Systematics of the Relhaniinae (Asteraceae-Gnaphalieae) in southern Africa: geography and evolution in an endemic Cape plant lineage.

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ABSTRACT

The Greater Cape Floristic Region (GCFR) houses a flora unique for its diversity and high endemicity. A large amount of the diversity is housed in just a few lineages, presumed to have radiated in the region. For many of these lineages there is no robust phylogenetic hypothesis of relationships, and few Cape plants have been examined for the spatial distribution of their population genetic variation. Such studies are especially relevant for the Cape where high rates of species diversification and the ongoing maintenance of species proliferation is hypothesised. Subtribe Relhaniinae of the daisy tribe Gnaphalieae is one such little-studied lineage. The taxonomic circumscription of this subtribe, the biogeography of its early diversification and its relationships to other members of the Gnaphalieae are elucidated by means of a dated phylogenetic hypothesis. Molecular DNA sequence data from both chloroplast and nuclear genomes are used to reconstruct evolutionary history using parsimony and Bayesian tools for phylogeny estimation. The subtribe is found to be non-monophyletic, and three independent Cape-centred clades are identified. These constitute the earliest-diverging lineages in the Gnaphalieae. Ancestral areas are reconstructed onto the tree using maximum likelihood and indicate a Cape origin and diversification for the Gnaphalieae, with subsequent, multiple dispersals out of southern Africa. A relaxed Bayesian clock is used to estimate dates for important events, calibrated using a combination of secondary age estimates, fossil pollen and geological events. The single inferred dispersal to Australasia, which may be responsible for the large diversity of the tribe there, is compared with other dated trans-Indian Ocean disjunctions, and results in a hypothesis of direct Miocene long-distance trans-oceanic dispersal facilitated by the West Wind Drift. Important directions for future biogeographic and systematic studies on the Gnaphalieae are identified.

One of the Cape clades identified in the above study, the *Stoebe* clade, is investigated using the same molecular markers and an additional chloroplast DNA region, in order to reconstruct relationships amongst the species and test generic circumscription. One genus, *Amphiglossa*, is found to be non-monophyletic and a recommendation is made to resurrect the genus *Pterothrix* in order to maintain monophyly. Relationships amongst subclades in the *Stoebe* clade can not be resolved, possibly due to a paucity of chloroplast DNA polymorphisms. Ancestral-state reconstruction using maximum likelihood indicates a high degree of homoplasy in all the macro-morphological characters used by previous workers to delimit the genera that fall within this polytomy (*Disparago*, *Elytropappus* and *Stoebe*). The evolution of these leaf, synflorescence and floral morphological characters is examined in order to shed light on the degree of homoplasy exhibited by each, and the patterns of character-state change across the tree. The analysis provides insight into patterns of morphological evolution, laying the groundwork for re-evaluation of characters previously thought to be homologous. The need for a rigorous, systematic anatomical study including all members of the clade is highlighted and some previously little-explored characters are identified that might provide greater future taxonomic insight.

Due to a poor palaeoclimatic record, the impact of Pleistocene climatic fluctuations on the palaeodistributions of plant species in the Greater Cape Floristic Region is largely unknown. One of the members of the *Stoebe* clade, the common and widespread shrub *Elytropappus rhinocerotis*, is examined using population genetic tools in order to explore the spatial distribution of genetic variation and to infer the degree and pattern of responses (range shifts, population extinction) to recent (Quaternary) climatic changes. A molecular fingerprinting tool, inter-simple sequence repeat (ISSR) PCR is used to examine populations from across the range of *E. rhinocerotis* in order to compare patterns of population genetic diversity with the long-noted and strong spatial patterns of plant species diversity in the CFR. A large amount of genetic variation is detected, which is apportioned largely amongst individuals within populations rather than amongst populations or regions. This is to be expected for an outcrossing and well-dispersed plant species. However, there is significant spatial structure and a very uneven distribution of diversity across the geographic range of *E. rhinocerotis.* Although areas that have high species-diversity also generally exhibited high genetic diversity, the eastern margin of the range and the Kamiesberg highlands both have a far higher relative genetic diversity in *E. rhinocerotis* than would be predicted by their relative species diversity. This indicates that the processes

producing greater leves of species diversity in some parts of the CFR are different than the processes responsible for high genetic variation within *E. rhinocerotis* populations. Geographic distance is a poor predictor of genetic distance between localities, especially towards the east of the range. This may be due to range alteration over the time-scale reflected by ISSR polymorphism. Inter-SSR variation declined from south to north in the western arm of the range, consistent with the prediction of Holocene aridification starting first and being most extreme in the north. Areas shown by the marker to harbour populations with high levels of variability include most parts of the eastern arm of the range, and the Kamiesberg highlands.

The present study constitutes a demonstration of the range of evolutionary questions that can be addressed using a range of molecular phylogenetic and population genetic techniques, and the elucidation of both deep and shallow evolutionary history of a single Cape floral lineage.

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CHAPTER 1

General introduction

1.1. Rationale

The Cape Floristic Region (CFR; Goldblatt, 1978), a tiny, roughly L-shaped portion of the southwest tip of Africa (Fig. 1-1) is globally unique (Bolus, 1886; Diels, 1908; Marloth, 1908; Goldblatt, 1978; White, 1983), having been recognised in the past as one of the world’s six floral kingdoms (Good, 1974; Takhatajan, 1969) despite being only 90,000 km^2 in area. With over 9,000 species of vascular plants (Goldblatt & Manning, 2000) it is comparable in diversity to equatorial rain forests (Cowling & Holmes, 2002) and recognised as one of the world’s biodiversity hotspots (Myers et al., 2000). Even more remarkable is the high level of species endemism (68.8 %; Goldblatt et al., 2005) which is more similar to oceanic islands than to other mainland areas (Linder, 2003).

In the past, common contemporary ecological causes were sought to explain this diversity and endemism. However, as noted by Cowling & Lombard (2002), unlike many other parts of the world, neither mean annual rainfall nor available energy explain diversity patterns in the CFR, and environmental heterogeneity is not markedly greater than in other Mediterranean-type climate zones. Increased understanding of the importance of ecological, geomorphic and climatic history in understanding biodiversity patterns (Ricklefs, 2004), together with the availability of tools for dating molecular phylogenetic hypotheses, has led to a shift in emphasis and more detailed examination of the evolutionary history of individual lineages. These studies attempt to infer the selective regime experienced by each lineage over time, the most appropriate way to study high species richness if the factors responsible for diversification differ among lineages (Verboom et al., 2003). Accumulation of knowledge about the histories of individual lineages may in future allow an accurate general synthesis of recruitment history into the flora and subsequent evolution of diversity.

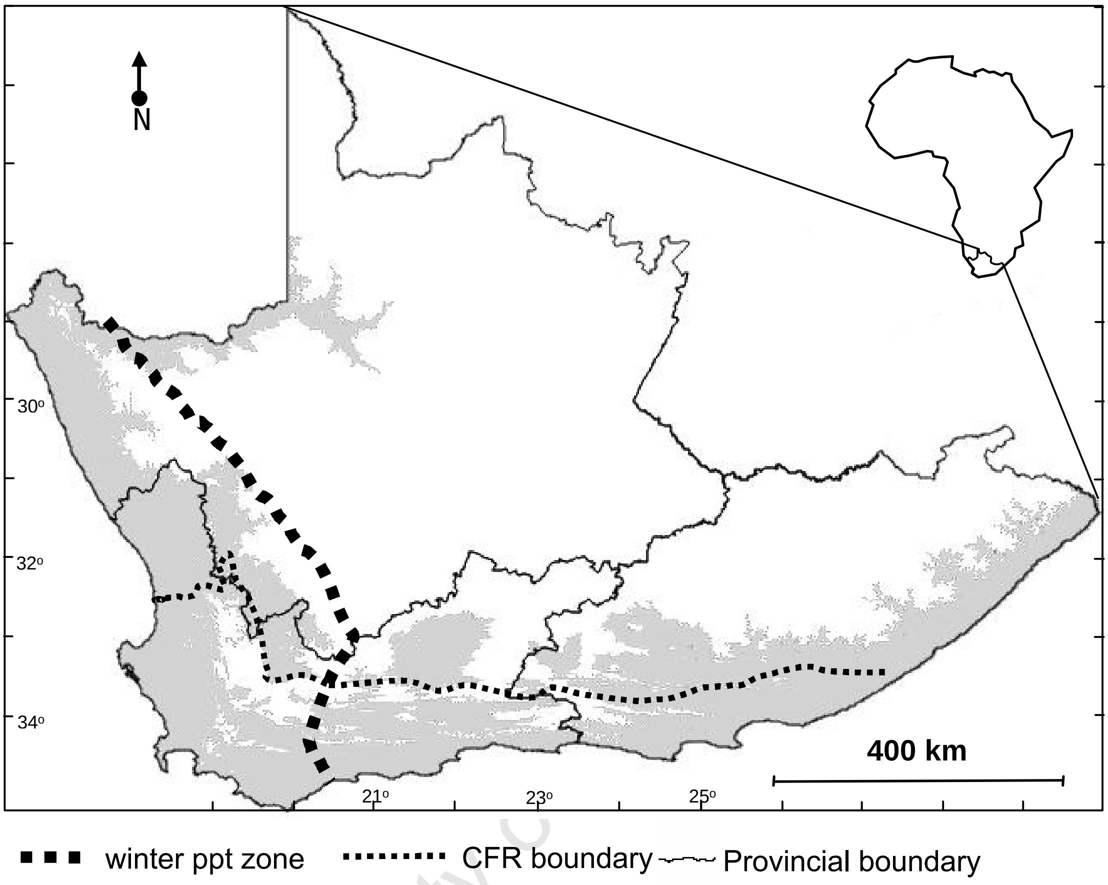


Fig. 1-1. The Cape Provinces of South Africa showing the extent of the region that receives precipitation (ppt) primarily in the winter months and the boundary of the Cape Floristic Region (CFR). Shaded areas lie below 800 m above mean sea level.

Focus on individual lineages is especially relevant for the Cape region because 50% of CFR species diversity is concentrated in just 33 of the largest Cape Floral Clades (Table 1 in Linder, 2003). Well known examples include *Erica* (ca. 660 spp. in the CFR); Proteae in the Proteaceae (ca. 260 spp.), the African Restionaceae (ca. 340 spp.) and the (sub)endemic families Bruniaceae (65 spp.) and Penaeaceae (23 spp.). These lineages, identified loosely as the ‘old Cape groups’ by Marloth (1929) or ‘typical Cape groups’ by Levyns (1964), give the flora its unique floristic makeup.

They tend to have a typical distribution within the CFR, with highest diversity in the southwest at the hook of the ‘L’ around the Caledon and Kogelberg mountains, and fewer species to the north and east (Levyns, 1952). Linder (2003) provided a formal definition for these “Cape Floral Clades”: they are lineages with the crown group or at least 50 % of species in the CFR and the sister group or paraphyletic basal elements also inside the CFR. These clades are thus presumed to have radiated within the region, indicating that relatively few lineage diversification events may be responsible for the uniqueness of the flora.

Many of the most important evolutionary and biogeographic questions pertaining to the history of the Cape flora were asked by early researchers in a series of discussions, reviews and studies between the late 1800’s and the late 1900’s (Levyns 1952, 1964; Adamson, 1938; Bolus, 1886; Weimarck, 1941; Taylor, 1978; Goldblatt, 1978). These include the origins of the Cape Floral Clades, the generation and persistence of species diversity, the historical extent of the winter rainfall region and the causes of regional differences in diversity and endemism across the CFR. These questions have since been addressed in various ways, but reconstruction of vegetation and environmental history from fossil data is difficult, due to both the paucity of suitable deposits and problems with dating (Levyns, 1952; Axelrod & Raven, 1978, Linder, 2003, Chase & Meadows, 2007). The observation of Levyns (1952) is still true today, that given this lack we are obliged to reconstruct the history of the Cape flora from the geographic distributions and evolutionary relationships of the modern taxa.

However, recent advances have provided powerful new tools for evolutionary analysis that were not available when these questions were first asked. Firstly, there has been development of robust statistical analytical methods and associated software for reconstructing both evolutionary relationships amongst extant taxa, and the history of character or distributional change (phylogenetic systematics). Secondly, the molecular revolution has provided a range of molecular markers that provide novel sources of information at all scales of evolutionary study. DNA sequence data routinely provides a relative wealth of characters for phylogeny reconstruction while allozymes, DNA haplotypes, microsatellites and DNA fingerprinting allow direct investigation of population-level processes.

Current biological diversity in the Cape and elsewhere is the result of interplay between the evolutionary forces of adaptation, speciation, persistence and extinction. Diversity and endemism are created, maintained, and destroyed by a range of different evolutionary and ecological processes. To examine these processes, studies need to focus at the scale appropriate to that process. To study the biogeographic origin of large lineages and their recruitment into the Cape flora requires a macroevolutionary approach at a taxonomic level higher than the lineage of interest. To examine the evolution of species diversity, morphological variation and habitat diversity, as well as the history of colonisation and establishment in new areas, studies must focus at the level of species or lower. Insight into the microevolutionary processes acting within and between populations, and the dynamics of population movements to track shifting climatic envelopes in changing environments, requires the use of populations and individuals as units of study. The history of these population-level interactions often leaves characteristic signatures in the genes of modern populations and the field of phylogeography has developed specifically to study genealogies in a geographical context. Where species have only recently diverged (as in many Cape groups) traditional species-level phylogenetic approaches may be uninformative; such recent divergences may be more appropriately studied with phylogeographic and population genetic analysis.

To understand the evolution of the Cape Flora we need to explore two separate processes: firstly, the generation and secondly, the maintenance of species diversity. To date, most studies have focussed on the processes which generated the diversity and we have very little understanding of the more recent conditions that have allowed these species to persist, or even (possibly) allowed the maintenance of high speciation rates. The more recent environmental history has influenced the geographical distribution patterns of species diversity in the Cape; this is best studied at the population level.

Robust species-level phylogenetic hypotheses are the baseline for evolutionary and biogeographic studies on the Cape flora. However, there is a notable paucity of spatial population genetic studies on Cape or even South African plants, with the only published studies known at the time of writing being those of Brain (1986; 1989) on *Acacia karoo* (Fabaceae; using allozymes); Caujape-Castells et al., (1999) on *Androcymbium* (Colchicaceae; allozymes); Barker et al. (1996) on hybridisation in *Aloe* (Asphodelaceae; RAPDs) and Ellis et al. (2007) on spatial genetic structure in *Argyroderma* (Aizoaceae; AFLPs).

The Asteraceae are the largest family in the region with a number of important Cape lineages, such as *Senecio*, the Calenduleae, the Arctotideae, the Ursiniinae, the *Amellus* group of genera, *Helichrysum* and the Relhaniineae. However, there are few phylogenetic studies on the family, even at the tribal level, and to date the only species-level molecular phylogenetic analysis on a Cape daisy group is for the Arctotideae (McKenzie et al., 2006; McKenzie & Barker, 2008; Funk & Chan, 2008). The present study aims to apply molecular techniques at all three of the taxonomic scales described above in order to examine the evolutionary, biogeographic and population history of members of the Cape daisy lineage Relhaniinae.

1.2. Background: the study group

Although southern Africa is a centre of diversity for the large daisy tribe Gnaphalieae, the only subtribe near-endemic to the region is the Relhaniinae. This group of predominantly ericoid shrubs was recognised as a subtribe by Anderberg (1991) on the basis of morphological cladistic analysis, in which he also identified two generic groups: the *Relhania* group (nine genera) and the *Metalasia* group (14 genera). Bayer et al.'s (2000) plastid DNA phylogeny of South African Gnaphalieae indicated that the Relhaniinae may be paraphyletic, and recovered a grade of three lineages, but had little statistical support. Koekemoer (2002) in an unpublished PhD thesis conducted a morphological cladistic analysis of the *Metalasia* group and proposed several new genera, but did not test generic circumscription. The Relhaniinae as circumscribed by Anderberg (1991) was listed by Linder (2003) as one of the larger Cape Floral Clades, although "this clade is still difficult to define, since no generic level analysis of the tribe exists that is both complete and robust" (Linder, 2003).

The subtribal classification of Anderberg (1991) needs to be re-assessed, as it is not congruent with the topologies found in molecular phylogenetic studies on tribe Gnaphalieae. These two studies independently examined the two largest centres of Gnaphalieae diversity: southern Africa (Bayer et al., 2000) and Australasia (Bayer et al., 2002). Implicit in their sampling is the assumption that the gnaphalioid floras of the two regions constitute independent monophyletic lineages, but this has never been tested. In addition, the monophyly of the genera included in the Relhaniinae has never been tested. Macromorphological characters used by previous authors to delimit genera appear to exhibit a high level of homoplasy and a species-level phylogeny is required to test generic limits and as a baseline for future evolutionary and taxonomic studies.

1.3. Background: the study region

1.3.1. Overview of the Cape region and flora

The Cape region is a small floristic or geographic province at the southwest corner of the African continent (Fig. 1-1) characterised by a fine-leaved, sclerophyllous temperate vegetation known as ‘fynbos’. The boundaries of the Cape appear be defined more or less by the distribution of winter moisture and/or by the distribution of coarse quartzitic sandstone-derived soils.

With other Mediterranean-type climate regions the Cape shares a high species-richness and an abundance of fire-survival mechanisms. The physical characteristics of the region have been reviewed in several publications (e.g. Taylor, 1978; Linder, 2003; Goldblatt & Manning, 2000; Goldblatt et al., 2005) so only a brief description is given here. The region is climatically, topographically and edaphically heterogeneous. The western arm of the Cape region (Fig. 1-1) has rainfall distributed mostly in the winter months while the eastern arm receives precipitation throughout the year but with peaks in spring and autumn. The southwestern part is the most mesic because precipitation decreases towards the north and becomes less reliable towards the east

(Cowling & Lombard, 2002). Montane areas receive more moisture than lowland sites, and the Fold Belt mountains orographically block rainfall from the interior arid plains. Inland and to the east, rainfall occurs in the summer months and the Cape flora is surrounded by other floras with summer-rainfall adapted taxa. The arid inland parts to the north (the Great Karoo) house a dry summer-rainfall shrub- and grassland while to the east, humidity increases and CFR vegetation grades into subtropical thicket in the Albany region.

The Cape Floristic Region (CFR; Goldblatt, 1978) is the more mesic southern part of the winter-moisture area and has been defined so as to incorporate the major portion of the fynbos vegetation type. This geographic delimitation has been important for quantifying floristics, which are complex as the region harbours a range of vegetation types and the ecotones between them, summarised in reviews by Cowling & Holmes (1992) and analyses by Low & Rebelo (1996) and Mucina & Rutherford (2006). Although precipitation appears to be the main factor affecting vegetation boundaries in the CFR, edaphic factors are also important and the distributions of vegetation types correspond very closely to those of soil types. These vegetation types have varying degrees of floristic similarity to each other and to neighbouring floras but the typical Cape elements are regarded as those belonging to the fynbos, renosterveld and succulent karoo (see below). These are also the main vegetation types pertinent to the present study.

Several outliers of Cape vegetation occur outside the CFR. These include parts of the Roggeveld Escarpment, the Great Escarpment in the Great Karoo, the Kamiesberg granitic highlands and the summits of the mountains in the Richtersveld desert at the extreme northwestern end of South Africa. These patches may be indicative of parts of the arid interior that due to their altitude or raised water tables offer sufficient winter moisture to sustain Cape elements. There are also outliers of Cape vegetation in the afromontane regions of the Drakensberg and the highlands of east Africa. The presence of these outliers has resulted speculation that the Cape flora was once more widespread than it is today (e.g. Levyns, 1938).

1.3.1.1. Fynbos:

Fynbos vegetation occurs in fire-prone areas of moderate to high rainfall, mostly on quartzitic sands but also with sufficient precipitation on richer, shale- or granite-derived soils and on coastal limestones. In structure the fynbos is predominantly a heathland of low sclerophyllous shrubs, graminoids and geophytes, marked by the near-absence of trees and a lack of dominance of any one species. Fynbos is structurally most closely akin to the kwongan of southwestern Australia, with which it also shares low-nutrient soils (and a scarcity of annual plants compared to most other Mediterranean-type climate floras). Floristically, however, fynbos has diverse affinities (Galley & Linder, 2006). Diversity characteristics and endemism are reviewed in Taylor (1978); Goldblatt (1978); Bond & Goldblatt (1984); Goldblatt & Manning (2000); Goldblatt et al., (2005); Linder (2003) and Born et al. (2006).

The most prominent plants in fynbos are non-Poaceae graminoids (mainly Restionaceae and Cyperaceae), proteoid shrubs, and microphyllous (‘ericoid’) much-ramified shrubs often from the Ericaceae, Thymeleaceae, *Metalasia* or *Stoebe*. Fynbos is more-or-less confined to the CFR, although as mentioned above there are small outliers in areas where altitude or raised water tables provide sufficient winter moisture (eg the Kamiesberg highlands), and where patches of quartzite-derived soils provide suitable edaphic habitat (eg in the Port Shepstone area in Kwa-Zulu Natal province to the east). All of the larger member-genera of the Relhaniinae are well-represented in the fynbos.

1.3.1.2. Renosterveld

Renosterveld vegetation (Cowling & Holmes, 1992; Cowling et al., 2005; Rebelo et al., 2006) is a fire-prone shrubland occurring in areas with rainfall intermediate between that of fynbos and succulent karoo. It occurs on the finer-grained (mostly shale- but also granite-derived and silcrete) clay-rich soils of the coastal platform and interior plains. It is structurally very similar to fynbos, being dominated by fine-leaved mainly asteraceous shrubs, with a (mainly C4) grass understory and a high diversity and biomass of geophytes (Boucher, 1980; Moll et al., 1984; McDowell & Moll, 1992). Although it shares many families and genera with fynbos, at the species level it is floristically distinct (Rebelo et al., 2006). It is characterised by the dominance of one member of the Relhaniinae, *Elytropappus rhinocerotis* or renosterbos. Several other Relhaniinae, notably in the genera *Relhania* and *Oedera* but also *Stoebe*, have endemic renosterveld species. This vegetation occurs throughout the CFR and often occurs as a transition between fynbos and succulent karoo; in addition, outliers of renosterveld-like vegetation occur in many areas outside of the CFR, usually in microsites with increased moisture availability relative to the surroundings (for example, on parts of the Great Escarpment in the Karoo). These outlying populations of renosterbos are often indicative of the presence of other Cape elements outside the CFR (Levyns, 1935).

1.3.1.3. Afrotemperate forest

Afrotemperate forest vegetation occurs mostly in small patches in the more mesic and fire-protected parts of the CFR. It grows on various soil types, from shallow to sandy humic sandstone-derived soils and also on shales and soils derived from Cape Granite (Mucina & Geldenhuys, 2006). Structurally it consists of evergreen tall multilayered closed-canopy rainforest, often with well-developed shrub and herb understoreys. Dominant trees include yellowoods (Podocarpaceae), *Ocotea*, *Olea*, *Pterocelastrus* and *Cunonia* (Mucina & Geldenhuys, 2006). There are no forest Relhaniinae but some *Stoebe* species occur at forest margins. Patches of this type of forest form an archipelago occurring throughout the southern CFR, reaching its greatest extent in the southern Cape along the narrow coastal strip in the Knysna-Tsitsikamma region. Towards the west, it is characterised by low species diversity, especially towards the furthest extent of its range (Levyns, 1938).

1.3.1.4. Succulent Karoo

The Succulent Karoo constitutes a biome of its own, coinciding largely with the region known as Namaqualand, and is a globally important biodiversity hotspot in its own right (Myers et al., 2000). Given its small size it may be the richest arid flora in the world (Hilton-Taylor, 1996) with endemism at 26 % (Driver et al., 2003). Succulent karoo vegetation is arid-adapted and does not provide sufficient biomass to sustain fires. Drought is the main disturbance factor and probably the strongest selective force. This vegetation type occurs in semi-desert areas where some to all of the precipitation in received in winter. It grows on a range of soils with the common feature being higher macro- and micro-nutrient availability than other vegetation types, due to a lack of leaching (Mucina et al., 2006). As a generalisation, a large proportion of the vegetation occurs on clay-rich shale- and granite-derived soils. Succulent karoo vegetation is characterised by the dominance of small shrubby or dwarf leaf-succulent taxa (most notably in the Aizoaceae [formerly Mesembryanthemoideae], but also in the families Euphorbiaceae, Crassulaceae, Asteraceae and Geraniaceae) and geophytes (Hilton-Taylor, 1996). Annuals are prominent mainly in the spring mass-flowering displays of weedy Asteraceae, of which the Relhaniinae genera *Rhynchopsidium* and *Leysera* are prominent. *Amphiglossa* is a succulent karoo genus while *Lachnospermum*, *Relhania* and *Oedera* have species in this vegetation type. The succulent karoo has strong floristic links to fynbos (see discussion on the Greater CFR, below) and occurs in a rough L-shaped region that lies immediately northwards of the L-shape characterising the Fynbos Biome.

1.3.2. The Greater Cape Floristic Region

The vegetation of the CFR is floristically very distinct from that of surrounding regions, with the possible exception of the Succulent Karoo to the north. Affinities of the Cape flora with that of the Succulent Karoo are complicated by the fact that significant pockets of succulent karoo vegetation occur within the CFR. When the neighbouring Namaqualand and Hantam-Roggeveld-Tanqua regions of predominantly Succulent Karoo vegetation are included in the concept of a Greater CFR (GCFR; Bayer, 1984; Born et al., 2006), the total proportion of endemic species increases from 68.8 % (Goldblatt et al., 2005) to 71.5% even though the total number of species only increases by ca. 3,000. The GCFR (Fig. 1-2) is edaphically, topographically and climatically diverse and the single most important defining feature appears to be the occurrence of rainfall mostly in the winter months, and a varying degree of summer aridity. Many CFR lineages extend into the GCFR and there are good arguments for the use of a GCFR concept (Bayer, 1984; Born et al., 2006). The concept of the ‘CFR’ was introduced above because floristic information is not yet available for the GCFR and most information on the Cape region was produced using the concept of the CFR. In the present study, however, the 'Cape region' is considered synonymous with the GCFR.

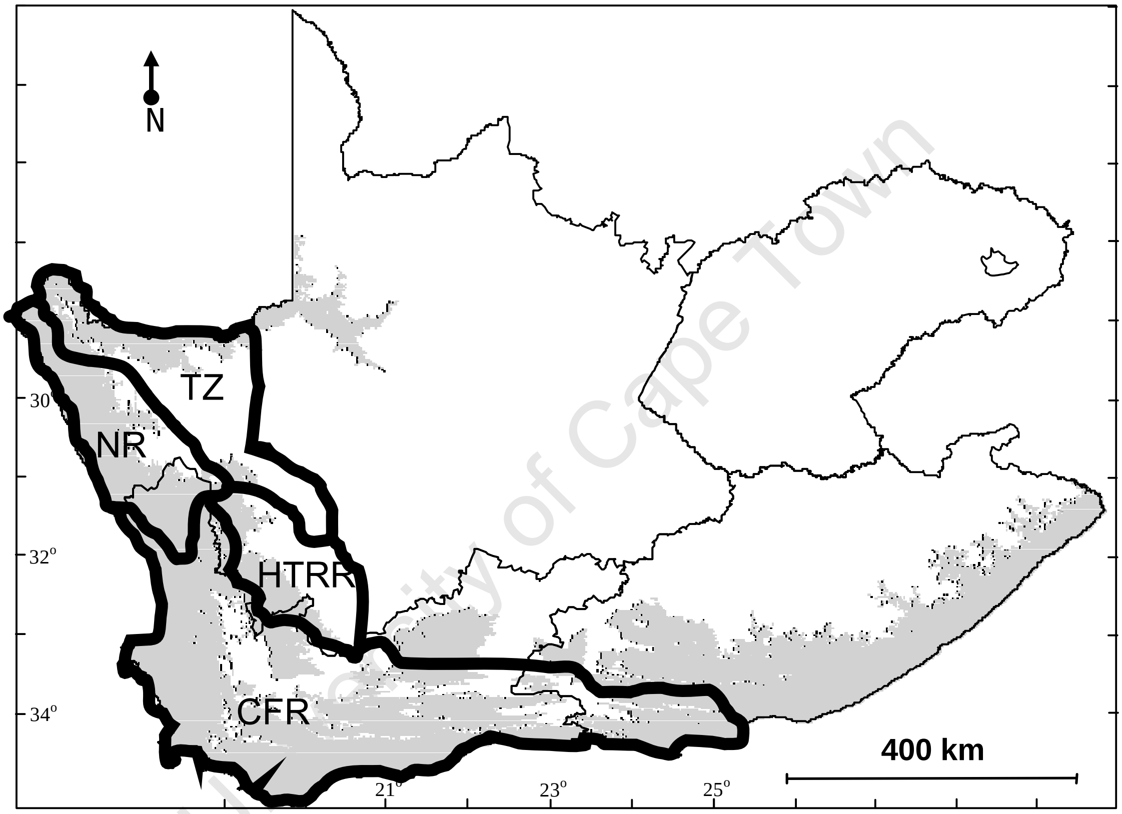


Fig. 1-2. Outline of the Greater Cape Floristic Region (GCFR) within South Africa, taken from Born et al. (2006). CFR – Cape Floristic Region; HTRR – Hantam-Tanqua-Roggeveld Region; NR – Namaqualand Region; TZ – Transition Zone. Shaded areas lie below 800 m above mean sea level.

1.4. Background: what are the causes of species richness and endemism of the Cape flora?

The origins and time scales of diversification in the Cape flora have been the subject of considerable discussion in the literature (e.g. Adamson 1958; Levyns 1964; Axelrod & Raven, 1978; Goldblatt, 1978; Linder et al., 1992; Linder, 2003, 2005; Cowling & Lombard, 2002; Dynesius & Jansson, 2000; Midgley & Roberts, 2001; Cowling et al., 2009; Verboom et al., 2009). A wide range of possible causes has been postulated. Several workers have emphasised current ecological, edaphic and climate conditions, while others have focussed on the importance of evolutionary, climatic and geographic history. It is still a matter of debate whether the species diversity is the result of recent, rapid radiations (Verboom et al., 2003, Richardson et al., 2001; Klak et al., 2003), steady diversification over long periods of time (Linder & Hardy, 2004) or a combination of these (Linder, 2008). However, it appears that Cape lineages have been recruited from disparate sources (Linder et al., 1992; Galley & Linder, 2006) and include groups with Gondwanan distributions (Goldblatt, 1978; Anderson et al., 1999), temperate Eurasian histories (Hurka et al., 2005), or temperate or tropical southern African affinities (Goldblatt, 1978; Linder et al., 1992).

Also of great importance is the question of whether species-rich Cape lineages are characterised by higher-than-usual speciation rates versus lower-than-average extinction rates (Cowling & Lombard, 2002; Linder, 2008; and see below). Although this is difficult to test in the absence of information on extinction rates, there are several hypotheses of common causes for increased speciation rates. These include the role of recurrent fire (Cowling, 1987); high topographical heterogeneity and gradients (Linder et al., 1992); high edaphic heterogeneity (Rebelo et al., 2006); creation of a novel adaptive zone by the inception of the summer-dry climate in the Late Miocene as a result of initiation of Benguela cold water upwelling along the Atlantic coastline of southern Africa (Siesser 1978; Siesser & Dingle 1981; Linder 2003; Levyns, 1964; Goldblatt, 1978); a combination of rapid late-Miocene climate change and uplift-mediated creation of novel edaphic habitats creating multiple new adaptive zones (Cowling et al, 2009); pollinator-mediated diversification (Johnson, van der Niet et al., 2006); low gene-flow distances (Goldblatt, 1997); and high climatic oscillations during the Pleistocene (Goldblatt, 1978; Midgley & Roberts, 2001).

If the large diversity is the result of increased speciation, another matter for debate is whether such radiations are the result of a common trigger for all lineages, or whether idiosyncratic, lineage-specific factors have been more important (Linder & Hardy 2004; Verboom et al., 2003). Multiple dated phylogenies have been compared to establish the range of dates for the onset of diversification in different lineages, looking for evidence of a common trigger for diversification. These studies have shown a range of dates from the Oligocene to the Pliocene (Linder, 2005) although the greatest concentration of dates is in the late Miocene (reviewed in Linder & Hardy, 2004). This is generally inconsistent with a common age for onset of diversification, although there are caveats about the comparability of many of these studies relating to dating methodology and species sampling.

In contrast to the above hypotheses, lower-than-average extinction rates may not require any specific event or special attribute of the lineages involved (beyond adaptation to their environment). Unusually high levels of species survival are hypothesised to be attributable to a relatively stable climatic history throughout the Neogene and during the Quaternary "ice ages" (Cowling & Lombard, 2002; Dynesius & Jansson, 2000), perhaps in conjunction with buffering of aridity in the Cape mountains due to southeaster montane precipitation (Deacon et al., 1992; Verboom et al., 2009). The tenet here is that a stable or only slightly variable climatic history allowed speciation processes to proceed unhindered, and did not provide selection against specialisation or small gene-flow distances, resulting in the ever-finer division of ecological niches in the Cape. Verboom et al. (2009) provide evidence for the possibility that BOTH factors, i.e. a long stable history and low extinction rates (for the fynbos) and rapid radiation (for the succulent karoo, with lineages radiating into the novel summer-arid adaptive zone), are responsible for the remarkable diversity of the GCFR.

From the above it can be seen that there are two directly opposite theories on the extent of Pleistocene climatic oscillations in the Cape. Goldblatt (1978) and Midgley & Roberts (2001) propose the ‘species pump’ hypothesis, that intermediate levels of climate oscillation promoted diversification by forcing dynamism in species ranges, resulting in isolate formation, allopatric speciation and subsequent secondary contact with associated hybrid speciation. Cowling & Lombard (2002) and Dynesius & Jansson (2000), take the opposite view, and propose ‘rampant gradual speciation’ facillitated by relatively stable climates buffered from extremes in temperature or precipitation. These oppposing views present very different ideas both of the extent of Milankovitch climatic oscillations and of the response of plant species to those oscillations. In the absence of sufficient palaeoclimatic data to distinguish these scenarios, we turn once again to the modern flora.

One of the most obvious reasons for the survival of species throughout the Quaternary in southern Africa is the fact that the area escaped Pleistocene glaciation. However, climates were not uniform in space or time during this period. It has long been noted (e.g. Levyns, 1938; Cowling & Lombard, 2002; Forest et al., 2007) that diversity and endemism of Cape lineages are higher in the southwest of the CFR, especially in the mountains of the Caledon and Kogelberg region. This is hypothesised to be due to higher palaeoclimatic stability (notably more reliable precipitation) in the southwest and especially in the mountains of the southwest, throughout the history of the lineages, preventing extinction of narrow endemics and allowing speciation in lineages with small gene-flow distances. It is clear that the history of species diversification in the region is complex and multi-faceted and will benefit from recognition that the climatologically diverse parts of the GCFR may have experienced different climatic histories. Determining the ways in which climates changed across the Cape is not only of academic interest; past climate changes, and the way organisms responded to them, may be substantial indicators of future, anthropogenically-driven changes.

One of the main hindrances to testing alternative hypotheses about the generation and maintenance of species diversity in the Cape is the paucity of good palaeoenvironmental data. Fossils do not preserve well in arid habitats. Many of the best southern African pollen records, for example, represent point sources on wetland or riverine sites, which cannot be extrapolated to the general climate of the time. Although there are other sources of data independent of moist conditions, there is a widely acknowledged paucity of accurately-dated fossil evidence on the paleo-climate of the region (eg Linder 2003; Chase & Meadows, 2007) and a great need for other sources of paleo-ecological information, such as can be found in phylogeographic patterns within extant taxa.

Patterns of distribution of genetic diversity in living species offer the potential to recover the history of their populations, due to the fact that past distributions, and fluctuations in population size, leave detectable signatures in the genes of extant populations (Avise et al., 1987). With the development of phylogeographic techniques, molecular tools now offer an independent source of data on the way plants responded to past climates. These techniques have been widely used in animal systems, and in the reconstruction of glacial recolonisation patterns for northern hemisphere plant species (eg Hewitt, 1999; 2000; Soltis et al., 1997; 2007). To date, intraspecific molecular techniques have not been applied to any southern African plant species in an explicitly phylogeographic context.

1.5. Problem statement

Current subtribal classification in the large daisy tribe Gnaphalieae does not appear to reflect evolutionary history, as the subtribes of Anderberg (1991) are not monophyletic. A robust phylogenetic hypothesis is required to test the monophyly of the Relhaniinae and its relationships within Gnaphalieae. The phylogeny is also needed in order to determine the biogeographic origin of the lineage in the Cape, and the direction of subsequent dispersals out of the region. Age estimates for important divergence events are needed to determine the timing of, and thus, geoclimatic background to, diversification and dispersal events. The origins and relationships of the gnaphaloid lineages in different parts of the world (southern Africa, the Mediterranean basin, the Americas and Australasia) are not well understood. Broader sampling across the Gnaphalieae will provide insight into the relationships and origins of taxa from southern Africa and other parts of the globe, and determine whether the Australasian and southern African gnaphalioid floras are monophyletic.

Subtribe Relhaniinae is recognised as one of the Cape Floral Clades housing a disproportionate amount of Cape species diversity. Diversification history in each of these clades needs to be examined to facillitate a future synthesis of the origin and evolution of the Cape Flora.

Within the Relhaniinae, genera are not monophyletic as currently circumscribed. Generic delimitation is difficult as there appears to be a high degree of homoplasy in the suite of macromorphological characters that have been used thus far. Phylogenetic resolution of the relationships amongst species will constitute a test of generic circumscription as it will identify the monophyletic entities that contain the generic type species. A species-level phylogenetic hypothesis will also allow the evolutionary history of characters used thus far in classification to be explored, in order to identify homoplasy (independent origins of apparently similar features, and reversals to ancestral states) in these characters.

One of the hypothesised causes of high diversity and endemism in the CFR is a relatively stable recent (late Quaternary) climatic history, with habitats being buffered against extremes of temperature and aridity. If this is true, members of Cape Floral Clades should have very stable distributional histories as they would not have had to undergo extensive migration or experienced much local extinction during Pleistocene climatic oscillations. The alternative hypothesis is that Milankovitch-driven shifts in climate and resulting orbitally-forced range dynamics (ORD; Dynesius & Jansson, 2000) have promoted highly dynamic distributions which promoted speciation in the Cape. The palaeoecological record is not detailed enough to distinguish between these hypotheses, but phylogeographic examination of population genetic signals in modern populations holds the potential to recover signals of past distributional history. This is an unexplored aspect of the history of the Cape flora which may provide new insights into the generation and persistence of species richness in the CFR. A member of the

Relhaniinae, *Elytropappus rhinocerotis*, has a distribution which largely matches that of the GCFR, an ecotonal ecology and putative representation in the fossil record, and so may be an ideal study subject for the examination of ORD in the Cape. Due to the paucity of fossil evidence in the Cape region, we currently have only limited understanding of the degree of Pleistocene climatic change here. Thus the role of past climatic oscillations in the generation and persistence of genetic and species-level diversity is poorly-understood in the Cape. Novel molecular techniques are now available to examine the recent population history of individual species. Applying these techniques, we can begin to examine the degree of historical fragmentation and the distributional history of Cape species. The relhaniinioid *Elytropappus rhinocerotis* is an ideal subject for examination of population history, due to its distribution in the Cape, its presence in the fossil record and its ecology.

CHAPTER 2

Evolutionary relationships of the Relhaniinae: Cape diversification and repeated out-of-southern-Africa dispersal in paper daisies (Gnaphalieae).

2.1. Introduction

Subtribe Relhaniinae was identified by Linder (2003) as tenth-largest of the Cape Floral Clades that are endemic or near-endemic to the Cape region and have typical patterns of diversity within the GCFR. These clades are hypothesised to have arisen and diversified in the Cape region. Understanding the origins, relationships and modes of diversification of all the individual Cape clades will be an important step towards a synthesis of the origins and maintenance of the unique diversity of the GCFR flora. To date, no phylogenetic study has included significant representation of Relhaniinae or produced a robustly-supported hypothesis of their relationships. However, the molecular phylogeny of Bayer et al., 2000 indicated that the Relhaniinae is likely to be polyphyletic and so broader sampling is required to ensure that all closely-related groups are represented.

Relhaniinae is a member of the large daisy tribe Gnaphalieae which is represented on most continents but forms a significant component of dry- and cool-temperate floras in the southern hemisphere. Tribe Gnaphalieae contains ca. 1240 species (Bayer et al., 2007) and has two main centres of diversity: southern Africa, where one quarter of the ca. 2 200 Asteraceae species are gnaphalioids (Koekemoer, 1996) and Australasia with nearly 500 species (Bayer et al., 2007). The remainder are found in South and Central America (ca. 100 species; Anderberg, 1991), the rest of Africa, the Mediterranean, Asia, and North America (Bayer et al., 2007). In the morphological cladistic analysis of Anderberg (1991), all the early-diverging lineages in the tribe come from southern Africa and from Australia. Bayer et al.’s (2000) molecular phylogenetic study did not include any Australasian taxa and indicated that the earliest diverging lineages come only from southern Africa and all belong to the near-endemic Cape subtribe Relhaniinae. Elucidation of the phylogenetic relationships in Relhaniinae is thus likely to be a key for understanding the early evolutionary history of the tribe as well as to providing insight into the origins of the unique Cape Flora of southern Africa. Inclusion of Australasian taxa will however also be required to ensure that all potential relationships at the base of the group are adequately inferred.

Lineages that today are endemic or near-endemic to the Cape fall into several groups based on their biogeographic relationships. Clade members or sister groups may be located in various other floristic provinces, such as (in southern Africa) the Karoo, grassland, afrotemperate, subtropical thicket; (in the rest of Africa) afroalpine, tropical and (further abroad), the Mediterranean flora, South and Central America, Eurasia and Australasia. These relationships reflect the area of origin of the ancestral taxon and / or the patterns of dispersal out of the Cape. Patterns that recur in many different groups have been summarised by biogeographic track analysis into the Gondwanan track, the African track and the boreal track (Linder et al., 1992). However, at the time of this track analysis, phylogenies were not available for many plant groups, and this can lead to misinterpretation of biogeographic patterns. For example, many distributions thought to be the result of typical Gondwanan vicariance have been shown to be the result of recent dispersal; these include lineages in Proteaceae (Barker et al., 2007) and Bombacaceae (Baum et al., 1998)). Only with a dated phylogeny can we tease out these patterns and distinguish early recruitment history into the Cape from post-Cape-diversification migration to other regions. Elucidating the geographical affinities and timing of diversification of Cape groups will eventually enable a synthetic answer to questions such as: do all Cape clades follow a similar recruitment history into the Cape? and what are the post-diversification patterns of dispersal out of the Cape?

The Gnaphalieae exhibit a distribution which could result from dispersal across all three of the tracks listed above. They are most diverse in South America, southern Africa and Australasia, a possible Gondwanan vicariant distribution. However, the daisy family as a whole is hypothesised to be younger than 50 million years ago (Ma; Funk et al., 2005), which is long after the full separation of the Gondwanan continents. The African track represents tropical affinities and genera such as *Helichrysum* are well represented in both the summer-rainfall tropical vegetation of southern Africa and the afromontane regions of the east African highlands. Relhaniinae also contains sub-afroalpine members. The boreal track is also represented, although these taxa represent only a small fraction of gnaphalioid diversity. Examples include the edelweiss, *Leontopodium* which is alpine and Himalayan. The present study aims to use a dated phylogeny to discern whether the Cape Gnaphalieae originated from one of these tracks or whether these distributions are the result of subsequent dispersal out of the Cape.

The present study builds on the analyses of Bayer et al. (2000; 2002) to construct a phylogenetic hypothesis for the Gnaphalieae with special focus on members of the Relhaniinae, but including non-relhaniinoid taxa from southern Africa, Australia and the Northern Hemisphere. Phylogenetic methods are used to examine the timing and geographic origins of the Gnaphalieae in the Cape, the timing of diversification of endemic Cape lineages, and the pattern and timing of dispersal out of the Cape. The timing and direction of trans-oceanic dispersal(s) in Gnaphalieae are examined, since the two major centres of diversity are on either side of the Indian Ocean, and samples are available for several Australasian taxa. There are many Cape groups with a trans-Indian Ocean disjunction, and these are compared in order to determine whether there is a general pattern in these dispersals, and to attempt to understand mechanisms that could cause such a pattern.

2.2. Materials and methods

2.2.1. Sampling

Outgroup taxa from other tribes in the Asteraceae were included for molecular clock calibration (see below) and non-relhanioid taxa from the Gnaphalieae were selected to represent the widest possible taxonomic and geographic coverage (Table 2-1). Field collection of leaf material was carried out in South Africa or, for some taxa, DNA was extracted from herbarium material at BOL. Sixty-seven new DNA sequences were generated for southern African taxa and the remainder obtained from published studies. Several taxa (marked with an asterisk in Table 1) are represented as composites of sequences from several different specimens.

Table 2-1. Specimen collection and voucher information. Localities listed without mention of a country are all in South Africa. CFR = Cape Floristic Region; NZ = New Zealand. Genbank Accession numbers starting with 'FM' were generated for this study and have not been previously published.

	Voucher details (if collected for this study)		**Genbank / EMBL accession numbers**			
Taxon	**Collector (and herbarium)**	**Collection locality**	***psbA-trnH***	***trnL* intron**	***trnL-trnF* spacer**	**ETS**
Actinobole uliginosum (A. Gray) H. Eichler	-	-	-	AF141736	AF141824	AF319667
Amphiglossa callunoides DC.	Bergh 1452a (NBG)	Uitenhague	FM173155	-	-	FM173123
Amphiglossa corrudifolia DC.	Koekemoer 1291 (BOL)	Prince Albert	FM173156	-	-	FM173124
Amphiglossa tomentosa (Thunb.) Harv.	Bergh 1332 (NBG)	Cederberg	FM173157	FM173184	FM173184	FM173125
Angianthus micropodioides (Benth.) Benth.	-	-	-	AF141695	AF141783	AF319671
Anisothrix kuntzei O. Hoffm.in Kuntze	-	-	-	AF098859	AF100522	-
Argentipallium obtusifolium (Sond.) Paul G.Wilson	-	-	-	AF141730	AF141818	AF319674
Argyroglottis turbinata Turcz.	-	-	-	AF141692	AF141780	AF319675
Arrowsmithia styphelioides DC	-	-	-	AF098809	AF100472	-
Aster novae-angliae L	-	-	-	U82018	U82019	AF319676
**Bryomorphe lycopodioides* (Sch. Bip) Levyns	Bergh 1155 (NBG)	Matroosberg	FM173158	AF098820	AF100483	FM173126
Chionolaena lavandulifolia (Kunth.) Benth. & Hook.	-	-	-	AY143593	AY143593	-
Craspedia variabilis J. Everett & Doust	-	-	EF187694	AF141713	AF141801	EF187619
Decazesia hecatocephala F. Muell.	-	-	-	AF141752	AF141840	AF319695
**Disparago ericoides* (Berg.) Gaertn.	Bergh 1143 (NBG)	Houw Hoek	FM173159	AF098821	AF100484	FM173127
**Dolichothrix ericoides* (Lam.) Hilliard & Burtt.	Bergh 180 (NBG)	Cederberg	FM173160	AF098822	AF100485	FM173128
**Edmondia sesamoides* (L.) Hilliard	Bergh 1130 (NBG)	Jonaskop	FM173161	AF098844	AF100507	FM173129
**Elytropappus rhinocerotis* (L.f.) Less.	Bergh 3.3; Bergh M1 (NBG)	Kamiesberg	FM173162	FM173185	FM173185	FM173130
Ewartia catipes (DC) Beauverd	-	-	-	AF141698	AF141786	AF319701
Ewartia sinclairii Cheeseman	-	-	AY611218	-	-	-
Felicia filifolia Burtt Davy	-	-	-	AF318120	AF318929	AF319703
Fitzwillia axilliflora (W. Fitz. ex Ewart & Jean White) P. S. Short	-	-	-	AF141708	AF141796	AF319704
Haeckeria ozothamnoides F. Muell.	-	-	-	AF141725	AF141813	AF319710
**Helianthus annuus* L.	-	-	X60428	U82038	U82039	AF319711
Helichrysum asperum (Thunb.) Hilliard & B. L. Burtt	Bergh 1165 (NBG)	Cederberg	FM173163	FM173186	FM173186	FM173131
**Helichrysum cylindriflorum* (L.) Hilliard & B. L. Burtt	Bergh 1063 (NBG)	Cederberg	-	AF098839	AF100502	FM173132
Helichrysum dockeri (F. Muell.) Benth.	-	-	-	AF318111	AF318922	AF319665
Helichrysum felinum Less.	Bergh 1194 (NBG)	Namaqualand	FM173164	FM173187	FM173187	FM173133
**Helichrysum lanceolatum* Kirk.	-	-	EF187698	AY606889	AY606900	EF187647
Ixiolaena tomentosa Sond. & Muell.	-	-	-	AF141704	AF141792	AF319717
Jacobaea argunensis (Turcz.) B. Nord.	-	-	-	AF468169	AF468169	-

*_Jacobaea maritima_ (L.) Pelser & Meijden	-	-	AY155647	AF460158	AF460105	-
Lachnospermum fasciculatum Baill.	Bergh 1105 (NBG)	Jonaskop	FM173165	-	-	FM173134
	Voucher details (if collected for this study)	**Genbank / EMBL accession numbers**	-	AF098824	AF100487	-
Taxon	**Collector (and herbarium)**	**Collection locality**	**_psbA-trnH_**	**_trnL_ intron**	**_trnL-trnF_ spacer**	**ETS**
*_Leontopodium alpinum_ Cass.	Bergh 1349 (NBG)	Switzerland	FM173166	AF141733	AF141821	FM173135
Leucochrysum stipitatum (F. Muell.) Paul G. Wilson	-	-	-	AF141722	AF141810	AF319722
*_Leysera gnaphalodes_ (L.) L.	Bergh 1441 (NBG)	Malmsbury	FM173167	AF098810	AF100473	FM173136
Metalasia acuta P. O. Karis	Bergh 1301 (NBG)	Cederberg	FM173168	-	-	FM173137
*_Metalasia densa_ (Lam) Karis	Bergh 1266 (NBG)	Pearly Beach	FM173169	AF098848	AF100511	FM173138
Metalasia galpinii L. Bolus	Bergh 1276 (NBG)	Langeberg	FM173170	-	-	FM173139
Millotia perpusilla (Turcz.) P. S. Short	-	-	-	AF141757	AF141845	AF319762
Millotia tenuifolia Cass.	-	-	-	AF318124	AF318933	AF319727
*_Monoculus monstrosus_ (Burm. f.) B. Nord	-	-	-	U82048	U82049	AF319733
*_Oedera squarrosa_ (L.) Anderb. & Bremer	Bergh 1065 (NBG)	Cederberg	-	AF098812	AF100475	FM173140
Ozothamnus whitei (N. T. Burb.) Anderb.	-	-	-	AF141748	AF141836	AF319737
Pentatrichia petrosa Klatt.	-	-	-	AF098817	AF100480	-
Petalacte coronata D. Don.	-	-	-	AF098843	AF100506	-
*_Phaenocoma prolifera_ (L.) D. don	Bergh 1206 (NBG)	Struisbaai	FM173171	AF098825	AF100488	FM173141
Pithocarpa pulchella Lindl.	-	-	-	AF141756	AF141844	AF319739
*_Plecostachys serpyllifolia_ (Berg.) Hilliard & B. L. Burtt.	Bergh 1271 (NBG)	Riviersonderend	FM173172	AF098849	AF100512	FM173142
Pogonolepis stricta Steetz	-	-	-	AF141678	AF141766	AF319745
Polycalymma stuartii F. Muell. & Sond.	-	-	-	AF141726	AF141814	AF319746
Pycnosorus globosus Bauer ex Benth.	-	-	EF187719	AF141680	AF141768	EF187625
Quinetia urvillei Cass.	-	-	-	AF141716	AF141804	AF319750
Relhania calycina (L.f.) L'Herit.	Karis 650 (NBG)		FM173173	-	-	FM173143
Relhania fruticosa (L.) Bremer	-	-	-	AF098813	AF100476	-
Relhania pungens L'Herit.	Karis 876 (NBG)		FM173174	-	-	FM173144
*_Rhynchopsidium pumilum_ (L.f.) DC.	Karis 770 (NBG)		FM173175	AF098811	AF100474	FM173145
Rosenia glandulosa Thunb.	-	-	-	AF098815	AF100478	-
Senecio burchellii DC	Bergh 1110 (NBG)	Jonaskop	FM173176	-	-	FM173146
Senecio cordifolius L. f.	-	-	-	AY952916	AY952916	-
Senecio vulgaris L.	-	-	-	DQ208177	-	AF319755
Siloxerus multiflorus Nees	-	-	-	AF318127	AF318936	AF319756
Sondottia glabrata P. S. Short	-	-	-	AF141738	AF141826	AF319758
*_Stoebe aethiopica_ L.	Bergh 1040 (NBG)	Cederberg	FM173177	AF098845	AF100508	FM173147
Stoebe cinerea Thunb.	Bergh 1210 (NBG)	Du Toit's Kloof	FM173178	FM173188	FM173188	FM173148
Stoebe cryptophylla Baker	Gehrke AF281 (NBG)	Madagascar	FM173179	-	-	FM173149
Stoebe kilimandscharica O. Hoffm.	Gehrke AF076 (NBG)	Kenya	FM173180	-	-	FM173150
Stoebe muirii Levyns	Bergh 1263 (NBG)	De Hoop	FM173181	-	-	FM173151
Stoebe passerinoides Willd.	Hedderson 15894 (NBG)	Réunion	FM173182	-	-	FM173152
Stuartina muelleri Sond.	-	-	EF187715	U82058	U82059	AF319760
Syncarpha canescens (L.) B. Nord.	Bergh 1222 (NBG)	Cape Town	FM173183	FM173189	FM173189	FM173153
Tagetes patula L.	-	-	-	U82060	U82061	AF319761
*_Vellereophyton dealbatum_ (Thunb.) Hilliard & B. L. Burtt.	Bergh 1256 (NBG)	Elim	-	AF098808	AF100471	FM173154

2.2.2. DNA isolation, amplification and sequencing

Leaf material was collected directly into silica gel. Approximately 30 mg of dried leaf material was ground with sterilized sand and/or liquid nitrogen and total genomic DNA isolated using the CTAB method (Doyle and Doyle, 1987). Extracts from problematic samples were cleaned through QIAGEN? QIAquick cleaning columns. DNA was suspended and diluted in Tris-EDTA buffer. PCR reactions were performed on a Hybaid PCR Sprint thermal cycler (Fisher Scientific International).

