Molecular Systematics, Biogeography and Dating of the tribe Haemantheae (Amaryllidaceae) and the Phylogeography of *Clivia*

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Ferozah Conrad
Cape Town, February 2008

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less"

Marie Curie 1(867-1934)

Abstract

The African tribe Haemantheae, belonging to the monocotyledonous family Amaryllidaceae, comprises six genera (*Gethyllis, Apodolirion, Haemanthus, Scadoxus, Clivia* and *Cryptostephanus*) with ca. 90 species. A phylogenetic hypothesis for the Haemantheae is presented as a basis for an enquiry into the generic and species relationships within the tribe. DNA sequence data from five plastid regions: the *rpoB-trnC* intergenic spacer, *trnL* intron, *trnL-F* intergenic spacer, the *rps16* intron, the *psbA-trnH* intergenic spacer and internal transcribed spacer region (*ITS*) have been collected and analysed for 62 taxa within this tribe using two outgroups within Amaryllidaceae. Combined parsimony and Bayesian analyses of the five plastid and one nuclear region indicated that *Scadoxus* and *Haemanthus* are monophyletic and resolved as sister clades to one another. The summer rainfall group of species within the genus *Haemanthus* is monophyletic. The genus *Apodolirion* is embedded within *Gethyllis* as has been previously suggested on morphological grounds. Both *Clivia* and *Cryptostephanus* resolved as monophyletic groups with *Cryptostephanus* placed as sister to *Clivia.* Character optimizations of 15 morphological characters were carried out and optimization of the character 'anther number' revealed the strongest evidence so far for not recognising the informally recognized *Gethyllis* 'villosa' group. Biogeographic analyses using the divergence/vicariance (DIVA) method were inconclusive in determining the ancestral node of Haemantheae as the phytogeographic areas occupied by the taxa were too widespread. Two methods, non parametric rate smoothing (NPRS) and a Bayesian method (implemented in BEAST) were used in the assessment of age estimates and divergence times within the Haemantheae. Due to a lack of fossil record for this group, a calibration point from Wikström *et al.* (2001) of 33 Ma was used, based on the split of Haemantheae and Hippeastreae. Results indicate a rapid diversification for the winter rainfall lineages of *Haemanthus* at around 5 Ma coinciding with the late Miocene/Pliocene and the aridification and formation of a Mediterranean-type climate in southwestern Africa. In contrast, *Gethyllis* reflects a gradual diversification from 20-8Ma before the onset of aridification and the establishment of the Mediterranean-type climate. Analyses of the *Clivia* populations reveal occasional haplotype sharing between *Clivia* species in those parts of the distribution range where they are sympatric. The interconnectedness of *C. gardenii* and *C. robusta* brings into question the recognition of these two entities as discrete species.

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CHAPTER 1. INTRODUCTION

The Cape Floristic Region, with the world's richest temperate flora, is only 90 000 km^2 in area, making up less than 4% of the total area of the southern African subcontinent but is home to >9000 species of plants. Distinctive features of this flora include the prevalence of fine-leaved sclerophyllous shrubs, very few trees and a remarkably large number of geophytes. Geophytes are primarily adapted to seasonally dry climates and escape the time of year unfavourable for growth by retreating to underground storage organs (Goldblatt and Manning 2000).

Geophytes are widespread throughout the world in many habitats but are typically most diverse and abundant in regions with a Mediterranean climate. The Cape Floristic Region stands out among these regions in the extraordinary richness of its geophyte flora. In a flora of 9000 species, some 1500, or 17% of the total flora, are geophytes (Manning *et al*. 2002).

In their study of geophyte diversity in the winter rainfall region of southern Africa, Procheş *et al.* (2005) noted that the western parts of the area are richer in geophytes than the east, a pattern that is common among geophytes from a range of families. They identified two distinct high diversity areas for Amaryllidaceae in southern Africa: one in the north-west, corresponding to the centre of diversity for the tribes Haemantheae and Amaryllideae, and one in the east, corresponding to the centre of diversity for the tribe Cyrtantheae.

The advantage of the geophytic habitat is the ability to survive short-term drought and respond rapidly to improved moisture conditions (Dafni *et al.* 1981, Rees 1989). In the winter-rainfall region (WRR) most geophytes are photosynthetically active in these cooler months (Rossa and von Willert 1999). The WRR, loosely defined as the area where more than 50% of total annual precipitation falls during the winter months (Bayer 1984, Jürgens 1991), stretches over large portions of three South African provinces and south western Namibia and incorporates the all-year rainfall area of the southern Cape. Ample and reliable winter rain - as experienced in the south western

WRR - is expected to promote the persistence of geophytes, and enable the evolution of small bulbs since opportunities for the replenishment of storage organs occur every year (Procheş *et al.* 2005). In the east, however, unreliable winter rains impose constraints on winter growing geophytes since prolonged dry spells may limit organ replenishment and compromise flowering, ultimately leading to the extinction of populations (Procheş *et al.* 2005). In the drier north western parts of the WRR, the uniquely reliable winter rainfall regime is thought to be a major driver of the extraordinary diversification of geophytes across a wide range of lineages (Procheş *et al.* 2005).

The core families in this group are those that develop either true bulbs, such as the amaryllis, and hyacinth families (Amaryllidaceae and Hyacinthaceae), or those that develop corms, mainly the iris and colchicum families (Iridaceae and Colchicaceae). The families Iridaceae and Hyacinthaceae are two of the 20 largest families in the Cape Flora based on species number. The Iridaceae is the third largest with 520 of the 661 species (or 79.1%) endemic to the region (Goldblatt and Manning 2000). Although the Amaryllidaceae are not amongst the largest families of monocotyledons in the Cape Florsitic Region, Hilton-Taylor (1996) reported that the family has a higher than expected number of endemic species in the Succulent Karoo region, which together with the Cape Floristic Region makes up the Greater Cape Floristic Region of southern Africa (Born *et al.* 2007, Jürgens 1991).

1.1. Haemantheae in sub-Saharan Africa

The Amaryllidaceae is one of the few families of the higher Asparagales well defined by a combination of umbel-like cymes, inferior ovaries and unique alkaloid chemistry (Meerow and Snijman 2006). The family consists of nine tribes according to the classification of Dahlgren *et al.* (1985). These are: Amaryllideae (African distribution except for *Crinum*), Haemantheae (African), Hippeastreae (neotropical/American), Lycorideae (Asian), Eucharideae (American), Stenomesseae (South American), Pancratieae (Old World), Narcisseae (west Mediterranean) and Galantheae (Mediterranean-Western Asiatic).

Partially following Hutchinson (1934, 1959), Traub (1963) included Alliaceae, Hemerocallidaceae and Ixioliriaceae as subfamilies, in the Amaryllidaceae. Two polyphyletic, informal taxa (infrafamilies) Amarylloidinae and Pancratiodinae, were erected in his subfamily Amarylloideae (Meerow 1995). Dahlgren *et al.* (1985) dispensed with any subfamilial classification above the level of tribe, and treated as Amaryllidaceae only those genera in Traub's Amarylloideae, also combining Stenomesseae and Eustephieae. Meerow (1985) resurrected Eustephieae from Stenomesseae and suggested the need for two new tribes, Calostemmateae and Hymenocallideae. Müller-Doblies and Müller-Doblies (1996) recognised ten tribes (among them Calostemmateae) and 19 subtribes; Meerow and Snijman (1998) recognise 13 tribes, with two subtribes only in the Amaryllideae (Meerow *et al.* 2000b).

1.1.1. Classification

For the purpose of this study the classification of Meerow *et al.* (1999b) has been followed. It includes all the baccate-fruited genera: *Haemanthus*, *Scadoxus*, *Gethyllis, Apodolirion*, *Cryptostephanus* and *Clivia*.

Four other infrafamilial classifications of Amaryllidaceae are those of Traub (1963), Dahlgren *et al.* (1985), Müller-Doblies and Müller-Doblies (1996) and Meerow and Snijman (1998). Traub (1963) recognised three different tribes: Haemantheae (*Haemanthus* and *Choananthus* (later reduced to *Scadoxus*)), Clivieae (*Clivia* and *Cryptostephanus*) and Gethylliae (*Apodolirion* and *Gethyllis*). Dahlgren *et al.* (1985) and Müller-Doblies and Müller-Doblies (1996) included *Cyrtanthus*, normally placed in Cyrtantheae, in the tribe Haemantheae, although Müller-Doblies and Müller-Doblies (1996) erected the monotypic subtribe, Cyrtanthinae, to accommodate it. A shared chromosome number with *Haemanthus* (2n=16 (Vosa and Snijman 1984)) and its strictly African range were the only evidence for uniting *Cyrtanthus* with the Haemantheae.

Meerow and Snijman (1998) recognised two tribes, Haemantheae and Gethyllideae, for the six genera. Haemantheae included *Clivia*, *Cryptostephanus*, *Scadoxus*, all with rhizomes or much reduced bulbs and *Haemanthus*, the only genus with well-developed

bulbs. *Cryptostephanus* is the only genus having seeds with a phytomelanous coat. Gethyllideae comprised two genera, *Gethyllis* and *Apodolirion*, both restricted to southern Africa, with elongated berries that differ from the globose berry found in the other baccate-fruited genera of Haemantheae (Meerow and Snijman 1998).

Recognition of Gethyllidaeae as a distinct tribe (Müller-Doblies and Müller-Doblies (1996 and Meerow and Snijman 1998) is not supported by molecular data; the two genera resolve as sister taxa, firmly embedded within Haemantheae (Meerow *et al.* 1999b, Meerow *et al.* 2000b).

Meerow *et al.* (1999b) used plastid sequence data (from the *rbcL* and *trnL-F* regions) for 48 genera of Amaryllidaceae and 29 genera of related Asparagalean families to re-examine the relationships of the genera previously placed in Haemantheae and Gethylleae. The results demonstrated that the baccate fruited tribe, Haemantheae comprises six genera (*Haemanthus*, *Scadoxus*, *Clivia*, *Cryptostephanus*, *Apodolirion* and *Gethyllis*) thereby supporting the monophyly of the tribe. The tribes Calostemmateae and Cyrtantheae remained as part of a polytomy inclusive of Haemantheae and the large Eurasian/America clade (Meerow *et al.* 1999b).

Meerow and Clayton (2004) further investigated Haemantheae using 19 species representing all the genera. The nuclear and combined nuclear and plastid matrices produced a well resolved tree comprising two clades. The smaller clade included *Clivia* and *Cryptostephanus* and the larger clade two subclades - one comprising *Haemanthus* and *Scadoxus* and the other *Apodolirion* and a paraphyletic *Gethyllis*. Meerow and Clayton (2004) supported the recognition of three subtribes in Haemantheae: Cliviinae D.Müll.-Doblies and U.Müll.-Doblies, Haemanthineae Pax and Gethyllidinae Dumort. A significant suggestion by Meerow and Clayton (2004) was that their results should be further augmented by increasing the depth of sampling for the genera in the tribe, as has been attempted for this study.

1.1.2. Morphology

Clivia (6 spp.) (Figure 1.1) and *Cryptostephanus* (4 spp.) are both evergreen, rhizomatous genera. *Clivia* is almost exclusively found in summer rainfall areas of southern Africa. The exception is *Clivia mirabilis,* which is found in the winter rainfall region of the Northern Cape. *Clivias* are understorey herbs growing predominantly in coastal and Afromontane forest. *Cryptostephanus* spp. are either savanna or forest herbs (Meerow and Clayton 2004) and the range of the genus extends from Namibia and Zimbabwe in southern Africa into Kenya in east Africa. Their chromosome numbers are x=11 and x=12 respectively.

Figure 1.1: Inflorescence and inflorescence colour variation in *Clivia*

Haemanthus (22 spp.) (Figure1.2) and *Scadoxus* (9 spp.) (Figure1.2) have long been recognised intuitively as sister taxa. Bjornstad and Friis (1972a, b) initially followed Baker's (1888, 1896) treatment of them as a single genus but then later separated them (Friis and Nordal 1976). Both genera have brush-like inflorescences, which are many-flowered, and their chromosome numbers are x=8 and x=9 respectively. *Scadoxus* spp. are mostly forest understorey herbs, some species of which do not form true bulbs. The genus is most common in the African tropics (Bjornstad and Friis 1972, Meerow and Clayton 2004). *Haemanthus*, with all species forming bulbs, is strictly southern African, and has species in both the summer and winter rainfall areas of the region (Snijman 1984).

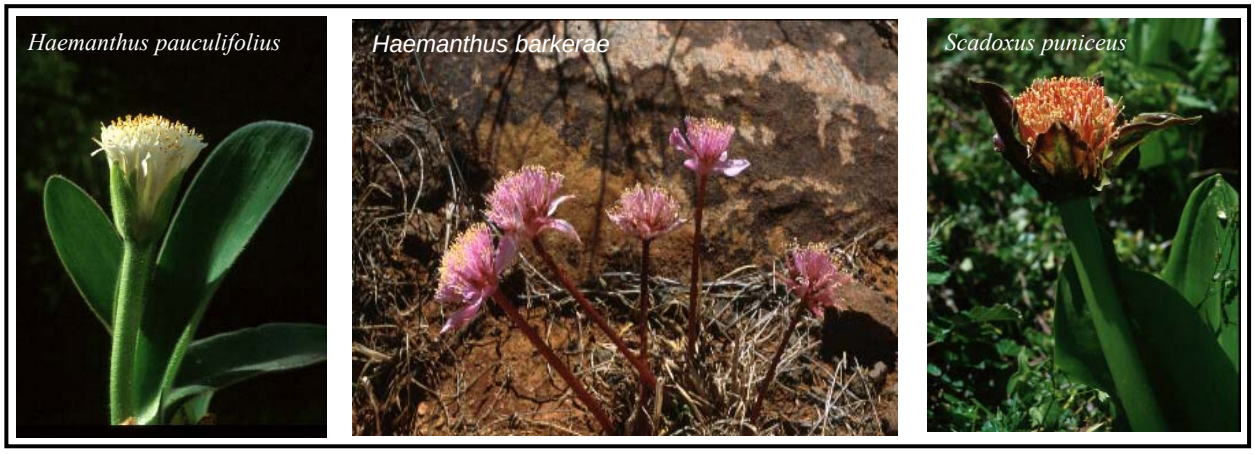


Figure 1.2: Habit variation in *Haemanthus* and *Scadoxus*

The most poorly understood members of the tribe are *Gethyllis* (32 spp.) (Figure 1.3 and 1.4) and *Apodolirion* (6 spp.) (Figure 1.4). These have a solitary flower with a subterranean ovary and they share the same chromosome number $x = 6$. *Apodolirion* is distinguished from *Gethyllis* by small differences in the anther attachment (Manning *et al*. 2002). *Gethyllis* is most common in the winter rainfall region of South Africa, occurring in the Western Cape Province and adjacent parts of southern Namibia, Northern and Eastern Cape Province, extending northwards to the Free State, North-West Province and southern Botswana. *Apodolirion* occurs in Mpumalanga, Free State and Eastern Cape as well as the Western Cape Province of South Africa.

Figure 1.3: Leaf position variation and fruit of *Gethyllis*

Figure 1.4: Anther number variation in *Gethyllis* and *Apodolirion*

The evolution of the bulb and rhizome in Amaryllidaceae has been interpreted differently by various authors prior to the availability of rigorous phylogenetic analyses. Takhtajan (1969) and Cronquist (1968) proposed that although most of the genera of Amaryllidaceae are true bulbous plants, it may reasonably be assumed that they originated from rhizomatous forms. The evolution of bulbs from rhizomes may have happened before or after the evolution of those features that distinguish Amaryllidaceae - primarily the inferior ovary and the umbelliform inflorescence surrounded by involucral bracts. If the development from rhizome to bulb has taken place within Amaryllidaceae, it is possible that the rhizomatous Amaryllidaceae possess other assumed primitive characters, although this is not a certainty as character evolution may not be correlated. (Björnstad & Friis 1972a).

More recently, on the basis of phylogenetic analysis using *rbcL* and *trnL-F* sequence data, Meerow *et al.* (1999b) considered the bulbless state plesiomorphic for the Amaryllidaceae. In their analysis of Haemantheae using ITS sequence data, Meerow and Clayton (2004) found, that *Clivia* and *Cryptostephanus* formed a clade representing entirely rhizomatous genera that never form bulbs. The *Clivia-Cryptostephanus* clade also resolved sister to the clade with the other four genera of Haemantheae that are almost entirely bulbous. Meerow and Clayton (2004) state that it is unclear whether bulbs form in *Scadoxus* only under certain environmental conditions or if bulb formation is limited to just certain species. In addition their analysis showed

that *Cryptostephanus* is the only genus that retained the plesiomorphic character of a phytomelanous testa on the seed.

Based on the results from the present, expanded molecular analysis, I shall examine the evolution of a selected group of plant traits. For example, it has been suggested that particular plant traits, for example storage organ size, are advantageous for the geophytic habit as they afford the ability to persist through short term drought, and respond rapidly when conditions alter (Dafni *et al.* 1981, Rees 1989). I shall therefore assess whether any particular plant traits are associated with species-rich clades. In a previous study on the Haemantheae, Meerow and Clayton (2004) used six characters. I shall re-examine five of the six and supplement this by assessing 15 characters in total in this study.

1.1.3. Biogeography and distribution

The Amaryllidaceae, with 59 genera and 860 spp., is chiefly a pantropical family with notable centres of diversity in South America (28 genera) and Africa (19 genera), with a further eight genera around the Mediterranean (Meerow and Snijman 1998). Only a single genus *Crinum* (Amaryllideae), with seeds apparently well adapted for dispersal over water, is represented in both the Old and New Worlds (Meerow *et al.* 2003).

Evidence from DNA sequence data places the most ancient (though not necessarily primitive) divergences within the family in Africa, which would suggest a West Gondwana origin (Fay *et al.* 1995, Meerow *et al.* 1999b), with West Gondwana defined as consisting of South America and Africa (McCarthy and Rubidge 2005). Climatic changes in southern Africa (Goldblatt 1978), which started in the late Eocene and became dramatic during the Oligocene and Miocene (Procheş *et al.* 2006), and geologic changes in South America (Meerow 1989) are thought to have been an important factor in the radiation of the family within its two main centres of diversity (Meerow and Snijman 1998).

In their investigations into the Cape flora's biogeography, Galley and Linder (2006) suggest that the Cape Floristic Region (CFR) comprises taxa that can be divided into three elements:

- Clades that are most diverse in the semi arid Succulent Karoo in the north and have outliers in the CFR
- Elements that are most species-rich in subtropical and tropical Africa and are shared with tropical Africa
- Cape clades, defined by Linder (2003) as the most inclusive clades, which have at least 50% of their species in the CFR and for which the basal nodes are optimized for being located in the CFR.

The African track (or distribution area of a taxon) has been stressed as evidence of a tropical African origin of the Cape flora (Levyns 1938, 1952, 1964; Axelrod and Raven 1978), as the number of taxa with tropical affinities is believed to outweigh those with Gondwanan links (Levyns 1964). Levyns (1964) suggested that the Cape flora is largely the result of southward migration from tropical Africa to the CFR, whereas Adamson (1958) and Wild (1964) suggested that the Cape Flora was once more widespread in Africa, and differentiated vicariously on the tropical mountains and within the CFR. A southward migration from tropical Africa has even been postulated for some taxa with current distributions in the CFR and Australasia (Levyns 1958, 1964). In contrast a northward dispersal is suggested for *Androcymbium* (Colchicaceae) from South Africa into the Mediterranean (Procheş *et al.*2006).

Galley and Linder (2006) found that the tropical African species of all groups for which adequately resolved phylogenetic hypothesis are available (eg. *Cliffortia*, *Aristea*, *Pelargonium*) are nested within otherwise Cape groups, thus leading to the rejection of the hypothesis of Levyns (1964) that Cape members of these groups are derived from the tropical African species.

Another study which refutes the hypothesis that north to south has been the prevailing direction of migration for taxa shared between the Cape and the Afromontane flora is that of Galley *et al.* (2006). They found that the most recent common ancestor of the four clades in their study (*Disa*, Irideae pro parte, *Pentaschistis* and Restionaceae) could be unambiguously traced to the Cape. Eighteen migrations from the Cape to the Drakensberg range and 12 from the Drakensberg to the rest of the Afromontane region north of the Limpopo River were documented. There were two migrations from the

Cape to areas north of the Limpopo River and migration events in the opposite direction were rare.

The genera of the Haemantheae have an interesting distribution pattern in that they include elements from both the Cape Floristic Region and tropical Africa thereby contributing to the debate on the historical affinities of the Cape clades and particularly their relationship to the afrotropics (Levyns 1957, Levyns 1963, Galley *et al*. 2006). Their distribution is as follows: *Apodolirion* is distributed through the eastern and southern parts of southern Africa (Figure 1.5). Most of the taxa from the genus *Gethyllis* occur in the Western Cape Province and adjacent parts of southern Namibia, and Northern Cape and Eastern Cape Provinces. Only one species extends northwards to the Free State, North-West Province and southern Botswana (Figure 1.6). *Haemanthus* occurs through most of southern Africa except the central Karoo in the Northern Cape Province, and extends into central Namibia and Limpopo Province, but is absent from Botswana and North-West Province (Snijman 1984; Figure 1.7). *Scadoxus* occurs throughout tropical Africa from Ethiopia southwards to the Western Cape Province, including the northern parts of Namibia and Botswana, throughout Limpopo Province, Gauteng and along the eastern coastal plain as far south as Bredasdorp in the Western Cape Province (Bjornstad and Friis 1972a) (Figure 1.9). *Clivia* occurs from Limpopo Province along the escarpment to coastal areas of the Eastern Cape Province, with one species occurring in the Northern Cape (Figure 1.8). *Cryptostephanus* is found in East & Central Africa, Angola and northern Namibia (Nordal 1982; Figure 1.10).

The two genera *Cryptostephanus* and *Scadoxus* have species occurring in both southern and tropical Africa. Even though *Clivia* is restricted to southern Africa, like *Cryptostephanus* it favours a tropical habitat; *Cryptostephanus* is found in tropical east and southern Africa and *Clivia* but for one species, occurs along the east coast of southern Africa in afromontane and inland coastal forests.
The distribution and relationship patterns reflected by Haemantheae, with genera in both southern and tropical Africa, are considered highly relevant to the current study on which direction plant lineages have spread through Africa

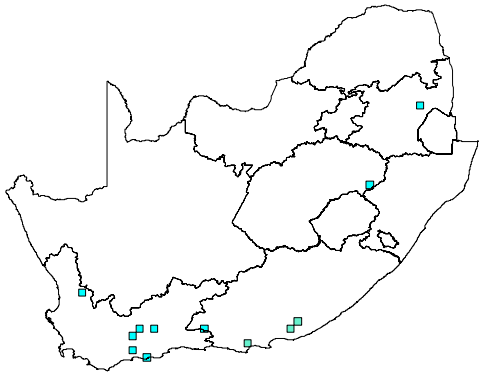


Figure 1.5: Distribution map of *Apodolirion* in South Africa, Swaziland and Lesotho

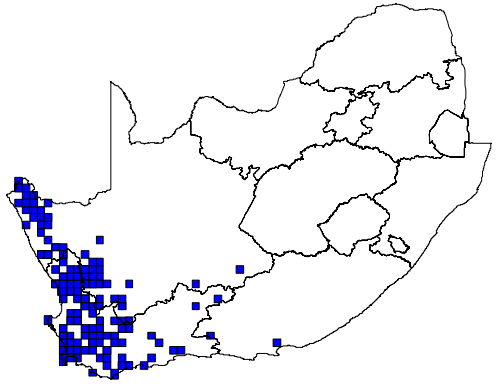


Figure 1.6: Distribution map of *Gethyllis* in South Africa, Swaziland and Lesotho

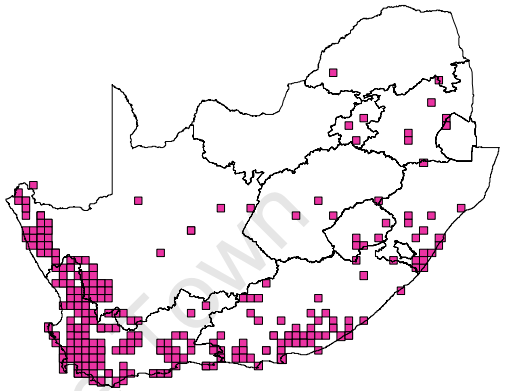


Figure 1.7: Distribution map of *Haemanthus* in South Africa, Swaziland and Lesotho

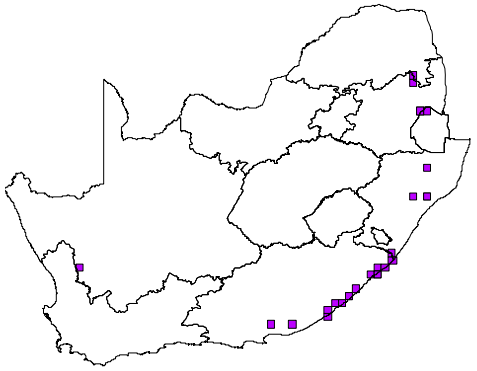


Figure 1.8: Distribution map of *Clivia* in South Africa, Swaziland and Lesotho

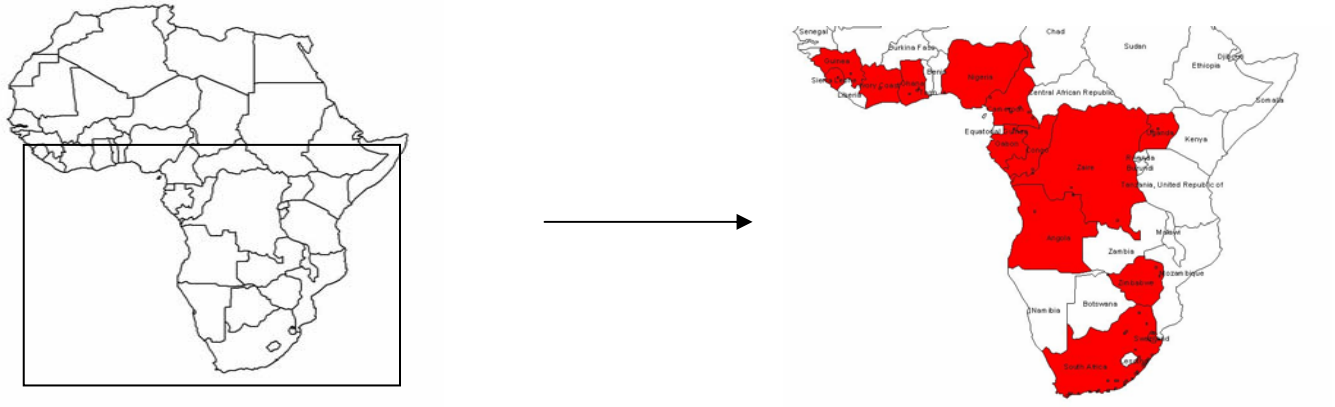


Figure 1.9: Distribution map of *Scadoxus* in Africa

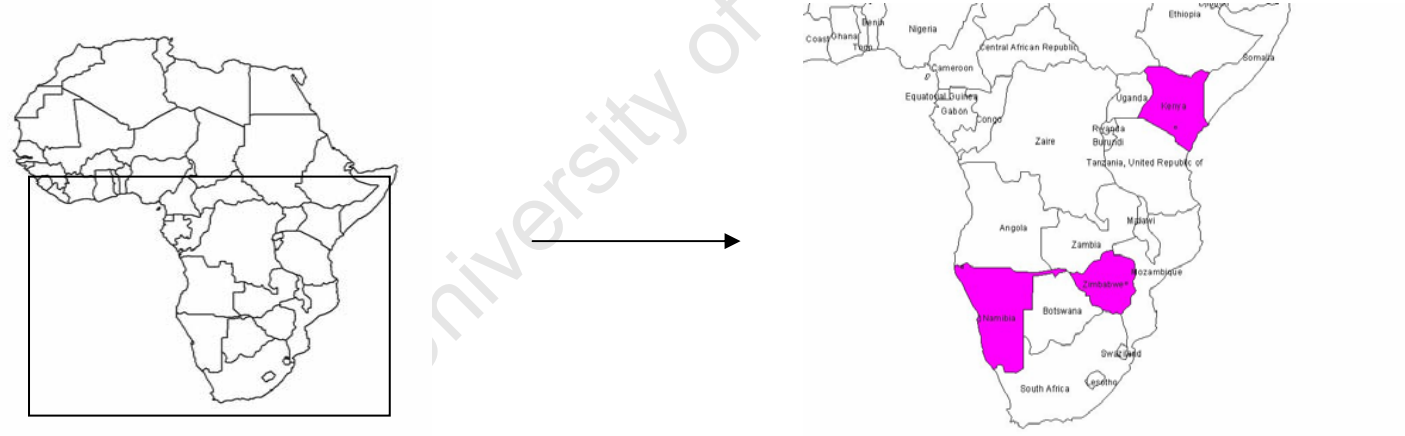


Figure 1.10: Distribution map of *Cryptostephanus* in Africa

Aridification in the Cape Region has been attributed to the separation of Antarctica from South America, which allowed the cold circum-Antarctic (Benguela) current to develop around 11-14 Ma ago. Such aridification may have been an important factor in initiating the transformation of the Miocene (26 Ma ago) subtropical forest to the fynbos vegetation of today (Coetzee 1983; Linder *et al.* 1992)

There is an east west gradient in the severity of the summer drought in southern Africa (Linder 2005). For several groups the greatest diversity and apparently the most recent radiations are situated in the more arid west (as reported for *Pelargonium* (Bakker *et al*. 2005), and for *Moraea* (Goldblatt *et al.* 2002)). Forest *et al.* (2007), in their study of phylogenetic diversity in the Cape Flora, found the western part of the Cape to be made up of relatively closely related genera, resulting from multiple radiations over at least the last 25 Ma. In the eastern part on the other hand, they found that the genera on average, are less closely related to one another. In addition Linder (2005) stated that there were no large recent radiations in the more mesic east.

Linder (2005) suggested that within the Cape Flora, species-poor clades are mostly restricted to forests and along permanent streams (e.g *Brabejum*, *Prionium*) and to fire protected habitats (e.g *Heeria*). These clades are largely absent from open, regularly burnt, heathy vegetation or open, succulent, semi-desert habitats dominated by species belonging to large species radiations.

Richardson *et al.* (2001) reported that diversification of the species-rich genus *Phylica* (a predominantly Cape-based genus of about 150 species) began approximately 7-8 Ma ago (late Miocene). Klak *et al.* (2004) showed recent (3.8-8.7 Ma ago) diversification in the Aizoaceae, which dominate the Succulent Karoo in terms of species numbers and density of coverage. Goldblatt *et al.* (2002) inferred from the dates on their phylogenetic tree that the main clades of the genus *Moraea,* one of the largest South African plant genera, diversified before the end of the Miocene (24-5.5 Ma).

Given that Haemantheae exhibits both species-rich and species-poor clades, this study investigates whether there is evidence of rapid diversification, as has been observed in other genera like *Phylica*. Determining whether these diversification events coincide

with a change in climate from moist, equable conditions that were prevalent in the Miocene, to summer dry conditions would contribute to further understanding of this group and the species-rich southern African flora more generally.

1.2. Phylogenetic relationships of Haemantheae

1.2.1. Generic level relationships

A previous study on Haemantheae by Conrad *et al.* (2006) with 43 taxa, representing all the genera and using DNA sequence data from five plastid regions, supported the stable classification of four (*Clivia*, *Cryptostephanus*, *Scadoxus* and *Haemanthus*) of the genera in this tribe. *Clivia* and *Cryptostephanus* resolved as reciprocally monophyletic sister taxa. *Scadoxus* and *Haemanthus* are monophyletic and resolved as sister clades to one another. The summer rainfall clade within *Haemanthus* is also monophyletic.

Furthermore, the analysis embedded *Apodolirion* within *Gethyllis,* although the species of *Apodolirion* are not monophyletic within *Gethyllis*. As currently circumscribed, *Apodolirion* is distinguished from *Gethyllis* by a difference in the attachment of the anthers. Anthers are uniformly basifixed in *Gethyllis* whereas the inner anthers of *Apodolirion* are basifixed and the outer are somewhat medifixed (Manning *et al*. 2002). Manning *et al*. (2002) note that this difference is arguably insufficient to separate the genera, but until strong evidence on their monophyly is available chose to maintain *Apodolirion* and *Gethyllis*. In this study a phylogeny of the tribe is reconstructed using both plastid and nuclear sequence data and a more representative sampling (64 taxa) of the tribe has been done (Chapter 2).

1.2.2. Phylogeography of Clivia

Indigenous to southern Africa, the genus *Clivia*, comprising six species, has been grown widely for more than 150 years in countries like the USA, China, Japan and Australia, where commercial production has been well developed.

Figure 1.11: Leaf and flower variation in *Clivia*

This genus is of great horticultural importance and is of particular interest owing to the discovery of a new species, *Clivia mirabilis,* in the winter rainfall Northern Cape Province of South Africa, in 2002. This prompted questions regarding the relationship of this taxon to the other five *Clivia* species, which are restricted to the summer rainfall regions of southern Africa: from the Eastern Cape northwards to Limpopo Province and Swaziland (Rourke 2002).

Wild populations of species occur in relatively small pockets, with distributions sometimes partially sympatric but often widely separated from each other. At present the genus appears to be in retreat, possibly caused by habitat fragmentation. *Clivias* are known to be intolerant of sunlight and thus Hammett (2002) suggested that the current distribution reflects the progressive destruction of forest vegetation which was formerly much more extensive than it is today. Snijman (2003) attributed the isolation of the winter-rainfall species of *Clivia* from those in the summer-rainfall regions to the establishment of a fire regime in the Cape region and recurring intense fires in recent times.

The impact of population fragmentation on genetic diversity depends on the level of gene flow among fragments. This in turn depends on the number of population fragments, dispersal ability of the species and geographic distribution or spatial pattern of populations (Frankham *et al.* 2002). *Clivia* is an ideal group for investigation due to its manageable size and also because it exhibits both a sympatric and an allopatric distribution. Haplotype networks will potentially give insights into

genetic variation within and between species as well as gene flow and direction of geneflow and spatial genetic structure within and between the populations.

C. nobilis occupies the southern-most part of the distribution range and *C. mirabilis* occupies the western-most part of the distribution range. In addition, *C. mirabilis* is also geographically isolated from the other five *Clivia* species which occur in partial sympatry. The distribution ranges of *C. miniata* and *C. caulescens* overlap in the Mpumalanga-Swaziland border region; *C. miniata* and *C. gardenii* overlap in KwaZulu-Natal; *C. miniata* and *C. nobilis* as well as *C. miniata* and *C. robusta,* overlap in the Eastern Cape.

In addition*, C. gardenii* and *C. robusta* also occur sympatrically in the Pondoland area (east coast of South Africa). The partial sympatry of these two species prompts questions about the discreteness of the populations in the areas of overlap, especially since *C. robusta* was considered to be a robust form of *C.gardenii* until its decription as a new species in 2004.

1.3. Thesis outline

This thesis presents a series of studies designed to address several questions pertaining to the tribe Haemantheae.

In Chapter 2, I undertake a phylogenetic analyis of the Haemantheae. Five plastid regions (*trnL* intron, *trnLF* intergenic spacer, *rps16* intron, *rpoBtrnC* intergenic spacer, *psbAtrnH* intergenic spacer) and one nuclear (ITS) region are used to determine intergeneric relationships and re-assess the existing classification. In addition to assessing generic delimitations, results from the phylogenetic recontruction were used to assess the evolution of selected traits in the tribe, as well as to investigate whether these are associated with any species-rich clades.

In Chapter 3, Dispersal-Vicariance Analysis (DIVA; Ronquist 1996, 1997) is used to better understand the biogeographical patterns inherent in the parsimony topology. An attempt to date the tribe is also undertaken using molecular dating techniques. Dating

is useful for many evolutionary investigations. Vinnersten and Bremer (2001) explored historical biogeographical questions in major clades of the Liliales and Linder (2003) attempted to relate speciation rate changes to paleo-environmental changes using molecular dating techniques.

As there has been no previous dating of the tribe, an estimated divergence time of Haemantheae will be presented. These data will also be used to consider whether there is evidence of rapid diversification in the species-rich genera *Haemanthus* and *Gethyllis*, and if so whether the rate of acceleration coincides with a change in climate from moist, equable conditions to dry, summer conditions.

