Phylogenetic affinities, species delimitation and adaptive radiation of New Zealand *Ranunculus*

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ABSTRACT

Ranunculus is the largest genus in the Ranunculaceae family and comprises *c.* 600 species. Its distribution is almost worldwide and the largest number of species occurs in temperate zones of North and South America, Europe, Asia, Australia, New Zealand, and in the alpine regions of New Guinea. In New Zealand the genus *Ranunculus* contains about 41 species and is found both in lowland and alpine environments. This thesis reports a phylogentic analysis of lowland and alpine New Zealand *Ranunculus*, an assessment of morphological variation and species boundaries among complex alpine species and examines evidence suggesting adaptive radiation of the alpine *Ranunculus* lineage.

Phylogenetic analysis suggests that New Zealand species of *Ranunculus* are not a monophyletic group. For some New Zealand species the closest affinities inferred from the analysis of nrDNA and cpDNA sequences are to species from other land masses such as Australia, the Northern Hemisphere, southern South America and islands in the southern Oceans. Contrary to Fisher's hypothesis (1965), the Andean South American *Ranunculus* in the section *Trollianthoideae* are not closely related to the New Zealand alpine group. The *Trollianthoideae* section was not monophyletic and the Peruvian-Ecuadorian species in it form a lineage sister to European alpine species. Instead, aquatic and sub-aquatic species from the Euro-Mediterranean region and southern South America and the Kerguelen Island were inferred as the closest relatives to the New Zealand alpine *Ranunculus*; albeit this relationship was weakly supported. Findings from this study suggest that colonisation of *Ranunculus* into the Southern Hemisphere has been a dynamic process and several long distance dispersal events and different colonisation routes have been used. Dispersal from New Zealand to Australia and vice versa, has also been inferred. Bird transportation and oceanic currents are speculated as being the most likely vectors for long dispersal for this group.

Morphological variability at the species level is a feature of several species of *Ranunculus* worldwide. In New Zealand, the alpine species *R. insignis* and *R. enysii* are characterised by extensive morphological variability across their distribution range. Currently, these two species include a number of geographically restricted forms that in earlier taxonomic treatments were considered as separate species. Analysis of qualitative and quantitative morphological characters using parametric and non-parametric statistical tests and multivariate analysis, habitat characterisation using environmental variables from the GIS database LENZ and molecular analyses of nrDNA and cpDNA sequences have provided a

framework for interpreting and understanding the nature of this phenotypic variation. An argument based on morphological, genetic and ecological support for the reinstatement of the species *R. insignis*, *R. lobulatus* and *R. monroi* is presented here. The last two species may correspond to lineages of recent origin. Hybridisation and introgression between *R. insignis* and *R. lobulatus* are suggested as being responsible for intermediate phenotypes found in areas where their distribution overlaps. Morphological variability in *R. enysii* is inferred to have had a complex origin. The species has a disjunct distribution and events of hybridisation and/or introgression with *R. monroi* and *R. gracilipes* seems to have occurred in some of the northern and southern populations, respectively. These hybrid lineages may have swamped out pure lineages of *R. enysii* and eliminated the ancestral phenotype. Studies including assessment of gene flow using microsatellites, phenotypic stability under common garden condition and pollination experiments will be necessary to further test these hypotheses. Contrary to the latter two species, *R. lyallii* is morphologically uniform across its distribution range but genetically diverse (11 haplotypes, one of them shared with *R. buchananii*). Morphological stability in this species is probably explained by morphological stasis and habitat specialisation.

The alpine *Ranunculus* group is outstanding in the New Zealand flora in terms of its great phenotypic and ecological diversity of its members. These two features plus the monophyletic nature of the group and its recent origin have suggested to previous researchers that the radiation of this group has been adaptive. Phylogenetic analysis of 20 *taxa* in this group using nrDNA and cpDNA sequences has shown that the group includes four lineages and that genetic diversity between the species forming each linage is low. This confirms findings from earlier studies by Lockhart *et al.* (2001). Cluster Analysis, multidimensional scaling analysis and histological and scanning microscopy observations of morphological and anatomical vegetative and reproductive characters were used to quantify the extent of morphological diversity in the group. Habitat diversity of this group was characterised using 16 environmental variables available from the GIS database LENZ and analysed using Canonical variates analysis. Although four habitat types were identified, there was no correlation between habitat and phenotype as predicted for an adaptive radiation. A number of alternative explanations for this lack of correspondence are discussed. The conclusion drawn from this study was that available data layers and resolution of LENZ limit the use of GIS databases for testing hypotheses of adaptation in the New Zealand Alps.

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I

The genus *Ranunculus; an overview*

INTRODUCTION

The genus *Ranunculus* was first described by Carl Linnaeus in 1753. The Latin name means 'little frog" and makes allusion to the wet habitats in which some species grow. It includes plants commonly known as buttercups, spearworts or water crowfoots. Many of these species are poisonous to cattle, horses and other livestock while some of them are very popular ornamental flowers with many cultivars selected for their large size and bright coloured flowers.

Since the first worldwide classification of *Ranunculus* by Candolle in 1824, the genus has been revised on several occasions but delimitation and classification at the generic and infrageneric level is still far from complete. Efforts have been frustrated mainly by the great number of species in the genus, the poor representation of specimens from several regional floras and the great variability and/or homoplasy of certain morphological characters. Nowadays, the use of molecular techniques and the establishment of international links of scientific cooperation are helping to untangle the phylogenetic affinities and complex evolutionary history of this genus. This chapter provides a brief overview on the taxonomy, phylogenetic affinities, ecology and speciation of the genus *Ranunculus* based on the literature available at the time this study was conducted.

The genus *Ranunculus* L.

Ranunculus L. is the largest genus in the Ranunculaceae family and comprises *c*. 600 species (Tamura 1993, Tamura 1995) and numerous microspecies and apomictic races (Hörandl *et al*. 2005). Its distribution is almost worldwide and the largest number of species occurs in temperate zones of North and South America, Europe, Asia, Australia, New Zealand, and in the alpine regions of New Guinea (Johansson 1998). A small number of species occur in tropical regions, but there they are restricted to high mountain environments (Tamura 1993, 1995).

Species of *Ranunculus* may be found in a variety of habitats such as forests, dry and damp meadows, wet soils, lake and river banks, and alpine heaths. Most of the species appear to have great ecological amplitude; however, habitat-specific species are not uncommon. Most *Ranunculus* are perennial herbaceous plants; yet annual or biennial species can also be found (Tamura 1995; Johansson 1998). Two life habits seem to prevail in the genus; geophilous

(*i.e.*, species with subterranean organs such as rhizomes, root-tubers or stolons) and hydrophilous (*i.e.*, species in aquatic environments with floating adaptations and usually heterophylly) (Tamura 1995).

Plant architecture is relatively constant within the genus. Many of the species form rosettes or a cluster of basal leaves, from which runners or stolons are generally produced. Leaves may be entire, compound or highly dissected. Flowers are single or aggregated forming a cyme. These are hermaphrodite, usually widely open and greenish or bright yellow. A few species have white or reddish flowers. The calyx may be formed by (3)-5-(7) sepals and the corolla by (0)-5-(12 or more) petals. Anthers and carpels may be few or very numerous and they are spirally arranged on the receptacle. The nectary gland, generally single, is located near the base of the petal and may be covered by a scale or naked. Achenes can vary from few to many, being smooth, hairy, winged or with tubercles or hooked spines. Some of these features are believed to facilitate dispersal either by abiotic or biotic agents (Judd *et al*. 2002). Figure 1 shows the typical structures of a *Ranunculus* plant.

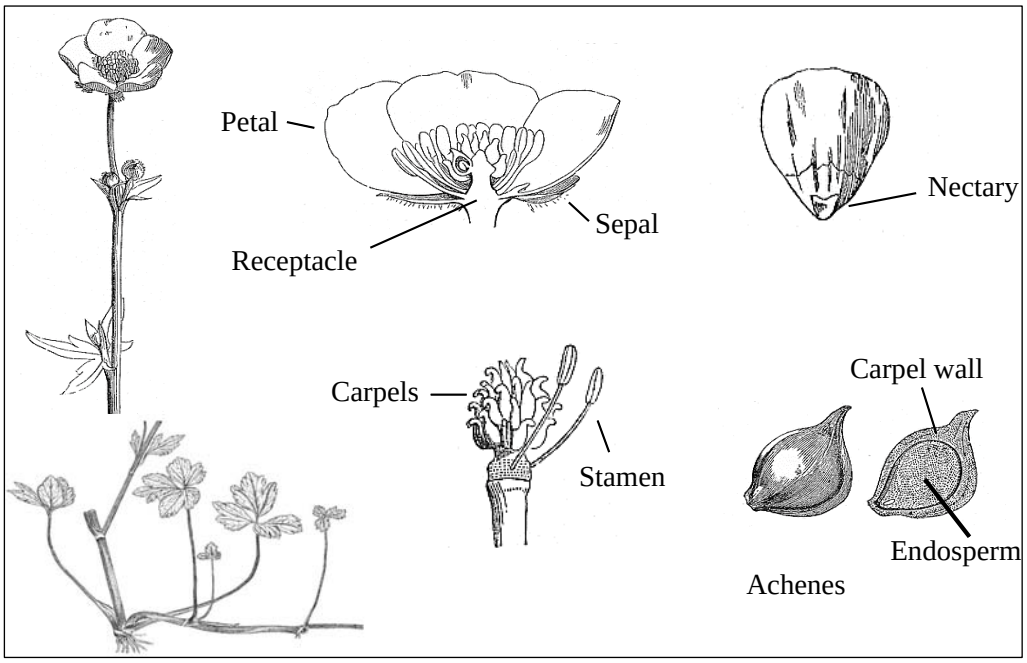


Figure 1: Plant habit and detail of the reproductive structures of *Ranunculus* (modified from Watson & Dallwitz 1992).

Taxonomy and phylogenetic relationships within *Ranunculus*

Following the most recent worldwide revision of the genus by Tamura (1995), *Ranunculus s.l.* can be subdivided into 7 subgenera; *Batrachium, Coptidium, Crymodes, Ficaria, Gampsoceras, Pallasiantha* and *Ranunculus,* and about 26 sections (Table 1). The subgenus *Ranunculus* is the most widespread and numerous in the genus and it includes a great diversity of species, some of them considered ancestral by Tamura (1995). This subgenus contains 20 sections and over 400 species. In his revision, Tamura also excluded several species from *Ranunculus* and assigned them to a number of genera, some of them monotypic, and deemed them as "satellite" to *Ranunculus*; *e.g*. *Aphanostema*, *Callianthemoides*, *Halerpestes* and *Krapfia*.

The most important morphological characters used by Tamura (1995) in his infrageneric classification of *Ranunculus* were those from the achenes. Leaf characters, on the contrary, have limited taxonomic value due to the great variability observed between and within species (Tamura 1995) or even within an individual. Leaves of some *Ranunculus* species are highly adaptable and their shape is easily affected by habitat conditions (Cook 1966). Recent phylogenetic studies of the genus using chloroplast restriction site analysis (Johansson 1998), nuclear ribosomal DNA sequences (Hörandl *et al* 2005) and chloroplast DNA sequences (Paun *et al*, 2005), however, have shown incongruence with this morphology-based classification and only few of the traditional subgenera and sections proposed by Tamura (1995) are monophyletic. Hörandl *et al.* (2005) suggested this incongruence is due to the great homoplasy found in some of the morphological characters used by Tamura (1995). The occurrence of widespread parallel evolution within the genus, and also in the Ranunculaceae, has also been observed by Hoot (1995) and Johansson (1998).

The phylogenetic analyses by Hörandl *et al*. (2005) and Paun *et al.* (2005) evidenced a large *Ranunculus* core clade from which the sections *Coptidium, Ficaria, Pallasiantha* and part of the section *Crymodes* are excluded (see Figure 2). This "core clade" includes at least 19 subclades; only some of them well resolved. A fairly similar topology was obtained when the ITS data set from Hörandl *et al.* (2005) was combined with Paun *et al.* (2005) plastid data set. The main difference between these two studies is that clades formed by the genera *Myosurus* and *Cerathocephala* (Figure 2) appear as a sister to the core *Ranunculus* clade.

Genus	Subgenus	Section	Distribution	Nos. species
Ranunculus	Coptidium	Coptidium	Europe, Asia, North America	1
	Pallasiantha	Pallasiantha	Eurasia, North America	1
	Crymodes	Crymodes	North America, Europe	4
	Ficaria	Ficaria	Europe, Asia, North America	5
	Gampsoceras	Gampsoceras	Turkey, Syria, Iran	1
	Batrachium	Batrachium	All continents	*c.* 30
	Ranunculus	Acetosellifolii	Europe	1
		Aconitifolii	Europe	3
		Acris	All continents	c. 150
		Casalea	North & South America	c. 10
		Chloeranunculus	Europe, North Africa	1
		Echinella	Eurasia, Africa, North America	*c.* 70
		Ficariifolius	Europe, Asia, North America	5
		Flammula	Eurasia, North America	*c.* 22
		Hecatonia	Eurasia North America	1
		Leptocaules	South America, Australia, NZ	*c.*13
		Leucoranunculus	Europe, Asia	4
		Micranthus	Europe, Asia, North Africa	2
		Physophyllum	Europe, North Africa	1
		Pseudoadonis	Australia, Tasmania, NZ	*c.* 15
		Ranuncella	Europe	6
		Ranunculastrum	Europe, Asia, North Africa	*c.* 70
		Ranunculus	Eurasia, North & S. America, Oceania	*c.* 160
		Thora	Europe	4
		Tuberifer	East Asia	2
		Xanthobatrachium	North & Southern Hemisphere	*c.* 8

Table 1: Subdivision of the genus *Ranunculus s.l.* following Tamura (1995).

A recent study by Lehnebach *et al.* (2007) (Appendix 1) suggests that the Andean genera *Laccopetalum* and *Krapfia* are closer to the *Ranunculus* core clade than *Myosurus* and *Cerathocephala*. The latter two appear sister to *Laccopetalum* and *Krapfia* in the phylogeny obtained by Lehnebach *et al.* (2007). Samples of these Andean genera were not available at the time studies of Hörandl *et al.* (2005) and Paun *et al.* (2005) were done.

As for the "satellite" genera, they all form a grade leading to the core *Ranunculus* clade, except for *Aphanostemma* which appears nested within the core clade (Figure 2). The split of the satellite genera from *Ranunculus* seems to have occurred *c.* 30mya during the Eocene and Oligocene (Paun *et al.* 2005). For more details on the phylogenetic affinities of *Ranunculus s.l.* and allied genera refer to the articles by Hörandl *et al.* (2005) and Paun *et al.* (2005) included in the Appendix 1.

Pollination, breeding systems and seed dispersal

Unlike many other biological aspects of the genus *Ranunculus*, its pollination, breeding systems and seed dispersal have been poorly studied. Much of the existing information is based on few observations and none of them in great detail. However, some general patterns can be observed.

Generalist pollination is common in many *Ranunculus* species (Pellmyr 1995). Insect pollination has been described in at least five *Ranunculus* species from the Northern Hemisphere (Steinbach & Gottsberger 1995), one species of the alpine region of central Japan (Yumamoto 1986) and in two species of the Chilean Andes (Riveros 1991). All these studies list insects from the orders Hymenoptera, Diptera and Coleoptera as the most common floral visitors. Dependence on pollinators to set fruit varies within the genus. Fisher (1965) noticed that some self-compatible New Zealand alpine *Ranunculus* are entirely dependent on pollinators to set fruit. Conversely, species of the Chilean Andes (Riveros 1991) and the Chilean-Argentinean Patagonia (Moore 1983) have overcome pollinator dependence by developing mechanisms of automatic self-pollination, delayed self-pollination or agamospermy. The latter is particularly important in the diversification of European *Ranunculus*; 800-900 microspecies have originated from apomictic species (Hörandl *et al.* 2005).

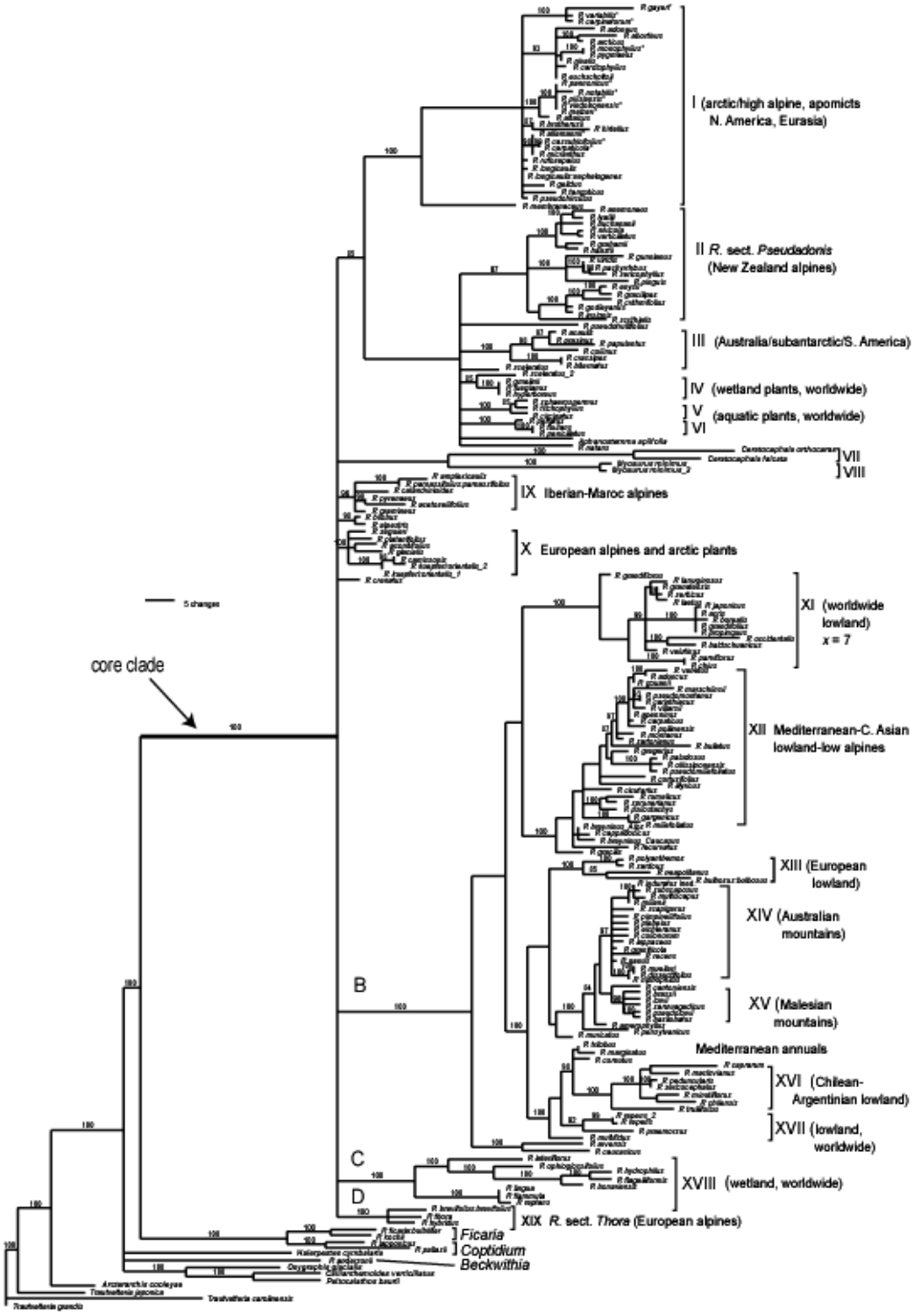


Figure 2: Phylogram of Bayesian inference analysis, posterior probability values ≥ 80 indicated on above the branches (from Hörandl *et al.* 2005).

In general, self-compatibility and inter-specific compatibility seem to be common in the genus. Rendle & Murray (1988) studied the breeding system of 13 New Zealand species of lowland *Ranunculus* by performing inter- and intra-specific pollinations. Their results showed that, despite the occurrence of pollen tube competition at the stylar channel, all crosses successfully set fruit. A similar situation has been described in many New Zealand alpine *Ranunculus* (Fisher 1965) and Australian alpine *Ranunculus* (Armstrong 2003).

The lack of reproductive barriers and visitation by generalist pollinators (all features that facilitates hybridisation and introgression) and agamospermy are regarded as key adaptations, promoting the species radiation observed in the genus (Pellmyr 1995). This also suggests that ecological factors, rather than reproductive isolation, play a key role in maintaining genetic isolation and species integrity (Armstrong 2003).

Information regarding the dispersal strategies and agents used by *Ranunculus* is also scant and speculation is abundant. The following are some examples briefly mentioned in the literature. Seed dispersal in *Ranunculus* seems to be limited, with most achenes unlikely to disperse beyond the maternal habitat, *i.e.*, 15 to 30 cm from the maternal plant (Scherff *et al.* 1994, Armstrong 2003). This may have the advantage of confining recruitment to parental habitats where survival of the seed is highest due to suitable habitat conditions (Armstrong 2003). But achenes are not always disseminated individually. In some species the entire fruit is scattered as the unit of dissemination (Tamura 1995), probably as a strategy to increase dispersal success and, therefore, the chance to colonize new habitats.

Achene flotation (hydrochory) is suspected to occur in aquatic species. In these species, achenes have an internal layer of spongy tissue with air spaces that could facilitate flotation (van der Pijl 1982). Since hydrochory may promote long-distance dispersal, the wide distribution of many aquatic *Ranunculus* has been attributed to this dispersal mechanism (Hörandl *et al.* 2005). Species in grasslands, meadows or forest floors appear to use animals as dispersal agents (epizoochory); and this would explain the presence of tubercles or hooked spines on the achene's testa (Paun *et al*. 2005). Such adaptations have been observed in many annual *Ranunculus* of the Mediterranean (Paun *et al*. 2005). Wind dispersal has not been reported in the literature yet, but its occurrence should not be discarded especially in those species with "winged" achenes. These wings are the result of the expansion of the external tissue of the style and the flattening of the achene's body towards the edges.

Other strategies such as geocarpy (the burying of the achenes near the mother plant) and amphicarpy (only a small number of achenes) have been observed in *Ranunculus* species inhabiting hostile environments, where chances of finding a suitable habitat for germination and establishment are low (van der Pijl 1982). Amphicarpy has been reported in species from the dry Andean regions of South America (van der Pijl 1982) and alpine species of New Zealand (Fisher 1965; Lockhart *et al.* 2001; P. Garnock-Jones pers. com.). Finally, Tamura (1995) also mentioned that achenes of some species may remain green long after being detached from the receptacle, perhaps due to a slow embryo development and a specialisation to humid habitats.

Hybrid speciation in *Ranunculus*

Hybridisation is an important evolutionary process in plants, and has not only been a source of novel ecological and morphological features (Stebbins 1974, Futuyma 1998, Barton 2001, Archibald *et al.* 2004) but also an important source of phenotypic convergence (Albach *et al.* 2004). Linder & Rieseberg (2004) affirm that hybrid speciation in plants can occur in at least two ways: diploid hybrid speciation and allopolyploid speciation. Diploid hybrid speciation results from a normal sexual event in which each gamete is haploid and from a different species. Diploid hybrids between species of similar sections and with overlapping distributions have been described in *Ranunculus* almost worldwide (Cook 1963, Fisher 1965, Briggs 1962, Lockhart *et al.* 2001, Armstrong 2003, Hörandl *et al.* 2005). Since hybrids produced by this process may have partial fertility or viability, introgression is likely to occur. Therefore, for speciation to occur, hybrids have to be isolated from parental species, for example in novel environments (Stebbins 1974, Linder & Riseberg 2004). Conversely, allopolyploidy is hybrid speciation between two species resulting in a new species that has the complete diploid chromosome complement of both parents. This process results in immediate speciation because any backcrossing to the diploid parents produces a high proportion of unviable or sterile triploid offspring. Allopolyploid origin has been reported for a number of aquatic and alpine *Ranunculus* species (Fisher 1965, Dahlgren & Cronberg 1996, Lockhart *et al.* 2001, Carter 2007).

Recent phylogenetic studies in the genus have confirmed that diploid hybridisation and polyploidy have been important events in the diversification, speciation and colonization of newly available habitats (Lockhart *et al.* 2001, Hörandl *et al.* 2005). In fact, it has been suggested that the diversification of the New Zealand alpine *Ranunculus* has been

accompanied by numerous hybridisation and allopolyploid events and the appearance of new alpine habitats during the Pliocene (Lockhart *et al.* 2001).

The New Zealand *Ranunculus*

About 41 species have been listed for the New Zealand archipelago (Garnock-Jones 1988, Heenan *et al.* 2006). They can be found across the entire altitudinal gradient of the country, from coastal and lowland environments to sub-alpine and alpine surroundings. Alpine *Ranunculus*, in particular, have been object of numerous studies (Allan 1926, Fisher 1965, Lockhart *et al.* 2001, Piripi 2003, Carter 2007, Norton *et al.* 2007). Two major contributions to the study of this group are the extensive monographic work produced by Fisher (1965) covering species distribution, morphological variation and hybridisation, and the molecular study by Lockhart *et al.* (2001) which examines phylogenetic affinities within the group and radiation and dispersal events using nuclear ribosomal and chloroplast DNA sequences. The latter study confirmed many of the hypotheses put forward by Fisher (1965), but also raised new questions requiring further study, such as the origin of the alpine group and phylogenetic affinities of the remaining lowland species, incongruence between morphology-based classification and genetic affinities in a number of species, and the factors that have promoted the diversification of the group.

Three main goals have been proposed for this study: to determine the phylogenetic affinities of New Zealand species of *Ranunculus*, to assess morphological diversity and delimit species boundaries of morphologically variable *taxa* within a selected group of New Zealand alpine *Ranunculus*, and finally, to examine the processes and factors that may have promoted the radiation and diversity observed in the New Zealand alpine *Ranunculus*. These goals will be addressed independently in the following three chapters of this thesis (Chapter II, III and IV). Finally, Chapter V presents the concluding remarks drawn from the study. Supplementary information on the genus and satellite genera is included in the Appendix section where relevant articles by the author, or in collaboration with other scientists, have been attached.

II

Phylogenetic affinities and Biogeographic patterns of *Ranunculus* in the Southern Hemisphere

INTRODUCTION

The origin of the New Zealand flora is a topic of great interest and numerous hypotheses have been proposed and explored through the years (Raven 1973, Wardle 1978, Pole 1994, Macphail 1997, Winkworth *et al.* 2000, Winkworth *et al.* 2005, Trewick *et al.* 2007). Currently, a growing number of molecular phylogenetic studies indicate that the origin of the New Zealand flora is multiple; with elements of Gondwanic origin (Schuettpelz *et al.* 2002, Wagstaff 2004, Schuettpelz & Hoot 2004, Knapp 2007), *in situ* diversification (Lockhart *et al.* 2001, Jakubowsky *et al.* 2005) and elements dispersed over long distances *e.g.* from South America (Wagstaff *et al.* 2000, Meudt & Simpson 2006), from the Northern Hemisphere (Winkworth 2000) and from the South Pacific and Australasian regions (Wagstaff & Wege 2002, Smissen *et al.* 2003). The importance of long-distance dispersal in shaping today's flora is well accepted (Winkworth *et al.* 2005). Transoceanic dispersal has been suggested for the ancestor of a number of plant genera now found in New Zealand including *Myosotis* (Winkworth *et al.* 2002), *Pachycladon* (Heenan & Mitchell 2003), *Ourisia* (Meudt & Simpson 2006), and several species of alpine *Ranunculus* (Lockhart *et al.* 2001).

The genus *Ranunculus* in New Zealand includes at least 41 species and three varieties (Garnock-Jones 1988, Heenan *et al.* 2006). Over 90% of them are endemic with only three species being found elsewhere: two species are shared with Australia and one with southern South America. The most recent flora treatment of the New Zealand *Ranunculus* has been prepared by Garnock-Jones (1988). In this treatment, New Zealand species were assigned to the subgenus *Ranunculus*, section *Ranunculus* and section *Epirotes*. Section *Ranunculus* included 18 species found in coastal, lowland and sub-alpine environments, whereas section *Epirotes* included 23 species, many of them restricted to alpine environments but with also a few species found in lowland habitats. The most recent morphology-based revision of the entire genus subdivided *Ranunculus* into 7 subgenera and 26 sections (Tamura 1995). New Zealand species of *Epirotes* were re-classified and included in the endemic section *Pseudoadonis* of lowland species and section *Acris* represented only by *R. reflexus*. The remaining species were not assigned to any section (Table 2).

Recent phylogenetic studies based on nuclear ribosomal and chloroplast DNA sequences have shown that New Zealand alpine members of the section *Pseudoadonis* form a monophyletic group within *Ranunculus* (Hörandl *et al.* 2005, Paun *et al.* 2005). No lowland representatives

Species	2n	Fisher (1965)	Garnock-Jones (1988)	Tamura (1995)
R. acraeus	?	n/a	n/a	n/a
R. buchananii	48	Epirotes Alpine	Epirotes Alpine	Pseudoadonis
R. crithmifolius	48			
R. enysii	48			
R. goodleyanus	48			
R. gracilipes	48			
R. grahamii	?			
R. haastii	48			
R. insignis	48			
R. lyallii	48			
R. nivicola	96			
R. pachyrrhizus	48			
R. piliferus	?			
R. pinguis	48			
R. scrithalis	?			
R. serycophyllus	48			
R. verticillatus	48			
R. viridis	?			
R. amphitricus	48			
R. acaulis	96	Epirotes lowland	Epirotes lowland	
R. macropus	96			n/a
R. limosella	48			
R. glabrifolius	96/144			
R. altus	48	n/a		
R. carsei	32	Chrysanthe	Ranunculus	
R. foliosus	48			
R. kirkii	48			
R. multiscapus	16			
R. recens	48			
R. royi	48			
R. reflexus	48			Acris
R. subscaposus	48			n/a
R. urvilleanus	16			
R. brevis	32			
R. cheesemanii	32			
R. maculatus	32			
R. membranifolius	32			
R. ternatifolius	32			
R. mirus	48			
R. simulans	?			
R. stylosus	?			

Table 2: Classification of New Zealand *Ranunculus* at the section level according to Fisher (1965), Garnock-Jones (1988) and Tamura (1995). Chromosome numbers are from Fisher (1965) and Hörandl *et al*. (2005). n/a: not assigned. ?: unknown

of the section *Pseudoadonis* or species of the section *Ranunculus* were included in any of these studies. Monophyly of the alpine members of the section *Pseudoadonis* was suggested before by Fisher (1965) based on their morphology. These species share morphological characters such as petals with several nectary glands and achenes with long persistent styles.

Lockhart *et al.* (2001) studied the phylogenetic affinities within the alpine species of *Ranunculus* using nrDNA and cpDNA sequences. That study concluded that the diversification of the group occurred after a long distance dispersal event to New Zealand and subsequent dispersal to alpine habitats in Australia and the sub-Antarctic islands of New Zealand. The origin of such founding dispersal in New Zealand was unclear at the time and two hypotheses were proposed. The first one claimed affinity with the extant Andean South American *Ranunculus* in the section *Trollianthoideae* (Fisher 1965, Wardle 1978, Ziman & Keener 1989). Similar to the New Zealand alpine *Ranunculus*, members of this section occupy high alpine habitats and have large flowers, turgid achenes and numerous nectary glands per petal. Alternatively, Raven (1973) proposed long distance dispersal to New Zealand from the Northern Hemisphere via New Guinea and Australia. As for the remaining New Zealand lowland and sub-alpine *Ranunculus*, no hypotheses have been proposed to explain affinities and origin.

The main goals proposed for this study are to (1) uncover the phylogenetic relationships within the New Zealand lowland and alpine species of *Ranunculus*, (2) determine the phylogenetic and biogeographic affinities of New Zealand *Ranunculus* and southern South American *Ranunculus* within a worldwide framework, and (3) infer the origin of the New Zealand alpine *Ranunculus*. In this study, earlier sequences obtained by Lockhart *et al*. (2001), Hörandl *et al.* (2005) and Paun *et al.* (2005) are supplemented with additional nrDNA ITS and cpDNA *mat*K sequences. These represent the remaining species of lowland and sub-alpine *Ranunculus* found in New Zealand, members of the Andean section *Trollianthoidea* and several North and southern South American species.

METHODS

Taxon sampling

In this study we sequenced and included 112 new samples representing *Ranunculus* species of Southern South America (Chile and Argentina), the Central Andes (section *Trollianthoideae* from Perú and Ecuador), North America, the Kerguelen Islands and the remaining New Zealand lowland and sub-alpine *Ranunculus* and two unnamed species (*R.* "celatus" and *R.* "induratus"). Three New Zealand lowland species were not included in this study, namely *R. mirus* and *R. simulans* and *R. stylosus*. Only herbarium material was available for these three species and DNA amplifications were not successful due to the age and poor preservation of the specimens. New Zealand members of the genera *Ceratocephala* (*C. pungens*) and *Myosurus* (*M. novae-zelandiae*), both in the Ranunculaceae and considered closely related to *Ranunculus* (Hörandl *et al.* 2005), were also included as outgroups. A sample of the genus *Hamadryas* from southern South America was also added as an outgroup. These new sequences were combined with those from Hörandl *et al.* (2005), Paun *et al.* (2005) and Lehnebach *et al.* (2007) resulting in two large matrices; 312 taxa for the ITS (84 of these were obtained in this study) and 132 taxa for the mat*K* matrix (16 of these were obtained in this study). Taxon names and voucher details for the new sequences have been listed in Appendix 2.

DNA isolation, amplification and sequencing

DNA extraction from fresh material used a protocol modified from Doyle & Doyle (1987). For herbarium material a DNeasy extraction kit (Qiagen) protocol was used. PCR amplification was carried out in a Biometra T1Thermocycler machine. The entire Internal Transcribed Spacer (ITS) region was amplified using the primers ITS4 and ITS5 (Appendix 3). A combination of internal primers was required for some samples, generally those from herbarium specimens. These internal primers are also listed in the Appendix 3. The entire *mat*K region (~2500 bp) with the intron and extron regions was amplified using the primers listed in Appendix 3. PCR conditions were 94°C 3 min for the first cycle; 94°C 30s, 55/50°C 30s, 48°C 1 min for the following 35 cycles; then 72°C 1min; 72°C 5min; and then cooled at 10°C. PCR products were quantified using 1% agarose gels and later purified using a Sap/Exo1 digestion method (2 μl Sap, 1 μl Exo1; cycle 37°C 30min; 80°C 15 min and then cooled at 10°C). Sequencing reactions were done in a 20 μl volume using Applied Biosystems Inc. standard protocols and the following cycle conditions: 96°C 30s, 50°C 15s, 60°C 4min, repeated for 27 cycles and then cooled at 10°C. Sequencing reactions were cleaned with the

magnetic bead-based technique using CleanSeq and a SPRI magnetic plate according to the manufacturer's instructions (Agencourt Bioscience Corp., Beverly, MA) and then sequenced using a capillary ABI3730 Genetic Analyzer, from Applied Biosystems Inc. by the Allan Wilson Centre Genome Service, Massey University, Palmerston North, New Zealand. Some sequencing reactions were precipitated following an EDTA/Sodium Acetate/Ethanol protocol; air dried and then sequenced.

Data analyses

Forward and reverse strand sequences were edited using Sequencher 4.2 (Genecode). Sequences were aligned using ClustalX (Thompson *et al*. 1997). Gaps were recoded using the simple gap coding method (Simmons & Ochoterena 2000) as implemented in GapCoder (Young & Healey 2003). Maximum parsimony (MP) analysis was performed for the ITS and matK data set using the software PAUP* 4.0b8 (Swofford 2000) using the heuristic search with 1000 replicates, random sequence addition, tree-bisection-reconnection (TBR) branch swapping, MULTRESS on (keeping multiple, equally parsimonious trees) bu saving only 10 trees each replicate. The unordered character states and equal character state weighting options were used in the analysis. A majority rule consensus tree was computed from 50 equally most parsimonious trees. The relative support for each node was examined with non-parametric bootstrapping (Felsenstein 1985; 100 replicates).

Phylogenetic reconstruction using Bayesian inference (BI) was done separately for both the ITS and plastid data set using MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Gaps were recoded for each dataset using the simple gap coding method (Simmons & Ochoterena 2000) as implemented in GapCoder (Young & Healey 2003) and then added as a second partition to the matrix. Four Markov Monte Carlo chains were run simultaneously starting from random trees. The analysis was run for 10,000,000 generations sampling a tree at every 1000 generations. A general time reversible model of substitution with gamma distribution was used as the most suitable model Modeltest3.6 (Posada & Crandall 1998). The trace files generated by Bayesian MCMC runs were analysed using the software Tracer. The first 200 trees were discarded as burn-in. A consensus tree was obtained for each data set and later combined into a single supernetwork using the z-closure rule as implemented in Splitstree (Huson & Bryant 2006). This method is helpful for visualising congruencies between data sets and identifying conflicts that might be explained by hybridisation (McBreen & Lockhart 2006).

RESULTS

The ITS sequences ranged from 380 to 608 basepairs in length, and the aligned data matrix included 658 bp. After coding the gaps and including them in the dataset a total of 804 characters were obtained; 339 were parsimony-informative. The MP analysis for the ITS dataset resulted in 50 most parsimonious trees of 2040 steps (CI= 0.38; RI= 0.87). The MP 50% majority rule consensus tree (not shown) and the Bayesian phylogram had a similar topology and showed high posterior probabilities (PP) and bootstrap (BS) values for the main clades. Posterior probability values ≥90% are indicated above the branches before the backslash symbol on the tree. Bootstrap values ≥ 50 are indicated above the branches after the backslash symbol. The tree has been divided for better representation and clades have been numbered from 1 to 11 and labelled as outgroup. The main differences between the MP tree and the Bayesian tree were the position of clade 8 formed by species from southwestern Europe and the Pyrenees and the species *R. gmelinii, R. crenatus* and *R. apiifolius*. In general, the topology of both trees is very similar to that reported before by Hörandl *et al.* (2005) and Paun *et al.* (2005). The most evident differences are the basal position of the section *Casalea* within the core *Ranunculus* clade, and the location of *Ceratocephala* and *Myosurus* outside the core *Ranunculus* clade. For this study, results and discussion are focused only on those clades containing New Zealand, Australian and South American species; *i.e.,* clades 1, 2, 6, 9, 10 and the outgroup. The remaining clades have been presented and discussed in more detail in Hörandl *et al.* 2005 (Appendix 1).

New Zealand *Ranunculus* do not form a monophyletic group in the ITS analysis and they occur in three different clades; *i.e.,* Clades 1, 2, and 6. The clade 1 (Figure 3A, PP= 98%, BS= 69%) includes species from Europe, North America, New Guinea, Australia, Tasmania and New Zealand. In this clade New Zealand is represented by ten species, two unnamed species (*R.* "celatus" and *R.* "induratus") and two samples of unknown identity, all of them from lowland and sub-alpine habitats. These species are contained within a well supported clade (PP= 85%, BS<50) that also includes most of the Australian and Tasmanian lowland species studied. One of the undescribed New Zealand *taxa* is sister to *R. eichlerianus*, endemic to the state of Victoria (Australia), and the second is sister to *R.* "induratus" from the South Island of New Zealand. This "New Zealand - Australia" clade is sister to a small clade of species from New Guinea and Asia. Both clades appear derived with respect to the North American species *R. pensylvanicus* and *R. muricatus* and sister to a clade of Euro-Mediterranean species (*e.g. R. bulbosus, R. sardous*).

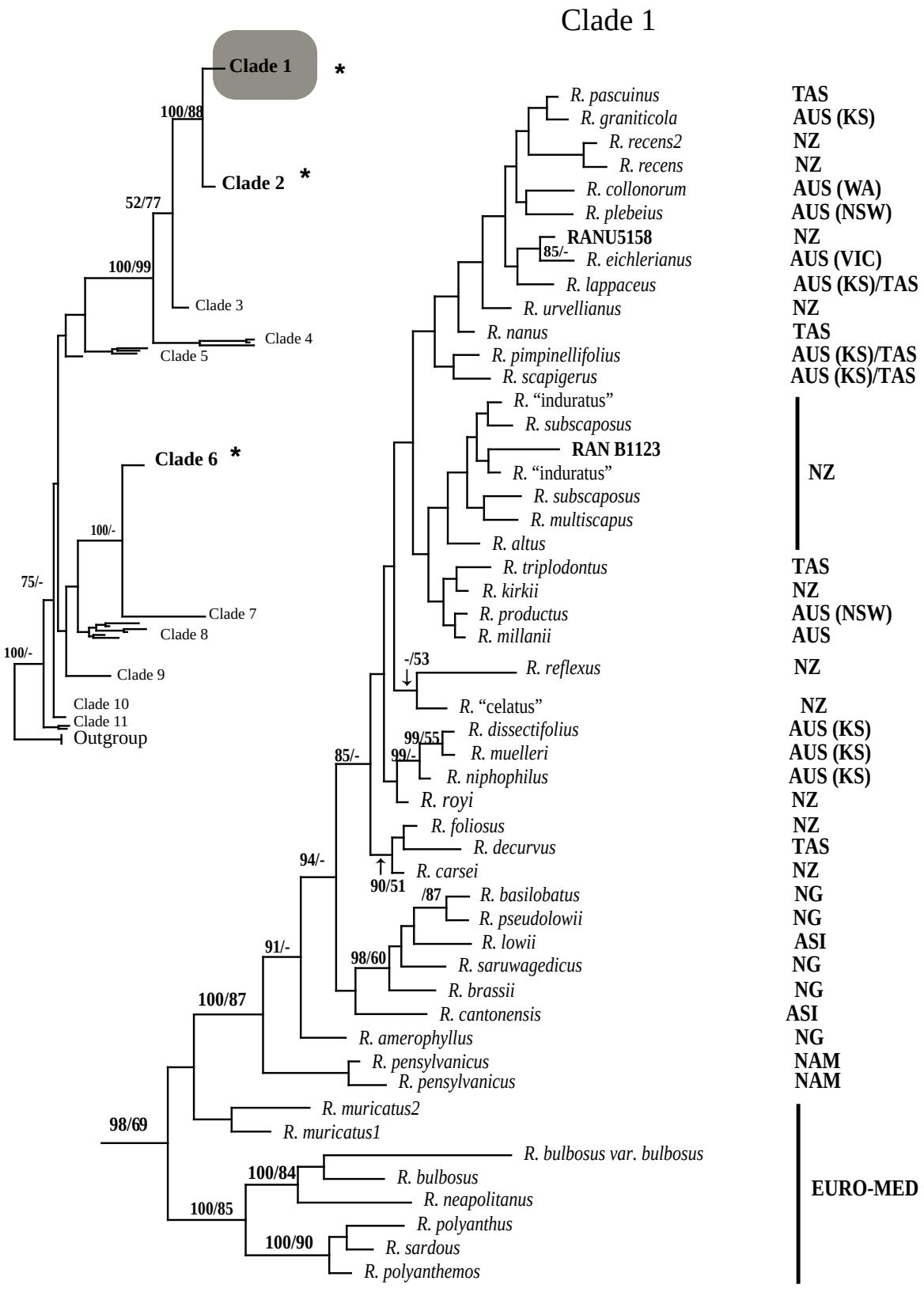


Figure 3A: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of nrDNA ITS sequences. Posterior probability values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches.

Clade 2 (Figure 3B) is sister to Clade 1 with high support and is very diverse, including species from North America, Europe, Mediterranean and Irano-Turian regions, Australia, South America and New Zealand. Sister to all the species in this clade is the South African species *R. multifidus*. The entire clade is weakly supported by the Bootstrap analysis (BS= 54%) but highly supported by Bayesian analysis (PP= 97%). Three smaller clades are found within Clade 2. The first clade, Clade 2A, is well supported and includes only southern South American species (PP= 100%, BS= 81%). These species form three lineages; one with two aquatic species shared by Chile and Argentina (*R. spegazzinii* and *R. trullifolius*) and three terrestrial species endemic to Argentina (*R. platensis*, *R. hillii* and *R. bonariensis* var. *phyteumifolius*). The other two lineages contain eight terrestrial species endemic to southern South America. One of them, *R. caprarum*, is only found in the Archipelago of Juan Fernández in the Pacific Ocean. Clade 2A is sister to a smaller and weakly supported clade, 2B, which contains several species from the Northern Hemisphere and one Australian endemic, *R. sessiliflorus*. The third clade within Clade 2, Clade 2C, includes five New Zealand species in two different lineages. The first lineage is strongly supported (PP= 100%, BS= 98%) and is formed by the sub-alpine and alpine species *R. cheesemanii*, *R. brevis*, *R. maculatus* and the rare *R. ternatifolius*. Sister to this clade are two northern South American species; *R. praemorsus* and *R. geranioides*. Sister to these two clades are *R. hispidus* and *R. fascicularis*, both native to North America. The grouping of these New Zealand+South America+North America clade is well supported by the Bayesian analysis (PP= 82%). The fifth New Zealand species in Clade 2C is the lowland *R. membranifolius* and it occurs in a basal branch with respect to *R. hispidus* and *R. fascicularis*. The above group is also well supported (PP= 100%, BS= 59%). Two samples of the Eurasian species *R. repens*, one of European origin and one collected in New Zealand, are sister to this group (PP= 84%, BS<50%).

◄ **Figure 3A**: cont. Origin of the sample is indicated next to species name and applies for the following figures. AJF: Archipelago of Juan Fernandez, ARG: Argentina, ASI: Asia, AUS (KS): Australia, Mount Kosciusko, AUS (MQ): Australia. Macquarie Island, AUS (NSW): Australia, New South Wales, AUS (VIC): Australia, Victoria, AUS (WA): Australia, Western Australia, CIRPOL: Circumpolar, EURO: European, KER: Kerguelen Island, MAC: Macaronesian, MED: Mediterranean, NAM: North America, NG: New Guinea, NZ: New Zealand, SAFR: South Africa, SSA: Southern South America, TAS: Tasmania, WW: Worldwide). Tree overview is presented in the upper left-corner. Location of the clade in the tree is indicated by the shading. Asterisks indicate clades containing New Zealand species.

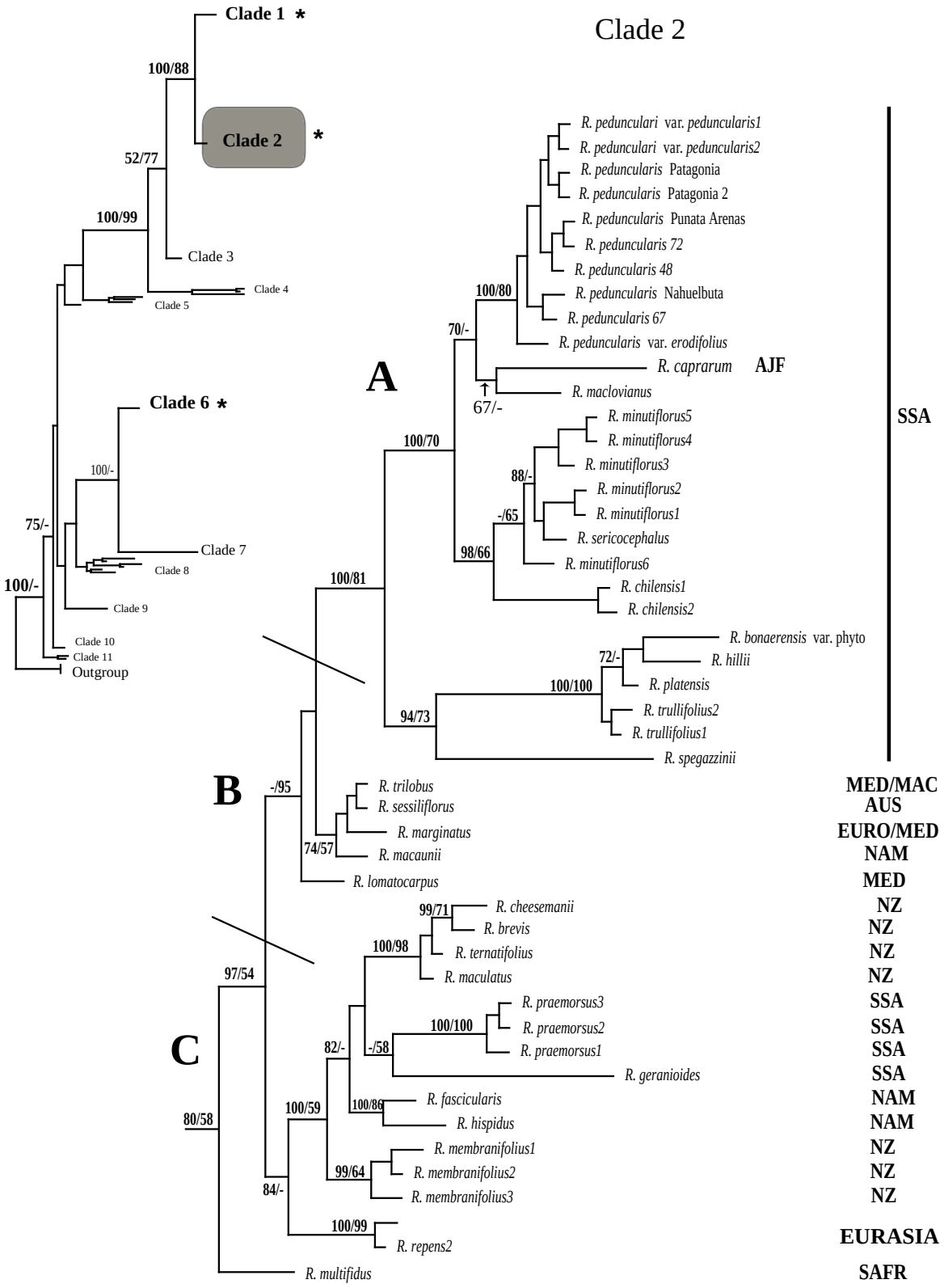


Figure 3B: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of nrDNA ITS sequences. Posterior probability values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches.

Clades 3, 4, 5 contain species from the Northern Hemisphere and Eurasia only, and these are dealt with in more detail in Hörandl *et al.* 2005 (Appendix 1). Clade 6 (Figure 3C) may be divided into two sub-clades, 6A and 6B. The first one includes all the New Zealand alpine *Ranunculus* of the section *Pseudoadonis sensu* Garnock-Jones (1988). They form a highly supported monophyletic group (PP= 100%, BS= 99%) that also includes two alpine species from Australia and Tasmania (*R. anemoneus* and *R. gunnianus*). Two well supported lineages of aquatic species were recovered as potential sister clades to the New Zealand alpine *Ranunculus*, although low BS or PP support was obtained for this relationship. The closest lineage contains three aquatic species from the European-Mediterranean region (*R. fluitans, R. penicillatus* and *R. peltatus*) and the second one contains species from southern South America (Patagonia) and the Kerguelen Islands in the Southern Ocean (*R. moseleyi* and *R. pseudotrullifolius*). These two lineages swap positions in the MP consensus tree, and the South America-Kerguelen Island lineage appears closer to the New Zealand alpines in the MP consensus tree than the European-Mediterranean lineage.

The second sub-clade, 6B (PP= 92%, BS<50%), is variable in composition and includes several species of aquatic or semi-aquatic habit occurring in North America, Eurasia, New Zealand, Australia, southern South America and some with circumboreal and worldwide distribution. New Zealand and Australian species form a well supported clade (PP= 100%, BS= 94%), with two New Zealand species on long branches, *i.e., R. macropus* and *R. limosella* (Figure 3C). The latter is uncommon in New Zealand and it is sister to the Australian *R. collinus*; however, low support was obtained for this relationship. Furthermore, *R. collinus* is not monophyletic and a second sample appears as sister species of *R. nanus* from Tasmania (PP= 97%, BS<50%). Paraphyly of *R. collinus* was also observed in the MP tree. *Ranunculus acaulis* is also paraphyletic and one accession from Stewart Island (New Zealand) groups with the sub-alpine species *R. papulentus* (Australia) and the lowland aquatic *R. prasinus* (Tasmania) (PP= 100%, BS<50%). The other accession of *R. acaulis* is from Chile and it is included in a well supported clade (PP= 100%, BS=100%) along with *R. crassipes* (Macquarie Island) and *R. biternatus* (Kerguelen Island and Patagonia). This clade is sister to the New Zealand+Australia clade with strong support (PP= 100%, BS= 94%). The relationship among three basal clades of 6B was locally unstable and collapsed as a trichotomy in the MP consensus tree. All the species in these clades are mainly found in the Northern Hemisphere or worldwide albeit one *taxon, R. fuegianus*. This species is endemic to

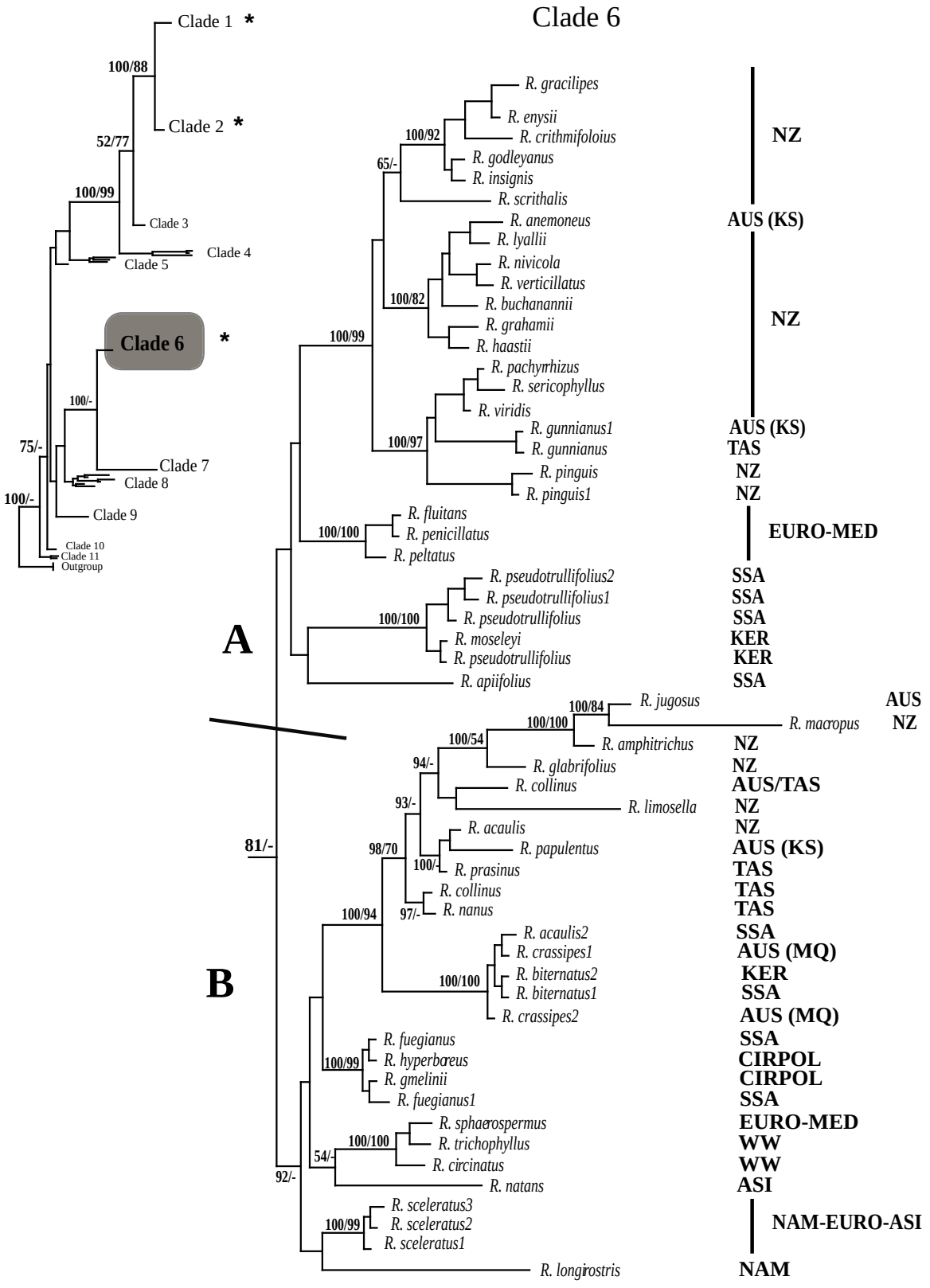


Figure 3C: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of nrDNA ITS sequences. Posterior probability values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches.

Patagonia in southern South America and in this study it groups with two circumpolar species *R. gmellini* and *R. hyperboreus* (PP= 99%, BS= 100%).

Clade 7 is sister to clade 6 and it is strongly supported in both analyses (PP+BS= 100%). It includes mainly species from the Northern Hemisphere and Central Asia and two semi-aquatic Peruvian species; *R. peruvianus* and *R. limoselloides*. These two taxa are supported as sister species with 100% BS and PP. Clade 8 includes about six species all from the Euro-Mediterranean region. For a detailed description of this clade see Hörandl *et al*. 2005 (Appendix 1)

Clade 9 is well supported (PP= 89%, BS= 100%) and includes several aquatic and semi-aquatic species found in the Northern Hemisphere and four South American species (*R. hydrophilus*, *R. psychrophilus*, *R. flagelliformis* and *R. bonariensis* var. *trisepalus*) (Figure 3D). These four species form a well supported clade (PP= 97%, BS= 100%) derived with respect to the species from the North Hemisphere. Clade 10 includes five species from section *Trollianthoideae* found in the Central Andes in Perú and Ecuador and the sole species of the Peruvian genus *Laccopetalum* (Figure 3D). These six species form a well supported monophyletic group (PP= 100%, BS= 97%) within a clade of European alpine species. This European-South American affinity is well supported by the Bayesian analysis (PP= 100%) but only weakly supported by the Bootstrap analysis (BS<50%). Clade 11 is sister to all other clades in the tree and includes two species found in the European Alps: *R. alpestris* and *R. bilobus*.

A series of smaller clades nested within the outgroup lead to the core *Ranunculus* clade (Figure 3E). Within these clades there are two clades of special interest for this study because they contain New Zealand species. One of these clades includes species of the genus *Myosurus* and *Ceratocephala* and their sister relationship to each other is well supported (PP= 98%, BS= 65%). Species of the genus *Myosurus* form a well supported clade (PP&BS= 100%), with the New Zealand endemic *M. novae-zelandiae* as sister to the North American species *M. minimus*. Species of the genus *Ceratocephala* also form a well supported monophyletic group (BS+PP= 100%) and the only New Zealand representative, *C. pungens*, appears in a more derived position and occurs on a longer branch than the Eurasian species included in this study. The other clade of interest contains species of the cosmopolitan genus *Halerpestes* and two accessions of *Ranunculus uniflorus*, endemic to the central and southern

Andes in South America. The support for this clade was high; PP= 100% and BS= 98%. Grouping of the *Halerpestes* species is well supported (BS+PP= 100%) and includes specimens of *H. cymbalaria* from Northern Argentina and Patagonia in southern South America and the endemic species *R. exilis*, restricted to salt lakes of the Atacama Desert in Chile. Both species are sister to the genus *Belckwithia* from North America. The remaining clades include several genera from North America, Eurasia, South Africa and South America. The sample of *Hamadryas* from southern South America groups with another South American monotypic genus, *Callianthemoides*, and the South African monotypic genus *Peltocalathos*, forming a well supported clade (PP= 100%, BS= 96%).

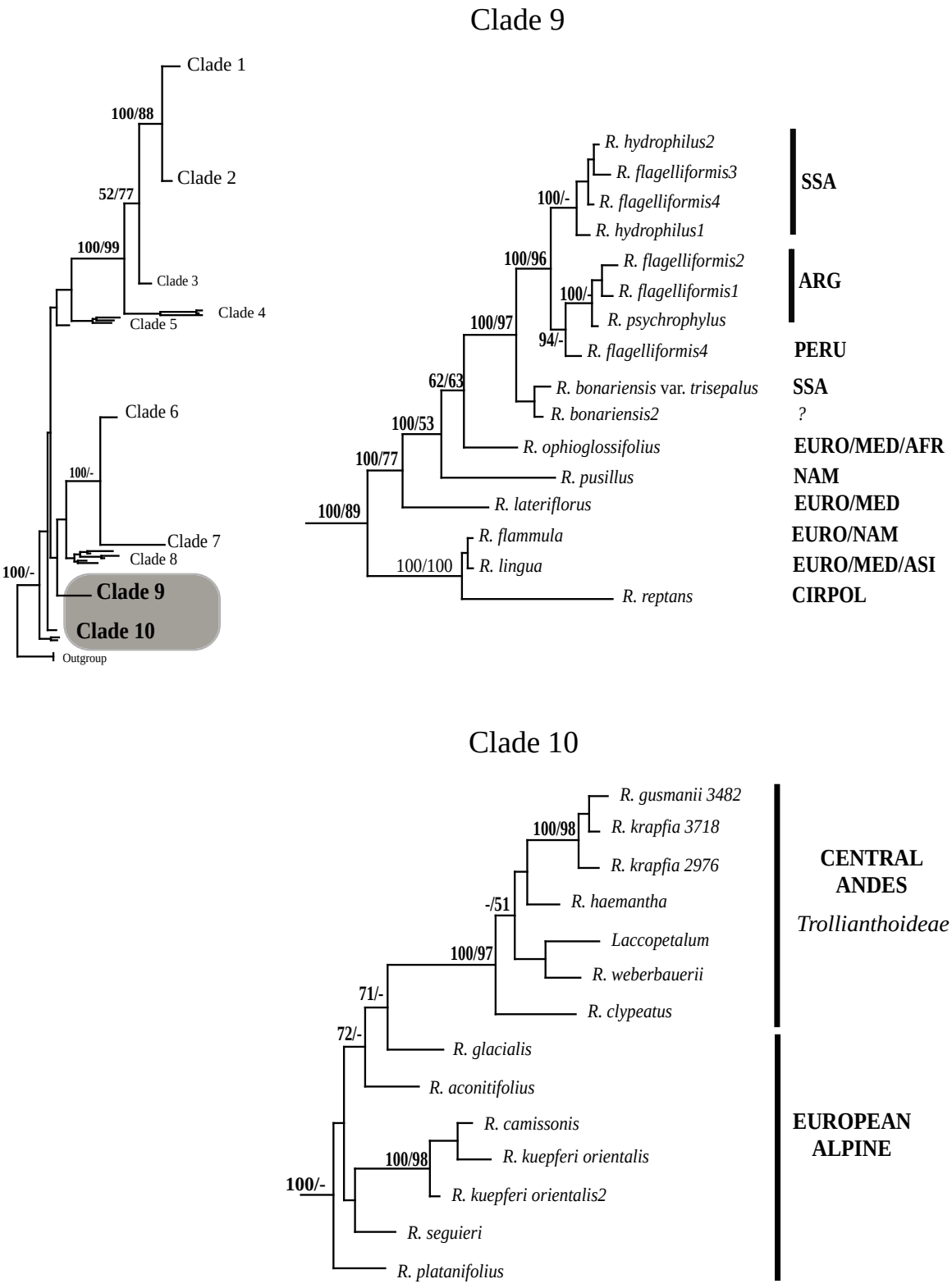


Figure 3D: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of nrDNA ITS sequences. Posterior probabily values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches.

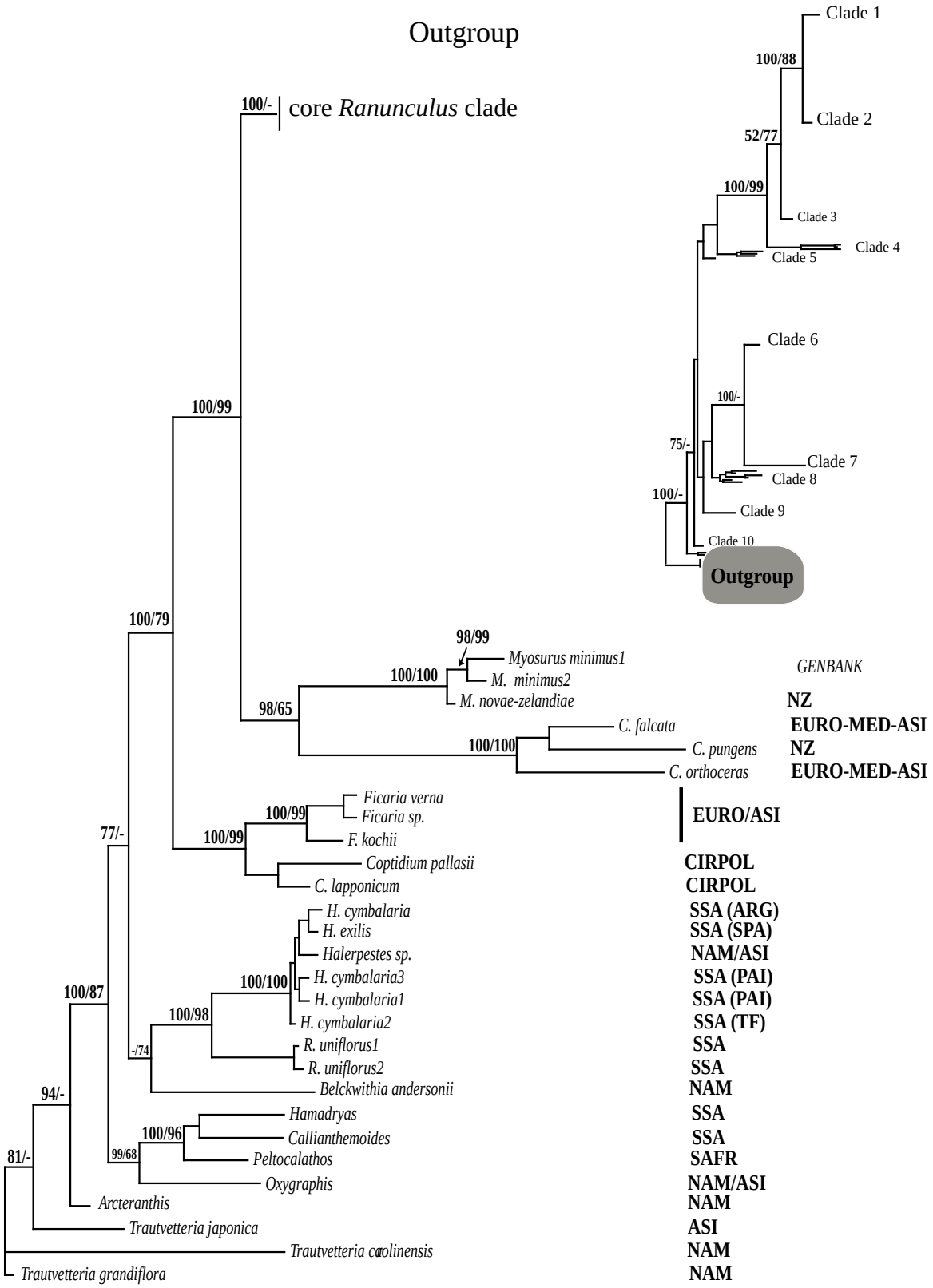


Figure 3E: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of nrDNA ITS sequences. Posterior probability values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches.

The mat*K* sequences ranged from 1420 to 1830 base pairs in length, and the aligned data matrix included 1946bp. After coding the gaps and including them in the dataset a total of 2031 characters were obtained; 480 were parsimony-informative. The MP analysis for the mat*K* data set resulted in 10 most parsimonious trees of 1383 steps. The MP 50% majority rule consensus tree (not shown) and the Bayesian phylogram (Figure 4A & B) had a similar topology. The only difference was the position of a clade containing 16 white flowering European species (indicated in Figure 4B). These *taxa* have low supported sister relationship to the largest clade found in Figure 4A in the MP tree. Bayesian and bootstrap support are high for the main clades. Bootstrap values ≥ 50 are indicated above the branches before the backslash symbol. Posterior probabilities ≥70% are indicated above the branches after the backslash symbol on the tree. This Bayesian phylogram will not be described in detailed since the number of species of interest for this study is low (highlighted in Figure 4A&B) and it has been described in more detailed by Paun *et al.* (2005) (Appendix 1). Furthermore this tree was only obtained to do the super network analysis detailed below.

Super-Network analysis of ITS and mat*K* trees

The super-network analysis showed that there is congruence between the ITS and the mat*K* gene trees and also supports the circumscription of the clades inferred from the MP and Bayesian analysis (Figure 5). The position of the root as inferred from the analysis of the ITS sequences has been indicated. The network shows reticulation at the base of most clades, *e.g.* Clade 1 and Clade 2 or Clade 3, while in some clades, for example Clade 6 and Clade 7, a star-like pattern is observed. Given the convergence between the ITS and mat*K* trees further discussion wil be focused on the more highly samples ITS tree.

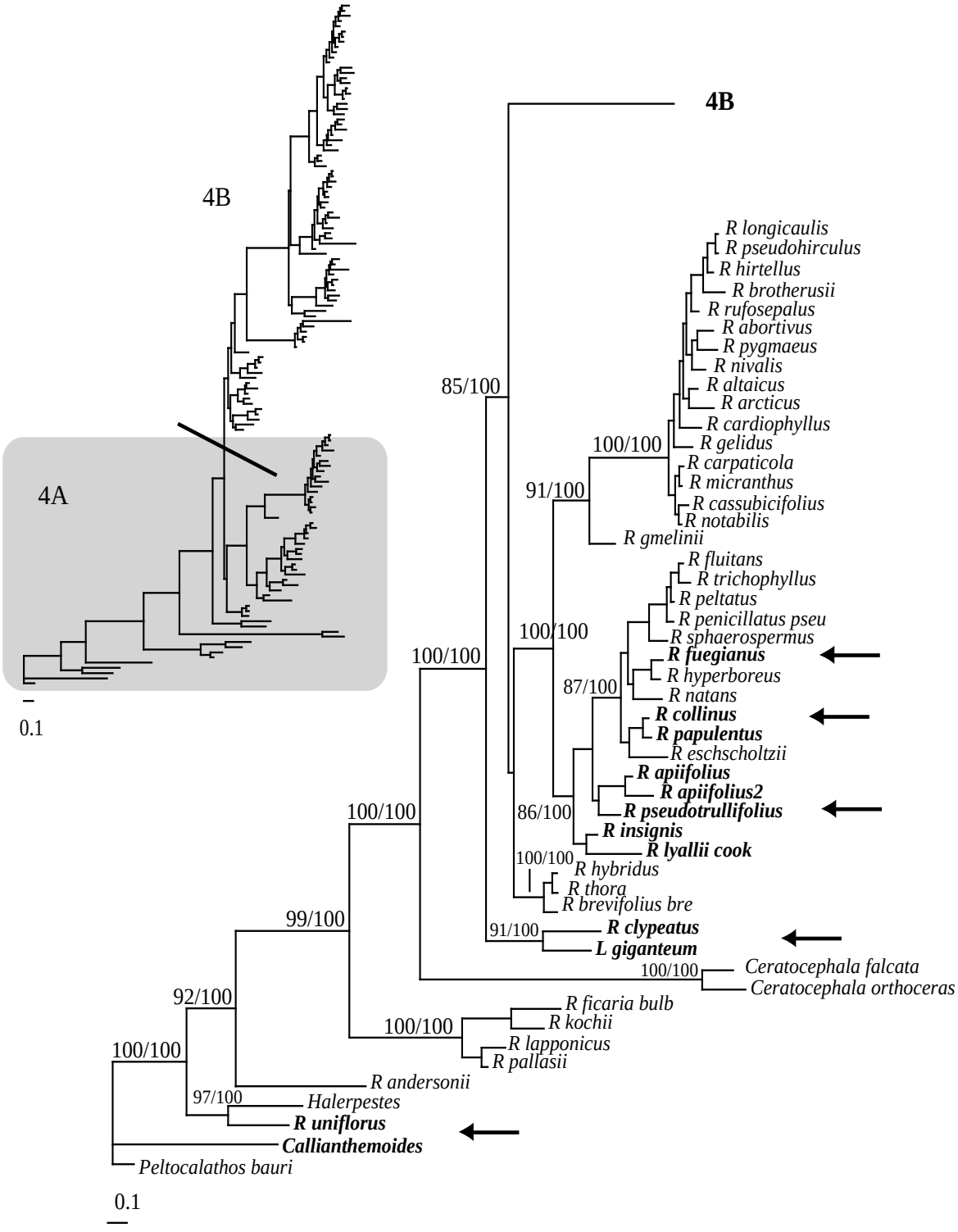


Figure 4A: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of cpDNA mat*K* sequences. Posterior probabilities values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches. Tree overview is presented in the upper left-corner. Species of interest for this study are highlighted and indicated by arrows.

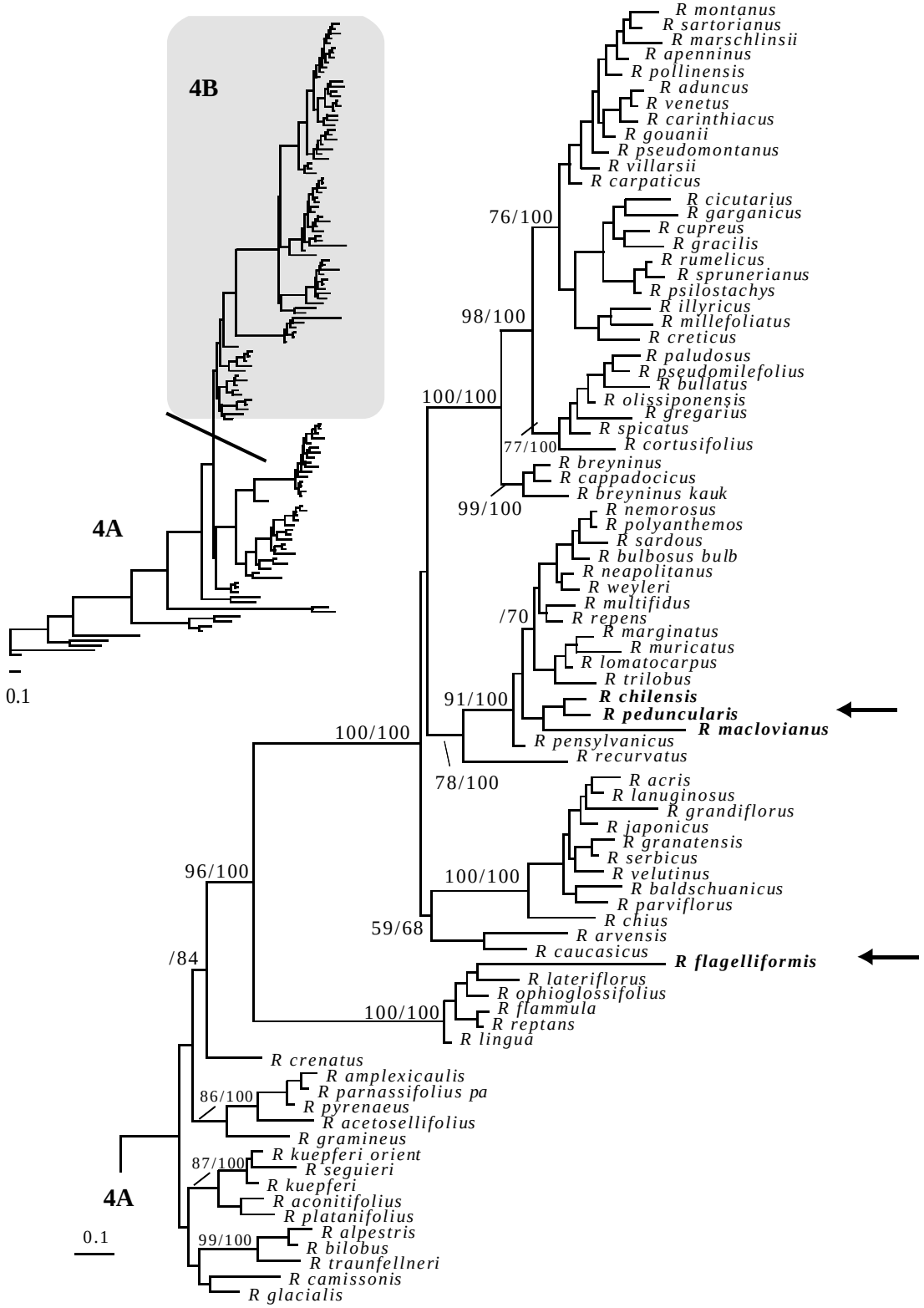


Figure 4B: Phylogenetic affinities of *Ranunculus* species and allied genera based on Bayesian analysis of cpDNA mat*K* sequences. Posterior probabilities values ≥ 50% (before slash) and bootstrap values ≥ 50% of maximum parsimony analysis (after slash) are indicated above the branches for the main clades only. Tree overview is presented in the upper left-corner. Species of interest for this study are highlighted and indicated by arrows.

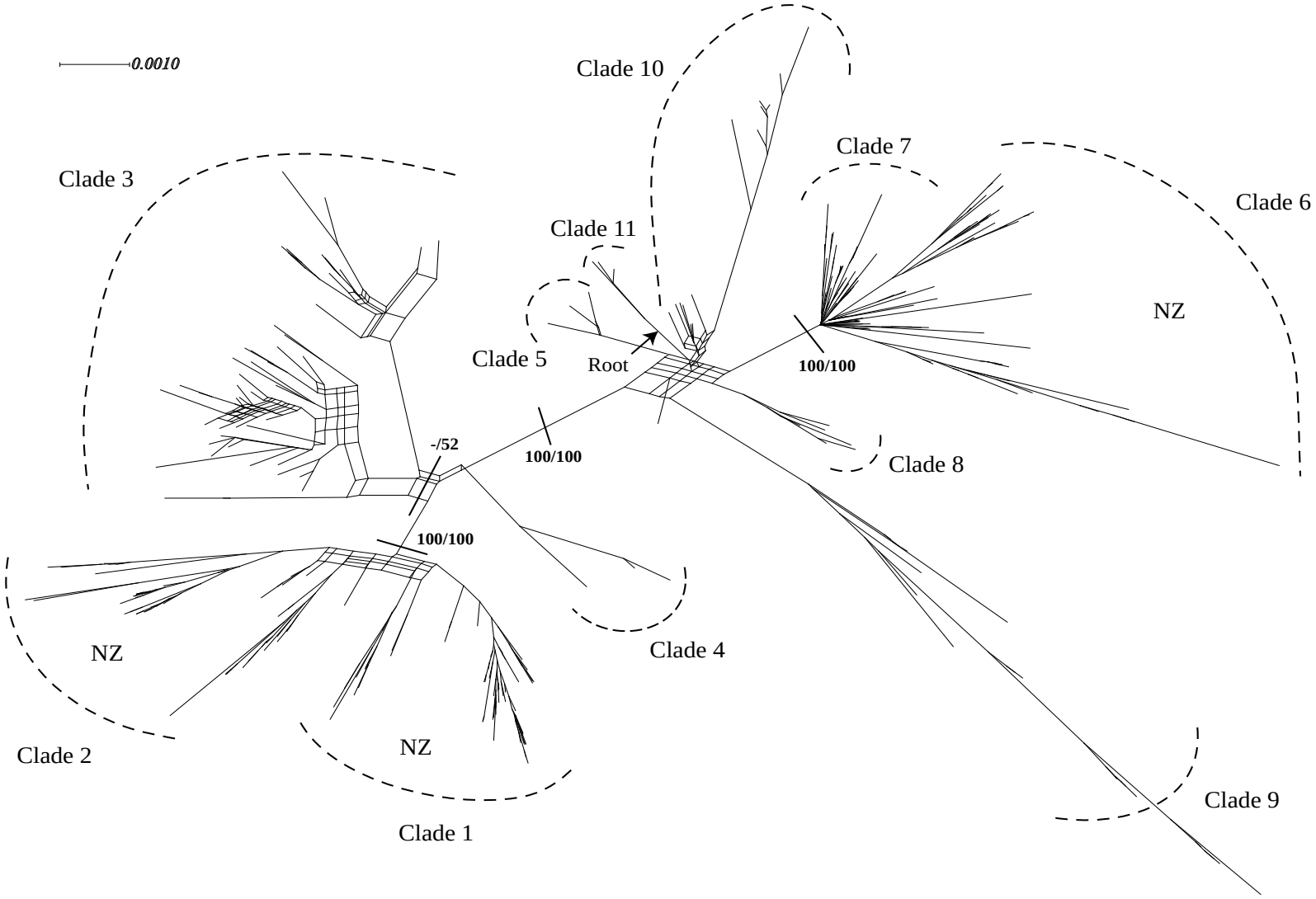


Figure 5: Supernetwork of the combined ITS and mat*K* Bayesian phylograms. Clades are numbered as in Figure 3. Posterior probabilities values and bootstrap values of maximum parsimony analysis for the main splits are indicated before and after the backlash, respectively. Clades including New Zealand species are indicated by NZ.

