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**PHYLOGENY OF THE GENERA *Karroochloa*, *Merxmuellera*
AND *Schismus* (Poaceae)**

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***Dissertation presented for the degree of Doctor of Philosophy in the Faculty of
Natural and Agricultural Sciences (Department of Plant Sciences: Genetics) at
the University of the Free State***

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Co-promotor: Prof H J T Venter***

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GRASS

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

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

Grass, so vagrant - does anything stray
With such gallant courage? The hardest way
Is coaxed and beguiled by the wayward grace
Of the constant friend of every space.

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 the grass

MABLE DUGGAN

Dedicated to my wife EDELGARD

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LIST OF ABBREVIATIONS

A	Adenine
AFLP	Arbitrary fragment length polymorphism
bp	Base pairs
C	Cytosine
°C	Centigrade
cm	Centimeter
CI	Consistency Index
CTAB	Cetyltrimethylammonium bromide
d	Genetic distance
DAF	Deoxyribonucleic acid (DNA) amplification fingerprinting
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetate
<i>ETS</i>	External transcribed spacer
F	Coefficient of similarity
Fig.	Figure
G	Guanine
g	Gram
HCl	Hydrochloric acid
KCl	Potassium chloride
<i>IGS</i>	Intergenic spacer
<i>ITS</i>	Internal transcribed spacer
km	kilometer
l	Liter

ln	Natural logarithm
<i>M</i>	Molar
mg	Milligrams
MgCl ₂	Magnesium chloride
<i>mM</i>	Milli molar
min.	Minute
m/m	Mass per mass
m/v	Mass per volume
n	Genetic chromosome number
NaCl	Sodium chloride
ng	Nanogram
nrDNA	Nuclear ribosomal DNA
PAUP	Phylogeny analysis using parsimony
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RDNA	Ribosomal DNA
RFLP	Restricted fragment length polymorphisms
s	Second
S	Similarity index
T	Thiamin
TAE buffer	Tris-acetic acid ethylenediamine tetra acetate buffer
TE buffer	Tris-ethylenediamine tetra acetate buffer
TBA	Tertiary butyl alcohol
Tris-HCl	Tris-hydrochloric acid
μl	Micro liter
μ <i>M</i>	Micro molar
UV	Ultraviolet
v/cm	Volts per centimeter
v/v	Volume per volume
x	Basic chromosome number
2n	Somatic chromosome number

2x-8x

2x(Diploid chromosome number) 3x-8x (Ploidy level)

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The grasses have received abundant scientific attention, both phylogenetic and otherwise. Only within the past fifteen years have cladistic methods been applied to questions of grass phylogeny and evolution (GPWG 2001). The historical prominence of the grasses as an object of botanical research reflects their almost ubiquitous biogeographical presence and their pervasive economic importance since the very beginnings of human civilisation. Approximately one-third of the world's dry land is covered by some species of Poaceae (Walter 1979), and the majority of the world's human population relies heavily (Pohl 1978), if not predominantly, on cereal grasses such as rice, maize and wheat for its daily sustenance.

According to Judd *et al.* (1999) there are about 650 genera and 8700 species of grasses in the world. The subfamilies and tribes are fairly uniformly distributed across the continents in broad climatic bands, but the genera, which are of more recent origin, tend to be restricted to a single continent (Clayton 1983). This is due to the fact that apparently the grasses began to diversify before oceans separated the continents.

In southern Africa, the grasses include 194 genera (rank second in number of genera for southern African flora), 967 species (rank seventh in number of species for southern African flora) and intraspecific taxa. One hundred and fifteen are naturalised and 847 are indigenous, including 329 endemic taxa (Gibbs Russell 1985).

1.2 THE SUBFAMILY ARUNDINOIDEAE

Most of the older taxonomic treatments of the Poaceae recognised six or seven major subfamilies of grasses, Bambusoideae Asch. & Graebn.,

Oryzoideae Burmeist., Pooideae Benth., Panicoideae A. Br., Arundinoideae Tateoka, Chloridoideae Rouy and Centothecoideae Soderstrom, which are further subdivided into 40 tribes consisting of 650 genera (Campbell 1985; Dahlgren *et al.* 1985; Watson *et al.* 1985; Clayton & Renvoize 1986; Renvoize & Clayton 1992; Watson & Dallwitz 1992; Judd *et al.* 1999).

Clayton and Renvoize (1986) in particular published a number of diagrams representing relationships based on their synthesis of knowledge at that time. These diagrams have served as a starting point for much subsequent work (GPWG 2001).

Phenetic analysis of the grass family, generally found groups consistent with the five or six subfamilies commonly recognised by the mid 1980's. Hilu and Wright (1982), in a cluster analysis of morphological and anatomical data, found eight major groups with strong support.

Watson *et al.* (1985) used the DELTA system to conduct comprehensive phenetic analysis of the grass family and their character list continues to be developed. Watson and Dallwitz (1992) initially recognised five subfamilies and subsequently updated their classification to include seven (Watson & Dallwitz 1999): these are Stipoideae Burmeist., Pooideae, Bambusoideae, Centothecoideae, Arundinoideae, Chloridoideae and Panicoideae.

The Arundinoideae is a very old taxon, the least specialised of the five subfamilies as described by Clayton and Renvoize (1986). The Arundinoideae is currently distributed mainly in the Southern Hemisphere and represents the basic stock from which the tropical savanna grasses evolved (Clayton & Renvoize 1986).

The Arundinoideae is considered to be very heterogeneous as well as taxonomically a difficult group to work with. This is due to the fact that there is a lack of reliable diagnostic features while being the least specialised of all the grass subfamilies (Conert 1987; Ellis 1987). This heterogeneity results from the inclusion of genera (and tribes) which do not fit well into other, well-defined subfamilies (Renvoize 1981). Many features, which are taxonomically discriminating in the other subfamilies, vary in this group and consequently

there is no clearly defined central core group, so the subfamily is probably polyphyletic (Ellis 1987).

The Arundinoideae is widely distributed, but do not show any physiological adaptations as a group, to specific environments and has mostly retained the apparently primitive C₃ photosynthetic pathway (Renvoize 1981). Grasses of this subfamily are widespread in the world, but the majority are distributed throughout the Southern Hemisphere (Gould 1968). A reason for this could be the climatic isolation from the continuous landmasses to the north (Goldblatt 1978). Most of the Arundinoideae species are perennial and only a few annuals have evolved (Conert 1987).

Several classifications for the grasses based on spikelet and inflorescence morphology were proposed in the 19th century (Pohl 1978; Calderón & Soderstrom 1980; Gould & Shaw 1983; Campbell 1985), with usually nine or ten tribes recognised.

Whether explicit or not, a different perspective on the evolution of grass and relationships within the grass family began to emerge at the end of the 19th century. Workers such as Celakovsky (1889), Goebel (1895) and Schuster (1910) carefully analysed spikelet structure and proposed that *Streptochaeta*, or something very similar were the most primitive grasses.

With the availability of leaf anatomical (Duval-Jouve 1875; Prat 1932), embryological (Tieghem 1897) and cytological (Avdulov 1931) data, a comprehensive reassessment of evolutionary relationships among grasses began. Additional data on embryo anatomy (Reeder 1957, 1961, 1962), starch grains (Tateoka 1962), lodicules (Jirásek & Jozifová 1968; Guédès & Dupuy 1976), and leaf anatomy (Brown 1958; Metcalfe 1960; Ellis 1980a, b, 1981a, b, 1982a, b, 1983a) accumulated and were also incorporated into evolutionary and classification schemes.

Several classification systems were published in the 20th century (Tateoka 1957; Prat 1960; Stebbins & Crampton 1961; Caro 1982; Clayton & Renvoize 1986; Tzvelev 1989; Renvoize & Clayton 1992; Watson & Dallwitz

1992). These classification systems are the major ones that are global in scope.

The number of subfamilies recognised ranges from two (Tzvelev 1989) to thirteen (Caro 1982). All but the Watson & Dallwitz (1992) classification, which is phenetic, were based on presumed evolutionary relationships. The major change was the subdivision of the old Festucoideae (or Pooideae) into several subfamilies. The Panicoideae was retained almost without modification.

In the present study the classification of Clayton and Renvoize (1986) will be used. This classification provides a broad definition for the tribe Arundineae that encompasses most of the genera in the subfamily.

This classification is, as noted earlier, based on embryological features, non-kranz leaf anatomy (including the presence of slender microhairs) and a generally simple spikelet structure and is, therefore, a broadly anatomical and morphological classification (Clayton & Renvoize 1986). This tribe is otherwise difficult to characterise, for it is heterogeneous with numerous isolated or weakly linked genera, whose relationships are highly conjectural. It is also difficult to categorise any of the features as primitive or advanced, and thus it is difficult to determine the direction of evolution (Clayton & Renvoize 1986).

Most of the species of *Merxmuellera* and *Karoochloa* were previously lumped into the genus *Danthonia* (Nees & Esenbeck 1841; Steudel 1855; Durand & Schinz 1895) and it is since 1969 and 1971 that species of *Danthonia* were allocated to the new genera *Karoochloa* and *Merxmuellera* (Conert & Türpe 1969; Conert 1971). All along the genus *Schismus* was regarded as very closely related to the genus *Karoochloa* although only one species of *Schismus* was originally assigned to the former genus *Danthonia* (Conert & Türpe 1974).

1.3 THE ARUNDINOIDEAE IN SOUTH AFRICA

There are six known floral Kingdoms in the world (Low & Rebelo 1996). South Africa is the only country to host an entire Kingdom, the Cape Floral Kingdom (Good 1974; Taylor 1978). One third of South Africa's plant species occurs in this Kingdom and the core of distribution of the three genera under

investigation is also in this Kingdom. The major vegetation type in this Kingdom is "fynbos".

Bond and Goldblatt (1984) listed almost 200 species of the family Poaceae for this region. Of these 200 species, almost all the endemic species belong to the subfamily Arundinoideae (Linder & Ellis 1990a). Therefore, it is not unexpected that arundinoids have developed specialised adaptations to cope with the Cape "fynbos" and the variety of niches in the Cape vegetation, with various structural and morphological adaptations which allow them to survive (Linder & Ellis 1990a).

1.3.1 *Karroochloa* Conert & Türpe

Karroochloa is a small southern African genus consisting of four species, two perennials and two annuals. All four species are endemic to southern Africa. The leaf blades are linear, up to 2 mm wide, flat, folded or rolled and not disarticulating. The inflorescences are paniculate and contracted (10-60 mm long) and more or less ovoid (Gibbs Russell *et al.* 1990).

The perennial species *K. curva* (Nees) Conert & Türpe and *K. purpurea* (L.f.) Conert & Türpe are adapted to specific environments (Conert 1971). *Karroochloa curva* grows on the lower levels of the south-western Cape Mountains, never exceeding 600 meters above sea level. *Karroochloa purpurea* occurs in mountainous habitats at altitudes between 2000 and 2300 meters. However, the two annuals, *K. schismoides* (Stapf ex Conert) Conert & Türpe and *K. tenella* (Nees) Conert & Türpe, are widely distributed (Conert 1971).

The four species of this genus were previously part of *Danthonia* but Conert and Türpe (1969) grouped them in a new genus *Karroochloa*. According to literature, six is the basic chromosome number (as *Danthonia* De Wet 1954a, 1960); Du Plessis & Spies 1988; Spies & Du Plessis 1988). All four species were investigated for this thesis.

1.3.2 *Merxmuellera* Conert

The first published report on *Merxmuellera* was that of Conert (1971). This is the largest and most interesting group amongst the species previously lumped into *Danthonia* (Conert 1971). Some of the species of this genus were originally also assigned to the genus *Rytidosperma* (Clayton & Renvoize 1986). This genus consists of perennials, which are caespitose. Eleven of the seventeen species are endemic to South Africa, one endemic to Zimbabwe and one to the Namibian desert. Four species inhabit the mountainous region on the border of South Africa with Lesotho (Gibbs Russell *et al.* 1990). The leaf blades are linear, 4-15 mm wide and nearly always rolled. The inflorescence is a single raceme up to 60 mm long (rarely observed in *M. disticha*) or paniculate and contracted (narrow, occasionally spike-like; usually longer than 60 mm, in contrast with *Karoochloa*) (Gibbs Russell *et al.* 1990).

In *Merxmuellera*, a basic chromosome number of six appears to be proven by various chromosome number reports on the genus (as *Danthonia* De Wet 1954a, 1960; Du Plessis & Spies 1988; Spies & Du Plessis 1988). Spies and Du Plessis (1988), however, reported on the possibility of a second basic chromosome number for the genus ($x=7$).

At present, 20 species are recognised in the genus *Merxmuellera*, two of which are only known from the mountains of Madagascar (Barker 1994). Seventeen of the 20 species were studied for this thesis.

1.3.3 *Schismus* P. Beauv.

Schismus is a small genus, comprising five species, and found throughout the world. *Schismus* species are tufted annuals or perennials, caespitose or decumbent. The leaf blades are linear to linear-lanceolate expanded or rolled, setaceous or glabrous. The inflorescences are contracted or spike-like panicles (Chippindall 1955; Gibbs Russell *et al.* 1990).

The type species, *S. barbatus* (Loefl. ex L.) Thell. grows in southern Africa as well as in northern Africa and Europe, ranging from the Canary Islands, southern France and Morocco to the Nile delta and from Arabia to the

Caucasus. The closely related *S. arabicus* ranges from the Himalayas to Greece in one direction and from Pakistan to the Nile delta in the other direction (Conert 1971).

Three more species are endemic to South Africa where they have adapted to extreme environmental conditions (Conert 1971; Conert & Türpe 1974). Three of the four African species were investigated for this thesis.

Although only one of the five species of this genus was originally described as a *Danthonia* species, the whole genus was later removed from *Danthonia* (Conert 1971; Conert & Türpe 1974). Conert and Türpe (1969) discovered a close relationship between *Schismus* and *Karoochloa*. This genus has a basic chromosome number of $x=6$ (Fariqi & Quirash 1979; Du Plessis & Spies 1988; Spies & Du Plessis 1988).

1.4 ECOLOGY

The vegetation of southern Africa is subdivided into seven biomes, namely Forest, Thicket, Savanna, Grassland, Nama Karoo, Succulent Karoo and "Fynbos" (Low & Rebelo 1996). The three genera under investigation mainly inhabit Grassland, Fynbos and Succulent Karoo, with a few species from the Nama Karoo (Gibbs Russell *et al.* 1990).

1.5 PLANT MORPHOLOGY

For practical purposes, characters of external morphology provide the prime base for recognition of genera, species and subspecies or varieties. In many families of flowering plants, these are the only characters that have been employed in the differentiation of taxa.

It has long been recognised that inflorescence and flower structures vary less with temporary environmental changes than most vegetative structures and thus are more reliable in taxonomy. In the system of Bentham and Hooker (1883) as modified by Hitchcock (1950), changes of spikelet structure and arrangement were used almost exclusively in the determination of grass subfamilies, tribes and genera. Vegetative shoot characteristics such as culm

height, leaf length, width and pubescence, and plant longevity (annual or perennial) have been utilised in species differentiation, but in this case the spikelet is considered to be the most important single classification. It is now known that frequently, external morphology is not a reliable indicator of phylogenetic relationships in the higher categories of classification.

Visible, external plant structure, however, remains the necessary basis for practical plant differentiation and identification. By necessity, morphological characteristics are and will continue to be used as the basis of species recognition.

1.6 LEAF ANATOMY

Structurally, the grass leaf blade is a complex organ, exhibiting a wide range of anatomical features and providing valuable additional taxonomic information. Despite this high degree of structural diversity, differences in leaf anatomy have proved to be systematically useful, and several anatomical characters are constant for, and vary between each of the major evolutionary lines and subfamilies of the Poaceae. Certain anatomical character combinations are diagnostic on subfamily level (Renvoize 1981; Watson *et al.* 1985). Taxonomically useful leaf anatomical characteristics of the Poaceae have been defined and illustrated by several authors (Ellis 1976, 1979; Clifford and Watson 1977) drawing freely on the work of Metcalfe (1960). These characters are derived from the leaf blade as viewed in transverse section and from the abaxial epidermis. Standardization of the leaf blade material studied is necessary owing to structural differences along the blade (Ellis 1976, 1979) and the level of insertion on a single tiller (Watson & Clifford 1976).

The anatomical character set used by Watson *et al.* (1986) includes most of the proven attributes and will help considerably with our knowledge of character distribution and variation. All modern taxonomic work on the Poaceae should include comparative leaf anatomical information in a form that it can be incorporated into this type of database, either visually in the form of photomicrographs, or by descriptions of these character states. All the studies

referred to under the subfamily discussion provide information of this sort and constitute the major source of taxonomically useful data on leaf blade anatomy. Ellis (1980a, 1980b, 1981a, 1981b, 1982a, 1982b, 1983a) and Barker and Ellis (1991) did an intensive study of the leaf anatomy of the genus *Merxmuellera*.

1.7 REPRODUCTIVE BIOLOGY

Grasses have developed a wide range of breeding behaviours (Connor 1979), broadly divisible into two opposite strategies. Some have countered the incestuous promiscuity of anemophily by developing a complex incompatibility system that ensures outbreeding (Heslop-Harrison & Heslop Harrison 1982); or, less often, by adopting dioecy. Others, particularly annuals, have reduced the uncertainty of anemophily by self-fertility or cleistogamy. The more extreme forms of inbreeding are invariably facultative and often mediated by environmental conditions, thus mitigating their restrictive effect on genetic diversity.

Cytogenetic systems are likewise extremely varied, with extensive development of polyploidy (Stebbins 1971); polyhaploidy and the reversion of polyploidy (Kimber & Riley 1963). Together these processes have produced systems of great flexibility, capable of responding conservatively or adaptively according to the exigencies of selection pressure. Their ability to proliferate segregate populations, which yet retain some capacity for gene exchange, has often created polymorphic complexes of fearsome taxonomic difficulty, but their versatility has been a potent factor in the success of the grasses (Stebbins 1985).

Not much research has been done on the reproductive structures of the Arundinoideae. Klopper *et al.* (1998) has done some work on the species of *Pentaschistus* and a report by Phillipson and Connor (1984) exists on the haustorial synergids in Danthonioid species. Prior to the present thesis no studies were carried out on the embryo sacs in the genera *Karroochloa*, *Merxmuellera* and *Schismus*.

1.8 CYTOGENETICS

Cytogenetical investigations were initiated to serve as an additional aid to morphological data in studies of taxonomy and phylogeny of the Poaceae (Pienaar 1955).

Several cytogenetic aspects, which can help in the unravelling of relationships between species of individuals in species, can be studied.

Cytogenetic aspects, which play a major role, are the following:

- meiotic behaviour (e.g. meiotic abnormalities such as univalents, laggard, chromosome bridges and micronuclei),
- chromosome pairing,
- chromosome size,
- polyploidy (Pienaar 1955).

Any data, which indicate differences between species, are of taxonomic significance, and thus constitute part of the evidence that may be used by taxonomists (Stace 1980).

Cytotaxonomy refers to the use of abovementioned characteristics and others, such as chromosome number and chromosome morphology, as data for classification (Jones & Luchsinger 1987). Despite certain limitations, cytogenetic investigations are an aid in establishing systematic and phylogenetic relationships among many species and genera and are of great value when used in conjunction with morphological, geographical and ecological studies (Pienaar 1955).

The value of cytotaxonomic data depends mainly on the material under investigation. For more than 70 years, cytogenetic data have played a major role in angiosperm evaluation and relationships (Raven 1975). From literature it appears as if grasses have a large diversity of chromosomal behaviour that raises many problems for those attempting to divide them into discrete species. Some 80% of the grasses investigated have a polyploid chromosome number (Clayton 1978).

Apomictic swarms are not unusual and over 2000 hybrids have been recorded, of which more or less 10% of the population is fertile (Clayton 1978).

The Russian cytogeneticist Avdulov did the first important work on grass cytogenetics in 1931. This study indicated that there was a correlation between the classification of grasses based on the size and number of chromosomes and the classification based on histology and anatomy. Both these classification systems differ from the classical system based on inflorescence characteristics (Stebbins 1956). Stebbins (1956) suggested the regrouping of grass tribes and genera, as proposed by Avdulov (1931). This was necessary due to the fact that all the characteristics studied reflect genetic and evolutionary relationships more effectively than the traditional system. Furthermore, this approach revealed a major division between tropical and temperate grasses (Renvoize 1981).

Previous studies indicated the primary chromosome number for Arundinoideae to be $x = 12$ (Clayton & Renvoize 1986). However, it is more likely that this is a secondary base number derived by polyploidy, since a number of arundinoid genera are now known with $n = 6$ (Roodt 1999):

- *Centropodia* (Du Plessis & Spies 1988).
- *Chaetobromus* (Du Plessis & Spies 1988; Spies & Du Plessis 1988; Spies *et al.* 1990).
- *Karoochloa* [(as *Danthonia*, De Wet 1954a, 1960); Du Plessis & Spies 1988; Spies & Du Plessis 1988].
- *Merxmuellera* [(as *Danthonia*, De Wet 1954a, 1960); Du Plessis & Spies 1988; Spies & Du Plessis 1988].
- *Pentameris* (Barker 1993).
- *Pseudopentameris* (Barker 1995b).
- *Schismus* (numerous reports, for example Faruqi & Quirash 1979; Du Plessis & Spies 1988; Spies & Du Plessis 1988).
- *Tribolium* [(Spies *et al.* 1992; Visser & Spies 1994c, d, e), not $x = 7$ as incorrectly reported by De Wet (1960). (As *Urochlaena*, Spies & Du Plessis 1988; Visser & Spies 1994c, d, e)].

Stebbins (1956), as well as Hunziker and Stebbins (1987), also considers $x = 6$ to be the basic chromosome number for Arundinoideae. A less

common base number in the subfamily is $x = 7$:

- *Dregeochloa* (Du Plessis & Spies 1988; Spies & Du Plessis 1988).
- *Merxmuellera* (Du Plessis & Spies 1988; Spies & Du Plessis 1988).
- *Pentaschistis* (Davidse *et al.* 1986; Du Plessis & Spies 1988; Du Plessis & Spies 1992; Klopper *et al.* 1998; Spies & Du Plessis 1988; Spies *et al.* 1994a).
- *Prionanthium* (Davidse 1988; Du Plessis & Spies 1988; Spies & Du Plessis 1988; Visser & Spies 1994e).
- *Pentameris* (Spies & Roodt 2001).

The use of cytogenetics as an aid to determine relationships between species of the grass genera is difficult, because two processes have blurred many interspecific boundaries: hybridisation and chromosome doubling or polyploidy (Stebbins 1956). According to Stebbins (1985), more than 80% of the grass taxa has undergone polyploidy sometime during their evolutionary history.

In order to explain the high frequency of polyploidy in the Poaceae and other plant groups, Stebbins (1985) proposed his "secondary contact hypothesis". According to this hypothesis taxa with "patchy" distributions would offer frequent opportunities for secondary contact and hybridisation between differentiated diploid populations. It is thus possible to maintain these gene combinations by the effect of polyploidy in the favouring of tetrasomic inheritance and preferential pairing of homologous chromosomes, as opposed to homoeologous chromosomes (Stebbins 1985). Polyploidy may occur in four types (Stebbins 1985):

- Multiples of the original low basic chromosome number.
- Multiples of the secondary basic chromosome number derived from the original numbers by an earlier cycle of polyploidy.
- Multiples of basic chromosome numbers, which are the lowest in the genus, but were derived from that of a pre-existing genus by a cycle of polyploidy in the distant past.

- Basic chromosome numbers derived through aneuploidy from secondary basic chromosome numbers (De Wet 1987).
- Accessory or B-chromosomes are relatively common in the grass family. Individuals with B-chromosomes tend to indicate an accumulation mechanism in the male, but not in the females (Jones 1975; Murray 1979). The most common accumulation mechanism in the grass family is the failure of separation at the first pollen mitosis (Jones & Rees 1982). From time to time B-chromosomes have been known to influence and regulate the amount of genetic variability within populations, by affecting chiasma frequency and homeologous chromosome associations. B-chromosomes may also affect chiasma formation by altering their distribution, especially in some cases of new polyploids (Hunziker & Stebbins 1987).