The 3’ portion of the external transcribed spacer (ETS) of nuclear ribosomal DNA was amplified using the primers AST1 (Markos and Baldwin, 2001) and 18S-ETS (Baldwin and Markos, 1998). The chloroplast *psbA-trnH*^{GUG} spacer was amplified using the primers *psbA*-F and *trnH*-R of Sang et al. (1997). The chloroplast *trnL*^{UAA} intron and the *trnL*^{UAA}-*trnF*^{GAA} intergenic spacer were amplified together using the “c” and “f” primers of Taberlet et al. (1991). The reaction mixtures consisted of 5.0 mM $MgCl_2$, dNTPs at 0.1mM each, primers at 0.5µM, 0.3µM and 0.33µM (ETS, *psbA-trnH* and *trnL-F*, respectively) and 0.75 (*psbA-trnH* and *trnL-F*) or 1.0 (ETS) unit(s) of Bioline BioTaq®. The ETS mixture also contained 2% DMSO. Reaction volumes were made up to 25 µl (ETS) or 30 µl (*psbA-trnH* and *trnL-F*) with sterilized Millipore^{TM} water, and included 3 – 4 µl of template DNA. Thermal profiles consisted of 2 minutes at 95^{o} C (ETS and *psbA-trnH*) or 97^{o} C (*trnL-F*) followed by 30 cycles of: a) 94^{o} C (ETS and *psbA-trnH*) or 97^{o} C (*trnL-F*) for 1 minute; b) 1 minute at 55^{o} C (ETS) or 52^{o} C (*trnL-F*); or 45 seconds at 54^{o} C (*psbA-trnH*) and c) 2 minutes (ETS and *trnL-F*) or 1 minute (*psbA-trnH*) at 72^{o} C. A final extension step at 72^{o} C lasted for 7 (ETS and *trnL-F*) or 8 (*psbA-trnH*) minutes.

Successfully amplified target DNA was cleaned in Microspin^{TM} columns using GFX Band Purification Kits from Amersham Biosciences UK or in QIAGEN? QIAquick PCR purification columns. Cycle sequencing used the following thermal profile: 25 cycles of: 30 seconds at 96^{o} C, 90 seconds at 50^{o} C and 4 minutes at 60^{o} C in tubes containing 1-3 µl of cleaned target DNA, 0.16 µl of primer, 2.0 µl of ABI PRISM® BigDye® Terminator v3.1 cycle sequencing reaction mix and 0.1 µl of 20% DMSO made up to 10 µl with sterilized Millipore^{TM} water. Sequenced fragments were visualised either on an ABI PRISM ® 377 capillary DNA Sequencer or an ABI

PRISM ® 3100 Genetic Analyzer. Each region was sequenced in both directions using the original PCR primers; chromatograms were checked and assembled with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Australia; Conor McCarthy, 1996) and Sequencher 4.5 (Gene Codes Corporation, 2005). Consensus sequences were aligned manually in MacClade 4.05 (Maddison and Maddison, 1992). Stretches of DNA that could not be unambiguously aligned across all taxa were recoded as missing data. Insertion/deletion (indel) events were coded independently as binary characters using the simple gap coding method of Simmons and Ochoterena (2000) implemented in Gapcoder (Young and Healy, 2003).

2.2.3. Phylogenetic analysis

Each of the four partitions (the *psbA-trnH* spacer*; trnL* intron*; trnL-trnF* spacer and the ETS) consisted of a stretch of nucleotides and the associated indel data and was initially analysed separately. Unweighted parsimony tree searching was conducted in PAUP ver 4.0 b10 (Swofford, 2002). Parsimony uninformative characters were excluded in order to standardize parsimony statistics. An initial shallow search was conducted with 10,000 random-addition replicates using NNI branch-swapping and saving only one tree per round of branch swapping. The resulting set of shortest trees was then subjected to TBR branch swapping while saving multiple trees. Node support was assessed via 1,000 non-parametric bootstrap replicates, each replicate saving a maximum of 500 trees based on a simple addition sequence with the TBR branch-swapping algorithm.

Simultaneous Bayesian inference of nucleotide substitution parameters and topology was performed in MrBayes v3.1 (Huelsenbeck and Ronquist, 2001). Indel characters were analysed according to the restriction site (binary) model with ascertainment (coding) bias accounted for using the ‘variable’ option (Ronquist et al., 2005). The default prior and likelihood settings were used for all remaining parameters except the nucleotide substitution model which was set to the general time-reversible model with an inverse gamma distribution of rate variation across sites (GTR+I+G; see Results). For partitioned analysis, substitution model parameters and rates of substitution were allowed to vary across partitions using ratepr = variable and the ‘unlink’ command.

The MCMC chain was run for up to 8,000,000 iterations, sampling parameters every 1,000 iterations. Each analysis used one cold chain and up to 11 heated chains, with chain heating parameter values between 0.06 and 0.20. Longer runs and greater numbers of heated chains were required to achieve convergence for the analyses combining more than two gene partitions. Convergence of the Markov chain and assessment of ‘burn-in’ duration was determined by examining the average standard deviation of split frequencies and by comparing likelihood values, parameter estimates and traces between samples from two simultaneous runs starting from different random starting trees in Tracer v1.3 (Rambaut and Drummond, 2003).

Additional assessment of convergence was based on the comparison of posterior probability (PP) consensus trees from different runs within and across analyses. At least three separate runs were conducted for each partition. Trees were visualized using the ‘compute consensus’ option in PAUP v4.0b10 (Swofford, 2002).

Tests of data combinability such as the incongruence length difference (ILD) test have been shown to have a high type I error rate (Planet, 2006; Yoder et al., 2001; Barker and Lutzoni, 2002) and to be especially difficult to interpret when comparing more than two data partitions (Planet, 2006), and when partitions are represented by different numbers of taxa, as in the current dataset. A more straightforward approach to detecting incongruence amongst data partitions was used: individual trees were compared for well-supported conflict (eg Wiens, 1998; Eldenäs and Linder, 2000) defined as a bootstrap value of = 75 % or a PP value = 0.95.

2.2.4. Molecular Age Estimation

BEAST v1.4.6 (Drummond and Rambaut, 2007) was used to estimate divergence times from all gene regions simultaneously and using only nucleotide data. This software uses a ‘relaxed phylogenetic’ model, where topology and branch lengths are estimated simultaneously from the data. The topology, including placement of the root node, is not specified *a priori* (Drummond et al., 2006). The partitioned BEAST .xml input file (Appendix 1) was created with BEAUti v1.4.6 (Drummond and Rambaut, 2007) and edited manually to allow parameters to be estimated independently amongst data partitions. The substitution model parameters were the same as in MrBayes (GTR+I+G) and the gamma distribution was modelled with four categories.

A log-likelihood ratio test rejected the strict clock model for our dataset ($p < 0.001$) so the relaxed Bayesian clock was implemented, with rates for each branch drawn independently from a lognormal distribution (Drummond and Rambaut, 2007). A Yule prior (constant rate of speciation per lineage) was set for the branch lengths and the 'mean.Rate' parameter had a uniform prior between 0 and 0.1. The 'coefficient of variation' had a uniform prior between 0 and 1.0 and the 'covariance' prior was uniform between -1.0 and 1.0. Runs were initiated on random starting trees. Initially, several short BEAST runs were performed to examine the MCMC performance. After optimal operator adjustment as suggested by the output diagnostics, two final BEAST runs each of 10,000,000 iterations were performed. Convergence was assessed as for MrBayes using Tracer v1.3 (Drummond and Rambaut, 2007). After discarding the first 1,000,000 samples as burn-in, the trees and parameter estimates from the two runs were combined. The samples from the posterior were summarized on the maximum clade credibility tree which is the tree that has the maximum sum of posterior probabilities on its internal nodes (Drummond et al., 2007) using TreeAnnotator v1.4.6 (Drummond and Rambaut, 2007) with posterior probability limit set to 0.5 and summarizing mean node heights. These were visualized using Figtree v1.1.2 (Drummond et al., 2007). Means and 95% higher posterior densities (HPD) of age estimates were obtained from the combined outputs using Tracer v1.3. The 95% HPD represents the shortest interval that contains 95% of the sampled values from the posterior (Drummond et al., 2007).

Calibration nodes were defined via appropriate taxon sets which were not constrained to be monophyletic. Prior age distributions with 'soft' bounds rather than 'hard' upper and lower cut-off bounds were used, as soft bounds allow the incorporation of more prior information about the timing of a divergence event (Yang and Rannala, 2006). Such prior distributions can be structured to better account for uncertainty both in the taxonomic placement of fossils and in the dates assigned to fossils or geological events, giving low but non-zero probabilities to times outside the most likely age boundaries. For example, since a fossil represents only the minimum age of the taxon (Hedges and Kumar, 2004), a distribution with a long 'tail' further back in time represents the diminishing probability that the divergence time may actually be much older than the fossil with greater accuracy than an arbitrary lower cut-off age. In addition, some leeway around the upper bound of the fossil age, rather than a hard cut-off, better incorporates the uncertainty associated with both the dating of the fossil and its assignment to a specific node in the tree. Hard, cut-off bounds assume that there is no error in either dating, taxonomic assignment of the fossil or correspondence between the fossil and the divergence it is assumed to represent. Hard cut-offs also have the drawback that when there is conflict amongst calibration points, age estimates for any ‘bad’ calibration points will be bound by the ‘hard’ limits of their priors, preventing the corrective influence of the (hopefully more accurate) remaining calibration points (Yang and Rannala 2006). Allowing the analysis occasionally to sample states with lower probabilities will also result in larger confidence intervals, more accurately reflecting the error associated with divergence time estimates.

The calibration of the root node (‘treemodel.Rootheight’ in BEAST) corresponds to the crown age of the large subfamily Asteroideae and was informed by a previous molecular dating study (Kim et al., 2005) which estimated this node to range from 35 – 39 Ma based on a slow and a fast rate calibration of *ndhF* from other angiosperm families, and from 26 – 29 Ma based on NPRS dating calibrated with an outgroup fossil. Secondary calibration dates compound several sources of error and should be implemented and interpreted with caution (Graur and Martin, 2004). For this reason, we used as our prior for this node a normal distribution with a mean at 32.5 Ma, the midpoint of Kim et al.’s (2005) dates, and a range spanning two standard deviations around this mean (95% confidence interval of 14.1 – 50.9 Ma; Fig. 2-1a).

Ambrosia is a genus in tribe Heliantheae (represented in our tree by *Helianthus*) and we used the earliest unambiguous *Ambrosia*-type pollen record, from the Beaverhead Basins flora of Montana (Leopold and MacGinitie, 1972; Becker, 1969), to calibrate the age of the node connecting *Helianthus* with *Tagetes* (tribe Tageteae). Becker (1969) and Graham (1996) state that this fossil is 22 – 30 (- 35) Ma. Uncertainty in the age and imprecision in nodal assignment were accommodated by specifying a lognormal prior distribution that covered a wider time range and allowed the date to

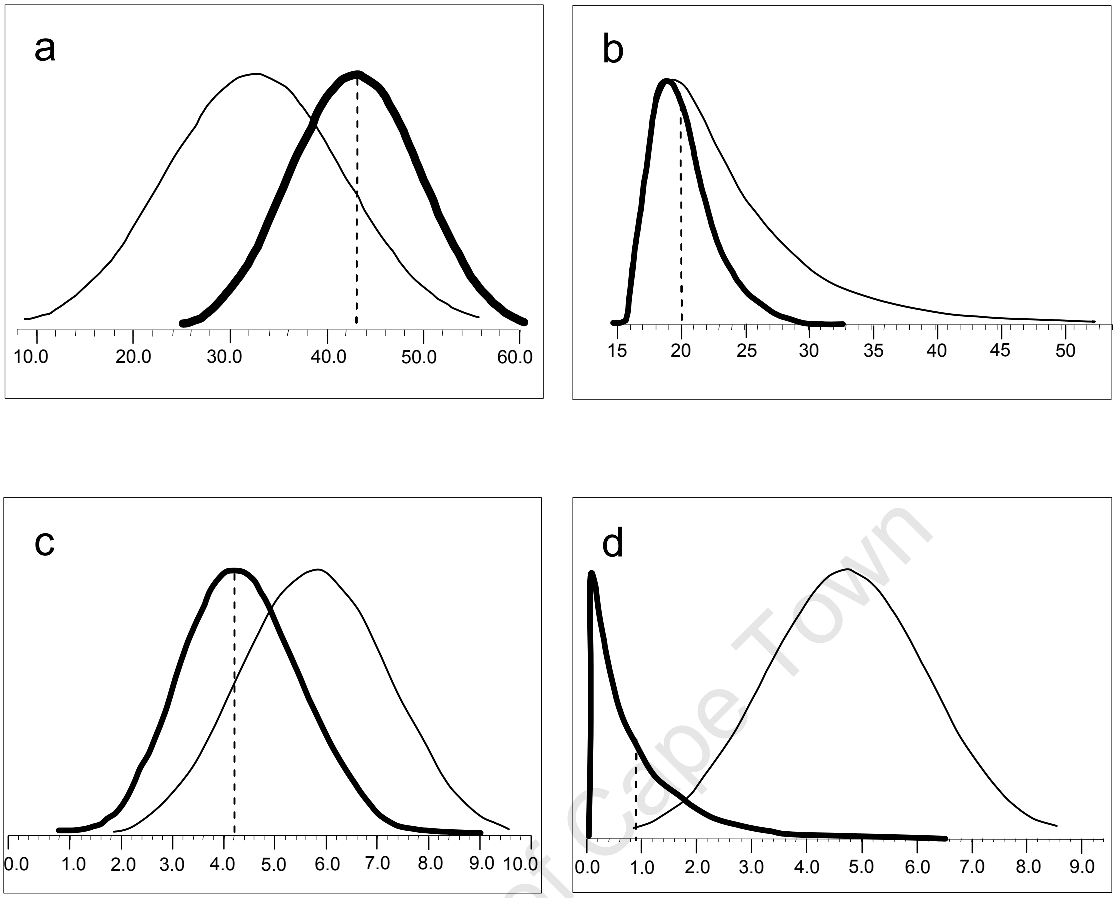


Fig. 2-1. Prior (thin line) and posterior (thick line) age distributions of calibration nodes in the BEAST uncorrelated relaxed clock analysis implementing calibration Scheme (a). The dashed line indicates the mean of the posterior distribution. The X-axes indicate time in millions of years before present. The Y-axes are scaled so that the two curves are of equal height. **a** – root node; **b** – age of the split between *Helianthus* and *Tagetes*; **c** – age of the limestone specialist *Stoebe muirii*; **d** – age of La Réunion endemic *Stoebe passerinoides.*

shift backwards in time (Fig. 2-1b). The 95% confidence interval for this prior lies between 16.9 and 44.1 Ma with the mean at 22.3 Ma.

The split between the range-restricted endemic limestone specialist *Stoebe muirii* and its closest sampled sister *S. aethiopica* is likely to have occurred only after exposure of the Agulhas limestones (the Bredasdorp formation; Hendey, 1983). These were laid down during a series of marine transgressions from around 20 Ma up to 10 Ma (and according to some authors, as late as 3 Ma; Siesser and Dingle, 1981; Hendey, 1983) and only exposed after regression (Siesser, 1972) which may have been related to continental uplift at around 5 Ma (Maud and Partridge, 1987). A normally-distributed prior is most appropriate for this kind of geological calibration (Ho, 2007) and so a normal distribution with mean at 6.0 and 95% confidence interval between 3.1 and 8.9 Ma was implemented as the prior for this node (Fig. 2-1c). The mean is shifted slightly backwards in time to allow, with lower probability, the possible occurrence of the habitat elsewhere before 5 Ma (e.g. the Elandsfontyn formation; Hendey, 1983). The prior also allows for the possibility that *S. muirii* may have speciated some time after the exposure of its habitat.

Stoebe passerinoides is endemic to high altitude heathlands on the volcanic island La Réunion where volcanism was initiated at ca. 5 Ma, building the massifs by ca. 2 Ma (McDougall, 1971; Gillot et al., 1994). The habitat itself may be older than La Réunion (Bell and Donoghue, 2005) so a normal prior distribution with a conservative 95% confidence interval between 1.8 and 7.7 Ma (mean of 4.75 Ma) was implemented for the split between this taxon and its sister *S. cryptophylla* from Madagascar. The older end of this range accommodates earlier volcanism in the Mascarene hotspot corresponding to the earliest estimated age of the shield volcano structure on the neighbouring island of Mauritius (McDougall and Chamalaun, 1969; Fig. 2-1d).

Given the potential errors associated with molecular age calibrations (Shaul and Graur, 2002; Heads, 2005), sensitivity to different calibration priors was tested using several alternative calibration schemes (see Table 2-3 in Results). The original calibration settings described above are referred to as Scheme (a). The influence of the root calibration was tested by using an uninformative root prior in Scheme (b). Scheme (c) has a lognormal root prior allowing for the age to shift backwards in time with diminishing probability. Scheme (d) implements the narrower range of dates for the root node reported in Kim et al. (2005). The calibration for the *Helianthus-Tagetes* split is based on a Chadronian *Ambrosia* pollen grain (Wing, 1987). The end of the Chadronian corresponds to the Eo-Oligocene boundary (Prothero and Berggren, 1992). If free of stratigraphic error, this fossil might provide a more precise minimum age bound. Additionally, this divergence may pre-date the origin of the *Ambrosia*-type lineage (Funk et al., 2005) in which case the fossil is likely to be younger than the actual divergence. The effect of this was explored by using a narrower lognormal prior distribution, shifted to the older end of the original prior in Scheme (e). Scheme (f) omits the two geological calibrations.

2.2.5. Ancestral area reconstruction

The fully-resolved BEAST maximum clade credibility tree was used for Maximum Likelihood (ML) ancestral area reconstruction. The advantages of this method over parsimony reconstruction are that it makes use of (in this case, time-proportional) branch lengths, and assigns relative probability values to nodal reconstructions. The Markov k-state one-parameter model (equal probability of change in both directions; Lewis, 2001) was implemented in Mesquite’s Ancestral State Reconstruction Packages (Maddison and Maddison, 2006). Areas were coded as a multistate character as follows: 0 – southern Africa, including Lesotho, Namibia, South Africa, Moçambique and Zimbabwe; 1 – the highlands of Kenya, Tanzania, Uganda, Malawi and the DRC, Madagascar and La Réunion; 2 – Eurasia including the Mediterranean region; 3 – Central America; and 4 – Australasia.

2.3. Results

2.3.1. Combining data partitions

Several instances of incongruent taxon placement at well-supported nodes were found in trees from different data sets. These all involved tip nodes and taxa which were either composites of sequences from different specimens, or were represented by only one of the gene regions. The incongruence is likely to be due to missing data, conflict between data partitions, or errors in Genbank sequences. These taxa (*Anaphalis margaritacea, Anaxeton arborescens, Anaxeton asperum, Athrixia capensis, Gnaphalium declinatum, Gnaphalium viscosum, Lasiopogon glomeratus, Pterygopappus lawrencii, Toxanthes perpusilla* and *Trichogyne ambigua*) were

Table 2-2. Taxa and character information for each gene region: number of included taxa, numbers of characters, parsimony informative (PI) characters, resolution and support for trees from each partition analysed separately, and from the combined analyses. BS = bootstrap percentage; PP = posterior probability of a node in MrBayes analysis.

	PLASTID					NUCLEAR	
	psbA-trnH	*trnL* intron	*trnL-F* spacer	combined *trnL-trnF* region	combined plastid	ETS	**ALL**
Total number of taxa	35	62	60	62	73	62	**73**
Number of ingroup taxa	32	53	53	53	63	55	**63**
Base-pairs in alignment	539	527	445	972	1511	486	**1997**
PI (%)	*65 (12.1)*	*44 (8.3)*	*75 (16.9)*	*119 (12.2)*	*184 (12.2)*	*223 (45.9)*	***407 (20.4)***
Indel characters	95	57	90	147	242	102	**344**
PI (%)	*37 (38.9)*	*19 (33.3)*	*25 (27.8)*	*44 (29.9)*	*81 (33.5)*	*39 (38.2)*	***120 (34.9)***
Total number of characters	634	584	535	1119	1753	588	**2463**
PI (%)	*102 (16.1)*	*63 (10.8)*	*100 (18.7)*	*163 (14.6)*	*265 (15.1)*	*262 (44.6)*	***527 (21.4)***
Parsimony statistics:							
Tree length	166	125	222	364	530	886	**1467**
CI	0.69	0.62	0.61	0.58	0.61	0.5	**0.52**
RI	0.84	0.80	0.77	0.76	0.78	0.78	**0.77**
RC	0.57	0.49	0.47	0.44	0.48	0.39	**0.40**
No (%) of nodes resolved in strict consensus*	12 (36.4)	20 (33.3)	24 (41.4)	27 (45.0)	22 (31.0)	31 (51.7)	**28 (39.4)**
No (%) of nodes with BS = 75*	11 (33.3)	5 (8.3)	11 (19.0)	16 (26.7)	17 (23.9)	21 (35.0)	**20 (28.2)**
No (%) of nodes with PP = 0.95*	13 (39.4)	9 (15.0)	13 (22.4)	24 (40.0)	23 (32.4)	28 (46.7)	**37 (52.1)**

* Percentages of nodes are based on the maximum possible number of internal branches (n-2 where n is the number of taxa included in the analysis). Strict consensus = strict consensus of all shortest trees in parsimony analysis.

removed from the analysis due to their potential to confound phylogenetic and branch length estimates, albeit at the expense of biogeographic and taxonomic coverage. Comparisons of support values showed no incongruence in the final 73-taxon data set.

2.3.2. Sequence characteristics and phylogenetic analyses

In the final alignment, individual regions are similar in length but differ greatly in their phylogenetic informativeness (Table 2-2). The ETS region provides nearly as many parsimony-informative (PI) characters as that of all the chloroplast data combined (262 versus 265) and nearly 45 % of ETS characters are PI compared with an average of only ca. 15 % for the plastid characters. Also, the ETS PI characters come mostly from nucleotide substitutions, while nearly a third of the plastid PI characters are derived from indel data. Given these facts, it is not surprising that the ETS region produces a better resolved and supported topology (Table 2-2) than any individual plastid partition. Combining the plastid data partitions reduces the proportion of resolved and supported nodes relative to the individual plastid regions, and combining ETS with the plastid data does the same relative to the ETS data alone (Table 2-2). This is likely to be due to the inclusion of an increased number of taxa with missing data. Partitioned Bayesian analysis with all regions gives the highest percentage of supported nodes (52.1%; Table 2- 2). This may be due to the difference between Bayesian PP values and the bootstrap (Simmons et al., 2004; Erixon et al., 2003; Alfaro and Holder, 2006) or to the use of a nucleotide substitution model in the Bayesian analysis, which incorporates homoplasy as well as rate variation across sites (Huelsenbeck and Crandall, 1997).

Although the actual models chosen for each gene region by MrModeltest 2.2 (Nylander, 2004) are F81+G (*psbA-trnH*) and GTR+ G (*trnL-trnF* intergenic spacer, *trnL* intron and ETS), a MrBayes run implementing these models returned the same topology and near-identical PP values to independent GTR+I+ G models for each region. The latter was implemented in the final analysis as Bayesian inference is more robust to model overspecification than underspecification (Huelsenbeck, 2004).
The BEAST maximum clade credibility tree with the highest posterior probabliity (Fig. 2- 3) has the same topology as the MrBayes tree, but several nodes which

received good support in the latter were not supported by the BEAST analysis. These were node B (the Gnaphalieae); nodes E and F, and node H (the Australasian taxa). Conversely, node L was well-supported in the BEAST analysis but only received a PP of 0.92 with MrBayes. Results may differ under the two models (ie unrooted vs. relaxed phylogenetic model; Drummond et al., 2006) but in this case the discrepancy appears to be due mainly to the exclusion of indel data from the BEAST analysis, as a MrBayes analysis without the indel data produced nodal PP values which were more similar to those of the BEAST analysis than of the original MrBayes analysis (results not shown).

2.3.3. Topology

Although tribe Gnaphalieae (node B, Fig. 2-2) is strongly supported in the MrBayes analysis (PP = 1.00), none of Anderberg's (1991) subtribes, indicated by uppercase letters after the taxon names in Fig. 2-2, are supported by the molecular data. The earliest-diverging gnaphaloid lineage (node K) was recovered and well-supported by all individual partitions and analyses and contains only southern African taxa. These are all either members of Anderberg's (1991) *Relhania* group of genera, or members of his paraphylum of early-diverging lineages termed by him the "basal group unassigned to any subtribe". This lineage is here called the '*Relhania* clade' (Fig. 2-2). Non-southern African taxa placed by Anderberg in the unassigned "basal group" fall within later-diverging lineages descended from node G (Fig. 2- 2). The remaining taxa from Relhaniinae *sensu* Anderberg fall into one of two well-supported (each PP = 1.0) clades (the '*Metalasia* clade' and the '*Stoebe* clade') that, together with the *Relhania* clade, form a largely southern African grade at the base of the Gnaphalieae. *Stoebe* species from the ericaceous belt associated with afroalpine habitats (*S. kilimandscharica*, *S. passerinoides* and *S. cryptophylla*) form a clade with PP of 1.0. The clade defined by node E (termed the 'rest of the Gnaphalieae' in Fig. 2- 2, with PP = 1.0) is sister to the *Stoebe* clade and contains taxa from Eurasia, Central America and Australasia, embedded in a southern African paraphylum (Fig. 2- 3). As found by previous authors (eg. Bayer et al., 2000; Galbany-Casals et al., 2004), sampled members of the large, cosmopolitan genus *Helichrysum* are not monophyletic. The southern African *Plecostachys* forms a well-supported (PP = 0.96) clade with the

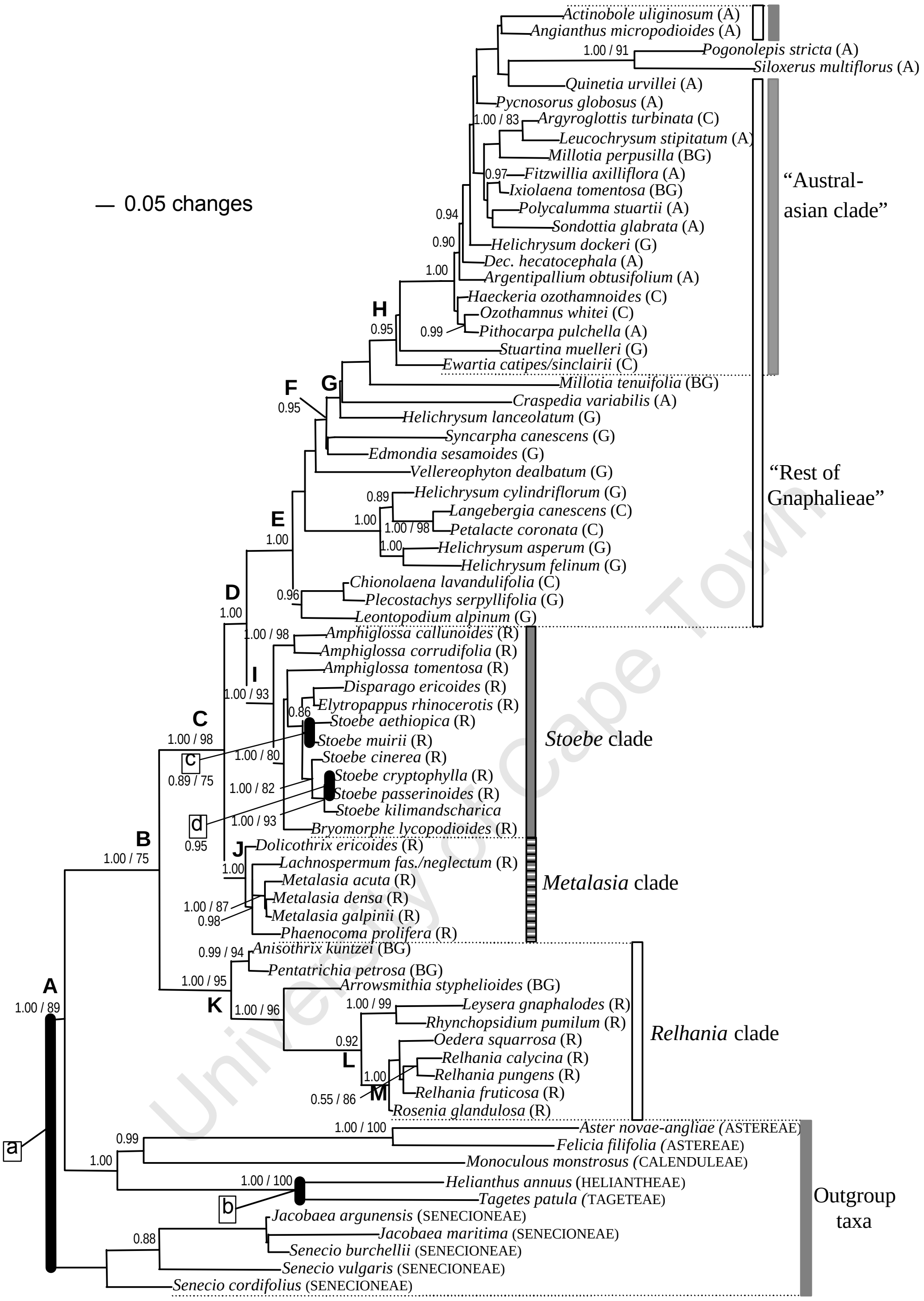
Eurasian *Leontopodium alpinum* and *Chionolaena lavandulifolia* from Mexico. Node F (PP = 0.95) indicates that the southern African taxa *Edmondia* and *Syncarpha* from Anderberg's (1991) Gnaphaliinae are most closely related to the Australasian taxa. Node G represents a large clade with unsupported internal relationships; these taxa occur only in Australasia. Despite lack of support at this node, a more exclusive Australasian clade (node H) is well-supported, indicating probable monophyly of this regional sample representing 15 of the 20 clades recovered in the consensus tree of Bayer et al. (2002). Although none of their clades was supported by bootstrap percentages = 50%, this is the best attempt at broad phylogenetic sampling of the Australasian Gnaphalieae, given current knowledge.

2.3.4. Estimates of divergence times

2.3.4.1. Parameters of the BEAST analysis

The following paragraph reports the mean (and 95% HPD) of parameter estimates combined from at least two separate BEAST analyses (minus the burn-in fraction). All parameter estimates are based on at least 450 (and often many more) independent samples from the posterior as estimated by Tracer v1.3. The mean number of new lineages arising from a single parent lineage per million years (Yule.birthrate) was 0.074 (0.068 – 0.11). The mean number of substitutions per site per million years (mean.Rate) across the whole tree was estimated o be 0.0018 (0.0011 – 0.0026). The mean branch rate under the relaxed clock model (ucld.mean) across all data partitions was 0.0020 (0.0017 – 0.0030) substitutions per site per million years and the standard deviation of this parameter (ucld.stdev) was 0.80 (0.67 – 0.95). The relative closeness of this estimate to 1.0 suggests rate heterogeneity across lineages (Drummond et al, 2007) which is confirmed by the non-zero value for the parameter 'coefficient of variation' (Drummond et al., 2007), estimated to be 0.82 (0.66 – 0.99). The parameter which measures the average autocorrelation of rates of evolution from parent to daughter lineages (covariance) was estimated to be 0.19 (0.03 – 0.37). If this value spans zero, daughter branches typically have rates which are very different to parent branches (Drummond et al. 2007) so the present dataset may exhibit some degree of rate autocorrelation (A. Drummond, pers. comm.).

Fig. 2-2. (opposite) Phylogram from partitioned MrBayes analysis. Numbers above branches are posterior probability (PP) values followed by bootstrap values, shown only for PP values = 0.85 and/or bootstrap values =75 %. Upper-case letters at nodes are referred to in the text. Lower-case letters in boxes indicate the calibration nodes (thick black lines) as in Fig. 2-1. Letters after the species names indicate subtribal affiliation (Anderberg, 1991): (A) – Angianthinae, (BG) – “basal group”; (C) – Cassiniinae, (G) – Gnaphalinae, (R) – Relhaniinae.



— 0.05 changes
Actinobole uliginosum (A)
Angianthus micropodioides (A)
1.00 / 91
Pogonolepis stricta (A)
Siloxerus multiflorus (A)
Quinetia urvillei (A)
Pycnosorus globosus (A)
1.00 / 83
Argyroglottis turbinata (C)
Leucochrysum stipitatum (A)
Millotia perpusilla (BG)
0.97
Fitzwillia axilliflora (A)
Ixiolaena tomentosa (BG)
Polycalumma stuartii (A)
Sondottia glabrata (A)
0.94
0.90
Helichrysum dockeri (G)
Dec. hecatocephala (A)
1.00
Argentipallium obtusifolium (A)
Haeckeria ozothamnoides (C)
Ozothamnus whitei (C)
0.99
Pithocarpa pulchella (A)
H
0.95
Stuartina muelleri (G)
Ewartia catipes/sinclairii (C)
Millotia tenuifolia (BG)
F
G
0.95
Craspedia variabilis (A)
Helichrysum lanceolatum (G)
Syncarpha canescens (G)
Edmondia sesamoides (G)
Vellereophyton dealbatum (G)
0.89
Helichrysum cylindriflorum (G)
1.00
Langebergia canescens (C)
1.00 / 98
Petalacte coronata (C)
E
1.00
1.00
Helichrysum asperum (G)
Helichrysum felinum (G)
0.96
Chionolaena lavandulifolia (C)
Plecostachys serpyllifolia (G)
D
1.00
Leontopodium alpinum (G)
1.00 / 98
Amphiglossa callunoides (R)
Amphiglossa corrudifolia (R)
I
Amphiglossa tomentosa (R)
1.00 / 93
Disparago ericoides (R)
Elytropappus rhinocerotis (R)
0.86
C
1.00 / 98
Stoebe aethiopica (R)
c
Stoebe muirii (R)
1.00 / 80
0.89 / 75
Stoebe cinerea (R)
Stoebe cryptophylla (R)
1.00 / 82
Stoebe passerinoides (R)
d
Stoebe kilimandscharica
1.00 / 93
0.95
Bryomorphe lycopodioides (R)
B
1.00 / 75
J
Dolicothrix ericoides (R)
1.00
Lachnospermum fas./neglectum (R)
Metalasia acuta (R)
1.00 / 87
Metalasia densa (R)
0.98
Metalasia galpinii (R)
Phaenocoma prolifera (R)
0.99 / 94
Anisothrix kuntzei (BG)
Pentatrichia petrosa (BG)
1.00 / 95
Arrowsmithia styphelioides (BG)
K
1.00 / 96
1.00 / 99
Leysera gnaphalodes (R)
Rhynchopsidium pumilum (R)
A
1.00 / 89
0.92
Oedera squarrosa (R)
L
Relhania calycina (R)
1.00
Relhania pungens (R)
0.55 / 86
M
Relhania fruticosa (R)
Rosenia glandulosa (R)
a
1.00 / 100
Aster novae-angliae (ASTEREAE)
0.99
Felicia filifolia (ASTEREAE)
1.00
Monoculous monstrosus (CALENDULEAE)
1.00 / 100
Helianthus annuus (HELIANTHEAE)
b
Tagetes patula (TAGETEAE)
Jacobaea argunensis (SENECIONEAE)
0.88
Jacobaea maritima (SENECIONEAE)
Senecio burchellii (SENECIONEAE)
Senecio vulgaris (SENECIONEAE)
Senecio cordifolius (SENECIONEAE)
"Austral-asian clade"
"Rest of Gnaphalieae"
Stoebe clade
Metalasia clade
Relhania clade
Outgroup taxa

2.3.4.2. Prior versus posterior distributions of calibration nodes

The posterior distributions of calibration node ages are shown in Fig. 2- 1. The posterior distribution most closely echoes the prior distribution for the age of the *Helianthus-Tagetes* split (Fig. 2-1b) but the others show a strong influence of their co-calibration points. The posterior distribution shifted backwards in time relative to the prior for the root node (Fig. 2-1a) and forward in time for the ages of the limestone endemic *Stoebe muirii* (Fig. 2-1c) and La Réunion endemic *Stoebe passerinoides* (Fig. 2-1d). The directions of the two latter shifts are consistent with the fact that geological calibrations represent oldest bounds on a node age. The calibration node for *S. passerinoides* is the only case where the shape of the posterior distribution is very different from that of the prior, perhaps due to the data and remaining calibrations overwhelming the prior in support of a very recent age.

2.3.4.3. Sensitivity analysis

When there is no constraint on the root node, the tree age is pushed backwards in time by the other calibrations, as shown in Scheme (b) in Table 2-3, with the root estimated to be over 80 Ma. Scheme (c) produced an intermediate set of divergence ages. Scheme (d), assuming no error in the dates from Kim et al. (2005), resulted in younger divergences across the tree. With Scheme (e), the narrower and older *Ambrosia* prior pushed this node and the root backwards in time but the remaining divergences became slightly younger. The youngest overall ages were obtained with Scheme (f) which omitted the two geological calibrations.

2.3.4.4. Mean age estimates for nodes of interest

The root node (the Asteroideae crown group) is estimated to be of Eocene age, with the majority of samples from the posterior falling in the Mid- to Late-Eocene (Table 2-4). The 95 % HPD for the age of Gnaphalieae spans the entire Eocene and Oligocene, with the mean in the Late Eocene. The estimated crown group ages of the three clades containing the former Relhaniinae are all in the Miocene. Although the *Relhania* clade is older than the *Stoebe* or *Metalasia* clades, the subclade containing *Relhania* (node L) also has a Miocene crown age. Node F falls in the Miocene and the radiation of the Australasian clade is estimated to have begun by the Mid-

Miocene.

Table 2-3. Results of the dating sensitivity analyses indicating prior distributions (PRIORS) on calibration nodes, and posterior distributions (POSTERIORS) on calibration nodes and two other nodes of interest (crown age of Gnaphalieae, and oldest age of the Australasian clade [node F]). Values are in millions of years before present and represent the mean and (in brackets) 95% HPD for each distribution. The initial calibration scheme (a) is shown for comparative purposes. For the priors, ‘N’ indicates a normal, ‘logN’ a lognormal, and ‘U’ a uniform distribution.

	(a) As described in Methods		(b) As for Scheme (a) but with an uninformative prior on the root node.	
	PRIORS	POSTERIORS	PRIORS	POSTERIORS
Root	N: 32.5 (14.1 - 50.9)	43.0 (29.3 - 56.6)	U: 0 - 1000	85.5 (36.7 - 145.2)
Ambrosia fossil	logN: 22.3 (16.9 - 44.1)	20.1 (15.8 - 25.8)	logN: 22.3 (16.9 - 44.1)	46.4 (33.5 - 67.8)
Gnaphalieae	-	34.5 (20.6 - 52.3)	-	72.2 (29.1 - 127.9)
Node F	-	15.6 (9.1 - 22.2)	-	31.3 (13.2 - 53.8)
Stoebe muirii	N: 6.0 (3.1 - 8.9)	4.2 (1.9 - 6.7)	N: 6.0 (3.1 - 8.9)	5.6 (3.0 - 8.3)
Stoebe passerinoides	N: 4.75 (1.8 - 7.7)	0.9 (5.4E-5 - 2.7)	N: 4.75 (1.8 - 7.7)	1.7 (5.2E-4 - 4.2)

	(c) As for Scheme (a) but with a logN prior allowing for a much older root node.		(d) As for Scheme (a) but with a narrower root prior corresponding closely to the range of estimates in Kim et al. (2005).	
	PRIORS	POSTERIORS	PRIORS	POSTERIORS
Root	logN: 33.1 (30.1 - 87.5)	57.2 (34.9-82.8)	N: 32.5 (26.2 - 38.8)	34.7 (28.8 - 40.3)
Ambrosia fossil	logN: 22.3 (16.9 - 44.1)	21.3 (15.9 - 28.6)	logN: 22.3 (16.9 - 44.1)	19.1 (15.9 - 23.3)
Gnaphalieae	-	48.3 (25.6 - 73.9)	-	28.5 (19.5 - 38.0)
Node F	-	21.3 (11.2 - 32.7)	-	13.4 (9.1 - 18.2)
Stoebe muirii	N: 6.0 (3.1 - 8.9)	4.9 (2.4 - 7.5)	N: 6.0 (3.1 - 8.9)	3.8 (1.7 - 6.0)
Stoebe passerinoides	N: 4.75 (1.8 - 7.7)	1.3 (1.4E-2 - 3.3)	N: 4.75 (1.8 - 7.7)	0.7 (1.9E-6 - 2.2)

	(e) As for Scheme (d) but *Ambrosia* with a narrower and older prior age distribution.		(f) As for Scheme (d) but leaving out the geological calibrations	
	PRIORS	POSTERIORS	PRIORS	POSTERIORS
Root	N: 32.5 (26.2 - 38.8)	37.2 (33.6 - 41.7)	N: 32.5 (26.2 - 38.8)	33.6 (27.7 - 39.4)
Ambrosia fossil	logN: 36.3 (33.9 - 41.7)	35.5 (33.3 - 37.9)	N: 22.3 (16.9 - 44.1)	19.1 (15.9 - 23.3)
Gnaphalieae	-	22.7 (15.6 - 30.1)	-	26.1 (17.2 - 35.9)
Node F	-	11.5 (7.1 - 15.6)	-	12.0 (7.9 - 16.5)
Stoebe muirii	N: 6.0 (3.1 - 8.9)	3.4 (1.5 - 5.7)	-	2.3 (0.6 - 4.1)
Stoebe passerinoides	N: 4.75 (1.8 - 7.7)	0.6 (9.5E-6 - 1.9)	-	0.2 (1.9E-7 - 0.8)

2.3.5. Ancestral area reconstruction

Conditional probabilities for ancestral states inferred with maximum likelihood represent the mapping confidence given the biogeographical data input into the analysis, and were all greater than or equal to 0.94 (those below 0.99 are indicated on Fig. 2-3). Biogeographic conclusions are least certain for more sparsely-sampled parts of the tree (ie the non-Relhaniinae). The earliest ancestral nodes of the Gnaphalieae (B – E) all optimize to southern Africa. The ancestors of the *Relhania*, *Metalasia* and *Stoebe* clades also optimize to southern Africa. There are four independent dispersals out of southern Africa: one to the afroalpine region in *Stoebe*, one to Australasia between nodes F and G, one to the Americas by *Chionolaena* and one to Eurasia by *Leontopodium*.

Table 2-4. Divergence age estimates in millions of years before present. Nodes are labelled as in Figs. 2-2 & 2-3. Values are the mean and 95 % HPD.

Node	Description	Mean	95% HPD
A	Root node	43.0	29.3 - 56.6
B	Gnaphalieae crown age	34.5	20.6 - 52.3
C	Gnaphalieae excluding *Relhania* clade	27.2	16.3 - 40.7
D	*Stoebe* clade + rest	24.1	13.9 - 34.9
E	Rest of Gnaphalieae (excluding *Stoebe* clade)	20.0	12.0 - 29.3
F	Australasian clade stem age	15.6	9.1 - 22.1
G	Australasian clade crown age	14.6	8.3 - 20.6
I	*Stoebe* clade crown age	12.2	5.3 - 20.0
J	*Metalasia* clade crown age	9.4	3.3 - 17.2
K	*Relhania* clade crown age	21.0	9.9 - 33.9
L	Sublade containing *Relhania*	13.6	6.4 - 22.3

2.4. Discussion

2.4.1.Topology and Systematics

2.4.1.1. Tribe Gnaphalieae

This study presents a robustly-supported phylogenetic hypothesis for relationships within Gnaphalieae, including representatives of 47 (25%) of the 185 currently-recognized genera in the tribe (Bayer et al., 2007) and members of all of Anderberg's (1991) subtribes except Loricariinae (see below). The analyses provide insight into relationships amongst regional Gnaphalieae from all continents, albeit with very sparse sampling from Eurasia and the Americas. Within the two largest areas of diversity, sampling is fairly representative for the Australian species but better for southern Africa.

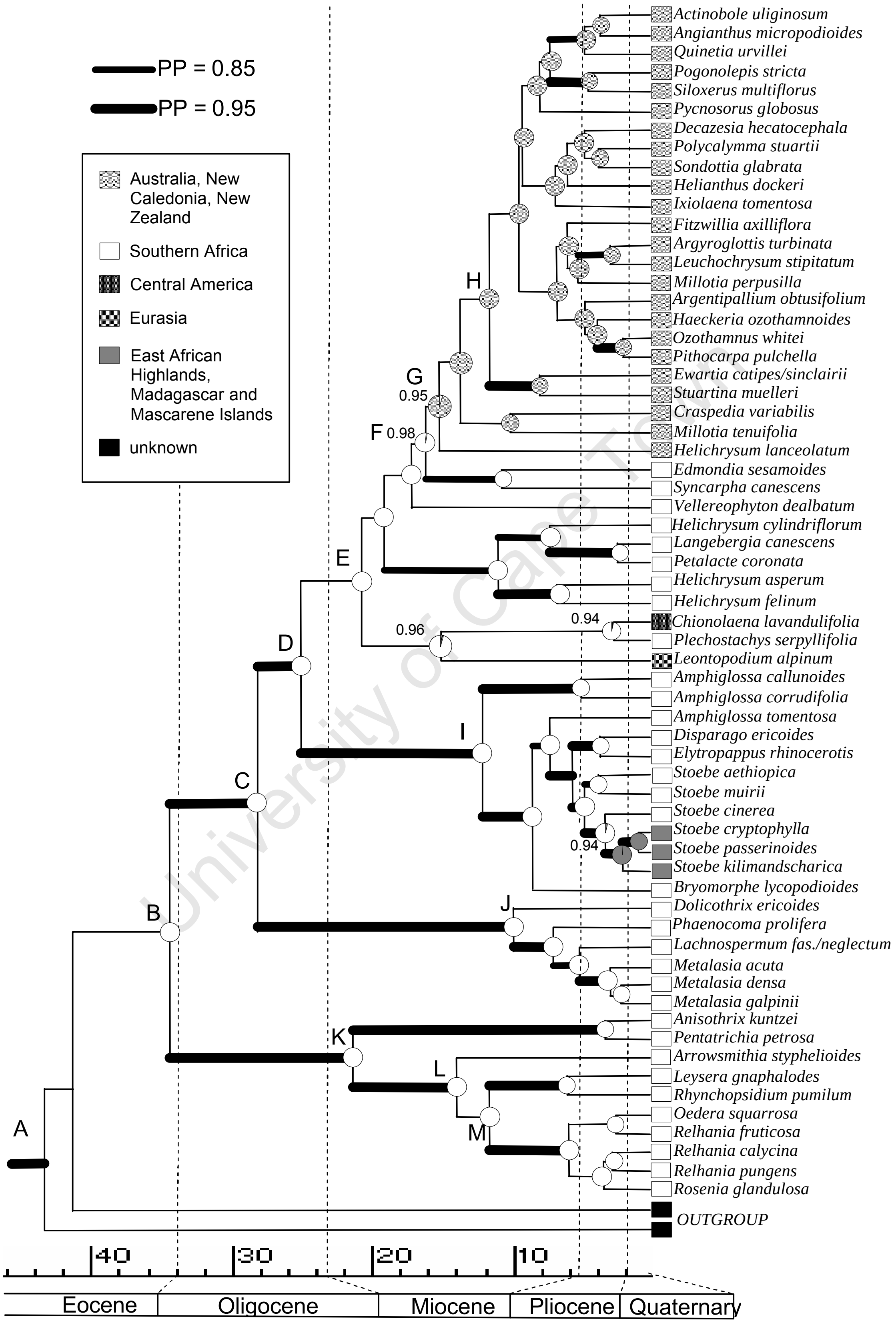
The tribe experienced its early diversification in southern Africa, resulting in at least three near-endemic basal lineages and a southern African paraphylum. Taxa from the rest of Africa, Eurasia, the Americas and Australiasia are embedded within this paraphylum, indicating multiple dispersals out of southern Africa after the initial diversification, apparently followed in many cases by radiations in other regions. For the southern African taxa, our topology is consistent with that of Bayer et al. (2000) but our addition of more DNA sequence data, and possibly the use of nucleotide substitution models in phylogenetic analysis, improved resolution and support. Relationships amongst the Australian and New Zealand taxa are poorly-resolved, and due to different taxon sampling cannot readily be compared with those found in previous analyses (Bayer et al., 2002; Breitweiser et al., 1999; Breitweiser & Ward, 2003). In the current tree, the species from Australasia are monophyletic, although support is lacking at node G. None of the above-mentioned studies included sufficient outgroup taxa to test for monophyly of the Australasian gnaphalioids.

