Evolution and Conservation of *Commidendrum* and *Elaphoglossum* from St Helena.

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Declaration

I declare that this thesis has been composed by myself and based on work, unless otherwise stated, that I have undertaken.

Antonia Eastwood, October 2002

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Abstract

St Helena is an isolated volcanic island (lat. 15° 56′S, long. 5°42′W) in the South Atlantic Ocean. The endemic flora of St Helena, comprised of 49 plant species, is considered to be one of the most threatened in the world. This thesis investigates the evolution and conservation of two threatened groups of plants endemic to St Helena: i) trees in the genera *Commidendrum* and *Melanodendron* (Asteraceae) and ii) epiphytic and terrestrial elaphoglossoid ferns in the genera *Elaphoglossum* and *Microstaphyla* (Lomariopsidaceae)

Chapter two investigates species relationships of *Commidendrum* and *Melanodendron* using the ITS region of ribosomal DNA. Despite showing a range of morphological and ecological variation the four species of *Commidendrum* form a closely related monophyletic group. *Melanodendron integrifolium* is sister to *Commidendrum* indicating that the two genera evolved from a common ancestor which arrived to St Helena via a single dispersal event. The role of heterochrony in the evolution of *Commidendrum* is discussed.

Chapter three investigates self-incompatibility and hybridisation in two of the most threatened *Commidendrum* species, *C. rotundifolium* and *C. spurium*. RAPD data indicated the presence of hybrids in the seed orchards of *C. rotundifolium* and *C. spurium*. Self-incompatibility in *C. rotundifolium* and *C. spurium* was investigated using a series of pollination experiments which examined pollen-stigma interaction at the stigma interface. Both *C. rotundifolium* and *C. spurium* possess a sporophytic self-incompatibility system, and poor seed viability in *C. rotundifolium* is due to a paucity of S-alleles. The conservations implications of this and inter-specific hybridisation are discussed.

Chapter four investigates the evolutionary relationships of the four elaphoglossid ferns, *E. dimorphum*, *E. nervosum*, *E. conforme* and *M. furcata* from St Helena using sequences of the chloroplast *trn*L intron (partial) and *trn*L-F intergenic spacer. The investigation revealed the close relationship of *E. nervosum*, *E. dimorphum* and *M. furcata*, whilst *E. conforme* was found to be distantly related. *Microstaphyla furcata* is shown to belong to *Elaphoglossum* confirming the previous transfer of this species to *Elaphoglossum bifurcatum*.

Species relationships of the endemic *Elaphoglossum*, and the extent and distribution of population genetic diversity were investigated using allozyme analysis in chapter five. As well as supporting the relationships of the taxa in the molecular phylogeny, the allozyme data suggest a hybrid origin of *E. dimorphum* between *E. nervosum* and *E. bifurcatum*. In addition the allozyme data revealed significant genetic differentiation in populations of *E. nervosum* and *E. bifurcatum* which should be taken into consideration in any future conservation programme.

To conclude, Chapter 5 is a general discussion on the evolution and conservation of island plants, highlighting my research findings from St Helena and comparing it to other studies.

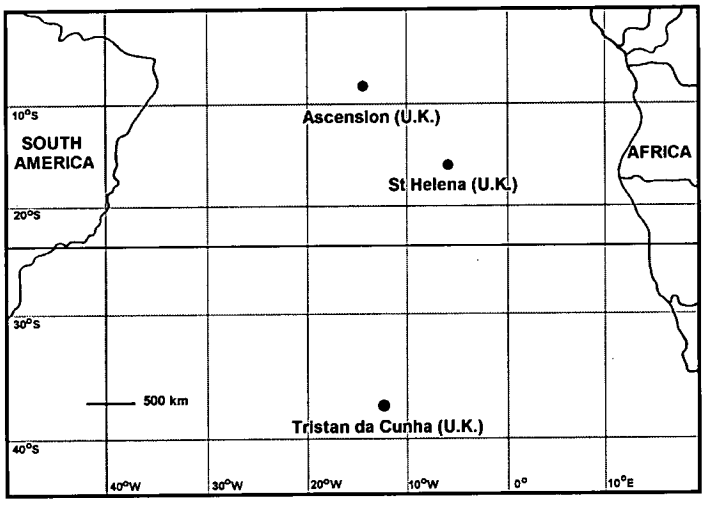
General introduction

1.1 St Helena

Geology, topography and climate

St Helena is a small (122km^2) volcanic island in the South Atlantic Ocean (lat. 15° 56′S, long 5°42′W). The island is extremely isolated, lying some 1, 931 km from Africa and 1,127 km from Ascension Island, the nearest land mass (Fig.1.1). St Helena is a dependent territory of the United Kingdom and has a population of about 5,500.

Fig. 1.1: Locality of St Helena in the South Atlantic Ocean.

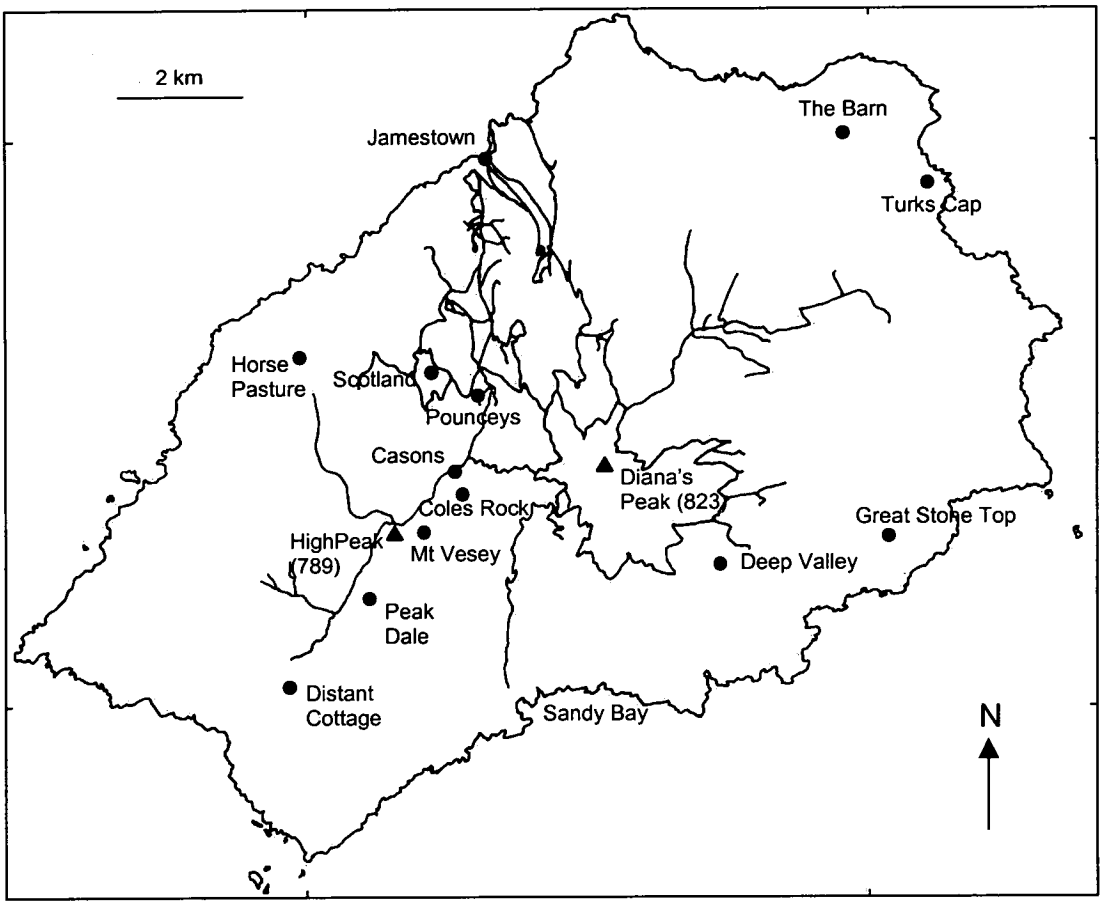


St Helena forms the top levels of a volcanic pile which rises more than 5 km above the ocean floor (Baker, 1970). The island was formed over a period of 7 million years by the coalescence of two basaltic shield-volcanoes beginning some 14.3 ± 1.0 million years ago (Baker, 1968; Baker, 1970). The complexity of the island's geomorphology which is apparent today is a result of late-stage volcanic activity, followed by uplift, subsequent erosion and subsidence (Nunn, 1994). Although the majority of the island is composed of alkali balsalts, erosion has exposed areas of trachytic-phonolitic rock formed by intrusions.

As a consequence of its geological history the topography of St Helena is remarkably varied and complex. The coast is composed of precipitous cliffs intersected by deep gorges and

valleys. Rolling hills, valleys and plains dominate the central uplands, which culminate in the sickle shaped central ridge of which Diana's Peak (823m) is the summit.

Fig. 1.2: Map of St Helena showing roads and some localities



St Helena is swept by the south-east trade wind which makes the climate temperate and equable, despite the tropical latitude (Cronk, 2000). The main current, the Benguela current, being wind driven is also from the south-east. The local climate on St Helena does, however, vary considerably, being influenced greatly by altitude. The coastal regions experience much drier, sunnier and warmer conditions than the central uplands and peaks. In Jamestown, on the coast, annual rainfall is *c.* 200 mm whilst on the peaks it is *c.* 1,200 (Cronk, 2000). The peaks (High Peak, Cuckold's Point, Diana's Peak and Mt Actaeon) are often shrouded in mist and can be 5°C-10°C cooler than Jamestown.

The island's vegetation

The vegetation of St Helena and its constituent flora has been greatly altered since the island's discovery in 1502 by the Portuguese, João da Nova. Unfortunately, very few reliable botanical records on the vegetation of St Helena exist before the 19th century and by then a large proportion of indigenous vegetation had already been destroyed (Cronk, 1984). An excellent historical account of the islands vegetation, ecology and destruction is given in Cronk (2000). From historical manuscripts, botanical records and the distribution of relict populations, Cronk (1989) developed a hypothetical reconstruction of the vegetation of St Helena prior to its discovery. Cronk divided the vegetation into 7 main types: 1) Tree-fern thicket, 2) Cabbage-tree woodland, 3) Moist gumwood woodland, 4) Dry gumwood woodland, 5) Ebony-gumwood thicket, 6) Scrubwood scrub and 7) Saline semi-desert. For a full list of the main and associated species see Cronk (1989). Today only approximately 1% of the island's cover is native vegetation. This indigenous vegetation, comprised largely of endemic species, occurs in a number of isolated patches at Diana's Peak, High Peak and Peak Dale. Remnants of Gumwood woodland, *Commidendrum robustum* (Roxb.) DC., which once would have covered St Helena at mid-altitudes, can now be only found at Peak Dale, restricted to around 1,000 individuals. These fragmented patches of vegetation are heavily invaded by exotic species such as *Phormium tenax* Forst (New Zealand Flax) and *Solanum mauritianum* Blanco. The non indigenous vegetation on St Helena is largely comprised of naturalised scrub, an invasive monoculture of *Phormium tenax*, forestry plantations and pastureland. In addition, there are also vast barren areas of heavily degraded and eroded slopes, known as the Crown Wastes (Fig. 1.3)

Fig. 1.3: The Crown Wastes, a vast area of heavily degraded and eroded slopes.

Origins and evolution of the endemic flora

The origins and evolution of the flora on oceanic islands and archipelagos have intrigued botanists since the days of European exploration and discovery in the 19th century. Isolated oceanic islands often have a unique flora comprising taxonomically isolated groups whose closest relatives are often difficult to elucidate. For example, although the flora of St Helena has relatively low species richness, with only 60 indigenous species, it contains 10 isolated endemic genera (Cronk, 1997).

According to Cronk (1997) there are two alternative hypotheses, with a continuum between the two extremes, to interpret the taxonomic isolation of insular endemics: paleo-endemism and neo-endemism. A paleo-endemic species is one whose endemism is attributed to the *ex situ* extinction of ancestral taxa and descendants from the original source area (Cronk, 1997). Paleo-endemics will tend to exhibit great taxonomic and geographic disjunctions and the sister group will be located far from the original source area. In contrast the endemism of a neo-endemic species is attributed to *in situ* evolution and, as a consequence, the sister group is likely to be still present in the original source area (Cronk, 1997). The essential distinction between the two types of endemism lies in the relative contribution of *in situ* evolution and *ex situ* extinction to the resulting endemism (Cronk, 1992).

Under the assumption that the endemic species of St Helena originated from Africa, Cronk (1987, 1992) indirectly inferred the ages of the endemics from geographical, taxonomic, paleo-climatic, paleo-ecological and ecological evidence. Table 1.1 below lists the endemics of St Helena according to the relictual hypothesis by Cronk. The ancient relics, or paleo-endemics (> 10 Myr BP), which includes the arborescent composites or cabbage trees, are isolated endemic genera whose closest extant relatives are South American or Australasian (Cronk, 1992). The ancestors of these ancient relics are thought to be South African in origin, which became extinct due to climatic change in the late Miocene (Coetzee, 1993). In contrast, the recent relics, or neo-endemics have close relatives in South Africa, with little taxonomic or geographical disjunction between them. They date from the late Pliocene or Pleistocene age (0-2.4 Myr BP) and are generally herbs of dry, arid habitats.

The presence of insular endemics on the Hawaiian Islands has been demonstrated to be due to rapid autochthonous evolution through speciation processes such as adaptive radiation (see Wagner & Funk, 1995). Carlquist (1995) contests the relictual hypothesis by Cronk and postulates that the patterns of evolution on Atlantic islands will change to resemble the

findings from the Hawaiian Islands as DNA analysis and cladistic methods are applied. However, as Cronk (1997) justifiably argues that the concept of relict endemism is independent of adaptive radiation as it is concerned with the source and coloniser lineages rather than post colonisation events. The nature of endemism on St Helena can, therefore, only be determined with extensive outgroup sampling and the application of a molecular clock. Under the concept of paleo-endemism the time of divergence between the island endemic and the closest extant relative would probably predate the formation of the island (Cronk, 1997).

Table 1.1: Types of St Helena endemics (adapted from Cronk, 1987 and 1992)

Genus	Species	Geography of related species
Recent relics (2.4 - 0 Myr BP)		
Chenopodium	1	Pantropical
Euphorbia	1	Pantropical
Hydrodea	1	S. Africa
Hypertelis	1	S. Africa
Bulbostylis	2	Africa
Osteospermum	1	S. Africa
Pelargonium	1	S. Africa
Eragrostis	1	S. Africa
Sub-ancient relics (10 - 2.4 Myr BP)		
Sium	2	Africa
Heliotropium	1	Pantropical
Acalypha	1	Mascarenes
Phylica	1	Mascarenes, S. Ocean Is.
Carex	2	African Mts, Mascarenes
Frankenia	1	Subtropics of S. hemisphere
Plantago	1	Pacific Is, S. America, Africa
Wahlenbergia	4	S. America, Australasia, S. Africa
Trochetiopsis	2	Mascarenes, Madagascar
Ancient relics (> 10 Myr Bp)		
Nesiota	1	Africa, Mascarenes
Nesohedyotis	1	India, Malaysia
Melissa	1	S. America
Commidendrum	4	Australasia, S. America
Melanodendron	1	Australasia, S. America
Trimeris	1	S. America, Pacific Is.
Lachanodes	1	S. America , Australasia
Pladaroxylon	1	S. America , Australasia
Petrobium	1	French Polynesia

Endemic flora and conservation

According to Cronk (2000) there are 49 endemic plant species on St Helena (not including Bryophyta), of which four are known to be extinct. The flora of St Helena is the most threatened in the world, with 41% of the indigenous species classified as threatened (Walter

& Gillett, 1998). Some of the endemic species are classified as Extinct in the Wild, according to the IUCN categories of threat (Hilton-Taylor, 2000). This includes the Bastard Gumwood, *Commidendrum rotundifolium* (Roxb.) DC. and the St Helena Olive, *Nesiota elliptica* (Roxb.) Hook.f., which can now only be found in cultivation. Other species, for example the St Helena Ebony, *Trochetiopsis melanoxylon* (Sol. ex Sims) Marais are Critically Endangered, restricted to only a few individuals in the wild. The main threats to the endemic flora of St Helena include small population sizes, habitat fragmentation, competition with exotic invasive flora, pests and diseases (exotic and indigenous) and grazing.

Fig. 1.4: Endemic vegetation at High Peak. Note encroachment of the invasive exotic *Solanum mauritianum* (top right)

The Environmental and Conservation Section (ECS) of the Agriculture and Natural Resources Department is responsible for individual species conservation as well as habitat management and restoration. The species team propagates Critically Endangered species (through seed and vegetative propagules) for reintroduction programmes, maintains several seed orchards, collects and stores germplasm for *ex situ* conservation and assists in reintroduction programmes. The habitat management team is responsible for the restoration and management of endemic habitats which includes Diana's Peak National Park and the Gumwood woodland at Peak Dale. For the past 7 years there has been an ambitious programme of weed eradication and habitat restoration in Diana's Peak National Park. Large tracts of impenetrable flax and other exotics have been cleared and tree-fern thicket has begun to regenerate into these areas. In 2000 an ambitious reforestation project, the Millennium Gumwood forest, was undertaken. Members of the public, school children and

representatives of community organisations were invited to plant a Gumwood at a prepared site at Horse Point. To date 368 trees have been planted at Horse Point.

1.2 The study groups: *Commidendrum* and *Elaphoglossum*

The threatened species investigated in this thesis can be split into two distinct groups, each with different life history characteristics. The first group are arborescent composites (Asteraceae) in the tribe Astereae. It includes all four species of *Commidendrum* DC. and the only species in the monotypic genus *Melanodendron* DC., *Melanodendron integrifolium* (Roxb.) DC. Both *Commidendrum* and *Melanodendron* are endemic genera to St Helena. *Melanodendron* has been included in this group as it is thought to be a close relative of *Commidendrum* (Carlquist, 1965; Bentham, 1873a).

The second group are terrestrial and epiphytic elaphoglossoid ferns (Lomariopsidaceae). It includes three *Elaphoglossum* Schott *ex* J. Smith species; *E. conforme* (Sw.) Schott, *E. nervosum* (Bory) H. Christ, *E. dimorphum* (Hook. & Grev.) Moore and one species of *Microstaphyla* Presl, *M. furcata* (L.f.) Fée. All these fern species are endemic to St Helena apart from *E. conforme* which is indigenous and whose distribution includes Ghana, Liberia, Tristan da Cunha and South Africa.

A list of the threatened species investigated in this thesis, with their conservation status according to the IUCN Categories of Threat (IUCN, 1994), is given in Table 1.2. The next two sections will examine these two groups of threatened species in detail, highlighting a number of biological (evolutionary and genetic) questions which may be relevant to their conservation on St Helena.

Table 1.2: List of study species and conservation status according to the IUCN Categories of Threat (1UCN, 1994), taken from and adapted from Walter & Gillett (1998) and Hilton-Taylor (2000)

Species	Conservation Status
Commidendrum robustum ssp. *robustum*	Endangered (EN, B1+2c)
Commidendrum robustum ssp. *gummiferum*	Extinct (EX)
Commidendrum rotundifolium	Extinct in the Wild (EW)
Commidendrum rugosum	Vulnerable (VU, D2)
Commidendrum spurium	Critically Endangered (CR, D)
Melanodendron integrifolium	Vulnerable (VU, D2)
Elaphoglossum conforme	non endemic
Elaphoglossum dimorphum	Critically Endangered (CR, D)
Elaphoglossum nervosum	Endangered (EN, D)
Microstaphyla furcata	Vulnerable (VU, D2)

The Commidendrum Group

The two endemic genera, *Commidendrum* and *Melanodendron*, are small trees and shrubs belonging to the Asteraceae family, tribe Astereae. The close affinity of *Commidendrum* with *Melanodendron* has been noted (Bentham, 1873a; Bentham, 1873b) and the two genera are thought to be closely related (Cronk, 2000). *Commidendrum* and *Melanodendron* exhibit a wide range of ecological and morphological variation which has, until even fairly recently, led some taxonomists to question the current classification of *Commidendrum* (Nesom, 1994). The taxonomic treatment of *Commidendrum* and *Melanodendron* used below is according to Cronk (2000).

Commidendrum robustum (Roxb.) DC. subsp. ***robustum*** Prod. 5: 344 (1836)
Vernacular name: Gumwood
Endangered (EN, B1 + 2c)

Synonyms:
Conyza robusta Roxb. in Beatson, Tracts: 304 (1816)
Aster roxburghii Hook.f., Hook. Ic. Pl. 11: t.1057 (1870)

Commidendrum robustum subsp. *robustum*, commonly known as Gumwood, is a small tree up to 8 metres high. It has pendulous white capitula (1.5cm across) and green/grey lanceolate leaves which are white floccose below.

Fig. 1.5: *Commidendrum robustum* from Peak Dale.

Inflorescences (one to a few capitula) are formed at terminal ends of branches. Figure 1.5 shows the inflorescence and leaves of *C. robustum* subsp. *robustum* from Peak Dale. *C.*

robustum subsp. *robustum* was once the main constituent of woodland that covered St Helena at mid-elevations (400-600m) (Cronk, 1986; Hemsley, 1885). For a full account of the decline of the St Helena Gumwood, *C. robustum*, see Cronk (1986). Today the species is considered Endangered according to the IUCN Categories of Threat (Hilton-Taylor, 2000). The only remnants of Gumwood woodland can be found between Luffkins and Peak Dale where there is a population of approximately 1,000 trees. In 1980, browsing and debarking by sheep threatened this population by preventing regeneration (Cronk, 2000). This was largely rectified by the construction of a fence which separated Peak Dale from the Crown Wastes. In the early 1990s the introduced scale insect, *Orthezia insignis* Browne, threatened to destroy this woodland (Smith, 1997) (Fig. 1.6). Fortunately, a biological control programme, using the agent *Hyperaspis panthera* (Coleoptera: Coccinellidae) was largely successful at controlling the scale insect.

Fig. 1.6: *Commidendrum robustum* infested with the introduced scale insect, *Orthezia insignis*.

There are a number of *C. robustum* populations at other localities on St Helena. However, these populations are very small (<25) and like the population at Peak Dale are heavily encroached by exotic species such as *Olea africana* (Black olive) and *Phormium tenax* (New Zealand flax). In addition, at Deep Valley and Maria's, there is morphological evidence to suggest that *C. robustum* hybridises with *Commidendrum rugosum*.

Over the last 20 years the ECS has replanted areas of degraded land, at Horse Point and High Peak, with *C. robustum* and augmented existing populations at Peak Dale and Deep Valley.

C. robustum (Roxb.) DC. subsp. ***gummiferum*** (Roxb.) Cronk Bull. Br. Mus. (Nat. Hist.), Bot. 25: 98 (1995)

Vernacular name: Burchell's gumwood or Cluster-leaved gumwood

Extinct (EX)

Synonyms:

Conzya gummifera Roxb. in Beatson, Tracts: 304 (1816)

Commidendrum gummiferum (Roxb.) DC., Prod. 5: 344 (1836)

Aster gummiferus Hook.f. [=*Commidendrum spurium*, according to Cronk, 2000] var. B, Hook.f., Hook. Ic. Pl. 11: t.1056 (1870)

Aster burchellii Hook.f., Hook. Ic. Pl. 11: t.1056 (1870)

Commidendrum burchellii (Hook.f.) Bentham & Hook.f. *ex* Hemsl., Bot. Voy. Challenger 1 (2): 71 (1884)

C. robustum (Roxb.) DC., sensu Hemsl., Bot. Voy. Challenger 1 (2): 71 (1884), pro parte.

This subspecies of *Commidendrum robustum* is now considered extinct (EX) (Cronk, 2000). However, as Cronk (2000) points out, a more accurate category would be subextinct as its genes have introgressed into *C. robustum* ssp. *robustum*. *Commidendrum robustum* subsp. *gummiferum* is similar to *C. robustum* subsp. *robustum* but slightly smaller. Its leaves are clustered towards the tops of branches, are cuneate-lanceolate and light green. The inflorescences have short peduncles, which bear drooping pedicels, each with a capitulum. It would have, at one time, grown locally on the upper parts of the gumwood zone, (400-700m) on the western fringes of the central ridge (Cronk, 2000). A number of individuals at Peak Dale still exhibit some of the characteristics of *C. robustum* ssp. *gummiferum*. At one time, prior to its introgression with *C. robustum* subsp. *robustum, C. robustum* subsp. *gummiferum* would have, no doubt, been ranked as a species (Cronk, 2000). Interestingly, although *C. robustum* subsp. *gummiferum* shows an intermediate morphology between *C. robustum* subsp. *robustum* and *C. spurium* (see table in Cronk, 2000, p.76.) no suggestion of a possible hybrid origin has been made.

Commidendrum rotundifolium (Roxb.) DC. Prod. 5: 344 (1836)

Vernacular name: Bastard gumwood

Extinct in the Wild (EW)

Synonyms:

Solidago rotundifolia Roxb. in Beatson, Tracts: 324 (1816)

Psiadia rotundifolia (Roxb.) Hook.f. *ex* Melliss, St Helena: 228, 186, t.41 (1875)

Commidendrum rotundifolium was thought to be extinct by the end of the 19th century until one individual was rediscovered in 1982 by a local naturalist (Cronk, 2000). This last known tree, which was growing on an inaccessible cliff at Horse Pasture, blew down in a gale in 1986. Fortunately, George Benjamin, the Conservation Officer at the time, managed to take cuttings and seed, and the species was propagated and established *ex situ* on the island. All the trees conserved *ex situ* at Pounceys (17) and Scotland (50+) are descendants from this last tree. Historical records, summarised by Cronk (2000) indicate that the species was already rare by the late 18th century and has therefore undergone a prolonged and severe population bottleneck. At one time *C. rotundifolium* would have been an associated species of dry gumwood (*C. robustum*) woodland (300-500m) and, also to some extent, the wet gumwood woodland which existed at higher elevations (500-650m) on St Helena (Cronk, 1989). *Commidendrum rotundifolium* is a small tree, up to three metres, although historical records suggest it once grew to seven metres. The inflorescence of *C. rotundifolium* is a subglobose corymb (Fig. 1.7) composed of hundreds of small capitula (3mm x 7mm).

Fig. 1.7: *Commidendrum rotundifolium.*

Leaves are subrotund to obovate-elliptical with long petioles, dark green and glabrous. The trees at Pounceys may be suffering from inbreeding depression as they lack vigour and flowering is poor and sporadic. They are heavily infested with black smut fungus and are

being attacked by white ants and other exotic pests. The fifty or so trees at the recently established seed orchard at Scotland were, until a bad winter in 2001, healthy and vigorous. Seed set in *C. rotundifolium* is very poor, with germination rates usually <1%, and this is a major impediment to any reintroduction or species recovery programme. The species may hybridise with *C. spurium*, and there is morphological evidence of hybrids and backcrosses in the seed orchards.

Commidendrum rugosum (Dryand.) DC. Prod. 5: 345 (1836)
Vernacular name: Scrubwood
Vulnerable (VU, D2)

Synonyms:
Conzya rugosa Dryand. in Aiton, Hort. Kew (ed. 1) 3: 184 (1789)
Aster glutinsosa Roxb. in Beatson, Tracts: 303 (1816)
A. rugosus (Dryand.) Melliss, St Helena: t.37 (in ic.) (1875)

Commidendrum rugosum, or Scrubwood, is the least threatened *Commidendrum* species on St Helena. It is a low growing shrub, easily recognised by its conspicuous large white capitula and extremely resinous, spathulate leaves (Fig. 1.8)

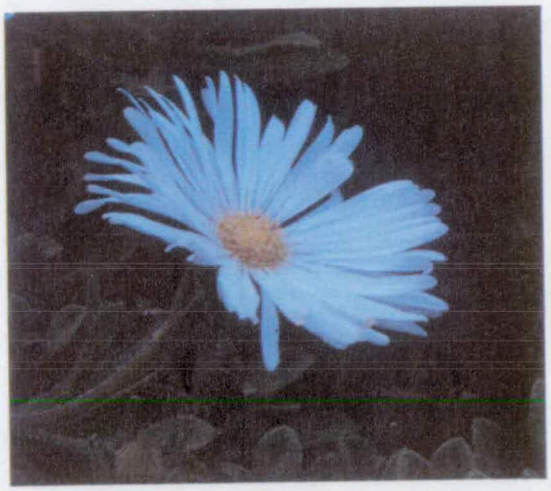


Fig. 1.8: *Commidendrum rugosum* inflorescence.

Scrubwood can be found at a number of localities on St Helena including Deep Valley, Distant Cottage and Man and Horse cliffs. It was once confined to cliffs but has now spread to the eroded slopes of the Crown Wastes following the extirpation of goats. The species is highly tolerant of wind, drought and salinity and formerly would have occupied a low

altitude zone from sea-level to 350m (Cronk, 1989). *Commidendrum rugosum* is not under immediate threat, however, it is classified as Vulnerable as populations tend to be localised, are small and depend on active conservation management to reduce invasive exotics. At localities where *C. rugosum and C. robustum* populations are in close proximity the two species hybridise.

Commidendrum spurium (G. Forst) DC. Prod. 5: 344 (1836)
Vernacular name: False Gumwood
Critically Endangered (CR, D)

Synonyms:
Solidago spuria G.Forst., Comment. Soc. Reg. Sci. Goetting. 9: 68 (1787)
Conzya cuneifolia Raeusch., Nom. ed.3: 240 (1797), non Lam. (1786) nec *Solidago cuneifolia* Roxb. in Beatson, Tracts: 324 (1816)
Aster gummiferus Hook.f., Hook. Ic. Pl. 11: t.1056 (1870)

Commidendrum spurium, the False gumwood, is a small tree up to 3m. The total population of *C. spurium* in the wild is eight and consists of one population of seven at Mt Vesey and an isolated individual at Coles Rock. According to Cronk (1989) *Commidendrum spurium* would have once been a constituent species of wet gumwood woodland, which existed on the slopes and cliffs just below the central ridge (500-650m) and a subdominant species of Cabbage-tree woodland (600-750m). The inflorescence of *C. spurium* is an erect corymb, composed of around 30 capitula (Fig. 1.9). Leaves are apetiolate, obovate-spathulate and pale green forming clusters at the tips of branches.

Fig. 1.9: *Commidendrum spurium*

Both sites of *C. spurium* are heavily encroached by invasive exotics such as *Phormium tenax* and *Solanum mauritianum*. The isolated individual at Coles Rock is nearing senescence and is heavily infested with the introduced scale insect, *Orthezia insignis* (Jacaranda bug). Seed set at Mt Vesey is reasonably good and seedlings have been observed in rock crevices and cleared soil. Unfortunately, no recent regeneration has been observed, possibly due to rabbit grazing. There are two *ex situ C. spurium* sites: at Pounceys and a recently established seed orchard at Mt Vesey. However, because of the risk of hybridisation with *C. rotundifolium*, the new seed orchard is only based on germplasm collected from Mt Vesey.

Melanodendron integrifolium (Roxb.) DC. Prod. 5: 280 (1836)
Vernacular name: Black cabbage tree
Vulnerable (VU, D2)

Synonyms:
Solidago integrifolia Roxb. in Beatson, Tracts: 323 (1816)

Melanodendron integrifolium, the Black cabbage tree, is a small spreading tree up to four metres with large leathery leaves clustered at the tips of branches. The inflorescence is a terminal corymb composed of around 25 capitula (Fig. 1.10). *Melanodendron integrifolium* is one of the dominant species of tree-fern thicket, which can be found along the central ridge at Diana's Peak, Mt Actaeon, Cabbage tree road and High Peak above 600m. In Diana's Peak National Park, the population exceeds 1,500 and may be closer to 3,000 individuals. Regeneration is good, particularly since the restoration of the park began over 8 years ago. Although not under any immediate threat the species is classified as Vulnerable as populations are localised, small and depend on active conservation management to reduce invasive exotics, such as *Phormium tenax*.

Fig. 1.10: *Melanodendron integrifolium* inflorescence. Note encroachment of *Phormium tenax* in the background.

The Elaphoglossum Group

The *Elaphoglossum* group includes three species of *Elaphoglossum*, two of which are endemic to St Helena and one species of *Microstaphyla*, *M. furcata*, which is also endemic. The relationship of *Microstaphyla* to *Elaphoglossum*, in particular to *E. dimorphum* and *E. nervosum* from St Helena, has long been debated (Mickel, 1980). The genus *Elaphoglossum*, which contains over 500 species, is remarkably uniform with regard to morphological characters. The majority of species have a characteristic simple blade, free veins and acrostichoid sori (Mickel & Atehortúa, 1980). *Microstaphyla furcata*, however, has a distinct pinnate frond, a distinction which, in the morphologically uniform genus *Elaphoglossum*, has warranted generic status by some taxonomists (Copeland, 1947; Maxon, 1923; Pichi Sermolli 1968, 1977). The original description of the genus *Microstaphyla* Presl was in fact based on this pinnate species from St Helena (Presl, 1849). However, Mickel (1980) argues, that based on morphological characters such as rhizome scales and habit, *E. dimorphum*, *E. nervosum* and *M. furcata* are closely related, and *M. furcata* should be treated as an *Elaphoglossum*, *E. bifurcatum* (Jacq.) Mickel. The taxonomic treatment of these elaphoglossoid ferns used below is according to Cronk (2000).

Elaphoglossum dimorphum (Hook. & Grev.) T. Moore Index Fil.: 8 (1857)
Vernacular name: Toothed tongue-fern
Critically Endangered (CR, D)

Synonyms:
Acrostichum dimorphum Hook. & Grev., Ic. Fil. 2: t.145 (1830)
Olfersia dimorpha (Hook & Grev.) Presl., Tent. Pterid.: 235 (1836)

Elaphoglossum dimorphum is a terrestrial fern, found growing on stone steps and surrounding rocks and banks. It is a small tufted plant easily recognised by its serrate/dentate leaf margin (Fig. 1.11). *Elaphoglossum dimorphum* is restricted to two localities within Diana's Peak National Park, Mount Actaeon and Cuckold's Point. The national park represents the largest area of tree fern thicket remaining on St Helena, covering approximately 1km^2. Occasional plants have also been noted further west on the central ridge, at High Peak and the Depot (Cronk, 2000), but the species has not been seen at these localities recently. As the total population of *E. dimorphum* is less than 50 individuals, it is classified as "Critically Endangered" (CR, D) according to the IUCN Categories of Threat

(IUCN, 1994). Its relationship to the other endemic elaphoglossoid ferns, *E. nervosum* and *M. furcata*, is uncertain with Mickel (1980) suggesting it may be a hybrid between the two species.

Fig. 1.11: *Elaphoglossum dimorphum*

Elaphoglossum nervosum (Bory.) H. Christ (Mon. Elaph.) Neue Denkschr. Allg. Schweiz. Ges. Naturw. 36: 50 (1899)

Vernacular name: Veined tongue-fern

Endangered (E, D)

Synonyms:

Acrostichum lanceolatum Roxb. in Beatson, Tracts: 296 (1816), non L. (1753)

Acrostichum nervosum Bory in Duperrey, Voy. 'Coquille', Bot. Crypt.: 252 (1829)

Acrostichum subdiaphanum Hook. & Grev., Ic. Fil.: t. 105 (1830)

Aconiopteris subdiaphana (Hook. & Grev.) Presl., Tent. Pterid.: 236, t.10, fig.17 (1836)

Aconiopteris nervosa (Bory) John Smith, Ferns Brit. & For.: 107 (1866)

Olfersia subdiaphana (Hook. & Grev.) T. Moore, Ind. Fil.: t.4. (1857)

Elaphoglossum nervosum, like *E. dimorphum*, is also restricted to Diana's Peak National Park, growing locally at Cuckold's Point, Diana's Peak and Mount Actaeon. The species is predominantly epiphytic, growing on the endemic tree fern, *Dicksonia arborescens* L'Héritier and the endemic tree, *Melanodendron integrifolium*. It is also occasionally found on rock faces and mossy banks. The total population of *E. nervosum* does not exceed 150 plants, and so the species is considered "Endangered" (E, D). A characteristic feature of this species is its prominent venation, making it unmistakable (Fig. 1.12).

Fig. 1.12: *Elaphoglossum nervosum*.

Elaphoglossum conforme (Swartz) Schott ex J. Smith Hook. Journ. Bot. 4: 148 (1841)

Vernacular name: Common tongue-fern

Non endemic

Synonyms:

Acrostichum conforme Swartz Syn. Fil.: 10, 192, t. 1(1) (1806)

The commonest *Elaphoglossum* species on St Helena is the indigenous, *E. conforme*, the Common tongue-fern. The distribution of *E. conforme* includes South Africa and Tristan da Cunha. On St Helena the species is restricted to Diana's Peak National Park where it is locally abundant, growing epiphytically on planted *Araucaria excelsa* and the endemic tree fern, *Dicksonia arborescens*. According to Mickel & Atehortúa (1980) *E. conforme* is taxonomically distant from the endemic *Elaphoglossum* species, *E. dimorphum* and *E. nervosum*.

