Further Studies With *Melianthus* L.: A Molecular Phylogeny, Evolutionary Patterns of Diversification in the Genus and Pollinator Syndromes

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A thesis presented in partial fulfillment for the degree of Master of Science
in Systematics and Biodiversity Science,
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University of Cape Town, South Africa

April 2003

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Abstract

A phylogeny was produced for the eight taxa comprising the largely South African genus *Melianthus* L. based on two plastid markers (*trn*L-F and *psb*A-*trn*H) and one nuclear marker (ITS). Topological comparisons with a tree based on an existing morphological data set revealed significant incongruence leading to a loss of resolution upon combination. Ultimately, the combined three-gene data tree was selected as the strongest phylogenetic estimate for *Melianthus* based on its better resolution and greater support levels. This tree confirms the monophyly of *Melianthus* with *M. major* being resolved as sister to the remainder of the genus. Within the remaining clade, *M. villosus* is resolved sister to a clade comprising two morphologically distinct subclades, one of these being noted for a western distribution (comprising *M. elongatus* and the *M. pectinatus* complex), the other being centred farther east (comprising *M. comosus* and the *M. dregeanus* complex).

A molecular clock analysis was employed to date the emergence of specific taxa and clades, while ancestral range and habitat reconstructions were performed to determine historical conditions under which these groups and their morphological novelties arose. A scenario depicting the evolution and diversification of *Melianthus* is developed against a backdrop of the paleo-history of southern Africa. The genus appears to have originated in the eastern part of South Africa during a mild, mesic Oligocene with subsequent westward expansion into drier habitats of Miocene and Pliocene origins.

Observations of bird visitors to a range of *Melianthus* species indicate that the genus employs a generalist pollination syndrome, thus refuting suggestions of a co-exclusive relationship with sunbirds (Nectariniidae). Nevertheless, the genus is undeniably ornithophilous. Nectar studies reveal interspecific variation in both the volume and concentration of nectar produced as well as in nectar colouration.

Key words: *molecular clock, speciation, ornithophily, nectar colouration, sunbirds.*

Contents

Acknowledgements

Financial assistance for the molecular sequencing, field collection and certain aspects of field observations for this study was kindly provided by the Department of Botany, University of Cape Town. In addition, I am deeply indebted to Michelle Kassner for a personal loan allowing me the opportunity to come to South Africa for the 16 months needed for this project.

I am sincerely indebted for the inspiration, guidance, focus and infinite patience of my supervisor, Dr. T. Verboom, without whom the completion of this project would not have been possible. Much thanks are also due to Dr. Verboom and Nicola Bergh during all aspects involved with field collections.

I wish to further thank Associate Professor T. Hedderson for additional supervisory advice, Tracey Nowell for laboratory guidance and the sequencing department at the University of Stellenbosch, Matieland.

Logistical and technical support was graciously offered by Gonzolo Aguiler, Terry Trindler-Smith, Des Barnes, Dawood Hattas, Sandy Smuts and the staff of the Bolus Herbarium and the Bolus Library.

Lastly, I also wish to thank my fellow classmates for the inspiration of their dedication, drive and commitment to their projects throughout our course of study. Best wishes in life always to Awot, Azieb, Mbaki, Mphamba, Ruvimbo, Tshifhiwa 'Senior' and Tshifhiwa 'Junior'.

Chapter 1: General Introduction

The family Melianthaceae Link is comprised of two equally small genera, *Melianthus* L. and *Bersama* Fres. (Phillips 1921, Watson and Dallwitz 1992, Ronse De Craene *et al.* 2001). The two genera are endemic to Africa, *Bersama* being confined to the east of the continent with a broad range from Ethiopia into the Eastern Cape of South Africa (Phillips 1921, Verdcourt 1956a, 1956b, Palgraves 2002) while *Melianthus* is more southerly in its distribution, being largely contained within South Africa (Phillips and Hofmeyer 1927b) with limited transgressions into Namibia (Merxmüller and Roessler 1968, Tansley unpublished 1983, Tansley and Schelpe 1984, Archer 1997, Dlamini unpublished 1999) and possibly Botswana. The closest relative of the family is believed to be the indigenous South African genus *Greyia* Hook. & Harv., which belongs to the unigeneric family, Greyiaceae Hutchinson (Leistner 2000, Ronse De Craene *et al.* 2001).

Although formally accredited to Linnaeus (1753), *Melianthus* was familiar to European taxonomists as early as the mid-1600s (Tansley unpublished 1983), with one of the first descriptions appearing in 1694 by Tournefort (Doweld 2001). For three centuries since, taxonomists have heatedly argued its circumscription from an ordinal, familial, generic and specific point of view (Doweld 2001, Ronse De Craene *et al.* 2001). With subspecies included, the total number of taxa in *Melianthus* is placed at eight although species number resides questionably at six (Dlamini unpublished 1999). As currently defined (Leistner 2000), the genus consists of *M. major* L., *M. villosus* Bolus, *M. pectinatus* spp. *pectinatus* (Harv.) Tansley and Schelpe, *M. pectinatus* Harv. spp. *gariepinus* (Merx. & Roessler) Tansley and Schelpe, *M. elongatus* Wijn., *M. comosus* Vahl, *M. dregeanus* ssp. *dregeanus* (Sond.) Tansley and Schelpe and *M. dregeanus* Sond. ssp. *insignis* (Kuntze) Tansley and Schelpe.

In general, plants in the genus are medium-sized shrubs with a near deciduous to semi-herbaceous habit. *Melianthus* bears imparipinnate, highly dissected, alternate foliage distinguished by conspicuous stipules (Watson and Dallwitz 1992). The toxic foliage has the reputation of a noxious aroma (Von Marilaun 1895, Marloth 1908,

1925) and is avoided by game, both domestic and wild, except during times of famine (Watt and Breyer-Brandwijk 1962). The genus shows a high degree of floral diversity with the 2-3.5 cm flowers either borne on terminal, tall, erect racemes held clear of the foliage, or axillary, short, lateral racemes tucked curiously beneath the leaves. The flowers are highly monosymmetric and consist of conspicuous coloured perianth segments, variably coloured maroon, red, orange and/or green, with the flowers resupinating during the maturation process. Floral parts are largely free and unfused although the central edge of the petals adhere to one another along their inside margins due to an intermingling of white crystalline hairs (Ronse De Craene *et al.* 2001). Sequential hermaphrodism in the form of protandry has been developed in the genus apparently to promote out-crossing, with dimorphic stamens maturing in pairs followed by a delayed maturation of the stigma.

The genus is characterised by the unusual distinction of nectar colouration with the nectar variably tan to black depending on the species (Marloth 1908, 1925). Despite bees visiting the flowers (Marloth 1908, Pooley 1998), most floral developments in the genus suggest a close association with ornithophily (Faegri and van der Pijl 1979, Rebelo 1987, Proctor *et al.* 1996) as evidenced by a copious production of dilute nectar with low sucrose content (Dlamini unpublished 1999, pers. observation).

The higher-level familial relationships of the genus have been adequately dealt with in recent molecular studies (Chase *et al.* 1993, Nickrent and Soltis 1995, Soltis and Soltis 1997, Soltis *et al.* 1997, APG 1998, Savolainen *et al.* 2000, Soltis *et al.* 2000) but no low-level molecular study explaining inter- and infra-specific relationships within *Melianthus* as yet exists. The most recent phylogenetic treatment for the genus is based on a cladistic analysis of morphological data (Dlamini unpublished 1999). Dlamini's (1999) phylogeny supports the monophyly of *Melianthus*, with *M. major* being resolved as sister to the rest of the *Melianthus* species. The remaining species divide into two main clades neatly distinguished by floral orientation, an erect-flowered clade (*M. villosus, M. elongatus, M. pectinatus, M. gariepinus*) versus a lateral-flowered clade (*M. comosus, M. dregeanus, M. insignis*). Whether low-level molecular studies would support Dlamini's hypothesis is unknown.

Hypotheses for the diversification of *Melianthus* are based on the notion of geographical isolation preventing gene flow (Dlamini unpublished 1999) although the

events leading to isolation of the species are unclear. Distributional ranges in the more highly localised taxa accord with specific sets of habitat variables while wider ranging taxa reflect a generalist strategy in straddling the edges of multiple ecological zones. The most widespread taxon in *Melianthus* is the arid-adapted *M. comosus*, which has a range stretching throughout much of central South Africa (i.e. the 'Karoo') and extending into Namibia (Dlamini unpublished 1999). The next largest range is found in *M. major*, which occurs from the southwestern edge of the Eastern Cape into the Western Cape (Phillips and Hofmeyer 1927b), seemingly avoiding the nutrient-poor conditions that typify the extreme SW Cape heathlands (i.e.'fynbos') biome. Three taxa are confined to the western part of South Africa and nearby Namibia. *Melianthus elongatus* has a localised range along the Quaternary sands that line the West Coast, its range extending from the mouth of the Orange River in the north to as far south as the Langebaan Peninsula. The *M. pectinatus* complex (*M. p.* ssp. *pectinatus* and *M. p.* ssp. *gariepinus*) occurs further inland and has a more highly localised range within southern Africa. Subspecies *pectinatus* occurs south of the Orange River to the edge of the arid Knersvlakte valley in the Northern Cape (Tansley and Schelpe 1984), while the range of subspecies *gariepinus* is restricted to the immediate margins of the Orange River (Archer 1997, Dlamini unpublished 1999). The remaining three species all have highly localised ranges in the eastern half of South Africa (Phillips and Hofmeyer 1927b). The most widespread of the three is *M. dregeanus* spp. *insignis*, which covers an area from the northwestern slopes of the Drakensberg massif in the Free State to Mpumalanga in the north. *Melianthus villosus* and *M. dregeanus* ssp. *dregeanus* are highly confined, with *M. villosus* being found along a narrow, mid-elevational belt on the northeastern slopes of the Drakensberg, and *M. dregeanus* spp. *dregeanus* being limited to isolated hill tops in the Eastern Cape Province, around Cathcart, Stutterheim and Komgha. (See distributions, Figure 1.1).

The greatest concentration of species diversity in *Melianthus* occurs along the Western side of South Africa, in the Northern and Western Cape, suggesting that diversification was stimulated by past aridification here, as has been demonstrated elsewhere in W. Cape taxa (Verboom 2000, Hartmann 2001, Richardson *et al.* 2001). Although species are considered geographically and ecologically separated (Dlamini unpublished 1999), overlap does occur along margin edges making the genus seem more parapatric than allopatric. With some range edges blurring, it is unclear then

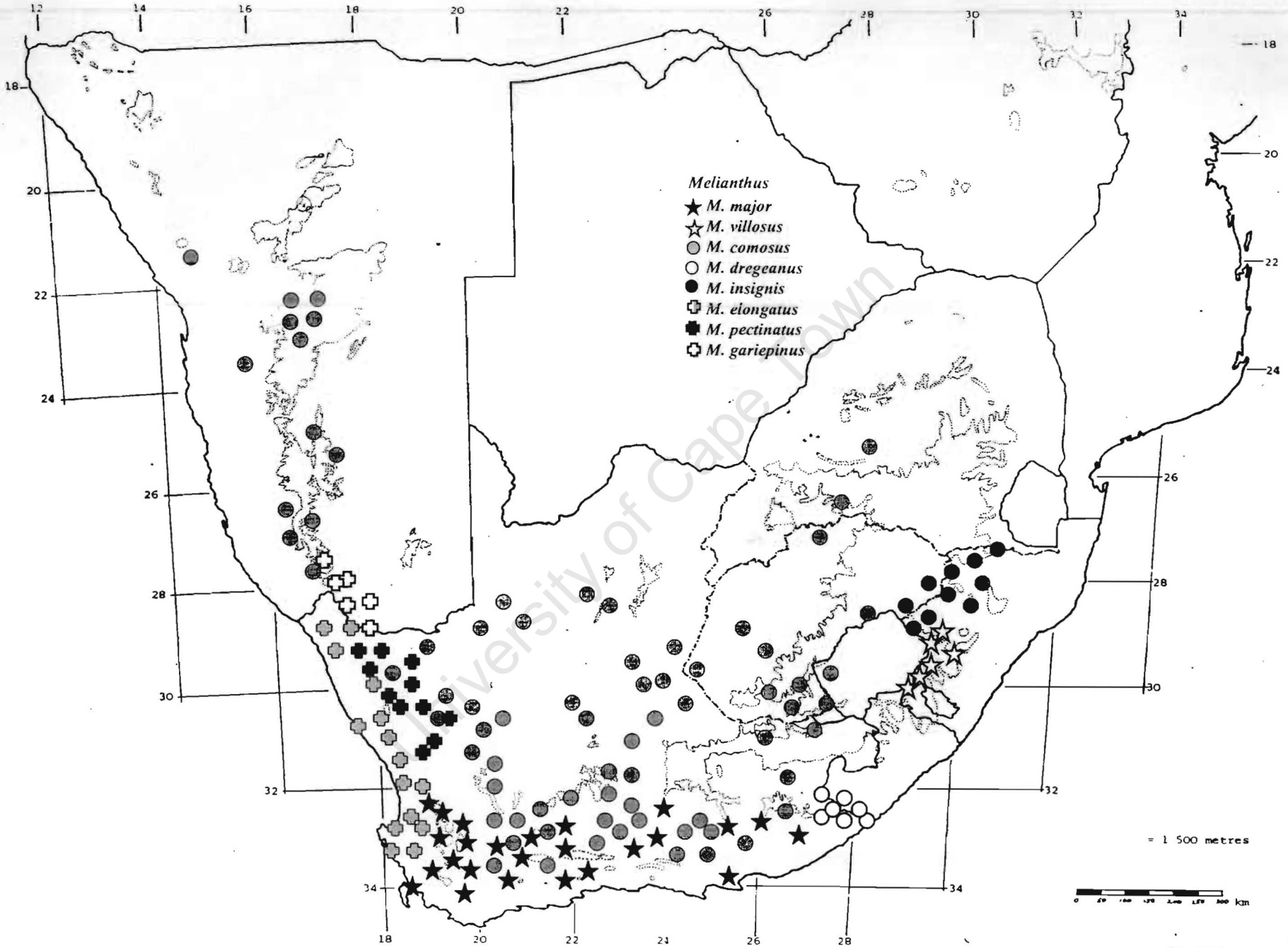


Figure 1.1: *Melianthus* distributions in southern Africa based on Bolus Herbarium collection and Dlamini's (unpublished 1999) grid maps for individual species.

whether the physical environment has been the ultimate stimulator of the morphological diversification typifying *Melianthus* species (Stebbins 1952) or have other factors come into play, such as pollinator specialisations (Johnson 1996)?

Objectives

Specific objectives of this thesis are as follows. Chapter 2 will attempt to produce a phylogeny for *Melianthus* based on molecular evidence to evaluate monophyly of the species. The results will be compared with Dlamini's morphological analysis, and the level of congruence assessed with a view to a combined analysis of these data (Kluge 1989, Bull *et al.* 1993). Using the best estimate of *Melianthus* phylogeny, the evolution of key morphological features will be evaluated using character optimisation methods. Following on from this, Chapter 3 will attempt to set up a spatio-temporal framework for better understanding of the historical circumstances under which *Melianthus* diversified. By using date estimations and palo-ecological reconstructions, the diversification of the genus relative to the influence of the physical and biotic environment will be investigated, touching on such clade-defining traits as novelties of floral morphology and flowering phenology. Chapter 4 will offer a brief synthesis of results including directions for future study.

Chapter 2: A Molecular Phylogeny of *Melianthus* L.

2.1 Introduction

The 'Burden' of Placing *Melianthus*: Historical Classifications

Planchon (1851): "Melianthus... *is one of those anomalous vegetable forms which puzzle the judgement of botanists by the very means that render them objects of eager and favourite inquiry."*

Melianthus L. is a small genus of shrubby, soft-wooded to semi-herbaceous plants bearing conspicuous stipules and deeply divided, imparipinnate foliage (Watson and Dallwitz 1992). The plants are noted as much for their diverse floral morphology (Ronse De Craene *et al.* 2001) as for the unpleasant odour emanating from the crushed foliage (Von Marilaun 1895). The highly zygomorphic blossoms are protandrous, resupinating at maturity from the production of copious dilute nectar, this most likely reflecting an adaptation for bird-pollination (Vogel 1954, Ronse De Craene *et al.* 2001). *Melianthus* belongs to the small African family Melianthaceae Link, which includes one other genus, the more widespread *Bersama* Fres (Phillips 1921).

Despite 300 years of recorded history, the hierarchical classification of *Melianthus* has long been considered 'the burden of the systematists' (Agardh 1859). Gürke (1896) was 'troubled' by the affiliations of the genus while Dahlgren (1980) expressed 'grave doubts' over its placement. Steyn *et al.* (1986) described the position of the family as 'a matter of dispute' while Archer (1997) referred to it as 'curious'. Takhtajan (1997) resorted to its isolation in the specially proposed order Melianthales, a motion Doweld (2001) seconded, lamenting *Melianthus* never held a 'stable and clear position within the angiosperms'. The prevalence of unease was perhaps best summarized by Ronse De Craene *et al.*'s (2001) use of the word 'enigmatic' to describe the perplexing genus.

None of this should come as a surprise given the misplacement of *Melianthus* since its taxonomic inception. Although the genus is formally accredited to Linnaeus, Tournefort (1694) was among the first to describe *Melianthus* (Doweld 2001), its 'fleur irrégulier' qualifying its placement in the second section of his eleventh class, an artificial assortment of complex flowered species from Ranunculaceae, Rutaceae

and Sapindaceae. Linnaeus (1753) digressed further, placing *Melianthus* in Class *XIV* of his *Didynamia: Gymnosperma* to reside uneasily amongst elements from Acanthaceae, Bignoniaceae, Gesneriaceae, Lamiaceae and Scrophulariaceae. Although placing *Melianthus* in the Geraniaceae, Adanson (1763) suggested a close affinity with Sapindaceae.

By the mid-1800s, two main schools of thought dominated, arguing for either a sapindaceous affinity (Reichenbach 1828, Planchon 1851, Harvey and Sonder 1860, Bentham and Hooker 1862-1867, Baillon 1874) or a rutaceous connection (de Jussieu 1789, de Jussieu 1825, Endlicher 1836-1840, Lindley 1846). The sapindalean argument would prove the most compelling, prevailing well into the late 1900s (Hutchinson 1926, Verdcourt 1956a, 1956b, Cronquist 1981, Dahlgren and van Wyk 1988, Thorne 1992).

At the familial level, Link (1831), followed by Planchon (1851), was the first to suggest the notion of Melianthaceae as a clearly circumscribed and 'natural family' (Doweld 2001) comprised solely of the two genera, *Melianthus* and *Bersama.* Bentham and Hooker (1862-1867) disagreed, however, linking both genera to *Greyia* in a conservatively defined Sapindaceae, a move supported by Baillon (1874). Radlkofer (1891) later shifted all three genera back to Melianthaceae although Gürke (1896) questioned the inclusion of *Greyia*. In keeping with Gürke's reasoning, Hutchinson (1926) placed *Greyia* in the newly proposed unigeneric family, Greyiaceae.

Whether Melianthaceae and Greyiaceae were related closely enough to warrant placement in the same family (Engler and Diels 1936, Phillips 1951, Erdtmann 1952, Lawrence 1963), in separate families (Hutchinson 1926, Heimsch 1942, Metcalfe and Chalk 1950, Scholz 1964 and Takhtajan 1969, Dahlgren 1980, Cronquist 1981, Steyn *et al.* 1986, 1986, Dahlgren and van Wyk 1988), within the same order (Hideux and Ferguson 1976, Dahlgren 1980, Dahlgren and van Wyk 1988) or even separate orders (Hutchinson 1926, Cronquist 1981, Doweld 1996a, 1996b, 1998, 2001, Takhtajan 1969, 1974, 1997), consumed taxonomists for much of the twentieth century.

The majority of conflict with historical Melianthaceae classifications can be attributed largely to a sole reliance on macromorphological details, which in itself is often ineffective for resolving phylogenetic relationships. Tenuous evidence from cytology (Federov 1969) and palynology (Erdtmann 1952) lent support to the concept

of *Melianthus, Bersama* and *Greyia* as comprising a single family within Sapindales. More detailed ontogeny/embryology (Metcalf and Chalk 1950, Khusalani 1963, Steyn *et al.* 1986, Hilger 1978, Danilova 1996, Doweld 1996a, Doweld 1996b, 1998, 2001, Dlamini unpublished 1999, Ronse De Craene *et al.* 2001) and histochemistry (Anderson and Koekemoer 1968, 1969a, 1969b, Agarwal and Rastogi 1976, Kelmanson *et al.* 2000) instead emphasized the distinctiveness between Melianthaceae and Greyiaceae. Indeed, the Russian systematists Takhtajan (1997) and Doweld (1996a, 1996b, 1998, 2001) deemed distinctions so pronounced as to warrant the placement of both into newly proposed orders, Melianthales and Greyiales.

Despite three centuries of morphological thought, the familial relations of *Melianthus* were not convincingly resolved until the rise of molecular systematics in the late 1900s. Phylogenies based on high-level molecular work not only supported the removal of the three genera from Sapindales but raised doubts over their distinctiveness. Using conserved gene regions such as plastid *rbc*L (Chase *et al.* 1993, APG 1998, Savolainen *et al.* 2000), nuclear18S (Soltis *et al.* 1997), combined *rbc*L-18S (Nickrent and Soltis 1995, Soltis and Soltis 1997) and combined *rbc*L-18S-*atpB* (Soltis *et al.* 2000), Melianthaceae was resolved in a single clade as sister to Greyiaceae plus a South American family, Francoaceae A. de Juss., with 100% jackknife support, a relationship tentatively suggested as early as 1874 by Baillon. The three genera also received novel placement in the order Geraniales (Savolainen *et al.* 2000, Soltis *et al.* 2000), although the position of the latter within the rosids remains unresolved and unsupported.

Addressing high-level relationships, previous molecular studies included no more than two representatives from the Melianthaceae with *Melianthus major* and *Bersama abyssinica* representing the two genera (Chase *et al.* 1993, Nickrent and Soltis 1995, Soltis and Soltis 1997, Soltis *et al.* 1997, APG 1998, Savolainen *et al.* 2000, Soltis *et al.* 2000). Consequently, no detailed low-level molecular phylogeny describing inter- and infra-specific relationships in *Melianthus* as yet exists.

Species Problems

Species level taxonomy has been equally contentious. Linnaeus described two species in 1753, *M. major* L. and *M. minor* L., distinguishing them by solitary versus paired stipules. Shortly thereafter, Vahl (1794) described another species, *M. comosus*

Vahl, which despite its uniqueness caused much confusion with *M. minor* since no type specimen existed for the latter.

Planchon (1851) proposed an unnecessary division of the genus into two genera, (*Melianthus* and *Diplerisma* Planch.), the idea subsequently being trounced by Bentham and Hooker (1862-1867). Planchon (1851) also published a new species, *M. himalayanus* Planch., based on a collection from India by Wallich. Hooker (1873) later decreed this as conspecific with *M. major,* the species apparently escaping into the wild in northern India shortly after its introduction as an ornamental by the English (Hooker and Thomson 1855).

In the Flora Capensis (Harvey and Sonder 1860), five species were recognised. Sonder listed *M. major*, began differentiating between *M comosus* Vahl and *M. minor* L. and described a new species, *M. dregeanus* Sond., from the Eastern Cape. A fifth species, *M. pectinatus* Harv., was introduced by Harvey (1860) in an appendix.

Hooker (1873) described a new species from Namaqualand, *M. trimenianus* Hook., based on a collection by Barkly of a plant with a reduced revolute leaflet seemingly pronounced to a degree of uniqueness for the genus. Bailey (1914) later reduced the name to synonymy under *M. pectinatus* Harv. suggesting Harvey had already accounted for the variability in leaf morphology.

By the end of the late 1800s, the number of taxa increased to seven after recent collections were made in the Drakensberg. Bolus (1896) described a distinct species, *M. villosus* Bolus, from the eastern Drakensberg while Kuntze (1898) described another species, *M. insignis* Kuntze, from the western slopes. Nevertheless, a review of the genus by Phillips and Hofmeyer (1927b) maintained the species number at six by sinking Kuntze's *insignis* to varietal status under *M. dregeanus,* an opinion protested by Dyer on at least two occasions (1952, 1959).

In 1968, Merxmüller and Roessler described what they believed to be a new species from Namibia, *M. gariepinus* Merx. & Roess. Phillips and Hofmeyer (1927b) had alluded earlier to its existence from collections made by Rose in the early 1900s but felt the material in their possession at that time was in too poor a state to reach a conclusion. Tansley (unpublished 1983) and Tansley and Schelpe (1984) felt *M. gariepinus* was most appropriate as a subspecies beneath *M. pectinatus* treating the *M. dregeanus* complex similarly by raising the variety *insignis* to subspecies level.

Despite Sonder's (1860) earlier attempt to distinguish *M. comosus* from *M. minor*, the matter was not addressed formally until 1985 when Brummit proposed to reject the confused epithet '*minor*' for either taxon. Instead, the previously published *M. comosus* Vahl (1794) was resurrected for the type specimen characterised by axillary located, lateral racemes, whereas the remaining taxon, typified by terminal erect racemes, would become known as *M. elongatus* Wijn.

Morphological Phylogeny

The first modern phylogeny explicitly addressing lower-level relationships in *Melianthus* was produced in 1999 by Dlamini (unpublished) based on a cladistic analysis of morphological data. His results resolve *M. major* as sister to the rest of *Melianthus*. The remaining species are divided into two main clades, one clade comprising *M. villosus* as sister to *M. elongatus* plus the *M. pectinatus* complex with a second clade composed of *M. comosus* plus the *M. dregeanus* complex. Based on morphometric analysis, Dlamini supported Dyer's (1952, 1959) opinion that *M. dregeanus* ssp. *insignis* warrants re-elevation to specific status based on differences in size of stipules, racemes and bracts, floral lobing and the attachment of the nectary. Dlamini also questioned whether a similarly detailed analysis of the *M. pectinatus* complex would elevate subspecies *gariepinus* to specific rank as well. With this thought in mind, the final species number of *Melianthus* would still seem to be in a state of flux. As currently defined (Leistner 2000) the species number resides questionably at six: however, this number could be raised to eight if the subspecies within the *M. pectinatus* and *M. dregeanus* complexes were elevated to species level.

In keeping with Dlamini's protocol, the subspecies names for the *M. dregeanus* and *M. pectinatus* complexes are herewith treated as species names, if only for the purposes of simplicity, e.g. *M. dregeanus* complex = *M. dregeanus* and *M. insignis*, and *M. pectinatus* complex = *M. pectinatus* and *M. gariepinus*.

Molecular Phylogeny

Controversy exists over the effectiveness of morphology for portraying phylogenetic history (Hedges and Maxson 1996) since some argue there is a qualitative difference between molecular and morphological data (Nei 1987, Sytsma *et al.* 1990, Avise 1994, Hedges and Sibley 1994). Phylogeny inference from morphology may be misleading due to subjective homology interpretation (Hedges and Maxson 1997) and the convergence of labile traits under the influence of

environmental selection (Stebbins 1952, Van Valen 1976, Andersson 1990). Conversely, molecular systematics allows for an 'objective' assessment of relationships independent of morphology (Givnish 1997). Also, molecular systematics generally produces a greater number of independent characters, which affords finer resolution and lower levels of homoplasy (Givnish and Sytsma 1997). Donoghue and Sanderson (1992) suggest that a sensible compromise between the two is to set aside morphological data until a molecular phylogeny is first obtained. Ultimately, pooling data sets will likely maximise the explanatory power (Kluge 1989, Kitching 1998, Page and Charleston 1998) of a phylogenetic analysis. Before combination though, an assessment of congruence between independent data sets is a prerequisite to ensure the underlying phylogeny is correctly interpreted (Miyamoto and Cracraft 1991, Bull *et al.* 1993, Hillis *et al.* 1996, Weins 1998).

