Testing the adaptive nature of morphological diversification in the hemiparasitic genus *Thesium* L. (Santalaceae)

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Thesium euphorbioides by GA Verboom

Plagiarism declaration

I know the meaning of plagiarism and declare that all of the work in this thesis, save for that which is properly acknowledged, is my own

Signed: _____ Date: _____

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Abstract

Much attention has been given to the potential role of edaphic heterogeneity in promoting functional divergence and even speciation in the CFR. The ability to tap into the resources of surrounding hosts may enable parasitic plants to overcome deficiencies in the availability of soil nutrients and, as such, parasitism may represent an adaptation to low-nutrient environments. The hemiparasitic genus *Thesium* (Santalaceae) offers an excellent opportunity for studying the potential role of edaphic heterogeneity in promoting divergence in specialized resource acquisition/utilization, and its consequences for speciation, in Cape plants. *Thesium* is a large, widely distributed genus of root hemiparasites in the CFR, occurring on all major substrates, although it seems to favour acidic, sandstone-derived soils. Within the CFR, *Thesium* shows remarkable diversity in growth form, particularly with regard to traits associated with carbon acquisition. This thesis tests the hypothesis that parasitism in *Thesium* represents a specialised foraging strategy for dealing with the nutrient poor soils found in the CFR. Specifically, I set out to test the hypothesis that *Thesium* represents a classic example of a Cape plant radiation with both functional and taxonomic diversification being driven by adaptation to environments of different fertility. The data are presented in two chapters, the first of which deals with the phylogeny of *Thesium* and the second with the functional significance of trait divergence. Results of a dating analysis on a molecular phylogeny comprising of 72 *Thesium* species suggests that the radiation leading to the extant diversity of Cape species occurred approximately 17 Mya. In chapter two, correlative analyses using both raw and phylogenetically independent (PIC) data show that divergence within the Cape clade in the degree of heterotrophy as measured using a series of traits, specifically leafiness, plant height and tissue chlorophyll and N content, reflects adaptation to edaphic environments which vary in terms of their nutritional properties. The fact that the radiation in Cape *Thesium* has been accompanied by morphological shifts, which are consistently associated with shifts to different environments, suggests that diversification in *Thesium* has been adaptive. I therefore propose that edaphic heterogeneity in the CFR has played a significant role in driving the morphological and taxonomic diversification of Cape *Thesium*. Parasitism in *Thesium* may thus represent a specialized foraging strategy for overcoming deficiencies in available nutrients in the nutrient poor environments of the CFR.

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Chapter 1

General Introduction

The Cape Floristic Region (CFR, Figure 1, Goldblatt& Manning 2000) of South Africa boasts a unique flora dominated by high numbers of endemic plant species (68.8%) from relatively few families and genera (Goldblatt & Manning 2002, Linder & Hardy 2004). Many of these plant lineages are considered to have speciated rapidly (Linder & Hardy 2004), with divergence being either adaptive or non-adaptive. Adaptive hypotheses highlight the role of differentiation associated with heterogeneity in water and nutrient availability throughout the region (Linder & Vlok 1991, Linder 2003, Verboom *et al.* 2004) or selection by pollinators (van der Niet & Johnson 2009, Johnson 2010), while non-adaptive hypotheses focus on differentiation in allopatry, typically associated with the varied topography of the region (Linder 2003). Coupled with the adaptive nature of speciation in some Cape lineages, many show significant divergence in morphological traits, including *Rhodocoma* (Restionaceae), *Cliffortia* (Rosaceae), *Leucospermum* (Proteaceae), *Aspalathus* (Fabaceae),*Muraltia* (Polygalaceae) (Rourke 1972, Linder & Vlok 1991) and *Ehrharta* (Verboom *et al.* 2004).

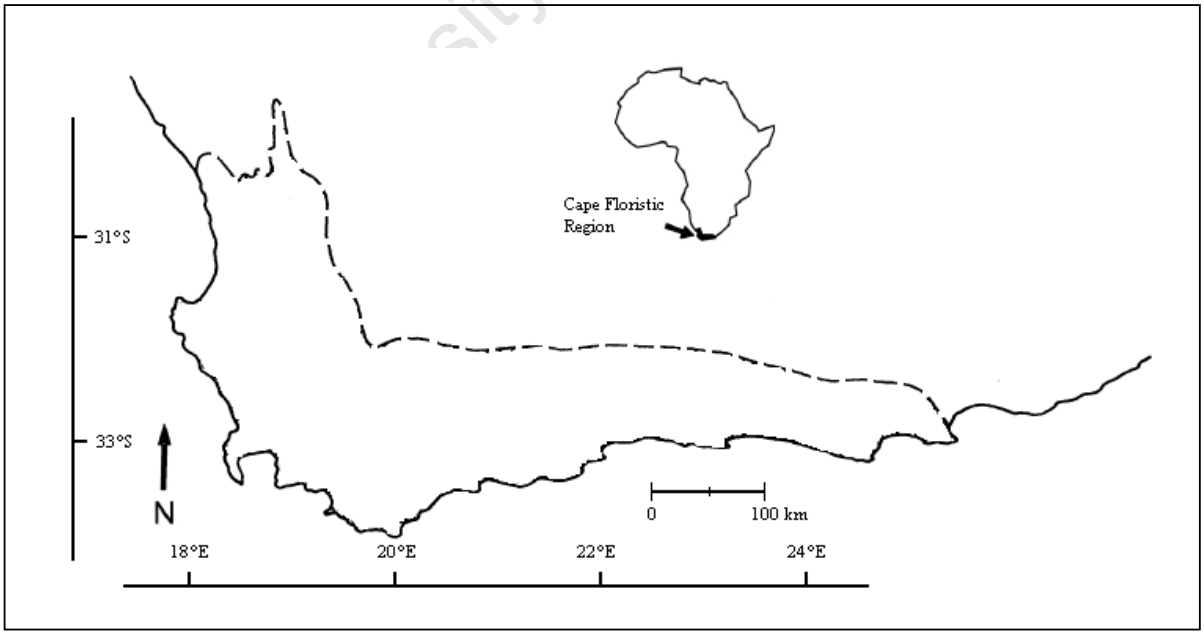


Figure 1. The extent of the Cape Floristic Region in the western part of South Africa (dotted line).

Much attention has been given to the potential role of edaphic heterogeneity in promoting functional divergence and even speciation in the CFR (Linder 1985, Cowling 1990, Cowling & Holmes 1992). This is supported by the presence of numerous substrate-specific endemics in the region (Cowling *et al.* 1992). Richards *et al.* (1997) found a strong association between plant community composition and soils with variable N and P availability, and proposed that differences in plant nutrient demand may be important in determining species' distributions. Verboom *et al.* (2004) demonstrated the role of edaphic adaptation in the radiation of the Cape genus, *Ehrharta*, arguing that substrate heterogeneity played a key role in directing the evolution of alternative growth forms and strategies in the genus. Similarly, morphological diversity between populations of *Argyroderma* was found to be significantly associated with edaphic habitat differences (Ellis *et al.* 2006), leading Ellis and co-workers to suggest that morphological divergence was an adaptive response to divergent selection imposed by variable edaphic environments.

The CFR is characterised by a mosaic of soils of variable fertility, though most of these soils are generally deficient in nitrogen (N, Stock & Lewis 1986) and phosphorus (P, Kruger *et al.* 1983), both of which are considered important determinants of the structure and function of the vegetation (Witkowski & Mitchell 1987, Richards *et al.* 1997, Cramer 2010). The primary determinants of the differences in nutrient status amongst soils are the substrates from which they are derived (McCarthy & Rubidge 2005). The sandstones of the Cape Fold Mountain belt (Table Mountain Group), for example, leach easily to give rise to sandy, low-pH soils that are characteristically deficient in plant available nutrients, particularly P (Lambrechts 1979, Specht & Moll 1983, Allsopp & Stock 1994). In contrast, the shales (Malmesbury, Bokkeveld,and Nama Groups) and granites (Cape Granite Suite and Namaqualand Complex) that dominate the coastal and lowland regions generate soils that generally have a much higher pH and are more fertile than those derived from sandstones(Lambrechts 1979, Specht & Moll 1983).

Terrestrial plants have developed a number of adaptations for overcoming nutrient limitation (Lambers *et al.* 2008), and many of these are expressed in plants native to the CFR (Lamont 1982). Some species, for example, have specialized root structures which enable them to mobilize P from insoluble complexes in soils having low P availability (Lambers *et al.* 2006). Within the CFR, such structures are present in Cyperaceae ('dauciform' roots, Shane *et al.* 2006) and Proteaceae ('proteaceous' roots, Shane & Lambers, 2005). A large number of taxa, for example, species of Ericaceae, form symbiotic associations with mycorrhizal fungi, enabling them to cope with the low level of nutrients available (Allsopp & Stock 1993). Lambers *et al.* (2008) suggest that soil infertility has been one of the strongest influences driving the evolutionary diversity of plants in nutrient poor systems, based on the high diversity of plants with specialised nutritional strategies in areas with the least fertile soils.

Parasitic plants are capable of making direct connections with their neighbouring plants via haustoria, through which they are able to obtain water, nutrients and photosynthate from their hosts (Kuijt 1969; Nickrent *et al.* 1998). Two major groups of parasitic plants occur: holoparasites, which are entirely dependent on host resources, and hemiparasites which are capable of obtaining some of their resources non-parasitically (Press 1989).This thesis addresses plants in the latter category. Both stem- (e.g. *Viscum*, Zuber 2004) and root-tapping (e.g. *Rhinanthus*, Press *et al.* 1988) hemiparasites exist. Because stem parasites, such as mistletoes, attach to their hosts above ground-level, they lack direct access to soil-based resources and consequently depend on their hosts for most of their nutritional requirements (Watson 2009). In contrast, root-tapping hemiparasites have access to both soil- and host-derived nutrients, and are therefore less dependent on their hosts for organic and inorganic nutrient supply. The ability to tap into the resources of surrounding hosts may, however, enable parasitic plants to overcome deficiencies in the availability of soil nutrients (Matthies 1995) and, as such, parasitism may represent an adaptation to low-nutrient environments.

Representatives of at least nine of the 14 families of parasitic plants occur in the CFR, with the majority of these parasitic species being root hemiparasites (Lamont 1982). Despite this relatively rich parasitic flora, little is known about the diversity and biology of parasitic lineages in the CFR. The santalaceous genus *Thesium* offers an excellent opportunity for studying the potential role of edaphic heterogeneity in promoting divergence in specialized resource acquisition/utilization, and its consequences for speciation, in Cape plants. Little is known about the relationships among *Thesium* species, or between the southern African species and the rest of the genus (Hendrych 1972, Der & Nickrent 2008). In addition, not much is known about the origin and diversification of the group. Hendrych (1972) proposed a Southern Hemisphere origin for the genus, with subsequent dispersal and diversification into Africa and Europe. This hypothesis, however, remains to be tested within a phylogenetic framework. Of the approximately 160 South African species, approximately half inhabit the nutrient-poor 'fynbos' environments of the CFR (Table 1, Goldblatt & Manning 2000). *Thesium* is widely distributed in the CFR, occurring on all major substrates, although it seems to favour acidic, sandstone-derived soils. A notable exception is a group of species from the section *Annulata* (after Hill 1915), which tend to favour the more nutrient rich, shale-derived soils of the low-land regions.

Within the CFR, *Thesium* shows remarkable diversity in growth form, particularly with regard to traits associated with carbon (C) acquisition. For example, *Thesium* species range in size, from large-bodied, woody individuals, to small-bodied, often yellow individuals that possess very few leaves. In contrast, floral variation seems more limited, with flowers in all species being minute and ranging in colour from yellow, to cream or white (Hendrych 1972). It has been proposed that parasitic plants of varying degrees of heterotrophy will also differ in terms of their photosynthetic capacities (Press 1989). Specifically, plants that depend on their hosts to meet their C requirements may be expected to show reduced investment in the structures and biochemistry associated with photosynthesis. Within *Thesium*, therefore,

interspecific variation in certain morphological and physiological attributes may reflect underlying variation in the degree to which C is acquired heterotrophically. In particular, it seems likely that leafier and/or more chlorophyll-rich individuals will have higher photosynthetic capacities reflecting a reduction in host dependence. Some capacity for autotrophic resource acquisition may enable hemiparasites to overcome nutritional deficiencies in their hosts (Atsatt 1970), which may only be possible when soil available nutrients are high. In contrast, the small-bodied, less leafy species of *Thesium* probably have lower photosynthetic capacities and are relatively more dependent on their hosts. Although some work has been done on the parasitic biology of *Thesium*, most studies have focussed on single species (Fer *et al.* 1993), or on the relationship between *Thesium* species and their hosts (Suetsugu *et al.* 2008, Dostalek and Munzbergova 2010). Little is known about the functional significance of trait variation in *Thesium*, or whether this trait variation represents adaptation to divergent nutritional environments in the CFR.

Table 1. Number of *Thesium* species from each major geographic region. Number in parentheses indicates the number of species in the CFR.

Region	Number of species
Southern Africa	160 (87)
Tropical Africa	82
Eurasia	79
South America	3
North Africa and Madagascar	13

The evolutionary ecology of parasites has been relatively understudied (Combes 2001, Poulin 2007) and, as a result, the factors of the physical environment that favour a parasitic lifestyle are poorly understood. This thesis tests the hypothesis that parasitism in *Thesium*

represents a specialised foraging strategy for dealing with the nutrient poor soils found in the CFR. Specifically, I set out to test the hypothesis that *Thesium* represents a classic example of a Cape plant radiation with both functional and taxonomic diversification being driven by adaptation to environments of different fertility. The primary data are presented in two chapters, the first of which deals with the phylogeny of *Thesium* and the second with the functional significance of trait divergence. In the first chapter, a phylogenetic hypothesis based on molecular sequence data for a sample of Cape species, as well as species from southern Africa, Tropical Africa and Eurasia, was used to explore the origin of Cape *Thesium* species, specifically testing the hypothesis of Hendrych (1972) that *Thesium* originated in the Southern Hemisphere, with subsequent migration north as well as speciation in southern Africa. This chapter also deals with the interspecific relationships within Cape *Thesium* with regards to the taxonomic groupings identified by Hill (1915).

In the second data chapter, I investigated morphological variation in *Thesium*, particularly in relation to divergence in foraging strategies among species found in the CFR. I evaluated whether divergence in C-acquisition traits is associated with divergence in edaphic environments. I also evaluated the degree of host dependence in *Thesium* species showing divergence in morphological traits. The main hypothesis I tested was that trait divergence in *Thesium* species is functionally linked to divergence in the degree of host dependence in *Thesium* species, and that this divergence represents adaptation to different nutritional environments. I further hypothesised that *Thesium* species are dependent on their hosts for organic C. If parasitism represents an adaptation to low soil nutrient availability, I predicted that the most heterotrophic species of *Thesium*, or species with the lowest photosynthetic capacity, will be associated with the most nutrient impoverished environments. Conversely, species that are less host dependent, owing to their higher photosynthetic capacities, will be associated with environments with higher nutrient availability.

Declaration of student contribution to published manuscript

Chapter 2, “Phylogenetics and biogeography of the parasitic genus *Thesium* L. (Santalaceae), with an emphasis on the Cape of South Africa”, was published in *The Botanical Journal of the Linnaean Society* (162:435-452, accepted January 2010). I carried out all of the data analysis and also wrote the paper. My co-authors, F Forest and GA Verboom provided advice and comments on the manuscript.

Chapter 2

Phylogenetics and biogeography of the parasitic genus *Thesium* L. (Santalaceae), with an emphasis on the Cape of South Africa

Abstract

Thesium is a large genus of parasitic shrubs belonging to tribe Thesieae of Santalaceae. It has a principally Old World distribution, with the greatest diversity being found in southern Africa. Little is known about the relationships within *Thesium* or its relationships with its closest relatives. In this chapter, I present a first estimate of species-level phylogenetic relationships in *Thesium* based on internal transcribed spacer (ITS) and *trnL–trnF* sequence data, and use this to explore the biogeographical history of the group. One hundred and four samples representing 72 *Thesium* species were included in a phylogenetic analysis. Plastid and combined data resolve *Thesium* as paraphyletic relative to *Thesidium* and *Austroamericium* with high posterior probability and bootstrap support. ITS sequence data place *Thesidium* as sister to a large *Thesium* clade, but with weak support. Ancestral range reconstruction and dating analysis suggest a southern African origin for the group, with a crown age of 39.1 ± 11.9 Mya, followed by dispersal into Europe and South America. A large clade of Cape species split in the Miocene from a clade comprising tropical species (25.5 ± 7.3 Mya) with the diversification of extant species beginning at 16.7 ± 6.3 Mya.

Introduction

Thesium L. is a large genus of predominantly perennial root-parasitic shrubs belonging to tribe Thesieae Reichenb. of Santalaceae. Species in the genus are often unattractive, having a yellowish colour and lacking leaves. The flowers are frequently small and creamy-white, and most species are probably pollinated by small bees and flies (Hendrych 1972).*Thesium* has a principally Old World distribution, with the greatest diversity being found in southern Africa. Of the *c*. 300 species of *Thesium* (Mabberley 2008), approximately 150 are native to southern Africa, a further 60 species occur in tropical and northern Africa, and the rest of the genus is primarily distributed in Europe and Asia. Only three species occur in South America, with two species centred in Brazil and a third native to Venezuela. *Thesium* is widely distributed in South Africa, with high densities of species in the Cape (or Cape Floristic Region; Goldblatt & Manning 2000). With 87 currently recognized species native to the CFR, including 35 endemics, *Thesium* is one of the largest genera in the region (Goldblatt & Manning 2002). Despite this diversity, *Thesium* has received little explicit systematic attention, either locally or globally. Besides providing insights into the evolution of *Thesium* itself, a sound systematic study of the genus promises to contribute to a growing body of work that is enhancing our understanding of the patterns and processes that underpin the richness of the Cape flora as a whole (Linder 2003, Galley & Linder 2006, Verboom *et al.* 2009).

For decades, the origin and evolution of the Cape flora has been the subject of some debate (see Levyns 1964, Axelrod & Raven 1978, Galley & Linder 2006,Galley *et al.* 2009). Levyns (1964) hypothesized a tropical African origin for many Cape lineages, with subsequent migration southwards into the Cape. Other authors have suggested a vicariance scenario, in which the Cape flora represents a relic of a previously widespread African flora (Wild 1968, King 1978). Recently, Galley *et al.* (2007) have shown that there is a general trend for unidirectional migration from the Cape into the Drakensberg (in the eastern part of South Africa) and thence northwards into tropical Africa. With strong affinities to other floristic

centres outside of the Cape region, *Thesium* is an ideal candidate for investigating the relationships of the Cape flora with other floras. Hendrych (1972) hypothesized that *Thesium* originated in southern Africa, subsequently migrating north into the rest of Africa and further into Europe and Asia. This hypothesis has not yet been evaluated in a phylogenetic context against the alternatives that: (i) *Thesium* biogeography is the result of vicariance, following isolation caused by climatic fluctuations, or (ii) *Thesium* originated in the north, migrating south with subsequent diversification in southern Africa.

Most systematic work on the genus has been based primarily on the morphological and geographical attributes of the species (Fig 1). Originally described by Linnaeus (1753), *Thesium* received limited attention until the 1800s, early treatments being hampered by poor species sampling and often focusing on particular geographical regions (Hendrych 1972). Throughout the first half of the 19th century, three infrageneric groups were recognized at sectional level, namely *Frisea* Endl., *Thesiosyris* Endl. and *Thesium* R.Br. (= *Euthesium* Benth.). These sections were broadly distinguished by floral morphology, especially flower shape and perianth structure, and geographical distribution, with *Thesium* being predominantly European and *Frisea* and *Thesiosyris* predominantly occurring in southern Africa.

