty associated with hybridisation (Abbott, 1992; Ellstrand & Schierenbeck, 2000). If so, it is possible that hybrid forms may have been best able to take competitive advantage of the novel microhabitats that arose in Pliocene and Pleistocene New Zealand. Alternatively, environmental changes may simply have provided new space for the survival of hybrid lineages by creating open niches in which hybrid types could have escaped competition from ancestral forms (Arnold, 1997; Rieseberg, 1997).

POLYPLOIDY. Polyploidy is considered to be an important phenomenon in the evolution of angiosperms – estimates suggest that perhaps 70% of flowering plant lineages have undergone polyploidisation during their evolution (Soltis & Soltis, 1999). However, the observed rarity of polyploidy in oceanic island floras suggests that this phenomenon has been of little importance in the evolution of island plant groups (Stuessy & Crawford, 1995; Crawford & Stuessy, 1997).

In New Zealand, polyploidisation has occurred in various plant groups, although few polyploid series – a group of closely related lineages (usually species or populations) which are primarily differentiated by ploidy level – have been reported (Hair, 1966; Connor, 1985). This observation is particularly noticeable in genera that elsewhere have numerous polyploid types; one example is the genus *Myosotis* (Grau, 1975; Beuzenberg & Hair, 1983). If polyploidy had played a significant role in the evolution of New Zealand plant groups it may be expected that

polyploids and, in particular, polyploid series would be more common. Rather the relatively limited occurrence of polyploidy in New Zealand plants suggests that this phenomenon has played only a minor role in the diversification of New Zealand plant genera (Raven, 1973).

6.2.5 The importance of environmental change in the evolution of alpine biodiversity in New Zealand

It is unclear whether environmental change merely creates or restricts suitable environments for speciation or whether it also biases the outcome of biological processes by directly affecting biotic factors such as competition and gene flow through pollen and seed dispersal. Certainly, the expansion of subalpine and alpine habitats in New Zealand during the Pliocene and Pleistocene could be expected to have provided numerous opportunities for species radiations to occur (Raven, 1972). It has been suggested that for many recent species radiations, both in New Zealand and elsewhere, the primary factor in morphological diversification has been geographical and ecological isolation rather than the formation of pre-zygotic barriers (Wardle, 1963; Crawford & Stuessy, 1997). Spatial isolation may be of particular importance for speciation in alpine habitats, where mountains could be expected to provide a significant geological barrier to gene flow between genetically similar and geographically close populations. One hypothesis suggests that following the onset of mountain building in New Zealand, allopatric populations became established and that these diversified into local, novel habitats (Wardle, 1963). The intense selective regimes imposed by fluctuating Pliocene and Pleistocene environments may have then resulted in morphological differentiation of geographically isolated populations without the development of genetic barriers. Alternatively, if population sizes were small, stochastic factors may also have been important in this process.

The cyclic nature of climate change during the Pliocene and Pleistocene may have been an important factor in the diversification of alpine plant lineages. Geological and palynological evidence indicates that the glacial and interglacial episodes of the last 2.5 MY resulted in repeated expansion and contraction of alpine habitats in New Zealand. Changes to the distributions of individual taxa and hence their interactions with related forms could also be expected to have accompanied climate fluctuations. Ehrendorfer (1958, 1959) and Stebbins (1984) have both suggested that the evolution of novel plant species is a cyclic process characterised by two stages (a) fragmentation of populations allowing for genetic differentiation in allopatry; (b) coalescence of population isolates leading to the formation of novel genetic variants through hybridisation, introgression and polyploidy. In particular the "secondary contact hypothesis" (Stebbins, 1984) emphasises the importance of Quaternary climate change in the diversification of plant groups in the Northern Hemisphere arctic-

alpine floras. Certainly this model is consistent with the available evidence for the evolution of New Zealand alpine plant genera.

The observation from molecular data that the extant diversity of New Zealand alpine plant groups can be correlated to late Tertiary and Quaternary events is consistent with results from genetic studies on continental floras of Europe and North America. These investigations tend to suggest that environmental change during the late Tertiary and Quaternary periods, both at global and local scales, has been of great importance in the development of the world's modern floristic biodiversity (Comes & Kadereit, 1998).

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APPENDIX I: *Myosotis* and outgroup accessions

APPENDIX TABLE 1
Details of accessions of *Myosotis* and outgroup taxa

Taxon	Location	Herbarium reference
Borago officinalis L.	Palmerston North, North Island, New Zealand (naturalised introduction)	MPN 24675
Echium vulgare L.	Lake Ohau, South Island, New Zealand (naturalised introduction)	MPN 24676
Eritrichium nanum (L.) Schrader ex Gaudin	Switzerland	Hertel 25764 [a]
Myosotidium hortensia (Decne) Baill.	Palmerston North, North Island, New Zealand	No voucher (nursery origin)
Myosotis abyssincia Boiss. & Reuter	Ethiopia	De Wilde 6944 [a]
Myosotis afropalustris C.H.Wr.	Natal, South Africa	K.Balkwill & M.J.Balkwill 5260 [a]
Myosotis albiflora Banks & Sol.	Punta Arenas, Chile	Voucher in prep.
Myosotis albo-sericea Hook. f.	Leaning Rock, South Island, New Zealand	Site voucher at CHR [b]
Myosotis alpestris F.W.Schmidt	Karten, Hochobir, Austria	F. Erhendorfer
Myosotis arnoldii L. Moore	Mt. Ben More, South Island, New Zealand	Site voucher at CANU
Myosotis arvensis (L.)Hill	Bavaria, Germany	H. Gröger 1018b
Myosotis australis R. Br.	Tasmania, Australia	MPN 24677
Myosotis australis R. Br	Mt. Kozciuscko, Australia	MPN 24678
Myosotis australis R. Br (identified as *M. saruwagedica*)	New Guinea	CHR 198545
Myosotis australis R. Br "Yellow"	Mt. Ben More, South Island, New Zealand	Site voucher at CANU
Myosotis brockiei L. Moore et M. Simpson	Cobb Gorge, South Island, New Zealand	Voucher in prep.
Myosotis cadmea Boiss.	Greece	Stainton 7320 [a]
Myosotis capitata Hook. f.	Cultivated (B. Rance)	Voucher in prep.
Myosotis colensoi (Kirk) Macbride	Castle Hill, South Island, New Zealand	Site vouchers at CANU and CHR
Myosotis congesta Shuttlew. ex Alb. & Reynier	Greece	Phitos M-33 [a]
Myosotis debilis Pomel	Spain	Gomez Vigide 317 [a]
Myosotis decumbens Host. ssp. *decumbens*	Alpi Maritime, Schönswetter & Tribsch	F. Erhendorfer
Myosotis discolor Pers.	Lake Lyndon, South Island, New Zealand (naturalised introduction)	Voucher in prep.
Myosotis exarrhena (R. Br.) F. Mueller	South-eastern NSW, Australia	CBG 9519354
Myosotis eximia Petrie	Reporoa Bog, North Island, New Zealand	MPN 24679
Myosotis explanata Cheesem.	Otira Valley, South Island, New Zealand	No voucher
Myosotis forsteri Lehm.	Poolburn Reservoir, South Island, New Zealand	MPN 24680
Myosotis goyenii Petrie	Broken River, South Island, New Zealand	Site voucher at CANU