In Chapter 4, phylogeographic techniques are used to investigate the spatial and temporal speciation in *Clivia*. Inter-and intra-specific variation of all six *Clivia* species across the distribution range is investigated and includes a haplotype network reconstruction of 87 *Clivia* individuals using two plastid noncoding sequence regions. In addition, the data generated will be used to establish whether diversification in the lineage is the result of allopatric speciation induced by habitat fragmentation of their forest habitats as hypothesised by Hammett (2002). The newly described *Clivia robusta* is included to elucidate its relationship with regard to the other five *Clivia* species, especially *C. gardenii*. In 2004 *C. robusta* was described (Murray *et al.* 2004) using nondiscrete morphological characters and distribution as criteria, even though the distribution of *C. gardenii* and *C. robusta* overlap to some extent. The controversy surrounding the description of *C. robusta* as a new species, brings into question the recognition of these two elements as discrete species when the interconnectedness of these two species is evident.

The final chapter of the thesis is a General Discussion in which I synthesise the outcomes from the previous three chapters and encompasses taxonomic recommendations and an hypothesis of the evolution of Haemantheae.

CHAPTER 2. MOLECULAR SYSTEMATICS AND CHARACTER EVOLUTION OF THE TRIBE HAEMANTHEAE (AMARYLLIDACEAE) USING CHLOROPLAST AND NUCLEAR SEQUENCE DATA

2.1. Introduction

The tribe Haemantheae, consisting of six genera, is one of nine tribes (sensu Dahlgren *et al.* 1985) in the monocotyledonous family Amaryllidaceae.

Various molecular studies have been carried out including the cladistic analysis of plastid (*rbcL* and *trnL-F*) sequence data by Meerow *et al.* (1999b), to establish the monophyly of Haemantheae (95% bootstrap support in the combined analysis). Their analyses did not support the recognition of Gethyllideae as a distinct tribe, as *Apodolirion* and *Gethyllis* resolve as sister taxa, firmly embedded within Haemantheae. In a study of 31 taxa of the Amaryllidaceae, using *matK* sequence data, Ito *et al.* (1999) used three genera (*Scadoxus*, *Haemanthus* and *Clivia*) as representatives of the tribe. These genera formed a monophyletic group (81% bootstrap support) with *Clivia* sister to a clade comprising both *Scadoxus* and *Haemanthus*.

Other studies of the family Amaryllidaceae include investigations by Meerow *et al*. (2000a), Meerow and Snijman (2001), Meerow *et al.* (2002) and Meerow *et al.* (2003) using sequence data from the choroplast *trnL-F* and nuclear *ITS* regions. Although there are several studies on other tribes in the Amaryllidaceae (eg. Amaryllideae (Snijman and Linder 1996 and Meerow and Snijman 2001) and Hymenocallideae (Meerow *et al.* 2002)), a recent study by Meerow and Clayton (2004) is the only one investigating Haemantheae. In their investigations of 19 species, representing all the genera of the tribe*, trnL-F* and nrDNA *ITS* sequence data were used. The most resolved and best-supported tree produced by the combined analysis, divided the tribe into two main clades with *Clivia* and *Cryptostephanus* both monophyletic and placed as sister genera to each other in the smaller clade. The second clade is divided into two subclades with *Haemanthus* and *Scadoxus* comprising the one, and *Apodolirion* and *Gethyllis* comprising the other.

Haemanthus and *Scadoxus* also resolved as sister genera in the analysis of Meerow *et al.* (1999b). In the past they have been treated as one genus (Baker 1888, Baker 1896, Bjornstad and Friis 1972a), but a cladistic analysis of 25 morphological characters (Nordal and Duncan 1984) upheld their delimitation as separate genera. Meerow and Clayton (2004) found the sister relationship of *Scadoxus* and *Haemanthus* to be well supported by the morphological synapomorphy of brush-like inflorescence, associated with the reduction in perianth size (in all species) and the dominance of the spathe bracts during anthesis (in at least some species of each genus (Nordal and Duncan 1984)).

As currently circumscribed, *Apodolirion* is distinguished from *Gethyllis* by a difference in the attachment of the anthers. In *Gethyllis* the anthers are uniformly basifixed whereas in *Apodolirion* the inner anthers are basifixed and the outer are somewhat medifixed (Manning *et al*. 2002). Although this difference is arguably insufficient to separate these genera, Manning *et al*. (2002) have chosen to maintain both *Apodolirion* and *Gethyllis* until a rigorous analysis of their relationship was done. The analysis of Conrad *et al.* (2006) showed species of *Apodolirion* to be embedded within *Gethyllis*, and the exemplars used did not resolve as monophyletic within *Gethyllis*. *Apodolirion* and *Gethyllis* share many specialised characters: solitary flowers, subterranean ovaries and elongated berries with numerous seeds. They also share the same basic chromosome number x=6 (Vosa 1986), which is unique in the tribe. The seeds of these two genera are small and hard, in contrast to the larger, water-rich, more or less fleshy seeds of the other genera. The scape remains inside the bulb of *Gethyllis* and *Apodolirion*, and both have fused spathe bracts (Meerow and Clayton 2004). In 1963, Traub expressed doubt about maintaining *Apodolirion* and *Gethyllis* as distinct genera, an argument also taken up to some extent by Hilliard and Burtt (1973). The ecological studies of Wilsenach (1965) showed little variation among the karyotypes of representatives from both genera (Meerow and Clayton 2004).

Previous studies of the Amaryllidaceae that included Haemantheae have comprised a limited number of samples only. Here the sampling is significantly increased (62 taxa) making this the most comprehensive study of Haemantheae to date. The objectives of

this study are to 1) reconstruct the phylogeny of the tribe using plastid and nuclear sequence data and 2) reassess Meerow and Clayton's (2004) generic classification of Haemantheae, with emphasis on the placement of *Apodolirion*.

In addition, a number of morphological traits within the tribe are investigated. The genera of the Haemantheae exhibit various interesting traits. All but three genera of the family Amaryllidaceae form tunicate bulbs, and all three exceptions (*Clivia*, *Cryptostephanus* and *Scadoxus*) occur in the tribe Haemantheae (Meerow and Snijman 1998). The primary function of geophytism is that of water storage and *Haemanthus* and *Gethyllis*, the bulbous genera, both occur mainly in the arid west regions of South Africa, where they are species-rich.

The use of micromorphological traits (e.g.leaf pubescence) as taxonomically significant characters is widely accepted (Weighlin 2002). Within the species-rich genus *Haemanthus* two states are recognised: hairs absent and single hairs. The question arises whether leaf pubescence is associated with species that occur in a specific rainfall region or whether it has evolved independently in different species, without any apparent link to climate.

A subterranean ovary, present in *Apodolirion* and *Gethyllis*, is also unique within the tribe (Meerow and Snijman 1998).

In total 15 morphological traits are investigated to identify potential morphological synapomorphies for the recovered clades and to explore whether particular plant traits appear to be associated with the species-rich clades.

2.2. Materials and Methods

2.2.1. Sampling

Three of the six *Apodolirion* species, two of the four *Cryptostephanus* species, 20 of the 32 *Gethyllis* species (two of which are undescribed), five of the nine *Scadoxus* species, five *Clivia* species and 21 of 22 *Haemanthus* species were included in the analysis. Thus 72% of all the species in the tribe were sampled.

The following five plastid regions: *trnL* intron*, trnL-F* intergenic spacer region, *rps16* intron, *rpoB-trnC* spacer region and *psbA-trnH* spacer region, as well as the *ITS* (internal transcribed spacer) nuclear region were sequenced for the 62 ingroup taxa of the Amaryllidaceae and two outgroup taxa.

Chloroplast and nuclear regions have proved to be a valuable source of phylogenetic information in angiosperms. Bakker *et al.* (1999) used the *trnL-F* intergenic spacer region for studies on *Pelargonium*, Reeves *et al.* (2001) used it for the molecular systematics of the Iridaceae, Sang *et al.* (1997) investigated *Paeonia* using the *trnL*-F and *psbA-trnH* regions and Ohsaka and Ohnishi (2000) used *rpoB-trnC* in the study of *Fagopyrum.* A phylogenetic analysis of the Primulaceae was carried out using *ITS* DNA sequence data (Martins *et al.* 2003), Trift *et al.* (2004) used *ITS, rps16* and *trnL-F* for *Dionysia* and McKenzie *et al.* (2006) used five chloroplast regions, among them *psbA-trnH, rps16,* as well as *ITS* in their study of Arctotidinae a subtribe of the Asteraceae.

Sprekelia and *Calostemma* were designated as the outgroup, based upon results of a larger analysis of DNA sequence data for the Amaryllidaceae (Meerow *et al.* 1999). Voucher and sequence data information are listed in Table 2.1.

2.2.2. Choice of Outgroup

Amaryllis, a genus from the African tribe Amaryllideae, has been used as an outgroup in several studies including the recent study of Meerow and Snijman (2006). In numerous investigations (Meerow *et al.* 2000b, Meerow *et al.* 1999a, Meerow *et al.* 1999b), the tribe Amaryllideae is placed as sister group to the rest of the Amaryllidaceae (including Haemantheae). This may imply that the Amaryllideae are not as closely related to the Haemantheae as are other tribes (like Calostemmateae) of the family.

In the combined *trnLF* and *rbcL* analyses of the Amaryllidaceae, Meerow *et al.* (1999b), reported the monophyly of Haemantheae and the sister relationship of Calostemmateae and Haemantheae. It may be deduced therefore that Calostemmateae

and Hippeastreae are more closely related to Haemantheae than Amaryllideae, and for this reason the genera *Calostemma* (Calostemmateae) and *Sprekelia* (Hippeastreae) were selected as outgroup taxa.

Calostemma, an Australasian genus, is currently being cultivated in the greenhouses in the Kirstenbosch Botanical Garden and leaf material was available for DNA extraction. *Sprekelia* is grown as an ornamental in South Africa and material was also easily available for DNA extraction. Three other genera *Lycoris* (Lycorideae), *Sternbergia* and *Narcissus* were also identified as potential outgroup genera but due to difficulties with amplification and sequencing only sequence data from *Calostemma* and *Sprekelia* were used.

Table 2.1: Haemantheae and outgroup species used in molecular analyses, with sequence data collected and voucher information. Where pressed herbarium vouchers were not available, digital images of the living plants were deposited in NBG. "NBG" indicates Compton Herbarium, South Africa with accession number in brackets; "ex hort. NBG" indicates Kirstenbosch Botanical Garden, South Africa with garden accession number; "-" indicates no sequence available; "partial sequence" indicates only a partial sequence available

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Apodolirion cedarbergense D.Müll.-Doblies	Dorse & Kragh s.n.; (NBG 160974)	√	√	√	√	√
Apodolirion lanceolatum (Thunb.) Baker	Nel s.n.; (NBG 90695)	√	√	√	√	√
Apodolirion macowanii Baker	Dold 447; (NBG 194539)	√	√	√	√	partial sequence
Clivia caulescens R.A. Dyer	Winter; ex hort. NBG 573/99	√	√	√	√	partial sequence
Clivia gardenii Hook.	Winter; ex hort. NBG 434/99	√	√	√	partial sequence	√
Clivia miniata (Lindl.) Regel	Winter; ex hort. NBG 442/99	√	√	√	partial sequence	partial sequence
Clivia mirabilis Rourke	Rourke 2220; (NBG 184720)	√	√	√	√	√
Clivia nobilis Lindl.	Winter; ex hort NBG 735/96	√	√	√	√	√

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Cryptostephanus haemanthoides Welw.	Luke & Luke 6398; ex hort. Quentin Luke, Kenya	√	√	√	√	partial sequence
Cryptostephanus vansonii Verdoorn	Duncan s.n.; (NBG 196209)	√	√	√	√	√
Gethyllis afra L.	Graham s.n.; (NBG 190585)	√	√	√	√	√
Gethyllis barkerae D.Müll.-Doblies	Liltved s.n.; (NBG)	√	√	√	√	partial sequence
Gethyllis britteniana Baker subsp. *britteniana*	Perry 1062; (NBG 134525)	√	√	√	√	partial sequence
Gethyllis britteniana subsp. *bruynsii* D.Müll.-Doblies	Liltved s.n.; (NBG)	√	√	√	√	partial sequence
Gethyllis campanulata L.Bolus	Snijman 1262; (NBG)	√	√	√	√	partial sequence
Gethyllis cavidens D.Müll.-Doblies	Liltved s.n.; (NBG)	√	√	√	√	partial sequence
Gethyllis ciliaris (Thunb.) Thunb. subsp. *ciliaris*	Duncan 364; ex hort. NBG 74/93	√	√	√	√	√
Gethyllis ciliaris subsp. *longituba* (L.Bolus) D.Müll.-Doblies	Liltved s.n.; (NBG)	√	√	√	√	partial sequence

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Gethyllis gregoriana D.Müll.-Doblies	Ex hort. E.G.H. Oliver	√	√	√	√	√
Gethyllis lanuginosa Marloth	Van Jaarsveld 4377; (NBG 190598)	√	√	√	√	partial sequence
Gethyllis lata L.Bolus subsp. *lata*	Liltved s.n.; (NBG)	√	√	√	√	partial sequence
Gethyllis lata subsp. *orbicularis* D.Müll.-Doblies	Lavranos & Bleck 24199; (NBG 147291)	√	√	√	√	√
Gethyllis sp.2	Liltved s.n.; (NBG)	√	√	√	√	√
Gethyllis linearis L.Bolus	Snijman 857; (NBG 151469)	√	√	√	√	partial sequence
Gethyllis marginata D.Müll.-Doblies	Liltved s.n.; (NBG)	√	√	√	√	partial sequence
Gethyllis multifolia L.Bolus	Townsend 130; (NBG 191659)	√	√	√	√	partial sequence
Gethyllis namaquensis (Schönl.) Oberm.	Manning 8/2000; (NBG 191661)	√	√	√	√	√

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Gethyllis sp.1	Liltved s.n.; (NBG)	√	√	√	√	partial sequence
Gethyllis roggeveldensis D.Müll.-Doblies		√	√	√	√	√
Gethyllis transkarooica D.Müll.-Doblies	Snijman 1907; (NBG 194552)	√	√	√	√	√
Gethyllis undulata Herb	Liltved s.n.; (NBG)	√	√	√	√	√
Gethyllis verrucosa Marloth	Liltved s.n.; (NBG)	√	√	√	√	√
Gethyllis verticillata R.Br. ex Herb.	Tait 76; ex hort. NBG 624/96	√	√	√	√	√
Gethyllis villosa (Thunb.) Thunb.	Williamson 4426; ex hort. NBG 768/91	√	√	√	√	partial sequence
Haemanthus albiflos Jacq.	Winter 498; ex hort. NBG 571/97	partial sequence	√	√	√	√
Haemanthus amarylloides Jacq. subsp. *amarylloides*	Snijman 599; (NBG 122774)	partial sequence	√	√	√	√

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Haemanthus amarylloides subsp. *polyanthus* Snijman	Snijman 1784; (NBG 176726)	√	√	√	√	√
Haemanthus barkerae Snijman	Snijman 2252; (NBG 133554)	√	√	√	√	√
Haemanthus canaliculatus Levyns	Snijman 1266; (NBG 167551)	√	√	√	√	√
Haemanthus carneus Ker Gawl.	Wisura1063; (NBG 8991)	√	√	√	√	√
Haemanthus coccineus L.	Snijman 1792: (NBG 176727)	√	√	√	√	√
Haemanthus crispus Snijman	Snijman 1771; (NBG 176725)	√	√	√	√	√
Haemanthus dasyphyllus Snijman	Snijman 1299; (NBG 151994)	√	√	√	√	√
Haemanthus deformis Hook. f.	Nichols 800; ex hort. NBG 1075/84	√	√	√	√	√
Haemanthus graniticus Snijman	Snijman 1782; (NBG 176730)	√	√	√	√	√

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Haemanthus humilis Jacq. subsp. *hirsutus* (Baker) Snijman	Van Jaarsveld 1834; ex hort. NBG 479/77	√	√	√	√	√
Haemanthus humilis Jacq. subsp. *humilis*	Van Jaarsveld 3325; (NBG 125072)	√	√	√	√	√
Haemanthus lanceifolius Jacq.	Snijman 1797a; (NBG 179724)	√	√	√	√	√
Haemanthus montanus Baker	GR Nichols 999; ex hort. NBG 672/88	√	√	√	√	√
Haemanthus namaquensis R.A.Dyer	Van Berkel 294; (NBG 121548)	√	√	√	√	√
Haemanthus nortieri Isaac	R. Jangle; ex hort. NBG 6/98	√	√	√	√	√
Haemanthus pauculifolius Snijman & A.E. van Wyk	R.J. Symonds; ex hort. NBG 3/98	√	√	√	√	√
Haemanthus pubescens L.f. subsp. *leipoldtii* Snijman	Snijman 432; (NBG 121704)	√	√	√	√	√
Haemanthus pubescens L.f. subsp. *pubescens*	Snijman 1787; (NBG 176731)	partial sequence	√	√	√	√

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Haemanthus pumilio Jacq.	Snijman 668; (NBG 126814)	√	√	√	√	√
Haemanthus sanguineus Jacq.	Snijman 210; (NBG 120915)	√	√	√	√	√
Haemanthus tristis Snijman	Manning 2242a; (NBG)	√	√	√	√	√
Haemanthus unifoliatus Snijman	Snijman 181; (NBG 122498)	√	√	√	√	√
Scadoxus membranaceus (Baker) Friis & Nordal	Roux 561; ex hort NBG 145/82	√	√	√	√	√
Scadoxus multiflorus (Martyn) Raf. subsp. *katharinae* (Baker) Friis & Nordal	Snijman & Manning 1878, (NBG 182877)	√	√	√	√	√
Scadoxus nutans (Bjornstad & Friis) Friis & Nordal	Ex hort R. Saunders; Nyanga, Zimbabwe	√	√	√	√	√
Scadoxus pole-evansii (Oberm.) Friis & Nordal	Ex hort R. Saunders; Nyanga, Zimbabwe	√	√	√	√	√

	Voucher/ accession	DNA sequence data				
		plastid regions				nuclear region
		rpoB-trnC intergenic spacer	*trnL-F* region	*rps16* intron	*psbA-trnH* intron	*ITS* region
Scadoxus puniceus (L.) Friis & Nordal	Wisura 1120; ex hort NBG 197/70	partial sequence	√	√	√	√
Calostemma purpureum R.Br.	Gibson 51; (NBG 201371)	√	√	–	√	partial sequence
Sprekelia sp.	*Ex hort* John Manning	√	√	√	√	√

2.2.3. DNA extraction

Total genomic DNA was extracted from 1.0 g fresh leaf or flower tissue or 0.2-1.0 g silica dried leaf or flower material using the 2X CTAB method of Doyle and Doyle (1987). Twenty to fifty nanograms of total genomic DNA were used as a template for Taq-mediated amplification. One hundred *µl* reactions contained Promega magnesium free thermophilic buffer (50nM KCl, 10mM Tris-HCl, 01% Triton X100), 3mM $MgCl_2$, 0.004% BSA (Savolainen *et al.* 1995), 0.2mM each dNTP, 100ng of each primer and 2.5U Taq polymerase.

2.2.4. PCR and DNA Sequencing

DNA amplification was carried out in a Gene Amp PCR System 9700 (Applied Biosystems Inc) with programmes tabulated in table 2.2.

Amplification of the *rpoB-trnC* intergenic spacer region was achieved using primers *rpoB*5' and *trnC*5' (Ohsaka and Ohnishi 2000). In instances where the region could not be amplified, internal primers (550F: 5' - ATT AAG TAC ATG CCG ATA CG – 3' and 550R: 5' – CGT ATC GGC ATG TAC TTA AT – 3') were designed to allow amplification of the region in two non-overlapping parts. Primers *rps*16F and *rps*162R (Oxelman *et al.* 1997) were used to amplify and sequence the *rps*16 intron. Primers 'c' and 'f' (Taberlet *et al*. 1991) were used to amplify the adjacent *trnL* intron and *trnL-F* intergenic spacer between the *trnL* and *trnF* exons. Primers *psbAF* and *trnHR* (Sang *et al*. 1997) were used to amplify the *psbA-trnH* intergenic spacer region and internal primers (570F: 5' – ATG TCC AAT AGA ATA TCT CG – 3'and 570R: 5' – CGA GAT ATT CTA TTG GAC AT – 3') were used to amplify the region in two parts where amplification of the entire region was unsuccessful. For the nuclear region the primers *AB101F* and *AB102R* (Baldwin *et al.* 1995) were used. If the complete region could not be amplified successfully, then internal primers *ITS* 5 and *ITS* 2 and *ITS* 3 and *ITS* 4 were used to amplify regions *ITS* 1 and *ITS* 2 respectively (Table 2.2).

For each of the above regions amplification primers were used as sequencing primers. Amplification products were purified using Qiaquick (Qiagen) spin columns

according to manufacturer's instructions and directly sequenced on an ABI 377 automated sequencer using standard dye-terminator chemistry following manufacturer's protocols (Applied Biosystems).

For assembly and editing of the complementary strands Sequencher version 4.1 (GeneCodes Inc.) was used and sequences were aligned by eye. Potentially parsimony-informative indels were coded as present (T)/absent (A) characters using the 'simple indel coding method' of Simmons and Ochoterena (2000). Parsimony and Bayesian phylogenetic analyses were performed using the software package PAUP* for Macintosh (v.4.0b10 Swofford 2001) and Mr Bayes v.3.1.1(Huelsenbeck and Ronquist 2001), respectively. The following matrices, each containing 64 taxa and including two outgroups, were analysed using parsimony and Bayesian inference:

- Individual matrices of the five plastid regions and one nuclear region
- Combined plastid matrix containing all plastid regions
- Combined plastid and nuclear matrix containing a combined data set of the plastid regions and a nuclear region

Table 2.2: Gene regions, programmes, primers and internal primers (where applicable) used in the study

Gene region	No of cycles	Programme	Primers	Internal primers
rpoB-trnC spacer	30 cycles First 5 cycles . Next 25 cycles	 denaturation: 94°C for 1 min. annealing: 52°C for 1 min. (with a decrease of 1 degree/cycle) extension: 72°C for 1 min. denaturation: 94°C for 1 min. annealing: 48°C for 1 min extension: 72°C for 1 min	*rpoB*5' and *trnC*5' (Ohsaka and Ohnishi 2000)	550F: 5' - ATT AAG TAC ATG CCG ATA CG – 3' 550R: 5' – CGT ATC GGC ATG TAC TTA AT – 3'
the *trnL-F* region	30 cycles	denaturation: 94°C for 1 min. annealing: 48°C for 1 min. extension: 72°C for 1 min.	Primers 'c' and 'f' (Taberlet *et al*. 1991)	
rps16 intron	30 cycles	denaturation: 94°C for 1 min. annealing: 48°C for 1 min. extension: 72°C for 1 min.	*rps*16F and *rps*162R (Oxelman *et al.* 1997)	
psbA-trnH spacer	28 cycles	denaturation: 94°C for 1 min. annealing: 52°C for 1 min. extension:72°C for 1 min.	*psbAF* and *trnHR* (Sang *et al*. 1997)	570F: 5' – ATG TCC AAT AGA ATA TCT CG – 3' 570R: 5' – CGA GAT ATT CTA TTG GAC AT – 3'
ITS region	30 cycles	denaturation: 94°C for 1 min. annealing: 52°C for 30s extension:72°C for 1 min.	*AB101F* and *AB102R* (Baldwin *et al.* 1995)	*ITS* 5 and *ITS* 2 *ITS* 3 and *ITS* 4

2.2.5. Phylogenetic Analysis

2.2.5.1. Parsimony

The data matrices were analysed using 1000 replicates of random taxon addition in order to maximize the chance of finding multiple islands of equally most parsimonious trees, tree bisection-reconnection (TBR) branch swapping, with MULPARS on. All character transformations were treated as equally likely (Fitch parsimony; Fitch 1971). A limit of five trees was set for each replicate to reduce time spent swapping on the large number of trees at or near the optimum. The consistency index (CI) and retention index (RI) were calculated for each analysis.

To assess internal support, 1000 bootstrap replicates were performed using simple taxon addition and TBR branch swapping with a tree limit of five trees per replicate. Only those groups with > 50% support, with the following categories: weak 50-74%, moderate 75-84% and strong 85-100% are indicated.

2.2.5.2. Partition Homogeneity Test

Congruence between the different data sets was tested using the partition homogeneity test (Farris *et al.* 1995) with 100 replicates, full heuristic search and random taxon addition. The partition homogeneity test is also referred to as the incongruence length difference (ILD) test. The power of the congruence tests stems from the assumption that there is a single phylogeny and that each data set being analysed should reflect that history, coupled with the improbability of obtaining similar phylogenies using chance alone. Congruence therefore validates a method of phylogenetic inference and a particular source of data (Page and Holmes 1998). $P \geq 0.05$ suggests congruency between the matrices and $P < 0.05$ suggests incongruence.

2.2.5.3. Bayesian Analysis

Analyses were carried out on the same two data sets used for parsimony analyses (i.e. combined plastid matrices and combined plastid and nuclear matrices) using the general time-reversal (GTR) model of DNA substitution and a discrete gamma distribution model of evolution with four rate classes. In order to explore the parameter space more thoroughly, Markov chain Monte Carlo simulations were run with four incrementally heated chains. Parameter values were not defined *a priori*, but

instead treated as unknown variables with uniform priors using the default settings. Using a random starting tree 5 000 000 generations were run. The Markov chain was sampled at intervals of 1000 generations to obtain 50 000 sample points. In order to test for convergence in the results, the Bayesian analyses were carried out multiple times, with models unlinked across partitions.

Stationarity was determined by plotting the natural logarithm of the likelihood (-inL) against generation for each analysis. Samples collected prior to stationarity and convergence, were discarded, as they contain no useful information concerning parameter values (Huelsenbeck and Ronquist 2001). The equilibrium samples were then used to generate 50% majority rule consensus trees in PAUP*, with the percentage of samples containing a particular group representing that group's posterior probability. These are known as the P values, and P ≥ 95% was considered evidence of significant support for a group. Groups contained in 90-94% of all the sampled trees were considered to be strongly supported (Leache and Reader 2002).

2.2.6. Character analysis

2.2.6.1. Morphological Data

Character optimization examines the evolution of characters and homology by plotting the characters on a tree obtained through analysis of combined molecular data or by scoring the morphological data. It allows reference of, for example how many times a character has evolved or whether there are any taxa in which the character has been lost.

Stevens (1991) discussed the delimitation of character states and the different approaches and opinions held in the literature as regards the treatment of characters as quantitative, qualitative, discrete and continuous data. He observed that many characters that are described as being either present or absent may actually represent quantitative variation.

In this study only discrete qualitative (e.g. presence or absence of phytomelan) and discrete quantitative characters (e.g. chromosome and stamen number) were used but no continuous quantitative characters. Where characters were variable in a species

they were coded as absent and present but the characters of leaf pubescence and inflorescence type was coded as multistate characters in terms of their variation (hairs: absent, single hairs, and T-shaped trichomes; inflorescence: brush type, tubular-flowered, and simple 1-flowered).

Meerow and Clayton (2004) investigated six characters in their investigation of 19 species (representing all genera) of the Haemantheae. This study re-examines five of the six characters of Meerow and Clayton (2004), and supplements them with 10 more morphological characters. A total of 15 characters were compiled using the literature (Dahlgren *et al.* 1985; Esler *et al*. 1999; Meerow 1995; Meerow and Clayton 2004; Meerow *et al.* 1999; Meerow *et al.* 2000b; Nordal and Duncan 1984; Snijman 1984; Snijman 2000; Telford 1987) and selected on the basis on their proposed taxonomic value in the classification system of Meerow and Clayton (2004).

Character descriptions and coding are provided in Appendix A and the matrix in Appendix B.

2.2.6.2. Choice of Tree

One of the most parsimonious trees of the combined plastid and nuclear analysis was used to optimize the selected characters, as it included data from both the plastid and nuclear regions. The sampling of this analysis included 64 taxa representing all six genera of the tribe and two outgroup taxa.

2.2.6.3. Character Optimization

The mapping of the characters was performed using PAUP* version 4.0b10 (Swofford 2002) with ACCTRAN character optimization, which favours reversals rather than parallelisms and with MacClade v4.01 (Maddison and Maddison 2001), which treats discrepancies between ACCTRAN and DELTRAN optimizations as ambiguities.

2.3. Results

2.3.1. Parsimony Analysis

***trnL-F* matrix**: Of the 873 characters included in the analysis, 75 (8.6%) were variable and 46 (5.3%) parsimony informative. Analysis of *trnL-F* sequences with equal weights resulted in 4910 equally parsimonious trees of 153 steps with CI and RI, 0.856 and 0.889, respectively. The strict consensus tree is illustrated in Figure 2.1 with bootstrap percentages indicated in bold below the branches, but groups with bootstrap percentages < 50% have nothing indicated. A poorly resolved tree was recovered with several polytomies. The winter rainfall *Haemanthus* species (with the exception of *H. montanus*) and the summer rainfall *Haemanthus* species (93% bootstrap support) formed two clades. *Cryptostephanus* (94% bootstrap support) as well as *Scadoxus* (90% bootstrap support) were monophyletic and *Gethyllis* and *Apodolirion* were placed in one clade with weak bootstrap support (55%).

***rps16* matrix**: Amplification of one of the outgroup taxa, *Calostemma purpurea*, was unsuccessful and this genus was therefore excluded. 809 characters were included in the analysis with 53 (6.6%) variable and 37 (5.7%) parsimony informative. Of the 4245 equally parsimonious trees recovered of 109 steps, the CI was 0.844 and RI 0.934. As with the *trnL-F* analysis, a polytomy was recovered in the strict consensus tree (Figure 2.2). *Clivia* and *Cryptostephanus* were unresolved and one clade, with two poorly resolved subclades, was recovered. One of the subclades consisted of a weakly supported (58% bootstrap support) monophyletic *Scadoxus* and an unresolved *Haemanthus* with the summer rainfall genera, except *H. montanus*, forming a distinct clade as it did in the *trnL-F* analysis, although it was weakly supported (60% bootstrap support) here. *Apodolirion* and *Gethyllis* formed the second subclade (91% bootstrap support) with *Apodolirion* firmly embedded within *Gethyllis*.

***rpoB-trnC* matrix**: Of the 917 characters included in the analysis, 70 (7.6%) were variable and 62 (6.8%) parsimony informative. 3550 trees were recovered with tree length 180 and CI and RI, 0.806 and 0.875, respectively. The strict consensus tree (Figure 2.3) formed two clades, with *Cryptostephanus* unresolved. The smaller clade

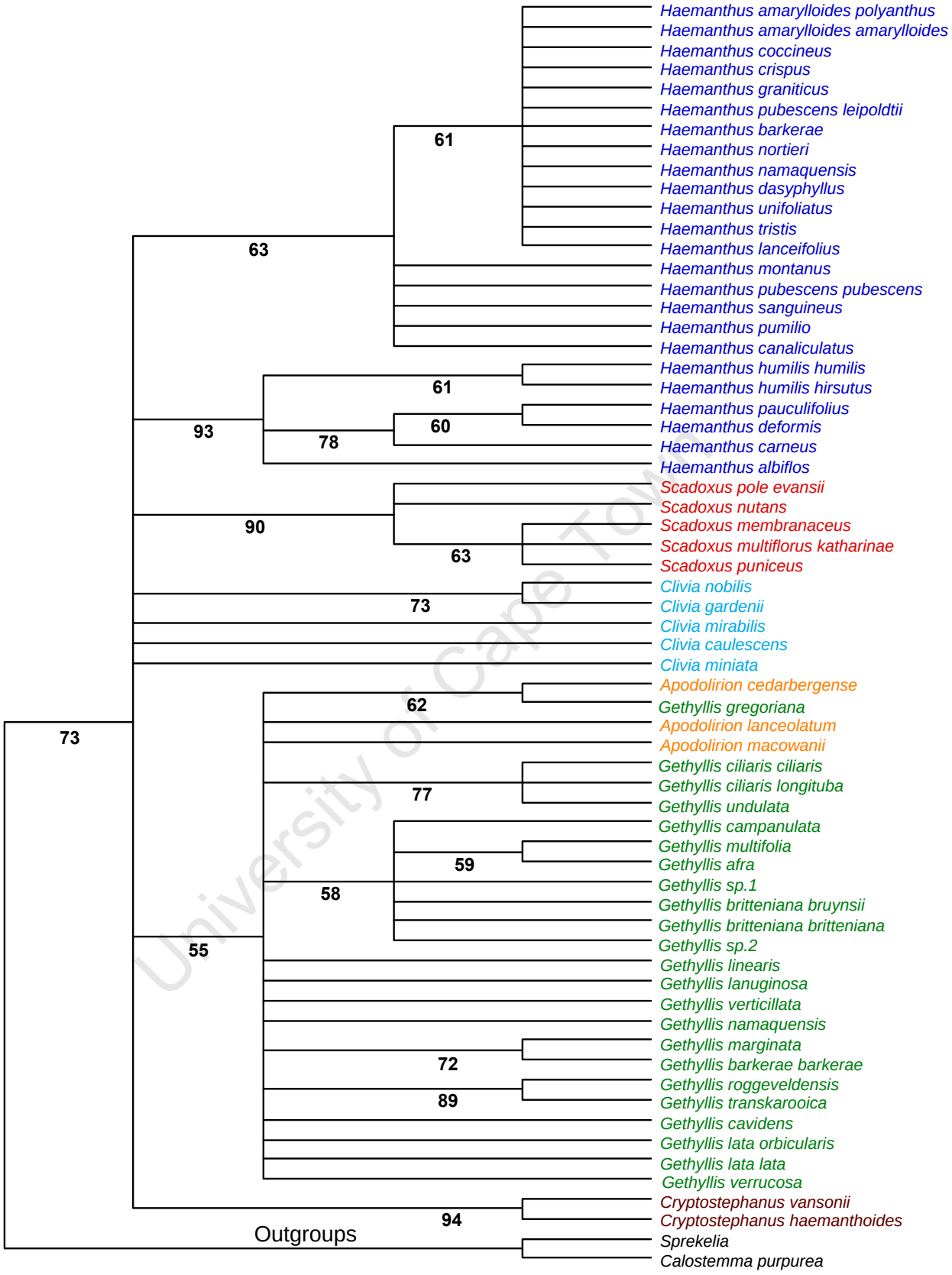


Figure 2.1: Strict consensus tree (CI = 0.856; RI = 0.889) of 4910 equally parsimonious trees (length = 153) using the *trnL-F* data set with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

Figure 2.2: Strict consensus tree (CI = 0.844; RI = 0.934) of 4245 equally parsimonious trees (length = 109) using the *rps16* data set with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

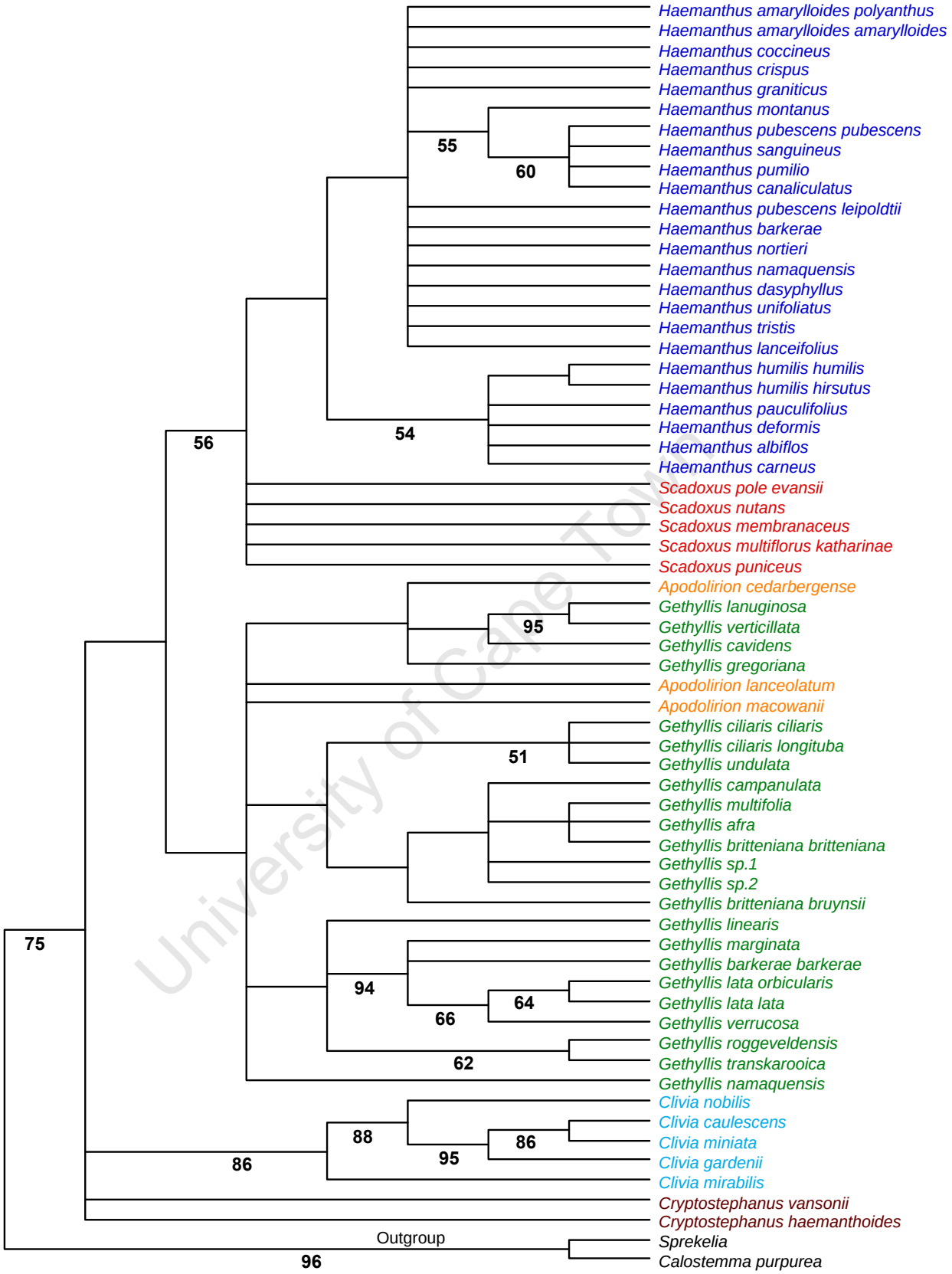


Figure 2.3: Strict consensus tree (CI = 0.806; RI = 0.875) of 3550 equally parsimonious trees (length = 180) using the *rpoB-trnC* data set with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

resolved a monophyletic *Clivia* with 86% bootstrap support. *Apodolirion* and *Gethyllis* comprised one of the subclades (no support indicated) and *Haemanthus* and *Scadoxus* make up the second subclade with poor (56%) support indicated. The summer rainfall *Haemanthus* species, with the exception of *H. montanus*, formed a distinct clade as in the *trnL-F* and *rps16* analyses (54% bootstrap support).