DISCUSSION

Phylogenetic affinities of New Zealand *Ranunculus*

New Zealand alpine and lowland *Ranunculus* do not form a monophyletic group and various affinities with species from Australia, New Guinea, North America, South America and the sub-Antarctic Islands were detected in this study. This conclusion is based on phylogenetic analyses of sequences representing all alpine and most lowland species of *Ranunculus* in New Zealand. Non-monophyly of New Zealand *Ranunculus* has been suggested before by Fisher (1965) and Wardle (1978). Furthermore, this finding was not unexpected based on results reported in earlier molecular studies by Lockhart *et al*. (2001) and Hörandl *et al.* (2005) that included a few representative *taxa* of the New Zealand species. Based on these results New Zealand *Ranunculus* can be divided into four groups.

The first group includes 10 of the 18 species included in the section *Ranunculus* by Garnock-Jones (1988) (Table 2). The phylogenetic analysis showed that species in this group are closely related to lowland and sub-alpine Australian *Ranunculus* from Mount Kosciusko and the Tasmanian mountains. Unlike most of these Australian species, the New Zealand species in this group are tetraploid (2n= 32) or hexaploid (2n= 48) except for the diploid *R. multiscapus*. The extent of genetic variability between these species is low, at least in the ITS marker, and this may suggest a recent origin of the group. Unfortunately this lack of variation also reduces the resolution and support for some of the clades. Nevertheless, several dispersal and *in situ* speciation events can be inferred within some clades based on the extent of sequence divergence and nesting patterns of relationship. Several of the New Zealand species in this group have an Australian sister species, some are nested within Australian species and some have a basal and others derived positions with respect to the Australian species. For instance, the threatened New Zealand species *R. recens s.l.* is nested among Australian species as is *R. kirkii*. Similarly, a New Zealand origin can be inferred for *R. decurvus* (Tasmania) which is nested among two New Zealand species (*R. carsei* and *R. foliosus*). Although the direction of dispersal is unclear, connection between New Zealand and Australia is also suggested by the sister relationship of the undescribed *taxa* labelled “Ranu5158” collected in the South Island with *R. eichlerianus* from Victoria state in Australia.

An *in situ* origin can be inferred for only two lineages, both of which are monophyletic (Fig. 3A): the sister species *R. reflexus* and the undescribed species *R.* “celatus” and the lineage formed by *R. altus*, *R. multiscapus*, *R. subscaposus*, *R.* “induratus” and the undescribed

specimen labelled “RanB1123”. Monophyly of the latter lineage provides support for the *in situ* origin of some endemic *Ranunculus* in New Zealand. The entire group (*i.e.*, New Zealand-Australia) is sister to a small clade of sub-alpine species from New Guinea-Asia.

The second group contains five species also included in the section *Ranunculus* by Garnock-Jones (1988) (Fig. 3B). All five species are tetraploid (2n= 32) as are the species they group with. This group includes one monophyletic lineage (*R. cheesemanii*, *R. brevis*, *R. ternatifolius* and *R. maculatus*) sister to a clade of two northern South American species; *R. preamorsus* and *R. geranioides*. Unlike these two species usually found in grasslands, the New Zealand species are restricted to wet habitats such as seepages, alpine bogs and swamps and bear solitary flowers. The fifth species in this group is represented by *R. membranifolius* and it may represent an earlier colonisation event into New Zealand from the Northern Hemisphere. This species has close affinity to the Eurasian species *R. repens* and is basal to two North American species. Genetic relatedness between *R. membranifolius* and *R. repens* was unexpected, especially when considering the morphological resemblance of *R. membranifolius* with *R. reflexus* found in Clade 1. It is likely that this phenotypic similarity is due to parallel evolution of their morphological characters.

The third group includes all five species in the lowland section *Epirotes sensu* Garnock-Jones (1988) (Fig. 3C). Including *R. amphitrichus* in the section *Pseudoadonis* as suggested by Tamura (1995) is not supported by this result. This group is not monophyletic and its closest affinities are with Australian species, principally Tasmania. Ploidy level within the group is high and 6-ploid, 8-ploid, 12-ploid and 18-ploid have been reported in Eichler & Walsh (2007). This trait is also shared with their Australian relatives (Eichler & Walsh 2007). Of the five species, an Australian origin can only be suggested for *R. acaulis* from Stewart Island. The origin of the remaining species is difficult to infer since two of them occur both in New Zealand and Australia and dispersal from Australia to New Zealand or vice versa, or both may have occurred. Unlike the species in Clade 1, genetic divergence between the species in this group is significant and most of the clades are well supported. This is particularly obvious in *R. macropus* and *R. limosella* found on particularly long branches suggesting considerable genetic differentiation after their origin. Genetic divergence, however, has not always been coupled with morphological divergence, and recognition of *R. macropus* from *R. amphitricus* and *R. glabrifolius* in the wild is not always easy because of their great phenotypic similarity. These morphological similarities are probably maintained by similar habitat conditions; all

three species are sub-aquatic and they are usually found in ponds or slow-flowing streams. Common ancestry of the entire group with a clade of sub-Antarctic species found in coastal aquatic habitats in southern South America and the Kerguelen islands was strongly supported.

The fourth group includes all the species assigned to the section *Epirotes* by Garnock-Jones (1988) or *Pseudoadonis* by Tamura (1995) (Fig. 3C). This group is monophyletic and two Australian alpine species have diverged from within this clade. Except for *R. nivicola* (12-ploid, 2n= 96) all other species in the group are hexaploid (2n= 48). Monophyly of this group has been reported before and its evolution has been described in detail by Lockhart *et al.* (2001). Phylogenetic affinities of this alpine group with the remaining New Zealand *Ranunculus*, however, remained uncertain. It is clear from the present study that none of the New Zealand lowland *Ranunculus* is related to this alpine group. In their study, Lockhart *et al.* (2001) described two hypotheses regarding the origin and affinities of this group. The first one was put forward by Fisher (1965) and predicts affinity with South American species in the section *Trollianthoideae* and the second one by Raven (1973) which suggests long distance dispersal from the Northern Hemisphere using New Guinea and Australia as stepping stones. Results from the present study did not provide support for either hypothesis. First, the section *Trollianthoideae* is not monophyletic and most of the species in the section appear basal within *Ranunculus* and closely related to species in the European Alps (Clade 10, Figure 3D). Secondly, this alpine group has no close affinity with New Guinean *Ranunculus* (but see above the first group discussed), as expected under Raven's hypothesis. A Northern Hemisphere origin, however, is not to be completely rejected. In fact, the closest sister clade of the New Zealand alpine *Pseudoadonis* contains three aquatic species from the Euro-Mediterranean region. Unfortunately this sister relationship has low BS and PP support. The second closest clade is formed by three aquatic species found in southern South America and the Kerguelen Islands and sub-Antarctic islands, but again support for these clades was low. Similar relationships have been obtained by Hörandl *et al.* (2005) using a smaller number of taxa and a slightly different approach in the data analysis where gaps were coded as a fifth character. Although there is not enough evidence to pinpoint closest relatives or the origin of this alpine group, there is strong evidence to believe it evolved from an aquatic ancestor. This is further suggested since this group is contained within a clade of aquatic to sub-aquatic species.

Taking into account the different affinities of New Zealand *Ranunculus* with overseas species, predicting the affinities of the three New Zealand species not included in this study is practically impossible. These species were included in the section *Ranunculus* by Garnock-Jones (1988). Only chromosome counts for *R. mirus* are available (2n=48). This species is found in alpine grasslands of the Nelson area in the South Island and morphologically resembles *R. reflexus* and *R. foliosus* both in Clade 1. The second species, *R. simulans*, is found in alpine bogs and seepages both in the North and South Island. This habitat is similar to that occupied by the monophyletic lineage formed by *R. maculatus*, *R. brevis*, *R. ternatifolius* and *R. royi* in Clade 2, but there is not further evidence to suggest relatedness of *R. simulans* to this group. The third species, *R. stylosus,* is endemic to Stewart Island and could also be related to this lineage. Morphological resemblance between *R. royi* and *R. stylosus* has been noticed before by Wilson & Garnock-Jones (1983); both species have solitary flowers but in *R. stylosus* the pedicel may have one or several bracts. However, as noticed before in *R. reflexus* and *R. membranifolius*, morphological similarity does not necessarily mean common ancestry.

Phylogenetic affinities of South American *Ranunculus*

Similar to New Zealand *Ranunculus,* South American species did not form a monophyletic group and affinities with species of New Zealand, Australia, North America and the Euro-Mediterranean region were inferred here. Furthermore, endemic species such as *R. semiverticillatus*, *R. exilis* and *R. uniflorus* fell outside the genus *Ranunculus* as delimited by Hörandl *et al* (2005). Most of the species occurring in southern Chile and Argentina formed a well supported clade. These species shared common ancestry with species such as *R. trilobus*, *R. marginatus* and *R. macaunii* from North America and the Euro-Mediterranean region. Species from this lineage have colonised different habitats in different regions of southern South America. For instance, evidence for colonisation of aquatic environments and a shift to terrestrial habit was observed in one of the earlier derived lineages within the group. The second lineage within this group, on the other hand, shows radiation into terrestrial habitats; grasslands/forest floor habitats and subalpine to alpine environments (*e.g. R. peduncularis*). An interesting hypothesis suggested by these analyses is a sister relationship between *R. maclovianus*, endemic to the Patagonian forests, and *R. caprarum,* endemic to Isla Alejandro Selkirk (= Masafuera) in the Archipelago of Juan Fernandez in the Pacific Ocean. This oceanic island is located c. 650kms from the continent and over 1900kms from Patagonia. Emergence of this island has been estimated at *c.* 1-2 million years ago (Stuessy & Taylor

1995). The relationship inferred between these two species is consistent with the connection between the Archipelago of Juan Fernández flora and the Chilean-Patagonian region suggested before by Takhtajan (1986).

A second group of South American species, *R. praemorsus* and *R. geranioides* (Clade 2B, Fig. 3B), is sister to a lineage of New Zealand species in Clade 2 (discussed in the previous section). These two species are found in northern South American (Argentina and Perú) and shared common ancestry with two North American species. Affinity between the species in this group is also supported by their chromosome number; 2n= 32.

The third group of South American species is found in Clade 6 (Fig. 3C). These appear closely related to the New Zealand alpine *Ranunculus*. However, support for this relationship is low. The semi-aquatic and aquatic species *R. appifolious* and *R. pseudotrullifolius* are found here. The first one is widespread in South America and has been recorded in wet habitats in Chile, Argentina, Brazil, Uruguay and Paraguay. The inclusion of *R. appiifolius* within *Ranunculus* has been questioned before by Tamura (1965) due to unique morphological characteristics of its flowers; small pink sepals and petals reduced to nectary scales. He considered *R. appiifolius* as 'primitive' and segregated it into the genus *Aphanostemma*. Similar to Hörandl *et al.* (2005), our results reject Tamura's reclassification of this *taxon* and support its inclusion in *Ranunculus*. The second species, *R. pseudotrullifolius*, is restricted to ponds and slow-flowing streams in the Chilean-Argentinean Patagonia and several circumpolar islands such as the Falkland Islands, the Kerguelen Island and Herald Islands. Only material from the Chilean-Argentinean Patagonia and Kerguelen Island were available for this study. The sample from the Kerguelen Island and a sample of *R. moseleyi* (endemic to the Kerguelen Island) appear sister to the Chilean-Argentinean accessions.

The fourth group in Fig. 3C is formed by the southern South American accessions of *R. acaulis* and *R. biternatus* and the Australian species *R. crassipes*. *Ranunculus acaulis* is aquatic and is generally found forming part of the riparian vegetation in coastlines or estuaries. The accession of *R. acaulis* from Chile did not group with the accession of *R. acaulis* from Stewart Island suggesting they belong to different evolutionary lineages. The Chilean accession grouped with *R. biternatus* and *R. crassipes*, from Macquarie Island, while

the New Zealand accession is sister to the Australian *R. papulentus*. This genetic difference is also supported by morphological vegetative characters.

The Chilean specimen is considerably bigger in size, the leaf lamina is wide, thin and divided into three leaflets with a dentated margin while the New Zealand accession is about 2cm tall, with short stems and a thick and waxy leaf lamina, also trifoliolate but with entire margins. Plants of New Zealand origin were grown over a year to investigate whether these characters were linked to habitat conditions but no major changes were observed (pers. obs.). An alternative explanation for this discrepancy could be that *R. acaulis*, as currently described in the Chilean Flora (Ruiz 2002) includes two entities: the one included in this study and *R. acaulis* as described in the New Zealand Flora by Garnock-Jones (1988). In fact, the New Zealand type has been collected before in Chile and several voucher specimens are stored in some Chilean Herbaria. The use of the names *R. biternatus* and *R. crassipes* also needs revision. The first name is used for a species restricted to *Nothofagus* forests in the Chilean-Argentinean Patagonia described by Smith in 1819. The specimen included in this study was collected from the same area as the type specimen used by Smith in 1819. The name, *R. crassipes* was used by Hooker in 1840 for the description of an aquatic to semi-aquatic species found in the Kerguelen Island (Garnock-Jones 1990). The name *R. biternatus* was used as a synonym for *R. crassipes* first by Cheeseman (1925) and then maintained under this name in the New Zealand flora (Garnock-Jones 1988) and in several other studies (Hennion & Couderc 1993, Hennion & Walton 1997). This has created considerable confusion on the identity of each species. Our findings suggest that *R. biternatus* is genetically similar to *R. crassipes* but a number of morphological and ecological traits can easily differentiate between them (ms. in preparation).

An unexpected finding is a close affinity of the Patagonian semi-aquatic species *R. fuegianus* with two aquatic species of circumboreal distribution, *R. hyperboreus* and *R. gmellinii*. Support for a close relationship of these three species is high and suggests recent dispersal to the Patagonian streams and ponds from places such as Iceland, Canada or Alaska. Morphological similarity between these three species is also remarkable, especially between *R. fuegianus* and *R. hyperboreus*.

Phylogenetic affinities with North American and European-Mediterranean species were also evident from a monophyletic lineage of four aquatic South American species: *R. boneaerensis*

var. *trisepalus*, *R. psicrophyllus*, *R. flagelliformis* and *R. hydrophilus*. The first species is widespread in South America and is found in Chile, Argentina, Brazil and Uruguay. It is possible that *R. boneaerensis* represents the first incursion of this aquatic lineage into South America from Euro-Mediterranean regions. This species is basal to a clade that includes accessions of *R. psicrophyllus* from Argentina, accessions of *R. flagelliformis* from Perú, Argentina and Chile and accessions of *R. hydrophilus* found in southern South America and the Falkland Islands. Unlike the other species in this clade, *R. flagelliformis* is not monophyletic. Northern accessions grouped with *R. psicrophyllus*, which is found in Bolivia and Argentina, while southern specimens grouped with *R. hydrophyllus*. It is likely that non-monophyly of the Chilean lineage of *R. flagelliformis* is caused by hybridisation events with *R. hydrophylus*. Hybridisation has been cited as a potential cause of paraphyly in other *Ranunculus* species before, *e.g.* Lockhart *et al.* (2001); however, this can not explain the sister relationship between *R. flagelliformis_3* from northern Chile populations (*e.g.* Tolhuaca) and *R. hydrophyllus*_2 from the Patagonia where *R. flagelliformis* does not occur. Sampling specimens of *R. hydrophyllus* from the Falkland Islands will probably help to unravel the relationship between these two species.

Finally, this study has showed the need for taxonomic revision of the South American *Ranunculus*. Four species currently treated as members of *Ranunculus* by the Chilean Flora (Ruiz 2002) have great affinity with genera outside the core *Ranunculus* clade as defined by Hörandl *et al.* (2005). The cosmopolitan species *R. cymbalaria* and *R. exilis*, endemic to high altitude salt-lakes in the Chilean-Argentinean Andes, are nested within the genus *Halerpestes*. These two species have been transferred from *Ranunculus* to *Halerpestes* by Tamura (1993) but this has not been acknowledged in the revision of the Chilean *Ranunculus* by Ruiz (2002). Species in this genus differ from *Ranunculus* in several anatomical characters of the achene the sclerenchyma layer containing the embryo is missing and it is replaced by a thin membrane of cells arranged in a jig-saw pattern and prominent veins running longitudinally along the achene. Similar achene features were observed in *R. uniflorus* found in Patagonia, suggesting relatedness between *R. uniflorus* and *Halerpestes*. Furthermore, common ancestry between these two taxa was evident from the Bayesian analysis (Figure 3E). Nomenclatural changes are needed for these species.

The fourth southern South American species that warrants nomenclatural changes is *R. semiverticillatus* (= *Callianthemoides semiverticillata*). Tamura (1993) excluded this species

from *Ranunculus* based in its achene morphology and the presence of thick tuber-like roots and elevated it to a monotypic genus. Similar to *Halerpestes*, the current revision of the Ranunculaceae in the Chilean Flora (Ruiz 1995) does not acknowledge this change. In the present study *C. semiverticillata* is sister to the southern South American genus *Hamadryas* and occupied a derived position with respect to the monotypic genus *Peltocalathos* from South Africa. Similar affinities were recovered by Hörandl *et al*. (2005) and Paun *et al.* (2005).

Southern Hemisphere *Ranunculus*, regional patterns of dispersal and dispersal vectors

The origin and primary differentiation of the Ranunculaceae family is thought to have occurred in the Northern Hemisphere after the break up of the super continent Pangaea about 180Mya (Ziman & Keener 1989). Although it has been impossible to pinpoint the origin of the ancestors of many of the tribes in the family, it is believed that diversification of *Ranunculus*, in the tribe Ranunculeae, occurred in the Euro-Mediterranean region and Eastern Asia (Ziman & Keener 1989). The date of origin of *Ranunculus* is unknown but Paun *et al.* (2005) have hypothesised that ancestors of the genus already had a wide distribution and ecological amplitude during the Oligocene. This coincides with the appearance of *Ranunculus* in the fossil record in Europe during the Oligocene Period, *c*. 37Mya (Pigg & DeVore 2005). During the Oligocene the land masses forming the super continent of Gondwanaland in the southern Hemisphere were still drifting apart but they were very close to reaching their present positions (Sanmartin & Ronquist 2004). South America was finally detached from Antarctica and drifted north towards North America allowing the Antarctic Circumpolar Current to flow, rapidly cooling the continent. The greatest differentiation of the genus, however, did not occur until the Miocene (10-7Mya) probably triggered by a period of global cooling (Paun *et al.* 2005). By this time, of the modern geologic features, only the land bridge between South America and North America was absent. Considering this paleogeographic scenario and the phylogenetic affinities of Southern Hemisphere *Ranunculus*, it is likely that colonization landmasses in the Southern Hemisphere by *Ranunculus* occurred post Gondwanaland break-up. A Northern Hemisphere origin has been suggested for other genera from the Ranunculaceae also found in the Southern Hemisphere, *i.e., Caltha* and *Anemone*, but unlike *Ranunculus* the origin and diversification of these genera have been set in the mid to late Cretaceous (*c*. 100mya) and it is believed they were spread via short-distance dispersal through the Northern Hemisphere and Gondwanaland since then (Schuettpelz *et al.* 2002, Schuettpelz & Hoot 2004).

Colonisation of *Ranunculus* into the Southern Hemisphere has occurred independently on several occasions and via different routes. Generally, dispersal has been followed by speciation events; *e.g*. the New Zealand alpine *Ranunculus* and the southern South American group. At least nine dispersal events from the Northern Hemisphere, principally Eurasia and the Euro-Mediterranean regions, to the southern landmasses is predicted from the findings of the present study.

It is likely that the ancestor of a number of sub-alpine and alpine New Zealand and Australian *Ranunculus* arrived from the Northern Hemisphere using southern Asia, New Guinea and Australia as "stepping stones". This route has been hypothesised before by Raven (1972) for several sub-alpine and alpine plants found in New Zealand and Australia and has been currently confirmed for a group of Australian *Ranunculus* (Armstrong 2003), *Corynocarpus* (Wagstaff & Dawson 2000), *Gunnera* (Wanntorp & Wanntorp 2003) and the Australian Chenopodiaceae (Kadereit *et al.* 2005). During the late Pliocene and Pleistocene (2-7Mya) the collision of the Australian plate with the Asian plate resulted in the uplift of mountains in Southeast Asia, New Guinea and Australia. Raven (1972) has proposed that the uplift of these mountain ranges would have created suitable habitats for the migration of sub-alpine and alpine plants between Asia and Australia. In the review by Winkworth *et al.* (2002) the stepping-stone hypothesis was not fully accepted due to the earlier divergence of New Zealand Alpine *Ranunculus* with reference to some Australian species. At the time of their review, however, the phylogeny of *Ranunculus* was far from complete and its diversity was largely underrepresented.

Direct long distance dispersal of *Ranunculus* from the Northern Hemisphere to New Zealand and Australia, *e.g*. from Eurasia, Euro-Mediterranean region and North America, seems to have occurred on several occasions. This is supported by the shared ancestry of *R. repens* (Eurasia) and *R. membranifolius* (New Zealand) and the sister relationship between *R. trilobus* (Euro-Mediterranean) and *R. sessiliflorus* (Australia). Long distance dispersal of plants in a single event has been considered unlikely by many scientists (Winkworth *et al.* 2002); however, recent phylogenetic studies of plant species with disjunct distribution seem to indicate the contrary. In fact, a revision by Sanmartin & Ronquist (2004) indicates that long distance dispersal has played a crucial role in development in the Southern Hemisphere biota. Direct long distance dispersal from Eurasia into Australia and New Zealand has also been

suggested, for example, to explain the bipolar distribution of *Scleranthus* (Caryophylaceae) by Smissen *et al*. (2003). These authors also suggested direct long distance dispersal to account for the bipolar distribution of *Ceratocepahala* (Ranunculaceae) which has two species native to Eurasia and a single endemic species in New Zealand. These three species were included in the present study as part of the outgroup (Figure 3E). Direct long distance dispersal from a North American ancestor is also likely to explain the origin of the sub-alpine and alpine New Zealand lineage formed by *R. maculatus*, *R. ternatifolius*, *R. brevis* and *R. cheesemanii*. Although seemingly an unlikely event, long distance dispersal from North America to the Southern Pacific has been used to explain the trans-Pacific disjunction and evolution of other plant groups (*e.g*. Chung *et al*. 2003). Furthermore, a recent revision by Mummenhoff & Franke (2007) reports direct long distance dispersal events between North America and New Zealand-Australia during the late Tertiary and Quaternary for the genera *Lepidium* (Brassicaceae) and *Microseris* (Asteraceae).

At least two other colonisation events from the Euro-Mediterranean and Circumboreal regions to the Southern Hemisphere, into the Sub-antarctic Islands in particular, were inferred from the present study. These events could explain the close affinity observed between the New Zealand alpine *Ranunculus* and two sub-aquatic *Ranunculus* in Patagonia and the Kerguelen Island with three aquatic Euro-Mediterranean species or the sister relationship between *R. fuegianus*, endemic to Patagonia, and the Circumboreal species *R. hyperboreus* and *R. gmelinii*. The latter three species share common ancestry with a small group of lowland semi-aquatic species found in New Zealand, Australia, southern South America and some of the Antarctic circumpolar Islands. Bipolar or "high-latitude" disjunct distributions at the species or species pairs level have been observed in numerous plant species (see review by Raven 1963) and they have been explained either by the origin on one side of the tropics and subsequent migration to the other in a single jump or by migration using mountain systems as stepping stones (Raven 1963). The morphological similarity observed within species or species pairs with bipolar disjunct distributions, however, suggest that they represent recent trans-tropical dispersal events (Moore *et al.* 2006), probably during the late Pliocene (Raven 1963). Fleming (1962) has referred to these connection as the Holartic elements of New Zealand Flora. Recent trans-tropical dispersal events may explain the great morphological similarity observed between the sister species *R. fueginaus* and *R. hyperboreus*. Plant dispersal, in the opposite direction, *i.e.*, from the Antarctic circumpolar region to the circumboreal region, however, has not been documented yet.

After arrival of *Ranunculus* in the Southern Hemisphere, dispersal has occurred in several directions and in multiple opportunities. Dispersal events from New Zealand to Australia and vice versa were inferred from this study for lowland, sub-alpine and alpine lineages. Dispersal across the Tasman along the west-wind drift has long been acknowledged (Raven 1973, Jordan 2001, McGlone *et al*. 2001). Dispersal against the westerly winds, however, has been considered less likely (Cook & Crisp 2005) and despite the evidence presented by Winkworth *et al.* (2002), Cook & Crisp (2005) believe dispersal from New Zealand to Australia, as indicated in the phylogenetic studies by Lockhart *et al.* (2001) and Winkworth *et al.* (2002) is unlikely. Phylogenetic patterns and the numerous dispersal events that can be inferred from this study, however, challenge Cook & Crisp's ideas and provide further support for dispersal from New Zealand to Australia. Colonisation of many islands in the Antarctic circumpolar region has also followed the dispersal of *Ranunculus* into the Southern Hemisphere probably assisted by the Antarctic Circumpolar Current. This may explain the presence of species such as *R. pseudotrullifolius* in Patagonia and on the Kerguelen Island and *R. crassipes* on the Macquarie and Kerguelen Islands. The presence of the Humboldt Current, parallel to the Chilean coastline and with a South to North direction, could have also assisted in the dispersal of the ancestor of *R. caprarum* in the Archipelago of Juan Fernandez from Patagonia.

Dispersal capability of *Ranunculus* seeds has been considered low (see Chapter I) and studies in North American and Australian alpine species suggest it is usually limited to within 15-30cm from the mother plant (Scheffer et al 1994, Armstrong 2003). Although these measurements may suggest that the type of long distance dispersal suggested above for *Ranunculus* is impossible, it is important to point out that the species studied by Scheffer *et al*. (1994) and Armstrong (2003) have achenes with smooth surfaces and therefore lack dispersal adaptations. Unfortunately, dispersal capability in *Ranunculus* species with achenes adapted to hydrochory (water transport) or epizoochory (animal transport) has never been studied. It is believed that hydrochory may promote long-distance dispersal and the wide distribution of many aquatic *Ranunculus* has been attributed to this dispersal mechanism (Hörandl *et al.* 2005). Achenes of many aquatic *Ranunculus* have an internal layer of spongy tissue with air spaces that facilitate flotation (van der Pijl 1982). Species in grasslands, meadows or forest floors, on the other hand, appear to use animals as dispersal agent and this would explain the presence of tubercles or hooked spines on the achene's testa (Paun *et al.* 2005). Such adaptations are typical of many annual *Ranunculus* of the Mediterranean (Paun *et*

al. 2005) and they are present in a number of lineages sister to the Southern Hemisphere *Ranunculus* (e.g. *R. muricatus* and *R. trilobus*).

The importance of birds in long distance dispersal of *Ranunculus*' achenes has not been suggested before but it has been inferred in several genera found in Hawaii (Carlquist 1981) and their effectiveness as dispersal vectors could be considerable. Winkworth *et al.* (2002) suggested transoceanic wanderers such as albatross, petrels and shearwaters, could be potential dispersal agents since they regularly travel between southern Pacific landmasses. A recent study on the migratory routes of sooty shearwaters using electronic tracking tags have demonstrated these birds cover incredibly large distances and different destinations on a yearly basis (Shaffer *et al.* 2006). Transportation of achenes of aquatic or semi-aquatic species on birds feet or plumage may explain, for example, the records of cosmopolitan species such as *R. trichophyllus* in many high altitude unconnected lakes along the Andes (Seimon *et al.* 2007) and all the way down to Patagonia (Moore 1981). If this dispersal mechanism is confirmed it could be considered highly effective. This may also explain, for instance, the colonisation of *R. trichophyllus* in recently deglaciated lakes in the Mount Everest region, a process that occurred within a 20 year period (Lacoul & Freedman 2006).

It is clear from this study that phylogenetic affinities of New Zealand and southern South American *Ranunculus* are multiple and that they have undergone incredible radiation and speciation after colonisation from the Northern Hemisphere. Colonisation of the Southern Hemisphere occurred on several occasions and via different routes and possibly by different vectors. Dispersal using mountain tops as stepping stones or direct dispersal aided by ocean currents or migratory birds are the most likely dispersal vectors. Future studies to investigate possible mechanisms of dispersal are needed (Knapp *et al*. 2007) as are studies using other nuclear and chloroplast markers. These will be essential for obtaining better resolution of affinities within regional groups of recent origin. The timing of these radiations and the factors promoting their speciation and morphological evolution are also of great importance for understanding the Southern Hemisphere flora. Understanding factors promoting speciation and morphological diversity of alpine New Zealand *Ranunculus* will be explored in the following chapters of this thesis.

III

Morphological variation and species delimitation in complex *Ranunculus* taxa of the New Zealand Alps

INTRODUCTION

Delimiting species is complicated in plants and particularly so when reproductive barriers are lacking and species are sympatric. The absence of reproductive barriers between species of many plant genera has demonstrated that the Biological Species Concept proposed by Mayr in 1963 can not be applied as easily applied to plants as it can be to animals. This concept defines a species as a group of interfertile individuals and, therefore, members of different species should not be able to interbreed. This is, however, only one of the over 20 species concepts or definitions put forward over the years (see review in Hey 2001, Mallet 2001). These include the Phenetic Species Concept, the Evolutionary Species Concept and the Ecological Species Concept, among others. The Phenetic Species Concept recognises species by morphological characters. Under this criterion species are groups of individuals with certain morphological characters. In practice, the phenetic concept measures as many characters as possible and then recognises phenetic clusters by multivariate statistics. These clusters approximate to a level of similarity sufficient to be called a species (Futuyma 1998, Judd *et al* 2002). The Evolutionary Species Concept proposed by Wiley in 1978, in contrast, emphasises recognition of evolutionary lineages, although it does not clearly describe how these lineages should be identified. In this concept, a species is a single lineage of ancestor-descendent populations which maintains its identity from other such lineages and each has its own evolutionary tendencies and historical fate. Unlike the concepts previously presented, in the Ecological Species Concept, a species is a set of organisms adapted to a particular set of resources, a niche, in the environment. According to this concept, populations form the discrete phenetic clusters that can be recognised as species because the ecological and evolutionary processes controlling how resources are divided up tend to produce those clusters (Judd *et al* 2002). Despite the number of studies and species concepts proposed there is still no consensus about species concepts in plants and even after years of discussion and theoretical developments criteria for the delimitation of plant species remains difficult.

Nowadays, after years of discussion and theories, identification and delimitation plant species is still difficult.

Furthermore, species boundaries may be extremely difficult to delimit in some groups due to the nature of phenotypic variation. Morphological variation at the species level in plants is the end result of multiple interactions between genetic, ecological, developmental and selection

processes. At the genetic level, plant phenotypes may be significantly affected by hybridisation which may promote the formation of novel or intermediate phenotypes (Stebbins 1957, Grant 1981, Linder & Riseberg 2004). Phenotypic differences may be enhanced even more by subsequent back-crossing (*i.e.*, introgression) between the resulting hybrids and their putative parents (*e.g*. Hedge *et al.* 2006). This is possible when the parental species and the hybrid progeny are partially or entirely sympatric and reproductive barriers are weak or absent and the resulting hybrid progeny show certain degrees of fertility. Polyploidy may also promote phenotypic variation in plants and its importance in plant evolution has recently received considerable attention (Soltis & Soltis 1999, Tate & Simpson 2003, 2004). Although it is generally assumed that polyploid individuals are larger and more robust than their diploid parental species, the opposite trend has also been observed (Stebbins 1957, Hörandl 2002, Tate & Simpson 2003, 2004) and polyploid individuals with reduced flower size and petal numbers, for example, have been described within some European *Ranunculus* species (Hörandl 2002). Polyploids of hybrid origin (allopolyploids) may also promote phenotypic variation but unlike diploid hybrids, allopolyploidy results in immediate speciation (Stebbins 1974, Linder & Riseberg 2004), with the resulting progeny being sometimes phenotypically more stable.

Depending on a plant's distribution and morphological plasticity, different patterns of morphological variation as responses to environmental conditions can also be detected, including clinal variation, formation of ecotypes, and phenotypic plasticity. Clinal variation is usually observed in species with continuous distributionS and occupying areas that range from one environmental regime to another, *e.g.* coastal-inland populations or altitudinal gradients (Stebbins 1957, Grant 1981, Ellison *et al.* 2004). Clinal morphological variation may be noticeable in all or only a couple of the plant characters, generally those of adaptive value (Stebbins 1957, Boyd 2002). Ecotypes, on the other hand, are usually observed in species with fragmented distribution, such as alpine plant species restricted to isolated mountain tops (Stebbins 1957, Grant 1981). Such ecotypes arise when morphological variation between these allopatric populations results from adaptation to local environmental conditions. The evolution of such phenotypes is promoted by selection and restricted gene flow among neighbouring populations, and also genetic drift when the populations are small (Grant 1981). Ecotypes can also be maintained even if gene flow is present, due to strong habitat selection, *e.g*. *Anthoxanthum* species adapted to toxic soils (Antonovics 2006). Finally, a species phenotype may dramatically change as a response to a heterogeneous and

changeable environment a feature known as phenotypic plasticity. Phenotypic plasticity of leaf characters in *Ranunculus* species is well known, particularly in semi-aquatic and aquatic species occurring in unpredictable habitats. Some typical examples are *Ranunculus repens* (Lynn & Waldren 2001) and *Ranunculus flammula* (Cook & Johnson 1968). These changes are generally an adaptive response to their environment and can be discrete or continuous.

To determine what criterion was using Fisher (1950) to define species within the alpine *Ranunculus* is not simple. Nevertheless, it is clear from his work that he recognised gene flow between the different entities and the extent of morphological variation within each entity. Morphological variation within some species of the New Zealand alpine *Ranunculus* is remarkable and eight "divergent patterns of variation" have been described by Fisher (1965). He also suggested that a number of these patterns occur in some species concurrently. For instance, after studying the distribution and variation of several morphological characters in *Ranunculus insignis* and *Ranunculus enysii*, Fisher (1965) concluded that patterns of clinal and "racial" variation occurred in the first species and patterns of sub-specific and "racial" variation in the second one. Less variable species such as *Ranunculus lyallii* were listed under the "uniform pattern" category, which he suggested was a consequence of habitat specialisation. Phylogenetic affinities within New Zealand alpine *Ranunculus* have been investigated using several molecular techniques by Lockhart *et al.* (2001), Piripi (2005) and Carter (2007). These studies have shown that *R. insignis*, *R. enysii* and *R. lyallii* are not monophyletic species. Whether this morphological variation is linked to their phylogenetic history is unknown.

The main goals proposed for this chapter are to investigate the extent of morphological variation within these three New Zealand alpine species of *Ranunculus* and assess the contribution of phylogenetic history and ecological variation to such patterns. To do this, I analysed and correlated quantitative and qualitative morphological characters, nuclear ribosomal and chloroplast DNA sequences and environmental data. This study will provide useful information to (i) assess whether the current species boundaries correspond to the morphological and genetic diversity of each species and to (ii) develop hypotheses explaining their evolutionary history.

METHODS

Study case 1: *Ranunculus insignis s.l.*

Ranunculus insignis is a common species in the alpine habitats of New Zealand. It is usually found in shady areas of sub-alpine scrub, snow tussock grasslands, herbfields and limestone cliffs in the North and South Island of New Zealand (Figure 6). The extent of morphological variation observed in *R. insignis* has been discussed in a number of studies since its description (Allan 1926, Fisher 1960, Fisher 1965). The species was originally described by J.D. Hooker using material collected from Mount Hikurangi in the North Island. Original localities mentioned in his description are all from the North Island (Ruahine Range, and Tongariro and Hikurangi Mountains). Later, in 1855, Hooker described *R. monroi*, and indicated the type localities as "Summit of McCrae's Run and Fairfield Downs", probably in the Inland Kaikoura Range, South Island. Morphological similarity between these two species and the discovery of new populations with individuals of intermediate phenotypes encouraged botanists such as Kirk and Cheeseman to redefine the boundaries between these two species. Kirk (1899) created *R. insignis* var. *lobulatus* (type: Mount Fyffe, Kaikoura Range) and circumscribed its occurrence to lowland and montane grasslands of the Upper Awatere River, Clarence River and Kaikoura Ranges and foothills in the South Island. This variety was given species status by Cockayne (1906). Cheeseman (1925), on the other hand, subdivided *R. monroi* into the varieties *dentatus* and *sericeus*. The first one included plants with ovate to ovate-lanceolate leaves with dense pubescence occurring in the Kaikoura Ranges, Mount Torlesse and Broken River, all from the South Island. *Ranunculus monroi* var. *sericeus* included plants with round-reniform, ovate or cordate leaves with the margin coarsely crenate or crenate-lobulate and achenes with silky hairs. This variety had a disjunct distribution and occurred in the Kaikoura Ranges (Marlborough) and Mount Peel (Canterbury, South Island).

Fisher (1965) assessed the stability of several vegetative and reproductive characters from wild and cultivated individuals in this species complex and concluded that "the overall variation pattern takes the form of an irregular cline" and that many of the characters used as diagnostic features in the former classifications are too variable and lack taxonomic value. However, he also stated that "most differences originally present were retained after two years of cultivation" and "significant modifications were only observed in overall size, and the texture and vesture of the leaves". Although his results suggest these morphological characters are genetically determined and not an environmental response, he concluded that

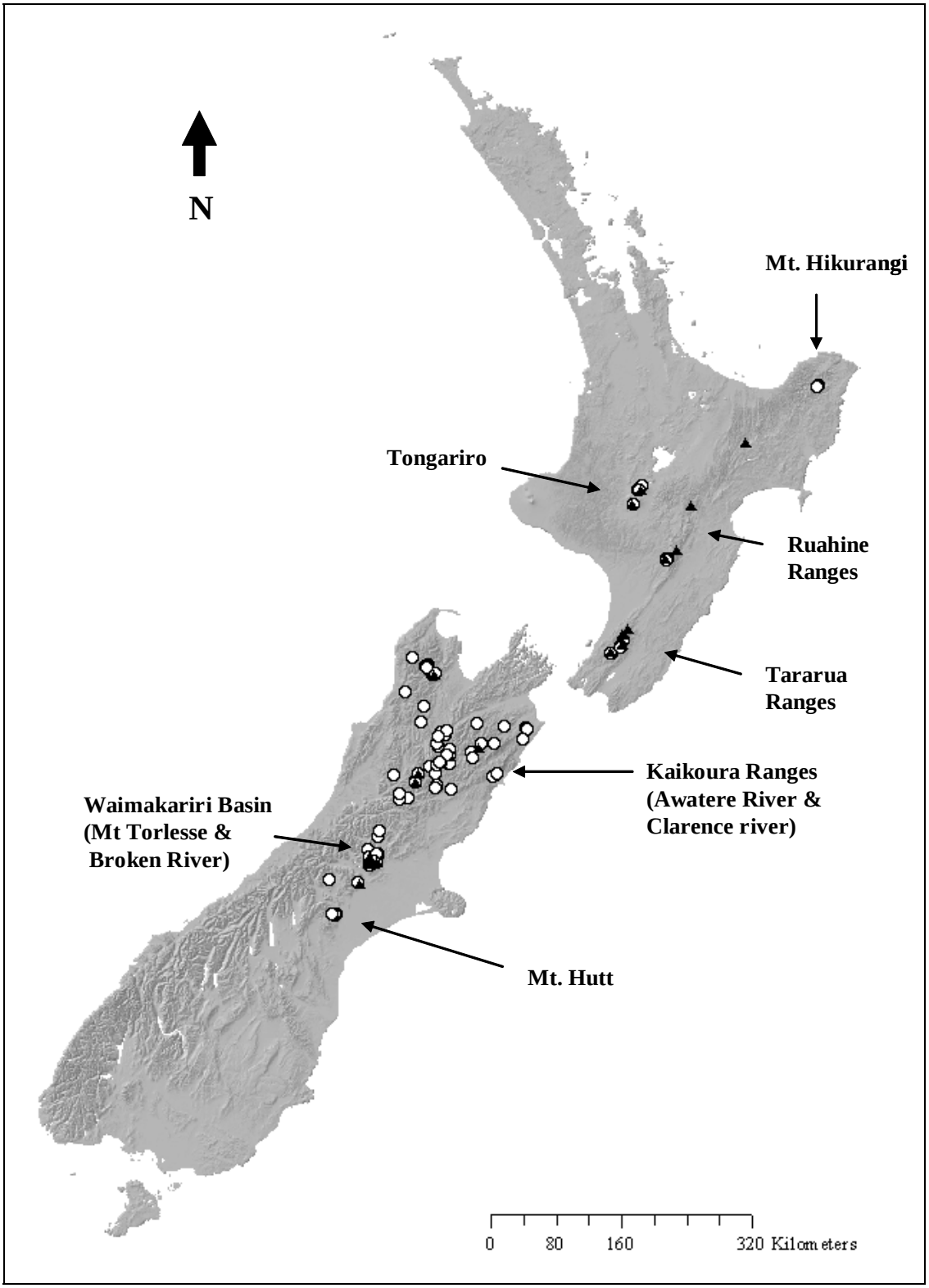


Figure 3: Distribution of *R. insignis s.l.*. Open circles indicate specimens used in the morphological analysis. Filled triangles indicate specimens included in the molecular analysis.

R. lobulatus and *R. monroi* should be considered part of *R. insignis*. The most up to date and currently accepted revision of the genus by Garnock-Jones (1988) follows Fisher's criterion and thus here the names *R. lobulatus* and *R. monroi* are considered as synonyms of *R. insignis*.

Study case 2: *Ranunculus enysii s.l.*

Ranunculus enysii is one of the most variable species of *Ranunculus* found in the South Island (Figure 7). It grows in sheltered places among tussock, scrub or in rocky clefts between 900-1500 m.a.s.l. As currently defined, it includes at least four variants based on leaf forms. The original description of *R. enysii* by Kirk (1880) was based on material from populations growing in the Trelissick Basin (*i.e.,* Waimakariri Basin). Plants with two different leaf types were recorded from this area: lamina divided into three simple leaflets or divided into five stalked leaflets. The other two forms were formerly known as *R. berggrenii* and *R. novae-zelandiae*; both names are currently regarded as synonyms of *R. enysii* (Fisher 1965, Garnock-Jones 1988). The name *R. berggrenii* was assigned to material collected in the Carrick Range near Cromwell. The most distinctive character of this form was the presence of entire, or slightly 2-3-lobed, orbicular to reniform leaves. The name *R. novae-zelandiae*, on the other hand, was used for specimens found in the Rock and Pillar Range, the Old Man Range and the Garvie Range. The leaf lamina of these specimens is 3-7 foliate or ternately divided and the lateral leaflets sessile with a slightly crenate to entire margin. In his study, Fisher (1965) widened the boundaries of Kirk's *R. enysii* to include *R. berggrenii* and *R. novae-zelandiae*. The presence of a distinctive nectary gland shape, digitate to semi-digitate glabrous leaves and a red pigmentation of the leaf veins were the characters in common used to group these species under *R. enysii*. He also indicated that the abundance of intermediate morphologies hinders subdivision of the group even at the sub-specific level. After growing the different forms for two years under common garden conditions, only minor changes in size, pigmentation and texture were observed by Fisher (1965) and he concluded that the variation observed must have a strong hereditary component. In this chapter the four forms will be referred to as 3WB and 5WB (the two forms of *R. enysii s.s.* from the Waimakariri Basin), R&P (*R. novae-zelandiae*) and BER (*R. berggrenii*). A fifth form collected in Bordland Burns (Southland) was recognised while doing this study and it will be indicated as BB.

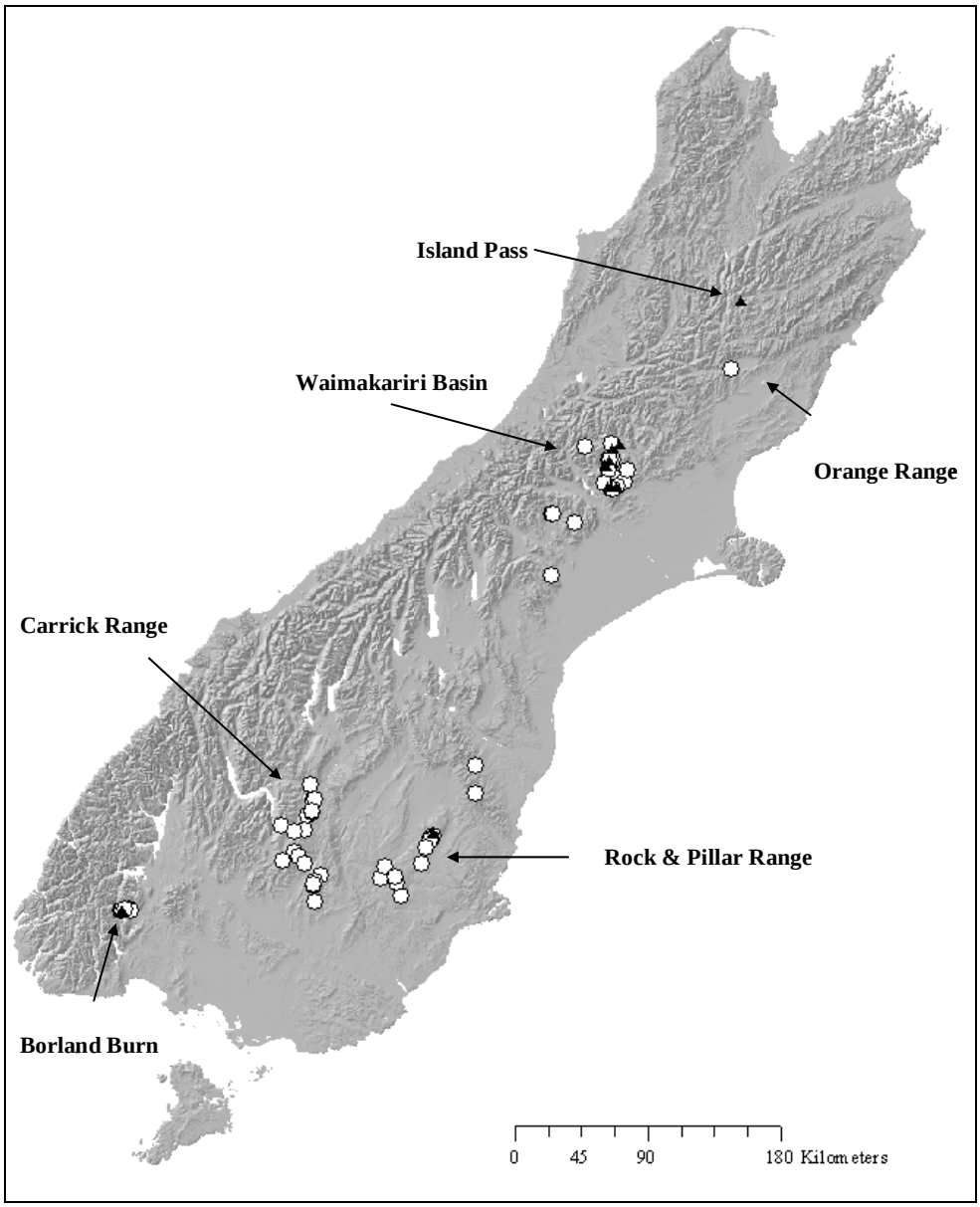


Figure 4: Distribution of *R. enysii s.l.* Open circles indicate specimens used in the morphological analysis. Filled triangles indicate specimens included in the molecular analysis.

Study case 3: *Ranunculus lyallii*

Ranunculus lyallii is one of the best known *Ranunculus* species from New Zealand. Although *R. lyallii* is locally common, its distribution is restricted to the higher rainfall areas throughout most of the South Island and Stewart Island (Figure 8). It is generally found growing by stream sides and wet sub-alpine to alpine scrub and grassland between 450-1500 m.a.s.l. Unlike *R. insignis s.l.* and *R. enysii s.l.*, *R. lyallii* is morphologically uniform and no substantial variation has been observed across its distribution. Only two varieties have been

distinguished in the past: var. *traversii* and var. *araneosa*. The first one is smaller with deeply crenate leaves and cream flowers while the second one is covered with white flaccid hairs (Allan 1961). Both varieties were regarded as localised hybrids of *R. lyallii* with *R. godleyanus* and *R. buchananii*, respectively, by Fisher (1965).

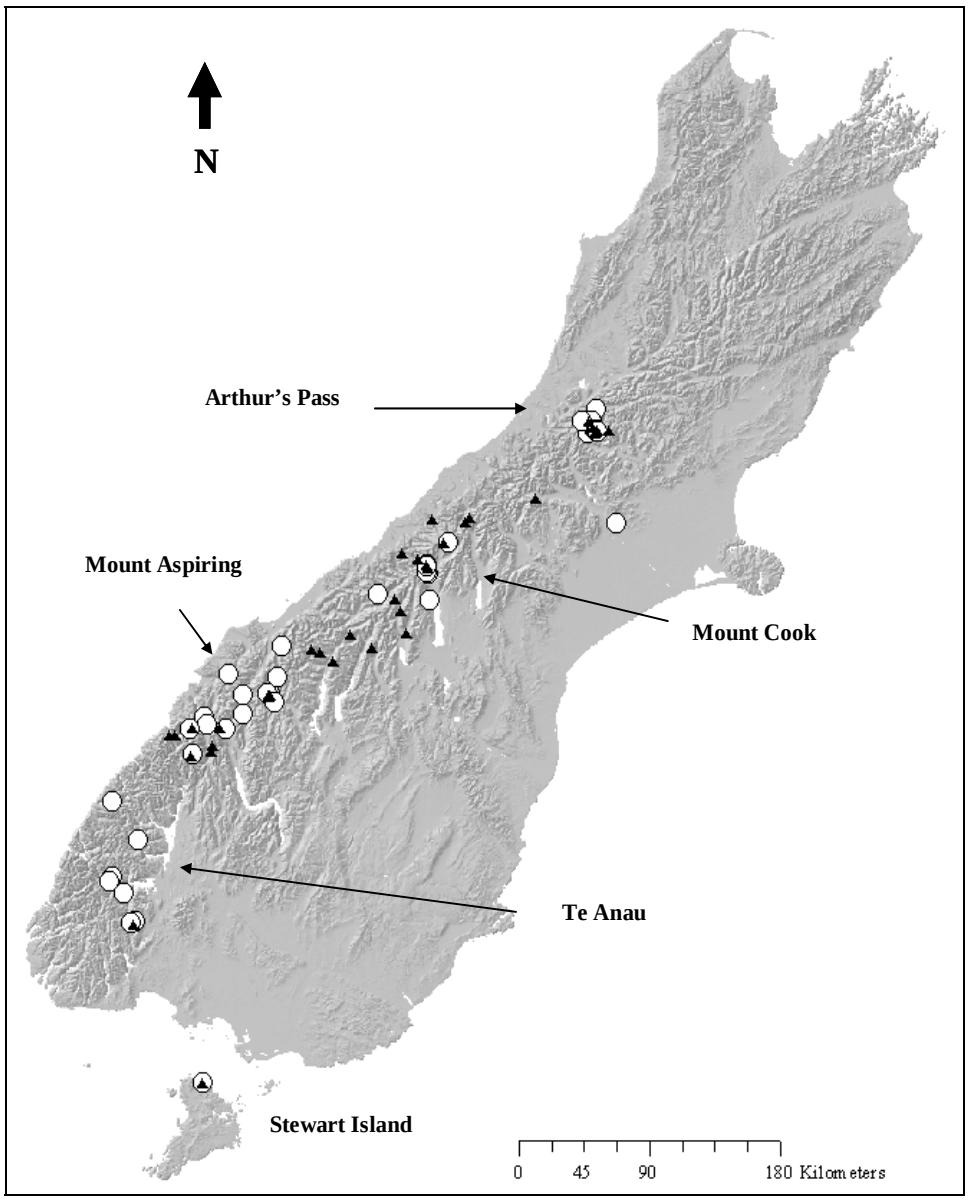


Figure 5: Distribution of *R. lyallii*. Open circles indicate specimens used in the morphological analysis. Filled triangles indicate specimens included in the molecular analysis.

Morphological variation

Samples for all three species were obtained from the main New Zealand herbaria (AK, CHR, MPN, OTA and WELT) and also collected from the wild. To gain a fair representation of the intraspecific morphological variation of each species, samples collected across the entire range of each species were included. The number of samples included in the morphological analysis was 146 for *R. insignis*, 83 for *R. enysii* and 41 for *R. lyallii*. Collection sites are indicated in Figures 6, 7 and 8 by open circles. A number of qualitative and quantitative morphological characters were scored from specimens bearing both floral and/or fruiting structures. Characters measured for each species are indicated in Tables 3, 4 and 5. Many of these characters have been previously regarded as taxonomically diagnostic and used in their formal taxonomic descriptions. Additionally, a number of other potentially informative characters were also included in the present study. Detailed observation of the material was assisted by the use of a dissecting microscope and measurements were made using a digital calliper. Leaf measurements were always made on the third leaf from the base. Floral characters were scored from either fresh specimens, ETOH (70%) fixed material or re-hydrated floral parts taken from voucher specimens. The latter were measured only when the herbaria granted permission for destructive sampling.

Phenetic analysis

After measuring each character, a data matrix was compiled for each species to assess character variation. Constant characters were identified and excluded from all the analyses. Characters excluded from the phenetic analysis of each species are indicated by an asterisk in Tables 3, 4 and 5. A distance matrix was obtained from the row data for each species using the Gower's Index as implemented in the software Le Progiciel R 4.0 (Casgrain and Legendre 2001). This index was preferred over other indices because it can handle "mixed data" such as binary nominal (*e.g.* presence/absence), multistate nominal (*e.g.* leaf shape), ordinal (*e.g.* shape of leaf apex) and continuous variables (*e.g.* leaf length) and missing values (Podani 1999). The dissimilarity matrix obtained was explored with cluster analysis and multidimensional scaling analysis using the software NTSYSpc, version 2.20 L (Rholf 2000). Individuals were grouped *a priori* according to forms (*R. insignis*, *R. enysii*) or geographic origin (*R. lyallii*). Support for maintaining these forms or genetic lineages as part of a single species was then explored.

Ranunculus insignis s.l.

	Qualitative characters		States
1	LS	Leaf shape	1: reniform, 2: elliptic, 3: cordiform
2	LH	Leaf hairs	0: present, 1: absent
3	HDIST	Hair distribution	1: entire lamina, 2: margin only
4	PEDH	Pedicel hairs	0: present, 1: absent
5	PEDCH	Colour pedicel hairs	1: red, 2: yellow, 3: white, 4: brown
6	LA	Leaf apex	1: rounded, 2: obtuse, 3: acute
7	LB	Leaf base	1: cordate, 2: rounded, 3: cuneate, 4: decurrent
8	LM	Leaf margin	1: crenate, 2: lobed, 3:dentate, 4:1-2, 5:1-3
9	HFLOS	Flowering stem hairs	0: present, 1: absent
10	CLS	Cauline leaves shape	1: linear, 2: linear/reniform, 3: linear/trifid, 4: trifid, 5: no leaves
11	ISHA	Inflorescence shape	1:compact; 2: lax
12	PB*	Petal base	1: acute
13	PA^	Petal apex	1: rounded; 2: emarginate
14	SNG^	Nectary gland shape	1: cup, 2: in three, 3: pocket
15	SEPH^	Sepals hair	0: present, 1: absent
16	HS^	Hairiness sepals	1: intense, 2: moderate, 3: sparse
17	HSC^	Sepal hair colour	1: reddish, 2: yellow, 3: white, 4: brown
18	ACHH^	Achene hairs	0: present, 1: absent
19	AH^	Achene hairiness	1: moderate, 2: intense, 3: sparse
20	FMOR^	Fruit morphology	1: rounded, 2: elongated
21	RECH^	Receptacle hairs	0: present, 1: absent
22	AMOR^	Achene morphology	1: obovate, 2: rounded, 3: oblong
23	SS^	Style shape	1: long/curved, 2: long/straight, 3: short/curved, 4: short/straight
	Quantitative characters		**Mean and ranges**
24	LL#	Leaf length (mm)	**54.7** (14 - 157)
25	LW#	Leaf width (mm)	**230** (7.8 - 57.8)
26	RL_W^	Ratio LL/LW	**1.2** (0.5 - 2.8)
27	NP^	Number of petals	**5** (4 - 11)
28	NS^	Number of sepals	**5** (4 - 9)
29	PL^	Petal length (mm)	**14.6** (7.3 - 23.8)
30	PW^	Petal width (mm)	**9.8** (3.3 - 19)
31	NNG*	Number nectary glands	1
32	NFLO	Number of flowers	**5** (1 - 30)
33	ABL#^	Achene body length (mm)	**1.9** (1 - 3)
34	SL#^	Style length (mm)	**2.2** (0.7-4.6)
35	RB_S^	Ratio ABL/SL	**1.13** (0.3 - 2.6)

Table 3: Qualitative and quantitative morphological characters studied in 151 specimens of *R. insignis s.l.* *characters excluded from all the analyses. #characters excluded from the Cluster and MDSC analyses. ^characters excluded from the DFA.

	Ranunculus enysii		
	Qualitative characters		**States**
1	LD	Leaf lamina division	1: entire, 2: in three, 3: in five
2	ATTL	Attachment of terminal leaflet	1: inapplicable, 2: pedicelled, 3: sessile
3	ATLL	Attachment of lateral leaflets	1: inapplicable, 2: pedicelled, 3: sessile
4	DLL	Division of lateral leaflet	1: inapplicable, 2: entire, 3:divided
5	LM	Leaf margin	1: crenate, 2: entire, 3:dentate
6	TLD	Terminal leaflet division	1: inapplicable, 2: simple, 3:divided
7	PS*	Petal shape	1: obovate
8	PA	Petal apex	1: rounded, 2:emarginate
9	PB*	Petal base	1: acute
10	NGS	Nectary gland shape	1: V shaped, 2: pocket, 3: cup, 4: 2-3
11	AMOR*	Achene morphology	1: rounded
12	SS	Style shape	1: curved, 2: erect
	Quantitative characters		**Mean and ranges**
13	LLMN	Length leaf mid nerve (mm)	**13** (2.8 - 52)
14	LLLP	Lateral leaflet petiolule length (mm)	**7.78** (1.6 - 31)
15	TLPL	Terminal leaflet petiolule length (mm)	**3.3** (1 - 11.4)
16	TLW	Terminal leaflet width (mm)	**13.3** (3.4 - 34.8)
17	NFLO	Number of flowers	**2** (1 - 5)
18	NP	Number of petals	**6** (2 - 15)
19	PL	Petal length (mm)	**9** (5.4 - 14.3)
20	PW	Petal width (mm)	**5.3** (2.8 - 10)
21	NS	Number of sepals	**7 (**3 - 5)
22	NNG*	Number of nectary gland	1
23	SL#	Style length (mm)	**0.9** (0.5 - 1.6)
24	ABL#	Achene body length (mm)	**1.6** (0.7 - 2.3)
25	RB_S	Ratio ABL/SL	**0.6** (0.26 - 1.5)

Table 4: Qualitative and quantitative morphological characters studied in 83 specimens of *R. enysii s.l.* *characters excluded from all the analyses. #characters excluded from the Cluster and MDSC analyses.

	Ranunculus lyallii		
	Qualitative characters		**States**
1	LS	Leaf shape	1: peltate, 2: reniform
2	LH	Leaf hairs	0: absent, 1:present
3	PEDH	Pedicel hairs	0: absent, 1:present
4	LM*	Leaf margin	1: crenate
5	FLOSH	Flowering stem hairs	0: absent, 1:present
6	CLS	Cauline leaves shape	1: trifid, 2: linear, 3: 1+2, 4: reniform + trifid, 5: reniform + linear
7	PS*	Petal shape	1: obovate
8	SS	Sepal shape	1: elliptic, 2: oblong
9	PB*	Petal base	1: acute
10	PA	Petal apex	1: rounded, 2: emarginate
11	NGS*	Nectary gland shape	1: pore
12	SEPH	Sepal hairs	0: present, 1: absent
13	AHS	Amount of sepal hairs	1: sparse, 2: moderate, 3: intense
14	CSH	Colour of sepal hairs	1: white, 2: yellow
15	AH	Achene hairs	0: absent, 1: present
16	AHAR	Achene hairiness	1: moderate, 2: intense, 3: sparse
17	FMOR	Fruit morphology	1: elongated, 2: rounded
18	RECH	Receptacle hairs	0: present, 1: absent
19	AMOR	Achene morphology	1: rounded, 2: oblong
20	SS	Style shape	1: long/straight, 2: long/curved, 3: short/straight, 4: short/curved
	Quantitative characters		**Mean and ranges**
21	LD	Leaf diameter (mm)	**133.6** (67.7 - 227.4)
22	NP	Number of petals	**14** (9 - 17)
23	PL	Petal length (mm)	**25.9** (18.7 - 35)
24	PW	Petal width (mm)	**14.3** (9.1 - 19.3)
25	NS	Number of sepals	**5** (3 - 6)
26	NFLO	Number of flowers	**9** (2 - 26)
27	ABL#	Achene body length (mm)	**2.3** (2 - 3)
28	SL#	Style length (mm)	**3.2** (1.9 - 5.3)
29	RB_S	Ratio ABL/SL	**0.8** (0.5 - 1.3)

Table 5: Qualitative and quantitative morphological characters studied in 41 specimens of *R. lyallii*. *characters excluded from all the analyses. #characters excluded from the Cluster and MDSC analyses.

Cluster analysis (CA) was done using the SAHN clustering algorithm (sequential, agglomerative, hierarchical, non-overlapping) and using the clustering method FLEXI. How well the dendrogram represented its corresponding pairwise distance matrix (Rohlf 2000) was evaluated by calculating the "cophenetic correlation coefficient". This coefficient was obtained by comparing the matrix of cophenetic values, calculated from the cluster analysis, and the original distance matrix using a Mantel Test. The modules COPH and MXCOMP in NTSYSpc were used for these analyses.

A non-parametric multidimensional scaling analysis (MDS) was conducted using the module MDSCALE in NTSYSpc. This is an ordination technique, like principal coordinates analysis or correspondence analysis which is useful for representing the dissimilarity among *n* objects or variables by *n* points in a *k*-dimensional space so that the interpoint distances in the *k*-dimensional space correspond as much as possible to the observed distances between the objects (Rohlf 2000). MDS has the desirable property that smaller interpoint distances tend to be preserved more faithfully than in a principal coordinate analysis. This is because MDS maximizes variances and gives greater weight to the larger distances (Rohlf 2000). The goodness of fit is given by a stress value ranging from 0 to 0.4, the closer to 0 the stress value is, the better the fit to the data (Rohlf 2000). A Minimum Spanning Tree was calculated from the distance matrix using the module MST of NTSYSpc and superimposed on the ordination. This calculation is useful to detect local distortions; *i.e.*, pairs of points which look close together in a plot but actually are far apart when other dimensions are taken into account (Rohlf 2000)

The following section applies to *R. insignis* only. Unlike the other two species, morphological variation in this species has been suggested to follow a North-South cline. If it is so, it would be expected to detect significant morphological overlap between the forms. Statistical difference of quantitative characters between the three groups distinguished by the MDS was assessed with the non-parametric Kruskal-Wallis test (K-W) using SPSS 13.0 (SPSS, Inc., Chicago, III.). This test uses ranked variables and its value is limited by the number of objects and the number of groups. The statistic obtained from this test may be used as an indication of how well characters differentiate between the groups resulting from the MDS. Characters with high K-W values may be considered more useful to differentiate between groups than those with lower K-W values. This test has proved useful in previous morphometric studies where data were not parametric (Ariati *et al.* 2007; Jolley & Klazenga 2007). Difference in mean

rank of the groups was tested using the Dunnet's C *post hoc* test for non-parametric data as implemented in SPSS 13.0. Qualitative characters, on the other hand, were explored using frequency histograms.

Twenty-one specimens with apparent intermediate phenotype between two forms of *R. insignis s.l.* were detected during the study. The identity of these intermediate specimens was assessed using a discriminate function analysis (DFA). This technique requires that the individuals be divided into groups *a priori*, and in this case, they were classified into three groups: "intermediate", "lobulatus form" and "insignis form". The DFA provides a set of weightings that allows the distinction of groups but also uses these weightings to assign specimens to groups. The DFA was used here to test whether the cases of interest (*i.e.*, intermediate) can actually be classified as predicted using the morphological characters measured or whether they belong to the form "insignis" or "lobulatus". This analysis also indicates the relative importance of each variable to differentiate between the groups (Dytham 2003). Variables with missing values were excluded from this analysis and are indicated in Table 3. Geographic distribution of these intermediate specimens with respect to their putative parental species is mapped in Figure 9.

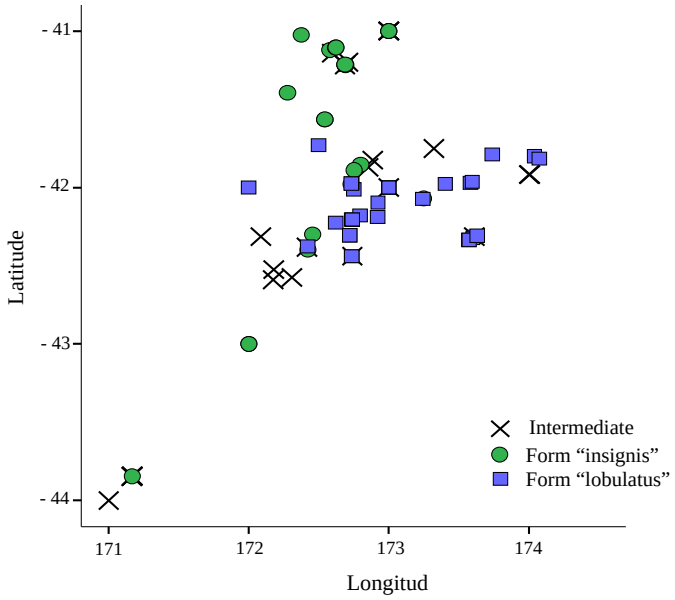


Figure 9: Geographic distribution of the intermediate specimens with respect to the forms "insignis" and "lobulatus" in the South Island of New Zealand.

Molecular analysis

Nuclear ribosomal DNA and chloroplast DNA sequences were obtained from leaf material collected from *R. insignis*, *R. lyalii* and *R. enysii* across their distribution range. Sequences from earlier studies (Lockhart et al 2001, Piripi 2005, Carter 2007) were also included in the analyses. The origin of all the accessions included in this study is illustrated in Figure 3, 4 and 5 by filled triangles. Individuals included in the molecular analyses that were also included in the morphological analysis are highlighted by labels in bold in the MP tree. Using the same specimen for both analyses would have been ideal. However, this approach was limited by the quality and preservation of the pressed material available. Thus not all genotyped samples were available for morphological analyses. The species R. scrithalis was used as outgroup in these analyses. This species is a member of the alpine *Ranunculus* but is member of a separate lineage (Lockhart *et al* 2001).

DNA Isolation, amplification and sequencing

DNA extraction from fresh material followed a protocol modified from Doyle & Doyle (1987) while a DNeasy extraction kit (Qiagen) was used for herbarium material. PCR amplification was carried out in a Biometra T1Thermocycler machine. The entire Internal Transcribed Spacer (ITS) region was amplified using the primers ITS4 and ITS5 described in Lockhart *et al.* (2001). Amplification of the junction of the inverted repeat A and small single-copy region (J_{SA}) of the chloroplast was done using the primers RERN and 151A published in Lockhart *et al.* (2001).

PCR conditions for ITS were 94°C 3 min for the first cycle; 94°C 30s, 55/50°C 30s, 48°C 1min for the following 35 cycles; then 72°C 1min; 72°C 5min; and then cooled at 10°C. These conditions were modified for J_{SA} as follows; 94°C 2 min for the first cycle; 94°C 1min, 55°C 1min, 72°C 1min for the following 35 cycles; then 72°C 5min. PCR products were quantified using 1% agarose gels and later purified using a Sap/Exo1 digestion method (10 U/ul, 1U/ul Exo1; cycle 37°C 30min; 80°C 15min and then cooled at 10°C). Sequencing reactions were done in a 20 µl volume using Applied Biosystems Inc. standard protocols and the cycle conditions: 96°C 30s, 50°C 15s, 60°C 4min., repeated for 27 cycles and then cooled at 10°C. Sequencing reactions were cleaned with the magnetic bead-based technique using the CleanSeq reagent and a SPRI magnetic plate according to the manufacturer's instructions (Agencourt Bioscience Corp., Beverly, MA) and then sequenced using a capillary ABI3730 Genetic Analyzer, from Applied Biosystems Inc. by the Allan Wilson Centre Genome

Service, Massey University, Palmerston North, New Zealand. Earlier sequencing reactions were precipitated following an EDTA/Sodium Acetate/Ethanol protocol, air dried and then sequenced.

Data analysis

Forward and reverse strand sequences were edited using Sequencher 4.2 (Genecode). Sequences were aligned using ClustalX (Thompson *et al.* 1997). Maximum parsimony (MP) analysis was performed for the ITS and J_{SA} data set using the software PAUP* 4.0b8 (Swofford 2000) using the heuristic search with 1000 replicates, random sequence addition, tree-bisection-reconnection (TBR) branch swapping, MULTRESS on (keeping multiple, equally parsimonious trees) bu saving only 10 trees each replicate. The unordered character states and equal character state weighting options were used in the analysis. The relative support for each node was examined with non-parametric bootstrapping (Felsenstein 1985; 100 replicates). The alpine species *R. scrithalis* was used as an arbitrary outgroup. Although it is part of the NZ alpine radiation it is genetically distant from all the other species. Phylogeographic patterns among cpDNA haplotypes were describe using TCS (Clements *et al.* 2000).

Habitat Characterisation and Geographic Distance

Collection details (*i.e.*, latitude and longitude) for all the specimens included in the morphological study were recorded from labels, herbarium databases and field collections. These points were used to obtain a number of environmental parameters for each site from the Land Environment of New Zealand database (LENZ) developed by Landcare Research. Data for 17 environmental variables were obtained from the layer LENZ level IV, which contains the most accurate environmental description than all the layers contained in LENZ. The variables investigated for each species are listed in Table 6. Prior to statistical analysis, data were standardised by calculating Z-scores using SPSS 13.0. Later, environmental variation for each species' habitat was assessed using Principal Component Analysis (PCA) using SPSS. Constant variables such as soil age and chemical limitation were not included in the analyses because they were invariable across the species distribution.

Geographic distances between collection sites were calculated with the option GeoDistances implemented in Le Progiciel R using the collection details of latitude and longitude registered for each sample.

Matrix Correlations

Correlation between the three data sets (morphology, habitat and geographic distance) was assessed using a Mantel test as implemented in NTSYSpc, module MXCOMP. The Mantel tests allows estimation of the association between two independent dissimilarity matrices describing the same set of entities and, by doing so, test whether the association between them is stronger than one would expect from chance (Sokal & Rohlf 1998). This test posits the null hypothesis that there is no association between the elements in one matrix and those in the other one (Sokal & Rohlf 1998). The test yields a standardised Mantel statistic, *Z*, which ranges between -1 and 1, and is equivalent to a Pearson product-moment correlation coefficient (Chen & Harvey 1999). Significance of the calculated *Z* is examined by using an approximate randomisation test. In this study significance of association between the matrices was determined using 9999 permutations. Two distance matrices having a Mantel *Z* with *P* value smaller than 0.05 were considered as significantly correlated. Correlations performed included: geographic distance versus morphology, habitat characteristics versus morphology and habitat dissimilarity versus geographic distance.

	Environmental Variables from LENZ (level IV)
1	Elevation (m)
2	Mean annual temperature (°C)
3	Minimal temperature of the coldest month (°C)
4	Mean annual solar radiation ($MJ/m^2/day$)
5	Winter solar radiation ($MJ/m^2/day$)
6	October vapour pressure deficit (kPa)
7	Water balance ratio
8	Soil water deficit (mm)
9	Slope (°)
10	Soil drainage (soil description)
11	Soil Age*
12	Chem_Limit*
13	Acid soluble phosphorus in the soil (Mg/100g)
14	Exchangeable calcium in the soil (Mg/100g)
15	Soil induration
16	Soil particle size (mm)

Table 6: List of variables obtained from the environmental layer IV of the LENZ database. The asterisk indicates constant variables excluded from the PCA.

RESULTS

Morphological analysis of *R. insignis s.l.*

The cluster analysis of the 124 specimens of *R. insignis s.l.* is shown in Figure 10. The cophenetic correlation between the morphological distance matrix and the tree matrix was 0.6, indicating a moderately good fit of the phenogram to the distance matrix. The analysis revealed three major groups (Figure 10). Group I is formed by two smaller sub-groups; IA and IB. The first one (IA) includes samples from the North Island and the Nelson-Marlborough area in the South Island. The subgroup IB includes specimens only from the South Island, particularly from the north eastern Nelson. Only three specimens in this sub-group are from the southern South Island; two from Mt. Peel and one from Broken River, Canterbury. Group II was also formed by two subgroups, IIA and IIB (Figure 10). Both subgroups contained specimens collected only in the South Island; in the central-east part of the Marlborough region. Group III was fairly homogeneous and only included specimens from the Canterbury region and one specimen from the Kaikoura Range.

Following the CA, specimens included in these three groups were assigned to the forms recognised previous to Fisher's reclassification and studied in the Multidimentional scaling analysis. The MDSC analysis had a low stress value (0.1). This analysis showed a clearer delimitation of the groups (Figure 11) than the cluster analysis. Proximity between each of the specimens is indicated by the superimposed Minimum Spanning Tree (red line in Figure 11). Cluster I includes almost exclusively specimens of the form insignis and three specimens of the form lobulatus. Two of the latter are from limestone cliffs near Flaxbourne River (Marlborough) and one from Mt. Peel, perhaps in southern Canterbury (geographical origin of this sample is uncertain since there is also a Mount Peel in the Nelson area; in addition the current geographical origin on the label of this voucher has been assigned by someone other than the collector and the labelling made many years after collection). These three samples were also grouped with specimens of the form insignis in the cluster analysis.

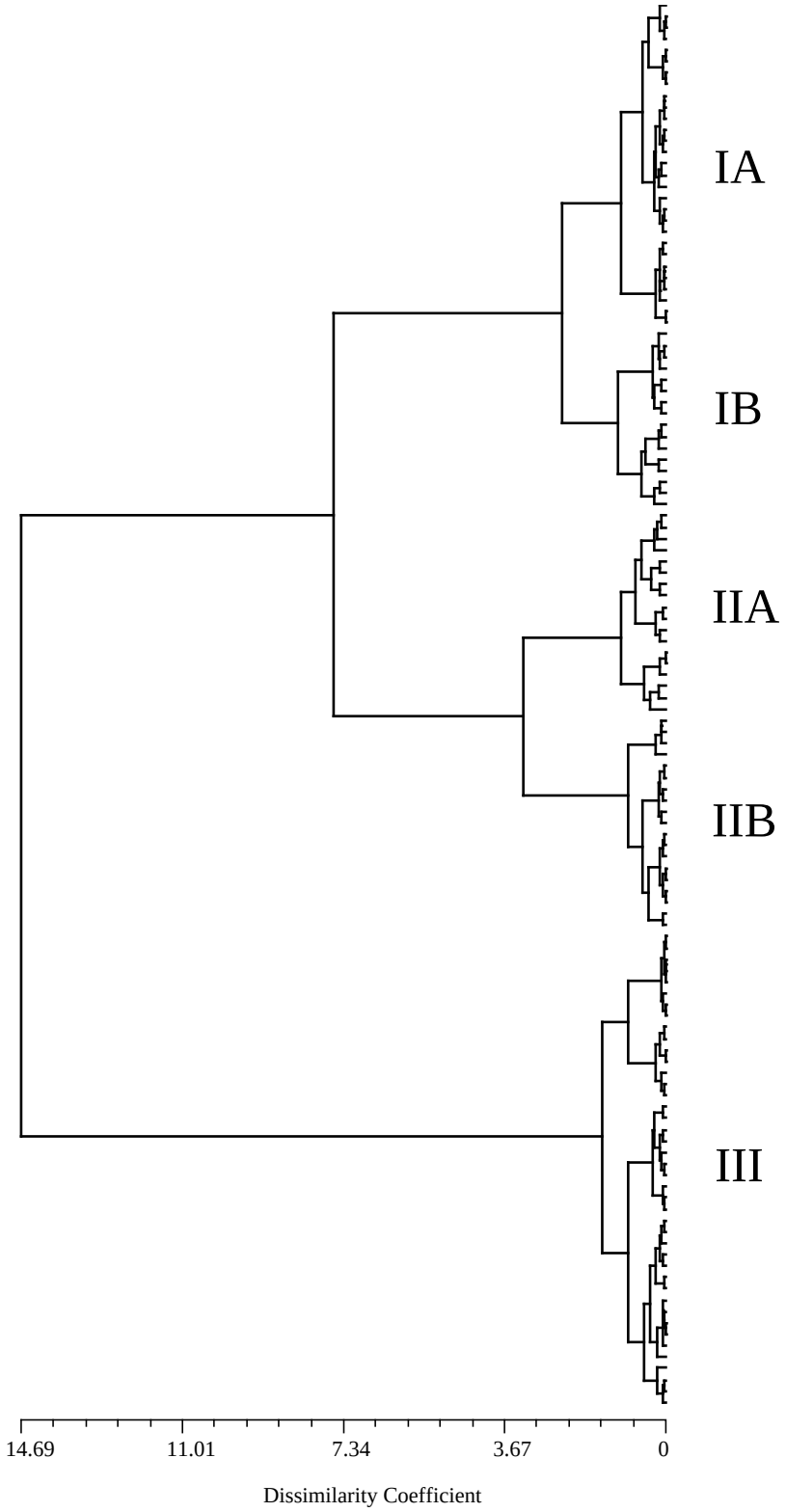


Figure 10: Cluster analysis of the 124 individuals of *R. insignis s.l.* based on morphological characters. Species cophenetic correlation coefficient = 0.6.

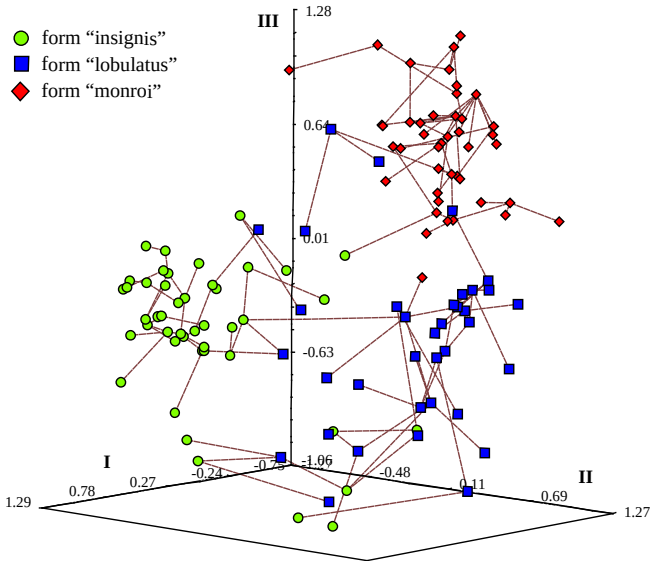


Figure 11: Multidimensional scaling ordination in three dimensions of the 124 specimens of *R. insignis s. l.* based in morphological characters. Different symbols indicate the *a priori* classification of the specimens. The Minimum spanning tree is indicated by the red line and indicates proximity between the points. Stress value = 0.6

Elements in Cluster II are more widespread than in Cluster I, and although it includes most of the specimens of the form lobulatus it also includes seven specimens of form insignis (all from southern Nelson) and one of the form monroi (from a limestone area near Castle Hill, Canterbury). This cluster is identical to the Group II obtained in the CA described earlier in Figure 10.

Most of the specimens of the form monroi measured are included in Cluster III along with four specimens of the form lobulatus and one of the form insignis. The first ones were collected from Mt. Murchison (Nelson), Lewis Pass and the Kaikoura Mountains (Marlborough), while the latter is from Ada Pass (Marlborough). Only one of the specimens of the form lobulatus, collected from the Kaikoura ranges, was also included in this group by the cluster analysis.

Character variation

The non-parametric K-W test found seven quantitative characters significantly different among the three groups (Table 7). Leaf size and number of flowers had the highest K-W value indicating their usefulness in differentiating between groups. Only two quantitative characters, number of petals and number of sepals, did not differ significantly between the groups. Many of the qualitative characters studied were shared, to a greater or lesser extent,

by the three groups distinguished in the MDS analysis. Only four characters (one vegetative and three reproductive) did not overlap between the groups (Figure 12). The frequency of specimens of one group sharing these characters with specimens from another group was generally low (1-2 individuals) except for the character “leaf shape” in the form lobulatus. This group had specimens with reniform and cordiform leaves (Figure 12).

Character	K-W	insignis v/s lobulatus	insignis v/s monroi	lobulatus v/s monroi
RL_W	87.95*	-0.14*	-1.09*	-0.95*
LL	43.22*	15.84*	31.48*	15.64*
LW	83.57*	28.74*	69.4*	40.65*
NP	1.16	n/a	n/a	n/a
NS	1.65	n/a	n/a	n/a
PL	42.12*	5.31*	7.14*	-5.31
PW	40.62*	4.15*	7.31*	3.17*
NNG	0.00	n/a	n/a	n/a
NFLO	50.67*	2.74	5.94*	3.2*
RB_S	13.33*	-0.87*	-0.64*	0.22

Table 7: K-W test and significance of each character for *R. insignis s.l.* Significance of the comparison is indicated by an asterisk. For character coding see Table 3.