APPENDIX TABLE 1
Details of accessions of *Myosotis* and outgroup taxa (Continued)

Taxon	Location	Herbarium reference
Myosotis incrassata Guss.	Greece	Merxmüller & Wiedmann 20130 [a]
Myosotis laxa Lehm. ssp. *caespitosa* (C.F.Schultz) Hyl.	Hopkins River, South Island, New Zealand	MPN 24681
Myosotis lithospermifolia Hornem.	Persia	Rechinger 6521 [a]
Myosotis macrantha (Hook. f.) Benth. et Hook. f.	Hooker Valley, South Island New Zealand	Site voucher at CANU
Myosotis macrosperma Engelm.	USA	Voucher in prep.
Myosotis matthewsii L. Moore	Cultivated (Percy's Reserve, Wellington)	Voucher in prep.
Myosotis sp. "Mossburn"	Mossburn, South Island, New Zealand	MPN 24682
Myosotis persoonii Rouy	Spain	Zubizarreta 5327 [a]
Myosotis petiolata Hook. f. var. *pottsiana*	Opotiki, North Island, New Zealand	MPN 24683
Myosotis sp. "Poolburn"	Poolburn Reservoir, South Island, New Zealand	MPN 24684
Myosotis propinqua Fisch. & Mey.	Persia	Rechinger 39832 [a]
Myosotis pulvinaris Hook. f.	Old Man Range, South Island, New Zealand	Voucher in prep.
Myosotis pygmaea var. *pygmaea*	Whanganui Inlet, South Island, New Zealand	MPN 24685
Myosotis scorpioides L.	Bavaria, Germany	Walter 183b [a]
Myosotis rakiura L. Moore	Curio Bay, South Island, New Zealand	MPN 24686
Myosotis refracta Boiss. ssp. *refracta*	Greece	Gröger 1463a [a]
Myosotis rehsteineri Wartm.	Bavaria, Germany	Dörr [a]
Myosotis ruscinonensis Rouy	France	Kunz& Reichstein M-310 [a]
Myosotis semiamplexicaulis DC.	South Africa	Acocks 21310 [a]
Myosotis stricta Link	Bavaria, Germany	Förther 7895 [a]
Myosotis sylvatica Ehr. ex Hoffm. ssp. *sylvatica*	Palmerston North, North Island, New Zealand (naturalised introduction)	MPN 24687
Myosotis tenericaulis Petrie	Volcanic Plateau, North Island, New Zealand	Voucher in prep.
Myosotis uniflora Hook. f.	Hopkins River, South Island, New Zealand	MPN 24688
Myosotis verna Nutt.	USA	Taylor 3489 [a]
Myosotis vestergrenii Stroh	Ethiopia	MO 3808817
Plagiobothrys albiflorus (Griseb.) R.L. Pérez-Mor.	Paso Cardenal Samore, Argentina	MPN24689
Symphytum × *uplandicum* Nyman	Wellington, North Island, New Zealand (naturalised introduction)	MPN 24690

Note: (a) Sample from the Staatsherbarium München [MSB].
(b) Collections have previously been made from this location. No voucher was taken for this study.

APPENDIX II: Australasian Apioideae and outgroup accessions

APPENDIX TABLE 2
Details of accessions of Australasian Apioideae and outgroup taxa

Taxon	Location	Herbarium reference
Aciphylla aurea W.R.B Oliver	Waipara, South Island, New Zealand	CHR 505826
Aciphylla aurea W.R.B Oliver	Mt. Cook, South Island, New Zealand	CHR 174473
Aciphylla colensoi Hook. f.	Ruahine Range, North Island, New Zealand	MPN 24691
Aciphylla dieffenbachii (F. Muell.) Kirk.	Cultivated (Landcare gardens)	CHR 512014 [a]
Aciphylla dissecta (Kirk) W.R.B Oliver	Tararua Range, North Island, New Zealand	MPN 24692
Aciphylla glacialis (F. Muell.) Benth.	Mt. Kozciuscko, Australia	MPN 24693
Aciphylla monroi Hook. f.	Porters Pass, South Island, New Zealand	CHR 511835
Aciphylla monroi Hook. f.	Craigeburn, South Island, New Zealand	CHR512015 (for ITS, Genbank No. U72374 [Mitchell *et al.*, 1998])
Aciphylla simplicifolia (F. Muell.) Benth.	Mt. Kozciuscko, Australia	MPN 24694
Aciphylla squarrosa J.R. et G. Forst.	unknown	direct submission to Genbank (U72379) by Mitchell *et al.*
Aciphylla subflabellata W.R.B Oliver	Cultivated (Percy's Reserve, Wellington)	Voucher in prep.
Aciphylla traversii (F. Muell.) Hook. f.	Cultivated (B. Rance)	Voucher in prep.
Aciphylla traversii (F. Muell.) Hook. f.	Cultivated (Otari Botanical Garden)	Voucher in prep.
Anisotome acutifolia (Kirk) Ckn.	Cultivated (B. Rance)	Voucher in prep.
Anisotome antipoda Hook. f.	Cultivated (B. Rance)	Voucher in prep.
Anisotome aromatica Hook. f.	Cultivated (Landcare Research Gardens)	No voucher (for ITS, Genbank No. U72374 [Mitchell *et al.*, 1998])
Anisotome aromatica Hook. f.	Mt. Hikurangi, North Island, New Zealand	Voucher in prep.
Anisotome cauticola J.W. Dawson	Cultivated (Percy's Reserve, Wellington)	Voucher in prep.
Anisotome filifolia (Hook. f.) Ckn. ex Laing	Porters Pass, South Island, New Zealand	CHR 467411
Anisotome flexuosa J.W. Dawson	Cultivated (Percy's Reserve, Wellington)	Voucher in prep.
Anisotome haastii (F Muell. ex Hook. f.) Ckn. et Laing	Cultivated (B. Rance)	Voucher in prep.
Anisotome intermedia Hook. f.	Cultivated (Otari Botanical Garden)	Voucher in prep.
Anisotome latifolia Hook. f.	Cultivated (B. Rance)	Voucher in prep.
Anisotome lyallii Hook. f.	Cultivated (B. Rance)	Voucher in prep.
Anisotome pilifera (Hook. f.) Ckn. et Laing	Two Thumb Range, South Island, New Zealand	CHR 469190
Anisotome pilifera (Hook. f.) Ckn. et Laing	Cultivated (B. Rance)	Voucher in prep.

