University of Cape Town

Phylogenetic relationships of the African species of the genus *Merxmuellera* Conert (Poaceae: Danthonioideae)

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In memory of my parents:

Grass

I have written of dawn, of the moon, and the trees;
Of people, and the flowers, and the song of bees.
But over these things my mind would pass,
And come to rest among grass

Grass so humble, that all things tread
Its tender blades. Grass - the bread,
The staff of life; a constant need
Of the man and beast - a power indeed...

God in His wisdom gave many friends
To grace our way, as along it wends
But the grandeur of many, my mind would pass,
And come to rest among grass...

- Mabel Duggan

Note

DNA sequences are available at GenBank or on request from Dr. Nigel Barker at the Department of Botany, Rhodes University.

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ABSTRACT

The subfamily Danthonioideae has been the subject of many investigations at both molecular and morphological level. DNA sequences have contributed towards our understanding of both the generic composition of the subfamily, the relationships within and between the subfamily and other grasses. All previous, anatomical, embryological and molecular studies have indicated that within the Danthonioideae, the African genus *Merxmuellera* Conert, is highly polyphyletic and needs urgent taxonomic attention. This genus comprises 17 African and two Madagascan species. This study expands on existing molecular (*rpo*C2 and ITS) and morphological data sets to further test the hypothesis of non-monophyly of *Merxmuellera sensu lato*. The results reveal that despite high levels of resolution, the morphological data yield poorly supported phylogenies due to high levels of homoplasy in the dataset. Analyses of the partitioned and combined molecular data sets provide topologies that are better supported and retrieve groups of genera in the whole study group but their relationships remains obscure. Topologies from the total evidence data set are well supported and indicate that within *Merxmuellera* s.l. there are four lineages, three of which are monophyletic: A monophyletic *Merxmuellera* s.s (*M. drakensbergensis, M. stereophylla, M. aureocephala, M. macowanii* and type species *M. davyi*) resolved basal to the rest of the danthonioid grasses; a monophyletic Cape *Merxmuellera* group (*M. decora, M. rufa, M. lupulina, M. arundinacea* and *M. cincta*); a monophyletic *M. rangei* - *M. papposa* group resolved outside the major clade containing the rest of the members of the subfamily.

Four species of *Merxmuellera* including *M. guillarmodiae; M. stricta, M. disticha* and *M. dura* are retrieved in a clade termed the '*Rytidosperma* Clade', which includes the Australasian genera *Rytidosperma,*

Austrodanthonia, Notodanthonia and Joycea as well as the African *Karroochloa, Schismus* and *Tribolium*;

This study thus, supports the hypothesis that *Merxmuellera* s.l. is polyphyletic and concludes that two new genera are recognisable within this genus, one comprising of the Cape *Merxmuellera* species and the other comprising of *M. rangei* and *M. papposa*. Furthermore, this study corroborates exclusion of the latter group as a separate genus in the Chloridoideae as hypothesised by previous studies. The monophyletic *Merxmuellera* comprises of the Drakensberg species and possibly the Madagascan species. Taxonomy of the four *Merxmuellera* s.l. species in the *Rytidosperma* clade remains uncertain and further work is needed in this clade before any meaningful conclusions about the interspecific relationships can be made.

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CHAPTER ONE

INTRODUCTION

1.1 General introduction

The grasses family, Poaceae, is one of the most important plant families, in numbers of individuals, biomass, area covered, diversity of habitats and value to man. As much as 30% of land area on earth is covered in natural grassland and savanna vegetation chiefly dominated by grasses (Walter, 1979). The family consists of approximately 10 000 species grouped into about 770 genera (Campbell, 1985, Dahlgren et al. 1985, Watson and Dallwitz, 1992, 1994; Renvoize and Clayton, 1992). In southern Africa, it consists of some 194 genera and 967 species and infraspecific taxa, of which 115 are exotic and 847 are indigenous, with 329 endemics (Gibbs Russell *et al.*, 1990).

In the past years, this family was allied to Cyperaceae and Juncaceae (Cronqist, 1981), but recent phylogenetic studies based on DNA data and morphology have indicated that it is allied to Restionaceae and Joinvilleaceae (Stebbins, 1982; Kellogg and Linder, 1995; Campbell and Kellogg, 1987; Linder and Rudall, 1993). However, Poaceae stands apart on numerous technical characteristics derived from embryology, and caryopsis and floral morphology. The embryo in grasses has a flat haustorial cotyledon and outer sheath, the coleoptile, that protects the plumule during germination (Negbi, 1984). A singled-seeded dry and indehiscent fruit with the pericarp fused to the seed coat in the hilar region are unique features of grasses (Dahlgren *et al.*, 1985). The inflorescence of grasses consists of units known as spikelets. The generalised spikelet consists of an assemblage of bracts borne on the rachilla. The basal-most bracts (glumes) are empty scales protecting the immature scales (Renvoize and Clayton, 1992) and the rest of the bracts (lemmae) are borne above with each holding a reduced floral axis bearing a prophyll-like structure called a palea. The lemma and the palea together

enclose two to three reduced perianth parts (lodicules), the androecium and the gynoecium together form a functional unit called a floret. There is a broad range of variation in the structure, size and shape of the spikelets and, this has been of great value in identification and classification for the grasses at all taxonomic levels.

1.2 Classification and the subfamily Danthonioideae

The monophyly of Poaceae has been extensively studied from basic morphology, but with addition of information using several other taxonomic techniques it has undergone several stages of reclassification (Gibbs Russell *et al.*, 1990). The first set of classification systems in grasses was largely based on inflorescence and spikelet morphology and these date as far back as 1814 when Robert Brown first circumscribed the grasses into two tribes, the Paniceae (equivalent of Panicoideae in modern classifications) and Poaceae (equivalent of Festucoideae of) based on spikelet morphology. He is the first to describe the grass spikelet and noted the basal reduction of the spikelets in the Paniceae and apical reduction in the Poaceae. In addition Brown (1814) noted that the Paniceae tend to prefer warm climatic conditions while the Poaceae prefer cool climates (Pohl, 1987).

Endlicher (1836 – 1840) subdivided the grasses into 13 tribes and did not recognise Brown's classification system. However, Brown's classification system was taken further in the late nineteenth century by Bentham and Hooker, (1883) and in the early twentieth century by Hitchcork, (1935). Bentham and Hooker, (1883) divided the grasses into two series, the Panicaceae and the Poaceae. The Panicaceae were distinguished by disarticulation below the spikelet and possession of a single terminal floret, and this included the traditional panicoid genera, as well as the Oryzeae, Tristegineae and Zoysieae (Pohl, 1987). The Poaceae were distinguished by disarticulation above the glumes and the terminal reduction of the florets and it included several of the traditional arundinoid genera, the bambusoids, and the chloridoids (Pohl, 1987). Hitchcork's

(1935) classification was more or less similar to that of Bentham and Hooker (1883), differing only in that Hitchcork's classification placed the bamboos and the pooids first and Adropogoneae were put last.

The next set of classifications systems were based on cytology and leaf anatomy, the most significant being that of Tateoka (1957), which marked the beginning of a detailed reassessment of evolutionary relationships among grasses. Addition of data from various aspects of biology including embryology (Reeder, 1962), starch grains (Tateoka, 1957), lodicules (Jirásek and Jozifová, 1968) and leaf anatomy (Metcalfe, 1960) resulted in a number of grass classification systems being published (e.g. Prat, 1960; Stebbins and Crampton, 1961; Caro, 1982; Clayton and Renvoize, 1986; Conert; 1987; Tsvelev, 1989; Watson and Dallwitz, 1992, 1994). The number of subfamilies recognised in these classifications systems ranged from two (Tsevlev, 1989) to 13 (Caro, 1982). The most significant change in several of these systems from that of Brown (1814) was the division of the Pooideae into several different subfamilies while the Panicoideae was retained (GPWG in press). In the recent literature, the Clayton and Renvoize, (1986) and Watson and Dallwitz, (1992, 1994) classification systems which recognised six and five subfamilies respectively, have been widely used (see Table 1.1 for comparison of these classifications systems). However, there have always been reports that some subfamilies are not sharply defined taxonomically as they lack meaningful diagnostic features to distinguish them from other subfamilies. For example, the Arundinoideae have always been considered morphologically heterogenous and a problematic subfamily (Renvoize,1981; Campbell, 1985; Clayton and Renvoize, 1986; Conert 1987; Ellis 1987 Kellogg and Campbell 1987; Watson 1990). Lack of reliable diagnostic features discriminating this subfamily has in most cases resulted in a number of conflicting tribal level classifications (e.g. Clayton and Renvoize, 1986; Conert, 1987; Watson,1990).

It is only in the past decade and a half that phylogenetic methods have been applied in an attempt to produce explicit phylogenetic hypothesis

within the grass family (e.g. Kellogg and Campbell, 1987; Davis and Soreng, 1993; Soreng and Davis, 1998; Hsiao *et al*, 1998; Barker *et al*, 1999).). Most of these studies have resulted in well supported relationships within Poaceae, the results of which led the Grass Phylogenies Working Group (GPWG in press) to attempt providing the first family-wide subfamilial classification of the grasses based on phylogenetic principles. Based on eight data sets (one structural, four plastome and three nuclear), the GPWG inferred a phylogeny from which twelve subfamilies were recognised. The major changes in the GPWG subfamily classification have been the break up of the Bambusoideae and Arundinoideae and the expansion of the Pooideae. In this classification, elements of the traditional Arundinoideae are now recognised as the Aristoideae, Arundinoideae s.s. and the newly erected subfamily Danthonioideae. In this study we follow this new classification to determine the taxonomy of *Merxmuellera* Conert in the context of the rest of the Danthonioideae.

The subfamily Danthonioideae has been elevated from Danthonieae (sensu Watson and Dallwitz, 1992, 1994), a tribe that has received considerable attention over the second half of the last century (e.g. De Wet 1954, 1956, 1960; Conert 1966, 1970, 1971, 1987; Conert and Tuerpe 1969; Connor and Edgar 1979; Verboom et al., 1994, Barker et al. 2000). Presence of haustorial synergids in the ovule and distant styles have been considered synapomorphies uniting this subfamily (Verboom et. al 1994) excluding *Merxmuellera rangei* in which these characteristic features were reported lacking. The generic composition of this subfamily has been recently established in a preliminary study by Barker *et al.* (2000), which recognised seven informal groups of genera (Table 1.2). This was based on a phylogeny derived from combined analysis of molecular (*rbcL*, *rpo*C2 and ITS) and morphological data sets. However, pending the redefinition of genera such as *Cortaderia* Stapf and *Merxmuellera*, a clear picture of the actual generic composition within this subfamily is still uncertain.

Table 1.1. Comparison of the current subfamily classification systems of Poaceae in use.

Subfamilies in bold indicate those subfamilies divided from Arundinoideae or Bambusoideae in the GPWG classification system. Abbreviations: GPWG = Grass Phylogenies Working Group

Clayton and Renvoize (1986)	Watson and Dallwitz (1992; 1994)	GPWG (in prep.)
Arundinoideae	Arundinoideae	Arundinoideae
		Aristoideae
		Danthonidoideae
Bambusoideae	Bambusoideae	Bambusoideae
		Puelioideae
		Ehrhartoideae
		Anomochlooideae
		Pharoideae
Chloridoideae	Chloridoideae	Chloridoideae
Panicoideae	Panicoideae	Panicoideae
Centothecoideae		Centothecoideae
Pooideae	Pooideae	Pooideae

Table 1.2. Current generic composition of Danthonioidae as circumscribed by Barker et al. (2000).

Group/Clade	Genus	Distribution
Rytidosperma clade	*Rytidosperma*	Australia
	Austrodanthonia	Australia
	Notodanthonia	Australia
	Tribolium	Australia
	Joycea	Australia
	Karroochloa	Africa
	Schismus	Africa
	Merxmuellera disticha	Africa
	M. guillarmoidae	Africa
	M. disticha	Africa
	M. dura	Africa
Pseudopentameris clade	*Pseudopentameris*	Africa
	Chaetobromus	Africa
	Plinthanthesis	Australia
Danthonia clade	*Danthonia*	South America
	Cortaderia	New Zealand
	Cortaderia	South America
Cortaderia "A" clade	*Lamprothyrsus*	South America
	Pentaschistis	Africa
Pentaschistis clade	*Pentameris*	Africa
	Prionanthium	Africa
	Chionochloa	New Zealand
Chionochloa clade	*Merxmuellera*	Africa
Basal *Merxmuellera* assemblage		

1.3 The genus *Merxmuellera* Conert

The genus *Merxmuellera* comprises seventeen species in southern African and two species endemic to Madagascar. It was first recognised by Conert (1970) and named in honour of Professor Herman Merxmüller, a distinguished contemporary German botanist who worked largely on the Flora of Namibia. In the original description of the genus, Conert (1970) recognised fourteen species previously included in the then broadly defined *Danthonia* DC. It was segregated on the basis that it deviates quite remarkably from *Danthonia* in many crucial features, especially of their floral morphology, making these two genera not even closely related (Conert, 1970). Most of the *Merxmuellera* species are tall and densely tufted perennials with persistent tough leaf-sheaths. Their leaf blades are usually narrow, permanently rolled or nearly so with the striations only conspicuous on the upper surface. The inflorescence is paniculate, contracted and occasionally spike-like (*M. disticha*) (Gibbs-Russell *et al.*, 1990). The spikelets are large, many-flowered, often gold-yellow in colour, with thin glumes and lemmas. Their glumes are only 1-nerved or rarely have a few additional lateral nerves while the lemmas are either irregularly pilose with hairs arranged in individual tufts or only near the margins. Based on these characters Conert (1971) mentioned that there is no genus among African grasses to which this genus shows any affinity, but their habit, formation of leaf sheaths and their tendency to unite the lateral lobes of the lemma with the lowermost part of the awn link *Merxmuellera* with *Chionochloa* in New Zealand and *Cortaderia* in South America.

In 1971, Conert segregated three more species from *Danthonia* and referred them to *Merxmuellera* as *M. decora*, *M. lupulina* and *M. rufa*. Although these three species share some characters typical of *Merxmuellera*, they are however, a morphologically distinct group within the genus. They can easily be distinguished from other species of *Merxmuellera* by their distinct bulbous bases that are deeply sunken into the ground (Linder and Ellis, 1990). In addition, the lower and sometimes

even the upper leaf sheaths are covered with woolly hairs on the outer surface. This characteristic woolliness is not found in any other *Merxmuellera* species (Chippindall, 1955). In 1975, Conert described an additional species of *Merxmuellera* and named it *M. guillarmoidae*. This particular species was described from the collections made by Killick in the Cathedral Peak in the eastern escarpment of Natal/Lesotho Drakensberg initially identified as *M. stricta*. Conert noted that specimens from this area differ from the typical *M. stricta* in length and arrangement of hairs on the lemma.

Merxmuellera setacea was the last species to be described in this genus by Barker and Ellis (1991). This species is distinguished from other members of the genus with its distinct thickened and swollen sheath base covering the rhizome and axilliary buds. Only the *M. lupulina, M. rufa* and *M. decora* group has swollen sheath bases but this differs from *M. setacea* in having basal sheaths that are densely woolly and unthicked. Furthermore, the lemma of *M. setacea* has one tuft of long, white, submarginal hairs on each side of the lemma body. Barker and Ellis (1991) noted that these tufts of hairs on the lemma link *M. setacea* to other southern African danthonioid genera such as *Karroochloa* Conert and Türpe, *Schismus* Beauv. and the chloridoid genera such as *Dregeochloa* Conert and *Centropodia* Reichenb. Although this character is present in neither *Pentaschistis* nor *Pentameris*, the anatomical investigation by Barker and Ellis (1991) places this species intermediate between these later genera and *Merxmuellera*. However its position in *Merxmuellera* was justified on the basis that it possesses typical *Merxmuellera* floral morphological characteristics such as spikelets with 3-10 florets, glumes variously nerved and lemmae which are irregularly pilose or with hairs arranged in tufts (Conert 1970, 1971)

1.3.1 Distribution of *Merxmuellera* species in southern Africa

The fynbos flora found in the winter rainfall areas of southern Africa is remarkably diverse in species composition and high levels of

endemism (Goldblatt and Manning, 2000). This floristic region is typically characterised by Proteaceae, Restionaceae and Ericaceae (Taylor, 1978). Although grasses are in most instances not considered very important in this region, they often exhibit interesting and unique adaptations and biology (Linder and Ellis, 1990) with many genera being endemic to the region. Many of these endemic grasses belong to the subfamily Danthonioideae. Although grasses rank very high in most floras, it is only the 13th largest family in the Cape floristic region (Goldblatt and Manning, 2000). Linder (1989) reviewed the phytogeographical patterns inherent in the grasses of the Cape Floristic Region, and showed that several taxa may help elucidate the origins and evolution of the Cape Flora. The genus *Merxmuellera* has its centre of diversity in this winter rainfall region of the Cape where many species are associated with the fynbos biome (Barker in Gibbs-Russell *et al.*, 1990). Some species do however occur further north in the summer rainfall areas in the sub-alpine and the alpine belt of the Drakensberg. The winter rainfall species, including *M. cincta* (Nees) Conert, *M. arundinacea* (Bergius) Conert, *M. decora* (Nees) Conert, *M. lupulina* (Thunberg) Conert, and *M. rufa* are endemic to the Cape flora. One of the Cape *Merxmuellera* species, *M. setacea,* is currently known to occur in two localities, being recorded only from Skurweberge on Blinkberg Pass in Wuppertal district as well as from its type locality in the Groot Winterhoek Wilderness area, north-west of Groen Mountain Suurvlakte plateau in the south-western Cape. *M. papposa* (Nees) Conert is also poorly collected and has so far been known to occur in few localities on dry, rocky river beds in the eastern Cape near Port Elizabeth. *M. dura* (Stapf) Conert and *M. rangei* (Pilg.) Conert are the two southern African *Merxmuellera* species that occur on dry rocky or sandy habitats in the Nama-Karroo in the north-western parts of the winter rainfall region of the Northern Cape. The distribution of *M. rangei* extends further north to Namibia in the Lüderitz-Süd district and in the Aus area as well as further south between Witputz and Lorelei (Ellis, 1982). Two species, *M. stricta* (Schrad.) Conert and *M. disticha* (Nees) Conert, are important

constituents of the Karrooid *Merxmuellera* Mountain Veld along the higher mountains of the False Karroo and the Central Upper Karroo (Acocks, 1975). They occur widely from Namaqualand in the north-east to the south western Cape, then eastwards to the north-eastern mountains from where the distribution continues in a northerly direction to the Drakensberg mountains in Lesotho and the eastern Free State (Ellis, 1980).

Other species including *M. drakensbergensis* (Schweick.) Conert, *M. stereophylla* (J. G. Anders.) Conert, *M. guillarmoidae, M. macowanii* (Stapf) Conert and *M. aureocephala* (J. G. Anders.) Conert are restricted to the summer rainfall region around the alpine areas of the Maloti Mountains in Lesotho and the Lesotho/Natal Drakensberg range along the eastern escarpment of southern Africa. *M. macowanii* and *M. drakensbergensis* are conspicuous elements of the alpine vegetation of this alpine belt (Anderson, 1960). They are adapted to marshy areas along streambanks and mud patch communities (Killick, 1963; Edwards, 1967) of the alpine belt along the summit of the high Drakensberg. On the other hand *M. stereophylla* has a restricted distribution, being only common in the dry alpine grassland of rocky basaltic areas as a crevice and ledge plant at altitudes above 2 000 m. *M. guillarmoidae* and *M. aureocephala* have a restricted distribution on ridges in the relatively lower altitudes of the Drakensberg around the Cathedral and the Cathkin Peak areas. The type species, *Merxmuellera davyi* (C. E. Hubbard) Conert, has a wide distribution extending from Marieskop in the Mpumalanga Drakensberg mountains in South Africa to Inyanga mountains of Zimbabwe and Mount Mulanje in Malawi (Conert, 1975).

Merxmuellera, as presently defined, is an African genus. However, Clayton and Renvoize (1986) included this genus in *Rytidosperma*, a concept that was rejected by Verboom, *et al.* (1996). Linder (1989) thus commented that until the generic limits in this group are defined, it becomes difficult to interpret correctly the phytogeographical import of the danthonioid grasses to the Cape flora.

1.3.2. Leaf anatomy studies

Ellis (1980a; 1980b; 1981a; 1981b; 1982; 1983) provided detailed accounts on the leaf anatomy of almost all the species of *Merxmuellera,* except *M. papposa*. In general, the observations made from those accounts have instigated some doubts on the current taxonomic status of some species as well as that of the genus itself. Several intraspecific anatomical variables within some members of this genus have been discovered of which some show close similarities to other species in the genus or even other species in other closely related genera. In this respect, *M. disticha* is considered the most morphologically distinct southern African representative of this genus (Ellis, 1980a). This species can be easily recognised by an inflorescence characterised by an oblong, uninterrupted, distichous spike. Studies on the leaf anatomy of *M. disticha* revealed that there are three distinct anatomical forms within this species including typical *M. disticha*, and Drakensberg and Alpine bog forms which also show distinct vegetative and habitat differences (1980a). According to Ellis (1980a), these anatomical forms are distinctly disjunct with characters being structurally different e.g. adaxial ribs, sclerenchyma girders, silica bodies and the bulliform cells. Moreover, each of these forms is characterised by a number of correlated characters, from both the leaf blade in section and the epidermis. Morphologically, the Drakensberg and the Alpine bog forms are characterised by 2-flowered spikelets, a character which links them with *Pentaschistis*, while the typical *M. disticha* has 3 or more florets per spikelet typical of the core *Merxmuellera*. The typical *M. disticha* and the Drakensberg form have lower glumes which are always distinctly 3-nerved (Chippindall, 1955), while the Alpine bog form has a single prominent nerve with 2 poorly developed lateral nerves (Ellis, 1980a).

This type of intraspecific variation existing in *M. disticha* has also been observed in *M. stricta*, a highly variable perennial, forming coarse, wiry tufts. Variation in *M. stricta* was reported earlier by Chippindall

(1955), who states that "There is a considerable variation in the plants referred to *Danthonia stricta*, and it is possible that they comprise more than one variety". Individuals of this species occurring in Namaqualand in the north-west can be easily confused with those of *M. dura*, but *M. stricta* can be distinguished by glabrous condition of the lemma at the point of insertion of the central awn (Ellis, 1980b). Consistent with their high morphological variability, Ellis (1980b) has also revealed that four anatomical forms exist within this species and these include, in addition to typical *M. stricta*, the Cathedral Peak, the Alpine bog and Drakensberg forms. These are also correlated with morphological and habitat differences. A situation similar to that observed in *M. disticha* exists where the typical *M. stricta* occurs in the north-east Cape mountains, while the Alpine and Drakensberg forms occur in the alpine region of Drakensberg similar to the other two forms of *M. disticha*. Ellis (1980b) indicated that the material from the alpine region of the Drakensberg cited by Conert (1975) as belonging to *M. guillarmoidae* falls into two of the anatomical forms, the Cathedral Peak and the Alpine bog forms and these closely resemble each other morphologically. However, Benesch (1995) kept these two forms (Cathedral Peak and Alpine bog forms) under *M. guillarmoidae*. The habitat preferences, growth form and leaf anatomy of the alpine form of *M. guillarmoidae* is shown to resemble the alpine bog form described in *M. disticha*. Ellis (1980b) observed that anatomically the two alpine forms of these two species bear similarities in the rib and furrow distribution and form, mesophyll configuration and epidermal structure. The pattern of arrangement of the various orders of vascular bundle along the width of the lamina varies in these two forms but corresponds with patterns observed in either typical *M. stricta* or typical *M. disticha.* Ellis (1980b) suggested that this observation indicates convergent evolution of these taxa in response to similar environmental conditions in these areas. There are some *Pentaschistis* species (e.g. *P. tysonii* Stapf) that display more leaf anatomical similarities with *M. stricta* than they do with typical *Pentaschistis* species. Ellis (1980b) noted this

as an indication that the generic limits of *Merxmuellera* may need redefinition before any further new species can possibly be described from these anatomical forms in this region.

The two conspicuous elements of the alpine vegetation of the Drakensberg, *M. drakensbergensis* and *M. stereophylla* similarly pose some taxonomic problems (Ellis, 1981a). Although geographic distribution of these two species overlaps to a great extent, they can, however, be distinguished morphologically and ecologically. *M. drakensbergensis* occurs on the moist areas along the streambanks and mud patch communities of the alpine belt along the summit of the Drakensberg while *M. stereophylla* on the other hand is restricted to the dry alpine grassland in the Drakensberg (Killick, 1978). Morphologically, *M. drakensbergensis* has olive-green soft leaves and grows to about 100 cm tall whereas *M. stereophylla* plants are shorter (80 cm high) and have grey-green leaves that are rigid and erect (Anderson, 1962). These two species are thought to be closely related to three other species of *Merxmuellera*: *M. macowanii, M. davyi* and *M. aureocephala,* the wiry, tufted and tussock forming perennial grasses occurring in the Drakensberg (Anderson 1962). The habitat preferences of *M. macowanii, M davyi* and *M. aureocephala* closely resemble the niches occupied by *M. drakensbergensis* and *M. stereophylla. M. macowanii*, similar to *M. drakensbergensis*, is frequent along mesic streambanks and in marshy areas of montane and subalpine belts of the Drakensberg, while *M. davyi* and *M. aureocephala*, similar to *M. stereophylla,* occupy xeric habitats on steep and rocky slopes of the mountain grassveld. Ellis (1980b, 1981a) examined the relationship between these five species based on anatomy and reported that two groups exist which are morphologically very similar, a group consisting of the *M. drakensbergensis and M. stereophylla,* and that consisting of *M. macowanii and M. davyi. M. drakensbergensis* and *M. stereophylla* are reported to show anatomical similarities with each other than to either *M. macowanii* or *M. davyi,* the leaf anatomy of the later two also showing close similarity.

The close anatomical similarity between *M. drakensbergensis* and *M. stereophylla* has led Ellis (1981a) to question their status at the specific rank, as there are no consistent and measurable structural differences in the leaf sections and the epidermides of these two species. The only difference noted was the cross sectional area and width of the leaf sections. This difference (although not consistent because several intermediates occur), has been associated with the fact that *M. drakensbergensis* generally has larger leaf blades than *M. stereophylla* (Ellis 1981a). However, this variation in size has been correlated with the number of vascular bundles in the leaf blades which are 12 in *M. stereophylla* and 13 or 15 in *M. drakensbergensis.* As a result, Ellis (1981a) indicated that anatomical characters appear to be in conflict with ecological and morphological characters thus bringing some doubt on holding these two species separate. Moreover, the type of arrangement of vascular bundles along the width of the lamina in these two species, characterised by the absence of third order vascular bundles between the lateral first order vascular bundles, is similar for both species. This condition has been observed in typical *M. stricta* (Ellis 1980b) and is thought to indicate a relationship of these species to *M. stricta* (Ellis, 1981a).

The almost identical situation to the one observed between *M. drakensbergensis* and *M. stereophylla* also exists between *M. macowanii* and *M. davyi.* Although these two species are morphologically and ecologically separable, anatomical evidence appears to be in conflict as the observations made suggest that these two species are very similar anatomically, both in leaf transverse section and in the abaxial epidermis (Ellis, 1981b). *M. davyi* only differs from *M. macowanii* in that is has narrow leaves with fewer vascular bundles per section (Ellis, 1981b). This apparent conflict of leaf anatomy with morphological and ecological indications has also called attention on the justification of the specific status of the species, thus also calling for their taxonomic re-assessment.