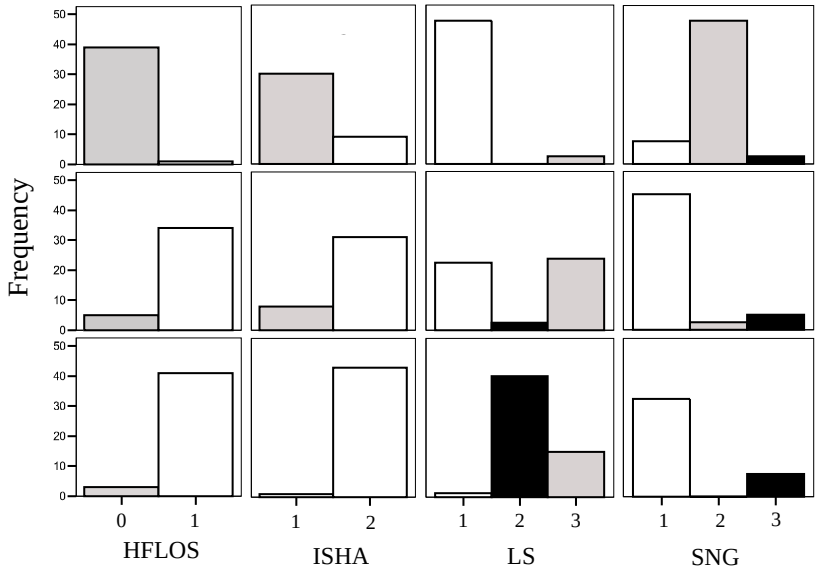


Figure 12: Frequency of four qualitative characters with the greatest degree of discontinuity between the three forms of *R. insignis s.l.* From top to bottom: forms insignis, lobulatus and monroi. For character coding and states see Table 3.

Discriminant analysis of intermediate individuals

The DFA re-classified 86.4% of the specimens correctly into the groups they were originally assigned. The most accurate classification was observed in the form lobulatus (92.9%) where only 2 specimens were assigned to other taxa (Table 8). As for the form insignis, 87% of the specimens were correctly classified and five specimens were assigned to the forms lobulatus (2 specimens) and Intermediate (3 specimens). About 76% of the specimens regarded as intermediate were correctly classified by the DFA and only five were assigned to other taxa; two specimens were grouped under insignis and three under lobulatus (Table 8).

The first two discriminant axes of the DFA accounted for 100% of the variation among the groups and clearly distinguished between the forms insignis and lobulatus (Figure 13). Intermediate specimens are scattered between these two groups, but relatively closer to the form insignis than to the form lobulatus. Characters that contribute more to the recognition of these groups are LH, HFLOS, LL and LW (Table 9).

	Predicted group membership			
Group	Intermediate	insignis	lobulatus	Total
Intermediate	16 (76.2%)	2 (9.5%)	3 (14.3%)	21
insignis	3 (7.7%)	34 (87.2%)	2 (5.1%)	39
lobulatus	2 (7.1%)	0 (0.0%)	26 (92.9%)	28

Table 8: Classification results of the DFA for the three groups: forms insignis and lobulatus and intermediate specimens. Values indicate number and percent of individuals assigned to each group.

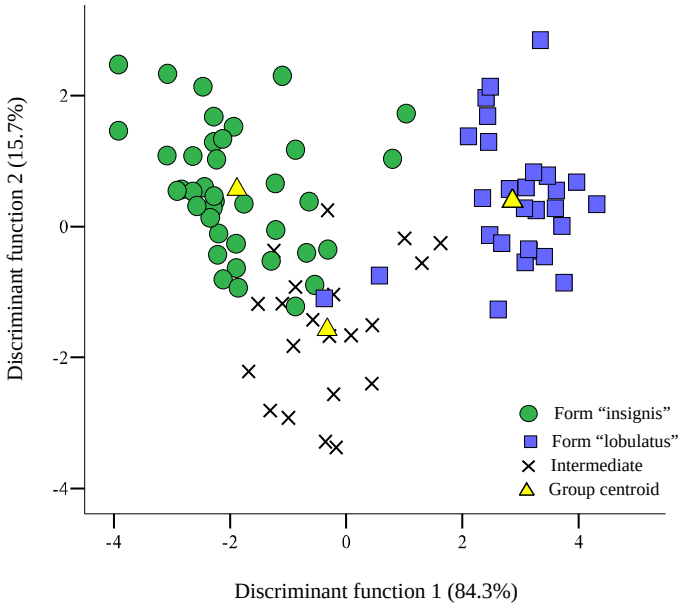


Figure 13: Discriminant function analysis of the intermediate individuals and putative parental species. Yellow triangles indicate group centroids.

Character	Function 1	Function 2
NFLO	0.224	0.085
LS	0.269	0.272
LH	**0.574**	0.251
HDIST	-0.080	-0.223
HPED	0.292	-0.217
CHPED	0.063	0.220
LA	0.043	0.020
LB	-0.195	-0.303
LM	-0.201	0.044
HFLOS	**0.852**	0.084
SCL	-0.127	-0.413
ISHA	0.127	-0.275
LL	**0.831**	**-0.578**
LW	**-1.308**	**1.176**

Table 9: Standardised canonical discriminant function coefficients for the groups made up by insignis, lobulatus and intermediate specimens. Variables with highest importance are highlighted.

Morphological analysis of *R. enysii s.l.*

The cluster analysis of the 83 specimens of *R. enysii s.l.* is shown in Figure 14. The cophenetic correlation coefficient of the morphological distance matrix and the tree matrix was 0.8, indicating a fairly good fit of the phenogram to the morphological distance matrix. The analyses revealed three main groups (Figure 14). Group I is formed exclusively by specimens of the type BER and includes all nine samples measured in this study. Dissimilarity coefficient is very low within these samples and high in reference to the other forms. Group II, conversely, included a mixture of specimens assigned to different leaf types. This group is divided into two subgroups (Figure 14). Subgroup IIA is formed almost entirely by specimens of the type 5WB and 3WB. Two specimens of the type BB from the Umbrella Mountains and Kakanui Mountains (Otago) and 3 specimens that were unable to be assigned to an *a priori* group from the Canterbury region were also included in this subgroup. The next subgroup, IIB, consists almost entirely of specimens of the type BB and a single specimen of the type R&P collected at the Rock and Pillar Range (Otago). Finally, Group III includes the great majority of the specimens of the R&P type, four specimens of 3WB from the Waimakariri Basin (Canterbury), and two specimens from Central Otago (Hector Mountains and Carrick Range) that were impossible to assign to the forms recognised before.

The multidimensional scaling analysis of the same 83 individuals had a low stress value (0.1) Similar to the cluster analysis, the MDS analysis clearly distinguished between specimens of type BER and the remaining samples. Specimens of type BER form a cohesive and distant group (Figure 15). Clustering of the remaining specimens was not as clear; yet three groups can be observed in the ordination space and they largely corresponded with the groups observed in the cluster analysis. A second MDS analysis, in which BER was excluded, produced a similar result but improved the spatial separation among these three clusters (Figure 16). All three clusters, however, are widely spread across the ordination space and a number of sub-clusters can be observed. Cluster I includes two sub-clusters. One of them is formed by all the specimens of the type BB and one specimen of the R&P type from the Rock and Pillar Mountains (Otago). The second sub-cluster contains specimens of the type 5WB and two unidentified specimens. These two sub-clusters are identical to the groups recovered in the cluster analysis in Figure 14. Cluster II is less cohesive than Cluster I suggesting greater morphological variation. Cluster II is formed by most of the type 3WB specimens from the Waimakariri Basin, four specimens of 5WB from Sugar Loaf Mountain and Mount Torlesse also in the Waimakairi Basin, one specimen of the type BB from the Kakanui Range (Otago)

and one undetermined specimen. Cluster III in the ordination includes all the specimens of the R&P type (except for the one in Cluster I), four specimens assigned a priori to 3WB from the Waimakariri Basin and two unidentified specimens. Clusters II and III were identical in composition to the Groups IIA and III observed in the cluster analysis (Figure 14).

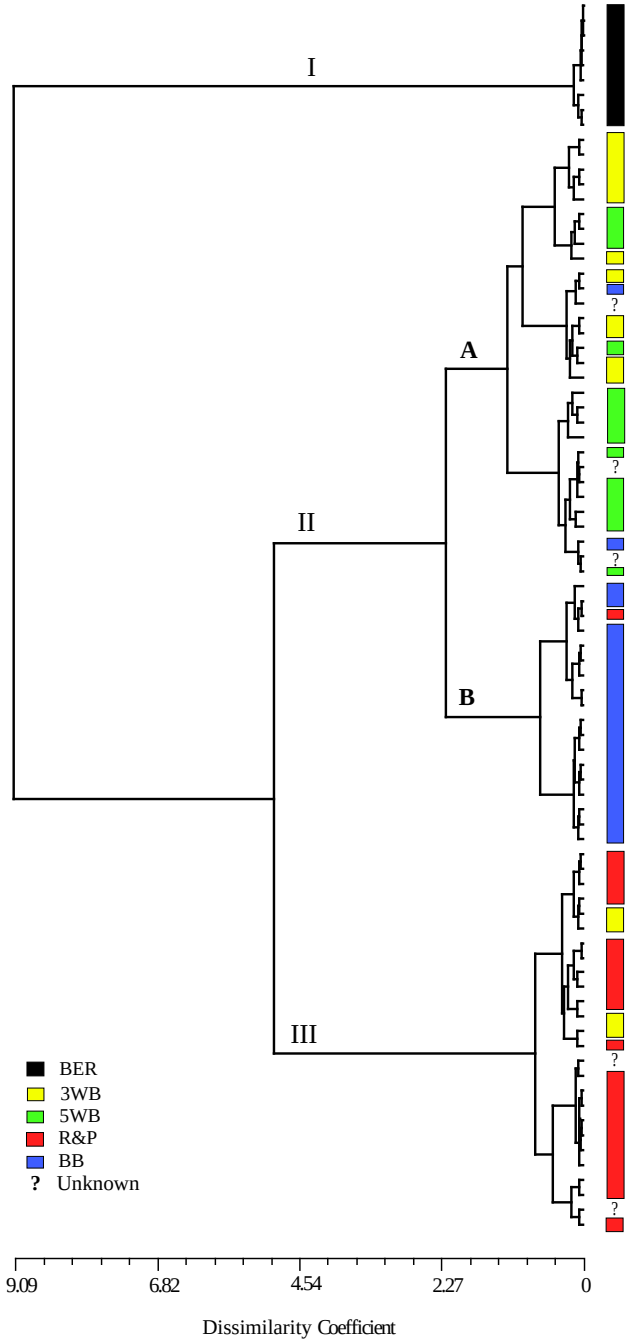


Figure 14: Cluster analysis of the 83 specimens of *R. enysii s.l.* based on morphological characters. Bars indicate *a priori* classification of the specimens according to leaf type (BER: black; 3WB: yellow; 5WB: green; BB: blue; R&P: red). Species Cophenetic correlation coefficient = 0.8

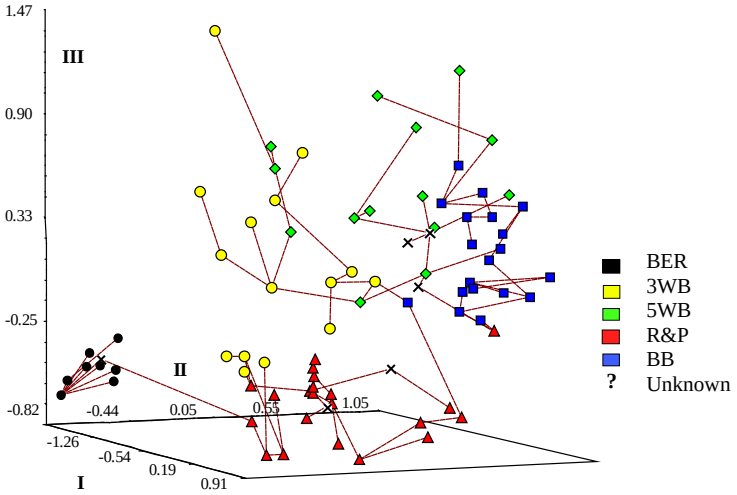


Figure 15: Multidimensional scaling ordination in three dimensions of the 83 specimens of *R. enysii s.l.* based in morphological characters. Different symbols indicate *a priori* classification of the specimens according to leaf type (BER: black; 3WB: yellow; 5WB: green; BB: blue; R&P: red). The Minimum spanning tree is indicated by the red line and indicates proximity between the points. Stress value = 0.1

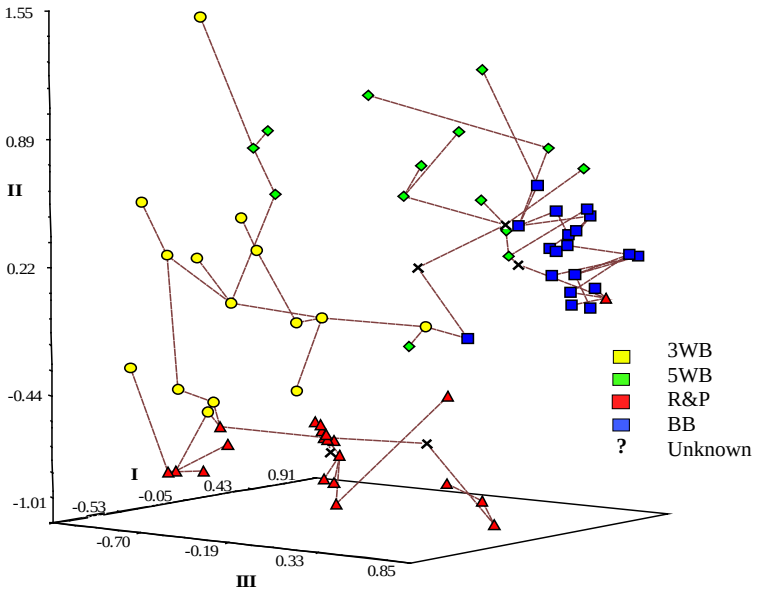


Figure 16: Multidimensional scaling ordination in three dimensions of *R. enysii s.l.* based in morphological characters. Specimens of the type BER excluded. Different symbols indicate *a priori* classification of the specimens according to leaf type (BER: black; 3WB: yellow; 5WB: green; BB: blue; R&P: red). The Minimum spanning tree is indicated by the red dashed line and indicates proximity between the points. Stress value = 0.1

Morphological analysis of *R. lyallii*

The cluster analysis of the 41 specimens of *R. lyallii* resulted in two major groups (Figure 17) and had a good cophenetic correlation coefficient (0.7). Cluster I contained specimens collected from populations neighbouring Arthur's Pass and Mount Aspiring. Two subgroups were observed in Cluster II. These two subgroups contain specimens from all four areas and, similarly to Cluster I, there is no geographic pattern within them. Furthermore, specimens from geographically distant populations were frequently grouped together (*e.g.* Arthur's Pass and Te Anau in Cluster II, Figure 17). Specimens collected in the Mount Cook area were restricted to Group II and usually grouped with specimens from geographically distant populations.

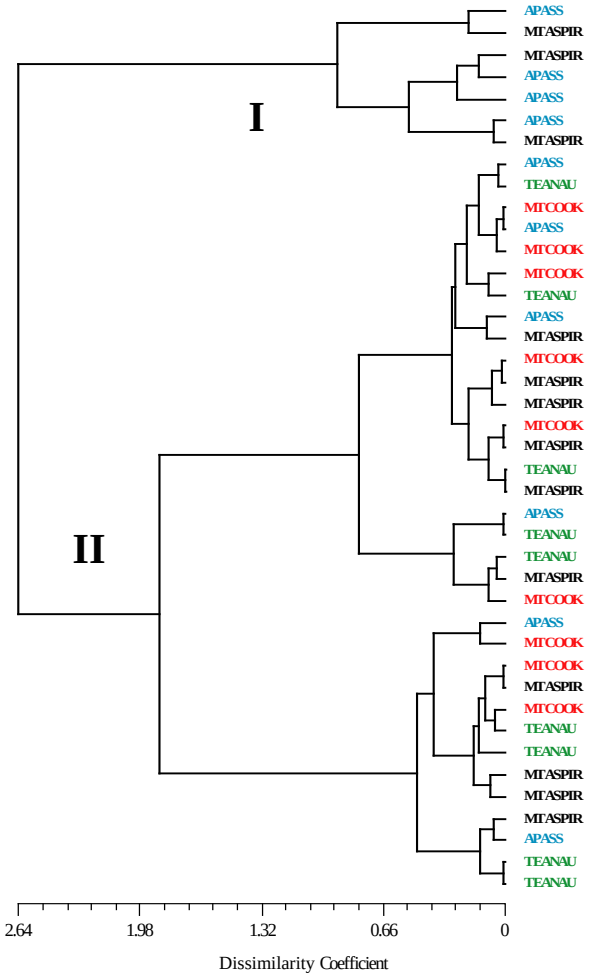


Figure 17: Cluster analysis of the 41 specimens of *R. lyalii*. based on morphological characters. Geographic origin of samples is indicated by fonts in different colour (APASS: Arthur's Pass, blue; MTCOOK: Mount Cook, red; MTASPIR, Mount Aspiring, black; TE ANAU: Fiordland and Stewart Island, green). Cophenetic correlation coefficient = 0.7

The MDS analysis of the same individuals produced a similar pattern to the one observed in the cluster analysis. The only evident difference with the CA was the splitting of the individuals forming Group I into two smaller subgroups (Figure 18). These two clusters are satellite to a bigger cluster with no apparent grouping pattern. The stress value obtained for this analysis was 0.1.

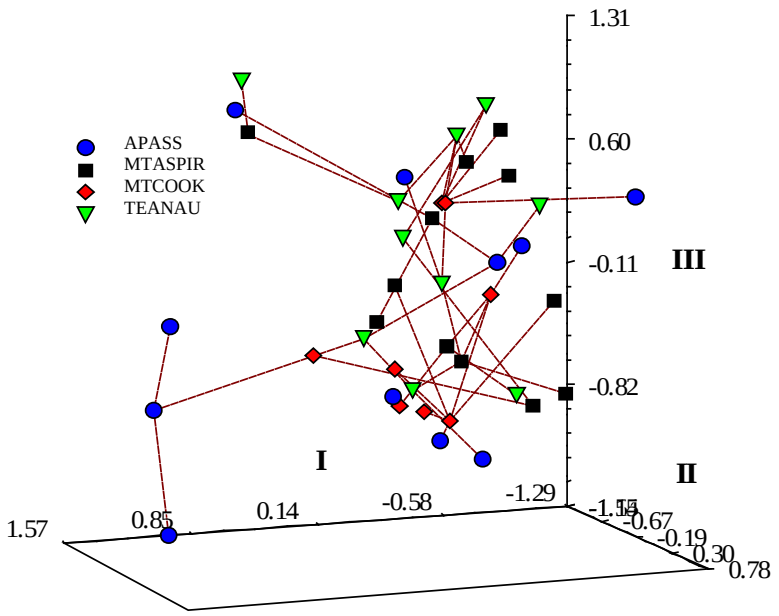


Figure 18: Multidimensional scaling ordination in three dimensions of the 41 specimens of *R. lyallii* based in morphological characters. Geographic origin of samples is indicated by fonts in different colour (APASS: Arthur's Pass, blue; MTCOOK: Mount Cook, red; MTASPIR, Mount Aspiring, black; TE ANAU: Fiordland and Stewart Island, green). The Minimum spanning tree is indicated by the red line and indicates proximity between the points. Stress value = 0.1

Molecular analysis *R. insignis s.l.*

The ITS sequence data set for the 29 specimens sequenced included 603 characters; 21 sites were variable but only two of them were parsimony informative (parallel bars on the tree, Figure 19A). Parsimony analysis divided *R. insignis s.l.* into two clades with low bootstrap support due to the low variation in the sequences (Figure 19A). The first clade includes specimens of the type insignis (green bar) and lobulatus (blue bar). The second clade contains only specimens from the Waimakariri Basin and Mount Hutt, where specimens matching the form monroi are generally found. Specimens in this clade differed from the previous one by the presence of two one base-pair substitutions at positions 43 and 177. Only one specimen assigned to the form monroi, Mount Hutt 2, appeared closer to the outgroup species *R. scrithalis* than to the in-group. The J_{SA} sequence data set contained 480 characters, of which 15 sites were variable but only 9 parsimony informative. Parsimony analysis of the cpDNA sequences also divided the specimens into two clades (Figure 19B), one includes all the specimens assigned to the form insignis and lobulatus (BS= 65%) and the second one all the specimens assigned the form monroi (BS= 62%). The first clade is characterised by a one base-pair substitution at the site 204 and the second one by a one base-pair substitution at the site 342. Within the first clade, the two specimens of the form lobulatus form a sub-clade with 61% BS. These two accessions contain a one base-pair substitution at the site 13. The clade containing the specimens of the form monroi also shows some substructure, and two sub-clades are contained within it (Figure 19B). One of these sub-clades has a high BS support (97%) and comprises only specimens from Mount Hutt. These are characterised by five one base-pair substitutions at the sites 9, 99, 131, 137 and 362. The other sub-clade (BS= 65%) contains four specimens from the Waimakariri Basin and all share a one base-pair substitution at the site 154.

Molecular analysis *R. enysii s.l.*

The ITS sequence data set for 17 specimens of *R. enysii s.l.* included 603 characters; 39 sites were variable but only 18 of them were parsimony informative (parallel bars on the tree, Figure 20A). Parsimony analysis divided *R. enysii* into two main clades (Figure 20A). The first clade (BS= 94%) contains specimens assigned to the forms BB and R&P, all sharing four one base-pair substitution at the sites 67, 411, 566 and 582.

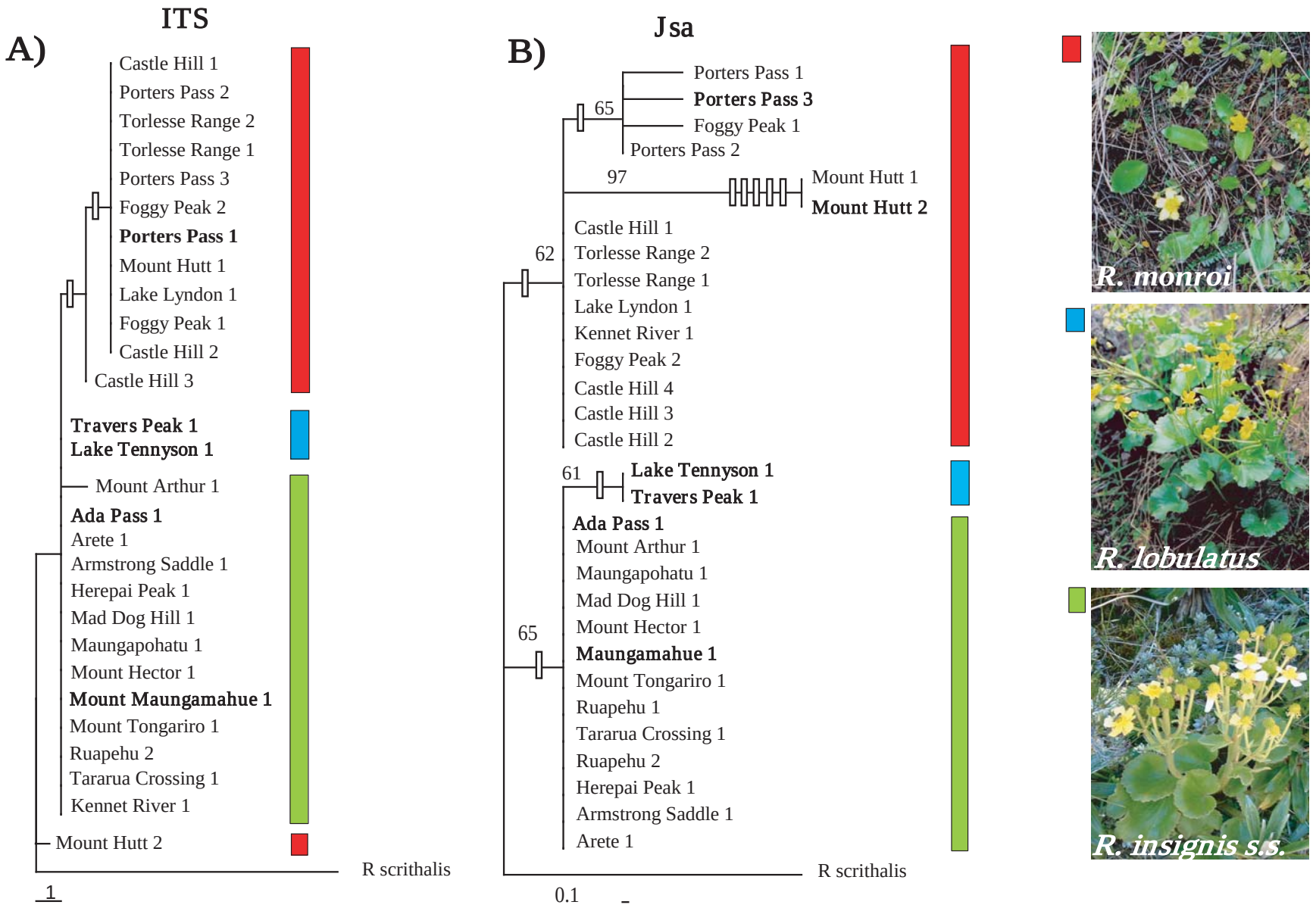


Figure 19: Maximum parsimony trees based on nrITS (A) and cpJ$_{SA}$ (B) sequences. Numbers above the branches indicate bootstrap values. Bars on branches indicate number of supporting parsimony informative sites. Colour bars indicate the three forms; monroi: red; lobulatus: blue; insignis: green. Labels in bold indicate specimen included in phenetic analysis.

The second main clade, with 67% BS support, differs by three one base-pair substitutions at the sites 102, 112 and 496. Within this clade a highly supported sub-clade (BS= 100%) includes all the specimens from the Waimakariri Basin (*i.e.*, types 3WB and 5WB) which share 10 one base-pair substitutions at the sites 12, 17, 25, 146, 177, 391, 412, 500, 517 and 531. A specimen from Island Pass (Marlborough), lacking these changes, is sister to this clade. The J_{SA} sequence data set contained 480 characters; 18 sites were variable but only 11 parsimony informative. The parsimony analysis of the cpDNA sequences also revealed two main clades (Figure 20B). The first one is a highly supported clade (BS= 99%) that includes two geographically distant specimens, Island Pass (Marlborough) and Nevis Valley (Otago). These two specimens share five one base-pair substitutions at the sites 72, 87, 101, 298 and 454. Sister to this smaller clade is a larger and well supported clade (89% BS), but not fully resolved. It includes specimens from the Waimakariri Basin, six of them forming a weakly supported sub-clade (65% BS) with specimens sharing a one base-pair substitution at the site 234. A second sub-clade with 77% BS is also found within this clade, and it includes specimens from the R&P and BB type. Specimens of these two forms shared a one base-pair substitution at the site 99 and formed a monophyletic group with 62% BS support.

Molecular analysis *R. lyallii*

The ITS sequence data set for *R. lyallii* (N=45) contained 603 characters; 39 sites were variable but only 18 of them were parsimony informative. The MP analysis divided the specimens into two clades, both with low Bootstrap support (Figure 21A). One of these clades contains specimens from populations in the central South Island (*i.e.*, Arthur's Pass and Mount Cook) while the other clade only included specimens from southern populations such as Mount Aspiring, Te Anau and Stewart Island. The J_{SA} sequence data set contained 477 characters; 19 sites were variable but only 10 parsimony informative. Results from the MP analysis of the J_{SA} sequences were similar to those from the ITS data set (tree not shown). However, the clade with specimens from Mount Aspiring area is sister to clades formed by the northern populations (*i.e.*, Arthur's Pass and Mount Cook) and not to the populations from Te Anau and Stewart Island as observed in the ITS (Figure 21A). Eleven J_{SA} haplotypes were detected in *R. lyallii* and their geographic distribution and network are shown in Figure 21B. Haplotype 2 is the most common and widespread and it is found in populations nearby Mount Cook and the north of Mount Aspiring.

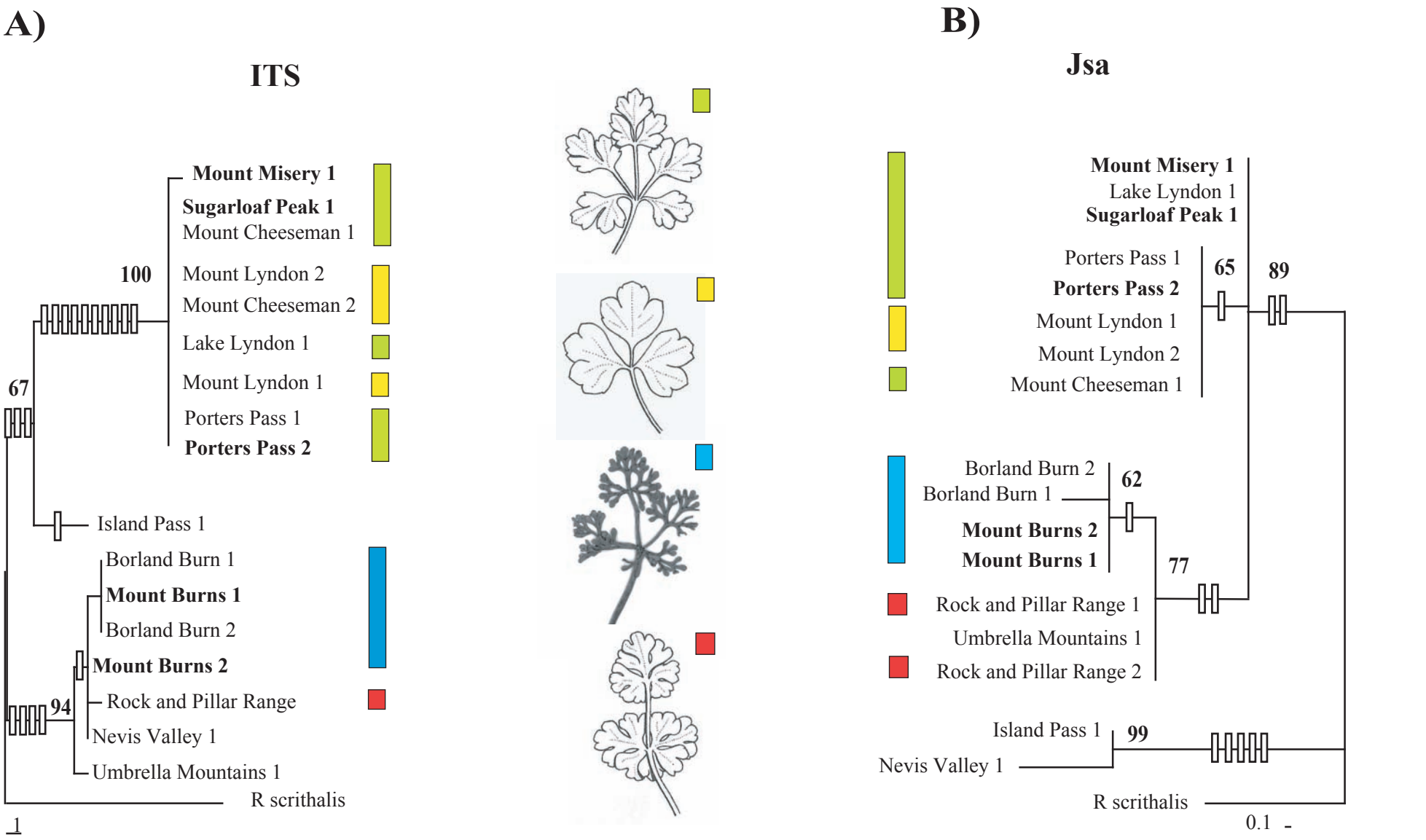
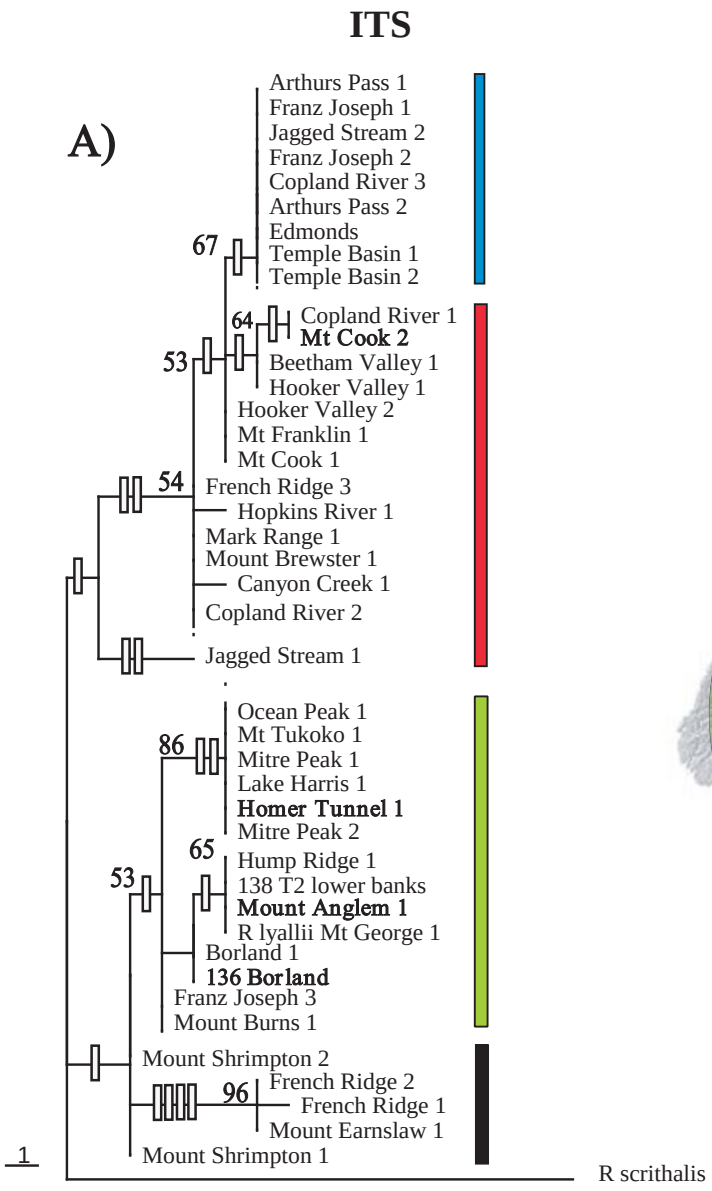
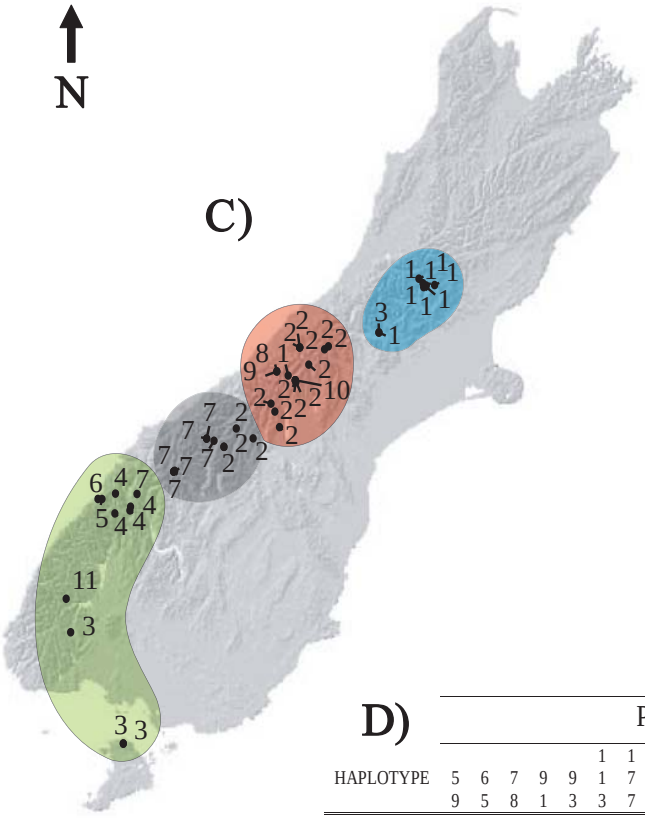
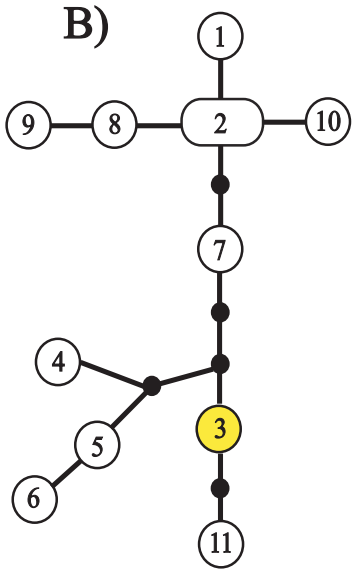


Figure 20: Maximum parsimony trees based on nrITS (A) and cpJsa (B) sequences. Numbers above the branches indicate bootstrap values. Bars on branches indicate number of supporting parsimony informative sites. The four leaf types are indicated by colour bars and illustrated.Green: 5WB; yellow: 3WB; blue: BB; red:R&P. Labels in bold indicate specimens included in the phenetic analysis.







D)

	Position of polymorphic sites																								
HAPLOTYPE	59	65	78	91	93	113	117	235	242	292	293	317	334	335	336	337	338	339	340	341	342	343	364	364	450
1	C	C	G	C	G	C	A	G	T	A	A	T	C	T	T	G	A	T	C	A	C	T	T	C	
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	
3	.	A	T	A	.	.	.	A	.	.	.	.	--	--	--	--	--	--	--	--	--	.	.	T	
4	.	A	T	A	.	T	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	
5	A	A	T	A	.	.	G	A	.	.	--	.	.	.	.	.	.	.	.	.	.	.	.	T	
6	A	A	T	A	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	
7	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	
8	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	
9	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	
10	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	
11	.	A	T	A	.	.	.	A	.	.	.	.	--	--	--	--	--	--	--	--	--	A	A	T	

Figure 21: Maximum parsimony trees based on nrITS (A) and haplotype network (B) based on cpJsa sequences of *R. lyallii* cpDNA haplotype distribution (C) and position of the polymorphic sites (D). Numbers above the branches indicate bootstrap values. Bars on branches indicate number of supporting parsimony informative sites. Coloured bars and shaded areas indicate the geographic origin of the samples. Numbers on the map represent the different haplotypes. Haplotype shaded yellow in the network is shared with *R. buchananii*. Labels in bold indicate samples included in the phenetic analysis.

The second most common haplotype is haplotype 7; this is found mainly in populations of Mount Aspiring and southern populations towards Te Anau. Haplotype 1 is practically restricted to populations north of the Waimakariri Basin, in Arthur's Pass, yet it is also found in one population of Mount Cook. A similar pattern is observed with Haplotype 3 which is shared by populations from Southland and Stewart Island, but it also found in one population at Jagged Stream (central Canterbury). Uncommon haplotypes, *i.e.*, recorded only once, were restricted to populations in Mount Cook (Haplotypes 8, 9 and 10) and Te Anau and Milford Sounds (Haplotypes 5, 6 and 11). Position of the polymorphic sites for each haplotype is indicated in the Figure 21D.

Habitat characterisation

Several analyses of Principal Components were run to characterise the habitats these taxa occupy. These analyses were run twice. First, all the variables obtained from the LENZ database were included in the analysis except for number 11 and 12 (Table 6). Variables with very low weights in the first three components of this full analysis were identified. Because these variables explained only a small fraction of the overall variation between sites they were excluded from the subsequent and final PCA.

Habitats R. insignis s.l.

The first three components of the PCA accounted for 80.8% of the variation among the sites. Eigenvalues, percentage of variance explained by the first five components and loading scores for each variable are listed in the Appendix 4. Variables strongly associated with the first three axes were: October vapour pressure, elevation, water balance ratio and annual temperature for Axis 1, soil induration and soil particle size for Axis 2 and annual solar radiation for Axis 3. The scatter-plot in Figure 22 represents the distribution of the three groups identified in the MDS (symbols) and the variation and direction of the environmental variables in the ordination space (arrows). The habitat occupied by the form insignis is the most diverse and at least three clusters are observed in the scatter plot (Figure 22). The first cluster (right top corner of the scatter-plot) represents sites associated with soils of large particle sizes and induration, high slope and elevation, and also high water balance ratio and cold temperatures. The second group represents sites in a completely opposite situation; *i.e.*, low slope and altitude and different soil features. The third group (in the upper left corner) represents sites under warm conditions (high annual temperature and solar radiation) and a higher October vapour pressure. The sites occupied by the form lobulatus are dispersed

between the left and right upper area of the scatter-plot forming two groups (Figure 22). The first group includes sites of warmer and drier conditions, while the second one includes sites at altitude, greater slopes, and greater soil particle size and induration. The habitats occupied by the form monroi are also variable but distributed along an environmental gradient with variation in temperature, solar radiation, elevation and October vapour pressure. A single exception was a site at Broken River that shares similar environmental conditions with the form insignis.

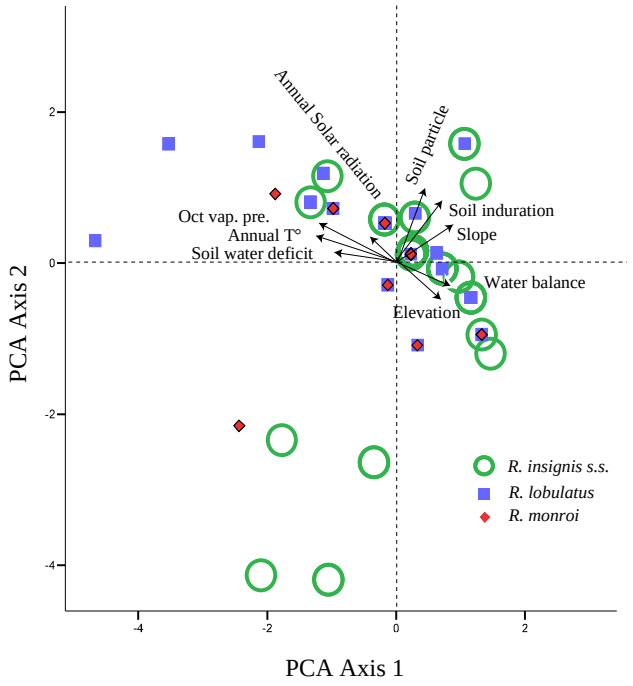


Figure 22: Loadings scores for each site on the first two PCA axes for the three forms of *R. insignis s.l.* Symbols represent sampling points. Vector directions/lengths represent eigenvectors of environmental variables for each axis.

Habitats R. enysii s.l.

The first three components of the PCA accounted for 85.3% of the variation among the sites. Eigenvalues, percentage of variance explained by the first five components and loading scores for each variable are presented in Appendix 5. Variables strongly associated with these three components were climatic variables (October vapour pressure, water balance ratio and annual solar radiation), and soil conditions (soil particle size, soil acidity and induration) for Axis 1; exchangeable calcium in the soil, slope and minimal temperature for Axis 2, and elevation and water deficit ratio for Axis 3. The distribution of the five groups is represented by

different symbols in the scatter-plot in the Figure 23, while variation and direction of the environmental variables in the ordination space is represented by arrows.

At least three of the forms occur in similar habitats; however, this overlap is not complete. The 5WB form is restricted to the upper half of the scatter plot and they represent warm sites (high solar radiation and temperature) with increased slopes and high soil drainage. The form 3WB is restricted to the upper right corner of the scatter-plot where the variables annual temperature and solar radiation, vapour pressure, humidity and soil drainage increase. The form BER is restricted to the lower half of the scatter-plot where environmental conditions such as elevation and slope are high and soil drainage and temperature are low. The habitats occupied by the forms BB and R&P are more diverse than the other three, and are distributed along an environmental gradient. The BB form occurs in habitats that grade from high to low soil particle size and induration, and water balance ratio. Finally, the habitats occupied by the form R&P fluctuates from low to high values of annual temperatures, slope, soil drainage, amount of exchangeable calcium in the soil and elevation.

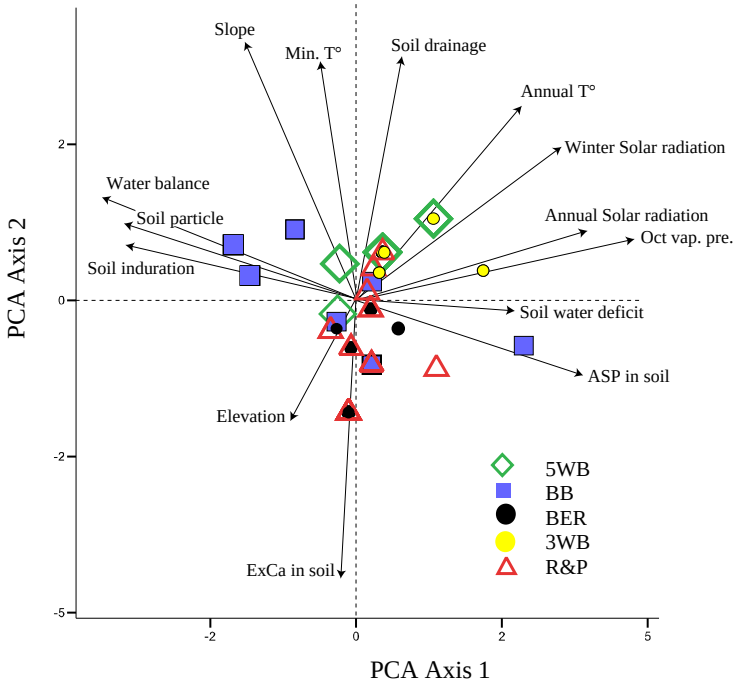


Figure 23: Loadings scores for each site on the first two PCA axes for the five forms of *R. enysii s.l.* studied. Symbols represent sampling points. Vector directions/lengths represent eigenvectors of environmental variables for each axis.

Habitats R. lyallii

The first three components of the PCA account for 82.3% of the variation among the habitats occupied by this species. Eigenvalues, percentage of variance explained by the first five components and loading scores for each variable are listed in the Appendix 6. Soil and climatic variables such as soil particle size and induration, slope, annual solar radiation and vapour pressure were the most important variables in Axis 1, elevation and minimal temperature in Axis 2 and the amount of exchangeable calcium in the soil in Axis 3. Figure 24 shows the habitat diversity of each region and the variation and direction of the environmental variables (arrows).

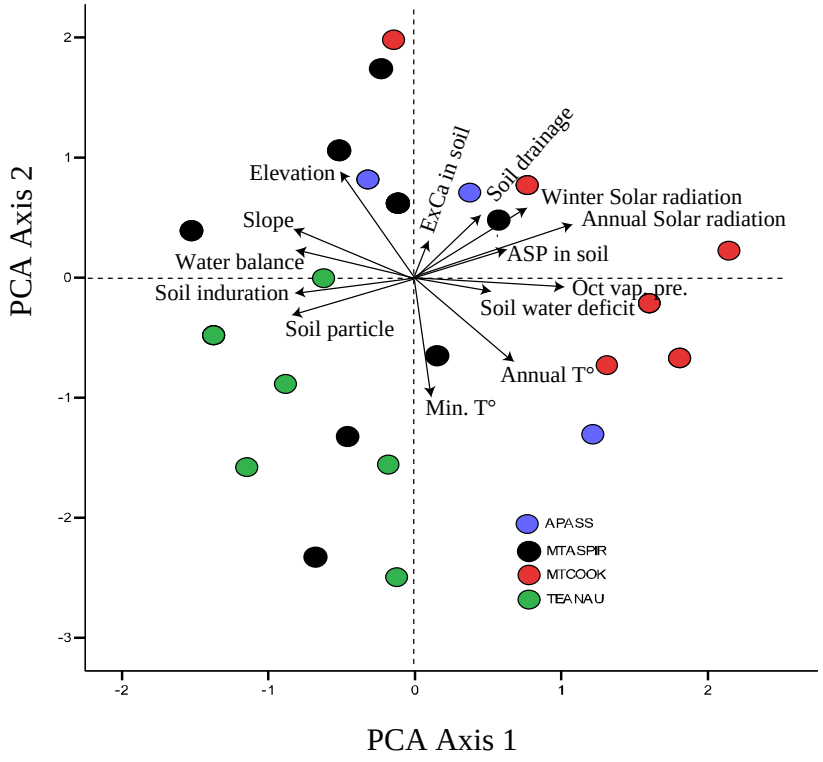


Figure 24: Loadings scores for each site on the first two PCA axes for *R. lyallii*. Symbols represent sampling points. Vector directions/lengths represent eigenvectors of environmental variables for each axis.

In general, the sites occupied by this species are quite variable. The most diverse habitats corresponded to those found in the area of Mount Aspiring, which are distributed across the entire ordination space (Figure 24). Habitats at Mount Cook and southern regions of Te Anau-Stewart Island are found at opposite ends of the ordination, mainly upper right corner and lower left corner, and they are associated with a gradient in soil and climatic variables. Conversely, the habitats occupied by *R. lyallii* at Arthur's Pass are more constant and include

sites associated with high elevations, low temperatures, high solar radiation and high soil drainage. However, some specimens are also found in habitats with low temperatures and high October vapour pressure (lower right corner of the scatter-plot in Figure 24).

Matrix correlations

Results for the Mantel tests to study the correlation between habitat and the different morphologies are presented in Table 10. Only correlations between the data matrices of *R. insignis s.l.* were statistically significant. Morphological divergence observed in the *R. insignis* complex was significantly correlated with geographic distance (Z= 0.18, P= 0.0001) and habitat (Z= 0.22, P= 0.0001).

R. insignis s.l.	Location	Habitat	Morphology
Location	-----	0.0001	0.0001
Habitat	0.27	-----	0.0001
Morphology	0.18	0.22	-----
R. enysii s.l.			
Location	-----	0.4904	0.6062
Habitat	-0.001	-----	0.0939
Morphology	-0.005	0.08	-----
R. lyalii			
Location	-----	0.8969	0.8039
Habitat	-0.03	-----	0.7629
Morphology	-0.02	-0.0629	-----

Table 10: Results of the Mantel tests for habitat, geographic distance and morphology for the three study cases: *R. insignis* s.l., *R. enysii* s.l. and *R. lyallii*. Numbers in the upper triangle are the probability that the observed Z is smaller than the random Z from 10000 permutations.

Discussion

Species delimitation and patterns of morphological variation in *R. insignis s.l.*

Results from the morphological and molecular analyses provide support to reject the grouping of *R. insignis*, *R. lobulatus* and *R. monroi* under a single species, *R. insignis*, as suggested by Fisher (1965). Quantitative characters such as leaf size, petal size, number of flowers per stem and the ratio of style/achene body length significantly differed between these three species. Several of these characters were considered to show a clinal pattern of variation by Fisher (1965), yet such pattern was not evident here. Although mean values of leaf size, petal size and number of flowers diminish from North to South mean differences between the three species are statistically different (Table 7) and do not follow the trend expected in a geographic cline (*i.e.,* overlap between *R. insignis* and *R. lobulatus* and overlap between *R. lobulatus* and *R. monroi*). Furthermore, clinal patterns of variation are generally described in species with continuous population systems with regular gene flow which helps to maintain the plants' phenotypes (Grant 1981), whereas these species are restricted to unconnected mountain tops.

Unlike quantitative characters, qualitative characters are considered more taxonomically informative (Stuessy 1994), yet studying them may be challenging. For instance, character state diversity in *Ranunculus* is very limited due to the great extent of homoplasy in vegetative and floral characters (Hoot 1995, Hörandl *et al*. 2005) and this reduces the number of informative characters considerably (*e.g*. sepal characters in this study). Also, qualitative characters can be difficult to score from dry pressed material since colour and shapes can be strongly distorted. This can explain the low frequency of uncommon states within these three species. Conversely, at least four quantitative characters were found to discriminate between these species size of the nectary gland, inflorescence shape, presence of hairs on the flowering stem, and leaf shape. Petals of *R. insignis* have a large (>3mm) and exposed nectary gland that appears to be formed by the fusion of three glands (Figure 25A). The nectary gland of *R. lobulatus* is smaller (~ 300µm), also naked but it is sunk forming a cup (Figure 25B) while the nectary gland of *R. monroi* is pocket shaped and also sunken in a pit, but only *c.* 100µm at its widest point (Figure 25C). Unfortunately, size and shape of the nectary glands is difficult to assess from dry pressed material.

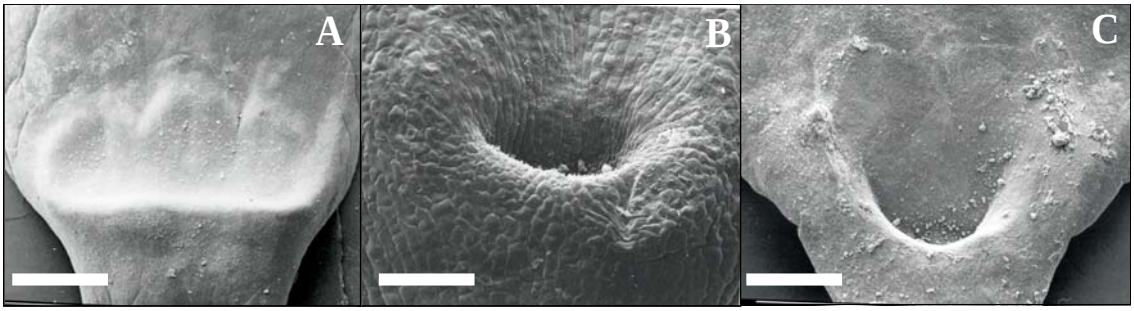


Figure 25: Variation in nectary gland morphology and size in the three forms of *R. insignis* s.l. (A: insignis, B: lobulatus, C: monroi; the bar indicates A: 1mm, B: 0.1mm, C: 0.5mm)

Inflorescence shape of all three species is distinct, and this character was considered less important by Fisher (1950). The inflorescence of *R. insignis* is compact with short-peduncled flowers, *R. lobulatus* has a lax inflorescence with long-peduncled flowers and *R. monroi* has a single flowering stem with one or two flowers. Hairy stems are typical of *R. insignis* and absent in *R. lobulatus* and *R. monroi*. Unlike the previous characters, leaf shape seems to be more plastic and two or three shapes were scored within some of the species (*e.g. R. lobulatus*).

Overall, the relationship of habitat conditions to the phenotypic diversity of this species was significant (Z= 0.22, P=0.001) but seems to be more evident in *R. lobulatus* than the other two species. For instance, environmental conditions are clearly different between the populations of *R. insignis* (Figure 22) occurring in the mountain tops of the North Island and those in the Nelson and Marlborough region in the South Island, however, no phenotypic divergence was noticed. Phenotypic stability between these populations was also pointed out by Fisher (1965). Similar to *R. insignis*, two habitat types were identified for *R. lobulatus* but, unlike *R. insignis*, phenotypic differences between specimens growing in these habitats were evident. Specimens of *R. lobulatus* growing in sites where the soil is scree or turf generally have smaller, hairier, more sub-obovate leaves than individuals growing among scrub and more stable soil. This phenotypic plasticity was obvious from populations sampled at Lake Tennyson (Kaikoura Mountains). The habitat occupied by *R. lobulatus* there grades from dense scrub by the lake shore to lower vegetation (tussock and patches of turf) on slopes with scree. Individuals at the base of these slopes are robust, subfructicose, with almost glabrous leaves and a lax cyme. Individuals on the scree and turf, on the contrary, have reduced size, leaves are hairier and arranged in a basal rosette and the flowering stem is erect, basal and

may contain one or two flowers only. Flowers, however, are morphologically identical to the flowers of the populations growing by the lake shore.

Phenotypic plasticity in *R. lobulatus* may explain the "continuum" reported by Fisher (1965), because some of its traits may also be observed in *R. monroi* at the Waimakariri Basin. Convergence of these phenotypic characters could also be responsible for the grouping of one specimen of *R. monroi* from Castle Hill with *R. lobulatus* and one specimen of *R. lobulatus* from the Kaikoura ranges with *R. monroi* in the cluster analysis (groups IIB and Group III in Figure 10, respectively) and the division of *R. lobulatus* into two groups as observed in the cluster analysis. Furthermore, it is likely that the variety *R. monroi* var. *sericeus*, described by Cheeseman (1925) and found in Mount Peel and the Kaikoura Ranges, was described using material that corresponded to this variety of *R. lobulatus.* The extent of phenotypic variation in *R. lobulatus* is also observed in the MDS analysis, where clustering of the specimens is less cohesive than the other two groups (Figure 11). Populations of *R. monroi* in the Canterbury region, on the other hand, are distributed across an environmental gradient and no morphological variation was observed.

Intermediate individuals between *R. insignis* and *R. lobulatus* in the Marlborough and Nelson regions and the northern Canterbury region may have resulted from hybridisation events, and possibly introgression, in contact zones where populations of both species are sympatric. The DFA clearly classifies these specimens as intermediate between their putative parental species (Figure 13) but morphologically closer to *R. insignis* than to *R. lobulatus*. This latter observation may suggest introgression in these contact zones. However, further genetic analyses including DNA fingerprinting techniques or microsatellites are required to test this hypothesis. A similar scenario has been recently uncovered for two *Phormium* species also found in the Marlborough and Nelson areas (Smissen *et al*. 2007).

Results from the phylogenetic analyses suggest these three *Ranunculus* species may represent lineages of recent origin or at incipient speciation. Sequences of *R. insignis* and *R. lobulatus* are identical in the ITS and only two one-base pair substitutions separate them from *R. monroi*. Low sequence variation may suggest a recent separation of these lineages followed by rapid morphological divergence, likely driven by habitat conditions (the correlation between habitat and morphology is significant in this group; Table 10). Such patterns of low genetic diversity and rapid morphological divergence has been described before in several

alpine plant species of New Zealand and Australia (see review in Winkworth *et al.* 2005) and the South American Andes (Schilling *et al.* 2000). Chloroplast sequences, instead, were more variable and several haplotypes were found. All specimens of *R. insignis* from the North and South Islands share the same haplotype and this is sister to the haplotype found in *R. lobulatus*, suggesting common ancestry between these two entities. Conversely, haplotypes found in *R. monroi* were numerous. None of these haplotypes is shared with the other two lineages, corroborating the absence of gene flow between them. However, these haplotypes are not unique to *R. monroi* and they are also shared with sympatric specimens of *R. enysii* (Lockhart *et al.* 2001, Piripi 2005) and *R. crithmifolius* (Lockhart *et al.* 2001, Piripi 2005). This is a finding consistent with hybridisation between these species. However, an hypothesis that this similarity represents retention of an ancestral haplotype still needs to be tested further.

Patterns of morphological variation and species delimitation in *R. enysii s.l.*

The extent of morphological variation observed in this group was considerable and at least five entities were distinguished. These five entities were geographically restricted and generally recognised at first sight by their size and division of the leaf lamina. However, when variation of characters such as leaf margin, width of the leaflets and insertion of lateral leaflets were considered, classification of variable specimens became impossible. An example of this are the four specimens labelled as "undetermined" in this study since *a priori* classification was impossible. These were later assigned to the 3WB and 5WB forms, R&P and BER by the CA and the MDS analysis. Morphological variation was also evident when group delimitation was attempted with both phenetic analyses; several specimens assigned *a priori* to a group were included into a different group. It is possible that some of the characters measured were not informative enough due to their variability or extent of phenotypic convergence.

Phenotypic convergence, for example, could explain the grouping of the trifoliolate specimens from the Waimakariri Basin (3WA) with specimens of the R&P form in the CA and MDS. Leaf lamina of these specimens is ternately divided, leaflets are sessile and, unlike the remaining specimens of the Waimakariri Basin, the margin of the lamina is entire. All these characteristics are typical of the R&P type. A similar situation is observed with the forms BB and 5WB (Figures 14, 15 and 16). These two groups appear closer to each other in both morphological analyses, and although they may be deemed different at first sight, their leaf

architecture is identical. The leaf lamina in both types is divided into five, with two of the four lateral leaflets petiolulate, the other two sessile and the terminal leaflet is supported by a long petiolule almost twice the length of the petiolule of the lateral leaflets. The terminal leaflet is tri-lobed in both forms. Phenotypic convergence due to environmental conditions is unlikely since these two forms occupy different habitats. A similar pattern was observed in the floral structures of these five forms. The shape of the nectary gland, for instance, is pocket-like in the types BB, R&P and BER. Only the nectary gland of the specimens of 3WB and 5WB was slightly different, v-shaped and surrounded by a raised flap-like structure.

The extent of morphological variation in this species complex is unique within the New Zealand alpine *Ranunculus* and the cause(s) promoting these patterns have remained unknown. Fisher (1965) put forward three possible sources of morphological variation in *R. enysii s.l.* plastic response to different habitat conditions, contact between previously separated races that have diverged morphologically, and hybridisation and introgression with sympatric species.

Morphological variation due to phenotypic plasticity has been reported in numerous species of *Ranunculus* before, but mainly in aquatic and semi-aquatic species (Lynn & Waldren 2001, Cook & Johnson 1968). These species are usually found in heterogeneous and changeable habitats and these phenotypic changes are highly adaptable and may be essential for their survival (*e.g.* Noel *et al.* 2007). Despite these five studied forms being geographically restricted and distant from one another, it was found that the habitats they occupy are only slightly different, and several soil and climatic variables overlap between them. Furthermore, there is no significant correlation between morphological divergence and habitat conditions or geographic distance (Table 10). These results may be sufficient to rule out the effect of habitat on phenotypic diversity in *R. enysii s.l.* and predict that significant features of morphological variation are genetically determined. This is further supported by Fisher's experiments on the stability of the different forms of *R. enysii s.s.* found at the Waimakariri Basin. These two forms were grown under common garden conditions for two years and no major changes were observed in the original phenotype.

The second hypothesis proposed by Fisher (1965) suggests morphological variation has been caused by secondary contact between allopatric races that have diverged morphologically. If this is true, populations geographically intermediate displaying all or at least some of these

phenotypes would be expected to occur between the Waimakariri Basin and Central Otago and Southland. Morphological variation in this study was assessed across the entire distribution of *R. enysii s.l.* and such populations were never detected. Furthermore, the distribution of this species is interrupted geographically by a gap of more than 200kms and gene flow between northern and southern populations in the South Island appears to be absent (*i.e.*, no chloroplast haplotypes are shared between them).

Phenotypic divergence after separation of the ancestral form of this species followed by hybridisation, and likely introgression, with other sympatric species is a plausible explanation for the morphological patterns of variation observed. As currently defined, *R. enysii* presents a disjunct distribution and it is found in the Waimakariri Basin (Canterbury) and Central Otago and Southland. This biogeographic pattern is common in many other plant species of the South Island and has been explained by glaciers wiping out all central populations (Heads 1998). It has been suggested that during the Pleistocene, glaciations fragmented the distribution of many species and the surviving populations were probably confined to isolated ice-free refugia such as mountain tops (Lockhart *et al.* 2001, Winkworth *et al.* 2005). This pattern of distribution is consistent with the similarity in the plant architecture observed before between northern and southern populations of *R. enysii s.l.* and the absence of gene flow between them. Analysis of the nrDNA and cpDNA sequences confirmed this species complex is not monophyletic and that four of these morphological entities belong to two evolutionary lineages. Absence of gene flow between these two lineages is further supported by AFLP fingerprinting data (Piripi 2005). The origin of the fifth form, BER, is still uncertain since no fresh material was available for molecular analysis due to conservation considerations.

Changes in the distribution of *R. enysii s.l.* and restriction of populations to glacial refugia may have brought the ancestral form of this species into contact with other *Ranunculus* species, that were otherwise allopatric. If so, hybridisation and introgression may have taken place in these refugia, in the absence of reproductive barriers. These interactions might then promote the origin of new or intermediate phenotypes, such as those found at the Waimakariri Basin. Morphological variation observed in these populations might be explained by hybridisation between *R. enysii s.s.* and *R. monroi*, and subsequent introgression of the fertile hybrids with their parental species. This is the third hypothesis put forward by Fisher (1965) and he referred to these populations as hybrid swarms. Gene flow between *R. enysii s.s.* and

R. monroi is also suggested by the presence of similar chloroplast haplotypes (Lockhart *et al*. 2001, Piripi 2005) and a relatively large number of polymorphic bands shared between them in AFLP analyses (Piripi 2005). If this interpretation is correct, the two forms of *R. enysii* s.s. found at the Waimakariri Basin may include several hybrid generations. From the two phenotypes found there, the three leaflets type seems to be the less stable since the progeny of self-pollinated plants shows greater variation in leaf morphology (Fisher 1965).

Similar events may explain the sources of morphological diversity observed in the populations from central Otago. However here, gene flow is suspected between *R. enysii* and sympatric populations of *R. gracilipes*. Hybrid swarms between these two species were also indentified by Fisher (1965) and gene flow between *R. enysii* from the type R&P populations and *R. gracilipes* has been reported before by Lockhart *et al.* (2001) and Piripi (2005).

Patterns of morphological variation and species delimitation in *R. lyallii*

Unlike the two previous study species, *R. lyallii* is morphologically uniform across its distribution but genetically very diverse. Both phenetic analyses showed no grouping by geographical areas while molecular analysis of nrDNA and cpDNA sequences do show such association. The absence of morphological variation in *R. lyallii* is unique within the alpine *Ranunculus* and has been considered by Fisher (1965) as a response to a 'greater than usual specialisation to habitat". This species is usually found growing by stream sides and wet-subalpine to alpine scrub and grasslands. Habitat characterisation using the information available from the LENZ data base did not support Fisher's hypothesis. On the contrary, habitat characterisation showed these habitats are quite variable even within the same area. It is important to keep in mind that the variables used to characterise these habitats may only account for general macro-environmental conditions and a different scenario could be detected if micro-environmental variables were included. Unfortunately, the LENZ database does not have the resolution to provide these detailed micro-environmental variables.

At least four major lineages were detected in the ITS data, corresponding with four geographical areas: Arthur's Pass, Mount Cook, Mount Aspiring and Te Anau-Stewart Island. Also 11 chloroplast haplotypes were found. Populations at Mount Cook and Te Anau-Stewart Island areas showed the highest diversity of haplotypes, with five and six haplotypes each, respectively. The extent of the genetic diversity of *R. lyallii* and the localised distribution of some of the haplotypes are consistent with theories of multiple populations surviving the Last

Glacial Maximum in isolated refugia (Lockhart *et al*. 2001, Winkworth *et al.* 2005, Shepherd *et al*. 2007). Although these theories suggest no gene flow between the refugia, two haplotypes were shared by these four areas (*e.g.* Haplotype 1 or Haplotype 3). A possible explanation for this pattern, besides gene flow, could be recent dispersal events between these populations or an ancestral polymorphism. It is also important and interesting to mention that *R. lyallii* is not monophyletic and affinities with *R. buchananii* have been detected before by Lockhart *et al.* (2001). One of the 11 haplotypes detected in this study, Haplotype 3 is shared with *R. buchananii*. This haplotype is mainly found in populations from Southland where the distribution of *R. lyallii* and *R. buchananii* overlaps, but also in Stewart Island and the Arthur's Pass area where *R. buchananii* is not found.

Concluding remarks

Results obtained in this chapter show that not all the species concepts presented before are fully applicable to all the entities in this group. Evidence of hybridisation, and perhaps also introgression, was detected in populations of *R. insignis* s.l. and *R. enysii* s.l. and at least two lineages were detected in *R. lyalii*. These results disagree with the biological species concept and the phylogenetic species concept. The extent of morphological intergradation observed in the phenotipically variable *R. enysii* s.l. also makes difficult to apply the phenetic species complex which aims for the recognition of discrete phenetic clusters. From the concepts presented in the introduction of this chapter, the ecological species concept seems to be the most applicable. Many of these forms seem to be associated to specific habitats and this promotes their reproductive isolation. Nevertheless, the combined approach taken in this study, including morphological, genetic and environmental data has proved useful to understand the patterns of morphological and genetic variation observed in these three species and develop hypothesis regarding their evolutionary history.

This study has provided morphological and genetic support to maintain *R. insignis*, *R. lobulatus* and *R. monroi* as different species and reject Fisher's hypothesis of clinal patterns of variation within the species complex. All three species possess a number of qualitative and quantitative characters that allow their successful identification. *Ranunculus lobulatus* and *R. monroi* may correspond to lineages of recent origin that have colonised slightly different habitats in the mountain tops of the South Island. Environmental differences between these habitats have a significant role in promoting phenotypic diversity in this group, and the existence of ecotypes is evident in *R. lobulatus*. Fixation of advantageous phenotypes in these

isolated populations could occur by restricted gene flow followed by selection. Recent speciation of *R. lobulatus* and *R. monroi* is supported by their genetic similarity and the lack of reproductive barriers. Absence of reproductive barriers is particularly evident in contact zones where populations of *R. lobulatus* and *R. insignis* occur sympatrically and a number of intermediate individuals of hybrid, or introgressed, origin were detected. However, the reproductive dynamic between these two species and their progeny in this contact zone, needs further sequence variation (using DNA fingerprinting or microsatellite techniques) and pollination studies.

The origin and the number of entities comprising the *R. enysii* complex were difficult to determine. Species boundaries within this complex are unclear due to the extent of phenotypic variation observed across its distribution. Nevertheless, five geographically restricted forms were identified; two in the central Canterbury Area (Waimakariri Basin), two in Central Otago (Rock and Pillar and Carrick Range) and one in Southland (Borland). Some of these forms occur in specific habitats while others have wider ecological amplitude. These environmental differences, however, are not significant enough to explain the morphological variation observed within the complex. Hybridisation and introgression, on the other hand, after major climatic and geographic events seem to be most plausible explanation for several phenotypes observed in this complex. Regular gene flow between *R. enysii* and *R. monroi* (central Canterbury) and *R. gracilipes* (Central Otago), for example, may have facilitated the formation of introgressed lineages with novel advantageous phenotypes that, over time, genetically swamped out pure lineages and eliminated the ancestral phenotype from the population. To further test this hypothesis, future studies including all different forms and inter-specific pollination experiments, assessment of the phenotypes under common garden conditions and fitness of the progeny will be necessary to disentangle the evolutionary history of this species complex.

Unlike the previous study cases, and in spite of its great genetic diversity and isolated populations with limited gene flow, *R. lyallii* is morphologically uniform across its distribution. The discordance between morphological and molecular divergence in this species may be attributable to morphological stasis. Morphological stasis has been proposed in a number of herbaceous and tree species with disjunct distributions; some of them are even paraphyletic (as occurs in *R. lyallii*) or polyphyletic in origin (see Nie *et al*. 2006). Stasis in morphology, and mainly ecologically significant traits, is usually explained by a relatively

constant environment and the action of stabilising selection on the phenotypes (Wen 1999, Nie *et al.* 2006). Habitat specialisation seems the most plausible explanation for the lack of clinal, ecotypic or plastic morphological variation within *R. lyallii*, and although environmental diversity between the sites where it occurs is sizeable, a detailed description and analysis of micro-environmental conditions could demonstrate the contrary. It is likely that the current state represents an optimal phenotype and any deviants from it could have a low fitness. If this is the case, this phenotype might be expected to have been maintained over time by stabilising selection, as long as a suitable habitat remains available (*e.g.* through the Pleistocene glacial/interglacial cycles, Winkworth *et al.* 2005). Future studies on the performance and fitness of this phenotype under different environmental conditions, detailed micro-sites descriptions and *in situ* eco-physiological studies would be ideal to test this hypothesis.

Taxonomic implications

The *R. insignis* complex consists of at least three entities which coincide with previous taxonomic treatments of the complex. The form lobulatus is morphologically distinct from *R. insignis* but it is little differentiated genetically at the ITS and J_{SA} loci. Thus, there is not compelling evidence yet to elevate this taxon status to species level. The occurrence of intermediate phenotypes between the forms lobulatus and insignis where their ranges overlap suggest gene flow between these two entities. However, this needs further study. On the other hand, the form monroi represents an independent lineage that is morphologically and genetically distinct and geographically isolated. Species status should be recognised for this entity and the name *R. monroi* should be reinstated.

As for *R. enysii* there was no clear evidence to support segregation of the different forms and restate past classifications. Morphological variation seems to be strongly affected by hybridisation events, either recent or ancient, and further research is needed. Till then, the current name and circumscription of the species should be maintained. *Ranunculus lyalii*, on the other hand, represents a genetically diverse species which is morphologically uniform across its distribution. These three study cases illustrate the complex nature of plant species and the inapplicability of a static species concept to define them.

IV

Phenotypic and habitat diversification: does the New Zealand Alpine *Ranunculus* group show features of an adaptive radiation?

INTRODUCTION

The evolutionary process by which distinct species arise is referred to as speciation. The ultimate result of this process is the establishment of reproductive barriers between previously interbreeding populations (Turelli *et al.* 2001). Geographic isolation between populations has been considered an important barrier to gene flow and based on the extent of spatial isolation four modes of speciation have been proposed; *i.e.,* allopatric, peripatric, parapatric and sympatric (Futuyma 1998). Allopatric speciation generally occurs when a physical barrier separates populations and prevents mating between their members. In the peripatric mode of speciation only a small subset of the population becomes isolated and gene flow with the main population is reduced and diversification is promoted. In the parapatric speciation, on the contrary, there is partial overlap between the incipient species, but individuals are more likely to mate with their geographic neighbours than with individuals at the opposite end of the population. Unlike the previous modes, for sympatric speciation to occur, geographic isolation is not required and speciation may occur by colonisation of a different niche within the same area by part of the population (*i.e.,* specialisation). Speciation may also occur directly by events such as hybridisation or polyploid formation, especially in plants with overlapping distributions (Grant 1981, Linder & Rieseberg 2004).

Although these speciation modes are well accepted, there has been a renewed interest in the contribution of ecological processes in the formation of new species (see review by Rundle & Nosil 2005). Unlike these previous modes, in ecological speciation, new species arise by the divergent selection that habitat and resources impose on populations. In this mode, gene flow between populations is hindered by ecologically-based divergent selection, *i.e.,* selection as a consequence of the interactions between individuals and their environment (Schluter 2000, Rundle & Nosil 2005). Reproductive isolation in this case evolves as a byproduct of the adaptation to different habitats (Levin 2004). Changes in habitat and resource utilisation can be significant promoters of divergent selection and they have been considered important speciation drivers in the evolution of flowering plants (Levin 2000, 2005). Divergent selection can arise by environmental differences such as habitat structure, climate, edaphic conditions, availability of resources and the set of predators/competitors present (Schluter 2000). These biotic and abiotic environmental differences may promote the ecological diversification of lineages and the diversification of the traits (phenotype) used to exploit the resources available (Schluter 2000). The evolution of ecological and phenotypic diversification within a lineage has been described as an adaptive radiation. Basically, it involves the differentiation

of a single ancestor into an array of species that inhabit a variety of environments and that differ in the morphological and physiological traits used to exploit the resources in those environments (Schluter 2000).

Adaptive radiation has been inferred in plant, animal and insect groups occurring mainly in Oceanic Islands. Some of the best known examples are the Hawaiian silversword alliance (Baldwin 1997), the Hawaiian lobeliads (Givnish *et al*. 1994, 1995), Darwin's Finches (Grant 1986), the West Indian *Anolis* lizards (Jackman *et al.* 1997), the drosophilid flies (Kambysellis & Craddock 1997) and Thomaisidae spiders in Hawaii (Garb 1999). Continental examples of adaptive radiation are less common but a few have been proposed, *e.g*. the *Aquilegia* (Hodges 1997), bromeliads of the genus *Brocchinia* (Givnish *et al*. 1997) and the genus *Lupinus* in the Andes (Hughes & Eastwood 2006). Adaptive radiations may be detected by the presence of four features: rapid speciation, recent common ancestry, phenotype-environment correlation and trait utility (Schluter 2000). The first feature, rapid speciation, is not a well defined concept but at least four standards might be used to detect it; three of them aim to detect episodes of branching in phylogenetic trees and the fourth, periods or lineages in which reproductive isolation evolves unusually rapidly (Schluter 2000). Recent common ancestry, on the other hand, is relatively easy to assess and it is generally inferred from phylogenetic studies. The presence of the remaining two features, phenotype-environment correlation and the usefulness of different traits to exploit resources in the different adaptive zones, is what makes a radiation "adaptive" (Schluter 2000). Evidence for phenotype-environment correlation is usually collected from field observations and morphological/anatomical comparative studies. Phenotypic differences between species must be genetically based and not a plastic response to divergent environmental signals during development (Schluter 2000). Some of the examples of phenotype and environmental correlations described in plant adaptive radiations are, for instance, the leaf shape and anatomy of the Hawaiian silversword alliance that correlates with a dry-wet continuum in their habitat (Baldwin 1997), the floral orientation, colour and nectary spur size associated with pollinator type in the *Aquilegia* (Hodges 1997), the plant habit and leaf type in the aquatic family Pontederiaceae (Barret & Graham 1997) or the trichomes in the *Brocchinia* bromeliads (Givnish *et al.* 1997). Testing the usefulness of these traits to exploit the different resources available and the fitness conferred to each species is the final step necessary to interpret a correlation between the phenotype and the environment as being adaptive (Schlutter 2000). This usually involves experimental studies in which traits are manipulated and the effect of such manipulations on

the species fitness is measured (Baldwin 1997, Givnish 1997, Schluter 2000, Givnish *et al.* 2004, Hodges & Arnold 2005).

The alpine *Ranunculus* group of the section *Pseudoadonis* has been regarded as a typical example of adaptive radiation in New Zealand (Fisher 1965). This group is an important element of the alpine flora and over 20 species have been recorded in the mountains of the North and South Island (Garnock-Jones 1988, Heenan *et al.* 2006). Of the four features normally characteristic of adaptive radiations, a single origin has been inferred for the section *Pseudoadonis*. Monophyly of the entire radiation has been inferred using nrDNA and cpDNA sequences by Lockhart *et al.* (2001) and it has been further confirmed in this study (Chapter II, Figure 3C). Molecular dating has suggested the group radiated within the last 3-5 Myrs (Pleistocene) after the arrival of a single founding ancestor in New Zealand (Lockhart *et al.* 2001), probably of aquatic habit (Chapter II). This period corresponded with the onset of the Southern Alps in New Zealand. Thus appearance of many novel alpine habitats has accompanied radiation of the group (Lockhart *et al.* 2001). This radiation was accompanied by a process of rapid morphological diversification when compared to the recent origin and low genetic divergence among the different species in the group (Lockhart *et al.* 2001).

The extent of morphological diversification, low genetic diversity and range of habitats occupied by alpine *Ranunculus* in New Zealand have led some researchers to speculate that the radiation of this plant group has been adaptive (Fisher 1965, Lockhart *et al.* 2001). However, phenotype-environment correlations have never been formally assessed in this group of species. Similarly, the adaptive significance of the different phenotypic traits has not been investigated. In this Chapter, morphological and habitat diversity of all New Zealand alpine *Ranunculus* will be studied. A comparative approach was used to address five questions:

- How phenotypically diverse is this group?
- Are vegetative characters more diverse than reproductive characters?
- Are closely related species morphologically similar?
- How different are the habitats these species occupy?
- Are morphologically similar species found in similar habitats?

METHODS

Phylogenetic affinities

Phylogenetic affinities within the group were based on the analysis of unpublished nuclear ribosomal DNA and chloroplast DNA sequences and those obtained from earlier studies (Lockhart *et al.* 2001; Piripi 2003; Carter 2007). Maximum Parsimony (MP) trees were built was performed separately for the ITS and J_{SA} data set using the software PAUP* 4.0b8 (Swofford 2000), using the heuristic search with 1000 replicates, random sequence addition, tree-bisection-reconnection (TBR) branch swapping, MULTRESS on (keeping multiple, equally parsimonious trees) but saving only 10 trees each replicate. The unordered character states and equal character state weighting options were used in the analysis. The relative support for each node was examined with non-parametric bootstrapping (Felsenstein 1985; 1000 replicates).