APPENDIX TABLE 2
Details of accessions of Australasian Apioideae and outgroup taxa (Continued)

Taxon	Location	Herbarium reference
Anisotome procumbens (F. Mueller) C.Webb	Hartz Mountain, Tasmania, Australia	HO 327798
Gingidia ensyii (Kirk) J.W. Dawson	Canterbury, South Island, New Zealand	CHR 512006 [a]
Gingidia flabellata (Kirk) J.W. Dawson	East Ruggedy, Stewart Island, New Zealand	CHR512011 (for ITS, Genbank No. U72374 [Mitchell *et al.*, 1998])
Gingidia montanum (Forst et Forst. f.) J.W. Dawson	Cultivated (B. Rance)	Voucher in prep.
Lecokia cretica, (Lam.) DC.	Ajlun, Jordan	ITS sequence from Downie *et al.* (1998). Genbank Nos. U78389 & U78449
Ligusticum scoticum L.	Massachusetts, USA	ITS sequence from Downie *et al.* (1998). Genbank No. U78357 & U78417
Smymium olusatrum	France	ITS sequence from Downie *et al.* (1998). Genbank No. U30594 & U30595

Note: (a) The ITS region of the same accession was also characterised by Mitchell *et al.* (1998). To confirm ambiguous positions in the sequence of these workers, the ITS locus of the sample was re-sequenced.

APPENDIX III: Oligonucleotide primers

APPENDIX TABLE 3
Oligonucleotide primers used for PCR amplification and/or DNA sequencing

Locus	Primer name	Primer sequence	Reference
Internal Transcribed Spacer (ITS) locus (nuclear)	ITS5	5' GGAAGTAAAAGTCGTAACAAGG 3'	White *et al.* (1990)
	ITS3	5' GCATCGATGAAGAACGTAGC 3'	
	ITS2	5' GCTACGTTCTTCATCGATGC 3'	
	ITS4	5' TCCTCCGCTTATTGATATGC 3'	
3' region of the *matK* locus (chloroplast)	tmK3R	5' GATTCGAACCCGGAACTAGTCGG 3'	Designed for this thesis (unpublished)
	tmK3AR	5' CGTACASTACTTTTGTGTTTMCG 3'	
	tK3MY1F	5' CCAATTATGCCAATGATTGCATC 3'	
	tK3MY1FB	5' CGATACTCTTCTTCCAATTATG 3'	
	tK3MY2F	5' CAATCAAAATCTTCTGGAATC 3'	
5' region of *ndhF* locus (chloroplast)	ND972F	5' GTCTCAATTGGTTATATGATG 3'	Olmstead & Sweere (1994) and designed for this thesis (unpublished)
	ND1318F	5' GGATTAACYGCATTTTATATG TTTCG 3'	
	ND1656F	5' ACTTTGTTTGTTGGATGTTTA 3'	
	ND1762R	5' CCGAAATAAGCTATACTGACT 3'	
	ND2110RM	5' CCCYABATATTTGATACCTTCKCC 3'	
Noncoding *psbA-trnK* intergenic spacer (chloroplast)	tmK3F	5' CCGACTAGTTCCGGGTTCGAATC 3'	Designed for this thesis (unpublished)
	PSBAR	5' CGCGTCTCTCTAAAATTGCAGTCAT 3'	
JSAD locus (chloroplast)	AFLP151	5' ATTATYAATGAAGGYAATACWAT ATATTTTC 3,	Lockhart *et al.* (in press) and designed for this thesis (unpublished)
	151A	5' GGTAATACTATATATTTTCTAAG 3'	
	ANIS151B	5' CACGATCCCTTTTTCTTG 3'	
	ANIS151C	5' CAGTATCAGGAAGAAGGAGAC 3'	
	ANIS151C2	5' CAGTATCAGGAAGAAGGAGAC 3'	
	REANA	5' TGAATACAAYTCTAACTAGCC 3'	
	REMYB	5' CAATCATTGGGTTTATAC 3'	
	JSAD3F	5' CTACTATTTAGGCAGAATRCC 3'	

APPENDIX IV: Oligonucleotides used in AFLP

APPENDIX TABLE 4
Oligonucleotides used in AFLP fingerprint analyses

Locus	Oligonucleotide name	Oligonucleotide sequence
Linkers	*Eco* RI linker:	
	Linker E1	5' CTCGTAGACTGCGTACC 3'
	Linker E2	5' AATTGGTACGCAGTCTAC 3'
	Mse I linker:	
	Linker M1	5' GACGATGAGTCCTGAG 3'
	Linker M2	5' TACTCAGGACTCAT 3'
Pre-selective amplification	*Eco* Pamp - A	5' GACTGCGTACCAATTCA 3'
	Mse Pamp - C	5' GATGAGTCCTGAGTAAC 3'
Selective amplification	*Eco* RI primers:	
	Eco RI - AGG	5' GACTGCGTACCAATTCAGG 3'
	Eco RI - ACC	5' GACTGCGTACCAATTCACC 3'
	Eco RI - ACG	5' GACTGCGTACCAATTCACG 3'
	Eco RI - AAT	5' GACTGCGTACCAATTCAAT 3'
	Eco RI - ATA	5' GACTGCGTACCAATTCATA 3'
	Mse I primers:	
	Mse I - CAC	5' GATGAGTCCTGAGTAACAC 3'
	Mse I - CAT	5' GATGAGTCCTGAGTAACAT 3'
	Mse I - CTC	5' GATGAGTCCTGAGTAACTC 3'
	Mse I - CTA	5' GATGAGTCCTGAGTAACTA 3'
	Mse I - CTG	5' GATGAGTCCTGAGTAACTG 3'

APPENDIX V: DNA Primers for AFLP derived markers

APPENDIX TABLE 5
Oligonucleotide primers used for PCR amplification and DNA sequencing of novel AFLP derived markers

Locus	Primer name	Primer sequence
MYOAT3	MYOAT3F	5' GTGCTGATCTACGGCACC 3'
	MYOAT3R	5' AGTGCTCTTCCTAGCTTATG 3'
MYOAT6	MYOAT6F	5' GGAAAGTGCAGGTTCATGC 3'
	MYOAT6R	5' CATACTTGGTAGTAACGGTG 3'
MYOGC5	MYOGC5F	5' GTCCTTGCAATAACAATGTA 3'
	MYOGC5R	5' CAACTATATGGATTCTAGTAT 3'