Anatomical examination of *M. aureocephala* (1981b) shows this species to be somewhat intermediate between *M. drakensbergensis-M. stereophylla* and *M. macowanii-M. davyi* groups, thus suggesting a possible link between these groups. Although the taxonomy of *M. aureocephala* is also a bit shaky, it however appears to be very important in understanding the relationships within this alpine *Merxmuellera* group. This species is only known from very few records, all of which are restricted areas in the subalpine belt of the Cathedral Peak and Cathkin Peak areas of the Drakensberg. Although it appears to be morphologically very similar to *M. davyi* except that it has larger spikelet parts and lanceolate glumes instead of narrowly lanceolate as is the case with *M. davyi*, the only diagnostic character distinguishing it from other four members in this group is that it flowers in winter while the rest flower in summer (Anderson, 1962). As a result, Ellis (1981b) has argued that it is possible that plants assigned to *M. aureocephala* may actually represent early or late flowering forms of some other *Merxmuellera* species in this summer rainfall area. The anatomical complexities in these summer-rainfall *Merxmuellera* species as noted by Ellis, may be an indication that this temperate region of high altitudes has only relatively recently been colonised by these typically winter rainfall grasses from the Cape region. The poor differentiation among the species may be the result of recent adaptive radiation of these species.

The anatomical studies on the summer rainfall *Merxmuellera* species (Ellis 1980a, 1980b, 1981a, 1981b) suggest that at least 12 entities can be recognised. This led Ellis (1981b) to suggest that the most practical taxonomic treatment of *Merxmuellera* species in the alpine region of the Drakensberg mountains appears to be recognising only two species, *M. stricta* and *M. disticha*, with numerous infraspecific taxa, possibly of subspecific rank. He further suggested that *M. guillarmoidae, M. macowanii, M. davyi, M. drakensbergensis* and *M. stereophylla* should be reduced to subspecific rank, and the anatomical forms of *M. stricta*

and *M. disticha* also be accorded taxonomic recognition with subspecific status.

There are also problems with the winter-rainfall area *Merxmuellera* species. Several studies have shown that the taxonomic treatment of *M. rangei* within *Merxmuellera* is doubtful. In keeping with all other species of *Merxmuellera*, *M. rangei* has a C_3 leaf anatomy, but despite this consistency, it is anatomically distinct (Ellis, 1982). The presence of adaxial parenchyma and the consequent development of the solid, cylindrical leaf form is atypical of *Merxmuellera* (Ellis 1983). In addition, it possesses three orders of vascular bundles which is atypical of the genus. These observations indicate that *M. rangei* probably occupies an isolated position within the genus and Ellis (1983) suggested its removal from Danthonioideae. This is consistent with the results from phylogenetic studies based on *rpo*C2 gene sequence where this species is resolved sister to the Danthonioideae showing a close relationship to *Centropodia* in the Chloridoideae (Barker *et al.* 1999). Similarly, Verboom et al. (1994) reported that this species lacks haustorial synergids, a character shown to be a synapomorphy of the danthonioid grasses.

1.3.3. Phylogenetic studies

Consistent with the morphological and anatomical diversity observed (Conert,1970, 1971; Benesh, 1995; Ellis, 1980a, 1980b, 1981a, 1981b 1983; Gibbs-Russell *et al.*, 1990), phylogenetic analysis of embryological data by Verboom *et al* (1994) has corroborated taxonomic problems of *Merxmuellera* among the Danthonioideae. Although this study was not based on a broad sample of the genus, their results demonstrated that *Merxmuellera* is probably paraphyletic or polyphyletic with species analysed being assigned to the different embryological groups. One group which they termed group B, comprised two *Merxmuellera* species, *M. arundinacea* and *M. rufa*, while other species were clustered in group D containing most of the core danthonioid genera. Species that were assigned to group D were shown to possess some apomorphic characters

typical of the core danthoinoid genera, while the group B species possess plesiomorphic characters suggesting their basal position in the danthonioid lineage.

Hsiao *et al.* (1998) published a molecular phylogeny for the subfamily using data from the Internal Transcribed Spacer (ITS) region of nuclear-encoded ribosomal DNA cistron. Only five species of *Merxmuellera* were included in the analysis but there were indications from the results on the possible non-monophyly of the genus. Analysis of this ITS data resolved *M. stricta* and *M. disticha* together with the Australian genera including *Rytidosperma*, *Notodanthonia* as well as the African *Karroochloa* and *Schismus*. *M. rangei* and *M. macowanii* were positioned sister to the rest of the Danthonioideae showing a closer relationship to *Centropodia* in the subfamily Chloridoideae.

The phylogenetic study by Barker *et al.* (1999) using data from the variable grass specific insert in the ribosomal polymerase subunit 2 (*rpoC2*) gene to examine relationships of the danthonioid genera in detail, was the first to include a broad sample of *Merxmuellera* in phylogenetic analyses. Similar to previous studies, all the phylogenies produced showed *Merxmuellera* to be polyphyletic as several species from this genus were scattered throughout the danthonioid lineage from basal to terminal clades, with one species, *M. rangei*, being resolved as more closely related to *Centropodia* and the chloridoids. In this study *M. macowanii* and *M. davyi* were resolved as a well-supported two taxon clade while the Cape *Merxmuellera* species including *M. cincta, M. arundinacea, M. setacea* and *M. rufa* formed the second most basal unresolved group. *M. guillarmoidae, M. disticha, M. dura* and *M. stricta* were associated with the terminal clade containing *Karroochloa, Schismus, Tribolium, Joycea, Austrodanthonia* and *Rytidosperma.*

Efforts to define the generic composition and relationships of the Danthonioideae by Barker *et al.* (2000) using data from morphology and molecules (ITS & *rpoC2*) have also more strongly indicated taxonomic problems of *Merxmuellera* as a genus. Although Barker *et al.* (2000)

criticised the results of the ITS study by Hsiao *et al.* (1998; 1999) by saying their results were possibly flawed by the use of inappropriate outgroup, and the problem of positional homology (alignment) in the *rpoC2* study by Barker *et al.* (1999), the polyphyly of *Merxmuellera* was however, still evident from their results. Their analyses of the combined data sets showed several *Merxmuellera* species as a paraphyletic assemblage of taxa at the base of the danthonioid lineage while other species were being resolved on terminal clades, similar to the observation made by earlier molecular phylogenetic studies of this subfamily.

1.4 Aims of the current study

There is evidence from anatomical, embryological and molecular studies on the possible polyphyly of the genus *Merxmuellera.* In an attempt to resolve the phylogenetic relationships of the African members of this genus in the context of the rest of the Danthonioideae, the fundamental aims of this thesis are to:

(i) Further test the hypothesis that within the subfamily Danthonioideae, the genus *Merxmuellera* is non-monophyletic.

(ii) Suggest monophyletic groups within the genus and recommend their recognition at the generic level to provide a taxonomy for the species of *Merxmuellera* based on new phylogenetic hypotheses.

(iii) Determine areas where relationships are not well defined and are in need of further investigations.

To achieve the above goals, we exploit data from morphology and anatomy along with sequence data from the entire ITS region and the *rpo*C2 gene, to explore the phylogenetic relationships of the African species of *Merxmuellera* in the context of the rest of the Danthonieae. Embryological data from Verboom *et al.* (1994) is also reanalysed together with morphological and anatomical data collected in this study.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Morphology and anatomical data

2.1.1 Sampling

A total of 44 species were sampled for morphology and anatomy (herewith referred to as morphology for convenience) in this study (Table 2.1). This includes all 17 southern African species of *Merxmuellera*, the remainder constituting representatives from other danthonioid genera. The Madagascan species of *Merxmuellera* were not included due to lack of suitable material for investigation. Sampling with regard to other taxa in the subfamily Danthonioideae was aimed at including representatives from seven groups of genera as circumscribed by Barker *et al.* (2000) (Table 1.2). Due to lack of material, some of the outgroup genera were not examined morphologically in this study, but were included in the molecular data sets to robustly determine the systematic position of *Merxmuellera* in the context of the rest of the Danthonioideae. Selection of representaive species from other danthonioid grasses included in the morphological data set was guided by availability of DNA sequences in subsequent analyses.

2.1.2 Source of material

Literature, dried herbarium material as well as material previously fixed in formalin-acetic acid alcohol (FAA) and stored in 70% alcohol (EtOH) were used to gather morphological data for phylogenetic analysis. Mst herbarium specimens used are housed in the Bolus Herbarium (BOL), while some were on obtained on loan from the National University of Lesotho, Roma Herbarium (ROML) (standard abbreviations as in Holmgren et al. 1990). Literature and herbarium material for some species were either inadequate or totally lacking. For those species, collecting trips to their known distribution areas were organised to supplement the available

material for detailed investigation. However, some of the species were

Table 2.1. List of species examined for morphology and anatomy

Taxon	Distribution
Austrodanthonia auriculata	Australia
Austrodanthonia laevis	Australia
Centropodia glauca	Africa
Chaetobromus involucratus subsp. *dregeanus*	Africa
Chaetobromus involucratus subsp. *involucratus*	Africa
Chionochloa macra	New Zealand
Chionochloa pallens	New Zealand
Chionochloa rigida	New Zealand
Cortaderia fulvida	New Zealand
Cortaderia richardii	New Zealand
Cortaderia selloana	SouthAmerica
Joycea pallida	Australia
Karroochloa purpurea	Africa
Karroochloa schismoides	Africa
Karroochloa tenella	Africa
Merxmuellera arundinacea	Africa
Merxmuellera aureocephala	Africa
Merxmuellera cincta ssp. cincta	Africa
Merxmuellera davyi	Africa
Merxmuellera decora	Africa
Merxmuellera disticha	Africa
Merxmuellera drakensbergensis	Africa
Merxmuellera dura	Africa
Merxmuellera guillarmoidae	Africa
Merxmuellera lupulina	Africa
Merxmuellera macowanii	Africa
Merxmuellera papposa	Africa
Merxmuellera rangei	Africa
Merxmuellera rufa	Africa
Merxmuellera setacea	Africa
Merxmuellera stereophylla	Africa
Merxmuellera stricta	Africa
Pentameris macrocalycina	Africa
Pentameris thuarii	Africa
Pentashistis aspera	Africa
Pentashistis curvifolia	Africa
Pseudopentameris caespitosa	Africa
Pseudopentameris macrantha	Africa
Rytidosperma nudiflorum	Australia
Rytidosperma pumilum	Australia
Schismus barbatus	Africa
Tribolium hispidum	Africa
Tribolium pusillum	Africa
Tribolium uniolae	Africa

not in flower during field visits for proper identification, and re-visits could not be effected as their flowering times were not overlapping with the study period. In such cases, illustrations and literature published by previous authors were used as the main source of data.

2.1.3 Preparation and examination of material

2.1.3.1 Morphology

Spikelet, floret and vegetative morphology were recorded from whole-mounts of dissected spikelets in glycerine. Whole-mounts from dried herbarium specimens were prepared by reconstituting the spikelets in soapy boiling water for about half an hour and carefully dissecting out under a Zeiss dissecting microscope. The dissected spikelets were placed on a microscope slide to which a drop of 50% glycerine had been added, and covered with a cover slip. Examination was done using both the Zeiss Standard 25 compound and Stemi SV 6 dissecting microscopes and drawings made using a *camera lucida*. Investigation of indumentum types on the spikelet parts were observed from dry specimens due to difficulties in observing them on wet mounts.

2.1.3.2 Anatomy

As Ellis (1979) pointed out, anatomical data is undoubtedly important in the jigsaw of complete systematic evidence in grasses as the usage of anatomical characters in conjunction with a wide spectrum of other diagnostic characters, tends to provide an essential ingredient of satisfactory treatment of grass taxonomy. Anatomical descriptions of all the *Merxmuellera* species have been published by Ellis in a series of papers except for *M. papposa*, the anatomy of which still remains unknown. For those taxa for which prepared anatomical slides or literature were not available for scoring data for phylogenetic analysis, leaf anatomy preparations were made from mid-portions of leaves fixed in the field in FAA and stored in 70% ethanol (EtOH). Where the fresh material was not available, herbarium material was re-hydrated in soapy

boiling water for about half an hour until specimens dropped to the bottom of the beaker. The specimens were removed from the beaker and washed in running water to remove detergent added when rehydrating and then placed in 50% EtoH for about thirty minutes. Using a new razor blade for every specimen, transverse sections of the leaves were prepared following the manual method of hand sectioning.

To investigate character variation of the leaf epidermis, abaxial and adaxial scrapes were prepared by scraping off both the leaf surfaces in a horizontal direction along the ribs with a sharp blade until only the epidermis remained. Both the transverse sections and the epidermal scrapes were subsequently stained in a mixture of Safranin and Alcian Blue (Tolivia and Tolivia 1987) and dehydration was performed in watchglasses following this sequence: 50% EtoH; 70% EtoH; 80% EtoH; 90% EtoH; and twice in 96% Etoh; Xylene, allowing the material to stand in each watchglass for about one minute. The sections and the epidermal scapes were mounted in Canada Balsam on a microscope slide and covered with a cover slip and dried in an oven maintained at a temperature of about 60^0 C.

Examinations of these anatomical preparations were made using a Zeiss Standard 25 compound microscope. Where possible, anatomical slides were photographed using a Leitz DIAPLAN compound mocroscope fitted with an Axiocam ZEIZZ digital camera. The hand-sectioning technique is very crude as the sections tend to be too thick for making good quality photographs. However, for the purpose of scoring up data for phylogenetic analysis in this study, these anatomical slides were adequate.

2.1.4 Character coding

Character coding for cladistic analysis involves investigating character variation within a group of taxa under study. The observed variation can then be partitioned into discrete characters and their component states, followed by construction of a data matrix in which

suitable states of each character are scored for each taxon. Bryant, (1989) discussed the importance of character analysis for phylogenetic inferences by arguing that it is a deductive process which can be tested by empirical observation of topographic and ontogenetic relationship and similarity between taxa, as opposed to the inductive nature of cladistic analysis. Although character analysis is fundamental to phylogenetic inferences, different authors have different perceptions on how to define and delimit characters and their component states. For example, Gift and Stevens, (1997), Stevens, (1991), and Thiele, (1993) prefer to code continuous and quantitative characters for phylogenetic analysis, while other authors prefer to delimit characters into discrete and qualitative states (Seberg, 1984; Baum, 1988). On the other hand some authors (e.g. Hawkins *et al.*, 1997) argue for the conventional approaches whereby they consider states as the forms of the "same thing" (the character). Other methods of character construction involve composite and reductive coding of characters for cladistic analysis (Wilkinson, 1995; Pliejel, 1995). Although each method has its own merits and disadvantages, it is sometimes difficult to stick to a particular coding approach. Each method can be applicable when trying to analyse some types of characters while other methods may not. For instance, attempts to increase the informativeness of a character by delimiting some intermediate states would definitely lead to multistate coding while in some cases nominal character coding (presence/absence) cannot be avoided.

In coding characters for this study all these approaches were used depending on the character being investigated. For multistate characters, an *apriori* hypothesis about character evolution was avoided by employing the non-additive approach (Fitch, 1971) which treats characters as unordered. Where discrete (Seberg, 1984; Baum, 1988) and conventional approaches (Hawkins *et al.*, 1997) to character coding were used, taxa lacking a specific feature were coded as inapplicable with respect to the variation in that feature being coded.

Characters were scored directly onto the Nexus data editor version 4.08 (Page, 2000) and a complete data set containing 76 characters was finally produced (Table 2.2).

2.1.5 Character descriptions

Anatomical characters

Most of the anatomical characters and their states were derived from the list produced by Ellis (1976, 1979).

1. Lamina outline: circular (0): expanded or permanently infolded (1)

There is a wide variation in lamina outline such that it is difficult to code the states precisely, especially from dry specimens which tend to roll their leaves when dry. This character is only included here to distinguish *M. rangei* and *M. papposa* with distinctly circular lamina outline (Fig. 2.2 A-B).

2. Ribs/ridges on the abaxial leaf surface: absent (0), present thoughout (1), slight undulations (2), only flanking midrib (3)

The abaxial and adaxial surfaces of the danthonioid grass leaf blades are generally longitudinally ribbed. The ribs, sometimes termed ridges (Arber, 1934; Wilson 1971), are usually developed in association with and adjacent to the vascular bundles and are often useful for diagnostic purposes (Ellis, 1979; Gordon-Gray and Ward, 1970). For the ribs to be considered present there must be corresponding furrows of various depths (refer to character 3) developed between vascular bundles and varying in location (characters 4) and transverse shape (character 5). They are generally characteristic of and more fully developed on the adaxial than the abaxial surface (Metcalfe, 1960). In most species abaxial ribs are usually absent (Fig. 2.3D). However, in few species abaxial ribs and furrows are developed associated with all vascular bundles (Fig. 2.3B) or only flanking midrib (Fig. 2.3C). An intermediate condition sometimes occurs where ribs appear as small undulations associated with vascular bundles (Fig. 2.3D). Care has to be taken when observing leaf sections obtained from herbarium material, as the presence of ribs or undulations

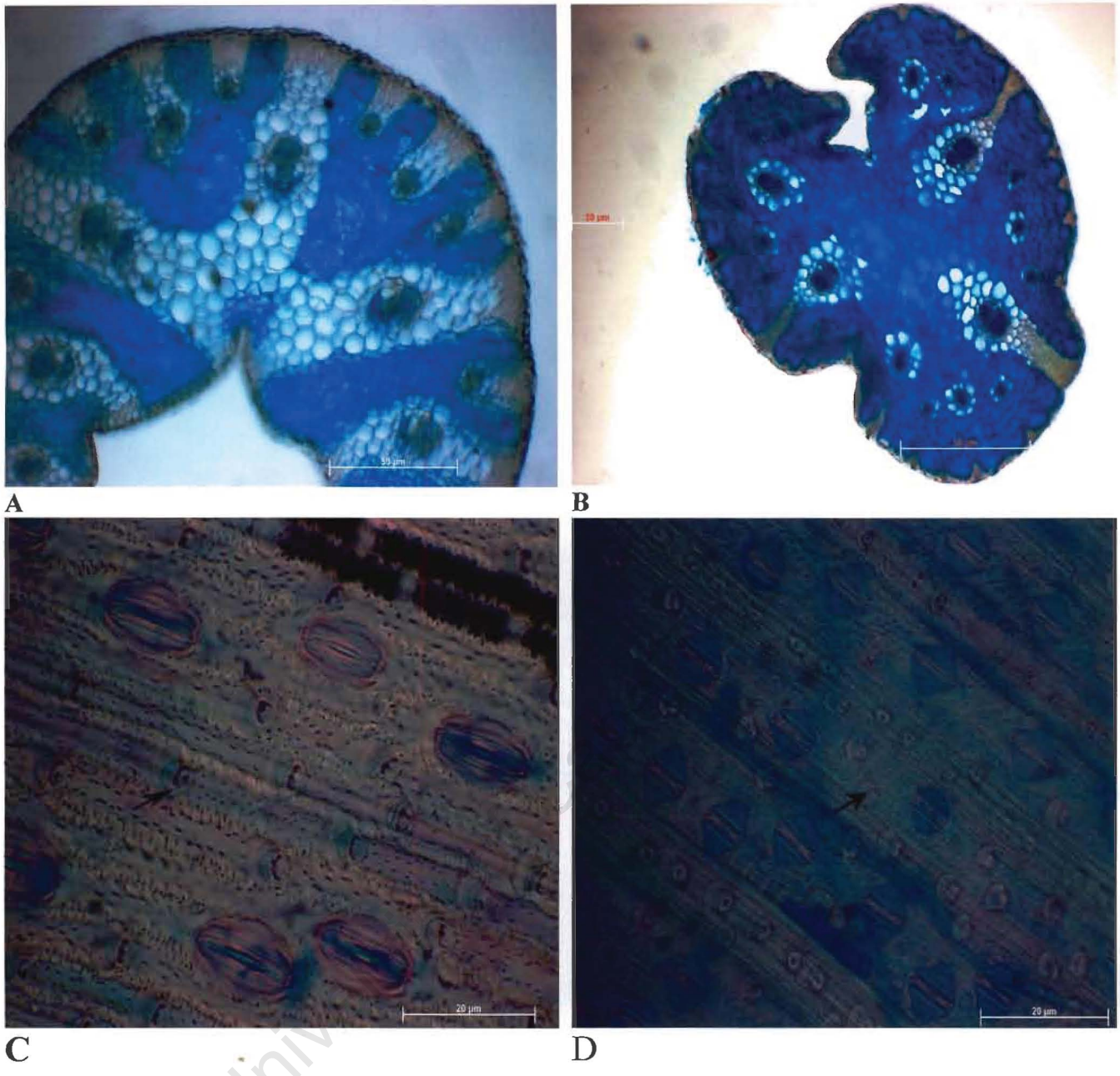


Fig. 2.2 Transverse leaf blade anatomy of *Merxmuellera papposa* (Barker & Mafa, 1760) (Fig.2A) and *M. rangei* (Pillans, 6022) (Fig. 2B). Note cylindrical lamina outline and a V shape groove on the adaxial surface in both species. Second order vascular bundles occur on the flanking sides of the third order vascular bundles towards the abaxial leaf epidermis. Also note pallisade-like chlorenchyma restricted to the abaxial surface consisting of regular sized colourless cells. Figs. 2C and 2D show the abaxial epidermides of *M. papposa* (Zeyher, 469) and *M. rangei* (Pillans, 6022) respectively. Note numerous and large stomata which appear somewhat inflated on narrow costal zones and the pitted epidermal cells shown with arrows.

on the abaxial leaf surface may be a result of material not being fully rehydrated.

3. Depth of adaxial furrows in comparison to the leaf thickness: deep furrows i.e. more than half the leaf thickness (0), shallow furrows i.e. less than a quarter of the leaf thickness (1), medium furrows i.e. a quarter to one half the leaf thickness (2).

The states in this character are arbitrarily subdivided. The depth of the larger ribs in the central region of the lamina between the margin and the median vascular bundle is regarded as leaf thickness. This character is correlated to character 2 above in that where ribs are always corresponding furrows developed between vascular bundles.

4. Distribution of adaxial furrows (Fig 2.3A-D): located between all vascular bundles (0); located between larger vascular bundles (1); furrows present only on either side of the median vascular bundle (2).

5. Shape of adaxial ribs over first order vascular bundles (Fig. 2.4A-D): obtuse i.e. apex rounded (0); flat-topped, square ribs i,e, apex flattened (1); triangular, i.e. apex pointed (2).

Variation in this character is usually constant within a species, but in a few species this character can vary within an individual plant making the delimitation of states difficult. In such cases a majority rule concept is adopted. If at least 80% of the ribs are of a particular state, that state is then coded for that species, otherwise it is coded polymorphic.

6. Median vascular bundle: indistinguishable from other first order vascular bundles (0); distinguishable by size from other first order vascular bundles (1).

Structurally, the midrib is similar to the lateral first order vascular bundles. However, in some species the midrib can be distinguished based on size relative to the lateral first order vascular bundles. Where a size difference occurs, the midrib is usually smaller and flanked by third order bundles. This state is constant within species, at least from the few specimens observed, but rather varies between species.

7. Vertical positioning of vascular bundles in the blade: all bundles

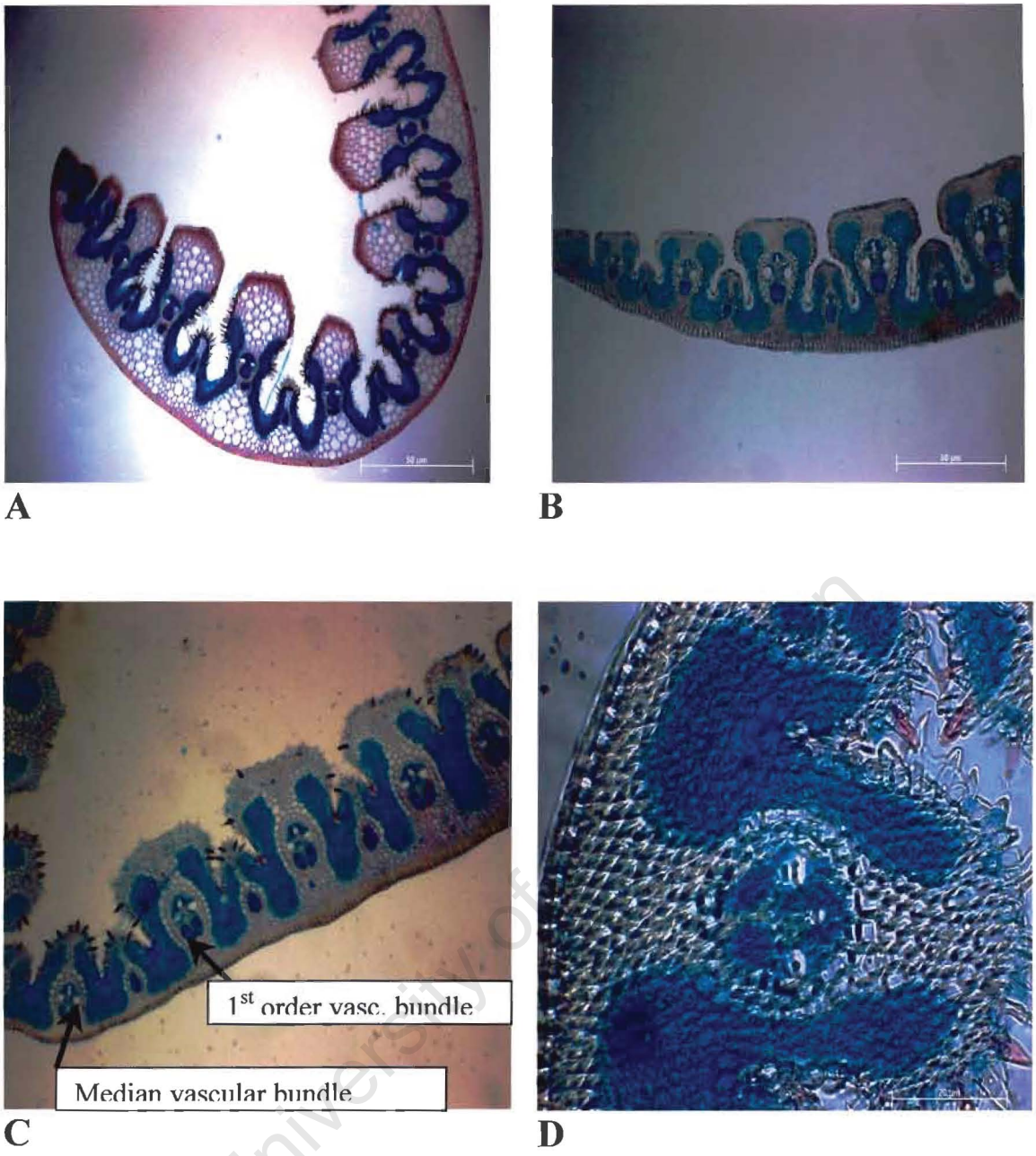


Fig. 2.3. Transverse leaf blade anatomy of *Merxmuellera cincta* (Crook, 2229) (Fig. 2.3A), *M. arundinacea* ((Linder, 5762) (Fig. 2.3B), *M. macowanii* (Linder 5094) (Fig. 2.3C) and *M. drakensbergensis* (Hilliard, 16440) (Fig. 2.3D). Note massive first order vascular bundles on the adaxial surface with apices triangular in *M. cincta,* and flat-topped in *M. arundinacea* and *M. macowanii,* and rounded in *M. drakensbergensis*. Ribs overlying the third order vascular bundles are smaller and rounded in shape on the apical region. Also note shape of groups of isodiametric chlorenchyma cells, and size difference between the median vascular bundles and the lateral first order vascular bundles (Fig. 2.3C).