The first comprehensive treatment of the genus was conducted by De Candolle (1857a, b). He based his subgeneric classifications on more material than had been examined by previous authors, examining all 112 species known at the time. Within a single year, De Candolle produced two treatments, first (1857a) dividing *Thesium* into five sections, but soon modifying this scheme and splitting the genus into six sections (Table 1). In his second scheme, De Candolle accepted the separation of the genus *Thesidium* from *Thesium*, as proposed by Sonder (1857). *Thesidium* is a small genus (approximately eight species) of parasitic shrubs endemic to the southern part of Africa, where its range overlaps completely with that of *Thesium*. It differs from *Thesium* in being monoecious and having unisexual,

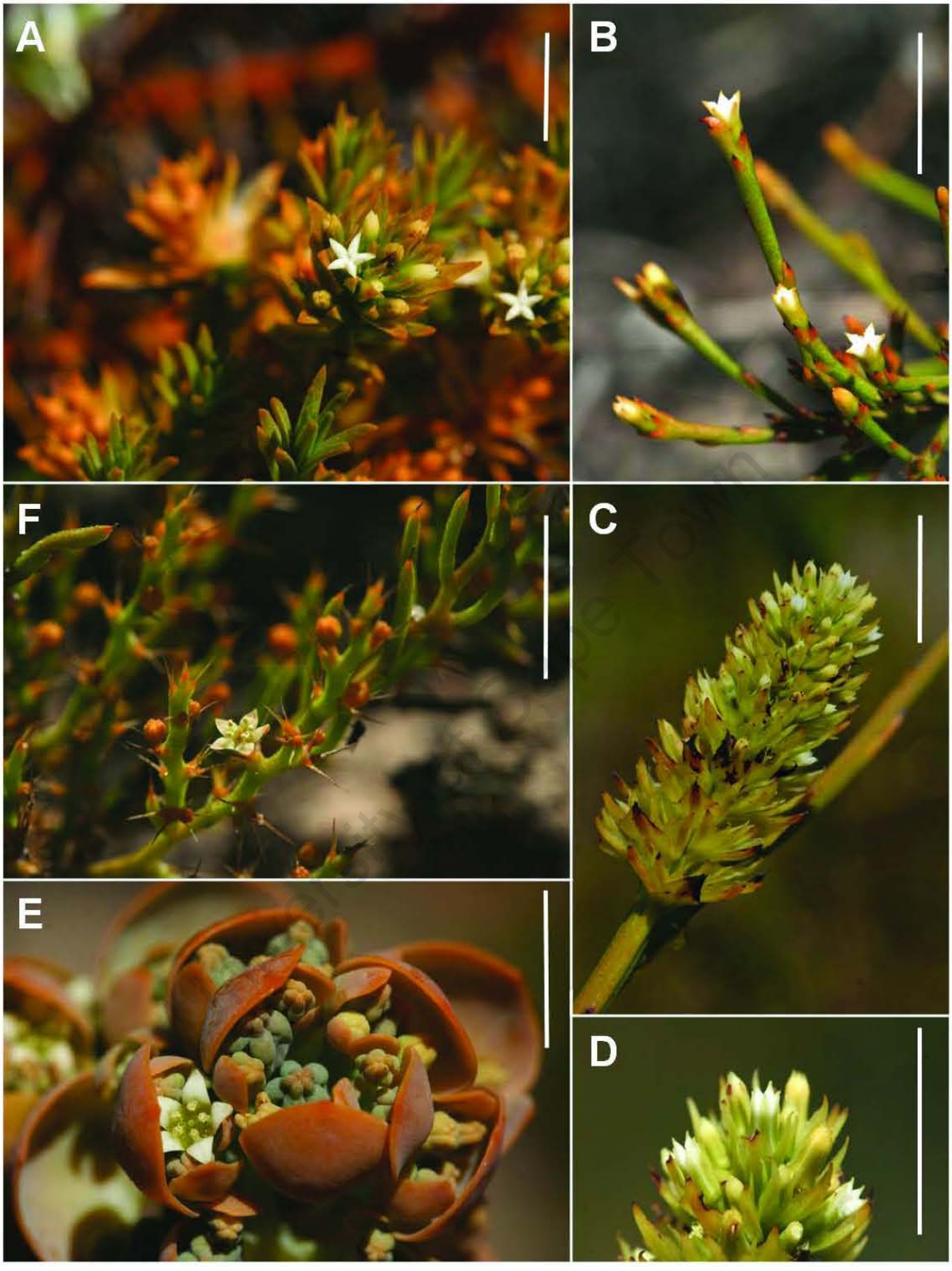


Figure 1. Species representing three of Hill's (1915) infrageneric groupings. Species shown are as follows: A, *Thesium carinatum* (section *Barbata*); B, *T. euphrasioides* (section *Barbata*); C, D, *T. spicatum* (section *Annulata*); E; *T. euphorbioides* (section *Imberbia*); F, *T. spinulosum* (section *Imberbia*). All scale bars, 1 cm. All photographs taken at Baardskeerdersbos,Western Cape, South Africa by GAVerboom.

tetramerous flowers. Recent molecular work (Der & Nickrent, 2008) has identified *Thesidium* as sister to *Thesium*, but limited sampling hampered the ability of these authors to evaluate the monophyly of the two genera. In his treatment of the genus, Hill (1915) created four new sections (Table 1, see also Fig 1). This treatment was, however, based on a limited geographical sample, as Hill focused on species from South Africa, and paid little attention to species found outside southern Africa. As a result of this sampling bias, Hill's treatment was not readily reconcilable with that of De Candolle. It was Pilger (1935) who first attempted to address the mismatch between these two treatments by examining a large number of *Thesium* species from across the distribution of the genus. In his treatment, *Thesium* was split into four sections (Table 1) which broadly agree with De Candolle's and earlier authors' works, but incorporate the groupings that Hill had devised for the South African *Thesium* species. The majority of species surveyed by Hill fall into Pilger's section *Frisea*, whereas some (from section *Imberbia*) fit into *Thesium* [as *Euthesium*] (Table 1).

The most recent comprehensive study of the genus is that of Hendrych (1972), who attempted to provide a broad outline of the group and revise the existing subgeneric Northern Hemisphere classifications. His study was, however, limited because of its focus on Northern Hemisphere species, paying little attention to southern African species. He offered little insight into the origin and evolution of the Cape members of *Thesium* or into the phylogenetic relationships within the genus. In addition, Hendrych focused solely on morphology and geography, not taking molecular data into account. Hendrych's treatment included three subgenera within *Thesium*: *Frisea*, *Thesium* and *Chrysothesium* DC. (Table 1). Hendrych (1963, 1972) also removed the three South American species from *Thesium* to a new genus, *Austroamericium*. This was based on their disjunct distribution relative to the rest of *Thesium* and their divergent morphological attributes, particularly fruit and floral morphology. Phylogenetic data have gained increasing importance in both the delimitation of higher taxa (Schrire & Lewis 1996, De Queiroz 2007) and in tracing the evolutionary histories of lineages, including their biogeographical origins (Avise 1994). In order to

Table 1. Broad level infrageneric classifications of *Thesium*, and geographic locality of each major grouping

De Candolle (1857)	Hill (1915)	Pilger (1935)	Hendrych (1972)	Geographic Locality
Sect. *Frisea*	Sect. *Annulata*	Sect. *Frisea**	Subgen. *Frisea***	Southern Africa
	Sect. *Penicillata*			Southern Africa
	Sect. *Barbata*			Southern Africa
Sect. *Euthesium*	Sect. *Imberbia*	Sect. *Euthesium*	Subgen. *Thesium*	Africa/Eurasia
	-Subsect. *Fimbriata*			
Sect. *Discothesium*	-Subsect. *Subglabra*			Africa/Eurasia
Sect. *Aetheothesium*	-Subsect. *Subglabra*			Africa/Eurasia
Sect. *Chrysothesium*		Sect. *Chrysothesium*	Subgen. *Chrysothesium*	Eurasia
Sect. *Psilothesium*		Sect. *Psilothesium*	Gen. *Austroamericum*	South America
			(Hendrych, 1963)	
Gen. *Thesidium*		Gen. *Thesidium*	Gen. *Thesidium*	Southern Africa
(Sonder,1857)				

* Hill's sections *Annulata*, *Penicillata*, *Barbata* retained as series of Pilger's Section *Frisea*
** Hill's sections *Annulata*, *Penicillata*, *Barbata* retained as sections of Hendrych's Subgenus *Frisea*

enhance the utility and predictive power of higher taxa, taxonomists set out to reclassify taxa that are found to be paraphyletic into monophyletic groupings (Backlund & Bremer 1998).

Phylogenetic studies involving *Thesium* are few, and those that exist generally focus at a higher taxonomic level (e.g. Der & Nickrent 2008) and, as such, sample a limited number of *Thesium* species. As a result, relationships amongst *Thesium* species remain poorly understood (Hendrych 1972), as is the relationship between *Thesium* and its closest relatives. In this chapter, I present a first estimate of species-level phylogenetic relationships in *Thesium* using plastid and nuclear molecular data with a particular focus on Cape species, and use this to explore the taxonomy and biogeographical history of the group, employing Bayesian- and parsimony-based phylogenetic analyses. Specifically, I aim to: (i) evaluate the relationships between *Thesium* and its segregate genera *Thesidium* and *Austroamericium* (*sensu* Hendrych 1963); (ii) assess relationships among *Thesium* species, especially in relation to existing infrageneric classifications; and (iii) evaluate, from a molecular phylogenetic perspective, Hendrych's southern origin hypothesis for the genus *Thesium*.

Materials and Methods

Taxon sampling

One hundred and four accessions (Appendix, Table 2), representing 72 *Thesium* species, were sampled for the phylogenetic analysis. Fifty-seven of the sampled species were from South Africa, with a further 14 species sampled from tropical Africa, Europe and Asia. Nine *Thesium* (seven Eurasian, two tropical African) and two *Austroamericium* species were sampled from herbarium specimens housed at the Royal Botanic Gardens, Kew (K; see Appendix). Three *Thesidium* species were also sampled. *Buckleya lanceolata* (Thesiae), *Exocarpus spartens* (Anthoboleae) and *Leptomeria cunninghamii* (Osyrideae) were sampled as outgroup taxa.

Table 2. Number of species sampled according to geographic area

Region	Number of species	Species Sampled
Southern Africa	160	57
Tropical Africa	82	16
Eurasia	79	7
S. America	3	2
N. Africa and Madagascar	13	0

DNA isolation, amplification and sequencing

Total DNA was extracted from silica-dried, field-collected samples or herbarium material using a modified version of the cetyltrimethylammonium bromide (CTAB) extraction protocol outlined in Doyle & Doyle (1987). Samples were ground using a small amount of fine silica in mortars preheated to 65 °C to facilitate grinding. DNA was precipitated in ethanol (for silica-dried samples) or isopropanol (for herbarium samples) at -20 °C for up to 10 days. Additional purification was performed using a caesium chloride/ethidium bromide gradient (Csiba & Powell 2006) and a dialysis procedure. All DNA samples were stored in the DNA bank at the Royal Botanic Gardens, Kew (http://data.kew.org/dnabank/homepage.html). One plastid region and one nuclear region were selected. The *trnL–trnF* region of the plastid genome (spacer and intron) was amplified in one reaction using the primers c and f designed by Taberlet *et al.* (1991). In some cases, amplification also required the use of the internal primers d and e. Polymerase chain reactions (PCRs) were performed in 25 µL volumes, containing 22.5 µL of ReddyMix PCR Master Mix (containing 2.5 mM $MgCl_2$; ABgene, Epsom, Surrey, UK), 0.5 µL of bovine serum albumin (BSA; 0.04%), 0.5 µL of each primer and 1 µL of DNA template. The ribosomal internal transcribed spacer (ITS) from the nuclear genome was generally amplified using primers 17SE and 26SE of Sun *et al.* (1994), but, when these reactions failed, the ITS region was amplified using the ITS4 and ITS5 primers (White *et al.* 1990). Each 25 µL reaction contained 21.5 µL of ReddyMix PCR Master Mix (containing 1.5 mM $MgCl_2$), 1 µL of dimethyl sulfoxide (DMSO), 0.5 µL of BSA, 0.5 µL of each primer and 1 µL of DNA template. Amplification of both regions employed the same thermal profile: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for

60 s, 45 °C for 60 s and 72 °C for 90 s, and completed with a final extension of 72 °C for 4 min. PCR products were then purified on columns (Nucleospin® Extract II minicolumn kit; Macherey-Nagel, Düren, Germany). Cycle sequencing was performed using the BigDye Terminator Cycle Sequencing kit (version 3.1; Applied Biosystems, Warrington, Cheshire, UK) following the manufacturer's protocol, and the same primers as used for amplification. Products from the cycle sequencing reactions were cleaned on a Biomek NXS8 automated workstation (Beckman Coulter, High Wycombe, Buckinghamshire, UK) and visualized on a 3730 DNA Analyser (Applied Biosystems). Complementary sequences were assembled and edited using Seqman (DNAStar Inc., Madison, WI, USA) and aligned in MegAlign (DNAStar Inc.) using the CLUSTALW (Thompson *et al.* 1994) alignment algorithm. Automatically aligned sequences were then exported into BioEdit v7.0 (Hall 1999) and the alignments were checked and edited by eye. Simple gap coding was implemented in GapCoder (Young & Healy 2003) to incorporate any insertion/deletion (indel) information present. Indels for which homology could not be confidently inferred (especially indels adjacent to homopolymers), and stretches of DNA sequence that could not be confidently aligned, were excluded from subsequent phylogenetic analyses. Stretches of sequence for which outgroup taxa could not be aligned to ingroup taxa with any confidence were treated as unknown for the outgroup taxa. All sequences generated as part of this study have been submitted to GenBank (Appendix), and aligned matrices to TreeBase (www.treebase.org: matrix number SN4795-25231).

Phylogenetic analyses and molecular dating

Phylogenetic relationships were inferred using both parsimony and Bayesian inference. Prior to combined analysis of the plastid and nuclear gene regions, separate parsimony analyses, comprising only terminals for which both regions were available, were conducted to allow for an evaluation of incongruence. These separate analyses included only those accessions for which both plastid and nuclear sequences were available (72 species). Combined analyses, however, included all available accessions. The incongruence length difference (ILD) test

(Farris *et al.* 1994), as implemented in PAUP 4.0b10 (Swofford 2002), was conducted to test for incongruence. Owing to the propensity of the ILD test to type I error (Cunningham 1997), conflict was considered to be significant if the *p*-value was below α = 0.01. Separate trees for each locus were also visually compared, with conflict considered to be supported when both conflicting nodes had bootstrap (BS; Felsenstein 1985) support greater than 75%.

Parsimony searches were conducted heuristically in PAUP 4.0b10 (Swofford 2002), with 10 000 random addition replicates, tree bisection–reconnection (TBR) branch swapping and MULTREES in effect. BS values were calculated on the basis of 500 replicates, each involving a heuristic search set-up as follows: simple addition sequence, TBR branch swapping and MAXTREES set to 500. Bayesian searches were conducted in MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001), with different DNA substitution models implemented for the different data partitions (i.e. ITS, *trnL–trnF* and indels). A GTR + I + G model (Yang 1994) was selected using ModelTest 3.7 (Posada & Crandall 1998) applied to both sequence partitions, and a Markov-k model (Lewis 2001) was applied to the coded indel data. The analyses were performed using two independent MCMCMC runs, each comprising four chains (one cold and three heated), and a random starting tree. Each chain was run for 10^6 generations, sampling every 100th generation, giving a total of 10 000 samples per run. Plots of the log-likelihood scores against generation time were generated in Tracer v1.3 (Rambaut & Drummond 2005) to determine when stationarity was achieved, and thus to estimate the 'burn-in' period. At the same time, the effective sample size (ESS) for each parameter of the models used was also employed to confirm sampling adequacy. Trees from the 'burn-in' were discarded prior to the calculation of posterior probabilities (PPs).

Molecular dating was conducted in BEAST v1.4.2 (Drummond & Rambaut 2007), using a log-normal relaxed clock. Dating was conducted on both nuclear and plastid data and on the combined data (see Pfeil 2009). Because the dataset comprised two loci, a mixed model was used, with separate models applied to nuclear and plastid datasets (GTR + I + G).

Molecular evolution model parameters were assigned flat priors, whereas tree priors were modelled according to a Yule speciation process. The analysis was calibrated by setting a single age prior estimated for the divergence between *Leptomeira* + *Exocarpus* and *Buckleya* + *Thesium*. A normal calibration prior with a mean of 73.65 and standard deviation of 7.00 was used, based on a separate dating of a higher level phylogenetic analysis (Der & Nickrent 2008). Calibration for that analysis was based on fossil evidence for the divergence between Santalaceae and Misodendraceae + Loranthaceae (see Vidal-Russell & Nickrent 2008). Again, log-likelihood scores were plotted against generation time, together with ESS values, and visualized in Tracer, with 'burn-in' trees discarded prior to generating the consensus tree.

For the purpose of biogeographic reconstructions, distributions of South African *Thesium* species were scored using Jordaan (2003), whereas non-South African species were characterized for broad geographical distribution (Steyermark 1951, Hendrych 1964, Polhill 2005). Ancestral character state reconstruction was conducted in Mesquite v2.5 (Maddison & Maddison, 2008), using likelihood optimizations according to Galley *et al.* (2009). Reconstruction was conducted on the tree with maximum PP obtained from the BEAST search. Nodes were reconstructed for a given area, if they were significantly present for that area and significantly absent for all other areas. Nodes that were significantly absent from all areas were treated as equivocal.

Results

Aligned matrices of ITS and *trnL–trnF* were 937 and 1272 bases long, respectively (Table 3). Separate analyses consisted of 72 ITS and *trnL–trnF* sequences, and combined analyses consisted of 91 ITS and 86 *trnL–trnF* sequences. ITS had higher ingroup sequence divergence (0–17.5% vs. 0–9.0%) and a higher percentage of potentially parsimony informative characters (31.5% vs. 21.8%) than *trnL–trnF*. The *trnL–trnF* region, however,

displayed the greatest variation in length and, consequently, had more indels (158 vs. 64). A summary of the tree statistics is shown in Table 3.

Table 3. Tree statistics for ITS, *trnL-trnF*, and the combined data set. Consistency indices (CI) are measured excluding parsimony uninformative characters. RI, retention index.

Marker	Number of sites	Parsimony Informative	CI	RI	Tree Length
ITS	937	295 (31.5%)	0.54	0.79	1033
trnL-trnF	1272	353 (21.8%)	0.65	0.83	833
Combined	2209	624 (28.3%)	0.54	0.80	2108

Separate analyses

The trees derived from the separate plastid and ITS analyses are presented in Figure 2. The plastid data resolve *Thesium* as paraphyletic relative to *Thesidium* and *Austroamericium* with high PP and BS support (PP = 1.00; BS = 100). In contrast, ITS sequence data place *Thesidium* as sister to a large clade consisting of all *Thesium* species sampled, but with weak support (PP = 0.53; BS < 50) for the monophyly of the latter. In general, the ITS tree is better supported, especially at deeper nodes, than the plastid tree, which is consistent with its higher proportion of potentially informative characters (Table 3). Results of the ILD test suggest that nuclear and plastid data are not significantly discordant (p> 0.01), although supported conflict (BS > 75) occurs in two areas. Firstly, the plastid data place *Thesium ericaefolium* A.DC. in a clade of species mostly drawn from Hill's section *Annulata* (BS = 88), whereas ITS places it as sister to a second accession of *T. ericaefolium* (BS = 96), both of these being resolved as sister to *T. glomeruliflorum* Sond. (BS = 97). Secondly, within a

ITS

trnL-trnF

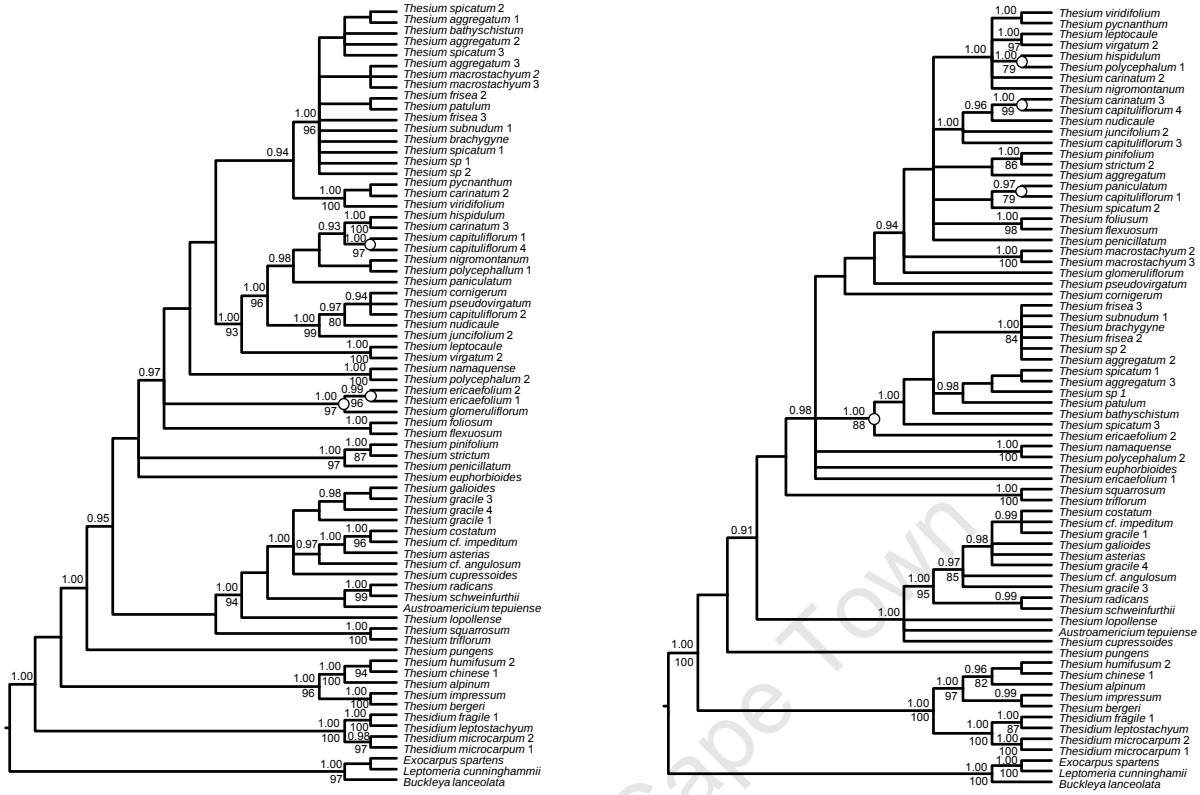


Figure 2. Consensus trees resulting from the separate Bayesian analyses of internal transcribed spacer (ITS) and *trnL–trnF* datasets. Only taxa with both plastid and ITS data available were included in these separate analyses. Posterior probabilities above 0.90 and bootstrap values above 75 are shown above andbelow the branches, respectively. Open circles indicate incongruent clades discussed in the text.