APPENDIX VI: Data files and phylogenetic analyses

APPENDIX TABLE 6
Data files used for specific analyses

Thesis section	Analysis	Data file name[a]
3.3.2	Maximum parsimony	MYOITSPAUP.nex; MYOmatKPAUP.nex
	Quartet puzzling	MYOITSPAUP.nex; MYOmatKPAUP.nex
3.3.3	Outgroup root placement (individual analyses)	*Borago*, BOITS.nex and BOmatK.nex; *Echium*, ECITS.nex and ECmatK.nex; *Eritrichium*, ERITS.nex and ERmatK.nex; *Myosotidium*, HOITS.nex and HOmatK.nex; *Plagiobothrys*, PLITS.nex and PLmatK.nex; *Symphytum*, SYITS.nex and SYmatK.nex
	Outgroup root placement (combined analyses)	*Echium*, ECH4MARKERS.nex; *Plagiobothrys*, PLA4MARKERS.nex
3.3.5	Quartet puzzling	MYO22PAUP.nex
	Myosotis sequence evolution rate estimate	"Test_Clock", MYO22TC.nex; Molecular clock test, MYO22PAUP.nex
	Dendroseris sequence evolution rate estimate	"Test_Clock", SANGTC.nex; Molecular clock test, SANGPAUP.nex
	Tests of variance	*M. albosericea*, ALBinfile; *M. exarrhena*, EXAinfile
3.3.6	Quartet puzzling	MYO22PAUP.nex
	M. persoonii placement on Southern Hemisphere radiation	Datafile, MYOAUSTPAUP.nex; Treefile, MYOAUST.tre
4.3.2	Maximum parsimony	APISITSALLPAUP.nex;
	Quartet puzzling	ANISITSPAUP.nex; ANISJSADPAUP.nex
4.3.4	Australasian Aploideae sequence evolution rate estimate	"Test_Clock", APISITSALLTC.nex; Molecular clock test, APISITSALLPAUP.nex
	Tests of variance	*Anisotome aromatica*, AROinfile; *Anisotome latifolia*, LATinfile
4.3.5	Outgroup root placement	*Lecokia*, LEC.nex; *Ligusticum*, LIG.nex; *Smyrnium*, SMY.nex
5.2.3	Figure 5.2	A, MYO22SD.nex; B, MYOAUSTRALSD.nex
	Figure 5.3	A, APISITSALLSD.nex; B, ANISITSSD.nex
	Figure 5.4	A, BALDWINALLSD.nex; B, BALDWININSD.nex
	Figure 5.5	A, SANGALLSD.nex; B, SANGINSD.nex

	Figure 5.7	A and B, MYOITSSD.nex
	Figure 5.8	A and B, MYOmatKSD.nex`
5.3.3	AFLP derived marker AT6 sequence alignment	MYOAT6.aln

Note: (a) Data files, except for MYOAT6.aln, are presented in Nexus format in Disc Appendix I. MYOAT6.aln is provided as a Word 97 document.

APPENDIX VII: Morphological data set for *Myosotis*

APPENDIX TABLE 7
Morphological character states

Taxon	Morphological characteristic							
	Perenniality	Flower colour	Flower shape	Corolla limb Ø (mm)	Position of anthers wrt corolla	Position of styles wrt corolla	Corolla length wrt calyx	Calyx length (mm)
Myosotis albiflora	Short-lived perennial	White	Tube	3.0	Included	Included	Approx. equal	3.5
Myosotis albosericea	Long-lived perennial	Yellow	Funnel	2.0	Partially included	Exserted	Longer	3.0
Myosotis alpestris	Long-lived perennial	Blue	Tube	8.0	Included	Included	Shorter	7.0
Myosotis arvensis	Biennial	Blue	Tube	3.0	Included	Included	Shorter	5.0
Myosotis australis	Short-lived perennial	White	Tube	1.8	Partially included	Included	Longer	3.0
Myosotis capitata	Short-lived perennial	Purple	Tube	2.5	Included	Exserted	Approx. equal	4.0
Myosotis congesta	Annual	Blue	Tube	1.0	Included	Exserted or equal	Longer	4.5
Myosotis decumbens subsp. *decumbens*	Long-lived perennial	Blue	Tube	7.0	Included	Included	Longer	6.0
Myosotis discolor	Annual	Blue	Tube	2.0	Included	Exserted or equal	Longer	4.5
Myosotis goyenii	Long-lived perennial	Cream	Funnel	3.0	Included	Exserted	Longer	4.0
Myosotis laxa var. *caespitosa*	Annual	Blue	Tube	3.5	Included	Included or equal	Approx. equal	1/2
Myosotis macrantha	Long-lived perennial	Brown-orange	Funnel	3.0	Exserted	Exserted	Longer	5.0
Myosotis matthewsii	Short-lived perennial	White	Bell	2.0	Exserted	Exserted	Shorter	7.5
Myosotis persoonii	Annual	Yellow	Tube	2.0	Included	Exserted or equal	Longer	1.5
Myosotis rakiura	Long-lived perennial	White	Tube	2.5	Included	Exserted	Shorter	3.0
Myosotis sylvatica	Short-lived perennial	Blue	Tube	7.0	Included	Included	Longer	4.5

APPENDIX TABLE 7
Morphological character states (Continued)

Taxon	Morphological characteristic								
	Calyx lobe length	Hairs on calyx base	Textured pollen?	Pollen grain size	Pollen type	Nutlet length (mm)	Bracts on cymes	Hairs on abaxial leaf surface	Basal leaf length (cm)
Myosotis albiflora	1/2	Appressed straight	Yes	Large	*australis* type	1.75	bractate	Appressed setiform	2.1
Myosotis albosericea	> 1/2	Appressed straight	Yes	Large	*uniflora* type	1.70	ebractate	Appressed setiform	3.0
Myosotis alpestris	1/2 - 3/4	Mixed	No	Small	*arvensis* type	2.50	ebractate	Straight	7.0
Myosotis arvensis	1/2	Spreading	No	Small	*arvensis* type	2.00	ebractate	Straight	8.0
Myosotis australis	1/4 - 1/2	Hooked	Yes	Large	*australis* type	1.50	ebractate	Straight	6.8
Myosotis capitata	> 1/2	Long silky	Yes	Large	*exarrhena* type	1.85	ebractate	Long silky	7.5
Myosotis congesta	1/2	Hooked	No	Large	*discolor* type	1.00	ebractate	Straight	4.0
Myosotis decumbens subsp. *decumbens*	1/2	Hooked	No	Small	*arvensis* type	2.00	ebractate	Appressed setiform	8.0
Myosotis discolor	1/2	Hooked	No	Large	*discolor* type	1.35	ebractate	Straight	4.0
Myosotis goyenii	> 1/2	Appressed straight	Yes	Large	*australis* type	2.20	ebractate	Appressed setiform	4.0
Myosotis laxa var. *caespitosa*	1/2	Appressed straight	No	Small	*arvensis* type	1.35	ebractate	Appressed setiform	8.0
Myosotis macrantha	> 1/2	Mixed	Yes	Large	*angustata* type	3.00	ebractate	Flexuous	7.5
Myosotis matthewsii	> 3/4	Appressed straight	Yes	Large	*uniflora* type	2.00	mixed	Appressed setiform	3.5
Myosotis persoonii	1/2	Hooked	No	Large	*discolor* type	1.35	mixed	Straight	4.0
Myosotis rakiura	> 1/2	Flexuous	Yes	Large	*australis* type	2.00	ebractate	Long silky	12.0
Myosotis sylvatica	1/2 - 3/4	Hooked	Yes	Small	*arvensis* type	1.85	ebractate	Straight	8.0

Numerical coding applied to the morphological character states or character classes.