According to Judd *et al.* (1999), "Eventual classification is (then) derived from phylogeny". Although confidence levels presumably increase the more gene sets are examined, phylogenies produced solely by molecular gene trees may not necessarily be synonymous with species trees (Wendel and Doyle 1998, Nicols 2001). Olmstead (1995) emphasises that gene trees provide the necessary 'hypotheses' for species circumscription but the more conventional criterion of diagnosability is still necessary for species delineation (Davis 200, Luer 2002).

Specific objectives of this study are to test the monophyly of *Melianthus* and its component taxa and to evaluate the existing morphological phylogeny in the light of molecular findings. Once the most convincing phylogeny is quantified, patterns depicting evolutionary character change can be more reliably inferred (Stevens 2001).

2.2 Materials and Methods

Sequencing

Twenty accessions representing all *Melianthus* species and subspecies plus four outgroup samples were sequenced (Table 2.1). Multiple outgroups were included to test the assumption of ingroup monophyly (Nixon and Carpenter 1993, Swofford *et al.* 1999). The majority of ingroup material was collected in the field throughout South Africa and Namibia. Multiple sampling was employed to test the monophyly of certain ingroup taxa exhibiting wide-ranging distribution (*M. comosus, M. major*) or morphologically divergent forms (*M. gariepinus, M. pectinatus*). In instances where wild-grown material was unobtainable, (*Bersama lucens, B. swinnyi, Grevia*

Table 2.1: List of samples used for DNA sequencing. Multiple accessions of single taxa are distinguished by numeric suffixes.

Taxon name sensu *Dlamini/ Sample No.*	*Voucher number*	*Collection locale*
Greyia flanaganii Bolus	*Henning* 32 (Kirstenbosch accession #444)	In cultivation, Kirstenbosch Botanical Garden.
Greyia radlkoferi Szyszyl.	*Henning* 31 (Kirstenbosch accession #203)	In cultivation, Kirstenbosch Botanical Garden.
Bersama lucens (Hochst.) Szyszyl.	*Henning* 34 (Kirstenbosch accession #439)	In cultivation, Kirstenbosch Botanical Garden.
Bersama swinnyi Phillips	*Henning* 35 (Kirstenbosch accession #441)	In cultivation, Kirstenbosch Botanical Garden.
Melianthus comosus Vahl 1	*Henning* 3	27-16 CA, Witputz Nord Farm, NW of Rosh Pinah, Namibia.
Melianthus comosus Vahl 2	*Henning* 34	31-19 AC, Nieuwoudtville, Calvinia.
Melianthus comosus Vahl 3	*Henning* 21	33-20 CC, Kogmanskloof, 5 km SW of Montagu.
Melianthus comosus Vahl 4	*Henning* 25	28-28 DA, Golden Gate Park in front of Brandweg Hotel.
Melianthus comosus Vahl 5	*Henning* 27	30-27 AC, 20 km S of Zastron on road to Rouxville.
Melianthus elongatus Wijn.	*Henning* 2	32-18 CC, Velddrif/Vredenburg road nr. junction with R27.
Melianthus insignus (Phill. & Hoff.) Tansley and Schelpe	*Henning* 24	28-28 DA, Golden Gate Park at the SW base of Brandweg Buttress.
Melianthus dregeanus (Sond.) Tansley and Schelpe	*Henning* 29	32-27 AC, 6 km NW of Cathcart on road to Windvoelberg, Farm 36.
Melianthus major L.1	*Henning* 1	33-20 CC, Kogmanskloof, 5 km SW of Montagu.
Melianthus major L.2, (green calyx form)	*Henning* 20	33-18 CD, gulley SE of Rhodes Memorial.
Melianthus major L.3, (purple stipe form)	*Henning* 16	32-18 DB, Piekenierskloof, besides entrance to Piekenierskloof Mountain Lodge.
Melianthus pectinatus (Harv.) Tansley and Schelpe 1, (ericoid leaf)	*Henning 9*	29-17 BD, 8 km west of Steinkopf on road to Port Nolloth.
Melianthus pectinatus (Harv.) Tansley and Schelpe 2, (typical leaf form)	*Henning* 13	30-18 CC, Anegas Farm, past Blouputs, on side of stream.
Melianthus gariepinus (Merxm. & Roess.) Tansley and Schelpe 1, (pendulous floral raceme, e.g. Merxm. & Roess. form)	*Henning* 6	27-16 CD, dry stream bed above borehole for Namuskluft campsite, SE of Rosh Pinah, Namibia.
Melianthus gariepinus (Merxm. & Roess.) Tansley and Schelpe 2, (erect floral raceme)	*Henning* 4	27-16 CD, besides dry stream bed at base of south-facing cliffs, 8 km past Macmillan's Pass heading towards Black Death River, nr. Rosh Pinah, Namibia.
Melianthus villosus Bolus	*Henning* 33 (Kirstenbosch accession #857)	In cultivation, Kirstenbosch Botanical Garden.

flanaganii, G. radlkoferi, and *M. villosus*), cultivated material was gathered from the Kirstenbosch Botanical Gardens in Cape Town, South Africa. All vouchers are housed in the Bolus Herbarium (BOL) at the University of Cape Town.

Total genomic DNA was extracted from fresh leaves wherever possible since conventional silica-dried material proved unsuccessful. In instances where immediate processing was not possible, material was collected for storage in 2X CTAB containing 1% polyvinylpyrrolidone (PVP) prior to extraction. The processing of ~20mg leaf samples for each taxon followed a CTAB DNA miniprep protocol modified from Gawel and Jarret (1991) and included the addition of 0.05-0.10mg PVP powder during the grinding process to override secondary compounds. Identical results were obtained during the grinding procedure whether tissues were frozen first with liquid nitrogen or ground directly in 2X CTAB solution; therefore, the latter procedure was preferred for simplicity.

Compensation for any limitations associated with single gene histories (Wendel and Doyle 1998) was addressed by sequencing both plastid and nuclear non-coding loci. Two plastid markers were used, the *trn*L-F intron-spacer region and the *psb*A-*trn*H intergenic spacer, with a nuclear marker obtained from the internal transcribed spacer (ITS) of nuclear rDNA. The ITS marker was chosen since it is reported to accumulate nucleotide changes more frequently than plastid DNA (Small *et al.* 1998). Amplification of the *trn*L-F intron-spacer region was achieved using the forward primer 'c' and the reverse primer 'f' (Taberlet *et al.* 1991). *Psb*A-*trn*H amplification was accomplished using forward primer *psb*AF and reverse primer *trn*HR (Sang *et al.* 1995). The internal transcribed spacer region encompasses the internal transcribed spacers ITS1 and ITS2, which flank either side of the 5.8S gene in 18S/26S nuclear rDNA. In order to capture the entire region, ITS amplification utilised the forward primer ITS5 and the reverse primer ITS4 (White *et al.* 1990). A complete list of primer details appears in Table 2.2.

Amplification reactions were prepared on ice based on a 50µl reaction volume comprising 33.50µl of sterilized water (dH_2O), 5.0µl of 50mM $MgCl_2$, 5.0µl of 10X DNA polymerase buffer (Bioline), 2µl of dNTP (10mM), 1µl of each primer (10µM), 0.5µl of *Taq* DNA polymerase (Bioline, 5U/µl, Bioline Ltd., London, UK) and 2µl of template DNA. In instances where extraction product proved exceptionally strong, <2µl of template DNA was used with extra dH_2O for compensation. The

Table 2.2: Primers used in PCR and sequencing reactions for *Melianthus* and 4 outgroups.

Region	Primer	Reading from 5' to 3'	Gene type
***trn*L-F**	Forward C	CGA AAT CGC TAG ACG CTA CG	plastid
"	Forward internal E	GGT TCA AGT CCC TCT ATC CC	"
"	Reverse F	TTT GAA CTG GTG ACA CGA G	"
***psb*A-*trn*H**	Forward *psb*AF	GTT ATG CAT GAA CGT AAT GCT C	"
"	Reverse *trn*H	CGC GCA TGG TGG ATT CAC AAA TC	"
ITS	Forward ITS5	GGA AGT AAA AGT CGT AAC AAG G	nuclear
"	Forward ITS3	GCA TCG ATG AAG AAC GCA GC	"
"	Reverse ITS4	TCC TCC GCT TAT TGA TAT GC	"
"	Reverse ITS2	GCT GCG TTC TTC ATC GAT GC	"

amplification of double-stranded *trn*L-F and *psb*A-*trn*H regions was accomplished via the polymerase chain reaction (PCR) using an initial denaturation period of 2 min at 94°C with 30 cycles of the following procedure: 60 sec at 94°C, 60 sec at 52°C, 2 min at 72°C, the final cycle followed by an 7 min extension period at 72°C. ITS amplification followed a similar procedure but with the initial annealing temperature raised to 55°C and the reaction mixture amended by substituting 1µl of dH_2O with 1µl of 100% dimethyl sulfoxide (Winship1987, Buckler *et al.* 1997).

Resultant PCR products were visualised on a 1% agarose gel stained with ethidium bromide to confirm successful amplification. PCR reactions were cleaned using a QIAquick® Qiaqen™ PCR Purification Kit (Qiagen GmbH, D-40724, Hilden, Germany). Sequencing reactions for single-stranded *trn*L-F DNA used forward primer 'c', reverse primer 'f' and, where necessary, internal forward primer 'e'. Single-stranded *psb*A-*trn*H DNA was accomplished with forward primer *psb*AF and reverse primer *trn*HR. Single-stranded ITS DNA used forward primers ITS5 and reverse primer ITS4. Secondary structure typical of ITS (Baldwin *et al.* 1995) necessitated the use of internal forward primer ITS 3 in all *Melianthus* samples and *Greyia* outgroups. One clade comprising *Melianthus elongatus*, *M. gariepinus* and *M. pectinatus* required the additional use of internal reverse primer 5.8S (Hershkovitz and Lewis 1996).

Reaction mixtures for cycle sequencing were prepared on ice using an ABI PRISM®BigDye™Terminator Cycle Sequence Kit (Applied Biosystems, Warrington, UK) based on a 10µl reaction volume made up of 2.84µl of dH_2O, 2.00µl of sequence terminator BigDye™ vers. 3.0, 3.00µl 2.5X cycle sequence buffer, 0.16µl of the respective primer and $\leq$2.00µl of the appropriate DNA product, the difference topped up with dH_2O. The ITS recipe differed by substitution of 0.5µl of 20% DMSO for 0.5µl of dH_2O. Cycle sequencing reactions consisted of 25 cycles of 30 sec at 96°C followed by 15 sec at 50°C and 4 minutes at 60°C. Both initial amplification and cycle sequencing reactions took place on a GeneAmp™PCR System 2700 machine (Applied Biosystems, Foster City, CA, USA). Cycle sequencing products were realised on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) by the University of Stellenbosch, Matieland, South Africa.

Alignment and Molecular Analysis

Forward and reverse chromatographs were assembled and edited in SeqMan II™ ver 2.04 (Lasergene software, DNASTAR Inc., Madison, WI, USA) before export to MegAlign™ (DNASTAR Inc.) for manual alignment on a Macintosh iMac computer. The somewhat conserved nature of *trn*L-F data (Soltis and Soltis 1998) allowed for more straightforward alignment than the more 'evolutionary plastic' *psba*A-*trn*H (Sang *et al.* 1997). An inversion 30 bp long was identified in the *psb*A-*trn*H data in a number of the *Melianthus* samples as depicted in Figure 2.1 (position 1929-1959, relative to *M .comosus*5, which was arbitrarily chosen as a reference point from the combined data Nexus file). Since short inversions in *psb*A-*trn*H are typically homoplasious in nature, and hence phylogenetically unreliable (Sang *et al.* 1997), the length of the inversion was defined as a character set to be excluded from future analyses. However, its actual presence/absence was still scored as a binary character (Jansen and Palmer 1987) appended to the end of the *psb*A-*trn*H data partition. ITS data proved equally variable during alignment. Areas in the outgroup too variable to meaningfully align against the ingroup were coded as '?' in the outgroup to reduce noise and facilitate searches (positions 1123-1195 and 1246-1313 in both *Greyia* species, relative to *M. comosus*5).

Since sequences of non-protein coding loci are not constrained by translation, insertions/deletions may be frequent (Hillis *et al.* 1996). Alignment required the insertion of 24 gaps in *trn*L-F, 29 in *psb*A-*trn*H and 25 in ITS for purposes of homology. Potentially informative indels appearing in unambiguously aligned areas were scored as presence/absence characters following a simple gap coding technique as outlined by Simmons and Ochoterena (2000) for assessing homology based on both indel position and length. Indels placed in untrimmed beginning and ending sections were meaningless and left uncoded. Resultant binary matrices were appended to the end of each respective gene matrix. The three individual partitions were then merged in Nexus format to provide a combined molecular data matrix of 2361 characters.

Pairwise sequence divergences were calculated as 'uncorrected p' values with sites containing missing and ambiguous data ignored for affected pair-wise distances.

Parsimony analysis with a heuristic search algorithm was performed in PAUP* 4.0b10 (Swofford 1998). Characters were treated as unordered and of equal weight (Fitch 1971) with gaps treated as missing. Searches were done using a random

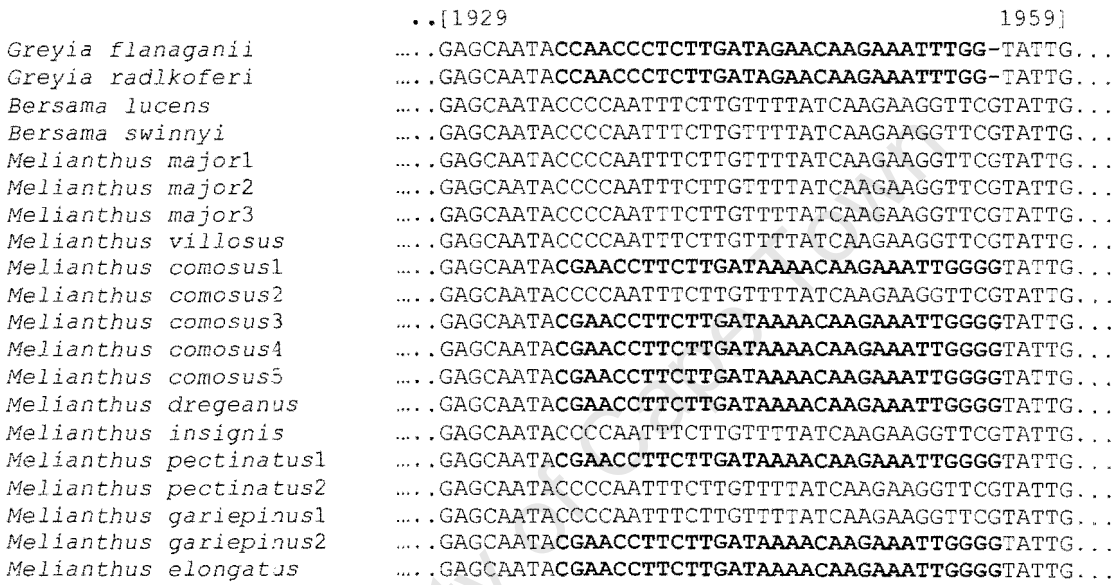


Figure 2.1: Distribution of a 30 bp inversion in the *psb*A-*trn*H sequences, which is indicated in bold highlight.

addition sequence with 10 000 replicates using the TBR (tree-bisection-reconnection) branch-swapping algorithm with the 'Multrees' option in effect. Multiple most parsimonious trees were summarised to a single strict consensus tree. Character support for nodes (Felsenstein 1985) was evaluated using 500 bootstrap replicates based on a simple addition sequence with 'Maxtrees' set to 1000. Polarity was established by rooting on *Greyia*, specifying the outgroup as a monophyletic sister group relative to the ingroup. Each gene region was initially analysed separately, then as a cpDNA analysis (Soltis *et al.* 1993), and finally as a combined molecular analysis (Kluge 1989) in order to check for visual discordance between topologies. Beginning and end segments of each gene matrix, although left untrimmed, were not excluded from analyses since they contained no phylogentically informative characters.

Hypotheses of incongruence were tested for statistical support using the Wilcoxon Sign-Rank (WSR) tests as employed by Templeton (1983). The Templeton test forces one data set to generate the topology from a consensus tree obtained from an alternative data partition. The resultant constrained tree is tested for 'goodness of fit' to see if it is significantly less parsimonious than the most parsimonious unconstrained tree.

Molecular-Morphological Analysis

For purposes of comparing the phylogenies based on molecular and morphological evidence, the morphological analysis conducted by Dlamini (unpublished 1999) was revisited and reanalysed. Prior to reanalysis, the following modifications were made to his data matrix:

1. Character 21 (petal fusion) was deleted since it describes the same state as character 18 (presence of crystalline hairs), the presence of the intertwining crystalline hairs causing the petals to adhere along their inner margins even though they are technically free.
2. Character 32 (number of ovules per locule) and character 40 (colour of sepals) were further refined for purposes of clearer species delineation.
3. Characters 47-49, concerning pollen morphology, were deleted due to the inability to provide personal verification for all species/forms involved.

In addition, two new characters were added describing both petal and nectar colouration. For purposes of clarity, all floral colours were qualified from fresh collected material by comparison against The Royal Horticultural Society's colour

charts (1966), the results listed in Table 2.3. Morphology character codes were manually entered at the end of the combined molecular matrix increasing the total number of characters to 2413. The modified character list can be seen in Table 2.4 with amended character numbers reflective of their new positions. The resultant data matrix appears in Table 2.5.

Terminals in the morphological matrix and an amended molecular matrix were reduced to a single representative each for the multiple sampled ingroup members *M. major* and *M. comosus*. The *M. pectinatus* complex (including *M pectinatus* and *M gariepinus*) was represented by four terminals corresponding to distinct morphologies. *M. pectinatus*1 exhibits a narrowed, ericoid, entire leaflet edge mistakenly attributed to improper herbarium mounting techniques by previous authors (Tansley and Schelpe 1984, Dlamini 1999 unpublished) versus the more typically serrate leaflet edge found in *M. pectinatus*2. *M. gariepinus*1 has variably erect flower spikes versus the distinctly pendulous flower spikes of *M. gariepinus*2. Outgroup samples were reduced to a composite representative for each genus (*Greyia* and *Bersama*).

Amended molecular and morphology partitions were analysed separately and as a total evidence approach using parsimony searches in PAUP* 4.0b10 (Swofford 1998) as outlined previously. Parameters were identical to those already established with the exception that four morphological characters were ordered (characters 2378, 2882, 2389, 2392) following Dlamini's protocol (1999 unpublished). Strict consensus topologies were used as constraints in the Templeton test (1983) to check for conflict between the molecular and morphology data partitions.

Character Reconstruction

Changes in morphological characters were reconstructed on the amended molecular topology using PAUP* 4.0b10 (Swofford 1998). Character optimisation methods were based on both accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN) models since a combination of models will provide the most conservative estimate (Givnish 1997). ACCTRAN tends to be the preferred method (de Pinna 1991), favouring character gains through quick changes allowing for subsequent reversals, whereas DELTRAN, with its own proponents (Swofford and Begle 1993), favours parallelisms through multiple gains by delaying change (Hillis *et al.* 1996). Character states appearing unequivocal under both models were used to distinguish terminals and clades. Reconstruction of ancestral conditions allows for

Table 2.3: Floral colours qualified by the Royal Horticultural Society's colour charts (1966) based on fresh *Melianthus* material collected as voucher specimens for sequencing purposes. *Melianthus insignis* is unreported since it was the only species collected before full bloom.

Taxa	Voucher # and locale	Sepal Colour	Petal Colour
***M. comosus*1**	Henning 3, Rosh Pinah, Namibia (27-16 CA)	Shrimp red 33.C with pea green 149.B apice and basal spot of oxblood red 183.B	Capsicum red 33.A distally with oxblood red 183.B base
***M. comosus*3**	Henning 21, Kogmanskloof (33-20 CC)	Faded mandarin red 41.D with pea green 149.B apice and basal spot of oxblood red 183.B	Signal red 43.A distally with oxblood red 183.B base
M. dregeanus	Henning 29, Cathcart (32-27 AC)	Brick red 35.A with pea green 149.B apice and basal spot of oxblood red 183.B	Ruby red 59.B distally with and oxblood red 183.B base
M. elongatus	Henning 2, Veldriff (32-18 CC)	Pea green 149.B withslight vermilion 41.D venation	Carrot red distally 29.A with oxblood red 183.B base, crown petals turkey red 33.D
***M. gariepinus*1**	Henning 6, Namaskluft, Namibia (27-16 CD)	Lettuce green 144.A	Faded scarlet to scarlet 43.D-43.B distally with oxblood red 183.B base
M. insignis	-	-	-
***M. major*3**	Henning 16, Piekenierskloof (32-18 DB)	Oxblood red 183.B	Oxblood red 183.B
***M. major*2**	Henning 20, University of Cape Town, in cult.	Scheele's green 144.B	Oxblood red 183.B
***M. pectinatus*2**	Henning 13, Anegas (30-18 CC)	Scheele's green 144.B with slight vermilion 41.D venation	Spanish orange 26.A to geranium lake 47.0 distally, oxblood red 183.B base, crown petals geranium lake 47.0
M. villosus	Henning 33, Kirstenbosch, in cult.	Scheele's green 144.B flushed with rhodamine purple 68.D	Saturn red 30.C with attenuating Scheele's green 143.D tip and oxblood red 183.B base.

Table 2.4: Amended characters and character states used for the phylogenetic analysis of *Melianthus* and the four outgroup taxa. Numbers accord with position in the amended molecular/morphology data partition. Dlamini's (unpublished 1999) original numbers placed in ( ). Ordered characters denoted by *. Characters modified from Dlamini's original matrix denoted by $^{+}$. New characters denoted by $^{++}$.

New No./ (Old No.)	Character State
2362.(1)	Stipules: 0 = paired, 1 = solitary, 2 = absent
2363.(2)	Stipules: 0 = prominent, 1 = small
2364.(3)	Leaves: 0 = simple, 1 = imparipinnate
2365.(4)	Leaves: 0 = with a musty smell, 1 = without a musty smell
2366.(5)	Leaf margins: 0 = dentate, 1 =serrate, 2 = entire
2367.(6)	Leaflet ventral indumentum: 0 = glabrous, 1 = villous
2368.(7)	Leaflet dorsal indumentum: 0 = glabrous, 1 = villous
2369.(8)	Rachis: 0 = winged, 1 = unwinged
2370.(9)	Leaf cell inclusions: 0 = raphides, 1 = styloids
2371.(10)	Inflorescence orientation: 0 = erect, 1 = lateral/pendulous
2372.(11)	Inflorescence position: 0 = terminal/sub-terminal, 1 = axillary
2373.(12)	Flower arrangement: 0 = alternate, 1 = opposite or whorled, 2 = irregular
2374.(13)	'Crown' of petals: 0 = present, 1 = absent
2375.(14)	Flower shape: 0 = actinomorphic, 1 = zygomorphic
2376.(15)	Odd sepal: 0 = one spurred, 1 = not spurred
2377.(16)	Lateral apices in odd sepal: 0 = present, 1 = absent
2378.(17)	*Petal size relative to odd sepal: 0 = <, 1 = equal to, 2 = >
2379.(18)	Petals adhere by woolly crystalline hairs: 0 = present, 1 = absent
2380.(19)	Lateral lobes: 0 = present, 1 = absent
2381.(20)	Petal outline: 0 = uniform, 1 = some petals with lateral appendages
2382.(22)	*Petal shape: 0 = elliptic, 1 = narrow-elliptic, 2 = very narrow elliptic
2383.(23)	Claws: 0 = glandular, 1 = eglandular
2384.(24)	Stamens: 0 = monomorphic, 1 = dimorphic
2385.(25)	Fruit dimensions: 0 = longer than wide, 1 = wider than long
2386.(26)	Fruit texture: 0 = membranous, 1 = leathery, 2 = woody capsules
2387.(27)	Fruit shape: 0 = acutely four winged, 1 = four rounded lobes
2388.(28)	Fruit/ovary indumentum: 0 = glabrous, 1 = villous
2389.(29)	*Ovary: 0 = 1-locular, 1 = 4-locular, 2 = 5 locular
2390.(30)	Ovule placentation: 0 = axial, 1 = basal, 2 = parietal
2391.(31)	Ovules outer integument: 0 = multiserial, 1 =uniserial
2392.(32)	*$^{+}$Numbers of ovules per locule: 0 = one, 1 = two, 2 = three or more
2393.(33)	Aril on seed: 0 = present, 1 = absent
2394.(34)	Position of the stipules: 0 = intrapetiolar, 1 = lateral
2395.(35)	Shape of the stipules: 0 = lanceolate, 1 =ovate
2396.(36)	Bract base: 0 = narrow, 1 = cordate
2397.(37)	Bract apex: 0 = acuminate, 1 = subacuminate to subulate
2398.(38)	Flower placement on peduncle: 0 = over its entirety, 1 = in upper half
2399.(39)	Indumentum on peduncle: 0 = glabrous, 1 = villous
2400.(40)	$^{+}$Colour of sepals: 0 = greenish, 1 = maroon, 2 = orange
2401.(41)	Outer sepals with a dark blotch: 0 = present, 1 = absent
2402.(42)	Lateral sepals: 0 =falcate, 1 = straight

Table 2.4 cont.