1.9 MOLECULAR SYSTEMATICS

The main purpose of any discipline in biological science is to analyse and determine genetic diversity and relationships between or within different species or populations (Weising *et al.* 1995). Cladistical evaluation of genetic variation has been increasingly complemented by molecular techniques in the past decade. Molecular markers based on polymorphisms which are found in proteins and DNA are used as an additional aid to taxonomy, phylogeny, ecology and genetics to either determine relationships between or in genera and species of grasses (Hsiao *et al.* 1999).

Molecular methods used for additional data in a taxonomic study include the following:

- restriction fragment length polymorphism (RFLP) in the nuclear and chloroplast genomes (Wang & Tanksley 1989),
- random amplified polymorphic DNA fragment patterns (RAPD) (Williams *et al.* 1990), DNA amplified fingerprinting,
- (DAF) (Weaver *et al.* 1995), arbitrary fragment length polymorphism,
- (AFLP) (Vos *et al.* 1995) and sequencing of various genes or DNA segments.

1.9.1 Random amplified polymorphic DNA (RAPD)

Molecular methods have become fundamental tools for plant biologists. These methods are useful for fingerprinting, phylogenetic studies, tagging genes and mapping of plant genomes. Several methods for comparing plants at molecular level have been developed, since the development of the polymerase chain reaction (PCR) (Mullis 1991). PCR amplifies specific portions of DNA which occur between sequences of synthetic DNA primers (Yu *et al.* 1993).

The development of the automated PCR technology supplies a new set of markers available to scientists interested in comparing organisms at molecular level especially the use of arbitrary primers to obtain random amplified polymorphic DNA (RAPD) markers. RAPD markers are obtained by PCR amplification of random DNA segments from single arbitrary primers (Williams *et al.* 1990). The arbitrary primers used for the RAPD PCR procedure are usually 9 to 10 base pairs in size. These primers have a CG content of 50% to 80% and do not contain palindromic sequences. The number of DNA fragments that are amplified is dependent on the primer and the genomic DNA used. A single nucleotide substitution in a primer can result in a complete change of the RAPD profile. This is an indication of the sensitivity of the technique. However, the method is not 100% reliable, because much larger numbers of fragments are observed when bacterial genomes are used as templates, than would be expected. Only DNA fragments within the size range of 100 to 3000 base pairs occur in DNA sequences and are amplified.

Polymorphisms for RAPD's may be due to single base pair changes, deletions of primer sites, insertions which increase the separation of primer sites over the 3000 base pairs limits and small insertions/deletions which result in changes in the size of the PCR product. The advantages using RAPD's are:

- universal set of primers can be used for all species,
- no probe libraries or primer sequence information are required,
- only the primer sequence information is needed for information transfer and the process can be automated.

A limitation in the use of the RAPD technique is that the markers are dominant DNA markers. This limitation can be overcome by using more than one closely related DNA marker.

Efficient use of RAPD markers requires quick DNA extraction, optimum amplification conditions and appropriate data analysis. RAPD's were successfully used:

- To develop molecular markers linked to a gene controlling fruit acidity in citrus (Fang *et al.* 1997);
- The analysis of tetraploid *Elymus* species (Sun *et al.* 1997);
- Population genetics of *Digitalis minor* (Sales *et al.* 2001).

Landry and Lapointe (1996) attempted to clarify some questions related to the application of RAPDs for phylogenetic reconstruction purposes. They found that by using more primers, stability increased. Landry and Lapointe (1996) indicated that at least 12 primers should be used to obtain a stable phylogeny. Their results also indicated that RAPD's should not be used to study phylogenetic relationships at higher taxonomic levels. In 1996, Klopper did a preliminary study on the genus *Pentaschistis* and indicated that RAPD's could have some potential in determining the phylogenetic relationships in the genus.

In this study the RAPD technique is used to determine the genetic variation in and between the species and to determine the phylogenetic relationships between 11 species of *Merxmuellera*, three species of the genus *Karoochloa* and three species of the genus *Schismus*.

1.9.2 Sequencing

Hamby and Zimmer (1988) and Doebley *et al.* (1990) published the first molecular phylogenetic data for the grass family, based respectively on ribosomal RNA and plastid gene *rbcL* (ribulose 1,5 bisphosphate carboxylase/oxygenase, large subunit) sequence data.

Barker (1995a) used data from chloroplast gene sequences, *rpoC2* and *rbcL*, to determine relationships among the genera and tribes of the subfamily Arundinoideae. The variable grass-specific region within the *rpoC2* gene was

used to indicate relationships between genera and tribes, and the more conserved *rbcL* gene was used to determine the tribal and subfamily relationships of the major groups in the grass family (Barker 1995a). Owing to the interdependence of the plastid data sets, the analysis of the combined data sets was recommended (De Queiroz 1993). Recognition, in the past, by some taxonomists (eg. Watson 1990) of Danthoieae and Arundineae as separate tribes, was supported by both the *rpoC2* and *rbcL* phylogenies (Barker 1995a).

Initially only restriction site mapping, which was easy to interpret, was reserved for phylogenetic analysis of data. Consequently, only moderately to slowly evolving DNA sequences have been widely used in plant phylogenetics (Chase *et al.* 1993; Hamby & Zimmer 1988, 1992). With the advent of polymerase chain reaction (PCR) technique DNA sequencing is now inexpensive and easy to use for phylogenetic studies at all taxonomic levels. This sequencing option offers increased precision and resolution by permitting more efficient homology assessment of molecular characters and character states than is possible by restriction site mapping. The primary challenge by using nucleotide characters for lower level phylogenetic studies is the identification of DNA regions which can be easily amplified and provide sufficient variation with a short sequence segment (Baldwin *et al.* 1995). An example of this is the internal transcribed spacers (*ITS*) region of 18-26S nuclear ribosomal DNA. Hsiao *et al.* (1999) inferred phylogenetic relationships within the grasses based on sequences of the *ITS* region of nuclear ribosomal DNA.

ITS regions include three components (Fig. 1.1): the 5.8 S subunit, an evolutionary highly conserved sequence region, two spacer regions designated *ITS1* and *ITS2*. *ITS* regions are part of the transcription unit of the nuclear ribosomal DNA (nrDNA). The spacer segments of the transcript are not incorporated in the mature ribosomes. *ITS1* and *ITS2* regions of the nrDNA transcript may play a role in the maturation of the nrRNA's.

Several characteristics of the *ITS* region promote its use for phylogenetic analysis: *ITS* region consists of very high repeated sequences in the plant

nuclear genome. The nrDNA repeat unit, including the subunits, *ITS1*, *ITS2* and the intergenic spacer (*IGS*). The nrDNA repeat unit is present in thousands of copies, arranged in tandem repeats of a chromosomal locus or at multiple loci (Rogers & Bendich 1987; Hamby & Zimmer 1992). This high copy number promotes detection by amplification, cloning and sequencing of nrDNA.

- This gene family undergoes rapid concerted evolution (Arrnheim *et al.* 1980; Hillis *et al.* 1991), via unequal crossing over and gene conversion, a property which promotes intragenomic uniformity of the repeat unit and accurate construction of species relationships from these sequences (Hamby & Zimmer 1992; Sanderson & Doyle 1992).
- The small size of the *ITS* region and the presence of highly conserved sequences flanking each of the two spacers makes this region easy to amplify.

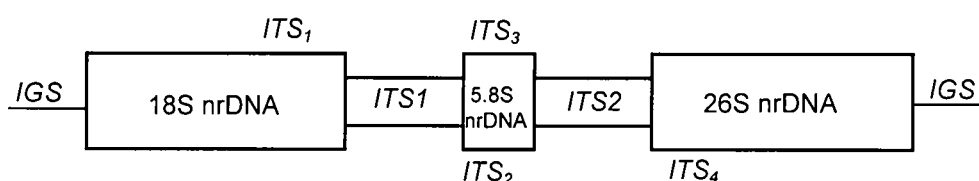


Figure 1.1. Repeat unit of 18-26S nuclear ribosomal DNA indicating the primer binding positions.

The *ITS* sequences of the following plant taxa were successfully investigated:

- Cordesse *et al.* (1993) sequenced the *ITS* region in Rice,
- Hsiao *et al.* (1995a, b) studied the phylogenetic relationships of 30 diploid species of *Triticeae* and *Pooideae* (Poaceae),
- Susanna *et al.* (1995) came to the conclusion that phylogenetic analysis of *ITS* sequence variation supports the monophyly of *Cardueae* (Asteraceae),
- Grebenstein *et al.* (1998) did the same with *Aveneae* (Poaceae) and other grasses as well as Guinea Yam species as deduced from *ITS1* and *ITS2* rDNA sequences,

- Twenty two diploid and tetraploid annual *Bromus* L. species of section *Bromus* (Poaceae) and three species belonging to other *Bromus* sections
- (Ainouche & Bayer 1997),
- Perennial and annual *Medicago* L. species (Diwan *et al.* 1997),
- *Eragrostis tef* (Zucc.) Trotter (Pillay 1997),
- *Abies* Mill. (Vendramin & Ziegenhagen 1997),
- *Lupinus* L. (Ainouche & Bayer 1999).
- Brochmann *et al.* (1998) analysed fifteen populations of *Saxifraga* by using random amplified polymorphic DNA (RAPD) and nucleotide sequences of the chloroplast gene *matK* and the internal transcribed spacers of nuclear ribosomal DNA (rDNA).

In this study sequences will be carried out on the internal transcribed spacer regions of ribosomal DNA in several species of the genera *Karroochloa*, *Merxmuellera* and *Schismus*.

1.10 PHYLOGENY

The phylogeny of the grasses has attracted much interest and published phylogenetic data have been based on both morphology data (Hilu & Wright 1982; Watson *et al.* 1985; Kellog & Campbell 1987) and molecular data of various kinds (Hamby & Zimmer 1988; Esen & Hilu 1989; Doebley *et al.* 1990; Hilu & Johansen 1991; Doyel *et al.* 1992; Davis & Soreng 1993; Cummings *et al.* 1994; Barker & Linder 1995; Hsiao *et al.* 1999). Many of these studies concentrated on determining the basal subfamily in the grasses and their relationship to the remaining subfamilies.

The main purpose of systematics is the phylogenetic reconstruction of the evolutionary processes, which generate biological diversity in the subfamilies, genera and species respectively. Molecular techniques in conjunction with morphology, cytology, ecology and leaf anatomy, have improved our ability to reconstruct the plant phylogeny (Soltis *et al.* 1992).

Phylogeny is thus the evolutionary history of an organism or taxonomic group (Häckel 1866). Existing species are the end products of a long process

of evolutionary diversification through polyploidisation, mutations, deletions and environmental conditions, which promote specific genotypes. The unique pattern of character inheritance and relationships with closely related species or individuals provides the basis for reconstructing phylogenetic history of genera or species.

Procedures for constructing phylogenetic hypotheses have been greatly developed by the discipline of phylogenetic systematics or cladistics (Hennig 1966), which is presently dominating the field of systematics (Hull 1989). In cladistics only synapomorphic derived characters are used as evidence to support hypotheses about phylogenetic relationships. Similarities due to the retention of symplesiomorphic characters are ignored because in determining relationships these characters are uninformative (Miyamoto & Cracraft 1991).

It is the ultimate goal of a phylogenetic evaluation to use various techniques to collect as much informative data to determine the phylogenetic relationships between the genera and species under investigation.

1.11 AIM OF THE STUDY

Almost a third of the world's representatives of the tribe Arundineae are indigenous to South Africa, and an ideal opportunity exists to study the phylogenetic relationships within the tribe, as well as within and between the genera *Karoochloa*, *Merxmuellera* and *Schismus*. Therefore, the aim of this study is to determine the phylogenetic relationships between the three genera and also between species of these genera. This is done by investigating cytogenetics and reproductive systems of the genera and species involved, as well as by using random amplified polymorphic DNA and sequencing of the *ITS* region of nrDNA. The data are combined to provide clearer indications of the phylogenetic relationships between genera and species.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Voucher herbarium specimens were collected in the field and stored in the Geo Potts Herbarium Bloemfontein (BLFU). Additional herbarium specimens for the morphological studies were borrowed from other herbaria in South Africa and are listed in Addendum A. These herbaria and their acronyms are listed in Table 2.1.

DNA Molecular Marker VI (pBR328 DNA cleaved with a mixture of BglI and HinfI) (Boehringer Mannheim Cat. no. 1062590) and Super Therm DNA polymerase (*Thermus aquaticus* polymerase) with 10X Buffer (Southern Life Biotechnology LPI-801, LPI-455) were respectively used as size standard marker and enzyme for the PCR reactions. Different primers were used for RAPD's (Operon Technologies, California) and the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Sciences, product number US 79765) were used during the sequencing study. All other chemicals used were of either analytical or electrophoretic grade.

Data on some of the *ITS* sequences were obtained from Genbank. These taxa were (accession numbers indicated in brackets): *Karoochloa purpurea* (AF019874), *Merxmuellera dura* (AF019872), *M. macowanii* (AF019863), *M. rangei* (AF019862), *M. setacea* (AF019867), *M. stricta* (AF019871), *Pentameris macrocalycina* (AF019864), *Pentaschistis aspera* (AF019865), *Prionanthium ecklonii* (AF019866) and *Schismus barbatus* (AF019873). The Genbank specimens will be referred to without any voucher numbers.

2.2 METHODS

2.2.1 Morphological descriptions and geographical distribution

Morphological descriptions are based on studies of the herbarium specimens mentioned in Addendum A. A stereo microscope was used to study sub-microscopic detail of the spikelets in particular. It was possible to plot the geographical distributions from locality information obtained from the herbarium specimen labels.

Table 2.1. Herbaria from which specimens were investigated.

BLFU	Department of Plant Sciences, University of the Free State, Bloemfontein, Republic of South Africa.
GRA	The Herbarium, Botanical Research Institute, PO Box 101, Grahamstown, South Africa.
NH	Botanical Research Unit, Natal Herbarium, Durban, Republic of South Africa.
NU	Botany Department, University of Natal, Pietermaritzburg, Republic of South Africa.
PRE	Botanical Research Institute, National Herbarium, Botanical Garden, Pretoria, Republic of South Africa.
STE	Government Herbarium, Botanical Research Unit, Stellenbosch, Republic of South Africa (now incorporated in the National Botanical Garden, Kirstenbosch).
STEL	Botany Department, University of Stellenbosch, Stellenbosch, Republic of South Africa.

2.2.2 Cytogenetics

2.2.2.1 Meiotic analysis

Young inflorescences were fixed in Carnoy's fixative [ethanol: chloroform: acetic acid - 6:3:1] (Carnoy 1886). The fixative was replaced by 70% (v/v) ethanol 24-48 hours after fixation. Anthers of the inflorescences were

squashed in 2% (m/v) aceto-carmine (Darlington & La Cour 1976) on a microscope slide. Contrast between cytoplasm and chromosomes was enhanced by adding a droplet of 45% (v/v) acetic acid, saturated with iron acetate, to the stain immediately before making the squash (Thomas 1940), whereafter the slide was gently heated over a spirit flame. Squashes were made according to the method of Darlington and La Cour (1976). The slides were made permanent by freezing them with liquid carbon dioxide (Bowen 1956), followed by dehydration in ethanol and mounting in Euparal.

Whenever possible, at least twenty cells of each of diakinesis, metaphase I, anaphase I and telophase I were examined per specimen. The haploid chromosome numbers, the presence of B chromosomes as well as the percentages of rod and ring bivalents and multivalents were recorded. In the case of metaphase I, anaphase I and telophase I, the number of chromosomal abnormalities (univalents, chromosome laggards and micronuclei) were recorded.

2.2.2.2 Microphotography

Microphotographs were made as follows: Photos were taken with a Nikon Microphot-FXA photomicroscope, using Pan-F 35-mm (ASA 50) black and white films. These films were developed for twelve minutes in Agfa Rodinol film developer, then rinsed in water for approximately 5 minutes. After fixing in Ilford rapid fixer for 10 minutes, the films were again rinsed in running water for 20 minutes. The films were then dried overnight.

From the films photomicrographs were developed on Ilford Multigrade paper using Ilfospeed developer. Development was stopped in water, to which some acetic acid was added and the photographs were then fixed with Ilford Hypam fixative, rinsed in water for 5 minutes and left face up, to dry.

The microphotographs depicting meiotic stages, abnormalities or certain behavioural trends during meiosis were mounted on herbarium sheets and are

stored in the Geo Potts Herbarium, Bloemfontein. Selections of these photographs, which best depict certain phenomena, are included in this thesis.

2.2.3 Embryo sac development

Inflorescence material from some of the specimens included in the meiotic and morphologic analysis was used for the study of embryo sac development. The material was again fixed in Carnoy's fixative (Carnoy 1886). Various stages of floral development were used for the embryo sac study. Ethyl alcohol (EtOH) and tertiary butyl alcohol (TBA) were applied to dehydrate the inflorescences (Table 2.2).

The material was left overnight in wax (60°C) for penetration, before being embedded in a paraffin wax (Merck and N.T. Laboratory Supplies). Sections (5-7 µm) were cut with a rotary microtome and these were affixed to pre-treated microscopic slides. The slides were pre-treated by covering them with a gelatine adhesive (5 g gelatine dissolved in 1 litre of warm distilled water, with 0.5 g chromium potassium sulphate added) and airdried before use (Jensen 1962).

Table 2.2. Dehydration procedure indicating the percentages of chemicals used and time of each dehydration step.

Step	H ₂ O	ETOH	TBA	Time (h)
1	70	30	0	1
2	50	50	0	1
3	30	50	20	1
4	15	45	40	1
5	5	25	70	1
6	0	15	85	1
7	0	0	100	2
8	0	0	100	2

The ribbons of sections were floated on water (45°C) and lifted onto the pre-treated slides (Jensen 1962).

Embryo sacs were stained with a modification (Spies & du Plessis 1986a) of the safranin (Johansen 1940) and fast green (Sass 1951) double staining techniques. This modification involves the following changes: the wax was removed in xylene (2 immersions of 10 minutes each), subsequently, slides were taken through xylene/ethanol (50:50), absolute ethanol and 70% ethanol for 5 minutes each and stained overnight in safranin (100 ml ethanol, 4 g sodium acetate dissolved in 100 ml water, 8 ml 40% formalin added to 4 g safranin, dissolved in 200 ml methyl cellosolve). The slides were rinsed in running water until all excess safranin was removed. Slides were destained in picro-ethanol (0.5 g picric-acid in 100 ml ethanol) for 15 seconds. Preparations were passed through ammonia-alcohol (3-4 drops ammonium hydroxide in 100 ml ethanol), for 1 minute and then through absolute ethanol (10 seconds).

Counter staining was done with fast green (0.33 g fast green in 100 ml ethanol) for 15 seconds, whereafter destaining in absolute ethyl alcohol in two successive immersions occurred. The first immersion was for one minute and the second until the preparation was sufficiently destained when observed under a microscope. After the slides had been rinsed in an ethanol/xylene solution (50:50) (for one minute) and in two successive immersions of xylene (five minutes each), eukitt was finally used for mounting the preparations (Spies & du Plessis 1986a). A modified version of Jensen's (1962) mounting method was used. This modification entails the following: a small amount of eukitt is applied to a cover slip, just enough to cover it without overflowing the edges of the slip. The prepared slide is removed from the xylene and carefully placed onto the cover slip. Microscopic examination was delayed for at least 24 hours.

A minimum of twenty embryo sacs per plant, representing different developmental stages, was studied for each specimen.

2.2.4 Molecular studies

Leaves from different specimens, collected in the field, were immersed and stored in a saturated sodium chloride and hexadecyl trimethyl ammonium bromide (CTAB) solution (Rogstad 1992).

2.2.4.1 DNA extraction

The CTAB method (Rogstad 1992) was used to extract DNA from ± 0.5 g of leaf material. The leaves were rinsed with distilled water and blotted with paper before the extractions were carried out in eppendorf tubes. In these tubes the material was ground to a fine powder in liquid nitrogen. The frozen tissue was then immediately incubated at 65°C for one hour, in 600 μ l CTAB extraction buffer [1% (m/v) CTAB, 50 mM Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.7 M NaCl] to which 1% (m/v) 2-mercapto-ethanol had been added just before use]. After one hour 600 μ l chloroform:iso-amylalcohol (24:1) was added, mixed thoroughly and the mixture centrifuged for five minutes at 3 000 g. The supernatant was transferred to a clean tube, and to this 600 μ l of cold (-20°C) absolute ethanol, containing 3 M sodium acetate (25:1) was added to precipitate the DNA. After one hour of incubation at 4°C, the mixture was centrifuged at 7 000 g for eight minutes. The supernatant was discarded and the DNA pellet washed twice with 70% (v/v) ethanol containing 10 mM ammonium acetate. After decanting the ethanol and evaporating any remaining ethanol, the DNA was dissolved in sterilised, distilled water (20-50 μ l, depending on the size of the pellet).

2.2.4.2 Taguchi optimisation

The optimisations of the PCR based RAPD analyses were done according to the Taguchi method (Cobb & Clarkson 1994). Accordingly four reaction components are varied in an orthogonal array by three different concentrations of each variable (Table 2.3). Thus the optimum concentration of each component can be calculated. The primer, dNTP, MgCl₂ and DNA con-

centrations were the variable components and DNA polymerase and buffer concentrations were kept constant.

Table 2.3. Components optimised by the Taguchi method (Cobb & Clarkson 1994).

Reactions	[Primer] 4.5pmol/ µl	[dNTP] 2mM/ µl	[MgCl ₂] 25mM/ µl	[DNA] 10ng/ µl
1	1	2	3	2
2	2	3	3	2.5
3	3	4	3	3
4	1	3	4	3
5	2	4	4	2
6	3	2	4	2.5
7	1	4	5	2.5
8	2	2	5	3
9	3	3	5	2

2.2.5 RAPD PCR

Optimized RAPD reaction volume was 25 µl and contained 2.5 µl of a 10x reaction buffer with 4 µl 25 mM MgCl₂, 2 µl 4 pmol primer, 4 µl of 2 mM dNTP mixture, 0.5 units *Taq* polymerase and 2 µl a 10 ng/µl DNA (diluted with sterile water). Sixteen primers were used, *OPA*₃ — 5'-AGT CAG CCA C-3', *OPA*₇ — 5'-GAA ACG GGT G-3', *OPA*₉ — 5'-GGG TAA CGC C-3', *OPB*₂ — 5'-TGA TCC CTG G-3', *OPB*₅ — 5'-TGC GCC CTT T-3', *OPC*₄ — 5'-CCG CAT CTA C-3', *OPC*₅ — 5'-GAT GAC CGC C-3', *OPC*₆ — 5'-GAA CGG ACT C-3', *OPC*₁₂ — 5'-TGT CAT CCC C-3', *OPF*₃ — 5'-CCT GAT CAC C-3', *OPF*₄ — 5'-GGT GAT CAG G-3', *OPF*₆ — 5'-GGG AAT TCG G-3', *OPF*₁₁ — 5'-TTG GTA CCC C-3', *OPF*₁₇ — 5'- AAC CCG GGA A-3', *OPG*₂ — 5'-GGC ACT GAG G-3' and *OPG*₅ — 5'-CTG AGA CGG A-3'. Standard amplifications were carried out

through an initial denaturation step of 94°C for 90 seconds and 36 amplification cycles of 94°C for 90 seconds, 34°C for 90 seconds, 72°C for 180 seconds. Reactions were cooled down to 4°C and stored at this temperature. They were heated to 65°C for 5 minutes prior to electrophoreses.

