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The spatial and temporal dynamics of diversification in *Tylecodon*, *Cotyledon* and *Adromischus* (Crassulaceae) in southern Africa.

University of Cape Town

by

Tracey L. Nowell

Thesis presented for the degree of Doctor of Philosophy
in the Department of Botany, Faculty of Science, University of Cape Town

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DECLARATION

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged below or in the appropriate section of this thesis.

- The majority of plant material used for DNA work was supplied by Peter V. Bruyns. All such material is indicated by collector number in Table 1.1 of Chapter 1.
- A large amount of the geographic data was supplied by Peter V. Bruyns in the form of an edited and supplemented version of data available on the PRECIS database.
- Geo-referencing of all accession data used in Chapter 4 was carried out by Matthew Lewis.
- Awot Kiflu helped with measurements for sympatry calculations used in Chapter 4.

Signed by candidate

Tracey L. Nowell

ABSTRACT

A molecular phylogenetic hypothesis was generated for *Tylecodon*, *Cotyledon* and *Adromischus* in order to estimate the timing and spatial dynamics of diversification across these three southern African genera. These data were used to investigate the correlates and consequences of adaptations to extreme aridity and summer drought in members of the group.

Molecular sequence data from three plastid regions (*trnL-F, psbA-trnH* and *rpoB-trnC*), together with nuclear ITS1 and 2 were produced and used to estimate phylogenetic relationships among and within the three focal genera. Bayesian and parsimony-based analyses of combined data supported the monophyly of *Cotyledon* and *Adromischus*, however *Tylecodon* was found to be polyphyletic as *T. racemosus* was recovered as sister to *Adromischus*. The level of genetic divergence exhibited by *T. racemosus* necessitated its elevation to a new genus, thus *Toelkenocodon* was erected to accommodate the lineage. Relationships within *Cotyledon* were resolved and well-supported, and some well-supported clades were recovered within *Tylecodon* and *Adromischus*. Broad patterns of flowering phenology and geographic distributions emerged across the genera. Resolution at the level of species was generally poor within *Tylecodon* and *Adromischus* due to low sequence divergence.

Divergence time estimation was performed to test hypotheses of climate-driven diversification in the genera focal to this study. Four methods were used: NPRS and Multidivtime, both of which assume autocorrelation of rates between ancestor-descendent lineages, a global molecular clock, and a relaxed phylogenetics approach that simultaneously estimates phylogeny and divergence times (BEAST). Topology based tests for significant shifts in diversification rate were used, and parsimony and likelihood ancestral reconstruction of the transition to succulent karoo endemism across the group was performed. Diversity within the genera was found to be relatively young, although there was variation among the dating methods, with NPRS and Multidivtime returning older dates for events than either the molecular clock or BEAST. Diversification events among

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and within *Tylecodon*, *Cotyledon* and *Adromischus* were coincident with major climatic events hypothesised to be drivers of speciation in the south-west of southern Africa during the late Miocene and early Pliocene. *Tylecodon* exhibits significantly higher rates of diversification than either *Cotyledon* or *Adromischus*. No significant shifts in diversification rates were detected across the phylogeny of the group and species accumulation appears to have been more or less linear throughout the separate histories of the genera. A clade of succulent karoo endemic species was identified in *Tylecodon* and the timing of the transition to this vegetation type occurred between 5.5 and 3.2 Myr, consistent with the establishment of the winter rainfall regime in the region. Data from a related study which involved estimating the timing of shifts to succulent karoo endemism in distantly related angiosperm groups corroborated this pattern. The majority of instances of endemism emerged during the last 5 Myr suggesting that the succulent karoo is considerably younger than the neighbouring fynbos biome.

Analyses of range size characteristics of *Tylecodon* and *Cotyledon* demonstrated that species of *Tylecodon* have significantly smaller ranges than species of *Cotyledon*. The hypothesis that range size is determined by plant size and the height at which seeds are released (the sum of vegetative height and inflorescence height) was tested and a positive relationship was found in *Tylecodon*, but not in *Cotyledon*. Further correlation-regression analyses revealed significant associations between the height of vegetative organs, inflorescences and the size of flowers in *Tylecodon*. Thus it was proposed that the proclivity of species with small ranges in *Tylecodon* is the result of limited dispersal. The expectation for the geographical mode of speciation under such a scenario is one of allopatry. Age-range correlations revealed that the predominant mode of speciation in both *Tylecodon* and *Cotyledon* is allopatric.

The hypothesis that *Cotyledon* and *Tylecodon* occupy different climatic niches was tested using using discriminant function analysis. The genera could be distinguished along an axis of increasing summer drought and potential evaporation. Species of *Tylecodon* occupy niches concentrated at the arid extremes of conditions where summer drought prevails: *Cotyledon* is

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largely absent from these areas. The shift into arid environments in *Tylecodon* coincides with the evolution of putative morphological adaptations, including leaf deciduousness and vegetative diminution which likely represent evolutionary innovations that enabled ancestral forms of *Tylecodon* to colonise new habitats created during the formation of the winter rainfall desert of the succulent karoo. Passive dispersal coupled with the morphological adaptations exhibited by species of *Tylecodon* have led to the strong association observed between range size and plant size, such that small plants are effectively isolated in microhabitats; a factor which is likely to enable differentiation as a result of highly restricted gene flow. The increased stature of plants of *Cotyledon* and the fact that inflorescence height is uncoupled from vegetative height, may lead to the relative mobility of propagules of members of the genus. Species can disperse more broadly, gene flow sustains the genetic integrity of species, and they are able to colonise new areas.

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I am extremely indebted to my supervisor Tony Verboom. He has been a tremendous source of ideas during the course of this work and has been on hand throughout to provide me with advice, support, and feedback. I gained considerable knowledge and learned many new skills through my interactions with Tony, and it is under his guidance that this project grew into something coherent.

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The thorough species sampling in this project is the result of extensive field collection by Peter Bruyns. I would like to thank him for sharing this material with me, and for useful comments on an earlier version of Chapter 1.

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INTRODUCTION

Southern Africa is a region of outstanding botanical diversity. It is home to three of the world's 34 biodiversity hotspots (Mittermeier et al., 2004). Two of these hotspots, namely the Cape Floristic Region (CFR) dominated by fynbos (sclerophyllous evergreen heath), and the succulent karoo, neighbour one another in south-western South Africa and collectively are known as the Greater Cape Floristic Region (GCFR: Jürgens, 1991, Born et al., 2007). In addition to their tremendous diversity, both floras have exceptionally high levels of endemism. The succulent karoo is dominated by dwarf succulent shrubs and is unique amongst the world's winter rainfall deserts on account of endemism and the high incidence of leaf succulence in the vegetation (Desmet & Cowling, 2004). The distribution of the GCFR concurs broadly with an area that experiences a Mediterranean-type climate. The fynbos-succulent karoo boundary is thought to be primarily determined by moisture availability (Cowling et al., 1997) such that the increased aridity of the succulent karoo results in plant spacing that is too great to carry fire (Rebelo et al., 2006).

The origins of the diverse flora of the fynbos biome are hypothesised to be the result of climate driven diversification. Levyns (1964) was one of the earliest proponents of the procession of aridification leading ultimately to the relictual distribution of temperate forest vegetation that we see today. It is thought that the progenitors of fynbos, having a higher tolerance for more arid conditions, replaced forest throughout much of its former range. In turn, as aridity intensified in what is now the succulent karoo region, fynbos became restricted to higher ground and succulent vegetation invaded lowland areas (Werger, 1983, Scott et al., 1997), thus implying a more recent origin for succulent karoo. Fynbos and succulent karoo experience different intensities of aridity yet are hypothesised to share high rates of speciation. Levyns (1964) attributed the latter to the shared seasonality, rather than to the total amount of rainfall received by the regions.

Floral components of fynbos have been the focus of considerable research into the patterns and processes of speciation (Linder, 2003 for a review). Molecular phylogenetic techniques have

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been applied to many of the lineages that characterise the vegetation. Research on these groups has been geared towards uncovering evolutionary relationships and the timing of diversification events. It is becoming increasingly apparent that fynbos elements have a long history of diversification (Linder, 2005) that began approximately 42 million years ago (Mya). The extent to which members of the succulent karoo share a common and contemporaneous history of diversification has received relatively little attention. Divergence time estimation carried out on members of Aizoaceae, the most speciose lineage that dominates much of the vegetation of the succulent karoo, revealed rates of diversification that are unsurpassed in plants, and suggested that rapid radiation had taken place in the group between 3.8 and 8.7 Mya (Klak et al., 2004).

Tylecodon, *Cotyledon* and *Adromischus*, three closely related taxa of Crassulaceae, are the focus of this study which investigates questions of speciation history. The biogeography of the genera, together with their contrasting range size characteristics, morphologies and life-history traits pose interesting questions for testing hypotheses regarding drivers of the diversification processes that have determined current diversity patterns in the group. *Cotyledon* is a small genus, comprising 11 species of leaf succulent shrubs and sub-shrubs distributed widely across southern Africa (Fig. 1). *Cotyledon barbeyi* and *C. orbiculata* are the only representatives found outside this region; *C. barbeyi* occurs from north-eastern South Africa to the Arabian Peninsula, and *C. orbiculata* is found throughout South Africa and extends northwards into southern Angola (van Jaarsveld & Koutnik, 2004). Species diversity within *Cotyledon* is relatively diffuse across its range and the highest concentration of species occurs along the coastal plains south of the Great Escarpment, between Robertson and East London. *Cotyledon orbiculata* is notable for its wide distribution which encompasses that of all other species in the genus. Five varieties are recognised within this species (Tölken, 1985) and, according to van Jaarsveld & Koutnik (2004), it is possibly the most variable species in southern Africa. All *Cotyledon* species have showy yellow to orange-red, pendulous flowers, often covered with a thick waxy bloom (Fig.1). In contrast, *Tylecodon* has many more species than *Cotyledon*, but occupies a restricted geographic area.

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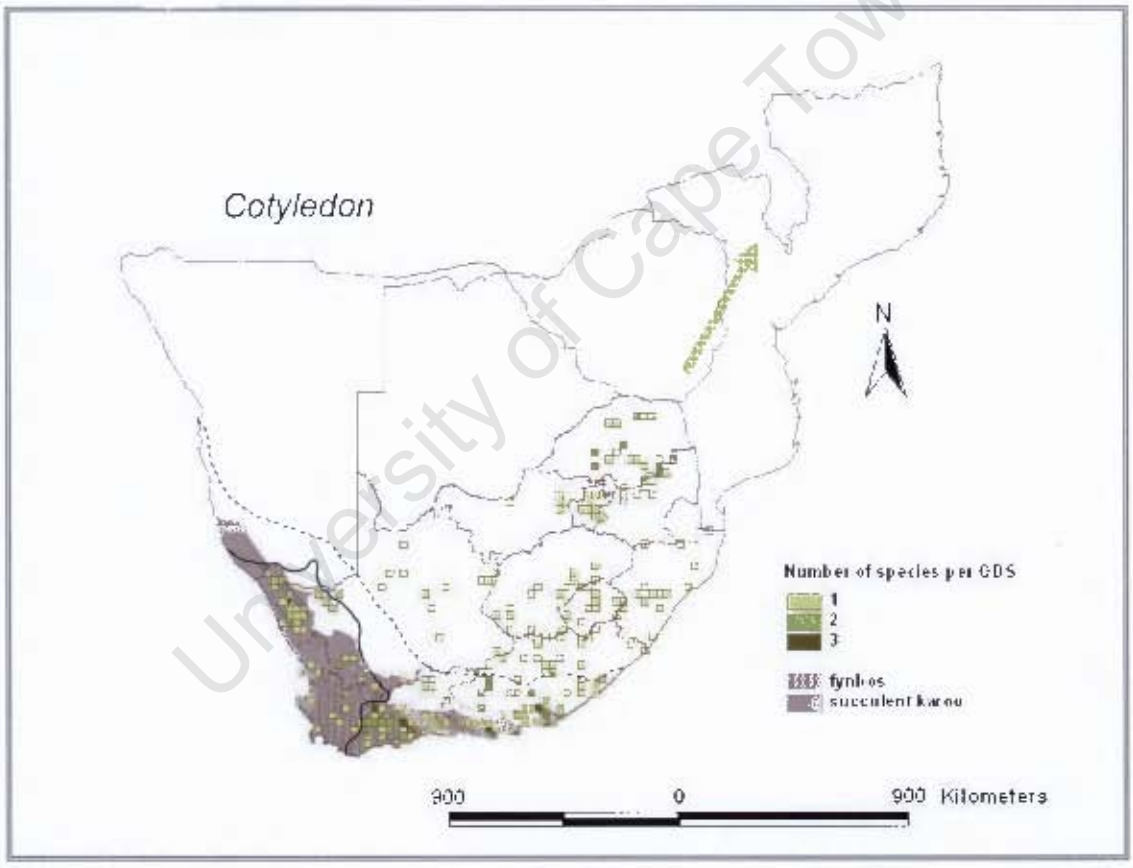

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Figure 1 *Above:* Distribution of *Cotyledon* in South Africa and Lesotho. Species richness shown per QDS is based on occurrence data. The green arrow indicates the continuation of the range of *C. barbeyi* north towards the Arabian Peninsula. The solid and dashed black lines indicate the boundaries of the winter- and summer-rainfall zones respectively (redrawn from Chase & Meadows, 2007).

Top left: Variation in leaf morphology within a single population of *C. orbiculata* in the Drakensberg Mountains. *Top right:* Inflorescence of *C. orbiculata*. Photographs by David Gwynne-Evans.

Most of the 46 species of *Tylecodon* are found within the winter rainfall zone of South Africa, with some ranging northwards into Namibia (Fig. 2); many species have highly restricted distributions. The genus has centres of diversity in the Richtersveld and Namaqualand where plants are strongly associated with rocky montane areas in gravelly and pebbly substrates (van Jaarsveld & Koutnik, 2004). Members of *Tylecodon* have seasonally deciduous, succulent leaves and succulent stems. Growth forms in the genus range from tiny, single-leaved plants with reduced pachycaulous stems and subterranean storage organs, to the dwarf succulent tree *Tylecodon paniculatus*, which can grow to over 2.5 metres tall (van Jaarsveld & Koutnik, 2004). Species of *Tylecodon* bear erect to spreading inflorescences and the tubular flowers are diverse in colour, ranging from white and pink to yellow, orange and red. *Adromischus* is a genus of dwarf shrubs with evergreen succulent leaves; in many species leaves are amassed in a basal rosette (Fig. 3). *Adromischus* shares several distributional characteristics with *Tylecodon*. Many of its 28 species are found within the winter rainfall zone of southern Africa; however it differs from *Tylecodon* in having several representatives restricted to aseasonal and summer rainfall areas. *Adromischus* has notable levels of species diversity in the south of its distribution from the Robertson Karoo and Little Karoo, eastwards to Willowmore and Graaff-Reinet, and to the north-west of its distribution in Namaqualand. Flowers are borne on spike-like inflorescences and the corolla tube is often green, while corolla lobes are variously coloured white, pink, maroon, or a combination thereof.

Cotyledon, *Tylecodon* and *Adromischus* are well-known taxonomically through the work of Tölken (1978 & 1985), and more recent synopses have been produced to include newly described species (Pilbeam et al., 1998; van Jaarsveld & Koutnik, 2004). Representatives of this southern African alliance of genera have been included in family-level analyses of relationships based on molecular data (van Ham & 't Hart, 1998, Mort et al., 2001). However, due to the taxonomic breadth of these studies, sampling within *Cotyledon*, *Tylecodon* and *Adromischus* has been very sparse. *Cotyledon* was the focus of a recent, well-sampled molecular phylogenetic study (Mort et

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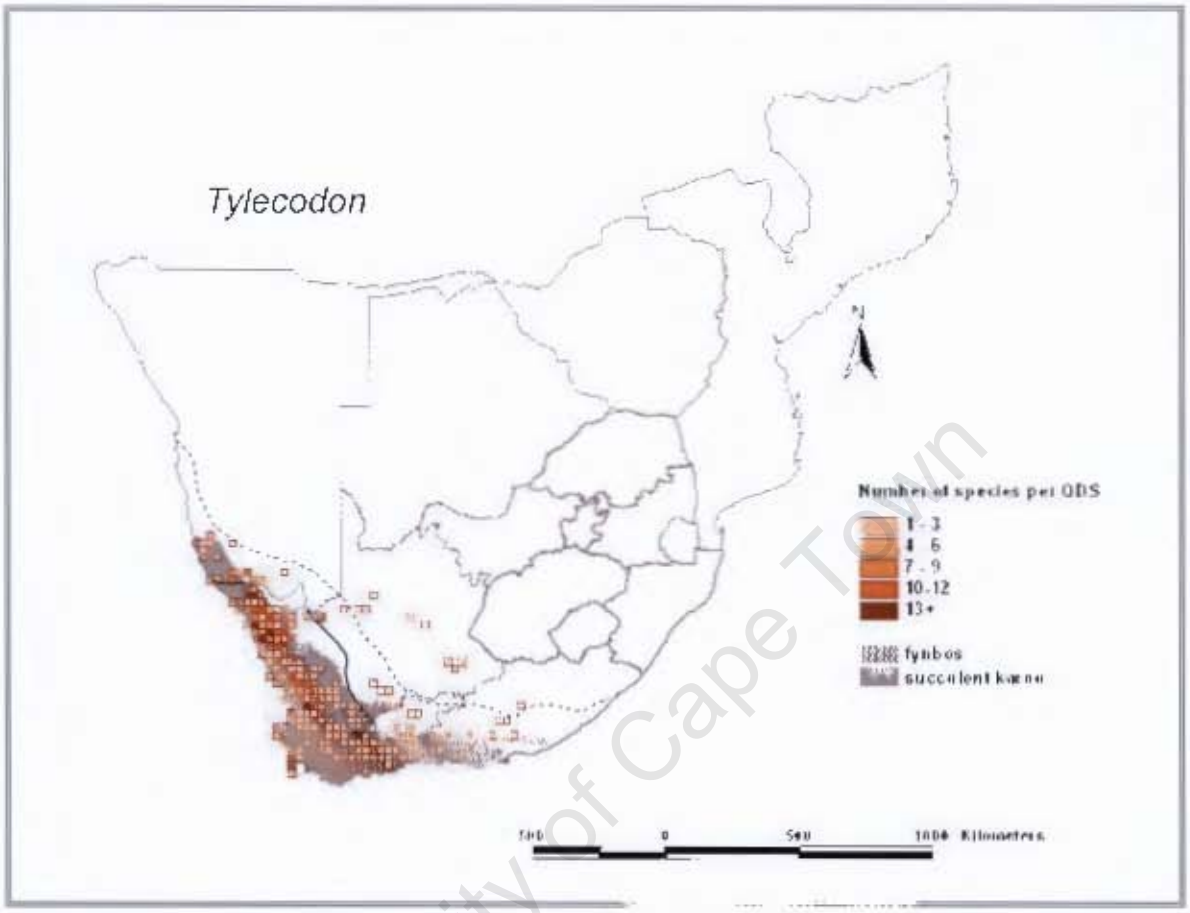

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Figure 2 *Above:* Distribution of *Tylecodon* in South Africa and Lesotho. Species richness shown per QDS is based on occurrence data. The solid and dashed black lines indicate the boundaries of the winter- and summer-rainfall zones respectively (redrawn from Chase & Meadows, 2007).

Left: Tylecodon paniculatus, the largest species in the genus, pictured here with withered leaves and showy red inflorescences.

Below: Tylecodon reticulatus. The species retains old inflorescences which are thought to protect the leaves from herbivores. Photograph by David Gwynne-Evans.

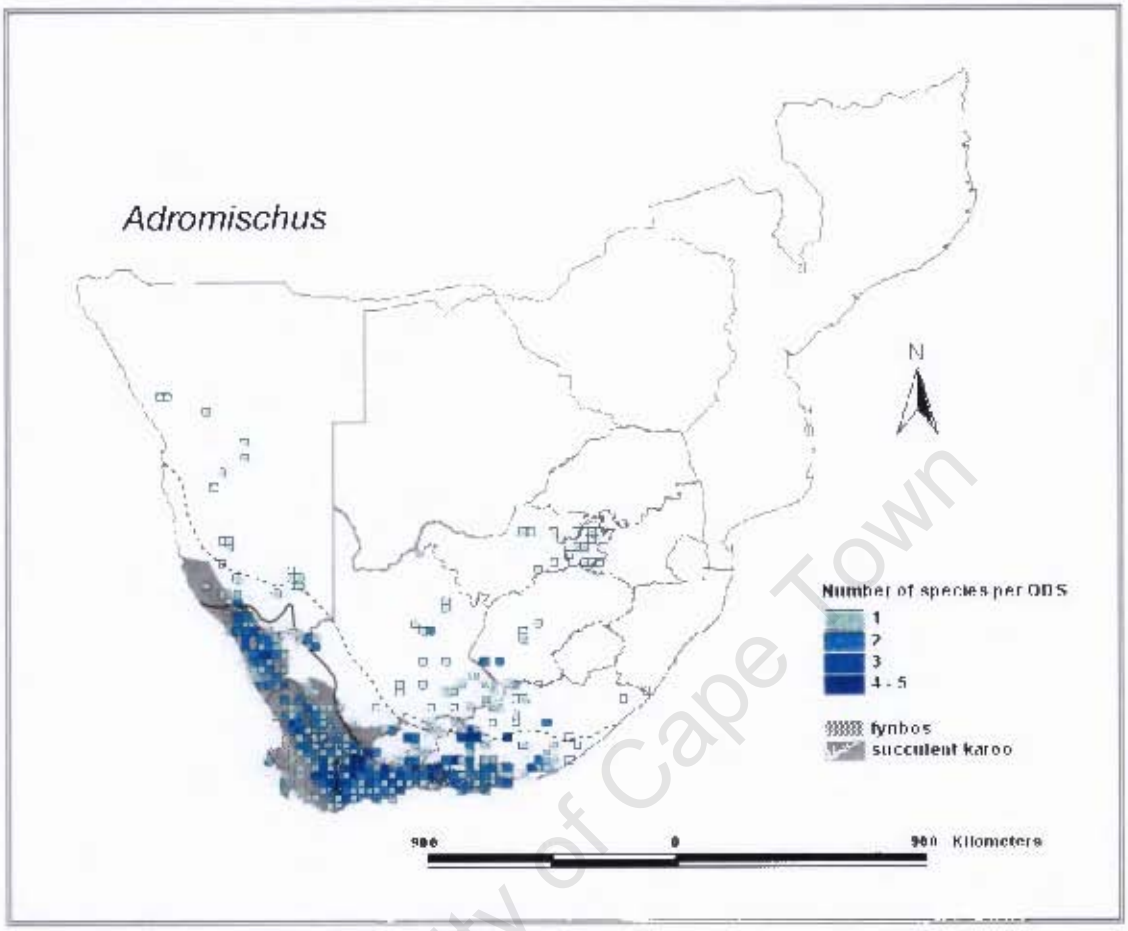

Figure 3. *Top:* Distribution of *Adromischus* in southern Africa. Species richness shown per QDS is based on occurrence data. The solid and dashed black lines indicate the boundaries of the winter- and summer-rainfall zones respectively (redrawn from Chase & Meadows, 2007).

Above left: Rosette-type growth form common among species of *Adromischus*.
Above right: Infructescence of *Adromischus* species showing dehiscent follicles.
Photographs by David Gwynne-Evans.

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al., 2005), but no equivalent hypotheses of evolutionary relationships exist for *Tylecodon* and *Adromischus*.

The first main aim of this work is to produce a phylogenetic hypothesis for *Cotyledon*, *Tylecodon* and *Adromischus*. Extensive species-level sampling is used in order to test the generic boundaries that are currently defined according to morphological characters (Tölken, 1978, 1985). Secondly, the timing of significant divergence events across and within the three genera will be investigated within the phylogenetic framework generated. Rates of diversification between genera will be calculated, and possible shifts in rates of diversification within clades will be tested. Temporal information will be used to test hypotheses of climate-driven diversification having occurred in this group of succulents. An increasing amount of corroborative evidence regarding palaeo-climates of south-western southern Africa is available (e.g. Zachos et al., 2001, deMenocal, 2004, and sources reviewed in Linder, 2003) and provides information on the important abiotic factors likely to be correlated with diversification in *Tylecodon, Cotyledon* and *Adromischus*. In particular, the role of increasing aridification and changes in rainfall seasonality is considered to be a potentially important determinant of the evolution of *Tylecodon* and *Adromischus*. Occurrence of a large number of species of these two genera in the succulent karoo is examined and the evolution of succulent karoo endemism in the group, combined with comparative information from distantly related groups (Verboom et al., in press), is used as a proxy to estimate the timing of the formation of the biome. The strong association between species of *Tylecodon* and the succulent karoo is of particular interest as the genus possesses morphological attributes such as deciduous leaves, swollen, subterranean stems and reduced plant size that are putative adaptations to increased aridity and severe summer drought. Such adaptations may play a role in determining gene flow and hence influence processes of differentiation in *Tylecodon*. Mechanisms by which features unique to *Tylecodon* may have led to increased diversification are investigated via examination of range sizes and their association with selected morphological characters to test the hypothesis that the evolution of these morphological characters is contemporaneous with increased aridity in the south-west of southern

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Africa and represent adaptations or pre-adaptations that enabled *Tylecodon* to exploit areas of extreme aridity and summer drought, and ultimately led to increased species numbers and rates of diversification in the genus.

Thus the four main components of this thesis are as follows:

- Estimation of phylogenetic relationships among and within the predominantly southern African genera of Kalanchoideae: *Tylecodon*, *Cotyledon* and *Adromischus*.
- Use of the phylogenetic hypothesis to evaluate current generic limits.
- Inference of a temporal scenario against which to test hypotheses of climatically-driven diversification of the three genera, and in the succulent karoo as a whole.
- Determination of the predominant geographic mode of speciation in *Tylecodon* and *Cotyledon*, and evaluation of the role of adaptations to aridity in driving speciation.

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CHAPTER 1

Cotyledon, *Tylecodon* and *Adromischus* (Kalanchoideae; Crassulaceae): phylogeny and generic delimitations inferred from nuclear and plastid DNA data.

INTRODUCTION

Cotyledon L., *Tylecodon* Tölken and *Adromischus* Lem. are closely related genera of the Crassulaceae (*Kalanchoe* clade sensu Mort et al., 2001) with a predominantly southern African distribution. Collectively they represent approximately one-sixth of the family's species diversity. Uncertainties regarding relationships in Crassulaceae have existed since De Candolle (1801) erected the family and result largely from the apparent multiple origins of sympetaly and variation in the number of staminal whorls found in members of the family ('t Hart & Eggli, 1995). Relationships within Crassulaceae have also been confounded by apparent convergence of vegetative characters due to a strong association with xeric habitats found in many representatives. Recently, molecular data have contributed to resolving higher-level taxonomic relationships and delimitation of the large, heterogeneous taxon *Sedum* (e.g. van Ham and 't Hart, 1998), together with the monophyly of other large and complex genera such as *Crassula*, *Sempervivum*, and *Kalanchoe* (Mort et al., 2001). These studies are largely consistent in recovering seven major clades within Crassulaceae. The ranking of these clades has recently been modified by Thiede & Eggli (2007), such that three subfamilies are recognised: the large and cosmopolitan Sempervivoideae, comprising five tribes; and the predominantly African subfamilies Crassuloideae and Kalanchoideae. The latter contains the three genera that are the focus of this study, together with *Kalanchoe*.

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Despite the taxonomic instability within Crassulaceae, the mutual affinities of *Cotyledon*, *Tylecodon* and *Adromischus* have been reiterated throughout the family's history. *Cotyledon* was first recorded in 1624 when Heurnius documented *Cotyledon orbiculata* from South Africa (van Jaarsveld and Koutnik, 2004). A monotypic *Cotyledon* was formally recognised in the first edition of *Species Plantarum* (Linnaeus, 1753), and nine species of *Cotyledon* were included in De Candolle's '*Crassuleae Legitimae*' (1828a). In 1852 Lemaire erected *Adromischus* to accommodate representatives with spike-like inflorescences and long corolla tubes. The name *Adromischus* was not generally used until Berger applied it in his monograph of the Crassulaceae in 1930. While Berger (1930) accepted the segregation of *Adromischus* from *Cotyledon*, both he and Schönland (1915) maintained *Cotyledon* as a mix of species with opposite-decussate and alternate leaves.

The current taxonomy of *Cotyledon*, *Tylecodon* and *Adromischus* follows Tölken's 1978 and 1985 treatments. In his re-evaluation of *Cotyledon* and *Adromischus* Tölken (1978) used leaf arrangement and leaf persistence (among other characters) to redefine the genera and to erect the genus *Tylecodon*. This new genus comprised 27 species with alternate, soft, deciduous leaves; 22 of these had previously been included in *Cotyledon,* and one in *Adromischus*, while four were newly described. Tölken retained *Adromischus* and *Cotyledon*, distinguishing them from *Tylecodon* by their stiff, evergreen leaves and from one another by the arrangement of their leaves. In *Adromischus* leaves are alternate, while in species of *Cotyledon* they are opposite-decussate. Thus, Tölken's new circumscription recapitulated De Candolle's (1828b) three informal groupings within *Cotyledon*, namely "Foliis oppositis; Foliis alternis, marcescentibus; Foliis alternis, persistentibus". Tölken divided *Adromischus* into five sections based on floral morphology, but no such subdivisions exist for *Cotyledon* or *Tylecodon*. *Cotyledon* and *Tylecodon* were the subjects of a recent synopsis by van Jaarsveld & Koutnik (2004), while *Adromischus* was similarly updated by Pilbeam et al. (1998).

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In addition to conclusions based on morphology, the close evolutionary affinities between *Cotyledon*, *Tylecodon* and *Adromischus*, together with their distinction from other members of Crassulaceae, has been supported by data from a broad range of sources. Uhl (1948) found that both *Cotyledon* and *Adromischus* have a haploid chromosome complement of nine, such that the genera cannot be distinguished cytologically. Mort et al. (2001) reconstructed the base chromosome number of Crassulaceae as eight, and nine was identified as the synapomorphous number of the *Kalanchoe* clade. A chemotaxonomic study of pigments contributing to red flower colour in representatives of Crassulaceae revealed that *Cotyledon* and *Tylecodon* share a very similar suite of anthocyanins, while *Crassula* shows a different pigment signature (van Wyk & Winter, 1995). Hideux (1981, in t'Hart & Eggli, 1995) analysed primarily palynological-based characters for members of Crassulaceae and identified the early divergence of a *'Crassula'*-lineage from the remainder of the family. Collectively, *Cotyledon*, *Adromischus* and *Kalanchoe* represented an African clade derived from the *'Crassula'*-lineage.

Despite the historical interest in *Cotyledon*, *Tylecodon* and *Adromischus*, the phylogenetic status of the genera, relationships among them, and interspecific relationships within genera remain unclear. *Cotyledon* was recently the subject of a comprehensively sampled species-level analysis reconstructing relationships using plastid and nuclear DNA data (Mort et al., 2005). *Cotyledon* was recovered as a strongly supported clade but there was little support for relationships within the genus. The current study is the first to use extensive sampling of all three genera for phylogenetic analyses. A molecular-based approach is implemented and aims to clarify phylogenetic uncertainty within and between *Cotyledon*, *Tylecodon* and *Adromischus* with near-complete species sampling. In addition, significant findings will be contextualised in terms of the geographical distribution of clades and patterns of flowering phenology across the genera. Plastid and nuclear DNA sequence data are generated and analysed to: a) test the monophyly of *Cotyledon*, *Tylecodon* and *Adromischus*; b) investigate evolutionary relationships amongst the study genera; c) elucidate relationships within each genus.

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METHODS

Taxon sampling

Accessions of *Cotyledon*, *Tylecodon* and *Adromischus* were sampled to attain as thorough specific- and intraspecific-level representation as possible in initial analyses. Members of *Kalanchoe* were included as representatives of the fourth genus of the Kalanchoideae. The more distantly related Sempervivoideae was represented by *Aeonium leucoblepharum*, and Crassuloideae was represented by species of *Crassula*. Details of accessions that represent a core sample of species across the genera of interest are provided in Table 1.1. The rationale and methodology for selection of the core samples is detailed under 'Parsimony analyses' later in the text. Taxonomic arrangement at the familial and subfamilial level, together with numbers of species currently recognised within genera, follow Thiede & Eggli (2007).

DNA extraction, amplification, and sequencing

DNA was extracted from fresh or silica-dried leaf material using a modified protocol based on that of Doyle and Doyle (1987), as outlined in Bruyns et al. (2005). To reconstruct relationships among the study group sequence data from four DNA regions were generated, namely: the plastid *trnL* ^{UAA} 5' exon – *trnF* ^{GAA} gene (including the *trnL* 5' exon, intron, the *trnL* ^{UAA} 3' exon and the *trnL* 3' – *trnF* intergenic spacer) (Taberlet et al., 1991); the plastid *psbA* – *trnH* intergenic spacer (Sang et al., 1997); the plastid *rpoB-trnC*^{GCA} (Shaw et al., 2005); and the nuclear-encoded internal transcribed spacers 1 and 2 (ITS1 and ITS2) flanking the 5.8s subunit of the 18-26s ribosomal RNA cistron (White et al., 1990). DNA regions were amplified using the following primer combinations: c and f (Taberlet et al., 1991) for the *trnL-F* region, *psbAF* and *trnHR* (Sang et al.,1997) for *psbA-trnH*, *rpoB* (Shaw et al., 2005) and *trnC*^{GCA} 'internal' (5' – CACAAAACAACAACTCAGGAC - 3') designed specifically for this group for the *rpoB-trnC*^{GCA} region, and its4 and its5 (White et al., 1990) for ITS.

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Amplifications were performed using 0.75 units of Super-Therm DNA polymerase in 30 μl volumes also containing 1X NH_4 buffer and 5 mM $MgCl_2$ (both supplied with polymerase), together with 0.1 mM of each dNTP, 0.3 μM of each primer, and 3 μl of DNA template. Polymerase chain reactions (PCRs) were performed using a GeneAmp® PCR System 2700 Version 2.07 (Applied Biosystems) set to the following thermal conditions: an initial denaturation at 94°C for 2 mins followed by 30 cycles of 94°C for 1 min., 52°C for 1 min., 72°C for 2 mins, with a final polymerisation step of 72°C for 7 mins. PCR products were purified using the GFX™ DNA and Gel Band Purification Kit (Amersham Biosciences).

Both strands of PCR products were cycle-sequenced as per the manufacturer's instructions using the ABI PRISM® Big Dye™ Terminator v3.1 Ready Reaction Kit (Applied Biosystems). Amplification primers were also used for cycle sequencing. Sequenced products were resolved on an ABI PRISM® 3100 Genetic Analyzer. Sequences were assembled and checked for inaccurate base calling using SeqMan II (LaserGene System Software, DNAStar, Inc.). Consensus sequences were aligned by eye using MegAlign (LaserGene System Software, DNAStar, Inc.) and alignments were trimmed in order to exclude messy ends. Only simple indels were found throughout the datasets, and indel presence/absence was coded in a binary manner using the 'simple indel coding' method of Simmons and Ochoterena (2000), and included in analyses.

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Parsimony analyses

Initial parsimony analysis was carried out on a 132-taxon dataset (Table 1.1) in which a number of species were represented by multiple accessions. This procedure was done to test the monophyly of these species such that those recovered as monophyletic could be reduced to a single representative in subsequent analyses. This way the core samples needed to obtain the most complete phylogenetic representation were identified using the smallest number of accessions.

Table 1.1. Details of the 132 accessions for which DNA data were generated and used for phylogenetic analyses in this work. Samples preceded by an * are those included in the 90-taxon 'core' dataset referred to in the text. Voucher specimens collected by PVB (Peter V Bruyns) and TLN (author) are all deposited at the Bolus Herbarium, The University of Cape Town. Others are as indicated. Sequence data generated for this study are indicated by 'x', missing data are indicated by '-' and accession numbers are given for sequences obtained from GenBank.