New No./ (Old No.)	Character State
2403.(43)	Odd sepal: 0 = saccate, 1 = not saccate
2404.(44)	Petal tip: 0 = spatulate, 1 = elliptic to lanceolate
2405.(45)	Indumentum of petal lamina: 0 =glabrous, 1 = villous
2406.(26)	Stamen orientation after pollination: 0 =upright, 1 =sideways, 2 = curled towards the odd sepal
2407.(50)	Nectary attached to: 0 = lateral sepal, 1 = odd sepal, 2 = all sepals
2408.(51)	Upper half of style: 0 = glabrous, 1 = villous
2409.(52)	Lower half of style: 0 = glabrous, 1 = villous
2410.(53)	Surface of seeds: 0 =smooth, 1 = verrucate
2411.(54)	Shape of seeds: 0 = globose, 1 = subglobose
2412.	$^{++}$Petal colouration: 0 = greenish/white, 1 = orange, 2 = maroon/red
2413.	$^{++}$ Nectar colouration: 0 = clear, 1 = tan, 2 = black

Table 2.5: Matrix of morphological character states based on that of Dlamini (unpublished 1999) with changes as specified in the text. Question marks denote inapplicable states for the taxa being addressed. Polymorphisms are placed inside { }.

```
                          [2362                                                2383]
Greyia                     2   ?  010{01}1  10{01}0   1  1 0  11211 0  01
Bersama                   {01}{01}110 0 {01}01 0 {01}{12}1{01}11211{01}01
Melianthus major           1   0 100  0  0  01 0  0   1  1 1  01001 0  10
Melianthus villosus        0   0 101  1  1  01 0  0   1  1 1  00000 1  20
Melianthus comosus         0   0 101  0  1  01 1  1   0  1 1  11101 0  10
Melianthus dregeanus       0   0 101  1  1  01 1  1   0  1 1  11001 0  10
Melianthus insignis        0   0 101  1  1  01 1  1   0  1 1  11001 0  10
Melianthus pectinatus1     0   0 102  1  1  01 0  0   1  0 1  00200 1  20
Melianthus pectinatus2     0   0 101  1  1  01 0  0   1  0 1  00200 1  20
Melianthus gariepinus1     0   0 101  1  1  01 0  0   1  0 1  00200 1  20
Melianthus gariepinus2     0   0 101  1  1  01 0  0   1  0 1  00200 1  20
Melianthus elongatus       0   0 101  1  1  01 0  0   1  0 1  00200 1  20

                          [2384                                      2413]
Greyia                     10 0  ?001121? ?  11?? 0  10101020021 0  0
Bersama                    10 2  10220000{01}1110 0  10110021121 0  0
Melianthus major           00 0  00100210 1  1110{01}11011010001 2  1
Melianthus villosus        00 0  01100111{01}1011 0  100110111110{01}2
Melianthus comosus         00 0  01100111 1  1001 2  00100011101 2  2
Melianthus dregeanus       01{12}11100111 1  1001 2  00000200110 2  1
Melianthus insignis        01{12}11100111 1  1001 2  10000210110 2  1
Melianthus pectinatus1     01 0  00100211 0  1010 0  10010110011{12}1
Melianthus pectinatus2     01 0  00100211 0  1010 0  10010110011{12}1
Melianthus gariepinus1     01 0  00100111 0  1010 0  10010110011{12}1
Melianthus gariepinus2     01 0  00100111 0  1010 0  10010110011{12}1
Melianthus elongatus       00{12}11100211 0  0110 0  10010100001{12}2
```

evaluation of polarity and direction of character state change (Kitching *et al.* 1998) offering the opportunity to hypothesize derived conditions within *Melianthus*.

2.3 Results

Molecular Data

Digital data matrices are included with this manuscript, see Appendix 1. Complemented sequence lengths of *trn*L-F ranges from 930-966 basepairs (bp) for *Melianthus* with 828-950 bp in the outgroups. The *psb*A-*trn*H sequences are 391-440 bp long in the outgroups and *M. major* while the remaining *Melianthus* species are shorter, having a range of 244-283 bp indicative of deletion events. ITS sequences range from 627-737 bp in *Melianthus* to 627-681 bp in the outgroups.

Results from pairwise sequence divergence are given in Table 2.6 as minimum-maximum ranges between *Melianthus* species, and between each outgroup and *Melianthus*. The difficulty in satisfactorily aligning certain sections in the ITS data set of the outgroup *Greyia* is qualified by *Greyia*'s high rate of divergence from *Melianthus*. Statistics describing the most parsimonious trees resulting from the analysis of individual partitions and from the combined analyses are listed in Table 2.7.

Trees obtained from the analyses of the three separate molecular data sets and a combined plastid analysis (Figure 2.2:A-D) receive high CI and RI values indicative of low levels of homoplasy (Table 2.7). Both the nuclear data set and the combined plastid data set show similar resolving power (nine well-supported nodes in ITS and eight well-supported nodes in cpDNA with ≥85% bootstrap support), which is greater than that found when either plastid region is analysed separately (five nodes in *trn*L-F and *psb*A-*trn*H each with ≥85% bootstrap support). Taken singly, neither plastid data set convincingly resolves the relationships among *M. comosus*, *M. elongatus* and the *M. dregeanus* and *M. pectinatus* complexes. Despite differences in resolution, the topologies of the four trees are relatively similar save for the following exceptions:

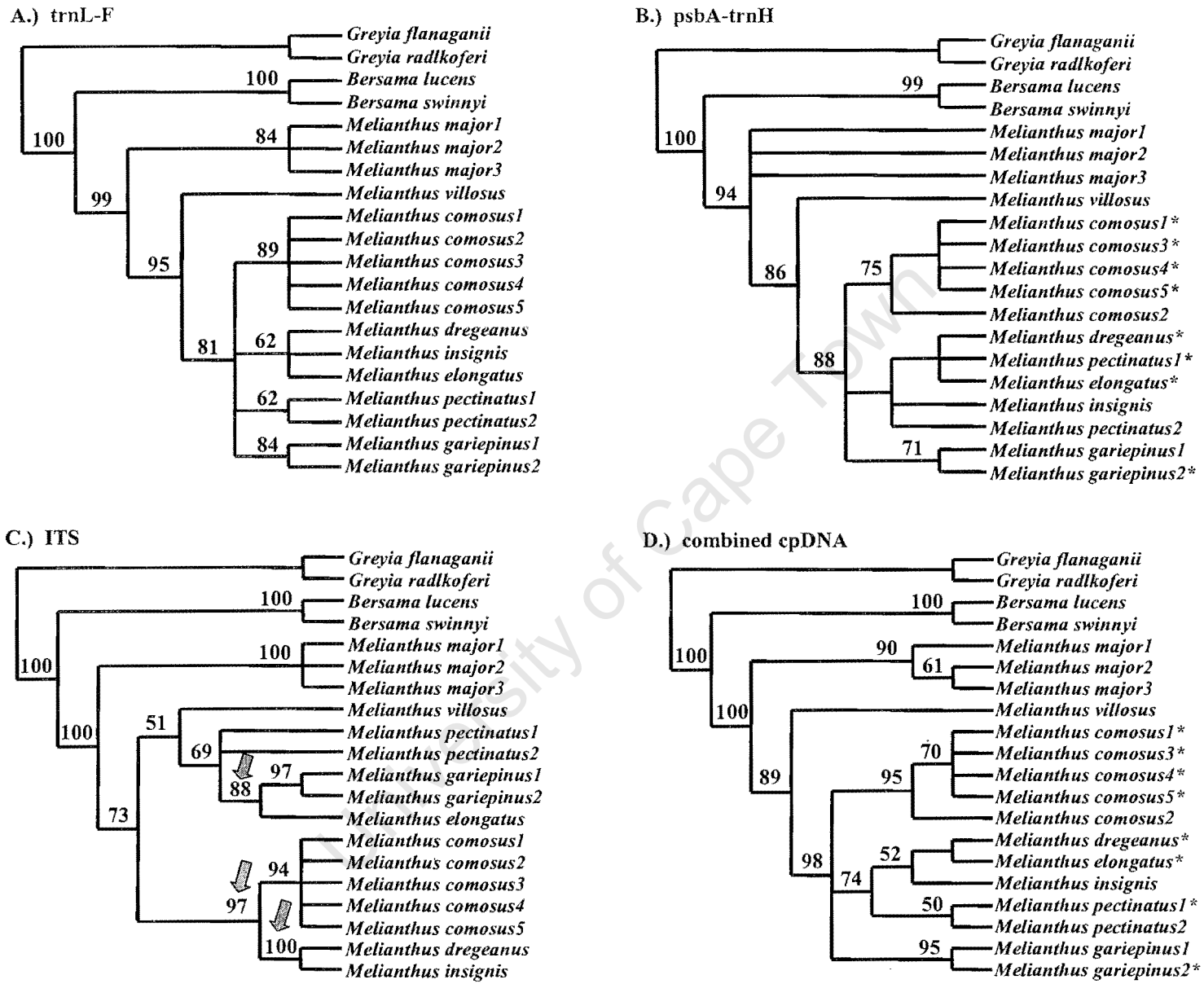
1.) *Trn*L-F creates a trichotomy made up of *M. elongatus*, *M. dregeanus* and *M. insignis* but this has poor support (62% bootstrap). In contrast, ITS identifies *M. elongatus* as the sister of *M. gariepinus* (88% bootstrap) and *M. dregeanus* plus *M. insignis* as the sister of *M. comosus* (97% bootstrap). *Psb*A-*trn*H splits the two *M. pectinatus* samples; one is included in a weakly supported

Table 2.6: Measurements of minimum to maximum sequence divergence based on pairwise distances in the molecular data partitions with the coded inversion excluded.

Region	Between *Melianthus* species	Between *Bersama* and *Melianthus*	Between *Greyia* and *Melianthus*
***trn*L-F**	0.00106 - 0.01176	0.01809 - 0.03071	0.04192 - 0.05432
***psb*A-*trn*H**	0.00452 - 0.04584	0.11682 - 0.13269	0.18843 - 0.23265
ITS	0.00410 - 0.01980	0.03636 - 0.08163	0.02924 - 0.13415
cpDNA	0.00170 - 0.01268	0.02160 - 0.04034	0.04057 - 0.07200
Complete molecular	0.00432 - 0.02043	0.05739 - 0.06852	0.10060 - 0.11011

Table 2.7: Statistical information describing tree produced by parsimony analyses of all data sets. CI=Consistency Index, RI=Retention Index, MP=most parsimonious. CI and RI values were calculated with uninformative characters excluded.

Data set	Parsimony-informative characters	No. of MP trees	Tree length	CI	RI	No. of nodes $\geq$ 85% bootstrap
***trn*L-F**	71	18	100	0.940	0.954	5
***psb*A-*trn*H**	78	5	106	0.972	0.978	5
ITS	180	18	269	0.892	0.922	9
cpDNA	149	3	207	0.952	0.963	8
Combined Molecular	329	2	483	0.905	0.928	10
Amended Molecular	91	1	443	0.926	0.783	6
Morphology	39	3	91	0.681	0.729	3
Amended Molecular/ Morphology	130	5	543	0.871	0.738	6



Figures 2.2A-D: Strict consensus of the most parsimonious trees for *Melianthus* and outgroups using PAUP* 4.0b10 analysis. A.) *trn*L-F data set, B.) *psb*A-*trn*H data set, C.) ITS data set, D.) combined cpDNA data set. Bootstrap support >50% is indicated above nodes. Taxa containing inversion indicated by *. Arrows in the ITS tree depict strongly supported nodes that are in conflict with weakly supported alternatives.

(<50% bootstrap) trichotomy along with *M. dregeanus* and *M. comosus* and the other occurs in a deeper trichotomy with the aforementioned group and *M. insignis*. The splitting effect can be directly attributed to the presence/absence scoring of the inversion despite *a priori* exclusion of the actual basepairs making up the inversion from the analysis. If the scoring for the inversion is removed from the analysis, the splitting of *M. pectinatus* is prevented, instead resolving *M. pectinatus*1 plus *M. pectinatus*2 as sisters in a collapsed polytomy with *M. elongatus*, *M. dregeanus* and *M. insignis*, (results not shown). The effect of scoring for the inversion also creates noise in the cpDNA analysis, linking *M. dregeanus* and *M. elongatus* as sister taxa but with less than 50% bootstrap support.

2.) The plastid markers handle the position of *M. villosus* differently from the nuclear marker. In both separate (*trn*L-F and *psb*A-*trn*H) and combined results (cpDNA), the plastid markers resolve *M. villosus* as sister to a clade comprising *M. comosus*, *M. dregeanus*, *M. elongatus, M. gariepinus, M. insignis* and *M. pectinatus* with good to strong support (81%, 88%, and 98% bootstrap respectively). In the fuller resolved ITS topology, *M. villosus* is instead placed as sister to the *M. pectinatus* complex plus *M. elongatus* but with weak support (51% bootstrap).

3.) The combined plastid analysis shows some resolution between the multi-sampled *M. major*, which is lacking in any single gene analysis.

Results from the Templeton test (Table 2.8) show neither the *psb*A-*trn*H nor the *trn*L-F data partitions as incongruent when constrained by the other single gene consensus topologies. Significant length increase does appear when the ITS data set is constrained by *trn*L-F (0.0008) and *psb*A-*trn*H (0.0001) topologies, which is directly attributed to the presence of several strongly supported nodes in the ITS tree (refer to Figure 2.2-C) that are contradicted by corresponding nodes in the plastid trees with weaker support. Since the results are consistently asymmetric, however, no real 'conflict' can be said to exist.

Pooling all genes into a combined molecular analysis results in the highest level of resolution and support (Figure 2.3). All the molecular analyses, whether separate or combined, are unambiguous in resolving *Melianthus* as a strongly supported monophyletic group, as does Dlamini's morphology.

The combined molecular analysis shows strong support for the basal position of *M. major* (98% bootstrap) as sister to the rest of *Melianthus*. The position of *M. villosus* as sister to a clade containing the KEC and WCC also receives good support (81%). Two monophyletic clades are resolved with good support (95% and 88% bootstrap), herewith referred to as the Karroid/Eastern clade (*M. comosus, M. dregeanus, M. insignis*) and the West Coast clade (*M. elongatus, M. gariepinus, M. pectinatus*). Within the Karroid/Eastern clade (KEC), *M. comosus* is resolved as sister to *M. dregeanus* plus *M. insignis* while the West Coast clade (WCC) contains a trichotomy comprising *M. pectinatus*1, *M. pectinatus*2 and a clade containing *M. elongatus* as sister to the two *M. gariepinus* samples. In both the cp DNA and combined topologies, *M. comosus*2 is consistently placed as sister to the remaining *M. comosus* samples and *M. major*1 is placed sister to *M. major*2 plus *M. major*3. The resolution of the multiple-sampled *M. major* and *M. comosus* unambiguously into exclusive clades in both the single gene and combined analyses (barring the exception of the unresolved *M. major* in the separate *psb*A-*trn*H topology), indicates geographic cohesion for these broader-distributed taxa, which were sampled throughout their range.

Molecular-Morphological Data

The amended molecular tree obtained from the reduction of terminals resulted in a topology identical to the combined molecular tree with one exception: the further resolution of *M. pectinatus*1 and *M. pectinatus*2 as monophyletic given moderate support (76% bootstrap).

Differences between the amended molecular and morphological trees relate to both the amount of resolution and topology (see Figure 2.4). The amended molecular topology resolves the *M. pectinatus* complex (comprising *M. pectinatus* and *M. gariepinus*) as paraphyletic by placing *M. elongatus* (62% bootstrap) as sister to *M. gariepinus*1 plus *M. gariepinus*2 with this clade in turn sister to *M. pectinatus*1 plus *M. pectinatus*2 (91% bootstrap). The morphological topology differs by showing no resolution among the samples representing the *M. pectinatus* complex, with *M.*

Table 2.8: Templeton WSR results testing for significant difference when the data partitions for each separate gene region are constrained by strict consensus topologies imposed from the other two gene analyses.

Data set	Tree length	No. of character changes	Z value	*p* value (*=significant)
***trn*L-F unconstrained**	100	-	-	-
-constrained by ITS strict consensus	105	4	-1.890	0.059
- constrained by *psb* strict consensus	102	2	-1.4142	0.500
ITS unconstrained	269	-	-	-
-constrained by *trn*L-F strict consensus	286	19	-3.3698	0.0008*
-constrained by *psb* strict consensus	305	25	-3.9951	0.0001*
***psb*A-*trn*H unconstrained**	106	-	-	-
-constrained by ITS strict consensus	111	4	-1.8889	0.0588
-constrained by *trn*L-F strict consensus	107	1	-1.0000	0.3173

Table 2.9: Templeton WSR results testing for significant difference in the amended molecular and morphological analyses when the data partition for one analysis is constrained by a strict consensus topology imposed from the other.

Data set	Tree length	No. of character changes	Z value	*p* value (*=significant)
Amended molecular unconstrained	443	-	-	-
-constrained by morphology consensus	450	11	-2.1106	0.0348*
Morphology unconstrained	95	-	-	-
-constrained by molecular consensus	100	8	-2.1213	0.0339*

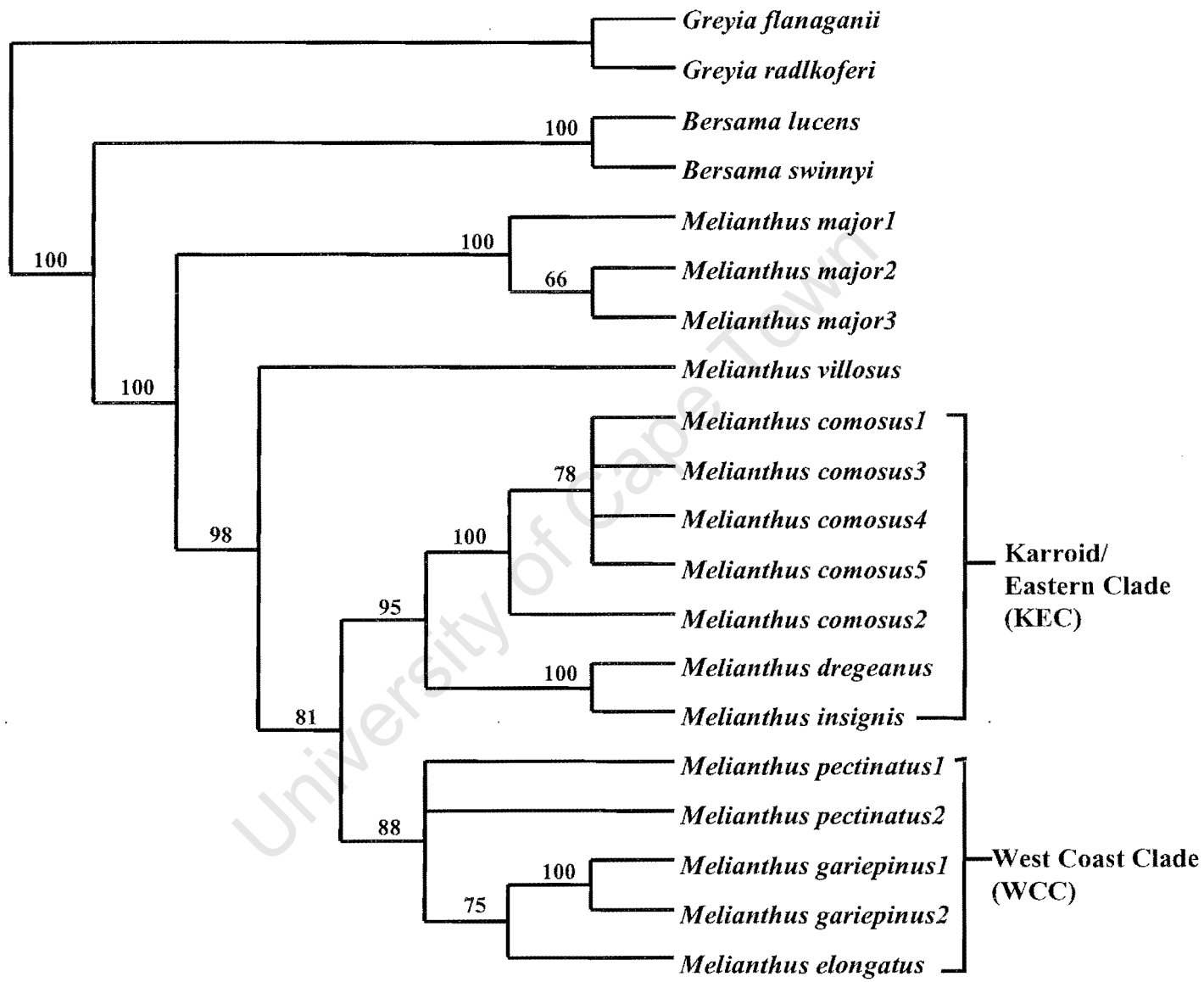
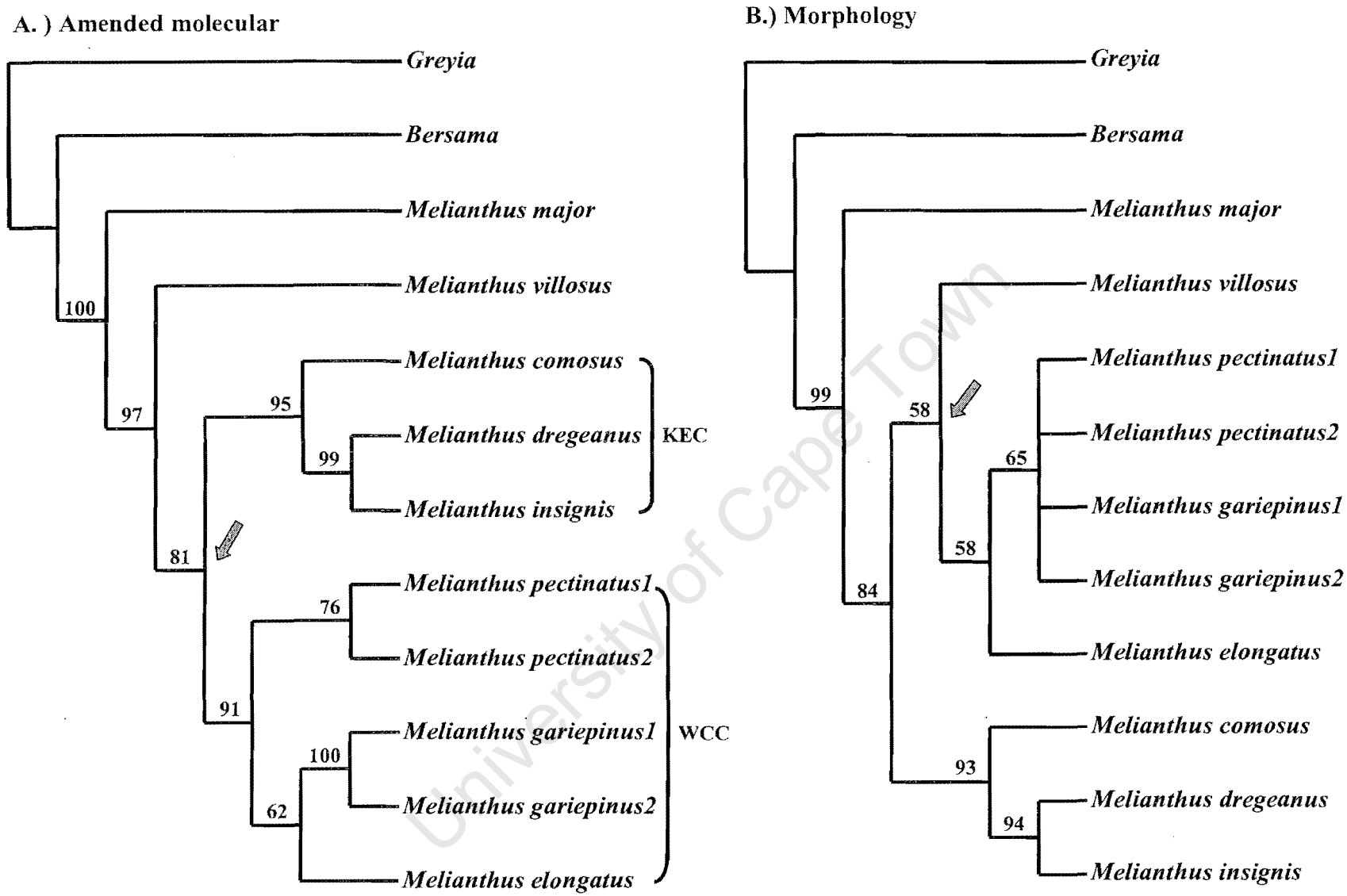


Figure 2.3: Strict consensus of combined molecular data set showing the greatest resolution and highest level of support of any of the analyses. Bootstrap support >50% is indicated above nodes.



Figures 2.4A-B: A.) Amended molecular and B.) morphological analyses, with arrows indicating conflict in the position of *M. villosus*. Bootstrap support >50% is indicated above nodes.

elongatus resolved as sister to the entire *M. pectinatus* complex (58% bootstrap). Admittedly, in both instances, the alternative relationships suggested for *M. elongatus* are weakly supported. Differences are also seen in the treatment of *M. villosus*. The amended molecular topology resolves *M. villosus* as sister to a clade comprising the KEC and the WCC with good support (81% bootstrap). The morphological topology, instead, is less decisive, weakly suggesting a placement of *M. villosus* as sister solely to the WCC (53% bootstrap).

Despite the slight differences between the amended molecular and morphology topologies, incongruence is indicated in the Templeton test (Table 2.9) with bidirectionally significant values showing reciprocal conflict. The result from the combination of the amended molecular and morphological data into a total evidence tree is shown in Figure 2.5. Resolution is poor, resulting in two polytomies: one comprising *M. villosus*, the KEC and the WCC, with the other polytomy made up of a largely unresolved WCC.

Character State Reconstruction

The results from character optimisation are shown in Table 2.10 with the main developments manually plotted on to the amended molecular topology seen in Figure 2.6. Several unequivocal characters separate the outgroups from the ingroup with the majority of phylogenetically informative changes occurring at the base of the tree. Pinnate leaves represent the ancestral condition for Melianthaceae with erect inflorescences bearing whorled to oppositely arranged flowers placed along the upper half of the raceme. The flowers are relatively actinomorphic bearing green sepals and elliptic-shaped, greenish-white petals as retained in the *Bersama* outgroup. *Melianthus*, as a derived ingroup, is characterized by dentate-serrate leaflets with a development towards highly zygomorphic flowers featuring enlarged sepals, dimorphic stamens and novel tan coloured nectar. The flowers are made up of modified narrowly elliptic petals conjoined along their inner edge by a fringe of intertwining hairs.

Several further synapomorphies readily distinguish the KEC from the WCC. Lateral racemes borne from the leaf axils typify the KEC, with the flowers arranged in an alternate fashion along the entire length of the peduncle. The flowers are characterised by reddish-orange coloured sepals with a basal maroon blotch. The WCC is instead characterised by a general retention of the symplesiomorphic erect

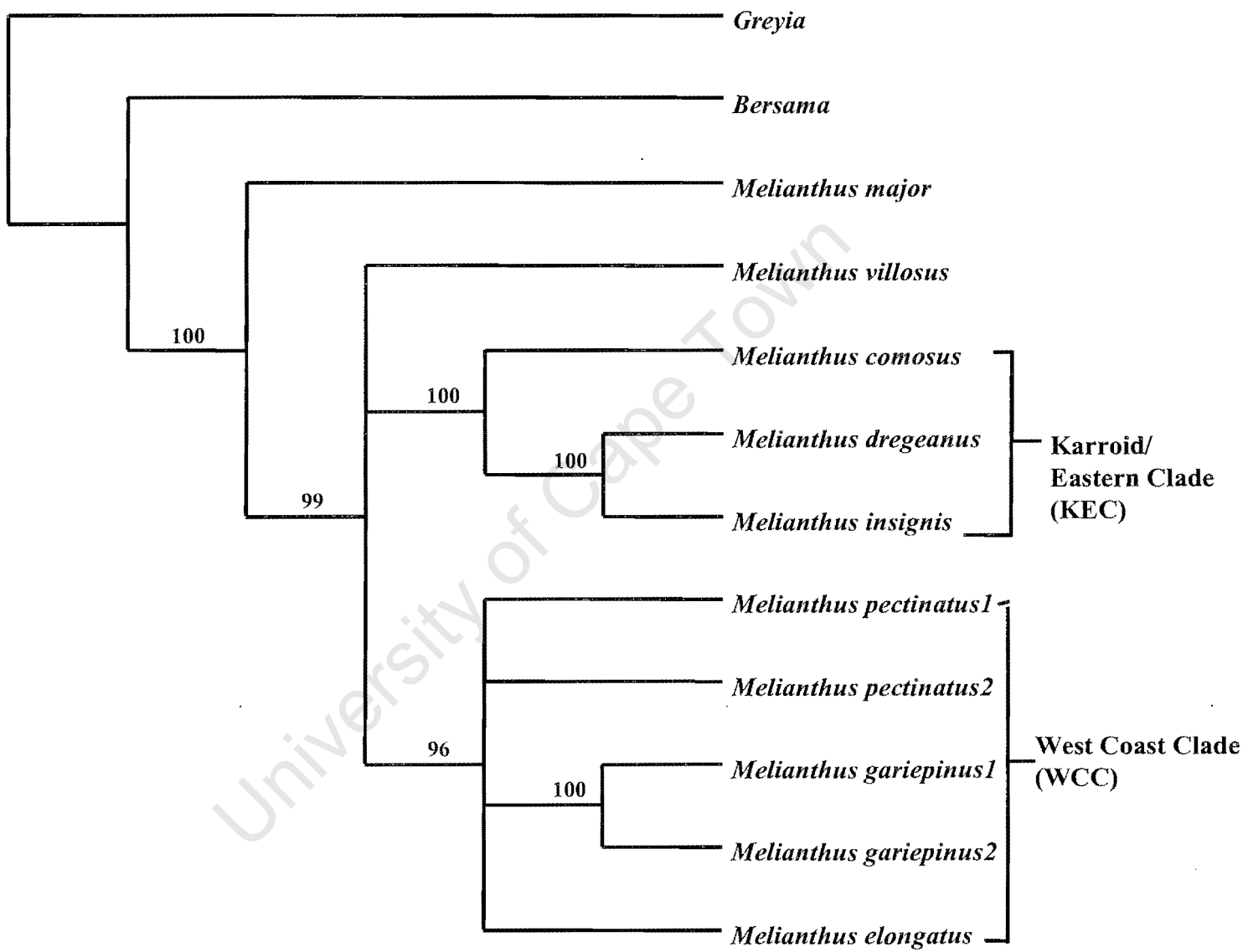


Figure 2.5: Topology resulting from amended molecular/morphological data showing poorer resolution. Bootstrap support >50% indicated above nodes.