Microstaphlya furcata (L.f.) Fée Mém. Soc. Nat. Strasbourg (Mém. Foug. 7) 5: 45, t.13 (1857)

Vernacular name: Mossy fern

Vulnerable (VU, D2)

Synonyms:

Adiantum furcatum L. f., Suppl. Pl. 447 (1781)

Osmunda bifurcata Jacq., Collect. 3: 282, t.20, fig.2. (1789)

Acrostichum bifurcatum Sw., Schrad. J. Bot. 1800 (2): 13 (1801), non *A. bifurcatum* Cav. (1789)

Microstaphlya bifurcata (Jacq.) Presl. Epimel. Bot.: 161 (1849)

Elaphoglossum furcatum (L.f.) Christ. Mono. Elaph.: 98, f. 50. (1899)

Elaphoglossum bifurcatum (Jacq.) Mickel , Britonnia 32: 116 (1980)

Microstaphyla furcata (L.f.) Fée has a wider distribution on St Helena than either *E. dimorphum* or *E. nervosum*. There are least ten populations of *M. furcata* on St Helena, all in the uplands at altitudes greater than 650m. The species is terrestrial, growing on shaded rocks, rock crevices, and earth banks. Although not under any immediate threat the species occupies less than 100km^2 and, would therefore, be classified as "Vulnerable" (VU, D2) (IUCN, 1994). The species has a distinct pinnate frond giving rise to its vernacular name, Mossy fern (Fig. 1.13).

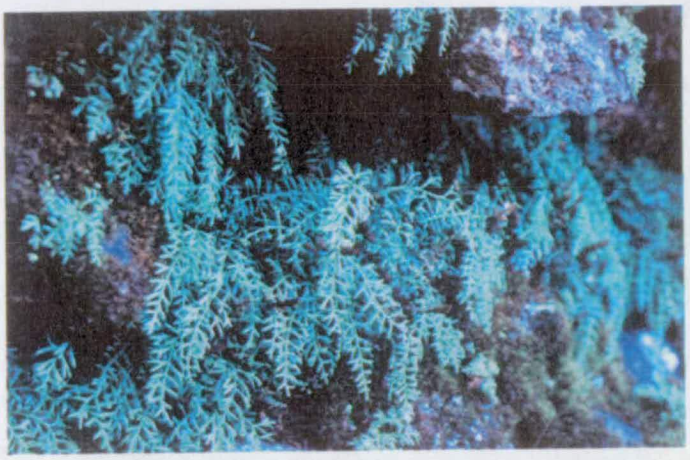


Fig. 1.13: *Microstaphyla furcata*.

1.3 Biological questions relevant to the conservation of *Commidendrum* and *Elaphoglossum*.

A number of evolutionary and biological questions arise from the previous section, which may be relevant to the conservation of these rare and threatened endemics. 1) What are the evolutionary (phylogenetic) relationships of the species within each group? 2) Do species within each study group hybridise? And if they do what is the significance of this to conservation management on St Helena? 3) What is the cause of poor seed viability in *Commidendrum rotundifolium*? 4) What is the extent and distribution of genetic diversity in these rare plant populations? The importance and relevance of these biological questions are

discussed below with examples of techniques (conservation tools) that may be used to answer them.

Evolutionary relationships

As Daugherty *et al.* (1990) has stated, taxonomic classification is a primary determinant of management priorities for endangered species. Outdated classifications may not reflect phylogenetic diversity and could lead to the misguided management of rare species (Avise, 1989, Daugherty *et al.* 1990, Hibbett & Donoghue, 1996, Rojas, 1992). This view is supported by Soltis and Gitzendanner (1999) who emphasise that it is phylogenetically based species for which one can ascertain i) distinguishing synapomorphy (-ies), ii) component populations and individuals, and iii) closest relatives, that should be the target of conservation efforts. A phylogenetic analysis of species within each study group on St Helena would provide a taxonomic framework on which biological research and conservation management can be founded. Questions relating to the evolutionary relationships of these taxa include: i) Do *Commidendrum* and *Melanodendron* form a monophyletic group with a common ancestor on St Helena or do they originate from two independent dispersal events ? ii) What is the relationship of *Microstaphyla furcata* to the other endemic elaphoglossoid ferns, *Elaphoglossum nervosum* and *E. dimorphum* ?

In the last twenty years systematists have increasingly used molecular markers as a source of characters for phylogenetic reconstruction. This has brought new insights into the relationships and evolution of plant taxa, particularly in taxonomically difficult groups, such as ferns (Gastony & Ungerer, 1997; Murakami *et al.* 1999). In addition the use of molecular phylogenetics has lead to a closer scrutiny of traditional morphological classifications, with reassessments of homoplasy in conventional characters and character homology using developmental techniques (Rudall, 2000). In recent years direct sequencing of chloroplast and nuclear DNA regions and genes, to infer phylogentic relationships, has largely replaced chloroplast DNA restriction site analysis, particularly with the widespread availability of automated sequencing (Soltis & Soltis, 1998). The choice of an appropriate gene or region for a phylogenetic analysis depends on a number of factors including i) the specific plant group and ii) the taxonomic level at which the study is to be conducted (see Soltis & Soltis, 1998 for an excellent review). For example, the fast evolving internal transcribed spacer (ITS) regions of ribosomal DNA have been used extensively to study speciation and evolution in island plants at the species level (Baldwin *et al.* 1998; Sang *et al.* 1994; Panero *et al.* 1999; Kim *et al.*, 1996) whilst the slower evolving chloroplast gene, *rbc*L is the

preferred gene at the family level or above (Soltis & Soltis, 1998; Michaels *et al.*, 1993; Kron & Chase, 1993).

Phylogenetic analysis could also assist in conservation planning by allowing the assessment of the biological distinctiveness of taxa as in the 'taxic diversity' measure of Vane-Wright *et al.* 1991 and the 'phylogenetic diversity' of Faith (1992). Byrne *et al.* (2001) recently conducted a phylogenetic analysis of two threatened Australian *Acacia* species, *A. sciophanes* and *A. lobulata*, and their widespread relatives using RFLP analysis of cpDNA. The study revealed that both threatened species were phylogenetically distinct despite being morphologically similar to their widespread relatives. In fact, *A. lobulata* represents an ancient lineage and most likely a relictual species.

Hybridisation

There is morphological evidence to suggest that species within each study group hybridise. For example there are a number of morphologically ambiguous individuals at *ex situ* localities and seed orchards of *C. rotundifolium* and *C. spurium*. Hybridisation within the *Commidendrum* group has implications for seed orchard management, reintroduction, and species recovery, particularly if the morphological integrity of these species is to be maintained. Within the *Elaphoglossum* group it has been suggested that *E. dimorphum* could be of hybrid origin between *E. nervosum* and *Microstaphyla furcata* (= *E. bifurcatum*) (Mickel, 1980). The possible hybrid origin of *E. dimorphum*, the rarest of the endemic *Elaphoglossum* species on St Helena could even lead to a review of the priority species for conservation.

Interspecific hybridisation in rare plants is often considered detrimental and has been highlighted by a number of authors (Rieseberg, 1991; Rieseberg & Gerber, 1995; Levin *et al.* 1996; Levin, 2000; Brochmann, 1984). Island plants are particularly susceptible to genetic assimilation through hybridisation because of small population sizes, the lack of reproductive barriers between congeneric species, invasion and colonisation of islands by closely related exotics and habitat disturbance (Rieseberg, 1991; Levin, 2000). However, as Arnold (1997) points out natural hybridisation and its relation to conservation can be viewed more positively. For example, hybridisation between rarer and more numerous taxa could potentially result in a genetic enrichment of the endangered form and may lead to elevated fitness in the rarer taxon (Arnold, 1997).

There are a number of molecular markers available to study hybridisation and species relationships in plants, the utility, ease of use and performance of which are discussed in Reiseberg & Ellstrand (1993) and Rieseberg & Brunsfeld (1992). They include allozymes (Murakami *et al.* 1999), RAPDs (Random Amplified Polymorphic DNA) (Sedgeley *et al.*, 1996; Crawford *et al.*, 1993; Hollingsworth *et al.*, 1998), fragment and restriction site analysis (Arnold *et al.*, 1991, Vogel *et al.*, 1998) and AFLPs (Amplified Fragment Length Polymorphism) (Beismann *et al.*, 1997; O'Hanlon *et al.*, 1999).

Poor seed viability in Commidendrum rotundifolium.

There could be a multitude of reasons for poor seed viability in a plant species, particularly in a rare and threatened one including: i) lack of pollinators, ii) germination conditions, iii) fitness of maternal plant and iv) source of pollen (availability of compatible mates in self-incompatible species). After closer examination of *C. rotundifolium* achenes it appears that poor seed viability is due to empty achenes (no embryos), and so germination conditions would seem to be the unlikely cause of poor seed viability. Neither is there any observational evidence to suggest a lack of pollinators (Eastwood, pers. obs.). However, propagation evidence (low but variable germination) in *C. spurium* and *C. rotundifolium* indicates that there may be a self-incompatibility system operating in these species which could affect seed viability. For example, germination in achenes collected from the population of *C. spurium* at Mount Vesey in 1999 was 40%. In contrast seed germination from the isolated tree at Oaklands (which is now dead) in 1999 was 8%. Self-incompatibility in plants is usually examined by conducting controlled pollinations (self and cross) and either calculating seed set or observing pollen tube growth in the pistil (Crowe, 1954; Reinartz & Les, 1994; Hiscock, 2000; Lewis *et al.*, 1988). Although more difficult to ascertain reduced fitness in parental trees of *C. rotundifolium* cannot be dismissed as a cause of poor seed viability.

Extent and distribution of genetic diversity

A knowledge of the extent and distribution of genetic diversity (neutral), can provide information on population structure, prevailing breeding systems, species relationships, hybridisation and patterns of migration (gene flow) essential to the design of successful conservation programmes (Holsinger, 1996). Neutral molecular marker data, however, cannot be used as a reliable predictor of adaptive variation (crucial to the long-term survival of an endangered species) or population viability and caution most be exercised in their

interpretation (Ennos *et al.* 1997; Hedrick, 2001; Holsinger, 1996; Lynch, 1996; Karhu *et al.*, 1996). Until fairly recently, allozymes were the main nuclear genetic marker used in population genetic analysis (Schaal *et al.*, 1991), their co-dominant mode of inheritance allowing estimations of allele and genotypic frequencies. Allozymes have been used extensively to gain an insight into the reproductive ecology of a range of plants with different life history traits and distributions (Hamrick & Godt, 1990) including ferns, rare plants and island endemics (Godt & Hamrick, 1999; Turner *et al.*, 2000; Genmill *et al.*, 1998; Rumsey *et al.*, 1999; Hooper & Haufler, 1997; Ranker, 1994; Crawford *et al.*, 1992). A major draw back of allozymes as genetic markers is the limited number of loci available (20-30) and their lack of variation in endangered species. A major advance in the development of suitable genetic markers for evolutionary genetic studies has been the discovery of highly variable loci, such as microsatellites (Schaal *et al.*, 1991). The introduction of highly variable loci such as microsatellites is having a large impact on the ability to resolve many questions in evolutionary and conservation biology (Hedrick, 1999). This may be particularly relevant in i) endangered species, which generally show low levels of allozyme diversity, ii) genotyping and paternity analysis studies and iii) population structure of clonal plants (Hedrick, 1999; Beaumont & Bruford, 1999) However, microsatellites require considerable development time, when compared with allozymes, are more costly and may not be transferable across related species (Edwards, *et al.*, 1996; Hughes, pers. comm.)

1.4 Objectives and thesis outline

This thesis investigates the evolution and conservation of two endangered groups of plants from St Helena; i) trees and shrubs in the genera *Commidendrum* and *Melanodendron* (Asteraceae) and ii) epiphytic and terrestrial elaphoglossoid ferns in the genera *Elaphoglossum* and *Microstaphyla*. The thesis is divided into four chapters, two chapters for each study group. The chapters were written as independent papers with the intention of publishing them in scientific journals, for example, Plant Systematics and Evolution. As a consequence a certain amount of repetition was inevitable, particularly in the introductory paragraphs.

The first main objective of the thesis was to gain an understanding of the phylogenetic relationships of the species in each of the study group. Chapter two investigates the

phylogenetic relationships and evolution of *Commidendrum* and *Melanodendron* using ITS (internal transcribed spacer) sequences of ribosomal DNA. A similar phylogenetic study is conducted in *Elaphoglossum* and *Microstaphyla,* but this time using sequences of the chloroplast *trn*L intron and *trn*L-F intergenic spacer (Chapter 4). It was intended that these phylogenetic studies would serve as a framework for more detailed research on aspects of reproductive biology, genetic diversity and population structure, all of which are essential in rare plant conservation.

Chapter three focuses on two of the most threatened *Commidendrum* species, *Commidendrum spurium* (CR) and *Commidendrum rotundifolium* (EW). This chapter attempts to answer specific questions relating to the reproductive biology of these species. The first objective was very specific: do the two species hybridise in the seed orchards and, if they do, what the implications are for conservation? The second objective was to investigate self-incompatibility in *C. rotundifolium* and *C. spurium* and its role in the low seed viability observed in *C. rotundifolium*. It is hoped that our study of the reproductive biology of *C. spurium* and *C. rotundifolium* will assist the CES on St Helena in seed orchard management and species recovery.

The last research chapter, chapter five, investigates species relationships of the endemic elaphoglossoid ferns, *Elaphoglossum dimorphum*, *E. nervosum* and *Microstaphyla furcata* in more detail using allozymes.

The final chapter concludes the thesis with a general discussion of my findings in relation to other studies conducted on other oceanic islands.

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Evolution of St Helena arborescent Astereae (Asteraceae): relationships of the genera *Commidendrum* and *Melanodendron*.

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Abstract

The phylogenetic relationships of endemic *Commidendrum* (four species) and *Melanodendron* (one species) from St Helena were inferred from sequences of ITS 1 and ITS 2 of ribosomal DNA. Despite showing a range of morphological and ecological variation, the four species of *Commidendrum*, *C. spurium*, *C. robustum*, *C. rotundifolium* and *C. rugosum* form a closely related monophyletic group with percentage sequence divergence between 0.2-0.9%. *Melanodendron integrifolium* is sister to *Commidendrum* indicating that the two genera evolved from a common ancestor which arrived in St Helena via a single dispersal event. The closest relatives of *Commidendrum* and *Melanodendron* appear to be South African, in the predominantly shrubby genus *Felicia*, although further sampling of South African Astereae is required. We discuss the evolution and adaptive radiation of these rare and threatened species with particular reference to the possible role of heterochrony.

Keywords: Island evolution, internal transcribed spacer, adaptive radiation, capitulum morphology, conservation, heterochrony.

2.1 Introduction

The 'gumwoods and 'cabbage trees' are considered to be among the botanical glories of St Helena (lat. 15° 56′S, long 5°42′W), an isolated volcanic island in the South Atlantic (Cronk, 2000). They include members of the endemic genera *Commidendrum* DC. (gumwoods and scrubwood) DC., and *Melanodendron* DC. (Black Cabbage tree), which are small trees or shrubs belonging to the Asteraceae family, tribe Astereae. *Commidendrum* is represented by four species whilst *Melanodendron* is monotypic with just one species, *M. integrifolium* (Roxb.) DC. The close affinity of *Commidendrum* with *Melanodendron* has been noted (Bentham, 1873a; Bentham, 1873b) and the two genera are thought to be closely related (Cronk, 2000).

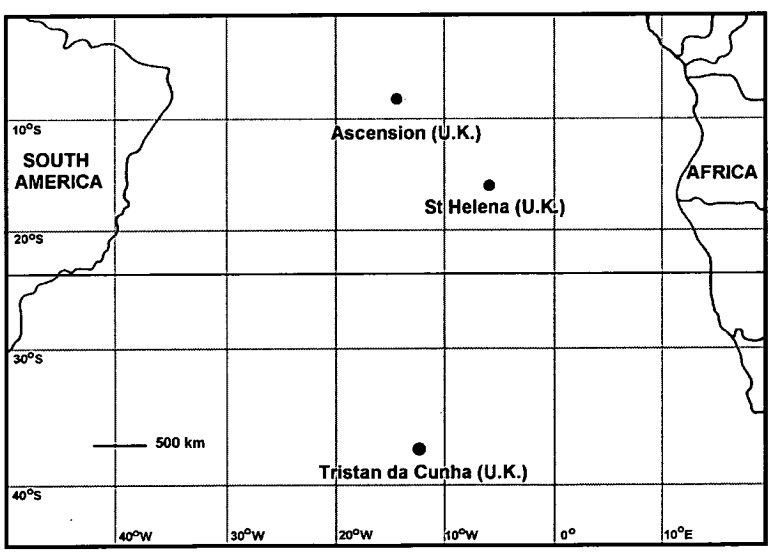
As with many insular endemics from isolated islands, the origin and evolution of *Commidendrum* and *Melanodendron* has attracted much interest and controversy from botanists and evolutionary biologists. The morphological divergence, and hence taxonomic isolation, of *Commidendrum* and *Melanodendron* within the Astereae subsequently led to speculation as to their closest continental relatives. Bentham (1873a), like Melliss (1875) considered the two genera to be of great antiquity, having taxonomic affinities with South American *Chiliotrichum* Cass., *Diplostephium* H.B. & K. and Australian *Olearia* Moench. A hypothesis for the taxonomic and geographic disjunction observed in these endemic composites was developed by Cronk (1987, 1992). Cronk proposed that the genera are ancient relicts dating from the Miocene, the ancestors of which became extinct in South Africa due to climate change in the late Miocene-early Pliocene. This contrasts with Carlquist (1965, 1974) who argued that the arborescent composites on St Helena are neo-endemics of recent origin. Carlquist (1965) postulated that they evolved from herbaceous composites, such as *Aster* L., acquiring woodiness as they radiated into a range of habitats and ecological niches. However, recent molecular evidence suggests that *Commidendrum* is part of a basal group of Astereae, closest to the southern African genera *Felicia* (83 species) Cass and *Amellus* L. (12 species) (Noyes & Rieseberg, 1999). This new insight was subsequently supported by Carlquist (2001) who analysed the wood anatomy of the St Helena Asteraceae and compared it with *Felicia amelloides* (DC.) Voss. He concluded that a close relationship between *Felicia* and *Commidendrum* and *Melanodendron* was justified on the basis of wood anatomy. It is interesting to note that Hemsley (1885, p.60), in his notes on the origins of the indigenous flora of St Helena, remarked that the "arboreous Compositae of St Helena differ little in floral structure from the Cape shrubby *Felicia*." Carlquist (2001)

also discovered that the wood of *Commidendrum* and *Melanodendron* lacked paedomorphic characters, a feature consistent with the evolution of woody stature from an herbaceous ancestor. He concludes that *Commidendrum* and *Melanodendron* evolved from an already woody (i.e. shrubby) lineage.

Molecular phylogenetics has provided new insights into the closest relatives of island endemics which are morphologically divergent from their continental progenitors (Baldwin *et al.*, 1991; Kim *et al.*, 1998; Francisco-Ortega *et al.*, 1997; Ballard & Systsma, 2000). There are also now many examples where the monophyly of insular plant lineages that exhibit extensive morphological and ecological variation have been tested (Baldwin & Robichaux, 1995; Givnish *et al.*, 1995; Sang *et al.*, 1994; Francisco-Ortega *et al.*, 2001; Panero *et al.*, 1999; Kim *et al.*, 1996).

However, most of these studies have predominantly focused on plant lineages exhibiting extensive radiations on the islands and archipelagos of Hawaii, Juan Fernandez and Macaronesia. St Helena does not have species rich radiations like the silversword alliance (*Argyroxiphium* DC., *Dubautia* Gaud., and *Wilkesia* A. Gray) on the Hawaiian archipelago. In contrast, eight of the ten endemic genera on St Helena are monotypic (Cronk, 2000). Also, unlike the archipelagos of Hawaii, Juan Fernandez and Macaronesia, St Helena is composed of just one island, only 122km². It is extremely isolated (Fig. 2.1), some 1,931km away from the nearest continent (Africa) and 1,127 km away from Ascension (the nearest land).

Fig. 2.1: Locality of St Helena in the South Atlantic Ocean.



St Helena was formed over a period of 7 million years by the coalescence of two basaltic shield-volcanoes beginning some 14.3 ± 1.0 million years ago (Baker, 1968; Baker, 1970). The topography of St Helena is remarkably varied and complex. The coast is composed of precipitous cliffs intersected by deep gorges and valleys. Rolling hills, valleys and plains dominate the central uplands, which culminate in the sickle shaped central ridge of which Diana's Peak (820m) is the summit. The climate on St Helena varies considerably, being influenced greatly by altitude. The coastal regions experience much drier, sunnier and warmer conditions than the central uplands and peaks. In Jamestown, on the coast, annual rainfall is *c.* 200 mm whilst on the peaks it is *c.* 1,200 (Cronk, 2000). The peaks (High Peak (789m), Cuckold's Point, Diana's Peak and Mt Actaeon) are often shrouded in mist and can be 5°C-10°C cooler than Jamestown.

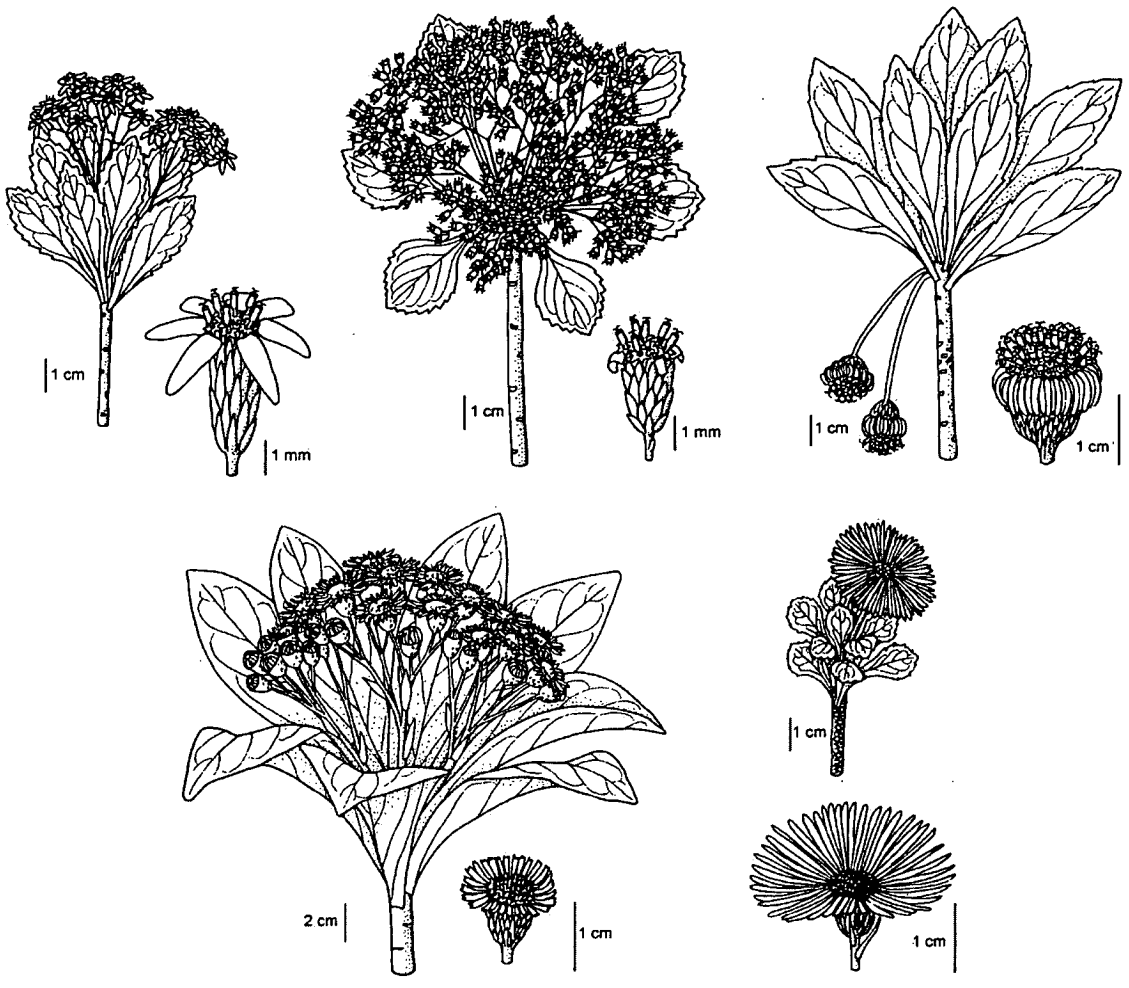
According to the reconstruction of the vegetation on St Helena, based on the extrapolation of present day distributions and historical accounts by Cronk (1989), *Commidendrum* and *Melanodendron* would have occupied a range of vegetation zones and habitats, as summarised in Table 2.1. All species would have been important structural elements of the original vegetation. *Commidendrum robustum* (Roxb.) DC., in particular, formed extensive woodlands in the middle altitudinal zone of St Helena and has numerous associated endemic insect species. *Commidendrum rugosum* (Dryand.) DC. presently occupies a wide range of habitats: steep coastal cliffs, deep gorges and the dry anthropogenic eroded wastelands known as the Crown Wastes. Conversely, *Melanodendron integrifolium* (Roxb.) DC.is restricted to the peaks along the central ridge of mountains in wet tree fern thicket. As well as occupying a range of habitats *Commidendrum* and *Melanodendron* display a wide range of morphological variation (floral and vegetative) as summarised in Table 2.1 and Fig. 2.2.

All the *Commidendrum* and *Melanodendron* species are threatened on St Helena and are listed in the 2000 IUCN Red List of Threatened Species (Hilton-Taylor, 2000). The species survive as small relict populations on cliffs or in fragmented patches of endemic vegetation heavily invaded by exotic species. One species, *Commidendrum rotundifolium* (Roxb.) DC., is Extinct in the Wild, according to the IUCN categories of threat (Hilton-Taylor, 2000) and is dependent on the horticultural skills of the Endemics Section (Department of Agriculture and Natural Resources, St Helena) for its survival. *Commidendrum spurium* (G. Forst.) DC., (Critically Endangered), is restricted to a small population of seven individuals near Mount Vesey and an isolated individual at Coles Rock. Both sites are heavily encroached by invasive exotics such as *Phormium tenax* Forst. and *Solanum mauritianum* Blanco.

Table 2.1: Morphological and ecological characteristics of *Commidendrum* and *Melanodendron*, * after Cronk, 1989, † after Calrquist, 2001

Species	Tree height (m)	No. capitula per inflor.	No. florets per capitula (disk:ray)	Leaf shape	Leaf indumentum	Ecology and distribution*	Mesomorphic ratio †
C. rugosum (Scrubwood)	1	1	c. 70: 50	Spathulate, revolute.	Tomentose, viscid, glandular	Dry slopes and coastal cliffs (0-350m). Highly tolerant to wind, drought and salinity.	39
C. robustum (Gumwood)	5-8	1- few	c.180: 40	Lanceolate, shallowly crenate-dentate	Pubescent above, floccose to tomentose below	Dry-moist gumwood woodland (300-650m). Drought resistant.	300
C. rotundifolium (Bastard Gumwood)	4-5	c. 300-1000	6-7: 0-6	Obovate-elliptical, serrate-dentate to subdentate	Glabrous, glandular	Once a component of dry gumwood woodland (300-500m). Drought resistant.	339
C. spurium (False Gumwood)	2-3	c.20	5-8: 5-8	Obovate-spatulate, shallowly dentate	Glabrous above, minutely pubescent below with glandular hairs	Once a component of wet Cabbage-tree woodland (600-750m). Mesic.	385
M. integrifolium Black cabbage tree	3-5	c. 20	50: 30	Entire, cuneate-lanceolate, revolute.	Glabrous above, glabrescent below	Tree fern thicket (700-820m). Drought intolerant.	611

Fig. 2.2: Floral diversity in *Commidendrum*, going clockwise from top left: *C. spurium*, *C. rotundifolium*, *C. robustum*, *C. rugosum* and *Melanodendron integrifolium*. Illustrated by Chistina Oliver.



The last woodland of Gumwood, *Commidendrum robustum* (Endangered), the national tree of St Helena, is located in Peak Dale. The woodland is composed of a population of approximately 1,000 trees, which regenerate well, especially since grazing was restricted. In the early 1990s the introduced scale insect, *Orthezia insignis* Browne, threatened to destroy this woodland (Smith, 1997). Fortunately, a biological control programme, using the agent *Hyperaspis panthera* (Coleoptera: Coccinellidae) was largely successful at controlling the scale insect. A number of small *C. robustum* populations exist at other localities, largely confined to cliff refugia. An account of the decline of *Commidendrum robustum* is given by Cronk (1986). *Commidendrum rugosum* and *Melanodendron integrifolium* have a wider

distribution in St Helena than the other species and are not under immediate threat. They are however, classified as Vulnerable because populations tend to be localised, small and depend on active conservation management to reduce invasive exotics.

There are three main objectives to our study. The first one is to confirm the close relationship of *Felicia* with *Commidendrum* as indicated by Noyes & Rieseberg (1999) with additional sampling of *Felicia* and other Astereae. The second is to determine whether *Commidendrum* and *Melanodendron* form a monophyletic group with a common ancestor on St Helena or whether they originate from two independent dispersal events involving related species. The third objective is to elucidate the phylogenetic relationships of the *Commidendrum* species to assist assessments of conservation priority for individual species and to determine patterns of evolutionary radiation. Phylogenetic relationships and evolutionary patterns were inferred from sequences of the internal transcribed spacer regions of nuclear ribosomal DNA (ITS). ITS was chosen for this study as a number of studies have demonstrated its utility in inferring phylogenetic relationships at the species/generic level in the Asteraceae (Baldwin, 1992; Francisco-Ortega *et al.*, 2001; Kim *et al.*, 1996)

2.2 Materials and Methods

Plant Material

A range of plant material, from different sources, including silica gel dried, herbarium vouchers and the living collections at the Royal Botanic Garden Edinburgh (RBGE) was used in the phylogenetic analysis (Table 2.2). The ingroup included all four species of *Commidendrum, Melanodendron integrifolium* and five species of *Felicia* from southern Africa. The five *Felicia* species in this study represent three of the six sections of *Felicia* (83 species) according to Grau (1973). The outgroup contained five species (from four genera) of Asteraceae in the tribe Astereae; *Aster vahlii* Hook. & Arn., *Baccharis* L. sp., *Olearia arborescens* Cockayne & Liang, *Olearia phlogopappa* (Labill.) DC. and *Chiliotrichum diffusum* (Forst.) O. Kuntze Voucher herbarium specimens of the silica gel dried material and from the RBGE living collections are held at the Royal Botanic Garden Edinburgh (E). The selection of outgroup taxa was based on the phylogenetic analysis of Astereae by Noyes & Rieseberg (1999). As a number of authors have suggested that *Commidendrum* and *Melanodendron* are specialised representatives of *Aster*, *Aster* was also included in the outgroup (Xiaoping & Bremer, 1993; Carlquist, 1965; Bremer, 1994).

Table 2.2: Details of the accessions/collecting numbers, localities, and habitat of the species used in the phylogenetic analysis. S= silica gel dried, H = herbarium specimen, LC = living collection.

Species	Accession/Collecting No.	Locality and habitat
Commidendrum robustum (Roxb.) DC.	AEAST72 (S)	St Helena, Peakdale, on steep rocky slopes and cliffs, 560m
Commidendrum rotundifolium (Roxb.) DC.	AEAST70 (S)	St Helena, Pounceys, *ex situ* site
Commidendrum rugosum (Dryand) DC.	19960944 (LC)	St Helena, opposite side of ridge to Great Hollow, below Bluepoint,
Commidendrum spurium (G. Forst.) DC.	AEAST21 (S)	St Helena, below Mount Vesey, on steep cliffs amongst *Phormium tenax*, 700m
Melanodendron integrifolium (Roxb.) DC.	V. Thomas No.1 (S)	St Helena, High Peak, in *Dicksonia arborecens* thicket and arborescent composites, 720m
Felicia aethiopica subsp. *ecklonis* (Less.) Grau	19780804 (LC)	South Africa, Cape Province, Bathhurst District, Coombs Valley
Felicia clavipilosa subsp. *transvaalensis* Grau	C.E. Moss Herbarium (dup.) K. Balkwill *et al.* 5981 (H)	South Africa, Transvaal, Pietersburg District, Blouberg Nature Reserve, on rocky quartzite ridges, 800m.
Felicia echinata Nees	19710299 (LC)	South Africa, Eastern Cape, between Port Elizabeth and Plettenberg Bay.
Felicia fruticosa subsp *brevipedunculata* (Hutchinson) Grau	C.E. Moss Herbarium (dup.) I.H. Hartley 873 (H)	South Africa, Cape Province, Victoria West District, Along roadside 15km south of Richmond on N1, 1300m
Felicia uliginosa Grau	19972678 (LC)	South Africa, Orange Free State, Mont aux Sources, in gravel over rock sheets, 3020m.
Aster vahlii Hook. & Arn.	E00096042 (H)	Chile, Region XII, Prov. Ultima Esperanza: Torres del Paine, *Nothofagus antartica* woodland, 75m.
Baccharis L. sp.	R.T. Pennington 1090 (S)	Peru, Dept. de Junin, Carpapata, 2200m
Olearia arborescens Cockayne & Liang	19840712 (LC) (18)	New Zealand, South Island, Otago, Mt Cook, *Nothofagus* forest, 3000ft
Olearia phlogopappa (Labill.) DC.	19961507 (LC) (19)	Australia, Tasmania, south end of Great Lake beside Lake Highway, on gravel, 965m.
Chiliotrichum diffusum (Forst.) O. Kuntze	19922585 (LC)	Chile, Región XI, Prov. de Aisén, Coyhaique, mixed open scrub, 450m.

DNA extraction and PCR

Total genomic DNA was extracted from one individual of each taxon using the modified CTAB procedure from Doyle & Doyle (1987). DNA extracted from herbarium specimens was left to precipitate in iso-propanol at -20°C for 2 days. The complete ITS region was amplified using the forward primer "ITS 5P" -5'-GGAAGGAGAAGTCGTAACAAG-3', modified from White *et al.* (1990) and the reverse primer "ITS4"- 5'-TCCTCCGCTTATTGATATGC-3' from White *et al.* (1990). Each PCR reaction contained: 2.5 µl x10 NH_4 buffer (Bioline, UK), 2.5 µl of 2mM dNTPs (Bioline, UK), 1 µl of 10 µM ITS-5P , 1 µl of 10 µM of ITS-4, 1 µl of 50mM magnesium chloride, 0.1 µl of Biotaq polymerase (Bioline, UK) and 1-2 µl DNA. The reaction volume was made up to 25 µl with sterile ultra-pure water.

The PCR was performed with the following conditions for all the taxa: i) one initial pre-step of 94°C for 3 minutes, followed by ii) 30 cycles of denaturation at 94°C (1 minute), annealing at 55°C (1 minute) and extension at 72°C (1 minute and 30 seconds) and iii) a terminal extension of 72°C for 5 minutes. After visualisation on 2% agarose gel the PCR products were purified using QIAquick™ purification columns supplied by Qiagen Ltd. UK.

The PCR products were sequenced directly using premixed Thermo Sequenase II reagent (Amersham Pharmacia, UK) and according to manufacturer's protocol. The ITS region was sequenced using the "5P" forward primer and the "ITS 4" reverse primer as above. In addition two internal primers, "ITS 2P"- 5'-GCTACGTTCTTCATCGATGC-3' reverse (modified from White *et al.* (1990) by Möller & Cronk, 1997) and "ITS 3P"- 5'-GCATCGATGAAGAACGTAGC-3' forward (reverse complement of "ITS 2P") were used to amplify ITS 1 and ITS 2 respectively. The sequencing reaction was conducted using the following PCR conditions: 25 cycles of denaturation at 96°C (10 seconds), annealing at 50°C (5 seconds) and extension at 60°C (4 minutes) and excess dye terminator removed following manufacturer's recommendations. Sequence analysis was conducted on an automated ABI Prism™ 377 sequencer.

Sequences were obtained for the majority of taxa by direct sequencing of the PCR product as described above. However, intra-individual ITS length polymorphism occurred in *C. rotundifolium*, *M. integrifolium* and *Felicia echinata*. There was one obvious length polymorphism (1 bp indel) in *Felicia echinata*, however the sequence was interpreted

satisfactorily by reading the forward and reverse sequences either side of the indel as in Denduangboripant *et al.* (2001). In *C. rotundifolium* and *M. integrifolium* this was not possible and so the PCR products were purified and ligated into plasmid vectors using the Topo TA Cloning kit (Invitrogen Co., CA, USA). Positive transformants were selected and cultured overnight in LB medium with 50 μg/ml ampicillin. Following a restriction analysis to check for inserts, the plasmids were purified using the QIAprep™ Spin columns supplied by Qiagen Ltd. UK and sequenced as above.

Sequence alignment and analysis

Sequences were initially edited using editing Sequence Navigator™ v.1.01 (Applied Biosystems) and subsequently exported into AutoAssembler™ v. 2.1 (Applied Biosystems) for final editing and assembling. As a confirmation the final sequence for each taxon was based on both forward and reverse sequence reactions. However, the final sequence for *F. echinata* is based on only one sequence reaction (either forward or reverse) due to the intra-individual length polymorphism. Indel bases in those taxa which showed intra-individual length polymorphism were coded as missing data. Intra-individual base polymorphism was combined into a single consensus sequence using IUB coding (R, Y, K, M). Sequences were aligned using ClustalX v.1.8 (Higgins *et al.* 1992) with some minor manual adjustment (Appendix 1). The percentage sequence divergence and G + C content was calculated within PAUP* (Swofford, 2001) version 4.0b7. MacClade version 3.05 (Maddison and Maddison, 1992) was used to calculate transition/transversion ratios.