(a) Perenniality.

Long lived perennial	0
Biennial	1
Annual	2
Short lived perennial	3

(b) Flower colour.

Blue	0
White	1
Yellow	2
Purple	3
Cream	4
Brown/orange	5

(c) Flower shape.

Tube	0
Funnel	1
Bell	2

(d) Corolla limb diameter (mm).

< 1.9	0
2.0 - 2.9	1
3.0 - 3.9	2
4.0 - 4.9	3
5.0 - 5.9	4
6.0 - 6.9	5
7.0 - 7.9	6
8.0 - 8.9	7

(e) Position of anthers with respect to corolla.

Included	0
Partially included	1
Excluded	2

(f) Position of styles with respect to corolla.

Included	0
Included or equal	1
Exserted or equal	2
Exserted	3

(g) Corolla length with respect to calyx.

Longer	0
Approximately equal	1
Shorter	2

(h) Calyx length (mm).

1.0 - 2.4	0
2.5 - 3.9	1
4.0 - 5.4	2
5.5 - 6.9	3
7.0 - 8.4	4

(i) Calyx lobe length (as proportion of calyx length).

≤ 1/2	0
range 1/2 – 3/4	1
≥ 3/4	2

(j) Types of hairs on calyx base.

spreading	0
straight/appressed	1
hooked	2
flexuous	3
long silky	4
mixed	5

(k) Surface texturing on pollen grains.

No	0
Yes	1

(l) Pollen grain size.

Large	0
Small	1

(m) Pollen type.

australis type	1
uniflora type	2
exarrhena type	3
angusta type	4
discolor type	5
arvensis type	6

(n) Nutlet length (mm).

< 1.25	0
1.26 - 1.50	1
1.51 - 1.75	2
1.76 - 2.00	3
2.01 – 2.25	4
2.26 – 2.50	5
2.51 – 2.75	6
< 2.76	7

(o) Bracts on cymes.

Yes	0
Mixed	1
No	2

(p) Basal leaf length (cm).

2.0 - 3.4	0
3.5 - 4.9	1
5.0 - 6.4	2
6.5 - 7.9	3
8.0 - 9.4	4
9.5 - 11.9	5

(q) Hairs on abaxial leaf surface.

spreading	0
appressed setiform	1
flexuous	2
long silky	3
straight	4

Morphological data for these analyses was collected from the following publications:

Costin *et al.* (1979); Curtis (1993); Harden (1990); Grau & Leins (1968); Grau & Merxmüller (1972) Grau & Schwab (1982); Jessop & Toelken (1986); Moore (1983); Moore (1961); Webb *et al.* (1988)

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Correspondence

The importance of dispersal and recent speciation in the flora of New Zealand

Comments on M. Poole (1994) The New Zealand flora – entirely long-distance dispersal? *J. Biogeogr.* **21**, 625–635 and letter from M. K. Macphail (1997) *J. Biogeogr.* **24**, 113–114.

The origins of the New Zealand flora have been the subject of considerable debate, particularly amongst botanists discussing the relative importance of plant dispersal in the Southern Hemisphere. Wardle (1963, 1968, 1978), although explaining some plant distributions by dispersal, has suggested that New Zealand's flora arose largely through diversification of taxa present on southern ocean lands following the break-up of the Gondwanan supercontinent. In contrast, Raven (1973) has proposed a main route of dispersal from the Northern Hemisphere via Australia for the (sub)alpine flora of New Zealand. Pole (1994) and Macphail (1997) have also argued for the general importance of Australia as a source for the New Zealand flora. However, their view is more extreme. They assume that New Zealand was completely submerged during the Oligocene and that all extant plant lineages in New Zealand (both alpine and lowland) have arrived from Australia by long distance dispersal since the Miocene. Their conclusion follows from observations that in New Zealand there is a poor match between extant species and those from the palynological and/or macrofossil record of the Tertiary period. Pole (1994) and Macphail (1997) argue that, if the current flora is truly of ancient origin, one must expect to find extant or morphologically similar forms in the Tertiary fossil record of New Zealand.

It is generally agreed that from the late Pliocene onward, New Zealand has undergone periods of great change, both geologically and climatically. Increased interactions between the Pacific and Australian tectonic plates led to the rapid uplift of the axial mountain ranges, possibly as early as 5 million years ago (Raven, 1973; Cooper & Millener, 1993). Profound climatic changes were initiated by a pronounced drop in the average temperature during the Late Pliocene, followed by marked temperature fluctuations during the Pleistocene resulting in the glacial and interglacial cycles of that period. Such changes were unlike the more stable conditions of the preceding 60 MY (Cooper & Millener, 1993). Emerging molecular studies on the New Zealand flora indicate that these phases of climatic and geological change were also times of intensified speciation in the New Zealand flora, and periods when long-distance dispersal had an increased importance in establishing trans-Tasman distributions between Australia and New Zealand. This is well illustrated by our recent studies on *Myosotis* L. (Boraginaceae).

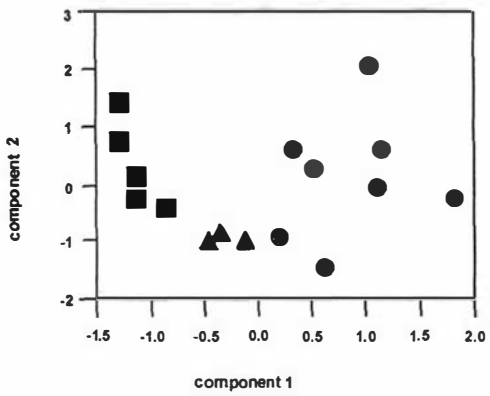

Figure 1 Scattergraph for a Principal Components Analysis (PCA) involving seventeen morphological characters which could be scored for sixteen representative taxa. Data points are coded according to the subgeneric groupings of Grau & Schwab, 1982. Section *Myosotis* (species are from Eurasia, North America and Africa) is indicated by black circles, Section *Exarrhena* discolor group (species are from Eurasia and Africa) are open and grey circles denote Section *Exarrhena* Austral group (species are from Australasia and South America). Data taken from Grau & Leins, 1968; Grau & Schwab, 1982, various regional floras not cited and from the authors' personal observations.