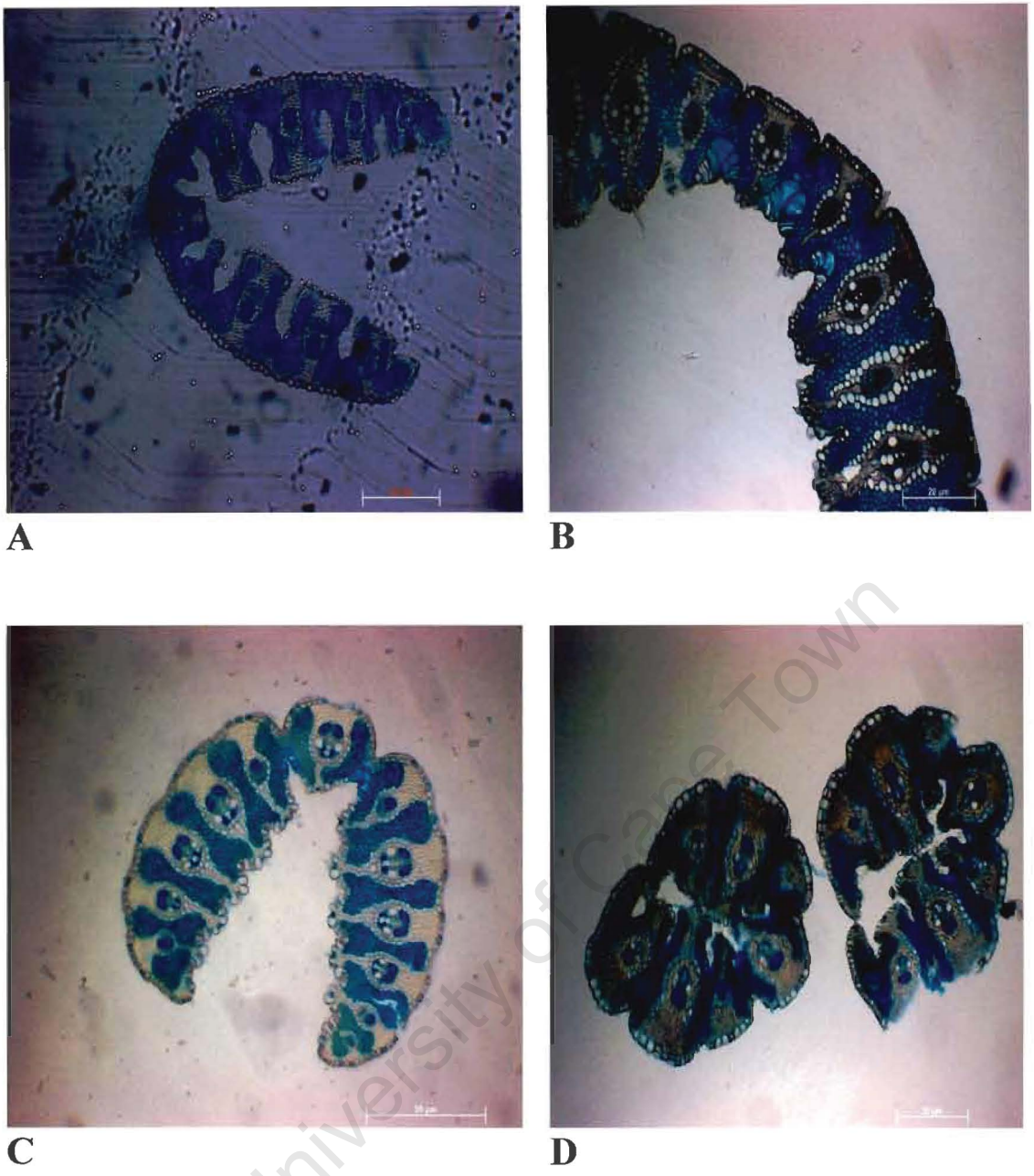


Fig. 2.4. Transverse leaf blade anatomy of *Merxmuellera disticha* (Linder, 5876) (Fig. 2.4A), *M. dura* (Linder, 5757) (Fig. 2.4B), *M. stricta* (Linder, 5428) (Fig. 2.4C) and *M. lupulina* (Pearson, 3529) (Fig. 2.4D). Note third order vascular bundles situated below deep furrows in *M. disticha*. Note abaxial furrows and shallow adaxial furrows with well developed bulliform cells in *M. dura*. Note adaxial and abaxial furrows only flanking midrib in *M. stricta* with poorly developed bulliform cells on the adaxial side. Note slight undulations associated with larger vascular bundles on the abaxial epidermal surface in *M. lupulina*.

centrally located (0); first order vascular bundles centrally located and third order vascular bundles abaxially positioned (1); all bundles located adaxially (2); all bundles located abaxially (3).

8. Second order vascular bundles: absent (0); present (1).

Presence of second order vascular bundles is usually rare in danthonioid grasses. They are structurally different from the first order bundles, but similar to third order bundles, differing only in size and usually occuring beneath the first order bundles or flanking the third order bundles (see Fig. 2.3A – B).

9. Shape of groupings of isodiametric chlorenchyma cells: X-shaped (0); W-shaped (1); Y-shaped (2); U-shaped (3). When the transverse leaf blade sections are stained with Safranin and Alcian blue the chlorenchyma cells appear in different shapes (Fig. 2.3 A – C).

10. Phloem : phloem completely surrounded by thick walled fibres (0); phloem adjoins the inner parenchyma sheaths (1).

In some species the phloem of the first order bundles may be surrounded by thick wall fibres whereas in some species these may divide the phloem into two resulting in a sclerosed phloem (character 11; Fig. 2.5). Where the phloem is not surrounded by fibres it is usually seen attached to the inner parenchyma sheath cells.

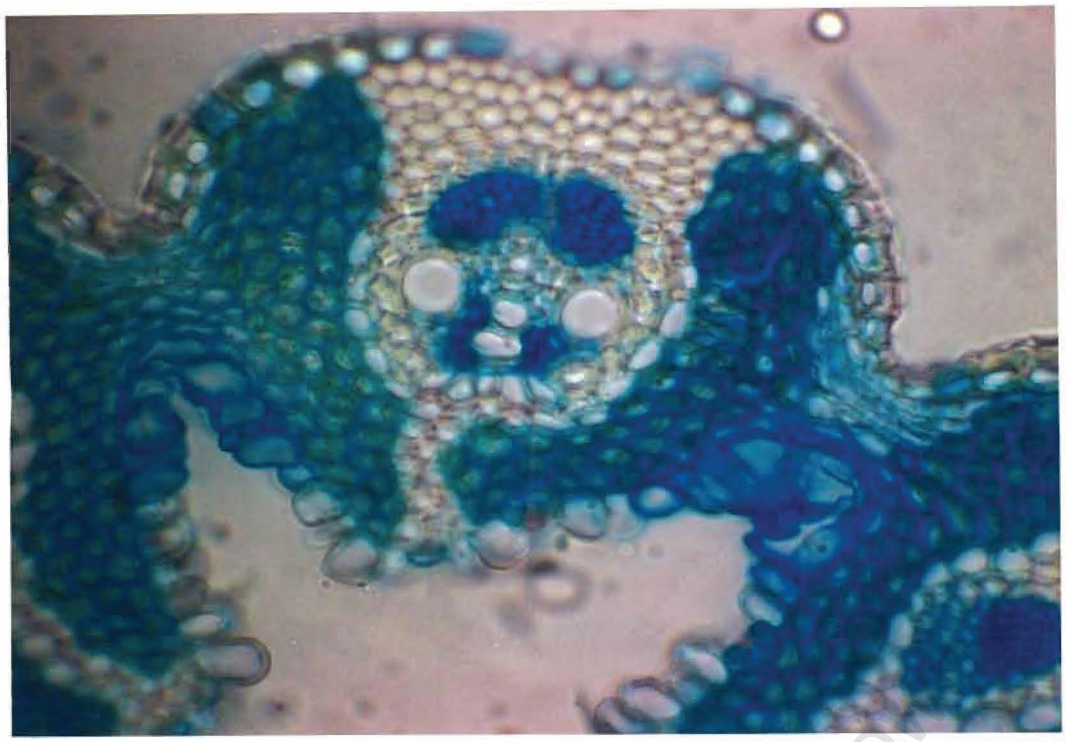
11. Sclerosed phloem: absent (0); present (1).

12. Outer bundle sheaths: complete (0); incomplete below and above (1); incomplete below (2); no bundle sheath surrounding the bundle (3).

The sclerenchyma girders on either side of the vascular bundles may interrupt the outer bundle sheath cells. If the interruption occurs both from the adaxial and the abaxial sides, the sheaths appear reduced to two lateral strips on either sides of the phloem (Fig. 2.6), or if interruption arises from the abaxial side it appears horse-shoe shaped thus coded incomplete.

13. Colourless cells in the mesophyll: absent (0); present (1).

In most species the chlorenchyma cells stain blue or green (cf. character



A

B

Fig. 2.5. Transverse leaf blade anatomy of *Merxmuellera stricta* (Linder, 5428) (Fig. 2.5A) and *M. macowanii* (Linder, 5762) (Fig.2.5B) showing details of the first order vascular bundle. Note the double bundle sheaths with the abaxial side of the inner sheath adjoining the phloem with sclerified tissue. Note the abaxial sclerenchyma girders interrupting the outer bundle sheaths. Also note large circular metaxylem vessels which are much wider than the bundles sheath cells.

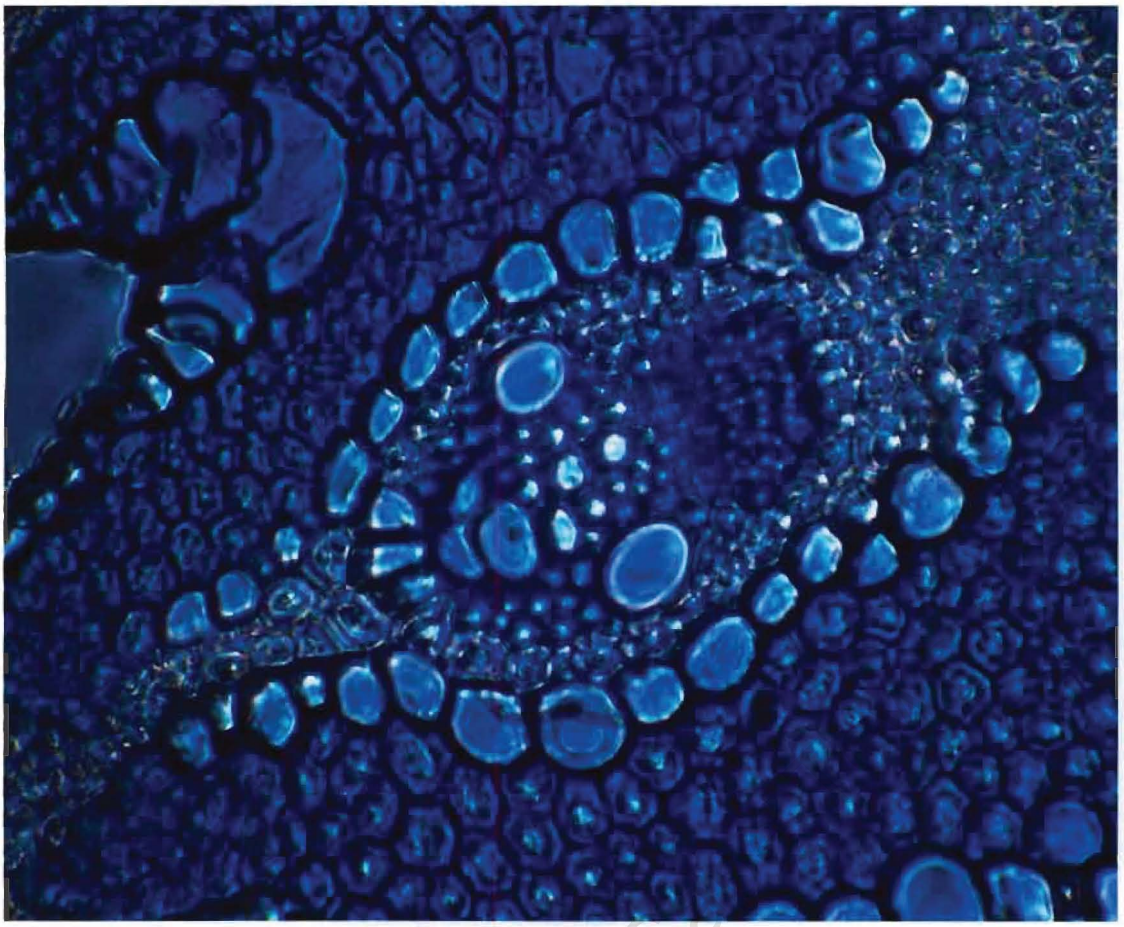


Fig. 2.6. Transverse leaf blade section of *Merxmuellera dura* (Linder, 5757) showing details of the first order vascular bundle. Note outer bundle sheath which is reduced to two lateral strips due to interruption of sclerenchyma girders from both the adaxial and and abaxial epidermal surfaces. Also note the metaxylem vessels with width equal or slightly greater than parenchyma sheath cells (cf. Fig. 2.5).

9) while in a few species the chlorenchyma appears as empty and somewhat inflated cells on the adaxial side of the first order vascular bundles (Fig. 2.2A – B).

14. Size of metaxylem vessels in relation to parenchyma sheath cells in transverse section: narrow vesels i.e. parenchyma sheath cells wider than vessels (0); wide vessels i.e. vessels with width equal to or slightly greater than parenchyma sheath cells (1); very wide vessels i.e. width of vessels very much more than that of parenchyma sheath cells (2).

15. Bulliform cells on the abaxial epidermis: absent (0); poorly developed (1); well developed (2).

These are groups of colourless cells forming part of the epidermis only differing from other epidermal cells in that they are often large and fan shaped, and are usually situated at the bases of the furrows. In some species these are large and conspicuous hence the state well developed (see Fig. 2.3B), while in some species they appear small and not very conspicuous (poorly developed).

16. Bulliform cells on the adaxial epidermis: absent (0); poorly developed (1); well developed (2).

17. Stomata on the abaxial epidermis: absent (0); present (1).

18. Stomata on the adaxial epidermis: absent (0); present (1).

19. Prickles on the abaxial epidermis: absent (0); present (1).

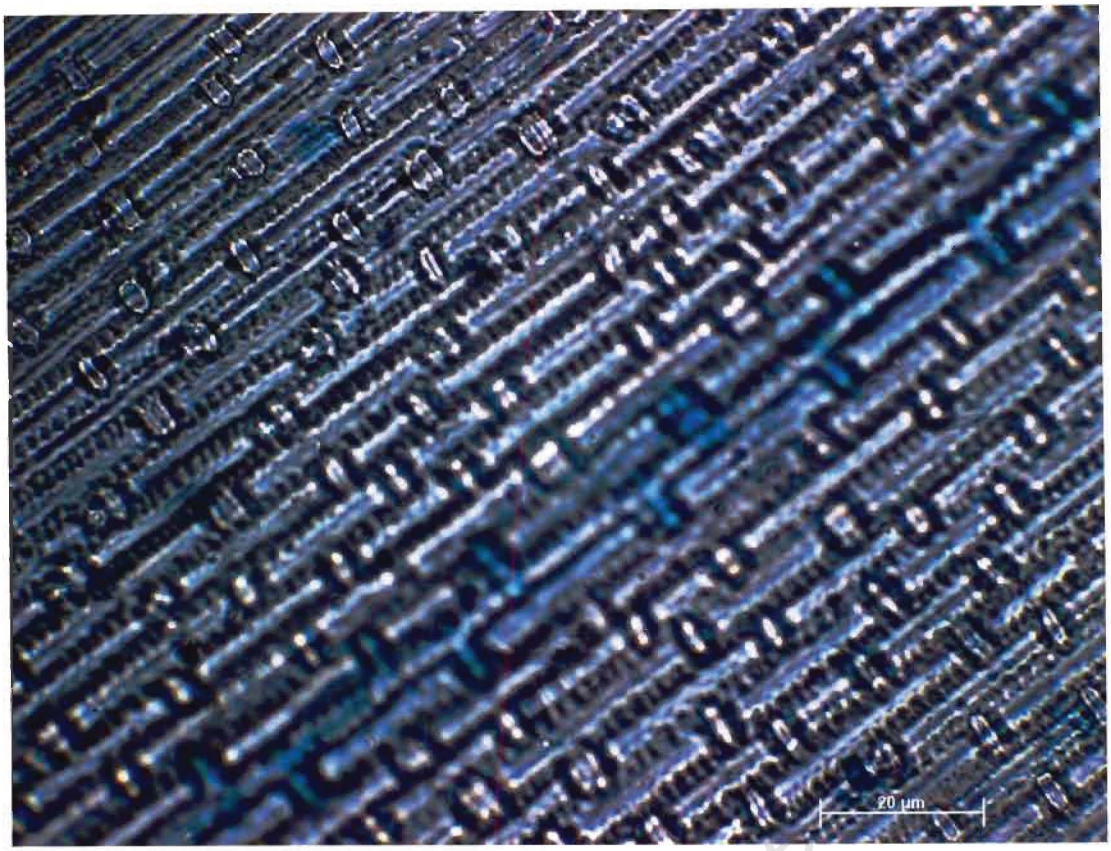
20. Prickles on the adaxial epidermis: absent (0); present (1).

21. Papillae on the abaxial epidermis: absent (0); present (1).

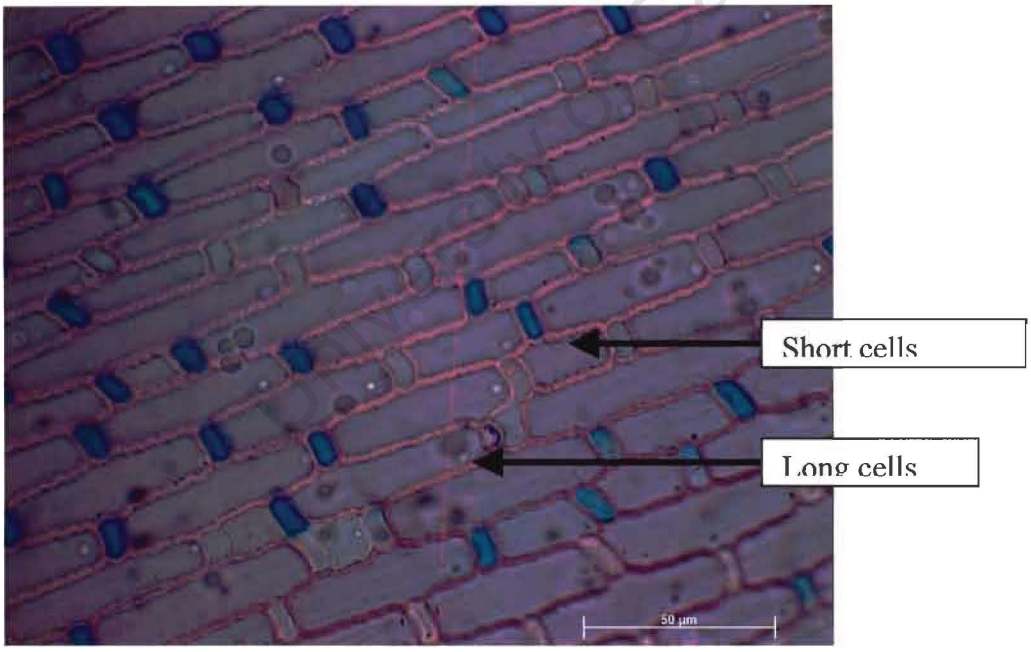
22. Papillae on the adaxial epidermis: absent (0); present (1).

23. Intercostal zones: differentiated (0); undifferentiated (1).

The epidermis of the grass leaf is divided into two zones, the intercostal and the costal zones. The costal zone is found opposite the leaf veins while the intercostal zone consists of long and short cells and is found between veins. The intercostal long cells are elongated and narrow with an undulating outline (Fig. 2.7A) or sometimes smooth and outwardly bowed appearing as if they are inflated (Fig. 2.7B). The short cells (Fig. 2.7B) are more or less isodiametric. When only the long cells are present



A



B

Fig. 2.7. - Abaxial leaf epidermides of *Merxmuellera drakensbergesis* (Hilliard, 16440) (Fig. 2.7A) and *M. decora* (Euston-Brown, 105/5) (Fig. 2.7B). Note narrow and long undulating intercostal cells in Fig. 2.7A and note outwardly bowed costal long cells in Fig. 2.7B. .

the epidermis is referred to as undifferentiated and differentiated when both types of cells occur.

Vegetative and floral morphology

24. Bulbous bases: absent (0); present (1)

Bulbous bases occur in some species of *Merxmuellera* and some allied danthonioid grasses from the Cape. These bulb-like structures are formed from swollen basal leaf sheath bases usually covered by a dense mass of wool appearing as dark-brown fibres in old specimens (character 26). At least one species of *Merxmuellera* bearing this structure does not show any indication of developing woolly hairs. In the field these geophytic structures are deeply sunken into the ground and are thought to be protecting young shoot bases against excessive evaporation in hot dry days and/or probably serve as protection against fire damage and predation (Linder and Ellis, 1990a).

25. Culm nodes : exposed (0); hidden (1).

Many species are 1 – 2 noded and these nodes are in most cases tightly covered by their leaf sheaths hence the state hidden is coded. However, some species are 2 – 3 noded with the upper nodes exserted just above the upper leaf sheaths and the state exposed is coded.

26. Basal leaf sheaths indumentum: woolly (0); glabrous (1); with stiff hairs (2); scaberulous (3).

27. Hairs inside the basal leaf sheath surface: absent (0); present (1)

28. Upper leaf sheaths: hairy (0); scaberulous (1); glabrous (2).

29. Sheath mouth: woolly beard (0); hairy beard (1); not bearded (2)

A bearded sheath mouth is common in danthonioid grasses. However, in some species these are borne as a dense intermingled mass of hairs hence the state woolly bearded is coded. In other species they are straight and easily seen as free.

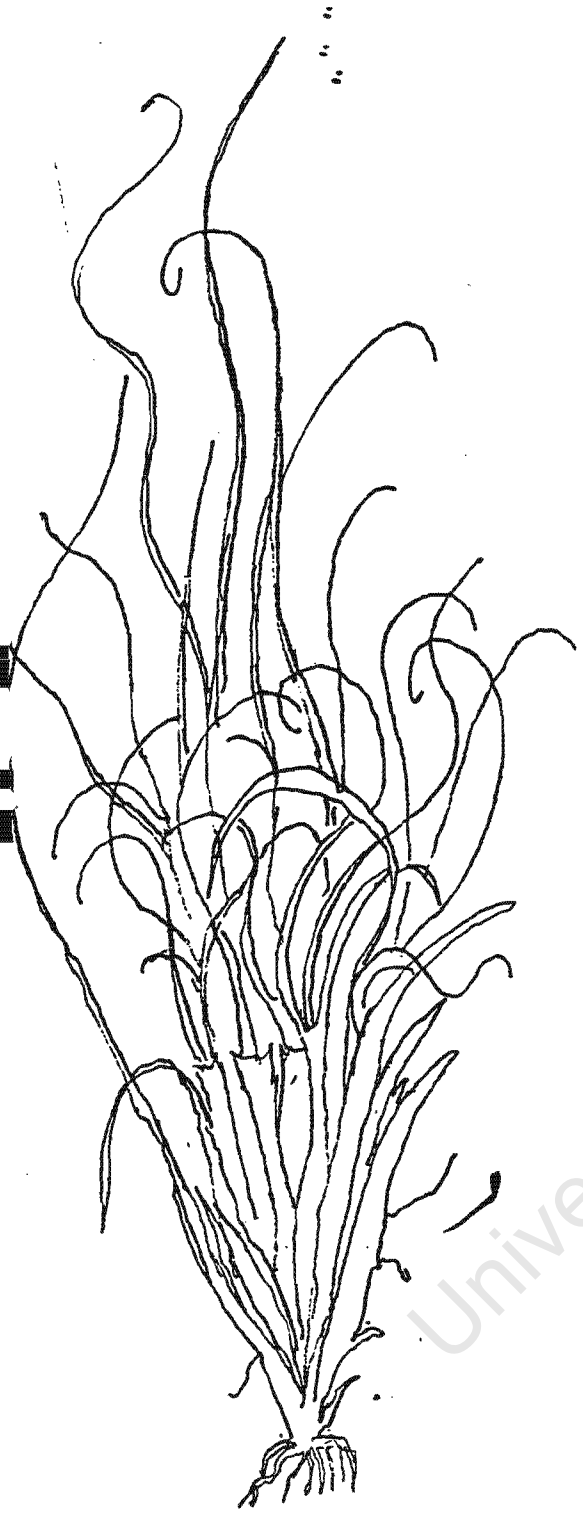
30. Behaviour of old leaves (Figs. 2.8A – B): *split* (0); *curl* (1).

Usually the old leaf blades for *M. drakensbergensis* and *M. macowanii* break off a little distance above the ligule and the remaining portion splits

Fig. 2.8 (next page). Old leaves variation.

A, *Pentaschistis curvifolia* (Crook, 1026);

B, *Merxmuellera drakensbergensis* (Mafa, 4)



A x 0.2

B x 0.2

along the middle nerve and the resultant halves recurve outwards at the apex (Anderson 1960). This character is distinct for these two *Merxmuellera* species. However, in some outgroup taxa especially in *Pentaschistis*, the old leaves break off anywhere above the sheath mouth and curl to form tight spirals

31. Leaf surface: hairy (0); scaberulous (1); glabrous (2).

32. Spikelet insertion: sessile (0); pedicellate (1); subsessile (2).

33. Spikelet arrangement: distichous (0); not distichous (1).

34. Pedicels : articulated (0); not articulated (1).

35. Tuft of hairs at the base of spikelets from the tips of the pedicels: absent (0); present (1); pedicels glabrous (2).

36. Number of flowers per spikelet: two (0); three to ten (1).

The number of florets in *Merxmuellera* varies from three to ten, and this is often characteristic for species. This variation overlaps greatly within *Merxmuellera* thus making it difficult to delimit the states precisely in an attempt to increase the informativeness of this character. We only use it here to distinguish this genus from *Pentaschistis* and *Pentameris* with two-flowered spikelets.

37. Glume length relative to spikelet: shorter than the spikelets (0); about equalling the spikelet (1); exceeding the spikelet (2).

38. Glume prickles: absent (0); present (1).

39. Glume bristles: absent (0); present (1).

40. Dorsal surface of the glume: pubescent (0); glabrous (1); with few long macrohairs (2); scabrid (3).

41. Glume markings: with purple colouring (0); without purple colouring (1).

42. Glume apex: acute (0); acuminate (1); long acuminate (2); awned (3).

43. Lemma markings: with purple colouring (0); without purple colouring (1).

44. Lemma awn: absent (0); present (1).

45. Awn position: terminal (0); sinus (1); absent (1).

46. Hairs inside the lemma at the point of awn insertion: absent (0);

present (0).

The glabrous condition of the lemma at the point of insertion of the central awn is a very common feature in the danthonioid grasses. However, in a few *Merxmuellera* species, a small patch of a few scattered hairs is present and usually restricted to an area where the lemma body tapers into an awn.

47. Hairs outside the lemma at the point of awn insertion: absent (0); present (1).

48. Lemma apex: unlobed (0); deeply bilobed (0); obscurely lobed (2).

Most species of *Merxmuellera* have a bilobed lemma with the awn emerging from the sinus, a feature that is generally typical of the danthonioid grasses. However, in some species lobes are not distinct as the central awn is fused to the lemma lobe for most of its length (at least 90%) hence we code state 2 to increase the informativeness of this character. Coding of this character links it with the character (49) which quantifies the degree of central awn adnation to the lemma lobes lengthwise. Where the lobe is partially fused to the awn column we delimit at least two states by observing whether the fusion is less than or more than halfway through the lemma lobe length from the point of awn insertion. This interval appears to be discontinuous in that either the fusion is way below half the lobe length or the lobe is almost completely fused.

49. Degree of lemma lobe fusion: free (0); partially adnate to less than half the lemma lobes (1); partially adnate to more than half the lemma lobes (2); completely adnate (3).

50. Lemma lobe bristles: absent (0); present and shorter than lobe length (1); present and equal or longer than lobe length (3).

51. Lemma inside: pubescent (0); glabrous (1)

52. Marginal fringe of hairs: absent (0); present (1).

This description outlined below refer to characters 52 to 55 and 59 (see Figs. 2.9 A – G)

The arrangement of hairs on the abaxial surface of the lemma has been

viewed as a useful taxonomic character in danthonioid grasses (e.g Vickery, 1956; Connor and Edgar, 1979; Conert, 1971; Conert and T rpe, 1969; Linder and Verboom, 1996; Linder and Davidse, 1997). Following Linder and Verboom (1996) for the description of this character, we distinguish the marginal hairs (Fig. 2.9B) as a long continuous line along the lemma margins. These hairs can sometimes be a little distance away from the margin. The longitudinal hairs are regarded as row of hairs along the nerves on the lemma back (Fig. 2.9A, E). In some species these hairs are borne from base to middle region of the lemma body while in some cases they reach the lemma apex just below the point of awn insertion. In some taxa these hairs are more or less the same length (character 59) or the upper row can be long appearing as if they are tufted. The lemma indumentum is regarded as tufted if it forms a group of compact hairs which may be variouly positioned on the lemma body (Figs. 2.9C – F, H). Investigation of this character has been made on whole mounts of the lemma using transmitted light due to difficulty of distinguishing different states when using incident light.