Phylogenetic analysis

The aligned sequence matrix was analysed using maximum parsimony (MP) with the branch-and-bound option in PAUP* version 4.0b7 (Swofford, 2001). Any regions of ambiguous alignment in the data matrix (bps: 16-17, 72-78, 128-131, 149-156, 203-205 and 460-473) were excluded from this and any further analyses. Individual gaps in the sequence data were treated as missing data. Indels were scored separately according to the simple gap coding method of Simmons and Ochoterena (2000) and added to a gap matrix at the end of the sequence data. In order to examine the effect of character re-weighting on tree topology an additional analysis was conducted using characters re-weighted by the mean value of the rescaled consistency index. Bootstrap values for each clade were calculated from 1,000 replicate parsimony analyses using the "branch and bound" option and "furthest" addition sequence of taxa. The decay index (Bremner support values) for each node was calculated using Autodecay 4.0.2. (Eriksson, 1999).

As a comparison to parsimony a maximum likelihood (ML) analysis was also conducted on the aligned data matrix. To test which model of DNA evolution best fitted our data we used the nested hierarchical approach in Modeltest version 3.0 (Posada & Crandall, 1998) within PAUP (Swofford, 2001). The evolutionary model of Tamura & Nei (1993), TrNef + G, was selected as it gave the best likelihood score out of the 56 DNA evolutionary models tested. The assumptions under this model are i) equal base frequencies ii) specified substitution rates (A-C = A-T = C-G = G-T = 1, A-G = 2.68, C-T = 5.23) and iii) Gamma distribution shape parameter of variable sites, G = 0.6413.

2.3 Results

Sequence analysis

A complete sequence for ITS1 and ITS2 was obtained for all the fifteen taxa in this study, although the last 25 base pairs of ITS 2 were not obtained for *F. echinata*. This gave an aligned matrix of 501 base pairs (excluding 5.8s). Three clones of *C. rotundifolium* and two of *M. integrifolium* were successfully sequenced. Efficiency of cloning was low in *M. integrifolium* resulting in a high number of false positive clones. The two clones of *M. integrifolium* that were sequenced were found to be identical. Both *C. rotundifolium* and *F. echinata* showed intra-individual length polymorphism (a single one base pair indel). In addition to intra-individual length polymorphism *C. rotundifolium* also showed intra-individual sequence polymorphism at four positions. The sequence characteristics for the taxa are shown in Table 2.3. The percentage sequence divergence within species of *Commidendrum* ranged from 0.2-0.9%, and between *Commidendrum* and *Melanodendron*, 3.6-3.9%.

Phylogenetic analysis

The MP analysis of ITS sequences only yielded two most parsimonious trees, 301 steps in length, one of which is represented as a phylogram in Figure 2.3. The strict consensus tree is represented as a cladogram in Figure 2.4. The two most parsimonious trees have a consistency index of 0.77 and a retention index of 0.75 (excluding ambiguous regions). The species of *Commidendrum* form a monophyletic group with high bootstrap support (97%). Further support for the monophyly of *Commidendrum* is provided by sequences from additional accessions of *C. robustum* and *C. spurium*. These additional accessions were not

included in the final analysis due to dubious provenance/identity. However, when included in the analysis the additional *C. spurium* accession forms a polytomy within the *Commidendrum* clade whilst the additional *C. robustum* accession groups with the *C.rugosum/C.robustum* group (see below).

Within *Commidendrum*, there is some support (52%) for a *C. rugosum* and *C. robustum* group, although this is based upon only one molecular synapomorphy. The relationship of *C. spurium* and *C. rotundifolium* within *Commidendrum* remains unresolved by the ITS data, as the polymorphic sites are autapomorphies. In the ITS phylogeny *Melanodendron integrifolium* is the sister group to *Commidendrum*, with 100% bootstrap support. The close relationship of *Melanodendron* to *Commidendrum* is indicated by the short branch length separating the two genera (15 steps). The reweighted parsimony analysis produced identical tree topologies (2 trees) to the unweighted analysis. The addition of the gap matrix had very little effect on the overall tree topology. The only difference was that in two of the six most parsimonious trees *Felicia aethiopica* no longer formed a sister group to the endemic St Helena taxa but instead formed a polytomy. The ML analysis produces a topology nearly identical to that in the MP strict consensus. The only difference is that *Aster vahlii* groups with *Olearia phlogopappa*.

The ITS phylogeny supports the close relationship of *Felicia* to *Commidendrum* and *Melanodendron* as first indicated by Noyes & Rieseberg (1999). Interestingly, of the five *Felicia* species analysed, *Felicia aethiopica* appears to be the sister species to *Commidendrum* and *Melanodendron*, but this relationship is only weakly supported by the bootstrap analysis (57%). The other four species of *Felicia* form a very weakly supported clade (51%). However, within *Felicia* there is strong support for the species' pairs, *F. fruticosa* and *F. echinata* (93%), and *F. clavipilosa* and *F. uliginosa* (100%). *Felicia fruticosa* and *F. echinata* are both in the section *Lignofelicia* according to Grau (1973). Likewise, *F. clavipilosa* and *F. uliginosa* are considered to be in the section *Felicia* (Grau, 1973). The branch length of the *F. clavipilosa* and *F. uliginosa* group is very long (39 steps), suggesting that these two species are phylogenetically distinct from the other three *Felicia*.

Table 2.3: Sequence and indel characteristics of i) the ingroup taxa: *Commidendrum* (4 species), *Melanodendron* (1 species) and *Felicia* (5 species) and ii) the outgroup taxa: *Olearia arborescens*, *O. phlogopappa*, *Baccharis* sp., *Aster vahlii* and *Chiliotrichum diffusum* for ITS1 and 2 of ribosomal DNA. In addition, sequence divergence (%) is given for i) St Helena taxa (*Commidendrum* and *Melanodendron*) and ii) between the St Helena taxa and the sister group *Felicia*.

Parameter	ITS 1	ITS 2	ITS 1 and ITS 2
Length range (total) (bp)	246-256	212-218	458-472
Length mean (total) (bp)	253.4	215	468.4
Length range ingroup (bp)	253-256	213-217	467-472
Length mean ingroup (bp)	254.3	214.7	469
Length mean outgroup (bp)	251.6	215.6	467.2
Aligned length	278	223	501
G + C content range (%)	0.47-0.55	0.53-0.58	0.5-0.56
G + C content mean (%)	0.5	0.55	0.52
Number of excluded sites	24	14	38
Number of indels (ingroup)	18	9	27
Number of indels (total)	24	12	36
Number of informative indels (ingroup)	7	2	9
Size of indels (ingroup)	1-6	1-2	1-6
Size of indels (total)	1-6	1-5	1-6
Number of sites after exclusion	254	209	463
Number of variable sites	103	81	184
Number of constant sites	151	128	279
Number of uninformative sites	42	34	76
Number of informative sites	61	47	108
Transitions (minimum)	81	66	147
Transversions (minimum)	61	34	96
Transitions/tranversions	1.33	1.94	1.53
Sequence divergence (St Helena) (%)	0-4.3	0-4.4	0.2-3.9
Sequence divergence (St Helena taxa/*Felicia*) (%)	9.9-21.2	8.2-15.5	10-17.6

Fig. 2.3: One of the two most parsimonious trees based on an analysis of ITS1 and ITS2 sequences (unambiguous regions only) of *Commidendrum*, *Melanodendron* and *Felicia*. The two trees are 301 steps long with a CI = 0.77 and RI = 0.75.

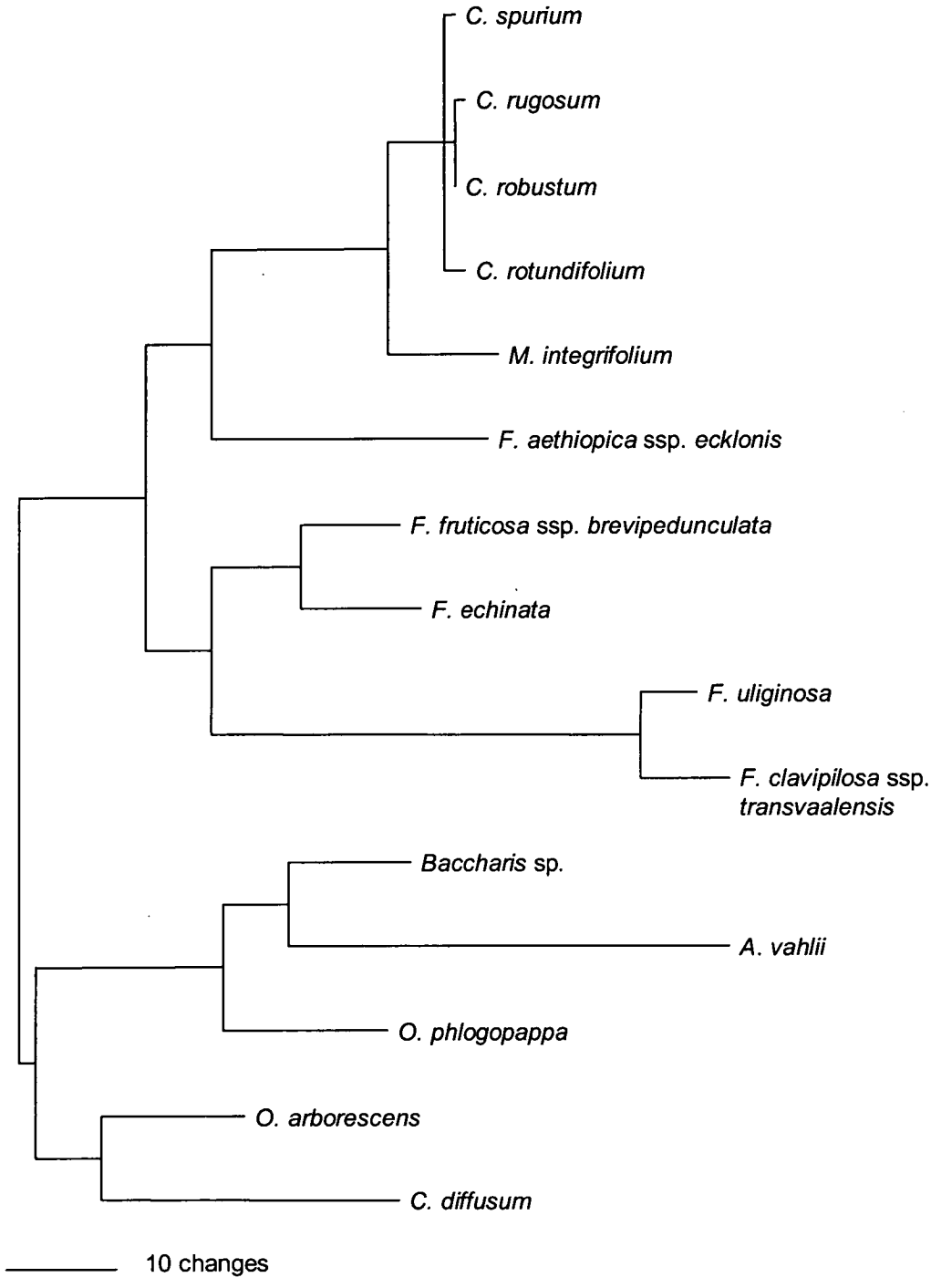
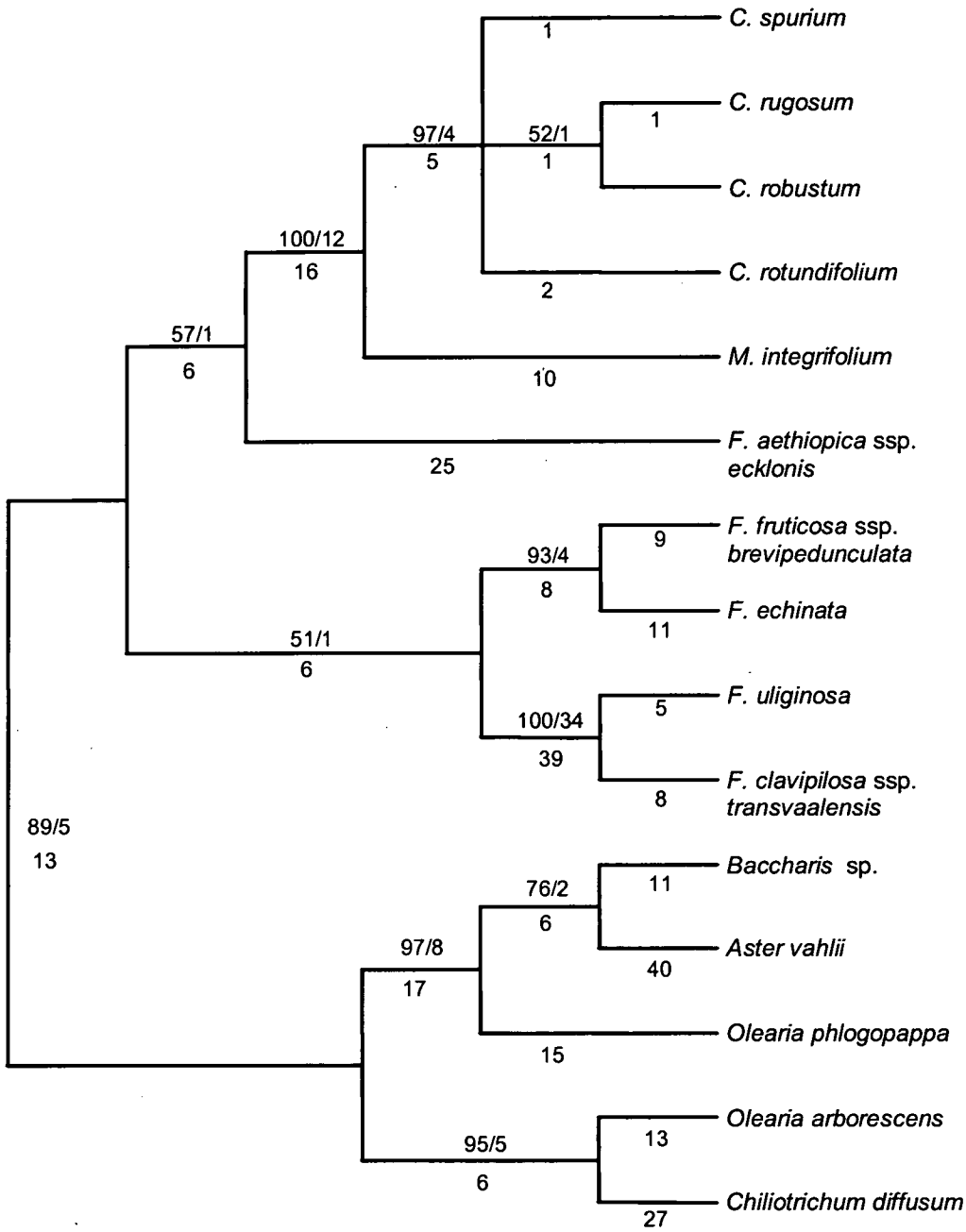


Fig. 2.4: The strict consensus tree of the two most parsimonious trees based on an analysis of ITS1 and ITS2 sequences (unambiguous regions only) of *Commidendrum*, *Melanodendron* and *Felicia*. The two trees are 301 steps long with a CI = 0.77 and RI = 0.75. The first figure above each branch is the bootstrap value (based on a 1,000 replicates) whilst the second figure is the decay index. Branch lengths are indicated below each branch.



2.4 Discussion

Relationships of Commidendrum and Melanodendron

The molecular phylogeny inferred from ITS sequences of rDNA strongly supports the monophyly of *Commidendrum*. Although the species display a range of morphological and ecological variation they form a very closely related group, as indicted by low sequence divergence and the lack of resolution in the phylogenetic trees. The ITS data did not resolve species relationships in *Commidendrum* adequately, although low support was provided for a *C. rugosum* and *C. robustum* clade. The presence of hybrids between C. *robustum* and *C. rugosum*, at sites where the two species occur in close proximity, also indicates a close genetic relationship. There is also morphological and recently, anatomical evidence from wood (Carlquist, 2001), to support a close relationship between *C. robustum* and *C. rugosum*. Both species have inflorescences composed of one to a few large capitula with many florets, as opposed to the highly branched corymbs of *C. spurium* and *C. rotundifolium*. They also share an unusual feature in their wood anatomy, not seen in the other *Commidendrum* species, the presence of elongate crystals in the ray cells (Carlquist, 2001). Although ITS data did not provide any phylogenetic evidence for a close relationship of *C. spurium* and *C. rotundifolium*, as opposed to the other species, the species do share some morphological and phenological features. The two species flower at the same time and both have an inflorescence composed of a branched corymb. They are also the only two species which will produce shoots from old wood (Cronk, 2000). A number of putative hybrids between *C. spurium* and *C. rotundifolium,* in the seed orchards on St Helena, were also recently confirmed using RAPDs (Chapter 3), providing further evidence for a close genetic relationship.

The close relationship of *Melanodendron* to *Commidendrum*, as demonstrated by the molecular phylogeny, is consistent with the hypothesis that the two genera originated from a common ancestor via a single dispersal event. A common origin for related endemic genera is more likely than multiple colonisations on small isolated islands, like St Helena, as the arrival of propagules is a very rare event (Kim *et al.*, 1996). The monophyly of related, but morphologically divergent taxa has been shown for a number of island plant groups (Kim *et al.*, 1996; Baldwin *et al.*, 1991; Givnish *et al.*, 1995). The close relationship of *Commidendrum* and *Melanodendron* is also supported by their shared wood anatomy, for example, in fibre dimorphism and homogeneous Type II wood rays (Carlquist, 2001).

Interestingly most Asteraceae have heterogeneous or paedomorphic wood rays (Carlquist, 2001). Carlquist (2001) also showed *Commidendrum* and *Melanodendron* had differences in wood anatomy, justifying their current treatment as two genera. The molecular phylogeny therefore wholly supports the anatomical work by Carlquist (2001).

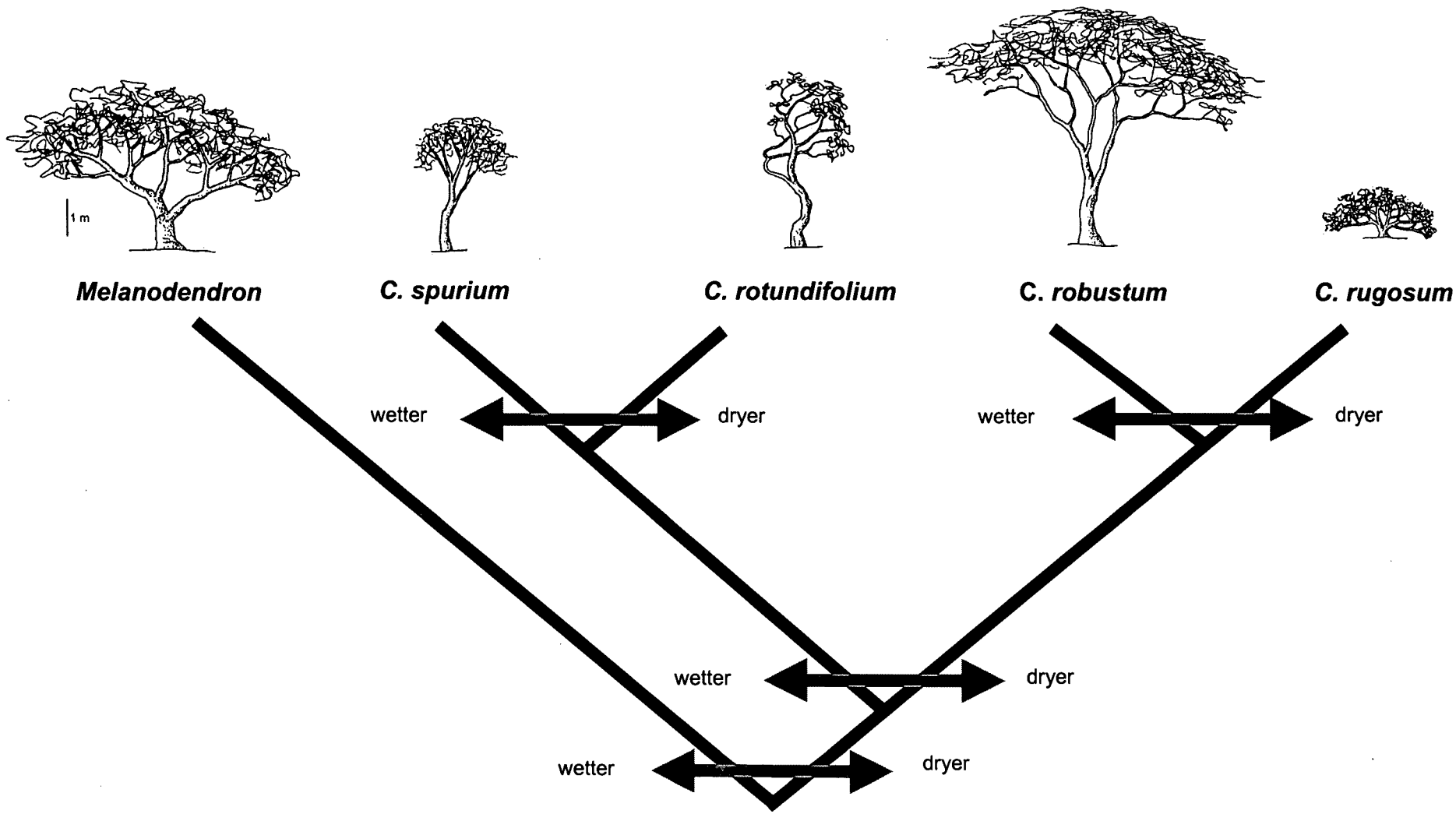
Adaptive radiation: ecological and morphological divergence

The low levels of phylogenetic divergence seen in *Commidendrum* together with evidence of hybridisation between species, strongly supports the hypothesis that the genus has undergone recent speciation. The four species of *Commidendrum* are distributed in a range of dry to mesic habitats ranging from the coastal cliffs and dry scrub of the lowlands (0-500m) to the wetter upland slopes and cliffs at mid to high elevations (500-700m). Our hypothesis is that the speciation of *Commidendrum* is ecological in origin, driven to a large extent by adaptation to different climatic zones (especially wet/dry environments) on St Helena. There is a dry-mesic gradient associated with the habitats of the four *Commidendrum* species, albeit overlapping to some extent, which provides evidence for minor ecological shifts. These ecological shifts are represented in the schematic diagram in Figure 2.5.

The first and major ecological shift in the evolution of the arborescent Astereae on St Helena would have, however, occurred in the divergence of *Commidendrum* (dryer) and *Melanodendron* (wetter) from a common ancestor (Fig. 2.5). *Melanodendron integrifolium* is confined to the wettest and coolest habitat on St Helena, tree fern (*Dicksonia arborescens*) thicket. On St Helena, tree fern thicket is restricted to the central ridge of mountains (700-823m), which are often mist covered. *Melanodendron integrifolium* is drought intolerant and shows characteristic features of a species adapted to wet sub-tropical conditions, with large wet-sclerophyll leaves and a high wood mesomorphic ratio (611) associated with mesic habitats (Carlquist, 2001). Vessel indicators of wood such as the mesomorphic ratio (vessel diameter x vessel element length divided by number of vessels per mm^2 of transection) tend to be sensitive indicators of ecology (Carlquist, 2001).

C. rugosum is restricted to the coastal cliffs, steep lowland valleys and dry scrub. It is the most drought tolerant of the four *Commidendrum* species, and has many associated apparently adaptive features, including small revolute, resinous leaves covered in glandular hairs. *Commidendrum rugosum* has the lowest wood mesomorphic ratio (39) of all the *Commidendrum* species (Carlquist, 2001) which is comparable to shrub species of dry

Figure 2.5: Schematic diagram of hypothesised relationships of *Commidendrum* and *Melanodendron* showing a possible scenario of possible divergent selection at each node. Wetter/Dryer refers to putative ecological shifts at nodes and not "characters" optimised on a tree. Illustrations of habit by Christina Oliver



habitats in southern California. The highly branched domed crown of *C. rugosum* is effective at condensing sea fogs, causing a pronounced stem-flow at the centre (Cronk, unpublished observation).

Although *C. rugosum* generally occurs at lower altitudes than *C. robustum*, *C. rugosum* is found on cliffs in the *C. robustum* zone. This has allowed the formation of hybrids at a number of localities. At a locality called Deep Valley, *C. rugosum* occupies the lower cliff faces (300-400) whilst *C. robustum* is found at the top of the cliffs (450-500m). *Commidendrum robustum* is also drought tolerant, but not to same extent as *C. rugosum*. The leaves of *C. robustum* are hairy above and white floccose/tomentose below, suggesting drought tolerance. The species, as the common name, Gumwood indicates, is resinous. In fact all the *Commidendrum* species are resinous to various degrees, with *C. rugosum* being the most resinous and *C. spurium* (False Gumwood) the least. Resin in plants may be associated with defence against insect herbivory, desiccation tolerance or prevention of UV light injury.

Information on the original distribution, ecology and habitat of *C. rotundifolium* (Extinct in the Wild) and *C. spurium* (Critically Endangered) is limited by the early disappearance of these species over their range and before the first records were made. As a consequence information on species' ecology is largely speculative and based on historical records and the extrapolation of extant distributions. *Commidendrum rotundifolium* is thought to have been an associated species of dry gumwood (*C. robustum*) woodland (300-500m) and, also, to some extent the wet gumwood woodland which existed at higher elevations (500-650m) (Cronk, 1989). The slopes and cliffs at mid-elevation subject to drought during the summer months and species would have had to be drought tolerant to some extent. *Commidendrum rotundifolium* has a mesomorphic ratio of 339, which suggests a mesic habitat according to Carlquist (2001). The broad dark green, glabrous leaves of *C. rotundifolium* may indicate a mesic habitat. However, it is resinous, a trait particularly evident on the upper surface of the leaves, which may impart some desiccation tolerance.

Commidendrum spurium is probably the only *Commidendrum* species of a true mesic habitat. In would have been a constituent species of wet gumwood woodland, which existed on the slopes and cliffs just below the central ridge (500-650m) and a sub dominant species of Cabbage-tree woodland (600-750m) (Cronk, 1989). The remnants of its former distribution can be still seen today at Mount Vesey (690m) and Coles Rock (640m). The

mesomorphic ratio of *C. spurium* is 385 (Carlquist, 2001), the highest of all the *Commidendrum* species and a clear indication of a wet forest species. The thin, light green leaves are typically mesomorphic.

Diversity in floral characters

As well as displaying a range of ecological adaptations to different climatic conditions on St Helena, *Commidendrum* also exhibits a range of floral diversity. A divergence in inflorescence and capitulum size occurs between *C. rugosum-C. robustum* (large capitula) and *C. rotundifolium-C. spurium* (small capitula), a possible indication of their relationships. *Commidendrum rugosum* and *C. robustum* both have one or a few terminal capitula composed of many florets. In contrast *C. rotundifolium* and *C. spurium* both have a corymb composed of many small capitula, each with a few florets. The ecological and evolutionary significance of this difference in inflorescence type is open to speculation. Burtt (1961) suggested a few important factors when considering the reduction trends (from a large capitulum to many small capitula arranged in a corymb) in the evolution of Asteraceae inflorescences: i) duration of flowering, ii) sex-ratios, iii) insect attack and iv) mechanical strength. These four factors all potentially affect the evolution of inflorescence type in *Commidendrum* and are briefly addressed below. The duration of flowering in *C. rotundifolium* of one terminal corymb is approximately 4-6 weeks whilst that of a terminal capitulum of *C. rugosum* is much less, approximately 1-2 weeks. *Commidendrum rotundifolium* and *C. spurium* only flower once a year at the beginning of the rainy winter season (April-June). This contrasts with *C. rugosum* and *C. robustum* which flower sporadically throughout the year, although still predominantly in April-July. The restricted flowering window of *C. rotundifolium* and *C. spurium*, just before the winter rains, may indicate drought intolerance of seedlings and selection towards inflorescence longevity and hence reproductive output. Furthermore, corymbose inflorescences, by promoting flower production over longer periods, may be advantageous in habitats subject to more rainy days. The proportion of female florets (ray) to hermaphrodite florets (disc) is greater in C. *rotundifolium* and *C. spurium* than *C. robustum* and *C. rugosum*, simply as a consequence of capitulum size. This would have the effect of increasing outbreeding (Burtt, 1961) possibly an important factor on islands. *Commidendrum rotundifolium* and *C. spurium* have been shown to be self-incompatible (Chapter 3) which supports the importance of outbreeding in these species. A defence against insect attack may have also been important in the evolution of a corymb from a single capitulum in *C. rotundifolium* and *C. spurium*. Capitula of *C.*

robustum and *C. rugosum* are heavily infested by seed eating larvae of the endemic moth, *Homoeosoma privata* Walker. The larvae can destroy entire seed heads before pupating and emerging from the capitula. In contrast the smaller capitula of *C. rotundifolium* and *C. spurium* are not infested. Bud infestation by insect larvae was shown to increase with capitulum size in a survey of 20 herbaceous Asteraceae species by Fenner *et al.* (2002). Burtt (1961) suggested that inflorescences arranged in large capitula on unbranched peduncles are more vulnerable to mechanical damage than coymbs containing the same number of florets. *Commidendrum spurium*, *C. rotundifolium* and *M. integrifolium* all have corymbs and occur at higher elevations on St Helena, and may therefore be more exposed to wind and rain damage.

Heterochrony and morphological diversity

Despite showing low levels of phylogenetic divergence in ITS sequences, *Commidendrum* species display a range of morphological diversity in habit, inflorescence type and leaf shape, as shown in previous sections. This could be attributed to changes in the relative timing and/or rate of development (heterochrony). A delay in the initiation (offset) or bringing forward (onset) of a specific development process, such as flowering time, as well as its rate can have dramatic effects on plant morphology (Li & Johnston 2000; Bateman 1994; Zopfi, 1995). Below we discuss the role that heterochrony may have had in the evolution of habit (and consequently life history) and inflorescence architecture in *Commidendrum*. The species of *Commidendrum* and *Melanodendron* all show a growth form which conforms to the modèle de Leewenburg (Hallé & Oldman, 1970), in which one to three side shoots develop below terminal inflorescences. The diversity in habit in *Commidendrum* could be a result of ontogenetic differences in the timing of the switch between the vegetative and reproductive phase. Flowering in *Commidendrum rugosum*, commences within 1-2 years of establishment and can occur when individuals are just 10 centimetres high. This precocious onset of reproduction, combined with short internodes, results in a dome shaped shrub up to one meter in height. In contrast, flowering in *Commidendrum robustum*, and hence reproductive maturity, does not commence until saplings are at least 2-3m tall (3-5 years after establishment), and already have a pronounced trunk. The evolution of habit in *Commidendrum* may be related to natural selection acting on life history traits such as flowering time. *Commidendrum rugosum* is a good coloniser of the barren, degraded slopes on St Helena known as the Crown Wastes. Although the barren habitats seen on St Helena today are man-made, evidence suggests that open habitats caused by wind erosion would have existed during the geological history of St Helena (Baker, 1970;

Muir & Baker, 1968). Plants which could colonise wind-swept and eroded cliffs quickly would be at an advantage. There would, therefore, have been strong selection for precocious reproduction and small stature as in *C. rugosum*. The evolution of *Cyanea* on the islands of Hawaii is postulated to have occurred through successive heterochronic events which have produced progressively more paedomorphic species (Lammers, 1990).

Heterochrony could also explain the dramatic differences seen in inflorescence architecture. The capitulum of Asteraceae is a racemose inflorescence, a condensed shoot, which has developed from an apical meristem (Burtt, 1977). In the formation of a capitulum the development of the internodes are suppressed or halted, an example of paedomorphosis of the progenesis type (Harris, 1999). The early stages of inflorescence development in *Lobelia tupa*, which has simple racemes up to 3m in height, displays a compact shape remarkably similar to that of a capitulum (Harris, 1999). Elongation of the raceme in *L. tuba* takes place late in ontogeny, long after the flowers are formed by the inflorescence apex. We postulate that in the development of *Commidendrum rugosum*, with a single large capitum, all the internodes of the inflorescence meristem are suppressed and so all floret primordia are aggregated into one large capitulum. However, in the branched corymbs of *C. roundifolium* and *C. spurium*, internodes of the primary inflorescence shoot (1st order) and subsequent axillary shoots (2nd to 5th branch orders) elongate, to produce a corymb. Suppression of internodes in the inflorescence meristem only occurs at later branch orders in the corymbose species resulting in a small aggregation of floret primordia, and therefore many small capitula. Differences in the degree of branching between species (*C. rotundifolium* and *C. spurium*) will depend on the number of shoots, which develop at each node. The major differences seen in the inflorescence architecture of *Commidendrum* could therefore be attributed to ontogenetic differences in the offset of inflorescence shoot elongation (earlier or later depending on patterns of evolution and ancestry).

Relationships with southern African Astereae

The ITS phylogeny confirms the close relationship of southern African *Felicia* to *Commidendrum* and *Melanodendron* as first indicated by Noyes and Rieseberg (1999). However, it is difficult to make inferences of the closest relative of *Commidendrum* and *Melanodendron* and the evolution of these genera on St Helena based on the samples in this study. Of the genera in the Astereae, the study by Noyes and Rieseberg narrowed the origins of *Commidendrum* and *Melanodendron* to South Africa, in particular to *Felicia* and *Amellus*.

Although this has been a considerable breakthrough in elucidating the closest relatives of these woody composites from St Helena more thorough sampling of *Felicia* (83 species), *Amellus* (12 species) and other closely related taxa is required. This is of particular importance as our work suggests that *Felicia* could be highly divergent, even paraphyletic. Further sampling of *Felicia* however, may show that this is simply an artefact of our limited sampling. Our phylogeny inferred from ITS does weakly suggest that *Commidendrum* and *Melanodendron* may be nested within *Felicia*, as a sister group of a Cape herbaceous species.

Conservation implications

Species conservation priorities are predominantly governed by the degree of threat to a species, as emphasised by the IUCN Red List of threatened species (Hilton-Taylor, 2000). Prioritising species and populations for conservation is an integral component of conservation management planning, ensuring the effective allocation of limited resources. Conservation prioritisation based on the degree of threat to a species uses the assumption that all species have equal biodiversity value. However, a number of authors argue that species do not have equal value and that measures should be employed to assess biological distinctness (Vane-Wright *et al.* 1991; May, 1990; Faith, 1992). The 'taxic diversity' measure of Vane-Wright *et al.* (1991) and the 'phylogenetic diversity' of Faith (1992) assess the biological distinctness of species or taxa directly from phylogenetic trees. By applying complementarity (Vane-Wright *et al.* 1991) or sub-set sampling (Faith, 1992) a sub-set of taxa can be selected from a group which maximises the proportion of diversity conserved. Preference is ultimately given to taxonomically or phylogenetically distinct species, i.e. basal species (Vane-Wright *et al.* 1991) or those with long branch lengths (Faith, 1992), over those species in speciose clades with closely related sibling taxa. Applying this approach to *Commidendrum* and *Melanodendron* on St Helena, priority would be given to *Melanodendron integrifolium* first, and then a single species of *Commidendrum*. From a molecular phylogenetic perspective, and using the approach of Vane-Wright *et al.* (1991) and Faith (1992), one could argue that conserving just one species of *Commidendrum*, for example, *C. rugosum* is all that is required to conserve the phylogenetic diversity of the St Helena clade. In contrast, to the selection of taxa based on phylogenetic distinction, Erwin (1991) proposes that recently and rapidly evolving taxa should be the focus of conservation efforts. He argues that these dynamic "evolutionary fronts" generate future biodiversity in contrast to relict endemics, which although phylogenetically distinct, represent the last foothold of past radiations. However, Rieseberg and Swensen (1996) do not support the

notion of prioritising species based on any phylogenetic criteria, and argue against placing priorities on unique and irreplaceable entities such as endangered species. In the case of *Commidendrum* and *Melanodendron* from St Helena, we also argue against prioritising species based on phylogenetic relationships alone. *Commidendrum* and *Melanodendron* represent the only extant adaptive radiation on St Helena, and all efforts should be made to conserve the representative ecological and morphological diversity in its entirety. Although *C. spurium* and *C. rotundifolium* are severely threatened and close to extinction they should not be left to undergo the same fate as two species of *Wahlenbergia*, Schrad., *W. burchelli* A. DC. and *W. roxburghii*, A. DC. now both extinct on St Helena. With only two species of *Wahlenbergia* now remaining, *W. angustifolia* (Roxb.) A.DC. and *W. linifolia* (Roxb.) A.DC., both of which are threatened, we can only speculate on the evolution and adaptive radiation of that genus.

2.5 Acknowledgments

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Incompatibility and hybridisation in *Commidendrum rotundifolium* and *C. spurium* from St Helena: implications for *ex situ* management and species recovery.

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Abstract

Randomly Amplified Polymorphic DNA (RAPD) was used to investigate five morphologically ambiguous individuals present in *ex situ* localities and seed orchards of *Commidendrum spurium* (Critically Endangered) and *C. rotundifolium* (Extinct in the Wild) from St Helena. RAPDs revealed that four of the individuals were hybrids, supporting morphological evidence, and one individual was a putative backcross. In order to investigate the causes of poor seed viability in *C. rotundifolium* and the possibility of a self-incompatibility system, experimental crosses were conducted and compared with *C. spurium*. Observations of pollen tube growth and behaviour at the stigmatic surface indicate that there is sporophytic self-incompatibility operating in both *C. rotundifolium* and *C. spurium*. Poor seed viability in *C. rotundifolium* can be explained by a paucity of *S*-alleles, as all individuals on St Helena are descendants from one tree. Self-incompatibility in *C. rotundifolium* and *C. spurium* has serious implications for species recovery and reintroduction. Inter-specific hybridisation is discussed as a means of introducing *S*- allele diversity into *C. rotundifolium* and saving it from inevitable extinction.

Keywords: Reproductive biology, *Commidendrum rotundifolium*, *Commidendrum spurium*, self-incompatibility, St Helena, endemics, hybridisation, conservation, seed orchard management, species recovery.