Myosotis is a genus with approximately 100 species and a worldwide distribution (Al-Shehbaz, 1991). Centres of diversity have developed in Western Eurasia & New Zealand. Grau & Schwab (1982) recognized three morphologically defined groups within the genus, two occurring in the northern hemisphere and Africa (section *Myosotis* and section *Exarrhena*, *discolor* group), the third being restricted to Australasia and South America (section *Exarrhena*, austral group). We report here a morphometric analysis (Fig. 1) which supports earlier observations that the austral taxa display greater variation than the other two groups (Grau & Leins, 1968; Grau & Schwab, 1982). Members of the austral group of section *Exarrhena* exhibit growth forms, floral and pollen characteristics not apparent in the remaining subgeneric groups. This greater morphological diversity has led to the suggestion that the austral group of *Myosotis* holds an ancestral position within the genus (Grau & Leins, 1968). However, we also report comparative DNA sequences from several chloroplast loci and a nuclear locus (Fig. 2). Analysis of these data show that, in comparison to the situation in representative northern hemisphere taxa, little genetic diversity exists within and between members of the austral group. Further, these molecular data, taken together with ITS and *mat*K sequences from more widely sampled taxa and outgroups (Winkworth *et al.* in prep) suggest that the *Myosotis* species studied from Australia, New Zealand, and South America are closely related and of northern origin, that their morphological diversification is recent and not of great antiquity, and that they have reached their widely disjunct distribution by long-distance dispersal.

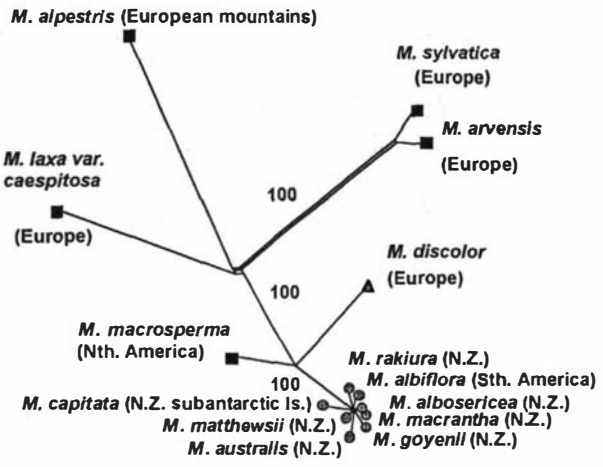

Figure 2 Splitsgraph (using Huson, 1998: SplitsTree2.1) made from a combined dataset of 2548 base pairs. Sequence data include the internally transcribed spacer region (ITS1, ITS2 and 5S gene) of the nuclear ribosomal DNA, the 3' region of the *mat*K gene, the 5' region of the *ndh*F gene and the *trn*K-*psb*A intergenic spacer of the chloroplast genome. Taxa are a selection from a more widely sampled set (Winkworth *et al.* in prep.); subgeneric groups are denoted as for Figure 1 and bootstrap values (250 replicates) for internal edges are given.

Our observations on *Myosotis*, indicating rapid morphological diversification, parallel those for other plant groups in New Zealand (*Hebe*, Wagstaff & Garnock-Jones, 1998; *Gingidia*, Mitchell *et al.*, 1998; *Carmichaelia*, Wagstaff *et al.*, 1999; *Sophora*, Hurr *et al.*, 1999; *Ranunculus*, Lockhart *et al.* submitted; *Anisotome* and *Aciphylla*, Winkworth *et al.* in prep) and from other island archipelagos (the Hawaiian Islands *Bidens*, Carr, 1987, and Silversword alliance Baldwin, Kyhos & Dvorak, 1990; the Juan Fernandez Islands *Robinsonia*, Sang *et al.*, 1994, and *Dendroseris* Sang *et al.*, 1995; and the Macaronesian Islands *Echium*, Böhle *et al.*, 1996 and *Sonchus* Kim *et al.*, 1996). Such diversification, i.e. in the situation of fast changing environments and newly opened island habitats, may be explained from recent observations made in plant developmental genetics (e.g. *Mimulus*, Bradshaw *et al.* (1995) and teosinte, Doebley *et al.* (1997)). These suggest that small amounts of genetic change at relatively few gene loci can underlie dramatic morphological differences in recently evolved plant species. Thus, it may not be suprising to find that little genetic change in neutral DNA markers has accompanied dramatic morphological evolution in island species.

Recent comparative DNA sequencing in several plant groups strongly support the palaeobotanical arguments of Pole (1994) and Macphail (1997) for the extraordinary evolutionary importance of recent long-distance dispersal in the origin of the extant New Zealand flora. Such an interpretation is clearly evident from molecular data for the (sub)alpine flora which has been strongly affected by climatic changes and the upheaval of the high mountains during the Plio- and Pleistocene. Presumably, long-distance dispersal also influenced the lower elevation flora of New Zealand during the earlier Tertiary when the distances between Australia, Tasmania, the Antarctic, and South America were smaller than today.

Nevertheless, despite the obvious importance of long distance dispersal, the unexpected rapid and profound morphological differentiation observed in all the genera studied should caution against overinterpreting the palaeobotanical evidence. Are apparent discontinuities always an indicator for the interruption of *in situ* lineages and new long-distance introduction? Many extinctions undoubtedly have occurred, but the complete disappearance of the terrestrial New Zealand biota during the Oligocene transgression and its *de novo* formation by long-distance arrivals in the Miocene as proposed by Pole (1994) certainly appears questionable. This is suggested, not only because of the uninterrupted fossil record of certain taxa given by Macphail (1997: Table 1), but also because of the occurrence of ancient austral groups at lower elevations in modern New Zealand. Examples include woody seed plants like certain members of Araucariaceae, Podocarpaceae, Proteaceae (Hoot & Douglas, 1998), Winteraceae (Suh *et al.*, 1993; Karol *et al.*, 1999) and *Nothofagus* (Manos, 1997), or animals (Cooper & Millener, 1993) such as *Leiopelma* (frogs), *Sphenodon* (tuatara), *Peripatus*, and some terrestrial gastropods. It is clearly more parsimonious on current information to interpret their presence as continuous lineages in the New Zealand region rather than as examples of long-distance dispersal (Linder & Crisp, 1995). Consequently, one has to assume the existence of at least a limited amount of terrestrial biota throughout the Tertiary in the general New Zealand area.

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SHORT COMMUNICATION

Markers derived from amplified fragment length polymorphism gels for plant ecology and evolution studies

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Abstract

We describe the types of polymerase chain reaction (PCR) markers that we have isolated using amplified fragment length polymorphisms (AFLP) in closely related taxa from diverse plant genera. With these markers, both inter- and intraspecific differences have been identified. The characterization of the nucleotide sequences and fragment length polymorphisms of such AFLP-derived PCR markers is promising for investigating the ecology and evolution of closely related plant taxa.