***psbA-trnH* matrix**: Of the 562 characters included in the analysis, 37 (6.6%) were variable and 27 (4.8%) parsimony informative. 4895 trees were recovered of tree length 96, CI 0.740 and RI 0.832. The poorly resolved strict consensus tree is shown in Figure 2.4. There were two clades worth mentioning. The first is the clade of the summer rainfall *Haemanthus* species (except *H. montanus*) , which formed a clade as in all the previous plastid analyses carried out. The second clade of *Haemanthus*, with bootstrap support of 78%, represents a group of species from the northwestern Cape and Namaqualand.

***ITS* matrix**: Of the 592 characters analysed, 139 (23%) were variable and 216 (36%) were parsimony informative. 4880 trees were recovered with 768 steps and CI and RI, 0.652 and 0.855, respectively. The strict consensus tree (Figure 2.5) formed a dichotomy consisting of one large clade, with several polytomies, sister *to Gethyllis britteniana* subsp. *britteniana*. *Haemanthus* formed a monophyletic genus (90% bootstrap support) and as in all the plastid analyses the summer rainfall species (including *H. montanus*) resolved as a distinct clade with 100% bootstrap support. *Scadoxus* resolved as a monophyletic genus (99% bootstrap support) and *Cryptostephanus* was placed in a subclade with two species of *Clivia*.

Amplification for 11 species of *Gethyllis* was unsuccessful for the ITS 2 region and only partial sequences were used in the analysis. Omitting the 11 taxa with partial sequences from the individual ITS analysis produced a *Gethyllis-Apodolirion* clade with bootstrap support of 90% compared to 56% when the taxa were included.

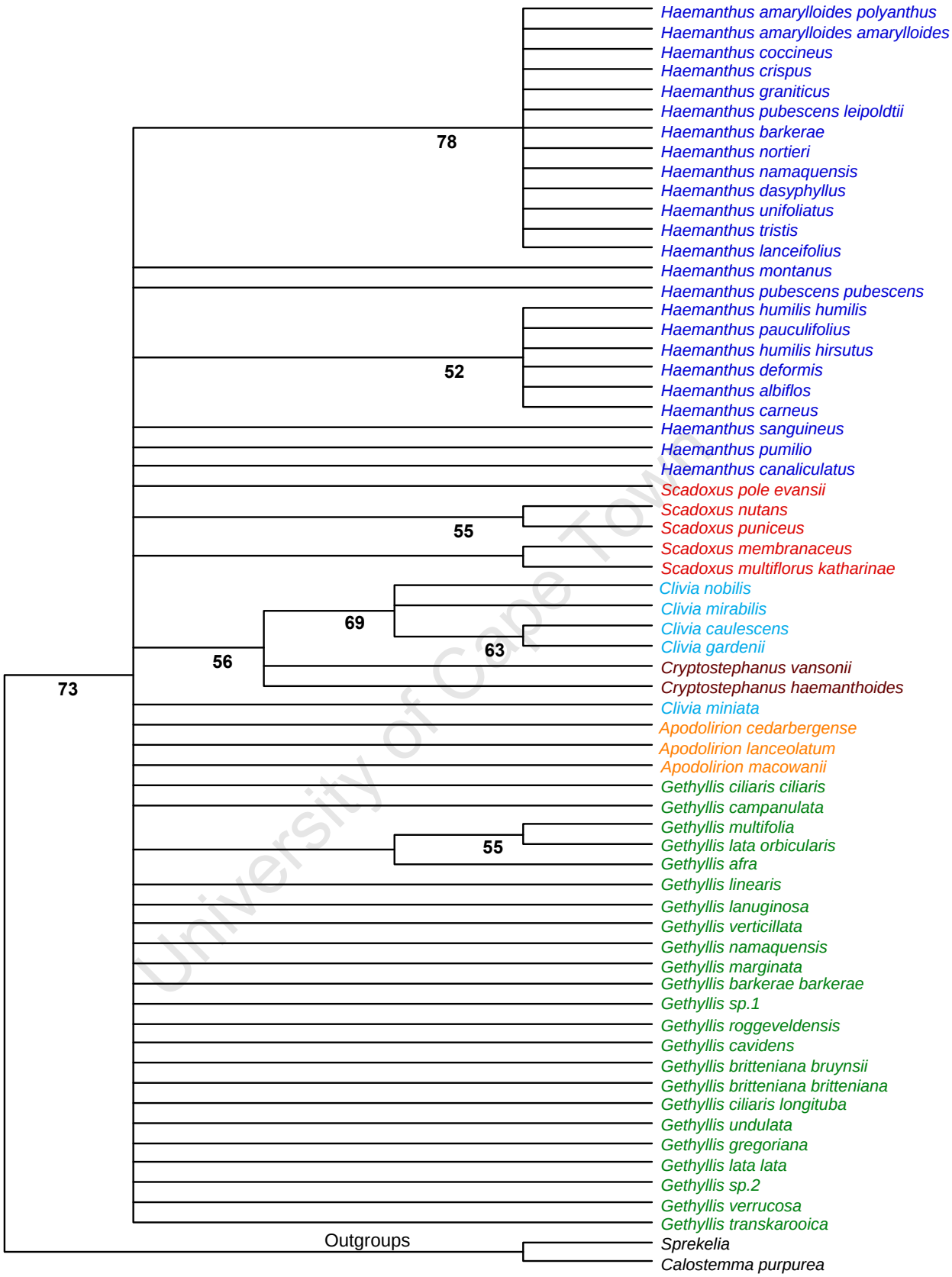


Figure 2.4: Strict consensus tree (CI = 0.740; RI = 0.832) of 4895 equally parsimonious trees (length = 96) using the *psbA-trnH* data set with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

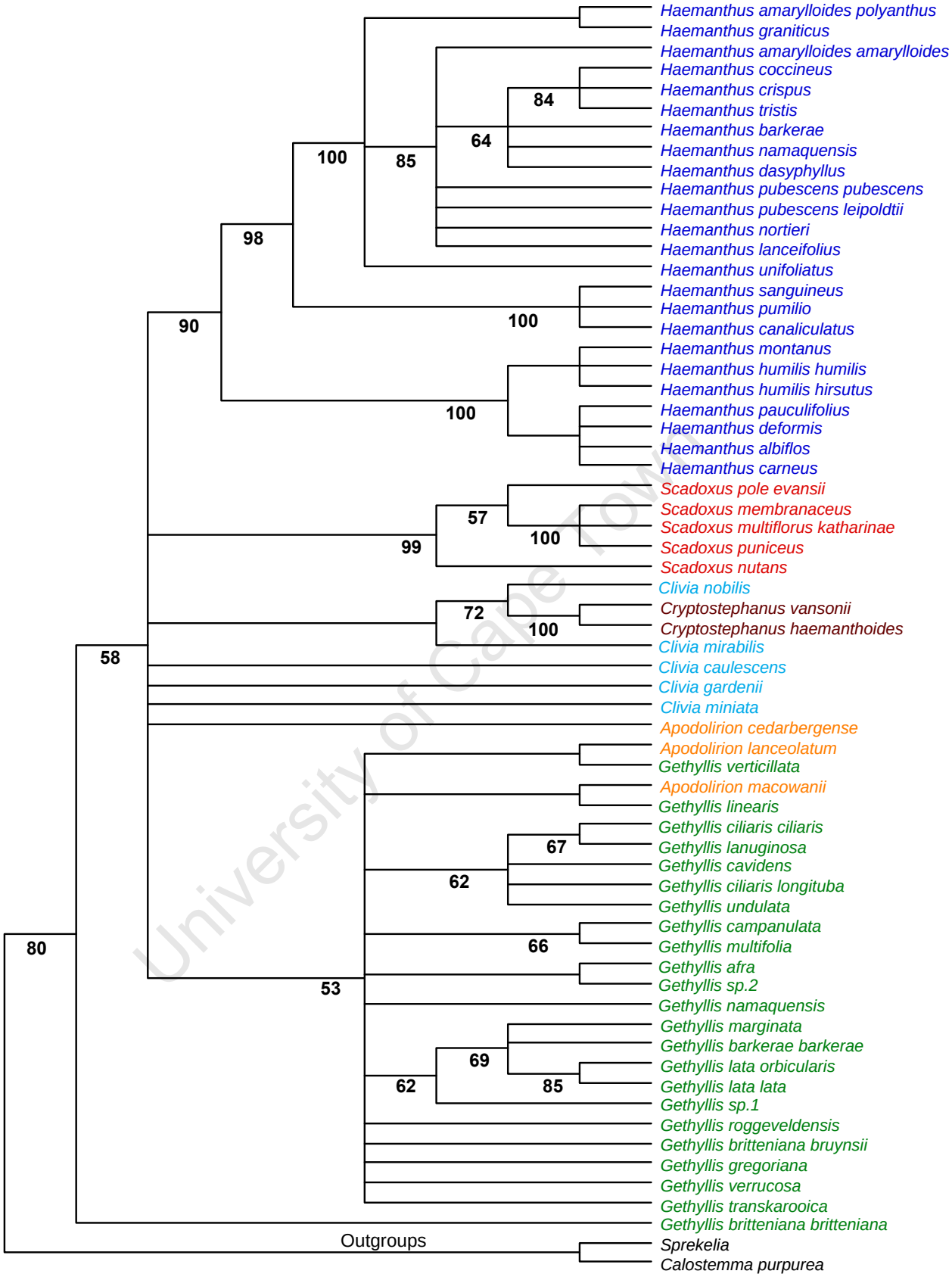


Figure 2.5: Strict consensus tree (CI = 0.652; RI = 0.855) of 4880 equally parsimonious trees (length = 768) using the *ITS* data set with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

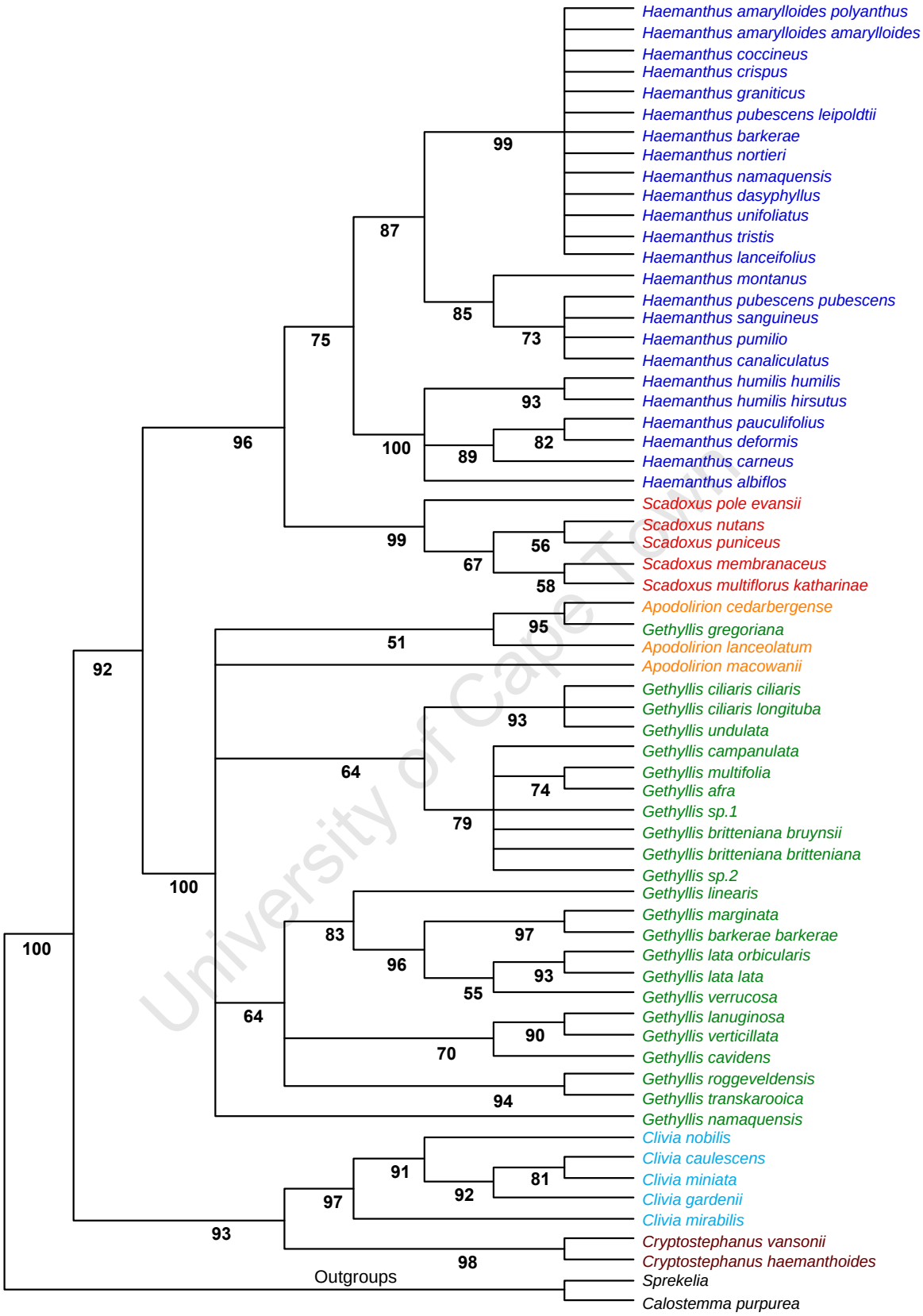


Figure 2.6: Strict consensus tree (CI = 0.77; RI = 0.86) of 4040 equally parsimonious trees (length = 567) using the combined plastid data sets with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

2.3.2. Partition Homogeneity Tests

Partition homogeneity (or ILD) tests were carried out for both the combined plastid and combined plastid and nuclear matrices. Dolphin *et al.* (2000) reported that noise (or random data) resulting from characters evolving at different rates could influence ILD tests leading to separate analysis of matrices. In this analysis, even though p= 0,01 was attained in both cases, suggesting incongruency, analyses combining the individual matrices were still carried out in order to assess all the evidence.

Combined plastid matrices: The total alignment length across all plastid regions was 3161bp, with 235 (7.43%) variable and 187 (5.91%) parsimony informative. Under parsimony 4040 trees were recovered (L = 567, CI = 0.77, RI = 0.86). The combined plastid analysis produced the best-resolved tree with ten nodes collapsing in the strict consensus tree (Figure 2.6). Two main clades were resolved. The larger clade comprised two subclades with *Haemanthus* and *Scadoxus* placed as sister genera to each other in the one with strong bootstrap support (96%). *Haemanthus* resolves as a monophyletic genus with moderate bootstrap support (75%) and the summer rainfall *Haemanthus* species, apart from *H. montanus*, formed a distinct clade with strong bootstrap support of 100%. *Scadoxus* was also monophyletic with 99% bootstrap support. *Gethyllis* and *Apodolirion* were placed together (100% bootstrap support) in the second subclade, with *Apodolirion* embedded within *Gethyllis*. The *Gethyllis-Apodolirion* clade was sister to the *Haemanthus-Scadoxus* clade. The second clade (93% bootstrap support) comprised a monophyletic *Clivia* (97% bootstrap support) placed sister to *Cryptostephanus*. *Cryptostephanus* resolved as a monophyletic genus with 98% bootstrap support.

Combined plastid and nuclear matrices: Of the 3753 characters included in the analysis, 374 (9.96%) were variable and 388 (10.33%) parsimony informative. 3845 trees were retrieved with length 1410, CI 0.667 and RI 0.828. The strict consensus tree illustrated in Figure 2.7 formed two clades. The larger clade consisted of two subclades; *Haemanthus* and *Scadoxus* in one, resolved as monophyletic genera with strong bootstrap support (97% and 100%, respectively). A poorly resolved *Apodolirion-Gethyllis* clade (89% bootstrap support) with *Apodolirion* embedded within *Gethyllis*, resolves sister to the *Haemanthus-Scadoxus* clade. The smaller, well

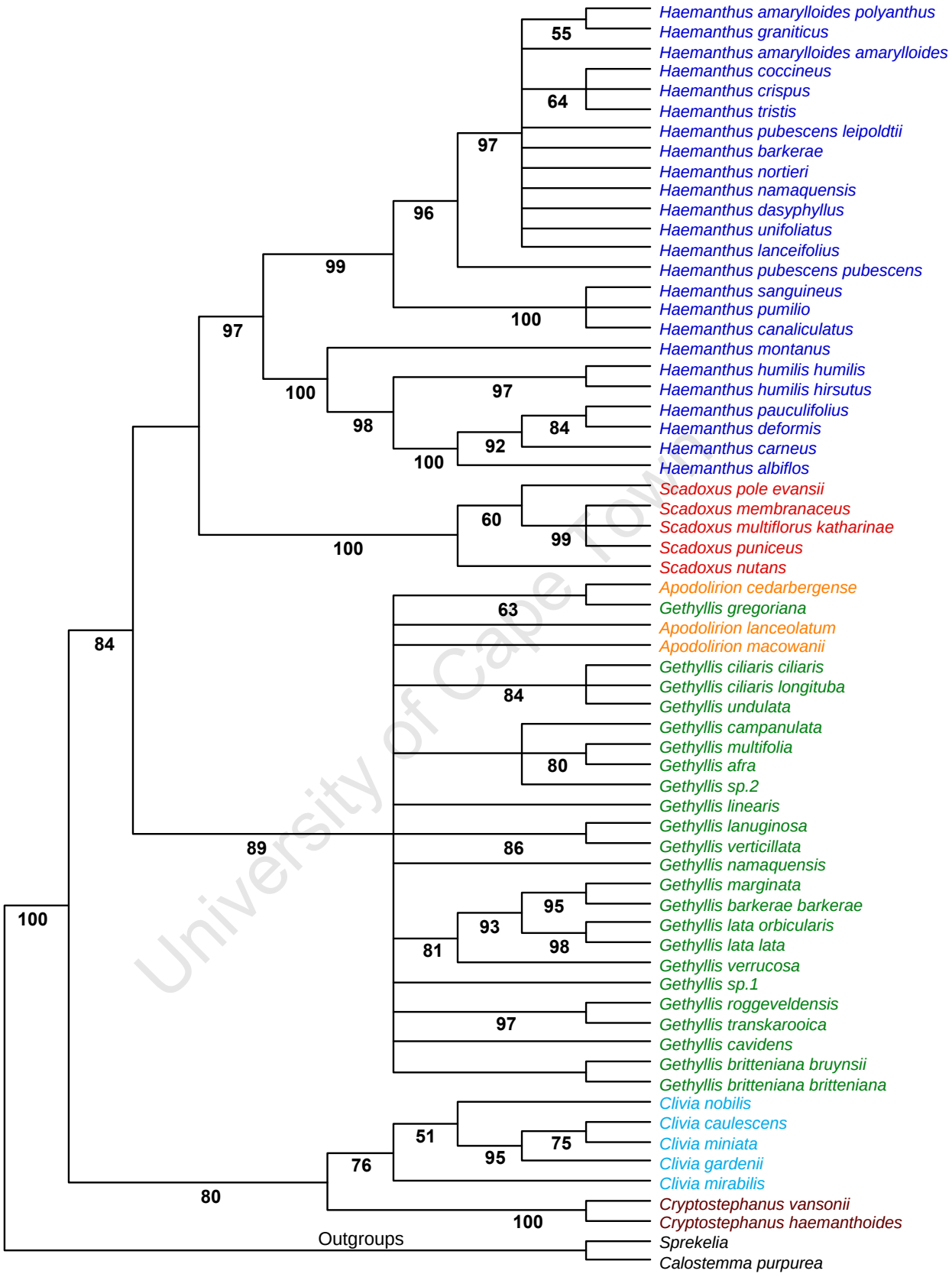


Figure 2.7: Strict consensus tree (CI = 0.667; RI = 0.828) of 3845 equally parsimonious trees (length = 1410) using the combined plastid and nuclear data sets with bootstrap values. Groups with bootstrap percentages < 50 are not indicated

resolved clade comprised a monophyletic *Clivia* (76% bootstrap support), sister to a clade comprising *Cryptostephanus* (100% bootstrap support).

Statistics for the Parsimony analyses are summarised in Table 2.3.

Table 2.3: Statistics for all the parsimony analyses of the plastid, nuclear, combined plastid and combined plastid and nuclear matrices

	Total no. of characters	Constant characters	Variable parsimony uninformative	Parsimony informative charcaters	No. of trees	Tree length	CI	RI
trnLF	873	742	81	50	4870	164	0.860	0.888
rps16	809	710	54	45	4300	120	0.850	0.934
rpoB-trnC	921	776	80	65	3500	198	0.818	0.877
psbA-trnH	562	495	40	27	4890	101	0.743	0.829
ITS	592	237	139	216	4830	768	0.652	0.855
Combined plastid matrices	3161	2719	255	187	3975	612	0.784	0.857
Combined plastid and nuclear matrices	3753	2991	374	388	3940	1410	0.667	0.828

Bootstrap support, for the tribe Haemantheae, ranged from weak to moderate (less than 50% to 80%) in the analyses of the individual plastid and nuclear datasets. In the combined plastid and combined plastid and nuclear parsimony analyses 100% bootstrap supports were obtained. This emphasizes the importance of a multigene dataset above a single dataset in obtaining a sound phylogeny.

2.3.3. Bayesian Analysis

combined plastid matrices: The majority rule tree (Figure 2.8) virtually mirrors the topology of the tree produced through parsimony analysis for the combined plastid matrices, with the majority of the clades reflecting high probabilities. *Haemanthus* and *Gethyllis* are better resolved in the Bayesian analysis as some nodes collapse with these genera in the strict consensus tree for the parsimony analysis. A high posterior probability value of $P = 99$ is obtained for the tribe.

The tribe resolved two clades. In the smaller clade a monophyletic *Clivia* was placed sister to *Cryptostephanus*. The larger clade formed two subclades: a *Gethyllis*-*Apodolirion* clade sister to a *Haemanthus*-*Scadoxus* clade, with *Haemanthus* and *Scadoxus* reciprocally monophyletic. As with the parsimony analysis, *Apodolirion* is firmly embedded within *Gethyllis*. Although a polytomy is present in the *Gethyllis*-*Apodolirion* clade, it is interesting to note the placement of *G. gregoriana* with two species of *Apodolirion*.

combined plastid and nuclear matrices: As with the combined plastid analyses, the maximum likelihood analysis for the combined plastid and nuclear matrices (Figure 2.9) shows a better resolved tree than that for the parsimony analysis, especially with regard to *Haemanthus* and *Gethyllis*. High probability values are reflected for *Cryptostephanus* (P =100), *Clivia* (P = 99), and *Scadoxus* (P = 100) and for Haemantheae (P =100).

As with the combined plastid analysis two clades are formed. *Clivia* and *Cryptostephanus* are placed as sister genera in the smaller clade. *Haemanthus*, *Scadoxus*, *Gethyllis* and *Apodolirion* are placed in the larger clade with *Haemanthus* and *Scadoxus* are recipricolly monophyletic in one of the subclades.

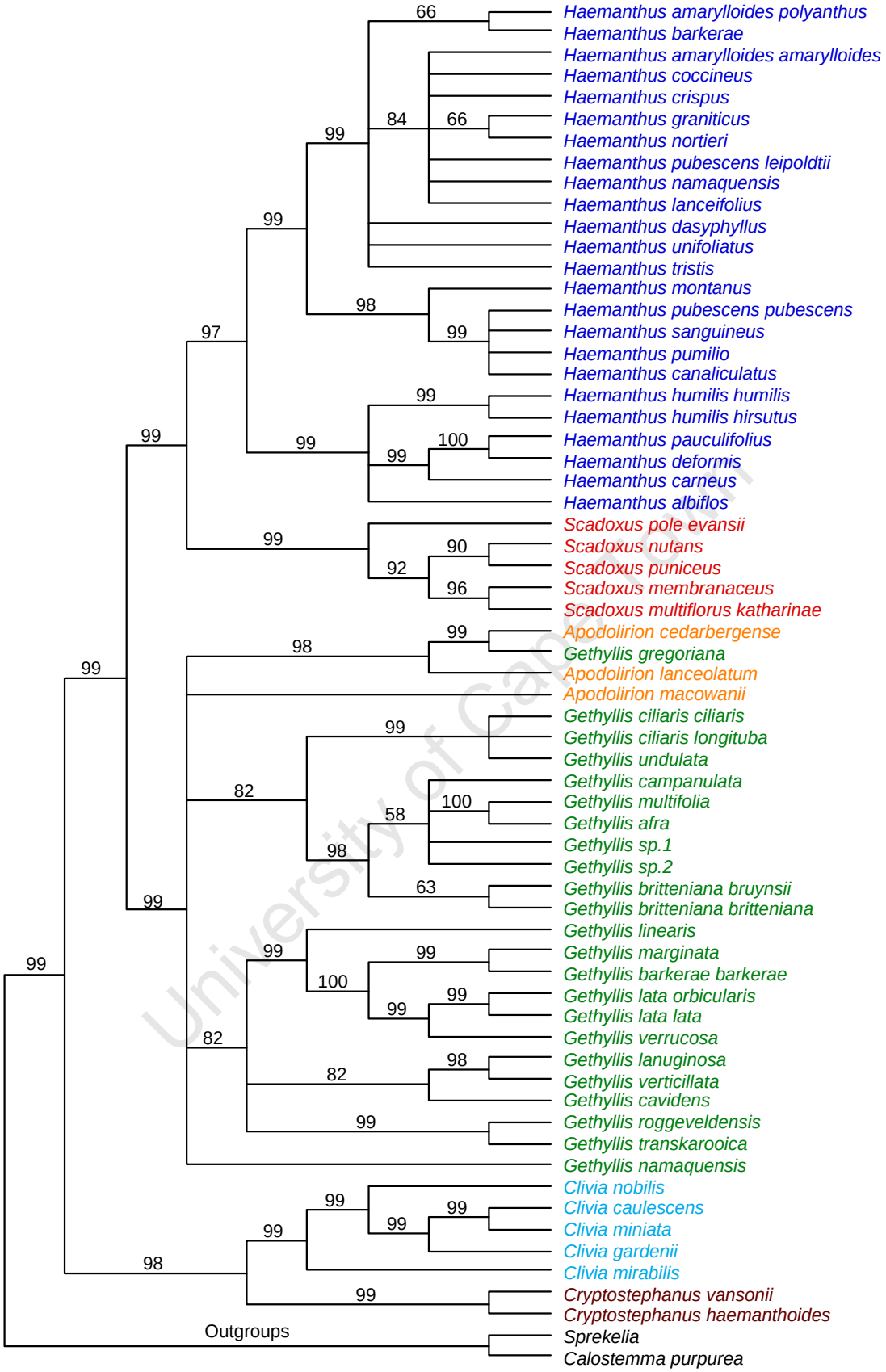


Figure 2.8: The 50% majority rule consensus tree of combined plastid regions using the Bayesian algorithm. Posterior probability values indicated above branches for values > 50%

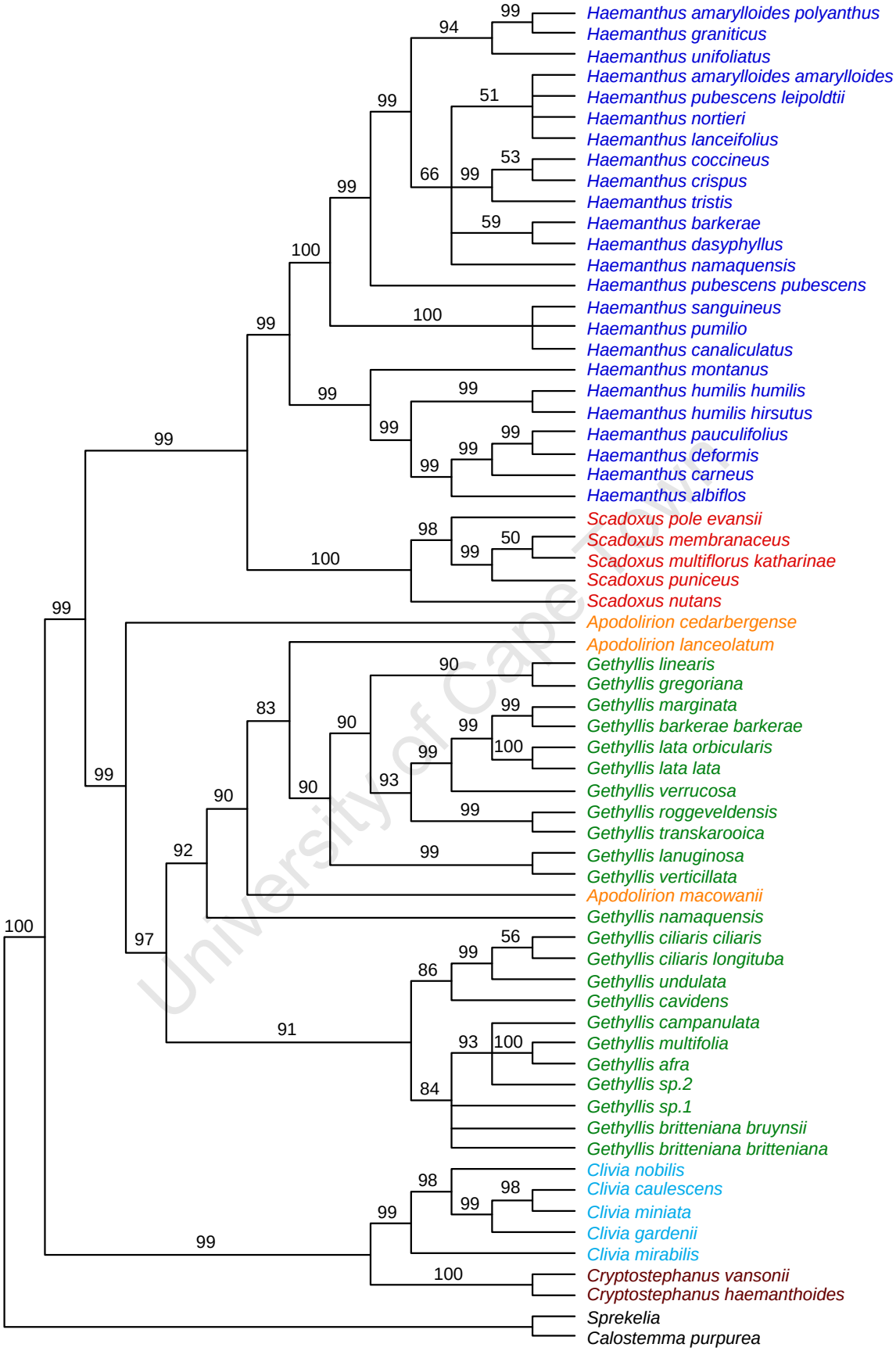


Figure 2.9: The 50% majority rule consensus tree of combined plastid and nuclear regions using the Bayesian algorithm. Posterior probability values indicated above branches for values > 50%

Summaries for the unlinked model parameters for both the combined plastid and combined plastid and nuclear matrices are presented in Table 2.4 and Table 2.5.

