

ORIGIN AND BIOGEOGRAPHY OF NEW ZEALAND

CRASPEDIA (COMPOSITAE: GNAPHALIEAE)

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Craspedia lanata on a marble landslip, Hidden Valley Stream,
subalpine, Westland, New Zealand

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ABSTRACT

Craspedia (Compositae: Gnaphalieae) is a genus of 23 species found only in Australia and New Zealand. New Zealand species of *Craspedia* have confusing and continuous character variation, with boundaries between species often indistinct and relationships difficult to elucidate. Taxonomic treatments in the genus so far have been regionally based, with the result that species between New Zealand and Australia have not been adequately compared. Phylogenetic analyses of ITS, ETS and *psbA-trnH* non-coding spacers show that New Zealand *Craspedia* is a monophyletic group nested within Australian *Craspedia*. This is consistent with a jump-dispersal event from Australia to New Zealand across the Tasman Sea.

The New Zealand lineage is identified as sister to one of two Australian lineages, which consists of mainly subalpine and alpine species found on the main divide of south eastern Australia and in Tasmania. An estimate of when New Zealand *Craspedia* diverged, using ITS substitution rates from other mainland/island disjunctions in Compositae, gave an approximate date of between 650,000 and 325,000 years ago. This is consistent with the New Zealand fossil pollen record, and with other molecular studies, in suggesting that the Pleistocene, a period of mountain building and climate change, has been an important factor in the evolution of the New Zealand herbaceous flora.

The two Australian lineages have not previously been recognised based on morphology and it is suggested they represent two independent species radiations into the Australian alpine zone.

Although the New Zealand clade is only partly resolved, the phylogenetic analyses of ITS and ETS indicate that some relationships are incongruent with those previously suggested by morphology and current species boundaries.

CHAPTER 1

Introduction

The genus *Craspedia* G.Forst. is part of the family Compositae, placed in tribe Gnaphalieae and subtribe Angianthinae. *Craspedia* is a group of erect, rosette-forming herbs with homogamous capitula, which are secondarily compound and borne on unbranched scapes. Most species are perennial with one species recorded as annual (Curtis 1963; Everett & Doust 1992a; Everett & Thompson 1992; Rozefelds 2002). The genus was based on *C. uniflora* G.Forst. from New Zealand. Twenty three species are currently accepted (Allan 1961; Everett & Doust 1992b; Everett 1999; Rozefelds 2002), six from New Zealand and 17 from Australia. The generic circumscription of *Craspedia* has formerly included species now assigned to the genus *Pycnosorus* Benth. by Everett & Doust (1992c), distinguished from *Craspedia* on the basis of character differences in chromosome number, partial heads, paleae, pappus and bracts.

Tribe Gnaphalieae (paper daisies or everlastings) has its greatest diversity in southern Africa, South America and Australia. The Angianthinae are the largest subtribe in the Australian Gnaphalieae, with about 60 of the 84 genera (Bayer et al. 2002). All genera of Angianthinae are endemic to Australia except *Craspedia*. A molecular phylogenetic analysis of the Australian Gnaphalieae by Bayer et al. (2002) placed south-eastern Australian perennial, shrubby and cushioned alpine members as the earliest split in the group and hypothesised that following diversification in south-east Australia, the Gnaphalieae radiated westward into the Eyrean interior zone (Schodde 1989) with increasing aridity during the Miocene. The analysis of Bayer et al. (2002) places *Craspedia* in a group of 28 predominantly annual Angianthinae genera. They diverge last in the phylogeny and this late divergence suggests that *Craspedia* is derived from dryland, annual ancestors that have moved recently into the temperate Bassian Zone (Schodde 1989) of south-east Australia and then into the alpine areas of the eastern uplands.

Species of *Craspedia* are found in a wide range of habitats from coastal to alpine and are generally plants of open areas, sometimes ruderal. Observations of some Australian species (pers. comm. Kuo-fang Chung) suggest they re-establish well after fire, e.g., in the

spring following the 2002/2003 summer fires on Mount Buffalo in Victoria, *Craspedia variabilis* densely covered slopes of burnt *Eucalyptus* woodland (Fig. 1).



Figure 1. *Craspedia variabilis* (in flower) covering slopes of Mount Buffalo in Victoria in the spring of 2003, following fires in mid Jan 2003 (photo Kuo-fang Chung).

In Australia non-alpine species of *Craspedia* are commonly found growing in association with forest habitats, whereas in New Zealand they are generally excluded from closed forests, although occasionally plants can be found in regenerating forest or in open subalpine forests. *Craspedia* is never seen in New Zealand or Tasmania in the large, widespread and dense populations that are characteristic of mainland Australia, both in forest and on alpine slopes (Fig. 1 & Plate 1C).

Craspedia grows in a wide range of soil types (sands, gravels, clays, earths and loams) derived from different geologies across a broad rainfall gradient in both countries. It appears to be intolerant only of extremely infertile and acidic soils. This is illustrated by the absence of *Craspedia* from parts of south western Tasmania, about 17% of Tasmania

(pers. comm. A. M. Buchanan), that is characterised by pre-Cambrian quartzose rocks, which produce infertile sandy acid soils that are wet and dominated by a heath vegetation known as ‘buttongrass moorland’ (Jackson 1999).

There are two centres of diversity in *Craspedia*, and both are associated with upland areas. One of these is in the Kosciuszko National Park alpine/subalpine area, where seven species are found. Thompson (1981) and Costin (2000) suggest these species could represent recent diversification into newly formed alpine niches with species maintained by strong habitat selection. The other centre is a larger area in the north-west of the South Island of New Zealand, which contains several species and about 20 distinct and localised entities (Druce 1993). The latter could reflect either an adaptive radiation or a polyphyletic assemblage of entities associated with variable geology, physiography and habitats, and particularly associated with the Tertiary limestone and marble belts in the region.

The distribution of *Craspedia* is shown in Figure 2. In Australia it has a latitudinal range of 24.91°S (Buckland Tablelands, Queensland) to 43.12°S (Tasmania). Only two species of *Craspedia* in Australia have distributions outside of eastern temperate eucalypt forest. One is *C. variabilis* occupying the south west of Western Australia, and the other is *C. haplorrhiza*, found in the semi-desert areas of north-west Victoria, New South Wales, Queensland and two localities in Western Australia: Mount Sandiman in the north and Kalgoorlie in the south. The Mount Sandiman and Kalgoorlie populations are both notably disjunct from eastern populations and the disjunction of the former is larger than the Tasman Sea barrier between Australia and New Zealand.

Most Australian non-alpine species are found in native grasslands and shrublands associated with *Eucalyptus* forests. Alpine species are characteristic of short grasslands, herbfields and flush habitats of the subalpine and alpine zones of Kosciuszko (Plate 1C & D) and Tasmania. Outside the Bassian zone *C. haplorrhiza* is characteristic of semi-desert steppes, acacia and low eucalypt mallee on the Darling and Riverina floodplains.

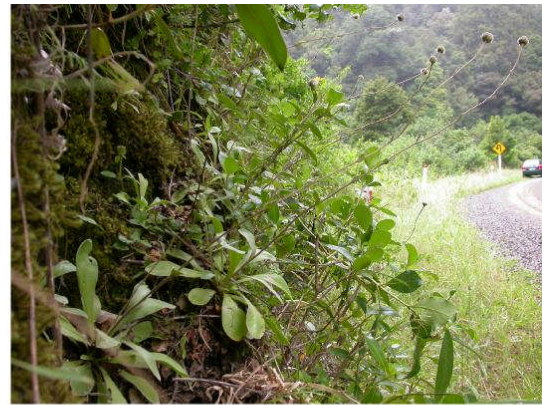


Figure 2. Distribution of *Craspedia* based on Australian Virtual Herbarium records and CHR herbarium records.

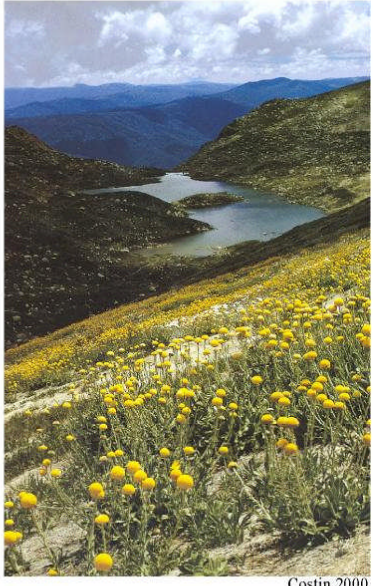
In New Zealand, *Craspedia* has a latitudinal range of 38.30° S (East Cape) to 52.34° S (Campbell Island). It is absent from the northern half of the North Island and it occurs on Campbell Island 660 km S of the South Island, and the Chatham Islands, 800 km E of East Cape, North Island — both notable disjunctions across ocean. In New Zealand, species exploit a wide range of open habitats, ranging from coastal sand dunes to alpine herbfield, and fellfield (Plate 1A), from greywacke rock scree (Plate 1E) to tall tussock grassland (Plate 1F) and forest margin banks (Plate 1B).



A



B



C

Costin 2000



D

Costin 2000



E

photo by I. Breitwieser



F

photo by P. N. Johnson

A *Craspedia* "elongata" on marble, Marino Mtns, N.W. Nelson, N.Z. **B** *C. minor* bank, Lake Waikaremoana, Urewera, N.Z. **C** *C. costiniana*, herbfield, Kosciuszko Nat. Park, Austr. **D** *C. alba* bog, Kosciuszko Nat. Park, Austr. **E** *C. incana* scree, Porters Pass, Canterbury, N.Z. **F** *C. lanata* talltussock, Otago, Pisa Range, N.Z.

Plate 1. Selection of species and species habitats of *Craspedia*.

The Australia/ New Zealand *Craspedia* distribution involves a notable disjunction of about 2000 km of ocean. There are two possibilities to account for this disjunction. One is a vicariant hypothesis, which suggests that *Craspedia* was present in this region about 85 million years ago (m.y.a.). before Antarctica/Australia and New Zealand rifted apart (Veevers et al. 1991), and therefore a more or less continuous distribution was broken by the rifting that created the Tasman Sea. The other involves more recent dispersal across the Tasman Sea barrier after its formation.

It is unlikely that a Gondwanan vicariant hypothesis can explain the disjunct distribution of *Craspedia* between Australia and New Zealand. The age at which Australia and New Zealand reached a maximum distance from each other by sea floor spreading is about 55 m.y.a. in the early Eocene (Veevers et al. 1991). The earliest records of a Compositae fossil (pollen) are about 30 m.y.a. in the middle Oligocene (Devore et al. 1995). The earliest records from New Zealand and Australia are also fossil pollen from the Oligocene. In New Zealand, which has near continuous Cenozoic terrestrial stratigraphic sequences (Fleming 1979; Lee et al. 2001), the first record of Compositae (as fossil pollen) is from the Late Oligocene (Couper 1953). Molecular phylogeographic evidence suggests that the Angianthinae are the result of an Australian Miocene radiation (Bayer et al. 2002). Therefore jump-dispersal over ocean is a more plausible hypothesis to explain the disjunction.

Evidence suggests that a large part of the New Zealand flora has originated from plant propagules crossing sea barriers since the rifting apart of the Australian/Antarctic landmass and New Zealand (Fleming 1979; Mildenhall 1980; Macphail 1997; Pole 1994, 2001). Trans-Tasman jump-dispersal of plant propagules to New Zealand from Australia has occurred throughout the Tertiary, as evidenced by the fossil record, which shows a non-random pattern of arrival of taxa in New Zealand post-dating that of Australia (Mildenhall 1980; Pole 1994; Macphail 1997).

More recently, molecular dating and observations of the level of genetic divergence of some herbaceous and sub-shrubby genera suggest that the arrival of these groups has been during the Late Tertiary and Quaternary, consistent with dispersal over ocean barriers after the breakup of Gondwana (Swenson & Bremer 1997; Breitwieser et al. 1999; Vijverberg et

al. 1999; Wagstaff et al. 1999; Wagstaff et al. 2000; Wagstaff & Wege 2002; Wagstaff et al. 2002; Winkworth et al. 2002; Perrie et al. 2003; Smissen et al. 2003). A number of authors (Breitwieser et al. 1999; Wagstaff et al. 1999; Wagstaff & Wege 2002; Wagstaff et al. 2002; Winkworth et al. 2002) suggest that the radiation of these genera has been in response to new environments created by extensive mountain building in the Late Pliocene and Pleistocene with concurrent cooling and episodes of glaciations.

Lloyd (1985) proposed that two types of species selection operate on islands like New Zealand (oceanic in character) where dispersal over sea barriers is the dominant means of acquiring biota: firstly by the non-random success of dispersal and establishment (immigration selection) and secondly by subsequent success in speciation and diversification, both involving selection for traits at a species level. One example he gave of the former is the high percentage of dioecy on islands due to bird dispersal, because of the correlation between dioecy and fleshy fruits (Bawa 1980). An example of the latter is the successful radiation of groups with simple, actinomorphic or weakly zygomorphic flowers such as *Hebe*, *Epilobium* and *Leptinella* in contrast to groups with strongly zygomorphic flowers such as *Mimulus*, *Utricularia* and *Chiloglottis* which have not speciated, correlated to the promiscuous, generalised pollinator fauna (Primack 1983; Lloyd 1985). This second type of species selection emphasises selection on ecological traits rather than specifically dispersal traits. Ancestors of species radiations are predicted to have pre-adapted traits (or key innovations), which confer an adaptive advantage and enable a coloniser to persist and move into new habitats.

Lloyd (1985) went so far as to predict that most traits seemingly peculiar to New Zealand have been acquired by species selection rather than autochthonous evolution at the individual level, although he named exceptions, e.g., insular woodiness and divaricating habit. To test species selection requires knowledge of where traits first arose, in New Zealand or outside New Zealand. This is not possible for *Craspedia*, as there is no evolutionary framework in which to assess the origin and direction of character evolution.

To test ideas on origin, character evolution and biogeography of genera requires the study of relationships between taxa. Estimating relationships and direction of evolution (a phylogeny) using morphology has often been problematic because of homoplasy

(independent origin or loss of traits across related taxa) and the difficulty in interpreting ancestral vs. derived character states. For example, different interpretations of character state polarity led Dawson (1971) and Webb (1986) to exactly opposite hypotheses of evolutionary relationships and biogeography in the Australasian Umbelliferae (*Aciphylla*, *Anisotome*, *Gingidia*, *Lignocarpa* and *Scandia*). Dawson hypothesised that the Australasian Umbelliferae were polyphyletic, with *Scandia* having a northern tropical origin and giving rise to *Gingidia* and *Lignocarpa* in New Zealand, and *Aciphylla* and *Anisotome* each having separate origins on the Chatham and Subantarctic islands respectively. Subsequently, *Gingidia* and *Aciphylla* independently dispersed across the Tasman Sea to Australia. Webb (1986), however, hypothesised that the five genera comprised a monophyletic group, with the origin of *Aciphylla*, *Anisotome* and *Gingidia* in Australia with subsequent independent dispersal of each genus to New Zealand, and then *Gingidia* giving rise to *Lignocarpa* and *Scandia* in New Zealand.

The New Zealand flora includes many examples of herbaceous and sub-shrubby plant groups with clusters of related species occupying varying open habitats, such as *Aciphylla*, *Bulbinella*, *Carex*, *Celmisia*, *Dracophyllum*, *Epilobium*, *Euchiton*, *Gentianella*, *Gingidia*, *Hebe*, *Leptinella*, *Pachycladon*, *Pimelea*, *Ranunculus*, *Raoulia* and *Wahlenbergia*. Many of these genera have confusing and continuous character variation, with boundaries between species often indistinct and relationships difficult to elucidate. Relationships with congeners in other regions of the south Pacific, in particular Australia, remain unclarified (Garnock-Jones & Breitwieser 1998). *Craspedia* fits into this pattern.

Difficulty in species recognition within *Craspedia* has long been noted (Allan 1961; Curtis 1963; Lander 1987; Breitwieser et al. 1999; Costin 2000; Rozefelds 2002). Treatments of the genus have so far been regionally based (Allan 1961; Curtis 1963; Everett & Doust 1992a; Everett & Thompson 1992), with the result that species have not been adequately compared between mainland Australia, Tasmania and New Zealand (Allan 1961; Breitwieser et al. 1999). Hooker (1853), Kirk (1899), Cheeseman (1925) and Cockayne (1928) all suggested that some taxa are possibly in both countries under different names, e.g., *C. lanata* var. *elongata* of New Zealand and *C. alpina* of Australia; and *C. robusta* var. *pedicellata* of New Zealand and *C. glauca* s.s. (as *C. glauca* var. *macrocephala*) of Australia.

Molecular sequencing technology has provided a new source of independent data to estimate phylogenetic relationships of morphologically similar plant groups between New Zealand and Australia. Molecular characters are less confounded by homoplasy than morphological or anatomical characters and can be used to estimate the dates of divergence of geographical disjunctions (Crawford 1992; Givnish & Sytsma 1997). For example, Wagstaff et al. (2002) used molecular sequences to clarify phylogenetic relationships in the New Zealand hebes (*Hebe* & related genera), from which they were then able to interpret the biogeography of the group. Estimates of divergence of major splits were done by calibrating base substitutions to the first appearance in the fossil record of *Hebe*, Scrophulariaceae and Lamiales. They concluded that the New Zealand hebes are monophyletic and derived from a single colonisation event via jump-dispersal from Australia or Eurasia to New Zealand in the Late Miocene (9.9 m.y.a.) Furthermore, subsequent to extensive adaptive radiation in New Zealand, they hypothesised ten jump-dispersal events from New Zealand across the Tasman Sea (east to west) to Australia and across the Pacific Ocean to various distant locations in different directions, e.g., Chatham Islands, New Guinea, Rapa Island, South America & New Zealand Subantarctic Islands.

For *Craspedia*, molecular phylogenetic analyses have so far included species only as part of wider studies into the phylogenetic relationships of the Gnaphalieae or Angianthinae (Breitwieser et al. 1999; Bayer et al. 2002). Breitwieser et al. (1999) included seven species of *Craspedia* (three Australian species and four New Zealand entities) and one of *Pycnosorus* in a molecular phylogenetic analysis of mostly New Zealand Gnaphalieae and distinguished separate New Zealand and Australian *Craspedia* lineages. They hypothesised that *Craspedia* in New Zealand is monophyletic and derived from a single founder event via jump-dispersal from Australia. Bayer et al. (2002) included one species of Australian *Craspedia* and two of *Pycnosorus* (one cited as *Craspedia*) in a study that included 69 genera of Australian Gnaphalieae. The results of this study were congruent with morphology in suggesting that *Pycnosorus* is sister group to *Craspedia*. The authors concluded that there was some evidence to suggest that *Craspedia* is a monophyletic group, “at least in part” (Bayer et al. 2002, p. 810), but only one true species of *Craspedia* was studied.

The present study, by inferring a phylogeny from a representative sample of *Craspedia* using molecular markers, will test the null hypothesis that New Zealand *Craspedia* is monophyletic, resulting from one colonisation event from an Australian progenitor (Breitwieser et al. 1999). Alternatively, are New Zealand species of *Craspedia* derived from multiple dispersal events from Australia and has there been subsequent ongoing trans-Tasman exchange between Australia and New Zealand (Fig. 3)?

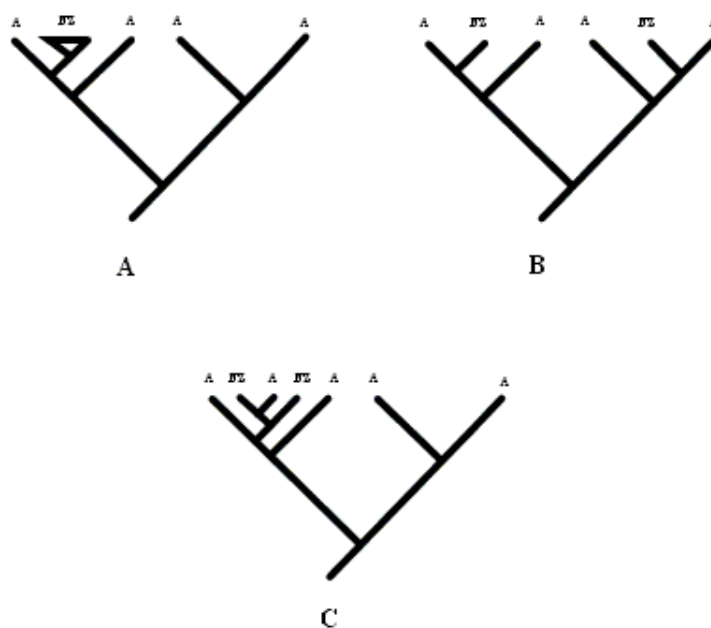


Figure 3. The expected tree topologies of the null and alternative hypotheses. New Zealand *Craspedia* is: **A.** monophyletic, consistent with one dispersal event; **B.** polyphyletic, consistent with two (or more) dispersal events; **C.** paraphyletic, consistent with dispersal from New Zealand back to Australia after establishing in New Zealand.

Establishing whether New Zealand *Craspedia* is monophyletic or polyphyletic will provide a framework for further exploration of morphological, ecological and geographical patterns at a finer scale and will contribute towards a comprehensive taxonomic treatment of the genus.

CHAPTER 2

Molecular data, phylogenetic inference and tree-building**2.1 Molecular data**

The non-coding intergenic spacers ITS (internal transcribed spacer) and ETS (external transcribed spacer) from the nuclear genome, and *psbA-trnH* from the chloroplast genome, were used in this study, providing two independent data sets.

The ITS region consists of ITS-1 and ITS-2, which are spacers between genes that code for ribosomal RNA subunits 18S, 5.8S and 26S (Fig. 4). The ITS region was used in this study for two principal reasons: (1) it has proved to be a variable molecular marker suitable for inferring phylogenetic relationships at lower taxonomic levels, i.e., the genus level and below (Baldwin et al. 1995), and (2) it has been established from a sample of seven species that ITS is variable enough to distinguish New Zealand and Australian lineages of *Craspedia* (Breitwieser et al. 1999).

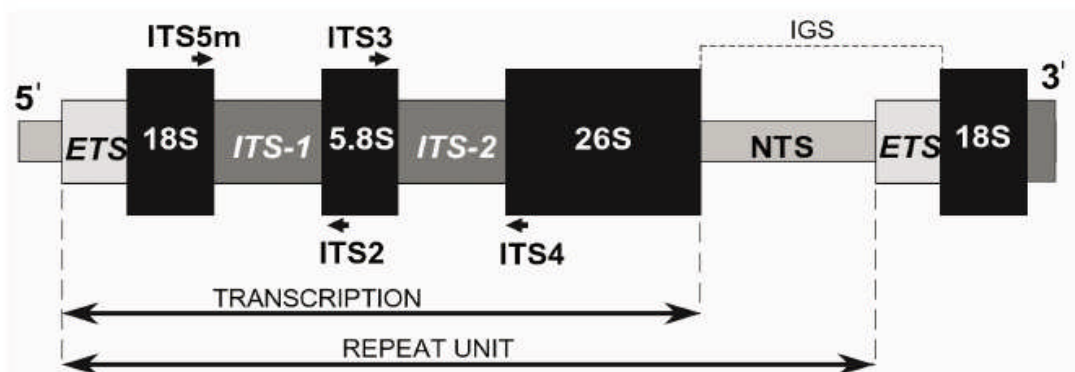


Figure 4. Basic structure of a repeat unit of nrDNA, ITS = internal transcribed spacer; ETS = external transcribed spacer; IGS = intergenic spacer; NTS = non-transcribed spacer. Genes are indicated in black. Not drawn to scale.

The ETS region (Fig. 4), also part of the rDNA gene family, has been chosen to supplement ITS. This marker has recently been shown to provide more variable characters than ITS (Baldwin & Markos 1998; Clevinger & Panero 2000; Markos & Baldwin 2001).

The use of ITS and ETS intergenic spacers for phylogenetic inference assumes that there is little variation in the tandem repeats of rDNA within genomes, due to concerted evolution. Concerted evolution occurs when differences among the rDNA repeats (which can exist in 100s to 1000s of copies) in the genome become homogenised to the same identical sequence type by DNA correction processes (Zimmer et al. 1980; Dover 1982).

This assumption however has been shown to be violated in certain circumstances, particularly associated with hybridisation and allopolyploidy. For example, Wendel et al. (1995) demonstrated that both parental rDNA repeat types or 'paralogues' existed in rDNA associated with allopolyploids of *Gossypium* suggesting that concerted evolution is not always complete. Incomplete homogenisation of multiple gene copies could confound phylogenetic inference due to the comparison of paralogues, which could result in the gene tree being incongruent with the species tree (Doyle 1992).

Paralogues are gene copies that are members of different gene genealogies (Fig. 5.). If concerted evolution did not take place amongst multiple copy genes, then gene copies if unlinked would diverge independently as separate gene lineages through acquiring mutations (or through the introduction of variants via hybridisation). Each gene lineage is related by orthologous relationships and will potentially recover phylogenetic species relationships. Figure 5 shows how comparing a mixture of paralogues could result in incongruent gene and species trees. Figure 5a shows an ancestral taxon in which a mutation within the rDNA repeats has produced paralogous genes variants X and Y. Genes X1, X2 and X3 form an orthologous group, the relationships paralleling speciation events resulting in species A, B and C. Similarly genes Y1, Y2 and Y3 form an orthologous group derived by the same speciation events as gene variant X. Both sets of orthologues recover the species phylogeny in Fig. 5b. However, paralogous genes from the two sets could be sampled by chance and result in incorrect inference of the species phylogeny. In Fig. 5c gene variants X1 and X3 are more closely related to each other (through the common inheritance of the same mutations) than gene variant Y2.

