MOLECULAR AND MORPHOLOGICAL PHYLOGENETIC ANALYSES OF *EUPHORBIA* L. (EUPHORBIACEAE) WITH AN EMPHASIS ON SOUTHERN AFRICAN REPRESENTATIVES

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ABSTRACT

The genus *Euphorbia* is morphologically diverse and nearly cosmopolitan. Both succulent and non-succulent species are found within the genus. Succulent species are found in most arid and semi-arid areas of the world, but show their greatest concentration and diversity of form in Southern Africa - especially the Cape Floristic Region. The monophyly and phylogenetic relationships of the genus *Euphorbia* were investigated based on morphology and on sequences of the nuclear ribosomal internal transcribed spacer (ITS) and chloroplast *psb*A-*trn*H intergenic spacer. Fifty-one species of *Euphorbia* and four outgroups from *Monadenium* and *Clutia* were sampled. Matrices were analysed using parsimony and maximum likelihood methods. Separate analyses of data partitions resulted in largely non-conflicting topologies; therefore the data sets were combined. The results showed that the genus *Euphorbia* is paraphyletic and four monophyletic groups with a number of putative synapomorphies defining each clade were strongly supported in most analyses. The Cape succulents fall into two well-supported clades. The results thus provided evidence for Cape radiations. Most analyses indicated that one of the Cape groups is sister to a group consisting of species mostly from Southern Africa (excluding the Cape Region). Increased taxon sampling is however needed to clarify relationships within the monophyletic groups. Morphological characters recovered broad groups within *Euphorbia*. Due to inadequate sampling in some sections, modifications in the present classification of *Euphorbia* are not suggested. The total evidence tree was used to explore morphological character evolution through character state optimisations. The presence of root tubers was homoplasious and this character state is hypothesized to have arisen at least five times under current sampling. Cylindrical stems, conspicuous leaves and absence of leaf spines were some of the pleisiomorphic states observed in some sampled members of the genus *Euphorbia*. Although the results obtained in the current study are preliminary, they have created further challenges for future studies of phylogenetic relationships and morphological character evolution in the genus *Euphorbia*.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The genus *Euphorbia* L. and the Cape Succulents

Euphorbia is the principal genus of the tribe Euphorbieae (with 11 genera), which in turn is the largest tribe of the family Euphorbiaceae (+/- 300 genera) (Dyer, 1975; Webster, 1975; Webster, 1994b). With about 2000 species (Everett, 1981; Carter, 2002) it is one of the largest genera of flowering plants, rivalling *Astragalus* L. (locoweed, 2000 species) in the Fabaceae and *Senecio* L. (groundsel, 2000 species) in the Asteraceae (Koutnik, 1996). The genus is nearly cosmopolitan and its habitat range is enormous, occurring from desert and semi-desert, through coastal dunes, garigue and maquis scrub, to cool temperate woodland where it occurs on damp fertile soils (Griffiths, 1992).

The genus *Euphorbia* consists mostly of monoecious plants. The important characters that distinguish the species of the genus include the vegetative morphology and the inflorescence structure (Webster, 1994a). The growth habit of *Euphorbia* includes herbs, shrubs and trees, usually with milky latex and sometimes succulent. These may be unarmed or spiny and the spines are diverse in nature. The leaves may be alternate or opposite and are often much reduced in succulent species, with or without stipules. The stipules, inserted at the base of the petiole, are a significant foliar structure in the genus (Ullarz, 1978 cited by Webster, 1994a), and these are sometimes spinescent. The stems may be smooth-cylindrical, tuberculate or they may have angles. The inflorescence (cyathium) is a cup shaped involucre with four or five nectar glands around the rim, alternating with five fringed lobes. The nectar glands are usually brightly coloured. The cyathia may be solitary or grouped.

The majority of the species in the genus are herbaceous and distributed in temperate and tropical zones. Succulent species (over 500) occur mainly in Africa, Madagascar, India,

Arabia and the Canary Islands, with a few species in Malaysia, Australia, and Central and tropical South America (Griffiths, 1992; Carter, 2002). In Southern Africa, there are about 200 species of succulent *Euphorbias* (White *et al.*, 1941). Nowhere worldwide have the species of *Euphorbia* reached the zenith of complexity in form that they display in South Africa and especially in the North, West and Eastern Cape Provinces (Williamson, 2000).

The succulent Euphorbieae are postulated to have migrated southwards and congregated in their variation, complexity and apparent adaptation to drought at the southern end of the African continent (Court, 1981). The Cape Region is situated at the southwestern tip of the African continent between latitudes 31° and 34° 30'S and it has a flora that is sharply distinct from the lands immediately surrounding it (Goldblatt and Manning, 2000). One of the four biomes of the Cape Region in which succulent *Euphorbias* are found is called Karroo steppe or succulent shrubland. This biome experiences a winter-wet (rainfall less than 200 mm per year) and summer-dry climatic regime. This is different from the rest of southern Africa (includes all countries south of the Kunene and Limpopo rivers), which receives summer rainfall and lacks nutrient-poor or nutrient-intermediate soils so characteristic of the Cape Region.

Succulent *Euphorbias* show a wide range of growth-form in the Cape Region. These range from leafy geophytes to shrubby, cylindrical-stemmed succulents with small caducuous leaves to forms with succulent tuberculate stems and minute leaves. These shrubs are mostly spineless and where spines are present, they are inflorescence spines. Trees are absent and the proportion of annuals is very low. The overwhelming proportion of shrubs is largely explained by the nutrient-poor quartzitic sand soils that must have raised local speciation rates because of the characteristic low vagility of the seeds in the majority of plants adapted to these soils (Goldblatt and Manning, 2000). Since the flowers in the genus *Euphorbia* are uniform, diversification of the plants in the Cape Region is not likely to be linked to diversity in pollination strategies.

The succulent species with paired-spines and angled stems (see below) have a much wider distribution, occurring in India, Arabia and throughout Africa (Transvaal, Mozambique, Zimbabwe, Swaziland, Natal, Namibia and adjacent Cape Province and Albany District) wherever climatic conditions are favourable to their growth (Croizat, 1965). Although the arrangement of the leaves or leaf rudiments at the apex of the tubercles or of their angular equivalents and the arrangement of peduncles and peduncular spines at the dividing points between tubercles are mostly constant in succulent *Euphorbias*, species from Madagascar do not fit into this pattern (White *et al.*, 1941). The stems of these species are mainly cylindrical and both the tubercles and spines are extremely variable and difficult to interpret.

Because of the diversity of succulent *Euphorbias* found in the Cape Region and the differences in distribution between the different spine types, Croizat (1965) suggested that the Cape *Euphorbias* might have multiple origins. One part was seen as belonging to the classic Cape Flora (i.e. derived in situ), the other part of a generalised South African flora. The current study tests this idea and in particular asks whether the Cape succulent species form one or a few monophyletic groups and if so how these are related to species from the rest of Southern Africa. Phylogenetic relationships of *Euphorbia* species with paired-spines and angled stems from Southern Africa are also evaluated in the current study, especially their relationships to those from India and Madagascar.

1.2 Infrageneric relationships of tribe Euphorbieae

The most recent classification of Euphorbieae (Webster, 1975; Webster, 1994b) recognises three subtribes: Anthosteminae (Baillon) Webster (both the staminate and pistillate flowers possess a perianth), Neoguillauminiinae Croizat (only the pistillate flower possess a perianth) and Euphorbiinae Hurusawa (characterised by lack of a perianth on both the staminate and pistillate flowers, although a rudimentary calyx-like structure is present below the pistillate flowers of a few species). Classification has been based mainly on the staminate calyces, petaloid appendages and gland characters. The

tribe Euphorbieae is homogeneous in the sense that all members possess the very peculiar inflorescence, the cyathium, distinct from all other inflorescence types seen in the family. The cyathium is thus a good contender for a synapomorphy, suggesting that members of the tribe have a common origin (Gilbert, 1994). Another similarity in the tribe is the frequency of succulence within the Euphorbieae, where it appears to have evolved several times independently (Gilbert, 1994).

Subtribe Euphorbiinae is the largest and most complex of the three subtribes. It is considered to include seven of the 11 genera in these subtribes, although some authors have subdivided the genus *Euphorbia* into many segregates. The seven genera according to the circumscription of Webster (1994b) are: *Euphorbia*, *Chamaesyce* Gray, *Cubanthus* (Boiss.) Millsp., *Monadenim* Pax, *Synadenium* Boiss., *Endadenium* Leach and *Pedilanthus* Necker ex Poit. The cyathial morphology of *Euphorbia* is relatively unspecialised. The cyathia are actinomorphic and generally possess one to five separate glands situated on the rim of the involucre, and this feature unites the genus (Webster, 1994a).

The genera segregated from *Euphorbia* are distinguished on the basis of involucral features with the exception of *Chamaesyce* (300 species world-wide) whose cyathium is similar to that of *Euphorbia*. The main difference is that the leaf veins are chlorenchyma-sheathed, the leaves are never alternate or whorled and the main axes cease growth above the cotyledons in genus *Chamaesyce* (Webster, 1994a). *Chamaesyce* is often treated as a subgenus of *Euphorbia* (Gilbert, 1987; Carter, 1988).

The genera *Synadenium* (20 species in Africa) and *Endadenium* (1 species in Angola) also possess actinomorphic cyathia. In *Synadenium* the cyathia possess five united glands that form a complete ring around the top, and in *Endadenium* the closed rim of the cyathium is not a gland but instead an apparently eglandular extension of the involucral wall with a ring of nectar-bearing depressions on the inside of the involucre. The genera *Pedilanthus* (15 species in Mexico), *Cubanthus* (3 species in Cuba) and *Monadenium* (70 species in Africa) possess zygomorphic cyathia. In *Pedilanthus* there are two to six

glands hidden within a nectar spur-like extension of the involucre; in *Cubanthus* there are two glands fused into a shield-like structure on the outside of the involucre; and in *Monadenium* the glands are united into a horse-shoe shaped structure (Leach, 1976; Rauh, 1984; Webster, 1994a). The genus *Monadenium* is similar in growth-form to the *Euphorbias* especially in the formation of thorns and spines and the reduction of foliage (Court, 1981).

Pax (1924), using the shape of involucral glands, treated the genera with continuous glands, *Synadenium*, *Monadenium* and *Stenadenium* (currently treated as a synonym of *Monadenium*) as an independent lineage different from *Euphorbia* in his phyletic graph of relationships. Pax and Hoffman (1931) proposed that *Euphorbia*, *Elaeophorbia* (sometimes recognised as distinct from *Euphorbia*), *Synadenium*, *Monadenium* and *Stenadenium* share a common ancestor. Croizat (1938) also questioned the polyphyly of *Euphorbia* based on the diverse origin of glands in Euphorbieae.

1.3 Taxonomic history of the genus *Euphorbia*

The origin of the name *Euphorbia* is historical. Around 1600 King Juba II of Mauritania discovered the first succulent species (probably *E. resinifera* Berg.) in the Atlas Mountains that he named Euphorbus, in honour of Euphorbus Musa his court physician (Rowley, 1984; Williamson, 2000; Carter, 2002). The literal meaning of this name is "well or adequately fed" (Williamson, 2000; Carter, 2002). The genus *Euphorbia* L. was first formally described by Linnaeus in 1753 based on *E. antiquorum* L. A year later other botanists, for example, Miller (1754) and Trew (1754), divided the genus into several smaller genera. The controversy of the monophyly of the genus *Euphorbia* has continued since then, and the issue as to whether *Euphorbia* should be recognised in its initial broad sense or be separated into many smaller genera has not been resolved (Steinmann and Porter, 2002).

Within *Euphorbia*, the common primary division employed is the rank of subgenus. Within the different subgenera, sections, subsections, series and subseries (in increasing taxonomic exclusivity) are all employed (Koutnik, 1996). A large genus like *Euphorbia* benefits from infrageneric categories as an aid in explaining hypothesised relationships among the many species (Kounik, 1996).

There are numerous taxonomic treatments of the genus *Euphorbia*, some covering one portion of the genus, others one geographical area (Rowley, 1985). Since these are numerous, only those focussing mostly on succulent species from Africa, Madagascar and India will be outlined below. These systems of classification have separated groups solely on the basis of vegetative characters, using habit, number of stem-angles, number of spines and prickles borne on the spine-shield (Carter, 1994); characters of the inflorescence and seed have been almost ignored.

The basic framework for the classification of *Euphorbia* L. provided by Boissier, (1862) for de Candolle's *Prodromus* is still in use today. Boissier recognised the genus *Euphorbia* with 723 species, of which fewer than 30 species are recognised as such today (Carter, 1994, Steinmann and Porter, 2002). The taxa of *Euphorbia* were allocated to 27 sections, numerous subsections and two "series": *Appendiculatae* and *Exappendiculatae*. The sections that are relevant to African succulent *Euphorbias* are *Arthrothamnus*, *Diacanthium*, *Euphorbium*, *Rhizanthium*, *Tirucalli*, *Lyciopsis* and *Tithymalus*. These sections were further divided into various subsections, for example, the section *Diacanthium* was further divided into *Biaculeatae* (species with each spiny shield bearing two spines) and *Triaculeatae* (species with one fused spine and two prickles). *Biaculeatae* was further divided into species with cylindrical as opposed to angled branches (two-angled versus three or more angled branches). South African taxa are prevalent in the section *Euphorbium*. Boissier's revision of *Euphorbia*, the last complete review of the genus, is famous for its judicious use of characters in defining species groups (Webster, 1987), although his sections were criticised by Bentham (1878) for being unequal in systematic value and number of species.

Bentham (1878) followed Boissier's treatment with the greatest change concerning the rank of Boissier's sections of *Euphorbia*. Bentham proposed a system containing only six sections, under which the majority of Boissier's sections were reduced to subsections. One of the six sections, *Euphorbium* (species with thick and succulent or rarely slender stem and branches, almost or quite without leaves), contained African succulent species.

Pax (1904) published a classification for species in section *Diacanthium*, based on the number of spines and prickles present in each spine shield (Carter, 1994) and other vegetative characters. The following names were given to his groups: *Monacanthae* (single spine), *Diacanthae* (*Biaculeate* Boiss.) with two spines, *Triacanthae* (*Triaculeate* Boiss.) with one spine, and two prickles, *Tetracanthae* with two spines and two prickles, and *Intermediae* with two spines, rudimentary prickles, and "upper stipular" spines on each side of the flowering-eye. *Intermediae* was the largest group and unrelated species were included in this group.

In 1907, Berger erected a classification system for succulent *Euphorbias*. He divided the succulents into 12 sections based on size, shape and number of branch-angles. Four of these sections (*Tithymalus*, *Arthrothamnus*, *Tirucalli* and *Diacanthium*) were similar to those proposed by Boissier (1862). Section *Diacanthium* was divided into *Teretes* (species with terete branches) and *Costatae* (species with angled-branches). Berger did not include single-spined species in his classification because they were not available at La Mortola garden where he was a curator (Carter, 1994).

In 1911 and 1912, N. E. Brown published a treatment of *Euphorbia* for the *Flora of Tropical Africa*. In this treatment, he favoured no formal sections within the genus. He used the characters of habit and spinescence (arrangement started with species with single spines first, followed by those with paired spines), and also distinguished between succulent and non-succulent species. He followed a similar pattern in 1915 for species from the regions covered by *Flora Capensis*. These treatments relied upon dried herbarium material that was often inadequate (Rowley, 1985).

Pax (1921) granted subsectional status to his groups when he classified the 109 African species known at that time. He recognised 21 sections as follows: *Anisophylum*, *Tenellae*, *Holsrianae*, *Pseudocalypha*, *Lyciopsis*, *Espinosae*, *Lignosae*, *Galarrhaei*, *Esulae*, *Trichadenia*, *Rhizanthium*, *Arthrothamnus*, *Tirucalli*, *Diacanthium*, *Anthacantha*, *Meleuphorbia*, *Dactylanthes*, *Medusae*, *Pseudeuphorbium*, *Pseudomedusea*, and *Treisia*. Some of the sections were further divided into subsections. The group *Rhizanthium* (Boiss.) was expanded to include one geophytic species and three non-geophytic species from Ethiopia. His previous section *Diacanthium* (Pax, 1904) was changed by removing herbs with tuberous roots from his former grouping in *Diacanthae* and adopted Berger's *Scolopendriae* as a subsection to house them (Carter, 1994). The subsection *Monacanthae* was partially absorbed into *Triacanthae*. Croizat (1972) criticised Pax's sections for not covering the entire field of *Euphorbia* classification. Pax's review of species throughout Africa has also been criticised as inconsistent and over-dependent on too few characters, many of which are seen now as unreliable (Gilbert, 1987).

In 1931, Pax and Hoffmann proposed 9 sections, split into about 50 subsections and a number of series. The sections *Arthrothamnus*, *Tirucalli*, and *Diacanthium* were reduced to subsectional status under section *Euphorbium* Boiss. All the succulent-stemmed species of the genus *Euphorbia* were included in this section. They used Berger's system to regroup the species and all of Berger's group names were also used.

White *et al.* (1941) denied that *Euphorbia* could be satisfactorily classified into subgeneric units having formal status. Thus, they grouped 41 South African species into 19 sections without formal names (Key 1–Key 19). Growth habit, the position of the capsule and the arrangement of the cyathia were used as important features in deciding relationships. Each group with its own special key-name is marked off from the others by a particular group of forms. White *et al.* (1941) were the first to diverge from the system of using vegetative characters as sectional indicators.

The first major conspectus to advocate the use of subgenus as the primary division of *Euphorbia* was published by Wheeler in 1943. He recognised eight subgenera, and out of

these, four subgenera contained succulent species (Table 1). These eight subgenera corresponded to the sections recognised by Pax and Hoffmann (1931). The eight subgenera he recognised are, in many features, profoundly unsatisfactory. For example, geophytes were conventionally placed within subgenus *Rhizanthium* although they had few features in common (Gilbert, 1987). Gilbert (1987) also suggested that the sectional nomenclature of subgenus *Tithymalus* is confused. Croizat (1972) also argued that Wheeler's subgenera are not critically delimited groups but that vague characters were used to bestow subgeneric status upon certain groups of species. For example, *Euphorbias* that are succulent and thorny (*Euphorbia* / *Tithymalus*) or *Euphorbias* that are thorny but not quite succulent (*Lyciopsis*); such assumptions rest on appearance and tradition.

Table 1: Four subgenera of succulent species recognised by Wheeler (1943) and their geographical areas.

Subgenera	Geographical distribution
Agaloma	Central America
Esula Pers.	Canary Islands and Africa
Euphorbia	Africa and India
Rhizanthium (Boiss.) L. C. Wheeler	Africa, primarily Southern Africa

Jacobsen (1954) combined the two systems of Berger (1907) and White *et al.* (1941) to form the basis of a new key, based solely on the character of the shoots, since he said classification based on the character of flowers was difficult. He divided succulent *Euphorbia* species into 28 groups. The species in group 9 (part) through group 19 are all Southern African and are confined to the Cape Province. Groups 20 to 28 contain species with paired spines formed from modified stipules. Koutnik (1996) said some of Jacobsen groups represent true genetic relationships while others are totally artificial, especially group 4. In this group, Jacobsen (1954) combined all species that have slender green stems but originate in a variety of geographic regions (Africa, Australia and South America).

Jacobsen (1960) divided the genus *Euphorbia* into sections and groups according to growth forms. The major groups were:
1) *Pedunculacanthae* (Group 1-Group 19) with thornless plants or plants with thorns that are formed by persisting and hardening peduncles and
2) *Stipulicanthae* with thorns mostly in pairs, derived from stipules. These groups were further separated into sections (Jacobsen, 1960).

In 1972, Croizat reviewed subgeneric classification of *Euphorbia*. His major contribution was to elevate subgenus *Chamaesyce* to generic rank. His argument was that in *Euphorbia* there are no clear cut subdivisions, thus he suggested just introducing subgenus *Euphorbia* with a key-note of characters followed by a pertinent list of sections, subsections and series. Croizat also suggested that subsections of Pax (1921) might be elevated to sections.

Gilbert (1987) did a review of the major infrageneric groups within the African species of *Euphorbia*. He recognised four major groups within the species of *Euphorbia* indigenous to mainland Africa as follows: *Chamaesyce*, *Esula*, *Euphorbia* and *Lacanthis*. He lumped the three genera *Eremophyton*, *Esula*, *Lyciopsis*, along with a significant proportion of "*Tithymalus* sensu Wheeler" into one subgenus *Esula*. The reason was that these subgenera were seen as inter-linked by intermediate species. Gilbert (1987) also provided a detailed overview of the group *Rhizanthium*, concluding that subgenus *Rhizanthium* as currently circumscribed is a heterogeneous assemblage of many unrelated species.

In 1994, Carter did a preliminary classification of *Euphorbia* subgenus *Euphorbia* and delimited two main sections: *Euphorbia* and *Tetracanthae* and several subsections (Table 2). Characteristics of the seed were used initially to separate these two sections. Section *Euphorbia* contained species with what she considered pleisiomorphic character states (habit, leaves, branches, spinescence, cyme arrangement, cyathial arrangement, capsule, female perianth) all with smooth, globose or subglobose seeds. Section *Tetracanthae* contained species with derived characters, all with tuberculate and more-or-less ovoid seeds. Within the classification there is some sharing of character states between

groupings and these were used to indicate trends in development (Carter, 1994). Some of the groupings were similar to those defined by previous authors.

Table 1.1 below summarises the taxonomic history of *Euphorbia*.

Table 1.1: Summary of the taxonomic history of the genus *Euphorbia*.

Classification by	Number of sections or groups	Succulent subgenera, sections or groups
Boissier (1862)	27sections, 2 series	***Arthrothamnus***, ***Diacanthium***, ***Euphorbium***, ***Rhizanthium***, ***Tirucalli***, ***Lyciopsis***, ***Tithymalus***
Bentham (1878)	6 sections	***Euphorbium***, ***Tithymalus***
Pax (1904)	1 section, 5 groups	***Diacanthium***: *Monacanthae*, *Diacanthae*, *Triacanthae*, *Tetracanthae*, *Intermediae*
Berger (1907)	12 sections	***Tithymalus***, ***Arthrothamnus***, ***Tirucali***, ***Diacanthium***, ***Anthacantha***, ***Meleuphorbia***, ***Dactylanthes***, ***Medusea***, ***Pseudeuphorbium***, ***Pseudomedusea***, ***Treisia***
N. E. Brown (1911-12, 1915)	No formal sections	
Pax (1921)	21 sections, various subsections	All 21 sections. ***Diacanthium***: *Diacanthae*, *Triacanthae*, *Tetracanthae*, *Scolopendriae*.
Pax and Hoffmann (1931)	9 sections	***Lyciopsis***, ***Pseudeuphorbium***, ***Euphorbium***: *Arthrothamnus*, *Tirucali*, *Diacanthium*, *Anthacantha*, *Meleuphorbia*, *Dactylanthus*, *Medusae*, *Pseudomedusae*, *Treisia*, ***Rhizanthium***, ***Tithymalus***, ***Esula***
White *et al.* (1941)	Groups without formal names	Key 1 to Key 19
Wheeler (1943)	8 subgenera	***Euphorbia***, ***Lyciopsis***, ***Esula***, ***Rhizanthium***
Jacobsen (1954)	28 groups with no formal ranking	Group 4, Group 6, Groups 9 (part) to 19 (Southern African *Euphorbias*). Groups 21 to 28 (*Euphorbia* subgenus *Euphorbia*)
Jacobsen (1960)	2 sections, various subsections	***Pedunculacanthae***: Groups 1-19 ***Euphorbia*** subsection ***Euphorbia***: a) *Teretes* b) *Angulatae* c) *Costatae* ***Monocanthium***, ***Triacanthium***, ***Tetracanthium***
Croizat (1972)	Sections based on previous authors	Genus ***Chamaesyce***
Gilbert (1987)	4 subgenera	***Chamaesyce***, ***Esula***, ***Euphorbia***, ***Lacanthis***

Table 1.1: continued

Carter (1994)	2 sections	***Euphorbia***: *Euphorbia, Ingentes, Spirales, Scolopendriae, Segmentes, Costatae* ***Tetracanthae***: *Sessiles, Pedicellares*

1.4 Problems with classifying *Euphorbia*

Attention was first directed to *Euphorbia* species more than 2500 years ago on account of the latex they contain (White *et al.*, 1941), and much has been written on the species of *Euphorbia*. There has also been a diversity of taxonomic treatments within the genus *Euphorbia* (as outlined above). However, a satisfactory, global, coherent infrageneric classification of the genus is lacking (Steinmann and Porter, 2000; Carter, 2002). Relationships of species within the genus and groups within the tribe are also uncertain. This is due to:

1) A large number of species. No one has been able to come to grips with all available material of both the Old World and the New World since Boissier (1862) (Gilbert, 1987).
2) Great morphological diversity. Thus, there is a profusion of intergrading and overlapping characters (Sherff, 1940 cited by Park and Elisens, 2000). Croizat (1965: 574) said "...the intraspecific combinations of characters are so intricate as to make it really difficult to identify a truly subgeneric taxon...thus it is difficult to feel adequately prepared to face the classification of *Euphorbia* with a reasonable hope of permanent success".
3) The genus being cosmopolitan, classifications have been done on the basis of local flora without comprehensive phylogenetic studies of the entire range of species. Species of some regions are less accurately understood than those of other regions. For example, the South African species for the most part have been carefully studied and are well illustrated (White *et al.*, 1941). A scheme that will work for species from one region (Old World) would be useless when extended to cover species from other regions (Gilbert, 1987).

4) Many species are represented in the herbarium by material that is wholly unsatisfactory for the worker intending to clear complex problems of *Euphorbia* systematics (Croizat, 1972).

1.5 Toward a phylogeny of *Euphorbia*: objectives of the present study

The reconstruction of phylogenetic relationships of organisms facilitates the testing of systematic hypotheses as well as organising of species into a formal classification (Nelson and Platnick, 1981; de Queiroz and Gauthier, 1994). Largely because of the problems encountered in the classification of *Euphorbia*, no phylogenetic hypotheses (either morphological or molecular) have been brokered at the genus level. A few species of *Euphorbia* from southern Africa have been included in a phylogenetic study at the tribal level done recently by Steinmann and Porter (2002). Because there are many species of *Euphorbia* in southern Africa, this study will necessarily be preliminary in investigating the phylogenetic relationships of the genus with sampling from as wide a range of species as possible.

In the current study, questions about the monophyly of various groups have been raised. This is because a phylogenetic approach demands that taxa be monophyletic and this concept was first formalised by Hennig (1966). A monophyletic group is defined as one that contains all and only the descendents of a common ancestor (Hennig, 1966) and is recognised because of derived characters shared by members of the group (synapomorphies).

It has been suggested by various authors (Court, 1981; Rauh, 1984, Carter, 1994) that succulence in *Euphorbia* has developed as a device for survival in arid regions. Carter (1994), in her preliminary classification of subgenus *Euphorbia,* suggested that within the genus *Euphorbia*, adaptation to increasingly arid habit conditions has involved an increase in succulence of the stem and branches, and a decrease in the size of the plant

body. Other suggested character changes associated with this increased adaptation occur in a reduction in leaf size, development of the spinescence, a reduction of the perianth lobe in the female flower, capsule shape and size, and features of the seed (Carter, 1994). Some of these morphological characters are investigated in this study in order to understand morphological character evolution in the genus *Euphorbia*. Carter (1994) also postulated that by comparing features of the least-succulent trees with those of the most succulent herbs, it is possible to hypothesise characters that have undergone change during adaptation to an arid environment.

The main objective of the current study was to determine phylogenetic relationships of the species of the genus *Euphorbia* from southern Africa. Both morphological and DNA sequence data are used to address the following questions:

1. Is the genus *Euphorbia* monophyletic i.e. are segregate genera such as *Monadenium* nested within the genus or not?
2. Do succulent species from the Cape Region form one or a few monophyletic groups, or are Cape succulents derived from a large number of lineages?
3. Do species with angled stems and paired stipular leaf basal spines from southern Africa form a monophyletic group? How are they related to similar species from India and Madagascar?
4. Are there some morphological characters that have undergone change during adaptation to arid environments within the genus *Euphorbia*? If so, which character states are pleisiomorphic and which ones are derived?

CHAPTER 2

MORPHOLOGY AND CLADISTIC ANALYSIS OF THE GENUS *EUPHORBIA* L.