Keywords: amplified fragment length polymorphisms (AFLP), direct repeats, plant molecular markers, sequence-characterized amplified region (SCAR)

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Introduction

The realization that the Quaternary has been a time of intense speciation and rapidly changing plant distributions globally (e.g. Comes & Kadereit 1998; Winkworth *et al.* 1999) has highlighted the need to identify fast-evolving regions of plant genomes for studying ecological and evolutionary processes. Restriction endonuclease-based methods such as restriction fragment length polymorphisms (RFLP) (Crawford *et al.* 1993; Mason-Gamer *et al.* 1995) and amplified fragment length polymorphisms (AFLP) (Mueller & Wolfenbarger 1999) provide one means for locating polymorphic regions (Lou & Boutry 1995; Lockhart & McLenachan 1997). Although AFLP is an extremely reliable method (Jones *et al.* 1998), our experience is that the effectiveness of AFLP is reduced when DNA quality is poor, presumably because of interfering plant metabolites and/or DNA degradation. This has limited our use of herbarium tissue and some material collected from natural populations.

However, we have taken advantage of the sensitivity of AFLP to locate polymorphic regions using a relatively small number of closely related species and/or intraspecific taxa for which high-quality DNA template can be obtained.

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Sequence-characterized amplified region (SCAR) markers (e.g. Melotto *et al.* 1996; Lockhart & McLenachan 1997; Schupp *et al.* 1999; Shan *et al.* 1999) were derived from AFLP profiles and used to rapidly analyse collections from natural populations and herbarium accessions. In this communication, we report the different types of SCAR markers that we have isolated using AFLP from diverse genera in the New Zealand flora.

Materials and methods

AFLP profiles were obtained for duplicate accessions (three to seven taxa) of the New Zealand plant genera: *Myrsine* (Myrsinaceae), *Nothofagus* (Nothofagaceae), *Rhopalostylis* (Palmae), *Phormium* (Phormiaceae) and *Myosotis* (Boraginaceae). AFLP reactions were performed as described in the Gibco BRL AFLP Analysis System I manual, with the modifications of Lockhart & McLenachan (1997). Electrophoresis and visualization of AFLP profiles in the present work used denaturing 5% polyacrylamide (PAA) gels and silver staining (Vos *et al.* 1995; Promega Corporation 1998). Previously, 4% SEPARIDE had also been used to successfully isolate an AFLP-derived chloroplast marker for direct sequencing studies in alpine *Ranunculus* (Lockhart & McLenachan 1997; Lockhart *et al.*, in press).

Selective AFLP primers were either AT rich or GC rich and these were arbitrarily chosen. Those which gave useful

Table 1 Overview of results at different stages in attempts to derive markers

Selective primer combinations used that gave AFLP profiles with polymorphic bands	No. of bands cut	No. of bands amplified	No. of bands sequenced	GenBank locus match	No. of useful PCR markers	Primers used to amplify polymorphic regions	No. of taxa investigated to date
Myrsine							
*Mse*I-CTA/*Eco*RI-AAA, AAT, ATA, ATT	17	12	10	none	1	(MdAf9F, MdAf9R)	25
*Mse*I-CAG/*Eco*RI-AAA, AAT, ACG, AGG	5	4	4	none	1	(MaAf5F, MaAf5R)	50
Nothofagus							
*Mse*I-CTA/*Eco*RI-ACG, AAT	13	9	9	none	1	(NmAf2F, NmAf2R)	230
Phormium							
*Mse*I-CTG/*Eco*RI-AGG, ACA	11	8	8	emb|AJ2906 64.1	1	(PtAf7oF, PtAf7oR PtAf7iF, PtAf7iR)	61 61
Rhopalostylis							
*Mse*I-CAT/*Eco*RI-AAT, ATT, AAA	5	5	4	none	1	(RbAf3F, RbAf3R)	15
*Mse*I-CTG/*Eco*RI-AGG, AGC, ACG, ACC	9	9	5	none			
*Mse*I-CTC/*Eco*RI-AGG, AGC, ACG, ACC	7	7	3	none			
Myosotis							
*Mse*I-CTG/*Eco*RI-AGG	1	1	1	none			
*Mse*I-CTC/*Eco*RI-ACC	2	2	2	none			
*Mse*I-CAC/*Eco*RI-ACG	1	1	1	none	1	(MyGC5F, MyGC5R)	22
*Mse*I-CTA/*Eco*RI-AAT	3	3	3	none			
*Mse*I-CAT/*Eco*RI-ATA	1	1	1	none			
*Mse*I-CAT/*Eco*RI-AAT	1	1	1	none	1	(MyAT3F, MyAT3R)	21

Most AFLP selective PCR primers that gave discrete profiles identified polymorphisms in different plant groups. Not all, but most gel cuts gave DNA that could be re-amplified with pre-amplification primers. Most fragments that could be re-amplified were cloned and sequenced in the pGEM®-T easy vector (Promega) using M13 forward and reverse sequencing primers. Not all clones made have been investigated. Larger cloned fragments, (those between 350 bp and 500 bp in size) were investigated. Primer pairs (20-25mers), and in a few cases nested primers, were designed and used to amplify products from genomic DNAs. Some of these products (~50%) showed no variability between accessions when run on PAA gels or when sequenced. Primer sequences for phylogenetically informative PCR markers shown in the table are available from p.j.lockhart@massey.ac.nz.

banding patterns are shown in Table 1. Polymorphic bands were cut from wet gels and eluted in sterile water. Most of these bands could be reamplifed (see Table 1) using *Mse*I and *Eco*RI preamplification primers (GIBCO BRL AFLP Analysis System I) freshly diluted from frozen stocks. Amplification products were purified using Qiaquick polymerase chain reaction (PCR) purification kits (Qiagen) and ligated into pGEM®-T easy vector (Promega) before cell transformation. The nucleotide sequences of cloned fragments were determined using the ABI377 sequencing protocol (Perkin Elmer). Primers were designed at the 5′ and 3′ ends of fragments to amplify by PCR the intervening regions from the genomic DNAs in other intraspecific or closely related taxa. This was done using a standard PCR protocol (Lockhart & McLenachan 1997).

Products were electrophoresed on agarose and/or PAA gels. When single bands were present, the DNA sequence of two to six accessions was determined using an ABI377 sequencing protocol to check for sequence variation. In some cases, where different alleles were suspected of being present, markers were characterized using dye-terminated single-base sequencing reactions. The fragment lengths of these products were visualized on a Li-cor 4200 DNA sequencer.

Results and Discussion

Table 1 provides an overview of our results at different stages in our attempts to find PCR markers for different plant groups. Table 2 and Fig. 1 show the variability observed with these markers in closely related taxa and/or populations studied to date. Variation has been detected in respect of the size, sequence and number of alleles amplified by the AFLP-derived primer pairs. All markers showed greater phylogenetic variation among New Zealand taxa than did sequences for the nuclear internal transcribed spacer region.