Table 2.10: Complete listing of unequivocal character changes under both ACCTRAN and DELTRAN algorithms. Node numbers correspond to Figure 2.6. . Nodes listed as * lack unambiguous synapomorphies.

Node 1 (ancestral conditions in Melianthacae):	-Imparipinnate foliage, stipules present, foliage without odour. -Erect inflorescence, flowers opposite or in whorls, in upper half of raceme, sepals green, petals free, petals larger than adaxial sepal, elliptically shaped, greenish/white in colour, nectary attached to all sepals, clear nectar. -Monomorphic stamens. -Fruit membranous, acutely four-winged, 2 ovules per locule, no aril present on seeds. -Styloids present.
→*Bersama*:	-Foliage without odour. -Fruit as woody capsule, unilocular, aril present.
Node 1→2 (emergence of *Melianthus*):	-Dentate leaf margin, foliage malodorous. -Flower irregular, odd sepal saccate, petals smaller than adaxial sepal, woolly crystalline hairs present, petals narrowly-elliptic, glandular claw, nectary attached to adaxial sepal, nectar tan. -Dimorphic stamens -More than two ovules per locule.
Node 2→*Melianthus major*:	-Glabrous leaf, solitary stipules. -Sepals green/maroon, lateral sepals falcate, petals red/maroon. -2 ovules per locule.
Node 2→3: Node 3→*Melianthus villosus*:	-Leaf margins serrate -Leaf villous. -Petals greenish/white and orange. -Nectar black.
Node 3→ 4:	*
Node 4→7 KEC:	-Nodding inflorescence, axillary, flowers alternate, flowers over entire length of peduncle, sepals orange, outer sepals with a dark mark, petals red/maroon. -Fruit with rounded lobes, 2 ovules per locule.
Node 7→ *Melianthus comosus*: **Node 7→ 8** **Node 8→ *Melianthus dregeanus***:	-Black nectar. -Leathery fruits. -Nectary attached to lateral sepals.
Node 4→5 WCC:	-Oval-shaped stipules. 'Crown' of petals, petals larger than adaxial sepal, petals red/orange. -Acutely lobed fruit.
Node 5→6 *Melianthus elongatus*:	-Nectary attached to lateral sepals, nectar black. -Fruit villous and woody, with rounded lobes.
Node 6→10 *Melianthus gariepinus*: **Node 9→ *Melianthus pectinatus*1**:	-2 ovules per locule -Entire leaflet, ericoid.

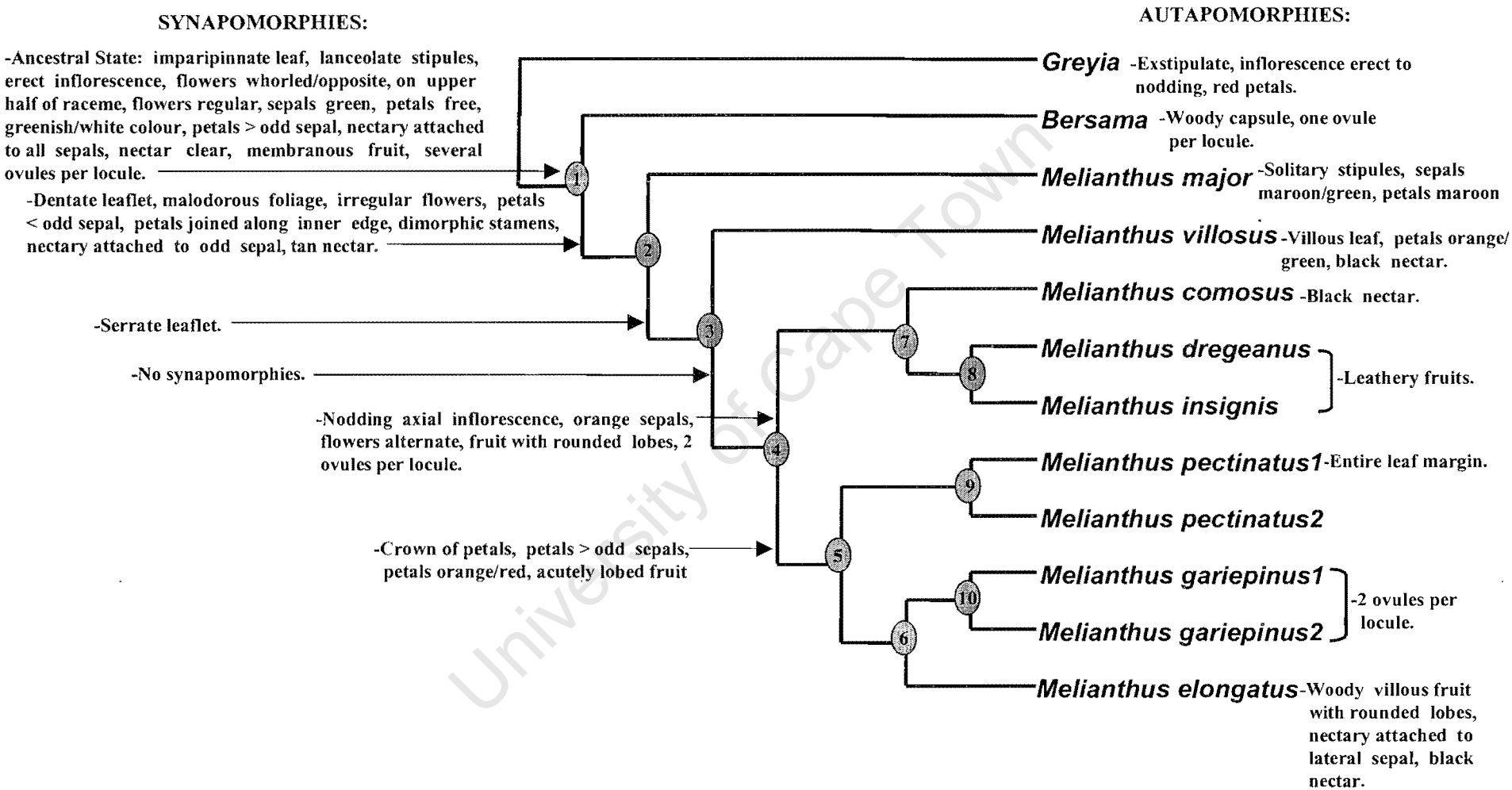


Figure 2.6: Amended molecular tree showing the emergence of major ancestral and derived morphological characters distinguishing terminals and clades. Character changes shown are unequivocal being resolved under both ACCTRAN and DELTRAN parsimony. Node numbers are listed inside '⓪' and correspond with Table 2.10, which lists the complete set of character changes.

flowered raceme with the whorled to opposite floral arrangement and green-coloured sepals. Distinguishing synapomorphies, however, are seen in the development of larger petals coloured variably orangish-red with the racemes topped by a concentrated crown of brighter red petals.

Under parsimony, optimisation of nectar colouration indicates three autapomorphic gains of black nectar. Although suboptimal, a single gain of black nectar with three reversals to tan nectar would only require one extra step.

2.4 Discussion

Previous molecular work involving *Melianthus major* sequences (Chase *et al.* 1993, Nickrent and Soltis 1995, Soltis and Soltis 1997, Soltis *et al.* 1997, APG 1998, Savolainen *et al.* 2000, Soltis *et al.* 2000) was based on conserved gene regions for addressing questions of high-level familial relationships. Inter- and infra-specific relationships within the genus itself were previously speculated on morphology alone. All the varying analyses done in this study, whether molecular or morphological, done singly or in combination, reaffirm Dlamini's (unpublished 1999) proposal of monophyly for *Melianthus*.

Choosing a Topology

Givnish and Sytsma (1997) report morphological data as more likely to 'corrupt' a precise analysis of detailed relationships than molecular data although excluding morphological characters may reflect a preconceived bias (Lee 1997). Hedges and Maxson (1996, 1997) argue otherwise and conclude the problem lies in the inequality of both types of data for dealing effectively with phylogenetic history. When differences exist between varying analyses, choosing an unequivocal topology backed by the most convincing support seems warranted.

The smaller number of phylogenetically informative characters in the morphology analysis results in a shorter tree with poorer support than that resolved by the molecular analyses. As well, the lower CI and RI values indicate more homoplasy capable of masking the true phylogeny (Givnish and Sytsma 1997, Kitching *et al.* 1998, Asmussen and Chase 2001). The asymmetrical incongruence between the varying molecular topologies due to the stronger support of the ITS data set can be viewed as 'soft' (Seelanan *et al.* 1997) and offers no deterrence to the combination of the gene partitions. Although conflict between the combined molecular topology and the morphological topology is barely significant, a total evidence aproach results in

uninformative polytomies. Therefore, the combined molecular data set by itself provides the most conclusive window into *Melianthus* speciation. Its higher probability for an accurate phylogenetic portrayal (Cunningham 1997, Givnish and Sytsma 1997) is supported by a greater number of independent parsimony informative characters, a longer tree length sporting high CI and RI values, and the resolution of more highly supported clades (10 clades ≥85% bootstrap). The placement of *M. villosus* as sister to a clade containing the KEC and WCC in the combined molecular topology is indicated with good support (81% bootstrap) whereas its placement is more equivocal in the morphology topology.

Testing hypotheses of evolution and relationship depends on the accuracy of the phylogeny itself (Baum and Larson 1991). In this study, character evolution was evaluated in terms of the better-resolved molecular topology since the presence of polytomies in the total evidence approach would have affected character optimisation. A polytomy in the weaker supported morphological topology would have also been problematic. The morphological topology provides an alternative albeit weaker hypothesis for the position of *M. villosus*, placing it as sister to the WCC. However, a lack of synapomorphies between the WCC and KEC offer little support for the placement. Unless stated otherwise, it is upon the combined molecular topology (refer to Figure 2.3) that the following discussed is based.

Relationships and Character Evolution

The divergence from a putative Melianthaceae ancestor gave rise to two lineages terminating in *Bersama* and *Melianthus*. Another split resulted in a branch terminating in *M. major*, as sister to the rest of *Melianthus*. From the next speciation event, the rather similar looking *M. villosus* arose as sister to a pair of well-defined groups, the Karroid/Eastern clade (KEC) and the West Coast clade (WCC). Both clades are morphologically and geographically cohesive.

Ancestral character state reconstruction suggests the ancestral condition for Melianthaceae consists of stipulate plants bearing pinnate foliage and erect inflorescences with whorled to oppositely arranged flowers placed along the upper half of the raceme. The flowers are relatively actinomorphic in appearance, unfused, with green sepals and elliptic-shaped, greenish-white petals. Flowers are 5-merous with the petal size larger than the adaxial (i.e. 'odd') sepal (Dlamini unpublished 1999). The nectary, which is attached to the base of all of the sepals, produces

colourless nectar. Membranous fruits are characterised by several ovules per locule and produce arilless seeds. This ancestral condition has been largely retained in most *Bersama* species with slight modifications such as woody fruits, a reduction to one ovule per locule and arillate seeds (Palgraves 2002).

Although the symplesiomorphic erect raceme is well represented within *Melianthus*, the individual flower dramatically diverged from *Bersama*. Notable changes include exaggerated developments in zygomorphy and colour differentiation of the sepals, petals and nectar. The changes in floral colouration can be positively correlated with adaptations to ornithophily (Vogel 1954, Skead 1967, Rebelo 1987, Proctor *et al.* 1996, Cheke *et al.* 2001) but the role of colouration in nectar, given the rarity of its occurrence (Olesen *et al.* 1998), is less clear. Tan coloured nectar represents the ancestral condition for *Melianthus*, as seen in *M. major*. As suggested by parsimony reconstruction, black nectar has arisen on three separate occasions in homoplasious fashion, in *M. villosus*, *M. comosus* and *M. elongatus*, with tan nectar retained in the *M. dregeanus* complex (*M. dregeanus*, *M. insignis*) and the *M. pectinatus* complex (*M. gariepinus*, *M. pectinatus*). Although a logical assumption, parsimony may not necessarily reflect how evolution takes place (Thorne 1996, Givnish 1997); therefore it is also worth considering the slightly less optimal notion that black nectar arose but a single time with three subsequent reversals to tan nectar.

Several autapomorphies distinguish *M. major* such as bold, glacous foliage and a dramatically enlarged, solitary stipule from a fusion of the two distinct stipules that otherwise typify the genus. *Melianthus villosus* most closely resembles *M. major* in overall size and appearance despite minor changes in floral colouration (see Table 2.3) and a villous covering for the leaves.

The WCC and the KEC are distinguished by further novelties in floral development (Phillips and Hofmeyer 1927b). The symplesiomorphic erect inflorescences of the WCC are borne from a terminal/subterminal position and generally held above the foliage, the flowers characterised by an increase in petal size and colouration. In contrast, the KEC features a unique shift in inflorescence position, placing the racemes in an axial position beneath the foliage in a lateral orientation. The WCC species retain an ancestral floral arrangement with the flowers whorled or opposite one another in the upper half of the raceme while the KEC shows a more derived condition with flowers alternating over the entire length of the lateral raceme from base to tip.

The KEC is comprised of three morphologically similar-looking species: *M. comosus* and the *M. dregeanus* complex (comprising *M. dregeanus* and *M. insignis*). Synapomorphies of reddish-orange coloured sepals with green-flushed apices and a basal maroon blotch unite the three, forming a polychromatic floral combination indicative of Faegri and van der Pijl's (1979) 'parrot colouration'. All three taxa show a reduction in number of ovules per locule to two. Black nectar and membranous inflated fruits distinguish *Melianthus comosus* from the tan nectar and derived leathery-textured fruits of the *M. dregeanus* complex. Superficially, an overall greater size difference further distinguishes the *M. dregeanus* complex, which is most pronounced in *M. insignis* (Phillips and Hofmeyer 1927b). Of the three taxa, the attachment of the nectary to the lateral sepals is unique to *M. dregeanus* (Dyer 1952, 1959, Dlamini 1999 unpublished) and differs both from *M. insignis* and *M. comosus* where the nectary is instead attached to the odd sepal.

The WCC is made up of *M. elongatus* and the *M. pectinatus* complex, comprising *M. gariepinus* and *M. pectinatus*. The flowers of the WCC are characterised by the synapomorphies of large recurved petals coloured variably orangish-red, with the distal end of the raceme sporting a congested 'crown' of closely-spaced, showier, brighter red flowers. Petal size increases up to two times the length of the odd sepal whereas in all other species the sepals are always more prominent. Fruiting structures are more useful for delineating the species within the WCC. *Melianthus elongatus* can be readily distinguished by its rounded fruits, which feature a derived villous covering, compared to the acutely lobed, nearly cross-shaped, smooth fruits of the *M. pectinatus* complex. Within the *M. pectinatus* complex, number of ovules per locule further distinguishes *Melianthus pectinatus* (more than two per locule) from *M. garipienus* (two per locule). Shape and number of leaflets were previously used to differentiate the *M. pectinatus* subspecies (Merxmüller and Roessler 1968) although Tansley (1983 unpublished) felt the characteristics were of questionable use since they nearly approximate clinal gradations. Nevertheless, the presence of a reduced leaflet with an entire, highly revolute edge seems unique to *M. pectinatus*1 (syn. '*M. trimeneanus* Hook.').

Species Status

Indications of monophyly for the multi-sampled *M. comosus* warrants mention since the widespread status of the species is unique in the genus, all other species

being relatively- to highly- localised. Sampling methods attempted to include representation from across the range of *M. comosus*, from Namibia to eastern, western and southern South Africa, their resolution, therefore, suggesting surprising cohesiveness in the species. Early divergence of the single sample from Namibia might indicate the onset of peripheral speciation although further sampling would be necessary to show the result is not artifactual. Although less widespread, relative cohesiveness was also shown in *M. major* based on three morphologically variable forms sampled from its northern, central range and eastern range.

Current circumscription (Leistner 2000) considers *M. insignis* as a subspecies of a broadly defined *M. dregeanus* complex following Tansley and Schelpe's treatment (1984), a proposal questioned most recently by Dlamini (unpublished 1999) who considers the two separate species, as did Dyer before him (1952, 1959). Despite being resolved as sister taxa by all the combined analyses, the infra-specific questions of mutual exclusiveness between *M. dregeanus* and *M. insignis* cannot be conclusively addressed due to the low sampling methods employed in this study.

A similar situation exists over the appropriateness of subspecific rank in the *M. pectinatus* complex. The position of *M. gariepinus* as sister to *M. elongatus* (75% bootstrap) raises doubts about the status of the *M. pectinatus* complex as currently defined. The latter situation could be dealt in one of three ways. Firstly, a highly conservative move would be to sink all three taxa into a single monophyletic species. This seems questionable given the distinction between *M. elongatus* and the *M. pectinatus* complex based on fruiting structure. Secondly, the present circumscription with two specific names could be maintained by considering the *M. pectinatus* complex as a 'paraspecies' (Crisp and Chandler 1996), a concept some systematists might find troublesome. Thirdly, Merxmüller and Roessler's (1968) original proposal, which afforded specific rank to subspecies *gariepinus*, could be reinstated. This latter option is worth investigating further. The few specimens of *M. gariepinus* seen in the field for this study, (near Rosh Pinah, Namibia), appeared completely distinct from *M. pectinatus* in both leaf and floral morphology. However, Archer's (1997) description and illustration of *M. gariepinus* more closely approximates *M. pectinatus* in appearance than anything seen by this author. The possibility exists, therefore, that *M. garipienus* is a very 'plastic' taxon exhibiting an enormous amount of clinal variation, possibly the result of varying degrees of aridity in its local microenvironment?

Subspecific ranking within the *M. pectinatus* complex (Tansley 1983 unpublished, Tansley and Schelpe 1984) was previously assigned on the grounds of 'disjunct distribution' with *M. gariepinus* reputedly restricted to an area north of the Orange River. This assumption has since been falsified with recent collections reported south of the river from the Richtersveld (Archer 1997, Dlamini unpublished 1999). Werger (1978) also questioned the perception of the Orange River as a true 'barrier' given the uniformity of vegetation found on either side of it. With the exceptional variation inherent in subspecies *gariepinus*, a more methodical morphological examination and subsequent molecular sequencing of populations from both sides of the river would be recommended.

2.5 Conclusions

Monophyly is confirmed for *Melianthus*. Given the distinctiveness of the genus, previous phylogenies based on morphology convey a similar underlying history as molecular phylogenies save for the placement of *M. villosus* and relationships within the *M. pectinatus* complex. The molecular phylogenetic estimate seems more robust in its support and resolution, especially concerning the position of *M. villosus*, thereby suggesting it would provide the more reliable hypothesis for studying evolutionary patterns in the genus. The emergence of two clades, a West Coast clade comprising *M. elongatus*, *M. gariepinus* and *M. pectinatus* and a Karroid/East Coast clade comprising *M. comosus, M dregeanus* and *M. insignis*, accords with distributional ranges and makes geographical sense with each clade distinguishable by a set of synapomorphies. Further sampling of *M. comosus* may be warranted to confirm the early divergence of the Namibian populations while a more detailed sampling of the *M. pectinatus* complex, specifically subspecies *gariepinus*, seems warranted for better understanding of its subspecific ranking. If this latter task is undertaken, perhaps Dlamini's (unpublished 1999) proposal of seven species for *Melianthus* will be countered by a change to eight?

Chapter 3: Biogeography and Diversification in *Melianthus* L.

3.1 Introduction

Cause and Effect?

Baum and Larson (1991): "A central axiom of the theory of natural selection is that evolutionary change is not random. Differential survival and reproduction of varying organisms is caused by the interaction between their heritable character variation and the environment. Different environments may favour the fixation of different traits."

Melianthus is a small African endemic genus centred in South Africa. Most species are localised to highly localised in their distributions (Dlamini unpublished 1999), the sole exception being the widespread *M. comosus*, which ranges across the border into Namibia and possibly Botswana. The majority of species are concentrated in the Northern and Western Cape with the genus exhibiting a high amount of morphological diversity given it modest size.

Lacking any fossil record, the origins of *Melianthus* are unclear. Likewise unknown are the circumstances and the time frame in which speciation may have occurred. Major clades and species can be readily distinguished by differences in plant size, leaf morphology, inflorescence orientation, floral colouration and flowering phenology. Being able to place the emergence of such traits within a spatio-temporal framework (Bakker *et al.* 1999, Richardson *et al.* 2001) may afford a better understanding of the evolution of the genus.

Ecoclimatic Effects

A review of the geologic history of southern Africa is necessary for any interpretation of the diversification of *Melianthus*. However, paleoclimatic history for the subcontintent tends to be patchy and in places is not clearly understood (Axelrod and Raven 1978, Werger 1978, Siesser 1980, Siesser and Dingle 1981, Deacon *et al.* 1992, Linder *et al.* 1992, Low and Rebelo 1996, Bakker *et al.* 1999, Meadows and Watkeys 1999, Richardson *et al.* 2001, Linder in press). Although early mountain formation in Southern Africa dates back to the Paleozoic (Deacon *et al.* 1992), much of the present landscape and climate of southern Africa can be attributed to disruption of Gondwanaland at the end of the Jurassic (Axelrod and Raven 1978).

Approximately 155 million years ago (mya), continental drift pushed West Gondwanaland (South America, Africa, Arabia) past East Gondwanaland (Madagascar, India, Antarctica, Australia) forcing the earth's major land blocks into new positions (Meadows and Watkeys 1999). This coincided with a rise in sea levels from displacement caused by the development of submerged mid-oceanic ridges, which presumably affected oceanic currents and influenced long-term climatic patterns (Meadows and Watkeys 1999). Nevertheless, the climate stayed relatively mild, humid and mesic all the way through the Paleocene and into the Eocene when change started to occur at the boundary with the Oligocene Era (Werger 1978, Dingle *et al.* 1983).

Residual effects from Gondwanaland fragmentation caused the final separation of Antarctica from South America approximately 30-35 mya, which was marked by significant global temperature declines (Dingle *et al.* 1983). This resulted in a cooling of the southern oceans detectable in detailed oxygen-isotope analyses of oceanic sediments (Shackleton 1986 as cited by Meadows and Watkeys 1999). The cooling promoted formation of the Antarctic Ice Cap during the early Oligocene (Werger 1978, Low and Rebelo 1996) resulting in dramatically lowered sea levels. Falling seas eventually impacted continental aridity (Haq *et al.* 1987) with dry summer conditions temporarily developing along the southwest of Africa (Linder in press). Although temperatures and rainfall started to increase globally by the late Oligocene, the recovery in southern Africa was mired by a growing trend towards amplified seasonality (Low and Rebelo 1996, Meadows and Watkeys 1999).

The Antarctic Ice Cap regained a secondary prominence in the mid-Miocene approximately 10 mya resulting in another sea level drop (Siesser and Dingle 1981) as temperatures declined worldwide (Low and Rebelo 1996, Meadows and Watkeys 1999). The upwelling of cold (Benguela) water off the western coast of southern Africa from 7-10 mya (Siesser 1980, Low and Rebelo 1996) coupled with localised high-pressure cells (Linder in press) are suggested to have caused the aridification of southwestern Africa, which reached its maximum aridity around 3 mya (Siesser 1980, Low and Rebelo 1996). Within the same area, a noticeable rainfall shift from a variably summer wet pattern to a consistently winter wet 'mediterranean' regime occurred approximately $\pm$5 mya (Linder *et al.* 1992, Bakker *et al.* 1999, Linder in press).

At a similar time, the warm Agulhas Current influenced the perpetuation of a subtropical climate along the east coast of South Africa from KwaZulu/Natal northwards with hot humid wet summers following mild dry winters (Axelrod and Raven 1978). The Eastern Cape marks a zone of transitional bimodality both in rainfall patterns and temperature extremes, the mild winters and summer rains of the easterly areas giving way to a near year-round rainfall season and cooler winters in the southwest (Deacon *et al.* 1992).

Further climatic degradation came from a great uplift in the interior plateau and its associated mountain ranges that started around the Miocene-Pliocene boundary (Werger 1978, Linder in press). The uplift was greatest in the east causing a westward tilt of the central plateau (Meadows and Watkeys 1999). Much of the interior of the country was subsequently left increasingly arid as clouds coming off the Indian Ocean shed the majority of their precipitation windward along coastal basins before moving inland (Linder in press).

Climate change has the potential to promote extinction and thus provide opportunities for the evolution of novel species (Stebbins 1952). In South Africa, increased aridity during the Cretaceous and Tertiary times are thought to have forced sub-tropical elements and rain-forest relicts from the older more mesic times to either retreat to equatorial positions or face extinction (Partridge 1997). It is possible that newly opened niches left room for a massive radiation of speciation by certain taxa able to adapt to the increasing aridity (Bakker *et al.* 1999) which paved the way for the emergence of the succulent vegetation of Namaqualand and the karroid scrubland of the interior plateau (Meadows and Watkeys 1999). Certain relictual taxa may have been able to persist in reduced 'refuge' spots indicative of former habitats (Linder *et al.* 1992). Examples of possible refugia from seasonal drought and aridity in and around southern Africa include oceanic islands, mountains, temperate coastal rainforest and riverbanks (Richardson *et al.* 2001).

In the context of this historical scheme of events, and noting the concentration of *Melianthus* diversity in the western part of South Africa, it is possible to hypothesis:

1.) *Melianthus* is ancestrally associated with a moist, aseasonal rainfall environment.

2.) Diversification of the genus coincided with the cooling and aridification events accompanying Antarctic Ice Cap formation either in the early Oligocene, the mid-to late-Miocene, or both.