2.1 Introduction

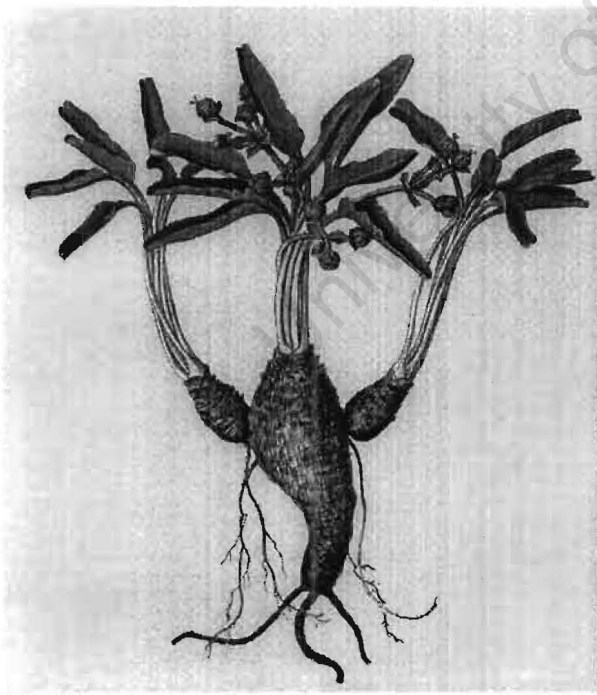
2.1.1 Morphological characteristics of the genus *Euphorbia*

The genus *Euphorbia* is morphologically diverse. The plants range from minute, creeping, non-succulent annuals to herbs, shrubs or trees that can be 10-20 metres tall. The stems can be smooth-cylindrical or tuberculate, they can be jointed or they may be more or less ribbed with angles. The cylindrical stems and branches may be irregularly dotted with isolated tubercles or with tubercles set apart from one another, but in more or less distinct spiral series. The leaves may be alternate or opposite, often much reduced in succulent species and with or without stipules. Many species have spines and these are diverse in nature (Figure 2.1b). Some spines (that may be paired) are hardened short shoots, some are sterile or persistent inflorescences, and others are leaf stipules that may be spinescent, and yet others are outgrowths of the basal rear of the leaves. The spines are mounted upon hardened sections of the tubercle surface that are called the spine shields. A sample of the diverse morphology of *Euphorbia* is illustrated in Figures 2.1a and 2.1b. In their non-blooming stage the succulent *Euphorbia* species resemble the cacti and are often mistaken for them (Jacobsen, 1954; Rauh, 1984).

Euphorbia species have in common the milky white sap, which in almost all cases is toxic on ingestion and highly irritant externally (Griffiths, 1992). In spite of the latex a few species are grazed by goats, sheep and rhinoceros (Williamson, 2000). A feature common to all the *Euphorbias* is the unique inflorescence (Figure 2.2; Everett, 1981; Williamson, 2000). The inflorescence is made up of a bundle of flowers called a cyathium (plural cyathia), which literally means a cup. The cyathium may be unisexual (when it contains only male flowers or only a single female flower) or bisexual (when it

Head clusters of the rhizomatous *E. tridentata* (Marx, 1992).

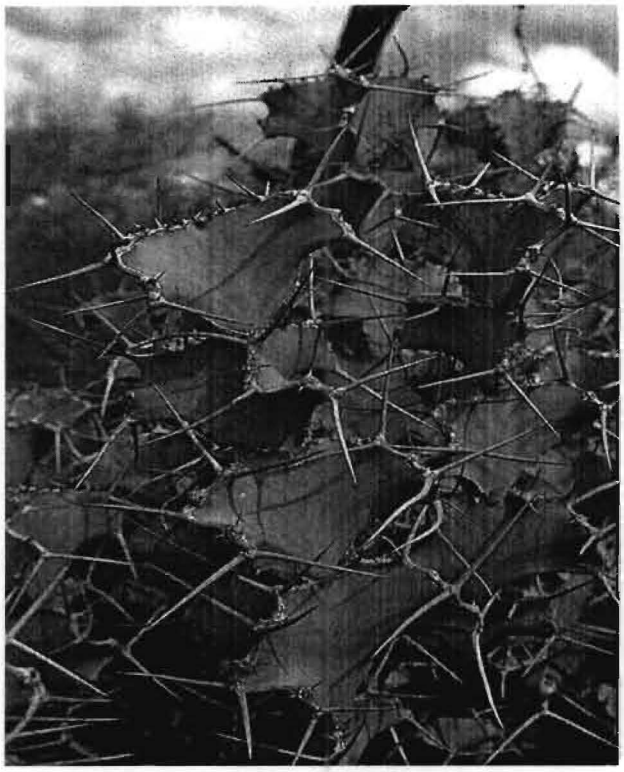
E. quadrialata – a species exhibiting the tree habit (Carter, 1987).



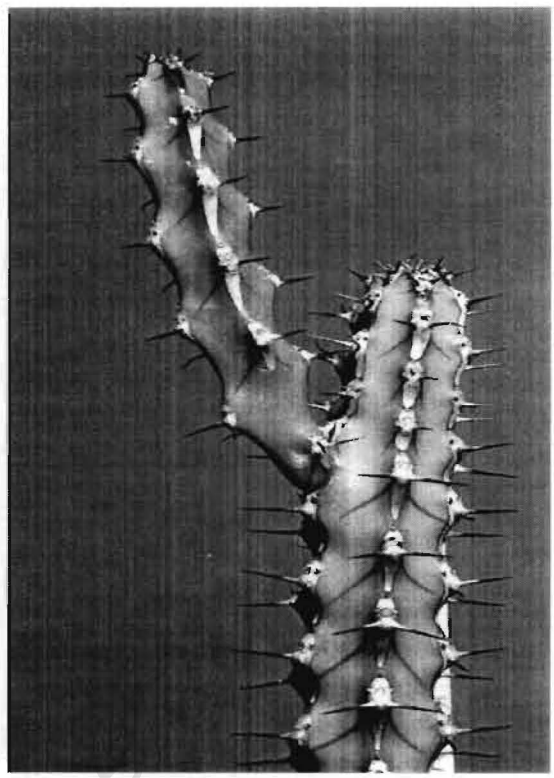
Tuberous rooted, *E. tuberosa* (Koutnik and van Jaarsveld, 1987).

E. decussata showing smooth-cylindrical and decussate stems (Williamson, 2000).

Figure 2.1a. Photographs illustrating the morphological diversity of the genus *Euphorbia.*

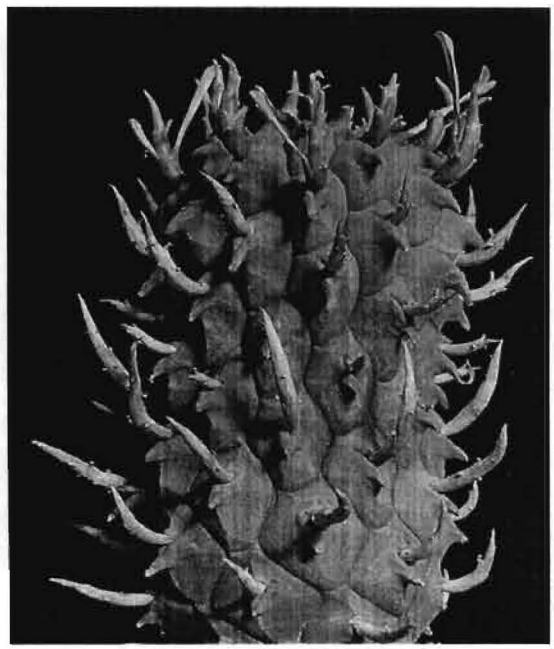


E. grandicornis subspecies *sejuncta* showing paired-spines and stipules (Fourie,1987).



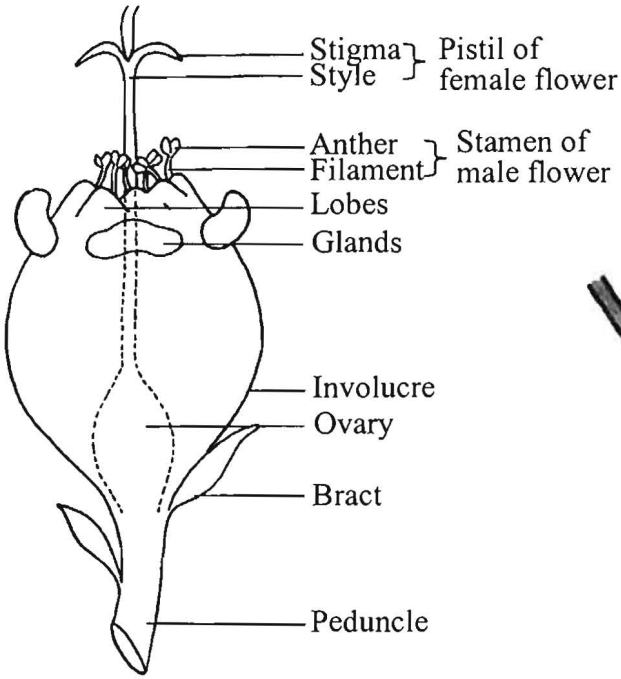
E. griseola subspecies *mashonica* showing paired spines with a horny base (*The Euphorbia Journal* **10:**188-220 [1996]).

Spine-tipped branches of *E. lignosa* (Koutnik,1988).

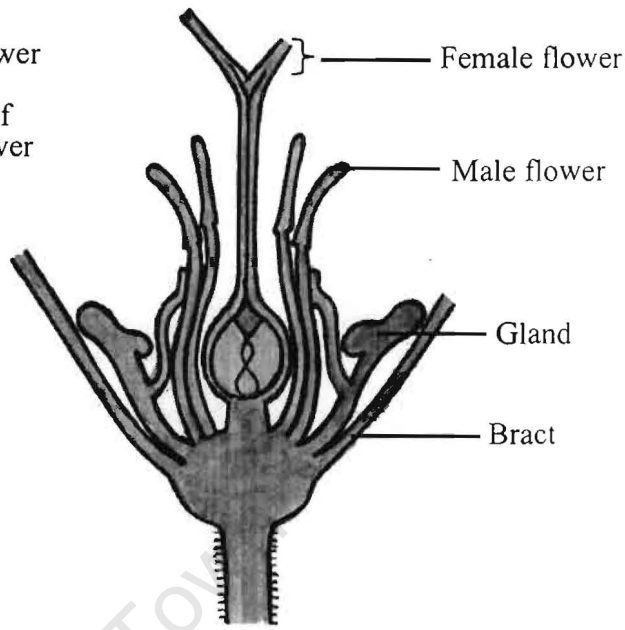


Inflorescence spines of *E. schoenlandii* (Koutnik, 1996).

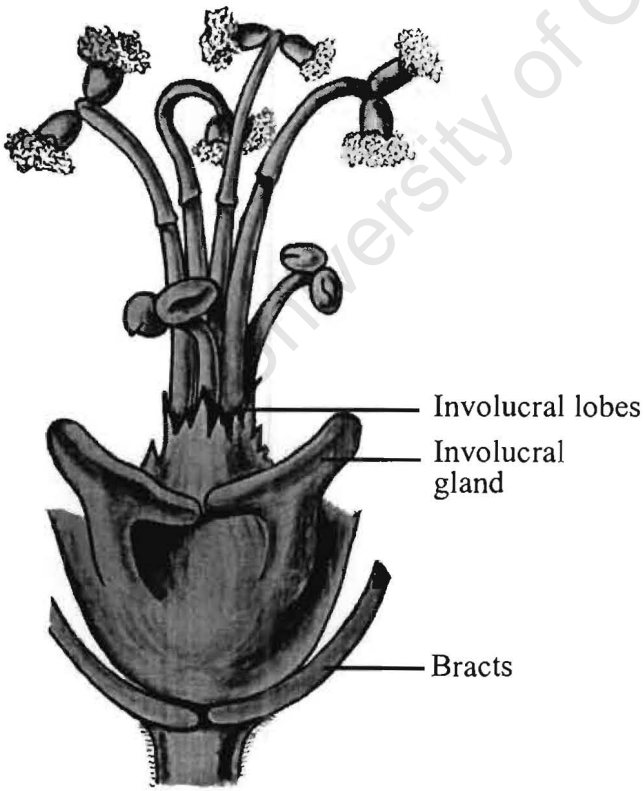
Figure 2.1b. Photographs showing the diverse nature of spines in the genus *Euphorbia.*



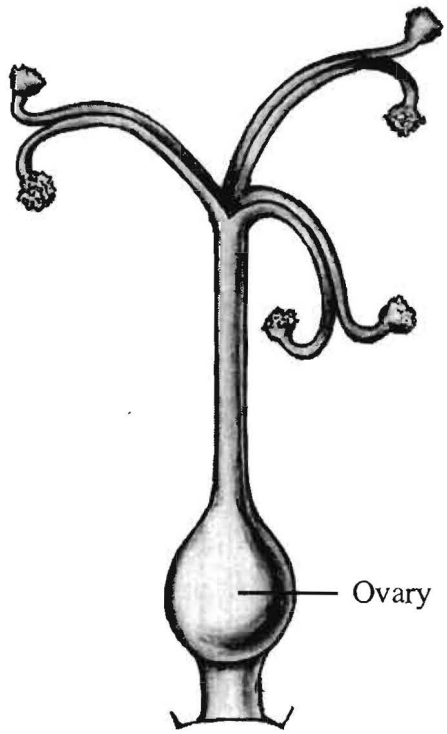
Cyathium (White and Sloane, 1939).



Cross section of a bisexual cyathium (Meijer, 1932).



Male cyathium (Meijer, 1932).



Female flower (Meijer, 1932).

Figure 2.2. Diagrams illustrating the inflorescence of *Euphorbia*.

contains both a female flower and a number of male flowers) (White *et al.*, 1941). Structurally the female flower is placed at the centre and consists of a pedicel with the ovary and three styles. The male flowers are placed around the female flower and consist of a single stamen on a pedicel. The female flowers usually mature before the male flowers and grow outwards from the involucre, thus avoiding self-pollination (Williamson, 2000). Some of the various cyathial forms are illustrated in Figure 2.2.

The fruit is a capsule with carpels that are 2-valved, separating at maturity from a persistent, central axis and opening along the inner face. The seeds may be smooth or may have a sculptured surface (Carter, 2002).

2.1.2 Phylogenetic relationships within the genus *Euphorbia*

The phylogenetic relationships within the genus *Euphorbia* have not been studied using cladistic methods. Phylogenetic studies using morphological characters were done at the subtribal level for New World Euphorbiinae by Park (1996) and for the major taxonomic groups of tribe Euphorbieae by Park and Elisens (2000). The diversity of the taxonomic treatments for the genus *Euphorbia* and reasons leading to this have already been outlined in the previous chapter. Tests of infrageneric boundaries within the genus *Euphorbia* using cladistic principles is a fundamental concern of systematists who specialise in *Euphorbia* and Euphorbiaceae (Park and Elisens, 2000). The current study will be the first to investigate the phylogenetic relationships of the genus *Euphorbia* using morphological characters and cladistic methods of analyses. The monophyly of the genus *Euphorbia* is also tested.

The gross vegetative and floral morphological characters have been used as diagnostic taxonomic features for interpreting the patterns of phylogeny within Euphorbiaceae (Webster, 1994a) and these have also received a great deal of attention in the genus *Euphorbia*. Classification in the genus *Euphorbia* is based primarily on floral structure (cyathial morphology is highly conserved). The flowers and their arrangement throughout the entire genus are so similar that, "although attempts have been made, no generally

acceptable basis has been found for dividing that vast and admittedly unwieldly group into a greater number of smaller genera" (Everett, 1981: 1296).

The vegetative morphology in the genus *Euphorbia* is highly plastic. This is one inherent problem in estimating phylogenetic relationships with morphological characters. However, in the current study vegetative characters including habit, stem, leaf and spine structure, together with characters of the inflorescence that are more stable in classifications (Carter, 1994) are used in an attempt to elucidate the phylogenetic relationships of the genus *Euphorbia*. These were selected on the basis that they are discrete and mostly easily codeable. Since they exhibit shared states among species they are also potentially phylogenetically informative.

2.1.3 Aims of the chapter

The aim of this chapter was to investigate the phylogenetic relationships of the genus *Euphorbia* using morphological characters. The morphological phylogeny was inferred using cladistic methods of analysis. The monophyly of the genus was also tested.

2.2 Materials and methods

2.2.1 Sampling of plants

Sampling of the genus *Euphorbia* was as broad as possible so as to represent much of the morphological diversity of the genus. Fifty-one species of *Euphorbia* and four outgroups were used in the current study. Because of the focus on the Cape succulents, forty-nine of the *Euphorbia* species sampled were succulent and only two *Euphorbia* species (*E. genistoides* P. J. Bergius and *E. helioscopia* L.) were non-succulent. Furthermore, most succulent plants sampled in this study were from southern Africa, with a few species from India and Madagascar. The *Euphorbia* species sampled belong to various sections and subsections recognised by Boissier (1862) and other subsequent workers discussed in Chapter 1. Table 2 shows the sampling of plants in relation to the distribution of diversity among some subgenera and sections in *Euphorbia*. Thus the sampling should permit tests of the monophyly of infrageneric taxa, as well as a preliminary assessment of phylogenetic relationships of the Cape species. Sampling of plants in this study depended on the availability of fresh material for molecular studies (Chapter 3).

Table 2: Sampling in relation to the distribution of diversity among some subgenera and sections in *Euphorbia*.

	Total number of species	Number of species sampled
Subgenus *Euphorbia*	250	19
Subgenus *Esula*	500	3
Section *Arthrothamnus*	20	7
Section *Denisophorbia*	20	1
Other subgenera and subsections	1 210	21

2.2.2 Outgroup selection

An outgroup is selected on the basis of possession of more inclusive synapomorphies shared with the ingroup (Nixon and Carpenter, 1993). The four outgroups were chosen from the genera *Monadenium* (*M. lugardae* N. E. Br. and *M. torrei* Leach) and *Clutia* L.

(*C. pulchella* L. and *C. pterogona* Müll. Arg.) because previous molecular and morphological studies at the tribal level have shown that *Monadenium* is closely related to *Euphorbia* (Park and Elisens, 2000; Steinmann and Porter, 2002) whereas *Clutia* (an African genus with the greatest diversity in South Africa) is more distantly related. *Clutia* belongs to subfamily Phyllanthoideae Ascherson.

2.2.3 Characters and character coding

In order to evaluate variation among members of the study group, morphological characters were obtained and scored from the taxonomic literature and by examination of live specimens whenever possible. All variation was initially considered potentially informative of relationship. An explicit protocol for the selection of characters is necessary to ensure maximum objectivity and repeatability of morphological analyses (Poe and Wiens, 2000).

Character coding for cladistic analyses begins by surveying characters throughout the study group and its outgroup (Stevens, 1991), the aim being to discover comparable features among the taxa in question (establish "one-to-one comparison" among the characters) thus proposing a primary homology (Brower and Schawaroch, 1996). This needs to be done accurately since the character coding represents the link between observation and analysis and greatly influences the results (Pleijel, 1995). Character construction (the partitioning of observed variation into discrete characters and character states) and data matrix construction then follows.

There have been debates on the aspects of coding procedures for characters and character states. There are two schools of thought on coding methods: those that advocate absence/presence coding and those that consider additivity and multi-state coding as appropriate for diagnosis of taxic relations (Kitching *et al.*, 1998). Absence/presence coding is invariably binary and contrasts presence against absence. The absence/presence approach has been criticised by Pimentel and Riggins (1987) because it treats character states independently, thus potentially introducing redundancy into the data and sacrificing information content. However, the advantage of this approach is that questionable

assumptions regarding both ordered and unordered observations are avoided since these will emerge as part of the results (Pleijel, 1995). Also fewest assumptions about character transformation are made. The absence/presence approach (Type C of Pleijel, 1995), binary (two possible states) approach and the multi-state coding methods were thus used in this study.

A total of 21 characters (20 qualitative) were identified comprising 19 binary characters (characters 1, 3-16, 18, 20 and 21) and two multi-state (three possible states) characters (characters 2, 17 and 19) that were unordered. The data matrix is provided in Table 2.1.

2.2.4 Character list and descriptions

A detailed discussion of each character is provided in order that the decisions associated with primary homology assessment are clear. This is important because the presentation of data, together with an explicit protocol for delimiting character states, is necessary to ensure that a cladistic analysis is consistent with the implicit assumption that all character states are discrete (Gift and Stevens, 1997).

Plant characters

1. Plant: (0) *annual*; (1) *perennial*

Perennials may be herbaceous, with only the underground portions living for several years or woody (Judd *et al.*, 1999). In the genus *Euphorbia*, most species are perennial and possess a woody habit (Park and Elisens, 2000) and few species are annuals, for example, *E. helioscopia*.

2. Growth habit: (0) *dwarf geophyte*; (1) *shrub*; (2) *tree*

The genus *Euphorbia* includes among its members a very varied assortment of plants, from annual dwarf herbs and tuberous rooted geophytes to shrubs and trees (Leach, 1976; Carter, 1994). All taxa sampled in this study are dwarf geophytes, shrubs or trees.

3. Plant: (0) *one or more tubers present*; (1) *tubers absent*

The roots of some *Euphorbia* species sampled in this study become greatly swollen and form enlarged tubers. These may be of surprising size when contrasted with the restricted aerial growth that may consist of a few herbaceous branches, or only a cluster of leaves and a few cyathia, for example, *E. silenifolia* (Haw.) Sweet and *E. tuberosa* L. These tuberous species represent another kind of succulence. The leaves die down during the dry season and the plant remains dormant underground waiting for another season (White *et al.*, 1941).

Stem characters

4. Stems: (0) *succulent*; (1) *non-succulent*

Only two *Euphorbia* species (*E. helioscopia* and *E. genistioides*) sampled in this study have non-succulent stems. Most species in the genus *Euphorbia* have succulent stems and consequently they are known as stem succulents (Rauh, 1984). In these, the stem acts as the main food and water storage organ. Carter (1994) suggested that an increase in succulence of the stem and branches is an adaptation to increasingly arid conditions in some cases.

5. Stems: (0) *cylindrical*; (1) *with angles*

Some *Euphorbia* species have smooth-cylindrical or tapering branches, while others have angled branches (White *et al.*, 1941). A tubercle (swelling of the tissue of the stems or branches at the base of a leaf) can merge with its neighbours above and below forming a ridge or an angle either vertically or spirally (White *et al.*, 1941). The prominence of the tubercles becomes modified into the prominence of the angle, and the angles are separated from those at the right and left by depressions, grooves or furrows, or by impressed lines (White *et al.*, 1941). Sometimes the tubercles remain fairly distinct in their positions along the angles, and the angles then appear to be toothed or deeply crenate; sometimes they blend so completely as to make the margins of the angles virtually even.

6. Stems (0) *angles 2-4*; (1) *angles 5 or more*

The number of angles is dependent on the leaf arrangement (Rauh, 1984). If the plant has a two-row leaf arrangement, that is, there are only two orthostiches (straight lines) then the stem has two angles (Rauh, 1984). Other species have many angles. For example, *E. mammillaris* L. may be 7 to 17 angled (White *et al.*, 1941). The stems of many species usually begin in decussate form, which leads to four angles. There seem to be two quite distinct arrangements, alternating and decussate, and the latter leads to the many-angled forms while the former gives rise to some of the cylindrical forms as in *E. lignosa* Marl.

7. Stems (0) *with tubercles*; (1) *without tubercles*

In the more highly succulent *Euphorbias*, the tubercles are often very prominent and they may cover the whole stem surface. These tubercles are formed from the leaf bases spreading downward on the stem to which it is fused. The leaf base, which may be very short, is clearly delineated by a lighter line against the rest of the tissue (Rauh, 1984). The tubercles frequently become thicker in their upper portion than at the base, and they can have a hooked or recurved appearance as in *E. hamata* (Haw.) Sweet and *E. schoenlandii* Pax. Because the leaves are pressed tightly one on the other, the tubercles cover the axes completely but are delineated clearly from one another. In species with a spiral leaf arrangement tubercles are usually hexagonal in shape whereas if the leaves are arranged in a straight line, as in *E. mammillaris*, they are square or rectangular. The tubercles always show the scar of the fallen leaf blade for a long time in the top portion of the stem. Each tubercle bears a leaf rudiment.

8. Central stem different from side branches: (0) *present* (1) *absent*

The main stem may be greatly reduced and fused with the root to form an enlarged main body as in *E. davyi* N. E. Br.

9. Stems: (0) *some stems rhizomatous*; (1) *none rhizomatous*

Some *Euphorbia* species colonise new areas with spreading rhizomes just beneath the surface of the soil, for example, *E. stapelioides* Boiss.

10. Branches tapering into "spine-like" tips: (0) *present*; (1) *absent*

Two species sampled in this study (*E. decussata* E. Mey. ex Boiss., *E. lignosa*) have branches that taper at their tips forming a spine-like projection (White *et al.*, 1941; Rauh, 1984).

Leaf characters

11. Leaves: (0) *succulent*; (1) *non-succulent*

Leaf succulence is present to only a slight degree in some *Euphorbia* and *Monadenium* species sampled in this study (Rowley, 1983; Park and Elisens, 2000).

12. Leaves: (0) *conspicuous*; (1) *reduced and scale-like*

Many species have normal, sometimes broad leaves that are usually deciduous, so the plants are leafless during most of the year and only new growth has leaves (Rauh, 1984). In other species, the leaf blades are small and short-lived or they are reduced to tiny rudiments, visible only with a magnifying glass, and the plants appear to be completely leafless during the whole year (Rauh, 1984).

13. Leaves: (0) *alternate*; (1) *opposite*; (2) *whorled*

Leaf spine characters

14. Stipules modified as spines: (0) *present*; (1) absent

Many *Euphorbia* species have stipules whereas they are absent in apparently related species. These are small appendages alongside the main leaves, which soon harden and after the leaf blades fall, the stipules may remain as small thorns (Rauh, 1984). Spines arising from the side or behind the leaf-base are common. There may be two, three or four of them (Uhlarz, 1978) although they normally appear in pairs on both sides of the base of the leaf. In subgenus *Euphorbia* stipules are mostly reduced to small prickles, much smaller than the main spines. Rarely they are comparable in size to the spines, sometimes they are present or absent (very rarely they can be slightly foliaceous); the

spines are sometimes fused and each leaf base is then associated with a single spine. In many other sections of *Euphorbia* the stipules are present but reduced to very small glands.

15. Dorsal spines: (0) *present*; (1) *absent*

Spines may also appear dorsally at the base of the leaf and sometimes these dorsal spines as well as stipular spines are present. These are secondary appendages of the leaf. Dorsal spines may be solitary or paired and are often large and very hard. In *E. grandicornis* Goeb. the dorsal spines can reach a length of up to 5 cm, whereas the stipular thorns are small and inconspicuous (White *et al.*, 1941; Rauh, 1984).

16. Spines: (0) *with horny base (spine shield) covering part of the tubercle*
(1) *without horny base*

In *Euphorbia,* spines around the leaf-base are often mounted upon hardened sections of the tubercle surface, which are called spiny shields (White *et al.*, 1941). Shields may be wholly detached from one another or united to form a continuous horny margin along the angles (Carter, 1994). The spiny shields surrounding the bases of leaves are common in *Euphorbia* subgenus *Euphorbia* (Carter, 1994).

Inflorescence characters

The unit of the inflorescence in the genera *Euphorbia* and *Monadenium* is called a cyathium (Figure 2.2). The cyathium consists of a cupular receptacle (involucre) with marginal lobes usually regularly alternating with glands, sometimes quite complex in structure (Gilbert, 1987). The glands secrete scented nectar to attract the pollinating insects (Court, 1981; Rowley, 1983). The distinguishing feature of *Euphorbia* and *Monadenium* is the shape and nature of their involucral nectar-secreting glands. In *Euphorbia*, the involucral unit has five lobes and five glands whereas in *Monadenium* the whole series is fused into one gland. In the genus *Monadenium* the nectary gland forms a rim around the flowers. This opens on one side (horse-shoe shape) where the exerted ovary curves downwards (Court, 1981).

There is no corolla although the coloured bracts or glands may sometimes resemble a flower. Each involucral lobe subtends a monochasium of male flowers (Schoute, 1937). The involucre usually encloses one female flower and many male flowers, which are arranged in five groups (Court, 1981). The male flower is always reduced to a single naked anther and the female flower consists of a naked stalked ovary consisting of 3 or more carpels (Gilbert, 1987). In some *Euphorbia* species, there is evidence of a female perianth while the male perianth is usually absent. The floral structure of the genus *Euphorbia* is not shared by any other genera in the family apart from a few closely related ones like *Monadenium*. The cyathium thus contrasts very strongly with inflorescence organization of the vast majority of other members of the family including *Clutia*.

17. Colour of involucral glands: (0) *red*; (1) *yellow-green*; (2) *white*

The colour of the glands makes the whole cyathium more conspicuous. Yellowish and yelow-green colours are vivid and attractive and these colours predominate throughout the genus *Euphorbia*. Involucres of other shades such as brown or red and white also occur (White *et al.*, 1941; Court, 1981).

18. Cyathia: (0) *solitary*; (1) *in groups on each peduncle*

The distribution of the cyathia upon the plants is very different in different species. Sometimes they occur singly directly on the stems or branches, while sometimes they are more regularly spaced along the upper part of the branches in racemes, or are clustered in cymes or umbels as in most spine-paired *Euphorbias* (White *et al.*, 1941).