Each reaction was duplicated in order to test the repeatability of results. The amplification products were separated on 1% (m/v) agarose gels with 1X TAE running buffer (40 mM Tris–acetate, 18.98 mM Acetic acid, 1 mM EDTA, pH 8.0), intercalated with ethidium bromide at 80 V for 2.5 hours and visualised by illumination with ultraviolet (UV) light. The gel was photographed and analysed.

2.2.5.1 Data analysis

Analysis of amplification products was done manually. The following criteria were considered:

- Number of fragments
- Repeatability of the reaction

Graphical representations of each of the analysed primers were created in this way. These representations were checked manually and scored for absence (0) or presence (1) of fragments.

2.2.5.2 Consistency test

Fragment sharing analyses were carried out for the RAPD data, by pairwise comparison of the samples according to the consistency formula of Nei and Li (1979).

$$F = 2(X_{1,2}) / (X_1 + X_2),$$

where $X_{1,2}$ is the number of shared fragments with similar molecular weights, X_1 is the total number of RAPD fragments in the one reaction, X_2 is the total number of RAPD fragments in the other reaction and F is the coefficient of similarity (Nei 1987). An F value of one will indicate that the samples are

identical, or fully repeatable, and lower values will indicate a lesser correspondence.

Genetic distances can be calculated by using the Nei-formula (Nei 1987):

$$d = -\ln (F),$$

where d is the genetic distance between two specimens.

2.2.6 Sequencing

2.2.6.1 *ITS* fragment amplification

Genomic DNA was used to amplify the DNA region between the 18S and 5.8S nrDNA genes (the *ITS1* region), as well as between the 5.8S and 26S nrDNA genes (the *ITS2* region), with the polymerase chain reaction. A small portion of the 5.8S gene was amplified in both cases as well, due to the annealing sites of the primers. The primers used for the PCR were ITS_L and ITS_2 (for *ITS1*) and ITS_3 and ITS_4 (*ITS2*) (White *et al.* 1990).

ITS_L 5'- TCGTAACAAGGTTTCCGTAGGTG-3'

ITS_2 5'- GCTGCGTTCTTCATCGATCG-3'

ITS_3 5'- GCATCGATGAAGAACGCAGC-3'

ITS_4 5'- TCCTCCGCTTATTGATATGC-3'

The PCR reactions were performed in a total volume of 50 μ l. The reactions were optimised according to the Taguchi method (Cobb & Clarkson 1994) (2.2.3.2).

The reactions were briefly centrifuged and placed in the Perkin Elmer GeneAmp PCR system 9600. An initial denaturation step at 94 °C was followed by 40 amplification cycles, each consisting of 30 seconds at 94 °C, 30 seconds at 50 °C and 90 seconds at 72 °C (Baldwin 1992).

The amplification products were separated on 1% (m/v) agarose gels as described in 2.2.4.

2.2.6.2 Sequencing

Using the system based on Sanger's *et al.* (1977) sequences were

carried out.

For each template to be sequenced the following were combined:

Sequence reagent pre-mix	8 μ l
Primer (50 pmol)	1 μ l
DNA template	10 ng/ μ l
Sterile water	10 μ l
Total volume	20 μ l

These reactions were placed in the Perkin Elmer thermal cycler with an initial denaturation step at 94°C for 1 min, followed by 25 amplification cycles, each consisting of 94°C for 30 sec., 50°C for 15 sec. and 60°C for four minutes.

After amplification, 7 μ l of 7.5 M ammonium acetate was added to each reaction, as well as 2.5 volumes (\pm 68 μ l) of 100 % (v/v) ethanol (-20°C). These reactions were mixed and placed on ice for at least 15 minutes. Each sample was then centrifuged for 15 minutes at 10 000 g, whereafter the supernatant was discarded and 250-500 μ l of 70% (v/v) ethanol (-20°C) was added to wash the pellet. The mixtures were centrifuged briefly, and after the supernatant was drawn off the pellets, were vacuum dried for three to five minutes and stored in this dry state at -20°C, till loaded on the gel. Prior to gel loading each pellet was resuspended in 4 μ l of formamide loading buffer, and then heated to 100°C for 2–5 minutes to denature. Samples of 1.5-2 μ l were loaded on a 6% polyacrylamide gel and separated for 4–6 hours on a ABI Prism™ 377 fluorescent sequencing system.

2.2.6.3 Sequence alignment

The $ITS_1 - ITS_2$ and $ITS_3 - ITS_4$ sequence combinations were aligned for each specimen, using the Sequence Navigator software (Applied Biosystems Inc., a Division of the Perkin Elmer Corporation) for an Apple Macintosh computer. The sequences were aligned using the comparative alignment option with a mismatch penalty of 5, gap penalty of 4 and gaps extend penalty

of 3. The *ITS1* and *ITS2* sequences of each specimen were then aligned using CLUSTAL W (Thompson *et al.* 1994) and MALIGN (Wheeler & Gladstein 1994). Final alignment was visually inspected and manually optimised for phylogenetic analysis.

2.2.7 Phylogenetic analysis

2.2.7.1 PAUP (Phylogenetic analysis using parsimony) analysis

Data were analysed with the computer program PAUP (version 3.1) by converting each data set (e.g. DAF fragment patterns or aligned sequences) into a datamatrix.

PAUP uses the principle of maximum parsimony, which searches for minimum length cladograms. HEURISTIC searches using RANDOM (200 replications) stepwise addition of taxa, followed by TBR (tree bisection-reconnection) branch swapping (STEEPEST DESCENT and MULPARS in effect) were used to find the most parsimonious cladograms. Topological constraints were not enforced and branches of zero length were collapsed to yield polytomies.

Searches were conducted to find multiple islands of equally parsimonious trees (Maddison 1991). This was done according to methods outlined in Olmstead and Palmer (1994).

Heuristic search options explores many trees but gives no guarantee that the trees found will in fact be the shortest for the data set (Kellogg & Watson 1993). The branch and bound and exhaustive search options were not considered due to their time consuming nature. Exhaustive searches are guaranteed to find the shortest trees, but become computationally prohibited if there are more than 11 taxa in the data set. The branch and bound algorithm, also guaranteed to find the shortest trees, is more efficient, but only for up to 30 taxa (Swofford 1993).

When dealing with DNA sequencing data each nucleotide position was scored as a uniformly weighted character, with gaps scored as missing data.

Sets of equally parsimonious trees were summarised using Strict, Semi-strict (combinable component) and Adams consensus trees. Multistate taxa were treated as uncertain. Uninformative characters were ignored and all characters were unordered (Fitch optimisation) with a weight of one. Furthermore, characters were mapped on the consensus cladograms using ACCTRAN (Accelerated Transformation) (Swofford & Maddison 1987), which prefers reversals to parallelisms (homoplasy) when both optimisations are equally parsimonious.

Statistics for evaluating and comparing the trees generated were created. Two values were calculated namely:

- CI (consistency index), which divides the minimum number of changes of characters on a tree by the actual number of changes (Kluge & Farris 1969; Farris 1989a, b), and,
- RI (the retention index), which corrects for the actual distribution of character states in the data matrix by subtracting both the minimum number of changes and the actual number of changes from the maximum number of changes possible (Farris 1989a, b).

Both these indices are measures of homoplasy and can be applied to individual characters or to entire trees. When these statistics are used to describe trees, only the phylogenetic informative characters are included.

All uninformative characters were excluded from the data matrices, due to the fact that these characters will inflate CI values by adding both one unit to the numerator and denominator in the calculations. These are both invariant (characters in which a single state is possessed by all groups under consideration), as well as uninformative [characters in which only one of the included taxa possesses a particular derived state (autapomorphy)] characters (Sanderson & Donoghue 1989).

By stepwisely increasing the length of the cladogram, with the Strict cladogram option, decay indices were obtained (Bremer 1988; Donoghue *et al.* 1992). Bootstrap values were calculated from 200 replicates (Felsenstein

1985), by using the general HEURISTIC search with TBR branch swapping and CLOSEST ADDITION sequence of taxa (STEEPEST DESCENT and MULPARS in effect). Bootstrapping phylogenies is a means of estimating the robustness of phylogeny reconstruction to sampling error (Sanderson & Doyle 1992). Hillis and Bull (1993) showed that bootstrapping provides a very conservative test of the accuracy of the cladogram, but that the absolute values may not be very meaningful.

Where applicable, some characters were excluded from the matrix where low CI: RI ratios were observed. Successive weighting (Farris 1969) was also applied to the characters to determine the effect that larger weights for certain less homoplasious characters would have on the parsimony of the phylogeny.

CHAPTER 3

MORPHOLOGY, DISTRIBUTION AND HABITAT

3.1 INTRODUCTION

Approximately 10% of all plant species in the world occur in South Africa. We are, therefore, responsible for caring for a sizeable portion of the Earth's plant wealth, even though this land surface is less than 1% of that of the globe. South Africa is also the only country to contain in total one of the world's six Floral Kingdoms, namely the Cape Floral Kingdom. One third of South Africa's plant species occur in this Kingdom. The vegetation of southern Africa is subdivided into seven biomes of which the three grass genera under investigation inhabit four of the seven biomes. The core of distribution is centered in the Cape Floral Kingdom with several species endemic to this Kingdom (Low & Rebelo 1996).

Natural hybridization is common in the grasses and the variability within a hybrid population increases (Ehrendorfer 1980). This level of genetic variability allows the grasses to take advantage of new habitats (Ehrendorfer 1980). Five kinds of hybridization namely occasional hybridization, recurrent hybridization, partial interbreeding, a complete local breakdown of reproductive isolation and the production of a new specific entity in plants, are distinguished (Reiger *et al.* 1976). This apparent ability to hybridize and to exploit the advantages of hybrid species complexes, with ranges of chromosome numbers and genomes, is ancient in the grasses (De Wet 1987). The phenomenon occurs in all subfamilies and is common in many genera such as *Merxmuellera*. Polyploidy is usually associated with hybridization. As many as 82% of grass species in southern Africa are of polyploid origin, indicating a high degree of hybridization (Spies *et al.* 1992).

Furthermore, in the Poaceae, with their highly specialized and reduced flowers, very fine morphological boundaries are often necessary to define differences between taxa. Anatomical data is, therefore, regarded as being of indisputable importance in the jigsaw of complete systematic evidence in this numerically large and important family (Ellis 1976).

Anatomical investigations of the grass leaf-blade have for a long time provided valuable taxonomic information. Presently, it is generally accepted that anatomical details, especially of the leaf-blade and embryo, when used in conjunction with a wide spectrum of other diagnostic characters, is an essential ingredient of any satisfactory treatment of grass taxonomy (Ellis 1976).

Surely it is each South African's responsibility to conserve this rich diversity. It is our duty to document and conserve the biological diversity as effectively as possible, so that we can appraise how effectively we are looking after it and what still needs to be done.

3.2 Synopsis of the taxa under investigation

Most *Merxmuellera* species exhibit great genetic and morphological variation (Spies & du Plessis 1988; Gibbs Russell *et al.* 1990). This variation within a species is particularly evident in *M. stricta*. This perennial species is a tufted grass, varying in height from 0.3 – 0.8 m and exhibiting a broad range of morphological forms. The inflorescence varies from 30 – 130 mm in length and is often branched at the base.

The genera *Karoochloa* and *Schismus* are tufted annuals or perennials, caespitose or decumbent. Their leaf blades are linear to linear-lanceolate, expanded or rolled, setaceous or glabrous. The inflorescence is contracted and spike-like. Genetic and morphological variations in the genera are low (Gibbs Russell *et al.* 1990).

The distribution maps of the species investigated may not indicate the total distribution areas because only specimens studied were indicated on these maps.

Karoochloa curva (Nees) Conert and Türpe is a stoloniferous, tufted, tussock-forming perennial grass. *Karoochloa curva* forms small tussocks of up to 0.5 m in diameter with leaves up to 0.25 m long arching outwards from the tuft base. This finding coincides with the findings of Conert and Türpe (1969). *Karoochloa curva* occurs along the Cape Fold belt from Cape Town and Cape Agulhas to near Port Elizabeth (Fig. 3.1). The altitude in this region ranges from sea level to 2200 m above sea level. The rainfall in this area is mainly in winter and varies between 200 - 2200 mm annually. *Karoochloa curva* is largely confined to soils derived from sandstone of the Cape Super Group and is frequently dominant along stream banks and in moist, shady areas. *Karoochloa curva* flowers from mid spring to the beginning of winter.

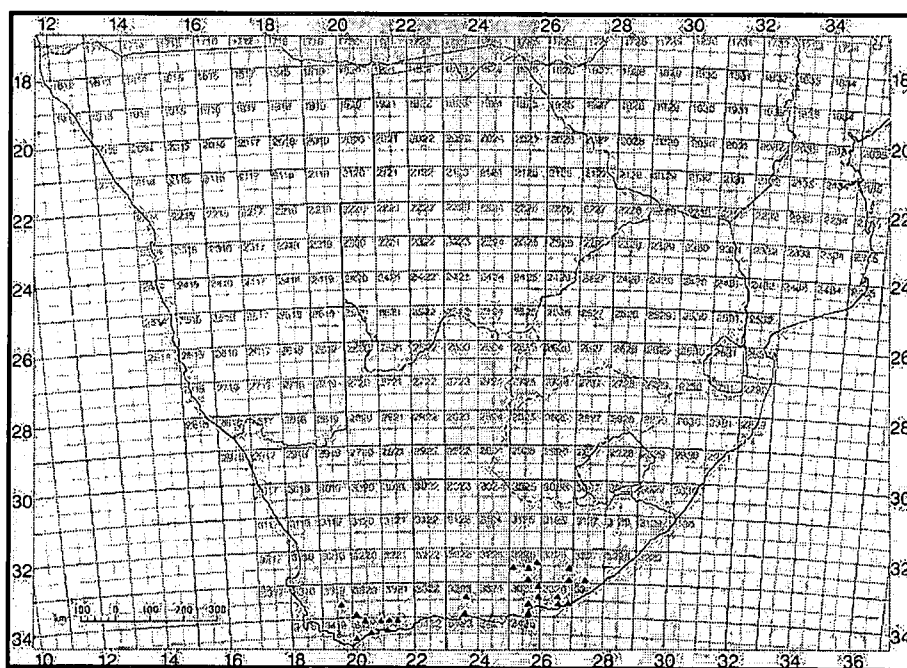


Figure 3.1 Geographical distribution of *Karoochloa curva*.

Karoochloa purpurea (L.f.) Conert and Türpe is a rhizomatous, tufted, tussock-forming perennial grass. *Karoochloa purpurea* forms small purplish tussocks of up to 0.3 m in diameter with leaves of up to 0.4 m long arching outwards from the tuft base. The abovementioned observations correlate with the measurements of the original description done by Conert and Türpe in

1969. *Karooochloa purpurea* is most commonly found at the Cape Fold Mountains from north of Nieuwoudtville to Cape Town and Cape Agulhas and eastwards to near Port Elizabeth. *Karooochloa purpurea*'s distribution further extends to the Drakensberg foothills of the Eastern Cape and KwaZulu-Natal (Fig. 3.2). The altitude varies from sea level to about 2200 m above sea level with an annual rainfall of between 200 - 2000 mm. Rainfall in the west is mainly in winter with a high summer rainfall component in the Drakensberg-Eastern Cape region. The soil in the Drakensberg region is often shallow, rocky and leached, derived from Karoo Sequence sediments and dolerite. In the western parts of the distribution area, the soil is mainly derived from sandstone of the Cape Super Group. *Karooochloa purpurea* flowers from mid winter to the end of summer.

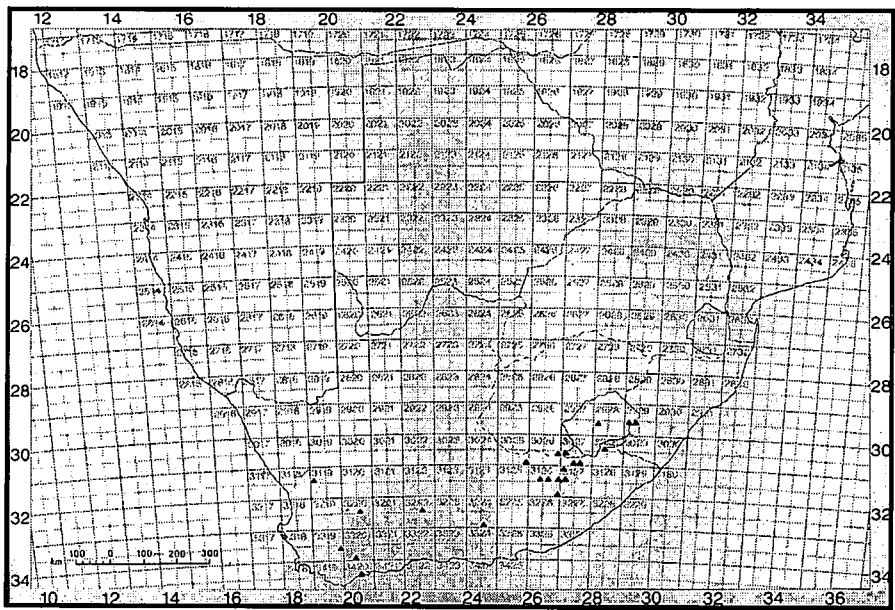


Figure 3.2 Geographical distribution of *Karooochloa purpurea*.

Karooochloa schismoides (Stapf ex Conert) Conert and Türpe is a wiry, tufted, tussock-forming annual grass. *Karooochloa schismoides* forms tussocks of up to 0.5 m in diameter with leaves of up to 0.6 m long arching outwards from the tuft base. These findings confirm the results of Conert and Türpe (1969). *Karooochloa schismoides* inhabits the Namaqualand-Kamiesberg-Roggeveld

region of the West Coast of South Africa and extends northwards into the southern parts of the Namib Desert of Namibia (Fig. 3.3). The altitude is mostly below 800 m with exceptions in the east where the relief may reach 1500 m. The rainfall is very low 50 - 300 mm annually and the rain falls mainly in the winter months. The area consists of rich soils that developed from the decay of granites and gneisses. In the south the Karoo Sequence shale and sandstone give rise to more skeletal soils. *Karoochloa schismoides* flowers from mid winter to mid autumn after sufficient rain.

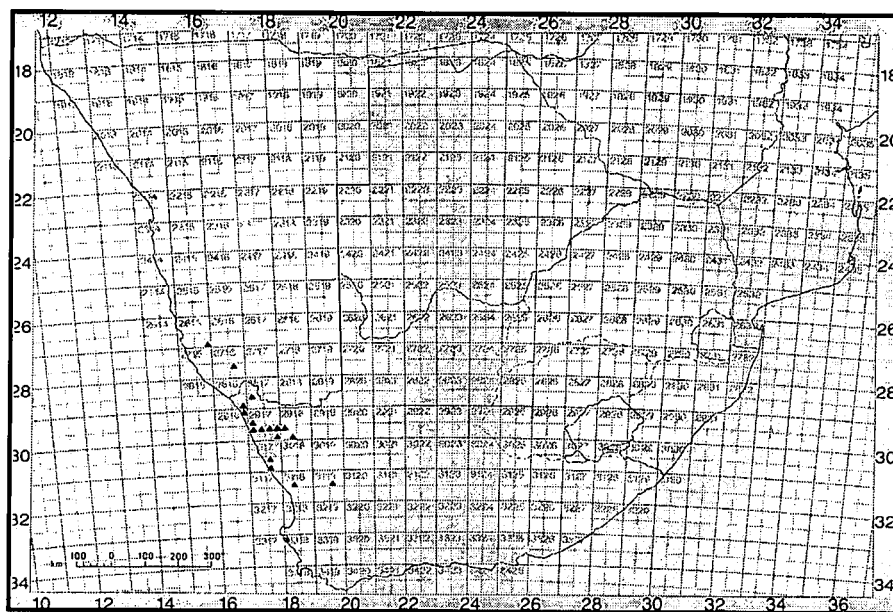


Figure 3.3 Geographical distribution of *Karoochloa schismoides*.

Karoochloa tenella (Nees) Conert and Türpe is a wiry, tufted, tussock-forming annual grass. *Karoochloa tenella* forms small tussocks of up to 0.2 m in diameter with leaves of up to 0.15 m long arching outwards from the tuft base. The abovementioned observations correlate with the observations done by Conert and Türpe (1969). *Karoochloa tenella* inhabits the Kamiesberg-Hantamberg-Roggeveld area and extends along the Cape Fold Belt from Nieuwoudtville to Cape Town and Cape Agulhas (Fig. 3.4). *Karoochloa tenella* is most frequent at altitudes of 1500 m in the east to 2200 m in the south of the distribution area. This area receives rain in the winter months and it varies

between 150 - 2200 mm per annum. *Karoochloa tenella* is common in disturbed sandy areas that arise from the decay of granites and gneisses as well as from the decay of sandstone of the Cape Super Group. Flowering depends on rainfall, but *K. tenella* mainly flowers from the end of winter to mid autumn.

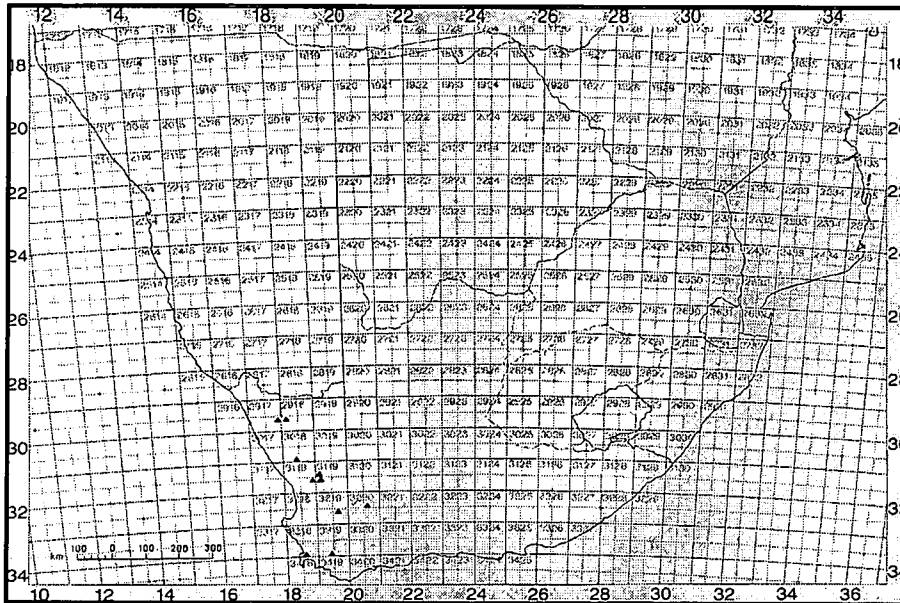


Figure 3.4 Geographical distribution of *Karoochloa tenella*.

Merxmuellera arundinacea (Berg). Connert is a robust, reedlike, perennial grass with large, lax tussocks of up to 1 m in diameter and leaves of up to 1 m long arching outwards from the tuft base. This finding coincides with the findings of Conert (1970). The *M. arundinacea* distribution area is along the Cape Fold Belt, north of Nieuwoudtville to Cape Town and Cape Agulhas and eastwards to near Port Elizabeth (Fig. 3.5). The altitude varies from sea level to 2200 m above sea level, with an annual winter rainfall of between 200 and 2200 mm. *Merxmuellera arundinacea* is largely confined to soils derived from sandstone of the Cape Super Group. Although the rainfall occurs in winter, this species is spring flowering.