Other factors that could have influenced spatial distributions in *Melianthus* are the historical role played by pollinators. Stebbins (1952) suggested vegetative differentiation is most often driven by ecological factors while pollinator preference is thought to influence floral divergence (see Johnson 1996). Pollination history might be a relevant factor in explaining floral divergence within the Melianthaceae since *Melianthus* is purportedly sunbird-pollinated (Scott-Elliot 1890, Von Marilaun 1895, Burtt Davey 1932, Vogel 1954) whereas *Bersama* is insect-pollinated (Phillips 1921, Verdcourt 1956b). Dlamini (unpublished 1999), influenced by Johnson (1996), suggested that interspecific floral differentiation within *Melianthus* might have been promoted by selection from unique pollinators specific within each species' range. This raises a third hypothesis:

3.) Pollinators were more important in driving the floral morphology that distinguishes species and clades in *Melianthus* whereas ecological factors influenced vegetative differentiation.

In order to evaluate these hypotheses, this study sets out to estimate the timing of speciation events in *Melianthus*, to determine its centre of origin and to establish the paleo-ecological changes accompanying its differentiation. The results of this will help to place the diversification of *Melianthus* species and clades within an eco-historical context.

Estimating Temporal Diversification Patterns

In the absence of dateable fossils, an alternative method for investigating temporal patterns of diversification in *Melianthus* uses phylogenetic trees, which provide an 'indirect' record of speciation (Barraclough and Nee 2001). Zuckerkandl and Pauling (1965) were the first to propose a means of dating evolutionary events by using calibrated differences in protein sequences. Ohta and Kimura (1971) followed with the proposal that most nucleotide substitutions in protein coding sequences are not functionally constrained and therefore accumulate at a relatively uniform rate (Bromham *et al.* 1999). The premise of neutrality added an invaluable theoretical underpinning for calculating divergence times based on DNA sequences in a method that soon became known as the 'molecular clock' (Freeman and Herron 2001).

Although subsequent evidence for variation in mutation rate (Gaut *et al.* 1993, Hillis *et al.* 1996, Sanderson 1997, 1998) appears to undermine the credibility of the molecular clock, Qiu *et al.* (1999) recently suggested otherwise. They hypothesize that problems of rate heterogeneity should be lessened due to the current practice of using combined data sets, which potentially alleviate unusual rate dynamics found in any single gene region. Even when likelihood models reject the assumption of rate constancy, methods such as non-parametric rate smoothing (NPRS) (Sanderson 1997, 1998) and semi-parametric rate smoothing (SPRS) (Sanderson 2002) have been proposed to circumvent the problem. NPRS allows for rate variation but assumes such changes are autocorrelated and inherited from an ancestral lineage through immediate descendants. SPRS, using a penalized likelihood model, was recently proposed since parametric methods are typically more statistically powerful than NPRS with absolute rate calculations.

Although the fossil record is typically fragmentary (Wray 2001), reference nodes for rate calibration are most commonly based on fossils or biogeographic events, which need careful placement (Baldwin and Sanderson 1998, Richardson *et al.* 2001). Higher-level clock analyses (Bremer 2000, Sanderson and Doyle 2001, Wikström *et al.* 2001, Bremer 2002) can also be used in the absence of suitable fossil evidence although one risks compounded error in the event of poor calculations. Despite potential problems with the molecular clock, Bromham *et al.* (1999) counter criticism by stating, "a sloppy clock is better than no clock". Imprecision, whether real or perceived, cannot deny the utility of clock-like evaluations in evolutionary biology, particularly in cases such as *Melianthus* where fossil evidence is lacking.

Reconstructing Ancestral Environments and Distributions

The classic adaptive radiation model (Simpson 1953) implies a direct response to a novel ecological or geographical setting will stimulate phylogenetic diversification. Such a model is thought to be a prime factor in explaining much of the flora of South Africa, in particular, the unique species-richness of the Cape Flora (Linder 1985). Assessing the biology of an ancestor relative to the historical environment in which it arose can help identify an "evolutionary point of origin" (Brooks and McLennan 1991), useful for understanding the 'how' and the 'why' of key traits (Givnish 1997). Many of the niches *Melianthus* favours are characterised by a combination of ecological variables unique to a specific location. Studying the

historical shifts of the genus, (as implied by phylogenies), in relation to reconstructed environments based on present day distribution patterns may shed light on the morphological strategies influenced by localised ancestral climates.

Distributional ranges may be established through dispersal or vicariance. Ronquist (1997) points out that impenetrable barriers in vicariant disruption seldom appear with sudden immediacy but more realistically build and subside gradually through the course of time. Vicariance also assumes the ancestral species were somehow more widespread than their descendants, which is rendered an "implausible paradox" by Bremer (1992). Suspected limitations can be countered by allowing for a more realistic model involving multiple dispersal events, each with a unique history (Ronquist 1997). 'Peripheral speciation' (Crisp and Chandler 1996) should also be taken into consideration since even without active vicariance events, gene flow will be limited across a species range (Ehrlich and Raven 1969) at both the 'margins' of its true geographical edge or in micro-environmental subtleties found there within (Van Valen 1976, Andersson 1990, Levin 1993).

3.2 Materials and Methods

Lineage Dating

For purposes of nodal dating, rate heterogeneity tests were employed to determine the applicability of a molecular clock model (Kimura 1980) to the full three-gene data set. Determination of the optimum model of sequence evolution was done using Modeltest 3.06 (Posada 2001) and PAUP* 4.0b10 (Swofford 1998) following a routine that compares 56 progressively more complicated evolutionary models. For this purpose one of the most parsimonious trees obtained from the combined molecular analysis in Chapter 2 was used as a framework for parameter estimation.

Once established, model parameters were fixed and used to test for a molecular clock assumption using maximum likelihood as implemented in PAUP* . A heuristic search was conducted using a random addition sequence with 10 replicates using TBR branch swapping with the Multrees option in effect. A double log likelihood ratio test (LRT) (Huelsenbeck and Rannala 1997) was used to evaluate whether molecular clock constrained trees, with a mid-point rooting, fit the data significantly worse than unconstrained trees. If no significant difference is detectable, the null hypothesis of rate constancy is accepted.

To calculate mean and standard error time estimates for each node, a bootstrap resampling procedure was used (Baldwin and Sanderson 1998) as originally laid out by Efron and Tibshirani (1993). For this purpose, 100 replicates were generated using the SEQBOOT programme within PHYLIP 3.6a3 (Felsenstein 2002) and evaluated in PAUP* under the optimal likelihood model. This yielded 100 length estimates for each branch from which a mean value ± two standard errors (±2SE) were calculated.

In the absence of fossil evidence for *Melianthus*, calibrating dates were obtained from Wikström *et al.* (2001), who used non-parametric-rate-smoothing and a three-gene data set (*rbc*L, *apt*B and nuclear 18S rDNA) to estimate the emergence of 560 angiosperm genera. Wikström *et al.* (2001) estimated branch lengths using three approaches: ACCTRAN and DELTRAN parsimony models and a maximum likelihood method. Since DELTRAN results consistently produced intermediate values for all 560 genera, date estimations were considered from ML and ACCTRAN results only, which suggested the divergence of Greyiaceae and Melianthaceae from putative ancestors occurred approximately 59 mya (ML) to 67 mya (ACCTRAN). Using these dates as a reference for calibration, branching times were thereby calculated for each subsequent node.

Ancestral Reconstructions

Putative ancestral distribution ranges were calculated using dispersal-vicariance analysis as implemented in DIVA 1.1 (Ronquist 1996). Reconstructions were done using the combined molecular topology pared down to 10 representatives, one for each *Melianthus* taxon and a composite for both *Bersama* and *Greyia*. Under dispersal-vicariance analysis, ancestral distributions are inferred from a three-dimensional cost matrix derived from a simple biogeographic model. Speciation is assumed to subdivide the ranges of widespread species into vicariant components with the optimal ancestral distributions considered those that minimize the number of implied dispersal and extinction events (Ronquist 1997).

Species distributions were based on herbarium records (BOL) and locality data from Dlamini (1999 unpublished) and Palgraves (2002) with a focus on the southern African subregion where *Melianthus* occurs. Areas used as units of analysis are politically defined, being the nine provinces of South Africa: Northern Cape, Western Cape, North West Province, Free State, Eastern Cape, Gauteng, KwaZulu/Natal, Limpopo and Mpumulanga, and four surrounding countries: Namibia, Botswana,

Lesotho, and Swaziland. To test the effect of alternative 'max area' settings, the analyses were repeated with 'max area' set at 10, 5, 3 and 2. Species were coded simply as present or absent in each area for ease of analysis.

In order to investigate historical changes in key ecological parameters, these were coded for extant taxa based on present distribution and reconstructed using parsimony. Parameters investigated and their unit states are listed in Table 3.1. Ecological profiles for extant taxa were inferred from the *South African Atlas of Agrohydrology and Climatology* (Schulze *et al.* 1997) based on current distribution ranges. Ecological parameters were treated as continuous or unordered discrete characters and optimised onto the pared down molecular topology using square-change parsimony (continuous characters) or Wagner parsimony (discrete unordered characters) as implemented in Maclade 4.0 (Maddison and Maddison 2000). Minimum and maximum values for each parameter were optimised separately with the subsequent range of values plotted onto the pared down molecular topology.

A test was undertaken to determine if the onset of flowering is correlated with the last recorded month of frost. Since both variables were treated as continuous (with numerically-coded months) it was possible to compare the variables using both conventional ahistorical correlation and PIC-based (phylogenetically independent contrasts) correlation. The PIC approach works on the principle of eliminating covariance due to inheritance by comparing a set of independent contrasts for significant correlation, and was performed using COMPARE 4.4 (Martins 1999) using an equal branch lengths model.

Visitor Observations

To test the hypothesis of pollinator specificity, casual observations were made of birds feeding from *Melianthus* under field conditions for all taxa excepting *M. insignis*, which was not seen in full flower. Recordings from *M. villosus* are based on a cultivated specimen at Kirstenbosch Botanical Garden, Cape Town, South Africa, and may not necessarily reflect visitors in its native range.

A more thorough visitor observation was undertaken 19-21 September 2003, at a field site in Kogmanskloof, approximately 5 km SW of Montagu in the Western Cape (33-20 CC). The site was chosen since it contained viable sympatric populations of both *M. comosus* and *M. major* in bloom simultaneously. In general, the two

species were separated by no more than 50-75 metres from one another, and in several instances *M. comosus* and *M. major* grew side-by-side.

Since the two species represent different floral morphologies for the genus, an *a priori* assumption was made that different pollinators would be involved in visitations. *Melianthus major* is characterised by elongated erect flowering racemes, to 100cm in length, which are held above the foliage, the flowers contained along the upper half of the raceme with the lower half functioning as a sturdy landing perch for birds when they initially land to feed. *Melianthus comosus* is instead typified by shortened lateral racemes, 5-10 cm in length, which are borne from the leaf axils and tucked beneath the foliage canopy. With the flowers borne along the entire length of the raceme, birds feed from them by perching on the woody branches of the shrub itself.

Two adjacent plots were delineated for observation purposes; each plot comprised of 10 bushes each, one exclusively containing *M. comosus* and one exclusively containing *M. major*. Visitor observations were recorded from 06H00-17H00 every 10 minutes from both sites for three consecutive days (33 hours) in total.

Nectar Analysis

Nectar samples were collected for all *Melianthus* species visited during the course of this study for purposes of qualification and quantification. Each species was sampled from a single collection site. Since no *ad hoc* preparation was done to exclude visitors prior to collection, the amounts extracted can be considered no more than a rough estimate. Precise measurements would require flowers to be covered for periods of 24-48 hours prior to nectar extraction (Baker and Baker 1983). Replicates were based on 10 sampled flowers taken one each from 10 different plants, in an attempt to detect variation within populations. The exception was *M. villosus* whose 10 replicates came from three flower spikes on a single multi-branched specimen in cultivation at Kirstenbosch Botanical Garden, Cape Town. *Melianthus insignis* was not seen in full bloom so is not included in the study. Nectar was extracted with a 10-100µl Finnpipette® micropipetter (Labsystems, Inc.) allowing for easy estimations of volume. Disposable tips were used between samplings to avoid cross contamination. Sucrose readings were calculated on a hand-held, temperature compensated refractometer (Atago CO. Ltd., 32-10 Honchu, Itubashi-ku, Japan), which measures nectar concentration in terms of equivalent percent sucrose ratios.

3.3 Results

Molecular clock test

Modeltest selected the GTR + G (General time reversible model plus estimated gamma distribution shape parameter) as the most appropriate model of sequence evolution resulting in four rated categories as listed:

1.) Nucleotide frequencies = 0.2883, 0.2068, 0.2189 and 0.2860.

2.) Substitution model matrix = 1.3441, 1.2654, 0.4264, 1.4035, and 2.2900.

3.) Proportion of invariant sites (PIV) = 0.0

4.) $\Gamma\alpha = 0.1974$.

Based on these estimated parameters under ML settings in PAUP*, a molecular clock assumption was accepted under the LRT given $-2\Delta lnL = 23.73$ ($0.1<p<0.5$, df = 18).

Age Estimates

Nodal age estimates are depicted in Table 3.2 with letters A-Q corresponding to labeled nodes on the combined molecular topology of Figure 3.1. Two sets of estimates are provided based on the divergence between *Greyia* and Melianthaceae as estimated by Wikström *et al.* (2001), at 59 mya (using a rate-smoothed ML tree) and 67 mya (using a rate-smoothed ACCTRAN parsimony tree). Based on these reference dates, the divergence between *Melianthus* and *Bersama* (Node P) is estimated to have occurred 28.00±0.56 mya (ML-based calibration) - 32.00±0.64 mya (ACCTRAN-based calibration). The deepest divergence within *Melianthus* (Node O), which lead to the separation of *M. major*, is dated at 11.71±0.40 mya - 13.29±0.45 mya with the next divergence (Node N) at 8.50±0.24 mya - 9.65±0.27 mya terminating in *M. villosus*. Separation of the West Coast clade (WCC) from the Karroid/Eastern clade (KEC) (Node M) occurred at 7.28±0.21 mya - 8.27±0.24 mya, the WCC being represented by symplesiomorphic erect flowered forms versus the derived lateral flowered developments of the KEC. The WCC began diverging (Node L) as early as 4.20±0.16 mya - 4.77±0.19 mya with the KEC quickly following suit (Node G) at 4.04±0.18 mya - 4.60±0.20 mya.

Using the ML calibration (59 mya), sequence divergence rates are estimated at 8.73×10^{-10} substitutions/site/year (s/s/y), while the ACCTRAN calibration (67 mya) suggests 9.91×10^{-10} s/s/y. Figure 3.2 summarizes nodal age estimations on a branch-proportional topology presented in the form of a chronogram with a geologic time scale reference.

Table 3.1: Ecological parameters used for ancestral state reconstruction featuring units of measurement with the variable coding methods indicated for each state.

Parameter	Units	Coding
Minimum midwinter (July) temperature	°C	continuous
Maximum midsummer (January) temperature	°C	continuous
Temperature range during flowering	°C	continuous
Season of rainfall	summer (Dec-Feb) = 1 autumn (Mar-May) = 2 winter (June-Aug) = 3 spring (Sep-Nov) = 4	discrete, unordered
Last recorded month of frost	calendar months (1=January, 2=February…)	continuous
Onset of flowering	calendar months (1=January, 2=February…)	continuous
Mean annual precipitation	mm	continuous

Table 3.2: Total age estimates (mean ± 2SE) in million years for each node based on 100 bootstrap replicates. Estimated ages are based on a rate-smoothed analysis by Wikström *et al.* (2001) who suggested the time of divergence of Greyiaceae and Melianthaceae at 59 mya (using a ML model) and 67 mya (using ACCTRAN parsimony). DELTRAN parsimony consistently gave intermediate results in Wikström *et al.*'s account and these are not shown. Letters correspond to nodes in Figure 3.1.

Node	Estimated age (ML)	Estimated age (ACCTRAN)
A	2.437±0.156	2.767±0.176
B	7.886±0.320	8.955±0.362
C	0.000	0.000
D	1.235±0.114	1.402±0.130
E	0.000	0.000
F	0.288±0.054	0.328±0.062
G	4.042±0.176	4.590±0.200
H	0.343±0.064	0.389±0.072
I	0.492±0.102	0.558±0.116
J	0.551±0.070	0.625±0.080
K	2.942±0.134	3.341±0.154
L	4.197±0.164	4.766±0.186
M	7.282±0.210	8.269±0.238
N	8.499±0.242	9.651±0.274
O	11.706±0.394	13.294±0.446
P	28.099±0.560	31.909±0.636
Q	59.000	67.000

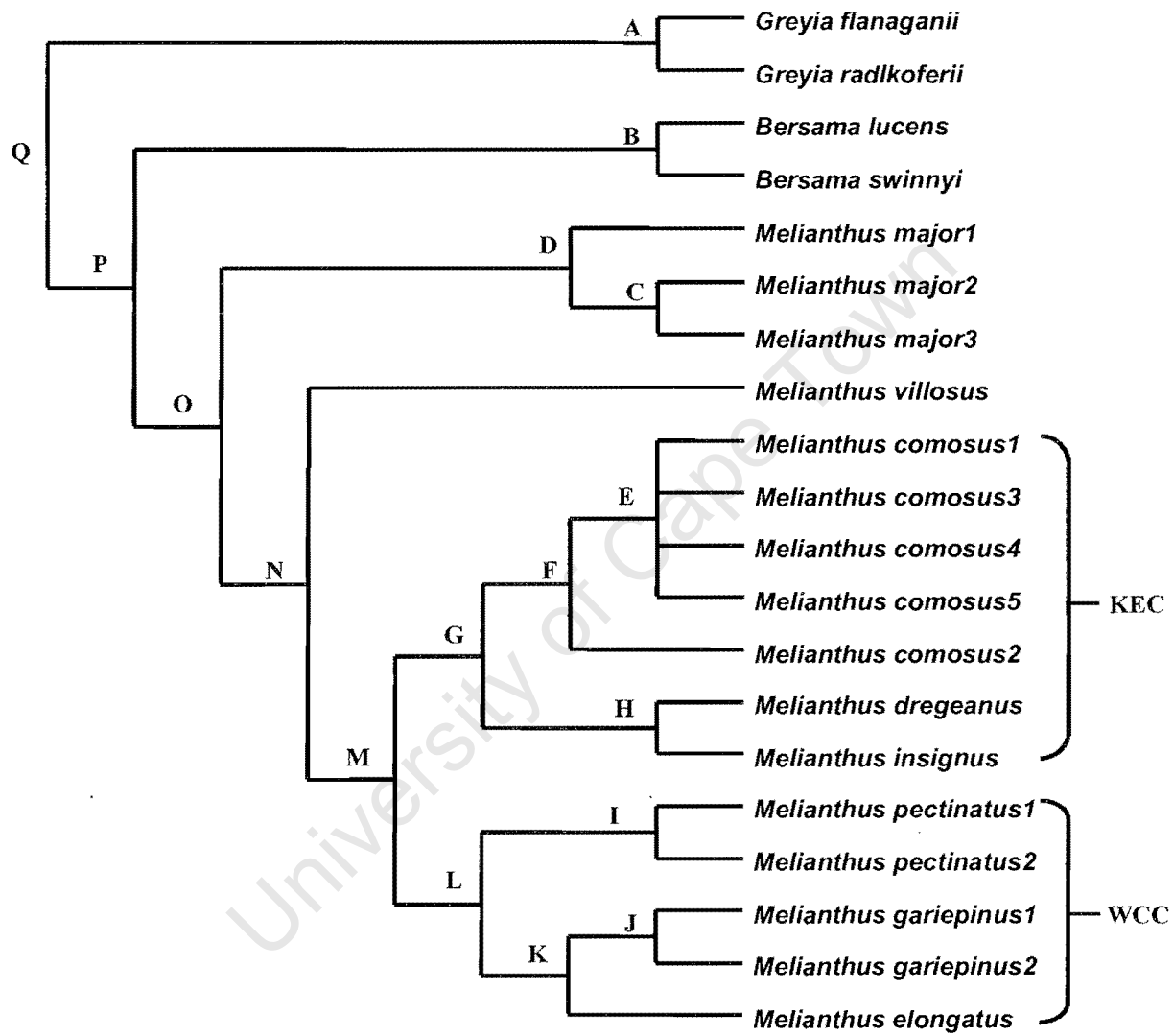


Figure 3.1: One of the two most parsimonious trees from the combined molecular analysis used for molecular clock analysis. Nodes are alphabetized and correspond to Table 3.2 depicting estimated branch lengths under a ML model.

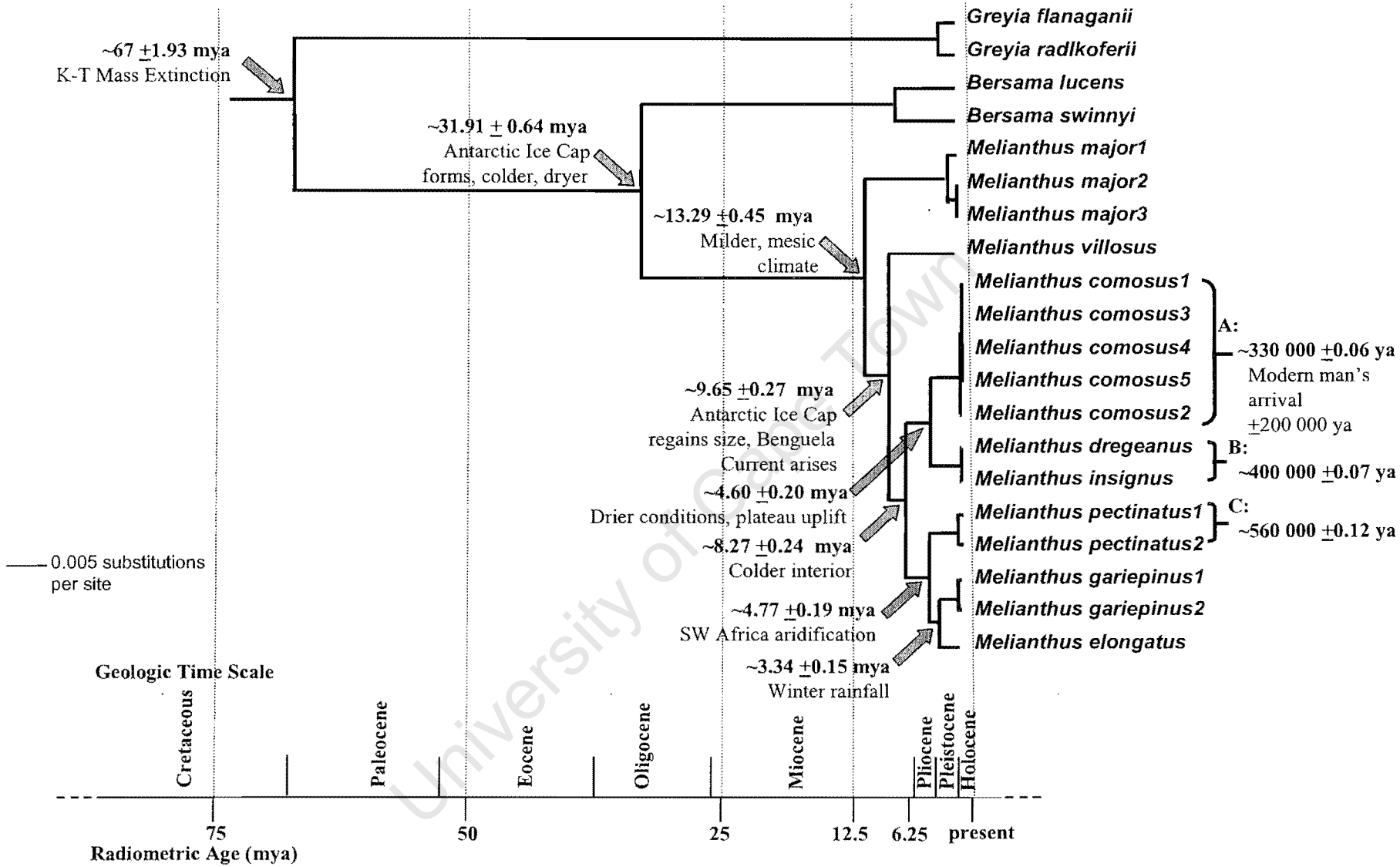


Figure 3.2: Time-calibrated phylogeny based on a molecular clock with branch lengths proportional to amount of sequence divergence. Times depicted derived from Wikström *et al.*'s (2001) rate-smoothed analysis with ACCTRAN-based branch length estimate suggesting the divergence of Melianthaceae and Greyiaceae at 67 mya. Arrows or brackets point to estimated nodal dates. Major historical events coinciding with speciation events are indicated in bold. A = divergence of *M. comosus2* from the rest of *M. comosus*, B = divergence between subspecies of *M. dregeanus* complex, C = divergence between 'ericoid' leaf versus typical leaf in *M. pectinatus*.

Geographical Distributions

The pared down topology used for DIVA analysis is shown in Figure 3.3 with numbered nodes corresponding to the results presented in Table 3.3. Ancestral area estimations when 'max areas' = 10 were highly equivocal at most nodes and are therefore not shown. Given the restricted ranges of most *Melianthus* species, the lower 'max areas' settings were considered more reasonable.

The most inclusive results pooled from 'max areas' settings at 5, 3 and 2 suggest that the family, Melianthaceae, arose in the eastern part of South Africa (Node 1) with the ancestral *Melianthus* inhabiting the Eastern Cape (Nodes 2-3). The start of a westward expansion is indicated with the range eventually broadening to include the Free State and the Northern Cape (Nodes 3-5). From this broad range emerge two clades, the WCC and the KEC. The range for the development of the WCC, (*M. elongatus* and the *M. pectinatus* complex), contracts to the Northern Cape (Nodes 6-7) while the diversification of the Karroid/Eastern clade, (*M. comosus* and the *M. dregeanus* complex), centres around the Eastern Cape, the Free State and Lesotho (Nodes 8-9).

Temperature

Reconstructions of minimum July (winter) and maximum January (summer) temperatures suggest the genus *Melianthus* arose in a climate of cool, barely frost-free winters (2-7°C) followed by hot summers (22-30°C) (see Figure 3.4). Two shifts into subzero winter environments are noted in *M. villosus* and *M. comosus*. The origin of the WCC is marked by a shift towards increased summer temperatures (24-30°C → 26-32°C) coupled with a milder winter (2-7°C → 4-9°C).

Flowering temperatures are based on extremes recorded during the main month of bloom (see Figure 3.5). Across the entire species distribution range, maximum temperatures during bloom time are relatively uniform both ancestrally (24-25 °C) and in present-day species (24-26 °C). In converse, minimum temperatures during flowering indicate a decline around the time *M. villosus* diverged with a sharper decline on the branch terminating in *M. comosus,* showing the ability of the latter to withstand fluctuations to zero °C when in bloom. An increase indicated in the minimum temperatures during the bloom period in the *M. dregeanus* complex can be attributed to a delay in the timing of flowering for that lineage.