19. Sexuality (0) *bisexual*; (1) *unisexual*; (2) *andro-polygamous*

In all cyathia both male and female florets are present. In the case where both mature, the cyathium is referred to as bisexual. However it is often the case that only one sex matures while the other aborts before maturity. Thus, some species have unisexual cyathia as found in dioecious situations. In the genus *Monadenium*, monoecious flower production is common and the genus *Clutia* is dioecious and rarely monoecious. Most species of

Euphorbia subgenus *Euphorbia* sampled in this study are andro-polygamous, i.e. the bisexual cyathium surrounds a male central cyathium.

20. Inflorescence modified as thorns: (0) *present*; (1) *absent*

Some *Euphorbia* species sampled in this study have thorns produced through the modification of the floral peduncles. These thorns are called "inflorescence thorns" because they are homologous with inflorescences or their withered and retained central axes (White *et al.*, 1941; Court, 1981; Trager, 1985). If they are sterile, they represent true thorns, because the vegetative point becomes a sharp hardened tip (Rauh, 1984) with a gradual drying out of the branch tips. This prevents the branch from forming a terminal bud and at the same time renders it somewhat woody and contracts the tip into a really sharp spine (Rauh, 1984). The fertile inflorescence thorns are, however, "pseudo-thorns"; they have a blunt tip because their vegetative point ends with the formation of a cyathium.

21. Persistent remains of peduncles: (0) *present*; (1) absent

Seven *Euphorbia* species (*E. multifolia* W. D. S, *E. filiflora* Marl., *E. lignosa*, *E. decussata*, *E. hallii* R. A. Dyer, *E. restituta* and *E. davyi*) sampled in this study have persistent peduncular remains that do not harden into thorns.

Table 2.1: Data matrix of character states for 21 morphological characters used in phylogenetic analysis of genus *Euphorbia*. "?" designates inapplicable states.

Taxa	Characters																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
E. arceuthobioides	1	1	?	0	0	?	1	1	1	1	0	1	1	1	1	?	1	1	1	1	1
E. tuberosa	1	0	0	0	0	?	1	1	0	1	1	0	2	1	1	?	1	1	1	1	1
E. silenifolia	1	0	0	0	0	?	1	1	1	1	1	0	2	1	1	?	1	1	1	1	1
E. nivulia	1	2	1	0	0	?	0	0	1	1	0	0	?	0	0	0	0	1	0	0	1
E. multifolia	1	1	0	0	0	?	0	1	1	1	1	0	2	1	1	?	1	?	0	1	0
E. filiflora	1	1	1	0	0	?	0	1	1	1	1	0	2	1	1	?	1	0	1	1	0
E. oxystegia	1	1	0	0	0	?	0	1	1	1	1	0	2	1	1	?	1	?	1	1	1
E. knuthii subspecies *knuthii*	1	1	0	0	1	0	0	1	0	1	0	1	1	0	0	0	1	1	2	1	1
E. corniculata	1	1	1	0	0	?	0	1	1	1	0	1	2	0	0	0	0	1	2	1	1
E. graniticola	1	1	1	0	1	1	0	0	1	1	0	0	1	0	0	0	1	1	2	1	1
E. helioscopia	0	1	1	1	0	?	1	1	1	1	1	0	0	1	1	?	1	1	0	1	1
E. genistoides	1	1	1	1	0	?	1	1	1	1	1	0	?	1	1	?	1	1	0	1	1
E. stapelioides	1	0	0	0	0	?	1	1	0	1	0	1	1	1	1	?	1	1	0	1	1
E. quadrata	1	1	0	0	0	?	1	1	1	1	1	0	0	1	1	?	1	1	0	1	1
E. hamata	1	1	1	0	1	0	0	1	1	1	0	0	?	1	1	?	1	0	1	1	1
E. schoenlandii	1	1	1	0	0	?	0	1	1	1	1	0	2	1	1	1	1	0	2	0	1
E. tridentata	1	0	0	0	0	?	0	1	0	1	0	1	2	1	1	?	2	0	1	1	1
E. subsalsa	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	1	1	1	1	1
E. stolonifera	1	1	1	0	0	?	1	1	0	1	1	1	0	1	1	?	1	1	2	1	1
E. aeruginosa	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	1	1	1	1	1
E. squarrosa	1	0	0	0	1	0	0	0	1	1	0	1	1	0	1	0	1	1	2	1	1
E. restituta	1	1	1	0	0	?	0	1	1	1	1	0	2	1	1	1	1	1	0	1	0
E. pillansii	1	1	1	0	1	1	0	1	1	1	0	1	2	1	1	1	1	1	1	0	1
E. grandicornis subspecies *sejuncta*	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	1	1	2	1	1

Table 2.1. continued

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
E. susannae	1	0	1	0	0	?	0	0	1	1	0	1	2	1	1	?	1	1	1	1	1
E. unicornis	1	1	1	0	0	?	0	1	1	1	0	1	2	0	0	0	0	1	2	1	1
E. contorta	1	1	1	0	1	1	0	1	1	1	0	0	2	0	0	0	0	1	2	1	1
E. malevola	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	0	1	2	1	1
E. vajravelui	1	2	1	0	1	0	0	0	1	1	0	1	1	0	1	0	1	?	2	1	1
E. elliotii	1	1	1	0	0	?	1	1	1	1	0	0	2	1	1	?	1	?	0	1	1
E. limpopoana	1	1	0	0	1	0	0	1	1	1	0	1	1	0	0	0	1	1	0	1	1
E. leistneri	1	1	1	0	0	?	0	1	1	1	1	0	2	1	1	?	1	1	0	1	1
E. lydenburgensis	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	1	1	2	1	1
E. mammillaris	1	1	1	0	1	1	0	1	1	1	0	1	2	1	1	1	1	0	0	0	1
E. neriifolia	1	2	1	0	1	1	0	1	1	1	0	0	?	0	0	0	0	1	1	1	1
E. griseola	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	1	1	2	1	1
E. lignosa	1	1	1	0	0	?	1	0	1	0	1	0	0	1	1	1	1	?	0	1	0
E. loricata	1	1	0	0	0	?	0	1	1	1	1	0	2	1	1	1	1	0	0	0	1
E. multiceps 1	1	1	1	0	0	?	0	0	1	1	0	1	2	1	1	1	1	0	0	0	1
E. multiceps 2	1	1	1	0	0	?	0	0	1	1	0	1	2	1	1	1	1	0	0	0	1
E. burmanii	1	1	1	0	0	?	1	1	1	1	0	1	1	1	1	?	1	1	1	1	1
E. decussata	1	1	1	0	0	?	1	1	1	0	0	1	1	1	1	?	1	1	1	1	0
E. exilis	1	1	1	0	0	?	1	1	1	1	0	1	1	1	1	?	1	?	1	1	1
E. ramulosa	1	1	1	0	1	0	0	1	1	1	0	1	1	0	0	0	0	1	2	1	1
E. mlangeana	1	1	1	0	1	0	0	0	1	1	0	1	1	0	1	0	1	1	2	1	1
E. lumbricalis	1	1	0	0	0	?	1	1	0	1	0	1	1	1	1	?	1	1	1	1	1
E. suffulta	1	1	1	0	0	?	1	1	1	1	0	1	1	1	1	?	1	0	1	1	1
E. waterbergensis	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	0	1	1	2	1	1
E. hallii	1	1	0	0	0	?	0	0	1	1	1	0	2	1	1	?	0	1	0	1	0
E. bruynsii	1	?	0	0	0	?	0	1	1	1	0	0	0	1	1	?	1	1	0	1	1
E. davyi	1	?	1	0	0	?	0	0	1	1	0	0	?	1	1	?	1	0	0	1	0
Monadenium lugardae	1	1	1	0	0	?	0	1	1	1	0	0	2	1	1	?	1	1	0	1	1
Monadenium torrei	1	1	1	0	0	?	0	1	1	1	0	0	2	0	0	?	1	1	0	1	1
Clutia pulchella	1	1	1	1	0	?	1	1	1	1	1	0	0	1	1	?	1	0	1	1	1
Clutia pterogona	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	?	2	0	1	1	1

2.2.5. Parsimony analyses

The morphological data were entered manually into MacClade version 4.0 (Maddison and Maddison, 2000) and saved as a Nexus file. Parsimony analyses of the morphological data were performed using the software package PAUP* version 4.0b10 (Phylogenetic Analyses Using Parsimony; Swofford, 1998) for Macintosh™. The data matrix was analysed using 1 000 replicates of random taxon-addition (branches having maximum length of zero collapsed to yield polytomies) to find islands of equally parsimonious trees. Searches used TBR (tree bisection-reconnection) branch swapping with MULPARS on and STEEPEST DESCENT options in effect. All character transformations were treated as equally likely and were unordered (Fitch parsimony; Fitch, 1971). Only two trees were saved at each replicate to minimise time spent swapping on islands of equally parsimonious trees (Maddison, 1991). All shortest trees found in the initial 1 000 replicates were then used as starting trees for a second round of heuristic search with TBR branch swapping and a tree limit of 20 000. Heuristic approaches to tree construction were used to search for the shortest trees because of the large size of the data set. Heuristic searches sacrifice the guarantee of optimality in favour of reduced computing time (Swofford *et al.*, 1996). The data matrices for morphological and the DNA data are included on a CD (Appendix 1).

2.2.5.1 Strict consensus tree

The shortest trees from the data set recovered from the different searches were pooled and used to generate a strict consensus tree, which provides the most conservative assessment of the agreement between trees (Page, 1989; Swofford, 1991). A strict consensus tree is derived by combining only those components that appear in all members of a set of fundamental cladograms (Kitching *et al.*, 1998).

2.2.5.2 Successive weighting

Successive weighting (Farris, 1969; Carpenter, 1988) was applied to the morphological data set using the maximum value of the rescaled consistency index with a base weight of 10. This procedure was designed to down-weight characters that are highly

homoplasious. Characters were reweighted until tree lengths were the same in two consecutive rounds. It is necessary that weights stabilize because they can affect the number and topology of the resulting cladograms (Kitching *et al.*, 1998).

2.2.5.3 Jackknife analysis

Internal support or robustness of the trees was assessed using 1 000 jackknife replicates (with 33.67 % character deletion) with simple taxon addition and a tree limit of ten trees per replicate. Jackknife randomly samples characters without replacement to form pseudoreplicate data sets that are smaller than the original (Kitching *et al.*, 1998). The support for a particular clade is the percentage of most parsimonious cladograms resulting from the pseudoreplicates in which a particular group is found (Kitching *et al.*, 1998). The following scheme of support was applied: Jackknife values of 50 – 74 % represent weak support, 75 – 89 % moderate support, and 90 – 100 % strong support.

2.2.5.4 Tree length, consistency and retention indices

The standard measures used in the current study to assess the quality of trees are the tree length (L), consistency, and retention indices. The retention index (RI; Farris, 1989) is the amount of similarity in a data matrix that can be interpreted as synapomorphy. The consistency index (CI; Kluge and Farris, 1969) is defined as the minimum number of character-state changes required by a particular data set (summed over all characters) divided by the total number of all state changes required to most parsimoniously fit all of the characters on the tree under consideration (Sanderson and Donoghue, 1989). If homoplasy is absent, the CI is 1.0 and it decreases toward 0 as homoplasy increases. CI is sensitive to the inclusion of unique derived (autapomorphic) characters that by definition are phylogenetically uninformative (Archie, 1989). In the current study, the CI excluding uninformative characters is reported.

2.3 Results

2.3.1 Equally weighted morphological data

Under equal character weights, 20 000 most parsimonious trees (L = 86, CI = 0.291, RI = 0.755) were retained (maximum trees was set to 20 000). The strict consensus of these is shown in Figure 2.3.

The relationships of some *Euphorbia* species are resolved and the analysis recovered some major groups (Figure 2.3). The ingroup taxa plus the outgroups *M. lugardae* and *M. torrei* form a monophyletic group, and this large clade (group A) is moderately supported (jackknife = 70 %). The sampled members of subgenus *Euphorbia* form a monophyletic group (clade D; Figure 2.3) and a number of clades are resolved within this. However, none of these relationships is supported (jackknife < 50 %). One of the outgroups *M. torrei* is weakly supported (jackknife = 52 %) as sister to subgenus *Euphorbia*. The relationships of the non-succulent *Euphorbia* species included in this study (*E. genistoides* and *E. helioscopia*) are unresolved, but outside a weakly supported succulent group (jackknife = 55%). The group (E) consisting of the two accessions of *E. multiceps* is also weakly supported (jackknife = 52 %).

2.3.2 Successively weighted morphological data

Trees stabilised after five rounds of successive weighting. In this analysis 886 most parsimonious trees were retained, (L = 168, CI = 0.565, RI = 0.918), the strict consensus of which is shown in Figure 2.4.

Resolution is considerably improved under weights, and the groups that are supported have jackknife values greater than 61 %. Subgenus *Euphorbia* and *M. torrei* that is nested within this subgenus in group G is generally resolved and moderately supported (jackknife = 77 %). Relationships among some sampled members of subgenus *Euphorbia* are unresolved, for example, *E. waterbergensis*, and *E. vajravelui*, *E. mlangeana* and *E. squarrosa* (Figure 2.4).

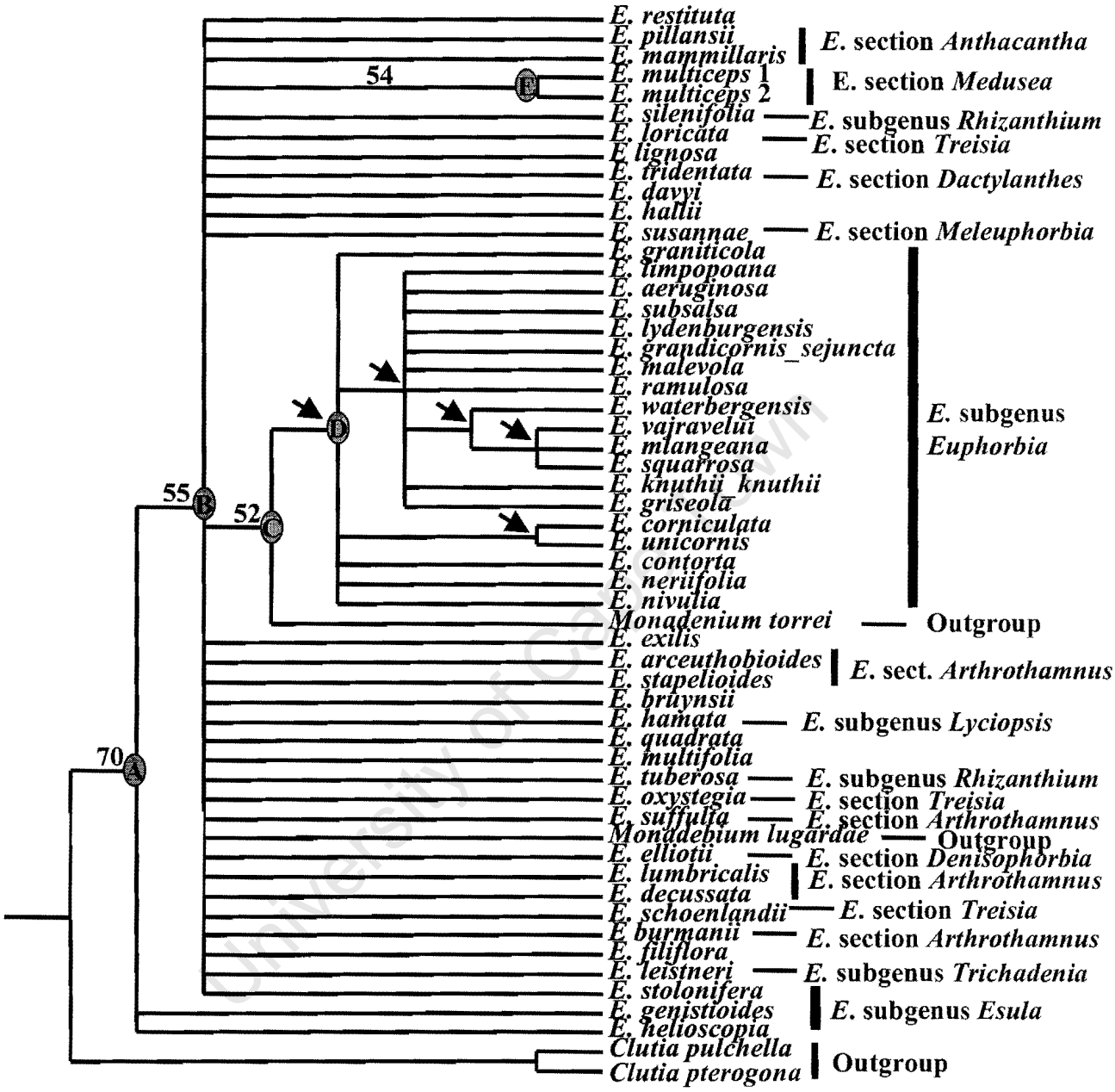


Figure 2.3. Strict consensus of 20 000 trees based on equally weighted parsimony analysis of the morphology data set. Jackknife values greater than 50 % are shown above the branches. Arrows indicate branches with less than 50 % support. A – E designate groups labeled for discussion in the text. The classification is derived from Boissier (1862) and subsequent workers. The classification of species not assigned to infrageneric groupings is unknown.

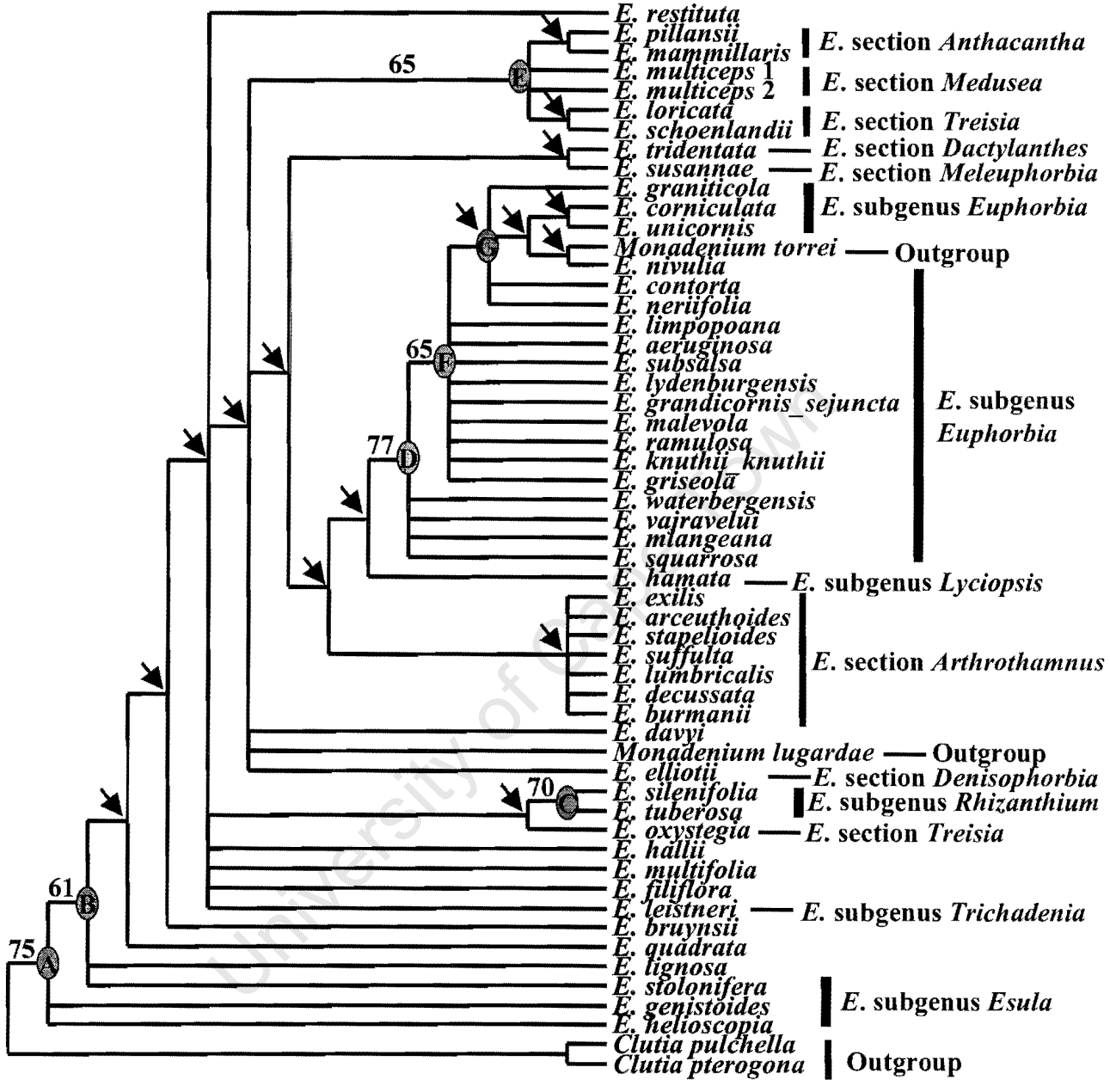


Figure 2.4. Strict consensus of 886 trees based on successively weighted parsimony analysis of the morphology data set. Jackknife values greater than 50 % are shown above the branches. Arrows indicate branches with less than 50 % support. A – G designate groups labeled for discussion in the text. The classification is derived from Boissier (1862) and subsequent workers. The classification of species not assigned to infrageneric groupings are unknown.

Two sampled members of subgenus *Rhizanthium* included in this study (*E. silenifolia* and *E. tuberosa*; Figure 2.4) are resolved as sister taxa and the relationship is moderately supported (jackknife = 70 %). The relationships of the non-succulent *Euphorbia* species are also unresolved as in the analysis of the equally weighted morphology data, but they are placed outside an unsupported clade comprising all the succulent species (Figure 2.3). The sampled members of sections *Treisia* (except *E. oxystegia*), *Medusea* and *Anthacantha* form a monophyletic group (clade E) that is weakly supported (jackknife = 65 %). Members of section *Arthrothamnus* included in this study form an unsupported monophyletic group whose internal relationships are unresolved (Figure 2.4).

2.4 Discussion

The morphological analyses show that genus *Euphorbia* is non-monophyletic as long as *Monadenium* is excluded. The monophyly of subgenus *Euphorbia* is also contradicted in the morphological analyses. This subgenus was recovered as monophyletic in the analysis of the equally weighted morphology data and non-monophyletic in the successively reweighted morphology data analysis where the outgroup *M. torrei* is nested within it. Although *M. torrei* has spines, it lacks the unique spine shield and the paired spines that are postulated synapomorphies for subgenus *Euphorbia* (Carter, 1994) implying that these are secondarily lost. Two sister taxa, *E. corniculata* and *E. unicornis* that were nested within subgenus *Euphorbia* occur as localised endemics in northern Mozambique.

Successive reweighting of characters resulted in a more resolved and better supported topology as compared to the tree recovered from equally weighted analysis (Figures 2.3 and 2.4). This might imply that the morphological data set is quite homoplasious as indicated by the very low CI (0.291) of the unweighted data. Although most nodes recovered from the successively reweighted analysis were not supported, the few that were supported had jackknife values above 60 % (Figure 2.4).

The relationship of two sister taxa (*E. silenifolia* and *E. tuberosa*) recovered in the analysis of the successively reweighted morphology data is supported (jackknife = 70 %; node C, Figure 2.3). These species belong to subgenus *Rhizanthium* and they are dwarf geophytes with succulent tuberous roots. The equally weighted analysis recovered two accessions of *E. multiceps* as sister taxa. There is a dispute as to whether these species should be treated as one or two different species because they are morphologically similar (P. V. Bruyns, personal communication).

The relationships of the two non-succulent species *E. genistoides* and *E. helioscopia* sampled in this study are resolved as "early diverging" or "basal" lineages, albeit with no support. Intensive sampling of the non-succulent species is thus needed in order to have a better insight of their phylogenetic relationships and how they are related to the succulent

species. Species belonging to section *Arthrothamnus* form an unsupported monophyletic group whose internal relationships are not resolved. These species are united by the presence of tiny leaves – a state that is apparently synapomorphic for the group.

Generally the morphological characters used in this study have managed to resolve relationships only among some broad groups of *Euphorbia*. This might be because the 21 morphological characters used were insufficient to resolve relationship among the species but useful in recovering the broad groups. The vegetative and inflorescence characters employed in the analyses are not highly variable among the *Euphorbia* species. This preliminary study thus indicates that the morphological characters of *Euphorbia* have a potential in phylogenetic reconstructions of the genus and has been useful in showing the broad relationships of *Euphorbia* species. In future, further detailed morphological studies using vegetative and inflorescence characters needs to be done in order to discern relationships of *Euphorbia* species.

It is difficult to make conclusions at this stage about the phylogenetic relationships of the genus *Euphorbia* with only morphological data since most groups recovered were either unsupported or had low support and the phylogenetic relationships of many species were not resolved. Further analyses using molecular data and the combined morphology and molecular data are presented in the forthcoming chapters in the hope that they might shed more light on the phylogenetic relationships of *Euphorbia* species within the broad groups recovered in this study and also solve the issue of the contradicted monophyly of subgenus *Euphorbia*.

CHAPTER 3

PHYLOGENY OF THE GENUS *EUPHORBIA* L. INFERRED FROM CHLOROPLAST (*psb*A-*trn*H) AND NUCLEAR (rRNA ITS) DNA SEQUENCES

3.1 Introduction

3.1.1 Utility of molecular evidence

There are some situations where morphological evidence alone will be inadequate for providing information on the phylogenetic relationships of a study group. For example, it may be difficult, as in this study, to score sufficient characters to resolve the relationships among the taxa. Molecular evidence may be used in this case and it may have the following advantages over morphology.

1) Molecular analysis can provide numerous independent characters that can improve the statistical consistency of the tree estimation process (Felsenstein, 1978). On the other hand, morphological analysis sometimes provides fewer characters, often of questionable homology (Miyamoto and Cracraft, 1991; Donoghue and Sanderson, 1992).
2) Morphology, unlike most DNA regions, is susceptible to convergent evolution by natural selection. Molecular data sets seem to contain less convergence than their morphological counterparts, but this may be due to differences in understanding of the genetic basis of convergence in molecules as opposed to morphology.
3) Molecular analyses are relatively free of the subjective kinds of character analysis that might mislead morphological analyses (Sytsma, 1990). The less ambiguous character state assignments in molecular data may reduce artifacts introduced through human error (Sanderson and Donoghue, 1989). In morphological data there might be a problem of character misclassification that in turn is a source of homoplasy.

Although much emphasis has been placed on the use of molecular versus morphological evidence, the primary goal in phylogenetic studies is to infer the single historical genealogy, that is, the true phylogeny of a group of organisms (Systma, 1990; de Queiroz *et al.*, 1995). The choice of appropriate DNA regions for phylogenetic reconstructions and the kind and methods of phylogenetic analyses are critical in inferring genealogy hence they are discussed below.

3.1.2 Choice of DNA region

DNA sequencing, with the advent of PCR technology, has rapidly become the major source of comparative molecular data (Olmstead and Palmer, 1994). The choice of molecule or DNA sequence to be examined is an important first step in any evolutionary or systematic study. Prior knowledge of rates of change, amounts of variation at different taxonomic levels and degree of expected homoplasy is essential before and after data are collected (Sytsma, 1990).

It is now widely accepted that multiple markers should be used to reconstruct the evolutionary history of a group. In the current study, two non-coding regions, the chloroplast *psb*A-*trn*H intergenic spacer and the ITS region of the nuclear rRNA cistron were utilised to infer relationships of the genus *Euphorbia*. Generally, non-coding regions display higher mutation rates than coding regions (Curtis and Clegg, 1984; Zurawski and Clegg, 1987; Clegg and Zurawski, 1991) and have the advantage of experiencing limited or no selective pressure (Palmer, 1987; Olmstead and Palmer, 1994). Insertions or deletions (indels) occur frequently in these non-coding regions and it has been shown that they are primarily synapomorphic characters in certain plant groups as, for example, in the genus *Paeonia* (Sang *et al.*, 1997). Although there are difficulties associated with non-coding sequence data, for example, alternative alignment possibilities of indels and regions of length mutations in which homology assessment is questionable (Kelchner, 2000), *psb*A-*trn*H and ITS have been shown to be useful in recovering phylogenies of plants at lower taxonomic levels.

The *psb*A-*trn*H intergenic spacer, an evolutionarily plastic region that can tolerate many indels (Aldrich *et al.*, 1988, Sang *et al.*, 1997), has been used as a phylogenetic marker to assess inter-specific relationships in plants. The *psb*A-*trn*H intergenic region lies close to the end of the large single-copy region of chloroplast DNA that is known to be a hot spot for length mutation (Aldrich *et al.* 1988).

Since chloroplast DNA genes are uniparentally transmitted, phylogenies derived from cpDNA sequences may or may not be congruent with overall organism history (Clegg and Zurawski, 1991), thus comparison with nuclear DNA phylogenies may be used as a means of identifying such problems (Wendel *et al.*, 1991) and verifying species relationships. The ITS region has been used for reconstructing lower level relationships among and within species in plants (Baldwin, 1992; Baldwin *et al.*, 1995; Sang *et al.*, 1995).