Table 2.4: Summary of model parameters for the Bayesian analysis for the combined plastid matrices. PSRF: Potential Scale Reduction Factor; Data partitions in parameter list: 1 = *trnL-F*, 2 = *rps16*; 3 = *rpoB-trnC*; 4 = *psbA-trnH*; 5 = *ITS*

			95%	confidence	interval	
Parameter	**Mean**	**Variance**	**Lower**	**Upper**	**Median**	**PSRF**
TL{all}	11.086845	1.830848	7.974000	13.509000	11.196000	1.005
r(A<->C){1}	0.154147	0.000973	0.095917	0.218689	0.153211	1.001
r(A<->G){1}	0.233044	0.001417	0.164465	0.311791	0.231240	1.000
r(A<->T){1}	0.050847	0.000186	0.027961	0.080871	0.049553	1.000
r(C<->G){1}	0.082958	0.000952	0.032945	0.152222	0.079606	1.000
r(C<->T){1}	0.331347	0.001844	0.249435	0.417603	0.330551	1.000
r(G<->T){1}	0.147656	0.001096	0.090819	0.219870	0.144935	1.003
r(A<->C){2}	0.243613	0.001963	0.162727	0.33450	0.241930	1.000
r(A<->G){2}	0.180412	0.001371	0.114837	0.258535	0.178225	1.000
r(A<->T){2}	0.046975	0.000261	0.020785	0.083560	0.045190	1.000
r(C<->G){2}	0.048187	0.000801	0.008093	0.116345	0.043302	1.000
r(C<->T){2}	0.302135	0.002446	0.209100	0.402503	0.301128	1.000
r(G<->T){2}	0.178679	0.001456	0.111155	0.259965	0.176170	1.000
r(A<->C){3}	0.196697	0.001042	0.138083	0.264214	0.195115	1.000
r(A<->G){3}	0.199947	0.000947	0.144033	0.264221	0.198503	1.000
r(A<->T){3}	0.039384	0.000113	0.021731	0.062838	0.038357	1.000
r(C<->G){3}	0.127511	0.001214	0.066725	0.202968	0.124994	1.001
r(C<->T){3}	0.297067	0.001559	0.222954	0.377575	0.296080	1.001
r(G<->T){3}	0.139394	0.000673	0.092872	0.193941	0.137992	1.000
r(A<->C){4}	0.139121	0.001632	0.074652	0.233059	0.133845	1.000
r(A<->G){4}	0.357459	0.003417	0.233110	0.466350	0.359611	1.002
r(A<->T){4}	0.053349	0.000317	0.024024	0.093313	0.051503	1.001
r(C<->G){4}	0.051087	0.000690	0.011616	0.112576	0.047149	1.001
r(C<->T){4}	0.259489	0.003012	0.170669	0.390107	0.252903	1.001
r(G<->T){4}	0.139494	0.001345	0.072450	0.215272	0.138045	1.000
pi(C){1}	0.174435	0.000158	0.150702	0.199867	0.174115	1.001
pi(G){1}	0.157462	0.000137	0.135220	0.180952	0.157190	1.000
pi(T){1}	0.302073	0.000225	0.273245	0.332170	0.301780	1.001
pi(A){2}	0.398099	0.000275	0.366124	0.430641	0.397982	1.000
pi(C){2}	0.122461	0.000116	0.102423	0.144410	0.122095	1.000
pi(G){2}	0.181925	0.000168	0.157456	0.208443	0.181584	1.000
pi(T){2}	0.297515	0.000238	0.267763	0.328339	0.297272	1.000

pi(A){3	0.365661	0.000231	0.336409	0.395881	0.365503	1.000
pi(C){3}	0.145753	0.000117	0.125404	0.167584	0.145509	1.000
pi(G){3}	0.163591	0.000131	0.141839	0.186580	0.163348	1.000
pi(T){3}	0.324995	0.000217	0.296584	0.354227	0.324873	1.000
pi(A){4}	0.312855	0.000353	0.276281	0.350011	0.312825	1.000
pi(C){4}	0.179263	0.000239	0.149560	0.210373	0.178951	1.000
pi(G){4}	0.160792	0.000233	0.132579	0.192729	0.160176	1.000
pi(T){4}	0.347090	0.000367	0.310010	0.385348	0.346904	1.000
alpha{1}	0.082863	0.000020	0.076529	0.091437	0.082483	1.002
alpha{2}	0.069878	0.000016	0.064244	0.077206	0.069563	1.003
alpha{3}	0.085366	0.000021	0.078701	0.095212	0.084902	1.004
alpha{4}	0.094392	0.001673	0.069884	0.196590	0.077215	1.000
pinvar{2}	0.705713	0.000968	0.644932	0.761917	0.706698	1.000
pinvar{4}	0.742196	0.001440	0.671329	0.815869	0.741169	1.000

Table 2.5: Summary of model parameters for the Bayesian analysis for the combined plastid and nuclear matrices. PSRF: Potential Scale Reduction Factor; Data partitions in parameter list: 1 = *trnL-F*, 2 = *rps16*; 3 = *rpoB-trnC*; 4 = *psbA-trnH*; 5 = *ITS*

			95% confidence interval			
Parameter	**Mean**	**Variance**	**Lower**	**Upper**	**Median**	**PSRF**
TL{all}	1.898986	0.363338	1.630000	2.109000	1.844000	1.000
r(A<->C){1}	0.046788	0.000258	0.024246	0.082921	0.044322	1.162
r(A<->G){1}	0.344309	0.142714	0.024086	0.865627	0.065473	2.400
r(A<->T){1}	0.012165	0.000034	0.004820	0.024053	0.011240	1.084
r(C<->G){1}	0.038089	0.001089	0.009161	0.141471	0.027945	1.012
r(C<->T){1}	0.483157	0.129633	0.031557	0.869202	0.623688	2.361
r(G<->T){1}	0.075492	0.003881	0.028994	0.270619	0.053652	1.017
r(A<->C){2}	0.234621	0.002615	0.148221	0.350022	0.229453	1.001
r(A<->G){2}	0.251331	0.003112	0.145123	0.363311	0.250684	1.002
r(A<->T){2}	0.055014	0.000406	0.022443	0.100480	0.052741	1.007
r(C<->G){2}	0.044284	0.000703	0.007365	0.108463	0.039564	1.000
r(C<->T){2}	0.235901	0.002421	0.151797	0.344613	0.231307	1.005
r(G<->T){2}	0.178849	0.001886	0.099726	0.270414	0.176657	1.001
r(A<->C){3}	0.079381	0.000457	0.048207	0.125934	0.076639	1.010
r(A<->G){3}	0.026087	0.000251	0.015503	0.039286	0.024022	1.004
r(A<->T){3}	0.009950	0.000023	0.004035	0.019287	0.009248	1.003
r(C<->G){3}	0.033905	0.000284	0.015209	0.060975	0.031552	1.005
r(C<->T){3}	0.820826	0.003360	0.754025	0.873953	0.828460	1.008
r(G<->T){3}	0.029852	0.000232	0.016834	0.046620	0.027821	1.002
r(A<->C){4}	0.184310	0.002506	0.098156	0.292734	0.180562	1.000
r(A<->G){4}	0.248890	0.004898	0.142938	0.421507	0.238460	1.004
r(A<->T){4}	0.051897	0.000399	0.020592	0.097973	0.049322	1.000

r(C<->G){4}	0.040398	0.000491	0.009374	0.094222	0.036550	1.001
r(C<->T){4}	0.384145	0.007752	0.207405	0.554422	0.385183	1.006
r(G<->T){4}	0.090361	0.000767	0.047590	0.156664	0.086540	1.000
r(A<->C){5}	0.121123	0.000225	0.093435	0.151993	0.120556	1.000
r(A<->G){5}	0.200805	0.000391	0.163372	0.240484	0.200434	1.001
r(A<->T){5}	0.123395	0.000340	0.089412	0.161448	0.122673	1.001
r(C<->G){5}	0.051961	0.000056	0.038372	0.067374	0.051627	1.007
r(C<->T){5}	0.405453	0.000720	0.354892	0.458999	0.404894	1.003
r(G<->T){5}	0.097262	0.000148	0.074882	0.122459	0.096789	1.003
pi(A){1}	0.370816	0.000285	0.338443	0.404562	0.370668	1.051
pi(C){1}	0.173936	0.000168	0.149278	0.199723	0.173781	1.076
pi(G){1}	0.150977	0.000216	0.123372	0.179614	0.150935	1.326
pi(T){1}	0.304271	0.000238	0.274631	0.334780	0.304106	1.002
pi(A){2}	0.390817	0.000277	0.358343	0.423156	0.390634	1.000
pi(C){2}	0.127760	0.000138	0.105287	0.151200	0.127520	1.001
pi(G){2}	0.179119	0.000163	0.154620	0.204989	0.178815	1.000
pi(T){2}	0.302304	0.000247	0.272352	0.333278	0.302106	1.000
pi(A){3}	0.369903	0.000245	0.339652	0.400860	0.369737	1.000
pi(C){3}	0.131733	0.000117	0.111301	0.153725	0.131407	1.000
pi(G){3}	0.176093	0.000149	0.152784	0.200555	0.175822	1.000
pi(T){3}	0.322271	0.000248	0.291498	0.353730	0.322200	1.001
pi(A){4}	0.305674	0.000370	0.268746	0.344263	0.305399	1.000
pi(C){4}	0.170837	0.000235	0.141753	0.201643	0.170540	1.000
pi(G){4}	0.176273	0.000261	0.144782	0.208176	0.176153	1.000
pi(T){4}	0.347215	0.000389	0.309438	0.386861	0.346944	1.000
pi(A){5}	0.169132	0.000157	0.145340	0.194419	0.168887	1.006
pi(C){5}	0.298653	0.000243	0.268554	0.329424	0.298543	1.010
pi(G){5}	0.338319	0.000286	0.305916	0.371441	0.338209	1.001
pi(T){5}	0.193897	0.000163	0.169822	0.219415	0.193570	1.000
alpha{1}	0.123874	0.000198	0.099513	0.150403	0.124034	1.471
alpha{2}	0.134642	0.000171	0.114600	0.161290	0.133682	1.004
alpha{3}	0.127173	0.000091	0.110497	0.146334	0.126790	1.006
alpha{4}	0.208983	0.002011	0.145053	0.314944	0.202116	1.000
alpha{5}	1.625300	0.370956	0.864057	3.118617	1.498302	1.000
pinvar{2}	0.671001	0.001150	0.605331	0.734715	0.671372	1.000
pinvar{4}	0.698807	0.001952	0.612557	0.788664	0.699304	1.001
pinvar{5}	0.133420	0.004735	0.009794	0.259711	0.135793	1.000

2.3.4. Character Analysis

Optimizations of characters one to fifteen (see Appendices A and B) are illustrated in Figure 2.10. *Clivia* and *Cryptostephanus* represent entirely rhizomatous genera (Fig 2.10 (1)) although rhizomes are also present in some species of *Scadoxus* (*S. nutans* in this study). Meerow *et al.* (1999b) considers this bulbless state plesiomorphic for the family. In this study rhizomes are synapomorphic for *Clivia* and *Cryptostephanus* but the analysis has not included representalives of Alliaceae and Agapanthaceae as outgroups as in Meerow *et al.* (1999b) study.

Bulbs consist of a few layers of tunics that sheath each other entirely, as in the *Gethyllis-Apodolirion* clade and the summer rainfall *Haemanthus* clade. In *H. albiflos* and *H. deformis* the tunics turn green and adopt a photosynthetic role when they are exposed to light (Snijman 1984). In the winter-rainfall *Haemanthus* species, the bulb tunics (Fig 2.10 (2)) are distichous and sheathe each other only towards the base, a state which is synapomorphic for this group. Bulb tunic characters are equivocal with *Sprekelia* as outgroup. If the analysis was expanded to include further outgroups, the distichous state would probably be shown to be unique in the family.

Clivia, *Cryptostephanus* and *Scadoxus* exhibit suberect (Fig 2.10 (3)) leaves. Although this trait is also dominant in *Haemanthus*, prostrate leaves have evolved at least eight times in *Haemanthus*. The leaf position character of spiralled leaves is characteristic of the *Gethyllis-Apodolirion* clade, having evolved once, but has been lost six times – three times to prostrate leaves and three times to suberect leaves.

Persistent, evergreen foliage is synapomorphic for *Clivia* and *Cryptostephanus*. This character state is also present in four summer rainfall species of *Haemanthus* (Fig 2.10 (4)). The deciduous habit resolves as plesiomorphic for the tribe.

Both synanthous-leaved (leaves are present at flowering) and hysteranthous-leaved (leafing and flowering occurs at separate times) species are represented among the geophytes. By nature of their evergreen habit *Clivia* and *Cryptostephanus* are both synanthous. Although deciduous, most *Scadoxus* species are synanthous except for two species (*S. pole evansii* and *S. puniceus*) which can be either synanthous or

hysteranthous (Fig 2.10 (5)). All the other genera of the tribe are hysteranthous but for three summer rainfall *Haemanthus* species (*H. albiflos*, *H. deformis* and *H. pauculifolius*). In this analysis, the result is equivocal as to whether the synanthous habit is apomorphic or plesiomorphic.

Leaf pubescence (Fig 2.10(6)) although uncommon in the tribe, has arisen in several species of *Gethyllis* and *Haemanthus*. Pubescens is synapomorphic for the clade of summer-rainfall species of *Haemanthus*. In addition, it appears to have evolved independently at least four times in the winter-rainfall taxa of *Haemanthus*. Weighlin (2002) distinguishes between two different types of trichomes in *Gethyllis*: simple and T-shaped. The analysis shows that simple trichomes are independently derived in *G. lanuginosa, G. roggeveldensis, G.gregoriana, G. campanulata* and the *G. ciliaris* clade which includes *G. ciliaris, G. undulata,* and *G. multifolia*. The unusual T-shaped (two-armed, equal-armed) trichomes are independently derived in *G. barkerae* and *G. verrucosa*. The unspecialized glabrous habit is plesiomorphic for the Haemantheae.

In *Apodolirion* and *Gethyllis* the scape is reduced and shorter than the bulb neck rendering it obsolete (Fig 2.10(7)), a synapomorphy for the *Apodolirion*-*Gethyllis* clade.

Haemanthus and *Scadoxus* exhibit brightly coloured fleshy spathe valves that enclose the flowers giving the inflorescence an appearance of a brush (Fig 2.10(8)). In addition the brush type inflorescence evolved independently once in the *Clivia*-*Cryptostephanus* clade, in *Clivia miniata*, while the other *Clivia* and *Cryptostephanus* species have tubular flowered inflorescences. The inflorescence of *Gethyllis* and *Apodolirion* are simple and one flowered. The reduction of the inflorescence to a solitary flower, in *Gethyllis* and *Apodolirion* is therefore an apomorphic condition within Haemantheae.

The position of the style in Haemantheae is straight and central for most species in Haemantheae (Fig 2.10(9)), although deflexed styles have evolved twice in the *Gethyllis* 'villosa' clade.

Gethyllis has been divided on the basis of floral morphology into two informal groups, "afra" and "villosa" (Manning *et al*. 2002) based on the sections proposed by Bolus, namely section *Orthostylis* and section *Clinostylis* respectively (Macowan and Bolus 1881). In the 'afra' group (e.g. *G. afra*, *G. ciliaris* subsp. *ciliaris*, *G. multifolia*) the filaments divide so there are more than six stamens; there are12 or more anthers per flower, up to 36 can be present and the flowers are fleshy, succulent and cup shaped. In the 'villosa' group (e.g. *G. verticillata*, *G. lanuginosa*, *G. linearis*) the style is at an angle; the stigma is large and capitate; there are six simple filaments and six anthers and the flowers are thin textured and salver shaped.

Flowers with six anthers (Fig 2.10(10)) are present in all but the *Gethyllis* 'afra' clade where the number of anthers is greater than six. Polyandry is thus synapomorphic for this clade although the character is secondarily lost in the undescribed *Gethyllis* sp.1. As the androecium of the polyandrous species is arranged in stamen bundles, it is likely that secondary polyandry occurs in *Gethyllis*, unlike polyandry in *Curculigo* in the family Hypoxidaceae (Kocyan 2007).

Clivia, *Cryptostephanus*, *Haemanthus* and *Scadoxus* have fewer than 10 ovules per locule, a condition which is plesiomorphic in the tribe. The derived condition of more than 10 ovules in the locule (Fig 2.10(11)) is the synapomorphic condition for *Gethyllis* and *Apodolirion*.

Only *Cryptostephanus* exhibits phytomelaniferous seeds. The presence of phytomelan in the outer integument has evolved only once in the tribe (Fig 2.10(12)).

The fruits of *Apodolirion* and *Gethyllis* are elongated berries rather than globose as in the other genera of Haemantheae (Fig 2.10(13)). Moreover, these two genera also share the same chromosome number (Fig 2.10(15)). Baccate fruits, however, are synapomorphic for the tribe as a whole. The seeds of *Gethyllis*, *Apodolirion*, *Haemanthus* and *Scadoxus* have green embryos, while the embryos of *Clivia* and *Cryptostephanus* are cream-coloured (Fig 2.10(14)), the plesiomorphic character for the tribe.

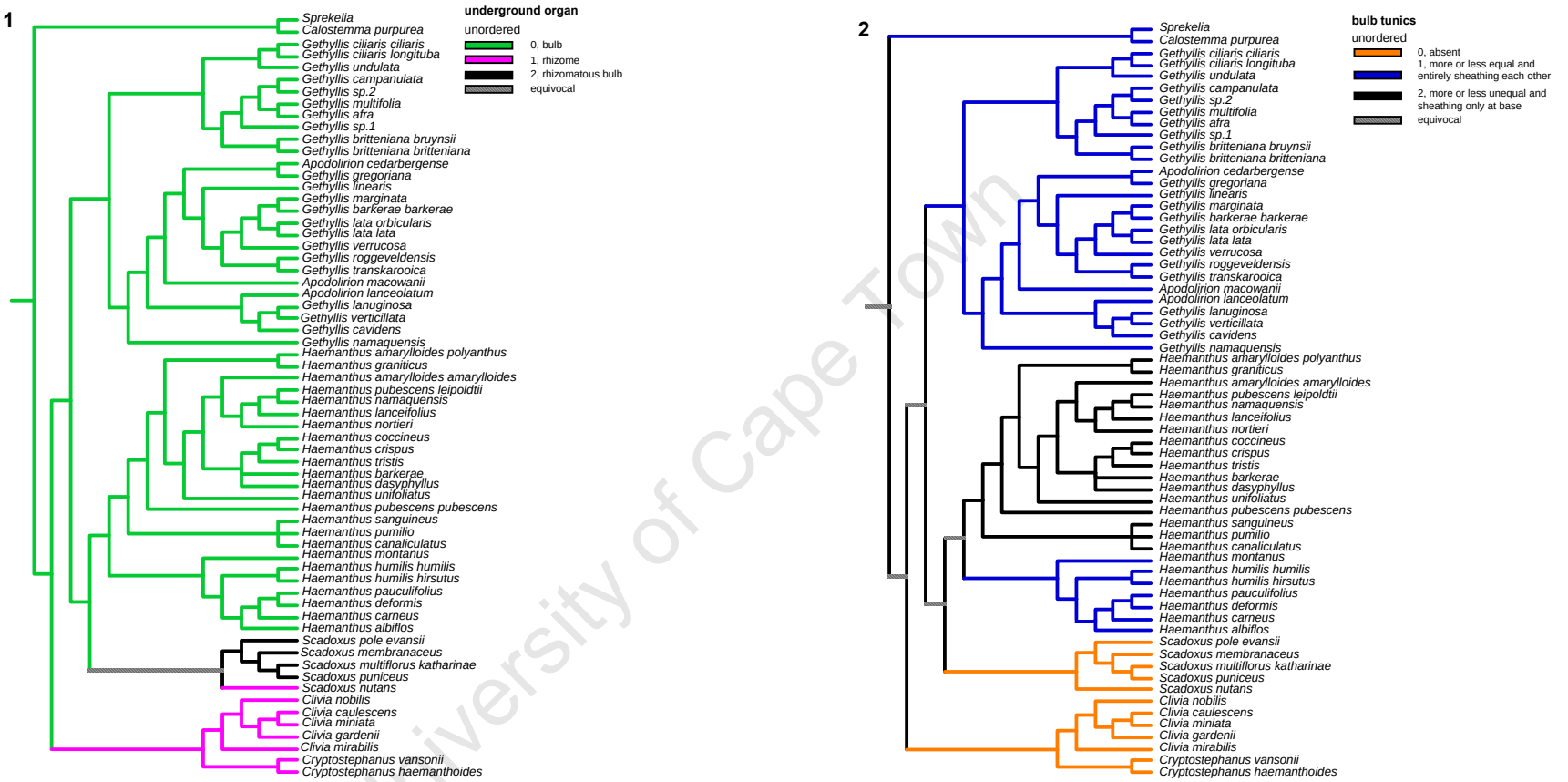


Figure 2.10: Character optimization of (1) underground organ: 0, bulb; 1, rhizome; 2, rhizomatous bulb; and (2) bulb tunics: 0, absent; 1, more or less equal and entirely sheathing each other; 2, more or less unequal and sheathing only at base; on the single most parsimonious tree found by parsimony analysis of the combined plastid and nuclear matrices

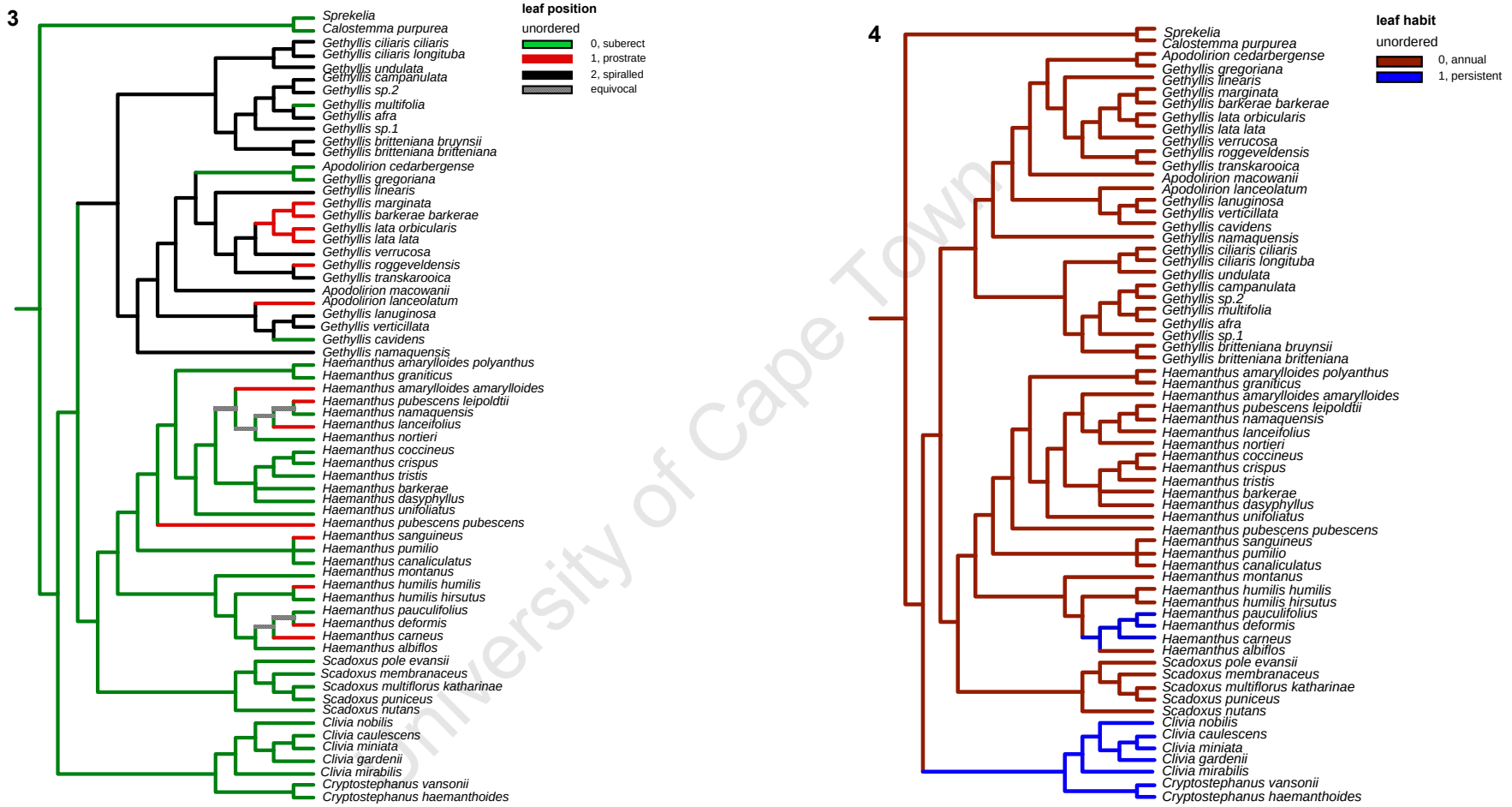


Figure 2.10: (cont.) Character optimization of (3) leaf position: 0, suberect; 1, prostrate; 2, spiralled; and (4) leaf habit: 0, annual; 1, persistent; on the single most parsimonious tree found by parsimony analysis of the combined plastid and nuclear matrices

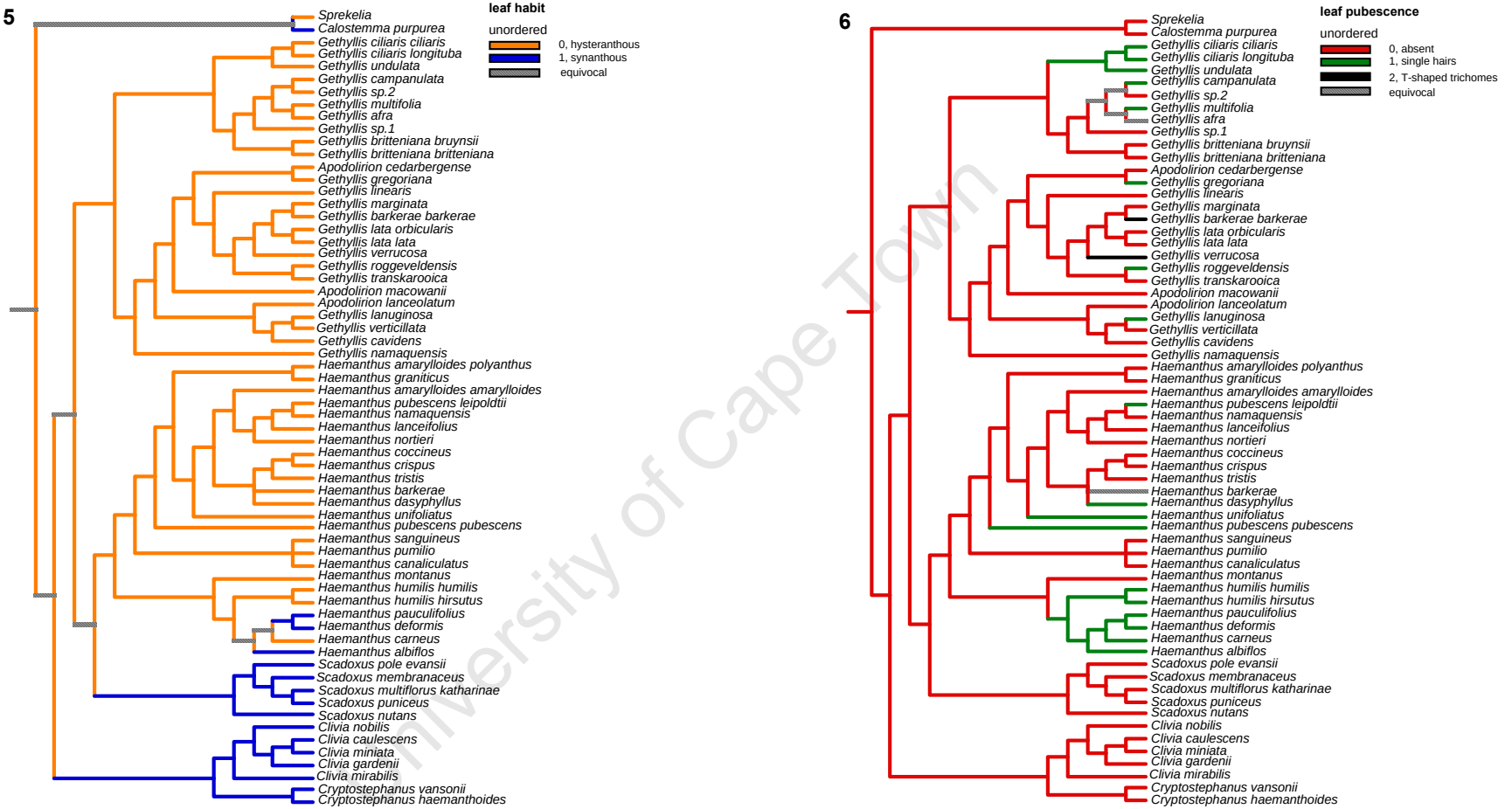


Figure 2.10: (cont.) Character optimization of (5) leaf habit: 0, hysteranthous; 1, synanthous; and (6) leaf pubescence: 0, absent; 1, single hairs; 2, T-shaped trichomes; on the single most parsimonious tree found by parsimony analysis of the combined plastid and nuclear matrices

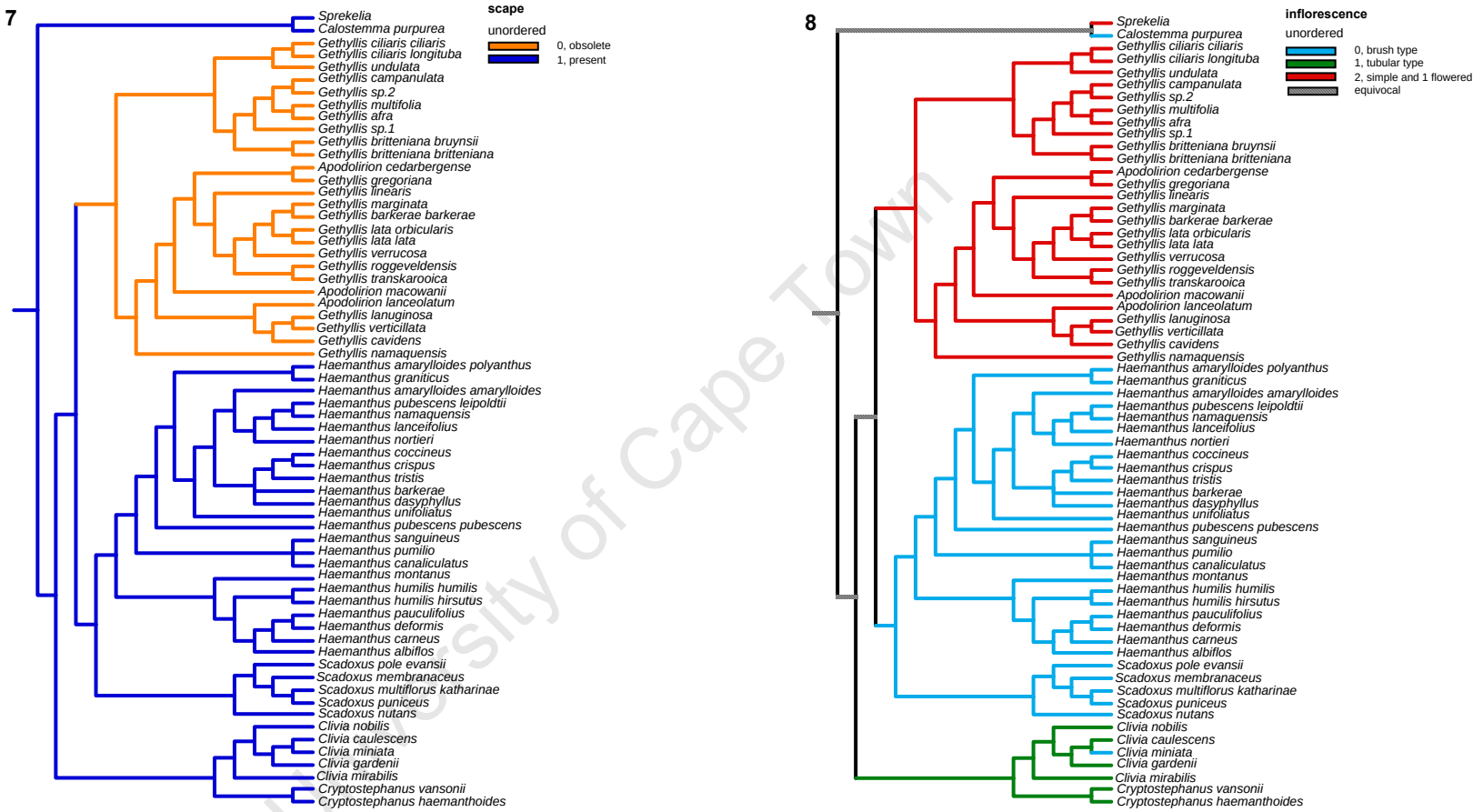


Figure 2.10: (cont.) Character optimization of (7) scape: 0, obsolete; 1, present; and (8) inflorence: 0, brushtype; 1, tubular type; 2, simple and 1 flowered; on the single most parsimonious tree found by phylogenetic analysis of combined plastid and nuclear matrices

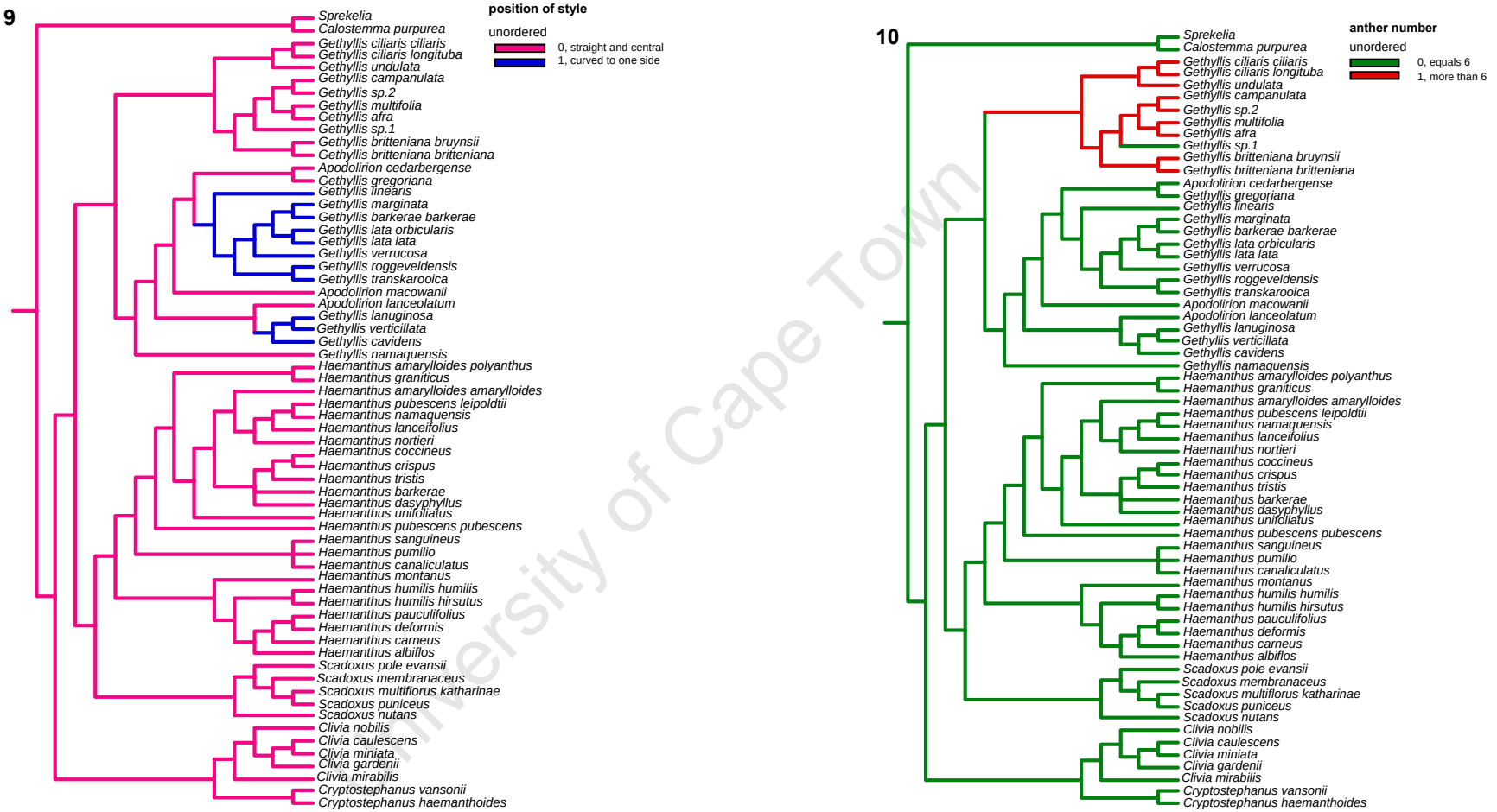


Figure 2.10: (cont.) Character optimization of (9) position of style: 0, straight and central; 1, curved to one side; and (10) anther number: 0, 6; 1, more than 6; on the single most parsimonious tree found by phylogenetic analysis of combined plastid and nuclear matrices

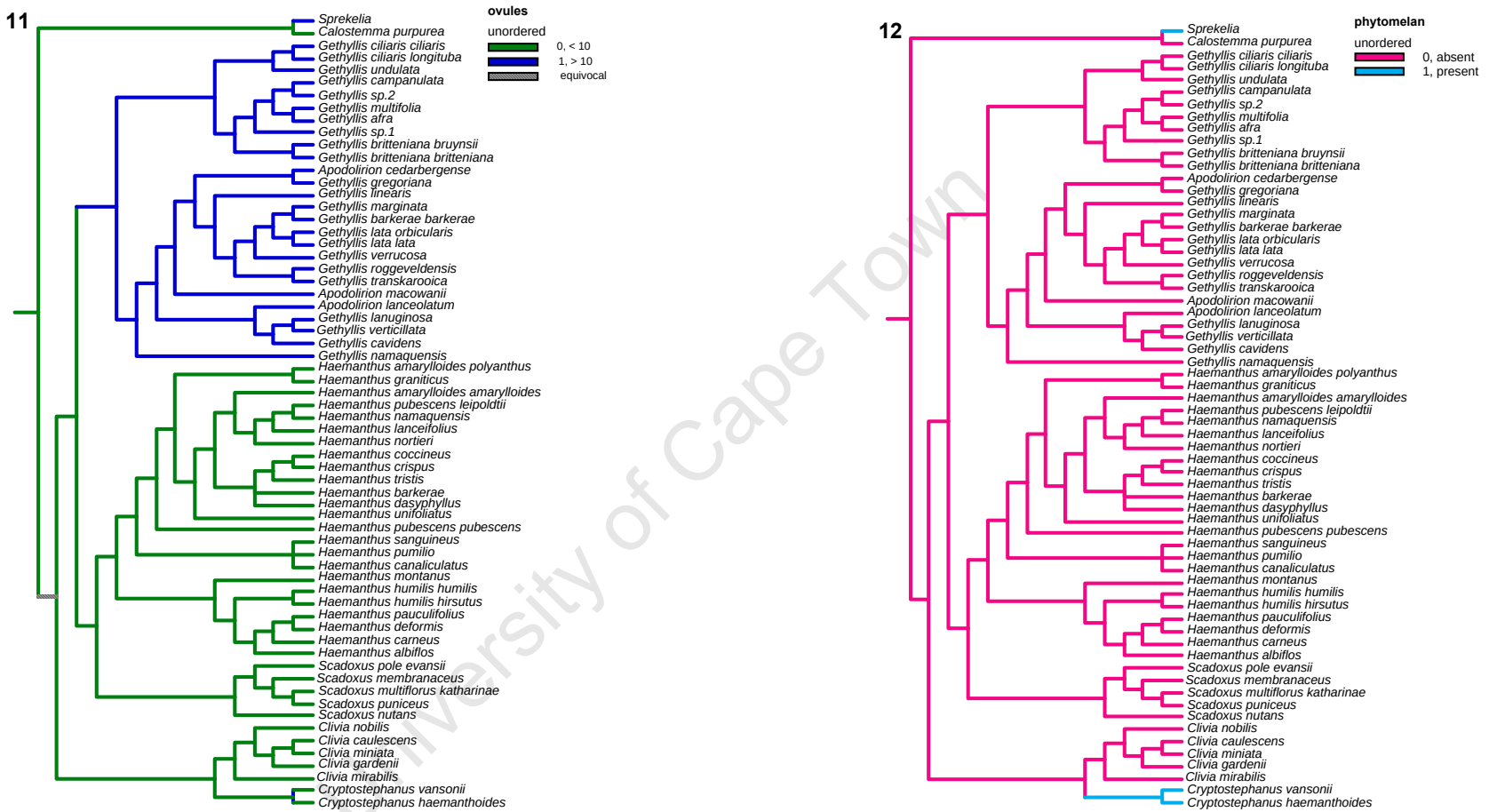


Figure 2.10: (cont.) Character optimization of (11) ovules:0, < 10; 1, > 10; and (12) phytomelan: 0, absent; 1, present; on the single most parsimonious tree found by phylogenetic analysis of combined plastid and nuclear matrices