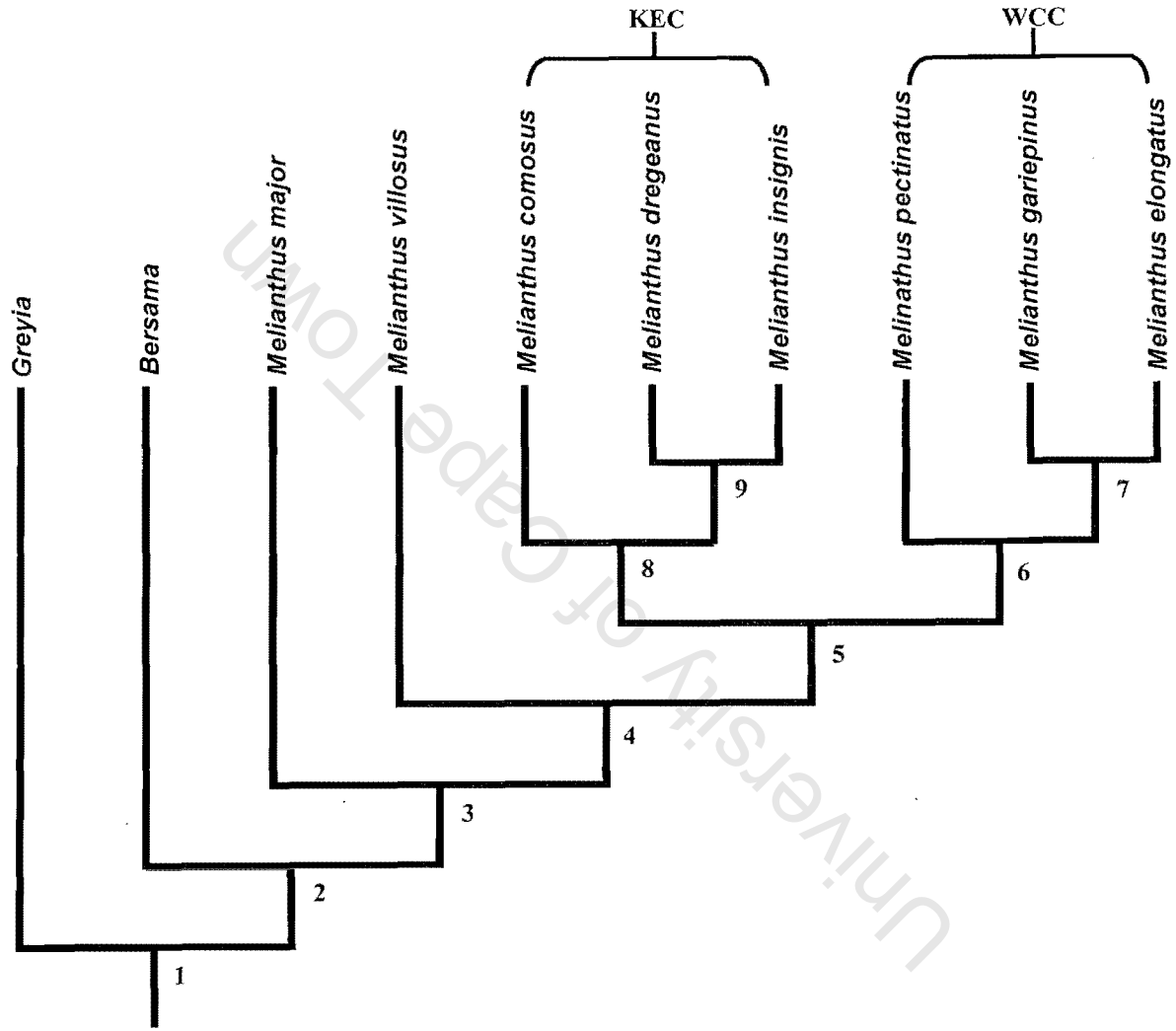


Figure 3.3: Pared down version of the combined molecular topology used for DIVA analysis. Numbers correspond to those listed in Table 3.3

Table 3.3: DIVA results for dispersal events in Melianthaceae. Node numbers correspond to those shown in Figure 3.3. The full set of possible ancestral areas resolved under all reconstructions is shown, with those resolved under the majority of reconstructions highlighted in bold. (NC=Northern Cape, WC=Western Cape, EC=Eastern Cape, FS=Free State, KZ=KwaZulu/Natal, LP=Limpopo, MP=Mpumulanga, LS=Lesotho, SW=Swaziland.)

	Max Areas = 5	**Max Areas = 3**	**Max Areas = 2**
No. of dispersals	26	28	29
Node 1. (Divergence of *Greyia* + Melianthaceae.)	**EC** FS LS KZ MP SW LP	**EC** FS LS KZ MP SW LP	**EC** FS LS KZ MP SW LP
Node 2. (Divergence of *Bersama* + *Melianthus*.)	**EC**	**EC**	**EC**
Node 3. (Ancestral *Melianthus*.)	**EC** FS WC LS	**EC** FS WC LS	**EC** FS LS
Node 4. (Ancestor of *Melianthus* excluding *M. major*.)	**NC EC FS**	**NC EC FS**	**EC FS**
Node 5. (Divergence of Karroid/Eastern clade + West Coast clade.)	**NC EC**	**NC EC**	**NC EC**
Node 6. (Divergence of West Coast clade)	**NC**	**NC**	**NC**
Node 7. (Ancestor of *M. gariepinus* + *M. elongatus*.)	**NC**	**NC**	**NC**
Node 8. (Divergence of Karroid/Eastern clade.)	**EC FS LS**	**EC FS LS**	**EC FS LS**
Node 9. (Ancestor of *M. dregeanus* + *M. insignis*.)	**EC** FS LS	**EC** FS LS	**EC** FS

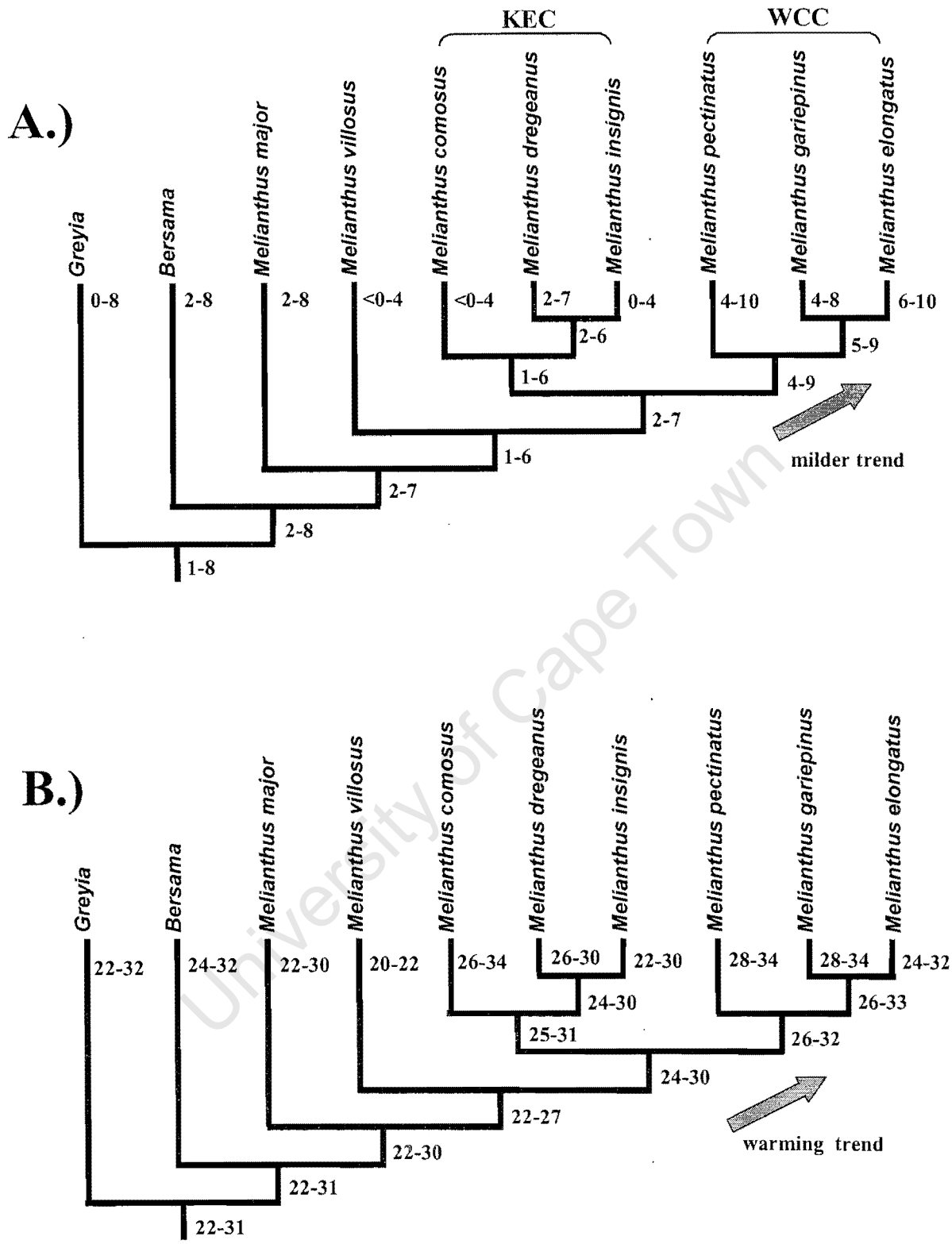


Figure 3.4A-B: Ancestral reconstructions based on conditions in present day species ranges showing minimum-maximum ranges. A.) July minimum temperature range (°C) during midwinter. B.) January maximum temperature range (°C) during midsummer. Arrows indicate developments. (KEC = Karroid/East Coast clade, WCC = West Coast clade.)

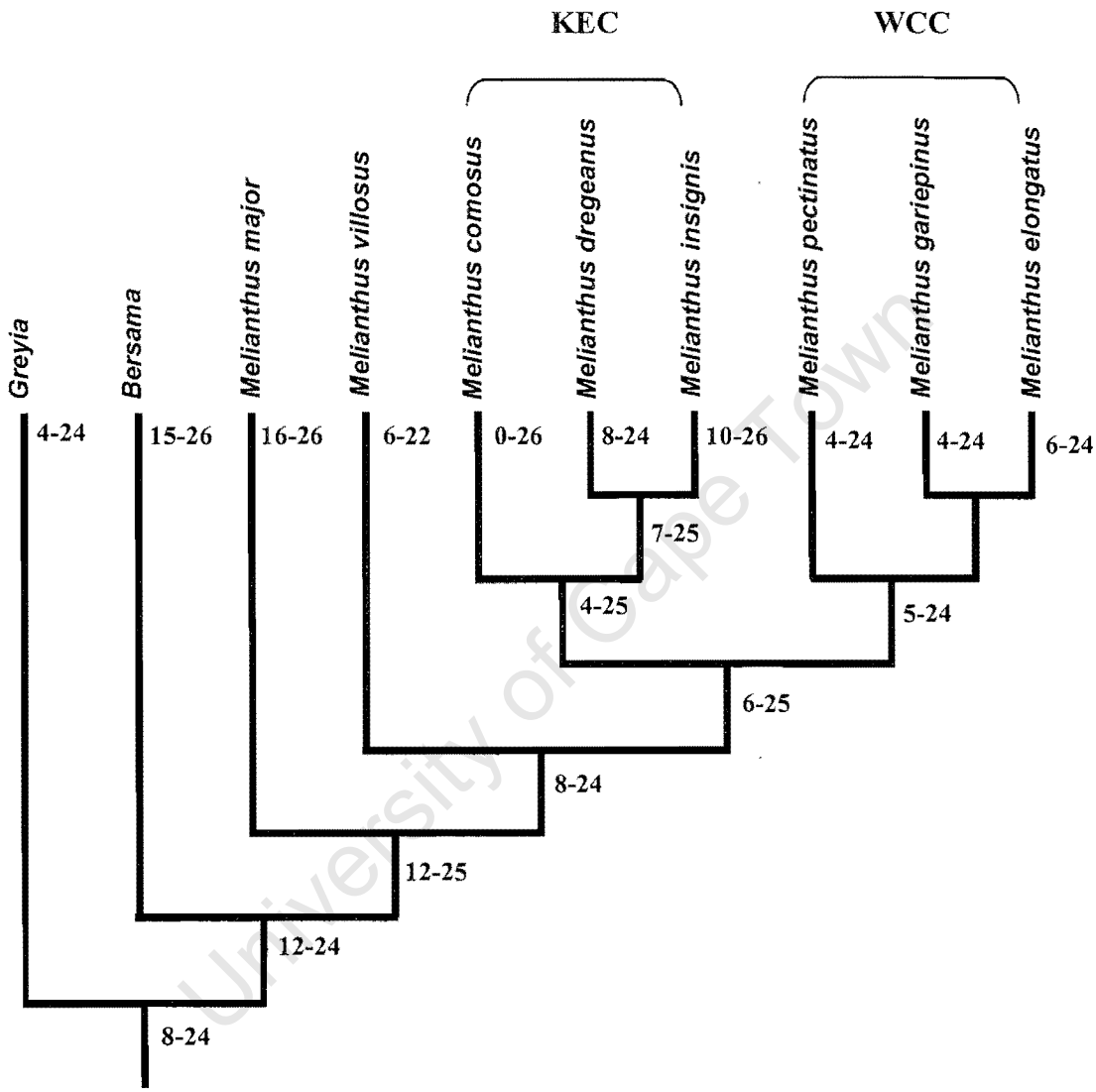


Figure 3.5: Ancestral reconstructions based on conditions in present day species ranges representing lowest mean minimum and highest mean maximum temperature encountered by species in their distribution ranges during their main month of bloom.

Floral Season and Frost

Figure 3.6A-B shows reconstructions for the month of first bloom and the last month of frost. With calendar months treated numerically, an ahistorical comparison between the two variables indicates a significant correlation, (Figure 3.7: $r = 0.729$, $0.1<p<0.5$, $p = 0.0167$, df = 8), which is also evident when PICs are used ($r = 0.727$, $0.1<p<0.5$, $p = 0.0172$, df = 8). Two outliers affecting a stronger regression fit in the ahistorical correlation are the delayed onset of bloom in *Bersama*, (which was treated as a composite genus and is influenced as much by rain patterns of the eastern coast as by temperature extremes), and the earlier bloom of *M. comosus*, (the only *Melianthus* species to begin flowering prior to the last frost date).

Rainfall

Reconstruction suggests Melianthaceae was ancestrally associated with summer to autumn rainfalls (see Figure 3.8A). A single shift to a winter rainfall pattern coincides with the emergence of the WCC in the Northern Cape. A year-round rainfall pattern appears twice, once on the branch leading to *M. major* and once on the branch leading to *M. dregeanus*. Due to their broader ranges, the rainfall patterns of *M. comosus* (summer/autumn/winter) and *M. major* (year-round) may not reflect an average condition for either taxon as a whole since each species straddles a series of different rainfall regimes.

Reconstruction of mean annual precipitation ranges (see Figure 3.8B) indicates a general trend towards increased dryness in *Melianthus*, culminating in the arid-adapted *M. comosus* on the one hand and the WCC on the other. From the initial point of divergence of *Bersama* and *Melianthus* (range 524-1105 mm), mean annual precipitation declines through to the divergence between the KEC and the WCC by about ~200-318 mm. From this point, the lower end of the range further drops by about ~250 mm on the branch terminating in *M. comosus*, with some areas receiving as little as 50 mm of rainfall a year. However, the upper end of the range does not change appreciably on this branch. The broad range of precipitation values in *M. comosus* is reflective of its wider distribution, indicating its presence in both drier and moister environments. The shift towards increased aridity reaches its zenith in the WCC. Following its divergence from the KEC, there is a general decrease in mean annual rainfall in the WCC, this being most dramatic on the branch terminating in *M. gariepinus*. This shows reduction in annual rainfall of up to ~250-500 mm, with less

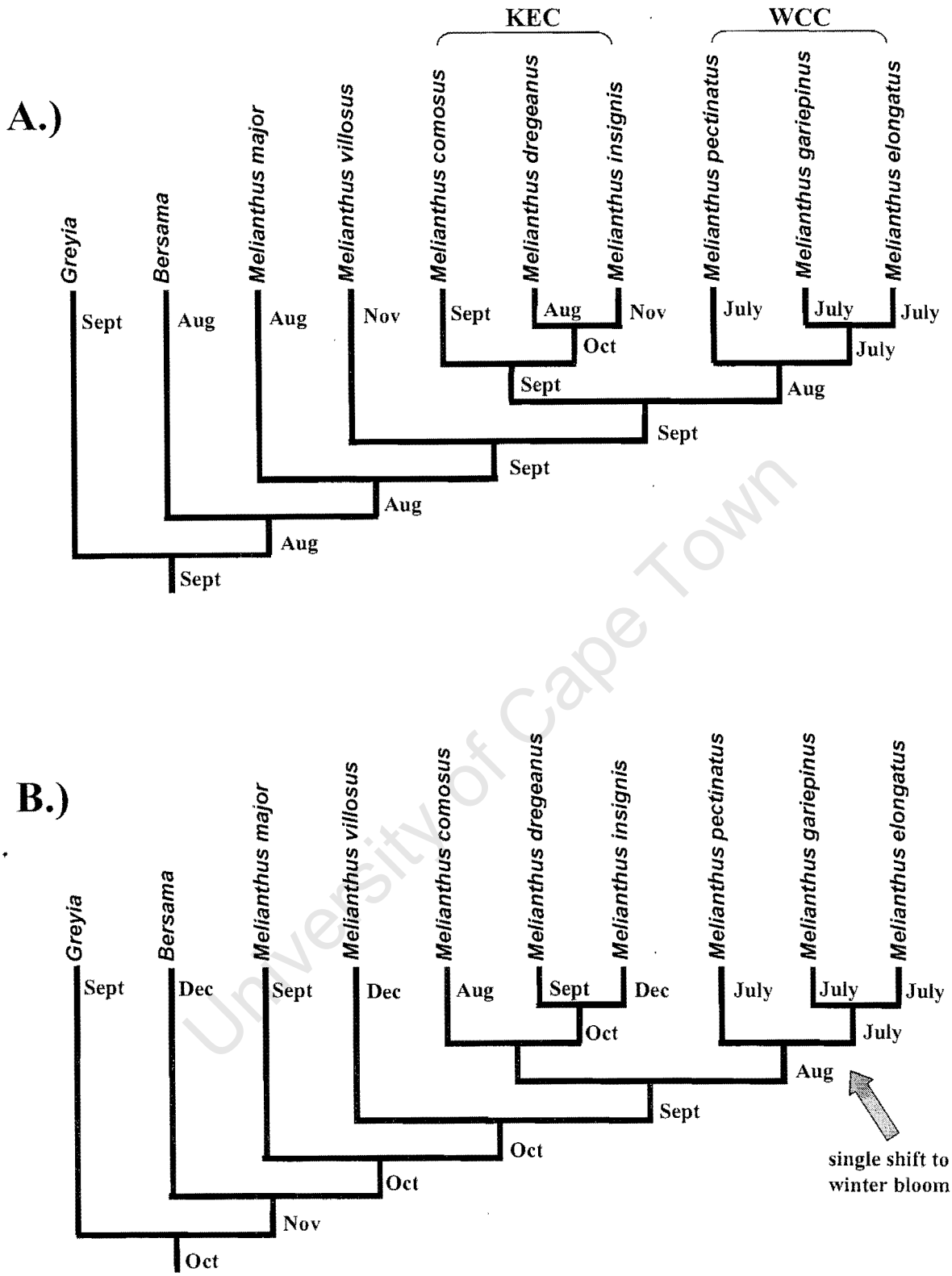


Figure 3.6A-B: Ancestral reconstructions based on conditions in present day species ranges. A.) Last month of recorded frost. B.) Month of first flowering. A single shift towards winter bloom is noted in the WCC. *Melianthus comosus* is the only species which begins to bloom before last recorded frost.

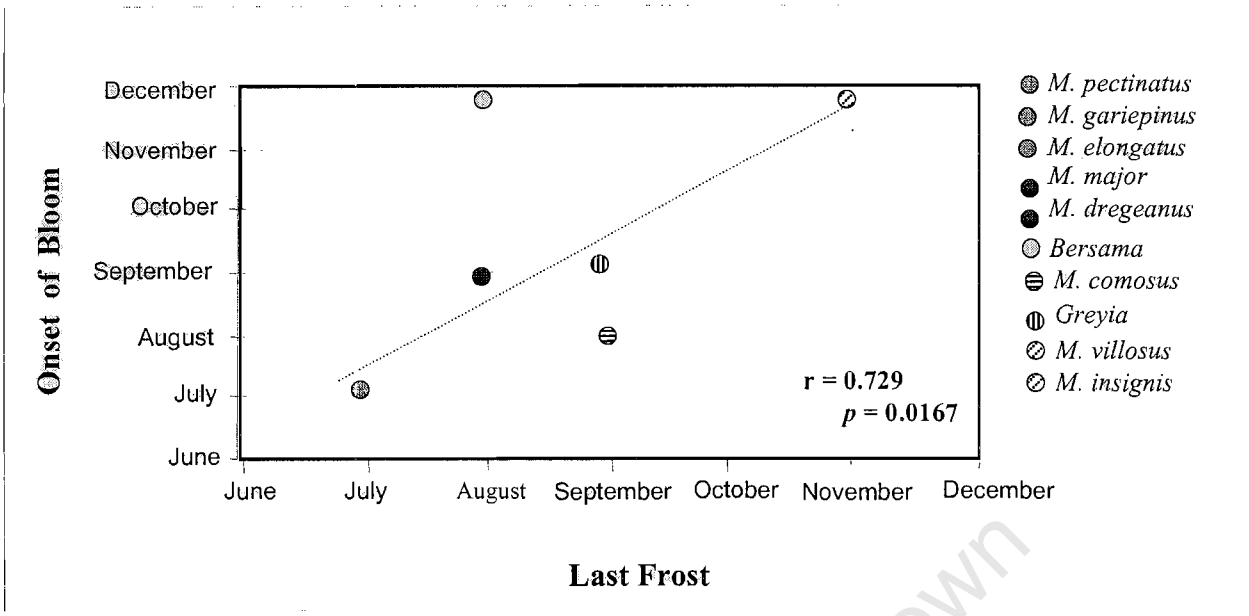


Figure 3.7: Comparison of the last date of frost and the onset of bloom. Outliers affecting a tighter fit are the composite *Bersama*, blooming much later than last month of frost and, more importantly, *M. comosus,* which is the only taxon shown that blooms before last month of frost. Results from PIC are similar, with $r = 0.727$, $p = 0.172$.

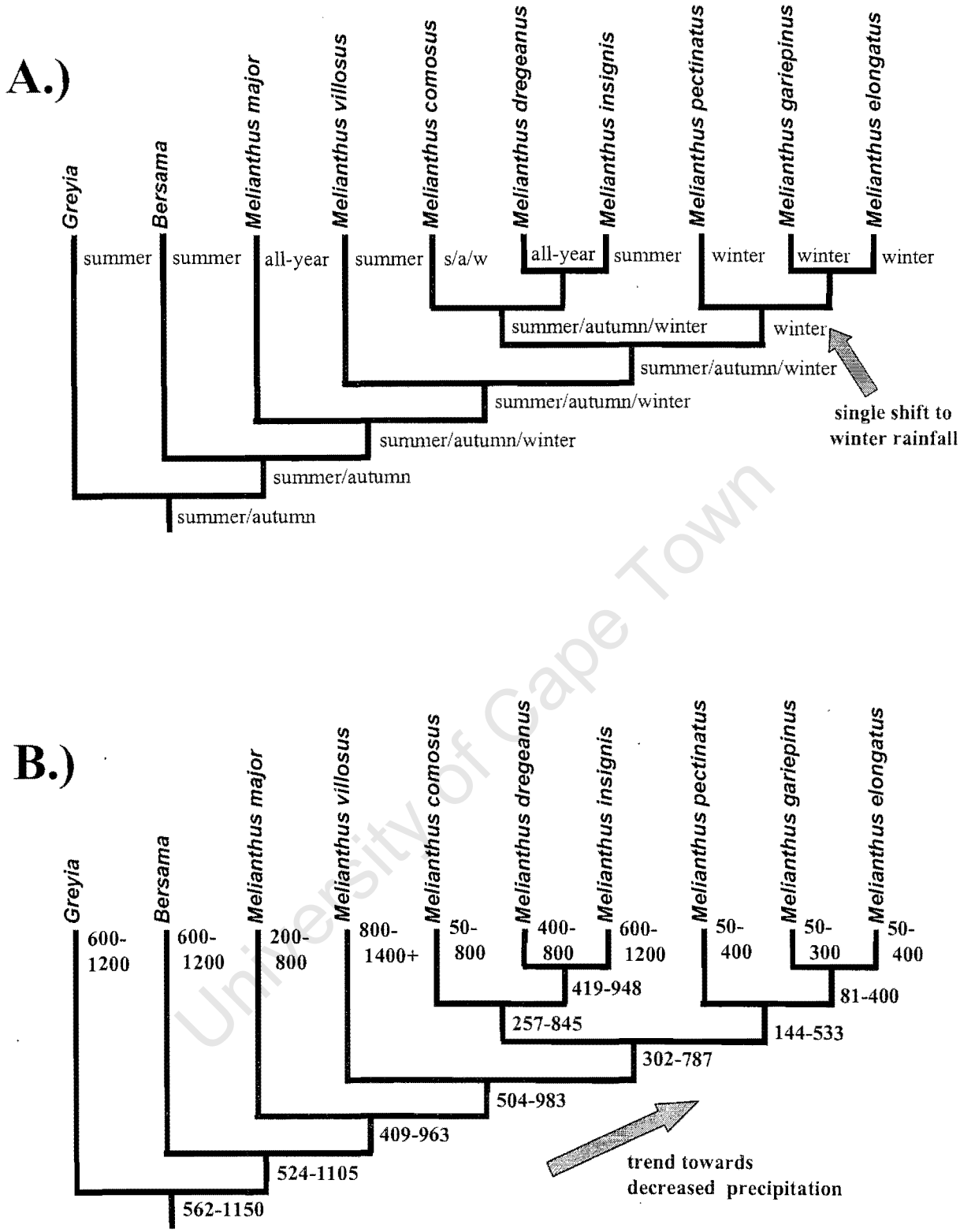


Figure 3.8A-B: Ancestral reconstructions based on conditions in present day species ranges. A.) Rainfall season . B.) Mean minimum-maximum range of annual precipitation in mm. A general trend towards decreased precipitation is noted.

than 50 mm of rainfall being recorded in some years. Conversely, both *M. major* and *M. villosus* reflect a preference for the more mesic habitats that are putatively ancestral. Although *M. major* does occur in habitats with as little as 200 mm of rainfall per anum, field observations indicate an association with riparian situations that obviates the need for higher rainfall under certain circumstances. As a counter trend towards the drying trend, a higher mean annual precipitation volume is indicated in the *M. dregeanus* complex, most markedly pronounced in *M. insignis*.

Visitor Observation Results

Results from casual visitor observations are listed in Table 3.4. A broad suite of 'potential' pollinators is indicated for *Melianthus* with sunbirds generally outnumbered by the more ubiquitous presence of Cape White Eyes, (*Zosterops pallidus*). Despite the broad distribution range of *Melianthus*, some species share similar visitors indicating a lack of co-exclusivity between visitor type and plant type in certain instances. More extensive observations comparing visitors to *M. comosus* and *M. major* at a sympatric site indicate a similar pattern (see Figure 3.9A-B) in showing White Eyes as the most abundant visitor to both *Melianthus* species, greatly exceeding the role of Lesser Double Collared Sunbirds in the total number of visits within this site. *Melianthus comosus* seems to attract a wider range of visitors overall than *M. major*, (11 species versus 6 species respectively). Lesser Double Collared Sunbirds in the study range would only visit *M. major* after exhausting other nearby nectar sources first, most notably *Lycium* spp., whose slender tubular flowers precluded visits by other pollinators in the vicinity, unlike the more readily accessible flowers of *Melianthus*. Throughout the period of study, the Lesser Double Collared Sunbird showed no feeding interest in *M. comosus* whatsoever, with an exclusive preference for *M. major*. Conversely, the following birds showed no interest in feeding from *M. major*: Mousebirds (Red Faced and Speckled), Cape Rock Thrush, Cape Sparrow, Cape Robin, and Cape Canary, instead feeding exclusively from *M. comosus*. Visitors shared by both *Melianthus* species include Cape White Eye, weavers (Southern Masked and Cape), Red Winged Starling and Cape Bulbul.