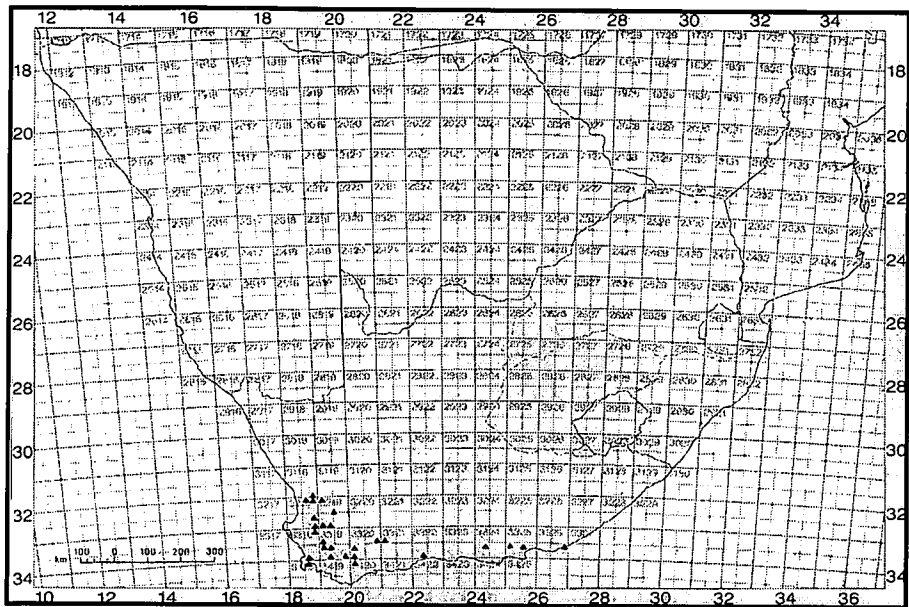


Figure 3.5 Geographical distribution of *Merxmuellera arundinacea*.

Merxmuellera aureocephala (J.G. Anders) Conert is a wiry, tufted, tussock-forming perennial grass. *Merxmuellera aureocephala* forms large, lax tussocks

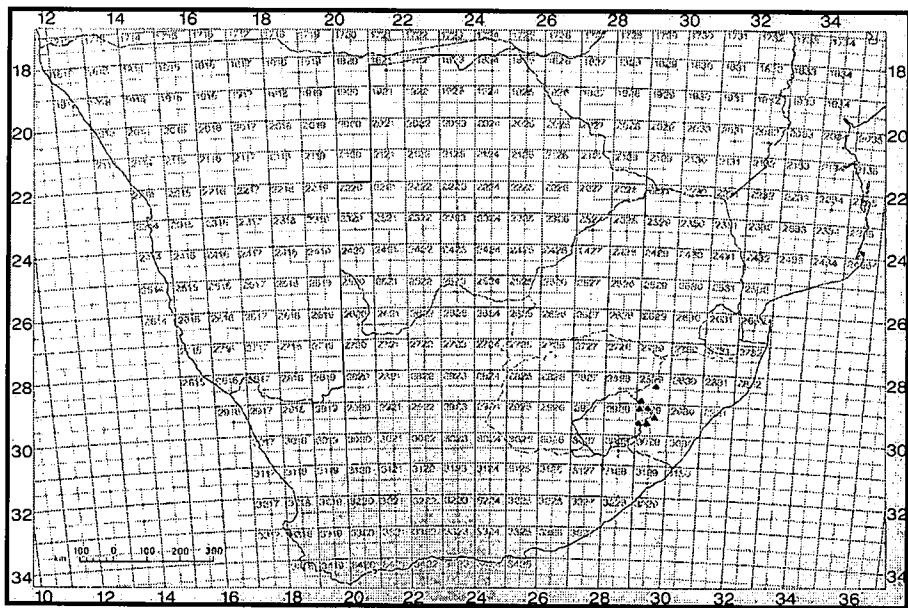


Figure 3.6 Geographical distribution of *Merxmuellera aureocephala*.

up to 0.3 m in diameter with leaves up to 0.4 m long arching outwards from the tuft base. This correlates with the observations of Conert (1970). *Merxmuellera*

aureocephala is confined to the Drakensberg mountain plateau and slopes of Lesotho and the adjacent KwaZulu-Natal. *Merxmuellera aureocephala* is mainly restricted to the Cathedral Peak, Cathkin Peak and Newcastle areas (Fig. 3.6). *Merxmuellera aureocephala* occurs at altitudes exceeding 1700 m above sea level. This area is quite moist with a summer rainfall of 575 to more than 1000 mm per annum. This mountainous area is characterized by soils that are shallow, rocky and leached. The geology is sandstone capped with basalt. *Merxmuellera aureocephala* flowers from mid winter to beginning of autumn.

Merxmuellera cincta (Nees) Conert is a robust, reedlike, perennial grass with large, lax tussocks of up to 1 m in diameter with leaves of up to 1 m long arching outwards from the tuft base. The results confirm the findings of Conert

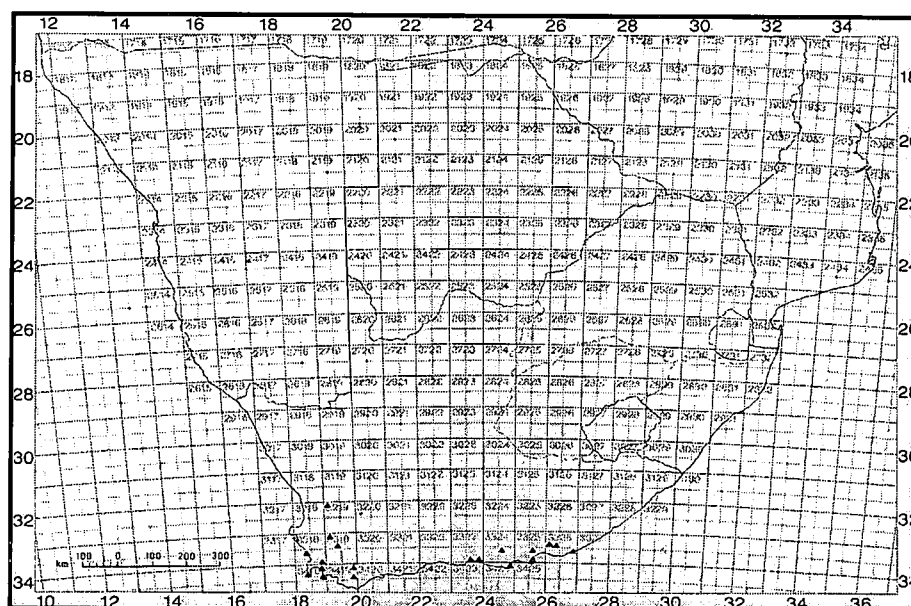


Figure 3.7 Geographical distribution of *Merxmuellera cincta*.

(1970). *Merxmuellera cincta* is confined to the Cape Fold Belt, north of Nieuwoudtville to Cape Town and Cape Agulhas and eastwards to Port Elizabeth (Fig. 3.7). The altitude ranges between 200 and 2200 m. Rainfall in this area reaches its climax in winter. The soil in this region is derived from

sandstone of the Cape Super Group. *Merxmuellera cincta* flowers in spring and summer.

Merxmuellera davyi (C.E. Hubb.) Conert is a wiry, tufted, perennial grass with large tussocks of up to 0.6 m in diameter with leaves of up to 0.6 m long arching outwards from the tuft base. The abovementioned results correlate with the observations in the original description done by Conert (1970). *Merxmuellera davyi* occurs on the Drakensberg mountains in Mpumalanga (Fig. 3.8) at altitudes above 2000 m (Conert 1975). This region receives 650 - 950 mm rain per annum, mostly in summer. *Merxmuellera davyi* is adapted to more xeric habitats and occurs on steep grassy slopes in rocky situations (Anderson 1962) and flowers in spring.

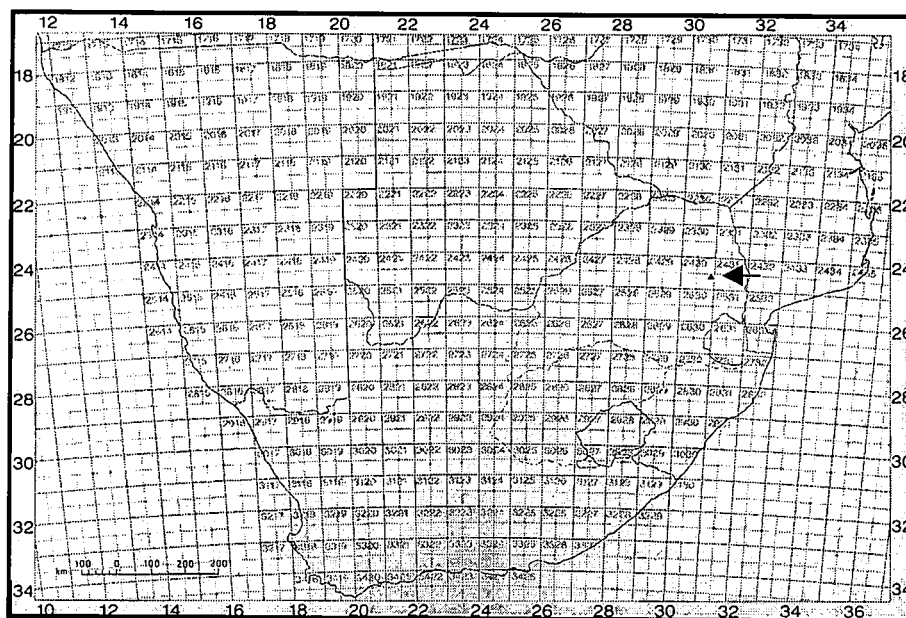


Figure 3.8 Geographical distribution of *Merxmuellera davyi*.

Merxmuellera decora (Nees) Conert is a robust perennial up to 2 m tall, leaves up to 1 m long and with a distinct bulbous base consisting of the old woolly leaf sheaths which are deeply sunken into the ground. This coincides with the findings of Conert (1970). *Merxmuellera decora* is confined to the southernmost parts of South Africa, mainly in the lowlands of the Western Cape, from Cape

Town to Knysna (Fig. 3.9). This area has a relief of 0 to 290 m. This area has a rainfall spectrum of 350 - 600 mm per annum, mainly in autumn, winter and spring. *Merxmuellera decora* is fire adapted and commonly occurs in firebreaks and is particularly conspicuous after burns. *Merxmuellera decora* occurs in sandy soils at the foothills of the mountains. *Merxmuellera decora* inhabits shallow sands, overlying limestone and associated calcretes, of the Bredasdorp formation at the foothills of the mountains. *Merxmuellera decora* flowers from spring to mid summer.

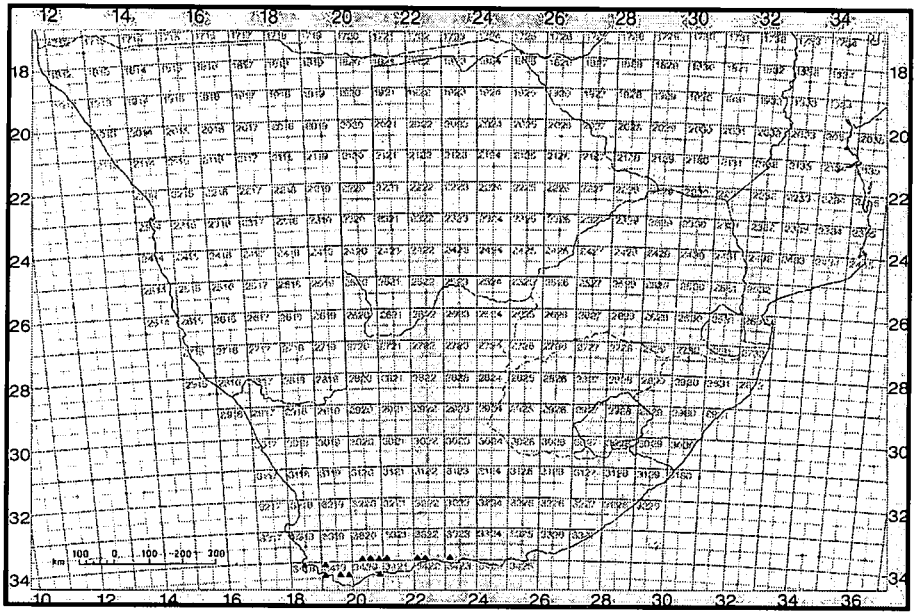


Figure 3.9 Geographical distribution of *Merxmuellera decora*.

Merxmuellera disticha (Nees) Conert is a wiry, tufted, tussock-forming perennial grass. *Merxmuellera disticha* forms tussocks of up to 0.1 m in diameter with leaves of up to 0.5 m long arching outwards from the tuft base. This coincides with the findings of Conert (1970). *Merxmuellera disticha* is the most distinctive southern African representative of the genus *Merxmuellera* and is found over extensive areas along the Kouga Mountains to Port Elizabeth and Grootrivierberge as well as from Steytlerville to Grahamstown and the Bushmans River Mouth on mountain slopes. *Merxmuellera disticha* extends to the Great Escarpment of the Eastern Cape from Barkley-East, Steynsburg and

Cradock-Middelburg areas and further north into the Drakensberg of Lesotho, KwaZulu-Natal and the Free State (Fig. 3.10). There is an extensive variation of altitude in which this species occurs from 200 to over 2200 m above sea level. The rainfall in this distribution area ranges from 350 - 600 mm with exceptions at higher altitudes of 1900 mm per annum. *Merxmuellera disticha* occurs in sandy soils of the Cape Super Group, calcareous, neutral to alkine, shallow sand overlying limestone, shallow rocky volcanic soils of the Stormberg plateau to shallow, rocky soils of the Drakensberg Mountains. *Merxmuellera disticha* flowers from mid spring to early winter.

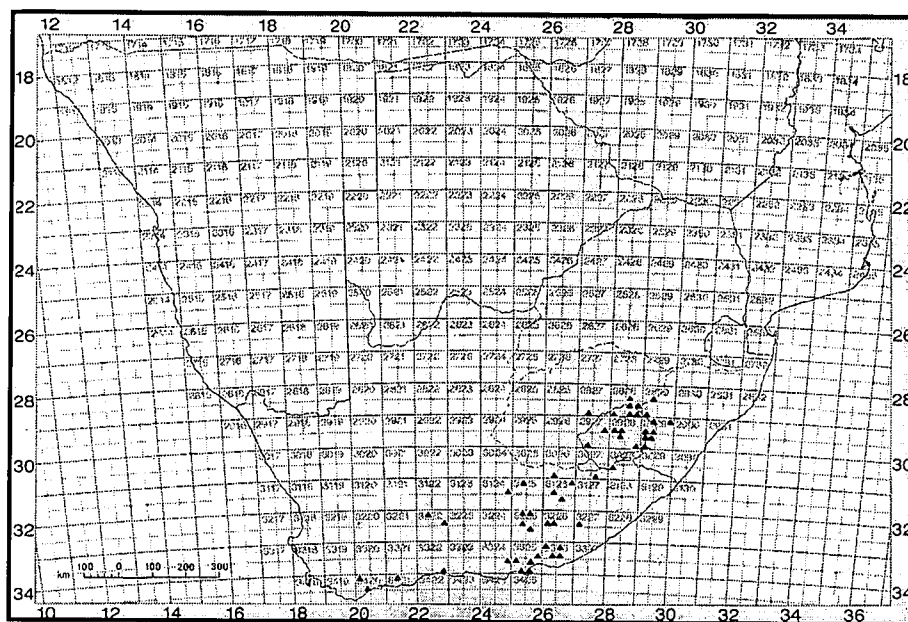


Figure 3.10 Geographical distribution of *Merxmuellera disticha*.

Merxmuellera drakensbergensis (Stapf) Conert is a tufted, rigid erect tussock-forming, perennial grass. The unbranched culms grow vertically up to 1 m tall and the leaves of 0.3 m are rigid and taper to a pungent apex. The abovementioned results correlate with the observations done by Conert (1970). *Merxmuellera drakensbergensis* occurs in mountain vegetation along the eastern escarpment of southern Africa, from the Barkley-East and the Maclear districts in the Eastern Cape Province along the Drakensberg mountains of KwaZulu-Natal and Lesotho to Mariepskop in Mpumlanga (Fig. 3.11) (Gibbs

Russell *et al.* 1990). The altitude of this area ranges between 1350 m to above 2000 m with an average summer rainfall of 450 - 600 mm per annum. At high altitudes the rainfall averages 1900 mm per annum. At these moist, high altitudes, *M. darkensbergensis* commonly occupies mesic situations in stream bank and mud patch communities (Killick 1963; Edwards 1967). *Merxmuellera drakensbergensis* flowers from mid spring to the beginning of autumn.

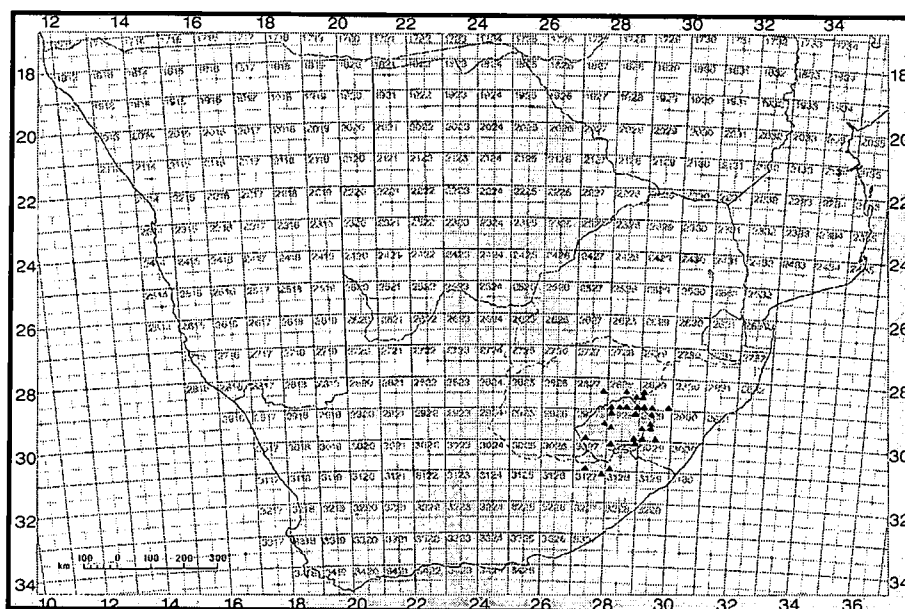


Figure 3.11 Geographical distribution of *Merxmuellera drakensbergensis*.

Merxmuellera dura (Stapf) Conert is a short, rhizomatous, tufted, perennial grass with tussocks of up to 0.2 m in diameter with leaves of up to 0.6 m long. The abovementioned results correlate with the observations done by Conert (1970). *M. dura* is largely confined to the Kamiesberg highlands around Leliefontein. Northwest Mountain Renosterveld grades with succulent Karoo and Fynbos. *Merxmuellera dura* is common in the Carnavan and Calvinia districts (Fig. 3.12). *Merxmuellera dura* occurs in sandy riverbeds or other habitats with accumulated, fine, loose sand. The distribution area can reach an altitude of 2200 m above sea level with winter rainfall that ranges from 200 - 2000 mm annually. *Merxmuellera dura* flowers from mid winter to the end of spring.

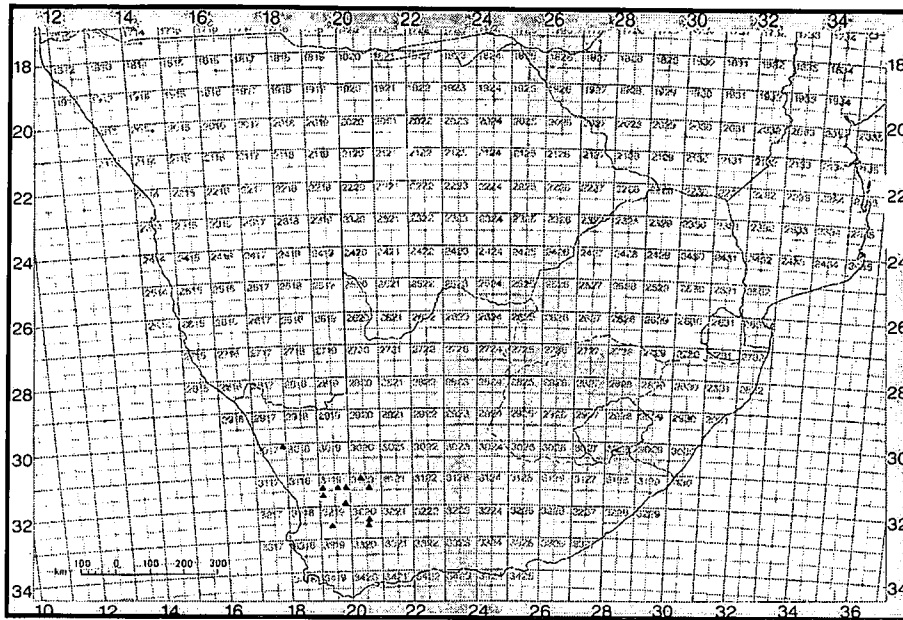


Figure 3.12 Geographical distribution of *Merxmuellera dura*.

Merxmuellera guillarmodiae (Conert) is a tufted, rigid, erect tussock-forming perennials grass. *Merxmuellera guillarmodiae*'s unbranched culms of 0.12 - 0.4 m tall grow vertically and the leaves are rigid and taper to a pungent apex. The leaves of up to 0.4 m are setaceous and tightly involute or canaliculate. This coincides with the findings in the description done by Conert in 1970. *Merxmuellera guillarmodiae* is confined to the Drakensberg mountain plateau and slopes of Lesotho and the adjacent KwaZulu-Natal mountains and is mainly restricted to the Cathedral Peak, Cathkin Peak and Newcastle areas (Fig. 3.13). *Merxmuellera guillarmodiae* occurs at altitudes from 1700 m above sea level. This area is quite moist with rainfall from 575 to more than 1000 mm per year. This area lies in the summer rainfall area. This mountainous area is characterized by soils that are shallow, rocky and leached. The geology is sandstone capped with basalt. *Merxmuellera guillarmodoae* flowers in summer.

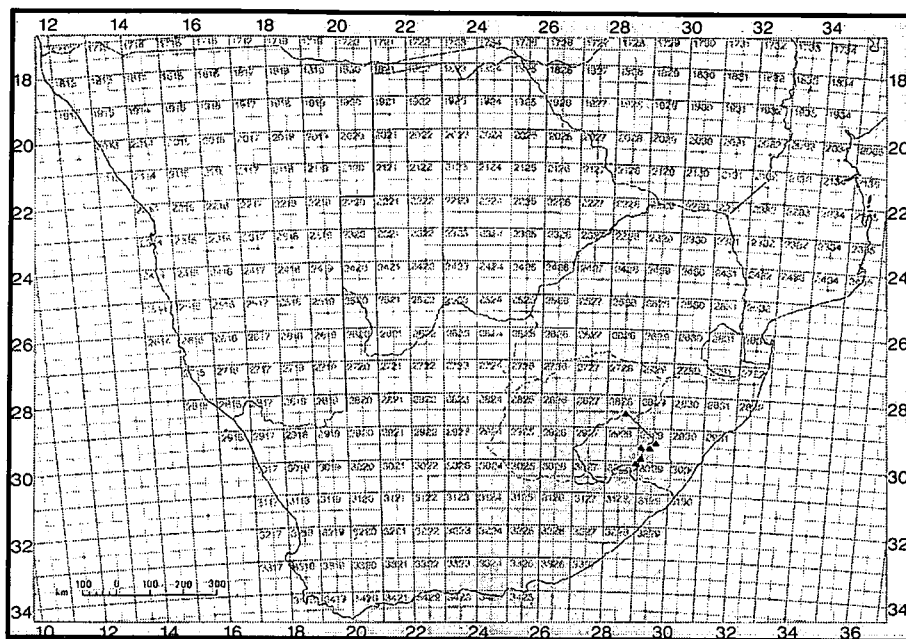


Figure 3.13 Geographical distribution of *Merxmuellera guillarmodiae*.