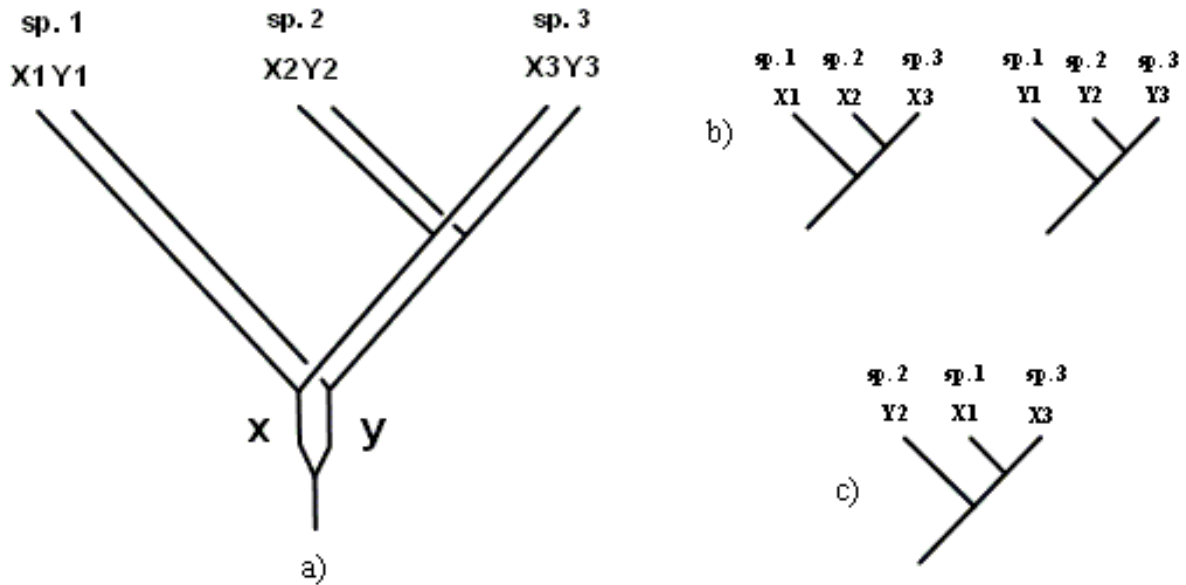


Figure 5. Orthologues and paralogues a) X & Y are two rDNA repeats that have diverged into separate gene lineages. b) orthologous sets X & Y both recover the species phylogeny. c) paralogous repeats from the two sets do not recover the species phylogeny.

An orthologous gene tree may also be incongruent with a species tree due to lineage sorting (Avice 1989; Doyle 1992).

Lineage sorting is the random sorting of intraspecific polymorphisms into different lineages at the time of speciation. The lineages of genes are separate from those of species and therefore the potential exists for incongruence between the two. The likelihood of lineage sorting is related to population demographics (Avice 1989). In Figure 6, the species tree is ((A B) C), whereas a species tree inferred from the gene tree X, Y and Z would be ((AC) B). Within the ancestral species of A, B and C at time zone 1, haplotypes X and Y diverge and then Z diverges from Y. The speciation event at time 2 results in species C and the ancestral species of A and B. Haplotype Z is inherited by species C and haplotypes X and Y are inherited by the ancestor species of A and B which is thus polymorphic for that gene. If extinction of one these haplotypes in the ancestor species of A and B (either X or Y) does not occur before the speciation event (at time 3) resulting in the species A and B, the gene tree will be incongruent with the species tree, as haplotype X (in species A) has a direct common ancestry with haplotype Z (in species C). Furthermore, in the case of three species, the two haplotypes that diverge first will not be the lineages from A and B two thirds of the time.

In particular, lineage sorting of chloroplasts (or chloroplast capture through introgressive hybridisation) can mislead phylogenetic inference at all taxonomic levels because chloroplasts are inherited as a single linkage group and therefore are insulated (from recombination) through generations, acting effectively as a single ‘character’ (Doyle 1992). In contrast nuclear genes are not as tightly linked and undergo recombination each generation, and therefore a higher likelihood exists of estimating the species tree by using a number of different nuclear markers, some of which will reflect the species phylogeny. Chloroplast gene trees are most useful when used with nuclear markers and morphological data, where incongruencies may aid in detecting past historical events, whether it be lineage sorting, occasional hybridisation (Sang 1997) or a reticulating pattern (Smitsen et al. 2004).

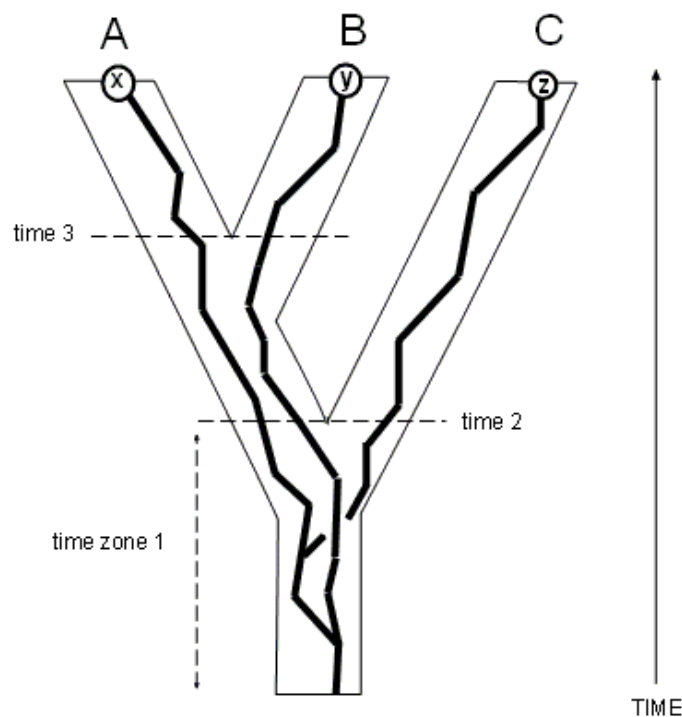


Figure 6. Lineage sorting. A phylogeny of three species (light lines) and a genealogy of gene haplotypes (dark lines). The species tree is ((A B) C) whereas the gene tree is ((AC) B).

Ideally phylogenetic inference of a species tree should be estimated using numerous nuclear gene regions and at least one chloroplast region in order to lessen the effects of inconsistent gene and species lineages due to lineage sorting and capture via hybridisation.

The chloroplast region used in this study is the non-coding intergenic spacer between the *psbA* and *trnH* genes, a short non-coding sequence. In *Nicotiana tabacum*, 'trnH' is immediately adjacent to the right member of the large inverted repeat in the single copy region (Wakasugi et al. 1998). In a number of studies *psbA-trnH* has been shown to be a faster-evolving chloroplast region than the *trnL-trnF* spacer and therefore suitable for phylogenetic inference at lower taxonomic levels (Sang 1997; Kim et al. 1999). However, in all the above studies, the *psbA-trnH* intergene is slower-evolving than ITS or ETS, and was less useful than these nuclear DNA intergenic spacers for resolving phylogenetic relationships at lower taxonomic levels. In the present study the *psbA-trnH* intergene was used because it would (1) provide an independent reconstruction of phylogeny in addition to ITS and ETS, and (2) show incongruence when compared to ITS and ETS (parentally inherited markers), which may suggest that the gene tree does not equate to the species tree.

2.2 Phylogenetic inference and tree building methods

The first numerical (algorithmic) inference of a phylogeny was of bees, using a clustering method with morphological characters (Michener & Sokal 1957). Phylogenetic tree building approaches first developed hand in hand with protein sequence analysis and population genetics in the 1960s (Zuckerlandl & Pauling 1962; Edwards & Cavalli-Sforza 1963, 1964; reviewed by Felsenstein 2004). Edwards and Cavalli-Sforza (1964) published the first parsimony tree in a paper on the gene frequencies of blood group alleles of human populations and introduced the likelihood method in the same paper.

Hennig (1966) introduced the explicit but non-numerical cladistic methodology of phylogenetic inference. His ideas developed the earlier work of Zimmerman (1930, 1931, 1933 & 1943). This method explicitly uses shared derived characters (synapomorphies) to identify monophyletic groups as a way of reconstructing evolutionary history using morphological characters. It is a point of contention whether parsimony is implied by Hennig's auxiliary principle as a way of deciding conflict (1966, p. 12). Further discussion can be found in Farris et al. (1970), Farris (1983) and Felsenstein (2004).

Maximum likelihood and parsimony are two commonly-used numerical tree-building methods to infer phylogenetic trees. Likelihood and parsimony are methods that use discrete characters and character states. Both methods are 'global' in that they relate all taxa simultaneously. They are also optimality methods, where all possible trees (bifurcating relationships) of the samples are evaluated. They use an algorithm as a tool in the evaluation of an objection function, i.e., ranking trees according to the optimality-criterion (Swofford et al. 1996). In contrast, the neighbour-joining method (Saitou & Nei 1987) is 'constructive', meaning it uses an algorithm to build one tree directly from pairwise-distances by following a series of steps dictated by the algorithm not according to an optimality-criterion, and therefore the algorithm has no process for ranking trees.

Phylogenetic inferences using parsimony has dominated systematic literature to date. Parsimony as implemented by the common software package Paup is the closest to traditional Hennig cladistics in philosophy because it is model free — it makes no explicit assumptions about evolutionary processes. However it does make the implicit assumption that evolution is parsimonious. It has a practical advantage over likelihood, especially with large data sets, in that it is relatively fast, allowing a more thorough search of tree topology. A parsimony analysis computes minimum length trees under restrictions on permissible character state changes (the optimality criterion). Minimum tree length implies the greatest amount of homology and least amount of homoplasy, i.e., changes due to common ancestry and not by reversals, parallelisms and convergences, for which another step is added to the tree (as an additional independent character change event).

Whereas parsimony apparently makes no explicit assumptions about the evolutionary process and operates on uncorrected data (the actual observed data), maximum likelihood and distance methods explicitly take advantage of what is known about nucleotide substitution processes and phylogeny and are model-based. Substitution models vary in sophistication and are attempts to correct data to give a better estimate of phylogeny. Model-based methods attempt to (1) correct for superimposed base changes and (2) correct for a defined amount of bias in the data depending on the model.

A list of commonly used substitution models for maximum likelihood and distance methods are:

Models that assume all nucleotides occur at equal frequencies (25%)

1. *The Jukes-Cantor (JC) model*

1. All substitutions are equally likely.
2. All nucleotides occur at the same frequency (25%).
3. One parameter: the rate of substitution (alpha).

2. *Kimura two parameter (K2P) model*

1. Transitions and transversions happen at different rates.
2. All nucleotides occur at the same frequency.
3. Two parameters: transition rate (alpha) and transversion rate (beta).

Models that allow the four nucleotides to be present in different frequencies

3. *Felsenstein (F84) & Hasegawa-Kishino-Yano (HKY85) models*

1. Two closely related models -- they use different calculations to model essentially the same thing.
2. Transitions and transversions occur at different rates.
3. Nucleotides occur at different frequencies.
4. Six parameters.

4. *General time reversible (GTR) model*

1. Assumes a symmetric substitution matrix (and thus is *time reversible*). In other words, A changes into T with the same rate that T changes into A.
2. Each pair of nucleotide substitutions has a different rate .
3. Nucleotides can occur at different frequencies.
4. Eight parameters.

Ignoring superimposed base changes and biases in sequence data can result in all tree-building methods being unreliable (Lockhart et al. 1994; Chang 1996). An example of inconsistency in parsimony is long-branch attraction (Felsenstein 1978; Lockhart & Cameron 2001) caused by failing to account for superimposed base changes and differences in substitution rates across lineages. This can result in a systematic error in parsimony trees, but also in trees estimated by uncorrected p-distances or distance methods and maximum likelihood when using simple models that makes few assumptions, e.g., Jukes-Cantor.

The method of maximum likelihood selects trees with the highest probability of producing the observed sequences under a selected model of DNA substitution. The highest (optimised) likelihood score is given to the tree that makes the observed data more likely to have arisen than other trees.

Maximum likelihood uses probability theory on substitutions on the basis that it is appropriate for data generated by a random process (Swofford et al. 1996). The likelihood score for a tree is a summation of all likelihoods calculated for each character state (assumed to be independent). All possible scenarios by which the tip sequences could have evolved are taken into account, some being more probable than others. The probability of each scenario is equal to the sum of the probabilities of the changes required by that scenario. This is why maximum likelihood is computationally slow, especially with large data sets and complex models.

A phylogenetic tree is a graphic representation of the genealogical relationships between entities. Parsimony and maximum likelihood methods assume that the data will conform to a bifurcating tree model, i.e., a “connected acyclic graph” (or a graph without cycles). When data do not conform to a tree-like model, criterion-based methods may provide some indication of a problem, such as near optimal trees that differ in topology and clades with low bootstrap values. This may indicate conflicting phylogenetic signal in the data. The program Splitstree using the split decomposition method (Bandelt & Dress 1992) is not restricted to a bifurcating tree model and can be used to test whether the data support a tree-like model and to explore conflicting signal. Splitstree is able to display conflicting signal graphically as a network (a graph with cycles).

A ‘split’ is the partition of the sample set into two mutually exclusive subsets. A polymorphic (binary state) character can thus be considered to define a ‘split’ in the sample set from which it was assayed (Huson 1998). For a quartet d of dissimilarities between pairs of taxa, there are three sums of the distances between each pair of taxa e.g. for four taxa a, b, c and d, the three distance sums are $d_{ab} + d_{cd}$, $d_{ac} + d_{bd}$, and $d_{ad} + d_{bc}$. The sum of the between-group distances can be expected to be larger than the sum of the within-group distances, but in practice split decomposition only assumes that for each group the sum of the within-group distances is at least not the largest of all sums collectively. A split is

described as ‘internal’ when both of the subsets it partitions contain at least two individual samples. Internal splits are also said to be parsimony-informative. When one of the subsets of a split contains only one individual sample, it can be described as ‘external’ or parsimony-uninformative. Two splits considered in tandem may be described as either compatible or incompatible. Compatible splits can be represented on the same bifurcating tree, while incompatible splits cannot. Incompatible splits describe conflicting hypotheses of relationship. Incompatible splits are displayed as a box in Fig. 7, where the alternative hypotheses of relationship, (AB) (CD) or (AC) (BD) are equally possible.

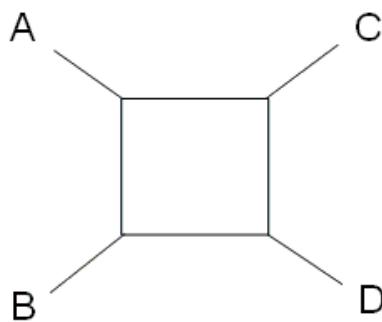


Figure 7. Split graph of incompatible splits between AB/CD and AC/BD.

The three methods outlined above are used in this study to estimate the phylogenetic relationships within *Craspedia* and in relation to outgroups. The three methods operate very differently and an investigative approach has been adopted in relation to tree-building, i.e., it is not assumed that there is one optimal method for phylogenetic data analysis.

CHAPTER 3

Materials and methods

The goal when reconstructing phylogeny is to use circumstantial evidence (present day DNA sequences, morphology, anatomy, etc.) to arrive at a hypothesis of the evolutionary history of a group of organisms. This requires (1) appropriate sampling of the study group and related taxa, (2) selection of characters that exhibit variation appropriate to the question posed, (3) definition of character states and (4) an appropriate tree-building method.

3.1 Sampling

3.1.1 Ingroup

Six factors have been taken into account for obtaining a representative sample of *Craspedia*: (1) the use of the taxonomic literature to identify species and species groups, (2) representation of the morphological diversity present in the genus, (3) geographic range (4) habitat diversity (5) altitudinal range and (6) to avoid sampling hybrids. With these considerations, a sample set of 32 species and informally named entities were chosen from *Craspedia* in New Zealand and Australia (Table 2). Collection localities are shown in Figure 8.

Since Allan's flora (1961) there have been 40 years of further discovery and collecting of *Craspedia*, firstly by Druce (1993) and secondly, by Breitwieser. Therefore sampling of New Zealand species and entities is based on Druce's checklist (1993), ensuring that all species of Allan (1961) except *C. major* were sampled. *C. major* was not sampled because Druce (1993) included it in *C. minor*. Other departures from Allan are: *C. minor* var. *viscosa* (Plate 1B) and *C. lanata* var. *elongata* are treated at specific rank (according to Druce 1993). No formal combination is available at specific rank for *C. lanata* var. *elongata*, and therefore this taxon is treated as an informal tagname, i.e., *C. "elongata"*. Because not all 55 species and informal entities could be sampled, Druce's checklist (Table 1) is divided into groups based on similar morphology from which samples were taken. Justification for sampling is provided in notes adjacent to groups. New Zealand material

of *Craspedia* was available from Breitwieser's collection at the Landcare Research nursery at Lincoln, supplemented by my own collections.

Table 1. Druce's 1993 checklist of accepted taxa. 'Bold type' indicates New Zealand *Craspedia* sampled for this study.

<i>Craspedia</i> taxa and entities (55)	morphological groups (6)
<p><i>C. incana</i> Allan <i>C. "incana Otago"</i> <i>C. "incana Marl"</i></p>	<p>incana group' lvs. densely covered in snow-white floccose tomentum, 3 disjunct similar ecological & morphological forms, (dry eastern alpine herbfield, fellfield & scree, high altitude). <i>C. incana</i> has notably large cypselas, and both it and <i>C. "incana Marl"</i> lack cypselas bifid hairs. All three were sampled on the basis of covering a wide allopatric geographic range, which is at high altitude.</p>
<p><i>C. lanata</i> var. <i>lanata</i> Allan <i>C. "elongata"</i> (no comb. avail. at sp. rank) <i>C. "ESI"</i> (eastern South Island)</p>	<p>'lanata group' lvs. dense-appressed to subappressed greyish white tomentum (eastern & main divide subalpine herbfield, grassland). Sampled on the basis to cover its wide geographic range.</p>
<p><i>C. "Havelock"</i> <i>C. "Clutha"</i> <i>C. "Heron"</i> <i>C. "Kaitorete"</i></p>	<p>'pattern group' lvs. with dense short appressed white or grey tomentum, red pollen sacs (except <i>C. "Havelock"</i>) (eastern, dry, river beds and terraces, coastal dunes). Sampled on the basis of its unusual lowland habitat, i.e., sand dune hollows.</p>
<p><i>C. minor</i> (Hook.f.) Allan <i>C. viscosa</i> Colenso <i>C. "coast"</i></p>	<p>'minor group' lvs. sparingly clad in subhispid hairs to glabrous, small glomerules and cypselas (lowland to subalpine, bank, cliff and rocky streamside, s.t. ruderal). Sampled both species on the basis of difference in leaf hairs.</p>
<p><i>C. robusta</i> var. <i>robusta</i> (Hook.f.) Cockayne <i>C. robusta</i> var. <i>pedicellata</i> (Kirk) Allan <i>C. "Skippers"</i></p>	<p>'robusta group' broad ovate lvs. glabrous to subhispid hairs, large glomerules (wet, coastal rock, sand dunes to alpine herbfield, of inland Otago, main divide, west coast & Stewart Island). Sampled because of its wide geographical range.</p>

<p><i>C. uniflora</i> var. <i>uniflora</i> G.Forst. <i>C. uniflora</i> var. <i>grandis</i> Allan <i>C. uniflora</i> var. <i>maritima</i> Allan <i>C. uniflora</i> var. <i>subhispidata</i> Allan <i>C. uniflora</i> var. "Charleston" <i>C. uniflora</i> var. "Hackett Limestone" <i>C. uniflora</i> var. "Haldon Hills" <i>C. "Mytton"</i> (=C. "marble") <i>C. uniflora</i> var. "Punakaiki" <i>C. uniflora</i> var. "N Canterbury White" <i>C. uniflora</i> var. "S Canterbury yellow" C. "Chatham" C. "Hikurangi" C. "Tararua" C. "Volcanic plateau"</p>	<p>'uniflora group' leaf margins distinctly white with tangled cottony hairs (with variation), surfaces covered in short hairs (lowland to alpine, coastal rocky areas, grassland/herbfield, banks and cliffs) Note: morphologically & ecologically variable.</p> <p>Sampled <i>C. uniflora</i> var. <i>uniflora</i> from Port Underwood based on its proximity to the type locality of <i>C. uniflora</i> G.Forst. Sampled <i>C. uniflora</i> var. <i>subhispidata</i> and <i>C. "Chatham"</i> on a geographical basis, as the former is found on Campbell Island c. 660 km S. of the South Island of New Zealand and the latter is found on the Chatham Islands 800 km E. of East Cape, North Island respectively.</p>
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<p>Tagnames not able to be grouped</p> <p>C. "Anglem" C. "Burgoo" C. "calcicole" C. "carpet" C. "Chalk" C. "Clara" C. "Fyfe narrow" C. "Garibaldi" C. "Goulard Downs" C. "Hackett" C. "Henderson" C. "Leatham" C. "long hairs" (=C. "LH Peel") C. "Loveridge" C. "Pikikiruna" C. "No Man" C. "Mararoa" C. "Owen" C. "serpentine" (=C. "Red Hills") C. "short hairs" C. "small bog" C. "tarn"</p> <p>C. "East Cape" C. "Rex"</p>	<p>C. "Anglem" restricted to Stewart Island (alpine fellfield). Sampled this entity as it was thought by Breitwieser and Druce to be close to species of Tasmanian <i>Craspedia</i>.</p> <p>C. "Garibaldi" lvs. arranged in a basal imbricate rosette, with dense subhispid hairs, cypselas with dense straight bifid hairs, restricted to sandstone on a ridge in N. W. Nelson. Sampled on the basis of its very distinctive morphology. The most distinctive New Zealand <i>Craspedia</i>.</p> <p>Note that <i>C. "East Cape"</i> and <i>C. "Rex"</i> are unknown. No herbarium vouchers with those names have been located and fieldwork has not established any candidates.</p>
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Sampling of Australian species is based on the accepted taxonomy, Curtis (1963), Everett & Doust (1992b); Everett (1999) and Rozefelds (2002). It was decided to collect nearly all species in Australia and to cover the geographic range as much as possible. Samples from Australia were collected on a field trip made in January 2003, where 14 of the 17 formally

described species were collected; *C. alpina* Hook.f., *C. alba* J.Everett & Joy Thomps., *C. aurantia* J.Everett & Joy Thomps., *C. canens* J.Everett & Doust, *C. coolaminica* J.Everett & Joy Thomps., *C. costiniana* J.Everett & Joy Thomps., *C. glabrata* (Hook.f.) Rozefelds, *C. glauca* s.s. (Labill.) Spreng., *C. lamicola* J.Everett & Joy Thomps., *C. leucantha* F.Muell., *C. maxgrayi* J.Everett & Joy Thomps., *C. paludicola* J.Everett & Doust, *C. preminghana* Rozefelds, *C. variabilis* J.Everett & Doust. Three species were not collected. *C. crocata* and *C. jamesii* were deliberately not collected, as *C. crocata*, *C. jamesii* and *C. aurantia* are considered to be a closely related group (pers. comm. N. Walsh 2003). *C. haplorrhiza* J.Everett & Doust, a species of semi-desert flood plains and damp depressions of semi-arid Australia, could not be collected due to severe drought in the summer of 2003. However, material was obtained and successfully extracted and sequenced from a pressed and dried specimen housed at the Melbourne Botanic Gardens Herbarium (MEL 235247). To obtain partial geographic representation of the widespread *C. variabilis*, an additional sample (MEL 2116666) of this species was obtained from pressed and dried material. The only major geographic area not sampled was south-west Western Australia, which is the westernmost extension of the distribution of *Craspedia* (*C. variabilis*). Plant material collected from Australia on the January field trip was deposited into silica gel for DNA analysis and was pressed and dried for herbarium vouchers.

Herbarium vouchers from both Australian and New Zealand collections are deposited at the Allan Herbarium, Lincoln. Duplicates of Australian collections are deposited at HO, MEL or NSW. Accessions sequenced for this study are shown in Table 2.

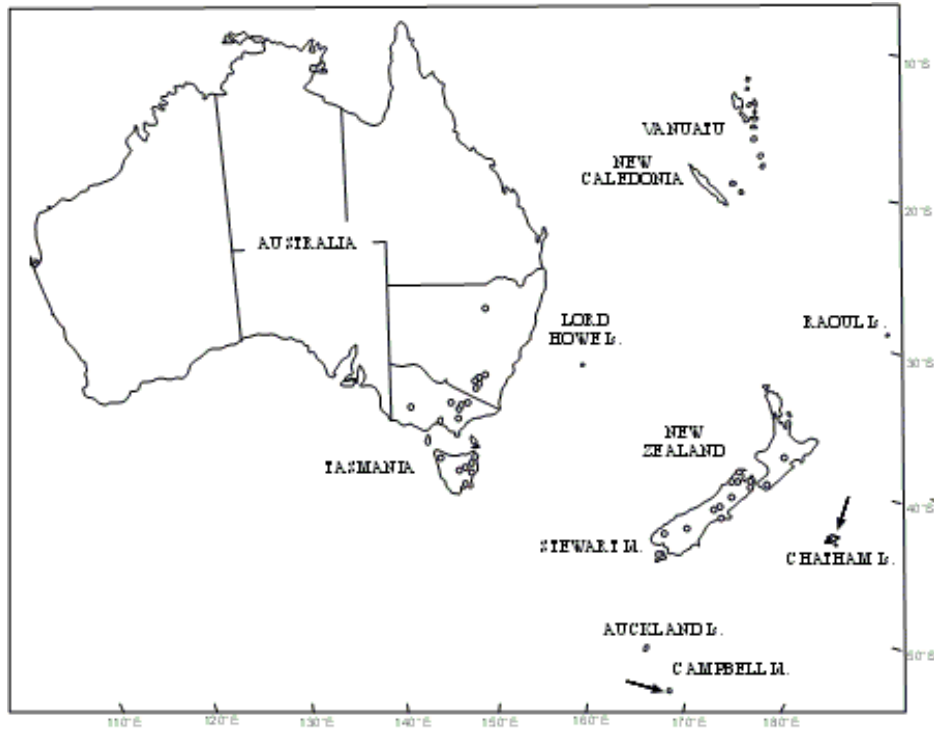


Figure 8. Collection localities for *Craspedia* sample set. Arrows indicate collections from Campbell Island and the Chatham Islands.

3.1.2 Outgroups

Outgroups to root trees were selected from recently published molecular phylogenies of the Australian and New Zealand Gnaphalieae (Breitwieser et al. 1999; Bayer et al. 2002). Four were selected: *Pycnosorus globosus* Benth. and *P. chrysanthes* (Schldl.) Sond., Australia; *Helichrysum lanceolatum* (Buchanan) Kirk, New Zealand; *Stuartina muelleri* Sond., Australia. *Pycnosorus* was chosen as it has been formerly included in *Craspedia* based on morphology (Bentham 1866) and has been identified by recent molecular studies as the genus sister to *Craspedia* (Breitwieser et al. 1999; Bayer et al. 2002). DNA sequences from the two species of *Pycnosorus* were similar and *P. chrysanthes* was excluded from further analyses. *Stuartina* is placed in an earlier divergence in comparison to *Craspedia* and *Pycnosorus* in Bayer et al.'s (2002) molecular phylogeny of Australian Gnaphalieae. *Helichrysum lanceolatum* is misplaced in *Helichrysum* (Ward & Breitwieser 1998) and is part of an almost exclusively endemic New Zealand clade of Gnaphalieae which includes the genera *Anaphalioides*, *Leucogenes*, *Rachelia* and *Raoulia* (Breitwieser et al. 1999).