Morphological characterisation

Plant habit and a number of macro-morphological characters were described based on observations made under the dissecting microscope of both fresh and herbarium material and the taxonomic literature available (Fisher 1965, Garnock-Jones 1988). Leaf anatomical characters were scored from histological preparations. Plant material was fixed in F.A.A. overnight and then dehydrated using ethanol series and embedded in Paraplast embedding medium. Sections of 10μm thick were obtained, stained with toluidine blue and later mounted in DPX. Achenes for SEM examination were treated with 1% Driselase for 24-48 hrs in order to remove the cuticle and parenchymatous cell layers and expose the cells of the carpel outer wall. After this enzymatic treatment, achenes were washed with distilled water and air dried. Samples were sputter-coated with gold and observed using a Cambridge 250 SEM. At least three achenes from different individuals collected across the distribution of each species were studied, if no variation was observed only one of them was photographed and included in the analysis. Petals bearing nectary glands were dehydrated using ethanol series and then sputter-coated with gold and observed using a Cambridge 250 SEM. Multiple samples were observed for the most and the least variable species in the New Zealand Alpine group sensu Fisher (1965). Variable species studied were *R. insignis s.l.* (151 voucher specimens and 25 fresh specimens), *R. enysii s.l.* (83 voucher specimens and 31 fresh specimens) and *R. gracilipes* (20 fresh specimens). Stability of the nectary gland shape was also assessed from species considered morphologically stable despite their genetic diversity, *e.g. R. lyallii* (41 voucher specimens and 15 fresh specimens). The New Zealand species *R. pinguis* and *R. viridis* and

the Australian *R. anemoneus* and *R. gunnianus*, also part of this radiation, were not included in the analysis because material was not available.

A total of 19 characters were investigated (Table 11) and, after coding, assembled into a data matrix (Table 12). All character states were treated as unordered and equally weighted. A similarity matrix that included all characters was calculated using the simple matching coefficient for multi-state data as implemented in the module SIMQUAL in the software NTSYSpc version 2.20L (Rohlf 2000). The matrix was explored with Cluster Analysis and Multidimentional scaling analysis using the software NTSYSpc. Vegetative and reproductive characters were also explored independently.

These characters were chosen because of their putative adaptive nature. For instance, leaf dissection and texture are involved in photosynthetic activity and temperature control (Stebbins 1974). Floral display and nectary glands correlate with a plant's reproductive success (van de Pijl 1973, Stebbins 1974). The presence of trichomes are involved in plant protection, water absorption and seed dispersal (Stebbins 1974, Werker 2000) and finally, the microsculpture of the seed surface aids may assitst dispersal, avoids seed contamination and controls seed surface temperature under insolation (Barthlott 1981).

Habitat types

Collection details (*i.e.*, latitude and longitude) for each taxon were recorded from herbarium specimen labels, herbaria databases and visited populations. A total of 769 points were gathered and included in the study. The Land Environment of New Zealand database (LENZ) developed by Landcare Research was then used to obtain a number of environmental parameters for each site. Data for 16 environmental variables were obtained from the layer LENZ level IV, which contains the most accurate environmental description of all layers contained within LENZ. The variables investigated are listed in Table 6 (Chapter III). Before statistical analysis, data were standardised by calculating Z-scores using SPSS 13.0. Habitat variation was assessed using Canonical Variates Analysis (CVA) using SPSS 13.0. This analysis maximizes the difference between groups rather than individuals as is the case with the PCA (Dytham 2003).

Habitat types identified by the Canonical Variates Analysis were circumscribed and the morphological groups within them assessed. Association with habitat was also explored by each character individually.

	Character	Character states and codes
1	Life form	cryptophyte (1); hemicryptophyte (2); chamaephyte (3)
2	Rhizome habit	creeping (1), erect (2)
3	Leaf outline	orbicular (1), reniform (2), cordiform (3), oblong (4), linear (5), peltiform (6), ovate (7)
4	Leaf dissection	dissected (1), entire (2)
5	Leaf hairs	always present (1), always absent (2), present/absent (3)
6	Leaf thickness	coriaceous (1), fleshy (2), membranaceous (3)
7	Palisade cells	two layer (1), three layers (2), four layers (3)
8	Inflorescence type	cyme (1), solitary (2)
9	Flower colour	yellow (1), white (2)
10	Nos. petals	5-9 (1), 10-15 (2), >15 (3)
11	Nos. glands	one (1), three (2)
12	Nectary shape	pocket (1), flap (2), open (3), horseshoe (4), cup (5)
13	Achene hairs	always present (1), always absent (2), present/absent (3)
14	Achene body	turgid (1), flattened (2)
15	Style length	≤ achene body (1), ≥ achene body (2)
16	Shape cells (achene surface)	elongated (1), irregular (2), polymorphic (3)
17	Outline anticlinal walls (achene surface)	sinuoused (1), straight (2)
18	Relief cell border (achene surface)	channelled (1), raised (2)
19	Curvature outer cell wall (achene surface)	convex (1), concave (2)

Table 11: Characters and character states recorded from 20 taxa of alpine *Ranunculus* of New Zealand.

RESULTS

Molecular analysis

The ITS data matrix included 173 sequences representing 20 taxa and a total of 604 characters, 112 of them were variable but only 79 parsimony informative. The MP analysis resulted in 10 most parsimonious trees of 172 steps (CI: 0.73; RI: 0.93). The MP 50% majority rule consensus tree is shown in Figure 26. Bootstrap values ≥ 50 are indicated above the branches. Rooting this tree at a basal polytomy leading to four distinct lineages, as in Lockhart *et al*. (2001), allows description of clades corresponding to groups previously identified and discussed by Lockhart *et al*. (2001). Four main clades were identified in the MP tree (numbered from 1 to 4 in Figure 26).

Clade 1 included sequences from 9 species *R. buchananii, R. lyallii, R. verticillatus, R. nivicola, R. piliferus, R. acraeus, R. haastii, R. grahamii* and one sequence of *R. insignis* that is nested within *R. verticillatus*. Clade 2 included sequences from only two species: *R. sericophyllus* and *R. pachyrrhizus*. Clade 3 grouped sequences of 9 taxa: *R. crithmifolius, R. gracilipes, R. godleyanus, R. monroi, R. lobulatus, R. insignis* and three forms of *R. enysii*; Porter Pass (PP), Borland Burns (BB) and Rock & Pillar (R&P). Clade 4 contained sequences of only one species, *R. scrithalis*. Within Clade 1, only *R. acraeus, R. piliferus* and *R. nivicola* appeared as monophyletic species, sequences of the remaining species occurred in up to three different sub-clades within Clade 1 (*e.g. R. lyallii* and *R. haastii*). *Ranunculus sericophyllus,* in Clade 2, is also paraphyletic and sequences of *R. pachyrrhizus* were nested within one of the two lineages of *R. sericophylus*. Two non monophyletic species were also observed within Clade 3, *i.e., R. enysii* and *R. monroi*. Two out of the three forms of *R. enysii* were recovered forming a monophyletic lineage; these were the forms found in Borland Burns and Rock & Pillar. Sequences of the third form, however, had a sister relationship with *R. crithmifolius*. As for *R. monroi*, only two sequences were found outside a sub-clade formed by the remaining sequences of *R. monroi*.

The J_{SA} data matrix included 173 sequences representing 20 *taxa* and a total of 513 characters, 57 of them were variable but only 48 were parsimony informative. The MP analysis resulted in 10 most parsimonious trees of 84 steps long (CI: 0.75; RI: 0.97). The MP 50% majority rule consensus tree is shown in Figure 27. Bootstrap values ≥ 50 are indicated above the branches. Four clades containing the same taxa as seen in the ITS tree were also recovered

here when using the chloroplast marker J_{SA}. The only exception was *R. nivicola* which was included in Clade 3 instead of Clade 1 (Figure 27, indicated by asterisk). Similar to the nuclear marker, the analysis of chloroplast sequences showed that *R. lyallii*, *R. haastii* and *R. monroi* are not monophyletic. Paraphyly was also detected in one other species; *R crithmifolius*. Finally, unlike what was observed in the analysis of the nuclear marker, *R. verticillatus* and *R. grahamii* formed well supported monophyletic lineages in the cpDNA tree. Although not monophyletic, *R. buchananii* cpDNA haplotypes were highly similar, unlike their corresponding ITS genotypes.

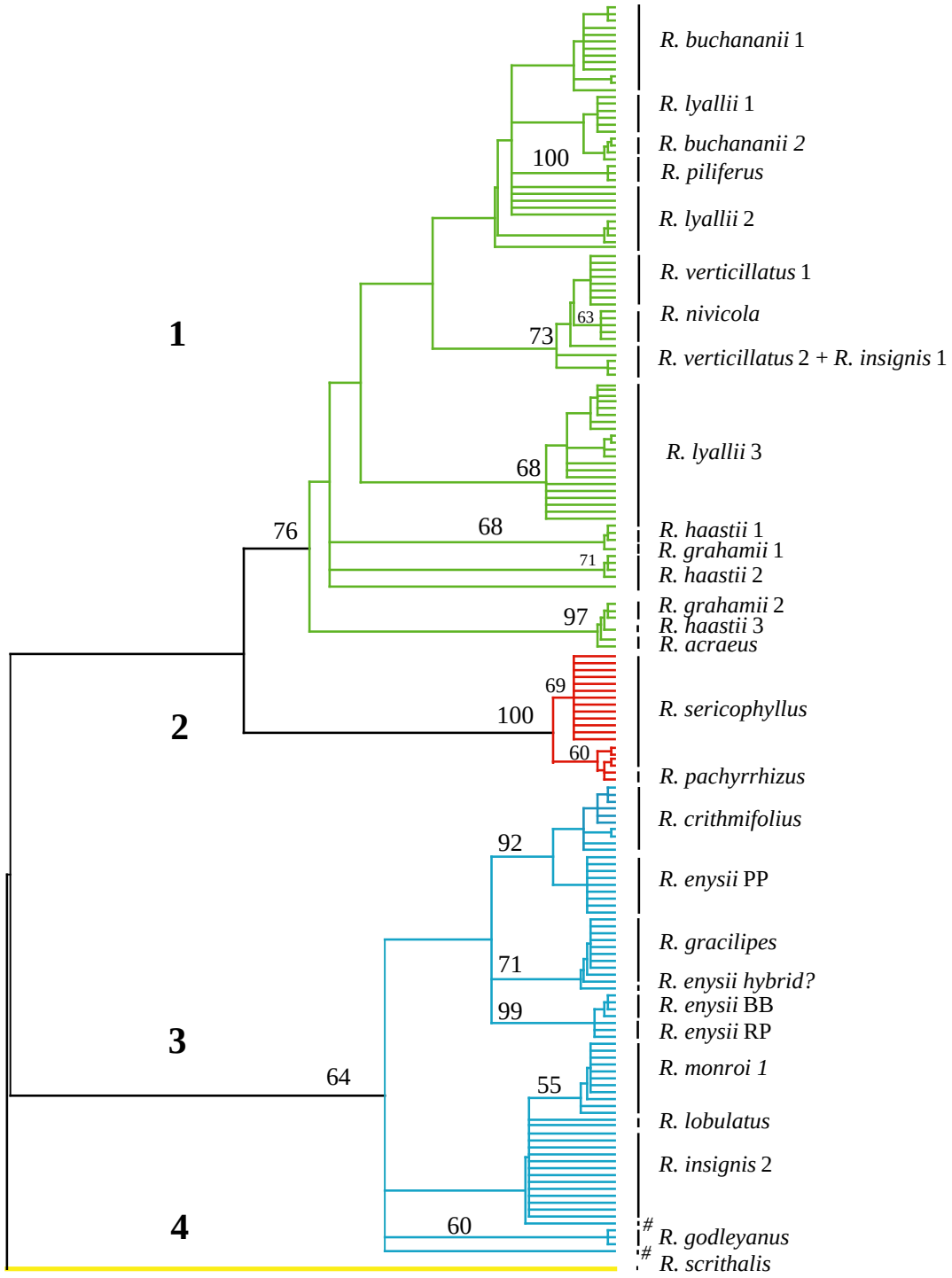


Figure 26: MP tree showing phylogenetic affinities of the New Zealand alpine *Ranunculus* based on nrDNA sequences (ITS). Bootstrap values >50% are indicated above the branches. Main clades are labelled 1, 2, 3 and 4 and highlighted. # indicates two specimens of *R. monroi*. Groups of non-monophyletic species are numbered.

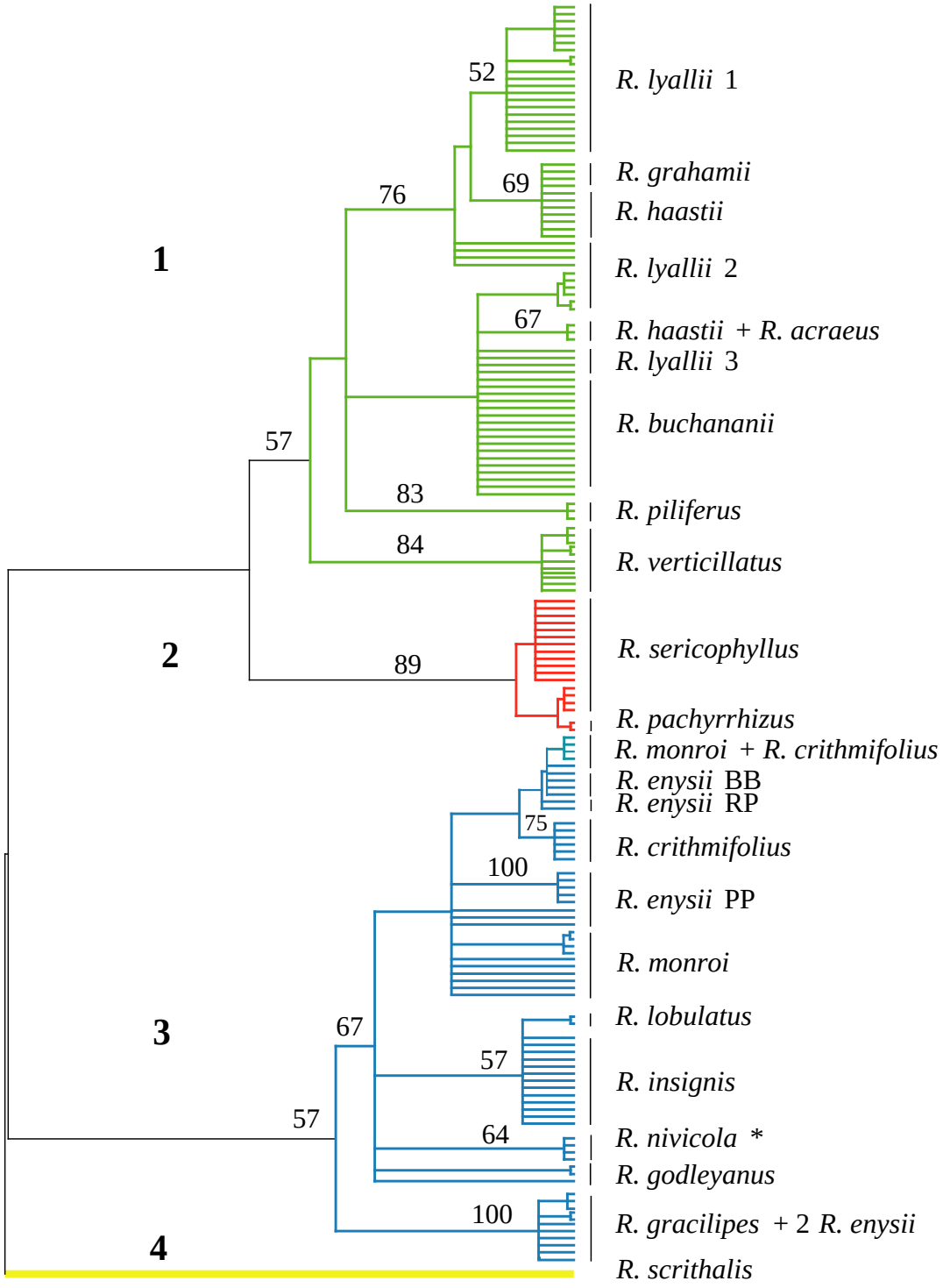


Figure 27: MP tree showing phylogenetic affinities of the New Zealand alpine *Ranunculus* based on cpDNA sequences (J_{SA}). Bootstrap values >50% are indicated above the branches. Main clades are labelled 1, 2, 3 and 4 and highlighted. * *R. nivicola* groups with species of the lineage 1 in the ITS tree in figure 26. Groups of non-monophyletic species are numbered.

Phenotypic diversity

The extent of morphological diversity of the group including vegetative and reproductive characters is represented in Figure 28. The 20 taxa studied are widely spread along the three dimensional space indicating that the extent of morphological diversity in the group is considerable. Separate analyses of this data set, *i.e.*, vegetative characters only or reproductive characters only, showed that morphological diversity of both character sets was equally large. Species appeared widely distributed within the three dimensional space in both MDSC plots (Figure 29A, B).

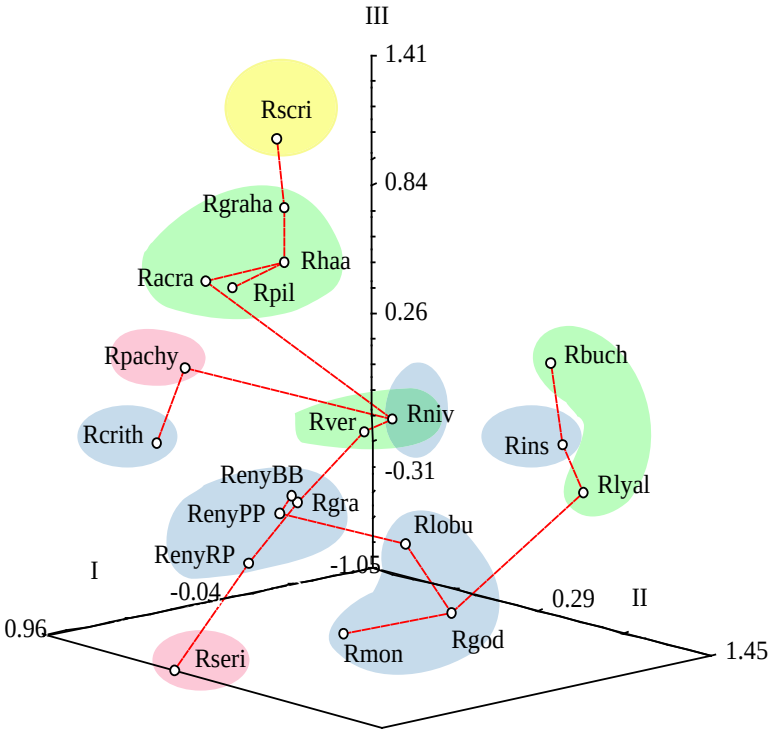


Figure 28: Multidimensional scaling ordination in three dimensions showing the morphological diversity of 20 taxa of alpine *Ranunculus* of New Zealand based on morphological and anatomical characters. Different colour shading indicate the phylogenetic lineage to which species belong according to the results of the ITS and J_{SA} sequence analysis. The Minimum spanning tree is indicated by the red dashed line and indicates proximity between the points. Racra: *R. acraeus*, Rbuch: *R. buchananii*, Rcrith: *R. crithmifolius*, RenyRP: *R. enysii* Rock & Pillar, RenyBB: *R. enysii* Borland Burns, RenyPP: R. *enysii* Porter's Pass, Rgod: *R. godleyanus*, Rgra: *R. gracilipes*, Rgraha: *R. grahamii*, Rhaa: *R. haastii*, Rins: *R. insignis*, Rlobu: *R. lobulatus*, R. lyal: *R. lyalii*, Rmon: *R. monroi*, Rniv: R. *nivicola*, Rpachy: *R. pachyrrhizus*, Rpil: *R. piliferus*, Rscri: *R. scrithalis*, Rseri: *R. sericophyllus*, Rver: *R. verticillatus*.

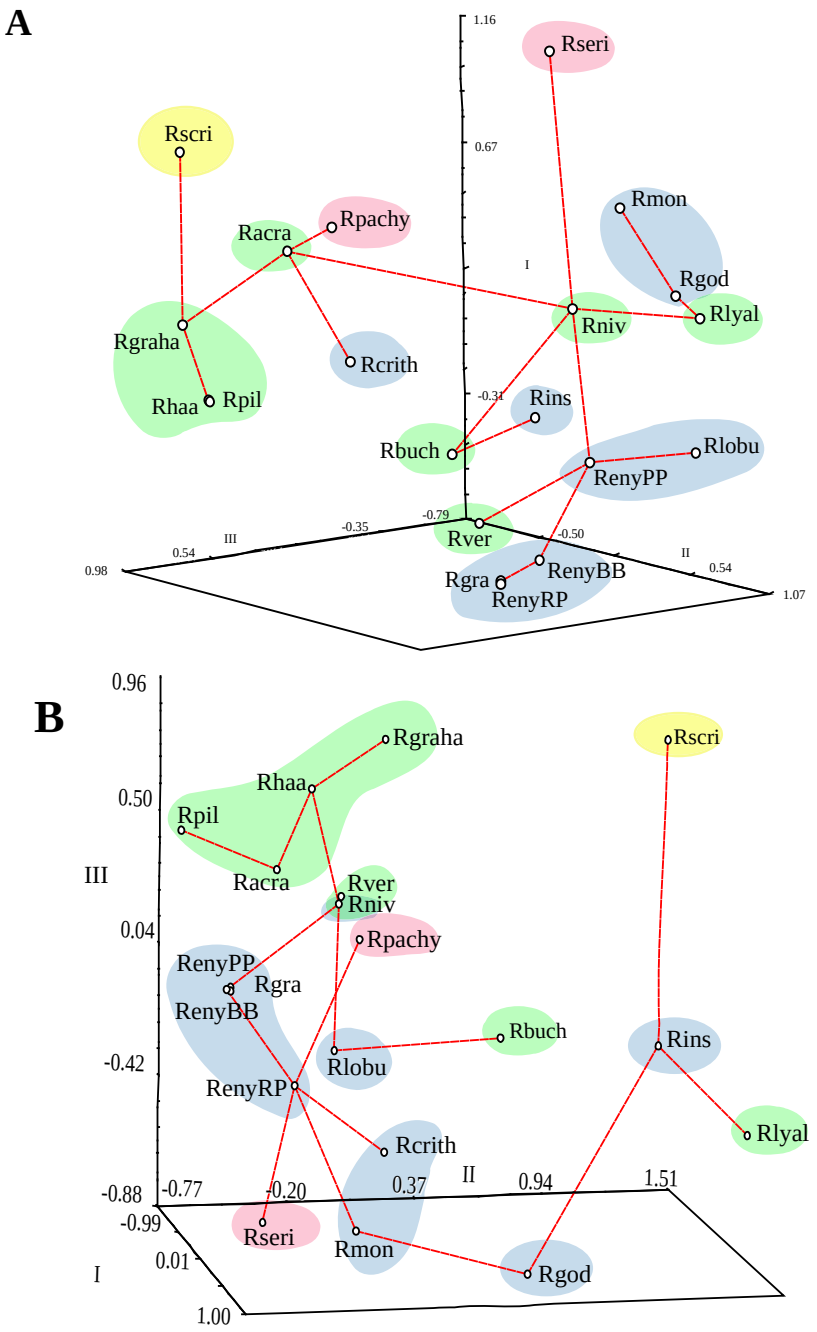


Figure 29: Multidimensional scaling ordination in three dimensions showing the morphological diversity of 20 taxa of alpine *Ranunculus* of New Zealand based on vegetative (A) and reproductive characters (B). Different colour shading indicates the phylogenetic lineage to which species belong according to the results of the ITS and J_{SA} sequence analysis. The Minimum spanning tree is indicated by the red dashed line and indicates proximity between the points. Racra: *R. acraeus*, Rbuch: *R. buchananii*, Rcrith: *R. crithmifolius*, RenyRP: *R. enysii* Rock & Pillar, RenyBB: *R. enysii* Borland Burns, RenyPP: R. *enysii* Porter's Pass, Rgod: *R. godleyanus*, Rgra: *R. gracilipes*, Rgraha: *R. grahamii*, Rhaa: *R. haastii*, Rins: *R. insignis*, Rlobu: *R. lobulatus*, R. lyal: *R. lyalii*, Rmon: *R. monroi*, Rniv: R. *nivicola*, Rpachy: *R. pachyrrhizus*, Rpil: *R. piliferus*, Rscri: *R. scrithalis*, Rseri: *R. sericophyllus*, Rver: *R. verticillatus*.

Phenetic analysis combining both vegetative and reproductive characters using Cluster Analysis showed that morphological similarity of species did not always indicate genetic relatedness as inferred from nITS or J_{SA} sequences (Figure 30). Morphological similarity between species from different lineages was very common. Some examples are the group formed by *R. crithmifolius* (Clade 3), *R. pachyrrhizus* (Clade 2) and *R. scrithalis* (Clade 4). Similarities incude same life form, leaf dissection and achene shape. Another example are *R. buchananii*, *R. lyallii* from Clade 1 and *R. insignis* from Clade 3, all three have similar type of rhizome, same life form and leaf thickening and hairy achenes. Morphological similarity among species genetically close was only apparent in some species (e.g. *R. grahamii*, *R. haastii*, *R. piliferus* and *R. acraeus* all from Clade 1 and the group formed by *R. enysii* and *R. gracilipes* from Clade 3, Figure 30).

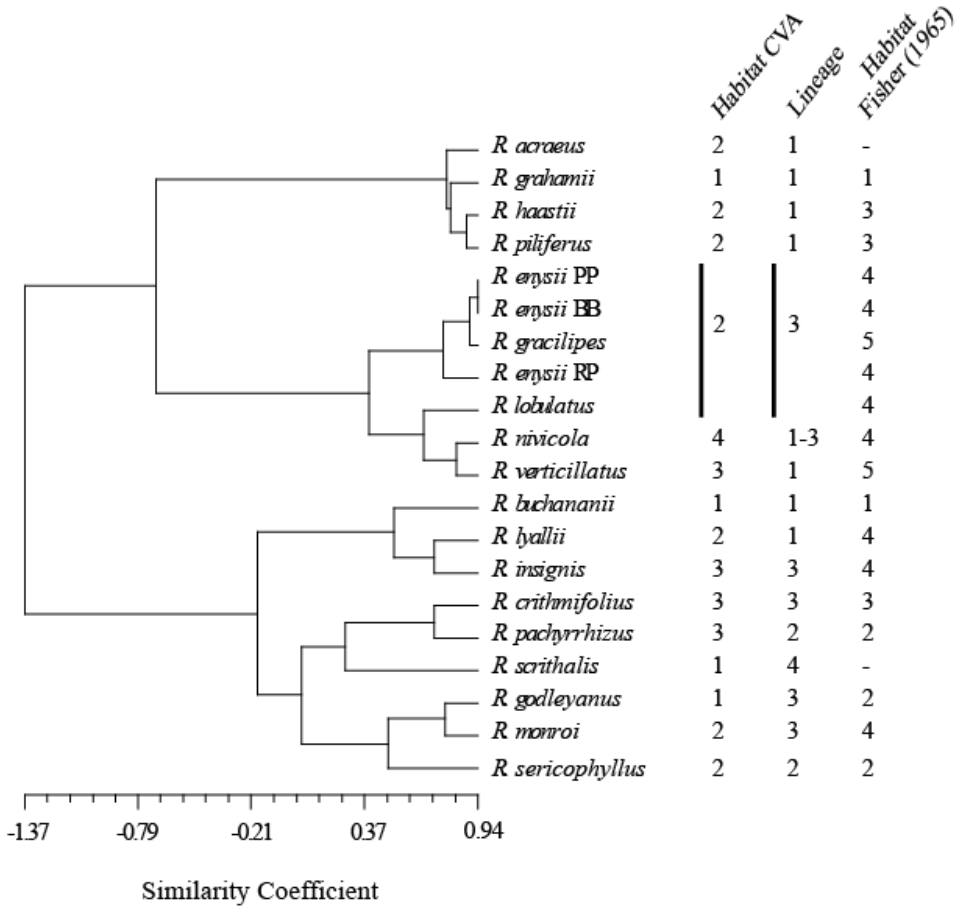


Figure 30: Cluster analysis of 20 alpine *Ranunculus* based on 19 morphological characters. The habitats predicted by the CVA of LENZ variables (habitat 1-4 as in Fig 34) and the genetic lineage inferred from ITS and J_{SA} sequences and the habitats described by Fisher (1: snowfield, 2: snowline fringe, 3: stony debries, 4: sheltered situations, 5: slowly draining habitats).

The diversity of potentially adaptive traits measured in this study was variable. Some traits varied little across the group while others varied considerably (Table 12). At least three life forms were detected in the group; hemicryptophyte, cryptophyte and chamaephyte. Most of the species in the group are hemicritophytes with resting buds at ground level; some examples of this life form are *R. lyallii*, *R. insignis* and *R. gracilipes*. Cryptophytes were represented by 5 species; all of them are rhizomatous species with resting buds below the soil surface and includes species such as *R. haastii* and *R. crithmifolius*. Only *R. pachyrrhizus* was considered a chamaephyte; this life form describes herbaceous plants with creeping or ascending shoots. Root type is correlated with the life forms and two states were detected: long creeping rhizomes and short and erect rootstock. Both states were observed in *R. sericophyllus* depending on plant size.

Leaf characters were the most diverse, particularly leaf shape, leaf texture and the number of cell layers of the palisade mesophyll. Leaf shape varied from peltiform in *R. lyalii* to reniform in *R. insignis* and linear-oblong in *R. gracilipes* (see Figure 31). Leaf texture varied from membranaceous to coriaceous while the number of cell layers in the palisade mesophyll fluctuated from 2, 3 layers to 4 layers. The number of cell layers in the palisade mesophyll was not necessarily related to leaf texture and in species, such as *R. verticillatus* with membranaceous leaves, the palisade mesophyll may contain up to four layers of cells.

Within the reproductive traits studied, nectary gland size and shape was surprisingly diverse (Figure 32). Size of nectary glands varied from *ca*. 300µm (*e.g. R. lobulatus* 13, Figure 32) to *ca*. 3mm in specimens of *R. insignis* (12, Figure 32) from the North Island. At least five types of nectary glands were detected: pocket type (*e.g. R. enysii* BB, *R. gracilipes* 3, 8 & 9 respectively, Figure 32), flap type (*e.g. R. godleyanus*, 7, Figure 32), open type (*e.g. R. lyalii*, 14, Figure 32), horseshoe type (*e.g. R. verticillatus*, 21, Figure 32) and cup type (*e.g. R. scrithalis*, 19, Figure 32). Other floral characters were more constant, *e.g.* flower colour (only 2 species with white flowers) and inflorescence types (solitary or compound cyme).

Achene microsculpture was also variable, especially the size of the carpel cells and the combination of anticlinal and periclinal cell walls (*e.g.* curvature, outline and relief) (Figure 33A-E). Two types of cell shapes were observed in the carpel wall, elongated (e.g. *R. buchananii*, 4, Figure 33A) and irregular (*R. monroi*, 36, Figure 33E). Both cell shapes can be observed in *R. lyalii* (34, Figure 33E). The relief of the cell borders and the curvature of the

periclinal cell walls were correlated and two states were observed, channelled cell boundaries and convex periclinal cell walls (*e.g. R. nivicola*, 26, Figure 33D) or raised cell boundaries and concave periclinal cell walls (*e.g. R. insignis*, 22, Figure 33C).

Taxon	Lineage	Habitat CVA	Characters																		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
R. acraeus	1	2	1	1	1	1	2	1	2	1	1	3	1	1	?	2	1	1	1	1	1
R. buchananii	1	1	2	2	2	1	1	2	1	1	2	3	1	2	3	1	2	1	1	1	1
R. grahamii	1	1	1	1	2	1	2	1	?	1	1	2	1	1	?	2	1	1	2	1	1
R. haastii	1	2	1	1	2	1	2	1	1	1	1	2	1	1	5	2	1	1	1	1	1
R. lyallii	1	2	2	2	6	2	2	2	2	1	2	2	1	2	3	1	2	3	2	2	2
R. piliferus	1	2	1	1	2	1	2	1	1	2	1	3	1	1	5	2	1	1	1	1	1
R. verticillatus	1	3	2	2	2	1	2	3	3	1	1	2	1	1	4	1	1	1	1	1	1
R. pachyrrhizus	2	3	1	1	1	1	2	2	3	2	1	2	1	1	3	1	1	1	1	2	2
R. sericophyllus	2	2	3	2	7	1	3	2	2	2	1	1	2	1	3	1	1	2	1	2	2
R. nivicola	1&3	4	2	2	1	1	2	2	2	1	1	2	1	1	3	1	1	1	1	1	1
R. crithmifolius	3	3	2	1	1	1	2	1	3	2	1	1	1	1	5	1	1	2	2	2	2
R. enysii PP	3	2	2	2	3	1	2	3	2	2	1	1	1	1	1	1	1	1	1	1	1
R. enysii RP	3	2	2	2	5	1	2	3	1	2	1	1	1	1	1	1	1	1	1	2	2
R. enysii BB	3	2	2	2	3	1	2	3	1	2	1	1	1	1	1	1	1	1	1	1	1
R. godleyanus	3	1	2	2	4	2	2	2	2	1	1	1	1	2	2	1	2	2	1	2	2
R. gracilipes	3	2	2	2	5	1	2	3	1	2	1	1	1	1	1	1	1	1	1	1	1
R. insignis	3	3	2	2	2	2	1	2	1	1	1	2	1	2	3	1	2	1	2	2	2
R. lobulatus	3	2	2	2	3	2	2	3	2	1	1	1	1	1	5	1	2	1	1	1	1
R. monroi	3	2	2	2	4	2	1	1	2	1	1	1	1	1	5	1	1	2	1	2	2
R. scrithalis	4	1	1	1	7	1	1	1	?	2	1	2	1	2	5	1	2	?	?	?	?

Table 12: Morphological and anatomical character and character-states recorded from 20 taxa of alpine *Ranunculus* of New Zealand. **1** Rhizome habit, **2** Life form, **3** Leaf outline, **4** Leaf dissection, **5** Leaf`hairs, **6** Leaf thickness, **7** Palisade cells, **8** Inflorescence type, **9** Flower colour, **10** Number of petals, **11** Number of nectary glands, **12** Nectary shape, **13** Achene hairs, **14** Achene body, **15** Style length, **16** Shape cells, **17** Outline anticlinal walls, **18** Relief cell border, **19** Curvature outer cell wall. For character states refer to Table 12. ?: missing. Lineage according to the analysis of ITS and J_{SA} sequences and habitats type from CVA.

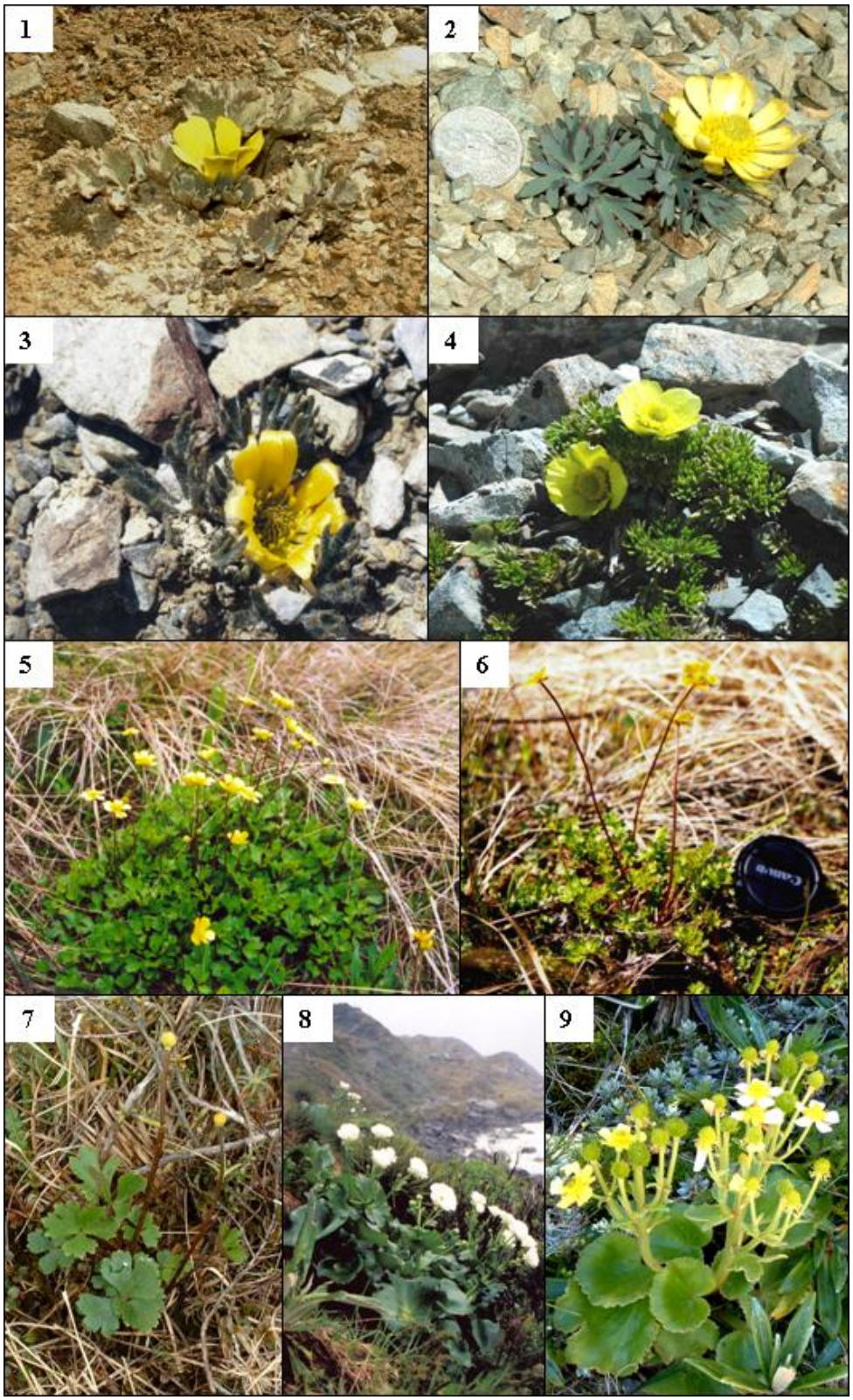


Figure 31: Diversity in plant habit observed within the New Zealand alpine *Ranunculus*. (1: *R. crithmifolius*, 2: *R. haastii*, 3: *R. scrithalis*, 4: *R. sericophyllus*, 5: *R. enysii*, 6: *R. gracilipes*, 7: *R.verticillatus*, 8: *R. lyallii*, 9: *R. insignis*)

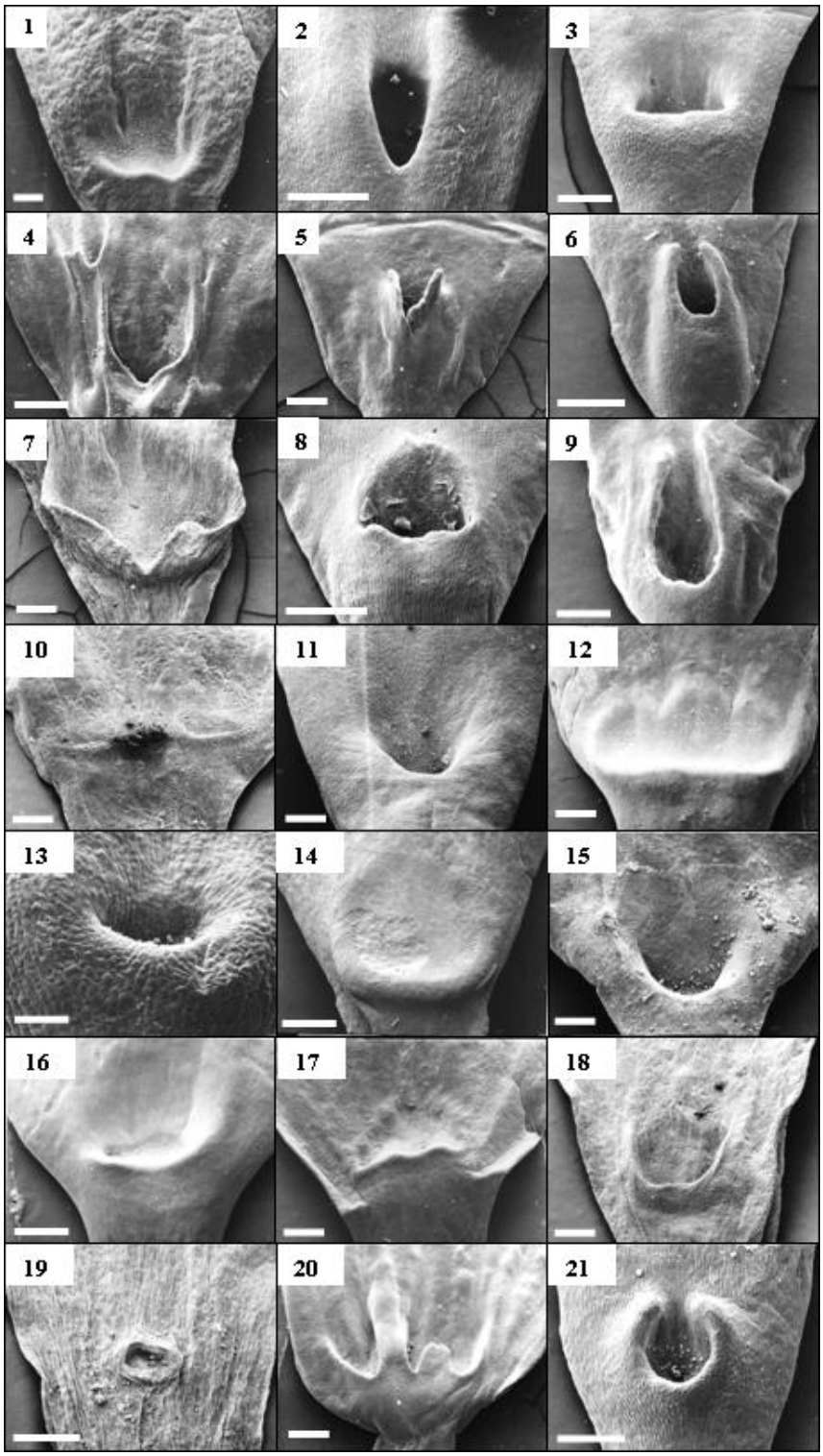


Figure 32: SEM micrographs showing the diversity of nectary gland shape and size of New Zealand alpine *Ranunculus*. Scale bar represents 500µm in all figures except for 13 (150µm) and 21 (250µm).1: *R. buchananii*, 2: *R. crithmifolius*, 3: *R. enysii* BB, 4-6: *R. enysii* PP, 7: *R. godleyanus*, 8-9: *R. gracilipes*, 10: *R. grahamii*, 11: *R. haastii*, 12: *R. insignis*, 13: *R. lobulatus*, 14: *R. lyallii*, 15: *R. monroi*, 16: *R. nivicola*, 17: *R. pachyrrhizus*, 18: *R. piliferus*, 19: *R. scrithalis*, 20: *R. sericophyllus*, 21: *R. verticillatus*.

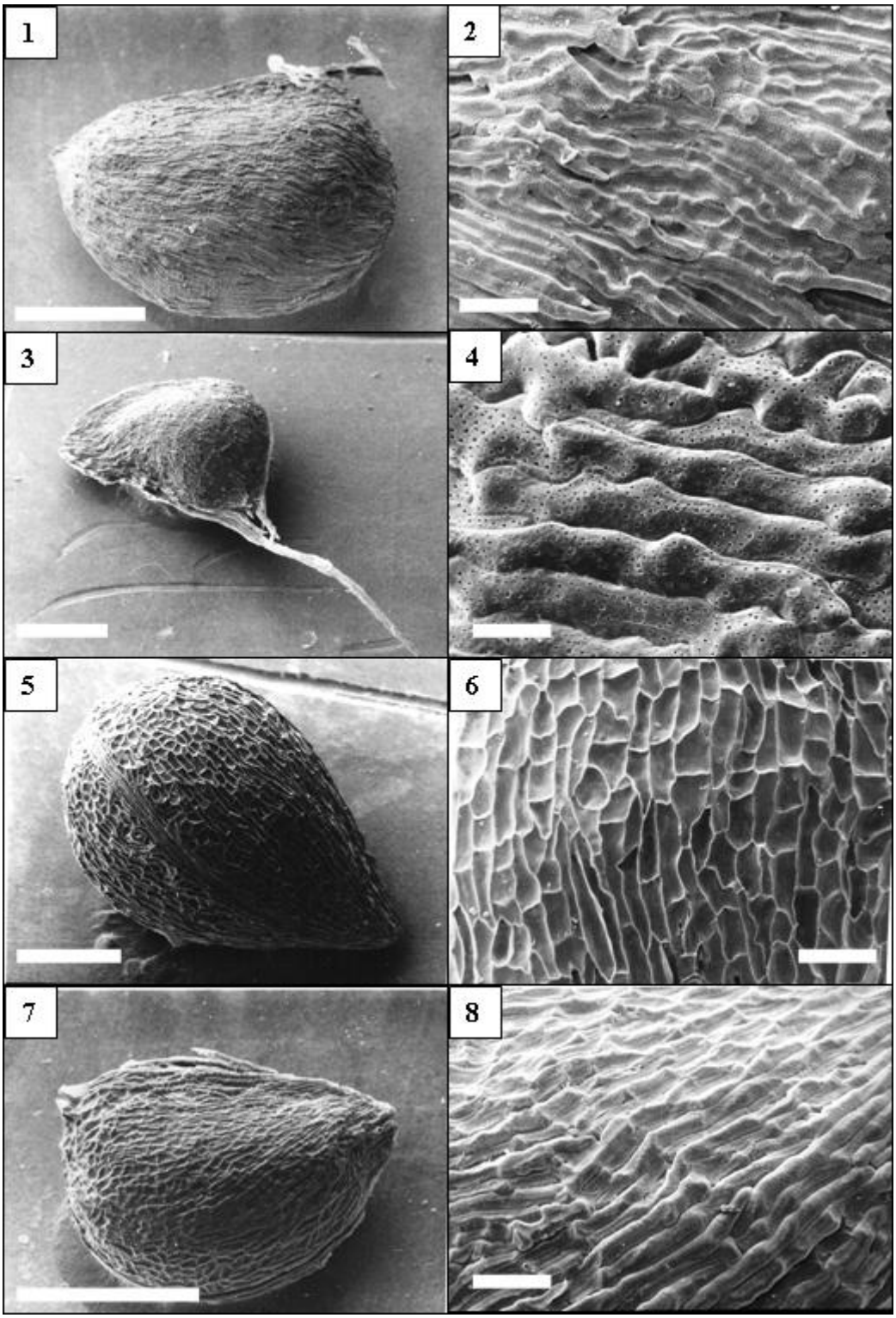


Figure 33A: Achene SEM micrographs of New Zealand alpine *Ranunculus* and detail of the carpel wall cells. 1 & 2: *R. acraeus*, 3 & 4: *R. buchananii*, 5 & 6: *R. crithmifolius*, 7 & 8: *R. enysii* Borland Burns. Scale bars indicate 1mm in panoramic micrographs, 40µm in 4, 100µm in 2 & 8 and 200µm in 6.

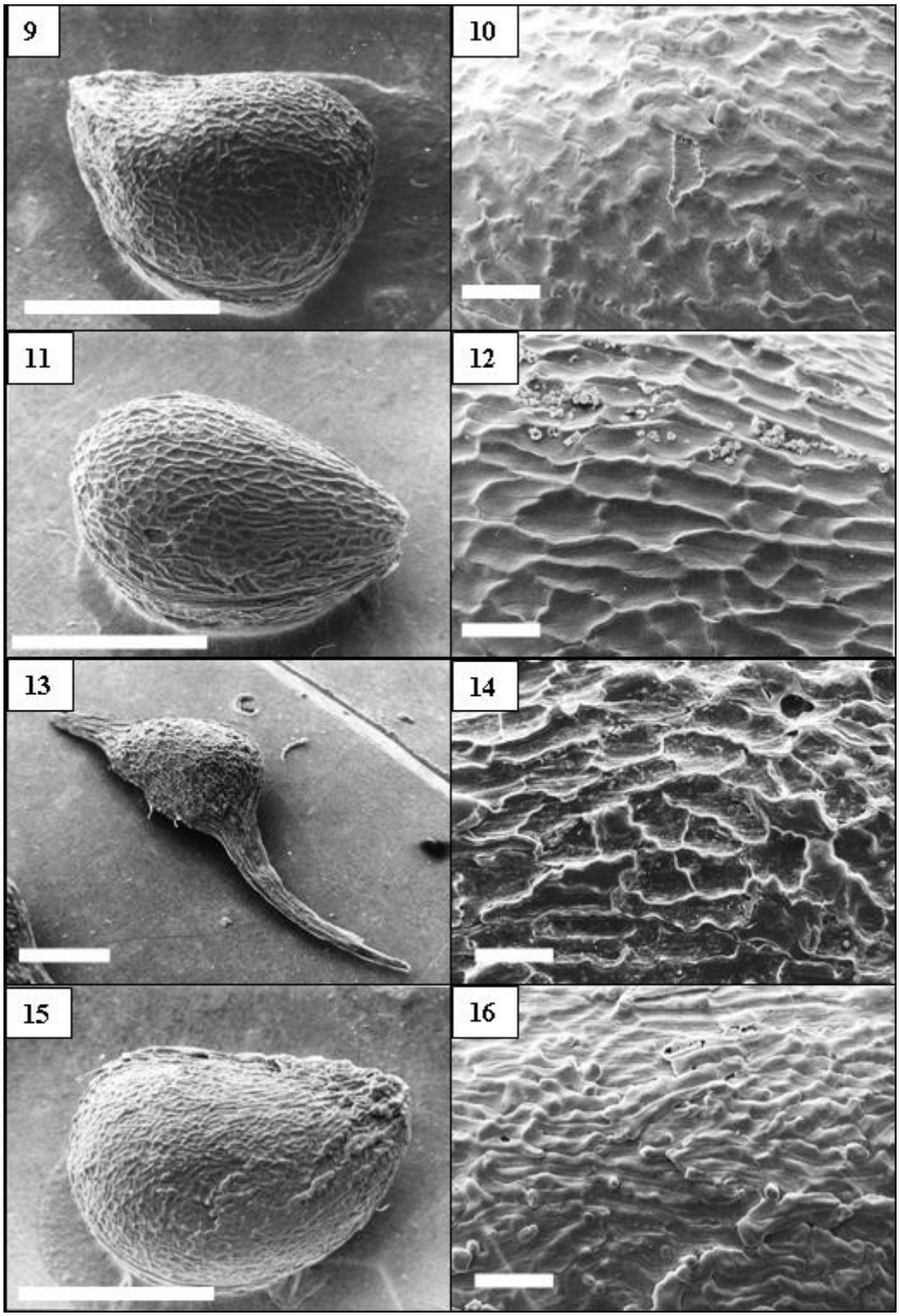


Figure 33B: Achene SEM micrographs of New Zealand alpine *Ranunculus* and detail of the carpel wall cells. 9 & 10: *R. enysii* Porter Pass, 11 & 12: *R. enysii* Rock and Pillar, 13 & 14: *R. godleyanus*, 15 & 16: *R. gracilipes*. Scale bars indicate 1mm in panoramic micrographs and 100µm in close ups.

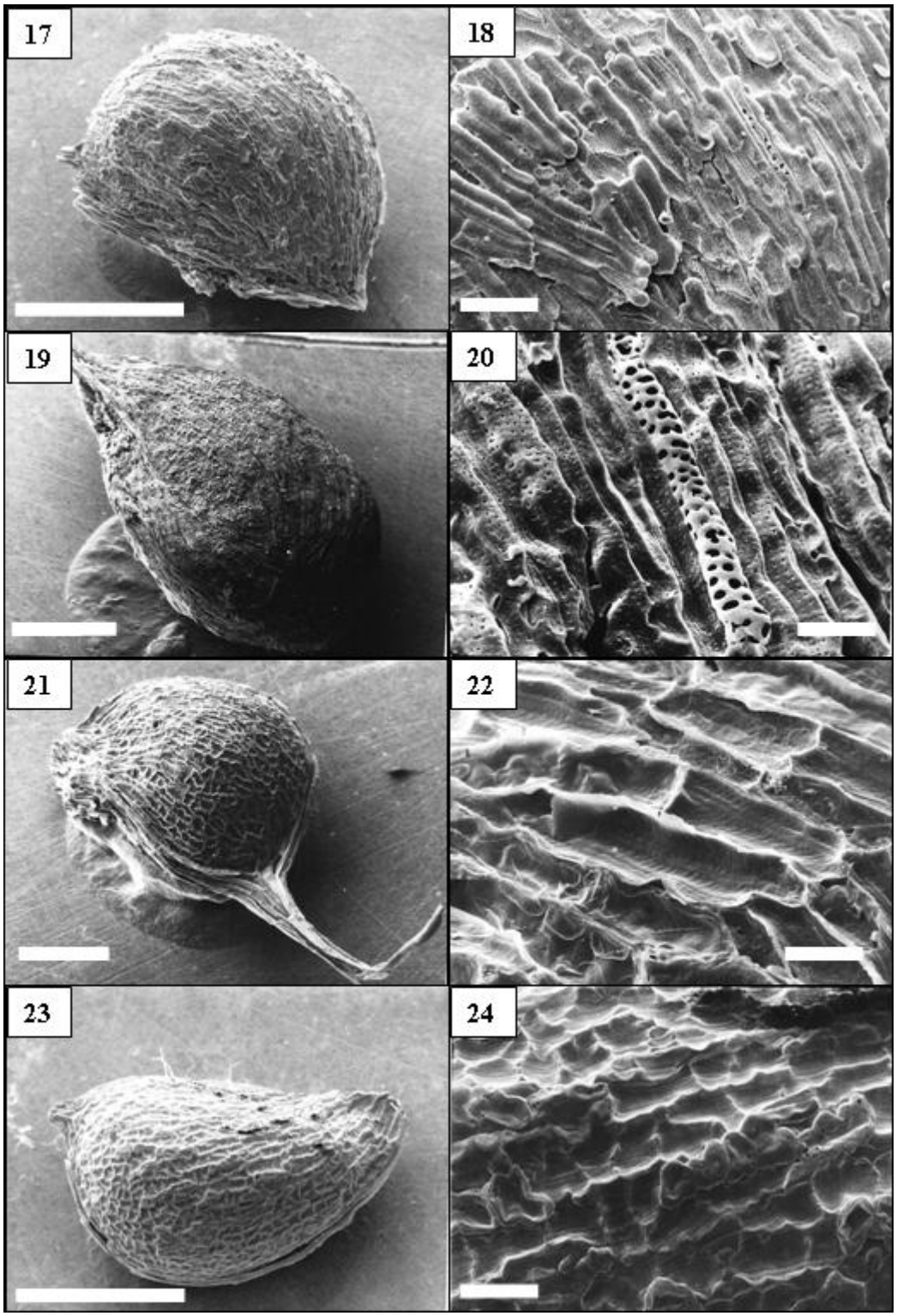


Figure 33C: Achene SEM micrographs of New Zealand alpine *Ranunculus* and detail of the carpel wall cells. 17 & 18: *R. grahamii*, 19 & 20: *R. haastii*, 21 & 22: *R. insignis*, 23 & 24: *R. lobulatus*. Scale bars indicate 1mm in panoramic micrographs and 40µm in 20 & 22 and 100µm in 18 and 24.

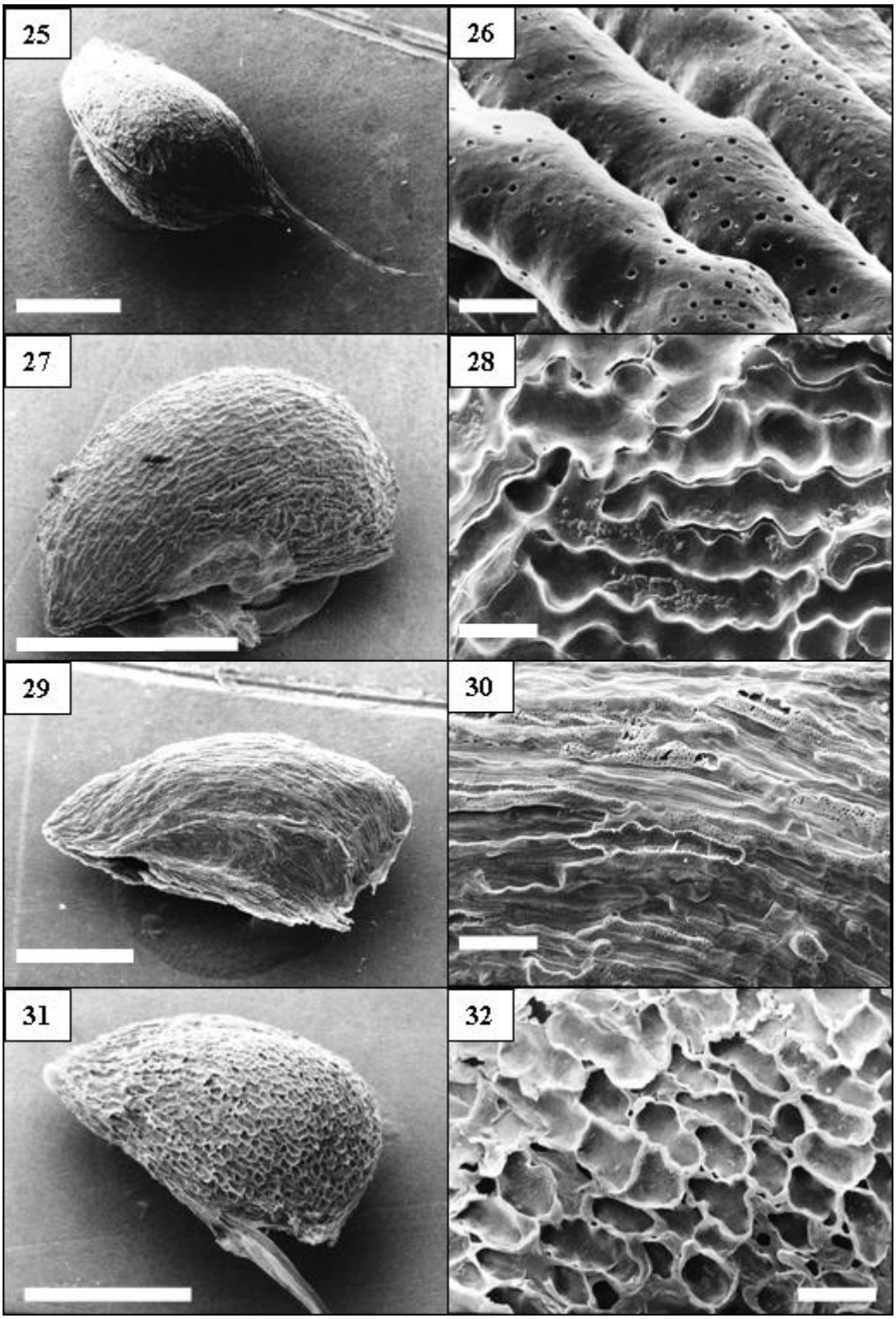


Figure 33D: Achene SEM micrographs of New Zealand alpine *Ranunculus* and detail of the carpel wall cells. 25 & 26: *R. nivicola*, 27 & 28: *R. pachyrrhizus*, 29 & 30: *R. piliferus*, 31 & 32: *R. sericophyllus*. Scale bars indicate 1mm in panoramic micrographs and 20µm in 26, 40µm in 28 & 30 and 100µm in 32.

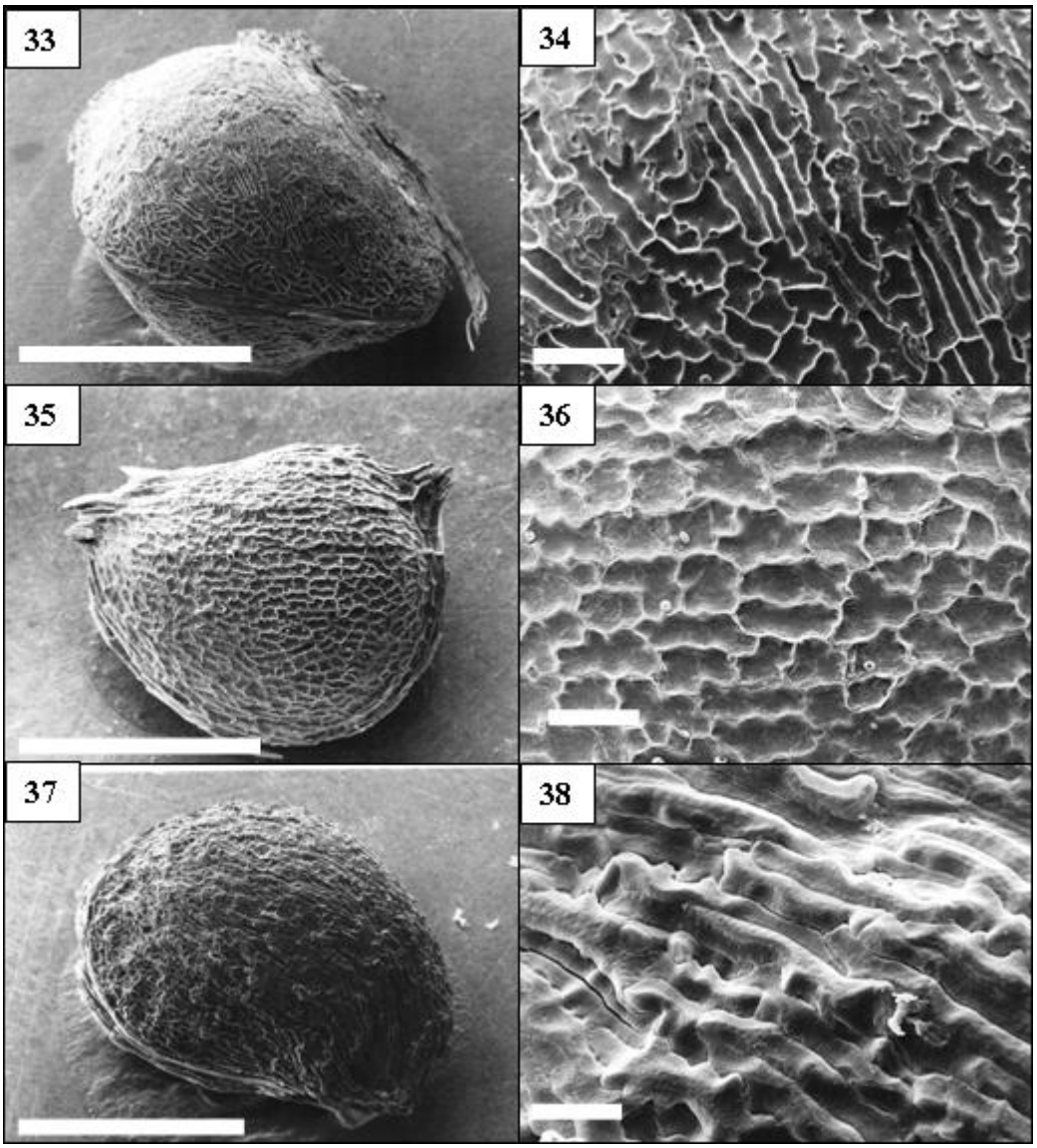


Figure 33E: Achene SEM micrographs of New Zealand alpine *Ranunculus* and detail of the carpel wall cells. 33 & 34: *R. lyallii*, 35 & 36: *R. monroi*, 37 & 38: *R. verticillatus* Scale bars indicate 1mm in panoramic micrographs, 40µm in 38 and 100µm in 34 and 36.

Habitats distinguished by LENZ

The first and second canonical variates accounted for 54% and 15.5% of the total environmental variation, respectively. The analysis showed that the sites occupied by each species are quite variable (open circles in Figure 34). However, the similarity of mean habitat values for each taxon (represented by the species centroids in Figure 34), suggests four distinct habitat types. The first canonical variates function distinguishes habitats 2, 3 and 4 and it is correlated with variables solar radiation and temperature. The second axis distinguishes between the habitats 1 and 2 and is strongly correlated with the variables of October vapour pressure and water balance ratio (Table 13). Habitat 1 contains five species; three species from Clade 1 (*R. grahamii*, *R. buchananii* and *R. lyallii*), one species from Clade 3 (*R. godleyanus*) and one (*R. scrithalis*) from Clade 4. Habitat 2 includes most taxa from Clade 3 (*R. lobulatus*, *R. monroi*, *R. gracilipes*, *R. crithmifolius* and the three forms of *R. enysii*), two species from Clade 2 (*R. pachyrrhizus* and *R. sericophyllus*) and three species from Clade 1 (*R. piliferus*, *R. acraeus* and *R. haastii*). Habitat 3 contains taxa that occur in both North and South Island, *i.e., R. insignis* from Clade 3 and *R verticillatus* from Clade 1. Habitat 4 is represented by one species, the allopolyploid species *R. nivicola*, endemic to the North Island.

Phenotypes are not explained by LENZ habitats

The overall phenotypic similarities observed between some taxa and represented by the MDSC clusters in Figure 28 showed no correspondence to any of the four distinct habitats. Three of the four habitats identified by the CV analysis are occupied by species representing different morphological groups (Figure 34). For example, habitat 1 is occupied by species representing morphological group A: *R. grahamii*, Group D: *R. buchananii* and *R. lyallii*, Group E: *R. scrithalis* and Group F: *R. godleyanus*. A similar pattern can be observed for habitat 2 and 3 (Figure 34). The lack of correspondence between habitat type and morphology was also evident when reproductive and vegetative characters were considered separately (Appendix 7). When each character was analysed individually, similar results were obtained (Table 12). A lack of correspondence between habitat and phenotype was also observed when using the habitat types described by Fisher (1965) (Figure 30).

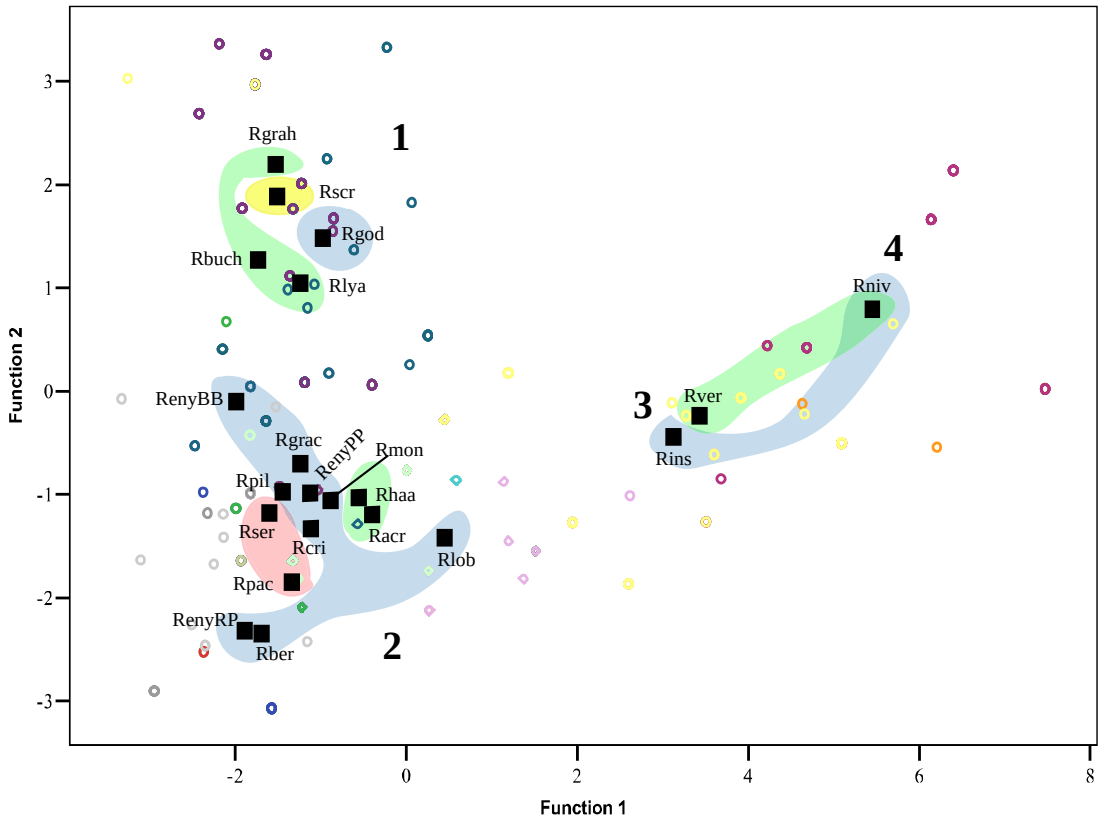


Figure 34: Canonical variates analysis of 16 environmental variables obtained from LENZ indicating the habitats occupied by 20 *taxa* of New Zealand alpine *Ranunculus*. Function 1: 54%; Function 2: 15.5%). Habitats detected by LENZ are indicated by numbers 1, 2, 3 and 4. Shading indicates the different morphological groups. Rscri: *R. scrithalis*, Rgraha: *R. grahamii*, Rhaa: *R. haastii*, Rpil: *R. piliferus*. Racra: *R. acraeus*, Rpachy: *R. pachyrrhizus*, Rcrith: *R. crithmifolius*, Rver: *R. verticillatus*, Rniv: R. *nivicola*, Rgra: *R. gracilipes*, RenyBB: *R. enysii* Borland Burns, RenyPP: R. *enysii* Porter's Pass, RenyRP: *R. enysii* Rock & Pillar, Rseri: *R. sericophyllus*, Rmon: *R. monroi*, Rlobu: *R. lobulatus*, Rgod: *R. godleyanus*, Rins: *R. insignis*, Rbuch: *R. buchananii*, R. lyal: *R. lyalii*.

DISCUSSION

The lack of correspondence between phenotype and habitat observed in this plant group might be explained in a number of ways; (i) the New Zealand alpine *Ranunculus* radiation is not adaptive, or (ii) alternatively there is adaptation of phenotypes but not those phenotypes measured here, and/or (iii) the data layers in LENZ do not capture the aspects of habitat most important for explaining ecological divergence of these species. A number of observations are relevant to interpreting these possibilities.

i) Evidence of adaptation in the New Zealand alpine *Ranunculus* radiation

As previously mentioned, a feature of the New Zealand alpine *Ranunculus* group is its considerable morphological diversity and low level of genetic divergence at neutral gene loci. Furthermore, within phylogenetic clades, there are dramatic changes in phenotypes suggesting that phenotypic evolution is not tightly constrained by phylogenetic affinity. Considerable phenotypic divergence among species genetically close has also been detected in other New Zealand alpine plant groups such as *Myosotis* (Winkworth *et al.* 2002), *Hebe* (Wagstaff & Garnock-Jones 1998), the Gnaphalieae (Breitwiesser *et al*. 1999) and *Anisotome* and *Acyphylla* (Winkworth 2000). Similar to the alpine *Ranunuclus*, morphological diversification in these plant groups is considerable and it has been accompanied by the development of strong ecological preferences, suggesting their radiation might also be adaptive.

Members of the alpine *Ranunculus* radiation may be found in at least five vegetation types in the New Zealand alpine system; subalpine scrub, tussock-herbfield, scree, fellfield and snowbanks (Mark & Adams 1986). Three of these habitats are shown in Figure 35. Species from all four lineages have independently colonised these niches, except for the sister species *R. pachyrrizus* and *R. sericophyllus* that are generally restricted to snowbanks. Colonisation of these vegetation types has corresponded with the parallel evolution of similar life forms in species from different lineages but occupying the same habitats; *e.g.* hemicryptophytes such as *R. lyallii*, *R. verticillatus* and *R. godleyanus* are found in scrub, tussock-herbfield and fellfields respectively. Cryptophytes such as *R. haastii* and *R. scrithalis* are found in scree and chamaephytes such as *R. pachyrrhizus* in the snowbanks. Colonisation of these niches also appears to be correlated with diversification of the root system of these species, *e.g.* scree specialist such as *R. haastii* possess a shallow woody creeping rhizome with long straight roots branching from it radially, *R. pachyrrizus* found in snowbanks has creeping or

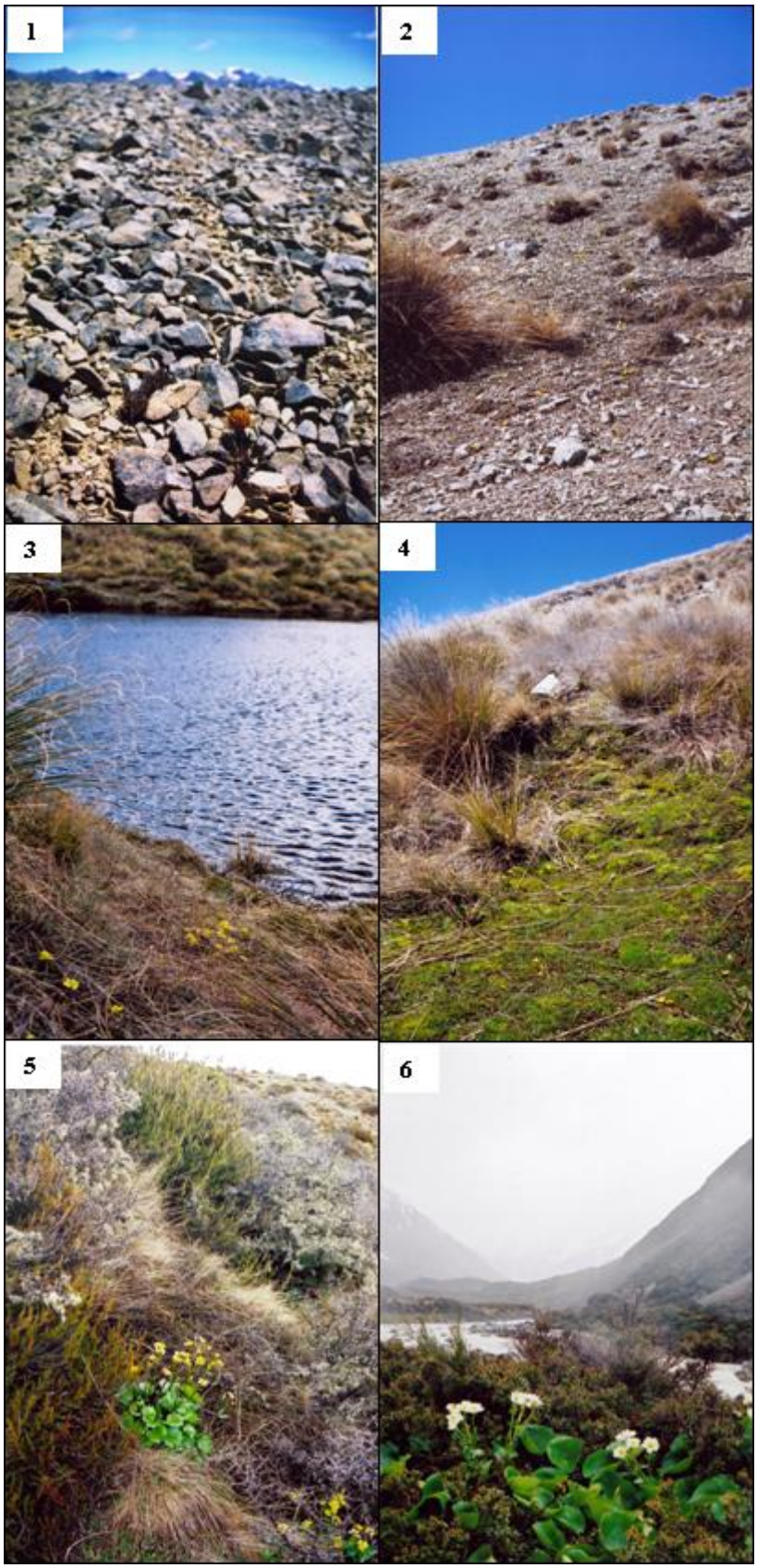


Figure 35: Habitats occupied by a selected group of alpine *Ranunculus* in the South Island. Scree (1: *R. haastii*, 2: *R. crithmifolius*), tussock-herbfield (3: *R. enysii* BB, 4: *R. gracilipes*), scrub (5: *R. lobulatus*, 6: *R. lyalii*).

ascending shoots and *R. nivicola* found in tussock-herbfield possess short and erect rootstock. Leaf dissection shows a similar pattern and species with entire leaves are found in scrub and those with dissected leaves are generally found in tussock-herbfields, scree and snowbanks. Correlation between niche and leaf texture is also noticeable. Most of the scree specialists have coriaceous leaves, snowbank and fellfield species have fleshy leaves, while scrub and tussock-herbfield species possess membranaceous leaves. Similar observations were made by Fisher (1965) and confirmed by Lockhart *et al.* (2001), who studied phylogenetic relationships within the group. These and the above observations support the inference of the New Zealand alpine *Ranunculus* radiation being adaptive. An association between phenotype and habitat, however, was not demonstrated in this study when using the LENZ database. However, a number of observations caution against over interpreting the findings from LENZ (described in the next section).

ii) Characters of adaptive significance

There is strong evidence for the adaptive significance of most of the phenotypic traits studied in the present work (van de Pijl 1973, Stebbins 1974, Barthlott 1981, Werker 2000). Hence, it was perhaps surprising that there was no association between these characters and the habitats detected by LENZ. Although it is possible the traits, while adaptive in other plant taxa are non-adaptive in New Zealand *Ranunculus*. Future studies of the alpine *Ranunculus* should explore a different set of traits and include anatomical and physiological characters. These might be important as it has been reported that adaptive radiations may only involve a few characters (Givnish 1998) and these may not be always conspicuous, *e.g*. tissue elastic properties in the Hawaiian silversword alliance (Robichaux & Canfield 1985) or trichome morphology for nutrient uptake in the bromeliads of the genus *Brocchinia* (Givnish *et al*. 1997).

iii) The limitation of LENZ in describing the habitats occupied by this radiation

Describing plant habitats using environmental variables from GIS databases is not new and such an approach has been used before to describe ecological diversification in members of an adaptive radiation in Hawaii (Friar *et al.* 2006). In New Zealand, the LENZ database has been successfully used to examine the effect of environment in the disjunct distribution of threatened species (Rogers & Walker 2005) and the loss of indigenous habitats (Walker *et al*. 2006). Recently, it has also been used to explore the role of the environment in the distribution of three alpine *Ranunculus* species in the North Island (Carter 2007), but unlike

the previous studies, this author questions the resolution of LENZ. The failure of the LENZ database to detect fine-scale habitat variation and the loss of resolution after extrapolating the data may be directly linked with the lack of habitat-phenotype correlation observed in this study. Climate layers used in LENZ are derived either directly or indirectly from mathematical surfaces that use information about the climate, location and elevation of a number of meteorological stations across New Zealand and the data for each variable at a specific location is predicted from the closest stations. These ideas are further supported by the failure of LENZ to detect highly distinctive habitats such as those occupied by the scree specialist species *R. haastii* (Fisher 1952).

The type of environmental parameters available from LENZ could have also influenced the results of this study. The variables included in LENZ mainly described climatic variables at a broader scale within a region. Parameters such as soil type, vegetation cover and variables such as pH, fertility and salinity indicators or other micro-habitat measurements are not available and they may provide a better characterization of the different habitats these *Ranunculus* species occupy. The role of fine-scale habitat variation has been recently described as an important speciation driver in the aizoaceous genus *Argyroderma* in South Africa (Ellis & Weis 2006). In this genus, colonisation of different edaphic microenvironments has been accompanied by morphological diversification of potentially functional traits. A similar example is represented by the radiation of members of the Proteaceae family in South Africa (Johnson 1996, Linden 2003). Soil conditions have been considered important in the radiation of the New Zealand species of *Pachycladon* (Heenan & Mitchell 2003) and species of *Helianthus* (Sambatti & Rice 2006). Unfortunately, a detailed micro-habitat description is lacking for the New Zealand *Ranunculus*, and this lack of knowledge identifies an important direction for future studies.

Vegetative and reproductive characters

It is also interesting to point out that morphological divergence in alpine *Ranunculus* is equally diverse when vegetative and reproductive character sets were analysed separately. Generally, determinants of speciation in plants are reflected in the patterns of radiation of their vegetative and floral character sets (Carson 1985). Thus, diversification of vegetative characters is expected to be greater than the diversification of reproductive characters when abiotic ecological conditions such as soil types and climate have promoted speciation in the group (Schluter 2000). Conversely, when a plant group's speciation has been promoted by

plant-pollinator interactions, vegetative characters are expected to be more stable. Well known examples are the orchid family or the columbines, which have radiated mainly in floral characters while their vegetative characters, in contrast, are exceptionally constant (Stebbins 1954, Johnson 1996, Hodges & Arnold 1995). The diversity in floral characters observed in the alpine *Ranunculus* such as inflorescence arrangement and nectary gland shape in particular (see Figure 32) is remarkable. Diversification of these floral traits does not comply with Carson (1985) and Schluter (2000) because it has been suggested that *Ranunculus* species are generally pollinated by unspecialised pollinators (Pellmyr 1995). In fact, many of the New Zealand alpine *Ranunculus* are visited by generalist pollinators such as syrphid flies (Fisher 1965). It is likely that floral diversification in this group has followed the diversification of vegetative characters after the species has established and adapted to their new habitats; diversification of reproductive characters has probably evolved as a mechanism of reproductive isolation. If this is true, the effectiveness of such a mechanism is worth testing, particularly when pollination systems and pollinators have been described as generalist and unspecialised in New Zealand (see review by Newstrom & Robertson 2005).