3.1 Introduction

The genus Commidendrum *on St Helena*

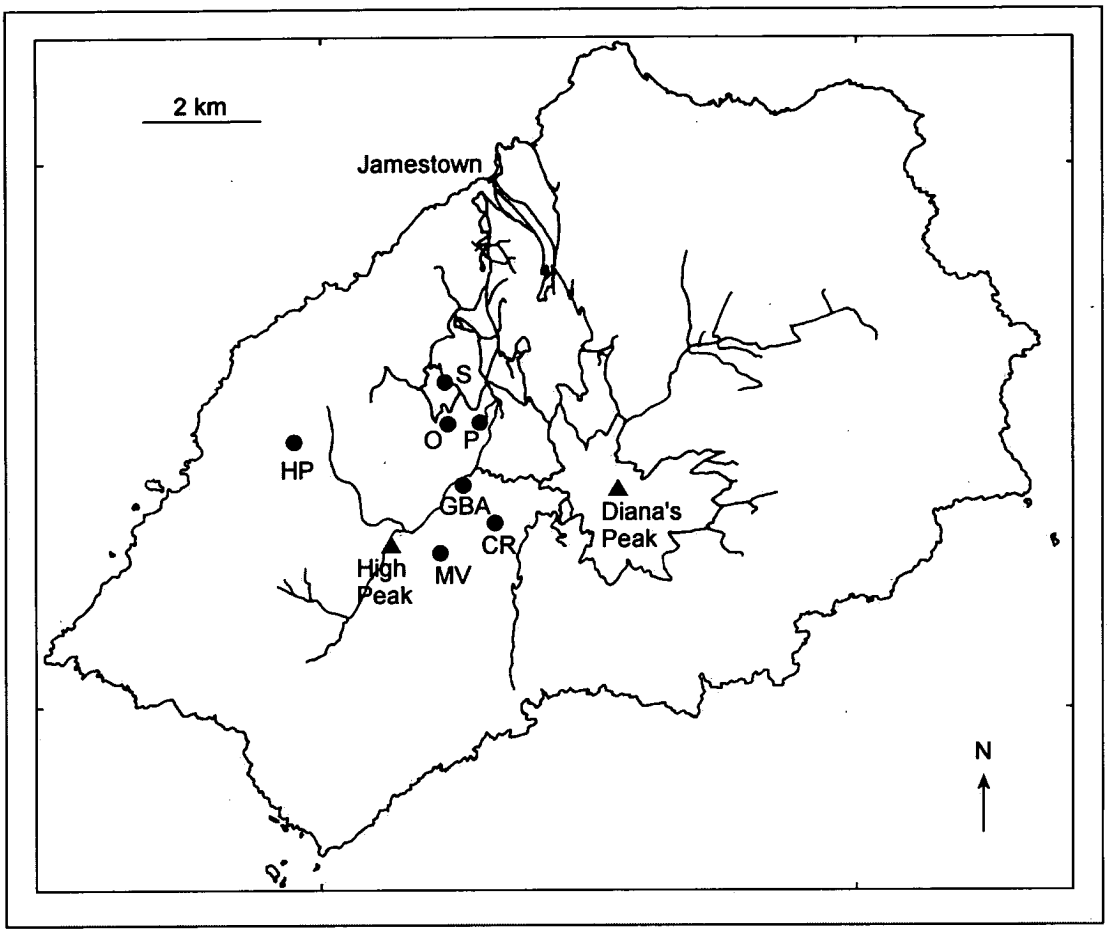
The endemic genus *Commidendrum* DC. (Asteraceae) from St Helena, an isolated volcanic island in the south Atlantic (lat. 15° 56′S, long. 5°42′W), consists of four very closely related species (Chapter 2). All the species are globally threatened and are listed in the IUCN Red List of Threatened Species (Hilton-Taylor, 2000). *Commidendrum rotundifolium* (Roxb.) DC. (Extinct in the Wild) and *C. spurium* (G. Forst.) DC. (Critically Endangered) are the most threatened, and, as a consequence, the Conservation and Environmental Section (CES) of the Agriculture and Natural Resources Department (ANRD) on St Helena have established seed orchards for *ex situ* conservation and species recovery. The *ex situ* and *in situ* sites used through out the text are shown in Fig. 3.1.

Conservation history

Commidendrum rotundifolium was thought to be extinct by the end of the 19th century until one individual was rediscovered in 1982 by a local naturalist (Cronk, 2000). This last known tree, which was growing on an inaccessible cliff at Horse Pasture (Fig. 3.1), blew down in a gale in 1986. Fortunately, George Benjamin, the Conservation Officer at the time, managed to take cuttings and seed, and the species was propagated and established *ex-situ* on the island. Historical records, summarised by Cronk (2000) indicate that the species was already rare by the late 18th century and has therefore been restricted to small populations for a prolonged period. The rarity of many of the endemics and the ecological degradation seen on St Helena today is largely a legacy of Portuguese (who discovered the island in 1502) and early British settlers (Cronk, 2000). Using historical records combined with the extrapolation of distribution data, Cronk (1989) postulated that *C. rotundifolium* was an associated species of dry gumwood (*C. robustum*) woodland (300-500m) and also to some extent the wet gumwood woodland which existed at higher elevations (500-650m) on St Helena.

There are only eight known individuals of *Commidendrum spurium* in the wild. Seven of the individuals make up a small relict population on steep cliffs below Mount Vesey (Fig. 3.1). The site is heavily encroached by invasive exotics such as *Phormium tenax* Forst. and *Solanum mauritianum* Blanco. In 1999 and 2000 seedlings were observed growing in rock crevices and adjacent cleared areas on the top of the cliff. However, these have not become established, possibly due to grazing by introduced rabbits.

Fig. 3.1: Map of St Helena showing the road network and the *in situ* and *ex situ* localities of *C. spurium* and *C. rotundifolium*. HP=Horse Pasture; O=Oaklands; P=Pounceys; S=Scotland; GBA=George Benjamin's Arboretum; CR=Coles Rock; MV=Mount Vesey.



The other individual of *C. spurium* occurs on a cliff face at Coles Rock (Fig. 3.1). The isolated site, like Mt Vesey, is heavily encroached by invasive exotics. The tree at Coles Rock is infested with the introduced scale insect, *Orthezia insignis* Browne, and the tree is nearing senescence. In 1986 a number of seedlings, which were growing in the rock crevices around the tree at Coles Rock, were collected, grown on and established at Pounceys (see Table 3.1 for description of *ex situ* sites on St Helena). Until 2000 there was another isolated individual of *C. spurium* at a locality called Oaklands (Fig. 3.1). However it became infested with stem rot and subsequently died, illustrating the vulnerability of this Critically Endangered species. According to Cronk (1989) *Commidendrum spurium* would have once been a constituent species of wet gumwood woodland, which existed on the slopes and cliffs just below the central ridge (500-650m) and a subdominant species of Cabbage-tree woodland (600-750m).

Ex situ *and propagation history*

Commidendrum rotundifolium

All the individuals of *Commidendrum rotundifolium* on St Helena originate from the one tree discovered in 1982 at Horse Pasture. There are two *ex situ* sites for *C. rotundifolium* on St Helena: Pounceys and Scotland (St Helena). For details on the *ex situ* sites and propagation history of *C. rotundifolium* see Table 3.1 and 3.2. The 17 trees at Pounceys are much smaller than early descriptions of the tree (1.3-2.5m in contrast to 7m) and smaller than the last tree (*c.* 4m) at Horse Pasture. Individuals also lack vigour, a possible indication of inbreeding depression. The trees are heavily infested with a range of exotic pests and pathogens including white ants (*Cryptotermes*). As all four species of *Commidendrum* are represented at Pounceys, and there is evidence of hybridisation (see below), a more isolated *C. rotundifolium* seed orchard was established at Scotland (locality of the ARND headquarters on St Helena) in 1998, on the grounds of the ANRD. It was hoped that the seed orchard at Scotland would be a source of pure bred propagules for a future reintroduction programme of *C. rotundifolium*. By the spring of 2000 some of the individuals sown in the spring of 1998, now between 0.75 and 1 metre high, had already started to flower. The majority of the individuals at Scotland resemble *C. rotundifolium* morphologically. However, it was noted that two saplings had leaves which had an intermediate morphology between *C. rotundifolium* and *C. spurium*. The young trees at Scotland are generally healthy and show vigorous growth.

Commidendrum spurium

There are three *ex situ* sites for *C. spurium* on St Helena: Pounceys, the George Benjamin Arboretum (GBA) at Casons, and Mt Vesey (see Table 3.1). Six of the *C. spurium* trees at Pounceys are the original seedlings that George Benjamin collected from Coles Rock in 1986. For the propagation history of *C. spurium* and the source of germplasm for the *ex situ* sites see Table 3.2. Two of the individuals at Pounceys and all the individuals at the GBA are morphologically ambiguous (Fig. 3.2) and appear to be of hybrid origin between *C. spurium* and *C. rotundifolium*. However, one of us (QC), at the time, regarded these individuals as just morphological variants of *C. spurium*. A new seed orchard for *C. spurium* was established in 1998 adjacent to the population at Mt. Vesey. However, due to concerns over the possible hybridisation of *C. rotundifolium* and *C. spurium* at Pounceys only plants raised from seed collected from the Mt Vesey population were utilised.

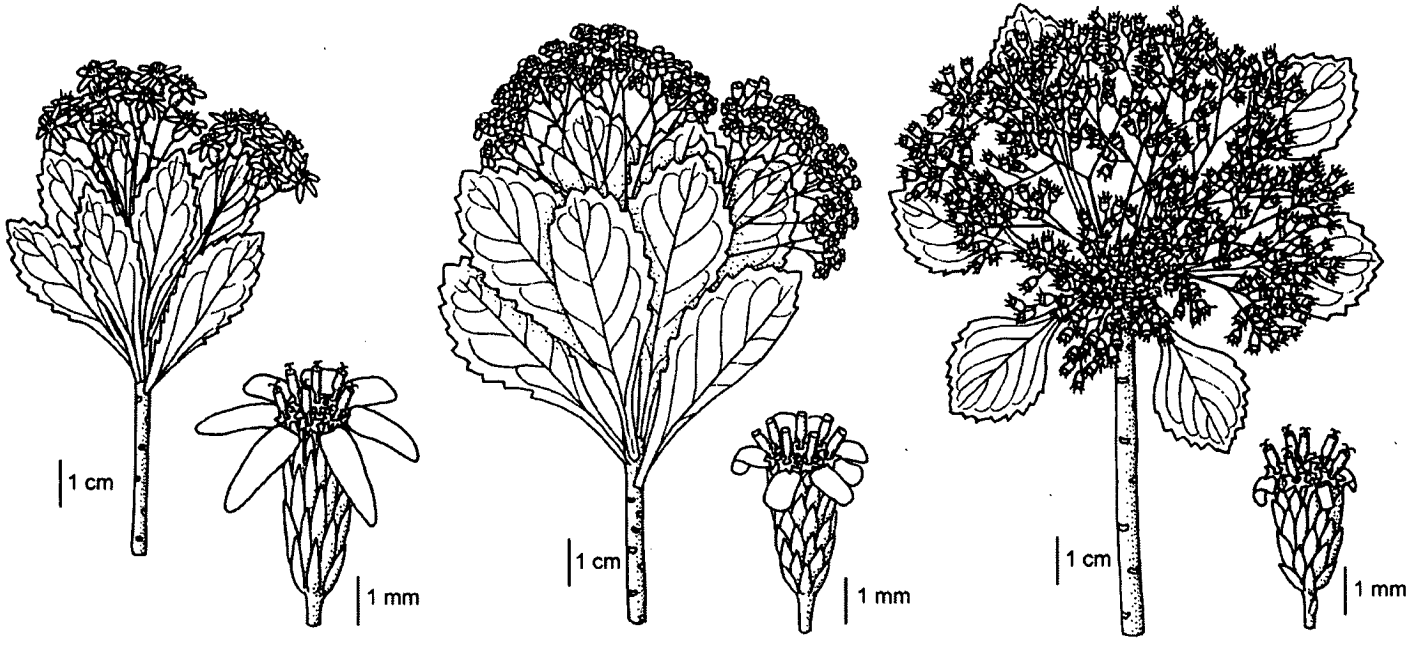
Table 3.1: Descriptive list of *in situ* and *ex situ* sites on St Helena used in text. HP=Horse Pasture, CR=Coles Rock, MV=Mt Vesey, O=Oaklands, P=Pounceys, S=Scotland, GBA= George Benjamin Arboretum and MVO= Mt Vesey seed orchard.

Localities	Description	Origin	Notes
HP	Single tree, source of all current *C. rotundifolium*.	wild	Died in 1986
CR	Single *C. spurium* tree	wild	
MV	Population of seven *C. spurium* individuals	wild	
O	Single *C. spurium* tree	wild	Died in 1999
P	*Ex situ* plot of land with a number of endemics. 17 C. *rotundifolium*, 8 *C. spurium* and 2 morphologically ambiguous individuals	*C. rotundifolium* -HP *C. spurium* – CR and P	See A-E and G-H in Table 3.2
S	*C. rotundifolium* seed orchard established in 1998. Over 50 accessions	Seed and cuttings collected from P	See F in Table 3.2 below
GBA	Arboretum promoting public awareness and education of St Helena endemics. 7 morphologically ambiguous individuals	Seed collected from P	See H in Table 3.2
MVO	*C. spurium* seed orchard, c. 20 accessions	Seed collected from MV	

Table 3.2: Source and propagation history of germplasm held *ex situ* on St Helena. HP=Horse Pasture, P=Pounceys, CR=Coles Rock, MV=Mt.Vesey, MVO=Mt Vesey seed orchard, S=Scotland, GBA=George Benjamin Arboretum. GB=George Benjamin, VT=Vanessa Thomas.

Germplasm	Germplasm source	Collected	Origin	Propagation Notes	Planted at	Notes
C. rotundifolium						
A	seed	by GB in 1983	HP	4 plants raised	P (21/05/84)	Flowered in 1985
B	cuttings	1982 and 1983	HP	1 cutting survived		
C	two seedlings	-	B	One seedling survived	P (29/03/83)	Flowered in 1984
D	Seed	from C in 1984	C	No germination		
E	Seed	from C in 1985	C	75% germination, only 13 survived	P	
F	seed and cuttings	by GB and VT 1986 & 1998	P	Seed germination poor but c. 50 plants raised.	S	Majority of individuals from 4 trees at P (tree nos. 1, 5, 6 and 7
C. spurium						
G	seedlings	by GB in 1986	CR	Six seedlings raised	P	
H	seed	GB	P (G)	Seed sown, resulting in 11 individuals.	P (4) GBA (7)	
I	seed	VT	MV	Good	MVO	

Fig. 3.2: From left to right: *C. spurium*, morphologically ambiguous individual and *C. rotundifolium*. Illustrated by Christina Oliver.



Evidence for self-incompatibility

With the exception of one tree in 1985 (see Table 3.2) seed germination in *C. rotundifolium* is usually very low (typically below 1%). For example, in one experiment, approximately 3,000 achenes, collected from Pounceys and Scotland in 1999 and 2000 respectively, were sown at the Royal Botanic Garden Edinburgh (RBGE), but only one seed germinated. Seed germination was also very low in the original tree at Horse Pasture (Cronk, pers. comm.) and as a consequence only four plants were raised although thousands of achenes were sown (Table 3.2). After closer examination of *C. rotundifolium* achenes it appears that low germination is due to empty achenes (no embryos).

The presence of seedlings at the population of *C. spurium* at Mount Vesey indicates that germination in this species is good. This is supported by propagation evidence from seed collected at Mount Vesey (Thomas, Endemics Section, pers. comm., 1999). Seed germination in seed collected from the largest tree (AEAST21) at Mount Vesey in 1999 was 40%. In contrast seed germination from the isolated tree at Oaklands (which is now dead) in 1999 was 8%. The low seed germination rate observed in the isolated tree at Oaklands indicates that there may be a self-incompatibility system operating in *C. spurium*.

Ex situ *conservation management of* C. spurium *and* C. rotundifolium*: the problems*

The first problem that impedes *ex situ* conservation, particularly seed orchard management, is the possibility that *C. rotundifolium* and *C. spurium* have hybridised. To maintain the morphological integrity of *C. spurium* in the new seed orchard at Mt Vesey only individuals originating from seed collected from the wild population at Mt Vesey have been utilised. No genotypes representing Cole's Rock or Oaklands have been introduced into the new seed orchard. The seed orchard therefore is unlikely to represent the full extant genotypic diversity in *C. spurium*. This could have serious implications for any future species recovery programme particularly if, as propagation evidence indicates, there is self-incompatibility system operating.

The second problem and large impediment to any species recovery program of C. *rotundifolium* is low seed viability. Propagation and preliminary germination experiments indicate that there is a self-incompatibility (SI) system operating in *C. rotundifolium* and *C. spurium*. The self-incompatibility system found in species of Asteraceae (Compositae) is

usually sporophytic stigmatic and multiallelic (de Nettancourt, 2001). Species which have undergone population bottlenecks, as *C. rotundifolium* and *C. spurium*, may have lost allelic variation at the SI locus. Because multiallelic self-incompatibility systems require a large number of incompatibility (S) alleles to maintain high levels of cross-compatibility, bottlenecks are expected seriously to reduce the potential for sexual reproduction in bottlenecked populations (Reinartz & Les, 1994).

The first main objective of our study was to determine, using RAPDs (Randomly Amplified Polymorphic DNA) as species markers, whether the morphological ambiguous individuals at Pounceys and the GBA are of hybrid origin between *C. rotundifolium* and *C. spurium* or morphological variants of *C. spurium*. The second objective was to investigate self-incompatibility in *C. rotundifolium* and *C. spurium* and its role in the low seed viability observed in *C. rotundifolium*. It is hoped that our study of the reproductive biology *of C. spurium* and *C. rotundifolium* will assist the CES on St Helena in seed orchard management and species' recovery.

3.2 Materials and methods

RAPD study

Plant material

To determine the identity of the morphologically ambiguous individuals leaves from 10 *C. spurium*, 12 *C. rotundifolium* and five morphologically ambiguous individuals were sampled in 1999 and 2000 and stored in silica gel. Details of the accession numbers and localities of the individuals sampled are listed in Table 3.3. Herbarium vouchers representing *C. spurium*, *C. rotundifolium* and the morphologically ambiguous individuals are held at the Herbarium (E) of Royal Botanic Garden Edinburgh (RBGE).

DNA extraction

Total genomic DNA was extracted from each individual using a modified CTAB procedure from Doyle & Doyle (1987). An additional washing step using 7.5 M ammonium acetate was included to remove excess polysaccharides (Weisling, *et al.* 1995). The quality and concentration (in ng) of the DNA was assessed by running the samples on 1% agarose, alongside the Hyperladder I supplied by Bioline, UK.

Screening of primers

A subset of *C. spurium* and *C. rotundifolium* samples were used to screen sixty 10-mer oligonucleotide primers from the Operon A, F and P kits (Operon Technologies). In addition 30 inter-SSR primers from the University of British Columbia, set #9 were screened. However, levels of inter-SSR polymorphism were found to be low and were thus abandoned in the screening phase. From the sixty RAPD primers screened, ten primers were selected which gave clear, reproducible species-specific bands. The primer sequences for the 10 selected primers are given in Table 3.4.

PCR

Each 25 µl PCR reaction contained: 2.5 µl x10 NH_4 buffer (Bioline, UK), 2.5 µl of 2mM dNTPs (Bioline, UK), 1.25 µl of 50mM magnesium chloride, 2.5 µl of 5µM primer, 0.1 µl of Biotaq polymerase (Bioline, UK), 5 µl DNA (1ng/µl) and 10.55 µl of sterile ultra-pure water. Amplification was carried out with the following PCR profile: i) one initial pre-step of 95°C for 2 minutes; ii) 2 cycles of 95°C (30 secs), 37°C (1 minute) and 72°C (2 minutes); iii) 2 cycles of 95°C (30 secs), 35°C (1 minute) and 72°C (2 minutes); iv) 41 cycles of 94°C (30 secs), 35°C (1 minute) and 72°C (2 minutes); v) a terminal extension of 72°C for 5 minutes. A negative control (with no DNA template) was included with the samples with each primer.

Scoring and analysis

The PCR products for each of the 27 individuals were separated using gel electrophoresis (3 hrs at 130V) on 1.6% agarose gels in TBE buffer and visualised using ethidium bromide. The presence (1) or absence (0) of strong reproducible bands was scored across each gel for each primer. Both polymorphic and monomorphic bands were scored. The analysis was conducted on the computer package R (Casgrain & Legendre, 2001). A similarity matrix was initially calculated using Jaccard's Coefficient from the presence/absence data matrix. This was subsequently converted to a distance matrix (D = 1-S). Distances between the individuals were examined in a multi-dimensional space using principal co-ordinate analysis (PCO).

Table 3.3: List of individuals and localities used in the RAPD study.

Author accession no.	Accession no. (St Helena)	Locality
C. spurium		
AEAST21	No. 5	Mt Vesey
AEAST89	-	Coles Rock
AEAST23	-	Oaklands
AEAST52	No. 5	Pounceys
AEAST47	No. 7	Pounceys
AEAST46	No. 3	Pounceys
AEAST35	No. 6	Pounceys
AEAST50	No. 4	Pounceys
AEAST48	No. 10	Pounceys
AEAST71	No. 9	Pounceys
Morphologically ambiguous		
AEAST22	No. 8	Pounceys
AEAST39	-	Casons
AEAST36	-	Casons
AEAST42	-	Casons
AEAST452	COMROT45	Scotland
C. rotundifolium		
AEAST31	No. 2	Pounceys
AEAST33	No. 14	Pounceys
AEAST54	No. 13	Pounceys
AEAST57	No. 7	Pounceys
AEAST70	No. 11	Pounceys
AEAST53	No. 5	Pounceys
AEAST49	No. 6	Pounceys
AEAST56	No. 15	Pounceys
AEAST58	No. 16	Pounceys
AEAST67	No. 12	Pounceys
AEAST68	No. 9	Pounceys
AEAST187	No. 1 or 18	Pounceys

Table 3.4: Primer sequences for the 10 RAPD primers (Operon) used

RAPD primer	Sequence 5'-3'
OPA-02	TGCCGAGCTG
OPA-08	GTGACGTAGG
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPF-02	GAGGATCCCT
OPP-04	GTGTCTCAGG
OPP-08	ACATCGCCCA
OPP-10	TCCCGCCTAC
OPP-13	GGAGTGCCTC
OPP-19	GGGAAGGACA

Compatibility study

Cross and self pollinations

To determine the role of self-incompatibility in the low reproductive success of C. *rotundifolium* a number of controlled pollinations (self and cross) were conducted on individuals at the seed orchard at Scotland, St Helena, between April and June 2000. For comparison, controlled pollinations were also conducted on individuals of *C. spurium* at Pounceys and in the wild population at Mt Vesey. Table 3.5 lists the different individuals and localities used in the pollination experiments. The individuals of *C. spurium* used in the compatibility study were all collected as seedlings around the single individual at Coles Rock and are therefore assumed to be selfed progeny. The inflorescence of *C. rotundifolium* and *C. spurium* consists of a corymb composed of many small radiate capitula (Figure 3.1). For both species, groups of developing capitula (bud stage) were bagged using small polythene pollination bags. In *C. rotundifolium* disk florets (hermaphrodite) open before the ray florets (female). To prevent contamination with self pollen, typewriting correction fluid (Tippex™) was applied on the developing buds of disk florets until the ray florets had opened and could be pollinated (Humphries cited in Alexander, 1979). In *C. spurium* the ray florets open slightly before the disk florets. However to prevent any contamination of ray florets with self pollen (before or after controlled pollination) the disk florets were removed just prior to opening. Pollination was conducted by gently dabbing a removed capitulum at anthesis (pollen donor) onto the stigmas of the ray florets (recipient). Following pollination, the pollination bags were replaced.

Fixing and staining of material for pollen tube observation

Capitula with pollinated ray florets were collected 1 day and 3 days after pollination and fixed for 24 hours in FPA solution (formalin 40%, concentrated propionic acid, 50% ethanol, 5:5:90 by volume). After fixation in FPA capitula were transferred into 70% ethanol for storage. The washing and staining protocol (using 0.1% aniline blue) for the detection of pollen tubes in the style by Dafni (1992) was modified for the small florets of *Commidendrum rotundifolium* and *C. spurium*. Squashed stigmas from at least three florets from one capitulum were viewed using a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) microscope under UV illumination.

Scoring of pollen-stigma interaction

The pollen-stigma interaction for each pollen grain was scored as one of the following categories: 1) no germination, 2) pollen grain germinates but pollen tube growth is arrested at the stigmatic surface or shortly after penetration, 3) pollen tube grows between stigmatic tissue and down the stigmatic branch towards the style and 4) pollen tube observed in the style. Crosses which appeared to have both compatible and incompatible pollen grains were repeated for confirmation. The percentage of pollen grains (mean) in category 3 and 4 (compatible) was calculated for each self or cross pollination. Pollinations which had 0-1% compatible pollen grains were classified as incompatible. Pollinations which had between 1-10% compatible pollen grains were classified as partially compatible. Pollinations which had >10% compatible pollen grains were classified as compatible.

Table 3.5: Individuals of *C. rotundifolium* and *C. spurium* used in controlled pollination experiments. Accession numbers in brackets refer to the maternal tree at Pounceys.

Accession no. (Authors)	Accession no. (St Helena)	Locality
C. spurium		
AEAST21	No. 5	Mt Vesey
AEAST181	No. 2	Mt Vesey
AEAST183	No.1	Mt Vesey
AEAST35	No. 6	Pounceys
AEAST46	No. 3	Pounceys
AEAST47	No. 7	Pounceys
AEAST52	No. 5	Pounceys
C. rotundifolium		
AEAST379	COMROT15 (No. 6)	Scotland
AEAST380	COMROT26 (No. 6)	Scotland
AEAST381	COMROT4 (No. 4)	Scotland
AEAST383	COMROT25 (No. 1)	Scotland
AEAST384	COMROT46 (No. 7)	Scotland
AEAST385	COMROT43 (No.1)	Scotland
AEAST386	COMROT28 (No. 6)	Scotland
AEAST387	COMROT29 (No. 5)	Scotland
AEAST390	COMROT14 (No. 1)	Scotland

3.3 Results

RAPD study

A total of 48 clear reproducible loci was scored for the ten primers, 23 of which were polymorphic and 25 monomorphic across the two species. The presence or absence of bands at each polymorphic locus for the individuals of *C. spurium*, *C. rotundifolium* and the morphologically ambiguous individuals is shown in Table 3.6. Nine of the polymorphic loci had bands which were specific to *C. rotundifolium* individuals and seven had bands exclusive to *C. spurium*. *Commidendrum rotundifolium* showed intra-specific polymorphism in six loci. This intra-specific polymorphism could be potentially informative regarding the parentage of any progeny. For example, AEAST67 (*C. rotundifolium*) has a band which is

absent in all the other *C. rotundifolium* and *C. spurium* individuals. However, two of the ambiguous individuals, AEAST22 and AEAST39, also have this band. Although not all the 17 individuals at Pounceys were analysed it is highly probable that AEAST 67 is the paternal parent of AEAST22 and AEAST39. The five morphologically ambiguous individuals showed polymorphism in six loci. At one of these loci two of these individuals possess bands not present in either *C. spurium* or *C. rotundifolium*. Although the *C. spurium* individuals are from three different localities on St Helena no intra-specific polymorphism was observed in the 48 loci scored. The banding profile obtained from the RAPD primer OPF-02 is shown in Figure 3.3. There is a clear additive banding pattern present in the morphologically ambiguous individuals. This clear additive pattern is also seen in 12 of the sixteen loci which had species specific bands (Table 3.6). In the other four loci, which show species-specific banding patterns, AEAST452 (maternal parent = *C. rotundifolium*) shows the same banding pattern as *C. rotundifolium*. In contrast the other four morphologically ambiguous individuals, AEAST22, AEAST39, AEAST36 and AEAST42 (maternal parent = *C. spurium*) share the same banding pattern as *C. spurium*. The relationship of the 27 individuals is represented as a PCO plot (first two principal co-ordinates) in Figure 3.4. Four of the morphologically ambiguous occupy an intermediate position between *C. spurium* and *C. rotundifolium*. The sample AEAST452 from Scotland, however, has greater affinity with *C. rotundifolium* and occupies a position intermediate between *C. rotundifolium* and the other four morphologically ambiguous individuals (possible backcross).

Table 3.6: Presence and absence of bands generated by 10 RAPD primers for *C. spurium*, *C. rotundifolium* and five morphologically ambiguous individuals (MAI).

	A2			A8	A18		A19				F2		P4	P8	P10	P13				P19			
C. spurium																							
AEAST21	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST89	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST23	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST52	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST47	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST46	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST35	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST50	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST48	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
AEAST71	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	1
MAI																							
AEAST22	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1
AEAST39	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1
AEAST36	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1
AEAST42	0	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1
AEAST452	0	1	0	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0
C. rotundifolium																							
AEAST31	0	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST33	0	0	1	1	1	0	0	0	0	1	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST54	0	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST57	0	0	1	1	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST70	0	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST53	0	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST49	0	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST56	0	0	1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST58	0	0	0	1	1	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST67	1	0	1	1	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST68	0	0	1	1	1	1	0	0	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0
AEAST187	0	0	0	1	1	1	0	0	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0

Fig. 3.3: RAPD banding profile of *C. spurium*, morphologically ambiguous individuals (MAI) and *C. rotundifolium* obtained using primer OPF-02. Band a is specific to *C. rotundifolium* and b is specific to *C. spurium*. The MAI have both a and b bands.

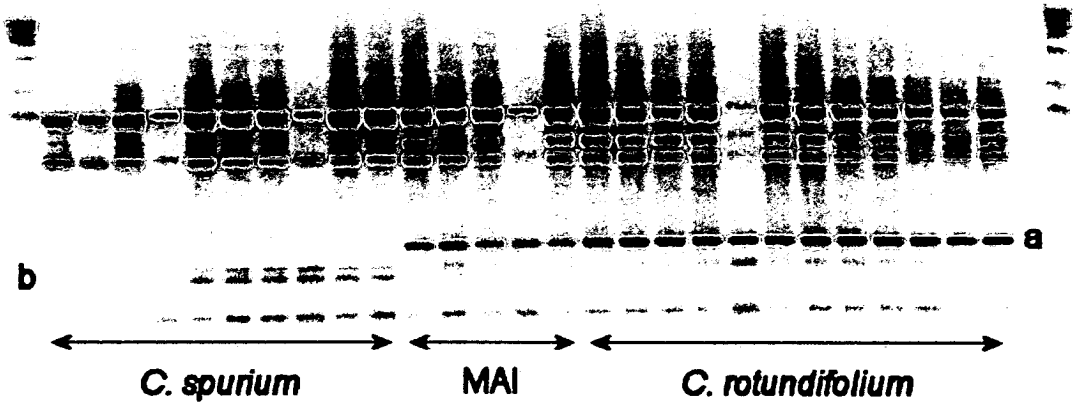
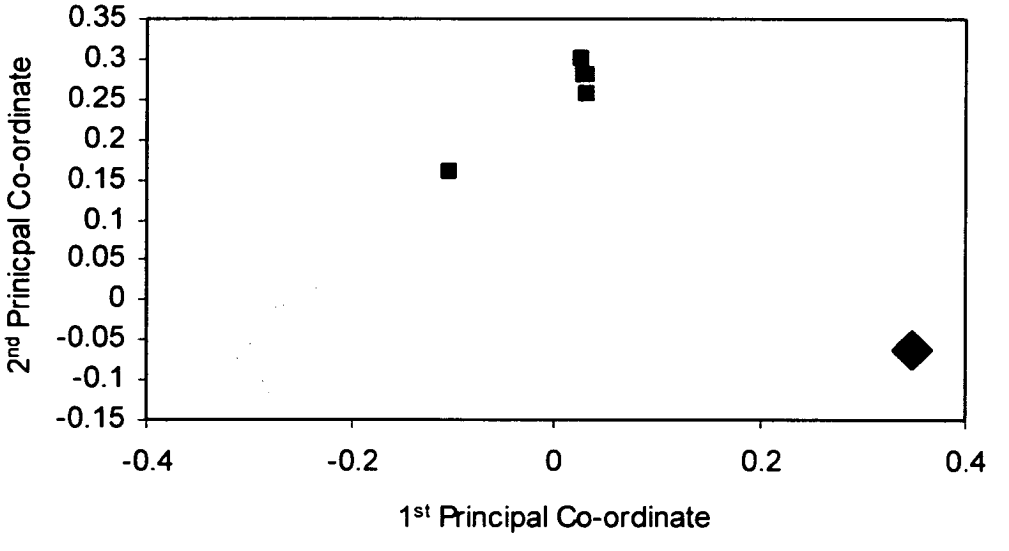


Fig. 3.4: Principal co-ordinate plot showing the relationships of *C. spurium* ◆, *C. rotundifolium* and morphologically ambiguous individuals ■ based on RAPD data.



Incompatibility in C. rotundifolium *and* C. spurium

Pollinations

In *C. spurium* 27 pollinations out of a total of 49 were conducted. It was not possible to conduct all the possible combinations due to difficulties in accessing some of the individuals at Mt Vesey and adverse weather conditions. In *C. rotundifolium*, 34 pollinations (self and cross) were successfully performed. The number of pollinations conducted on *C. rotundifolium* at Scotland was limited by tree maturity. The majority of trees in the seed orchard were between 1-2 years old when the pollination study was conducted. As a consequence only a small proportion had begun to flower in 2000. In addition some of these were not in flower at the same time also limiting the number of cross pollinations possible.

Incompatibility in C. spurium *and* C. rotundifolium

When self-pollinated the majority of *C. spurium* and *C. rotundifolium* individuals exhibited a SI response at the pollen-stigma interface, indicating sporophytic self-incompatibility (Table 3.7 and 3.8). In a typical SI response pollen grains germinate but are quickly arrested at the stigmatic surface or shortly after penetration (Fig. 3.5A). Pollen tube arrest was often accompanied with callose deposition in the papillae cells of the stigma. Two individuals of *C. spurium*, AEAST21 and AEAST35, showed partial self-compatibility, where 1.1% and 5.2% of pollen tubes penetrated into the stigmatic tissue and grew along the stigma arm. Of the 21 cross pollinations conducted in *C. spurium*, 14 were compatible and four were incompatible (Table 3.7). Figure 3.5B shows a compatible response in *C. spurium* (AEAST35 pollinated with AEAST21 from Mt Vesey). After a compatible pollination, pollen tubes grow into the stigmatic tissue and along the stigma arm towards the style. In some compatible pollinations, pollen tubes were visible in the style. All the *C. spurium* pollinations conducted between AEAST21 at Mt Vesey and the genotypes at Pounceys, apart from one, which was partially compatible (see Table 3.7), were compatible. The genotypes AEAST47 and AEAST52 showed reciprocal partial-compatibility between each other. AEAST46 showed reciprocal differences in incompatibility with other individuals at Pounceys. When genotypes at Pounceys are pollinated with AEAST46 they all show an incompatible reaction (Table 3.7 and Fig. 3.5C). However, when AEAST46 is pollinated with AEAST52 there is a compatible reaction and a partially compatible reaction with AEAST47. When AEAST46 is pollinated with AEAST35 there is an incompatible reaction.

The percentage of compatible pollen grains (mean) in crosses, which were considered compatible (>10%), varied greatly and was between 30.3% and 83.4%. The percentage of compatible pollen tended to be lower in crosses between Mt Vesey and Pounceys, and reflected low pollen germination.

Table 3.7: Results of self and cross pollinations conducted on *C. spurium* individuals from Pounceys (P) and the wild population at Mt Vesey (MV). + denotes a compatible cross; +/- denotes a partially compatible cross; - denotes a incompatible cross; / not obtained (see methods for explanation). Pollen recipients are down the left hand column, pollen donors across the top. Shaded box represents crosses conducted within a sib-family.