The internal transcribed spacers are part of the nuclear rDNA transcript but are not incorporated into ribosomes (Soltis and Soltis, 1998). The ITS region includes two internal transcribed spacers (ITS 1 and ITS 2) and an evolutionarily highly conserved sequence, the 5.8S subunit. ITS 1 is located between the small subunit (16S-18S) and 5.8S rRNA cistronic regions, and ITS 2 is located between the 5.8S and large subunit (23S-28S) rRNA cistronic region. The two spacers and the 5.8S subunit are collectively known as the ITS region (Baldwin *et al.*, 1995). The basic structure of this region is shown in Figure 3.1.

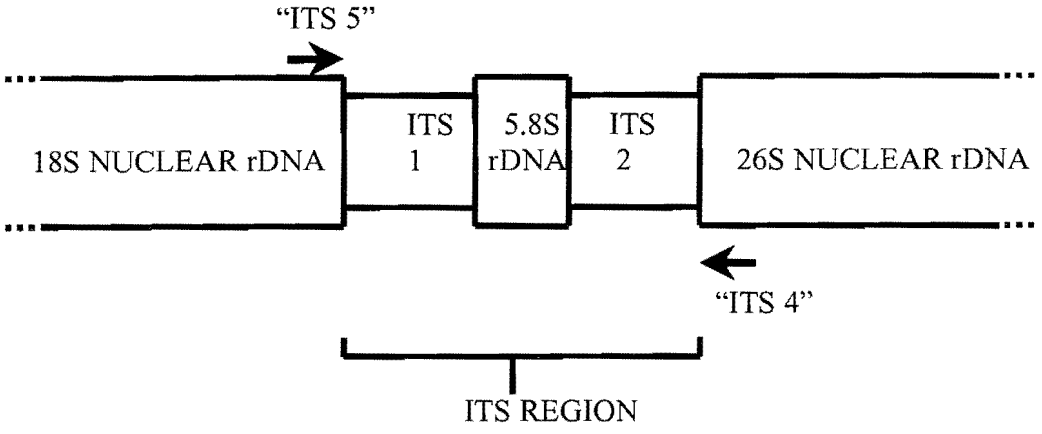


Figure 3.1: The organisation of the entire ITS region of the 18S/26S nuclear ribosomal DNA (nrDNA) repeats. Arrows indicate orientation and approximate positions of primer sites. Primer names ("ITS 4" and "ITS 5") are from White *et al.* (1990). Diagram redrawn from Baldwin *et al.* (1995).

The ITS region is small in most angiosperms (less than 700 base pairs) and highly repeated in the plant nuclear genome. The region thus has the advantage that it is easily PCR-amplified and sequenced with conserved primers positioned in the gene regions (Baldwin *et al.*, 1995; Liston *et al.*, 1996). Also, the relatively high rates of nucleotide substitution in the internal transcribed spacers permit systematic comparisons of relatively recently diverged taxa (Liston *et al.*, 1996). Another advantage is that the region usually undergoes rapid concerted evolution via unequal crossing over and gene conversion (Hillis *et al.*, 1991; Arnheim, 1983) and this property contributes to accurate reconstruction of species relationships from these sequences (Sanderson and Doyle, 1992; Baldwin *et al.*, 1995).

3.1.3 Combined versus separate analysis

Questions and controversies arise whenever multiple studies have been conducted to address the same problem. In this study multiple data sets represent different genomes and different kinds of data (sequence data and morphological data). There have been debates on whether data from these multiple data sets should be combined or analysed separately. In the combined or "total" evidence approach (Kluge, 1989; Kluge and Wolf, 1993), all the available data is combined into a single matrix before phylogenetic

analyses. Phylogenetic information from all characters is considered simultaneously and conflict between individual characters can be assessed (Larson, 1994). The consensus approach (Miyamoto and Fitch, 1995) involves analysing data sets separately and the different phylogenetic estimates compared. This method seeks similarities between independent analyses for phylogenetic corroboration. The conditional combination approach (Bull *et al.*, 1993; de Queiroz, 1993; de Queiroz *et al.*, 1995) involves combining data except when heterogeneity between data sets yields significantly different phylogenetic estimates that are too great to be explained by sampling error of either taxa or characters (Bull *et al.*, 1993; Huelsenbeck *et al.*, 1996). Heterogeneity may then be attributable to different branching histories for the different loci or to features of gene evolution such as lineage sorting (Rieseberg and Soltis 1991; Wendel and Doyle, 1998).

Legitimate arguments exist on both sides of the debate. The key arguments for the combined approach are that it minimises sampling error and maximises the "explanatory power" of the data (Kluge, 1989). Combined data analyses may reveal groups not present in any of the separate trees and may also resolve conflicts among trees from separate analyses (de Queiroz *et al.*, 1995). The underlying argument is that with an increasing number of characters the phylogenetic signal is more likely to assert itself over the noise in separate data sets, resulting in a more accurate estimate of the true phylogeny (Barrett *et al.*, 1991; de Queiroz, 1993).

Some arguments given against combining data sets are that, one data set may have inordinately great influence on an analysis, simply by virtue of having a large number of characters (Hillis, 1987) that might be misleading in some way. Other arguments concern the impact of putting together a "bad" data set that does not have the ability to accurately reflect true phylogenetic relationships and a "good" data set with the ability (Bull *et al.*, 1993). Conducting separate analyses ensures that data sets that strongly contradict each other will each provide a phylogenetic estimate that reveals the contrasting relationships. Also, in plants contrasting histories of different genomes (uniparentally inherited and nuclear) may be revealed especially in cases of hybridisation and cytoplasmic

introgression events that occur at a higher frequency than nuclear introgression (Doyle, 1992; Wendel and Doyle, 1998), resulting in discordance among phylogenies.

Since in a given case both combined and separate analyses can have advantages, some authors have concluded that, the "best" method of analysis in a given instance may depend on the relative importance given the resolving power versus avoidance of error (Bull *et al.*, 1993; de Queiroz, 1993; de Queiroz *et al.*, 1995). In this study separate analysis of the ITS and psbA-trnH data sets and the combined analysis of these data sets were performed since comparison of individual analyses showed little or no conflict (as determined by inspection of strict consensus trees and comparison of jackknife support). These approaches also have potential benefits as outlined above.

3.1.4 Aims of the chapter

Since the morphological analyses managed to delineate some broad groupings in *Euphorbia*, one of the aims of the current chapter is to use DNA sequence data in an attempt to gain additional insights into the phylogenetic relationships of the genus *Euphorbia*. The monophyly of the genus *Euphorbia* and of some broad groupings recovered in the morphological phylogeny, for example subgenus *Euphorbia,* is also tested. The other aim is to test Croizat's (1965) idea, based on the diversity of succulent *Euphorbias* in the Cape Region, that the flora of Southern Africa is divided into two - a Cape Flora and a generalised South African element (see Chapter 1). The particular issue to be addressed is whether succulent species from the Cape Region form one or a few monophyletic groups, or whether these Cape succulents are derived from a large number of lineages.

3.2 Materials and Methods

3.2.1 Sampling of plants and data sets

The same sampling strategy utilised in Chapter 2 was also used in this chapter. Some subsections of *Euphorbia* were not included because sequences for taxa in these subsections were difficult to obtain and also due to limited project time (6 months) and resources.

The ITS data set included 43 species (4 outgroups), whereas that from the *psb*A-*trn*H spacer included 51 species (4 outgroups). Twelve *Euphorbia* species were included in the *psb*A-*trn*H spacer data but not included in the ITS and four species were included in the ITS that were not included in *psb*A-*trn*H spacer (Table 3.1). This was because of problems of obtaining "clean" sequences for one region for these taxa. All samples were vouchered with herbarium specimens (Table 3.1).

Table 3.1: *Euphorbia* species sampled in the molecular study and their geographical distribution. The availability of sequences for each gene region is indicated by x whereas unavailability is indicated by -.

Taxon	Geographical distribution	Voucher (in Bolus)	Sequences for ITS	*psb*A-*trn*H
Ingroup				
E. arceuthobioides Boiss.	Cape Province	PVB 9070	x	x
E. tuberosa L.	Cape Province	PVB 9075	x	x
E. silenifolia (Haw.) Sweet	Cape Province	PVB 9069	-	x
E. vajravelui Binojk. & Balakr.	Southern India	PVB 5891	x	x
E. nivulia Buch.-Ham	Madagascar	PVB 5869	x	x
E. graniticola Leach	Mozambique	PVB 7398	x	-
E. multifolia W. D. S	Cape Province	PVB 2791	x	x
E. filiflora Marl.	Cape Province	PVB 5156	x	x
E. oxystegia Boiss.	Cape Province	PVB 6342	x	x
E. exilis Leach	Cape Province	PVB 9117	x	x
E. elliotii Leandri	Madagascar	PVB 5964	x	x
E. bruynsii Leach	Cape Province	PVB 1814	x	x
E. suffulta Bruyns	Cape Province	PVB 3142	x	x
E. knuthii subspecies *knuthii* Pax	Mozambique	PVB 4466	x	x
E. stapelioides Boiss.	Northern Cape	PVB 3947	x	x
E. quadrata Nel	Cape Province	PVB 3936	x	x
E. hamata (Haw.) Sweet	Cape Province	PVB 4347	x	x
E. schoenlandii Pax	Cape Province	PVB 6135	x	x
E. lumbricalis Leach	Cape Province	PVB 1083	x	x
E. stolonifera Marl.	Cape Province	PVB 3938	x	x
E. aeruginosa Schweickerdt	Cape Province	PVB 7473	x	x
E. corniculata R. A. Dyer	Mozambique	PVB 7714	x	x
E. squarrosa Haw.	Cape Province	PVB 7480	x	x
E. restituta N. E. Br.	Cape Province	PVB 6129	-	x
E. unicornis R. A. Dyer	Mozambique	PVB 8554	x	x
E. grandicornis subspecies *sejuncta* Goeb.	Natal / Mozimbique	PVB 8543	x	x
E. susannae Marl.	Cape Province	PVB 945	-	x
E. lydenburgensis Schweickerdt & Letty	Transvaal	PVB 6615	x	x

Table 3.1. continued

E. ramulosa Leach	Mozambique	PVB 8540	x	x
E. contorta Leach	Mozambique	PVB 7732	x	x
E. mammillaris L.	Cape Province	PVB 6702	-	x
E. neriifolia L.	India	PVB 5917	x	x
E. griseola Pax	Botswana	PVB 7375	x	x
E. leistneri R. H. Archer	Namibia	PVB 5598	x	x
E. waterbergensis R. A. Dyer	Northern Province	PVB 6539	x	x
E. limpopoana (Carter) Leach	S. E. Botswana, Zimbabwe, N. E. South Africa	PVB 7474	x	-
E. malevola Leach	Zimbabwe	PVB 7757	x	x
E. mlangeana Leach	Malawi	PVB 8602	x	x
E. lignosa Marl.	S. W. Africa, Namaqualand	PVB 4184	-	x
E. loricata Lam.	Cape Province	PVB 6165	-	x
E. multiceps 1 A. Berger	Cape Province	PVB 9162	-	x
E. multiceps 2 A. Berger	Cape Province	PVB 9168	-	x
E. burmanii E. Mey. ex Boiss.	Cape Province	PVB 9172	x	x
E. decussata E. Mey. ex Boiss.	Cape Province	PVB 9163	x	x
E. hallii R. A. Dyer	Cape Province	PVB 896	-	x
E. davyi N. E. Br.	Cape Province	-	-	x
E. tridentata Lam.	Cape Province	PVB 7118	-	x
E. pillansii N. E. Br.	Cape Province	PVB 7332	-	x
E. subsalsa Hiern	Angola, Namibia	PVB 4102	x	-
E. genistoides P. J. Bergius	Cape Province	PVB 9314	x	x
E. helioscopia L.	KwaZulu Natal (Europe)	PVB 9315	x	x
Outgroups				
Monadenium torrei Leach	Northern Mozambique	PVB 8553	x	x
Monadenium lugardae N. E. Br.	Transvaal, Botswana	PVB 7470	x	x
Clutia pulchella L.	KwaZulu Natal	PVB *sn.*	x	x
Clutia pterogona Müll. Arg.	KwaZulu Natal	PVB 9313	x	x

3.2.2 DNA extraction, amplification and sequencing

Total genomic DNA was extracted from approximately 150 mg fresh plant material using a modified version of the CTAB protocol described by Doyle and Doyle (1987). 2 % PVP was added to aid in the inactivation of unusual plant chemistries. Extraction was carried out in Eppendorf tubes and DNA was precipitated using isopropanol.

The Polymerase Chain Reaction (PCR) was used to amplify the entire ITS region using the forward primer ITS 5 and the reverse primer ITS 4 (White *et al.*, 1990; Table 3.2) and the *psb*A-*trn*H spacer region using the forward primer *psb*AF and the reverse primer *trn*HR (Sang *et al.*, 1997).

Table 3.2. Primers used in PCR and sequencing of the genus *Euphorbia*.

DNA sequences	Primers	
ITS (White *et al.*, 1990)	ITS 4	5'TCC TCC GCT TAT TGA TAT GC3'
	ITS 5	5'GGA AGT AAA AGT CGT AAC AAG G3'
*psb*A-*trn*H (Sang *et al.*,	*psb*AF	5'GTT ATG CAT CGT AAT GCT C3'
1997)	*trn*HR	5'CGC GCA TGG ATT CAC AAA TC3'

Double-stranded DNA amplifications were performed in a 36 µl volume containing 22.27 µl sterile water, 3.75µl 10 X DNA polymerase buffer, 3.75 µl 50 µmol/l $MgCl_2$, 1.5 µl 10 mmol each deoxynucleotide triphosphate (dNTP), 1.12 µl of each primer (100 ng/µl), 0.225 µl 5u/µl taq DNA polymerase (Promega, Madison, Winconsin, USA), and template DNA.

Thermocycling was conducted on a DNA thermal cycler programmed as follows: 30 cycles of 94 °C for 3 min (to denature double-stranded template DNA), 52 °C for 1 min (to anneal primers to single-stranded template DNA), 72 °C for 2 min (to extend primers) and a final cycle at 72 °C for 7 min (to allow completion of unfinished DNA strands). Negative controls (all components except DNA) were included in each set of samples to check for contaminants.

The resulting PCR products were run on a 1 % (w/v) agarose gel stained with ethidium bromide and visualised on a UV Trans-illuminator. The PCR products were then cleaned of excess primers and dNTPs by using the QlAquick Qiagen™ PCR Purification Kit (Qiagen GmbH, D-40724, Hilden, Germany) following the manufacturers' instructions.

Purified PCR products were sequenced using the ABI PRISM® BigDye™ Terminator Version 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). The PCR amplification primers were used as sequencing primers. Cycle sequencing reactions were prepared on ice and done in 10 µl reactions by combining 2 µl terminator mix, 2 µl 2.5X cycle-sequencing buffer, 0.16 µl primer, 3.84 µl sterile water, and 2 µl cleaned PCR product. Cycle sequencing was conducted on an Applied Biosystems Gene Amp® PCR system 2700 machine programmed as follows: 25 cycles of 96 °C for 30 sec, 50 °C for 15 sec, and 60 °C for 4 min. The cycle sequencing products were resolved on an ABI PRISM® 3100 Genetic Analyser (Foster City, CA, USA) run by the University of Stellenbosch (South Africa) gene sequencing facility, according to the manufacturers instructions.

Automated sequence output files were edited using the SeqMan module of the Lasergene System Software Package (DNA STAR Inc., 1994). The base positions in the forward (5'-3') and reverse (3'-5') sequences were checked for agreement between the two strands and assembled to make consensus sequences.

The strength of any phylogenetic estimation rests on the accuracy of character homology assessment (Kelchner, 2000). Careful alignment is required so that comparable positions may provide the basis for the establishment of hypotheses of primary homology (Simmons and Ochoterena, 2000). Alignments of consensus sequences were performed manually with the MegAlign program (Lasergene Software Package, DNA STAR Inc., 1994) by initially aligning two sequences by eye and subsequently adding the rest of the sequences. Alignment by eye is necessary because one can recognise indel events such as unique indels, direct repeats, inverted repeats and homopolymer length polymorphisms, which must be aligned differently.

Because of high divergence in the DNA sequences and the large number of taxa included in the study, alignment was problematic for certain highly variable regions of both ITS and *psb*A-*trn*H sequences. The difficulties mostly occurred with aligning the outgroup *Clutia* to the ingroup taxa. Problematic areas in the data sets were excluded from the phylogenetic analyses due to uncertainty about homology (Swofford, *et al.*, 1996; Asmussen and Chase, 2001). In order to assess the utility of the *psb*A-*trn*H intergenic spacer for further studies, 20 ingroup taxa (whose relationships were unresolved in the analysis of the *psb*A-*trn*H data set that included 51 species) from one of the Cape groups and two outgroups (*E. genistoides* and *E. stolonifera* Marl.) from a sister clade were aligned separately.

The plastid *psb*A-*trn*H region was length-variable and thus numerous gaps were introduced to align the sequences. DNA sequences of the ITS region had limited length variation, thus few indels were introduced. A gap (indel) is defined as a non-terminal run of "-" characters in a sequence usually introduced when a sequence is aligned to other sequences (Young and Healy, 2002). The gaps produced during the alignment of sequences represent hypothesised evolutionary events and are therefore potential phylogenetic characters. Thus they were included in the analyses in this study.

The indels were identified and coded as present or absent using the computer programme Gap Coder (Young and Healy, 2002) designed to add indel characters to a DNA sequence matrix and prepare it for input to PAUP*. The program uses the "simple gap coding" method of Simmons and Ochoterena (2000). Simple coding was done manually for the 20 ingroup taxa of one of the Cape groups (C) since there were only 13 indels. The outgroups for the Cape clade were not coded. Simple indel character coding is conservative and easy to implement. Simple indel coding is implemented by coding all gaps that have different 5' and / or 3' termini as separate presence or absence characters. Whenever gaps from different sequences may be a subset of other gaps, sequences that have longer completely overlapping gaps (i.e., extending to or beyond both the 5' and 3' termini of the gap being coded) are coded as inapplicable for the gap character being coded (Simmons and Ochoterena, 2000). Leading and trailing indels, which are generally

artifacts of aligning sequences with different 5' and 3' termini (Simmons and Ochoterena, 2000), were replaced with "?" characters.

The corresponding positions in the gap-coded matrices for regions that were problematic to align together with uninformative gaps were also excluded from the analyses. The use of gaps as characters may be difficult for methods of phylogenetic analyses that require a specific model of molecular evolution, insofar as models commonly used in ML calculations do not deal with indels (Thorne and Kishino, 1992). Gaps were thus excluded in the ML analyses in this study.

3.2.3 Cladistic analyses of molecular data

3.2.3.1 Data sets

Four matrices including indel characters were constructed with the aligned ITS and *psb*A-*trn*H sequences:

i) A matrix consisting of separate ITS sequences for 43 taxa.

ii) A matrix consisting of separate *psb*A-*trn*H sequences for 51 taxa.

iii) A matrix consisting of the combined ITS and *psb*A-*trn*H sequences for 43 taxa (39 common taxa for both regions and 4 taxa from ITS) from the genus *Euphorbia*.

iv) A matrix consisting of *psb*A-*trn*H sequences for 20 taxa from Cape clade C.

All parsimony analyses were performed using the software package PAUP* version 4.0b10 (Phylogenetic Analyses Using Parsimony; Swofford, 1998) for Macintosh™. Tree searches were conducted on the separate ITS and *psb*A-*trn*H data sets, and subsequently on both data sets combined since the data sets were found to be nearly perfectly in agreement and contained no strongly supported incongruent groups.

Each data matrix was analysed using 1 000 replicates of random taxon-addition (branches having maximum length of zero collapsed to yield polytomies) to find islands of equally parsimonious trees. Searches used TBR (tree bisection-reconnection) branch swapping with MULPARS on and the STEEPEST DESCENT options in effect. All character

transformations were treated as equally likely and were unordered (Fitch parsimony; Fitch, 1971). Only two trees were saved at each replicate to minimise time spent swapping on islands of equally parsimonious trees (Maddison, 1991). All shortest trees found in the initial 1 000 replicates were then used as starting trees for a second round of heuristic search with TBR branch swapping. Heuristic approaches to tree construction were used to search for the shortest trees because of the large size and complexity of the data matrices.

Internal support or robustness of the trees was assessed using 1 000 jackknife replicates (with 33.67 % character deletion) with simple taxon addition, one round of heuristic search, and a limit of ten trees per replicate. The support levels of groups with less than 50 % were not reported. The same scheme of support described in Chapter 2 was also applied in the current study.

Maximum likelihood methods of phylogenetic inference for nucleotide data (Felsenstein, 1981) evaluate a hypothesis about evolutionary history in terms of the probability that a proposed model of the evolutionary process and the hypothesized history would give rise to the observed data (Swofford *et al.*, 1996; Lewis, 1998). One of the perceived strengths of the ML method is the ease with which hypotheses can be formulated and tested (Huelsenbeck and Crandall, 1997). Models that are commonly used in conjunction with the ML criterion allow each branch to have its own probability of change, thus allowing for variation in rates of evolution across the tree.

The ML approach has two distinct advantages over parsimony. The first advantage is that ML explicitly incorporates a model of substitution into the estimation procedure whereas parsimony methods incorporate variations of these models implicitly. Secondly the optimality scores produced by ML approaches do not depend on particular combinations of ancestral character states at the internal nodes of the tree thus eliminating the ancestral states as parameters of the model (Lewis, 1998). Rather, parsimony tree lengths are based on one or a few maximally parsimonious states at the internal nodes of the tree thus

increasing the number of parameters to be estimated which might lead to inconsistent estimation (Lewis, 1998).

Since nucleotide sites evolve independently, the likelihood of each site is calculated separately and these likelihoods are then combined into a total value at the end. Phylogenies are then inferred by finding those trees that yield the highest likelihoods.

Maximum likelihood analyses of the data sets were performed using PAUP*. The ML analyses were performed using the general time-reversible (GTR) model of nucleotide substitution (Yang, 1994) that estimates independent probabilities for the six possible substitutions (A to C, A to G, A to T, C to G, C to T and G to T) in a symmetric rate matrix and also allows unequal base frequencies. To compensate for among-site rate heterogeneity some proportion of nucleotide sites (P_{inv}) was modeled as completely resistant to change while substitution in the remainder was assumed to follow a gamma (Γ) distribution with shape parameter, alpha (α; Yang, 1993). Because obtaining the likelihoods by integrating over the gamma distribution is computationally intensive (Yang, 1993), the gamma distribution was divided into four rate categories by finding boundaries in the distribution such that each category has equal probability (Yang, 1994). The median of each category was used to represent all of the rates within that category (Swofford *et al.*, 1996).

The rate matrix, (P_{inv}) and α were estimated simultaneously, using the discrete approximation of Yang (1994; implemented in PAUP*) with four rate categories and empirically observed base frequencies. Model parameters were estimated for a randomly chosen parsimony tree from each data set and fixed for subsequent analyses. The ML heuristic search strategy consisting of 1 000 random addition sequence replicates with MULPARS and STEEPEST DESCENT options in effect and with TBR branch swapping.

Support for the estimated phylogenies using the ML approach, was determined using the jackknife with 100 replicates as implemented in PAUP* (Swofford, 1998). The Fast

stepwise addition search strategy was implemented since swapping would have required time beyond the constraints of this study.

3.3 Results

3.3.1 Parsimony analysis of the ITS data set

The longest sequence for the ITS region was 745 base pairs (*E. grandicornis* subspecies *sejuncta*, *E. subsalsa* and *E. unicornis*) whereas the shortest sequence was 525 bp (*Clutia pulchella*). Most of the indels included in the analysis consisted of one to three base pairs. Analysis of the nrDNA ITS region for 43 taxa included 751 characters, of which 457 (61 %) were variable and 331 (44 %) were parsimony informative. In this analysis 24 most parsimonious trees were retained, (L = 1211, CI = 0.53, RI = 0.82), the strict consensus of which is presented in Figure 3.1.

The genus *Euphorbia* is non-monophyletic with respect to the outgroup *Monadenium* and this large group (A) is well supported (jackknife = 100%; Figure 3.1). The phylogeny recovered from ITS data is well resolved. For discussion, a number of major groups are designated (A, B, C, D, E_1, F, F_1, G and H) in Figure 3.1. Clade B is a weakly supported (jackknife = 52 %) grouping of two non-succulent plants and one succulent plant (*E. stolonifera*) belonging to subgenus *Esula*. Clade C contains plants from the Cape Region and is composed of representatives from various sections and subsections of the genus *Euphorbia*. Clade E_1 also consists of plants from the Cape Region but from section *Arthrothamnus*. These two highly supported (jackknife = 100 %) Cape clades are resolved as sister taxa but with no jackknife support. *E. leistneri* is sister to the two Cape groups but this relationship is also not supported. A group (F) comprising *Monadenium*, subgenus *Euphorbia* and *E. elliotii* is strongly supported (jackknife = 100 %), but the sister group relationship of *Monadenium* and subgenus *Euphorbia* has weak support (jackknife = 56 %; Figure 3.1). Subgenus *Euphorbia* (Clade F_1) is composed of plants mainly from southern Africa (excluding the Cape Floristic Region) and India. This clade is strongly supported (jackknife = 100%). Within subgenus *Euphorbia* two major monophyletic groups (G and H) are resolved and well supported (jackknife = 98 % and

76 % respectively). The monophyly of a group (D) comprising *Monadenium* and all ingroup taxa except subgenus *Esula* is poorly supported (jackknife = 60 %).

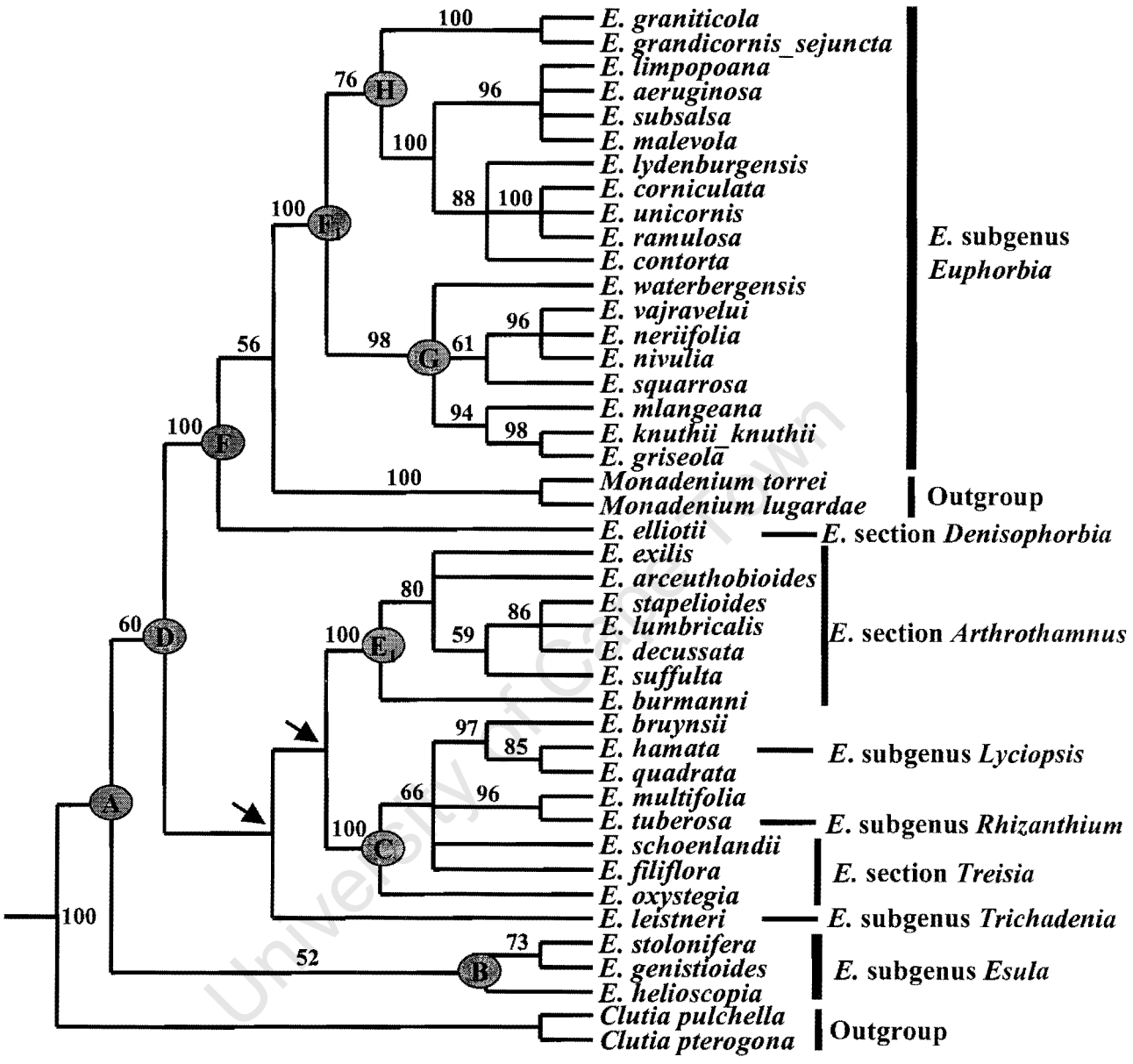


Figure 3.1. Strict consensus of 24 trees based on unweighted maximum parsimony analysis of the ITS data set. Jackknife values greater than 50 % are shown above the branches. Arrows indicate branches with less than 50 % support. A-H designate groups labeled for discussion in the text. The classification is derived from Boissier (1862) and subsequent workers. The classification of species not assigned to infrageneric groupings are unknown.