Summary

The comparative approach taken in this study using molecular, morphological and environmental data has provided an initial insight into the extent of genetic, morphological and habitat diversity of the New Zealand alpine *Ranunculus* and provided the first objective test of the adaptive nature of this radiation. However, while the extent of morphological diversity in vegetative and reproductive character sets studied here and the evolution of similar morphologies in similar habitats suggest that abiotic and biotic interactions have played an important role in the diversification of this group clear correspondence between habitat and phenotype was not demonstrated using LENZ. Future studies including a different range of anatomical characters of putative adaptive value, measurement of eco-physiological and reproductive performance, differential gene expression and a detailed fine-scale habitat characterisation for each species across their distribution ranges are needed to further test the importance of ecological drivers for explaining the nature of this radiation.

V

Concluding remarks

Understanding the origin of New Zealand biota has been a topic of much interest for biogeographers (Raven 1973, Wardle 1978, Pole 1994, Macphail 1997, Winkworth *et al.* 2000, Winkworth *et al.* 2005, Trewick *et al*. 2007). The use of molecular techniques to decipher the phylogentic affinities of New Zealand plants has provided valuable information about the origin, colonisation dynamics and evolution of New Zealand flora (*e.g.* Winkworth *et al.* 2000, Lockhart *et al.* 2001, Winkworth *et al.* 2005). Until now, most of these studies have focused on small plant groups with representatives mainly found in Australasia and South America (*e.g*. Wagstaff *et al.* 2000, Wagstaff & Wege 2002, Smissen *et al.* 2003, Wagstaff 2004, Jakubowsky *et al.* 2005, Meudt & Simpson 2006, Knapp 2007). Unlike these plant groups, the genus *Ranunculus* comprises about 600 species and has a worldwide distribution (Tamura 1995, Hörandl *et al.* 2005, Paun *et al.* 2005) which increases the opportunity to uncover diverse patterns of colonisation and speciation.

The phylogenetic study of New Zealand *Ranunculus* reported in this thesis suggests affinities of New Zealand species with species from Australia, the Northern Hemisphere, southern South America and islands in the Southern Ocean. An important conclusion is the inference of multiple events of dispersal between New Zealand and other landmasses. This finding highlights the importance of long distance dispersal in the colonisation of the New Zealand archipelago by *Ranunculus* and the importance of dispersal in the origins of New Zealand and other Southern Hemisphere floras (*e.g.* Lockhart *et al.* 2001, Winkworth et al. 2002; Winkworth *et al.* 2005, Meudt & Simpson 2006, Knapp *et al*. 2007, and others). This biogeographic pattern of many events of transoceanic dispersal involving a single genus has not been reported for other members in the *Ranunculaceae* (see Schuettpelz *et al.* 2002, Schuettpelz & Hoot 2004) bu in other plant groups from New Zealand (*e.g*. Lloyd *et al*. 2006, Wagstaff *et al*. 2006). Unpublished results for other worldwide distributed genera in New Zealand are consistent with this pattern (Richard Gardner, pers. comm.). It is likely that the phylogenetic study of other speciose and worldwide distributed groups found in New Zealand such as *Carex*, *Uncinia* and the *Poaceae* family might show similar patterns as those observed in *Ranunculus* (Kerry Ford, pers. comm).

Results reported in this thesis also suggest that the ancestor of a number of sub-alpine and alpine New Zealand *Ranunculus* have arrived from the Northern Hemisphere using southern Asia, New Guinea and Australia as "stepping stones" as hypothesised by Raven (1972). However, there is also evidence to support (and insufficient evidence to reject) an hypothesis

for direct long distance dispersal of *Ranunculus* from the Northern Hemisphere to New Zealand and Australia on several occasions. Events of dispersal from New Zealand to Australia and vice versa were also inferred from this study and they support previous claims of dispersal against the predominant circumpolar westerly winds by Lockhart *et al*. (2001).

Patterns of morphological and genetic variation observed among a selected group of New Zealand alpine *Ranunculus* suggest that hybridisation, and possibly introgression, have greatly contributed to the evolution and ecology of the group. Such suggestions are not new (see Fisher1965; Lockhart *et al.* 2001), however, the phylogenetic analyses of a much larger number of samples reported in this thesis, which confirm the clade structure and geographic distributions of genotypes previously noted in Lockhart *et al*. (2001), give weight to these suggestions. It is possible that hybridisation has been important both for the origins of some of the *taxa* studied here and the morphological variability observed across their distribution ranges (*e.g*. *R.enysii s.l. and R. insignis s.l.*). Further work, however, is still needed to test these hypotheses. Analyses with genome wide markers and the recent development of a parametric test to distinguish hybridisation from lineage sorting (Joly *et al*. submitted) should help in this respect.

Environmental changes during the Pleistocene are believed to have played an important role in determining distribution and speciation patterns in the evolution of New Zealand flora in general (Wardle 1963). It has been hypothesised that during this period glaciations fragmented the distribution range of many species and the surviving populations were confined to isolated ice-free refugia such as mountain tops (Lockhart *et al.* 2001, Heenan and Mitchell 2003; Winkworth *et al.* 2005). It is likely that several species, otherwise allopatric, were confined to similar ice-free areas facilitating gene flow between them. If this is so, some lineages such as those currently observed in *R. enysii* may be the outcome of ancient hybridisation events. Although hybridisation events may be readily detected using molecular markers, dating such events is more problematic (Comes & Kadereit 1998).

Similar to other alpine plant groups in New Zealand, phenotypic diversity of the alpine *Ranunculus* group is considerable and suggests the radiation of this group has been adaptive. Adaptive radiation have been inferred for several plant groups in other island environments and inferred for the genus *Pachycladon* in New Zealand (Heenan & Mitchell 2003). Numerous observations on phenotypic variation and habitat diversity suggest the radiation of

the alpine *Ranunculus* group is adaptive. However, an objective test of this using the environmental database LENZ could not demonstrate this. Arguments were presented which lead to the conclusion of a lack of resolution in the available data layers. It is thought that fine-scale habitat descriptions are needed to test a hypothesis of adaptive radiation.

Future studies in this group need to address (i) the extent of gene flow between geographically isolated populations of the same species, (ii) hybridisation and introgression between species with overlapping distributions and, (iii) the evolution of ecophysiological traits and their correlation with fine scale habitat characteristics. All such studies will be required to obtain information critical to understanding the evolution and diversity of this genus in New Zealand.

REFERENCES

Abbott, R.J. 1992. Plant invasions, interspecific hybridisation and evolution of new planta taxa. *Trends in Ecology and Evolution* 12: 401-405.

Albach, D.C.; Martinez-Ortega, M.M. and M.W. Chase. 2004. *Veronica*: parallel morphological evolution and phylogeography in the Mediterranean. *Plant Systematics and Evolution* 246: 177-194.

Allan H.H. 1926. Epharmonic response in certain New Zealand species, and its bearing on taxonomic questions. *The Journal of Ecology* 14(1) 72-91.

Allan, H.H. 1961: Flora of New Zealand. Vol. I. Government Printer. Wellington. New Zealand.

Antonovics, J. 2006. Evolution in closely adjacent plant populations: long-term persistence of pre-reproductive isolation at a mine boundary. *Heredity* 97: 33-37.

Archibald, J.K.; Wolfe, A.D. and S.D. Johnson. 2004. Hybridisation and gene flow between a day- and night-flowering species of *Zaluzianskya* (Schrophulariaceae *s.s.*, tribe Manuleeae). *American Journal of Botany* 91: 1333-1344.

Ariati, S.R.; Murphy, D.J.; Gardener S. and P.Y. Ladiges. 2007. Morphological and genetic variation within the widespread species *Acacia victoriae* (Mimosaceae). *Australian Systematic Botany* 20(1): 54-62.

Armstrong, T.J.B. 2003. Hybridisation and adaptive radiation in Australian alpine *Ranunculus*. PhD Thesis. Australian National University. Australia.

Baker, M.A. and R.A. Johnson. 2000. Morphometric analysis of *Escobaria sneedii* var. *sneedii*, E. *sneedii* var. *leei*, and *E. guadalupensis* (Cactaceae). *Systematic Botany* 25(4): 577-587.

Baldwin, B.C. 1997. Adaptive radiation of the Hawaiian silversword alliance: congruence and conflict of phylogenetic evidence from molecular and non-molecular investigations. In: *Molecular evolution and adaptive radiation,* Givnish, T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 104-128.

Barret, S.C.H. and S.W. Graham. 1997. Adaptive radiation in the aquatic plant family Pontederiaceae: insights from phylogenetic analysis. In: *Molecular evolution and adaptive radiation.* Givnish, T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 225-258.

Barthlott, W. 1981. Epidermal and seed surface characters of plants: Systematic applicability and some evolutionary aspects. *Nordic Journal of Botany* 1: 345-355.

Barton, N.H. 2001. The role of hybridisation in evolution. *Molecular Ecology* 10: 551-568.

Bernardello, G.; Anderson, G.J.; Stuessy, T.F. and Crawford D.J. 2006. The angiosperm flora of the archipelago Juan Fernandez (Chile): origin and dispersal. *Canadian journal of Botany* 84: 1266-1281.

Berry, P.E.; Hahn, W.J.; Sytsma, K.J.; Hall, J.C. and M. Austin. 2004. Phylogenetic relationships and biogeography of *Fuchsia* (Onagraceae) based on non-coding nuclear and chloroplast DNA data. *American Journal of Botany* 91(4): 601-641.

Boyd, A. 2002. Morphological analysis of Sky Island population of *Macromeria viridiflora* (Boraginaceae). *Systematic Botany* 27(1): 116-126.

Breitwieser, I.; Glenny, D.S.; Thorne, A.; and S.J. Wagstaff. 1999. Phylogenetic relationships in Australasian Gnaphalieae (Compositae) inferred from ITS sequences. *New Zealand Journal of Botany* 37: 399-412.

Briggs, B. 1962. Interspecific hybridization in the *Raunculus lappaceus* group. Evolution 16: 372-390.

Carlquist, S. 1981. Chance Dispersal: Long-distance dispersal of organisms, widely accepted as a major cause of distribution patterns, poses challenging problems of analysis. *American Scientist* 69: 509-516.

Carson, H.L. 1985. Unification of speciation theory in plants and animals. *Systematic Botany* 10: 380-390.

Carter, R.J. 2007. An investigation into the evolutionary relationships of the North Island alpine *Ranunculus*. MSc Thesis. Massey University, Palmerston North. New Zealand.

Casgrain, P. and P. Legendre. 2001. The R package for multivariate and spatial analysis, Version 4.0. User's Manual. University of Montreal, Montreal. Canada.

Cheeseman, T.F. 1925. Manual of the New Zealand Flora. Government Print. New Zealand.

Chen, Y and H.H. Harvey.1999. Spatial structuring of length-at-age of the benthivorous white sucker *(Catostomus commersoni)* in relation to environmental variables. *Aquatic Living Resources* 12(6): 351-362.

Chung K.; Peng, C.; Downie, S.R.; Spalik, K. and B.A. Schaal. 2005. Molecular systematics of the Trans-Pacific alpine genus *Oreomyrrhis* (Apiaceae): phylogenetic affinities and biogeographic implications. *American journal of Botany* 92(12): 2054-2071.

Cockayne, L. 1906. Notes on the subalpine scrub of Mount Fyffe (Seward Kaikouras). *Transactions of the New Zealand Institute* 38: 361-374.

Coleman, M. and R.J. Abbott, 2003. Possible causes of morphological variation in an endemic Moroccan groundsel (*Senecio leucanthemifolius* var. *casablancae*): evidence from chloroplast DNA and random amplified polymorphic DNA markers. *Molecular Ecology* 12: 423-434.

Comes, H.P. and J.W. Kadereit. 1998. The effect of Quaternary climatic changes on plant distribution and evolution. *Trends Plant Sci* 3: 432-438.

Cook, C.D.K. 1963. Studies in *Ranunculus* subgenus *Batrachium* (DC.) A. Gray. II. General morphological considerations in the taxonomy of the subgenus. *Watsonia* 5: 294-303.

Cook, C.D.K. 1966. A monographic study of *Ranunculus* subgenus *Batrachium* (DC.) A. Gray. *Mitteilungen der Botanischen Staatssammlung München* 6: 47-237.

Cook, L.G. and M.D. Crisp. 2005. Directional asymmetry of long-distance dispersal and colonization could mislead reconstructions of biogeography. *Journal of Biogeography* 32: 741-754.

Cook, S.A.; M.P. Johnson. 1968. Adaptation to heterogeneous environments. I. variation in heterophylly in *Ranunculus flammula* L. *Evolution* 22: 496-516.

Dahlgren, G. and L. Svensson. 1994. Variation in leaves and petals of *Ranunculus* subgenus *batrachium* on the Aegean Islands, analysed by multivariate analysis. *Botanical Journal of the Linnaean Society* 14: 253-270.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Dytham, C. 2003. Choosing and using statistics: a biologist's guide. Blackwell Publishing, Malden, MA.

Eichler, J. and N.G. Walsh. 2007. Ranunculaceae. In: *Flora Australia*, Volume 2. Winteraceae to Platanaceae. Australian Biological Resources Study, CSIRO Publishing

Ellis A.G. and A.E. Weis. 2006. Coexistence and differentiation of 'flowering stones': the role of local adaptation to soil microenvironment. *Journal of Ecology* 94 (2): 322-335.

Ellison, A.M.; Buckley, H.L.; Muller, T.E. and N.J. Gotelli. 2004. Morphological variation in *Sarracenia purpurea (Sarraceniaceae):* geographic, environmental, and taxonomic correlates. *American journal of Botany* 91(11): 1930-1935.

Felsenstein J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Fisher, F.J.F. 1952. Observation on the vegetation of screes in Canterbury, New Zealand. *Journal of Ecology* 40: 156-167.

Fisher, F.J.F. 1960. Leaf shape in *Ranunculus insignis*. *New Zealand Journal of Science* 3: 685-693.

Fisher, F.J.F. 1965: The alpine *Ranunculi* of New Zealand. New Zealand Department of Scientific and Industrial Research bulletin 165. DSIRO Publishing, Wellington. New Zealand.

Fleming, C.A. 1962. New Zealand Biogeography- A palaeontologist's approach. Tuatara 10: 53-104.

Friar, E.A.; Prince, L. M.; Roalson, E.H; McGlaughlin, M.E.; Cruse-Sanders, J.M.; De Groot, S.J. and J.M. Porter. 2006. Ecological Speciation in the East Maui–endemic *Dubautia* (Asteraceae) species. *Evolution* 60 (9): 1777-1792.

Futuyma, D.J. 1998. Evolutionary Biology. Sinauer associates, Inc. Massachusets.

Garb, J. 1999. An Adaptive Radiation of Hawaiian Thomisidae: Biogeographic and Genetic Evidence. The Journal of Arachnology 27: 71-78.

Garnock-Jones, P. 1988. Ranunculaceae. In: *Flora of New Zealand*, Volume IV. Webb, C.J.; Sykes, W.R. & P.J. Garnock-Jones (Eds). Manaaki Whenua Press, Christchurch.

Garnock-Jones, P.J. 1990. Typification of *Ranunculus* names in New Zealand (Ranunculaceae). *New Zealand Journal of Botany* 28: 115-123.

Givnish, T.J. 1997. Adaptive radiation and molecular systematics: aims and conceptual issues. In: *Molecular evolution and adaptive radiation.* Givnish, T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 1-54.

Givnish, T.J. 1998. Adaptive plant evolution on islands: classical pattern, molecular data, new insights. In: *Evolution on islands*. Grant, P.R. (Ed.). Oxford University Press, Oxford. Pp. 281-304.

Givnish, T.J.; Montgomery, R.A. and G. Goldstein. 2004. Adaptive radiation of photosynthetic physiology of the Hawaiian Lobeliads: light regimes, static light responses, and whole-plant compensation points. *American Journal of Botany* 91(2): 228-246.

Givnish, T.J.; Sytsma K.J.; Smith, J.F.; Hahn. W.J.; Benzing, D.H.; and E.M. Burkhardt. 1997. Molecular evolution and adaptive radiation in *Brocchinia* (Bromeliaceae: Pitcairnioideae) atop tepuis of the Guayana shield. In: *Molecular evolution and adaptive radiation.* Givnish T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 259-311.

Givnish, T.J.; Sytsma, K. J.; Smith, J.F. and W.J. Hahn. 1995. Molecular evolution, adaptive radiation, and geographic speciation in *Cyanea* (Campanulaceae, Lobelioidae). In: *Hawaiian biogeography—evolution in a hot-spot archipelago.* W. Wagner, L. & V.A. Funk (Eds.). Smithsonian Institution Press. Washington D.C. Pp. 288-337.

Goldblatt, P. and J.C. Manning. 1996. Phylogeny and speciation in *Lapeirousia* subgenus *Lapeirousia* (Iridaceae). *Annals of the Missouri Botanical Garden* 83: 346-361.

Grant, P.R. 1986. Ecology and Evolution of Darwin's finches. Princeton University Press. Princeton.

Grant, V. 1981. Plant speciation. Columbia University Press. New York.

Heads, M. 1998. Biogeographic disjunction along the alpine fault, New Zealand. *Biological Journal of the Linnaean Society* 63: 161-176.

Hedge, S.G.; Nason, J.D; Clegg, J.M. and N.C. Ellstrand. 2006. The evolution of California's wild radish has resulted in the extinction of its progenitors. *Evolution* 16(6): 1187-1197.

Heenan, P.B. and A.D. Mitchell. 2003. Phylogeny, biogeography and adaptive radiation of *Pachycladon* (Brassicaceae) in the mountains of South Island, New Zealand. *Journal of Biogeography* 30: 1737-1749.

Heenan, P.B.; Lockhart, P.J.; Kirkham, N.; McBreen, K. and D. Havell. 2006. Relationships in the alpine *Ranunculus haastii* (Ranunculaceae) complex and recognition of *R. piliferus* and *R. acraeus* from southern New Zealand. *New Zealand Journal of Botany* 44: 425-441.

Hennion, F. and D.W.H. Walton. 1997. Ecology and seed morphology of endemic species from Kerguelen Phytogeographic Zone. *Polar Biology* 18 (4): 229-235.

Hennion, F. and H. Couderc. 1993. Cytogenetical variability of *Ranunculus* species from Iles Kerguelen. *Antarctic Science* 5: 37-40.

Hodges, S.A. 1997. Rapid radiation due to a key innovation in columbines (Ranunculaceae: *Achilegia*). In: *Molecular evolution and adaptive radiation.* Givnish, T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 391-406.

Hodges, S.A. and M.L. Arnold. 1995. Spurring plant diversification: are floral nectar spurs a key innovation. *Proceedings of the Royal Society of London* B, *Biological Sciences* 262: 343-348.

Hoot, S.B. 1991. Phylogeny of the Ranunculaceae based on epidermal microcharacters and macromorphology. *Systematic Botany* 16: 741-755.

Hoot, S.B. 1995. Phylogeny of the Ranunculaceae based on preliminary *atpB, rbcL* and 18S nuclear ribosomomal DNA sequence data. *Plant Systematics and Evolution, Suppl.* 9: 214-251.

Hörandl, E. 2002 Morphological differentiation within the *Ranunculus cassubicus* group compared to variation of isozymes, ploidy levels, and reproductive systems: implications for taxonomy. *Plant Systematics and Evolution* 233: 65-78.

Hörandl, E.; Paun, O.; Johansson, J.T; Lehnebach, C.; Armstrong, T.; Chen, L and P. Lockhart. 2005. Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. *Molecular Phylogenetics and Evolution* 36: 305-327.

Huelsenbeck, J.P. and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

Hufford, L. and M. McMahon. 2004. Morphological evolution and systematics of *Synthyris* and *Basseya* (Veronicaceae): a phylogenetic analysis. *Systematic Botany* 29: 716-736.

Hughes, C. and R. Eastwood. 2006. Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the Natural Academy of Science* 103(27): 10334-10339.

Huson, D.H. and D. Bryant. 2006. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23:254-267.

Jackman, T.; Losos, J.B. and D.T.J. Littlewood. 1997. Phylogenetic studies of convergent adaptive radiations in Caribbean Anolis lizards. In: *Molecular evolution and adaptive radiation.* Givnish, T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 535-558.

Jakubowsky, G.; Kiehn, M.; Samuel, R. and T.F. Stuessy. 2005. Evolution of *Nertera* (Rubiaceae) with focus on the Tristan da Cunha archipelago. International Botanical Congress, Vienna.

Jensen, U. and B. Greben. 1984. Serological aspects and plylogenetic relationships of the Magnoliidae. *Taxon* 33: 563-577.

Johansson, J.T. 1995. A revised chloroplast DNA phylogeny of the Ranunculaceae. *Plant Systematics and Evolution, Suppl.* 9: 253-261.

Johansson, J.T. 1998. Chloroplast DNA restriction site mapping and the phylogeny of Ranunculus (Ranunculaceae). *Plant Systematic and Evolution* 213: 1-19.

Johnson, S.D. 1996. Pollination, adaptation, and speciation models in the Cape Flora of South Africa. *Taxon* 45: 59-66.

Jolley, H. and N. Klazenga. 2007. A morphometric study of *Breutelia pendula* and *B. elongata* (Bryophyta, Bartramaniaceae). *Australian Systematic Botany* 20(1): 82-91.

Jorgensen, S. and R. Mauricio. 2005. Hybridization as a source of evolutionary novelty: leaf shape in a Hawaiian composite. *Genetica* 123: 171-179.

Judd, W.; Campbell, C.S.; Kellogg, E.A.; Stevens, P.S. and M.J. Donoghue. 2002. Plant systematics: a phylogenetic approach. Sinauer Associates. Massachusetts.

Kadereit, G.; Gotzek, D.; Jacobs, S. and H. Freitag. 2005. Origin and age of Australian Chenopodiaceae. *Organisms, Diversity and Evolution* 5: 59-80.

Kambysellis, M.P. and E.M. Craddock. 1997. Ecological and reproductive shifts in the diversification of the endemic Hawaiian *Drosophila*. In: *Molecular evolution and adaptive radiation.* Givnish, T.J. & K.J. Sytsma (Eds.). Cambridge University Press. Pp. 475-510.

Kirk, T. 1880. Descriptions of the New Zealand Flowering Plants. *Transactions of the New Zealand Institute* 12:393-397

Kirk, T. 1899: Students' flora of New Zealand. Government Printer, Wellington.

Knapp, M. 2007. Discontinuous distributions of iconic New Zealand plant taxa and their implications for Southern Hemisphere biogeography. PhD Thesis, Massey University, Palmerston North. New Zealand.

Knapp, M.; Stöckler, K., Havell, D.; Delsuc, F.; Sebastiani, F. P.J Lockhart. 2005. Relaxed Molecular Clock Provides Evidence for Long-Distance Dispersal of *Nothofagus* (Southern Beech). PLoS Biology 3(1): e14.

Kosuge, K. and M. Tamura. 1986. Isozymic variation of glutamate dehydrogenase in the Ranunculaceae. *Acta Phytotxonomica Geobotanica* 37: 167-179.

Lacoul, P. and B. Freedman 2006. Recent observation of a proliferation of *Ranunculus trichophyllus* Chaix. in high-altitude Lakes of the Mount Everest region. *Arctic, Antarctic, and Alpine Research* 38(3): 394-398.

Lehnebach, C.A.; Cano, A.; Monsalve, C.; McLenachan, P.; Hörandl, E. and P. Lockhart. 2007. Phylogenetic relationships of the monotypic Peruvian genus *Laccopetalum* (Ranunculaceae). *Plant Systematics and Evolution* 264 (1-2): 109-116.

Levin, D. 2000. The origin, expansion, and demise of plant species. Oxford University Press.

Levin, D. 2003. Ecological speciation: lessons from invasive species. *Systematic Botany* 28: 643-650.

Levin, D. 2004. Ecological Speciation: The Role of Disturbance. *Systematic Botany* 29(2): 225-233.

Lihova, J.; Kucera, J.; Perny, M. and K. Marhold. Hybridization between two polyploid *Cardamine* (Brassicaceae) species in north-western Spain: discordance between morphological and genetic variation patterns. *Annals of Botany* 99: 1038-1096.

Linder, C.R. and L.H. Rieseberg. 2004. Reconstructing patterns of reticulate evolution in plants. *American journal of Botany* 91: 1700-1708.

Linder, H.P. 2003. The radiation of the Cape flora, Southern Africa. *Biological Reviews* 78: 597-638.

Lockhart, P.; McLechnanan, P.A.; Havell, D.; Glenny, D.; Huson, D. and U. Jensen. 2001. Phylogeny, dispersal and radiation of New Zealand alpine buttercups: molecular evidence under split decomposition. *Annals of the Missouri Botanical Garden* 88: 458-477.

Lynn, D.E. and S. Waldren. 2001. Morphological variation in populations of *Ranunculus repens* from the temporary limestone lakes (Turloughs) in the west or Ireland. *Annals of Botany* 87: 9-17.

Macphail, M.K. 1997. Comment on "M. Pole (1994) The New Zealand flora– entirely long-distance dispersal? Journal of Biogeography 21: 625-635" *Journal of Biogeography* 24: 113-114.

Mark, A.F. and N.M. Adams. 1986. New Zealand Alpine Plants. Reed Methuen Publishers Ltd. Auckland.

McBreen K. and P.J. Lockhart. 2006. Reconstructing reticulate evolutionary histories of plants. *Trends in Plant Science* 11(8): 338-404.

McGlone, M.S.; Duncan, R.P. and P.B. Heenan. 2001. Endemism, species selection and the origin and distribution of the vascular plant flora of New Zealand. *Journal of Biogeography* 28: 199-216.

Meimberg, H.; Wistuba, A.; Dittrich, P. and G. Heubl. 2001. Molecular phylogeny of Nepenthaceae based on cladistic analysis of plastid *trn*K intron sequence data. *Plant Biology* 3: 164-175.

Meudt, H.M. and B.B. Simpson. 2006. The biogeography of the austral, subalpine genus *Ourisia* (Plantaginaceae) based on molecular phylogenetic evidence: South America origin and dispersal to New Zealand and Tasmania. *Biological Journal of the Linnaean Society* 87: 479-513.

Mitchell, A.D. and S.J. Wagstaff. 2000. Phylogeny and biogeography of the Chilean *Psudopanax laetevirens. New Zealand Journal of Botany* 38: 409-414.

Moore, D. M. 1983. *Flora of Tierra del Fuego*. Oswestry, Shropshire, A. Nelson, St. Louis, Missouri Botanical Garden.

Moore, D.M.; Tye, A. and R.K. Jansen. 2006. Patterns of long-distance dispersal in *Tiquilia* subg. *Tiuilia* (Boraginaceae): implications for the origins of amphitropical disjunts and Galapagos Islands endemics. *American Journal of Botany* 93(8): 1163-1177.

Mummenhoff, K. and A. Franzke. 2007. Gone with the bird: late tertiary and quaternary intercontinental long-distance dispersal and allopolyploidization in plants. *Systematics and Biodiversity* 5(3): 255-260.

Newstrom, L. and A. Robertson. 2005. Godley Review: Progress in understanding pollination systems in New Zealand. *New Zealand Journal of Botany* 43: 1-59.

Nie, Z.; Sun, H.; Beardsley, P.M.; Olmstead, R.G. and J. Wen. 2006. Evolution of biogeographic disjunction between eastern Asia and eastern North America in *Phryma* (Phrymaceae). *American Journal of Botany* 93(9): 1343-1356.

Noel, F.; Machon, N. and E. Porcher. 2007. No genetic diversity at molecular markers and strong phenotypic plasticity in populations of *Ranunculus nodiflorus*, an endangered plant species in France. *Annals of Botany* 99: 1203-1212.

Norton, D.A.; Dickinson, Y. and M. van Leeuwen. 2007. Ecology of a *Ranunculus lyallii* population at its dryland distributional limit in Canterbury, New Zealand. *New Zealand Journal of Botany* 45: 81-85.

Patterson, T.B. and T.J. Givnish. 2004. Geographic cohesion, chromosomal evolution, parallel adaptive radiations, and consequent floral adaptations in *Calochortus* (Calochortaceae): evidence from a cpDNA phylogeny. *New Phytologist* 161(1): 253-264.

Paun, O.; Hörandl, E.; Lehnebach, C.; Johansson, J.T. and P. Lockhart. 2005. Phylogenetic relationships and biogeography of Mediterranean and European alpine *Ranunculi*

(Ranunculaceae) inferred from nrITS and plastid sequence data. *Taxon* 54 (4): 911-930.

Pellmyr, O. 1995. Pollination biology. *In*: Natürliche Pflanzenfamilien. Hiepko, P. (Ed.) Duncker & Humblot, Berlin. Pp. 160—184.

Pigg, K.B. and M.L. DeVore. 2003. *Paleoactaea gen. nov*. (Ranunculaceae) fruits from the Paleogene of North Dakota and the London Clay. *American Journal of Botany* 92:1650-1659.

Piripi, M.M. 2005. Evolutionary relationships of the Castle Hill buttercup (*Ranunculus crithmifolius* subspecies *paucifolius*). MSc Thesis, Massey University, Palmerston North. New Zealand.

Podani, J. 1999. Extending Gower's general coefficient of similarity to ordinal characters. *Taxon* 48: 331-340.

Pole, M. 1994. The New Zealand flora- entirely long-distance dispersal? *Journal of Biogeography* 21: 625-635.

Posada, D. and K.A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817-818.

Raven, P.H. 1963. Amphitropical relationships in the floras of North and South America. *Quarterly Review Biology* 38: 151-177.

Raven, P.H. 1972. Evolution and endemism in the New Zealand species of *Epilobium*. In: *Taxonomy, Phytogeography and Evolution*. Valentine, D.H. (Ed.). Academic Press, London. Pp. 259-274.

Raven, P.H. 1973. Evolution of the subalpine and alpine plant groups in New Zealand. *New Zealand Journal of Botany* 11: 177-200.

Rendle, H. and B.G. Murray. 1988. Breeding systems and pollen behaviour in compatible and incompatible crosses in New Zealand species of *Ranunculus* L. *New Zealand journal of Botany* 26: 467-471.

Riveros, M. 1991. Biologia Reproductiva en especies vegetales de dos comunidades de la zona templada del sur de Chile, 40°S, PhD Thesis. Universidad de Chile, Santiago. Chile.

Robichaux, R.H. and J.E. Canfield. 1985. Tissue elastic properties of eight Hawaiian Dubautia species that differ in habitat and diploid chromosome number. *Oecologia* 66: 77-80.

Rogers, G. and S. Walker. 2005. Is *Pittosporum patulatum* Hook. F. threatened by pest herbivory in eastern South Island, New Zealand? *New Zealand Journal of Ecology* 29(1): 11-28.

Rohlf, F.J. 2000. NTSYSpc: Numerical Taxonomy and Multivariate Analysis System, Version 2.2. Applied Biostatistics Inc., New York.

Ruiz, E. 2002. Ranunculaceae. In: Flora de Chile Vol.2, Fasc. 1. Marticorena, C. & R. Rodríguez (Eds). Universidad de Concepcion. Concepcion. Chile

Rundle, H.D. and P. Nosil. 2005. Ecological speciation. *Ecology Letters* 8: 336-352.

Sambatti, J.B.M and K. Rice. 2006. Local adaptation, patterns of selection, and gene flow in the Californian serpentine sunflower (*Helianthus exilis*). *Evolution* 60(4): 696-710.

Sanmartin, I., and F. Ronquist. 2004. Southern hemisphere biogeography inferred by event-based models: plant versus animal patterns. *Systematic Biology* 53(2): 216-243.

Scherff, E.J.; Galen, C. and M.L. Stanton. 1994**.** Seed dispersal, seedling survival and habitat affinity in a snowbed plant: limits to the distribution of the snow buttercup, *Ranunculus adoneus*. *Oikos* 69 (3): 405-413.

Schilling, E.E.; da Costa F.B.; Lopes N.P. and P.J. Heise. 2000. Brazilian species of *Viguiera* (Asteraceae) exhibit low levels of its sequence variation. *Edinburgh Journal of Botany* 57(3): 323-332.

Schluter, D. 2000. The ecology of adaptive radiation. Oxford University Press, New York.

Schuettpelz, E. and S.B. Hoot. 2004. Phylogeny and biogeography of *Caltha* (Ranunculaceae) based on chloroplast and nuclear DNA sequences. *American journal of Botany* 91(2): 247-253.

Schuettpelz, E.; Hoot, S.B.; Samuel, R. and F. Ehrendorfer. 2002. Multiple origins of Southern hemisphere *Anemone* (Ranunculaceae) based on plastid and nuclear sequence data. *Plant Systematics and Evolution* 231: 143-151.

Seimon, A.; Halloy, S.R.P.; and T.A. Seimon. 2007. Recent observation of a proliferation of *Ranunculus trichophyllus* chaix. in high-altitude lakes of the Mount Everest region: Comment. *Arctic Antarctic and Alpine Research* 39 (2): 340-341.

Shaffer, S.A.; Tremblay, Y.; Weimerskirch, H.; Scott, D.; Thompson, D.R.; Sagar, P.M.; Moller, H.; Taylor, G.A.; Foley, D.G.; Block, B.A. and Costa, D.P. 2006. Migratory shearwaters integrate oceanic resources across the Pacific Ocean in an endless summer. *PNAS* 103(34): 12799-12802.

Shepherd, L.D.; Perrie, L.R. and P.J. Brownsey. 2007. Fire and ice: volcanic and glacial impacts on the phylogeography of the New Zealand forest fern *Asplenium hookerianum. Molecular Ecology* 16: 4536-4549.

Simmons, M.P. and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49 (2): 369-381.

Smissen, R.D. and P.B. Heenan. 2007. DNA fingerprinting supports hybridisation as a factor explaining complex natural variation in *Phormium* (Hemerocallidaceae). *New Zealand Journal of Botany* 45: 419-432.

Smissen, R.D.; Garnock-Jones, P.J. and G.K. Chambers. 2003 Phylogenetics analysis of ITS sequences suggests a Pliocene origin for the bipolar distribution of *Scleranthus* (Caryophyllaceae). *Australian Systematic Botany* 16: 301-315.

Sokal, R. R. and F.J. Rholf. 1998. Biometry. W. H. Freeman and Company, New York

Soltis; D.E. and P.S. Soltis. 1999. Polyploidy: Recurrent formation and genome evolution. *Trends in Ecology and Evolution* 14: 348-352.

Stebbins, L.G. 1950. Variation and evolution in Plants. Columbia University Press, New York.

Stebbins, L.G. 1974. Flowering plants: evolution above the species level. Harvard University Press, Massachusetts

Steinbach, K. and G. Gottsberger. 1995. Phenology and pollination biology of five *Ranunculus* species in central Germany. *Plant Systematics and Evolution, Suppl.* 9: 319-323.

Stuessy, T.F. 1994. Case studies in plant taxonomy: exercises in applied pattern recognition. Columbia University Press, New York.

Stuessy, T.F. and C. Taylor. 1995. Evolucion de la Flora Chilena. In: Flora de Chile, Vol. 1. Marticorena, C. & R. Rodriguez (Eds.). Universidad de Concepcion, Concepcion. Chile.

Swofford, D. L. 2000. PAUP*: Phylogenetic analysis using parsimony and other methods. Ver. 4.0b6. Sinauer Associates, Sunderland, MA.

Takhtajan, A. 1986. Floristic Regions of the World. University of California Press.

Tamura, M. 1993. Ranunculaceae. *In*: The families and genera of vascular plants. Kubitzki, K. (Ed.). Springer-Verlag, Berlin.

Tamura, M. 1995. Angiospermae. Ordnung Ranunculales. Familie Ranunculaceae. II. Systematic Part. In: *Natürliche Pflanzenfamilien*. Hiepko, P. (Ed.) Duncker & Humblot, Berlin. Pp. 223-519.

Tate, J.A. and B.B Simpson. 2004. Breeding system evolution in *Tarasa* (Malvaceae) and selection for reduced pollen grain size in the polyploid species. *American Journal of Botany* 91: 207-213.

Tate, J.A. and B.B. Simpson. 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany* 28: 723-737.

Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F. and D.G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

Trewick, S.A.; Paterson, A.M. and H.J. Campbell. 2007. Hello New Zealand. *Journal of Biogeography* 34(1): 1–6.

Turelli, M.; Barton, N.H. and J.A. Coyne. 2001. Theory and speciation. *Trends in Ecology and Evolution*. 16(7): 330-343

Utelli, A.B; Roy, B.A and M. Baltisberger. 2000. Molecular and morphological analyses of European *Aconitum* species (*Ranunculaceae*). *Plant Systematics and Evolution* 224: 195-212.

van der Pijl, L. 1982. Principles of dispersal in higher plants. Springer-Verlag. Berlin

Vijverberg, K.; Kuperus, P.; Breeuwer, J.A.J. and K. Bachmann 2000. Incipient adaptive radiation of New Zealand and Australian *Microseris* (Asteraceae): an amplified fragment length polymorphism (AFLP) study. *Journal of Evolutionary Biology* 13: 997-1008.

Wagstaff, S.J. 2004. Evolution and biogeography of the austral genus *Phyllocladus* (Podocarpaceae). *Journal of Biogeography* 31: 1569-1577.

Wagstaff, S.J. and M.A. Dawson. 2000. Classification, origin, and patterns of diversification of *Corynocarpus* (Corynocarpaceae) inferred from DNA sequences. *Systematic Botany* 25 (1): 134-149.

Wagstaff, S.J. and P.J. Garnock-Jones. 2000. Patterns of diversification in *Chionohebe* and *Parahebe* (Scrophulariaceae) inferred from ITS sequences. *New Zealand Journal of Botany* 38: 389-407.

Wagstaff, S.J. and J. Wege. 2002. Patterns of diversification in New Zealand Stylidiaceae. *American Journal of Botany* 89(5): 865-874.

Wagstaff, S.J.; Martinsson, K. and U. Swenson. 2000. Divergence estimates of *Tetrachondra hamiltonii* and *T. patagonica* (Tetrachondraceae) and their implications for austral biography. *New Zealand Journal of Botany* 38: 587-596.

Wagstaff, S.J.; Breitwieser, I. and U. Swenson. 2006. Origin and relationships of the austral genus *Abrotanella* (Asteraceae) inferred from DNA sequences. *Taxon* 55: 95-106.

Walker, S.; Price, R.; Rutledge, D.; Stephens, T. and W.G. Lee. 2006. New Zealand Journal of Ecology 30(2): 169-177.

Wanntorp, L. and H. Wanntorp. 2003. The biogeography of *Gunnera* L.: vicariance and dispersal. *Journal of Biogeography* 30: 979-987.

Wardle, P. 1978. Origin of the New Zealand mountain flora, with special reference to trans-Tasman relationships. *New Zealand Journal of Botany* 16: 535-550.

Watson, L. and M.J. Dallwitz. 1992. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 1st June 2007. http://delta-intkey.com'.

Wen, J. 1999. Evolution of eastern Asian and eastern North American disjunct distributions of flowering plants. *Annual Review of Ecology and Systematics* 30: 421-455.

Werker, E. 2000. Seed Anatomy. Gebrüder Borntraeger. Berlin.

Winkworth, R.C. 2000. Evolution of the New Zealand alpine flora: origins, diversification and dispersal. PhD Thesis. Massey University, Palmerston North. New Zealand.

Winkworth, R.C.; Grau, J.; Robertson, A.W. and P.J. Lockhart. 2002a. The origins and evolution of the genus *Myosotis* L. (Boraginaceae). *Molecular Phylogenetics and Evolution* 24: 180-193.

Winkworth, R.C.; Wagstaff, S.J.; Glenny, D. and P.J. Lockhart. 2005. Evolution of the New Zealand mountain flora: origins, diversification and dispersal. *Organisms, Diversity and Evolution* 5: 237-247.

Winkworth, R.C; Wagstaff, S.J.; Glenny, D. and P.J. Lockhart. 2002b. Plant dispersal N.E.W.S. from New Zealand. *Trends in Ecology and Evolution* 17(11) 514-520.

Young, N.D. and J. Healey. 2003. GapCoder automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4:6.

Yukoyama, J.; Susuki, M.; Iwatsuki, K. and M. Hasebe. 2000. Molecular phylogeny of *Coriaria*, with special emphasis on the disjunct distribution. *Molecular Phylogenetics and Evolution* 14(1): 11-19.

Yumamoto, T. 1986. The ecological pollination syndromes of insect-pollinated plants in an alpine meadow. *Ecological Research* 1: 83-95.

Ziman, S.N. and C.S. Keener. 1989. A geographical analysis of the family Ranunculaceae. *Annals of the Missouri Botanical Garden* 76: 1012-1049.

Appendix 1: Articles published during the production of this thesis.

Hörandl, E.; Paun, O.; Johansson, J.T; Lehnebach, C.; Armstrong, T.; Chen, L and P. Lockhart. 2005. Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. *Molecular Phylogenetics and Evolution* 36: 305-327.

Paun, O.; Lehnebach, C.; Johansson, J.T., Lockhart, P. And E. Hörandl. 2005. Phylogenetic relationships and biogeography of Mediterranean and European alpine *Ranunculi* (Ranunculaceae) inferred from nrITS and plastid sequence data. *Taxon* 54 (4): 911-930.

Lehnebach, C.A.; Cano, A.; Monsalve, C.; McLenachan, P.; Hörandl, E. and P. Lockhart. 2007. Phylogenetic relationships of the monotypic Peruvian genus *Laccopetalum* (Ranunculaceae). *Plant Systematics and Evolution* 264 (1-2): 109-116.

Lehnebach, C.A.; Rivero, M.; Ezcurra, C. & P.J. Lockhart. 2007. A new northern limit for the distribution of the Patagonian buttercup *Ranunculus spegazzinii* (Ranunculaceae) in Chile. Gayana 64 (2): 237-240.

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Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis

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Abstract

Ranunculus is a large genus with a worldwide distribution. Phylogenetic analyses of c. 200 species of *Ranunculus* s.l. based on sequences of the nrITS using maximum parsimony and Bayesian inference yielded high congruence with previous cpDNA restriction site analyses, but strongly contradict previous classifications. A large core clade including *Ranunculus* subg. *Ranunculus*, subg. *Batrachium*, subg. *Crymodes* p.p., *Ceratocephala*, *Myosurus*, and *Aphanostemma* is separated from *R.* subg. *Ficaria*, subg. *Pallasiantha*, subg. *Coptidium*, subg. *Crymodes* p.p., *Halerpestes*, *Peltocalathos*, *Callianthemoides*, and *Arcteranthis*. Within the core clade, 19 clades can be described with morphological and karyological features. Several sections are not monophyletic. Parallel evolution of morphological characters in adaptation to climatic conditions may be a reason for incongruence of molecular data and morphology-based classifications. In some mountainous regions, groups of closely related species may have originated from adaptive radiation and rapid speciation. Split decomposition analysis indicated complex patterns of relationship and suggested hybridization in the apomictic *R. auricomus* complex, *R.* subg. *Batrachium*, and the white-flowering European alpines. The evolutionary success of the genus might be due to a combination of morphological plasticity and adaptations, hybridization and polyploidy as important factors for regional diversification, and a broad range of reproductive strategies.

Keywords: Evolution; Molecular phylogenetics; *Ranunculus*; Ranunculaceae; Split decomposition

1. Introduction

Ranunculus (buttercups) represents the largest genus within Ranunculaceae, comprising ca. 600 species (Tamura, 1995) and distributed on all continents. Most species are found in temperate to arctic/subantarctic zones; in the tropics they are rare and restricted to high mountain areas. Buttercups are established in various terrestrial or aquatic habitats from lowlands to high alpine zones, and terrestrial species are often specialized in extreme, mainly cold/humid, but sometimes also xeric conditions. Various morphological adaptations and different reproductive strategies such as vegetative reproduction (stolons, bulbils), self-compatibility (e.g., in water-buttercups, Cook, 1966; in high alpine species, Pickering, 1997a,b; Riveros, 1991), and agamospermy (*R. auricomus* complex; see, e.g., Nogler, 1984; *R. parnassifolius* L. and *R. kuepferi* Greut. et Burdet; Huber, 1988) may be important factors for their ability to colonize habitats at higher altitudes and latitudes. Within certain mountain systems, high species diversity is

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observed (e.g., Briggs, 1960; Eichler, 1958; Fisher, 1965; Landolt, 1954, 1956).

Polyploidy and hybridization may be regarded as important factors for speciation and evolutionary success of buttercups. Basic chromosome numbers are $x = 8$ or $x = 7$; the latter is rare and usually regarded as the derived type (e.g., Goepfert, 1974). Based on both basic numbers, polyploidy is frequent and found in approximately 40% of the species included here, which may serve as a rough estimate for the genus. Chromosome numbers up to $2n = 128$ (16-ploid based on $x = 8$) are known (see survey in Table 2). Autopolyploidy has been suggested for, e.g., *R. cassubicifolius* W. Koch (Hörandl and Greilhuber, 2002). Allopolyploid origin of species has been documented for many taxa (e.g., *R. cantoniensis* DC., Okada, 1984, 1989; *R. kuepferi*, Huber, 1988; *R. nivicola* Hook, Fisher, 1965; *R. peltatus* Moench s.l. and *R. penicillatus* (Dum.) Bab., Dahlgren and Cronberg, 1996; polyploid microspecies of the apomictic *R. auricomus* complex, Rousi, 1956). Hybridization occurs within many groups, including water-buttercups (*R.* subg. *Batrachium*; Cook, 1963), the alpine species of New Zealand (*R.* sect. *Pseudadonis*; Fisher, 1965; Lockhart et al., 2001), the Australian *R. lappaceus* group (Briggs, 1962), *R.* sect. *Flammula* (Gornall, 1987), and the European *R. montanus* (Landolt, 1954, 1956) and *R. polyanthemos* groups (Baltisberger, 1980). Nevertheless, hybridization between species of different sections is rare and has been documented only for some white-flowering alpine species of Europe belonging to sects. *Aconitifolii* and *Ranuncella* (Huber, 1988), and between *R.* subg. *Pallasiantha* and subg. *Coptidium* (Nilsson, 2001).

Generic delimitation and infrageneric classification of *Ranunculus* is still under consideration. Previous classifications were based mainly on characters of the achenes (shape of body and beak of achenes, pericarp structure, and indumentum), the shape of the receptacle, floral morphology (number of sepals and honey-leaves, here called petals, gloss and colour of petals, and shape of nectary), life form, and the root system (either uniform or dimorphic with fibrous and tuberous roots). Leaf characters can vary considerably within sections (from undivided-peltate to strongly dissected) and are often obvious adaptations to habitats (e.g., strongly dissected leaves in water-buttercups; Cook, 1966) and thus of limited value for infrageneric classifications. Infrageneric taxa rarely have exclusive diagnostic morphological characters but are rather characterized by a combination of features.

In the first worldwide classification of Candolle (1824) based on 159 species, *Ranunculus* was classified within tribe Ranunculeae, excluding *Myosurus*, *Ceratocephala*, and *Ficaria*, and subdivided into sections *Batrachium*, *Ranunculastrum* (incl. *Krapfia*), *Thora*, *Hecatonia*, and *Echinella* using features of achenes, roots, and flowers. Later worldwide classifications differ considerably among authors; e.g., Prantl (1887), mainly relying on fruit anatomy, excluded *Oxygraphis*, and subdivided *Ranunculus* into sections *Ceratocephala*, *Ficaria*, *Marsypadenium*, *Hypolepium*, *Thora*, *Physophyllum*, and *Butyranthus*. Tamura's surveys (1993, 1995) are the only modern worldwide classifications and are based mainly on achene characters. He excluded several small "satellite" genera previously described under *Ranunculus* (*Aphanostemma*, *Arcteranthis*, *Callianthemoides*, *Ceratocephala*, *Cyrtorhyncha*, *Halerpestes*, *Kumlienia*, *Oxygraphis*, and *Peltocalathos*; all classified in subtribe Ranunculinae; Table 1). *Ranunculus* s.str. is subdivided into seven subgenera (*Pallasiantha*, *Coptidium*, *Ficaria*, *Batrachium*, *Crymodes*, *Gampsoceras*, and *Ranunculus*), and subg. *Ranunculus* is subdivided into 20 sections (Table 1). Tamura's (1995) classification differs considerably from regional treatments, such as that of Ovczinnikov (1937) for the Flora of USSR, Whittemore (1997) for the Flora of North America, and Tutin and Cook (1993) for Flora Europaea (Table 1) which reflects the uncertainty about relationships within the genus.

Previous molecular phylogenies of *Ranunculus* s.l. using cpDNA restriction site analysis of 78 species (Johansson, 1998) and a *matK–trnK* analysis of 133, mainly European species (Paun et al., in press) inferred relationships that were not congruent with previous classifications. Within subg. *Ranunculus*, only few of the traditional sections were confirmed as monophyletic. Overall at the species level, relationships remained poorly resolved in Johansson (1998).

Sequences of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA is today among the most employed markers for phylogenetic studies on the generic/subgeneric level and has shown to be informative in buttercups even at the specific and intraspecific level (Lockhart et al., 2001; Winkworth et al., in press). In combination with data from the chloroplast genome and other external evidence, this nuclear marker also offers insights into reticulate patterns caused by hybridization. In allopolyploids, rDNA sequences may show either additivity of genomes of the parents or only one repeated rDNA type due to concerted evolution (see review in Soltis et al., 2003). Using the appropriate tools to analyze conflicting phylogenetic signals, such as split decomposition (e.g., Lockhart et al., 2001) can give further insights into possible reticulate relationships within the genus.

The main goals of the present study are (1) to analyze phylogenetic relationships of *Ranunculus* s.l. with an appropriate marker and to compare results with previous classifications, (2) to study possible effects of hybridization and allopolyploidy on the phylogeny of the genus, and (3) to understand better the factors for the evolutionary success of the genus. A detailed treatment of morphological characters, regional groups, biogeo-

Table 1
Survey of modern classifications of *Ranunculus* and allied genera (Ranunculeae, after Tamura, 1995). *Xanthobatr* = *Xanthobatrachium*

Tamura (1995) (worldwide)	Ovczinnikov (1937) (USSR)	Whittemore (1997) (North America)	Tutin and Cook (1993) (Europe)	Number of species	Distribution (after Tamura, 1995)
Tribus Ranunculeae					
Subtrib. Trautvetteriinae					
Trautvetteria[a]	*Trautvetteria*	*Trautvetteria*		3	N. America, SE. Asia
Subtrib. Myosurinae					
Myosurus[a]	*Myosurus*	*Myosurus*		15	All continents
Subtrib. Ranunculinae					
Kumlienia		*R.* subg. *Ranunculus* sect. *Pseudaphanostemma*		1	N. America
Arcteranthis[a]		*R.* subg. *R.* sect. *Arcteranthis*		1	N. America
Halerpestes[a]	***Halerpestes***	*R.* subg. *R.* sect. *Halodes*	*R.* subg. *Ranunculus* sect. *Halodes*	10	Asia, America[c]
Oxygraphis[a]	***Oxygraphis*** subg. *Euoxygraphis*	*R.* subg. *Oxygraphis*		4	Asia, N. America
Peltocalathos[a]				1	South Africa
Callianthemoides[a]				1	S. America
Cyrtorhyncha[a]		*R.* subg. *R.* sect. *Cyrtorhyncha*		1	N. America
Paroxygraphis				1	Asia (Himalaya)
Hamadryas				6	S. America
Aphanostemma[a]				1	S. America
Ranunculus[a]	***Ranunculus***			600	all continents
1. subg. *Coptidium*[a]	*R.* subg. *Auricomus* sect. *Coptidium*	*R.* subg. *Coptidium*		1	Europe, Asia, N. America
2. subg. *Pallasiantha*[a]	*R.* subg. *Auricomus* sect. *Coptidium*	*R.* subg. *Pallasiantha*	*R.* subg. *R.* sect. *Pallasiantha*	1	Europe, Asia, N. America
3. subg. *Ficaria*[a]	***Ficaria***	*R.* subg. *Ficaria*	*R.* subg. *R.* sect. *Ficaria*	5	Europe, Asia, N. America
4. subg. *Batrachium*[a]	***Batrachium***	*R.* subg. *Batrachium*	*R.* subg. *Batrachium*	30	Worldwide
5. subg. *Crymodes*[a]	***Oxygraphis*** subg. *Crymodes*	*R.* subg. *Crymodes*	*R.* subg. *R.* sect. *Crymodes*	4	Europe; N. America
6. subg. *Ranunculus*[a]			*R.* subg. *Ranunculus*	550	All continents
sect. *Ranunculus*[a]	*R.* subg. *Au.* sect. *Euauricomus*	- sect. *Epirotes*	- sect. *Auricomus*, sect. *Insulares*	160[b]	Eurasia, America, Oceania
sect. *Flammula*[a]	*R.* subg. *Au.* sect. *Flammula*	- sect. *Flammula*	- sect. *Flammula*	22	Eurasia, N. America
sect. *Hecatonia*[a]	*R.* subg. *Hecatonia*	- sect. *Hecatonia*	- sect. *Hecatonia*	1	Eurasia, N. America[c]
sect. *Xanthobatr.*[a]	*R.* subg. *Auricomus* sect. *Xanthobatrachium*	- sect. *Hecatonia*	- sect. *Xanthobatrachium*	8	Northern hemisphere
sect. *Thora*[a]	*R.* subg. *Thora*		- sect. *Thora*	4	Europe
sect. *Aconitifolii*[a]			- sect. *Aconitifolii*	3	Europe
sect. *Ranuncella*[a]			- sect. *Ranuncella*	6	Europe
sect. *Leucoranunculus*[a]			- sect. *Leucoranunculus*	4	Europe, Asia
sect. *Physophyllum*[a]			- sect. *Physophyllum*	1	Europe, northern Africa
sect. *Acetosellifolii*[a]			- sect. *Acetosellifolii*	1	Europe
sect. *Chloeranunculus*[a]			- sect. *Ranuncella*	1	Europe, northern Africa
sect. *Ficariifolius*				5	E. Asia
sect. *Casalea*[a]				10	America
sect. *Tuberifer*				2	E. Asia
sect. *Pseudadonis*[a]				15	New Zealand, Australia
sect. *Acris*[a]	*R.* subg. *Chrysanthe*	- sect. *Ranunculus*	- sect. *Ranunculus*	150	All continents
sect. *Echinella*[a]	*R.* subg. *Pachyloma*	- sect. *Echinella*	- sect. *Echinella*	70	Eurasia, Africa, N. America
sect. *Micranthus*[a]	*R.* subg. *Micranthus*		- sect. *Micranthus*	2	Europe, N. Africa, Asia

(*continued on next page*)

Table 1 (*continued*)

Tamura (1995) (worldwide)	Ovezinnikov (1937) (USSR)	Whittemore (1997) (North America)	Tutin and Cook (1993) (Europe)	Number of species	Distribution (after Tamura, 1995)
sect. *Leptocaules*[a]				7	S. America, New Zealand, Australia
sect. *Ranunculastrum*[a]	*R.* subg. *Ranunculastrum*		- sect. *Ranunculastrum*	70	Europe, Asia, N. Africa
7. subg. *Gampsoceras*				1	Asia
Ceratocephala[a]	***Ceratocephala***	*R.* subg. *Ceratocephala*	***Ceratocephala***	4	Eurasia, northern Africa[c]
Laccopetalum				1	S. America
Krapfia				8	S. America

Bold, genera that have been treated as *Ranunculus* by various authors; underlined, sections or subgenera of *Ranunculus* that have been treated as genera by various authors.

[a] Material here included.

[b] Plus ca. 900 agamospecies (*R. auricomus* complex).

[c] Naturalized in other continents.

graphical aspects, and a definitive classification, will be presented in forthcoming papers.

2. Materials and methods

2.1. Materials

Sampling of species followed a strategy to (1) include materials from all continents, and (2) include representatives of all subgenera and sections of *Ranunculus* s.str. after the classification of Tamura (1995). With respect to taxonomic groups, the material is complete except for the monotypic *R.* subg. *Gampsoceras* from Iran, and the small sections *Tuberifer* and *Ficariifolius* from Eastern Asia. For the apomictic *R. auricomus* complex, which comprises around 800–900 microspecies, the sampling strategy was to include (1) all diploid sexual species hitherto known (*R. cassubicifolius*, *R. carpaticola* Soó, *R. notabilis* Hörandl et Guterm.; see Hörandl, 2004), and (2) to include 2–3 apomictic representatives of the four main morphological groups or grades of the complex (*R. cassubicus*, *R. fallax*, *R. monophyllus*, and *R. auricomus* s.str.; Ericsson, 2001; Hörandl and Gutermann, 1998). Included were also most of the DNA extractions used in Johansson (1998), sequences from Lockhart et al. (2001), and Armstrong et al. (2004). For some widespread and critical species, more than one accession was sequenced, but in case of identical sequences only one was used for phylogenetic reconstruction. Altogether, c. 200 species (some only treated as subspecies by other authors) were included in the final analyses (Table 2). All genera from subtribe Ranunculinae that are so far available were treated as the ingroup (see Table 1).

Both the morphological studies of Tamura (1995) and the molecular studies at the family level by Johansson and Jansen (1993), Johansson (1995), and Hoot (1995) indicate that the widespread genus *Myosurus* (classified in subtribe Myosurinae after Tamura, 1995) is the closest relative of *Ranunculus* s.l. Sister to this *Myosurus–Ranunculus* clade is the Eastern Asian-North American genus *Trautvetteria* (subtribe Trautvetteriinae after Tamura, 1995; see also Ro et al., 1997). Both genera were used as outgroups in our study. Sequences for more distantly related genera (i.e., *Cimicifuga*, *Trollius*, *Anemone*, *Anemonopsis*, and *Caltha*) were difficult to align unambiguously with *Ranunculus* species and were not used.

2.2. DNA extraction, sequencing, and alignment

At the Institute of Botany of the University of Vienna, DNA extraction was performed as in Doyle and Doyle (1987); some samples were extracted using the DNeasy extraction kit (Qiagen), following the manufac-

Table 2
Materials used in this study

Genus	Species	Author	Add. prov.	Provenance of sample used/source	Collector(s)	Herbarium or collection No.	Herbar	GenBank Accession No.	Chromosome No. (2n)	Source of chromosome No.
Aphanostemma (=*R. apiifolius*)	*apiifolia*	(Pers.) St.-Hil.		Chile	C. Lehnebach	s.n.	VALD	AY680092	32	1
Arcteranthis (=*R. cooleyae*)	*cooleyae*	(Vasey & Rose) Greene		Canada	U. Jensen	28432	MPN	AY680201	16	2
Callianthemoides (=*R. semiverticillatus*)	*semiverticillatus*	(Philippi) Tamura	(2)	Argentina	C. Lehnebach	s.n.	VALD	AY680199	—	—
Ceratocephala (=*R. falcatus*)	*falcata*	(L.) Pers.		Iran	K.H. Rechinger	50857	W	AY680191	40	4
Ceratocephala (=*R. testiculatus*)	*orthoceras*	DC.		Austria	E. Hörandl	3837	WU	AY680190	14	4
Halerpestes (=*R. cymbalaria*)	*cymbalaria*	(Pursh) Greene	(2)	cult. Rezia BG	J.T. Johansson	204	LD	AY680196	16	4, 3
Myosurus	*minimus*	L.		Genbank				U96041, U96042	16	1
Myosurus	*minimus_2*	L.		Genbank				AJ347913	16	1
Oxygraphis	*glacialis*	(Fischer ex DC.)	Bunge	Alaska	R. Elven et al.	SUP02-184	O	AY680198	16	4, 3
Peltocalathos (= *R. baurii*)	*baurii*	(McOwan) Tamura		South Africa	L. Mucina	030103/22	WU	AY680200	—	—
Ranunculus	*abortivus*	L.		USA, Connecticut	J.T. Johansson	186	CONN	AY680048	16	4, 1
Ranunculus	*acaulis*	DC.		Australia, Tasmania	D. Havell	24592	MPN	AF323319	48	1
Ranunculus	*acetosellifolius*	Boiss.		cult. Gothenburg BG	J.T. Johansson	s.n.	—	AY680075	16	3
Ranunculus	*aconitifolius*	L.		cult. Copenhagen BG	J.T. Johansson	274	LD	AY680081	16	4, 3
Ranunculus	*acris*	L.	(1)	cult. Bonn BG	J.T. Johansson	194	CONN	AY680167	14	4, 3 (also $2n = 28$, 16)
Ranunculus	*adoneus*	A. Gray		USA, Colorado	F. Ehrendorfer	FER70	WU	AY680030	16	2
Ranunculus	*aduncus*	Gren. & Godr.		Italy	E. Hörandl	6818	WU	AY680088	16	1
Ranunculus	*allemannii*	Br.-Bl.		Austria	E. Hörandl	6687	WU	AY680039	48*	3
Ranunculus	*alpestris*	L.		cult. Rezia BG	J.T. Johansson	242	LD	AY680078	16	4, 3
Ranunculus	*altaicus*	Laxm.	(1)	Russia, Altai	A. Tribsch	9545	WU	AY680112	16	1
Ranunculus	*amerophyllus*	F.Muell.		Papua New Guinea	P. van Royen	8105969	CBG	AY680146	—	—
Ranunculus	*amplexicaulis*	L.		cult. Lund BG	J.T. Johansson	222	LD	AY680071	16	1
Ranunculus	*andersonii*	A. Gray	(1)	cult. Gothenburg BG	J.T. Johansson	s.n.	—	AY680197	—	—
Ranunculus	*anemoneus*	F. Muell.		Australia (NSW)	A.J. Whalen	9704217	CBG	AF323273	—	—
Ranunculus	*apenninus*	(Chiov.) Pign.		Italy	E. Hörandl	6069	WU	AY680091	16	1
Ranunculus (= *R. affinis*)	*arcticus*	Richards.		cult. Devonian BG	J.T. Johansson	239	LD	AY680049	48	4
Ranunculus	*arvensis*	L.		cult. Kiel BG	J.T. Johansson	180	CONN	AY680177	32	4, 3, 1
Ranunculus	*baldschuanicus*	Regel ex Kom.		cult. Copenhagen BG	J.T. Johansson	272	LD	AY680174	14, 28	4, 1
Ranunculus	*basilobatus*	H. Eichler ex P.van Royen	New Guinea	K. Kerenga	358347	CANB	AY680131	—	—	
Ranunculus	*bilobus*	Bertol.		Italy	E. Hörandl	4574	WU	AY680077	16	3
Ranunculus	*biternatus*	Sm.		Chile	C. Lehnebach	s.n.	VALD	AY680061	48	1, 10
Ranunculus	*bonariensis*	Poir.	(1)	Chile	C. Lehnebach	s.n.	VALD	AY680183	—	—
Ranunculus (=*R. acris* ssp. *borealis*)	*borealis*	Trautv.		USSR	Unknown	420039	CANB	AY680168	14, 14, 28	3, 1
Ranunculus	*brassii*	H. Eichler		New Guinea	R.D. Hoogland & R. Schodde	91542	CANB	AY680127	—	—
Ranunculus	*brevifolius* ssp. *brevifolius*	Ten.		cult. Gothenburg BG	J.T. Johansson	s.n.	—	AY680187	16	3
Ranunculus	*breyninus*	Cr.		Austria (loc. class.)	E. Hörandl	5249	WU	AY680115	16	4, 3

(*continued on next page*)

Table 2 (*continued*)

Genus	Species	Author	Add. prov.	Provenance of sample used/source	Collector(s)	Herbarium or collection No.	Herbar	GenBank Accession No.	Chromosome No. (2n)	Source of chromosome No.
Ranunculus (=*R. oreophilus*)	*breyninus_K*	Cr.		Georgia (Caucasus)	E. Hörandl	8361	WU	AY680116	—	—
Ranunculus	*brotherusii*	Freyn		Nepal	M. Staudinger	484280	LI	AY680037	—	—
Ranunculus	*buchanani*	Hook. f.	(1)	New Zealand	D. Glenny	509922	CHR	AF323280	48	5
Ranunculus	*bulbosus* ssp. *bulbosus*	L.		Sweden	J.T. Johansson	s.n.	—	AY680124	16	3
Ranunculus	*bullatus*	L.		Greece	E. Hörandl & W. Gutermann	7191	WU	AY680114	16	4, 3
Ranunculus	*calandrinioides*	Oliver		cult. Gothenburg BG	J.T. Johansson	240	LD	AY680073	16	
Ranunculus (= *Beckwithia camissonis*)	*camissonis*	Aucl.		USSR	R. Koropewa	s.n.	W	AY680083	—	—
Ranunculus	*cantoniensis*	DC.		cult. Tokyo BG	J.T. Johansson	197	LD	AY680126	16, 32*	1
Ranunculus	*cappadocicus*	Willd.		Georgia, Kaukasus	E. Hörandl	8269	WU	AY680117	16	4
Ranunculus	*caprarum*	Skottsb.		Juan Fernandez Isl.	A. Landero	9355	OS	AY680151	—	—
Ranunculus	*cardiophyllus*	Hook. f.		cult. Gothenburg BG	J.T. Johansson	HZ 86-29	—	AY680045	32, 64	2, 4
Ranunculus	*carinthiacus*	Hoppe		Austria	E. Hörandl	4096	WU	AY680093	16	6, 3
Ranunculus	*carpaticola*	Soó		Slovakia	E. Hörandl	8483	WU	AY680041	16	9
Ranunculus	*carpaticus*	Herbich		Romania	O. Paun	s.n.	WU	AY680096	16	3
Ranunculus	*carpinetorum*	Hörandl & Guterm.		Austria	E. Hörandl	5588	WU	AY680031	32*	7
Ranunculus	*cassubicifolius*	W. Koch		Germany	E. Hörandl	8477	WU	AY680040	16	7
Ranunculus	*caucasicus*	MB.		Georgia, Kaukasus	E. Hörandl	8259	WU	AY680178	16	3, 1
Ranunculus	*chilensis*	DC.		Chile	C. Lehnebach	s.n.	VALD	AY680157	—	—
Ranunculus	*chius*	DC.		Greece	W. Gutermann	34758	WU	AY680176	14	4, 1
Ranunculus	*cicutarius*	Schlecht.		Iran	H. Akhani	320156	LI	AY680103	—	—
Ranunculus	*circinatus*	Sibth.		Germany	H. Lehmann	24674	MPN	AF323321	16	3
Ranunculus	*collinus*	DC.		cult. Canberra BG	Crisp & Telford	2227	CAN	AY680059	48	1
Ranunculus	*collonorum*	Endl.		Australia, WA	T.R. Lally	468358	CANB	AY680139	16	8
Ranunculus (= *R. lomatocarpus*)	*cornutus*	DC.		Azerbeidschan	G. Schneeweiß	6806	WU	AY680153	—	—
Ranunculus	*cortusifolius*	Willd.		cult. Halle BG	J.T. Johansson	237	LD	AY680101	16	4
Ranunculus	*crassipes*	Hook. f.		Australia, Macquarie Island	D. Bergstrom	MPN28433	MPN	AY680060	—	—
Ranunculus	*crenatus*	Waldst. & Kit.	(1)	Austria	E. Hörandl	2818	WU	AY680086	16	4, 3
Ranunculus	*crithmifolius* ssp. *crithmifolius*	Hook. f.		New Zealand	D. Glenny	509774	CHR	AF323311	48	5
Ranunculus	*dissectifolius*	Benth.		Australia, NSW	E.M. Canning	54437	CBG	AY680144	—	—
Ranunculus	*eichleranus*	Briggs		Australia, Vic.	C. Totterdell	349737	CANB	AY680138	16	8
Ranunculus	*enysii*	T.Kirk	(1)	New Zealand	D. Glenny	509805	CHR	AF323316	48	1
Ranunculus	*eschscholtzii*	Schlecht.		Canada	U. Jensen	UJ8	MPN	AY680050	32, 48	4
Ranunculus	*ficaria* ssp. *bulbilifer*	Lambinon		Sweden	J.T. Johansson	s.n.	—	AY680192	24, 32, 32	3, 4
Ranunculus	*flagelliformis*	Sm.	(2)	Peru	P. Gute, G. Müler	309853	LI	AY680182	48	1
Ranunculus	*flammula*	L.		cult. Oldenburg BG	J.T. Johansson	193	CONN	AY680185	32	4, 3
Ranunculus	*fluitans*	Lam.		Sweden	J.T. Johansson	s.n.	—	AY680069	16, 24, 32	3
Ranunculus	*fuegianus*	Speg.		Chile	C. Lehnebach	s.n.	VALD	AY680064	48	10
Ranunculus	*garganicus*	Ten.		Italy	W. Gutermann	34974	WU	AY680107	16	1
Ranunculus	*gayeri*	Soó		Austria	W. Gutermann	23542	WU	AY680028	32*	7
Ranunculus	*gelidus*	Kar. & Kir.		Xinjiang, China	B. Wang	28426	MPN	AY680054	16	2
Ranunculus	*glacialis*	L.		Sweden	J.T. Johansson	s.n.	—	AY680082	16	4, 3
Ranunculus	*gmelini* ssp. *gmelini*	DC.		USA, Alaska	C. Schröck	454907	LI	AY680063	16, 32, 64, 64	3, 2, 4

Ranunculus	*godleyanus*	Hook. f.		New Zealand	D. Glenny	499398	CHR	AF323309	48	5
Ranunculus	*gouanii*	Willd.		cult. Schachen	J.T. Johansson	s.n.	—	AY680098	16	4
Ranunculus	*gracilipes*	Hook. f.		New Zealand	D. Glenny	529048	CHR	AF323314	48	1
Ranunculus	*gracilis*	Schleich.		Greece	J.T. Johansson	s.n.	—	AY680120	16	3
Ranunculus	*grahami*	Petrie		New Zealand	A.C. Archer	217997	CHR	AF323286	—	—
Ranunculus	*gramineus*	L.		cult. Krefeld BG	J.T. Johansson	s.n.	—	AY680076	16	4, 3
Ranunculus	*granatensis*	Boiss.		unknown	J.T. Johansson	266	LD	AY680165	28	4, 3
Ranunculus	*grandiflorus*	L.		Georgia (Kaukasus)	E. Hörandl	8271	WU	AY680053	14,28	1
Ranunculus	*grandifolius*	C.A.Mey.		Xinjiang, China	B. Wang	28422	MPN	AY680169	28	1
Ranunculus	*graniticola*	Melville	(2)	Australia, NSW	A.J. Whalen	9704209	CBG	AY680141	16	4
Ranunculus	*gregarius*	Brot.		cult. Berlin-Dahlem BG	J.T. Johansson	232	LD	AY680100	16	4
Ranunculus	*gunnianus*	Hook. f.		Australia, NSW	Donaldson, S.	9501186	CBG	AF323298	48	1
Ranunculus	*haastii* ssp. *haastii*	Hook. f.		New Zealand	D. Glenny	532284	CHR	AF323285	48	5
Ranunculus	*hirtellus*	Royle		Nepal	F. Tod	372997	LI	AY680038	32	1
Ranunculus	*hybridus*	Biria		cult. Gothenburg BG	J.T. Johansson	s.n.	—	AY680189	16	1
Ranunculus	*hydrophilus*	Bunge		Chile	C. Lehnebach	s.n.	VALD	AY680181	32	10
Ranunculus	*hyperboreus*	Rottb.		Sweden	J.T. Johansson	s.n.	—	AY680065	32	3, 1
Ranunculus	*illyricus*	L.		Sweden	Lundgren	s.n.	—	AY680119	16, 32, 32	3, 4
Ranunculus	*induratus*	ined.		New Zealand	P.G. Jones	n/n	MPN	AY680125	—	—
Ranunculus	*insignis*	Hook. f.		New Zealand	D. Glenny	24605	MPN	AF323306	48	4
Ranunculus	*japonicus*	Thunb.		China	XieLei	XL200348	WU	AY680164	14	1
Ranunculus	*kochii*	Ledeb.		cult. Gothenburg BG	J.T. Johansson	s.n.	—	AY680193	—	—
Ranunculus	*kuepferi* ssp. *orientalis_1*	W. Huber		Italy	P. Schönswetter & A. Tribsch	2213	WU	AY680084	24, 32, 40*	1
Ranunculus	*kuepferi* ssp. *orientalis_2*	W. Huber		Austria	E. Hörandl	4336	WU	AY680085	24,32,40*	1
Ranunculus	*laetus*	Salisb.		Nepal	E. & J. Hüttinger	372985	LI	AY680172	28	4
Ranunculus	*lanuginosus*	L.		unknown	J.T. Johansson	255	LD	AY680163	28	4, 3 (also $2n = 32$)
Ranunculus	*lappaceus*	Sm.		Australia, NSW	I. Crawford	9909805	CBG	AY680140	16	4, 8
Ranunculus (= *Coptidium lapponicum*)	*lapponicus*	L.		Sweden	J.T. Johansson	s.n.	—	AY680194	16	4, 3 (also $2n = 32$)
Ranunculus	*lateriflorus*	DC.		cult. Catania BG	J.T. Johansson	235	LD	AY680179	16	4, 3
Ranunculus	*lingua*	L.		cult. Lund BG	J.T. Johansson	s.n.	—	AY680184	128	4, 3, 1
Ranunculus	*longicaulis*	C.A.Mey.		Pakistan	A. Millinger	470564	LI	AY680051	32	1
Ranunculus	*longicaulis var. nephelogenes* (= *R. nephelogenes*)	Edgeworth			Q.S. Zheng	28420	MPN	AY680052	—	—
Ranunculus	*lowii*	Stapf		Malaysia, Sabah	C.B. Christie	n/n	MPN	AY680128	—	—
Ranunculus	*lyallii*	Hook. f.		New Zealand	M.A. Steel	24603	MPN	AF323277	48	5
Ranunculus	*maclovianus*	Urv.		Chile	C. Lehnebach	s.n.	VALD	AY680158	48	10
Ranunculus	*marginatus*	Urv.		cult. Copenhagen BG	J.T. Johansson	286	LD	AY680150	32, 16	4, 3
Ranunculus	*marschlinsii*	Steud.		Corse	E. Hörandl	6981	WU	AY680089	16	3
Ranunculus	*melzeri*	Hörandl & Guterm.		Austria	E. Hörandl	6013	WU	AY680036	—*	—
Ranunculus	*membranaceus*	Royle		China, Tibet	Q.S. Zheng	28419	MPN	AY680056	—	—
Ranunculus	*micranthus*	Nutt.		USA, Ohio	A. Lonsing	50563	LI	AY680042	16	2
Ranunculus	*millanii*	F. Muell.	(2)	Australia, NSW	R.H. Jones	9400244	CBG	AY680134	16	1
Ranunculus	*millefoliatus*	Vahl		cult. Graz BG	J.T. Johansson	293	LD	AY680108	16, 32, 16	3, 4
Ranunculus	*minutiflorus*	Bert. ex Phil.	(1)	Chile	C. Lehnebach	s.n.	VALD	AY680156	32	10
Ranunculus	*monophyllus*	L. s.l.		Unknown	J.T. Johansson	s.n.	—	AY680043	32*	3
Ranunculus	*montanus*	Willd. s.str.		Austria	E. Hörandl	666	WU	AY680094	32*	4, 3

(*continued on next page*)

Table 2 (*continued*)

Genus	Species	Author	Add. prov.	Provenance of sample used/source	Collector(s)	Herbarium or collection No.	Herbar	GenBank Accession No.	Chromosome no. (2n)	Source of chromosome No.
Ranunculus	*muelleri*	Benth.		Australia, NSW	G. Stewart	8500935	CBG	AY680143	16	8
Ranunculus	*multifidus*	Forssk.		South Africa	L. Mucina	031102/7	WU	AY680162	—	—
Ranunculus	*multiscapus*	Hook. f.		New Zealand	D. Havell	n/n	MPN	AY680133		
Ranunculus	*muricatus*	L.		cult. Siena BG	J.T. Johansson	210	LD	AY680148	48, 32, 48,64	4, 3
Ranunculus	*nanus*	Hook. f.		Australia, TAS	R. Burns	9400881	CBG	AY680142	16	1
Ranunculus	*natans*	C.A.Mey.		Russia	A. Tribsch	9558	WU	AY680113	16,32	1
Ranunculus (=*R. bulbosus* ssp. *aleae*)	*neapolitanus*	Ten.		Greece	J.T. Johansson	224	LD	AY680123	16	3
Ranunculus	*niphophilus*	Briggs		Australia, NSW	S. Donaldson	9704590	CBG	AY680145	16	1
Ranunculus	*nivalis*	L.		Sweden	J.T. Johansson	s.n.	—	AY680046	40, 48, 56, 48	3, 4
Ranunculus	*nivicola*	Hook. f.		New Zealand	P.J. Lockhart	24608	MPN	AF323308	96*	5
Ranunculus	*notabilis*	Hörandl & Guterm.		Austria	E. Hörandl	5612	WU	AY680033	16	7
Ranunculus	*occidentalis var. hexasepalus*	Nutt.	(1)	Canada	U. Jensen	28429	MPN	AY680171	28	4
Ranunculus	*olissiponensis*	Pers.		Spain	W. Gutermann	37407	WU	AY680109	—	—
Ranunculus	*ophioglossifolius*	Vill.		cult. Nantes BG	J.T. Johansson	208	LD	AY680180	16	4, 3
Ranunculus	*pachyrrhizus*	Hook. f.		New Zealand	D. Havell	24598	MPN	AF323295	48	5
Ranunculus	*pallasii*	Schlecht.		Alaska	R. Elven & al.	SUP02-175	O	AY680195	32	3
Ranunculus	*paludosus*	Poir.		Greece	W. Gutermann	34754	WU	AY680102	16, 32	3
Ranunculus	*pannonicus*	Soó		Austria	E. Hörandl	5564	WU	AY680032	32*	7
Ranunculus	*papulentus*	Melville		cult. Canberra BG	J.T. Johansson	760141p	—	AY680058	96	1
Ranunculus	*parnassifolius* ssp. *parnassifolius*	L.		France/Spain	G. Schneeweiß	6509	WU	AY680072	16	3
Ranunculus	*parviflorus*	L.		cult. Copenhagen BG	J.T. Johansson	287	LD	AY680175	28, 32	4, 3
Ranunculus	*peduncularis*	Sm.		Chile	C. Lehnebach	s.n.	VALD	AY680154	48	10
Ranunculus	*peltatus:peltatus*	Moench		cult. Nantes BG	J.T. Johansson	206	LD	AY680068	16, 32, 48*, 16	3, 4
Ranunculus	*penicillatus: pseudofluitans*	(Dum.) Bab.		England	G. Dahlgren	BE9	LD	AY680070	24,32,40*	3
Ranunculus	*pensylvanicus*	L. f.		USA	V. Zila	447002	LI	AY680147	16	2
Ranunculus	*pilisiensis*	Soó		Hungary	E. Hörandl	6600	WU	AY680034	32*	7
Ranunculus	*pimpinellifolius*	Hook. f.		Australia, NSW	R.O. Makinson	9106374	CBG	AY680136	16	1, 8
Ranunculus	*pinguis*	Hook. f.		New Zealand	V. Nicolls	24590	MPN	AF323299	48	5
Ranunculus	*platanifolius*	L.		Norway	J.T. Johansson	277	LD	AY680080	16	3
Ranunculus	*plebeius*	DC.		Australia, NSW	I. Crawford	9914363	CBG	AY680137	16	8
Ranunculus	*pollinensis*	Chiovenda		Italy	E. Hörandl	8247	WU	AY680097	32	3
Ranunculus	*polyanthemos*	L.	(1)	Austria	E. Hörandl	5130	WU	AY680121	16	3
Ranunculus	*praemorsus*	H.B. & K. ex DC.	(1)	Argentina	L. & F. Ehrendorfer	FER19	WU	AY680161	—	—
Ranunculus	*prasinus*	Menadue		Australia, Tasmania	F.E. Davies	8900255	CBG	AY680057	48	1
Ranunculus (=*R. acris* ssp. *borealis*)	*propinquus*	C.A.Mey.		China, Xinjiang, Qinghe County	B. Wang	28424	MPN	AY680170	28	3
Ranunculus	*pseudohirculus*	Schrenk ex F.E.L. Fischer & C.A. Mey.		Russia	A. Tribsch	9593	WU	AY680111	—	—
Ranunculus	*pseudolowii*	H. Eichler		Papua New Guinea	J.F. Veldkamp	403273	CANB	AY680130	—	—
Ranunculus	*pseudomillefoliatus*	Grau		Spain	G. Schneeweiß et al.	7253	WU	AY680110	—	—
Ranunculus	*pseudomontanus*	Schur		Slovakia	E. Hörandl	5904	WU	AY680090	16	3

Ranunculus	*pseudotrullifolius*	Skottsb.		Chile	C. Lehnebach	s.n.	VALD	AY680203	48	10
Ranunculus	*psilostachys*	Griseb.		cult. Lund BG	J.T. Johansson	219	LD	AY680106	16, 32	4, 3
Ranunculus	*pygmaeus*	Wahlenb.		Sweden	J.T. Johansson	s.n.	—	AY680044	16	4, 3
Ranunculus	*pyrenaeus*	L.		Spain	G. Schneeweiß et al.	6498	WU	AY680074	16	3
Ranunculus	*recens*	T. Kirk		New Zealand	D. Havell	n/n	MPN	AF323320	48	1
Ranunculus	*recurvatus*	Bong		USA, Connecticut	J.T. Johansson	185	CONN	AY680118	32	4
Ranunculus	*repens*	L.	(2)	Sweden	J.T. Johansson	s.n.	—	AY680160	32	3
Ranunculus	*repens_2*	L.		China	B. Wang	28421	MPN	AY680152	32	3
Ranunculus	*reptans*	L.		Switzerland	Y. Willi	br3	Z	AY680186	32, 48, 32	3, 4
Ranunculus	*rufosepalus*	Franch.		Pakistan	A. Millinger	392897	LI	AY680047	—	—
Ranunculus	*rumelicus*	Griseb.		Greece	Snogerup	5993b	LD	AY680104	16, 24	3
Ranunculus	*sardous*	Cr.	(1)	Sweden	J.T. Johansson	s.n.	—	AY680122	16, 32	4, 3
Ranunculus	*sartorianus*	Boiss. & Heldr.		cult. Copenhagen BG	J.T. Johansson	271	LD	AY680095	16, 32	6, 3
Ranunculus	*saruwagedicus*	H. Eichler		Papua New Guinea	Korner	294212	CANB	AY680129	—	—
Ranunculus	*scapigerus*	Hook. f.		Australia, NSW	P. Gilmour	8702252	CBG	AY680135	16	1, 8
Ranunculus	*sceleratus*	L.		Germany	H. Lehmann	24589	MPN	AF323322	16, 32, 32	3, 4
Ranunculus	*sceleratus_2*	L.		British Columbia	U. Jensen	28428	MPN	AY680062	16, 32, 32	3, 4
Ranunculus	*scrithalis*	P.J. Garnock-Jones		New Zealand	P.J. Lockhart	24600	MPN	AF323305	—	—
Ranunculus	*seguieri* ssp. *seguieri*	Vill.		cult. Gothenburg BG	J.T. Johansson	226	LD	AY680079	16	3
Ranunculus	*serbicus*	Vis.		cult. Mühlhausen BG	J.T. Johansson	249	LD	AY680166	28	4, 3
Ranunculus	*sericocephalus*	Hook. f.		Chile	C. Lehnebach	s.n.	VALD	AY680155	48	10
Ranunculus	*sericophyllus*	Hook. f.		New Zealand	D. Glenny	530524	CHR	AF323288	48	5
Ranunculus	*sphaerospermus*	Boiss. & Blanche		Turkey	Dahlgren	B87B	LD	AY680066	16	4, 3
Ranunculus	*sprunerianus*	Boiss.		Greece	J.T. Johansson	230	LD	AY680105	16, 32	3
Ranunculus	*subscaposus*	Hook. f.		New Zealand: Auckland islands	P. J. Garnock-Jones	n/n	MPN	AY680132	—	—
Ranunculus	*tanguticus*	(Finet & Gagnep.) Hao	(2)	China	X.Q. Zhao	28417	MPN	AY680055	—	—
Ranunculus	*thora*	L.		cult. Lund BG	J.T. Johansson	223	LD	AY680188	16	4, 3
Ranunculus	*trichophyllus*	Chaix		Greece	G. Dahlgren	B23	LD	AY680067	32	4, 3
Ranunculus	*trilobus*	Desf.		cult. Antwerpen BG	J.T. Johansson	217	LD	AY680149	32, 48	3, 4
Ranunculus	*trullifolius*	Hook. f.		Chile	C. Lehnebach	s.n.	VALD	AY680159	—	—
Ranunculus	*variabilis*	Hörandl & Guterm.		Austria	E. Hörandl	5641	WU	AY680029	32*	7
Ranunculus	*velutinus*	Schur		cult. Rotterdam BG	J.T. Johansson	270	LD	AY680173	14	4, 3
Ranunculus	*venetus*	Huter ex Landolt		Italy	W. Gutermann	35349	WU	AY680087	32	3
Ranunculus	*verticillatus*	Eastw.		New Zealand	D. Glenny	530493	CHR	AF323304	48	5
Ranunculus (= *R. grenieranus*)	*villarsii*	DC.		Austria	E. Hörandl	664	WU	AY680099	16	4, 6
Ranunculus	*vindobonensis*	Hörandl & Guterm.		Austria	E. Hörandl	6602	WU	AY680035	32*	7
Ranunculus	*viridis*	H.D. Wilson & P.J. Garnock-Jones		New Zealand	D. Havell	s.n.	—	AF323297	—	—
Trautvetteria	*carolinensis*	Vail		Genbank				U96035, U96036	—	—
Trautvetteria	*grandis*	Honda		cult. California BG	J.T. Johansson	82.1322	—	AY680202	28	1
Trautvetteria	*Japonica*	Sieb. & Zucc.		Genbank				U96037, U96038	14	1

Add. Prov., number of additional sequences from other provenances (see Materials and methods); BG, Botanical Garden; n/n, voucher not yet numbered. s.n., sine numero; herbarium acronyms after Index herbariorum, see http://www.nybg.org/bsci/ih/ih.html; an asterisk following chromosome number indicates an allopolyploid species excluded in the MP analysis shown in Fig. 2.