Most of the South American taxa were placed by Anderberg (1991) in the subtribes Gnaphaliinae and Loricariinae. Although many taxa from the Gnaphaliinae are included in the present analysis, sequence data could not be obtained from any member of Loricariinae except *Pterygopappus lawrencii* from Tasmania. Both Bayer et al. (2002) and Breitweiser et al. (1999) found good support for the inclusion of this species with the Australasian taxa. The present study confrims this, but *P. lawrencii* was removed from the final analysis as its position was unstable within the Australasian clade. Anderberg (1991) placed the Loricariinae as (unsupported) sister to Relhaniinae, based on morphological cladistic analysis. However, the current study and the results of Bayer et al. (2000; 2002) show that the subtribes of Anderberg (1991) are not monophyletic. Relhaniinae is clearly not monophyletic, while the grade of southern African lineages descended from node E in Figs. 2-1 and 2-2 consists of both Gnaphaliinae and Cassiniinae. The Australasian clade (node G) contains members of Gnaphaliinae, Cassiniinae, the “basal group”, and Angianthiinae. Elucidation of subtribal relationships will require greater sampling from across the tribe (Bayer et al., 2007), and especially from poorly-sampled regions such as South and Central America. Greater geographic sampling for several large, widely-distributed and possibly polyphyletic genera will also be key to full understanding of gnaphalioid relationships (ie *Anaphalis* [ca. 110 spp.], *Gnaphalium* [ca. 80 spp.], *Helichrysum* [ca. 600 spp.], and *Pseudognaphalium* [ca. 90 spp.]).

2.4.1.2. Subtribe Relhaniinae

Early diversification produced the lineages that Anderberg (1991) assigned to the Relhaniinae. This subtribe is well-represented in the present analysis, as the eight unsampled genera share well-established morphological synaporphies with genera in the *Metalasia* clade or the clade subtended by node M (Anderberg, 1991; Karis, 1990). Thus further sampling is unlikely to change the finding that this Cape Floral Clade is not monophyletic, consisting instead of at least three independent lineages.

Fig. 2-3. (Opposite). Maximum clade credibility tree from relaxed phylogenetic analysis in BEAST. The timescale is in millions of years before present with ages from Gradstein et al. (2004). Squares represent geographic areas of extant taxa; circles represent geographical areas of ancestral taxa as inferred by maximum likelihood (ML) reconstruction. Numbers next to nodes indicate the ML probability of that state if it is less than 0.999. Line thickness indicates branch support.


PP = 0.85
PP = 0.95
▨ Australia, New Caledonia, New Zealand
☐ Southern Africa
▦ Central America
▩ Eurasia
■ East African Highlands, Madagascar and Mascarene Islands
■ unknown
A
B
C
D
E
F 0.98
G
0.95
H
I
J
K
L
M
0.96
0.94
0.94
Actinobole uliginosum
Angianthus micropodioides
Quinetia urvillei
Pogonolepis stricta
Siloxerus multiflorus
Pycnosorus globosus
Decazesia hecatocephala
Polycalymma stuartii
Sondottia glabrata
Helianthus dockeri
Ixiolaena tomentosa
Fitzwillia axilliflora
Argyroglottis turbinata
Leuchochrysum stipitatum
Millotia perpusilla
Argentipallium obtusifolium
Haeckeria ozothamnoides
Ozothamnus whitei
Pithocarpa pulchella
Ewartia catipes/sinclairii
Stuartina muelleri
Craspedia variabilis
Millotia tenuifolia
Helichrysum lanceolatum
Edmondia sesamoides
Syncarpha canescens
Vellereophyton dealbatum
Helichrysum cylindriflorum
Langebergia canescens
Petalacte coronata
Helichrysum asperum
Helichrysum felinum
Chionolaena lavandulifolia
Plechostachys serpyllifolia
Leontopodium alpinum
Amphiglossa callunoides
Amphiglossa corrudifolia
Amphiglossa tomentosa
Disparago ericoides
Elytropappus rhinocerotis
Stoebe aethiopica
Stoebe muirii
Stoebe cinerea
Stoebe cryptophylla
Stoebe passerinoides
Stoebe kilimandscharica
Bryomorphe lycopodioides
Dolicothrix ericoides
Phaenocoma prolifera
Lachnospermum fas./neglectum
Metalasia acuta
Metalasia densa
Metalasia galpinii
Anisothrix kuntzei
Pentatrichia petrosa
Arrowsmithia styphelioides
Leysera gnaphalodes
Rhynchopsidium pumilum
Oedera squarrosa
Relhania fruticosa
Relhania calycina
Relhania pungens
Rosenia glandulosa
OUTGROUP
40
30
20
10
Eocene
Oligocene
Miocene
Pliocene
Quaternary

Relhania and relatives fall into the earliest-diverging lineage of the tribe, the *Relhania* clade, while the remaining taxa belong to the *Metalasia* and *Stoebe* clades. The phylogeny thus provides smaller, well-defined clades within the Relhaniinae for future species-level analysis, although future studies should include specimens of *Trichogyne* and *Lasiopogon*, identified by Bayer et al. (2000) as possible close relatives.

The *Metalasia* and *Stoebe* clades are not supported as sisters despite sharing the complex character of anomalous secondary stem thickening, and this character should be investigated further, with broader taxonomic sampling.

Instead of a single Cape radiation there are at least three independent, smaller Cape-centred diversifications associated with the former Relhaniinae, as the *Relhania* group of genera (node L), the *Stoebe* clade and the *Metalasia* clade are all Cape-centred, with crown ages in the Late Miocene. Independent diversification in these three groups may thus have occurred at roughly the same time, consistent with the hypothesis that a common environmental trigger might be responsible for the diversification of the species-rich Cape clades. Although several dated Cape diversifications are older or younger than the Late Miocene (Linder, 2005), this is amost commonly-inferred time for the onset of many Cape radiations (Linder, 2005), e.g. Cape *Indigofera* (13.1 Ma; Schrire et al., 2003), *Ehrharta* (9.8 Ma; Verboom et al., 2003); *Muraltia* clade II (19.3; Forest et al., 2007). All these groups have substantial endemism in summer-arid and/or fire-prone habitats and it is possible that their diversification around this time is linked to the establishment of these conditions. In this regard, it is interesting to note that *Amphiglossa* s.s. (*A. callunoides* + *A. corrudifolia*) is one of the most species-poor lineages within the *Stoebe* clade and is confined to river beds, presumably due to the presence of a higher water table. It constitutes the earliest-diverging lineage in the clade which may be the result of the ancestor being confined to more mesic environments and consistent with a scenario of radiation into the developing Mediterranean-type climate of the Cape in the Late Miocene, as was found by Verboom et al. (2003) for *Ehrharta*.

Each of these newly-identified, smaller Cape Floral Clades within the former Relhaniinae provides and independent test of the causes of diversification in the region. Such studies will require a detailed species-level analysis of each clade to examine the mode and timing of its diversification in the Cape.

Sparse sampling in the current study precludes the testing of rates of diversification within each clade. From the tree in Fig. 2-3 it appears that most of the diversification in Gnaphalieae has been relatively recent (i.e. since the Miocene). However, there is still little information on rates of extinction and how these might have changed through time. The stem lineages of al Cape members of Gnaphalieae were present in the region for substantial periods of time prior to the development of the summer-arid climate (nodes B – D in Fig. 2-3). From this it can be hypothesised that the success of Gnaphalieae in the Cape region may be due to pre-adaptation in the ancestral taxa for summer-arid conditions, as well as the arid environments later colonised with such success in central Australia.

2.4.1.3. Proposed taxonomic changes to subtribe Relhaniiinae

The taxonomy of the Relhaniinae sensu Anderberg (1991) clearly requires revision. Description of the *Relhania* clade and Relhaniinae s.s. awaits greater sampling. However, the *Stoebe* and *Metalasia* clades are shown to be independent monophyletic entities and should recognised in order to uphold monophyly. I propose two new subtribes, the Stoebinae and the Metalasiinae, each named after the largest genus in the clade. Note that although these subtribes are described below, they will be formally (validly) published elsewhere.

Both the Stoebinae and the Metalasiinae share the following characteristics: they are thin-barked woody shrubs or sub-shrubs, with leaves small, entire, sessile, involute, adaxially white-tomentose, abaxially frequently with a skin-like indumentum and glabrous or variously hairy, linear to oblanceolate (rarely deltoid), apiculate to mucronate, frequently twisted and often borne in fascicles. Capitula are terminal, borne singly or grouped into synflorescences; small, generally cylindrical, few- to many- flowered, mostly discoid, with white, pink or purple (rarely colourless or pale yellow) corolla. Involucral bracts are imbricate, increasing in size from the outside inwards; outermost bracts are often leaf-like. Style branches are linear, truncate and terminally penicillate with two inner parallel stigmatic surfaces. Anthers are apically appendaged, basally with branched tails. Pappus bristles are caducous or persistent, nude to barbellate to long-plumose. Achenes are cylindrical to slightly angled, frequently ribbed, glabrous, papillose or villous.

The following characters separate the two groups:

1. Apices of inner or all involucral bracts expanded, petaloid (opaque and coloured pink, white or [rarely] various shades of yellow); pappus bristles nude to barbellate, caducous……..…………..….…………..….**Metalasiinae**
2. Apices of involucral bracts undifferentiated, pale straw-coloured; chartaceous or cartilaginous; pappus bristles long-plumose from the base or in the upper part only, usually persistent…....……………….…………………**Stoebinae**

Metalasiinae Bergh.

Type: *Metalasia* R. Br.

Leaves usually eglandular, capitula solitary or in loose to dense terminal umbelliform to almost spherical synflorescences, free or fused together by hairs, discoid (disciform in one species, *Phaenocoma prolifera*). Lamina of the inner or all involucral bracts frequently opaque, and coloured white, pink or (rarely) various shades of yellow. Florets 3 – 50 (but see *Phaenocoma*). Pappus bristles caducous, nude or scabrid to barbellate; apically usually slightly broadened and serrate to dentate, rarely entire, flattened or sometimes flat, often thicker and rounded to clavate at the apex, opaque white or occasionally coloured or translucent.

Member genera: *Atricantha* Hilliard & Burtt; *Calotesta* Karis, *Dolicothrix* Hilliard & Burtt, *Hydroidea* Karis, *Lachnospermum* Willd., *Metalasia* R. Br., *Phaenocoma* D. Don, *Planea* Karis.

Stoebinae Bergh.

Type: *Stoebe* L.

Branches often thin and wiry, rarely plants spinescent or stoloniferous. Leaves eglandular or glandular with sessile or stalked glands. Capitula solitary, or few to many together in loose to dense terminal globular, cylindrical or leafy, spicate synflorescences, homogamous, discoid or heterogamous, radiate, 1 – 15 flowered. Outermost involucral bracts leafy, becoming scarious or cartilaginous and increasing in size inwards, sometimes with a conspicuous white-tomentose abaxial patch. Pappus of bristles or rarely wanting, persistent, shaft ± terete, barbellate below and plumose to long-plumose apically or plumose for entire length. Ray florets (if present) female or sterile, three-lobed, bilabiate or pseudobilabiate, with short lamina, pink or white. Member genera: *Amphiglossa* DC. , *Bryomorphe* Harv., *Disparago* Gaertn., *Elytropappus* Cass., *Stoebe* L..

2.4.2. Divergence times and biogeography

2.4.2.1. Calibration schemes and age estimates

The sensitivity analysis indicates that it is mainy the older nodes that are affected by different calibration schemes, and that these node ages rely heavily on the root node calibration to keep them in the younger range of age estimates. Despite this, the estimate of an Eocene age for the stem divergence between Gnaphalieae and other members of subfamily Asteroideae (root node, A) is older than would be predicted by many authors, who estimate that the entire family Asteraceae is of Eocene or even Oligocene age (Raven and Axelrod, 1974; Graham, 1996; Bremer and Gustafsson, 1997; Funk et al., 2005; Anderberg et al., 2007). However, this young age for the family is based largely on the absence of pre-Oligocene fossils, and Turner (1977) and Muller (1970) have argued that the Oligo-Miocene fossil presence of Asteraceae indicates not the origin but the increase in abundance of the family, since the modern tribes are all represented in their current geographic positions. Thus this perhaps controversially old date for the root node is not actually contradicted by any good fossil evidence, and an even older age for node A (calibration schemes b and c) might also be possible. The youngest age estimate for the root node in the current study was obtained with calibration scheme (f), showing the importance of the root calibration and that the geological calibration points serve to push the age of the root node backwards in time.

The calibration priors for the root were derived secondarily from the previous dating exercises of Kim et al. (2005). However, potential pitfalls of using such secondarily-derived calibration ages were taken into account in several ways. Firstly, the prior distribution was set to span a much broader age range than the estimates of Kim et al. (2005): their estimate using both a slow and a fast estimated rate of angiosperm cpDNA molecular evolution ranged from 35 – 39 Ma, while their NPRS dates calibrated with an outgroup fossil ranged from 26 – 29 Ma. I implemented a normal prior distribution centred at the midpoint of this range of dates (i.e. the midpoint between 26 and 39 Ma) and with a 95% confidence interval from 14.1 to 50.9 Ma. Secondly, although I report the means of the posterior age estimates, the inferences in the following section take into account the full 95% HPD of the posterior ages. For example, I discuss below the Miocene diversification of several Cape groups. These all have mean ages in the Miocene, but their 95% HPD also fall within the Miocene epoch, with the exception that the upper end of the interval in two of the clades spans the start of the Pliocene. Point date estimates are never considered to be particularly accurate in the present paper. For this reason, the age estimates from calibration scheme (a) are considered fairly conservative. In the absence of compelling evidence that any of the alternative calibration schemes are more accurate, the results focus on the posterior distributions generated with this scheme, and the alternative calibration schemes are presented in order to show the sensitivity of these dates to alternative settings.

2.4.2.2. Age of the Gnaphalieae

The crown age of tribe Gnaphalieae (node B) is estimated to be 34.5 (20.6 – 52.3) Ma, indicating that early diversification occurred around the Late Eocene. All calibration schemes except for Scheme (b) with no constraint on the root, place this diversification at or after the peak in global temperatures around 50 Ma (Zachos et al., 2001) which presaged global climatic cooling and, in Africa, the subsequent expansion of open habitats together with the development of arid-adapted vegetation

(Bobe, 2006).

2.4.2.3. Early divergence in Gnaphalieae: the southern African lineages

Early diversification of the tribe occurred in southern Africa, and due to fairly good taxonomic sampling this inference is unlikely to be changed by future analyses of the *Stoebe* or *Metalasia* clade. However, several unsampled Mediterranean and Eurasian genera from Anderberg's "basal group" (*Athrixia, Phagnalon* and *Aliella*) are affiliated with *Anisothrix* and *Pentatrichia* in the *Relhania* clade (N. Montes-Moreno, pers. comm.; Fig. 2-2). Since this lineage represents the earliest split in the tribe, inclusion of these taxa in ancestral area analysis might indicate a different geographic origin for the Gnaphalieae.

Another factor influencing the inferred point of origin of the tribe is the geographic distribution of the sister group. The sister relationship of Gnaphalieae remains poorly tested (Funk et al., 2005, Bayer et al., 2007). Despite strong support for Anthemidae + Astereae as sister to Gnaphalieae in the analysis of Panero & Funk (2008), each of these tribes is represented by only three species. Clarification of the relationships at the base of Gnaphalieae might produce novel insights into where the tribe arose. However, even if the ancestor of the tribe occurred outside southern Africa, the region has clearly played a major role in the evolution of the tribe. In this regard, there are parallels with the geographic history of several related lineages in Asteraceae. The splits at the base of subfamily Asteroideae leading to the tribes Anthemideae, Senecioneae [especially subtribes Senecioninae and Othonninae] and Calenduleae, as well as *Corymbium* and Cichorioideae s.s. are all postulated to have had a southern African origin and/or diversification (Funk et al., 2005; Oberprieler, 2005; Pelser et al., 2007; Himmelreich et al., 2008). Most of these groups have substantial diversity within the region but have also dispersed out of southern Africa and independently radiated in the Mediterranean, Australia and the Americas. The southern African region has thus been an important cradle of diversification for a large part of the daisy family, as well as an 'evolutionary springboard' from which multiple lineages colonized the rest of the world.

2.4.2.3. Dispersals out of the southern African region

After initial diversification in Gnaphalieae, there were multiple dispersals out of southern Africa. Several of these have been captured in the current study but there are additional, independent dispersal events which we have not sampled, suggesting that the observed events (discussed below) under-represent the true number. For example, the genera *Leysera* and *Ifloga* each have a southern African-centred distribution with one or more species in the Mediterranean region (Bremer, 1978; C. H. Trisos et al., in prep.), indicating at least two additional dispersal events to the Mediterranean region. Directional dispersal from southern Africa to the Mediterranean may have been especially prevalent in Gnaphalieae. The current study indicates that the tree of Mediterranean *Helichrysum* produced by Galbany-Casals et al. (2004) has a southern African root. If this is true, their phylogeny indicates at least five dispersal events from southern Africa to the Mediterranean Region, and one from there to Asia.

Four of the species of the otherwise Cape-centred *Stoebe* clade occur in the ericaceous vegetation which characterizes the lower margin of the afroalpine zone (Hedberg, 1970). Three of these species were sampled: *S. kilimandscharica* from east Africa; *S. passerinoides* from Réunion, and the Madagascan *S. cryptophylla*. The unsampled taxon, *S. pachyclada*, is likely on morphological grounds to be closely related to *S. cryptophylla* (Koekemoer, 2002) and the afroalpine distribution thus appears to be the result of a single, recent dispersal event out of the Cape (Fig. 2-3) dated between 2.0 (0.4 – 4.0) Ma and 3.5 (1.1 – 6.0) Ma. This is consistent with the results of Galley et al. (2006), who showed overwhelmingly unidirectional dispersal from the Cape to the Afrotemperate and afroalpine regions during the last 17 Ma, and provides strong evidence to disprove the hypothesis of Levyns (1964) that *Stoebe* arose “in the north, leaving behind scattered records of its journey southwards, and finally undergoing intensive speciation in the south-west”. A peripatric speciation model following dispersal to East Africa seems most probable in *Stoebe*, as each sub-afroalpine region (mainland Africa, Madagascar, and La Réunion) has its own endemic species or species pair. The estimated age of 0.9 Ma for *S. passerinoides* is consistent with the 2 Ma age of La Réunion’s alpine zone postulated by McDougall (1971) and Gillot et al. (1994).

Three other dispersals out of Africa are evident (Fig. 2-3). Dispersal to Eurasia is inferred for the ancestor of *Leontopodium* and this event is potentially as old as the node uniting *Leontopodium* with *Plecostachys* and *Chionolaena,* dated to the middle Miocene. Dispersal to Central and South America by *Chionolaena* appears to have been more recent, in the Pliocene or later (Fig. 2-3). The reconstruction indicates that the common ancestor of *Chionolaena* and *Plecostachys* was located in southern Africa, implying dispersal directly from Africa to Central America. However, sparse sampling of Eurasian and American taxa means that dispersal to the Americas via Europe or elsewhere cannot be excluded. With no information on the monophyly of the South American taxa, it also cannot be determined whether the dispersal by *Chionolaena* represents the founding of the South American everlasting flora (a 'single origin' hypothesis) or whether it is only one of many colonisations of that continent, perhaps from multiple different regions and at different times ('multiple origins' hypothesis). The only other South American taxon known by the present author to have been included in molecular phylogenetic analysis is *Lucilia araucana* from Argentina, placed by Anderberg (1991) in the Gnaphaliinae. In the analysis of Breitweiser et al. (1999), *L. araucana* was strongly supported as sister to a New Zealand species, which together with the results of the current study supports the 'multiple origins' hypothesis for Gnaphalieae from the Americas.

2.4.2.4. Trans-Indian Ocean dispersal

The gnaphalioid taxa of Australia and New Zealand appear to have been founded by a single dispersal event from Africa, since they all share a common ancestor at node G (Figs. 2-2 and 2-3), and this lends support to a 'single origin' hypothesis for the Australasian taxa (Bayer et al., 2002).

The results presented here support direct long-distance dispersal to Australia across the Southern Indian Ocean, because the Australasian taxa are embedded within a grade of African lineages (nodes E – F; Fig. 2-3) not within a Eurasian clade as might be expected if they reached Australia via stepping-stone dispersal though Asia. The same geographic pattern was recovered by Galbany-Casals et al. (2004) in a study of mainly Mediterranean gnaphalioids. Although the taxa they sampled are mostly different to those in the present study, they recovered clades which match those found in Fig. 2-1, and showed, with good support (BS = 80 % at all relevant nodes), that Australian and New Zealand taxa are sister to species from the Cape, while Eurasian species are embedded in a separate southern African grade. If the Australian species are the result of such long-distance dispersal, they might be expected to have inherited the ability to disperse long distances, and indeed Australian gnaphalioids have clearly achieved the ca. 2,000 km oceanic crossing from Australia to New Zealand multiple times (e.g. Breitweiser et al., 1999; Breitweiser and Ward, 2003).

The dispersal to Australia is estimated to have occurred between 14.6 (8.3 – 20.6) and 15.6 (9.1 – 22.1) Ma, in the Miocene (Fig. 2-3; Table 2-4) with the oldest estimate (Table 2-3, Scheme [b]) at 31.3 (13.2 – 53.8). This is consistent with the fossil record which suggests that Asteraceae arrived in Australia well after the isolation of the continent more than 35 Ma (Crisp et al., 2004; Stover and Partridge, 1973; Martin, 1973). Novel evidence of long-distance trans-oceanic dispersal has opened new discussion on the mechanisms involved (Knapp et al., 2005; de Queiroz, 2005; Sanmartín et al., 2007). Long-distance dispersal by wind has been implicated in the distribution of Southern Hemisphere extra-tropical lichens, bryophytes and pteridophytes (Muñoz et al., 2004) and may be facilitated by the westerly winds, which increase in force with greater latitude in the Southern Ocean (‘roaring forties’, ‘furious fifties’ and ‘shrieking sixties’). Although seeds in Gnaphalieae can be small and light, and possess a pappus which might enhance wind dispersal, it is unknown whether they could be blown across the ca. 8000 km of ocean separating Africa from Australia. Another possibility is oceanic rafting, which would be facilitated by the

Table 2-5. (opposite) Mean ages and likely directions of trans-Indian Ocean dispersals. Molecular age estimates were derived from the original reference, in which case the methods are outlined in the third column, or from the relaxed Bayesian analyses of Verboom et al. (2009).

Inferred direction of movement	**Family**	**Phylogeny and age estimate based on:**	**Taxa involved**	**Age estimate (Ma)***	**Reference**
From Australasia to Africa	Poaceae	Chloroplast and nuclear DNA sequences	Ehrharteae	38.7	Verboom et al. (2003) Verboom et al. (2009)
	Proteaceae	Chloroplast DNA sequences and fossil-calibrated relaxed Bayesian clock	*Adenanthos* - Leucadendrinae	34.25	
			Aulax - *Petrophile*	55.95	Sauquet et al. (in press)
	Iridaceae	Chloroplast DNA sequences; NPRS calibrated with an estimate from a prior analysis.	*Patersonia* - *Geosiris*	51	Goldblatt et al. (2002)
	Restionaceae	Chloroplast DNA sequences	Basal split	52.57	Linder et al. (2003) Verboom et al. (2009)
	Cyperaceae	Chloroplast DNA sequences	Reticulate-sheathed *Tetraria* clade	9.2	
			Capeobolus - *Cyathocoma*	1.86	
			Non-reticulate-sheathed *Tetraria* clade	12.76	
			Neesenbeckia punctoria	10.22	Verboom (2006) Verboom et al. (2009)
From Southern Africa to Australasia	Chenopodiaceae	Chloroplast and nuclear DNA sequences; strict clock analysis calibrated with Chenopodiaceae macrofossils and fossil pollen.	*Sarcocornia*	2.9	Kadereit et al. (2005; 2006)
	Brassicaceae	ITS sequences and a local molecular clock calibrated using fossil data.	*Lepidium*	0.55	Mummenhoff et al. (2004)
	Zygophyllaceae	Chloroplast DNA sequences and relaxed Bayesian clock	*Zygophyllum*	4.53	Bellstedt et al.(in press)
	Cyperaceae	Chloroplast DNA sequences	*Trianoptiles* - *Carpha*	13.03	Verboom (2006) Verboom et al. (2009)
	Asteraceae	This study	Australasian *Gnaphalieae*	15.1	This study
	Asteraceae	Nuclear ITS DNA sequences; NPRS dating	*Leptinella* - *Soliva* - *Cotula*	7.7	Oberprieler (2005) Himmelreich et al. (2008)
	Geraniaceae	Chloroplast, mitochondrial and nuclear DNA sequences	*Pelargonium havlase* - *P. australe* clade	6.11	Bakker et al. (2004; 2005) Verboom et al. (2009)
	Poaceae	Chloroplast and nuclear DNA sequences	*Rytidosperma*	1.77	Verboom et al. (2006) Verboom et al. (2009)

* Dispersal of a disjunct clade could have occurred anywhere along the branch between the stem and crown nodes. Where age estimates existed for both stem and crown nodes, we used the mean of the total range (ie the range from the youngest estimate for the crown node to the oldest estimate for the stem node). If stem node age was not available we took the oldest estimated age for the crown node.

West Wind Drift (WWD), a large surface current flowing from west to east in the Southern Ocean. West Wind Drift would take roughly a year to float objects between South Africa and Australia (Waters and Roy, 2004). Tolerance to sea-water in the seeds of Gnaphalieae is unknown, but flotation for a year seems unlikely. Other mechanisms are possible, such as rafting above the water on floating vegetation, or attachment to the feet or feathers of migrating birds, as shown for *Lepidium* (Brassicaceae, Table 5; Carlquist, 1983; Mummenhoff et al., 2004). *Lepidium* has mucilaginous seeds, as does *Zygophyllum*, another successful colonizer of Australia from Africa (Beier et al., 2003; Table 2-5). Inter-continental dispersal in *Sarcocornia* (Table 2-5) and well-dispersed relatives in the Chenopodiaceae is likely to be facilitated by their adaptations to dispersal by salt water and water birds (Kadereit et al., 2006). However, long-distance trans-oceanic dispersal has been inferred for many terrestrial organisms that possess no obvious adaptations to facilitate such dispersal (e.g. de Queiroz, 2005; Sanmartin et al., 2007; Muñoz et al., 2004; Barker et al., 2007). A survey of the literature was conducted for other Southern Hemisphere plant groups which exhibit trans-Indian Ocean disjunction, in which the direction of dispersal could reasonably be inferred and for which dated phylogenetic hypotheses are available. The age of each dispersal event was measured as the mean of the estimated ages of the nodes at either end of the branch along which the disjunction occurred (Table 2-5). In ten angiosperm families (including the present Gnaphalieae example), evidence was found for 17 disjunctions whose estimated ages ranged from 0.5 to 55.9 Ma (Table 2-5). This rules out Gondwanan vicariance scenarios because Africa had separated from other Gondwanan land masses by 100 Ma (Scotese, 2008). If the original study did not provide evidence for the direction of dispersal across the Indian Ocean, this was inferred from the phylogeny using a strict parsimony criterion (minimizing the number of dispersals). Nine of the dispersal events are from Australasia to Africa and occurred between 1.8 and 55.9 Ma (mean and standard deviation 29.6 ± 21.3 Ma). The remaining eight are in the opposite direction and occurred between 0.5 and 15.1 Ma (6.5 ± 5.2 Ma). These means are significantly different (t-test, $p = 0.007$). Thus dispersals from Australia to Africa have been ongoing since the Eocene, while movements in the opposite direction have only occurred since the start of the Miocene epoch.

There are several other groups which exhibit dispersal eastwards across the Indian Ocean from southern Africa: the grass genus *Pentaschistis* (Galley and Linder, 2007); *Wurmbea* in the Colchicaceae (Case et al., 2008); *Gunniopsis* in the Aizoaceae (Klak et al. 2003); *Cymbonotus* (Asteraceae; Funk et al., 2007); *Leptinella* (Asteraceae; Himmelreich et al., 2008) and Senecioneae (Asteraceae; four dispersals from Africa to Australasia; Pelser et al., 2007). These were excluded from the analysis because age estimates are not yet available. However, the authors of these studies all estimate the dispersals to be Miocene or younger (Vinnersten and Bremer, 2001; A. Case, pers. comm.; C. Klak, pers. comm.; V. Funk, pers. comm.; P. Pelser, pers. comm.). Assuming that this pattern is not due to biased extinction or different ages of continental habitats, the fact that eastwards dispersal occurs only after the Oligocene might implicate the West Wind Drift (WWD) in these events, because full establishment of this surface circumpolar current only occurred after the deepening of oceanic passageways separating Antarctica from South America ca. 41 Ma (Scher and Martin, 2001) and from Australia ca. 35 Ma (Stickley et al., 2004). The WWD is independently invoked in the gnaphalioid dispersal if, as proposed by Bayer et al. (2002), initial colonization was to the Bassian biotic region of southeastern Australia and Tasmania. This is because WWD is likely to raft propagules from southern Africa not to the closest landfall (Western Australia) but to these south-easternmost regions (New South Wales and Tasmania; Waters and Roy, 2004). This raises the possibility of oceanic rafting as a primary factor in long-distance Angiosperm colonization of Australia from southern Africa.

From the above it can be hypothesised that, although the two main centres of Gnaphalieae diversity, southern Africa and Australia, have similar numbers of species, they have very different histories of species accumulation. In southern Africa, species may have been accumulating since the Eocene, while in Australia the diversity appears to be the result of rapid radiation from perhaps a single ancestor that colonised the continent only in the Miocene. This suggests that speciation/extinction ratios amongst the two regions are very different and opposite to those for the

Restionaceae (Linder et al., 2003).

2.4.3. Conclusions

Tribe Gnaphalieae may have originated in southern Africa in the Eocene and this region has served as a cradle of gnaphalioid diversification, accounting for the large number of species represented in the southern African region. The former Cape Floral Clade Relhaniinae is shown to be paraphyletic with members falling into the three earliest-diverging lineages of the tribe: the *Relhania* clade, the *Metalasia* clade and the *Stoebe* clade. These all had southern African ancestors, and the Cape-centred clades all began diversifying in the Miocene. A well-sampled phylogenetic hypothesis for the tribe and its closest relatives, including taxa from the Americas, is required for further biogeographic insight and to provide the framework for future evolutionary studies. However, the present study indicates that dispersal out of southern Africa has been ongoing since the early Miocene. Only one successful dispersal event around 15 Ma is required to explain the presence of the tribe in Australasia, and diversity here may be the result of rapid radiation. West-to-east trans-Indian Ocean dispersal appears to have been occurring only since the Miocene, potentially implicating the WWD in long-distance plant dispersal.

CHAPTER 3

Species-level relationships in the *Stoebe* clade: generic circumscription and the evolution of macromorphological characters.

3.1. Introduction

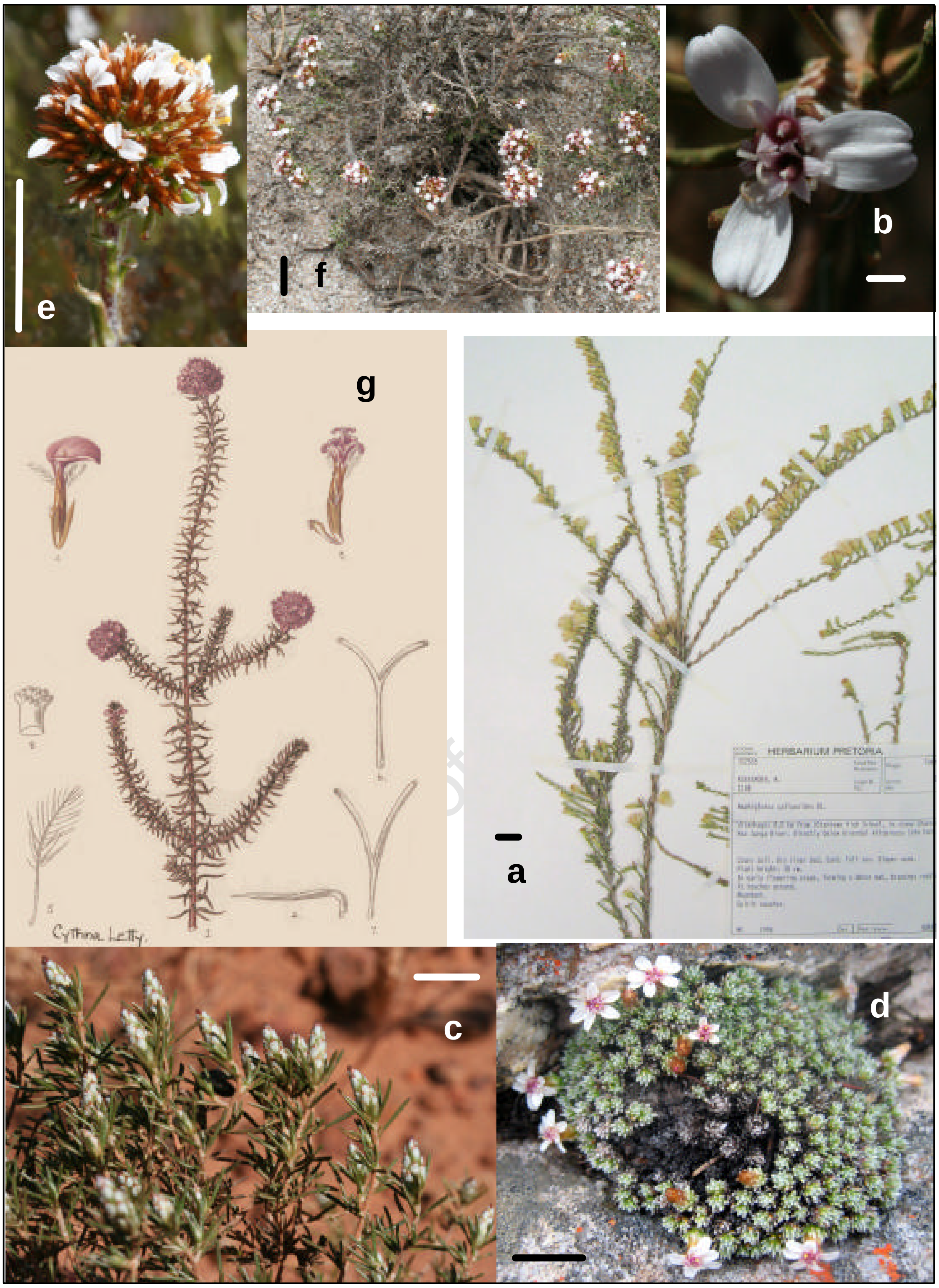
3.1.1. Rationale

The goal of modern taxonomy is to produce predictive, stable classifications that reflect true evolutionary history. In the absence of a phylogenetic tree of relationships and with no independent information on the evolutionary relationships amongst species, competing classification schemes are in essence subjective decisions as to which characters exhibit more or less homoplasy. These competing hypotheses can only be tested with a well-supported and resolved phylogenetic hypothesis for the group of interest. Supraspecific taxa that correspond to monophyletic entities can then be identified from the phylogeny, which can also be used to examine the evolutionary histories of morphological, anatomical and life history attributes. Character transformations can thus be placed in their evolutionary context, and homoplasious transitions identified.

Currently, there is no hypothesis of phylogenetic relationships amongst species in the *Stoebe* clade. This is the Cape Floral Clade identified in Chapter 2 of the current study. Prior to this analysis, the taxa in the *Stoebe* clade were thought to belong to Anderberg's (1991; 1994) *Metalasia* group of genera, together with the large genus *Metalasia* and several smaller allied genera. Phylogenetic analyses have been conducted for the entire *Metalasia* group, using genera as terminals and characters derived from morphology (Anderberg, 1991; Koekemoer, 2002). However, the *Metalasia* group is not monophyletic; the use of genera as terminals does not allow testing of generic monophyly, and the small number of characters obtainable from morphological data makes a critical evaluation of competing hypotheses of character homology difficult. It is now possible to conduct a phylogenetic analysis at the level of species, using molecular data to obtain statistical measures of node support.

The five currently-recognised genera in the Stoebe clade include a total of 62 species. These genera (*Amphiglossa*, *Bryomorphe*, *Disparago*, *Elytropappus* and *Stoebe*; Table 3-1) are distinguished by a suite of morphological characters that occur in various combinations and exhibit a large degree of homoplasy amongst genera (Lessing, 1832; De Candolle, 1838; Harvey & Sonder, 1894; Levyns, 1935; 1936; 1937; Koekemoer, 2002). A species-level phylogeny using an independent set of characters to reconstruct the tree will test whether the homoplasy is due to non-monophyly of genera or alternatively, to morphological characters being highly homoplasious with parallel series of character variation in each genus. The following section is a brief overview of the current taxonomy of the clade.

Fig. 3-1. (opposite) Illustration of morphological variation in *Amphiglossa, Bryomorphe* and *Disparago*. (a) – herbarium specimen of *Amphiglossa callunoides* showing stoloniferous growth form and solitary capitula. (b) Capitulum of *A. tomentosa* to show radiate head with three ray and several disc florets. (c) Side view branch tips of *A. tomentosa* to show arrangement of heads and the white tomentose patches on the involucral bracts. (d) – *Bryomorphe lycopodioides*, entire plant. (Photo: R. de Roo). (e) *Disparago anomala*, globose secondary arrangements of heads, each head with one ray and one disc floret. (f) – *D. ericoides*, habit. (g) – illustration of *D. tortilis* showing 1, flowering twig; 2, twisted leaf; 3, discoid head; 4, radiate head; 5, pappus bristle; 6 & 7, style branches; and 8, tip of style branch. (Scale bars all 1 cm except in (b) = 1 mm. All scans and photographs by N. G. Bergh unless otherwise indicated).


e
f
b
g
Cythna Letty
HERBARIUM PRETORIA
a
c
d

3.1.2. Taxonomic background:

The CFR members of the *Stoebe* clade were most recently revised by Goldblatt & Manning (2000); their nomenclature is followed hereafter for these species, while the latest published revision is followed for those occurring outside of the CFR.

***3.1.2.1. Amphiglossa* DC.** Type: *A. corrudifolia* DC

The genus *Amphiglossa* as currently circumscribed has 11 species and was most recently revised by Koekemoer (1999). Capitula in *Amphiglossa* are either radiate (possessing both ray and disc florets; Fig. 3-1b) or discoid (possessing only disc florets). *Amphiglossa* species are distinguished from other members of the *Stoebe* clade by their generally widely-spaced leaves, relatively large capitula borne singly or few together at the branch tips (Fig. 3-1c) and containing three or more florets, and usually thick, cartilaginous involucral bracts with a patch of white tomentose hairs on the abaxial surface (Fig. 3-1c). In addition, the florets are generally more slender than those in other genera and the pappus bristles are long-plumose all the way to the base. It is primarily a genus of arid habitats, occurring mainly in the Northern Cape, Namibia, and the Little Karoo.

***3.1.2.2. Bryomorphe* Harv.** Type: *B. lycopodioides* (Sch. Bip) Levyns

This tiny, monotypic genus looks like a large moss when sterile (Fig. 3-1d). The numerous florets per capitulum (seven to nine pink disc and six to seven white ray florets) give this species the most typical ‘daisy-like’ flowerheads, as all other species in the *Stoebe* clade have fewer rays. Disc florets are tubular with large, reflexed corolla lobes. It grows on thin sheets of soil on boulders at moderate to high altitudes in the Western Cape mountains.

***3.1.2.3. Disparago* Gaertn.** Type: *D. ericoides* (Berg.) Gaertn.

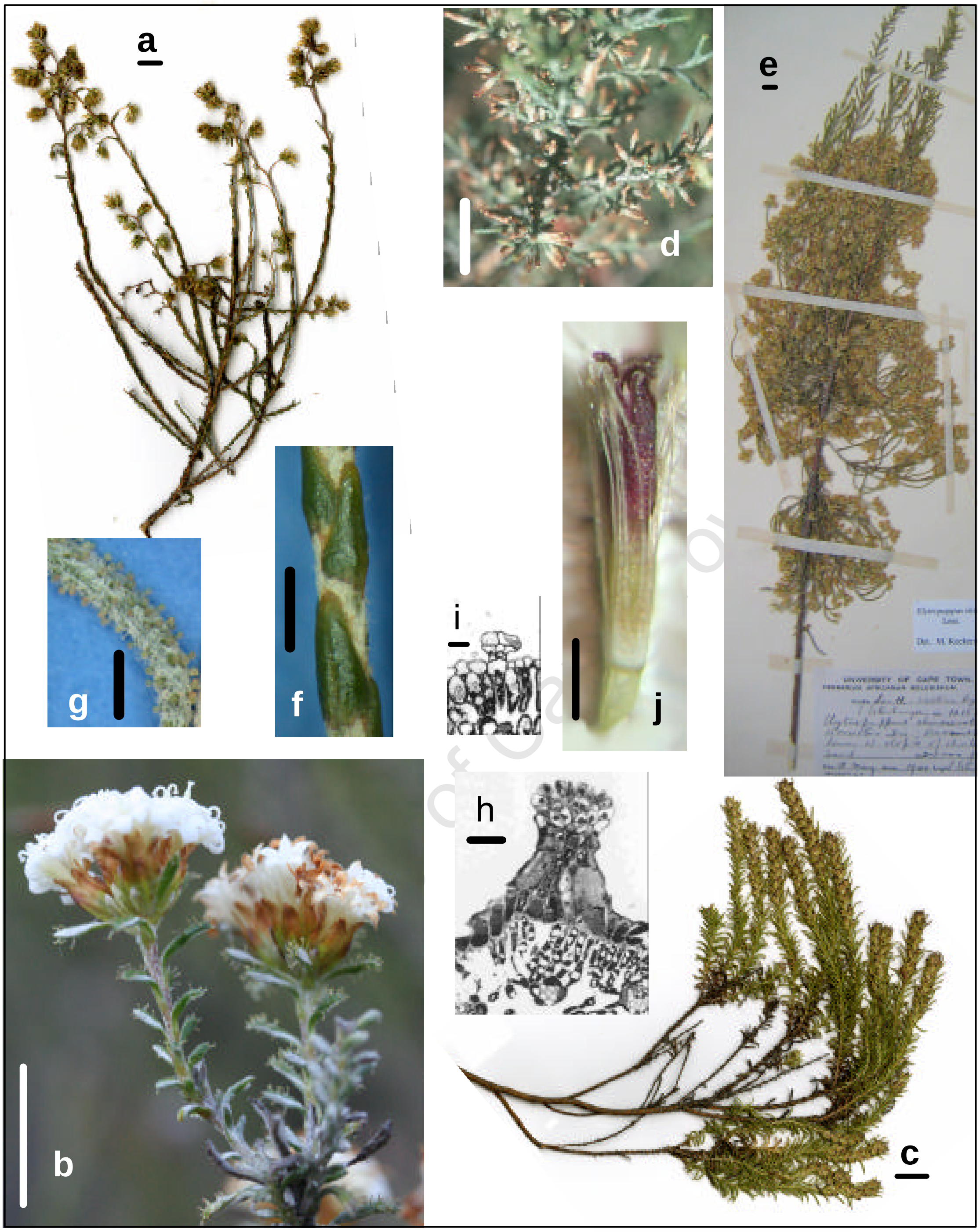
Disparago is a group of erect or sprawling shrublets defined by the presence of both ray and disc florets in the same capitulum (with the exception of *D. tortilis*, see below). It is distinguished from *Amphiglossa*, which has several taxa with radiate heads, by the much smaller heads arranged into rounded synflorescences (Fig. 3-1e) and by the pappus bristles which are plumose only apically (Fig. 3-1g$_5$). Pappus bristles in *Disparago* are also flattened and more robust than in *Amphiglossa* although in the former the pappus is frequently reduced or absent on the ray or (more rarely) on both types of floret. *Bryomorphe* also has rays and discs but is distinguished from *Disparago* by its cushion-like, mossy habit, as well as many more florets per capitulum. Most commonly, a capitulum in *Disparago* consists of one (female or sterile) ray floret and one bisexual disc floret but heads can contain up to three ray florets and five disc florets. The condition in *D. tortilis* is that of a single floret per capitulum which is either a ray or a disc floret, but heads are grouped into secondary heads which contain both types of floret (Fig. 3-1g). The massing of capitula into various types of secondary or even tertiary structures is a feature of species in *Disparago, Elytropappus* and *Stoebe*. These structures will be termed 'synflorescences' in this study. In *Disparago,* the synflorescence is generally a compact globe borne at the branch tips (Fig. 3-1e).

The corolla lobes of the disc florets in *Disparago* are generally erect and the base of the style usually possesses a waxy disc. This disc, often coloured yellow, has been proposed to be a nectary (Koekemoer, 2002) although this has not been confirmed. Disk florets are functionally male in several species (Koekemoer, 1991; 1993). Outer involucral bracts can be variously leafy but the inner bracts are thin and pale brown and glabrous. The most recent treatment was by Koekemoer (1993) who recognized nine species in four subgeneric sections. In her unpublished thesis, Koekemoer (2002) elevated these sections to genus status, creating the names *'Laevicarpa', 'Gonygyloglossa'* and *'Monticapra'*. Species are distributed mainly in the Western Cape with extensions into the Eastern Cape along the eastward-trending Cape Fold Belt, with one species (*D. tortilis* [DC] Sch. Bip) extending eastwards to Kwa-Zulu Natal.

***3.1.2.4. Elytropappus* Cass.** Type: *E. hispidus* (L.f.) Druce

All included species are erect shrublets with more-or-less glandular leaves. The glands are either multicellular and very obvious (Fig. 3-2g,h) or microscopically small and embedded within the indumentum (Fig. 3-2f,i). Heads are homogamous and contain two to eight disc florets. The disc florets in *Elytropappus* are campanulate and white (Fig. 3-2b) or tubular and plum-red with erect corolla lobes (Fig. 3-2j). Outer involucral bracts can be leafy but the inner bracts are thin and pale brown. Heads are either arranged in loose groups at the branch tips or arranged in more complex compound synflorescences (Fig. 3-2a-e). There is no disc at the base of the style and the pappus is of the *Disparago* type. The most recent revision of the genus is by Levyns (1935) who recognized eight species in three subgeneric sections. Koekemoer (2002) in her unpublished PhD thesis elevated each of these sections to genus resulting in the new names '*Dicerothamnus'* (for *E. rhinocerotis* and *E. adpressus*) and '*Myrovernix'* (for species with stalked glands and plum-red florets). She also transferred to '*Myrovernix*' the only member of *Stoebe* with stalked glands, *Stoebe intricata*.This transfer was formalised by Goldblatt & Manning (2000) in the new combination *Elytropappus intricata* (Levyns) Koekemoer. In the same PhD thesis Koekemoer described two new species of *Elytropappus*. Species currently recognised as members of *Elytropappus* (Goldblatt & Manning, 2000) occur mainly in the Western Cape with one species (*E. rhinocerotis* [L.f.] Less.) extending to the marginal areas of the winter-rainfall region (the Richtersveld, along the Great Escarpment and extending eastwards to East London).