Cape-dominated clade which is, in general, poorly resolved, the plastid data place *T. hispidulum* Lam. as sister to *T. polycephallum* Schltr. (BS = 79), *T. paniculatum* L. as sister to one sample of *T. capituliflorum* Sond. (BS = 79) and *T. carinatum* A.DC. as sister to another sample of *T. capituliflorum* (BS = 99). In contrast, ITS places *T. hispidulum* as sister to *T. carinatum* (BS = 100) and *T. capituliflorum* as sister to *T. capituliflorum* (BS = 97). Since this incongruence was localised in nature, being limited to the terminal branches in the separate gene trees, a combined plastid/nuclear analysis was deemed justifiable.

Table 4. Results of separate dating analyses (MYA). Numbers in parentheses below regions indicate the number of taxa used.

Comment	ITS (72)	*trnL-trnF* (72)	Combined (72)	Combined (104)
Origin	42.7 ± 13.8	37.2 ± 14.2	35.9 ± 11.5	39.1 ± 11.9
Divergence between Eurasian and *Thesidium* species	36.6 ± 12.6	24.1 ± 12.1	28.5 ± 9.9	29.8 ± 10.7
Cape origin (max age)	25.6 ± 9.9	30.1 ± 13.1	22.2 ± 18.6	25.5 ± 7.3
South American divergence	10.1 ± 4.4	8.9 ± 7.7	9.6 ± 4.9	13.4 ± 6
Diversification within Cape clade	17.2 ± 6.9	22.9 ± 11.4	13.9 ± 4.9	16.7 ± 6.3

Combined analyses

Combining ITS and plastid data generally strengthened nodal support values (Fig 3). As in the separate plastid analysis, both *Thesidium* and *Austroamericium* are nested within *Thesium*, the monophyly of each being strongly supported (Fig 3: PP = 1.00, BS = 100). Within *Thesium*, two principal clades are resolved with varying degrees of support (Fig 3). Clade 1 (PP = 1.00, BS = 100) comprises two well supported clades: a Eurasian clade (PP = 1.00, BS = 100) and the *Thesidium* clade (PP = 1.00, BS = 100). Clade 2, with high posterior and low BS support (PP = 1.00, BS < 75), is dominated by two principal clades: a Cape clade (PP = 1.00, BS < 75) and a strongly supported Tropical clade (PP = 1.00, BS = 100), including the two sampled South American species of *Austroamericium*. Completing Clade 2 is a South African-centred grade comprising *T. pungens* + *T. spinulosum* (PP = 1.00, BS < 50) and *T. triflorum* + *T. squarrosum* (PP = 1.00, BS < 50). Deep relationships within the Cape clade are poorly resolved and unsupported, possibly reflecting rapid diversification. Some assemblages are, however, resolved, although support is weak to moderate. These include a clade (PP = 0.78, BS = 0.90) of species from Hill's section *Annulata* and a clade of 'leafy' *Thesium* species, essentially representing Hill's section *Barbata* (PP = 0.79, BS < 50). Where species were represented by multiple accessions, these generally grouped together in the same major clade, but often failed to form sister relationships with conspecific accessions (Fig 3).

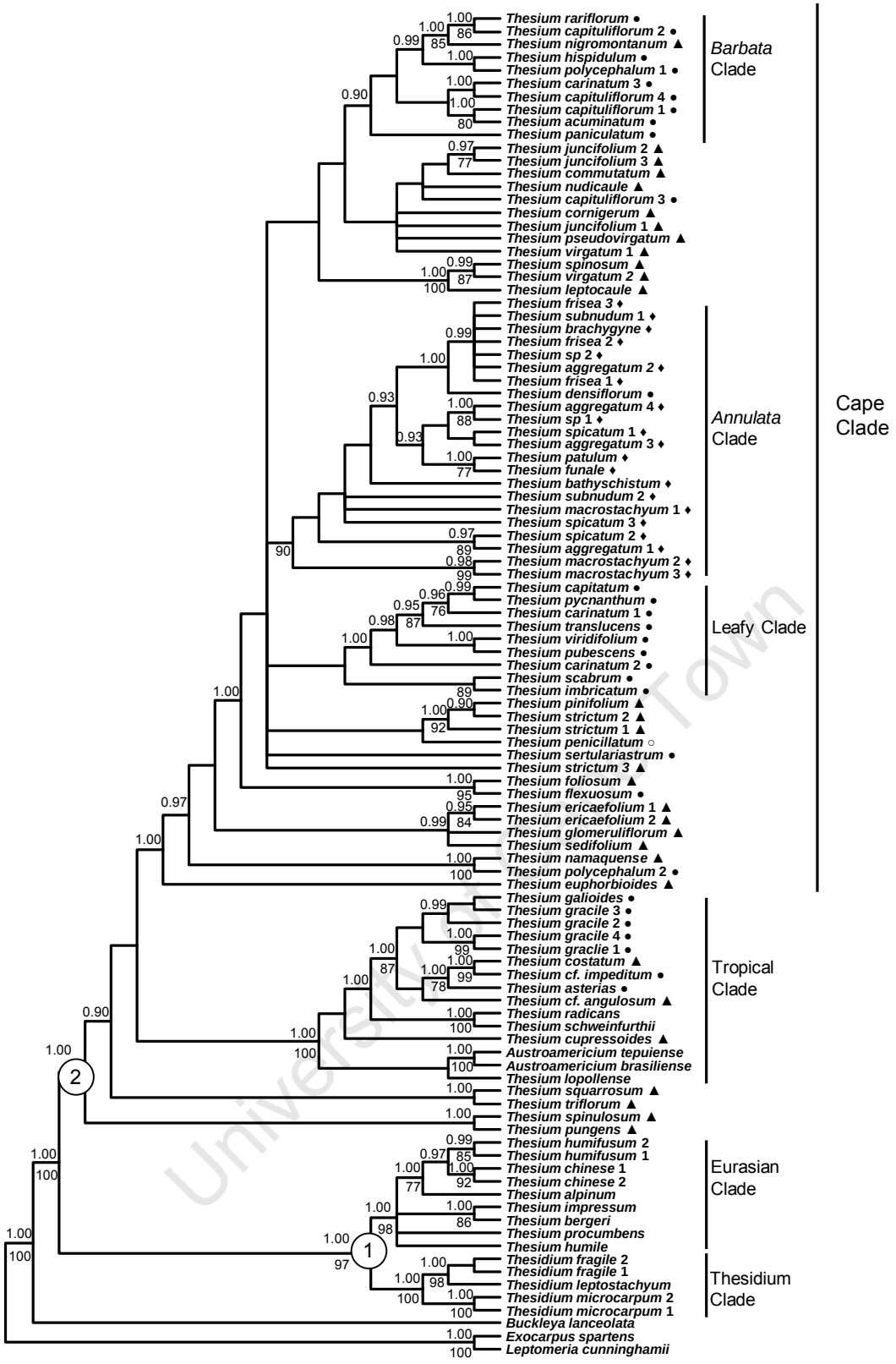


Figure 3. Consensus tree of the combined internal transcribed spacer (ITS) and *trnL–trnF* Bayesian analysis. Posteriorprobabilities above 0.90 and bootstrap values above 75 are indicated above and below the branches, respectively. Clades referred to in the text are labelled. Symbols next to South African species' names indicate the subgeneric classification based on Hill (1915) and are coded as follows: ●, section *Barbata*; ▲, section *Imberbia*; ○, section *Penicillata*; ♦, section,*Annulata*.

Biogeography and dating

In general, nodal age estimates are consistent across analyses based on separate gene regions, and with those based on the combined data with full and reduced taxon sampling (Table 4). Broad-scale ancestral range reconstruction (Fig 4) suggests a southern African origin for the genus, with subsequent dispersal into the rest of Africa and single dispersal events to South America and Europe. The age of the crown node of *Thesium* (Fig 4: Node 1) is dated to the late Eocene (39.1 ± 11.9 Mya). Within *Thesium*, the divergence of the Eurasian clade from *Thesidium* dates to the Oligocene, 29.8 ± 10.7 Mya (Fig 4: Node 2), and the origin of the Cape clade to the Miocene (25.5 ± 7.3 Mya; Fig 4). The crown age for the group is 21.3 ± 8.0 Mya. The latter is succeeded by a relatively recent diversification event, beginning at 16.7 ± 6.3 Mya (Fig 4: Node 5). The South American clade appears to have diverged from its tropical African sister around 13.4 ± 6.0 Mya (Fig 4: Node 4).

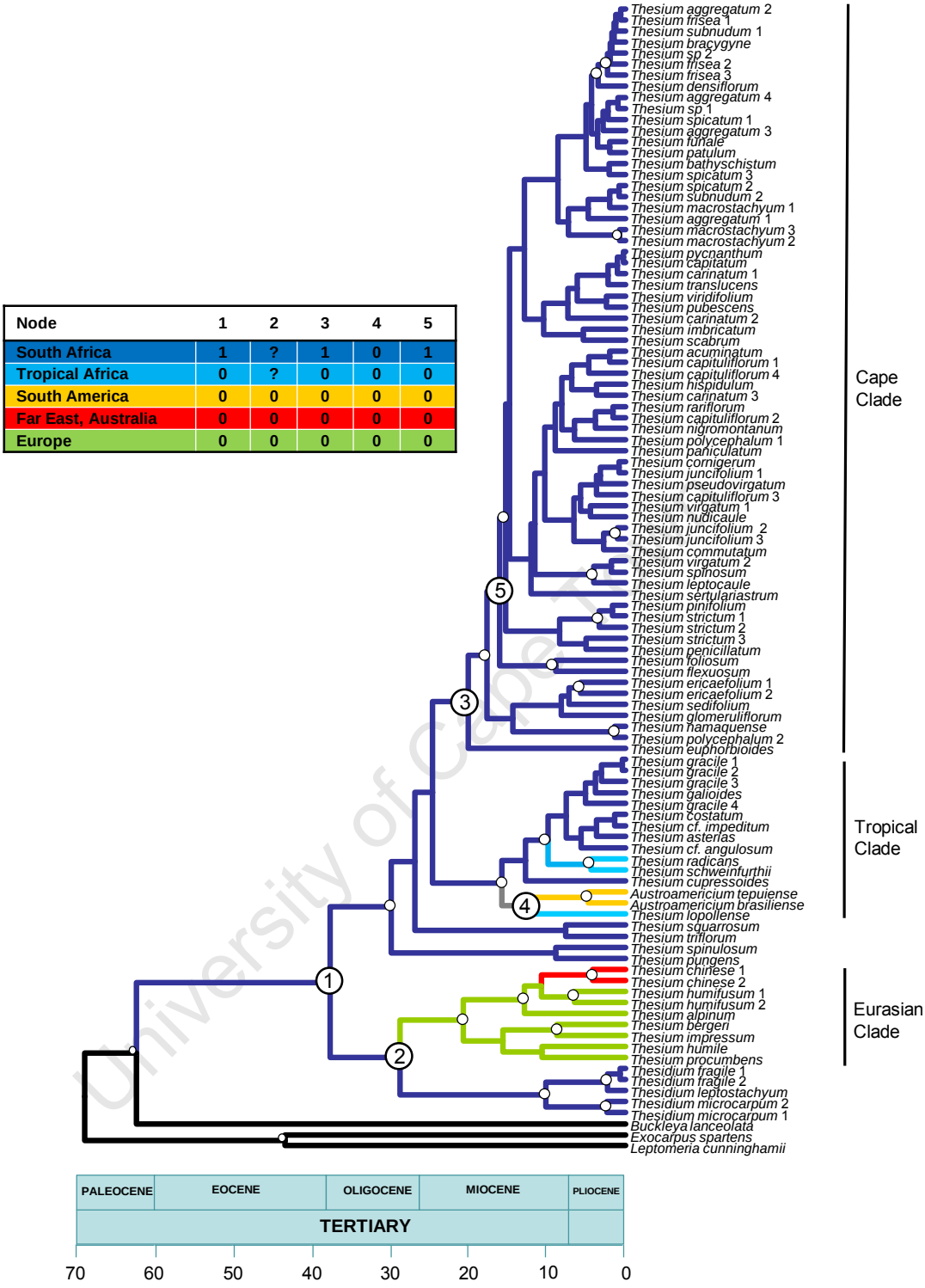


Figure 4. Ancestral area reconstruction (maximum likelihood) on the chronogram produced using a relaxed, log-normal clock (implemented in BEAST; see text for details). Selected clades are numbered for reference. Grey lines correspond toequivocal branches. Open circles indicate nodes with posterior probability (PP) > 0.95.

Discussion

In combination, ITS and *trnL–trnF* yielded a well resolved and, in places, strongly supported phylogenetic hypothesis for the genus *Thesium*. The reciprocal monophyly of *Thesium*, *Thesidium* and *Austroamericium* is contradicted, suggesting a need for generic realignment, although the formalization of this lies outside the scope of this study. Finally, ancestral area reconstruction supports the notion of a southern African origin for the group, with subsequent dispersal out of Africa, and rapid speciation in the Cape.

Concerns have been raised over the use of ITS in phylogenetic studies because of the risk of paralogy associated with the region (Álvarez & Wendel 2003). A lack of strong and widespread conflict between ITS and plastid data (Fig 2), however, suggests that paralogy is not a major problem in this study. Where conflict is observed, three possible explanations exist. Firstly, incongruence may be the result of sampling error (Hipp*et al.* 2004). Alternatively, lineage sorting (Avise 1994, Maddison 1997) and hybridization (Mckinnon *et al*. 1999) may cause discordance between datasets, the former being particularly influential where effective population sizes are large relative to the time since divergence (Maddison & Knowles 2006). In the current study, the majority of conflict between markers is restricted to geographically distinct clades (Fig 2), and the taxa involved typically have broad distributions. This, coupled with the relatively recent nature of the group (especially in terms of Cape species, see below; Fig 4), suggests that lineage sorting may account for most of the observed conflict. Recent divergence probably also explains the observed pattern of species-level paraphyly (Fig 3). In addition, many of the species represented here as paraphyletic have broad geographical distributions (Goldblatt & Manning 2002). Thus, large effective population sizes, coupled with recent divergence, may not have allowed enough time for fixation to occur at the nuclear and plastid loci used in this study (Avise 2000).

Both plastid and combined data reject the reciprocal monophyly of *Thesium* and *Thesidium* (Figs 2, 3), suggesting, instead, that *Thesidium* is embedded within *Thesium*, being sister to

the Eurasian *Thesium* clade. Although ITS identifies *Thesidium* as sister to a larger *Thesium* clade, this relationship is unsupported statistically (Fig 2). Backlund & Bremer (1998) proposed that the primary principle in determining the taxonomic ranking for a set of organisms is monophyly. In order to ensure generic monophyly, two options are available: (i) to sink *Thesidium* into *Thesium*, retaining it as a section in the genus (*Hagnothesium sensu* De Candolle 1857a), or (ii) to elevate Eurasian *Thesium* species to the genus level. There is strong morphological support for the maintenance of *Thesidium* as a separate genus. Specifically, *Thesidium* species are dioecious plants, with tetramerous flowers, traits not seen among *Thesium* species. Such morphological differences between the two genera support the principle of maximizing diagnosability (Backlund & Bremer 1998). In contrast, the morphological differences between European and African *Thesium* species are not as pronounced. All of the Eurasian species sampled in this study come from Hendrych's subgenus *Thesium*, which is characterized by campanulate perigonia, glabrous perianth margins and a pencil of hairs attaching anthers to the perianth lobes (Hendrych 1972). These morphological characters are not unique to this group, however, with similar floral morphologies being found in South African *Thesium* species (in Hill's section *Imberbia*). Given the lack of morphological exclusivity between Eurasian and southern African taxa, coupled with the strength of the support for *Thesidium* being nested within *Thesium*, I feel, on current evidence, that they would be best maintained within *Thesium*. This suggests that *Thesidium* should be reduced to a subgenus within *Thesium*. However, a lack of full sampling of species from the Eurasian subgenus and the absence of a comprehensive comparative morphological study make any final judgement on this potential change premature.

Hendrych (1963) segregated the South American *Thesium* species as a new genus, *Austroamericium*, based on their apparent morphological distinctiveness and their geographical disjunction from the rest of the *Thesium* (all Old World). Molecular evidence presented here contradicts Hendrych's view, however, suggesting that *Austroamericium* is

strongly (PP = 1.00, BS = 100) nested within a predominantly tropical African *Thesium* clade. Hendrych (1972) cited floral structure as a key diagnostic feature of *Austroamericium*, with these species having, among other features, funnel-shaped perianth tubes, with the anthers close to the free lobes of the perigonium. He also suggested that pear-shaped fruits that retain the dried lobes of the perigonium after they have formed and have raised ribs connected by reticulated veins were unique to these South American species. In addition, he cited an annual life history, in conjunction with scale-shaped leaves, as supporting their exclusion from the rest of *Thesium*. Many of the characters identified as characteristic of *Austroamericium* by Hendrych are, however, seen in tropical African *Thesium* species. For example, many tropical African species are annual, with scale-like leaves, and some species also have ribbed fruit, with vein reticulation (e.g. Group 4, in Polhill 2005). Although a more thorough morphological investigation is required, such superficial morphological similarity between South American and tropical African taxa, combined with the molecular evidence outlined here, supports the re-inclusion of these species in *Thesium*.

Amongst the South African species, relationships generally do not correspond well with Hill's (1915) infrageneric groupings, although some consistencies are evident. Section *Imberbia* forms a paraphyletic grade, which suggests that its morphological characteristics may represent plesiomorphic states. These species have glabrous or fringed perianth margins and hairs attaching the anthers to the perianth segments. Hill's section *Barbata* is represented by two clades within the larger Cape clade. One clade (PP = 0.79, BS < 50) comprises a group of leafy species (Fig 3: Leafy clade), and the second is sister to a clade (PP = 0.90, BS < 50) of *Imberbia* species. The Cape section *Annulata* receives moderate support (PP = 0.78, BS = 90, Fig 3) and is distinguished morphologically by having anthers free from the perianth lobes (i.e. without attaching hairs) and a characteristic ring of hairs at the throat of the perianth tube (Hill 1915). The inclusion of *T. densiflorum* in the *Annulata* clade is exceptional, this species being assigned to section *Barbata* by Hill. Both sections *Barbata* and *Annulata* have a dense apical beard on their perianth lobes, but differ in the

presence of attaching hairs behind the anthers in section *Barbata* species. As well as sharing its range with many species from section *Annulata*, some individuals of *T. densiflorum* have been noted to lack attaching hairs behind their anthers (T. Moore, *pers. observ.*). This species also shares a similar floral arrangement to many species from section *Annulata*, with flowers arranged in 'dense rounded heads at the ends of branches' (Levyns 1950). The only South African representative of the section *Penicillata* arises as sister to a clade comprising section *Imberbia* species. Overall, the lack of support for the monophyly of Hill's infrageneric groupings suggests that a new system is required. Increased genetic and taxonomic sampling is required to shed further light on the relationships within *Thesium* (*cf*. Albach & Chase 2004).

Ancestral area reconstruction (Fig 4) supports Hendrych's (1972) hypothesis of a southern African origin for the genus. As such, the results presented here mirror a biogeographical pattern shown by several other groups (Galley *et al*. 2007), which exhibit a clear northward dispersal trend out of southern Africa. Based on my limited sampling of the Eurasian species, the occurrence of the genus in Eurasia is apparently the result of a single dispersal event from Africa to Europe and thence to Asia (Fig 4). Of course, limited taxon sampling may exert a major effect on ancestral reconstruction ancestral reconstruction (Salisbury & Kim 2001) and here the limited number of Eurasian species sampled (see Table 2) may impact on the reconstruction of the basal node.