3.3.2 Parsimony analysis of the *psb*A-*trn*H data set

The longest sequence for the *psb*A-*trn*H spacer region was 803 bp (*E. elliotii*) whereas the shortest sequence was 381 bp (*E. helioscopia*). Numerous indels were present in this data set, the longest of which was 12 bp (5'ACTAATTTTGTA3') and occurred at position 948-961 relative to *E. filiflora* (arbitrarily chosen as the reference taxon). This was present in *E. bruynsii*, *E. davyi*, *E. filiflora*, *E. hallii*, *E. hamata*, *E. lignosa*, *E. multiceps* 1 and 2, *E. quadrata*, *E. restituta*, and *E. shoenlandii*. The *psb*A-*trn*H data set included 1341 characters, of which 602 (45 %) were variable and 333 (25 %) were parsimony informative. In this analysis 385 591 most parsimonious trees were retained, (L = 964, CI = 0.63, RI = 0.87), the strict consensus of which is presented in Figure 3.2.

Some of the groups (A, B, C, E_1, F and F_1) identified in the ITS analysis were also recovered in the analysis of the *psb*A-*trn*H data. The relationships among the groups are unresolved in the *psb*A-*trn*H topology (Figure 3.2). The two monophyletic groups (G and H) nested within subgenus *Euphorbia* recovered in the ITS analysis were not recovered in the *psb*A-*trn*H analysis (Figure 3.2) mainly because few taxa for this group were included in the latter analysis. However, this may reflect a different gene history or, more likely given the weak support in the *psb*A-*trn*H tree, poor resolving power on the part of that marker. Because of increased taxon sampling in the Cape group C, some relationships of species from various sections and subgenera are resolved in this group. For example, two species of subgenus *Rhizanthium* are resolved as sister taxa (Figure 3.2). *E. leistneri* is supported as sister to one of the Cape clades (E_1) and the relationship between these Cape clades is unresolved (Figure 3.2).

Apart from the differences in the taxa included, the *psb*A-*trn*H topology is nearly identical to the ITS topology. However, there are differences in the support of some internal nodes, for example, clade B has moderate support (jackknife = 80 %) in the *psb*A-*trn*H tree compared to the low support (jackknife = 52 %) in the ITS tree. Also, the Indian *Euphorbias* (*E. vajravelui*, *E. neriifolia* and *E. nivulia*; Figure 3.2) nested within subgenus *Euphorbia* are highly supported (jackknife = 96 %) as monophyletic in the ITS tree compared to the weaker support (jackknife = 69 %) in *psb*A-*trn*H tree.

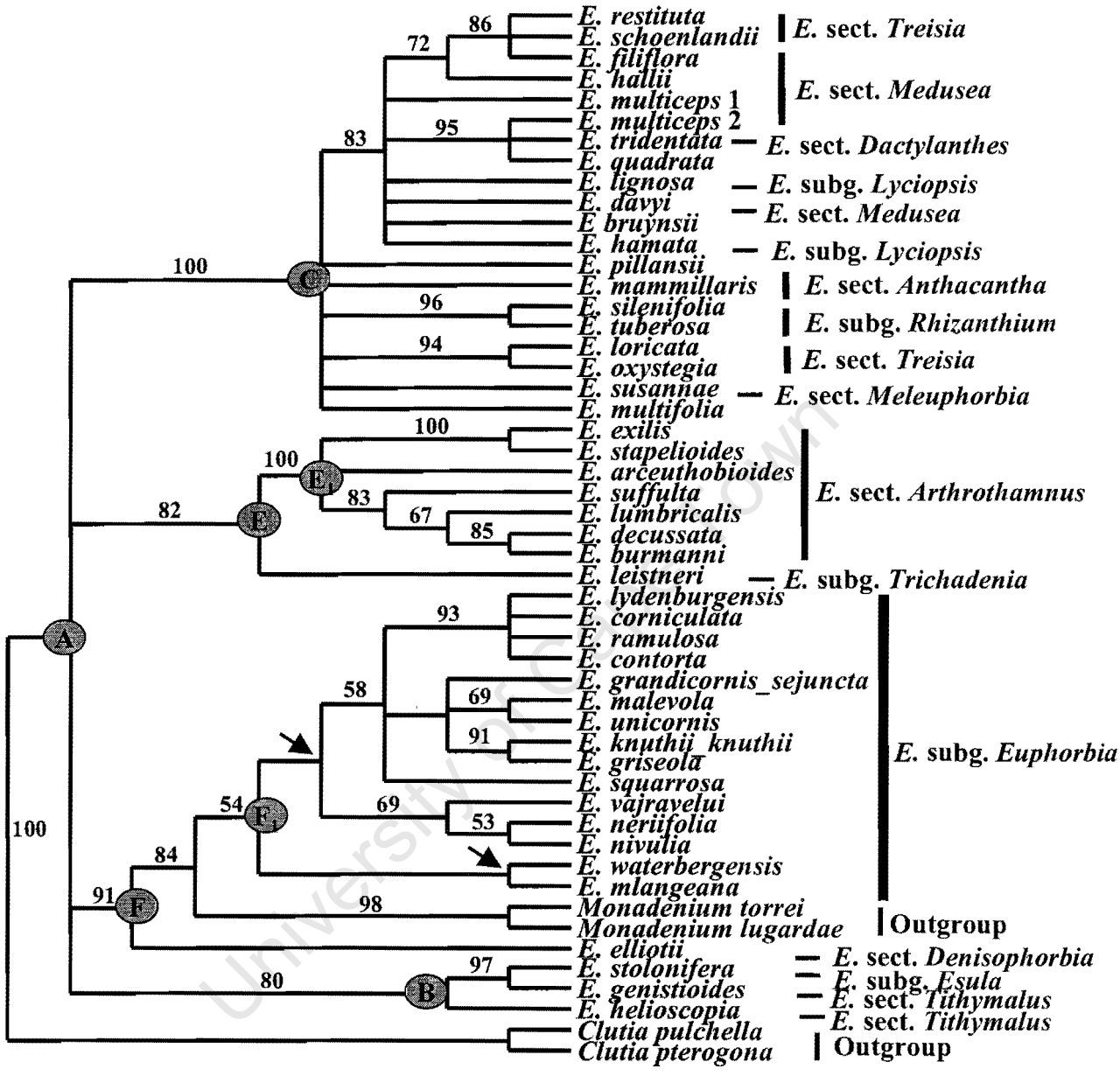


Figure 3.2. Strict consensus of 385 591 trees based on equally weighted parsimony analysis of the *psb*A-*trn*H region. Jackknife values greater than 50 % are shown above the branches. Arrows indicate branches with less than 50 % support. A-F designate major groups labeled for discussion in the text. The classification is derived from Boissier (1862) and subsequent workers. The classification of species not assigned to infrageneric groupings are unknown.

3.3.3 Parsimony analysis of the combined ITS and *psb*A-*trn*H data sets

Analysis of the two regions for the 39 common taxa and 4 taxa from ITS included 2092 characters, of which 1043 (50 %) were variable and 651 (31 %) were parsimony informative. In this analysis 468 most parsimonious trees were retained, (L = 2886, CI = 0.55, RI = 0.82), the strict consensus of which is presented in Figure 3.3.

Some of the groups (A, B, C, E, E_1, F, F_1, and H) identified in the independent analyses of ITS and psbA-trnH data were also recovered in the analyses of the combined data (Figures 3.1-3.3). The topology of the combined data is generally well resolved and strongly supported (26 nodes have jackknife ≥ 75 %) as compared to the independent tree structures. For example, clade H is strongly supported in the combined tree (jackknife = 99%), moderately supported (jackknife = 76 %) in the ITS data and unresolved in the *psb*A-*trn*H data (Figures 3.1-3.3). Also the strongly supported (jackknife = 91 %) sister taxa, *E. filiflora* and *E.schoenlandii* in the combined data were moderately supported (jackknife = 86 %) in the *psb*A-*trn*H data and unresolved in the ITS data (Figures 3.1-3.3).

There are however, disagreements in some parts of the combined tree with the independent analyses. For example, clade G, which is strongly supported by ITS data breaks down in the combined analysis (Figure 3.3). Also, the relationships among the major groups that were resolved in the ITS data are unresolved in the combined analysis (Figure 3.3). The placement of *E. burmanii* as sister to all other sampled taxa of section *Arthrothamnus* that is strongly supported (jackknife = 100 %) by ITS data is contradicted in the combined data where *E. burmanii* is sister to *E. lumbricalis* and *E. decussata* although this relationship is weakly supported (jackknife = 58 %; Figures 3.1 and 3.3). Because disagreements mostly occurred with the ITS analysis, the combined tree generally provided the same structure as the *psb*A-*trn*H analysis.

3.3.4 Parsimony analysis of the *psb*A-*trn*H data set for the Cape clade

The *psb*A-*trn*H data set for Cape clade C whose relationships were largely unresolved in the overall analysis included 780 characters, of which 118 (15 %) were

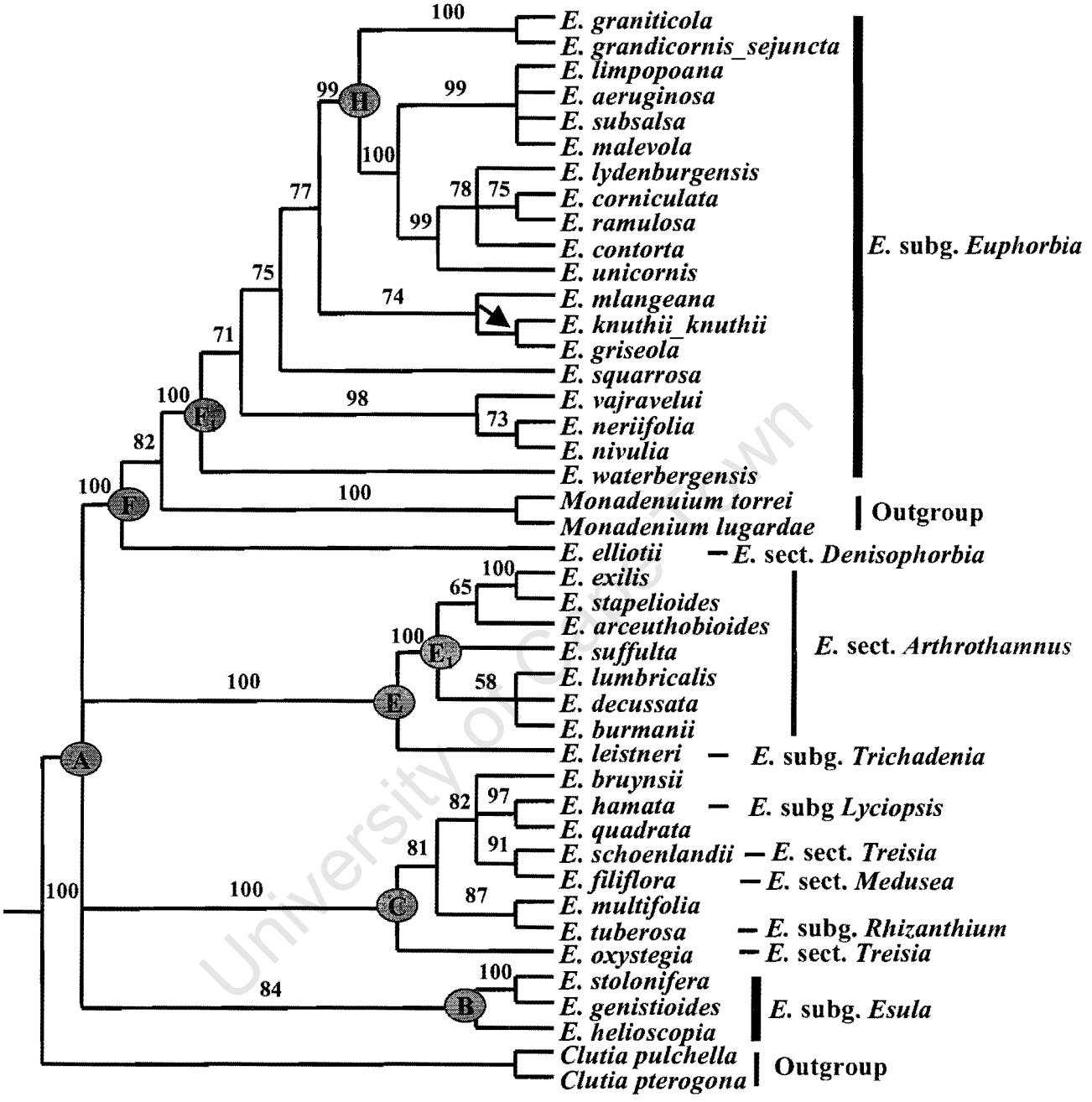


Figure 3.3. Strict consensus of 468 trees based on unweighted maximum parsimony analysis of the combined ITS and *psb*A-*trn*H data sets. Jackknife values greater than 50 % are shown above the branches. Arrows indicate branches with less than 50 % support. A-F and H designate groups labeled for discussion in the text. sect. = section, subg. = subgenus.The classification is derived from Boissier and subsequent workers. The classification of species not assigned to infrageneric groupings are unknown.

variable and 70 (9 %) were parsimony informative. Thirteen indels were identified and the longest deletion was 58 bp shared by *E. bruynsii* and *E. tridentata* relative to position 464-522 of *E. filiflora*. In the analysis of *psb*A-*trn*H data 247 most parsimonious trees were retained, (L = 1054, CI = 0.72 and RI = 0.88), the strict consensus of which is presented in Figure 3.4.

The detailed analysis of 20 taxa from this group resulted in a more resolved tree. This is probably because more characters were obtained in the detailed analysis since more of the sequenced region was alignable across these 20 taxa. Three monophyletic groups (B, C and D) are nested within the entire Cape clade (A) that is strongly supported (jackknife = 100 %; Figure 3.4). Clade B is strongly supported (jackknife = 100 %) whereas clade C and a clade comprising *E. hamata*, the sister taxa *E. oxystegia* and *E. loricata* and clade D_2 are weakly supported (jackknife = 54 % and 68 % respectively; Figure 3.4). Additional relationships recovered include the resolution of *E. pillansii* and *E. multifolia* as sister taxa with a weak jackknife support of 60 %. *E. quadrata* and *E. multiceps* 2 were also resolved as sister taxa with a jackknife support of 71 % and these were in turn sister to *E. tridentata* (Figure 3.4).

Table 3.3: Statistics from equally weighted parsimony analyses of ITS, *psb*A-*trn*H, combined ITS and *psb*A-*trn*H, and *psb*A-*trn*H Cape group data sets. CI = consistency index and RI = retention index.

Analysis	Number of taxa	Number of trees	Tree length	Total characters	Informative characters	CI	RI
ITS	43	24	1211	751	331	0.53	0.82
*psb*A-*trn*H	51	38 591	964	1341	333	0.63	0.87
combined ITS and *psb*A-*trn*H	43	468	2886	2092	651	0.55	0.82
*psb*A-*trn*H Cape clade	20	247	1054	780	70	0.72	0.88

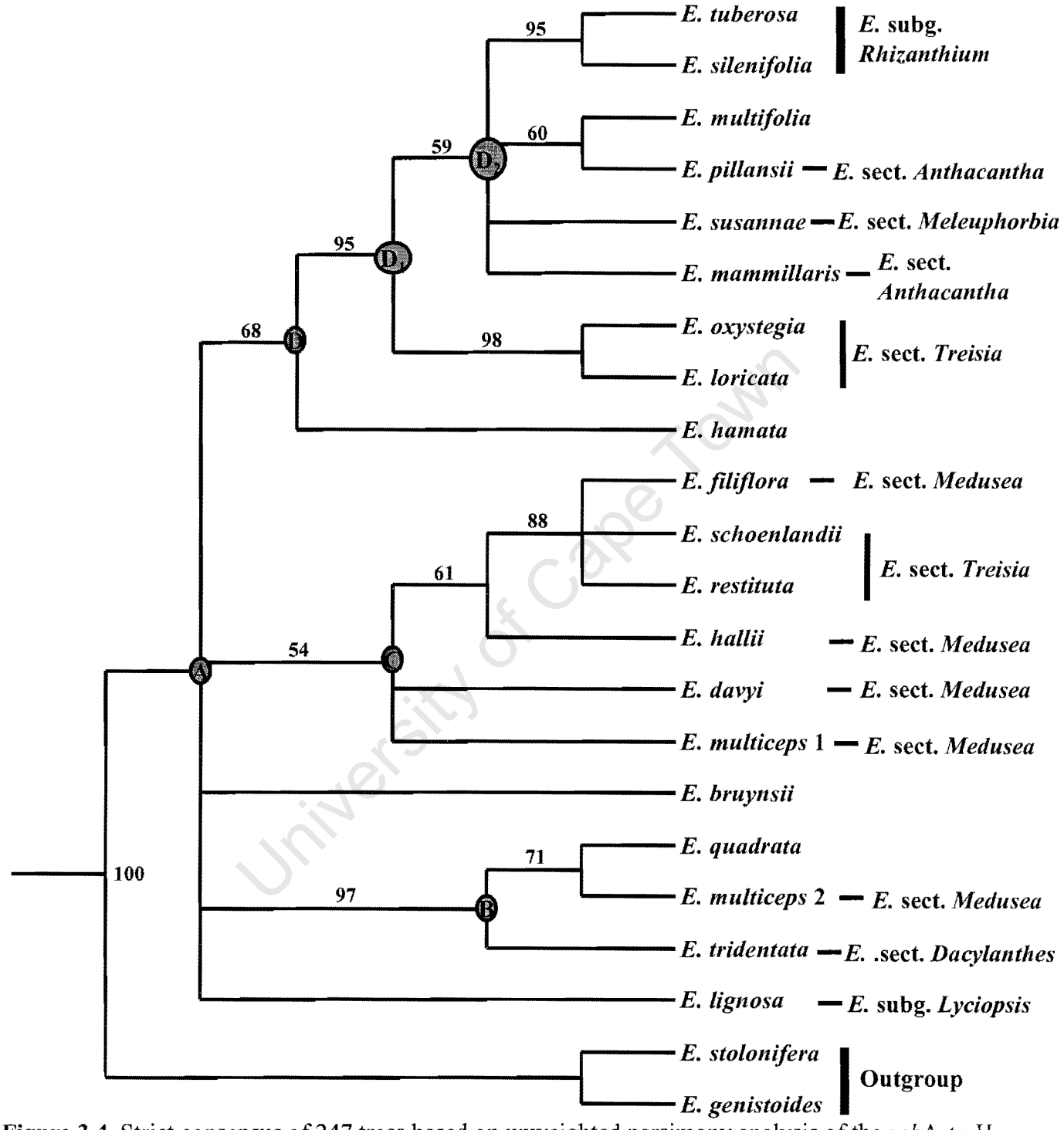


Figure 3.4. Strict consensus of 247 trees based on unweighted parsimony analysis of the *psb*A-*trn*H data sets for the 20 taxa from one of the Cape clade. Jackknife values >50 % are depicted above the branches. A-D designate groups labeled for discussion in the text. sect. = section, subg. = subgenus. The classification is derived from Boissier (1862) and subsequent workers. The classification of species not assigned to infrageneric groupings are unknown.

3.3.5 Maximum likelihood analyses of ITS, *psb*A-*trn*H and, combined ITS and *psb*A-*trn*H data sets

The models derived from each of the data sets are summarised in Table 3.4. The ML model parameters show that the substitution rates, proportion of invariant sites and gamma shape parameter estimates are different for the two DNA regions (Table 3.4). Single trees for each of the data set were found under the ML model and these are presented in Figures 3.5-3.7, with the following Ln likelihoods: ITS data = –6131, *psb*A-*trn*H data = –5172 and the combined data = –11 718 (Table 3.4). The values of the shape parameter for the two gene regions are closer to 1.0 in the ITS and *psb*A-*trn*H data sets (Table 3.4) implying that there is little disparity in relative rates. However the value of the shape parameter for the combined data (0.7) implies a high disparity in relative rates, as might be expected from combining regions evolving under different modes. ITS is G-C rich with the following empirical base frequencies: A = 0.225; C = 0.302; G = 0.282 and T = 0.191 whereas *psb*A-*trn*H is A-T rich with the following empirical base frequencies: A = 0.355; C = 0.093; G = 0.121 and T = 0.431.

Relationships among the major groups recovered in the parsimony and ML analyses of all the data sets are largely congruent with each other, but a few differences are apparent. The relationships amongst the major groups that were unresolved in the parsimony analyses (Figures 3.2 and 3.3) are resolved in all the ML analyses (Figures 3.5-3.7). The Cape clade (E_1) plus *E. leistneri* is critically resolved as sister to a group comprising subgenus *Euphorbia*, *Monadenium* and *E. elliotii*. This relationship is strongly supported (jackknife = 92 %; Figures 3.7) in the combined ML analysis, weakly supported (jackknife = 71 %; Figure 3.5) in the ITS analysis and unsupported in the *psb*A-*trn*H analysis (Figure 3.6). The major conflict amongst the ML analyses is the rooting of the outgroup *Clutia* to the ingroup taxa in the ML ITS analysis (Figure 3.5). The ML analyses of *psb*A-*trn*H and combined data sets supports Clade B as sister to other Cape group C (jackknife = 78 % and 67 % respectively; Figures 3.6 and 3.7). Most of the analyses except the ITS parsimony analysis support the placement of *E. leistneri* as sister to only one Cape clade (E_1).

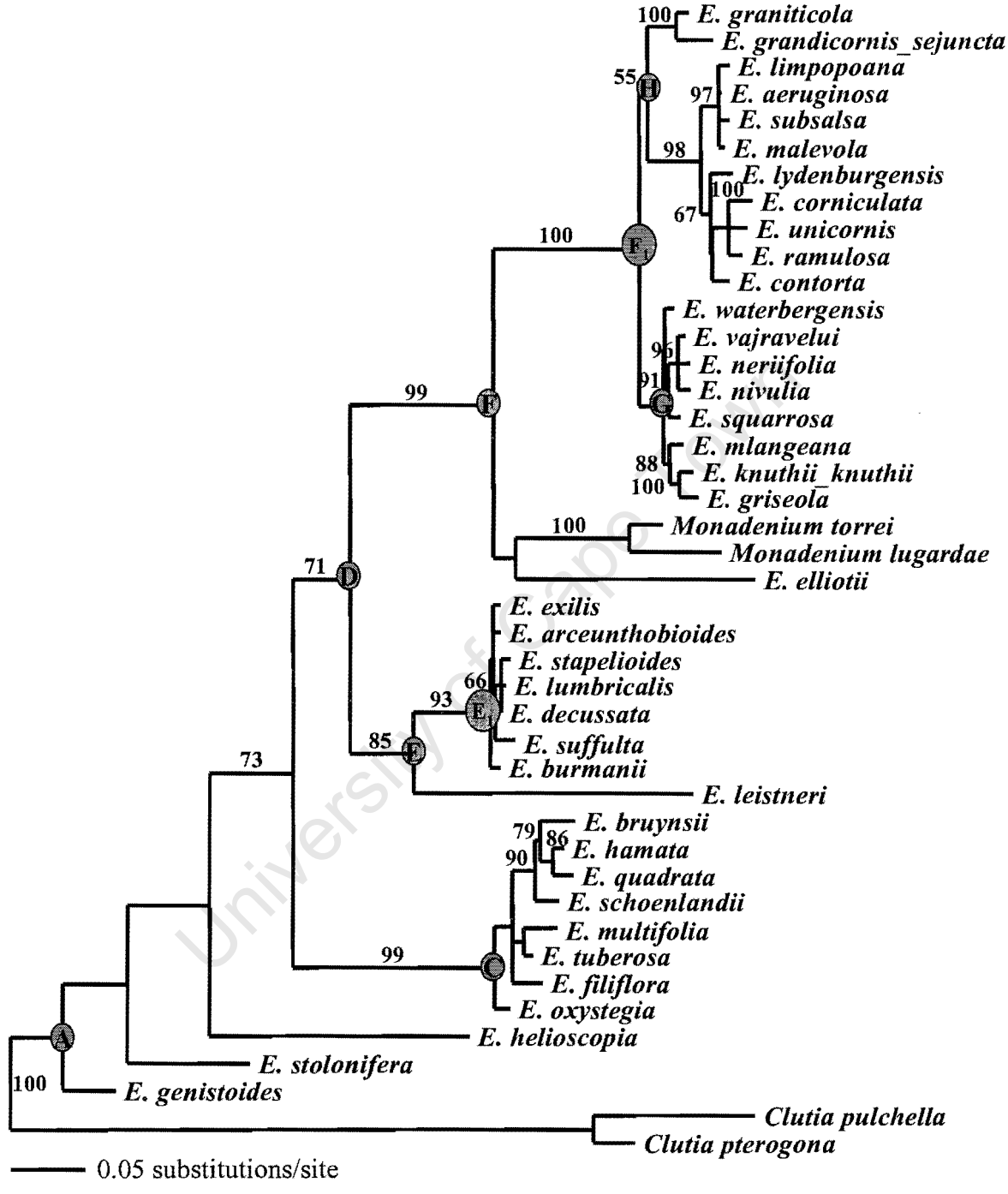


Figure 3.5. Phylogram of one maximum likelihood tree obtained from the ITS analysis, showing branch lengths. A-H designate groups labeled for discussion in the text. Jackknife values >50 % are shown above the branches.

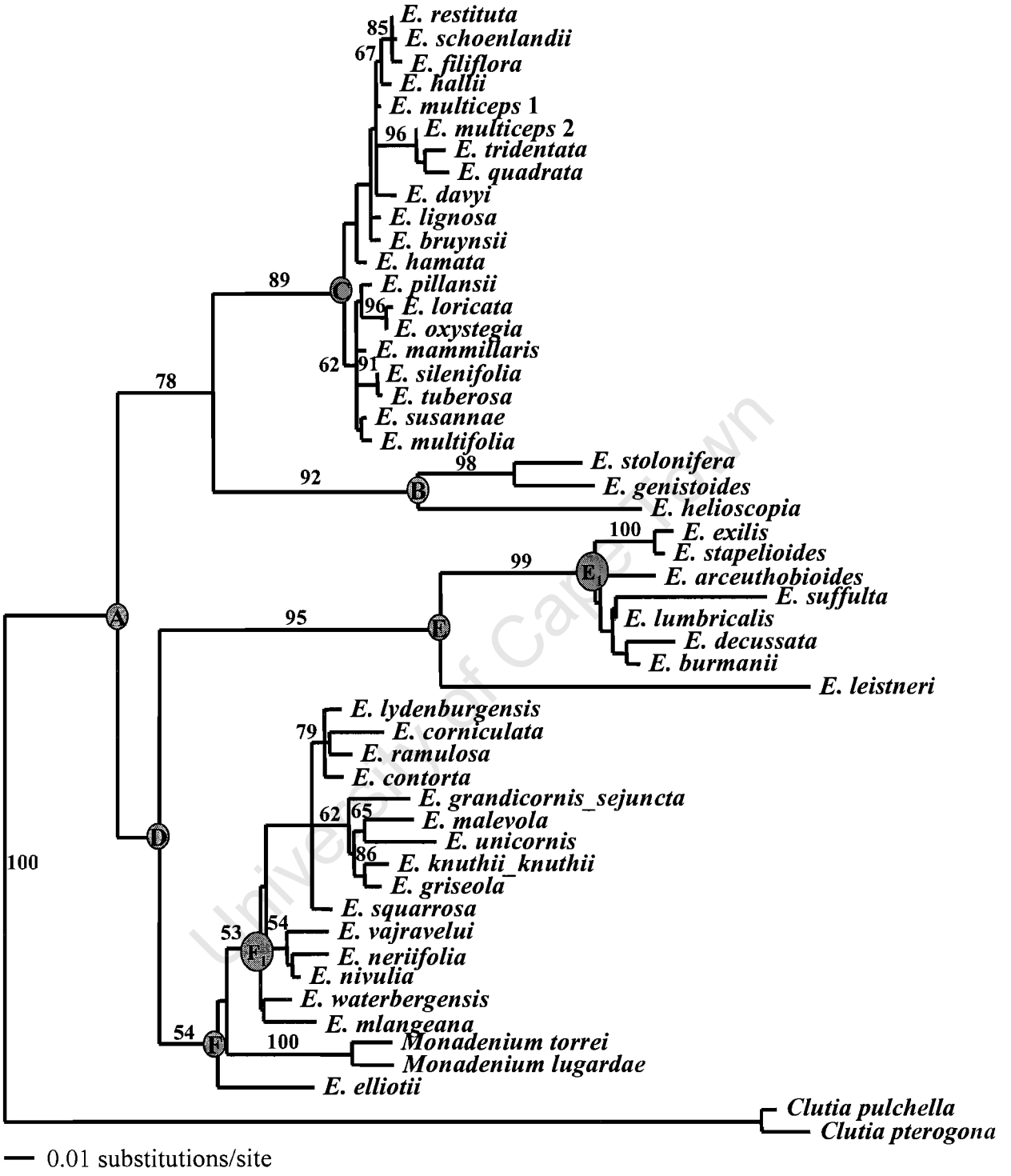


Figure 3.6. Phylogram of one maximum likelihood tree obtained from the *psb*A-*trn*H analysis, showing branch lengths. A-F designate groups labeled for discussion in the text. Jackknife values > 50 % are shown above the branches.