Fig. 3-2. (opposite) Morphological variation in *Elytropappus*. (a) – pressed specimen of *Elytropappus scaber* showing paniculate synflorescence (scan: M. Smith). (b) – *E. hispidus* flowering twig showing long-stalked leaf glands. (c) - pressed specimen of *E. longifolius* showing dense spicate synflorescences (scan: M. Smith). (d) – *E. rhinocerotis* capitula at anthesis. (e) - herbarium specimen of *E. rhinocerotis* showing massing of capitula in unstructured synflorescence. (f) - leaves of *E. rhinocerotis* (scale bar = 1mm) (g) - stalked glands on tomentose leaf of *E. gnaphaloides* (scale bar = 1mm) (h) - T/S of long stalked gland on leaf of *E. longifolius* (scale bar = 20 µm) (i) T/S of gland on the leaf of *E. rhinocerotis* (scale bar 5 µm). (j) – plum-red floret of *E. rhinocerotis.* (Scale bars all represent 1 cm except as noted; leaf anatomical sections from Koekemoer, 2002, Rand Afrikaans University Website. Photos: N. G. Bergh).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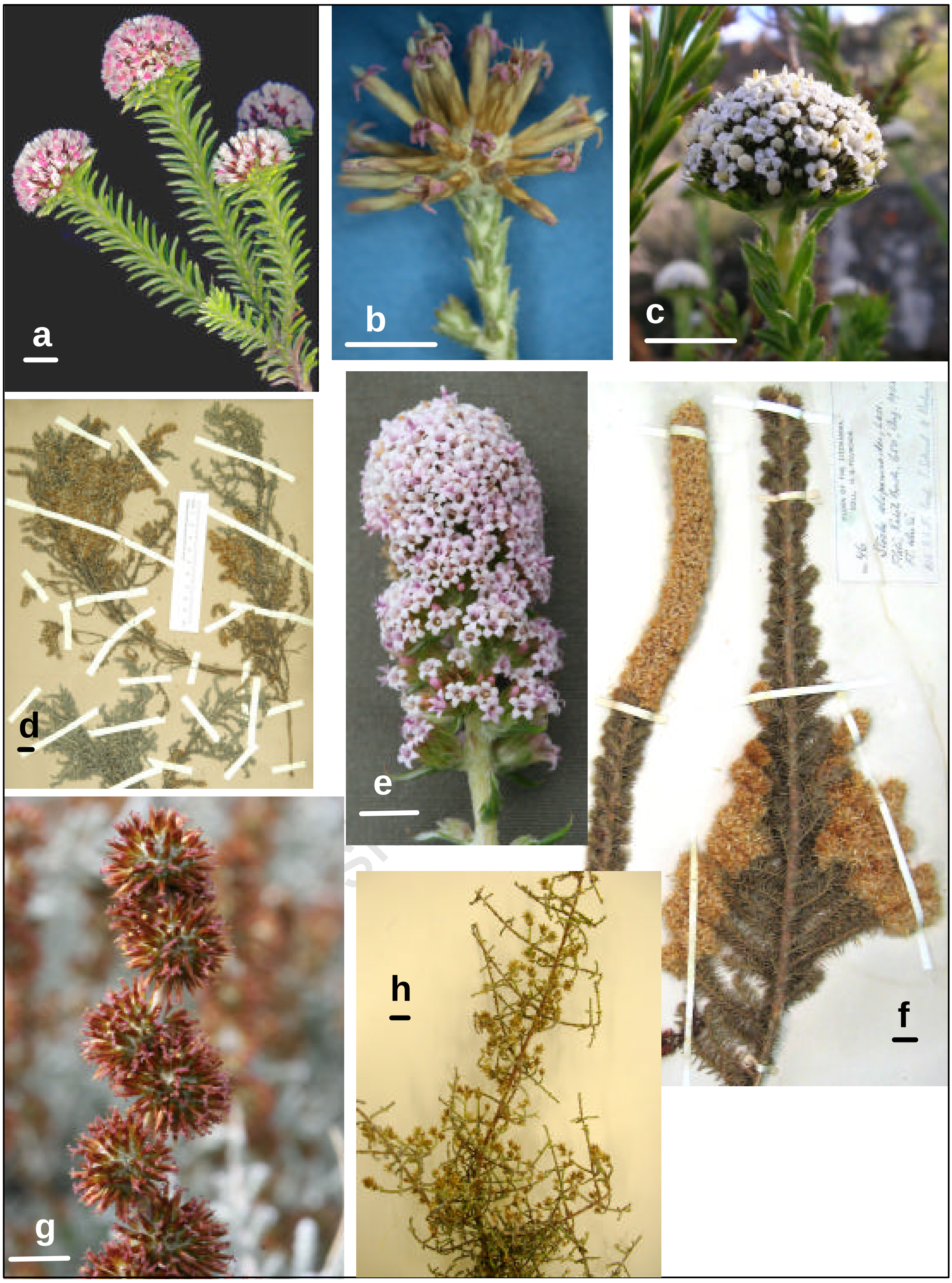
3.1.2.5. Stoebe **L.** Type: *S. aethiopica* L.

This genus of erect or sprawling shrublets is defined by having capitula with only a single disc floret. Apart from floret number, the genus is fairly diverse and has morphological similarities with most other genera in the *Stoebe* clade. Shoots can be all of one kind or differentiated into long and short shoots (brachyblasts), a character found also in *Amphiglossa*. Leaves can be glabrous or variously hairy. With the transfer of *S. intricata* to *Elytropappus* (Koekemoer, 2002; Goldblatt & Manning, 2000), *Stoebe* no longer has stalked glands. Synflorescences vary from compact, rounded, terminal globes (Fig. 3-3a, c) to complex spicate (Fig. 3-3e-g) or loosely paniculate structures with thousands of tiny capitula (Fig. 3-3d). Outer involucral bracts can be leafy but the inner bracts are generally thin and pale brown or opaque white. Florets are white, pink or dark plum-red in colour, with or without a 'nectary' disc and with corolla lobes either erect or reflexed. The pappus is of the *Disparago* type but is absent in a few species.

Fig. 3-3. (Opposite). Illustration of morphological variation in *Stoebe*. (a) – flowering twig of *S. rosea* to show dense globose synflorescence (from www.plantweb.co.za). (b) – sparsely-headed synflorescence of *S. fusca*. (c) – compact globose synflorescence of *S. aethiopica* (photo: K. Davis). (d) – herbarium specimen of *S. kilimandscharica* showing loose, paniculate synflorescence (scan courtesy of the Institute for Systematic Botany, Zürich). (e) – elongated synflorescence of *S. capitata*; this species has both globose and oblong synflorescences. (f) – elongated, dense spicate synflorescence of *S. alopecuroides* illustrated on a scanned herbarium specimen (leaf brachyblasts also evident). (g) – linear, spicate aggregation of globose synflorescences in *S. incana* (photo: M. N. Britton). (h) – divaricate branching and arrangement of capitula on a twig of *E. intricata* (formerly *S. intricata*). (Scale bars all 1 cm; photographs by N. G. Bergh unless otherwise indicated).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The species currently in *Stoebe* originally belonged to three genera, *Stoebe*, *Seriphium* L. (type: *S. cinereum* L.) and *Perotriche* Cass. (type: *P. tortilis* Cass.). Linnaeus (1753) distinguished *Seriphium* and *Stoebe* on synflorescence shape: *Stoebe* had rounded, terminal synflorescences while those in *Seriphium* were spicate or paniculate. The allocation of species to these two genera has since changed according to the characters used by different authors: synflorescence structure and leaf morphology (Linnaeus, 1753); presence / absence of an annulus (Lessing, 1832; De Candolle, 1838) and floret characters (Koekemoer, 2002). Levyns (1937) noted two informal groups based on the two main types of disc florets in the *Stoebe* clade: 1) white / pink florets, usually with relatively large reflexed corolla lobes and possessing a ‘nectary’ disc at the base of the style, reminiscent of the disk florets of *Disparago*. These taxa usually have terminal, globose synflorescences and lack brachyblasts; 2) plum-red tubular florets, usually with small erect lobes and no waxy disc, most similar to those in *Elytropappus*. Levyns (1937) also recognised the frequent association between plum-red florets and possessing brachyblasts. However, the character associations described above are not always present, as several species have various combinations of floret colour, corolla lobe orientation, presence of the ‘nectary’ disc and brachyblasts.

Perotriche was erected for species which are identical to *Stoebe* except in their lack of a pappus (currently *S. schultzii* Levyns, *S. montana* Schltr. ex Levyns, some specimens of *S. capitata* P. J. Berg. and several species not recognised by Goldblatt & Manning, 2000). The most recent published revision of *Stoebe* is by Levyns (1937) who recognized 34 species and sunk *Perotriche* into *Stoebe*, which by then also included *Seriphium*.

Although most species currently in *Stoebe* are confined to the Western Cape province, there are several taxa which are more widely-spread (*S. plumosa* Thunb.– to Angola; *S. vulgaris* Levyns – to Zimbabwe); and several that do not occur in the CFR. These comprise the group of species from the afroalpine regions (shown to be monophyletic in the analysis of Chapter 2): *S. kilimandscharica* O. Hoffm. (in the East African highlands); and *S. cryptophylla* Baker, *S. pachyclada* Humbert, and *S. passerinoides*

Willd. (on Madagascar or La Réunion).

3.1.3. Questions

Given the complexities in generic delimitation and the homoplasy of morphological characters outlined above, the present chapter sets out to address the following questions:

1. Does the current taxonomy adequately represent patterns of evolutionary relationship in the clade? Specifically, are genera monophyletic?
2. What is the evolutionary history of each of the main morphological characters that have been used in classification?
3. Are there morphological characters that have a low-level of homoplasy, and so can be used as taxonomic markers?
4. What is the best scheme of generic delimitation for the *Stoebe* clade?

3.2. Methods

3.2.1. Sampling:

Near-complete species-level sampling was achieved by supplementing field-collected material with DNA from herbarium specimens (Table 3-2), although not every region was successfully amplified for every specimen. Where possible, at least two accessions of each species were included in order to check for monophyly of molecular haplotypes. Young leaves free of any sign of disease or insect damage were selected from plants in the field and dried immediately on silica gel. Approximately 30 mg of dried leaf material was ground with sterilized sand and/or liquid nitrogen and total genomic DNA isolated using the CTAB method of Doyle & Doyle (1987). Extracts from problematic samples and herbarium material were cleaned through QIAGEN? QIAquick cleaning columns. DNA was suspended and diluted in TE. PCR reactions were performed on a Hybaid PCR Sprint thermal cycler (Fisher Scientific International) and products checked on 1% agarose gels stained with ethidium bromide.

3.2.2. Choice of molecular markers:

Species-level molecular phylogenetic studies in the Gnaphalieae are hampered by lack of resolution (Bayer et al., 2000; 2002; Breitweiser et al., 1999; Galbany-Casals et al., 2004). The most informative marker used so far in Gnaphalieae is the external transcribed spacer (ETS) of the 18S – 26S nuclear ribosomal repeat. This marker was most informative across genera and even within some genera in the framework study detailed in Chapter 2. The internal transcribed spacer (ITS) of the same nuclear ribosomal repeat has also been used in phylogenetic studies of Gnaphalieae (Breitweiser et al., 1999; Galbany-Casals et al., 2004) but was not explored as a marker in the present study, for the following reasons. Comparison of the studies of Bayer et al. (2002) and Breitweiser et al. (1999) indicates that ITS may be less polymorphic than the ETS. Also, ITS is likely to be inherited as a single linkage group with the ETS (Hamby & Zimmer, 1992) and so will not provide an independent, unlinked test of phylogenetic relationships. In contrast, a more useful test of phylogenetic congruence is to compare a chloroplast gene tree with the ETS tree to provide signal from independent loci, and also information on hybridisation events (if any). Nuclear markers are inherited biparentally, and chloroplasts maternally in most angiosperms so conflicting signals of relationship between the two genomes might point to hybridisation events or other causes of incongruence.

Several chloroplast markers, either previously used in Gnaphalieae or reported in the literature to be informative in angiosperms at the species level, were screened. Two potentially very informative chloroplast spacer regions, *trnD-trnT* and *trnS-trnG* (Shaw et al., 2005), were not screened, as an examination of their position in the chloroplast genome indicated that they are interrupted by Asteraceae-specific chloroplast inversions (Jansen & Palmer 1987). The remaining plastid regions were amplified according to standard protocols. These are the *rpoB-trnC* spacer (using the primers trnC^{GCA}R and rpoB from Shaw et al., 2005, modified from Ohsako & Ohnishi, 2000), the *matK* gene (using the primers trnK-3914F and trnK-2R from Johnson & Soltis, 1995), the *trnS-trnfM* spacer (using the primers trnS^{UGA} and trnfM^{CAU} from Demesure et al., 1995), the *trnT-trnL* spacer (using the a and b primers from Taberlet et al. 1991), and the *psbA-trnH* and *ycf6-psbM* spacers (see below for primers). An additional nuclear locus that is inherited independently of the ETS and has shown phylogenetic utility in *Oxalis* was also screened, the putatively single-copy nuclear gene ncpGS (chloroplast-expressed glutamine synthetase; using the primers GScp687f and GScp994r; Emshwiller & Doyle, 1999). Each of these gene or spacer regions was initially amplified in five species from the *Stoebe* clade (*E. adpressus*, *E. hispidus*, *S. gomphrenoides*, *S. vulgaris* and *E. rhinocerotis*) and one outgroup taxon (*Dolicothrix ericoides*; see Chapter 2). Successfully amplified samples were sequenced in both directions, aligned manually and examined for phylogenetic utility.

3.2.3. Polymerase chain reaction amplification and sequencing:

The regions ultimately chosen for amplification (see Results) were the 3’ portion of the nuclear ribosomal external transcribed spacer (ETS), the plastid *psbA-trnH*^{GUG} spacer and the plastid *ycf*6-*psbM* spacer. The 3’ ETS was amplified using the primers AST1 (Markos, 2001) and 18S-ETS (Baldwin & Markos, 1998). The *psbA-trnH* spacer was amplified using the primers psbA-F and trnH-R of Sang et al. (1997). The primers ycf6F and psbMR from Shaw et al. (2005) were used to amplify the *ycf*6-*psbM* spacer. The reaction mixtures consisted of 5.0 mM (*psbA-trnH* and ETS) or 6.0 mM (*ycf6-psbM*) $MgCl_2$, dNTPs at 0.1mM (*psbA-trnH* and ETS) or 1.2 mM (*ycf6-psbM*) each, primers at 0.5µM, 0.3µM and 0.6µM (ETS, *psbA-trnH*, and *ycf6-psbM* respectively). Per reaction, 0.75 (*psbA-trnH* and *ycf6-psbM*) or 1.0 (ETS) unit(s) of Bioline BioTaq® were used. The ETS mixture also contained 2% DMS0. Reaction volumes were made up to 25 µl (ETS and *ycf6-psbM*) or 30 µl (*psbA-trnH*) with sterilized Millipore^{TM} water, and included 3 – 4 µl of template DNA. Thermal profiles consisted of 2 minutes at 95^{o} C followed by 30 cycles of: a) 94^{o} C (ETS and *psbA-trnH*) or 95^{o} C (*ycf6-psbM*) for one minute; b) 1 minute at 55^{o} C (ETS and *ycf6-psbM*) or 45 seconds at 54^{o} C (*psbA-trnH*) and c) 2 minutes (ETS and *ycf6-psbM*) or 1 minute (*psbA-trnH*) at 72^{o} C. A final extension step at 72^{o} C lasted for 7 (ETS) or 8 (*psbA-trnH* and *ycf6-psbM*) minutes.

Successfully amplified target DNA was cleaned and sequenced by Macrogen Inc., Korea (protocols at http://www.macrogen.com), or the sequencing reactions were performed as described below, and sequenced fragments visualised on either an ABI PRISM ®377 capillary DNA Sequencer or an ABI PRISM®3100 Genetic Analyzer. Target DNA was cleaned in Microspin^{TM} columns using GFX Band Purification Kits from Amersham Biosciences UK or in QIAGEN? QIAquick PCR purification columns. Cycle sequencing used the following thermal profile: 25 cycles of: 30 seconds at 96^o C, 90 seconds at 50^o C and 4 minutes at 60^o C in tubes containing 1-3 µl of cleaned target DNA, 0.16 µl of primer, 2.0 µl of ABI PRISM® BigDye® Terminator v3.1 cycle sequencing reaction mix and 0.1 µl of 20% DMSO made up to 10 µl with sterilized Millipore^{TM} water.

3.2.4. Sequence assembly and alignment:

Each region was sequenced in both directions using the original PCR primers; chromatograms were checked and assembled with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Australia; Conor McCarthy, 1996) and Sequencher 4.5 (Gene Codes Corporation, 2005). Consensus sequences were aligned manually in Maclade 4.05 (Maddison & Maddison, 1992) and Bioedit 7.0.5.3 (Hall, 1999). Stretches of DNA that could not be unambiguously aligned across all taxa were recoded as missing data. Insertion/deletion (indel) events were coded independently as binary characters using the simple gap coding method of Simmons & Ochoterena (2000), implemented in Gapcoder (Young & Healy, 2003). The resulting matrix of indel characters was examined and only phylogenetically informative and unambiguously assignable (ie not overlapping) indels were included in the analysis.

3.2.5. Phylogenetic analysis:

Phylogenetic analyses were initially performed on each gene locus independently. Datasets were assessed for incongruence by comparing trees for conflicting nodes that received support equal to or greater than 75% bootstrap or 0.95 posterior probability (PP), as in Wiens (1998). The two chloroplast regions were initially analysed separately to examine the degree of phylogenetic resolution provided by each. They were then analysed in combination and finally, all datasets were combined into a single analysis. This combined analysis included only the 46 specimens for which sequence data are available for all three gene regions.

Table 3-1. Summary of main features of genera in the *Stoebe* clade.

	***Amphiglossa* (11 spp.)**	***Bryomorphe* (1 sp.)**	***Disparago (9 spp.)***	***Elytropappus* (9 spp.)**	***Stoebe* (32 spp.)**
Glands on leaves and stems	Absent	Absent	Absent	Stalked glands present and conspicuous in all but two species; these possess small, sessile glands.	Absent.
Synflorescence structure	Capitula solitary in the leaf axils or one to few capitula loosely grouped at the branch tips	Capitula solitary at branch tips.	Three or more (up to + 50) capitula grouped into tight globular terminal synflorescences	Few capitula aggregated into a rounded, terminal head (*E. hispidus*) or one to few capitula loosely grouped at the branch tips, terminal branches arranged to form a paniculate synflorescence, or groups compactly arranged to form a leafy spike.	Few to many capitula grouped into tight globular terminal synflorescences, or this aggregation elongated to form a dense spike, or groups of capitula borne in whorls along a main axis to form a 'beaded' spike, or one to few capitula loosely grouped at the branch tips, terminal branches arranged to form a paniculate synflorescence.
Involucral bracts	Basally thickened, hard and cartilaginous; most species outer bracts abaxially with a patch of short white-tomentose hairs towards the apex	Thin, pale brown	Thin, pale brown.	Thin, pale brown.	Thin, pale brown or opaque white.
Possession of ray florets	Present in four species	Present	Present in all species	Absent	Absent
Number of florets per head	Three to 12	12 to 14	One to eight.	Two to eight	One

	***Amphiglossa* (11 spp.)**	***Bryomorphe* (1 sp.)**	***Disparago (9 spp.)***	***Elytropappus* (8 spp.)**	***Stoebe* (33 spp.)**
Colour of disc floret corolla	White or pale pink	Dark pink	White or various shades of pink.	Plum-red, white in *E. hispidus.*	White, various shades of pink, plum red or pale yellow (in one species)
Orientation of corolla lobes	Reflexed	Reflexed	Erect, reflexed in one species.	Erect, reflexed in *E. hispidus* and *E. longifolius.*	Erect or reflexed
'Nectary' disc	Present	Present	Present in all except *D. kolbei*	Absent in all except *E. hispidus*	Present or absent.
Pappus	Delicate bristles long-plumose from base; bristles rounded in T/S	Slender, shortly plumose to barbellate bristles, rounded in T/S	Robust bristles plumose apically and barbellate basally. Flattened in T/S. Absent or reduced on ray florets of some species; entirely absent in *D. anomala*	Robust bristles plumose apically and barbellate basally. Flattened in T/S	Robust bristles plumose apically and barbellate basally, flattened in T/S. Absent in two spp.

Table 3-2. Sampled species, collector's name and accession number for each specimen; herbarium where voucher is lodged and locality where specimen was collected (if collected for this study). Provinces of South Africa: EC - Eastern Cape; FS - Free State; KZN - Kwa-Zulu Natal; NC - Northern Cape; WC - Western Cape. Collectors: BG: B. Gehrke; EE: E. Esterhuysen; DGE: D. Gwynne-Evans; MK: M. Koekemoer; NGB: N. G. Bergh; NH: Nick Helme; TAH: T. A. Hedderson; TTS: T. Trinder-Smith.

Taxon	**Collector , voucher locality**	**Collecting locality**
Amphiglossa callunoides DC	NGB 1452a (NBG)	Uitenhague, EC
Amphiglossa celans Koekemoer	MK 1099 (PRE)	-
Amphiglossa corrudifolia DC.	MK 1291 (PRE)	-
Amphiglossa grisea Koekemoer	MK 1111(PRE)	-
Amphiglossa perotrichioides DC.	MK 608(PRE)	-
Amphiglossa rudolphii Koekemoer	MK 1214(PRE)	-
Amphiglossa tecta (F. Brusse) Koekemoer	MK 405(PRE)	-
Amphiglossa tecta (F. Brusse) Koekemoer	MK 1066(PRE)	-
Amphiglossa tecta (F. Brusse) Koekemoer	Brusse 5629 (BOL)	-
Amphiglossa thuja (Merxm.) Koekemoer	MK 1200(PRE)	-
Amphiglossa tomentosa (Thunb.) Harv.	NGB 1322	Katbakkies Pass, WC
Amphiglossa tomentosa (Thunb.) Harv.	NGB 1332 (NBG)	Niewoudtville, WC
Amphiglossa tomentosa (Thunb.) Harv.	NGB 1342	Pakhuis Pass, Cederberg, WC
Amphiglossa tomentosa (Thunb.) Harv.	NGB 1369	Koingnaas, NC
Amphiglossa triflora DC.	Gubb 12223	-
Amphiglossa triflora DC.	MK 1064(PRE)	-
Bryomorphe lycopodioides (Sch. Bip) Levyns	TTS 376 (BOL)	Matroosberg, WC
Bryomorphe lycopodioides (Sch. Bip) Levyns	NGB 1155	Cederberg, WC
Disparago anomala Schltr. ex Levyns	NGB 1224	Houwhoekberg, WC
Disparago anomala Schltr. ex Levyns	NGB 1258	Struisbaai, WC
Disparago barbata Koekemoer	EE 28119	-
Disparago ericoides (Berg.) Gaertn.	NGB 1143	Jonaskop, WC
Disparago gongyloides Koekemoer	NGB 1386	Groot Winterhoek, WC
Disparago kolbei (H. Bol.) Hutch.	NGB 1419	Swartberg Pass, EC
Disparago kraussii Sch. Bip.	NGB 1262	De Hoop, WC
Disparago laxifolia DC.	NGB 1412	Kogelberg, WC
Disparago pilosa Koekemoer	MK 361(PRE)	-
Disparago pilosa Koekemoer	EE 33928	-
Disparago tortilis (DC.) Sch. Bip.	NGB 1451	Uitenhague, EC

Elytropappus adpressus Harv.	NGB 605	Swartberg Pass, EC
Elytropappus adpressus Harv.	NGB 1315	Skurweberg, WC
Elytropappus aridus Koekemoer *nom. ined.*	MK 1289(PRE)	-
Elytropappus glandulosus Less.	NGB 1096	Caledon Swartberg, WC
Elytropappus gnaphaloides (L.) Levyns	NGB 1203	Houwhoekberg, WC
Elytropappus gnaphaloides (L.) Levyns	NGB 1215b	Houwhoekberg, WC
Elytropappus gnaphaloides (L.) Levyns	NGB 1275	Garcia's Pass, Langeberg, WC
Elytropappus gnaphaloides (L.) Levyns	NGB 1336a	Botterkloof Pass, N. Cederberg, WC
Elytropappus hispidus (L.f.) Druce	NGB 1009	Sneeuberg, Cederberg, WC
Elytropappus hispidus (L.f.) Druce	NGB 1043	Matroosberg, WC
Elytropappus hispidus (L.f.) Druce	NGB 1409	Sneeuberg, Cederberg, WC
Elytropappus intricata (Levyns) Koekemoer	NGB 1293	Swartberg Pass, EC
Elytropappus intricata (Levyns) Koekemoer	NGB 1325	Niewoudtville, WC
Elytropappus longifolius (DC.) Levyns	NGB 1248	Silvermine, Cape Town, WC
Elytropappus longifolius (DC.) Levyns	NGB 1444	Silvermine, Cape Town, WC
Elytropappus rhinocerotis (L.f.) Less	NGB Co1	Kamiesberg, NC
Elytropappus rhinocerotis (L.f.) Less	NGB Co2	Sterkstroom, EC
Elytropappus scaber (L.f) Levyns	NGB 1006	Sneeuberg, Cederberg, WC
Elytropappus scaber (L.f) Levyns	NGB 1250	Silvermine, Cape Town, WC
Elytropappus sp. nov. Koekemoer	MK 611(PRE)	-
Elytropappus sp. nov. Koekemoer	MK 1202(PRE)	-
Stoebe aethiopica L.	NGB 1040 (NBG)	Cederberg, WC
Stoebe aethiopica L.	NGB 1416	Kanetberg, Langeberg, WC
Stoebe alopecuroides (Lam.) Less.	NH 5522	Tsitsikamma, EC
Stoebe alopecuroides (Lam.) Less.	NGB 1283	Robinson's Pass, Mossel Bay, EC
Stoebe capitata P.J. Bergius	NGB 1188	Slopes of Du Toit's Berg, WC
Stoebe cinerea (L.) Thunb.	NGB1185	Slopes of Du Toit's Berg, WC
Stoebe cinerea (L.) Thunb.	NGB 1210 (NBG)	Houwhoekberg, WC
Stoebe cryptophylla Baker	BG AF281	Madagascar
Stoebe cryptophylla Baker	Kew DNA Bank	-
Stoebe cyathuloides Schltr.	NGB 1236	Baardskeerdersbos, WC
Stoebe cyathuloides Schltr.	NGB 1429	Soetanysberg, Elim, WC
Stoebe fusca (L.) Thunb.	NGB 1318	Katbakkies Pass, WC
Stoebe gomphrenoides P.J. Bergius	NGB 1439a	Riverlands Nature Reserve, WC
Stoebe gomphrenoides P.J. Bergius	NGB 1439b	Riverlands Nature Reserve, WC

Stoebe incana Thunb.	NGB 1228	Houwhoekberg, WC
Stoebe kilimandscharica O. Hoffm.	BG AF076	Mount Kenya, Kenya
Stoebe leucocephala DC.	NGB 1404	Pakhuis Pass, Cederberg, WC
Stoebe leucocephala DC.	NGB 1437	Riverlands Nature Reserve, WC
Stoebe microphylla DC.	NGB 1415	Kanetberg, Langeberg, WC
Stoebe montana Schltr. ex Levyns	MK 360(PRE)	-
Stoebe muirii Levyns	NGB 1263	De Hoop, WC
Stoebe muirii Levyns	NGB 1422	Stilbaai, WC
Stoebe nervigera (DC.) Sch. Bip.	MK 1284(PRE)	-
Stoebe passerinoides Willd.	TAH 15821 (NBG)	Piton de Fournaise, La Reúnion
Stoebe passerinoides Willd.	TAH 15894	Cirque Salazine, La Reúnion
Stoebe phyllostachya (DC.) Sch. Bip	NGB 1457	Garcia's Pass, Langeberg, WC
Stoebe phyllostachya (DC.) Sch. Bip	NGB 1458	Grootvadersbos, Langeberg, WC
Stoebe plumosa (L.) Thunb.	NGB 1242	Baardskeerdersbos, WC
Stoebe plumosa (L.) Thunb.	NGB 1284	Robinson's Pass, Mossel Bay, EC
Stoebe prostrata L.	NGB 1163	Table Mountain, Cape Town, WC
Stoebe prostrata L.	NGB 1253	Constantiaberg, Cape Town, WC
Stoebe rosea Wolley-Dod	NGB 1445	Constantiaberg, Cape Town, WC
Stoebe rugulosa Harv.	NGB 1270	Swellendam, WC
Stoebe saxatilis Levyns	NGB 1245	Hex River Berg, Worcester, WC
Stoebe schultzii Levyns	NGB 1308	Soetanysberg, Elim, WC
Stoebe spiralis Less.	NGB 1187	Slopes of Du Toit's Berg, WC
Stoebe vulgaris Levyns	NGB 1079	Zastron, FS
Stoebe vulgaris Levyns	NGB 1080	Zastron, FS
Stoebe vulgaris Levyns	NGB 1081	Hogsback, EC
Stoebe vulgaris Levyns	DGE 2145	Umtamvuna Gorge, KZN

Unweighted parsimony tree searching was conducted in PAUP ver 4.0 b10, including only parsimony informative (PI) characters in order to compare parsimony statistics such as the consistency index (CI). An initial shallow search performed 10 000 random-addition replicates using NNI branch-swapping and saving a single tree per round of branch swapping. The resulting set of shortest trees was subjected to TBR branch swapping while saving multiple trees. Node support was assessed via 1000 non-parametric bootstrap replicates, each replicate saving a maximum of 500 trees based on a simple addition sequence with the TBR branch-swapping algorithm. Trees were rooted on *A. callunoides* and *A. corrudifolia* based on the results of the broader phylogenetic analysis in Chapter 2.

The most appropriate model of nucleotide substitution for each dataset was the HKY+I+G model (the HKY model of Hasegawa et al., 1985, with the addition of a parameter for a proportion of invariable sites and for gamma-distributed rate variation) or one slightly less complex, as assessed using the Akaike Information Criterion (Akaike, 1974) implemented in MrModeltest v2.2 (Nylander, 2005). Simultaneous Bayesian inference of nucleotide substitution parameters and topology was performed in MrBayes v3.1 (Huelsenbeck and Ronquist 2001). The binary insertion/deletion (indel) characters were coded as restriction data and analysed with a gamma-shaped prior distribution of rate variation across characters, and with “coding = variable” to indicate to the software that the characters are only detectable if the states vary across taxa. The nucleotide substitution model was set to the HKY+I+G for all DNA regions. For partitioned analysis, substitution model parameters and rates of substitution were allowed to vary across partitions using ratepr = variable and the ‘unlink’ command. The MCMC chain was run for up to 10 000 000 iterations, sampling parameters every 1000 iterations. Each analysis used one cold chain and up to 11 heated chains, with chain heating parameter values between 0.06 and 0.20. Longer runs and greater numbers of heated chains were required to achieve convergence for the analyses combining more than two gene partitions. Convergence of the Markov chain and assessment of ‘burn-in’ duration was determined by finding the point in the run where the average standard deviation of split frequencies (ASDSF) between topologies dropped below 0.01 and by comparing likelihood values, parameter estimates and traces from independent runs (starting from different random starting trees) in Tracer v1.3 (Drummond and Rambaut 2007). Additional assessment of convergence was based on the comparison of posterior probability (PP) consensus trees from different runs within and across analyses. At least two runs were conducted for each partition.

3.2.6. Choice of morphological characters:

Morphological characters selected for ancestral state reconstruction are those that have been previously used in generic delimitation and/or have been highlighted as taxonomically useful by previous workers (Lessing, 1832; De Candolle, 1838; Harvey & Sonder, 1894; Levyns, 1935; 1936; 1937; Koekemoer, 2002). As is true for Gnaphalieae as a whole (Anderberg, 1994), floral characters are the main source of taxonomic information for genera in the *Stoebe* clade. Most of the morphological characters examined relate to the number and type of floret. The following characters were defined and coded for all species.

a. Presence or absence of large, conspicuous (visible to the naked eye) stalked glands on the leaves and/or stems.
b. Synflorescence arrangement. This character is extremely difficult to code as there is little data with which to assess character transformations, and the homology of apparently equivalent states is uncertain. For example, many species in *Elytropappus* and *Stoebe* have loose arrangements of several capitula on short branchlets, aggregated at the ends of the branches (Fig. 3-2a,e; Fig. 3-3 d,h). Should this be considered a compound paniculate synflorescence, or does it represent the absence of any secondary structure beyond the grouping of the few heads at the ends of the branchlets? Should the arrangement of heads into a spicate, elongate series of rounded glomerules, as found in many *Stoebe* species (Fig. 3-3g), be considered homologous to a compact spike (Fig. 3-3e,f), or an extended version of the terminal globose synflorescence? Coding of different states of this character requires a primary assessment of homology. Although many authors have done this [Linnaeus (1753) distinguished two types of synflorescence arrangement for species currently in *Stoebe*: capitula massed into solitary, rounded heads terminating

the branches, or capitula arranged in a spike-like structure or scattered on the branches. Koekemoer (2002) also coded synflorescence arrangement as a binary character, in this case either capitula solitary or capitula in secondary arrangements], there is not sufficient information on the ontogeny or genetic basis of synflorescence structure to make this assessment objectively. The greatest insight into this character may come from the phylogeny. Nevertheless, in a first attempt to map the range of diversity, synflorescence arrangement was coded as follows: none (no secondary arrangement of capitula, i.e. heads solitary); loose (capitula in small groups at the branch tips, these arranged loosely into paniculate-type tertiary structures); compact spike (in which heads are tightly aggregated but the overall structure has an elongate, spicate structure) and compact globe (in which the capitula are arranged into a small or large, globose secondary head).

c. Presence or absence of ray florets.
d. The number of florets in a head. This character was given four states, with species possessing either one, two – three, four – seven, or more than seven florets.
e. Colour of the disc floret corolla. This character was coded only for disc florets as not all taxa possess rays. The states were white and/or pink, dark plum-red, or pale yellow.
f. Corolla lobe orientation. The size of the lobes was not taken into account, and lobe orientation was coded as either erect or reflexed.
g. Presence or absence of the ‘nectary’ disc in any florets in the capitulum.
h. Presence or absence of a pappus on the disc florets.

This is the list of characters for which ancestral state analysis was conducted. Several additional, potentially phylogenetically informative characters from features of the leaves, indumentum and other parts were also coded for use only in phylogeny estimation. The full list of characters, character coding and resulting data matrix is given in Appendix 2. The state of each character for each species was determined by reference to the taxonomic literature on the group and confirmed by examination of several dried specimens of each species. These characters were included together with the molecular characters in a combined parsimony analysis. Multistate characters were input as unordered into this analysis. Parsimony tree searching was conducted as described above.

Several characters which have been used in taxonomy of the group were not traced onto the tree. Possession of brachyblasts was not reconstructed as this character was not defined clearly enough in a structural sense by previous authors to enable it to be coded unambiguously for all taxa. Leaf size, shape, orientation and indumentum were also not reconstructed. Achene characters were also not examined as these have tended to vary within genera.

3.2.7. Reconstruction of ancestor states for morphological characters:

The characters were reconstructed onto a single tree using Mesquite v. 2.5 (Maddison & Maddison, 2006) and maximum likelihood optimisation. The tree was chosen randomly by selecting the last tree sampled by the MrBayes Markov chain for the combined analysis of all DNA regions. This gives an arbitrary resolution of the polytomies in the consensus tree, but supported nodes are indicated in all figures, and interpretations are made taking nodal support into account (see Discussion). Character histories were traced on this tree using maximum likelihood in the Ancestral State Reconstruction packages in Mesquite v.2.5 (Maddison & Maddison, 2006) and assuming equal rates of transition for all characters (the Markov k-state model, Lewis, 2001).

3.3. Results

3.3.1. Choice of DNA regions:

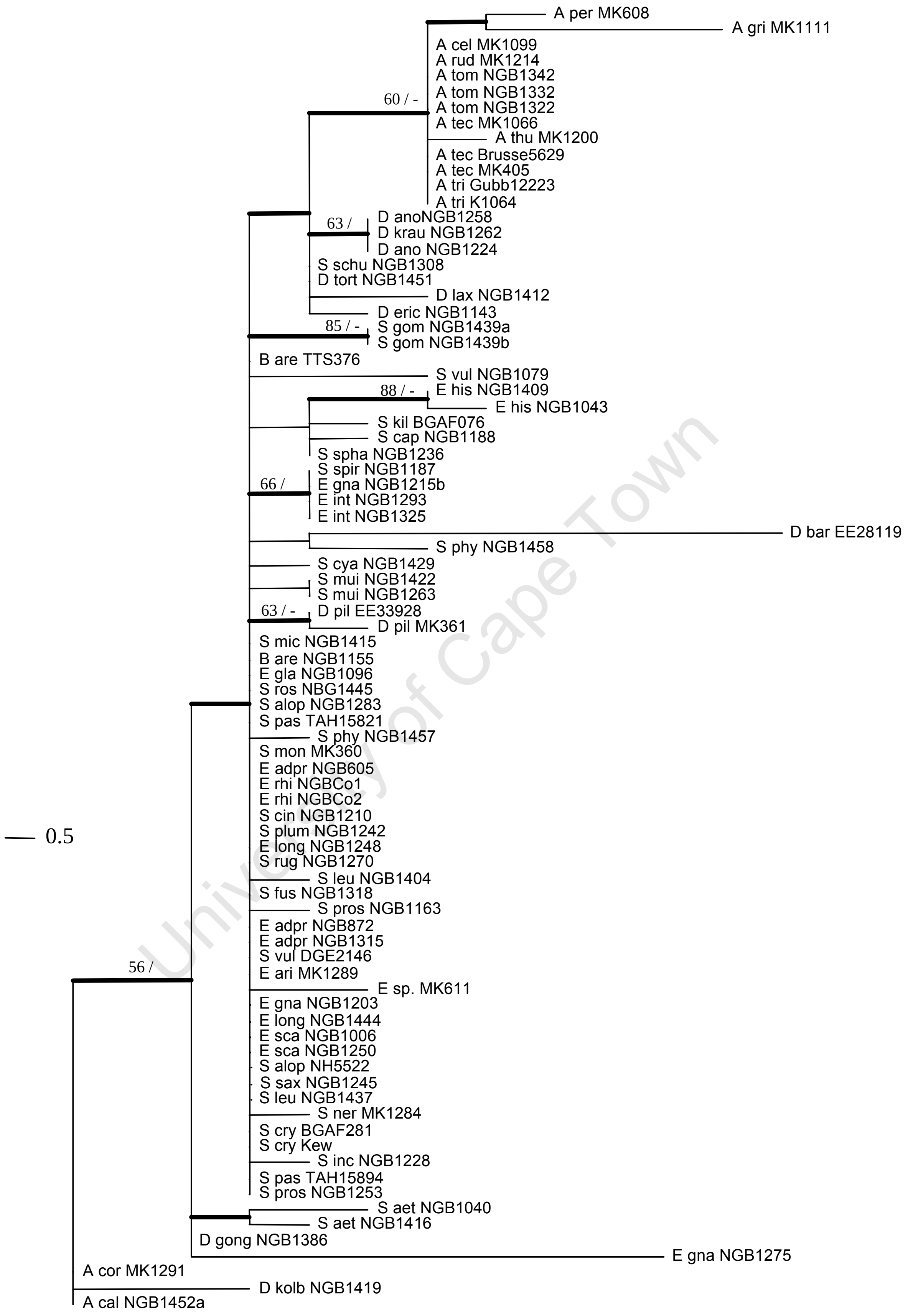
Of the DNA regions screened, *rpoB-trnC* and ncpGS could not be successfully amplified. The *matK* gene was not phylogenetically informative (only seven phylogenetically informative characters [PIC’s] across an aligned length of 1445 nucleotides for the five species screened). The *trnS-trnfM* spacer produced multiple bands. These were separated by gel electrophoresis, cleaned, sequenced and BLASTed against existing published sequences. The shorter band could not be matched to any existing sequence but the longer band matched the *trnS*, *ycf9* and *psbZ* genes of Asteraceae taxa on genbank. However, this region was not informative across the species screened. The *trnL*^{UAA}-*trnF*^{GAA} region was also uninformative at the species level, an unsurprising result given the sparseness of PIC's across the Gnaphalieae for this region (Chapter 2). Within the *Stoebe* clade, only 3 DNA substitutions and 2 indel characters were parsimony-informative in an alignment of 841 characters from 17 taxa so this region was not sequenced further. The *trnT-trnL* region was informative at the generic level but provided no information within the *Stoebe* clade. The *psbA-trnH* and *ycf6-psbM* spacers were the most informative of the screened plastid markers.

3.3.1.1. Plastid regions

3.3.1.1.1. The *psbA-trnH* region:

The dataset for this region consists of 83 accessions from 62 species with an aligned length of 483 nucleotide (18 of which are PI) and 2 PI indel characters, bringing the total proportion of PIC's to 4.5%. Parsimony searches including only the PIC's found 1 000 700 shortest trees of length 24, CI of 0.875, RI of 0.947 and RC of 0.829 (Fig. 3-4). There is no support for any groupings above the level of species. In the *psbA-trnH* gene tree, 19 species are represented by multiple accessions. Two of these group together with good statistical support, but the remainder are part of the large polytomy characterising all samples in the tree

Fig. 3-4. (opposite). One of the shortest trees from parsimony analysis of the *psbA-trnH* data. Numbers above branches indicate bootstrap / posterior probabilities. Only bootstrap values above 50 % and PP values above 0.75 are shown. Branches indicated with thicker lines are retained in the parsimony strict consensus tree.


A per MK608
A gri MK1111
A cel MK1099
A rud MK1214
A tom NGB1342
60 / -
A tom NGB1332
A tom NGB1322
A tec MK1066
A thu MK1200
A tec Brusse5629
A tec MK405
A tri Gubb12223
A tri K1064
D anoNGB1258
63 /
D krau NGB1262
D ano NGB1224
S schu NGB1308
D tort NGB1451
D lax NGB1412
D eric NGB1143
85 / -
S gom NGB1439a
S gom NGB1439b
B are TTS376
S vul NGB1079
88 / -
E his NGB1409
E his NGB1043
S kil BGAF076
S cap NGB1188
S spha NGB1236
S spir NGB1187
66 /
E gna NGB1215b
E int NGB1293
E int NGB1325
D bar EE28119
S phy NGB1458
S cya NGB1429
S mui NGB1422
S mui NGB1263
63 / -
D pil EE33928
D pil MK361
S mic NGB1415
B are NGB1155
E gla NGB1096
S ros NBG1445
S alop NGB1283
S pas TAH15821
S phy NGB1457
S mon MK360
E adpr NGB605
E rhi NGBCo1
E rhi NGBCo2
S cin NGB1210
0.5
S plum NGB1242
E long NGB1248
S rug NGB1270
S leu NGB1404
S fus NGB1318
S pros NGB1163
E adpr NGB872
E adpr NGB1315
S vul DGE2146
56 /
E ari MK1289
E sp. MK611
E gna NGB1203
E long NGB1444
E sca NGB1006
E sca NGB1250
S alop NH5522
S sax NGB1245
S leu NGB1437
S ner MK1284
S cry BGAF281
S cry Kew
S inc NGB1228
S pas TAH15894
S pros NGB1253
S aet NGB1040
S aet NGB1416
D gong NGB1386
E gna NGB1275
A cor MK1291
D kolb NGB1419
A cal NGB1452a

3.3.1.1.2. The *ycf6-psbM* region*:*

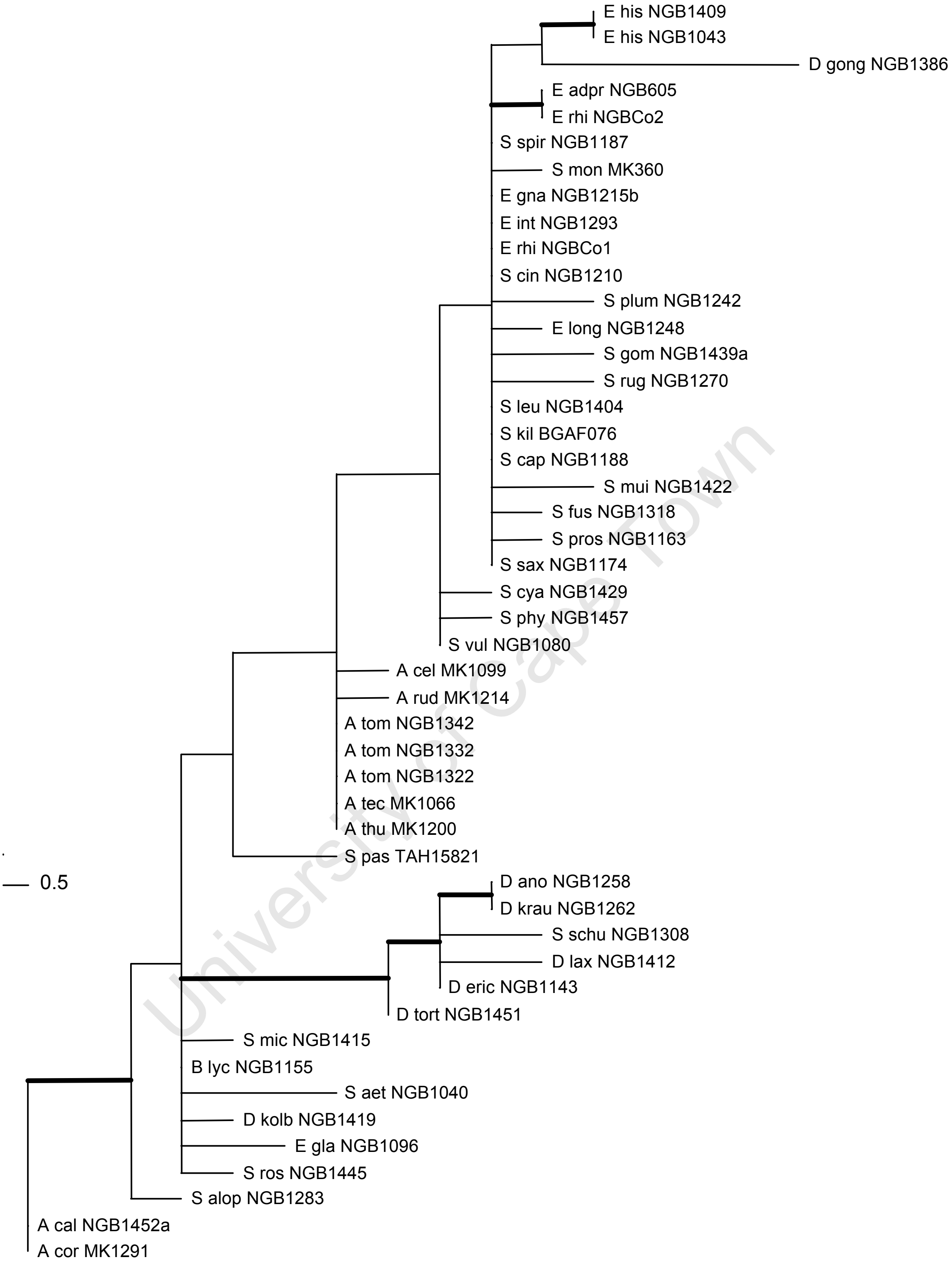
The dataset consists of 48 accessions from 43 species with an aligned length of 652 nucleotide (8 of which are PI) and 6 PI indel characters. Parsimony searches including only the PIC's found 56,734 shortest trees of length 19, CI of 0.737, RI of 0.917 and RC of 0.675. Since only 1.5 % of the characters were parsimony informative for this region, it is not surprising that no well-supported nodes were recovered (Fig. 3-5).

3.3.1.1.3. Combined plastid data:

Loci on the chloroplast genome are usually inherited as a single, non-recombining unit in plants. The different plastid regions should thus reflect the same evolutionary history, justifying combined analysis. Forty-six accessions from 42 species were sequenced for both *psbA-trnH* and *ycf6-psbM*. The two loci were concatenated into a single dataset containing just these taxa and the indels recoded, resulting in a dataset with an aligned length of 1140 characters (1132 nucleotide and eight indel) of which 25 were PI (17 nucleotide and eight indel, giving a total proportion of 2.2 % PICs). A parsimony search on only the PI characters found 72 shortest trees of length 33 steps, CI of 0.818, RI of 0.943 and RC of 0.772 (Fig. 3-6).

Parsimony bootstrap analysis supported the separation of *A. corrudifolia* + *A. callunoides* as a lineage on its own branch (Fig. 3-6). The rest of the *Amphiglossa* species formed a clade (BS of 82 %) which is supported by BS of 76 % as the sister to a clade containing the species D. *ericoides*, *D. laxifolia*, *D. tortilis*, *D. anomala* and *D. kraussii* (arrow in Fig. 3-6). This latter clade is supported by a BS of 95 % and will be referred to as the 'core *Disparago* clade'. It also contains a *Stoebe* species, *S. schultzii*. MrBayes analysis showed little support for any groupings, although the 'rest of *Amphiglossa*' clade was recovered (PP of 0.96; Fig. 3-6).

Fig. 3-5. (opposite) One of the shortest trees from parsimony analysis of the *ycf6-psbM* data. No bootstrap values above 50 % were found. Branches indicated with thicker lines were recovered in the parsimony strict consensus tree.


E his NGB1409
E his NGB1043
D gong NGB1386
E adpr NGB605
E rhi NGBCo2
S spir NGB1187
S mon MK360
E gna NGB1215b
E int NGB1293
E rhi NGBCo1
S cin NGB1210
S plum NGB1242
E long NGB1248
S gom NGB1439a
S rug NGB1270
S leu NGB1404
S kil BGAF076
S cap NGB1188
S mui NGB1422
S fus NGB1318
S pros NGB1163
S sax NGB1174
S cya NGB1429
S phy NGB1457
S vul NGB1080
A cel MK1099
A rud MK1214
A tom NGB1342
A tom NGB1332
A tom NGB1322
A tec MK1066
A thu MK1200
S pas TAH15821
0.5
D ano NGB1258
D krau NGB1262
S schu NGB1308
D lax NGB1412
D eric NGB1143
D tort NGB1451
S mic NGB1415
B lyc NGB1155
S aet NGB1040
D kolb NGB1419
E gla NGB1096
S ros NGB1445
S alop NGB1283
A cal NGB1452a
A cor MK1291

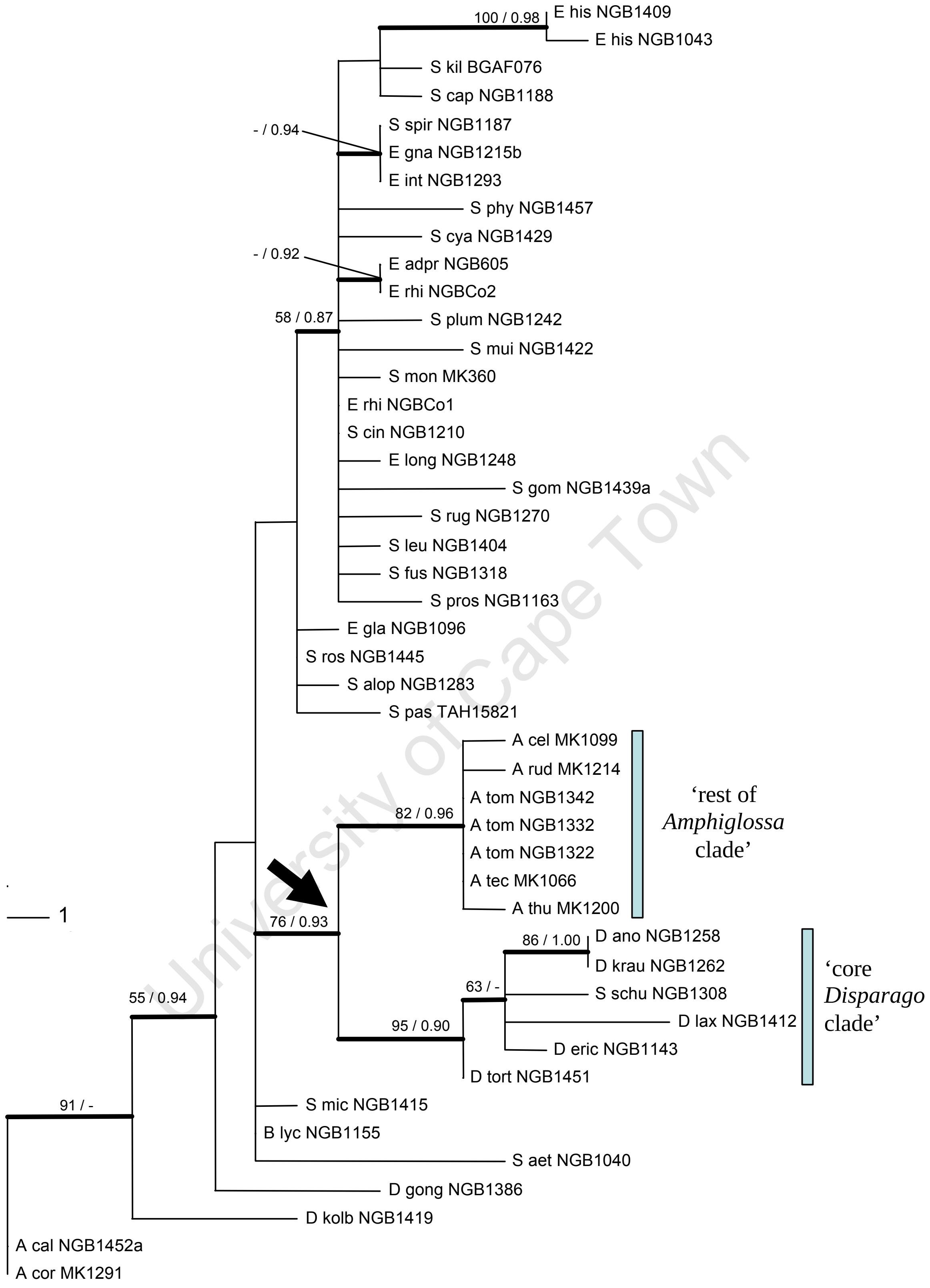
3.3.1.1.4. Multiple accessions from single species:

The multiple accessions from the same species either grouped together with high support or were unresolved but with no support for alternative relationships, so there is no evidence from individual or combined analysis of the plastid data of non-monophyly of any species samples.