The South American *Thesium* split from their African relatives approximately 13.4 Mya (Fig 4). The exact location of the common ancestor is unclear from likelihood optimizations (Node 4: Fig 4), although a tropical African distribution seems intuitive. Moreover, parsimony optimization suggests a tropical African reconstruction for this node (data not shown). This date is far too recent to be the result of vicariance associated with the break-up of Gondwana (approximately 90 Mya, Sanmartín & Ronquist 2004), as suggested by Hendrych (1972), but instead indicates the more likely involvement of long-distance dispersal. A large

number of plant and animal genera display trans- Atlantic disjunctions (Thorne 1973) and, in a review of plants displaying trans-Atlantic distributions, Renner (2004) found that, of the 11 genera studied, seven were best explained as recent (< 10 Mya) dispersal events across the Atlantic. She suggested that strong oceanic currents, rather than dispersal by wind or birds, may effectively transport 'rafts' of plant material across the Atlantic. The strongly supported monophyly of the South American *Thesium* species, nested within a tropical African clade, suggests a single, relatively recent dispersal event from tropical Africa to South America.

High *Thesium* diversity in the Cape appears to be the result of recent diversification within a single lineage (Fig 4). The relative antiquity of this Cape clade matches that of several other Cape groups, for example Rhamnaceae (Richardson *et al.* 2001), Irideae (Goldblatt *et al.* 2002), *Ehrharta* (Verboom *et al.* 2003), African Restionaceae (Linder *et al.* 2003), *Pelargonium* (Bakker *et al.* 2005) and *Muraltia* (Forest *et al.* 2007). The radiation of the group, however, only occurred subsequently in the Miocene (16.7 ± 6.7 Mya). A similar pattern was observed in the South African *Pelargonium* (Bakker *et al.* 2005), which shows recent diversification events in the Miocene and Pliocene. Climate has been recognized as a key driver in the recent radiations of many Cape lineages (Richardson *et al.* 2001, Verboom *et al.* 2003, Bakker *et al.* 2005, McKenzie & Barker 2008, Verboom *et al.* 2009) and, in particular, the large climatic fluctuations experienced during the early-late Miocene are suggested to have stimulated speciation in the Cape winter rainfall region at that time (Verboom *et al.* 2003).

Parasitic plants may be affected by changes in climate, both directly through impacts on their own physiology and indirectly through impacts on their hosts (Phoenix & Press 2005). Thus, range expansions and contractions of both parasites and/or their hosts during periods of climatic fluctuation may have promoted speciation in parasitic lineages. Miocene climatic changes were accompanied by major geomorphic events which significantly altered the substrates available for plant colonization, providing novel habitats (Cowling *et al.* 2009).

Many studies have revealed the effect of nutrient availability on parasite success and fecundity, with parasitic plants grown under high nutrient conditions generally being larger and having higher reproductive outputs (Salonen & Puustinen 1996). Thus, the exposure of novel, relatively nutrient-rich substrates (Cowling *et al.* 2009) may have provided opportunities for both *Thesium* species and their hosts to diversify into novel, unexploited niches. Some Cape species, particularly from section *Annulata* (e.g. *T. funale* L. and *T. patulum* A.W.Hill) are found almost exclusively on clay-rich lowland soils that were exposed relatively recently during the mid- to late-Miocene (Cowling *et al.* 2009). Such large-scale changes in climate and geology may have played an influential role in the evolution of *Thesium*, but little is known about the roles climate and geology play in the evolution of a parasitic lifestyle in plants. This study serves as a preliminary framework for future research, not only on the phylogenetics and biogeography of the genus *Thesium*, but also for the understanding of the evolution of the species-rich Cape region as a whole.

Appendix 1. Genbank Accession number and collection details forsamples used in phylogentic analyses.

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
GU294634	GU256819	*Thesium brachygyne*	southern Africa	ABLouw 11371, BOL	WC, South Africa
GU294659	GU256852	*Thesium aggregatum 2*	southern Africa	ABLouw 12062, BOL	Capricorn Park, WC, South Africa
GU294633	GU256818	*Thesium nudicaule*	southern Africa	ABLouw 12249, BOL	WC, South Africa
GU294635	GU256820	*Thesium hispidulum*	southern Africa	ABLouw 9440, BOL	TulbaghValley, WC, South Africa
GU294632	GU256817	*Thesium subnudum 1*	southern Africa	ABLouw 9563, BOL	TulbaghValley, WC, South Africa
GU294638	GU256823	*Thesium lopollense*	tropical Africa	ANGOLA 1959, BOL	Southern Angola
GU294641	GU256827	*Thesium patulum*	southern Africa	DGEvans 25/11/08/1, BOL	Stellenbosch, WC, South Africa
GU294640	GU256826	*Thesium frisea 2*	southern Africa	FForest CP 3, NBG	Cape Peninsula, WC, South Africa
GU294682		*Thesium pubscens*	southern Africa	FForest CP 4, NBG	Cape Peninsula, WC, South Africa
GU294607	GU256784	*Thesidium leptostachyum*	southern Africa	FForest 1, NBG	WC, South Africa, WC, South Africa
	GU256794	*Thesium carinatum 1*	southern Africa	FForest 594, NBG	Cederberg, WC, South Africa
	GU256813	*Thesium strictum 1*	southern Africa	FForest 668, NBG	TableMountain, WC, South Africa
	GU256825	*Thesium aggregatum 4*	southern Africa	FForest 669, NBG	TableMountain, WC, South Africa
GU294616	GU256793	*Thesium viridifolium*	southern Africa	FForest 680, NBG	CapePeninsula, WC, South Africa
GU294646	GU256832	*Thesium aggregatum 1*	southern Africa	FForest 694, NBG	De Hoop, WC, South Africa

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
GU294643	GU256829	*Thesium nigromontanum*	southern Africa	FForest 702, NBG	Potberg, WC, South Africa
GU294683		*Thesium commutatum*	southern Africa	FForest 716, NBG	De Hoop, WC, South Africa
	GU256866	*Thesium funale*	southern Africa	FForest 732, NBG	Salmonsdam, WC, South Africa
GU294624	GU256804	*Thesium leptocaule*	southern Africa	FForest 768, NBG	Baviaanskloof, WC, South Africa
GU294644	GU256830	*Thesium spicatum 2*	southern Africa	FForest 850, NBG	Prince Albert's Pass, WC, South Africa
GU294610	GU256787	*Thesium squarrosum*	southern Africa	FForest 851, NBG	Uniondale, WC, South Africa
GU294612	GU256789	*Thesium namaquense*	southern Africa	FForest 896, NBG	Spektakel Pass, NC, South Africa
GU294613	GU256790	*Thesium polycephalum 2*	southern Africa	FForest 911, NBG	Spektakel Pass, NC, South Africa
GU294642	GU256828	*Thesium spicatum 1*	southern Africa	FForest 950, NBG	Landroskop, WC, South Africa
GU294647	GU256833	*Thesium cornigerum*	southern Africa	FForest 952, NBG	Landroskop, WC, South Africa
GU294614	GU256791	*Thesium euphorbioides*	southern Africa	FForest 953, NBG	Landroskop, WC, South Africa
GU294674		*Thesium humile*	Europe	M.M. Abd el Ghani sn	no locality (Kew DNA bank 1858)
GU294602	GU256780	*Thesium humifusum 2*	Europe	Chase1881	Sierra Neveda, Spain (Kew DNA bank 1881)
GU294598	GU256776	*Thesium impressum*	Asia	K 36056	Van Dist., Turkey
GU294639	GU256824	*Austroamericium tepuiense*	South America	K 36057	Parana, Brazil
GU294680		*Austroamericium brasiliense*	South America	K 36058	Bahia, Brazil

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
GU294599	GU256777	*Thesium alpinum*	Europe	K 36059	Vitosa Mt., Bulgaria
GU294600	GU256778	*Thesium bergeri*	Europe	K 36060	Sterea Ellas Div., Greece
GU294672		*Thesium procumbens*	Europe	K 36061	Erzurum, Turkey
GU294601	GU256779	*Thesium radicans*	Tropical Africa	K 36062	Jabal Qahar, Saudi Arabia
GU294673		*Thesium chinese 2*	Asia/Australia	K 36063	Nedezhdensky Dist., Eastern Russia
GU294603	GU256781	*Thesium chinese 1*	Asia/Australia	K 36065	LakeInverell, NSW, Australia
GU294605	GU256782	*Thesium schweinfurthii*	Tropical Africa	K 36066	Kasungami, Zaire
GU294669	GU256863	*Buckleya lanceolata*	Asia/America	K 36067	Sado-Ga-Sima Niigata Prefecture, Japan
GU294604		*Thesium humifusum 1*	Europe		no locality (Kew DNA bank 3687)
	GU256834	*Thesium juncifolium 1*	southern Africa	Muasya&Striton 4081, BOL	WC, South Africa
GU294652	GU256844	*Thesium capituliflorum 4*	southern Africa	Muasya&Striton 4083, BOL	WC, South Africa
GU294617	GU256797	*Thesium pycnanthum*	southern Africa	MNBritton 1904/082, BOL	Jonkershoek, Cape Town, South Africa
	GU256795	*Thesium translucens*	southern Africa	MNBritton 1904/083, BOL	Jonkershoek, Cape Town, South Africa
GU294648	GU256835	*Thesium pseudovirgatum*	southern Africa	MNBritton 1904/084, BOL	Jonkershoek, Cape Town, South Africa
GU294649	GU256836	*Thesiumaggregatum 3*	southern Africa	MNBritton 1904/085, BOL	Jonkershoek, Cape Town, South Africa
	GU256841	*Thesium rariflorum*	southern Africa	Moore 100, BOL	Houwhoek, WC, South Africa

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
	GU256796	*Thesium capitatum*	southern Africa	Moore 111, BOL	Jonkershoek, WC, South Africa
	GU256805	*Thesium spinosum*	southern Africa	Moore 114, BOL	De Hoop NR, WC South Africa
GU294611	GU256788	*Thesium triflorum*	southern Africa	Moore 128, BOL	Graaf Reinet, EC, South Africa
	GU256838	*Thesium virgatum 1*	southern Africa	Moore 14, BOL	Harold Porter BG, WC, South Africa
	GU256839	*Thesium macrostacyhum 1*	southern Africa	Moore 140, BOL	Baardskeerdersbosch, WC, South Africa
	GU256807	*Thesium sedifolium*	southern Africa	Moore 146, BOL	Baardskeerdersbosch, WC, South Africa
GU294627	GU256811	*Thesium spinulosum*	southern Africa	Moore 148, BOL	Baardskeerdersbosch, WC, South Africa
	GU256845	*Thesium acuminatum*	southern Africa	Moore 149, BOL	Red Hill, Cape Town, South Africa
GU294679		*Thesium densiflorum*	southern Africa	Moore 152, BOL	CampsBay, Cape Town, South Africa
	GU256808	*Thesium scabrum*	southern Africa	Moore 155, BOL	CampsBay, Cape Town, South Africa
GU294645	GU256831	*Thesium sp 1*	southern Africa	Moore 16, BOL	Sir Lowry's Pass, WC, South Africa
GU294655	GU256848	*Thesium capituliflorum 3*	southern Africa	Moore 165, BOL	Grootwinterhoek Mts, WC, South Africa
GU294656	GU256849	*Thesium macrostacyhum 2*	southern Africa	Moore 166, BOL	Grootwinterhoek Mts, WC, South Africa
GU294618	GU256798	*Thesium carinatum 2*	southern Africa	Moore 167, BOL	Grootwinterhoek Mts, WC, South Africa
GU294657	GU256850	*Thesium macrostacyhum 3*	southern Africa	Moore 168, BOL	Grootwinterhoek Mts, WC, South Africa
	GU256843	*Thesium capituliflorum 2*	southern Africa	Moore 169, BOL	Grootwinterhoek Mts, WC, South Africa

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
GU294636	GU256821	*Thesium panicluatun*	southern Africa	Moore 23, BOL	Pilaarkop, WC, South Africa
GU294619	GU256799	*Thesium foliosum*	southern Africa	Moore 41, BOL	ThumbPeak, WC South Africa
GU294620	GU256800	*Thesium pinifolium*	southern Africa	Moore 43, BOL	ThumbPeak, WC South Africa
GU294676		*Thesium sertulariastum*	southern Africa	Moore 45, BOL	Prince Alberts Pass, WC South Africa
GU294621	GU256801	*Thesium glomerulifolium*	southern Africa	Moore 46, BOL	Prince Alberts Pass, WC South Africa
GU294622	GU256802	*Thesium strictum 2*	southern Africa	Moore 48, BOL	Prince Alberts Pass, WC South Africa
GU294615	GU256792	*Thesium galioides*	southern Africa	Moore 50, BOL	Prince Alberts Pass, WC South Africa
GU294675		*Thesium juncifolium 3*	southern Africa	Moore 54, BOL	Grootvadersbos, WC South Africa
GU294653	GU256846	*Thesium juncifolium 2*	southern Africa	Moore 62, BOL	Grootvadersbos, WC South Africa
GU294654	GU256847	*Thesium bathyshcistum*	southern Africa	Moore 87, BOL	Heuningberg NR, Bredasdorp, South Africa
GU294623	GU256803	*Thesium ericaefolium 2*	southern Africa	Moore 89, BOL	Potberg, WC, South Africa
	GU256837	*Thesium subnudum 2*	southern Africa	Moore 96, BOL	Kogelberg, WC South Africa
GU294626	GU256809	*Thesium frisea 3*	southern Africa	NGB 1616, BOL	Drakensberg, KZN, South Africa
GU294668	GU256862	*Thesium gracile 4*	southern Africa	TLNowell s.n., BOL	SaniPass, KZN, South Africa
	GU256810	*Thesium imbricatum*	southern Africa	TTrinder-Smith 423, BOL	Drakensberg, KZN, South Africa
GU294665	GU256858	*Thesium gracile3*	southern Africa	TTrinder-Smith 424, BOL	Drakensberg, KZN, South Africa

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
GU294664	GU256857	*Thesium asterias*	southern Africa	TTrinder-Smith 432, BOL	Oribi Gorge, KZN, South Africa
GU294661	GU256854	*Thesium cf. angulosum*	southern Africa	Verboom 1025, BOL	Garden Castle NR, KZN, South Africa
GU294666	GU256859	*Thesium cupressoides*	southern Africa	Verboom 1026, BOL	Garden Castle NR, KZN, South Africa
GU294662	GU256855	*Thesium costatum*	southern Africa	Verboom 1037, BOL	Garden Castle NR, KZN, South Africa
GU294663	GU256856	*Thesium cf. impeditum*	southern Africa	Verboom 1043, BOL	Wakkestroom, MPA, South Africa
GU294667	GU256860	*Thesium gracile1*	southern Africa	Verboom 1054a, BOL	Barberton, MPA, South Africa
	GU256861	*Thesium gracile 2*	southern Africa	Verboom 1054b, BOL	Barberton, MPA, South Africa
GU294629	GU256814	*Thesium penicillatum*	southern Africa	Verboom 1140, BOL	Hottentots Hollands NR, WC, South Africa
GU294651	GU256842	*Thesium polycephalum 1*	southern Africa	Verboom 1142, BOL	Springbok, NC, South Africa
GU294608	GU256785	*Thesidium micorcarpum2*	southern Africa	Verboom 1149, BOL	Kamannassieberg, WC, South Africa
GU294609	GU256786	*Thesidium micorcarpum 1*	southern Africa	Verboom 1150, BOL	Kamannassieberg, WC, South Africa
GU294625	GU256806	*Thesium virgatum 2*	southern Africa	Verboom 1153, BOL	Kamannassieberg, WC, South Africa
GU294630	GU256815	*Thesium flexuosum*	southern Africa	Verboom 1156, BOL	Kamannassieberg, WC, South Africa
GU294670	GU256864	*Exocarpus spartens*	Australia	Verboom 1273, BOL	Western Australia
GU294671	GU256865	*Leptomeria cunninghamii*	Australia	Verboom 1274, BOL	Western Australia
GU294658	GU256851	*Thesium sp 2*	southern Africa	Verboom 1290, BOL	Du ToitsKloof Mountains, WC, South Africa

trnL-trnF	ITS	Species	Distribution	Voucher	Collection Locality
GU294677		*Thesium strictum 3*	southern Africa	Verboom 1295, BOL	Betty's Bay, WC South Africa
GU294631	GU256816	*Thesium ericaefolium 1*	southern Africa	Verboom 1296, BOL	Betty's Bay, WC South Africa
GU294650	GU256840	*Thesium capituliflorum 1*	southern Africa	Verboom 1297, BOL	Betty's Bay, WC South Africa
GU294660	GU256853	*Thesium spicatum 3*	southern Africa	Verboom 1300, BOL	Betty's Bay, WC South Africa
GU294681		*Thesidium fragile 2*	southern Africa	Verboom 1305, BOL	Betty's Bay, WC South Africa
GU294637	GU256822	*Thesium carinatum 3*	southern Africa	Verboom 1311, BOL	Betty's Bay, WC South Africa
GU294628	GU256812	*Thesium pungens*	southern Africa	Verboom 1340, BOL	Namaqualand, WC, South Africa
GU294606	GU256783	*Thesidium fragile 1*	southern Africa	Verboom 912, BOL	Kamannassieberg, WC, South Africa
GU294678		*Thesium frisea 1*	southern Africa	ABLouw WV14, BOL	WC, South Africa

Chapter 3

The role of edaphic variation in shaping divergence in carbon acquisition traits in the hemiparasitic genus *Thesium* L. (Santalaceae) in the Cape Floristic Region

Abstract

Parasitism represents a specialized mode of foraging for water and nutrients among flowering plants and may be a strategy to overcome resource constraints in low nutrient environments. It remains unclear whether the edaphic environment plays a role in the divergence in the level of heterotrophy (degree of host dependence) among closely related hemiparasitic species. Twelve species of *Thesium* native to the Cape Floristic Region (CFR) of South Africa were sampled and divergence in carbon acquisition-associated traits was evaluated and correlated with divergence in the edaphic environments. The isotopic signatures of *T. capitatum and T. nigromontanum* individuals differed significantly when grown withC_3 and C_4 hosts ($F_{5,24}$ = 6.41, $p < 0.01$), confirming that both utilised heterotrophically-acquired C. The resolution of the isotopic data was, however, inadequate to permit an evaluation of the relative importance of heterotrophically-derived C between the two parasite species. Under greenhouse conditions, the leafier *T. capitatum* individuals displayed significantly higher rates of photosynthesis than the non-leafy *T. nigromontanum* ($F_{5,38}$ = 8.29, $p < 0.05$). *Thesium* species displayed significant divergence in carbon acquisition traits, specifically height, leafiness and chlorophyll content, and variation in these traits was associated with divergence in soil physical properties and nutrient availability. Species of *Thesium* with lower photosynthetic capacities appeared to be restricted to less productive, more nutrient poor sites, where competition for light and resources is low. In contrast, taller, more autotrophic species might be able to capitalise on higher photosynthetic capacities, enabling them to withstand competition in more productive systems.

Introduction

Parasitic plants are able to acquire some of the resources they require for their growth and reproduction from other plants, attaching to either the roots or the shoots of their hosts through structures called haustoria (Kuijt 1969, Nickrent *et al.* 1998). Parasitism is believed to have arisen 12-13 times within flowering plants (Westwood *et al.* 2010) and it has been estimated that at least 1% of angiosperms are parasitic (Musselman & Press 1995). In general, parasitic plants are able to obtain water, nutrients and carbon (C, Ehleringer *et al.* 1985, Press 1989) from their hosts, with the degree of host-dependence with respect to these resources differing significantly among species (Nickrent *et al.* 1998). Holoparasites lack chlorophyll, and often have reduced or lack functional leaves, and are thus almost entirely dependent on their hosts for C(Shen *et al.* 2007). Hemiparastic plants, on the other hand, are parasites which have the capacity to assimilate their own C, but which nonetheless acquire some of their C from their hosts heterotrophically (Tennakoon & Pate 1996).

The Cape Floristic Region (CFR, Goldblatt & Manning 2000) is renowned for its high floristic diversity and levels of endemism (Goldbaltt & Manning 2000, Linder 2003, Verboom *et al.* 2009). In addition, the region shows remarkable geo-physical heterogeneity (Goldblatt &Manning 2002; Linder 2003). Although typified by nutrient poor soils (Stock & Lewis 1986), the substrates of the CFR are highly variable (Kruger *et al.* 1983), with major differences being driven by the underlying geology. Little empirical work has been done to quantify the differences in nutritional status of the soils derived from different substrates. Fynbos soils are, however, known to be particularly deficient in nitrogen (N) and phosphorus (P) (Witkowski and Mitchell 1987), both of which are key resources for plant growth and may be critical in determining plant community structure and function (Kruger *et al.* 1983). In addition, soil pH has been demonstrated to have a significant effect on the ability of plants to take up nutrients from the soil. Similarly, soil electro-conductivity (EC) and cation exchange

capacity (CEC) are important in determining the availability of nutrients to plants (Tisdale *et al.* 1993), thus playing an important role in determining plant success and distributions. High edaphic heterogeneity has long been considered important in promoting speciation in the region (Cowling 1990, Verboom *et al.* 2004). Richards *et al.* (1997) proposed that spatial variation in soil nutrient availability may be important in explaining landscape-level species distributions and community composition in nutrient-poor systems such as those dominating the CFR. Little work has been done to investigate the role of soil nutrients in determining the distribution of parasitic plants in the region or the role soil nutrients play in determining divergence in nutrient acquisition strategies CFR species.