Taxon	Voucher Herbarium, collector	DNA region *trnL-F*	*psbA*	*rpoB*	ITS
Kalanchoideae A. Berger					
Adromischus Lem.					
**A. alstonii* (Schönl. & Bak.f.) C.A. Sm.	PVB 9265	x	x	x	x
**A. bicolor* P.C. Hutch.	PVB 9797	x	x	x	x
**A. caryophyllaceus* (Burm.f.) Lem.	PVB 8219a	x	x	x	x
**A. cooperi* (Bak.) Berger	PVB 9325	x	x	x	x
A. cristatus (Haw.) Lem. var. *mzimvubuensis*	PVB 8928	x	x	x	x
**A. cristatus* (Haw.) Lem. var. *schonlandii* (Phill.) Tölken	PVB 8942	x	x	x	x
**A. fallax* Tölken	PVB 2997	x	x	x	x
**A. filicaulis* (Ecklon & Zeyher) C.A. Smith subsp. *filicaulis*	PVB 9880	x	x	-	-
**A. filicaulis* (Ecklon & Zeyher) C.A. Smith subsp. *filicaulis*	PVB 9918	x	x	-	-
A. filicaulis (Ecklon & Zeyher) C.A. Smith subsp. *marlothii* (Schonland) Tölken	TLN 280	x	x	-	-
**A. hemisphaericus* (L.) Lem.	Parker sn	x	x	x	x
**A. humilis* (Marloth) V. Poelln	PVB 4868	x	x	x	-
**A. inamoenus* Tölken	PVB sn	x	x	x	x
A. cf. inamoenus Tölken	PVB sn	x	x	x	-
**A. leucophyllus* Uitew.	PVB 3738	x	x	x	-
**A. liebenbergii* P.C. Hutch.	PVB 9183	x	x	x	x
**A. liebenbergii* P.C. Hutch. 'orientalis'	PVB 4401	x	-	x	-
**A. maculatus* (Salm-Dyck) Lem.	TLN 281	x	x	x	x
**A. mammillaris* (L.f.) Lem.	PVB 8981	x	x	x	x
**A. marianiae* (Marloth) Berger	PVB 9493a	x	x	x	x
A. marianiae (Marloth) Berger	PVB 9145a	x	x	x	-
A. marianiae (Marloth) Berger	PVB 9176	x	x	x	-
A. marianiae (Marloth) Berger	PVB 9928	x	x	x	-
A. marianiae (Marloth) Berger 'hallii'	PVB 9872	x	x	x	-
A. marianiae (Marloth) Berger 'hallii'	PVB 9493a	x	x	x	-
A. marianiae (Marloth) Berger 'immaculatus'	PVB 9849	x	x	x	-
A. marianiae (Marloth) Berger 'immaculatus'	PVB 9901	x	x	x	-
A. marianiae (Marloth) Berger 'herrei'	PVB 3877	x	x	x	-
**A. maximus* P.C. Hutch.	PVB 9082	x	x	x	x
**A. montium-klinghardtii* (Dinter) Berger	PVB 9235	x	x	x	x
**A. nanus* (N.E. Br,) V. Poelln.	PVB 9493	x	x	x	x
**A. phillipsae* (Marloth) V. Poelln.	PVB 6079	x	x	x	x
**A. roaneanus* Uitew.	PVB 9924	x	-	x	x
**A. schuldtianus* (V. Poelln.) V. Poelln. Subsp. *Schuldtianus*	PVB 8334	x	x	x	x
**A. schuldtianus* (V. Poelln.) V. Poelln. subsp. *brandbergensis* B.Nord & Van Jaarsv.	PVB 2838	x	-	x	-
**A. sphenophyllus* C.A. Sm.	PVB 9326	x	x	x	x
**A. subviridis* Tölken	PVB 7939	x	x	x	x
**A. triflorus* (L.f.) Berger	PVB 9767	x	x	x	x
**A. triflorus* (L.f.) Berger	PVB sn	x	x	x	x
**A. trigynus* (Burch.) V. Poelln.	PVB 9404	x	x	x	-
**A. umbracticola* C.A. Sm.	PVB 7000	x	x	x	x
A. spp.	PVB 5021	x	x	-	-
Cotyledon L.					
**C. adscendens* R.A. Dyer	van Jaarsveld sn	-	AY596345	x	AY596330
**C. barbeyi* Schweinf. Ex Barker	PVB 6570a	x	x	x	x
**C. campanulata* Marloth	PVB 1786	x	x	x	x
**C. cuneata* Thunb.	TLN 271	x	x	x	x
C. cuneata Thunb.	PVB 8870	x	x	x	-

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Table 1.1. continued

Taxon	Voucher Herbarium, collector	DNA region *trnL-F*	*psbA*	*rpoB*	ITS
**C. eliseae* Van Jaarsv.	HBG 17212	AY692295	AY596338	x	AY596323
C. orbiculata L.	PVB 9326	x	x	-	-
**C. orbiculata* L. var. *orbiculata*	PVB 9225	x	x	x	x
C. orbiculata L. var. *orbiculata*	TLN 272	-	x	-	-
C. orbiculata L. var. *spuria* (L.) Tölken	TLN 273	-	x	x	-
C. orbiculata L. var. *spuria* (L.) Tölken	TLN 274	-	x	x	-
**C. papillaris* L.f.	PVB 9900	x	x	x	x
C. papillaris L.f.	PVB 8400a	x	x	x	-
C. papillaris L.f.	PVB 9020	x	x	x	-
C. papillaris L.f.	PVB 9850	x	x	x	-
C. tomentosa Harv. subsp. *tomentosa* Tölken	PVB 7046	x	x	x	-
**C. tomentosa* Harv. subsp. ladismithensis (Poelln.) Tölken	PVB 7657	x	x	x	x
**C. velutina* Hook.f.	PVB 8413	x	x	x	x
**C. woodii* Schönland & Baker f.	PVB 7094	x	x	x	x
***Tylecodon* Tölken**					
**T. albiflorus* Bruyns	PVB 7516	x	x	x	-
**T. aridimontanus* G. Will.	Tribble 2941	x	x	x	-
**T. atropurpureus* Bruyns	PVB 2658	x	x	x	x
**T. aurusbergensis* G. Will. & Van Jaarsv.	Lavranos sn	x	x	x	-
**T. bayeri* Van Jaarsv.	PVB 1490	x	x	x	x
**T. bayeri* Van Jaarsv.	PVB 2664	x	x	x	x
**T. buchholzianus* (Schuldt & P. Stephan) Tölken	PVB 1491	x	x	-	-
**T. cacalioides* (L.f.) Tölken	PVB 8940	x	x	x	x
**T. cordiformis* G. Will.	PVB 9095	x	x	x	x
**T. decipiens* Tölken	PVB 9137	x	x	x	x
**T. ellaphieae* Van Jaarsv.	PVB 3238	x	x	x	-
**T. faucium* (Poelln.) Tölken	PVB 2542	x	x	x	-
**T. fragilis* (R.A. Dyer) Tölken	PVB 6711	x	x	-	x
**T. grandiflorus* (Burm.f.) Tölken	PVB 6721	x	x	x	-
**T. hallii* (Tölken) Tölken	PVB 9254	x	x	x	-
**T. hirtifolius* (W.F. Barker) Tölken	PVB 8905	x	x	x	x
**T. kritzingeri* Van Jaarsv.	PVB sn	x	x	x	-
**T. leucothrix* (C.A. Sm) Tölken	PVB 3730	x	x	x	x
T. leucothrix (C.A. Sm) Tölken	PVB 7347	x	x	x	-
T. leucothrix (C.A. Sm) Tölken	PVB 8870	x	-	-	-
**T. longipes* Van Jaarsv. & G. Will.	PVB 9124	x	x	x	x
**T. nolteei* Lavranos	PVB 9084	x	x	x	x
**T. occultans* (Tölken) Tölken	PVB 6806	x	x	x	x
**T. paniculatus* (L.f.) Tölken	TLN 152	x	x	x	x
**T. pearsonii* (Schönland) Tölken	PVB 9086	x	x	x	x
**T. pusillus* Bruyns	PVB 2668	x	x	x	x
**T. pygmaeus* (W.F. Barker) Tölken	PVB 1087	x	x	x	x
T. racemosus (Harv.) Tölken;	PVB 2769	x	x	-	-
**T. racemosus* (Harv.) Tölken;	PVB 9865	x	x	x	x
T. reticulatus L.f.	PVB 3884	x	x	x	-
**T. reticulatus* (L.f.) Tölken subsp. *reticulatus*	PVB 9143	x	x	-	x
T. reticulatus (L.f.) Tölken subsp. *reticulatus*	PVB 9085	x	x	-	-
**T. reticulatus* L.f. subsp. *phyllopodium* Tölken	PVB 2817	x	x	x	x
T. reticulatus L.f. subsp. *phyllopodium* Tölken	PVB 6332	x	x	x	-
**T. rubrovenosus* (Dinter) Tölken	PVB 4658	x	x	x	-
**T. scandens* Van Jaarsv.	PVB 9090	x	x	x	x
**T. schaeferianus* (Dinter) Tölken	CM 1999	x	x	x	x
**T. similis* (Tölken) Tölken	PVB 4609	x	x	x	x
**T. singularis* (R.A. Dyer) Tölken	PVB 8839	x	x	x	x
**T. stenocaulis* Bruyns	PVB 7577	x	x	x	x
**T. striatus* (Hutchison) Tölken	PVB 7501	x	x	x	x
**T. striatus* (Hutchison) Tölken	PVB 9177	x	x	x	-
**T. suffultus* Bruyns ex Tölken	PVB 6373	x	x	x	x
**T. sulphureus* (Tölken) Tölken	PVB 5244	x	x	x	x
**T. tenuis* (Tölken) Bruyns	PVB 3219	x	x	x	-
**T. torulosus* Tölken	PVB 1391	x	x	x	x
**T. tribblei* Van Jaarsv.	PVB 8263	x	x	x	x
**T. tuberosus* Tölken	PVB 2660	x	x	x	-

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Table 1.1. continued

Taxon	Voucher Herbarium, collector	DNA region *trnL-F*	*psbA*	*rpoB*	ITS
**T. ventricosus* (Burm.f.) Tölken	TLN 133	x	x	-	x
**T. viridiflorus* (Tölken) Tölken	PVB 9119	x	x	x	x
**T. wallichii* (Harv.) Tölken subsp. *wallichii*	PVB 9826	x	x	x	-
T. wallichii (Harv.) Tölken subsp. *ecklonianus* (Harv.) Tölken	PVB 8915	x	x	x	-
Tylecodon spp.	PVB 7936	x	x	x	-
Tylecodon spp.	PVB 8265	x	x	x	-
Tylecodon spp.	PVB 8841	x	x	x	-
Kalanchoe Adans.					
**K. elizae* Berger	PVB 7743	x	x	x	x
**K. gracilipes* (*Kitchinga gracilipes* Baker)	PVB 6232	x	x	x	x
**K. humilis* Britten	PVB 7749	x	x	-	x
K. cf. umilis Britten	PVB sn	x	x	-	-
K. lanceolata (Forssk.) Pers.	PVB 9732	x	x	-	-
K. lateritia Engl.	PVB 7651	x	x	-	-
K. latisepala N.E.Br.	PVB 7749	x	x	-	-
K. marmorata Baker	PVB sn	x	x	-	-
K. rotundifolia (Haw.) Haw.	PVB 9346a	x	x	-	-
K. cf. sexangularis N.E.Br.	PVB 8750	x	x	x	-
Sempervivoideae Arn.					
**Aeonium leucoblepharum* Webb ex A.Rich.	PVB 8432	x	x	-	x
Crassuloideae Burnett					
Crassula L.					
C. barbata Thunb.	PVB 9173	x	x	-	-
C. fascicularis Lam.	TLN 161	x	x	-	-
C. multiceps Haw.	PVB 9269	x	x	-	-
C. sericea Schönland	TLN 135	x	x	-	-
**C. pellucida* L.	TLN 174	x	x	-	x
**C. rupestris* Thunb.	TLN 153	x	x	-	x

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This reduced redundancy in datasets and facilitated more thorough tree searches in subsequent analyses. A core set of 90 taxa was identified. Parsimony-based searches were also used to detect conflict among data partitions for a 71-taxon set, comprising accessions for which data from all four DNA regions were available. Sets of common taxa were used to identify topological variation resulting from different histories among DNA regions that was not confounded by variation in taxon sampling across data partitions. Conflict among data partitions was assessed on a node-by-node basis by comparing topologies generating from separate analyses of each partition. This approach allowed the nodes involved in incongruent reconstructions to be identified, and levels of support for such relationships to be assessed. Formal measures of incongruence such as the incongruence length difference (ILD; Farris et al., 1994) do not facilitate

such investigation. In addition, ILD has been criticised for its high type I error rate (e.g. Cunningham, 1997; Darlu & Lecointre, 2002; Barker & Lutzoni, 2002), and has been found to be a poor indicator of dataset combinability (Barker & Lutzoni, 2002). Only conflicting relationships that attained Jackknife (JK) percentages ≥ 75% were considered. Four taxa were identified as potentially problematic and final parsimony and Bayesian tree searches were carried out both with these taxa included (90 taxa) and with them excluded (86 taxa).

Parsimony analyses were carried out using PAUP* 4.0b10 (Swofford, 2002). Heuristic searches were performed with the following search options implemented: 10,000 random addition sequence replicates with three trees held at each step, tree-bisection-reconnection (TBR) branch-swapping performed on all trees, with no more than three trees saved from each replicate. Characters were given equal weight and states were unordered. Nodal support was evaluated using the jackknife (JK) emulating Jac resampling (Farris et al., 1996), also implemented in PAUP*. Ten thousand jackknife replicates were generated, deleting 36.79% of the characters at each replicate (Farris et al., 1996), with TBR branch-swapping performed at each replicate.

Bayesian inference

In order to perform mixed-model phylogenetic analyses on combined data, models of nucleotide evolution were selected for each data partition using Modeltest version 3.06 (Posada & Crandall, 1998). One of the most parsimonious trees (MPTs) recovered from parsimony analysis of combined plastid data was used as the input topology for selecting a model for combined plastid data, similarly, one of the MPTs from parsimony of analysis of ITS data was used for selecting the model for the ITS partition. Huelsenbeck and Rannala (2004) found that using a model that assumes unnecessary parameters has a negligible effect on posterior probability (pp) estimation, while under-specification of an evolutionary model leads to over-estimation of the pp that the tree is correct. They therefore recommended that "...the model should be as complex as possible while still allowing parameters to be identified." (2004: 912). Pol (2004) demonstrated that hierarchical likelihood ratio tests (hLRTs) are sensitive to the addition/removal sequence of

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parameters. Given that hLRTs implemented in Modeltest (Posada & Crandall, 1998) evaluate models from the simplest to the more complex, the procedure has a tendency to select less complex models. The AIC, on the other hand, evaluates competing models simultaneously and shows no such tendency (Posada & Buckley, 2004). Models selected by the Akaike Information Criterion (AIC) implemented in Modeltest were applied to subsequent Bayesian analyses.

Mixed-model phylogenetic analyses were performed on combined data for the 90- and 86-taxon datasets using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). This programme implements a Metropolis-coupled Markov chain Monte Carlo (MC^3) algorithm to sample the posterior probability distribution of trees for a given dataset. Two simultaneous analyses of 6 x 10^6 generations were undertaken, each running four Markov chains of which three were heated to a setting of 0.2. Model parameters for each data partition were sampled every 100 generations under a General Time-Reversible model of nucleotide evolution, using empirical base composition, with rate heterogeneity among sites modelled by a gamma distribution (GTR+G). In addition, a proportion of invariant sites was estimated for the *psbA-trnH* and the *rpoB-trnC* plastid regions (GTR+I+G). To estimate the number of generations required for the chains to converge on the pp distribution (ppd) of trees, log-likelihood values of sampling points were plotted, and the average standard deviation of split frequencies (ASDSF) and the convergence diagnostic: potential scale reduction factor (PSRF) were examined following each MCMC run, as outlined in Ronquist et al. (2005). Trees from the burn-in phase of analyses, i.e. those that did not converge on the ppd of trees, were discarded. Remaining trees were combined to produce a composite sample for the combined data. The pp of clades was estimated by calculating a 50% majority-rule consensus tree in PAUP* 4.0b10 (Swofford, 2002).

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RESULTS

Parsimony Analyses

Alignment of sequence data resulted in a combined dataset of 2833 characters. Collectively the plastid partitions contributed 2163 aligned nucleotide positions and 19 insertion-deletion characters (indels). Plastid DNA sequence data were generated for a total of 132 accessions, representing 97 species (Table 1.2). There were 821 bases and seven indels available from the *trnL-F* region for 128 accessions, 467 bases and seven indels from the *psbA-trnH* region for 128 accessions, and 875 bases, five indels from the *rpoB-trnC* region for 100 accessions. Alignment of ITS data for 71 taxa contributed 670 nucleotide characters but no informative indels.

Jackknife (JK) analysis of the initial 132-taxon dataset recovered most species as monophyletic, including the highly variable, widely distributed taxon, *Cotyledon orbiculata* (Fig. 1.1). Monophyletic taxa were pruned to a single representative. Exceptions to this were *Adromischus triflorus*, *A. liebenbergii*, *Tylecodon reticulatus* and *T. striatus*, each recovered as polyphyletic. An accession representing each phylogenetic position for these taxa was retained in subsequent analyses. As the 10 species of *Kalanchoe* formed a clade and relationships within the genus are beyond the scope of this investigation, only three were retained. Similarly, *Crassula* was reduced to two accessions, giving a dataset of 90 taxa. There was limited resolution following analysis of the 132-taxon set (based on *trnL-F* and *psbA-trnH* data only) however, nodes that were resolved concurred strongly with those present following analyses of the smaller datasets, descriptions of which follow. In addition, support observed in the 132-taxon set was generally similar, albeit lower in many instances, as that in subsequent analyses.

Conflict among data partitions

Analysis of each plastid data partition recovered poorly resolved and largely congruent topologies, with no supported conflicts (trees not shown). All three datasets, each of 71 taxa, were therefore combined for assessing conflict between plastid and ITS data partitions.

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Computed JK consensus trees are shown in Figure 1.2., and a brief description of conflicting phylogenetic positions follows. Jackknife support for each relationship is given in parentheses.

Table 1.2. Summary of number of species and accessions for which DNA data were generated for phylogeny reconstruction, as detailed in Table 1.1.

Subfamily Genus	No. of species	No. of species represented in current study	Total no. of accessions available	No. of accessions per DNA region			
				trnL-F	*psbA*	*rpoB*	ITS
Kalanchoideae							
Cotyledon	11	10	19	15	19	14	10
Tylecodon	46	43	55	55	54	48	31
Adromischus	27	27	41	41	38	35	24
Kalanchoe	~ 144	10	10	10	10	3	3
Sempevivoideae							
Aeonium	~ 36	1	1	1	1	0	1
Crassuloideae							
Crassula	~ 195	6	6	6	6	0	2
Totals		97	132				

Adromischus

ITS recovers *A. fallax* as sister to *A. phillipsae* (90), both being outside the *A. nanus* - *A. bicolor* clade (86); in contrast, plastid data place *A. fallax* as sister to *A. umbracticola* + *A. schuldtianus* (90), nested within the *A. nanus* – *A. bicolor* clade (80).

Tylecodon

(i) The ITS data place *T. stenocaulis* as sister to *T. ventricosus* + *T. striatus* (86), while plastid data recover *T. stenocaulis* as sister to *T. leucothrix* (95), and include *T. ventricosus* + *T. striatus* within a clade of seven other taxa (*T. cacalioides* – *T. nolteei* clade) (93).

(ii) ITS places *T. fragilis* as sister to *T. schaeferianus* (96), and plastid data recover *T. fragilis* as sister to *T. decipiens* (89).

(iii) ITS resolves *T. suffultus*, *T. occultans*, *T. reticulatus* ssp *reticulatus* and *T. nolteei* as members of the *T. cordiformis* – *T. pusillus* clade (72), with *T. occultans* being placed sister to *T. reticulatus* ssp *phyllopodium* (87). In contrast, analysis of plastid data recovers these taxa within the *T. cacalioides* – *T. ventricosus* clade (93), retaining *T. reticulatus* ssp. *phyllopodium* as a member of the *T. cordiformis* – *T. pusillus* clade (76).

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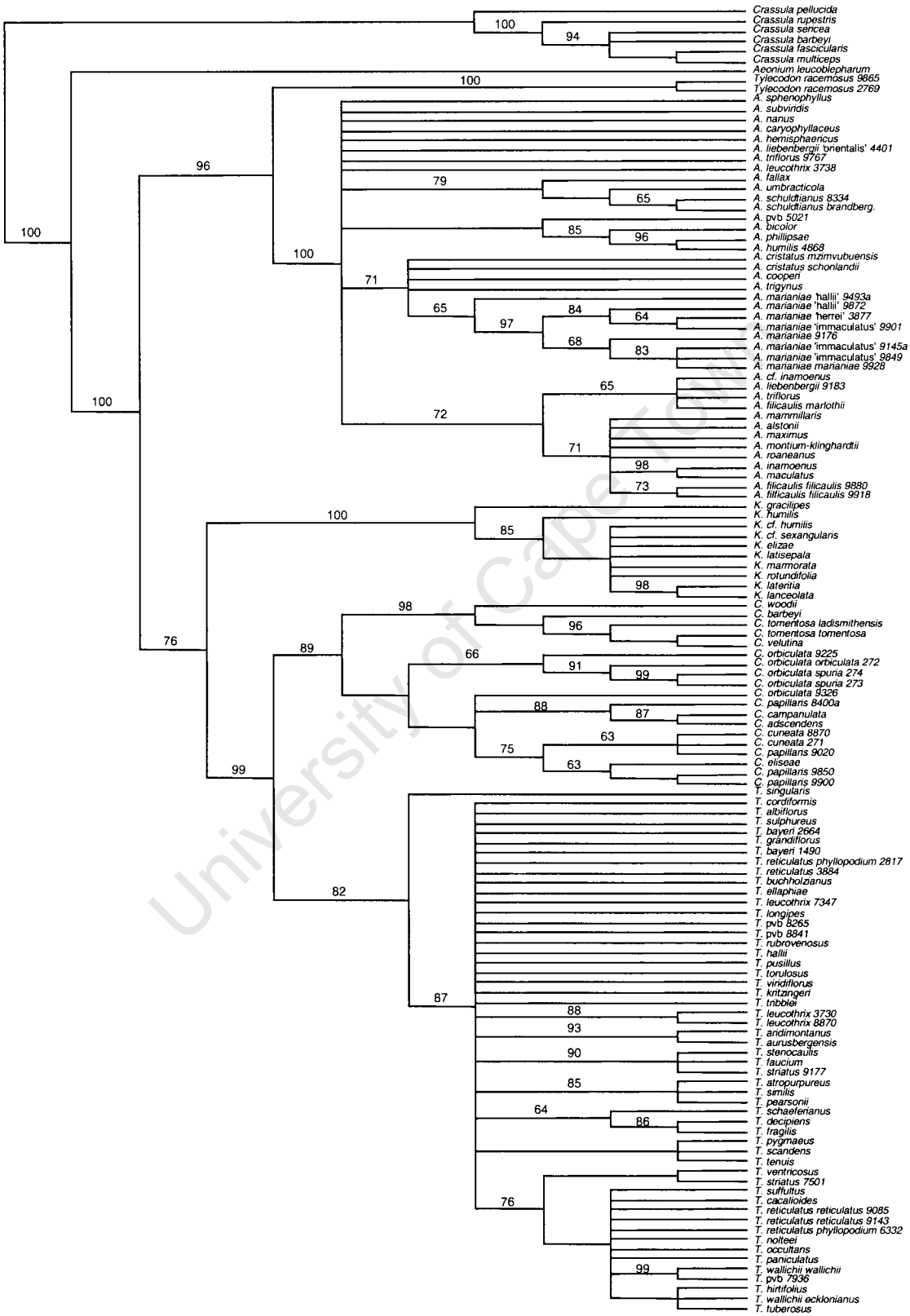

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Figure 1.1. Jackknife consensus tree recovered following analysis of plastid data (*trnL-F* and *psbA-trnH*) for 132 taxa. Several taxa are represented by multiple accessions in order to determine whether or not they represent monophyletic groups. Jackknife values of ≥ 63% are shown above branches.

Applying the 75% JK criterion to the conflicts outlined above identifies *A. fallax*, *T. stenocaulis*, *T. fragilis* and *T. occultans* as potentially problematic for inclusion in an analysis of combined ITS and plastid data. Subsequent analyses were carried out with these taxa included (90-taxon set), and with them excluded (86-taxon set). The former comprised 10 of the 11 *Cotyledon* species (10 accessions), 43 of the 46 *Tylecodon* species (45 accessions), and 27 of the 28 species of *Adromischus* (29 accessions), together with three species of *Kalanchoe*, *Aeonium leucoblepharum*, and two species of *Crassula*. Topological information from the strict consensus trees computed from results of parsimony analysis is incorporated into Figures 1.3 and 1.4. Tree statistics are shown in Table 1.3, and reporting of results is combined with those from Bayesian inference that follows.

Bayesian inference: generic relationships and monophyly

Trees sampled after the ASDSF stabilised at < 0.03 and the PSRF was at or close to zero (after approximately 4,000,000 generations) were retained for both taxon sets. Independent runs produced highly similar results and so trees from only one run were used. Thus for both taxon sets a 50% majority rule consensus tree was computed from the remaining 20,000 trees considered to be a good representation of the posterior probability distribution of trees (Figs. 1.3 & 1.4). Posterior probabilities ≥ 0.90 are reported in parentheses.

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Table 1.3. Tree statistics of combined plastid and ITS dataset analysed for 90 and 86 taxa. PICs – parsimony informative characters, MPTs – most parsimonious trees, CI – consistency index, RCI – rescaled consistency index.

Taxon set	Characters included	Characters variable	PICs	MPTs	Tree length	CI	RCI
90	2852	872	494	939	1631	0.556	0.500
86	2852	865	490	5250	1596	0.567	0.510

Parsimony and Bayesian reconstructions of phylogeny are highly congruent, with the exception of the position of *Kalanchoe* (Figs. 1.2 & 1.3). Parsimony recovers *Kalanchoe* as sister to a well-supported clade (JK=91%) containing *Cotyledon*, *Tylecodon* and *Adromischus*, whereas Bayesian analysis recovers *Adromischus* as sister to a clade comprising *Kalanchoe* and *Cotyledon* + *Tylecodon* which attains pp=0.92.

Eliminating taxa previously identified as problematic from Bayesian analysis has no effect on the deep relationships in the phylogeny. Variation in topology occurs only within genera and differs between the taxa involved. *Adromischus fallax* is strongly supported as sister (pp=1.00) to *A. phillipsae* + *A. humilis*, and its inclusion results in the sister pair being placed within the *A. subviridis* – *A. humilis* clade. When *A. fallax* is omitted *A. phillipsae* + *A. humilis* is sister to all other species of *Adromischus* – a relationship also highly supported (pp=1.00). Under parsimony all nodes relevant to this conflict are unresolved. Within *Tylecodon*, removing *T. stenocaulis* has no effect on relationships between remaining clade members. *Tylecodon occultans* is sister to *T. suffultus* + *T. reticulatus* ssp *reticulatus* + *T.nolteei*, forming a clade that is nested within the *T. ventricosus* – *T. tuberosus* clade. When *T. occultans* is removed, the clade to which it belonged is placed as sister to a correspondingly reduced *T. ventricosus* – *T. tuberosus* clade with high pp (1.00). In addition, JK support for the reduced clade increases from 70 to 81%. Omitting *T. fragilis* has no effect on support for the clade to which it belonged - it remains high (pp=0.96), while JK support increases from < 50% to 78%.

Relationships within Kalanchoideae

Kalanchoideae are monophyletic and support for this is strong (pp=1.00; Fig. 1.2). Generic-level relationships within the subfamily are resolved and also attain strong support from the data. The monophyly of *Cotyledon*, *Adromischus* and most of *Tylecodon* is strongly supported (pp=1.00). In turn, the sister relationship between *Cotyledon* and *Tylecodon* attains high probability (pp=1.00). The strongly supported (pp=1.00) placement of *T. racemosus* as sister to *Adromischus*, renders *Tylecodon,* as currently circumscribed, polyphyletic.

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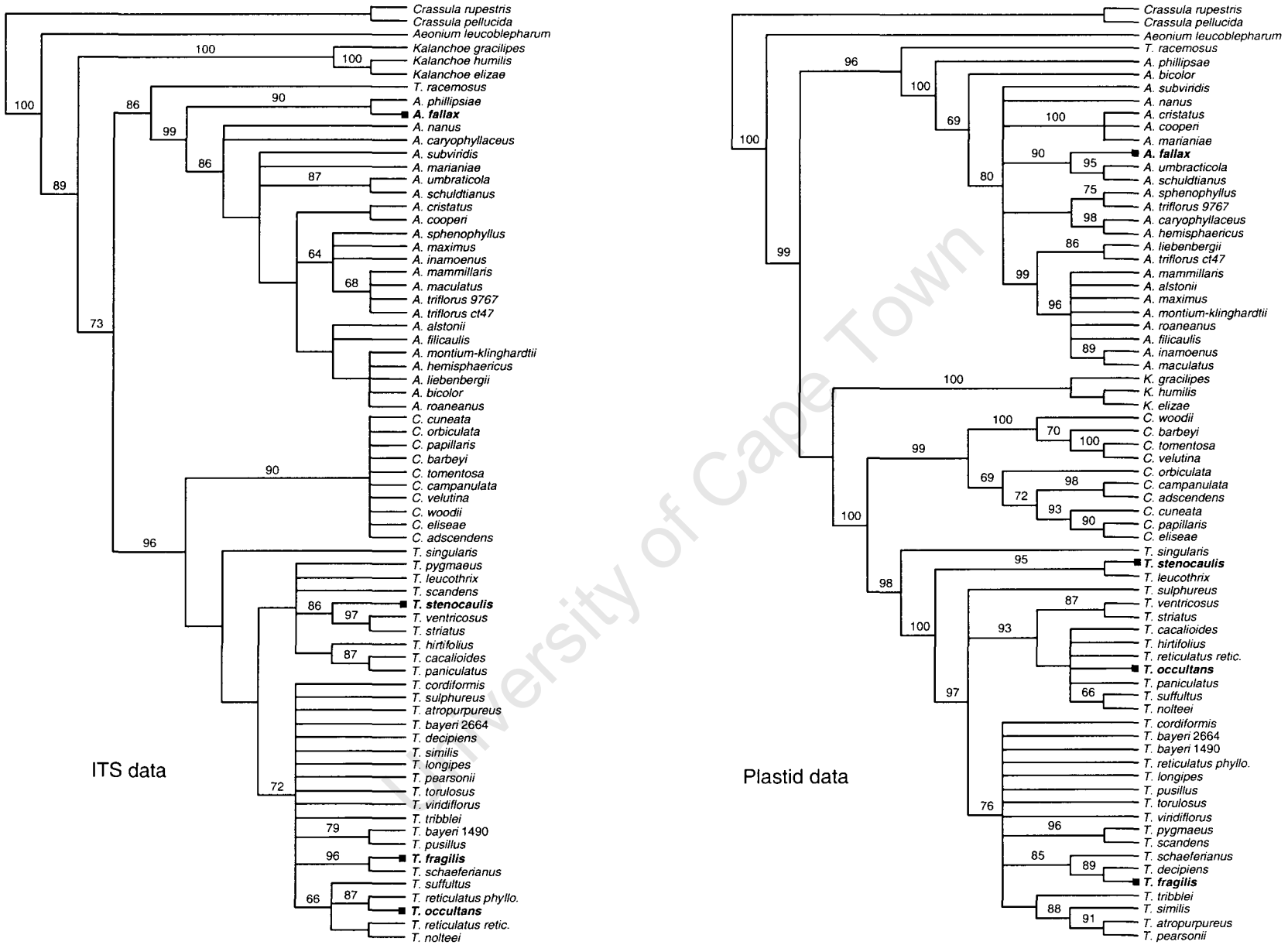

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Figure 1.2. Jackknife consensus trees from analysis of ITS (left-hand tree) and plastid data (right-hand tree) for a common set of 71 taxa. Nodes with a group frequency of ≥ 50% are shown as resolved, and JK values of ≥ 63% are given above branches. Species which have conflicting relationships between the two topologies are indicated by a solid square on the branch.

In order to assess the genetic divergence of *Tylecodon racemosus* from other taxa focal to this study, genetic distances (uncorrected p distances) between pairwise species comparisons of sequence data were calculated in PAUP (Swofford, 2002). Pairwise distances between species were calculated for species of *Cotyledon*, *Tylecodon*, and *Adromischus*, and between members of these genera and *T. racemosus*. *Tylecodon racemosus* was found to be a highly divergent lineage (Table 1.4). Genetic distances between species within each genus are small (0.012 – 0.014), while the distances between genera range from 0.029 to 0.066. *Tylecodon racemosus* is at least as different from *Tylecodon*, *Cotyledon* and *Adromischus* as each is from the other (0.054-0.068).

Table 1.4. Summary of pairwise genetic distances calculated between species of *Cotyledon, Tylecodon, Adromischus*, and between these genera and *Tylecodon racemosus*. Values are uncorrected 'p' distances.

Comparison	Average genetic distance	+/- stdev
Within *Cotyledon*	**0.012**	0.006
Within *Tylecodon*	**0.013**	0.011
Within *Adromischus*	**0.014**	0.006
Cotyledon – Tylecodon	**0.029**	0.009
Cotyledon – Adromischus	**0.066**	0.037
Tylecodon - Adromischus	**0.058**	0.006
T. racemosus - Cotyledon	**0.068**	0.013
T. racemosus - Tylecodon	**0.059**	0.007
T. racemosus – Adromischus	**0.054**	0.006
T. racemosus – all	**0.059**	0.009

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Species-level relationships

Relationships within *Cotyledon* are completely resolved. Two strongly supported clades are recovered within the genus, each with pp=1.00. *Cotyledon woodii* is placed as sister to *C. barbeyi* + *C. tomentosa* + *C. velutina* (p=1.00). The latter two species form a highly supported pair (p=1.00). The second clade comprises *C. campanulata* + *C. adscendens* (pp=1.00) recovered as sister to the remaining four species. Within this clade *C. papillaris* + *C. eliseae* is highly supported (pp=1.00), with *C. cuneata* sister to this pair. The latter relationship attains high JK support (93%),

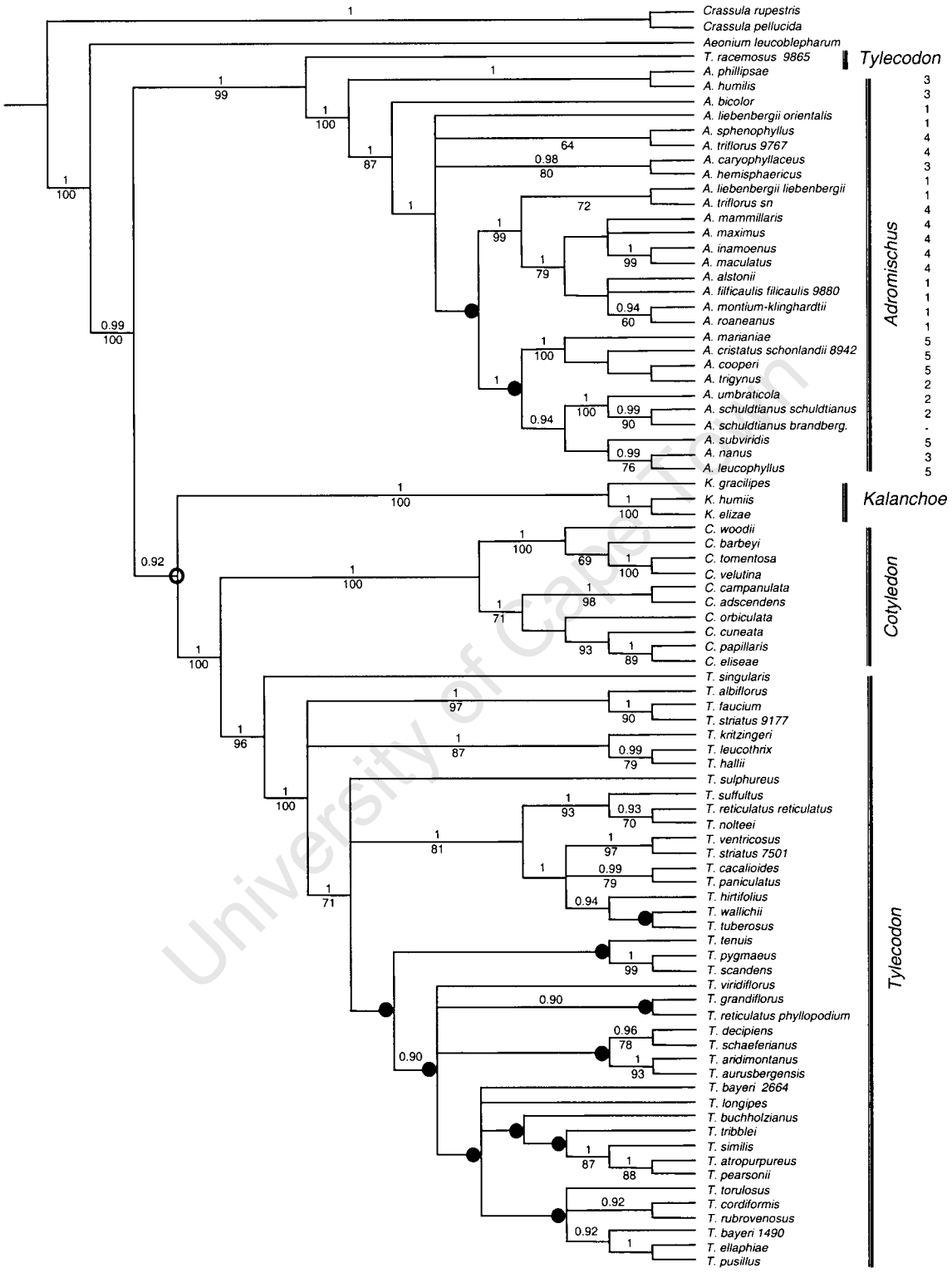

Figure 1.3. Majority rule consensus tree (50%), computed from 20,000 trees following Bayesian analysis of combined ITS and plastid data for 86 taxa. Four taxa involved in conflicting resolutions from ITS and plastid datasets were omitted from these analyses. Posterior probabilities of clades are given above branches and JK percentages from parsimony analysis are given below branches. Nodes marked with black dots are unresolved in the parsimony strict consensus tree, and that marked with an open circle conflicts with the parsimony reconstruction (see text). Numbers opposite species of *Adromischus* indicate the section in which Tölken (1985) placed each species: **1** *Adromischus*; **2** *Borealí*; **3** *Brevipedunculati*; **4** *Incisilobati*; **5** *Longipedunculati*.

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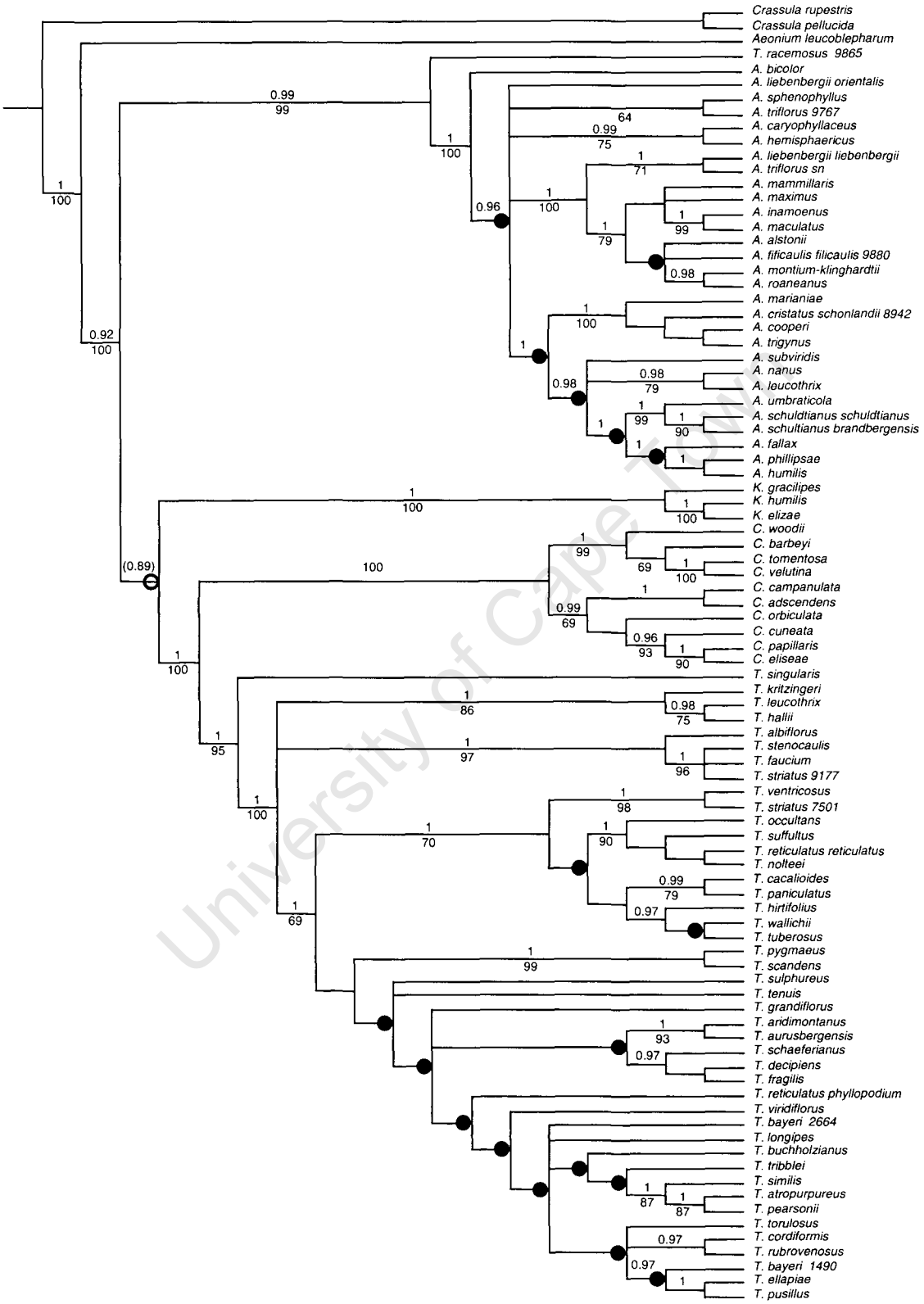

Figure 1.4. Majority rule consensus tree (50%) computed from 20,000 trees following Bayesian analysis of combined ITS and plastid data for 90 taxa. Taxa involved in conflicts between data partitions are included in this analysis. Posterior probabilities of clades are given above branches and JK percentages from parsimony analysis are given below branches. Nodes marked with black dots are unresolved in the strict consensus tree, and that marked with an open circle conflicts with the parsimony reconstruction (see text).

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but is not supported in Bayesian reconstructions. *Cotyledon orbiculata* is recovered as sister to these species, although the relationship is unsupported.

Resolution within *Tylecodon* is poor; however some structure is recovered. *Tylecodon singularis* is recovered as sister to all other species of *Tylecodon,* and four main clades that attain support are identified. Two such clades emerge, and while they receive high support (pp=1.00), there is no information regarding their affinities to the remaining species in the genus. The first clade finds *T. albiflorus* as sister to *T. faucium* + *T. striatus* 9177, while in the second clade *T. kritzingeri* is recovered as sister to *T. leucothrix* + *T. hallii* (pp=1.00) Two larger clades that group most other species are recovered. The *T. suffultus* – *T. tuberosus* clade houses 10 species, with pp=1.00. Within this *T. suffultus* is resolved as sister to *T. reticulatus* ssp *reticulatus* + *T. nolteei* (pp=0.93) and the other clade, supported by pp=1.00 contains *T. ventricosus* + *T. striatus* (pp=1.00), *T. cacalioides* + *T. paniculatus* (pp=0.99), and finally *T. hirtifolius* as sister to *T. wallichii* + *T. tuberosus*. The latter pair is unsupported. Finally, a clade of 20 species is recovered and supported with pp=0.90. Whilst there is little structure within this clade, four species pairs are reliably identified (i.e. pp ≥ 0.95), namely *T. decipiens* + *T. schaeferianus* (pp=0.96), *T. aridimontanus* + *T. aurusbergensis* (pp=1.00), *T. atropurpureus* + *T. pearsonii* (pp=1.00), and *T. ellaphiae* + *T. pusillus* (pp=1.00). An additional strongly supported pairing between *T. pygmaeus* and *T. scandens* (pp=1.00) is recovered and falls outside any of the clades described.