3.3.1.2. The ETS region:

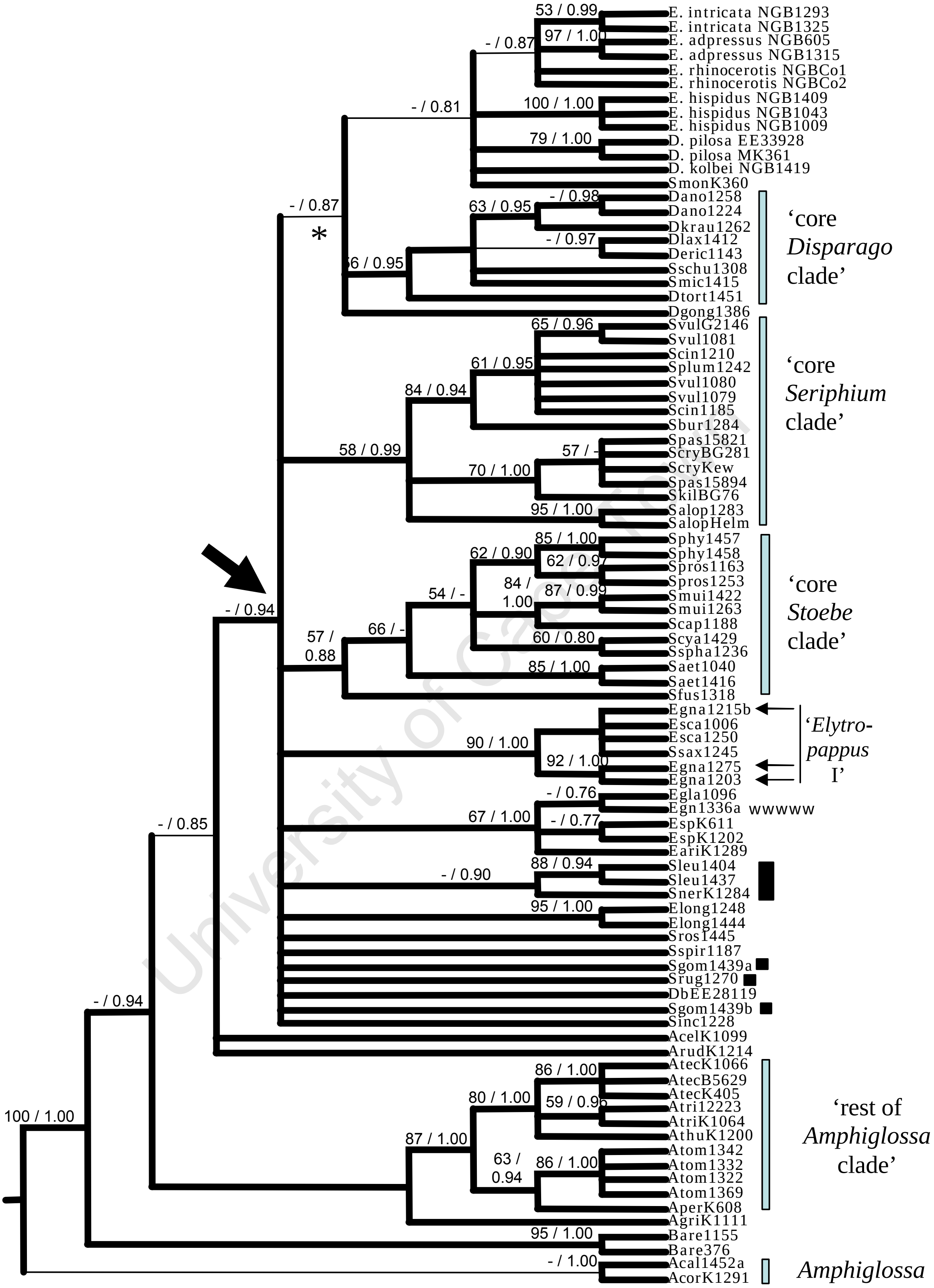
The ETS dataset consists of 90 accessions from 57 species with an aligned length of 489 characters (481 nucleotide and eight indel). There is a much greater proportion of parsimony-informative sites (21.1 %) in the ETS than in the plastid dataset, as well as a larger absolute number of PICs (95 nucleotide and eight indel characters). A parsimony search on only the PI characters found 579 193 shortest trees of length 253 steps, CI of 0.534, RI of 0.797 and RC of 0.425. Bayesian analysis did not achieve convergence if the indel characters were included. This is probably because the indel characters are specified as a separate partition with its own parameters, and eight binary characters do not provide sufficient information for estimation of parameters and relationships across 90 terminals. For the final Bayesian analysis, only nucleotide substitution data were input. The MCMC chain was run for 19,000,000 generations and the first 2,000,000 generations discarded as burn-in.

Fig. 3-6. (opposite) One of the shortest trees from parsimony analysis of the combined plastid data. Numbers above branches indicate bootstrap support measures followed by posterior probabilities. Only bootstrap values above 50 % and PP values above 0.75 are shown. Branches indicated with thicker lines were recovered in the parsimony strict consensus tree. The arrow is referred to in the text.


100 / 0.98
E his NGB1409
E his NGB1043
S kil BGAF076
S cap NGB1188
- / 0.94
S spir NGB1187
E gna NGB1215b
E int NGB1293
S phy NGB1457
S cya NGB1429
- / 0.92
E adpr NGB605
E rhi NGBCo2
58 / 0.87
S plum NGB1242
S mui NGB1422
S mon MK360
E rhi NGBCo1
S cin NGB1210
E long NGB1248
S gom NGB1439a
S rug NGB1270
S leu NGB1404
S fus NGB1318
S pros NGB1163
E gla NGB1096
S ros NGB1445
S alop NGB1283
S pas TAH15821
A cel MK1099
A rud MK1214
A tom NGB1342
82 / 0.96
A tom NGB1332
A tom NGB1322
A tec MK1066
A thu MK1200
'rest of *Amphiglossa* clade'
1
76 / 0.93
86 / 1.00
D ano NGB1258
D krau NGB1262
63 / -
S schu NGB1308
55 / 0.94
95 / 0.90
D lax NGB1412
D eric NGB1143
D tort NGB1451
'core *Disparago* clade'
91 / -
S mic NGB1415
B lyc NGB1155
S aet NGB1040
D gong NGB1386
D kolb NGB1419
A cal NGB1452a
A cor MK1291

The Bayesian majority-rule consensus tree has greater resolution than the parsimony consensus and is depicted in Fig. 3-7. The ETS data recovered several internal clades but could not resolve a large backbone polytomy which included *Disparago*, *Elytropappus* and *Stoebe* (arrow, Fig. 3-7). The lineage containing *A. callunoides + A. corrudifolia* is well-supported and the two *Bryomorphe* accessions are recovered as the next-diverging lineage, sister to the 'rest of *Amphiglossa*' and the polytomy described above. These 'rest of *Amphiglossa*' species, with the exception of the unplaced *A. grisea, A. celans* and *A. rudophii*, group together with BS of 87% and PP of 1.00. The 'core *Disparago* clade' recovered in the plastid analyses is also supported in Bayesian (but not parsimony) analysis of the ETS data (BS of only 56 % but PP of 0.95) and also includes *S. microphylla*, which was unplaced in the plastid analysis. The Bayesian consensus tree suggests, but with no support, that the 'core *Disparago* clade' forms part of a larger grouping containing most of the remaining *Disparago* species as well as *E. intricata, E. adpressus, E. rhinocerotis* and *E. hispidus* (asterisk, Fig. 3-7). A small clade containing three of the four accessions of *E. gnaphaloides* together with *E. scaber* and *S. saxatilis* ('*Elytropappus* clade I') has a BS of 90 % and PP of 1.00 but its relationship to other species is not recovered. Several of the *Stoebe* species with small-lobed, plum-coloured florets lacking the 'nectary' disc (*S.*

Fig. 3-7. (opposite) Majority-rule consensus of 34 000 trees from Bayesian analysis of ETS data. Numbers above branches indicate bootstrap support measures followed by posterior probabilities, with only bootstrap values above 50 % and PP values above 0.75 shown. Thin lines represent branches not present in the parsimony strict consensus tree. Note that in the parsimony strict consensus, the *S. gomphrenoides* accessions (bars) form a clade together with the *S. leucocephala* accessions, *S. rugulosa* and *S. nervigera*. Other annotations are referred to in the text.


53 / 0.99
97 / 1.00
- / 0.87
E. intricata NGB1293
E. intricata NGB1325
E. adpressus NGB605
E. adpressus NGB1315
E. rhinocerotis NGBCo1
E. rhinocerotis NGBCo2
100 / 1.00
E. hispidus NGB1409
E. hispidus NGB1043
E. hispidus NGB1009
- / 0.81
79 / 1.00
D. pilosa EE33928
D. pilosa MK361
D. kolbei NGB1419
SmonK360
- / 0.98
Dano1258
Dano1224
63 / 0.95
Dkrau1262
- / 0.87
- / 0.97
Dlax1412
*
Deric1143
56 / 0.95
Sschu1308
Smic1415
Dtort1451
Dgong1386
'core Disparago clade'
65 / 0.96
SvulG2146
Svul1081
Scin1210
61 / 0.95
Splum1242
Svul1080
84 / 0.94
Svul1079
Scin1185
Sbur1284
'core Seriphium clade'
57 / -
Spas15821
ScryBG281
58 / 0.99
70 / 1.00
ScryKew
Spas15894
SkilBG76
95 / 1.00
Salop1283
SalopHelm
85 / 1.00
Sphy1457
62 / 0.90
Sphy1458
62 / 0.97
Spros1163
Spros1253
84 / 1.00
87 / 0.99
54 / -
Smui1422
Smui1263
- / 0.94
Scap1188
66 / -
60 / 0.80
Scya1429
'core Stoebe clade'
57 / 0.88
Sspha1236
85 / 1.00
Saet1040
Saet1416
Sfus1318
Egna1215b
Esca1006
'Elytro-pappus I'
90 / 1.00
Esca1250
Ssax1245
92 / 1.00
Egna1275
Egna1203
- / 0.76
Egla1096
Egn1336a wwwww
- / 0.85
67 / 1.00
- / 0.77
EspK611
EspK1202
EariK1289
88 / 0.94
Sleu1404
- / 0.90
Sleu1437
SnerK1284
95 / 1.00
Elong1248
Elong1444
Sros1445
Sspir1187
Sgom1439a
Srug1270
DbEE28119
Sgom1439b
- / 0.94
Sinc1228
AcelK1099
ArudK1214
86 / 1.00
AtecK1066
AtecB5629
80 / 1.00
AtecK405
59 / 0.96
Atri12223
'rest of Amphiglossa clade'
100 / 1.00
AtriK1064
87 / 1.00
AthuK1200
Atom1342
63 / 0.94
86 / 1.00
Atom1332
Atom1322
Atom1369
AperK608
AgriK1111
95 / 1.00
Bare1155
Bare376
- / 1.00
Acal1452a
AcorK1291
Amphiglossa

burchellii, S. cinerea, S. plumosa and several accessions of *S. vulgaris*) group together (BS of 84 %; PP of 0.94) and form part of a larger clade containing the afroalpine taxa, which share this floret morphology, and *S. alopecuroides*, which does not. This larger clade has a high posterior probability and is termed the 'core *Seriphium* clade' hereafter, as it contains most of the species allocated to the concept of *Seriphium* by Koekemoer (2002). Almost all of the *Stoebe* species with pale, campanulate florets and nectary discs form a clade; this receives no appreciable support but is labelled the 'core *Stoebe* clade' in Fig. 3-7 as it contains many of the taxa in Koekmoer's (2002) narrower concept of *Stoebe*, as well as the type, *S. aethiopica*. Within this clade, there is very strong support for a sister relationship between *S. capitata* and *S. muirii* (BS of 84%, PP of 1.00).

3.3.1.2.1. Multiple accessions of a single species:

In the ETS analysis, 23 species are represented by two to four accessions. For 15 of these, the species is recovered as monophyletic with PP > 0.95 (and 0.94 in the case of *S. leucocephala*). Although they are all recovered as monophyletic in the parsimony analyses, the BS values for these 15 clades range from 53 to 100 %. For the remaining eight species represented by multiple accessions, seven of them may be recovered as monophyletic with the addition of more data because monophyly is not contradicted by any significant PP or BS values, and the multiple samples are generally placed within an unresolved group including accessions from one or a few closely-related species. The lack of support here appears to be due to lack of sufficient informative characters to exclude other, closely-related species from the subclade. The remaining species, *E. gnaphaloides*, is represented by four specimens. Two of these, NGB1275 and NGB1203 (indicated by small arrows in Fig. 3-7) form a clade supported by a PP of 1.00 and BS of 92 %. Accession NGB1215b is placed in the well-supported '*Elytropappus* I clade' (PP of 1.00 and BS of 90 %) together with the two previously-mentioned specimens as well as *E. scaber* and *S. saxatilis*. These three accessions of *E. gnaphaloides* could well be monophyletic, as the relationships within the clade are unresolved. The fourth accession (NGB1336a; waved line in Fig. 3-7) is placed within a fairly well-supported clade (PP of 1.00 but BS of 67 %) also containing *E. glandulosus*, *E. aridus* and *E. sp. nov*. The phylogenetic positions of these two clades are unresolved on the backbone polytomy, so it is possible that they will fall together with the addition of more data. The taxa in these two clades all share numerous morphological synapomorphies, such as plum-red florets, glandular leaves and heads arranged in open, paniculate synflorescences and many of them were placed by Koekemoer (2002) into the novel genus '*Myrovernix*', together with *E. longifolius* which is unplaced in our tree, and *E. intricata* which appears to be more closely related to *E. rhinocerotis*. However, *E. gnaphaloides* is a very variable taxon which may require closer morphological and anatomical investigation.

3.3.2. Combinability of data partitions and combined topology:

There is potential conflict between the plastid and ETS data in the relationships of the 'core *Disparago* clade'. The plastid bootstrap analysis provided a support measure of 76 % for the sister relationship of this clade to the 'rest of *Amphiglossa*' clade (arrow, Fig. 3-6) while ETS provided a PP value of 0.94 (arrow, Fig. 3-7) for the inclusion of the 'core *Disparagos*' with *Elytropappus* and *Stoebe*, and placed the 'rest of *Amphiglossa*' as sister to this. These are both marginal support values, and in neither case is the relationship supported by all analyses. Apart from this, there are no highly-supported (BS = 75%; PP = 0.95) incongruent nodes in the plastid versus ETS trees. For the plastid data, however, there is generally not enough support to properly assess incongruence.

The partitions were combined to create a single matrix. The proportion of phylogenetically informative sites is ten times greater in the ETS dataset than in the combined plastid dataset, and the absolute number of PI characters is five times greater for ETS, so the topology may be greatly influenced by the nuclear gene. This combined dataset consists of 46 accessions from 42 species with an aligned length of 1629 characters (1613 nucleotide and 16 indel) of which 94 were PI (80 nucleotide and 22 indel). A parsimony search on only the PI characters found 597 shortest trees of length 207 steps, CI of 0.527, RI of 0.734 and RC of 0.386. The Bayesian consensus tree is presented in Fig. 3-8 as this tree resolves more nodes than the parsimony strict consensus tree. Bayesian analysis of the combined data required very long runs (> 10,000,000 generations) to achieve convergence, and was characterised by the Markov chain appearing to jump between islands with different likelihoods. The consensus tree presented in Fig. 3-8 was taken from the last portion of a very long run which appeared to reach stationarity at the island with the highest likelihood.

Parsimony analysis recovered the *A. callunoides* + *A. corrudifolia* lineage (node B; BS = 100 %) but the remaining backbone relationships are unresolved, including the position of *Bryomorphe*. Bayesian analysis, however, placed a PP of 1.00 on the node supporting *Bryomorphe* as sister to the remaining taxa (node C). The 'rest of *Amphiglossa'* species formed a clade (BS = 78 %, PP = 1.00). Unsurprisingly, the placement of this clade sister to 'core *Disparago*' agrees with that of the ETS tree, but here there is good PP support for this relationship, as well as additional support at node D for the inclusion of the 'core *Disparago* clade' with *Elytropappus* and *Stoebe* species. The 'core *Disparago*' clade was (BS of 84 % and PP of 1.00) includes both *Stoebe schultzii* and *S. microphylla*. Bayesian (but not parsimony) analysis also gives strong support to a clade containing the remaining *Stoebe* taxa as well as several *Elytropappus* species (node E). Within this group, many relationships are unresolved but two clades are well-defined and supported by Bayesian (but not bootstrap) measures. These are, firstly, the same 'core *Seriphium* clade' recovered in the ETS analysis and secondly, the 'core *Stoebe* clade' (PP = 0.99) sensu Koekemoer (2002). *Elytropappus* is not recovered as monophyletic, several accessions are united with *Stoebe* species by node E, while *E. rhinocerotis, E. adpressus, E. intricata* and *E. hispidus* appear to belong in a lineage (although not receiving any statistical support) with closer affinity to *Disparago* (Fig. 3-7).

Several sister-species relationships received good support, such as those between *S. capitata* + *S. muirii* (BS = 88 %, PP = 1.00), and *S. cinerea* + *S. plumosa* (BS=95 %; PP = 1.00). The positions of several *Stoebe* (*S. gomphrenoides, S. rugulosa, S. leucocephala, S. rosea, S. spiralis, S. monticola* and *E. intricata*), *Disparago* (*D. gongyloides* and *D. kolbei*) and all of the *Elytropappus* species remain uncertain.

The matrix of 16 coded morphological characters resulted in much-reduced resolution and support when analysed together with the DNA sequence data (results not shown), so these characters were not used in further phylogenetic estimation.

3.3.2.1. Multiple accessions of the same species:

Very few species are represented by multiple accessions sequenced for all three gene regions. Two of the three (*A. tomentosa*, three accessions and *E. hispidus*, two accessions) are monophyletic (PP of 1.00 in both cases and BS of 100 % for the latter). One of the accessions of *E. rhinocerotis* is sister to *E. adpressus* (PP of 1.00, BS of 54 %) while the other forms a polytomy with this *E. rhinocerotis* / *E. adpressus* lineage and *E. intricata*. In the ETS gene tree, the two accessions of *E. adpressus* were highly supported as monophyletic; the two accessions of *E. intricata* were monophyletic (high PP but negligable BS) and the two accessions of *E. rhinocerotis* were part of a polytomy with these two species. These three species are probably very closely related. A sister relationship between *E. rhinocerotis* and *E. adpressus* is supported on morphological grounds (habit and most morphological features, as evinced by the common names 'renosterbos' and 'wyfie-renosterbos' ["wife of the rhinoceros-bush"], respectively. *Elytropappus intricata* is likely to be sister to the renosterbos lineage. A close relationship between these three species has not previously been proposed and merits further investigation (see Discussion).

3.3.3. Reconstruction of ancestor states for characters used in generic classification:

3.3.3.1. Presence of stalked, multicellular glands on the leaves and stems:

On the tree chosen for reconstruction of ancestral states, glands evolved independently between three and five times (Fig. 3-9). Once gained, no clades appear to have lost glands. The species of *Elytropappus* possessing glands (*E. glandulosus*, *E. hispidus, E. longifolius*, *E. gnaphaloides, E. intricata*, as well as the unsampled *E. scaber*) are not monophyletic in the combined analysis although their monophyly is not contradicted by any well-supported nodes.

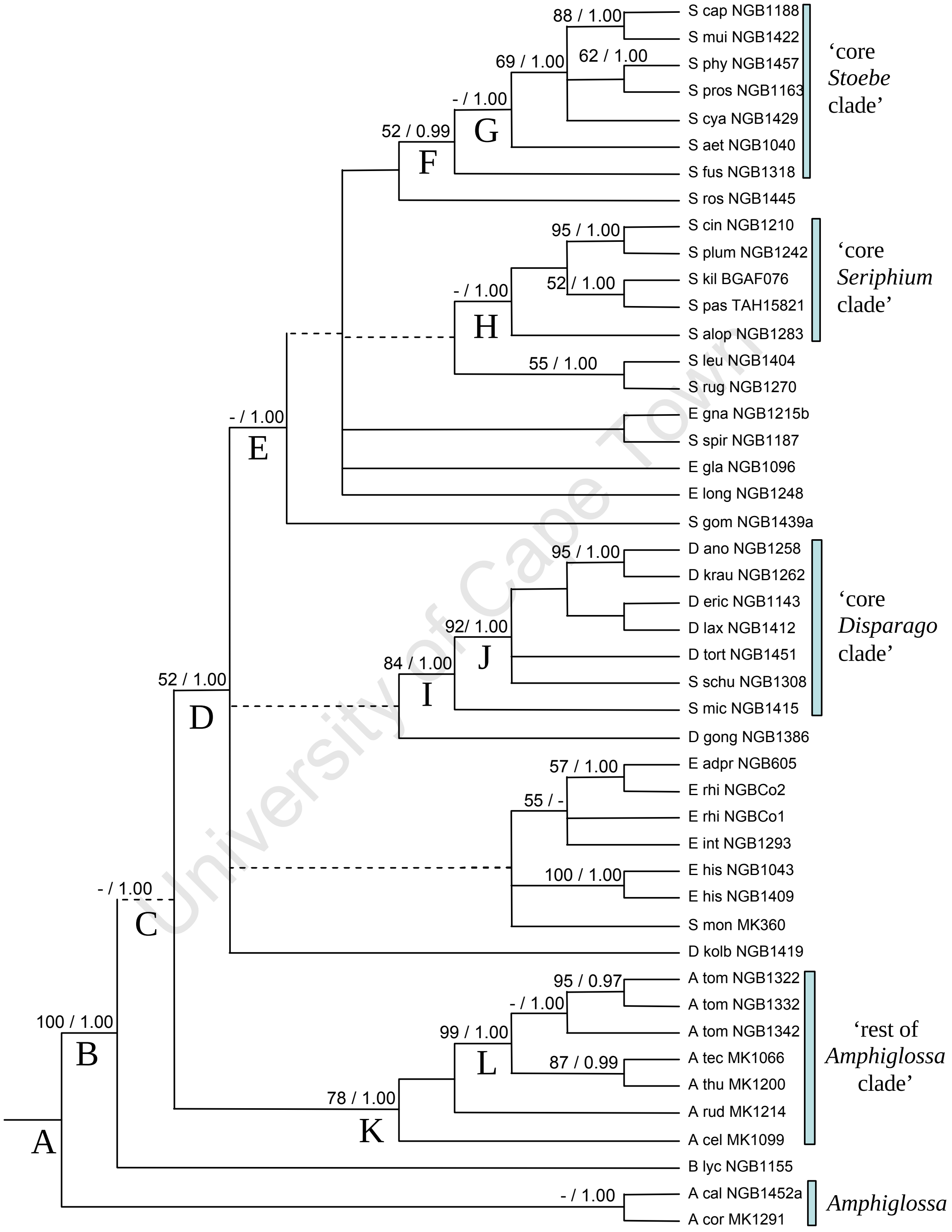
3.3.3.2. Synflorescence structure:

The early-diverging lineages all have heads borne singly or in small groups at the branch tips without a complex synflorescence structure, and this is the inferred state for the ancestor (Fig. 3-10). Compact, globose and tightly-fused synflorescences are inferred to have evolved after node D, with a reversion to the ancestral state in the clade containing *E. rhinocerotis*. Within this clade, *E. hispidus* then re-evolves the round, compact synflorescence. The compact globe appears to have elongated once to form the compact spike found in many members of the 'core *Seriphium* clade' (node H), and this elongation also occurred independently at least three more times. An open, more paniculate synflorescence shape is inferred to have arisen three times, once from a globe-shaped synflorescence in *S. rugulosa* and twice from the compact spike.

3.3.3.3. Possession of ray florets:

The ancestor of the *Stoebe* clade is inferred to have had rays (node A, Fig. 3-11) which were subsequently lost in the ancestor of the 'rest of *Amphiglossa*' clade (node K), as all the early divergences lead to rayless species. Within the 'rest of *Amphiglossa* clade', one taxon, *A. tomentosa*, regained the rayed condition. Progressing up the backbone of the tree from node A to node D, ancestors appear to have a successively lower chance of possessing ray florets, and after the divergence of the 'core *Disparago* clade' (node D), rays are permanently lost. Rays are also lost twice independently within the 'core *Disparago* clade'.

Fig. 3-8. (opposite) Majority-rule consensus of all trees sampled from the posterior distribution in a partitioned Bayesian analysis including all three datasets (plastid regions *psbA-trnH* and *ycf6-psbM* and the nuclear ETS). Numbers above branches indicate bootstrap support measures followed by posterior probabilities. Only bootstrap values above 50 % and PP values above 0.75 are shown. Dotted branches collapse in the strict parsimony consensus.


88 / 1.00
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S pros NGB1163
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S cya NGB1429
G
52 / 0.99
S aet NGB1040
F
S fus NGB1318
'core *Stoebe* clade'
S ros NGB1445
95 / 1.00
S cin NGB1210
S plum NGB1242
52 / 1.00
S kil BGAF076
- / 1.00
S pas TAH15821
H
S alop NGB1283
'core *Seriphium* clade'
55 / 1.00
S leu NGB1404
S rug NGB1270
- / 1.00
E gna NGB1215b
S spir NGB1187
E
E gla NGB1096
E long NGB1248
S gom NGB1439a
95 / 1.00
D ano NGB1258
D krau NGB1262
D eric NGB1143
92/ 1.00
D lax NGB1412
84 / 1.00
J
D tort NGB1451
52 / 1.00
S schu NGB1308
I
S mic NGB1415
'core *Disparago* clade'
D
D gong NGB1386
57 / 1.00
E adpr NGB605
55 / -
E rhi NGBCo2
E rhi NGBCo1
E int NGB1293
100 / 1.00
E his NGB1043
E his NGB1409
- / 1.00
S mon MK360
C
D kolb NGB1419
95 / 0.97
A tom NGB1322
- / 1.00
A tom NGB1332
100 / 1.00
99 / 1.00
A tom NGB1342
B
L
A tec MK1066
87 / 0.99
A thu MK1200
78 / 1.00
A rud MK1214
'rest of *Amphiglossa* clade'
A
K
A cel MK1099
B lyc NGB1155
- / 1.00
A cal NGB1452a
A cor MK1291
Amphiglossa

3.3.3.4. Number of florets per capitulum:

The ancestral state is likely to have been possession of more than seven florets in a capitulum (Fig. 3-12) and there appears to be a progression towards fewer florets throughout the history of the group, perhaps linked to the loss of rays. The number of florets is inferred to have become smaller during the evolution of the 'rest of *Amphiglossa*' clade but higher numbers re-evolved twice within this clade. The 'core *Disparago* clade' also exhibits a reduction in floret number, with three independent reductions to a single floret per capitulum. From node E all taxa are inferred to have inherited a single floret per capitulum, although there is at least one, and possibly two to three, independent reversals to a larger number of florets, in *E. glandulosus*, *E. gnaphaloides* and *E. longifolius*.

3.3.3.5. Colour of disc floret corolla:

White or pink petals are inferred to be ancestral (Fig. 3-13). Plum red florets appear to have evolved separately up to four times, and to have been lost again in *E. hispidus*. The single case of a yellow corolla, in *S. spiralis*, appears to have arisen from a white or pink corolla, although this ancestral node is equivocal.

Figs. 3-9 – 3-16. (following pages). Maximum likelihood ancestral character-state reconstructions. Letters at nodes correspond to those in Figs. 3-4 – 3-8. Only those nodes marked with a letter or an asterisk received appreciable statistical support. Inset in Fig. 3-14: erect corolla lobes in (a) *S. incana*, (b) *S. nervigera* and (c) *S. rosea*. Reflexed lobes (d) in *S. prostrata*. Drawings of florets with pappus removed from Levyns (1937). Inset in Fig. 3-15: gynoecium of floret of (a) *S. incana*, disc absent and (b) *S. prostrata*, disc present and base of style swollen (from Levyns, 1937).

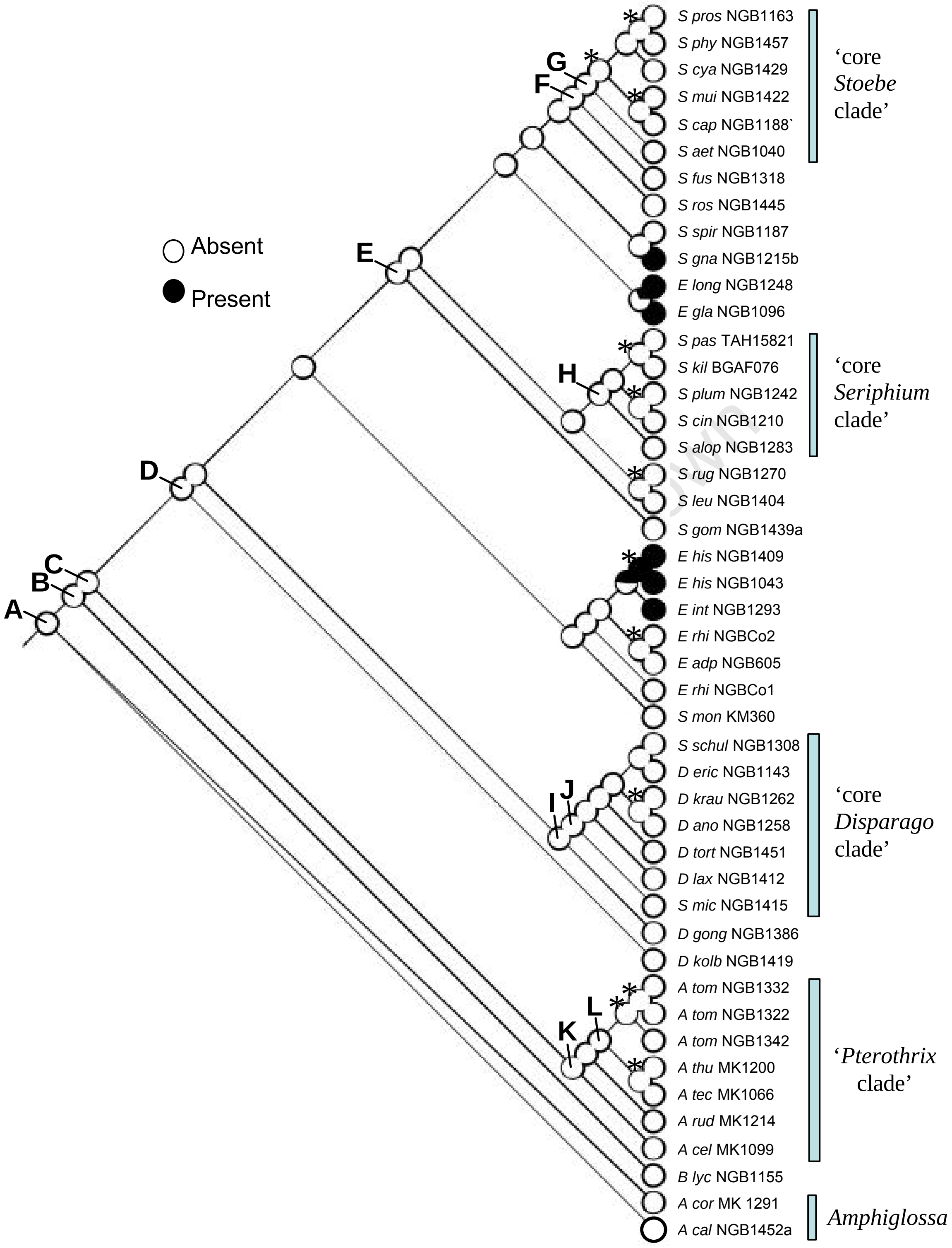

Fig. 3-9. Presence of large, conspicuous leaf glands

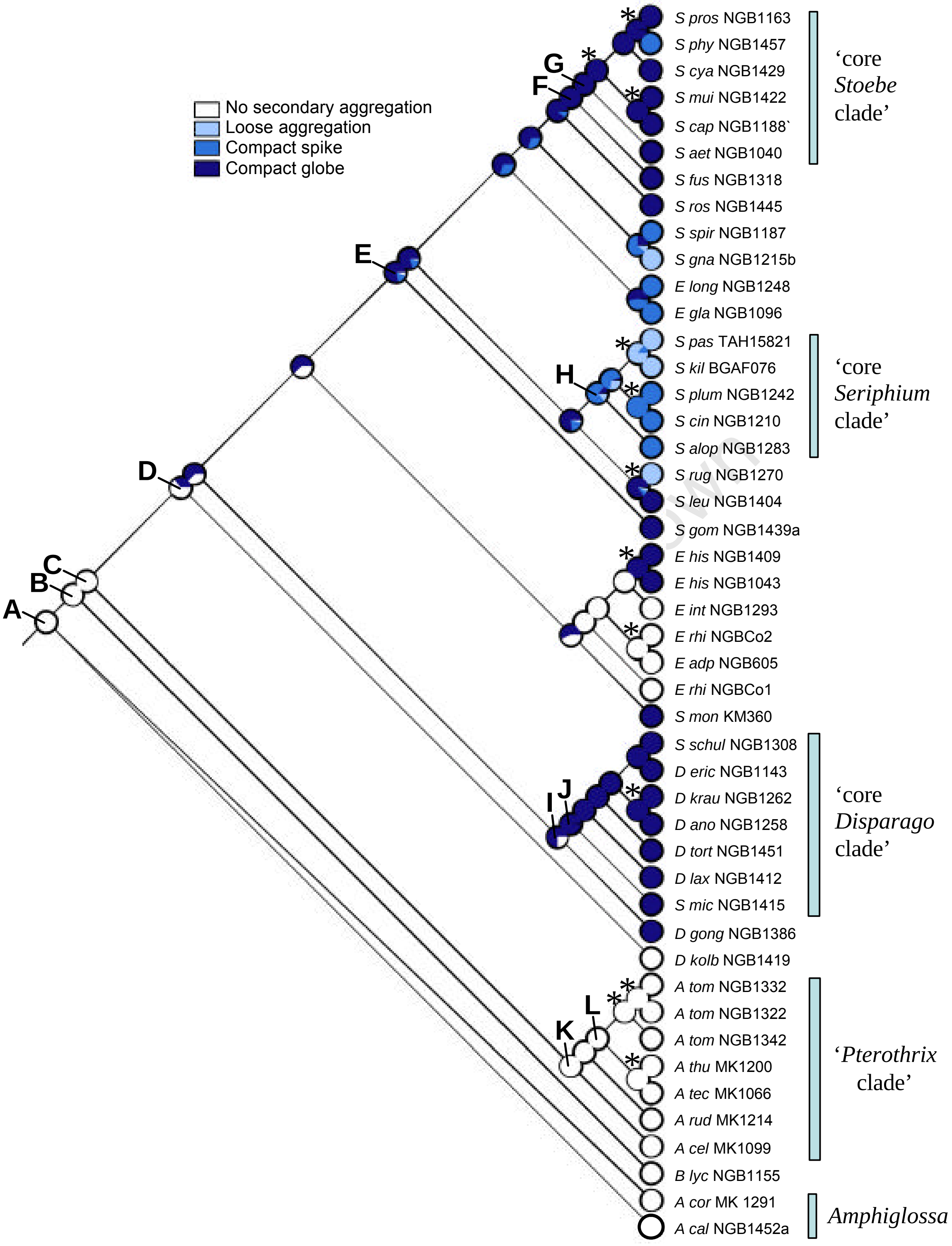

Fig. 3-10. Synflorescence arrangement

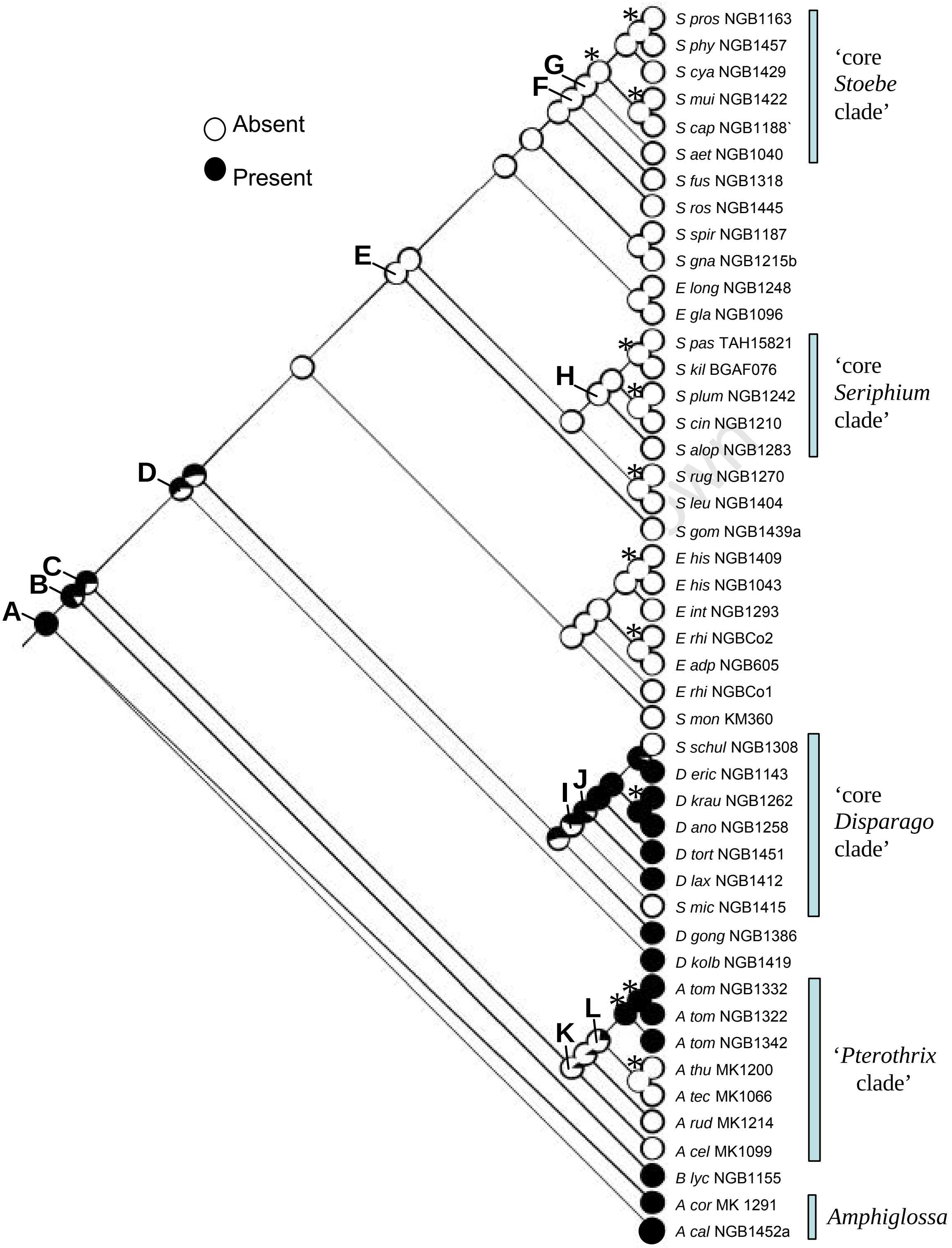

Fig. 3-11. Ray florets

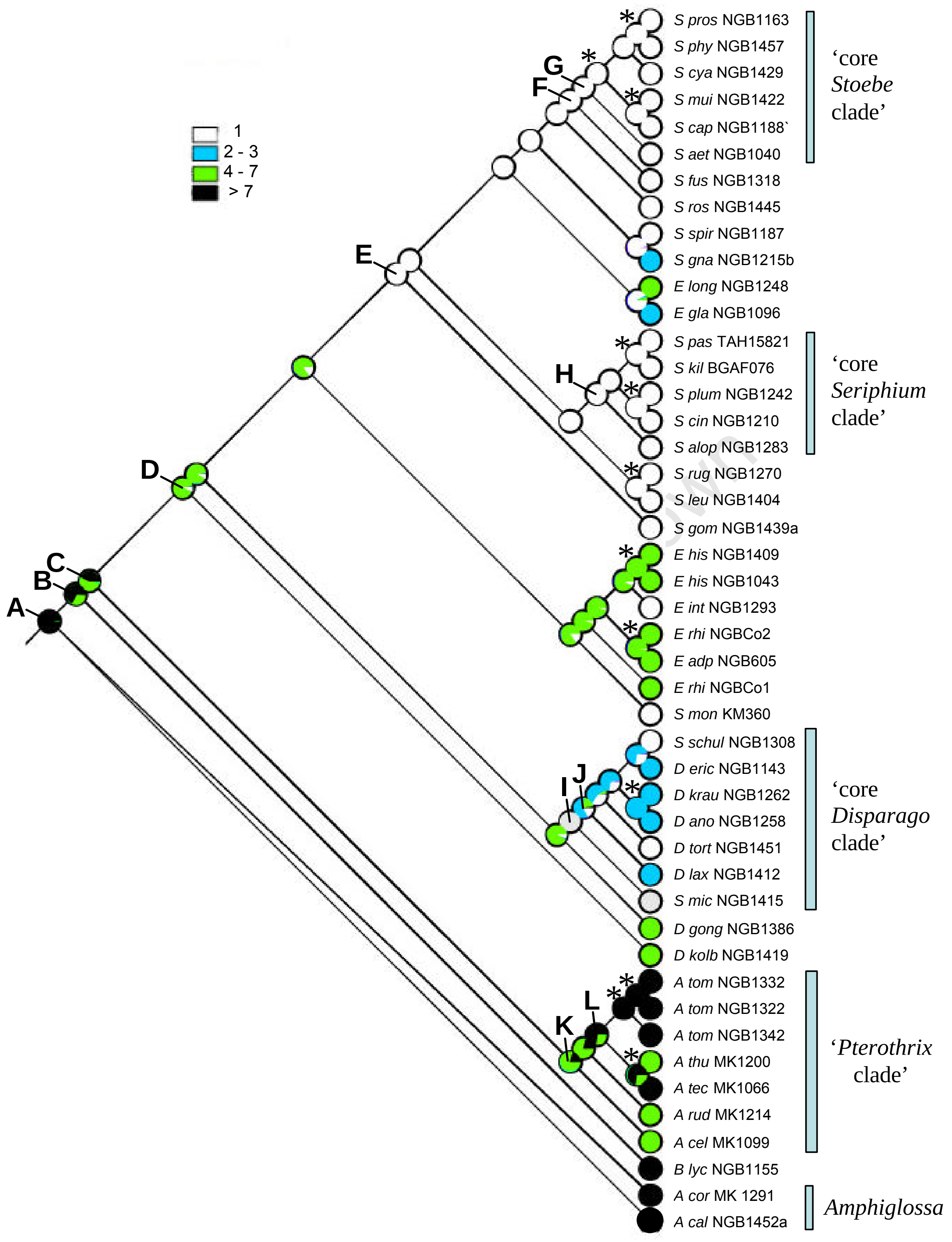

Fig. 3-12. Floret number

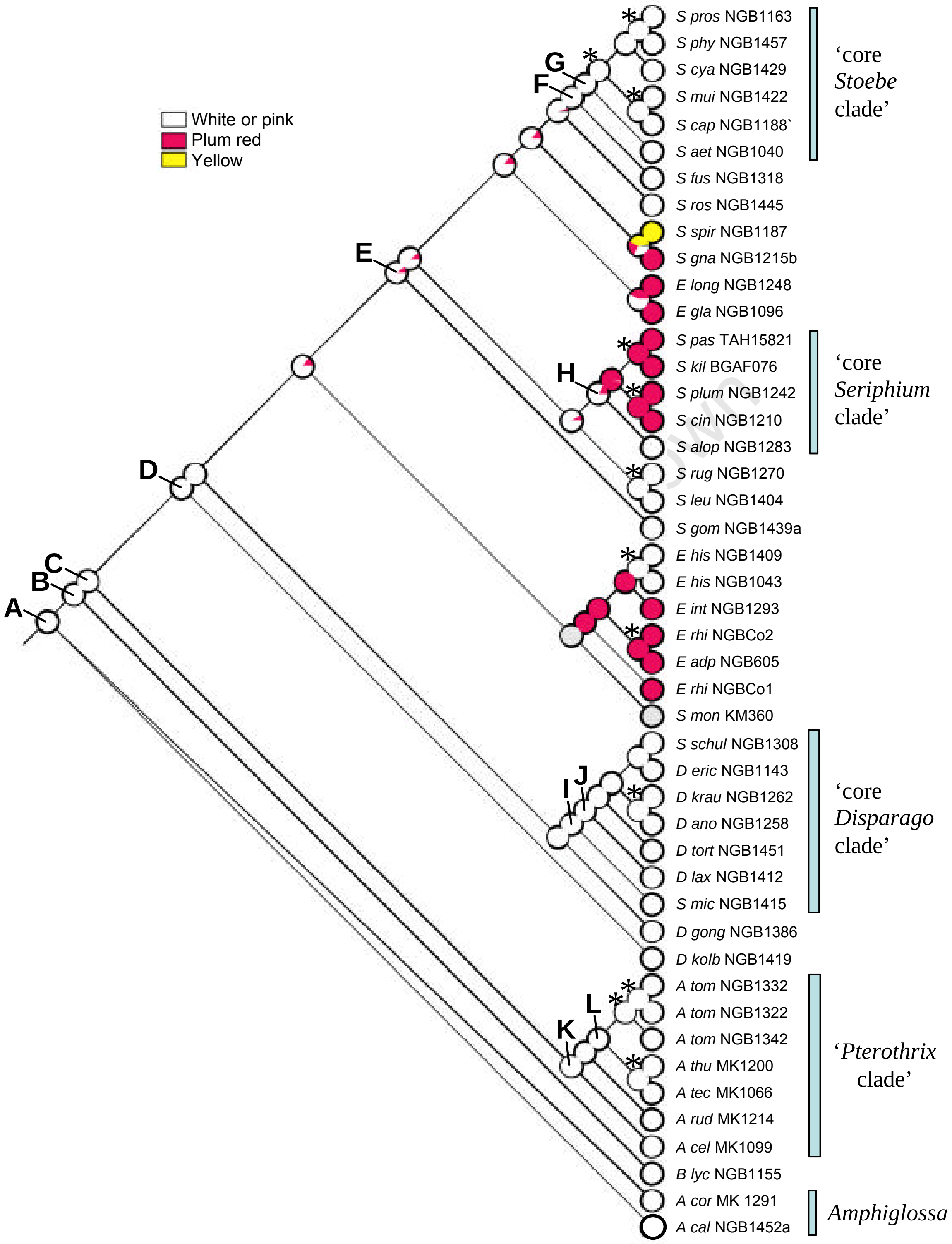

Fig. 3-13. Disc floret colour

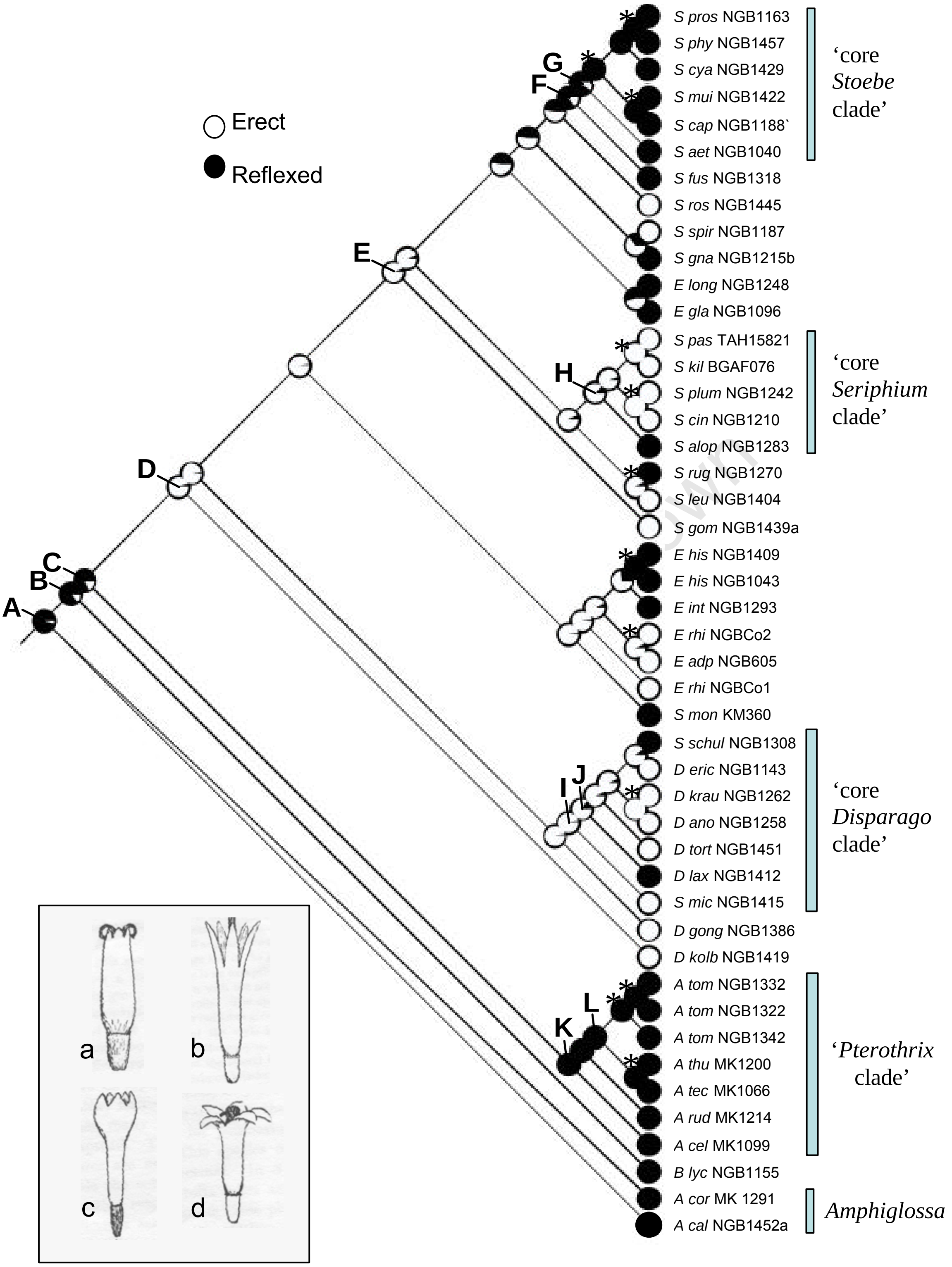

Fig. 3-14. Corolla lobe orientation

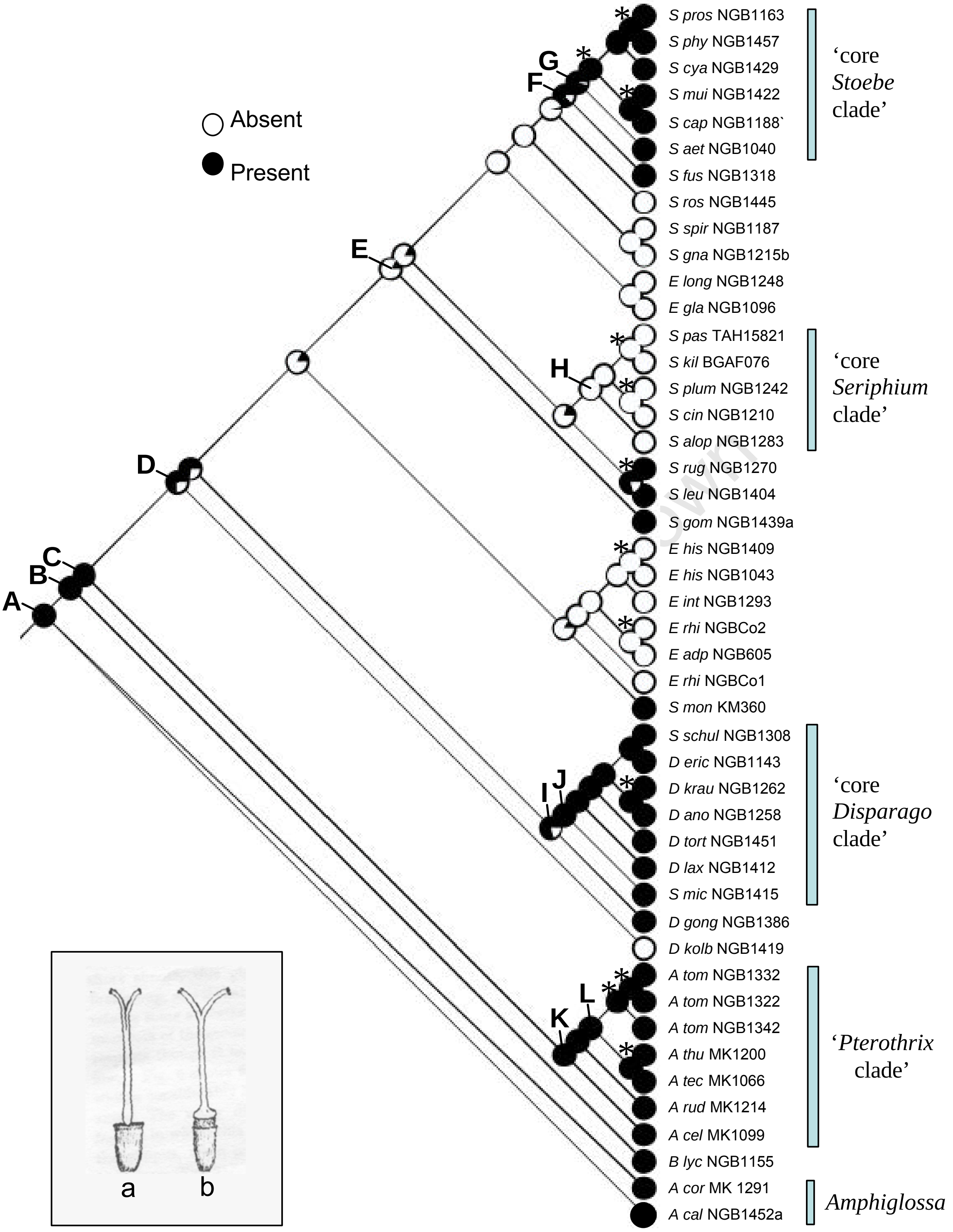

Fig. 3-15. Possession of a waxy 'nectary' disc.