53. Marginal lemma hair tufts: absent (0); present (1).

54. Basal lemma hair tuft: absent (0); present (1).

55. Apical lemma hair tuft: absent (0); present (1).

56. Upper lemma hair tuft: shorter than lobes (0); equal or longer than lobes (1).

57. Lemma: clasping palea (0); not clasping palea (1).

58. Lemma hairs: tapering (0); clavate (1).

59. Longitudinal hairs: hairs of equal length (0); upper lemma hairs longer (1)

60. Palea indumentum between the keels: absent (0); hairy (1); scabrous (2)

The palea indumentum may be separated into three characters (60, 61 and 62). The first character (60) describes the presence of long hairs between the palea keels (Fig. 2.10E). In species where this indumentum is present, there is substantial variation: in some species only few hairs

Fig. 2.9 **A** - **H** (next page) Abaxial lemma surface showing arrangement of hairs and the degree of adnation of lemma lobes to the central awn.

A *Merxmuellera rufa* (Adamson, 2097);

B *Merxmuellera stereophylla* (Killick, 1317);

C *Merxmuellera papposa* (Barker and Mafa, 1760);

D *Merxmuellera macowanii* (Mafa, 2);

E *Centropodia glauca* (Dinter, 6681);

F *Merxmuellera stricta* (Linder, 5766);

G *Pseudopentameris macrantha*;

H *Merxmuellera drakensbergensis* (Hillard & Burt, 6440).

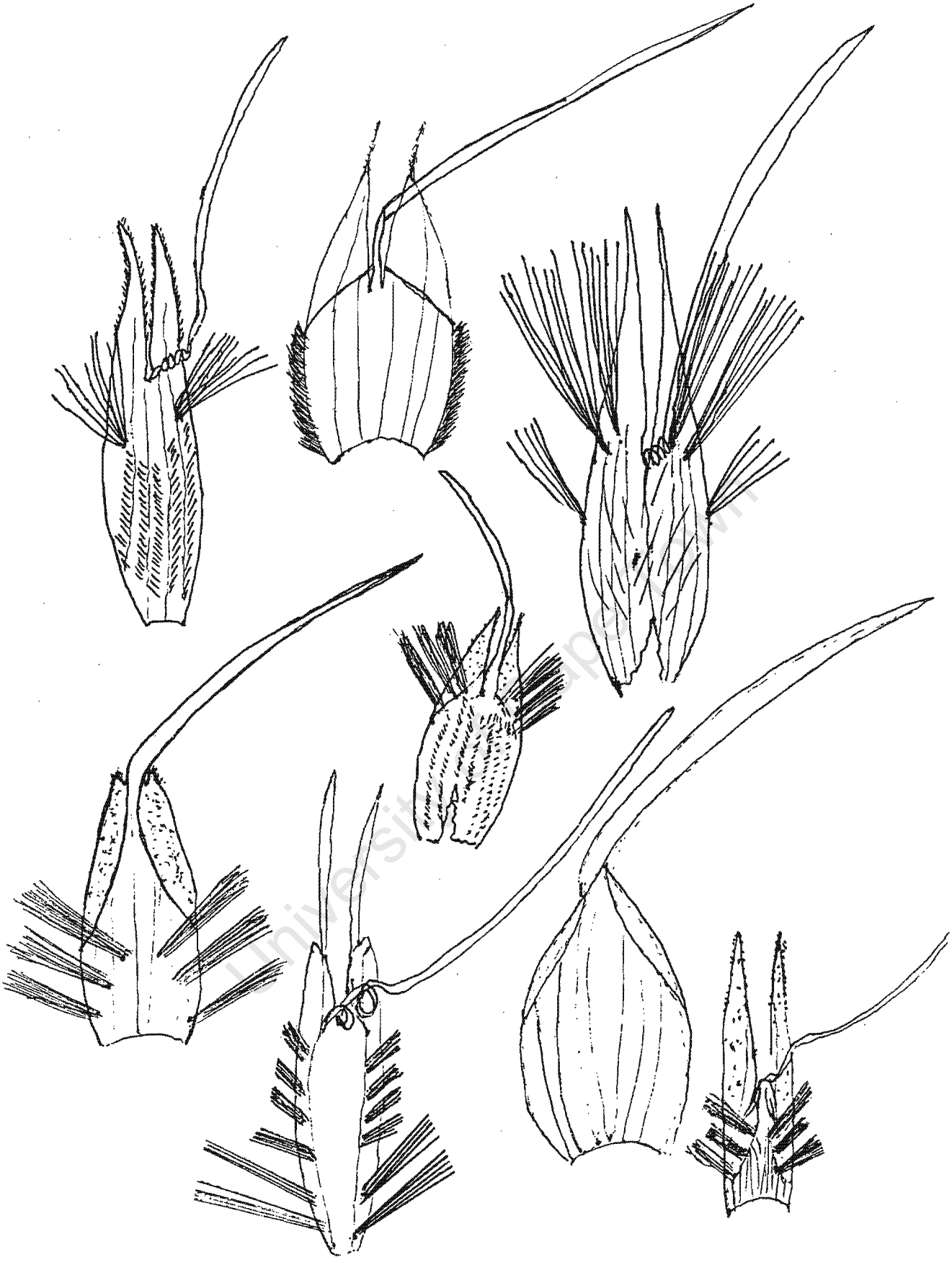


Fig. 2.10. **A** - **G** (next page) Palea showing variation in the apices, indumetum between flaps and keels, rachilla and the callus hairs.

A, *Merxmuellera rufa*;

B, *Merxmuellera stricta* (Linder, 5766);

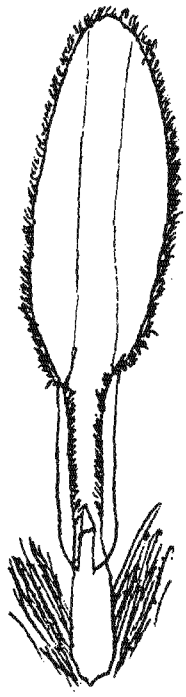
C, *Merxmuellera lupulina* (Pillans, 9591);

D, *Merxmuellera dura* (Linder, 5421);

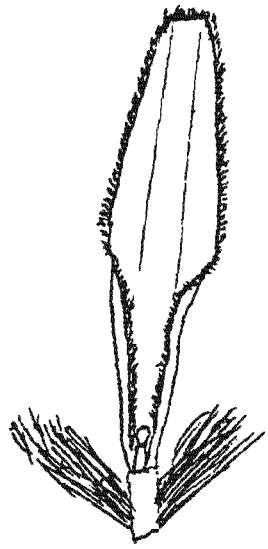
E, *Centropodia glauca* (Dinter, 6681);

F, *Chiochloa rigida*;

G, *Merxmuellera papposa* (Barker and Mafa, 1760).



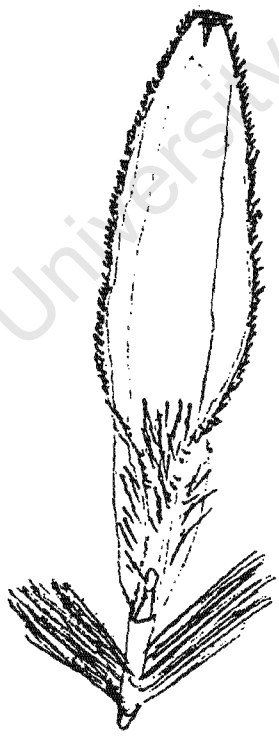
A



B

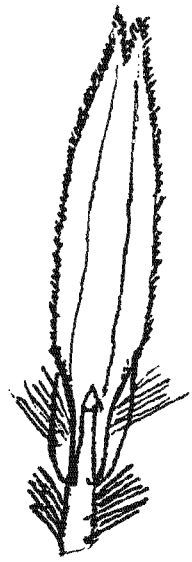
C

D



E

F



G

5 mm

are present while in others a dense indumentum may be observed. In several species this indumentum may be observed on the lower half of the palea, and rarely goes up to the palea apex. The second character (61) describes the presence of an indumentum between the keel and the palea margin. The third character (62) describes the presence or absence of cilia on the palea keels, which are usually present, but varies in their density. If there are only few hairs that occur sporadically leaving gaps from which the shiny part of the keel can be seen, we then regard this state as glabrous as opposed to the state where the cilia can be seen as dense.

61. Palea flaps: hairy (0); glabrous (1); scabrous (2) (Fig. 2.10E, F).

62. Palea margins: glabrous to sparsely scabrid (0); densely scabrid (1).

63. Palea apex (Figs. 2.10A –G): pointed (0); blunt (1); incised (2); bilobed (3).

64. Palea keels: reaching apex (0); not reaching apex (1).

Palea keels stopping a little distance from the palea apex has been surveyed earlier by Linder and Verboom (1996) and shown to be a synapomorphy of *Pentaschistis*.

65. Callus length relative to rachilla length: shorter (0); about equal (1); longer (2)

66. Callus hairs: absent (0); reaching rachilla internode (1); not reaching rachilla internode (2).

67. Rachilla internode: glabrous (0); hairy (1)

68. Hairs on the apical region of the ovary : absent (0); present (1).

The genus *Pentameris* has deciduous hairs on the apical region of the ovary. This character is thus considered important in separating this genus from the rest of the danthonioids as well its closely related genera, *Pseudopentameris* and *Pentaschistis*, which have glabrous ovaries.

Embryological characters

This section describes variable features of the integuments, micropyle and the synergids in the megagametophyte. Species data for these

embryological characters are obtained from Verboom *et al.* (1994) and describes variation only for those taxa that were included in their analysis. The remainder are coded as having missing data for these characters.

69. Haustorial synergids: Haustoria absent (0); haustoria present but weakly developed, not protruding through the micropyle (1); haustoria present and strongly developed, protuding through micropyle (2).

70. Inner integument and micropyle: micropyle broad or expanded such that the inner integument is discontinuous at the micropylar end (0); micropyle constricted, inner integument complete (1).

71. Starch in the synergids (excludes starch elsewhere in the megagametophyte): starch absent, if present it is very finely granular (0); starch present as large globular grains (1).

72. Thickening of inner integument around micropyle: one or two cells thick (0); more than two cells thick (1).

73. Outer integument: reduced to a collar surrounding the base of the ovule on the side of its attachment to the placenta, or at most may cover the chalazal half of the ovule, micropyle endostomic (0); covered the entire ovular surface, micropyle exostomic (1).

74. Thickness of nucellar epidermis at micropyle: epidermis one or two cells thick (0); epidermis more than two cells thick (presence of a nucellar cap) (1).

74. Thickening of micropylar margin of the outer integument: absent (0); present (1).

75. Orientation of micropyle: micropyle not conspicuously oblique (0); micropyle visibly oblique (1).

76. Apomictic embryo sacs: no apomixis observed (0); apomictic embryo sacs observed in at least some embryo ovules (1).

Table 2.2. Data matrix of 76 morphological, anatomical and embryological characters used in the phylogenetic analysis of 44 species sampled. Characters numbers (top row) correspond to those indicated in the description of characters in the text. The first 67 characters are anatomical and morphological; the last 9 characters are embryological obtained from Verboom et al. (1994). Missing data are indicated by "?", polymorphic characters by "*" and inapplicable characters by "-".
Note: a taxon lacking a specific feature is coded "inapplicable" with respect to the variation in that feature being coded. Abbreviations: Chaetobromus inv. ssp. inv = *Chaetobromus involucratus* subspecies *involucratus; Chaetobromus inv.* ssp. *dreg.* = *Chaetobromus involucratus* subspecies *dregeanus; Merxmuellera cincta* ssp. *cinc.* = *Merxmuellera cincta* subspecies *cincta*.

Taxon name	Character state							
	10	20	30	40	50	60	70	76
Centropodia glauca	01101000-1	0000121111	000102002-	0111002103	1011110001	11000-1011	1010000010	110010
Merxmuellera disticha	0011011031	0201020101	011103001-	200--02002	1111100021	11110-00-2	0020000201	000100
Merxmuellera macowanii	020*011020	1101020001	0111111220	2111112001	1111100102	00010-10-2	2120?00???	??????
Merxmuellera davyi	0200011020	0101020?01	0111111?1	0111?11?0?	?1?1100101	0?101010-?	?120100???	??????
Merxmuellera aureocephala	0202011020	1101020001	011111101-	0111112001	1111100101	01100-10-0	0120100???	??????
Merxmuellera drakensbergensis	0200011020	1202020001	0111111210	2111112001	1111100101	00101010-0	2120000???	??????
Merxmuellera stereophylla	0200010020	1201020001	011111122-	2111012011	1211100121	10001010-0	1120000???	??????
Merxmuellera stricta	0120200001	1102111?00	011103020-	2111112001	1111100021	01110-10-0	0110200201	010000
Merxmuellera guillarmoidae	0001200001	0101001101	010101021-	0111111100	0101000310	01010-10-2	0100200???	??????
Merxmuellera dura	0101110001	0101020000	000101021-	0111111113	0101101011	11010-00-0	0120100201	010000
Merxmuellera rangei	1---0-1120	0010001100	000101020-	211101?001	1111100001	1110110001	0010200??	??????
Merxmuellera decora	0101100001	0001111100	000000000-	1111212011	1211100011	11000-1010	0100210???	??????
Merxmuellera lupulina	0100000001	0101021101	010000000-	101-110??1	1011100002	11000-1011	0100000???	??????
Merxmuellera rufa	0200000031	0102021100	000100020-	201-212000	0011100011	11000-?011	010?000201	101111
Merxmuellera papposa	1---001120	1110011111	000101020-	1111012101	1111100011	1110111001	1120110???	??????
Merxmuellera arundinacea	0001001011	0102020001	001103022-	1111112010	1311101021	10000-1001	0110000100	111011
Merxmuellera cincta ssp. *cincta*	0002001011	0112020001	001102000-	0111112103	0211100111	11000-1011	0110000???	??????
Merxmuellera setacea	0200001011	1100010101	0010?10??-	0111?11?0?	01?1100?1	11100-1?00	0??1?00???	??????
Chionochloa macra	0001000010	0112000001	011111022-	1111210001	1011100001	11000-1000	0120000???	??????
Chionochloa pallens	0201003010	0102000001	011112002-	1111?10001	1?11100?11	11000-1000	0120000???	??????

Chionochloa rigida	0201003010 0101000001 011112002- 0111010001 1011100021 11000-1000 0130100??? ??????
Cortaderia selloana	02012000*1 1102001001 0001??002- 2111?12111 1211000300 01010-00-0 2110100201 000100
Cortaderia fulvida	0????????? ?????????? ???111022- 2111112010 1211000300 01010-10-0 2100100??? ??????
Cortaderia richardii	0200210120 1112201101 100111022- 2111112010 1211000300 01010-10-0 0120100??? ??????
Tribolium uniolae	0200000031 0001021010 011101021- 200--10101 0000-----0 11000-1100 0110100??? ??????
Tribolium pusilla	0100000?1 1001221100 010101021- 201--10112 0301000--0 10000-1101 0010200201 000000
Tribolium hispida	0201000011 0001021100 000101001- 101--?2102 0000-----0 11000-1001 0120100??? ??????
Schismus barbata	0100010?1 0001001?10 000101021- 1011012103 0000-----0 11000-1100 1121200??? ??????
Pentaschistis curvifolia	0000000010 1101020001 0011000112 1111000113 0211100022 11000-1001 1021200??? ??????
Pentaschistis aspera	0201203?0 1102020101 001102022- 1111002103 0111100022 11000-1000 10200?0??? ??????
Joycea pallida	0110201021 0101021?10 000101021- 2111012103 0201100011 11000-1001 0120000201 0?0101
Pentameris macrocalycina	00000130*1 0101020001 0?11010202 2111002101 1211100022 11000-1001 0021001110 101100
Pentameris thuarii	0200000131 0102020011 0001020112 1111002113 0211100022 11000-1001 0021001010 010110
Pseudopentameris macrantha	0201000131 0*01020000 000101021- 2111002101 0211100021 01000-1000 1020200201 000100
Pseudopentameris caespitosa	0101010031 0*01020100 000101001- 0111202101 0211100021 01000-0000 1020200??? ??????
Karroochloa purpurea	0200200?1 0001021101 ?0?101021- 0111011103 0011100001 01111110-0 0120200201 000100
Karroochloa tenella	0---0100-1 0001021101 000101001- 1111012111 0011100001 11111110-0 1010210201 000000
Karroochloa schismoides	0100010021 0002021101 ?00101001- 1111012103 0011100011 11000-1011 1000100201 000000
Chaetobromus inv. ssp. inv.	0110103121 0100020101 000102021- 1110012103 0111110021 01000-10-0 1011200201 000100
Chaetobromus inv. ssp. dreg.	0110000?1 0101011?01 000102001- 1110012103 0211110022 01000-10-1 1000200201 000100
Rytidosperma nudiflorum	0102000?0 0000000?01 1111???0?- 0111?1*000 0111100111 01110-00-0 0020000??? ??????
Rytidosperma pumilum	0-2-0000-0 0000020101 0011???0?- 0111?1*000 0111110301 1?111000-0 1020000??? ??????
Austrodanthonia auriculata	0????????? ?????????? ???102?0?- 1111?12001 1111110021 01111110-1 0100200??? ??????
Austrodanthonia laevis	0????????? ?????????? ???1?2?01- 1111?12001 0111100021 01111110-0 1120200??? ??????

2.2 Molecular data

With the advent of polymerase chain reaction (PCR) technology for deoxyribonucleic acid (DNA) amplification, sequencing has now become sufficiently inexpensive and easy to use for phylogenetic studies at all taxonomic levels. Consequently, the field of plant molecular systematics has rapidly advanced in obtaining and utilising nucleotide sequences for addressing a wide variety of phylogenetic and evolutionary questions. Given the current emphasis on DNA sequences for phylogeny estimation, the primary challenge to using nucleotide characters for lower level phylogenetic studies is the identification of easily amplifiable and relatively rapidly evolving, but unambiguously alignable, DNA regions that can provide sufficient, suitable variation within a short sequence segment (Baldwin *et al.* 1995). Although sequence data from the chloroplast genome is still being actively collected and used in phylogeny estimation, investigators are now exploring nuclear gene sequences to compare their topologies with chloroplast-based topologies. It is now a common practise to apply multiple data sets to a group of taxa because there is a growing consensus that reliance on a single data set often results in insufficient phylogenetic resolution or misleading inferences. As a result this study exploited two genes, the nuclear sequences from the internal transcribed spacer region (ITS) and the variable grass-specific insert in the chloroplast RNA polymerase subunit 2 (*rpo*C2), which have been previously used to study phylogenetic relationships in the subfamily Danthonioideae.

2.2.1 Utility of *rpo*C2 and ITS sequences data

2.2.1.1 The variable grass specific insert in the chloroplast ribosomal polymerase subunit 2 (*rpo*C2) gene.

Most chloroplast genes are slowly evolving (e.g. *rbcl*) and thus limited in their utility in resolving lower taxonomic questions. However, the insert of the plastid *rpo*C2 has been reported to be highly variable and hence useful in its systematic applicability at lower taxonomic levels (Cummings *et al.*, 1994; Barker *et al.*, 1999). The *rpo*C2 gene is located in chloroplast

genome and is part of the *rpo*BC operon containing *rpo*C $B/C_1/C_2$ genes coding for RNA polymerase β subunits (Igloi *et al.*, 1990). Igloi *et al.* (1990) were the first to locate, and establish its nucleotide sequences from maize (*Zea mays*) chloroplast RNA polymerase. This operon in general is part of a large inversion with respect to tobacco and spinach chloroplast genomes and is flanked by *trn*C and *rps*2 genes. The *rpo*C2 insert consists of short repeat motifs (c. 21 bp) termed heptameric repeats (Igloi *et al.* 1990). These heptameric repeats are thought to have arisen through slipped strand mispairing (Igloi, *et al.* 1990; Cumming *et al.*, 1994), an activity that has been postulated to be of major significance in gene and genome evolution (Levinson and Gutman, 1987). Although the systematic applicability of *rpo*C_2 is limited at the high level due to its high variability and has not been used in many systematic studies so far, Barker *et al.* (1999) have used it for elucidating the generic relationships of the subfamily Danthonioideae. Most recently Barker *et al.* (2000) have also used it to establish the relationships and composition of the Danthonioideae, and its resolution has been greatly enhanced when used in combination with the nuclear genes (e.g. ITS).

2.2.1.2 The internal transcribed spacer region

The internal transcribed spacer region is part of the transcriptional unit of the 18S – 26S eukaryotic nuclear-encoded ribosomal DNA cistron (nrDNA), but the spacer segments of the transcript are not incorporated into mature ribosomes (Baldwin *et al.* 1995). There are three components making up the this region (Figure 2.1): the evolutionarily highly conserved 5.8S rRNA gene; and the two spacers which are referred to as ITS-1 and ITS-2. The two spacers and the 5.8S subunit are collectively known as the internal transcribed spacer (ITS) region.

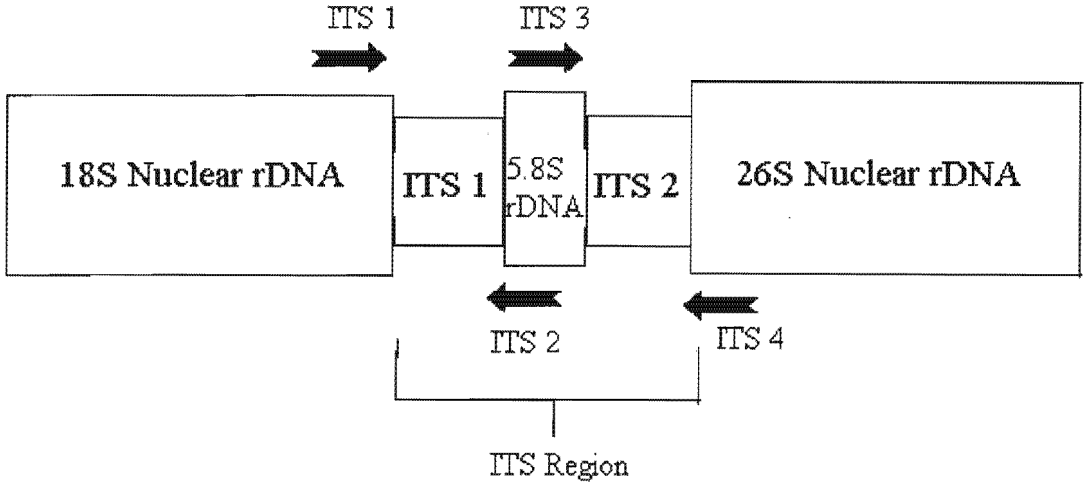


Figure 2.2. Organization of the entire internal transcribed spacer regions (ITS) of the 18S to 26S nuclear-encoded ribosomal DNA cistron (nr(DNA).

The length of each of these spacers is about 300 base pairs in angiosperms (Baldwin *et al.,* 1995). Baldwin *et al.* (1995) reviewed the utility of the ITS region in plant molecular systematics in depth and has outlined several properties making this region very useful for addressing lower-level phylogenetic questions in angiosperms. The first reason for promoting its use in phylogenetic analyses in angiosperms is the high copy number of tandem repeats in the entire nrDNA at a chromosomal locus or at multiple loci (Roger and Bendich 1987; Hamby and Zimmer, 1992). This high copy number eases detection, thus permitting systematic comparisons of relatively recently diverged taxa (Liston *et al.*, 1996). In addition the ITS region can be easily amplified from total DNA and sequenced with conserved primers positioned in the cistronic regions (Baldwin *et al.,* 1995). The second reason, and the most important from the viewpoint of phylogeny reconstruction, is that the spacer undergoes rapid concerted evolution through unequal crossing-over and gene conversion (Arnheim *et al.*, 1980; Apples & Dvorak, 1982; Baldwin *et al.*, 1995) and can therefore resolve lower-level relationships than the slow evolving chloroplast genes. Thirdly, the ITS region in angiosperms is small in size, less than 700 base pairs, and the presence of highly conserved

sequences flanking each of the two spacers make this region easy to amplify (Baldwin *et al.,* 1995).

2.2.2 DNA sampling strategy

Sequence data for the chloroplast *rpo*C2 gene and rDNA (ITS) for most species of *Merxmuellera* and numerous other danthonioid grasses have been previously published (Barker *et al.*,1999, 2000; Hsiao *et al.*, 1998). Molecular data reported in this study utilises and expands on these published sequences data (Table 2.3). Barker made sequences from representatives of other genera in the subfamily available for simultaneous analysis with *Merxmuellera*. No fresh material or material dried with silica gel was available for *M. aureocephala, M. papposa, M. stereophylla* and *M. drakensbergensis* and attempts to amplify *rpo*C2 gene and the entire ITS region from herbarium specimens failed. Field collections for new material for these taxa were undertaken to obtain fresh material for molecular study and voucher specimens were deposited in appropriate herbaria. The material collected for this purpose was dried in the field using silica gel as described by Chase and Hills, (1987). However, *M. aureocephala* was not in flower at the time field collections were made since flowers are necessary for proper identification and as such this species was not included in the molecular analyses.

2.2.3 DNA extraction

The modified hot CTAB method of DNA extraction by Doyle and Doyle (1987) was followed to extract DNA from both fresh and dried leaf material. Following this method, approximately 1 to 2 centimetre (cm) length of the leaves was cut into small shavings and ground in the mortar and pestle in the presence of 1 millilitre (ml) CTAB extraction buffer to which 1 drop of B-Mercaptoethanol was added. The fluid was placed into a 1.5 ml eppendorf tube and incubated in the 60^{0} water bath for approximately 30 minutes (min). A volume of about 500 µl of chloroform: isoamyl alcohol (CIA) was added. The suspension was shaken vigorously

and centrifuged for 1 minute at 13,000 revolutions per minute (rpm). The supernatant was centrifuged again for 1 min at 13,000 rpm, 600 µl supernatant (the top layer above the band of cell debris) was transfered to a clean 1.5 ml eppendorf tube and mixed with 400 µl of ice-cold isopropanol and this was left in ice for 10 min. After centrifuging again at 13,000 rpm for 10 minutes, the supernatant was then carefully poured out leaving a small grey-white pellet of DNA behind. To wash all the salts from the pellet, 750 ml of 70% ethanol was added and the tube was gently inverted a few times before pouring the ethanol out. A small piece of paper towel was used to remove as much of the traces of ethanol as possible from the tube and then left open in the draft at the fume hood mouth to air dry the pellet. The eluted DNA was re-suspended in 300 µl of distilled water and was then readily available for amplification.