The five sections of *Adromischus* recognised by Tölken are indicated on Fig. 1.3. There is reasonable correspondence between his morphology-based classification and the molecular data analysed here. *Adromischus phillipsae* and *A. humilis* are sister species, both from Section *Brevipedunculati* (3), (pp=1.00) this pair being recovered as sister to the rest of *Adromischus*. The subsequent divergence of *A. bicolor* leaves a well-supported clade (pp=1.00) within which two main clades are partly resolved. The *A. liebenbergii* – *A. roaneanus* clade (pp=1.00) contains members of Sections *Adromischus* (1) and *Incisilobati* (4) only. The second main group, *A. marianiae* – *A. leucophyllus* (pp=1.00) is further resolved as the *A. marianiae* – *A. trigynus* clade

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(pp=1.00) containing members of Sections *Boreali* (2) and *Longipedunculati* (5), and the *A. umbraticola* – *A. leucophyllus* clade (pp=0.94) comprising members of Sections *Boreali*, *Brevipedunculati*, and *Longipedunculati*.

DISCUSSION

Phylogenetic relationships estimated from data from the nuclear and plastid loci used in this study are highly congruent indicating that they share a common evolutionary history. Thus hybridisation and paralogy are not of major concern in the interpretation of relationships in this group given these data. Several supported conflicts do emerge, however most conflict lacked clear support. Nuclear ITS data could not be produced for 20 of the terminals, mostly species of *Tylecodon*, in the 90-taxon dataset using standard sequencing techniques as there was evidence of multiple signals in sequence traces. The ITS region is a popular source of information in plant phylogenetic inference. As a result of its widespread application the potential for incorrect inference has been uncovered and the region's utility for phylogenetic reconstruction questioned (Álvarez and Wendel, 2003). The region occurs in multiple and tandemly repeated 18S-26S rDNA arrays that can evolve independently and thus blur or even obscure phylogenetic relationships at the organismal level (Álvarez and Wendel, 2003). Without knowing whether the ITS data generated here represent orthologous or paralogous loci, the assumption must be that no one set of data are more correct than the other, and topological inconsistencies are interpreted as indicating potential biologically significant processes (Wendel and Doyle, 1998). It has been suggested that plastid DNA alleles coalesce faster than nuclear alleles due to their smaller effective population sizes (Wendel and Doyle, 1998). In modelling this process Moore (1995) proposed that in order to match the level of confidence in the congruence of a single organellar tree, 16 independent nuclear gene trees would be required. This level of DNA sampling is impractical for the current investigation and as analyses of nuclear and plastid data produce generally concordant topologies on supported nodes, whereas most conflict lacks clear support.

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Caution has been exercised in interpreting the phylogenetic discord found in this study and the small amount present is not sufficient to undermine the value of this phylogenetic hypothesis.

Including *Adromischus fallax* in analyses clearly confounds relationships between *A. phillipsae* + *A. humilis* and the remaining members of the genus. When *A. fallax* is included the three species form a clade which is of relatively recent origin, nested within a larger well-supported clade (*A. marianiae – A. humilis*, Fig. 1.4). When *A. fallax* is omitted the remaining two species form a pair whose lineage diverged very early in the history of the genus (Fig. 1.3). It is not surprising that *A. fallax, A. phillipsae* and *A. humilis* form a clade. Tölken (1985) placed them in Section *Brevipedunculati* as they all have softly herbaceous leaves - unusual in the genus. *Adromischus fallax* is a little known species, restricted to a single mountain top near Graaff-Reinet in the Eastern Cape Province. Additional field collected material is needed to establish whether the individual sequenced for this study is of hybrid origin, or whether the seemingly different histories result from the species being the product of hybridisation. It would be interesting to clarify the timing of the evolution of softly herbaceous leaves in the group as, although they are not seasonally deciduous, they closely resemble the leaves of species of *Tylecodon*, and those of the new genus described in the next chapter (*Toelkenocodon*).

The data are inconclusive regarding the relationship between *Kalanchoe* and the remaining genera of Kalanchoideae. Plastid and combined data recover *Kalanchoe* as sister to *Cotyledon* + *Tylecodon*, with *Adromischus* sister to these three genera. These findings are consistent with those of a family-wide analysis by Mort et al. (2001). Similarly, van Ham and 't Hart (1998) recovered *Adromischus* as an earlier diverging lineage, sister to a clade comprising *Cotyledon*, and *Kalanchoe* + *Bryophyllum* (the latter has since been included in *Kalanchoe:* Thiede & Eggli, 2007). Their inferences were based on a single representative of each genus and *Tylecodon* was not included, thus direct comparison between their work and the current study is somewhat limited. In contrast to analysis of plastid and combined data, ITS data place *Kalanchoe* as sister to a clade comprising *Cotyledon*, *Tylecodon* and *Adromischus. Kalanchoe* is a large and variable

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group comprising some 144 species distributed throughout the Old World tropics, especially Africa and Madagascar (Mabberley, 1997). Only 13 species occur within southern Africa (Tölken, 1985) and the monophyly of the genus has yet to be tested with thorough sampling and molecular phylogenetic analyses. The uncertainty regarding the relationships of the genus within Kalanchoideae may well result from the low numbers of species represented in this and other studies (e.g. Mort et al., 2001). Species of *Kalanchoe* included here are however, recovered as monophyletic and are not nested within any of the focal study genera. Increased sampling in future investigations will help to clarify the situation. *Kalanchoe* is known to be of polypoid origin (Mort et al., 2001): thus the conflict between nuclear and plastid data revealed here may well indicate historical hybridisation.

The monophyly of *Cotyledon, Tylecodon* and *Adromischus* recovered by molecular data in this study concurs almost completely with the most inclusive generic-level classifications based on morphology (Tölken 1978, 1985; van Jaarsveld & Koutnik, 2004). That *Tylecodon racemosus* is not included in the *Tylecodon* clade is an unexpected finding. Two accessions of *T. racemosus* from separate localities were used to check the validity of this finding. Their monophyly was strongly supported by both ITS and plastid data, independently (JK=100%). All data partitions, whether analysed singly or in combination, using parsimony and Bayesian methods, recovered *T. racemosus* as sister to *Adromischus*. Morphology unambiguously places *T. racemosus* within *Tylecodon*, and no mention of suspicion regarding its taxonomic position has been made in treatments detailing the taxon (Tölken 1978, 1985; van Jaarsveld & Koutnik, 2004). It shows little morphological resemblance to *Adromischus* with which it shared a MRCA, and in the context of the southern African members of the Kalanchoideae, molecular evidence regarding the relative divergence of *T. racemosus* is clear. There is little doubt that the taxon should be elevated to the rank of genus. Morphological interpretation is potentially more challenging and is the subject of the following chapter.

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There is little topological agreement between this study and that of Mort et al. (2005) regarding relationships within *Cotyledon.* Although the phylogeny of *Cotyledon* is fully resolved in their study, the only relationships that attain bootstrap (BS) values > 50% are the pairing of the two subspecies of *C. tomentosa* (BS=97%), and the recovery of *C. orbiculata* var. *oblonga* as sister to these (BS=61%). In contrast, the data analysed here produce a well supported topology. Direct comparison of the two reconstructions is somewhat hampered by differences in sampling within *Cotyledon orbiculata.* Mort et al. (2005) include representatives of all varieties of *C. orbiculata,* while only *C. orbiculata* var. *orbiculata* and *C. orbiculata* var. *spuria* were available for the current work. More detailed sampling within the highly variable *C. orbiculata*, together with analysis of additional sequence data are needed to produce a more robust phylogenetic hypothesis for the genus. There is concordance between geography and the two main clades recovered in *Cotyledon* (Fig. 1.5). The 'eastern', *C. barbeyi* - *C. velutina* clade is distributed mostly east of Cape Agulhas. *Cotyledon barbeyi* is its northern-most member and is a widespread, common species found from KwaZulu-Natal, South Africa northwards to the Arabian Peninsula. A 'western' clade also emerges: the *C. campanulata* – *C. eliseae* clade, with representatives found in Namaqualand, ranging south to the coast and eastwards to East London. There is substantial overlap between the eastern and western clades in the southern-most part of the genus' range between Cape Agulhas and East London, and *Cotyledon orbiculata* of the western clade is widespread and occurs throughout the ranges of all other species of *Cotyledon*. The geographic mingling of representatives of these two divergent clades suggests that species of *Cotyledon* are relatively mobile, dispersing into new areas and expanding their current ranges to overlap with members of the more distantly related clade.

Several geographically cohesive clades emerge from analyses of *Tylecodon*. The *T. albiflorus* – *T. striatus* clade comprises species restricted to succulent karoo vegetation in the Little Karoo Centre (van Wyk & Smith, 2001) and the Ceres Karoo, with *T. striatus* extending northwards into the Roggeveld Mountains (Fig. 1.6). A Gariep Centre (sensu van Wyk & Smith, 2001) clade is recovered and represents almost half of the species of *Tylecodon*. The diversity of the Gariep

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clade is concentrated in the Richtersveld, while some species range eastwards along the Orange River Valley, and others extend north into Namibia. The Gariep Centre coincides with an area that receives regular, coastal fog that contributes significantly to annual precipitation (Williamson, 2000). Within the Gariep Centre clade two strongly supported groups emerge: the narrowly distributed montane sisters: *T. aridimontanus* and *T. aurusbergensis*, and a clade of coastal species, namely *T. schaeferianus*, *T. decipiens* and *T. fragilis*. All species, except *T. aridimontanus,* have subterranean tubers. *Tylecodon kritzingeri* and *T. hallii*, also found in the Richtersveld, are recovered within the same clade as *T. leucothrix*, which is distributed in the distant Little Karoo Centre. This is a considerable disjunction between closely related species. Several explanations are equally plausible given that no decisive evidence exists: extinction of intervening species or populations, seed dispersal, and post-speciation range shifts. Interestingly, four of the six most widespread species of *Tylecodon*, namely *T. wallichii, T. paniculatus, T. reticulatus* (currently ssp. *reticulatus*) and *T. ventricosus*, are confined to one clade. There is no obvious unifying feature exclusive to these species that might account for their relatively widespread distributions. They are amongst the larger species in the genus, and this may confer increased dispersal distances, although other species of similar stature are not widespread. The remaining members of this clade are restricted to the Knersvlakte Centre (*T. nolteei* and *T. suffultus*), northern Namaqualand (*T. tuberosus* and *T.hirtifolius*), while *T. cacalioides* is restricted to the Little Karoo Centre.

Adromischus is noteable for the high incidence of close relatives that are geographically widely separated (Fig. 1.7). Two main, well-supported clades account for the majority of species, namely the *A. liebenbergii – A. roaneanus* clade and the *A. marianiae – A. leucophyllus* clade. Disjunctions are prevalent in the latter. For example, *A. nanus* occurs from the Richtersveld to Springbok (Pilbeam et al. 1998) and its sister is found between Roberston, Montagu, and around the Little Karoo. *Adromischus umbraticola* is also part of this clade and is found on the Highveld around Pretoria and the Soutpansberg and Blouberg, while its closest living relative, *A. schuldtianus,* occurs in Namibia. As with some members of *Tylecodon*, these disjunctions

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suggest mobility of seeds and, in the case of species of *Adromischus* which propagate readily from fallen leaves, asexual propagules. Given the current data however, extinction and post-speciation range shifts cannot be ruled out. The second of the two main clades contains species with somewhat more cohesive distributions. *The A. mammillaris – A. maculatus* subclade is found mainly in the Western Cape Province, and the *A. alstonii – A. roaneanus* clade ranges from the Western Cape Province north into the Richtersveld.

Different patterns of flowering phenology emerge across the three genera investigated here. Coincident with the east-west split in the two main clades of *Cotyledon* is the separation of flowering time. Members of the western clade flower mostly in spring and early summer, with the exception of *C. eliseae*, which flowers in January (Fig. 1.5). In contrast, species of the eastern clade exhibit greater variation, with flowering occurring from late summer through to winter. Across the entire distribution of *Cotyledon* flowering occurs after the main rainfall season and is likely to be synchronous with a greater availability of pollinators in the dry season when many other plants have finished flowering (van Jaarsveld & Koutnik, 2004). A very broad phenological sequence is apparent across the phylogeny of *Tylecodon*, with a shift from spring to late summer flowering in more recently diverged clades (Fig. 1.6). This coincides with the evolution of a speciose clade with many representatives distributed in Namaqualand and the Richtersveld area (*T. viridiflorus – T. pusillus* clade). Most members of this clade flower during early to mid summer. There is very little phylogenetic structure within the clade of late summer flowering species, but it is interesting that flowering times between well-supported sister species identified by these analyses, both in this and other clades, are more often than not asynchronous. In some instances, such as with *T. pygmaeus* and *T. scandens,* species are sympatric and they flower from November to January, and February to March, respectively. Others that are allopatric also separate their flowering times. *Tylecodon ellaphiae* from the north-eastern Richtersveld flowers in January and February whereas its sister, *T. pusillus* found in the south-eastern Richtersveld, flowers from March to June. Across the succulent karoo, which is home to many species of *Tylecodon*, succulents tend to flower after the herbaceous annuals of the region. Succulents are

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able to separate growth and flowering due to the availability of water stored in the plant body, and the shift in flowering may be an adaptation to reduce competition for pollinators (Esler, 1999). Across *Adromischus* flowering times are relatively conserved; some flower in November-December, others in December-January. As with *Tylecodon*, there are temporal shifts in flowering between several sister species. Unfortunately flowering times are not known for several species of *Adromischus*, but in sister pairs for which times are known, flowering rarely overlaps. *Adromischus nanus* found east of the Richtersveld flowers during November and December, while *A. leucophyllous* of the Little Karoo, flowers in January and February. Likewise, *A. caryophyllaceus* reported from the Little Karoo to Hermanus flowers from January to March, whereas its sister *A. hemisphaericus,* found in the south-western Cape and north to the Knersvlakte, flowers in early summer. Very little is known about pollination in the southern African members of Kalanchoideae and so the phylogenetic context of phenological patterns presented here offers considerable scope for comparative analysis of sister pairs within these genera. Combining investigations of population genetic structure with those of habitat isolation and the natural history of pollination would provide valuable insight into the processes determining reproductive isolation and speciation across this group of succulents.

Analyses of data from two independent loci generated for this study recover highly concordant estimates of phylogeny for *Cotyledon*, *Tylecodon* and *Adromischus*. Testing current generic limits with extensive species-level sampling and molecular sequence data identified *Tylecodon racemosus* as having a separate evolutionary history, sufficiently divergent from all currently described clades to necessitate its elevation to the rank of genus: *Toelkencodon*. The morphologically cryptic nature of this taxon's divergence highlights the importance of using DNA data as a source of information for phylogenetic inference that is independent of the confounding effects of morphological convergence. In light of these findings, the value of thorough sampling when investigating species-level relationships cannot be over-emphasised. Further data are required to produce more robust estimates of relationships within clades, however the phylogeny presented here provides a great deal of information on the evolutionary history of *Cotyledon*,

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Tylecodon, *Adromischus* and *Toelkenocodon*. Relationships are sufficiently resolved and well-supported to facilitate the investigations of the temporal and spatial patterns of diversification that have occurred across these southern African genera of Kalanchoideae; analyses that are the subject of Chapters 3 and 4.

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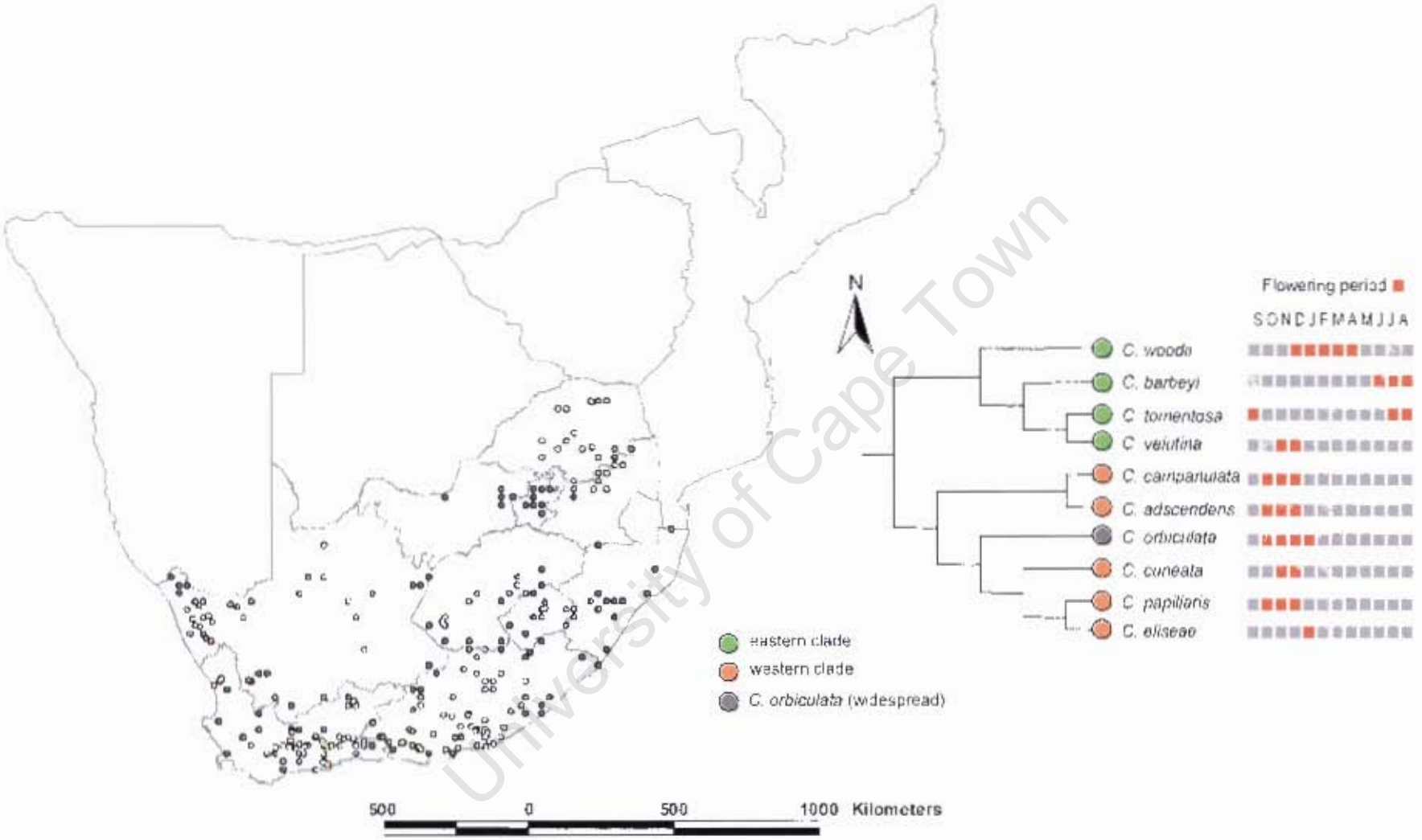

Figure 1.4. Geographical distribution of clades and flowering phenology in *Cotyledon* in South Africa and Lesotho.

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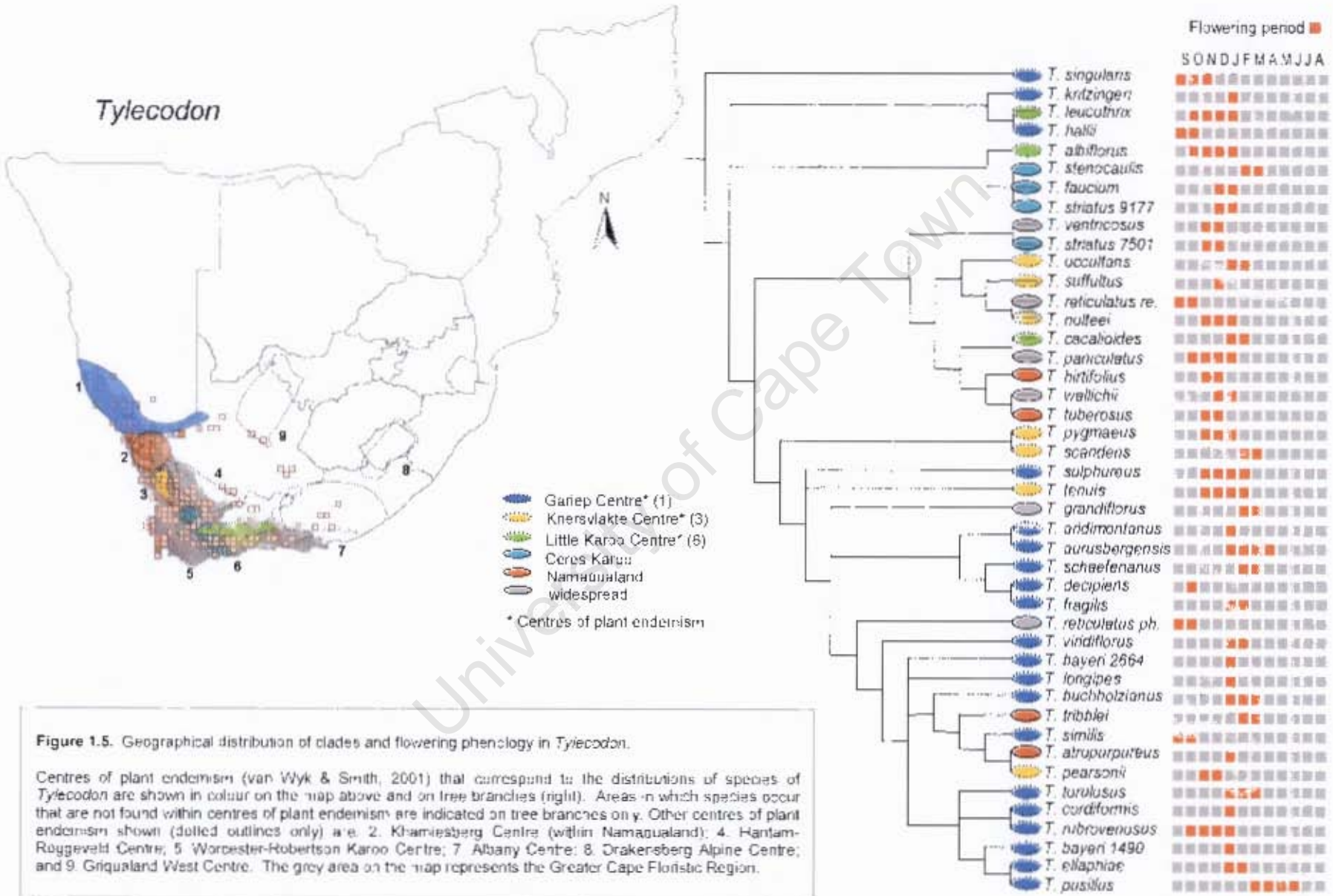

Figure 1.5. Geographical distribution of clades and flowering phenology in *Tylecodon*.

Centres of plant endemism (van Wyk & Smith, 2001) that correspond to the distributions of species of *Tylecodon* are shown in colour on the map above and on tree branches (right). Areas in which species occur that are not found within centres of plant endemism are indicated on tree branches only. Other centres of plant endemism shown (dotted outlines only) are: 2. Khamiesberg Centre (within Namaqualand); 4. Hantam-Roggeveld Centre; 5. Worcester-Robertson Karoo Centre; 7. Albany Centre; 8. Drakensberg Alpine Centre; and 9. Griqualand West Centre. The grey area on the map represents the Greater Cape Floristic Region.

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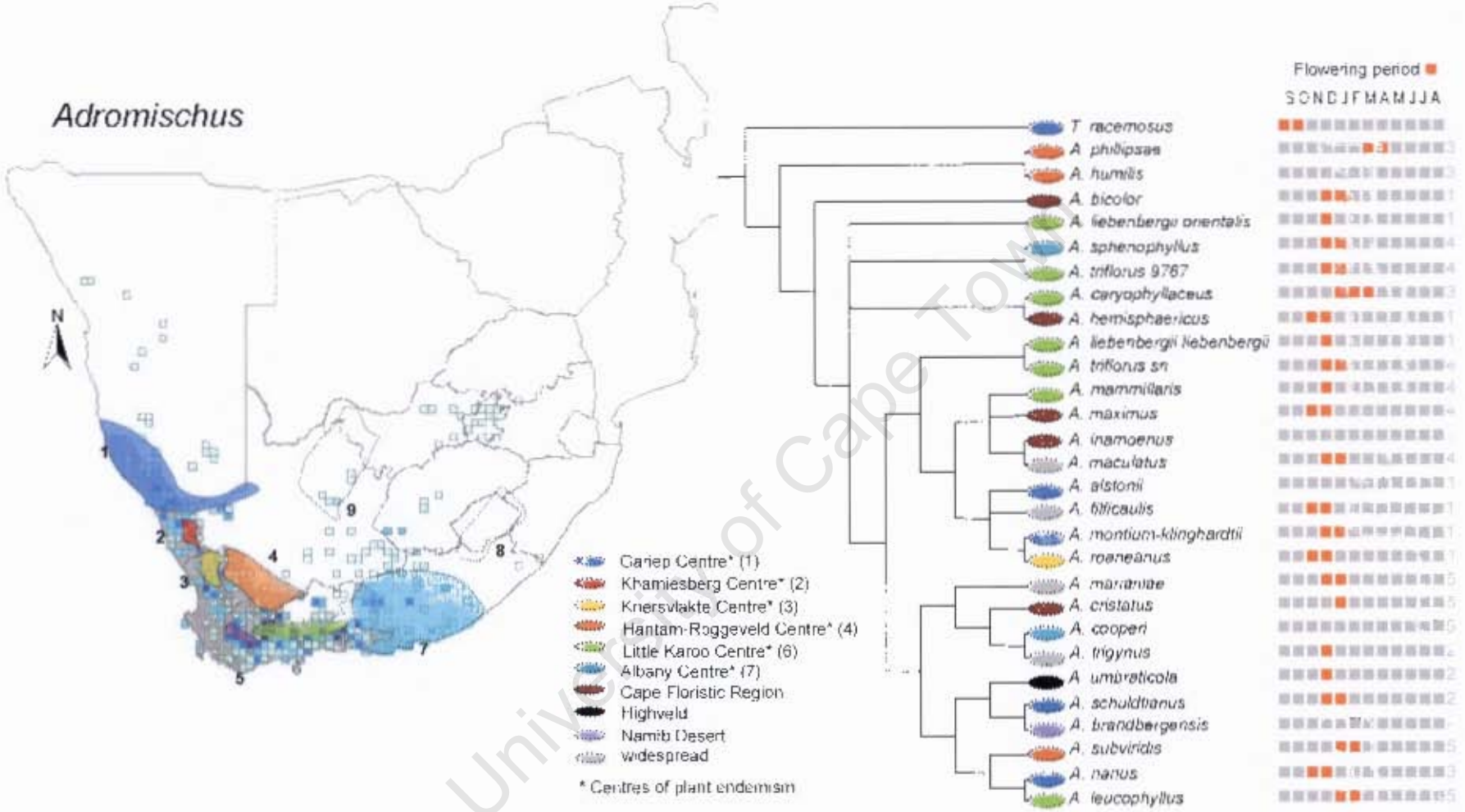

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Figure 1.6. Geographical distribution of clades and flowering phenology in *Adromischus*. Numbers to the right of the flowering period chart indicate the Section to which each belongs (Tölken, 1985): 1. Adromischus; 2. Bunelli; 3. Brevipedunculati; 4. Incisilobati; 5. Longipedunculati. Flowering times are not known for the species which lack orange squares.

Centres of plant endemism (van Wyk & Smith, 2001) that correspond to the distributions of species of *Adromischus* are shown in colour on the map above and on tree branches (right). Areas in which species occur that are not found within centres of plant endemism are indicated on tree branches only. Other centres of plant endemism indicated by dotted outlines only are: 8. Drakensberg Alpine Centre; and 9. Griqualand West Centre. The grey area on the map represents the Greater Cape Floristic Region.

CHAPTER 2

Toelkenocodon, a new genus of southern African Crassulaceae.

INTRODUCTION

Tylecodon is a genus of seasonally deciduous, dwarf, succulent shrubs distributed in the winter rainfall region of south-western southern Africa. Many species of *Tylecodon* are restricted to succulent karoo vegetation. Information on the taxonomy of the genus, together with that of its sister *Cotyledon*, has been recently updated to include the many species described since Tölken erected *Tylecodon* in 1978 (van Jaarsveld & Koutnik, 2004). The genus comprises 46 species, and is one of four genera of Kalanchoideae, the subfamily of predominantly African representatives of Crassulaceae. Kalanchoideae also includes *Adromischus* with 28 species, having near equal representation in the winter and summer rainfall regions of southern Africa, and *Cotyledon* with 11 species, found mostly in summer rainfall areas. Members of Kalanchoideae are easily distinguished from other members of Crassulaceae by the possession of a well-developed corolla tube (Thiede & Eggli, 2007). While the aforementioned genera are pentamerous, *Kalanchoe* – the fourth member of the subfamily - has flowers in four parts. *Kalanchoe* is a variable and widespread genus comprising around 150 species distributed in summer rainfall areas.

Tylecodon, *Cotyledon* and *Adromischus*, were the focus of a recent, near completely sampled species-level phylogeny that used plastid and nuclear DNA data to test the monophyly of and elucidate relationships within the three genera (Chapter 1). This molecular study revealed that *Tylecodon* is polyphyletic as *Tylecodon racemosus* is recovered as sister to *Adromischus*. This relationship is very strongly supported by data from both independent loci. Analyses of genetic

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distances within and among genera (Table 1.4, Chapter 1) indicate that *T. racemosus* is as different from *Adromischus* as it is from either *Tylecodon* or *Cotyledon*, arguing for its elevation to the rank of genus. Morphologically *T. racemosus* closely resembles species of *Tylecodon*. It has soft, seasonally deciduous leaves, a pachycaulous base, and erect to spreading inflorescences of 1-6 monochasia. *Adromischus* is a morphologically coherent genus which differs from *T. racemosus* in having stiff persistent leaves, often with a waxy bloom and horny margin, spike-like inflorescences, and corolla lobes joined by a thin membrane. The seemingly cryptic nature of features that might be unique to *T. racemosus* necessitates further examination of the distribution of morphological characters across members of the Kalanchoideae. Thus the aims of this chapter are two fold: to determine whether *T. racemosus* has unique features, and to describe it formally as a new genus.

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METHODS

Specimens of *T. racemosus* (hereinafter referred to as *Toelkenocodon*) held at the Compton Herbarium (NBG), Kirstenbosch, were examined in order to identify morphological characters by which the taxon might be distinguished. In addition, a re-evaluation of putative synapomorphies for existing genera of the Kalanchoideae was performed by extracting information, pertinent at the generic level, from Tölken (1978, 1985), van Jaarsveld & Koutnik (2004), and Thiede & Eggli (2007). Characters selected and the character states determined for each genus are provided in Tables 2.1 and 2.2, respectively. Binary and multistate characters were coded, and polymorphic character states, non-applicable characters and missing information were treated as unknown (?). Character reconstruction was performed using a summary tree topology (Fig. 2.1) of Kalanchoideae under Fitch parsimony (Fitch, 1971 as implemented in PAUP* 4.0b10: Swofford, 2002). The multistate character (character 6) was treated as unordered and the delayed transformation (DELTRAN) character optimisation criterion was employed (Swofford & Maddison,

1987, also implemented in PAUP* 4.0b10). *Aeonium* (Sempervivoideae) was used as the outgroup.

Table 2.1. Generic-level characters recorded across genera of Kalanchoideae and *Aeonium*

1.	Leaves spirally arranged (0), leaves decussate (1)
2.	Leaves deciduous (0), leaves persistent (1)
3.	Transition from leaves to bracts on peduncle abrupt (0), transition gradual (1)
4.	Flowering whilst functional leaves present (0), or after leaves have withered (1)
5.	Inflorescence pendent (0), erect-spreading (1)
6.	Number of floral parts 5 (0), or 4 (1), 6+ (2)
7.	Petals free (0), fused (1)
8.	Membrane at corolla lobe sinus (0), membrane absent (1)
9.	Corolla lobes as long or longer than tube (0), lobes shorter (1)
10.	Filaments fused to corolla (0), filaments free (1)
11.	Filaments with hairs where attached to corolla (0), filaments glabrous (1)
12.	Anthers exerted (0), or included (1)
13.	Follicle dehisces along entire ventral suture (0), or at apex of follicle only (1)
14.	Nectary scales free (0) or fused to carpel, cup-like (1)

Table 2.2. Matrix of character states for genera of Kalanchoideae and *Aeonium*. Missing, non-applicable and polymorphic states (indicated below by *) were scored as "?".

Genus	Character states
Aeonium	01?01 201?1 1100
Toelkenocodon	00101 01110 1100
Adromischus	01001 01010 1* 00
Kalanchoe	11001 11110 1* 00
Cotyledon	11100 01100 0001
Tylecodon	00011 01110 0010

Leaves whorled in *C. orbiculata* var. *flanaganii*
Flowers pendulous in *A. phillipsae*.

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RESULTS AND DISCUSSION

Unique synapomorphies are found for all genera of Kalanchoideae, except *Toelkenocodon* (Fig. 2.1). Seasonally deciduous leaves have evolved independently in *Tylecodon* and *Toelkenocodon*: thus the character can no longer be viewed as a unique morphological synapomorphy of *Tylecodon*.

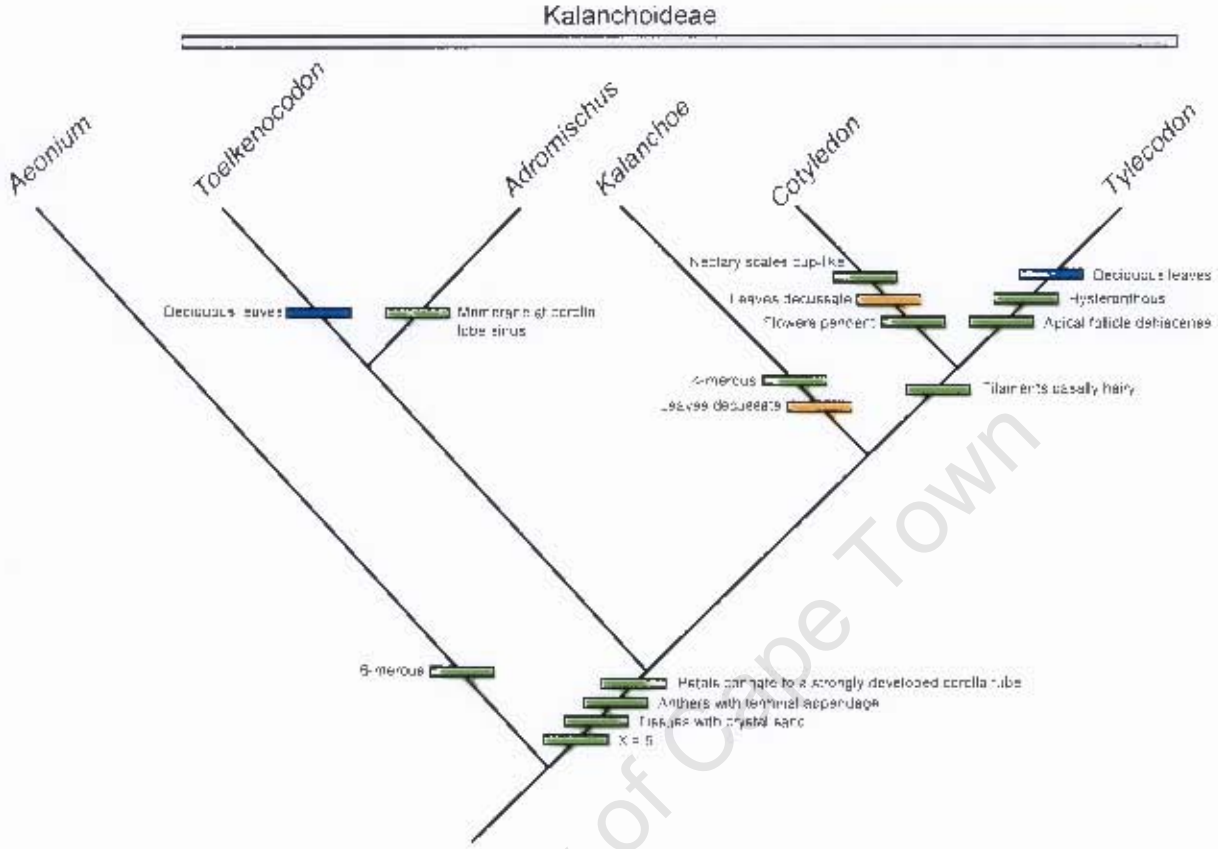

Figure 2.1. Summary cladogram showing the distribution of putative synapomorphies for genera of Kalanchoideae, including the new genus *Toelkenocodon*. *Aeonium* (Sempervivoideae) is included as the outgroup. Synapomorphous characters are shown as green bars. Characters that, in combination with others, represent a unique suite of characters for a given genus are shown as blue and orange bars.

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Despite the apparent lack of unique synapomorphies in *Toelkenocodon*, the fact that this taxon may represent a new genus has been recognised previously. In September 1939 J.P. Roux collected and illustrated a specimen of *Toelkenocodon* (687). The specimen was accompanied by a description written by P. Kles in 1940 (Fig. 2.2). The writer clearly thought that it should be treated as a new genus, although there was no explicit mention as to why they believed this to be the case. The most striking feature of the illustration is that flowering is synchronous with the presence of green, physiologically active leaves. Sixteen of the 17 specimens examined for the current study had flowers and leaves simultaneously, and one had fruits and leaves

simultaneously. Members of *Tylecodon s.str.* often exhibit some temporal overlap between leaves and inflorescences, but leaves are frequently in a withered state by the time flowers are fully developed, and the plants are more usually hysteranthous. In addition, none of the specimens of *Toelkenocodon* examined had a tuft of hairs where filaments are connate with the corolla tube: a putative synapomorphy for *Cotyledon* and *Tylecodon* (Thiede & Eggli, 2007). Thus *Toelkenocodon* can be defined by a unique suite of morphological characters. It has seasonally deciduous leaves and flowering is synanthous. The inner surface of the corolla is glabrous and no tuft of hairs is present where the filaments are connate with the corolla tube. In addition members of the genus generally flower in September and October. This is considerably earlier than most species of *Tylecodon* with which it is likely to be confused.

While the lack of phylogenetic informativeness of monotypic genera is undesirable (Schrire & Lewis, 1996), broadening the circumscription of *Adromischus* to accommodate *T. racemosus* (*Toelkenocodon*) would weaken considerably the predictive value of *Adromischus* as currently circumscribed. On the strength of molecular evidence presented in Chapter 1 a new genus is erected to accommodate this highly divergent taxon. The differentiation of *Toelkenocodon* from other genera in Kalanchoideae is supported by a unique combination of morphological characters.

The name *Toelkenocodon* is proposed in recognition of Helmut Tölken whose exhaustive work on Crassulaceae has contributed tremendously to our knowledge and understanding of the family. The latter part of the name relates to its former taxonomic placement within *Tylecodon* and to the genetic nature of the evidence that uncovered its evolutionary history.