1, Tropicos database (http://mobot.mobot.org/cgi-bin/search_vast); 2, Whittemore (1997); 3, Jalas and Suominen (1989); 4, Goepfert (1974); 5, Fisher (1965); 6, Landolt (1956); 7, Dobes and Vitek (2000); 8, Briggs (1960); 9, Hörandl and Greilhuber (2002); and 10, Moore (1983).

turer's instructions with minor modifications. Polymerase chain reactions (PCR) were performed in a 90 μl Ready PCR Master Mix with 2 μl (40 pmol) primers and 4 μl dimethyl sulfoxide (DMSO), the latter added to reduce secondary structure problems. PCR amplification was carried out in a Programmable Thermal Controller PTC-100 (MJ Research, Margaritella, BioTrade, Vienna, Austria). The entire ITS1-5.8S-ITS2 region was amplified (595–604 bp) using primers from Sun et al. (1994; AB101 and AB102). Some samples, mainly from herbarium material, were amplified using internal primers ITS 5, 2, 3, and 4 (Baldwin et al., 1995). PCR conditions were first cycle 95 °C for 4.3 min, 45 °C for 1 min, and 72 °C for 1 min, for the following 35 cycles 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min, then extension at 72 °C for 10 min PCR products were purified on 1% agarose gels using the QIAquick purification kit from Qiagen, Margaritella, Vienna, and Austria. For cycle sequencing a 10 μl volume containing 2 μl of BigDye Terminator RR mix (Applied Biosystems, Vienna, Austria), 1 μl primer (5 pmol), 6 μl of purified DNA template, and 1 μl ddH_2O were used. Cycle sequencing conditions were 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, repeated for 25 cycles and then cooled at 4 °C (GeneAmp PCR System 9700, Applied Biosystems). Sequencing was performed on an ABI PRISM 377 DNA Sequencer (ABI) following the protocol provided by the manufacturer (ABI). Both forward and reverse strands were sequenced and combined using the program Autoassembler version 1.4.0 (Applied Biosystems). At the Allan Wilson Centre in New Zealand, almost identical protocols were followed in obtaining ITS sequences. Minor differences included no DMSO for PCR and 20 μl reaction volumes. Forward and reverse strand sequences were edited using Sequencer 4.2 (Genecode).

An initial multiple sequence alignment was made using the progressive alignment procedure of CLUSTALX (Thompson et al., 1994) and then refined by hand to minimize the number of independent indels. Alignment was straightforward and required few and only short indels (aligned matrix submitted to GenBank).

2.3. Ambiguities and heteroplasmy in sequences

In 42 species ambiguous sites occurred (in most species only 1–3, in one 12). Ambiguities were most common in samples extracted from herbarium materials and in regions at the end of the forward and reverse sequences where the overlap of the two strands was incomplete. Combination of the forward and the reverse strand usually revealed no additive sites even in high polyploid or allopolyploid species. Therefore, ambiguous sites were not treated as phylogenetically informative, and excluded from the analyses.

2.4. Parsimony analyses

Maximum parsimony (MP) analyses were undertaken using PAUP* version 4.0b8 (Swofford, 2001) using the heuristic search with 1000 replicates, random sequence addition, tree-bisection-reconnection (TBR) branch swapping, MULTREES on (keeping multiple, equally parsimonious trees) but saving only 10 trees each replicate to reduce time spent in swapping on large numbers of suboptimal trees. Each nucleotide position was treated as an unordered, multistate character of equal weight (Fitch parsimony; Fitch, 1971). Indels (which typically were overlapping and only 1–2 bp in length, only one indel was 3 bp) were treated as single characters. This treatment assumed that each indel pattern represented a single evolutionary event, and had the effect of retaining phylogenetic information without overemphasizing their influence on the data's phylogenetic structure (see discussion in Simmons and Ochoterena, 2000). Coding gaps as a fifth character was preferred over treatment of gaps as missing data as indels of our data appear highly informative, resulting overall in higher levels of non-parametric bootstrap support (Felsenstein, 1985) at different levels of taxonomic relationship (results not shown). A majority rule consensus tree was computed from 600 equally most parsimonious trees (not shown). Internal support was assessed using non-parametric bootstrap analysis (Felsenstein, 1985). Bootstrap values (1000 replicates) are shown on the corresponding clades of a majority rule consensus tree of Bayesian inference analysis (Fig. 1). To test the influence of hybridization on the analysis, an alternative parsimony analysis with the same settings excluding 16 species of allopolyploid origin (see introduction and Table 2) was performed and is shown in Fig. 2.

2.5. Bayesian inference

Prior to tree building, a General Time Reversible model of substitution (GTR) with gamma distribution was identified as the best fitting symmetrical model for the sequence data (Modeltest version 3.0; Posada and Crandall, 1998). Phylogenetic reconstruction using Bayesian inference (BI) was performed with the program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Four Markov Monte Carlo chains were run simultaneously starting from random trees. The analysis was run for 2,000,000 generations sampling a tree at every 500 generations. The 3601 trees sampled after reaching stationary phase (after c. 200,000 generations) were collected and used to build a majority rule consensus tree with PAUP 4.0b8 (Swofford, 2001) which is shown as a phylogram with Posterior probability values in Fig. 1.

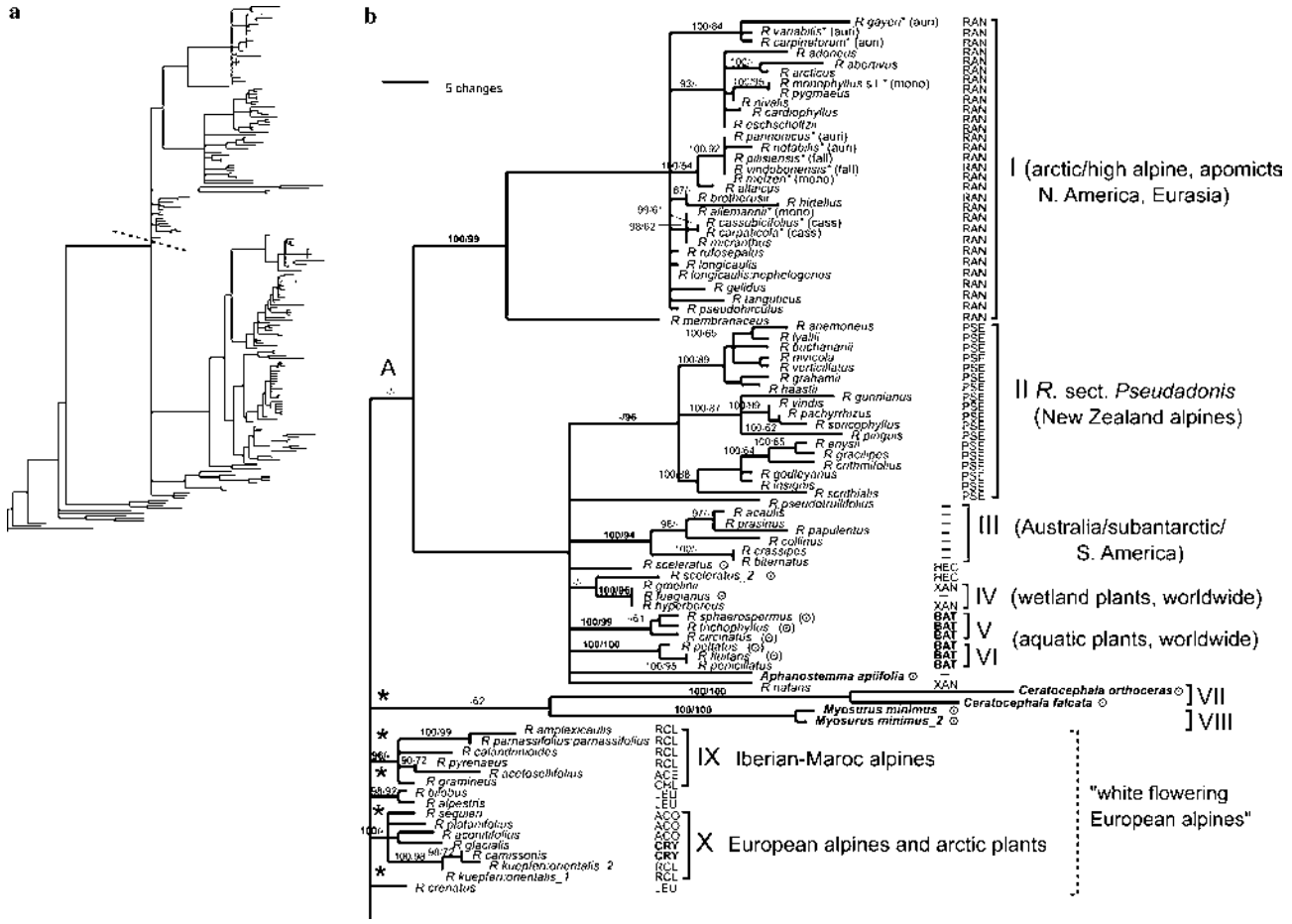

Fig. 1. Phylogram obtained from Bayesian inference analysis; (a) overview of the tree, the dashed line indicates the split of the two parts that are shown in b and c; (b) upper part of the tree; large asterisks indicate branches that were united in one clade in the maximum parsimony analysis (bootstrap support ⩽50%); (c) lower part of the tree; (b,c) posterior probability values ⩾95% (before slash) and bootstrap values ⩾ 50% of maximum parsimony analysis (after slash) are given above branches. The brackets with Roman numerals or generic or subgeneric names indicate corresponding clades in the maximum parsimony analysis. ⊙, annual; (⊙) annual or perennial; all other species are perennial. Three-letter acronyms indicate subgenera (bold) and sections of subg. *Ranunculus* after Tamura (1995); order from the top to the bottom of the tree. RAN = sect. *Ranunculus* sensu Tamura; PSE, sect. *Pseudadonis*; HEC, sect. *Hecatonia*; XAN, sect. *Xanthobatrachium*; BAT, subg. *Batrachium*; LEU, sect. *Leucoranunculus*; RCL, sect. *Ranuncella*; ACE, sect. *Acetosellifolii*; CHL, sect. *Chloeranunculus*; ACO, sect. *Aconitifolii*; CRY, subg. *Crymodes*; ACR, sect. *Acris* sensu Tamura; ECH, sect. *Echinella*; RST, sect. *Ranunculastrum*; PHY, sect. *Physophyllum*; LEP, sect. *Leptocaules*; MIC, sect. *Micranthus*; FLA, sect. *Flammula*; CAS, sect. *Casalea*; THO, sect. *Thora*; FIC, subg. *Ficaria*; COP, subg. *Coptidium*; and PAL, subg. *Pallasiantha*. Species without acronym are not mentioned in Tamura (1995). Species names in bold belong to other genera (after Tamura, 1995). *Species of the *R. auricomus* complex (collective groups in parentheses: cass, *cassubicus*; fall, *fallax*; mono, *monophyllus*; and auri, *auricomus* s.str.).

2.6. Split decomposition

Reticulate relationships as a consequence of hybridization and/or allopolyploidy can result in phylogenetic signals that are poorly represented by a bifurcating evolutionary model (Winkworth et al., in press). In an ITS sequence alignment, whose sequences have been subject to concerted evolution and/or gene conversion, these signals will be represented by incompatible site patterns across some sequence positions. Split decomposition is a network method that will display complex patterns of sequence variation that are unable to be shown by a bifurcating evolutionary model. This approach can indicate whether the data structure is bifurcate (visualized as a tree-like splits graph) or reticulate (visualized as a network-like splits graph). In the latter case, support for different relationships is identified by visualizing the splits across sets of parallel lines. This concept is illustrated in Fig. 3, where splits have been highlighted with dashed lines. For more comprehensive explanations of the method, see Huson (1998), Lockhart et al. (2001), and Winkworth et al. (in press).

For some data sets, splits graphs are helpful for interpreting whether unresolved branches in bifurcating trees are caused by incompatible site patterns or from having too few informative sites. Split decomposition has been used by Lockhart et al. (2001) and Winkworth et al. (in press) to analyze hybridization and evolutionary traits in New Zealand alpine buttercups. Unfortunately due

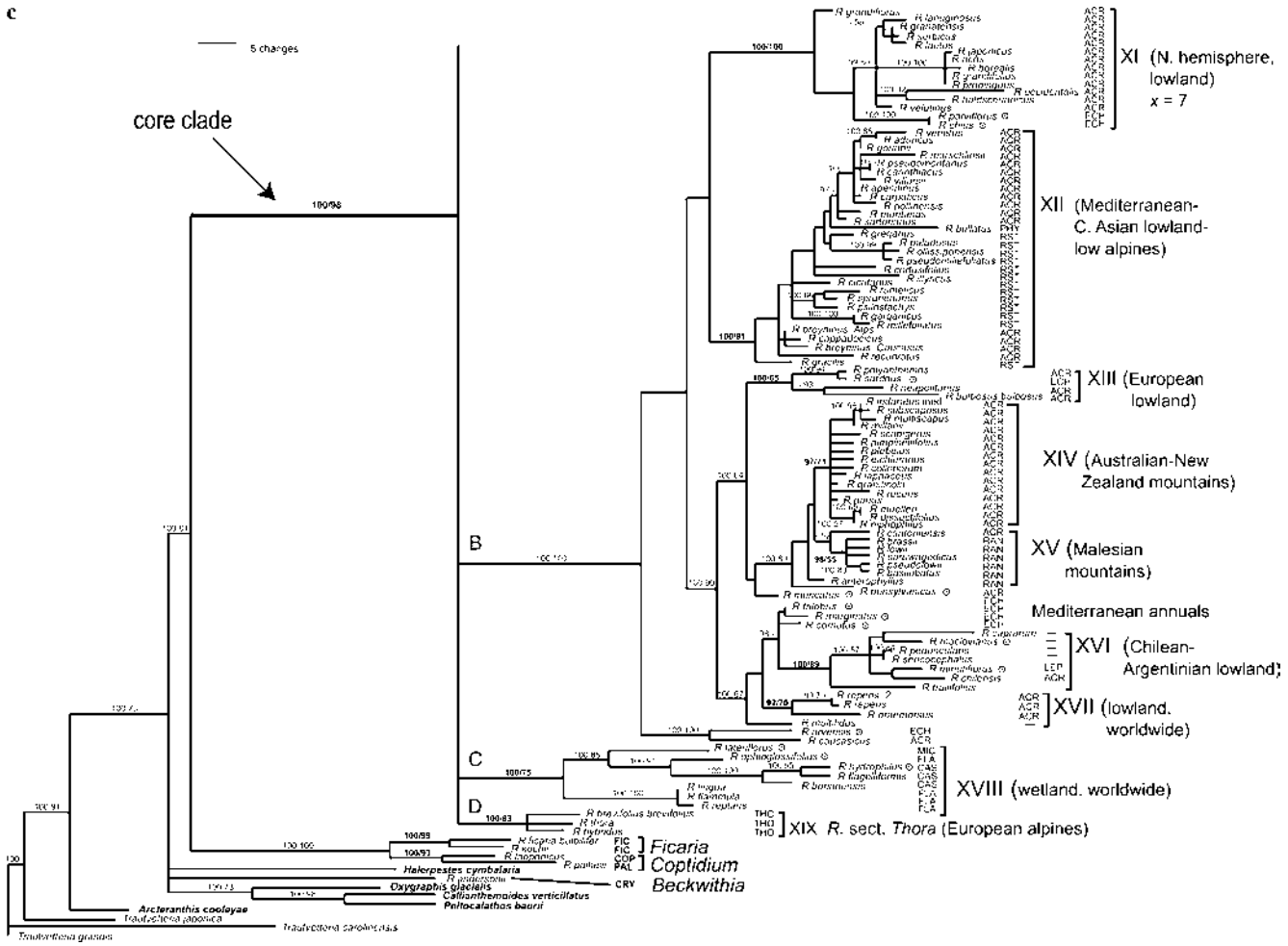

Fig 1. *(continued)*

to technical limitations in the standard implementation of the method, it is only useful for studying small data sets (up to 20 samples) where the sequences are not significantly divergent from each other (see Winkworth et al., in press, for discussion). For this reason, our analyses were performed only on selected groups of species that can be regarded as closely related because of morphology and monophyly in cpDNA restriction site analyses (Johansson, 1998): *Ranunculus* subg. *Batrachium*, the *R. auricomus* complex, and the white-flowering European alpine species (*R.* sects. *Ranuncella*, *Aconitifolii*, *Leucoranunculus*). In all these groups prior, external information from karyology, crossing experiments, and morphology indicated intensive hybridization and/or allopolyploidy.

The analyses were performed with the program SplitsTree 4.0 (Huson, 1998) using *p*-distances (Hamming distances) with gaps and ambiguous sites coded as missing data. Estimates of how well the splits graphs represented information in the sequences were made by comparing *p*-distances with splits graph distances (Winkworth et al., in press). Fit values ranging from 0 to 100% indicate how well the graph represents the information contained in the data.

3. Results

The length of the ITS sequences included in the final data matrix ranged from 595 to 604 bp, the aligned data matrix consisted of 641 bp. Heteroplasmic additivity of genomes, as documented for other genera (e.g., Kim and Jansen, 1994; Sang et al., 1995; Wu and Huang, 2004) for other genera in cases of hybridization and/or allopolyploidy, was not a common feature of our *Ranunculus* data. Evidence of heteroplasmy is apparent in intra- and interspecific comparisons of ITS sequences from the New Zealand *Pseudadonis* group. The only allopolyploid species in this group (*R. nivicola*), however, has an ITS sequence almost identical to that of the putative paternal parent (*R. verticillatus* Eastw.). Buttercups that are potentially hybrid in origin may have ITS sequences subject to gene conversion and homogenization as also observed by Mor-

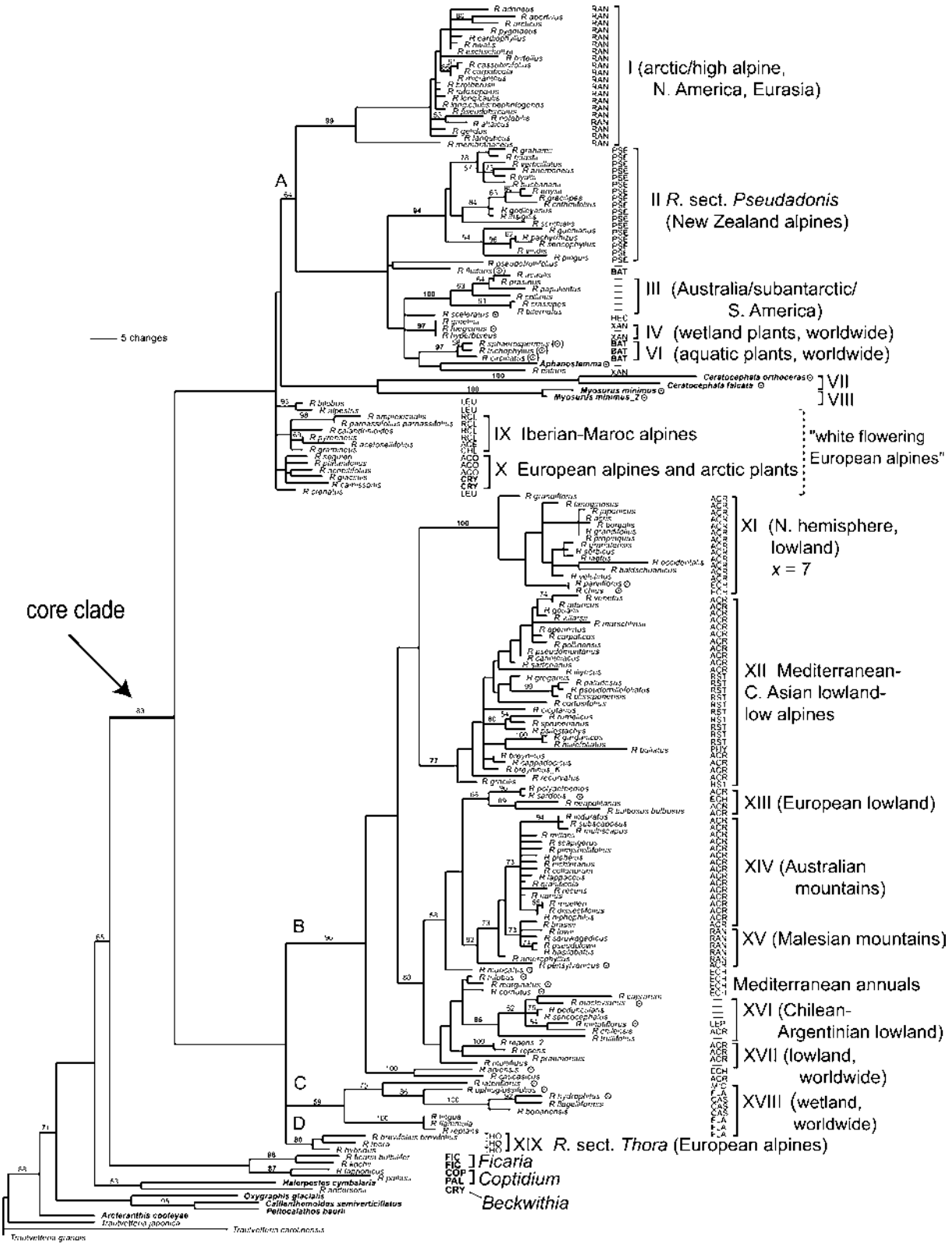

Fig. 2. Phylogram of a 50% majority rule consensus tree reconstructed using maximum parsimony; 16 allopolyploid taxa have been excluded (see Table 2). Bootstrap values are given above branches. Designations of clades, abbreviations and symbols as in Fig. 1.

rell and Rieseberg (1998) in diploid *Gilia* species of hybrid origin and Chase et al. (2003) in allopolyploid *Nicotiana* species.

By treating gaps (6.7% of the total characters) as the 5th base, 390 (61%) positions varied and 284 (44.3%) of these were potentially parsimony informative. Parsi-

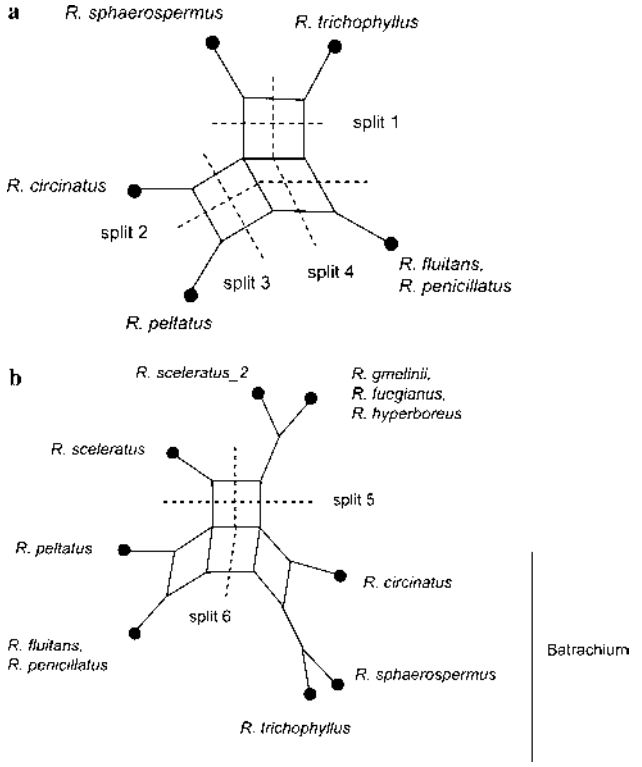

Fig. 3. (a) Splits graph of *R.* subg. *Batrachium* (clades V and VI in MP and BI trees). There are four internal splits that partition taxa into different groups in this graph. Two of these splits (3 and 4) are supported by indel patterns. They do not appear in the splits graph if it is constructed without indels. (b) Closely related aquatic buttercups (sect. *Hecatonia*, sect. *Xanthobatrachium*) have been included in this second analysis. The additional splits 5 and 6 indicate reticulate relationships between the sections. Branch lengths have equal weights in both graphs.

mony analysis yielded 600 most parsimonious trees of 1637 steps with a consistency index (CI; Kluge and Farris, 1969) of 0.39 and a retention index (RI; Farris, 1989) of 0.85.

The MP majority rule consensus tree (not shown) and BI phylogram yielded overall congruent topologies. The phylogram of BI is selected for presentation of the complete data set because of higher resolution in corresponding clades at lower levels (Fig. 1). In corresponding clades, bootstrap values of MP are usually lower than posterior probability values of BI. This observation is consistent with other empirical observations (e.g., Huelsenbeck et al., 2002) and reflects that these values measure different statistical properties of phylogenetic trees (e.g., Alfaro et al., 2003). From a practical point of view, bootstrap values are more conservative and might fail to support a true node, whereas posterior probability values are more liberal and may fail to reject a false node (Archibald et al., 2003). Therefore, only posterior probabilities $\geqslant 95\%$ are shown in Fig. 1. The alternative MP analysis excluding hybrid taxa yielded 1480 most parsimony trees with 1559 steps, CI = 0.403 and RI = 0.842. The topology of the tree is very similar to the MP analysis of the complete set of taxa in having a higher resolution of large subclades of the core clade (A–D), but also here clade A remained with bootstrap support <50 (Fig. 2). Usually only congruent clades with high support in all analyses were considered for discussion.

Split decomposition analyses (Figs. 3 and 4) indicated reticulate relationships for all analyzed groups where hybridization had been suggested in the past. As an

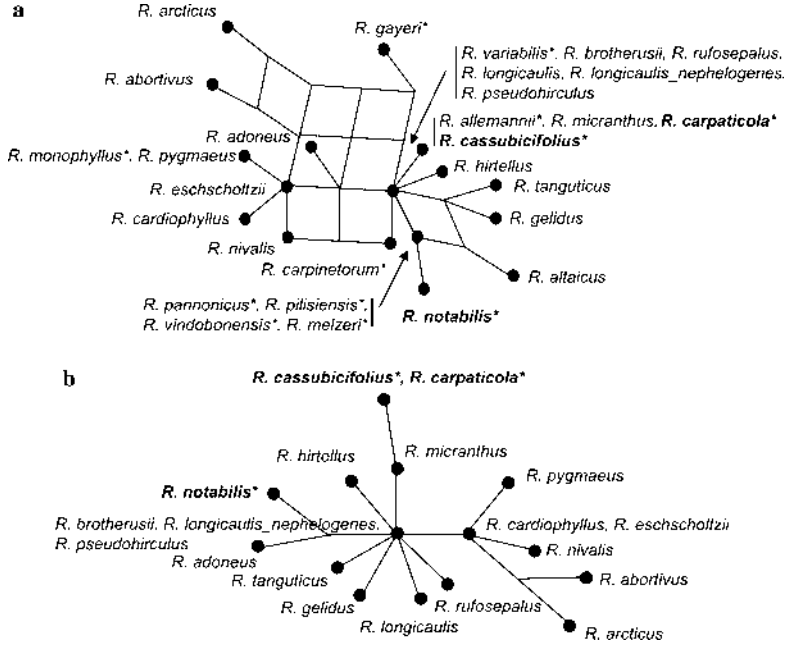

Fig. 4. Splits graphs of clade I in MP and BI. (a) All species of the apomictic *R. auricomus* group (marked with an asterisk; diploid sexual taxa in bold) have been included, Fit = 58.6%. (b) splits graph of the whole clade excluding allopolyploid agamospecies of the *R. auricomus* complex (diploid sexual taxa in bold) *R. altaicus*, Fit = 61.2%. Branch lengths have equal weights in both graphs.

example, the splits in Fig. 3a show that there is support in the data for grouping (1) *R. sphaerospermus*, *R. trichophyllus* (split 1); (2) *R. sphaerospermus*, *R. trichophyllus*, *R. circinatus* (split 2); (3) *R. circinatus*, *R. peltatus* (split 3); and (4) *R. trichophyllus*, *R. fluitans*, *R. penicillatus* (split 4). Patterns of reticulation were also apparent in split decomposition analyses of the mainly diploid white-flowering European alpine species (not shown). Considering that our ITS sequences had low divergence values, reticulations are probably not due to multiple substitutions, but more likely to hybrid relationships.

4. Discussion

Our nrITS phylogeny strongly contradicts previous morphology-based taxonomies (see Figs. 1 and 2; Table 1). Instead, it shows a high level of congruence with a recent phylogenetic reconstruction based on chloroplast DNA restriction site analyses (Johansson, 1998). For the following discussion, Tamura's (1995) classification and nomenclature is followed throughout. Most clades found in both MP and BI analyses (numbered in Roman numerals) can be characterized by morphological and/or karyological features reported in the literature and might represent a basis for a forthcoming revised classification (Figs. 1 and 2).

4.1. The core clade and allied genera

Our data show a large core clade (including *Ranunculus* subg. *Ranunculus* with all sections; see Table 1), *R.* subg. *Batrachium*, *R.* subg. *Crymodes* p.p., *Myosurus* —although defined as outgroup!—*Ceratocephala*, and *Aphanostemma*, that is in the complete data set (Fig. 1) separated with high bootstrap (98%) and posterior probability support (100%) and a very long branch length (35 changes). A series of smaller clades, including *R.* subg. *Ficaria*, *R.* subg. *Coptidium*, and *R.* subg. *Crymodes* p.p. (*R. andersonii* A. Gray) and most of the genera recognized by Tamura (1995) (*Halerpestes*, *Oxygraphis*, *Callianthemoides*, *Peltocalathos*, and *Arcteranthis*) form a grade leading to the core *Ranunculus* clade. This topology is congruent with results from Johansson (1998) and also with preliminary results from sequences of the *matK* region (Paun et al., in press). Fruit anatomy supports the restriction of the genus *Ranunculus* to the core clade: all taxa in the core clade have achenes with a sclerenchymatous layer (in *Myosurus* only weakly developed; Lonay, 1901; Tamura, 1995), which is not found in the taxa outside the core clade (with the exception of *Ficaria*).

Ranunculus pallasii Schlecht. and *R. lapponicus* L. are found together in a well supported clade and share several characters such as three sepals and a spongy tissue in the achenes. Hybridization between these two species is frequent in Nordic areas (*R.* ×*spitsbergensis* Hadac; Nilsson, 2001) further supporting uniting them into the genus *Coptidium* Nym. Of the non-core *Ranunculus*, *Ficaria* is the only exception with a well-developed sclerenchymatous layer in the fruit (Lonay, 1901). As suggested by many previous authors (e.g., Ovczinnikov, 1937, Table 1; Janchen, 1949) it is well supported as a separate genus by other distinct features (achenes with a long-cuneate base and only a rudimentary beak, three sepals, strongly dimorphic roots, and a single cotyledon; Förster, 1997). *Ranunculus andersonii*, previously classified in *R.* subg. *Crymodes*, has an utriculous fruit unlike all other Ranunculi in the core clade which have achenes (Benson, 1948). This observation supports the argument to elevate this species into a distinct genus *Beckwithia* Jeps., as already proposed by Janchen (1949). The other allied genera (*Halerpestes*, *Callianthemoides*, *Peltocalathos*, *Oxygraphis*, and *Arcteranthis*) are characterized by longitudinal, parallel, and non-reticulate prominent veins on the achenes. These are character combinations not found in any of the core species.

4.2. Core clade, subclades (A–D)

Within the core *Ranunculus* clade, the relationships of larger clades remain largely unresolved and are not completely congruent between parsimony analysis and Bayesian inference. In parsimony analyses, clade A (Fig. 2) included also some smaller clades that remained unresolved in Bayesian analysis (marked by asterisks in Fig. 1; bootstrap support of the clade in MP <50%; see Fig. 2). Both analyses left at least four unresolved major clades (MP, A+*, B-C; BI, B-D). The species within each of the major clades (A–D) generally share ecological features: clade (A) comprises mainly arctic or high alpine species, specialized to cold and extreme habitats; see (sub)clades I, II and III, IX, X. Moreover, there is a high frequency of polyploids, specializations in the reproductive system (apomixis in the *R. auricomus* group, clade I), and strong adaptations to aquatic habitats in "subg. *Batrachium*" clades V, VI, and sect. *Xanthobatrachium* (clade IV). The second main clade (B), which is well supported in both analyses, comprises mainly lowland species (clade XI) or if orophytic, then rather subalpine and specialized to grassland habitats, such as the *R. montanus* group. In clade B we find also many annuals (*R.* sect. *Echinella*) or perennials with tuberous roots (*R.* sect. *Ranunculastrum*), both mainly distributed in the meridional zones. The last two well-supported (PP = 100%) clades (C and D) comprise creeping wetland plants (C), and a group of European low alpine species (D).

These large clades are neither well characterised morphologically nor karyologically; there is a tendency for swollen achenes in clades A and D, and laterally compressed achenes in clades B and C, but other features

are not yet known. Thus, the ecological features within these major clades might be rather a result of convergent evolution and adaptations to certain habitats than of common ancestry.

At a lower taxonomic level, the trees resulting from MP and BI are largely congruent but, for the most part, do not correspond with sections of Tamura (1995). Only three sections are confirmed as monophyletic groups (*R.* sect. *Pseudadonis*, clade II; *R.* sect. *Casalea*, monophyletic within clade XVIII, and *R.* sect. *Thora*, clade XIX). Altogether, 19 clades (indicated with Roman numerals) were found in both analyses and are largely congruent with the clades found in Johansson (1998). These clades can be characterized with morphological and karyological data.

4.3. Subclades (I–XIX) in the core clade

Clade I corresponds to the core of Tamura's (1995) section "*Ranunculus*" (the name should be replaced by sect. *Auricomus* Spach, because the generitype of *Ranunculus* L. is *R. acris* L.; see Linnaean Plant Names Database, 2003). This clade comprises perennials with fibrous or string-like roots, divided basal leaves that differ considerably from the reduced, linear-segmented stem leaves, and swollen, smooth, glabrous or pubescent, bordered achenes with beaks usually much shorter than the body of the achene. At present, this clade includes only species from the Northern Hemisphere; the inclusion of ca. 40 species from the Malesian islands and ca. 20 species from Australia and New Zealand in this section by Tamura (1995) is not supported by our data (see clade XV, Fig. 1).

Within clade I, the microspecies of the predominantly apomictic *R. auricomus* complex are nested, but they do not form a monophyletic group. Diploid sexual species of the complex (*R. cassubicifolius*, *R. carpaticola*, and *R. notabilis*) appear in two distinct subclades and are very distinct, an observation consistent with the genetic divergence of sexual ancestors of the complex, as already shown by Hörandl (2004) with isozyme data. The four morphological collective groups or grades within the complex (*cassubicus*, *fallax*, *monophyllus*, and *auricomus* s.str.; Hörandl, 1998; Ericsson, 2001) are not confirmed as monophyletic groups except the "*cassubicus*" group (Fig. 1). Polytomies in clade I might be well due to conflicting sites of hybrid taxa. The apomictic *R. auricomus* group is represented by an extreme network-like splits graph (Fig. 4a). The fit statistic for this representation is relatively low (58.6%) indicating that there are incompatibilities in the data too complex for representation on a splits graph or a bifurcating tree. Interestingly, an analysis of clade I taxa including only sexual members of the section (all but *R. altaicus* Laxm.) yields a tree-like splits graph (Fig. 4b). Although the fit statistic remained low for this graph (61.2%) the reduced complexity of the figure indicates that much of the incompatibility among taxa in this clade arises from the presence of the allopolyploid apomictic species. The excluded diploid sexual *R. altaicus* has incompatibilities that are most likely due to multiple substitutions. As suggested by splits graph analysis in Figs. 4a and b and by an MP analysis without the *R. auricomus* agamospecies (Fig. 2), inclusion of the allopolyploids reduces bifurcating resolution within clade I of the MP and BI trees. This is a finding consistent with an expectation of hybrid origins for these taxa.

Clade II is one of the few which corresponds to a section (*R.* sect. *Pseudadonis*) defined by Tamura (1995). Fisher (1965) recognized the alpine species from New Zealand as a distinct natural group of perennials characterized by swollen, smooth or rugulose achenes with beaks usually at least as long as the fruit body, and often an increased number of nectaries. Geographical isolation followed by a strong adaptive radiation in the Alps of New Zealand (Lockhart et al., 2001) may have been strong factors for the distinctness of this group.

Clade III comprises a group of Australian taxa (unassigned by Tamura, 1995), some of them with a subantarctic to Southern South American distribution (*R. acaulis* DC., *R. biternatus* Sm.). The clade comprises annuals and perennials with small petals and swollen achenes.

The two samples of *Ranunculus sceleratus* L., the only species of Tamura's (1995) *R.* sect. *Hecatonia*, appear on two unresolved branches indicating that this widespread and polymorphic species shows also considerable ITS variation. One sample indicates a close relationship to a group of wetland buttercups (*R.* sect. *Xanthobatrachium*, clade IV). Tamura (1995) restricted *R.* sect. *Xanthobatrachium* to the Northern Hemisphere, but the inclusion of the Patagonian *R. fuegianus* Speg. in clade IV indicates a worldwide distribution. Species of clade IV are characterized by creeping or floating stems, a more or less pronounced heterophylly, very small, swollen, smooth achenes with short beaks, and enlarged receptacles after anthesis. The relationships with *Aphanostemma apiifolia* (Pers.) St.-Hil. (see below) and the water-buttercups (clades V and VI) need to be studied.

Ranunculus subg. *Batrachium* (clades V and VI in Fig. 1) is clearly nested within the core clade of *Ranunculus*, confirming results from chloroplast DNA data (Johansson, 1998; Paun et al., in press). Morphologically this group of widespread, annual to perennial water-plants is traditionally defined by transverse (horizontal) ridges on the pericarp, white dull flowers, and a pronounced heterophylly (Cook, 1963, 1966). In fact, none of these features is unique within the subtribe or even within *Ranunculus* s.str.: less pronounced ridges on the achenes are found also in *R. sceleratus* and might be an adaptation to aquatic habitats because these ridges are weak and provide easy breaking zones that permit a quick

rehydration of the dried fruits (Cook, 1963). Dull petals are found in several sections of *Ranunculus* and are due to a lack of a reflecting starch layer that causes the gloss in most yellow-shining buttercups (Parkin, 1928). Heterophylly is found also in *R.* sect. *Xanthobatrachium* (e.g., Young et al., 1995), in the *R. auricomus* group (e.g., Hörandl et al., 2001), and, in a less pronounced form, in more distant groups, e.g., the *R. acris* complex (Coles, 1971). In aquatic buttercups heterophylly has been developed to the extreme with laminate and capillary leaves on the same plant in some of the species; leaf shape is partly dependent on environmental conditions and highly adaptive (Cook, 1966). The nectary scale is reduced compared to other buttercups, and within aquatic buttercups the reduction of the nectary reaches complete loss of its function (Dahlgren, 1992). In this respect and in the light of our molecular analyses, *R.* subg. *Batrachium* (clades V + VI) represents most probably a highly specialized group of *Ranunculus* that does not merit generic status, as already suggested by Prantl (1887) and Cook (1963).

Unlike other molecular studies (Johansson, 1998; Paun et al., in press), tree building analyses of the ITS data did not yield the water-buttercups as monophyletic (see clades V and VI in Fig. 1). Hybridization is frequent within this group and allopolyploid origin has been suggested, e.g., for *R. penicillatus* s.l. and *R. peltatus* (Cook, 1966; Dahlgren and Cronberg, 1996). Removal of these taxa does not improve the situation, but splits off *R. fluitans* (Fig. 2) confirming complex relationships among all wetland plants in clade A. Split decomposition of aquatic buttercups (Fig. 3) confirms highly reticulate relationships among all the taxa involved, which may have also caused the split into two clades. For the species belonging to sect. *Batrachium* (Fig. 3a) the splits graph indicates that the *p*-distances (Hamming distances) calculated from the data do not fit well on a bifurcating tree with respect to the pattern of nucleotide substitution. Fig. 3b includes other closely related aquatic buttercups. This latter figure provides an explanation for why *Batrachium* does not appear as a monophyletic group in the BI and MP trees. That is, whilst there are patterns in the data that support monophyly for all species of *Batrachium* (identified by split 5) there are also some patterns in the data indicating that the group is not monophyletic (identified by split 6). The significance of these incompatibilities is not clear with current data. Thus, we caution against overemphasizing that the group is not monophyletic in the ITS trees.

Also the inclusion of *Ceratocephala* (Clade VII) and *Myosurus* (Clade VIII) in the core clade must be taken with caution. Both lineages are long branched and form a polytomy in BI analyses and a poorly supported clade (bootstrap <50%) in the MP analyses. Johansson (1998) found considerable length mutations in the plastid genome, making it impossible to align the two genera with other Ranunculi; *matK–trnL* sequence data also suggest an alternative relationship to that inferred from ITS data. In analyses of the latter, both genera are with high bootstrap/PP support sister to the core clade (Paun et al., in press). *Ceratocephala* has been treated as a separate genus by most authors (except Whittemore, 1997; Table 1) because of specific fruit characters (fruiting head falling off as a whole and not splitting into achenes; achenes with lateral bulges and a very large beak). *Myosurus* was always treated as a separate genus because of its linear leaves, scapose stems, sepals with a spur-like structure, reduced petals, strongly elongated receptacle, tail-like fruit, flattened and ridged achenes, secondary pendulous ovules and is even classified in another subtribe (Myosurinae) by Tamura (1995). Nevertheless, Janchen (1949) proposed a close relationship of *Myosurus* to *Ranunculus*, classifying both of them in the large subtribe Ranunculinae. He regarded *Myosurus* as very derived because of such features as annual life form and secondary pendulous ovules. The molecular data and some morphological features (achenes with a sclerenchymatous layer) suggest that both genera originated from *Ranunculus*, but considerable branch lengths in our ITS trees, and several derived morphological features indicate that evolution of both genera may be very advanced or may have involved processes more complex than that of their relatives in the nrITS tree. The grouping of *Ceratocephala* and *Myosurus* in one clade, which is weakly supported in MP but not in BI, might be due to long branch attraction, caused by the successive random reversals of base substitutions together with other mutations, which can force even distantly related taxa together. The distinct morphological and karyological features clearly separate them from each other and justify treating them as separate genera.

The monotypic, South-American genus *Aphanostemma* is also nested within the core clade and either in a large unresolved clade with clades II–VI (Fig. 1), or sister to *R. natans* C.A. Mey. (Fig. 2). *Aphanostemma apiifolia* (=*Ranunculus apiifolius*) is an annual growing in ephemeral ponds, characterized by a reduction of petals to nectary scales, but with a typical *Ranunculus* achene (with a sclerenchymatous layer and without longitudinal ridges). With our data, this species also seems to have evolved out of *Ranunculus* and specialized in floral features. It can hardly be regarded as a primitive species as suggested by Tamura (1995). Prantl (1887) suggested a close relationship with *R. sceleratus* and *R. abortivus* L. because of the small petals (shorter than sepals), a concept more in line with our results.

Clades IX and X in Fig. 1 and the unresolved branches of *R.* sect. *Leucoranunculus* comprise European alpine species with white or pale yellow flowers (without a light-reflecting starch layer), and more or less fleshy roots (“white-flowering European alpines”).

Prantl (1887) united these species under *R.* sect. *Hypolepium*, and in Johansson's (1998) cpDNA restriction site analyses these taxa appeared in one weakly supported clade (BS = 64). A close relationship is also indicated by hybridization over the sections (Huber, 1988) and similar karyotypes (Goepfert, 1974; Diosdado and Pastor, 1993). Clade IX corresponds to a group of species having undivided, linear to ovate leaves and with parallel venation, doubled nectary scales, and elongated receptacles in fruit. *Ranunculus acetosellifolius* and *R. gramineus* have each been classified in monotypic sections; the former because of tuberous roots and hastate leaves, the latter because of yellow flowers and sculptured achenes. Our molecular data suggest a close relationship of both species with sect. *Ranuncella* sensu Tamura (1995). Moreover, all species in clade IX occur in the Iberian Peninsula and in the mountains of Maroc (Atlas), further supporting a common geographical origin.

Clade X is a mixture of Tamura's (1995) *R.* sect. *Aconitifolii*, *R.* subg. *Crymodes* p.p. and one species of *R.* sect. *Ranuncella* (*R. kuepferi*). Tamura's (1995) subg. *Crymodes* includes *R. glacialis* L. (an arctic and high alpine species of Europe and Greenland), *R. camissonis* Aucl. (Beringian region) and the North American *R. andersonii* (Rocky Mountains; = *Beckwithia*); the latter is found outside the core clade (see above). This classification was mainly based on only one common feature, sepals persisting during the fruit stage and surrounding the achenes like a cup. Our phylogenies indicate this is a case of convergence and perhaps an adaptation to extremely cold temperatures to protect the young achenes. In fact, not only the cpDNA data from Johansson (1998), but also fruit anatomy supports the complete split of the subgenus observed here (see above). On the other hand, a close relationship of *R. glacialis* and sect. *Aconitifolii* (group X), which was already proposed by Janchen (1949), is confirmed by (rare) hybridization of *R. glacialis* and *R. aconitifolius* L. (Huber, 1988), and by such common features as distinctly bordered or winged achenes. A problematic taxon within this clade is the polyploid and apomictic (aposporous) *R. kuepferi* subsp. *orientalis* W. Huber from the Alps, which resembles morphologically the diploid *R. pyrenaeus* L. (Pyrenees) and has been classified within sect. *Ranuncella* (Tutin and Cook, 1993). suggest a close relationship with *R.* sect. *Aconitifolii* (clade X). Both subspecies of *Ranunculus kuepferi* hybridize with the members of this section both in the field and in experimental crosses (Huber, 1988). Based on our results, it is hypothesized that the tetraploid *R. kuepferi* originated from hybridization between a diploid *kuepferi*-ancestor and a diploid member of sect. *Aconitifolii*, and overcame hybrid sterility via apomixis.

Ranunculus crenatus Waldst. & Kit. does not appear in a clade with *R. bilobus* Bertol. and *R. alpestris* L. (all sect. *Leucoranunculus* sensu Tamura, 1995), which may correspond to Baltisberger's (1994) two subunits within the section, one with a distinct nectary scale (*R. crenatus*, *R. magellensis* Ten.) and one without a nectary scale, but only a ridge (*R. alpestris*, *R. bilobus*, and *R. traunfellneri* Hoppe).

Removal of allopolyploid *R. kuepferi* from MP analysis (Fig. 2) does not increase resolution of relationships of white flowering European alpines. Split decomposition of these species yielded network-like splits graphs with and without *R. kuepferi* subsp. *orientalis*, indicating that conflicting sites and a possibly complex pattern of relationships exist between some of the remaining diploid species.

In the second large clade (B), none of Tamura's sections are monophyletic. Most striking is the extreme split of *R.* sect. *Echinella*, a morphologically well-defined group of annuals with spiny or tuberculate fruits. In our trees, a well-supported clade (XI) appears, also found by Johansson (1998), and consists of members of *R.* sect. *Acris* and *R.* sect. *Echinella*. All species in this clade have a basic chromosome number $x = 7$, whereas all other *Ranunculi* have $x = 8$ (see also Table 2). Also *Ceratocephala* has $x = 7$, but Goepfert (1974) has shown that the karyotype of *Ceratocephala* is unique within the subtribe in having five metacentric chromosomes, whereas all other *Ranunculi* s.str. have a maximum of four; species of clade XI have only two to three and a predominance of acrocentric chromosomes. These findings suggest a parallel reduction of the basic chromosome number from eight to seven rather than a closer relationship.

Clade XI comprises widespread species of lowlands, mainly of grassland and forest habitats; the two annual Mediterranean "Echinellas," *R. parviflorus* L. and *R. chius* DC., prefer humid, shady habitats in cultivated land and might have evolved out of this group. Morphologically, clade XI can be well defined by compressed, bordered achenes, terete pedicels, erect sepals and glabrous receptacles. Thus, it might be argued to restrict *R.* sect. *Acris* to this well-defined clade as opposed to the heterogeneous and obviously polyphyletic large section "*Acris*" sensu Tamura (1995). This section should be named *R.* sect. *Ranunculus* because *R. acris* L. is the generitype of *Ranunculus* L. (see Linnaean Plant Names Database, 2003).

Clade XII comprises mainly lowland to subalpine, Mediterranean and Central European species, previously referred to *R.* sects. "*Acris* " sensu Tamura ("*R. montanus* group") and *Ranunculastrum*. The so-called *R. montanus* group (Landolt, 1954, 1956) comprises species of low alpine grasslands, distributed in the European mountain system (Pyrenees, Apennines, Alps, Carpathians, Dinarids, and Balkans). The group is characterized by fleshy string-like roots, a elongated rhizome, glabrous receptacles except an apical bundle of hairs, and compressed, bordered achenes. The group is not monophyletic, because *R. breyninus* Cr. (=*R. oreo-*

philus MB.; for nomenclature see Aeschimann and Heitz, 1996) and *R. cappadocicus* Willd. are split off from the others. *Ranunculus breyninus* was included by Landolt (1954, 1956) in the *R. montanus* group but already recognized as distinct from the others in having an apically hairy rhizome, a hairy receptacle throughout, and swollen achenes. This species has its centre of morphological diversity in the Caucasus region (Hörandl, unpubl.), whereas the rest of the *R. montanus* group has diversified within Europe.

Within clade XII, between the *R. montanus* groups (BI) or intermingled with them (MP) we find most species of Tamura's (1995) sect. *Ranunculastrum*, characterized mainly by perennial life form, dimorphic, fibrous and often strongly tuberous roots, a postfloral elongated receptacle, and strongly compressed, tuberculate, spiny or foveolate achenes. These species occur mainly in the lowlands or in low mountain areas of the meridional zone with a tendency to dry, grassy or deserted habitats. These "Mediterraneans" and the *R. montanus* group s.l. together form a well-supported clade that was also found by Johansson (1998). They share also a similar karyotype (Goepfert, 1974). These groups may have had a common, mediterranean to southwestern Asian origin, and perhaps diversified later with adaptations of the underground parts to either dry lowland or mesic subalpine habitats.

The sister clade to this mainly Eurasian clade (XI, XII) consists of three smaller subclades (XIII–XVII). Clade XIII comprises European perennial grassland or forest species that are characterized by sulcate pedicels, hairy receptacles, and compressed, bordered achenes with short beaks. A close relationship between the *R. polyanthemos* group and *R. bulbosus* is confirmed by a similar karyotype (Goepfert, 1974); experimental crosses yielded hybrids which had at least a reduced fertility (Baltisberger, 1981).

Clade XIV includes lowland to subalpine species distributed from Australia (*R. lappaceus* group; Briggs, 1960, 1962) to New Zealand and is characterized by compressed, bordered achenes with long beaks (ca. as long as fruit body). A close relationship among these taxa supports a model of recent diversification, as earlier proposed by Briggs (1962). Previous studies (Briggs, 1960, 1962) also suggest that hybridization and adaptation may be important factors in the evolution of the *R. lappaceus* group. Further investigation of reticulate evolution and adaptive divergence among these alpine and subalpine habitat specialists, including split decomposition analysis, is presented in Armstrong et al. (2004).

Clade XV comprises seven species from the Malesian mountains having ± swollen fruits and shorter beaks, and a tendency to scapose stems. Resolution within these groups is low and indicates that adaptive radiation and rapid speciation led to the diversification in these regions (in Malesia ca. 40 species reported by Eichler, 1958). The South-Eastern Asian allopolyploid *R. cantoniensis* is here sister to all remaining taxa in clade XV with weak to no support, indicating a possible geographical connection. Removal of *R. cantoniensis* does not change the topology of the remaining clade (Fig. 2). The relationships of the two Australasian clades with the North American *R. pensylvanicus* L. f. need to be studied.

As sister to clade XV an only partly resolved group of mainly Mediterranean species, previously referred to *R.* sect. *Echinella*, is found. The taxa share pericolpate pollen (*R. cornutus* group sensu Santisuk, 1979 contrary to the "*parviflora* group" with periporate pollen that is found in clade XI).

The sister to this Mediterranean "*cornutus*" group is a well-supported clade of Chilean to Argentinian taxa (clade XVI). The geographical grouping is quite contradictory to previous classifications: Tamura (1995) assigned *R. chilensis* DC. to sect. *Acris*, *R. minutiflorus* Bert. ex Phil. to *R.* sect. *Leptocaules*, which he regarded as a Southern hemispheric group of annuals. Skottsberg (1922) hypothesized a relationship of *R. caprarum* Skottsb. (endemic to Juan Fernández islands) to the New Zealand alpine species (*R.* sect. *Pseudadonis*) because of the habit and its unusual large fruits, or to species from Hawaii. The geographical grouping of the taxa in clade XVI is supported by common morphological features such as rather narrow petals (oblong to oblong-spatheolate, in most other buttercups of the core clade obovate); the achenes, regardless of remarkable differences in size, are compressed and have distinct margins or wings tapering into broad beaks, and a fine foveolate surface. These fruit characters confirm a close relationship to the "*cornutus* group" and also to clades XIV–XVI, whereas a close relationship to New Zealand alpines can be clearly rejected; possible relationships to Pacific species need to be studied (material not yet available).

The European species of clade XVII are temperate forest and grassland plants, morphologically similar to clade XIII, in all likelihood another case of parallel evolution. Crosses between *R. repens* (XVII) and *R. bulbosus* (XIII) yielded no seed set, whereas crosses of species within clade XIII (*R. repens* × *R. polyanthemos*) showed at least some fertility (Coles, 1973). The inclusion of the South American *R. praemorsus* H.B. & K. ex DC. in clade XVII indicate a worldwide distribution of the group. *Ranunculus multifidus* Forssk., a species widespread in Africa, is sister to the clade and represents the "*cornutus*" group. Association of this species with the Chilean clade and the "*repens*" clade indicates a geographical connection between these South American, African, and Eurasian taxa.

Sister to all remaining taxa in clade B is a well-supported clade of the annual *R. arvensis* L. (*R.* sect. *Echinella*) and the perennial *R. caucasicus* MB. (*R.* sect.

Acris). *Ranunculus arvensis* has the typical flat spiny fruit of sect. *Echinella*, but with a very peculiar xylem anatomy in having a pinnate venation of the pericarp with several ramifications, a characteristic not found in other species of sect. *Echinella* or other European Ranunculi (Trzaski, 1999). The pollen is periporate as in *R. parviflorus* and *R. chius*. The species is most probably isolated from the other annuals and clusters with *R. caucasicus*, which resembles morphologically the species in clade XVII, only because its closer (Asian?) relatives are not included here.

Clade XVIII is supported in both parsimony and Bayesian analyses and comprises three sections: the monotypic *R.* sect. *Micranthus* (Eurasia), *R.* sect. *Flammula* from the northern hemisphere (not monophyletic here), and *R.* sect. *Casalea* from South America (here confirmed as monophyletic). The three sections consist of creeping or procumbent, annual or perennial wetland plants with undivided leaves, very small, more or less swollen achenes and tiny beaks. Moreover, sections *Flammula* and *Micranthus* share a similar karyotype (D'Ovidio and Marchi, 1990); for the South American taxa, karyological data are not yet available.

Clade XIX corresponds to *R.* sect. *Thora*, a well-defined small group of low alpine European species characterized by uniform tuberous roots, the lack of basal leaves, undivided or tripartite, somewhat bluish-green stem leaves, pale yellow flowers, and swollen, glabrous, unbordered achenes.

4.4. Character evolution

The main reason for incongruence of molecular data and previous classifications may relate to homoplasy found with morphological characters used to delimit infrageneric taxa. The most striking example is perhaps the combination of spiny or tuberculate, compressed achenes and annual life form, that was regarded as characteristic of *R.* sect. *Echinella*, but our data indicate that it has evolved independently several times. The split of the section is confirmed by the occurrence of two different pollen types (Santisuk, 1979). Most of these species occur in the meridional zone, and therefore, these features are most probably adaptations to Mediterranean climate and predominantly epizoochorous seed dispersal. Tuberous roots are also found on several positions of the tree in *Ficaria*, *R.* section *Thora*, sect. *Physophyllum*, sect. *Acetosellifoli*, and in most species of sect. *Ranunculastrum*. Again these species are mainly found in the Mediterranean, where tuberous roots as a reserve for nutrition might help to survive the dry summer period in these areas. Fleshy, string-like roots are found in most of the "satellite" genera (*Arcteranthis*, *Oxygraphis*, *Peltocalathos*, and *Callianthemoides*) and may be a primitive feature, which has since developed to pronounced tubers (*Ficaria*) under certain environmental conditions. Another example is a potential to heterophylly found in several groups, which might have been a prerequisite for colonizing aquatic habitats and has been developed to the extreme in *R.* sect. *Xanthobatrachium*, and *R.* subg. *Batrachium*. The affinity to aquatic habitats might have promoted long-distance dispersal and the wide distribution of many of these groups.

Other morphological characters used for previous classifications, e.g., the various types of nectary scales (Benson, 1940), glossy petals (Parkin, 1928), or the xylem structure of the achenes (Trzaski, 1999), do not show a clear correlation to the groups found in the molecular data. Morphological reductions, such as development of a single cotyledon in *R. ficaria*, *R. glacialis*, or *R. parnassifolius*, happened in parallel several times. It seems that each group of buttercups has developed its own special combination of features in adaptation to certain habitats, but it is difficult to find any taxon combining a set of "primitive" characters. Moreover, many morphological characters, such as fruit anatomy and pollen types, have not yet been scored in all taxa; therefore, it is difficult to find reliable characters that can be used for classifications.

Our results confirm the importance of karyological features for the understanding of natural groups in buttercups, as seen in the clear separation of the *R. acris* group with $x = 7$ from the other Ranunculi with $x = 8$. This resembles the molecular phylogeny of *Anemone*, another genus with R-type chromosomes in Ranunculaceae (Tamura, 1995), where even the main clades correspond to groups with basic chromosome numbers (Schuettpelz et al., 2002). Further studies are needed to confirm whether groups of karyotypes, as defined by Goepfert (1974), are congruent with molecular data.

4.5. Speciation and diversification

Our analyses confirm that hybridization and polyploidy are likely to be the main evolutionary factors for diversification and speciation within certain groups, especially in regions of higher altitudes and latitudes. Hybridization might predispose colonizing populations to rapid diversification under disruptive selection and thus promote adaptive radiations (e.g., Seehausen, 2004). A high frequency of polyploids, especially of high polyploids in the arctic and subantarctic/high alpine zones follows a general trait, supporting a hypothesis of greater colonizing abilities in colder climates of polyploids and a higher capacity of habitat differentiation (Soltis et al., 2003). Nevertheless, it remains uncertain whether polyploidy had an impact on the origin of major groups within *Ranunculus*. In almost all highly diversified groups (except *R.* sect. *Pseudadonis* in the Alps of New Zealand) diploid species or at least diploid cytodemes of species are still present. Alternative analyses with and without allopolyploids have not changed

dramatically the topology of the trees, but still left polytomies on the basis of major clades (Figs. 1 and 2). These observations and partly incongruent tree topologies of nrITS and plastid data sets (Paun et al., in press) suggest reticulate relationships, probably on the diploid and on the polyploid level, in the history of the genus. Similar conclusions have been drawn from recent sequence analyses of e.g., the genus *Achillea* (Guo et al., 2004).

The general success of *Ranunculus* in respect to species diversity and distribution might be mainly due to a combination of (1) high morphological plasticity including a genetic flexibility for a rapid adaptation to new habitats, thus permitting development of various eco- and phenotypes; (2) hybridization and polyploidy as factors for diversification, and (3) a broad range of reproductive systems including vegetative growth, autogamy, apomixis and combinations thereof, enabling species to colonize various habitats, especially in regions with colder climates. A parallel example with regard to factors (2) and (3) for speciation and diversification might be the genus *Antennaria* (Asteraceae), distributed in temperate to arctic zones of the Northern Hemisphere and of South America, and consisting of diploids and polyploid apomictic complexes. Similar to *Ranunculus*, ITS sequence analyses in *Antennaria* showed incongruence with previous morphology-based classifications presumably due to convergent evolution of characters in adaptation to arctic or alpine habitats; polyploidy and apomixis were also important factors for diversification (Bayer et al., 1996).

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References

Aeschimann, D., Heitz, C., 1996. Typifications of *Ranunculus villarsii* DC. and of *Ranunculus breyninus* Crantz (Ranunculaceae). Candollea 51, 95–98.

Alfaro, M.E., Zoller, S., Lutzoni, F., 2003. Bayes or Bootstrap? A simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic relationships. Mol. Biol. Evol. 20, 255–266.

Archibald, J.K., Mort, E.M., Crawford, D.J., 2003. Bayesian inference of phylogeny: a non-technical primer. Taxon 52, 187–191.

Armstrong, T., Hörandl, E., Paun, O., Johansson, J.T., Lehnebach, C., Lockhart, P., 2004. A molecular phylogeny of /Ranunculus/ and the relationships of Malesian taxa (Ranunculaceae). Sixth International Flora Malesiana Symposium 2004, Programme and Abstracts, Los Baños, Philippines, p. 30.

Baldwin, A., Sanderson, B., Porter, M., Wojciechowski, J.M., Campbell, C., Donoghue, M., 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. 82, 247–277.

Baltisberger, M., 1980. Die Artengruppe des *Ranunculus polyanthemos* L. in Europa. Ber. Schweiz. Bot. Ges. 90, 143–188.

Baltisberger, M., 1981. Verwandtschaftsbeziehungen zwischen der Gruppe des *Ranunculus polyanthemos* L. und *R. repens* L. sowie Arten der Gruppe des *R. acris* L. und des *R. bulbosus* L. Bot. Helv. 91, 61–74.

Baltisberger, M., 1994. *Ranunculus cacuminis* and *R. crenatus*, representatives of the *R. alpestris*-group Ranunculaceae on the Balkan Peninsula. Pl. Syst. Evol. 190, 231–244.

Bayer, R., Soltis, D.E., Soltis, P.S., 1996. Phylogenetic inferences in *Antennaria* (Asteraceae) based on sequences from nuclear ribosomal DNA ITS. Am. J. Bot. 83, 516–527.

Benson, L., 1940. The North American subdivisions of *Ranunculus*. Am. J. Bot. 83, 516–527.

Benson, L., 1948. A treatise on the North American Ranunculi. Am. Midl. Nat. 40, 1–261.

Briggs, B.G., 1960. *Ranunculus lappaceus* and allied species of the Australian mainland. I. Taxonomy. Proc. Linn. Soc. N.S.W 84, 295–324.

Briggs, B.G., 1962. Interspecific hybridization in the *Ranunculus lappaceus* group. Evolution 16, 372–390.

Candolle, A., de, 1824. Prodromus Systematis Naturalis Regni Vegetabilis. Treuttel et Würz, Paris, France.

Chase, M.W., Knapp, S., Cox, A.V., Clarkson, J.J., Butsko, Y., Joseph, J., Savolainen, V., Parokonny, A.S., 2003. Molecular systematics, GISH and the origin of hybrid taxa in *Nicotiana* (Solanaceae). Ann. Bot. London 92, 107–127.

Coles, S.M., 1971. The *Ranunculus acris* L complex in Europe. Watsonia 8, 237–261.

Coles, S.M., 1973. *Ranunculus bulbosus* L in Europe. Watsonia 9, 207–228.

Cook, C.D.K., 1963. Studies in *Ranunculus* subgenus *Batrachium* (DC.) A. Gray. II. General morphological considerations in the taxonomy of the subgenus. Watsonia 5, 294–303.

Cook, C.D.K., 1966. A monographic study of *Ranunculus* subgenus *Batrachium* (DC). A. Gray. Mitt. Bot. Staatssammlung München 6, 47–237.

Dahlgren, G., 1992. *Ranunculus* subgenus *Batrachium* on the Aegean islands and adjacent areas: nectary types and breeding system. Nord. J. Bot. 12, 299–310.

Dahlgren, G., Cronberg, N., 1996. Species differentiation and relationships in *Ranunculus* subgenus *Batrachium* (Ranunculaceae) elucidated by isozyme electrophoresis. Symb. Bot. Upsal. 31, 91–104.

Diosdado, J., Pastor, C.J., 1993. Karyological studies of some orophyte taxa of the genus *Ranunculus* L. from the Iberian Peninsula. Bot. J. Linn. Soc. 111, 23–35.

Dobes, C., Vitek, E., 2000. Documented Chromosome Number Checklist of Austrian Vascular Plants. Museum of Natural History Vienna, Vienna, Austria.

D'Ovidio, R., Marchi, P., 1990. DNA content, karyotype structure analysis and karyotype symmetry in *Ranunculus* L. (Ranunculaceae). Italian species belonging to sections *Flammula* (Webb) Benson and *Micranthus* (Ovcz.) Nyarady. Caryologia 43, 99–115.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

Eichler, H.J., 1958. Revision der Ranunculaceen Malesiens. Bibl. Bot. 124, 1–110 (7 tables).

Ericsson, S., 2001. 8. *Ranunculus auricomus* complex. In: Jonsell, B., Karlsson, T. (Eds.), Flora Nordica, Chenopodiaceae to Fumariaceae, vol. 2. The Bergius Foundation, Stockholm, Sweden, pp. 237–255 (see also pp. 382–397).

Farris, J.S., 1989. The retention index and rescaled consistency index. Cladistics 5, 417–419.

Felsenstein, J., 1985. Confidence limits on phylogenetics: an approach using the bootstrap. Evolution 39, 783–791.

Fisher, F.J.F., 1965. The Alpine *Ranunculi* of New Zealand. DSIR, Wellington, New Zealand.

Fitch, W.M., 1971. Toward defining the course of evolution: minimal change for a specific tree topology. Syst. Zool. 20, 406–416.

Förster, P., 1997. Seedlings of the tribus Ranunculeae DC. and the tribus Adonideae Kunth (Ranunculaceae). Flora 192, 133–142.

Goepfert, D., 1974. Karyotypes and DNA content in species of *Ranunculus* L. and related genera. Bot. Notiser 127, 464–489.

Gornall, R.J., 1987. Notes on a hybrid spearwort, *Ranunculus flammula* L. × *R. reptans* L. Watsonia 16, 383–388.

Guo, Y.-P., Ehrendorfer, F., Samuel, R., 2004. Phylogeny and systematics of *Achillea* (*Asteraceae-Anthemideae*) inferred from nrITS and plastid *trnL-F* DNA sequences. Taxon 53, 657–672.

Hoot, S.B., 1995. Phylogeny of the Ranunculaceae based on preliminary *atpB*, *rbcL* and 18S nuclear ribosomal DNA sequence data. Pl. Syst. Evol. (Suppl. 9), 214–251.

Hörandl, E., 1998. Species concepts in agamic complexes: applications in the *Ranunculus auricomus* complex and general perspectives. Folia Geobot. 33, 335–348.

Hörandl, E., 2004. Comparative analysis of genetic divergence among sexual ancestors of apomictic complexes using isozyme data. Int. J. Pl. Sci. 165, 615–622.

Hörandl, E., Jakubowsky, G., Dobes, C., 2001. Isozyme and morphological diversity within apomictic and sexual taxa of the *Ranunculus auricomus* complex. Pl. Syst. Evol. 226, 165–185.

Hörandl, E., Gutermann, W., 1998. Der *Ranunculus auricomus* complex in Österreich: 1. Methodik; Gruppierung der mitteleuropäischen Sippen. Bot. Jahrb. 120, 1–44.

Hörandl, E., Greilhuber, J., 2002. Diploid and autotetraploid sexuals and their relationships to apomicts in the *Ranunculus cassubicus* group: insights from DNA content and isozyme variation. Pl. Syst. Evol. 234, 85–100.

Huber, W., 1988. Natürliche Bastardierungen zwischen weißblühenden *Ranunculus*-Arten in den Alpen. *Veröff.* Geobot. Inst. ETH Stiftung Rübel Zürich 100, 1–160.

Huelsenbeck, P.J., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Huelsenbeck, P.J., Larget, B., Miller, R.E., Ronquist, F., 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. Syst. Biol. 51, 673–688.

Huson, D.H., 1998. SplitsTree: analysing and visualizing evolutionary data. Bioinformatics 14, 68–73.

Jalas, J., Suominen, J., 1989. Nymphaeaceae to Ranunculaceae. Atlas Florae Europaeae, vol. 8. The Committee for Mapping the Flora of Europe, Helsinki, Finland.

Janchen, E., 1949. Die systematische Gliederung der Ranunculaceen und Berberidaceen. Denkschr. Österr. Akad. Wiss., Math. –Naturwiss Kl. 108, 1–82.

Johansson, J.T., Jansen, R.K., 1993. Chloroplast DNA variation and phylogeny of the Ranunculaceae. Pl. Syst. Evol. 187, 29–49.

Johansson, J.T., 1995. A revised chloroplast DNA phylogeny of the Ranunculaceae. Pl. Syst. Evol. (Suppl. 9), 253–261.

Johansson, J.T., 1998. Chloroplast DNA restriction site mapping and the phylogeny of *Ranunculus* (Ranunculaceae). Pl. Syst. Evol. 213, 1–19.

Kim, K.J., Jansen, R.K., 1994. Comparison of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*, Asteraceae): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. Pl. Syst. Evol. 190, 157–185.

Kluge, A., Farris, J.S., 1969. Quantitative phyletics and the evolution of anurans. Syst. Zool. 18, 1–32.

Landolt, E., 1954. Die Artengruppe des *Ranunculus montanus* Willd. in den Alpen und im Jura (zytologisch-systematische Untersuchungen). Ber. Schweizer Bot. Gesell. 64, 9–83.

Landolt, E., 1956. Die Artengruppe des *Ranunculus montanus* Willd. in den Pyrenäen und anderen europäischen Gebirgen westlich der Alpen. Ber. Schweizer Bot. Gesell. 66, 92–117.

Lockhart, P., McLechnanan, P.A., Havell, D., Glenny, D., Huson, D., Jensen, U., 2001. Phylogeny, dispersal and radiation of New Zealand alpine buttercups: molecular evidence under split decomposition. Ann. Missouri Bot. Gard. 88, 458–477.

Linnaean Plant Names Database, 2003. The Natural History Museum, London. Available from: <http://internt.nhm.ac.uk/cgi-bin/Botany/linnaean/>.

Lonay, H., 1901. Contribution a l' anatomie des Renonculacees. Structure des pericarps et des speroderms. Arch. Bot. 8, 1–55.

Moore, D.M., 1983. Flora of Tierra del Fuego. Oswestry, Shropshire, A. Nelson, St. Louis, Missouri Botanical Garden, USA.

Morrell, P.L., Rieseberg, L.H., 1998. Molecular tests of the proposed diploid hybrid origin of *Gilia achilleifolia* (Polemoniaceae). Am. J. Bot. 85, 1439–1453.

Nilsson, Ö, 2001. *Ranunculus lapponicus*, *Ranunculus pallasii* and *Ranunculus* ×*spitsbergensis*. In: Jonsell, B., Karlsson, T. (Eds.), Flora Nordica, Chenopodiaceae to Fumariaceae, vol. 2. The Bergius Foundation, Stockholm, Sweden, pp. 232–234.

Nogler, G.A., 1984. Genetics of apospory in apomictic *Ranunculus auricomus*: 5. conclusion. Bot. Helv. 94, 411–423.

Okada, H., 1984. Polyphyletic allopolyploid origin of *Ranunculus cantoniensis* (4×) from *R. silerifolius* (2×) × *R. chinensis* (2×). Pl. Syst. Evol. 148, 89–102.

Okada, H., 1989. Cytogenetical changes of offsprings from the induced tetraploid hybrid between *Ranunculus silerifolius* ($2n = 16$) and *R. chinensis* ($2n = 16$) (Ranunculaceae). Pl. Syst. Evol. 143, 89–102.

Ovczinnikov, P.N., 1937. *Ranunculus*. In: Komarow, W.A. (Ed.), Flora URSS, vol. VII. Ranales and Rhoeadales. Botanicheskii Institut Akademii Nauk USSR, Moscow, USSR, pp. 351–509 (English translation 1970).

Parkin, J., 1928. The glossy petal of *Ranunculus*. Ann. Bot. 42, 739–755.

Paun, O., Lehnebach, C., Johansson, J.T., Lockhart, P., Hörandl, E., In press. Phylogenetic relationships and biogeography of /Ranunculus/ and allied genera (Ranunculaceae) in the Mediterranean region and in the European Alpine System. Taxon 54.

Pickering, C.M., 1997a. Breeding systems of Australian *Ranunculus* in the alpine region. Nord. J. Bot. 17, 613–620.

Pickering, C.M., 1997b. Reproductive strategies and constraints of alpine plants as illustrated by five species of Australian alpine *Ranunculus*. Opera Bot. 132, 101–108.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Prantl, K., 1887. Beiträge zur Morphologie und Systematik der Ranunculaceen. Bot. Jahrb. Syst. 9, 225–273.

Riveros, M., 1991. Biologia Reproductiva en especies vegetales de dos comunidades de la zona templada del sur de Chile, 40'S. PhD Thesis, Universidad de Chile, Santiago, Chile.

Ro, K.E., Keener, C.S., McPheron, B.A., 1997. Molecular phylogenetic study of the Ranunculaceae: utility of the nuclear 26S ribosomal DNA in inferring intrafamilial relationships. Mol. Phylog. Evol. 8, 117–127.

Rousi, A., 1956. Cytotaxonomy and reproduction in the apomictic *Ranunculus auricomus* group. Ann. Bot. Soc. Zool.-bot. Fenn. Vanamo 29 (2), 1–64 (1 table).

Sang, T., Crawford, D.J., Stuessy, T.F., 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. USA 92, 6813–6817.

Santisuk, T., 1979. A palynological study of the tribe Ranunculeae (Ranunculaceae). Opera Bot. 48, 1–74.

Schuettpelz, E., Hoot, S.B., Samuel, R., Ehrendorfer, F., 2002. Multiple origins of southern hemisphere *Anemone* (Ranunculaceae) based on plastid and nuclear sequence data. Pl. Syst. Evol. 231, 143–151.

Seehausen, O., 2004. Hybridization and adaptive radiations. Trends Ecol. Evol. 19, 198–207.

Simmons, M., Ochoterena, P.H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.

Skottsberg, C., 1922. Ranunculus caprarum. In: Skottsberg, C. (Ed.), The Natural History of Juan Fernandez and Easter Island, Part 2. Almqvist & Wiksells, Uppsala, Sweden, pp. 123–124.

Soltis, D.E., Soltis, P.S., Tate, J.A., 2003. Advances in the study of polyploidy since plant speciation. New Phytol. 161, 173–191.

Sun, Y., Skinner, D.Z., Liang, G.H., Hulbert, S.H., 1994. Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theor. Appl. Genet. 89, 26–32.

Swofford, D.L., 2001. PAUP*: phylogenetic analysis using parsimony. Version 4. Computer software. Sinauer Associates, Sunderland, MA.

Tamura, M., 1993. Ranunculaceae. In: Kubitzki, K., Rohwer, J.G., Bittrich, V. (Eds.), The Families and Genera of Vascular Plants. 2. Flowering Plants. Dicotyledons, Magnoliid, Hamamelid, and Caryophyllid Families. Springer, Berlin, Germany, pp. 563–583.

Tamura, M., 1995. Angiospermae. Ordnung Ranunculales. Fam. Ranunculaceae. II. Systematic Part. In: Hiepko, P. (Ed.), Natürliche Pflanzenfamilien, second ed., 17aIV,. Duncker & Humblot, Berlin, Germany, pp. 223–519.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Trzaski, L., 1999. Xylem distribution in the achene of some European *Ranunculus* species as a taxonomical criterion of *Ranunculus* genus. Phytomorphology 49, 241–251.

Tutin, T.G., Cook, C.D.K., 1993. *Ranunculus*. In: Tutin, T.G., Heywood, V.H., Burges, N.A., Valentine, D.H., Walters, S.M., Webb, D.A. (Eds.), Flora Europaea I: Psilotaceae to Platanaceae, second ed. Cambridge University Press, Cambridge, UK, pp. 269–290.

Whittemore, A., 1997. *Ranunculus*. In: Flora of North America Committee (Eds.), Flora of North America North of Mexico, vol. 3. Magnoliophyta: Magnoliidae and Hamamelidae, Oxford Univ. Press, New York, pp. 88–135.