Table 2. Accessions sequenced for this study.

Taxa or "entity"	Origin	Collector	no.	date	Herbarium no.
	AUSTRALIA				
<i>Craspedia alpina</i>	Tasmania, Hobart, Mt. Wellington	KAF & AMP	20/03	13/01/03	CHR 565519
<i>C. alba</i>	Victoria, Snowfields, Pretty Valley	KAF & AMP	2/03	4/01/03	MEL 2172914
<i>C. aurantia</i> (NSW)	N.S.W. Mt. Kosciuszko	KAF & AMP	5/02	30/12/02	CHR 489399
<i>C. aurantia</i> (Vict)	Victoria, Bogong High Plains (Doust 1102)	S. McLoughin	1a.	4/02/97	CHR 487571
<i>C. canens</i>	Victoria, Traralgon, railway reserve	KAF & AMP	13/03	5/01/03	MEL 2172917
<i>C. coolaminica</i>	N.S.W., Coolamine Plains	KAF & AMP	10/03	1/01/03	CHR 489419
<i>C. coolaminica</i>	Tasmania, Mt. Rufus	KAF & AMP	25/03	25/01/03	CHR 565524
<i>C. costiniana</i>	N.S.W., Mt. Kosciuszko	KAF & AMP	6/02	30/12/02	CHR 489414
<i>C. glabrata</i>	Tasmania, Ben Lomond National Park	KAF & AMP	17/03	17/01/03	CHR 565516
<i>C. glauca</i> s.s.	Tasmania, Eaglehawk Neck	KAF & AMP	21/03	15/01/03	CHR 565520
<i>C. haplorrhiza</i>	N.S.W., NW Plains, 16.7 km N of Namoi River bridge	R.G. Coveny	12650	21/08/87	MEL 235247
<i>C. lamicola</i>	N.S.W. Mt. Kosciuszko	KAF & AMP	7/02	30/12/02	CHR 489422
<i>C. leucantha</i>	N.S.W. Mt. Kosciuszko	KAF & AMP	11/03	2/01/03	CHR 489421
<i>C. maxgrayi</i>	N.S.W. Mt. Kosciuszko	KAF & AMP	4/02	30/12/02	CHR 489415
<i>C. paludicola</i>	Victoria, near Gisborne, Caulder HWY	KAF & AMP	1/02	27/12/02	MEL 2172915
<i>C. preminghana</i>	cult. Royal Tasmanian Botanical Gardens	KAF & AMP	19/03	13/01/03	HO 441463
<i>C. variabilis</i> (NSW)	N.S.W., Kosciuszko alpine HWY	KAF & AMP	3/02	29/12/02	CHR 489420
<i>C. variabilis</i> (Vict)	Victoria, Grampians, 4 km N of Zumsteins	S. Parfett	270	09/09/01	MEL 2116666
<i>C. "Tunbridge"</i>	Tasmania, Campbell Town, Golf course	KAF & AMP	27/03	8/02/03	CHR 489423
<i>Pycnosorus chrysanthes</i> *	Victoria, Kamarooka State Park	KAF & AMP	2/02	28/12/02	MEL 2172916
<i>P. globosus</i>	N.S.W., between Hie Hie and Narrabri				CBG 9313737
	NEW ZEALAND				
<i>C. incana</i>	Canterbury, Porters Pass	KAF	39/03	6/04/03	CHR 489426
<i>C. minor</i>	Marlborough, Waima River	KAF	2/WA	15/11/97	CHR 515394
<i>C. viscosa</i>	Wairarapa, Aorangi Range	IB	2055	11/12/97	CHR 516289

<i>C. robusta</i> var. <i>robusta</i>	Fiordland, Lake Wapiti	KAF	118/98	12/04/98	CHR 516277
<i>C. uniflora</i> var. <i>uniflora</i>	Marlborough, Port Underwood	B. Molloy		18/03/98	CHR 516278
<i>C. uniflora</i> var. <i>subhispida</i>	Campbell Island, Mt. Honey	C.D. Meurk		23/12/98	CHR 537470
<i>C.</i> “Anglem”	Stewart Island, Mt. Anglem	D. Glenney	6353	1/02/96	CHR 509876
<i>C.</i> “Chatham”	Chatham Islands	D. Glenney	7028	5/12/97	CHR 530699
<i>C.</i> “elongata”	Canterbury, Lake Tennyson	IB	2033	13/03/96	CHR 514376
<i>C.</i> “elongata”*	N.W. Nelson, Marino Mtns	KAF	46/98	16/02/98	CHR 515916
<i>C.</i> “Garibaldi	N.W. Nelson, Garibaldi Ridge	IB	2104	4/03/98	CHR 516320
<i>C.</i> “incana Marlborough”	Marlborough, below Barefell Pass	IB & R. Vogt	1091	9/12/95	CHR 514277
<i>C.</i> “incana Otago”	Otago, Old Man Range	G. Bawden		Feb/96	CHR 514269
<i>C.</i> “Kaitorete”	Canterbury, Kaitorete Spit	IB	2036	1997	-
<i>Helichrysum lanceolatum</i>	Auckland, Waitakere Range, Piha	R. Smissen	Q11418719	13/12/02	CHR 569869
<i>Stuartina muelleri</i>	Canterbury, Banks Peninsula, Port Hills	KAF	42/03	25/05/03	CHR 489452

An asterisk (*) indicates a sample sequenced but not included in analyses, as it is identical or very similar to that of another accession.

KAF & AMP indicates K.A. Ford & A.M. Purves; IB indicates I. Breitwieser.

3.2 Extraction and amplification of DNA

3.2.1 DNA extraction

Extraction of DNA was carried out using young leaf material from either fresh leaves, silica gel dried leaves or herbarium specimens, using a Qiagen Dneasy plant mini kit following the manufacturer's recommendations (see appendix 1 for protocol). Some modification was required for a few samples that were difficult to amplify. In these cases a phenol/chloroform procedure was implemented to clean up extractions and resulted in successful amplification (Sambrook et al. 1989).

3.2.2 Amplification of DNA using the Polymerase Chain Reaction (PCR)

PCR was carried out on the three regions of DNA and sequenced with Dye Terminator Cycle Sequencing chemistry. The following primers were used to amplify the target DNA regions used in the study.

Nuclear DNA primers:

ITS (ribosomal DNA intergenic spacers ITS1 & ITS 2 and the intervening rDNA gene 5.8S)

forward ITS 5' GGAAGTAAAAGTCGTAACAAGG 3' (White et al. 1990)

reverse ITS 4' 5' TCCTCCGCTTATTGATATGC 3' (White et al. 1990)

ETS (3' end of the external transcribed spacer of rDNA)

forward ETS-1 5' CGCATCGTTCGGTGCATTCTGGC 3' (Bayer et al. 2002)

reverse 18S-IGS 5' GAGACAAGCATATGACTACTGGCAGGATCAACCAG (Baldwin et al. 1998).

Chloroplast DNA primers:

psbA-trnH (intergenic spacer in cpDNA)

forward *psbA* 5' GTTATGCATGAACGTAATGCTC 3' (Sang 1997)

reverse *trnH* 5' CGCGCATGGTGGATTCACAAATC 3' (Sang 1997)

PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences (Mullis & Faloona 1987). The procedure uses two oligonucleotide primers that hybridise two opposite strands and flank the region of interest in the target DNA. An exponential increase of the target DNA fragment results from a repetitive series of cycles involving template denaturation, primer annealing and the extension of annealed primers by DNA polymerase.

A Qiagen *Taq* DNA polymerase kit was used to amplify dsDNA. For a single 20 μ l reaction mix the following was used: 8.3 μ l of sterile water; 2.0 μ l 10x PCR buffer (1.5MM MgCl₂ in 2.0 μ l rxn); 2.5 μ l 2M dNTPs (deoxynucleosidetriphosphates) in equimolar ratio; 4.0 μ l 5xQ buffer; 1.0 μ l of each 10pM primer; 0.2 μ l of *Taq* DNA polymerase (5units/ μ L) and 1 μ l of genomic DNA. In general 10x dilution of the extraction product was used as template DNA. Further dilutions 100x, 500x and 1000x often resulted in successful amplification after failure of 10x dilutions. Negative controls were included in each set of amplications.

The thermal reactions were carried out for all three markers as follows:

1. 94°C 3min – denature DNA
2. 94°C 30sec
3. 48°C 30sec – annealing
4. 72°C 1min – *Taq* pol. extension
5. 72°C 1min – final completion phase
6. 4°C on hold

Cycle steps 2. to 4. for 35 times (c. 2.0hrs).

Prior to sequencing, the PCR product was purified (to get rid of excess primers, dNTPs and salts) using an exonuclease I/shrimp alkaline phosphatase digest (SAP). For a single reaction the following was added: 1 μ l SAP (1.0unit/ μ L) and 1 μ l Exol (10units/ μ L). The thermal reaction was incubated at 37°C or 30 min then the enzymes denatured at 80°C for 15min.

3.3 Cycle sequencing

Both strands of the PCR products were cycle sequenced, using ABI Big Dye 3.1 chemistry (20 μ l (1/4) reaction). The primers used for the amplification were also used for the sequencing reactions plus primers ITS2 and ITS3.

ITS 2 = 5' gCTgCgTTCTTCATCgATgC 3' (White et al. 1990)

ITS 3 = 5' gCATCgATgAAgAACgCAgC 3' (White et al. 1990)

The following reaction mix was used: 3.0 μ l 5x sequence buffer; Big 3.0 μ l Big Dye Terminator v3.1; 3.2 μ l (3.2pM) of primer; between 2.0 and 5 μ l of DNA template, and sterile water to make up a final volume of 20 μ l.

The thermal reactions were carried out for all three markers as follows:

1. 96°C 10sec
2. 50°C 5sec
3. 60°C 4min

cycle steps 1. to 3. 27 times.

3.4 Precipitation (purifying extension products)

The labelled DNA samples were subsequently purified by ethanol precipitation. To each tube was added 2 μ l 3M sodium acetate pH5.2 and 50 μ l of chilled 95% ethanol. The tubes were mixed gently and then centrifuged at 13000rpm for 30 minutes. The 95% ethanol was then removed using an automatic pipette. The process was repeated using 125 μ l of chilled 70% ethanol and centrifuging at 13000rpm for 10 minutes. In this final step, as much ethanol as possible was removed from each tube using an automatic pipette, taking care not to disturb the DNA pellets. The tubes were left inverted and uncapped to air dry overnight. In the morning the tubes were capped and sent for sequencing by courier to the Allan Wilson Centre Genome Service at Massey University. The products were separated on an ABI 3731 genetic analyser.

3.5 Sequence editing

Sequence fragments were edited and a consensus sequence assembled with the aid of Sequencher 3.0 software (Gene Codes, Ann Arbor, Michigan, USA). Boundaries of the spacer regions were determined from published studies (ITS, Baldwin et al. 1995; ETS, Bayer et al. 2002 & *psbA-trnH*, Sang et al. 1997). Sequencher allows the visual comparison of multiple fragments of DNA resulting from amplification using different primer combinations. Sometimes a base is ambiguous on one sequence but not the other, and a consensus from a number of fragments can be used for the final single DNA sequence used in analyses.

3.6 Characters, homology and sequence alignments

Characters (morphological, anatomical, nucleotides, etc.) are generally interpreted as *independent* variables whose values (states) are mutually exclusive. DNA sequences are made up of the base residues A (adenine), T (thymine), C (cytosine), and G (guanine). For maximum parsimony and maximum likelihood analyses, base residues are treated as discrete unordered multistate characters.

Homology of characters is fundamental to phylogenetic inference. This term was first defined by Richard Owen (1843) a British palaeontologist, as the "same organ under every variety of form and function". A more contemporary and precise interpretation, applied to molecular sequence data, would be that two nucleotides are homologous if they are descended from a common ancestral nucleotide. Non-random patterns in homologous character data are due to inheritance of modifications and allow recovery of evolutionarily related groups. For sequence data, positional homology is inferred from similarity between sequences, manually or by an alignment program using an algorithm.

In this study the program Clustal X version 1.8 (Thompson et al.1997) was used to align sequences. Alignment programs use an algorithm designed to maximise percent base sequence *similarity* while minimising the number of gap events. Because the sequences differed in length, gaps were placed to obtain positional homology of characters. Alignments were then checked manually.

Clustal creates a multiple alignment in four steps:

- 1) aligns each sequence to each other sequence in a series of pairwise alignments;
- 2) creates a distance matrix from pairwise alignments;
- 3) uses the distance matrix to create a neighbour-joining guide tree;
- 4) uses the guide tree based on pairwise similarity to add sequences progressively to the alignment.

The neighbour-joining guide tree is rooted at the midpoint of the longest path through the tree to determine the direction of evolution, and the sequences are progressively added to the alignment matrix. The program algorithm starts with the two most similar sequences (which are fixed in relation to one another) and then adds the next most similar sequence(s) etc... Simultaneous multiple alignment (using dynamic programming) is a possibility but is too computationally intensive to be generally used (Swofford et al. 1996). The Clustal progressive pairwise method is a heuristic alternative.

3.7 Phylogenetic analyses

Inferences of the phylogeny used three different methods — parsimony, maximum likelihood as it is implemented by PAUP version 4.0b10 (Swofford 2002) and Splitstree 4.0b5 (Huson & Bryant 2004).

Gaps (-) and ambiguous character states (which were coded as a '?') were treated as missing data. For parsimony analyses informative indels were coded as binary. An informative indel is one that is shared by more than one taxon. Informative indels were not able to be included in maximum likelihood analyses, as they do not conform to the assumptions of the sequence evolution model implemented by the program.

Heuristic search strategies were employed in all parsimony and maximum likelihood analyses as implemented by PAUP version 4.0b10 (Swofford 2002), as the size of the data set (35 samples) made exhaustive searches prohibitive due to the enormous amount of computing time required. Heuristic searches do not examine and score all the trees, thus

speeding up computing time. They attempt to avoid looking at tree topologies that are suboptimal, but are prone to falling into “local optima”, this being particularly true of stepwise addition and star decomposition, two heuristic search methods known as greedy algorithms. Branch swapping algorithms are less prone to local optima as they try random branch-swaps. It should be noted therefore that heuristic searches do not guarantee that the globally optimal tree will be found.

Whether the ITS, ETS and *psbA-trnH* sequences could be combined into a single data set was assessed using the partition-homogeneity test (Farris et al., 1994,) implemented by PAUP version 4.0b10 (Swofford 2002). This tests for significant incongruence between data sets.

For parsimony analyses all characters were equally weighted. Data sets were analysed by the Wagner parsimony optimality criterion using the heuristic search option of PAUP 4.0b10 (Swofford 2002), with ‘collapse-maximum-branch-length-if-zero’ option. Starting sub-optimal neighbour-joining trees were obtained via stepwise addition using uncorrected p-distances, and the searches for optimal trees were carried out under the parsimony criterion by the branch-swapping algorithm TBR (tree-bisection-reconnection). DELTRAN character optimisation was used instead of ACCTTRAN, which in this version of PAUP* sometimes reports incorrect branch lengths for trees rooted using an outgroup (see <http://paup.csit.fsu.edu/problems.html>).

Maximum likelihood analyses were analysed using options available in PAUP 4.0b10 (Swofford 2002). Each data set was analysed under a simple and a complex substitution model in order to test consistency and explore possible systematic error due to violations of the assumptions made by these models. The substitution models Jukes-Cantor (Jukes & Cantor 1969) and general time reversible (Lanave et al. 1984, Rodriguez et al. 1990) were chosen. Jukes-Cantor, the simple substitution model, assumes only that the bases occur with equal frequency (0.25). GTR, the most complex model, takes into account unequal base frequencies and different substitution rates among bases. An extra parameter ‘gamma’, which takes into account site-to-site rate variation, was added to GTR.

The rooting of trees was determined via outgroup comparison. The outgroup taxa are

considered to reflect the ancestral states of the characters, and as a result, the characters of the ingroup become polarised and the eventual monophyly of (parts of) the ingroup can be determined.

The program Splitstree 4.0b5 (Huson & Bryant 2004) was used to analyse and represent the data under the splits decomposition method (Bandelt & Dress 1992; Huson 1998, Lockhart et al. 2001) implemented under a parsimony criterion. The primary purpose for using Splitstree was to test how well the data conformed to a tree-like model and to investigate conflicting signal. Bootstrap analysis was used to identify internal splits with high support.

3.8 Testing the reliability of inferred phylogenetic trees

3.8.1 PTP test (permutation tail probability)

The PTP is a test for phylogenetic signal (Archie 1989) and measures whether the data is significantly non-random, i.e., greater than that would be expected from data generated randomly. A permutation is done by reordering the data and destroying any correlation among characters to that expected by chance alone. A permutation tail probability is the proportion of data sets with as good or better measure than the real data. In this study, 100 random permutations were carried out for each of the three data sets.

3.8.2 Measure of fit between trees and data

Measures of fit between trees and data include the consistency index (CI). The CI is the minimum number of steps needed to represent the data divided by the actual number of steps. This measure can also be used excluding autapomorphies (character changes that are unique for only one individual). The CI is negatively correlated to the number of taxa and the number of characters. The RI (Retention index) is considered to be a better measure of evidential support for groups than the CI. This measure can be thought of as the proportion of similarities on a tree that are interpreted as synapomorphies (Farris 1989). The RC (rescaled consistency index) was used in this study as it excludes autapomorphies and incorporates the RI.

3.8.3 Measure of branch support

The statistical support for each internal branch of the tree can be estimated by bootstrap (Felsenstein 1985). The 'bootstrap' commonly applied to phylogenetic analysis is a non-parametric resampling and re-analysis of the data with replacement many times (x100 or x1000) using the same tree-building method as the original analysis (Swofford et al. 1996).

Bootstrapping is a measure of how well the data supports internal tree branches. The percentage of times a given internal branch is recovered in the trees built from the pseudosample data sets is termed the bootstrap support (BS) for that branch. The results are depicted in a consensus tree in which only branches of at least 50% BS are represented. Consequently, these consensus trees are not necessarily fully resolved (where internal branches are supported with < 50% BS), with portions of the tree collapsed into polytomies.

A low bootstrap value can be caused by either a low number of supporting characters in the original dataset such that in pseudosamples they are missed or because conflicting signal in the data supports alternative bifurcating topologies.

In this study bootstraps searches were performed with 100 replicates using a HEURISTIC search strategy, SIMPLE addition sequence of the taxa and TBR branch swapping with a MAXTREE limit of 100 per replicate. Only clades with at least 50% frequency were retained in trees (50% majority rule) and a clade is considered to be well supported with a bootstrap value of 95% or higher.

3.9 Dating the Australian/New Zealand divergence

A calibration of pairwise sequence divergences with absolute time was made by comparison of ITS sequences from *Craspedia* with those from the Juan Fernandez Islands genera *Dendroseris* and *Robinsonia* (Compositae) (Sang et al. 1994, 1995). By using an age of 4 MY for the Juan Fernandez Islands, Sang et al. (1994, 1995) estimated evolutionary rates for *Dendroseris* and *Robinsonia* of, 3.94×10^{-9} and 7.83×10^{-9} substitutions per site per year respectively. Applied to *Craspedia* these substitution rates gave an estimated divergence time between New Zealand and Australian *Craspedia* of

325,000 to 644,000 thousand years. These figures are based on the smallest pairwise sequence divergence between New Zealand and Australian *Craspedia*, i.e., 0.00508 between *C.* “Garibaldi” and *C. aurantia* (NSW) and *C. variabilis* (Vict).

CHAPTER 4

Results

Sequences of ITS, ETS and *psbA-trnH* were obtained for a total of 32 species and undescribed entities belonging to *Craspedia* (ingroup) and species of *Helichrysum*, *Pycnosorus* and *Stuartina* (outgroup taxa).

4.1 Alignments

4.1.1 ITS non-coding spacers (ITS 1 & ITS 2) plus the 5.8S gene

The ITS data set (including outgroup) comprised 642 characters. Alignment of the ingroup sequences required 24 indels, involving single or double base pairs. There are only three informative indels and they were included in the parsimony analysis coded as binary characters. Sequence length varied from 623 bp to 640 bp. Sequence length in *Craspedia* varied from 635 bp (*C. alpina* & *C. haplorrhiza*) to 638 bp (all N.Z. samples). All samples of Australian *Craspedia* with the exception of *C. alpina* and *C. haplorrhiza* were 637 bp in length. The pairwise sequence divergence within *Craspedia* ranged from 0 to 3.6 % and the mean sequence divergence was 1.0%. Table 3 gives a summary of pairwise sequence divergence for all data sets and main clades.

4.1.2 ETS non-coding spacer

The ETS data set (including outgroup) comprised 508 characters. Alignment of the ingroup sequences required 9 single base pair indels. The only three informative indels were included in the parsimony analysis coded as binary characters. Sequence length varied from 498 bp to 501. Sequence length in *Craspedia* varied from 498 bp to 501 bp. The pairwise sequence divergence within the ingroup ranged from 0 to 6.4% and the mean sequence divergence was 2.5% (Table 3).

4.1.3 *psbA-trnH* non-coding spacer

The *psbA-trnH* data set comprises 579 characters (not including 24 characters deleted from the analysis, bp 513-537, see Appendix 3 for alignment). This data set has a low amount of variation, high A-T base composition, and four regions consisting of perfect and imperfect repeats which appear to be hotspots for indel events. Alignment of the ingroup sequences required 26 indels ranging from 1 to 130 bp in length. The sequence length varied from 337 bp in *C. haplorrhiza* to 550 bp in *C. minor* (a difference of 213 bp). The pairwise sequence divergence within the ingroup ranged from 0 to 2.0% and the mean pairwise sequence divergence was 0.57% (Table 3).

Considerable manual adjustment was required to align the *psbA-trnH* sequences. This was due to A/T rich repeats. The Clustal program (Thompson et al. 1997) does not cope well with repeats, especially when there is a considerable difference in base pair composition as is the case with this data set. In manually adjusting the alignment, a conservative approach was taken, meaning that the creation of synapomorphies was avoided if at all possible. Three regions in the *psbA-trnH* region had to be aligned manually by eye, base positions 166-190 (24 bps), 446-493 (47 bps) and 513-537 (24 bps). The last section (513-537) could not be aligned with confidence and has been excluded from the analyses.

The outgroup was reduced to *Helichrysum lanceolatum* and *Stuartina muelleri* as *Pycnosorus globosus* was excluded from the data set because of lack of data. Six large indels are present in the *psbA-trnH* region of *P. globosus* resulting in a large difference in sequence length compared with the rest of the data set. The sequence length of *P. globosus* is 283 bp — the next longest sequence length is *C. haplorrhiza* with 337 bp and all other *Craspedia* are 488 bp or higher (a difference of 205 or more bp). The difference in sequence length between *Pycnosorus* and the ingroup can easily be detected on 1.5% agarose gel (Fig. 9B). The largest indel in *P. globosus* is 105 bp from position 301 to 406 and the average indel length is 47 bp.

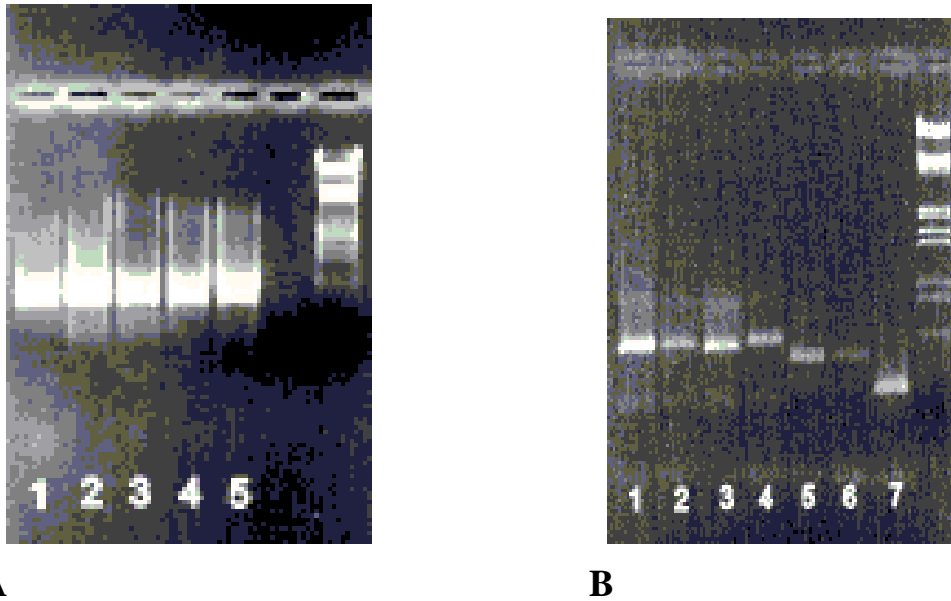


Figure 9. A. Amplification of ITS visualised on 1.5% agarose gel, five samples and negative control. **B.** Amplification of *psbA-trnH*, note smaller fragment sizes of *Stuartina muelleri* lanes in 5 & 6 and *Pycnosorus globosus* in lane 7 compared with samples of *Craspedia* in lanes 1, 2, 3, &4.

Although indels were not included in the *psbA-trnH* analyses the following observations were made. Three informative single base pair indels are congruent with the substitution data, with two supporting the monophyly of *Craspedia* (one insertion and one deletion) and the other (a deletion) supporting the monophyly of New Zealand *Craspedia* plus *C. leucantha* (from Australia). In contrast the four sites of indels of various lengths are incongruent with the substitution data. Two sites in particular appear to be insertion hot spots (based on outgroup comparison). At these sites insertions are composed of imperfect nearly pure A/T repeats.

A similar pattern was found in a study of *Taraxacum* (Mes et al. 2000), in non-coding chloroplast DNA (including *psbA-trnH*) where variable lengths of repeated segments were found to be homoplasious in relation to substitutions, but single base pair indels were congruent. The authors hypothesised that indel events were caused by two different processes: the large indels were inverted repeats associated with stabilised hairpin structures, whereas the single base pair indels were single sequence repeats caused by replication slippage. On this basis I decided not to include any indels in the analyses of the *psbA-trnH* data set.