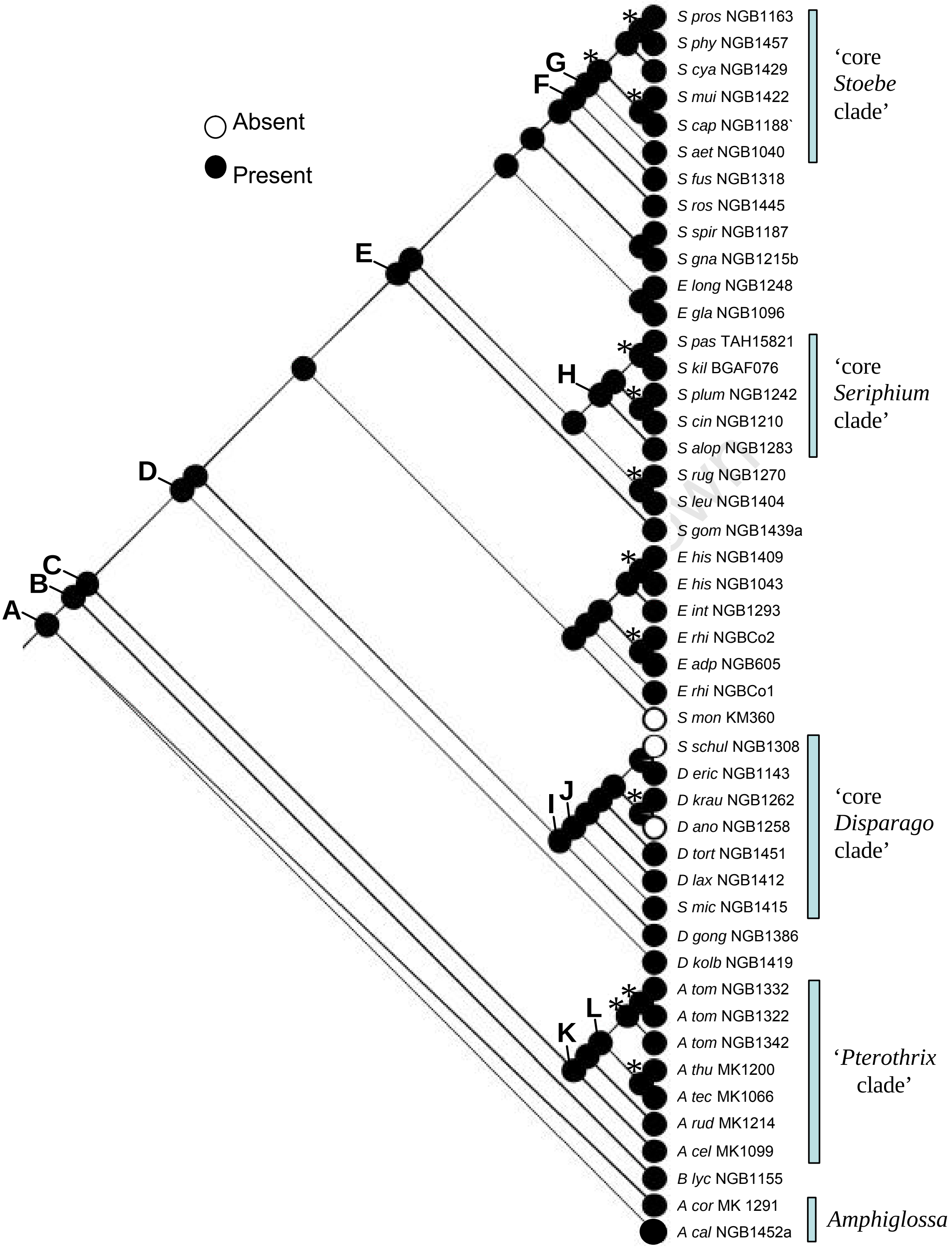

Fig. 3-16. Pappus

3.3.3.6. Orientation of the corolla lobes (erect or reflexed):

The ancestor of the *Stoebe* clade is inferred to have had reflexed corolla lobes, and this condition persisted in the ancestors of *Bryomorphe* and the 'rest of *Amphiglossa*' (Fig. 3-14). The ancestor of the 'core *Disparago* clade' and of all remaining species (node D), however, is inferred to have had erect corolla lobes. This condition is then retained, but reversions to the reflexed condition appear to have been frequent. For example, there are nine independent reversions to reflexed lobes with this topology. Two of these occur in the 'core *Disparago* clade' and one is inferred for the ancestor of the 'core *Stoebe* clade' and *S. fusca* (node F). Except for the basal switch, no instance of a shift back to erect lobes from reflexed lobes is inferred.

3.3.3.7. Possession of a 'nectary' disc at the base of the style:

The ancestral condition is inferred to be presence of the 'nectary' disc (Fig. 3-15). The disc appears to have been lost once in *D. kolbei* and again independently between nodes D and E. The placement of *D. kolbei* is uncertain. Given the lack of resolution in the spine of the tree it could branch off anywhere between nodes D and E. A position closer to node E could result in inference of only a single loss of the disc here. There are several subsequent instances of the disc being regained, for example two independent inferred gains, in *S. gomphrenoides* and the lineage containing *S. leucocephala* and *S. rugulosa*, and an additional regaining of the disc in the 'core *Stoebe* clade' at node F.

3.3.3.8. Presence or absence of a pappus:

This feature has not been used in generic circumscription as loss of the pappus is rare. Nevertheless, the analysis suggests that each loss occurred independently: twice in the 'core *Disparago* clade' and once in *S. montana* (Fig. 3-16).

3.4. Discussion

3.4.1. Topology and systematics

The robustly-supported nodes in the combined consensus tree indicate a set of phylogenetic relationships which is at odds with some aspects of the current generic taxonomy. *Amphiglossa* is clearly not monophyletic and the relationships amongst taxa currently in *Disparago, Elytropappus* and *Stoebe* are complex and require re-examination. All the main characters used in delimitation of these genera exhibit a large degree of homoplasy. The character reconstruction exercise highlights the need for a systematic, species-level study of morphology and anatomy in *Disparago, Elytropappus* and *Stoebe*. This was begun by Koekemoer (2002) who, as a result, identified several clades which have a degree of correspondence to the subclades identified in the present molecular study. However, since Koekemoer (2002) did not include all members of these genera, and since the phylogenetic hypotheses presented in Figs. 3-7 and 3-8 of the current study do not resolve the relationships amongst the subclades or the positions of all species, these relationships are still not adequately understood.

The lack of phylogenetic resolution does not appear to be the result of character conflict between the two different genomic partitions, because combining partitions increased both resolution and support. Instead, it is very likely that insufficient characters were sampled, especially from the chloroplast genome. Additional plastid loci should be sequenced for all taxa and independent low-copy nuclear markers screened. If increased resolution were the only aim, sequencing the ITS region would make sense. However, since the ITS and the ETS are likely to form a single linkage group, a phylogeny based on the signal from this gene region only would be a gene tree, which might differ from the species tree for several reasons (e.g., Pamilo & Nei, 1988). Nevertheless, even one resolved gene tree would be a useful start in clarifying relationships within this difficult group. Also, despite the negative effect on phylogenetic signal of including the morphological data, morphological characters might prove extremely informative after more careful examination of morphology, anatomy and cytology.

Another possible reason for the polytomy subtending these three genera is that they are the result of a rapid radiation, during which there was no time for the accumulation of neutral molecular mutations. It is curious that molecular and morphological data appear to mirror each other well, in the sense that many of the unplaced taxa have unusual combinations of morphological features. For example *S. rosea* has all the attributes of the 'core *Stoebe* clade' except that it lacks large, spreading corolla lobes; *S. spiralis* has all the attributes of the 'core *Seriphium* clade' except for the plum-red corolla colour. Both these species are unplaced. Other unplaced species which lack the specific morphological synapomorphies for the 'core *Stoebe*' and 'core *Seriphium*' clades (see below) are *S. montana, S. leucocephala, S. rugulosa*, *S. gomphrenoides*, *S. nervigera* and *E. intricata*.

3.4.2. Taxonomy

The two cases presented above (lack of data versus rapid radiation) have different implications for future taxonomic decisions regarding *Disparago, Elytropappus* and *Stoebe*. If rapid radiation underpins the evolution of species diversity in these three genera, even large numbers of additional characters are unlikely to resolve relationships at the base of this clade, and it may never be possible to assign all taxa to diagnosable subclades. The best taxonomic solution would then be to sink both *Disparago* and *Elytropappus* into *Stoebe*, which is the older name (Linnaeus, 1753).

The most desirable taxonomic outcome is to be able to identify well-supported smaller monophyletic units which would best represent biological diversity. There are indications that this might be possible with the addition of more data, because several subclades (the 'core *Stoebe*', 'core *Disparago*' and 'core *Seriphium*' clades) are consistently recovered and well supported in the combined analysis and in some of the separate analyses. There are also other, tentatively recovered groupings, such as the larger group containing *Disparago* and several *Elytropappus* species in the ETS tree.

The tendency of multiple accessions of a single species to form a clade provides some incidental support for the current alpha-taxonomic treatment of many entities within the *Stoebe* clade, although a proper assessment will require detailed specimen-level investigation. There is no indication of hybrid origin of any species.

3.4.2.2. Taxa currently in Amphiglossa:

The type, *Amphiglossa corrudifolia*, falls into a lineage (with *A. callunoides*) that is recovered and supported in all analyses. The remaining species, however, fall into a well-supported clade (the 'rest of *Amphiglossa*'), the placement of which varies in analyses from different gene partitions, although the different placements are never well supported. Although the relationship between the 'rest of *Amphiglossa*' and the remaining taxa is unresolved, the two *Amphiglossa* clades are never placed in a sister relationship and their paraphyly is well supported. I propose that the two species in the earliest-diverging lineage be retained in *Amphiglossa* while, for the remaining species, I propose the resurrection of an earlier name, *Pterothrix* DC (see below). These two lineages are easily distinguished by *Amphiglossa* s.s. having capitula borne singly rather than in groups, much thinner involucral bracts lacking the white-tomentose patch, and by narrowly deltoid, bright-green leaves. *Amphiglossa callunoides* and *A. corrudifolia* are distinguished from each other by a single floret character (presence or absence of a small lobe or lobes in the sinus of the ray floret corolla tube) and by geographic distribution: *A. callunoides* occurs on gravelly stream banks of rivers arising from the Groot Winterhoek, Baviaanskloof and Kouga mountains near the coast in the Eastern Cape, while *A. corrudifolia* is known from a dry river bed north of the Swartberg Mountains and disjunctly in the Northern Cape. The unusual distribution of the latter species may be an artifact of under-collection, and more observations and vouchers of species in this genus are required to elucidate their ecology, distribution, conservation status and taxonomic distinctness.

The remaining nine species currently in *Amphiglossa* were not all included in the analysis, and several were unplaced in the ETS phylogeny. They nevertheless possess numerous synapomorphies that are very likely to unite them in future analyses. *Amphiglossa* s.l. was initially erected as two separate genera, *Amphiglossa* and *Pterothrix* DC (type: *P. spinescens* DC, now synonymised with *A. triflora* DC), by De Candolle in 1838. The two genera were separated on the basis of heterogamous or homogamous, respectively, heads. The new concept of *Pterothrix* proposed will include species both with and without ray florets. A sister-relationship between *Pterothrix* and *Disparago*, as suggested by the plastid data, may be reasonable if the presence of rays in both lineages is symplesiomorphic. However, the two genera are clearly distinguished morphologically and ecologically and are not placed close together in the total evidence tree. More data may clarify the relationships of *Pterothrix* with other members of the *Stoebe* clade.

Pterothrix will contain nine species: the eight included in the ETS analysis as well as *A. susannae*. *Pterothrix* can be distinguished by its large, turbinate capitula with thick, pale brown to reddish cartilaginous involucral bracts usually with a patch of white tomentose hairs on the abaxial surface (Fig. 3-1c). These proposed taxonomic changes are presented in Section 3.4.4. below.

3.4.2.3. ***Bryomorphe*****:**

The individual florets in *Bryomorphe* are perhaps most similar to those in *Disparago*, but the pappus bristles in *Bryomorphe* are more slender and less flattened than in *Disparago*. This, and the solitary flowerheads, suggest affinity with *Amphiglossa* s.s. The retention of *B. lycopodioides* in a distinct genus is supported due to its placement on an isolated branch in the phylogeny, diverging between *Amphiglossa* s.s. and the rest.

3.4.2.4. ***Disparago*****,** ***Elytropappus*** **and** ***Stoebe*** **(node D*):***

Several well-supported nodes uphold clades which contain members of more than one of these genera. Some clades with synapomorphic combinations of characters are both well-supported and morphologically diagnosable (the ‘core *Disparago* clade’, ‘core *Stoebe* clade’ and ‘core *Seriphium* clade’) but in every case, other taxa, placed outside the clade, also possess the relevent synapomorphies, and/or the clade contains one or a few species which do not possess the defining synapomorphies. Thus taxonomic subdivision within the clade subtended by node D is not supported. It is possible that additional molecular or morphological data will allow recognition of monophyletic entities with minimal transfers of species between these genera. If so, the ‘core’

Disparago, Seriphium and *Stoebe* clades may form the nuclei of such new genera.

3.4.3. Evolution of characters in the *Stoebe* clade:

Each of the characters traced over the phylogeny is discussed in turn below. Although a single tree was used to trace the evolution of these characters, the inferences would be equally valid for alternative topologies, as nodal support is taken into account in the interpretations.

3.4.3.1. Presence of stalked, multicellular glands on the leaves and stems:

Unfortunately, there is no support for any exclusive relationship between the taxa possessing this character, and so no support for the proposed genus '*Myrovernix*' (Koekemoer, 2002). The single remaining species with stalked glands, *E. hispidus*, has white, campanulate florets and may not be related. It is possible that with more resolution, a monophyletic '*Myrovernix*' will be recovered. Stalked glands will then have evolved twice in the *Stoebe* clade. The glands in *E. hispidus* tend to have longer stalks and smaller heads than those in the '*Myrovernix*' taxa. However, the high PP support at node E of the combined tree (Fig. 3-8) suggests that *E. gnaphaloides*, *E glandulosus* and *E. longifolius* are more closely related to species currently in *Stoebe* then to the remaining glandular taxa. *Stoebe saxatilis* may also be related to the glandular taxa as the ETS data placed it in a well-supported clade (PP = 1.00) with *E. gnaphaloides* and *E. scaber*. Although *S. saxatilis* does not possess stalked glands, its capitula are very similar to these taxa.

In many trees, *E. intricata* is grouped with *E. rhinocerotis* and *E. adpressus*. The latter two species have tiny, sessile glands with a completely different cellular structure to those in '*Myrovernix*' (Fig. 3-2; Koekemoer, 2002). Detailed anatomical examination of the glands in the *Stoebe* clade is warranted to determine whether they are homologous, and such examination may provide novel taxonomic characters.

The function of the glands is unclear. The '*Myrovernix*' type glands and those found in *E. adpressus* and *E. rhinocerotis* result in the plants being very sticky, and the resin might have a role in deterring herbivores (see below), promoting flammability (Bond

& Midgley, 1995) or reducing water loss (*E. rhinocerotis* plants have been seen to exude resin on very hot days; N. G. Bergh, pers. obs.).

3.4.3.2. Secondary aggregation of the capitula (synflorescence structure):

From the ancestral condition of solitary heads, the first synflorescence development appears to have been aggregation into a terminal globe, and the reconstruction suggests that this condition is ancestral to all other types of synflorescence arrangement. The evolution of the compact spike from the terminal globe might be achieved by elongation or duplication of the synflorescence axis. The dense spicate structures found in the 'core *Seriphium* clade' (Fig. 3-3g) appear to have evolved separately from those found in *E. longifolius* and *E. glandulosus* (Fig. 3-2c), and should probably be considered non-homologous structures. On closer examination, it can be noted that the former have a globose substructure lacking in the latter.

The more loosely aggregated synflorescence types are more complex, but those in the afroalpine *Stoebe* species in the 'core *Seriphium* clade' (Fig. 3-3d) appear to be very different from those in *E. rhinocerotis* and *E. adpressus* (classified as 'no secondary aggregation' in Fig. 3-10 and illustrated in Fig. 3-2e). An ontological study of the development of these types of synflorescences may be the best method for determining how the different structures evolved.

3.4.3.3.Possession of ray florets:

This character is very labile and potentially misleading for generic classification. *Disparago*, which was previously defined by the possession of rays, is shown to include species ('core *Disparago* clade') which lack rays. *Disparago tortilis* epitomises lability in this character. Each synflorescence of this species (Fig. 3-1g) contains both ray and disc florets, but individual capitula contain either a ray or a disc floret. Presumably, some heads lose the ancestral ray while others lose the ancestral disc floret, although it is also possible that the single floret in a *D. tortilis* head has the capacity to develop either into a ray or a disc.

Rays may bear a cost, either via transpirational water loss or the attraction of floral predators. Ray florets have been gained and lost several times in the Asteraceae as a whole, and the family possesses at least four different types of ray florets (bilabiate; unilabiate / pseudobilabiate; three-lobed (true ray) or four-lobed ray; ligulate / five-lobed ray). Each type of ray floret may represent a separate independent evolution of the ray lamina in response to selection for structures to attract pollinators. Ray florets in *Amphiglossa* are bilabiate or pseudobilabiate while *Disparago* has three-lobed (ie true) rays Koekemoer (1993, 1999). Ancestral character-state reconstruction favours independent gains of rays in *Pterothrix* and in the 'core *Disparago* clade'; a systematic anatomical investigation of the ray lamina and other ray floret features might provide evidence for multiple independent origins of rays in the *Stoebe* clade. Despite overall lability of this character, the clade subtended by node E is defined by the absence of ray florets.

3.4.3.4.Number of florets per capitulum:

This character is also very labile but seems to exhibit an evolutionary trend of reduction, as floret number is reduced to one multiple times, especially within the 'core *Disparago* clade'. The character is plastic even within species in this group, as several characteristically two-flowered species have been found with only one floret, and three-flowered heads have occasionally been noted (Levyns, 1936). The loss of the ray floret from a head characteristically containing one ray and one disc floret leads naturally to the condition of a solitary disc floret per head, so reduction in floret number is also linked to ray floret presence or absence. Capitula with a single disc floret may have arisen independently from different ancestral states in *S. microphylla* and *S. schultzii* versus the remaining *Stoebe* species. In the first two, the ancestral state is inferred to be a head with one disc and one ray floret, and they may each have lost the ray floret independently. For the remaining *Stoebe* species, the condition is inferred to have arisen as a result of reduction in the number of disc florets in a discoid head. The progression towards fewer florets throughout the history of the group may be related to selection for seed-predator avoidance. This speculation is based on the observation that a high proportion of the ovules in several species are parasitised by gall midges (N. Bergh, pers. obs., J. Hoffmann, pers. comm.). If the involucre serves as a barrier to seed predators, having only a single ovary within each involucre decreases the reward for the predator and cost to the plant for each successful penetration of an involucre by a gall midge. The extremely thick, often almost bone-like involucral bracts in '*Pterothrix*' and is another mechanism to reduce apparency to insects.

3.4.3.5. Possession of a 'nectary' disc at the base of the style:

Despite frequent losses and gains of this character over the tree, it is fairly constant within the well-supported subclades. Loss and the subsequent regaining of the disc may reflect switches between different pollination syndromes, although the utility of the disc in pollination needs to be explored. Discs are largest at anthesis after which they shrivel; they are often not evident in older florets (N. Bergh, pers. obs.). This supports the hypothesis that the disc functions in attracting pollinators, as it would not benefit the plant to attract insects once fertilisation has occurred. However, as for many Cape floral groups, there have been no studies on the breeding systems or compatibility systems of members of the *Stoebe* clade. For this reason, we have no information on the degree to which these species are dependant on pollinators, if at all, or on which pollination syndromes might be involved. Although insects appear to be the most obvious pollinators, due to the size and arrangement of the florets and inflorescences, and to observations of insects visiting flowers (ants and flies; N. Bergh, pers. obs.) in the absence of such studies it is impossible to say how important they might be in reproduction.

3.4.3.6. Colour of disc floret corolla:

This is another character too labile for use in generic circumscription. Again, phylogenetic uncertainty hampers ancestral state reconstruction, but there are potentially four different origins of the plum-red floret. This warrants further research to determine whether the plum-red floret is homologous or if there are actually four different types. Differences in the corolla tube and in the relative sizes of the corolla lobes may be informative, as noted by Levyns (1935, 1937). Some species with plum-red florets (eg *E. rhinocerotis*) also possess trichomes abaxially on the corolla lobes, a feature which has not been previously recorded, presumably because these trichomes mimic the fresh pollen grains which are also found at the tip of a floret at anthesis.

Possession of plum-red florets is always associated with absence of a ‘nectary’ disc and (with the exception of *E. longifolius*, which is equivocal for this character) with having erect corolla lobes. Possession of white or pink florets is however not always associated with the alternative states for these characters (ie having reflexed corolla lobes and the presence of the ‘nectary’ disc). It seems very likely that all these characters are involved in pollination. Although no pollination studies have been conducted, possession of a nectary and white or pink petals is likely to be linked to insect pollination. Absence of the nectary and plum-red floret colour may be associated with wind-pollination. Pollination studies on this group are extremely difficult due to the small floret size and tight aggregation of individual florets into primary heads and synflorescences.

3.4.3.7. Orientation of the corolla lobes (erect or reflexed):

This character varies within most clades, and, as described above, is not always consistent with the other putatively pollination-associated characters. Several *Stoebe* species have erect lobes but possess nectary discs (*S. schultzii, S. microphylla, S. montana, S. leucocephala* and *S. gomphrenoides*) although these species all have large corolla lobes. *Stoebe spirale* and *S. rosea* have erect lobes which are small with a pale corolla (pale yellow and pink, respectively) and these species lack the disc. *Stoebe alopecuroides* has reflexed lobes which are large and white and no ‘nectary’ disc. Reflexion of the lobes may have a function independent of pollinator attraction or be selectively neutral, or may have been too coarsely defined in our reconstruction (for example, species with erect lobes should perhaps have been separated into those with large and those with small lobes; see Fig. 3-14 a - c). A re-examination of the reflexion of the corolla lobes as well as other features of the floret, is recommended.

3.4.3.8. Presence or absence of a pappus in the disc florets:

This character is not useful for generic circumscription but is nevertheless interesting because there are taxa within both *Disparago* and *Stoebe* that lack a pappus. However, these taxa all fall outside the larger ‘mainly *Stoebe*’ clade subtended by node E, and the propensity for loss of the pappus seems to be associated mainly with the ‘core *Disparago* clade’ where it has occurred twice independently. In addition, pappus loss or reduction is plastic within species in the ‘core *Disparago*’ clade. *Disparago laxifolia*, *D. tortilis*, *D. kraussii* and *D. ericoides* usually (but not always) lack the pappus, or show reduction in the number of pappus bristles on the ray floret (Levyns, 1936). It is possible that if this character were systematically examined in all members of the *Stoebe* clade, greater within-species variability would be discovered. If so, this would provide evidence for combining *D. kraussii* and *D. anomala*, as the separation of these species is based solely on the absence of the pappus in the latter. These two species are otherwise indistinguishable, grow in similar habitats and have concurrent flowering times. They should probably be united, possibly as subspecies of *D. kraussii*, although multiple losses of the pappus in different populations would render D. *kraussii* subsp. *anomala* polyphyletic and so a meaningless taxon. This is a question which might best be answered with a population-level study and a thorough examination of within-species variation in *D. kraussii* and *D. anomala*.

From a phylogenetic perspective, the rarity of complete pappus loss in the *Stoebe* clade may be indicative of *S. montana* being more closely related to the ‘core *Disparago* clade’ than is currently understood. Such a relationship is not contradicted by any node support statistics.

3.4.4. Taxonomic implications

Taxonomies constitute scientific hypotheses of relationships and so may legitimately be considered ‘work in progress’. Nevertheless, taxonomic treatments have a wide range of users, and taxonomic changes result in considerable work for the systematics community and others. It is therefore desirable that changes be made conservatively to reflect only those relationships which are likely to be borne out by future data. For these reasons, I recommend formal recognition only of those clades recovered by more than one genetic locus and/or receiving good statistical support. It is also advantageous if formal taxa are morphologically diagnosable. The following diagnoses constitute my taxonomic reccommendations at this time and will be formalised in valid publication elsewhere. Taxonomic changes to *Stoebe*, *Disparago* and *Elytropappus* are postponed until further data is available. However, paraphyly of

Amphiglossa is well-supported and merits division of this genus.

***Amphiglossa* DC.**

Type: *A. corrudifolia* DC

Stoloniferous or rhizomatous shrublets with thin wiry branches growing in sandy or riparian habitats. Leaves bright green, narrowly triangular, acute, sessile, appressed except when old and subtending a brachyblast, imbricate on brachyblasts. Capitula solitary at the ends of brachyblasts or in the axils of the leaves, radiate, heterogamous, 8-10-flowered and with equal numbers of ray and disc florets in a head. Ray florets white, female, 4 or 5, lamina up to 1.5 mm long, 1-3-lobed. Disc florets white, bisexual. Cypselas subterete, ca. 1 mm long, papillose, developing in 3-5 florets (usually rays), released in a cluster with interlocking pappus bristles at maturity. Pappus bristles free, 18-23, barbed in lower ~1/5th, delicately long-plumose above. Distribution: narrowly distributed in the Eastern Cape from Uitenhague westwards through Baviaanskloof to the Kouga mountains NW of Joubertina, north of the Swartberg mountains near Prince Albert and disjunctly in the Northern Cape province.

Included species:

Amphiglossa callunoides DC.

Amphiglossa corrudifolia DC.

***Pterothrix* DC.**

Type: *Pterothrix spinescens* DC.

Erect woody, much-branched subshrubs with wiry branches. Leaves linear, deltoid or narrowly lanceolate, abaxial surface dark green, arachnoid or glabrous, adaxial surface white-tomentose, margins involute, tips mucronate, acute or rounded. Capitula radiate or discoid, homogamous or heterogamous, solitary or in small groups of distinct units at the branch tips, ray florets 3 – 12, female, disc florets 3-12, bisexual, 5-lobed. Involucral bracts in several series, outermost chaffy or foliaceous often with a distinct white-tomentose patch on the upper abaxial surface, inner longer, involucral bract bases often thickened and cartilaginous. Disc present at the base of the style in all florets. Cypselas small, papillose, released together with the interlocking pappus bristles. Pappus bristles 18-32, densely and delicately long-plumose, free or rarely fused at the base. Distribution: Cape Provinces of South Africa (one species over entire Highveld) and Namibia.

Included species:

Pterothrix celans (Koekemoer) Bergh
Pterothrix grisea (Koekemoer) Bergh
Pterothrix perotrichioides (DC.) Harv.
Pterothrix rudolphii (Koekemoer) Bergh
Pterothrix susannae (Koekemoer) Bergh
Pterothrix tecta F. Brusse
Pterothrix thuja Merxm.
Pterothrix tomentosa (DC.) Bergh
Pterothrix spinescens DC.

3.4.4. Conclusions

The use of DNA sequences from two plastid and one nuclear locus results in a phylogenetic hypothesis for the *Stoebe* clade that suffers from lack of resolution. This is manifest in a backbone polytomy subtending the genera *Disparago, Elytropappus* and *Stoebe*. Although some subclades corresponding to a 'core *Disparago*', 'core *Seriphium*' and 'core *Stoebe*' clade are recovered and well-supported, the relationships amongst these subclades are not resolved, and many individual species in the three genera are unplaced. Taxonomic recommendations for these three taxa are postponed as there is sufficient evidence to indicate that more sequence data might help to resolve relationships. The phylogeny does however indicate the paraphyly of the genus *Amphiglossa* which I suggest be segregated into *Amphiglossa s.s.* and *Pterothrix*. The evolutionary history of several characters currently used in generic delimitation is elucidated, and a large degree of homoplasy is found in all of these characters. Future taxonomic work can build on the increased understanding of character evolution gained via the molecular phylogenetic approach. The present analysis points the way for detailed future anatomical studies and character re-evaluation.

CHAPTER 4

An examination of population history using a survey of current spatial patterns of genetic variation in renosterbos (*Elytropappus rhinocerotis*).

4.1. Introduction

In the Cape region, vegetation boundaries are correlated with precipitation (Cowling & Holmes, 1992), and so plant species ranges are likely to have been affected by past alteration in rainfall regimes. Although southern Africa was not glaciated during the Quaternary, temperatures during the Last Glacial Maximum (LGM, 24—18 thousand years ago) are thought to have been reduced by ca. 5-6 °C (Heaton & al., 1983, Talma & Vogel, 1992), coupled with alteration in both amount and seasonality of precipitation (van Zinderen-Bakker, 1976; Barrable & al., 2002).

Palaeoclimatic data indicate that changes in precipitation regime may not have been uniform across the area, especially between the current winter and all-year rainfall zones (Fig. 4-1; Meadows & Baxter, 1999; Barrable & al., 2002). However, knowledge of late Quaternary environmental change in southern Africa is limited because continuous and precisely-dated palaeoenvironmental records are scarce (Meadows & Sugden, 1993) compared with, for example, temperate areas of the northern hemisphere (Bennett, 1997). Molecular data potentially provide an independent means of inferring population history, because historical patterns of gene flow as well as population events such as bottlenecks and long-distance colonisation leave their signatures in the genetic structure of modern populations (Avise & al., 1987).

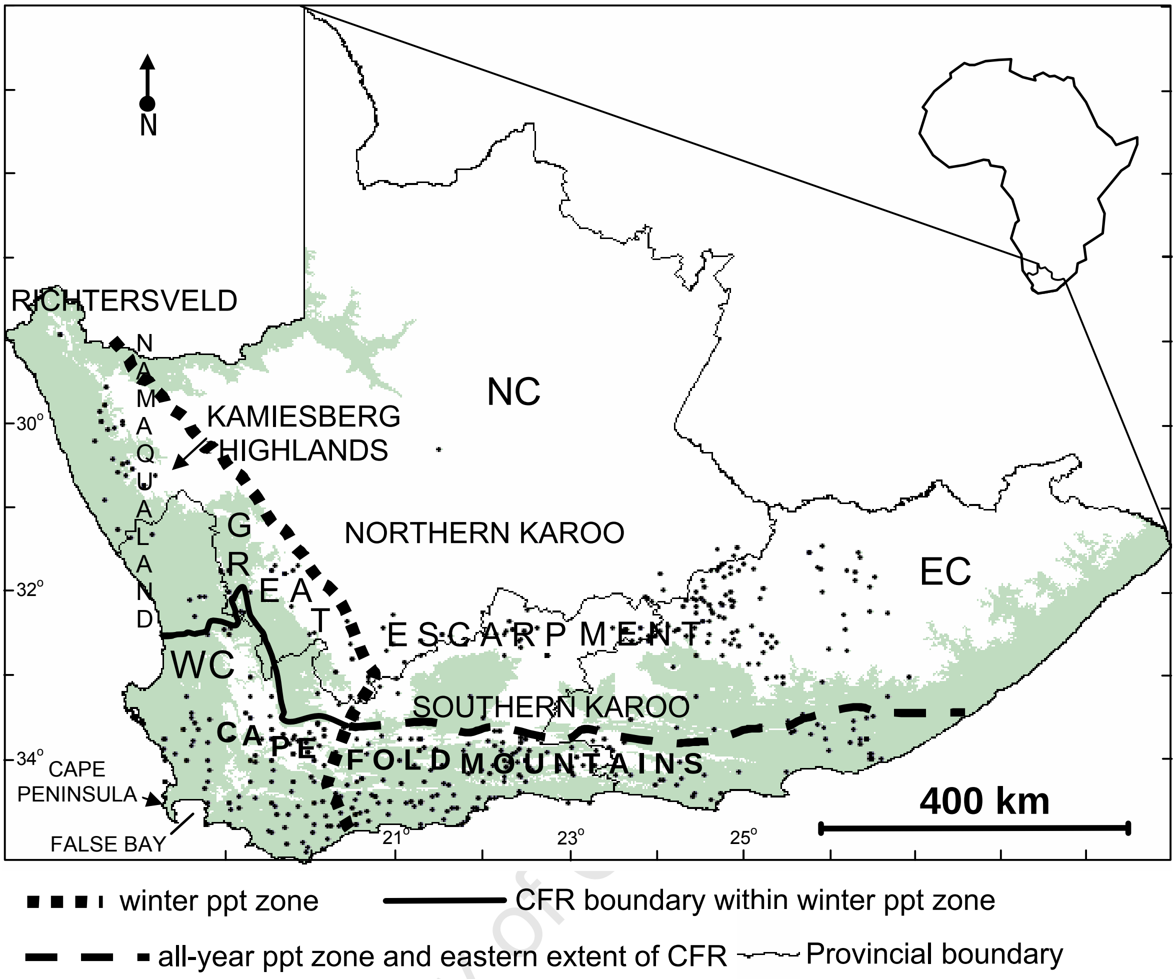

Fig. 4-1. The location of the Cape Provinces of South Africa on the African continent (WC, Western Cape; EC, Eastern Cape; NC, Northern Cape. Grey shading indicates areas with elevation less than 800m above mean sea level. Dots indicate records of *E. rhinocerotis* occurrence as mapped by the PRECIS and Acock's databases (see Acknowledgements). Inland and to the east of the winter precipitation zones (shown with thick solid and broken lines), rainfall occurs predominantly in the summer months. 'CFR' = Cape Floristic Region; 'ppt' = precipitation.

This chapter documents the use of the PCR - based hypervariable inter-simple sequence repeat (ISSR) technique (Zietkiewicz & al., 1994), which produces dominant markers from multiple anonymous sites located between microsatellite repeats in the plant nuclear genome (Wang & al., 1994), to examine spatial genetic structure in *Elytropappus rhinocerotis* (L.f.) Less.. This species, commonly known as 'renosterbos', is a long-lived woody shrub which is the dominant member of the vegetation type renosterveld (Cowling & Holmes, 1992). The adult plant is drought-resistant due to a deep taproot (Scott & van Breda, 1937), but the seedlings are killed by even slight moisture stress (Levyns, 1956). During its juvenile phase, it is also intolerant of shading and so does not survive competition from other plant species (Levyns, 1956).

Renosterbos is widespread across the CFR lowlands, and its distribution is roughly congruent with that of the GCFR in South Africa (see Fig. 1-2). However, it also occurs in smaller populations outside the main GCFR, in more arid parts of the Cape provinces (Fig. 4- 1). Here it is confined to sites where aridity is probably ameliorated by high elevation, raised water tables or orographic precipitation. For example, at the extreme north of its range in the mountainous Richtersveld desert, less than 10 plants were observed on the summit of the Vandersterrberg (1380m). In the Great Karoo, population occur on the Great Escarpment in the grassland biome.

The distribution of renosterbos suggests that it requires more moisture than succulent karoo plant species but less than most fynbos taxa (Levyns, 1938; Cowling & Holmes, 1992) and it is often found in an ecotone between the two vegetation types. Since vegetation records of past climate change are most sensitive near ecotones (Peteet, 2000), the ecotonal ecology of renosterbos may make it a good general model species for examining distributional history.

Renosterbos may also be one of the few Cape plants that is represented in the fragmentary palaeoenvironmental record. Being putatively wind-pollinated (M. Koekemoer, pers. comm.) it is thought to be one of the sources of the *Stoebe*-type pollen found in Quaternary palynological sediments from around southern Africa (eg

Deacon & al., 1983; Meadows & Sugden, 1988; Bousman & al., 1988; Scott & Bousman, 1990; Scott & al., 2004).

There is at present in the western arm of renosterbos' distribution, an increase in both temperature and aridity moving from the Cape Peninsula in the extreme south, towards the Richtersveld in the north (Fig. 4-1). However, this aridity gradient may not always have been present, and Namaqualand appears to have been substantially wetter during the phases of the last glacial period (van Zinderen-Bakker, 1976; Cockroft & al., 1987, Meadows & Baxter, 2001). Palynological evidence from the last glacial period, including records of *Stoebe*-type pollen, indicates that between approximately 70-17 thousand years (ky) ago, CFR elements existed far to the north of their current extent (Shi & al., 1998, 2000; Dupont & al., 2005). Midgley & Roberts (2001) used bioclimatic envelope modelling to estimate the extent of fynbos and succulent karoo vegetation types over the last 21 ky. Their model predicts that renosterveld may have been present throughout the Richtersveld at the LGM. If this is correct then increasing Holocene aridity over the last ca. 12 ky must have subsequently caused large-scale extinction of renosterbos populations in this area, leading to the current highly fragmented distribution there. Such a demographic scenario should result in northern Namaqualand populations showing signals of a genetic bottleneck (reduced genetic variation and greater isolation among populations) relative to southwestern populations. The vegetation of the southern part of the western arm may not have been affected to the same degree by Holocene aridity (Meadows & Baxter, 1999) and the current regional pattern of higher species diversity and endemism in the southwest is hypothesised to be the result of greater historical climatic stability in this part of the CFR (Cowling & Lombard, 2002; Midgley & Roberts, 2001).

In contrast, the climatic history of the eastern arm of the distribution range (from Cape Agulhas eastwards) is more difficult to reconstruct. Despite indications of some alteration to both temperature and precipitation regimes (Deacon & al., 1983; Martin, 1968; Partridge & al., 1999) and reduced diversity and endemism in typical fynbos lineages, conditions may never have been harsh enough to cause extinction of renosterbos populations. Complex climatic regimes as well as the nature of palaeoclimatic data make it difficult to predict past distribution changes of any species in the eastern arm, which will also be influenced by ecological interactions such as competition. Such interactions may have been more important during times (and in regions) where climatic constraints were less severe.

Several authors have used dominant, multilocus, anonymous markers to examine historical demography and detect refugial or recently founded populations (eg Clausing & al., 2000; Hess & al., 2000; Amsellem & al., 2000; Stehlik & al., 2001; Holderegger & al., 2002; Stehlik & al., 2002; Knowles & Richards, 2005). Since genetic diversity takes a long time to accumulate and is sensitive to reductions in population size (Widmer & Lexer, 2001), areas which have housed large, stable populations for long periods of time should be characterised by greater variability than areas which have experienced drastic or persistent reduction in numbers (Nei, 1975). In addition to having higher diversity, refugial areas are also likely to be genetically distinct (Ferris & al., 1999) due to isolation from other populations. In contrast, localities which have been recently colonised should possess a reduced subset of the loci present in the source populations (Hewitt, 1996). Localities from across the range of *E. rhinocerotis* were surveyed in order to answer the following questions:

1. What are the patterns of genetic diversity and relatedness across the entire distribution range? and do these mirror species-level patterns?
2. Does the western arm of the distribution exhibit a gradient of genetic variation indicating greater recent bottlenecks in the north than in the south?
3. Which areas harbour higher-than-average levels of ISSR variability, indicating that they may have housed large, stable populations of *E. rhinocerotis* throughout the period of time reflected by the genetic marker?

4.2. Methods

4.2.1. Sampling, DNA extraction and ISSR amplification

In order to achieve the most comprehensive survey of spatial genetic structuring in *E. rhinocerotis*, multiple localities were sampled across the entire known distribution range of the species, including isolated ‘edge’ regions, and attempting to prevent the occurrence of large unsampled intervening areas where the species does occur (Fig. 4-2). This entire-range sampling allows the inference of broad-scale patterns of spatial genetic variation (Arafeh & Kadereit, 2006), albeit at the cost of denser within-population sampling. In total, 107 samples and 26 localities were included in the analysis (Table 4-1).

Young shoots were collected into silica gel for drying prior to DNA extraction by the CTAB method of Doyle & Doyle (1987) and a single voucher specimen per locality was deposited in the Compton Herbarium (NBG), Cape Town (Table 1). Initial screening of ISSR primers included eight renosterbos samples from four populations spanning a wide geographic range. Nine ISSR primers were screened from the University of British Columbia Biotechnology Laboratory Primer set 9. Three primers were selected based on their ability to produce a large number of discrete bands that were polymorphic across the sample specimens. The primer sequences are (GA)$_8$-A (primer 812); (AG)$_8$-YC (primer 835) and (CA)$_8$-RT (primer 846). Amplifications were performed in either a Hybaid PCR Sprint (Fisher Scientific International) or an ABI GeneAmp® (Applied Biosystems) thermal cycler. Reaction volumes were made up to 25 ?l with PCR-grade autoclaved water. Reaction mixtures consisted of 1X reaction buffer, $MgCl_2$ at 3mM (primers 812 and 835) or 2.5mM (primer 846); 0.15, 0.1 and 0.2 mM of each dNTP (primers 812, 835 and 846, respectively); 0.4 µM of primer and 0.03, 0.04 and 0.024 units of Biotaq^{TM} (Bioline) per µl (primers 812, 835 and 846, respectively). The
thermal cycler profiles consisted of an initial denaturation for 1.5 min. at 94^o followed by 35 cycles of: 1 min. at the primer-specific annealing temperature, 1 min. at 72^o and 30 sec. at 94^o. This was followed by 2 min. at 52^o and 3 min. at 75^o. The annealing temperatures for the three primers were 54^o, 50^o and 53^o for primer 812, 835 and 846, respectively.

4.2.2. Band visualization and assessment of locus identity.

Banding profiles were visualised by electrophoresis in 5% TBE buffer using 1.5% (weight by volume) 12 - lane agarose gels. Gels were run at 65 - 80 mV until the bromophenol blue loading-buffer dye band had advanced 8 cm from the wells (approximately 3 hours), stained with Ethidium Bromide and photographed under UV light. Multiple gel images were printed at different levels of contrast and brightness to facilitate detection of both bright and faint bands (Zietkiewicz & al., 1994). For each primer, one or two individual plants that exhibited a range of fragment sizes were chosen as reference samples and their banding profiles were used in lieu of size standards. The sizes of bands in reference sample profiles were measured against Eco-RV – digested ? DNA size standards in a separate gel. Extracted *E. rhinocerotis* DNA samples were labelled with a random letter code. This resulted in sample identity being unknown so that band scoring was completely objective. In each scoring gel, reference samples occupied three lanes while samples to be scored were run in two non-adjacent replicate lanes per gel, similar to van der Kloet & Paterson (2000). Each fragment size was assumed to represent a separate ISSR locus and individuals were coded for the phenotype band present (1) or absent (0) at each locus. Reproducibility of fragments was tested by rigorous replication of all steps in the generation and visualisation of ISSR bands. Each primer-sample combination was repeated in at least two and up to 20, separate PCRs, and run out in at least two separate scoring gels.

4.2.3. Data analysis

Multivariate analyses were used to measure broad patterns of spatial genetic structure across the distribution range and included all 107 samples. Pairwise distance metrics used were the index of Jaccard (1908) which takes into account only shared band presence, and the simple matching coefficient (SM) which uses both shared presences and absences (Sneath & Sokal, 1973), both calculated in NTSYS-2.1 (Rohlf, 2000). The observed proportion of bands not shared by two accessions were calculated for all pairwise comparisons in Splitstree 4 v. 4.6 (Huson & Bryant, 2006), where this metric is referred to as the ‘uncorrected P-value’. The dis-/similarity matrices were clustered

Table 4-1. Sampling localities for *E. rhinocerotis*. The columns are: locality codes (Loc. ID), no. of sampled individuals (n), sampling localities and provinces (EC--Eastern Cape; NC--Northern Cape; WC--Western Cape), region to which locality was assigned for genetic diversity calculations (see Fig. 4-2), voucher number [all vouchers deposited at the Compton Herbarium (NBG)] and locality co-ordinates in decimal degrees.

Loc. ID	n	Locality, province	REGION	Voucher no.	Co-ordinates (long / lat)
BARD	3	Baardskeerdersbos, WC	Southcentral	*Bergh 301*	19.58 / -34.59
BAV	5	Baviaanskloof, western end, EC	Southcoast	*Bergh 238*	23.58 / -33.49
BEAU	5	Beaufort West, Karoo, WC	Eastcentral	*Bergh 795*	22.56 / -32.2
CATH	1	Cathcart, north of Stutterheim, EC	--	*Bergh 746*	27.11 / -32.32
CORN	1	Cornellsberg, Richtersveld, NC	Richtersveld	*Desmet 3160*	17.18 / -28.57
EYAM	1	Eyams, Richtersveld, NC	Richtersveld	*Desmet 3273*	17.62 / -29.35
GAR	5	Garcia's Pass, Muiskraal, WC	Southcentral	*Bergh 270*	21.22 / -33.92
GRA	5	Coombs, near Grahamstown, EC	Southeast	*Bergh 728*	26.78 / -33.29
HANK	3	Hankey, near East London, EC	Southeast	*Bergh 692*	24.82 / -33.91
JON	10	Jonaskop, near Villiersdorp, WC	Jonaskop	*Bergh 871*	19.52 / -33.92
KOMS	3	Komsberg Pass, Roggeveld, NC	Roggescarp	*Bergh 374*	20.76 / -32.67
KOU	5	Koue Bokkeveld, WC	Swartland	*Bergh 201*	19.26 / -32.82
LEL	8	Leliefontein, Kamiesberg, NC	Leliefontein	*Bergh 76*	18.08 / -30.31
LION	3	Little Lion's Head, Cape Town, WC	Cape Peninsula	*Bergh 992*	18.35 / -34.01
MALM	5	Malmesbury, WC	Swartland	*Bergh 880*	18.72 / -33.47
MENS	5	Farm Mensieskraal, NC	Northescarp	*Bergh 62*	19.31 / -31.73
MID	5	Middelpos, Roggeveld scarp, NC	Roggescarp	*Bergh 990*	20.25 / -31.91
NBETH	3	Nieuw-Bethesda, Sneeuberge, EC	Northeast	*Bergh 817*	25.06 / -31.97
NIEU	3	Nieuwoudtville Waterfall, NC	Northescarp	*Bergh 980*	19.12 / -31.32
PLET	3	Plettenberg Bay, South coast, EC	Southcoast	*Bergh 478*	23.57 / -33.96
RHOD	4	Rhode's Memorial, Cape Town,WC	Cape Peninsula	*Bergh 950*	18.46 / -33.96
SPEK	5	Spektakel Pass, Namaqualand, NC	Northnamaqua	*Bergh 319*	17.71 / -29.7
STERK	5	Sterkstroom, Stormberg mts, EC	Northeast	*Bergh 751*	26.66 / -31.63
SWART	3	Swartberg Pass, WC	Eastcentral	*Bergh 614*	22.11 / -33.38
TOE	3	Farm Toeneus, Kamieskroon, NC	Northnamaqua	*Bergh 964*	17.82 / -30.22
VAN	5	Vandersterrberg, Richtersveld, NC	Richtersveld	*Bergh 966*	17.07 / -28.43
Total:	**107**	**26 populations**			

using UPGMA (NTSYS-2.1) and Neighbour Joining (NJ; Splitstree 4 v. 4.6). The goodness-of-fit of the UPGMA dendrograms was measured by their cophenetic correlation with the original dissimilarity matrix in NTSYS-2.1. The robustness of the NJ groupings was quantified with 1000 bootstrap replicates in Splitstree 4 v. 4.6 (Huson & Bryant, 2006). Principal co-ordinates analysis (PCoA) scatterplots based on Jaccard’s index and the SM coefficient were produced in NTSYS-2.1 (Rohlf, 2000).