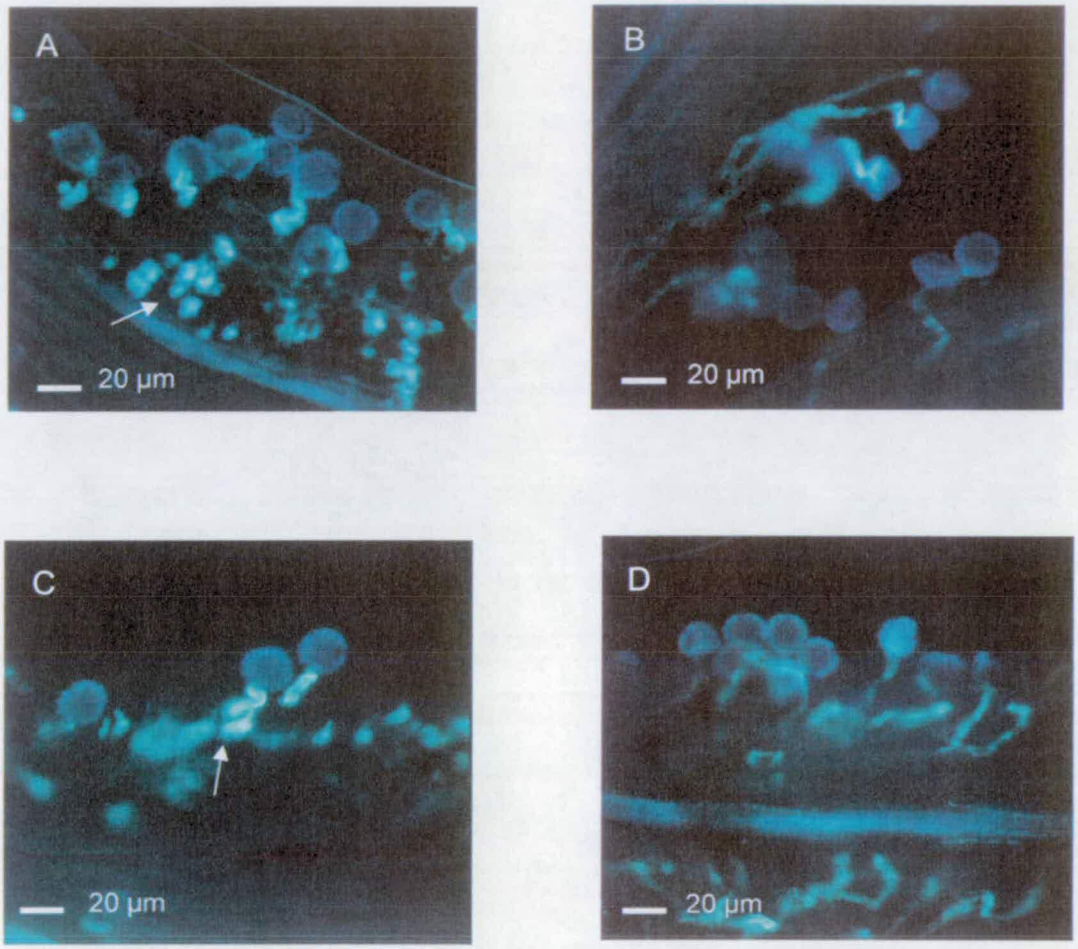
	21MV	35P	46P	47P	52P	181MV	183MV
21MV	+/-	+	+	+/-	/	+/-	+
35P	+					+	/
46P	/					/	/
47P	+					/	/
52P	+					/	/

Table 3.8: Results of self and cross pollinations conducted on *C. rotundifolium* at the seed orchard at Scotland. + denotes a compatible cross; - denotes a incompatible cross; / not obtained (see methods for explanation). Pollen recipients are down the left hand column, pollen donors across the top.

	379	380	381	385	387	390	383	384	386
379	-	/	-	-	-	/	/	-	/
380	/	-	-	/	-	-	-	/	-
381	/	-	-	/	-	-	/	/	-
385	+	/	-	-	-	/	/	+	/
387	/	-	-	-	-	-	-	/	-
390	/	-	-	-	-	-	-	/	/

In contrast to *C. spurium* only two of the cross pollinations conducted in *C. rotundifolium* were compatible (Table 3.8). In both cases the maternal parent was AEAST385 and the pollen parents were AEAST379 and AEAST384. Figure 3.5D shows the compatible reaction at the stigma-pollen interface in AEAST385 when crossed with AEAST379.

Fig. 3.5: Fluorescence micrographs of compatible and incompatible reactions at the pollen-stigma interface of *C. rotundifolium* and *C. spurium*. A) Self-incompatible reaction in self-pollinated *C. rotundifolium*. In pollen grains which germinate the pollen tube is quickly arrested at the stigmatic surface. Note deposition of callose in the papillar cells (arrow). B) Compatible reaction in cross pollinated *C. spurium* (AEAST35 x AEAST21). Pollen tubes grow into the stigmatic tissue and down the stigma arm towards the style. C) An incompatible reaction in cross pollinated *C. spurium* from Pounceys (AEAST47 x AEAST46). Note deposition of callose in the papillar cell adjacent to the pollen grain (arrow). D) One of the two compatible pollinations in *C. rotundifolium* (AEAST385 x AEAST379).



3.4 Discussion

Reproductive Biology in Commidendrum

Hybridisation in C. rotundifolium *and* C. spurium

The RAPD data provide strong evidence that the morphologically ambiguous individuals at Pounceys and Casons are hybrids between *C. rotundifolium* and *C. spurium*, and not morphological variants of *C. spurium*. Evidence from the RAPD data suggests that the morphologically ambiguous sapling at the *C. rotundifolium* seed orchard (Scotland) is a backcross between a *C. spurium* x *C. rotundifolium* hybrid (paternal parent) and *C. rotundifolium* (maternal parent).

The absence of RAPD polymorphism in *C. spurium* may be an artefact of primer selection rather than a lack of variation in the species. Primers for this study were specifically selected to show species-specific bands rather than intra-specific variation. Intra-specific variation in *C. spurium* was observed with several primers during the initial screening process. Although *C. rotundifolium* has undergone a single-founder population bottleneck intra-specific variation occurred at six RAPD loci. Some of this intra-specific variation could be potentially useful in calculating the parentage of the hybrids and of *C. rotundifolium* genotypes at Scotland. At one locus two of the hybrid plants, AEAST39 and AEAST36, had bands which were not present in either of the parental species examined. A probable explanation for this is that not all the 17 possible *C. rotundifolium* parents from Pounceys were examined in the RAPD study.

The lack of reproductive barriers between closely related species and genera has also resulted in the formation of hybrids on Hawaii (Carr & Kyhos, 1981; Carr & Kyhos, 1986), the Juan Fernandez Islands (Crawford *et al.* 1993) and in Macaronesia (Brochmann, 1984). The close phylogenetic relationship between species of *Commidendrum* has been shown using sequences of the internal transcribed spacer regions of ribosomal DNA (Chapter 2). Further support for the close relationship between *C. rotundifolium* and *C. spurium* is provided by the RAPD data. For example, of the 48 loci scored in this study 25 were monomorphic across *C. spurium* and *C. rotundifolium*. In addition, there is also

morphological evidence for hybridisation between the other two *Commidendrum* species, *C. robustum* (Roxb.) DC.and *C. rugosum* (Dryand) DC. (Eastwood, pers. obs.).

Incompatibility in Commidendrum spurium *and* C. rotundifolium

Evidence for sporophytic SI

Our study provides strong evidence for the presence of sporophytic SI in *Commmidendrum rotundifolium* and *C. spurium.* Sporophytic SI is widespread in the Asteraceae (Richards, 1997) and has been studied in a number of species (Abbott & Forbes, 1993; Crowe, 1954; Hiscock, 2000a). Interestingly, Baker's Law (Baker, 1955) predicts that plant species which have established through long-distance dispersal, such as species of *Commidendrum*, are more likely to be self-compatible than self-incompatible. However, a number of insular species of Asteraceae including *Argyroxiphium* DC., *Dubautia* Gaud. and *Wilkesia* A. Gray from Hawaii (Carr *et al.*, 1986) and *Dendroseris* D. Don from the Juan Fernandez islands (Anderson, *et al.* 2001) have been shown to be self-incompatible.

Partial self-compatibility in C. spurium

Two individuals of *C. spurium* on St Helena showed partial self-compatibility. This supports the propagation evidence which indicates that there is a level of self compatibility in isolated individuals of *C. spurium* at Coles Rock and Oaklands. Variation in individual self-compatibility has also been recorded in the threatened species, *Aster furcatus*, where most individuals showed some level of partial self-compatibility (Reinartz & Les, 1994). Flexibility within the SI response, whereby self seed is produced by normally SI individuals (although seed set after crossing is considerably greater) is a well known phenomenon termed pseudo self-compatibility (de Nettancourt, 1977; Levin, 1996) or 'leakiness'. Pseudo self-compatibility, from the physiological or genetic breakdown of SI can occur naturally or be induced (de Nettancourt, 2001) as in the technique of bud pollination which is used to produce hybrid seed in plant breeding programmes. The existence of pseudo self-compatible individuals is rare in natural populations of *Senecio squalidus* (Hiscock, 2000a). However, forced inbreeding generated individuals at a frequency of approximately 11%, which were pseudo self-compatible (Hiscock, 2000a). The author of this study suggests that *S. squalidus* is heterozygous at loci which modify S gene action. Levin (1996) has suggested that genetic pseudo self-compatibility is a key reproductive strategy enabling SI species to negotiate

population bottlenecks without the complete dissolution of self incompatibility. There is evidence, however, that in populations which have undergone severe bottlenecks, as in *Aster furcatus*, there may be a total breakdown of the SI system which ultimately can lead to self compatible individuals (Reinartz & Les, 1994). *Aster furcatus* appears to be actively evolving self-compatibility to ensure a modest seed set in bottlenecked populations (Reinartz & Les, 1994). These authors predict a poorly functioning or non-functional incompatibility system in rare, self-incompatible plants.

Reciprocal differences in compatibility

There were differences in cross compatibility of the four siblings at Pounceys (selfed progeny from isolated individual at Coles Rock) resulting in three main compatibility classes: i) reciprocal compatibility, ii) reciprocal incompatibility and iii) reciprocal difference in compatibility. This is consistent with a sporophytic SI controlled by a single *S* locus, comprised of two alleles segregating in a Mendelian fashion. To produce these compatibility classes the individual at Coles Rock must be heterozygous at this locus. As in the diallel crosses of siblings from two selfed progenies of *Senecio squalidus* (Hiscock, 2000b), the reciprocal differences in compatibility seen in *C. spurium* can be explained by the dominance of one *S* allele in the stigma and codominance in the pollen. The hierarchical dominance relationship amongst *S*-alleles found in many sporophytic species (Richards, 1997) increases the number of compatible crosses (Byers & Meagher, 1992), particularly amongst siblings. We have attempted to allocate genotypes to the four individuals at Pounceys, based on the pattern of compatibility classes observed (Table 3. 9).

Compatibility between Mt Vesey and Pounceys

All the reciprocal crosses which were conducted between AEAST21 at Mt Vesey and the individuals at Pounceys, with the exception of AEAST21 x AEAST47 (see below), were compatible indicating that the two sites do not share any S-alleles. The compatible cross between AEAST35 (Pounceys) and AEAST181 (Mt Vesey) also supports this. One individual at Mt Vesey, AEAST183, was compatible as a pollen donor with AEAST21 whilst the other individual tested, AEAST181, was partially compatible. The partial compatibility between AEAST21 and AEAST181 at Mount Vesey indicates that the two plants share S-alleles, and are probably closely related.

Table 3.9: Genetic interpretation of the three compatibility classes observed in four individuals from Pounceys (self progeny from one isolated individual at Coles Rock). Pollen recipients are down the left hand column, pollen donors across the top.The reciprocal differences in incompatibility can be explained by the dominance of the b allele in the stigma but its codominance in the pollen.

	AEAST35 SbSb	AEAST47 Sa Sa	AEAST52 SaSa	AEAST46 SaSb
AEAST35 SbSb	-	+	+	-
AEAST47 SaSa	+	-	-	-
AEAST52 SaSa	+	-	-	-
AEAST46 SaSb	-	+	+	-

Unexpected crossing anomalies: possibility of an additional gametophytic G gene

Although the presence of two alleles (one which shows dominance in the stigma and codominance in the pollen) at a single S locus largely explains the compatibility classes observed at Pounceys, there were a number of anomalies. Firstly, two of the individuals, AEAST47 and AEAST52, showed reciprocal partial compatibility. If under our assumption these two individuals are homozygous (*Sa*Sa), then we would predict that they would be cross-incompatible. In addition a cross, AEAST46 x AEAST47, which was expected to be compatible, was only partially compatible.

The presence of unexpected crossing anomalies, for example, compatible or semi-compatible crosses in otherwise fully incompatible groups has been recorded by a number of authors in the Brassicaceae and Asteraceae (Table 4 in Lewis *et al.*, 1988; Hiscock, 2000b, Zuberi & Lewis, 1988). These anomalous results are predominantly confined to sib-matings rather than self pollinations (de Nettancourt, 2001). Extensive progeny analysis of sib-families in *Brassica campestris* (Zuberi & Lewis, 1988) and *Raphanus sativus* (Lewis *et al.*, 1988) has revealed the presence of an additional gametophytic gene, *G*, operating alongside the long established *S* gene. In this gametophytic /sporophytic incompatibility system, some crosses which were predicted to be incompatible (possessed the same *S* phenotype) were in fact compatible, due to different G alleles present in the stigma and pollen.

As well as the presence of a *G* gene in *Commidendrum*, there are many other possible explanations for the reciprocal partial compatibility observed in AEAST47 and AEAST52, including i) pollen contamination, ii) point mutations which affect the specificity of the *S* gene in different siblings (de Nettancourt, 2001), iii) presence of another *S*-allele and iv) environmental. However, without a detailed genetic analysis conducted in a controlled environment all these explanations are open to question, particularly as the sporophytic SI has been shown to be complex in other species.

A further anomaly

Under our genetic interpretation for the individuals at Pounceys (Table 9) the partial compatibility between AEAST46 and AEAST47, rather than the expected full compatibility cannot be explained. Partial compatibility with AEAST47 as the pollen donor occurs also with and AEAST21 at Mt Vesey. The partial compatibility class assigned to the cross AEAST21 x AEAST46, however, requires verifying because unfortunately it is based on only three florets for one capitulum. Environmental and physiological factors such as pollen fertility (age of pollen) and the physiological state of plants on the day of pollination may have affected compatibility. The percentage of compatible pollen in the crosses which were considered compatible (>10%) varied greatly and ranged from 30.3% to 83.4%. Hiscock (2000a) found much lower rates of pollen penetration in crosses of *Senecio squalidus*, approximately 10% in a ray cross and 22% in a disk cross, which was later attributed to poor pollen viability. However, it is unlikely that pollen viability of AEAST47 is responsible for the partial cross compatibility with AEAST46, as the number of compatible pollen grains was very high when AEAST35 was crossed with pollen from AEAST47 (74.8%).

Another factor which may affect the number of compatible pollen grains classified may be the pollen load which is applied to the stigma during pollination, although this would have to be tested. Zuberi & Lewis (1988) advocate the use of the restricted pollination technique to ensure all the pollen grains are in contact with the stigma. This was essential in their study to distinguish late geminated compatible pollen from incompatible pollen with its short non-penetrating tube. The crosses using AEAST47 as a pollen donor would have to be verified and our genetic interpretation may have to be modified.

Conservation implications and recommendations

Hybridisation as a threat to rare plants

The threat of extinction by interspecific hybridisation of rare plants has been highlighted by a number of authors (Rieseberg, 1991; Rieseberg & Gerber, 1995; Levin *et al.* 1996; Levin, 2000; Brochmann, 1984). Island plants are particularly susceptible to genetic assimilation through hybridisation because of small population sizes, the lack of reproductive barriers between congeneric species, invasion and colonisation of islands by closely related exotics and habitat disturbance (Rieseberg, 1991; Levin, 2000).

There is currently no threat of hybridisation of *C. rotundifolium* and *C. spurium* in the wild as *C. rotundifolium* is restricted to *ex situ* sites. However, as the reintroduction of endangered plants on Hawaii has shown, the choice of future reintroduction sites for *C. rotundifolium* and *C. spurium* could be critical. When two of the most endangered species in Hawaii, *Hibiscadelphus giffardianus* and *H. hualalaiensis*, were transplanted into the same site they produced a vigorous hybrid swarm which was considered to threaten the survival of *H. giffardianus* (Mehrhoff, 1996). The recent establishment of species specific seed orchards for *C. rotundifolium* and *C. spurium* on St Helena will prevent further hybridisation and introgression between the species. If the morphological integrity of *C. rotundifolium* and *C. spurium* is to be maintained in the seed orchards then individuals showing intermediate morphology should be eliminated. In addition no further seed should be collected directly from Pounceys and used as a future source of germplasm. However, to maximise the genetic diversity maintained *ex situ* cuttings of all the representative genotypes at Pounceys should be taken, propagated and established at Mt Vesey and Scotland respectively. This is particularly imperative in *C. rotundifolium* where the original 17 plants are nearing senescence and are threatened by insect attack.

Incompatibility and the conservation of threatened island plants

The critical number of *S*-alleles needed for a small population to be viable and remain functional in a species with sporophytic self-incompatibility is three (de Nettancourt, 2001; Imrie *et al.*, 1972). Imrie *et al.* (1972) calculated the rate of loss of S-alleles by genetic drift in *Carthamus flavescens* (sporophytic, multiallelic monofactorial self-incompatiblity system with hierarchical dominance), and showed that four was the maximum number of alleles that could be maintained in a population of 32 plants originally segregating for six alleles. A population of 16 individuals was unable to maintain the critical number of three S-alleles in *C. flavescens*. Although the number of S-alleles in the seven individuals of *C. spurium* at Mt

Vesey is not known, it is unlikely to be greater than three, particularly as two individuals probably share S-alleles. In fact, the whole population at Mount Vesey is probably composed of closely related individuals, particularly as when the population was first rediscovered in 1995 there were only four individuals (Cronk, 2000) indicating that three of the individuals are progeny of the original four. Despite the fact that pseudo self-incompatibility, the dominance hierarchy of *S*-alelles and the suspected presence of a gametophytic *G* gene may increase levels of compatibility in *C. spurium* it is unlikely that these are sufficient to maintain a viable population at Mt Vesey. It is essential that individuals with distinct *S*-alleles are introduced to the population to ensure cross compatibility between mates as in Jackson *et al.* (1997) and the population is increased to at least 50 individuals. The genotypes and cross compatibility of the individuals introduced to Mount Vesey should be established prior to any reintroduction to ensure successful regeneration. In addition to the individuals at Pounceys, there are eight young saplings of *C. spurium* in cultivation at RBGE, the progeny of the tree at Oaklands which died in 1999. These should be repatriated to St Helena to use in any future species recovery.

Although, not all reciprocal pollinations were conducted in *C. rotundifolium*, the results in cross compatibility were in stark contrast to *C. spurium*. Of the 28 cross pollinations conducted in *C. rotundifolium* only two were compatible. Both of these were when AEAST385 was the pollen recipient. The low number of compatible crosses between *C. rotundifolium* individuals at Scotland provides strong evidence that poor seed set in *C. rotundifolium* is due to a paucity of *S*-alleles in the species. As all the individuals of *C. rotundifolium* are descendants from only one individual, it is likely that there are only two S-alleles present in *C. rotundifolium*, assuming no new *S*-alleles have been generated through mutation as in *Raphanus sativus* (Lewis *et al.*, 1988). Ideally the cross compatibilities between all the individuals at Scotland and Pounceys should be established so that any population which is reintroduced back into the wild is founded on compatible mating groups.

Hybridisation as a conservation tool

Intentional hybridisation could be beneficial in rare taxa which have lost genetic variation through genetic drift and show detrimental signs of inbreeding depression (Allendorf *et al.*, 2001; Arnold, 1997). *Trochetiopsis* x *benjaminii* Cronk, a vigorous hybrid between the St Helena endemic *Trochetiopsis ebenus* Cronk (Critically Endangered) and *Trochetiopsis erythroxylon* (G. Forst.) Marais (Extinct in the Wild) threatens to replace *T. erythroxylon* on St Helena and in cultivation in the UK (Cronk, 2000). *Trochetiopsis erythroxylon*, Redwood,

is close to extinction and shows signs of inbreeding depression (Rowe, 1995; Cronk, 2000). The only way of saving *T. erythroxylon* from extinction would be to conduct a controlled back-cross breeding programme (Cronk, 2000). Although *C. rotundifolium* may exhibit signs of inbreeding depression (Cronk, 2000), this has not been verified empirically. The paucity of *S*-allele variation (see below for discussion) does threaten the survival of *C. rotundifolium* and will severely impede any reintroduction or species recovery. Unless new *S*-alleles are introduced into *C. rotundifolium*, the species will become more and more difficult to maintain *ex situ* and it is highly likely to become extinct. The simplest method of introducing allelic diversity into *C. rotundifolium* would be to conduct a controlled back-cross breeding programme with *C. spurium* and to establish an experimental population located some distance away from Mt Vesey. Hybridisation between populations lacking in *S*-allele diversity has been conducted in a few plants species with self-incompatibility (Jackson *et al.*, 1997; DeMauro, 1994 cited in Guerrant, 1996). However, these have only been conducted between threatened populations of the same species, not between different species. The arguments for and against using hybridisation as a conservation biology tool, although usually confined to populations and subspecies is still being debated by conservation biologists and authorities (O'Brien & Mayr, 199; Allendorf *et al.*, 2001; Guerrant, 1996; Arnold, 1997).

The main concern over founding populations composed of individuals from different genetic sources is that it may lead to outbreeding depression, the reduced fitness of offspring following intraspecific hybridisation (Templeton, 1986; Leberg, 1993). Neither the hybrids between *C. rotundifolium* and *C. spurium* nor the backcrossed individuals show any of signs outbreeding depression. Outbreeding depression is most common when the genetic divergence between populations is high (Frankham, 1995). Although morphologically distinct, species of *Commidendrum* are very closely related as shown by ITS (Chapter 2) and supported by RAPDs. In addition, with a self-incompatible species threatened with extinction, such as *C. rotundifolium*, there may not be an option but to take a pragmatic approach. We, like Barrett & Kohn (1991), advocate the use of more experimental methods in conservation programmes.

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Comparison of molecular and morphological data on St Helena: *Elaphoglossum*.

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Abstract

The endemic elaphoglossoid ferns, *Elaphoglossum dimorphum*, *E. nervosum*, and *Microstaphyla furcata* of St Helena, form a closely related group within section *Lepidoglossa* when analysed phylogenetically using sequences from the chloroplast *trn*L intron (partial) and *trn*L-F intergenic spacer. There is hardly any *trn*L-F sequence divergence between the species, in fact sequences of *E. nervosum* and *E. dimorphum* are identical, implying that the three species are the result of a recent radiation. *Microstaphyla furcata*, traditionally placed in its own genus, is clearly shown to belong to *Elaphoglossum* confirming the previous transfer of this species to *Elaphoglossum bifurcatum*. The results are consistent with the possible origin of *E. dimorphum* as a hybrid between *E. bifurcatum* and *E. nervosum*. The potential conflict between phylogenetic and morphological distinctness in determining species conservation priorities is discussed.

Key words: *Elaphoglossum*, *Microstaphyla*, St Helena, phylogeny, morphological divergence, conservation priorities, *trn*L intron, *trn*L-F spacer, molecular systematics, Lomariopsidaceae, ferns.

4.1. Introduction

St Helena is a small oceanic island (15°56'S, 5°42'W) in the South Atlantic Ocean. This isolated volcanic island, has a remarkable flora with 49 endemic plant species, 13 of which are ferns (Cronk, 2000). Of the 13 endemic fern species, 11 of them merit inclusion in the IUCN Red List of Threatened Plants (Walter and Gillett, 1998). The main threat to these endemic ferns is the encroachment of habitat from invasive exotic species such as *Phormium tenax* Forst. (New Zealand Flax). Three of the endemic ferns are in the family Lomariopsidaceae and include two species of *Elaphoglossum* Schott ex J. Smith, *E. dimorphum* (Hook. and Grev.) Moore and *E. nervosum* (Bory) Christ, and the related *Microstaphyla furcata* (L.f.) Fée.

The relationship of *Microstaphyla* to *Elaphoglossum*, in particular to *E. dimorphum* and *E. nervosum* from St Helena, has long been debated (Mickel, 1980). The genus *Elaphoglossum*, which contains over 500 species, is remarkably uniform with regards to morphological characters. The majority of species have a characteristic simple blade, free veins and acrostichoid sori (Mickel & Atehortúa, 1980). *Microstaphyla furcata*, however, has a distinct pinnate frond, a distinction which, in the morphologically uniform genus *Elaphoglossum*, has warranted generic status by some taxonomists (Copeland, 1947; Maxon, 1923; Pichi Sermolli 1968, 1977). The original description of genus, *Microstaphyla* Presl, was in fact based on this pinnate species from St Helena (Presl, 1849). *Microstaphyla* later came to include another two species, *M. moorei* (Britton) Underwood (Underwood, 1905) and *M. columbiana* Maxon (Maxon, 1923) but both were transferred into *Peltapteris* (Gómez, 1975) and subsequently into *Elaphoglossum* (Mickel, 1980).

The blade of *E. dimorphum*, as its vernacular name, the Toothed tongue-fern, suggests, has a toothed margin. In fact, Fée (1852; 1857) proposed that *E. dimorphum* and *Microstaphyla furcata* were the same species, having found intermediates between the two. However, despite the fact that the species occur sympatrically at some localities we have not found evidence of any intermediate forms on St Helena, and like Hooker (1861), reject this hypothesis. *Elaphoglossum nervosum*, the Nerved tongue-fern, has an entire blade with prominent veins. These veins fork near the margin, and then unite laterally to form a commissural vein. In his discussion on the relationships of dissected elaphoglossoid ferns Mickel (1980) concludes, based on morphological characters such as rhizome scales and habit, that *E. dimorphum*, *E. nervosum* and *M. furcata* are closely related, arguing that frond

architecture is a weak taxonomic character at the generic level. Mickel (1980) suggests that *E. dimorphum* arose as a lacerated form of *E. nervosum*, the more extreme dissected form being *M. furcata*, but does not dismiss the possibility of *E. dimorphum* being a hybrid of *E. nervosum* and *M. furcata*. He also suggests they could be forms of the same species. Like Christ (1899) and Christensen (1906), Mickel (1980) treats *Microstaphyla* under *Elaphoglossum*, as *E. bifurcatum* (Jacq.) Mickel. In contrast Cronk (2000), in his endemic Flora of St Helena, maintained the generic distinction.

Unfortunately, these interesting ferns, which have stimulated much interest from taxonomists and evolutionary biologists, are severely threatened on St Helena. *Elaphoglossum dimorphum* is restricted to two localities within Diana's Peak National Park, Mount Actaeon and Cuckold's Point. The national park represents the largest area of tree fern thicket remaining on St Helena, covering approximately 1km^2. Occasional plants have also been noted further west on the central ridge, at High Peak and the Depot (Cronk, 2000), but the species has not been seen at these localities recently. *Elaphoglossum dimorphum* is a terrestrial fern, found growing on stone steps and surrounding rocks and banks. As the total population of *E. dimorphum* is less than 50 individuals, it is classified as "Critically Endangered" (CR, D) according to the IUCN Categories of Threat (IUCN, 1994).

Elaphoglossum nervosum, like *E. dimorphum*, is also restricted to Diana's Peak National Park, growing locally at Cuckold's Point, Diana's Peak and Mount Actaeon. The species is predominantly epiphytic, growing on the endemic tree fern, *Dicksonia arborescens* L'Héritier and the endemic tree, *Melanodendron integrifolium* (Roxb.) DC. It is also occasionally found on rock faces and mossy banks. The total population of *E. nervosum* does not exceed 150 plants, and so the species is considered "Endangered" (E, D)

Microstaphyla furcata has a wider distribution on St Helena than either *E. dimorphum* or *E. nervosum*. There are least ten populations of *M. furcata* on St Helena, all in the uplands at altitudes greater than 650m. The species is terrestrial, growing on shaded rocks, rock crevices, and earth banks. Although not under any immediate threat the species occupies less than 100km^2 and would therefore be classified as "Vulnerable" (V, D) (IUCN, 1994)

The use of morphology in reconstructing phylogenies of ferns is often complicated by the lack of phylogenetically informative characters (Haufler & Ranker, 1995). For example, taxonomic complexity in the cheilanthoid ferns is attributed to morphological homoplasy by

convergent evolution to xeric habitats (Gastony & Rollo, 1995). The lack of informative characters coupled with disagreement by authors in character interpretation has led to taxonomic controversy in a number of fern groups (Crane *et al.* 1995, Hauk, 1995). This has led fern systematists to search for new sources of characters in molecular data, including restriction site and nucleotide sequence data, to infer phylogenetic relationships. The use of molecular data to infer phylogeny has yielded valuable insights into the relationships and evolution of ferns, some with taxonomic implications (Gastony & Ungerer, 1997; Hasebe *et al.* 1994, Murakami *et al.* 1999).

In angiosperms, the comparatively slowly evolving chloroplast *rbc*L gene, which encodes the large subunit of ribulose 1,5-biphosphate carboxylase/oxygenase, has been used primarily to infer phylogenies at higher taxonomic levels, especially at the interfamily or ordinal level (Soltis & Soltis, 1998). However, in ferns, it appears to have a wider range of applicability, and has been shown to resolve closely related genera (Gastony & Ungerer, 1997) and species within genera (Haufler & Ranker, 1995; Hauk, 1995, Murakami *et al.*, 1999). However, in *Elaphoglossum, rbc*L failed to resolve closely related species (Mickel, pers. comm., 2001) as it did in closely related species of *Botrychium* (Ophioglossaceae) (Hauk, 1995). Nucleotide sequences of the non-coding regions of *trn*L-F gene have been widely applied to resolve phylogenetic relationships in many groups of angiosperms at the generic level. However, their application in phylogenetic reconstruction in ferns has been limited, possibly due to limitations of the "universal" primers of Taberlet *et al.* (1991) commonly used. Hauk *et al.* (1996) found that sequence divergence rates in the *trn*L-*trn*F intergenic spacer in Ophioglossaceae were five times greater than in *rbc*L.

This study uses the phylogenetic analysis of sequences from two non-coding regions of chloroplast DNA, the *trn*L intron (partial) and *trn*L-F intergenic spacer, to infer relationships in the endemic elaphoglossoid ferns from St Helena.

4.2. Materials and Methods

Plant material

The plant material used in this study was all dried in silica gel, apart from *Rumohra adiantiformis* (G. Forster) Ching which was taken from the living collection at the Royal Botanic Garden, Edinburgh (RBGE). The ingroup included the three endemic elaphoglossoid species from St Helena and the indigenous, but not endemic, *Elaphoglossum conforme* (Swartz) Schott ex J. Smith. As a comparison, six other species of *Elaphoglossum* from East

Africa were included in the ingroup, as well as *E. semicylindricum* (Bowdich) Benl from Madeira. The 11 *Elaphoglossum* species in the ingroup represent four out of the nine sections of *Elaphoglossum* (*Lepidoglossa* Christ, *Eximia* Mickel & Atehortúa, *Setosa* Christ, and *Elaphoglossum*) according to Mickel & Atehortúa (1980). Table 4.1 lists the species used in the phylogenetic analysis with details of locality and habitat. Voucher herbarium specimens of the taxa from St Helena and *Rumohra adiantiformis* are held at the Royal Botanic Garden Edinburgh (E). Voucher specimens for the species analysed from East Africa are with Andreas Hemp at the University of Bayreuth (UBT) in Germany. *Rumohra adiantiformis* (G. Forster) Ching was chosen to represent the outgroup as recent molecular and morphological studies have shown it to be closely related to *Elaphoglossum* (Hasebe *et al.*, 1995; Pryer *et al.*, 1995).

DNA extraction and PCR

Total genomic DNA was extracted from one individual of each taxon using the modified CTAB procedure from Doyle & Doyle (1987). Two non-coding regions of chloroplast DNA using the polymerase chain reaction were amplified: 1) the intron, of the *trn*L (UAA) gene, and 2) the intergenic spacer between the *trn*L (UAA) 3' exon and *trn*F (GAA). To amplify these regions the universal primers d, e and f of Taberlet *et al.* (1991) were used. The usual c primer of Taberlet *et al.* (1991) was replaced with FERN1, 5' GGC AGC CCC CAR ATT CAG GGR AACC 3', a primer designed for *Dryopteris* at the Natural History Museum, London. The FERN1 primer lies within the intron of the trnL (UAA) gene, approximately 50-80 base pairs downstream from the trnL (UAA) 5' exon. Each PCR reaction contained: 2.5 µl x10 NH_4 buffer (Bioline, UK), 2.5 µl of 2mM dNTPs (Bioline, UK), 1 µl of 10 µM forward primer (FERN1 or E), 1 µl of 10 µM of reverse primer (D or F) , 1 µl of 50mM magnesium chloride, 0.1 µl of Biotaq polymerase (Bioline, UK) and 1-2 µl DNA. The reaction volume was made up to 25 µl with sterile ultra-pure water.

The PCR was performed with the following conditions for all the taxa: i) one initial pre-step of 94°C for 4 minutes, followed by ii) 30 cycles of denaturation at 94°C (45 seconds), annealing at 55°C (45 seconds) and extension at 72°C (3 minutes) and iii) a terminal extension of 72°C for 10 minutes. After visualisation using on 2% agarose gel the PCR products were purified using QIAquick™ purification columns supplied by Qiagen Ltd., UK. An annealing temperature of 55°C was required for high specificity to avoid multiple bands. However, this temperature resulted in no product for the *trn*L intron in *M. furcata* and *E. subcinnamomeum*. The annealing temperature was therefore reduced to 51°C in these two

Table 4.1: List of species with collection, locality and habitat details used in phylogenetic analysis.

Species	Collecting no.	Locality and habitat
Elaphoglossum conforme (Swartz) ex Smith	AEAST211	St Helena, Diana's Peak National Park, Mt Actaeon: Epiphyte on *Dicksonia arborescens*. 780m
Elaphoglossum dimorphum (Hook. & Grev.) Moore	AEAST302	St Helena, Diana's Peak National Park, Mt Actaeon: On rock face with *M. furcata* and *E. nervosum*. 780m.
Elaphoglossum nervosum (Bory) Christ	AEAST358	St Helena, Diana's Peak National Park, Mt Actaeon: Epiphyte on *Dicksonia arborescens*. 780m.
Microstaphyla furcata (L. f.) Fée	AEAST721	St Helena, Diana's Peak National Park, Cuckold's Point: On large rock boulder, amongst flax with *E. nervosum* amd *M. furcata*. 750m.
Elaphoglossum angulatum (Blume) Moore	AHEMP7	Mt Kilimanjaro, between Weru-Weru and Lanza river: Epiphyte in montane *Ocotea-Podocarpus* forest, 2320m.
Elaphoglossum aubertii (Desv.) Moore	AHEMP8	Mt Kilimanjaro, between Weru-Weru and Lanza river: Epiphyte in montane *Ocotea-Podocarpus* forest, 2200m.
Elaphoglossum deckenii (Kuhn) C. Chr.	AHEMP6	Mt Kilimanjaro, between Weru-Weru and Lanza river: Epiphyte in montane *Ocotea-Podocarpus* forest, 2250m.
Elaphoglossum lastii (Bak.) C. Chr.	AHEMP23	Mt Kilimanjaro, Marangu gate, epiphyte on *Agauria salicifolia* in *Agauria-Macaranga* forest, 1800m.
Elaphoglossum semicylindricum (Bowdich) Benl.	JVOGE-ELA-7	Madeira
Elaphoglossum spathulatum (Bory) Moore	AHEMP1	Mt Kilimanjaro, on boulders in stream bed, 1700m, Weru-Weru river system
Elaphoglossum subcinnamomeum (Christ) Hieron	AHEMP19	Mt Kilimanjaro Maua: semishaded rock in small river gorge, 2800m.
Rumohra adiantiformis (G. Forster) Ching	19932232	South Africa, cultivated at RBGE.

species. This lower temperature resulted in two PCR products, seen as one bright band (~600 bp) and a faint band (~420 bp) when visualised on a 2% agarose gel.The remaining PCR product (20 µl) was loaded and visualised on 1% low melting point agarose (Sigma). The bright band, corresponding to the *trn*L intron in the other *Elaphoglossum* species, was extracted using the QIAquick gel extraction kit (by Qiagen Ltd. UK) and sequenced directly.

The PCR products were sequenced directly using premixed Thermo Sequenase II reagent (Amersham Pharmacia, UK) and according to manufacturer's protocol. The sequencing reaction was conducted using the following PCR conditions: 25 cycles of denaturation at 96°C (10 seconds), annealing at 50°C (5 seconds) and extension at 60°C (4 minutes). DyeEx ™ spin columns from Qiagen Ltd. were used according to recommended protocol for dye terminator removal of the sequence reactions. For confirmation both forward and reverse sequences were performed for each taxon. Sequence analysis was conducted on an automated ABI Prism™ 377 sequencer.

Sequence alignment and analysis

Sequences were initially edited using editing Sequence Navigator™ v.1.01 (Applied Biosystems) and subsequently exported into AutoAssembler™ v. 2.1 (Applied Biosystems) for final editing and assembling. Sequences were aligned using ClustalX v.1.8 (Higgins *et al.* 1992) with some minor manual adjustment (Appendix 2). The first 30 bp downstream from the FERN1 primer were unreadable and were therefore excluded from any sequence statistics calculations and the phylogenetic analysis. Sequence divergence between the taxa was calculated within PAUP version 4.0b7 (Swofford, 2001) whilst the transition/transversion ratios were calculated using MacClade version 3.05 (Maddison & Maddison, 1992).

Phylogenetic analysis

A phylogenetic analysis was performed on the aligned data matrix, using the branch-and-bound option in PAUP (Swofford, 2001) version 4.0b7. Gaps in the sequence data were treated as missing data. Insertion/deletion (indels) events were scored according to the simple gap coding method of Simmons & Ochoterena (2000) and added to a separate gap matrix at the end of the sequence data. Ambiguous regions which yielded a number of alternative interpretations were excluded in the analysis (see Appendix 2, bps: 705-730). Bootstrap values for each clade were calculated from 10,000 replicate parsimony analyses

using the "branch and bound" option and "furthest" addition sequence of taxa. The decay index for each individual clade was calculated using Autodecay 4.0.2 (Eriksson, 1999).

4.3. Results

Sequence analysis

A complete sequence for the intergenic spacer and a partial intron sequence of the *trn*L-F gene were obtained for all the 12 taxa in this study. This gave an aligned data matrix of 960 base pairs. The sequence and indel characteristics are shown in Table 4.2.