	ITS	ETS	<i>psbA-trnH</i>
<i>Craspedia</i> + outgroup	2.14	3.85	0.85
<i>Craspedia</i> (ingroup)	1.00	2.53	0.57
<i>Craspedia</i> (ingrp.) excl. <i>C. haplorrhiza</i>	0.84	2.33	-
AUS 1 (incl. <i>C. haplorrhiza</i>)	0.72	1.27	-
AUS 1 (excl. <i>C. haplorrhiza</i>)	0.26	0.37	-
AUS 2	0.42	1.25	-
NZ	0.19	0.19	0.16

Table 3. Summary of % mean pairwise sequence divergence for each data set and main clades.

4.2 Phylogenetic analyses

In all analyses *Craspedia* (the ingroup) is monophyletic, supported by a bootstrap of 100%. All heuristic searches ran to completion. Values from each bootstrap analysis are plotted onto the corresponding strict consensus tree.

	ITS	ETS	ITS/ETS	<i>psbA-trnH</i>
aligned length	644	507	1151	581
no. constant characters	511	369	876	542
no. of parsimony uninform. chars.	55	62	121	21
no. of parsimony inform. chars.	78	76	154	17
% parsimony inform. chars.	12	15	13	2.9
% GC content	48	44	46	22

Table 4. Sequence characteristics for ITS, ETS, and *psbA-trnH* sequences (incl. outgroup taxa).

4.2.1 ITS non-coding spacers

The Permutation test under a parsimony criterion suggests that there is considerable non-random structure in the ITS spacers ($P = 0.01$). This indicates there is significant phylogenetic signal in the data.

The parsimony analysis conducted on the data set with a heuristic search found 105,511 equally parsimonious shortest trees with a best score of 172. After collapsing branches if minimum length is zero and discarding duplicates from the original 105,511 trees, there were 612 distinct trees from which a strict consensus tree and a 50% majority rule bootstrap were calculated (Fig. 10). Rescaled consistency index (RC) = 0.838 (CI=0.901; RI=0.930; CI excluding uninformative characters = 0.848).

Four clades of *Craspedia* were recognised (Fig. 10) in a strict consensus tree, although the order of divergence was unresolved, resulting in a polytomy. Three of the clades are Australian and the fourth is composed of all the New Zealand samples. Two Australian clades (AUS 1 and AUS 2) contain the majority of Australian species, and the third is monotypic containing *C. haplorrhiza* on a long branch. The three main clades (AUS 1, AUS 2 & NZ) are supported by bootstrap values of 72%, 89% and 88% respectively.

A maximum likelihood analysis, (with GTR + gamma model) using a heuristic search, found 102 trees with a likelihood score of 1865.55. A strict consensus and a 50% majority rule bootstrap tree were calculated (Fig. 11). Bootstrap values for AUS 2 and NZ clades are the same as the parsimony bootstrap values, whereas the bootstrap value for AUS 1 is significantly lower than the parsimony value, i.e., 53% compared with 72% (Figs. 10 & 11). Trees with Jukes-Cantor and GTR models were identical in topology to the tree under GTR+G. The topologies of the parsimony and maximum likelihood strict consensus trees are congruent with the exception of *C. haplorrhiza*. In the parsimony tree, *C. haplorrhiza* is a separate lineage with AUS 1, AUS 2 and NZ in an unresolved polytomy, whereas in the maximum likelihood tree, *C. haplorrhiza* is placed as sister to the AUS 1 clade. The bootstrap value for this clade is low at 53%.

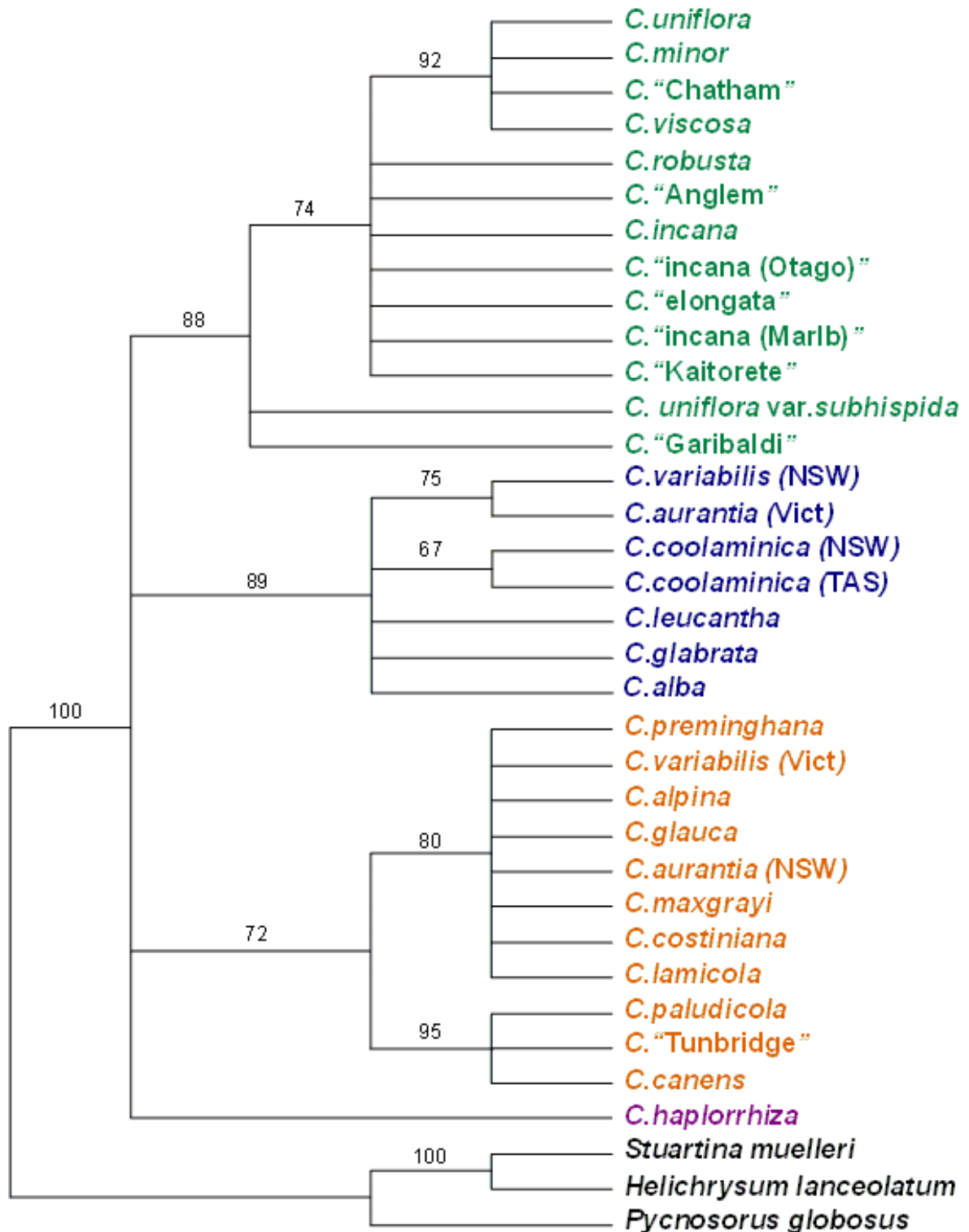


Figure 10. ITS parsimony strict consensus tree with bootstrap values above lines. Main clades are: AUS 1 = ORANGE; AUS 2 = BLUE; NZ = GREEN & *C. haplorrhiza* = PURPLE.

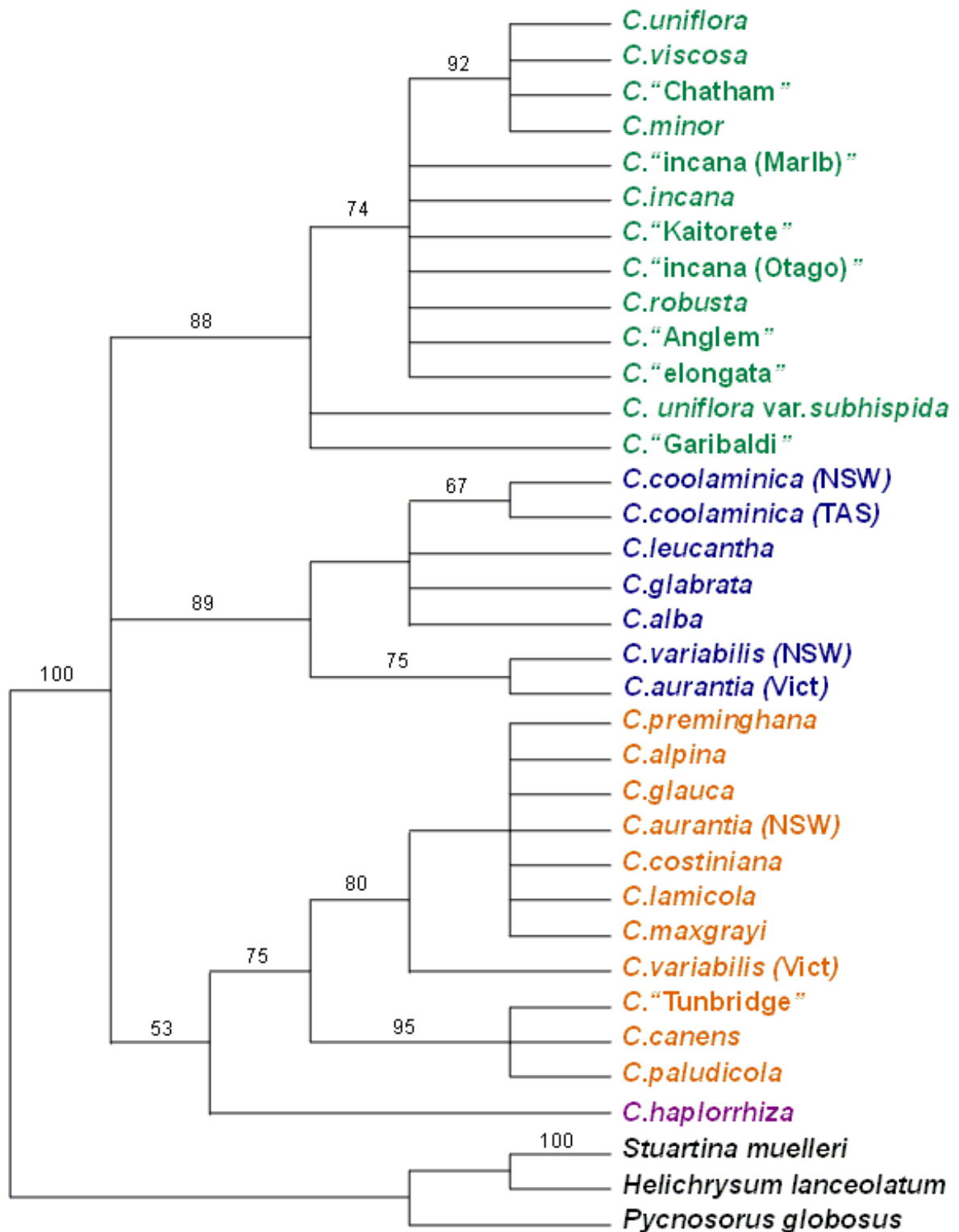


Figure 11. ITS maximum likelihood (GTR+G) strict consensus tree with bootstrap values above lines. Main clades are: AUS 1 = ORANGE; AUS 2 = BLUE; NZ = GREEN & *C. haplorrhiza* = PURPLE.

4.2.2 ETS non-coding spacer

The Permutation test under a parsimony criterion suggests that there is considerable non-random structure in the ETS spacer ($P = 0.01$).

The parsimony analysis conducted on the data set with a heuristic search found 48,682 equally parsimonious shortest trees with a best score of 183. After collapsing branches if minimum length is zero and discarding duplicates from the original 48,682 trees, there were 2 distinct trees from which a strict consensus and a 50% majority rule bootstrap were calculated (Fig. 12). Rescaled consistency index (RC) = 0.791 (CI=0.864; RI=0.915; CI excluding uninformative characters = 0.774).

The four clades in the ITS strict consensus tree are also present in the ETS strict consensus. However, unlike the ITS parsimony and maximum likelihood trees, the order of divergence is resolved. The AUS 1 clade is sister to the AUS 2/NZ clade. *C. haplorrhiza* is sister to the rest of *Craspedia*. Bootstrap values are higher than the ITS values for the three main clades AUS 1, AUS 2 and NZ— 95%, 99% and 93% respectively. The bootstrap value for the AUS 2/NZ clade is moderately high at 91%. The *Craspedia* clade (minus *C. haplorrhiza*) is not well supported (70% bootstrap support).

The maximum likelihood analysis using the GTR+G model with a heuristic search found one tree with a likelihood score of 1682.12. (Fig. 13). Bootstrap values in general are slightly lower than for the parsimony tree. The topology of the likelihood and parsimony trees is congruent with the exception of the placement of *Craspedia haplorrhiza*. In the parsimony tree *C. haplorrhiza* is sister to the rest of *Craspedia*, whereas in the maximum likelihood tree it is sister to the AUS 2/NZ clade. However the *C. haplorrhiza*/AUS 2/NZ clade is not well supported (60% bootstrap support). This result is also incongruent with ITS likelihood tree, which places *C. haplorrhiza* as sister to AUS 1. Maximum likelihood analysis with GTR (without gamma) produced a tree topology identical to that of GTR+G. However a Jukes-Cantor model produced a topology identical to that of the parsimony analysis. (A substitution rate could not be found for ETS.)

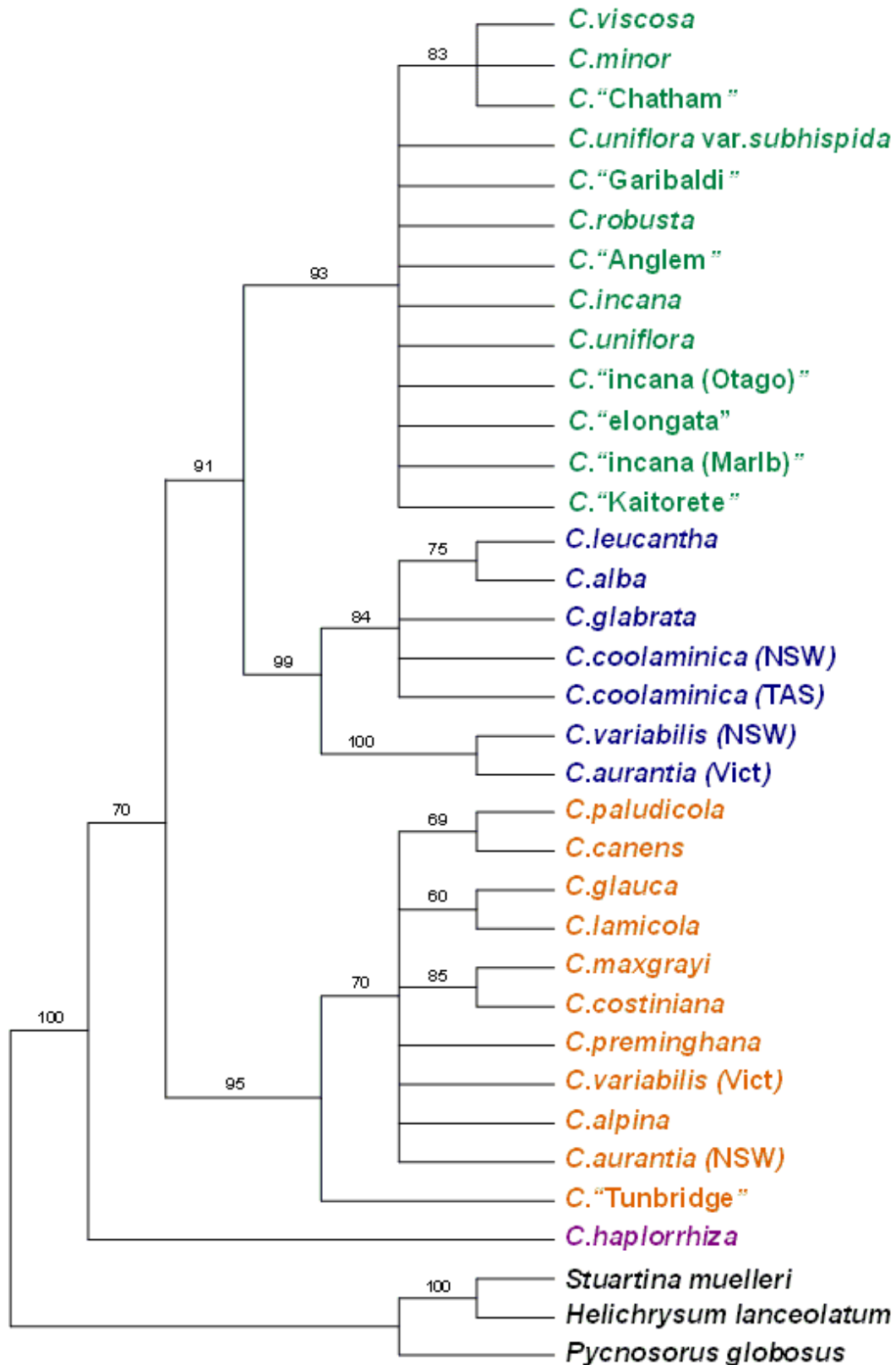


Figure 12. ETS parsimony strict consensus tree with bootstrap values above lines. Main clades are: AUS 1 = ORANGE; AUS 2 = BLUE; NZ = GREEN & *C. haplorrhiza* = PURPLE.

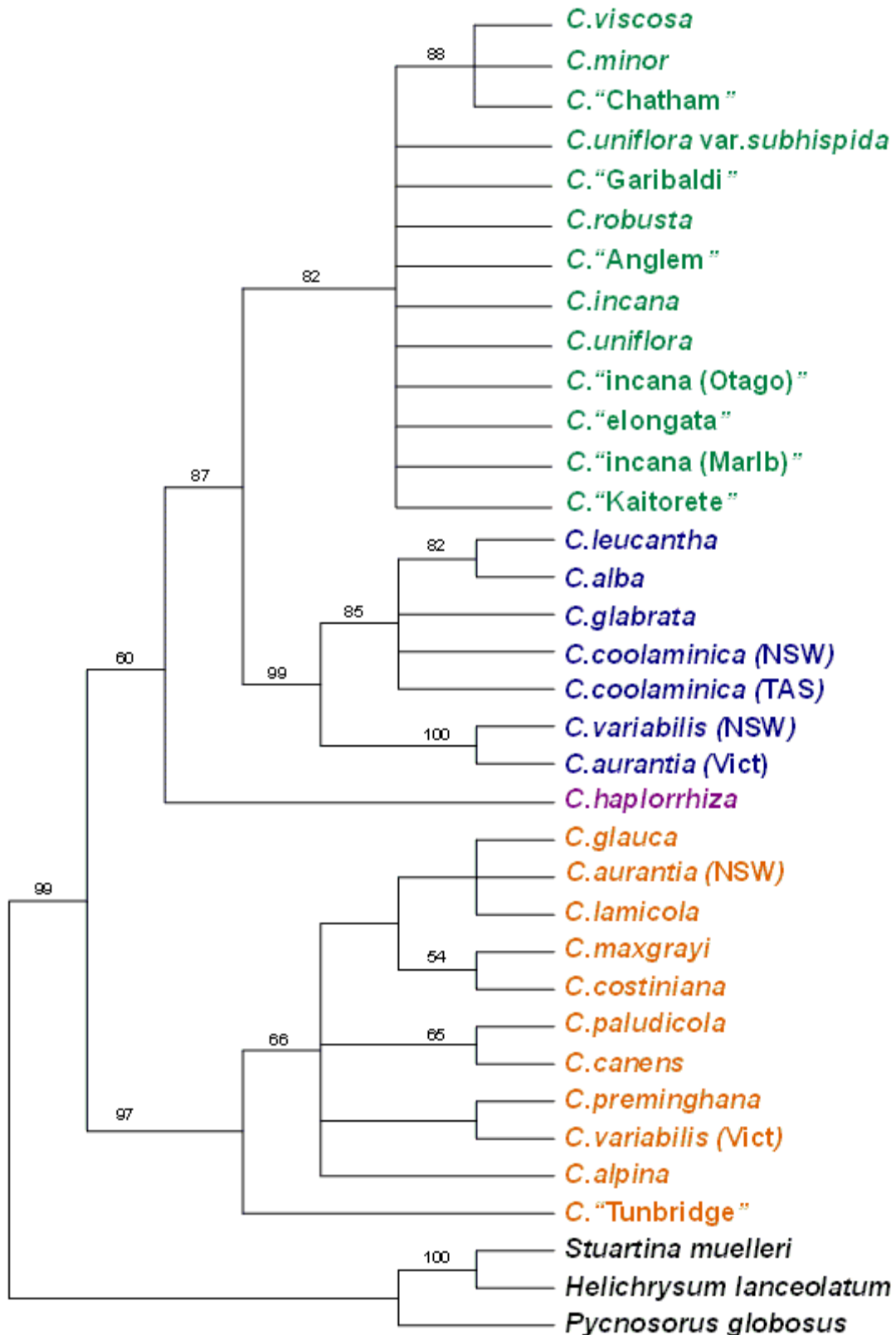


Figure 13. ETS maximum likelihood (GTR+G) strict consensus tree with bootstrap values above lines. Main clades are: AUS 1 = **ORANGE**; AUS 2 = **BLUE**; NZ = **GREEN** & *C. haplorrhiza* = **PURPLE**.

4.2.3 Combined ITS and ETS data sets

A partition-homogeneity test found that the ITS and ETS data sets were not significantly incongruent ($P = 0.5$) and therefore they were combined. The combined data set comprised a total of 1150 characters. Figure 14 shows the parsimony and maximum likelihood strict consensus trees for the combined ITS and ETS data set.

The parsimony analysis conducted on the ITS/ETS data set (incl. indels) with a heuristic search found 3992 equally parsimonious trees with a best score of 361. After collapsing branches if minimum length is zero and discarding duplicates from the original 3992 trees there were 17 distinct trees from which a strict consensus and 50% majority rule bootstrap were calculated. Rescaled consistency index = 0.801 (CI=0.872; RI=0.918; CI excluding uninformative characters =0.799).

Bootstrap values obtained for lineages in the combined ITS/ETS analysis are generally higher than those obtained from analysis of ITS or ETS data individually (Table 5). The three main lineages AUS 1, AUS 2 and NZ that are present in the ITS and ETS analyses are also present in the combined analysis and supported with higher bootstrap values — 99%, 100% and 100% respectively. The AUS 2/NZ clade is also supported by a higher bootstrap value, 96%. The ITS/ETS strict consensus tree is better resolved than either ITS or ETS individually.

Craspedia haplorrhiza increases homoplasy in both the individual ITS and ETS analyses, and rescaled consistency indices and bootstrap values increase with its exclusion. An increase in bootstrap support is particularly noticeable for the AUS 1/AUS 2/NZ clade in the ETS analyses and the AUS 1 and AUS 2 clades in the ITS analyses. However, the effect is less pronounced with the combined ITS and ETS data set. Table 5 shows a comparison of rescaled consistency indices and bootstrap values for parsimony analyses, with and without *C. haplorrhiza*.

	RC	bootstrap AUS 1	bootstrap AUS 2	bootstrap NZ	bootstrap AUS 2/NZ	bootstrap AUS 1, 2 & NZ
ETS	0.791	95	99	93	91	70
ETS (minus <i>C.haplorrhiza</i>)	0.825	98	99	96	94	100
ITS	0.838	72	89	88	-	100
ITS (minus <i>C.haplorrhiza</i>)	0.857	82	97	85	<50	100
comb.	0.801	99	100	100	96	100
comb. (minus <i>C.haplorrhiza</i>)	0.808	100	100	99	98	100

Table 5. Summary of rescaled consistency indices for ITS, ETS and *psbA-trnH* parsimony analysis and bootstrap values for major clades with and without *Craspedia haplorrhiza*.

The maximum likelihood analysis using a GTR+G model with a heuristic search found 6 equally likely trees with a best score of 3623.06. A strict consensus and a 50% majority rule bootstrap were calculated (Fig. 14). The strict consensus tree is highly congruent with that from the parsimony analysis but with the exception of *C. haplorrhiza*, which is placed as in the ITS maximum likelihood tree, sister to the AUS 1 clade. This group is not supported in the bootstrap analysis and collapses, i.e., it has below 50% bootstrap support. The other two main clades, AUS 2 and NZ, have high bootstrap support, 100% and 95% respectively, comparable to the parsimony bootstrap values. The AUS 2/NZ clade has 87% bootstrap support, which is lower than in the parsimony analysis. Maximum likelihood analysis with GTR (without gamma) produced a tree topology no different to that of GTR+G. However a Jukes-Cantor model produced a topology identical to that of the parsimony.

4.2.4 Further resolution within the three main *Craspedia* clades

Within the three main clades there is further support for phylogenetic relationships, particularly within the two Australian clades, AUS 1 and AUS 2, and to a lesser extent in the New Zealand clade (Fig. 14).