An analysis of molecular variance (AMOVA) was used to examine genetic structure measured as the partitioning of genetic variation within and among groups in ARLEQUIN V.2.0 (Excoffier & al., 1992; Schneider & al., 2000). The three localities represented by a single sample each were excluded from AMOVA analysis. Levels of significance of the variance components were assessed via 10 000 nonparametric randomisation permutations as implemented in ARLEQUIN. Four different grouping structures were assessed. Firstly, samples were grouped by locality to assess the amount of ISSR variance within the average locality. Secondly, the distribution range was divided into eastern and western arms, corresponding to the summer arid zone (western arm; Fig. 4-1) and the all-year rainfall zone (eastern arm). The third and fourth AMOVA structures estimated the amount of variation partitioned across groupings that were suggested by the multivariate analyses (see Results).

4.2.4. Analysis of regional patterns of ISSR variation

The entire-range sampling strategy precludes inference of population-level parameters due to low numbers of samples for each locality. However, the AMOVA indicated that nearly 80% of the variation measured in the entire species sample is represented within localities (see Table 4-2 and Results). In renosterbos, any given locality is thus largely representative of the total variation (Gregorius, 1988). Although statistically significant, differentiation among localities is small and so we considered it biologically reasonable to group individuals from neighbouring localities into ‘regions’ for further analysis. The rationale for grouping samples into regions is to increase sample sizes so that diversity estimates are based on larger (and equal) numbers of individuals. Grouping samples into regions also allows estimation of genetic relatedness of populations from different regions, providing an overview of spatial structure. Regional groupings were made up of samples from nearest-neighbour localities less than 250km apart. To maintain equal sample sizes, some individuals were excluded from the regional analyses. Samples to be excluded were chosen randomly.

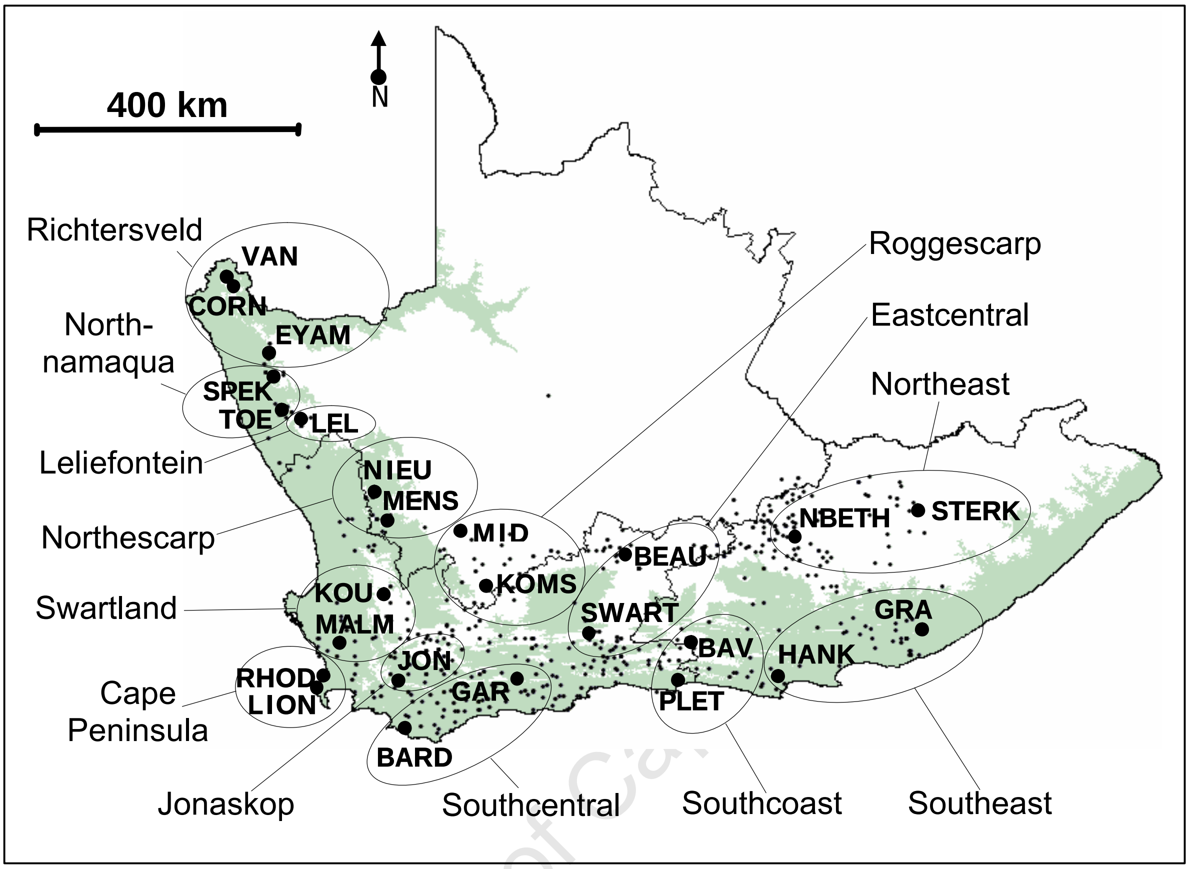

Fig. 4-2. The Cape Provinces of South Africa showing sampled localities, indicated by black dots with names in upper case. Grey shading indicates areas with elevation less than 800m above mean sea level. The regions into which samples were grouped for regional analyses are indicated by the ellipses. Names of regions are given in lower case with initial capitalised letter.

Any specific arrangement of individuals into a regional grouping structure may not be congruent with the overall pattern of genetic structure. It is also possible that different arrangements of localities into regions might produce different results. To test this, diversity calculations were repeated using multiple different nearest-neighbour allocations of individuals to regions. For example, in the eastern arm, samples can be grouped into regions which include only localities aligned along two east-west axes, one for the coastal and one for the inland mountain ranges. Alternatively, there could be several groupings, each running north-south from the escarpment to the coastline. Multiple types of grouping arrangements were tested, as well as multiple different arrangements within either east-west or north-south types of groupings. A similar range of types of arrangements was also tested for the western arm of the range. All regional grouping structures resulted in the same patterns of range-wide diversity, which are all consistent with the conclusions drawn (see Discussion), so results are presented for only a single grouping arrangement, i.e. that depicted in Fig. 4-2. This arrangement includes 103 *E. rhinocerotis* individuals grouped into 13 regions.

The frequency of band presence at each locus was calculated among samples from each region. This produced interval (as opposed to binary) data for each locus which was used to calculate a pairwise Euclidean distance matrix and clustered using UPGMA in NTSYS-2.1 (Rohlf, 2000). A Principal Components Analysis (PCA; Hotelling, 1933) as implemented by STATISTICA version 6.1 was performed on the interval data and the results mapped for visual inspection using synthesis mapping, as in Piazza & al. (1981) and Cavalli-Sforza & al. (1994). This is a technique for graphical display of complex multivariate data. Each map represents one PC axis with the position of a sample on that axis indicated by greyscale shading. The shading scale was chosen by dividing each PC axis into nine segments and assigning each segment a shade from white at one extreme of the axis to black at the other. The applicable greyscale shade was then mapped onto the geographic centre of each region, which is the point midway between all localities included in that region, weighted according to number of samples from each locality (as in Templeton & al., 1995). Values were interpolated onto the map space between geographical centres using inverse weighting of the distance to the three nearest neighbour geographical centres in ArcView GIS 3.3 with a Spatial Analyst extension (Environmental Research Institute, Inc.).

Measures of phenotypic rather than genetic diversity were used in order to reduce the number of genetic assumptions required (e.g. no analysis assumed Hardy-Weinberg genotype proportions). The following diversity measures were calculated: the percentage of loci that are polymorphic (P) for each region; Shannon's (1949) diversity index (H') calculated as in King & Schaal (1989); and the simple matching coefficient as calculated in NTSYS-2.1 (Rohlf, 2000). The SM was used to calculate the average pairwise distance between all individuals in a region (Amsellem & al., 2000).

4.2.5. Isolation-by-distance analysis.

Isolation-by-distance (IBD) between individual samples was tested via Mantel's (1967) test using NTSYS-2.1 PC (Rohlf, 2000). Significance was determined by comparing the observed Mantel test statistic M_R with the distribution of values obtained from 10 000 random Monte Carlo permutations of one of the matrices (Rohlf 2000) in the same software. Great-circle geographic distances between localities were calculated in Arcview 3.3. Geographic distances between individuals from the same locality were set at zero. The IBD analysis was conducted for all samples as well as for the eastern and western arms of the distributions. In order to prevent the use of arbitrary geographic boundaries, localities in the extreme southwest of the distribution were included in both 'western arm' and 'eastern arm' analyses.

Directional autocorrelation between genetic and geographic distance was examined over eight distance classes of 250 km each, as in Oden & Sokal (1986). The Mantel statistic M_R was calculated for each geographic distance-class matrix versus the ISSR distance matrix, tested for significance as described above, and plotted against geographic distance. Significance values were corrected using the sequential Bonferroni technique of Holm (1979).

4.3. Results.

4.3.1. Band replicability across PCRs and gels

The number of bands obtained per ISSR primer is unusually high (Wolfe & Liston, 1998) and more comparable to that found in typical AFLP studies. However, all bands were present in all replicates; these results may be attributable to species-specific SSR abundance and genomic distribution and/or the particular band-detection methods employed in this study. Only bands that are always very faint when they are detectable vary in their observable presence, and this may be related to the limitations of agarose gel visualisation. These faint bands were not included in the analyses and all scored ISSR loci were reproducible between template concentrations, gels and replicate PCR's. For some samples, such as the five reference samples, this involved successful reproduction of identical banding patterns in over 20 PCR reactions and 50 scoring gels. The dataset consisted of 220 reliable ISSR loci overall, ranging from ca. 300 to ca. 2000 base pairs in length. Of these, 79 come from primer 812, 73 from primer 835 and 68 from primer 846. All 220 bands were included in analyses as the statistical measures employed make minimal assumptions about the genetic nature of the ISSR bands (Lynch & Milligan, 1994). No two individuals share the same ISSR banding pattern. Only one band is present in every individual, making the directly observed proportion of polymorphic loci 99.5%. The proportion of bands present in > 5 and < 95% of the sample is 0.73. Several loci are unique to a single locality (data not shown), but none of these is present in more than two individuals from that locality, and possessing unique loci is correlated with sample size so is probably not informative.

4.3.2. Analysis of all individuals

Fig. 4-3 shows the unrooted NJ tree based on P distances of Splitstree 4 v.4.6 (Huson & Bryant, 2006), with geographically coherent clusters labelled. The only locality for which all sampled individuals group together is PLET from the south coast of the Eastern Cape Province. This locality is genetically distinct in all multivariate analyses, and although it is represented by only three samples, many other localities have similarly few samples which do not group together or form a distinctive cluster. Several MENS samples from the northern CFR are also quite distinct, as are five of the ten samples from JON near the southwest of the Cape Fold mountain chain. There is also a grouping which includes five of the seven samples from the Cape Peninsula (localities RHOD and LION) as well as two samples from nearby KOU. However, the cluster that has greatest geographical coherence contains 93% of the individuals from the extreme northwest of the range (localities VAN, SPEK, EYAM, CORN and TOE). These localities all occur to the north of the Kamiesberg massif. Samples from LEL, collected on the Kamiesberg itself, are distributed throughout the tree, frequently grouping with samples from the far eastern or southern extreme of the range.

Although none of these clusters has bootstrap support above 50%, these same groupings were recovered in all multivariate analyses: NJ (Fig. 4-3), UPGMA and PCoA (data not shown). The UPGMA topologies from both the Jaccard and SM coefficient are essentially the same as that in Fig. 4-3. The cophenetic correlations of the UPGMA dendrograms are r = 0.526 (Jaccard's index) and r = 0.634 (SM coefficient). The first three axes of the principal co-ordinate scatterplots summarised 7.4% (Jaccard's index) and 10.6% (SM) of the variation in the data. The main feature of all the individual-level multivariate analyses, clearly visible in Fig. 4-3, is that there is very little geographical structuring of genetic variation in *E. rhinocerotis*. No localities or regions form well-supported groupings, and most clusters consist of individuals from geographically distant localities across the distribution range.

4.3.3. Analysis of Molecular Variance (AMOVA).

The AMOVA indicates that nearly 80% of ISSR variance is represented among individuals within localities (Table 4-2A.). However, there was no significant apportionment of variance between the summer-arid and the all-year rainfall zones (Fig. B within Table 4-2). There is statistical support for the stronger association of LEL with eastern localities: if LEL is included with the eastern rather than western samples, the west versus east component, though very small, acquires statistical

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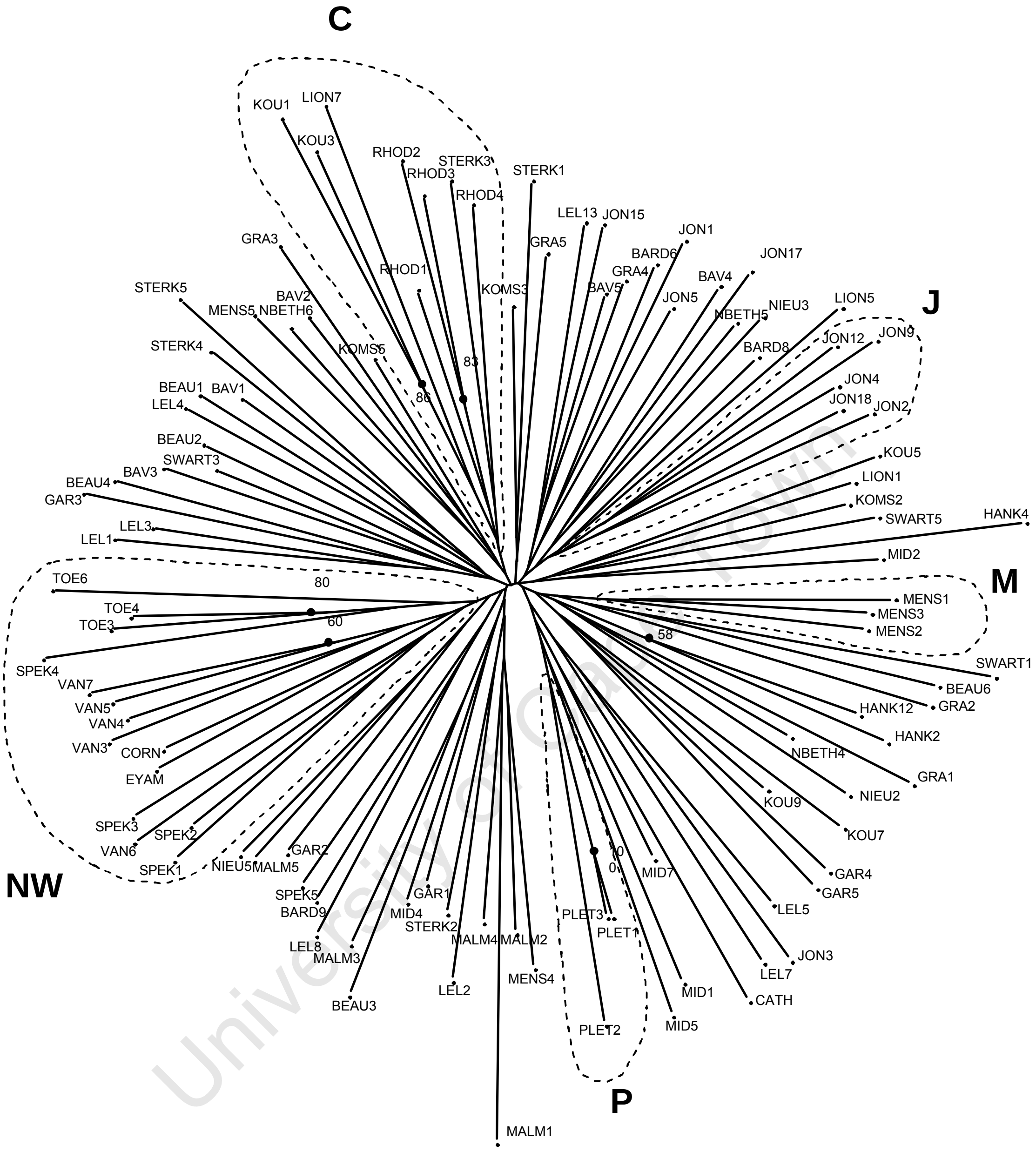
Fig. 4-3. (opposite) Unrooted neighbour-joining tree of all 107 renosterbos accessions based on P distances as calculated in Splitstree 4v.4.6. Clusters enclosed in dotted lines indicate localities or regions whose samples group together (C= five of the seven samples from the Cape Peninsula and two samples from the nearby locality KOU; J= five of the ten samples from locality JON; M = three of the five samples from locality MENS; NW = 14 of the 15 samples from the northwesternmost localites, ie. those north of LEL; and P = the three samples from locality PLET). Bootstrap values are shown only for those nodes which received > 50% support (1000 replicates).



significance (Table 4-2B). The strongest genetic disjunctions suggested by the multivariate analyses occur between northwestern localities (those north of LEL), and all others, and between PLET and surrounding localities. In order to quantify these breaks, we performed AMOVAs with localities divided accordingly (Table 4- 2C and

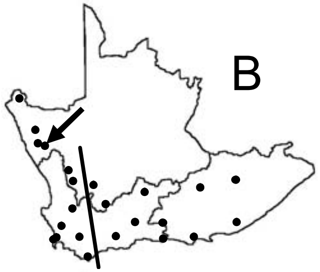
D). These arrangements showed the greatest structure, with nearly 6% of the variance attributable to the division between northwestern localities and the rest, and 7.8% attributable to a division of localities into northwestern versus PLET versus the rest. AMOVA was also used to examine ISSR structure within the eastern and western arms of the range separately, divided according to Fig. B in Table 4-2. The amount of ISSR variation apportioned among western localities only (excluding LEL) is 23.32%, while that apportioned amongst eastern localities only (including LEL) is 17.87% (each separate AMOVA with $p < 0.0001$). If LEL is excluded from the analysis entirely, the separate values are 23.32% amongst western, and 19.99% amongst eastern localities (again, all $p < 0.0001$).

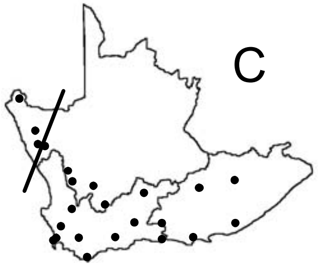
4.3.4. Isolation-by-distance analyses.

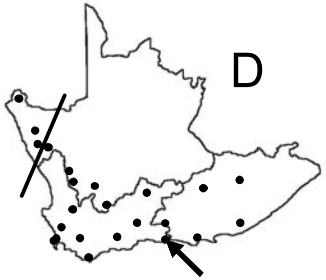
The Mantel test indicates a statistically significant but negligible trend of increasing genetic distance with untransformed geographic distance between individual *E. rhinocerotis* plants ($M_R = 0.13$, $p = 0.001$). The spatial autocorrelation analysis (Fig. 4-4) shows that the slight effect of decreasing relatedness with distance operates most strongly over distances of about 250 km, but once samples are more than 750 km apart this relationship is no longer significant (filled symbols in Fig. 4-4). This indicates that samples which are more than 750 km apart are as distinct as, for example, samples 1500 km apart. The pattern of IBD holds true for the western and eastern arms separately (broken lines, Fig. 4-4), but the trend is stronger and operates over greater distances in the western arm.

Table 4-2. (opposite) AMOVA design and results. All statistical significance values were determined by comparison against 10 000 random permutations of samples and localities, while keeping sample numbers at each locality constant. Western grouping: VAN, SPEK, TOE, LEL, NIEU, MENS, KOU, MALM, RHOD, LION, JON, BARD. Eastern grouping: MID, KOMS, GAR, SWART, BEAU, BAV, PLET, HANK, NBETH, GRA, STERK.

	Source of variation	d.f.	Sum of squares	Variance components	% total variation	? - statistics	p
A.	**Total (23 localities) ---**						
	Among localities	22	359.56	1.28	20.98	0.209	<0.0001
	Among individuals	185	892.96	4.83	79.02	-	-
B.	**Western (12 localities) vs Eastern (11 localities) ---**						
	Among West vs. East	1	18.60	0.01	0.18	0.002	0.177
	Among localities within groups	21	340.95	1.28	20.87	0.210	<0.0001
	Within localities	185	892.96	4.83	78.95	0.210	<0.0001
	Western vs Eastern localities but with LEL (arrow, Fig. B) included with Eastern localities--------------------------------------						
	Among West vs. (East + LEL)	1	23.76	0.06	0.94	0.009	0.009
	Among localities within groups	21	335.79	1.25	20.39	0.206	<0.0001
	Within localities	185	892.96	4.83	78.67	0.213	<0.0001
C.	**Northwestern grouping (localities VAN, SPEK & TOE) vs all other localities---**						
	Among Northwest vs. Rest	1	33.47	0.39	6.04	0.060	0.001
	Among localities within groups	21	326.08	1.19	18.61	0.200	<0.0001
	Within localities	185	892.96	4.83	75.34	0.250	<0.0001
D.	**Northwestern grouping vs locality PLET (arrow) vs the rest of the sample ---**						
	Among groupings	2	55.23	0.50	7.78	0.078	<0.0001
	Among localities within groups	20	304.32	1.14	17.62	0.191	<0.0001
	Within localities	186	892.96	4.83	74.6	0.254	<0.0001







Leliefontein clusters together with the geographically distant Northeast region. The cophenetic correlation of this dendrogram with the original distance matrix is 0.92. The values of H', P and average SM for each region are shown in Fig. 4- 7. The different measures agree well in the ranking of regions by ISSR diversity. There is a very uneven distribution of variation across the range of the species, but most of the

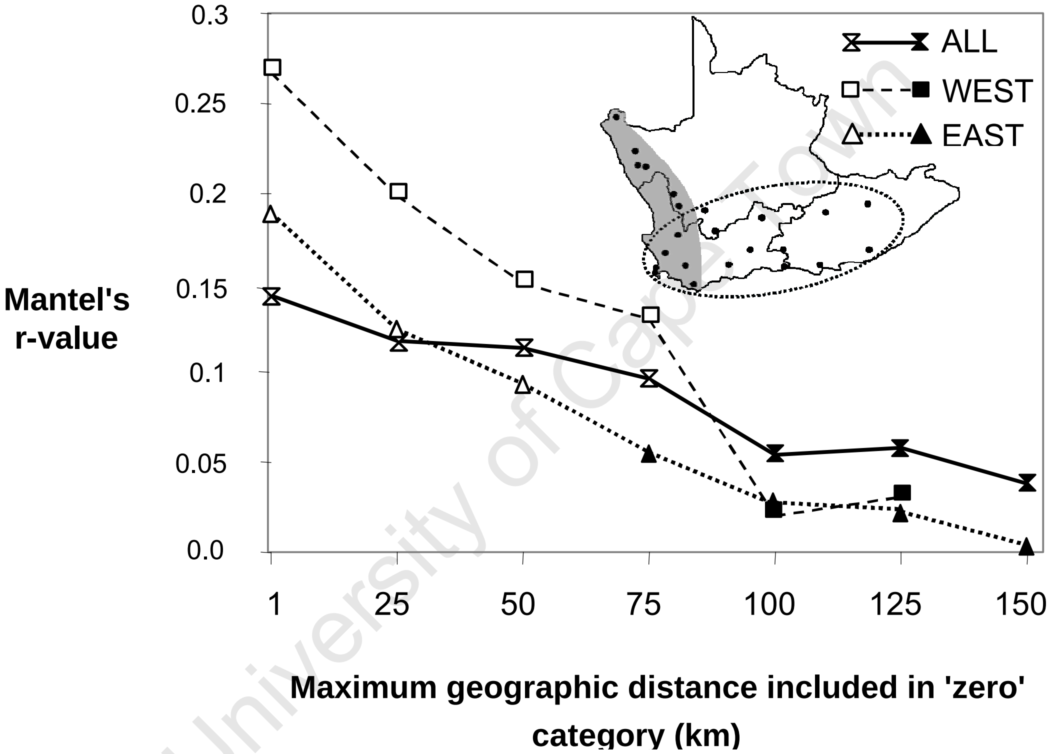


Fig. 4-4. Spatial correlogram of Mantel’s r-statistic with increasing geographic distance. Open symbols indicate statistical significance (p<0.01) after sequential Bonferroni correction. Closed symbols indicate correlations which are not significant even at p = 0.05. “ALL” indicates all localities included in analysis; “WEST” indicates localities from the western arm of the distribution only (indicated in the figure by the shaded region) and “EAST” indicates only localities from the eastern arm of the distribution (those included in the ellipse in the inset figure.)

4.3.5. Regional patterns: synthesis mapping.

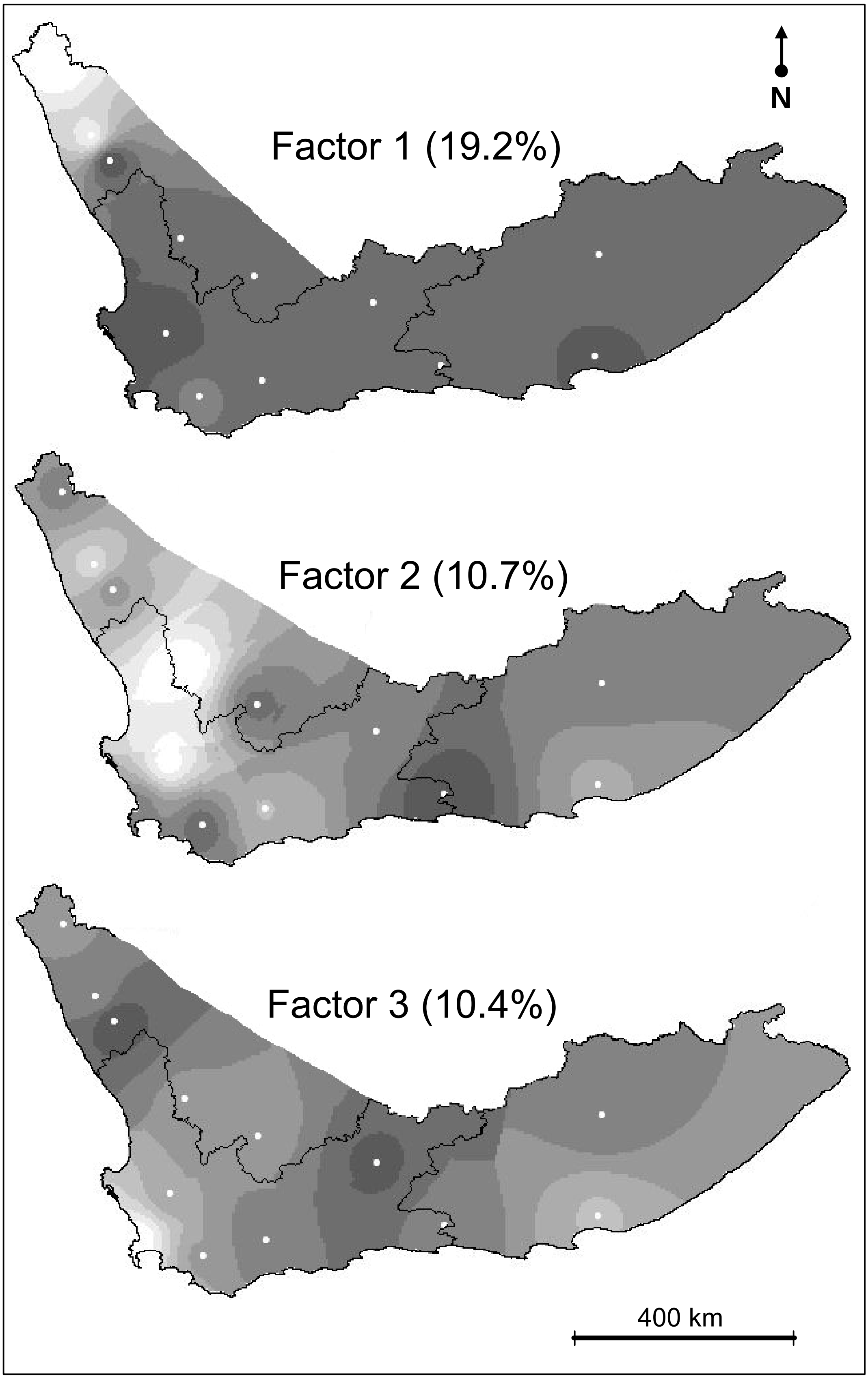
Fig. 4-5 shows synthesis maps of band frequencies plotted onto the centroid of each region. The first three Principal Component axes account for 40.3% of the variation. In each map, the shading indicates the position of a region from black at one extreme of the axis to white at the other. The shades are independent in each map and indicate only similarity amongst regions for the particular PC axis being represented. Actual values of the factor co-ordinates are not relevant to interpreting the figures and only indicate whether regions have similar or different scores for each factor. Each axis needs to be interpreted independently and indicates the degree to which a region is different from other regions in terms of the variance explained by that axis only (Cavalli-Sforza & al., 1994). By far the strongest signal in the regional PCA is the distinctness of the northwestern localities, which accounts for most of the variation in the most influential axis, Factor 1, and may therefore account for most of the variance in the data. Leliefontein is distinct from its neighbours on all three axes. Factor 2 indicates distinctness of regions Swartland and Northescarp, which occur on the same end of this axis as Northnamaqua. An ISSR discontinuity between the central part of the eastern arm and the regions to the west and east of this is also evident on Factor 2 around the Southcoast region. Principal component 3 echoes this ISSR discontinuity, but places it more strongly on the Eastcentral region and also shows a grade in affinity between a highly distinctive Cape Peninsula region and other regions in the southwest.

4.3.6. Regional patterns: spatial structure and ISSR diversity.

The grouping of localities into regions increased the apportionment of ISSR variance within groups, as measured by AMOVA, to 87.38% ($p < 0.0001$) within the average region. The proportion of ISSR variability among individuals within regions as estimated by H' (King & Schaal, 1989) is 71.3%.

Fig. 4-6 shows the UPGMA cluster diagram of regions. The greatest distance in the phenogram separates the northwestern grouping (Richtersveld and Northnamaqua) from the rest. The association of the Kamiesberg locality with southern localities, which was found with individual-level analyses, is true at the regional level also, as regions with below-average diversity measures occur in the western arm of the range (regions Richtersveld, Northnamaqua, Northescarp, Jonaskop and the Cape Peninsula). However, Leliefontein and Swartland, also from the western arm, have the two highest diversity measures. Roggescarp and Southcoast are the two regions from the eastern arm with below-average diversity; all other regions from the eastern arm have values that fall above the mean.

Fig. 4-5. (opposite). Synthesis maps of the first three principal components factor scores of ISSR band frequencies for each region. The centroid of each region (its geographic location weighted by sample number at each locality) is indicated with a white dot. Shading in each map represents the range of factor co-ordinate values, arbitrarily set at black for highest and white for lowest values, interpolated via inverse distance weighting to the three nearest neighbours.


N
Factor 1 (19.2%)
Factor 2 (10.7%)
Factor 3 (10.4%)
400 km

4.3.7. Is there a north-south gradient of genetic variation in the western arm?

Within the western arm, ISSR diversity does appear to decrease as one moves northwards, but then increases due to the high-diversity region Leliefontein (Fig. 4-7). To put a statistical value on this observation, Shannon's index and average SM dissimilarity per region were regressed against geographic distance from False Bay (Fig. 4-1) for all the regions from the western arm (Richtersveld, Northnamaqua, Leliefontein, Northescarp, Swartland, Cape Peninsula and Jonaskop). There is no significant decrease in diversity moving northwards. However, when Leliefontein is excluded from the analysis, a significant negative correlation exists between genetic diversity and distance from False Bay ($R^2 = 0.81$, $p < 0.01$ for Shannon's index and $R^2 = 0.65$, $p < 0.02$ for SM) despite the reduction in degrees of freedom resulting from removal of a data point.

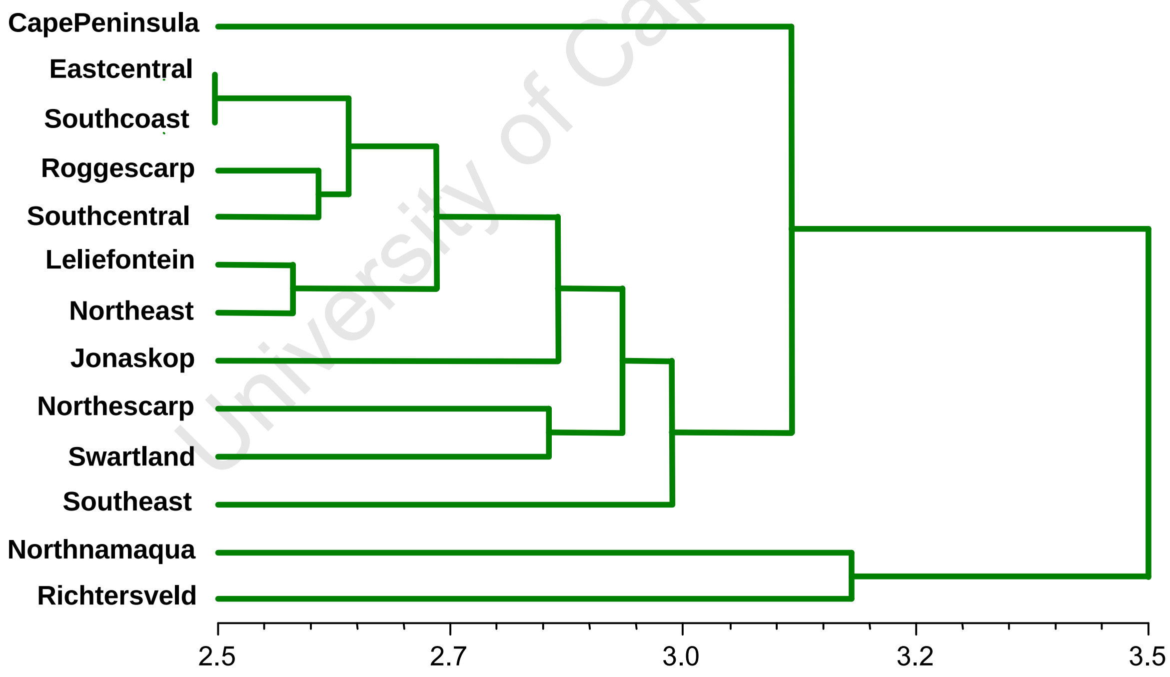


Fig. 4-6. UPGMA clustering of regions (see Fig. 4-2) based on Euclidean distances calculated from band frequencies at each locus for each region. The cophenetic correlation is r = 0.918.

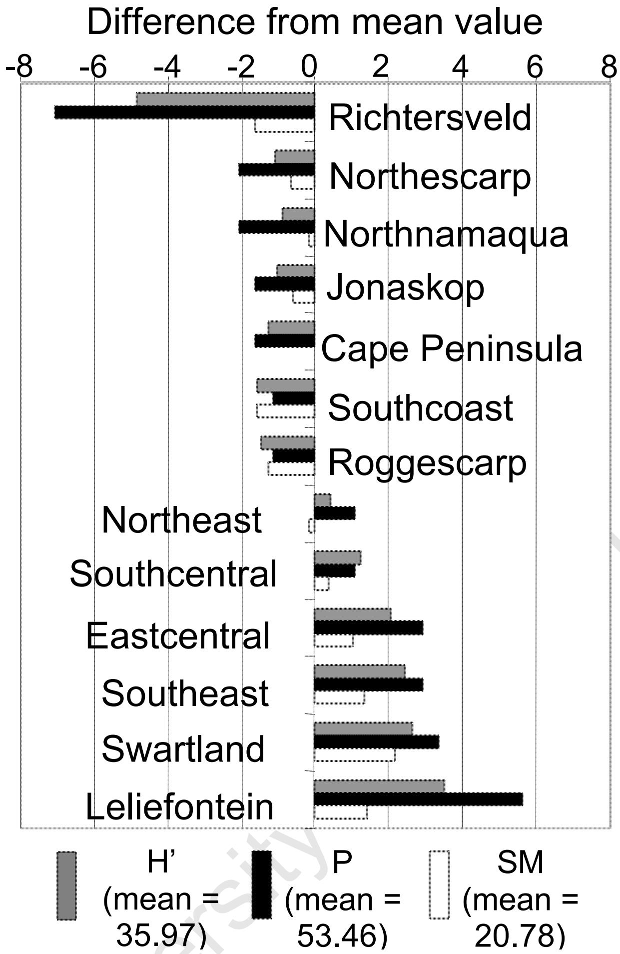


Fig. 4-7. Diversity measures for regions (each regions consists of eight individuals and from of one- to several localities, as shown in Fig. 4-2). Each bar represents the difference between the region's value and the mean value for all regions. P - the proportion of loci which are polymorphic within a region; H'- Shannon's index based on the natural logarithm, and pairwise SM - the mean pairwise dissimilarity between individuals from a region, expressed as a percentage.

4.4. Discussion

Despite low numbers of samples from each locality, this study achieved range-wide sampling of the species' distribution which allowed examination of broad-scale patterns of spatial genetic structure. This makes it one of the first studies of spatial population genetic structure on a Cape plant taxon (as mentioned in Chapter 1). Most of the existing studies focussed on smaller spatial scales than are addressed here, with the notable exception of the allozyme study of *Acacia karoo* (Brain 1986; 1989). Renosterbos samples produced many more ISSR bands than are usually reported in the literature (e.g., Wolfe & Liston, 1998) and one possible reason for this is that the species might be a high polyploid. However, little chromosomal study has been performed on the *Stoebe* clade and none on renosterbos. The diploid chromosome number is 16 for all examined members both the *Stoebe* and *Metalasia* clades (6 spp. examined; Hedberg & Hedberg 1977; Nordenstam 1969; Karis 1989). All of the species examined were diploid, but future work should focus on collecting chromosomal data for renosterbos as well as all other members of the *Stoebe* clade. Renosterbos harbours a large amount of ISSR variation, and this variation is not strongly structured according to the current geographical distribution of its populations. The lack of strong geographic structure is probably compounded by high levels of recombination among ISSR loci. Such recombination could be a product of high levels of gene-flow and high outbreeding rates, and is to be expected for hypervariable, recombining, multilocus and dominant markers in plant species which are outcrossing and well-dispersed (Nybom, 2004). Both pollen and seeds will contribute to dispersal of nuclear loci such as ISSRs (Wang & al., 1994) and several characteristics of *E. rhinocerotis* are likely to increase average dispersal distances. Firstly, wind-dispersal is likely to be an efficient method for translocating both pollen and seeds; secondly, adult renosterbos plants have a very large seed output (potentially thousands per plant per year); and thirdly, there is an initial period of physiological seed dormancy which spreads germination over several years after seed is shed (Levyns, 1929). As a result, only geographic structure that is either recent or persistent (or both) is likely to be reflected in the ISSR data.

4.4.1. Patterns of genetic diversity and relatedness across the range

To a large degree, localities are representative of the total variation in the species, and both AMOVA and H' gave roughly the same proportions for this measure. This type of spatial structure (high within-population variability) is also supported by the results of individual-level multivariate analyses, with the low cophenetic correlations for the UPGMA dendrograms (Rohlf, 2000), small proportion of the variation explained by PCoA, and low bootstrap values (NJ). Shared band absences may be informative, as indicated by the marginally better fit of the SM phenogram to the data, as compared to the Jaccard phenogram, and as proposed by Rieseberg (1996) for within-species comparisons.

Despite the low level population genetic structure, a significant amount of ISSR variation is still allocated among groups, whether these are localities or regions. This indicates a degree of spatial ISSR structure in *E. rhinocerotis*, which is clear from the AMOVA and from the fact that all analyses recovered the same geographic pattern. This pattern is essentially the distinctness of the samples from the extreme northwest of the range, the uniqueness of PLET samples, and the strong association of LEL samples (or the region Leliefontein as a whole) with samples from the far eastern part of the distribution range. However, this pattern is not congruent with the current geographical arrangement of populations, and this is lent statistical support by the fact that there is only a very weak signal of IBD. What little signal of IBD is present in the data is due to differences between individuals, so grouping localities into geographic regions is not likely to violate the spatial structure that is present. Regional groupings allowed more robust sample sizes for diversity estimates, and more rigorous examination of how ISSR diversity varies from region to region. The ranking of regions by amount of ISSR diversity was in agreement across different diversity estimators (Fig. 4-7). Although some of the areas of highest population genetic diversity mirror patterns of species-level diversity in the Cape, several regions of very high ISSR diversity are not particularly rich in species. Especially marked is the large amount of population genetic diversity in the Kamiesberg and the far east of the range, areas of low species-diversity for Cape lineages. The following sections discuss the possible causes for the ISSR patterns in the western and eastern arms of the range separately.

4.4.2. The western arm of the distribution

This part of the range exhibits a very strong signal of genetic disjunction between northernmost localities and all the rest.

Except for Leliefontein, ISSR diversity decreases moving northwards, as expected if diversity is linked to the increasing aridity gradient in the western arm. The data are thus consistent with the scenario of Holocene aridity beginning in the north and spreading southwards. The palaeoclimatic models predict a greater degree and earlier onset of aridification in the north (van Zinderen-Bakker, 1976), which would have caused vicariance and population-size bottlenecks in populations whose ancestors had previously enjoyed more mesic conditions in Namaqualand during the last glacial period. The distinctness of the northwestern populations is thus consistent with long-term and persistent isolation, which may well date back to the start of the Holocene. An alternative scenario, which does not require a change in precipitation in the northwest, is that northwestern populations are the result of long-distance colonisation events, perhaps from the Kamiesberg, with subsequent differentiation by genetic drift in isolation. If this were the case, northwestern localities would be expected to carry a reduced subset of the bands present in the source localities, and to show greatest genetic affinity with these source populations. Such clear indications of sources for the northwestern localities are not evident in our data; but if colonisation events happened sufficiently far back in time, genetic association between source and sink localities might not be detectable, especially with high recombination rates amongst phylogenetically unordered markers. It may not be possible to distinguish vicariance from ancient dispersal, but it would be expected that if Richtersveld samples were derived from multiple independent samples, they would collectively harbour greater ISSR diversity, and show lower genetic similarity to each other, than is observed. Alternatively, ISSR variation may simply reflect current ecological conditions, with the correlation of diversity and aridity in the western arm arising from populations being smallest and most isolated in the most arid parts of the range (i.e. those from the most extreme environment in the Richtersveld). Unfortunately, it is not possible to test whether unordered markers such as ISSRs reflect current conditions or historical demographic processes, and diversity differences amongst localities may simply reflect ongoing population size effects. Correlation of genetic variation with population size is well documented in the conservation genetic literature (e.g. Frankham, 1996). Some of the northern localities, especially VAN, are very small, while LEL, the genetic exception, has potentially a very large effective size. However, population sizes were not quantified in the present study.

The greater IBD in the western arm than the eastern arm, as indicated both by the Mantel test and by the greater apportioning of AMOVA variation amongst western localities, may indicate greater site-fidelity here. Populations in the western arm thus appear to have existed in their current localities for longer periods than those in the east, perhaps due to a lack of suitable sites for colonisation.

The fact that the strongest genetic disjunction occurs between LEL and TOE, and that the region Leliefontein has the highest levels of ISSR diversity as estimated by all three diversity measures, indicates that LEL is unaffected by whatever factors influenced the northwestern populations. The Kamiesberg highlands, due to their altitude, experience substantially reduced temperature and increased precipitation relative to the surrounding plains (Schulze, 1995). Despite being only 48 km from TOE, LEL is ca. 700 m higher, and amelioration of aridity with increasing altitude is a well-known ecological phenomenon. For this reason, Midgley & Roberts' (2001) model predicts that fynbos would survive on the top of the Kamiesberg even during the hottest and driest period of the current interglacial (the Holocene Altithermal ca. 8-4 ka; Partridge, 1993). This serves as additional support for the hypothesis that aridity is a strong reason for the genetic distinctiveness of the northwestern localities.

The high ISSR diversity evident in LEL may also be in part a result of Kamiesberg populations acting as a sink for propagules from multiple surrounding areas, due to the better habitat offered by the moister highland areas. The high diversity of such a locality may then be the result of its members having genetic affinities with a wide range of source localities. If this is the case, the ISSR data indicates source localities to the south and east of LEL, and this is consistent with the current prevailing wind direction in the region (Desmet & Cowling, 1999), which is likely to move seeds in a predominantly northerly direction.

The uniqueness and high ISSR diversity of LEL samples are also consistent with the inference that this area provided stable habitat for renosterbos throughout the Holocene. It may be that the greater genetic affinity of LEL with southeastern samples is due to greater opportunity for gene exchange between southeastern populations and LEL than between the southeast and the shrinking, isolated northern populations. However, it is interesting that LEL shows genetic affinity with populations from the far eastern end of the range (region Northeast containing localities STERK and NBETH) rather than with closer localities such as the Great Escarpment (Fig. 4-6). The latter are the most obvious means of connecting LEL to the eastern arm via gene flow, but such a Great-Escarpment gene-flow corridor is not indicated by the data. It is thus tempting to hypothesise a historical expansion of renosterbos into the Northern Karoo, connecting the Kamiesberg highlands directly with northeastern localities such as STERK. A paleoclimatic incursion of the winter-rainfall region into what is currently the summer-rainfall interior of South Africa (Northern Karoo; Fig. 4-1) has previously been hypothesised by Van Zinderen Bakker (1976), and Dowson (1988) suggested that the reduced temperatures of the last glacial may have allowed an eastward and northward shift in the winter-rainfall vegetation types. The possibility of such a shift, however, remains speculative without further paleoclimatic data.

The summit of the Kamiesberg houses outliers of the Cape flora (Marloth, 1908; Pearson, 1912; Adamson, 1938; Weimarck, 1941; van-Wyk & Smith, 2001) which have been hypothesised to be the remnants of a once more widespread CFR (e.g. Rourke, 1990). The alternative explanation is that they are the result of independent long-distance colonisation events from the south. The high diversity and affinity of Leliefontein samples with eastern localities data may be more consistent with longstanding isolation of a large, genetically diverse population, perhaps even with an historical extension of the range into the Northern Karoo (Fig. 4-1) as discussed above, and the present study may thus represent the first genetic data indicating that the Kamiesberg is a historical refugium for Cape floral elements in arid Namaqualand. In this regard the ISSR data also lend support to the idea that the CFR may in the past have extended further to the north than it does at present.

The isolation of populations in Namaqualand, especially in the far north, may provide greater opportunities for population divergence and isolate speciation relative to other parts of the range. However, this may be balanced by a greater chance of extinction of small populations in the arid west.

4.4.3. The eastern arm of the range

As discussed above, there is no evidence of a gene flow route between the Kamiesberg and the southeast via the Great Escarpment. Fig. 4-5 shows that there is no detectable genetic connectivity between the regions that make up this putative 'corridor'. Instead of a pattern of east-west connectivity along the Great Escarpment, the dendrogram in Fig. 4-6 indicates that Great Escarpment regions such as Roggescarp, Eastcentral and Northeast are closest to various other regions (respectively, Southcentral, Southcoast, and Leliefontein). Similarly, there is no detectable east-west corridor linking regions along the south coast. This lack of correlation between geographic proximity and genetic relatedness is clear from the low level of IBD. Either patterns of gene flow are not easily predictable from geography, or other factors, such as historical range shifts or genetic recombination of ISSR markers, have obscured or prevented the development of such geographically predictable patterns.

In the eastern arm, regions are generally characterised by high levels of ISSR variation and there are no areas which are very distinct in this regard, unlike the Kamiesberg in Namaqualand. This may indicate either high historical population sizes or good gene flow amongst localities, or both, for most localities in the southwestern Cape and at the eastern extreme of the species' range. However, the central area (region Southcoast comprising localities PLET and BAV) and also region Jonaskop have lower ISSR variability, while the southwest and the far eastern part of the range have very high diversity estimates in many grouping arrangements tested.

The southwestern-most Cape is predicted to have experienced relatively mesic climatic conditions throughout the late Quaternary, due to the area remaining within the influence of moisture-bearing westerlies at both glacial and inter-glacial extremes (Meadows & Baxter, 1999). Jonaskop may be an exception to this as it occurs in the rainshadow on the north-facing slopes of part of the Cape Fold Mountain belt and houses an ecotonal vegetation ranging from fynbos on the summit to succulent karoo vegetation in the arid Little Karoo at the base. The effects of aridity may explain why it has much lower levels of variation than other southwestern regions. Parts of the Great Escarpment may represent plausible long-term habitat for renosterbos due to their higher altitudes attenuating any arid periods. The far eastern arm of the range has not been explicitly considered as a potential long-term refuge for Cape floral elements, allthough there is palaeoecological evidence of *Stoebe*-type pollen presence throughout the late Quaternary in places (Scholtz, 1986; Bousman & al., 1988; Meadows & Sugden, 1988; Scott & al., 2005). These deposits might have been produced by the summer-rainfall relative, *Stoebe vulgaris* Levyns, but there are additional indications that *E. rhinocerotis* has had a long history in the area that today forms its eastern extent. Levyns (1935) speculated that renosterbos originated in the eastern part of the range, since it’s phenology appears to conform more closely to the current climate in the east. She also described renosterbos as being ecologically more ‘at equilibrium’ with other components of the eastern vegetation, while in the west it often dominates renosterveld communities and has a more ‘invasive’ character. More tellingly, *E. rhinocerotis* has evolved a close association only in the eastern part of its range with a flightless insect, the locust *Lentula obtusifrons* Stål., which has no other food source (Smit, 1935). Although there is no information on the paleodistribution of *L. obtusifrons*, it has not been collected west of 21^{o} E longitude (S. Antunes and M. Picker, unpublished data). Such an obligate association argues for a long sympatric history, possibly only in the east of the renosterbos’ range.