Table 4.2: Sequence and indel characteristics for 11 species of *Elaphoglossum* (ingroup) and *Rumohra adiantiformis* (outgroup) based on *trn*L_{UAA} partial intron and *trn*L_{UAA}-*trn*F_{GAA} intergenic spacer. In addition, sequence divergence (%) is given for i) the endemic St Helena taxa (*E. dimorphum*, *E. nervosum* and *M. furcata*) and ii) between the endemic St Helena taxa and the other *Elaphoglossum* species in the ingroup.

Parameter	Partial intron	Intergenic spacer	Partial intron + spacer
Length range (total) (bp)	474-589	308-321	795-906
Length mean (total) (bp)	552.7	315.3	868
Length range ingroup (bp)	531-589	311-318	839-906
Length mean ingroup (bp)	559.8	314.8	874.6
Length outgroup (bp)	474	321	795
Aligned length	610	350	960
G + C content range (%)	45.1-48.2	48.3-52.0	47.8-48.9
G + C content mean (%)	47.2	50.5	48.4
Number of excluded sites	53 (9%)	26 (7%)	79 (8%)
Number of indels (ingroup)	12	12	24
Number of indels (total)	18	16	34
Number of informative indels (ingroup)	9 (50%)	6 (37.5%)	15 (44.1%)
Size of indels (ingroup)	1-36	1-6	1-36
Size of indels (total)	1-55	1-11	1-55
Number of sites after exclusion of ambiguous	557	324	881
Number of variable sites	129 (23.2%)	95 (29.3%)	224 (25.4%)
Number of constant sites	428 (76.8%)	229 (70.7%)	657 (74.6%)
Number of uninformative sites	98 (17.6%)	65 (20.1%)	163 (18.5%)
Number of informative sites	31 (5.6%)	30 (9.3 %)	61 (6.9%)
Transitions (unambiguous)	35	27	66
Transversions (unambiguous)	29	17	48
Transitions/tranversions	1.21	1.6	1.36
Sequence divergence (St Helena) (%)	0	0	0
Sequence divergence (St Helena/African *Elaphoglossum*) (%)	1.7-5.9	1.7-7.3	1.9-6.4

The sequences of *E. dimorphum* and *E. nervosum* were identical. *Microstaphyla furcata* differed only in two autapomorphic indels, one 8bp in length the other 1bp (in ambiguous

region and therefore excluded from the analysis), While these three species had no divergence, the divergence between the other ingroup taxa ranged from 1.3-7.2%. The pairwise sequence divergences between *Elaphoglossum semicylindricum* and *E. deckenii* (Kuhn) C. Chr., and the St Helenian endemics are 2.37% and 1.85% respectively. *Elaphoglossum semicylindricum* and *E. deckenii* are considered to be in the same section (Lepidoglossa) as the St Helenian endemics, but different subsections according to Mickel & Atehortúa (1980). The average pairwise sequence divergence in the ingroup was 4.7 %. Both the intron (partial) and spacer sequences provided a substantial number of phylogenetically informative sites, 30 and 31 respectively. The two chloroplast regions also provided a number of phylogenetically informative insertion/deletion events (indels).

Phylogenetic analysis

The parsimony analysis of unambiguously aligned *trn*L-F sequences yielded only one most parsimonious tree, represented as a cladogram in Fig. 4.1. The tree has a length of 261 steps with a consistency index of 0.931 and a retention index of 0.885 (including all characters). The three endemic elaphoglossoid ferns from St Helena, *E. nervosum*, *E. dimorphum* and *M. furcata* form a well supported monophyletic clade, with 100% bootstrap support. The two species, *E. semicylindricum* from Madeira and *E. deckenii* from East Africa, are the sister group to the St Helenian endemics. The other species of *Elaphoglossum* of St Helena, the indigenous *E. conforme*, is distantly related to *E. nervosum*, *E. dimorphum* and *M. furcata*. The parsimony analysis of unambiguously aligned trnL-F sequences combined with the gap matrix also yielded one tree of 300 steps (CI = 0.923, RI = 0.787). The tree is represented as a phylogram in Fig. 4.2. The topology of the tree is largely congruent with the most parsimonious tree based on sequences alone (Fig. 4.1). The position of *E. lastii* (Bak.) C. Chr., is however, different in the two trees. In Figure 2 (sequences and gap matrix) *E. lastii* forms a clade with *E. angulatum* and *E. conforme*, with all the other species forming a monophyletic sister group. However, this grouping is not statistically supported and collapses in the bootstrap analysis. When sequences are analysed alone as in Figure 4.1, *E. angulatum* (Blume) Moore and *E. conforme* form a small clade whilst *E. lastii* and the other *Elaphoglossum* species form another clade.

The sections of *Elaphoglossum* according to Mickel & Atehortúa (1980) are indicated on Figure 4.2. The clade which includes the St Helenian endemics (*M. furcata*, *E. dimorphum* and *E. nervosum*) and their sister group (*E. semicylindricum* and *E. deckenii*) correspond to the subsections *Pilosa* Christ and *Polylepidea* Christ of section *Lepidoglossa* respectively.

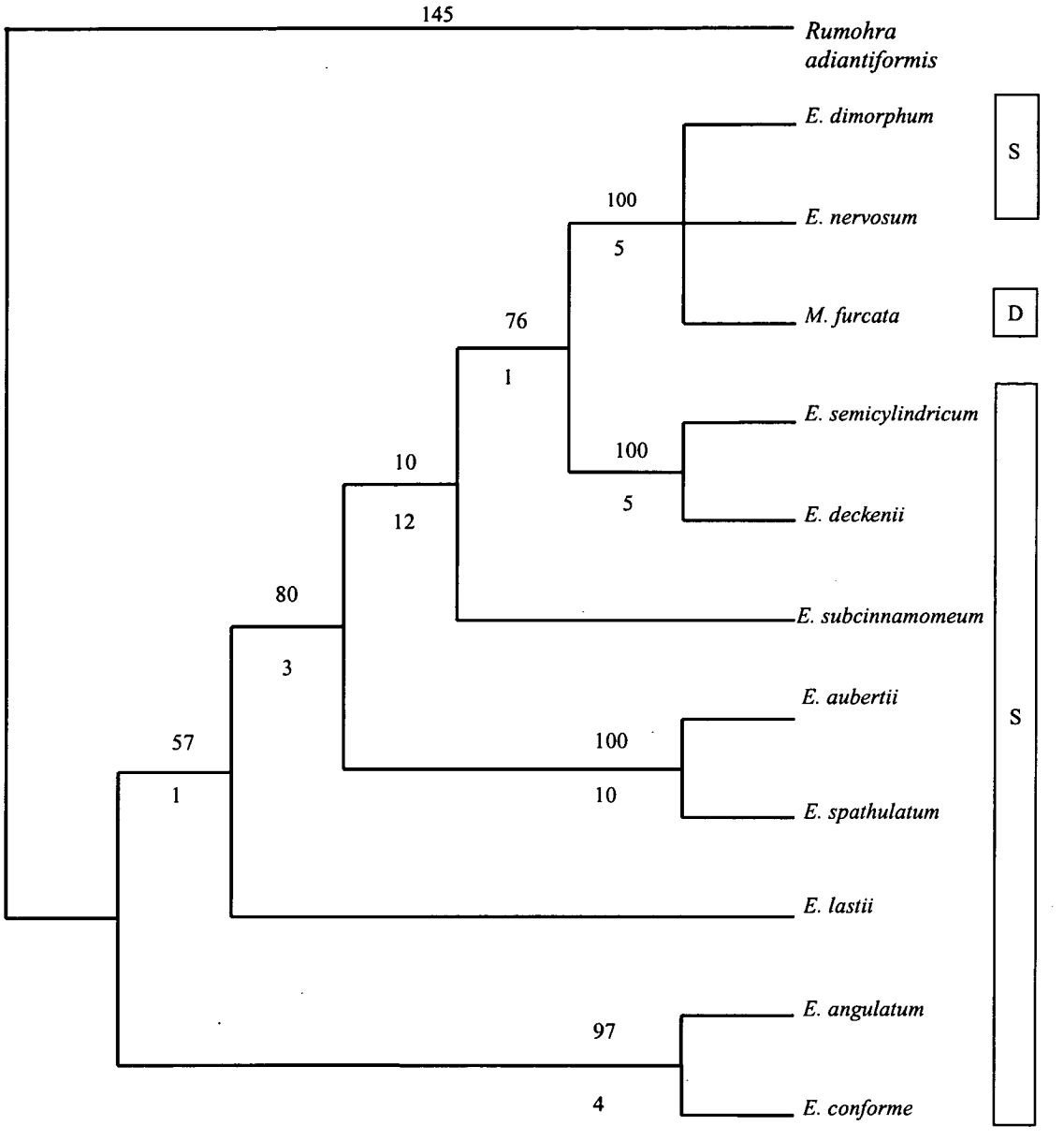

Fig. 4.1: A cladogram of the single most parsimonious tree based on an analysis of sequences of the *trn*L intron (partial) and *trn*L-F intergenic spacer in 11 species of *Elaphoglossum* and *Microstaphyla furcata*. The tree has a length of 261 steps; CI = 0.931; RI = 0.885. Bootstrap values (1000 replicates) are shown above each branch. Decay indices for each clade are shown below. S = simple leaves, D = divided leaves.

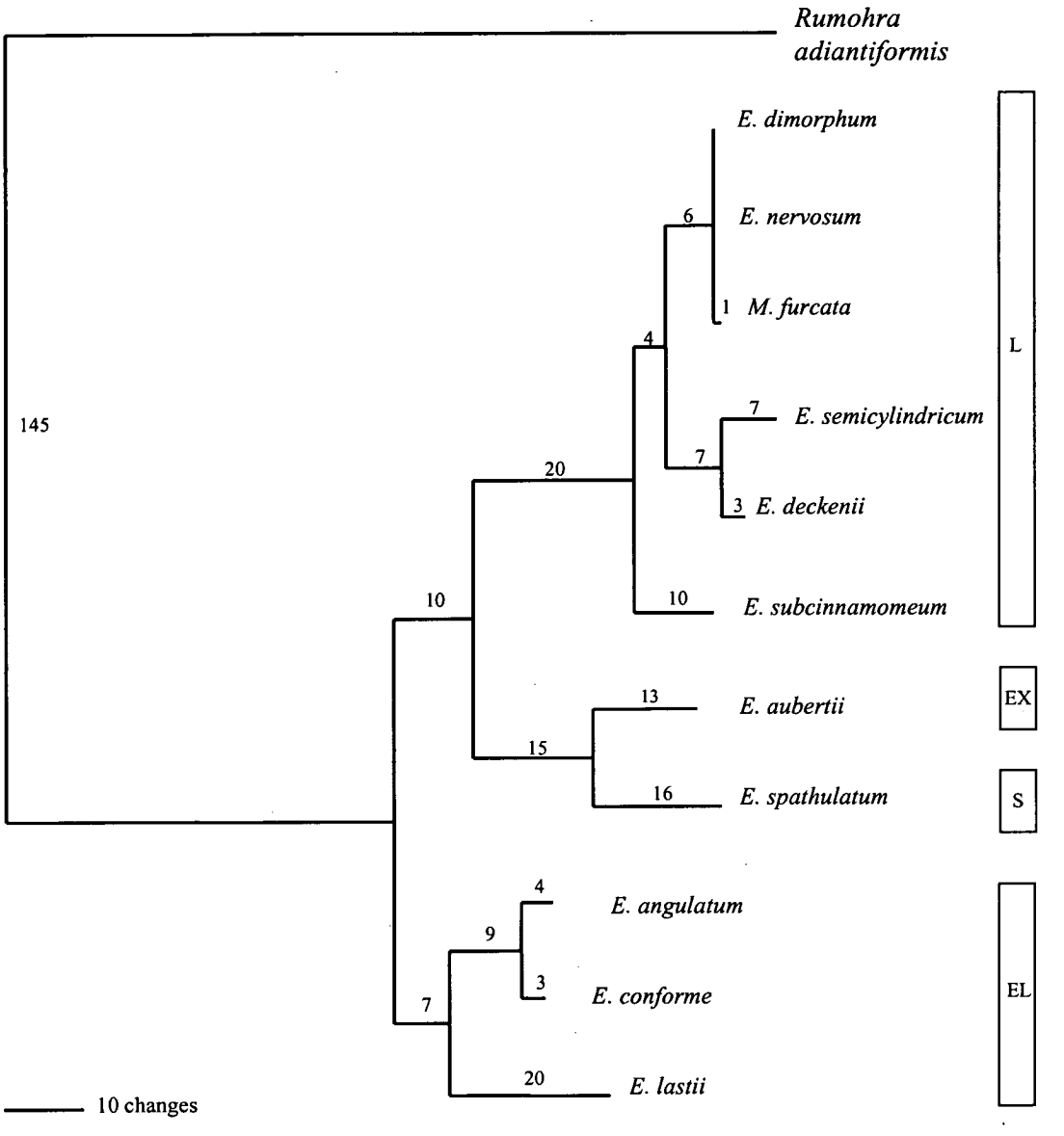

Fig. 4.2: The single most parsimonious tree of 10 *Elaphoglossum* species and *Microstaphyla furcata* based on *trn*L partial intron and *trn*L-F intergenic sequences including a gap matrix. The tree has a length of 300 steps, a CI = 0.923 and a RI = 0.875. Branch lengths are indicated above each branch. The different sections of *Elaphoglossum* according to Mickel & Atehortúa (1980) are indicated with L=*Lepidoglossa* Christ, EX= *Eximia* Mickel & Atehortúa, S=*Setosa* Christ, and EL= *Elaphoglossum*.

Elaphoglossum subcinnamomeum is also considered to be in subsection *Pilosa* according to Mickel & Atehortúa (1980), however, in the *trn*L-F phylogeny it is more distantly related to the St Helenian endemics than *E. semicylindricum* or *E. deckenii*.

4.4. Discussion

Species relationships

The molecular phylogeny clearly indicates that *E. dimorphum*, *E. nervosum* and *M. furcata* are a closely related group, strongly supporting Mickel's (1980) hypothesis of their close relationship. The evidence from molecular data is thus consistent with Mickel's (1980) treatment of *Microstaphyla furcata* under *Elaphoglossum*, as *Elaphoglossum bifurcatum* (Jacq.) Mickel. As a result of these findings *M. furcata* will be now be referred to as *E. bifurcatum*.

The low levels of sequence divergence between the endemic *Elaphoglossum* species indicate recent speciation events on St Helena from a common ancestor, which was probably African in origin. This event must have occurred less than 14 million years ago, the estimated age of St Helena from potassium-argon dating (Baker, 1967). The common ancestor speciated into a number of ecological niches with distinct blade morphology: epiphytic *E. nervosum* with an entire blade and prominent venation; terrestrial *E. dimorphum* with a toothed margin; and terrestrial *E. bifurcatum* with a distinct pinnate frond, the cause of much taxonomic debate. Extensive ecological and morphological variation (which can obscure phylogenetic relationships, particularly with continental relatives) with little genetic divergence has been reported for a number of insular plant groups (Sang *et al.*, 1994; Baldwin, 1998). Marked morphological differences can arise through changes in one or a few genes, for example, two mutant alleles of the *Unifoliata* gene dramatically effect the pinnate leaf of pea (*Pisum sativum*) (DeMason & Schmidt, 2001). The majority of species of Aspleniaceae are pinnatifid but a small number have an entire leaf. In a recent molecular phylogeny of Aspleniaceae, using chloroplast *rbc*L nucleotide sequences, neither simple nor pinnatifid leaf were found to be phylogenetically informative (Murakami *et al.* 1999). Interestingly, the genus *Neottopteris*, which was defined by its special simple leaf was paraphyletic and formed a clade with *Asplenium prolongatum* whose leaves are pinnatifid 3-4 times. Evidence from the molecular phylogeny of *Elaphoglossum* on St Helena and that by Murakami *et al.* (1999) on Aspleniaceae indicate that leaf dissection is a weak taxonomic character in ferns at the generic level, as suggested by Mickel (1980).

There are two hypotheses concerning the relationships of *E. dimorphum*, *E. nervosum* and *E. bifurcatum*. The first, as suggested by Mickel (1980), is that *E. dimorphum* is of hybrid origin involving *E. nervosum* and *E. bifurcatum*. The intermediate morphology of *E. dimorphum* supports this hypothesis. Although there have been no studies on the inheritance of chloroplast DNA in *Elaphoglossum*, maternal inheritance has been reported in *Asplenium* (Vogel et al., 1998) and in the cheilanthoid ferns (Gastony and Yatskievych, 1992). The *trn*L-F sequences of *E. dimorphum* and *E. nervosum* are identical, which does not conflict with the hypothesis of a hybrid origin of *E. dimorphum*. If *E. dimorphum* is of hybrid origin, then *E. nervosum* would be the maternal parent. In a recent *rbc*L phylogeny of Aspleniaceae by Murakami *et al.* (1999), sequences of *A.* x *kenzoi*, which is thought to be hybrid of *A. prolongatum* and *A. wrightii* from Yaku Island, were found to be identical to *A. prolongatum*, indicating that *A. prolongatum* is the maternal parent. Further analysis using allozymes revealed that the paternal parent of *A.* x *kenzoi* was *A. antiquum* (*Neottopteris antiqua*), not *A. wrightii*. The molecular phylogeny first indicated the close relationship of *A. prolongatum* (pinnate) to *A. antiquum* (simple blade), despite their different gross morphologies. There is no reproductive evidence to support the hypothesis that *E. dimorphum* is of hybrid origin. The spores are fully fertile (unlike those of the hybrid *A. x kenzoi*) and progeny show no segregation into the putative parental types. Nor have we observed any morphological evidence for segregation in the field.

The second hypothesis explaining the relationships of these three taxa is that they have undergone a recent divergence. Although the chloroplast *trn*L intron and *trn*L-F spacer regions are non-coding they may not evolve fast enough to differentiate between *E. dimorphum* and *E. nervosum*. A molecular phylogeny of *Commidendrum* (Asteraceae), a genus endemic to St Helena, using the internal transcribed spacers of nuclear ribosomal DNA also failed to resolve relationships completely, indicating recent speciation and radiation (Chapter 2). The lack of phylogenetic resolution using molecular characters from conventional chloroplast and nuclear markers has been an obstacle in inferring species relationships in a number of insular plant groups (Baldwin *et al.*, 1998).

A further study is planned, using allozyme analysis and cytological investigations, to extend this investigation of the relationships of these endangered ferns from St Helena.

Utility of* trn*L intron and* trn*L-F spacer sequences

The sequence divergence values among the 11 *Elaphoglossum* species (representing four sections) were relatively high (average value 4.7%). There are no published sequence divergence values of *rbc*L in *Elaphoglossum* at present and so no direct comparisons can be made. Sequence divergence rates in *rbc*L for other intrageneric fern studies vary considerably; from an average of 0.6 % *rbc*L in *Botrychium* subgenus *Botrychium* (Hauk, 1995), 1.87% in sister species of *Polypodium* (Haufler & Ranker, 1995) and 9.8% between different sections of *Trichomanes* (Hymenophyllaceae) Dubuisson (1997). Differences between sequence divergence values at a given rank will vary greatly in different groups of plants depending on different rates of mutation and contrasting concepts of taxonomy (Hauk, 1995). Hauk *et al.* (1996) found that sequence divergence in the *trn*L-F intergenic spacer was three to five times higher than *rbc*L in 40 species of Ophioglossaceae. In addition to higher sequence divergence rates the *trn*L-F spacer also revealed 20 phylogenetically informative indels which reduced the number of parsimonious trees and provided greater resolution (Hauk *et al.* 1996). The *trn*L-F intron (partial) and spacer successfully resolved relationships between most of the *Elaphoglossum* species in this study, indicating its applicability to the whole genus. It was however, unable to resolve the close relationship between *E. dimorphum*, *E. nervosum*, and *E. bifurcatum*, which may reflect their recent speciation and/or hybridisation.

The use of non-coding regions of *trn*L-F gene has been limited in ferns. Although the objective of our study was primarily to investigate the phylogenetic relationships of the endemic elaphoglossoid taxa from St Helena it has revealed the utility of these non-coding regions of the chloroplast gene in resolving relationships between species of *Elaphoglossum*, and probably in other fern genera. It would be particularly interesting to investigate the phylogenetic relationship of South American *Elaphoglossum* species, in particular those in section *Lepidoglossa*, subsection *Pilosa*, with the St Helenian endemics. The location of the FERN1 primer used to sequence the *trn*L intron is positioned in the intron rather than the exon, and so only a partial intron sequence was obtained for each taxon. Although this provided useful phylogenetic signal, it would be advantageous for future studies to design fern specific primers that sit in the *trn*L exon.

Implications for conservation

As Daugherty *et al.* (1990) has stated, taxonomic classification is a primary determinant of management priorities for endangered species. Outdated classifications may not reflect phylogenetic diversity and could lead to the misguided management of rare species (Avise, 1989, Daugherty *et al.* 1990, Hibbett & Donoghue, 1996, Rojas, 1992). This is particularly relevant for cryptic species, but could conversely be as important when devising conservation strategies for endemic island plant groups whose phylogenetic relationships may be obscured by extensive morphological or ecological divergence. As Soltis & Gitzendanner (1999) emphasised, phylogenetically based species, for which one can ascertain i) distinguishing synapomorphy (-ies), ii) component populations and individuals, and iii) closest relatives, should be the target of conservation efforts. Byrne *et al.* (2001) recently conducted a phylogenetic analysis of two threatened Australian *Acacia* species, *A. sciophanes* and *A. lobulata*, and their widespread relatives using RFLP analysis of cpDNA. The study revealed that both threatened species were phylogenetically distinct despite being morphologically similar to their widespread relatives. In fact *A. lobulata* represents an ancient lineage and most likely a relictual species.

It has been suggested that cryptic species should be conserved on the basis of their phylogenetic divergence (even though morphological divergence is limited). However, arguments for phylogenetic divergence would tend to diminish the value of recently diverged island species which may be morphologically distinctive. Clearly there may be a conflict between conservation on the basis of phylogenetic history versus morphological diversity. Erwin (1991) goes so far as to recommend the prioritisation of recent evolution ("evolutionary front") in order to secure future diversity. In the case of "*Microstaphyla*" the morphological feature of dissected fronds is of great interest within the genus, arguably justifying conservation prioritisation. However, in terms of evolutionary history there is no justification for its conservation as an endemic genus "*Microstaphyla*" (i.e. *E. bifurcatum*) as opposed to *E. nervosum* or *E. dimorphum*.

Species conservation priorities, based around a taxonomic, often typological framework, are predominately governed by the degree of threat to a species as emphasised by the IUCN Red Lists of threatened species (Walter & Gillett, 1998; IUCN, 1996; Oldfield *et al.* 1998; Hilton-Taylor, 2000). Species conservation priorities are usually assigned with the assumption that all species have an equal biodiversity value. However, this is obviously not the case and to address this issue a number of authors have proposed the use of phylogenetic

or taxonomic diversity to select taxa for conservation priority (Vane-Wright *et al.*, 1991; Faith, 1992). In terms of rarity-based prioritisation *E. dimorphum* is clearly the most threatened. However, if *E. dimorphum* is of recent hybrid origin involving *E. nervosum* and *E. bifurcatum* then its conservation priority based on genetic divergence would be lower.

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Relationships and genetic diversity of endemic *Elaphoglossum* from St Helena

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Abstract

Allozyme polymorphism was used to 1) investigate the relationships of three threatened species of *Elaphoglossum* from St Helena, *E. nervosum*, *E. bifurcatum* and *E. dimorphum*, and 2) to estimate levels of genetic diversity and its partitioning among populations. Despite showing morphological and ecological variation, the three species are closely related with high genetic identities. Evidence from two enzyme loci suggests that *E. dimorphum* is of hybrid origin involving *E. nervosum* and *E. bifurcatum*. Levels of genetic diversity were low in the three species, but comparable with insular endemic angiosperms. Populations of *E. nervosum* and *E. bifurcatum* showed significant genetic differentiation which should be taken into account in any conservation programme.

Keywords: *Elaphoglossum*, St Helena, isozyme analyse, island endemics, genetic diversity, conservation.

5.1 Introduction

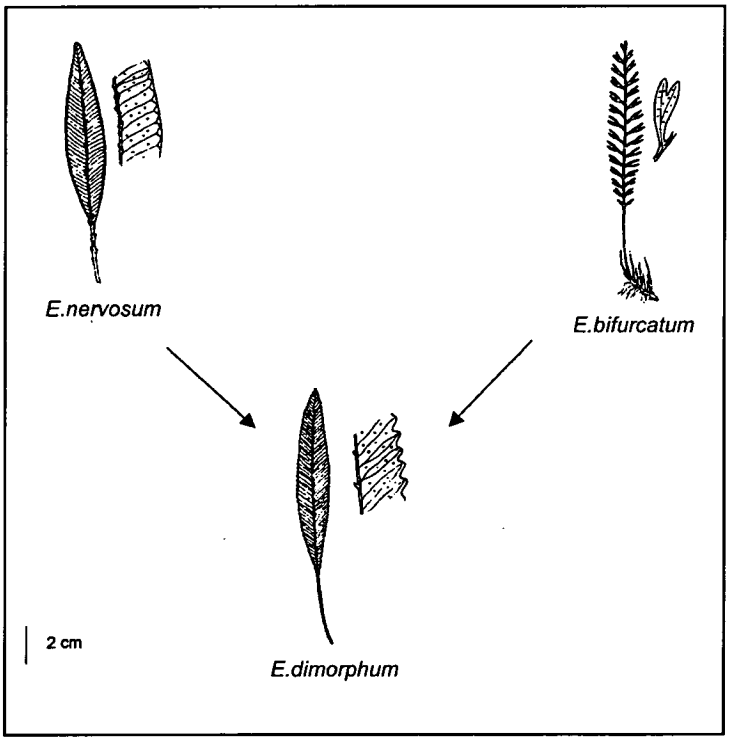
An understanding of a species' ecology, life history traits, breeding strategy and population genetic structure is a prerequisite of any effective conservation strategy. The preservation of genetic diversity, and hence the evolutionary potential of a species is one of the primary goals of conservation (Avise, 1994). In order to conserve the range of genetic diversity within a species empirical research is ideal, if not always available. Allozyme polymorphism has been used to investigate genetic diversity and its partitioning, species relationships, gene flow, breeding systems and hybridisation in a number of endangered plant species (Young *et al.*, 1999, Rumsey *et al.*, 1999, Case *et al.*, 1998, Gemmill *et al.*, 1998). However, there are only a few examples in the literature of investigations of the genetic diversity of threatened island ferns (Ranker, 1994).

This study uses isozyme electrophoresis to investigate the genetic diversity and relationships of three endemic *Elaphoglossum* species from the island of St Helena (15°56'S, 5°42'W), an isolated oceanic island in the South Atlantic Ocean. The three species, *E. nervosum* (Bory) Christ, *E. dimorphum* (Hook. & Grev.) Moore and *E. bifurcatum* (Jacq.) Mickel are all listed in the 1997 IUCN Red List of Threatened Plants (Walter and Gillett, 1998). The main threat to these endemic ferns is the encroachment of habitat by exotic invasive species such as *Phormium tenax* Forst., New Zealand flax. *Elaphoglossum nervosum* (Endangered) is restricted to a number of small populations along the central ridge of mountains in Diana's Peak National Park. *Elaphoglossum nervosum* is predominantly an epiphyte, on two endemic tree species, *Dicksonia arborescens* L'Héritier and *Melanodendron integrifolium* (Roxb.) DC., although it is also occasionally found on rock faces and mossy banks. *Elaphoglossum dimorphum* is restricted to two localities along the central mountain ridge in Diana's Peak National Park. It formerly occurred on High Peak and the Depot (Cronk, 2000). The total number of individuals does not exceed 50 and so the species is considered Critically Endangered. At these two sites *E. dimorphum* is found on stone steps, adjacent rocks and mossy banks growing in close proximity to *E. nervosum* and *E. bifurcatum*. At one of the sites *E. dimorphum* and *E. nervosum* grow together on a rock face. *E. bifurcatum* (Lower Risk) has a more widespread distribution on St Helena and is found at a number of localities above 650m. Like *E. dimorphum* it is terrestrial and is found growing on shaded rock faces and mossy banks. A preliminary cytological investigation obtained a chromosome count of $2n = 82$ for *E. dimorphum* and *E. bifurcatum* (Eastwood, unpublished data), the diploid number for *Elaphoglossum* (Lovis, 1977). These preliminary counts are from root tips of a single individual (sporophyte) cultivated at RBGE (*E. dimorphum*=20000254; *E.*

bifurcatum=20000257). The material in cultivation at RBGE originates from spores collected from two plants both from Mt Actaeon, Diana's Peak National Park.

A recent molecular study on the endemic *Elaphoglossum* species from St Helena, *E. bifurcatum*, *E. nervosum* and *E. dimorphum*, using the chloroplast region *trn*L-F, revealed that the species form a closely related monophyletic group (Chapter 4). These new data are in concurrence with the treatment of *Elaphoglossum* by Mickel and Atehortúa (1980) and the treatment of *Microstaphyla furcata* (L.f.) Fée under *Elaphoglossum* (Mickel, 1980). The three species vary in blade morphology and habitat preferences but show similarities in their rhizome and rachis scales, blade dots and gametophyte morphology. There is a gradation in the degree of blade dissection from *E. nervosum*, which has an entire blade, to *E. bifurcatum*, which has a pinnate blade. *E. dimorphum* has an intermediate blade morphology with a shallowly-lacerated margin. In his review of the relationships of dissected elaphoglossoid ferns, Mickel (1980) postulated that *E. dimorphum* could be a hybrid of *E. nervosum* and *E. bifurcatum*, as illustrated in Figure 5.1.

Fig. 5.1: The possible hybrid origin of *E. dimorphum* on St Helena (drawings taken from Mickel, 1980), showing individual fronds and frond detail.



There were two main objectives of this study: 1) to investigate the levels and patterns of genetic diversity in *E. bifurcatum*, *E. nervosum* and *E. dimorphum* using allozyme polymorphism and 2) to elucidate the relationships among the three species. An understanding of species relationships and the genetic structure of populations will allow conservation authorities on St Helena to make informed decisions on species and population management.

5.2 Materials and methods

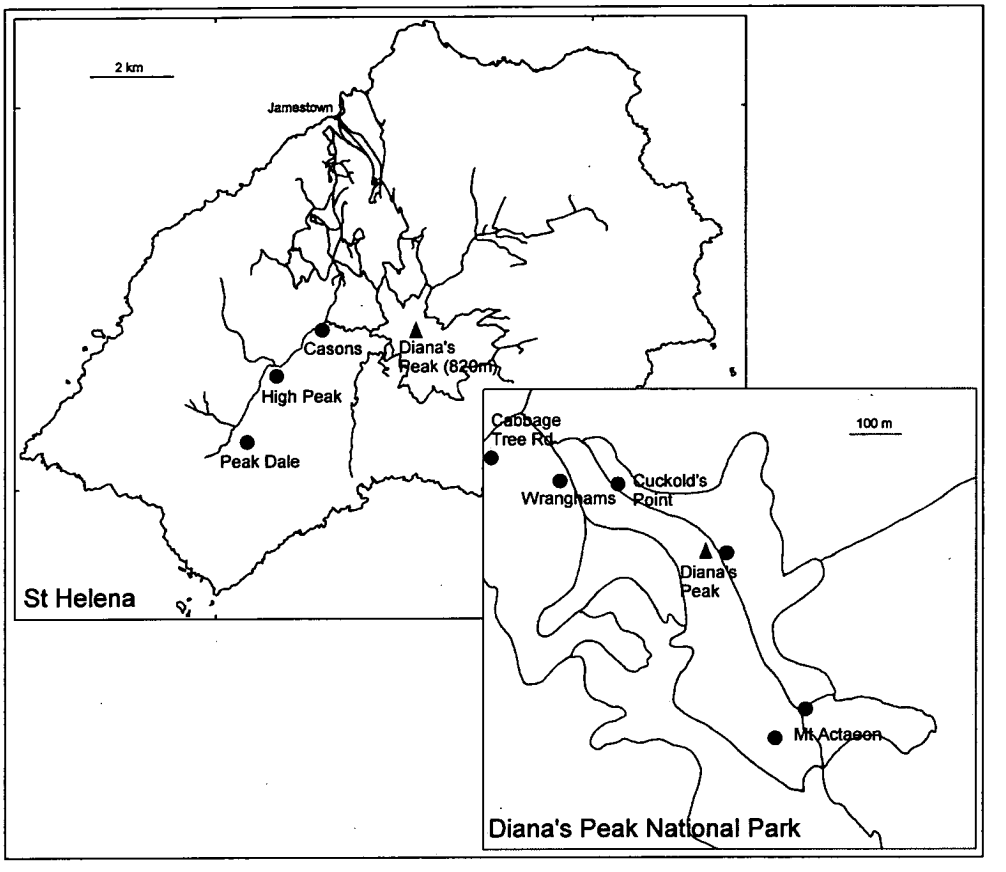
Sampling

Plants were sampled from populations of the three endemic taxa; *Elaphoglossum bifurcatum*, *E. nervosum*, and *E. dimorphum* over two consecutive years, 1999/2000. A list of the species, localities and sample sizes is given below in Table 5.1. Figure 5.2 is a map showing the localities of the populations. Samples of *E. dimorphum* and *E. nervosum* were taken from all the populations currently known on St Helena. In the case of the epiphyte, *E. nervosum*, only one individual per tree was sampled to avoid re-sampling the same genet.

Table 5.1: Collection localities and sample sizes used to study allozyme polymorphism in *Elaphoglossum bifurcatum*, *E. nervosum* and *E. dimorphum*.

Species	Locality and site number	Sample size (n)
E. bifurcatum	Cabbage Tree Road	35
	Casons	52
	Cuckold's Point A	34
	Cuckold's Point B	9
	Wranghams	19
	Mt Actaeon	20
	High Peak A	25
	High Peak B	12
	Peak Dale	25
		231
E. nervosum	Diana's Peak	9
	Cuckold's Point	15
	Cabbage Tree Road	16
	Mt Actaeon	12
	SE slopes of Mt Actaeon	29
		81
E. dimorphum	Mt Actaeon	34
	Cuckold's Point	12
		46

Fig. 5.2: Map of St Helena and Diana's Peak National Park showing roads (or paths) and localities of *E. nervosum*, *E. dimorphum*, and *E. bifurcatum* populations collected in this study.



Extraction, Storage, and Isozyme electrophoresis

Samples were stored in plastic bags, and where possible, kept cool for the duration of the return trip back to the UK. Once in the UK, small amounts of leaf tissue (30mg) were ground on ice in 80-100µl of Tris-HCl grinding buffer with 4% w/v PVP (pH 7.5). The Tris-HCl grinding buffer was made up according to Soltis *et al.* (1983) with the addition of 10% v/v DMSO and 0.25% v/v 2-mercaptoethanol. The crude extracts were absorbed onto filter wicks, frozen rapidly and stored at –80°C.

Allozyme polymorphism was investigated using standard starch gel electrophoresis using crude extracts as described in Wendel & Weeden (1990) and Soltis *et al.* (1983). The nine enzyme systems which resolved and were informative are as follows: Malate dehydrogenase (MDH), Phosphoglucoisomerase (PGI), 6-Phosphogluconate dehydrogenase (6-PGD), Aspartate amino-transferase (AAT), UTP-glucose-1-phospate uridyltransferase (UGGP),

Triosephosphate isomerase (TPI), Phosphoglucomutase (PGM), Acid Phosphatase (ACP) and Isocitrate dehydrogenase (IDH).

To optimise resolution of the nine enzyme systems, two electrode/gel buffers were used. Morpholine citrate (System 11) was used to resolve IDH, 6-PGD, MDH, PGM and UGPP, whist Lithium borate (System 7) was used for AAT, PGI, TPI and ACP. All the starch gels were made at 13.7%. The morpholine citrate buffer was made according to Wendel & Weeden (1990) with a pH range of 6.4-7.4. The Lithium borate gel and electrode buffer was modified from Soltis *et al.* (1983)

Samples of the different species and populations were run together on the same gel and a standard marker was placed on each gel so that comparisons between gels could be made. In the absence of progeny arrays the interpretation of the zymograms were based upon established models of quaternary structure, number of loci and subcellular compartmentalisation (Gastony & Darrow, 1983; Weeden & Wendel, 1990)

Data analysis

A number of genetic diversity parameters were estimated for each population: *(P)*, the proportion of polymorphic loci; *(A)*, the mean number of alleles per locus; *(Ap)*, the mean number of alleles per polymorphic locus and (H_E), the expected heterozygosity. Genetic identities for each species were estimated using Nei's (1972) genetic identity values. The genetic distances for species and populations used in the Neighbour Joining algorithm were based on Nei's (1972) genetic distance. The genetic diversity calculations were conducted using the population genetic package GDA (Lewis & Zaykin, 2001). Wrights *F* statistics (Wright, 1969, 1978) were used to investigate population genetic differentiation and were calculated using FSTAT 2.9.3.1 (Goudet, 2001), which uses Weir & Cockerham's (1984) estimation of F_{ST} (θ) and F_{IS} (f). Bootstrapping over all loci (15, 000 replicates) was used to test whether the estimates of F_{ST} and F_{IS} were significantly different from zero (random mating).

5.3 Results

Species relationships

Of the nine enzyme systems that were screened, 16 putative loci resolved consistently and clearly. Isozyme phenotypes for *E. bifurcatum*, *E. dimorphum* and *E. nervosum* are shown in Fig. 5.3. and Table 5.3. An interpretation of putative loci (numbers 1-3) and alleles (a-c) is provided for each enzyme apart from TPI which showed a fixed pattern in all three species. In total there were five polymorphic loci (loci with > 1 allele) in *E. bifurcatum,* compared with only two in *E. nervosum*. At two enzyme loci, MDH-1 and UGPP-2, additive allelic patterns were observed in *E. dimorphum*. In MDH-1 all *E. dimorphum* individuals appeared to be fixed heterozygotes, possessing both of the species-specific alleles from the putative parental species, *E. bifurcatum* and *E. nervosum*. In UGGP-2 the majority of *E. dimorphum* individuals (31) were heterozygotes although some individuals (15) were homozygotes for the c allele.