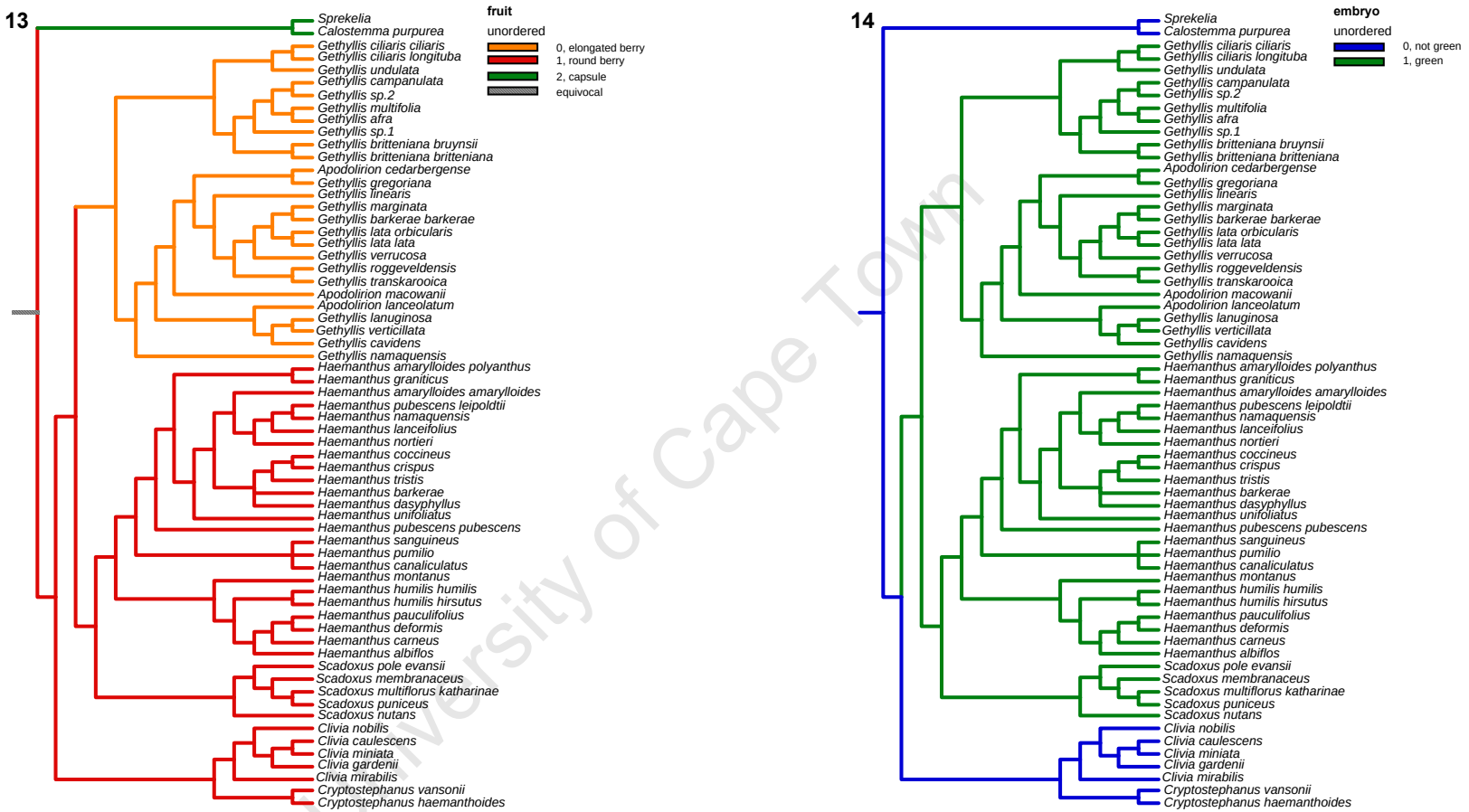


Figure 2.10: (cont.) Character optimization of (13) fruit: 0, elongated berry; 1, round berry; 2, capsule; and (14) embryo: 0, not green; 1, green; on the single most parsimonious tree found by phylogenetic analysis of combined plastid and nuclear matrices

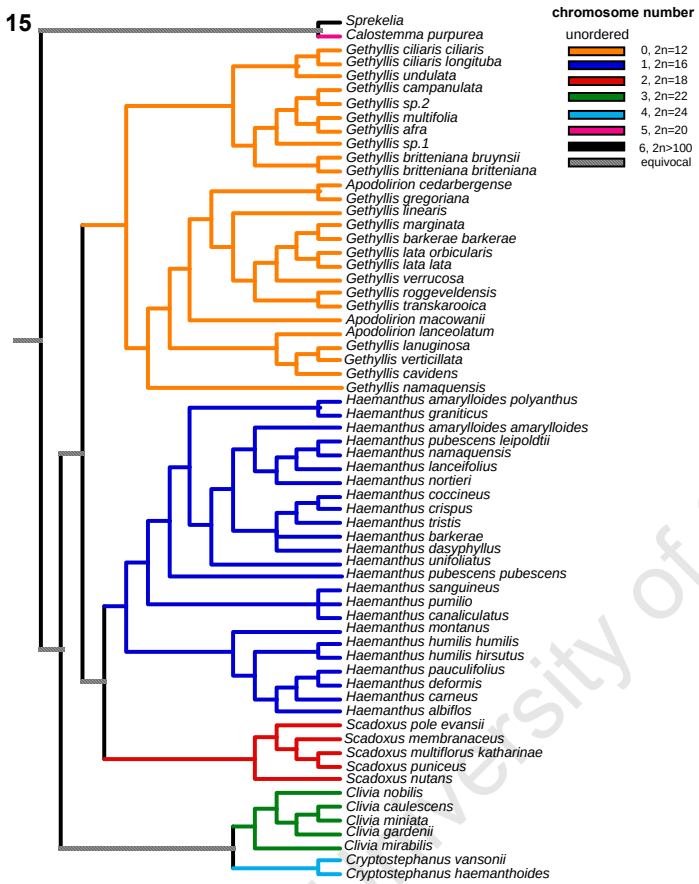


Figure 2.10: (cont.) Character optimization of (15) chromosome number: 0, 2n=12; 1, 2n=16; 2, 2n=18; 3, 2n=22; 4, 2n=24; 5, 2n=20; 6, 2n>100; on the single most parsimonious tree found by phylogenetic analysis of combined plastid and nuclear matrices

2.4. Discussion

With the trend toward multiple gene analysis, one method to determine whether datasets can be combined is the partition homogeneity (or ILD) test. In this study, the results from these tests reveal whether there is incongruence among the plastid matrices and also between the plastid and nuclear matrices. It is unlikely that incongruence among chloroplast regions would result from different histories since they are inherited as a unit and as sampling error is a possible reason for incongruence it is not advisable to partition the datasets. Using simulations, Dolphin *et al.* (2000) reported that noise (or random data) could influence results so that separate analyses of two matrices (representing a similar or identical underlying topology but whose characters have evolved at different rates) are carried out. Similarly Barker and Lutzoni (2000) state that decisions with regard to data combinability would be misleading if based on the ILD only.

Another method to determine combinability of datasets involves comparing nodes of independent trees to test for incongruence (Mason-Gamer and Kellogg 1996).

When there is more than one dataset, as in the case with this study, Reeves *et al.* (2001) suggested that they be combined for optimal result. Kluge (1989), Dubuison *et al.* (1998) and Seelanan *et al.* (1997) termed this the 'total evidence' approach.

Using computer simulations, Wiens (1998) explored the effect of including taxa with missing data on the accuracy of phylogenetic analysis. In general, his results supported the inclusion of taxa with missing data in parsimony analysis, but he suggested that this should not be done uncritically. The alternative, where taxa with incomplete data are excluded would hugely impact the taxonomic scope of any study and thereby decreasing the accuracy of the phylogenetic reconstruction. Fortunately, in this study, missing data was limited to taxa within a closely related group. For many of the *Gethyllis sp*. only partial ITS sequences were retrieved, and including these taxa despite partial sequences was considered a more beneficial trade-off than deleting them from the dataset entirely.

Of the five individual gene analyses, the *rpoB-trnC* intergenic spacer region produced the best-resolved tree and the *psbA-trnH* region the poorest resolved tree. In a recent study of Aizoaceae using *psbA-trnH,* Klak *et al.* (2004) found informative variation in this group, whereas Khunou (*pers. comm.*) found little or no informative variation in her study of *Carpobrotus*, a genus belonging to the same family using the *psbA-trnH* region. However, informative variation was reported for this region in studies by Miller *et al.* (2003) (Fabaceae, Solanaceae), Aldrich *et al.* (1988) (Fabaceae) and Sang *et al.* (1997) (Paeoniaceae).

The phylogenetic tree produced with the combined plastid matrices is better resolved than the tree produced with the combined plastid and nuclear analysis especially as regards the *Gethyllis-Apodolirion* clade. This may be attributed to the fact that amplification for 11 species of *Gethyllis* was unsuccessful for the ITS 2 region and only partial sequences were used in the analysis.

Haemanthus and *Scadoxus* are both monophyletic and resolve as sister clades in the combined plastid (Fig 2.6) and combined plastid and nuclear (Fig 2.7) analyses. These results compare favourably with the investigations of Ito *et al.* (1999). They sampled 31 of the 59 genera in Amaryllidaceae, including *Clivia*, *Haemanthus* and *Scadoxus* for the matK gene. Their results resolved two African clades, with *Clivia*, *Haemanthus* and *Scadoxus* (72% bootstrap support) in one, and *Haemanthus* and *Scadoxus* resolving as sister genera with 98% bootstrap support in the parsimony analysis. Using *trnL-F* and *ITS* sequence data, Meerow and Clayton (2004) also resolved *Haemanthus* and *Scadoxus* as sister genera in both the ITS and combined analyses.

In previous classifications (Björnstad and Friis 1972a, Björnstad and Friis 1972b), *Haemanthus* and *Scadoxus* were considered to be one genus but subsequently they have been split into two separate genera on grounds of morphology (differences in the leaf foliage, Friis and Nordal 1976) and cytology (*Scadoxus* has a chromosome number of x=9 and *Haemanthus* x=8, Vosa and Marchi 1980). In all analyses, both combined and separate plastid and nuclear analyses, the molecular data supported the reciprical monophyly of *Haemanthus* and *Scadoxus*.

Of the 21 *Haemanthus* species used in this study, 15 occur in the winter rainfall region and only six in the summer rainfall region. The summer rainfall *Haemanthus* species (*H. humilis* subsp. *humilis*, *H. humilis* subsp. *hirsutus*, *H. pauculifolius*, *H. deformis*, *H. carneus*, *H. albiflos*) with the exception of *H. montanus,* consistently group together in all the analyses. This split between Eastern Cape (summer rainfall) and Western Cape (winter rainfall) species of *Haemanthus* was also observed in the phylogenies of Meerow and Clayton (2004). The four species of *Haemanthus* used in their study form two clades - one consisting of summer rainfall species and the other winter rainfall species.

In southern Africa, there is an east to west gradient in the severity of the summer drought; for several groups (*Pelargonium* - Bakker *et al.* (2003); *Moraea* - Goldblatt *et al.* (2002)) the greatest diversity and apparently the most recent radiations are situated in the more arid west (Linder 2005). Richardson *et al.* (2001) reported that diversification of the species-rich genus *Phylica*, a predominantly Cape based genus of about 150 species, began approximately 7-8 Ma ago (late Miocene). Klak *et al.* (2004) showed recent (3.8-8.7 Ma ago) diversification in the Aizoaceae, which dominate the Succulent Karoo (an arid region along the west coast of southern Africa) in terms of species numbers and density of coverage. Goldblatt *et a.l* (2002) inferred from the dates on their phylogenetic tree that the main clades of the genus *Moraea,* one of the largest South African plant genera of the Iridaceae, evolved before the end of the Miocene. With regards to *Haemanthus*, Snijman (1984) postulated that taxa requiring summer rain were restricted eastwards, while the increasing aridity in the west favoured rapid speciation and gave rise to many of the specialized features in the species in that area.

One such specialization is the presence of large (100-130mm in diameter) bulbs with distichous tunics that sheathe only at the base. Three genera exhibit bulbs: *Haemanthus*, *Gethyllis* and *Scadoxus*. It is unclear whether bulbs form in *Scadoxus* only under certain conditions or if bulb formation is limited to certain species (Meerow and Clayton 2004). *Haemanthus* and *Gethyllis* are most species-rich in areas with hot, dry summers and the presence of this underground organ for the storage of food reserves is essential for surviving seasonal conditions unfavourable for growth. Plants that have adopted this strategy produce seasonal flushes of above ground parts

that last through the growing season but die back completely to the subterranean perennating parts during periods of unfavorable growth. Procheş *et al.* (1995) showed that larger storage organs are more likely to occur in the Cape Floristic Region where rainfall is less abundant or less predictable although other factors may also influence this. *Haemanthus* for example, although shown to possess large bulbs, vary depending on where they are found. *Haemanthus* species in the eastern parts generally have smaller bulbs (±80mm in diameter) than the species in the west. While the main function of geophytism is that of water storage (Rees 1989) it is clear that geophytism also presents an advantage in mineral ion storage (Ruiters and McKenzie 1994). Even though my analysis suggests that the rhizome is a derived character it is important to note that in terms of the family Amaryllidaceae as a whole and its outgroups Agapanthaceae and Alliaceae, it is not. Meerow *et al.* (1999b) consider the rhizomatous condition to be the plesiomorphic state for the Amaryllidaceae. In contrast to the bulbous species of Haemantheae, only one rhizomatous species (*Clivia mirabilis*) is found in the semi-arid northwestern Cape, suggesting that this feature has been evolutionary unsuccessful in that region. This lends support to the hypothesis that in *Haemanthus* and *Gethyllis*, occupying predominantly the arid west regions, the evolution of the derived state of bulbs has been an adaptation that has been pivotal to their success.

The prostrate leaf syndrome, characterized by few (often two) flattened leaves, pressed to the ground, is present in *Haemanthus* (Snijman 1984, Esler *et al.* 1999) as well as in *Gethyllis*.This strategy has been interpreted as advantageous in avoiding herbivory or competition from neighbours, in creating a CO_2-rich environment underneath the leaves, or in regulating evapotranspiration (Esler *et al.* 1999). Cramer *et al.* (2007) investigated the utilization of soil derived CO_2 for photosynthesis and found that the prostrate leaves of *Brunsvigia orientalis* (Amaryllideae) allowed a small proportion of the total photosynthetic CO_2 to be derived from the soil, and consequently reduced water loss. Coiled wiry leaves (*Gethyllis villosa, Gethyllis afra*) are almost exclusively associated with sparse vegetation on rocky sites, especially gravel plains. Coiling may well be an adaptation to reduce wind damage to the relatively large photosynthetic surface required to maximize photosynthetic activity in the cool growing season of the winter-rainfall region of southern Africa (Procheş *et al.* 2006).

Leaves of *Haemanthus* and *Gethyllis* species are frequently pubescent, unlike the species of the other genera where leaves are glabrous. It is thought that leaf pubescence is advantageous in Haemantheae, and the family as a whole where species occur in semi-arid conditions, as the silvery white appearance causes a large proportion of the light to be reflected from the leaves keeping transpirations and therefore water loss, to a minimum; more experimental evidence for this is needed (Manning *et al.* 2002). In *Gethyllis* two different trichomes are present: simple and T-shaped (Weighlin 2002). The T-shaped trichomes are two-armed and equal-armed and are unique to the family. They occur in groups of 2-3 in *G. barkerae* and *G. villosa* (*G. barkerae* was included in this study) and in groups of 5-6 or more in other species (Weighlin 2002).

In a synanthous phenology, leaves are present at flowering whereas with the hysteranthous condition the flowers and leaves appear in separate seasons (Dafni *et al.* 1981b). The difference between synanthous and hysteranthous geophytes has been attributed to the need to optimise the allocation of resources under different environments (Dafni *et al.* 1981b). Procheş *et al.* (2006) found this to be true in their study of a few Cape geophytes (*Geissorhiza*, *Gladiolus*, *Moraea*, *Watsonia*, *Eriospermum*, *Cyrtanthus*, *Haemanthus*) where hysteranthous species occured mainly in areas of scarce and more strictly seasonal rainfall. In this study too, *Haemanthus* and *Gethyllis* species that are distributed mainly in the arid west regions of southern Africa exhibit hysteranthy. Through the absence of leaves during flowering and fruiting, the main storage function has therefore been transferred from the leaves to the bulb, an adaptation adopted by plants exposed to extreme conditions as a survival strategy. It is possible that in the western arid regions of South Africa, hysteranthy enabled *Gethyllis* and *Haemanthus* not only to survive the hot dry summers, but also to thrive.

Burtt (1970) proposed that the subterranean ovary was a preadaptation to the hysteranthous leaf habit. However, the results (Fig. 2.10 (5) and Fig. 2.10 (7)) show that this sequence is not unequivocal. Two scenarios are possible: either the hysteranthous habit evolved earlier than the subterranean ovary and manifested itself in many species of *Haemanthus* and all species of *Gethyllis* and *Apodolirion* or else

the hysteranthous habit evolved in parallel in *Haemanthus* and the *Gethyllis*-*Apodolirion* clade. It is thus not possible to determine whether the change to the hysteranthous habit preceded the evolution of the subterranean ovary or not.

Johnson and Bond (1994), in their study of flowers and butterfly pollination in the South African fynbos, recognize four floral types: classic-type, brush-type, flag-type and horizontal in their investigations into red flowers and pollinators. Both *Haemanthus* and *Scadoxus* exhibit red, brush-type flowers which is an effective character in butterfly pollination. Red flowers are typical of bird-pollinated flowers and although Johnson and Bond (1994) state that this floral morphology excludes birds, Manning *et al.* (2002) report visits to red-flowered species of *Haemanthus* by sunbirds and the large satyrid butterfly known as the Pride of Table Mountain, *Aeropetes tulbaghia*. Brush-type flowers are effective as they exploit the habit of the *Aeropetes* butterfly of swooping down on flowers without landing. During these 'inspection' visits which outnumber feeding visits, pollen is brushed on to the butterfly (Johnson and Bond 1994). The pink flowers of *Haemanthus* are probably pollinated by bees (Snijman 1984, Manning *et al.* 2002).

It is interesting to note that there is a higher allocation of resources for the production of seedlings in terms of the presence of numerous ovules in *Apodolirion*, *Gethyllis*, where solitary flowers are present, and *Cryptostephanus*. In genera where flowers are many (*Clivia, Haemanthus, Scadoxus*), fewer than ten ovules are present in the ovary. It is also possible that the chances of the summer flowering *Gethyllis* being pollinated in the summer-dry areas are less than those of *Haemanthus* (autumn flowering in the summer-dry area to early summer flowering in the summer-rainfall region), therefore many seeds can potentially be produced from relatively few visits to a single flower that has many ovules per ovary.

In the combined plastid and combined plastid and nuclear trees, *Apodolirion* is firmly embedded in the *Gethyllis*-clade. The analysis resolves *Apodolirion* as polyphyletic and embedded within *Gethyllis*. The shared morphological characters between *Gethyllis* and *Apodolirion*, coupled with the molecular evidence unequivocally suggest that this clade represents a single genus in need of reclassification. Doubt about the distinctiveness of these two genera was repeatedly expressed by Traub

(1963), Wilsenach (1965) (based on karyology), Hilliard and Burtt (1973) and more recently by Meerow and Clayton (2004).

Optimisation of the character 'anther number' (Fig. 10 (10)) only partly retrieves the "afra' and "villosa" groups. This is the strongest evidence so far for not recognising this infrageneric taxonomic division. The "afra" group usually has more than six filaments and anthers per flower, the style is straight and has a small stigma, and the flowers are fleshy, succulent and cup shaped. The "villosa" group has six simple filaments and six anthers, the style is curved sideways and has a large capitate stigma, and the flowers are thin textured and salver shaped. My results (combined plastid analysis) partially retrieve the "afra" group (*G. ciliaris* subsp. *ciliaris, G. undulata, G. ciliaris* su*bsp. longituba, G. campanulata, G. sp2, G. multifolia, G. afra, G.* sp1, *G. britteniana* subsp. *bruynsii, G britteniana* subsp. *britteniana*). There are two exceptions. *G.* sp.2 and *G. gregoriana,* which unlike the other *Gethyllis* species with six stamens, have a straight, central style. This condition seems to be independently derived in *G.* sp.2, unlike in *G. gregoriana* which resolved with *Apodolirion cedarbergense* in a separate clade. The affinity between *Apodolirion cedarbergense and G. gregoriana* has never been considered before.

The "villosa" group (*G. linearis, G. marginata, G. barkerae* subsp. *barkerae, G. lata* subsp. *orbicularis, G. lata* subsp. *lata, G. verrucosa, G. roggeveldensis, G. transkarooica, G. lanuginosa, G. cavidens* and *G. verticillata*) formed a distinct clade, with the curved style as synapomorphic. It is interesting to note that *G. namaquensis,* a species previously placed in the monotypic genus *Klingia,* is placed as sister to the 'villosa' group. *Klingia* was separated from *Gethyllis* on the basis of its wide cup or corona formed by the fusion of six staminal filaments (Obermeyer 1980), a structure not found in *Gethyllis* as it was understood at the time (Schönland 1949). Its position precludes the recognition of formal subgeneric groups in a combined *Gethyllis-Apodolirion*.

In the combined plastid and combined plastid and nuclear analyses with parsimony and maximum likelihood, *Cryptostephanus* resolved as sister to *Clivia* forming a clade of rhizomatous genera that never form bulbs. In addition to the absence of a bulb, *Cryptostephanus* exhibits the presence of the phytomelanous testa which

Meerow and Clayton (2004) consider to be plesiomorphic. Since phytomelan encrusted seeds is an ancestral state of the order Asparagales, Meerow *et al.* (1999b) inferred that this trait has been lost five times in the family Amaryllidaceae. *Cryptostephanus* is the only genus of the tribe to retain this phytomelanous state (Meerow and Clayton 2004, Rasmussen *et al*. 2006).

In their study of the tribe Amaryllideae, Snijman and Linder (1996) suggested that seedling vigor, associated with green embryos (all genera in the Amaryllideae possess a green embryo), probably contributed to widespread radiation of many ancestral species of the group throughout sub-Saharan Africa during the early Tertiary when seasonally moist subtropical/tropical conditions prevailed. They postulated that a key innovation was the development of a seed with a large green integument and stomatose testa within the subtribe Amaryllidinae during the subsequent aridification of Africa and the inception of extreme summer aridity in south western Africa during the Pleistocene (Snijman and Linder 1996). Four genera in the Haemantheae *Gethyllis, Apodolirion, Haemanthus* and *Scadoxus* share the synapomorphic condition of green embryos. Two of these genera are species-rich and occur predominantly in the semiarid winter rainfall region of south western Africa. As in the Amaryllideae, this adaptation of a large, water-rich seed with a green embryo possibly contributed to the success of these genera during the change to increasingly severe environmental conditions in south western Africa.

A chromosome number of $x = 11$ is considered plesiomorphic in Amaryllidaceae due to its broad occurrence in many of the tribes of the family (Goldblatt 1976, Meerow 1984). *Clivia*, with somatic chromosome number x=11, resolved in combined plastid and plastid and nuclear analyses as sister to *Cryptostephanus,* which is characterized by a somatic chromosome number of x=12 (Gouws 1949). Vosa and Snijman (1984) found *Haemanthus* to be chromosomally uniform. In addition, it has been shown that *Haemanthus* originated from the genus *Scadoxus* by a chromosome translocation which resulted in a disploid reduction of the chromosome number from x=9 to x=8 (Vosa and Snijman 1984, Vosa and Marchi 1980).

Givnish *et al.* (2006) in their study of the phylogenetic relationships of monocots and the occurrence of concerted convergence, comment on net venation and fleshy fruits

evolving simultaneously (i.e. at the same node) in many taxa (Arecales, Zingiberales, Amaryllidaceae). In other cases the evolution of fleshy fruits slightly lags behind that of net venation. In Haemantheae the evolution of fleshy fruit precedes the evolution of net venation, a character limited to *Scadoxus*. Their study shows that fleshy fruits and net venation have even stronger patterns of correlated evolution with shady conditions than with each other. In almost every case, the evolution of net venation and fleshy fruit is associated with life in forest understories, whereas their loss is associated with open habitats (Givnish *et al.* 2006). While net venation in *Scadoxus* is associated with forest habitat, not all the forest dwelling species in Haemantheae have this, e.g. some species of *Clivia.* These, however, may occupy the partially open places in forests.

2.5. Conclusion

The molecular data (plastid and nuclear) have clearly defined the generic delimitations within the tribe, producing a well-resolved phylogeny with the comprehensive sampling used in this analysis. This phylogeny is consistent with generic delimitations as proposed by Meerow and Clayton (2004). In summary the combined analyses resolve *Haemanthus* and *Scadoxus* as reciprocally monophyletic and the *Haemanthus*-*Scadoxus* clade sister to a clade comprising *Apodolirion* and *Gethyllis*. A polyphyletic *Apodolirion* is firmly embedded within *Gethyllis*. *Clivia* and *Cryptostephanus* comprise the second, smaller clade and are both monophyletic. These results therefore support the recognition of the three subtribes in Haemantheae: Cliviinae D. &U. M.-D., Haemanthinae Pax, and Gethyllidinae Dumort as proposed by Meerow and Clayton (2004).

Based on morphological grounds and the molecular results produced here, it is recommended that the status of *Apodolirion* as a separate genus be changed and that it be sunk into *Gethyllis*.

Particular plant traits have been identified that can be associated with *Haemanthus* and *Gethyllis,* the two species-rich genera of the tribe: the presence of a bulb, leaf pubescence and the occurrence of annual leaves, hysteranthy and the presence of

green embryos. These specializations equip plants to survive unfavourable conditions in the arid west regions of southern Africa and may have contributed to radiation of these genera.

CHAPTER 3. BIOGEOGRAPHICAL PATTERNS AND ESTIMATED DIVERGENCE TIMES WITHIN HAEMANTHEAE

3.1. Introduction

The potential geographic range of any taxon is limited by the suitability of environmental factors. Within these zones of ecological tolerance, historical elements also shape geographic distribution. The success of countless alien plants and animals around the world is testimony to the fact that not all habitats suitable for a species are naturally occupied by that species. Thus, whether or not a species occurs in a particular area is a function not only of ecology, but also of historical demographic and dispersal patterns, which themselves are influenced by the proximity and spatial relationships of habitable environments. The study of such historical factors is the focus of molecular biogeography (Avise 2004).

According to Weimark (1941), Levyns (1952), Axelrod and Raven (1978) and Taylor (1978), the Cape Floristic Region is considered to be one of the oldest on the African continent, and possibly covered a much wider area in the past than it does now. Hypotheses to explain its origin are numerous and have been the subject of many debates. One of the core bulbous families within the Cape Floristic Region is the Amaryllidaceae, with 59 genera and about 850 spp worldwide, and 16 genera and 103 species in the Cape (Manning *et al.* 2002). This monocotyledonous family has notable centres of diversity in South America and Africa, with a further eight genera around the Mediterranean (Meerow and Snijman 1998). Only a single genus *Crinum* (± 65 species (Fangan and Nordal 1993); tribe Amaryllideae), with seeds well adapted for oceanic dispersal (fleshy, floating and salt resistant), is represented in both the Old and New Worlds (Africa, Asia, America, and Australia).

Evidence from DNA sequence data places the most ancient (though not necessarily primitive) lineages within the family in Africa (Fay *et al.* 1995, Meerow *et al.* 2000b), which would suggest a West Gondwana origin. Climatic changes in southern Africa (Goldblatt 1978) and geologic changes in South America (Meerow 1989) are thought

to have been important factors in the radiation of the family within its two main centres of diversity (Meerow and Snijman 1998).

The African tribe Haemantheae is one of eight within the Amaryllidaceae. It has an interesting distribution pattern in that the genera represent elements from both the Cape Floristic Region and tropical Africa, contributing to the debate on the historical affinities of the Cape clades (Galley and Linder 2006). Distribution information (with maps) is discussed in Chapter One. Only one other biogeographic study of the Haemantheae by Meerow and Clayton (2004) has been carried out. In their analysis, which included 19 of the ca. 78 species, they found the tribe to have an eastern South African origin, with several subsequent dispersal events to the winter rainfall region.

The first objective of this chapter is to investigate the biogeographical patterns of the tribe Haemantheae, to propose an hypothesis to explain its distribution and to assess the geographical origin of the tribe using the dispersal-vicariance method (DIVA; Ronquist 1996).

The time and place of the first appearance of the flowering plants have long been debated. There is good fossil evidence that early angiosperms, including a number resembling modern magnolias (*Magnolia*), were present in the Early Cretaceous, 130 million years ago (Ma), in the tropics of southern Laurasia and northern Gondwana (MacDonald 2003, Barry-Cox and Moore 2005). Evidence of these earliest angiosperms comes from fossilized leaves, stems, fruits, pollen and (very rarely) flowers. There has been much study of modern plant morphology and genetic structure in order to determine which living species might be closely related to the ancient ancestors of the angiosperms. Despite intensive efforts for over 200 years, scientists have still not reached consensus on which type of plant was the ancestor to the angiosperms, and where and when the angiosperms first evolved (MacDonald 2003).

Many of the modern plant families appeared in the Middle to late Cretaceous. From the Early Tertiary, angiosperms have diversified to comprise 90% or more of the Earth's total flora today (MacDonald 2003).

In the absence of fossil records, phylogenetic trees based on DNA sequence data enable estimation of divergence times under the assumption that sequences have a uniform rate of evolution. In these circumstances, a known date of divergence for a given pair can then be used to calculate a rate of substitution (the calibration rate), which can be applied to dating other nodes (Rambaut and Bromham 1998). However, the use of a molecular clock to place absolute dates on lineage divergence times is limited by the validity of applying a calibration rate from one part of the tree to date other nodes. Uniform rates of change across a tree cannot be assumed as lineage specific rate variation has been demonstrated for many taxonomic groups including plants (Gaut *et al*. 1992). Incorrectly assuming the clock may lead to spurious date estimates (Takezaki *et al*. 1995), and thus any analysis must incorporate explicit means to evaluate rate constancy, and, if necessary, to deal with rate-variable data. Graur and Martin (2004) further discusses the generation of inaccurate divergence time estimates, due to improper methodology, based on a single calibration point.

Various authors have proposed alternative algorithms that are designed to produce ultrametric trees without assuming a global molecular clock (Sanderson 1997; Rambaut and Bromham 1998; Thorne *et al*. 1998; Huelsenbeck *et al*. 2000). One method for producing an ultrametric tree is Sanderson's nonparametric rate smoothing (NPRS) method, which assumes that evolutionary rates are autocorrelated in time (Sanderson 1997). This means that substitution rates are assumed to be inherited and limits are put on the rate changes from an ancestral to a descendant lineage (Sanderson 1997). For each branch in a given tree the local rate of molecular evolution is estimated, and then the sum of the differences between the local estimated rates is minimized for ancestor and descendant branches across the tree (Sanderson 1997).

A second method, a relaxed phylogenetics approach in which the phylogeny and divergence dates are co-estimated under a relaxed molecular clock, is implemented in the application BEAST (Bayesian Evolutionary Analysis Sampling Trees) (Drummond and Rambaut 2006). The Bayesian relaxed clock method models the molecular rate among lineages as varying in an auto correlated manner, with the rate in each branch being drawn (*a priori*) from a parametric distribution whose mean is a function of the rate on the parent branch.

The second objective of this chapter is therefore to use Sanderson's method on nonparametric rate smoothing (NPRS; Sanderson 1997) and a relaxed molecular clock (Drummond and Rambaut 2003; BEAST) to estimate a divergence time for the tribe Haemantheae in order to consider whether there is evidence of rapid diversification in the winter rainfall lineages of genus *Haemanthus* and the genus *Gethyllis,* whether the onset of radiation of the two winter rainfall clades occur at the same time, and if so, whether the rate of acceleration coincides with a change in climate from moist, equable conditions to dry, summer conditions. In addition, a comparison of the estimated divergence times of the summer versus the winter rainfall clades will be undertaken.

3.2. Materials and Methods

3.2.1. Dispersal vicariance analyses

To assign distributions to the internal nodes in the tree, the DIVA (dispersal vicariance analysis) programme was used (Ronquist 1996, 1997). The programme optimises distributions for each node of the tree by favouring vicariance events and minimising the number of assumed dispersals and extinctions. Between the nodes of the given tree, DIVA assigns a cost of one to changes in distribution interpreted as due to extinction or dispersal but no cost to changes caused by vicariance events (Ronquist 1996, 1997).

Distribution data are based on herbarium specimens housed at the Compton Herbarium (NBG), Cape Town and the National Herbarium (PRE), Pretoria. Twelve areas in total were defined. Areas within the Cape Region were defined according to the six phytogeographic centres of Goldblatt and Manning (2000) and Manning *et al.* (2002) and areas outside the Cape Region were based on the definition of Goldblatt *et al.* (2002) (Table 3.1).

The resulting distribution matrix, containing 62 taxa (outgroups excluded) and 12 areas, was too large to be read by DIVA (error message reported). For this reason the optimization was performed in three steps. Analysis A and Analysis B (Figure 3.1)

were processed separately and subsequently reduced to single branches and the results used as input in Analysis C. For simplicity the tree subdivisions were carried out according to the clades representing genera. Analysis A represents the genus *Haemanthus*, Analysis B the *Apodolirion*-*Gethyllis* clade and Analysis C involved the three outstanding genera plus Analysis A and B, reduced to a single branch each. For all analyses (A, B and C), optimizations were performed using a limit of eight areas (option 'maxareas=8' in command 'optimize') to reduce ambiguities at more basal nodes of the tree. One of the most parsimonious trees from the combined plastid and nuclear analysis (see Chapter two) was arbitrarily chosen for the optimization of the 12 geographical areas. DIVA requires a fully bifurcated tree and since this tree contained three trichotomies, these were arbitrarily resolved in MacClade (version 4.01; Madison and Madison 2001). The results in the deeper nodes of interest here were not affected by arbitrarily resolving the trichotomies because the three nodes where these polytomies are found (all in Analysis A) gave the same optimizations regardless of which topology was used.

The topology used here is an incomplete species level phylogenetic analysis. There is little relevance in assessing the ancestral distributions of nodes close to the terminals because these are likely to change with the addition of taxa, thus only deeper nodes will be discussed further. However the sampling is representative of all six *Clivia* species, with populations from the most northerly range in Swaziland/Mpumalanga, the southerly Eastern Cape and the westerly Nieuwoudtville, Northern Cape regions included in the study.