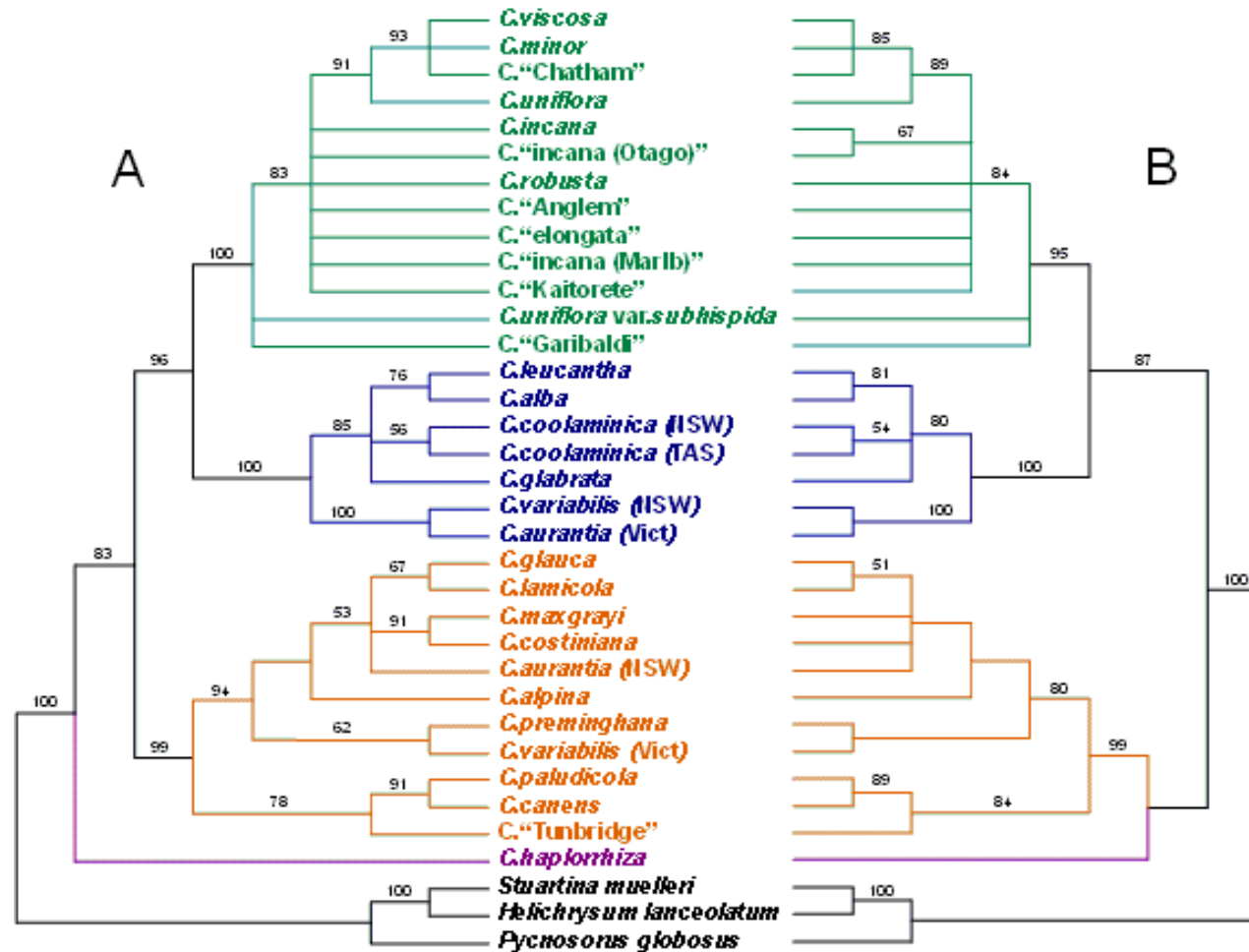


Figure 14. ETS & ITS combined A Parsimony strict consensus tree. B Maximum likelihood (GTR+G) strict consensus tree. Main clades are: AUS 1 = **ORANGE**; AUS 2 = **BLUE**; NZ = **GREEN** & *C. haplorrhiza* = **PURPLE**. Bootstrap values above lines.

4.2.4.1 New Zealand clade

In the New Zealand clade the earliest split is an unresolved polytomy between the North West Nelson endemic *Craspedia* “Garibaldi”, the subantarctic Campbell Island species *C. uniflora* var. *subhispida* and the rest of New Zealand *Craspedia*. *Craspedia* “Chatham”, *C. minor*, *C. uniflora* and *C. viscosa* form a monophyletic group, which is nested within the main New Zealand *Craspedia* grade with bootstrap values of 91% and 89%. *C. uniflora* is sister to an unresolved polytomy consisting of *C.* “Chatham”, *C. minor* and *C. viscosa*. Relationships otherwise are unresolved, with the seven New Zealand *Craspedia* in the main grade forming a polytomy. *C. uniflora* is polyphyletic, as *C. uniflora* var. *subhispida* and *C. uniflora* var. *uniflora* do not share a direct common ancestor.

4.2.4.2 Australian clades

There are major splits supporting phylogenetic relationships within both the Australian lineages AUS 1 and AUS 2.

In the AUS 1 clade the first split is the lowland species *Craspedia canens*, *C. paludicola* and an unnamed Tasmanian entity *C.* “Tunbridge” from the rest of the AUS 1 clade. This lowland clade is supported by moderate bootstrap values in the parsimony and maximum likelihood analyses, 78% and 84% respectively. Most of the signal for this clade is coming from ITS region and in the ITS bootstrap analysis this clade has 95% support. The ETS supports *C.* “Tunbridge” as sister to the rest of the AUS 1 clade and *C. paludicola* and *C. canens* are weakly supported sister species nested in the AUS 1 clade.

The other side of the ITS/ETS AUS 1 split is better supported in the combined analysis, with bootstrap values of 94% and 80% (parsimony & ML respectively). The first split within this group is between lowland species *C. preminghana* and *C. variabilis* (Vict) and a group of almost entirely alpine species (the exception being *C. glauca* s.s). The alpine group is not supported (BS = <50%), and the Kosciuszko clade consisting of *C. aurantia* (NSW), *C. costiniana*, *C. maxgrayi*, *C. lamicola* plus *C. glauca* s.s. is weakly supported in the parsimony analysis (53%) and not supported in the maximum likelihood analysis (BS = <50%).

In the AUS 2 clade the main internal split is between *C. aurantia* (Vict) and *C. variabilis* (NSW), and *C. alba*, *C. coolaminica*, *C. glabrata* and *C. leucantha*. Both groups are well supported by high bootstrap values, particularly *C. aurantia* (Vict) and *C. variabilis* (NSW).

On the other side of the main AUS 2 split, *C. alba*, *C. glabrata* and *C. leucantha* are all alpine species with various restricted distributions, and *C. coolaminica* is a widespread montane/subalpine species of the mainland eastern highlands and Tasmania. The low support for the mainland and Tasmanian *C. coolaminica* is caused by conflicting signal in one character in the ETS data — a split supporting *C. coolaminica* (NSW) and *C. leucantha* vs. the rest of the clade. The sister species relationship between *C. alba* and *C. leucantha* is moderately supported, and is also affected by the homoplasy noted above. The relationship between these weakly supported clades is unresolved in the strict consensus trees.

The results indicate there are two polyphyletic (possibly paraphyletic) Australian species *C. aurantia* and *C. variabilis*, i.e., they both occur in the two main Australian clades.

4.2.5 *psbA-trnH* non-coding spacer

The Permutation test under a parsimony criterion suggests that there is considerable non-random structure in the *psbA-trnH* spacers (P=0.01).

The *psbA-trnH* and ITS/ETS data sets passed a partition-homogeneity test (P = 0.12) and therefore are not significantly incongruent. I did not, however, combine the *psbA-trnH* data set with those of ITS and ETS, on the basis that nuclear and chloroplast DNA are from different genomes.

The parsimony analysis (excl. indels) found with a heuristic search 28 equally parsimonious trees of 46 steps. (Fig. 15). Rescaled consistency index = 0.9638 (CI=0.9750; RI=0.9885; CI excluding uninformative characters =0.9474). A maximum likelihood analysis using GTR+G found 1 tree with a likelihood score of 993.43. The maximum likelihood analysis produced a tree identical to the parsimony tree, irrespective of model (JC or GTR) or the parameter gamma.

There are 14 *Craspedia* chloroplast haplotypes in the parsimony and maximum likelihood analyses. Eight of the 13 New Zealand accessions have an identical chloroplast haplotype and the other five have accumulated 1 or 2 apomorphic base changes. Twelve of the 19 Australian haplotypes are identical and the seven others have accumulated up to three apomorphic base changes. Although there is a large difference in sequence length, *C. haplorrhiza* comes out with the rest of *Craspedia*.

New Zealand *Craspedia* is a monophyletic group nested in Australian *Craspedia* and is derived from a common ancestral haplotype with the Australian species, *C. leucantha*. *Craspedia leucantha* is sister to New Zealand *Craspedia*. The New Zealand plus *C. leucantha* clade is supported by three base substitutions and one single bp indel (not incl. in analyses). The New Zealand clade is supported by three base substitutions. The rest of the Australian species form a paraphyletic polytomy. Similarly there is no resolution in relationships between the six New Zealand haplotypes.

4.2.6 Split decomposition analyses

Split decomposition of the *psbA-trnH* data set (excluding outgroup) is consistent with a bifurcating tree, with no incompatible splits in the data (Fig. 16).

However, there is conflict in the ITS and ETS data sets, as was suspected from alternative hypotheses in the placement of *C. haplorrhiza* between the parsimony and maximum likelihood trees, and in differences in bootstrap values and consistency indices with and without *C. haplorrhiza*. This conflict is displayed as boxes (incompatible splits) in the split graphs (Fig. 17 & 18.). The split graphs of both ITS and ETS with *C. haplorrhiza* are mostly consistent with a bifurcating tree model with some moderate amount of conflicting signal mostly caused by *C. haplorrhiza*, particularly in the ITS graph. When *C. haplorrhiza* is removed, both the ITS and ETS graphs are highly consistent with a bifurcating tree model. Only one conflicting split remains in the ETS split graph tree (Fig. 18B). This involves a split between *C. aurantia* (Vict), *C. variabilis* (NSW) and AUS 1 with the rest of AUS 2 and the NZ clade.

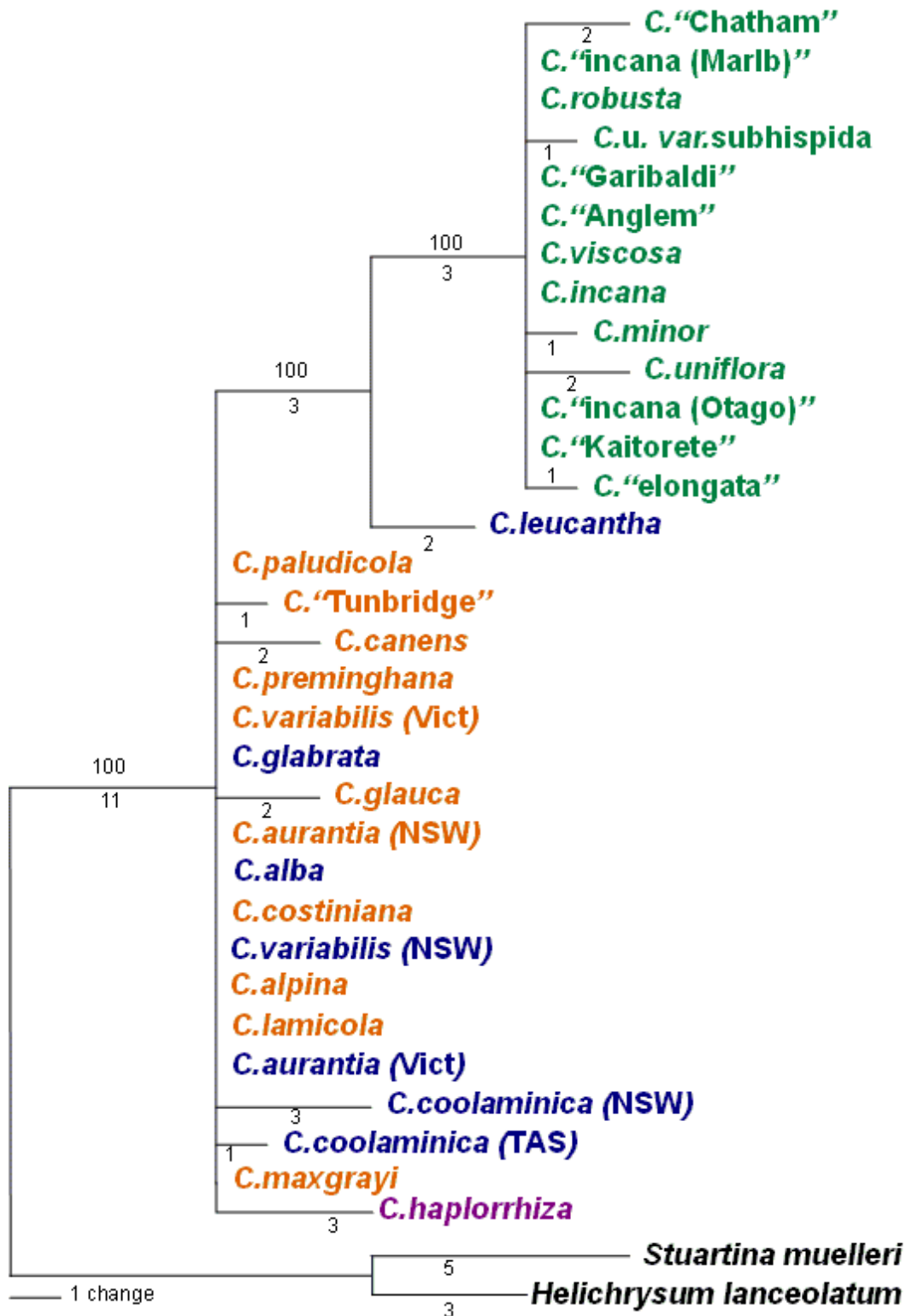


Figure 15. *psbA-trnH* parsimony tree 1. Colours represent the main clades distinguished by ITS & ETS: AUS 1 = ORANGE; AUS 2 = BLUE; NZ = GREEN & *C. haplorrhiza* = PURPLE. Bootstrap values above lines and branch lengths below.

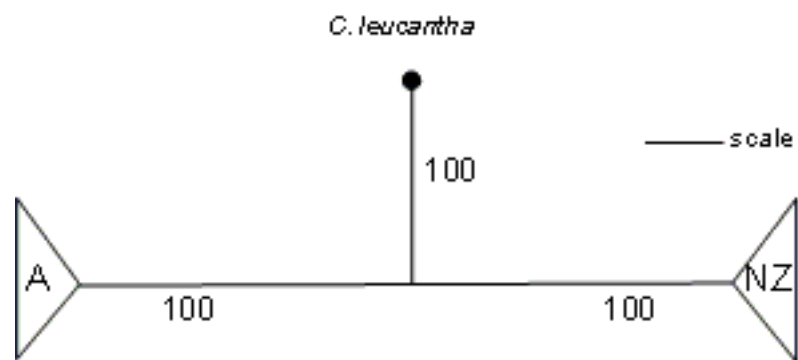


Figure 16. *psbA-trnH* splits graph of *Craspedia* (ingroup). A = Australian *Craspedia* except *C. leucantha* and NZ = New Zealand *Craspedia*. Bootstrap values included. Scale = 1 change.

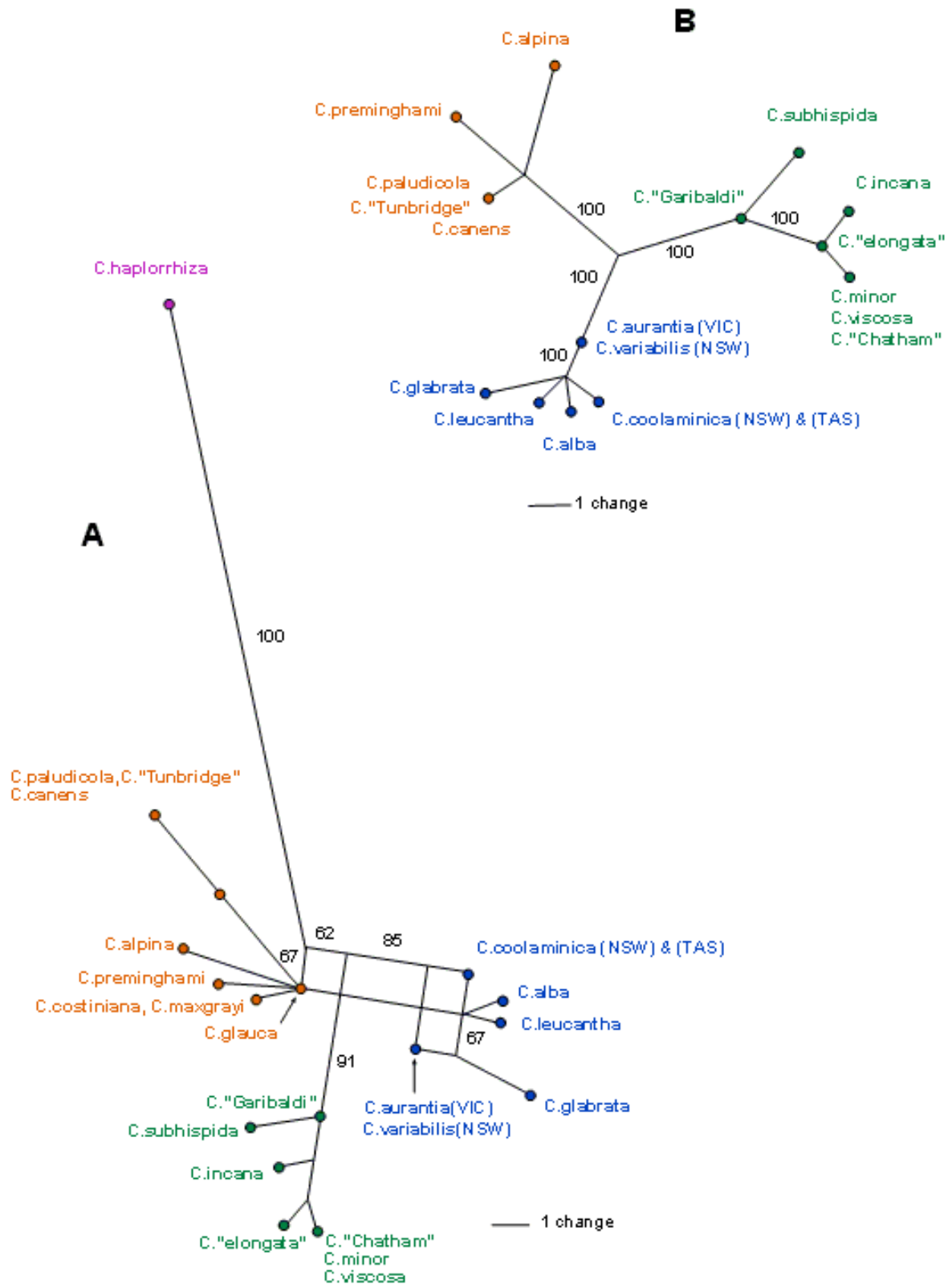


Figure 17. ITS split graph (parsimony criterion) A *Craspedia* (ingroup). B *Craspedia* (ingroup excluding *C. haplorrhiza*). Bootstrap values for internal splits in black. Main clades are: AUS 1 = ORANGE; AUS 2 = BLUE; NZ = GREEN & *C. haplorrhiza* = PURPLE.

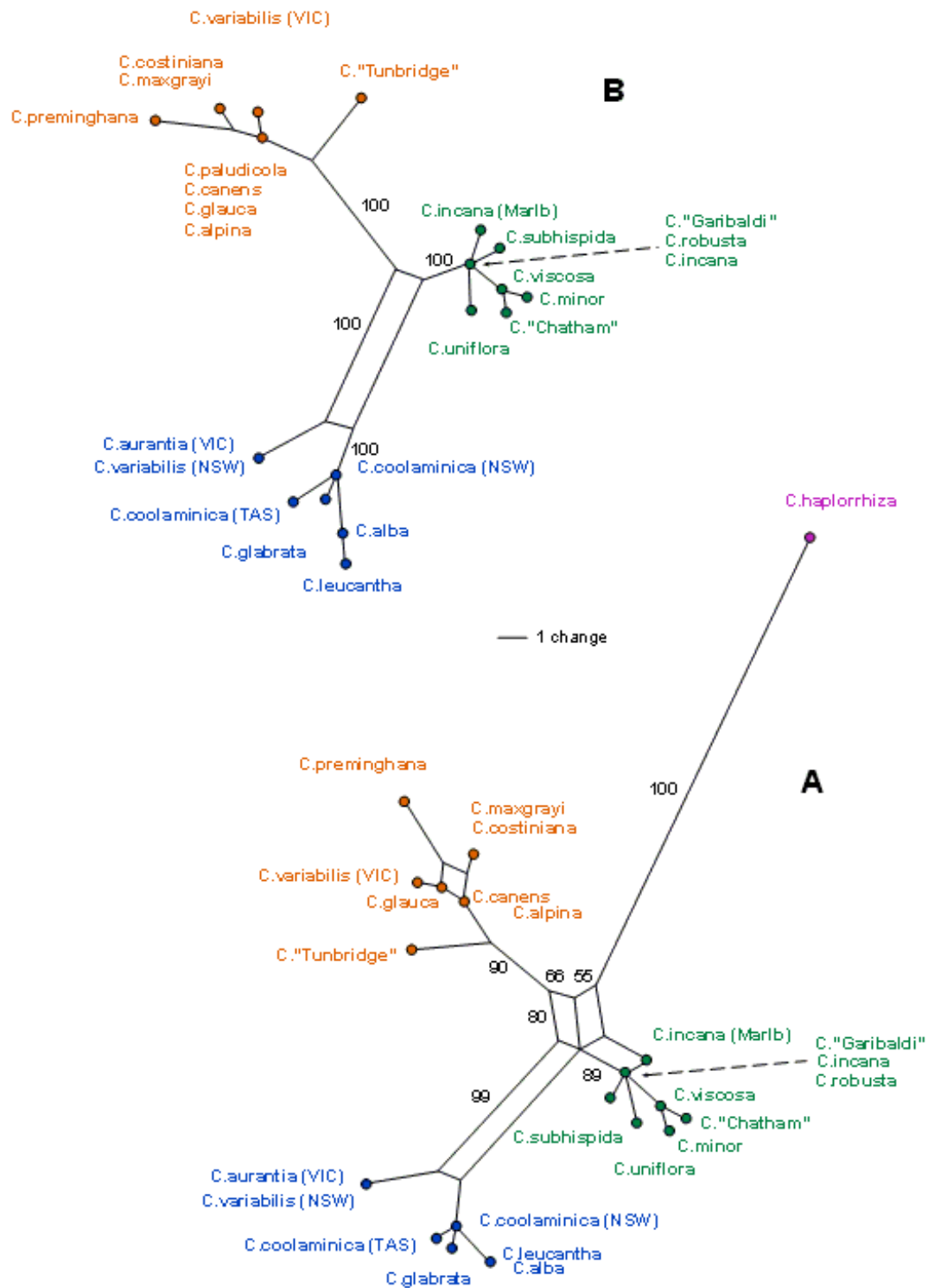


Figure 18. ETS split graph (parsimony criterion) A *Craspedia* (ingroup). B *Craspedia* (ingroup excluding *C. haplorrhiza*). Bootstrap values for internal splits in black. Main clades are: AUS 1 = ORANGE; AUS 2 = BLUE; NZ = GREEN & *C. haplorrhiza* = PURPLE.

CHAPTER 5

Discussion

5.1 Congruence of results from tree-building methods

The two bifurcating tree building methods used for this study, parsimony and maximum likelihood, produced near-congruent tree topologies from analyses of the ITS, ETS and *psbA-trnH* data sets. The only exception is the placement of *C. haplorrhiza* in both the ETS and ITS analyses. Otherwise tree topology and branch length are congruent for both methods and for different substitution models used with maximum likelihood.

The split decomposition analysis of the *psbA-trnH* data set produced a graph that was consistent with a bifurcating tree model with no incompatible splits present (Fig. 15). The split decomposition analyses of the ITS and ETS data sets produced graphs that were consistent with a bifurcating tree model, but contained a low amount of conflict, which showed up as boxiness in the graphs. Once *C. haplorrhiza* was removed from analyses the boxiness disappeared (Fig. 16b & 17b). Split decomposition displayed graphically the conflicting signal caused by *C. haplorrhiza*, which was suspected as the source of homoplasy from the comparison of bootstrap and rescaled consistency index values with and without this taxon included in analyses (Table 5).

The only incongruence between methods and models involved one species, *Craspedia haplorrhiza*, in both the ITS and ETS analyses, resulting in three competing hypotheses. The competing hypotheses are: *C. haplorrhiza* is sister to the rest of *Craspedia* (ETS parsimony & ITS/ETS parsimony), sister to the AUS 1 clade (ITS ML GTR model & ITS/ETS ML GTR model), sister to the AUS 2/NZ lineage (ETS ML GTR model), or part of a polytomy with AUS 1 and AUS 2 clades (ITS parsimony). *C. haplorrhiza* increases homoplasy in both data sets. Consistency indices and bootstrap values increase with its exclusion (Table 5). This is due to a lack of signal and conflict, which is most likely caused by a saturation of substitutions along this branch relative to the rest of *Craspedia*. The long branch of *C. haplorrhiza* probably reflects a higher substitution rate in this species, as *C. haplorrhiza* is the only annual species in *Craspedia*. It has been well

documented that annuals have significantly higher substitution rates (Ainiouche & Bayer 1999; Andreasen & Baldwin 2001) than related perennials and evidence suggests this results from differences in generation time (Andreasen & Baldwin 2001). The difference in the topologies in the combined ITS/ETS parsimony and maximum likelihood strict consensus trees, is most easily explained by the GTR substitution model correcting for the attraction of the long branch of *C. haplorrhiza* to the long branch leading to the outgroup.

The phylogenetic relationships of *Craspedia haplorrhiza* are uncertain, i.e., whether *C. haplorrhiza* is sister to the rest of *Craspedia* or included within the main *Craspedia* clade. The *psbA-trnH* analysis includes *C. haplorrhiza* within an unresolved paraphyletic Australian grade. However, there is little variation in this region (with only six informative sites) and furthermore *Pycnosorus* was not included, due to large indels which resulted in no informative sites to compare in relation to *Craspedia*. The phylogenetic relationships of *C. haplorrhiza* therefore remain uncertain and further data are required to resolve its relationship relative to the main *Craspedia* clade.

5.2 Molecular markers

There was enough variation in the ITS and ETS sequences for the purpose of this study, which was to resolve relationships within *Craspedia* in order to identify lineages at a trans-Tasman scale. The *psbA-trnH* sequences were not variable enough to resolve relationships within *Craspedia* and did not resolve the deepest split uncovered by the nuclear rDNA spacers, i.e., the (AUS 1)/ (AUS 2, NZ) split. However, the results from the *psbA-trnH* spacer are congruent with the ITS/ETS spacers in supporting the monophyly of New Zealand *Craspedia*. The New Zealand species of *Craspedia* have inherited a unique haplotype in common with the Australian species, *C. leucantha*. Therefore there is strong support for the monophyly of New Zealand *Craspedia* based on two independent data sets.