A common feature of most of the polymorphic regions that we have identified is the presence of small direct repeats. Such repeats have also been observed elsewhere in fragments derived from fingerprint profiles. In some cases the genomic regions showed elevated substitution rates (Lou & Boutry 1995; Lockhart *et al.*, in press). In *Rhopalostylis* a single primer pair amplified multiple fragments (Fig. 1b), one of which contained both inverted and direct repeats. In *Phormium*, no repeats were observed; however, the variable region in the derived AFLP marker showed a GenBank match (Expectation value = $3e^{-07}$) to a truncated retrotransposon.

Recently, in genome mapping studies, the use of SCAR markers has been cautioned against since primers may amplify homologous regions (of identical or near identical size) from different chromosomes (Shan *et al.* 1999). However, such markers can be useful for molecular ecology, since they allow characterization of genomic complexity. Phylogenetic analyses of an earlier derived chloroplast

Table 2 Observations on derived markers which appear phylogenetically useful in New Zealand taxa

	Myosotis	*Rhopalostylis*	*Nothofagus*	*Myrsine*	*Phormium*
Marker	MyAT3F	RbAf3	NaAf2	MaAf5	PtAf
Initial observation on agarose and/or PAA gels	multiple products of different sizes (Fig. 1a)	multiple products of different sizes (Fig. 1b)	single or doublet products (Fig. 1c)	single or doublet products	single band only observed in closely related taxa
Suspected complexity of PCR product(s)	co-amplification of multilocus alleles	co-amplification of multilocus alleles	co-amplification of single locus alleles	co-amplification of single locus alleles	unique allele type
Observed variation in taxa investigated to date	unique and shared haplotypes in 22 closely related species	two distinct haplotypes separating sub-species	five distinct haplotypes in 230 accessions of *N. menziesii*	putative hybrid alleles in population 3 visualised on Li-cor (Fig. 1d)	direct sequencing identifies eight haplotypes in 61 accessions

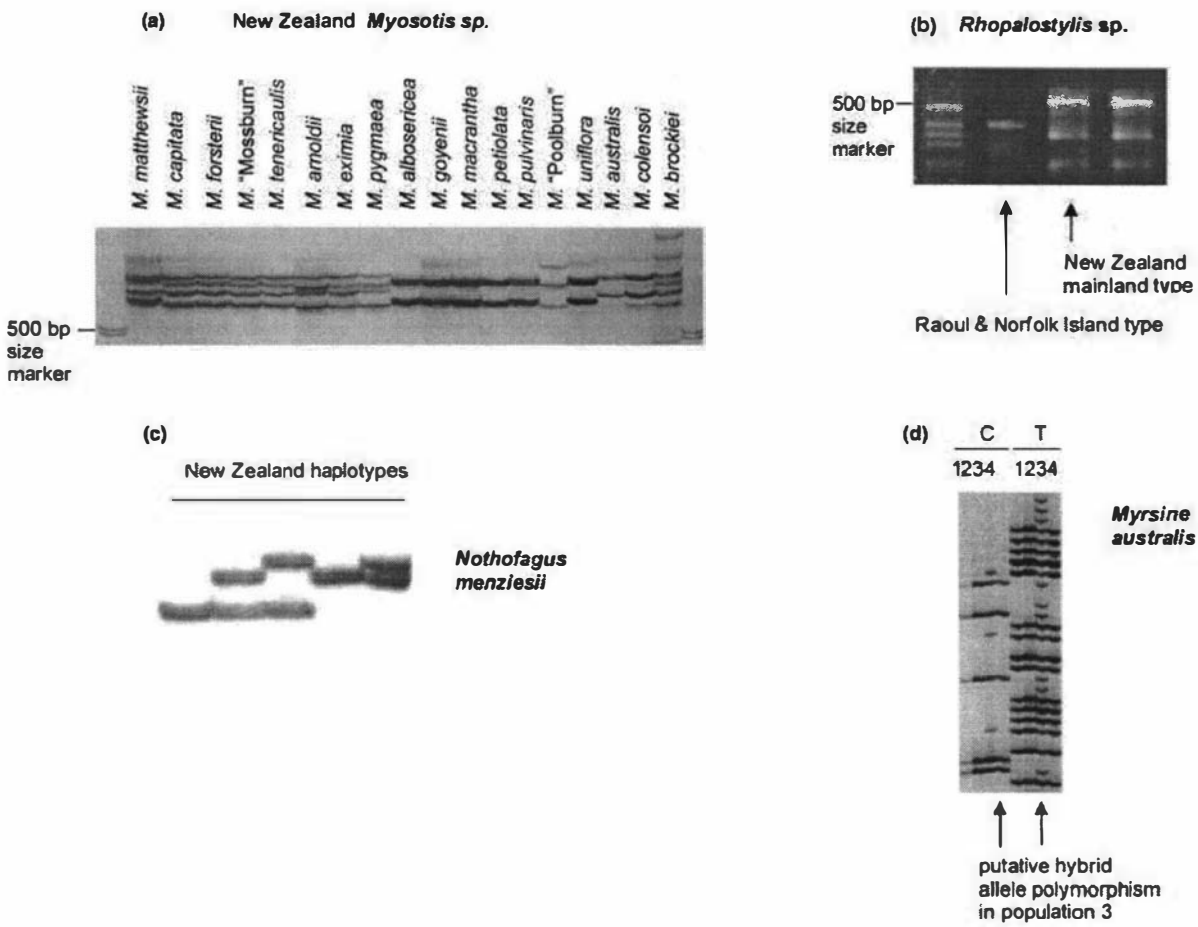

Fig. 1 (a) Silver-stained 5% PAA gel showing haplotypes in 18 different species of *Myosotis* which are genetically very similar (Winkworth *et al.* 1999) (b) ethidium bromide-stained 1% agarose gel showing banding patterns for New Zealand species of *Rhopalostylis*; (c) silver-stained 5% PAA gel showing banding patterns in individuals from different New Zealand populations of *Nothofagus menziesii*; (d) Li-cor single-base sequencing gel (ddCTP and ddTTP reactions) showing banding patterns in individuals from different New Zealand populations of *Myrsine australis*.

SCAR marker (Lockhart *et al.*, in press) and those being investigated in our present ongoing studies (unpublished observations), demonstrate that AFLP markers can be informative for understanding the evolutionary histories of populations and species.

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This research forms part of molecular systematic studies investigating the origins and distributions of endemic New Zealand flora. K. Stöckler is a PhD student studying New Zealand forest flora including *Nothofagus* and *Myrsine*. R. Winkworth is a PhD student studying alpine genera including *Myosotis*. K. McBreen is a postdoctoral researcher studying *Phormium*. P. McLenachan is a molecular biologist with interests in genome evolution. S. Zauner is a PhD student studying comparative genome evolution. P. Lockhart is a lecturer in Bioinformatics and Advanced New Zealand Botany. His research interests include sequence evolution and the development of phylogenetic methodologies.