Plants in the CFR have developed a number of strategies to cope with the low nutrient availability (Lamont 1982), including cluster roots (e.g. Proteaceae), mychorrizal associations (e.g. Ericaceae), carnivory (e.g. Droseraceae) and parasitism (e.g. Viscaceae). Representatives of at least nine of the 14 families of parasitic plants occur in the CFR, with the majority of these parasitic species being root hemiparasites (Lamont 1982). *Thesium* is a large genus of predominantly root-hemiparasites from the family Santalaceae. It has a principally Old World distribution, with its highest diversity centred in southern Africa, and a high concentration of species in the CFR. Although there has been little experimental work testing the host preferences of *Thesium*, members of the genus are considered to be generalist parasites, tapping into a wide range of host species (Visser, 1981). Dostálek and Münzbergová (2010) found that *Thesium* species were attached to 94 % of all species in surveyed plots. In a field study, Suetsugu *et al.* (2008) found that *Thesium chinense* parasitized 22 plant species from 11 plant families. They also found that *Thesium chinense* generally preferred the Poaceae, suggesting some post-attachment selectivity. *Thesium* species occur on all the major substrate types in the CFR, ranging from the richer, shale-derived soils to the highly leached sandstone-derived soils. The genus displays remarkable diversity in morphology (Fig 1), but little is known about what determines the distributions of

individual *Thesium* species within the CFR and, more particularly, whether there is a link between morphological divergence and the degree of heterotrophy.

Parasitic plants are often associated with highly disturbed, low fertility and low productivity environments (Matthies 1995, Watson 2009), which suggests that there are constraints on

Figure 1. Four species of *Thesium* from the Cape Floristic Region (CFR)of South Africa, displaying the wide range of morphological variation found in the genus. Species can be grouped into three broad categories: small and leafless such as A, *T. capituliflorum*; B, *T.*

virgatum; densely leafy such as C, *T. translucens*; and tall and leafy such as D, *T. euphorbioides*. All images from Bardskeerdersbos, WC by GAVerboom.

the parasitic lifestyle. One apparent cost is that associated with the development of a functional haustorium (Press 1989, Cameron & Seel 2007), which accounts for a large proportion of below-ground respiration, and thus has a large effect on the C balance of the plant, accounting for up to 40% of below ground respiration (Press *et al.* 1991). Haustoria are also often short-lived and lack secondary thickening, adding to the carbon cost of producing them (Pate 2001).Also, the greater the dependence on a host, the more important is the need for a suitable host, i.e. one that is capable of providing the required amount C and other nutrients to sustain itself and the parasite. Host quality may thus be a major determinant of parasitic plant distribution (Watson 2009). A further cost may be that the parasite eventually competes with its hosts for resources (Matthies 1995; Keith *et al.* 2004), limiting the size to which a parasite can grow. Because of the costs associated with a hemiparasitic lifestyle, hemiparasitism may only be beneficial in certain habitats. Specifically, heterotrophy may be most beneficial in systems in which non-host-derived resources (e.g. soil nutrients) are scarce (Smith 2000), and where competition for nonhost-derived resources is low, as hemiparasites still require access to light, soil nutrients and inorganic C in order to complete their lifecycles. Matthies (1995) suggests that the balance between heterotrophy and competition could restrict hemiparasites to habitats of relatively low productivity, such as those dominating the CFR in South Africa and Western Australia (Lambers *et al.* 2008). The association between parasitic plants and soil nutrients may be most applicable to root hemiparasites. Stem parasites, which do not have access to soil nutrients, are more constrained by the nutrient contents of their hosts (Watson 2009), whereas root parasites have access to the soil nutrient pool and can tap into multiple hosts. This may explain why South African mistletoes show their greatest diversity in mesic, nutrient rich savannas with nearly nine times as many species occurring here, as are found in nutrient-deficient Cape fynbos (Dean *et al.* 1994). Root hemiparasitic plants interact with

their host plants both directly, through parasitism, and indirectly, through competition for resources (Smith 2000). In general, parasites possess poorly developed root systems (Seel *et al.* 1993a). For example, Mann & Musselman (1981) found that root parasites produce fewer haustoria and have larger root systems in fertilised pots and may thus be less heterotrophic under conditions when soil resources are high. This may be in order to save the resources required for haustorial production. The balance between conservation of resources in haustorial production and competition for resources may make strong host dependence feasible only in nutrient limited systems, where competition is low and the pay-off for tapping into host root systems is highest.

An increased degree of heterotrophy allows for a reduced investment in metabolic pathways and the morphological structures required for nutrient uptake and metabolism and autotrophic C acquisition (Press 1989). As a result, the leaves in many parasitic angiosperms are reduced or lost (Nickrent *et al.* 1998). When present, leaves of hemiparasites often differ structurally from those of their free living relatives (Press 1989). In a survey of South African parasitic plants and their host, de la Harpe *et al.* (1981) found that parasites had chlorophyll concentrations ranging between 1 and 11 mg g^{-1} dry mass whereas non-parasitic plants had an average chlorophyll concentration of 24.7 mg g^{-1} dry mass. In addition, the photo-assimilatory capacity of leaves in hemiparasites is often lower than that in non-parasitic plants (Press 1989). Shah *et al.* (1987) reported poorly developed mesophyll in the hemiparasite *Striga hermontheca*, contributing to it having photosynthetic rates as low as 2.5 μmol m^{-2} s^{-1}, half those reported for their hosts . Press *et al.* (1988) measured rates of between 2.1 and 7.5 μmol m^{-2} s^{-1} for eight root hemiparasitic species. The observed morphological differences that *Thesium* species display in their foliar traits could be linked to differences in their ability to acquire C autotrophically. Plants with a greater capacity for autotrophic C gain might be considered less dependent on their hosts, and so less heterotrophic. Thus, understanding how *Thesium* species differ in terms of traits such

as plant size, leafiness, and chlorophyll content could provide and provide an indirect measure of how dependent these species are on their hosts.

Because hemiparastism may be most beneficial in low-nutrient systems, we hypothesised that species of *Thesium* with the most strongly hemiparasitic strategies (i.e. small-bodied species with, low chlorophyll content and reduced leaves) will tend to occur on the most nutrient deficient soils. Conversely, *Thesium* species characterised by greater leafiness and/or greater foliar N and chlorophyll content will be associated with soils having higher nutrient availability. In addition, we hypothesised that leafier and/or greener species of *Thesium* might have higher photosynthetic capacities than non-leafy species making them less reliant on host-derived C. This study set out to (i) quantify variation in C acquisition-associated traits between *Thesium* species from the CFR, and, using these traits, to verify the existence of distinct heterotrophy strategies; (ii) assess experimentally whether *Thesium* species that display trait divergence also vary in physiological processes such as photosynthetic and transpiration rates; and (iii) evaluate, from an evolutionary perspective, whether these heterotrophy-associated traits are associated with edaphic and environmental variation.

Materials and Methods

Field data collection and species sampling

I sampled twelve morphologically diverse species of *Thesium* from sites on and around the Cape Peninsula in the south-western part of the CFR, spanning a range of soil types (Appendix 2A). In order to capture variation between populations, each species was sampled from at least three separate localities, with whole plants of three individuals of each species being sampled at each locality. In addition to plant material, 500 g of soil was taken from the top 20 cm of soil directly beneath each of the individuals sampled. Field sampling

took place between October 2008 and February 2009 and again between November and December 2009.

Plant trait analysis

Maximum plant height was measured *in situ* for all individuals sampled. In order to characterise leafiness and photosynthetic area, leaves were detached from the shoots to allow leaf area (LA) and stem area to be measured separately. Shoot material was photographed by placing shoots between two sheets of glass along with a reference square of 1 cm^2 and a ruler. Images were imported into Photoshop v 7.0 (Adobe Systems Inc., USA), and the area of the plant material (in pixels) measured using the "Magic Wand" tool with reference to an object of known area. The maximum stem diameter (SD) of each shoot sample was measured using digital callipers and a leafiness index (LA/SD) calculated. Shoot chlorophyll concentration was measured using the protocol outlined by Hiscox & Israelstam (1979).Briefly, 10 g of fresh plant material was cut fine and placed into 7 mL of dimethyl sulfoxide (DMSO) and incubated at 60°C for approximately 4 h, or until plant tissue was cleared. After incubation, a further 3 mL of DMSO was added and the DMSO extract used for spectrophometric analysis at both 645 and 663 nm. The chlorophyll concentration was calculated using the equations of Arnon (1949). Plant tissue was dried for approximately 48 h at 70 °C, and crushed into a fine powder using a ball mill (MM200, Retch, Haan, Germany) and tissue N and C determined using mass spectrometry, with leaf and stem material were analysed separately. 2.8 ± 0.2 mg of milled leaf or stem material of each sample was weighed into an 8 x 5 mm tin capsule (Elemental Microanalysis Ltd, Okehampton, UK). The samples were then combusted in a Thermo Flash EA 1112 series elemental analyzer coupled to a Delta Plus XP isotope ratio mass spectrometer via a Thermo Finnigan Conflo III control unit (Thermo Electron Corporation, Milan, Italy). Sucrose and two additional in-house standards and were used to calibrate the results.

Soil analysis

Soil samples were air dried and sieved through a 2 mm sieve prior to analysis. Soil pH was measured by shaking 2 g of material in 20 mL 1 M KCl at 180 rpm for 60 min, centrifuging at 10 000 *g* for 10 min and measuring the pH of the supernatant. Available [P] was determined by extracting 2 g of soil in Bray II solution (Bray & Kurtz 1945) which was filtered through Whatman No.2 filter paper. The filtrate was analyzed colorimetrically using the Malachite Green method (Motomizu *et al.*1983). Total [P] was determined according to Bray & Kurtz (1945) using ICP-AES analysis. Exchangeable cations were displaced from 10 g of sample with 25 mL of 0.2*M* ammonium acetate. The samples were filtered through Whatman No. 2 filter paper and made to 200 mL before the concentrations of Ca, K, Mg and Na were determined using ICP-AES analysis. The concentrations of these cations along with the concentration of H^+ ions were used to calculate soil T-value. The T-value is a measure of soil cation exchange capacity (CEC), which is the maximum quantity of total cations that a soil can hold, for a given pH value. It is a useful measure of soil fertility and plant nutrient availability (Tisdale *et al.* 1993). Total soil N was determined using mass spectrometry as outlined above, using*ca*. 32 mg of sieved soil per sample.

Habitat specificity

In order to determine whether *Thesium* species in the CFR display affinity for particular substrates, all 87 CFR species were coded for their presence or absence on four major substrate groupings in the region. Species scoring was based on collection information of species in the Pretoria(PRE) and Bolus(BOL) herbaria, as well as habitat descriptions found in Goldblatt & Manning (2000). A species was scored as being associated with a particular substrate if more than 10% of herbarium records were sampled from that substrate type. The numbers of species expected to occur on each substrate were then determined on the basis

of the area of the CFR (percentage) covered by each substrate, and a χ^2 test performed to test whether the observed associations deviated significantly from chance expectation.

Trait-environment associations

Trait and edaphic variation between *Thesium* species was evaluated using nested-design ANOVA, with populations of individuals being nested within species and post-hoc Tukey Honest Significant Difference (HSD) tests used to identify significant differences. Relationships between *Thesium* traits and environmental variables were evaluated using standard correlation-regression analyses performed in STATISICA 9.0 (Statsoft Inc., 2009). Given that differences in morphology between species might reflect differences in degree of host dependence, the first factor (PC) derived from a principal components analysis (PCA, Fig 4) based on all of the measured plant variables was used as an index of heterotrophy (HET I). In the same way, a PCA was conducted using soil pH, EC and T-value to produce a soil fertility axis. However, because pH appeared to vary along a different axis (Appendix 4), the PCA was repeated with T-value and EC and the first PC of this PCA used as an index of soil fertility (SF I). Soil N, P and pH were used as independent measures of soil fertility.

In order to account for non-independence of species due to phylogenetic relatedness, all correlations were evaluated using both raw species trait values (TIPs) and using phylogenetic independent contrasts (PICs, Felsenstein 1985). For this purpose, 100 phylogenetic trees were sampled from the posterior distribution of the BEAST analysis presented in chapter 2, which contained sequence data from over 70 *Thesium* species. These trees were then pruned to include only the 12 species for which trait data were available. These trees were incorporated into the PIC analyses, which were conducted in R 2.10.1 (R Foundation for Statistical Computing), incorporating the PICANTE analysis package (Kembel *et al.* 2010).

Pot experiment

Seedlings of *Thesium nigromontanum* Sond. species were collected from a recently burnt site on the top of the Franschhoek Pass (33.92° S; 19.16°E), while *Thesium capitatum* L. seedlings were collected from post-burn vegetation on the Vijoen's Pass (34.10°S; 19.05°E), in the Western Cape, South Africa. Vouchers of both species have been deposited in BOL. *T. capitatum* is a leafy shrub, growing up to 40 cm tall, which occurs primarily on sandy slopes throughout the Western Cape. *T. nigromontanum* is a slender, generally low-growing shrub, with very few leaves, occurring primarily on sandstone flats and slopes in the Western Cape. Seedlings were transplanted into pots filled with acid-washed sand. Each pot was fertilized with slow-release fertilizer (Phostrogen: Beyer Garden, Cambridge) having an N:P:K ratio of 15:7:15, which was reapplied every two months. Plants were kept in a greenhouse at the University of Cape Town from October 2009 and watered for 5 min once a day using an automated irrigation system. After allowing parasite seedlings to establish for four months, one third of the pots were sown with seeds of C_4*Zea mays* L.*,* one third with seeds of a native C_3 grass *Ehrharta erecta* Lam., while in one third of the pots *Thesium* individuals were left without a host. Grasses were selected as hosts because (i) they are easy to germinate and propagate, (ii) recent evidence suggests that Poaceae is a preferred host group of *Thesium* (Suetsugu *et al.* (2008), and (iii) grasses show the required variation in photosynthetic system required for this experiment. Host plants were allowed to germinate and grow for another six weeks, after which pots were moved into a phytotron growth chamber. Chambers were maintained at a maximum temperature of 25 ºC and approximately 50% humidity during the day, with 14h of daylight. Night-time temperatures were maintained at 16 ºC. Parasite and host gas exchange measurements, including photosynthesis (*A*) and transpiration (*E*), were conducted using a LiCor-6400 IRGA (LiCor Instruments, Lincoln, NE, USA), using a 6400-07 Needle chamber. CO_2 concentration in the cuvette was maintained at approximately 420 ppm, and humidity maintained at approximately 50% RH. Shoot tissue from five individuals of each parasite species for each

treatment was then sampled, oven-dried at 70°C and analysed for isotopic signatures using mass spectrometry as outlined above. The proportion of C (after Press *et al.* 1987) derived heterotrophically for each species was calculated using the equation:

$$\frac{P_M - P_{NH}}{M - P_{NH}}$$

where P_M is the $\delta^{13}C$ of the parasite attached to *Z. mays*, P_{NH} is the $\delta^{13}C$ of the parasite without a host and M is the $\delta^{13}C$ of *Z. mays*. Gas exchange, shoot N and isotopic signature between species grown with or without either *Z. mays* or *E. erecta* were evaluated using factorial ANOVA, with parasite species set as a random factor. Tukey HSD tests were performed to evaluate differences between treatments.

Herbarium Sampling

In order to test whether host $\delta^{13}C$ had a significant effect on the $\delta^{13}C$ of the parasite, ten species of *Thesium* from the C_4 grassland region of South Africa were sampled from herbarium sheets housed in BOL (Appendix 2B), and their $\delta^{13}C$ signature compared to the 12species collected fresh from the C_3-dominated fynbos vegetation of the CFR. Three individuals per species were sampled, and their shoot isotopic signatures measured according to the protocol outlined above.

Results

Plant traits

Field sampled material showed significant differences between *Thesium* species with respect to heterotrophy-associated traits (Fig 2). Plant height varied significantly between species ($F_{11,96}$= 38.66, $p < 0.01$), ranging from 12 cm in *T. capituliflorum* to 129 cm in *T. strictum*. Plant leafiness (LA/SD) also differed significantly ($F_{11,96}$ = 33.49, $p < 0.01$), as did

chlorophyll content ($F_{11,24}$ = 4.63, p <0.01) and leaf N ($F_{9,14}$ = 5.43, p < 0.01). Using TIP data, plant height showed a significant positive association with chlorophyll content and a weak but non-significant positive association with leafiness (Fig 3).

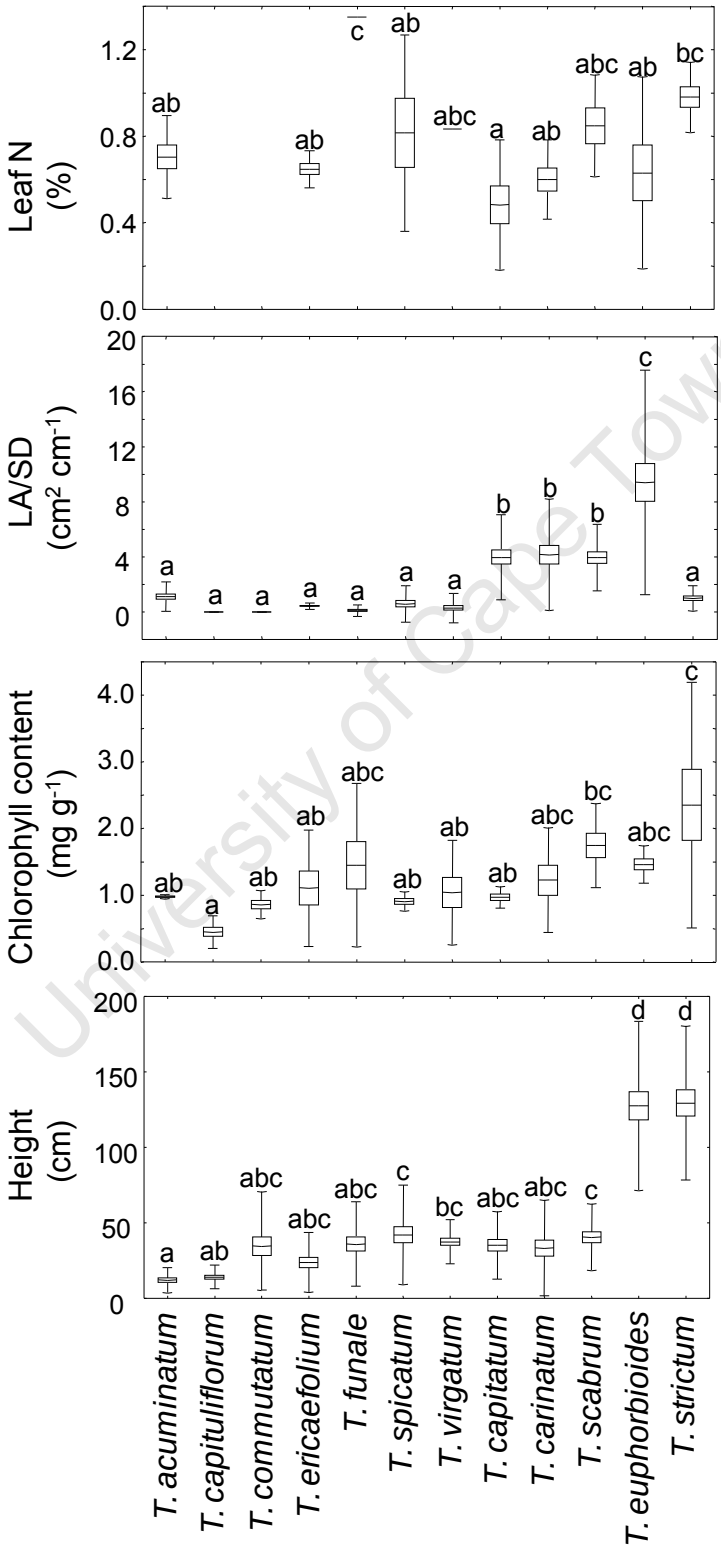


Figure 2. Box and whisker plots showing variation in traits between 12 species of *Thesium* sampled from the CFR. Lines represent means, boxes standard error, and whiskers standard deviation. Letters represent significant differences (α < 0.05) between groups based on a post-hoc Tukey HSD test on ANOVA results.