Merxmuellera lupulina (Thunb.) Conert is a strong perennial of 0.4 m tall with a distinct bulbous base consisting of the old woolly leaf sheaths that are deeply sunken into the ground. This coincides with the findings in the original description done by Conert in 1970. *Merxmuellera lupulina* predominantly occupies the Western Cape Forelands from just north of Piketberg, to Somerset West, mainly on the lowlands and low hills (Fig. 3.14). The altitude does not exceed 500 m above sea level with an average winter rainfall of 300 mm - 600 mm. *Merxmuellera lupulina* occurs in sandy soils derived mainly from Malmesbury Group shales, Caper Granite suite and Klipheuwel formation shales. *Merxmuellera lupulina* appears to be more frequent in firebreaks after a recent fire. *Merxmuellera lupulina* flowers from mid spring to mid summer.

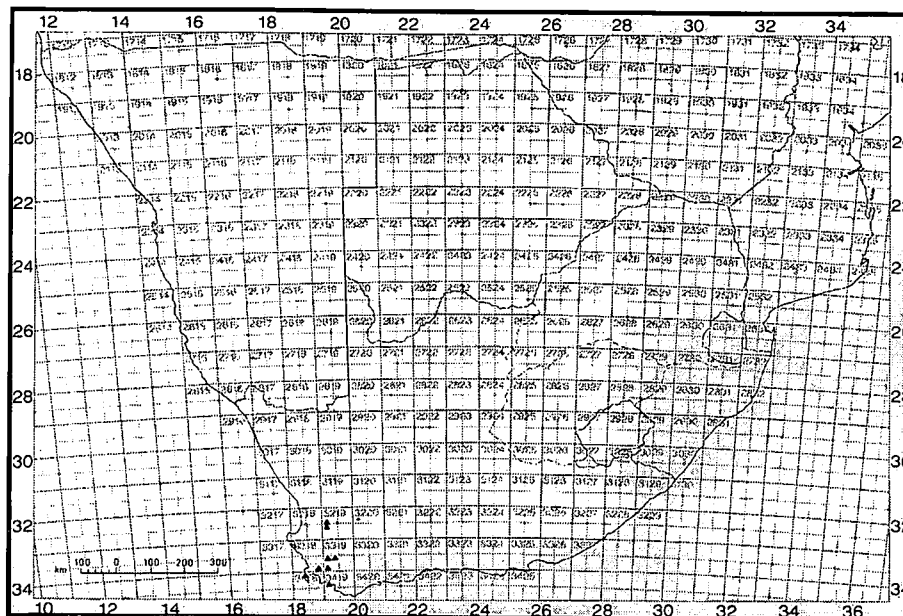


Figure 3.14 Geographical distribution of *Merxmuellera lupulina*.

Merxmuellera macowanii (Stapf) Conert is a wiry, tufted, tussock-forming perennial grass. *Merxmuellera macowanii*, in particular, forms large, lax tussocks of up to 0.6 m in diameter with leaves of up to 1 m long. The abovementioned results correlate with the observations done by Conert (1970). *Merxmuellera macowanii* arching outwards in mountain vegetation along the eastern escarpment of southern Africa, from the Drakensberg Mountains in Mpumalanga southwards as far as the Witteberge, Stormberge and Amatole Mountains in the Eastern Cape Province (Fig. 3.15). *Merxmuellera macowanii* is frequent at altitudes of 1500 m to 3000 m and is also found in the midlands of KwaZulu-Natal at lower altitudes. Rainfall in this region ranges from 450 to 1900 mm at the highest altitudes. *Merxmuellera macowanii* is frequently dominant along stream banks and in marshy areas of the montane and subalpine belt of the Drakensberg (Killick 1963; Edwards 1967) but is nevertheless, a xeromorphic grass with sclerophyllous leaves. Soils are very clayey, black vertic or near vertic, mostly of montmorillonitic types. *Merxmuellera macowanii* flowers from mid winter to mid summer.

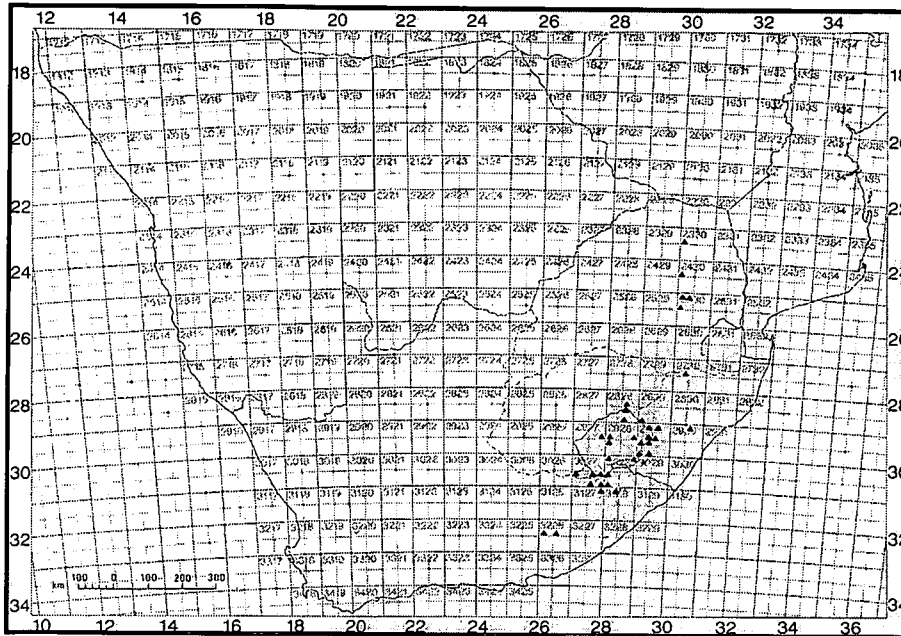


Figure 3.15 Geographical distribution of *Merxmuellera macowanii*.

Merxmuellera papposa (Nees) Conert is a robust perennial of up to 0.5 m tall. This finding coincides with the findings in the description of Conert (1970). *Merxmuellera papposa* occurs in the Uitenhage area (Fig. 3.16), with an altitude not exceeding 800 m. This area receives up to 1000 mm rain per annum mainly

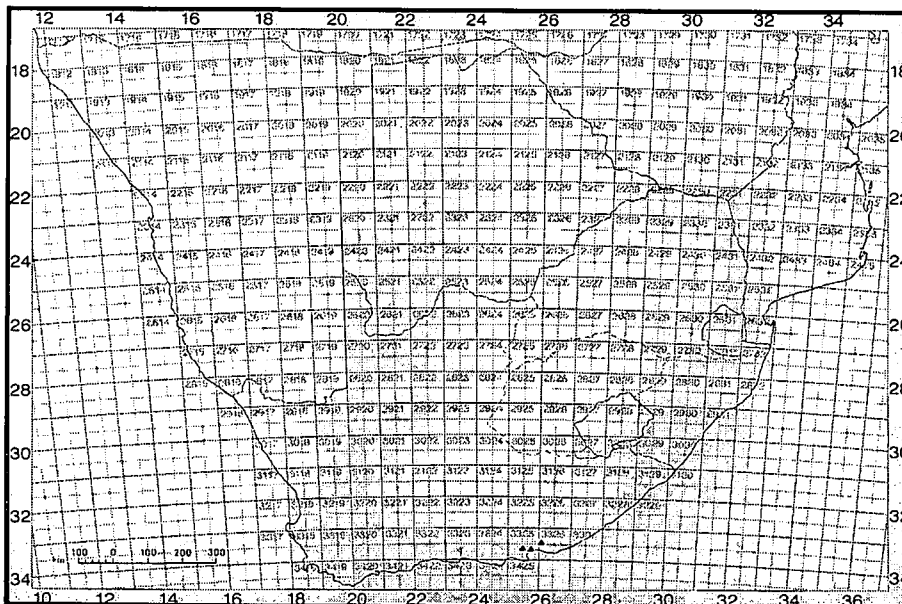


Figure 3.16 Geographical distribution of *Merxmuellera papposa*.

in winter. The soil is mainly derived from Cape Super Group, sandstone and Enon conglomerates in the Uitenhage Group. *Merxmuellera papposa* is a very localized summer flowering species of the genus *Merxmuellera*.

Merxmuellera rangei (Pilg.) Conert is a small tufted, tussock-forming perennial grass, with tussocks of up to 0.1 m in diameter with leaves of up to 0.14 m long. The abovementioned results correlate with the observations done by Conert (1970). This region receives between 200 and 290 mm rain per annum and the altitude is mostly below 800 m. *Merxmuellera rangei* occurs in dry, sandy habitats of granite 'koppies' and dry watercourses in southwestern Namibia (Fig. 3.17). Flowering depends on rainfall, but this species mainly flowers in spring after sufficient rain.

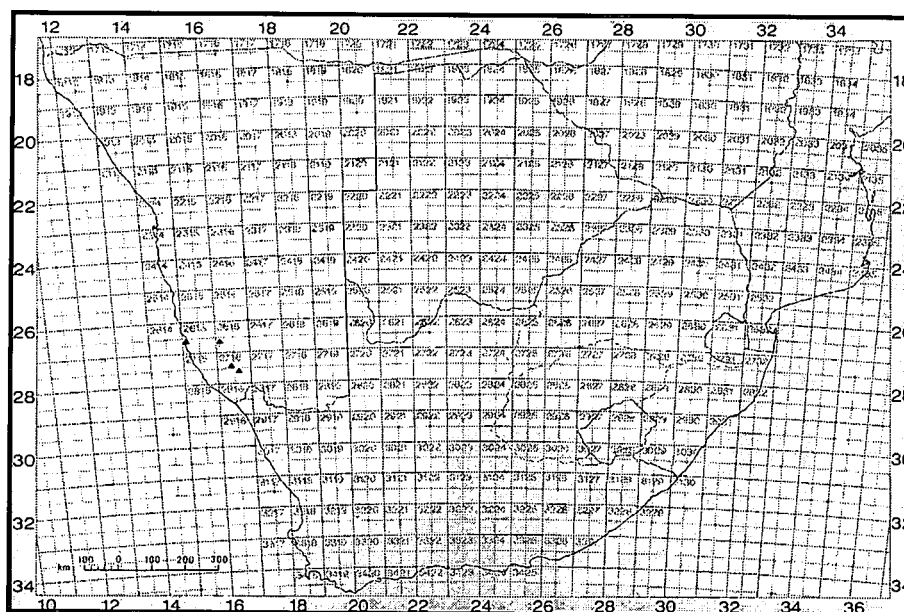


Figure 3.17 Geographical distribution of *Merxmuellera rangei*.

Merxmuellera rufa (Nees) Conert is a strong perennial of up to 0.4 m tall with a distinct bulbous base consisting of the old woolly leaf sheaths, which are deeply sunken into the ground. The observed data correlates with the original description done by Conert (1970). *Merxmuellera rufa* is confined to the moist southern parts of South Africa and is found from the Olifantsriver mouth to

Muizenberg and the West Coast lowlands as well as on the Elim Flats in the Western Cape (Fig. 3.18). The rainfall ranges between 200 and 2000 mm annually in this region. The altitude in this region varies between sea level and 2200 m above sea level. The soil is derived from a variety of depositional landscapes with gravelly, lateritic and seasonally waterlogged soils. Most of these soils are of tertiary origin, being aeolian and podsolized, but some are derived from the Cape Granite Suite and Table Mountain Group sandstone. *Merxmuellera rufa* commonly occurs in firebreaks especially after a recent fire. *Merxmuellera rufa* flowers from spring to mid summer.

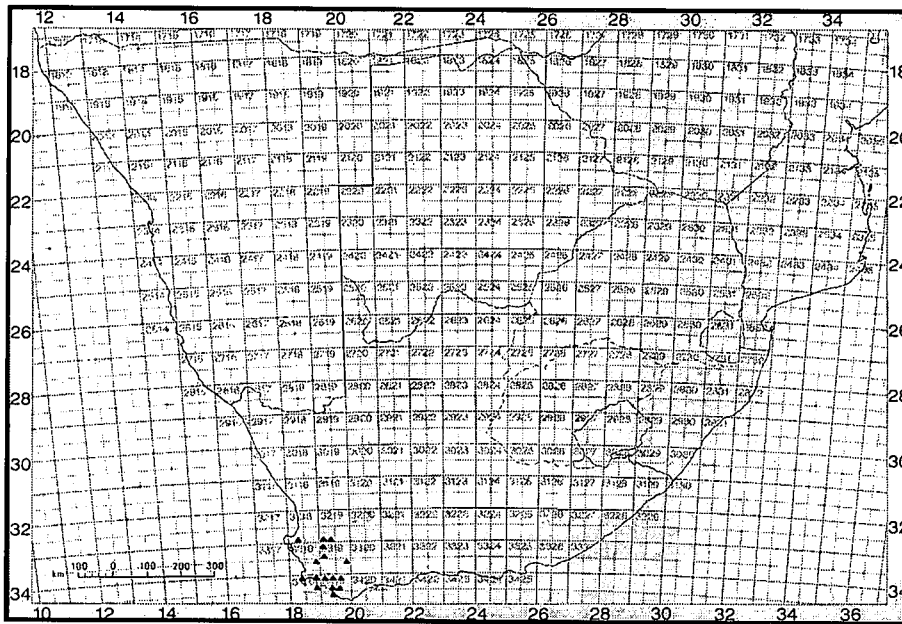


Figure 3.18 Geographical distribution of *Merxmuellera rufa*.

Merxmuellera setacea Barker & Ellis is a caespitose, rhizomatous perennial grass. *Merxmuellera setacea* forms bulb-like structures covered with swollen leaf sheath bases. Leaves can reach a length of up to 0.15 m. This finding coincides with the results in the original description done by Barker and Ellis (1991). *Merxmuellera setacea* is confined to the Worcester, Wupperthal area. The altitude varies between 1000 and 1500 m above sea level, with an average annual winter rainfall of between 450 and 600 mm. *Merxmuellera setacea* is

largely confined to soils derived from sandstone of the Cape Super Group. Although the rainfall occurs mainly in winter this species is summer flowering.

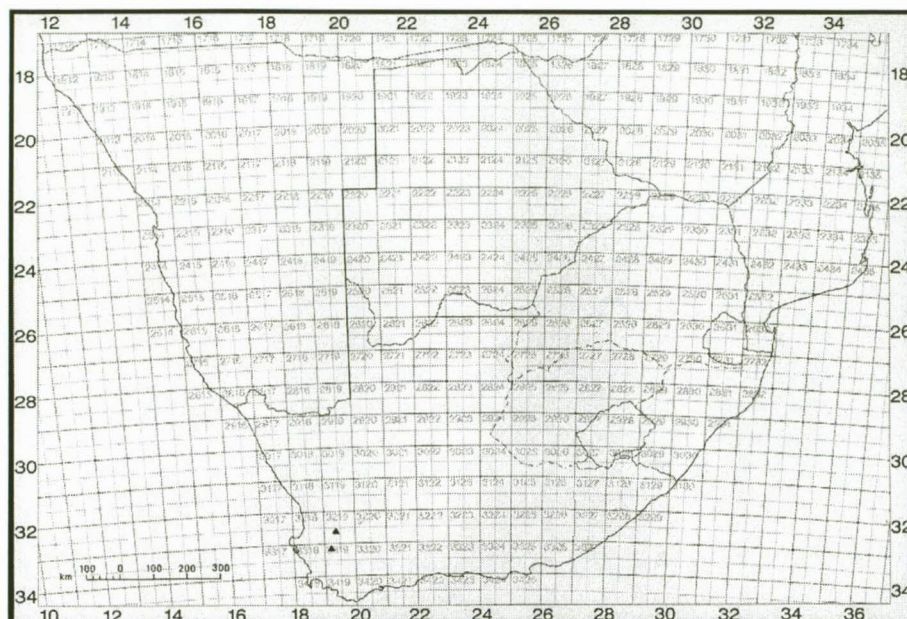


Figure 3.19 Geographical distribution of *Merxmuellera setacea*.

Merxmuellera stereophylla (Stapf) Conert is a wiry, tufted, rigidly erect, tussock-forming, perennial grass with unbranched culms growing vertically up to 0.8 m tall and the leaves are rigid and tapering to a pungent apex with a length of up to 0.36 m. The leaves are setaceous and tightly involute or canaliculate. The abovementioned results correlate with the observations done by Conert (1970). This species has a more limited distribution, being found only in the Drakensberg areas of KwaZulu-Natal and Lesotho (Fig. 3.20). The altitude of this area ranges from 1350 to above 2000 m with an average rainfall of 450 600 mm per annum. At the higher altitudes the average rainfall is 1900 mm per annum. It is at these altitudes with the high rainfall that the specimens of *Merxmuellera stereophylla* are commonly found. This region mainly receives its rain in summer. *Merxmuellera stereophylla* flowers from mid summer to mid autumn.

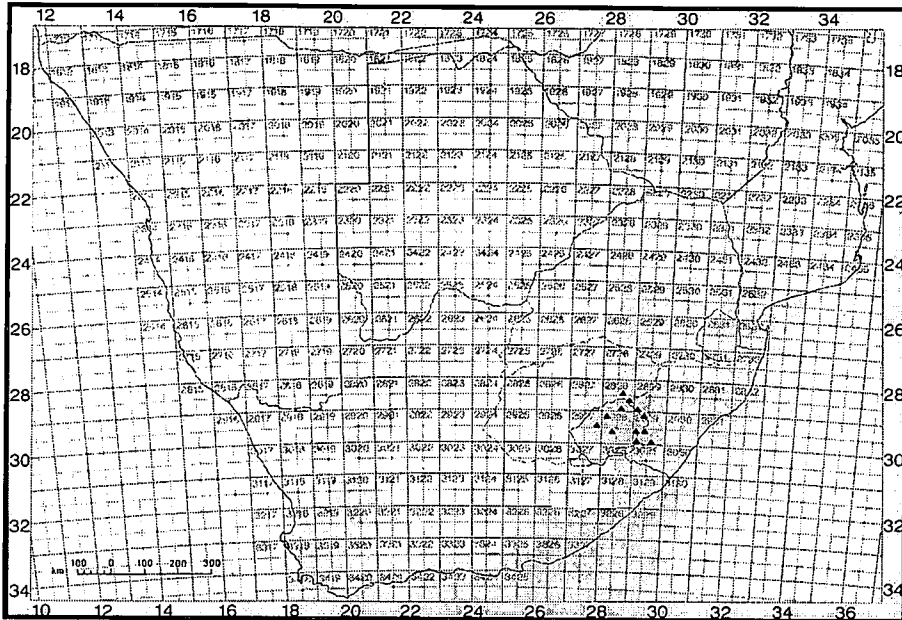


Figure 3.20 Geographical distribution of *Merxmuellera stereophylla*.

Merxmuellera stricta (Schrad.) Conert is a variable perennial, forming coarse,

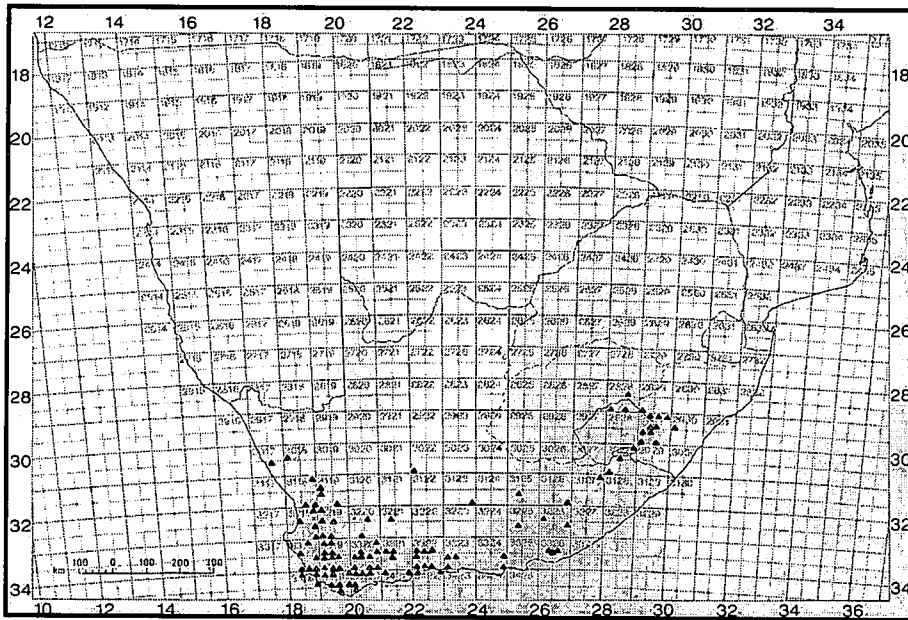


Figure 3.21 Geographical distribution of *Merxmuellera stricta*.

wiry tufts. The abovementioned results correlate with the observations done by Conert (1970). *Merxmuellera stricta* inhabits the Namaqualand-Kamiesberg-

Roggeveld region and southwards to the Cape Fold Mountains. The distribution of this species extends to the Drakensberg of the Eastern Cape and KwaZulu-Natal (Fig. 3.21). The altitude varies from sea level to about 2200 m above sea level with an annual rainfall of between 200 - 2000 mm. Rainfall in the west is mainly in winter with a high summer rainfall component in the Drakensberg-Eastern Cape region. The soil in the Drakensberg region is often shallow, rocky and leached, derived from Karoo Sequence sediments and dolerite. In the western point of this distribution, the soils are mainly derived from sandstone of the Cape Super Group. *Merxmuellera stricta* flowers from the beginning of spring to the beginning of autumn.

Schismus barbatus (Loefl. Ex L.) Thell is a wiry, tufted, tussock-forming annual grass, forming small tussocks of up to 0.3 m in diameter with leaves of up to 0.5 m, arching outwards from the tuft base. This coincides with the findings of Conert and Türpe (1974). *Schismus barbatus* inhabits Namibia, the Namaqualand-Kamiesberg-Roggeveld region and southwards to the Cape Fold Mountains. The distribution of this species extends to the

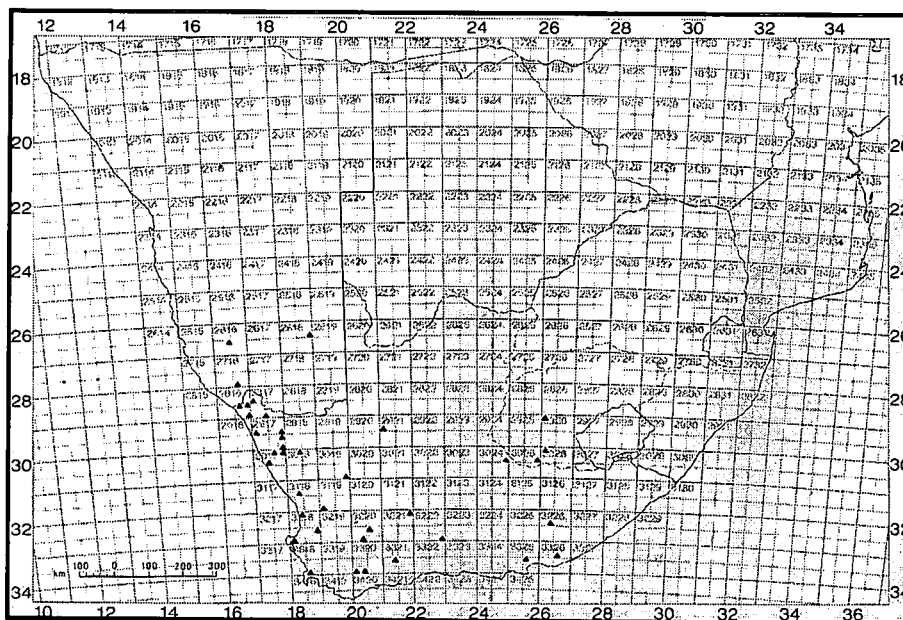


Figure 3.22 Geographical distribution of *Schismus barbatus*.