Table. 2.3. List of ITS and *rpo*C2 sequences used in the analysis along with voucher details. If the ITS and the *rpo*C2 genes were sequenced from different voucher specimens, their collectors details are provided above and below each other respectively. Species listed as ex cult. New Zealand were provided to N. P. Barker by R. Buxton from the Botanic Garden at Lincoln, New Zealand and their garden numbers starts with a letter G.
Abbreviations: ex cult. = ex cultivated; n.s = not sequenced; n.p = sequences not published

Species name	**Voucher**	**Geographical distribution**	**GenBank number ITS**	**GenBank number *rpo*C2**
Austrodanthonia auriculata (J. M. Black) H. P. Linder	Linder 5569	Australia	n.p.	n.s.
Austrodanthonia caespitosa (Gaulich) H. P. Linder		Australia	n.p.	n.s.
Austrodanthonia laevis (Vickery) H.P.Linder	Linder 5633	Australia	AF019875	U96313
Centropodia glauca (Nees) T.A.Cope	Barker 967 Linder 5410	Africa	AF019861	 U92265
Chaetobromus involucratus subsp. (Schrad.) Nees *dregeanus* Verboom	Barker 978	Africa	n.p.	U92483
Chaetobromus involucratus subsp. (Schrad.) Nees *involucratus*	Barker 1715	Africa	n.p.	n.p.
Chionochloa rigida (Raoul) Zotov	Linder 5710	New Zealand	n.p.	n.s.
Chionochloa flavescens Zotov		Ex cult. New Zealand	n.p.	n.s.
Chionochloa frigida (Vickery) Conert	Jacobs 7935	New Zealand	AF019868	n.s.
Chionochloa macra Zotov	Hsiao 475278	Ex cult. New Zealand	n.p.	U92701
Chionochloa pallens Zotov	Hsiao 475279	Ex cult. New Zealand	n.p.	n.s.
Cortaderia araucana Stapf	G 7162	Ex cult. New Zealand	n.p.	n.s.
Cortaderia archboldii (Hitchc.) Connor & Edgar	Marsen 115	South America	n.p.	n.s.
Cortaderia bifida Pilg.	Lyle 1497	New Zealand	n.p.	AF355988
Cortaderia columbiana (Pilg.) Pilg.	Lyle & Cerillo 920	South America	n.p.	AF355991

Cortaderia fulvida (Buchan.) Zotov	G. 5088	New Zealand	n.p,	U93359
Cortaderia hapalotricha (Pilg.) Conert	Lyle 1525	South America	n.p.	AF355989
Cortaderia jubata Lemoine ex Carrière	Lyle 1315	South America	n.p.	AF355987
Cortaderia nitida (Kunth) Pilg.	Lyle 1434	South America	n.p.	AF355990
Cortaderia richardii (Endl.) Zotov	Hsiao 191	South America	n.p.	n.s.
Cortaderia rudiuscula Stapf	G 11157	Ex cult. New Zealand	n.p.	AF355992
Cortaderia selloana (Schult.& Schult. f.) Asch. & Graeb.	Robinson, s.n.	South America	AF019812	U93360
Cortaderia sericantha (Steud.) Hitchc.	Lyle 1128	South America	n.p.	AF355986
Cortaderia splendens Connor	G 10872	Ex cult. New Zealand	n.p.	AF355994
Cortaderia turbaria Connor	G 17358	Ex cult. New Zealand	n.s.	AF355995
Danthonia californica (Thunb.) Munro ex Macoun	Curto	South America	AF019813	n.s.
Danthonia scheiderii Pilg.	Soreng, 5638	Himalaya	n.p.	n.p.
Danthonia secundiflora J. & C. Presl	Lyle 1617	South America	n.s	U93361
Danthonia spicata Roem. and Schult.	Kellogg, s.n.	South America	n.p.	U93662
Danthonia vestita Pilg.	Chase, MWC 8878	South America	n.p.	n.p.
Joycea pallida (R.Br.) Linder	Linder 5564	Australia	AF019880	U94394
Karroochloa purpurea (L.F.) Conert and Tuerpe	Linder 5360	Africa	n.p.	U94824
Lamprothyrsus peruvianus Hitchc.	Lincoln, N.Z	New Zealand	AF019874	U94952
Merxmuellera arundinacea (Berg.) Conert	Barker 1017	Africa	n.p.	U94953
Merxmuellera cincta subsp.(Nees) Conert *cincta* (Nees) Conert	Barker 1160	Africa	n.p.	U94954
Merxmuellera cincta (Nees) Conert subsp. *sericea* N. P. Barker	Barker 1545	Africa	n.p.	AF355985
Merxmuellera davyi (C.E. Hubb.) Conert	Barker 942	Africa	n.p.	U94955
Merxmuellera decora (Nees) Conert	Barker 1168	Africa	n.p.	AF355984
Merxmuellera disticha (Nees) Conert	Barker 1002	Africa	n.p.	U94956
Merxmuellera drakensbergensis (Shweick.) Conert	Mafa 3	Africa	n.s.	n.p

Merxmuellera dura (Stapf) Conert	Barker 983	Africa	AF019872	U94957
Merxmuellera guillarmoidae Conert	Barker 1009	Africa	n.p.	U95075
Merxmuellera lupulina (Thunb.) Conert	Linder 7004	Africa	n.s	AF355983
Merxmuellera macowanii (Stapf) Conert	Kew 142-8301715 Barker 1008	Africa	AF019863	U95076
Merxmuellera papposa (Nees) Conert	Barker and Mafa 1760	Africa	n.p.	n.p.
Merxmuellera rangei (Pilg.) Conert	Barker 960	Africa	AF019862	U95077
Merxmuellera rufa (Nees) Conert	Barker 1149	Africa	n.p.	U95078
Merxmuellera setacea N.P.Baker	Barker 987	Africa	AF019867	U95079
Merxmuellera stereophylla (Anderson) Conert	Mafa 4	Africa	n.s.	n.p.
Merxmuellera stricta (Schrad.) Conert	Barker 1159	Africa	AF019871	U95080
Notochloe microdon (Benth.) Domin	Dalby 94/15 Watson n.s.	New Zealand	AF019869	U95126
Notodanthonia gracilis (Hook. f.) Zotov	Linder 5683	Australia	n.p.	n.s.
Pentameris macrocalycina (Steud.) Schweick.	Barker 1164	Africa	AF019864	n.p.
Pentameris thuarii Beauv.	Linder 5456	Africa	n.s	U95127
Pentaschistis aristidoides (Thunb.) Stapf.	Barker 1158	Africa	n.s.	n.p.
Pentaschistis aspera (Thunb.) Stapf	Barker 1165	Africa	AF019865	U95128
Pentaschistis capillaris (Thunb.) McClean	Linder 5439	Africa	n.s.	n.p.
Pentaschistis curvifolia (Schrad.) Stapf	Barker 1165	Africa	n.s	U95129
Pentaschistis patula (Nees) Stapf.	Barker 5432	Africa	n.s.	n.p.
Pentaschistis lima (Nees) Stapf.		Africa	n.s.	n.p.
Pentaschistis pyrophyla Linder	Linder 5509	Africa	n.s.	n.p.
Pentaschistis densifolia (Nees) Stapf	Barker	Africa	n.s.	n.p.
Pentaschistis triseta (Thunb.) Stapf	Linder 5435	Africa	n.s.	n.p.
Pentaschistis velutina Linder	Linder 5446	Africa	n.s.	n.p.
Plinthanthesis paradoxa (R. Br.) S.T.Blake	Jacobs 7773	Australia	n.p.	U95361
Prionanthium ecklonii (Nees) stapf	Linder 5402	Africa	AF019866	U95362
Pseudopentameris brachyphylla (Stapf.) Conert	Barker 1669	Africa	n.s.	n.p.

Pseudopentameris caespitosa N. P. Barker	Barker 1668	Africa	n.s.	n.p.
Pseudopentameris macrantha (Schrad.) Conert	Linder 5470	Africa	n.p.	U96307
Rytidosperma nudiflorum (Morris) Connor & Edgar	Linder 5693	Australia	AF019876	U96314
Rytidosperma oreoboloides (F. Muell.) H. P. Linder	Chase, MWC 8877	Australia	n.p.	n.s.
Rytidosperma pumilum (Kirk) Linder	Linder 5747	Australia	AF019878	U96312
Schismus barbartus (Loefl. Ex L.) Thell.	Linder 5359	Africa	AF019873	U96308
Tribolium hispidum (Thunb.) Desv.	Linder 1740	Africa	n.p.	n.s.
Tribolium pusillum (Nees) H.P. Linder	Linder 5402	Africa	n.p.	U96311
Tribolium uniolae (L.f.) Renvoize	Barker 1163	Africa	n.p.	U96310

2.2.4 DNA amplification

The rpoC2 gene and the ITS region were amplified by polymerase chain reaction (PCR). The *rpo*C2 primers designed by Barker *et. al.* (1999) using published rice and maize *rpo*C2 sequences (Igloi *et al.* 1990) were used to amplify the *rpo*C2 gene for all the taxa sampled for this gene in this study. These primers are called "*rpo*C2-U" (upstream = 5'-TTTGATTTGTTAGCGAAAAAGCG-3') and the *rpo*C2-D (downstream = 5'-ATCCACTCCAATAATACTATTGTC-3'). A double stranded DNA fragment for the entire ITS region, which consisted of the ITS1 and ITS2 regions was amplified using ITS primers, ITSL (5'-TCGTAACAAGGTTTCCGTAGGTG-3') designed by Hsiao et al. (1998) for ITS1 and ITS2 (TAGAATTCCCCGGTTCGCTCGCCGTTAC) designed by Sun *et al.* (1994). The two internal primers Danth 5.8SF (forward: 5'-GACTCTCGGCAACGG-3') and Danth 5.8SR (reverse: 5-TTTGGCGTGACGCCC-3') designed by Barker (unpublished) were also used to sequence the coding regions of the 5.8s rDNA

A 100 µl PCR reaction was run for each sample. As the concentration of magnesium in the reaction mixture is critical for successful PCR, care has to be taken for its optimization. In this study we prepared four PCR reactions of different magnesium concentrations which ran concurrently for total DNA amplified. 4 µl, 8 µl, 12 µl and 16 µl $MgCl_2$ was added from the first microtube to the fourth making up a final concentration of 1 to 4 mM reaction mix respectively. A stock solution for each primer was made up by diluting1 µl of each primer with 200 µl of deionised water. For each primer used, the stock concentration was 40 mM. A mixture of dNTPs (10 µl each with an initial concentration of 0.525mM) was prepared and diluted with 160 µl dH_2O. Within each tube the same amounts following were placed: 4 µl each primer, 10 µl thermophilic DNA polymerase 10X Mg free buffer; 4 µl dNPT mix; 5 µl template DNA which was taken directly from the total DNA extracted and 0.4 µl Taq DNA polymerase (Promega). In general, each reaction was made up to 100 µl with H_2O.

These settings worked well for most samples amplified for the *rpo*C2 gene. However, numerous failures for ITS amplification were usually encountered. There are claims that betaine improves PCR in DNA amplification and to solve the high rate of amplification failure for ITS, 20 µl of 5M Betaine was added to each reaction mixture before the final making up to 100 µl with water. This approach worked well for most reactions attempted. All the PCR reaction mixtures were overlaid with a drop of mineral oil to prevent evaporation during amplification. The PCR reactions performed in a Hybaid PCR Sprint thermal cycler exposed to the following PCR profile: denaturing step of 95°C for 45 seconds (sec); annealing step of 50°C for 45 sec; extension step of 72°C for 3 minutes. At the end of the last cycle for each gene, a final extension step of 72°C for 10 min was allowed. Following this PCR profile, *rpo*C2 gene was cycled 35 times and 40 cycles for ITS.

2.2.4.1 Agarose gel electrophoresis, DNA visualisation and cleaning

To find out whether the DNA has been successfully amplified, PCR products were run on 1% agarose and visualized on an ultra-violet (UV) transilluminator. To prepare a 1% agarose gel, 0.5 mg of agarose powder was dissolved completely by boiling it in 50 ml of 1x Tris-boric acid EDTA (TBE) buffer solution in a microwave for about 3 min, swilling the tube ocassionally. When fully dissolved, the solution was cooled down under running water for few minutes and stained with 15-30 µl of ethidium bromide for visualisation purposes under UV light. The gel was then poured into the casting tray with combs arranged to create wells for loading the gel. After the gel was completely set i.e. forming a semi-opaque white colour, about 15 microliters (µl) of the PCR product mixed with a blue loading buffer on the parafilm strip was loaded into a well on the gel and run for 20 to 30 min. The stained PCR product was visualised using UV transilluminator. The successful reactions were pooled out and cleaned using the QIAquick PCR purification kit from Qiagen (protocol in Appendix I), eluting the purified product with dionised water.

2.2.5 DNA sequencing

The purified double stranded PCR products were directly cycle sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing kit. Sequencing for clean *rpo*C2 PCR products was done using the primers called *rpo*C2-1 (5'-CGGAATTCTTTACGTAGAAATACTA-3') and *rpo*C2-2 (5'-CGGTCGACTTGTTCCTCGATGCTCAA-3') obtained by Barker (1999) from Kellogg (USA). The clean PCR products for ITS were directly sequenced with the same amplification primers ITSL, ITS2 and the two internal primers 5.8SF and 5.8SR. The success of the cycle sequencing was confirmed by electrophoresing 1 µl the cycle sequence products on a 1% agarose gel as described above and visualised using UV transilluminator.

Successful cycle sequencing products were precipitated out following the manufacture's instructions and were dried in a rotary evaporator at 60°C under vacuum. The products were run on an ABI 377 autosequencer. The raw data files were assembled and edited using the Sequencher 3.0 software package (Gene codes corporation) accelerated by power Macintosh.

2.2.6 Sequences alignment

Sequence alignment is a crucial step in any molecular phylogenetic study, and is essentially a problem of homology (Swofford and Olsen, 1990; Miyamoto and Cracraft, 1991; Wheeler *et al.*, 1995; Barker *et al.*, 1999). Molecular sequence alignment is in essence a procedure by which we can recognise and describe potential homology among nucleotide or amino acid positions. The objective of this step is to establish provisional (putative or primary) homology statements across taxa (Mindell, 1991) in the form of bases in the data matrix, which can be subjected to some form of phylogenetic analysis. In sequence alignment the primary homology (sensu dePinna, 1991) is generally established through the computation of a pairwise similarity cost function. That is, for effective phylogenetic analysis of DNA sequences, it is absolutely necessary that

the sequences be aligned in such a way that homologous nucleotide base positions within homologous genes are compared (Mindell, 1991).

To achieve the above, the sequences were entered into and manually aligned using the DNA And Protein Sequence Alignment (DAPSA) programme written by E. H. Harley (Department of Chemical Pathology, University of Cape Town). The alignment of sequences for the *rpo*C2 data presented and analysed in this study is slightly different from that of Barker *et al.*, (1999; 2000) in length. In this study sequences for *rpo*C2 gene were re-aligned by eye to remove the deletions that were hypothesised to ensure positional homology of nucleotides with respect to sequences for taxa in other tribes. Therefore, the nucleotide homology remains the same but the sequences are somewhat shorter than in these previous studies.

2.3 Phylogenetic analysis

Morphological and the molecular data sets obtained were analysed individually and in combination under maximum parsimony method as implemented in Phylogenetic Analysis Using Parsimony (PAUP*) test version 4.0b4a (Swofford, 1997) on an Apple Macintosh. Since this study aimed at testing the monophyly of *Merxmuellera*, all the representative genera of Danthonioideae included were allowed to resolve simultaneously with the ingroup and cladograms were rooted with *Centropodia glauca* (subfamily Chloridoideae). This species is an immediate sister to the danthonioid lineage (Barker *et al.*, 1995, 1999; Hsiao *et al.*, 1998, 1999) and has been previously used to root cladograms in the phylogenetic studies of the Danthonioideae (Barker *et al.*, 2000).

2.3.1 Searching for most parsimonious trees

Methods for finding most parsimonious or minimum length tree(s) fall into two categories as discussed by Swofford and Olsen (1990). The first category includes the exact methods (exhaustive, branch-and-bound) that quarantee the discovery of all optimal trees. Exhaustive searches examines all possible fully resolved unrooted cladograms for all the taxa included, and length of each is calculated. The branch-and-bound method closely resembles the exhaustive search, but differs in that it does not require every completed cladogram to be examined individually. This method begins the calculations using the heuristic method employing the branch-swapping algorithm, the cladogram of which is retained as a reference length or upper bound for use during subsequent cladogram construction. These methods are only usable if the data sets are of small to medium size (8 to 20 taxa).

The second category is for larger data sets in which heuristic methods must be employed. Heuristic searches differ in that there is no quaranttee that the shortest tree has been found. Most searches contain two elements: In the first search, a tree estimate is obtained by an algorithmic approach. In the second search, the branches of the tree are moved around in search of a more optimal solution. Maddison (1991) suggested that during tree searches, there usually exist a collection of most parsimonious trees that can be partitioned into islands based upon the length of trees and the number of rearrangements by which trees differ. Maddison, (1991) defines the island of trees as a collection of trees that are less than or equal specified length that are topologically similar enough to one another, each tree being connected to every other tree in the island through a series of trees, and each one differing from the next by a single rearrangement of branches. As result trees in different islands for a given data matrix may have different implications for character evolution. Maddison, (1991) noted that the possibility of presence of multiple islands among the set of most parsimonious trees is evident if the number of taxa in a data matrix exceeds twenty and the

preliminary tree searches indicate low retention indices (less than about 0.67).

All the data sets reported here are included large number of terminals and hence prohibit the use of methods in the first category for finding trees that are equally parsimonious. This conditions is also indicative of the possibility of multiple islands in data matrices. To explore the possibility of multiple islands of trees (Maddison, 1991), preliminary heuristic searches were performed on all the data sets using 200 random addition sequences with tree bisection and reconnection (TBR) branch-swapping algorithm while keeping only one most parsimonious tree for each replicate (MULPARS off). The size and the composition of the islands, and the number of times they were rediscovered were determined by observing the status report in PAUP. All the data sets resulted in at least three islands of most parsimonious trees. These initial trees were used as the starting trees to conduct a more thorough heuristic tree searching using TBR branch-swapping algorithm, steepest descent options in effect, collapsing branches with maximum length of zero and saving all most parsimonious trees (MULPARS on). Trees found in the searches were saved up the maximum that could be retained in computer memory.

2.3.1.1 Separate analyses

Three data sets were used for cladistic analysis in this study:

1. ITS
2. *rpo*C2
3. morphology (including anatomy and embryology)

These data sets were analysed individually, giving equal weights to all the characters. Multistate characters were treated as unordered (non-additive, Fitch 1971) to avoid apriori assumptions regarding character evolution.

2.3.1.2 Successively character weighting

Morphological characters were successively weighted to compare the topology that they can provide since they are relatively limited in number to topologies of the molecular data which are relatively large when analysed under equal weights.

Using the optimal trees obtained in the analysis of the unweighted morphological data, characters were assigned new weights by their rescaled consistency indices (base weight = 1) following the successive reweighting approach of Farris (1969). Characters were reweighted reiteratively until the topology of the tree found stabilised. At each round of successive weighting, was on 100 replicates of heuristic searches were used under the same tree searching conditions set for the unweighted morphological data. The shortest trees found were used as starting trees for the next round of a thorough heuristic tree searching. These trees were then saved and characters were weighted equally to check on how long they are when characters are weighted equally and mapped onto them. This exercise was undertaken to indicate whether these trees represent a subset of the shortest trees obtained by the unweighted analysis, or if they are different trees. Character distribution were optimised under ACCTRAN procedure in MaClade version 3.07 (Maddison and Maddison, 1992).

2.3.1.3 Bootstrap and Decay analyses

One of the most important requirements in phylogenetic analysis is to statistically assess the reliability of individual internal nodes of an estimated tree. There are many methods that have been suggested and used for this purpose. These include the rasampling techniques, which include parsimony jacknifing (Mueller and Ayala, 1982; Dodds, 1986) and bootstrap (Felsenstein, 1985; Sanderson, 1989). The other method is Bremer support which does not involve resampling of the data (Bremer, 1988).

Bootstrap and jacknife are the two branch support methods which estimate the variance of sampling distribution by repeatedly re-sampling data from the original data set. However, these two methods differ in the way in which re-sampling is performed. For bootstrap, characters are resampled with replacement from the original data set until a pseudoreplicate data set containing the original number of observations is obtained (Felsenstein, 1985; Sanderson, 1989). For each pseudoreplicate, the most parsimonious trees are obtained and the degree of conflict assessed by means of a majority rule consensus tree which shows groups found in more than 50% of all the trees retained. Support for each of these groups is then interpreted as the frequency by which individual groups occur in the pseudoreplicates. In contrast, jacknife re-samples the original data set by dropping a certain proportion of characters at a time and re-computing the estimate from the remaining observations. Since the jacknife is applied without replacement as in boostrap, the pseudoreplicate data sets are usually smaller than the original data and the aim here is to achieve better variance estimates that might otherwise be possible from small samples. There is usually a very slight difference in the results provided by these two methods, but the bootstrap method has been much used in literature and was preferred in this study. The following full heuristic search parameters in PAUP were used for this purpose: 100 replicates of random addition sequence, TBR branch-swapping algorithm, MULPARS and Steepest option in effect.

Assessment of confidence limits on phylogenies based on bootstrap has been much criticised (Hillis and Bull, 1993; Kluge and Wolf, 1993). The argument is that during bootstrap analysis not all the characters are sampled, and as such the proportions of the pseudoreplicates created during boostrap analysis may differ to a great extend with the actual replications. As a result the strength of support for each resolved clade was further evaluated by generating decay indices that are used as a measure of support for individual clades (Bremer, 1988; Donoghue *et al.* 1992). This method was originally proposed by Farris *et al.* (1982) for

distance analyses and by Bremer (1988) for parsimony analyses and is profoundly different from jacknife and bootstrap approaches that involve perturbation of the original data set. The Bremer support method uses all the characters to examine the number of steps needed to collapse a node in the consensus tree of the near most parsimonious tree. Bremer support analysis was performed on AutoDecay ver 4.0 (Erickson 1998) as implemented in PAUP*. AutoDecay programme helps in performing decay analysis of nodes in a cladogram or consensus tree using the reverse constraint option in PAUP. To obtain each decay index, the setup command was executed to read consensus trees saved for each data set to create constraint trees for the nodes. The heuristic searches to determine the decay indices were performed following these parameters: 10 replicates of random addition sequence per reverse constraint tree, TBR branch-swapping algorithm and saving multiple trees (MULTREES on). The decay indices were then extracted with extract decay indices in AutoDecay. The difference in length between the original and the contraint searches represent the decay index for each node evaluated.

2.3.1.4 Combined analyses

Whether or not to combine different data sets for phylogenetic analysis has been a controversial subject in the field of systematics. Many systematists have expressed different opinions in whether data sets collected from the common group of taxa should be:

(i). analysed individually (Pesole *et al.*, 1991; Shaffer *et al.*, 1991; Swofford, 1991; Marshall, 1992; de Queiroz, 1993)

(ii). combined (total evidence) prior to phylogenetic analysis (Miyamoto, 1985; Kluge, 1989; Barrett *et al.* 1991; Donoghue and Sanderson 1992)

or

(iii) analysed separately before combining the independent estimates using consensus (taxonomic congruence) methods (Adams, 1972; Mickevich, 1978; Nelson, 1979; Hillis, 1987; Swofford, 1991; de Queiroz, 1993)

Advocates of the principle of total evidence argue that combined inference from multiple data sets may potentially yield the strongest estimate of phylogeny. This is based on the grounds that certain characters are useful in the resolution of some nodes on the cladogram but uninformative for others hence combining two or more data sets may improve the resolution of the whole tree. In addition, it is argued that weak, but true phylogenetic signals may be present in different data sets, but signal within any single data set may be masked by noise and with combining data sets the signals may be additive and rise above the noise (e.g. Barrett *et al.* 1991). Contrary to the total evidence approaches of analysing multiple data sets, advocates of separate analysis of multiple data sets appeal to the importance of independence among data sets. Their argument relies on the recognition that different data sets are governed by different phylogenetic histories as well as different rates of character evolution, thus combining data sets may violate assumptions underlying each process. A third approach to the treatment of multiple data sets is that of assessing data sets statistically for phylogenetic incongruence (Bull *et al.* 1993). This approach has been widely followed by many authors because not only addressing the question of combining data sets, by analysing data sets separately and testing them for taxonomic incongruence. Under this approach we gain insights on how much each data set contributes to a resolution of a particular node in the combined analysis (Nixon and Carpenter 1996).

Two combinations of data sets were constructed. The first included only the molecular data sets (ITS and *rpo*C2) while the second included molecular data and morphological data sets (total evidence data set). These data sets included only those outgroup taxa from which both of their sequences were available. Tree searching for the combined data sets was performed in the same manner as conducted in separate analyses.

To assess topological conflicts (incongruence), competing clades obtained from the most parsimonious trees for individual data sets the

bootstrap and the decay indices supporting them were compared. If the bootstrap support values for the competing clades was identified to be 65% or more and the decay indices were at least 2, then conflict was considered significant and suggested for further testing using other statistical tests of incongruence. What is a good boostrap or good decay support can be a very debatable statement as they are just the statistical measures of support. In this study the threshold of two steps in decay analysis is chosen because support of one step is the minimum for the node to be considered existing hence we chose the next value as an initial step to consider carrying-out other more robust statistical tests incongruence if there is a topological conflict among data sets. As for bootstrap, a 65% threshold is chosen because it is a value somewhere halfway between a 50% support which is considered weak, and the maximum support (100%) a node may be offered. It is sometimes the case that a decay value of for example, two steps, chosen here as the threshold, maybe offered to a node receiving a low bootstrap value. This is a flaw of decay analysis due to the fact that this analysis tend sometimes overestimate support when the heuristic methods used fail to find the optimal solution (Oxelman *et al.*, 1999). This is a very controversial issue that this study may not contest at the present moment.

CHAPTER THREE

RESULTS

3.1 Individual data sets

3.1.1 Morphology

3.1.1.1 Unweighted morphological data

Cladistics analysis of the unweighted morphological data for forty-four taxa, using *Centropodia glauca* to root the cladograms, resulted in four most parsimonious trees {length (l) = 467 steps, consistency index (c.i) = 0.2313 and retention index (r.i)= 0.5069} (Table 3.1). One of the most parsimonious trees with bootstrap values superimposed above branches on nodes with more that 50% support is presented in Fig. 3.1. Results obtained from bootstrap and decay analyses indicate that only two nodes (4 and 40) are strongly supported (bootstrap = 75, 77; decay index = 4, 5 respectively), while nodes 1, 21, 31, 33, 34 and 41 have weak to moderate support. The rest of the nodes have very little support. Only two nodes (node 7 and node 36) collapse in the strict consensus of the four most parsimonious trees.

Monophyly of most genera included in analysis is not contradicted except for *Karroochloa, Tribolium, Pentaschistis, Pentameris* and *Merxmuellera*. The data set resolves two subspecies of *Chaetobromus* in a weakly supported clade outside the rest of the taxa contained in node 2. Of the remaining taxa in node 2, *Karroochloa schismoides* is resolved sister to the rest, followed by a well supported clade 4 (bootstrap = 75; decay = 4) containing *M. rangei* and *M. papposa* characterised by a synapomorphy of circular lamina outline. *M. guillarmoidae* and *M. dura* are resolved together in clade 9 with low support and are sister to clade 10. Within clade 10, *M. stricta* is sister to the Australasian genus, *Austrodanthonia,* represented by two species, *A. auriculata* and *A. laevis*. Three species of *Tribolium* (*T. uniolae, T. pusilla* and *T. hispida*) are resolved together with *Schismus barbatus* in clade 13 with low boostrap

but decay support of 2 steps above the minimum length of most parsimonious trees found.

Clade 17 resolves *Joycea pallida* sister to three species of *Merxmuellera* (*M. rufa, M. lupulina* and *M. decora*) contained in clade 18. The internal structure within this *Merxmuellera* group has low support, but it is characterised by their distinct bulbous bases that are densely woolly, atypical of the genus.

The monophyly of *Pseudopentameris* in clade 21 represented by two species (*P. macrantha* and *P. caespitosa*) is weakly supported and is sister to the remaining taxa contained in clade 22. This clade resolves two nodes, 23 and 28. Clade 23 contains species of *Pentameris, Pentaschistis* and the two reed-like species of *Merxmuellera* (*M. arundinacea* and *M. cincta*). *M. cincta* and *M. arundinacea* are resolved together (node 19) as a weakly supported clade sister to *Pentaschistis aspera*. Clade 28 resolves *M. disticha* sister to the rest of the taxa contained in clade 29. *M. setacea* is resolved in clade 30 sister to a moderately supported clade (bootstrap = 60; decay index = 2) containing two species of *Rytidosperma*. The monophyly of *Chionochloa* is moderately supported (bootstrap = 67%; decay index = 2 step; node 33) and is sister to clade 35 containing the Drakensberg *Merxmuellera* species with *Cortaderia* embedded within. This clade has low support and resolves *M. davyi, M. aureocephala* and *M. drakensbergensis* paraphyletic on the basal nodes. Within this clade the species of *Cortaderia* are retrieved as a monophyletic group in node 40 (bootstrap = 77; decay = 5).