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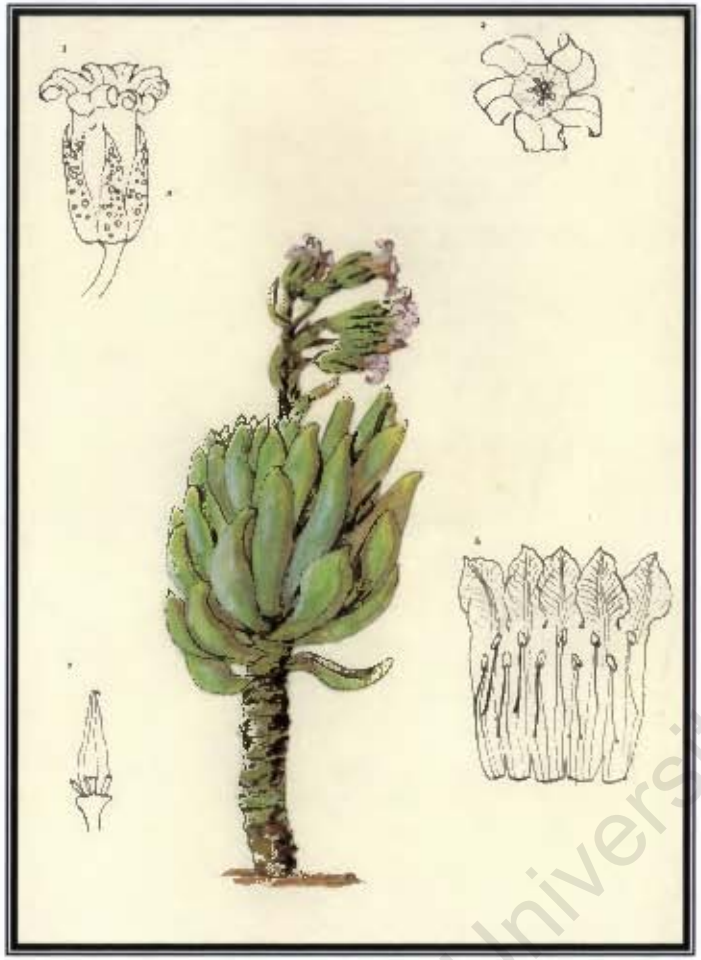
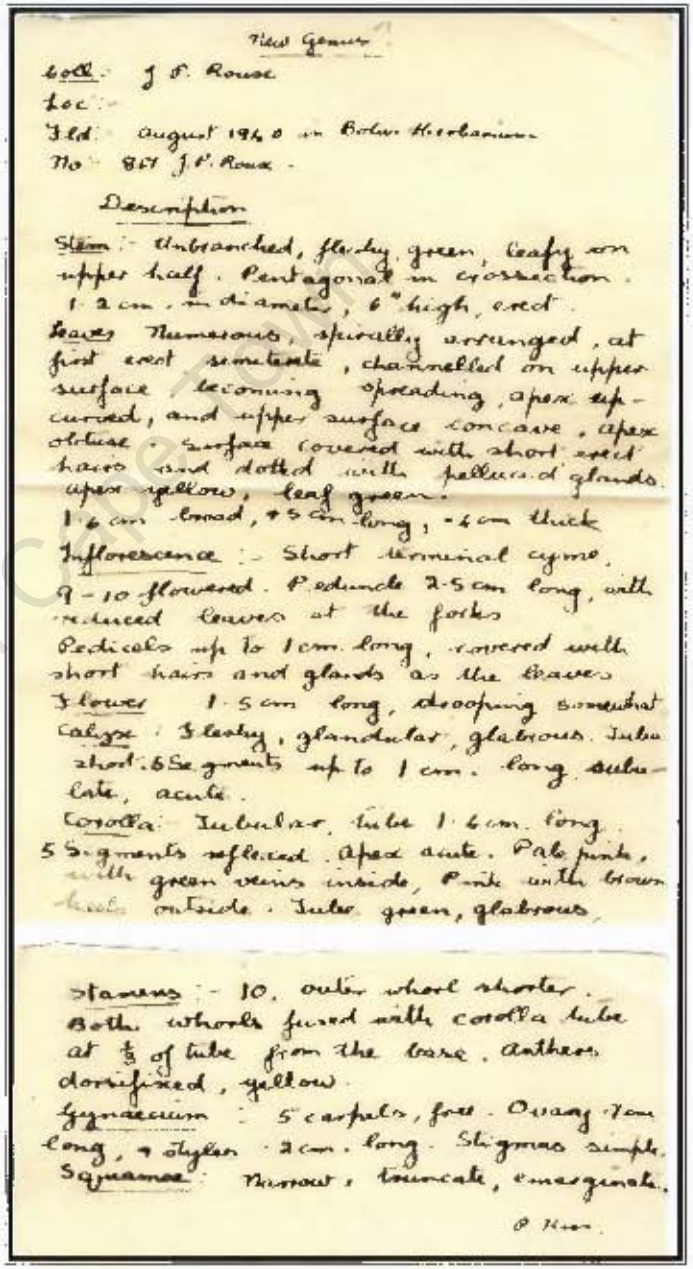


Figure 2.2. Illustration by J.P. Roux of a specimen of *Tylecodon racemosus* collected by the same in 1939. The description which accompanies the specimen in the Compton Herbarium (NBG) at Kirstenbosch clearly states that P. Kies believed this to be a new genus. The question-mark was added in pencil sometime later.



New Genus

Coll: J P. Roux

Loc:

Fld: August 1940 in Bolus Herbarium

No: 861 J.P. Roux.

Description

Stem:- Unbranched, fleshy green, leafy on upper half. Pentagonal in cross section. 1·2 cm. in diameter, 6" high, erect.

Leaves Numerous, spirally arranged, at first erect semitate, channelled on upper surface, becoming spreading, apex up-curved, and upper surface concave, apex obtuse. Surface covered with short erect hairs and dotted with pellucid glands. Apex yellow, leaf green.

1·6 cm broad, 7·5 cm long; ·4 cm thick

Inflorescence:- Short terminal cyme, 9-10 flowered. Peduncle 2·5 cm long, with reduced leaves at the forks

Pedicels up to 1 cm. long, covered with short hairs and glands as the leaves

Flower 1·5 cm long, drooping somewhat

Calyx: 5 fleshy, glandular, glabrous. Tube short: 5 Segments up to 1 cm. long, subulate, acute.

Corolla: Tubular, tube 1·4 cm. long. 5 Segments reflexed. Apex acute. Pale pink, with green veins inside, Pink with brown [illegible] outside. Tube green, glabrous.

Stamens:- 10, outer whorl shorter. Both whorls fused with corolla tube at ⅓ of tube from the base. Anthers dorsifixed, yellow.

Gynaecium: 5 carpels, free. Ovary ·7 cm long, 1 stylar ·2 cm. long. Stigmas simple.

Squamae: Narrow, truncate, emarginate.

P Kies.

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Toelkenocodon *T.L.Nowell* & *P.V.Bryuns,* gen.nov. (Kalanchoideae, Thiede &Eggli, 2007; Crassulaceae DC. in Lam. & DC.).

Tylecodon valde affine a quo differt ferens flores et foliis simul et glabris interiorbus tubo corollae et filamentis.

TYPE - *Toelkenocodon racemosus (Harv.)* T.L.Nowell & P.V.Bryuns (=*Tylecodon racemosus* (Harv.))

Deciduous dwarf succulent shrublets. Erect stems retaining withered leaves, later with pale brown peeling bark and white leaf scars. *Leaves* linear to rarely almost orbicular, more or less abruptly cuneate, acute to obtuse, terete or slightly grooved to dorsiventrally flattened, glandular-pubescent to glabrous, green to grey-green, often with large translucent papillae. *Inflorescence* a dense terminal thyrse with monochasia. *Flowers* glandular-pubescent to glabrous, peduncle green. *Calyx* glandular-pubescent to glabrous, pale green; lobes lanceolate-triangular, acute and usually with a colourless apex. *Corolla* glandular-puberulous to glabrous on outside; tube cylindrical but slightly broadened at mouth, glabrous inside, pale green; lobes recurved, white more or less tinged pink. *Anthers* more or less included. *Squamae* oblong-cuneate to almost square, entire to deeply emarginated, slightly fleshy, pale yellow.

Distribution: A single species found in southern Namibia and the Northern Cape of South Africa.

Etymology: Toelkenocodon is formed from Helmut Tölken, honouring his contribution to the taxonomy of Crassulaceae, from *Tylecodon*, the genus in which it was placed previously, and 'codon' in reference to the DNA data that informed the authors of its new rank.

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Toelkenocodon racemosus (*Harv.*) *T.L.Nowell, & P.V.Bryuns,* comb. nov.

Tylecodon racemosus (Harv.) Tölken in Bothalia 12: 380 (1978). Type: Cape, between Kaus, Natvoet and Doornpoort, Drège s.n. (S, lecto; BM;K).

Cotyledon racemosa Harv. in F.C. 2: 375 (1862), pro parte, excl. specimen b; N.E. Br. In Gdnr's Chron. Ser. 3, 51: 348 (1912); Schonl. in Rec. Albany Mus. 3: 149 (1915); V. Peolln. in Reprium nov. Spec. Regni veg. 42: 22 (1937).

C. chloroleuca Dinter ex Friedr. In Mitt. Bot. StSamml., München 3: 597, one fig. (1960); in F.S.W.A. 52: 8 (1968). Type: South West Africa/Namibia, 20km north of Sendelingsdrift, *Herre* in SUG20039 (M, holo.).

Deciduous dwarf succulent shrublets to 50 cm tall. Freely to sparsely branched, erect stems retaining withered leaves, later with pale brown peeling bark and white leaf scars. *Leaves* linear, oblanceolate to obovate rarely almost orbicular, (10-)20-45(-60) x (2-)5-25(-35) mm, more or less abruptly cuneate, acute to obtuse, terete or slightly grooved to dorsiventrally flattened, glandular-pubescent to glabrous, green to grey-green, often with large translucent papillae. *Inflorescence* a dense terminal thyrse with 1-6 monochasia, each with 1-4(-6) flowers, glandular-pubescent to glabrous; peduncle 30-50(-80) mm long, green; pedicels 5-12 mm long. *Calyx* 7-12mm long, glandular-pubescent to glabrous, pale green; lobes lanceolate-triangular, acute and usually with a colourless apex. *Corolla* glandular-puberulous to glabrous on outside; tube cylindrical but slightly broadened at mouth, 7-10 mm long, glabrous inside, pale green; lobes 4-6 mm long, recurved, white more or less tinged pink. *Anthers* 1.1-1.8 mm long. *Squamae* oblong-cuneate to almost square, 1-1.8 x 0.6-1 mm, entire to deeply emarginated, slightly fleshy, pale yellow.

Distribution and ecology: Found to the north from Witputz in southern Namibia, southwards to Wildepaardehoek Pass, near Springbok, Namaqualand, and east to near Steinkopf (Fig. 2.3).

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Plants are most frequently found in mountainous areas on gravely slopes and rocky outcrops, on cooler, well-drained, south-facing slopes. Distributed in areas receiving > 50% of rainfall during winter months, with average annual rainfall ranging from 25-200 mm. Plants almost exclusively confined to succulent karoo vegetation. Flowering: September, October.

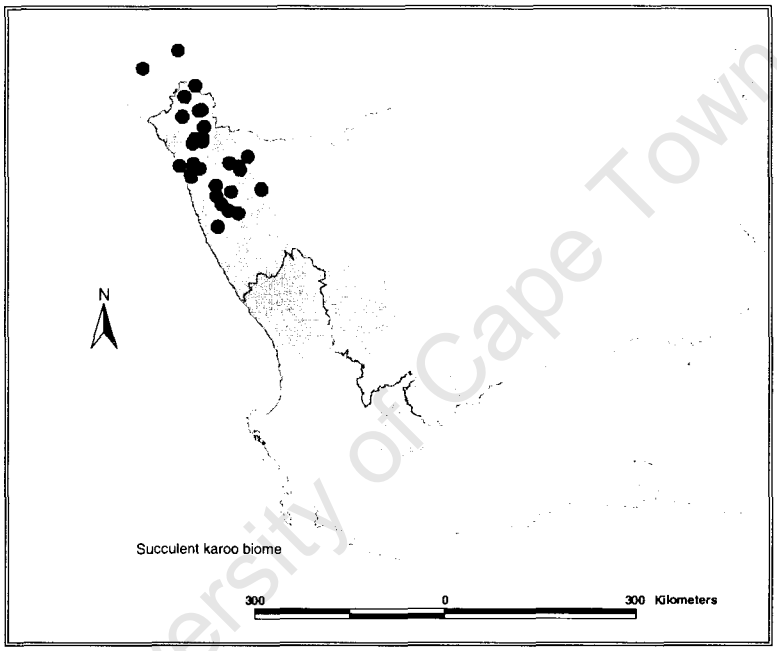

Figure 2.3. Distribution of *Toelkenocodon racemosus.*

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Additional material examined:

NAMIBIA – Lorelei, Rosh Pinah, 8 September 1987, P.V. Bruyns 2769a (NBG). Sonberg, Rosh Pinah, 3 September 2000 (fruiting), P.V. Bruyns 8840 (NBG). Ratelpoort, 8 August 1979, E. van Jaarsveld 4002 (NBG).
SOUTH AFRICA – Komaggas, 9 September 1950, H. Hall sn (NBG). Karrachab Poort, 1 December 1976, P.V. Bruyns 1390 (NBG). Brakputs, Springbok, 3 June 1980, E. van Jaarsveld 5314 (NBG). Gifberg, south of Port Nolloth, 27 September 1969, W. Wisura 682 (NBG). Gemsbokvlei, Augrabies, 30 March 1971, W. Wisura 1305 (NBG). Doornpoort, Namaqualand, 29 October 1949, R.H. Compton sn. (NBG). Oemsberg, Vioolsdrif, 1992, G. Williamson 4437 (NBG). Stinkfonteinberg, Vioolsdrif, 1992, G. Williamson 4479 (NBG). Tafelkop Pass, Richtersveld, 15 October 1988, G. Williamson 3936 (NBG). South of Port Nolloth, 23 September 1953, H. Hall sn (NBG). South west of Eksteenfontein, 3 December 1974, W. Wisura sn (NBG). Doringwater, Eenriet, 11 November 1991, J. Lavranos sn (NBG).

CHAPTER 3

Diversification in *Tylecodon*, *Cotyledon,* and *Adromischus* and the timing of the formation of the succulent karoo biome.

INTRODUCTION

The vegetation of the winter rainfall region of South Africa and Namibia is dominated by the fynbos and succulent karoo biomes (as defined in Mucina and Rutherford 2006). Both vegetation types boast remarkable species richness and endemism (Myers et al., 2000; Mittermeier et al., 2004) and there is evidence that several of their constituent plant groups have undergone rapid radiation (see Linder 2003 for review; Klak et al, 2004). Fynbos and succulent karoo vegetation border one another throughout much of their geographical range, and collectively they form the Greater Cape Floristic Region (Jürgens, 1991 & 1997). These vegetation types form phytogeographic units that have affinities with the Afrotemperate flora, and recent analyses of floristic elements corroborate the sharp distinction of fynbos and succulent karoo from the rest of the flora of southern Africa (Born et al, 2007). Given the present day correlation between high diversity, endemism, and geography it is logical to seek environmental factors that explain the historical patterns of diversification in these biomes (Barraclough et al., 1998; Linder, 2003).

Subtropical and tropical woodland vegetation is believed to have dominated much of southern Africa in the Tertiary (Coetzee, 1978). During this era the African subcontinent migrated north, temperatures fell, and the seasonality of rainfall changed (Siesser, 1980). Extinction of woodland vegetation ensued, and is thought to have triggered the expansion and diversification of the Cape floral elements that survived the changing conditions. Thus a 'tabula rasa' hypothesis (Linder and Hardy, 2004) has been invoked to explain simultaneous speciation across Cape floral groups, stimulated by the availability of new niches in aridifying

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habitats (Levyns, 1964; Linder, 1985; Linder et al., 1992; Goldblatt & Manning, 2000; and Linder & Hardy, 2004). Climatic changes in the region are strongly correlated with the glacial evolution of Antarctica (Coetzee, 1978 & 1983; Zachos et al., 2001). Planktonic assemblages, increased rates of sedimentation, and higher organic carbon content in deep sea sediments off the coast of Namibia indicate greater primary and secondary production during the early Late Miocene (12-10 million years ago [Mya]: Siesser, 1980). Together with the presence of diatoms characteristic of upwelled waters, evidence suggests that the Benguela Current (the eastern-most section of the South Atlantic Gyre) was initiated around this time (Siesser, 1980). Conditions incidental upon the Benguela Current have greatly influenced the climate, and therefore the vegetation of south-western southern Africa. Perhaps the most significant effect on the types of vegetation and their distributions is the drop in onshore precipitation resulting in aridification, and ultimately the establishment of the Mediterranean-type climate ca. 5 Mya, that prevails in the region today (Marlow et al., 2000).

Environmental change that accompanies variation in climate has been hypothesised to stimulate organismal diversification (Vrba, 1985; Marlow et al., 2000; Midgley et al., 2001). The species richness of fynbos is thought to be the product of fluctuating climate acting on a flora distributed across rugged topography, diverse soil-types and complex rainfall gradients (Kruger & Taylor, 1979; Goldblatt & Manning, 2000; Linder, 2003). Within this system recurrent fire induces local extinctions and is believed to promote allopatric speciation (Cowling, 1987). Contrary to long standing views on the evolution of the Cape flora (reviewed in Linder, 2003) recent findings point to a gradual accumulation of diversity beginning in the Oligocene, possibly earlier (Restionaceae, see below), and may be explained by lineages radiating at different times in response to different drivers (Linder, 2005). Evidence for this comes from analyses of molecular data for species-level phylogenies of Cape floral clades. Timings for diversification events include those of Restionaceae which began between 42 and 20 Mya (Linder et al., 2003), diversification within *Protea* occurred between 10 and 37 Mya (Barraclough and Reeves, 2005), while *Phylica* radiated 7-8 Mya (Richardson et al., 2001) and Heliophileae diverged as recently as 5.4 - 3.7 Mya (Mummenhof et al., 2005). Work on *Pelargonium* (Bakker et al., 2005) indicates that the genus was present in the Cape Floristic

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Region 30 Mya, with a radiation occurring in what is now the winter-rainfall area 22 - 16 Mya. This was followed by proliferation of a clade of xerophytes 18 - 11 Mya and the subsequent diversification of a clade of geophytes around 10 Mya. The geophyte clade has centres of diversity in Namaqualand in the succulent karoo. Nested radiations in *Pelargonium* indicate that adaptations that enabled species to survive and diversify in increasingly arid conditions, is derived in the group (Bakker et al., 2005). Similarly, molecular evidence from species of *Ehrharta* points to a Late Miocene radiation out of mesic mountain habitats onto the newly seasonally arid coastal plains of the succulent karoo (Verboom et al., 2003).

The extent to which the origins and evolutionary history of diversity of the succulent karoo corresponds to that of fynbos is largely unquantified. Treatment of species distributed mainly within the succulent karoo is often incidental with work that focuses on the evolution of fynbos elements (Bakker et al., 2005; Mummenhoff et al., 2005; and Goldblatt et al., 2002). This confounds inference of the ages of succulent karoo lineages as the pertinent nodes are not dated. Studies that have dated both fynbos and succulent karoo lineages (Bakker et al., 2005; Verboom et al., 2003) find support for Levyns' (1964) assertion that succulent karoo vegetation is younger than fynbos. In turn, their findings corroborate Werger's (1983) proposal that dwarf succulent shrubland has invaded areas made available by the retreat of sclerophyllous vegetation to higher altitudes, as it tracked more mesic conditions. Analysis of molecular evidence for a remarkably rich, predominantly succulent karoo group (Aizoaceae) revealed that more than 1500 species have evolved in the last 9 Myr or so (Klak et al., 2004). So there are indications that the temporal dynamics of the succulent karoo do not mirror those of the fynbos, and represent a system that is, to some extent, independent from its sclerophyllous neighbour. To establish whether this pattern is general, or restricted to a handful of plant lineages will require the estimation of divergence times across a wide phylogenetic sample of groups with at least some representatives in the succulent karoo and fynbos.

Approximately half of the species of *Tylecodon* are endemic to the succulent karoo. Many other members, together with species of *Adromischus,* have at least part of their range within

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the biome. Detailed information on the distributions of *Tylecodon* and *Adromischus*, together with the availability of a comprehensively sampled species-level phylogeny (Chapter 1), provide an appropriate system with which to explore the dynamics of diversification in the succulent karoo biome. 'A flora cannot evolve, it can only reflect the sum diversities accumulated by its evolving lineages' (Linder, 2005: 540). Thus dates generated in this study of the Kalanchoideae will be combined with data from a study which extends this work to more distantly related taxa (Verboom et al., in press; see Appendix 1 for manuscript) in which a common method of divergence time estimation is applied to a selection of published phylogenies that contain representatives in the succulent karoo. This will provide age estimates for lineages that are more comparable across groups as variation introduced by different dating algorithms is eliminated. Divergence times of nodes for which the reconstructed ancestral vegetation was succulent karoo will be used as a proxy for the formation of the biome. Such an approach assumes that the plants occupy the same habitat that they did when they diverged, and that their appearance in the phylogeny post-dates the establishment of succulent karoo vegetation. Combining information from distantly related lineages endemic to the succulent karoo will strengthen correlative inference regarding the formation of the biome.

This chapter will address the following specific questions: a) When did *Tylecodon*, *Cotyledon* and *Adromischus* diverge and diversify? b) Is there evidence of increased lineage diversification rates coincident with the intensification of the Benguela upwelling at around 12-10 Mya, or the onset of the true Mediterranean-type climate approximately 5 Mya? c) How old is the succulent karoo, and is the shift to succulent karoo simultaneous across lineages such that the 'tabula rasa' hypothesis is applicable to this biome?

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METHODS

Divergence time estimation

Divergence times were estimated for Kalanchoideae applying a global molecular clock (Felsenstein, 1981) and two methods that relax the clock using the assumption that rates between ancestor-descendent branches are autocorrelated. These were nonparametric rate smoothing (NPRS: Sanderson, 1997) and Bayesian MCMC-based Multidivtime estimation (Thorne et al., 1998; and Thorne & Kishino, 2002). In addition, ages were estimated using Bayesian Evolutionary Analysis Sampling Trees (BEAST: Drummond and Rambaut, 2006) - a method that simultaneously estimates phylogeny and divergence times. All procedures used the molecular data generated as detailed in the previous chapter.

Calibrating divergence times.

Kalanchoe and *Sedum* are estimated to have diverged 25-29 Mya; this range encompasses the dates published in Wikström et al. (2001) for three methods of branch length estimation. To incorporate further uncertainty into divergence time estimates the 95% confidence intervals of the prior distribution on the calibration node were set as ± 1.96*SE (assuming a normal distribution and df = ∞). A standard error of 3 Myr was used (i.e. bootstrap standard error estimate given in Wikström et al., 2001) and applied as ± 6 Mya (rounding up) to the range of age estimates. This gave a lower bound of 19 Myr and an upper bound of 35 Myr. The mean (27 Myr) of the 25-29 Myr range mentioned above was used for Bayesian-based methods. *Aeonium leucoblepharum* represents Sempervivoideae in this study.

Dating methods

Clock and NPRS procedures were performed using two datasets and a combination thereof: 1288 bps of plastid *trnL-F* and *psbA-trnH* data for 82 taxa; 670 bps of ITS for 67 taxa; and a subset of 65 taxa with 1958 bps of these data combined. Parameters of molecular evolution for each of the partitions were estimated using Modeltest version 3.06 (Posada and Crandall, 1998). Branch length optimisation was carried out with each dataset using the selected model parameters and the Bayesian maximum clade credibility tree (henceforth referred to as the

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Bayesian summary tree) as a topological constraint, under a maximum likelihood (ML) criterion, both with and without a molecular clock enforced (Felsenstein, 1981), as implemented in PAUP version 4.10b10 (Swofford, 2002). Log-likelihood ratio tests (LRTs: Felsenstein, 1988) were used to test for significant deviation from clocklike behaviour. P-values are obtained by comparing the difference in log-likelihoods (Λ) to critical values of the Chi-square (X^2) distribution with (n-2) degrees of freedom, both with and without a clock enforced; (Λ, calculated as 2 [$\ln L_{clock}$ - $\ln L_{noclock}$]). Log-likelihood ratio tests were performed with *Crassula* included and excluded. Error associated with branch length assignment, and ultimately node ages, was estimated via 100 bootstrap replicates as for branch length optimisation described above, implementing a heuristic search, with data added 'as is' and no branch swapping performed. The 100 bootstrap replicates of the ultrametric tree were used to scale divergence times. Similarly, divergence times were profiled across 100 bootstrap replicates of the phylogram produced without a clock, using rate smoothing (Sanderson, 1997) under Powell's optimisation algorithm implemented in r8s version 1.6 (Sanderson, 2003). Both procedures were performed using the 35 Myr and 19 Myr calibrations for the eight nodes selected to represent the first branching event in each genus and the beginning of putative radiations (as detailed in Table 3.3).

Bayesian relaxed clock age estimation was performed using Multidivtime (Thorne et al., 1998; and Thorne & Kishino, 2002). Model parameter estimation, branch length optimisation and calculation of a variance-covariance matrix were performed on plastid, ITS and combined data partitions separately using a common 65-taxon set and the Bayesian summary tree. Procedures were implemented as described in the Multidivtime 'readme' files (Thorne and Kishino, 2002) and the step-by-step manual compiled by Rutschmann (2005). Parameters for the F84 + gamma model of molecular evolution were estimated with the baseml module of PAML (Yang, 1997) and the resulting formatted substitution file was used to optimise branch lengths and estimate a variance-covariance matrix using estbranches (Thorne et al., 1998). Log-likelihood values reported by baseml and estbranches were used to check the performance of the likelihood optimisation. Five Bayes MCMC runs were performed: four were run for 10^6 generations with the Markov chain sampled every 100 generations, starting

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with the 10^4+1 generation. Estimates of divergence times were compared across the four runs as a proxy for assessing whether the chains were converging on the same posterior distribution of node ages. The prior for genes having the same tendency to change rate (commonbrown) was set to 1 for two runs, and 0 for two runs. The prior for the mean and standard deviation of the Brownian motion constant (brownmean and brownsd) were set to either 0.4 or 0.6 for these runs. A final extended MCMC analysis of 10^6 generations, sampled every 400 cycles after a burnin of 10^5 cycles, was performed with commonbrown=1, brownmean and brownsd=0.6. Brownmean was set such that the product of brownmean and the a priori expected number of time units between the root and tips (rttm) lay between 1 and 2 (Thorne and Kishino, 2002). The rttm and standard deviation (rttmsd) were set to 2.7 time units (where 1 time unit is 10 Myr), with upper and lower age constraints set to 1.9 and 3.5 time units, respectively. The mean and sd of the prior distribution for the rate of molecular evolution at the root node were set to 0.9, calculated as the overall mean of the three weighted median amounts of molecular evolution (from ITS, *psbA*, and *trnL-F*) between *Aeonium* (the ingroup root) and the ingroup tips, using the genetic distance data generated by estbranches (Thorne & Kishino, 2002).

Simultaneous estimation of phylogeny and divergence times was carried out on the 65-taxon combined dataset (as with Multidivtime), and on an 84–taxon dataset for the plastid partition (*trnL-F* and *psbA*), using BEAST version 1.4 (Drummond & Rambaut, 2007). BEAST XML input files were produced for the two plastid regions combined, and for trnL-F, psbA and ITS data separately, after specifying priors using the Bayesian Evolutionary Analysis Utility (BEAUti, distributed with BEAST). A combined BEAST XML input file was created by hand from the *trnL-F*, *psbA* and ITS XML files in order to perform mixed model analysis. Priors were set as follows: a general time-reversible model of nucleotide substitution was assumed with gamma-distributed rate variation with four rate categories, and a proportion of invariant sites (GTR+Γ+I). The uncorrelated lognormal distribution (UCLN, as recommended by Drummond et al., 2006) was selected to model rates on branches, and the time to the most recent common ancestor (t_{MRCA}) for the ingroup subsets (i.e. everything except *Crassula*), of the combined- (65 taxa) and plastid- (84 taxa) datasets, was specified with a mean of 27 and

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standard deviation of 4.865, using a normal distribution prior. The ingroup was assumed to be monophyletic, and a Yule tree prior was used. All other priors were left at default settings. Tuning of the parameter operators was set to 'auto-optimize'. Four independent MCMC analyses were each run for 10^6 cycles, and operators were adjusted manually after each successive analysis according to suggestions in the operator performance report in order to improve convergence and mixing of subsequent MCMC chains. Convergence was assessed using Tracer version 1.2 (Rambaut & Drummond, 2003). A final analysis of 10^7 generations was performed to increase the effective sample size (ESS) of all parameters to above the suggested value of 200. Results of the final MCMC run were summarised using TreeAnnotator (distributed with BEAST).

Shift(s) to succulent karoo in distantly related lineages

The procedures described here for *Tylecodon*, *Cotyledon* and *Adromischus* were applied to an array of angiosperm groups, as summarised in Table 3.1. Divergence time estimation was carried out across these distantly related lineages in order to establish whether a shift to succulent vegetation was synchronous across independent phylogenetic groups. This work has been written-up separately and accepted for publication as Verboom et al. (In press). A brief description of the study is given here and the manuscript is provided in Appendix 1. Criteria for selecting the plant groups detailed in Table 3.1 were that a) the group had at least one lineage endemic to the succulent karoo and, b) a reasonably densely sampled, molecular-based species level phylogeny was available. Divergence time estimation was carried out using BEAST version 1.4.6 (Drummond & Rambaut, 2007), following the methodology outlined previously in this chapter. Uncertainty surrounding calibration times differed slightly from the method described in this chapter and was applied as a 95% confidence interval arbitrarily defined as (mean age – [0.2 x mean age], mean age + [0.2 x mean age]). Succulent karoo endemism was reconstructed using the parsimony criterion and BEAST-generated topologies for each group. The age of the root of the first unequivocal transition to succulent karoo vegetation in each of the clades or lineages was taken as the date of interest.

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Table 3.1. Distantly related angiosperm groups used to estimate the age of the succulent karoo. Complete details can be found in the manuscript provided in Appendix 1

Taxonomic group	No. of species in group	No. of SK endemics	Age prior for group (Myr)	Dates of transitions to succulent karoo (Myr)	Data source
Ehrharta	20	3	40.0	10.0, 1.7, 1.1, 0.2	Verboom et al. 2003
Heliophileae	50	9	21.0	0.71	Mummenhof et al. 2005
Kalanchoideae	77	24	27.0	14.8, 8.7, 1.7, 1.1, 1.0, 0.6, 0.4	current study
Melianthus	8	2	63.0	13.4	Linder et al. 2006
Moraea	75	11	25.5	14.0, 13.0, 10.4, 9.0, 8.0, 5.5, 3.8, 1.93	Goldblatt et al. 2002
Muraltia	75	2	18.0	5.3, 3.3	Forest et al. 2007
Pelargonium	142	29	42.5	17.4, 17.1 14.7 13.1. 9.3, 5.8, 1.0	Bakker et al. 2005
Tribolium	23	4	14.0	3.8, 1.8, 1.4, 0.4	Verboom et al. 2006
Zaluzianskya	23	6	12.0	4.5	Archibald et al. 2005

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Accumulation of lineages through time

Diversification rates (r) were calculated using a maximum likelihood estimator (Magallón and Sanderson, 2001) r = [ln (n) – ln 2] / time. Diversification was measured from the deepest bifurcation in *Tylecodon*, *Cotyledon,* and *Adromischus* to the tips, thus treating each as a crown group (Magallón & Sanderson, 2001). Cumulative log-lineage-through-time (LTT) plots were produced by hand for each genus from the Multidivtime and BEAST chronograms by counting the number of branches present at each successive node, beginning at the node representing the divergence of each genus from its sister, and working towards the terminals of that genus. The natural logarithm (ln) of the number of lineages was plotted against node ages.

Variation in diversification rates

Whole-tree topological tests, implemented in SymmeTree (Chan & Moore, 2005) were used to test the null hypothesis that there are no significant differences in diversification rates across the phylogeny of *Cotyledon*, *Tylecodon* and *Adromischus.* Tests were performed using the Bayesian maximum clade credibility tree (fully resolved), and the 50% majority rule consensus tree (with polytomies, as shown in Chapter 1, Fig. 1.3) for the 86-taxon set

generated with combined ITS and plastid data (Chapter 1) . To accommodate topological uncertainty present in the latter tree 10^5 random resolutions of polytomies were performed under a taxon-size sensitive, equal rates Markov (TSS-ERM) model of random branching. For both topologies, P-values for whole-tree test statistics were calculated with reference to null distributions produced from 10^5 simulated trees generated under an ERM model of branching.

Succulent karoo vegetation-type reconstruction

The biome in which each of 1710 geo-referenced accessions of *Cotyledon*, *Tylecodon* and *Adromischus* occur within South Africa was compiled from VEGMAP version 4.0b (Mucina and Rutherford, 2004) using Arcview GIS 3.2. These data were then summarised to a character state representing each species' range as either endemic to the succulent karoo (1), or not endemic to the succulent karoo (0). Ancestral character state reconstruction was performed using the Bayesian maximum clade credibility tree and both a parsimony criterion (using equal branch lengths) and the likelihood function (using Bayesian branch lengths); likelihood calculations were carried out using the Markov k-state one parameter (Mk1) model. All procedures were implemented in Mesquite version 1.12 (Maddison and Maddison, 2006).

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RESULTS

Divergence time estimates

Log(ln)-likelihood ratio tests (LRT; Table 3.2) indicated significant rate heterogeneity present in all data partitions, both with *Crassula* included and excluded. A strict molecular clock was thus rejected in all cases. A clock approach was still included in the study to aid evaluation of relative branch-length allocation across the tree by the various dating methods employed here.

Table 3.2. Results of Log-likelihood ratio tests used to detect significant deviations from clock-like behaviour within data partitions. Tests were performed with *Crassula* included (upper value) and excluded (lower value).

Data partition	Taxa (n)	Λ	P value
Plastid	**82**	**232.3**	**< 0.001**
	80	**213.9**	**< 0.001**
ITS	**67**	**536.9**	**< 0.001**
	65	**358.8**	**< 0.001**
Combined	**65**	**217.8**	**< 0.001**
	63	**178.9**	**< 0.001**

A summary of time estimates for selected nodes of *Adromischus*, *Cotyledon*, and *Tylecodon*, produced using all four dating techniques is provided in Table 3.3. Mean node ages and 95% credibility intervals (CI) or highest posterior densities (HPDs) generated using Multidivtime and BEAST are shown in Figure 3.1. Mean age ranges and standard deviations of six nodes dated using the clock and NPRS are also represented on these plots for visual comparison, and are shown on the trees in Figures 3.3 and 3.4. Comparing ages returned from the four divergence time estimation methods employed here, enforcing a clock consistently returns the youngest mean dates (Fig. 3.1). Multidivtime estimation represents the other extreme, with all mean ages being older than the clock, NPRS- and BEAST-derived dates. BEAST age estimates lie between those of NPRS and the clock, and error associated with mean node ages is considerably smaller than the error estimated using Multidivtime.

Effects of the various branch length transformation methods adopted here are illustrated in Figure 3.2. The Bayesian summary phylogram, with unconstrained branch lengths, is provided as a benchmark against which to compare chronograms. Comparison suggests that the effects on branch lengths fall into two groups. The clock- and BEAST-derived chronograms more closely resemble the phylogram with greater branch length allocated to the deeper nodes of the tree. Non-parametric rate smoothing and Multidivtime methods allocate a considerably greater proportion of time to terminal branches. The relative allocation of time to deeper nodes of the tree is presented numerically in Table 3.4.

In order to simplify presentation of results from dating exercises only age estimates produced using Multidivtime and BEAST will be considered further. These methods represent one that

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Table 3.3. Timing of diversification events in *Cotyledon*, *Tylecodon* and *Adromischus* estimated using a forced global clock (Clock); nonparametric rate smoothing (NPRS); Multidivtime Bayesian relaxed clock estimation; and BEAST Bayesian relaxed phylogenetics. Age estimates are given for the eight nodes common to both the Bayesian maximum clade credibility tree and the BEAST 50% majority rule consensus tree. Clock and NPRS mean ages and standard deviations (±sd) are given for each of the three data partitions analysed using both the 19- and 35 Myr calibration points. Node age estimates were obtained from plastid and combined data using BEAST, and from combined data only using Multidivtime. Nodes 1-8 are indicated on the trees provided in Figs. 3.3 and 3.4.

NODE	Clock		NPRS		Multidivtime	BEAST
	mean node age (±sd)		mean node age (±sd)		mean node age (95% CI)	mean node age (95% HPD)
	19Myr	35 Myr	19 Myr	35 Myr		
***Toelkenocodon – Adromischus* (1)**						
plastid	7.9 (1.2)	14.5 (2.3)	10.5 (0.7)	19.3 (1.3)	-	**14.8** (6.4 – 24.2)
its	5.2 (1.0)	9.6 (1.9)	9.3 (2.6)	17.2 (4.9)	-	-
combined	**6.4** (0.7)	**11.9** (1.3)	**8.3** (1.2)	**15.2** (2.2)	**19.5** (12.9 – 28.2)	**12.6** (5.3 – 20.4)
***Adromischus* (2)**						
plastid	4.1 (0.8)	7.5 (1.4)	8.6 (0.7)	15.8 (1.2)	-	**10.3** (4.3 – 16.8)
its	1.6 (0.4)	2.9 (0.7)	6.8 (2.7)	12.6 (5.0)	-	-
combined	**2.8** (0.4)	**5.2** (0.7)	**6.3** (1.1)	**11.4** (1.9)	**11.0** (5.8 – 18.3)	**7.6** (3.2 – 13.1)
***Adromischus* (3)**						
plastid	2.4 (0.4)	4.5 (0.8)	5.5 (0.8)	10.1 (1.2)	-	**6.5** (2.7 – 10.9)
its	1.0 (0.2)	1.8 (0.4)	4.3 (2.3)	7.9 (4.4)	-	-
combined	**1.6** (0.2)	**2.9** (0.4)	**3.8** (0.8)	**6.9** (1.2)	**7.7** (3.6 – 13.7)	**4.3** (1.7 – 7.3)
***Cotyledon – Tylecodon* (4)**						
plastid	4.6 (0.8)	8.5 (1.5)	8.8 (0.7)	16.3 (1.3)	-	**11.4** (4.8 – 18.4)
its	2.6 (0.5)	4.8 (1.0)	7.2 (2.3)	13.3 (4.4)	-	-
combined	**3.6** (0.4)	**6.7** (0.8)	**6.9** (1.1)	**12.6** (2.2)	**13.5** (7.8 – 21.4)	**9.1** (3.5 – 15.0)
***Cotyledon* (5)**						
plastid	2.6 (0.6)	4.7 (1.2)	7.0 (1.1)	13.0 (2.0)	-	**7.5** (2.8 – 12.8)
its	0.5 (0.1)	0.9 (0.3)	3.6 (2.1)	6.7 (3.9)	-	-
combined	**1.3** (0.3)	**2.4** (0.5)	**4.6** (1.2)	**8.4** (2.0)	**7.4** (3.1 – 13.8)	**4.0** (1.3 – 7.2)
***Tylecodon* (6)**						
plastid	3.2 (0.6)	5.9 (1.1)	7.5 (0.8)	13.9 (1.4)	-	**8.7** (3.7 – 14.5)
its	2.1 (0.4)	3.9 (0.8)	6.4 (2.2)	11.9 (4.3)	-	-
combined	**2.8** (0.3)	**5.2** (0.6)	**5.9** (1.0)	**10.9** (1.9)	**11.7** (6.5 – 19.2)	**7.0** (2.8 – 12.1)
***Tylecodon* (7)**						
plastid	2.1 (0.5)	3.9 (0.9)	6.6 (0.8)	12.2 (1.4)	-	**7.2** (3.0 – 11.9)
its	1.4 (0.3)	2.6 (0.6)	4.8 (2.0)	8.9 (3.8)	-	-
combined	**2.0** (0.3)	**3.7** (0.6)	**4.6** (0.9)	**8.1** (1.7)	**9.9** (5.1 – 16.9)	**4.9** (1.9 – 8.4)
***Tylecodon* (8)**						
plastid	0.9 (0.2)	1.6 (0.4)	3.9 (0.8)	7.1 (1.4)	-	**4.2*** (1.6 – 7.1)
its	0.7 (0.2)	1.3 (0.3)	3.7 (1.6)	6.9 (3.0)	-	-
combined	**0.8** (0.2)	**1.5** (0.3)	**2.9** (0.7)	**5.4** (1.4)	**5.5** (2.4 – 10.3)	**2.4** (0.8 – 4.0)

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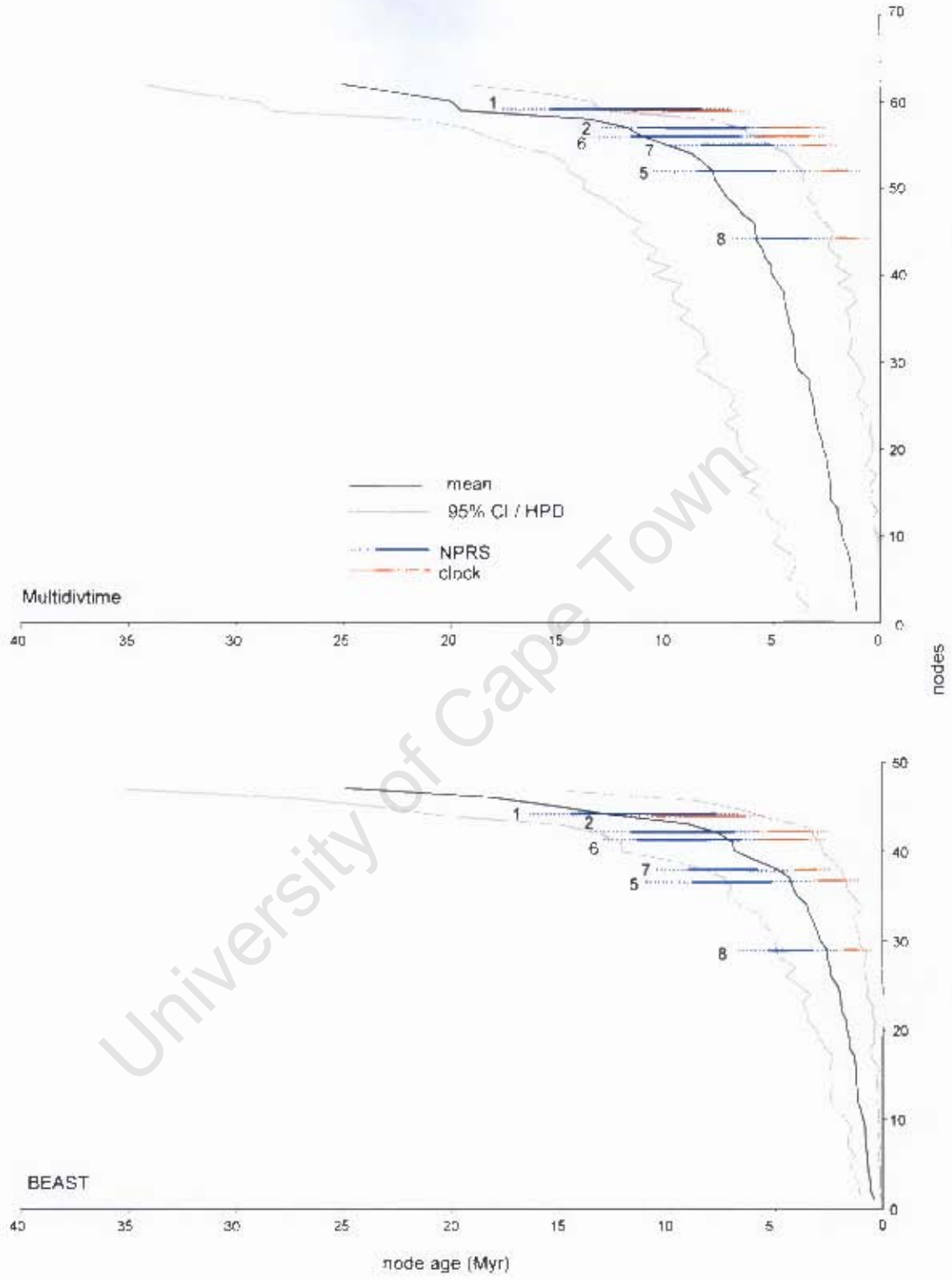

Figure 3.1. Node age estimates produced from analyses of combined data for a 63-taxon set using Multidivtime (using Bayesian summary tree for 65 taxa, but with *Crassula* removed) and BEAST. Mean node ages (black lines) are flanked by the upper and lower bounds of their 95% CI / HPD (grey lines). Node numbers on the y-axis were allocated to chronologically ordered ages to facilitate plotting these data. Mean and error estimates were available for all 62 internal nodes following estimation by Multidivtime. This information was available for 54 internal nodes using BEAST as HPDs were only computed for nodes with > 0.50 posterior probability. Clock and NPRS-derived date-ranges are shown for selected nodes; orange bars represent ages scaled from trees produced under a clock assumption, and blue bars represent ages profiled from rate smoothed (NPRS) bootstrapped trees. The mean-range (solid lines) and 95% CI (dotted lines) are shown. Numbers next to the bars correspond to nodes indicated on trees in Figures 3.3 & 3.4 and in Table 3.3.