The ETS and ITS regions are evolving approximately 4.5 and 2 times faster respectively than the *psbA-trnH* region as shown by pairwise sequence divergences of uncorrected p-distances (Table 3). This produced trees that resolved three main clades within *Craspedia*, one in New Zealand and two in Australia (Fig. 14). All New Zealand species of *Craspedia* are nested within an Australian clade. There was a high degree of congruence between the

ITS and ETS gene trees. The ETS sequences provided more variable characters and an increase in phylogenetic resolution, as has been reported previously (Baldwin & Markos 1998; Clevinger & Panero 2000; Markos & Baldwin 2001). In this study ETS resolved and strongly supported one of the Australian *Craspedia* clades (AUS 2) as the most closely related to the clade of New Zealand *Craspedia*. The combined ETS and ITS analyses gave an increase in resolution (within the main clades) and higher bootstrap values for the three main clades and sister group relationship between the New Zealand clade and the Australian AUS 2 clade.

Results of the *psbA-trnH* analyses indicate that the New Zealand lineage has inherited a haplotype from a common ancestor with the Australian species *C. leucantha* (Fig. 15). This haplotype is unique to the New Zealand lineage and *C. leucantha*, at least within the present sample. This can be most parsimoniously explained by lineage sorting (Avice 1989; Doyle 1992), a process whereby chloroplast genomes (which are a single linkage group) or nuclear genes are randomly sorted into different lineages at the time of speciation (Fig. 6). This assumes that the ancestral species of New Zealand *Craspedia* and the AUS 2 lineage was polymorphic for the common chloroplast haplotype (which is present in the majority of *Craspedia*) and at least one other haplotype. This assumption is not unreasonable as chloroplast polymorphism is commonly documented in populations of extant species (see review by Soltis et al. 1992). Through stochastic lineage (see Chapter 2 p. 14) sorting only New Zealand *Craspedia* and *C. leucantha* have inherited the rare haplotype.

An alternative explanation to the above is that the N.Z./ *C. leucantha* haplotype evolved in New Zealand, and therefore *C. leucantha* is indicative of a back dispersal event and chloroplast capture. If this happened, then the dispersal event must have occurred early in the evolution of the group in New Zealand, as *C. leucantha* shares only a subset of synapomorphies with New Zealand *Craspedia*. This requires, firstly, the extinction of the rDNA genes of the back disperser (maternal parent) in Australia through introgressive hybridisation, and secondly, the extinction of some ancestral chloroplast diversity in New Zealand. This explanation is compatible with the genetic disjunction having evolved at a trans-Tasman scale and the interesting possibility of gene flow across the Tasman Sea

between New Zealand and Australian *Craspedia*. However, this is a less parsimonious explanation than lineage sorting and seems much less likely.

5.3 Origin of New Zealand *Craspedia*

The null hypothesis, that New Zealand *Craspedia* is monophyletic, consistent with one dispersal event from an Australian progenitor (Breitwieser et al. 1999), cannot be rejected. All three data sets, ETS, ITS and *psbA-trnH*, are consistent with the hypothesis that New Zealand *Craspedia* is a monophyletic group derived from a single jump-dispersal and founder event to New Zealand, originating from south-east Australia and radiating into many habitats (Breitwieser et al. 1999).

Data from ITS and ETS uncovered sub-generic relationships in *Craspedia* previously not detected using morphological characters (Fig. 14). The New Zealand lineage was identified as most closely related to one of two Australian lineages (AUS 2), consisting of four species, *C. alba*, *C. glabrata*, *C. coolaminica* and *C. leucantha*, and two samples identified as *C. aurantia* and *C. variabilis*, which are found in montane to alpine habitats along the main divide of eastern Australia and in Tasmania. A crude estimate of the time of arrival based on ITS substitution rates calculated from other island/mainland disjunctions in the Compositae (Sang 1994, 1995), is between 650,000 and 325,000 years ago, within the Quaternary Period. The estimate of time of arrival in New Zealand does not conflict with Bayer et al. (2002), who suggest that the Australian Gnaphalieae evolved and radiated in the Miocene correlated with the onset of aridity in the Australian interior. Based on this estimate, the arrival and radiation of species of *Craspedia* in New Zealand occurred during a period of fluctuating climate, with numerous glacial and interglacial cycles (Salinger & McGlone 1989), and after the main uplift of the southern alps, which took place in the Pliocene and the early Quaternary, about 3 to 1.5 m.y.a. (Suggate et al. 1978). This would have provided a founder population with abundant open habitats in which to colonise and disperse. Compositae pollen (not distinguishable to generic rank), and similarly pollen of Cyperaceae and Poaceae, although recorded in the Oligocene from New Zealand (Couper 1953), became much more abundant from the Middle Pliocene onwards (Mildenhall 1980). Furthermore, pollen of a number of genera characteristic of open habitats is first recorded in the Pliocene and Quaternary, e.g., *Bulbinella*,

Colobanthus, *Gentianella*, *Euphrasia*, *Forstera*, *Hebe*, *Pimelea*, *Ranunculus* and *Wahlenbergia*. The results of the present study are consistent with the New Zealand fossil pollen record (Mildenhall 1980; Macphail 1997) and other molecular studies (Breitwieser et al. 1999; Wagstaff et al. 1999, 2000, Wagstaff & Wege 2002; Wagstaff et al. 2002; Lockhart et al. 2001; Winkworth et al. 2002; Heenan et al. 2003), in suggesting that the Pleistocene, a period of mountain building and climate change, has been an important factor in the evolution of the New Zealand shrubby and herbaceous flora.

Most of the morphological (pers. obs.) and ITS/ETS sequence diversity (Fig. 14) in the AUS 2 lineage is restricted to the mainland Australian eastern uplands: *C. leucantha* (endemic to the Kosciuszko alpine zone), *C. alba* (found mainly in the Kosciuszko alpine zone, but also with two populations documented outside of this area 100 km south in the Victorian Alps), and *C. aurantia* (Vict) and *C. variabilis* (NSW), both sampled from montane and subalpine areas in Victoria and N.S.W respectively. The exceptions are *C. coolaminica*, which is widespread throughout the subalpine areas of the eastern uplands and in Tasmania, and *C. glabrata*, which is endemic to alpine Tasmania and the only species absent from the mainland. Evidence from the *psbA-trnH* parsimony tree (Fig. 15) suggests that species of New Zealand *Craspedia* share a rare chloroplast haplotype with *C. leucantha*, a species endemic to the Kosciuszko alpine zone. No other species of Australian *Craspedia* was sampled with this haplotype. The evidence presented above from both the nuclear and chloroplast non-coding spacers and morphology, suggests that New Zealand *Craspedia* probably originated from the mainland Australian uplands rather than Tasmania.

There are three alternative jump-dispersal routes to New Zealand via which *Craspedia* could have arrived: firstly, directly across the 2000 kms of Tasman Sea, secondly, indirectly over a greater distance of subantarctic waters via Campbell Island and thirdly and intuitively less probable, by another indirect route via New Caledonia and a hypothetical island arc connecting to New Zealand in the Miocene (Lee et al. 2001) (see Fig. 1 for map).

In the ITS/ETS strict consensus trees (Fig. 14) and the ITS and ETS splitstrees (Fig 17 & 18) the first divergence in New Zealand *Craspedia* is a soft polytomy between *C.*

“Garibaldi” (from N.W. Nelson in the South Island), *C. uniflora* var. *subhispidata* (from the subantarctic Campbell Island) and the rest of New Zealand *Craspedia*. Therefore the evidence is equivocal as to whether dispersal of the ancestral species of New Zealand *Craspedia* came via Campbell Island or further north across the Tasman Sea.

Although there is evidence for an early Miocene island arc connecting New Zealand with New Caledonia (Herzer et al. 1997) and offering a northern dispersal route by short hops, this appears an unlikely option for *Craspedia*. Firstly, evidence from the present study supports an origin from Australian mountain species and a Pleistocene arrival in New Zealand (650,000 to 325,000 years). Secondly, New Zealand *Craspedia* is concentrated in the South Island and is absent from the upper North Island above latitude 38° S (Allan 1961; Druce 1993) as well as from New Caledonia (Guillaumin 1948). A later Pliocene or Pleistocene route from the north also seems unlikely. Unlike some other Australasian Gnaphalieae, e.g., *Anaphalioides* (Glenny 1997) and *Euchiton* (Ward et al. 2003), *Craspedia* has not reached the Papua New Guinea highlands which are of similar age to the New Zealand alps (Ollier 1986), and is not found north of 24.91°S (Buckland Tablelands) in Queensland. Although not conclusive (because of the possibility of extinction) this strongly suggests that dispersal and colonisation has not been from the north, which would also imply at least some semi-tropical tendencies (if in the Miocene), of which there are none in New Zealand or Australian *Craspedia*.

Although the molecular evidence is equivocal, a direct route from mainland Australia to New Zealand is favoured over a longer indirect route via Campbell Island, as *Craspedia* is restricted to Campbell Island, and its absence from other New Zealand sub-Antarctic islands, especially the Auckland Islands (c. 310 km away), suggests a relatively recent chance dispersal event from the main islands of New Zealand. A number of other studies of New Zealand/subantarctic island plant disjunctions also suggest that dispersal has occurred from New Zealand to the subantarctic islands, e.g., *Gentianella* (Glenny 2003), *Hebe* (Wagstaff et al. 2002) and *Ranunculus* (Lockhart et al. 2001).

It is very difficult to determine which passive dispersal mode is more likely correctly to explain the arrival of *Craspedia* in New Zealand—dispersal by wind or bird carriage seem the only likely options, as *Craspedia* cypselas have no obvious adaptations to ocean drift,

i.e., thick protective shell impervious to sea-water, loose airy layers or bladders, which would provide buoyancy. Although the Compositae pappus is an adaptation to wind dispersal, *Craspedia* in Australia and New Zealand has a pappus that easily breaks away from the fruit (pers. obs.), and therefore do not seem particularly well adapted to be blown long distances. Cypsela size varies in Australia from 1.0 to 5.0 mm in length and species in the AUS 2 lineage vary from 1.0 to 3.0 mm in length (Everett & Doust 1992b; Everett 1999; Rozefelds 2002). In New Zealand the mean length of cypselas is 2.5 mm (unpublished data Breitwieser & Ford). An exception, however, is the New Zealand *C. minor* group (consisting of *C. minor*, *C. viscosa* & *C. "Chatham"*), which has significantly smaller cypselas (mean length = 1.56 mm) with a high pappus length to fruit length ratio (Breitwieser & Ford, unpublished data) and may be better adapted to longer jump-dispersal events. *C. minor* and *C. viscosa* have relatively large geographic ranges and *C. "Chatham"* represents one of two jump-dispersal events from New Zealand to distant islands. However, contrary to what would be expected, i.e., an ancestral position in the New Zealand clade, this group is nested in a derived position and therefore increased adaptation for wind dispersal may have evolved within New Zealand.

The other option, bird carriage, requires that a cypselas is either ingested, attached or caught in mud on the feet or legs of birds. The first option is the most unlikely as *Craspedia* cypselas seem ill-adapted to bird dispersal via ingestion. They are brown, inconspicuous fruits covered in long, stiff, bifid hairs and glandular hairs that secrete a sticky mucilage. It is perhaps more likely that the bifid hairs and/or the sticky mucilage could aid in attaching or sticking to bird feathers. The muddy feet option is conceivable, as New Zealand species of *Craspedia* are most closely related to an Australian lineage (AUS 2) in which three of the five species (*C. alba*, *C. leucantha* and *C. glabrata*) are characteristic of subalpine and alpine riparian habitats (Plate 1D). If the ancestral habitat of New Zealand *Craspedia* was riparian, propagules could have arrived on the muddy feet of birds (or perhaps been defecated after accidental ingestion in water), as has been attributed to genera such as *Carex*, which are associated with riparian habitats and have many species which appear to have no obvious fruit adaptations correlated to dispersal (Catling et al. 1990). Many upland bog species of *Carex* have relatively large fruits (mean length = 3.0mm, Moore & Edgar 1970).

An ancestral ‘riparian’ habitat for New Zealand *Craspedia* would make the dispersal of propagules by muddy bird feet from mountain riparian habitats a possibility. Cypselas of the ‘minor group’, *C. minor*, *C. viscosa* and *C. “Chatham”*, with their small size and long pappus, suggest wind dispersal may be more likely to explain how *C. “Chatham”* arrived in the Chatham Islands (c. 800 km east of East Cape). However, this does not apply to *C. uniflora* var. *subhispida*, which has dispersed to Campbell Island (660 km south of N.Z.), one of several subantarctic island groups south of New Zealand, in spite of having larger cypselas (2.0-2.4 mm long).

The colonisation of New Zealand by a single founder population, few dispersal events to islands (two) and no back dispersal to Australia, suggest that dispersal of *Craspedia* propagules over large distances is rare. Clearly *Craspedia* is not well adapted to long jump-dispersal across oceans even with the aid of a dominant (roaring 40s) westerly wind flow across the Tasman Sea. Bird carriage or wind dispersal are both possible explanations as to the mode of dispersal of *Craspedia* propagules from Australia to New Zealand. It is difficult to decide between the two options due to the impossibility of obtaining direct evidence.

Based on the results of this study from ITS and ETS, white flower colour and lanate leaves have evolved more than once (Fig. 14). Previous suggestions of shared Australian and New Zealand taxa are based on superficial similarity. For example, one pair of taxa, *C. lanata* var. *elongata* (N.Z.) and *C. alpina* (Austr.), which were suggested to be shared between New Zealand and Australia (Hooker, 1853; Cheeseman, 1925; Kirk, 1899; Cockayne, 1928) is not monophyletic. The two taxa belong to the least related clades, NZ and AUS 1, in the inferred *Craspedia* phylogeny, suggesting convergent evolution correlated to habitat. *C. lanata* var. *elongata* and *C. alpina* are both lanate-leaved plants with white flowers, from alpine areas in the New Zealand southern alps and Tasmania respectively. White flower colour and lanate leaves are strongly correlated with alpine habitats in Australia but not in New Zealand. *C. robusta* var. *pedicellata* (N.Z.) and *C. glauca* s.s. (Austr.), also suggested as shared taxa, has not been tested as *C. robusta* var. *pedicellata* was not included in the study.

5.4 Evolution and biogeography within New Zealand

There is little resolution in any of the data sets from which to infer relationships and biogeography within New Zealand *Craspedia*. Variation in the New Zealand ITS and ETS sequences is less than in the Australian ones (Table 3), as would be expected if the New Zealand lineage diverged recently from an Australian lineage. However, at least as much or more morphological and habitat diversity exists in New Zealand as compared with Australia (pers. obs.) despite three divergent lineages in the latter. The observation of small amounts of sequence divergence in contrast to large amounts of morphological divergence has been made for other New Zealand species radiations after continental/island jump-dispersal events, e.g., an almost exclusively endemic New Zealand clade of Gnaphalieae which includes the genera *Anaphalioides*, *Leucogenes*, *Rachelia* and *Raoulia* (Breitwieser et al. 1999), *Hebe* (Wagstaff et al. 2002) and *Myosotis* (Winkworth et al. 2002), and also for other islands, such as the Hawai'i Madiinae (Baldwin 2003), *Dendroseris* and *Robinsonia* in the Juan Fernandez Islands (Sang et al. 1994, 1995) and *Argyranthemum* in Macronesia (Francisco-Ortega et al. 1997).

This rapid morphological divergence points to rapid radiation of *Craspedia* in New Zealand, by either adaptive radiation and/or through founder events and genetic drift in a physiographically variable landscape. Breitwieser et al. (1999) suggested that much of the morphological variation in New Zealand *Craspedia* is phenotypic and habitat induced.

The first split in New Zealand *Craspedia* in the ITS/ETS parsimony strict consensus tree is between *C. uniflora* var. *subhispidata*, *C. "Garibaldi"* and the rest of New Zealand *Craspedia* (forming a polytomy). Two groups are distinguished in New Zealand *Craspedia*: one contains the majority of species and entities sampled (7 out of 13) in an unresolved polytomy, and the other is a monophyletic group nested within this polytomy, consisting of three taxa, *C. minor*, *C. viscosa*, *C. uniflora*, and one informal entity, *C. "Chatham"*. *C. uniflora* is placed sister to *C. minor*, *C. viscosa* and *C. "Chatham"*. Allan (1961) tentatively included *C. "Chatham"* in *C. uniflora* var. *uniflora*, but Druce (1993) regarded it a separate species; a close relationship with *C. minor* and *C. viscosa* has not before been suggested. The ITS/ETS sequences of *C. "Chatham"* and *C. viscosa* are identical. A relationship between *C. uniflora* var. *uniflora* and *C. minor* and *C. viscosa* was

also unexpected, and has not been previously suggested based on morphology. The ITS/ETS tree indicates that *C. uniflora* is polyphyletic, as *C. uniflora* var. *uniflora* (collected near the type locality) and *C. uniflora* var. *subhispidata* do not share a common ancestor. Although there is little resolution within species of New Zealand *Craspedia*, the present study indicates that the current species boundary of *C. uniflora* is misplaced due to superficial morphological similarity.

The phylogeny suggests that *C. minor*, *C. uniflora*, *C. viscosa* and *C. “Chatham”* have diverged from the main New Zealand *Craspedia* lineage and speciated into subalpine, montane and lowland habitats, including a jump-dispersal event over 800 km ocean to the Chatham Islands. *Craspedia minor*, *C. viscosa* and *C. “Chatham”* to varying extents exhibit ruderal tendencies (adaptation to persistent disturbance), and are commonly found on open rocky ground, streamside, bank and cliff habitats (based on CHR herbarium records) (e.g., Plate 1B). As discussed earlier *C. minor*, *C. viscosa* and *C. “Chatham”* have two distinguishing traits that may correlate with dispersability, and which appear to have evolved in New Zealand, i.e., small cypsela size and a high pappus to cypsela length ratio (unpublished data, Breitwieser & Ford). If increased dispersability has evolved within New Zealand this is contrary to Lloyd’s hypothesis of ‘immigration selection’, which predicts dispersal traits to be selected for at the species level from outside New Zealand (Lloyd 1985).

5.5 Evolution and biogeography within Australia

ITS and ETS trees support two main Australian clades, which contain most of the Australian species. These two lineages have not previously been recognised based on morphology (Curtis 1963; Everett & Doust 1992a; Everett & Thompson 1992; Rozefelds 2002). They are largely sympatric in south-east Australia (including Tasmania). A possible third lineage is monotypic and comprises the semi-desert species *C. haplorrhiza*, the only *Craspedia* recorded as an annual (Everett & Doust 1992a). The ITS/ETS parsimony strict consensus tree supports this species as the earliest divergence in the tree, whereas the maximum likelihood has this taxon as sister to the AUS 1 clade (with no bootstrap support).

In contrast to *Craspedia* in New Zealand, phylogenetic analyses of ETS and ITS data (Fig. 14) suggest that species of Tasmanian *Craspedia*, i.e., *C. alpina*, *C. glauca*, *C. paludicola*, *C. preminghana* and *C. “Tunbridge”*, are a polyphyletic assemblage of species that are conspecific with or related to mainland Australian species. The direction of dispersal is difficult to determine from the analyses, as the relationships between species are generally not well resolved. However, as evidence suggests that *Craspedia* has originated from semi-desert ancestors of mainland Australia (Bayer 2002), it would be logical to suspect that generally the direction of dispersal has been from mainland Australia to Tasmania. Five of the seven species (including *C. paludicola*, collected from Tasmania but not sequenced in this study) and one un-named entity are conspecific with a mainland species or more closely related to a mainland species than to any other Tasmania species. The exceptions are the Tasmanian endemic *C. glabrata* and the widespread *C. coolaminica*, which could share a common ancestor in Tasmania. Relationships between the AUS 2 taxa are not resolved enough to suggest one scenario over the other. There is no evidence of a species radiation in Tasmania, in contrast to New Zealand, and some species in Tasmania parallel their Australian mainland relatives in habitat preference, e.g., *C. alpina*, *C. “Tunbridge”*, *C. glabrata* and *C. coolaminica*. The exceptions are *C. glauca* s.s., which occurs in coastal shrubland and forest along the east coast of Tasmania, in contrast to its Kosciuszko alpine relations, and *C. preminghana*, which is found localised on steep basalt cliffs with coastal vegetation in north-west Tasmania, in contrast to its grassland sister species, *C. variabilis* (Vict).

The biogeographic pattern of Tasmanian *Craspedia* can be explained by either multiple jump-dispersal events or vicariance. Disjunctions appear at different depths in the tree, but this does not necessarily favour a dispersal hypothesis, as Tasmania has been connected to mainland Australia at least six times during the Pleistocene glaciations, a period of about 1.7 million years based on evidence from isotopic oxygen deficit in foraminifera (Shackelton et al. 1973, 1976).

Thompson (1981) and Costin (2000) suggested that the Kosciuszko alpine species, *C. alba*, *C. aurantia*, *C. costiniana*, *C. jamesii*, *C. lamicola*, *C. leucantha* and *C. maxgrayi*, could represent a recent diversification into alpine niches maintained by habitat selection, possibly suggesting sympatric speciation. The idea that the Kosciuszko alpine species

constitute a monophyletic group is not supported by the results of this study. Some of the Kosciuszko alpine species, *C. aurantia*, *C. costiniana*, *C. lamicola* and *C. maxgrayi*, form a weakly supported group (also including the Tasmanian species *C. glauca* s.s.) and diverge in the AUS 1 clade from the Tasmanian sister species *C. alpina* (Fig. 5A & B). Other Kosciuszko alpine species are not included in this group, i.e., *C. alba* and *C. leucantha*, which are members of the AUS 2 clade. These two species share at least some gross morphological similarities and habitat preferences that distinguish them from the AUS 1 Kosciuszko alpine species. They are small, non-robust with relatively short scapes, and white-flowered, whereas the rest of the Kosciuszko alpine species are tall, robust with long scapes, and yellow-flowered. The former occupy riparian habitats whereas the latter are plants of drier habitats, such as grasslands, herbfields on slopes, and rocky outcrops (Costin, 2000). *Craspedia glabrata* of Tasmania, also a member of the AUS 2 clade, is comparable to *C. alba* and *C. leucantha* in gross morphology and habitat; it is also a small, non-robust white-flowered species, occupying moist, poorly-drained soils and swampy areas or occasionally grassland (Rozefelds, 2002).

The ITS/ETS trees (Fig. 14) identified two polyphyletic species, *C. aurantia* and *C. variabilis*. They both occur in the two main Australian clades, AUS 1 and AUS 2. *Craspedia aurantia* (Vict) and *C. variabilis* (NSW), however, are probably mis-identified. They differ in their combined ITS/ETS sequences by only one base change, and are well supported as a monophyletic group within the AUS 2 clade. Further sampling and examination is required. It is possible, however, that they represent an unrecognised cryptic species in the eastern Australia uplands.

Within the AUS 1 clade there is a discernable split between lowland and alpine taxa. *Craspedia* “Tunbridge”, *C. canens*, *C. paludicola*, *C. variabilis* (Vict) and *C. preminghana* are all lowland taxa, in contrast to *C. alpina*, *C. aurantia* (NSW), *C. costiniana*, *C. maxgrayi*, *C. lamicola* and *C. glauca*, which are all (with the exception of *C. glauca*) alpine taxa. The AUS 2 clade is mostly subalpine/alpine species, although the distribution of the ‘*C. aurantia* (Vict)/*C. variabilis* (NSW)’ pair is largely unknown. *C. aurantia* (Vict) was collected from a subalpine or alpine site on the Bogong Plains near Falls Creek in Victoria, and *C. variabilis* (NSW) was collected from a road cutting in *Eucalyptus* forest at lower altitudes in Kosciuszko National Park. In the AUS 1 clade the phylogeny suggests

that the direction of evolution is from lowland to upland, as the earliest splits are between lowland species. The evidence is equivocal for the AUS 2 clade and more sampling may be required, particularly to establish distribution of the '*C. aurantia* (Vict)/*C. variabilis* (NSW)' entity. The relationships of the alpine species in both clades are largely unresolved, indicating recent diversification. Phylogenetic evidence strongly suggests that the two Australian clades represent separate species radiations into the Australian alpine zone.

5.6 Conclusion

In this study, a phylogeny of *Craspedia* was inferred from ITS, ETS and *psbA-trnH* non-coding spacers. By establishing that New Zealand *Craspedia* is monophyletic, further taxonomic, morphological and biogeographical investigation can proceed within a known phylogenetic framework within New Zealand. Previously, it was thought that some New Zealand taxa shared a closer relationship with Australian taxa, e.g., *C. "elongata"* and *C. alpina*. However the inferred phylogeny suggests that morphological similarity between at least these two entities is due to convergent evolution, probably correlated to habitat.

Although the New Zealand clade is only partly resolved, the phylogenetic analyses of ITS and ETS indicate some relationships are incongruent with those previously suggested by morphology and question the current species boundaries of *C. uniflora*.

The phylogenetic analyses of ITS and ETS also provide a preliminary framework in which to test ideas on character evolution in New Zealand. Lloyd's 'immigration selection' hypothesis predicts that dispersal traits are selected for at the species level from outside New Zealand. Based on the results of this study, it is suggested that the *C. minor* group may have evolved increased dispersability within New Zealand, possibly correlated with a radiation into subalpine, montane and lowland ruderal habitats.

ITS and ETS support two divergent lineages of *Craspedia* in Australia, which contain most Australian species. These lineages have not previously been recognised based on morphology and it is suggested they represent two independent species radiations into the Australian alpine zone. One of the lineages, consisting of mostly subalpine and alpine

species, was identified as sister to the New Zealand lineage. The divergence of these two lineages was estimated to be between 650,000 and 325,000 years ago, within the Pleistocene. These results are consistent with the New Zealand fossil pollen record and with other molecular studies in suggesting that the Pleistocene, a period of mountain building and climate change, has been an important factor in the evolution of the New Zealand herbaceous flora.

Phylogenetic analyses of ETS and ITS suggest that, in contrast to *Craspedia* in New Zealand, species of Tasmanian *Craspedia* are a polyphyletic assemblage of species that are conspecific with or more closely related to mainland Australian species. There is no evidence of a species radiation in Tasmania on the scale seen in New Zealand, and some species in Tasmania parallel their Australian mainland relatives in habitat preference.

5.7 Future Research

Future research should aim at resolving phylogenetic relationships within *Craspedia* in New Zealand by using higher resolution markers such as ISSRs or AFLPs. This could help clarify species boundaries and phylogeographic patterns, particularly in North West Nelson, where there are several species and about 20 distinct localised entities of *Craspedia*.