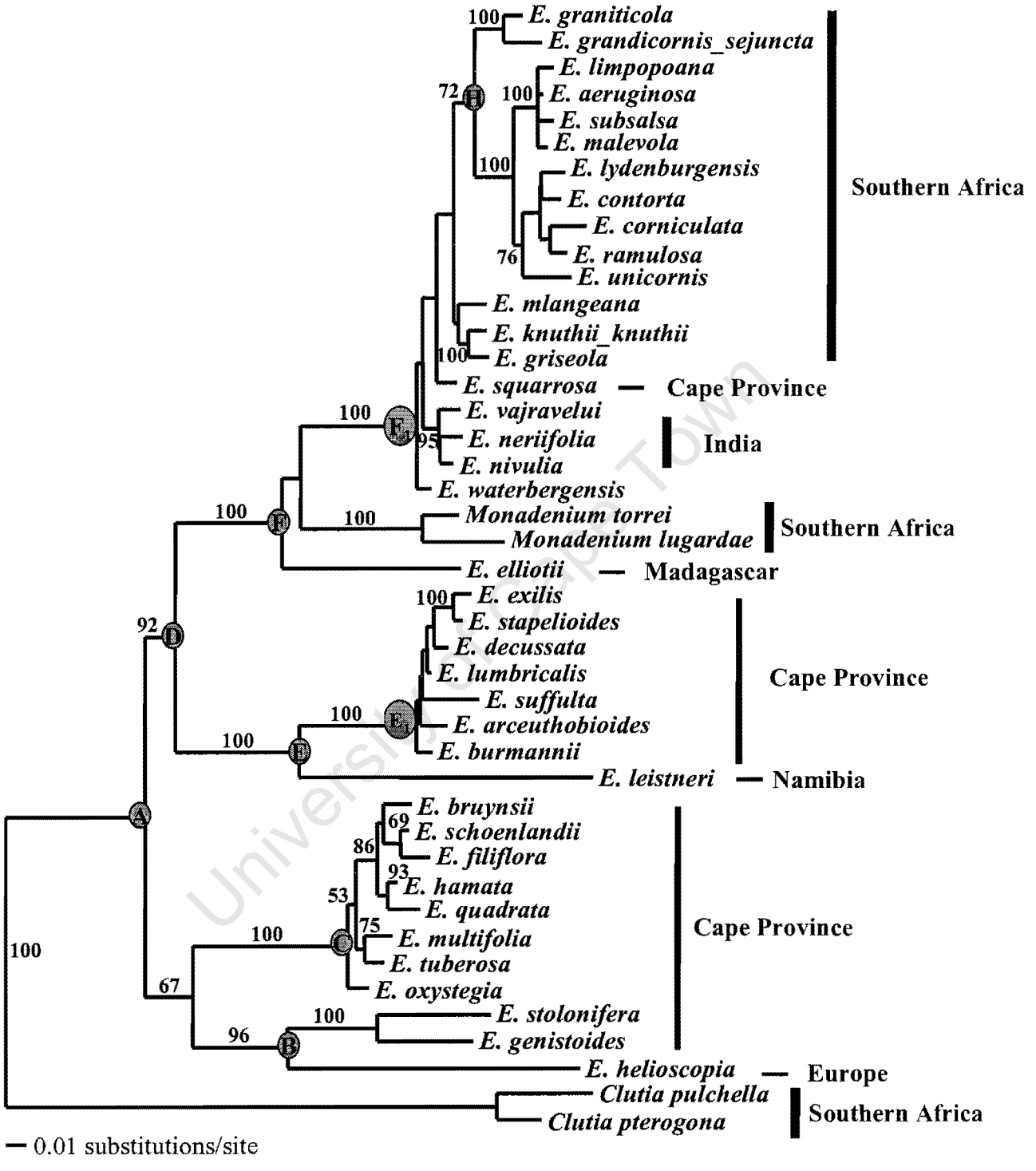


Figure 3.7. Phylogram of one maximum likelihood tree obtained from the combined ITS and *psb*A-*trn*H analysis, showing branch lengths. A-F and H designate groups labeled for discussion in the text. Jackknife values >50 % are shown above the branches.

There are also slight differences in the support of the internal nodes for the parsimony and ML analyses (Table 3.5). Generally the parsimony analyses have more internal nodes with moderate to high jackknife support as compared to the ML analyses. However, this may be due to the "fast" branch swapping approaches used in the ML jackknife analyses.

The phylogenies recovered from ML analyses of the three data sets also showed heterogeneity in branch lengths (Figures 3.5-3.7). The ML analyses of *psb*A-*trn*H, ITS and the combined data sets showed that short branches subtend species within the Cape group C whereas those in the other major Cape clade (E_1) generally have long branches. The Ml analyses also showed that within each Cape group there is quite a bit of branch length variation. The ML analyses for the three data sets also showed that taxa in Group B have long branches (Figures 3.6 and 3.7).

Table 3.4: Summary of the ML GTR + G + I[a] model parameters for ITS, *psb*A-*trn*H and combined ITS and *psb*A-*trn*H data sets. The G-T substitution type was arbitrarily set to 1.

Data set	Ln likelihood	P_{inv}	Gamma shape	A-C	A-G	A-T	C-G	C-T	G-T
ITS	-6131	0.253	1.118	0.697	2.172	1.1434	0.700	3.776	1.0
*psb*A-*trn*H	-5172	0.0	0.917	1.198	0.654	0.495	1.444	0.912	1.0
combined	-11 718	0.081	0.739	0.853	1.409	0.980	0.997	2.015	1.0

[a] General time reversible with an estimated gamma distribution shape parameter and proportion invariant sites.

Table 3.5: Comparison of support levels (percentages) for key nodes under alternative data and methods of analyses. x represent collapsed nodes.

Node	ITS parsimony	ITS ML	*psb*A-*trn*H parsimony	*psb*A-*trn*H ML	Combined parsimony	Combined ML
A	100	100	100	100	100	100
B	52	x	80	92	84	96
C	100	99	100	89	100	100
D	60	71	x	<50	x	92
E_1	100	93	100	99	100	100
F	100	99	91	54	100	100
F_1	100	100	x	53	100	100
G	98	91	x	x	x	x
H	76	55	x	x	99	72

3.4 Discussion

3.4.1 Utility of the ITS and *psb*A-*trn*H gene regions

The ability to sequence both strands of the entire ITS region and *psb*A-*trn*H intergenic spacer with only two primers as shown in the current study will facilitate the use of these regions in a broader comparative sequence analyses of the genus *Euphorbia*. The length of the ITS region varied from about 525 to 745 bp and that of the intergenic spacer (*psb*A-*trn*H) from 381 to 803 bp although most sequences had lengths between 600 and 750 bp. The *psb*A-*trn*H sequences are longer than normally reported in other studies (e.g. 281-324 bp in *Paeonia* species; Sang *et al.* 1997). The size of these regions make them useful especially in comparing recently diverged taxa, as there are sufficient informative sites for the generation of robust phylogenetic hypotheses.

Indels occurred frequently in the *psb*A-*trn*H spacer region and were phylogenetically useful. For example, the insertion of 9 bp (5'TTGATATTC3') present only in *E. stolonifera* and *E. genistoides* between positions 916 and 917 relative to *E. filiflora* is a synapomorphy for this pair. The detailed analysis of the *psb*A-*trn*H data for one of the Cape groups had 13 indels that are synapomorphic. Some of these indels were not codable for all the sampled *Euphorbia* species and outgroups. For example, the insertion of 24 bp (5'CTTCTACCTTTTTAAATTTAAATC3') present only in *E. quadrata* and *E. hamata* between position 558 and 559 relative to *E. filiflora* is a synapomorphy uniting these species. Since more of the sequence is alignable within the Cape clade, even more substitution characters are available suggesting that the region will be of considerable utility in continued studies of these Cape radiations.

3.4.2 Comparison of data sets

Phylogenies reconstructed from both ITS and *psb*A-*trn*H regions were generally well resolved due to the abundance of informative characters (Table 3.3). The ITS data set had a greater percentage (44 %; Table 3.3) of informative characters as compared to the other data sets, and thus had potentially more phylogenetic signal. The ITS data set also had the

greatest percentage (61 %) of variable characters. Despite many indels the *psb*A-*trn*H data set had the fewest (45 %) variable characters. The relatively high proportion of variable characters in the two regions render them extremely useful for indicating relationships within the genus *Euphorbia*. Combining the molecular data sets resulted in the tree having more resolved nodes (26) with moderate support (jackknife ≥ 75 %). This supports the notion of enhanced phylogenetic signal in the combined data set. Independent analyses of the molecular data sets showed little conflict in the topologies recovered. In all the data sets the retention indices were high (RI > 0.82) suggesting that a majority of synapomorphous change was retained.

3.4.3 Relationships of genus *Euphorbia*

The current study showed that the genus *Euphorbia* as currently circumscribed is non-monophyletic (Figures 3.1-3.3 and 3.5-3.7) since the outgroup *Monadenium* is nested within it. These results agree with previous findings (Steinmann and Porter, 2002). This is not surprising since the gross morphology of *Monadenium* is so overwhelmingly similar to the genus *Euphorbia* as not to bear being extricated from it (Croizat, 1972). Croizat (1972) also suggested that the genera *Monadenium*, *Synadenium* and *Euphorbia* are morphogenetically, phylogenetically and even biogeographically a single one. The major difference between the genera *Euphorbia* and *Monadenium* is that *Euphorbia* is characterised by a number (2-8, usually 5) of separate involucral glands in place of the single organ of *Monadenium* (Leach, 1976).

Four clades that are supported were recovered in most analyses of this study. These are two Cape clades (discussed below), and subgenera *Esula* and *Euphorbia*. Species of subgenus *Euphorbia* sampled in this study were mainly from Southern Africa and India. Most of the analyses have shown that *E. elliotii* from Madagascar is sister to *Monadenium* and subgenus *Euphorbia*. *E. elliotii* is spineless and possesses cylindrical stems. These characteristics are different from those of subgenus *Euphorbia* where most species possess the putative synapomorphy of paired-spines mounted on a horny-base (Carter, 1994) and the stems are angled. Further intensive sampling of species from

Madagascar with similar characteristics is required in order to confirm the results of this study.

Relationships within subgenus *Euphorbia* were resolved in most analyses and a number of clades were identified within this subgenus (Figures 3.1-3.3; 3-5-3.7). It is difficult at this stage to discuss the relationships of some of these clades because sampling was not representative of the two major sections and various subsections delineated by Carter (1994) using morphology. It is however important to note that some clades, for example the clade consisting of Indian *Euphorbias* (*E. vajravelui*, *E. neriifolia* and *E. nivulia*) were recovered and supported in all the analyses. This suggests that increased sampling of the under-sampled subsections as well as some subsections recognised by Carter (1994) and not included in this study might yield a comprehensive evolutionary hypothesis for subgenus *Euphorbia*.

Subgenus *Esula* was resolved and supported as monophyletic in most of the analyses in the current study except the ML analysis of the ITS data set (Figure 3.5). Two of the species sampled from this subgenus (*E. genistoides* and *E. helioscopia*) are non-succulents whereas the other species, *E. stolonifera*, is succulent. The relationships of these three species are discussed in Chapter 5. Because of limited taxon sampling in this clade the relationships of succulents versus non-succulents cannot be discussed convincingly at this stage.

3.4.4 Cape lineages in *Euphorbia*

Two monophyletic groups (clades C and E_1) consisting entirely of species from the Cape Region have been recovered in all analyses of different data sets in the current study. The parsimony analyses were unable to resolve the relationships of these two convincingly, but the ML analyses showed that *E. leistneri* plus Cape clade E_1 is sister to subgenus *Euphorbia* plus *E. elliotii* plus *Monadenium* (Clade F; Figures 3.5-3.7). These results have thus given insights into the probable relationships of some species from the Cape Region to those from the generalised South African Flora. The other Cape clade (C) was resolved and supported as sister to subgenus *Esula* in two of the ML analyses (Figures

3.6-3.7). This is not surprising since all the species in these two clades are from the Cape Region. It is not clear from this study whether the two Cape clades recovered from the analyses are derived from different lineages i.e. whether this represents two separate "Cape radiations"? However, suggestions are that there have been at least two radiations of Euphorbia in the Cape regions, and further study of these would be of considerable value in elucidating the origin of rich flora of this region. Additional sampling of non-Cape groups is also needed to determine whether or not the two Cape clades arose within single or distinct lineages. Adding critically selected taxa from the Cape groups would probably result in substantial improvement in resolution of the relationships within these Cape clades and possibly reveal other such clades.

Detailed analysis of one of the Cape clades (C) gave additional insight in terms of the relationships of species in this clade (Figure 3.4). Three clades were recovered in this analysis (Figure 3.4). However section *Treisia* occurs in two clades (C and D) although these are weakly supported (jackknife = 54 % and 68 % respectively). Two species of subgenus *Rhizanthium* (*E. tuberosa* and *E. silenifolia*) are sister taxa and strongly supported (jackknife = 95 %; Figure 3.4) thus confirming the morphological findings (Figure 2.4) and relationships recovered by the parsimony analysis of the *psb*A-*trn*H data set. Two accessions of *E. multiceps* sampled in this study are morphologically similar and were recovered as sister taxa in the morphological analysis (Figure 2.3) but they were not resolved as such in this analysis (Figure 3.4). A detailed discussion of relationships of species in this clade is provided in Chapter 5.

3.4.5 Comparison of phylogenies recovered in the current study

The reconstructions of *Euphorbia* phylogeny from the ITS and *psb*A-*trn*H data sets in the parsimony analysis are largely similar in this study, and thus corroborate evidence of their phylogenetic signal. The overall structure of both analyses is the same (Figures 3.1 and 3.2) and when differences do occur, these generally involve groups that lack jackknife support in one or both analyses. Both kinds of data are useful in estimating phylogenetic relationships. However, there was decreased resolution in some parts of the combined tree, for example, clade G that is strongly supported (jackknife = 98 %) in the

ITS data (Figure 3.1) breaks down in the combined analysis (Figure 3.3). This was unexpected and might be attributed to the *psb*A-*trn*H data that was difficult to align in some regions especially to the outgroup *Clutia* although some of these ambiguous regions were excluded in the analyses. In the current study, all the other analyses strongly support the placement of *E. leistneri* as sister to clade E_1 except the parsimony analysis of the ITS region (jackknife < 50 %; Figure 3.1). The relationship of *E. leistneri* (from Namibia) to species from the Cape Region is not surprising on geographical grounds.

The ML analysis of the ITS data set (Figure 3.5) conflicted with all the other analyses in terms of the rooting of the outgroup *Clutia* and the relationships of subgenus *Esula* is not supported. However, ML analyses of *psb*A-*trn*H and, the combined ITS and *psb*A-*trn*H data sets (Figures 3.6 and 3.7) positioned subgenus *Esula* (clade B) as sister to clade C thus giving insight of the relationships. This relationship is unresolved in the phylogenies recovered using the parsimony analyses of three data sets (Figures 3.1-3.3). The ML analyses generally resolved phylogenetic relationships of *Euphorbia* species among and within groups, for example, small supported clades nested within subgenus *Euphorbia* (Figures 3.5-3.7). Intensive sampling however needs to be done especially with species from subgenus *Esula* in order to verify the relationships shown in the ML analyses.

The current study showed that for the two different DNA regions employed, there is a difference in branch lengths between the two Cape groups (Figures 3.5-3.7). Clade C generally exhibited short branch lengths whereas clade E_1 exhibited long branches. These results provide some evidence that clade C radiated more recently than clade E_1 (assuming no bias in taxon sampling). Subgenus *Esula* also had long branch lengths and these might have resulted from sparse sampling (only three species were sampled in this subgenus). Relative rate test could be used to test if there is a significant difference in terminal branch lengths but because of low sampling this might not yield any significant results.

CHAPTER 4

COMBINED ANALYSIS OF MORPHOLOGICAL AND MOLECULAR DATA: "TOTAL EVIDENCE" AND CHARACTER EVOLUTION

4.1 Introduction

4.1.1 Total evidence approach

Combining molecular and morphological data sets in a single analysis (the total evidence approach) has led to debates in systematics as already discussed in the previous chapter. The main argument against the total evidence approach is that the phylogenetic signal of larger data sets will overwhelm that of smaller data sets (Miyamoto, 1985). However, Eernisse and Kluge (1993) counter the criticism by suggesting that character covariation of the different data sets is more important than the number of characters in each data set. Despite the much larger number of molecular characters potentially available, the addition of a few morphological characters to such an analysis might be decisive in choosing among a set of trees (Donoghue *et al.*, 1989). In this study the molecular and morphological data were combined in a single phylogenetic analysis.

4.1.2 Character optimisations and evolution

To fully understand the evolutionary history of the genus *Euphorbia*, and particularly to understand patterns and causes of character change, it is necessary to know not only the character states of living taxa, but also their ancestors. Since adequate fossils (fossil taxa may have a significant effect on character optimisation; Donoghue *et al.*, 1989) are not available for many taxa or characters the alternative way of inferring ancestral character states is mapping the character states of living organisms onto phylogenies using the

method of parsimony (Madison and Madison, 1992; Swofford and Madison, 1992). Character state reconstruction provides a powerful mechanism for studying many facets of the evolutionary process (Swofford and Madison, 1992). Carter (1994) suggested some evolutionary trends within subgenus *Euphorbia* based on morphology (see Chapter 1) and hypothesised that some morphological characters have undergone change as an adaptation to arid environments. However discussions on character state evolution as inferred from character optimisations on phylogenetic trees are entirely lacking for both subgenus *Euphorbia* and the entire genus. It is hoped that in the current study character optimisations would shed light on, and provide an understanding of, character evolution of this morphological diverse genus.

4.1.3 Aims

The aims of this chapter were:

1) To perform a phylogenetic analysis of the combined molecular and morphological data sets using the parsimony method.
2) To test the monophyly of the genus *Euphorbia* and the monophyly of various sections within the genus.
3) To evaluate morphological character evolution by optimising characters that have been hypothesised to have undergone change in their particular environments, on the total evidence tree.

4.2 Materials and methods

4.2.1 Data set and parsimony analysis

The combined molecular data set with 43 species, together with the morphological data was used in the current analyses. Twelve *Euphorbia* species for which only morphological data and *psb*A-*trn*H data were available, were excluded. The combined analysis of the morphological and molecular data sets was performed in PAUP* version 4.0b10 (Swofford, 1998) as in the previous chapters. A strict consensus tree was computed and support for the recovered phylogeny was assessed using jackknife analysis as in the previous chapters.

4.2.2 Character optimisations

To assess homology statements and the hypotheses of character evolution, character optimisations were done on one of the most parsimonious trees recovered using the combined morphological and molecular data sets. The total evidence data set was used because it is well resolved and contains a higher number of supported nodes as compared to the separate data sets. Character optimisations were performed in MacClade version 4.0 (Maddison and Maddison, 2000). Where reconstructions differed between accelerated and delayed transformation, they were treated as equivocal.

4.3 Results

4.3.1 Parsimony analysis of the total evidence data set

The combined molecular (ITS and *psb*A-*trn*H) and morphology data sets for the 43 taxa included 2113 characters, of which 1064 (50.3 %) were variable and 668 (31.6 %) were parsimony informative. In this analysis 24 most parsimonious trees were retained (L = 2255, CI = 0.64, RI = 0.82). The strict consensus tree is presented in Figure 4.1.

The phylogeny recovered from the total evidence tree is well resolved. The topology is nearly identical to the topology of the combined molecular data sets. The genus *Euphorbia* is non-monophyletic with respect to the outgroup *Monadenium* and this large group (A) is highly supported (jackknife = 100 %; Figure 4.1). Some of the groups (B, C, D, E, E_1, F, F_1 and H) identified in the molecular analyses of data sets in the previous chapter were also recovered in the analysis of the total evidence data (Figure 4.1). A number of clades are also resolved within them. Most groups are well supported with jackknife values of 100 % (e.g. clades C, E_1 and F_1; Figure 4.1).

The relationships among the groups are resolved in the current analyses. Section *Arthrothamnus* (from the Cape Region) and *E. leistneri* are resolved as sister to group F comprising subgenus *Euphorbia*, *Monadenium* and *E. elliotii*. This relationship is however, not supported. Group C is resolved and supported (jackknife = 65 %) as sister to group D (Figure 4.1). Clade B is also resolved but outside a weakly supported succulent group (jackknife = 65 %). Generally there is an improvement in the support of some clades as compared to the results of the separate molecular analyses and the morphological analyses.

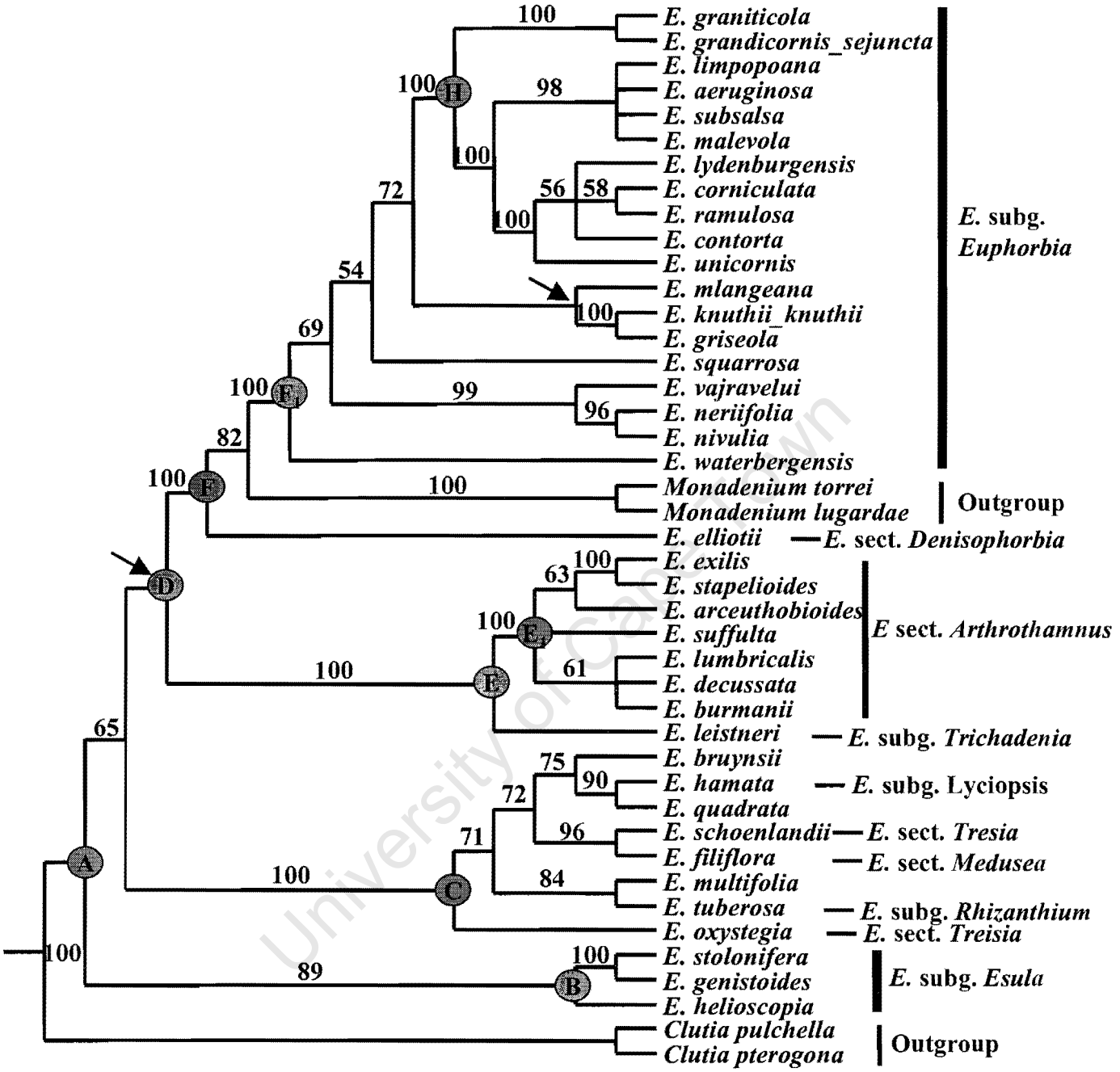


Figure 4.1. Strict consensus of 24 trees based on the equally weighted parsimony analysis of the Combined molecular (ITS and *psb*A-*trn*H) and morphology data sets. Jackknife values greater than 50 % are shown above the branches. Arrows indicate branches with jackknife values less than 50 %. A-F and H designate major groups labeled for discussion in the text. sect. = section, subg. = subgenus. The classification is derived from Boissier (1862) and subsequent workers. The classification of species not assigned to infrageneric groupings are unknown.

4.4 Discussion

4.4.1 Phylogeny recovered from the total evidence approach

The topology recovered from the analysis of the combined morphology and molecular data sets is well resolved and the support levels are similar to those in the combined molecular tree. The main difference is the improved resolution (weakly supported though) at the base of the total evidence tree. However, it seems that the recovered topology was more dependent on the numerous molecular characters than the few morphological ones since previous results of this study showed that only broad groups were resolved in morphological phylogenies.

The results confirm that the genus *Euphorbia* is non-monophyletic. The relationships of *Euphorbia* and *Monadenium* have been discussed in the previous chapter. Subgenus *Euphorbia* was recovered as a monophyletic group that is strongly supported and a number of well-supported clades were nested within. For example, the clade containing the Indian species *E. vajravelui*, *E. neriifolia* and *E. nivulia* was highly supported (jackknife = 99 %). The current results support Carter's (1994) conclusion from studies of morphology that subgenus *Euphorbia* encompasses one of the well-defined groups within the genus. The relationships of species in this subgenus and their implications for classification are discussed in Chapter 5.

Clade B was resolved and strongly supported (jackknife = 89 %; Figure 4.1). Two species in this clade are non-succulent and they grouped together with the succulent *E. stolonifera*. Although the monophyly of this group is upheld in the current study, too few non-succulent species were included to make any definitive statements. There is need to revisit the sampling strategy and include more non-succulent species in order to understand the relationships of species within this clade and also with the succulent species in particular. This relationship of succulent and non-succulent species has implications in understanding morphological character evolution (discussed below).

The current study also shows that species from the Cape Region do not form a monophyletic group, thus indicating that several distinct "elements" occur in the region. However, two distinct groups that are monophyletic were recovered in the analysis of the total evidence data set. *E. leistneri* plus one of the Cape groups (section *Arthrothamnus*) is resolved as sister to group F, which is composed mostly of species from the rest of South Africa and other Southern African countries. *E. squarrosa* from the Cape Province is nested within subgenus *Euphorbia* (F_1). Although the relationship of section *Arthrothamnus* to group F is not supported, the results have provided additional insight in understanding the relationships of the Cape species among themselves and also with other taxa from outside the Cape Region. These relationships were unresolved in the parsimony analyses of the molecular data sets and the morphological analyses of the previous chapters. This suggests an increase in the phylogenetic signal of the total evidence data set. Further studies with intensive sampling are required in order to confirm the current findings and to understand the evolutionary relationships of the Cape groups to the rest of Southern Africa and the potential diversification of the *Euphorbia* species in the Cape Region.

Generally the results obtained in this study contradict the belief by some authors, for example, Hedges and Maxson (1996) that molecular and morphological data provide a greater contribution to evolutionary studies if they are treated independently: molecular data for estimating the phylogeny and morphological data for mapping evolutionary changes on that phylogeny. The current study however supports the view of Axsmith *et al.* (1988) that the use of DNA sequences to the exclusion of all classical morphological data is short-sighted. These results thus lead to the recommendation that molecular and morphological data sets should be combined in cladistic analyses irrespective of the fact that there are only a few morphological characters.

4.4.2 Morphological character evolution

Character evolution was evaluated in this study by optimising some morphological characters that are frequently used in the classification of *Euphorbia* onto the total evidence tree. These character optimisations are discussed individually.