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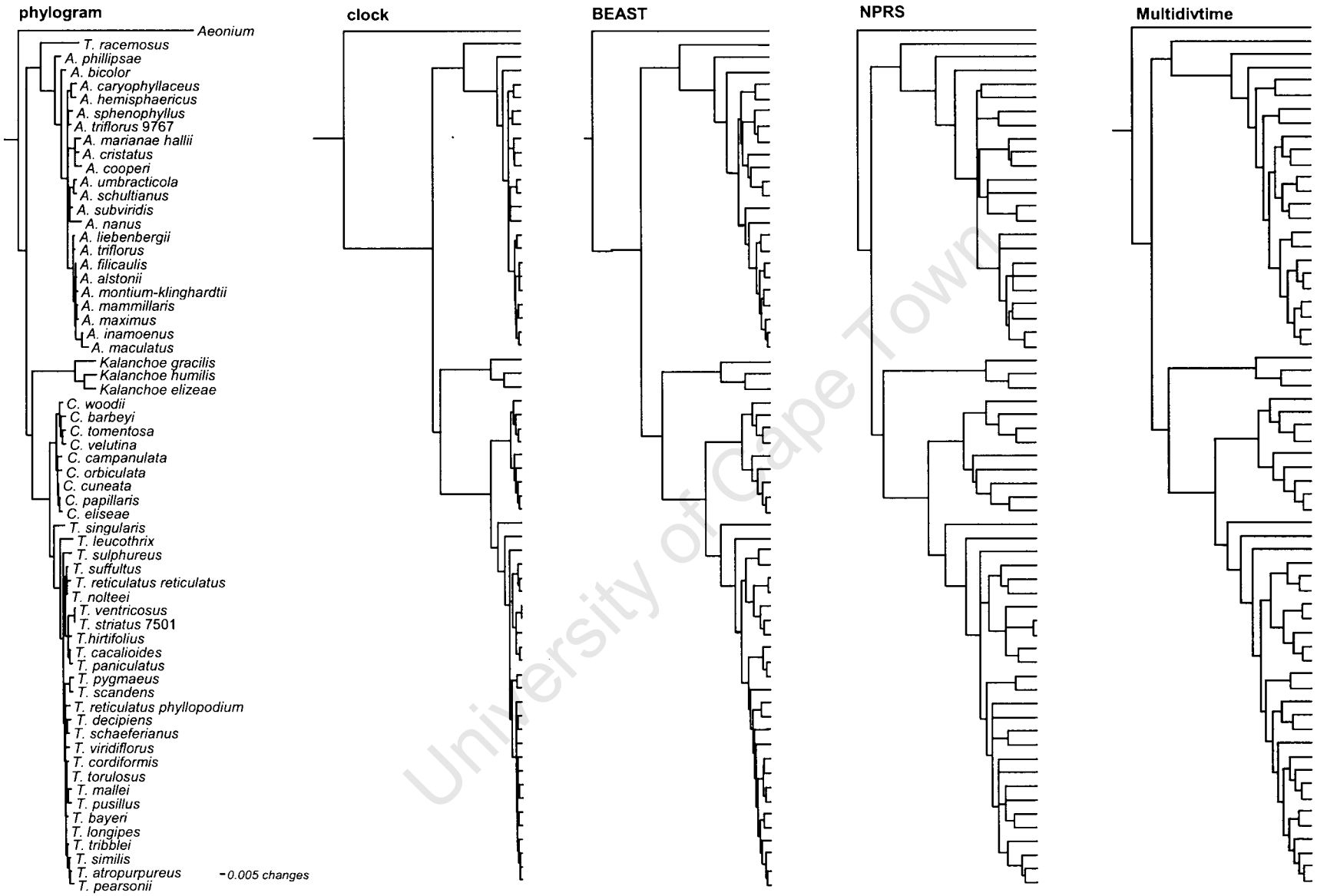

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Figure 3.2. Relative time allocation in chronograms produced using the dating techniques indicated above each tree. The untransformed Bayesian maximum clade credibility phylogram (far left) is shown for comparison. Chronograms are drawn such that the branch leading to *Aeonium* is the same length in each case, with all other branches scaled accordingly.

assumes autocorrelation and one that does not, while both take models of molecular evolution into account. In addition, of the methods that accommodate rate heterogeneity, they returned the extremes of dates, and provide estimates for all nodes in the phylogeny (except for the 10 nodes with pp < 0.50 following analysis using BEAST). Chronograms annotated with node ages and the putative timing of initiation of the Benguala upwelling and establishment of the Mediterranean-type climate are illustrated in Figures 3.3 and 3.4.

Table 3.4. Proportion of time allocated between ingroup root and first branching event in each genus across the methods of dating used in this study. Methods are ordered from most to least time allocated to deeper nodes (cf Fig. 3.2).

Dating technique	Percentage of time between root and first branching point			
	Adromischus	***Tylecodon***	***Cotyledon***	**Average**
Clock	88	88	96	**91**
BEAST	68	71	84	**74**
Multidivtime	55	52	69	**59**
NPRS	44	48	41	**44**

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Accumulation of lineages through time

Per-lineage diversification rates estimated for *Tylecodon*, *Cotyledon,* and *Adromischus* are summarised in Table 3.5. *Tylecodon* exhibits the highest diversification rates, irrespective of the dataset or estimation method used. Combined data recover rates that are very similar within each method – Multidivtime estimates for all genera fall between 0.09 and 0.12 species Myr^{-1}, while BEAST estimates range from 0.15 to 0.19 species Myr^{-1}. More thorough taxon sampling, mostly of species of *Tylecodon,* increases the rate difference between *Tylecodon* (0.16 species Myr^{-1}), *Cotyledon* (0.09 species Myr^{-1}), *Adromischus* (0.11 species Myr^{-1}).

Log-lineages-through-time (LTT) plots are provided in Figure 3.5. Visual comparison of the plots suggests that the accumulation of diversity in all three genera has been more or less linear throughout their history, at least since the first branching event in each. Whole-tree tests of topology detected no significant shifts in diversification rate within the ingroup. Although significant shifts were detected in initial tests they did not persist once *Crassula* and *Aeonium* were excluded, suggesting that differential rates found to be significant initially

Table 3.5. Summary of estimates of per-lineage diversification rates (per million years, Myr^{-1}) for *Tylecodon*, *Cotyledon* and *Adromischus*. Dates were generated using combined data for 65 species (58 ingroup species) analysed with BEAST and Multidivtime, and using plastid data for 84 species (77 ingroup species) analysed with BEAST only.

Genus	Dating method; data partition	No. of species per genus	Divergence date in Myr,	Diversification rate: Species Myr^{-1}
Tylecodon	BEAST; plastid	46	8.8	0.155
	BEAST; combined		7.0	0.194
	Multidivtime; combined		11.7	0.116
Cotyledon	BEAST; plastid	10	7.7	0.091
	BEAST; combined		4.0	0.175
	Multidivtime; combined		7.4	0.094
Adromischus	BEAST; plastid	28	11.0	0.106
	BEAST; combined		7.6	0.153
	Multidivtime; combined		11.0	0.106

resulted from undersampling in the outgroup. Plots of the accumulation of lineages within *Tylecodon* and *Adromischus* track each other for much of the time, and this pattern is robust to taxon-sampling and dating method. There is a phase dated at 4, 3 and 2 Myr (indicated by red arrows on Fig. 3.5) during which the curves of the two genera cross. After this phase *Tylecodon* continues to accumulate species, and outstrips *Adromischus*. This pattern is most pronounced following analysis of plastid data using BEAST (green plots) which reflects the improved sampling of *Tylecodon* in this dataset. There is a 4 – 6 Myr delay (depending on dataset and method) prior to the first bifurcation in *Cotyledon*. This post-dates that of its sister genus, *Tylecodon,* by 1.2 – 3 Myr. During this phase, up to 2.5 – 4.2 Myr, the plot of *Cotyledon* more closely resembles that of *Adromischus*. Thereafter rates are very similar to *Tylecodon* and *Adromischus*, particularly with BEAST analysis of the combined data.

Succulent karoo vegetation-type reconstruction

According to parsimony reconstruction the unequivocal shift to succulent karoo in *Tylecodon* occurred along the branch leading to the *T. tenuis* – *T. pearsonii* clade (Fig. 3.6) at around 6.4 Mya (Multidivtime), 4.2 Mya (BEAST, plastid data), and 3.2 Mya (BEAST combined data). Such a transition may have occurred as early as 7.1 Mya (Multidivtime). The likelihood of succulent karoo being the ancestral vegetation type is represented diagrammatically at

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selected nodes in Figure 3.6. Likelihood reconstruction of the ancestral vegetation-type of the ingroup as a whole is inconclusive - the MRCA is as likely to be endemic to the succulent as not. This also applies to the MRCA of *Tylecodon*. Within *Tylecodon* the likelihood of a succulent karoo endemic ancestor fluctuates until it reaches a value of 0.69 at the node bounding the *T. suffultus* – *T. pearsonii* clade. Thereafter the likelihood of the ancestor being succulent karoo endemic approaches, and remains close to 1.00. Shifts to succulent karoo endemism in various angiosperm groups range from 17 – 0.2 Mya (Table 3.1). The greatest proportion of these vegetation transitions occurred during the last 5 Myr. A near equal number of shifts are older and occurred between 5 and 17 Mya (Fig. 3.7).

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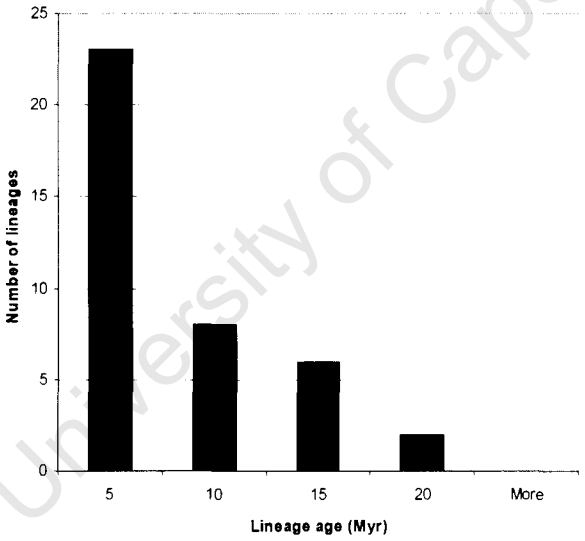

Figure 3.7. Number of lineages entering the succulent karoo at various intervals during the last 20 Myr.

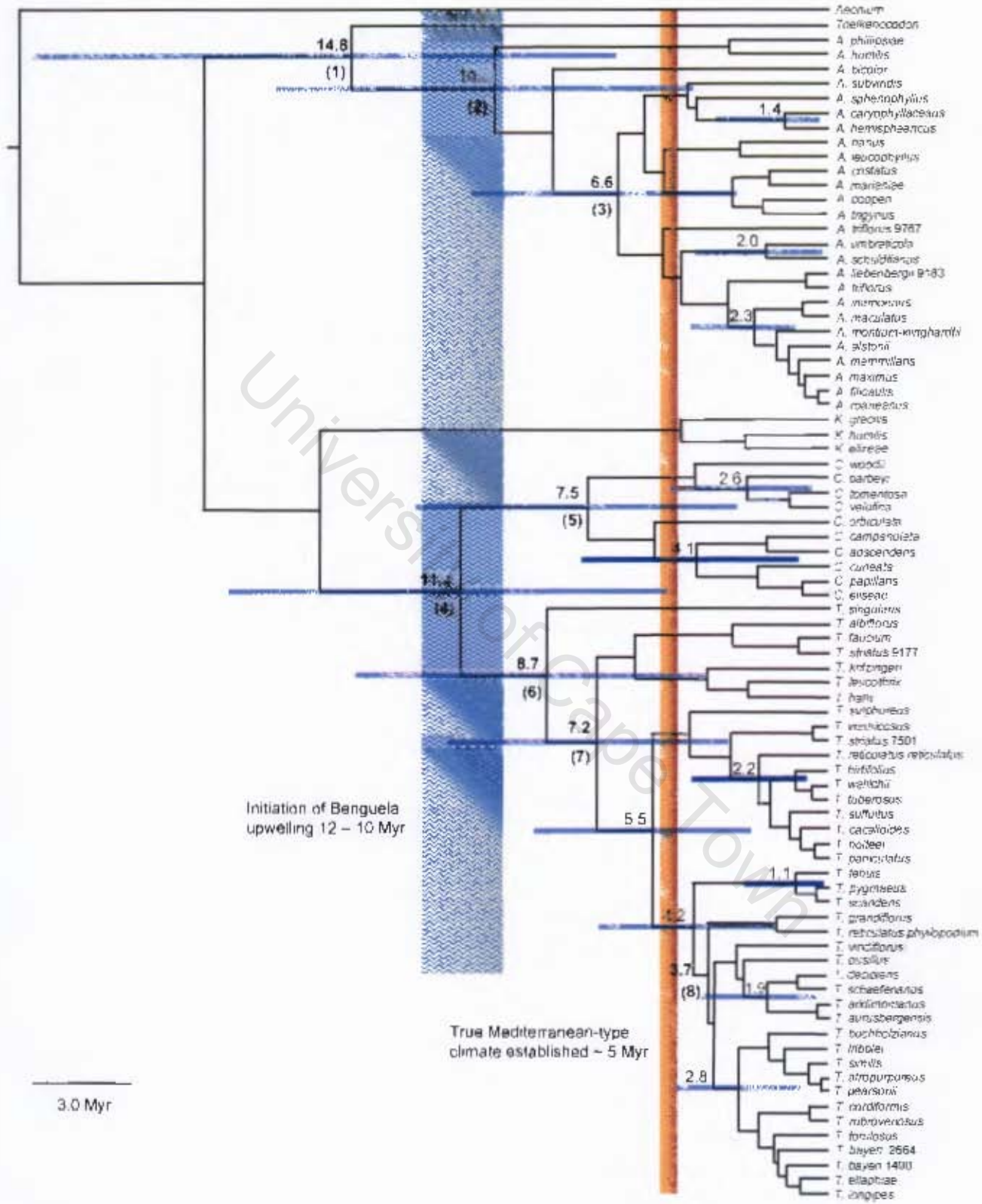

Figure 3.3. Chronogram produced from analysis of plastid data for 84 taxa performed using BEAST. Mean ages (value above branch) and their 95% HPD (blue bars) are shown for a selection of nodes. Values in parentheses correspond to node numbers shown in **Table 3.3** - the nodes dated using all four techniques. No 95% HPD is shown for node 8 as this relationship attained a posterior probability of < 0.50. The outgroup *Crassula* has been omitted from tree drawing.

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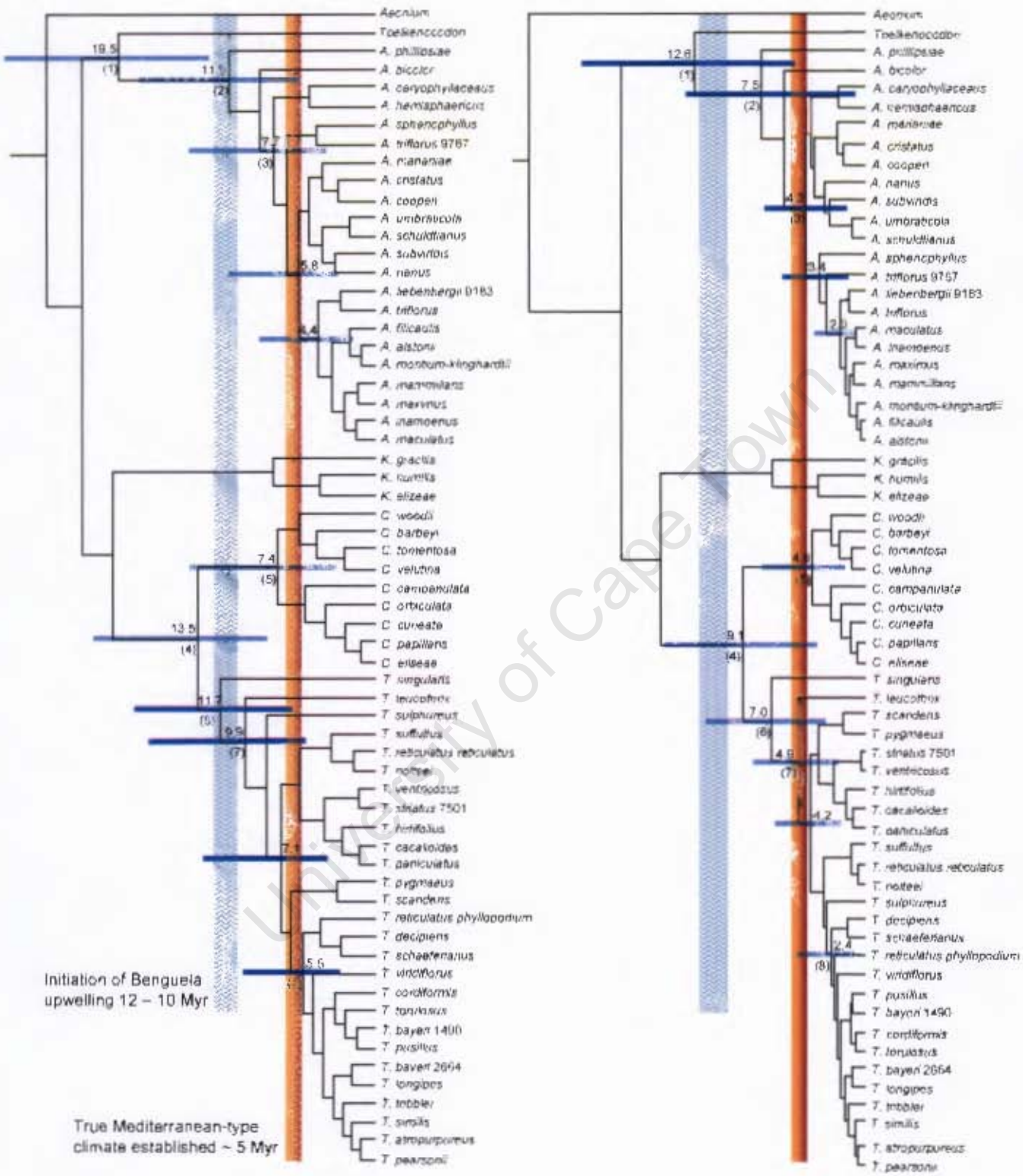

Figure 3.4. Chronograms produced from Bayesian analyses of combined data for 65 taxa. Mean ages and their 95% CI / HPD (blue bars) are shown for a selection of nodes. Values in parentheses correspond to node numbers shown in Table 3.3 – the nodes dated using all four techniques. **Left tree**: Divergence time estimates produced using Multidivtime and the Bayesian maximum clade credibility tree. **Right tree**: Divergence times and topology estimated simultaneously using BEAST. The outgroup *Crassula* has been omitted from tree drawing.

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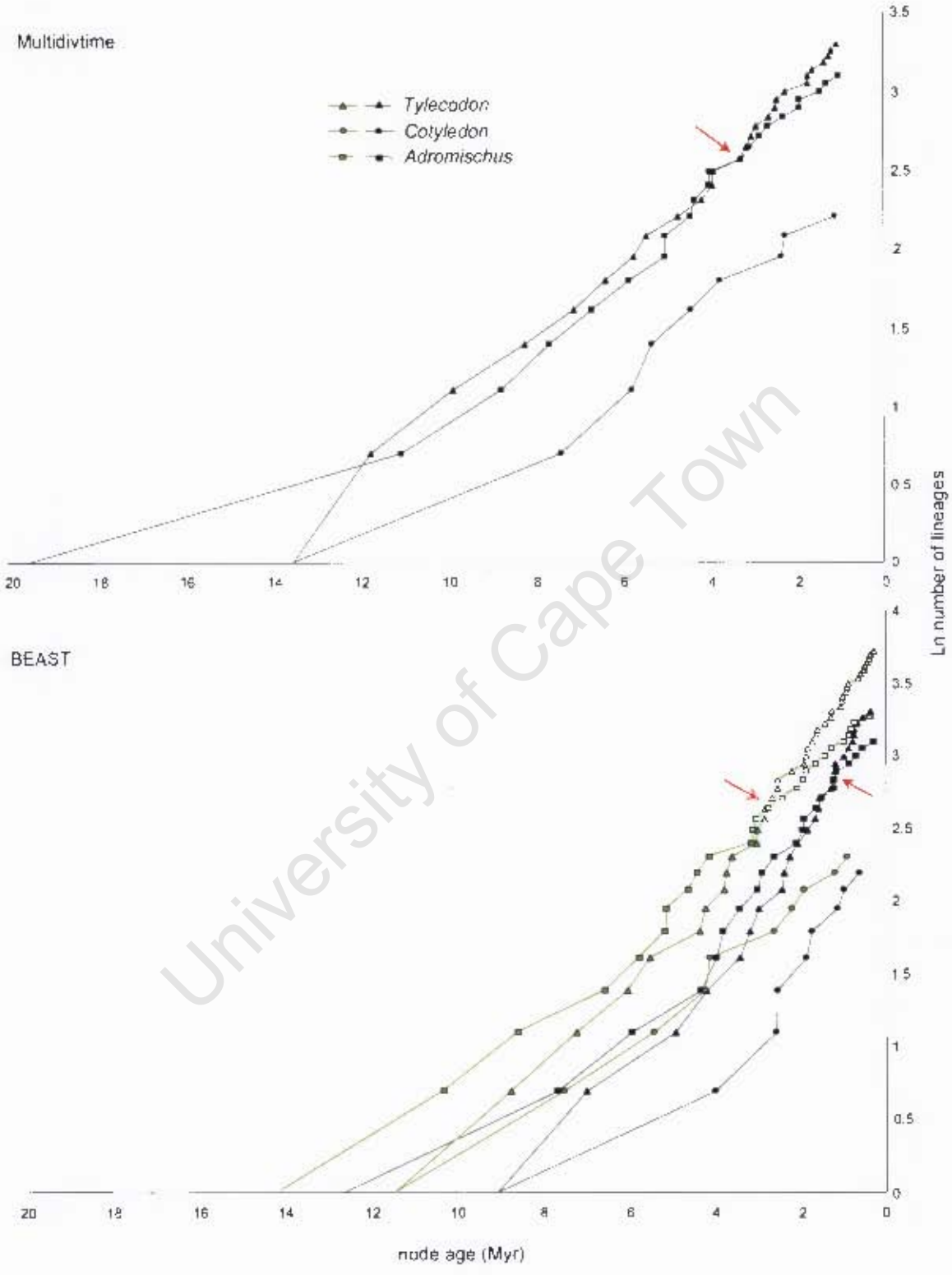

Figure 3.5. Species accumulation curves (log-lineages-through-time, LTT) for *Adromischus*, *Tylecodon* and *Cotyledon*. Divergence time estimates were produced from analyses of combined data for 65 taxa using Multidivtime and BEAST (black lines), and from analysis of plastid data for an 84-taxon dataset using BEAST (green lines). Plots begin at the time when each genus diverged from its sister.

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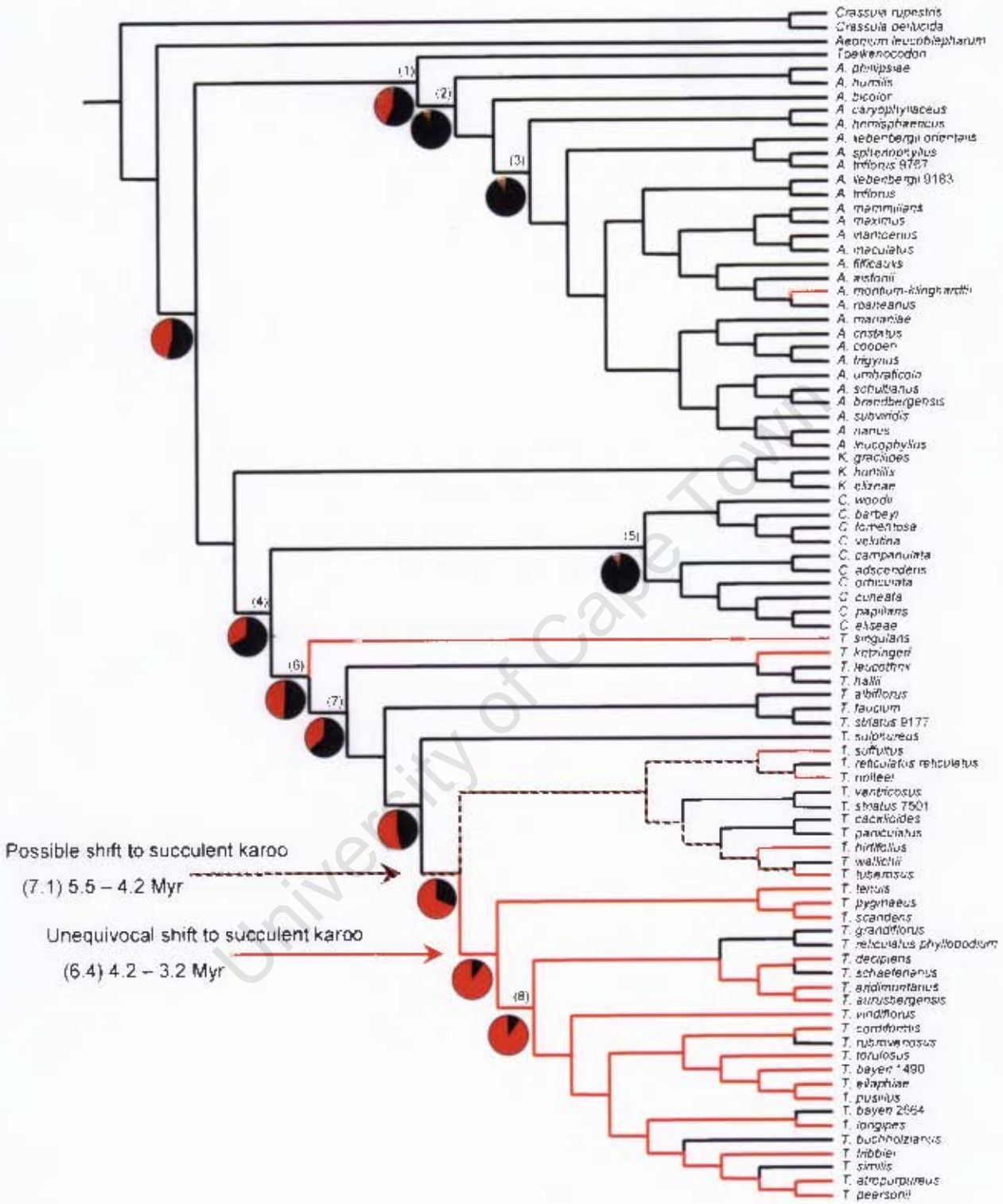

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Figure 3.6. Ancestral vegetation type reconstruction for taxa included in this study. Reconstructions were performed under parsimony and likelihood using the Bayesian summary tree comprising 86 taxa (Chapter 1). Orange indicates succulent karoo endemism, black indicates vegetation-type other than succulent karoo. Orange/black stripes highlight branches for which the parsimony reconstruction is equivocal. The proportion of likelihood attributed to each of these character states is illustrated by pie charts at a selection of nodes (same colour scheme). Numbers in parentheses above branches refer to the nodes for which dates were estimated using the four dating techniques detailed in Table 3.3. Timings of the shift to succulent karoo given next to the arrows are listed as: (Multidivtime) BEAST plastid – BEAST combined.

DISCUSSION

The species diversity within *Tylecodon*, *Cotyledon* and *Adromischus* is comparatively young. This is especially true when estimation methods do not assume autocorrelation, although estimates are fairly robust to methodology. Increases in diversity coincide remarkably with major climatic shifts in south-western southern Africa. The start of the diversification process in Kalanchoideae is synchronous with the establishment of the Benguela upwelling 12 – 10 Myr (Siesser, 1980): all major extant lineages diverged at, or near, this time (Figs. 3.3 & 3.4). In turn, species diversity within these lineages increased considerably following the onset of the Mediterranean-type climate around 5 Mya. This increased diversification phase is also visualised in Figure 3.5 (BEAST LTT plots) which highlights a slight upturn in diversification at around 4.5 – 5.5 Mya in *Tylecodon* and *Adromischus.* No significant shifts in diversification rates were, however, detected at these or any other points in the phylogeny. Tests used for detecting significant tree imbalance rely on the suitability of null models, and the equal-rates-Markov (ERM) model has been suggested to be a poor fit to many applications for which it is used (Mooers & Heard, 1997). These authors advocate use of the proportional-to-distinguishable-arrangements (PDA) model for cases in which tree shape may be affected by climate change. The PDA model (implemented in SymmeTREE; Chan & Moore, 2005) was applied to the topology of *Tylecodon* and *Cotyledon*, using *Kalanchoe* as the root, and results remained non-significant. Given the apparent rate constancy of diversification through time in the group it is necessary to explore possible processes by which this pattern may have arisen.

The shift to succulent karoo endemism occurred fairly early in the evolutionary history of *Tylecodon*, possibly as early as 5.5 Mya. This is taken as a strong indication that the vegetation type we see in the area today had established by this time. Whilst most succulent karoo endemism is relatively young in *Tylecodon*, two considerably older lineages are endemic to the region. *Tylecodon singularis* diverged from other members of the genus around 9 Mya and *Toelkenocodon* (almost endemic to succulent karoo) diverged from the MRCA it shared with *Adromischus* approximately 15 Mya. Given the relative antiquity and

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isolated phylogenetic positions of these taxa it is possible that occupation of succulent karoo habitats has occurred since each diverged from a MRCA. The timing of shifts to succulent karoo in *Tylecodon* and its relatives is likely to represent clade-specific responses to changing environments. It is therefore necessary to draw on information from a broader phylogenetic base in order to estimate the timing of the formation of this biome.

By far the highest frequency of transitions to succulent karoo in the angiosperm groups dated occured during the last 5 Mya (Fig. 3.7). While 17 Myr is somewhat older than the hypothesised climatic changes that led to the establishment of the Mediterranean climate, it is still remarkably young. Early transitions to succulent karoo are estimated to have occurred in *Pelargonium, Moraea, Melianthus* and *Kalanchoideae*. Transitions dated for the related study (Appendix 1) were based on stem node dates which give older ages than if crown node dates are used. It is difficult to use the latter in cases where the transition to succulent karoo occurs in a single species as the crown age is zero. In addition, as discussed for *T. singularis* and *Toelkenocodon*, occupation of succulent karoo habitat may represent a post-speciational shift in these lineages, with adaptations to the current environment occurring via anagenesis. Alternatively, the antiquity of these lineages may reflect the inadequacies of calibration dates as these studies are all limited by the lack of available fossil evidence (discussed later). Whatever the caveats surrounding the accuracy of dates applied, the emergent story is that the succulent karoo is a young biome; younger than the neighbouring fynbos, which may date back to the Early Oligocene (Linder, 2005). The marked increase in the number of lineages making the transition to succulent karoo during the last 5-10 Mya is consistent with climatic trends documented for this period. The climate history of the region was not a linear trend of cooling but was interspersed with colder excursions that would have influenced the environments of the Greater Cape Floristic Region considerably. Wind-borne dusts recovered in marine sediments off the coast of Namibia provide a continuous record of sea surface temperatures (SST) for the last 4.6 Myr. Evidently there was a drop of around 10°C during the last 3.2 Myr which marked the onset of a prolonged period of cooling coincident with the onset of Northern Hemisphere glaciation (Marlow et al., 2000). Events such as this impacted on the mean climates experienced in the southern Africa, and likely intensified the process that

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began in the region some 10 Mya. Patterns of lineage splitting and extinction in Kalanchoideae are interpreted against this historical climatic evidence.

The chronological sequence of shifts into succulent karoo presented here suggests a gradual process of occupation of the biome between 17 – 5 Mya, with subsequent radiations occurring in some lineages. This overall pattern of transitions is not entirely consistent with the tabula rasa hypothesis, but rather suggests the gradual clearing of the table, at least initially. Plants requiring more mesic conditions were increasingly marginalised, giving lineages such as *Tylecodon*, *Moraea* and *Pelargonium*, having or evolving suitably adapted growth forms, the opportunity to enter the arena. Once established in the biome the extent to which lineages diversified was determined largely by the interaction between the traits they carried (phylogeny) and the new, relatively stable conditions that prevailed in the region.

The log-linearity of species accumulation curves for *Tylecodon*, *Cotyledon* and *Adromischus* (Fig. 3.5) implies underlying exponential growth rates; pure birth processes consistent with little or no extinction (Nee, 2006). Whilst it may be unrealistic to dismiss extinction, Nee (2006) states that the fit of the pure birth model to a given dataset is implicit in the log-linearity of curves produced from the data. This suggests that the southern African representatives of Kalanchoideae, restricted predominantly to the winter rainfall region, are undergoing a phase of steady radiation. The genera are still in a bottom-heavy pre-equilibrium stage of their evolution (Gould et al., 1977), and such a pattern ties in well with conditions in the winter rainfall region where climatic stability during the Pleistocene '...has relaxed the selection against specialisation and traits associated with poor dispersal...' (Dynesius & Jansson, 2000: 9119). Vrba (1985) suggested that speciation simply does not occur unless forced to by environmental change. Species are evolutionarily conservative, and therefore far more likely to track a shifting habitat than evolve to occupy a new one. The fossil record for plants is relatively poor, and so there is no paleontological evidence against which current diversity and distributions can be contextualised. The fossil record for many mammal groups is, by contrast, quite rich and has been interpreted in light of knowledge of shifting climates

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(deMenocal, 2004; and Brehrensmeyer, 2006). The overarching finding of these works is one of climate-driven speciation and extinction.

How can speciation driven by climatic change be reconciled with speciation coincident with stability in the winter rainfall region of southern Africa? It appears to be a question of the relative timing of events. The process of aridification and transition from aseasonal to seasonal rainfall in the area is well documented (Siesser, 1980, Zachos et al, 2001). A general cooling trend followed the climatic optimum of the late Middle Miocene (17 – 15 Mya) with the re-establishment of the Antarctic ice sheet and concomitant upwelling of the Benguela Current (10 Mya: Siesser, 1980). Climatic changes are unlikely to have been instantaneous but would rather have altered selective regimes experienced by resident plant populations over a long period. Some populations may have been wiped out early in the process while others persisted in marginal habitats. Extinction of the most arid-sensitive species would render areas available for occupation. The plants that invaded these areas were a sample of those that were in the right place at the right time, possessing the right adaptations, or adaptive potential. The likely scenario for *Tylecodon* and *Adromischus* is one of ancestral species climatically 'forced' to diverge as aridity impinged upon their environment, possibly fragmenting ancestral ranges. Summer-drought squeezed out poorly adapted lineages that died out or, where possible, retreated to higher ground. Thus a change in the mean climatic conditions ensued producing a turnover pulse of extinction and subsequent diversification (sensu Vrba, 1985). Once the summer-arid climate was established the region became relatively stable, buffered from high amplitude Milankovitch oscillations by virtue of its geographical position (Dynesius & Jansson, 2000). Under these conditions, species of *Tylecodon* and, to a lesser extent, *Adromischus* underwent specialisation within the newly occupied areas. The process of differentiation is likely to have been reinforced by the inherently poor dispersal ability of members of these genera (Chapter 4).

Overall rates of diversification in the focal genera of this study differ. When interpreted in a spatial context the consistently higher rate found in *Tylecodon* is striking. The genus has accumulated four times the number of species of its sister *Cotyledon*, despite occupying only

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15% of the land area. Diversification rates calculated on a per km^2 basis are 8.8×10^{-7} species per km^2 per Myr for *Tylecodon*, and 7.3×10^{-8} species per km^2 per Myr for *Cotyledon* and *Adromischus*. The strong link between *Tylecodon* and the succulent karoo points to an environmental cause for its high extant diversity. Such a conclusion is logical given that the area is famous for its diversity and high levels of endemism generally (Desmet & Cowling, 2004). *Adromischus* too has many representatives within the succulent karoo but is not confined to the area in the same way that *Tylecodon* is. Hence the diversity within *Tylecodon* appears to be the result of derived morphological characteristics in the clade interacting with the abiotic environment. This process results in the partitioning of available land, with subsequent genetic differentiation and the formation of new species. These biological features are explored in the next chapter.

Methodological considerations

Invoking autocorrelation of rates, as done by NPRS and Multidivtime, greatly increases the proportion of time allocated to shallower nodes of the tree for this group. This leads to older age estimates of species. It is reasonably well documented that NPRS tends to overestimate node ages (Martin et al., 2004; and Linder et al. 2006). Hugall and Lee (2004) noted that NPRS lowered the ratio between deeper and shallower nodes of a tree, and the pattern produced in *Adromischus*, *Tylecodon* and *Cotyledon* is consistent with their findings. Multidivtime has a similar though less pronounced effect on time estimates. It is reasonable to conclude for this phylogeny at least, that autocorrelation consistently overestimates the age of species, independent of whether or not the techniques include models of molecular evolution.