Table 3.1. Tree statistics describing the trees obtained from the cladistic analyses of separate and combined data sets based on both the informative and the uninformative characters.

Data set	No. of taxa	No. of informative characters	No. of optimal trees	Tree length	CI	RI
Morphology (unweighted)	44	76	4	467	0.2313	0.5069
*Morphology (weighted)	44	76	3	470	0.230	0.503
ITS	58	195	40	2188	0.4824	0.7159
rpoC2	64	132	23859	1704	0.5154	0.8440
Combined molecular (rpoC2 + ITS)	48	299	12	1128	0.4231	0.7107
Combined molecular and morphology	49	306	16	1567	0.5910	0.6234

*Note: statistics from the analysis of unweighted morphological data provided is that obtained after mapping the weighted trees onto the unweighted characters to check on whether trees found in the former analysis are different or a subset of the unweighted trees retrieved in the unweighted analysis.

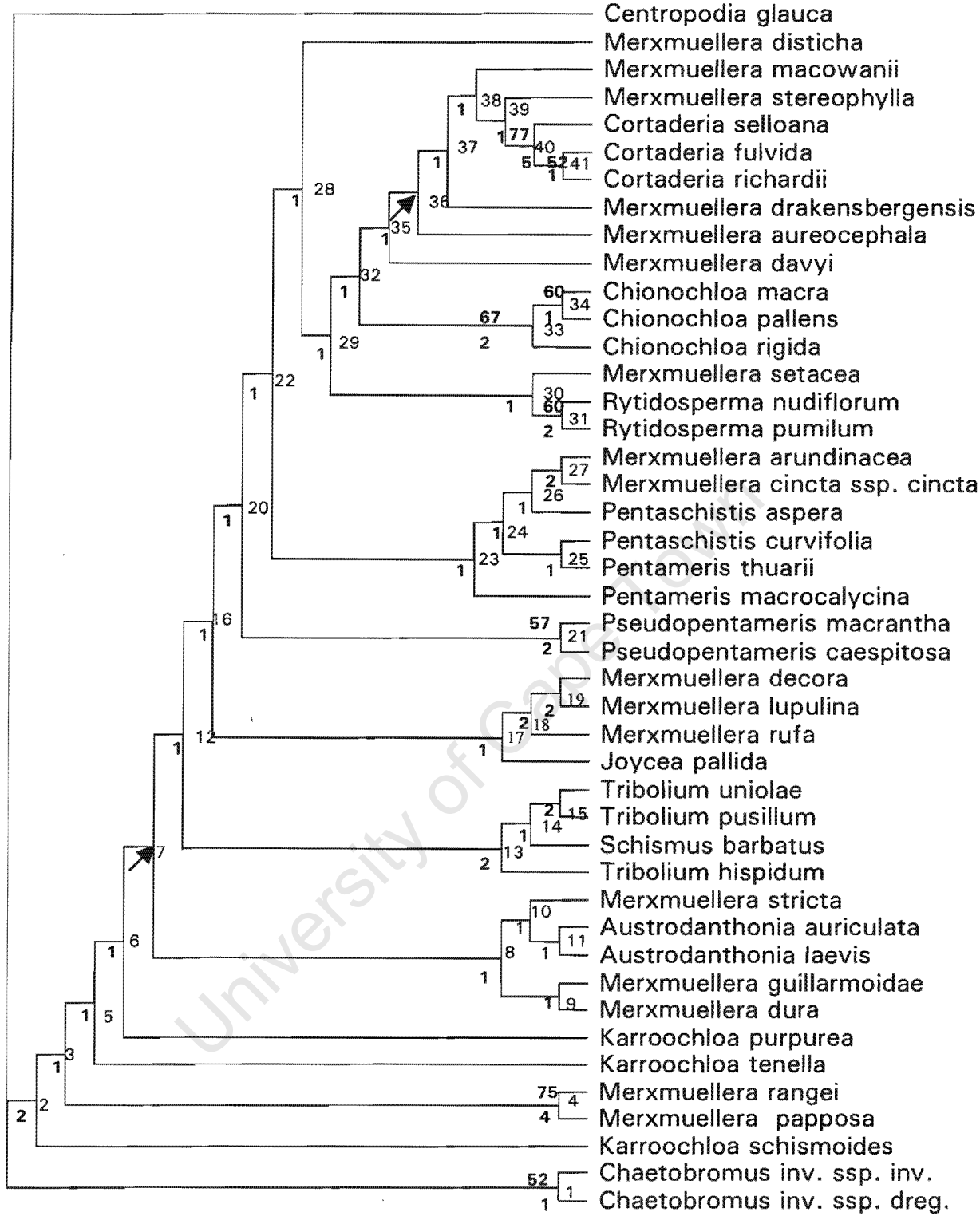


Fig. 3.1. One of the four most parsimonious trees obtained from heuristic search of the unweighted morphological data (l = 467; c.i. = 0.23131; r.i. = 0.5069). Numbers in bold below branches indicate decay support values. Numbers in bold above branches indicate bootstrap support values on nodes with more than 50% support. Abbreviations: inv. = involucratus; dreg = dregeanus; ssp. = subspecies

3.1.1.2 Successively weighted morphological data

Successive weighting of the morphological data located three most parsimonious trees after two rounds of successive weighting (l = 50; c.i. = 0.3671; r.i. = 0.6564). When these trees are mapped onto the unweighted data, the length of the successively weighted trees are three steps longer (l = 470) than the length of trees found in the unweighted analysis, and consistency index and the retention index are almost similar 0.230 and 0.503 respectively (Table 3.1). This is an indication that these trees retrieved in the weighted analysis are not different trees from the shortest trees found in the unweighted analysis, but a subset of the shortest trees in the latter analysis. One of the most parsimonious trees is presented in Fig. 3.2 and Fig. 3.3 shows all possible character changes under ACCTRAN optimisation. Only one node (node 27) collapses in the strict consensus of three most parsimonious trees obtained, thus the strict consensus tree is highly resolved. Topologically, the weighted morphological data differs from the unweighted data at one point: *Merxmuellera* species from the Drakensberg are grouped together in a moderately supported clade (bootstrap = 58%) in the weighted data. Phylogenetic structure within this clade is not well supported except for weak support for the sister relationship between *M. davyi* and *M. aureocephala* (bootstrap = 55%). In general, there is an increase in support for most of the clades retrieved compared to the results of the unweighted morphological data. Support for the monophyly of *Chaetobromus* in node 1 is increased (bootstrap = 76%), and relationship between *M. rangei* and *M. papposa* is strongly supported (bootstrap = 86). A clade containing *Tribolium* and *Schismus* is also well supported (bootstrap = 71%; node 8). The monophyly of geophytic *Merxmuellera* species in node 18 has very little support, similar to the results of unweighted morphological data, but the relationship between *M. decora* and *M. lupulina* has weak support (bootstrap = 52%). Support for the monophyly of *Pseudopentameris* represented by two species in this analysis is high (bootstrap = 71%; node 21). Support for the monophyly

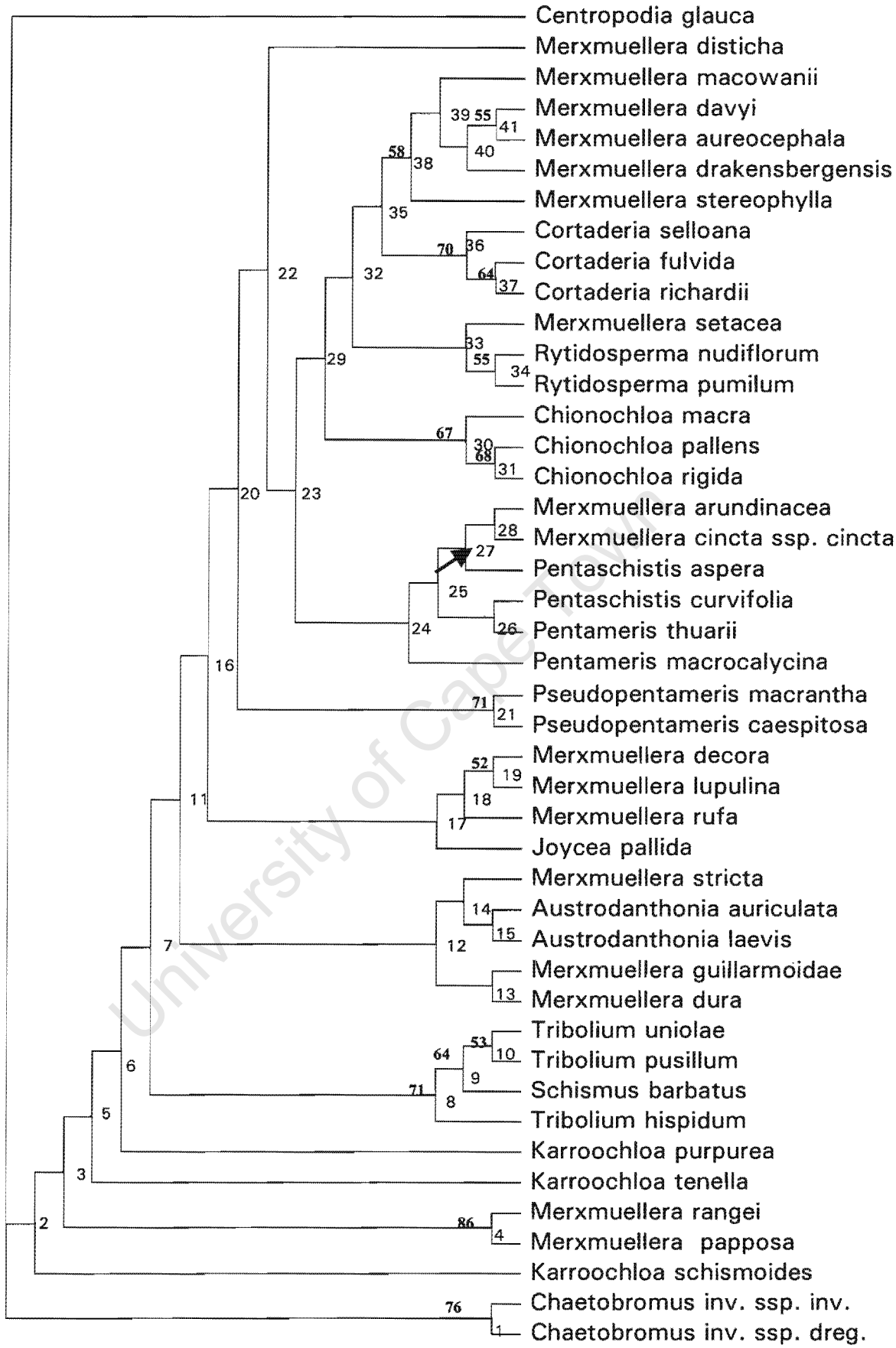


Fig. 3.2. One of the three most parsimonious trees obtained from heuristic search of successively weighted morphological data (l = 470; c.i. = 0.230; r.i. = 0.503). Numbers in bold above branches indicate bootstrap support values on nodes with more than 50% support.

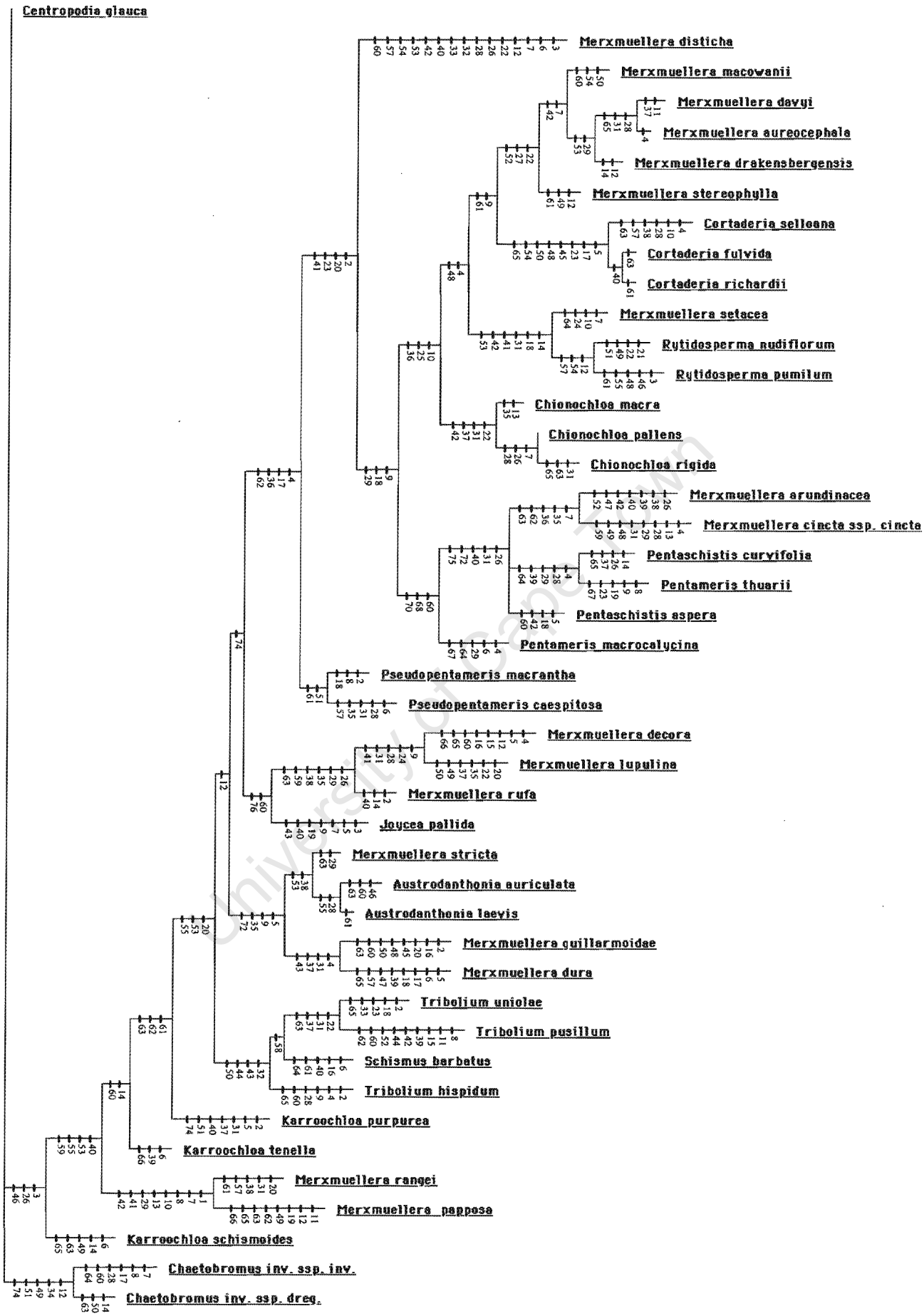


Fig. 3.3. Possible character state changes mapped on one of the most parsimonious trees found in heuristic tree searching on the successively weighted morphological data. Character numbers are as in the text

of *Chionochloa* in node 30 represented by three species is similar to that obtained in the analysis of the unweighted morphological data, but incongruent on the phylogenetic structure within this clade. In this analysis, *C. pallens* and *C. rigida* are retrieved as sister taxa in node 31 with moderate support (bootstrap = 68%). Support for a sister relationship between *Rytidosperma pumilum* and *R. nudiflorum* is decreased in this analysis (bootstrap = 55%). Support for the monophyly of *Cortaderia* is increased (bootstrap = 70%), but its sister relationship to the Drakensberg *Merxmuellera* group is very low.

3.1.2 Molecular

3.1.2.1 *rpoC2* data

With the exeption of *M. drakensbergensis, M. stereophylla* and *Joycea pallida* from which partially sequences for the *rpo*C2 gene were obtained, the unaligned sequences for the whole study group ranged from 407 bp in *Pseudopentameris caespitosa* to 571 in *M. papposa*. The alignment of the *rpoC2* sequences was 708 bp long of which 132 (19%) were phylogenetically informative (Table 3.1). Cladistic analysis of the *rpoC2* data was terminated due to computer memory overflow when a maximum number of 23859 most parsimonious trees were saved (l = 1704, c.i. = 0.6998, r.i. = 0.8440). One of the most parsimonious tree is presented in Fig. 3.4 with bootstrap and decay support values shown above and below the branches respectively. The branch lengths, based on th included number of nucleotide changes under ACCTRAN optimisation, are shown above the branches.

Topology of one of the most parsimonious trees provided in Fig. 3.4 shows that twelve nodes collapse in the strict consensus tree. Nine major clades are retrieved of which five are strongly supported (bootstrap >82, decay index >3 steps; Table 3.2) while the rest had little support. In general the monophyly of most genera is not contradicted except for *Cortaderia, Merxmuellera, Rytidosperma* and *Danthonia*. For the species of *Merxmuellera* sequenced for the *rpoC2* gene, the results from this

analysis as shown in Fig. 3.4 indicate the existence of three principal monophyletic clades: (1) *M. rangei – M. papposa* clade (node A), (2) the *M. davyi – M. drakensbergensis* clade (node C) which includes the summer rainfall *Merxmuellera* species from the Drakensberg excluding *M. stereophylla* resolved in node E with *Danthonia vestita*, and (3) *M. arundinacea – M. lupulina* clade (node I) which includes all the winter rainfall species from south western Cape. The rest of the species of *Merxmuellera* are resolved basal to the rest of the closely related genera contained in clade Q referred to as the *Rytidosperma* clade (sensu Barker *et al.*, 2000).

M. rangei and *M. papposa* are resolved as well supported two-taxon clade outside the rest of the genera in the subfamily Danthonioideae (Fig. 3.4; node A) with moderate support (bootstrap = 67%, decay index = 3). Within node B, eight major clades are retrieved, most of which group closely related genera together (Fig. 3.4; Table 3.2). The summer rainfall *Merxmuellera* species including *M. drakensbergensis, M. macowanii* and *M. davyi* from the Drakensberg form a well supported clade (Fig. 3.4; node C; Table 3.2) sister to the rest of the taxa. Within this group *M. drakensbergensis* is sister to *M. macowanii* and *M. davyi* with moderate support (bootstrap = 64; decay index = 2 steps). Only four nucleotide changes have occurred along the branch subtending these three taxa while five changes are shown in the *M. rangei-M. papposa* lineage. However, one species from the Drakensberg, *M. stereophylla*, does not group together with the rest of the members from this region, but is placed sister to *Danthonia vestita* in clade E with low bootstrap and decay support (bootstrap <50%; decay index =1 step).

The remaining taxa are resolved in clade F with moderate support (bootstrap = 66%; decay = 2 steps). Within this clade, African genera including *Pentameris, Prionanthium* and *Pentaschistis* are resolved as a well supported clade (node G; Fig.3.4; Table 3.2) sister to the rest of the taxa. The robustness of clade G is correlated with relatively long branches (12 nucleotide changes) subtending this clade. Clade I resolves species

of *Merxmuellera* from the Cape as a monophyletic group. Bootstrap and decay support for this clade is very low. Within this clade, the geophytic *Merxmuellera* species including *M. lupulina, M. decora* and *M. rufa* are grouped together as a weakly supported subclade (bootstrap = 60%; decay index = 2), and are sister to a subclade grouping the caespitose *Merxmuellera* species (*M. arundinacea, M. setacea* and *M. cincta*) with low bootstrap (<50%) and decay support (1 step). Clade J is moderately supported (bootstrap = 65%; decay = 4 steps) and resolves *Chionochloa* represented by one taxon (*C. macra*) in the *rpo*C2 sequence data sister to the rest of the taxa. The next clade K is strongly supported (bootstrap = 86%; decay = 6) and it resolves into two nodes (Q and L). Node L has a weak support and divides into two nodes, M and N. The South American species of *Cortaderia* and *Lamprothyrsus* are resolved together in clade M with a weak support (Fig. 3.4; Table 3.2). Clade N is strongly supported by bootstrap (85%) but moderately so by decay (3 steps) and groups all the New Zealand species of *Cortaderia*, the Australasian genera *Notochloe, Plinthanthesis* as well as two species of *Danthonia* from South America. Node P is strongly supported clade (bootstrap = 99%; decay = 9 step) and resolves *Pseudopentameris* and *Chaetobromus.* The monophyly of *Chaetobromus* is strongly supported by 98% bootstrap and 6 steps in decay analysis. Bootstrap analysis supports the monophyly of *Pseudopentameris* (83%), but the node requires only three steps to collapse above the length of the optimal trees. Clade Q referred to as the *Rytidosperma* clade (sensu Barker et al. 2000) is weakly supported and resolves *M. disticha, M. dura* together with Australasian genera *Rytidosperma, Joycea* and *Austrodanthonia* as well as the African genera *Karroochloa* and *Tribolium* and *Schismus barbatus* and the Himalayan *Danthonia scheiderii*. Relationships within this clade have low support except for the strongly supported relationship of *M. guillarmoidae* and *M. stricta* (bootstrap = 89%; decay index = 6; node T) and the strongly supported monophyly of *Tribolium* (bootstrap = 99%; decay = 5 steps).

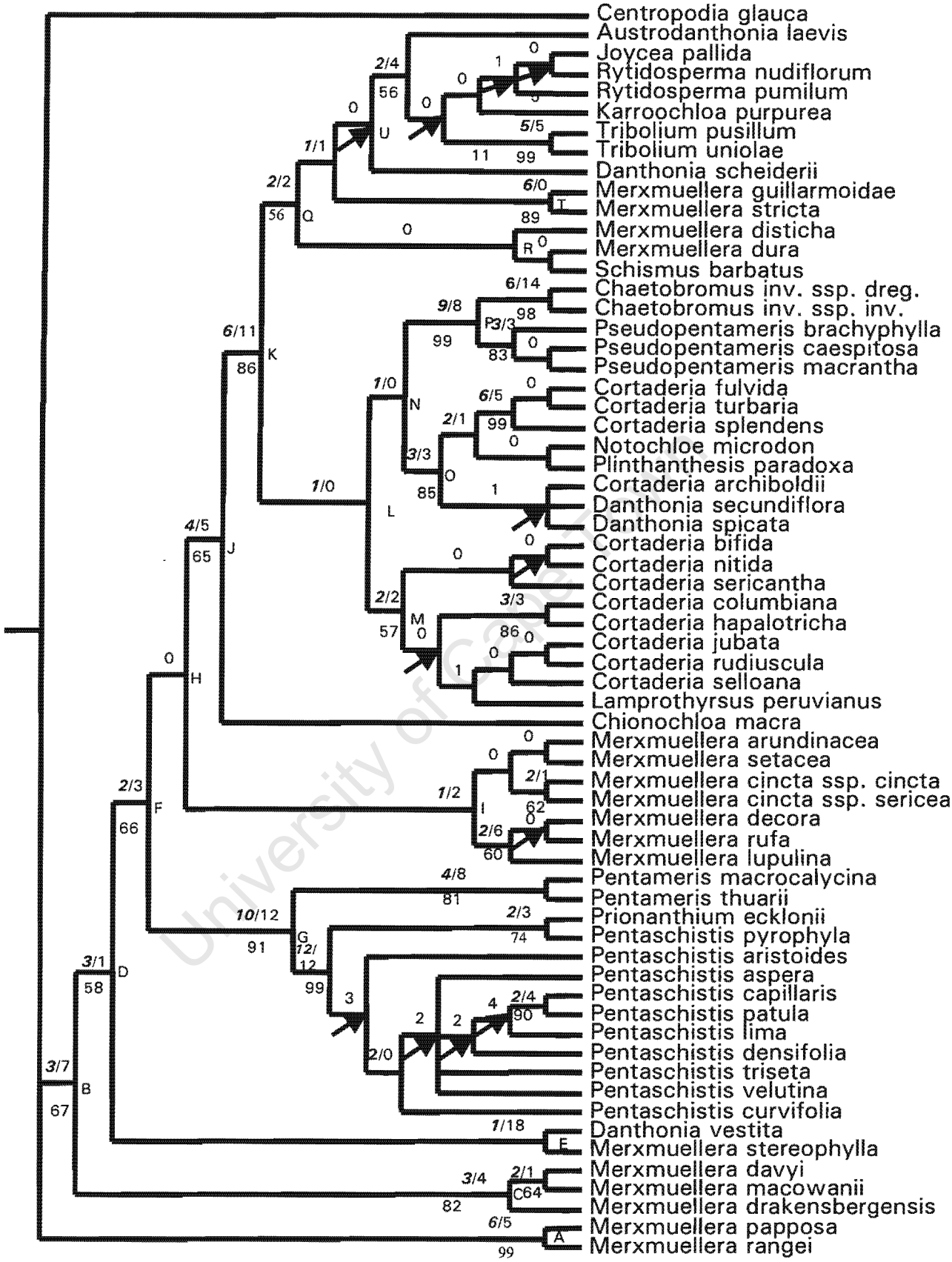


Fig. 3.4. One of the fundamental trees from a set of 23895 shortest trees obtained from the heuristic search of the rpoC2 sequence data. The first numbers in bold and italics above branches are decay support values. The second number above branches separated by a slash are branch length values obtained under ACCTRAN optimisation procedure. Arrows show nodes that collapse in the strict consensus of the shortest trees found. Letters represent node numbers referred to in the text. Abbreviations: ssp. = subspecies; inv. = involucratus; dreg. = dregeanus.

Table 3.2. Major clades retrieved from cladistic analysis of the *rpoC2* data set.

Genera and infraspecific names are abbreviated as follows: *Chaet. dreg.* = *Chaetobromus involucratus subspecies dregeanus*; Cort. = *Cortaderia*; Pent. = *Pentameris*; Psch = *Pentaschistis;* Lamp. = *Lamprothrsus;* Merx. = *Merxmuellera.* Pseu. = *Pseudopentameris*; Aust. = *Austrodanthonia;* Dant. = *Danthonia;* Schi. = *Schismus*. n.s. refers to nodes with less than 50% boostrap.

Clade	Clade designation	Bootstrap (%)	Decay index
1. *Merx. rangei – Merx. papposa*	A	99	6
2. *Merx. drakebsbergensis – Merx. davyi*	C	82	3
3. *Merx. stereophylla – Dant. vestita*	E	n.s.	1
4. *Pent.macrocalycina – Psch. curvifolia*	G	91	10
5. *Merx. lupulina – Merx. arundinacea*	I	n.s.	1
6. *Cort. bifida – Lamp. peruvianus*	M	57	2
7. *Cort. fulvida – Dant. spicata*	O	85	3
8. *Chaet. dreg. – Pseu. macrantha*	P	99	9
9. *Aust. Laevis – Schi. barbatus*	Q	56	2

Table 3.3. Major clades retrieved from cladistic analysis of the ITS data set.

Generic and infraspecific names are abbreviated as follows: *Chaet. dreg.* = *Chaetobromus involucratus* subspecies *dregeanus*; Cort. = *Cortaderia*; Pent = *Pentameris*; Psch = *Pentaschistis;* Merx. = *Merxmuellera*; Pseu. = *Pseudopentameris*; Aust. = *Austrodanthonia;* Dant. = *Danthonia;* Schi. = *Schismus*; Chio. = *Chionochloa.* n.s. refers to nodes with less than 50% boostrap.