Further investigation into chloroplast phylogeography between Australia and New Zealand is another interesting avenue to explore if a more variable marker could be found.

In Australia, interesting questions remain in regards to the '*C. aurantia* (Vict)/*C. variabilis* (NSW)' clade, such as, its distribution and whether it is morphologically or cytologically distinct. Further sampling of this entity and of *C. variabilis* might possibly uncover more ITS/ETS sequence variation.

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Appendix 1

Extraction of DNA

1. Fresh or dried plant material was ripped up and snap frozen in liquid nitrogen. The frozen material was ground to a fine powder using a mortar and pestle and collected in a 2.0ml eppendorf tube.
2. 4 μ l of Rnase A stock solution (100mg/ml) and 400 μ l of AP1 lysis buffer was added to the tube. The mixture was incubated for 10 minutes at 65°C, and mixed twice by inverting tube.
3. 130 μ l of AP2 buffer was then added to the lysate, mixed, and incubated on ice for 5 minutes. Afterwards the lysate was centrifuged for 5 minutes at 13,000rpm.
4. The supernatant was added to a QIAshredder spin column sitting in a 2ml collection tube. The lysate in the spin column was centrifuged for 2 minutes at 13,000rpm. The flow through fraction in the collection tube was transferred to a new 2.0ml microcentrifuge tube, taking care not to disturb the pellet in the base of the tube.
5. To the cleared lysate was added 1.5 volumes of AP3/E buffer, usually this was between 645 - 675 μ l of buffer depending on the amount of lysate. The AP3/E buffer was pipetted directly onto the cleared lysate and mixed immediately.
6. 650 μ l of the mixture was then added to the Dneasy spin column sitting in a 2.0ml collection tube and centrifuged for 1 minute at 8000rpm. The flow through was discarded, and this step was repeated with the remaining mixture.
7. 500 μ l of buffer AW was added to the spin column, which was placed in a new 2.0ml collection tube, then centrifuged for 1 minute at 8000rpm. The flow through

was discarded. 500µl of buffer AW was again added to the spin column and centrifuged for 2 minutes at 13,000rpm to dry the spin column membrane.

8. The spin column was carefully removed from the collection tube to avoid carry over of ethanol and placed into a new 2.0ml microcentrifuge tube.
9. 100µl (or 50) of preheated (65°C) buffer AE was pipetted directly onto the membrane of the spin column. The column was then incubated for 5 minutes at room temperature, and then centrifuged for 1 minute at 8000rpm to elute total DNA.

To estimate that a reasonable amount of DNA of high molecular weight was extracted, 5.0µl of DNA and 2.0µl of loading buffer was electrophoresed in 0.5xTBE on a 1.5% agarose gel for approximately 1.0 hour at 70 volts and visualised by staining in ethidium bromide.

APPENDIX 2: ITS

	10	20	30	40	50	60	70	80
Stu.muel	TCGAACCCTG	CAAAGCAGAA	CGACCCGTGA	ACATGTAACT	ACTACCGGAC	AACATAGGGA	TTGAGCTTTT	GTTTGATCCT
Heli.lanC....-	..-T...G.	..-...-...	.C.....-
Pyc.globACG.	...GA....A..
C.haplorrhizaAC	.A.....G.T...C.	.CAGA....
C.paludicolaACG.T.	...T...C.	.CACA....W
C."Tunbridge"ACG.T.	...T...C.	.CACA....
C.canensACG.T.	...T...C.	.CACA....W
C.preminghamiACG.T...C.	.CMCA....
C.variabil(VIC)ACG.T...C.	.CCCA....
C.alpinaY....ACG.T...C.	.CACA....
C.glaucaACG.T...C.	.CMCA....
C.aurantia(NSW)ACKG.T...C.	.CACA....
C.maxgrayiACTG.T...C.	.CACA....
C.costinianaACTG.T...C.	.CACA....
C.lamicolaACG.T...C.	.CACA....
C.leucanthaACG.T...	...T...C.	.CACA....
C.glabrataACG.T...	...T...C.	.CACA....
C.variabil(NSW)ACG.T...	...T...C.	.CACA....
C.aurantia(VIC)ACG.T...	...T...C.	.CACA....
C.coolamin(NSW)ACG.T...	...T...C.	.CACA....
C.coolamin(TAS)ACG.T...	...T...C.	.CACA....
C.albaACG.T...	...T...C.	.CACA....
C.subhispidiaACT.G.	...G....	...T...C.	.CACA....
C."Garibaldi"ACG.	...G....	...T...C.	.CACA....
C.robustaACG.	...G....	...T...C.	.CACA....
C."Anglem"ACG.	...G....	...T...C.	.CWCA....
C.viscosaACG.	...G....	...T...C.	.CACA....
C.incanaACG.	...G....	...T...C.	.CACA....
C.minorACG.	...G....	...T...C.	.CACA....
C."Chatham"ACG.	...G....	...T...C.	.CACA....
C.unifloraACG.	...G....	...T...C.	.CACA....
C."incana(Otago)"ACG.	...G....	...T...C.	.CACA....
C."Kaitorete"ACG.	...G....	...T...C.	.CACA....
C.elongataACG.	...G....	...T...C.	.C.CA....
C."incana(Marlb)"ACG.	...G....	...T...C.	.C.CA....

	90	100	110	120	130	140	150	160
Stu.muel	TATTTGGCCT	TTGCCGATGT	GCGTTTCGAGA	CTCCTTAGGG	ATGACAGGAT	GTCACATTGG	CATACTAACC	AACCCCGGCA
Heli.lan	..-G.-....	...T.....A.A....CC.....-....
Pyc.glob	..-G.TT...	...T.A....	.T.....C...A..
C.haplorrhiza	.T-G.C....	...T.A....	.T.....CTT..T.....	-.T.....	..A.....
C.paludicola	.T-G.T....	...T.A....	.T.....	A...CTT..Y	-TT.....	..A.....
C."Tunbridge"	.T-G.C....	...T.A....	.T.....	A...CTT..C	-TT.....	..A.....
C.canens	.T-G.Y....	...T.A....	.T.....	A...CTT..Y	-TT.....	..A.....
C.preminghami	.T-G.C....	...T.A....	.T.....	...T.CTTR.	-TT.....	..A.....
C.variabil(VIC)	.T-G.C....	...T.A....	.T.....	M..Y.CTT..Y	-TT.....	..A.....
C.alpina	.T-G.C....	...T.A....	.T.....CTT..	-TT.....	..A.....
C.glauca	.T-G.C....	...T.A....	.T.....	...Y.CTT..	-TT.....	..A.....
C.aurantia(NSW)	.T-G.C....	...T.A....	.T.....CTT..	-TT.....	..A.....
C.maxgrayi	.T-G.C....	...T.A....	.T.....CTT..	-TT.....	..A.....
C.costiniana	.T-G.C....	...T.A....	.T.....CTT..	-TT.....	..A.....
C.lamicola	.T-G.C....	...T.A....	.T.....CTT..	-TT.....	..A.....
C.leucantha	.T-G.C....	...T.A....	.T...A...CTT..	-T.....	..C.....
C.glabrata	.T-G.C....	...T.A....	.T...A...CTT..	-T.....	..A.....
C.variabil(NSW)	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C.aurantia(VIC)	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C.coolamin(NSW)	.T-G.C....	...T.A....	.T...A...CTT..	-T.....	..A.....
C.coolamin(TAS)	.T-G.C....	...T.A....	.T...A...CTT..	-T.....	..A.....
C.alba	.T-G.C....	...T.A....	.T...A...CTT..	-T.....	..A.....
C.subhispida	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C."Garibaldi"	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C.robusta	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C."Anglem"	.T-G.C....	...T.A....	.T.....	...Y.CTT..	-T.....	..A.....
C.viscosa	.T-G.C....	...T.A....	.T.....	...T.CTT..	-T.....	..A.....
C.incana	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C.minor	.T-G.C....	...T.A....	.T.....	...T.CTT..	-T.....	..A.....
C."Chatham"	.T-G.C....	...T.A....	.T.....	...T.CTT..	-T.....	..A.....
C.uniflora	.T-G.C....	...T.A....	.T.....	...T.CTT..	-T.....	..A.....
C."incana(Otago)"	.T-G.C....	...T.A....	.T.....CTT..	-T.....	..A.....
C."Kaitorete"	.T-G.C....	...T.A....	.T...K.CTT..	-T.....	..A.....
C.elongata	.T-G.Y....	...T.A....	.T.....	...T.CTT..	-T.....	..A.....
C."incana(Marlb)"	.T-G.C....	...T.A....	.T.....	...T.CTT..	-T.....	..A.....

	170	180	190	200	210	220	230	240
Stu.muel	CGGAAT-GTG	CCAAGGAAAA	TTTAACTTAA	AGAATGGTTG	GTTTCATGAA	CTCCCCTTTT	GCGGTGCGCT	CATGAAATTC
Heli.lan-TA..T	R.....C.T
Pyc.glob-T	.A.....	...T...A.T	...T...A.T	..AY.....	T.....T.A.C.T
C.haplorrhiza-TGT	.A.....CA.TT	..A.....C	A.....T...TC.A
C.paludicola-TTT	.A.....TA.TT	..AT.....T...CTC.A
C."Tunbridge"-TTT	.A.....TA.TT	..AT.....T...CTC.A
C.canens-TTT	.A.....TA.TT	..AT.....T...CTC.A
C.preminghami-TTT	.A.....TA.TT	..A.....T...CTC.A
C.variabil(VIC)-TTT	.A.....TA.TR...T	..AY.....T...CTC.A
C.alpina-TTT	.A.....TAATT	..A.....T...C--.A
C.glauca-TTT	.A.....TA.TT	..A.....T...CTC.A
C.aurantia(NSW)-TTT	.A.....TA.TY...T	..A.....T...CTC.A
C.maxgrayi-TTT	.A.....TA.TT	..A.....T...CTC.A
C.costiniana-TTT	.A.....TA.TT	..A.....T...CTC.A
C.lamicola-TTT	.A.....TA.TTC..T	..A.....T...CTC.A
C.leucantha-T.T	.A.....TA.TT	..A.....T...TC.A
C.glabrata-T.TTA.TC..T	..A.....T...TC.A
C.variabil(NSW)-T.T	.A.....TA.TC..T	..A.....T...TC.A
C.aurantia(VIC)-T.T	.A.....TA.TC..T	..A.....T...TC.A
C.coolamin(NSW)-T.T	.A.....TA.TT	..A.....CT...TC.A
C.coolamin(TAS)-T.T	.A.....TA.TT	..A.....CT...TC.A
C.alba-T.T	.A.....TA.TT	..A.....T...	G.....TC.A
C.subhispida-T.T	.A...C..TA.TT	..A.....T...TC.A
C."Garibaldi"-T.T	.A...C..TA.TT	..A.....T...TC.A
C.robusta-T.T	.A...C..TA.TT	..A.....T...	..C...TC.A
C."Anglem"	.R.....-T.T	.A...C..TA.TT	..YA.....T...	..C...TC.A
C.viscosa-T.T	.A...C..TA.TT	..A.....T.T.	..C...TC.A
C.incana-T.T	.A...C..TA.TT	..CA.....T...	..C...TC.A
C.minor-T.T	.A...C..TA.TT	..A.....T.T.	..C...TC.A
C."Chatham"-T.T	.A...C..TA.TT	..A.....T.T.	..C...TC.A
C.uniflora-T.T	.A...C..TA.TT	..A.....T.Y.	..C...TC.A
C."incana(Otago)"-T.T	.A...C..TA.TT	..CA.....T...	..C...TC.A
C."Kaitorete"-T.T	.A...C..TA.TT	..YA.....T...	..M...TC.A
C.elongata	.A.....-T.T	.A...C..TA.TT	..A.....T...	..C...TC.A
C."incana(Marlb)"	.A.....-T.T	.A...C..TA.TT	..A.....T...	..C...TC.A

	410	420	430	440	450	460	470	480
Stu.muel	CGTCTGCCTG	GGCGTCAAGC	ATCGTGTGCG	CCCCCACCAC	TCCTCCCTTA	GTGGATGTTT	GGCGTGGGGG	CGGAGATTGG
Heli.lan-.....	..--.T....	T.T.....
Pyc.globA.T.T	C.A..T....	.A.A.....	..T.....A....
C.haplorrhizaA.-....A	C.A.....--	.A.....	ATA.....T....
C.paludicolaA.-....A	C.A.....	.A.....	.TA.....
C."Tunbridge"A.-....A	C.A.....	.A.....	.TA.....
C.canensA.-....A	C.A.....	.A.....	.TA.....
C.preminghamiA.T.-...A	C.A.....	.A.....	.TA.....K....
C.variabil(VIC)A.Y.-...A	C.A.....	.A.....	.TA.....
C.alpinaA.T.-...A	C.A.....	CA.....	.TA.....T....
C.glaucaA.T.-...A	C.A.....	.A.....	.TA.....T....
C.aurantia(NSW)A.T.-...A	C.A.....	.A.....	.TA.....T....
C.maxgrayiA.T.-...A	C.A.....	.A.....	.TA.....T....
C.costinianaA.T.-...A	C.A.....	.A.....	.TA.....T....
C.lamicolaA.T.-...A	C.A.....	.A.....	.TA.....T....
C.leucanthaA.-....A	C.A.....	.A.....	.TA.....
C.glabrataA.-....A	C.A.....	.A.....	.TA.....
C.variabil(NSW)A.T.-...A	C.A.....	.A.....	.TA.....
C.aurantia(VIC)A.T.-...A	C.A.....	.A.....	.TA.....
C.coolamin(NSW)A.Y.-...A	C.A.....	RA.....	.TA.....K....
C.coolamin(TAS)A.-....A	C.A.....	.A.....	.TA.....
C.albaA.-....A	C.A.....	.A.....	.TA.....
C.subhispidiaA.-....A	C.A.....	.A.....	.TA.....
C."Garibaldi"A.-....A	C.A.....	.A.....	.TA.....
C.robustaA.-....A	C.A.....	.A.....	.TA.....
C."Anglem"A.-....A	C.A.....	.A.....	.TA.....
C.viscosaA.-....A	C.A.....	.A.....	.TA.....
C.incanaA.-....A	C.A.....	.A.....	.TA.....
C.minorA.-....A	C.A.....	.A.....	.TA.....
C."Chatham"A.-....A	C.A.....	.A.....	.TA.....
C.unifloraA.-....A	C.A.....	.A.....	.TA.....
C."incana(Otago)"A.-....A	C.A.....	.A.....	.TA.....
C."Kaitorete"A.-....A	C.A.....	.A.....	.TA.....
C.elongataA.-....A	C.A.....	.A.....	.TA.....
C."incana(Marlb)"A.-....A	C.A.....	.A.....	.TA.....

	490	500	510	520	530	540	550	560
Stu.muel	TCTCCC GTTT	CTTTGATACG	GTTGGCCAAA	ATAGGAGTCC	CCATCGATGG	ACGCACGACT	AGTGGTGGTT	GACAAAACCT
Heli.lan-.....	-.....
Pyc.globAA.....T.....
C.haplorrhizaCA.....T.....	T.....T.T..C.....
C.paludicolaC.....T.....	T.....T.C.....
C."Tunbridge"C.....T.....	T.....T.C.....
C.canensC.....T.....	T.....T.C.....
C.preminghamiC.....T.....	T.....T.CT.A..
C.variabil(VIC)C.....T.....	T.....T.C.....
C.alpinaC.....T.....	T.....T.C.....
C.glaucaC.....T.....	T.....T.C.....
C.aurantia(NSW)C.....T.....	T.....T.C.....
C.maxgrayiC.....T.....	T.....T.C.....
C.costinianaC.....T.....	T.....T.C.....
C.lamicolaC.....T.....	T.....T.C.....
C.leucanthaC.....T.....	T.....T.C.....
C.glabrataC.....T.....T	T.....T.C.....
C.variabil(NSW)C.....T.....	T.....T.C.....
C.aurantia(VIC)C.....T.....	T.....T.C.....
C.coolamin(NSW)C.....T.....	T.....T.C.....
C.coolamin(TAS)C.....T.....	T.....T.C.....
C.albaC.....T.....	T.....T.C.....
C.subhispidiaC.....T.....	T.....T.C.....
C."Garibaldi"C.....T.....	T.....T.C.....
C.robustaC.....T.....	T.....T.	..A.....C.....
C."Angle"C.....T.....	T.....T.	..A.....C.....
C.viscosaC.....T.....	T.....T.	..A.....C.....
C.incanaC..A..T.....	T.....T.	..A.....C.....
C.minorC.....T.....	T.....T.	..A.....C.....
C."Chatham"C.....T.....	T.....T.	..A.....C.....
C.unifloraC.....T.....	T.....T.	..A.....C.....
C."incana(Otago)"C.....T.....	T.....T.	..A.....C.....
C."Kaitorete"C.....T.....	T.....T.	..A.....C.....
C.elongataC.....T.....	T.....T.	..A.....C.....
C."incana(Marlb)"C.....T.....	T.....T.	..A.....C.....

	570	580	590	600	610	620	630	640
Stu.muel	TCGTCTTGCG	TTGTGCGTCT	TGAGTCGTAC	GGGAAGATCT	CTTTAAAGAC	CCCAATGCGT	TGTCTTTC-G	ATGACGCTTC
Heli.lanA.....T..T-
Pyc.globT.A.	.A.....TT	A..TG.....	.A.A.....C.T..-A....
C.haplorrhizaTG.	.A.....T.	.A.T.T...TT..T..	.AC...CA-	G....A....
C.paludicolaG.	.A.....T.	.A.T....TTT..T..	.AC...A-A....
C."Tunbridge"G.	.A.....T.	.A.T....TTT..T..	.AC...A-A....
C.canensG.	.A.....T.	.A.T....TTT..T..	.AC...A-A....
C.preminghamiG.	.A.....T.	.A.T....M	.A.....T..T..	.A....A-A....
C.variabil(VIC)G.	.A.....T.	.A.T....T	.A.....WT..T..	.AY....A-A....
C.alpinaG.	.A.....T.	.A.T....T	.A.....T..T..	.A....A-A....
C.glaucaG.	.A.....T.	.A.T....T	.A.....T..T..	.A....A-A....
C.aurantia(NSW)G.	.A.....T.	.A.T....T	.A.W.....T..T..	.A....A-A....
C.maxgrayiG.	.A.....T.	.A.T....T	.A.....T..T..	.A....A-A....
C.costinianaG.	.A.....T.	.A.T....T	.A.....T..T..	.A....A-A....
C.lamicolaG.	.A.....T.	.A.T....T	.A.T.....T..T..	.A....A-A....
C.leucanthaG.	.A.....T.	.A.T....T	.A.....C..T..	.AC...A-A....
C.glabrataG.	.A.....T.	.A.T....T	.A.....C..T..	.AC...A-A....
C.variabil(NSW)G.	.A.....T.	.A.T....T	.A.....C..T..	.AC...A-A....
C.aurantia(VIC)G.	.A.....T.	.A.T....T	.A.....C..T..	.AC...A-A....
C.coolamin(NSW)G.	.A.....T.	.A.T....T	.AR.....C..T..	.AY....A-A....
C.coolamin(TAS)G.	.A.....T.	.A.T....T	.AR.....C..T..	.AC...A-A....
C.albaG.	.A.....T.	.A.T....T	.A.....C..T..	.AC...A-A....
C.subhispidiaG.	.A.....T.	.A.T....TT..T..	.AC...AAA....
C."Garibaldi"G.	.A.....T.	.A.T....TT..T..	.AC...AAA....
C.robustaG.	.A.....T.	.A.T....TT..T..	.AC...AAA....
C."Anglem"G.	.A.....T.	.A.T....TY..T..	.AC...AAA....
C.viscosaG.	.A...T..T.	.A.T....TT..T..	.AC...AA	...G.A....
C.incanaG.	.A.....T.	.A.T....TY..T..	.AC...AAA....
C.minorG.	.A...T..T.	.A.T....TT..T..	.AC...AA	...G.A....
C."Chatham"G.	.A...T..T.	.A.T....TT..T..	.AC...AA	...G.A....
C.unifloraG.	.A...T..T.	.A.T....TT..T..	.AC...AA	...R.A....
C."incana(Otago)"G.	.A.....T.	.A.T....TC..T..	.AC...AAA....
C."Kaitorete"G.	.A.....T.	.A.T....TY..T..	.AC...AAA....
C.elongataG.	.A.....T.	.A.T....TY..T..	.AC...AAA....
C."incana(Marlb)"G.	.A.....T.	.A.T....TH..T..	.AC...AAA....

Stu.muel	GA
Heli.lan	..
Pyc.glob	..
C.haplorrhiza	..
C.paludicola	..
C."Tunbridge"	..
C.canens	..
C.preminghami	..
C.variabil(VIC)	..
C.alpina	..
C.glauca	..
C.aurantia(NSW)	..
C.maxgrayi	..
C.costiniana	..
C.lamicola	..
C.leucantha	..
C.glabrata	..
C.variabil(NSW)	..
C.aurantia(VIC)	..
C.coolamin(NSW)	..
C.coolamin(TAS)	..
C.alba	..
C.subhispidia	..
C."Garibaldi"	..
C.robusta	..
C."Angle"	..
C.viscosa	..
C.incana	..
C.minor	..
C."Chatham"	..
C.uniflora	..
C."incana(Otago)"	..
C."Kaitorete"	..
C.elongata	..
C."incana(Marlb)"	..

APPENDIX 3: ETS

	10	20	30	40	50	60	70	80
Stu.muel	ATCATTTTGG	CTATTGGCGG	CTTGCCCTCGC	ATTTGAGCTT	-GGTGGGTGG	TTGATAG-TT	GGGTGGTTTC	GGGGATGTCT
Heli.lan	??????????	??????????	?????.A...	-.....C..	CC.T...-..CAA.
Pyc.glob	G.....C...T..T..	-...T.....	C..T...-C.A
C.haplorrhiza	G.....	...A...TT.T.T	..A.....	-...T.....	C..C...-C.A.....
C.paludicola	G.....	...AC..TT.	...A..T..	?...T.....	C..T...?..A...T.
C."Tunbridge"	G.....	...AC.ATT.	...A..T..	-...T.....	C..T...-..A.....
C.canens	G.....	M..AC..TT.	...A..T..	-...T.....	C..T...-..A...T.
C.preminghami	G.....	...AC..T-	...A..T..	T...T.....	C..T...-..A.....
C.variabil(VIC)	G.....	...AC..TT.	...A..T..	T...T.....	C..T...-..A.....
C.alpina	G.....	.C.AC..TT.	...M..T..	-...T.....	C..T...-..A...Y.
C.glauca	G.....	...AC..TT.	...A..T..	T...T.....	C..T...-..A...Y.
C.aurantia(NSW)	G.....	...AC..TT.	...A..T..	T...T.....	C..T...-..A.....
C.maxgrayi	G.....	...AC..TT.	...A..T..	-...T.....	C..T...T..A.....
C.costiniana	G.....	S..AC..TT.	...W..T..	-...T.....	C..T...T..A...Y.
C.lamicola	G.....	...AC..TT.	...A..T..	T...T.....	C..T...-..A.....
C.leucantha	G.....	.A.A...TT.	...A..GT..	...T.....	-...T.....	C..T...-C.A...AT.
C.glabrata	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C..T...-C.A...AT.
C.variabil(NSW)	G.....	...A...TT.	...A..T..	...T...A.	-T...T.....	C..T...T..A...AT.
C.aurantia(VIC)	G.....	...A...TT.	...A..T..	...T...A.	-T...T.....	C..T...T..A...AT.
C.coolamin(NSW)	G.....	...A...TT.	...M..T..	...T.....	-...T.....	C..T...-C.A...AT.
C.coolamin(TAS)	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C..T...-C.A...AT.
C.alba	G.....	...A...TT.	...A..GT..	...T.....	-...T.....	C..T...-C.A...AT.
C.subhispida	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C."Garibaldi"	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C.robusta	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C."Anglem"	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C.viscosa	G.....	...A...TTA	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C.incana	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C.minor	G.....	...A...TTA	...A.T.T.	...T.....	-...T.....	C.AT...-C.A.....
C."Chatham"	G.....	...A...TTA	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C.uniflora	G.....	S..A...TT.	...A..T..	...T.....	-...T.....	C.AT...AGC.A.....
C."incana(Otago)"	G.....	...A...TT.	...A.T.T.	...T.....	-...T.....	C.AT...-C.A.....
C."Kaitorete"	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C.elongata	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....
C."incana(Marlb)"	G.....	...A...TT.	...A..T..	...T.....	-...T.....	C.AT...-C.A.....

	170	180	190	200	210	220	230	240
Stu.muel	ACCTTTTCAG	TCTTGTGTCA	ATGCTTGTAT	CGCATTGAAT	GCACGGTGGG	TTTCTTGTGT	TGCATTCCCTA	ATTGATGGTA
Heli.lanC.....G.....
Pyc.globC.....	.T....C..T..	T..G....CC	A..TT.....A.G....	.GC.....
C.haplorrhizaC.....	.T...CTA..G....TC	...T.....C	.A...G....	.G.....
C.paludicolaC.....	.T.....G....C	...T.....A...G....	.G.....
C."Tunbridge"C.....	.T.....TG....C	...T.....A...G....	.G.....C.
C.canensC.....	.T.....G....C	...T.....A...G....	.G.....
C.preminghamiC.....	.T.....G....C	...T.....A...G....	.G.....
C.variabil(VIC)C.....	.T.....G....C	...T.....A...G....	.G.....
C.alpinaC.....	.T.....G....C	...T.....A...G....	.G.....
C.glaucaC.....	.T.....G....C	...T.....A...G....	.G.....
C.aurantia(NSW)C.....	.T.....G....C	...T.....A...G....	.G.....
C.maxgrayiC.....	.T.....G....C	...T.....A...G....	.G.....
C.costinianaC.....	.T.....G....C	...T.....A...G....	.G.....
C.lamicolaC.....	.T.....G....C	...T.....A...G....	.G.....
C.leucanthaC.....	.T.....TG....C	...T.....	CA-.G....	.G.....
C.glabrataC.....	.T.....C....	..TG....C	...T.....	CA-.G....	.G.....
C.variabil(NSW)C.....	.T.....TG....C	...T.....	CA-.G....	.G.....
C.aurantia(VIC)C.....	.T.....TG....C	...T.....	CA-.G....	.G.....
C.coolamin(NSW)C.....	.T.....TG....C	...T.....	CA-.G....	.G.....
C.coolamin(TAS)C.....	.T.....TG....C	...T.....	CA-.G....	.G.....
C.albaC.....	.T.....TG....C	...T.....	CA-.G....	.G.....
C.subhispidiaC.....	.T.....G....C	...T.....A...G....	.G.....
C."Garibaldi"C.....	.T.....G....C	...T.....A...G....	.G.....
C.robustaC.....	.T.....G....C	...T.....A...G....	.G.....
C."Anglem"C.....	.T.....G....C	...T.....A...G....	.G.....
C.viscosaC.....	.T.....G....C	...T.....A...G....	.G.....
C.incanaC.....	.T.....G....C	...T.....A...G....	.G.....
C.minorC.....	.T.....G....C	...T.....A...G....	.G.....
C."Chatham"C.....	.T.....G....C	...T.....A...G....	.G.....
C.unifloraC.....	.T.....G....C	...T..G...A...G....	.G.....
C."incana(Otago)"C.....	.T.....G....C	...T.....A...G....	.G.....
C."Kaitorete"C.....	.T.....G....C	...T.....A...G....	.G.....
C.elongataC.....	.T.....G....C	...T.....A...G....	.G.....
C."incana(Marlb)"C.....	.T...C....G....C	...T.....A...G....	.G.....