Nectar Evaluation

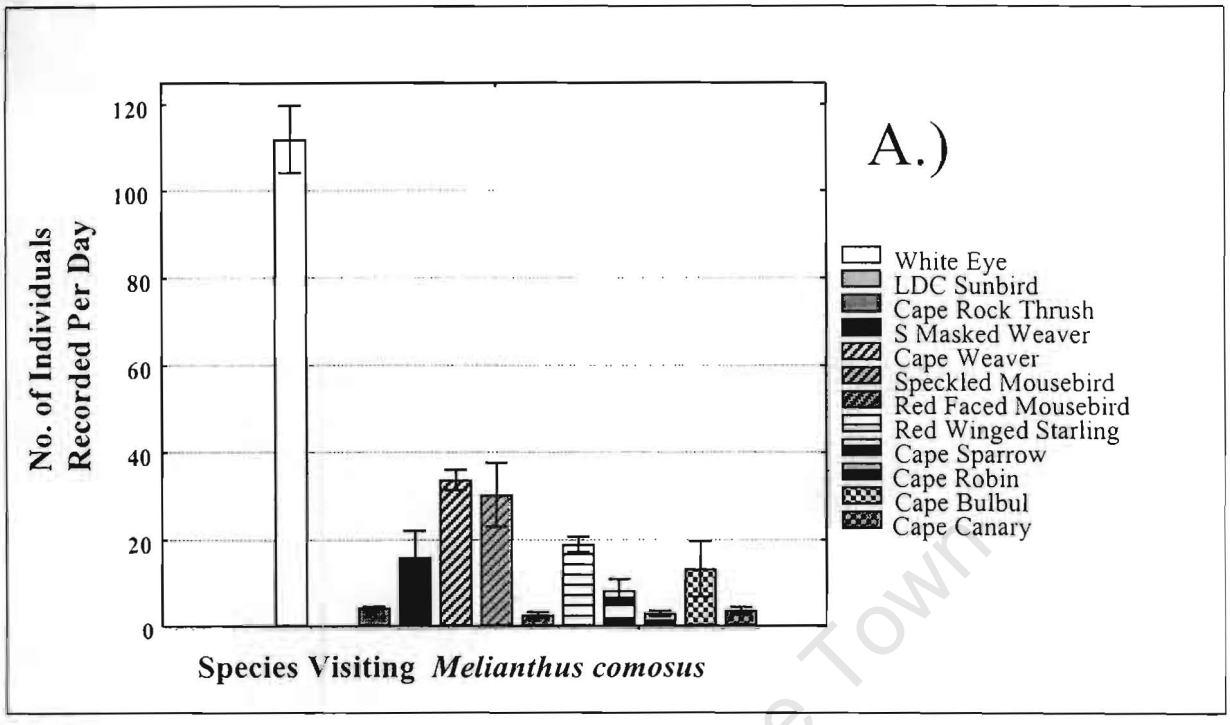
Differences in the sucrose concentration (see Figure 3.10A) and the volume of nectar (see Figure 3.10B) produced by the different *Melianthus* species were assessed. *Melianthus* falls within the category of hexose-rich nectar with a mean sucrose

Table 3.4: Casual observations of avian visitors to *Melianthus* species. With the exception of *M. villosus*, all observations were done under field conditions from multiple locations. *Melianthus villosus* was observed in cultivation at Kirstenbosch Botanic Gardens, Cape Town. Since *M. insignis* was not observed in full flower, it was excluded from list. No pollinators were actually observed feeding at *M. gariepinus* although Dusky sunbirds were in the vicinity and flowers showed signs of recent visitation through depleted nectar levels, therefore Dusky sunbirds are unreliably inferred (*X inferred).

Species	*M. major*	*M. villosus*	*M. elongatus*	*M. pectinatus*	*M. gariepinus**	*M. comosus*	*M. dregeanus*
Cape White Eye (*Zosterops pallidus*)	X	X	X	X	-	X	X
Lesser Double Collared Sunbird (*Cinnyris chalybeus*)	X	X	X	X	-	X	X
Greater Double Collared Sunbird (*Cinnyris afer*)	X	-	-	-	-	-	X
Amethyst Sunbird (*Chalcomitra amethystina*)	X	-	-	-	-	-	-
Orange-breasted Sunbird (*Anthobaphes violacea*)	-	-	-	-	-	X	-
Dusky Sunbird (*Cinnyris fuscus*)	X	-	X	X	-*X inferred	X	-
Malachite Sunbird (*Nectarinia famosa*)	X	X	-	X	-	-	X
Cape Rock Thrush (*Monticola rupestris*)	-	-	-	-	-	X	-
Southern Masked Weaver (*Ploceus velatus*)	X	-	-	-	-	X	-
Cape Weaver (*Ploceus capensis*)	X	X	-	-	-	X	X
Speckled Mousebird (*Colius striatus*)	-	-	-	X	-	X	-
Red Faced Mousebird (*Colius indicus*)	-	-	-	-	-	X	-
Red Winged Starling (*Onychognathus morio*)	X	-	X	X	-	X	-
Pied Starling (*Spreo bicolour*)	-	-	X	-	-	X	-

Table 3.4 cont.

Species	*M. major*	*M. villosus*	*M. elongatus*	*M. pectinatus*	*M. gariepinus**	*M. comosus*	*M. dregeanus*
Pale Winged Starling (*Onychognathus nabouroup*)	-	-	X	X	-	X	-
Cape Sparrow (*Passer melanurus*)	-	-	-	-	-	X	-
Cape Robin (*Cossypha caffra*)	-	-	-	-	-	X	-
Cape Bulbul (*Pycnonotus capensis*)	X	X	X	X	-	X	-
Black-eyed Bulbul (*Pycnonotus capensis*)	X	-	-	-	-	X	X
Cape Canary (*Serinus flaviventris*)	-	-	-	-	-	X	-



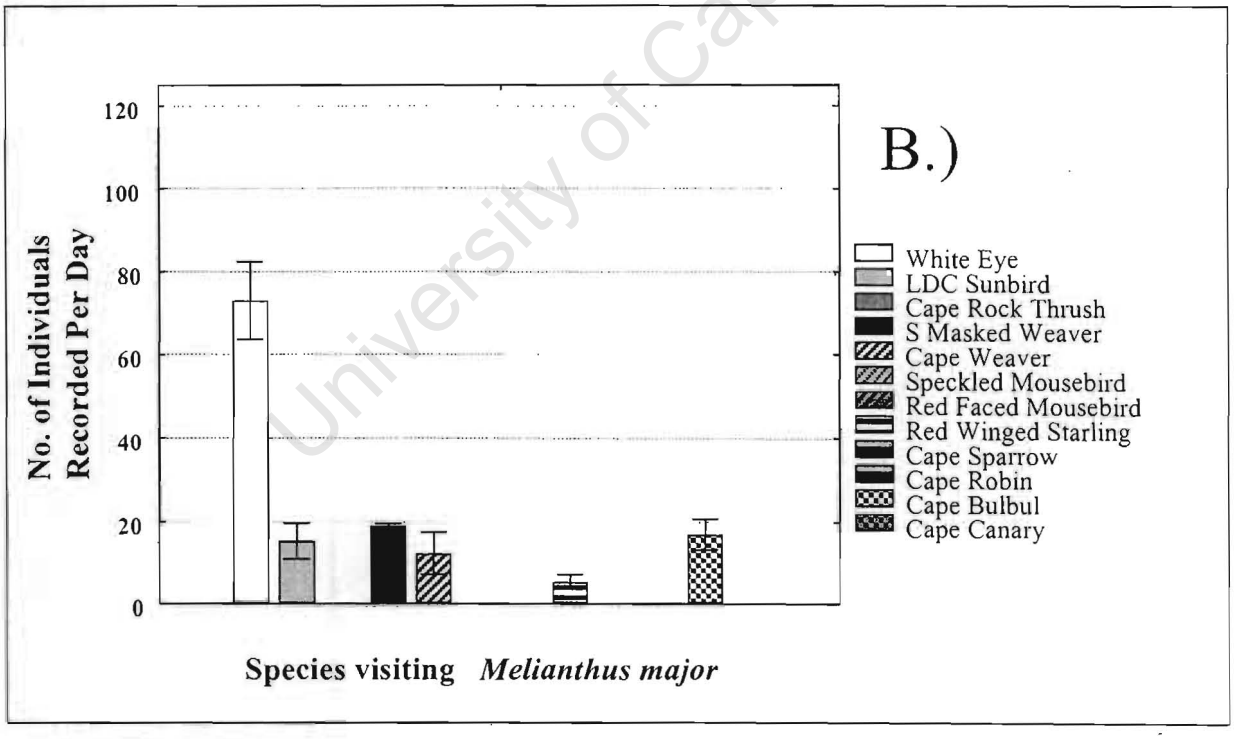


Table 3.9A-B: Mean number of observed daily visitors (±SE) to *M. comosus* and *M. major* based on 3 days (33 hours) of observation at a field location at Kogmanskloof, near Montagu, Western Cape Province. The site was selected because it contained viable sympatric populations of both species adjacent to one another. Each area observed was delineated to contain 10 study plants. Observations were made 19-21 September 2003. Visitors were recorded every 10 minutes from 06H00-17H00 with results pooled to a daily mean for purposes of presentation.

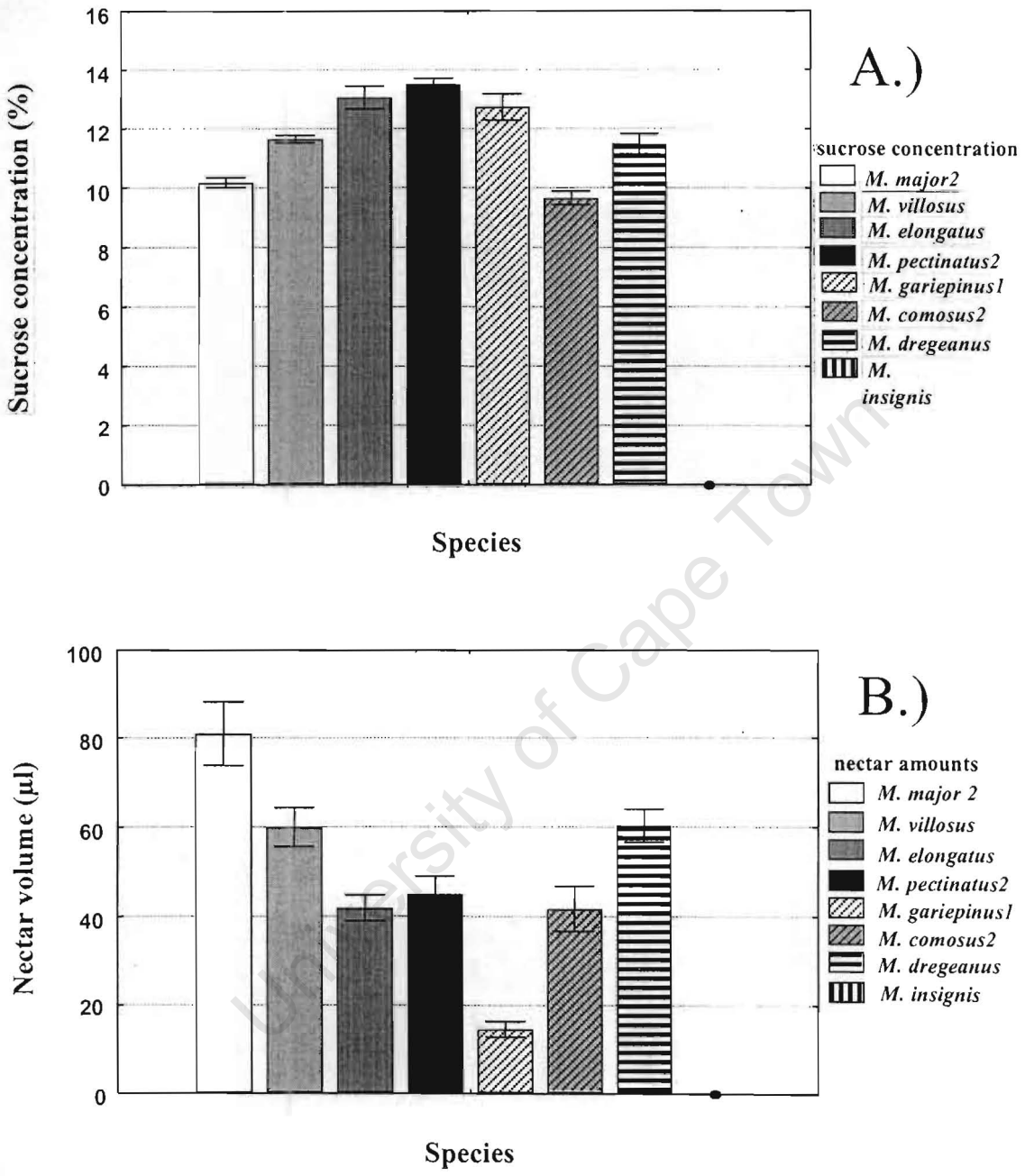


Figure 3.10A-B: A.) Mean nectar sucrose concentration (±SE) and B.) mean total volume of nectar (±SE) present in flowers from *Melianthus* collected in the field. The value for each species are based on 10 replicates with each flower coming from a separate plant when feasible, except for *M. villosus*, which was taken from a single cultivated plant.

concentration of 11.74% across all species sampled, (*M. comosus*2 minimum value = 9.5%; *M. pectinatus*2 maximum value = 13.5%, with 10.0-49.9% = 'hexose-rich') indicating a low value consistent with ornithophily syndromes. In general, the WCC shows a higher mean sucrose concentration (13.1%) than the KEC (10.5%) with the *M. major/M. villosus* grade giving intermediate values (~11.0%). A mean nectar volume of 50µl is found overall in *Melianthus* nectar with a minimum of 16µl in *M. gariepinus*1 and a maximum of 80µl in *M. major*2.

An inverse correlation between nectar volume and sucrose concentration is indicated in the WCC and the KEC. In general, the WCC shows a lower mean nectar volume (36µl) with a higher mean sucrose concentration (13.1%) than the KEC, which shows a higher mean volume (52µl) and a slightly lower mean sucrose concentration (10.5%). Although based on a single population of each species, these results seem reasonable, suggesting that lower amounts of nectar production might be associated with a higher sucrose level while higher amounts of nectar volume become more dilute.

3.4 Discussion

Leakey and Lewin 1996: *"The earth history is seldom one of gradualistic progression, as Lyell and Darwin so fervently desired, but rather one of sporadic and spasmodic convulsions."*

Melianthaceae is postulated to be a post-Gondwanaland African endemic comprising two small genera, *Bersama* and *Melianthus*. Within *Melianthus*, most species are confined to South Africa, where they have localised (*M. elongatus*, *M. major*) to highly localised (*M. dregeanus* and *M. pectinatus* complexes, *M. villosus*) ranges. The exception to this pattern is the widespread *M. comosus*, which extends as far northwards as northern Namibia (Dlamini unpublished 1999). To a large extent, the diversification of the genus seems to coincide with purported paleo-ecological changes characterising the Oligocene, Miocene and Pliocene (see Figure 3.2).

The Historical Context For *Melianthus* Evolution:

K-T Origins

Ancestral reconstructions based on a molecular phylogeny postulate the origin of Melianthaceae in the eastern part of South Africa. By using a rate-smoothed analysis with ML and ACCTRAN-based branch estimates, Wikström *et al.* 2001 dated the divergence of Melianthaceae from Greyiaceae at 59 – 67 mya, during the

boundary between the Cretaceous and Tertiary periods (Axelrod and Raven 1978). Based on fossil evidence indicating a predominantly woody flora (Axelrod and Raven 1978, Cronquist 1981), it is hypothesised that a mild, mesic, aseasonal climate prevailed globally at the time. The reconstructions presented here identify such environments as being ancestral in Melianthaceae, (high annual rainfall), but suggest seasonality with wet, hot summers followed by dry, cool winters.

Oligocene Events

The initial divergence of ancestral *Melianthus* from *Bersama* probably occurred during the mid-Oligocene (~28±0.56 mya - 32±0.64 mya) in an era of climatic deterioration marked by temperature declines and decreased rainfall due to Antarctic Ice Cap formation (Dingle *et al.* 1983). Ecological reconstructions in this study reflect a transition to dryer conditions with a decline in mean annual precipitation. Diversification in *Melianthus*, therefore, is postulated to have been driven by drought avoidance and temperature control in what was becoming a seasonably more taxing environment (Dingle *et al.* 1983).

Melianthus is characterised by a shrubby, semi-herbaceous, sparser habit (Phillips and Hofmeyer 1927b) compared to the taller, woody, densely foliaged trees comprising *Bersama* (Phillips 1921, Phillips and Hofmeyer 1927a, Verdcourt 1956a, 1956b, Palgraves 2002). The pinnate foliage of the family is further modified and divided in *Melianthus* than it is in *Bersama* (Palgraves 2002), which may help to reduce leaf temperatures and facilitate heat transport through alleviation of rapid temperature fluctuations (Gutschik 1999), thereby lessening water stress (Chabot and Hicks 1982). A winged rachis, as is most prominently developed in *Melianthus*, is necessary for increased mechanical support for the finer dissected foliage (Niklas 1999). In addition, the ability of the conduplicate leaves of *Melianthus* to fold upwards diurnally during hot periods ameliorates the impact of solar radiation, presumably assisting in the regulation of evapotranspiration rates to maintain turgidity (Körner 1999). Compared to the evergreen woody habit of *Bersama*, *Melianthus* developed a variably deciduous to semi-herbaceous nature. Dormancy in the genus occurs as a seasonal reaction to factors of coldness (as in *M. villosus*) and aridity (as in all other species) and would function as a resource reallocation for purposes of stress alleviation (Chabot and Hicks 1982).

'Pre-adaptations' to environmental changes (Brooks and McLennan 1991) may have predisposed ancestral *Melianthus* for eventual Miocene range expansion

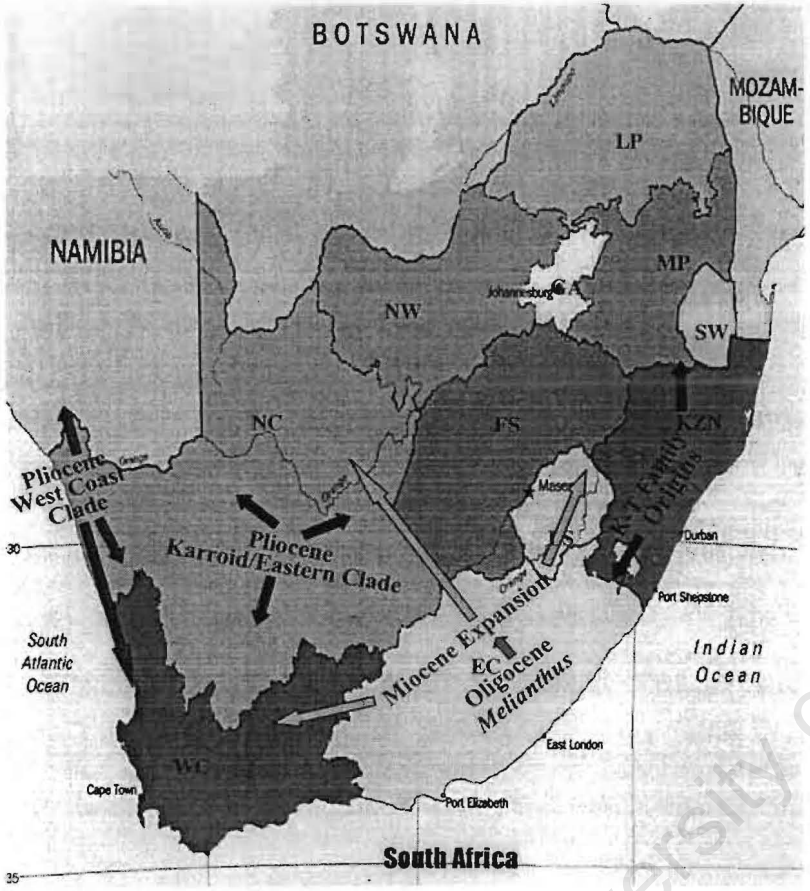
westwards into cooler, more arid zones, as depicted in Figure 3.11. Lacking such innovations, *Bersama* spread northwards instead into more benign environments (Verdcourt 1956a, 1956b) with a purported '*Bersama*-like' fossil (Hamilton 1968) dating to ±25 mya being reported from Mount Elgon in the Ugandan mountains.

Miocene Events

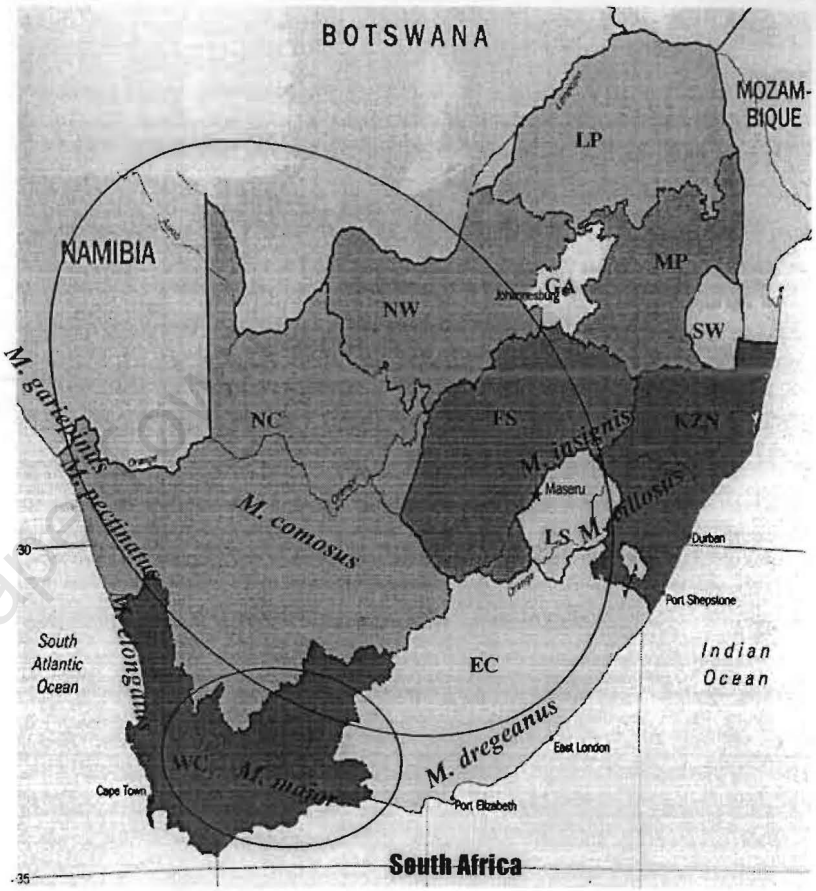
A putative origin of *M. major* in the Eastern Cape concurs with Dlamini's (1999 unpublished) hypothesis, which suggested the same, date estimations suggesting the emergence of *M. major* during the middle of the Miocene era (11.71±0.40 mya - 13.29±0.45 mya). The combination of tall stature, large leaves and uniquely glaucous foliage (van der Walt 2000) distinguishes *M. major* from the rest of *Melianthus*.

Global climatic patterns were mesic throughout the early-to-mid Miocene (Dingle *et al.* 1983) and mild enough to promote a mixture of subtropical vegetation throughout much of South Africa (Meadows and Watkeys 1999). Nevertheless, ecological reconstructions indicate an overall precipitation decline in the Eastern Cape, which would have encouraged the retreat of *M. major* into the damper habitat of drainage lines and streamside locations where it currently resides. It is unclear whether the bolder stature of the species is an ancestral state, with its retention being permitted by the shift to riparian conditions, or whether it instead arose as a novelty associated with such habitats. The warmer temperatures indicated by reconstructions presumably would have had greater solar impact given South Africa was still positioned 15° further north than its present position (Meadows and Watkeys 1999). Reconstructions of rainfall patterns for *M. major* suggest a tri-season rain pattern (spring/summer/autumn) in the Eastern Cape. The glaucousness of the leaves in *M. major* could be perceived as a dual purpose 'shield', affording plants the capacity to deflect solar radiance during hot, sunnier times while shedding water more efficiently during rainy periods to minimize solute loss (Gutschick 1999).

The origin of *M. villosus* coincides with a northward expansion from the Eastern Cape into the Free State during the mid-Miocene (8.50±0.24 mya - 9.65±0.27 mya). Ecological reconstructions suggest a decline in temperatures but an increase in precipitation during its development, indicative of its eventual move into the summer-wet regime of the Drakensberg Mountains. *Melianthus villosus* features a dense covering of stellate pubescence on its foliage and stems (Dyer 1952) and nearly



A.) Directions of ancestral expansion.



B.) Centres of present ranges.

Figure 3.11A-B: Species locations, past and present.A.) Depiction of ancestral locations with arrows indicating future range expansion. B.) Present locations of *Melianthus* species with a rough indication of expansiveness of range in *M. comosus* and *M. major*. (NC = Northern Cape, WC = Western Cape, NW = North West Province, FS = Free State, EC = Eastern Cape, GA = Gauteng, KZN = KwaZulu/Natal, MP = Mpumulanga, LP = Limpopo, LS = Lesotho, SW = Swaziland.)

approximates the stature of *M. major*. The thermo-regulatory benefit of nonglandular trichomes (Ehleringer 1981) in *M. villosus* can be viewed in two ways: as either a reaction to the climatic deterioration characterising much of the later Miocene (Dingle *et al.* 1983, Linder *et al.* 1992) or alternatively, as a more recent development caused by a colder present environment than the frost-free conditions it originally evolved in. Together, *M. major* and *M. villosus* form a grade favouring comparatively mesic conditions that approximate the ancestral preference of *Melianthus*.

Miocene/Pliocene Boundary Events

The initiation of the cold Benguela Current off the coast of SW Africa in the late Miocene created a far-reaching trend towards drier conditions along western coastal regions (Siesser and Dingle 1981). Stebbins (1952) hypothesized that aridification stimulates speciation with Goldblatt and Manning (2000) noting the dramatically drying conditions were the single most important factor affecting major vegetation change on the subcontinent. Further drying influence was felt inland due to an enormous uplift of the central plateau. This caused areas west of the Drakensberg to aridify as clouds moving over the massif were encouraged to shed moisture largely on its eastern face (Meadows and Watkeys 1999), a trend of events favouring the moisture-loving *M. villosus*. Ecological reconstructions suggest the mild winters and hot summers in the interior of the continent were affected by a developing trend towards decreasing precipitation and cooler conditions approaching the Pliocene. Two *Melianthus* clades diverged at this time (7.28±0.21 mya - 8.27±0.24 mya), the Karroid/Eastern clade (KEC) and the West Coast clade (WCC), which diversified in the Eastern Cape to the Northern Cape respectively. Both the KEC and WCC are typified by a lower stature than the taller *M. major/M. villosus* grade and feature highly dissected, reduced foliage, the KEC being further distinguished by a novel shift of flowers to an axillary position beneath the foliage.