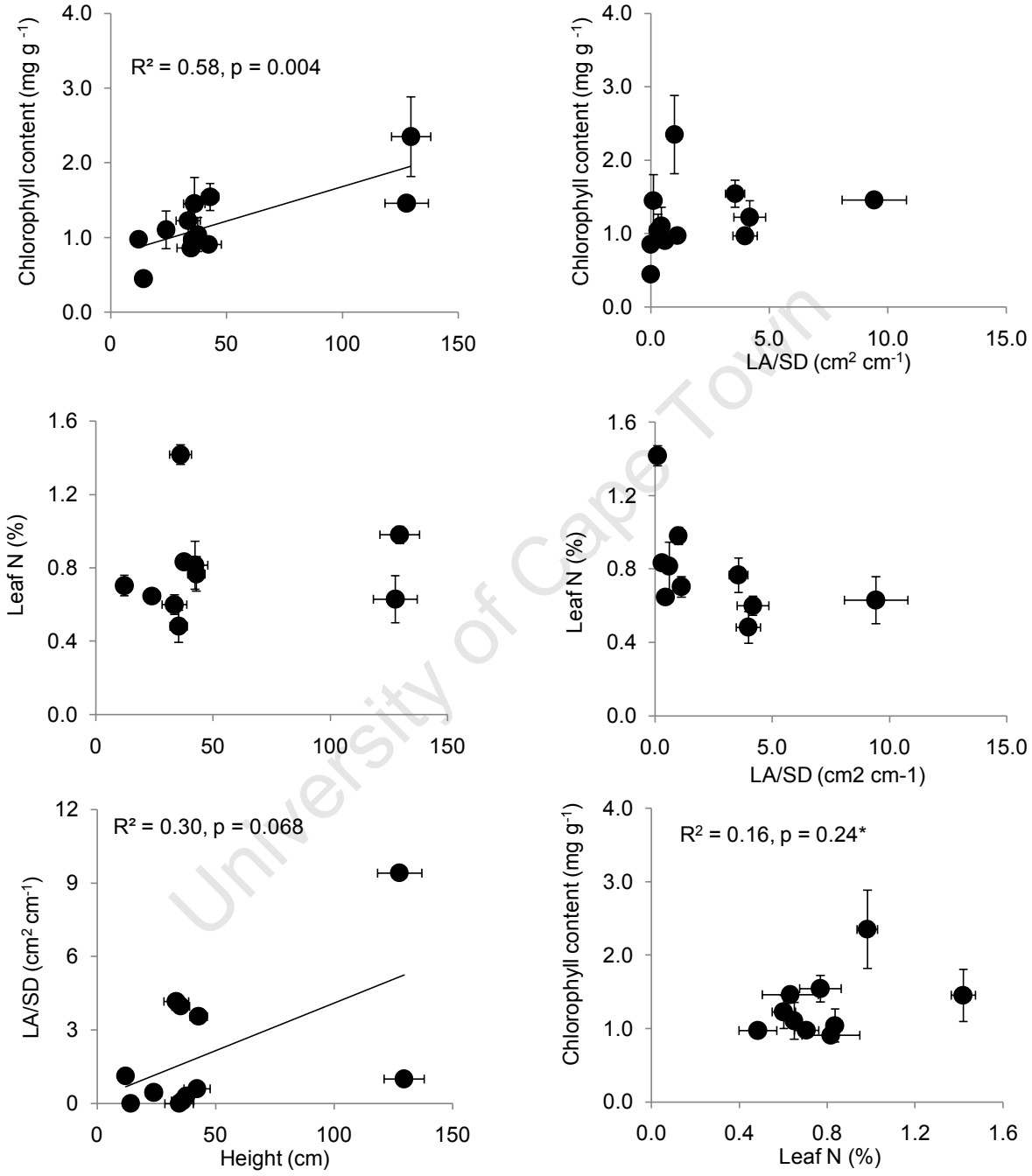


Figure 3. The relationships between mean trait values for twelve *Thesium* species sampled from the CFR, based on TIP values. For each species, three individuals were sampled from each of three separate localities (n=9). Error bars represent standard errors. Correlation statistics shown are based on TIP values. Asterisks after p-values indicate significant PIC correlations (α = 0.05).

Although two species differed markedly in height from the rest (*T. strictum* and *T. euphorbioides*), the trends did not change if the height axes in these figures were logged. Using PIC data, height did not correlate significantly with any other trait variable. In contrast, Chlorophyll content showed a significant association with Leaf N when evaluated using PIC (Fig 3).

A PCA of the trait data revealed the presence of three loose groupings (Fig 4), comprising i) the two tallest species (> 1 m in height,*T. strictum* and *T. euphorbioides*), ii) the three leafiest, shorter (< 1 m tall, with LA/SD values between 3-4 cm^2 cm^{-1}) species (*T. carinatum, T. capitatum* and *T. scabrum*) and iii) the remaining seven species which were less than 1m tall and had LA/SD values of less than 3 cm^2 cm^{-1}. The first two factors captured80.20% of the variance in the data, the first PC (HET I) being most strongly associated with plant height (R^2 = -0.89, $p < 0.001$) and chlorophyll content (R^2 = -0.72, $p < 0.001$). This factor was then used as an additional trait variable in subsequent analyses.

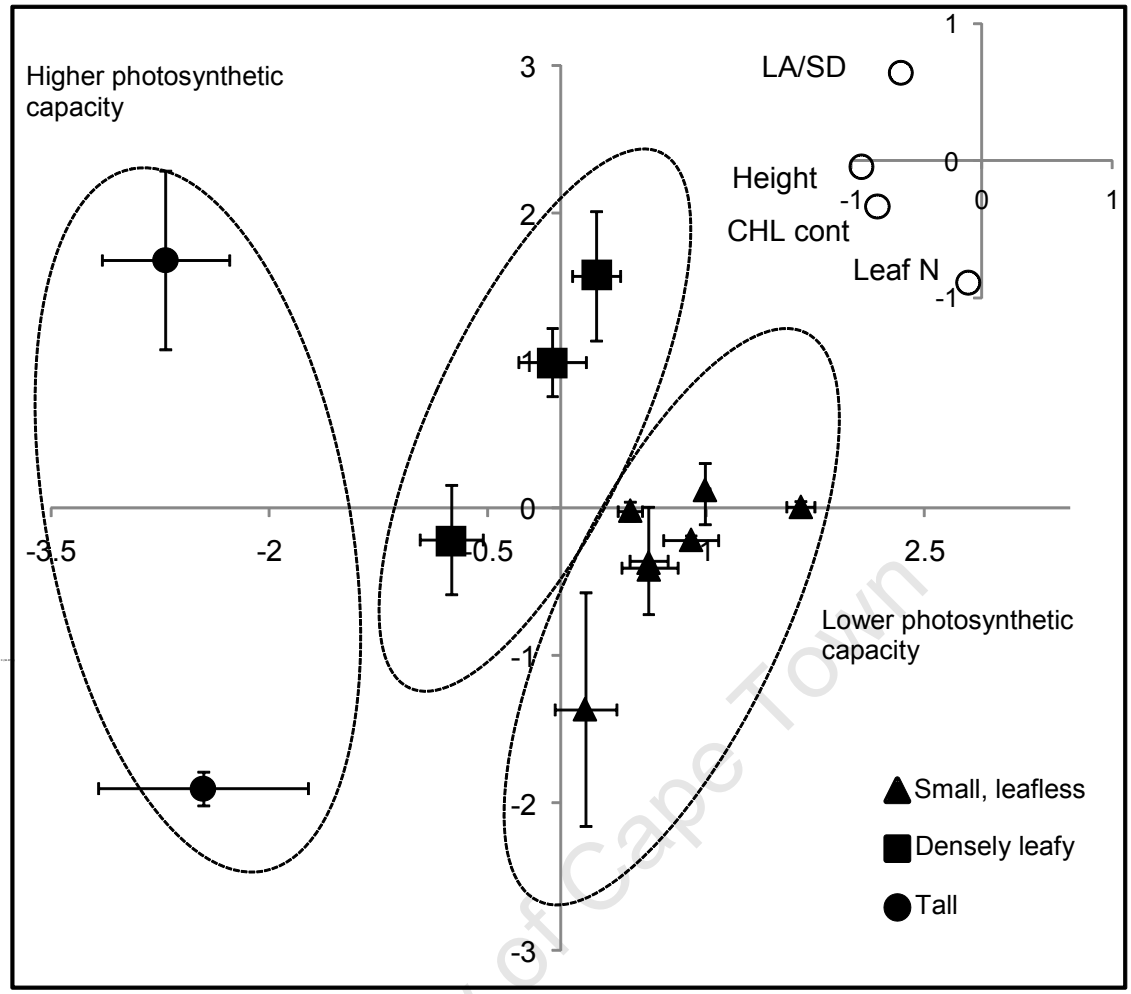


Figure 4. Principal component analysis based on four carbon acquisition-associated trait variables. Symbols represent different 'strategies', corresponding to groupings identified in Figure 1. Inset figure shows the relationship between the trait variables used in the analysis, and their contribution to each axis of the PCA.

Habitat specificity

The distribution of *Thesium* species on the different substrate types within the CFR differed significantly from what would be expected if species were distributed randomly with respect to substrate (Table 1), with more *Thesium* species than expected occurring on sandstone. In contrast, fewer species than expected occur on shale. In addition, species differed significantly in their edaphic niches (Table 2), with only available P not differing significantly

between species. The soils on which all species were found were oligotrophic, having relatively low values of N, P, EC and T-value. Soils were generally acidic, with pH values between approximately 3.0 and 5.5. Soil pH was not significantly associated with either soil total P or soil N but was significantly associated with soil available P (Appendix 3). The combined soil fertility index (SF I), the first PC based on a PCA of T-value EC (Appendix 4) captured 72.16 % of the total variance, and was strongly correlated with T-value (R^2 = 0.42,

Table 1. The observed number of species of *Thesium* on each major substrate in the CFR based on collection information of species from the Pretoria (PRE) and Bolus (BOL) herbaria, as well as habitat descriptions found in Goldblatt & Manning (2000). Expected numbers of species for each substrate determined on the basis of the area of the CFR (percentage) covered by each substrate. Observed numbers of species differed significantly from expected (X_3^2 = 12.32, p< 0.01). Areas of each substrate from Hoffmann *et al.* (unpublished).

	Area (%)	**Observed**	**Expected**
Sandstone (Table Mountain group)	46.34	73	61
Shale	24.98	17	33
Quaternary System	15.81	19	21
Other[1]	12.87	23	17

[1] Cape Granite, Limestone, Calcareous , Silcrete

p < 0.01) and weakly correlated with EC (R^2 = 0.30, p < 0.1).

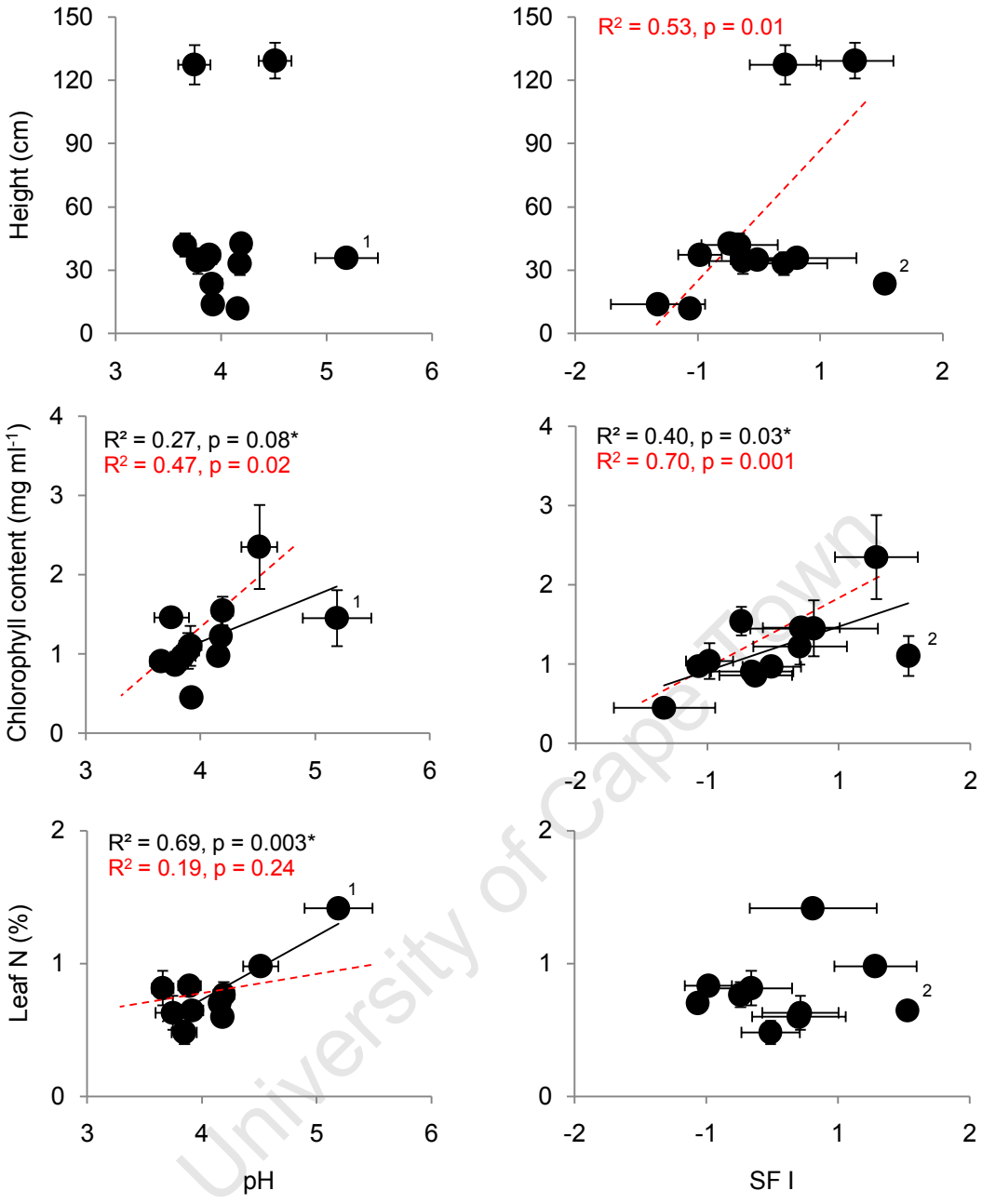


Figure 5. The relationships between pH and soil fertility (SF I) and carbon acquisition-associated traits based on field samples obtained for 12 Cape *Thesium* species, based on TIP values. SF I represents the first principal component of a PCA based on EC and T-values for each species (Appendix 4). For each species, three individuals were sampled from each of three separate localities (n=9). Error bars represent standard errors. Correlation statistics shown are based on TIP values. Asterisks after p-values indicate significant PIC correlations (α = 0.05, see Appendix 1). Dashed lines and statistics presented in red type describe relationships with *T. funale* (1) or *T. ericaefolium* (2)omitted.

Trait-environment associations

There was general agreement between TIP and PIC correlations (Appendix 1), although in five instances PICs revealed significant relationships that were not significant when evaluated using TIPs. However, in none of these cases did the nature of the relationships change (Appendix 1). Likewise, on only two occasions did TIP comparisons identify significant relationships that were not significant when PICs were used, and again the nature of the relationships did not change between analyses (Appendix 1). Using TIPs, soil pH was significantly associated with leaf N (Fig 5), but with no other plant traits. With PICs, however, soil pH also showed a significant association with chlorophyll content (Appendix 1). Neither soil N nor P were significantly associated with any plant traits, whether evaluated using TIPs or PICs (Appendix 1). SF I was, however, significantly associated with chlorophyll content (Fig 5), and HET I (Fig 6) using TIP analysis. In addition, soil EC was significantly associated with both chlorophyll content and HET I when tested using PICs.

Table 2. Differences in soil properties for each species base on a nested design ANOVA based on field-collected soil samples for 12 CFR *Thesium* species. For each species, three individuals were sampled from each of three separate localities (n=9). Asterisks indicate significant p-values at p< 0.05.

Soil Variable	***df***	**F-stat**	**p-value**
pH	11, 72	38.31	0.000*
EC	11, 72	1.96	0.045*
T-value	11, 72	19.87	0.000*
Total P	11, 72	8.64	0.000*
Bray II P	11, 72	1.15	0.335
Soil N	11, 72	2.79	0.004*

Two species were found to have a significant impact on the outcome of TIP correlations owing to their high leverage (Figs 5, 6). When *T. funale* was removed from the analyses, the associations between soil pH and chlorophyll content were strengthened. In contrast, the

association between soil pH and leaf became non-significant. Similarly, the associations of SF I with height, chlorophyll content and HET I were strengthened when *T. ericaefolium* was excluded.

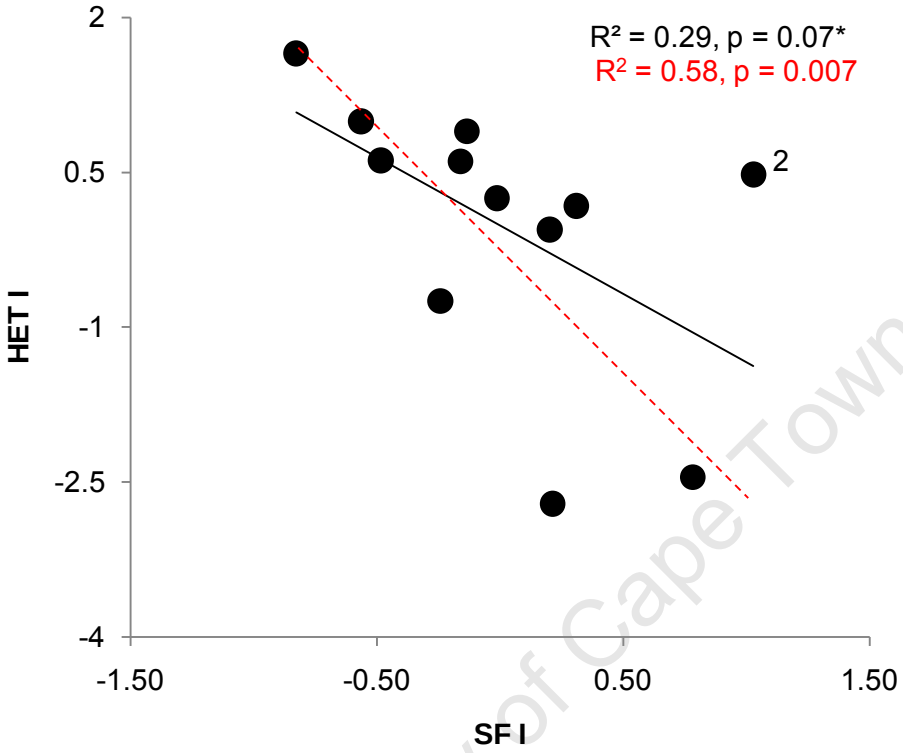


Figure 6. The relationship between heterotrophy (HET I) and soil fertility (SF I) across 12 Cape *Thesium* species , based on TIP values. HET I represents the first principal component of a PCA based on a suite of traits used as proxies for degree of heterotrophy (Figure 4). SF represents the first principal component of a PCA based on EC and T-values for each species (Appendix 4). Error bars represent standard errors. The dashed line and statistics presented in red type describe the relationship with*T. ericaefolium* (2) omitted.

Host carbon contribution and parasite gas exchange characteristics

Species sampled from grassland sites displayed higher tissue $\delta^{13}C$ values (mean ± standard error: -25.46 ± 0.53‰) compared with species sampled from fynbos communities in the CFR (28.10 ± 0.30‰), this difference being significant (Table 3, $t_{12} = 4.4$, $p < 0.01$). Mean photosynthetic rates were significantly higher in *T. capitatum* than in *T. nigromontanum*

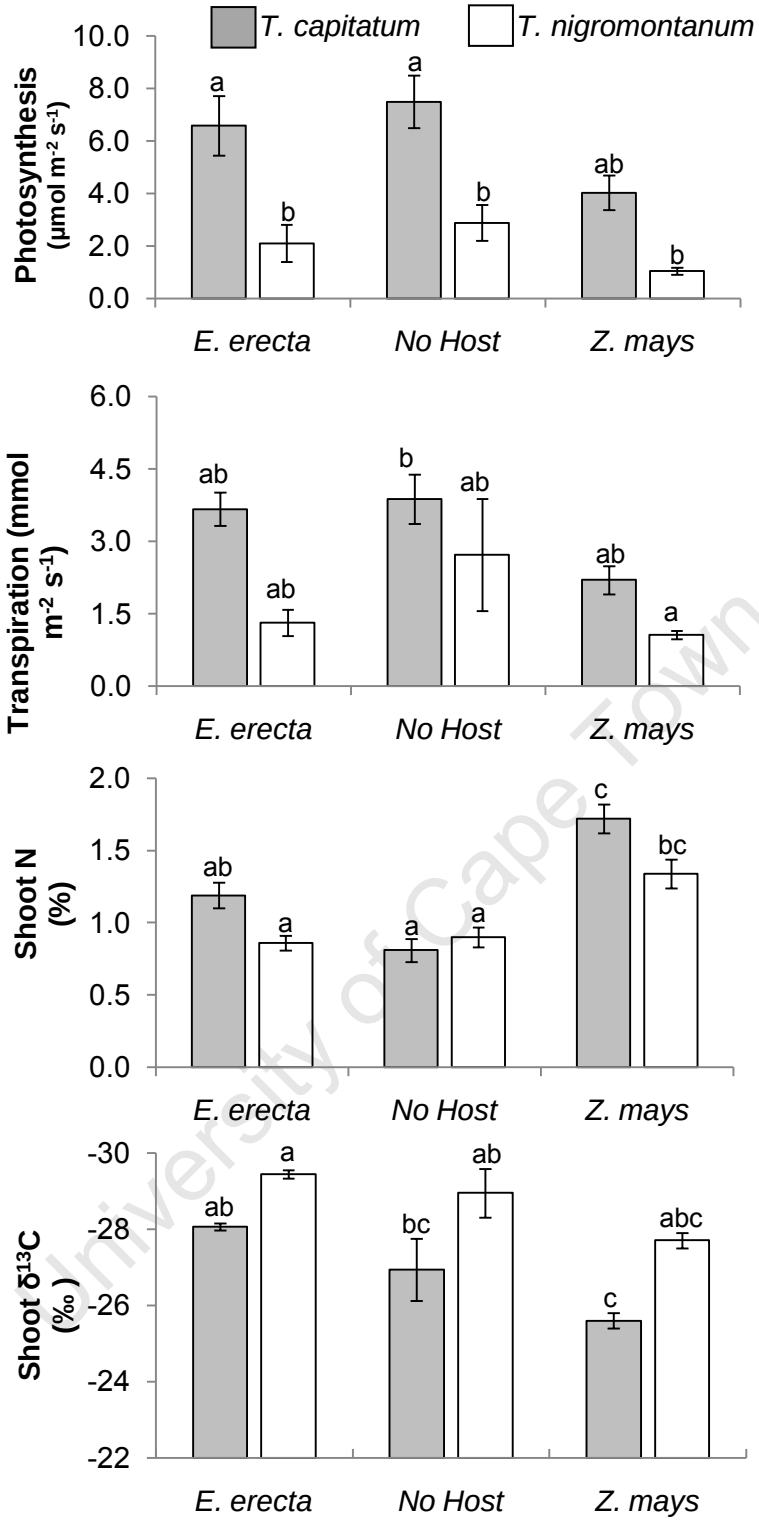


Figure 7. Gas exchange, tissue N and $\delta^{13}C$ characteristics of *Thesium capitatum* and *T. nigromontanum* individuals, grown without a host and attached to *Zea mays* (C_4) and *Ehrharta erecta* (C_3). Bars represent standard errors. Letters represent significant differences between treatments based on a post-hoc Tukey HSD test, after a factorial-design ANOVA was performed. Differences are significant at $p < 0.05$.