Winkworth, R.C., Bryant, D., Lockhart, P.J., Havell, D., Moulton, V., in press. Biogeographic interpretation of Split Graphs: Least Squares Optimization of Branch Length. Syst. Biol.

Wu, M.J., Huang, T.-C., 2004. Taxonomy of the *Euphrasia transmorrisonenis* complex (Orobanchaceae) in Taiwan based on nrITS. Taxon 53, 911–918.

Young, J.P., Dickinson, T.A., Dengler, N.G., 1995. A morphometric analysis of heterophyllous leaf development in *Ranunculus flabellaris*. Int. J. Pl. Sci. 156, 590–602.

Phylogenetic relationships and biogeography of *Ranunculus* and allied genera (Ranunculaceae) in the Mediterranean region and in the European Alpine System

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Ranunculus s.l. shows a considerable species diversity and degree of endemism in the Mediterranean region and occurs with various life forms from the lowlands to the highest mountains. Based on a sampling from all continents, sequences of the ITS of nrDNA, the plastid *matK*, and the adjacent *trnK* regions were analysed using maximum parsimony and Bayesian inference. Both separate and combined analyses of the two datasets yielded a large core clade of *Ranunculus* excluding *Ficaria*, *Coptidium*, and the extraeuropean genera *Beckwithia*, *Callianthemoides*, *Halerpestes*, and *Peltocalathos*. The *Ceratocephala-Myosurus*-clade is sister to the core *Ranunculus* in the plastid and the combined datasets on very long branches, thus supporting a classification of *Ceratocephala* and *Myosurus* as separate genera. Within *Ranunculus* s.s., eight well supported and highly consistent clades correspond either to widespread ecological groups (wetlands, high altitudes/latitudes) or to regional (mainly European) geographical groups. Alpine Mediterranean buttercups belong to orophytic clades, most species of which also occur in the European alpine system; others show widespread northern hemisphere distributions. Only one Mediterranean clade is restricted to the Iberian Peninsula and adjacent regions. Present distribution patterns and molecular data support a hypothesis of an origin of alpine buttercups from lowland ancestors of the same geographical region. At lower altitudes, the predominant life forms, i.e., therophytes and geophytes, evolved multiple times suggesting parallel adaptations to the Mediterranean climate. Geophytes differentiated into an eastern and western Mediterranean group, and are most closely related to the subalpine, non-monophyletic "*R. montanus*" group, thus supporting a hypothesis of a common lowland ancestor. Tentative estimates for divergence times of the major clades in *Ranunculus* s.l. were made based on an age calibration for the *Ranunculus-Xanthorhiza*-split, using *matK* sequences and penalized likelihood analyses. The results from this study suggest that the split of allied genera from *Ranunculus* s.s. occurred during the Eocene and Oligocene, with the core clade of *Ranunculus* being c. 24.0 Myr old. Diversification of *Ranunculus* s.s. into main ecological/geographical clades took place in the late Miocene, and speciation within the Mediterranean groups during the Pliocene and Pleistocene. Diversification of life forms at lower altitudes occurred mainly during or after the establishment of the Mediterranean climate. Island endemics of Macaronesia and Crete are probably rather young descendents of neighbouring geographical groups. Diversification of alpine groups took place at different geological times, but is in general correlated with periods of colder climate. The high diversity of buttercups is likely a consequence of the broad spectrum of different habitats in the Mediterranean region.

KEYWORDS: life forms, molecular clock, molecular phylogenetics, *Ranunculus*.

INTRODUCTION

Ranunculus (buttercups) represents the largest genus within Ranunculaceae, comprising ca. 600 species (Tamura, 1995) and is distributed on all continents. Most species occur in temperate to arctic/subantarctic zones; nevertheless, the genus shows a considerable diversity in the Mediterranean with c. 160 species, c. 78 of them being endemic to the region (Greuter & al., 1989; excluding microspecies of the apomictic *R. auricomus* complex). Also in other continents, several buttercups grow in regions with a Mediterranean climate (e.g., Cape region of South Africa, California, Florida, Central Chile).

Buttercups occur in all regions and altitudinal zones of the Mediterranean. In the lowlands, various morpho-

logical adaptations have been developed to accommodate the summer-dry and winter-rainy climate of the region: many species have dimorphic roots, partly fibrous, partly strongly tuberous, the latter being a reserve for nutrition. This enables the plants to develop flowering stems quickly in the short spring season, whereas in the hot and dry summer period the plants usually wither completely. A few species use the rainy period in fall for flowering (e.g., *R. bullatus*). Another strategy is found in numerous annuals of the Mediterranean region. These flower rapidly, set seed and wither before the summer. Geophytic and therophytic life forms are in general frequent in the Mediterranean flora, especially in the patchy vegetation of the Mediterranean lowlands (pastures, understory of olive plantations, macchie, phrygana, etc.). Aquatic *Ranunculus* species, which have a cosmopolitan distribution, as well as species that prefer forest habitats are found in this area. Several species, including many endemics, occur in the alpine zone above the treeline, which ranges in the Mediterranean region from 2000 m in the North (46° latitude) to 3000 m in the South (30° latitude; Blondel & Aronson, 1999). At these altitudes, buttercups show similar features and flowering times to most species of the temperate European mountain system.

Relationships and taxonomy of Mediterranean *Ranunculus* species are uncertain, highlighting the problem that there is not yet an accepted classification of the genus worldwide. A comparison of various infrageneric taxonomic concepts is available in Hörandl & al. (2005). The most recent worldwide taxonomy by Tamura (1993, 1995) has classified the Mediterranean taxa on various taxonomic levels. This treatment included *Ceratocephala* which was regarded as distinct from *Ranunculus*. It is a small genus of annuals with very large fruit beaks, mainly distributed in the meridional zone (sensu Meusel & al., 1965) with a single species in southern New Zealand (Garnock-Jones, 1984, as *Ceratocephalus*). Tamura (1993, 1995) also considered *Ficaria*, a temperate to meridional group of species with three sepals, tuberous roots and beakless, stalked fruits, but regarded it as a subgenus of *Ranunculus*. Other taxa studied include those placed in *Ranunculus* s.s., section *Ranunculastrum* and sect. *Echinella*. The former are characterized by tuberous roots and elongated fruits, and the latter are annuals with spiny or tuberculous fruits. These taxa are all endemic to the Mediterranean or largely distributed within this region. The alpine species of the Mediterranean have localized or disjunct, usually endemic geographic distributions. They belong to different sections: the yellow-flowering species were referred to the so-called *R. montanus*-group (Landolt, 1954, 1956), which is distributed in the European Alpine system (Pyrenees-Alps-Apennines-Carpathians-Dinarids-Balkans), and partly to the Caucasus region; the group as a whole was classified under *R.* sect. *Acris* by Tamura (1995). Other examples of alpine endemics are *R. brevifolius* (*R.* sect. *Thora*), *Ranunculus pyrenaeus*, *R. amplexicaulis*, *R. parnassifolius* subsp. *parnassifolius* (all *R.* sect. *Ranuncella* after Tamura, 1995). Four small sections are endemic or subendemic to the Mediterranean: section *Insulares*, comprising *Ranunculus weyleri*, a local endemic from Mallorca, and the Sardinian endemic *R. cymbalariifolius* (Tutin & Cook, 1993, in *Flora Europaea*), and the monotypic sections *Acetosellifoli* (Iberian peninsula), *Physophyllum* (omnimediterranean), and *Chloeranunculus* (extending to Central France) sensu Tamura (1995). Altogether, Tamura's (1995) classification reflects a high morphological diversity of buttercups in the Mediterranean region (see examples in Fig. 1).

Previous molecular studies using cpDNA restriction sites (Johansson, 1998) and ITS sequences (Hörandl & al., 2005) have produced tree topologies that are incongruent with previous classifications. The results from these studies suggest general widespread ecological and regional geographical groups. The "Mediterranean" sections (i.e., sections *Echinella*, *Ranunculastrum*, *Acris*, *Ranuncella*, and *Leucoranunculus*) were not found to be monophyletic. On the sectional level, hybridization (as suggested by split decomposition analysis) has led to reticulate relationships in many groups (Hörandl & al., 2005). This is a finding that cautions against over interpreting bifurcating tree topologies which are based on analyses of a single nuclear marker. Studies with additional independent DNA markers are helpful in this respect (Holland & al., 2004), and in particular, inclusion of plastid markers allows for more precise estimates of species divergence times (e.g., Knapp & al., 2005) which can facilitate understanding of the genus. Sampling for the Euro-Mediterranean region is, in contrast to other continents, representative enough to address biogeographical questions. Thus, the aim of the present study is (1) to evaluate the phylogenies revealed by ITS with highly informative and uniparental inherited plastid markers, (2) to clarify relationships of the (Euro)-Mediterranean taxa within a worldwide framework, (3) to evaluate life form strategies in the region, and (4) to develop hypotheses for the spatial-temporal development of the (Euro)-Mediterranean buttercup flora in context of the geological history and ecology of the region.

MATERIALS AND METHODS

Materials. — Materials are a subset of those used in Hörandl & al. (2005) plus six additional taxa (*R. creticus*, *R. cupreus*, *R. kuepferi* subsp. *kuepferi*, *R. serpens*

Fig. 1. Representatives of Mediterranean to C. European *Ranunculi* and allied genera. A, *Ranunculus thora* (alpine limestone scree, Alps Maritimes); B, *R. seguieri* (alpine limestone scree, Alps Maritimes); C, *Ceratocephala falcata* (open places in cultivated land, W. Turkey; D, *R. millefoliatus* (open places in pastures, Sicily); E, *R. aduncus* (subalpine grassland, Alps Maritimes); F, *R. cortusifolius* (*Laurus* forest, Tenerife). Photo credit: Franz Hadacek.

subsp. *nemorosus*, *R. spicatus*, and *R. weyleri*). Sampling comprises altogether 133 species including representatives from all sections (except two small Eastern Asian ones), and all continents. Taxa that are not of specific interest for the present paper were excluded (temperate to arctic allopolyploid taxa of the *R. auricomus* complex, and several taxa from the southern hemisphere). Almost all ITS sequences were taken from Hörandl & al. (2005), whereas all plastid sequence data (except for the outgroup) are newly reported here (Appendix 1). According to the delimitation of the Mediterranean region after Blondel & Aronson (1999: fig. 1.5), we include here 30 species endemic or subendemic to the Mediterranean region and one endemic to Macaronesia, plus 47 species non-endemic occurring in the region. Materials, vouchers, and GenBank accession numbers are documented in Appendix 1. Selection of *Trautvetteria* as an outgroup, which is the closest relative to the *Ranunculus*-*Myosurus* clade, followed the findings and reasoning of Hörandl & al. (2005).

DNA extraction, sequencing, and alignment. — DNA extraction and sequencing was performed according to the protocol described by Hörandl & al. (2005), except that for amplification of the *matK* region, 0.4% BSA (Bovine Serum Albumin Acetylated) was used instead of DMSO. The primers given in Table 1 amplified the *matK* region and also a part of the flanking *trnK* intron. A multiple sequence alignment was performed for an initial group of sequences using the progressive alignment procedure of CLUSTAL X (Thompson & al., 1994). Additional taxa were also subsequently added into this aligned matrix, which was refined manually to minimize the number of independent indels (data matrix has been submitted to GenBank).

Table 1. Sequences of the primers used for amplification of *matK* and flanking *trnK* intron region. The starting point is given with the first bp of *matK*.

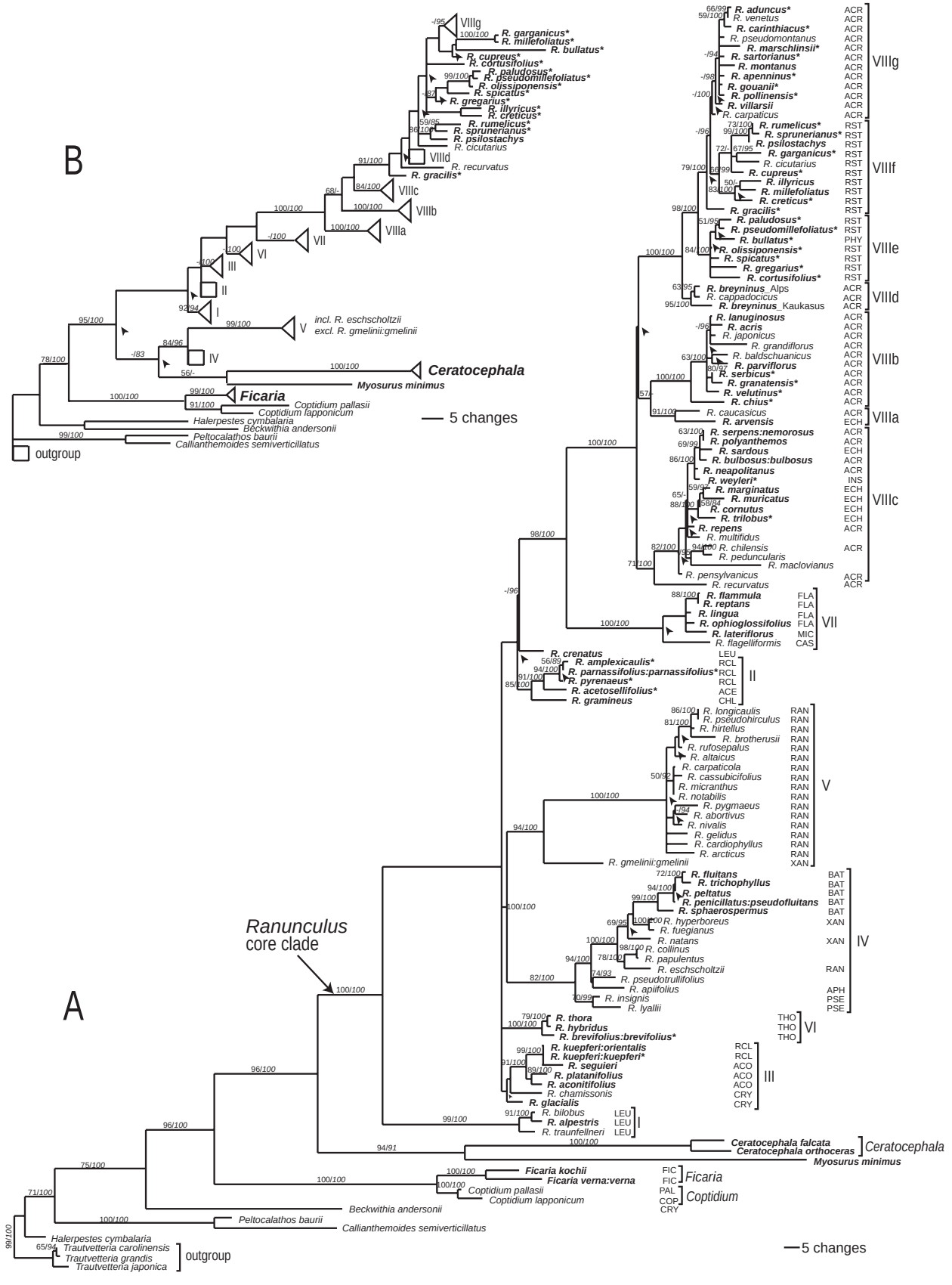
Name	Starting point	Sequence (5'-3')
*trnK*710f	-24	GTATCGCACTATGTWTCATTTGA
*trnK*345f	321	GTRGAAATTCCATTTKCA
*matK*700f	714	CTACGTAAAMAATCTTMTCATTT
*trnK*3ar	1400	CGTACASTACTTTTGTGTTTKCG
*matK*1360r	1438	CCAAGAATTCCGAACCCAAYCTTT
*trnK*3r	1951	GATTCGAACCCGGAACTAGTCGG

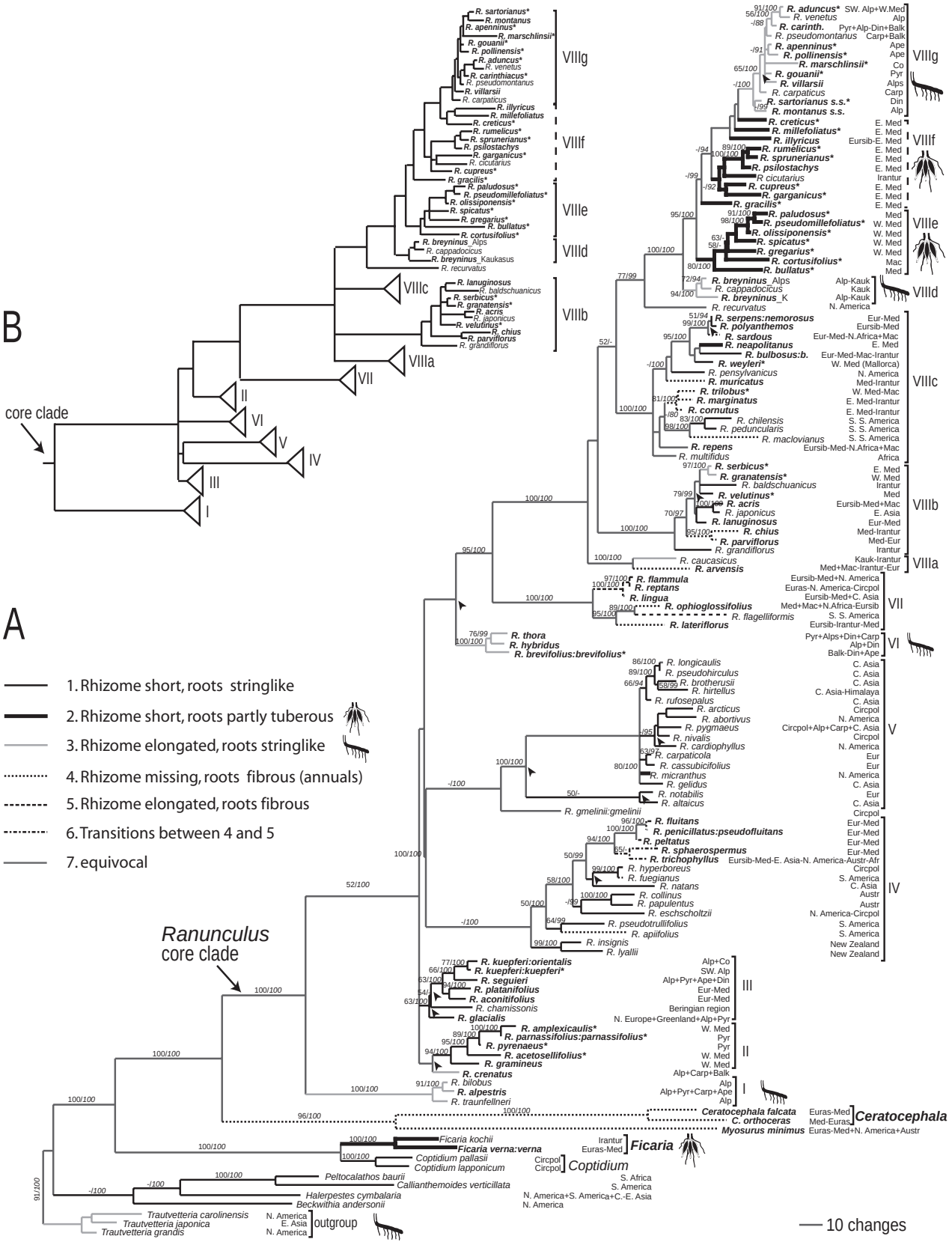
Parsimony-informative insertions and/or deletions (indels) in the *matK*/*trnK* intron regions (see Appendices 2 and 3, the latter in the online version of *Taxon*) were coded as present-absent characters (following the recommendations of Simmons & Ochoterena, 2000; Andersson & Chase, 2001) and used in further parsimony analyses (in total by coding the indels, 20 parsimony-informative characters were added). The singular case of an insertion (positions 1689–1701 in Appendix 3, available on-line) requiring parallel or reversal events detected for group VIIIf was coded separately for each type of insertion. Alignment, gap treatment and analyses of the ITS dataset were done as in Hörandl & al. (2005).

Parsimony analyses (MP). — Maximum parsimony analyses were undertaken using PAUP* version 4.0b8 (Swofford, 2001). Heuristic searches were performed using 2000 replicates, random sequence addition, (TBR) branch swapping, Multrees on, but saving only 10 trees for each replicate to reduce the time spent in swapping large numbers of suboptimal trees. All characters were equally weighted and treated as unordered (Fitch parsimony; Fitch, 1971). Majority rule consensus and strict consensus trees were computed from all equally most parsimonious trees. Besides standard tree parameters (tree length, consistency index, CI, and retention index, RI), internal support for individual branches was estimated using non-parametric bootstrapping (Felsenstein, 1985) with 2000 replicates, and simple sequence addition. Bootstrap values (BP) are shown on the corresponding branches of the phylograms (Figs. 2 and 3A).

Bayesian inference (BI). — Phylogenetic reconstruction was also undertaken using Bayesian inference as it was found to be relatively efficient and accurate in analysing large *Ranunculus* datasets (e.g., Hörandl & al., 2005). It was performed with the program MrBayes v3.0 (Huelsenbeck & Ronquist, 2001). Four Markov Monte Carlo chains were run simultaneously starting from random trees for 2,000,000 generations, sampling a tree

Fig. 2 (next page). Phylograms for 50% majority rule consensus trees based on parsimony analyses of the *matK/trnK* (A) and the ITS dataset (B, condensed form). Bootstrap (before slash), and posterior probability values (from the Bayesian analysis, after slash, in italics) for corresponding clades are indicated above branches. Arrowheads mark branches that collapsed in the strict consensus tree. Species occurring in the Mediterranean are marked in bold face, endemics with an asterisk (delimitation of the Mediterranean and adjacent regions after Blondel & Aronson, 1999). Three-letter acronyms indicate genera, subgenera and sections of *Ranunculus* after Tamura (1995). ACE = *R.* sect. *Acetosellifolii*; ACO = *R.* sect. *Aconitifolii*; ACR = *R.* sect. *Acris* sensu Tamura; APH = *Aphanostemma*; BAT = *R.* subg. *Batrachium*; CAS = *R.* sect. *Casalea*; CHL = *R.* sect. *Chloeranunculus*; COP = *R.* subg. *Coptidium*; CRY = *R.* subg. *Crymodes*; ECH = *R.* sect. *Echinella*; FIC = *R.* subg. *Ficaria*; FLA = *R.* sect. *Flammula*; HEC = *R.* sect. *Hecatonia*; INS = *R.* sect. *Insulares*; LEP = *R.* sect. *Leptocaules*; LEU = *R.* sect. *Leucoranunculus*; MIC = *R.* sect. *Micranthus*; PAL = *R.* subg. *Pallasiantha*; PHY = *R.* sect. *Physophyllum*; PSE = *R.* sect. *Pseudadonis*; RAN = *R.* sect. *Ranunculus* sensu Tamura; RCL = *R.* sect. *Ranuncella*; RST = *R.* sect. *Ranunculastrum*; THO = *R.* sect. *Thora*; XAN = *R.* sect. *Xanthobatrachium*. Species without acronyms are not mentioned in Tamura (1995).


B
VIIIg
R. garganicus*
R. millefoliatus*
R. bullatus*
R. cupreus*
R. cortusifolius*
R. paludosus*
R. pseudomillefoliatus*
R. olissiponensis*
R. spicatus*
R. gregarius*
R. illyricus*
R. creticus*
R. rumelicus*
R. sprunerianus*
R. psilostachys
R. cicutarius
VIIId
R. recurvatus
R. gracilis*
VIIIc
VIIIb
VIIIa
VII
VI
III
II
I
V
incl. R. eschscholtzii
excl. R. gmelinii:gmelinii
IV
Ceratocephala
Myosurus minimus
Ficaria
Coptidium pallasii
Coptidium lapponicum
Halerpestes cymbalaria
Beckwithia andersonii
Peltocalathos baurii
Callianthemoides semiverticillatus
outgroup
5 changes
A
Ranunculus
core clade
R. aduncus*
R. venetus
R. carinthiacus*
R. pseudomontanus
R. marschlinsii*
R. sartorianus*
R. montanus
R. apenninus*
R. gouanii*
R. pollinensis*
R. villarsii
R. carpaticus
R. sprunerianus*
R. psilostachys
R. garganicus*
R. cicutarius
R. cupreus*
R. illyricus
R. millefoliatus
R. creticus*
R. gracilis*
R. paludosus*
R. pseudomillefoliatus*
R. bullatus*
R. olissiponensis*
R. spicatus*
R. gregarius*
R. cortusifolius*
R. breyninus_Alps
R. cappadocicus
R. breyninus_Kaukasus
R. lanuginosus
R. acris
R. japonicus
R. grandiflorus
R. baldschuanicus
R. parviflorus
R. serbicus*
R. granatensis*
R. velutinus*
R. chius*
R. caucasicus
R. arvensis
R. serpens:nemorosus
R. polyanthemos
R. sardous
R. bulbosus:bulbosus
R. neapolitanus
R. weyleri*
R. marginatus
R. muricatus
R. cornutus
R. trilobus*
R. repens
R. multifidus
R. chilensis
R. peduncularis
R. maclovianus
R. pensylvanicus
R. recurvatus
R. flammula
R. reptans
R. lingua
R. ophioglossifolius
R. lateriflorus
R. flagelliformis
VIIIg
VIIIf
VIIIe
VIIId
VIIIb
VIIIa
VIIIc
VII
R. crenatus
R. amplexicaulis*
R. parnassifolius:parnassifolius*
R. pyrenaeus*
R. acetosellifolius*
R. gramineus
II
R. longicaulis
R. pseudohirculus
R. hirtellus
R. brotherusii
R. rufosepalus
R. altaicus
R. carpaticola
R. cassubicifolius
R. micranthus
R. notabilis
R. pygmaeus
R. abortivus
R. nivalis
R. gelidus
R. cardiophyllus
R. arcticus
R. gmelinii:gmelinii
V
R. fluitans
R. trichophyllus
R. peltatus
R. penicillatus:pseudofluitans
R. sphaerospermus
R. hyperboreus
R. fuegianus
R. natans
R. collinus
R. papulentus
R. eschscholtzii
R. pseudotrullifolius
R. apiifolius
R. insignis
R. lyallii
IV
R. thora
R. hybridus
R. brevifolius:brevifolius*
VI
R. kuepferi:orientalis
R. kuepferi:kuepferi*
R. seguieri
R. platanifolius
R. aconitifolius
R. chamissonis
R. glacialis
III
R. bilobus
R. alpestris
R. traunfellneri
I
Ceratocephala falcata
Ceratocephala orthoceras
Ceratocephala
Myosurus minimus
Ficaria kochii
Ficaria verna:verna
Ficaria
Coptidium pallasii
Coptidium lapponicum
Coptidium
Beckwithia andersonii
Peltocalathos baurii
Callianthemoides semiverticillatus
Halerpestes cymbalaria
Trautvetteria carolinensis
Trautvetteria grandis
Trautvetteria japonica
outgroup
5 changes


B
core clade
I
II
III
IV
V
VI
VII
VIIIa
VIIIb
VIIIc
VIIId
VIIIe
VIIIf
VIIIg
A
1. Rhizome short, roots stringlike
2. Rhizome short, roots partly tuberous
3. Rhizome elongated, roots stringlike
4. Rhizome missing, roots fibrous (annuals)
5. Rhizome elongated, roots fibrous
6. Transitions between 4 and 5
7. equivocal
Ranunculus
core clade
Ceratocephala
Ficaria
Coptidium
outgroup
10 changes

every 200 generations. General time reversible model of substitution with gamma distribution and invariable sites (GTR+I+G) was selected by Modeltest version 3.0 (Posada & Crandall, 1998) to be best fitting model to the ITS dataset (Hörandl & al., 2005). This was also the case for the *matK* dataset which was separately analysed with Modeltest 3.0.

Combination of the ITS and plastid datasets. — In our present study, we have adopted the approach of Muellner & al. (2003) regarding combining data partitions when gene trees show topological congruence as indicated by high bootstrap support. Significant incongruence (P = 0.001) among the datasets was yielded by an incongruence length difference test (ILD, Farris & al., 1995; using 1000 resamplings under the parsimony criterion, the tree-bisection-reconnection branch swapping algorithm, simple addition sequence, Multrees on, with the number of trees retained in each replicate limited to 10). Nevertheless, taxa that account for the non-homogeneity between the two partitions vary usually within the same clade, and thus this incongruence does not affect the major relationships amongst clades which is the main focus of our study. Siddall (1997) points out that the ILD test does not actually reveal the amount of incongruence, and can be insensitive to small but significant topological differences suggested by the different datasets. Thus, in agreement with the argumentation of e.g., Muellner & al. (2003), we see no objection to combine our *matK* and ITS data for a total evidence approach (Kluge, 1989; Nixon & Carpenter, 1996; Acevedo-Rosas & al., 2004; Berry & al., 2004) for developing hypotheses for higher level taxonomic relationships and biogeographic patterns in *Ranunculus*.

For the Bayesian inference analyses of the combined dataset, four different partitions (ITS, *trnK* intron region before *matK*, *matK* itself and *trnK* intron preceding *matK*) were predefined, and during the analysis the program was set to estimate unlinked model parameters for each different partition (Nylander & al., 2004). Altogether 9001 trees were sampled after reaching stationary phase (after c. 200,000 generations), and from these a majority rule consensus tree was constructed using PAUP 4.0b8 (Swofford, 2001). Bayesian phylograms were constructed with MrBayes implementing the option "sumt contype=halfcompat" (Fig. 3B).

Character evolution of underground parts. — As a step towards evaluating the relationship of life forms with phylogeny, features of underground organs were scored for all taxa on herbarium specimens in WU, W and in the field. Initial separate character scores were made for rhizomes (absent/elongated/short, i.e., ± globose) and roots (fibrous/string-like, including fleshy but elongated roots/tuberous, i.e., short fusiform roots). Character state assignments for internal tree nodes were then optimized on the combined molecular tree using MacClade vers. 4.0 (Maddison & Maddison, 2000). This study revealed five highly correlated character associations representing functional units corresponding to life forms: rhizomes absent, roots fibrous (therophytes); rhizomes elongated, roots fibrous (hydrophytes); rhizomes short, roots tuberous (reserve function; geophytes); rhizomes elongated, roots stringlike (strong anchoring function; hemicryptoyphytes); rhizomes short, roots stringlike (all-rounder type; hemicryptophytes). These combined character states, which are likely to be more informative than the separated traits (see, e.g., Kirchoff & al., 2004) were mapped onto the combined molecular tree (Fig. 3A).

Molecular clock analyses. — Unfortunately, no pre-quaternary fossils are known for *Ranunculus* or any closely related lineages. Therefore, we have chosen the age interval of Wikström & al. (2001) for the split of *Ranunculus* (Ranunculeae) and *Xanthorhiza* (Dichocarpeae) as a basis for our age calibration. We have used *matK* rather than ITS sequences for our age estimates because of the more conserved evolution of this gene over the evolutionary divergences studied. Because *matK* is part of the plastid DNA, its analysis also avoids the problems of recombination and concerted evolution that are typically associated with interspecific hybridisation, a phenomenon that appears to be common in the genus (Hörandl & al., 2005). Although a few studies report the possibility of frequent chloroplast exchange among taxa resulting from hybridization (Mort & al., 2001; Acevedo-

Fig. 3 (previous page). Phylogram for the 50% majority rule consensus trees of the combined plastid and ITS dataset based on (A) parsimony analyses, and (B, reduced form) Bayesian inference. Bootstrap values (before slash) and posterior probability values (after slash, in italics) for corresponding clades are indicated above branches. Arrowheads mark branches that collapsed in the strict consensus tree. Designation of Mediterranean taxa as in Fig. 2. Acronyms for geographic distribution (delimitation of the Mediterranean and adjacent regions after Blondel & Aronson, 1999, modified): Alp, Alps; Ape, Apennines; Austr, Australia; Balk, Balkan; C. Asia, Central-Asian mountains and highlands; Circpol, arctic-circumpolar; Carpathians, Carp; Co, Corse; Din, Dinarids; Eur, temperate to boreal Europe; Euras, Eurasia; Eursib, Euro-Siberian (temperate to boreal zone); Irantur, Irano-Turanian; Kauk, Caucasus region; Mac, Macaronesia; Med, Mediterranean; Pyr, Pyrenees+Cantabrian range. The region with the largest distribution area is given first; "+" indicates disjunctions, "-" a more or less continuous distribution. Only the native range is given. Distributional data after Ovczinnikov (1937), Meusel & al. (1965), Hultén & Fries (1986), Greuter & al. (1989), Jalas & Suominen (1989), Iranshar & al. (1992), and Whittemore (1997).

Rosas & al., 2004), this is unlikely to affect our inferences of divergence times amongst more distantly related taxa, since hybridisation in genus *Ranunculus* appears more restricted to closely related species (Hörandl & al., 2005). Third, for *Xanthorhiza*, 1263 bp of the *matK* region were available from GenBank. Thus the *matK* region that we were able to analyze was much longer than that of the ITS region, and consequently smaller variances are expected to be associated with molecular clock estimates. For our dating study, we used a subset of taxa, which included representatives from all major clades identified in the combined gene analysis. We excluded *Trautvetteria* because of a high percentage (ca. 40%) of missing data in the selected region. With this reduced dataset, a new heuristic search in parsimony was run with the same settings as above.

Penalized likelihood (PL; Sanderson, 2002; Sanderson & al., 2004) analysis was performed using the r8s program, version 1.60 (Sanderson, 2003) on the phylogram of the majority rule consensus tree based on 99 equally most parsimonious trees. The relaxed clock method of PL allows different rates of substitution for each branch of the tree, however it restricts the rate difference allowed between neighboring branches. Unlike nonparametric rate smoothing (NPRS, Sanderson & al., 2004), PL assigns a penalty for rapid rate changes among branches based on a smoothness parameter. We used the cross-validation approach in r8s to infer the optimal level of rate smoothing for our data, which confirmed the assumed non-clock like rates. To prevent the optimization algorithm from converging on a local optimum, the PL searches were started at ten different initial time estimates (num_time_guesses=10) and were restarted ten times for each guess (num_restarts=10). A Truncated Newton (TN) method was then used to assign dates onto the initial phylogram (Sanderson, 2002). To explicitly incorporate the uncertainty of the primary age calibration, we constrained the age of the *Xanthorhiza-Ranunculus* split to a range of 51–66 Myr as reported by Wikström & al. (2001).

RESULTS

The aligned plastid dataset had a length of 1578 bp for the *matK* gene (individual sequence length ranging from 1515 bp for *R. carpaticola* to 1545 for *R. granatensis*). A 26 bp untranslated region preceding the coding region and a 340 bp region of the *trnK* intron after *matK* gene were also considered in our analysis. The non coding regions contained several informative gaps (Appendices 2 and 3, see online version). Parsimony analysis with gaps coded as binary characters based on 501 parsimony-informative characters yielded 7660 most parsimonious trees with a length of 1497 steps, a CI of 0.61 and a RI of 0.9. A phylogram based on the 50% majority rule consensus tree is shown in Fig. 2A (relatively few nodes collapsed in the corresponding strict consensus tree; these nodes have been marked with arrowheads). Bayesian inference revealed the same tree topology, with overall high posterior probability (PP) support for well supported clades found in the maximum parsimony analysis.

The ITS dataset ranged from 595 to 604 bp; the aligned data matrix consisted of 641 bp. The separate ITS analysis yielded 5250 most parsimonious trees of 1277 steps (CI = 0.43, RI = 0.82); the majority rule consensus tree has a very similar topology to that shown in Hörandl & al. (2005) and is shown here in a condensed form. Some polytomies appear in the strict consensus form of this tree (indicated on Fig. 2B).

The topologies of the ITS and *matK-trnK* consensus trees are highly congruent with respect to separation of allied genera on long basal branches (*Beckwithia*, *Callianthemoides*, *Coptidium*, *Halerpestes*, *Ficaria*). The main difference is that with the ITS data alone, the *Ceratocephala-Myosurus* clade is nested within the core *Ranunculus* clade, although with low bootstrap and posterior probability support. With the plastid dataset, the *Ceratocephala-Myosurus* clade is sister to the core clade. Within the core clade, both ITS and plastid data yielded a set of congruent well-supported clades (I–VIII) comprising very stable sets of species corresponding to ecological or morphological groups (see discussion); some of these clades are characterized by indels, such as clades V, VII, VIII, VIIIc, VIIId–g, VIIIf. The relationships of these clades to each other are partly incongruent or unresolved. Some weakly supported relationships within the core clade suggested by the majority rule consensus ITS tree, collapsed in the strict consensus tree (Fig. 2B), and were unresolved in a more extensively sampled ITS dataset (Hörandl & al., 2005). These putative relationships were also unresolved in the *matK-trnK* tree. That is, Clade I appears on a basal branch in the core clade in *matK-trnK* but is sister to the clade comprising clades II–III, VI–VIII in ITS, but again without boostrap support. Clade VI is on an unresolved branch in the plastid tree but sister to VII+VIII in ITS. The subdivision of clade VIII differs with respect to the position of clade VIIIa (basal in ITS, but not in *matK-trnK*) and VIIIb (sister to VIIIc–g in ITS, but to VIIIa in *matK-trnK*).

Maximum parsimony analysis of the combined dataset yielded 340 most parsimonious trees with a CI of 0.50 and a RI of 0.86 (Fig. 3A). Of the 2595 characters, 730 were parsimony-informative. Again, the clades found in the combined analyses remain very stable, and tree topologies are similar to the plastid tree, the main difference being that clade II remains on an unresolved

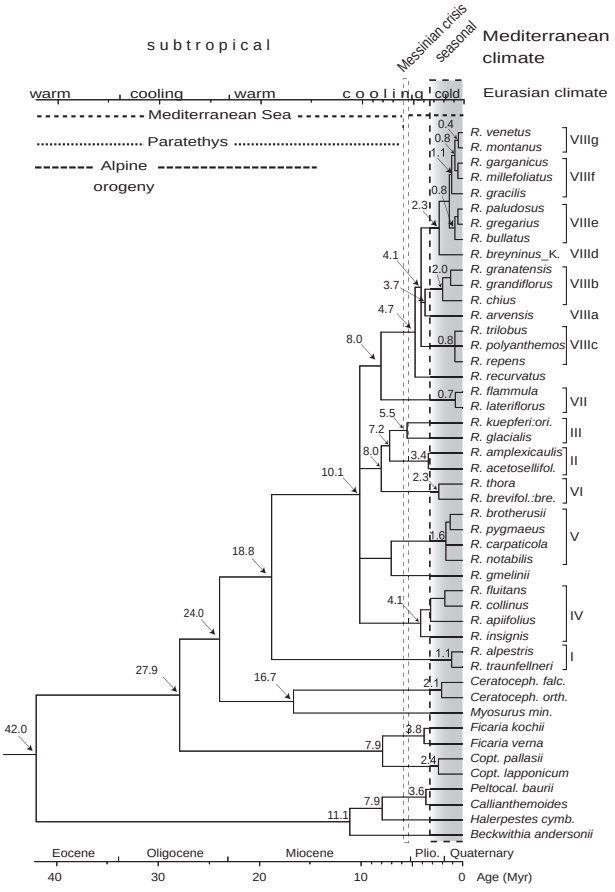


Fig. 4. Chronogram of the reduced dataset with age estimates for nodes of main clades. Climatic data after Suc (1984) and Frakes & al. (1992). The period of seasonal Mediterranean climate is shaded in grey.

branch, and clade VIIIa is basal within clade VIII. Bayesian inference analysis (Fig. 3B) yields a topology similar to MP, the main differences are again the position of clade II, and an unresolved polytomy on the base of clade VIII.

The tree reconstructed from the reduced *matK* dataset used for age estimates is similar to the tree obtained for the complete plastid DNA dataset, but leaves clades IV and V unresolved, and unites clades II, III, VI (Fig. 4). The cross-validation analysis in PL revealed a low to medium value for rate smoothing (3.50), which is not surprising considering some very long branches present in all trees (i.e., the branch of *Ceratocephala* or *Myosurus*). For the *matK* region selected (ca. 80% of total *matK* region) the analysis indicated an average substitution rate of 2.2×10^{-9} per site per year, with a standard deviation of 0.68×10^{-9}. Although the split between *Xanthorhiza* and *Ranunculus* was initially constrained to 51–66 Myr, the algorithm pushed the age of the split toward the maximum value; therefore, the split of *Xanthorhiza* from the genera of Ranunculeae sensu Tamura included here was estimated as 66.0 Myr old (around the turn of Upper Cretaceous/Paleocene). Results of the age estimate analysis for the major clades of *Ranunculus* are presented as a chronogram, calibrated against the geological timescale (Willis & McElwain, 2002; see Fig. 4).

DISCUSSION

Relationships, delimitation, and history of genera. — Our conclusions are based largely on analyses of a combined ITS and *matK* dataset. These data provide for the most robust analysis, and we focus on clades congruent in all analyses. Because of the issue of taxonomic uncertainty surrounding and within genus *Ranunculus*, our discussion first considers the major phylogenetic features of the genus before discussing specific Mediterranean lineages.

Both MP and BI of the combined dataset revealed a well-supported large core clade (BP and PP = 100), including *Ranunculus* s.s. (incl. subg. *Batrachium*, *Aphanostemma*, and subg. *Crymodes* p.p.), but excluding *Ceratocephala*, *Myosurus*, *Ficaria*, *Coptidium*, and the extraeuropean genera *Beckwithia*, *Callianthemoides*, *Halerpestes*, and *Peltocalathos*. An alternative definition of the "core *Ranunculus*" excluding clade I, which appears on a long basal branch (*R. alpestris* group), has only low bootstrap support (52). Molecular data and the lack of distinct morphological features of clade I support its inclusion into *Ranunculus*, which corresponds to the generic concept as already discussed in Hörandl & al. (2005). The most important difference to this study is that *Ceratocephala* and *Myosurus*, now being on a basal branch as sister to the *Ranunculus* core clade, can be alternatively classified to retain a monophyletic genus *Ranunculus*.

Arguments against inclusion of *Ceratocephala* into *Ranunculus* s.s. are (1) the occurrence of three indels (each three bp in length) present in the plastid data that are specific for *Ceratocephala*, plus considerable length mutations in the plastid genome, making it impossible to align the genus with other *Ranunculi* using cpDNA restriction site analysis (Johansson, 1998); (2) morphological features: *Ceratocephala* has been treated as a separate genus by most authors because of specific fruit characters (fruiting head falling off as a whole and not splitting into achenes; achenes with lateral bulges and a very large beak); and (3) differences in karyotype: *Ceratocephala* has five metacentric and two telocentric chromosomes ($x = 7$), thus deviating from *Ranunculus* s.s. with a maximum of four metacentric chromosomes and a predominant basic number of $x = 8$ (Goepfert, 1974).

Evidence also favours *Myosurus* being excluded from *Ranunculus* s.s. It is a genus with c. 15 annual species and a worldwide, mainly temperate distribution, that taxonomically has been always kept separate from *Ranunculus*. Tamura (1995) classified *Myosurus* in a different subtribe (Myosurinae) from *Ranunculus* because of distinct morphological (Hörandl & al., 2005) and chromosome features (a combined *Thalictrum-Ranunculus* type; Tamura, 1995). The ITS data and some morphological features (achenes with a sclerenchymatous layer) suggest that both genera originated from *Ranunculus*, but considerable branch lengths in both the ITS and plastid trees, and several derived morphological features cast doubt on this suggested phylogenetic relationship. The analyses of the plastid and combined sequences, plus distinct morphological and karyological features clearly separate them from the core clade, and from each other. This evidence justifies their classification as separate genera, as already suggested by Janchen (1949).

The exclusion of *Ficaria* is well supported in all molecular datasets and a consequence of the concept discussed above. Inclusion of this genus and its sister clade, the Nordic *Coptidium* (for discussion of generic concept see Hörandl & al., 2005) into *Ranunculus* would result in a very large, morphologically heterogeneous genus. *Ficaria* is well defined as a separate genus by distinct morphological features (achenes with a long-cuneate base and only a rudimentary beak, three sepals, strongly dimorphic roots with pronounced tubers, and a single cotyledon; Förster, 1997). *Ficaria* is an Eurasian genus, with many meridional species such as *Ficaria kochii* (distributed from Turkey to the Irano-Turanian region) and *Ficaria ficarioides* Bory & Chaub., distributed in the Eastern Mediterranean. The furthest range expansion to temperate and boreal zones is observed in the tetraploid bulbil-forming *Ficaria verna* subsp. *verna* (= *Ranunculus ficaria* subsp. *bulbilifer*; for nomenclatural background see Laegaard, 2001), suggesting a southern origin of the genus.

For the other taxa outside the core clade, the generic concept discussed in detail in Hörandl & al. (2005) is well supported by the *matK-trnK* and combined datasets.

Survey of the geographical-ecological groups in *Ranunculus* s.s. — The topology of the core clade in the combined analysis shows eight well-supported clades (I–VIII), which are very stable in their species composition and represent widespread ecological groups (wetland plants: IV, VII; higher latitudes/altitudes: V, higher altitudes: I, II, III, VI), only the four latter being also regional geographical groups (European alpines). Clade VIII is very heterogeneous, but comprises in general terrestrial species with a predominantly temperate to meridional distribution. The incongruence in the relationships of clades to each other in the different analyses may be due to (1) lack of extinct ancestral species in the dataset, and (2) complex relationships of ancestors due to hybridization. Overlaps of distribution areas of species were probably present at all times during the evolution of the genus. In present buttercups, hybridization and polyploidy are common features (Hörandl & al., 2005). Since clades I–VIII are not geographically separated, either the ancestors had wide distributions and diversified in parallel in different continents, or the lineages within the clades had an ability for long-distance dispersal; both features are observed in many present-day species.

The European alpine *Ranunculi* (clades I, II, III, VI). — Clade I is found on a long basal branch of the core clade. Its divergence from the other *Ranunculi* appears to have occured in the early Miocene. At that time, the old-tertiary Alpine orogeny had already formed the main European mountain belts (Tyrrhenids, Alps, Dinarid-Hellenids, Balcanids, continued by Pontids, Taurus, and Caucasus, separated from each other and from Northern Europe by the Paratethys; e.g., Thenius, 1977; Smith & al., 1994); this finding supports, therefore, an hypothesis for an early origin of orophytic groups. A basal position of the *R. alpestris* group within *Ranunculus* is consistent with the form of its nectary being a simple ridge (Hörandl, unpubl.; see also Baltisberger, 1994), since this structure is regarded as the most primitive form of the organ within the genus (Benson, 1940; Hörandl, unpubl.) and resembles the early ontogenetic stage of a transverse zone in petals of *Ranunculus* s.l. (Erbar & al., 1998). The separation of *R. crenatus* from the *alpestris* group as already suggested by Baltisberger (1994) is again confirmed by our molecular data. Clade I comprises here the more widespread *R. alpestris* (Alps, Pyrenees, C. Apennines), and narrowly distributed endemics in the southern Alps (*R. traunfellneri*, *R. bilobus*).

Clades II+III are probably closely related because of present hybridization patterns among members of the clades (Huber, 1988) and common peculiar characters (cup-like doubled nectary scales, and reticulate veins on the achenes). Hybridization among ancestors could have caused reticulate relationships, which results either in a collapse of branches on bifurcating trees (see Hörandl & al., 2005) or in incongruent positions in nuclear and plastid datasets. The present distributional patterns (clade II in southwestern Europe, clade III mainly in Central and Northern Europe) could also reflect a geographical connection in the late Miocene, when a continuous landmass existed from the Iberian Peninsula to Central and Northern Europe, whereas eastern and southeastern Europe was still split by remnants of the Paratethys (Smith & al., 1994).

Clade II (excluding *R. crenatus*, which is not consistently found in this clade), is a southwestern Mediterranean group characterized by white flowers, undivided, entire, linear to ovate or hastate leaves with a parallel venation and comprises most of *R.* sect. *Ranuncella* and the monotypic sections *Acetosellifolii* and *Chloeranunculus*. All species except *R. gramineus* are endemic to the mountains of the Iberian peninsula, but also the latter (distributed from NW Africa to temperate France, Central Italy, and Corsica, with a broad altitudinal and ecological amplitude) has its centre of distribution in the Iberian peninsula. The basal position of *R. gramineus* strongly supports an origin of the clade from lowland ancestors in the Iberian Peninsula, probably in the Miocene (split of clade II and III 7.2 Mya). The only taxon reaching the Alps, *R. parnassifolius* subsp. *heterocarpus* Küpfer, is a tetraploid apomict and therefore most likely a derivative of the diploid sexual subspecies of *R. parnassifolius* endemic to the Iberian Peninsula.

Clade III (with 100 PP, but only weak bootstrap support) consists of species with a centre of distribution in Central Europe, whereby most of the species reach the northern regions of the Mediterranean and the Pyrenees, but cannot be regarded as typical Mediterranean elements. This group is morphologically heterogeneous which is probably a result of an extreme adaptive radiation, as also observed in the New Zealand alpine species (Lockhart & al., 2001). *Ranunculus aconitifolius* and *R. platanifolius* are broad-leaf tall herbs with multi-branched inflorescences growing in the montane to subalpine forest zone of temperate (boreal) to submeridional Europe. The disjunct arctic-alpine *R. glacialis* and its relative *R. chamissonis* (often regarded only as a subspecies or variety of the former) endemic to the Beringian region are adapted to extreme high latitudes and altitudes and cold climates with a small habit, unbranched inflorescences, somewhat succulent leaves, and cup-like persistent sepals protecting the fruits. Our data suggest a considerable age for the divergence time of the lineage leading to *R. glacialis* (5.5 Mya; end of Miocene) in a period of cooler climate with glacial events at higher latitudes (5.5–5.35 Mya; Frakes & al., 1992). The species could have had already a pre-Quaternary northern distribution as hypothesized by Santisuk (1979), which could have become disjunct in glacial periods with a separation of the Beringian *R. chamissonis*. An alternative explanation based on AFLP variation patterns (without dating) is that the present disjunct distribution of *R. glacialis* is a result of postglacial colonization out of a centre in the Alps/Tatra mountains (Schönswetter & al., 2003). Because of the restriction to high-alpine habitats, stepwise migration in the lowlands is unlikely. Mechanisms of long-distance dispersal need to be studied.

Most striking is the inclusion of the alpine *R. kuepferi* in clade III, which is morphologically most similar to *R. pyrenaeus* (clade II) and has often been treated only as a subspecies of the latter (see synonymy in Greuter & al., 1989). Ancient lineage sorting within the *pyrenaeus-kuepferi*-ancestor could explain the unexpected tree topology. Nevertheless, hybridization of *R. kuepferi* with other species of clade III both in the field and in experimental crosses (Huber, 1988) support a close relationship. *Ranunculus kuepferi* subsp. *kuepferi*, the diploid sexual cytodeme, is endemic to the Alps Maritimes, whereas the tetraploid apomictic subsp. *orientalis* is more widespread in previously glaciated parts of the Alps, but occurs also locally in Corsica (Huber, 1989). *Ranunculus seguieri* is also a high-alpine species with a centre of distribution in the Alps (subsp. *seguieri*) and geographical separation of subspecies in the Pyrenees, Central Apennines, and the Dinarids, thus suggesting an allopatric speciation process.

The group diversified in the late Miocene, either from a northern ancestor and subsequent radiation in Central Europe, or from a lowland ancestor in Central Europe with subsequent altitudinal differentiation and pre- or postglacial colonization of the North. Present distribution patterns make the latter scenario more likely, but as exemplified with *R. glacialis*, the lack of information about pre-glacial distribution, the degree of extinction in the North during ice-ages and the potential for long-distance dispersal make biogeographical reconstructions difficult.

The relationships of *R.* sect. *Thora* (clade V, PP and bootstrap 100) remain unclear, because the basic position to clades VII+VIII in the combined MP analysis is neither supported by bootstrap analysis nor by Bayesian inference (unresolved), nor in the ITS data of Hörandl & al. (2005). This morphologically well-defined clade (see Hörandl & al., 2005) shows again a pre-glacial origin for diversification in the temperate to Mediterranean mountain belt.

The ancestors of the temperate alpine flora have been either regarded as descendants from the North or as migrants from lower altitudes (e.g., Stebbins, 1984). In our dataset, none of the three alpine clades (I, II, III, VI), nor the subalpine *R. montanus* group (clades VIIId, g; see below) has a species with a doubtless arctic origin. Only the arctic-alpine *R. pygmaeus* is nested within a northern group (clade V). These observations suggest that most alpine buttercups have originated more likely from temperate lowland ancestors with subsequent adaptation to high altitudes than from Northern ancestors. Similar conclusions have been drawn for other taxa with an Alpine-Mediterranean distribution (*Anthyllis*, *Pritzelago*, *Soldanella*), based on studies of diversification on the intraspecific level in the Pleistocene (Comes & Kadereit,

2003). Our age estimates suggest origin of clades II, III, VI in the late Miocene and their diversification in the Pliocene, which means clearly pre-glacial speciation events. This is consistent with the idea that polyploidy, a trait often correlated with degree of glaciation (Stebbins, 1984) and predominant in endemics of formerly glaciated areas (e.g., Brochmann & al., 2003), is very rare in these groups. Polyploidy occurs only in the above-mentioned subspecies of *R. kuepferi* and *R. parnassifolius* plus in *R. montanus* s.s. (all distributed in the formerly glaciated Alps), but not in the southern European alpine species.

Widespread ecological groups in *Ranunculus* (IV, V, VII, VIII). — Clades IV and V appear consistently as sister groups and are well supported (PP and BP values 100) both in MP and BI. Clade IV includes the water-buttercups (*R.* subg. *Batrachium* sensu Tamura 1995, here a well-supported (BP = 94, PP = 100) monophyletic group, contrary to Hörandl & al. (2005), and is sister to other aquatic species from temperate Eurasia and South America. The two New Zealand alpine species appear here on a basic branch of clade IV, which is characterised by a 6 bp insertion in the *trnK* sequences (positions 1835–1842 in Appendix 3, available on-line).

Clade V, excluding the more distant *R. gmelinii*, is well supported and characterized by 3 and 9 bp deletions in the plastid dataset (see Appendix 3). It comprises terrestrial northern hemispheric, temperate to arctic or alpine species, many of them occurring in the mountains of Central Asia. The age estimate of the crown group suggests recent diversification during the Pleistocene, which seems plausible regarding the distribution of many species in previously glaciated areas. At present no Mediterranean species are included here, but Greuter & al. (1989) report endemics of the predominantly apomictic *R. auricomus* complex, which is consistently found in this clade (here represented by diploid sexual *R. cassubicifolius*, *R. carpaticola*, and *R. notabilis*).

Clade VII is well supported by 100% bootstrap and posterior probability values and a nine bp deletion in the *trnK* sequence (positions 1652 to 1667 in Appendix 3). It includes creeping wetland plants of muddy or boggy habitats distributed from arctic to meridional Eurasia and South America, thus forming again a worldwide group. Most species are widespread (*R. reptans*, arctic to meridional Eurasia; *R. flammula*, boreal to meridional Eurasia and North America) and also the mediterranean *R. ophioglossifolius* expands far into the temperate zone, northwards to the southern British Islands and to Gotland (Jalas & Suominen, 1989). As in other aquatic species (see clade IV), long-distance dispersal is common and was probably important during the origin and history of the clades.

The "wetland" species in clades IV and VII are not monophyletic, but evolved probably with parallel adaptations to humid or aquatic habitats. Clade V has a tendency to high latidudes/altitudes and cooler climates. Development of an annual life form in clades IV and V, and also separation of a high alpine group in New Zealand on the basis of clade IV, indicate an ability to adapt to different habitats.

As sister to clade VII species appears a well-supported clade VIII (bootstrap and posterior probability = 100) comprising species from the meridional zone of Eurasia, including most of the Mediterranean endemics, and some species from temperate North America, South Africa and southern South America. The subdivision of the clade (a–g) follows neither previous classifications (see Fig. 2A), nor clear geographical groupings (Fig. 3A).

The Mediterranean annual buttercups. — The split of section *Echinella* already observed by Johansson (1998) and Hörandl & al. (2005) is confirmed by our dataset. The morphological features characterising the section (spiny to tuberculate fruits, and annual life form) are both highly homoplasious within the genus. Annual life form is readily interpreted as an adaptation to short flowering season in spring, when still sufficient humidity is available from the winter rainfalls, with rapid seed set and death of the plant before the summer drought. Spiny fruits are observed in various plant groups in summer-dry or arid climates (e.g., *Medicago*, *Astragalus*) and might be adaptations to epizoochorous seed dispersal.

Ranunculus arvensis (clade VIIIa) has the typical flat spiny fruit of sect. *Echinella*, but with very peculiar features such as a pinnate venation of the pericarp with several ramifications (Trzaski, 1999), and a papillose seed surface (Hörandl, unpubl.), characteristics not found in other species of sect. *Echinella* or other European *Ranunculi*. The pollen is periporate as in *R. parviflorus* and *R. chius*. The species is isolated from the other annuals and clusters with *R. caucasicus*, which resembles morphologically the species in a well supported clade VIIIb, probably only because the closer (Asian?) relatives of *R. arvensis* are not included here.

The annuals *R. chius* and *R. parviflorus* appear in a well-supported clade (VIIIb; BP and PP = 100), comprising all *Ranunculi* with the basic chromosome number $x = 7$. The other species in the clade are temperate to meridional perennial grassland and forest species from whole Eurasia, including some widespread species (*R. acris*).

The Mediterranean to Central Asian *R. cornutus*-group is sister to a clade of southern South American taxa (*R. chilensis*, *R. maclovianus*, *R. peduncularis*), both nested within a well-supported clade VIIIc (BP and PP = 100) comprising species from all continents. The relationships of the *R. chilensis*-*cornutus*-clade remain unresolved against the "*polyanthemos*" group occurring

mainly in temperate forests, which includes also one North American species (*R. pensylvanicus*). The latter and the appearance of *R. multifidus*, a species widespread in Africa, on an unresolved branch confirms a worldwide distribution of the clade. A larger ITS dataset also positioned Australasian and Malesian alpine taxa in this clade (Hörandl & al., 2005). The species in this clade have quite different ecological preferences, ranging from temperate forests (e.g., *R. polyanthemos*) to wet places (*R. sardous*) to arid habitats (*R. cornutus*). Many species have large distribution areas.

Our analyses suggest that the Mediterranean annuals originate from multiple parallel events of evolution in groups of various perennial taxa, some of which are not closely related to temperate Eurasian taxa. A similar pattern in the evolution of short-lived groups from perennials is observed in the Northern hemispheric genus *Androsace* (Schneeweiss & al., 2004). The divergence of annual buttercups appears to have initiated in the Pliocene, perhaps as a consequence of a general aridity in this period (e.g., Schwarzbach & al., 1974). Diversification of annuals is more or less parallel to the establishment of the Mediterranean climatic conditions, which probably promoted the therophytic life form.

The Mediterranean lowland to subalpine perennials. — Most of the Mediterranean perennials appear in a well-supported clade (BP+PP = 100) comprising the subclades VIIId–g, which are also characterized by a common 5 bp insertion (positions 1737–1748) and a 5 bp deletion (positions 1661–1667) in *trnK* (Appendix 3). On a basal branch appears the *R. breyninus* group (VIIId, BP = 74, PP = 100), distributed in the high mountain ranges from the Caucasus region to the Alps. *Ranunculus breyninus* (= *R. oreophilus* MB.) was included in the *R. montanus*-group by Landolt (1954, 1956) but already recognized as distinct from the others in having an apically hairy rhizome, a hairy receptacle throughout, and swollen achenes. This species together with *R. cappadocicus* has its centre of morphological diversity in the Caucasus region (Ovczinnikov, 1937; Hörandl, unpubl.), whereas the rest of the *R. montanus* group, characterized by glabrous rhizomes, an only apically hairy receptacle, and ± flattened achenes, has diversified within the European Alpine system.

The sister to the *R. breyninus* clade (VIIId) is subdivided into three groups: clade VIIIe is a moderately supported group (BP = 80, PP = 100) of western Mediterranean species (*R. olissiponensis*, *R. spicatus*, *R. pseudomillefoliatus*), two omnimediterranean ones (*R. bullatus*, *R. paludosus*), and the Macaronesian endemic *R. cortusifolius*. The group named VIIIf is not strictly monophyletic, but it has a six bp indel in the *trnK* intron at the same position, but with two different sequences (Appendix 3). The group comprises Eastern Mediterranean species and the Irano-Turanian *R. cicutarius*, and is only weakly separated from the rest of the *R. montanus* group, which appears as monophyletic (VIIIg) but with low support (BP < 50, PP = 100). Our data and also morphological features suggest inclusion of *R. marschlinsii* (endemic to Corsica) and *R. carpaticus* (Carpathians) into the *R. montanus* group (both species were not included by Landolt, 1954, 1956).

A classification of perennials according to underground parts is not supported. The combination of short rhizomes and tuberous roots appears to be homoplasious and is again probably only a rather recent adaptation to the summer-dry Mediterranean climate at lower altitudes (geophytes). The geographical differentiation in an eastern/western group reflects the general biogeographical division of the Mediterranean basin (Blondel & Aronson, 1999). Elongated rhizomes+stringlike roots appear often in species of higher altitudes, such as the "*montanus*" group (VIIId, g), sect. *Thora* (clade VI), and the *R. alpestris* group (clade I; see Fig. 3A), and can be interpreted as adaptations to steep slopes. It must be emphasized that the *R. montanus* group is not really high alpine, but predominantly occurring in mesic subalpine to low alpine grassland and tall-herb communities and growing in such habitats also often below the treeline. This ecological behaviour supports a hypothesis of a common origin of tuberous and rhizomatous species out of a lowland ancestor. Within the *R. montanus* group, the species with narrow distribution areas form geographical groups (*R. venetus*, *R. aduncus*: southwestern Alps; *R. pollinensis*, *R. gouanii*: Apennines), but this is not consistent in BI and the whole group shows no clear geographical pattern. The isolated distribution of most of the alpine species, and the weak ecological differentiation among the species suggest a predominantly allopatric speciation process as it is likely for most other alpine plants in southern Europe (Comes & Kadereit, 2003; Vargas, 2003). The rather young age (0.4 Myr) is consistent with generally weak morphological differentiation amongst taxa, which would justify also a classification on the subspecific level.

Our data suggest a common ancestry of the geophytic lowland and the hemicryptophytic subalpine buttercup flora in the late Pliocene. The subsequent morphological adaptation to different altitudes and climatic conditions happened probably shortly after the establishment of the Mediterranean climate between 3.2 to 2.8 Mya (Suc, 1984).

Island endemism and the case of *R. cortusifolius*. — In all of the five groups (VIIIc–g), taxa endemic to the Mediterranean region occur, some of them with narrow distribution areas either in high mountains of the mainland (e.g., *R. apenninus*, *R. pollinensis*) or on islands (e.g., *R. weyleri*: Mallorca; *R. cupreus*:

Crete; *R. cortusifolius*: Macaronesia). Mainland mountain endemics are usually related to morphologically similar species (e.g., in the *R. montanus* group, see discussion above). Island endemics, as sampled so far (three of eleven after Tutin & al., 1993) are either nested within a group of species of the same phytogeographical region (*R. cupreus*, E-Mediterranean; *R. cortusifolius*, W-Mediterranean), or in groups of widespread species (see *R. weyleri*). Contrary to mountain endemics, the three taxa included here are morphologically quite distinct from their close relatives, which is a general trait of island endemics (Baldwin & al., 1998).

The Macaronesian endemic *R. cortusifolius* (VIIIe) deserves special attention. The species was formerly regarded as most closely related to *R. creticus* (endemic to Crete, Eastern Aegean islands and adjacent Turkey, growing in gorges and on shaded rocks) based on the similarity of a large habit, tuberous roots, large palmately lobed leaves, and large flowers (Lowe, 1857). This was regarded as an example of Macaronesian-Eastern Mediterranean floristic connections (Bramwell & Richardson, 1973). Our data do not confirm this hypothesis, similar to observations in other genera, where close relationships of Macaronesian taxa to geographical distant taxa based on morphological studies were often not supported by molecular data (Andrus & al., 2004). Similarities in habit and leaf shape are homoplasious and probably an adaptation to shaded habitats and/or a more oceanic climate on islands. A tall, broad-leaved habit is also observed in some other island endemics (e.g., *R. caprarum* Skottsb. on Juan Fernández Islands, or *R. mauiensis* A. Gray on Hawai'i). *Ranunculus cortusifolius* is a tall herb growing in the *Laurus* forest vegetation of the Canarian Islands; on Madeira and the Azores giant forms occur in moist ravines and gorges near waterfalls (Lowe, 1857). The Macaronesian *Laurus* vegetation is regarded as a relict of a subtropical Tertiary flora (e.g., Bramwell, 1976; Willis & McElwain, 2002). Nevertheless, our molecular data and also the presence of tuberous roots even at moist habitats suggest that *R. cortusifolius* is not a palaeoendemic element of Tertiary subtropical forests, but rather a young colonizer (estimated diversification of clade VIIIe in the Pleistocene, 0.8 Mya) and descendant of western Mediterranean geophytic buttercups. This example and other age estimates of island endemic buttercups (all < 0.8 Myr) are consistent with a general pattern of neo-endemism and dynamic speciation processes that are predominant in the Mediterranean area (Thompson, 2005).

Historical survey and conclusion. — The selection of the maximum age for the basal *Xanthorhiza*-*Ranunculus* split is reasonable because Wikström & al. (2001, 2004) regard ages of terminal nodes in the family tree generally as underestimates. However, the inferred dates using the PL method must be taken tentatively, as relaxed clock methods are known to perform poorly in the absence of multiple date calibrations (Gaut, 1998; Sanderson, 2004; Wikström & al., 2004; Knapp & al., 2005). Thus, accuracy of age estimates on terminal nodes should be taken with caution. For example, an age of 0.9 Myr has been estimated for the split of *R. notabilis* and *R. carpaticola* based on genetic distances inferred from isozyme data (Hörandl, 2004), which fits that this age is younger than the split for clade V (1.6 Mya, Fig. 4). Lockhart & al. (2001) suggest an age of 5 Myr for the diversification of the New Zealand alpine species and this estimate is older than is suggested from our PL (i.e., 4.1 Myr) estimates. The origin of many clades within *Ranunculus* corresponds to the Late Tertiary/Quarternary period (Fig. 4), which was a period of dramatic and global climatic change, when diversification is thought to have occurred in many island and continental floras (e.g., Comes & Kadereit, 1998; Winkworth & al., 2002). Thus, our temporal reconstruction is similar to findings with molecular studies on other plant groups.

Based on the age estimate of 66 Myr for the split of tribe Ranunculeae from *Xanthorhiza*, the extraeuropean genera are separated from the other ranunculaean taxa in the Eocene (42 Mya). This was a period of continental isolation, with the formation of the Tethys, the Atlantic, and the Panama corridor (Smith & al., 1994). For the ancestor of the narrowly distributed monotypic genera (*Peltocalathos*: Cape region; *Callianthemoides*: southern S. America; and *Beckwithia andersonii*: E. North America), a basic pattern of vicariance is likely, followed by much more recent diversification to derive extant taxa (Fig. 4). We refrain here from a more detailed historical reconstruction for the tribe because of incomplete sampling of other allied genera.

A brief hypothetical outline of the history of the tribe in Eurasia shows that main diversification periods are roughly correlated with climatic periods (Fig. 4). We hypothesize that the ancestor of *Ranunculus* s.s. had a wide distribution and ecological amplitude in late Oligocene. The split of the *Ficaria*-*Coptidium* stem group appears during a period of cooler climate, and replacement of tropical vegetation by warm/cool evergreen and deciduous forest biome in southern Europe (Willis & McElwaine, 2002). The split of the *Myosurus*-*Ceratocephala* stem group took place during a subsequent period of warming, but species within *Ceratocephala* differentiated much later after the establishment of the Mediterranean climate (3.2–2.8 Mya, Suc, 1984). The main differentiation of *Ranunculus* s.s. into ecological groups (widespread wetland: IV, VII; higher latitudes/altitudes: V; orophytes (II, III, VI), and also the *Ficaria*/*Coptidium* split is observed in the late Miocene between 10 and 7 Mya, a period of global cool-

ing. In southern Europe, tropical elements disappeared, and a winterwet biome with an evergreen forest and shrub vegetation established, whereas in Central and Northern Europe a cool-temperate biome with deciduous forests was predominant (Willis & McElwaine, 2002). Climatic changes probably promoted not only a separation of groups with respect to latitude, but also in altitude within the already existing European Alpine belt. It is uncertain whether the desiccation of the Mediterranean Sea during the Messinian crisis (5.96–5.33 Mya, Krijgsman & al., 1999) had an impact on the buttercup flora, but land connections and a more continental climate and could have promoted invasion of near East Asian elements. The late Miocene to early Pliocene is a period of cyclic warming-cooling, followed by a general decrease of temperature and an increase of aridity in Pliocene (Crowley & North, 1991; Frakes & al., 1992); the palm-line moved in Europe southwards to c. 45° latitude, and climate was similar to present (Schwarzbach, 1974). In this period diversification of *Ficaria* and the widespread clades II, IV and VIII started. The differentiation of the Mediterranean lowland flora into annuals, tuberous geophytes at lower altitudes and subalpine rhizomatous hemicryptophytes (clades VIIId–g) happened after the establishment of the Mediterranean seasonal climate and a complex mosaic of vegetation types 2.8–3.2 Mya (Suc, 1984), speciation within these groups took place mainly during the Pleistocene.

Especially for the alpine taxa, the origin and diversification of each of the clades during different time periods obstructs phytogeographic generalizations. Orophytic species have probably originated from lowland ancestors of the same region (I, II, III, VI, VIIIg), and most species of the European alpine system show closer relationships to Mediterranean than to northern European taxa. A geographic separation is observed on terminal branches within these clades but is not pronounced between them (only clade II being a regional geographical group within the European mountain system). The separation of landmasses by the Mediterranean Sea is perhaps too weak for a long-term geographical isolation of major groups. The pre-glacial age of most clades and missing information about the distribution of ancestors because of the lack of pre-quaternary fossils makes it difficult to develop biogeographical hypotheses from present distribution patterns, as exemplified by clade III. Tertiary ancestors of the present Mediterranean alpine buttercups could have become extinct in glaciated areas (Pyrenees, Alps, northern Europe). Therefore, it is hard to say whether present distributions reflect relictual pre-glacial patterns or inter- or postglacial dispersal. Nevertheless, a common pattern for the groups of higher altitudes/latitudes is probably that diversification took place during periods of a cooling of the climate. This resembles hypotheses for the alpine *Primula* sect. *Auricula*, where simulation studies showed that speciation took place rather in glacial periods at lower altitudes than in interglacial periods in high-altitude interglacial refugia (Kadereit & al., 2004).

At lower altitudes, the cooling of the climate caused largely a replacement of the Eu-mediterranean vegetation by deciduous forests (Blondel & Aronson, 1999). Migrations and range expansion/restrictions parallel to climatic fluctuations were frequent in the Pleistocene (Blondel & Aronson, 1999; Vargas, 2003). For distributional patterns in the lowlands, human influence must be also regarded as an important factor. Domestication of animals and cultivation of plants started in the Mediterranean region already about 10,000 years ago (Blondel & Aronson, 1999). Deforestation by fire and grazing led to a replacement of the natural forest vegetation by a more patchy landscape of sclerophyllous woodland remnants, pastures, olive plantations, cultivated land, etc., thus providing various new habitats for the herbaceous flora. Anthropogenic range expansions are documented for annual buttercups (*R. arvensis*, *R. muricatus*, *R. parviflorus*, *R. sardous*; Jalas & Suominen, 1989).

Each lineage within *Ranunculus* shows a different age, distribution and diversification pattern, also suggesting that the species have also their own individual biogeographical histories. Phylogeographical studies on the intra- and infraspecific levels in other genera corroborate this general observation in the Mediterranean (e.g., Vargas, 2003). The high species diversity and the considerable degree of endemism in *Ranunculus* reflect the general biodiversity of the Mediterranean region. Representing only a small part of the Earth's dry land, it harbours about 10% of the world's total vascular plants species diversity, of which more than 50% are endemic (Blondel & Aronson, 1999). This richness is probably due to (1) the complex and frequent changes of the geomorphology and climate in the history of the Mediterranean that promoted evolutionary processes and speciation, (2) its geographical position as contact zone for the floras of neighbouring Europe, Asia, and Africa, but also including highly isolated areas such as islands and high mountains; and (3) the extraordinary diverse geomorphology, including a 48,000 km long and highly structured coastline, a high altitudinal range, and a patchy-like human-influenced lowland vegetation, thus providing a broad spectrum of habitats. For a genus such as *Ranunculus*, which has in general a high morphological plasticity and ability to adapt to different niches (Hörandl & al., 2005), the Mediterranean region is obviously an El Dorado for diversification and speciation.

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LITERATURE CITED

Acevedo-Rosas, R., Cameron, K., Sosa, V. & Pell, S. 2004. A molecular phylogenetic study of *Graptopetalum* (Crassulaceae) based on ETS, ITS, *rpl16*, and *trnL-F* nucleotide sequences. *Amer. J. Bot.* 91: 1099–1104.

Andersson, L. & Chase, M. W. 2001. Phylogeny and classification of Marantaceae. *Bot. J. Linn. Soc.* 135: 275–287.

Andrus, N., Trusty, J., Santos-Guerra, A., Jansen, R. K. & Francisco-Ortega, J. 2004. Using molecular phylogenies to test phytogeographical links between East/South Africa-Southern Arabia and the Macaronesian islands - a review, and the case of *Vierea* and *Pulicaria* section *Vieraeopsis* (Asteraceae). *Taxon* 53: 333–346.

Baldwin, B. G., Crawford, D. J., Francisco-Ortega, J., Kim, S.-C., Sang, T. & Stuessy, T. F. 1998. Molecular phylogenetic insights on the origin and evolution of oceanic island plants. Pp. 410–441 in: Soltis, P. S., Soltis, D. E. & Doyle, J. J. (eds.), *Molecular Systematics of Plants, II. DNA Sequencing.* Kluwer, New York.

Baltisberger, M. 1994. *Ranunculus cacuminis* and *R. crenatus*, representatives of the *R. alpestris*-group (Ranunculaceae) on the Balkan Peninsula. *Pl. Syst. Evol.* 190: 231–244.

Benson, L. 1940. The North American subdivisions of *Ranunculus*. *Amer. J. Bot.* 83: 516–527.

Berry, P. E., Hahn, W. J., Sytsma, K. J., Hall, J. C. & Mast, A. 2004. Phylogenetic relationships and biogeography of *Fuchsia* (Onagraceae) based on noncoding nuclear and chloroplast DNA data. *Amer. J. Bot.* 91: 601–614.

Blondel, J. & Aronson, J. 1999. *Biology and Wildlife of the Mediterranean Region.* Oxford Univ. Press, Oxford.

Bramwell, D. 1976. The endemic flora of the Canary Islands; distribution, relationships, and phytogeography. Pp. 207–240 in: Kunkel, G. (ed.), *Biogeography and Ecology in the Canary Islands.* Junk, The Hague.

Bramwell, D. & Richardson, I. B. K. 1973. Floristic connections between Macaronesia and the East Mediterranean region. *Monogr. Biolog. Canarienses* 4: 118–125.

Brochmann, C., Gabrielsen, T. M., Nordal, I., Landvik, J. Y. & Elven, R. 2003. Glacial survival or tabula rasa? The history of North Atlantic biota revisited. *Taxon* 52: 417–450.

Comes, H. P. & Kadereit, J. W. 1998. The effect of Quaternary climatic changes on plant distribution and evolution. *Trends Plant Sci.* 3: 432–438.

Comes, H. P. & Kadereit, J. W. 2003. Spatial and temporal patterns in the evolution of the flora of the European Alpine System. *Taxon* 52: 451–462.

Crowley, T. J. & North, G. R. 1991. *Paleoclimatology*. Oxford Univ. Press, New York.

Erbar, C., Kusma, S. & Leins, P. 1998. Development and interpretation of nectary organs in Ranunculaceae. *Flora* 194: 317–332.

Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. 1995. Constructing a significance test for incongruence. *Syst. Biol.* 44: 570–572.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Fitch, W. M. 1971. Toward defining the course of evolution: minimal change for a specific tree topology. *Syst. Zool.* 20: 406–416.

Förster, P. 1997. Seedlings of the tribus Ranunculeae DC. and the tribus Adonideae Kunth (Ranunculaceae). *Flora* 192: 133–142.

Frakes, L. A., Francis, J. E. & Syktus, J. I. 1992. *Climatic Modes of the Phanerozoic*. Cambridge Univ. Press, Cambridge.

Garnock-Jones, P. J. 1984. *Ceratocephalus pungens* (Ranunculaceae): a new species from New Zealand. *New Zealand J. Bot.* 22: 135–137.

Gaut, B. S. 1998. Molecular clocks and nucleotide substitution rates in higher plants. Pp. 93–120 in: Hecht, M. K., MacIntyre, R. J. & Clegg, M. T. (eds.), *Evolutionary Biology*, vol. 30. Plenum Press, New York.

Goepfert, D. 1974. Karyotypes and DNA content in species of *Ranunculus* L. and related genera. *Bot. Notiser* 127: 464–489.

Greuter, W., Burdet, H. M. & Long, G. 1989. *Med-Checklist 4. Dicotyledones (Lauraceae-Rhamnaceae).* Conservatoire et Jardin botaniques, Geneve.

Holland, B. R., Huber, K. T., Moulton, V. & Lockhart, P. J. 2004. Using consensus networks to visualize contradictory evidence for species phylogeny. *Molec. Biol. Evol.* 21: 1459–1461.

Hörandl, E. 2004. Comparative analysis of genetic divergence among sexual ancestors of apomictic complexes using isozyme data. *Int. J. Plant Sci.* 165: 615–622.

Hörandl, E., Paun, O., Johansson, J. T., Lehnebach, C., Armstrong, T., Chen, L. & Lockhart, P. 2005. Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. *Molec. Phylog. Evol.* 36: 305–327.

Huber, W. 1988. Natürliche Bastardierungen zwischen weißblühenden *Ranunculus*-Arten in den Alpen. *Veröff. Geobot. Inst. ETH Stiftung Rübel Zürich* 100: 1–160.

Huber, W. 1989. *Ranunculus kuepferi* Greuter & Burdet in Korsica (Gruppe *R. pyrenaeus* L.). *Candollea* 44: 630–637.

Huelsenbeck, P. J. & Ronquist, F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17: 754–755.

Hultén, E. & Fries, M. 1986. *Atlas of North European Vascular Plants north of the Tropic of Cancer*, vol. 1. Koeltz, Königstein.

Iranshar, M., Rechinger, K. H., Jr. & Riedl, H. 1992. Ranunculaceae. Pp. 1–249 in: Rechinger, K. H., Jr. (ed.), *Flora Iranica*, vol. 171. Akad. Druck- u. Verlagsanstalt, Graz.

Jalas, J. & Suominen, J. 1989. *Nymphaeaceae to Ranunculaceae. Atlas Florae Europaeae*, vol. 8. The Committee for Mapping the Flora of Europe, Helsinki.

Janchen, E. 1949. Die systematische Gliederung der Ranunculaceen und Berberidaceen. *Österr. Akad. Wiss, Math.-Naturwiss. Kl., Denkschr.* 108: 1–82.

Johansson, J. T. 1998. Chloroplast DNA restriction site mapping and the phylogeny of *Ranunculus* (Ranunculaceae). *Pl. Syst. Evol.* 213: 1–19.

Kadereit, J. W., Griebeler, E. M. & Comes, H. P. 2004. Quaternary diversification in European alpine plants: patterns and process. *Phil. Trans. Roy. Soc. London B* 359: 265–274.

Kirchoff, B. K., Richter, S. J., Remington, D. L. & Wisniewsky, E. 2004. Complex data produce better characters. *Syst. Biol.* 53: 1–17.

Kluge, A. G. 1989. A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). *Syst. Zool.* 38: 7–25.

Knapp, M., Stöckler, K., Havell, D., Delsuc, F., Sebastiani, F. & Lockhart, P. J. 2005. Relaxed molecular clock provides evidence for long-distance dispersal of *Nothofagus* (Southern Beech). *PLoS Biology. Jan*; 3(1):e14.

Krijgsman, W., Hilgen, F. J., Raffi, I., Sierro, F. J. & Wilson, D. S. 1999. Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400: 652–655.

Laegaard, S. 2001. *Ranunculus ficaria*. Pp. 230–231 in: Jonsell, B. & Karlsson, T. (eds.), *Flora Nordica*, vol. 2. Chenopodiaceae to Fumariaceae. The Bergius Foundation, Stockholm.

Landolt, E. 1954. Die Artengruppe des *Ranunculus montanus* Willd. in den Alpen und im Jura (zytologisch-systematische Untersuchungen). *Ber. Schweizer Bot. Gesell.* 64: 9–83.