Further DIVA analysis was carried out using delineated rainfall regions in southern Africa in an attempt to better clarify the biogeography of Haemantheae (sources: School of Bioresources Engineering and Environmental Hydrology, University of Natal Pietermaritzburg, South Africa and Climatological Atlas of Africa (Jackson 1961)). Seven areas were defined (Figure 3.2) and the resulting distribution matrix, containing 62 taxa (outgroups excluded) was once again too large to be read by DIVA (error message reported). Again the optimization was performed in three steps as with the previous DIVA analysis (Figure 3.1).

Table3.1: Phytogeographic areas used in DIVA analysis (based on Goldblatt and Manning (2000), Manning et al. (2002))

DIVA	Centres	Definitions
A	Namaqualand and southern Namibia (NAM)	Namaqualand and southern Namibia
B	Northwestern Cape (NW)	Bokkeveld, Gifberg, Cedarberg and Piketberg to Hex River and western Langeberg
C	Southwestern Cape (SW)	West coast, Hottentots Holland, Riviersonderend, Klein River, part of Bredasdorp plains, Potberg
D	Langeberg (LB)	Langeberg and part of the plains south of Langeberg
E	Agulhas plain (AP)	South coast from Gansbaai to Mosselbaai,
F	Southeastern Cape (SE)	Outeniqua, Tsitsikamma, Baviaanskloof to Great Winterhoek, coast from Mosselbaai to Port Elizabeth
G	Karoo Mountains (KM)	Witteberg, Klein and Groot Swartberg, Kamanassie, western part of Baviaanskloof
H	Roggeveld Centre (RC)	Roggeveld Escarpment and Tanqua Karoo
I	Eastern South Africa (ESA)	Port Elizabeth to KwaZulu-Natal
J	Northern South Africa (NSA)	KwaZulu-Natal to Limpopo River (South Africa-Zimbabwe border), including Swaziland, Mpumalanga, Waterberge, Magaliesberge
K	Southern tropical Africa (STA)	Limpopo River to northern border of Kenya
L	Central South Africa (CSA)	Great Karoo to border of Botswana

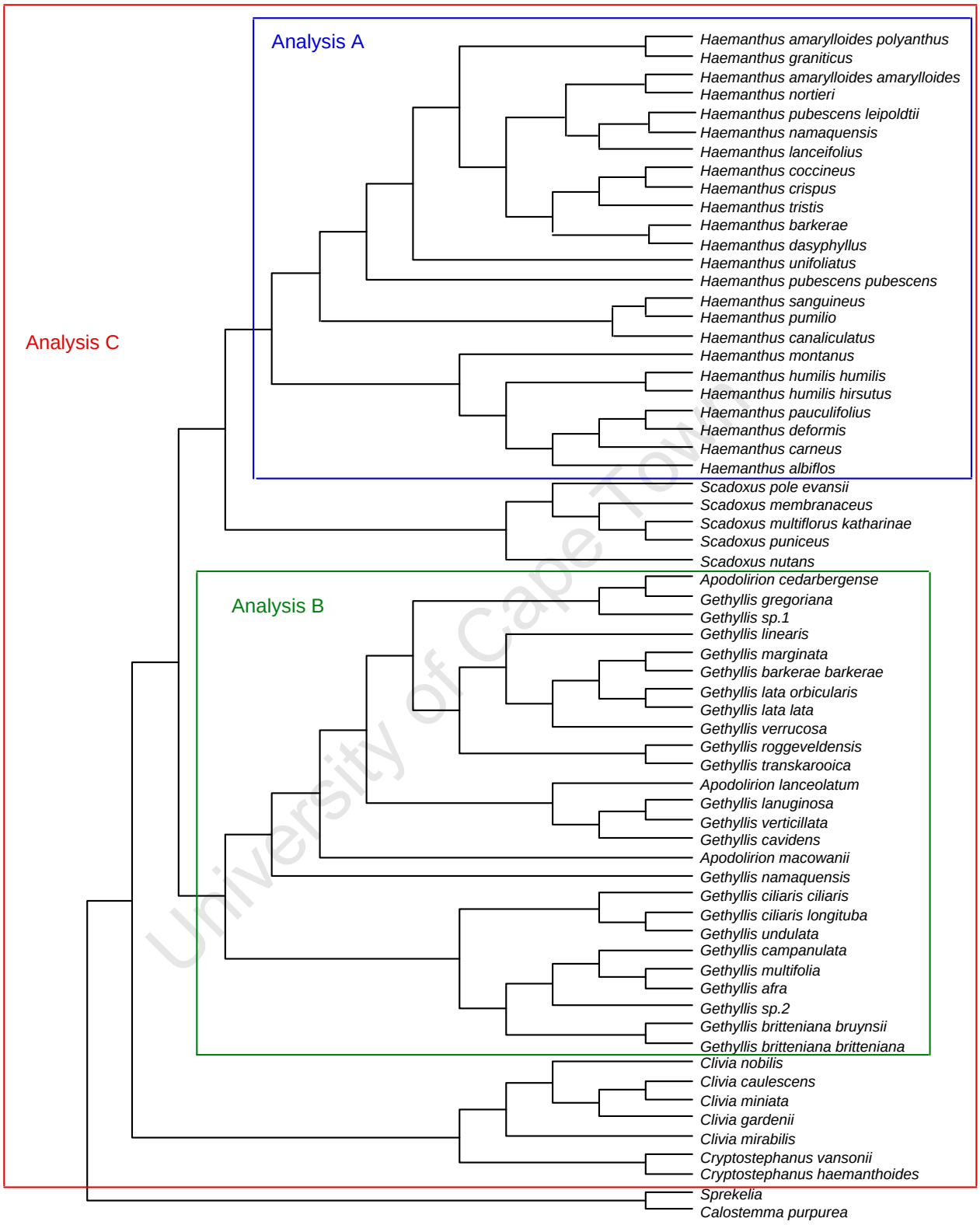


Figure 3.1 An illustration of Analysis A, B and C

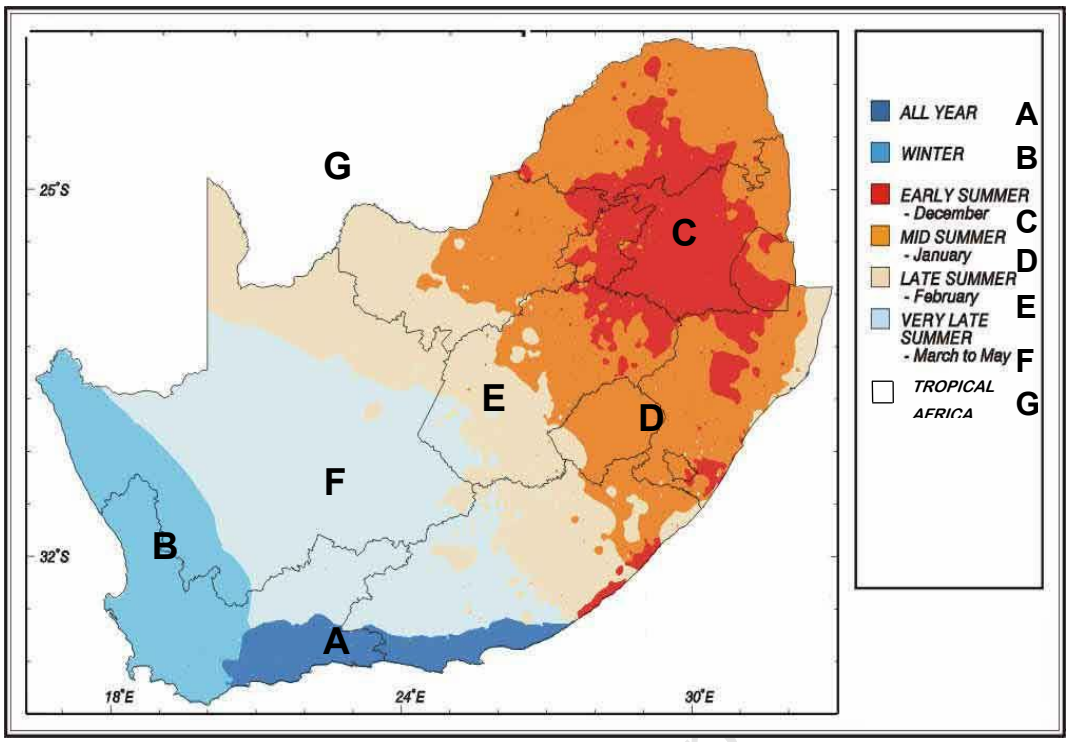


Figure 3.2: Map of rainfall seasonality of South Africa, Swaziland and Lesotho with rainfall regions used in DIVA analyis indicated. (Adapted from source- School of Bioresources Engineering and Environmental Hydrology, University of Natal Pietermaritzburg, South Africa)

3.2.2. Dating

Phylogenetic dating requires at least one reference node calibrated by at least one reference fossil (or other calibration points such as oceanic islands, other molecular estimates, etc). The reference node(s) is used for rate calibration and, with an observed rate for the tree (or part of the tree), the age of other nodes of the tree may be estimated. Furthermore, the reference fossils (or other calibration points) must be sufficiently well known to be attached to a given node of the phylogeny with confidence.

Due to the absence of fossils for Amaryllidaceae, the only possible approach is to use results from previous dating of the angiosperms. In 2001, Wikström *et al.* carried out a global analysis of angiosperm families using all available fossil data and estimated divergence times based on divergence in DNA sequence data using non-parametric rate smoothing. This provides a context for at least estimating the likely age of a node.

One of the most parsimonious trees found in the combined analysis based on the five DNA regions was arbitrarily chosen to infer divergence times - the same tree used for the dispersal-vicariance analyses (see above). An estimate from Wikström *et al.* (2001) was used, where they reported the split between *Clivia* (the only genus of the tribe Haemantheae represented in their study) and *Hippeastrum* to be 33 Ma. *Clivia* and *Hippeastrum* represent the family Amaryllidaceae in their study. This estimate was used as the calibration point of the stem node of Haemantheae.

Rate heterogeneity across lineages was evaluated using the likelihood ratio test (Felsenstein 1988). The comparison of the difference between the likelihood scores of the tree with and without an enforced molecular clock, multiplied by two, was compared with a χ^2 distribution. For a fully resolved tree (no polytomies), the number of degrees of freedom is *N*-2, where *N* is the number of taxa, and corresponds to the number of internal branches (Saunderson and Doyle 2001). Three trichotomies are found in the tree used and the number of degrees of freedom is therefore 59 (*N*=64-2-3). The likelihood ratio test showed highly significant rate heterogeneity among lineages for this data set ($p<0.001$).

Maximum likelihood (ML) branch lengths were optimised for all DNA regions simultaneously using the general time-reversible (GTR) model of DNA evolution, determined to be the best model of DNA substitution by the software Modeltest (version 3.6; Posada and Crandall 1998); all models tested by default by the programme were considered. Proportion of invariable sites and an alpha shape parameter for the gamma distribution to account for among-site rate heterogeneity were estimated from the data (Yang 1993; GTR+ I+Γ). Values estimated from the data, as provided by Modeltest, were used for the optimization of branch lengths. Only ML branch lengths were calculated because the maximum parsimony optimization does not take into account the uncertainties in the mapping estimation (Nielsen 2002). The resulting tree was made ultrametric using the NPRS method (Sanderson 1997) as implemented in the programme TreeEdit v1.0a10 (Rambaut and Charleston 2001; available at http:/evolve.zoo.ox.ac.uk/software/TreeEdit). Confidence intervals (95%) on the age

estimate for each node were also calculated. 100 bootstrap trees were generated in PAUP*. Using TreeEdit (Rambaut and Charleston 2001) 100 mean branch lengths from the terminals to the nodes were obtained for each node. The standard deviation of the 100 mean branch lengths for each node provides the standard error in mean branch length due to character sampling.

For the relaxed molecular clock, the ages of the most recent common ancestors (MRCAs) were estimated using Bayesian inference as implemented by the programme BEAST (version 1.4; Drummond and Rambaut 2006). The date estimates were made under a general time-reversible (GTR) model of nucleotide substitution. Rate variation among sites was modeled using a discrete gamma distribution with four rate categories. Parameters were sampled once every 1000 generation for 1,000,000 Markov Chain Monte Carlo (MCMC) steps for six separate analyses optimizing parameters as suggested after every analysis. A final analysis of 10,000,000 MCMC steps was carried out and convergence of the chains to the stationary distribution was checked by visual inspection of plotted posterior estimates using the programme TRACER (version 1.4; Drummond and Rambaut 2004). The effective sample size (ESS) of the sampled parameters was well in excess of 100, an indication that the MCMC chain has been run long enough. The information from a sample of trees produced by BEAST was summarized onto a single target tree in Tree Annotator (http://beast.bio.ed.ac.uk/) and viewed, including summary information, in Fig Tree (http://beast.bio.ed.ac.uk/FigTree).

3.3. Results

3.3.1. Dispersal-vicariance analyses

The results of the dispersal-vicariance optimization for the first DIVA analyis are shown in Figure 3.3. For Analysis A, 57206 alternative equally optimal reconstructions, involving six dispersals and three vicariance events, were obtained and the root node was assigned only one optimization with a combination of 11 areas. The ancestral origin of the summer rainfall clade is assigned a combination of three areas: Eastern South Africa (ESA), Northern South Africa (NSA) and Central South Africa (CSA). For Analysis B,

DIVA produced 16 alternative equally optimal reconstructions involving three dispersal and one vicariance event, with the root node assigned one optimization with a combination of 11 areas. Analysis C produced 8192 alternative equally optimal reconstructions involving no dispersal or vicariance events. The root node was assigned one optimization containing a combination of 12 areas. *Scadoxus* is rooted in Southern tropical Africa (STA) and the ancestral origin of *Clivia* and *Cryptostephanus* were assigned three possible optimizations: North Western Cape (NW) /South Tropical Africa (STA), Eastern South Africa (ESA)/ South Tropical Africa (STA) and North Western Cape (NW)/, Eastern South Africa (ESA)/ South Tropical Africa (STA).

In the second DIVA analysis (Figure 3.4), Analyis A produced 35 alternative, equally optimal reconstructions with four dispersal events and five vicariance events (involving more than two areas). The root node of *Haemanthus* is assigned three different optimizations. Analysis B produced only one optimal reconstruction, with one dispersal event and no vicariance events. The root node is assigned one ancestral area, the winter rainfall region (B). For Analysis C, 1728 alternative equally optimal reconstructions were produced with 20 dispersal events and 136 vicariance events, involving more than two areas. The root node is assigned one optimizations, with a combination of all seven areas.

Scadoxus is assigned one possible optimization: Tropical Africa (G). The *Gethyllis-Apodolirion* clade also shows one possible optimization for its ancestral area: Winter (B).

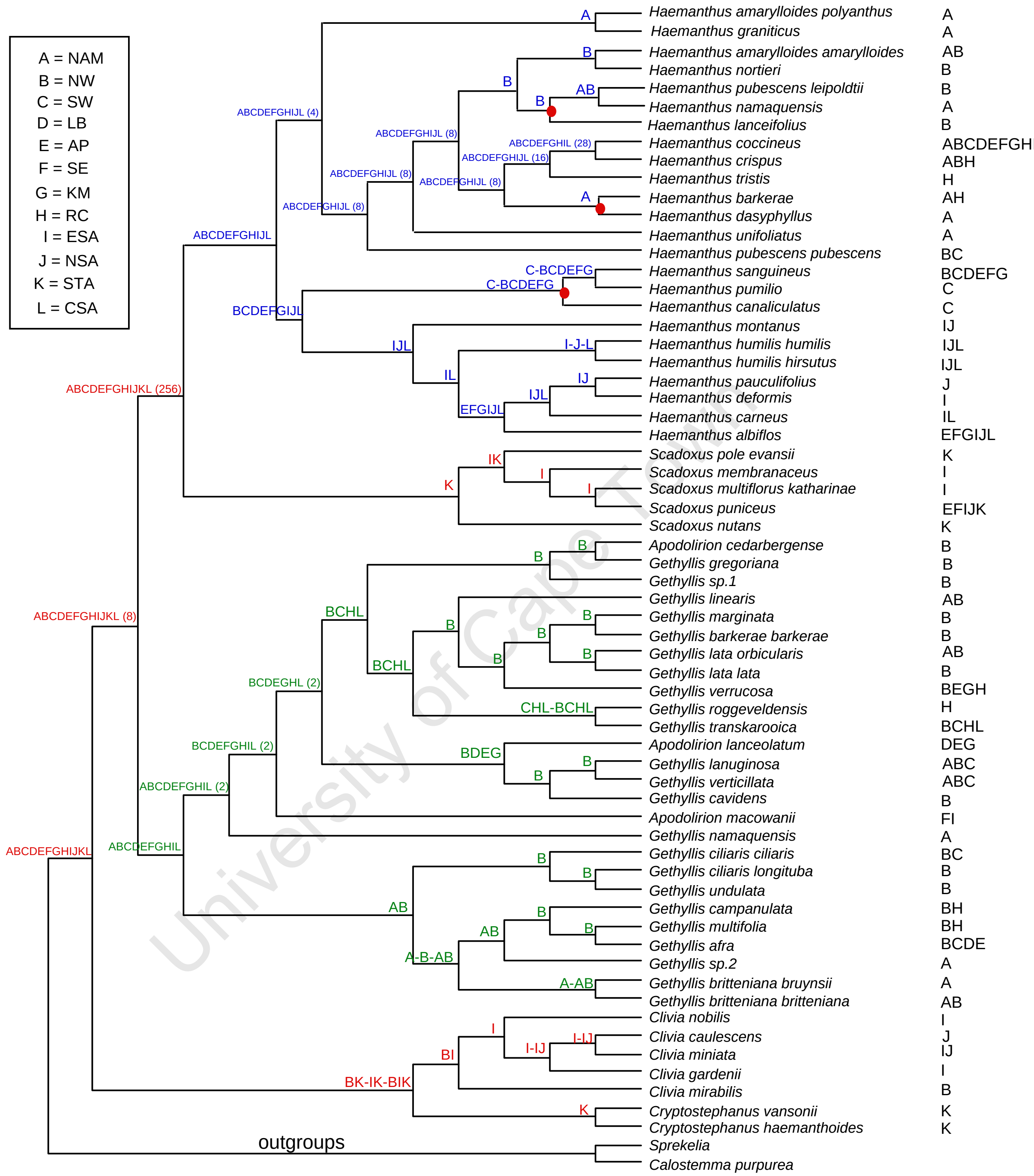


Figure 3.3: Optimization of ancestral areas. Red dots indicate clades that resolved arbitrarily. The numbers in brackets indicate the number of optimizations possible at the node

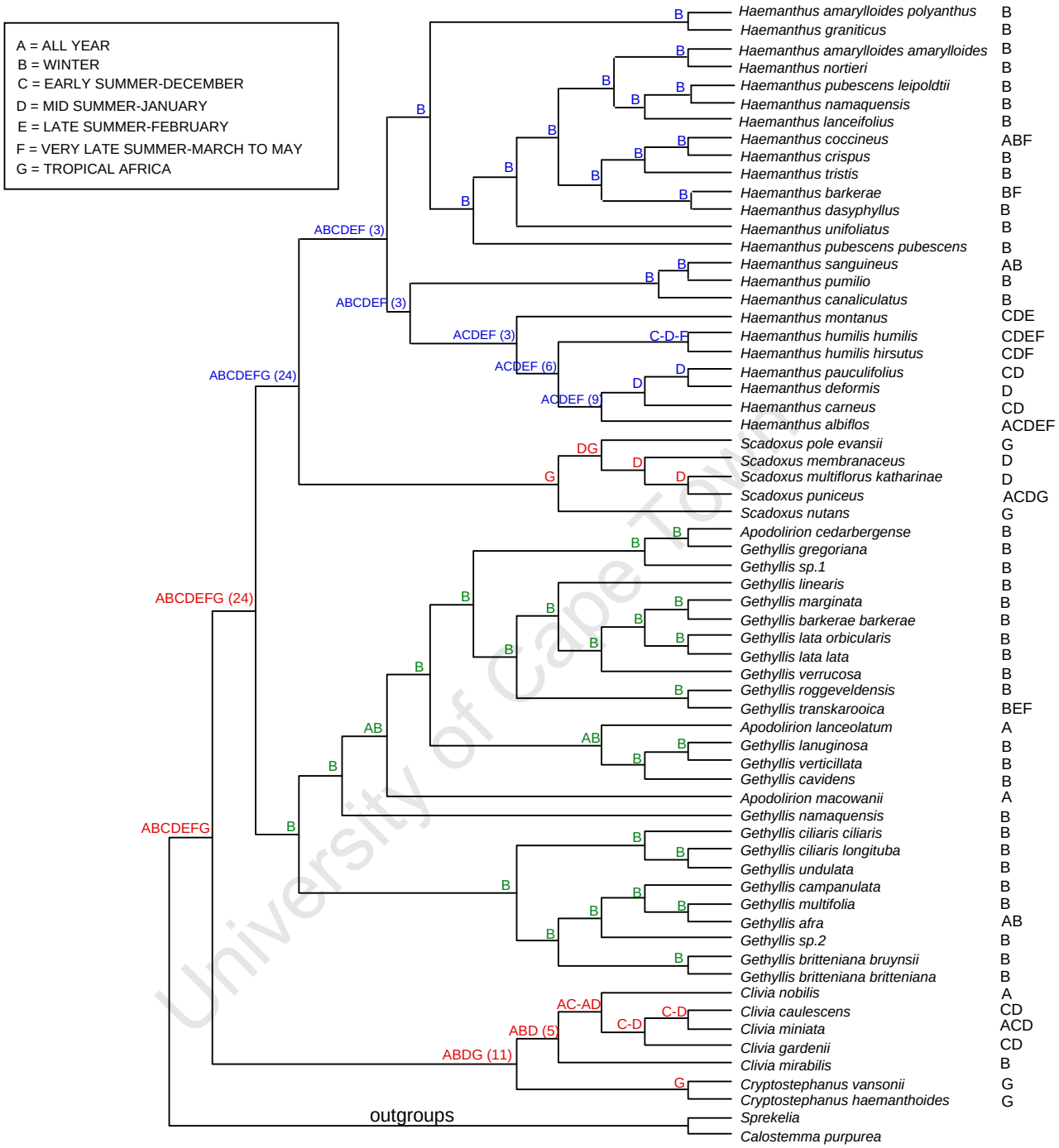


Figure 3.4: Optimization of ancestral areas based on rainfall. The numbers in brackets indicate the number of optimizations possible at the node

3.3.2. Dating

Age estimates of the NPRS analysis are presented in Figure 3.5. In this analysis it is suggested that Haemantheae started to diverge in the early Oligocene. The split between the clade comprising *Clivia* and *Cryptostephanus* and the clade comprising *Gethyllis*, *Apodolirion*, *Haemanthus* and *Scadoxus* possibly occurred in the early Oligocene (28 Ma). *Clivia* split from the *Cryptostephanus* in the late Oligocene (25 Ma) and at 23 Ma there was a divergence between *Gethyllis-Apodolirion* and *Haemanthus-Scadoxus*.

Figure 3.6 depicts a chronogram with the age estimates obtained using a Bayesian inference implemented in BEAST. Here it is suggested that estimated ages of *Gethyllis-Apodolirion* and *Haemanthus* are both in the Early Miocene at 21.5 Ma and 16.6 Ma, respectively. *Haemanthus* and *Scadoxus* diverge at 22.7 Ma, also in the Early Miocene.

For *Clivia*, the date of divergence of the winter-rainfall *C. mirabils* from the summer rainfall species is estimated at 15.9 and 15.6 Ma for NPRS and BEAST respectively.

The two models used in this study show similar results and table 3.2 lists the divergence times for the six genera of Haematheae and the outgroups. The NPRS model shows date estimations with 95% confidence intervals and the Bayesian model (implemented in BEAST) shows date estimations with 95% highest posterior densities (HPD) intervals. The 95% HPD is the shortest interval that contains 95% of the sampled values (Drummond and Rambaut 2006).

Table 3.2: Divergence time estimations with 95% confidence intervals and 95% highest posterior densities (HPD) interval for the divergence time estimates. Data in red indicate where both NPRS and BEAST dating models produce the same divergence dates for genera

	Analysis			
	NPRS		BEAST	
Clade	**Estimated age (Ma)**	**Bootstrap estimate of standard error (Ma)**	**Estimated age (Ma)**	**95% HPD interval**
Cryptostephanus	9	0	9.4	2.6 - 17.6
Scadoxus	17	0	11.7	5.3 - 19.4
Clivia	17	0	15.6	8.2 - 24.1
Haemanthus	18	3.76	16.6	10.7 - 22.8
Gethyllis- Apodolirion	21	3.77	21.5	15.1 - 27.9
Haemanthus-Scadoxus	23	4.17	22.7	16.0 - 29.2
Cryptostephanus- Clivia	25	2.88	25.5	16.9 - 33.6
Gethyllis-Apodolirion/ Haemanthus-Scadoxus	25	5.72	27.9	21.5 - 37.5
Gethyllis-Apodolirion-Haemanthus-Scadoxus/ Clivia- Cryptostephanus	28	8.07	32.5	27.8 - 37.5
Haemantheae/Outgroup	33	9.03	39.1	29.4 - 52.1

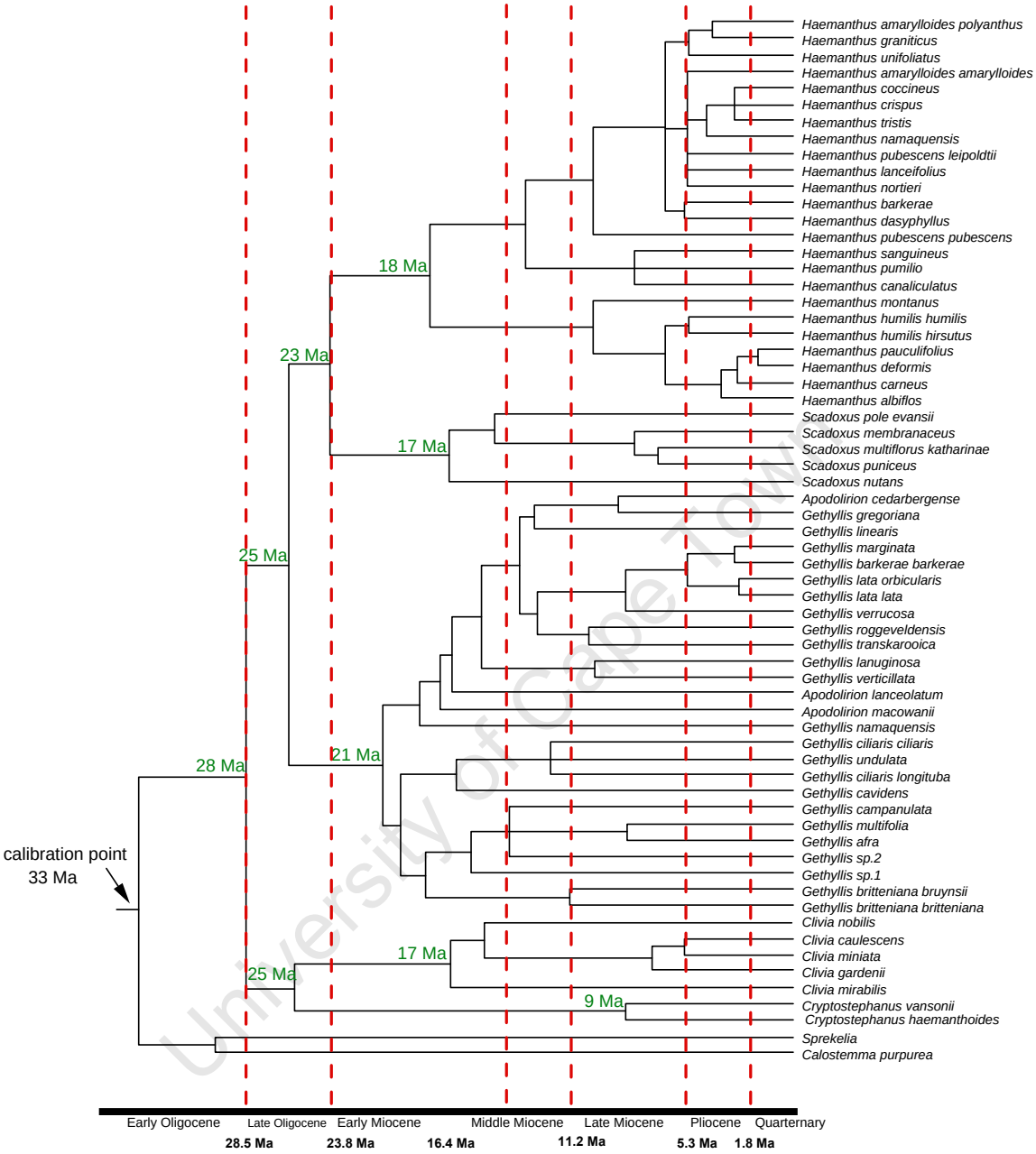


Figure 3.5: Chronogram obtained using one of the most parsimonious tree from the combined plastid and nuclear analysis of DNA sequence data sets and made ultrametric with the NPRS method (Sanderson 1997). The geological timescale is shown at the bottom (Palmer and Geissman 1999. Dates in millions of years ago (Ma)

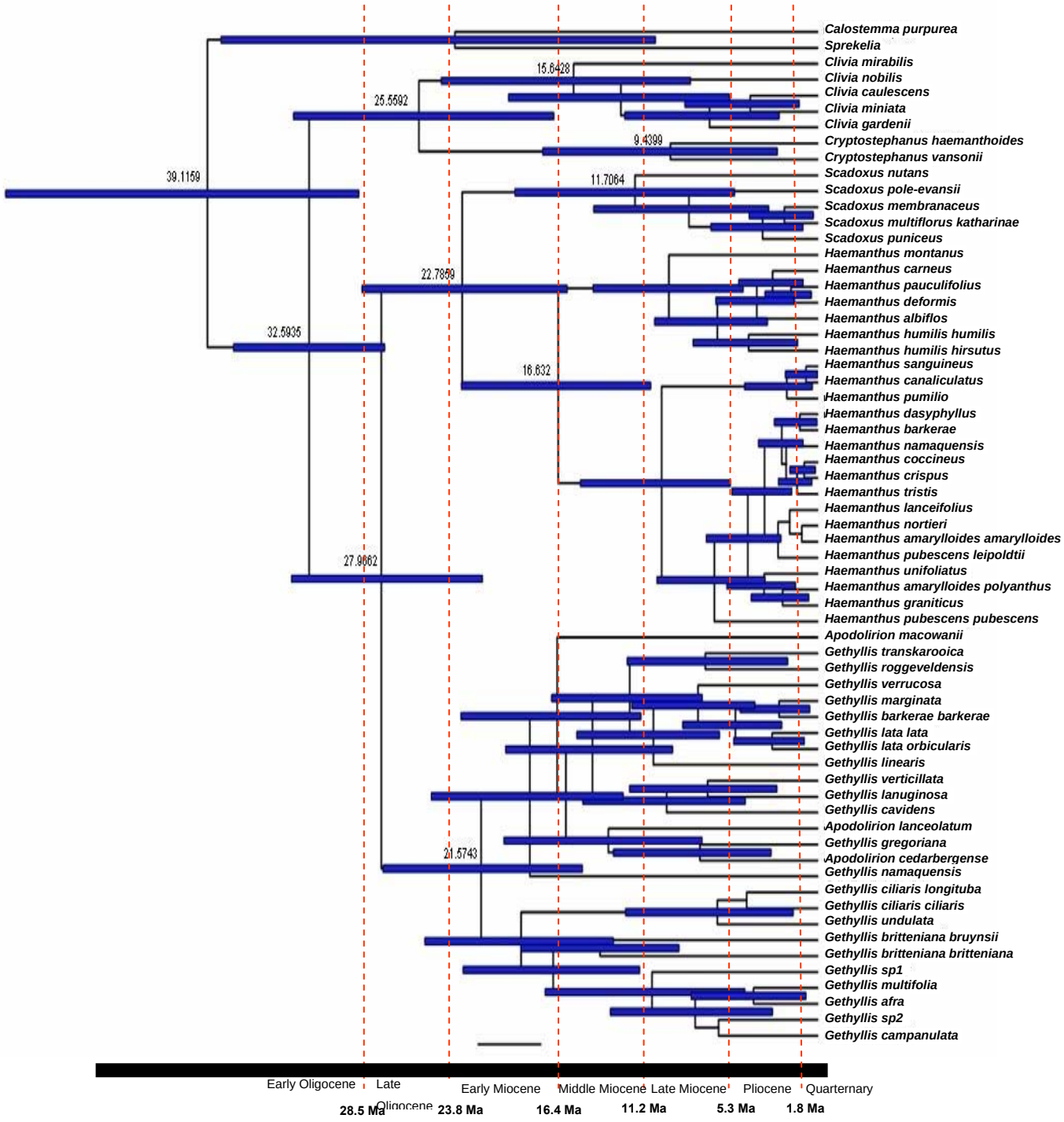


Figure 3.6: Chronogram obtained using one of the most parsimonious trees from the combined plastid and nuclear analysis of DNA sequence data sets using BEAST (Drummond and Rambaut 2006). The blue bars represent the 95% HPD interval for the divergence time estimates. The geological timescale is shown at the bottom (Palmer and Geissman 1999). Dates in millions of years ago (Ma)

3.4. Discussion and Conclusion

All results are based on the combined plastid and nuclear phylogenetic analyses of Haemantheae presented in Chapter Two. Although the sampling for the tribe is incomplete (62 species out of ± 90) all genera are well represented and it is unlikely that additions will produce substantially different results.

The results obtained from the dispersal-vicariance analyses using DIVA (Ronquist 1996) are of little help in determining the ancestral area at the root node of the Haemantheae as the areas indicated are widespread. The root node is assigned all 12 areas when no maximum is set for the number of areas allowed. The DIVA analysis using rainfall regions did not prove more useful as the areas were once again too widespread. Ronquist (1996) points out that 'optimizations become less reliable as you approach the root node' and in DIVA this is evident since the root node distribution includes most or all the areas occupied by the terminals, because of the increased uncertainty of the area optimization towards the root node.

However, the DIVA results using rainfall regions clearly indicate a marked divergence into a winter rainfall (B) and a summer rainfall clade (ACDEF) in the higher nodes of *Haemanthus* and the ancestral area of *Gethyllis* is assigned one optimization of winter rainfall (B) only.

Wikström *et al.* (2001) used *Clivia* and *Hippeastrum* to represent the family Amaryllidaceae in their dating of the Angiosperm tree. As these two genera do not belong to the same tribe (*Clivia* to Haemantheae and *Hippeastrum* to Hippeastreae) the 33 Ma estimate they suggest for the split between these two species, was used to calibrate the stem node of the Haemantheae. Figure 3.5 illustrates the chronogram with age estimates for the different genera using the NPRS method of Sanderson (1997).

The early to middle Miocene (23.8-11.2 Ma) saw the climatic shift from summer-wet to summer-dry conditions. The geological uplift of southern Africa occurred mostly in the east and the south Atlantic cooled further and marine upwelling commenced along the west coast, leading to arid conditions in the west (McCarthy and Rubidge 2005). The middle to late Miocene (16.4-5.3 Ma) saw the formation of an ice cap on

Antarctica which led to a major cooling of the Benguela current and desertification of the Namib which may have effected change in the vegetation (Coetzee 1983). Further uplift occurred (± 5Ma), especially in the east. The eastern escarpment began to trap moisture, increasing the rainfall gradient across southern Africa and causing more arid conditions to develop in the interior, resulting in the formation of the Kalahari Desert (McCarthy and Rubidge 2005). The pronounced sea level regression recorded on the South African continental shelf near the Miocene/Pliocene boundary (Siesser and Dingle 1981) may have enhanced coastal dry conditions too.

Based on calibration of the molecular clock using the NPRS ultrametric tree and Bayesian inference using BEAST, the estimated age of *Gethyllis* (*Gethyllis-Apodolirion* clade) is ± 21 Ma for both NPRS and the Bayesian analysis, and that of *Haemanthus*, the other species-rich genus in the tribe, is 18 Ma for NPRS and 16 Ma for Bayesian analysis. Both these estimated ages coincide with the early Miocene (23.8 – 16.4 Ma). *Haemanthus* saw a rapid radiation in the last 6 Ma but *Gethyllis* experienced a more gradual diversification over from ± 21 Ma - 10 Ma.