Nei's (1972) genetic identity between *E. nervosum* and *E. dimorphum* is 0.96, between *E. nervosum* and *E. bifurcatum* 0.86, and between *E. bifurcatum* and *E. dimorphum* 0.91.

Genetic diversity

Table 5.2 shows estimates of five population genetic diversity parameters for *E. bifurcatum* and *E. nervosum*. These genetic diversity parameters was not be estimated in *E. dimorphum* due to uncertainty regarding the designation of loci and alleles and apparent deviation from disomic mendelian inheritance.

E. bifurcatum and *E. nervosum* show low levels of population genetic diversity in all the estimated diversity parameters. The percentage of polymorphic loci in *E. bifurcatum* and *E. nervosum* populations is 8% and 1% respectively. The expected heterozygosity or gene diversity is 2% in *E. bifurcatum* and 0.2 % in *E. nervosum*.

Table 5.2: Population level genetic diversity parameters for endemic *Elaphoglossum bifurcatum* and *E. nervosum*. *(P)* is the proportion of polymorphic loci; *(A)* the mean number of alleles per locus; *(Ap)* the mean number of alleles per polymorphic locus and *(H_E)* the expected heterozygosity.

Species	No. of pops.	Mean *N*	*P*	*A*	*Ap*	H_E
E. bifurcatum	9	25.6	0.08	1.10	2.38	0.02
E. nervosum	5	16.2	0.01	1.01	2	0.002

Genetic structure of populations

A cluster analysis of populations of *E. nervosum*, *E. dimorphum* and *E. bifurcatum* based on Nei's (1972) genetic distance is shown in the unrooted neighbour joining tree in Figure 5.4. The two populations of *E. dimorphum* lie between the populations of *E. nervosum* and those of *E. bifurcatum*.

The population of *E. nervosum* which is found on the SE slopes of Mt Actaeon is genetically distinct from the other *E. nervosum* populations. This high level of population genetic divergence reflects the fixation of alleles at one enzyme locus, PGM-2. Four populations of *E. nervosum* are fixed for the common allele b, whereas the population below Mt Actaeon is fixed for the a allele.

Fig. 5.3: Isozyme phenotypes of *Elaphoglossum bifurcatum*, *E. dimorphum*, and *E. nervosum* for 9 enzyme systems. An interpretation of putative loci (numbers) and respective alleles (small letters) is given for all enzyme systems apart from TPI which showed a fixed pattern in all species. The striped band in MDH-2 indicates a co-migrating band in *E. nervosum* and *E. bifurcatum*. HTD = Heterodimer. Capital letters indicate single enzyme phenotypes. Anode towards top of figure.

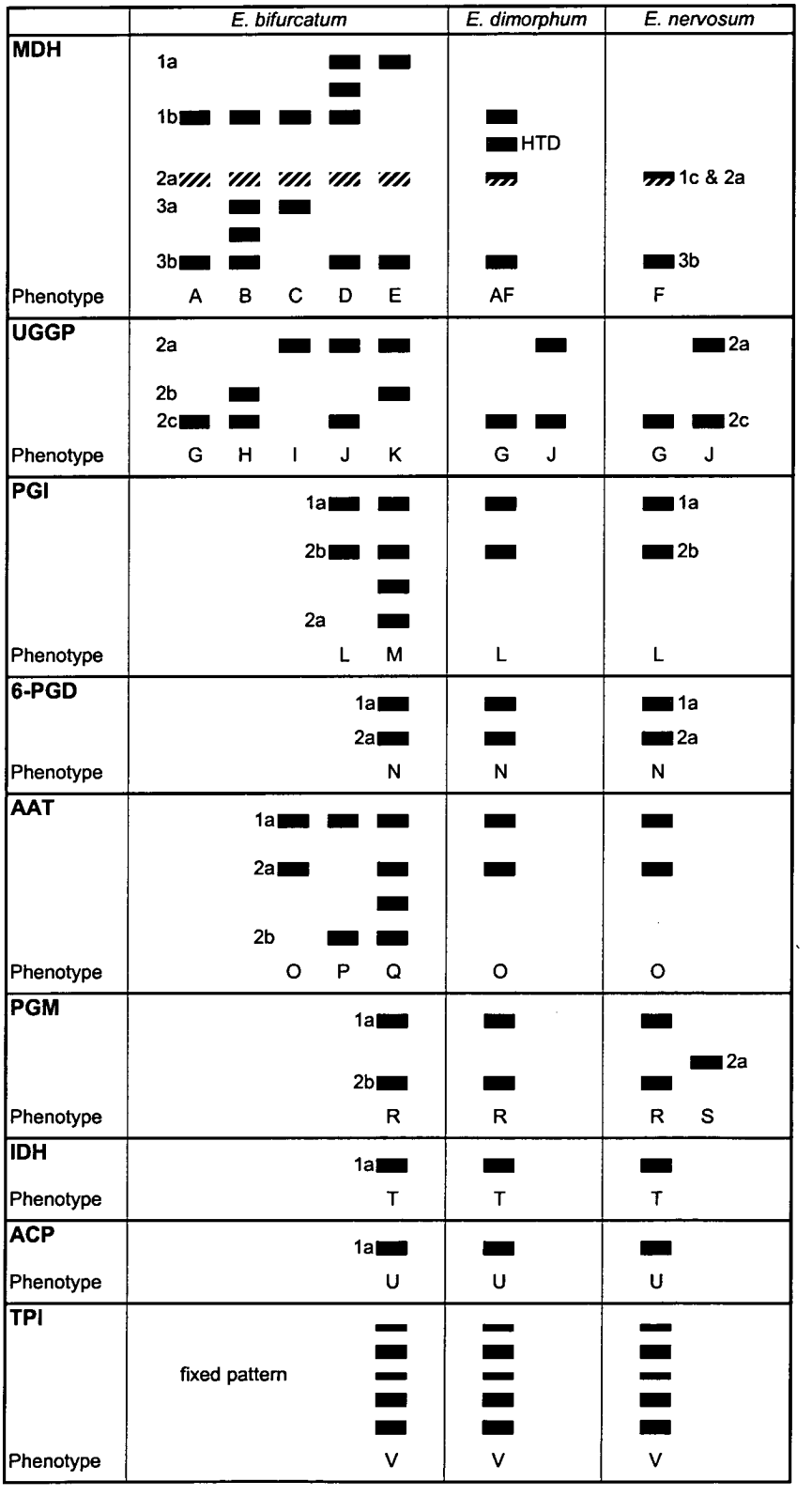
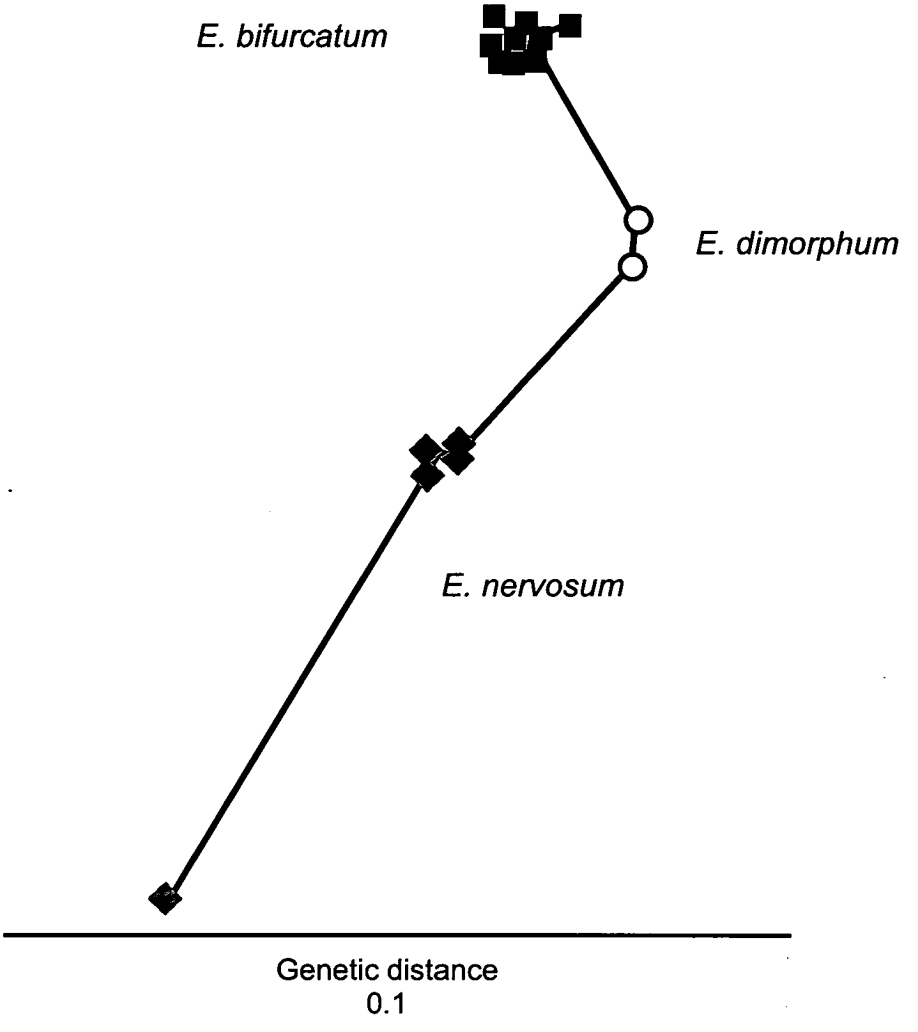


Table 5.3: Single locus phenotypes and their distribution in populations of *E. bifurcatum*, *E. dimorphum* and *E. nervosum*.

		Single-enzyme phenotypes								
Population	n	MDH	UGGP	PGI	6-PGD	AAT	PGM	IDH	ACP	TPI
E. bifurcatum										
Cabbage Tree Road	35	A=21 B=13 C= 1	G=12 J=8 I=1	L=35	N=35	O=35	R=35	T=35	U=35	V=35
Casons	52	A=49 D=2 E=1	G=50 H=2	L=47 M=5	N=52	O=47 P=1 Q=4	R=52	T=52	U=52	V=52
Cuckold's Point A	34	A=34	G=26 H=3 J=5	L=34	N=34	O=34	R=34	T=34	U=34	V=34
Cuckold's Point B	9	A=9	G=5 H=4	L=9	N=9	O=9	R=9	T=9	U=9	V=9
Wranghams	19	A=19	G=11 H=2 I=1 J=5	L=19	N=19	O=19	R=19	T=19	U=19	V=19
Mt Actaeon	20	A=20	G=10 I=1 J=8 K=1	L=20	N=20	O=20	R=20	T=20	U=20	V=29
High Peak A	25	A=25	G=25	L=25	N=25	O=25	R=25	T=25	U=25	V=25
High Peak B	12	A=12	G=12	L=12	N=12	O=7 P=1 Q=4	R=12	T=12	U=12	V=12
Peak Dale	25	A=23 D=2	G=25	L=25	N=25	O=25	R=25	T=25	U=25	V=25
E. dimorphum										
Mt Actaeon	34	AF=34	G=10 J=24	L=34	N=34	O=34	R=34	T=34	U=34	V=34
Cuckold's Point A	12	AF=12	G=5 J=7	L=12	N=12	O=12	R=12	T=12	U=12	V=12
E. nervosum										
Diana's Peak	9	F=9	G=9	L=9	N=9	O=9	R=9	T=9	U=9	V=9
Cuckold's Point A	15	F=15	G=13 J=2	L=15	N=15	O=15	R=15	T=15	U=15	V=15
Cabbage Tree Road	16	F=16	G=16	L=16	N=16	O=16	R=16	T=16	U=16	V=16
Mt Actaeon	12	F=12	G=12	L=12	N=12	O=12	R=12	T=12	U=12	V=12
SE slopes of Mt Actaeon	29	F=29	G=29	L=29	N=29	O=29	S=29	T=29	U=29	V=29

The high population differentiation of two populations in *E. nervosum* is also seen in the highly significant F_{ST} value of 0.96 ($p<0.01$), where F_{ST} (Wright, 1969, 1978) represents genetic differentiation among populations. Low, but significant, genetic differentiation is also present in populations of *E. bifurcatum*, where the F_{ST} is 0.1 ($p<0.01$). Estimates of F_{IS}, deviation from random mating within populations, were not significantly different from zero (*E. bifurcatum*, $F_{IS}=0.039$, $p<0.01$; *E. nervosum*, $F_{IS}=-0.04$, $p<0.01$).

Fig. 5.4: A neighbour joining tree of *E. nervosum* , *E. dimorphum* ○ and *E. bifurcatum* populations based on Nei's (1972) genetic distance.



5.4 Discussion

Species relationships

The genetic identities observed between *E. nervosum*, *E. dimorphum* and *E. bifurcatum* are high when compared with other species of congeneric homosporous ferns (Soltis & Soltis, 1990a). The mean genetic identity, for example, between six North American species of *Polystichum* was 0.524 (Soltis *et al.* 1990). However, these values are based on continental fern taxa with comparably wide geographic distributions. The high genetic identities between the three endemic *Elaphoglossum* species, a reflection of low levels of species' allozyme divergence, are comparable with average genetic identities amongst congeneric angiosperm species from other oceanic islands (Ito *et al.*, 1998; Baldwin, 1998; Kim *et al.*,

1999). The low levels of genetic divergence between the three endemic *Elaphoglossum* species indicate that the species have undergone a recent, rapid speciation. Although the species are closely related they display distinct morphological and ecological variation. The distinctness of the pinnate blade of *E. bifurcatum* has led to taxonomic confusion in the past, and the species has been placed in ten different genera (Mickel, 1980). The genus *Microstaphyla* Presl is, in fact, based on this unusual pinnate *Elaphoglossum* from St Helena.

Evidence from two polymorphic loci, MDH-1 and UGGP-2, supports the hypothesis that *E. dimorphum* is of hybrid origin involving the two parental taxa, *E. nervosum* and *E. bifurcatum*. In addition the two distinct *E. dimorphum* phenotypes at UGGP-2 indicate that the hybrid species may have multiple origins. However, as *Elaphoglossum nervosum* has very little allozyme polymorphism and essentially contains only a subset of the alleles found in *E. bifurcatum* (not including MDH-1), further genetic analysis with a more polymorphic marker would be desirable. The fixed heterozygosity at MDH-1 suggests that *E. dimorphum* is a segmental allopolyploid. However, a chromosome count of 2n=82 has been obtained for *E. dimorphum* indicating that the species is a diploid. This count, based on a single cell, needs to be confirmed, but on the available cytological evidence *E. dimorphum* is not a polyploid. *Elaphoglossum dimorphum* is fully fertile and has been propagated successfully from spores at the Royal Botanic Garden Edinburgh. This suggests that *E. dimorphum* individuals are not F_1 hybrids, although an analysis of plants raised from spores should be conducted for confirmation. The cytological evidence conflicts with the allopolyploid explanation of fixed heterozygosity, indicating that alternative explanations, such as apomixis, have to be considered (e.g. Schneller *et al.*, 1998).

The three *Elaphoglossum* species appear to have originated from a common ancestor, which consequently diverged into two distinct species, one an epiphyte, *E. nervosum*, and the second a terrestrial species, *E. bifurcatum*, with a distinct pinnate blade. The putative hybrid species, *E. dimorphum*, between *E. bifurcatum* and *E. nervosum*, has a limited distribution restricted to two sites on stone steps, the surrounding boulders and banks. From observations in the field, the two progenitor taxa, *E. nervosum* amd *E. bifurcatum* only occur together at these two sites in Diana's Peak National Park. The rarity of *E. dimorphum* could, therefore, be attributed to a lack of opportunity for hybridisation. Once formed, the distribution of *E. dimorphum* could be restricted by the availability of suitable intermediate habitat and by competition from its progenitor species.

Genetic diversity

Plant species with restricted geographical distributions, such as endemics, generally exhibit lower levels of genetic variability than widespread species (Hamrick & Godt, 1990; Karron, 1991). According to the analysis by Hamrick & Godt (1990), endemic plant populations are polymorphic at approximately 26.3% of their loci and have a mean genetic diversity (heterozygosity) of 6%. *Elaphoglossum bifurcatum* and *E. nervosum*, in comparison, have much lower levels of genetic diversity within populations. Such low levels of genetic diversity have been observed in other insular endemics (Crawford *et al.*, 1992; Gemmill, 1998). There are exceptions though, as illustrated by the rare Hawaiian fern, *Adenophorus periens* L.E. Bishop, where populations were found to be polymorphic at 80% of their loci and expected heterozygosity was over 20% (Ranker, 1994).

The low levels of genetic diversity in insular endemics can be attributed to founder effects in the progenitor species associated with long distance dispersal and establishment on isolated islands. The subsequent founding of new populations, which may include speciation, could likewise deplete genetic variation by loss of alleles (Crawford *et al.*, 1992). *Elaphoglossum bifurcatum* (2%) has ten times the amount of expected heterozygosity than *E. nervosum* (0.2%). The distribution of *E. bifurcatum* is much more extensive on St Helena than *E. nervosum* and population sizes are much larger. Larger population sizes are suggested as an explanation for higher levels of allozyme diversity in certain species of the silversword alliance (Witter & Carr, 1988). Small, fragmented populations, as in *E. nervosum*, are prone to genetic drift and hence loss of genetic diversity. Tree-fern thicket, the habitat of *E. nervosum*, once covered a greater area on St Helena than today (Cronk, 2000). Even in relatively recent history (1950s) swathes of endemic tree fern thicket were cleared from the mountain slopes and re-planted with a monoculture of New Zealand Flax, *Phormium tenax*. Large reductions in population sizes or genetic bottlenecks, followed by subsequent re-colonisation, could contribute to the lower levels of genetic diversity observed in *E. nervosum* compared with *E. bifurcatum*.

Genetic structure

Despite having low levels of genetic diversity, populations of *E. nervosum* and *E. bifurcatum* show significant genetic differentiation and should be considered in any conservation strategy. The low, but significant, levels of population differentiation in *E. bifurcatum* may be due to habitat fragmentation. The endemic vegetation on St Helena exists as isolated

fragments surrounded by naturalised exotic species which may prevent gene flow between populations. In contrast, *Elaphoglossum nervosum* showed high levels of population differentiation over very short distances (200m) which suggests a lack of gene flow between adjacent populations. High levels of population differentiation, as in *E. nervosum*, have also been reported in *Dryopteris expansa* (C. Presl) Fraser-Jenkins & Jermy (Soltis & Soltis, 1990b) and in this case, was due to the fixation of LAP alleles in different populations. To ensure a range of allelic diversity is conserved in *E. nervosum* and *E. bifurcatum*, key habitats should be actively managed to ensure minimal encroachment by invasive exotics. In addition any germplasm collection for *ex situ* conservation should sample a large number of populations to ensure any population differentiation is encountered for.

This work demonstrates the value of genetic studies of rare and threatened taxa in conservation planning, has helped to elucidate the relationships between the species, supports the proposal that *E. dimorphum* is of hybrid origin and highlights the high levels of genetic differentiation in *E. nervosum*.

5.5 Acknowledgements

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General Discussion

6.1 Oceanic islands; uniqueness and threats

Oceanic islands, by their very nature, offer great opportunities for the study of evolution and have for a long time been recognised as natural laboratories due to their isolation and diversity of species and habitats (Emerson, 2002; Darwin, 1859; Wallace, 1880; Carlquist, 1965, 1974; Stuessy & Ono, 1998; Baldwin *et al.*, 1998). Oceanic islands have characteristically high levels of plant endemism, for example, 94.4% of the angiosperm species on the Hawaiian Islands are endemic (Carlquist, 1974). The flora of St Helena, although relatively species poor, also has high levels of plant endemism (81.7%) including 10 endemic genera (Cronk, 1997). The high level of endemism on Hawaii has been demonstrated to be due to rapid autochthonous evolution through speciation processes such as adaptive radiation (see Wagner & Funk, 1995). However, the endemic flora on St Helena is considered to be part of a relictual series, whereby the endemic species are relics of differing ages ranging from the Miocene to Pleistocene (Cronk, 1987; Cronk, 1992). Carlquist (1995) however, contests the relic hypothesis by Cronk and postulates that the patterns of evolution on Atlantic islands will change to resemble the findings from the Hawaiian Islands as DNA analysis and cladistic methods are applied.

Unfortunately a large proportion of island endemics are threatened, as exemplified by the Juan Fernandez Islands where over 70% of the endemic species of angiosperms are classified as threatened (Stuessy *et al.* 1992). On St Helena all the endemic plant species are classified as threatened, with the possible exception of the endemic tree fern, *Dicksonia arborescens* (Lower Risk) (Cronk, 2000). The high proportion of threatened insular endemics (one in three of all threatened plants are island endemics: Groombridge, 1992) has been attributed to a number of intrinsic and extrinsic (anthropogenic) factors including: limited species distributions, small population sizes, introduction of alien plants and animals, and the clearing of indigenous vegetation for farming, firewood and urban development (Rieseberg & Swensen, 1996; Cronk, 1997). Unfortunately, St Helena epitomises all the current challenges facing conservation biologists working on islands; extensive environmental degradation, habitat fragmentation, species on the verge of extinction, invasive plants, introduced pests and pathogens, and the lack of resources and trained staff (Smith, 1997; Maunder *et al.*, 1995; Cronk, 2000). In addition the lack of infrastructure, airport and

political will power (St Helena is a dependent territory of the UK and heavily dependent on development aid) combined with logistical difficulties has limited plant evolution and conservation studies on the island with a few notable exceptions (Cronk, 1984; Rowe, 1995; Carlquist, 2001; Richardson, 1999). This is in stark contrast to the large number of studies conducted on the islands and archipelagos of Hawaii, Juan Fernandez, and Macaronesia (Wagner & Funk, 1995; Stuessy & Ono, 1998). This thesis has attempted to redress this imbalance in island biology research with a series of evolutionary and reproductive biology studies in the endemic species of *Commidendrum* (Asteraceae), *Melanodendron* (Asteraceae) and *Elaphoglossum* (Lomariopsidaceae) from St Helena. A brief discussion of my findings in relation to other studies, particularly on island evolution and conservation, is given below.

6.2 Evolution and speciation on oceanic islands

The majority of evolutionary studies on oceanic islands have focused on the archipelagos of Hawaii, Juan Fernandez and Macaronesia and have been predominantly on angiosperms (Baldwin *et al.*, 1998; Baldwin *et al.*, 1991; Kim *et al.*, 1998; Givnish *et al.*, 1995; Francisco-Ortega *et al.*, 2001; Panero *et al.*, 1999; Sang *et al.*, 1994). These archipelagos often show extensive radiations as exemplified by the silversword alliance on Hawaii (Baldwin, 1997). This contrasts to St Helena where eight of the ten endemic genera on St Helena are monotypic (Cronk, 2000). Although St Helena endemics have been included in a number of broad phylogenetic studies (Richardson, 2001; Bakker et al., 1998; Noyes & Rieseberg, 1999) no studies have focused specifically on the evolution and speciation of endemic plant groups on St Helena. The work presented in this thesis is the first study to investigate evolution and speciation of two very distinct plant groups from St Helena; i) arborescent composites in the endemic genera *Commidendrum* and *Melanodendron* and ii) epiphytic and terrestrial elaphoglossoid ferns in the genera *Elaphoglossum* and *Microstaphyla*.

One of the emerging trends to come out of the large number of studies of evolution and speciation on oceanic islands is the monophyly of related taxa despite the fact that many of the plant lineages often exhibit extensive morphological and ecological diversity (Baldwin *et al.* 1998). There are of course a number of exceptions to this trend, for example *Lavatera* (Malvaceae) and the Gonosperminae (Asteraceae) from the Canary Islands (Ray, 1995; Francisco-Ortega, *et al.*, 2001). Like the many studies on other oceanic islands, the arborescent composites, *Commidendrum* and *Melanodendron*, two closely related genera

from St Helena, were also shown to be monophyletic, that is they have originated from a common ancestor via a single dispersal event (Chapter 2). In addition, species within *Commidendrum* were shown to be monophyletic with very little sequence divergence between them. In the elaphoglossoid ferns, the endemic species *Elaphoglossum dimorphum*, *E. nervosum* and *Microstaphyla furcata* were shown to be monophyletic, despite the distinct frond morphology of *M. furcata* (Chapter 4). *Microstaphyla furcata*, traditionally placed in its own genus, was shown to be an *Elaphoglossum* and confirmed the previous transfer of this species to *Elaphoglossum bifurcatum*. The close relationship of the endemic *Elaphoglossum* species is also supported by allozyme data (Chapter 5), with genetic identities ranging from 0.86-0.96. The indigenous *Elaphoglossum*, *E. conforme*, is distantly related to the endemic *Elaphoglossum* species, indicating independent origins of this species and the endemic *Elaphoglossum* species. Extensive morphological and ecological variation coupled with low phylogenetic divergence between related taxa, as seen in the St Helena endemics, has been observed in other island endemics and has been attributed to recent and rapid speciation (Francisco-Ortega *et al.*, 1997; Baldwin, 1997).

As well as testing the monophyly of island endemics some phylogenetic studies have provided new insights into the closest relatives of island endemics which are morphologically divergent from the continental progenitors (Baldwin, *et al.* 1991; Kim *et al.*, 1998; Francisco-Ortega *et al.*, 1997; Ballard & Systema, 2000). Until fairly recently the closest relatives of *Commidendrum* and *Melanodendron*, due to their taxonomic divergence, were only speculated. However, a broad phylogenetic study of the Astereae using ITS by Noyes & Rieseberg (1999) indicated that *Commidendrum* was part of a basal group of the Astereae, closest to the South African genera *Felicia* and *Amellus*. Further sampling of *Felicia* and other Astereae in our study supported the findings of Noyes & Rieseberg (1999) (Chapter 2) although more sampling of the Asteraeae is needed to confirm this. The phylogenetic study on the elaphoglossoid ferns indicated that the origins of the endemic *Elaphoglossum* on St Helena are also African although this needs further testing.

The phylogenetic studies on these two study groups from St Helena have been invaluable to elucidate species relationships, particularly in the ferns, and gain an insight into the patterns of evolution and origins of plants on St Helena. The study has also provided a systematic framework which future conservation and research efforts can be built around. However, due to the limited sampling of African Astereae these findings neither support nor refute the hypothesis of relic endemism on St Helena (Cronk, 1987; 1992).

6.3 Hybridisation in oceanic island plants

Many species of island endemic lineages, despite often having striking morphological and ecological differences, have been found to be inter-fertile, an indication of their genetic cohesiveness (Baldwin *et al.*, 1998; Carr & Kyhos, 1986; Francisco-Ortega, *et al.*, 1997; Francisco-Ortega *et al.*, 2000; Crawford *et al.*, 1993; Brochmann, 1984). In fact natural hybridisation and introgression is postulated to be of great importance in the evolution and speciation of some Hawaiian and Macaronesian lineages (see Arnold, 1997 for a review).

Natural hybrids between related taxa can occur in species which are sympatric and can result in hybrid swarms as seen on the Canary Islands (Francisco-Ortega *et al.*, 2000). On St Helena, there is morphological evidence to suggest that *Commidendrum rugosum* and *C. robustum* hybridise where the two species are sympatric (Eastwood, pers. obs.). If reproductive isolation from the progenitor taxa follows hybridisation, for example, through polyploidy, it can lead to speciation (Arnold, 1997). Evidence from allozymes (Chapter 5) indicates that *Elaphoglossum dimorphum* is of a hybrid origin between the progenitor species *E. nervosum* and *E. bifurcatum*. The rarity of *E. dimorphum* (CR) could therefore be attributed to a lack of opportunity for hybridisation on St Helena.

Natural hybrids can also occur between related taxa which are not sympatric if natural isolating barriers (ecological and geographical) break down as seen on many oceanic islands (Brochmann, 1984; Francisco-Ortega, 2000; Rieseberg & Gerber, 1995; Mehrhoff, 1996). On St Helena, hybridisation between the endemic *Trochetiopsis ebenus* and *T. erythroxylon* (Rowe, 1995) was shown to occur when the two species were brought into cultivation at the same *ex situ* locality. Similarly in this thesis (Chapter 3) hybridisation between *Commidendrum spurium* and *C. rotundifolium* were shown to occur at Pounceys, an *ex situ* locality where both species are planted. Hybridisation between these two closely related and threatened species has many implications for conservation, particularly for seed orchard management and species recovery (see also below).

6.4 Self-incompatibility and conservation of threatened plants

Baker's Law (Baker, 1955) predicts that plant species which have established through long distance dispersal, such as insular species, are more likely to be self-compatible than self-incompatible. The majority of endemic species on Hawaii and the Juan Fernandez Islands are self-compatible, with the notable exceptions of members of the Hawaiian silversword alliance (*Argyroxiphium* DC., *Dubautia* Gaud. And *Wilkesia* A. Gray) and *Dendroseris* D. Don (Juan Fernandez Islands) (Carr *et al.*, 1986; Anderson, *et al.*, 2001). Research in this thesis showed that *Commidendrum rotundifolium* (EW) and *C. spurium* (CR) are self-incompatible (Chapter 2). Detailed crossing experiments in these two threatened plants, the first study of its kind to be conducted on island endemics, showed that poor seed viability in *C. rotundifolium* was due to a paucity of *S*- alleles (Chapter 2). This has serious implications for the species recovery programme of *C. rotundifolium*, justifying a more pragmatic approach to ensure its long term survival. This may even include using hybridisation as a conservation tool (with *Commidendrum spurium*), to introduce S- allele diversity, as well as conducting controlled pollinations. The presence of a self-incompatibility system in *C. spurium* also has many implications for both seed orchard and small population management. For example, isolated individuals and populations may have to be augmented with compatible individuals to ensure regeneration and long term survival. The findings of my research into the reproductive biology of *C. rotundifolium* and *C. spurium* are being directly incorporated into the species recovery plans for these two species.

6.5 Genetic diversity

There have been a large number of studies looking at the genetic diversity of insular endemics using allozymes. However, with the exception of two known studies, (Ranker, 1992; Ranker, 1994) these have been confined to angiosperms (Witter & Carr, 1988; Crawford *et al.*, 1992; Kim *et al.*, 1999; Gemmill *et al.*, 1998; Ito *et al.*, 1998; Crawford *et al.*, 2001). Recent reviews of the levels of diversity within species and populations of insular endemics (angiosperms) on Hawaii (de Joode & Wendel, 1992) and the Juan Fernandez Islands (Crawford *et al.*, 2001) show that insular endemics have very low levels of diversity compared with widespread taxa or even endemic plant species in general. For example, the range of expected heterozygosity (in populations) of species on the Juan Fernandez Islands

was between 0.00-0.18 (Crawford *et al.*, 2001). Factors most frequently cited for the low levels of genetic diversity in insular endemics include: bottlenecks associated with long-distance dispersal to islands, establishment of colonising ancestors on islands, and drift and inbreeding in small populations (de Joode & Wendel, 1992). In the only known study of a rare endemic island fern, *Adenophorus periens*, L.E. Bishop, from Hawaii, levels of genetic diversity were high, with expected heterozygosity in populations exceeding 0.2 (Ranker, 1994). The obscure high levels of genetic diversity in *A. periens* were attributed to an outcrossing mating system and perennial life cycle in the species. Levels of genetic diversity were also found to be high in the related but widespread endemic ferns, *A. tamariscinus* (Kaulf.) Hook & Grev. and *A. tripinnatifidus* Gaud. The allozyme study on the endemic *Elaphoglossum* species is the first one to investigate genetic diversity in ferns from St Helena, allowing comparisons with insular endemics from other oceanic islands to be made. Levels of genetic diversity in populations (expected heterozygosity) of *Elaphoglossum nervosum* (0.002) and *E. bifurcatum* (0.02) from St Helena were shown to be very low, but comparable with the majority of other island endemics. The genetic diversity in populations of *E. bifurcatum* was ten times that of *E. nervosum* and can be attributed to the restricted distribution of the latter species on St Helena. Despite having low levels of genetic diversity, populations of *Elaphoglossum bifurcatum* and *E. nervosum* show significant genetic differentiation which should be taken in to account in devising a conservation strategy. For example, to conserve the maximum amount of genetic diversity for *ex situ* conservation spores should be collected from a large number of populations.

6.6 Conclusion

Despite the challenges of working on isolated islands the work presented in this thesis has highlighted how invaluable integrated and applied research can be in increasing our understanding of the biology of rare, threatened island endemics. Only through an understanding of species relationships, patterns of evolution, ecology and reproduction in rare plants can we make informed conservation decisions and plan effective species recovery programmes.