Drakensberg of the Eastern Cape and KwaZulu-Natal (Fig. 3.22). *Schismus barbatus* also inhabits parts of North Africa, the Middle East and Southwestern Asia. It is the most widely disturbed species studied in this thesis. The altitude varies from sea level to about 2200 m above sea level with an annual rainfall of between 50 - 2000 mm. Rainfall in the west is mainly in winter with a high summer rainfall component in the Drakensberg-Eastern Cape region. The soil in the Drakensberg region is often shallow, rocky and leached, derived from Karoo Sequence sediments and dolerite. In the west of this distribution area, the soil is mainly derived from sandstone of the Cape Super Group. *Schismus barbatus* flowers from winter to the beginning of summer.

Schismus inermis (Stapf) C.E. Hubb. is a wiry, tufted, tussock-forming annual, forming small tussocks of up to 0.5 m in diameter with leaves of up to 0.3 m long, arching outwards from the tuft base. The abovementioned results correlate with the observations done by Conert and Türpe (1974). *Schismus inermis* occurs in the Kamiesberg, Hantamsberge and Roggeveld. This species extends further along the Cape Fold Belt from Cape Town and Cape Agulhas to near Port Elizabeth (Fig. 3.23). The relief of this region varies between sea

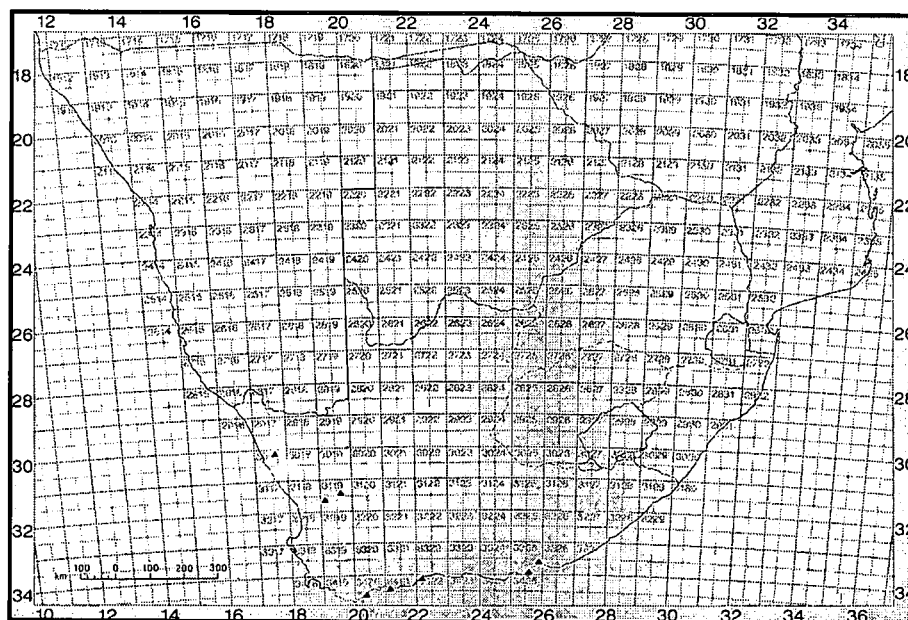


Figure 3.23 Geographical distribution of *Schismus inermis*.

level to 2200 m above sea level. This area mainly receives rain in winter and it varies between 150 - 2000 mm annually. The soil types vary from granites and gneisses that decay to form rich soils derived from sandstone of the Cape Super Group. *Schismus inermis* is frequently found on dense grassy mountain slopes and rocky areas. *Schismus inermis* flowers from the beginning of winter to the end of summer.

Schismus scaberrimus Nees is a wiry, tufted, tussock-forming perennial grass, forming small tussocks of up to 0.5 m in diameter with leaves of up to 0.2 m long, standing upright from the tuft base. This coincides with the findings of Conert and Türpe (1974). This species occurs in the area below the escarpment at elevations ranging from 0 - 600 m that represents extremely arid vegetation of the low-lying parts of Namaqualand and the Tanqua Karoo. Rainfall ranges from 50 - 200 mm per annum, occurring in winter. Granites and gneisses decay to form rich soils. In the southeast the Karoo Sequence chases and sandstone gives rise to more skeletal soils. *Schismus scaberrimus* flowers in spring.

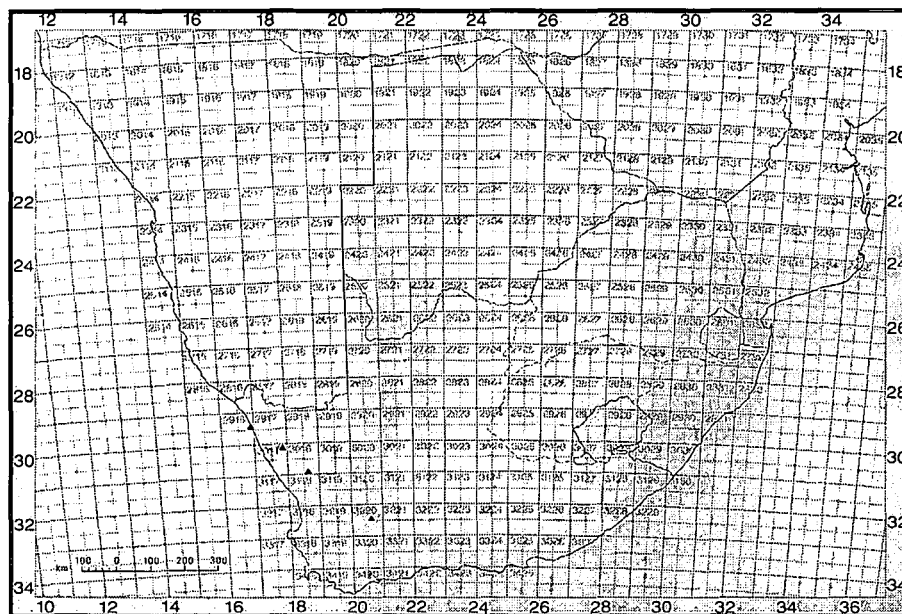


Figure 3.24 Geographical distribution of *Schismus scaberrimus*.

3.3 Cladistical analysis based on morphological characteristics

Phenetic taxonomy characters are ideally simple empirical entries. They consist solely of observation and are termed 'unit characters'. These are defined by 1:1 correspondence, and are called 'isologous'. The central role of homology determination is not made explicit, and this failing is to be overcome by using a large enough data set (Sneath & Sokal 1973).

In cladistic studies, characters are complex. They have a large theoretical content, and may be regarded as 'low-level hypotheses' (Neff 1986). The theoretical content is contained in the hypotheses of polarity and homology. There is a close link between homology, synapomorphy and cladograms. A phylogenetic tree (cladogram) is a representation of the historical course of speciation. Therefore, sufficient to follow the history of evolution on both the species and supraspecific levels of biological organization. The history of speciation might be recovered when speciation is coupled with character modification or when the rate of speciation does not proceed faster than the rate of character evolution.

The initial or primitive character state during evolution is termed plesiomorphous. There are several ways in which the plesiomorphous state can be defined. Plesiomorphous is the state that is found or has been inferred to be present in the most recent ancestor of the taxon under investigation. It could also be that state which defines a group larger than and containing the group to be characterised (Brady 1983).

The character derived from the plesiomorphous state is termed apomorphic. This occurs in one or more species in the taxon under investigation, or defines the group to be characterised (Brady 1983). Plesiomorphy and apomorphy are thus relative concepts, with the plesiomorphic conditions defined as preceding the apomorphic state in time.

This process is known as reconstructing phylogenetic (genealogical) relationships. Such a reconstruction is a hypothesis and is subjected to further rigorous testing (Wiley 1981).

3.4 Discussion

Today it is known that external morphology is frequently not a reliable indicator of phylogenetic relationships in the higher categories of classification (GPWG 2001). Visible, external plant structure however, remains the necessary basis for practical plant differentiation and identification. By necessity, morphological characteristics are and will continue to be used as the basis of species recognition (Soderstrom *et al.* 1990). Forty-one (Addendum B) characteristics were selected from the morphology data. This selection was essential, as not all the measurements were suitable for the phylogenetic analysis. Measurements like plant and spike length, for example, were so variable (genera overlapped for many of these measurements) that it was impossible to consider them for inclusion in the analysis.

The main purpose of this study was not to analyse known data but to compare other data sets with the present results to see if a clear picture could be obtained for the relationships between the genera and species studied. This was essential because some of the species in the genera are so closely related morphologically that presently it is impossible to identify some of the species in the field. As some of the species in the genera *Karoochloa* and *Merxmuellera* have been previously lumped in the genus *Danthonia* (Nees & Esenbeck 1841; Steudel 1855; Durand & Schinz 1895) before 1971, it was endeavored to determine the relationships between these genera. The genus *Schismus* was included because it had always been in close association with some of the *Danthonia* species, although only one species was included in the former genus *Danthonia* and later with some of the *Karoochloa* species (Conert & Türpe 1974). It was therefore necessary to investigate other techniques that could supply more evidence on the status of these three genera and their species.

Of the three genera studied, 64% of the species are endemic to the Cape Floral Kingdom and 96% are endemic to southern Africa. Considering *Karoochloa*, the distribution of its two perennial species is parallel to one another with some overlap in certain localities. *Karoochloa curva* is distributed from the Cape peninsula through the coastal regions, eastwards to the

Drakensberg of Lesotho, the Free State and Kwazulu-Natal provinces of South Africa. *Karoochloa purpurea* inhabits the same area but more inland parallel to the distribution of *K. curva*. These two species are thus concentrated in the moist, eastern locations of southern Africa.

Karoochloa's two annual species, *K. schismoides* and *K. tenella* also indicate an area of overlap, but inhabit areas which spread away from each other. *Karoochloa schismoides* inhabits the Northern Cape Province of South Africa and extends into the dryer Namibia, whereas *K. tenella* also inhabits the Northern Cape Province but spreads southwards into the Western Cape Province of South Africa. The cladogram separates the four species into two clades based on their annual and perennial status (Fig. 3.25 Clade 1).

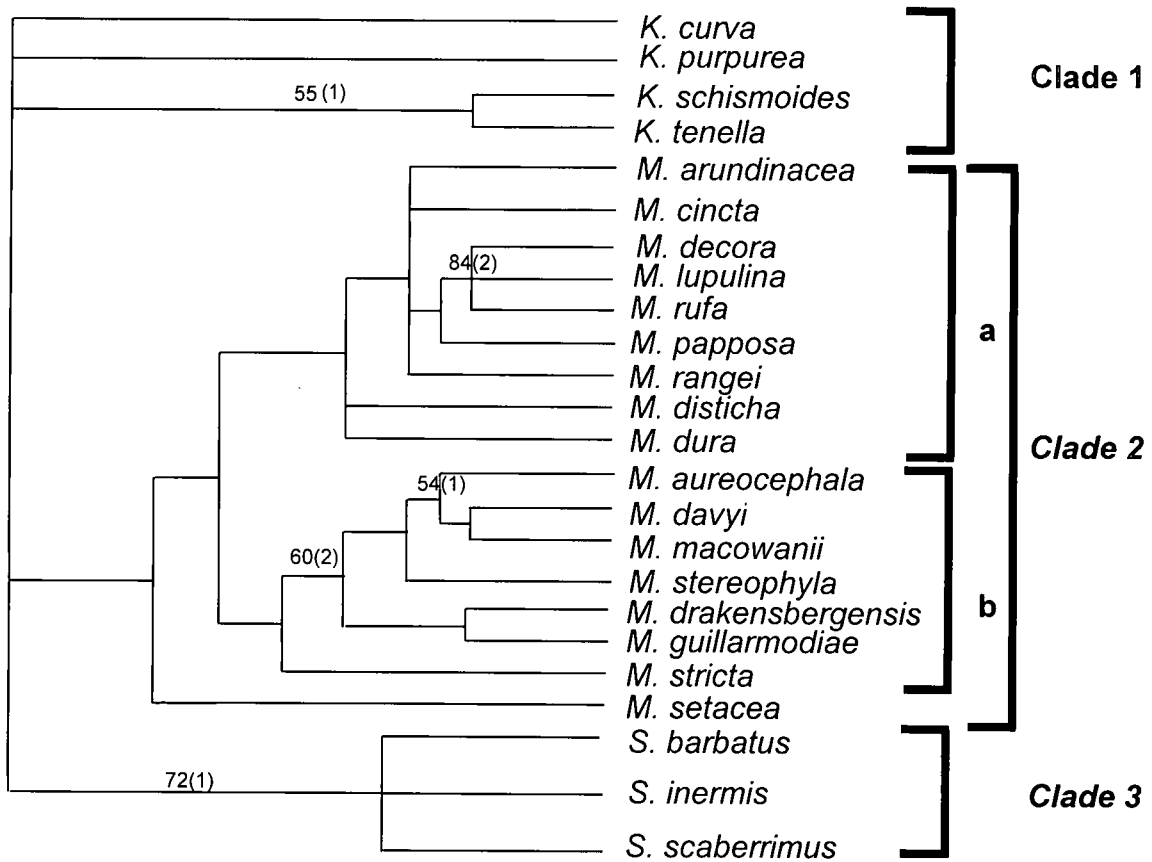


Figure 3.25. The phylogenetic relationship of 24 species of the three genera under investigation. The tree had a length of 46 and a consistency index of

0.56 (RI = 0.74 and RC = 0.42). Bootstrap values are indicated above lines and Bremer support in parenthesis.

Morphologically *Merxmuellera* shows a monophyletic origin. In the genus the species were grouped mainly into two groups. The one group includes all the species of the Cape floristic Kingdom as well as the species *M. disticha* that had a wide distribution region. This group includes the following species, namely *M. arundinacea*, *M. cincta*, *M. decora*, *M. lupulina*, *M. rufa*, *M. papposa*, *M. rangei*, *M. disticha* and *M. dura* (Fig. 3.25, Clade 2a).

The second group includes all the species in the Drakensberg region, namely *M. aureocephala*, *M. davyi*, *M. macowanii*, *M. streophyla*, *M. drakensbergensis*, *M. guillarmodiae*, and *M. stricta* (Fig. 3.25, Clade 2b).

Merxmuellera disticha and *M. stricta* had a wide distribution that links the two cores of distribution. These two species reveal similar ecological amplitudes with *M. stricta* dominating the area east of Summerset West and *M. disticha* dominating the region west of the town. It is interesting to note that these two species are incorporated into two sister clades (Fig. 3.25, Clade 2 & 3) of *Merxmuellera* and are not grouped together.

In the group of the Cape Floral Kingdom (Fig. 3.25, Clade 2) there are three species namely, *M. rufa*, *M. lupulina* and *M. decora* that are very closely related as can be seen from the terminal polytomy with a bootstrap value of 48, in the phylogram. The distance data shows that there is no difference between the three species studied (Addendum C). These three species inhabit the most southern parts of South Africa at low altitudes and require a fire stimulus to produce reproductive structures. In the Drakensberg group there are two groups of species that show a close relationship namely, *M. dayvi* and *M. macowanii* (Fig. 3.25 Clade 3) and *M. drakensbergensis* and *M. guillarmodiae* (Fig. 3.25, Clade 3). In these two cases no bootstrap support of over 50% was found. If considering the distance data there was a very small difference of 0.03 between *M. drakensbergensis* and *M. guillarmodiae*, whereas no difference was observed between *M. macowanii* and *M. davyi* and a 0.03 difference was

observed between the previous two species and *M. aureocephala* (Addendum C).

The new addition by Barker and Ellis (1991) to the genus *Merxmuellera*, namely, *M. setacea* appears to be morphologically separate (Fig 3.25 Clade 2). Although morphologically unique, it was still included in the *Merxmuellera* grouping, indicating that it is rightfully a species of the genus, although basal to the *Merxmuellera* clade. The distance data emphasizes this basal position with a slightly closer relationship to *Merxmuellera* than to *Karoochloa* (Addendum C).

The genus *Schismus* appears to also be monophyletic and the grouping is supported by a bootstrap value of 72. The species also appear morphologically similar. The distance data indicates that *S. barbatus* is closer related to *S. inermis* than to *S. scaberrimus* (Addendum C), although not enough to give branches in the phylogram.

3.5 Conclusions

Apparently the species in the genus *Karoochloa* morphologically converge, therefore the poor resolution of the phylogram. The main characteristic that divides the genus into two groups is the perennial and annual status of the species. When considering the distribution areas of these species it is necessary to test the annual status of the species *K. schismoides*. This species inhabits the most arid region of the Northern Cape Province of South Africa and Namibia although it is morphologically very similar to *K. curva* and therefore it is possible that this species is forced to be annual. To test this hypothesis, specimens of the annual species *K. schismoides* need to be grown under optimal conditions to determine whether it is annual or perennial.

In the genus *Merxmuellera* a strong grouping of the species *M. rufa*, *M. lupulina* and *M. decora* occurs. Because of the polytomy nature of this sub-clade (Fig. 3.25 Clade 2a) as well as the distance data (Addendum C), the three species may prove to be included into a single species. This is also the only species in the genus *Merxmuellera* that requires a fire stimulus to reproduce.

Merxmuellera setacea may be the species linking the two genera *Merxmuellera* and *Karoochloa* to each other. The species is very localised and therefore if the chromosomes are compatible it may be a hybrid between a *Merxmuellera* species, in this case possibly *M. rufa*, *M. decora* or *M. lupulina* and a *Karoochloa* species possibly *K. shismoides* or *K. tenella*.

If we look at the distribution areas of these species involved it is most likely that it can be a hybrid between the species *M. rufa* or *M. lupulina* and *K. tenella*.

Schismus pleuropogon may be a once off hybrid because only one specimen was collected by Stapf (1916) who did the original description and this species could not be found again at the locality indicated in the species description. No herbarium specimens were available, therefore this species was not included in this study.

CHAPTER 4

CYTOGENETIC STUDY

4.1 INTRODUCTION

The significance of chromosome numbers in grass taxonomy became apparent after the publication by Avdulov (1931). Based on the assumption that the Bamboos were primitive, Sharma (1979) proposed that $n=6$ was the ancestral basic chromosome number for grasses, and that the present variation in basic chromosome numbers was the result of polyploidy and aneuploidy.

According to Stebbins (1956) the Arundinoideae, or reed grasses, which retained the apparently primitive chromosome condition of a basic number of $x=6$ or 12, remained primitive in some other respects, such as the lack of reduction in the spikelet, and preserved a considerable diversity in epidermal and anatomical structures.

The basic chromosome number in *Danthonia* appears to be twofold. Calder (1937) observed species with 42 chromosomes and suggested that the basic number for these species is $x=7$. Other counts by the same author and also by Stebbins and Löve (1941) indicated that a basic number of $x=6$ is also present. Two species from South Africa *D. curva* Nees (= *Karoochloa curva*) and *D. disticha* Nees (= *Merxmuellera disticha*) with $2n=2x=12$ proved the presence of a basic chromosome number of $x=6$ as well.

This cytogenetic investigation was initiated to determine the chromosome numbers of several species that have never been studied before and to serve as an additional aid to the morphological data set in this study.

4.2 RESULTS AND DISCUSSION

The basic chromosome number (x) is an important criteria in the grouping of grass genera into tribes and subfamilies. This is actually or

theoretically the lowest gametic chromosome number in the species or group of related species (Rieger *et al.* 1976).

Although polyploid chromosome series exist in most large grass genera, a single basic chromosome number, with a few exceptions, exists for a genus. The great majority of grasses have chromosome numbers in multiples of $x=6$, 7, 9 or 10. Calder (1937) demonstrated that the large undefined genus *Danthonia*, of which the three genera under investigation were part of at that time, consists of species with a basic chromosome number of $x=6$ and $x=7$. Spies and Du Plessis (1988) also reported a basic chromosome number of $x=7$ for *M. dura* in the genus *Merxmuellera*. This observation is based on the investigation of one specimen with a very high ploidy level ($2n=56$).

In this thesis, numerous specimens of the genera *Karoochloa*, *Merxmuellera* and *Schismus* (Table 4.1) were examined and none indicated a basic chromosome number of $x=7$. Unfortunately no specimen of the species *M. dura* was investigated due to the lack of sufficient cytogenetic material.

Therefore, the South African specimens of the former genus *Danthonia*, have only one basic chromosome number, i.e. $x=6$. The non South African species in *Danthonia* were responsible for the second basic chromosome number of $x=7$. The chromosome count described by Spies and Du Plessis (1988) in the genus *Merxmuellera* may represent a miscount because of the high ploidy level (perhaps $2n=54$ instead of the described $2n=56$).

Table 4.1. List of *Karoochloa*, *Merxmuellera* and *Schismus* specimens studied with their somatic chromosome numbers.

VOUCHER NUMBER	2n
<i>K. curva</i>	
Spies 4518	12
<i>K. purpurea</i>	
Spies 3370, 4536 & 4542	12

<i>K. tenella</i> Spies 4350	12
<i>K. schismoides</i> Spies 2826, 2937, 2976, 3081, 3357, 3371, 3382, 4276	12
<i>M. decora</i> Spies 4407, 4458	48
<i>M. disticha</i> Spies 4751	24
<i>M. drakensbergensis</i> Spies 4676, 4687	36
<i>M. lupulina</i> Spies 4601	48
<i>M. macowanii</i> Spies 4682, 4724, 4727, 4757	48
<i>M. rufa</i> Spies 4402	48
<i>M. stricta</i> Davidse 33333, 33939, 34091, Spies 3695, 4684	24
Davidse 33347, 34110, 34121, 34028, Spies 3147, 3469, 3618, 3637, 3839, 4339, 4351	36
<i>S. barbatus</i> Davidse 34039, Spies 4237, 4277, 4278, 4285, 4289, 4215, 4366, 4523, 4524, 5284, 5342	12
<i>S. inermis</i> Davidse 33758, Spies 4575	12

S. scaberrimus

Spies 4660, 4661

12

Spies *et al.* (1992) stated that about 82 % of the grasses are polyploid. Polyploidy is usually associated with hybridisation and is recognised as an important evolutionary process in the Poaceae. Meiotic analysis of the specimens in the genera *Karoochloa*, *Merxmuellera* and *Schismus*, as well as previous literature (De Wet 1954a, 1960; Spies & Du Plessis 1986b, 1988) revealed gametic chromosome numbers of six and multiples of six (Figs. 4.1-4.3).

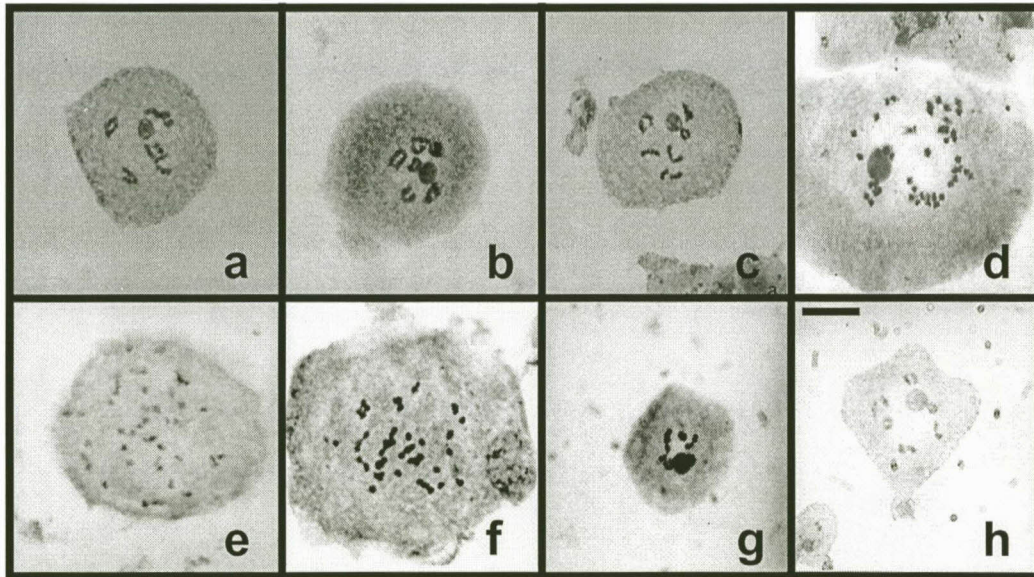


Figure 4.1. Meiotic chromosomes in *Karoochloa*, *Merxmuellera* and *Schismus*. **a.** *K. curva*, *Spies 4518*, diakinesis, $n = 6, 6_{IIR}$; **b.** *K. purpurea*, *Spies 3370*, diakinesis, $n = 6, 6_{IIR}$; **c.** *K. tenella*, *Spies 4350*, diakinesis, $n = 6, 6_{IIR}$; **d.** *M. lupulina*, *Spies 4601*, diakinesis, $n = 24$; **e.** *M. macowanii*, *Spies 4727*, diakinesis, $n = 24$; **f.** *M. rufa*, *Spies 4402*, diakinesis, $n = 24$; **g.** *S. barbatus*, *Davidse 34039*, diakinesis, $n = 6, 4_{IIR}2_{ICh}$; **h.** *S. scaberrimus*, *Spies 4661*, diakinesis, $n = 6, 4_{IIR}2_{ICh}$.