Clock-like evolution was rejected for members of Kalanchoideae analysed here however the distribution of branch lengths produced under rate constancy most closely resembles that of the unconstrained phylogram (Fig. 3.2). Ho et al. (2005) assessed the performance of various time estimation methods on simulated data and concluded that if evolution of sequence data is clock-like or nearly so then precision, and possibly accuracy of substitution rates are improved by assuming a clock. Linder et al. (2005) advocate using a molecular clock whenever possible, mainly because of its robustness to under-sampling of taxa. A local clock

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model (Rambaut & Bromham, 1998; and Yoder & Yang, 2000) may offer better precision of time estimates for *Adromischus*, *Tylecodon* and *Cotyledon*, given that different rates can be assigned to them, and to *Crassula*, *Aeonium* and *Kalanchoe*. Branch length optimisation in BEAST is not constrained by autocorrelation, and is not, therefore, a priori prone to overestimation of node ages. The method accommodates uncertainty surrounding calibration times with phylogenetic uncertainty. This allows calibration information to be applied to data without introducing false precision associated with point calibrations - an implementation that has been strongly criticised (Graur & Martin, 2004; Heads, 2005). There are few published studies against which to compare the performance of Bayesian relaxed phylogenetics. However, the uncorrelated relaxed clock models implemented in BEAST performed well in simulations, even when data were evolved under a model of rate constancy (Drummond et al., 2006). Thus this method is favoured as the biologically most meaningful technique applied to diversification events in *Adromischus*, *Tylecodon* and *Cotyledon* and hence for distantly related lineages (Appendix 2). Correlates and potential causes of divergence events are interpreted accordingly. Dates obtained from analysis of plastid data offer inference from near complete sampling (84 taxa and 1288 base pairs) while analysis of combined data provide time estimates based on two genome sources and more sequence data, but with fewer taxa (65 taxa, and 1958 base pairs). It is likely then that divergence events occurred between those estimated with these two datasets.

The importance of fossil evidence as a source of minimum ages of clades for dating exercises cannot be overemphasised (Thorne & Kishino, 2002; Magallón, 2004). Fossils assigned reliably within a phylogenetic context are scarce (Magallón & Sanderson, 2001). In a re-evaluation of fossil evidence of the angiosperms, Crepet et al (2004) estimated that Crassulaceae diverged from other clades in the Saxifragales around 70 Mya. An attempt was made in this study to apply the age of the fossil taxon *Tarahumara sophiae* discovered in Mexican strata deposited approximately 70 Mya, and assigned to Haloragaceae of the Saxifragales (Hernández-Castillo & Cevallos-Ferriz, 1999). Unfortunately DNA sequences for members of Crassulaceae could not be aligned with those from extant species of *Haloragis* as they were too divergent. Hence it was necessary to apply calibration dates that are

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extrapolated from scant palaeontological evidence to finer resolution phylogenies (i.e. Wikström et al., 2001). Error inherent in the fossil record is therefore compounded (Pulquério & Nichols, 2006), and interpretation of the tempo of evolutionary events in this group is carried out with such caveats in mind. Despite their inaccuracies, such data are invaluable to studies of this nature as they provide the only temporal anchor currently available for *Adromischus*, *Tylecodon* and *Cotyledon,* together with many other southern African plant groups.

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CHAPTER 4

The geography of speciation and range size determinants in *Tylecodon* and *Cotyledon*.

INTRODUCTION

Southern Africa boasts a rich flora that represents some 40% of the entire African flora (Linder et al., 2005). Genera centred in the fynbos and succulent karoo biomes contribute disproportionately to the high species to genus ratio of the region (Gibbs Russell, 1985), and as a result of the dominance of a few very speciose families with high levels of endemism, namely Aizoaceae, Crassulaceae and Apocynaceae, the vegetation of the succulent karoo is a phylogenetically non-random assemblage (Cowling & Hilton-Taylor, 1994). The high representation of these families in the biome suggests either a long history of speciation, or elevated rates of speciation. Remarkably high speciation rates, outstripping those of taxa on oceanic islands, have been found in members of Aizoaceae (Klak et al., 2004) and, as demonstrated in Chapter 3, *Tylecodon* has speciated at a notably higher rate than its sister *Cotyledon*. More than half of the species of *Tylecodon* are endemic to the succulent karoo; in the context of differences in land area occupied by each genus, the high diversification rate found in *Tylecodon* is extraordinary. The 46 species of *Tylecodon* occur within an area approximately 180 000 km^2, while *Cotyledon* is distributed across an area six to seven times larger.

High rates of diversification in two succulent karoo-centred plant groups have been attributed to morphological key innovations thought to confer selective advantages for surviving the rigours of an arid environment. The evolution of wide-band tracheids in Ruschioideae (Aizoaceae), together with leaf and capsule morphology, are thought to have been pivotal to the recent and rapid radiation of the clade (Klak et al., 2004). Tuber fattening in the geophytic

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lineage of xerophytes of *Pelargonium* is purported to explain the subsequent radiation of the group in Namaqualand (Bakker et al., 2005). The mechanisms by which these innovations have led to high diversity has been alluded to but rarely thoroughly explored. Identifying characteristics common to members of speciose clades is an important component of revealing the mechanisms that underlie the generation of diversity. It has been argued however, that establishing a link between such characters and the diversification process is difficult, if not impossible, and renders this approach untestable (Cracraft, 1981; Slowinski & Guyer, 1993). Using sister groups mitigates this problem to some extent as comparison of numbers of species in clades is relative, being between two contemporaneously diverging lineages thus eliminating the need for conjecture regarding the significance of absolute clade sizes among distantly related groups (Cracraft, 1981). Sister group comparisons are used here to investigate some of the correlates of characters unique to each of *Cotyledon* and *Tylecodon* as a means of exploring mechanisms by which these genera have diverged in their evolutionary trajectories. In particular, this chapter explores the association between selected morphological adaptations to extreme aridity exhibited by species of *Tylecodon* and increased rates of speciation in the genus. The aim is to test the hypothesis that limitations to dispersal imposed by reduced plant size and photosynthetic capacity are a primary determinant of range size in *Tylecodon*, and have had consequences for gene flow, and ultimately speciation in the genus.

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Plant succulence is strongly associated with limited water availability (von Willert et al., 1992) so, by virtue of their phylogenetic history, species of *Cotyledon* and *Tylecodon* are adapted to survive periodic drought. Morphological and life-history traits of members of *Tylecodon* appear to represent further specialisation that enables the plants to tolerate extreme aridity. Leaf deciduousness, hysteranthy, development of pachycauls (sensu Rowley, 1987), geophytism, diminution of vegetative organs and the general tendency towards reduced surface area to volume ratios, are strategies for conserving moisture during periods of low extrinsic availability, and for reducing the risk of overheating of the plant body. The distribution of many diminutive growth forms, particularly those of 'stone plants' (e.g. *Conophytum*, *Argyroderma*: Aizoaceae) is suggested to be linked to the occurrence of coastal fog that

penetrates much of the succulent karoo: condensed fog can provide significant moisture supplements to small plants (Midgley & van der Heyden, 1999). In addition, the strong association that many dwarf succulent species have with rock crevices and rocky habitats may relate to the fact that rock surfaces promote the condensation of fog and thus increase water availability to nearby plants through run-off (von Willert et al., 1992).

The absorbance of photosynthetically active radiation (PAR) increases with increasing degrees of succulence resulting in reduced carbon gain (Von Willert et al, 1992). This has repercussions for growth and reproductive effort. Bakker et al. (2005) postulated that the trade-off for improved water storage conferred by fattened tubers in species of *Pelargonium* was that of lower seed production and dispersal, which ultimately led to restricted gene flow. There is a high proclivity of passive dispersal in the karoo generally, and it has been proposed that propagules landing close to parent plants have a selective advantage in these arid environments (Esler, 1999). Short dispersal distances may also reflect physiological constraints imposed by succulent growth forms, as suggested for *Pelargonium* (Bakker et al., 2005). Irrespective of the evolutionary history of such dispersal mechanisms, the most likely determinant of dispersal distance is the height at which propagules are released from the parent plant. Seeds of *Tylecodon* and *Cotyledon* have no adaptations for long-range dispersal; they are usually < 1 mm in size and are scattered as wind-blown dust or by raindrops hitting follicles (personal observations after Hoffman and Cowling, 1987). The significant association between growth form and endemism in broken, rocky habitats of the succulent karoo (Cowling & Hilton-Taylor, 1994, refs therein; Hilton-Taylor, 1996) suggests that dispersal distances in *Tylecodon* may be strongly influenced by topography. All else being equal, the shorter the plant, the shorter its dispersal distance. If *Tylecodon* is effectively a succulent karoo specialist then, given the size of this biome, there should be no correlation between species age and range size.

Reduced dispersal ability is often associated with species having small ranges and concomitantly small population sizes. In turn, small population size is associated with low density (Gaston, 1997). These characteristics can impact upon gene flow and ultimately, rates

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of speciation. High levels of centre-mediated gene flow in widespread, common species are believed to prevent differentiation of individuals that occupy the periphery of the species' or population's range (Gavrilets et al., 2000). In the absence of such cohesive gene flow - as in range-restricted, rare populations of low density - individuals at the periphery of the population that may be surviving in sub-optimal conditions are able to diverge and adapt to the local environment (Gavrilets et al, 2000). Similarly, dispersal to new environments, although rare, can lead to new divergent populations and incipient species, as founders are not constrained by the homogenising effects of gene flow. This process is basically peripatric speciation as defined by Mayr (1963). Such a scenario was invoked to explain diversification in *Argyroderma* (Ellis et al., 2006). This small genus of ruschioids (Aizoaceae) is restricted to the unique quartz gravel habitat of the Knersvlakte within the succulent karoo biome. Following an investigation of the mechanisms of divergence in *Argyroderma,* Ellis et al. (2006) and Ellis & Weis (2006) reported a process of differentiation in allopatry at extremely fine spatial scales. Hydrochastic capsules ensure highly localised dispersal in members of the genus (Ellis et al., 2006) and restricted gene flow is maintained by edaphic specialisation and divergent habitat selection, while post-speciational flowering time shifts have maintained differentiation upon secondary contact between species (Ellis et al., 2006; Ellis & Weis, 2006).

In addition to seed-mediated gene flow, pollen movement between populations can greatly influence processes of differentiation. Pollination syndromes are associated with different effective spatial scales due to the foraging distances of pollinators. Species of *Cotyledon* exhibit classic bird-pollination characteristics, namely pendulous, tubular flowers that are red to yellow in colour and produce copious amounts of nectar (van Jaarsveld & Koutnik, 2004). Most species of *Tylecodon* are thought to be insect-pollinated (van Jaarsveld & Koutnik, 2004) and a few specific plant-pollinator interactions have been observed. *Tylecodon hallii* is pollinated by a pollen wasp (*Masarina tylecodoni*: Gess et al., 1998) and *T. cacalioides* is pollinated by a long-proboscid horse-fly (*Philoliche tumidifacies*: Gess, 2001). Interestingly, two of the widespread species of *Tylecodon* (*T. paniculatus* and *T. grandiflorus*) are pollinated by sunbirds (Gess, 2001; van Jaarsveld & Koutnik, 2004). The effect of pollination syndrome on gene flow was the focus of a recent study of two species of *Streptocarpus* in South Africa:

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one sunbird-pollinated (*S. dunnii*), the other (*S. primulifolius*) pollinated by a species of nemestrinid fly (Hughes et al., 2007). Greater pollen-mediated gene flow was observed in the sunbird pollinated species, and is believed to account for the higher levels of genetic cohesion between populations of *S. dunnii*. Although information on the pollination biology of succulent karoo groups is scarce, the pollinator fauna is documented to share the high levels of endemism found in the flora of the region (Esler, 1999). Short distances involved in insect pollination in *Tylecodon* may well serve to reinforce patterns of fine-scale differentiation that arise from localised dispersal.

In a system of reduced or no gene flow resulting from short dispersal distances the prediction for the predominant mode of speciation in *Tylecodon* is one of allopatry, as found to be the case in *Argyroderma* (Ellis et al., 2006). Information on the spatial distribution of species combined with species-level phylogenies that trace the historical sequence of lineage splitting can be used to infer geographical modes of speciation (Barraclough & Vogler, 2000). Lynch (1989) developed a method that involved measuring species range areas, areas of overlap, and distances separating non-overlapping species' ranges, for ever-more inclusive groups of a cladogram, working from the tips to the deeper nodes of the tree. Several workers have elaborated upon Lynch's method. Barraclough et al. (1998) incorporated explicit expectations into plots of the degree of sympatry against node height under the null model that geographic ranges are random with respect to phylogeny. Initially, models simulating cladogenesis under several modes of geographic speciation, with varying degrees of random range shifts, were generated as a means of assessing whether current species' ranges retained any phylogenetic pattern (Barraclough & Vogler, 2000). More recently, randomisation tests have been introduced. Species ranges are randomly shuffled amongst the tips of the phylogeny to test whether closely related species display a geographic pattern of overlap that is significantly different from that of randomly chosen species (Fitzpatrick & Turelli, 2006; Perret et al., 2007). Such an approach is adopted in the current study. To date, broad-scale investigations into modes of speciation have focussed on animal groups (Lynch, 1989; Chesser and Zink, 1994; and Fitzpatrick & Turelli, 2006) and the work of Perret et al. (2007) on Gesneriaceae is the only example currently known of these methods being applied to

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plants. This work on *Cotyledon*, *Tylecodon* and *Adromischus* represents a significant addition to the body of evidence on geographic modes of speciation in plants. Plants are amongst the least vagile organisms in terms of reproductive individuals and therefore provide data on modes of speciation that are likely to differ from those of highly mobile animals.

In an effort to understand the processes driving elevated rates of diversification in *Tylecodon*, this chapter will address the following specific questions. 1) Does *Tylecodon* occupy significantly different climatic environments from its sister *Cotyledon*? 2) Do the two genera differ in terms of their range sizes, and can this difference be associated with traits reflecting adaptations to aridity? 3) Is there evidence to suggest that speciation in *Tylecodon* and *Cotyledon* has been predominantly allopatric?

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METHODS

Climatic niche characterisation and trait reconstruction procedures were carried out for the two focal genera of this study – *Cotyledon* and *Tylecodon*, together with *Adromischus* and *Toelkenocodon*. The latter, more distantly related genera were included to faciliate more reliable inference of ancestral character states across the phylogeny of the southern African representatives of the clade. Interpretation of results is restricted to *Tylecodon* and *Cotyledon*.

Climatic niche characterisation

Climatic data for a total of 1652 geo-referenced localities of *Tylecodon* (665), *Cotyledon* (273), *Adromischus* (680), and *Toelkenocodon* (34) in South Africa were extracted from Schulze (1997). Monthly climatic variables selected were: means of daily minimum and maximum temperatures (T_{min} and T_{max}, ºC), median rainfall (mm), and potential evaporation (A-pan equivalent, PE_{apan}, mm). Data were not available for Namibian records.

Discriminant analysis (DA) was used to evaluate whether the genera differ with respect to the climatic variables selected, and to identify which variables are associated with the geographic distributions of members of *Tylecodon* and *Cotyledon*. The DA was performed using the 48

climatic variables listed above for all 1652 localities (all 3 genera) and 938 localities (*Tylecodon* and *Cotyledon* only) using SPSS (1997). The efficacy of discriminant functions at predicting group membership was assessed by reclassifying records to a group according to their discriminant functions (Tabachnick & Fidell, 1996). The proportion of correct classifications was determined and a value greater than 33% (assuming equal probability of a record being classified into any group) was taken to indicate that discriminant functions satisfactorily predicted group membership. Cross-validation of the reclassification rates was performed by removing each record in turn. By convention, only loadings of variables ≥ 0.33 were interpreted as informative (Tabachnick & Fidell, 1996).

Trait reconstruction

Mean annual rainfall (MAP), calculated as the average value for all records of each species, was used in place of discriminant function (DF) scores for climate reconstructions, to avoid interpretation being confounded by the somewhat abstract nature of DF scores. In adopting such an approach aridity is used to represent the separation of *Tylecodon* from *Cotyledon* along an axis of rainfall amount and seasonality, and high potential evaporation. The appropriateness of using MAP for this purpose was assessed by linear regression-correlation analysis of discriminant function scores on MAP.

In order to assess associations between levels of aridity, as explained above, range size and putative adaptations in *Tylecodon*, ancestral character states were reconstructed using Fitch (categorical characters: Fitch, 1971) or squared change parsimony (continuous characters: Huey & Bennett, 1987; Maddison, 1991) as implemented in Mesquite version 1.12 (Maddison & Maddison, 2006). Three categorical characters were scored as present (1) or absent (0) for all species of the four genera represented in the BEAST plastid topology (Chapter 3, Fig. 3.3), namely a) leaf deciduousness, b) plants predominantly restricted to growing in rock crevices, c) possession of subterranean storage organs, defined from a functional standpoint, as having roots or stems that store water, that are at least half buried below the substrate surface. Three continuous characters - mean annual rainfall (mm), range-size (km^2) and vegetative height (mm) - were reconstructed in the same way.

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Associations of vegetative plant size, inflorescences and flowers

Characteristics that may be linked to different pollination syndromes were tested using linear regression and correlation analysis (Microsoft ® Office Excel, 2003). Possible relationships of vegetative height with inflorescence length and floral tube length were tested to determine whether smaller plants produced shorter inflorescences and smaller flowers.

Range sizes and measures of sympatry

Age-range correlations (ARCs) were performed using the plastid topology and age estimates produced in Chapter 3 using BEAST. This topology provided the most complete taxon sampling and set of age estimates with which to analyse pattern. *Tylecodon striatus* 9177, representing a new undescribed species, was excluded from ARCs as distribution data were only available for the species, since found to be paraphyletic (Chapter 1). Distribution data were used for the species as is and only the phylogenetic position of *T. striatus* 7501 was included in analyses. *Tylecodon, Cotyledon* and *Adromischus* were represented by 40, 10 and 26 species, respectively, together with the monotypic *Toelkenocodon*.

Range area measurements were performed using ArcView GIS 3.2. Species range maps were produced from a total of 1048 geo-referenced records of *Cotyledon* and *Tylecodon* using two methods of reconstructing species' ranges. Minimum convex polygons were created around dot distribution data for each species using a convex hull extension (ArcView GIS 3.2) – this represents species 'extent of occurrence' (EOO: Gaston, 1994). Secondly, a 10 km radius buffer was added to each locality point as a finer scale representation of range akin to the 'area of occupancy' (AOO: Gaston, 1994). Areas, overlaps and distances (m^2 and km) were measured adopting the UTM (Universal Transverse Mercator) coordinate system and WGS84 (World Geodetic System 1984) map datum. The following measurements were recorded: a) range area, measured as (i) the total area of each species' polygon, and (ii) the total area of all buffered points of each species, measuring overlapping areas only once; b) the area of overlap, if any, for every pairwise comparison between species (within genera), for polygons and buffers. Range-size distributions were plotted for each genus. As range sizes of *Cotyledon* and *Tylecodon* are not normally distributed, differences between group medians

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were tested using the Mann-Whitney U test executed in Statistica version 7.0 (Statsoft Inc., 2004). The relationships of range size with species age and height of seed release (calculated as the sum of the vegetative height and inflorescence height) were tested using linear regression and correlation analysis (Microsoft ® Office Excel, 2003).

The degree of sympatry between all pairwise species comparisons was calculated as the proportion of the smaller species' range that the overlap represented (Chesser & Zink, 1994; Barraclough and Vogler, 2000; Fitzpatrick & Turelli, 2006; Perret et al. 2007). This provides a sympatry index that ranges from 0 (allopatric) to 1 (completely sympatric). Estimates of the average overlap between species since time of speciation were calculated for each genus following the 'nested averages of pairwise overlaps' methods developed by Fitzpatrick & Turelli (2006: 603-4, equation 1). Alternative methods, such as those employed by Barraclough & Vogler (2000) and Perret et al., (2007) have been strongly criticised as they use the union of clade-member ranges as a proxy for ancestral ranges that involve comparisons spanning ever-deeper nodes of a phylogeny (Chesser & Zink, 1994; Losos & Glor, 2003). Calculating nested averages of pairwise overlaps accounts for the phylogenetic nesting of species, does not attempt to reconstruct ancestral ranges, and overcomes the fact that re-using species in multiple pairwise comparisons violates the assumption of independence (Felsenstein, 1985, in Fitzpatrick & Turelli, 2006). In addition, the nested averages method is less affected by incomplete taxon sampling (Fitzpatrick & Turelli, 2006). The method is therefore better suited to the current study which utilises a topology that is resolved according to maximum clade credibilities (Chapter 3) several of which attain very low posterior probabilities, and must be interpreted with caution.

Mean range overlaps were plotted against node age and linear regression lines were fitted. To test the null hypothesis that present-day ranges contain no information regarding the geography of speciation, null distributions of slope and intercept were simulated by randomising the degree of sympatry (Fitzpatrick & Turelli, 2006). Values in a degree of sympatry matrix were reshuffled 10,000 times and slope and intercept were calculated following each rearrangement. Departures from random were tested using a two-tailed

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significance test, calculated as 2 x the proportion of randomisations that gave a value more extreme than that observed (Fitzpatrick & Turelli, 2006; Perret et al., 2007). Plots of sympatry against node-age provide a summary of the geographic mode of speciation (Barraclough & Vogler, 2000). The most recently diverged taxa reflect the predominant mode of speciation such that speciation in allopatry will give intercept values significantly closer to 0 than expected by chance, whereupon increasing sympatry with increasing node age is characteristic of post-speciational range shifts. Higher incidence of speciation in sympatry will give intercepts closer to 1.

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RESULTS

Climatic niche characterisation

Tylecodon and *Cotyledon* showed significant separation along a discriminant function axis defined by climatic variables (Wilks' Lambda 0.452, $p < 0.001$, df = 39). The high discriminatory power of the function is illustrated in Figure 4.1. Loadings of correlations between climate variables and the discriminant function (Appendix 2) suggest that *Tylecodon*

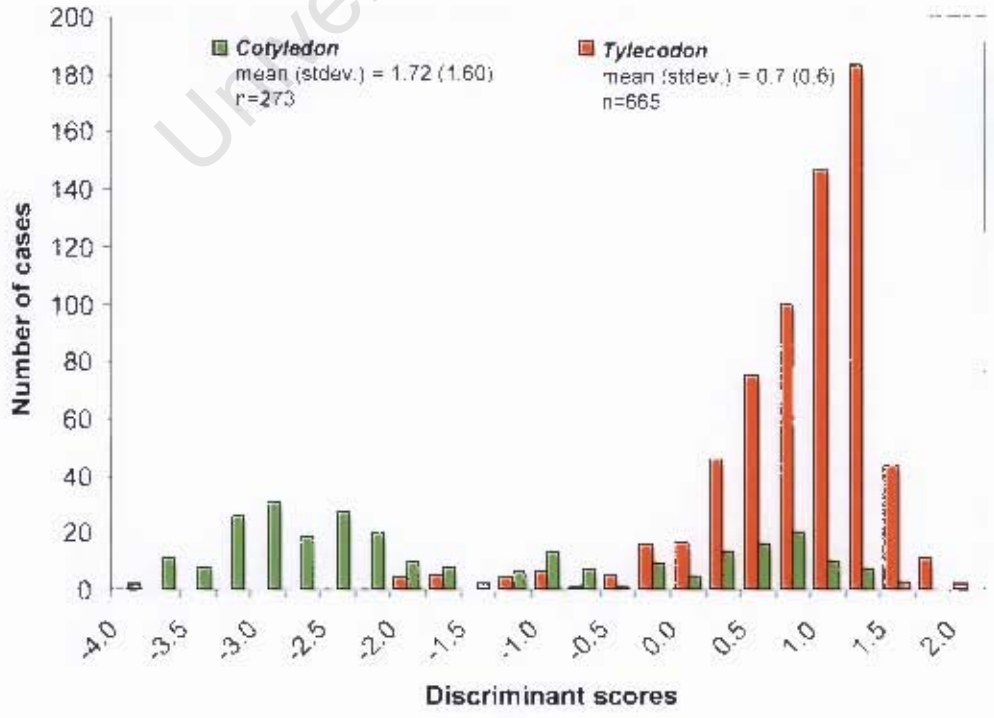

Figure 4.1. Discriminant function plot showing the separation of group means with respect to selected climatic variables.

and *Cotyledon* occupy climatic niches that are highly distinct from one another. *Tylecodon* (group mean = 0.7) is strongly associated with low rainfall and high potential evaporation during the summer months (November to March). *Cotyledon*, although exhibiting greater spread along the discriminant axis, is associated with the occurrence of summer rainfall and lower potential evaporation during this period. Only loadings of > 0.50 were interpreted. A complete list of climatic variables and their loadings, together with details of cross-validation of the reclassification of cases based on the discriminant function is given in Appendix X.

Trait reconstruction

DF scores and MAP are highly significantly correlated (Fig. 4.2, $R^2 = 0.5438$; $p = << 0.001$) thus MAP was substituted for DF scores in order to reconstruct ancestral climate niches. The MRCA of the southern African genera of Kalanchoideae occupied environments receiving 234-300mm of MAP. A shift to more arid environments is evident in *Tylecodon*, with MAP < 233 mm being ancestral (Fig. 4.3). The appearance of geophytism and leaf deciduousness is coincident with this shift to increased aridity. Leaf deciduousness has evolved independently in *Toelkenocodon*, this also being coupled with a shift to very arid sites (40-105 mm rainfall). In contrast, *Cotyledon* shows a shift to more mesic conditions, with most extant species associated with MAP > 300mm. Species of *Adromischus* occur across the gamut of variation in rainfall levels observed across *Cotyledon* and *Tylecodon*.

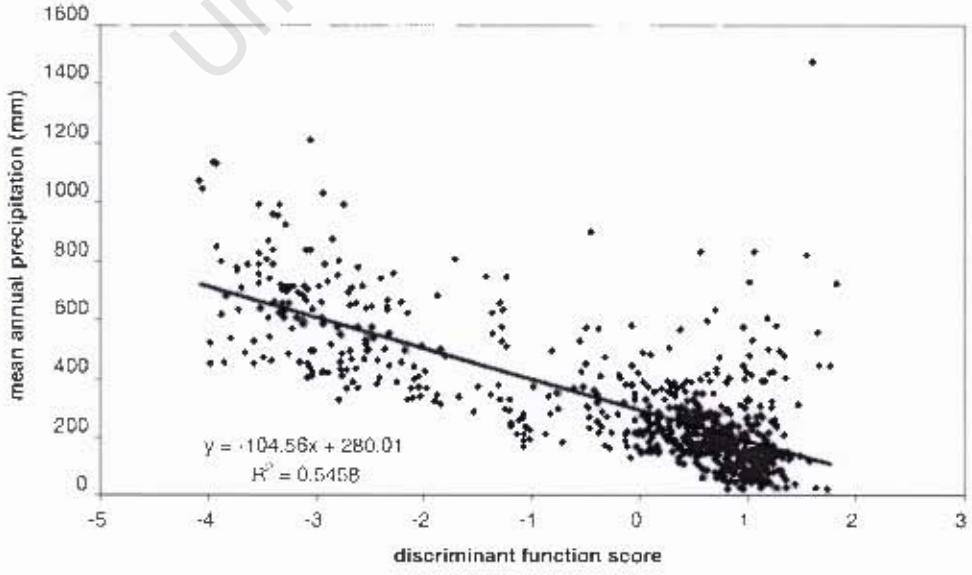

Figure 4.2. Regression of mean annual precipitation on the discriminant function score of selected climatic variables used in DA ($p = << 0.001$).

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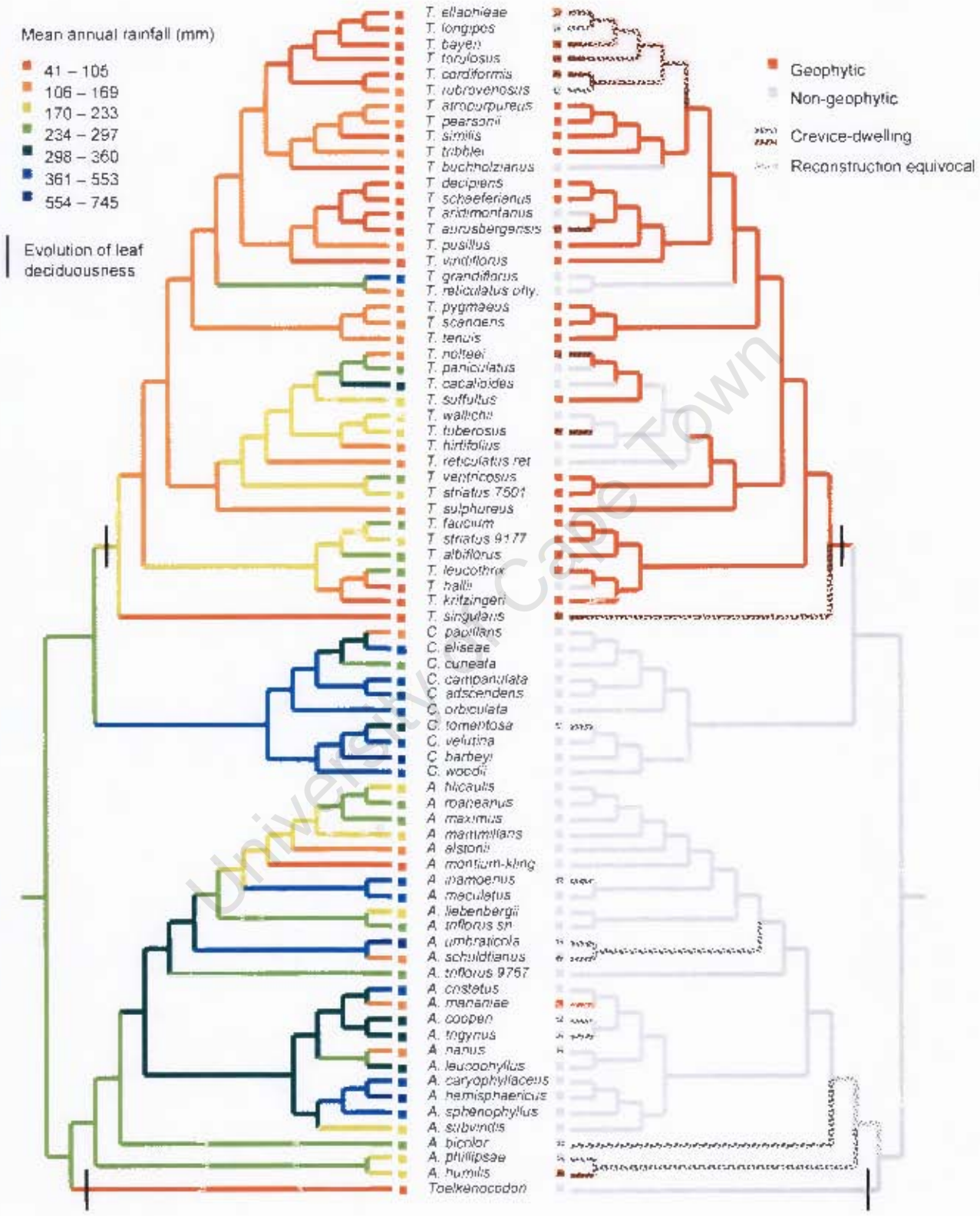

Figure 4.3. Ancestral character state reconstructions using parsimony. **Left-hand tree** shows mean annual rainfall. Note that at > 360 mm of rainfall the range given is larger to simplify representation. **Right-hand tree** illustrates reconstructions of geophytism and crevice-dwelling across all genera. Evolution of leaf-deciduousness is indicated by vertical black bars

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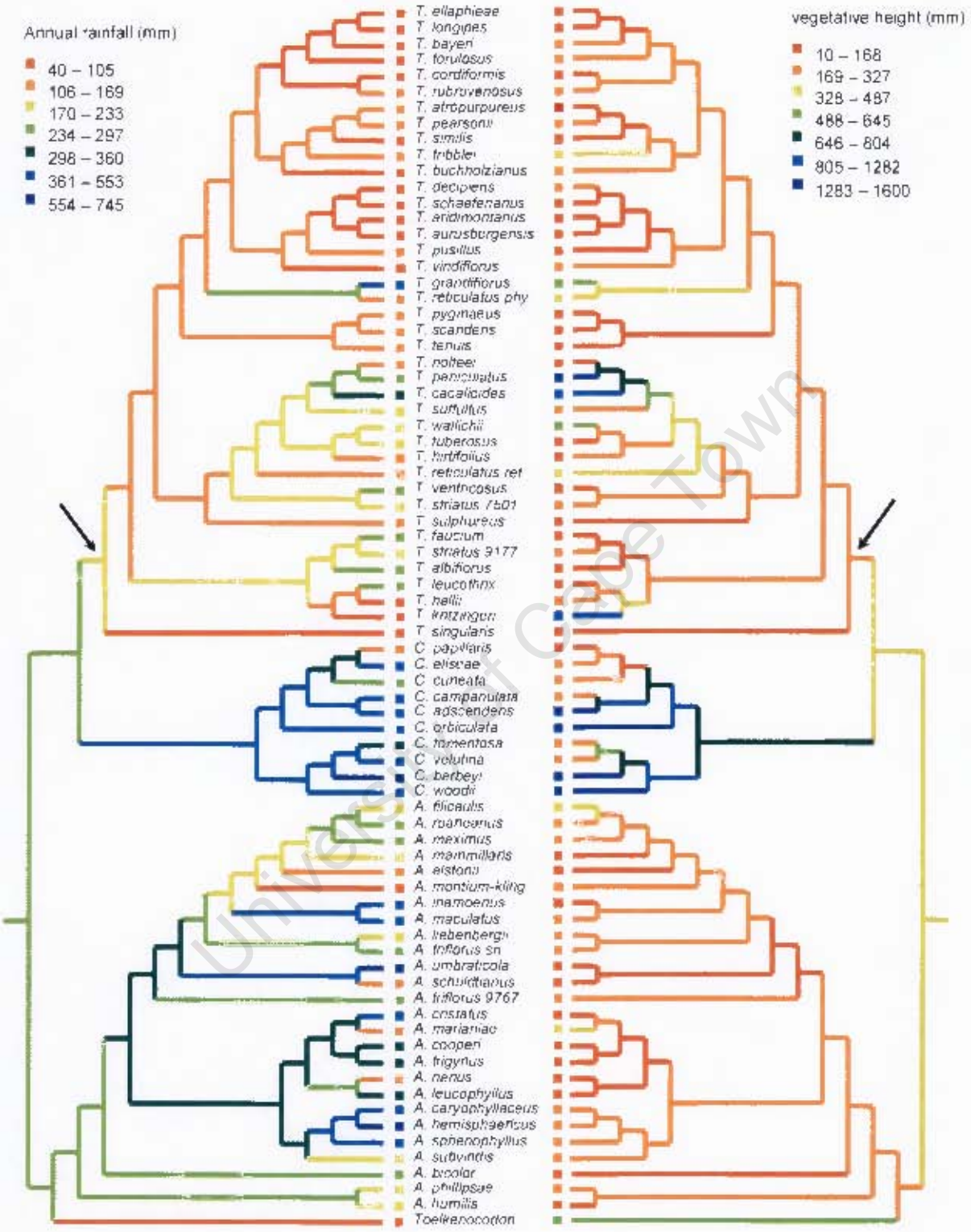

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Figure 4.4. Ancestral character state reconstructions using parsimony. **Left-hand tree** shows mean annual rainfall **Right-hand tree** illustrates reconstruction of vegetative plant height, used as a measure of diminution. Black arrows indicate coincident shifts to greater aridity and reduced plant size. Note that at > 360 mm of rainfall and plant height > 804 mm categories are larger to simplify presentation.

Reconstruction of vegetative height indicates a shift towards diminutive plants in *Tylecodon*, after it diverged from *Cotyledon*. Once again, the appearance of this morphological adaptation in *Tylecodon* coincides with the shift to increasing levels of aridity (Fig. 4.4).

Range sizes in *Cotyledon* and *Tylecodon*

Range sizes in *Tylecodon* are generally smaller than in *Cotyledon*. The Mann-Whitney U test returned a significant difference between the range sizes of *Cotyledon* and *Tylecodon* when ranges were represented by polygons ($Z = 2.77$; $p = 0.006$) but not when buffers were used ($Z = 1.18$; $p = 0.24$). Polygons produce a frequency distribution dispersed across six orders of magnitude while this is narrowed to only three orders using buffers (Fig. 4.5).

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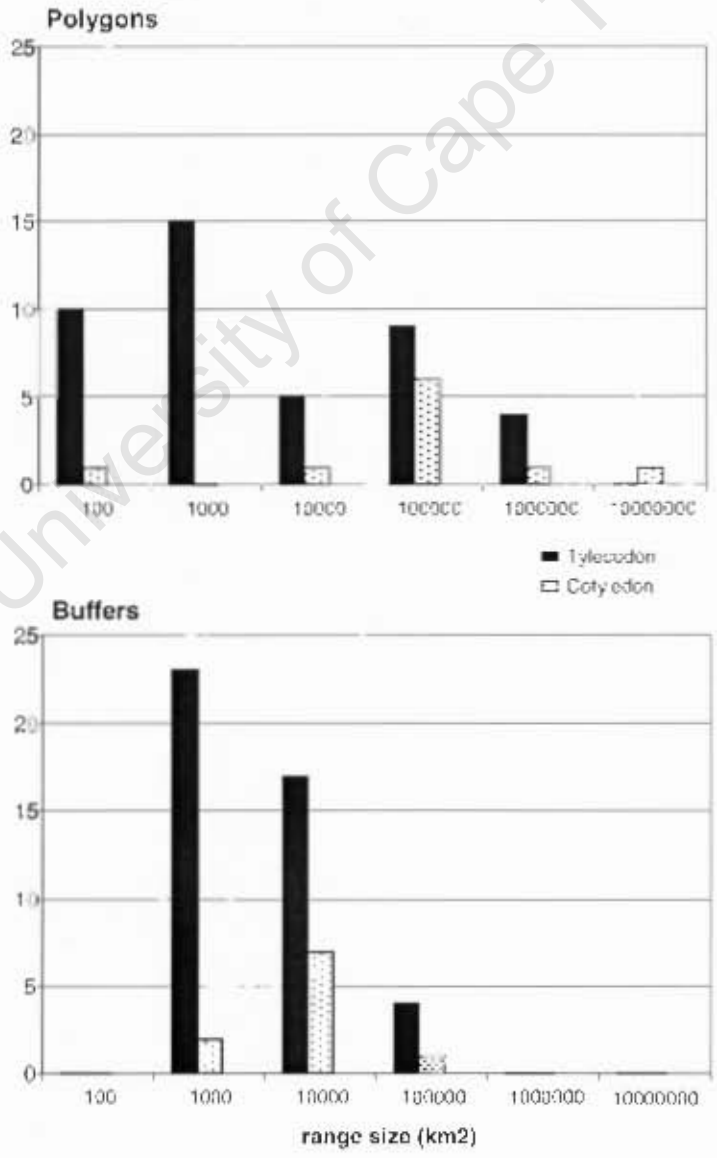

Figure 4.5. Range size distributions of *Tylecodon* and *Cotyledon* inferred on the basis of buffers and polygons.

The distribution of range sizes in *Cotyledon* and *Tylecodon* overlap considerably, more so when represented by buffers. *Tylecodon* has the highest frequency of species in the smallest size class, while most species of *Cotyledon* fall into the middle size class. When polygons are used *Tylecodon* has a high frequency of range sizes not found in *Cotyledon*.

Correlates of range size

Plots of species' range sizes against species' ages are illustrated in Figure 4.6. Linear regression and correlation analysis for *Cotyledon* indicates a significant relationship, with range size tending to increase with the inferred age of the species when buffer ranges are used ($R^2 = 0.442$, $p = 0.04$). This relationship is significant only at $\alpha=0.1$ when polygons are used ($R^2 = 0.336$, $p = 0.08$). There is no relationship between species age and range size in *Tylecodon* (Fig. 4.6).