Table 3. Shoot carbon isotope ($\delta^{13}C$) signatures for 22 *Thesium* species sampled from two major biomes in South Africa. The 12 fynbos species correspond to the species sampled in this study, while the grassland species were sampled from herbarium material housed in BOL. Values in bold type indicate mean $\delta^{13}C$ values of species sampled from each region.

Locality	Species	$\delta^{13}C$ (SE) ‰
Fynbos (C3)	*T. acuminatum*	-28.37 (0.81)
	T. capitatum	-28.11 (0.81)
	T. capituliflorum	-29.66 (0.63)
	T. carinatum	-28.56 (0.53)
	T. commutatum	-29.06 (0.81)
	T. ericaefolium	-27.08 (0.81)
	T. euphorbioides	-27.28 (0.81)
	T. funale	-28.38 (0.63)
	T. scabrum	-27.85 (0.57)
	T. spicatum	-27.87 (0.57)
	T. strictum	-27.07 (0.63)
	T. virgatum	-27.94 (0.57)
	Mean	**-28.10 (0.30)**
Grassland (C4)	*T. angulosum*	-26.37 (0.84)
	T. asterias	-26.07 (0.07)
	T. costatum	-24.77 (0.83)
	T. cupressoides	-27.28 (0.54)
	T. gracile	-27.18 (1.58)
	T. magalisbergensis	-25.05 (0.29)
	T. racemosum	-23.99 (0.88)
	T. transvaalsense	-21.50 (1.31)
	T. triflorum	-26.91 (1.06)
	T. imbricatum	-25.43 (0.17)
	Mean	**-25.45 (0.53)**

across all treatments ($F_{5,38} = 8.29$, $p < 0.01$), except when *T. capitatum* was grown with *Z. mays* (Fig 7), although photosynthetic rates did not differ significantly between treatments for either species (Fig 7). Transpiration rate also differed between treatments ($F_{5,38} = 3.49$, $p < 0.01$), but the post hoc test revealed that the only significant difference was between *T. capitatum* individuals grown without a host and *T. nigromontanum* species grown with a maize host (Fig 7).

Shoot tissue N content was significantly different for both species across treatments (Fig 7, $F_{5,24} = 14.51$, $p < 0.01$), with both species having significantly higher tissue N concentrations

when attached to *Z. mays* hosts compared to unattached individuals and individuals attached to *E. erecta*. The tissue N concentrations of individuals attached to *E. erecta* did not differ from those of unattached individuals. Treatments also differed significantly in shoot $\delta^{13}C$ (Fig 7, $F_{5,24} = 6.41$, $p < 0.01$), with *Z. mays*-attached individuals of *T. capitatum* having significantly higher values than those of individuals attached to *E. erecta* or unattached individuals. In contrast, *T. nigromontanum* individuals did not differ significantly across treatments in terms of $\delta^{13}C$ values. In both species, however, there was a trend for a decrease in $\delta^{13}C$ between individuals attached to *E. erecta* and individuals attached to *Z. mays*, with unattached individuals having intermediate $\delta^{13}C$ values.

Discussion

Hemiparasites are often poorly represented in nutrient rich systems, tending to favour disturbed, nutrient poor habitats (Matthies 1995, Watson 2009). The CFR has a disproportionate number of *Thesium* species for its area relative to the rest of South Africa (85 out of approximately 160 species, Goldblatt & Manning 2000). Furthermore, within the CFR, more *Thesium* species are associated with nutrient-deficient sandstone-derived substrates than would be expected if species were distributed randomly with respect to subtsrate, while fewer than expected species are associated with the more nutrient-rich shale (Table 1). One possible explanation for this bias is that parasitism offers greater benefits in nutrient poor systems (e.g. Matties 1995). Alternatively, parasitic plants may be less efficient in using available soil resources in more nutrient rich systems, causing them to be competitively excluded from the more nutrient rich environments (Matthies 1995). Hemiparasites are not entirely dependent on their hosts, retaining their capacity to acquire some of their resources (e.g. light, water, nutrients) autotrophically. Thus host plants not only represent a source of resources for their parasites, but also a source of competition for water, light and soil nutrients.

The data presented in this study reveal significant interspecific variation in traits that are associated with variation in photosynthetic capacity. Taller plants tend to possess more chlorophyll and also have higher leaf N concentrations (Figs 3, 4). Tissue N is related to photosynthetic capacity because the majority of the proteins of the Calvin-cycle and thylakoids account for the bulk of shoot N (Evans 1989). In contrast, small-bodied *Thesium* species tend to have relatively low concentrations of chlorophyll and may thus be expected to have lower photosynthetic capacities. For example, *T. capituliflorum* is the smallest species in this study and displays the lowest mean chlorophyll concentration (0.45 mg g^{-1}), nearly half that of the next largest species. *Thesium* species also differed significantly in leafiness (Fig 2), though this was not well correlated with other autotrophy-linked traits (Fig 3). The absence of leaves may also limit the photosynthetic capacity of plants, because stem photosynthesis is generally lower than leaf photosynthesis (Nilsen *et al.* 1993, Tinoco-Ojanuren 2008, Yiotis *et al.* 2008). This divergence in photosynthetic capacity between *Thesium* species is demonstrated by the differences in photosynthetic rates between *T. capitatum* and *T. nigromontanum* (Fig 7). The leafier *T. capitatum* had significantly higher photosynthetic rates across all treatments, except when grown with a maize host. Photosynthesis in the unattached plants was 2.5 times higher in *T. capitatum*. These variations in trait-associated differences in photosynthetic capacity appear to reflect variation in C-foraging strategies amongst species of *Thesium*, with larger, leafier species of *Thesium* having higher photosynthetic capacities than small-bodied, more aphyllous species, the latter having lower concentrations of chlorophyll and N in their tissues.

Larger-bodied *Thesium* species, which also have greater photosynthetic capacity (Figs4, 7) may be better competitors for resources than the smaller-bodied species, which have lower tissue chlorophyll and N concentrations. This may enable them to perform better in more

eutrophic sites. In contrast, smaller-bodied species, with lower photosynthetic capacities may be restricted to low-nutrient, low-biomass sites in which competition for resources, such as water, light and soil nutrients is low (Pennings & Callaway 2002). In sites where host biomass is high, the negative effects of competition for light and other resources might outweigh the benefits of being parasitic, only allowing the most autotrophic of parasite species to compete. Also, larger-bodied species may have more developed root systems, enabling them to compete better with their neighbours than smaller, more heterotrophic species. Unfortunately, the relationship between above- and below- ground biomass in *Thesium* has received relatively little explicit attention.

Despite possessing some capacity for autotrophy, hemiparasitic plants have been shown to obtain a significant proportion of their C needs via parasitic uptake. For example, Ducharme and Ehleringer (1996) estimated that the facultative hemiparasite *Castilleja linariifolia* derived on average 40% of its C heterotrophically. The use of C_4 hosts as a 'biological tracer' of C supply has been used in previous host-parasite research (Press *et al.* 1987). Plants grown with a C_4 host are expected to have a higher $\delta^{13}C$ values relative to free-living parasites if they obtain a significant proportion of their C from their host. Our data provide evidence for heterotrophic acquisition of C in *Thesium* (Fig 7), with *T. capitatum* individuals showing a tracer effect in two directions. Individuals attached to a *Z. mays* host displayed significantly higher tissue $\delta^{13}C$ values than individuals attached to *E. erecta*. What is not clear from these data, however, is whether the two species of *Thesium* studied differ with respect to the proportion of C acquired parasitically, though my data do suggest that species differ in their ability to manufacture their own photosynthate. Based on the data presented here, *T. capitatum* and *T. nigromontanum* individuals attached to *Z. mays* hosts derive *ca*.7-9 % of their C parasitically. The use of host C by *Thesium* in this study corroborates findings by Fer *et al.* (1993) who used a ^{14}C tracer to demonstrate that *Thesium humile* obtained at least some of its C from its wheat host.

Supporting our conclusion that *Thesium* plants obtain at least some of their C heterotrophically, the survey of tissue $\delta^{13}C$ values for species from C_4 grassland sites and fynbos (the dominant vegetation type in the CFR) species provides corroborative evidence for the use of organic C by *Thesium* species (Table 3). The signal observed could be interpreted as the grassland species using predominantly C_4 hosts and therefore possessing higher $\delta^{13}C$ values than species from the C_3-dominated fynbos. There are, however, two potentially confounding factors in this comparison. Firstly, the comparison of grassland and fynbos species represents a single phylogenetic contrast (the grassland species are probably related, as are the fynbos species: see chapter 2), such that the observed association of foliar $\delta^{13}C$ values with vegetational association could be incidental, rather than causal. Secondly, differences in climate, particularly in rainfall seasonality, between grassland and fynbos environments may cause foliar $\delta^{13}C$ to differ between regions (Amundson *et al.* 1994). Rainfall seasonality has a significant effect on plant WUE (Voltas *et al.* 2008) efficiency, and may therefore have a significant effect on plant $\delta^{13}C$ signatures (Farquhar *et al*. 1989).Taken with the results from the greenhouse experiment, however, the observed pattern in $\delta^{13}C$ across biomes is consistent with the expectation that *Thesium* species are dependent on their hosts for organic C.

Although *Thesium* species do appear to differ in terms of the edaphic environments they occupy (Table 2), there is no clear association between *Thesium* traits and soil N and total and available P (Appendix 1). This may be the result of these species' ability to acquire some of their requirements for N and P from their hosts (Watson 2009, Dostálek & Münzbergová 2010).Jiang *et al.* (2004) found that attached *Rhinanthus minor* individuals displayed significantly higher tissue N concentrations than unattached individuals. Both *T. capitatum* and *T. nigromontanum* individuals displayed significantly higher tissue N

concentrations when attached to maize compared to unattached individuals (Fig 7), which could be due to *Thesium* species abstracting nutrients from their hosts (Press 1995). This tissue N enrichment was not, however, evident when individuals were grown with *E.erecta*. One reason for this difference could be the difference in size between hosts, the*Z. mays* plants being much larger than the *E. erecta* individuals. *Thesium* individuals grown with *Z. mays* may have had more opportunity for form successful haustorial connections because of the greater abundance of host root material in the pots. Parasite success on a host has been shown to be a function of the physical structure of the host's roots, as well as the host's ability to defend itself (Cameron *et al.* 2006, Jiang *et al.* 2008). Despite this, neither species had a significant effect on either *Z. mays* or *E. erecta* in terms of their gas exchange properties (data not shown), suggesting that the negative effect of the parasites on either host was relatively low. This is supported by the relatively small amount of C that appears to be heterotrophically-derived for each species.

Much of the research in parasitic plant biology has focussed on the influence of soil nutrients on parasitic growth in the absence of a host (Seel *et al.* 1993b) under different nutrient treatments (Salonen & Puustinen 1996), and under elevated CO_2 (Matthies & Egli 1999). Davies & Graves (2000) found that P supply had a significant effect on both host and parasite growth, with high P treatments increasing growth of parasitized hosts by 112% and reducing parasite biomass by 90% compared with low-P treatments. Seel *et al.* (1993a) supplied unattached *Rhinanthus minor* L. parasites with inorganic N, P and potassium, and found that only P resulted in a significant increase in growth of the parasite. Soil pH has also been identified as an important determinant of the distribution of the hemiparasitic *Rhinanthus minor* L., which has been found to occur on a wide range of substrates throughout its range, but is absent from sites with a pH lower than 5 (Westbury 2004). Few studies have attempted to quantify differences in heterotrophy-associated traits between closely related parasitic species and test whether these differences are associated with

differences in the edaphic environments occupied by these species. In the current study, I find no clear association between *Thesium* trait divergence and either soil N or total P. Plant available P is, however, significantly associated with soil pH (Appendix 3) and could thus be important, indirectly, in determining the distributions of *Thesium* species. My results support the suggestion that divergence in C acquisition traits in *Thesium* is associated with variation in the physical and chemical properties of the soils inhabited by different parasite species, with *Thesium* species with potentially higher photosynthetic capacities tending to occur on soils having higher pH, higher EC and higher cation exchange capacity (Figs 5, 6). The heterogeneity of soil types in the CFR, and their associated differences in fertility, might have played an important role in promoting the high morphological diversity that is observed in *Thesium* species in the region.

Conclusion

The twelve species of *Thesium* that were sampled in this study displayed significant variation in traits associated with their ability to produce autotrophic C. These traits were significantly correlated with edaphic variables important for determining the availability of soil nutrients to plants. The fact that associations are also seen when PICs are used indicates that historical shifts in plant traits have been consistently associated with edaphic niche shifts. I propose that divergence in traits associated with photosynthetic capacity in *Thesium* is the result of adaptation to environments of different fertility. This study corroborates previous studies that have highlighted the importance of soil fertility in the CFR in driving morphological diversification in Cape plants, and may provide insight into the high species diversity of *Thesium* in the CFR.

Appendix 1. a) Correlations between plant trait and edaphic variables based on phylogenetic independent contrast (PICs) analyses, using 100 randomly sampled trees from a posterior distribution of 5000 trees from chapter 2. b) Pearson product-moment correlations describing the associations between plant traits and edaphic variables (TIPs). Bold type indicates correlations significant at $p < 0.05$.

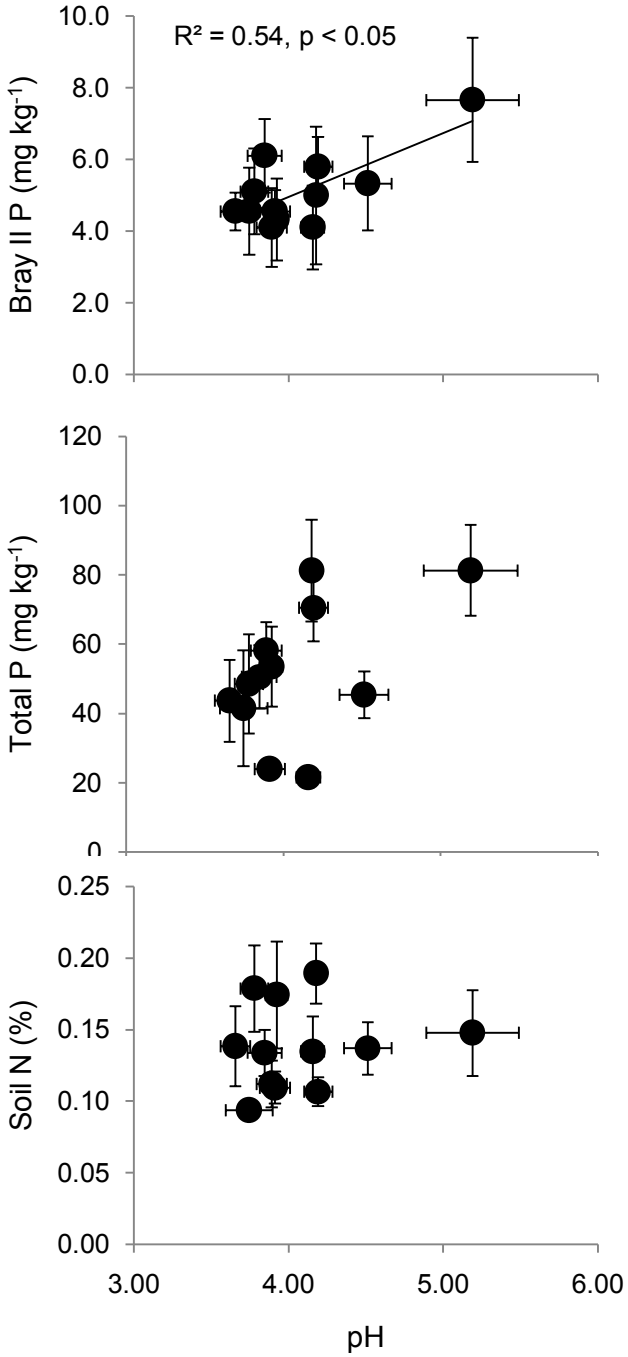
	Plant variable				
Soil Variable	Height (cm)	LA/SD ($cm^2\ cm^{-1}$)	CHL ($mg\ g^{-1}$)	Leaf N (%)	HET I
a) **TIPS**					
pH	-0.04	-0.09	**0.62**	**0.69**	0.53
Total P	0.02	-0.20	-0.11	-0.24	-0.17
Bray II P	0.05	-0.15	0.31	0.24	0.23
Soil N	-0.07	-0.38	-0.34	-0.57	-0.41
SF I	0.36	-0.01	**0.62**	0.35	0.55
EC	0.14	0.23	**0.69**	0.59	**0.66**
T-value	-0.06	0.01	0.45	0.48	0.36
b) **PICS**					
pH	0.06	-0.23	0.52	**0.83**	0.41
Total P	-0.06	0.06	0.15	0.40	0.04
Bray II P	0.04	-0.02	0.36	0.59	0.26
Soil N	-0.38	-0.38	-0.33	0.13	-0.44
SF I	0.46	0.12	**0.63**	0.13	**0.59**
EC	0.32	0.18	0.51	-0.05	0.52
T-value	-0.05	-0.24	0.12	0.00	0.05

Appendix 2A. Sample localities for species used in this study and voucher numbers for specimens housed in the Bolus herbarium (BOL).