4.4.2.1 Growth habit

The growth habit in the genus *Euphorbia* ranges from trees, shrubs to dwarf geophytes. Figure 4.2 suggests that dwarf geophytes arose independently in two Cape groups and also in subgenus *Euphorbia*. The three geophytes sampled under the current study (*E. tuberosa*, *E. stapelioides* and *E. squarrosa*) are from the Cape Region and they occur mostly in dry sandy and stony areas (White *et al.*, 1941). Goldblatt and Manning (2000) suggested that geophytes in the Cape Region are primarily adapted to seasonally dry climates and they escape the time of the year unfavourable for growth by retreating to underground storage organs. Under current sampling trees are found within subgenus *Euphorbia*. The Indian trees sampled in this study grow in different environments. *E. vajravelui* occurs in forest areas at altitudes of about 1000 – 2000 metres (Binojkumar and Balakrishnan, 1991) whereas *E. neriifolia* and *E. nivulia* are commonly found in dry rocky places.

4.4.2.2 Root tubers

Tuberous roots are regarded as a kind of succulence in the genus *Euphorbia* (e.g. White *et al.* 1941, Rauh, 1984). When this character is optimised on the preferred tree (Figure 4.3), absence of root tubers is pleisiomorphic under current sampling. The *Euphorbia* species sampled in the current study show that the presence of root tubers has multiple origins and it is thus postulated to have arisen five times. Root tubers appear to have arisen in one of the Cape clades (section *Arthrothamnus*) with *E. hamata*, *E. schoenlandii* and *E. filiflora* representing a reversal to the absence of root tubers in their common ancestors. In the other Cape clade the presence of root tubers arose independently in two of the sampled members (*E. stapelioides* and *E. lumbricalis*). This character state is a good synapomorphy for section *Arthrothamnus*. These *Euphorbia* species occur mostly in the succulent shrubland biome of the Cape Region where winter rainfall is below 200 mm annually and summers are normally dry (Goldblatt and Manning, 2000). Figure 4.3 also shows that the presence of root tubers arose independently in two sampled members of subgenus *Euphorbia* (*E. squarrosa* and *E. knuthii* subspecies *knuthii*). *E. knuthii* subspecies *E. knuthii* is common and widespread in the Delagoa Bay region of Mozambique, particularly in low-lying, black turf areas. Dense populations are also

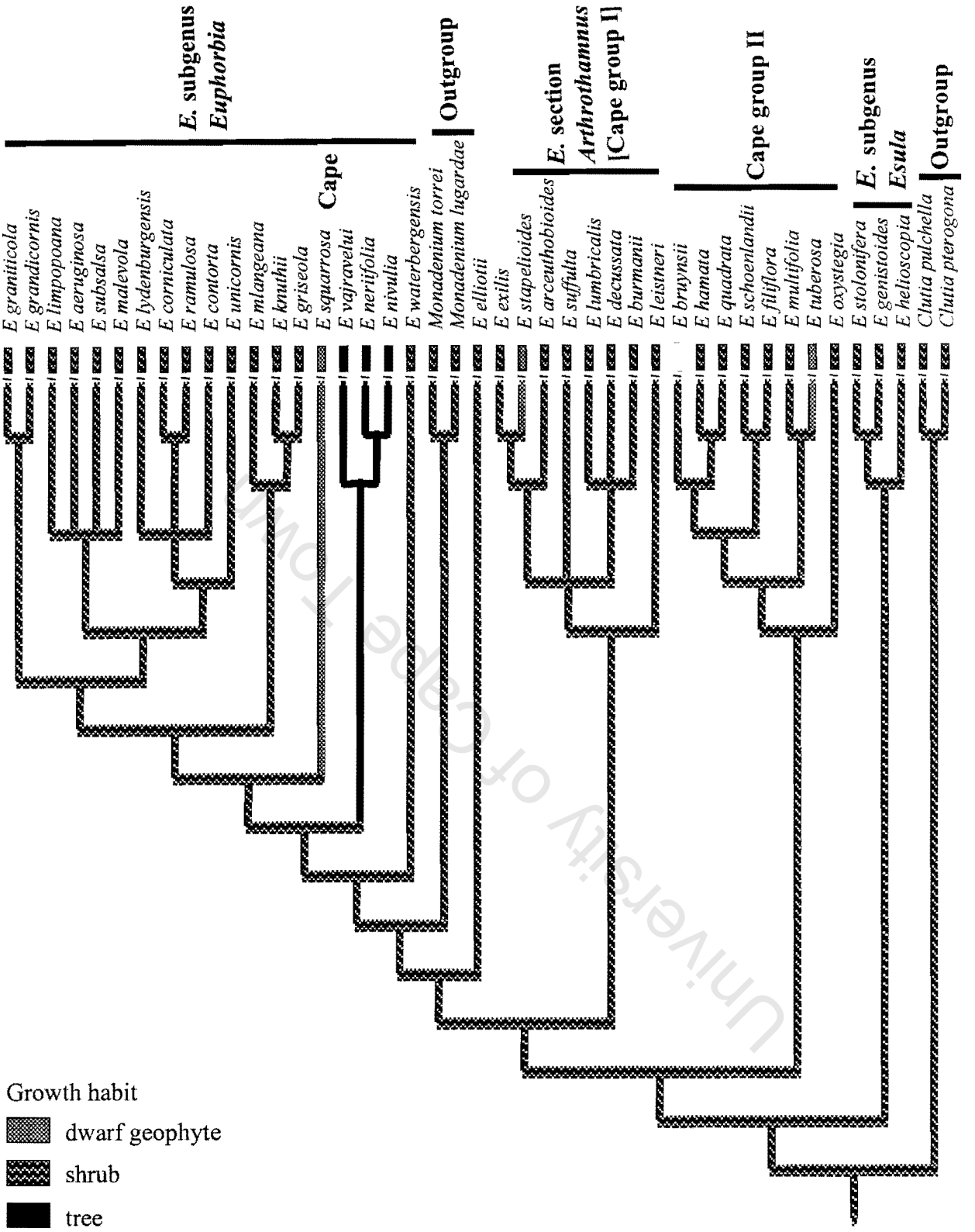


Figure 4.2. Optimisation of morphological character 2: growth habit.

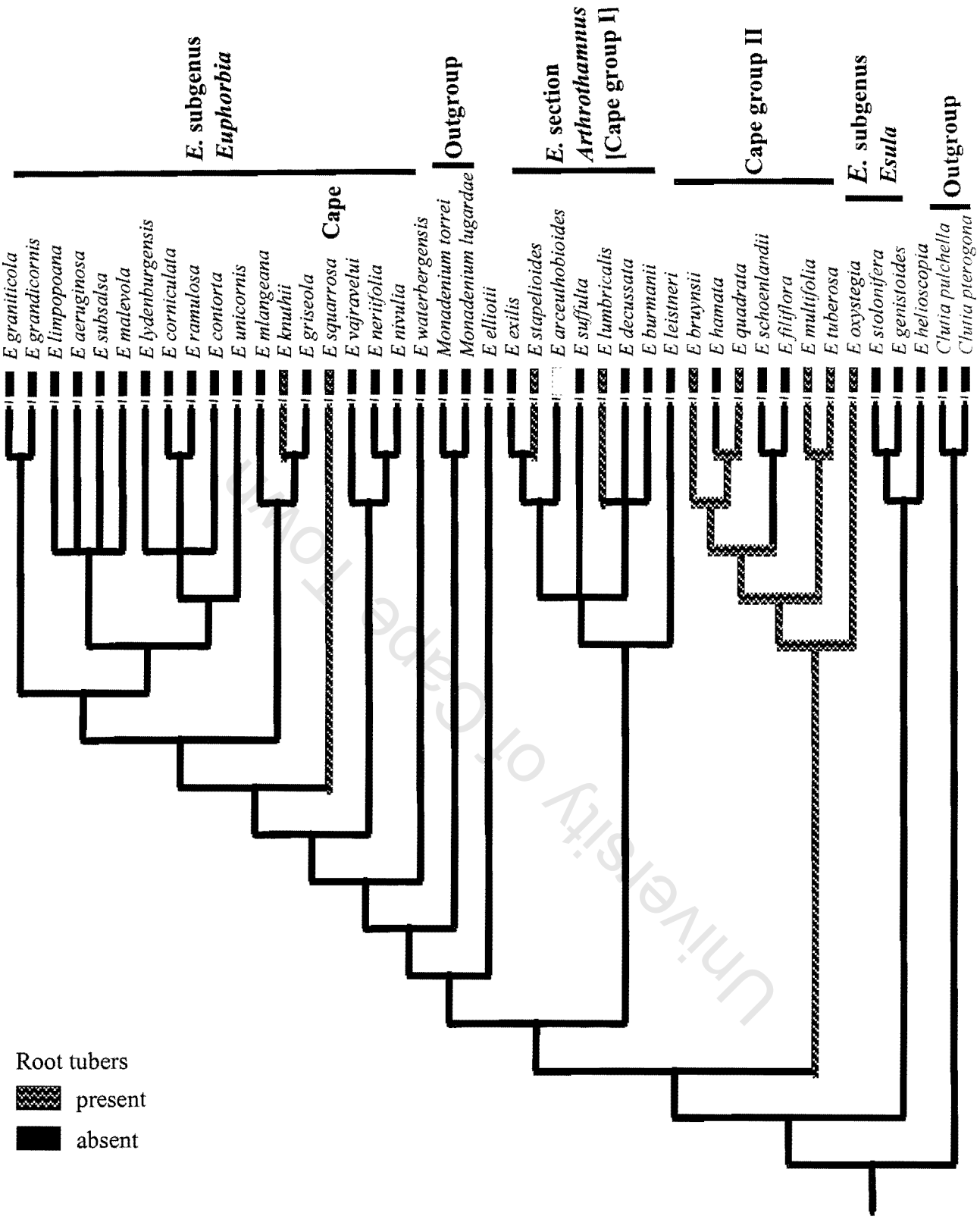


Figure 4.3. Optimisation of morphological character 3: root tubers.

found in some of the extensive seasonal vleis between Boane and Catuane in Mozambique (Leach, 1973).

4.4.2.3 Stem succulence

Non-succulent stems were optimised as the plesiomorphic condition in the current study as (Figure 4.4). Under current sampling of taxa the most parsimonious solution shows that stem succulence arose twice within *Euphorbia* species whilst the less parsimonious solution shows that stem succulence arose once with a reversal to non-succulent stems in *E. genistoides* and *E. helioscopia*. Carter (1994) hypothesised that succulence in the genus *Euphorbia* has developed as a device for survival in arid regions. The species sampled in this study in which stem succulence is present are from varied habitats ranging from Cape Region (with dry summer) to Southern African countries (with wet summer) and forest areas of India. White *et al.* (1941) hypothesised that stem succulence is associated with branch succulence leading to a decrease in the size of the plant body. This study does not support this hypothesis, since shrubs and trees sampled in this study had succulent stems.

4.4.2.4 Shape of the stem

Cylindrical stems, is a plesiomorphic feature of most *Euphorbia* species from the Cape Region (except *E. hamata*) sampled in the current study (Figure 4.5). Angular stems arose in subgenus *Euphorbia* with *E. corniculata*, *E. unicornis* and *E. nivulia* representing reversals to cylindrical stems. Angular stems are probably synapomorphic for subgenus *Euphorbia* albeit with reversals. Since angular stems also arose in *E. hamata*, it is postulated that this character state arose twice under current sampling.

4.4.2.5 Leaf size

Carter (1994) suggested that the large and persistent leaves of some trees and shrubs have given way in most species to very small or minute leaves that are quickly deciduous as an adaptation to arid conditions. When leaf size is optimised on the preferred tree (Figure 4.6), conspicuous leaves, is plesiomorphic under current sampling. It is hypothesised that reduced and scale-like leaves arose three times i.e. in *E. stolonifera*, subgenus *Euphorbia*

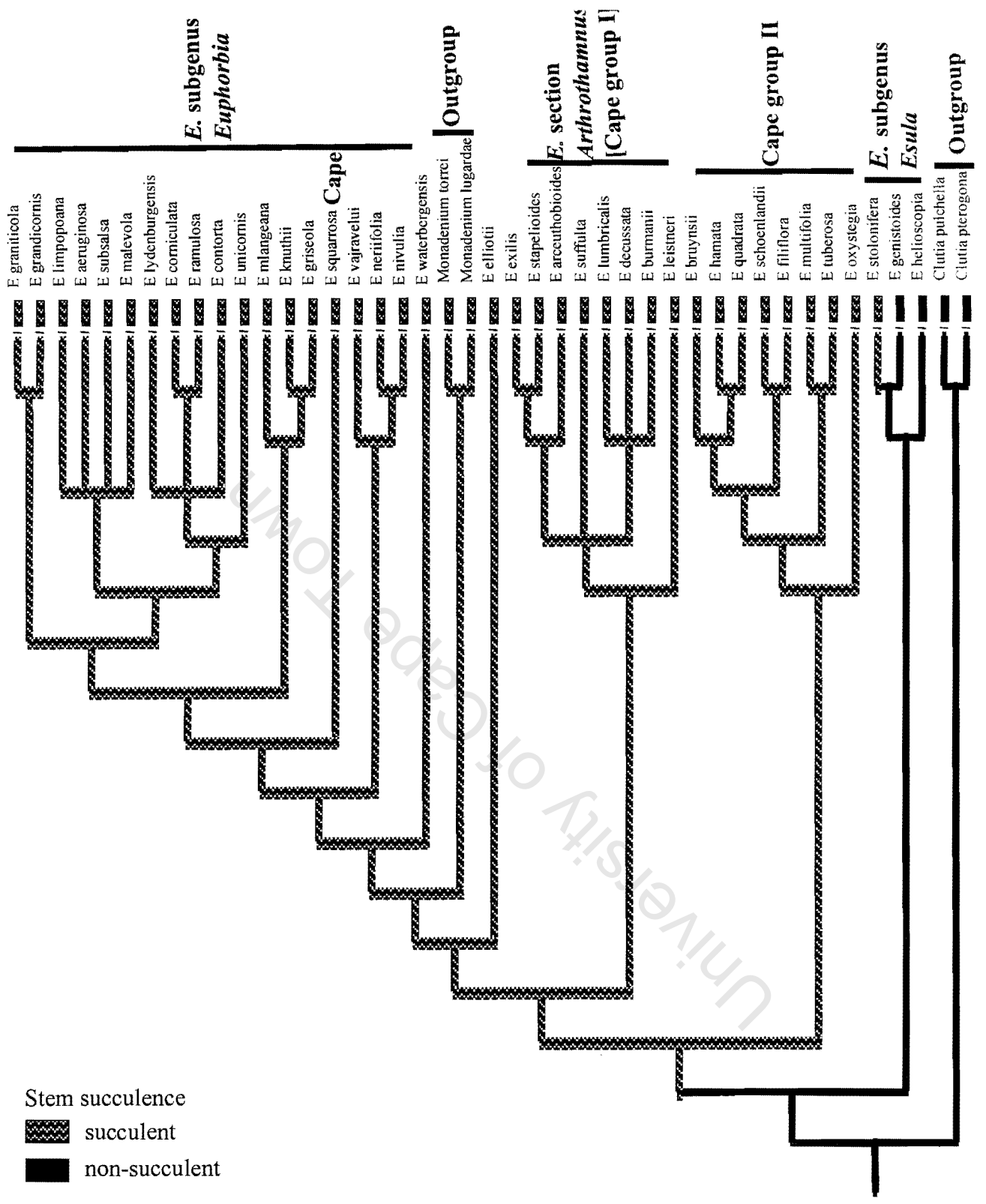


Figure 4.4. Optimisation of morphological character 4: stem succulence.

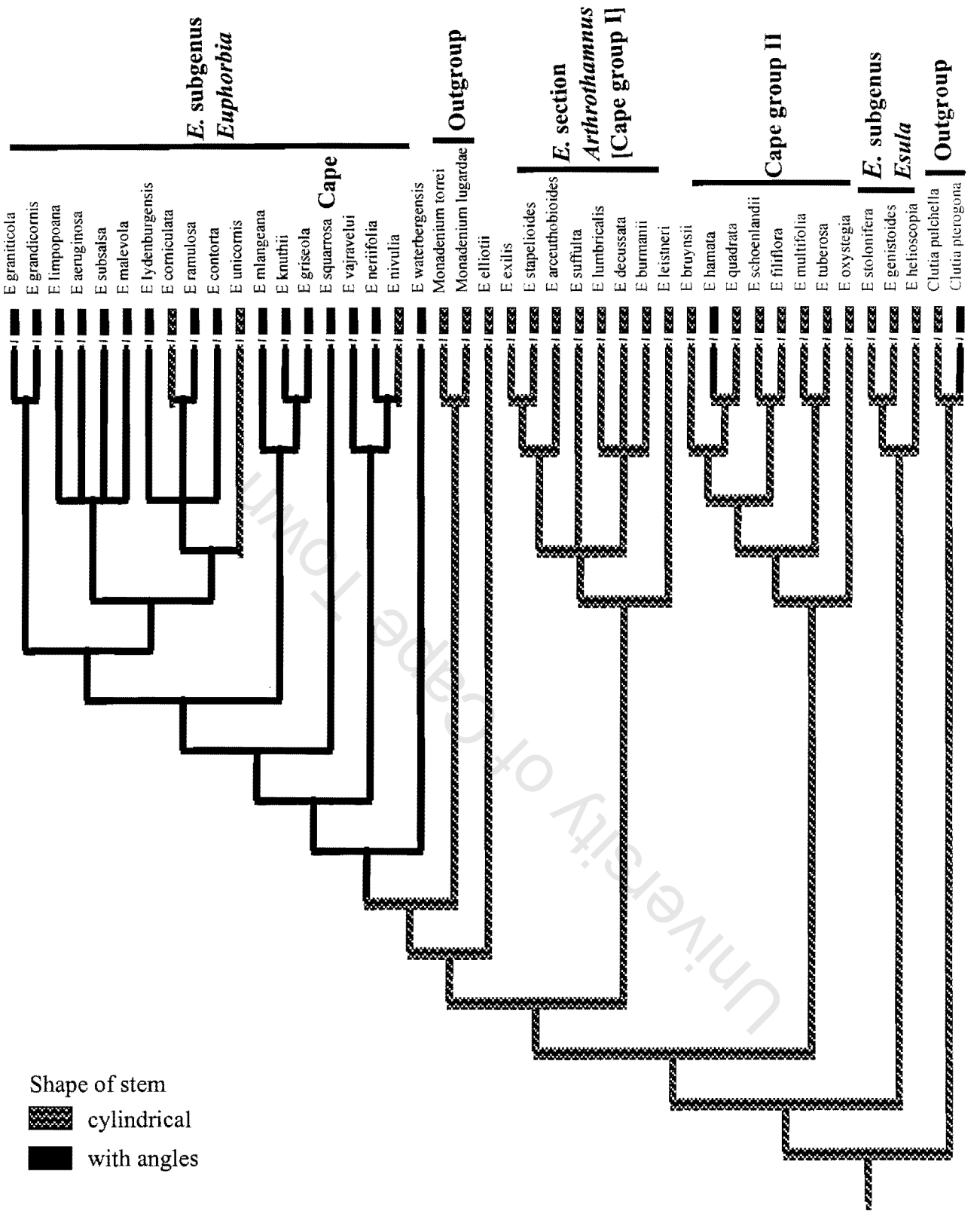


Figure 4.5. Optimisation of morphological character 5: shape of the stem.

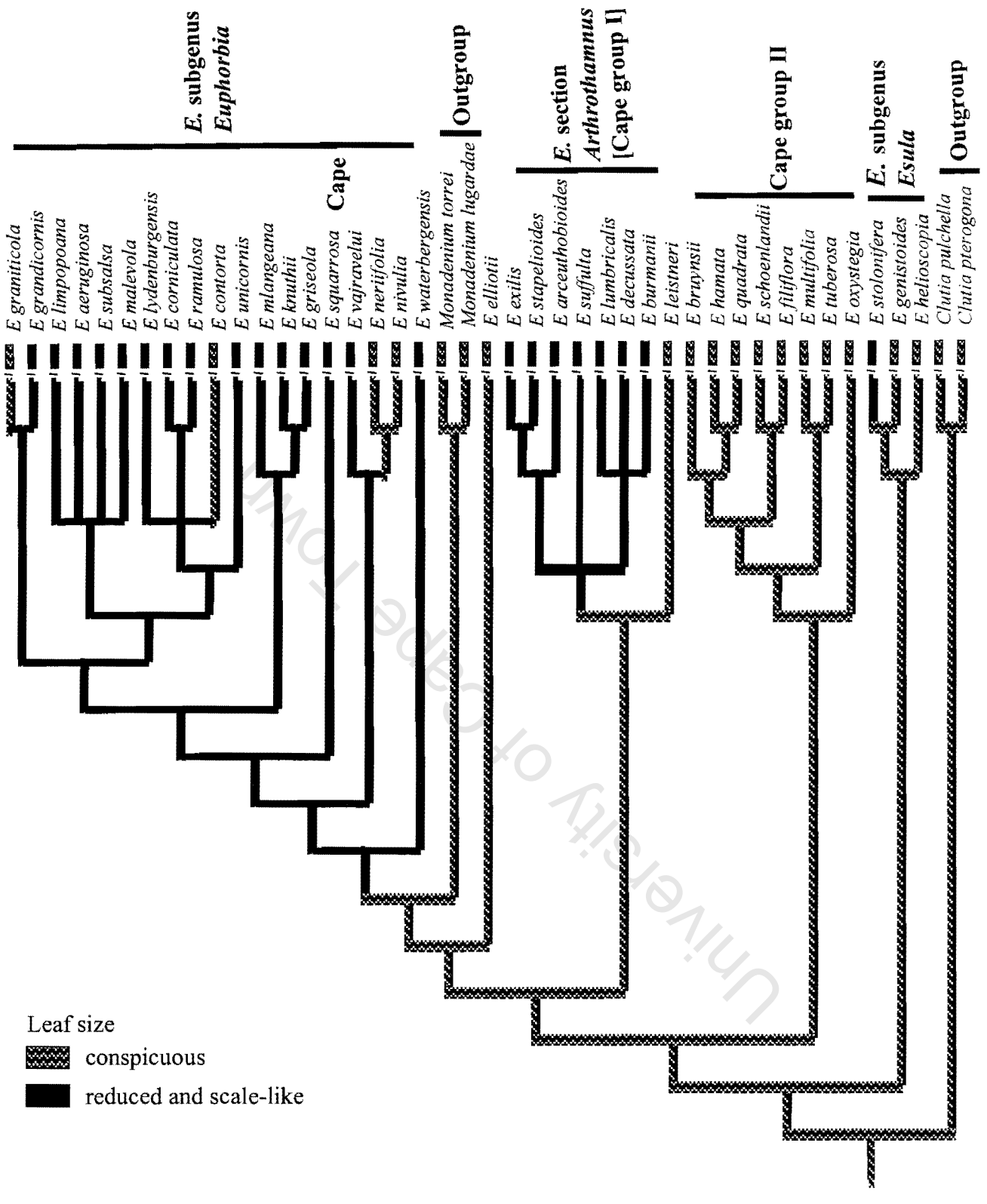


Figure 4.6. Optimisation of morphological character 12: leaf size.

(with reversals to conspicuous leaves in *E. graniticola*, *E. contorta*, *E. neriifolia* and *E. nivulia*) and section *Arthrothamnus*. This character is a good synapomorphy for section *Arthrothamnus*. The other clade from the Cape Region has species with large leaves.

4.4.2.6 Leaf spines

The absence of spines modified from stipules (stipular spines) is plesiomorphic under current sampling, whereas the presence of stipular spines is a synapomorphy for subgenus *Euphorbia* (Figure 4.7). The presence of stipular spines is normally associated with the presence of dorsal spines in subgenus *Euphorbia* (Figure 4.8). Under current sampling it is suggested that the presence of dorsal spines arose three times although this character is optimised equivocally at the common node in most species of subgenus *Euphorbia* (with a reversal to absence in *E. mlangeana*; Figure 4.8). The leaf spines in general are thus synapomorphic for the subgenus. Stipular and dorsal spines are usually mounted on a horny pad (the spine-shield) that surrounds the base of the leaf scar. The presence of the spine-shield in subgenus *Euphorbia* is diagnostic (Carter, 1988). The *Euphorbia* species with paired-spines usually have angled stems (Figure 4.5).

Carter (1994) also hypothesised that the development of spinescence in the genus *Euphorbia* is a character associated with increased adaptation to arid conditions. The spiny species of subgenus *Euphorbia* have a wide distribution occurring throughout Africa wherever climatic conditions are favourable to their growth as well as in some countries of southern Asia such as India (White *et al.*, 1941).

4.4.2.7 Sexuality

White *et al.* (1941) hypothesised that the unisexual habit among *Euphorbia* species is possibly a later phase of evolution than the bisexual habit. This is because some vestiges of an ovary are found in the cyathia of male unisexual plants. Sometimes the female flower is suppressed in some of the cyathia of normally bisexual species. The most interesting instance of this takes place in subgenus *Euphorbia* although there is no complete uniformity.

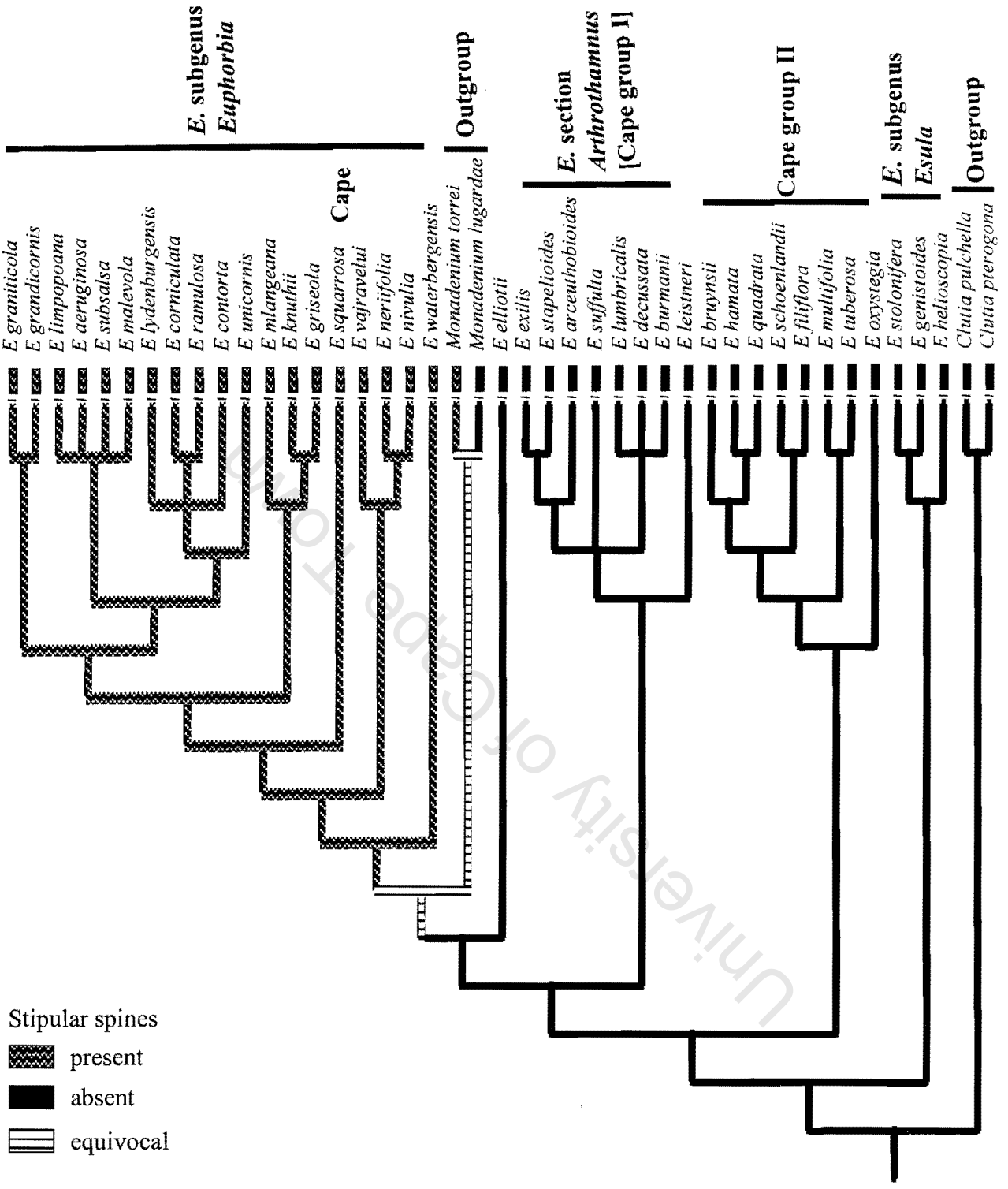


Figure 4.7. Optimisation of morphological character 14: stipules modified as spines.