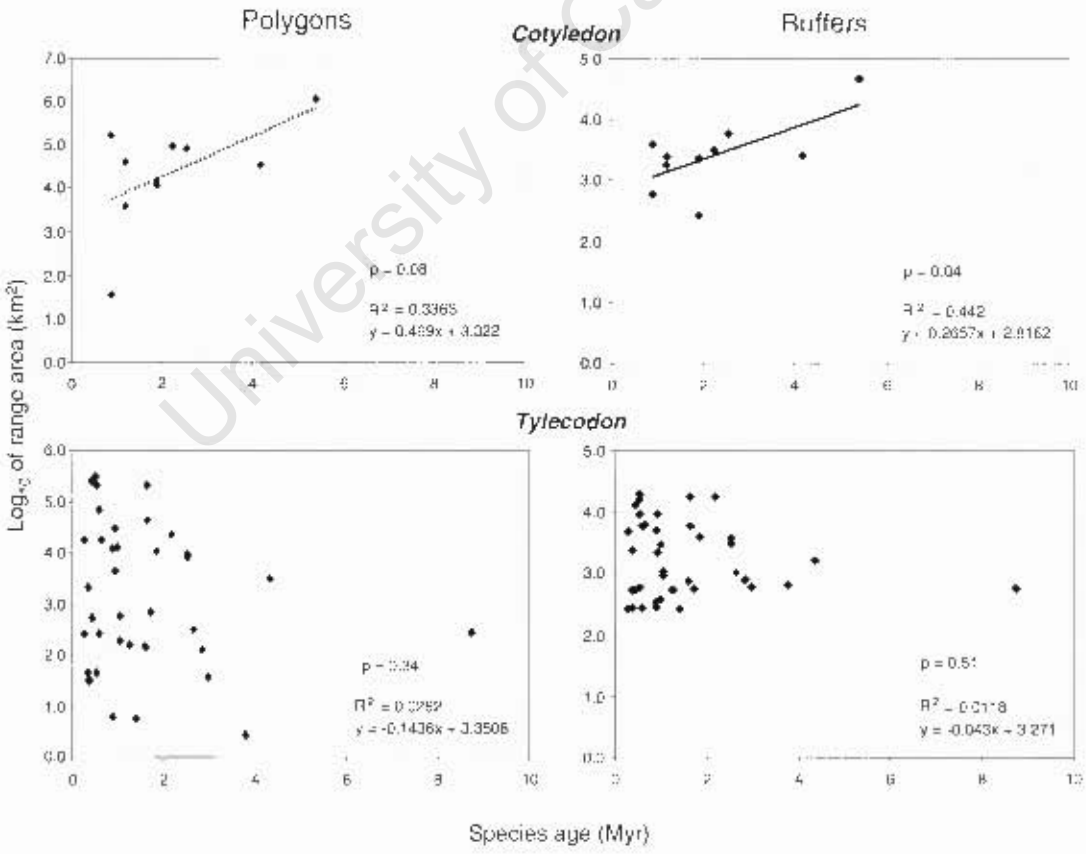

Figure 4.6. Linear regress on and correlation analysis of range size and species age.

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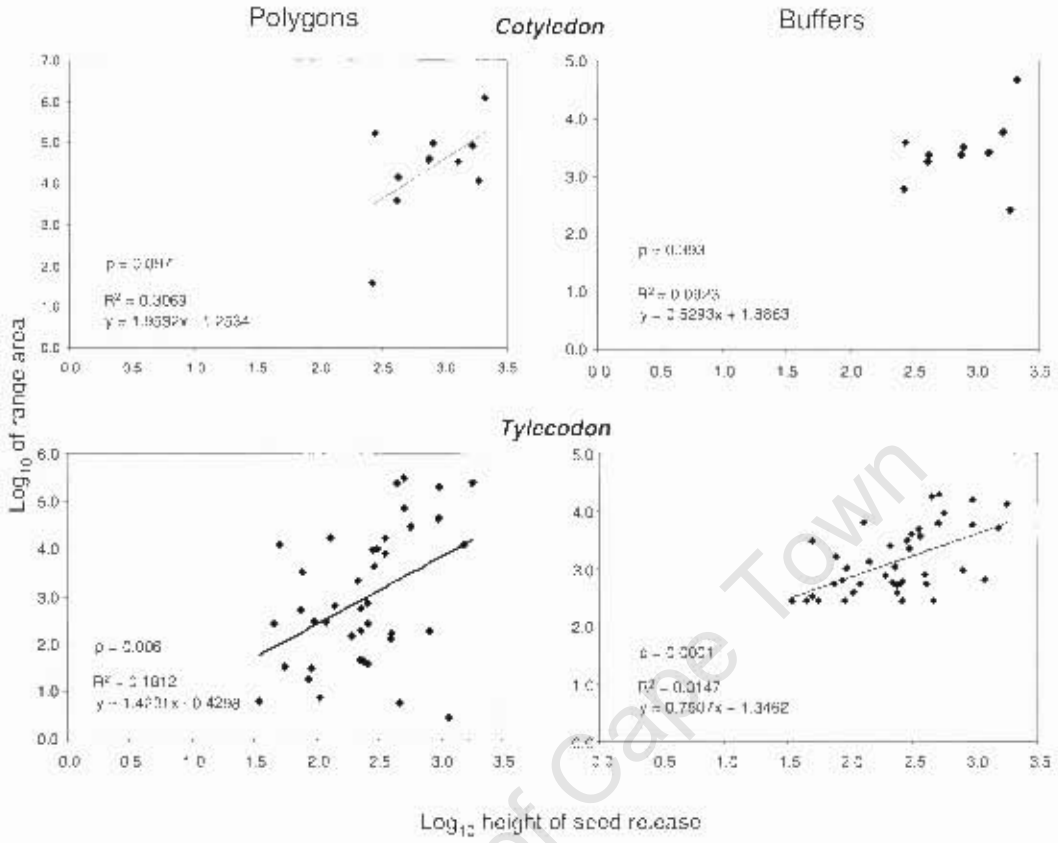

Figure 4.7. Linear regression and correlation analysis of range size and height at which seeds are released. Seed release height is calculated as the sum of vegetative height and length of the inflorescence.

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In both genera range size is correlated with height of seed release (Fig. 4.7). The association is weak in *Cotyledon* but highly significant in *Tylecodon*, irrespective of how ranges are represented. The R^2 value for *Tylecodon* under polygon range reconstruction is low (buffers: $R^2 = 0.3147$, $p = 0.0001$; polygons: $R^2 = 0.1812$, $p = 0.006$).

Age-range correlations

Plots of degree of sympatry against node age are shown in Figure 4.8. Observed values of intercept, slope, and p-values obtained from comparison against null distributions of the same, are detailed on each graph. Plots are largely consistent with phylogenetic signal being retained in species' distributions. Qualitatively, *Cotyledon* shows a pattern consistent with predominantly allopatric speciation. The observed intercept is significantly smaller than expected from randomised ranges (at α=0.1 only; p=0.09) using polygons, but not at all

significant using buffer ranges. Sympatry increases with node age, indicative of post-speciational range shifts and expansion, and the slope is significantly greater than that expected at random (buffers: p=0.007). The notable levels of sympatry (> 0.5) found in *Cotyledon* at coarse scale (polygons) are restricted to older nodes, for example *C. orbiculata*, that diverged > 5 Mya. *Tylecodon* shows a very different pattern through time with many instances of sympatry at coarse scale in recently diverged species (< 2 Myr). The observed intercept of 0.52 is significantly greater compared to random (p=0.0008). Equally, there is evidence for allopatric speciation over the same time period, with the pattern tending to blur

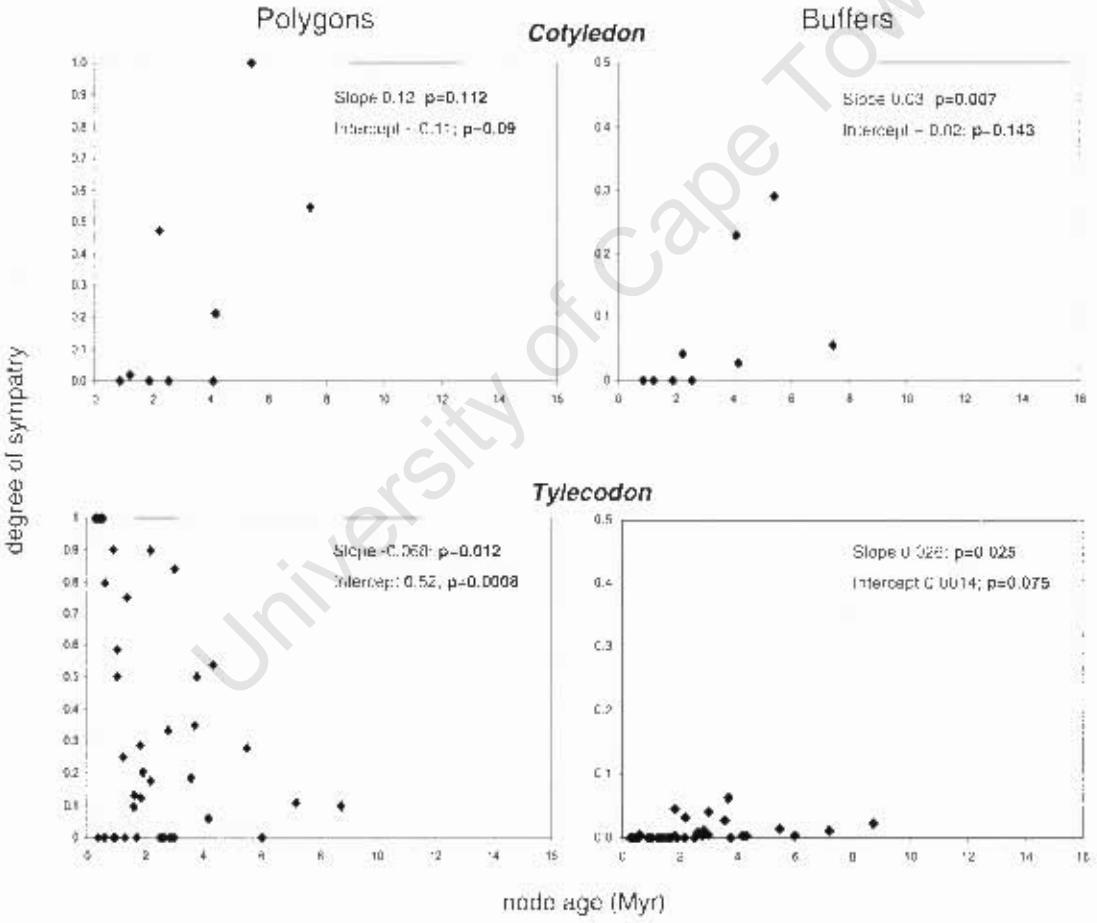

Figure 4.8. Age-range correlations in *Tylecodon* and *Cotyledon*, plotted as the degree of sympatry against node age.

with increasing node age, possibly due to range movements. At fine-scale (buffers) however, there is no evidence for sympatric speciation in *Tylecodon*. This pattern remains consistent through time, with only very low levels of sympatry across some nodes > 2 Myr old.

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Associations of vegetative plant size, inflorescences and flowers

In *Tylecodon* there is a significant relationship between vegetative plant size and inflorescence length (Fig. 4.9, p = 0.019) and in turn, flower size (floral tube length) is very strongly correlated with inflorescence height (Fig. 4.10, p = 0.0009). In contrast, these vegetative and floral characters show no association in *Cotyledon* (Figs 4.9 & 4.10).

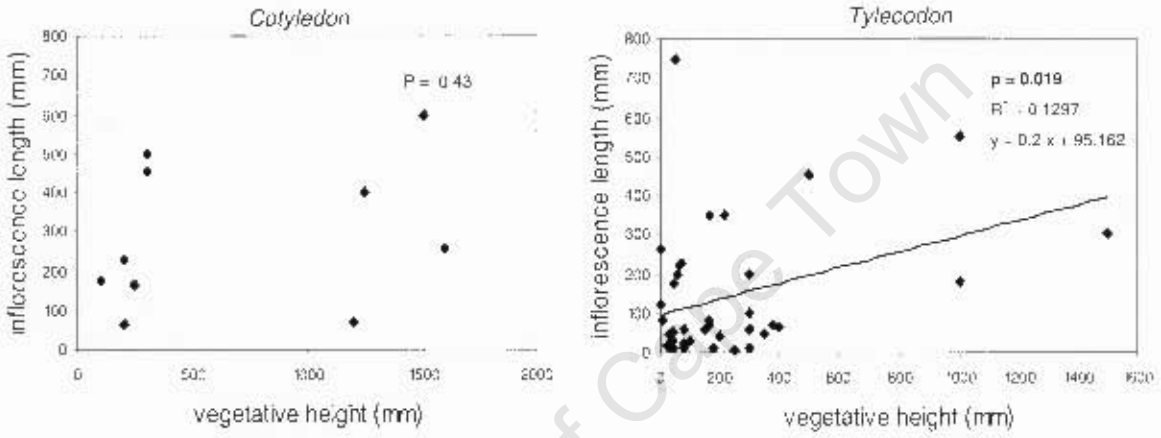

Figure 4.9. Linear regression-correlation of inflorescence length and vegetative height.

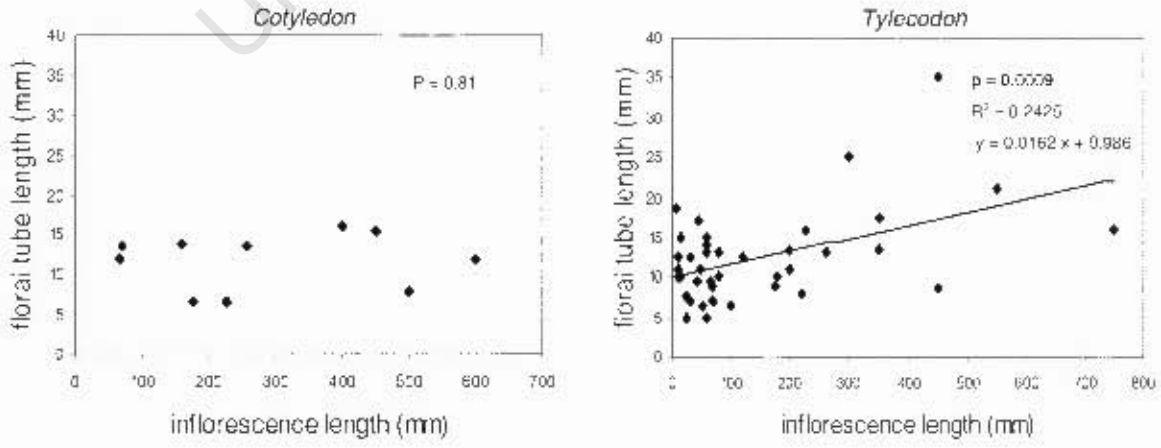

Figure 4.10. Linear regression-correlation of floral tube length on inflorescence length.

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DISCUSSION

Based on climatic niche characterisation presented here *Tylecodon* is a specialist of extremely arid areas that experience severe summer-drought but reliable winter rainfall. Specialisation comes in the form of morphological adaptations that bestow increased water-use-efficiency and avoidance of overheating. Such adaptations include deciduous leaves, development of a geophytic habit, pachycauls, and reductions in plant size. Coupled with seed traits associated with passive dispersal, the adaptations present in species of *Tylecodon* have led to the strong association between range size and plant size, such that small plants are effectively trapped in microhabitats and rarely colonise new areas. When colonisation does occur it is followed by differentiation through highly restricted gene flow. *Cotyledon* on the other hand is not as constrained by its environment. Inflorescence height is uncoupled from vegetative height and so species can disperse more broadly and are able to colonise new areas. The relative mobility of propagules in members of *Cotyledon* results in gene flow which sustains genetic integrity of species.

Thresholds of total annual rainfall, combined with its seasonality, demarcate the broad-scale distribution of *Tylecodon* and *Cotyledon*. Occupation of areas experiencing summer drought is derived in the group. The common ancestor of *Cotyledon* and *Tylecodon* is likely to have occurred in areas receiving between 230- and 300-mm of rainfall annually. Extant species of *Tylecodon* are tightly bound to areas experiencing summer drought and receiving less than 230 mm of rainfall per year (Fig. 4.3 & 4.4). Conversely, *Cotyledon* is more or less absent from these areas and is generally found in localities receiving > 360 mm of rainfall per year. The absence of thorough representation of species of *Kalanchoe* and its omission from trait reconstructions make deeper level interpretations within the subfamily tentative. Species of *Kalanchoe* are found in summer rainfall areas and, given that the winter rainfall system has only come into being during the last 12-10 Myr, summer or aseasonal rainfall is likely to be the ancestral climate of Kalanchoideae.

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The shift into arid environments in *Tylecodon* coincides with the evolution of leaf deciduousness and vegetative diminution in the genus. These morphological adaptations probably represent evolutionary novelties that enabled ancestral forms of *Tylecodon* to colonise new habitats created during the formation of the winter rainfall desert of the succulent karoo. Leaf deciduousness has arisen twice in members of Kalanchoideae examined here, both in *Tylecodon* and *Toelkenocodon* (Fig. 4.3). The 'all-cell succulent' type of leaves in these genera are cheap in terms of structural carbon inputs and can grow rapidly (Midgley & van der Heyden, 1999); they are, however, unable to withstand variations in turgor (von Willert et al., 1992) and so are not adapted to tolerate drought. Species of *Cotyledon* have xeromorphic-type leaves with thickened epidermal cells and epicuticular waxes that protect against excessive water loss and insolation during the summer months. Leaves of this kind maintain their shape during shrinkage through water loss by means of a specialised internal structure and so avoid damage (von Willert et al., 1992). Although it has been suggested that drought-deciduous species have temperature-sensitive leaves and respond to high temperatures by morphological changes such as abscission, rather than by physiological acclimation (e.g. Smith & Nobel, 1977), Wand et al. (2001) found no evidence for this in the succulent karoo species investigated. Unfortunately, plants were not subjected to water stress in her study and, given that high temperatures and water stress are rarely uncoupled in the succulent karoo, interpretation of the findings is limited. Leaf senescence in drought- versus seasonally-deciduous plants appears to have different triggers. In *Tylecodon paniculatus* leaf fall is controlled by photoperiod (Von Willert et al., 1992). As day-length increases leaf-water is reabsorbed by the stem and the leaves wither and die. This strategy ensures that leaves are lost prior to the hottest periods, so avoiding heat damage to the plant and excessive water loss via transpiration. The production of new leaves in *T. paniculatus* occurs prior to the winter rains and the water necessary for this growth is relocated from stem reserves (Von Willert et al., 1992). Storing water in the plant body in this way uncouples growth from extrinsic water availability enabling leaves to develop prior to the winter rains. Mild winter temperatures, unique to the succulent karoo winter rainfall desert, allow growth to continue during the wet season with very low risk of frost damage (Esler et al., 1999).

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Vegetative persistence appears to be favoured at the expense of dispersability in species of *Tylecodon*. Adaptations determining evolutionary processes in *Tylecodon* appear to mirror those hypothesised to have occurred in the geophytic clade of *Pelargonium* (Section Hoarea: Jones & Price, 1996, in Bakker et al., 2005). Tuber-fattening, lack of internode elongation, limited branching, and the evolution of deciduous leaves was linked to increased levels of aridity, and the concomitant reduction in carbon gain, enforced by these morphological traits, was purported to have led to lower seed production and dispersal, and consequently, to restricted gene flow in the group (Bakker et al., 2005). Minute seeds are common to both *Cotyledon* and *Tylecodon* (Van Jaarsveld & Koutnik, 2004) and so the strong positive correlations between height of reproductive and vegetative structures in the latter, that are not found in *Cotyledon,* suggest that allometric constraints may be limiting dispersal (Samson & Werk, 1986). In the absence of measures of relative allocation of biomass in species of the two genera, this interpretation is speculative. It has been demonstrated empirically however, that reproductive effort is often strongly correlated with plant size; a relationship which is linear to curvilinear, becoming asymptotic at group-dependent size thresholds (Reekie, 1999). That range size does not increase with increasing species age in *Tylecodon* is strong evidence for dispersal-restricted range expansion in the genus, while correlations between range size and the height of seed-release point to the mechanisms that determine this process. The frequent association of members of *Tylecodon* with rocky terrain is likely to exacerbate effects of constraints on reproductive effort, given that once seeds are released, the closer they are to the ground, the more likely they are to become trapped against nearby boulders and in crevices. Data relating species range sizes to the topography of their habitat would be a valuable component of comparative analyses that focussed on different range-size characteristics in closely related species.

The over-arching pattern of speciation in *Tylecodon* and *Cotyledon* is one of allopatry. Allopatric speciation is cited as the null hypothesis against which all other modes of speciation must be tested (Futuyama & Mayer, 1980). The underlying process is one of random drift and mutation (Gavrilets, 2003). Mayr was one of the longest standing advocates of allopatric speciation. He believed that the initiation of the speciation process could always be explained

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by geographic isolation, and ecological specialisation was only important in maintaining discontinuities that arose during physical separation of populations and incipient species (Mayr, 1942). A model of geographic isolation and the corollary of genetic processes are particularly germane to *Tylecodon* and *Cotyledon*.

Geographic modes of speciation inferred in this study are clearly influenced by the scale at which species' ranges are represented. Given the large disparity between methods for *Tylecodon* this factor warrants further interpretation. There are notable levels of sympatry (> 0.5) in recently diverged species of *Tylecodon* at coarse scale, but the pattern completely breaks down at a smaller scale. This may indicate finer partitioning of the landscape that is homogenised by polygons, or point to under-collecting in the genus. Species with low local abundances characteristic of many species of *Tylecodon*, tend to be recorded less frequently, irrespective of the number of sites in which they actually occur (Gaston, 1997). In addition to many of the species being small, plants are often tucked away in rock crevices, or camouflaged against a background of gravel and pebbles. *Tylecodon pygmaeus* and *T. scandens* are almost indistinguishable amongst the mosaic of quartz patches on the Knersvlakte in southern Namaqualand, South Africa. Their cryptic nature makes collecting a formidable task and it is likely that this results in their under-representation in distribution data. Differences can also be attributed to mapping compromises suggested by Lynch (1989). Shaded distribution maps and polygons that summarise distributions are the traditional methods used for investigating modes of speciation but may not reflect actual co-occurrence (Barraclough & Vogler, 2000). If for instance, ranges of species of *Tylecodon* interdigitate more than those of its sister due to features of the landscape in which they occur, sympatry will be greatly over-estimated when using polygons. Disparities found between ranges of *Tylecodon* that result from the methods of representation highlight the importance of considering fine-scale distribution patterns in addition to those generated using summary approaches (Barraclough and Vogler, 2000).

The evolutionary trajectory of *Tylecodon* differs greatly from that of its sister *Cotyledon*. Evidence presented here points to species of *Tylecodon* being specialists of the succulent

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karoo, adapted to survive harsh summer drought in microhabitats. The morphological adaptations that confer increased survival, indirectly constrain the movement of reproductive propagules. As seed dispersal is passive or via wind and raindrops, the effective distances of dispersal are a function of inflorescence height. Dispersal capacity may be further limited by the rocky terrain in which many of the plants occur. Seed mediated gene flow is therefore largely a local phenomenon, such that populations and incipient species are effectively caught in rarity 'traps' (Chown, 1997). Pollen movement is determined by the pollinating fauna which, based on inflorescence architecture, is limited to insects in all but the largest species of *Tylecodon*. This system of pollination is associated with shorter foraging distances (i.e. than vertebrate-effected pollination) which ensures that pollen transfer if effected over short distances. Populations of species of *Tylecodon* are then able to differentiate in allopatry, via drift and subsequent adaptation to microhabitats and competition for pollinators, ensuring morphological and phenological shifts between closely related taxa. Ultimately these mechanisms have led to the relatively high rates of speciation found in *Tylecodon*.

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SUMMARY

This study set out to produce a phylogenetic hypothesis for the southern African representatives of Kalanchoideae, and to test the current generic limits of *Tylecodon*, *Cotyledon* and *Adromischus*. Spatial and temporal aspects of diversification were explored in this phylogenetic context, and mechanisms by which the different patterns of diversity in the genera may have come into being were investigated.

Parsimony and Bayesian analyses of plastid and nuclear DNA sequence data recovered a well-resolved and highly supported hypothesis of intergeneric relationships for the members of Kalanchoideae sampled here, however, whether or not *Tylecodon*, *Cotyledon* and *Adromischus* represent a southern African clade remains open. Nuclear ITS data support this relationship, whereas plastid data recover an alternative arrangement in which *Adromischus* is sister to a clade comprising *Kalanchoe*, *Tylecodon* and *Cotyledon*. Morphological divergence, biogeography and polyploidy documented in species of *Kalanchoe* suggest that the group is sufficiently different from *Tylecodon*, *Cotyledon* and *Adromischus*. Thus uncertainty regarding the affinities of *Kalanchoe* to the remaining genera of Kalanchoideae does not compromise the validity of the relationships resolved in the three genera that are the focus of this study. Further investigations aimed at sampling the full gamut of variation in *Kalanchoe* are required to resolve these conflicts. Understanding of the evolutionary history of this large and variable group would be enhanced by cytogenetic studies that may identify possible candidates of ancient hybridisation(s). Generic monophyly concordant with morphology-based studies was confirmed for *Cotyledon* and *Adromischus*, however *Tylecodon* was found to be polyphyletic as molecular data recovered *Tylecodon racemosus* as sister to *Adromischus*. As a result of the high levels of genetic and morphological divergence of this taxon from *Adromischus*, it was elevated to the rank of genus (Chapter 2).

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Species-level relationships were only partially resolved in *Tylecodon* and *Adromischus*, while analyses recovered a fully resolved and well-supported phylogeny for *Cotyledon*. Further sampling within the varieties of *C. orbiculata*, together with additional DNA data is required to resolve conflicts between the findings of this study and that of Mort et al. (2005). Although some clades within *Tylecodon* and *Adromischus* were without structure, relationships across the phylogeny were sufficiently well resolved to identify geographically coherent clades in all three genera, together with interesting phenomena of flowering time shifts in *Tylecodon* and *Cotyledon*. Members of the most speciose clade of *Tylecodon*, found mainly in Namaqualand and the Richtersveld regions of South Africa, have apparently shifted flowering times from early to late summer: a possible strategy for reducing competition for pollinators. Flowering in *Cotyledon* occurs after the main rainfall season which varies across its range. In addition to broad patterns across genera, robust sister pairs were identified in all three genera and offer considerable opportunity for comparative analyses aimed at determining the relative importance of isolation and ecology in processes of divergence.

The divergent nature of *Toelkenocodon* revealed by molecular data is not reflected in its morphology. The taxon is virtually indistinguishable from members of *Tylecodon,* yet it shared its most recent common ancestor with *Adromischus*. The strength of the molecular evidence was sufficient to warrant the taxon being given the rank of genus, and the name *Toelkenocodon* was proposed (Chapter 2). The cryptic nature of any morphological distinction from members of *Tylecodon*, and the lack of shared similarities with *Adromischus,* required that putative synapomorphies for genera of Kalanchoideae be re-evaluated in order to identify characters that might be unique to *Toelkenocodon*. It was found that *Toelkenocodon* exhibits a unique combination of characters that distinguish it from others members of Kalanchoideae: plants are synanthous, and have deciduous leaves and a glabrous inner corolla, with no tuft of hairs where the filaments are connate with the corolla tube.

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A comparison of the effects of four dating techniques on branch length transformation formed a large component of Chapter 3. Nodes were dated using a global molecular clock and two methods that accommodate rate heterogeneity among branches by assuming autocorrelation of rates between ancestor and descendant branches: NPRS and Bayesian-based Multidivtime estimation. A relaxed phylogenetics approach, which simultaneously estimates phylogeny and divergence times, implemented in BEAST, was also applied to these data. Both methods that invoke autocorrelation estimated ages older than those of the clock and BEAST, with the effect being most pronounced towards the tips of chronograms. Clock-like behaviour was rejected for these data, and node ages estimated using BEAST were selected as the most meaningful for interpretation, primarily because the technique does not assume autocorrelation.

The timing of diversification in *Tylecodon*, *Cotyledon* and *Adromischus* was found to be coincident with major climatic shifts known to have affected the south-west of southern Africa during the late Miocene and Pliocene. Genera diverged around 12 – 10 Mya, coincident with the Benguela upwelling and initiation of a period of marked aridity. *Toelkenocodon* diverged somewhat earlier at around 14.8 Mya. The majority of lineage-splitting within genera occurred after the true Mediterranean climate was established in the region, with most species being less than 5 Myr old. Lineages-through-time plots illustrated a more or less linear process throughout the history of *Tylecodon*, *Cotyledon* and *Adromischus*. However, *Tylecodon* has diversified at a higher rate than the other two genera. A shift to succulent karoo vegetation occurred in *Tylecodon* between 5.5 and 3.2 Mya in what is currently the most species rich clade in the genus. The consensus from other groups is largely concordant with the findings in Kalanchoideae. Most species or clades entered the succulent karoo during the last 5 Myr, suggesting that this vegetation-type is considerably more recent than the neighbouring fynbos vegetation. Some groups exhibited a shift to succulent karoo as early as 17 Mya. The antiquity of these shifts may result from the use of stem node ages, or the possible inaccuracy of available calibration dates.

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The final component of this thesis explored the spatial dynamics of diversification in *Cotyledon* and *Tylecodon* and their relationship to traits associated with adaptation to an extremely arid environment. Mechanisms by which *Tylecodon* might have diversified substantially faster than its sister, *Cotyledon* were investigated. Analyses of range size characteristics of *Tylecodon* and *Cotyledon* demonstrated that *Tylecodon* has significantly smaller ranges than *Cotyledon*. The hypothesis that range size is determined primarily by plant size and the height at which seeds are released (calculated as the sum of vegetative height and inflorescence height) was tested. These variables were found to be positively correlated in *Tylecodon*, but not in *Cotyledon*. Additional correlation-regression analyses revealed significant associations between the height of vegetative organs, inflorescences and the size of flowers in *Tylecodon*. Such associations were not found in *Cotyledon*. Thus it was proposed that the proclivity of species with small ranges in *Tylecodon* is the result of limited dispersal. Age-range correlations revealed that the predominant mode of speciation in both *Tylecodon* and *Cotyledon* is allopatric.

The hypothesis that *Cotyledon* and *Tylecodon* occupy different climatic niches was tested using discriminant function analysis. The genera differ in their tolerances of summer drought and potential evaporation. Species of *Tylecodon* occupy niches concentrated at the arid extremes that experience summer drought, whereas *Cotyledon* is largely absent from these areas. The shift into more extreme arid environments in *Tylecodon* coincides with the emergence of morphological characters such as leaf deciduousness, development of a pachycaulous habit, subterranean storage organs and reduced vegetative plant size – characters that may represent evolutionary novelties that enabled ancestral forms of *Tylecodon* to colonise new habitats created during the formation of the winter rainfall desert of the succulent karoo, approximately 5 Mya. Short dispersal distances coupled with the morphological adaptations exhibited by species of *Tylecodon* are hypothesised to have resulted in the strong association between range size and plant height, with smaller plants having smaller ranges, such that they are confined to microhabitats. Effective isolation of populations enables differentiation to occur as gene flow is highly restricted. The increased stature of plants of *Cotyledon* and the fact that inflorescence

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height is uncoupled from vegetative height, results in the relative mobility of propagules of members of the genus. Species can disperse more broadly with gene flow sustaining the genetic integrity of species. The effects of pollinator-mediated gene flow are likely to reinforce dispersal-mediated gene flow, given that genera exhibit divergence in pollination syndromes.

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APPENDIX 1

N.B. This manuscript, submitted to Molecular Phylogenetics and Evolution, is an extension of the ideas and work presented in Chapter 3 of this thesis. T.L. Nowell and G.A. Verboom are joint-principal authors.

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Origin and diversification of the Greater Cape flora: Ancient species repository, hot-bed of recent radiation, or both?

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ABSTRACT

Like island-endemic taxa, whose origins are expected to antedate the appearance of the islands on which they occur, biome-endemic taxa should be younger than the biomes to which they are endemic. Accordingly, the ages of biome-endemic lineages may offer insights into biome history. In this study, we used the ages of multiple lineages to explore the origin and diversification of two southern African biomes whose remarkable floristic richness and endemism has identified them as global biodiversity hotspots (succulent karoo and fynbos). We used parsimony optimization to identify succulent karoo- and fynbos-endemic lineages across 17 groups of plants, for which dated phylogenies had been inferred using a relaxed Bayesian (BEAST) approach. All succulent karoo-endemic lineages were less than 17.5 My old, the majority being younger than 10 My. This is largely consistent with suggestions that this biome is the product of recent radiation, probably triggered by climatic deterioration since the late Miocene. In contrast, fynbos-endemic lineages showed a broader age distribution, with some lineages originating in the Oligocene, but most being more recent. Also, in groups having both succulent karoo- and fynbos-endemic lineages, there was a tendency for the latter to be older. These patterns reflect the greater antiquity of fynbos, but also indicate considerable recent speciation, probably through a combination of climatically-induced refugium fragmentation and adaptive radiation.

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1. Introduction

The high species richness and specific and generic endemism of the Cape flora have been well documented (Goldblatt and Manning, 2000), the area being identified as a global biodiversity hotspot (Myers et al., 2000). The historical events underlying the genesis of this diversity, as well as the time frame over which it occurred, have been the subject of considerable discussion in the literature (e.g. Adamson, 1958; Levyns, 1964; Axelrod and Raven, 1978; Linder et al., 1992; Linder, 2003, 2005; Cowling et al., this volume). Margaret Levyns (1964) was the first to suggest that the remarkable plant species diversity of the western half of the Cape Floristic Region (CFR, Goldblatt and Manning, 2000) was the result of elevated speciation following the onset of arid climates

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in the area. The strongly winter-rainfall, summer-arid climate that characterises much of the region today, is thought to have been initiated around the end of the Miocene, as a consequence of changes in the extent of Antarctic glaciation (Zachos et al., 2001), sea surface temperatures (Siesser, 1980; Zachos et al., 2001) and the strength and position of high-pressure cells over the southern oceans (Siesser, 1980; Dieckmann et al., 2003; Linder, 2003). This may have led to widespread extinction in the flora at the time, opening an array of empty niches into which lineages that were pre-adapted to survive summer aridity were able to diversify. Whilst this version of events has been reiterated by several subsequent workers (e.g. Linder et al., 1992; Goldblatt and Manning, 2000) and supported by molecular dating studies demonstrating end-Miocene radiations in certain lineages (Richardson et al., 2001; Verboom et al., 2003), there are indications that the full picture is more complex. First, new molecular dating studies suggest that several Cape lineages originated and started to diversify well before the end-Miocene (Linder and Hardy, 2004; Bakker et al., 2005; Linder, 2005), when climates were presumably moister and more aseasonal than they are at present. Second, whilst the western-CFR undoubtedly receives most of its rain during winter, the intensity of summer drought varies substantially in space, suggesting differential selection for an arid-adapted flora. For example, moisture carried by the summer south-easterlies ensures that high-altitude environments throughout the CFR experience reduced summer moisture deficits (Deacon et al., 1992), and the latter may consequently act as refugia for moisture-loving species. It is precisely these environments that support the greatest fynbos plant species richness as well as the highest concentrations of local endemics, a pattern that may partly be a result of reduced extinction in the past (Cowling and Lombard, 2002). It is also in these environments that most of the region's palaeoendemic taxa occur (Linder et al. 1992).

Elucidating the origins and diversification of the Cape flora has not been helped by a failure to distinguish it from the adjacent succulent karoo flora. Linder (2003) pointed out that several of the "Cape floral clades", lineages which originated in the CFR and have experienced most of their evolution there, are most difficult to separate from the lineages that occur along the arid west coast and in the succulent karoo. Indeed, many of the Cape clades (e.g. *Ehrharta*, Relhaniinae, Arctoteae, Heliophileae, *Pelargonium*, *Lampranthus*) contain large numbers of species which are native and, in some cases endemic, to the succulent karoo. Consequently, some authors have advocated a broader concept of the Cape flora (Greater Cape Floristic Region, GCFR: Jürgens, 1991; Born et al., 2006) embracing both fynbos and succulent karoo biomes. The floristic affinity between the fynbos and succulent karoo is perhaps unsurprising since these biomes share a predominantly winter-rainfall regime. Nonetheless, the physiognomies and ecologies of their floras reflect contrasting climatic experiences, the succulent karoo being drier overall and experiencing more acute summer aridity (Milton et al., 1997; Cowling, 1998; Mucina and Rutherford, 2006). Consequently, whereas the fynbos biome is dominated by heathy, evergreen shrubs, the succulent karoo flora reflects a diversity of adaptations for surviving extreme seasonal drought. Dominant life forms include annuals, geophytes and succulents (Milton et al., 1997; Cowling, 1998). In contrast to the fynbos biome which comprises a mixture of young and ancient lineages (Linder, 2005), possibly due to the presence of long-term climatic refugia, the succulent karoo biome is probably of recent origin (Scott et al., 1997), its appearance being tied to the appearance of acutely summer-arid climates in the late Miocene. Accordingly, we predict that the age distributions of fynbos- and succulent karoo-endemic lineages should differ, with succulent karoo specialists consistently being of late Miocene age or younger, and fynbos lineages showing a broader range of ages. Also, excepting plant groups which entered the GCFR recently, lineages having both fynbos and succulent karoo specialists should show a tendency for their fynbos elements to be older. Existing studies provide some support for these predictions. For example, the ruschioid Aizoaceae, the dominant succulent component of the succulent karoo flora, originated ca. 3.8–8.7 Mya, whence it has given rise to over 1500 species (Klak et al., 2004). In *Pelargonium*, the age of the succulent-karoo centred "xerophytic clade" is inferred to be somewhat older (18 My), but it is nested within a broader, fynbos-centred winter-rainfall clade dated at about 22 My (Bakker et al., 2005). A similar pattern is apparent in *Ehrharta*, whose late-Miocene radiation in the succulent karoo appears to have emerged from within an older fynbos clade (Verboom et al., 2003).

In this paper, we test the predictions set out above by comparing the ages of fynbos- and succulent karoo-endemic lineages across 17 groups of plants for which molecular sequence data were available. Our general approach involves: (i) inferring a topology and dating all the nodes using a single analytical method, (ii) using ancestral character state reconstruction to infer which lineages are endemic to either biome, and (iii) to determine and compare the ages of these lineages. The logic underlying the use of biome-endemic lineages as proxies for estimating the ages of the biomes themselves is straightforward: presumably, a lineage endemic to a particular biome did not exist prior to the appearance of that biome.

2. Materials and methods

2.1. Taxon sampling

The 17 plant groups included in this study (Table 1) were selected on the basis of (i) the availability of appropriate DNA sequence alignments, (ii) reasonably dense species sampling, and (iii) the inclusion of one or more species endemic to the succulent karoo or fynbos biomes sensu Mucina and Rutherford (2006). Twelve of the data sets have already been published or are currently in press, whilst the remainder are close to publication. The lineages sampled are evenly balanced between monocots ($n = 9$) and eudicots ($n = 8$), the latter comprising mostly rosids ($n = 6$).

2.2. Phylogeny inference and molecular dating

Prior to dating analysis, taxa reflecting conflict amongst markers were pared from each data set. Overall, there were three such cases: (i) *Tribolium pusillum* was removed from the *Tribolium* data set (nuclear ribosomal ITS vs *trnL–trnF* conflict: Verboom et al., 2006), (ii) *Zaluzianskya mirabilis* was removed from the *Zaluzianskya* data set (nuclear ribosomal ITS vs plastid conflict: Archibald et al., 2005), and (iii) a clade of seven species (*Jamesbrittenia burkeana*, *Jamesbrittenia crassicaulis*, *Jamesbrittenia jurassica*, *Jamesbrittenia pristisepala*, *Jamesbrittenia silenoides*, and *Jamesbrittenia stricta*) was removed from the *Jamesbrittenia* data set (nuclear GScp vs plastid conflict: G.A. Verboom, unpublished data).