	250	260	270	280	290	300	310	320
Stu.muel	TCGTTTGATG	AATTGATGTC	CATTTT-CGT	CTTGCGTTGC	ACATGCGATG	TGAGATGAAC	CTTAAAGCAG	CCTTTTTGAT
Heli.lan	..A.....	T....--...T..C..G....
Pyc.glob	T..A....C.	T.....-T.....	T....T..C.	CA..G.....	T.G..T...T	A....G....
C.haplorrhizaA.T....	A....T...	..C...C..A.....	.A...T...TG....
C.paludicola	C.....T....	A.....-A....T..TTG....
C."Tunbridge"	C....C....T....	A.....-T..TTG....
C.canens	C.....T....	A.....-A....T..TTG....
C.preminghami	C.?..CA...T....	A.....-A....T..TTG....
C.variabil(VIC)	C....C....T....	A.....-A....T..TTG....
C.alpina	C....C....T....	A.....-A....T..TTG....
C.glauca	C.?..C....T....	A.....-A....T..TTG....
C.aurantia(NSW)	C.A..CR...T....	A.....-A....T..TTG....
C.maxgrayi	C.A..CA...T....	A.....-A....T..TTG....
C.costiniana	C.A..CA...T....	A.....-A....T..TTG....
C.lamicola	C.A..C....T....	A.....-A....T..TTG....
C.leucantha	C.....T....	A.....-TA.....T...T	..G..G....
C.glabrata	C.....T....	A.....-A.....T...T	..G..G....
C.variabil(NSW)	C.....T....	A.....-T...T	..G..G....
C.aurantia(VIC)	C.....T....	A.....-T...T	..G..G....
C.coolamin(NSW)	C.....T....	A.....-A.....T...T	..G..G....
C.coolamin(TAS)	C.....	..C.T....	A.....-A.....T...T	..G..G....
C.alba	C.....T....	A.....-TA.....T...T	..G..G....
C.subhispidia	C.....T....	A.....-A.T...T	..G..G....
C."Garibaldi"	C.....T....	A.....-T...T	..G..G....
C.robusta	C.....T....	A.....-T...T	..G..G....
C."Anglem"	C.....T....	A.....-T...T	..G..G....
C.viscosa	C.....T....	A.....-T...T	..G..G....
C.incana	C.....T....	A.....-T...T	..G..G....
C.minor	C.....T....	A.....-T...T	..G..G....
C."Chatham"	C.....T....	A.....-T...T	..G..G....
C.uniflora	C.....T....	A.....-T...T	..G..G....
C."incana(Otago)"	C.....T....	A.....-T...T	..G..G....
C."Kaitorete"	C.....T....	A.....-T...T	..G..G....
C.elongata	C.....T....	A.....-T...T	..G..G....
C."incana(Marlb)"	C.....T....	A.....-T...T	..G..G....

	330	340	350	360	370	380	390	400
Stu.muel	CCACTCTTTT	GATGCACACG	CATTGATTTT	GTGGCCCAT	GAGTGTGCT	TGGTCTCATG	GATATGGAAC	CTT-GTGTGG
Heli.lanT.....-
Pyc.globA....T..T.CA.C..T....A...C-
C.haplorrhizaA...C	.G.....C.....GT.GC.	A...A...CA....	..C-.....
C.paludicolaA....	.G....C..T.GC.	A.....C-
C."Tunbridge"A....	.G....C..C.....T.GC.	A.....C-
C.canensA....	.G....C..T.GC.	A.....C-
C.preminghamiA....	.G....C..T.GC.	A.....Y-
C.variabil(VIC)A....	.G....C..T.GC.	A.....C-
C.alpinaA....	.G....C..T.GC.	A.....C-
C.glaucaA....	.G....C..T.GC.	A.....T-
C.aurantia(NSW)A....	.G....C..	K...T.GC.	A.....Y-
C.maxgrayiA....	.G....C..	T...T.GC.	A.....C-
C.costinianaA....	.G....C..	T...T.GC.	A.....C-
C.lamicolaA....	.G....C..T.GC.	A.....T-
C.leucanthaAA....	.G.....C.....T.GC.	AT...A...C-
C.glabrataAA....	.G.....C...CT.GC.	AT...A...C-
C.variabil(NSW)A....	.G....T..C...AT.GC.	AT...A...C-
C.aurantia(VIC)A....	.G....T..C...AT.GC.	AT...A...C-
C.coolamin(NSW)AA....	.G.....C...CT.GC.	AT...A...C-
C.coolamin(TAS)AA....	.T.....C.....T.GC.	AT...A...CC.....
C.albaAA....	.G.....C...CT.GC.	AT...A...C-
C.subhispidiaA....	.G.....C...CT.GC.	A...A...C-
C."Garibaldi"A....	.G.....C...CT.GC.	A...A...C-
C.robustaA....	.G.....C...CT.GC.	A...A...C-
C."Anglem"A....	.G.....C...CT.GC.	A...A...C-
C.viscosaA....	.G.....C...CT.GC.	A...A...C-
C.incanaA....	.G.....C...CT.GC.	A...A...WC-
C.minorA....	.G.....C...CT.GC.	A...A...C-
C."Chatham"A....	.G.....C...CT.GC.	A...A...C-
C.unifloraA....	.G.....C...CT.GC.	A...A...C-
C."incana(Otago)"A....	.G.....C...CT.GC.	A...A...WC-
C."Kaitorete"A....	.G.....C...CT.GC.	A...A...C-
C.elongataA....	.G.....C...CT.GC.	A...A...C-
C."incana(Marlb)"A....	.G.....C...CT.GC.	A...A...C-

	410	420	430	440	450	460	470	480
Stu.muel	GCATGGGATC	TTTTATTAGT	TCCTT-GTTT	-GCCCTAGTA	AAGCTTTTT-C	AAATACGATT	TCCTATCATG	TGCT-AATGT
Heli.lanC....-.....-.....T-.....CT.-.C..
Pyc.globCGG...A-.....-.....T-.....G...C	CTT.-.GC..
C.haplorrhizaA..C..TG...A-.....-.....T-.....G...CTT.T.GC..
C.paludicolaA..C..GG...A-.....-.....R.T-.....G...CTT.-.GCA.
C."Tunbridge"A..C..GG...A-.....-.....T-.....G...CTT.-.GCA.
C.canensA..C..GG...A-.....-.....T-.....G...CTT.-.GCA.
C.preminghamiA..C..GG...A-.....-.....K.T-.....G.A..CK.	.TT.-.?CA.
C.variabil(VIC)A..C..GG...A-.....-.....T-.....G...CTT.-.GCA.
C.alpinaA..C..GG...A-.....-.....T-.....G...CTT.-.GCA.
C.glaucaA..C..GG...A-.....-.....T-.....G...CTT.-.TCA.
C.aurantia(NSW)A..C..GG...A-.....-.....T-.....G...CTT.-.TCA.
C.maxgrayiA..C..GG...A-.....-.....T-.....G...CTT.-.TCA.
C.costinianaA..C..GG...A-.....-.....T-.....G...CG.	.TT.-.TCA.
C.lamicolaA..C..GG...A-.....-.....T-.....G...CTT.-.TCA.
C.leucanthaA..C..G...A-.....-.....A.T-.....G...CTT.-.GC..
C.glabrataA..C..G...A-.....-.....A.T-.....G...CTT.-.GC..
C.variabil(NSW)A..C..G...A-.....-.....A.T-.....G...C	CTT.-.GC..
C.aurantia(VIC)A..C..G...A-.....-.....A.T-.....G...C	CTT.-.GC..
C.coolamin(NSW)A..C..G...A-.....-.....A.T-.....G...CTT.-.GC..
C.coolamin(TAS)A..C..G...AC....	C.....A.TT.G...CTT.-.GC..
C.albaA..C..G...A-.....-.....A.T-.....G...CTT.-.GC..
C.subhispidiaA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C."Garibaldi"A..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C.robustaA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C."Anglem"A..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C.viscosaA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C.incanaA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C.minorA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C."Chatham"A..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C.unifloraA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C."incana(Otago)"A..C..TC...A-.....-.....T-.....G...CTT.-.GC..
C."Kaitorete"A..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C.elongataA..C..GC...A-.....-.....T-.....G...CTT.-.GC..
C."incana(Marlb)"A..C..GC...A-.....-.....T-.....G...CTT.-.GC..

	490	500	
Stu.muel	GGTAGGCTAT	ATCGAATATG	AATGCTAC
Heli.lan
Pyc.globT...R....
C.haplorrhiza	.T...A..CT....
C.paludicola	.T...A..CT....
C."Tunbridge"	.C...A..CG....
C.canens	.T...A..CG....
C.preminghami	.T...A..C
C.variabil(VIC)	.T...A..C
C.alpina	.T...A..CG....
C.glauca	.T...A..CR....
C.aurantia(NSW)	.T...A..CG....
C.maxgrayi	.T...A..CG....
C.costiniana	.T...A..CG....
C.lamicola	.T...A..CG....
C.leucantha	.T...A..CG....
C.glabrata	.T...A..CG....
C.variabil(NSW)	.T...A..CG....
C.aurantia(VIC)	.T...A..CG....
C.coolamin(NSW)	.T...A..CG....
C.coolamin(TAS)	.T...A..CG....
C.alba	.T...A..CG....
C.subhispida	.T...A..CG....
C."Garibaldi"	.T...A..CG....
C.robusta	.T...A..CG....
C."Angle"	.T...A..CG....
C.viscosa	.T...A..CG....
C.incana	.T...A..CG....
C.minor	.T...A..CG....
C."Chatham"	.T...A..CG....
C.uniflora	.T...A..CG....
C."incana(Otago)"	.T...A..CG....
C."Kaitorete"	.T...A..CG....
C.elongata	.T...A..CG....
C."incana(Marlb)"	.T...A..CG....

	90	100	110	120	130	140	150	160
Stu.muel	TTTGAACTAA	AAAAGGAGCA	ATAA-TGCC	TCTTGTTTTA	TCAAGAGGGA	AGCTATTGCT	CCTTTTTTTA	TTTACTTACA
Heli.lan
Pyc.globG.
C.haplorrhiza	GC.T...	...A.AA.	A.....C	..-T.....G.
C.paludicola	GC.T...	...A.AA.	A.....C	..-T.....G.
C."Tunbridge"	GC.T...	...A.AA.	A.....C	..-T.....G.
C.canens	GC.T...	...A.AA.	A.....C	..-T.....G.
C.preminghami	GC.T...	...A.AA.	A.....C	..-T.....G.
C.variabil(VIC)	GC.T...	...A.AA.	A.....C	..-T.....G.
C.alpina	GC.T...	...A.AA.	A.....C	..-T.....G.
C.glauca	GC.T...	...A.AA.	A.....C	..-T.....G.
C.aurantia(NSW)	GC.T...	...A.AA.	A.....C	..-T.....G.
C.maxgrayi	GC.T...	...A.AA.	A.....C	..-T.....G.
C.costiniana	GC.T...	...A.AA.	A.....C	..-T.....G.
C.lamicola	GC.T...	...A.AA.	A.....C	..-T.....G.
C.leucantha	GC.T...	...A.AA.	A.....C	..-T.....G.
C.glabrata	GC.T...	...A.AA.	A.....C	..-T.....G.
C.variabil(NSW)	GC.T...	...A.AA.	A.....C	..-T.....G.
C.aurantia(VIC)	GC.T...	...A.AA.	A.....C	..-T.....G.
C.coolamin(NSW)	GC.T...	...A.AA.	A.....C	..-T.....G.
C.coolamin(TAS)	GC.T...	...A.AA.	A.....C	..-T.....----
C.alba	GC.T...	...A.AA.	A.....C	..-T.....G.
C.subhispidia	GC.T...	...A.AA.	A.....C	..-T.....G.
C."Garibaldi"	GC.T...	...A.AA.	A.....C	..-T.....G.
C.robusta	GC.T...	...A.AA.	A.....C	..-T.....G.
C."Anglem"	GC.T...	...A.AA.	A.....C	..-T.....----
C.viscosa	GC.T...	...A.AA.	A.....C	..-T.....G.
C.incana	GC.T...	...A.AA.	A.....C	..-T.....G.
C.minor	GC.T...	...A.AA.	A.....C	..-T.....G.
C."Chatham"	GC.T...	...A.AA.	A.....C	..-T.....G.
C.uniflora	GC.T...	...A.AA.	A.....C	..-T.....G.
C."incana(Otago)"	GC.T...	...A.AA.	A.....C	..-T.....G.
C."Kaitorete"	GC.T...	...A.AA.	A.....C	..-T.....G.
C.elongata	GC.T...	...A.AA.	A.....C	..-T.....G.
C."incana(Marlb)"	GC.T...	...A.AA.	A.....C	..-T.....G.

	170	180	190	200	210	220	230	240
Stu.muel	T-----	-----	-----	-----	-----	-----AA	TTTCTTTAAA	AGTAACAAGG
Heli.lan	.-----	-----	-----	-----	-----	-----
Pyc.glob	.-----	-----	-----	-AATTTTTTT	ATTTACTTAC	-ATAATAT..	-----
C.haplorrhiza	.TAGAT----	-----	-----TAGA	TAATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.paludicola	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C."Tunbridge"	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.canens	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.preminghami	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.variabil(VIC)	.TAGAT----	-----TTAC	TTAGATTAGA	TAATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.alpina	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.glauca	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.aurantia(NSW)	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.maxgrayi	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.costiniana	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.lamicola	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.leucantha	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.glabrata	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.variabil(NSW)	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.aurantia(VIC)	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.coolamin(NSW)	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.coolamin(TAS)	-----	-----	-----	-----	-----AAAA	TAGAATAA..T
C.alba	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TAGAATAA..T
C.subhispidia	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C."Garibaldi"	.TAGATAAATT	TTTTATTTAC	TTAGATTAGA	TAATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C.robusta	.TAGATAAATT	TTTTATTTAC	TTAGATTAGA	TAATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C."Anglem"	-----	-----	-----	-----	-----AAAA	TGGAATAA..T
C.viscosa	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C.incana	.TAGATAAATT	TTTTATTTAC	TTAGATTAGA	TAATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C.minor	.TAGATAAATT	TTTTATTTAC	TTAGATTAGA	TAATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C."Chatham"	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C.uniflora	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C."incana(Otago)"	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C."Kaitorete"	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C.elongata	.TAGAT----	-----	-----	-AATTTTTTT-	ATTTACAAAA	TGGAATAA..T
C."incana(Marlb)"	.TAGATAAATT	TTTTATTTAC	TTAGATTAGA	TAATTTTTTT-	ATTTACAAAA	TGGAATAA..T

	250	260	270	280	290	300	310	320
Stu.muel	ACTTTTTATA	GTTT-----	--GGTTGGAT	TCGCGTATTT	TTTCTTTGTA	TTTTTTCATT	TATATTATAG	GTTTCTATT-
Heli.lan	-----	-----C.....-
Pyc.glob	-.....	-----	-----	-----	-----	-----	-----
C.haplorrhizaTATAGT	TT.....C.A.G.A..--	-----	-----
C.paludicola	-----	--.....C.....-
C."Tunbridge"	-----	--.....C.....-
C.canens	-----	--.....C.....-
C.preminghami	-----	--.....C.....-
C.variabil(VIC)	-----	--.....C.....-
C.alpina	-----	--.....C.....-
C.glauca	-----	--.....C.....-
C.aurantia(NSW)	-----	--.....C.....-
C.maxgrayi	-----	--.....C.....-
C.costiniana	-----	--.....C.....-
C.lamicola	-----	--.....C.....-
C.leucantha	-----	--.....C.....-
C.glabrata	-----	--.....C.....-
C.variabil(NSW)	-----	--.....C.....T
C.aurantia(VIC)	-----	--.....C.....-
C.coolamin(NSW)	-----	--.....C.....T
C.coolamin(TAS)	-----	--.....C.....-
C.alba	-----	--.....C.....-
C.subhispidia	-----	--.....C.....T
C."Garibaldi"	-----	--.....C.....T
C.robusta	-----	--.....C.....T
C."Anglem"	-----	--.....C.....T
C.viscosa	-----	--.....C.....T
C.incana	-----	--.....C.....T
C.minor	-----	--.....C.....T
C."Chatham"	-----	--.....C.....T
C.uniflora	-----	--.....C.....C.....T
C."incana(Otago)"	-----	--.....C.....T
C."Kaitorete"	-----	--.....C.....T
C.elongata	-----	--A.....C.....T
C."incana(Marlb)"	-----	--.....C.....T

	330	340	350	360	370	380	390	400
Stu.muel	-----	TCTATTCTAT	ATCTTTTTCT	TAATCTTTTA	AGAAGTTTTA	TTTCCT----	--ATTCAATT	TCAATCGAAA
Heli.lan	-----C.....	T.....A----	-----
Pyc.glob	-----	-----	-----	-----	-----	-----	-----	-----
C.haplorrhiza	-----	-----	-----	-----	T.-----	-----	-----	-----
C.paludicola	-----CTATTC.....	T.....A----	-----
C."Tunbridge"	-----CTATTC.....	T.....A----	-----
C.canens	-----CTATTC.....	T.....A----	-----
C.preminghami	-----CTATTC.....	T.....ATTTC	CA.....
C.variabil(VIC)	-----CTATTC.....	T.....A----	-----
C.alpina	-----CTATTC.....	T.....A----	-----
C.glauca	-----CTATTC.....	T.....A----	-----
C.aurantia(NSW)	-----CTATTC.....	T.....A----	-----
C.maxgrayi	-----CTATTC.....	T.....A----	-----
C.costiniana	-----CTATTC.....	T.....A----	-----
C.lamicola	-----CTATTC.....	T.....A----	-----
C.leucantha	-----CTATT	...G.....	...C.....	T.....A----	-----
C.glabrata	-----CTATTC.....	T.....A----	-----
C.variabil(NSW)	CTATTCTATTC.....	T.....A----	-----
C.aurantia(VIC)	-----CTATTC.....	T.....A----	-----
C.coolamin(NSW)	CTATTCTATTC.....	T.....A----	-----
C.coolamin(TAS)	-----CTATTC.....	T.....A----	-----
C.alba	-----CTATTC.....	T.....A----	-----
C.subhispidia	CTATTCTATTC.....	T.....A----	--.G.....C...
C."Garibaldi"	CTATTCTATTC.....	T.....A----	--.G.....C...
C.robusta	CTATTCTATTC.....	T.....A----	--.G.....C...
C."Anglem"	CTATTCTATTC.....	T.....A----	--.G.....C...
C.viscosa	CTATTCTATTC.....	T.....A----	--.G.....C...
C.incana	CTATTCTATTC.....	T.....A----	--.G.....C...
C.minor	CTATTCTATTC.....	T.....A----	--.G.....C...
C."Chatham"	CTATTCTATTC.....	T.....A----	--.G.....C...
C.uniflora	CTATTCTATTC.....	T.....A----	--.G.....C...
C."incana(Otago)"	CTATTCTATTC.....	T.....A----	--.G.....C...
C."Kaitorete"	CTATTCTATTC.....	T.....A----	--.G.....C...
C.elongata	CTATTCTATTC.....	T.....A----	--.G.....C...
C."incana(Marlb)"	CTATTCTATTC.....	T.....A----	--.G.....C...

	410	420	430	440	450	460	470	480
Stu.muel	AT-AGATAAA	A-TTTTTAAT	TTTGCTTATT	TATTA--CT-	-----	-----	-----	-----
Heli.lan	..-.....	.A-.....	-----	-----	-----	-----
Pyc.glob	-.A...A--	-----	-----	-----	-----	-----	-----
C.haplorrhiza	-----	.A.-----	-----	-----	-----	-----	-----	-----
C.paludicola	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C."Tunbridge"	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	A-ATTAT---
C.canens	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.preminghami	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	A-ATTATT--
C.variabil(VIC)	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	A-ATTATT--
C.alpina	..-.....	.A.....	...T.....TT..A	TTATT-CT--	-----	-----	--ATTATT--
C.glauca	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.aurantia(NSW)	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.maxgrayi	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.costiniana	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.lamicola	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.leucantha	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	-----	A A-ATTATT--
C.glabrata	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.variabil(NSW)	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.aurantia(VIC)	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.coolamin(NSW)	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.coolamin(TAS)	..-.....	.A.....	...T.....TT..A	TTAGT-----	-----	-----	-----
C.alba	..-.....	.A.....	...T.....TT..A	TTATT-----	-----	-----	-----
C.subhispidia	..-.....	.AA.....	...T.....TTA.A	TTATT-CTTA	TTTATTATT-	-----	ATATTATTAA
C."Garibaldi"	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	-----	A ATATTATTAA
C.robusta	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	-----	A ATATTATTAA
C."Angle"	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	-----	A ATATTATTAA
C.viscosa	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	--AA-TATT-	ATATTATTAA
C.incana	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	-----	A ATATTATTAA
C.minor	..-.....	.AA.....	...T.....TTA.A	TTATT-CTTA	TTTATTATTA	-T-ATTATTA	ATATTATTAA
C."Chatham"	..-.....	.AA.....	...T.....TTA.A	TTATTA---A	--TATTA--A	TTAA-TATT-	ATATTATTAA
C.uniflora	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	--AA-TATT-	ATATTATTAA
C."incana(Otago)"	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	--AATTATTA	ATATTATTAA
C."Kaitorete"	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	--AATTATTA	ATATTATTAA
C.elongata	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	--AATTATTA	ATATTATTAA
C."incana(Marlb)"	..-.....	.AA.....	...T.....TTA.A	TTATT-----	-----	-----	A ATATTATTAA

	490	500	510	520	530	540	550	560
Stu.muel	-----	-----TTT	ATTTT-----	-----	-----	-----ATAA	ATAAGAACTA	GAAAATAGTA
Heli.lan	-----	-----	-----	-----	-----	T.....
Pyc.glob	-----	---T-CT..-	-----	-----	-----	-----
C.haplorrhiza	-----	-----	-----	-----	-----	-----
C.paludicola	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C."Tunbridge"	----AATTAT	TATTACT...TAAAT	TTATTTTATAA	ATTT-----	-----
C.canens	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.preminghami	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.variabil(VIC)	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.alpina	----AATTAT	TATTACT...TAAAT	TTCTTT----	-----	-----
C.glauca	----AATTAT	TATTACT...TAAAT	TTCTTT----	-----	-----
C.aurantia(NSW)	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.maxgrayi	----AATTAT	TATTACT...TAAAT	TTCTTT----	-----	-----
C.costiniana	----AATTAT	TATTACT...TAAAT	TTCTTT----	-----	-----
C.lamicola	----AATTAT	TATTACT...TAAAT	TTCTTT----	-----	-----
C.leucantha	----AATTAT	TATTACT...	...TAATT	-----	-----	-----
C.glabrata	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.variabil(NSW)	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.aurantia(VIC)	----AATTAT	TATTACT...TAAAT	TTATTT----	-----	-----
C.coolamin(NSW)	----AATTAT	TATTACT...TAAAT	TTATTTTATA-	-TT-----	-----G.
C.coolamin(TAS)	----AATTAT	TATTACT...TAAAT	TTATTTTATAA	-TTT-----	-----
C.alba	----AATTAT	TATTACT...TAAAT	TTCTTT----	-----	-----
C.subhispidia	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C."Garibaldi"	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C.robusta	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C."Anglem"	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C.viscosa	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C.incana	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C.minor	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C."Chatham"	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C.uniflora	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----
C."incana(Otago)"	TATTAATTAT	TATTACT...	...TAATT	TT-----	----TATTTT	AATTTT....
C."Kaitorete"	TATTAATTAT	TATTACT...	...TAATT	TT-----	----TATTTT	-----
C.elongata	TATTAATTAT	TATTACT...	...TAATT	TT-----	----TATTTT	-----
C."incana(Marlb)"	TATTAATTAT	TATTACT...	...TAATT	TT-----	-----	-----

	570	580	590	600
Stu.muel	GA-----	GGGGCGGATG	TAGCCAAGTG	GATCAAGGCA GTG
Heli.lan	-.TAGTAGA-
Pyc.glob	..-----
C.haplorrhiza	..-----G
C.paludicola	..-----G
C."Tunbridge"	..-----G
C.canens	..-----G
C.preminghami	..-----G
C.variabil(VIC)	..-----G
C.alpina	..-----G
C.glauca	..-----G
C.aurantia(NSW)	..-----G
C.maxgrayi	..-----G
C.costiniana	..-----G
C.lamicola	..-----G
C.leucantha	..-----G
C.glabrata	..-----G
C.variabil(NSW)	..-----G
C.aurantia(VIC)	..-----G
C.coolamin(NSW)	..-----G
C.coolamin(TAS)	..-----G
C.alba	..-----G
C.subhispidia	..-----G
C."Garibaldi"	..-----G
C.robusta	..-----G
C."Anglem"	..-----G
C.viscosa	..-----G
C.incana	..-----G
C.minor	..-----G
C."Chatham"	..-----G
C.uniflora	..-----G
C."incana(Otago)"	..-----G
C."Kaitorete"	..-----G
C.elongata	..-----G
C."incana(Marlb)"	..-----G