The estimated ages of the winter rainfall *Haemanthus* clade and that of the predominantly winter rainfall *Gethyllis-Apodolirion* clade do not coincide with one another. The estimated age of the *Gethyllis-Apodolirion* clade is ± 21 Ma (both NPRS and the Bayesian analysis) which coincides with the middle Miocene (23.8-11.2 Ma) and the onset of arid conditions in the west. The estimated age of the winter rainfall *Haemanthus* clade is more recent at ±14 Ma, coinciding with the middle to late Miocene (16.4-5.3 Ma).

The estimated ages of *Clivia* and *Scadoxus*, both species-poor genera, are within the same epochs as *Haemanthus* and *Gethyllis-Clivia* at 17 Ma and 15 Ma, and *Scadoxus* 17 Ma and 11 Ma for NPRS and Bayesian analysis, respectively.

It is interesting to note that Givnish *et al.* (2006) found the most recent instances of concerted convergence in fleshy fruits and net venation, in *Griffinia*, *Proiphys-Scadoxus* (Amaryllidaceae) and *Curculigo* (Hypoxidaceae), occurred in the last 5-10 Ma.

Tertiary fossil deposits from the south Western Cape area (Noordhoek) (Coetzee 1983) indicate the presence of woodland vegetation and thus of a moister and more

equable Tertiary rainfall regime than what is currently prevalent. The decline of this woodland element in association with the onset of a seasonally arid climate may have favoured the diversification of lineages (like *Haemanthus*) that were able to adapt to these new conditions (Linder *et al.* 1992).

The early Pliocene, at about 5 Ma, saw the onset of aridification and the establishment of a true Mediterranean climate. Several studies (Richardson *et al.* 2001-*Phylica*;. Klak *et al.* 2004-Aizoaceae; Goldblatt *et al.* 2002-*Moraea* and Verboom *et al.* 2003 *Ehrharta*) report diversification during the same epoch.

In a study of the evolution of diversity of the Cape flora, Linder (2005) suggested that there was no single obvious trigger for the radiation of the Cape flora as there was a gradual transformation in the climate, from a tropical to a warm temperate forest climate and eventually to a summer-dry Mediterranean type climate. This corresponds with the great spread in dates of the initiation of the radiation of the various lineages. Linder (2005) also suggested that the evolution of key innovations allowing the exploitation of new seasonally arid, fire prone habitats might have been the crucial factor that allowed some groups to radiate.

In 2004, Klak *et al.* proposed that several morphological characters constitute key innovations that facilitated the major radiation of the core of the Ruschioideae and stated that climatic and ecological factors alone where not responsible for the radiation in this group, supporting the suggestion of Linder (2005) concerning the evolution of key innovations. In addition, Linder (2005) proposed that where numerous lineages appear to have radiated extensively, and possibly undergone a remarkable increase in speciation rate in a given area at more or less the same time, then the explanation for this diversity should be found in the environment. He also stated that radiation in a single clade may be explained by features unique to the clade as is the case with Klak *et al.*'s (2004) research on the Ruschioideae.

In the tribe Haemantheae, results indicate a rapid diversification for the winter rainfall lineages of *Haemanthus* at around 5 Ma coinciding with the late Miocene/ Pliocene and aridification and formation of a Mediterranean-type climate. In particular, it is interesting that the only clade in the Haemantheae that has species which are fire

specialists is in *Haemanthus* and this lineage, comprising *H. sanguineus, H. pumilio* and *H. canaliculatus*, is estimated to have diversified between the late Miocene and Pliocene. *Gethyllis* on the other hand reflects a gradual diversification from 20-8 ma, before the aridification and the establishment of the Mediterranean-type climate.

Galley and Linder (2006) date the migration of elements of the Cape flora to the tropical Afrotemperate regions at between 0.54 and 10 Ma ago. They argue that such migrations are congruent with the recent formation of the uplands of tropical Africa, which dates to the Miocene with further upliftments in the Pliocene and Pleistocene. Two genera from the Haemantheae, *Scadoxus* and *Cryptostephanus*, have species occurring in both southern and tropical Africa. It is interesting to note that the diversification for the two species of *Cryptostephanus* and for three species of *Scadoxus* takes place during this period. It is possible that this diversification was triggered by their establishment in more suitable habitats.

Bakker *et al*. (2005) found similar results with *Pelargonium*. They indicated that the East African disjunction between *Pelargonium* species may be correlated with Pleistocene upliftment events in East and Southern Africa, which provided a high-altitude corridor via the afromontane forests through otherwise tropical latitudes (Bakker *et al*. 2005, Linder 2003).

However, the results obtained for this study are not sufficiently informative to shed light on migration patterns of the tribe as debated by Levyns (1952) and more recently by Galley and Linder (2006), or to contribute towards the debate on the affinities of the Cape clades.

The interpretation of the biogeography and dating results for this study are broad and are limited by a few factors. Firstly, the method of inferring ancestral areas yields information that is too broad to make logical deductions. Secondly, the molecular dating techniques are by no means ideal. Bell and Donoghue (2005), in their study of divergence times of the Dipsacales, found great variation in age estimates based on different methods and on different gene regions, suggesting that estimates based on a single method applied to a single gene be treated very cautiously. Linder *et al.* (2005) discovered in their investigation of three different methods in the African

Restionaceae, a linear relationship between the degree of under estimation of test node ages and the distances from the calibration points for NPRS and the Bayesian analysis. This justifies the suggestion made by Shaul and Graur (2002) that when using NPRS and Bayesian analysis the calibration nodes should be situated within the group. Lee (1999) recommended multiple calibration points to protect against errors in single calibration points. These are but two of the numerous recommendations found in the literature. Poor fossil record of the Cape flora also means there are no independent corroborations of the suggested divergence times.

CHAPTER 4. SYSTEMATICS AND PHYLOGEOGRAPHY OF *CLIVIA*

4.1. Introduction

Clivia is of enormous horticultural importance and has been cultivated extensively for more than 150 years in the Far East (China and Japan), Europe, the USA, New Zealand, Australia and the United Kingdom. Different market trends can be observed in these regions. The biggest market in Europe is Belgium where compact, small, broadleaved greenhouse and pot-plants are cultivated. The Far East is by far the biggest market in the world with the emphasis on the foliage, and broadleaved and variegated plants are cultivated as greenhouse and pot-plants. In South Africa, broad and variegated leaved plants are grown but there is particular interest in flower form and colour. The Australian and New Zealand market require broad and variegated foliage and different flower colour variations. The youngest but fastest growing market is the USA where broadleaved and variegated greenhouse and pot-plants are cultivated (Swanevelder 2003).

This horticultural significance has led to a large international commercial market. The estimated annual production revenue (which includes sale of seedlings) is listed in Table 4.1. Swanevelder (2003), who conducted extensive research as no official statistics are available, still believes that these figures are underestimates.

Table 4.1: Estimated annual production revenue for Clivia (Swanevelder 2003)

Country/Region	Production	Revenue in US Dollars
Australia & New Zealand	< 1 million plants	1-5million
South Africa	1-5 million plants	1-5million
USA	1-5 million plants	50-100million
Europe	> 1 million plants	8-15million
Far East	> 2 million plants	200million

The genus *Clivia* comprises six species: *C. miniata* (Lindl.) Regel*, C. nobilis* Lindl., *C. gardenii* Hook, *C. caulescens* R.A.Dyer, *C. robusta* B.G. Murray, Ran, de Lange, Hammett, Truter *et* Swanevelder and *C.mirabilis* Rourke. Of the six, five species (*C.*

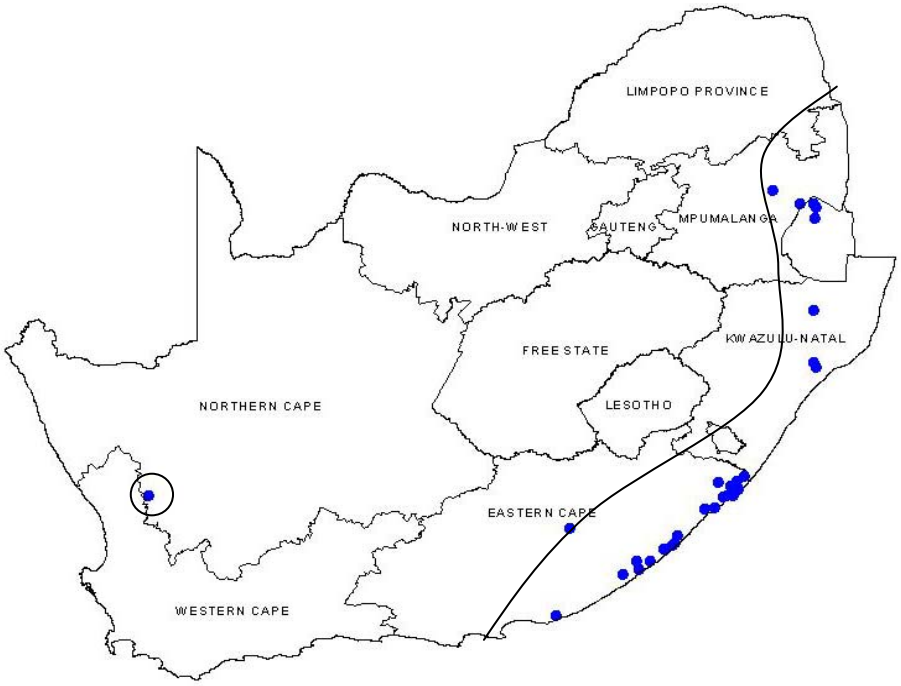


Figure 4.1: Distribution map of Clivia. Blue circles indicate populations included in the study

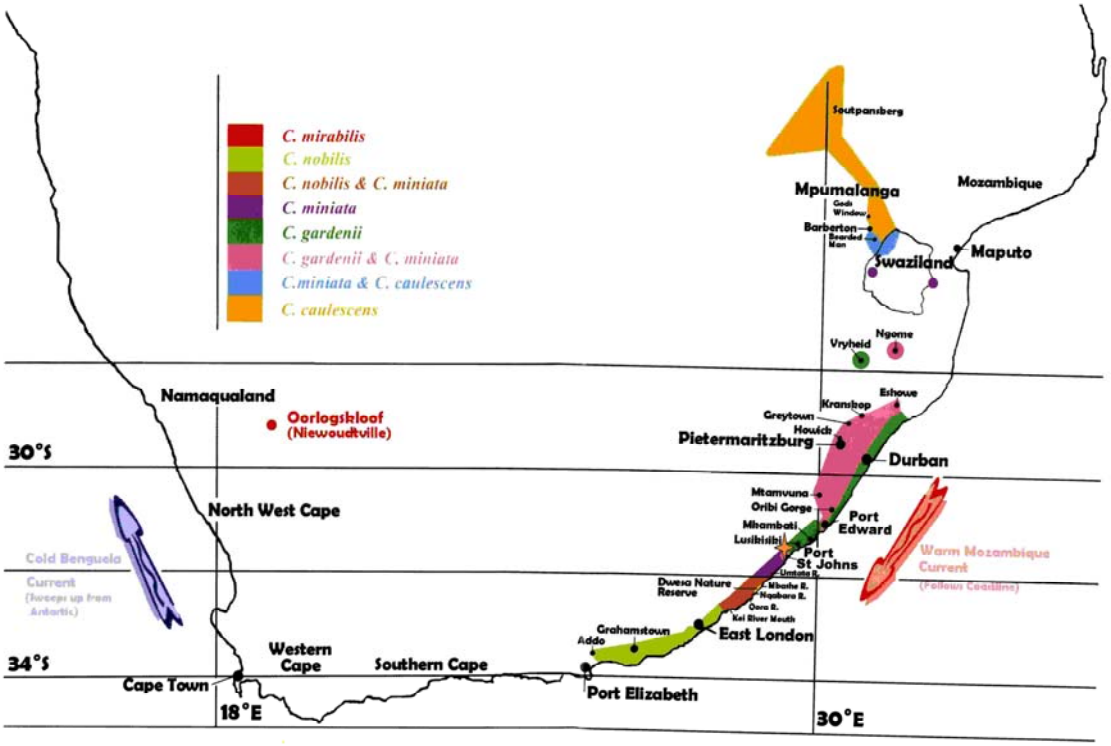


Figure 4.2: The known distribution of *Clivia* species (orange star = *C. robusta*). The map also indicates where the species grow sympatrically. Source: Clivia Five, 2003. pg 96-97

miniata , *C. nobilis*, *C. gardenii*, *C. caulescens* and *C. robusta*) are found in coastal and inland Afromontane forest along the east coast of southern Africa, from the Eastern Cape northwards to Limpopo Province and Swaziland (Rourke 2002). One species (*C. mirabilis*) was discovered in 2002, in the Oorlogskloof Nature Reserve, in a semi arid valley in the Northern Cape.

The discovery of *Clivia mirabilis,* in a climate and locality so different from that previously known for *Clivia*, has prompted phylogeographic questions regarding this genus. *Clivia* has always been seen as belonging to densely forested, subtropical environments experiencing a summer rainfall, dry winter climatic regime. *C. mirabilis*, in direct contrast, occurs remotely in the arid Northern Cape with its strictly winter rainfall regime, isolated from the other *Clivia* species (Rourke 2002). The distribution ranges of the tubular-flowered *Clivia* species (*C. nobilis*, *C. gardenii*, *C. caulescens* and *C. robusta*) are parapatric in relation to one another (they do not occupy the same geographical ranges but the ranges are contiguous (Wiley 1981)) while the distribution range of the brush-type flowered *C. miniata* is partly sympatric (populations are found together in one part of the distribution range but apart in another (Wiley 1981)) with all the tubular-flowered species, except for *C. mirabilis*.

In terms of morphology, *C. miniata* is distinct and easy to identify but the morphological differences between the tubular-flowered species are subtle and geographical data have often been favoured in the identification of species.

In 2005, *Clivia robusta* from Pondoland, Transkei, along the east coast of South Africa was described. Murray *et al* (2004) used karyological, morphological and distribution pattern data to distinguish *Clivia robusta* from *Clivia gardenii,* the species to which it is most closely related.

Advances in molecular biology and their application at the population level have provided us with the opportunity to explore the extent and distribution of genetic variation at a wide range of loci within plant species. Amplification and sequencing can be used to characterize the plastid DNA haplotypes present in a population or species and to reconstruct the gene phylogeny that relates them. In the light of population genetic theory it is often possible to make inferences about the

evolutionary and ecological processes that have moulded the observed genetic structure (Avise 1994). Although the nucleotide substitution rate of plant chloroplast DNA is lower than that of nuclear DNA, nucleotide variation of non-coding regions in chloroplast DNA can be used at the intraspecific level because of its considerably higher evolutionary rate than the gene-coding region (Fujii *et al.* 1997).

Population genealogies are often multifurcated, with descendant genes coexisting with persistent ancestors and recombination events (in nuclear loci) producing reticulate relationships. Unrooted networks are more sensitive at resolving relationships among closely related haplotypes (Excoffier *et al.* 1992) because they assess the distribution and relationships of haplotypes among the localities without assuming bifurcation events. Several individuals sharing the same haplotypes can thus be joined by a single node, which at the population level is clearly a more accurate way of reflecting the relatedness among the lineages.

Several algorithms are available for haplotype network reconstruction and the two used in this study are summarised briefly:

- The median joining (MJ) network method

  (Network http://www.fluxus-engineering.com) This method can handle large sets of data and also multistate data (Bandelt *et al* 1999). It begins by combining the minimum spanning trees within a single network. With a parsimony criterion median vectors (representing missing intermediates) are added to the network.
- Statistical parsimony

  (TCS: http://bioag.byu.edu/zoology/crandall_lab/programs.htm.) It begins by estimating the maximum number of differences among haplotypes as a result of single substitutions with a 95% statistical confidence (parsimony limit/parsimony connection limit). After this, haplotypes differing by one change are connected and then two and so on, until all the haplotypes are connected in a single network or the parsimony connection limit is reached.

Both methods require the absence of recombination which restricts the application of these methods at a population level.

In addition to haplotype reconstruction, *F*-statistics for the analyses of population structure, Analysis of Molecular Variance (AMOVA, Exoffier *et al.* 1992), Spatial analysis of molecular variance (SAMOVA, Dupanloup *et al*. 2002) and a coalescent model, MIGRATE were used in this population level study.

The most frequently used measure for describing population structure is the *F*-statistic, a measure of population differentiation first developed by Sewall Wright (1965). Since the original work of Wright, several authors have proposed methods to estimate F_{ST} leading to a number of F_{ST} analogues such as G_{ST} (Nei 1987), θ (Weir and Cockerham 1984), R_{ST} (Slatkin 1985) and φ_{ST} (Excoffier et al. 1992). F_{ST} analogues are dependent on the within population genetic variation (Hedrick 2005), therefore high levels of genetic variation rather than low levels of variation lead to lower F_{ST} estimates (Meirmans 2006).

The AMOVA approach has been widely used for the hierachical analysis of the genetic diversity in a set of sampled populations. Set criterion (e.g ecological, linguistic, or cultural) is often used to define *a priori* groups.

In addition SAMOVA analyses were also carried out. SAMOVA is based on a simulated annealing procedure that aims at maximising the proportion of total genetic variance due to differences between groups of populations and will define the strongest structure of populations based solely on genetic data (Dupanloup *et al*. 2002). SAMOVA does not require that groups be defined *a priori*.

With the advent of methods based on coalescent theory (coalescence: merging of ancestral lineages going back in time), tools now exist that allow us to estimate migration patterns without the unrealistic assumption of symmetry of migration rates or equal population sizes (Beerli 1998, Beerli and Felsenstein 2001). MIGRATE is a maximum likelihood method based on coalescence. It uses a Markov chain Monte Carlo approach and sample geneologies that are then used to find the maximum likelihood estimate of a full migration matrix with population sizes.

Based on the recent description of two new species of *Clivia*, its unusual distribution pattern, its horticultural significance and growing commercial market, the genus makes for an interesting population level case study.

The aim of this chapter is firstly to elucidate the species level relationship of *Clivia* in the light of the recent description of *Clivia robusta*, or swamp *Clivia* – a species that grows sympatrically with *C.gardenii* and was formerly considered to be a robust form of *C. gardenii.* A haplotype network reconstruction, various F_{ST} analogues and a coalescent based model were carried out to obtain a better understanding of the evolutionary relationships by exploring phylogeographic patterns within the *Clivia* species and among them.

With regards to biogeography, *Clivia mirabilis* is the only species in this genus which is found in the winter rainfall region of South Africa and, based on an earlier study by Conrad *et al*. (2003), is placed as sister to all of the summer rainfall species. This prompts questions regarding the evolution of the genus, particularly whether the disjunction to the Northern Cape is due to vicariance or dispersal and whether *C. mirabilis* shows reduced genetic diversity. In addition, partial sympatry between *C. miniata* and the tubular flowered species also prompts questions about the discreteness of the populations in the areas of overlap.

4.2. Materials and Method

4.2.1. Sampling

Of the 107 individuals sampled, 89, representing 33 populations across the distribution range, were successfully amplified for three plastid regions: the *rpoB-trnC* intergenic spacer, *trnL* intron, *trnL-F* intergenic spacer. Leaf material from all the localities cited by Murray *et al.* (2004) in their recent description of *Clivia robusta*, was included in the analysis.

Selection of DNA regions for analysis was based on the broader study of the tribe, where the *trnL-F* region and the *rpoB-trnC* intergenic spacer produced the most variation. Voucher and population locality information are listed in Table 4.2.

Table 4.2: Kirstenbosch Botanical Garden (NBG) accession information and population locality details for all *Clivia* individuals used in the analyses

Population	Haplotype network reference	Individuals in population	Locality	Grid	Collector's details	Kirstenbosch accession number
Clivia caulescens	Caul01-03	3	locality1 – Mpumalanga	2531CC	JWI 569	NBG 441/99
Clivia caulescens	Caul04-05	2	locality 2 – Mpumalanga	2530DA	JWI 57	NBG 433/99
Clivia caulescens	Caul06-09	4	locality 3 – Swaziland	2531CD	JWI 573	NBG 573/99
Clivia caulescens	Caul10	1	locality 4 – Swaziland	2631AB	ALL	NBG 321/00
Clivia caulescens	Caul11-14	4	locality 5 – Mpumalanga	2531CC	JWI 567	NBG 439/99
Clivia gardenii	Gard15-18	4	locality 1 – KZN	2731CD	JWI 565	NBG 437/99
Clivia gardenii	Gard19-21	3	locality 2 – KZN	2831CD	JWI 559	NBG 430/99
Clivia gardenii	Gard22-25	4	locality 3 – EC	3129BB	JW557	NBG 517/98
Clivia gardenii	Gard26-29	4	locality 4 - KZN	2831CD	JWI 562	NBG 434/99
Clivia gardenii	Gard33-36	4	locality 5 – EC	3129BB	Ex hort Fred van Niekerk	
Clivia gardenii	Gard37	1	locality 6 – EC	3129BB	Ex hort Fred van Niekerk	
Clivia gardenii	Gard38	1	locality 7 – EC	3129BD	Ex hort Len Chiazzara	
Clivia robusta	Robu30-32	3	locality 1- EC	3129BD	JWI 579	NBG 314/00
Clivia robusta	Robu 39	1	locality 2 – EC	3129BD	JW557	NBG 517/98
Clivia robusta	Robu 40	1	locality 3 – EC	3129BC	Venter 3864	PRE 556875

Population	Haplotype network reference	Individuals in population	Locality	Grid	Collector's details	Kirstenbosch accession number
Clivia robusta	Robu 41	1	locality 4 – EC	3129BB	J.T. Truter 4072	
Clivia robusta	Robu 42	1	locality 5 – EC	3129BD	JPR 2180	NBG 313/00
Clivia robusta	Robu 43	1	locality 6 - KZN	3129BD	JWI 554	NBG 514/98
Clivia robusta	Robu 44	1	locality 7 – EC	3129BB	JPR 2145	
Clivia nobilis	Nobi45-47	3	locality 1 - EC	3326DA	JWI 525	NBG 598/97
Clivia nobilis	Nobi48-51	4	locality 2 – EC	3227DD	JWI 467	NBG 717/96
Clivia nobilis	Nobi52-54	3	locality 3 – EC	3228BD	JWI 500	NBG 573/97
Clivia nobilis	Nobi55-57	3	locality 4 – EC	3228CC & CD	JW 534	NBG 606/97
Clivia nobilis	Nobi58-59	2	locality 5 – Transkei	3228CB	JWI 485	NBG 735/96
Clivia nobilis	Nobi60	1	locality 6 – EC	3326AA	JWI 466	NBG 716/90
Clivia miniata	Mini61-63	3	locality 1- Natal	2831CD	JWI	NBG 435/99
Clivia miniata	Mini64-67	4	locality 2 - EC	3129CB	JWI553	NBG 513/98
Clivia miniata	Mini68-69	2	locality 3 – EC	3228BB	JWI 545	NBG 524/98
Clivia miniata	Mini70-73	4	locality 4 - Swaziland	2531CD	JWI 570	NBG 442/99
Clivia miniata	Mini74-76	3	locality 5 - EC	3228BC	JWI 470	NBG 720/96
Clivia miniata	Mini77-80	4	locality 6 – EC	3228CA	JPR 2195	NBG 509/00
Clivia miniata	Mini81-82	2	locality 7 – KZN	3030CB	S.Venter	
Clivia mirabilis	Mira83-87	5	Oorlogskloof Nature Reserve, Northern Cape	3119AC	JPR 2220	NBG 35/04

4.2.2. DNA extraction, PCR and DNA sequencing

Protocols as described in Chapter two were followed.

4.2.3. Phylogenetic and phylogeographic analyses

A heuristic search was performed using the parsimony algorithm of the software package PAUP* for Macintosh (Phylogenetic Analysis using Parsimony v.4.0b 10, Swofford 2000). To assess internal support 1000 bootstrap replicates were performed using simple taxon addition and TBR branch swapping with a tree limit of ten trees per replicate. *Cryptostephanus vansonii* Verdoorn was designated as the outgroup taxon based on the larger analysis in chapter two.

All cladistic analyses were performed using 1000 replicates of random taxon addition, tree bisection-reconnection (TBR) branch swapping, with MULPARS on. All character transformations were treated as equally likely (Fitch parsimony; Fitch 1971). A limit of ten trees was set for each replicate to reduce time spent swapping on large numbers of trees at or near the optimum.

To investigate genetic relationships among haplotypes, networks were constructed separately for both the individual regions (*trnLF* and *rpoB-trnC*) and combined data matrices using TCS (Clement *et al.* 2000) and median joining networks using Network version 4.1.0.0 (Bandelt *et al.* 1999) and for all the individuals (n=89).

To allow an assessment of the degree of differentiation among the sampling areas, a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.* 2002) was conducted *post hoc* identification of clades in the network. The SAMOVA takes into consideration geographic locations of sampling and sequence data to identify groups of populations that are geographically homogenous and maximally separated from each other. It aims to maximize the proportion of genetic variation due to differences between groups based on simulated annealing procedures (Dupanloup *et al.* 2002). As such, this analysis was useful for statistically differentiating between historically isolated groups in the network (Tolley *et al.* 2006). It also incorporates traditional F-statistics (F_{CT}, F_{SC}, F_{ST}) in recognising population substructure. F_{CT} is the proportion

of total genetic variance due to the differences between groups of populations; F_{SC} reveals the degree of differentiation between populations within groups; F_{ST} shows the genetic variation between subpopulations relative to the total population. One hundred simulated annealing processes were performed for each possible number of populations, ranging from two through to eight populations for the *trnLF* and combined datasets.

Haplotype diversity (*h*) and nucleotide diversity (π) among groups were calculated in Arlequin ver 2.0 (Schneider *et al.* 200). The distribution of variation within and between assemblages was investigated by an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) as implemented in Arlequin ver 2.0 (Schneider *et al.* 2000).

To assess the genetic divergence among these groups, F_{ST} and Φ_{ST} were estimated. F_{ST} takes into account only the differences in haplotype frequencies in the different populations, while Φ_{ST} takes into account both the haplotype frequency and nucleotide diversity (Hurwood and Hughes 1998, Beheregaray and Sunnucks 2001).

To estimate maximum likelihood (ML) migration rates among the populations of the six *Clivia* species I used MIGRATE version 2.1.3. (Beerli 1997-2004). This approach, based on coalescence using Markov Chain Monte Carlo (MCMC) searches, takes both history and asymmetrical gene flow into account, unlike migration-drift equilibrium classical approaches and allows simultaneous estimation of population growth or decline (Beerli and Felsenstein 2001). Several runs were performed with different combinations of short and long chains.

4.3. Results

4.3.1. Parsimony and Network analyses

Although individual datasets were analysed, only results from the combined analysis will be discussed as both the *trnLF* and *rpoB-trnC* datasets represent chloroplast loci. Since the chloroplast is inherited as a unit, all loci by definition share the same history, and any differences must be due to positional sampling effects or

compositional bias. It is therefore appropriate to combine chloroplast loci, as separate analyses are difficult to justify.

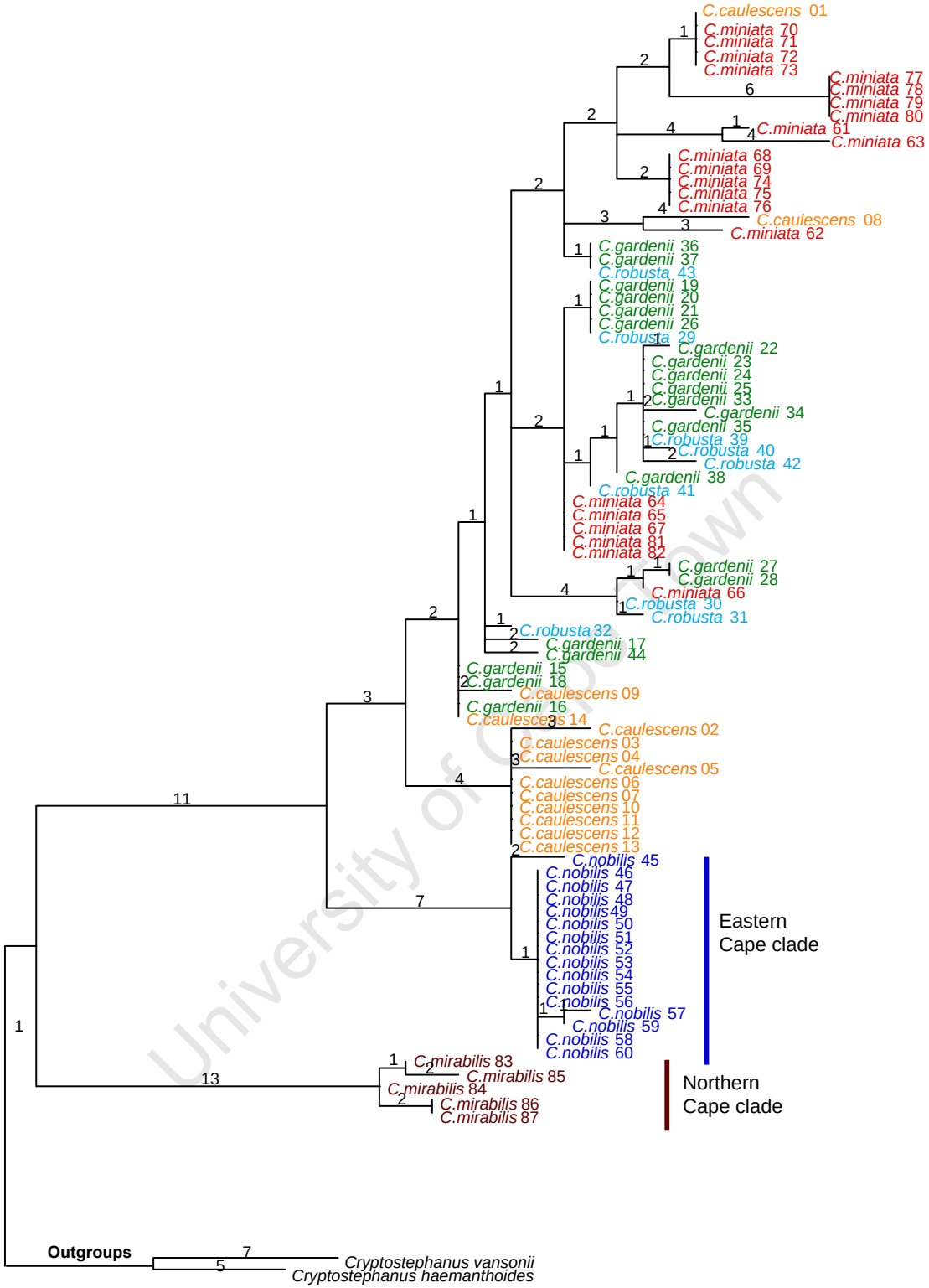
Haplotype reconstruction software Network (Bandelt *et al.* 1999) was unable to execute data matrices consisting of more than 1000 characters. An alternative software TCS (Clement *et al.* 2000) was utilised, as the combined datasets consisted of more than 1000 characters.

Of the 1732 characters included in the combined analysis 1667 were constant, 15 variable characters were parsimony-uninformative and 50 were parsimony informative. 3780 equally parsimonious trees were recovered with tree length 104, CI 0.644 and RI 0.920. Two clades were recovered in one of the equally parsimonious trees (Figure 4.3). One clade consisted of a monophyletic *C. mirabilis* and the other is further subdivided into two subclades comprising a monophyletic *C. nobilis,* sister to a clade consisting of the other four species. This split reflects the two lineages of *Clivia*; one lineage representing the winter rainfall/Northern Cape lineage and the other the predominantly summer rainfall/east coast lineage.

Haplotype network reconstructed for the combined analysis is shown in Figure 4.4. Haplotypes of *C. mirabilis* occur in close proximity to each other. Sharing of haplotypes occur between *C. miniata* and *C. caulescens*; *C. gardenii* and *C. robusta*; and *C. miniata* and *C. robusta*.

4.3.2. Molecular Diversity Analyses

The SAMOVA was run on the combined (*trnLF* and *rpoB-trnC*) datasets to investigate the variation within and between the species. Although table 4.3 only shows the structure for eight different groups, 24 groups were analysed in total. SAMOVA did not produce any significant results with the F_{CT} results increasing constantly without any noteworthy increase at any particular groupings.This may suggest that no isolation of the lineages has occurred with respect to the sequences used here and that variation in these data between the species is as great as the variation within species. None of the groups proposed by SAMOVA reflect the haplotype networks obtained.



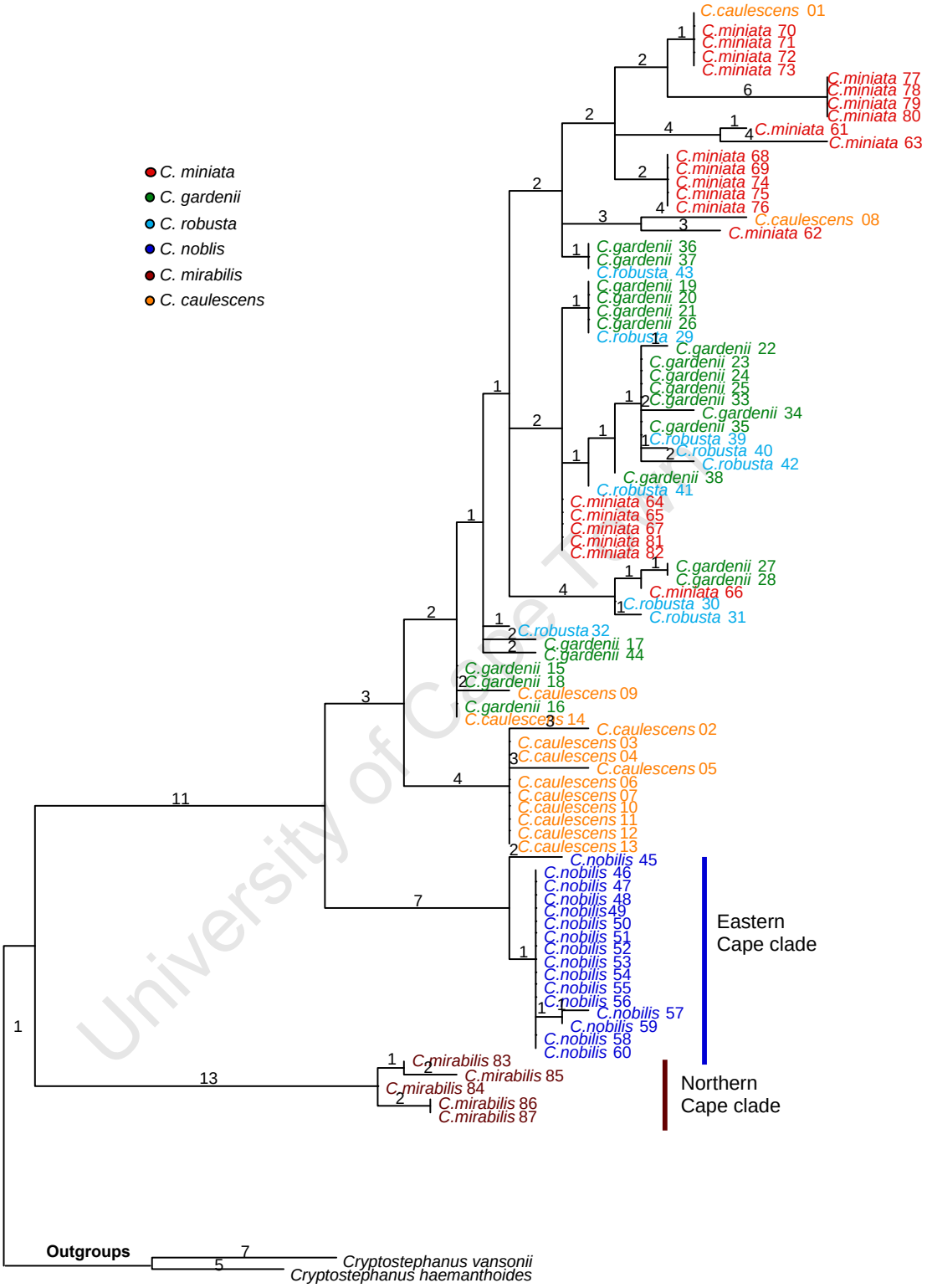


Figure 4.3: One of 208 equally parsimonious trees from combined analysis with branch lengths indicated above the branches

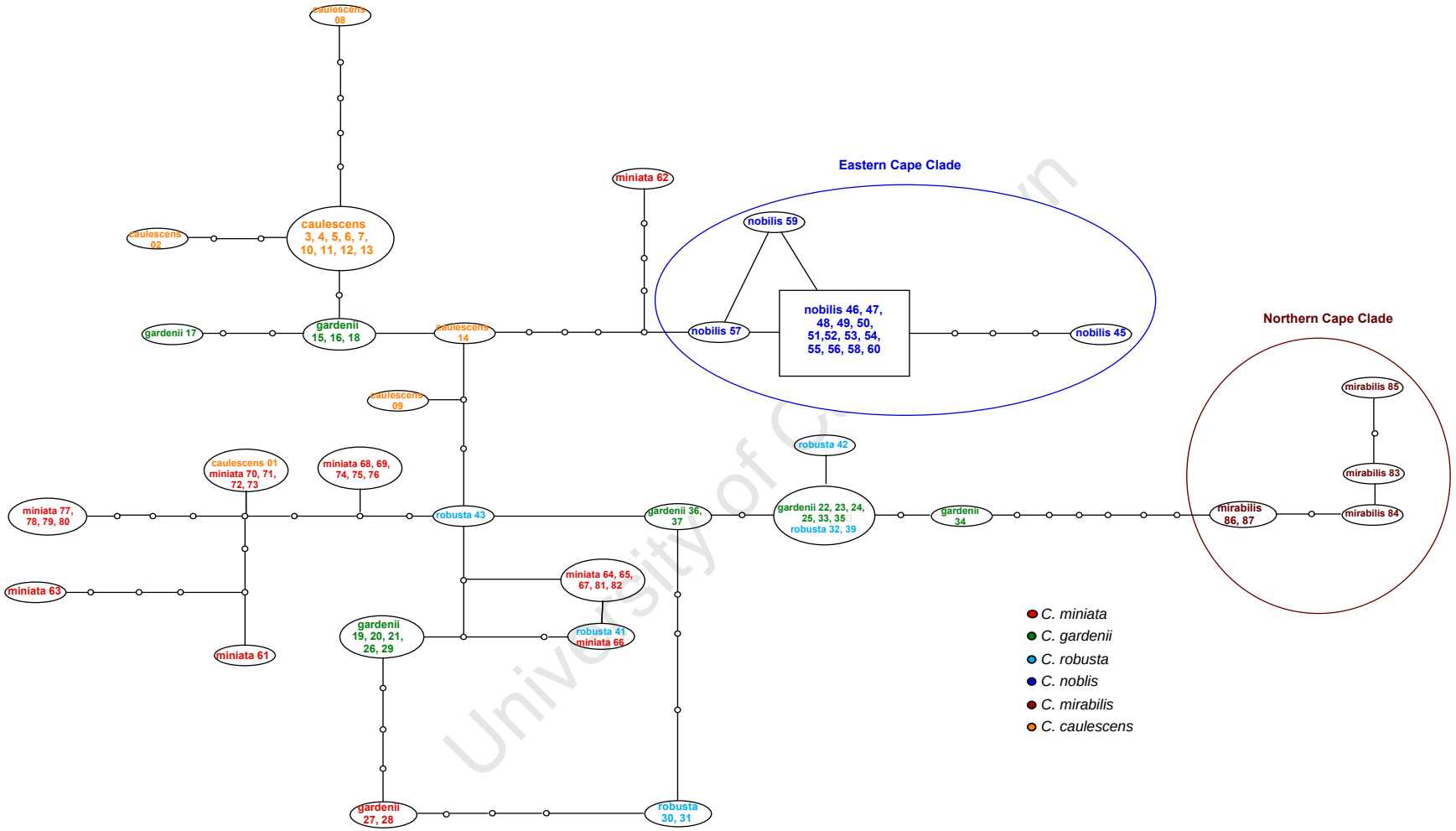


Figure 4.4: Haplotype network from TCS using combined plastid data sets. Circles surround species. Missing intermediate occur on the branches linking haplotypes

Due to the small sample sizes from many localities and the lack of obvious criteria for the definition of groups of populations, individuals were assigned to groups on the basis of their taxonomic classification i.e. groups were assigned according to species. For the six groups reflecting the six species, AMOVA revealed variation among populations at 33.92%, half of that of the within population variation (66.08%). The groups revealed a low degree of genetic subdivision with only 23% genetic variation between the groups (species) for the combined analysis (F_{ST} 0.23424 $p < 0.0001$; Φ_{ST} 0.49853 $p < 0.0001$ respectively). Haplotype and nucleotide diversity are highest for *C. miniata* but this could be caused by sampling bias (Table 4.4 and Table 4.5). Nucleotide composition (Table 4.6) is similar between the different groups for both datasets.