In order to ensure that node ages obtained from analyses of the various data sets were maximally comparable, an identical analytical procedure was applied throughout. All data sets were analysed using a relaxed Bayesian approach as implemented in BEAST version 1.4.6 (Drummond and Rambaut, 2007). We favoured BEAST over nonparametric rate smoothing (NPRS, Sanderson, 1997), penalised likelihood (PL, Sanderson, 2002) or the relaxed Bayesian approach as implemented in Multidivtime (Thorne and Kishino, 2002) because, unlike these other methods, BEAST does not assume rate autocorrelation which may systematically distort branch lengths, reducing the ratio of deep to shallow nodes (Hugall and Lee, 2004; Martin et al., 2004). Instead, BEAST accommodates among-branch rate variation by allowing each

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Table 1
Phylogenetic data sets included in this study, indicating source publications and DNA loci used

Lineage	Data source	DNA loci	Calibration node	Age prior (mean) (My)	Calibration reference	Age posterior (mean) (My)
Ehrharta	Verboom et al. (2003)	*trn*L–*trn*F, ITS	Divergence between *Microlaena stipoides–Tetrarrhena* and *Zotovia–Ehrharta* clades	40	Bouchenak-Khelladi (2007)	41.9
Haemantheae	F. Conrad (unpublished data)	*trn*L–*trn*F, rps16, *psb*A–*trn*H	Crown node of Haemantheae	6	Forest et al. (2007b)	5.9
Heliophileae	Mummenhoff et al. (2005)	*trn*L–*trn*F, ITS	Divergence between Cleome and rest of Brassicaceae	21	Wikström et al. (2001)	22.6
Isolepis–Ficinia	M. Muasya (unpublished data)	*rps*16, ITS	Divergence between Hypolytreae and rest of Cyperaceae	44	Bremer, 2002	39.4
Jamesbrittenia	G.A. Verboom (unpublished data)	*rps*16, *psb*A–*trn*H, GScp	Crown node of Manuleeae	10	Forest et al. (2007b)	10.2
Melianthus	Linder et al. (2006)	*trn*L–*trn*F, *psb*A–*trn*H, ITS	Divergence between Greyiaceae and Melianthaceae	63	Wikström et al. (2001)	60.3
Moraea	Goldblatt et al. (2002)	*rbc*L, *trn*L–*trn*F, *rps*16	Divergence between *Ferraria* and *Moraea–Homeria* clade	25.5	Goldblatt et al. (2002)	26.3
Muraltia	Forest et al. (in press)	*trn*L–*trn*F, *atp*B–*rbc*L, ITS	Divergence between *Muraltia* and *Polygala*	18	Forest et al. (2007b)	18.5
Oxalis	K. Oberlander (unpublished data)	ITS, *trn*L–*trn*F, *trn*S–*trn*G	Divergence between *Oxalis* and *Averrhoa*	47	Wikström et al. (2001)	47
Pelargonium	Bakker et al. (2004, 2005)	*trn*L–*trn*F, ITS	Divergence between *Geranium* and *Pelargonium*	42.5	Wikström et al. (2001)	43
Pentaschistis	Galley and Linder, 2007	*trn*L–*trn*F, *trn*T–*trn*L, *atp*B–*rbc*L, rpl16, *trn*D–*psb*A	Crown node of Danthonieae	14	Bouchenak-Khelladi (2007)	13.9
Restionaceae	Linder et al. (2003)	rbcL, trnL–trnF	Divergence between Anarthriaceae and Restionaceae	91	Bremer, 2002	91.5
Satyrium	Van der Niet et al. (in press)	*mat*K, *trn*K intron, *trn*S–*trn*G, *trn*L–*trn*F	Divergence between Disperis and Satyrium	27.8	This study[a]	31.2
Schoeneae	Verboom (2006)	*rbc*L, *trn*L–*trn*F, *rps*16	Divergence between Hypolytreae and rest of Cyperaceae	44	Bremer, 2002	46
Tribolium	Verboom et al. (2006)	*trn*L–*trn*F, ITS	Crown node of Danthonieae	14	Bouchenak-Khelladi (2007)	14.3
Zaluzianskya	Archibald et al. (2005)	*rpl*16, *trn*L–*trn*F, ITS	Stem node of Manuleae	10	Forest et al. (2007b)	9.56
Zygophyllum	D. Bellstedt (unpublished data)	*rbc*L, *trn*L–*trn*F	Divergence between Zygophylloideae and Larrioideae	38.2	This study[b]	38.2

Details pertaining to calibration of the BEAST analyses are also provided.

[a] Calibration of the *Satyrium* phylogeny necessitated that the crown node of *Satyrium* first be dated via a higher-level (Orchidaceae-wide) dating analysis. For this purpose, we conducted a BEAST analysis on the combined *rbc*L + *mat*K data set used by Ramírez et al. (2007), to which we added seven species of *Satyrium*, thereby ensuring that the crown node of the *Satyrium* was sampled. This analysis was set up in an identical manner to other BEAST analyses run for this paper (see Section 2) except that calibration priors were applied to two nodes, using fossil data described by Ramírez et al. (2007): (i) a lognormal prior, with offset = 93.0 My, mean = 2.60 My and standard deviation = 1.0 My, was applied to the crown node of Orchidaceae. (ii) A lognormal prior, with offset = 15.0 My, mean = 0.96 My and standard deviation = 1.0 My, was applied to the crown node of Goodyerinae. Lognormal priors were used to reflect the fact that fossils indicate minimum ages of the lineages they represent.

[b] Calibration of the *Zygophyllum* phylogeny necessitated that the stem node of Zygophylloideae first be dated via a higher-level dating analysis. For this purpose, a parsimony search, done using PAUP version 4.0b10 (Swofford, 2002), was used to produce an *rbc*L phylogeny for 103 species comprising (for source sequence data, see Electronic supplementary material, Appendix A): *Gingko biloba*, 97 species representing all angiosperm families found in the GCFR, one species from Krameriaceae, two representatives of Zygophyllaceae subfamily Larrioideae (sister to Zygophylloideae), and two *Zygophyllum* species. Branch lengths on this tree were then estimated using maximum likelihood (PAUP), under an appropriate sequence evolution model (selected using Modeltest version 3.06: Posada and Crandall, 1998). Following rejection of a clock (df = 99, $P < 0.01$), a chronogram was produced using penalized likelihood (Sanderson, 2002), as implemented in r8s version 1.71 (Sanderson, 2006), with a smoothing parameter (λ) of 316.22 (determined by cross validation). The following references were used to calibrate the tree: the first occurrence of tricolpate pollen at 125 Mya (Anderson et al., 2005), set as a fixed age; the occurrence of African Restionaceae at 61 Mya (Linder et al., 2003), set as a minimum age constraint; the occurrence of genistoid legumes at 56 Mya (Lavin et al., 2006), set as a minimum age constraint; and the inferred age of *Phylica* 12 Mya (Richardson et al., 2001), set as a maximum age constraint.

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branch to draw its rate from a discretized lognormal distribution, whose shape is estimated as part of the analysis (Drummond et al., 2006).

In contrast to other dating methods, BEAST simultaneously estimates topology along with the node ages, allowing sequence divergences to inform topology estimation (Drummond et al., 2006). All BEAST analyses were run in the absence of topological constraints except where these were necessary to ensure resolution of the calibration node. Since most data sets comprised multiple loci (Table 1), mixed models were used, with a separate model applied to each locus. Molecular evolution model parameters used flat priors, whilst tree priors were modeled according to a Yule speciation process. All analyses were calibrated by setting an age prior on a single node for which an age estimate was available from a higher-level dating analysis (Table 1). Whilst indirect calibrations of this type have been vigorously criticised because they compound error (Graur and Martin, 2004; Hedges and Kumar, 2004), they usually represent the only point of reference where appropriate fossils do not exist. Since this was true for most of the groups included in this study, we decided to employ indirect calibrations throughout, in order to ensure that the error associated with each of our node age estimates was of a uniform nature. In all cases, node age priors were modeled as a normal distribution having its mean equal to the node age suggested by the higher-level dating analysis and a 95% confidence interval arbitrarily defined as (mean age - [0.2 x mean age], mean age+[0.2 x mean age]).

Posterior distributions for each parameter were obtaining using a Monte Carlo Markov Chain (MCMC) which was run for 10–20 million generations, and sampled every 1000th generation. Inspection of the results using Tracer version 1.3 (Rambaut and Drummond, 2006) confirmed that stationarity was achieved in all cases and that sample sizes were adequate. Trees were summarized as maximum clade credibility trees using the TreeAnnotator program which forms part of the BEAST package, and visualized using FigTree version 1.0 (Rambaut, 2006). In each case, the first 30% of samples was discarded to avoid sampling the burn-in phase.

2.3. Reconstruction of biome endemism

In order to identify which lineages were ancestrally endemic to the succulent karoo or fynbos biomes (hereafter termed succulent karoo- and fynbos-endemic lineages), each species placed within the ingroup in each tree was scored for three characters: (i) species present (1) or absent (0) in the succulent karoo biome, (ii) species present (1) or absent (0) in the fynbos biome, and (iii) species present (1) or absent (0) elsewhere (i.e. outside the fynbos or succulent karoo). Biome definitions followed Mucina and Rutherford (2006), and scoring was based on field observations as well as habitat and distribution descriptions reported in the literature (Marais, 1970; Goldblatt, 1977, 1979, 1981, 1986, 1987; Van der Walt 1981; Van der Walt and Vorster, 1981, 1988; Snijman, 1984; Gibbs-Russell et al., 1990; Hilliard 1994; Linder and Ellis, 1990; Dlamini, 1999; Linder and Davidse 1997; Linder and Kurzweil, 1999; Rourke, 2002; Germishuizen and Meyer, 2003; Galley and Linder, 2006). Based on this scoring system, each species had one of seven possible states for the three-character combination (100, 010, 001, 110, 101, 011, 111), with succulent karoo- and fynbos-endemics being represented as "100" and "010", respectively. In order to allow for polymorphisms at ancestral nodes and to avoid all-zero reconstructions, these combinations were then converted to a single seven-state "polymorphism" character and reconstructed in Mesquite version 2.0 (Maddison and Maddison, 2007) using parsimony-based Sankoff optimization as recommended by Hardy and Linder (2005). The state transition costs were defined by a stepwise matrix identical to that shown in Hardy and Linder's (2005) Fig. 2.

2.4. Age comparison of succulent karoo- and fynbos-endemic lineages

Frequency distributions of stem node ages were generated for the full sets of succulent karoo- and fynbos-endemic lineages across all 17 data sets. We did not consider crown node ages as many of the biome-endemic lineages were single species and so lacked meaningful crown nodes. Since the resulting lineage age distributions deviated substantially from normality, comparison of the ages of succulent karoo- and fynbos-endemic lineages was done using Mann–Whitney's U test. To test whether trees containing both succulent karoo- and fynbos-endemic lineages consistently revealed the fynbos-endemic lineages to be older, the oldest succulent karoo- and fynbos-endemic lineages from each data set were compared in a pairwise manner, using Wilcoxon matched pairs test. This test has the strength that, because comparisons are drawn within each individual tree, it is partly robust to calibration error. Finally, although we had expected the oldest fynbos-endemic lineage in any given tree generally to antedate the oldest succulent karoo-endemic lineage, this expectation really only applies to groups that entered the GCFR (i.e. fynbos plus succulent karoo biomes) prior to the genesis of the succulent karoo. Therefore, for all trees containing both succulent karoo- and fynbos-endemic lineages, we subtracted the age of the oldest succulent karoo-endemic lineage from that of the oldest fynbos-endemic lineage, and plotted this difference against the age of the oldest GCFR-endemic lineage in that tree. The resulting relationship was evaluated using linear correlation. All statistical tests described in this section were conducted in STATISTICA version 7.0 (Statsoft Inc., 2004).

3. Results

All of the phylogenetic trees obtained using BEAST were topologically similar to those previously produced using other analytical methods and reported in the literature. Also, in all cases the posterior age estimate on the calibration node closely matched the age prior (Table 1). Amongst the various data sets, the ingroup stem node ages varied considerably, ranging from 1.6 My in Heliophileae to 91.5 My in Restionaceae + Anarthriaceae (Figs. 1 and 2).

All of the groups surveyed contained at least one fynbos-endemic lineage, whereas four (Restionaceae, *Satyrium*, Schoeneae, *Zygophyllum*) did not contain any succulent karoo-endemic lineages. Reconstructions on some nodes were equivocal and in some cases the way these were resolved dictated the number of succulent karoo- and/or fynbos-endemic lineages present, as well as their ages. For the purpose of this paper, we treated these nodes as uncertain and thus not indicative of biome-endemism. This had the effect of favouring multiple, younger, biome-endemic lineages over somewhat fewer, older such lineages (DELTRAN-like). We recognise that where an interpretation of "succulent karoo-endemic" or "fynbos-endemic" was possible on a set of equivocally resolved branches, this often meant that such endemism could be interpreted as arising further back in time. Generally, however, the temporal discrepancy was small, usually being less than 2 My and in one case 3.2 My. Larger discrepancies were evident in three instances. First, the branch (Fig. 1, node marked X) subtending the winter rainfall *Pelargonium* clade (*sensu* Bakker et al., 2005) was resolved as either fynbos- or succulent karoo-endemic. Depending on how this is resolved, the age of the oldest fynbos- or succulent karoo-endemic lineage in *Pelargonium* can be pushed back to 26.2 My. Second, several of the deeper nodes in *Satyrium* were resolved as either fynbos-endemic or as occurring outside the GCFR (Fig. 1, node marked Y). Under the first interpretation, the age of the oldest fynbos-endemic lineage in *Satyrium* is pushed back to 25.8 My. Finally, in *Ehrharta* an equivocal reconstruction (succulent karoo-

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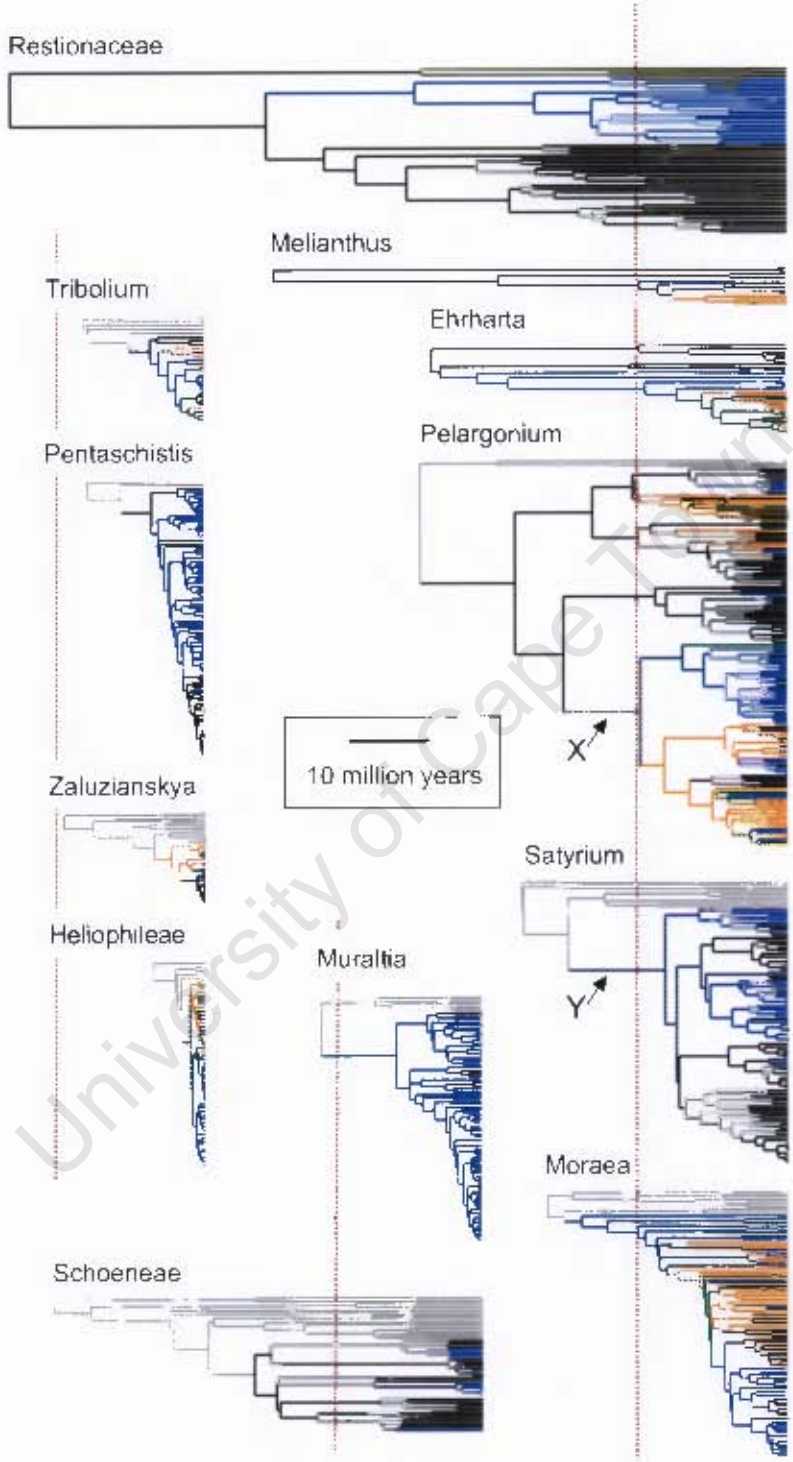

Fig. 1. BEAST chronograms for the 12 published data sets included in this study, with branches coloured to reflect reconstructions of biome-endemism: yellow, succulent karoo-endemic; blue, fynbos-endemic; green, GCFR-endemic; black, not endemic to the GCFR; grey, not included in reconstruction. Mixed-colour lines indicate equivocal reconstructions. The stippled red line passing vertically through each tree reflects the 17.36 Mya level, the estimated stem node age of the oldest succulent karoo-endemic lineage.

endemic or GCFR-endemic) along the spine of the *Ehrharta longiflora*–*Ehrharta triandra* clade may push succulent karoo-endemism in this clade back to 6.4 My.

Treating equivocal reconstructions as not indicative of biome-endemism, we identified 55 succulent karoo-endemic lineages and 41 fynbos-endemic lineages across all 17 data sets. The age distributions of both showed a predominance of lineages less than 20 My old (Fig. 2), although three fynbos lineages were substantially older (*Ehrharta*, 40.9 My; *Moraea* 26.3 My; African Restionaceae, 61.3 My). Only six succulent karoo-endemic lineages were

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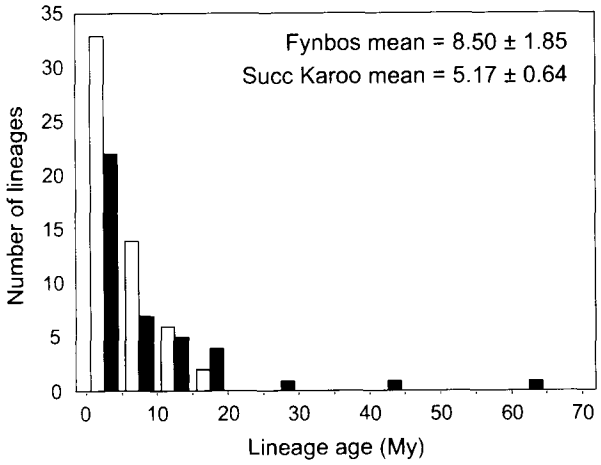

Fig. 2. Stem node age distributions of succulent karoo- (open bars) and fynbos-endemic (solid bars) lineages, obtained when equivocal reconstructions are treated as not indicative of biome-endemism. For each lineage type, the mean stem node age±standard error is indicated.

older than 10 My, the oldest of these being dated at 17.36 My. On average, the succulent karoo-endemic lineages were younger (Fig. 2), although the difference was not significant ($Z = 0.755$, $P = 0.45$). Eight of the 13 groups that contained both succulent karoo- and fynbos-endemic lineages (*Ehrharta*, *Isolepis-Ficinia*, *Melianthus*, *Moraea*, *Muraltia*, *Pentaschistis*, *Oxalis*, and *Tribolium*), had a single fynbos-endemic lineage which was older than all of the succulent karoo lineages included (Fig. 3), the latter commonly being nested within the former. These patterns were robust to alternative resolutions at equivocal nodes. The remaining groups (Haemantheae, Heliophileae, *Jamesbrittenia*, *Pelargonium*, *Zaluzianskya*) had an older succulent karoo-endemic lineage, although in *Pelargonium* an older fynbos lineage was possible if the branch subtending the winter-rainfall clade was (Fig. 1, node marked X) interpreted as fynbos-endemic, as suggested by Bakker et al. (2005). Considered over all 13 groups, therefore, there was a tendency for the fynbos-endemic lineages to be older than the oldest succulent karoo-endemic lineages, although this was significant only at the $\alpha = 0.1$ level ($Z = 1.852$, $P = 0.06$). This trend was strengthened when the winter-rainfall *Pelargonium* clade was assumed to have a fynbos origin ($Z = 2.341$, $P = 0.019$). Interestingly, with the exception of *Pelargonium*, all the groups that countered this trend lacked evidence of a deep evolutionary history in the GCFR, the ages of their oldest GCFR-endemic clades being 5.22 My or less. Thus, the difference between the age of oldest fynbos-endemic lineage and that of the oldest succulent karoo-endemic lineage showed a tight, positive relationship with the first time of appearance of a lineage endemic to the GCFR (Fig. 4). Again, this relationship is strengthened if a fynbos origin is assumed for the winter-rainfall *Pelargonium* clade ($R^2 = 0.814$, $P < 0.0001$).

4. Discussion

The patterns presented in this paper suggest that age estimates of biome-endemic lineages can provide useful insights into biome history. All lineages identified as ancestrally endemic to the succulent karoo were younger than 17.5 My, the vast majority being less than 10 My old. This is consistent with a mid- to late-Miocene origin for this biome. In contrast, at least a few fynbos-endemic lineages extend much further back in time, suggesting a much deeper history for the fynbos. This is corroborated by a consistent tendency for groups with a deep history in the GCFR to produce fynbos-endemic lineages before they give rise to succulent karoo specialists. We argue that the fynbos and succulent karoo floras have rather different diversificational histories, and that this distinction is important when exploring the origins of the Greater Cape flora.

Our failure to identify any succulent karoo-endemic lineages older than 17.5 My supports Levyns (1964) interpretation of the succulent karoo as a comparatively young biome. Most likely, the appearance of the succulent karoo was associated with increased aridification and the establishment of a pronouncedly summer-arid climate along the west coast of southern Africa. Since this is thought to have started about 14 to 10 Mya (Siesser, 1980; Dieckmann et al., 2003; Linder 2003), however, our identification of a few succulent karoo-endemic lineages dating back to the early- or mid-Miocene is at odds with current palaeoclimatic reconstructions. Prior to this time, climates are thought to have been warmer and moister, supporting a dry, subtropical, woodland flora (Coetzee, 1978, 1983). Besides possible errors associated with chronology and interpretation of palaeoenvironmental records, we can think of at least three factors that may explain this inconsistency: (i) error associated with chronogram calibration and branch length estimation, (ii) our use of stem node rather than crown node ages, and (iii) the possibility that apparent succulent karoo-endemic lineages initially inhabited nonsucculent karoo vegetation.

In running the dating analyses for this paper, we purposefully employed a single calibration and branch length estimation procedure in order to ensure that the results were maximally comparable across lineages. Whilst this approach may minimize systematic biases, we readily acknowledge that there remains substantial scope for error. For example, in the case of all succulent karoo-endemic nodes having a mean age greater than 10 My, the 95% confidence intervals were broad enough to allow for the possibility that these nodes were less than 10 My old (Appendix A), in many cases substantially so. Thus we cannot exclude the possibility that these lineages are actually much younger than our analyses suggest. There is also considerable room for error in the inference of ancestral habitats, as well as our interpretation of precisely at which node habitat switches occurred. As noted by Frumhoff and Reeves (1994), we can never be entirely certain that the progenitors of an apparently succulent karoo-endemic lineage did not evolve in a different habitat, our inference of a succulent karoo-endemic ancestor being an artefact of the reconstruction method. Also, since we consistently associated habitat shifts with stem rather than crown nodes, the timing of such shifts would be pushed backwards, potentially making them appear older than they really are.

Notwithstanding the slight mismatch between palaeoclimatic reconstructions and the inferred ages of the oldest succulent karoo-endemic lineages, a total absence of succulent karoo-endemic lineages older than 17.5 My identifies this flora as being relatively young, its appearance and diversification probably being stimulated by adaptation to the summer-arid "adaptive zone" *sensu* Simpson (1953) that appeared in the late Miocene. The high richness and endemism of the succulent karoo flora (Cowling, 1998), then, identifies this biome as an arena or hot-bed of recent and relatively rapid radiation, probably following a classic Simpson (1953) adaptive radiation model. This is most spectacularly illustrated by the ruschioid Aizoaceae (not included in this study) which are suggested to have given rise to more than 1500 species inside the last 3.8–8.7 My (Klak et al., 2004), at least some of this speciation being the result of adaptation along habitat gradients (Ellis and Weis, 2005; Ellis et al., 2006).

The consistent youth of succulent karoo-endemic lineages contrasts with the pattern in fynbos, for which we identified five ancestrally-endemic lineages older than 17 My, three of these

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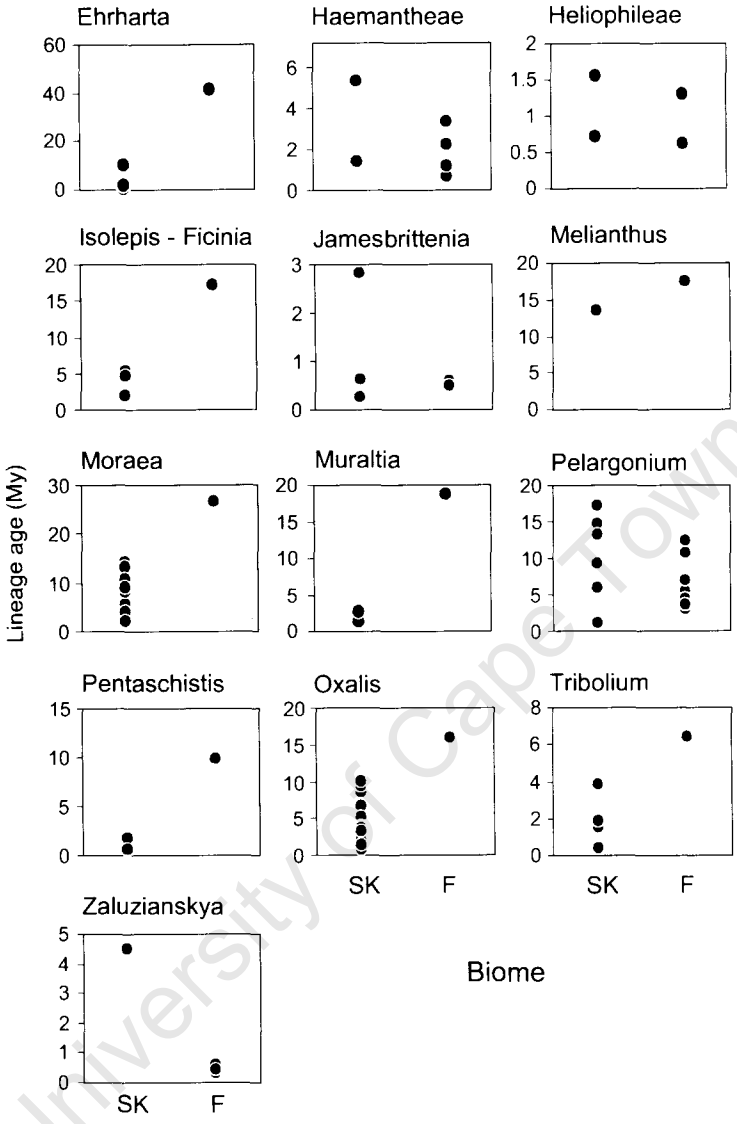

Fig. 3. Comparison of estimated mean stem node ages of succulent karoo- (SK) and fynbos-endemic (F) lineages in each of the trees in which both types of endemism occur. Equivocal reconstructions have been treated as not indicative of biome-endemism.

being substantially older. Included amongst these is the African Restionaceae clade (61.3 My), whose members constitute the dominant element of fynbos vegetation, and have been used to characterize it (Campbell, 1985). Although these age estimates again reflect considerable error, the 95% confidence intervals on most of these estimates reject the possibility that they arose after the mid-Miocene (Appendix A). Indeed, in the case of *Ehrharta* and Restionaceae, an origin after the Oligocene seems very unlikely. The same is probably true for the fynbos-endemic family Bruniaceae for which Quint and Classen-Bockhoff (2004) inferred a stem node age of 59.7–99.5 My. Further support for the relative antiquity of fynbos (compared to succulent karoo) is provided by a group-specific comparison of fynbos- and succulent karoo-endemic lineages. In groups having both fynbos- and succulent karoo-endemic lineages, we identified a consistent tendency for fynbos-endemic lineages to be the older, except in groups whose first appearance in the GCFR post-dated the end-Miocene origin of the succulent karoo (Haemantheae, *Jamesbrittenia*, *Zaluzianskya*). In these younger groups there is obviously no basis for expecting a bias in either direction.

The antiquity of some key fynbos lineages has been interpreted as evidence for the antiquity of the biome itself (e.g. Bakker et al., 2005; Linder 2005). This in turn implies the existence of long-term stable refugia in which fynbos vegetation has been able to persist not only through the Quaternary (Dynesius and Jansson, 2000; Jansson and Dynesius, 2002) but possibly the entire Cainozoic. The older elements in fynbos (e.g. Restionaceae, Bruniaceae, basal nodes in *Ehrharta*) are largely endemic to the sandstones of the Cape Fold Mountains, identifying the latter as potential refugia. Consistent with this, plant species richness (as well as rare species richness) in the western CFR is positively correlated with altitude, possibly due to reduced extinction in montane sites (Cowling and Lombard 2002). It is also in moist environments within the Cape Fold mountains that most of the region's palaeoendemics occur (Linder et al., 1992). Thus, we suggest that it is the moister, cooler, and less seasonal environments of the Cape Fold mountains that have acted as long-term refugia for the long-term persistence of fynbos.

Paired with evidence showing more-or-less linear species accumulation through time (Linder and Hardy, 2004), the potential

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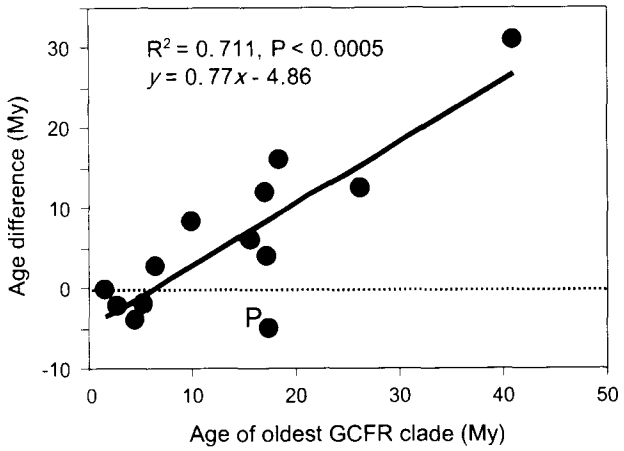

Fig. 4. Plot of the stem node age difference between the oldest fynbos-endemic lineage and the oldest succulent karoo-endemic lineage, against the age of the oldest GCFR-endemic clade present in each of the trees in which both succulent karoo- and fynbos-endemic lineages occur. Points above the stippled horizontal line represent groups in which fynbos-endemism is older, and points below the line groups in which succulent karoo-endemism is older. The point representing *Pelargonium* is indicated by the letter "P". Equivocal reconstructions have been treated as not indicative of biome-endemism.

antiquity of fynbos partly supports the view that its high species richness is the product of a long history of speciation (Adamson, 1958). Whilst the antiquity of certain key fynbos elements may identify this biome as ancient, however, it does not imply that all of its constituent lineages are ancient, nor does it preclude the possibility that some of its current species diversity is the product of recent radiation. Indeed, our data reveal the existence of large numbers of comparatively young fynbos-endemic lineages, some of which (e.g. *Pentaschistis*, *Muraltia*) diversified inside the last 15 My, possibly in response to Miocene–Pliocene climate change. In contrast to the succulent karoo, whose diversification may fit a simple Simpsonian radiation model, climate change may have affected fynbos speciation in two distinct ways. First, the pronounced seasonality of lowland environments in the CFR suggests that, like the succulent karoo, these environments may have experienced high end-Miocene extinction followed by adaptive radiation, possibly in response to their high substrate diversity and landscape evolution (Verboom et al., 2004; Cowling et al., this volume). Second, climatically-induced fragmentation of montane refugia, both at the end of the Miocene and later, may have promoted speciation in allopatry, in a similar manner to that described for other systems (e.g. Roy, 1997; Knowles, 2001). Unfortunately, whilst expedient, our use of Mucina and Rutherford's (2006) fynbos definition (which encompasses mountain and lowland fynbos, as well as lowland renosterveld) does not offer the resolution necessary to test these ideas.

The Greater Cape flora displays a diversity of speciational histories which need to be teased-apart if the origins of this flora are to be fully understood. Whereas we view the succulent karoo flora as a relatively recent phenomenon, the product of perhaps 10 My of evolution, our data support Linder's (2005) deduction that the fynbos flora comprises a mix of ancient and recently radiated groups, indicating a somewhat more complex diversification history. Consequently, the processes underlying the diversities of these two biomes may differ slightly, a point which we believe has been inadequately acknowledged in the past. We suggest that, within the CFR, there is a need to distinguish amongst the lowland and montane communities as these may have experienced different diversification histories.

5. Uncited references

Forest et al. (2007a).

Appendix A

Stem node ages of lineages identified by parsimony optimization as fynbos- and succulent karoo-endemic

Group	Biome endemism	Stem node age (My)	95% Confidence interval (My)
Ehrharta	Fynbos	40.89	31.58, 41.25
	Succulent karoo	10	5.16, 11.27
		0.20 (6.38)	0.01, 1.71 (2.9, 8.32)
		1.66	0.46, 4.31
		1.1	Not estimated
Haemantheae	Fynbos	2.05	0.72, 2.86
		0.54	0.11, 1.36
		1.03	0.46, 2.21
		3.15	Not estimated
	Succulent karoo	5.22	3.81, 6.19
		1.25	0.55, 1.81
Heliophileae	Fynbos	1.29 (2.60)	Not estimated (0.93, 3.68)
		0.62*	0.26, 1.07
	Succulent karoo	1.55 (2.60)	0.71, 2.51 (0.93, 3.68)
		0.71	0.29, 1.20
Isolepis	Fynbos	17.07 (18.63)	12.45, 26.57 (14.84, 30.97)
	Succulent karoo	5.16	2.40, 13.80
		4.4	2.69, 8.61
		1.78	1.00, 5.40
Jamesbrittenia	Fynbos	0.58 (0.78)	0.20, 1.11 (0.50, 1.75)
		0.56*	Not estimated
		0.48	Not estimated
	Succulent karoo	2.81	2.42, 5.81
		0.26	0.07, 1.47
		0.59	0.37, 1.34
		0.24	Not estimated
Melianthus	Fynbos	17.26	5.71, 26.78
	Succulent karoo	13.4	3.57, 15.27
Moraea	Fynbos	26.33	19.10, 30.24
	Succulent karoo	13.91 (15.41)	Not estimated (11.67, 21.01)
		5.47*	2.48, 10.44
		3.8	1.56, 6.65
		12.93 (14.26)	Not estimated (9.24,17.37)
		10.40*	7.61, 14.49
		7.96*	4.00, 9.27
		8.96*	Not estimated
		1.93*	0.39, 3.02
Muraltia	Fynbos	18.49	14.15, 21.35

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Appendix A (*continued*)

Group	Biome endemism	Stem node age (My)	95% Confidence interval (My)
	Succulent karoo	1.08	1.06, 2.80
		2.51	1.30, 3.97
Oxalis	Fynbos	15.75	14.50, 30.88
	Succulent karoo	5.45	4.89, 12.51
		9.81 (11.25)	9.77, 20.02 (10.90, 22.42)
		8.34*	7.58, 15.97
		6.60*	5.00, 11.17
		9.33*	8.63, 18.26
		9.81*	9.77, 20.02
		2.71	2.58, 8.17
		4.97	Not estimated
		5.12	3.97, 8.88
		1.81	1.15, 4.04
		1.31	1.16, 3.81
		0.7	0.68, 2.96
		1.93	1.16, 3.53
		1.3	0.97, 3.12
		3.78	1.20, 5.55
		3.57	2.53, 8.09
		3.24	2.22, 6.76
Pelargonium	Fynbos	10.64	3.72, 13.63
		2.83	Not estimated
		5.47	0.41, 5.54
		12.23 (26.20)	5.41, 16.48 (10.63, 28.50)
		0.98	0.19, 3.15
		5.79	Not estimated
		6.96	1.89, 7.04
		4.12	Not estimated
		4.29	Not estimated
		3.45	1.00, 4.30
	Succulent karoo	17.36 (18.15)	Not estimated (7.82, 22.4)
		14.67*	4.76, 15.29
		13.12	3.81, 14.37
		9.25	3.41, 11.88
		17.09 (26.2)	7.02, 19.99 (10.63, 28.5)
Pentaschistis	Fynbos	9.91	9.82, 15.76
	Succulent karoo	1.56	0.83, 3.15
		0.5	0.3, 1.42
Restionaceae	Fynbos	61.31	31.69, 65.44
Satyrium	Fynbos	14.38 (25.78)	8.30, 18.4 (13.47, 26.26)
		9.62*	Not estimated
		2.24*	0.24, 2.56
		2.97*	1.39, 4.79
		2.24*	Not estimated
Schoeneae	Fynbos	8.89	4.25, 13.63
		12.76	6.20, 20.92
		10.22	3.83, 15.91
		1.13	0.61, 3.58
Tribolium	Fynbos	6.39	4.41, 10.14
Zaluzianskya	Fynbos	3.79 (5.41)	1.08, 4.95 (3.34, 8.14)
		1.44*	0.20, 2.21
		0.36	0.08, 1.22
		1.8	0.79, 3.03
		0.27	0.19, 0.87
		0.56	0.13, 0.74
		0.41	Not estimated
	Succulent karoo	4.45	2.82, 5.93
Zygophyllum	Fynbos	2.18 (5.34)	Not estimated (Not estimated)

In some cases, biome-endemism may be interpreted as older (indicated in parentheses) depending on how equivocally reconstructed nodes are resolved. Asterisks indicate biome-endemic lineages that fuse with other biome-endemic lineages when equivocal branches are resolved in favour of earlier biome-endemism.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.ympev.2008.01.037.

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APPENDIX 2

Tables 1 and 2 provide details of the discriminant function analysis used for climatic niche characterisation in Chapter 4.

Table 1. Predicted group membership following cross-validation of groups based on the discriminant functions. Percentages of correctly reclassified cases are shown in bold type.

Group	**Predicted group membership - %** (number of cases)		**Total cases**
	Tylecodon	*Cotyledon*	
Tylecodon	**95.2 (633)**	4.8 (32)	665
Cotyledon	30.0 (82)	**70.0 (191)**	273

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Table 2. Loading matrix of correlations between predictors and the discriminant function. Abbreviations describe variables per month, either as numerals following the variable, or a three letter abbreviation preceding the variable: Potential evaporation$_{(A\ pan)}$ (e.g. APAN 03G, for March); Average precipitation (e.g. novavgprecip, for November); Average minimum and maximum monthly temperature (e.g. TMIN04 and TMAX04, for April). Variables followed by (a)' were not used in analysis.

Predictor variable	Correlation
maravgprecip	-0.837
febavgprecip	-0.816
APAN03G	0.766
novavgprecip	-0.747
APAN02G	0.746
janavgprecip	-0.726
decavgprecip	-0.722
APAN12G	0.701
APAN01G	0.683
octavgprecip	-0.642
APAN11G	0.572
apravgprecip	-0.545
APAN04G	0.481
TMAX03	0.412
TMAX02	0.407
TMAX04	0.316
TMAX01	0.303
TMIN06(a)	0.299
TMAX11	0.279
APAN10G	0.266
TMIN07(a)	0.246
TMAX12	0.237
sepavgprecip	-0.221
TMIN05(a)	0.216
APAN07G	-0.209
junavgprecip	0.196
julavgprecip	0.180
TMAX05	0.172
APAN06G	-0.169
APAN08G	-0.159
TMIN10	-0.150
TMIN04(a)	0.139
augavgprecip	0.131
TMIN08(a)	0.117
TMAX10	0.116
TMAX06	0.116
TMIN12(a)	-0.115
TMIN01	-0.106
TMIN11(a)	-0.094
TMAX08	-0.071
TMIN09(a)	-0.060
APAN05G	0.050
TMAX09	-0.047
TMIN03(a)	0.033
TMAX07	0.031
APAN09G	0.022
mayavgprecip	0.015
TMIN02	0.010

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