Landolt, E. 1956. Die Artengruppe des *Ranunculus montanus* Willd. in den Pyrenäen und anderen europäischen Gebirgen westlich der Alpen. *Ber. Schweizer Bot. Gesell.* 66: 92–117.

Lockhart, P., McLechnanan, P. A., Havell, D., Glenny, D., Huson, D. & Jensen, U. 2001. Phylogeny, dispersal and radiation of New Zealand alpine buttercups: molecular evidence under split decomposition. *Ann. Missouri Bot. Gard.* 88: 458–477.

Lowe, R. T. 1857. On *Ranunculus creticus*, *R. cortusaefolius*, and *grandifolius* of authors. *J. Bot. & Kew Gard. Misc.* 9 (65): 65–75.

Maddison, D. R. & Maddison, W. P. 2000. *MacClade 4: Analysis of Phylogeny and Character Evolution, Version 4.0*. Sinauer Associates, Sunderland, Massachusetts.

Meusel, W., Jäger, E. & Weinert, E. 1965. *Chorologie der zentraleuropäischen Flora*. G. Fischer, Jena.

Mort, M. E., Soltis, D. E., Soltis, P. E., Francisco-Ortega, J. & Santos-Guerra, A. 2001. Phylogenetic relationships and evolution of Crassulaceae inferred from *matK* sequence data. *Amer. J. Bot.* 88: 76–91.

Muellner, A. N., Samuel, R., Johnson, S. A., Cheek, M., Pennington, T. D. & Chase, M. W. 2003. Molecular phylogenetics of Meliaceae (Sapindales) based on nuclear and plastid DNA sequences. *Amer. J. Bot.* 90: 471–480.

Nixon, K. C. & Carpenter, J. M. 1996. On simultaneous analysis. *Cladistics* 12: 221–241.

Nylander, J. A. A., Ronquist, F., Huelsenbeck, J. P. & Nieves-Aldrey, J. L. 2004. Bayesian phylogenetic analysis of combined data. *Syst. Biol.* 53: 47–67.

Ovczinnikov, P. N. 1937. *Ranunculus*. Pp. 351–509 in: Komarov, W. A. (ed.), *Flora URSS*, Vol. VII. Ranales and Rhoeadales, Botanicheskii Institut Akademii Nauk USSR, Moscow. [English translation 1970.]

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

Sanderson, M. J. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molec. Biol. Evol.* 19: 101–109.

Sanderson, M. J. 2003. *r8s: Analysis of Rates ("r8s") of Evolution (and other stuff)*. Version 1.60. Computer software. http://ginger.ucdavis.edu/r8s/

Sanderson, M. J., Thorne, J. L., Wikström, N. & Bremer, K. 2004. Molecular evidence on plant divergence times. *Amer. J. Bot.* 91: 1656–1665.

Santisuk, T. 1979. A palynological study of the tribe Ranunculeae (Ranunculaceae). *Opera Bot.* 48: 1–74.

Schneeweiss, G. M., Schönswetter, P., Kelso, S. & Niklfeld, H. 2004. Complex biogeographic patterns in *Androsace* (Primulaceae) and related genera: evidence from phylogenetic analyses of nuclear Internal Transcribed Spacer and plastid *trnL-F* sequences. *Syst. Biol.* 53: 856–876.

Schönswetter, P., Paun, O., Tribsch, A. & Niklfeld, H. 2003. Out of the Alps: colonization of Northern Europe by east alpine populations of the glacier buttercup *Ranunculus glacialis* L. (Ranunculaceae). *Molec. Ecol.* 12: 3373–3381.

Schwarzbach, M. 1974. *Das Klima der Vorzeit*, ed. 3. Enke Verlag, Stuttgart.

Siddall, M. E. 1997. Prior agreement: arbitration or arbitrary? *Syst. Biol.* 46: 765–769.

Simmons, M. & Ochoterena, P. H. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Syst. Biol.* 49: 369–381.

Smith, A. G., Smith, D. G. & Funnell, B. M. 1994. *Atlas of Mesozoic and Cenozoic Coastlines*. Cambridge Univ. Press, Cambridge.

Stebbins, G. L. 1984. Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. *Bot. Helv.* 94: 1–14.

Suc, J.-P. 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature* 307: 429–432.

Swofford, D. L. 2001. *PAUP*: Phylogenetic Analysis Using Parsimony*. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura, M. 1993. Ranunculaceae. Pp. 563–583 in: Kubitzki, K., Rohwer, J. G. & Bittrich, V. (eds.), *The Families and Genera of Vascular Plants. 2. Flowering Plants. Dicotyledons, Magnoliid, Hamamelid, and Caryophyllid Families*. Springer, Berlin.

Tamura, M. 1995. Angiospermae. Ordnung Ranunculales.

Fam. Ranunculaceae. II. Systematic Part. Pp. 223–519 in: Hiepko, P. (ed.), *Natürliche Pflanzenfamilien*, ed. 2, 17aIV. Duncker & Humblot, Berlin.

Thenius, E. 1977. *Meere und Länder im Wechsel der Zeiten*. Springer, Berlin.

Thompson, J. D. 2005. *Plant Evolution in the Mediterranean.* Oxford Univ. Press, New York.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Trzaski, L. 1999. Xylem distribution in the achene of some European *Ranunculus* species as a taxonomical criterion of *Ranunculus* genus. *Phytomorphology* 49: 241–251.

Tutin, T. G. & Cook, C. D. K. 1993. *Ranunculus*. Pp. 269–290 in: Tutin, T. G., Heywood, V. H., Burges, N. A., Valentine, D. H., Walters, S. M. & Webb, D. A. (eds.), *Flora Europaea I: Psilotaceae to Platanaceae*, ed. 2. Cambridge Univ. Press, Cambridge.

Vargas, P. 2003. Molecular evidence for multiple diversification of alpine plants in Mediterranean Europe. *Taxon* 52: 463–476.

Whittemore, A. 1997. *Ranunculus*. Pp. 88–135 in: Flora of North America Committee (eds.), *Flora of North America North of Mexico*, vol. 3. Magnoliophyta: Magnoliidae and Hamamelidae. Oxford Univ. Press, New York.

Wikström, N., Savolainen, V. & Chase, M. W. 2001. Evolution of the angiosperms: calibrating the family tree. *Proc. Royal Soc. London, ser. B* 268: 2211–2220.

Wikström, N., Savolainen, V. & Chase, M. W. 2004. Angiosperm divergence times: congruence and incongruence between fossils and sequence divergence estimates. Pp. 142–165 in: Donoghue, P. C. J. & Smith, M. P. (eds.), *Telling the Evolutionary Time—Molecular Clocks and the Fossil Record.* CRC Press, London.

Willis, K. J. & McElwain, J. C. 2002. *The Evolution of Plants.* Oxford Univ. Press, New York.

Winkworth, R. C., Wagstaff, S., Glenny, D. & Lockhart, P. J. 2002. Plant dispersal N.E.W.S. from New Zealand. *Trends Ecol. Evol.* 17: 514–520.

Appendix 1. Materials used in this study (BG = Botanical Garden).

Species (synonym); **country**; **collector, collection number and herbarium**; **ITS GenBank no.**; ***matK* GenBank no.**

Beckwithia andersonii (A. Gray) Jeps. (*Ranunculus andersonii*); cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680197; AY954238. *Callianthemoides semiverticillatus* (Philippi) Tamura (*R. semiverticillatus*); Argentina; *C. Lehnebach s.n.* VALD; AY680199; AY954236. *Ceratocephala falcata* (L.) Pers. (*R. falcatus*); Iran; *K.H. Rechinger, Jr. 50857* W; AY680191; AY954229. *C. orthoceras* DC. (*R. testiculatus*); Austria; *E. Hörandl 3837* WU; AY680190; AY954230. *Coptidium lapponicum* (L.) Tzvelev (*R. lapponicus*); Sweden; *J.T. Johansson s.n.* -; AY680194; AY954234. *C. pallasii* (Schlecht.) Tzvelev (*R. pallasii*); Alaska; *R. Elven & al. SUP02-175* O; AY680195; AY954233. *Ficaria kochii* (Ledeb.) Iranshar & Rech.f. (*R. kochii*); cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680193; AY954231. *F. verna* Huds. ssp. *verna*[1] (*R. ficaria* subsp. *bulbilifer*); Sweden; *J.T. Johansson s.n.* -; AY680192; AY954232. *Halerpestes cymbalaria* (Pursh) Greene (*R. cymbalaria*); cult. Rezia BG; *J.T. Johansson 204* LD; AY680196; AY954237. *Myosurus minimus* L.; Genbank; AJ347913; AF007947, AJ414344. *Peltocalathos baurii* (McOwan) Tamura (*R. baurii*); South Africa; *L. Mucina 030103/22* WU; AY680200; AY954235. *Ranunculus abortivus* L.; Connecticut; *J.T. Johansson 186* CONN; AY680048; AY954126. *R. acetosellifolius* Boiss.; cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680075; AY954226. *R. aconitifolius* L.; cult. Copenhagen BG; *J.T. Johansson 274* LD; AY680081; AY954217. *R. acris* L.; cult. Bonn BG; *J.T. Johansson 194* CONN; AY680167; AY954199. *R. aduncus* Gren. & Godr.; Italy; *E. Hörandl 6818* WU; AY680088; AY954143. *R. alpestris* L.; cult. Rezia BG; *J.T. Johansson 242* LD; AY680078; AY954221. *R. altaicus* Laxm.; Russia; *A. Tribsch 9545* WU; AY680112; AY954116. *R. amplexicaulis* L.; cult. Lund BG; *J.T. Johansson 222* LD; AY680071; AY954223. *R. apenninus* (Chiov.) Pign.; Italy; *E. Hörandl 6069* WU; AY680091; AY954150. *R. apiifolius* Pers. (*Aphanostemma apiifolia*); Chile; *C. Lehnebach s.n.* VALD; AY680092; AY954140. *R. arcticus* Richards. (*R. affinis*); cult. Devonian BG; *J.T. Johansson 239* LD; AY680049; AY954125. *R. arvensis* L.; cult. Kiel BG; *J.T. Johansson 180* CONN; AY680177; AY954193. *R. baldschuanicus* Regel ex Kom.; cult. Copenhagen BG; *J.T. Johansson 272* LD; AY680174; AY954195. *R. bilobus* Bertol.; Italy; *E. Hörandl 4574* WU; AY680077; AY954220. *R. brevifolius* ssp. *brevifolius* Ten.; cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680187; AY954212. *R. breyninus* Cr. (*R. oreophilus*); Austria (loc. class.); *E. Hörandl 5249* WU; AY680115; AY954172. *R. breyninus* Cr. (*R. oreophilus*); Georgia; *E. Hörandl 8361* WU; AY680116; AY954174. *R. brotherusii* Freyn; Nepal; *M. Staudinger 484280* LI; AY680037; AY954119. *R. bulbosus* ssp. *bulbosus* L.; Sweden; *J.T. Johansson s.n.* -; AY680124; AY954188. *R. bullatus* L.; Greece; *W. Gutermann 30068* & *E. Hörandl 7191* WU; AY680114; AY954161. *R. camissonis* Aucl. (*Beckwithia camissonis*); U.S.S.R.; *R. Koropewa s.n.* W; AY680083; AY954218. *R. cappadocicus* Willd.; Georgia; *E. Hörandl 8269* WU; AY680117; AY954173. *R. cardiophyllus* Hook.; cult. Gothenburg BG; *J.T. Johansson HZ 86-29* GB; AY680045; AY954124. *R. carinthiacus* Hoppe; Austria; *E. Hörandl 4096* WU; AY680093; AY954145. *R. carpaticola* Soó; Slovakia; *E. Hörandl 8483* WU; AY680041; AY954111. *R. carpaticus* Herbich; Romania; *O. Paun s.n.* WU; AY680096; AY954154. *R. cassubicifolius* W. Koch; Germany; *E. Hörandl 8477* WU; AY680040; AY954112. *R. caucasicus* MB.; Georgia; *E. Hörandl 8259* WU; AY680178; AY954192. *R. chilensis* DC.; Chile; *C. Lehnebach s.n.* VALD; AY680157; AY954179. *R. chius* DC.; Greece; *W. Gutermann & al. 34758* WU; AY680176; AY954201. *R. cicutarius* Schlecht.; Iran; *H. Akhani 320156* LI; AY680103; AY954167. *R. collinus* DC.; cult. Canberra BG; *Crisp & Telford 2227* CAN; AY680059; AY954137. *R. cornutus* DC. (*R. lomatocarpus*); Azerbeidschan; *G. Schneeweiss 6806* WU; AY680153; AY954178. *R. cortusifolius* Willd.; cult. Halle BG; *J.T. Johansson 237* LD; AY680101; AY954160. *R. crenatus* Waldst. & Kit.; Austria; *E. Hörandl 2818* WU; AY680086; AY954228. *R. creticus* L.; Greece, Crete; *E. Hörandl 8518* W; AY954239; AY954163. *R. cupreus* Boiss. & Heldr.; Greece, Crete; *E. Hörandl 8666* W; AY954240; AY954164. *R. eschscholtzii* Schlecht.; Canada; *U. Jensen UJ8* MPN; AY680050; AY954127. *R. flagelliformis* Sm.; Peru; *P. Gute, G. Müler 309853* LI;

[1]For nomenclature see Laegaard, 2001

Appendix 1 (continued).

Species (Synonym); **Country**; **Collector, collection number and herbarium**; **ITS GenBank no.**; ***matK* GenBank no.**

AY680182; AY954208. *R. flammula* L.; cult. Oldenburg BG; *J.T. Johansson 193* CONN; AY680185; AY954204. *R. fluitans* Lam.; Scania; *J.T. Johansson s.n.* -; AY680069; AY954129. *R. fuegianus* Speg.; Chile; *L. & F. Ehrendorfer s.n.* VALD; AY680064; AY954136. *R. garganicus* Ten.; Greece; *W. Gutermann & al. 34974* WU; AY680107; AY954165. *R. gelidus* Kar. & Kir.; Xinjiang, China; *B. Wang 28426* MPN; AY680054; AY954114. *R. glacialis* L.; Sweden; *J.T. Johansson s.n.* -; AY680082; AY954219. *R. gmelinii* ssp. *gmelinii* DC.; U.S.A., Alaska; *C. Schröck 454907* LI; AY680063; AY954128. *R. gouanii* Willd.; cult. Schachen; *J.T. Johansson s.n.* -; AY680098; AY954151. *R. gracilis* Schleich.; Greece; *J.T. Johansson s.n.* -; AY680120; AY954171. *R. gramineus* L.; cult. Krefeld BG; *J.T. Johansson s.n.* -; AY680076; AY954227. *R. granatensis* Boiss.; unknown; *J.T. Johansson 266* LD; AY680165; AY954197. *R. grandiflorus* L.; Georgia; *E. Hörandl 8271* WU; AY680053; AY954203. *R. gregarius* Brot.; cult. Berlin-Dahlem BG; *J.T. Johansson 232* LD; AY680100; AY954159. *R. hirtellus* Royle; Nepal; *F. Tod 372997* LI; AY680038; AY954120. *R. hybridus* Biria; cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680189; AY954211. *R. hyperboreus* Rottb.; Sweden; *J.T. Johansson s.n.* -; AY680065; AY954135. *R. illyricus* L.; Sweden; *Lundgren s.n.* -; AY680119; AY954162. *R. insignis* Hook.f.; New Zealand; *D. Glenny 24605* MPN; AF323306; AY954141. *R. japonicus* Thunb.; China; *XieLei XL200348* WU; AY680164; AY954200. *R. kuepferi* ssp. *kuepferi* W. Greuter & Burdet; Italy; *E. Hörandl 9525* WU; AY954241; AY954214. *R. kuepferi* ssp. *orientalis* W. Huber; Austria; *E. Hörandl 4336* WU; AY680085; AY954213. *R. lanuginosus* L.; unknown; *J.T. Johansson 255* LD; AY680163; AY954194. *R. lateriflorus* DC.; cult. Catania BG; *J.T. Johansson 235* LD; AY680179; AY954209. *R. lingua* L.; cult. Lund BG; *J.T. Johansson s.n.* -; AY680184; AY954206. *R. longicaulis* C.A.Mey.; Pakistan; *A. Millinger 470564* LI; AY680051; AY954117. *R. lyallii* Hook. f.; New Zealand; *M.A. Steel 24603* MPN; AF323277; AY954142. *R. maclovianus* Urv.; Chile; *C. Lehnebach s.n.* VALD; AY680158; AY954181. *R. marginatus* Urv.; cult. Copenhagen BG; *J.T. Johansson 286* LD; AY680150; AY954177. *R. marschlinsii* Steud.; Corse; *E. Hörandl 6981* WU; AY680089; AY954147. *R. micranthus* Nutt.; U.S.A., Ohio; *A. Lonsing 50563* LI; AY680042; AY954113. *R. millefoliatus* Vahl; cult. Graz BG; *J.T. Johansson 293* LD; AY680108; AY954166. *R. montanus* Willd. s.s.; Austria; *E. Hörandl 666* WU; AY680094; AY954149. *R. multifidus* Forssk.; South Africa; *L. Mucina 031102/7* WU; AY680162; AY954183. *R. muricatus* L.; cult. Siena BG; *J.T. Johansson 210* LD; AY680148; AY954191. *R. natans* C.A.Mey.; Russia; *A. Tribsch 9558* WU; AY680113; AY954134. *R. neapolitanus* Ten. (*R. bulbosus* ssp. *aleae*); Greece; *J.T. Johansson 224* LD; AY680123; AY954187. *R. nivalis* L.; Sweden; *J.T. Johansson s.n.* -; AY680046; AY954123. *R. notabilis* Hörandl & Guterm.; Austria; *E. Hörandl 5612* WU; AY680033; AY954115. *R. olissiponensis* Pers.; Spain; *W. Gutermann 37407* WU; AY680109; AY954157. *R. ophioglossifolius* Vill.; cult. Nantes BG; *J.T. Johansson 208* LD; AY680180; AY954207. *R. paludosus* Poir.; Greece; *W. Gutermann & al. 34754* WU; AY680102; AY954155. *R. papulentus* Melville; cult. Canberra BG; *J.T. Johansson 760141p* -; AY680058; AY954138. *R. parnassifolius* ssp. *parnassifolius* L.; France/Spain; *G. Schneeweiss & al. 6509* WU; AY680072; AY954224. *R. parviflorus* L.; cult. Copenhagen BG; *J.T. Johansson 287* LD; AY680175; AY954202. *R. pedunculatus* Sm.; Chile; *C. Lehnebach s.n.* VALD; AY680154; AY954180. *R. peltatus* ssp. *peltatus* Moench (*Batrachium peltatum*); cult. Nantes BG; *J.T. Johansson 206* LD; AY680068; AY954131. *R. penicillatus* ssp. *pseudofluitans* (Dum.) Bab. (*Batrachium penicillatum*); England; *G. Dahlgren BE9* LD; AY680070; AY954130. *R. pensylvanicus* L. f.; U.S.A.; *V. Zila 447002* LI; AY680147; AY954190. *R. platanifolius* L.; Norway; *J.T. Johansson 277* LD; AY680080; AY954216. *R. pollinensis* Chiovenda; Italy; *E. Hörandl 8247* WU; AY680097; AY954152. *R. polyanthemos* L.; Austria; *E. Hörandl 5130* WU; AY680121; AY954185. *R. pseudohirculus* Schrenk ex F.E.L. Fischer & C.A. Mey.; Russia; *A. Tribsch 9593* WU; AY680111; AY954118. *R. pseudomillefoliatus* Grau; Spain; *G. Schneeweiss & al. 7253* WU; AY680110; AY954156. *R. pseudomontanus* Schur; Slovakia; *E. Hörandl 5904* WU; AY680090; AY954146. *R. pseudotrullifolius* Skottsb.; Chile; *C. Lehnebach s.n.* VALD; AY680203; AY954139. *R. psilostachys* Griseb.; cult. Lund BG; *J.T. Johansson 219* LD; AY680106; AY954170. *R. pygmaeus* Wahlenb.; Sweden; *P. Larson & A. Granberg 9345* WU; AY954242; AY954122. *R. pyrenaeus* L.; Spain; *G. Schneeweiss & al. 6498* WU; AY680074; AY954225. *R. recurvatus* Bong; Connecticut; *J.T. Johansson 185* CONN; AY680118; AY954175. *R. repens* L.; Sweden; *J.T. Johansson s.n.* -; AY680160; AY954182. *R. reptans* L.; Switzerland; *Y. Willi br3* Z; AY680186; AY954205. *R. rufosepalus* Franch.; Pakistan; *A. Millinger 392897* LI; AY680047; AY954121. *R. rumelicus* Griseb.; Greece; *Snogerup 5993b* LD; AY680104; AY954168. *R. sardous* Cr.; Sweden; *J.T. Johansson s.n.* -; AY680122; AY954186. *R. sartorianus* Boiss. & Heldr. (incl. *R. ruscinonensis*); cult. Copenhagen BG; *J.T. Johansson 271* LD; AY680095; AY954148. *R. seguieri* ssp. *seguieri* Vill.; cult. Gothenburg BG; *J.T. Johansson 226* LD; AY680079; AY954215. *R. serbicus* Vis.; cult. Mühlhausen BG; *J.T. Johansson 249* LD; AY680166; AY954196. *R. serpens* ssp. *nemorosus* (DC.) G. Lopez Gonzalez (*R. nemorosus*); Austria; *E. Hörandl 9522* WU; AY954243; AY954184. *R. sphaerospermus* Boiss. & Blanche (*Batrachium sphaerospermum*); Turkey; *G. Dahlgren B87B* LD; AY680066; AY954132. *R. spicatus* Desf. s. l.; cult. Wisley Bot. Garden; *J.T. Johansson s.n.* LD; AY954244; AY954158. *R. sprunerianus* Boiss.; Greece; *J.T. Johansson 230* LD; AY680105; AY954169. *R. thora* L.; cult. Lund BG; *J.T. Johansson 223* LD; AY680188; AY954210. *R. traunfellneri* Hoppe; Austria; *E. Hörandl 2518* WU; AY954245; AY954222. *R. trichophyllus* Chaix (*Batrachium trichophyllum*); Greece; *G. Dahlgren B23* LD; AY680067; AY954133. *R. trilobus* Desf.; cult. Antwerpen BG; *J.T. Johansson 217* LD; AY680149; AY954176. *R. velutinus* Schur; cult. Rotterdam BG; *J.T. Johansson 270* LD; AY680173; AY954198. *R. venetus* Huter ex Landolt; Italy; *W. Gutermann & al. 35349* WU; AY680087; AY954144. *R. villarsii* DC. (*R. grenieranus*); Austria; *E. Hörandl 664* WU; AY680099; AY954153. *R. weyleri* Mares; Mallorca; *M. Mus Hö9521* WU; AY954246; AY954189. *Trautvetteria carolinensis* Vail; Genbank; U96035, U96036; AF007946. *T. grandis* Honda; Genbank; -; AF007945. *T. japonica* Sieb. & Zucc.; Genbank; U96037, U96038; AF007944. *Xanthorhiza simplicissima* Marshall; Genbank; -; AB069848.

Appendix 2. Indels present outside the *Ranunculus* core clade.

	Character Number (5'-3')				
	matK			***trnK* intron**	
Species	0429 – 0333	1518 – 1522	1528 – 1532	1621 – 1650	1798 – 1806
Core-clade *Ranunculus* species	RATTA	T---C	C---T	T-ATGT-----------------------A	C-------T
Ceratocephala falcata	A---A	TTTTC	CACCT	T-ATGT-----------------------A	C-------T
Ceratocephala orthoceras	A---A	TTTTC	CACCT	T-ATGT-----------AAATATAATATAT	C-------T
Myosurus minimus	GA-TG	T---C	T---T	T-ATGT-----------------------C	C-------T
Ficaria kochii	AATTA	T---C	C---T	TCATGATTGATTAGGAGATTCCGTAAATGA	C-------T
Ficaria verna subsp. *verna*	AATTA	T---C	C---T	TCATGATTGATTAGGAGATTCCATAAATGA	C-------T
Coptidium pallasii	GATTA	T---C	C---T	TCATGATTGATTAGGAGATTCCGTAAATGA	C-------T
Coptidium lapponicum	GATTA	T---C	C---T	TCATGATTGATTAGGAGATTCCGTAAATGA	C-------T
Peltocalathos baurii	GATTA	C---C	C---T	T-ATGT------------------AAATTT	CTAGAGACT
Callianthemoides semiverticillatus	?????	C---C	C---T	T-ATGT-------------TATGTAAATTT	CTAGAGACT
Halerpestes cymbalaria	GATTA	C---C	C---T	T-ATGT-----------------------A	C-------T
Beckwithia andersonii	AATTA	T---C	C---T	T-TTGT------------------AAATGT	C-------T

Appendix 3. Indels present inside the *Ranunculus* core clade.

Character Number (5'-3')

Species	Clade	matK 0399–0427	matK 0634–0641	matK 0674–0681	matK 1436–1440	matK 1515–1519	trnK intron 1652–1667	trnK intron 1671–1682	trnK intron 1689–1701	trnK intron 1725–1731	trnK intron 1737–1743	trnK intron 1813–1819	trnK intron 1835–1842
R. aduncus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. venetus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. carinthiacus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. pseudomontanus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A---------TTA	A------T	TCTTATT	CAGAATT	A------C
R. marschlinsii	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTA-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. sartorianus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. montanus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. apenninus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	T---A	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. pollinensis	VIIIg	AATAGAAAAA------------------T	A------C	A------A	T---A	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. gouanii	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. villarsii	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. carpaticus	VIIIg	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. cupreus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----CAATAAA	A------T	TCTTATT	CAGAATT	A------C
R. garganicus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACATC-----A	GATGAAAGAATT	A-----CAATAAA	A------T	TCTTATT	CAGAATT	A------C
R. cicutarius	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----CAATAAA	A------T	TCTTATT	CAGAATT	A------C
R. rumelicus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----CAAGAAA	A------T	TCTTATT	CAGAATT	A------C
R. sprunerianus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----CAAGAAA	A------T	TCTTATT	CAGAATT	A------C
R. psilostachys	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----CAAGAAA	A------T	TCTTATT	CAGAATT	A------C
R. millefoliatus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----ATTGTTA	A------T	TCTTATT	CAGAATT	A------C
R. illyricus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----ATTGTTA	A------T	TCTTATT	CAGAATT	A------C
R. creticus	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----ATTGTTA	A------T	TCTTATT	CAGAATT	A------C
R. gracilis	VIIIf	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----------A	A------T	TCTTATT	CAGAATT	A------C
R. paludosus	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----------A	A------T	TCTTATT	CAGAATT	A------C
R. pseudomillefoliatus	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----------A	A------T	TCTTATT	CAGAATT	A------C
R. bullatus	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	A-----------A	A------T	TCTTATT	CAGAATT	A------C
R. olissiponensis	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. spicatus	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. gregarius	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. cortusifolius	VIIIe	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. breyninus_Alps	VIIId	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. cappadocicus	VIIId	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTA	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. breyninus_Kaukasus	VIIId	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTC-----A	GATGAAAGAATT	T-----------A	A------T	TCTTATT	CAGAATT	A------C
R. lanuginosus	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. acris	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	A-----------A	A------T	T-----T	CAGAATT	A------C
R. japonicus	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. chius	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. parviflorus	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. grandiflorus	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. caucasicus	VIIIa	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTACTA	GATTAAAGAATT	A-----------A	A------T	T-----T	CAGAATT	A------C
R. arvensis	VIIIa	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTACTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. baldschuanicus	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. serbicus	VIIIb	AATAGAAAAAGGTAAAGAAATAGAAAAAT	A------C	A------A	TTTAA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. granatensis	VIIIb	AATAGAAAAAGGTAAAGAAATAGAAAAAT	A------C	A------A	TTTAA	ATTTC	ATATAACTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. velutinus	VIIIb	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATAAAATTTCTTCCTA	GATTAAAGAATT	T-----------A	A------T	T-----T	CAGAATT	A------C
R. recurvatus		AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAATTTCTTCCTA	GATGAAAGAATT	A-----------A	A------T	T-----T	CAGAATT	A------C
R. pensylvanicus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	GATTAAAGAATT	A-----------A	A------T	T-----T	CAGAATT	A------C
R. trilobus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTGA	ATTTC	ATGTAACTTTTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. marginatus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. cornutus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. repens	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. multifidus	VIIIc	CATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. serpens:nemorosus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. polyanthemos	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. sardous	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. neapolitanus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. bulbosus:bulbosus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. weyleri	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. muricatus	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	ACTGAAAT	T-----T	CAGAATT	A------C
R. chilensis	VIIIc	AATAGAAAAA------------------T	A------C	A------A	TTTAA	ATTTC	ATGTAACTTCTTCCTA	G----------T	A-----------A	A------T	T-----T	CAGAATT	A------C

Appendix 2. Continued.

Species	Group	Character Number (5'-3') — *matK*	*trnK intron*
		0 0 0 0 1 1 1 1	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
		3 4 6 6 6 4 4 5 5	6 6 6 6 6 7 7 7 7 8 8 8 8
		9 2 3 4 7 8 3 4 1 1 5	6 7 8 8 0 2 3 3 4 1 1 3 4
		9 7 4 1 4 1 6 0 5 9	2 7 1 2 9 1 5 1 7 3 3 9 5 2
R. peduncularis	VIIIc	AATAGAAAAA----------T A------C A-----AA TTTAA ATTTC	ATGTAACTTCTTCCTA G----------T A----------A A------T T-----T CAGAATT A-----C
R. maclovianus	VIIIc	AATAGAAAAA----------T A------C A------A TTTAA ATTTC	ATGTAACTTCTTCCTA G----------T A----------A A------T T-----T CAGAATT A-----C
R. flammula	VII	AATAAAAAAA----------T A------C A------A TTTGA ATATC	A---------TTCCTA AATTAAAGAAGA T----------A A------T T-----T T-----T A-----C
R. reptans	VII	AATAAAAAAA----------T A------C A------A TTTGA ATATC	A---------TTCCTA AATTAAAGAAGA T----------A A------T T-----T T-----T A-----C
R. lingua	VII	AATAAAAAAA----------T A------C A------A TTTGA ATATC	A---------TTCCTA AATTAAAGAAGA T----------A A------T T-----T T-----T A-----C
R. ophioglossifolius	VII	AATAAAAAAA----------T A------C A------A TTTGA ATCTC	A---------TTCCTA AATTAAAGAAGA T----------A A------T T-----T T-----T A-----C
R. flagelliformis	VII	-ATAGAAAAA----------T A------C ???????? TTTGA ATCTC	A---------TTCCTA AATTAAAGAAGA T----------A A------T T-----T T-----T A-----C
R. lateriflorus	VII	AATAAAAAAA----------T A------C A------A TTTGA ATCTC	A---------TTCCTA AATTAAAGAAGA T----------A A------T T-----T T-----T A-----C
R. carpaticola	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. cassubicifollius	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. micranthus	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. gelidus	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. notabilis	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. altaicus	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC TGAAGCCAATTTT A------T T-----T C-----T A-----C
R. longicaulis	V	A----------T A------C A------A TTTGA A---C	ATGTAACCTCTTCCTA GATTAATGAAGC TGAAGCCAATTTT A------T T-----T C-----T A-----C
R. pseudohirculus	V	A----------T A------C A------A TTTGA A---C	ATGTAACCTCTTCCTA GATTAATGAAGC TGAAGCCAATTTT A------T T-----T C-----T A-----C
R. brotherusii	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC TGAAGCCAATTTT A------T T-----T C-----T A-----C
R. hirtellus	V	A----------T A------C A------A TTTGA A---C	ATGTAACCTCTTCCTA GATTAATGAAGC TGAAGCCAATTTT A------T T-----T C-----T A-----C
R. rufosepalus	V	A----------T A------C A------A TTTGA A---C	ATGTAACCTCTTCCTA GATTAATGAAGC TGAAGCCAATTTT A------T T-----T C-----C A-----C
R. pygmaeus	V	A----------T A------C A------A TTTGA A---A	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. nivalis	V	A----------T ATTACTAC A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. abortivus	V	A----------T ATTACTAC A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. cardiophyllus	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCTTA GATTAATGAAGC T----------T A------T T-----T C-----T A-----C
R. arcticus	V	A----------T A------C A------A TTTGA A---C	ATGTAACTTCTTCCTA GATTAATGAAGC T----------T A------T C-----T C-----T A-----C
R. gmelinii:gmelinii	V	AAATGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC T----------T A------T T-----T C-----T A-----C
R. eschscholtzii	IV	AATYGAAAAA----------T ???????? ???????? TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. fluitans	IV	AATGGAAAAA----------T A------C A------A TTTGA AGATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. penicillatus:pseud.	IV	AATGGAAAAA----------T A------C A------A TTTGA AGATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. peltatus	IV	AATGGAAAAA----------T A------C A------A TTTGA AGATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. trichophyllus	IV	AATGGAAAAA----------T A------C A------A TTTGA AGATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. sphaerospermus	IV	AATGGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. natans	IV	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. hyperboreus	IV	AATGGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC T----------T A------T T-----T C-----T A-----C
R. fuegianus	IV	AATGGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. collinus	IV	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. papulentus	IV	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. pseudotrullifolius	IV	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. apiifolia	IV	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T A-----C
R. insignis	IV	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC C----------T A------T T-----T C-----T ATTTATAC
R. lyallii	IV	?????????????????????????????? ???????? ???????? TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGC A----------T A------T T-----T C-----T ATTTATAC
R. thora	VI	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------T A------T T-----T C-----T A-----C
R. hybridus	VI	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------T A------T T-----T C-----T A-----C
R. brevifolius:brev.	VI	AATAGAAAAA----------T A------C A------A TTTGA ATCTC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------T A------T T-----T C-----T A-----C
R. kuepferi:orientalis	III	AATAGAAAAA----------T A------C ATTTTAAA TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. kuepferi:kuepferi	III	AATAGAAAAA----------T A------C ATTTTAAA TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. seguieri	III	AATAGAAAAA----------T A------C ATTTTAAA TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. platanifolius	III	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. aconitifolius	III	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. chamissonis	III	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. glacialis	III	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. bilobus	I	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------C A------T T-----T C-----T A-----C
R. alpestris	I	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------C A------T T-----T C-----T A-----C
R. traunfellneri	I	AATGGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTT------A GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. amplexicaulis	II	AATAGAAAAA----------T A------C A------A TTTTA ATATC	ATGTAACTTCTTCCTA AACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. parnassifolius:parn.	II	AATAGAAAAA----------T A------C A------A TTTTA ATATC	ATGTAACTTCTTCCTA AACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. pyrenaeus	II	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA AACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. acetosellifolius	II	AATAGAAAAA----------T A------C A------A TTTTA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T-------ATTTA A------T T-----T C-----T A-----C
R. gramineus	II	AATAGAAAAA----------T A------C A------A TTTGA AGATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------A A------T T-----T C-----T A-----C
R. crenatus	II	AATAGAAAAA----------T A------C A------A TTTGA ATATC	ATGTAACTTCTTCCTA GACTAAAGAAGA T----------C A------T T-----T C-----T A-----C

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Phylogenetic relationships of the monotypic Peruvian genus *Laccopetalum* (Ranunculaceae)

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Abstract. *Laccopetalum giganteum* (Ranunculaceae) is a rare endemic buttercup from the montane regions of the Peruvian Andes. The systematic position of this monotypic genus within Ranunculaceae was investigated using cpDNA *mat*K sequence data. Our findings indicate that *L. giganteum* forms a highly supported clade together with *Krapfia*. Several morphological characters are shared by these genera; large subglobose and fleshy flowers, presence of androgynophore with a separated staminal and carpellate region and many tiny achenes. The *Laccopetalum-Krapfia* clade is sister to the core *Ranunculus* group with a high bootstrap support. The number of sepals and similar characteristics of achene morphology support an affinity of *Laccopetalum* with *Ranunculus* s.s tr.

Key words: Cordillera de los Andes, *Krapfia*, *Laccopetalum giganteum*, *mat*K, phylogeny, *Ranunculus*, Ranunculaceae, South America, taxonomy.

Introduction

The giant Peruvian buttercup, *Laccopetalum giganteum* (Wedd.) Ulbr., is the only species in the genus *Laccopetalum* Ulbr. (Ranunculaceae). This genus was originally described by Ulbrich (1906) using material collected in 1861 by Antonio Raimondi in Northern Perú (Provincia de Pataz, Departamento de la Libertad). The species is endemic to the Peruvian Andes and it is restricted to habitats on the mountain tops (c. 4000 to 4800 m) such as rock-clefts and stony places (Fig. 1). The plant is a robust perennial with fleshy, coriaceous, sinuate-dentate or serrate leaves with a thick whitish cuticle, possibly an adaptation to the harsh and xerophytic environment where it grows. The flower is greenish, single, terminal and large (10–15 cm width) and protandrous (Ulbrich 1922). Due to the inaccessibility of such habitats and the difficulty in collecting

Fig. 1. Habitat of *Laccopetalum giganteum* in the mountains of the Peruvian Andes (Cordillera Pelagatos, Pallasca, Ancash), c. 4600–4800 m. Photograph A. Cano

plants, Ulbrich (1906) believed its conservation would not be an issue. However, *L. giganteum* has been recently considered critically endangered by the Institute of Natural Resources (INRENA) of Perú after applying the IUCN Red List criteria. Overcollection by the local community for its medicinal properties has become the main conservation threat. Flowers are collected, cooked and used as an infusion to treat lung and throat illnesses. A mixture of *L. giganteum* flowers and salt is also believed to improve cattle fecundity (Brack 1999).

The systematic position of *L. giganteum* within the Ranunculaceae and its phylogenetic relationships are unclear. Before Ulbrich (1906), the giant Peruvian buttercup was assigned to *Ranunculus* (*R. giganteum*) by Weddell (1861). However, the presence of an androgynophore and the number of deep nectary pores in the petals, generally up to 30, makes it different from *Ranunculus* and all other genera of the tribe Ranunculeae. In fact, Ulbrich (1906) based the genus name on this last feature (*Laccopetalum* from the Greek *lakkos*: a pit and *petalon*: a petal). This character was also used by Janchen (1949) to justify the subtribe Laccopetalinae, which contained only *L. giganteum*, within Ranunculaceae.

In the most recent worldwide treatment of the Ranunculaceae (Tamura 1993a, 1995), *Laccopetalum* was placed in the tribe Ranunculeae, subtribe Ranunculinae, and was treated as a "satellite" genus to *Ranunculus* together with 14 other genera (e.g. *Ceratocephala*, *Krapfia*, *Halerpestes*, *Callianthemoides*, and others). In his treatment, Tamura alleged nectary number was not a suitable character to define the subtribe Laccopetalinae since petals of other endemic Peruvian Ranunculaceae (i.e. some *Krapfia* species) and several New Zealand alpine *Ranunculus* (e.g. *R. grahamii* and *R. sericophyllus*) also have more than one nectary gland. He suggested that the petal of *Laccopetalum* could be derived from that of *Ranunculus* species with a single nectary by "multiplication". Tamura (1995) also hypothesised that "*Laccopetalum* was connected to *Ranunculus* through the genus *Krapfia*". *Krapfia* is a genus endemic to the high mountain tops of the Andes in Ecuador, Bolivia, Perú and Colombia and comprises about eight species. It shares with *Laccopetalum* the presence of an androgynophore with a separated staminal and carpellate region, and many xerophytic adaptations such as coriaceous leaves, thick sepals and fleshy receptacles.

Recent efforts to reveal the phylogenetic relationships within the Ranunculaceae (Hoot 1991, 1995; Johansson 1995) and the genus *Ranunculus* in particular (Hörandl et al. 2005; Paun et al. 2005) have shown that many of the genera included in the subtribe Ranunculinae, and previously described under *Ranunculus*, form a grade leading to a core *Ranunculus* clade. These phylogenetic analyses based on nuclear and chloroplast sequences have helped to identify the importance of certain morphological characters for phylogenetic analyses, and the directions of character evolution. In this article we analysed sequences from our previous studies with new molecular data to clarify the systematic position of this rare Peruvian buttercup within the Ranunculaceae.

Material and methods

Taxon sampling. Ranunculaceae is represented by 26 genera out of the c. 59 included in the family by

Tamura (1995). Within the genus *Ranunculus*, 14 taxa were chosen to represent each of the main sub-clades of the core *Ranunculus* group as defined by Hörandl et al. (2005). *Ceratocephala* and *Myosurus* were also included in our analysis; these genera represent 2 sub-clades of the core *Ranunculus* group obtained by Hörandl et al. (2005). To understand the relationship of *Laccopetalum* with the genus *Krapfia*, we sequenced and included in this analysis *Krapfia clypeata* (Ulbr.) Standl. et Macbr. Following Hoot (1995), *Glaucidium* and *Hydrastis* were used as outgroups. Taxon names, voucher data, and GenBank accession numbers are listed in Appendix 1. Samples of *Laccopetalum* and *Krapfia* were collected in the field, preserved in silica gel and vouchered as herbarium specimens. Voucher specimens of *L. giganteum* used for morphological studies are listed in Appendix 2.

DNA isolation, amplification and sequencing. DNA extraction from fresh material followed the protocol modified from Doyle and Doyle (1987) or, in the case of herbarium material, the DNeasy extraction kit was used (Qiagen). PCR amplification was carried out in a Biometra T1Thermocycler machine. The entire *mat*K region (~2500 bp) with intron and exon region was amplified using the primers listed in Table 1. PCR conditions were 94°C 3 min for the first cycle; 94°C 30 s, 55/50°C 1 min, 72°C 2 min for the following 35 cycles; then 72°C 5 min; and then held at 10°C. PCR products were purified using a Sap/Exo1 digestion method (2 µl Sap, 1 µl Exo1; cycle 37°C 30 min; 80°C 15 min and then cooled at 10°C). Sequencing reactions were done in a 20 µl volume using Applied Biosystems Inc. standard protocols and sequenced using a capillary ABI3730 Genetic Analyzer, from Applied Biosystems Inc.

Data analysis. Forward and reverse strand sequences were edited using Sequencer 4.2 (Gene-code). Sequences were aligned using ClustalX (Thompson et al. 1997). Sites containing gaps and ambiguous characters were removed. To find the most appropriate DNA substitution model the Akaike Information Criterion (AIC) and the Hierarchical Likelihood Ratio Tests (hLRTs) were calculated using Modeltest 3.6 (Posada and Crandall 1998). Both tests selected the $TVM + G_4$ (transversion model plus gamma) as the best-fit model. The data set was then analysed under a heuristic maximum likelihood (ML) criterion using PAUP* (Swofford 2001) with the tree-bisection-reconnection (TBR) branch swapping option. The relative support for each node was examined with non-parametric bootstrapping (Felsenstein 1985; 100 replicates).

Results

The *Laccopetalum trn*K region sequenced was 2622bp. The sequence was submitted to GenBank, accession number DQ400695 along with the *K. clypeata* sequence (DQ490058). Both sequences translated with no frameshifts or stop codons in the protein coding region and alignment with the sequences from GenBank was unambiguous. An exception concerned the *Myosurus mat*K sequence (AJ414344) which presented some difficulty when aligned against the remaining taxa due to a frameshift insertion. The aligned final data matrix used for tree building contained 43 taxa, 825 positions (362 variable sites and 217 parsimony informative sites) and no indels.

ML analysis produced three equally likely trees. A consensus network of these trees is presented in Fig. 2. *Laccopetalum* is sister to

Table 1. Sequences of the primers used for amplification of *mat*K and flanking *trn*K intron region

Name	Sequence (5′-3′)	Primer source
*trn*K5f	GGGTTGCGAACTCAACGGTAGAG	This study
*trn*K580r	TTTGTTTGTTATACGATACCAA	This study
*mat*K710f	GTATGGCACTATGTWTCATTTG	Paun et al. 2005
*trn*K1457r	CATGAAAATTATATAGGAAC	This study
*mat*K3ar	CGTACASTACTTTTGTGTTTNC	Paun et al. 2005
*trn*K3r	GATTCGAACCCGGAACTAGTCGG	Paun et al. 2005
*trn*K1859f	GGAATCAAATGTTAGAAAAT	This study

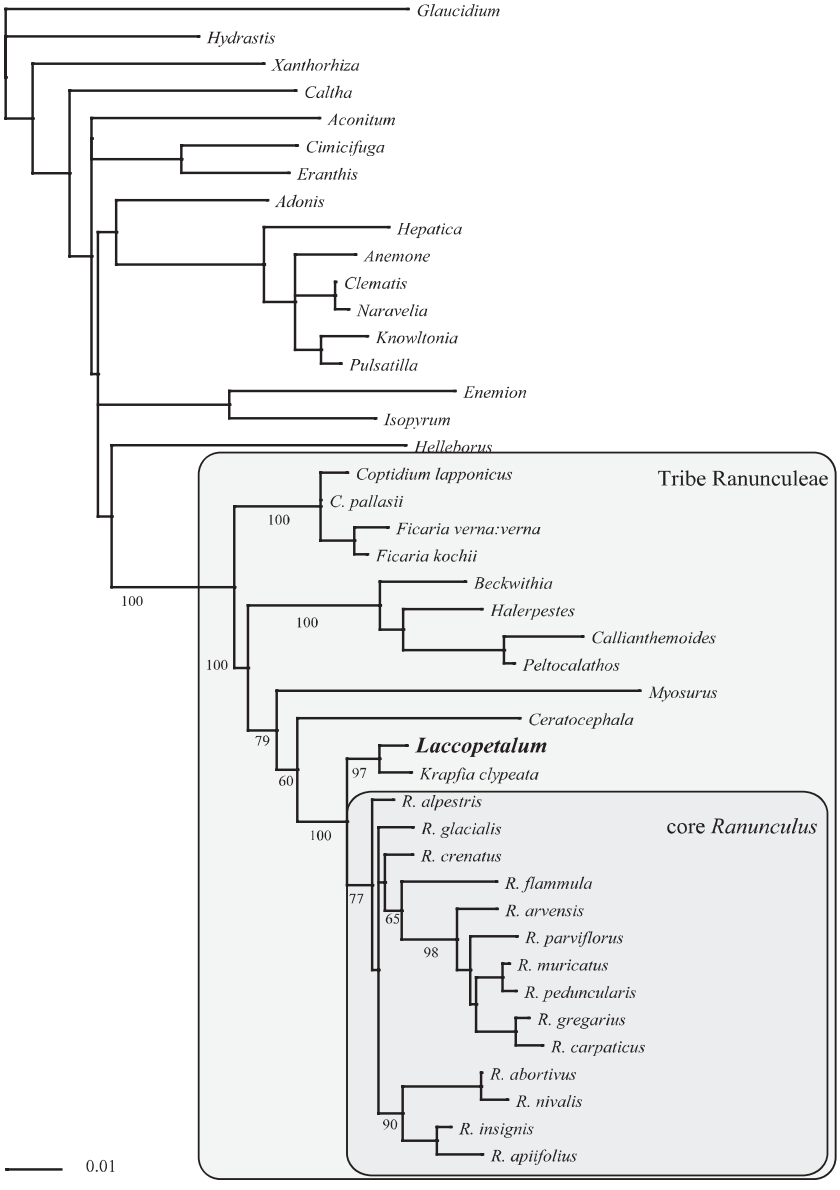

Fig. 2. Phylogram based on ML analysis of the *mat*K/*trn*K dataset. Bootstrap values for the main clades of *Ranunculus* and allied genera are indicated below the branches

Krapfia clypeata (BS = 97%), this clade is sister to the core *Ranunculus* clade (BS = 100%) as defined by Hörandl et al. (2005). The genera *Ceratocephala* and *Myosurus* are sister to the *L. giganteum-K. clypeata* clade and the core *Ranunculus* clade. Allied *Ranunculus* genera (sensu Hörandl et al. 2005) of the tribe Ranunculeae (sensu Tamura 1995) form two clades {*Beckwithia*, *Halerpestes*, *Callianthemoides*, *Peltocalathos*} and {*Coptidium* and *Ficaria*}, both with 100% bootstrap support. The Ranunculeae form a long branch and are strongly supported as a monophyletic group (BS = 100%).

Discussion

Our phylogenetic analysis shows that *Laccopetalum* forms a clade with *Krapfia*; both are sister to the core *Ranunculus*. The affinities

Fig. 3. Detail of a flower of *Laccopetalum giganteum* (bar = 5 cm). Photograph A. Cano

between *Laccopetalum* and *Krapfia* or *Ranunculus* respectively were previously hypothesised but not fully understood. Tamura (1995) suggested that *Laccopetalum* was related to *Ranunculus* through the genus *Krapfia*. Common ancestry between *Laccopetalum* and *Krapfia* is supported from our sequence analyses and also by several floral morphological characters; e.g. subglobose flowers, thick and concave sepals and petals, more than one nectary gland per petal, large number of carpels (>400) and stamens on a clavate fleshy receptacle (androgynophore) with a smooth free zone separating the staminal and carpellate part (Figs. 3 and 4). Palynological studies by Santisuk (1979) and Nowicke and Skvarla (1995) also noticed the affinity between *Laccopetalum* and *Krapfia*; both genera have pantoporate pollen grains.

Ceratocephala and *Myosurus* which were previously considered sister to the core *Ranunculus* (Paun et al. 2005) are in our phylogeny more distantly related than *Laccopetalum* and *Krapfia*. Their distinctive fruit morphology, karyotype and cpDNA sequence characteristics, however, suggest an earlier divergence from the core *Ranunculus* (see also discussion in Paun et al. 2005). *Laccopetalum* and *K. clypeata,* on the contrary, share with the core *Ranunculus* group the presence of 5–6 sepals and achenes with similar morphological and anatomical characteristics; ovate or round body and a distinct beak and lateral faces without longitudinal ridges. This hypothesis is also strongly supported in our analysis by a 100% bootstrap value, and relatively short branches compared with the remaining genera of Ranunculeae.

Our results have a number of taxonomic implications. Based on the phylogenetic evidence presented here and the many morphological characters shared by *Laccopetalum* and *Krapfia* with the core *Ranunculus* group, it may be appropriate to expand the core clade to include *Laccopetalum* and *Krapfia*. The presence of an androgynophore with a separated

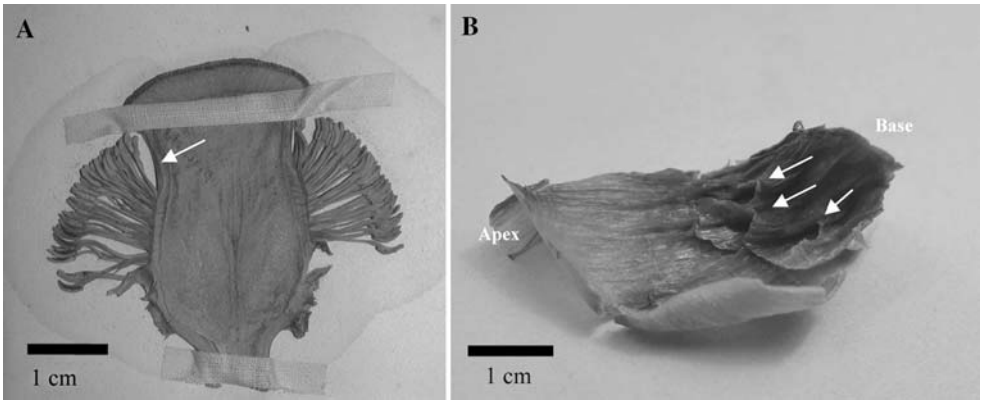

Fig. 4. Longitudinal section of the androgynophore of *Laccopetalum giganteum* (**A** white arrow indicates free zone) and half of a petal with nectary glands (**B** white arrows) from *Lopez 1189* **USM** and *Cano et al. 15196* **USM**, respectively

staminal and carpellate region may be a useful diagnostic morphological character to support this section. Alternatively, *Laccopetalum* and *Krapfia* might be considered as the sister genera of the core *Ranunculus*. Further studies, including all genera of Ranunculeae, plus additional material from *Krapfia*, are required before final taxonomic conclusions can be drawn.

Previous studies aiming to understand phylogenetic affinities and evolution in the Ranunculaceae (e.g. Hoot 1991, 1995; Johansson 1995, 1998) and within *Ranunculus* in particular (e.g. Lockhart et al. 2001, Hörandl et al. 2005, Paun et al. 2005) have demonstrated how parallel evolution of morphological characters or rapid morphological divergence between closely related taxa have misled evolutionary hypotheses and many taxonomic treatments. Both patterns were observed again in our study; multiple nectary glands have evolved separately in *Laccopetalum* and New Zealand alpine *Ranunculus*. However, it is important to note that their shape and distribution in the petal are very different. Nectary glands in *Laccopetalum* are sunken in channels longitudinally distributed in the petal; this opening is delimited by raised v-shaped margins (Fig. 4B). They are located in the upper third of the petal. In the New Zealand alpine *R. sericophyllus*, the nectary glands are open elliptic pits located at the base of the petal. None of these morphologies match with the typologies proposed by Benson (1940) for North American *Ranunculus*. Unfortunately, there were no flowers of *K. clypeata* available to study, but Tamura (1995) describes the nectary of this species as a transversely elongated gland covered with a scale. Whether this corresponds to only one nectary gland or several glands fused together is not certain but has been suggested to occur in other *Krapfia* species such as *K. polystachya* (Santisuk 1979). As for the second pattern, great morphological disparity can be observed between *L. giganteum* and *K. clypeata*. The former is a robust plant of almost 70 cm height with large *Agave*-like leaves and large flowers while the second species reaches only c. 20 cm, has peltiform villous leaves and considerably smaller flowers and a different nectary type. Understanding the drivers of morphological innovation and ecological diversification in *Ranunculus* species from alpine areas of New Zealand and South America is our ongoing subject of study.

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Appendix 1. List of taxa included in this study and GenBank accession numbers and authors

Aconitum apoiense Nakai, AB038175, Kita and Kato 2001. *Adonis vernalis* L., AJ414340, Sun et al. 2001. *Anemone flaccida* F. Schmidt., AB110530, Miikeda et al., unpublished. *Beckwithia andersonii* (A. Gray) Jeps., AY954238, Paun et al. 2005. *Callianthemoides semiverticillatus* (Phillipi) Tamura, AY954236, Paun et al. 2005. *Caltha palustris* L., AB069845, Adachi et al., unpublished. *Cerathocephala orthoceras* DC., AY954230, Paun et al. 2005. *Cimicifuga acerina* (Siebold et Zucc.) Tanaka, AF353578, Song et al. 2001. *Clematis fusca* Turcz., AB110535, Miikeda et al., unpublished. *Coptidium lapponicus* (L.) Tzvelev, AY954234, Paun et al. 2005. *Coptidium pallasii* (Schlecht.) Tzvelev, AY954233, Paun et al. 2005. *Enemion raddeanum* Regel., AB069846, Adachi et al., unpublished. *Eranthis hyemalis* (L.) Salisb., AJ414342, Sun et al. 2001. *Ficaria kochii* (Ledeb.) Iranshar et Rech.f., AY954231, Paun et al. 2005. *Ficaria verna* Huds. Spp. *verna*,

AY954232, Paun et al. 2005. *Glaucidum palmatum* Siebold et Zucc., AB069850, Adachi et al., unpublished. *Halerpestes cymbalaria* (Pursh) Greene, AY954237, Paun et al. 2005. *Helleborus viridis* L. subsp. *occidentalis*, AJ414339, Sun et al. 2001. *Hepatica americana* (DC.) Ker Gawl., AF542590, Hilu et al. 2003. *Hydrastis canadensis* L., AB069849, Adachi et al, unpublished. *Isopyrum fumarioides* L., AJ414343, Sun et al. 2001. *Knowltonia* sp., AB110533, Miikeda et al., unpublished. *Krapfia clypeata* (Ulbr.) Standl. et Macbr. (syn: *Ranunculus clypeatus* (Ulbr.) Lourteig), Perú, Sanchez et al. 11173 (F, CPUN, MPN); DQ490058. *Laccopetalum giganteum* (Wedd.) Ulbr.; Perú; Cano et al. 15196 USM; DQ400695. *Myosurus minimus* L., AJ414344, Sun et al. 2001. *Naravelia laurifolia* Wall., AB110526, Miikeda et al., unpublished. *Peltocalathos baurii* (McOwan) Tamura, AY954235, Paun et al. 2005. *Pulsatilla cernua* (Thunb.) Berchtold et Presl, AB110531, Miikeda et al., unpublished. *Ranunculus abortivus* L., AY954126, Paun et al. 2005. *R. alpestris* L., AY954221, Paun et al. 2005. *R. apiifolius* (Pers.) St.-Hill, AY954140, Paun et al. 2005. *R. arvensis* L., AY954193, Paun et al. 2005. *R. carpaticus* Herbich, AY954154, Paun et al. 2005. *R. crenatus* Waldst. et Kit, AY954228, Paun et al. 2005. *R. flammula* L., AY954204, Paun et al. 2005. *R. glacialis* L., AY954219, Paun et al. 2005. *R. gregarius* Brot., AY954159, Paun et al. 2005. *R. insignis* Hook. f., AY954141, Paun et al. 2005. *R. muricatus* L., AY954191, Paun et al. 2005. *R. nivalis* L., AY954123, Paun et al. 2005. *R. parviflorus* L., AY954202, Paun et al. 2005. *R. peduncularis* Sm., AY954180, Paun et al. 2005. *R. repens* L., AY954182, Paun et al. 2005. *Xanthorhiza simplicissima* Marshall, AB069848, Adachi et al., unpublished.

Appendix 2. Voucher specimens of *Laccopetalum giganteum* used for morphological studies

Perú, Departamento de Ancash, Provincia de Pallasca: Nevada de Pelagatos, alt. 4.600 m, leg. Arnaldo Lopez (1189), 30 August 1955, **USM**. Nevada de Pelagatos, alt. 4600 – 4700 m, leg. Ferreyra et Lourtieg (18591 A), 27 July 1976, 58167 **USM**. Conchucos, Co. Huabumbo, alt. 4400 m, leg. unknown, 23 January 1976, 173140 **USM**.

References

Benson L. (1940) The North American subdivisions of *Ranunculus*. Amer. J. Bot. 83: 516–527.

Brack A. (1999) Diccionario Enciclopédico de Plantas útiles del Perú. Editorial Bartolomé de Las Casas, Cusco.

Doyle J. J., Doyle J. L. (1987) A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem. Bull. 19: 11–15.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791.

Hilu K. W., Borsch T., Muller K., Soltis D. E., Soltis P. S., Savolainen V., Chase M. W., Powell M. P., Alice L. A., Evans R., Sauquet H., Neinhuis C., Slotta T. A. B., Rohwer J. G., Campbell C. S., Chatrou L. W. (2003) Angiosperm phylogeny based on *mat*K sequence information. Amer. J. Bot. 90: 1758–1776.

Hoot S. B. (1991) Phylogeny of the Ranunculaceae based on epidermal microcharacters and macromorphology. Syst. Bot. 16: 741–755.

Hoot S. B. (1995) Phylogeny of the Ranunculaceae based on preliminary *atp*B, *rbc*L and 18S nuclear ribosomomal DNA sequence data. Pl. Syst. Evol. Suppl. 9: 214–251.

Hörandl E., Paun O., Johansson J. T., Lehnebach C., Armstrong T., Chen L., Lockhart P. (2005) Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. Molec. Phylogenet. Evol. 36: 305–327.

Janchen E. (1949) Die systematische Gliederung der Ranunculaceen und Berberidaceen. Denkschr. Akad. Wiss. Wien Math.-Naturw. Kl. 108: 1–82.

Johansson J. T. (1995) A revised chloroplast DNA phylogeny of the Ranunculaceae. Pl. Syst. Evol. Suppl. 9: 253–261.

Johansson J. T. (1998) Chloroplast DNA restriction site mapping and the phylogeny of Ranunculus (Ranunculaceae). Pl. Syst. Evol. 213: 1–19.

Kita Y., Kato M. (2001) Infrafamilial phylogeny of the aquatic angiosperm Podostemaceae inferred from the nucleotide sequences of the *mat*K gene. Pl. Biol. 3: 156–163.

Lockhart P., McLenachan P. A., Havell D., Glenny D., Huson D., Jensen U. (2001) Phylogeny, dispersal and radiation of New Zealand alpine buttercups: molecular evidence under split decomposition. Ann. Missouri Bot. Gard. 88: 458–477.

Lourteig A. (1956) Ranunculaceas de Sudamerica Tropical. Mem. Soc. Ci. Nat. La Salle. 16: 125–220.

MacBride J. F. (1937) Ranunculaceae, Flora of Perú. Field Museum of Natural History; Botanical Series. 13: 639–661.

Nowicke W. J., Skvarla J. J. (1995). Pollen morphology. I. General Part. In: Hiepko P. (ed.) Natürliche Pflanzenfamilien, ed. 2, 17aIV. Duncker et Humblot, Berlin. pp. 129–159.

Paun O., Lehnebach C., Johansson J. T., Lockhart P., Hörandl E. (2005) Phylogenetic relationships and biogeography of Mediterranean and European alpine Ranunculi (Ranunculaceae) inferred from nrITS and plastid sequence data. Taxon 54: 911–930.

Posada D., Crandall K. A. (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Santisuk T. (1979) A palynological study of the tribe Ranunculeae (Ranunculaceae). Opera Bot. 48: 1–74.

Song B. H., Wang X. Q., Li F. Z., Hong D. Y. (2001) Further evidence for paraphyly of the Celtidaceae from the chloroplast gene *mat*K. Pl. Syst. Evol. 228: 107–115.

Sun H., McLewin W., Fay M. (2001) Molecular phylogeny of *Helleborus* (Ranunculaceae), with an emphasis on the East Asian-Mediterranean disjunction. Taxon 50: 1001–1018.

Swofford D. L. (2001) PAUP*: Phylogenetic Analysis using Parsimony. Version 4.0b10 Sinauer Associates, Sunderland, Massachusetts.

Tamura M. (1993a) Ranunculaceae. In: Kubitzki K., Rohwer J. G., Bittrich V. (eds.) The families and genera of vascular plants. 2. Flowering plants. Dicotyledons, Magnoliid, Hamamelid, and Caryophyllid Families. Springer, Berlin, pp. 563–583.

Tamura M. (1993b) New species and combinations in the Ranunculaceae. Acta Phytotax Geobot. 44: 27–28.

Tamura M. (1995) Angiospermae. Ordnung Ranunculales. Fam. Ranunculaceae. II. Systematic Part. In: Hiepko P. (ed.) Natürliche Pflanzenfamilien, ed. 2, 17aIV. Duncker and Humblot, Berlin, pp. 223–519.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876–4882.

Ulbrich E. (1906) Ranunculaceae. In: Urban I. (ed.) Plantae novae andinae imprimis Weberbauerianae I. Bot. Jahrb. Syst. 37: 404.

Ulbrich E. (1922). Ranunculaceae novae vel criticae V. Notizbl. Bot. Gart. Berlin-Dahlem 8: 251–272.

Weddell H. A. (1861) Ranunculaceae. In: Bertrand P. (ed.) Chloris Andina 2, pp. 303–305.

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A NEW NORTHERN LIMIT FOR THE DISTRIBUTION OF *RANUNCULUS SPEGAZZINII* LOURTEIG (RANUNCULACEAE) IN CHILE

EXTENSION DEL RANGO DE DISTRIBUCION DE RANUNCULUS SPEGAZZINII *LOURTEIG (RANUNCULACEAE) EN CHILE*

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RESUMEN

Se informa la colecta de *Ranunculus spegazzinii* Lourteig en la IX Región de Chile (38°S). Este nuevo registro extiende el límite norte de distribución de esta especie en Chile en c. 1400 km. Además se presenta un mapa con los puntos de colecta de *R. spegazzinii* e imágenes del nectario y aquenio obtenidas en el microscopio electrónico de barrido.

Ranunculus L. is the largest genus in the Ranunculaceae family. It comprises about 600 species (Tamura 1993, 1995) and its distribution is almost worldwide. In Chile, *Ranunculus* includes 22 native species (Ruiz 2001). They are usually found in moist habitats such as streams or lake banks, wetlands and forests; in lowland, sub-alpine and alpine environments. The distribution of this genus in Chile extends from the Altiplano in the northern Andes to the cold Patagonian streams and ponds in the South. It is in Patagonia where most of the Chilean *Ranunculus* species occur, many of them growing sympatrically.

Ranunculus spegazzinii Lourteig is one of the many native buttercups found in Patagonia. The species is characterized by the glabrous habit and large creeping stems. Leaves are homomorphous with ovate lamina, basally truncated and only slightly 3-5-lobed or crenate apex. Flowers are single, yellow, with calyx and corolla 5-6-merous and less than 10 finely foveolated carpels. The oblong-lanceolate petals bear one nectary gland only, and this is located in the upper half of the petal. The nectary is small and pocket-like (Fig 1 A), with the nectary scale tightly covering the nectary gland (Fig. 1 B). The achene testa is reticulated with irregular and not isodiametric cells (Fig. 1 C). Anticlinal cell walls are raised, straight or slightly curved and periclinal cell walls are concave and with no apparent ornamentation (Fig. 1 D). The latter characters confer the foveolate surface pattern observed in the achenes of this species.

In Chile, the distribution of *R. spegazzinii* is remarkably limited and it has been collected only in Torres del Paine National Park, XII Region (51°03'S) (Fig. 2, point 4). In Argentina, on the contrary, *R. spegazzinii* has a widespread distribution and it has been collected in the southern provinces of Neuquén, Río Negro and Chubut (Lourteig 1984, Zuloaga & Morrone 1999) (Fig. 2, points 1, 2, 3). A recent expedition to the Cordillera de los Andes in the IX Region of Chile, part of a broader study on the phylogenetic affinities of Southern South American *Ranunculus*, has evidenced the occurrence of *R. spegazzinii* in this region. This collection extends the northern limit of distribution for this species in Chile in almost 1400 km. This location, however, is relatively close to the northern limit of distribution previously recorded for this species in Argentina (Fig. 2, point 1).

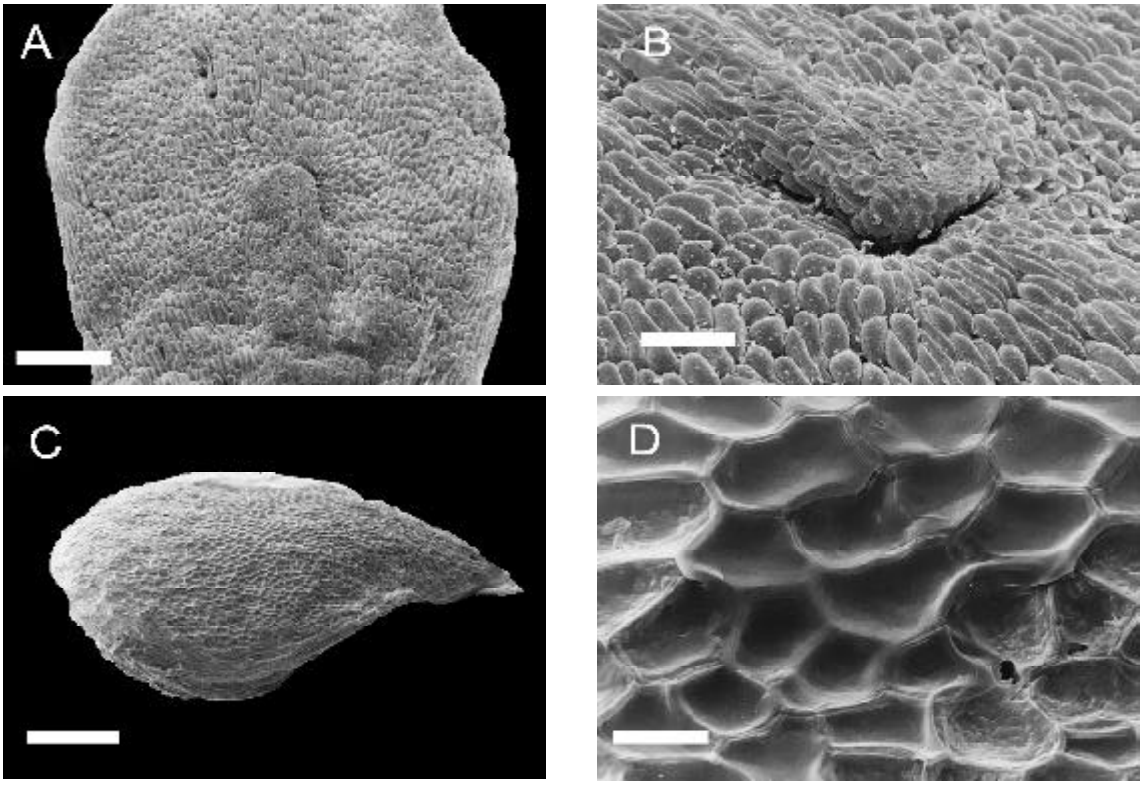

FIGURE 1. SEM micrographs of *Ranunculus spegazzinni* petal with nectary (A), close up of nectary scale (B), achene (C) and achene surface (D). Scale bars: A= 100µm; B= 50 µm; C= 500 µm; D= 20 µm). A and B from Lehnebach s.n. (MPN); C and D from Lehnebach & Ezcurra s.n. (MPN).

FIGURA 1. Fotografía de microscopía electrónica de barrido del pétalo (A), nectario (B), aquenio (C) y superficie del aquenio (D) de *Ranunculus spegazzinni*. Escala: A= 100µm; B= 50 µm; C= 500 µm; D= 20 µm). A y B de Lehnebach s.n. (MPN); C y D de Lehnebach & Ezcurra s.n. (MPN).

METHODS

Species identification and determination of collection details were assisted by descriptions available from the literature (Lourteig 1951, 1984, Ruiz 2001) and the study of material from past collections found in the herbaria CONC and SI. Flowering and fruiting individuals of *R. spegazzinii* were collected and fixed in 50 % ethanol. Later, petals were dehydrated using ethanol series of 50, 70, 85 and 100 %. After dehydration, petals were critical point dried and sputter-coated with gold and observed under the SEM (Cambridge 250). Achenes were etched with Driselase 1 % for 24 h to expose cell microcharacters, rinsed with distilled water and then air dried. Achenes were sputter-coated with gold and observed under the SEM.

SPECIMENS STUDIED

ARGENTINA: Neuquén. Dpto. Aluminé: brazo muerto de poca profundidad del arroyo Calfiquitra. SW lago Ruca Choroi. Parque Nacional Lanin. 02-II-1968. Eskuche & Klein 218 (SI). Rio Negro. Dpto. San Carlos de Bariloche: Llao-Llao. 12-III-2004 Lehnebach & Ezcurra s.n. (MPN).

CHILE: IX Region. Provincia de Cautín, cuesta Fusta, close to Quimquén, pond by the road, 1160masl. 38°40'S-71°22'W. 01-II-2006. Lehnebach s.n. (MPN). XII Region, Provincia de Ultima Esperanza, Parque Nacional Torres del Paine, Lago Skottsberg 500m W, 100 masl. 51°03'S-73°05'W. 11-III-1998. Elvebackk & Bjerke 98:477 (CONC).

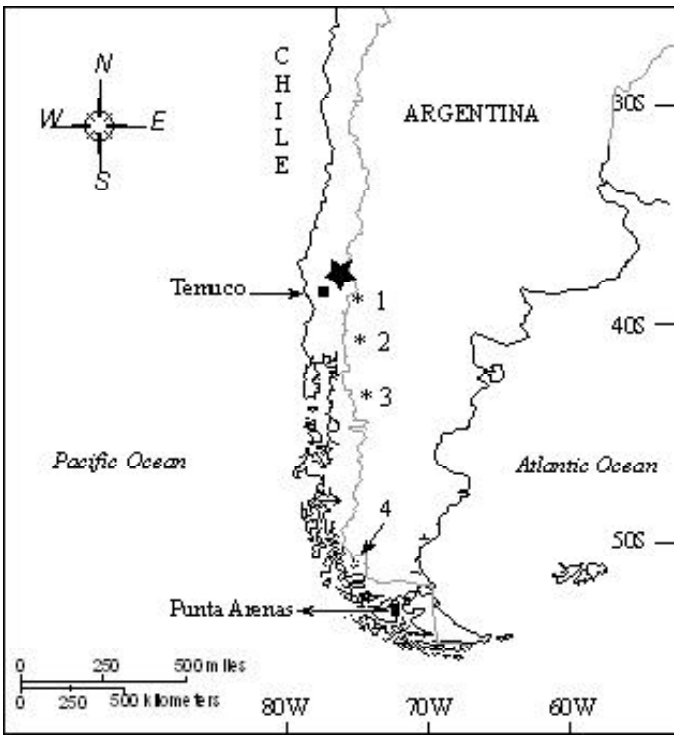

FIGURE 2. New collection of *Ranunculus spegazzinii* in the IX Region of Chile (★) and previous collections in Argentina and Chile (1: Neuquén, 2: Rio Negro, 3: Chubut, 4: Torres del Paine National Park).

FIGURA 2. Nueva colecta de *Ranunculus spegazzinii* en la IX Región de Chile (★) y previas colectas en Argentina y Chile (1: Neuquén, 2: Rio Negro, 3: Chubut, 4: Parque Nacional Torres del Paine).

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REFERENCES

LOURTEIG, A. 1951. Ranunculáceas de Sudamérica templada. Darwiniana 9(3-4): 445-608.

LOURTEIG, A. 1984. Ranunculaceae. En M.N. Correa (Ed.), Fl. Patagónica, Colección Científica Instituto Nacional Tecnología Agropecuaria 8(4a): 284-322.

RUIZ, E. 2001. Ranunculaceae. In C. Marticorena & R. Rodríguez (Eds). Flora de Chile 2: 40-84.

TAMURA, M. 1993. Ranunculaceae. En K. Kubitzki, J. G. Rowher & V. Bittrich (Eds.), The families and genera of vascular plants. pp. 563-583. Springer-Verlag, Berlin.

TAMURA, M. 1995. Systematic Part. In P. Hiepko (Ed.), Die Natürliche Pflanzenfamilien: Ranunculaceae. Duncker & Humboldt, Berlin. pp. 223-519.

ZULOAGA, F.O. & O. MORRONE. 1999. Ranunculaceae. In F. Zuloaga & O. Morrone (Eds.). Catálogo de las Plantas Vasculares de la Argentina: Dicotyledoneae. Monographs in Systematic Botany from the Missouri Botanical Garden 74: 964-974.

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Appendix 2: Collection details of Ranunculus species sequenced in chapter II and not published before (ITS and mat*K*) are included in CD attached

Appendix 3: Sequences of the primers used for amplification of the internal transcriber spacer (ITS) and chloroplast regions J_{SA} and *mat*K-*trn*K.

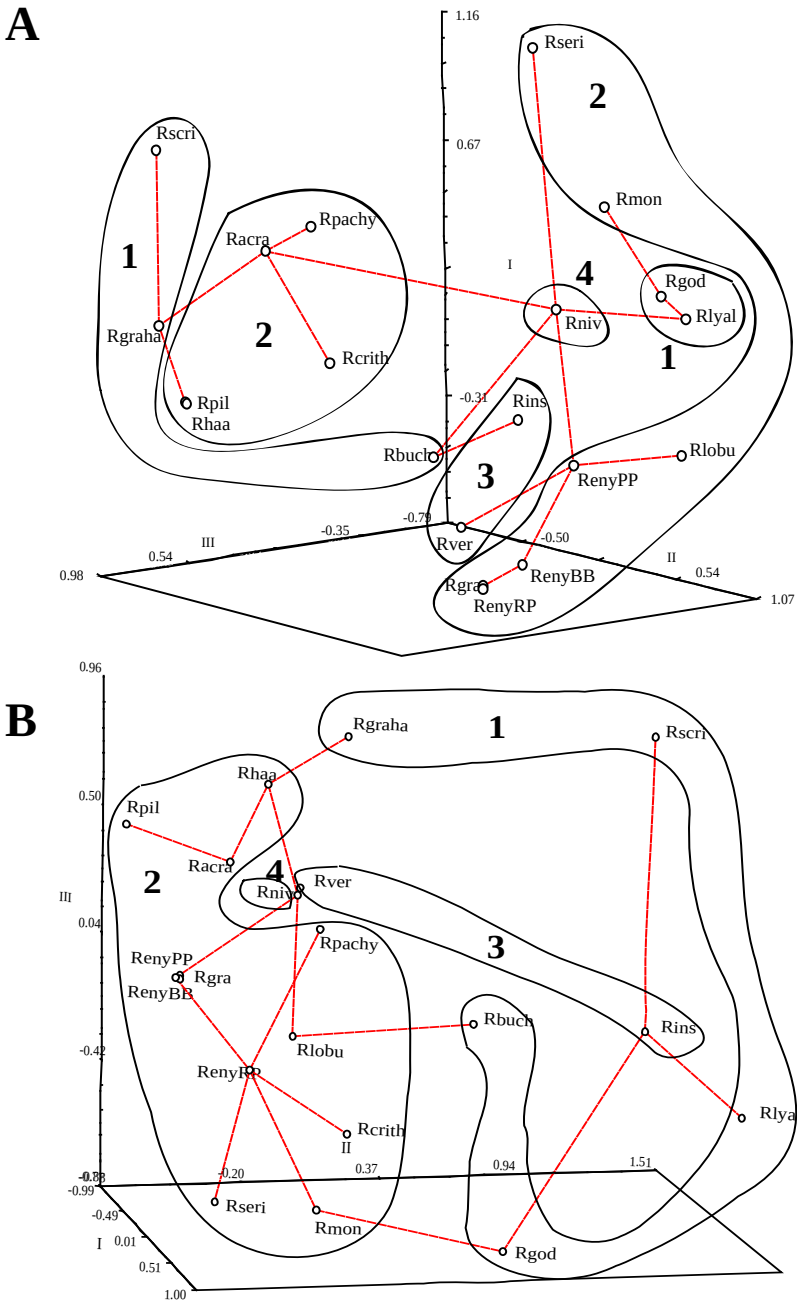
Name	Sequence (5'-3')	Primer source
	ITS	
ITS2	GCTACGTTCTTCATCGATGC	White *at al* 1990
ITS3	GCATCGATGAAGAACGTAGC	White *at al* 1990
ITS4	TCCTCCCGTTATTGATATGC	Baldwin 1992
ITS5	GGAAGTAAAAGTCGTAACAAGG	Baldwin 1992
	J_{SA}	
RERN	ATTATYAATGAAGGYAATACWATATATTTTC	Lockhart *et al.* 2001
151A	CAAATTCCAATGACCAAATAGTTCG	Lockhart *et al.* 2001
	*mat*K - *trn*K	
*trn*K5f	GGGTTGCGAACTCAACGGTAGAG	This study
*trn*K580r	TTTGTTTGTTATACGATACCAA	This study
*mat*K710f	GTATGGCACTATGTWTCATTTG	Paun *et al.* 2005
*trn*K1457r	CATGAAAATTATATAGGAAC	This study
*mat*K3ar	CGTACASTACTTTTGTGTTTNC	Paun *et al*. 2005
*trn*K3r	GATTCGAACCCGGAACTAGTCGG	Paun *et al*. 2005
*trn*K1859f	GGAATCAAATGTTAGAAAAT	This study

Appendix 4: Eigen values, percentage of variance explained by the first five components and loading scores for the environmental data of *R. isignis s.l.*

Appendix 5: Eigen values, percentage of variance explained by the first five components and loading scores for the environmental data of *R. enysii s.l.*

Appendix 6: Eigen values, percentage of variance explained by the first five components and loading scores for the environmental data of *R. lyalii s.l.*

Appendix 7: Multidimensional scaling ordination in three dimensions showing the morphological diversity of 20 *taxa* of alpine *Ranunculus* of New Zealand based on vegetative (A) and reproductive characters (B). Habitats detected by the CVA are indicated by the black line. The minimum spanning tree is indicated by the red dashed line and indicates proximity between the points. Rscri: *R. scrithalis*, Rgraha: *R. grahamii*, Rhaa: *R. haastii*, Rpil: *R. piliferus*. Racra: *R. acraeus*, Rpachy: *R. pachyrrhizus*, Rcrith: *R. crithmifolius*, Rver: *R. verticillatus*, Rniv: R. *nivicola*, Rgra: *R. gracilipes*, RenyBB: *R. enysii* Borland Burns, RenyPP: R. *enysii* Porter's Pass, RenyRP: *R. enysii* Rock & Pillar, Rseri: *R. sericophyllus*, Rmon: *R. monroi*, Rlobu: *R. lobulatus*, Rgod: *R. godleyanus*, Rins: *R. insignis*, Rbuch: *R. buchananii*, R. lyal: *R. lyalii*.



Files in attached CD

Chapter II

Matrices sequences Chapter II (Matrix ITS_gap_coded.txt; Matrix matK_coded.txt)

Chapter III

Environmental data chapter III

Morphology distance matrix (*R. insignis s.l.*, *R. enysii s.l.* and *R. lyallii*)

Voucher specimens studied in Chapter III

Matrices sequences chapter III

Chapter IV

Matrices sequences chapter IV

Environmental data Chapter IV

Collection details Alpine *Ranunculus* specimens

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