Clade	Clade designation	Bootstrap (%)	Decay index
1. *Merx. rangei – Merx papposa*	A_1	100	7
2. *Merx. macowanii – M. davyi*	A_2	99	5
3. *Chaet. dreg. – Pseu. macrantha*	C	99	9
4. Cort. fulvida – Plin. paradoxa	F	88	5
5. *Cort. araucana – Dant. californica*	G	n.s.	1.
6. *Pent. macrocalycina – Psch. aspera*	J	97	8
7. *Merx. arundinacea – Merx. rufa*	L	n.s.	1
8. *Chio. flavescens – Chio. rigida*	M	99	6
9. *Aust. auriculata – Schi. barbatus*	N	n.s.	1

3.1.2.2 ITS data

With an exception of *Rytidosperma oreoboloides* and *Danthonia vestita* for which only the ITS 1 sequences were obtained, the entire ITS region for the whole study group ranged from 438 bp in *Cortaderia columbiana* and *C. nitida* to 614 bp in *Pseudopentameris macrantha*. The alignment of the entire ITS sequences was 677 base pairs long. Of the 288 (43%) variable sites, 195 (29%) were parsimony informative (Table 3.1). Cladistic analysis of the ITS data set yielded 40 most parsimonious trees each of length 2188 steps, with a consistency index of 0.4824 excluding uninformative characters and retention index of 0.7159. One of the most parsimonious trees is presented in Fig. 3.5 with bootstrap and decay values superimposed below and above branches respectively. Branch lengths obtained under ACCTRAN optimisation procedure are also shown above the branches. In the strict consensus, nine out of 42 nodes resolved are collapsed as shown in Fig. 3.5. The topology of the ITS sequence data as presented in Fig. 3.5 shows that twenty three nodes have bootstrap support above sixty five percent, and only twenty four nodes have decay support of at least two steps above the minimum length of the most parsimonious trees.

As with the *rpo*C2 data the monophyly of most genera retrieved in the ITS data is not contradicted except for *Merxmuellera*, *Cortaderia*, *Danthonia* and *Austrodanthonia*. In this analysis, the Drakensberg *Merxmuellera* species retrieved by the *rpo*C2 data as the basal-most taxa in the Danthonioideae, are clustered with the *M. rangei* – *M. papposa* clade (node A). The relationship betweent these two group receives very little support. Support for the clade containing the rest of the taxa (node B) is very strong in ITS compared to the *rpo*C2. Within this clade the basal-most taxa include the African genera *Chaetobromus* and *Pseudopentameris* retrieved as a strongly supported clade (Fig. 3.5; Table 3.3, node C). The next clade, D, is very weakly supported and resolves two nodes, E and H. Clade E has low support, and also resolves two very weakly supported nodes, F and G. In clade F, *Notochloe microdon* and

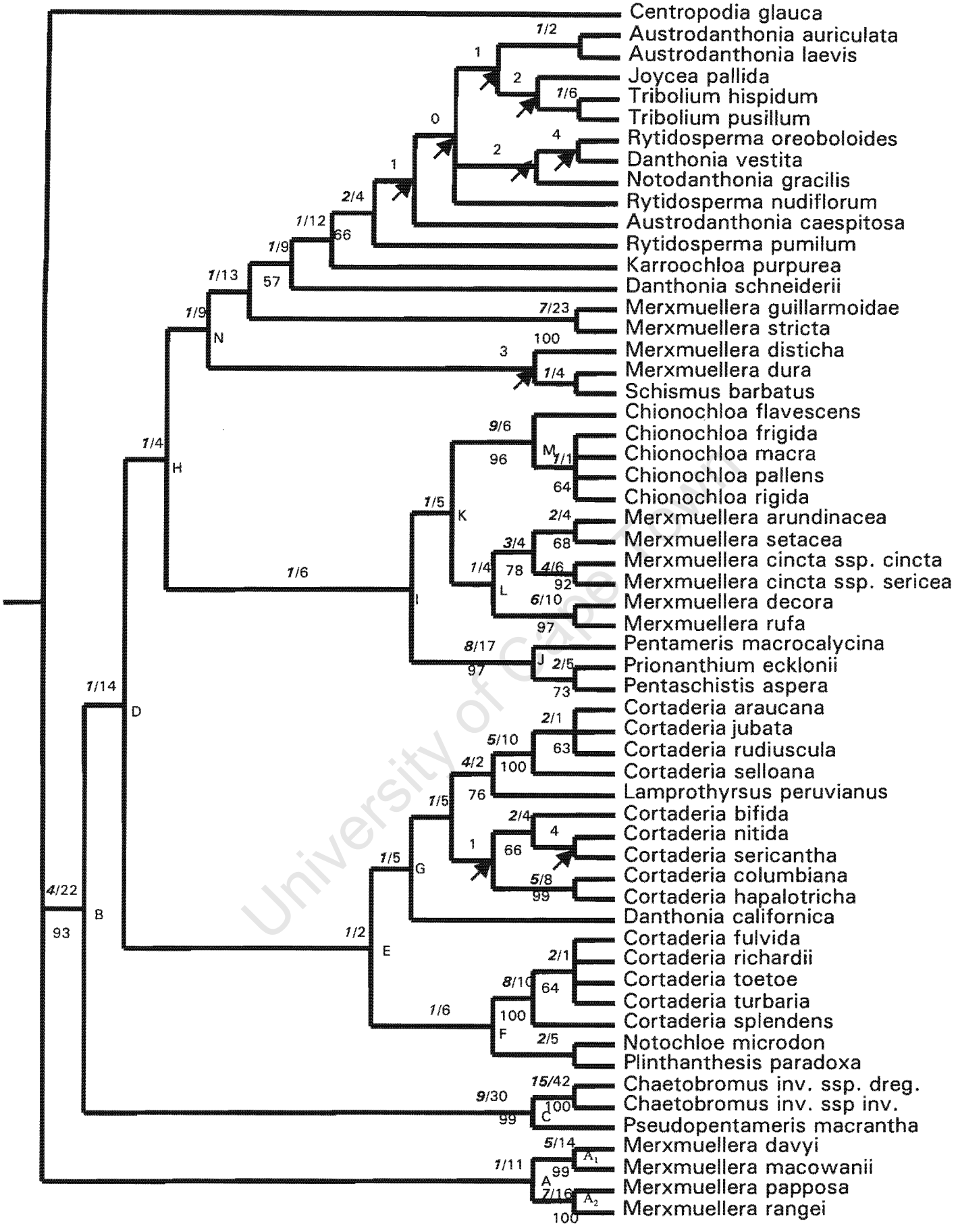


Fig. 3.5. One of the fundamental trees from a set of forty most parsimonious trees obtained from the heuristic search of the ITS sequence data. Numbers in bold and italics above the branches are the decay values and the second numbers separated by a forward slash are the branch length values determined by ACCTRAN optimization. Bootstrap percentages are shown below branches on nodes with more than 50% support. Arrows indicate nodes that collapse in the strict consensus. Letters are clade numbers referred to in the text. Abbreviations: ssp. = subspecies; inv. = involucratus; dreg. = dregeanus.

Plinthanthesis are grouped together and are sister to a strongly supported clade (bootstrap = 100; decay index = 8 step) containing the New Zealand species of *Cortaderia*. Clade G has weak support and resolves *Danthonia californica* sister to the South American species of *Cortaderia* and *Lamprothyrsus*. The next largest clade, H, has low support, and resolves two nodes (I and N) with very low support. Within node I, the relationship between *Pentameris, Prionanthium* and *Pentaschistis* in node J is very strong (bootstrap = 97%; decay = 8 steps). The Cape species of *Merxmuellera* are grouped together in a weakly supported clade sister to a strongly supported clade M containing species of *Chionochloa* (bootstrap = 96%; decay index = 9 steps). The phylogenetic structure within the Cape *Merxmuellera* species is well supported. Support for the monophyly of species of *Merxmuellera* characterised by bulb-like bases represented by *M. decora* and *M. rufa* in this analysis is very high (bootstrap = 97%; decay = 6 steps). In addition, a subclade containing the caespitose species of *Merxmuellera* is good (78 % bootstrap; decay index of 3 steps). Relationships of *Merxmuellera* species included in the *Rytidosperma* clade (node N) are similar to that observed in the *rpo*C2 analysis, but there is an increase in support for the clade containing *M. guillarmoidae* and *M. stricta* (bootstrap = 100%; decay index = 7 step).

3.2 Combined data sets

3.2.1 Combined molecular (rpoC2 + ITS)

Cladistic analysis of the combined *rpoC2* and ITS data for those ingroup taxa for which both data sets were available yielded twelve most parsimonious trees with a length of 1128 steps, consistency index of 0.6232 and retention index of 0.7107. One of the twelve most parsimonious trees selected at random is presented in Fig. 3.6 with bootstrap and decay support values shown below and above branches respectively. The branch length values obtained under ACCTRAN optimisation procedure are shown above the branches. Only three nodes collapse in the strict consensus tree. In general, the topology is largely

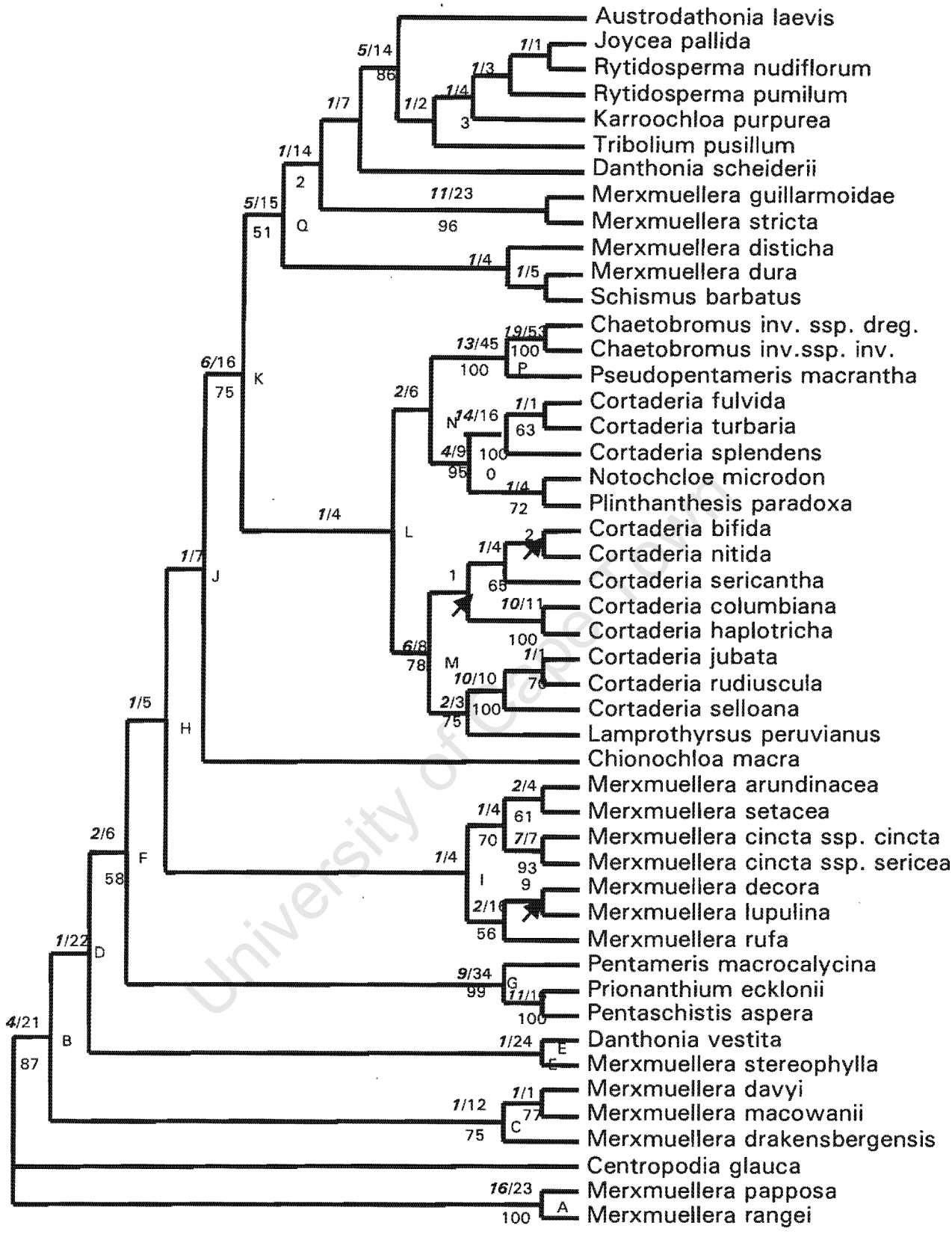


Fig. 3. 6. One of the fundamental trees from a set of 12 most parsimonious trees (l = 1128; c.i. = 0.6232; r.i = 0.7107) found in heuristic searches of the combined molecular (rpoC2 + ITS) data sets. The first numbers in bold and italics above branches indicate decay indices and the second numbers separated by a forward slash indicate branch lengths values determined using ACCTRAN optimization. Bootstrap percentages on nodes with more than 50% support are shown below branches. Alphabetical letters designate the node numbers described in text. Arrows indicate nodes that collapse in the strict consensus tree Abbreviations: ssp = susbspecies; inv. = involuctratus;dreg. = dregeanus.

compatible with the topology obtained from the *rpo*C2 analysis. Placement of *M. rangei* and *M. papposa* as a monophyletic clade outside the rest of the danthonioid grasses is congruent with the ITS and the *rpo*C2, but there is increase in support for this relationship in this analysis (bootstrap = 100%; decay = 16 steps). Compared to the *rpo*C2 data, a clade containing the rest of the danthonioid grasses (node B) is well supported (bootstrap = 87%; decay = 4 steps), but there is generally loss of support for subsequent basal nodes retrieved. However, both *rpo*C2 and ITS data sets complement each other in support of the majority of the more terminal clades. Support for the major clades retrieved is indicated in Table 3.4.

3.2.2 Combined molecular and morphological data (Total evidence).

Cladistic analysis of the combined molecular and morphological data resulted in sixteen most parsimonious trees (L = 1657; C.I. = 0.5910; R.I. = 0.6823). One of the most parsimonious trees with branch support and branch length values superimposed onto it is presented in Fig. 3.7. Only three nodes collapse in the strict consensus tree and these are shown by arrows in Fig. 3.7. The greatest amount of phylogenetic resolution from this analysis entails the delimitation of nine major groups within the subfamily Danthonioideae (Fig. 3.7; Table 3.5). Most of these groups are also retrieved in the analyses of the partitioned and combined molecular data sets. In this analysis there is improvement in clade support as well as increase in branch lengths compared to partitioned and combined molecular data sets. Although differing in the species composition, the general grouping of genera is similar to that observed in the *rpo*C2 (Fig. 3.4), ITS (Fig. 3.5) and the combined molecular data sets (Fig. 3.6) but differs markedly from morphological data. However, sister group relationships between some of the major clades retrieved in this analysis differ from those found with other data sets. Three monophyletic groups within *Merxmuellera* are retrieved similar to the *rpo*C2 and combined molecular data sets. The placement of *M. rangei* and *M.*

papposa as a lineage outside the rest of the danthonioid grasses is consistent with the *rpo*C2 and combined molecular data sets. Monophyly of all the Drakensberg species of *Merxmuellera* (including *M. stereophylla* shown to be sister to *D. vestita* by the *rpo*C2 and combined data sets) is strongly supported (bootstrap = 88%; decay index = 4 step). The placement of this group sister to the rest of the members of the subfamily is well supported (bootstrap = 80%; decay = 4 step). The sister group relationship of *Chionochloa* to the winter rainfall *Merxmuellera* species from the Cape is consistent with the ITS data (Fig. 3.7). The *Pentameris-Pentaschistis* clade is resolved sister to taxa in the remaining clades. Contrary to the *rpo*C2 (Fig. 3.4) and combined molecular analyses (Fig. 3.6), *Chaetobromus-Pseudopentameris* clade is positioned sister to a clade containing the South American *Cortaderia* species and the New Zealand species of *Cortaderia.* The sister group relationship of these two *Cortaderia* clades is consistent with the results from the ITS data set but contradicts other data sets. However, this relationship is strongly supported in this analysis (Fig. 3.7; Table 3.5). The placement of the *M. disticha, M. dura, M. guillarmoidae* and *M. stricta* together with other closely related genera (*Austrodanthonia, Rytidosperma, Karroochloa, Joycea* and *Schismus*) is congruent with the partitioned and combined molecular data sets, but there is difference in the phylogenetic pattern within this *Rytidosperma* clade. In this analysis, *M. disticha* is the most basal taxon in the *Rytidosperma* clade, while the remaining species of *Merxmuellera* are grouped together in a weakly supported clade with *Danthonia schneiderii* embedded within them. However, support for the relationship between *M. stricta* and *M. guillarmoidae* is very high (Fig. 3.7; Table 3.5), but the sister group relationship between this clade and *D. scheideri* contradicts the results from other data sets. This conflict is nevertheless given little support.

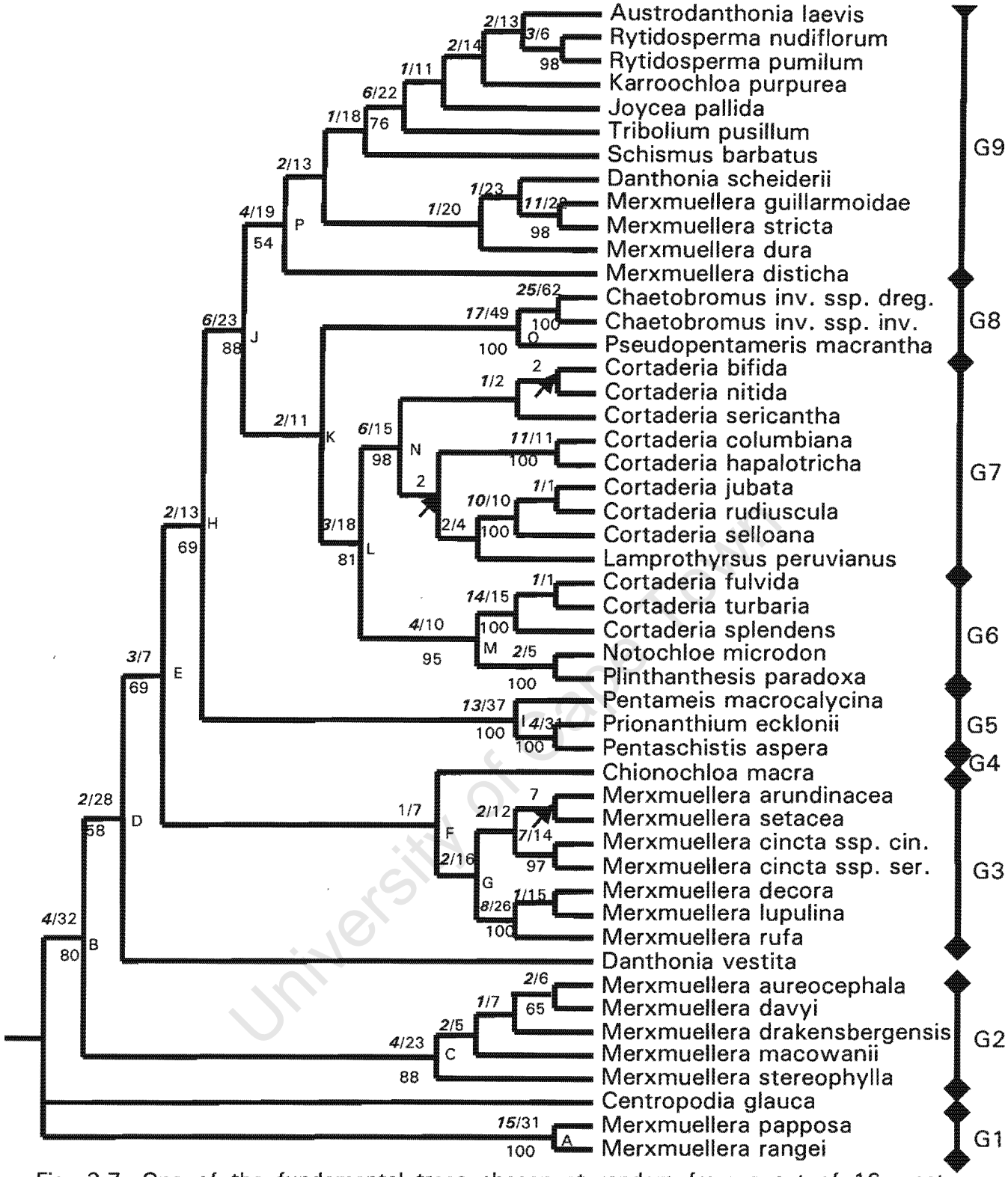


Fig. 3.7. One of the fundamental trees chosen at random from a set of 16 most parsimonious trees (l = 1567; c.i. = 0.5910; r.i. = 0.6823) obtained from the heuristic search of the combined molecular (rpoc2 + ITS) and morphological data sets (Total Evidence). The first numbers in bold and italics above branches indicate decay support values and the second numbers separated by a forward slash indicate branch lengths values determined using ACCTRAN optimization. Bootstrap percentages on nodes with more than 50% support are shown below branches. Letters indicate node numbers described in the text. Abbreviations: ssp = subspecies; inv. = involucratus; dreg. = dregeanus; ser. = sericea; cin. = cincta. Number on the right defining major clades are as follows: G1 = *M. rangei* – *M. papposa* clade, G2 = Drakensberg *Merxmuellera* clade, G3 = Cape *Merxmuellera* clade, G4 = *Chionochloa* clade (= clade M in ITS tree), G5 = *Pentaschistis* clade, G6 = South American *Cortaderia* clade, G7 = New Zealand *Cortaderia* clade, G8 = *Pseudopentameris* clade, G9 = *Rytidosperma* clade.

Table 3.4. Major clades retrieved from cladistic analysis of the combined molecular (ITS + *rpoC2*) data set.

Genera and infraspecific names are abbreviated as follows: *Chaet. inv.* ssp. *dreg.* = *Chaetobromus involucratus subspecies dregeanus*; Cort. = *Cortaderia*; Pent = *Pentameris*; Psch = *Pentaschistis;* Merx. = *Merxmuellera.* Pseu. = *Pseudopentameris*; Aust. = *Austrodanthonia;* Lamp. *Lamprothyrsus.* Dant. = *Danthonia;* Schi. = *Schismus.* Plin. *Plinthanthesis.* n.s. refers to nodes with less than 50% boostrap.

Clade	Clade designation	Bootstrap (%)	Decay index
Merx. rangei – Merx. papposa	A	100	16
Merx. davyi – Merx. drakensbergensis	C	75	1
Pent. macrocalycina – Psch. aspera	G	99	9
Merx. arundinacea – Merx. rufa	I	<50	1
Cort. bifida – Lamp. peruviana	M	78	6
Cort. fulvida – Plin. paradoxa	O	95	4
Chae.dreg. – Pseu. macrantha	P	100	13
Aust. laevis – Schi. barbata	Q	51	5

Table 3.5. Major clades retrieved from cladistic analysis of the combined molecular and morphological data sets.

Genera and infraspecific names are abbreviated as follows: *Chaet. inv.* ssp. *dreg.* = *Chaetobromus involucratus subspecies dregeanus*; Cort. = *Cortaderia*; Pent = *Pentameris*; Psch = *Pentaschistis;* Merx. = *Merxmuellera.* Pseu. = *Pseudopentameris*; Aust. = *Austrodanthonia;* Lamp. *Lamprothyrsus.* Dant. = *Danthonia;* Schi. = *Schismus.* Plin. *Plinthanthesis.* n.s. refers to nodes with less than 50% boostrap and or decay index of less than 2 steps

Clade	Clade designation	Bootstrap (%)	Decay index
Merx. rangei – Merx. papposa	A	100	15
Merx. aureocephala – Merx. stereophylla	C	88	4
Merx. arundinacea – Merx. rufa	G	ns	2
Pent. macrocalycina – Psch. aspera	I	100	13
Cort. fulvida – Plin. paradoxa	M	94	4
Cort. bifida – Lamp. peruviana	N	98	6
Chaet. Dreg. – P. macrantha	O	100	17
Aust. laevis – Merx. disticha	P	57	4

CHAPTER FOUR

DISCUSSION

4.1 Phylogenetic resolution and Support: General findings

Cladistic analyses of morphological data alone are problematic as they provided poorly supported trees (Fig. 3.1, 3.2). Inspection of character distribution on the cladogram of the weighted morphological data (Fig. 3.3) indicates that there are few synapormorphies in the data set and many characters are highly homoplasious. This observation is corroborated by the low consistency indices (Table 3.1), as well as low bootstrap percentiles. In general, there is a high level of noise in the morphological data.

These statistical results obtained from analyses of the morphological data are comparable to those of Barker *et al.* (2000). They mentioned that the unstable topology obtained in their results from the morphological data could have been due to inadequate sampling of species and/or substantial within-genus variation. While species sampling for morphology is considered adequate in this study in testing the monophyly of *Merxmuellera*, unstable topologies obtained from analyses of morphological data can be associated with lack of non-homoplasious synapormophies uniting all the Cape *Merxmuellera* species, as they are widely distributed throughout the cladogram while the Drakensberg *Merxmuellera* species are brought together in the analysis of the weighted data. Furthermore, several characters that are informative within the genus as well as within the rest of the study group do not co-vary, thus contributing to the instability of the cladograms. Since topologies provided from the analysis of morphological data alone are unstable, they are unreliable in forming the basis for inferring the systematic position of *Merxmuellera* in the danthonioid grasses.

The results obtained from analyses of partitioned molecular (*rpo*C2 and ITS) and combined analyses including morphology are better supported

than analyses of morphology alone, thus providing better inferences of the phylogenetic position of this genus in the context of the rest of the Danthonioideae (Figs 3.4 - 3.7). In agreement with earlier reports from anatomy (Ellis, 1980 – 1983), embryology (Verboom, *et al.* 1994) and molecular phylogenetic studies (Hsiao *et al.*, 1998; Barker *et al.*, 1999, 2000), the current study support the contention that the genus *Merxmuellera* as currently recognised is not monophyletic. The results from this study do not support Barker, *et al.* (2000) on grouping the Cape *Merxmuellera* species and the Drakensberg *Merxmuellera* as a paraphyletic assemblage basal to the rest of the Danthonioideae. The major contribution from this study is the identification of three distinct monophyletic groups within the current broadly defined *Merxmuellera*: (1) *M. rangei – M. papposa* clade, (2) *Merxmuellera* s.s (includes all the summer rainfall species of *Merxmuellera* occurring largely in the Drakensberg, and (3) Cape *Merxmuellera* clade (includes all the winter rainfall species of *Merxmuellera* from the south western Cape) while the rest of the species are retrieved together with closely related genera in the broadly defined *Rytidosperma* clade consistent with Barker *et al.* (1999, 2000).

Although the *M. rangei* clade and the Drakenberg clade appears to be clustered together in the ITS analysis, the only difference between this analysis and other analyses with respect to members of these clades is in the placement of the outgroup. Looking at the support for the node holding the two clades, it is possible that when forcing the topology of the tree, that node may collapse and allow the outgroup to placed anywhere between them.

Regarding the rest of the danthonioid taxa, the results from this study identified several groups of genera within the subfamily Danthonioideae (Table 4.1) and most of these groups correspond to the informal groups circumscribed by Barker *et al.* (2000). Both the *rpoC2* and the ITS analyses hypothesise similar grouping of genera, but are incongruent at some points on their relationships. This incogruence indicates the need for

a major decision on which of these should be preferred as better estimation of the phylogeny for our study group. The relationships between these groups of genera are better supported in the *rpo*C2 data while the ITS data are not very informative about their relationships as indicated by lack of support on the more basal nodes and low consistency index. Strong hypotheses are usually preferred than weak ones as a reliable estimation of relationships within a character set (Farris, 1983, Felsenstein, 1985, Sanderson, 1995; Davis *et al.* 1998). Without engaging into philosophical debates on whether or not high levels of data consistency and robustness of support can be argued for as accurate estimation of hypothesised phylogenies (Carpenter, 1992; Hillis and Bull, 1993; Kluge and Wolf, 1993; Sanderson, 1995; Givnish and Sytsma, 1997), it is simply noted that low support for the basal nodes in the ITS data set correlates with low consistency index indicative of high homoplasy (0.4824).

The combined molecular data set (Fig. 3.6) provided the same phylogenetic hypothesis as the *rpo*C2 data but with low support on basal nodes and low consistency index (0.4232) suggesting that there is conflict among data set in the resolution of some nodes. Although the *rpo*C2 and the ITS data sets corroborate each other on the general grouping of most genera, it is possible (not with great certainty since the constraint analyses were not performed) that the *rpo*C2 data is overwhelming the ITS data on the relationships the relationships between these groups, thus indicating the strength of plastid gene in resolving higher level relationships than the nuclear genes. This observation is not surprising as nuclear genes such as the ITS are limited in their use for resolving higher taxonomic relationships compared to chloroplast genes which are relatively slow evolving and useful at a higher classification level (Baldwin, *et al.*, 1995; Liston *et al.*,1996). However, reliance on one gene for inferring relationships can be misleading hence inference of phylogenetic relationships among taxa from the combined data sets is a

more reliable approach in favour of complementarity between data sets (Miyamoto, 1985).