Effective population size, F_{ST} values and migration rates obtained are summarized in Table 4.6 and 4.7. Several combinations of short and long chains were carried out with 17 short and 3 long producing the optimal results with no error values reported. The estimates obtained for effective population size indicate *C. caulescens* to be the biggest and *C. nobilis* the smallest. Migration between the species was bidirectional with very high past gene flow rates observed between *C. robusta* and *C. gardenii*, within *C. mirabilis* and within *C. nobilis*.

Table 4.3: Results from a spatial analysis of molecular variance (SAMOVA) showing *F* values given different numbers of groupings for the combined datasets. Sets of lineages that were combined within the groups are indicated

2 groups 1. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1 2. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6, miniata1, miniata2, miniata3, miniata4, miniata5, miniata6, miniata7, mirabilis1	F_{SC} = 0.660 F_{ST} = 0.886 F_{CT} = 0.664
3 groups 1. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, , miniata1, miniata2, miniata3, miniata4, miniata5, miniata6, miniata7, mirabilis1 2. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6 3. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	F_{SC} = 0.506 F_{ST} = 0.858 F_{CT} = 0.714
4 groups 1. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, miniata1, miniata2, miniata3, miniata4, miniata5, miniata7, mirabilis1 2. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1 3. miniata6 4. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	F_{SC} = 0.424 F_{ST} = 0.853 F_{CT} = 0.745
5 groups 1. mirabilis1 2. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1 3. miniata6 4. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6 5. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, miniata1, miniata2, miniata3, miniata4, miniata5, miniata7	F_{SC} = 0.361 F_{ST} = 0.845 F_{CT} = 0.757
6 groups 1. mirabilis1 2. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1 3. gardenii2, gardenii3, gardenii4, , gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11gardenii12, gardenii13, gardenii14, miniata1, miniata2, miniata3, miniata5, miniata7 4. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6 5. miniata6 6. miniata4	F_{SC} = 0.297 F_{ST} = 0.839 F_{CT} = 0.770
7 groups 1. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1 2. miniata4 3. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6 4. mirabilis1 5. gardenii2, gardenii3, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11gardenii12, gardenii13, miniata1, miniata2, miniata3, miniata5, miniata7 6. miniata6	F_{SC} = 0.218 F_{ST} = 0.828 F_{CT} = 0.780

7. gardenii4, gardenii5, gardenii14	
8 groups 1. gardenii4, gardenii5, gardenii14 2. mirabilis1 3. gardenii7, gardenii13 4. gardenii2, gardenii3, gardenii6, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, miniata1, miniata2, miniata3, miniata5, miniata7 5. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6 6. miniata4 7. miniata6 8. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	F_{SC} = 0.180 F_{ST} = 0.826 F_{CT} = 0.788

Table 4.4: Molecular diversity indices for the 6 groups (species) for the combined dataset. Significance values (*p*) are given in brackets

Group	No. of individuals	No. of haplotypes	Molecular diversity indices	
			Haplotype diversity (*h*)	Nuceotide diversity (π)
Caulescens	14	6	0.6813 (0.1316)	0.002078 (0.001247)
Gardenii	21	9	0.8571 (0.0466)	0.002874 (0.001682)
Robusta	9	8	0.7222 (0.1592)	0.000933 (0.000886)
Nobilis	16	4	0.3500 (0.1478)	0.000388 (0.000388)
Miniata	22	9	0.8788 (0.0364)	0.003343 (0.001843)
Mirabilis	5	4	0.9000 (0.1610)	0.001449 (0.001082)

Table 4.5: Nucleotide composition for combined datasets from AMOVA

Combined datasets		
Caulescens	**Gardenii**	**Robusta**
C:15.20%	C:15.27%	C:15.41%
T:31.88%	T:31.92%	T:31.67%
A:36.50%	A:36.58%	A:36.66%
G:16.42%	G:16.24%	G:16.26%
Nobilis	**Miniata**	**Mirabilis**
C:15.23%	C:15.22%	C:15.27%
T:31.86%	T:31.89%	T:31.88%
A:36.71%	A:36.67%	A:36.69%
G:16.20%	G:16.21%	G:16.16%

Table 4.6: Effective population size (expressed as $\theta = 2N_e\mu$) and F_{ST} values

	θ	F_{ST}
caulescens (1)	0.023259	0.00888
gardenii (2)	0.016958	0.00281
robusta (3)	0.007471	0.00326
nobilis (4)	0.001986	0.00091
miniata (5)	0.005124	0.00187
mirabilis (6)	0.006533	0.00156

Table 4.7: Migration rates estimated via ML with MCMC searches (using MIGRATE)

Direction of migration	Migration rate
M21	247.716904
M31	247.716904
M41	247.716904
M51	1057.06
M61	247.716904
M12	4445.21
M32	21598.1
M42	1822.47
M52	1822.47
M62	1822.47
M13	266.551971
M23	1298.19
M43	266.551971
M53	266.551971
M63	266.551971
M14	1234.05
M24	1234.05
M34	1234.05
M54	1234.05
M64	2289.35
M15	302.433968
M25	741.581994
M35	302.433968
M45	302.433968
M65	302.433968
M16	589.847565
M25	589.847565
M36	589.847565
M46	589.847565
M56	1091.98

4.4. Discussion and Conclusion

The phylogeny reconstructed from the combined dataset has short internal branches and weak bootstrap support, while most of the polytomies show sharing of haplotypes (Figure 4.3).One likely scenario is that the genus has recent origins, a phenomenon not uncommon in the region. Results in chapter 3, suggest 17 Ma (NPRS) and 15.6 Ma (BEAST) for the genus. Richardson *et al.* (2001) dated the genus *Phylica* using island species and other genera from the same tribe and reported a radiation date of 7-8 Ma and Klak *et al.* (2004), in their study of the family Aizoaceae, reported a radiation date of 3-4 Ma.

Phylogeography relies on interpreting patterns of congruence, or lack of congruence between geographical distribution of the haplotypes and their geneological relationships. When clades of closely related haplotypes are geographically restricted or occur in close proximity, congruence exists (Schaal *et al.* 1998). Using parsimony, results from the phylogenetic analysis for the combined datasets (Figure 4.3), showed two clades. One clade consisted of *C. mirabilis*, the only monophyletic species of the six in the genus, the other clade divided into two subclades. One of the subclades comprised a monophyletic *C. nobilis,* sister to a clade consisting of a combination of the other four species. A haplotype network reconstruction showed the same pattern. The incongruence between the phylogeographic patterns, the currently accepted taxonomy and geography suggest that there may be ancestral polymorphisms present or incomplete lineage sorting in the genus.

An alternative explanation for the sharing of haplotypes between species is hybridization. References to artificial hybrids are made in the literature by Rourke (2003) and Koopowitz (2002). Natural interspecific hybridization in the genus has rarely been recorded. In 2006, Swanevelder *et al.* formally described a natural *Clivia* hybrid *Clivia* x *nimbicola,* an intermediate between *C. caulescens* and *C. miniata*, growing sympatrically with *C. caulescens* and *C. miniata* and confined to the Barberton area of endemism on the border of South Africa and Swaziland.

In the northern part of the Eastern Cape *C. miniata* and *C. robusta* grow sympatrically and share haplotypes in the haplotype network reconstructed from the

combined datasets. However, they do not share the same flowering times: *C. miniata* flowers in June and *C. robusta* in September which makes hybridization unlikely although it cannot be ruled out completely since *C. miniata* has been known to flower sporadically throughout the year. In Mpumalanga *C. miniata* and *C. caulescens* grow sympatrically and they are also observed to share haplotypes but these two species have different pollinators; the butterfly for *C. miniata* and the sunbird for *C. caulescens*, again making hybridization unlikely, but not impossible. Although the presence of ancestral polymorphisms and incomplete lineage sorting are possible options to explain haplotype sharing for these sympatrically occurring individuals, it is difficult to discern which of the possibilities are is likely for *C. miniata*. This aside, haplotype sharing is clearly evident in these sympatrically occurring species.

Haplotype sharing is also observed between *C. gardenii* and *C. robusta* in the Eastern Cape where they occur sympatrically. The interconnectedness between these two species brings into question the recognition of these two elements as discrete species since *C. robusta* was considered a 'robust' form of *C. gardenii* until it was formally described in 2004 by Murray *et al.*

C. mirabilis (Northern Cape) and *C. nobilis* (Eastern Cape) show the most discrete haplotypes, probably as a result of highly restricted gene flow. *C. mirabilis* occurs in the western most part of the distribution range and *C. nobilis* in the southern most part of the range.

Dupanloup *et al.* (2002) states that the SAMOVA model allows one to define the strongest structure of populations in genetic terms but that the identification of the correct number of groups depends critically on the degree of differentiation between groups. SAMOVA reveals no significant groupings of the populations, suggesting a lack of genetic structure. AMOVA was therefore structured to reflect the six species of *Clivia* as groups. The results showed low genetic variation among groups and high variation within groups and a low degree of genetic subdivision, implying once again a lack of genetic structure and the likelihood that no isolation of the lineages has occurred in these data. All analyses, including the statistical analyses, therefore support the likelihood of incomplete lineage sorting present in *Clivia*.

With the discovery of *Clivia mirabilis* in the Northern Cape in 2002, the question arose whether the genus once occupied a wider range spanning the Eastern and Western Cape. Evidence from the coalescent model, MIGRATE, supports the hypothesis that the genus formerly occupied a wider range spanning the Eastern and Western Cape with past flow gene rates recorded between *C. mirabils* and *C. nobilis*, the most southerly species of *Clivia* and the closest geographically to *C. mirabilis*. Subsequent fragmentation of this distribution range may have been precipitated by the increase in aridity experienced in the Northern and Western Cape during the late Miocene (15-8 Ma) with subtropical elements giving way to fynbos elements. This may have caused the range of *Clivia* to retreat and may account for only one lineage, now represented by *C. mirabilis*, occupying a semi-arid habitat in the Northern Cape.

Although long distance dispersal should be considered, it is highly unlikely for two reasons. Firstly, *Clivia* have heavy fleshy berries that make them unsuitable for wind dispersal, and secondly, while dispersal of seeds by frugivorous birds between adjacent forest patches is likely, dispersal over 800km of arid country does not appear very probable (Rourke 2002). Morever, no frugivorous birds are known to migrate between the Eastern Cape and Northern Cape (Snijman 2002).

An alternate scenario is that *Clivia mirabilis* is a relictual population that successfully colonised a previously unoccupied habitat in the Northern Cape. Tolley *et al.* 2006, in their study of the biogeography of dwarf chameleons suggested that climatic fluctuations have created islands of differing vegetation types, some of which may have persisted as isolated patches for long periods of time. The considerable climatic fluctuation throughout the Pliocene and into the Quaternary caused vegetational changes in the region which are thought to be more complex than a simple reduction of forest and establishment of fynbos and other mesic and arid vegetation types (Midgley *et al.* 2001, Barrable *et al.* 2002).

In 2003, Snijman proposed that recurring intense fires in the fynbos have served to isolate *Clivias* in the Northern Cape from those along the east coast of South Africa. In this scenario it seems that *C. mirabilis* has persisted at Oorlogskloof for hundreds of generations untouched by fires that probably destroyed its ancestors which once occupied the southern Cape during more favourable times (Snijman 2003).

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

The most recent investigation into the Haemantheae was carried out by Meerow and Clayton in 2004.They used *trnL-F* and nrDNA ITS in their study of 19 species of the tribe. The present study increased the sampling considerably to 62 species (with 72% of the Haemantheae represented) and supplements the molecular data available with the addition of three more plastid datasets. This comprehensive investigation reinforced evidence for the monophyly of the tribe and enabled a reassessment of the generic classification of Haemantheae using the well-resolved phylogeny obtained.

Meerow *et al.* (1999b) resolved a monophyletic Haemantheae in their investigation of the family Amaryllidaceae, using *rbcL* and t*rnL-F* sequence data. Ito *et al.* (1999) also established a monophyletic Haemantheae using *matK* sequence data but only three genera. Although bootstrap support for the individual datasets, in this investigation, ranged from weak to moderate, 100% bootstrap support for the tribe was obtained in both the combined plastid and combined plastid and nuclear parsimony analyses. Similarly, the Bayesian analyses produced high probability values in the combined plastid (*P*=99) and combined plastid and nuclear datasets (*P*=100) where the topologies mirrored those of the parsimony analyses.

Haemanthus, *Scadoxus, Clivia* and *Cryptostephanus* emerged as well-defined genera. In the combined plastid and nuclear datasets, the strict consensus tree resolved the tribe into two clades. The smaller clade comprised a reciprocally monophyletic *Clivia* and *Cryptostephanus* with bootstrap support of 80% and 100%, respectively. The larger clade consisted of two subclades: a *Gethyllis-Apodolirion* and a *Haemanthus-Scadoxus* clade. *Haemanthus* and *Scadoxus* were reciprocally monophyletic and resolved as sister genera with 97% and 100% bootstrap support. *Apodolirion* is firmly embedded within *Gethyllis*. This result supports the recognition by Meerow and Clayton (2004) of three subtribes, Cliviinae, Haemanthinae and Gethyllidinae.

Within *Haemanthus*, well supported sister clades are resolved that correspond to summer rainfall and winter rainfall species.

Uncertainty about the distinctiveness of *Gethyllis* and *Apodolirion* was repeatedly expressed by Traub (1963) and other taxonomists 40 years ago, and more recently by Meerow and Clayton (2004). As currently circumscribed, *Apodolirion* is distinguished from *Gethyllis* by a difference in the attachment of the anthers. My analysis show *Apodolirion* to be embedded within *Gethyllis*, and the *Apodolirion* species included here do not form a monophyletic group within *Gethyllis*. *Apodolirion* and *Gethyllis* share many specialized characters: solitary flowers, subterranean ovaries and elongated berries with numerous seeds. They also share the same basic chromosome number x=6 (Vosa 1986), which is unique in the tribe. The shared morphological characters between *Gethyllis* and *Apodolirion* and the molecular evidence of *Apodolirion* embedded within *Gethyllis*, suggest that this clade represents a single genus and a reclassification of the genus is needed.

Another interesting association is that of *Gethyllis gregoriana* and *Apodolirion cedarbergense*. They share a combination of characters unlike that found in species of the 'afra' group (characterised by six or more anthers) and the 'villosa' group (characterised by a laterally curved style). This suggests that the current informal subgeneric groupings can no longer be applied.

Plants in arid conditions often develop specializations to enable them to survive these unfavourable conditions. *Haemanthus* and *Gethyllis,* the two species-rich genera of Haemantheae with distribution ranges mainly in the arid west regions of southern Africa, exhibit several synapomorphic characters, namely a true bulb, leaf pubescence and hysteranthy. These features may not only have contributed to the survival of these species but may have potentially contributed to the radiation of these taxa.

For the dispersal-vicariance analyses using DIVA (Ronquist 1996), the root node of the tribe Haemantheae is assigned all areas used in the study and alternate analysis using rainfall regions did not prove more useful as the areas were once again too widespread. In their biogeographic analysis of the tribe, using 19 species, Meerow and Clayton (2004) rooted the tribe in eastern South Africa. Although the sampling in the present study is more extensive, the areas that are covered by the additional taxa are too many. Future work on the biogeography of the tribe would benefit from

reducing the areas assigned to the analysis and by including regions in East Africa so as to consider the tropical distribution of *Scadoxus* and *Cryptostephanus.*

Although it is highly speculative to do molecular dating without a well-corroborated fossil record, this study attempted molecular dating for the tribe Haemantheae for the first time. Rutschmann (2006) reviews the more common methods used today for estimating divergence times and rates of molecular evolution and these two methods were used here: non-parametric rate smoothing (a method that corrects for rate heterogeneity) and Bayesian implementation of rate variation implemented in BEAST.

It is interesting to note that the radiations of *Haemanthus* and *Gethyllis* did not coincide and therefore were not triggered by one climatic event as was initially hypothesised. A rapid diversification for the winter rainfall lineages of *Haemanthus* took place around 5 Ma, coinciding with the late Miocene/ Pliocene and the aridification and formation of a Mediterranean-type climate. *Gethyllis* on the other hand reflected a gradual diversification from 20-8 Ma, before the aridification and the establishment of the Mediterranean-type climate. This supports the now generally accepted view that the diversity in the Cape region has evolved from several independent radiations interspersed over a long period of time.

Ideally more dating methods should be tested, but the preliminary results obtained for the two methods offer good insights into the diversifications and radiations of the Haemantheae.

The debate on the definition of a species and how to determine species boundaries or species delimitations has been waging for decades and is one that will probably never be resolved. Preliminary DNA sequencing results for the phylogeography of *Clivia* suggests that only two species of the six in the genus *Clivia* are 'true species' in this case referring to monophyletic species, namely *C. mirabilis* and *C. nobilis*. The monophyly of *C. caulescens, C. gardenii*, *C. robusta* and *C. miniata* was not established using two plastid regions. However, the possibilty that the haplotypes which *C. miniata* shares with other taxa (*C. gardenii* and *C. caulescens*) is due to hybridisation where their populations overlap cannot be eliminated.

Additional sequencing is required to shed more light on the infraspecific relationships as well as the species delimitations of this genus. Isolation with Migration (IM) is a coalescent based model recently extended to to include multiple loci (Hey and Nielsen 2004). In addition, it is able to estimate population divergence, along with the population sizes before and after divergence, and the migration rate between populations after divergence. It is also able to infer an asymmetric division of the ancestral population at the time of speciation, with subsequent linear growth of each population to its current size making it a valuable tool in phylogeographic studies (Hey and Nielsen 2004).

Two scenarios have been proposed to understand how *Clivia mirabilis,* in the Northern Cape, came to be isolated from the other five *Clivia* species which occur along the east coast of southern Africa, the nearest being *C. nobilis* some 800km away. No records of wild *Clivia* exist in the southern Cape, despite more than a century of botanical exploration. The first scenario was long distance dispersal through seed but this was ruled out by Rourke (2002). Instead he speculated that *C. mirabilis* is relictual, a survivor of the past climatic history when subtropical vegetation covered much of the interior of South Africa. Dating of the tribe revealed the estimated divergence of *C. mirabilis* from the summer rainfall *Clivia* species to be about 16 Ma (17 Ma for NPRS and 15.6 Ma BEAST). This coincides with the Miocene and the increase in aridification that eliminated subtropical vegetation leaving surivivors to adapt to the emergence of an increasingly dry climate. A second scenario, proposed by Snijman (2003), is that the impact of fire on the Cape forests since the development of the Mediterranean-type climate in the south western Cape, and the inability of *Clivia* to cope with fire have been major factors that led to its current distribution pattern.

In conclusion, both Procheş et al. (2006) and Barraclough (2006) suggested that a stable climate with reliable seasonal rainfall is possibly the cause for the high diversity of geophytes in the Cape. The exact mechanisms, however, of how it affects speciation or extinction is still unclear (Barraclough 2006) but with the advent of new technologies better insights into the evolution of diversity may be revealed.

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Appendix A

Character optimizations of 15 morphological characters of Haemantheae were carried out using the phylogenetic tree obtained from the combined plastid and nuclear parsimony analysis. Morphological characters were compiled from the literature (Dafni *et al.* 1981; Dahlgren *et al.* 1985; Esler *et al*. 1999; Meerow 1995; Meerow and Clayton 2004; Meerow *et al.* 1999; Meerow *et al.* 2000; Nordal and Duncan 1984; Snijman 1984; Snijman 2000; Telford 1987) and the characters were treated as unordered and equally weighted in all analyses.

In general, Stevens (1991) has observed that many characters which are described as being either present or absent may actually represent quantitative variation.
For this study where characters are variable in a species they were coded as absent and present.

1 Underground organ: bulb (0), rhizome (1), rhizomatous bulb (2)

The majority of species in Haemantheae have true bulbs. These usually have a neck of old leaf bases and they sometimes divide into clusters. In *Gethyllis* they often have conspicuous basal sheaths surrounding the foliage leaves. Rhizomes are short or long and erect. *Scadoxus* exhibits a polymorphic state where rhizomes occur in some species and rhizomatous bulbs (not 'true' bulbs) occur in others.

2 Bulb tunics: absent (0), more or less equal and entirely sheathing each other (1), more or less unequal, distichous and sheathing only at base (2)

Bulbs are lacking in *Scadoxus*, *Clivia* and *Cryptostephanus.*
In *Haemanthus* bulb tunics are either approximately equal and more or less entirely sheathe each other (*Haemanthus humilis, H. carneus, H. montanus, H. albiflos* and *H. deformis*) or they are distichous and sheathe each other only towards the base (Snijman 1984).
Bulb tunics in *Gethyllis* and *Apodolirion* consist of few layers only that sheathe each other entirely.

3 Leaf position: suberect (0), prostrate (1), spiralled (2)

Leaves in *Apodolirion* are spiralled (*A. macowanii*), prostrate (*A. lanceolatum*) or suberect (*A. cedarbergense*).

In *Gethyllis* the leaves are more often spiralled (*G. afra, G. britteniana* subsp. *britteniana, G. campanulata, G. ciliaris, G. lanuginosa, G. linearis, G. transkarooica, G. verticillata, G. verrucosa*) and prostrate in a few spcies (*G. sp1*, *G. roggeveldensis, G. barkerae, G. lata* subsp. *lata*, *G. roggeveldensis).*

Some species of *Haemanthus* (*H. amarylloides* subsp. *amarylloides, H. pubescens* subsp. *pubescens, H. pubescens* subsp. *leipoldtii, H. sanguineus, H. deformis*, *H. humilis* subsp. *humilis, H. carneus, H. lanceifolius*) have prostrate leaves (Snijman 1984).

Cryptostephanus, *Clivia* and *Sprekelia* have suberect leaves (Meerow *et al.* 2000).

4 Leaf habit: annual (0), persistent (1)

A strictly annual growth pattern, in which just one set of foliage leaves is produced and shed each year, is found in most species. The leaves may be present during or before flowering. Other members exhibit evergreen leaves that persist for two or more years.

5 Leaf habit: hysteranthous (0), synanthous (1)

Clivia and *Cryptostephanus* are species with synanthous leaves so that the flowers co-occurr with the leaves. Three species of *Scadoxus* in this study are synanthous and two (*S. pole evansii* and *S. puniceus*) are either synanthous or hysteranthous. *Gethyllis* and *Apodolirion* are all hysteranthous and also *Haemanthus* but for *H. pauculifolius*, *H. deformis* and *H. albiflos*.

6 Leaf pubescence: absent (0), single hairs (1), T-shaped trichomes (2)

Leaf pubescence in Haemantheae is uncommon and the leaves are mostly glabrous. In *Gethyllis* the pubescence occurs as simple trichomes which are unicellular, uniseriate, unbranched (*G. lanuginosa, G. roggeveldensis, G.gregoriana, G.*

campanulata, G. ciliaris, G. multifolia) and as T-shaped trichomes which are two-armed and with the arms equal (*G. barkerae, G. verrucosa*) (Weighlin 2002).
The leaf margins in *Haemanthus deformis, H. albiflos* and *H. carneus* are always fringed. *H. humilis* is rarely entirely glabrous and *H. pubescens* is usually pubescent (Snijman 1984).

7 Scape: obsolete (0), present (1)

In *Apodolirion* and *Gethyllis* the scape is reduced and included in the bulb neck, rendering it obsolete. A stout, fleshy, solid, well-exserted scape is present in the other genera. In *Clivia* and *Cryptostephanus*, it is shorter than the leaves and compressed with two sharp edges and in the *Haemanthus* and *Scadoxus* it is elliptical in cross section.

8 Inflorescence: brush type (0), tubular type (1), simple (2)

The inflorescence is a pseudo-umbel with several helicoid cymes. The shape of the individual flowers determines the overall shape of the inflorescence which can be divided into three types. The brush-type inflorescence has flowers which are tightly clustered together and these are characterised by brightly coloured, persistent spathe bracts which often form part of the pollinator attraction system. The tubular type of inflorescence consists of clusters of tubular flowers in which the stamens are included or only slightly exserted from the tube. The flowers may be pendulous or laxly spreading. In contrast to the rest of the Haemantheae, all the species of *Gethyllis* and *Apodolirion* have slightly reduced simple inflorescences consisting of a solitary flower.

9 Position of the style: straight and central (0), curved to one side (1)

The style is exserted. It can be straight and stout as long as the stamens, or longer than the stamens as in *Gethyllis lanuginosa*. It can also be curved with a broad stigma.

10 Anther number: six (0), more than six (1)

In *Apodolirion* six anthers occur in two or a solitary, linear series. They are basifixed in the inner series and dorsifixed in the outer series. The number of anthers in *Gethyllis* ranges from 6-60.

Clivia, *Cryptostephanus* and both genera representing the outgroup (*Sprekelia*, *Calostemma*) have six anthers. In *Haemanthus* and *Scadoxus* six anthers occur which are dorsifixed and versatile (Snijman1984, Snijman 2000, Manning *et al*. 2002).

11 Ovules: <10 (0), >10 (1)

The number of ovules per locule may either be many (more than 10); 5-6 ovules per locule in collateral pairs; or a solitary ovule per locule. Only in *Gethyllis* and *Apodolirion* are more than 10 ovules present per locule.

12 Phytomelan: absent (0), present (1)

Phytomelan is a component of the cell wall of the seed's testa that is black in colour and deposited during carbohydrate consuming processes. It is absent from the testa of most genera of Haemantheae except *Cryptostephanus* and in the outgroup *Sprekelia.* In the monocots generally phytomelan is only known in the Asparagales (Dahlgren *et al.* 1985).

13 Fruit: elongated berry (0), round berry (1), capsule (2)

The fruits of Haemantheae are all indehiscent. They may be clavate, cylindric, often red-spotted aromatic and edible or ovoid to globose. Dehiscent capsules are present in the outgroup and they are considered the basic fruit type for the family by Dahlgren *et al.* 1985. The elongated berries of *Gethyllis* and *Apodolirion* have a translucent membrane and soft endosperm.

14 Embryo: not green (0) green (1)

The fruit of *Gethyllis* and *Apodolirion* have green embryos as do those of *Haemanthus* and *Scadoxus*. *Clivia* and *Cryptostephanus* have a cream-coloured embryo (pers. comm. Graham Duncan) and also the outgroup taxon *Sprekelia*.

15 Chromosome number: 2n=12 (0), 2n=16 (1), 2n=18 (2), 2n=22 (3), 2n=24 (4), 2n=20 (5), 2n>100 (6)

Chromosome numbers range from 2n = 12 to 2n = 24 (Meerow and Snijman 1998, Meerow 1995). Goldblatt (1974) considered 2n = 22 (diploid $x = 11$) the basic chromosome number for the tribe.

Appendix B

Matrix of the 15 morphological characters used in the analysis

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sprekelia	0	1	0	0	0	0	1	2	0	0	1	1	2	0	6
Calostemma	0	1	0	0	1	0	1	0	0	0	0	0	2	?	5
Haemanthus amarylloides subsp. *polyanthus*	0	2	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus amarylloides subsp. *amarylloides*	0	2	1	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus coccineus	0	2	0/1	0	0	0/1	1	0	0	0	0	0	1	1	1
Haemanthus crispus	0	2	0	0	0	0/1	1	0	0	0	0	0	1	1	1
Haemanthus graniticus	0	2	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus montanus	0	1	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus pubescens subsp. *pubescens*	0	2	1	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus pubescens subsp. *leipoldtii*	0	2	1	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus humilis subsp. *humilis*	0	1	1	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus humilis subsp. *hirsutus*	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus pauculifolius	0	1	0	1	1	1	1	0	0	0	0	0	1	1	1
Haemanthus barkerae	0	2	0	0	0	0/1	1	0	0	0	0	0	1	1	1
Haemanthus sanguineus	0	2	1	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus pumilio	0	2	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus nortieri	0	2	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus namaquensis	0	2	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus deformis	0	1	1	1	1	1	1	0	0	0	0	0	1	1	1
Haemanthus albiflos	0	1	0	1	1	1	1	0	0	0	0	0	1	1	1
Haemanthus dasyphyllus	0	2	0	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus unifoliatus	0	2	0	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus tristis	0	2	0	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus carneus	0	1	1	0	0	1	1	0	0	0	0	0	1	1	1
Haemanthus lanceifolius	0	2	1	0	0	0	1	0	0	0	0	0	1	1	1
Haemanthus canaliculatus	0	2	0	0/1	0	0	1	0	0	0	0	0	1	1	1

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Scadoxus pole evansii	2	0	0	0	0/1	0	1	0	0	0	0	0	1	1	2
Scadoxus nutans	1	0	0	0	1	0	1	0	0	0	0	0	1	1	2
Scadoxus membranaceus	2	0	0	0	1	0	1	0	0	0	0	0	1	1	2
Scadoxus multiflorus subsp. *katharinae*	2	0	0	0	1	0	1	0	0	0	0	0	1	1	2
Scadoxus puniceus	2	0	0	0	0/1	0	1	0	0	0	0	0	1	1	2
Clivia nobilis	1	0	0	1	1	0	1	1	0	0	0	0	1	0	3
Clivia mirabilis	1	0	0	1	1	0	1	1	0	0	0	0	1	0	3
Clivia caulescens	1	0	0	1	1	0	1	1	0	0	0	0	1	0	3
Clivia gardenii	1	0	0	1	1	0	1	1	0	0	0	0	1	0	3
Clivia miniata	1	0	0	1	1	0	1	0	0	0	0	0	1	0	3
Apodolirion cedarbergense	0	1	0	0	0	0	0	2	0	0	1	0	0	1	0
Apodolirion lanceolatum	0	1	1	0	0	0	0	2	0	0	1	0	0	1	0
Apodolirion macowanii	0	1	2	0	0	0	0	2	0	0	1	0	0	1	0
Gethyllis ciliaris subsp. *ciliaris*	0	1	2	0	0	1	0	2	0	1	1	0	0	1	0
Gethyllis campanulata	0	1	2	0	0	1	0	2	0	1	1	0	0	1	0
Gethyllis multifolia	0	1	0	0	0	1	0	2	0	1	1	0	0	1	0
Gethyllis linearis	0	1	2	0	0	0	0	2	1	0	1	0	0	1	0
Gethyllis lanuginosa	0	1	2	0	0	1	0	2	1	0	1	0	0	1	0
Gethyllis afra	0	1	2	0	0	0/1	0	2	0	1	1	0	0	1	0
Gethyllis verticillata	0	1	2	0	0	0	0	2	1	0	1	0	0	1	0
Gethyllis namaquensis	0	1	2	0	0	0	0	2	0	0	1	0	0	1	0
Gethyllis marginata	0	1	1	0	0	0	0	2	1	0	1	0	0	1	0
Gethyllis barkerae subsp. *barkerae*	0	1	1	0	0	2	0	2	1	0	1	0	0	1	0
*Gethyllis sp.*1	0	1	2	0	0	0	0	2	0	0	1	0	0	1	0
Gethyllis roggeveldensis	0	1	1	0	0	1	0	2	1	0	1	0	0	1	0
Gethyllis cavidens	0	1	0	0	0	0	0	2	0	0	1	0	0	1	0
Gethyllis britteniana subsp. *bruynsii*	0	1	2	0	0	0	0	2	0	1	1	0	0	1	0
Gethyllis britteniana subsp. *britteniana*	0	1	2	0	0	0	0	2	0	1	1	0	0	1	0
Gethyllis ciliaris subsp. *longituba*	0	1	2	0	0	1	0	2	0	1	1	0	0	1	0
Gethyllis undulata	0	1	2	0	0	1	0	2	0	1	1	0	0	1	0
Gethyllis gregoriana	0	1	0	0	0	1	0	2	0	0	1	0	0	1	0
Gethyllis lata subsp. *orbicularis*	0	1	1	0	0	0	0	2	1	0	1	0	0	1	0
Gethyllis lata subsp. *lata*	0	1	1	0	0	0	0	2	1	0	1	0	0	1	0

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
*Gethyllis sp.*2	0	1	2	0	0	0	0	2	0	1	1	0	0	1	0
Gethyllis verrucosa	0	1	2	0	0	2	0	2	1	0	1	0	0	1	0
Gethyllis transkarooica	0	1	2	0	0	0	0	2	1	0	1	0	0	1	0
Cryptostephanus vansonii	1	0	0	1	1	0	1	1	1	0	0	1	1	0	4
Cryptostephanus haemanthoides	1	0	0	1	1	0	1	1	1	0	0	1	1	0	4