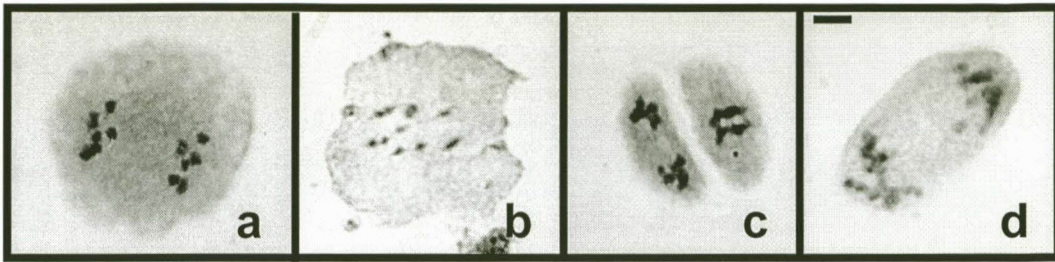


Figure 4.2. Meiotic chromosomes in *Karroochloa* and *Schismus*. **a.** *S. barbatus*, Davidse 34039, anaphase I, $n = 6$; **b.** *S. scaberrimus*, Spies 4661, anaphase I, $n = 6$; **c.** *K. purpurea*, Spies 3370, anaphase II, $n = 6$; **d.** *S. inermis*, Spies 4575, anaphase II, $n = 6$.

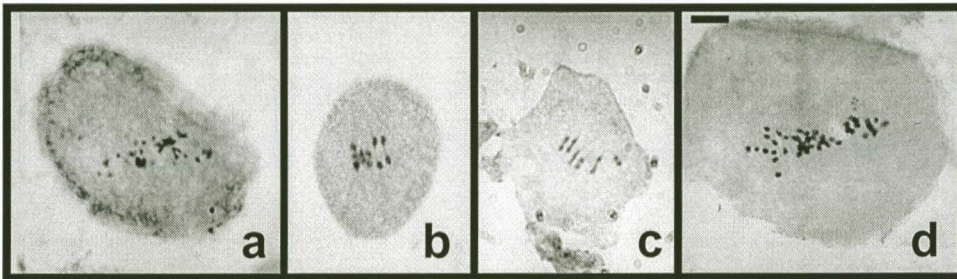


Figure 4.3. Meiotic chromosomes in *Merxmuellera* and *Schismus*. **a.** *M. macowanii*, Spies 4727, metaphase I, $n = 24$; **b.** *S. barbatus*, Spies 4523, metaphase I, $n = 6$; **c.** *S. scaberrimus*, Spies 4660, metaphase I, $n = 6$; **d.** *M. decora*, Spies 4407, metaphase I, $n = 24$.

All the specimens studied from the genera *Karroochloa* and *Schismus* were diploid, whereas the specimens of the genus *Merxmuellera* were at high ploidy levels. In addition to the high ploidy levels observed in *Merxmuellera*, two reports on diploid specimens were published, *M. arundinacea* (De Wet 1960) and *M. disticha* (De Wet 1954a). This study describes the first chromosome number reports for *M. drakensbergensis*, *M. lupulina*, *M. macowanii* and *M. rufa*.

All the specimens of *Karroochloa* and *Schismus* studied, revealed diploid chromosome numbers. This confirms previous reports but higher ploidy levels

were published previously: *K. purpurea* (Spies & Du Plessis 1986b [2n=24]; De Wet 1954a [2n=24]), *K. schismoides* (Du Plessis & Spies 1988 [2n=24]), *K. tenella* (De Wet 1960 [2n=24]), *S. barbatus* (Du Plessis and Spies 1988 [2n=24, 36]) and *S. scaberrimus* (Spies and Du Plessis 1988 [2n=24, 36]).

Higher ploidy levels were detected in some of the cells of the specimens under investigation (*M. stricta*, Spies 3637; *S. scaberrimus*, Spies 4660, *S. inermis*, Davidse 3358). This could be the result of cell fusion (Spies and van Wyk 1995), which is common in the genus *Merxmuellera* but this is a first report for the genus *Schismus*. Although numerous cells had undergone cell fusion in the genera *Merxmuellera* and *Schismus*, polyploids were only observed in the genus *Merxmuellera*, thus indicating that cell fusion may be present but does not always lead to high ploidy levels.

The species in the three genera under investigation vary from young to old polyploid complexes. Published data in conjunction with the cytogenetic data in this thesis was insufficient to determine the polyploid status of *K. curva*, *M. arundinacea*, *M. disticha* and *S. inermis*. However, *K. purpurea*, *K. tenella*, *K. schismoides*, *S. barbatus* and *S. scaberrimus* represent young polyploid complexes. In a young polyploid complex diploidy prevails, but higher levels of ploidy do occur (Grant 1981). It is important to note that a limited number of specimens were studied in some of the species. More specimens of these species should be collected to determine their polyploid complex status. Thus more specimens should be studied to verify this hypotheses.

Polyploidy prevails in the mature polyploid complexes with a few cases of diploidy. No mature polyploid complexes were observed. In old polyploid complexes only polyploidy levels are observed (Grant 1981). All the specimens studied as well as published data indicate that *M. cincta*, *M. decora*, *M. disticha*, *M. drakensbergensis*, *M. dura*, *M. lupulina*, *M. macowanii*, *M. rangei*, *M. rufa*, and *M. stricta* represent old polyploid complexes. It is important to note that a limited number of specimens were studied in some of the old polyploid species and that in these cases more specimens need to be investigated to confirm these hypotheses.

B-chromosomes were observed during this study in *K. purpurea* (Spies 2473), *M. stricta* (Spies 3637), *M. decora* (Spies 4407) and *Schismus inermis* (Spies 4575). B-chromosomes are usually smaller than the normal A-chromosome complement and do not display Mendelian inheritance. They often exhibit non-disjunction during mitotic anaphase. Therefore, within an individual, their frequencies vary from one organ to another. A study done by Bosemark (1957) showed variation in B-chromosomes, not only between flowers, but also within flowers and individual anthers. The mechanism underlying this variation is not known. It may result from the loss of the B-chromosome at some stage during early development of the panicles combined with similar events in pre-meiotic mitosis in the anthers. In great numbers B-chromosomes could reduce fertility and diminish growth. These chromosomes are not known to carry any genes with major effects (Jones & Rees 1982).

Various meiotic chromosome abnormalities were observed. Univalents were observed in some of the studied specimens *K. curva* (Spies 4518), *M. stricta* (Spies 3637) and *M. disticha* (Spies 4751). Chromosome laggards were observed in anaphase I and/or II in *M. decora* (Spies 4407), *M. disticha* (Spies 4751), *M. stricta* (Davidse 34091), *S. inermis* (Spies 4575). A few micronuclei were observed in *M. decora* (Spies 4407), *M. stricta* (Spies 3637), *M. macowanii* (Spies 4727) and *S. inermis* (Spies 4575). Anaphase I bridges were observed in *M. decora* (Spies 4407). This is most probably the result of paracentric inversion. The frequency of these abnormalities was so low that it should not affect the fertility of the specimens.

During this study embryo sacs of three *Karoochloa* (Figs. 4. 9-11), four *Merxmuellera* (Figs. 4. 12-15) and two *Schismus* (Figs. 4. 16 & 17) species were investigated.

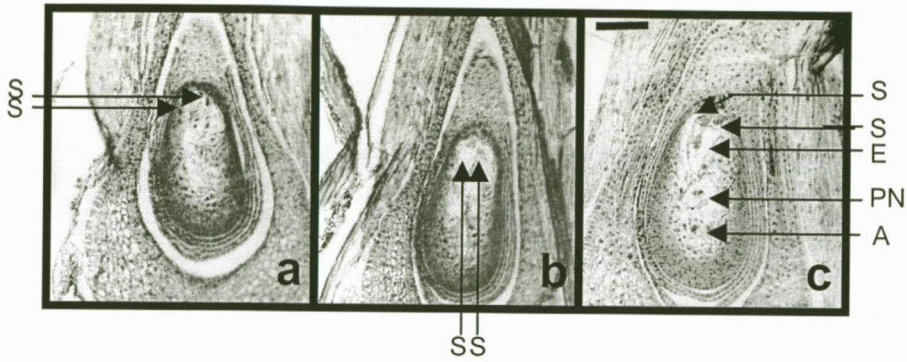


Figure 4.4. Embryo sac of *Karroochloa curva*. **a.** Two synergids (S). **b.** Two synergids (S). **c.** Mature embryo sac with two synergids (S), egg cell (E), polar nuclei (PN) and antipodals (A). *Polygonum* type embryo sac. (a-c: Spies 4518).

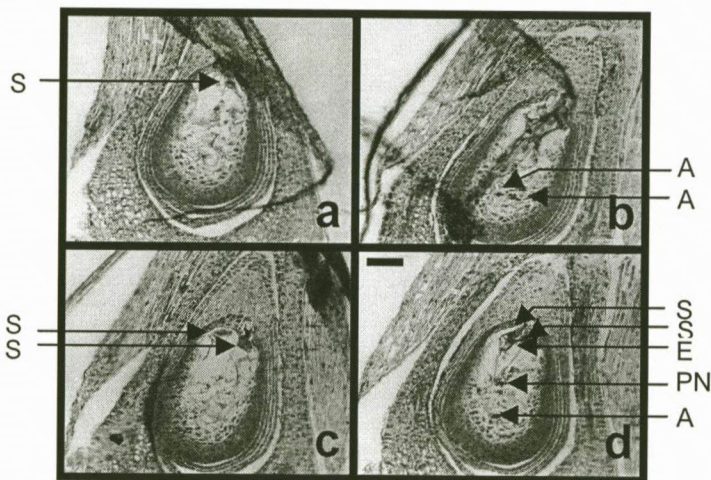


Figure 4.5. Embryo sac of *Karroochloa purpurea*. **a.** Egg cell (E). **b.** Two synergids (S). **c.** Mature embryo sac with two synergids (S), egg cell (E) and antipodal (A). **d.** Mature embryo sac with a synergid (S), polar nuclei (PN) and antipodals (A). *Polygonum* type embryo sac. (a-d: Spies 4536).

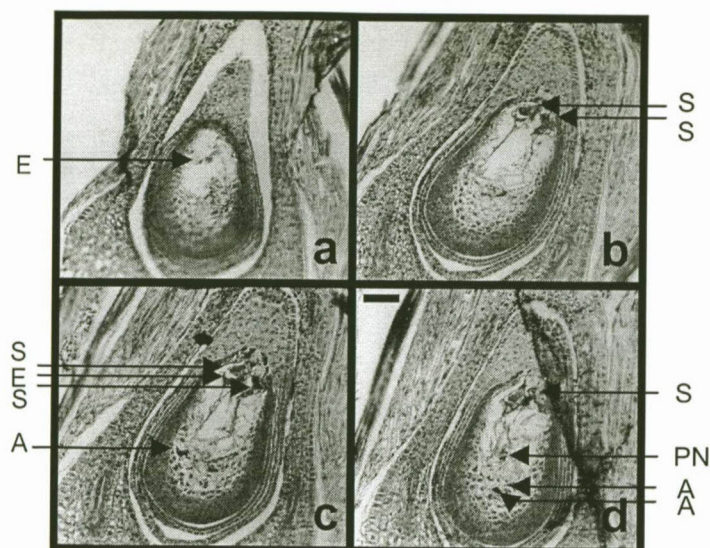


Figure 4.6. Embryo sac of *Karroochloa tenella*. **a.** Synergid (S). **b.** Two antipodals (A). **c.** Two synergids (S). **d.** Mature embryo sac with two synergids (S), egg cell (E), polar nuclei (PN) and antipodal (A). *Polygonum* type embryo sac. (a-d: Spies 4350).

All specimens studied had *Polygonum*-type embryo sacs. The only species with prominent integuments were *Karroochloa curva* (Fig 4.4), *K. tenella* (Fig 4.5), *K. schismoides* (Fig. 4.6) and *Merxmuellera disticha* (Fig 4.7). The specimens of *Karroochloa* were the only ones with both inner and outer integuments. The inner integument is shorter than the outer (Fig. 4.4 – 4.6). *Merxmuellera disticha* had only one integument (Fig. 4.7).

The ovules of *Karroochloa*, *Merxmuellera* and *Schismus* are hemi-anatropous and borne on an axile placenta. These results correspond with results obtained in the literature.

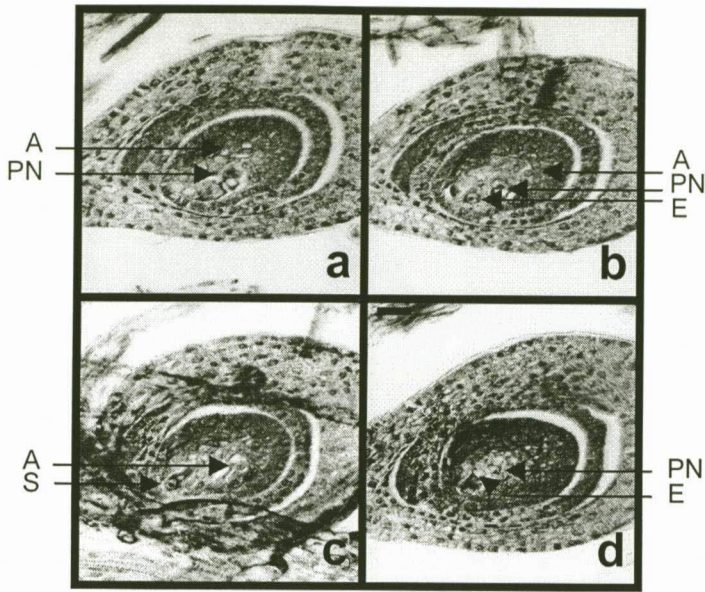


Figure 4.7. Embryo sacs of *Merxmuellera disticha*. **a.** Antipodal (A) and polar nuclei (PN). **b.** Egg cell (E), polar nuclei (PN) and antipodal (A). **c.** Synergid (S) and antipodal (A). **d.** Egg cell (E), polar nuclei (PN). *Polygonum* type embryo sac. (a-d: Spies 4751).

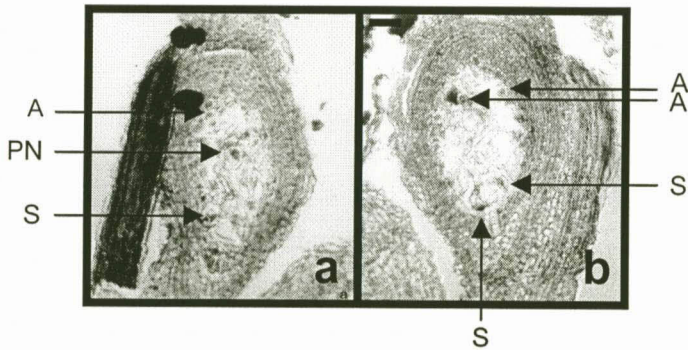


Figure 4.8. Embryo sac of *Merxmuellera drakensbergensis*. **a.** Synergid (S), polar nuclei (PN) and antipodal cell (A). **b.** Two synergids (S) and antipodal cells (A). *Polygonum* type embryo sac. (a-b: Spies 4676).

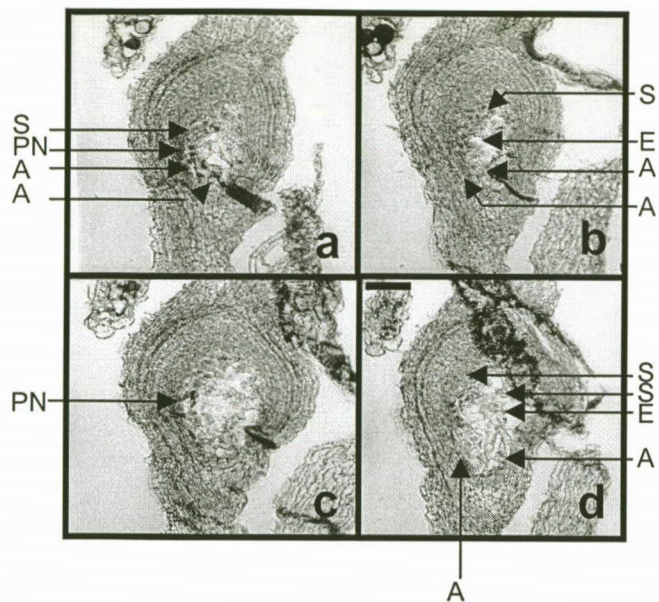


Figure 4.9. Embryo sac of *Merxmuellera macowanii*: **a.** Synergids (S), Polar nuclei (PN) and antipodal cell (A). **b.** Synergids (S), egg cell (E) and antipodal cell (A). **c.** Polar nuclei (PN). **d.** Synergid (S), egg cell (E) and antipodal cell (A). *Polygonum*-type embryo sac (a-d: Spies 4727).

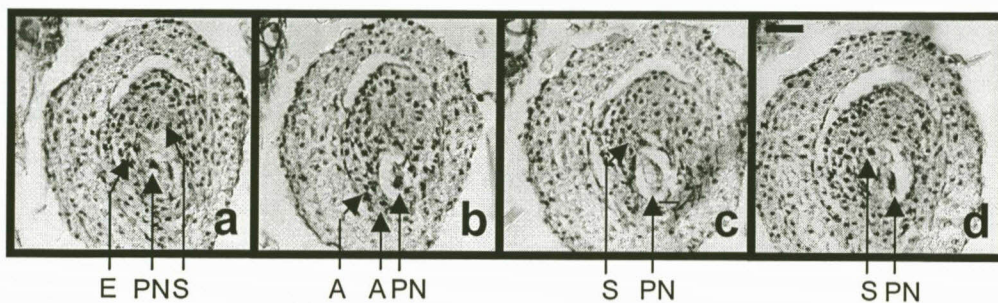


Figure 4.10. Embryo sac of *Merxmuellera rufa*: **a.** Macrospore mother cell (MM); **b.** Mature embryo sac with two synergids (S), egg cell (E), polar nuclei (PN) and antipodals (A). *Polygonum*-type embryo sac (a-b: Spies 4402).

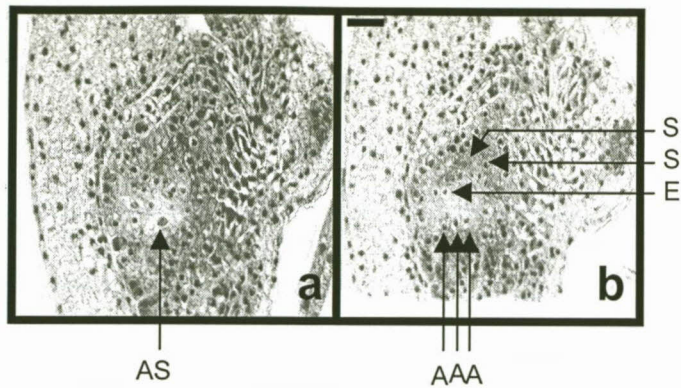


Figure 4.11. Embryo sac of *Schismus barbatus*: **a.** Archespore (AS). **b.** Three antipodal cells (A) and polar nuclei, synergids (S), egg cells (E) and polar nuclei (PN). *Polygonum*-type embryo sac (**a-b**: Davidse 34039).

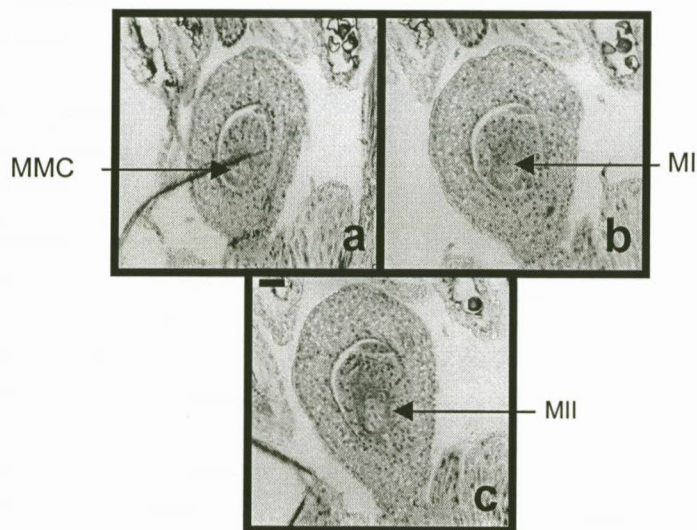


Figure 4.12. Embryo sac of *Schismus scaberrimus*: **a.** Macrospore mother cell (MMC); **b.** Meiosis I (MI); **c.** Meiosis II (MII). Indication of a *polygonum* embryo sac type. (**a-c**: Spies 4660).

Anatropy is most common among the angiosperms (Davis 1966). Two hundred and four families are exclusively anatropous (Davis 1966), whereas 20 families are either orthotropous or atropous (Bor 1978).

Embryo sac development in all the species studied indicated evidences of the monosporic type of embryo sac and results in a mature embryo sac with

eight nuclei. This form of embryo sac development represents the *Polygonum*-type (Schnarf 1929; Maheswari 1950; Johri 1965).

Although the embryo sacs were of the *Polygonum*-type, variations in embryo sac structure were still observed between the genera *Karroochloa*, *Merxmuellera* and *Schsimus*.

The embryo sac structure of the genus *Karroochloa* (Figs. 4.4-4.6) was the most conserved with a large variation in embryo sac structure observed in the genus *Merxmuellera* (Figs. 4.7-4.10). The overall size of the mature embryo sacs differed and thickened cell walls of the egg apparatus and polar nuclei were observed in some species (Fig. 4.6).

Several species of *Cortaderia* (Philipson 1978), were the first report of synergids with haustorial projections in monocotyledons. The development of synergid haustoria is age-related, although they are usually present once the "mature embryo sac" stage (Maheshwari, 1950) has been reached and persist into the early stages of embryo development. However, there is a danger that they may be overlooked in immature material. Although we studied the embryo sacs meticulously, no evidence was found of haustorial synergids as was expected by Philipson and Connor (1984) for the African danthonioid segregated genera with a west Gondwanaland history.

4.3 CONCLUSIONS

The evolutionary history of most grass genera is not simple but a highly interacting system. Most of the common grass species did not evolve from a single ancestral type, but consist of gene combinations of various widely differing ancestors (Stebbins 1956).

All plants studied had a basic chromosome number of $x=6$. Morphological similarities and dissimilarities, although useful, do not satisfactorily clarify the apparent relationship between the genera under investigation.

Hybridisation and polyploidy appears to be the driving force of evolution in the genus *Merxmuellera*.