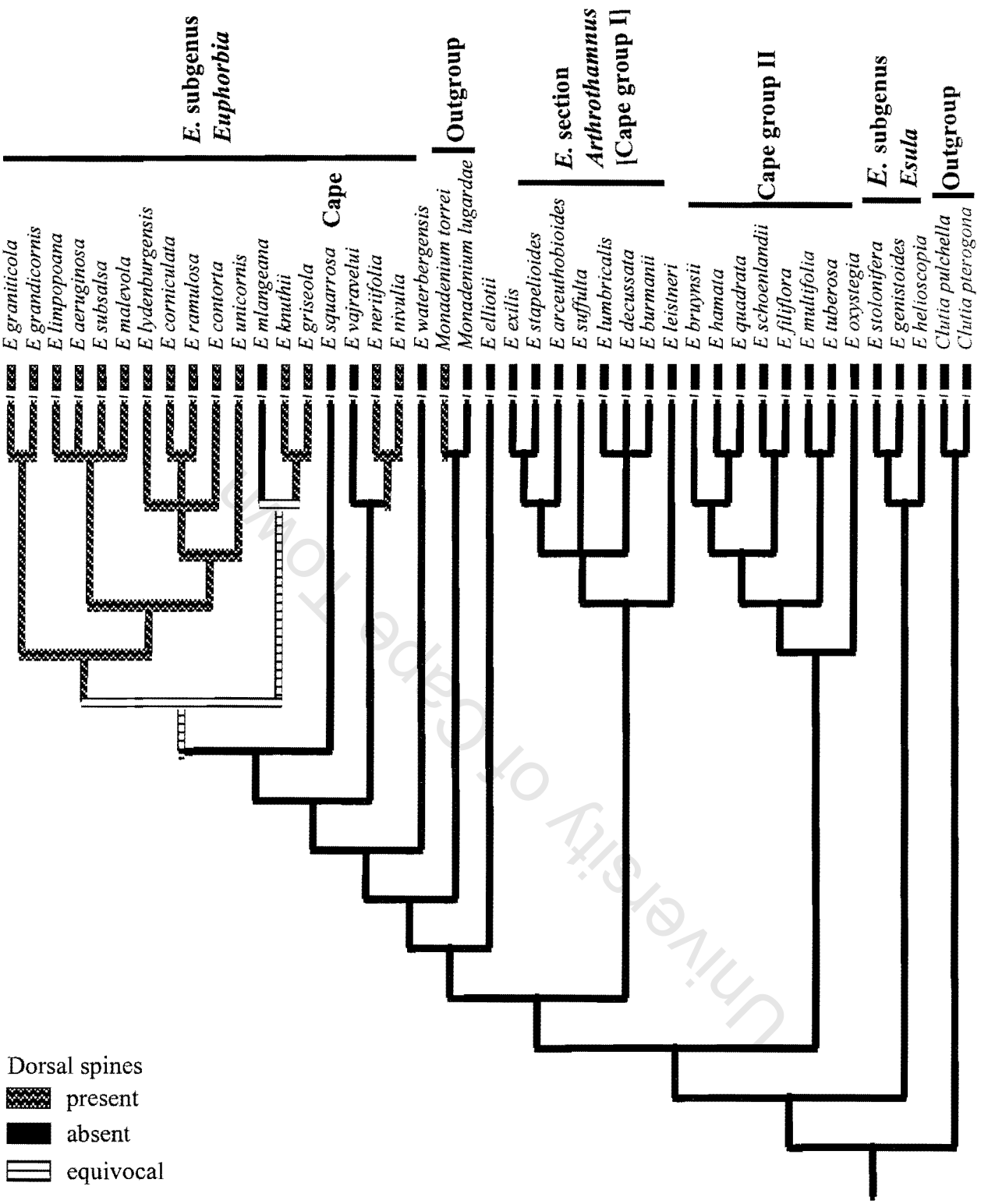


Figure 4.8. Optimisation of morphological character 15: dorsal spines.

Bisexual, unisexual and andro-polygamous character states are optimised on the preferred tree (Figure 4.9) as equivocal. Most species of subgenus *Euphorbia* are andro-polygamous and this state might be synapomorphic. The unisexual character state is present in three sampled members (*E. neriifolia*, *E. subsalsa* and *E. aeruginosa*) of this subgenus whereas the bisexual character state is present in two sampled members (*E. nivulia* and *E. limpopoana*; Figure 4.9). The unisexual and bisexual character states are generally homoplasious among the sampled taxa in the current study.

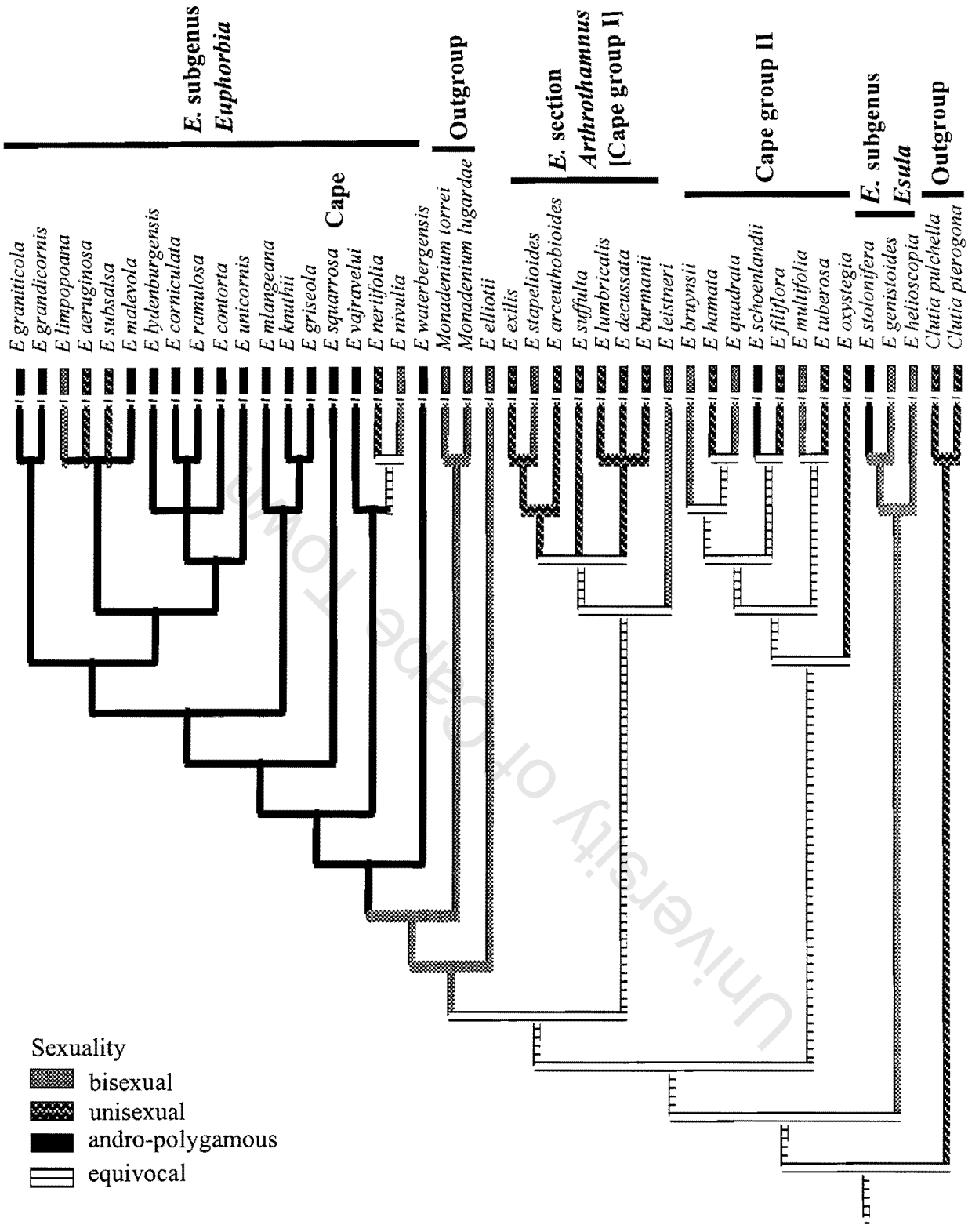


Figure 4.9. Optimisation of morphological character 19: sexuality.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 General Discussion

The current study is the first to investigate phylogenetic relationships of the genus *Euphorbia* using both molecular and morphological data. A better understanding of these relationships emerged from this study using different kinds of data sets in separate and multiple analyses. The monophyly of *Euphorbia* as currently circumscribed is strongly contradicted by the molecular (Figures 3.1-3.3, 3.5-3.7 and 4.1) as well as morphological data (Figures 2.3 and 2.4). The outgroup *Monadenium* was nested within the ingroup taxa and *M. lugardae* and *M. torrei* were strongly supported as sister taxa only in the molecular and total evidence analyses. These results confirm the findings of Steinmann and Porter (2002) that the tribe Euphorbiinae is monophyletic and the genus *Euphorbia* is paraphyletic. The present sampling does not very rigorously test the monophyly of Euphorbiinae since only *Clutia* was included to represent the non-Euphorbiinae. The significant morphological differences between the genera *Euphorbia* and *Monadenium* lie in the structure of the cyathia as discussed in the previous chapters. *Euphorbia* species usually have about five separate nectar glands and the whole cyathium is radially organised, whereas the cyathia of all *Monadenium* species have a dorsiventral structure with a single fused nectar gland (Leach, 1976; Court, 1981; Rauh, 1984).

Various monophyletic groups within *Euphorbia* were also identified in this study and the relationships of species within these clades are discussed below. Some monophyletic groups of major interest are, clade B consisting of only three species belonging to subgenus *Esula*, two clades (C and E_1) containing species from the Cape Floristic Region and a clade (F_1) containing species belonging to subgenus *Euphorbia* (with a number of

clades nested within this subgenus) mostly from Southern Africa with a few species from India. The resolved relationships among the groups indicated that clades B and C are sister groups and *E. leistneri* plus Cape clade E_1 is sister to a group comprising *E. elliotii*, the *Monadenium* species and subgenus *Euphorbia*. Although the relationships between the two Cape clades were unclear in some analyses, suggestions are that these two clades have been derived from two separate lineages.

5.1.1 Congruence of trees recovered from different analyses

In order to address the possible concern of reconstructing a misleading phylogeny using only one method, parsimony and ML methods were used in this study and the results compared. The phylogenies recovered from molecular data sets using these methods of analysis were generally congruent with each other (Figures 3.1-3.3; 3.5-3.7) suggesting the usefulness of the methods in testing phylogenetic hypothesis. The only conflicts in the data sets involved the rooting of the outgroup *Clutia* to the ingroup taxa and the relationships of subgenus *Esula* that are not supported in the ITS ML analysis (Figure 3.5). The ML analyses of the molecular data sets were fully resolved and thus provided better insights into relationships among groups. For example, *E. leistneri* plus section *Arthrothamnus* was well supported as sister to subgenus *Euphorbia* (Figures 3.5-3.7).

Congruence of phylogenies recovered from different analyses has been discussed in detail in the previous chapters thus major points are highlighted in the current chapter. Separate molecular analyses produced fully resolved trees that were largely congruent with each other (in areas where the same taxa were employed) with moderate to high jackknife support for most nodes (Figures 3.1, 3.2, 3.5 and 3.6). The only significant discrepancy was that *E. leistneri* was sister to the two Cape clades in the ITS phylogeny (Figure 3.1) whereas in most other analyses *E. leistneri* was placed as sister to clade E_1 (Figures 3.2-3.3; 3.5-3.7 and 4.1).

A few broad groups (most of them not supported) were recovered using the successively weighted morphology data (Figure 2.4). This suggests that the few morphological characters (21) used were insufficient in showing phylogenetic relationships of species

but useful in showing broad groups. The low CI in the equally weighted morphology data set indicates substantial homoplasy in the morphological characters available for the present study. Low CI (0.34) was also obtained in previous morphological studies for the tribe Euphorbieae (Park and Elisens, 2000).

The combined molecular data set produced an even more fully resolved tree with strong support for most nodes (Figures 3.3, 3.7) thus confirming the increased explanatory power of the data set (Kluge, 1989; Nixon and Carpenter, 1996) and that a more accurate estimate of the true phylogeny was reconstructed (Barrett *et al.*, 1991; de Queiroz, 1993). The total evidence tree was highly congruent with the combined molecular tree and it also recovered relationships among groups. This also supports the idea that the molecular and morphological characters are complementary to each other and thus when combined provide an increase in phylogenetic signal. Some of the conflicting relationships observed in the current study between separate analyses were resolved in the simultaneous analyses of the combined data sets.

Areas of incongruence were however noted between the ITS analysis (Figures 3.1 and 3.5) and the simultaneous analyses of combined data sets (Figures 3.5, 3.7 and 4.1). For example, as noted previously clade G was strongly supported in the ITS analyses and broke down in the simultaneous analyses of the combined data sets. This might be attributed to the fact that there were more sampled taxa for the ITS analyses in this clade as compared to *psb*A-*trn*H data set (clean sequences for four taxa were difficult to obtain). This might also alternatively reflect a different gene history or, more likely poor resolving power on the part of the *psb*A-*trn*H marker. Other differences in all the phylogenies mostly occurred in the support of the nodes with the combined molecular tree having the greatest number of supported nodes and the total evidence tree having the most resolved topology (weakly supported though) at the base of the tree thus achieving the aims of simultaneous analyses i.e. to produce the phylogenetic hypothesis with maximum explanatory power and support.

Because the results from the current study have shown that the phylogenetic reconstructions of the genus *Euphorbia* using the ITS and *psb*A-*trn*H molecular markers were largely congruent in both separate and combined analyses, this suggests the usefulness of both markers and also that the same history was tracked. The usefulness of the ITS region within the tribe Euphorbieae was also confirmed by Steinmann and Porter (2002). Also the abundance of informative characters in both data sets suggests the potential of these molecular markers for future studies.

5.1.2 General relationships within the genus *Euphorbia*

5.1.2.1 *Euphorbia* subgenus *Esula*

Subgenus *Esula* is the largest subgenus currently recognised within *Euphorbia*, containing about 500 species (Wheeler, 1943), and is also considered the most taxonomically difficult. Three species (*E. genistoides*, *E. helioscopia* and *E. stolonifera*) belonging to this subgenus were sampled in this study. These plants lack a tuberous root, have smooth-cylindrical stems (succulent in *E. stolonifera*), conspicuous leaves (except *E. genistoides*), are spineless and their cyathia are in groups. These plants have characters that are considered plesiomorphic for the genus *Euphorbia* (Carter, 1994) and some of these characters have been shown to be such in the current study. The relationships of these three species were well supported in most analyses. If branches that are weakly supported are considered unreliable and ignored, clade B would be considered as sister to clade C and the relationship is moderately to strongly supported (Figures 3.6 and 3.7). Two species of subgenus *Esula* (*E. genistoides* and *E. stolonifera*) are from the Cape Region: thus their relationship to other species from the Cape Region is not surprising. However, intensive sampling for both succulent and non-succulent plants needs to be done in order to confirm the monophyly of the group and the relationships with other subgenera and sections of *Euphorbia*.

5.1.2.2 *Euphorbia* section *Arthrothamnus*

Constituting one of the clades from the Cape Region, section *Arthrothamnus* consists mostly of plants that are unisexual and have smooth stems and tiny leaves that are

arranged opposite one another along the stems. Optimisation of the character state presence of tiny leaves has shown that this state arose in section *Arthrothamnus* and is synapomorphic for the clade. *E. leistneri* from Namibia is strongly supported as sister to section *Arthrothamnus* although it possesses tuberculate stems and conspicuous leaves. This relationship is also supported by a recent study of the tribe Euphorbieae done by Steinmann and Porter (2002). They proposed that section *Arthrothamnus* should be restricted to about 20 species in South Africa and Namibia. These are dioecious, dichotomously branching shrubs, with photosynthetic articulate branches and small, opposite leaves. The current study has also shown that section *Arthrothamnus* is more likely sister to subgenus *Euphorbia* (Figures 3.5, 3.6, 3.7 and 4.1) rather than to the other clade from the Cape Region.

5.1.2.3 Clade C

Clade C consists of various sections and subsections of genus *Euphorbia* from the Cape Region. Members of sections *Dactylanthes*, *Medusea*, *Meleuphorbia*, *Treisia* and *Anthacantha* sampled in this study all fall into this clade. Plants from these sections have succulent tuberculate-stems that are cylindrical, conspicuous leaves and most species possess glandular involucral processes. Four species from subgenera *Rhizanthium* and *Lyciopsis* were also sampled in this study. Generally this clade consists mostly of shrubs and dwarf geophytes. Optimisation indicates that root tubers arose among the species sampled in this clade with *E. filiflora*, *E. hamata* and *E. schoenlandii* representing a reversal to the absence of root tubers in their common ancestor.

The *psb*A-*trn*H data set had data for more members of this clade (clean ITS sequences were difficult to obtain) thus most of the discussion here is based on this phylogeny (Figure 3.2) and that of the detailed *psb*A-*trn*H analysis (Figure 3.4). The relationship of *E. loricata* and *E. oxystegia* is strongly supported (jackknife = 94 %, 98%; Figures 3.2 and 3.4 respectively). These species are morphologically similar and have persistent spiny flower stalks and three bracts beneath the cyathia.

The species *E. restituta* and *E. schoenlandii* are sister taxa (jackknife = 86 %, 88%; Figures 3.2 and 3.4 respectively) and *E. hallii* is sister to these species (jackknife = 72 %, 61%; Figures 3.2 and 3.4 respectively). The stems of these species are tuberculate, the flower stalks persistent, and the cyathia bisexual with small processes on the glands. An interesting feature of these species is the presence of a groove on the tubercle above the leaf scar. This groove is seen also in *E. fasciculata* (not sampled in this study), and only these four species from Namaqualand possess this feature (Koutnik, 1996). *E. filiflora*, a member of *Euphorbia* section *Medusea* also occurred together with *E. restituta* and *E. schoenlandii* although this species is morphologically different. The relationship of *E. filiflora* and *E. schoenlandii* the only representatives of this clade that had all data is also strongly supported in the combined molecular analysis (jackknife = 91 %; Figure 3.3) and the combined molecular and morphological analysis (jackknife = 96 %; Figure 4.1).

The relationships of two species belonging to subgenus *Rhizanthium* (*E. silenifolia* and *E. tuberosa*) have been strongly supported (jackknife = 96 %, 95%; Figures 3.2 and 3.4 respectively). This relationship was also supported in the successively weighted morphological analysis (jackknife = 70 %; Figure 2.4). These species are dwarf geophytes characterised by the presence of a tuberous root. *E. silenifolia* and *E. tuberosa* also possess large leaves, a feature that optimises as plesiomorphic under current sampling. Gilbert (1987: 236) hypothesised that species in subgenus *Rhizanthium* are highly specialised and "...must be considered to be towards the end of their evolutionary lines i.e. a grade rather than a clade". The current evidence suggest that they do form a clade: thus, intensive sampling from this clade together with morphological character optimisations might be able to confirm this hypothesis.

The results from the detailed analysis have shown that some sections are non-monophyletic. For example, sampled members of section *Treisia* occurred in clades C and D (Figure 3.4). These results support Gilbert's (1987) prediction that there does not appear to be any important discontinuity between species in various sections thus leading to one taxonomic problem of the genus *Euphorbia*. Generally the relationships of species

shown in this clade suggest that increased taxonomic sampling might be helpful in revealing some relationship of species in the under-sampled groups.

5.1.2.4 *Euphorbia* subgenus *Euphorbia*

The species of subgenus *Euphorbia* sampled in this study form a monophyletic group that is highly supported in all molecular and total evidence analyses (Figures 3.1-3.3; 3.5-3-7) and weakly supported in the equally weighted morphological analysis (Figure 2.3). Various sections of subgenus *Euphorbia* are also monophyletic and nested within this subgenus. Similar results for different species were obtained by Steinmann and Porter (2002) using ITS and *ndh*F molecular markers. Species are united in this subgenus by having spines formed from modified stipules and optimisation under current sampling shows the presence of stipular spines as a synapomorphy for this clade (Figure 4.7). These spines are always in pairs even in those species that just have one spine. For example, *E. unicornis* and *E. neriifolia* actually have a fused pair (Carter, 1994). The spine-shield is present at the base of each leaf and it is a hypothesised synapomorphy for subgenus *Euphorbia*.

The outgroup *Monadenium* and one of the ingroup taxa *E. elliotii* are also strongly supported as sister to subgenus *Euphorbia*. *E. elliotii* belongs to section *Denisophorbia* which consists of about 20 species mostly confined to Madagascar. This bisexual shrub has cylindrical stems and conspicuous leaves. The current study has shown that cylindrical stems and conspicuous leaves are plesiomorphic features for the genus *Euphorbia* (Figures 4.5 and 4.6). The relationship of *E. elliotii* to subgenus *Euphorbia* is not surprising because it was proposed as a subsection of section *Euphorbia* by Leandri (1957) and was elevated to the rank of section by Croizat (1972). However, it is difficult to make conclusions concerning these relationships since only one species was sampled in this study.

As mentioned in previous chapters, definite inferences concerning the monophyly of taxa currently recognised within subgenus *Euphorbia* cannot be made in this preliminary

study. This is because of inadequate sampling of members from most of these groups. Intensive sampling of taxa from various sections and subsections of this subgenus is thus required in order to understand further phylogenetic relationships of subgenus *Euphorbia*.

5.1.3 Taxonomic implications

Problems encountered in the classification of the genus *Euphorbia* for example, non-monophyly of section Treisia and genus *Euphorbia* have been highlighted in this study. Although various monophyletic groups including the well-defined subgenus *Euphorbia* were recovered in the current study, there is not enough evidence to suggest changes in the present classification of the genus *Euphorbia*. There is however a possibility that the two monophyletic groups from the Cape Region are derived from two different lineages and this is potentially important in the study of Cape radiations (see below). This study is preliminary and intensive sampling in the various sections of the recovered monophyletic groups need to be done in order to suggest modifications in the current classification of the genus *Euphorbia*. However, a number of authors have suggested splitting the genus *Euphorbia* because of its classification problems (see Chapter 1). For example, Carter (1994: 378) stated that the subgenus *Euphorbia* "could be separated as a genus in its own right" and Gilbert (1987) suggested changes in rank within *Euphorbia*. Recently Steinmann and Porter (2002: 479) disagreed that the genus *Euphorbia* should be divided and were of the opinion that "the best solution to the problem of Euphorbiinae classification is to expand *Euphorbia* to encompass all members of the tribe". There are taxonomic consequences of restricting *Euphorbia* to subgenus *Euphorbia*. For example, approximately 90 % of the species currently in the genus would need to be accommodated in other genera whose boundaries and circumscription would be vague and certainly debated for some time (Steinmann and Porter, 2002). Nonetheless the current study shows that molecular data are likely to be extremely helpful in this process, and the morphological synapomorphies already identified for some groups suggest that it would be possible to diagnose most segregate taxa.

5.1.4 Hypothesised origin of *Euphorbia* and the Cape radiations

Because of the limited sampling of species and the bias towards southern African *Euphorbia* species in the current study, it is not possible to comment strongly on the origin and biogeographic relations of the genus *Euphorbia*. Gilbert (1987) suggested that subgenus *Euphorbia* is isolated in terms of possible relationships thus making it difficult to indicate with confidence any group from which it might have been derived. On the other hand, Carter (1994) postulated that subgenus *Euphorbia* may have originated in Asia and migrated to northern Africa at about the time the continents were breaking up and Madagascar became separated. As the continent became drier, development and proliferation of the genus within Africa occurred. This hypothesis was based on the evidence that subgenus *Euphorbia* is related to a small group of fleshy stemmed shrubs from the evergreen forests of India and Malaysia. Future studies involving intensive sampling from the hypothesised related groups of the genus *Euphorbia* should lead to definite conclusions about biogeographic relationships.

Two monophyletic groups from the Cape Floristic Region were recovered in most analyses of this study and the results also suggested the possibility of at least two radiations of *Euphorbia* species in the Cape Region. Clade C has been suggested to have radiated more recently than Clade E_1 (Figures 3.5-3.7) in the current study. There might be one or more factors that have promoted rapid evolution and diversification within these Cape lineages. Goldblatt and Manning (2000) hypothesised that the diversification of species in the Cape Region is explained by the fairly dry climate of the region (largely semi-arid) and the impoverished sandstone soils of the Cape mountain ranges. This climatic regime and these soils are different from those in other areas outside the Cape Region as discussed earlier on. The largest variety and density of *Euphorbia* species are found in the transitional belts between the Karroid areas and other vegetation types (Croizat, 1965). In the riverine forests the succulent *Euphorbias* are mostly confined to rocky slopes and hills. There seem to be few morphological characters that occur in the succulent *Euphorbias* from the Cape Region that are absent in other *Euphorbia* species outside the Cape Region under current sampling. These few characters include, the presence of tuberous roots, cylindrical stems and absence of leaf spines. The presence of

tuberous roots is hypothesised as a survival strategy in the arid regions (e.g. Carter, 1994). Thus with the current evidence it is not possible to suggest what actually has driven speciation and diversification observed in the extant members of the genus. Increased sampling of taxa in the recovered Cape clades might be able to shed more light on the issue of the diversification of *Euphorbia* species in the Cape Region.

5.2 Problems encountered during the study and future directions

The sampling of succulent *Euphorbia* presented in this study did not cover all the subsections recognised by Boissier (1862) and subsequent workers although it is the first to attempt any broad sampling of the morphological diversity of the genus. Some sections were omitted in this study due to unavailability of fresh material or because some material proved intractable for molecular analyses. Thus there is need for more extensive sampling in future studies. Selecting taxa from the under-sampled sections and subsections of the various recovered monophyletic groups might reveal further relationships among the *Euphorbia* species and their biogeographic history. As many outgroups as possible from both the closely related and distantly related genera, for example, species in tribe Hippomaninae and Hurea should also be included. These outgroups may give insights into morphological character evolution of this diverse and interesting genus.

The sequences of *trn*L-*trn*F intergenic spacer that are probably the most frequently used non-coding region of cpDNA in phylogenetic studies (Taberlet *et al.*, 1991) were used in the preliminary study. These sequences did not show enough variation to resolve relationships of about eight species of *Euphorbia* and they had also the problem of homopolymers. The plastid *psb*A-*trn*H and nuclear ITS markers were more variable and had higher numbers of informative sites and thus were used in the current study. Since the results presented in this study showed the usefulness of the ITS and *psb*A-*trn*H molecular markers in recovering *Euphorbia* phylogeny, these molecular markers may be

used in future studies. The detailed analysis of the Cape group using *psb*A-*trn*H has also indicated the potential of this marker for future studies of the Cape radiations.

Morphological work also provided some insights into relationships and character evolution of the genus *Euphorbia*. Detailed morphological studies were not completed because of the limited project time. Future morphological studies involving detailed characters of the cyathia and seed that have been shown to be stable in the classification of the tribe Euphorbieae might show interesting results of the phylogenetic relationships of the genus *Euphorbia*. Research on the anatomy of the stems and leaves would also likely lead to further character discovery and insights into morphological and anatomical evolution.

5.3 Conclusions

This thesis presents preliminary investigations of the phylogenetic relationships of the genus *Euphorbia* using cladistic methods. ITS and *psb*A-*trn*H sequences were useful in recovering well resolved phylogenies that are largely congruent with each other whilst the morphological data recovered only a few broad groups. The questions posed at the beginning (see Chapter 1) were mostly answered in this study and a better understanding of phylogenetic relationships emerged with the following conclusions: The genus *Euphorbia* is paraphyletic, with *Monadenium* nested within it. Four monophyletic groups that were recovered in most analyses are: subgenus *Esula*, subgenus *Euphorbia* (a number of monophyletic groups were nested within this subgenus) and two Cape clades (section *Arthrothamnus* and a clade consisting of representatives of various sections and subsections of *Euphorbia*). Section *Arthrothamnus* was sister to a group comprising *E. elliotii*, the *Monadeniums* and subgenus *Euphorbia* in most analyses. The other Cape clade was sister to subgenus *Esula* although this is contradicted by the total evidence topology. This sister relationship is apparent only when the *psb*A-*trn*H data (in combination with ITS or not) are subjected to ML analysis. The results suggest at least two radiations of *Euphorbia* species in the Cape Region.

Some monophyletic groups recovered in this study were well supported with a number of putative synapomorphies defining each clade. Subgenus *Euphorbia* is widely distributed (from Cape Province to Asian sub-continent) and the presence of paired-spines mounted on a horny base define the group. Leaf spines were absent in the *Euphorbia* species from the Cape Region. Some of these species had inflorescences modified as spines and others were spineless. Although there is generally strong support for the various monophyletic groups and a number of morphological features that define each group, there is not enough evidence to suggest changes in the current classification of the genus *Euphorbia*. The phylogenetic relationships inferred in this study might improve with increased taxon sampling of the various monophyletic groups and the results might be useful in yielding a comprehensive hypothesis for the genus *Euphorbia*.

The results from the current study have also contributed to the understanding of the evolution of some morphological characters that have been hypothesised (e.g. Carter, 1994) to undergo change during adaptations to arid environments. For example, optimisations suggested that succulence of the stem arose twice and the presence of root tubers arose multiple times in the species sampled in this study. Cylindrical stems, conspicuous leaves and absence of leaf spines were plesiomorphic states observed in some sampled members of the genus *Euphorbia*.

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Appendix 1

Data sets referred to in the text are stored as Nexus files on a CD included with this thesis for use with PAUP* (Swofford, 1998). Character sets are defined and details of exclusion sets are included.