6.7 References

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Appendix 1: Aligned ITS1 and ITS2 matrix of *Commidendrum* and *Felicia*

COMSPU = *Commidendrum spurium*, COMRUG = *C. rugosum*, COMROB = *C. robustum*, COMROT = *C. rotundifolium*, MELINT = *Melanodendron integrifolium*, FELFRU = *Felicia fruticosa*, FELAET = *F. aethiopica*, FELECH = *F. echinata*, FELULI = *F. uliginosa*, FELCLA = *F. clavipilosa*, BACSPP = *Baccharis* sp. OLEARB = *Olearia arborescens*, OLEPHL = *O. phlogopappa*, CHIDIF = *Chiliotrichum diffusum*, ASTVAH = *Aster vahlii*.
Regions of ambiguous alignment = * (bps: 16-17, 72-78, 128-131, 149-156, 203-205, 460-473)

```
[                  10        20        30        40        50        60        70        80        90]
[                  .         .         .         .         .         .         .         .         .
             [ITS1
                           **                                                  *******
COMSPU       TCGAAACCTGCATA-G-CAGAACGACCCGCGAACATGTTATAACAACCAGGTGGCAGGA-TGGGTTGGT--TCATTTC----GATCCTCT
COMRUG       TCGAAACCTGCATA-G-CAGAACGACCCGCGAACATGTTATAACAACCAGGTGGCAGGA-TGGGTTGGT--TCATTTC----GATCCTCT
COMROB       TCGAAACCTGCATA-G-CAGAACGACCCGCGAACATGTTATAACMACCAGGTGGCAGGA-TGGGTTGGT--TCATTTC----GATCCTCT
COMROT (con) YCGAAACCTGCATA-G-CAGAACGACCCGCGAACATGTTATAACAACCAGGTGGCAGGA-TGGGTTGGT--TCATTTC----GATCCTCT
MELINT       TCGAAACCTGCATA-GCCAGAACGACCCGCGAACGTGTTATAACAACCAGGTGGCAGGA-TGGGTTGGT--TCATTTC----GATCCTCT
FELFRU       TCGAAACCTGCAAA-GGCAGAACGACCCGCGAACATGTTATAACAACCACGTGCCAGGA-TGGGTCAGT--TTATTCT----GATCCTCT
FELAET       TCGAAACCTGCAAA-G-CAGAACGACCCGTGAACATGTTAAAACAACCATGTGCCAGGAGTGG-TTGGT--TTATTCC----GATCCTGC
FELECH (con) TCGAAACCTGCAAAAG-CAGAACGACCCGTGAACATGTTATAACAACCATGTGCCAGGA-TGGTTTGGT--TTATTCT----GATCCTCT
FELULI       TCGAAACCTGCAT--GGCAGAACGACCCGCGAACATGTTATAACAGACGCTTCCCAGGA-TGGATTGGT--TAA--C-------TCCTCT
FELCLA       TCGAAACCTGCAT--GGCAGAACGACCCGTGAACATGTTATAACATATGCTTCCCAGGA-TGGATTGGT--TAA--C-------TCCTTT
BACSPP       TCGAAGCCTGCAAA-G-CAGAACGACCCGCGAACACGTTATAACAGCCA--TGCCAAGA-TGGGTCGGGCGTCA----GTTCGATCCTCG
OLEARB       TCGAAGCCTGCAAA-G-CAGAACGACCCGTGAACATGTTATAACAACCACGTGCCAGGA--GGGTCGGGCGTCA----GTCAGATCCTCG
OLEPHL       TCGAAGCCTGCAAA-G-CAGAACGACCCGTGAACATGTTATAACAACCA--TGCCAGGA-TGGGTTGGGCGTTA----GCTCGATTCTCG
CHIDIF       TCGTAGCCTGCAAA-G-CAGAACGACCCGTGAACATGTTATAACAACAATGTGCCAAGA--GGGTTGGGCGTCA----GTCCGATCCTCG
ASTVAH       TCGAAGCCTGCAAA-G-CA-AACGACCCGCGAACATGTTACAACAAC--CTTGCCATGA--GGTTCGGGC-TTT----GTTCGATCCTCA
```

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 [                  100        110        120        130        140        150        160        170        180]
 [                  .          .          .          .          .          .          .          .          .]
                                                     ****                  ********
COMSPU        -----TGTCACACCGTTGATGTGCGTTCTTGATCACCCTG-TTGGGGCCTCTTGGA--CGTTGCATCG-ACATAACAAAACCCCGGCACA
COMRUG        -----TGTCACACCGTTGATGTGCGTTCTTGATCACCCTG-TTAGGGCCTCTTGGA--CGTTGCATCG-ACATAACAAAACCCCGGCACA
COMROB        -----TGTCACACCGTTGATGTGCGTTCTTGATCACCCTG-TTGGGGCCTCTTGGA--CGTTGCATCG-ACATAACAAAACCCCGGCACA
COMROT        -----TGTCACACCGTTGATGTGCGTTCTTGATCACCMTG-TTGGGGCCTCTTGGA--CGTTGCATCG-ACATAACAAAACCCCGGCACA
MELINT        -----TGGCACACCGTGGATGTGCGTTTTTGATCACCCTG-TTGGGGCCTCTTGGA--CGTTGCATCGGACATAACCAGACCCCGGCACA
FELFRU        -----TGGCGCACCGTCGACGTGCGTTCTTGATCACCCCG-TTGGGGTCTCTCGAA--TGTTGCTAAG-ACGTAACAAAACCCCGGCACG
FELAET        -----TGGCACACCGTTGATCTTCGTCCTTGATAACCCTG-TTAGGGTCTCTTGGA--TGTTGCTTTG-ACGTAACAAAACCCCGGCACT
FELECH        -----TGGCGCACCGTCGATGTGCGTTTTTGATCACCCTG-TTRGGGTCTCTTGAA--CGTTGCTATG-ACGTAACAAAACCCCGGCACG
FELULI        CTCCCTGGGATGCTGTCAAAGTGCGTTATTGGTCACC-TCATTGGGGTGTCTCG--TGCGTTCCTTTG-ACTTAACAAAACCCCGGCACG
FELCLA        CTCCCCGGGATGCTGTCAAAGTGCGTTATTGGTCACC-TCATTGGGGTGTCTCG--TGCGTTCCTTTG-ACTTAACAAAACCCCGGCACG
BACSPP        -----TGGCACACCGTCGATGTGCGTCCCTGAGGACCCG--TTGGGGCCTCTTGGT--CGTTGCTTTG-ACGTAACAAAACCCCGGCACG
OLLARB        -----GGCCACGCTGTCGATGTGCGTTCTTGATGACC-TC-TCGGGGCCTCTTGGA--CGTCGCGTCG-GCATAACAAAACCCCGGCACG
OLEPHY        -----TGGCACACCGTCGATGTGCGTCCTTGATGACCC---TTTGGGCTTCTTGGT--CGCTTTTTCG-ACGTAACAAAACCCCGGCACG
CHIDIF        -----TGGCACGCTGTCGATTTGCGTTCATGATGACC-TC-TCGGGGAAAGATGGA--YGTCGCATCG-GCATAACAAAACCCCGGCACG
ASTVAH        -----TGGCACACCGTTGATGTGATGCC-TATTGACCC---TTTGGGTCT-TTGGT--TGTTGCACTG--CGTAACAAAACCCCGGCACG

 [                  190        200        210        220        230        240        250        260        270]
 [                  .          .          .          .          .          .          .          .          .]
                                  ***
COMSPU        AGTTGTGCCAAGGAAAAATTAA-C-TAAAGAACGGC-TCGTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAATGTCGCTT-TTT
COMRUG        AGTTGTGCCAAGGAAAAATTAA-C-TAAAAAACGGC-TCGTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAATGTCGCTT-TTT
COMROB        AGTTGTGCCAAGGAAAAATTAA-C-TAAAAAACGGC-TCGTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAATGTCGCTT-TTT
COMROT        AGTTGTGCCAAGGAAAAATTAA-C-TAAAGAACGGC-TCGTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAATGTCGCTT-TTT
MELINT        AGTTGTGCCAAGGAAAAATTAA-C-TAAAGAACGACATCGTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAATGTCGCTT-TTT
FELFRU        GGATGTGCCAAGGAAAAATAAA-C-TGAAGAATGGC-TCTTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GAAATGTGGCTTCTTT
FELAET        GGTTGTGCCAAGGAAAA-TTAAAT-TGAAGAATGGC-TCGTTCCATGATTT-CTCGTTC-GCGGTGAACTCAT-GGATTGTGGCTTCTTT
FELECH        GGATGTGCCAAGGAAAA-ATAAAC-TGAAGAATGGC-TCGTTTCATGATATGCCCGTTC-GCGGTGTGCTCAT-GAAAGGTGGCTTCTTT
FELULI        GAATGTGCCAAGGAAAA-TTAAACA-GAAGGATCGC-TCGTTCTTTGACGC-TCCGTTTTGCGGTGTGCTCTT-TGAACGTGGCTTCTTT
FELCLA        GAATGTGCCAAGGAAAA-TTAAACAT-AAGGATCGC-TCGTTCTTTGACGC-ACCGTTTTGCGGTGTGCTCTT-TGAACGTGGCTTCTTT
BACSPP        GGATGTGCCAAGGAAAT-TTAAAT-TGAAGAATGGC-TCGTTCCATGATGT-CCCGTTC-GCGGTGTTCTCAT-GGAGCGTGGCTTCTTT
OLEARB        GGACGTGCCAAGGAAAA-TTAA-C-TGAAGAATGGC-TCTTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAACGCGGCTTCTTT
OLEPHL        GGATGTGCCAAGGAAAT-TTAAAT-TGAAGAATGGC-TTGTTCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGAGCGTGGCTTCTTT
CHIDIF        GGATGTGCCAAGGAAAG-TTAAACA-GAAGAATGGC-TCGTTCCATGATGT-CCCGTCT-GCGGTGTGCTCAT-GSAACGTGGCTTCTTT
ASTVAH        GGATGTGCCAAGGAAAC-TTAAATTTAAGAATTGCC-TCC-TCCATGATGT-CCCGTTC-GCGGTGTGCTCAT-GGGGCGTCGCTTCTTT
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[                  280        290        300        310        320        330        340        350        360]
[                  .          .          .          .          .          .          .          .          .]
                ITS1][5.8S

COMSPU      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
COMRUG      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
COMROB      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
COMROT      GTAATCASAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGRTGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
MELINT      GTAACCACAAACGACTCTCGGCAACGGATATCTCGGCTCASGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
FELFRU      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
FELAET      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
FELECH      ?TAATCACAAACGACTCTCGGCAACAGATATCTCGGCTCACGCATCGATGAA?AACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
FELULI      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
FELCLA      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
BACSPP      GTAATCACAAACGACTCTCGGCAACGGATATSTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
OLEARB      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
OLEPHL      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
CHIDIF      GTAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA
ASTVAH      ATAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGA

[                  370        380        390        400        410        420        430        440        450]
[                  .          .          .          .          .          .          .          .          .]
                                                                                    5.8S]      [ITS2

COMSPU      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
COMRUG      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACGGCATCGCGTC
COMROB      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCRCGTC
COMROT      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTT
MELINT      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCCAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
FELFRU      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCTGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
FELAET      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
FELECH      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGGCCGAAGCCATTCGGCTGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
FELULIG     ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGCCTGCCTGGGCGTCACG-CATCTCGTC
FELCLA      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGCCTGCCTGGGCGTCACG-CATCTCGTC
BACSPP      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCTGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
OLEARB      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCTTTTGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCTCGTC
OLEPHL      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
CHIDIF      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCTTTTGGCCGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
ASTVAH      ATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCTGAGGGCACGTCTGCCTGGGCGTCACG-CATCGCGTC
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[                460       470       480       490       500       510       520       530       540]
[                .         .         .         .         .         .         .         .         .]
                 **************
COMSPU      GCCCCCACCCAG-CCCTCTTTT-GGGATGCTTGGTT-GGGAGCGGATATTGGTCTCCCGTTCTTCATTGAGCGGTTGGCCAAAACGAGAG
COMRUG      GCCCCCACCCAG-CCCTCCTTT-GGGATGCCTGGTT-GGGAGCGGATATTGGTCTCCCGTTCTTCATTGAGCGGTTGGCCAAAACGAGAG
COMROB      GCCCCCACCCAG-CCCTCCTTT-GGGATGCCTGGTT-GGGAGCGGATATTGGTCTCCCGTTCTTCATTGAGCGGTTGGCCAAAACGAGAG
COMROB      GCCCCCACCCAG-CCCTCCTTT-GGG-TGCCTGGTT-GGGAGCGGATATTGGTCTCCCGTTCTTCATTGAGCGGTTGGCCAAAACGAGAG
MELINT      GCCCCCACCTAG-CCCTCCTTT-GGGATGCCTGGTT-GGGAGCGGATATTGGTCTCCCGTTCCTCATTGAGCGGTTGGCAAAAATAAGAG
FELFRU      GCCCCCACCTT--TCCTTCTTT-GCGAAGACTGGTT-GGGAGCGGATATTGGTCTCCCGTTCCTCACCGAGCGGTTGGCCAAAATAAGAG
FELAET      GCCCCCACCATG-CCATCCTTT-G-GATGATCGGTT-GGGGGCGGATATTGGTCTCCCGTTCCTCACCGAGTGGTTGGCCGAAATAAGAG
FELECH      GCCCCCACCAA--TCCTTCTTTTGCGAAGACTGGTT?GGGAGCGGATATTGGTCTCCCGTTCCTCACCGAGCGGTTGGCCAAAATAAGAG
FELULI      GCCCCCACCAT-CTTTTCCTCT-GGGAAGATTGGTT-TGGAGCGGATATTGGTCTCCCGTTCCTCACCGAGCGGTTGGCCGAAATAAGAG
FELCLA      GACCCCTCCAT-CTTATCCTCT-GGGAAGATTGGTT-GGGGGCGGATATTGGTCTCCCGTTCCTCACCGAGCGGTTGGCCGAAATAAGAG
BACSPP      GCTCCCACCAA--CCCTTCCTTCGGGATACTTGGCT-GGGGGCGGATACTGGTCTCCCGTTCCTCAACGAGCGGTTGGCCAAAATAAGAG
OLEARB      GCCCCCACCATG-CCTCCCTTT-GGGATGCTTGGCTAGGGGGCGGATACTGGTCTCCCGTTCCTCACCGAGCGGTTGGCCAAAATAAGAG
OLEPHL      GCTCCCACCAT--TCCTTCTTTCGGGATGCTTGGTT-GGGGGCGGATACTGGTCTCCCGTTCCTCATCGGGCGGTTGGCCAAAATAAGAG
CHIDIF      GCCCCCACCATG-CCTTCCTTTTGGAATGCTTGGC--GGGGGCGGATACTGGTCTCCCGTTCCTAACCGAGCGGTTGACCTAAATATGAG
ASTVAH      GCTCCCACCTAA-CACTCCTTTTGGGATGATTGGTT-TGGGGCGGATATTGGTCTCCCGTTTTTCACGGAGTGGTTGGCCAAAATAAGAG

[                550       560       570       580       590       600       610       620       630]
[                .         .         .         .         .         .         .         .         .]

COMSPU      TCCCCTTCGACGGACACACGACTAGTGGTGGTTGATAAAACCCTGAATTGC-GTCGCGTGTCTCGTCGCAAGGGTGTATCTTAATAGACC
COMRUG      TCCCCTTCGACGGACACACGACTAGTGGTGGTTGATAAAACCCTGAATTGC-GTCGCGTGTCTCGTCGCAAGGGTGTATCTTAATAGACC
COMROB      TCCCCTTCGACGGACACACGACTAGTGGTGGTTGATAAAACCCTGAATTGC-GTCGCGTGTCTCGTCGCAAGGGTGTATCTTAATAGACC
COMROT      TCCCCTTCGACGGACACACGACWAGTGGTGGTTGATAAAACCCTGAATTGC?GTCGCGTGTCTCGTCGCAAGGGTGTATCTTAGTAGACC
MELINT      TCCCCTTCGACGGACACACGACTAGTGGTGGTTGATAAAACCCTGAATCGC-GTCGCGTGTCTCGTTGCAAGGGTGTATCTTAATAGACC
FELFRU      TCCCTTTCGACGGACGCACGACTAGTGGTGGTTGACAAAACCCTGAATCGT-GCCGTGTGTCTCGTCGCAAGGGTGTATCTTAATAGACC
FELAET      TCCCCTTTGACGGACGCACGACTAGTGGTGGTTGATAAAACCCTGAATCGC-GTCGTGTGTCTCGTC-CAAGTGTGTGTCTTAATAGACC
FELECH      TCCCTTTTGACGGACGCACGACTAGTGGTGGTTGACAAAACCTTGAATCGT-GTCGTGTGTCTTGT?GCAAGGGTGTACCTTAA?AGACC
FELULI      TCCTCTTAGACGGACGCACGACTTGTGGTGGTTGACAAAACCTTGAATCGT-GTCGTGTGTCTTGT?GCAAGGGTGTACCTTAA?AGACC
FELCLA      TCCCCTTAGACGGACGCACGACTTGTGGTGGTTGACAAAACCGTGAATCGC-GTCGTGTGTCTTGTCTCAAGGGATTATCTTAACAGACC
BACSPP      TTCCTTTTGACGGACGCACGACTAGTGGTGGTTGACAAAACCCGGAATCGT-GACGTGTGTCTCGTCAAAAGGGTGCATCTTAATAGACC
OLEARB      TCCCCTTCGACGGACGCACGACTAGTGGTGGTTGACAAAACCCAGAATCGG-GTCGCGTGTCTTGTCGCAAGGGTGTATCTTAATAGACC
OLEPHL      TCTTCTTTGACGGACGCACGACTAGTGGTGGTTGACAAAACC-GGAATCGT-GTCGTGTGTCTTGAATAAAGGGTGCATCTTAATAGACC
CHIDIF      TCTCCTTCGACGGAAGCACGACTAGTGGTGGTTGACAAAACCCAGATTCGA-GTCGTGTGTCTCGTCGCAAGGGTTTGTCTCAACAGACC
ASTVAH      TCCCTTTTGACAGGAACACGACTAGTGGTGGTTGACAAAACCCAGAATTTT-GTTGTGTGTCTTGCCAAGATGGTGCATCTTAATAGACC
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[                    640        650        660        670        680        690        700        710        720]
[                    .          .          .          .          .          .          .          .          .]
                                           ITS2]

COMSPU        C-AATGTGTCGTCTTGTAACGACACTTCGACCG        01000010l?011010100011111000100010000
COMRUG        C-AATGTGTCGTCTTGTAACGACACTTCGACCG        01000010l?011010100011111000010001000
COMROB        C-AATGTGTCGTCTTGTAACGACACTTCGACCG        01000010l?011010100011111000100010000
COMROT        C-AATGTGTCGTCTTGTAACGACACTTCGACCG        01000010l?011010100011111010101010000
MELINT        C-AACGTGTCGTCTTGTAACGACACTTCGACCG        01000010l?011010000001111000101010000
FELFRU        C-AACGTGTCGTCTTGCGACGGCGCTTCGACCG        01000010l?011010100011110100010001000
FELAET        C-AATGTGTCGTTTTTT-ATGACACTTCGACCG        01000001l?011010110011110110010011001
FELECH        ??????????????????????????????????      01000010l?011010110010101000?000?00?
FELULI        C-AACGTGTC--CTTAC-ACGACACTTCGACCG        1?0000101?1?010011101100100010001101
FELCLA        C-AGCGTGTC--CTTAC-ACGACACTTCGACCG        1?0000101?1?010011011100100010001101
BACSPP        C-AATGCGTTGTCATGTAACGACGCTTCGACCG        010010100000101011001110100010001000
OLEARB        CCAACGTGTCGTCTTGTAACRAC?CTTCTWTYK        010001?000001010110011101000000000000
OLEPHL        C-AAGGCGTTGTCATATGACGATGCTTCGACCG        010010100000101011001110100011001000
CHIDIF        CCAATGTGTCGTCTTGTAACGACGCTTCGACCG        010001?000001010111011101001100000000
ASTVAH        C-AATGCGTTGTC-----ATGACGCTTCGACCG        011101?001001011?1001110100010001010?
```

Appendix 2: Aligned *trn*L intron and *trn*L-F intergenic spacer matrix of *Elaphoglossum*

RUMADI = *Rumohra adiantiformis*, ELADIM = *Elaphoglossum dimorphum*, ELANER = *E. nervosum*, MICFUR = *Microstaphlya furcata*, ELASEM = *E. semicylindricum*, ELADEC = *E. deckenii*, ELASUB = *E. subcinnamomeum*, ELAAUB = *E. aubertii*, ELASPA = *E. spathulatum*, ELAANG = *E. angulatum*, ELALAS = *E. lastii*, ELACON = *E. conforme*.

Regions of ambiguous alignment = * (bps: 705-730)

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[                  10        20        30        40        50        60        70        80        90]
[                  .         .         .         .         .         .         .         .         .]

           [trnL intron (partial)
RUMADI     AAA-----TCTTGTATTACTCAGATAAATTTCGGGCGATGAGGCGAGATAGGTACAGAGACTCGATGGGGGCCATTCCAACGAACGAG--
ELADIM     AAATCTTGTCTTGTATTACTCAAATAAATTTTGG-CAATGAGGCGAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELANER     AAATCTTGTCTTGTATTACTCAAATAAATTTTGG-CAATGAGGCGAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
MICFUR     AAATCTTGTCTTGTATTACTCAAATAAATTTTGG-CAATGAGGCGAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELASEM     AAATCTTGTCTTGTATTACTAAAATAAATTTTGG-CAATGAGGCGAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELADEC     ??????????????????????????????????????????????????GAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELASUB     AAATCTTGTCTTGTATTACTCAAATAAATTTTGG-CCATGAGGCGAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELAAUB     AAATCTTGTCTTGTATTAGTCAAATAAATTTTGG-CAATTAGACAAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELASPA     AAATCTTGTCTTGTATTACTCAAAGAAATTTTGG-CAATTAGGCAAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELAANG     AAATCTTGTCTTGTATTACTCAAATAAATTTTGG-CAATGAGGCAAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELALAS     AAATCTTGTCGTGTATTACTCAAATAAATTTTGG-CAATGAGGCAAAATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
ELACON     AAATCTTGTCTTGTATTACTCAAATAAATTTTGG-CAATGAGGCAAGATAGGTACAGAGGCTCGATGGGGGCCATTCCAACGAGCAATAT
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[                   100        110        120        130        140        150        160        170        180]
[                   .          .          .          .          .          .          .          .          .]

RUMADI      ----------------------CAATTTGTT-----------------------AGTTACTAGTTCTAGAGAGAACTGAATATAGAAC
ELADIM      GTTAGTCATAT--------TTAGTTATTAATT-----------------------AGTTAATAGTTCTAGAAAGAGCTGAATATCGAAC
ELANER      GTTAGTCATAT--------TTAGTTATTAATT-----------------------AGTTAATAGTTCTAGAAAGAGCTGAATATCGAAC
MICFUR      GTTAGTCATATTTAGTTATTTAGTTATTAATT-----------------------AGTTAATAGTTCTAGAAAGAGCTGAATATCGAAC
ELASEM      GTTAGTCATAT--------TTAGTTATTAATT-----------------------AGTTAATAGTTCTAGAAAGAGCTGAATATCAAAC
ELADEC      GTTAGTCATAT--------TTAGTTATAAATT-----------------------AGTTAATAGTTCTAGAAAGAGCTGAATATCAAAC
ELASUB      GTTAGTCATAT--------TTAGTTATTAATT-----------------------AGTTAATAGTTCTAGAAAGAACTGAATATCGAAC
ELAAUB      GTTAGTCATAT------------TTATTAATT-----------------------AGTTGATAGTTCTAAAAAGAACTGAATATCGAAC
ELASPA      GTTAGTCATAA------------TTATTAATT-----------------------AGTTAATAGTTCTGAAAAGAACTGAATATCGAAC
ELAANG      GTTAGTCATAT------------TTATTAATTATATGTTAGTCATATTTATTAATT----AATAGTTCTGGAAAGAACTGAATATAGAAC
ELALAS      GTTAGTCATAT-----------------------------------TTATTAATT----AATAGTTCTGGAAAGAACTGAATATATAAC
ELACON      GTTAGTCATAT-----------------------------------TTATTAATT----AATAGTTCTGGAAAGAACTGAATATAGAAC


[                   190        200        210        220        230        240        250        260        270]
[                   .          .          .          .          .          .          .          .          .]

RUMADI      TGTTTTGTTTGGTTAACCGCATGAGGTATAATGTATAAGAGCGAGACCTAGTAACGAAATAAAATTTCACTTTT---------AAGTTG-
ELADIM      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAATATTGATGCTTATTGAAAAGAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
ELANER      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAATATTGATGCTTATTGAAAAGAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
MICFUR      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAATATTGATGCTTATTGAAAAGAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
ELASEM      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAATATTGATGCTTATTGAAAAAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
ELADEC      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAATATTGATGTTTATTGAAAAAAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
ELASUB      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAATATTGATGCTTATTGAAAAAAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
ELAAUB      TGTTTTGTTTGGTTAATTGCATGAAGTATAATCTATAAGATTGAGGCTTATTGAAGAAAGTAAATTTTGTCTTTCCTCGTTTGAAGTTGG
ELASPA      TGTTTTGTTTGGTTAATTGCATGAAATATAATCTATAAGATTTAGGCTTATTGAAGAAAGTAGGGTTTGTCTTTCCTCGTTT--------
ELAANG      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAAGATTGAGGCTTATTGAAGAAAGTAAATTTTGTCTTTCCTCGTTTGAAGTTGG
ELALAS      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAAGATTGAGGCTTATTGAAGAAAGTAGATTTTGTCTTTCCTCGTTTGAAGTTGG
ELACON      TGTTTTGCTTGGTTAATTGCATGAAGTATAATCTATAAGATTGAGGCTTATTGAAGAAAGTAAATTTTGTCTTTCCTCGTTTGAAGTTGG
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[                  280        290        300        310        320        330        340        350        360]
[                  .          .          .          .          .          .          .          .          .]

RUMADI     ----------------------------------------------------TTTTATTA----CTT------AGTGGTGAAACACTA
ELADIM     GACCAGACCCCTCGGTTTGAGGCCAGCATTCAATTACAAAAGCAGGACAGTCTCTTTTATTA-------------CGTCGTAAAGCATTA
ELANER     GACCAGACCCCTCGGTTTGAGGCCAGCATTCAATTACAAAAGCAGGACAGTCTCTTTTATTA-------------CGTCGTAAAGCATTA
MICFUR     GACCAGACCCCTCGGTTTGAGGCCAGCATTCAATTACAAAAGCAGGACAGTCTCTTTTATTA-------------CGTCGTAAAGCATTA
ELASEM     GACTAGACCCCTCGATTTGAGGCCAGCATTCAATTACAAAAGCAGGACAGTCTCTTTTATTA-------------CGTCGTAAAGCATTA
ELADEC     GACCAGACCCCTCGATTTGAGGCCAGCATTCAATTACAAAAGCAGGACAGTCTCTTTTATTA-------------CGTCGTAAAGCATTA
ELASUB     GACCAGACCCCTCGGTTTGAGGCCAGCATTCAATTACAAAAGCAGGACAGTCTCTTTTATTA-------------CGTCGTAAAGCATTA
ELAAUB     GACCAGACCCCTCGGTTTGAGGCCAGCATTAAGTTACAAAATCAGGACAATCTCTTTTATTA-------------CGTAGTAAAGCATTA
ELASPA     ------------------GAGGCCAGCATTAAGTTACAAAATCAGGACAATCTCTTTTATTA-------------CGTAGTAAAGCATTA
ELAANG     GACCAGACCCCTCGCTTTGAGGCCAGCATTCAGTCACAAAATCAGGACAATCTCTTTTATTACTCTCTTCTATTACGTAGTAAAGCATTA
ELALAS     GATCAGACCCCTCGGTTTGAGGACAGCATTCAGCCACAAAATTGGGACAATCTCTTTTATTACTCTCTTTTATTACGTAGTAAAGCATTA
ELACON     GACCAGACCCCTCGGTTTGAGGCCAGCATTCAGTCACAAAATCAGGACAATCTCTTTTATTACTCTCTTCTATTACGTAGTAAAGCATTA
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[                  370        380        390        400        410        420        430        440        450]
[                  .          .          .          .          .          .          .          .          .]

RUMADI     GGTAGACCCTCTCTACTAC-AGCTAATATACATACTTCTATCTTATTTGTTGTGGGATCCCAATTCATTTTGGCGAAAGCTGTTAAACAA
ELADIM     GATAGACTCTTTCTACTAC-TGCTAATCTACATATTTATATCTTAGATATTGTAGGATCTCACTTAATTTAGACAAAAACGCTTAA----
ELANER     GATAGACTCTTTCTACTAC-TGCTAATCTACATATTTATATCTTAGATATTGTAGGATCTCACTTAATTTAGACAAAAACGCTTAA----
MICFUR     GATAGACTCTTTCTACTAC-TGCTAATCTACATATTTATATCTTAGATATTGTAGGATCTCACTTAATTTAGACAAAAACGCTTAA----
ELASEM     GATAGACTCTTTCTACTGC-TGCTAATCTACATATTTATATCTTAGATATTGTAGGATCTCACTCAATTTCGACAAAAACGATTAA----
ELADEC     GATAGACTCTTTCTACTAC-TGCTAATCTACATATTTATATCTTAGATATTGTAGGATCTCACTTAATTTCGACAAAAACGATTAA----
ELASUB     GATAGACTCTTTCTACTAC-TGCTAATCTACATATTTATATATTAGATATTGTAGGATCTCACTTAATTGTGACAAAAACGCTTAA----
ELAAUB     GATAGACTCATTCTACTAC-TGCTAATCTACATATTTCTATCTTATATATTGTAGTATCTCACTTAATTTTGACAAAAACGCTTAACTAA
ELASPA     GATAGACTCTTTCTACTAC-TGTTAATCTACATATTTCTATCTTATATATTGTATGATCTCACTTAATTTCGACAAAAACGCTTAATTAA
ELAANG     GATAGACTCTCTCTACTACATGCTAATCTACATATTTCTATCTTAGATATTGTAGGATCTCAGTTAATTTCGACAAAAACGCTTAACTAA
ELALAS     GATAGACTCTCTCTACTAC-CGCTAATCTACATATTTCTATCTTAGATATTGTAGGATCTTACTTAATTTTGACAAAAACGCTTAACTAA
ELACON     GATAGACTCTCTCTACTACATGCTAATCTACATATTTCTATCTTAGATATTGTAGGATCTCACTTAATTTCGACAAAAACGCTTAACTAA
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[                     460       470       480       490       500       510       520       530       540]
[                       .         .         .         .         .         .         .         .         .]

RUMADI     GCGAGCCAGTAACGAAAAACAAAACTCTCGCGAATGGAGGTAGAGTCCCATTCCACGCACTTAATAAAAGTGAAGAA----TAGCGGCGC
ELADIM     GCGAGTCGGTAACAAAAAGTAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTAATAGAAGTAAAGAAGAAATAGCGGCGC
ELANER     GCGAGTCGGTAACAAAAAGTAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTAATAGAAGTAAAGAAGAAATAGCGGCGC
MICFUR     GCGAGTCGGTAACAAAAAGTAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTAATAGAAGTAAAGAAGAAATAGCGGCGC
ELASEM     GCGAGTCGGTAACAAAAAGCGATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTGATAGAAGTAAAGAAAAAATAGCGGCGC
ELADEC     GCGAGTCGGTAACAAAAAGCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTGATAGAAGTAAAGAAGAAATAGCGGCGC
ELASUB     GCGAGTCGGTAACAAAAAGCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTAATAGAAGTAAAGAAGAAATAGCGGCGC
ELAAUB     GCGAGTCGGTAACAAAACTCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTGATAGAAGTAAAGAAGAAATAGCGGCGC
ELASPA     GCGAGTCGGTAACAAAAAGCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTAATAGAAGTAAAGAAGAAATAGCGGCGC
ELAANG     GCGAGTCGGTAACAAAAAGCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACAGACTTAATAGAAGTAAATAAAAAATAGCGGCGC
ELALAS     GTGAGTCGGTAACAAAAAGCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACGGACTTAATAGAAGTAAAGAAAAAATAGCGGCGC
ELACON     GCGAGTCGGTAACAAAAAGCAATACTCTCGTGAATGGAGGTAGAGTCCTGCTCCACAGACTTAATAGAAGTAAATAAAAAATAGCGGCGC
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[                     550       560       570       580       590       600       610       620       630]
[                       .         .         .         .         .         .         .         .         .]
              trnL intron   ][               trnL-F exon                      ][trnL-F intergenic spacer

RUMADI     AAGTT-GCAGTAGAACGAAAATCCGTTGGTTTCACACAGAACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----AACTAT-----
ELADIM     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAC
ELANER     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAC
MICFUR     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAC
ELASEM     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAC
ELADEC     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAATGA-----GGCCATATTAC
ELASUB     AAGTCCACAGTAGAACGAAAATCCGTTG-TTTCACG--AGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAC
ELAAUB     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAAGGAAAGGGGGCCATATTAC
ELASPA     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAAGGA-----GGCCATATTAC
ELAANG     AAGTC-AAAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAC
ELALAS     AAGTC-ACAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGCCATATTAA
ELACON     AAGTC-AAAGTAGAACGAAAATCCGTTGGTTTCACA--GGACCGTGAGGGTTCAAGTCCCTCTATCCCCAACGA-----GGSTATATTAC
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[                640       650       660       670       680       690       700       710       720]
[                .         .         .         .         .         .         .         .         .]
                                                                                 ****************
RUMADI      -TTAGTTAACTCATTTCAGGTTGTTTGGATCCGCATATTTTGTTCGGAA-GTGAAATACGTAAACCACTCTAATTCCTTTTT-GTCTGGT
ELADIM      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACTAAATACTTAAACCATCCTAAA----TTCT-GTTTGAT
ELANER      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACTAAATACTTAAACCATCCTAAA----TTCT-GTTTGAT
MICFUR      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACTAAATACTTAAACCATCCTAAA----TTCT-GTTGGAT
ELASEM      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACAAAATACCTAAACCATCCTAAA----TTCT-GTTTGAT
ELADEC      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACAAAATACCTAAACCATCCTAAA----TTCT-GTTTGAT
ELASUB      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACAAAATAACTAAACCATCCTAAA----TTCT-GTTTGAT
ELAAUB      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACAAAATGTGTAAACCACCCTAAA----TTCT-GTTTAGT
ELASPA      ATCAGTTAACCTATTTTAGGCTGTTTGAATCCGCAGAATTTGGTCGGTTTACAAAATGTGTAAACCACCCTAAA----TTCT-GTTTAGT
ELAANG      ATTAGTTAACCTATTTTAGGCTGTTTGAATCCGCATAATTTGGTCGGTTTACAAAATATGTAAACCACCCTAAAATAGCTTT-GTCTGGT
ELALAS      ATCAGTTAACCTATTTTAGGCTATTTGAATCCGCATAATTTGGTCGGTTTACAAAATATGTAAACCACCCTAAATTATGTTT-GTCTGGT
ELACON      ATTAGTTAACCTATTTTAGGCTGTTTGAATCCGCATAATTTGGTCGGTTTACAAAATATGTAAACCACCCTAAAATAGCTTT-GTCTGGT
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[                730       740       750       760       770       780       790       800       810]
[                .         .         .         .         .         .         .         .         .]
                 **********
RUMADI      CTTTCATA--AAGAGTTTGCAAGTGAGCCATTCAGGCCTT-TAATATGCATGAATGGCTCAATCTGGCCCTCCCCCGTCCATACTTTACT
ELADIM      TTTTTTTTT-AATAGTTTGTAAGTGAGCCATTCATACCTT-TCATGTGCATGAATGGCTCAATCTATAGCCCC-TAGCCTATACTTTAGT
ELANER      TTTTTTTTT-AATAGTTTGTAAGTGAGCCATTCATACCTT-TCATGTGCATGAATGGCTCAATCTATAGCCCC-TAGCCTATACTTTAGT
MICFUR      TTTTTTTTTTAATAGTTTGTAAGTGAGCCATTCATACCTT-TCATGTGCATGAATGGCTCAATCTATAGCCCC-TAGCCTATACTTTAGT
ELASEM      TCTTTTTT--AATAGTTTGTAAGTGAGCCATTCATACCTT-TAATGTGCATGAATGGCTCAATCTATAACCCC-TAGCCTATACTTTAGT
ELADEC      TTTTTTTTT-AATACTTTGTAAGTGAGCCATTCATACCTT-TAATGTGCATGAATGGCTCAATCTATAACCCC-TAGCCTATACTTTAGT
ELASUB      TTTTTTTTT-TAAAGTTTGTAAGTAAGCCATTCATACCAT-TAATGTGCATGAATGGCTCAATCTATAGCCCC-TAGCTTATACTTTAGT
ELAAUB      TTCTTAAA--AATAGTTTGTAAGTGAGCCATTCATACCTT-TAATGTGCATGAATGGCTCAATCTAGAGCCCCACCCCCTATACTTTAGT
ELASPA      TTTTTAAA--ACTATTTTGTAAGTGAGCCATTCATACCTT-TAATGTGCATGAATGGCTCAATCTAGAGCCCCACCCCCTA-----TAGT
ELAANG      CTTTTAAA--AATAGTTTGTAAGTGAGCCATTCATACCTTCTAATGTGCATGAATGGCTCCATCTAGAGACCC--CCCCCATACTTTAGC
ELALAS      TTTTTAA---AATAGTCTGTAAGTAAGCCATTCATACCTTCTAATGTGCATGAATGGCTCCATCTAGAGCCCC--CCCCTATACTTTAGT
ELACON      CTTTTAAA--AATAGTTTGTAAGTGAGCCATTCATACCTTCTAATGTGCATGAATGGCTCCATCTAGAGACCC-CCCCCCATACTTTAGT
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[                  820        830        840        850        860        870        880        890        900]
[                  .          .          .          .          .          .          .          .          .]

RUMADI      TTATTTACTACAAGCGATACTTTATTAGACATATCTGTAAAAGCGAGATCAACGGTTTGACTAGGTTAAAAAATTA-------CAGTTGA
ELADIM      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAGAGTCAGATTAGCGG-----------TAAAAAAATA--GACTAAAGTTGA
ELANER      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAGAGTCAGATTAGCGG-----------TAAAAAAATA--GACTAAAGTTGA
MICFUR      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAGAGTCAGATTAGCGG-----------TAAAAAAATA--GACTAAAGTTGA
ELASEM      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAGAGTCAGATTAGCGG-----------TAAAAAAATA--GACTAAAGTTGA
ELADEC      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAGAGTCAGATTAGCGG-----------TAAAAAAATA--GACTAAAGTTGA
ELASUB      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAGAGTCAGATTAGCAG-----------TAAAAAAATA--GACTAAAGTTGA
ELAAUB      CTTTTTA-TAAGAGCAATATTGTATTAAACGTATTTATAAGAGTCAGATTAGCAA-----------TAAAAAAAAAAA---TAAAGTTGA
ELASPA      CTTTTTA-TAAAATCAATATTGTATTAAACGTATTTATAAGAGTCAGATTAGCAA-----------TAAAAAAAAAAAGACTAAAGTTGA
ELAANG      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAAAGTCAGATTAGCGG-----------TAAAAAAAAAAAGACTAAAGTTGA
ELALAS      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAAAATCAGATTAGCGG-----------TAAAAAAGAA----CTAAAATTGA
ELACON      TTTTTTGCTAAAAGCAATATTGTATTAAACATATTTATAAAAGTCAGATTAGCGG-----------TAAAAAAAAAA-GACTAAAGTTGA
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[                  910        920        930        940        950        960        970        980        990]
[                  .          .          .          .          .          .          .          .          .]
                                          trnL intergenic spacer                 ]
RUMADI      AAAATAGACTTAACTGA----TTTCTAGTTAACCCTTATCTGTATCTGTAATTGAGTTGG
ELADIM      AAAATCTACTTAATTAA----TTTCTAGTTAACTTTTATTCGTATTCGTAAATAAGTTAG
ELANER      AAAATCTACTTAATTAA----TTTCTAGTTAACTTTTATTCGTATTCGTAAATAAGTTAG
MICFUR      AAAATCTACTTAATTAA----TTTCTAGTTAACTTTTATTCGTATTCGTAAATAAGTTAG
ELASEM      AAAATCTACTTAATTAA----TTTCTAGTTAACTTTTATTCCT------AAATAAGTTAG
ELADEC      AAAATCTACTTAATTAA----TTTCTAGTTAACTTTTATTCGT------AAATAAGTTAG
ELASUB      AAAATCTACTTAATTAATTAATTTCTAGTTAACTTTTATTCGT------AAATAAGTTAG
ELAAUB      GAGATTTACTTAATTAA----TTTCTAGTTAACTTTTATTCGT------AAATAAGTTAG
ELASPA      AAGATTTACTTAATTAA----TTTCTAGTTAACTTTTATTCGT------AAATAAGTTAG
ELAANG      AAAATCTACTTAATTGA----TTTCTAGTTAACTTTTATTCGT------AAATAAGTTGG
ELALAS      AAAATCTACTTAATTGA----TTTCTAGTTAACTTTTATTCGT------AAATAAGTTGG
ELACON      AAAATCTACTTAATTGA----TTTCTAGTTAACTTTTATTCGT------AAATAAGTTGG
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[                    1000      1010      1020      1030      1040      1050      1060      1070      1080]
[                    .         .         .         .         .         .         .         .         .]
                [          gap matrix                ]
RUMADI          101??0101011101011111000001????10
ELADIM          01010010000??111011001100010100?10
ELANER          01010010000??111011001100010100?10
MICFUR          01000010000??111011001100010100?10
ELASEM          01010010000??111011001100010100?11
ELADEC          ??010010000??111011001100010100?11
ELASUB          01010010000??111001001100010100?01
ELAAUB          010?1010000??110010001000110010011
ELASPA          010?1010010??110011001001110000011
ELAANG          010?10010000000001100?10010000011
ELALAS          010??10100000010011000?10010?01?11
ELACON          010??101000000000110001000100000111
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