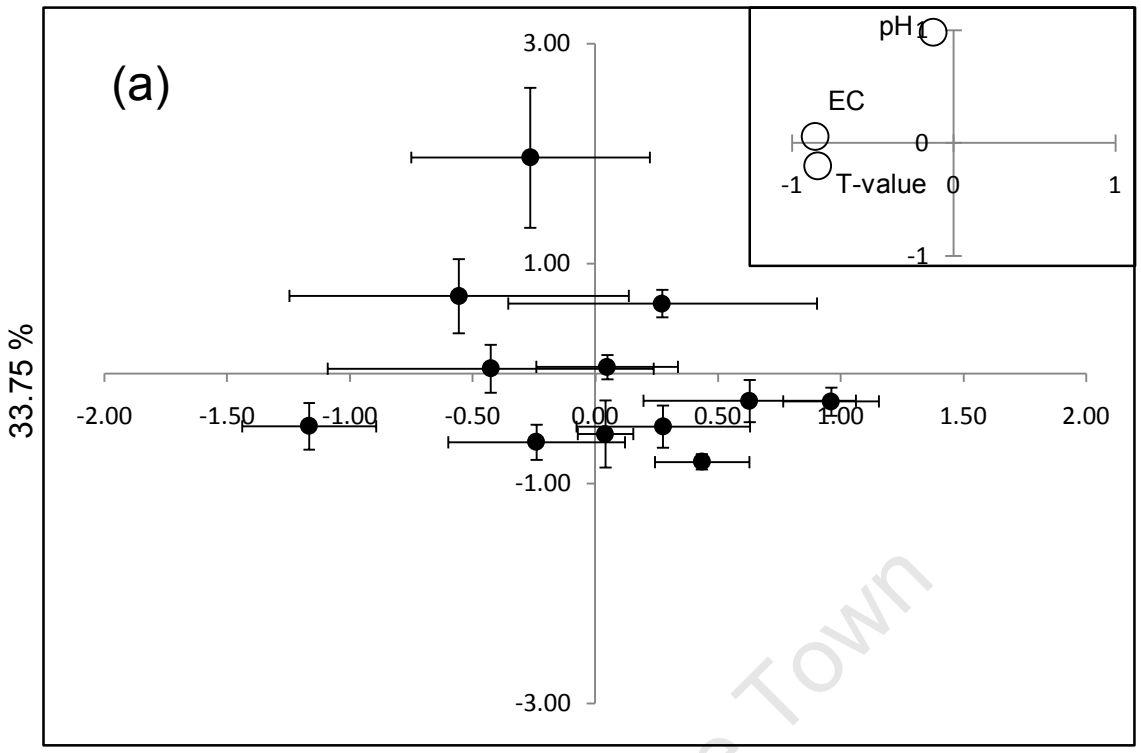
Species	Locality	Voucher	GPS
Thesium acuminatum	Cape Point	Moore 123	34.56°S ; 19.58°E
Thesium acuminatum	Red Hill	Moore 149	34.57°S ; 19.56°E
Thesium acuminatum	Silvermine	Moore 172	34.57°S ; 19.56°E
Thesium capitatum	Jonkershoek	Moore 111	34.56°S ; 19.57°E
Thesium capitatum	Landdros Kop	Moore 126	34.56°S ; 19.57°E
Thesium capitatum	Baarskeerdersbosch	Moore 139	34.57°S ; 19.56°E
Thesium capituliflorum	Franschhoek	Moore 116	34.32°S ; 18.96°E
Thesium capituliflorum	Table Mountain	Moore 130	34.32°S ; 18.96°E
Thesium capituliflorum	Red Hill	Moore 150	33.96°S ; 18.39°E
Thesium carinatum	Helderberg	Moore 112	34.23°S ; 18.41°E
Thesium carinatum	Franschhoek	Moore 115	34.23°S ; 18.41°E
Thesium carinatum	Cape Point	Moore 122	34.23°S ; 18.41°E
Thesium commutatum	Houwhoek	Moore 101	33.90°S ; 19.16°E
Thesium commutatum	Baarskeerdersbosch	Moore 133	33.90°S ; 19.16°E
Thesium commutatum	Buffelstal	Moore 97	33.90°S ; 19.16°E
Thesium ericaefolium	Baarskeerdersbosch	Moore 132	33.90°S ; 19.16°E
Thesium ericaefolium	Hermanus	Moore 170	33.90°S ; 19.16°E
Thesium ericaefolium	Silvermine	Moore 171	34.04°S ; 18.87°E
Thesium euphorbioides	Baarskeerdersbosch	Moore 137	34.04°S ; 18.87°E
Thesium euphorbioides	Landdros Kop	Moore 142	34.42°S ; 19.22°E
Thesium euphorbioides	Buffelstal	Moore 99	34.20°S ; 19.16°E
Thesium funale	Solole (Glen Cairn)	Moore 127	34.20°S ; 19.16°E
Thesium funale	Table Mountain	Moore 129	34.20°S ; 19.16°E
Thesium funale	Red Hill	Moore 151	34.04°S ; 18.87°E
Thesium scabrum	Helderberg	Moore 113	34.02°S ; 18.4°E
Thesium scabrum	Franschhoek	Moore 119	34.05°S ; 18.99°E
Thesium scabrum	Baarskeerdersbosch	Moore 141	33.93°S ; 18.39°E
Thesium spicatum	Houwhoek	Moore 105	34.18°S ; 18.38°E
Thesium spicatum	Sir Lowry's Pass	Moore 107	34.18°S ; 18.38°E
Thesium spicatum	Franschhoek	Moore 117	34.20°S ; 18.40°E
Thesium strictum	Houwhoek	Moore 106	34.10°S ; 18.44°E
Thesium strictum	Cape Point	Moore 121	34.10°S ; 18.44°E
Thesium strictum	Camps Bay	Moore 153	34.15°S ; 18.94°E
Thesium virgatum	Franschhoek	Moore 120	34.15°S ; 18.39°E
Thesium virgatum	Bardskeerdersbosch	Moore 131	33.95°S ; 18.40°E
Thesium virgatum	Lions Head	Moore 156	34.56°S ; 19.58°E

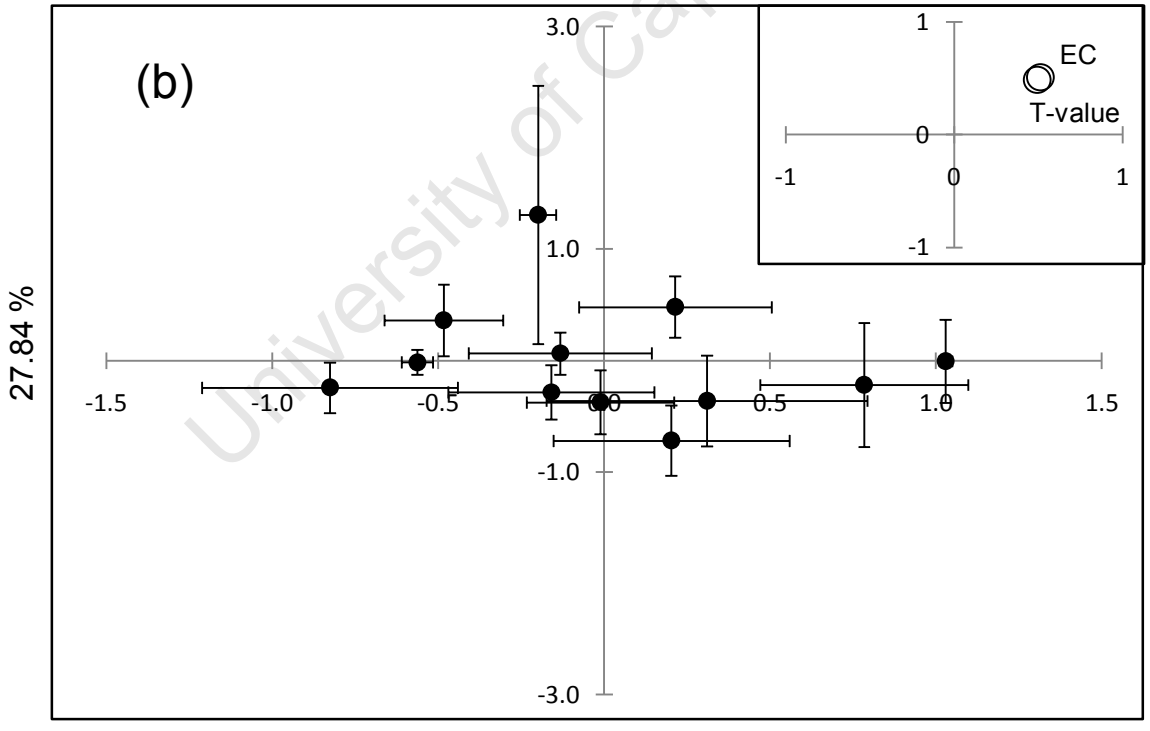
Appendix 2B Specimens sampled from the Bolus herbarium (BOL) for $\delta^{13}C$ isotope analysis

Species	Locality	Bolus Reference/ Voucher Number
T. asterias	Swaziland	BOL 43616
T. asterias	Kwa Zulu Natal	BOL 43617
T. asterias	Drakensberg, KZN	TTS 432
T. imbricatum	Andriesberg, EC	BOL 42715
T. imbricatum	Nieuweveld Mts, EC	BOL 42716
T. imbricatum	Lesotho	BOL 42719
T. triflorum	Barberton, MPA	BOL 8206
T. triflorum	Barberton, MPA	BOL 8207
T. triflorum	Free State	BOL 8208
T. gracile	Sani Pass, KZN	TLN S/n
T. gracile	Barberton, MPA	TV 1054
T. gracile	Cathedral Peak, KZN	TTS 424
T.cupressoides	Cathedral Peak, KZN	Esterhysen 17358
T.cupressoides	Cathedral Peak, KZN	Esterhysen 17358
T.cupressoides	Drakensberg, KZN	TV 1026
T. costatum	Lesotho	Compton 21249
T. costatum	Eastern Free State	Stauffer 1963
T. costatum	Lesotho	BOL 8094
T. magalismontanum	Waterberg, Gauteng	BOL 42777
T. magalismontanum	Magalisberg,Gauteng	BOL 42776
T. magalismontanum	Doornkraal, KZN	BOL 42775
T. angulosum	Kwa Zulu Natal	BOL 8203
T. angulosum	Drakensberg, KZN	BOL 8195
T. angulosum	Drakensberg, KZN	BOL 8196
T. racemosum	Swaziland	BOL 8810
T. racemosum	Belfast, KZN	BOL 8109
T. racemosum	Drakensberg, KZN	BOL 8107
T. transvaalense	Free State	BOL 67761
T. transvaalense	Krugersdorp, Gauteng	BOL 42783
T. transvaalense	Rustenberg, Gauteng	BOL 42753



Appendix 3. The relationships between soil pH and soil N and P values, based on field samples obtained for twelve species of *Thesium* from the CFR, using TIP values. For each species, three individuals were sampled from each of three separate localities (n=9). Error bars represent standard errors. Significant linear correlations are included.





Appendix 4. Principal component analyses based on soil variable data obtained for 12 *Thesium* species sampled from the CFR, with pH, EC and T-value (a) and just EC and T-value (b). Inset figure shows the relationship between the trait variables used in the analysis, and their contribution to each axis of the PCA.

Chapter 4

Synthesis

The rather non-descript morphology of *Thesium*, which is probably a direct consequence of its parasitic lifestyle, has resulted in the genus being largely overlooked by researchers in the Cape flora (Visser 1981, Goldblatt and Manning 2002).The genus is, however, highly variable in growth form, with some species being small-bodied, yellow and leafless, and others being tall and sometimes leafy. At the same time, although some ecological (Suetsugu *et al.* 2008, Dostalek & Munzbergova 2010) and physiological (Fer *et al.* 1993) work has been conducted on northern hemisphere relatives of South African *Thesium* species, almost no work has been done locally (de la Harpe *et al.* 1981, Visser 1981).

Few studies of Cape plant groups have explicitly tested both the pattern of, as well as the evolutionary forces, responsible for radiation (but see Verboom *et al.* 2004, Ellis *et al.* 2006). Building on earlier work exploring the adaptive radiation of Cape plant groups, the aim of this thesis was to test the hypothesis that the species richness of *Thesium* in the CFR is a consequence of adaptive radiation. In order to test this over-arching hypothesis, I set out to show that (i) the genus has undergone a radiation in the CFR and (ii) that diversification has been associated with adaptive divergence in traits associated with divergence in the degree of parasitism between species.

An adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage (Schluter 1996, 2000). Schluter (2000) identifies four features that are necessary for the detection of an adaptive radiation. These are common ancestry, phenotype-environment correlation, trait utility and rapid speciation. There is relatively strong support for the presences of a Cape *Thesium* clade, which is sister to a clade of species

from outside of the CFR and Tropical Africa (Chapter 2). The origin of Cape-based species occurred in the Miocene, approximately 26 Mya, which is congruent with the origins of several other groups of Cape plants (Richardson *et al.* 2001, Verboom *et al.* 2003, Bakker *et al.* 2005, Forest *et al.* 2007). The radiation leading to the extant diversity of Cape*Thesium* species occurred subsequently, however, beginning approximately 17 Mya. A similar pattern is seen in Cape *Pelargonuim* species (Bakker *et al.* 2005). In addition, I found that *Thesium* is likely to have originated in southern Africa, with subsequent dispersal into Europe and South America. This supports the hypothesis of Hendrych (1972), who proposed a Southern Hemisphere origin for the genus. The analysis presented in this thesis, however, may be influenced by our heavy sampling bias towards southern African species and may require further validation with more representative sampling.

There is good evidence to show that divergence within the Cape clade in the degree of heterotrophy as measured using a series of traits, specifically leafiness, plant height and tissue chlorophyll and N content, reflects adaptation to edaphic environments which vary in their nutritional properties (Chapter 3). According to the phylogenetic methodology of testing for adaptation proposed by Baum and Larson (1991), a character can be considered an adaptation if it provides current utility to the organism and if it has been generated under natural selection for its current function. Pot experiments indicated that *Thesium* species with divergent traits, particularly plant size and leafiness, had significantly different photosynthetic capacities, with the larger, leafier *T. capitatum* having consistently higher photosynthetic rates than the smaller, leafless *T. nigromontanum*. This suggests that the observed divergence in these traits does indeed have an effect on physiological performance. Further, within the CFR *Thesium* is associated with the nutrient-poor sandstone substrates more frequently than expected at random, and interspecific variation in C-acquisition traits is also associated significantly with edaphic variables. Specifically, larger-bodied, leafier, more chlorophyllus species tend to inhabit environments of higher soil fertility, while the, smaller-

bodied, more achlorophyllous species tend to occupy the most nutrient-poor environments. These associations persisted when phylogenetic history was taken into account (PIC analyses), suggesting that shifts in these traits are consistently associated with shifts in environments of varying fertility. For example the split between the Leafy and *Annulata* clades (Fig 1, see Chapter 2) appears to be associated with a shift in soil pH, with leafy species tending to occur on soils with marginally higher soil pH.

The fact that the radiation in Cape *Thesium* has been accompanied by morphological shifts, which are consistently associated with shifts to different environments, suggests that diversification in *Thesium* has been adaptive. I therefore propose that edaphic heterogeneity in the CFR has played a significant role in driving the morphological and taxonomic diversification of Cape *Thesium*. It is, however, important to note that there are approximately80 *Thesium* species in the CFR, of which only 45 have been sampled in this study, and only 12 have been used in the investigation of trait divergence (Fig 1). In addition, major morphological differences between species appear to have arisen early on in the diversification of the group (Fig 1). For example the Leafy clade, appears to have arisen approximately 11 Myr ago, with subsequent morphological differentiation being limited. This suggests that factors other than adaptive divergence might be driving diversification within lineages of uniform morphology. One possible explanation for divergence within these lineages might be the relatively short dispersal distances of many species. Many Cape *Thesium* species appear to have elaiosomes on their seeds (Hendrych 1972), which may allow for their burial by ants (myrmecochory) and enable their seeds to escape from burning during fire (Hughes & Westoby 1990, Bond and Slingsby 1983). Low dispersal distances in ant dispersed lineages (Anderson 1988), might restrict gene flow among populations, thereby promoting speciation (Lengyel *et al.* 2009). Very little work has been done on the seed biology of *Thesium*, however, and so for the moment this scenario remains speculative.

Conclusion

Thesium species display remarkable diversity in traits associated with C-acquisition, and thus appear to vary in their degree of host dependence, with more parasitic species tending to favour more nutrient poor environments. This may be due to their ability to acquire some of the resources they need for growth and reproduction from their hosts, thus overcoming deficiencies in available soil nutrients. As such, parasitism in *Thesium* may represent a specialized foraging strategy for overcoming the constraints imposed by low soil fertility, with the diversity of *Thesium* in the CFR being driven by the diversity of edaphic environments found in the region.

Future research

This study focuses on the abiotic factors that control the distribution and drive the functional trait divergence of *Thesium*. However, other abiotic factors (e.g. disturbance),as well as biotic factors (e.g. suitability of hosts), may also play an important role in driving the evolution of diversity in *Thesium*. Host quality, for example, has been shown to be important in determining the distribution of other parasitic species (Watson 2009**,** Meulebrouck *et al.* 2009).

Parasitic plants are often absent from undisturbed habitats (Kuijt 1969). The major disturbance in Mediterranean-type ecosystems, such as the Fynbos biome of the CFR, is fire

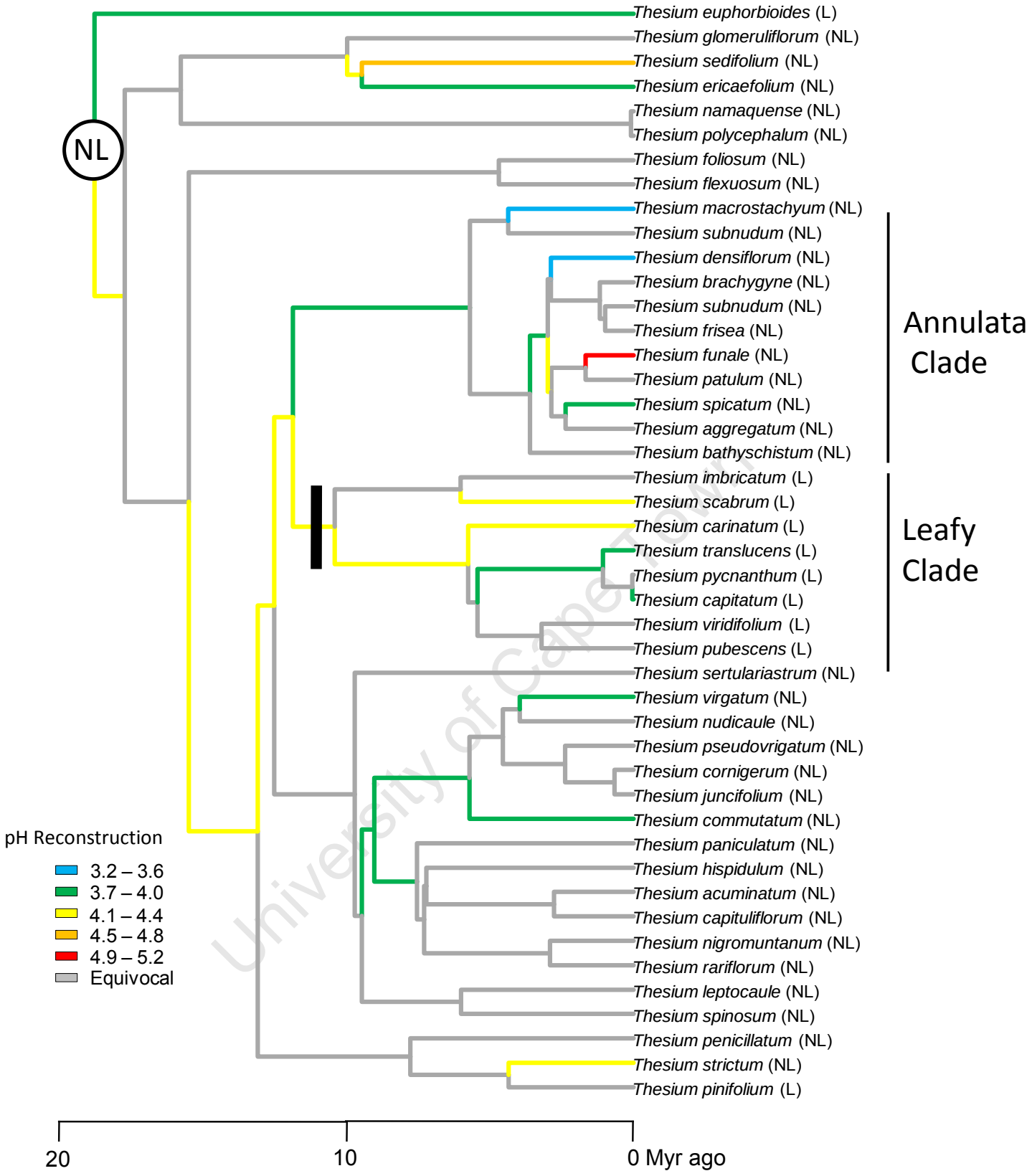


Figure 1. Squared change ancestral character state reconstruction of pH across the Cape clade (Chapter 2). All duplicate accessions were removed prior to analysis. The black bar indicates transition from a non-leafy (NL) to a leafy (L) ancestor based on Maximum Likelihood (MK-1) reconstruction. The basal reconstruction was for a non-leafy ancestor to the group.

(Naveh 1990). Although very little work has been done on the response of *Thesium* species to fire, preliminary work suggests that there is a strong association between *Thesium* abundance and vegetation age at a local scale (T. Moore unpublished). Fire has a major effect on both the biotic and abiotic environment, through its effects on vegetation cover and, consequently, light availability and competition for water and nutrients (Keeley & Fotheringham 2000). In addition, fire accelerates the mineralization of organic matter, increasing the availability of inorganic nutrients immediately after fire (Dunn & Debano 1977, Brown & Mitchell 1986, Stock & Lewis 1986).The reduction of competition for resources may be a key reason for the association between *Thesium* abundance and vegetation age.

In this study, I have used morphological traits as proxies of degree of heterotrophy in *Thesium*. More direct measures of degree of host dependence, such as ^{14} Cand radioactive amino acid labelling experiments (e.g. Fer *et al.* 1993), might provide clearer insight into divergence in degree of host dependence in *Thesium*. In addition, I have focussed on *Thesium* species from the nutrient poor CFR. An investigation into the role of abiotic factors in determining the distributions of other parasitic species might reinforce the patterns of species distributions that we have observed.

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