Pliocene Events

Further climatic deterioration dictated much of the Pliocene with aridification events intensifying in southwestern Africa. Geographical reconstructions indicate the WCC diversified in the Northern Cape during this period, giving rise to three new taxa in the span of a few million years: *M. pectinatus* (~4.20±0.16 mya - 4.77±0.19 mya), and *M. elongatus* and *M. gariepinus* (~2.94±0.13 mya - 3.34±0.15 mya). Ecological reconstructions portray a dryer 'mediterranean-like' climate with a dramatically decreasing mean precipitation accompanied by a single shift to winter

rainfall. With the region noted for a prolonged summer drought, the maximum water allocations needed for flower and nectar production can only be achieved during the winter-wet periods, providing the necessary incentive for the evolution of winter flowering that typifies most of the West Coast flora (Goldblatt and Manning 2000), including the WCC. Tentative nectar analysis reveals the WCC produces less nectar volume with a higher sucrose content than other *Melianthus* species, perhaps indicative of the lower mean annual precipitation in this range.

Increasing aridity as one heads northwards may have possibly stimulated further morphological change in the WCC. The area west of Steinkopf in the Northern Province (29-17 BD), which experiences more intense summer drought than areas further south and east, features a *M. pectinatus* subpopulation with the recent development (~0.50±0.10 mya - 0.56±0.12 mya) of a pronounced 'ericoid' leaflet. Unlike the erect symplesiomorphic inflorescences of *M. elongatus* and *M. pectinatus*, the more northerly *M. gariepinus* shows great plasticity in inflorescence orientation ranging from the symplesiomorphically erect forms (*M. gariepinus*1) reported by Archer (1997) to the stiffly pendulous forms (*M. gariepinus*2) reported by Merxmüller and Roessler (1968). The variably pendulous inflorescence may be an attempt to ameliorate the greater aridity and solar effects recorded in its present range, where mean annual precipitation is lower (≤50-300mm per annum) than in any other WCC member.

The KEC also produced three taxa during the Pliocene, *M. comosus* (~4.04±0.18 mya - 4.60±0.20 mya) and *M. dregeanus* and *M. insignis* (~0.34±0.06 mya - 0.39±0.07 mya). Geographical reconstructions suggest an eastern origin centred in the Free State, Lesotho and the Eastern Cape. The similar-looking plants are characterised by lateral inflorescences tucked beneath the foliage. Compared to the plesiomorphic condition of elongated erect spikes with a whorled-to-opposite floral arrangement, the novel floral shift in the KEC was accompanied by a shortening of the raceme and the development of an alternate floral arrangement. Weight constraints from gravity would have predicated raceme shortening once it assumed a lateral position with the alternate floral arrangement necessary to accommodate the size of the flowers. Reconstructions support a decrease in mean annual precipitation over most of the Karoo with a drastic drop in winter temperatures resulting from the increased elevational gradients due to the central plateau uplift.

Flowering phenology in *Melianthus* seems to be based on a combination of two main factors, water availability and ambient temperature. *Melianthus comosus* is unique in commencing bloom prior to the end of frost (see Figure 3.8), a feature which may explain its ability to colonise large tracts of the central plateau of the country where winter bloom would be favoured by water relations though generally prohibited by winter frost. In *M. comosus*, the threat of potential frost damage to the flowers is ameliorated beneath a sheltering microclimate of the foliage. This novel combination of winter bloom plus the floral shift could have functioned as a 'key innovation' (*sensu* Givnish 1997) allowing access to previously unavailable resources (Baum and Larson 1991, Brooks and McLennan 1991, Givnish 1997), thereby moving *M. comosus* into a new adaptive zone by exploitation of the environmental homogeneity of the Karoo.

Herbivory

Several biotic factors have potentially influenced *Melianthus* evolution, some of which are difficult to place within a specific time frame. The toxic foliage (Agarwal and Rastogi 1976, Kelmanson *et al.* 2000) of *Melianthus* with its malodorous, petrol-like aroma was damned as 'repulsive to the human olfactory organ' by Von Maurilan (1895). Increased herbivory pressure on *Melianthus* would have played a key role in the development of the secondary compounds, specifically bufadienolides (Anderson and Koekemoer 1968, 1969a, 1969b), which characterize the genus. Although secondary compounds are usually sequestered within leaf vacuoles (Glasby 1991), a villous mixture of glandular and nonglandular trichomes on the calyces, pedicels and floral bracts of the *M. major/M. villosus* grade united chemical protection (Werker 1993) with a mechanical defense mechanism (Ehleringer 1981). Megaherbivores such as perissodactyls and artiodactyls (Meadows and Watkins 1999) appeared throughout southern Africa on the edge of the Eocene/Oligocene boundary and would have frequented the drainage lines and water sources (Owens-Smith and Danckwerts 1996) probably favoured by *Melianthus* thus making them as potentially damaging as the phytophagous insects in existence since the Jurassic. Despite the toxicity of the genus to most predators (Watt and Breyer-Brandwijk 1962), large seed losses to bruchid beetles are evident in *M. major* (pers. observation), presumably due to its glabrous ovary, which offers little protection compared to the pubescent or woody fruits that typify much of the rest of the genus.

Pollination Syndromes

Climatic factors may explain the shifts in inflorescence position and flowering phenology in *Melianthus* but pollination syndromes are potentially more effective in explaining floral morphology (Johnson 1996). Since most biotic pollinator schemes are insect orientated (Gentry 1974, Chapman 1998), an increase in floral complexity is usually associated with specialized 'intelligent' insect lineages, such as bees, butterflies and sphingid moths (Heinrich 1979, Proctor *et al.* 1996, Chapman 1998). Some of the duller colouration in the flowers typifying the earliest diverging *Melianthus* species (*M. major* and *M. villosus*) might suggest an ancestral predilection for insect pollination, as is found in *Bersama* (Verdcourt 1956a, 1956b). However, the majority of floral modifications in *Melianthus* species indicate a commitment to ornithophily: sturdy perches to support heavier pollinators, suitable orange/red floral colouration for the greater avian sensitivity to longer wavelengths (Richards 1986), a lack of floral scent, a thickened perianth texture to deflect feeding damage, a cup-like calyx to hold copious amounts of nectar needed for meeting the greater energy requirements of larger visitors, and a greatly increased distance between the stigma and style in relation to the nectary (Skead 1967, Faegri and van der Pijl 1979, Rebelo 1987, Proctor *et al.* 1996, Cheke *et al.* 2001). The sucrose concentrations of *Melianthus* nectar are relatively low, falling within the category 'hexose-rich' as designated by Baker and Baker (1983), most closely approximating the level of fruit juices, which deters entomophily while attracting frugiverous visitors as well as true nectivores (Getliffe Norris 1989, Proctor *et al.* 1996). Even from a rough estimate, the sheer volume of nectar production, with flowers capable of producing $\geq$80µl in some species, re-emphasizes the need for synchronicity between floral production, maximum water availability and benign temperatures in *Melianthus*.

Aridity causes a decline in the number of avian pollinators able to live year-round in western South Africa (Skead 1967, Cheke *et al.* 2001). Therefore, the avian visian signal in *Melianthus* species occurring in these drier areas needed to become more acute for effective long-distance signaling. This may explain the evolution of the enlarged exerted petals and the showier red-petaled crown typifying the WCC, and the prominent reddish-orange coloured calyces characteristic of the KEC. Determinants for calyx versus corolla modifications are unclear but presumably involve a resource allocation cost for the most effective method that ensures

reproductive success while minimising mechanical damage from surrounding vegetation or degradation from unfavourable environmental factors.

Colouration in nectar, a peculiarity of the genus, which generally rare in angiosperms (Olesen *et al.* 1998), adds another variable, with *Melianthus* exhibiting both tan and black nectar. Since avian colour perception is particularly keen to polychromatism (Faegri and van der Pijl 1979), black coloured nectar in two of the *Melianthus* species (*M. elongatus* and *M. comosus*) could form part of the collective avian visual signal affording heightened contrast against the reddish-orange flowers. This contrast may have been progressively selected by birds, and perhaps indirectly formed a cryptic deterrent against entomophily at the same time since insects are unable to effectively discriminate between red (Proctor *et al.* 1996, Chapman 1998), brown and black colouration (Waddington 1983).

The principle pollinators of *Melianthus* are generally cited as sunbirds, the Nectariniidae (Scott-Elliot 1890, Von Marilaun 1895, Burtt Davey 1932, Vogel 1954). With *Melianthus* lacking any perianth fusion that would dictate a degree of specificity, the notion of co-exclusivity with sunbirds seems questionable, however, especially given the number of references listing alternative visitors (Marloth 1908, 1925, Skead 1967, Maclean 1993). Casual observations across a range of *Melianthus* locations (see Table 3.4) indicate a broad suite of birds feed from the plants suggesting a generalist pollination syndrome. Along with sunbirds (Lesser Double Collared, Greater Double Collared, Dusky, Malachite), a combination of Cape White Eyes, bulbuls, Cape Sparrows, starlings, weavers, and more rarely mousebirds, feed from the flowers *collectively*, with some of the visitors, such as White Eyes, recorded from nearly every *Melianthus* species observed.

A more detailed study between *M. major* and *M. comosus* (see Figure 3.9) lends further support to the hypothesis that *Melianthus* behaves as a generalist in terms of its pollination syndrome. Weakening of the pedicel due to resupination of the flower from the weight of accumulated nectar causes the flower in *Melianthus* to tip forward under the least amount of pressure. Pollen is readily dusted onto the heads of short-beaked visitors, turning even the most unlikely of candidates into potential pollinators. Given their 'rougher' feeding technique, therefore, the short-beaked opportunists like the Cape White Eyes might seem even more ideal as pollinators than the specialised long-billed Nectariniidae.

Cheke *et al.* (2001) state: "there are no recorded instances of any one species of Nectariinidae being intimately connected with any one particular species of flowering plant", which these initial field observations for *Melianthus* further confirm. This counters suggestions (Dlamini unpublished 1999, as derived from Johnson 1996) that specialisation to specific pollinators may account for the floral divergence in *Melianthus*. Rather, the types of environment in which pollination occurs may drive floral divergence.

Dispersal Mechanisms

Dispersal mechanisms for *Melianthus* have yet to be studied but presumably the seeds are spread passively. The genus would hardly seem a suitable candidate for biotic dispersal mechanisms since their dry membranous to woody fruits and simple seeds offer little incentive to either frugivores or herbivores. Lacking a myxocarpic response neither would the seeds affix easily to animals for passive transport (Bouman and Meeuse 1992). Although humification through ant-dispersal reputedly plays an important role in the Karoo (Esler 1999), *Melianthus* seeds show no elaisome adaptations to promote myrmecochory. Modern man arrived in South Africa approximately ~200 000 ya (Smith 1999) with all indigenous people reporting abundant medicinal uses for *Melianthus* as treatments for snakebite, backaches, bruises, ring worm and rheumatism (Smith 1895, Watt and Breyer-Brandwijk 1962, Iwu 1999, Van Wyk and Gericke 2000). Early human inhabitants would have been prone to travel along the drainage lines *Melianthus* favours in search of water and game and may have been responsible for minor seed dispersal. However, man's arrival post-dated the westward expansion of *Melianthus* by tens of millions of years, so anthropogenic dispersal would have had negligible impact on species distributions.

More logistically feasible is the idea of the Orange River as an abiotic dispersal agent. From its inception in the Lesotho highlands, the spread of the Orange River from the east to the northwest across the Karoo is suggestive of a 'dispersal corridor' (Vernon 1999) as it drains the western slopes of the Drakensberg, the Free State and the Northern Cape (Helgren 1979). With the open landscape of the Karoo and a predictable hydrological regime characterized by late summer floods (Palmer *et al.* 1999), a diplochorous seed dispersal mechanism for Miocene *Melianthus* might be likely, combining elements of barochory (passive weight dispersal) with a secondary hydrochorous phase (Campbell *et al.* 2002). Thus, exploitation of the Orange River as

a crucial disperser may explain the emergence of the WCC in the northwest corner of the Northern Province and the dramatic spread of the KEC's *M. comosus* across the Karoo.

Current Distribution Ranges in *Melianthus*

Geographical isolation is believed to account for the integrity of the various *Melianthus* species (Dlamini unpublished 1999). Ancestral reconstructions of *Melianthus* suggest allopatry was prevalent when speciation took place. However, the development of key novelties, (e.g. winter bloom, floral shifts, drought tolerance mechanisms), as manifested in *M. comosus*, subsequently promoted broad range expansion to such an extent that the genus could now be considered parapatric.

This study emphasises that adaptations to historical climate was a principal factor during the divergence of *Melianthus* with current day species largely locked into ranges determined by evolutionary habitat preferences. Dlamini (unpublished 1999) also raises the importance of edaphic factors, mentioning soil types as specific to each species. The most distinct of these are shown by *M. elongatus*, which inhabits the Quaternary calcified sands of the West Coast, and *M. villosus*, which inhabits basaltic formations along the eastern face of the Drakensberg. However, the remaining species are found on variably shale to granite-based soils indicating edaphic generalism. Rather more relevant in determining distribution patterns is a combination of factors involving water and temperature (Dlamini unpublished 1999), especially in relation to bloom time. Thus, *M. major* and *M. villosus*, which are largely confined to moister sites that are buffered against climatic change (Linder *et al.* 1992), behave as 'relictual' species (Richardson *et al.* 2001), their needs being rooted in a more mesic Oligocene past. Subsequently, *Melianthus villosus* has become restricted to a limited mid-elevation range on the wet face of the Drakensberg more by a need for a favourable high rainfall regime with a cost-resource appropriate temperature than by a specific soil type, with its spread being checked by increased extremes at both higher elevations and lower elevations. Likewise, *M. major* has been forced to retreat into riparian situations, not through a need for a specific soil type, but rather to obviate the drying conditions within its range, its spread into the more arid regions of the interior limited by its outsized stature. More recent speciation events, such as those responsible for the divergence of the WCC and the KEC, (save for *M. insignis*), were directly influenced by the dryer prevailing environments of the late

Miocene and early Pliocene. The aridification trends induced appropriate morphological changes, resulting in lowered height, further leaf dissection and summer dormancy.

Threat of winter frost keeps the WCC from expanding eastwards into the Karoo, primarily because their unprotected erect inflorescences would be subjected to frost damage during their winter bloom period. Northern expansion in the WCC may be limited by the extreme aridity of the Namib Desert. Southward expansion in the *M. pectinatus* complex is halted by the arid Knersvlakte valley on the edge of the Great Escarpment while *M. elongatus* is able to spread further south to the Langebaan Peninsula, using the Quaternary sands along the coastline as a corridor. Recent developments in the KEC (*M. dregeanus* and *M. insignis*) are still inflexible in their bloom time limiting them to a specific water regime at mid-altitudes in order to achieve an appropriate cost-ratio benefit allowing survival. The topographic homogeneity of the Karoo encouraged the greater vagility of *M. comosus* based on the key innovation of a shift in inflorescence position combined with a winter blooming period, thus allowing exploitation of a previously unavailable new adaptive zone

Granted ecological conditions greatly limit *Melianthus* in their native habitats, the genus seems more adaptable than perceived, implied by the success of most species in the horticulture industry, e.g. *M. major* and *M. comosus* (Everett 1981, Bailey 1914, Huxley 1992, van der Walt 2000), *M. villosus* (Pooley 1998), *M. pectinatus* (Bailey 1914, Elliovson 1955) and *M. gariepinus* (Archer 1997).

The hypothesis that floral diversification between *Melianthus* was driven by preferential selection from specific pollinators unique to each species' range (Dlamini unpublished 1999) remains to be thoroughly tested. However, the idea seems unlikely in view of the generalist pollination syndrome indicated by the broad suite of birds visiting the plants and by a complete lack of floral fusion within *Melianthus* that would have promoted specificity.

3.5 Conclusions

Diversification in *Melianthus*, although modest, correlates with the purported paleoclimatic events affecting southern Africa. Ancestral reconstructions suggest an origin in the Eastern Cape under initially moist mild conditions that gradually deteriorated into the colder and drier events that characterise the Oligocene Ice Cap formation. Miocene expansion may have been encouraged by a temporary mild

inclusion with the Orange River acting as a possible westward dispersal corridor. The earliest diverging taxa, *M. major* and *M. villosus*, retain properties of their ancestral environments, hence behave as 'relictual' species. The WCC diversified in the arid environment characterising the Pliocene events in southwestern Africa. The limited distributions of most of the taxa may be attributed to ecological constraints based largely on water availability and temperature in relation to soil types and floral phenology. Hence, although most of the species might be categorized as eco-specialists, *M. comosus* acts more as a generalist in its life history. Adaptation to bird pollination probably had strong influence on most of the floral divergence of *Melianthus* from *Bersama*. Tentative indications suggest that a suite of multiple bird lineages, comprising true nectivores, frugivores and opportunists, could act as effective pollinators for *Melianthus* making the genus a generalist in terms of its pollination syndrome. Initial indications from nectar evaluation suggest the largest volume of nectar is produced by the more mesic *M. major/M. villosus* grade with nectar production lowest in the WCC, correlating with a lower mean annual precipitation depicting the WCC environment. An inverse correlation between nectar volume and sucrose concentration (Cruden and Hermann 1983) is also indicated in the KEC and the WCC although further evaluation with a broad sampling is required for verification. Field experiments linking phenotypic variation to interspecific differentiations in reproductive success and survivorship (Schluter 2000) are warranted.

Chapter 4: General Conclusion and Future Directions

Following on the heels of Dlamini's (unpublished 1999) morphological assessment, this study confirms the monophyly of *Melianthus*. By sequencing data from multiple markers and revisiting Dlamini's morphological data set, it was possible to compare topologies obtained from several data sets for visual and statistical congruence both separately and in various combinations. Where multiple topologies exist, choosing the tree with the clearest resolution and highest support seems warranted (Cunningham 1997, Givnish and Systma 1997). In this study, it was argued that the combined molecular data set provides the strongest phylogenetic estimate of *Melianthus* relationships, having more resolution and greater support levels (10 nodes with $\geq$85% bootstrap) than any of the trees based on single genes, the combined plastid data, morphology or total evidence. Some authors argue that bias is introduced by leaving morphological data out of an analysis (Kluge 1989), yet in this study the inclusion of morphology resulted in uninformative polytomies, hence justifying its exclusion.

Although relatively similar, the topologies obtained from the combined molecular and the morphological data sets differ most noticeably in their treatment of *M. villosus*. Dlamini's morphological topology places *M. villosus* as sister to the WCC but with weak support (58% bootstrap). The combined molecular topology, instead, places *M. villosus* more decisively as sister to a clade comprising both the KEC (*M. comosus* and the *M. dregeanus* complex) and the WCC (*M. elongatus* and the *M. pectinatus* complex) with good support (81% bootstrap). Granted Dlamini's hypothesis, placing *M. villosus* with other seemingly similar erect-inflorescence taxa in the WCC, seems reasonable from a floral morphology perspective, it lacks geographic sense when considered both in terms of present species distributions and ancestral range reconstructions.

Monophyly of two widespread species, *M. major* and *M. comosus*, supports their recognition at species level, the low variation in the latter perhaps reflect the recency of spread. However, the specific status of the *M. dregeanus* complex (including *M. insignis*) and the *M. pectinatus* complex (including *M. gariepinus*) is open to question.

From a taxonomic perspective, the apparent paraphyly of *M. pectinatus* can be addressed in three ways: (1) maintain the status quo and consider *M. pectinatus* a 'paraspecies' (Crisp and Chandler 1996), (2) conservatively sink all three taxa into a 'metaspecies' for the purpose of enforcing monophyly (Crisp and Chandler 1996), or (3.) elevate *M. gariepinus* to specific status as originally proposed by Merxmüller and Roessler (1968), thus creating three species instead of two for the WCC. Given the age of diversification for *M. gariepinus* (~4 - 5 mya), it seems reasonable to argue that enough time has elapsed to assure its genetic isolation, thus supporting its recognition at species level. However, a full assessment of species state for *M. gariepinus* will require a more comprehensive biosystematic sampling, involving collections from both sides of the Orange River.

As with *M. pectinatus*, the subspecies status afforded the taxa in the *M. dregeanus* complex is open to question (Dlamini unpublished 1999), prompting Dlamini's proposal to raise *M. dregeanus* and *M. insignis* to specific status based on morphological differences (see also Dyer 1952, 1959). This deserves further exploration, particularly given the 400 km disjunction between the subspecies despite the recency of divergence (~0.34±0.06 mya - 0.39±0.07 mya).

Although geographical isolation has been cited as the principal mechanism maintaining genetic integrity between sister taxa (Dlamini unpublished 1999), the evidence for this seems thin given the overlap along range edges and the synchronous bloom periods in closely related species, such as those belonging to the WCC. Testing the potential for reproductive compatibility may be warranted, particularly in sympatric locations such as that used for field observations in Chapter 3. Bailey (1914) mentions '*M. intermedius*', a hybrid between *M. comosus* and *M. major*, existed in American horticulture but whether it was the result of a naturally occurring or man-made cross is unclear.

Ecological and temporal reconstructions suggest *Melianthus* species mirror the historical environments in which they arose. *Melianthus* probably originated in the eastern parts of South Africa during the mid-Oligocene in a mild, moist period. Current habitat preferences of the earliest diverging species in *Melianthus*, *M. major* and *M. villosus,* suggests that these over-sized taxa behave as relicts from a mesic past. The moisture needs of *Melianthus major* force it into riparian habitats, while *M. villosus* restricted to a narrow, mid-elevational belt of the eastern Drakensberg in a summer-wet environment. Confinement to aseasonally moist microclimates has

previously been cited (Linder *et al.* 1992) as evidence of a 'relictual' status in ancient, taxonomically isolated African genera such as *Brabejum* (Protaceae), *Hartogiella* and *Maurocenia* (Celastraceae), *Heeria* (Anacardiaceae), *Metrosideros* (Myrtaceae), *Platylophus* (Cunoniaceae), and *Smellophyllum* (Sapindaceae). Eventual Ice Cape formation led to exaggerated seasonality accompanied by cooler temperatures and increasing dryness, which would have promoted the shrubbier, semi-herbaceous, seasonally deciduous nature of *Melianthus* accompanied by its more highly dissected foliage than that seen in its sister genus, *Bersama*. Range expansion in *Melianthus* may have been encouraged by a temporary mild, mesic interlude in the early-to-mid Miocene, with possible utilization of the Orange River as an important westward dispersal corridor. Subsequent diversification in the WCC occurred under the decidedly more arid conditions characterising the drier Miocene/Pliocene boundary. Although coinciding with the same aridification events that purportedly lead to 'explosive radiation' in other western South African taxa (Verboom 2000, Goldblatt *et al.* 2001, Hartmann 2001, Richardson *et al.* 2001), the WCC responded in decidedly more modest fashion by producing only three new taxa here.

Due to the energy expense associated with flowering, bloom time in *Melianthus* appears to be rather labile, being dictated by water and temperature constraints. Winter bloom in the WCC occurs as a response to the winter rainy period that characterises the West Coast climate. Due to the mild winter temperatures, bloom occurs unhindered, with the WCC taxa all retaining an ancestral condition of erect inflorescences held above the foliage. In the case of *Melianthus comosus*, winter bloom evolved in conjunction with a novel lateral floral position beneath the foliage canopy, forming a favourable microclimate that alleviates frost damage. The two traits may function as a 'key innovation' (*sensu* Givnish 1997) possibly allowing *M. comosus* the opportunity to exploit the extensive central plateau of South Africa.

This study argues that most morphological differences characterising *Melianthus* can be viewed as a reaction to the physical environment. However, other characteristics unique to the genus may be more effectively explained by biotic interactions. The petrol-like aroma of the toxic foliage (Von Marilaun 1985) presumable arose as an herbivory defense while floral morphology, beyond inflorescence orientation in this case, might be more effectively explained in terms of pollinator preference (Johnson 1996). The latter introduces the possibility that speciation in *Melianthus* may have been driven by pollinator specificity (Dlamini

unpublished 1999). That the floral divergence in *Melianthus*, as it relates to its sister genus *Bersama*, indicates a strong correlation with ornithophily (Faegri and van der Pijl 1979, Rebelo 1987, Proctor *et al.* 1996, Cheke *et al.* 2001) is not in dispute. What is unclear, however, is who the avian pollinators of *Melianthus* are. Most indications point to an association with sunbirds, the Nectariniidae, (Scott-Elliot 1890, Von Marilaun 1895, Burtt Davey 1932, Vogel 1954), but suggestions of co-exclusivity between sunbirds and *Melianthus* seem dubious (Marloth 1908, 1925, Skead 1967, Maclean 1993). Data presented here indicate, instead, that *Melianthus* behaves as a generalist in terms of its pollination syndrome. Such a strategy may be necessitated by a lack of consistency associated with most sunbird lineages (Cheke *et al.* 2001) plus the overall reduction in the number of specialised nectar feeders able to effectively survive year-round within the drier ranges much of *Melianthus* inhabits (Skead 1967). Therefore, it seems more likely that it is the habitat that predicates the floral morphology in *Melianthus*.

Future Areas For Exploration

It is hoped that this thesis reflects the inherent potential of *Melianthus* as an object for further studies on any of a number of fronts. Since more questions have potentially been raised than answered, further avenues to explore are listed below.

i Nectar colouration:

Reconstructions presented in this thesis suggest that in *Melianthus* black nectar arose multiple times, or possibly once (less parsimonious), from an ancestral tan coloured nectar. The adaptive benefits (if any) of colouration in nectar remain unclear, but on current evidence, its role as part of a collective avian signal seems most likely. Further investigation into the role of nectar colouration may be worth pursuing if only for its presumed rarity (Olesen *et al.* 1998). Besides Olesen *et al.*'s (1998) report of red nectar in *Nesocodon* spp. from the Mauritius Islands, yellow nectar was found in *Aloe striata* and brown nectar was observed in both *Grevillea robusta* and *Phormium tenax*, which are all ornithophilous taxa. The syndrome of nectar colouration, therefore, may not be as rare as speculated. HPLC analysis of nectar from other angiosperm lineages featuring colouration in nectar would be useful to determine if any specific chemical composition recurs.

ii Water relations:

The prodigious nectar production in *Melianthus* (overall mean volume of ~50µl across all species sampled) paired with its variably arid habitat suggests that water relations, particularly in relation to nectar output, are worthy of investigation. Initial indications point to different clades producing differing volumes and sucrose concentrations in nectar, possibly reflective of the conditions in the environments they occupy. Verifying this hypothesis with further sampling seems warranted.

iii Pollinator specificity:

Further field observations of *Melianthus* across its range are required to corroborate the hypothesis this study presents that the genus employs a generalist pollination strategy. Locating and observing sympatric populations of *Melianthus* at additional localities will also be helpful in determining whether certain birds, even as a collective guild, associate with particular *Melianthus* species, or whether specificity of any type is completely lacking.

iiiv Reproductive success

Studies testing the physiological implications of having erect/exposed inflorescences versus lateral/concealed inflorescences with respect to temperature differentiation and its effect on floral life history would be worthy of pursuit to try and quantify the notion that the floral strategy employed by *M. comosus* is somehow more beneficial than that, of say, *M. major* in relation to reproductive capacity. Seed set experiments may also help indentify whether *M. comosus* is able to bring a greater number of seeds to maturation through alleviation of insect predation, as hypothesized, than currently seen in *M. major* and perhaps other more restricted taxa as well.

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Appendix

Digital data matrices are provided with this thesis in the form a CD diskette. Character sets are defined on the diskette including all exclusions used during analyses.