The presence of *Stoebe*-type pollen and renosterbos charcoal fossils imply that *E. rhinocerotis* may have been widespread across large parts of the eastern arm during the late Quaternary (Martin, 1968; Deacon & al., 1983; Scholtz, 1986). However, climatic fluctuations may have prevented continuous presence of renosterbos in the central part of the region. This central part not only has lower ISSR diversity, but also is genetically distinct. Locality PLET is an extreme genetic outlier amongst all renosterbos localities. The reasons for this are unclear, and it would be difficult to support speculation based only on the three PLET samples. However, regional diversity statistics for the centre of the eastern arm calculated without PLET but using, for example, samples from BAV and HANK, still find a reduction in ISSR variability here. Thus there may be a real historical reason for lower diversity in the southern coastal region. Palynological and other evidence indicates that afrotemperate forests were greatly restricted during the last glacial period when *Stoebe*-type pollen was abundant (Butzer & Helgren, 1972; Martin, 1968; Scholtz, 1986). However, increases in moisture during phases of the Holocene and the resulting expansion of forests (Martin, 1968; Partridge & al., 1999) may have displaced the shade-intolerant renosterbos (Levyns, 1956) from the southern coastal mountains. Subsequent contraction of these forests due to late-Holocene aridification, together with anthropological deforestation, could have resulted in later recolonisation by renosterbos and the more recent populations could have lower genetic variation.

Greater isolation among western than among eastern localities (separate AMOVAs; Fig. 4-6) is consistent with a scenario in which some eastern populations only recently colonised their current positions, since this would not allow time for isolation by distance to become established.

4.4.4. Conclusions

The question of whether *E. rhinocerotis* populations experienced a small or a large amount of Pleistocene distributional change can not be answered by the current data set. However, it can be used to identify parts of the range that appear to have experienced relatively greater or lesser historical climatic alteration. Relative amounts of genetic diversity can be compared across the range, and these indicate that historical population range dynamics are likely to have been very different in different parts of the GCFR, pointing to a complex array of climatic histories.

To the extent that ISSR patterns reflect history, in the western arm, climatic changes were either of greater amplitude or of consistent direction while in the east, climatic conditions may have oscillated between dry/wet conditions with no consistent directionality to the change. This may be the reason for what appears to be a clearer picture of demographic history in the genetic structure of western localities. Although the diversity patterns are consistent with a long stable climatic history in the extreme southwest, in the rest of the western arm, populations may be restricted both geographically and in terms of size. The most obvious explanation for this is reductions in population sizes due to aridity, both today and historically. The Kamiesberg sample is the exception that seems to prove the rule. In the eastern arm, populations appear less restricted in terms of size but distributional shifts may have played a greater role, and here it is hypothesised that biotic interactions may have been more important here. The identification of potential ‘refugia’ at the eastern end of the species’ distribution range represents a novel hypothesis for the CFR.

Higher diversity and potentially, climatic stability in the southwest of the GCFR and in parts of the mountains of the great escarpment support the important role of topographic heterogeneity in the history of the Cape flora. The interaction of topography and climate is likely to be especially important, with altitude and orographic effects providing important climatic refugia. In this regard, continental uplift may have been an important factor in the history of the flora due to its role in the generation of topographic heterogeneity in southern Africa.

Phylogenetically unordered, dominant markers such as ISSRs have limited utility for phylogeographic inference. Nevertheless, this research constitutes a first examination of the distribution of genetic variation in a member of the unique Cape flora. The results are largely consistent with predictions of how palaeoclimatic changes might have structured genetic variation in renosterbos. Hypotheses are presented for putative changes in distribution of this typical Cape plant species in response to late-Quaternary climate changes in the Cape provinces of South Africa. These hypotheses may be tested by future phylogeographic, population genetic or palaeoecological research in the region.

CHAPTER 5

General Discussion

The work presented in the preceding chapters constitutes the study of a typical Cape lineage at three different evolutionary scales and so provides an in-depth examination of one of the relatively few diversification events responsible for the uniqueness of the Cape Flora. At each scale, a range of questions which can best be answered at that level and with appropriate molecular and analytical tools, are addressed. The population-level study is contextualised by the species-level phylogenetic analysis which places *E. rhinocerotis* amongst its closest relatives. This species-level study is contextualised by the broader analysis which identifies the clade to which those species belong and explores the older biogeographic history of the group. The different levels of resolution provide a detailed examination of the history of the lineages, allowing insights into the origin of the ancestral taxon, the geographic location and timing of subsequent diversification events and range alterations, and the response of populations to fluctuating climatic conditions.

The main hypotheses addressed in the thesis are summarised below.

5.1. Hypotheses addressed

1. The subtribal classification of the large daisy tribe Gnaphalieae, based on the morphological cladistic analysis of Anderberg (1991) does not reflect evolutionary relationships and needs to be re-examined in light of a robust phylogenetic hypothesis.
2. Tribe Gnaphalieae arose in Southern Africa, and Southern African Gnaphalieae constitute a monophyletic group.
3. Australasian Gnaphalieae constitute a monophyletic group.
4. Australasian gnaphalioids are derived, via dispersal and subsequent diversification in Australia and surrounds, from an ancestor that occurred in southern Africa. Alternatively, this dispersal may have been from the Mediterranean basin or Eurasia.
5. The Cape Floral Clade, subtribe Relhaniinae, is not monophyletic as currently

circumscribed and requires a re-assessment of relationships, taxonomy, biogeography and evolution.

6. The afroalpine members of the Relhaniinae constitute the remnants of a northern origin and migration southwards via the East African Highlands into the Cape, with subsequent diversification in the region. The afroalpine taxa are thus the earliest-diverging lineages (Levyns, 1964). Alternatively, Relhaniinae originated in the Cape, and the afroalpine members are the result of one or several more recent northwards dispersal, as found for other Cape taxa with afroalpine members (Galley et al., 2006).
7. As currently circumscribed (Anderberg, 1991; Koekemoer, 2002), informal supra-generic groupings (the *Relhania* group and the *Metalasia* group) within the Relhaniinae are not monophyletic.
8. As currently circumscribed, genera within the Cape-centred *Stoebe* clade are not monophyletic.
9. Lack of generic monophyly is due to a high level of homoplasy in the macromorphological characters used thus-far to delimit genera. Using only these characters, there may be no scheme of generic classification that produces monophyletic and diagnosable genera, with the possible and unsatisfactory alternatives of either ‘lumping’ all species into one very large genus, or finely ‘splitting’ them into many very small or monotypic genera.
10. The current distributions of plant populations in the CFR are the result of interplay between edaphic factors and climate, with the latter changing in response to orbital forcing such that species’ ranges have been altered during the Late Quaternary.
11. Plant populations from different parts of the GCFR have responded in different ways to Pleistocene climatic oscillations, reflecting non-uniform climatic responses to orbital forcing across the region.
12. Patterns of species diversity across the CFR mirror patterns of genetic diversity within a typical Cape taxon, *Elytropappus rhinocerotis*, consistent with the hypothesis that macro- and micro-evolutionary processes have operated in similar ways across the landscape.
13. The degree of distributional perturbation in the Cape may have been far lower

than that in glaciated areas such as North America and Europe, and most Cape species have been able to persist more-or-less in their current distributions throughout the Pleistocene. Speciation processes have thus been able to proceed relatively unhindered in those parts of the GCFR most buffered from climatic (especially precipitation) alteration. As a result, these parts of the CFR harbour the highest levels of species diversity and endemism, and also the highest levels of within-population genetic diversity in widespread taxa.

5.2. Examination of the evolution and relationships of a Cape Floral Clade: the endemic Cape lineages in Gnaphalieae.

This thesis represents the first single study to examine a Cape plant lineage at a range of evolutionary scales and to examine multiple aspects of diversification in the group, especially in relation to biogeographic history in the Cape, timing of diversification, evolution of morphological diversity and the evolution of geographic range at the population level. Each of the finer-scale studies is contextualised within a broader analysis.

5.3. Biogeography of Gnaphalieae

5.3.1. Sampling

The phylogenetic hypothesis presented in Chapter 2 represents the first analysis with broad sampling across the tribe, including taxa from both of the two major centres of diversity (southern Africa and Australasia). This allows a first examination of the origins and broad biogeographic relationships within Gnaphalieae. However, global sampling is poor and the lack of taxa from the Americas is a significant gap. Future studies should included more Gnaphalieae from the northern hemisphere in order to properly reconstruct relationships and biogeographic pathways. The circumscription of the large, polyphyletic genus *Helichrysum* is a key focus for future progress on Gnaphalieae systematics. Another focal area which requires greater sampling is the elucidation of the sister relationships of the tribe. Although the members of the earliest-diverging lineage (the *Relhania* clade) are known, identification of the sister group is required to determine the origin of Gnaphalieae, and when and how the ancestral lineage was recruited into the Cape flora.

5.3.2. Phylogeny

This study presents a phylogenetic hypothesis of the broad relationships in the tribe, with good statistical support at most nodes. This has not been possible in previous analyses of the Gnaphalieae. It was achieved here by sampling larger numbers of characters, through the use of DNA sequence data and the coding of indel characters. The use of nucleotide-substitution models in phylogeny reconstruction also appears to have improved the phylogenetic estimate.

Good sampling of the southern African taxa formerly included in the Relhaniinae allowed for rigorous delimitation of clades within this former subtribe, and demonstrated the relationships amongst the early-diverging lineages of Gnaphalieae.

5.3.3. Molecular age analysis and Cape diversification

There has been no previous estimate of the age of the Gnaphalieae, nor of the ages of the main diversification and dispersal events in the tribe. The relaxed Bayesian molecular clock is probably the most sophisticated method available thus far for molecular dating, as it models rate heterogeneity across branches without the assumption of rate autocorrelation, as well as allowing probability distributions for calibration ages that incorporate information about uncertainty in both the age and taxonomic assignment of the calibration information. Calibration points for the molecular age estimates were based on several different types of ages, both fossil and geological. Although none of these calibrations are particularly robust, taken together (especially given a degree of congruence between their independent estimates of divergence ages) they may provide increased confidence in the final estimates.

The study demonstrates at least three independent and temporally close relhaniinioid diversifications in the Cape. These are dated to the late Miocene, the time when several other Cape clades also appear to have been radiating. Although molecular age estimates have broad confidence intervals, these dates may implicate a common ecological background for radiations in the Cape in the late Miocene. These results suppport suggestions by previous authors that diversification in the Cape may be linked to the opening of summer arid and / or fire prone habitats, promoting the proliferation especially of lineages capable of producing woody perennials with strategies for avoiding or tolerating summer drought and surviving fire.

5.3.4. Biogeography

Globally sparse sampling means that biogeographic inferences across the tribe as a whole need to be interpreted cautiously. Nevertheless, the analysis allowed clear inferences of biogeographic history of the Gnaphalieae in the Cape region, and demonstrated that the Cape is not only an isolated hotspot of diversity but also a source for lineage dispersal and subsequent diversification in other parts of the world.

Future work on the tribe should focus on elucidating the basal relationships in the Gnaphalieae as well as fleshing out the broader phylogenetic relationships withing the tribe, mainly the phylogenetic position or positions of the South American taxa and of large, polyphyletic genera such as *Helichrysum*. Well-resolved phylogenetic hypotheses for all the Cape clades within the former Relhaniinae will provide further independent tests of speciation history and should include examination of tempo of diversification and the role of ecological factors, especially substrate specificity and climate tolerances, in speciation. Similar types of studies within the Australasion Gnaphalieae should provide interesting comparisons on rates of diversification and ecological speciation. Generic circumscription within all three Cape clades will also be improved if well-resolved species trees can be supported. If resolution of species relationships is not possible, due to very recent divergence, gene-tree approaches based on the coalescent might prove the only way to reliably infer species trees. The lack of sufficient resolution in molecular phylogenies might also require a return to the use of detailed anatomical studies to provide sufficient characters for species-level phylogeny reconstruction.

5.4. Evolution and generic delimitation in the *Stoebe* clade.

5.4.1. Sampling

Near-complete species-level sampling was achieved for members of the *Stoebe* clade, although fewer species were sampled for all gene regions. The final analysis is thus not based on a complete species sample. Nevertheless, sampling is broadly distributed across all groups and so is probably sufficient to allow a broad test of generic monophyly.

5.4.2. Phylogeny

Unlike previous studies addressing circumscription in *Stoebe* and relatives, this analysis was based on a rigorously-defined ingroup. However, lack of phylogenetic resolution greatly hampered the degree to which genera could be fully circumscribed and hypotheses of character evolution evaluated. This lack of resolution may point to rapid radiation in the group or may indicate the need for more DNA sequence data. Given the extreme lack of resolution in the chloroplast data, it seems worthwhile to sequence and re-analyse with an additional chloroplast marker before proposing changes to generic circumscription of the taxa currently in *Stoebe*, *Disparago* and Elytropappus.

The analytical examination of the evolution of morphological characters has not been performed before for the group and paves the way for future detailed anatomical studies. However, this reconstruction of ancestral character states only traced character history for a few characters already indicated to be homoplasious. A rigorous and systematic examination of characters is required. Detailed anatomical and ontological studies are needed to establish homologies in characters such as synflorescence arrangement and floret morphology, and to identify potentially novel characters. A well-resolved phylogenetic hypothesis is probably the most powerful tool available for assessing homologies. In Chapter 2, characters of the involucral bracts and pappus that distinguish the Metalasiinae and Stoebiinae, were identified for the first time as a result of the recovery of these clades in the phylogeny. Resolution of the backbone polytomy within the *Stoebe* clade may be the most effective means of examining character evolution and identifying taxonomically useful characters.

5.5. Palaeogeography of renosterbos

5.5.1. Examination of population-level relationships in the Cape flora

The palaeogeographic study represents one of the first population genetic studies on a

Cape plant taxon, and the first to use genetic tools to attempt to examine population history. Although ISSR's are a useful tool with several advantages, they have limited utility for inferring phylogeographic history, as they are phylogenetically unordered and genomically anonymous. Nevertheless, they allowed a survey of the distribution of genetic variation across the geographic range of a Cape plant species whose distribution is congruent with that of the flora of the GCFR. As such the distribution of within-species diversity could be compared with the distribution of species-level diversity across the CFR, and is indicated to show some, but not complete correspondence. Although the regions with the highest species diversity (southwestern Cape mountains) also have high genetic diversity in *E. rhinocerotis*, populations from other regions exhibit equally large or even greater genetic diversity. This indicates that for this species at least, the processes which have shaped genetic diversity (recent events and microevolutionary processes) operate differently across space than the processes which shape the distribution of species diversity in the Cape (older events, operating over longer timescales, and macroevolutionary processes). Alternatively, *E. rhinocerotis* may be an 'atypical' member of the Cape flora, since it occurs on shale- and granite-derived soils on the lowlands and in the interior, while the most species-rich parts of the CFR are in the mesic southwestern mountains on quartz-rich sandstone-derived soils.

Attempts to infer population distributional history from the current distribution of population genetic relatedness indicated that different parts of the GCFR may have experienced different recent climatic histories and highlighted the importance of topography-mediated climatic buffering in the region. Although *E. rhinocerotis* populations appear to have been impacted by aridity, populations still persist even in the most arid parts of the GCFR, and these are inferred to be relictual rather than the result of recent dispersal, indicating that although Holocene climate perturbations have affected this species, they have not been sufficient to cause complete extinction, even in marginal locations. This further indicates that although the GCFR region was affected by Holocene climatic fluctuations, these were weak enough to allow evolutionary processes that contribute to species diversity to continue relatively unhindered. This is consistent with the hypothesis of a stable climatic history allowing speciation to proceed unhindered in the GCFR as one of the contributing factors to the regions' high diversity and endemism.

The data presented for *E. rhinocerotis* is more consistent with the 'rampant gradual speciation' hypothesis than that involving a 'species pump' because, for this apparently well-dispersed species, recent climatic perturbations do not appear to have been strong enough to have created complete genetic isolation of populations across the range. Of course, subsequent changes might have re-connected populations once isolated, but there is no indication in the ISSR data that any such isolates are on a separate evolutionary trajectory (the requirement for speciation). In terms of future research on this topic, identification of different parts of the range that may have experienced differing degrees of climatic stability offers important insights into the complexity of historical Cape habitats and highlights geographic areas and distributions of interest.

Future phylogeographic studies on renosterbos or other Cape plants should make use of DNA sequence data, which will allow many novel phylogeographic analyses including the use of coalescent theory-based tools. Future population-level and gene-tree based studies will also increasingly require more information on the breeding systems and reproductive ecology of Cape plant taxa, as well as the examination of chromosomal evolution. Future phylogeographical work may also benefit from a focus on more 'typical' Cape taxa that occur on the substrates of the Cape fold mountains. However, narrowly-distributed taxa offer less information on past ranges than widely-distributed ones. A key area of study for future within-population studies is also whether the Cape Flora in the past occupied a greater area than it does today, for example did it extend onto the central plateau (what is currently the Great Karoo)?

6. Summary and conclusions

- Tribe Gnaphalieae probably originated in southern Africa, and specifically in the Cape approximately 40 Ma.
- The ancestor of Gnaphalieae may have been present in the region prior to diversification, although this remains to be properly tested and requires

elucidation of the basal branching relationships and identification of the sister lineage to Gnaphalieae.

- Early diversification of the tribe occurred in southern Africa and accounts for the large diversity of gnaphaloids in this region.
- Dispersal out of the Cape region was ongoing from the early Miocene and resulted in the establishment of the tribe in the northern hemisphere and in Australia. The Australasian taxa may be the result of rapid diversification after a single dispersal event across the Indian Ocean around 15 Ma.
- West-to-east trans-Indian Ocean long-distance dispersal appears to have occurred in many angiosperm lineages only since the Miocene, potentially implicating the West Wind Drift as a dispersal agent.
- Members of the former Relhaniinae form the three earliest-diverging lineages in the tribe (the *Relhania* clade, the *Metalasia* clade and the *Stoebe* clade). All three had Cape ancestors and began diversifying in the Miocene, in accordance with divergence times in many other Cape clades, and consistent with the hypothesis of a common environmental trigger for many of the Cape speciations.
- Ancestors of all of the Cape clades in the former Relhaniinae were present in the region prior to their diversification, and probably before the development of what is recognised as the Cape Flora of today. This implies that the ancestral gnaphaloid in the Cape was pre-adapted for colonisation of summer-arid habitats, consistent with the radiation of the clades into the developing summer-dry and / or fire-prone habitats of the GCFR.
- The *Stoebe* clade is identified as the Cape clade containing *Elytropappus rhinocerotis* and its closest relatives. Non-cape *Stoebe*s are shown to be the result of recent (Pliocene) long-distance dispersal to the afroalpine region, followed by dispersal and colonisation to the Mascarenes.
- *Amphiglossa* is shown to be non-monophyletic and the resurrection of the genus *Pterothrix* is proposed to accommodate the shrubby, arid-adapted species which form the most species-rich clade of the former genus.
- Despite the identification of a ‘core *Disparago*’, ‘core *Seriphium*’ and ‘core *Stoebe*’ clade, lack of phylogenetic resolution results in the postponement of

further taxonomic recommendations in the *Stoebe* clade.

- The evolutionary history of several characters currently used for generic delimitation in these groups indicated a large degree of homoplasy in all characters.
- Characters of the corolla tube and pappus morphology were shown to be potentially good markers for future taxonomic work on this group of genera, but require re-evaluation and careful systematic study.
- The closest relatives of *Elytropappus rhinocerotis* are *E. adpressus* and *E. intricata*. Representatives of these taxa should be included as outgroups in future phylogeographic work on the species.
- The survey of genetic variation in *E. rhinocerotis* illustrates the complexity of different climatic regimes within the GCFR and indicates that populations in the western and eastern parts of the region may have experienced very different climatic histories as a result of Pleistocene climatic oscillations.
- This indicates that not all parts of the GCFR respond in the same way to climate change, which has implications for conservation planning in the context of future climate change.
- In the western arm of the GCFR, climatic changes were either of greater amplitude or of consistent direction while in the east, climatic conditions may have oscillated between dry/wet conditions with no consistent, long-term directionality to the changes. This may be the reason for what appears to be a clearer picture of demographic history in the genetic structure of western localities.
- In the western arm, populations appear to be restricted both geographically and in terms of size. The most obvious explanation for this is reductions in population sizes due to aridity, both today and historically. Samples from the Kamiesberg highlands are the exception that seems to prove the rule.
- In the eastern arm, populations appear less restricted in terms of size but distributional shifts may have played a greater role, and biotic interactions may have been more important.
- Locations of putative refugia from past climate change, indicated by higher-than-average levels of genetic variation, are consistent with predictions that the

southwestern corner of the CFR was buffered from past climatic oscillations. However, the Kamiesberg Highlands and the far eastern extent of the CFR also appear to have housed large, stable renosterbos populations over the period of time reflected by the genetic marker. In this the intraspecific patterns of genetic variation are at odds with regional patterns of species diversity and endemism.

- The distribution of genetic variation within a single plant species whose distribution closely corresponds to that of the GCFR is similar to, but not exactly matching, the distribution of species diversity for the flora as a whole. Although the southwestern corner of the GCFR harbours a high degree of genetic diversity for *E. rhinocerotis* and this diversity does decrease moving northwards and eastwards, other parts of the GCFR not noted for their high levels of species diversity (notably the Kamiesberg highlands and the extreme east of the range) harbour very high levels of ISSR diversity.
- This research constitutes a first examination of the distribution of genetic variation in a member of the unique Cape flora. Hypotheses are presented about putative changes in distribution of this typical Cape plant species in respone to late-Quaternary climate changes in the Cape provinces of South Africa. These hypotheses should be tested by future phylogeographic, population genetic or palaeoecological research in the region, especially applying recently-developed phylogeographic methods based on coalescent modelling of DNA sequences from multiple loci.

The present study constitutes a demonstration of the range of evolutionary questions that can be addressed in a single lineage at a range of evolutionary scales with appropriate molecular tools and techniques. A synthesis across the three scales of sutdy highlights the role of climatic aridification at all evolutionary levels in the history of Cape Gnaphalieae. The ancestor of the tribe may have been pre-adapted for life in habitats with low humidity or even extreme aridity, given the success of many descendant lineages in summer-dry, alpine or arid climates (eg Mediterranean regions; alpine and sub-alpine regions of the African Highlands, the Andes and the Himalaya; and the central Australian desert). Early diversification coincides with global climatic cooling (Zachos et al., 2001) ca. 50 Ma and the subsequent opening of arid habitats in Africa (Bobe, 2006). Although this has not been tested, increased diversification in the early gnaphaloid lineages appears to be coincident with the development of the summer-arid Cape climate in the Miocene. This may have provided novel adaptive zones suitable for colonisation by these lineages. The colonisation of and rapid radiation in Australia is another example of the ability of gnaphaloid ancestors to exploit various arid environments. Ability to survive aridity may also explain much of the colonisation ability of the group, for example dispersal northwards through Africa may be linked to periods of aridity which produced an ‘arid corridor’ from the southwestern part of the continent to the Horn of Africa (Bobe, 2006). The earliest-diverging lineage in the *Stoebe* clade, *Amphiglossa* s.s., occurs in dry river beds while more recently-evolved lineages have colonised a range of habitats, including the semi-arid Succulent Karoo. This may be indicative of some progression from more mesic to more arid environments within this Cape clade. The interplay between the opposing forces of aridity as a novel adaptive niche and threats to plant survival during drought conditions may explain much of the pattern of species diversity in the Greater Cape Floristic Region today. Edaphic specialisation is also likely to be important and may have a complex interaction with climate. The GCFR taxon *Elytropappus rhinocerotis* appears to have been a successful coloniser of marginal habitats and to have had its population history influenced by Holocene orbital forcing, perhaps most strongly via the influence of such forcing on the aridity component of climate.

CHAPTER 6

Reference List

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APPENDICES

Appendix 1: input .xml file for BEAST analysis of Gnaphalieae implementing calibration Scheme (a). Note: the sequence alignment sections are not included, for the sake of brevity.

```
<?xml version="1.0" standalone="yes"?>
<!-- Generated by BEAUTi v1.4.6                                              -->
<!--       by Alexei J. Drummond and Andrew Rambaut                          -->
<!--       Department of Computer Science, University of Auckland and        -->
<!--       Institute of Evolutionary Biology, University of Edinburgh        -->
<!--       http://beast.bio.ed.ac.uk/                                        -->
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      <!-- The list of taxa analyse (can also include dates/ages).
-->
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      <taxon idref="Ixi_tom_TRN_ETS"/>
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      <taxon idref="Sto_cry_PSB_ETS"/>
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      <taxon idref="Ang_mic_TRN_ETS"/>
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      <taxon idref="Fit_axi_TRN_ETS"/>
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      <taxon idref="Hel_doc_TRN_ETS"/>
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      <taxon idref="Ixi_tom_TRN_ETS"/>
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      <taxon idref="Cra_var_TRN_ETS"/>
      <taxon idref="Dec_hec_TRN_ETS"/>
      <taxon idref="Edm_ses_PSB_TRN_ETS"/>
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      <taxon idref="Fit_axi_TRN_ETS"/>
      <taxon idref="Hae_ozo_TRN_ETS"/>
      <taxon idref="Hel_doc_TRN_ETS"/>
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            <taxon idref="Mil_per_TRN_ETS"/>
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            <taxon idref="Ozo_whi_TRN_ETS"/>
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            <taxon idref="Stu_mue_PSB_TRN_ETS"/>
            <taxon idref="Syn_can_PSB_TRN_ETS"/>
      </taxa>


      <!-- The sequence alignment (each sequence refers to a taxon above).
-->
      <!-- ntax=73 nchar=539
-->
      <alignment id="PSBalignment" dataType="nucleotide">

      </alignment>


      <!-- The sequence alignment (each sequence refers to a taxon above).
-->
      <!-- ntax=73 nchar=527
-->
      <alignment id="TRNLalignment" dataType="nucleotide">

      </alignment>

<!-- The sequence alignment (each sequence refers to a taxon above).         -->
      <!-- ntax=73 nchar=448
-->
      <alignment id="TRNIGSalignment" dataType="nucleotide">

      </alignment>

<!-- The sequence alignment (each sequence refers to a taxon above).         -->
      <!-- ntax=73 nchar=488
-->
      <alignment id="ETSalignment" dataType="nucleotide">

      </alignment>

      <!-- The unique patterns for all positions
-->
      <!-- npatterns=288
-->
      <patterns id="PSBpatterns" from="1">
            <alignment idref="PSBalignment"/>
      </patterns>

      <!-- The unique patterns for all positions
-->
```

```
      <!-- npatterns=227
-->
      <patterns id="TRNLpatterns" from="1">
            <alignment idref="TRNLalignment"/>
      </patterns>

      <!-- The unique patterns for all positions
-->
      <!-- npatterns=302
-->
      <patterns id="TRNIGSpatterns" from="1">
            <alignment idref="TRNIGSalignment"/>
      </patterns>

      <!-- The unique patterns for all positions
-->
      <!-- npatterns=390
-->
      <patterns id="ETSpatterns" from="1">
            <alignment idref="ETSalignment"/>
      </patterns>



      <!-- A prior on the distribution node heights defined given
-->
      <!-- a Yule speciation process (a pure birth process).
-->
      <yuleModel id="yule" units="substitutions">
            <birthRate>
                  <parameter id="yule.birthRate" value="1.0" lower="0.0"

upper="1000000.0"/>
            </birthRate>
      </yuleModel>
      <!-- This is a simple constant population size coalescent model
-->
      <!-- that is used to generate an initial tree for the chain.
-->
      <constantSize id="initialDemo" units="substitutions">
            <populationSize>
                  <parameter id="initialDemo.popSize" value="100.0"/>
            </populationSize>
      </constantSize>

      <!-- Generate a random starting tree under the coalescent process
-->
      <coalescentTree id="startingTree" rootHeight="32.5">
            <constrainedTaxa>
                  <taxa idref="taxa"/>

                  <tmrca monophyletic="false">
                        <taxa idref="Helianthus-Tagetes"/>
                  </tmrca>
                  <tmrca monophyletic="false">
                        <taxa idref="Limestone"/>
                  </tmrca>
```

```
                  <tmrca monophyletic="false">
                        <taxa idref="Reunion"/>
                  </tmrca>

            </constrainedTaxa>
            <constantSize idref="initialDemo"/>
      </coalescentTree>

      <treeModel id="treeModel">
            <coalescentTree idref="startingTree"/>
            <rootHeight>
                  <parameter id="treeModel.rootHeight"/>
            </rootHeight>
            <nodeHeights internalNodes="true">
                  <parameter id="treeModel.internalNodeHeights"/>
            </nodeHeights>
            <nodeHeights internalNodes="true" rootNode="true">
                  <parameter id="treeModel.allInternalNodeHeights"/>
            </nodeHeights>
      </treeModel>

      <speciationLikelihood id="speciation">
            <model>
                  <yuleModel idref="yule"/>
            </model>
            <speciesTree>
                  <treeModel idref="treeModel"/>
            </speciesTree>
      </speciationLikelihood>

      <!-- The uncorrelated relaxed clock (Drummond, Ho, Phillips & Rambaut,
2006) -->
      <discretizedBranchRates id="branchRates">
            <treeModel idref="treeModel"/>
            <distribution>
                  <logNormalDistributionModel meanInRealSpace="true">
                        <mean>
                              <parameter id="ucld.mean" value="0.0012"
lower="0.0"

upper="100.0"/>
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                        <stdev>
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upper="10.0"/>
                        </stdev>
                  </logNormalDistributionModel>
            </distribution>
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            </rateCategories>
      </discretizedBranchRates>

      <rateStatistic id="meanRate" name="meanRate" mode="mean" internal="true"
external="true">
```

```
            <treeModel idref="treeModel"/>
            <discretizedBranchRates idref="branchRates"/>
      </rateStatistic>

      <rateStatistic id="coefficientOfVariation" name="coefficientOfVariation"

mode="coefficientOfVariation" internal="true" external="true">
            <treeModel idref="treeModel"/>
            <discretizedBranchRates idref="branchRates"/>
      </rateStatistic>

      <rateCovarianceStatistic id="covariance" name="covariance">
            <treeModel idref="treeModel"/>
            <discretizedBranchRates idref="branchRates"/>
      </rateCovarianceStatistic>

      <!-- The general time reversible (GTR) substitution model
-->
      <gtrModel id="PSBgtr">
            <frequencies>
                  <frequencyModel dataType="nucleotide">
                        <alignment idref="PSBalignment"/>
                        <frequencies>
                              <parameter id="PSBgtr.frequencies" dimension="4"/>
                        </frequencies>
                  </frequencyModel>
            </frequencies>
            <rateAC>
                  <parameter id="PSBgtr.ac" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAC>
            <rateAG>
                  <parameter id="PSBgtr.ag" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAG>
            <rateAT>
                  <parameter id="PSBgtr.at" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAT>
            <rateCG>
                  <parameter id="PSBgtr.cg" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateCG>
            <rateGT>
                  <parameter id="PSBgtr.gt" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateGT>
      </gtrModel>

            <!-- The general time reversible (GTR) substitution model
-->
      <gtrModel id="TRNLgtr">
            <frequencies>
                  <frequencyModel dataType="nucleotide">
                        <alignment idref="TRNLalignment"/>
                        <frequencies>
```

```
                              <parameter id="TRNLgtr.frequencies"
dimension="4"/>
                        </frequencies>
                  </frequencyModel>
            </frequencies>
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upper="Infinity"/>
            </rateAC>
            <rateAG>
                  <parameter id="TRNLgtr.ag" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAG>
            <rateAT>
                  <parameter id="TRNLgtr.at" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAT>
            <rateCG>
                  <parameter id="TRNLgtr.cg" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateCG>
            <rateGT>
                  <parameter id="TRNLgtr.gt" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateGT>
      </gtrModel>

      <!-- The general time reversible (GTR) substitution model
-->
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                        <alignment idref="TRNIGSalignment"/>
                        <frequencies>
                              <parameter id="TRNIGSgtr.frequencies"
dimension="4"/>
                        </frequencies>
                  </frequencyModel>
            </frequencies>
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upper="Infinity"/>
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                  <parameter id="TRNIGSgtr.ag" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAG>
            <rateAT>
                  <parameter id="TRNIGSgtr.at" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAT>
            <rateCG>
                  <parameter id="TRNIGSgtr.cg" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateCG>
            <rateGT>
```

```
                  <parameter id="TRNIGSgtr.gt" value="1.0" lower="0.0"
upper="Infinity"/>
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      </gtrModel>

      <!-- The general time reversible (GTR) substitution model
-->
      <gtrModel id="ETSgtr">
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                        <alignment idref="ETSalignment"/>
                        <frequencies>
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                        </frequencies>
                  </frequencyModel>
            </frequencies>
            <rateAC>
                  <parameter id="ETSgtr.ac" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAC>
            <rateAG>
                  <parameter id="ETSgtr.ag" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAG>
            <rateAT>
                  <parameter id="ETSgtr.at" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateAT>
            <rateCG>
                  <parameter id="ETSgtr.cg" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateCG>
            <rateGT>
                  <parameter id="ETSgtr.gt" value="1.0" lower="0.0"
upper="Infinity"/>
            </rateGT>
      </gtrModel>



      <!-- site model
-->
      <siteModel id="PSBsiteModel">
            <substitutionModel>
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            </substitutionModel>
            <gammaShape gammaCategories="4">
                  <parameter id="PSBsiteModel.alpha" value="0.5" lower="0.0"

upper="100.0"/>
            </gammaShape>
            <proportionInvariant>
                  <parameter id="PSBsiteModel.pInv" value="0.5" lower="0.0"
upper="1.0"/>
            </proportionInvariant>
      </siteModel>
```

```
            <!-- site model
-->
      <siteModel id="TRNLsiteModel">
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            </substitutionModel>
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upper="100.0"/>
            </gammaShape>
            <proportionInvariant>
                  <parameter id="TRNLsiteModel.pInv" value="0.5" lower="0.0"
upper="1.0"/>
            </proportionInvariant>
      </siteModel>

      <!-- site model
-->
      <siteModel id="TRNIGSsiteModel">
            <substitutionModel>
                  <gtrModel idref="TRNIGSgtr"/>
            </substitutionModel>
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upper="100.0"/>
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            <proportionInvariant>
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upper="1.0"/>
            </proportionInvariant>
      </siteModel>

      <!-- site model
-->
      <siteModel id="ETSsiteModel">
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            </substitutionModel>
            <gammaShape gammaCategories="4">
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upper="100.0"/>
            </gammaShape>
            <proportionInvariant>
                  <parameter id="ETSsiteModel.pInv" value="0.5" lower="0.0"
upper="1.0"/>
            </proportionInvariant>
      </siteModel>


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            <patterns idref="PSBpatterns"/>
            <treeModel idref="treeModel"/>
            <siteModel idref="PSBsiteModel"/>
```

```
      <discretizedBranchRates idref="branchRates"/>
</treeLikelihood>

<treeLikelihood id="TRNLtreeLikelihood">
      <patterns idref="TRNLpatterns"/>
      <treeModel idref="treeModel"/>
      <siteModel idref="TRNLsiteModel"/>
      <discretizedBranchRates idref="branchRates"/>
</treeLikelihood>

<treeLikelihood id="TRNIGStreeLikelihood">
      <patterns idref="TRNIGSpatterns"/>
      <treeModel idref="treeModel"/>
      <siteModel idref="TRNIGSsiteModel"/>
      <discretizedBranchRates idref="branchRates"/>
</treeLikelihood>

<treeLikelihood id="ETStreeLikelihood">
      <patterns idref="ETSpatterns"/>
      <treeModel idref="treeModel"/>
      <siteModel idref="ETSsiteModel"/>
      <discretizedBranchRates idref="branchRates"/>
</treeLikelihood>


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      <mrca>
            <taxa idref="Gnaphalieae"/>
      </mrca>
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</tmrcaStatistic>
<tmrcaStatistic id="tmrca(GnaphsexRelhanias)">
      <mrca>
            <taxa idref="GnaphsexRelhanias"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(Helianthus-Tagetes)">
      <mrca>
            <taxa idref="Helianthus-Tagetes"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(Limestone)">
      <mrca>
            <taxa idref="Limestone"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(Metalasiaclade)">
      <mrca>
            <taxa idref="Metalasiaclade"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
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      <mrca>
```

```
            <taxa idref="Outgroups"/>
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      <treeModel idref="treeModel"/>
</tmrcaStatistic>
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      <mrca>
            <taxa idref="Ozthree"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
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      <mrca>
            <taxa idref="Relhaniaclade"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
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      <mrca>
            <taxa idref="RestexStoebes"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(Reunion)">
      <mrca>
            <taxa idref="Reunion"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(Stoebeclade)">
      <mrca>
            <taxa idref="Stoebeclade"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(StoebesandRest)">
      <mrca>
            <taxa idref="StoebesandRest"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>
<tmrcaStatistic id="tmrca(ZNode)">
      <mrca>
            <taxa idref="ZNode"/>
      </mrca>
      <treeModel idref="treeModel"/>
</tmrcaStatistic>


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      </scaleOperator>
      <scaleOperator scaleFactor="0.2431" weight="1">
            <parameter idref="PSBgtr.ag"/>
      </scaleOperator>
      <scaleOperator scaleFactor="0.3508" weight="1">
            <parameter idref="PSBgtr.at"/>
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```
</scaleOperator>
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      <parameter idref="PSBgtr.gt"/>
</scaleOperator>
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</scaleOperator>
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</scaleOperator>


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      <parameter idref="TRNLgtr.ag"/>
</scaleOperator>
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      <parameter idref="TRNLgtr.at"/>
</scaleOperator>
<scaleOperator scaleFactor="0.23" weight="1">
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</scaleOperator>
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      <parameter idref="TRNLgtr.gt"/>
</scaleOperator>
<scaleOperator scaleFactor="0.3616" weight="1">
      <parameter idref="TRNLsiteModel.alpha"/>
</scaleOperator>
<scaleOperator scaleFactor="0.33" weight="1">
      <parameter idref="TRNLsiteModel.pInv"/>
</scaleOperator>

<scaleOperator scaleFactor="0.35" weight="1">
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</scaleOperator>
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      <parameter idref="TRNIGSgtr.ag"/>
</scaleOperator>
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      <parameter idref="TRNIGSgtr.at"/>
</scaleOperator>
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</scaleOperator>
<scaleOperator scaleFactor="0.2944" weight="1">
      <parameter idref="TRNIGSgtr.gt"/>
</scaleOperator>
<scaleOperator scaleFactor="0.3460" weight="1">
      <parameter idref="TRNIGSsiteModel.alpha"/>
</scaleOperator>
<scaleOperator scaleFactor="0.1763" weight="1">
      <parameter idref="TRNIGSsiteModel.pInv"/>
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</scaleOperator>

<scaleOperator scaleFactor="0.43" weight="1">
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</scaleOperator>
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      <parameter idref="ETSgtr.ag"/>
</scaleOperator>
<scaleOperator scaleFactor="0.75" weight="1">
      <parameter idref="ETSgtr.at"/>
</scaleOperator>
<scaleOperator scaleFactor="0.37" weight="1">
      <parameter idref="ETSgtr.cg"/>
</scaleOperator>
<scaleOperator scaleFactor="0.75" weight="1">
      <parameter idref="ETSgtr.gt"/>
</scaleOperator>
<scaleOperator scaleFactor="0.75" weight="1">
      <parameter idref="ETSsiteModel.alpha"/>
</scaleOperator>
<scaleOperator scaleFactor="0.3145" weight="1">
      <parameter idref="ETSsiteModel.pInv"/>
</scaleOperator>

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      <parameter idref="ucld.mean"/>
</scaleOperator>
<scaleOperator scaleFactor="0.75" weight="3">
      <parameter idref="ucld.stdev"/>
</scaleOperator>

<upDownOperator scaleFactor="0.5708" weight="3">
      <up>
            <parameter idref="ucld.mean"/>
      </up>
      <down>
            <parameter idref="treeModel.allInternalNodeHeights"/>
      </down>
</upDownOperator>
<swapOperator size="1" weight="30" autoOptimize="false">
      <parameter idref="branchRates.categories"/>
</swapOperator>
<scaleOperator scaleFactor="0.75" weight="3">
      <parameter idref="yule.birthRate"/>
</scaleOperator>
<scaleOperator scaleFactor="0.8264" weight="3">
      <parameter idref="treeModel.rootHeight"/>
</scaleOperator>
<uniformOperator weight="30">
      <parameter idref="treeModel.internalNodeHeights"/>
</uniformOperator>
<subtreeSlide size="3.22" gaussian="true" weight="15">
      <treeModel idref="treeModel"/>
</subtreeSlide>
<narrowExchange weight="15">
      <treeModel idref="treeModel"/>
</narrowExchange>
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      <wideExchange weight="3">
            <treeModel idref="treeModel"/>
      </wideExchange>
      <wilsonBalding weight="3">
            <treeModel idref="treeModel"/>
      </wilsonBalding>
</operators>

<mcmc id="mcmc" chainLength="10000000" autoOptimize="true">
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                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="PSBgtr.ag"/>
                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="PSBgtr.at"/>
                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="PSBgtr.cg"/>
                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="PSBgtr.gt"/>
                  </jeffreysPrior>


                  <jeffreysPrior>
                        <parameter idref="TRNLgtr.ac"/>
                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="TRNLgtr.ag"/>
                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="TRNLgtr.at"/>
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                  <jeffreysPrior>
                        <parameter idref="TRNLgtr.cg"/>
                  </jeffreysPrior>
                  <jeffreysPrior>
                        <parameter idref="TRNLgtr.gt"/>
                  </jeffreysPrior>


                  <jeffreysPrior>
                        <parameter idref="TRNIGSgtr.ac"/>
                  </jeffreysPrior>
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```

```
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                        <jeffreysPrior>
                              <parameter idref="TRNIGSgtr.gt"/>
                        </jeffreysPrior>

                        <jeffreysPrior>
                              <parameter idref="ETSgtr.ac"/>
                        </jeffreysPrior>
                        <jeffreysPrior>
                              <parameter idref="ETSgtr.ag"/>
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                        <jeffreysPrior>
                              <parameter idref="ETSgtr.at"/>
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                        <jeffreysPrior>
                              <parameter idref="ETSgtr.cg"/>
                        </jeffreysPrior>
                        <jeffreysPrior>
                              <parameter idref="ETSgtr.gt"/>
                        </jeffreysPrior>




                        <normalPrior mean="32.5" stdev="3.2">
                              <parameter idref="treeModel.rootHeight"/>
                        </normalPrior>
                        <logNormalPrior mean="2.0" stdev="0.7" offset="15.0"

meanInRealSpace="false">
                              <statistic idref="tmrca(Helianthus-Tagetes)"/>
                        </logNormalPrior>
                        <normalPrior mean="4.75" stdev="1.5">
                              <statistic idref="tmrca(Reunion)"/>
                        </normalPrior>
                        <normalPrior mean="6.0" stdev="1.5">
                              <statistic idref="tmrca(Limestone)"/>
                        </normalPrior>
                        <speciationLikelihood idref="speciation"/>
                  </prior>
                  <likelihood id="likelihood">
                        <treeLikelihood idref="PSBtreeLikelihood"/>
                        <treeLikelihood idref="TRNLtreeLikelihood"/>
                        <treeLikelihood idref="TRNIGStreeLikelihood"/>
                        <treeLikelihood idref="ETStreeLikelihood"/>
                  </likelihood>

            </posterior>
            <operators idref="operators"/>
            <log id="screenLog" logEvery="1000">
                  <column label="Posterior" dp="4" width="12">
                        <posterior idref="posterior"/>
                  </column>
                  <column label="Prior" dp="4" width="12">
                        <prior idref="prior"/>
                  </column>
                  <column label="L(PSBtree)" dp="4" width="12">
```

```
            <likelihood idref="PSBtreeLikelihood"/>
      </column>
      <column label="L(TRNLtree)" dp="4" width="12">
            <likelihood idref="TRNLtreeLikelihood"/>
      </column>
      <column label="L(TRNIGStree)" dp="4" width="12">
            <likelihood idref="TRNIGStreeLikelihood"/>
      </column>
      <column label="L(ETStree)" dp="4" width="12">
            <likelihood idref="ETStreeLikelihood"/>
      </column>
      <column label="Root Height" sf="6" width="12">
            <parameter idref="treeModel.rootHeight"/>
      </column>
      <column label="Rate" sf="6" width="12">
            <rateStatistic idref="meanRate"/>
      </column>
</log>
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      <posterior idref="posterior"/>
      <prior idref="prior"/>
      <likelihood idref="likelihood"/>
      <rateStatistic idref="meanRate"/>
      <parameter idref="treeModel.rootHeight"/>
      <tmrcaStatistic idref="tmrca(Gnaphalieae)"/>
      <tmrcaStatistic idref="tmrca(GnaphsexRelhanias)"/>
      <tmrcaStatistic idref="tmrca(Helianthus-Tagetes)"/>
      <tmrcaStatistic idref="tmrca(Limestone)"/>
      <tmrcaStatistic idref="tmrca(Metalasiaclade)"/>
      <tmrcaStatistic idref="tmrca(Outgroups)"/>
      <tmrcaStatistic idref="tmrca(Ozthree)"/>
      <tmrcaStatistic idref="tmrca(Relhaniaclade)"/>
      <tmrcaStatistic idref="tmrca(RestexStoebes)"/>
      <tmrcaStatistic idref="tmrca(Reunion)"/>
      <tmrcaStatistic idref="tmrca(Stoebeclade)"/>
      <tmrcaStatistic idref="tmrca(StoebesandRest)"/>
      <tmrcaStatistic idref="tmrca(ZNode)"/>
      <parameter idref="yule.birthRate"/>

      <parameter idref="PSBgtr.ac"/>
      <parameter idref="PSBgtr.ag"/>
      <parameter idref="PSBgtr.at"/>
      <parameter idref="PSBgtr.cg"/>
      <parameter idref="PSBgtr.gt"/>
      <parameter idref="PSBsiteModel.alpha"/>
      <parameter idref="PSBsiteModel.pInv"/>

      <parameter idref="TRNLgtr.ac"/>
      <parameter idref="TRNLgtr.ag"/>
      <parameter idref="TRNLgtr.at"/>
      <parameter idref="TRNLgtr.cg"/>
      <parameter idref="TRNLgtr.gt"/>
      <parameter idref="TRNLsiteModel.alpha"/>
      <parameter idref="TRNLsiteModel.pInv"/>

      <parameter idref="TRNIGSgtr.ac"/>
      <parameter idref="TRNIGSgtr.ag"/>
```

```
                  <parameter idref="TRNIGSgtr.at"/>
                  <parameter idref="TRNIGSgtr.cg"/>
                  <parameter idref="TRNIGSgtr.gt"/>
                  <parameter idref="TRNIGSsiteModel.alpha"/>
                  <parameter idref="TRNIGSsiteModel.pInv"/>

                  <parameter idref="ETSgtr.ac"/>
                  <parameter idref="ETSgtr.ag"/>
                  <parameter idref="ETSgtr.at"/>
                  <parameter idref="ETSgtr.cg"/>
                  <parameter idref="ETSgtr.gt"/>
                  <parameter idref="ETSsiteModel.alpha"/>
                  <parameter idref="ETSsiteModel.pInv"/>


                  <parameter idref="ucld.mean"/>
                  <parameter idref="ucld.stdev"/>
                  <rateStatistic idref="coefficientOfVariation"/>
                  <rateCovarianceStatistic idref="covariance"/>

                  <treeLikelihood idref="PSBtreeLikelihood"/>
                  <treeLikelihood idref="TRNLtreeLikelihood"/>
                  <treeLikelihood idref="TRNIGStreeLikelihood"/>
                  <treeLikelihood idref="ETStreeLikelihood"/>

                  <speciationLikelihood idref="speciation"/>
            </log>
            <logTree id="treeFileLog" logEvery="1000" nexusFormat="true"

fileName="frame_02_June_1.trees" sortTranslationTable="true">
                  <treeModel idref="treeModel"/>
                  <discretizedBranchRates idref="branchRates"/>
                  <posterior idref="posterior"/>
            </logTree>
            <logTree id="substTreeFileLog" logEvery="1000" nexusFormat="true"

fileName="frame_02_June_1(subst).trees" branchLengths="substitutions">
                  <treeModel idref="treeModel"/>
                  <discretizedBranchRates idref="branchRates"/>
            </logTree>
      </mcmc>

      <report>
            <property name="timer">
                  <object idref="mcmc"/>
            </property>
      </report>

</beast>
```

Appendix 2: list of characters and morphological data matrix used for analyses in Chapter 3.

List of characters:

1	Leaf orientation (0 = completely adpressed; 1 = erect and straight; 2 = spreading / curled / curved / reflexed, twisted)
2	Presence of conspicuous stalked glands (0 = absent; 1 = present)
3	Synflorescence shape (0=no secondary structure; 1 = loosely aggregated; 2 = compact spike; 3 = compact globe)
4	Synflorescence (0 = solitary; 1 = compound)
5	Degree of fusion amongst capitula in smallest synflorescence unit (0 = free; 1= loosely fused; 2 = completely fused)
6	No. of heads in smallest synflorescence unit (0 = <10; 1 = 10-19; 2 = 20-40; 3 = >/=40)
7	Average no. of florets per head (0 = 1; 1 = 1.5-2; 2 = 2.5-6; 3= >6)
8	Ray florets (0 = absent; 1 = present)
9	Disc floret shape (0 = filiform; 1 = linear/tubular; 2 = cup-shaped; 3 = campanulate)
10	Disc corolla lobe size relative to tube width (0 = short; 1 = long)
11	Disc corolla lobe orientation (0 = erect; 1 = reflexed)
12	Disc floret colour (0=white; 1=pink; 2=plum red; 3=yellow / pale brown)
13	Pappus (0 = absent; 1 = present)
14	Brachyblasts (0 = absent; 1 = present)
15	'Nectary' disc (0 = absent; 1 = present)

```
Matrix
     Acal1452a  100000310110111
     AcelK1099  100000200110101
     AcorK1291  100000310110111
     ArudK1214  200000200110101
     AtecK1066  200000300110101
     AthuK1200  000000200110101
     Atom1322   100000310110101
     Atom1332   100000310110101
     Atom1342   100000310110101
     Bare1155   100000313110101
     Dano1258   203122113000001
     Deric1143  203111113100101
     Dgong1386  203112211000101
     Dkolb1419  000000212000100
     Dkrau1262  203123113100101
```

```
Dlax1412   203112111110101
Dtort1451  203122013100101
Eadpr605   000000201001100
Egla1096   212100101011100
Egna1215b  211000101011100
Ehis1043   213120203110100
Ehis1409   213120203110100
Elong1248  212100203011100
ErhiCo1    000000201001100
ErhiCo2    000000201001100
Saet1040   103122003110101
Salop1283  202112003110110
Scap1188   203123003110101
Scin1210   202012001001110
Scya1429   203122003110101
Sfus1318   203111003110101
Sgom1439a  003123003100101
Sint1293   010011001011100
SkilBG76   2010??001001110
Sleu1404   203112001100101
Smic1415   003111001000101
SmonK360   203??00??1?001
Smui1422   203112003110101
Spas15821  001000001001100
Sphy1457   202001003110101
Splum1242  102000001001110
Spros1163  203112003110101
Sros1445   203113002000100
Srug1270   101010001110101
Sschu1308  003112003110001
Sspir1187  202010001002100
```