The topology obtained from the analysis of the molecular and morphological data (total evidence) is highly supported (Fig. 3.7) compared to other data sets. Although there are some arguments that inclusion of morphology data into molecular data sets can mislead hypotheses provided by the molecular data sets (Hedges and Maxson, 1986; Givnish and Sytsma, 1997), the results from this study do not support this contention but recommend use of all available data in cladistic analysis in favour of complementarity among data sets observed through both nodal resolution and support. Higher support provided by this data set allows us to form a more powerful basis of inferring well supported phylogenetic hypotheses to consider the systematics of *Merxmuellera* in the context of the rest of the Danthonioideae. Unless otherwise stated, the discussions to follow are largely based on the topology provided by the results of the combined molecular and morphological data as shown in Fig. 3.7.

Table 4.1. Comparison of groups of genera delimited in this study to that circumscribed by Barker et al. (2000).

Groups delimited in this study	Barker *et al.* (2000) informal grouping
Merxmuellera rangei - *M. papposa* clade	*Chloridoid*
Drakensberg Merxmuellera clade	Basal *Mermxuellera* assemblage
Cape *Merxmuellera* clade	Basal *Merxmuellera* assemblage
Chionochloa clade	*Chionochloa* clade
Pentameris – Pentaschistis clade	*Pentaschistis* clade
South American Cortaderia, Danthonia clade	*Danthonia* clade
New Zealand Cortaderia, Lamprothyrsus and *Plintanthesis* clade	*Cortaderia "A"* clade
Chaetobromus - *Pseudopentameris* clade	*Pseudopentameris* clade
Rytidosperma clade	*Rytidosperma* clade

4.2 Monophyly of *Merxmuellera*

At a higher level classification, the monophyly of *Merxmuellera* is not supported, thus supporting the results of Hsiao, *et al.* (1998) and Barker *et al.* (1999, 2000). Given the morphological and anatomical diversity in this genus, it would be surprising indeed if this study would unite all the species of *Merxmuellera* s.l. as one monophyletic group that can be defined on morphological or even anatomical grounds. While several genera were included in this analyis, the major focus was not to resolve relationships within the whole subfamily but to adequately test the monophyly of *Merxmuellera* in an attempt to detect groups within the genus that can be formally recognised in a higher taxonomic classification.

Merxmuellera s.s.

Simultaneous analysis of the molecular and morphological data (Fig. 3.7) provides good support (bootstrap = 88%; decay index = 4 steps) for the monophyly of *Merxmuellera* s. s. This comprises five species mostly from the summer rainfall region of the Drakensberg (*M. drakensbergensis, M. stereophylla, M. macowanii, M. aureocephala* and *M. davyi*) as the earliest diverging lineage within the Danthonioideae (Fig. 3.7).

The basal position of this clade of *Merxmuellera* s.s. is in agreement with Barker, *et al.* (1999, 2000). This study however, does not support their inclusion as a paraphyletic assemblage together with other species of *Merxmuellera* from the Cape. Although their analyses were not inclusive of all members of the genus, their results showed that the Drakensberg species represented by *M. macowanii* and the type species *M. davyi* were always retrieved as a well-supported basal-most clade while the Cape species were placed as the second unresolved basal group. However, this group is distinct in the total evidence tree obtained in this study (Fig. 3.7), corroborating the suggestion by Anderson (1962) that this is a morphologically coherent group. *Merxmuellera* s.s as circumscribed here is defined by dense hairs on the inner surfaces of the basal leaf sheaths. In addition, the leaf blades are minutely hairy or scabrid on the inner surfaces just above the ligule. Fertile lemma with three distinct tufts of hairs arranged in an oblique row on either side of the central nerve is characteristic for most members of this group (Fig.2.9D, H). In *M. macowanii, M. davyi* and *M. drakensbergensis* these tufted hairs are equally spaced with the lowermost tuft being placed closer to the lemma margin, while in *M. aurocephala* the lowermost tuft is a bit more distant from the upper two tufts (Fig. 2.9D). However, the arrangement of lemma indumentum in *M. stereophylla* is exceptional. The lamma back is glabrous, but characterised by long soft hairs along the margin as a continuous band from base to the apex without any definite formation of tufts (Fig. 2.9B). These marginal hairs are longer and dense

from base to middle and somewhat shorter and sparse from middle to apex.

The internal phylogeny of *Merxmuellera* s.s is weakly supported, and the two groups that have been hypothesised to exist within the Drakensberg *Merxmuellera* species are not corroborated in this study. Anatomical investigations by Ellis (1981a, b) and Benesch (1995) suggested that *M. stereophylla* and *M. drakensbergensis* are anatomically similar to each other while the *M. macowanii, M. aureocephala* and *M. davyi* group resemble one another anatomically. Failure to retrieve these two is not surprising owing to insufficient overlap between the three data sets included in the total evidence analysis. For example, the placement of *M. aureocephala* in this lineage is supported only by morphological characters, while the inclusion of *M. drakensbergensis* and *M. stereophylla* is supported by morphology and partial sequences from the *rpo*C2 gene. However, the strong support for this clade leaves no doubt as to its actual existence. In order to elucidate the phylogenetic structure within and to test the hypothesis of the existence of two closely related groups entails addition of data for those taxa with missing data. This group also does not agree with Ellis's (1981b) suggestion that all these species should be treated as subspecies of *M. stricta* and *M. disticha*. In all the phylogenies produced in this study none of these species in the *Merxmuellera* s.s. shows any relationship to either *M. stricta* or *M. disticha*. Their treatment as a morphologically coherent group separate from the latter two species is highly supported in this study.

Based on the morphological criteria we can only speculate about the membership of two Madagascan species of *Merxmuellera*, *M. ambalavoensis* and *M. tsaratananensis*, which were not included in any of the analyses due to lack of adequate material. In the recent revision of these two species as well as the Drakensberg species, Benesch (1995) noted a great morphological and ecological overlap. In common with other members of this clade, their lemma backs are also characterised by three tufts of hairs arranged in an oblique row, typical of the Drakenberg group.

Benesch (1995) noted that individuals assigned *M. tsaratananensis* could not be easily separated from *M. macowanii* and *M. davyi* morphologically. In *M tsaratananensis* the old leave blades break a little distance above the ligule similar to *M. macowanii* and *M.davyi*. In addition, the lemma lobes of *M. tsaratananensis* are almost completely adnate to the central awn, a character that is typical of *M. macowanii*. Ecologically, *M. macowanii* and *M. tsaratananensis* overlap markedly in that they are both alpine grasses always growing in muddy patches along streambanks in association with *Sphagnum*. Furthermore, Benesch (1995) also reported that although there are slight anatomical differences, which could be due to environmental factors between *M. ambalavoensis* and *M. aureocephala*, individuals assinged to any of these species are not easily separable on morphological and ecological grounds. The shape and length of the lemma as well as the arrangement of tufts of hairs on the lemma back are similar in *M. ambalavoensis* and *M. aureocephala*. Ecologically, both species are xerophytic and only differ in their flowering times which is winter in *M. aureocephala* and in summer for *M. amabalavoensis*. Using these definitions of lemma indumentum type and the overlapping ecology, it seems likely that *M. ambalavoensis* and *M. tsaratananensis* are also members of *Merxmuellera* s.s., thus making *Merxmuellera* s.s comprise seven species.

M. rangei – M. papposa clade

This two-taxon clade, well supported in the partitioned and combined data sets, is consistently placed outside the rest of the Danthonioideae and is best considered as a separate lineage. Anatomically and morphologically, *M. rangei* and *M. papposa* are very distinct within the genus as well as among the subfamily, confirming their isolated position in the danthonioid grasses. Similar to the anatomical observations made by Ellis (1982) on *M. rangei*, the transverse leaf blade anatomy shows that *M. papposa* also possesses three orders of vascular bundles and a solid circular leaf outline with the adaxial surface reduced to a small V

shaped groove atypical of the danthonioid grasses (Fig. 2.2A-B). In addition, the mesophyl of *M. rangei* and *M. papposa* consists of a palisade-like abaxial chlorenchyma restricted to the area adjacent to the abaxial epidermis while most of the adaxial surface is occupied by large and somewhat inflated colourless cells (Fig. 2.2A-B). The colourless cells appear much thinner than the outer vascular bundle sheath cells which are thick and conspicuously larger than the mesophyll cells. The abaxial epidermis structure of these two species is exceptionally distinct in that it consists of numerous and large unprotected triangular shaped stomata across narrow costal zones (Fig. 2.2C-D). While these species are xerophytic in habitat preference, Ellis (1982) has pointed out that these epidermal modifications are difficult to explain in grasses from this type of habitat especially when other structures are usually modified to reduce water loss. Most danthonioid grasses from xeric habitats lack stomata on the abaxial epidermis, but if present, they are borne at the bases of furrows covered by a mass of interlocked hairs to protect their exposure to direct sunlight as a modification to water loss reduction (Ellis, 1982). The pattern displayed by *M. rangei* and *M. papposa* shows that they probably don't use this modification, and Ellis (1982) has postulated that development of thick cuticle indicated by pitted epidermal cells (see Figs. 2.2C – D) may be a different adaptation by these taxa to water loss reduction with the stomata only opening occasionally.

These observations are in agreement with previous expanded molecular phylogenetic studies (e.g Hsiao *et al.*, 1998; Barker *et al.*, 1999) which showed that *M. rangei* occupies a position outside the danthonioid lineage and shows affinity to *Centropodia* and the chloridoids. Furthermore, Verboom *et al.* (1994) reported the presence of haustorial synergids in the megagametophyte of the danthonioid grasses, a character that has been accepted to be a synapomorphy for this group in the current subfamilial classification of the grasses (GPWG in press), but *M. rangei* was shown to lack this character. The state of this character has not been studied in *M. papposa,* but it is possible to infer based on its

close relationship to *M. rangei* that this species might also be lacking this typical synapomorphy uniting the danthonioid grasses. While lack of this character by *M. rangei* and possibly its close relative *M. papposa* provides good evidence for their position outside this subfamily, it does not provide evidence for their relationship to the chloridoids as shown by Hsiao *et al.* (1998) and Barker, *et al.* (1999). However, development of colourless cells and mesophyl that is not composed of tightly packed, angular, isodiametric chlorenchyma cells have been shown by Ellis (1982) to be common to *Dregeochloa* in the subfamily Chloridoideae thus showing an affinity of these two taxa to this lineage. In other danthonioid grasses the mesophyl is composed of small, tightly packed, angular and isodiametric chlorenchyma cells grouped in different shapes (see Figs. 2.3A-C, 2.4A - B).

Morphologically, *M. papposa* and *M. rangei* are also very very similar and not easily separable and show affinity to some extent to *Centropodia glauca.* Their abaxial lemma surfaces have three tufts of long hairs on either side of the central nerve, borne on the apical region of the lemma body just below the point of awn insertion and exserted beyond the lemma lobes (Fig. 2.9C). The rest of the lemma body is densely covered with short hairs without any obvious arrangement. This arrangement of lemma indumentum is similar to *Centropodia glauca*. This suite of anatomical and morphological characters for *M. rangei* and *M. papposa* undoubtedly provides support for their exclusion from the subfamily Danthonioideae, in line with suggestions made in earlier studies as well as their position observed in current study.

Cape Merxmuellera clade

This is the second largest monophyletic group within *Merxmuellera* s.l comprising six species including *M. arundinacea, M. setacea, M. cincta, M. rufa, M. lupulina* and *M. decora*, all of which are endemic to the Cape floristic region. Results from the analyses of the partitioned molecular and combined data sets are highly congruent with each other in

retrieving these species as monophyletic. However, this clade has a bootstrap support less than 50% in all analyses but requires at least 2 step to collapse in the total evidence topology.

There is no obvious morphological or anatomical synapomorphy defining this clade. In most species in this clade, the lemma backs are covered the full length by longitudinal lines of hairs along the nerves from base to middle region. While this is not unique in the subfamily, it does distinguish this group from the Drakenberg species. However, *M. setacea* has a glabrous lemma back with a single marginal tuft on either side of the central nerve similar to *M. dura*.

In all analyses, members of this clade are constantly retrieved as two subgroups: *M. rufa, M. decora* and *M. lupulina* subgroup (termed *M. lupulina* subgroup for convenience), and *M. arundinacea, M. cincta* and *M. setacea* subgroup (termed *M. arundinacea* subgroup for convenience). The *M. lupulina* subgroup is a morphologically and anatomically distinct subgroup within the Cape *Merxmuellera* group as well as within the genus in general. Development of bulbous bases which are densely woolly on the outer surfaces of basal leaf sheath sheaths are typical of this subgroup. These are deeply sunken into the ground and have been postulated as a means of defence against predation and/or protecting the innovation shoots against excessive evaporation or fire damage (Tsvelev, 1976; Linder, 1989; Barker and Ellis, 1990a). Furthermore, the abaxial leaf epidermis consists of characteristic outwardly bowed costal long cells appearing as if they are inflated (Fig. 2.7B). Ellis, (1983) emphasized that epidermal structure as well as the sclerenchyma girders of cellulose only staining green with fast safranin is exceptional in the genus where scelerenchyma is always lignified. On the other hand, the *M. arundinacea* subgroup are caespitose grasses and survive interfires by developing knotty tillering with long and stout innovation buds that allow them to coppice from base after fire. While it is clear that these two subgroups have apomorphies distinguishing them from each other, the question stands as whether to recognise each as a separate group that can be

warranted generic status or to recognise them as one large group that can be warranted generic status. The *M. arundinacea* subgroup has little support in the total evidence analysis as in other analyses to recognise it separately. Whereas the large clade including all the Cape *Merxmuellera* is weakly supported by bootstrap, it however receives a minimum decay support of 2. Since strongly supported nodes are preferred than the weakly supported ones (Farris, 1983; Felsenstein, 1985; Sanderson, 1995), we are compelled to give recognition to the large clade inclusive of all the Cape *Merxmuellera* than to recommend generic status to indivudual subgroups within.

The relationship of the Cape *Merxmuellera* group to other lineages is uncertain as different data sets place them differently. The ITS and the combined molecular and morphologically data sets show this group as more closely related to *Chionochloa* (Fig. 3.5, 3.7 respectively), but this relationship has very low support. On the other hand *rpo*C2 and the combined molecular analyses places it between the latter genus and the *Pentameris – Pentaschistis clade* (Figs. 3.4, 3.6). These incongruent relationships are however, not strongly supported in any analyses. Few characters support the relationship between members of this clade and *Chionochloa* as earlier indicated by Conert (1971) although he was referring to all species of *Merxmuellera* s.l. In common, they are both characterised by non-synapomorphic characters: expanded leaves and lemma backs which are densely hairy, but the hairs in *Chionochloa* are only arranged in three rows and cover the entire length of the lemma body.

The Cape *Merxmuellera* species show more affinity to some members of the *Pentameris - Pentaschistis* clade comprising the African genera *Pentameris, Prionanthium* and *Pentaschistis* as indicated by *rpo*C2 and the combined molecular data sets (Figs 3.4, 3.6). The link between these clades is supported by morphological adaptations they have in common, at least for some members. The Cape *Merxmuellera* species and some species of *Pentaschistis* share some common interfire survival strategies

such as development of geophytic structures and development of knotty tillering to allow coppicing from the base after fires (e.g Linder, 1989; Linder and Ellis, 1990a; Davidse, 1988; Barker and Ellis, 1988). This alliance contradicts Conert (1971) who argued that there is no genus of the African grasses to which *Merxmuellera* shows affinity but shows affinity to *Chionochloa* and the South American *Cortaderia* species. However, the *Pentameris – Pentaschistis* clade can still be distinguished from the Cape *Merxmuellera* group by presence of two florets per spikelet, differentiated multicellular glands, small rachilla extension above the upper floret and the palea features (Davidse, 1988) Cytologically, *Pentaschistis* and *Prionanthium* have the chromosome base number of n = 7 or 13 and n = 7 respectively (Davidse, 1988) while the Cape *Merxmuellera* species as well as other species in *Merxmuellera* s.l. generally have chromosome base number of n = 6 (De Wet, 1954, 1960; Spies and Du Plessis, 1988).

Rytidosperma clade

Four species of *Merxmuellera* s.l. inclinding *M. dura, M. disticha, M. guillarmoidae* and *M. stricta* are consistently placed in this clade in all analyses together with the Australasian genera, *Rytidosperma, Austrodanthonia, Notodanthonia, Joycea* and the African genera *Karroochloa, Tribolium* and *Schismus*, and *Danthonia scheiderii* from Himalaya. This clade correponds to the *Rytidosperma* clade in the total evidence tree based on ITS, *rbcl*, *rpo*C2 and morphology by Barker *et al.* (2000).

Relationships within this clade are not clear as there is no single morphological or anatomical character defining it. Whereas Clayton and Renvoize (1986) lumped several of genera in this clade including *Merxmuellera* under *Rytidospema*, Linder and Verboom (1996) have kept these apart and even segregated several smaller genera such as *Joycea, Thonandia* and the revival of *Notodanthonia* leaving *Merxmuellera* and the reduced *Rytidopserma* as recongnisable genera. Since this clade is

undersampled for several of the genera, their monophyly cannot be confidently challenged in the present study. However, the placement of some *Merxmuellera* species in this clade is not surprising owing to the fact that the redelimitation of *Rytidosperma* by Linder and Verboom (1996) was bases largely on lemma indumentum variation. The species of *Merxmuellera* included in this clade have tufted hairs on the lemma but vary from the rest of members in this clade in that these hairs do not form transverse rows as is typical with genera such as *Rytidosperma* and *Karroochloa*. For example, *M. disticha* and *M. dura* possess one basal tuft of hairs on either side of the central nerve and a fringe of marginal hairs differentiating them from other *Merxmuellera* species as well as other genera in the *Rytidosperma* clade. The relationship between *M. stricta* and *M. guillarmoidae* is not surprising owing to their close morphological and anatomical similarities, especially the indumentum type. Four to six tufts of hairs located near the lemma margin define these two species. Although Conert (1975) separated *M. guillarmoidae* from *M. stricta* based on the number of tufts arguing that *M. guillarmoidae* has five tufts and six in *M. stricta*, observations made from this study indicate that the number of tufts in these species is continuous, varying from four to six, hence making this separation questionable.

While the position of these *Merxmuellera* s.l. species in this clade is consistent with previous molecular phylogenies, it is difficult to make any sensible suggestions about their taxonomic treatment due to undersampling in this clade. Sampling of more species from the other genera included in this clade is needed before reaching any taxonomic conclusions about the fate of the *Merxmuellera* species placed here.

4.3 Generic limits

With the exception of Clayton and Renvoize (1986) who attempted to lump *Merxmuellera* s.l. into the broadly defined *Rytidosperma* concept which included genera such as *Karroochloa* and *Erythranthera*, the generic limits within *Merxmuellera* s.l. as currently recognised has been

questioned. There has not been a single study that argued for the monophyly of *Merxmuellera* as currently recognised since its original description by Conert (1970).

In the current systematic approaches, delimitation of natural taxonomic units for classification at any taxonomic level is largely based on the cladograms produced from cladistic analysis (Humphries and Funk, 1984; Linder, 1988). The aim of the exercise being to identify monophyletic groups for formal naming, thus establishing classification that are maximally stable (Linder, 1991). Barker, *et al.* (2000) informally grouping several of the *Merxmuellera* species into paraphyletic assemblages, cladism would reject this approach from the view that paraphyletic taxa are not real phylogenetic units and usually lead to confusion about both the character distribution and relationships of taxa as opposed to monophyletic groups (Donoghue and Cantino, 1988; Humphries and Chappill, 1988; Schrire and Lewis, 1996; Backlund and Bremer, 1998). Upon discovery of paraphyletic taxa, systematists are inclined to dividing them into several more narrowly circumscribed monophyletic taxa.

From the results of the current study, the monophyly of *Merxmuellera* s.l. has been thoroughly tested in a larger data set of the danthonioid grasses. Although there are areas that are not clearly defined by the data sets, it is unsatisfactory to continue recognising a large single *Merxmuellera* s.l. as opposed to recognition of multiple genera. Using the monophyly criterion, identification of three monophyletic groups within the *Merxmuellera* s.l. in this study provides good evidence for redelimitation of this genus into at least three separate genera despite limited morphological character support for monophyly of some of the groups retrieved. Strong support for the monophyly of *Merxmuellera* s.s. is corroborated by the synapomorphy of presence of dense hairs inside the basal leaf sheaths. Other characters include the arrangement of lemma back indumentum into three tufts of hairs arranged in an oblique row from base to apex of the lemma body. This character exclude *M.*

stereophylla which shows a different pattern. Inclusion of the Madagascan species into *Merxmuellera* is most likely owing to their greater similarities to several members of this group than to any other danthonioid grasses.

Exclusion of *M. papposa* and *M. rangei* from the Danthonioideae is well supported based upon their distinct floral and foliar anatomy. However their inclusion into the Chloridoideae is still not yet satisfactory, as a result of lack of clear morphological and anatomical characters that defines them in the context of chloridoid grasses. Their inclusion into that subfamily is largely based on the their position closer to the Chloridoideae as revealed by Hsiao *et al.* (1998) and Barker, *et al.* (1999).

Although, there are no clear synapomorphies characterising the Cape *Merxmuellera* group, their recognition is largely based on their retrieval as a single monophyletic taxa in all analysis. It is possible that within this group, the two subgroups can be recognised as separate smaller genera based on their different morphological adaptations to interfire survival strategies. However, due to lack of support for the *M. arundinacea* subgroup these cannot be confidently regarded these as separate genera but rather recognise them as one genus based upon the lemma indumentum which covers most of the lemma back and arranged in longitudinal rows thus distinguishing them from the rest of the members in *Merxmuellera*

Four species, *M. disticha, M. dura, M, stricta* and *M. guillarmoidae* cannot be accommodated in any one of these groups, as they make up a paraphyletic group within the *Rytidosperma* clade. The decision to accord formal status to this group necessitates recognition of either one paraphyletic group or two monotypic (*M. disticha, M. dura*) and one monophyletic group (*M. guillarmoidae* and *M. stricta*). Since neither paraphyletic taxa nor monotypic higher level taxa are desirable (Donoghue and Cantino, 1988; Humphries and Chappill, 1988; Schrire and Lewis, 1996; Backlund and Bremer, 1998), with the latter increasing taxonomic redundancy (Backlund and Bremer, 1998), both approaches are

unsatisfactory. It is obvious that formal recognition of this group of *Merxmeullera* species in the *Rytidopsperma* clade at a higher level classification is questionable. I therefore recommend that this group be informally recognised as a separate group of taxa from either the *Merxmuellera* s.s., the Cape *Merxmuellera* group nor the *M. rangei – M. papposa* to which they show no phylogenetic affinity. Their formal recognition will depend on the outcomes of further work within the *Rytidosperma* clade any researcher would wish to undertake.

CHAPTER FIVE

CONCLUSIONS

In order to adequately test the monophyly of the genus *Merxmuellera* in this study the approach was to include morphological data and molecular data of representatives from the informal groups circumscribed by Barker *et al.* (2000) in the subfamily Danthonioideae and use *Centropodia glauca* to root all the phylogenies produced. The results from the analyses of morphological data alone were found unreliable in inferring a robust systematic position of *Merxmuellera* in the context of the rest of the genera in the subfamily due to low support and low consistency indices obtained. The results from the analysis of partitioned molecular data sets provided better insights on the systematic position of *Merxmuellera* s.l. although unclear relationship for some of the groups within *Merxmuellera* to other groups of genera in the subfamily masked a clear phylogenetic picture of the genus from which conclusions can be inferred. This problem could be associated with a limited overlap of taxa between data sets. The combined analysis of molecular and morphology data set provided better results with well supported relationships for most groups retrieved. Based on the evidence provided by the total evidence data, generic status may be warranted to the following three monophyletic group of *Merxmuellera* as follows:

(a). The *Merxmuellera* s.s. clade, comprises of all the five Drakensberg species and the two Madagascan endemic species, *M. tsaratananensis* and *M. ambalavoensis.* This clade is characterised by dense hairs on the inner surface of the basal leaf sheaths and minute hairs on the inner surface of the leaf blade above the ligule. In addition, lemma indumentun is characterised by three tufts of hairs arranged in an oblique row on either side of the central nerve except for *M. stereophylla*. This group is restricted to the alpine areas of the Drakensberg and Madagascar where different species occupy xerophytic and mesophytic habitats.

(b). The Cape *Merxmuellera* clade, comprises *M. arundinacea, M. cincta, M. setacea, M. rufa, M. lupulina* and *M. decora*. This clade shares longitudinal rows of hairs borne on the lemma back from the base to the middle region. This group is restricted to the winter rainfall areas of the Cape where they occur in association with the fynbos biome.

(c). The *M. rangei – M. papposa* clade, comprising *M. rangei* and *M. papposa* are defined by solid cylindrical lamina outline, development of colourless cells and mesophyl that is not composed of tightly packed, angular, isodiametric chlorenchyma cells. In addition, lack of haustorial synergids in *M. rangei*, and possibly in *M. papposa* as postulated from their close relationship, warrant their exclusion from the Danthonioid lineage and these should be accorded a generic status in the Chloridoideae. Their inclusion in the chloridoids is based on Barker *et al.* (1999), Hsiao *et al.* (1998) as well some morphological characters they share with *Centropodia glauca*. *M. rangei* occurs in Nama Karroo to Namibia while *M. papposa* is known only in Baviaanskloof near Port Elizabeth in the Eastern Cape.

The relationships of four species included in the *Rytidosperma* clade are not resolved and are in need of further investigation within the context of the rest of the genera included in this clade.

Future prospects

Systematics of the Danthonioideae

Identification of monophyletic groups within the genus *Merxmuellera* that can be accorded formal generic status, has improved our understanding of the generic composition within the Danthonioideae. However, generic relationships within subfamily are still obscure in many areas due to lack of a robust support. Additional molecular sequences especially from other chloroplast genes such as the rbcl are needed to robustly increase support for the more basal nodes in an attempt to elucidate confidently the relationships between the groups delimited.

Lower level taxonomic studies

This study has revealed four groups within the genus *Merxmuellera* s.l. three of which are clearly monophyletic. However, the wider focus of the study in determining groups of taxa within the genus that can be recognised at a higher taxonomic level has precluded investigation of finer details within such groups. Further studies within each group would be valuable in determining the actual species composition and relationships as well as the taxonomic status of some taxa as the anatomical studies by Ellis (1980a; 1980b; 1981a; 1981b; 1982; 1983) questioned the status of some species at the specific level.

Biogeography

Biogeographical studies are inherently important in revealing historical interpretation for some evolutionary and geological adaptations observed in taxa. This study does not provided indications on evolutionary complexes that may be observed in the group. It thus leaves a scope for further work on the whole subfamily and as well as its component genera.

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Appendix I QIAquick PCR purification protocol using microcentrifuge.

Note: all centrifuge steps are at 13, 000 rounds per minute

1. Determine the volume of each PCR reaction.and place each in a QIAquick 2 ml spin column.
2. Add five volumes of PB buffer to one volume of PCR reaction.
3. Place each spin column on a microcentrifuge and centrifuge for approximately one minute to bind DNA to the column membrane.
4. Discard the flow through from the collecting tubes and place the spin column back into the same tubes.
5. To wash DNA, add 0.75 ml of PE buffer to which 96% ethanol has been added and centrifuge for one minute.
6. Discard the flow through and centrifuge for an extra one minute.
7. Place QIAquick spin columns in a new microfuge tube.
8. To elute DNA from the QIAquick spin column membrane, add 50 microlitres of deionised water to directly on the centre of the QIAquick membrane and centrifuge for one minute.
9. The double stranded DNA fragments are now ready for sequencing.