To conclude it can be stated that:

1. All the species studied have a definite basic chromosome number of six.
2. The genus *Merxmuellera* represents an old polyploid complex, with high ploidy levels.
3. *Karroochloa* and *Schismus* represent young polyploid complexes with only diploids in this study but polyploids were reported in other studies (Spies and Du Plessis 1988).
4. Chromosomal behaviour during meiosis suggest a hybrid origin for *M. stricta* and *M. disticha*.
5. All the species studied had an anatropous ovule.
6. The embryo sacs develop from monosporic macrospores.
7. The embryo sac is, therefore, of the *Polygonum*-type.
8. The embryo sacs of *Karroochloa*, *Merxmuellera* and *Schismus* differ.
9. The conservative embryo sac structure in the genus *Karroochloa* is a further indication of the close relationship between the species of the genus as was indicated by the morphological data in chapter three. Thus it is most likely that the perennial species *K. curva* and annual *K. schismoides* as well as perennial *K. purpurea* and annual *K. tenella* must be incorporated into only two genera as was postulated in Chapter three.

The cytogenetic results reveal a complex genetic composition of the three genera under investigation, especially *Merxmuellera* with its high chromosome numbers and indications of hybridization. Cytotaxonomy should be used as a guideline and ultimately be used in conjunction with morphology, anatomy and molecular studies.

A more complete study of all the species is needed, but there are already indications that the reproductive structure is useful in the taxonomical distinction of the different genera, but not between the species investigated.

CHAPTER 5

RANDOM AMPLIFIED POLYMORPHIC DNA

5.1 INTRODUCTION

The genetic structure of plant populations reflect the interaction of different processes including long-term evolutionary history of species like shift in distribution, habitat fragmentation, mutations, genetic drift, mating systems, gene flow and selection (Slatkin 1987, Schaal *et al.* 1998).

All these factors can lead to complex genetic structuring within populations, which is often difficult to resolve. Nevertheless, the development of a number of different DNA markers has provided powerful tools for the investigation of genetic variation within a species and can facilitate understanding of such complexities (Mitton 2000).

Random amplified polymorphic DNA (RAPD) technology via the polymerase chain reaction (PCR) has fast become a means of investigating genetic diversity within and between populations and has been applied to many plant species (Nebauer *et al.* 1999, 2000).

In spite of this, the variation statistics used to estimate and partition genetic variation in natural populations cannot be applied easily to RAPD data obtained from outcrossing species because complete genotypic determination is largely hampered by their dominant nature (Isabel *et al.* 1999). During recent years several strategies have been proposed (Lynch & Milligan 1994; Apostol *et al.* 1996; Stewart & Excoffier 1996) to minimise the effect of RAPD dominance.

Although all these problems exist with the technique, RAPD's were successfully used to develop molecular markers linked to a gene controlling fruit acidity in citrus (Fang *et al.* 1997) and in the analysis of tetraploid *Elymus* species (Sun *et al.* 1997).

Landry and Lapointe(1996) tried to clarify some questions related to the application of RAPD's for phylogenetic reconstruction purposes. They found that by using more primers, stability had increased. They also indicated that at least 12 primers should be used to obtain a stable phylogeny. Their results further indicated that RAPD's should not be used to study phylogenetic relationships at higher taxonomic levels. Klopper (1996) did a preliminary study on the genus *Pentascistis* and indicated that RAPD's could have some potential in determining the phylogenetic relationships in the genus. Therefore, with these successes in mind, this technique was used in the present thesis to collect additional information on the genera studied which may help in unravelling the genera's inter and intra phylogenetic status.

RAPD data will be analysed by a cladistic framework (Borowsky *et al.* 1995), which considers each RAPD fragment as an independent character, coded as either being present or absent for every specimen. A data matrix was assembled to enable a cladistic analysis (Addendum D).

In this study the RAPD technique is used to determine the genetic variation in and between the species and to determine the phylogenetic relationships between three species of the genus *Karoochloa*, eleven species of *Merxmuellera* and three species of the genus *Schismus*.

5.2 RESULTS

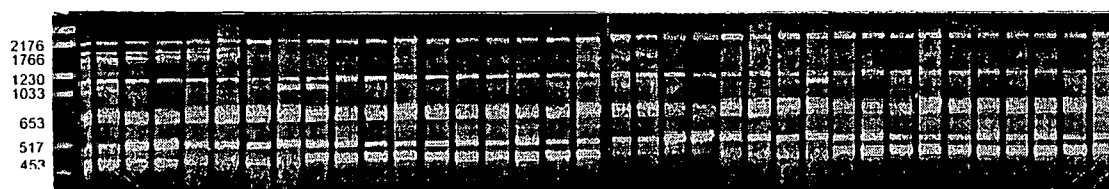
A hundred and forty primers were tested on two species of each genus to get an indication of which primers give repeatable results in these three genera. From these primers thirty-three were selected, which gave good fragment reproducibility and a simple gel profile. All the specimens for this study were then included in the final screening for suitable primers to be included in the RAPD analysis and a selection of sixteen primers (Table 5.1) were made from the thirty-three primer subset. Attention was paid to primers with simple profiles to minimize interpretation mistakes, which could influence the final analysis of the species' phylogenetic relationships.

Table 5.1 Number of fragments that show variation.

PRIMER	NUMBER OF FRAGMENTS	% OF FRAGMENTS THAT VARIED
OPA 3	11	45%
OPA 7	9	44%
OPA 9	11	45%
OPB 2	10	40%
OPB 5	10	50%
OPC 4	8	37%
OPC 5	11	54%
OPC 6	10	50%
OPC 12	9	87%
OPF 3	9	90%
OPF 4	12	83%
OPF 6	6	75%
OPF 11	12	90%
OPF 17	9	71%
OPG 2	10	83%
OPG 5	14	93%

A total of 161 discrete fragments ranging in size from 298 base pairs (bp) to 2176 bp, were amplified with the 16 primers selected. This gave an average of 9 fragments per primer per specimen.

From the 161 fragments, 65% indicated variation for the species and genera under investigation. The selected primers and their products are shown in Figs. 5.1 – 5.16).



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.1. RAPD profile obtained with OPA3. M. Molecular weight marker; 1. *K. curva*; 2. *K. purpurea*; 3. *K. schismoides*; 4. *M. arundinacea*; 5. *M. cincta*; 6. *M. decora*; 7. *M. disticha*; 8. *M. drakensbergensis*; 9. *M. dura*; 10. *M. guillarmodiae*; 11. *M. lupulina*; 12. *M. stricta* 1 (Spies 4684); 13. *M. rufa*; 14. *M.*

stereophylla; 15. *M. stricta* (Spies 4351); 16. *S. barbatus*; 17. *S. inermis*; 18. *S. scaberrimus*.

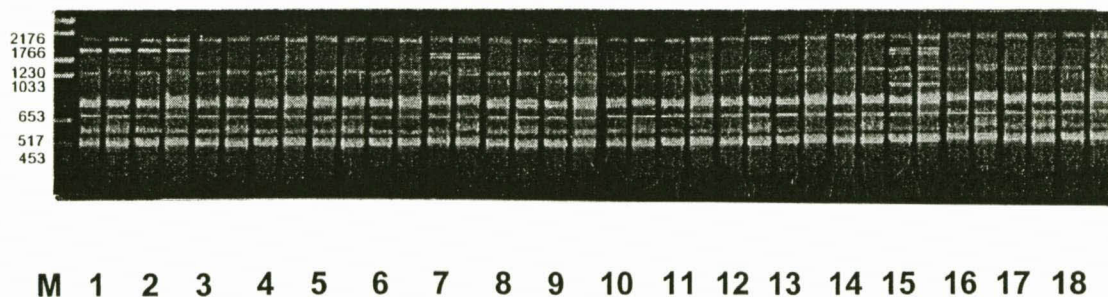


Figure 5.2. RAPD profile obtained with *OPA7*. (For legend, see Fig. 5.1).

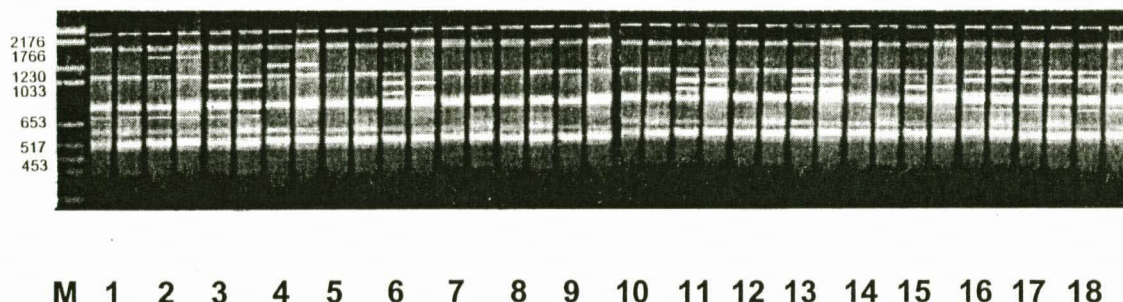


Figure 5.3. RAPD profile obtained with *OPA9*. (For legend, see Fig. 5.1).

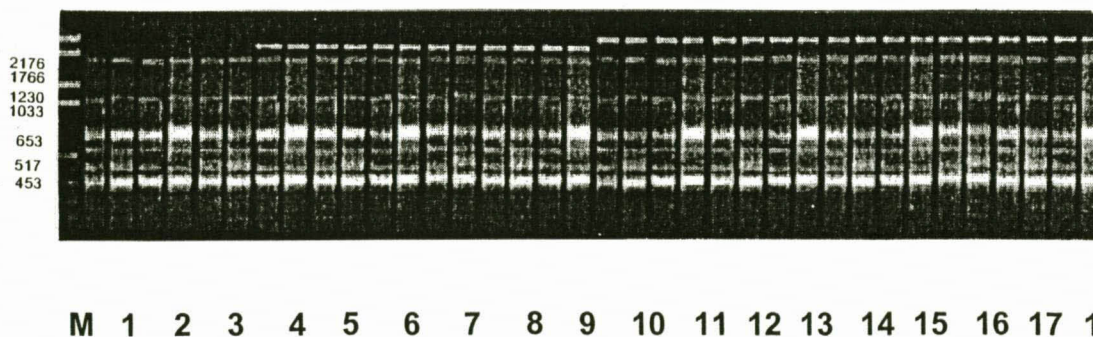
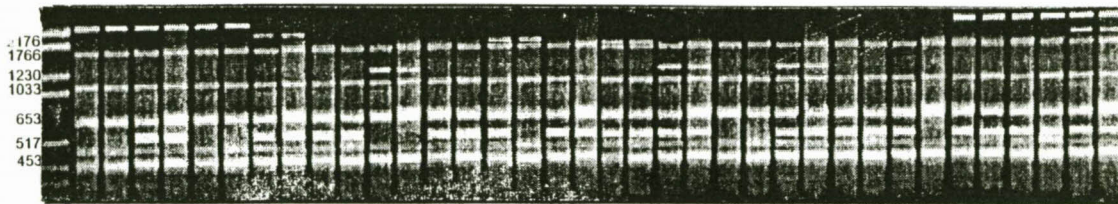
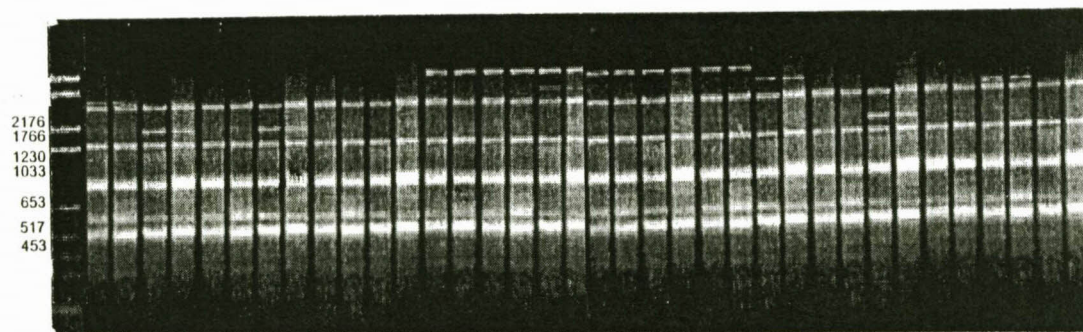


Figure 5.4. RAPD profile obtained with *OPB2*. (For legend, see Fig. 5.1).



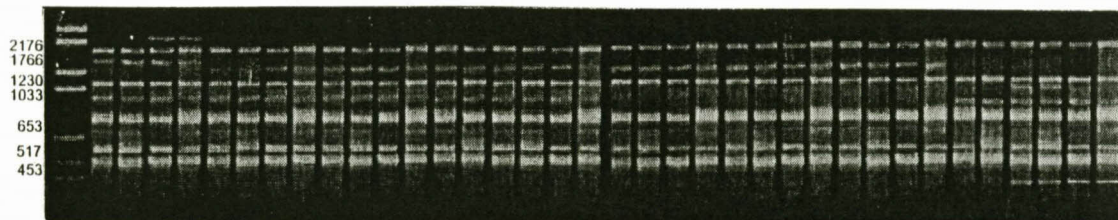
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.5. RAPD profile obtained with *OPB5*. (For legend, see Fig. 5.1).



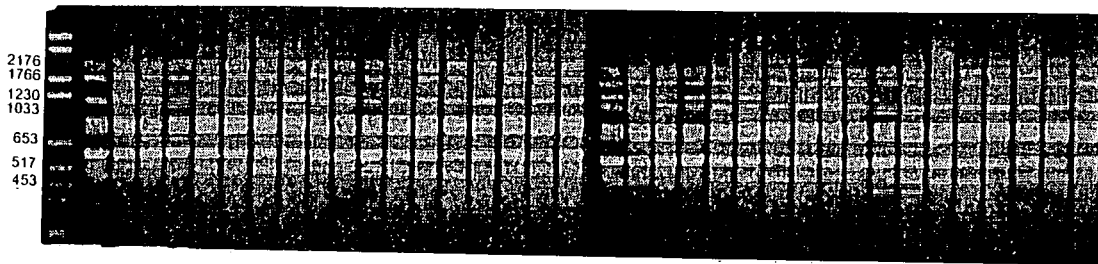
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.6. RAPD profile obtained with *OPC4*. (For legend, see Fig. 5.1).



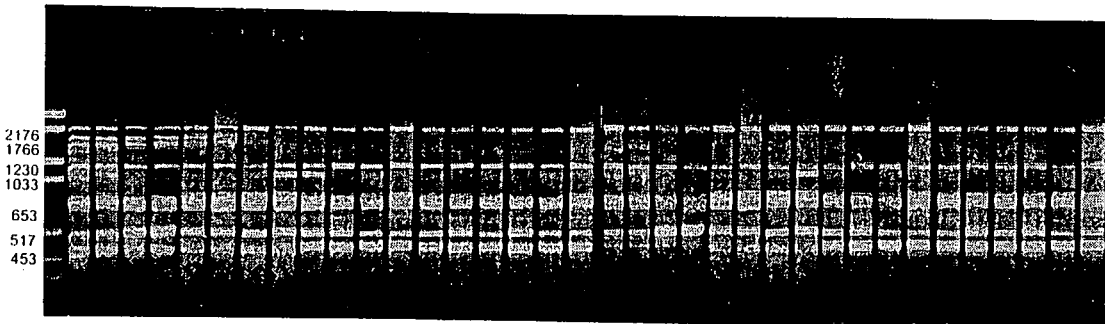
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.7. RAPD profile obtained with *OPC5*. (For legend, see Fig. 5.1).



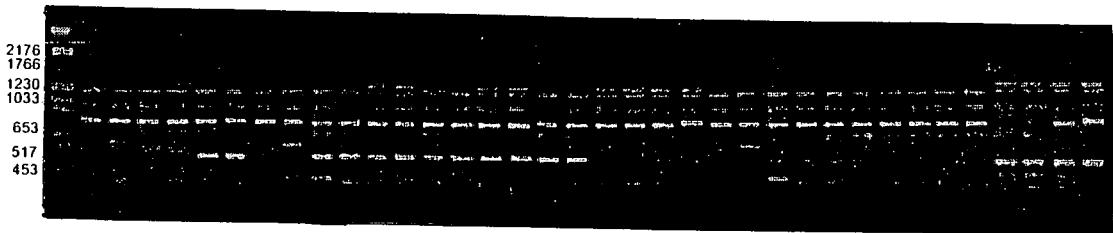
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.8. RAPD profile obtained with *OPC6*. (For legend, see Fig. 5.1).



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.9. RAPD profile obtained with *OPC12*. (For legend, see Fig. 5.1).

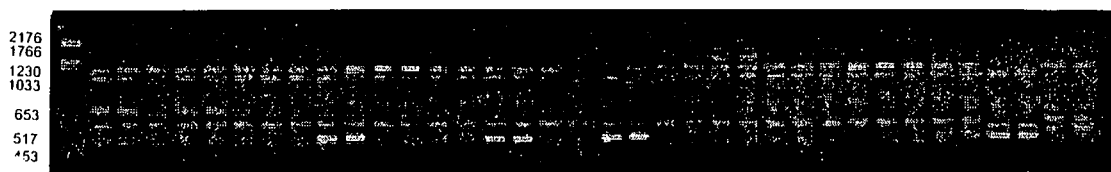


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

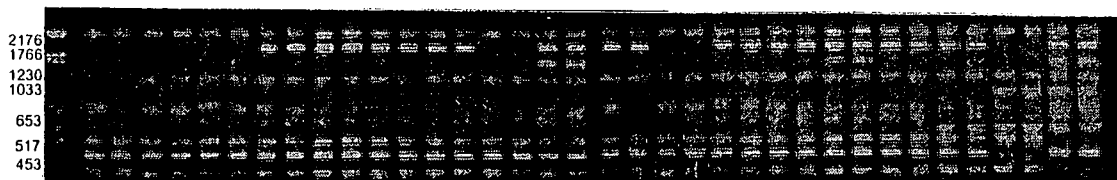
Figure 5.10. RAPD profile obtained with *OPF3*. (For legend, see Fig. 5.1).



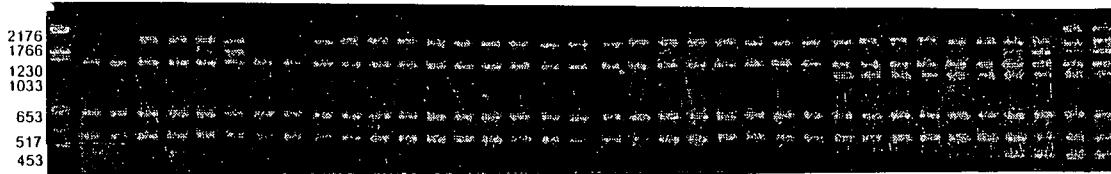
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 Figure 5.11. RAPD profile obtained with *OPF4*. (For legend, see Fig. 5.1).



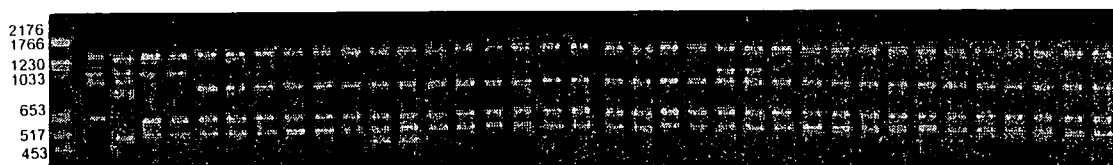
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 Figure 5.12. RAPD profile obtained with *OPF6*. (For legend, see Fig. 5.1).



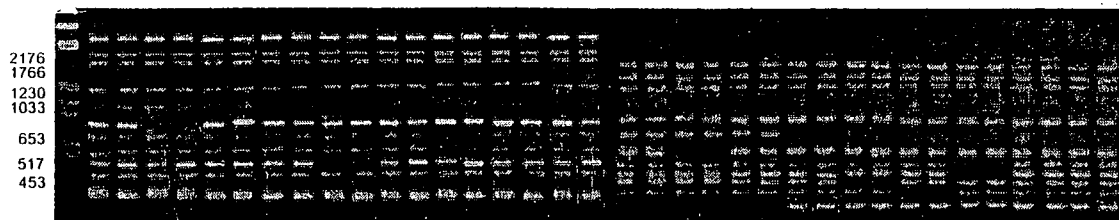
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 Figure 5.13. RAPD profile obtained with *OPF11*. (For legend, see Fig. 5.1).



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 Figure 5.14. RAPD profile obtained with *OPF17*. (For legend, see Fig. 5.1).



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 Figure 5.15. RAPD profile obtained with *OPG2*. (For legend, see Fig. 5.1).



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 Figure 5.16. RAPD profile obtained with *OPG5*. (For legend, see Fig. 5.1).

5.3 DISCUSSION

The PCR based RAPD procedure is a sensitive technique (Sambrook *et al.* 1989), and control reactions should be included. Fragments present in both the negative control reaction and the reactions containing DNA, should not be used for analysis (Yu *et al.* 1993). Some primers will give fragment patterns without DNA templates, resulting from the formation of primer multimers (Williams *et al.* 1990).

Landry and Lapointe (1997) found the interpretation of an absent RAPD fragment problematic. They indicated two ways of interpreting these results, i) either the fragment was present in an ancestral organism and it was lost or ii) the particular fragment was never present in any ancestor. Two individuals lacking one fragment as a result of shared loss should thus be considered more similar, but this case is far less likely than the second possibility. The more related the taxa, the more likely shared loss is true. RAPD analysis is thus more accurate in investigating relationships among populations of a single species or very closely related species than between less related species (Gonzalès & Ferrer 1993).

According to Landry (1996) at least twelve primers should be used to obtain a stable phylogeny. Numerous results were obtained in this study, and the stability of cladograms can be tested. Various aspects of "stability" could be tested, for example the effect of more primers (or "more data") on the resolution of the cladogram, on the tree length and on the stability of OTU's within a cladogram. Thus it is possible to determine the influence of each primer on the data set used and to test Landry's findings statistically.

Different numbers of primers were used in a series of cladograms. For example all cladograms were determined for the data obtained for only one primer. Then twenty random combinations of two primers were used to construct cladograms. This continued until the data of 16 primers were used. These results are listed in Addendum E. The average values of the following formulas were used to determine the stability of the different primer

combinations:

Resolution of the cladogram: Resolution in a cladogram is inhibited by homoplasy and this results in polytomy in consensus cladograms. The higher the degree of dichotomy in a cladogram, the more effective the resolution. In a cladogram with only dichotomous branches, the ideal resolution will be when the number of dichotomous branches equals the number of OTU's minus one (meaning all branches are dichotomous). Therefore, the formula,

$$R = D / (T - 1),$$

where R is the resolution, D is the number of dichotomous branches and T equals the number of taxa or OTU's, was derived. R represents a value between 0 and 1; the closer R is to one, the closer the cladogram is to full resolution.

The effect of the number of primers on the R-value of the cladograms indicate a linear correlation between the resolution and the number of primers used to resolve the phylogenetic relationships between the species investigated (Fig. 5.17). The number of primers influenced the resolution. Therefore care must be taken to screen a large amount of primers in the initial primer screening from which the final data set is assembled to be used in the phylogenetic analysis.

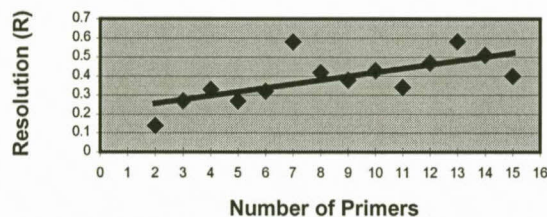


Fig. 5.17. Effect of number of primers on the resolution (R) factor (Addendum F).

Tree length: In a two-way comparison of tree lengths, the difference in the length of two cladograms (dTL) was used. However, this value may be high and the different proportions may skew a comparison of R with dTL. Therefore it was decided to use the formula,

$$L = 1 - (dTL/xTL),$$

where L represents the tree length factor and xTL represents the average tree length. Once again an L-value of 1 will represent cases where there is no difference between the lengths of two cladograms. When the L-values for the different number of primers were compared a linear increase in tree length in comparison to number of primers was found.

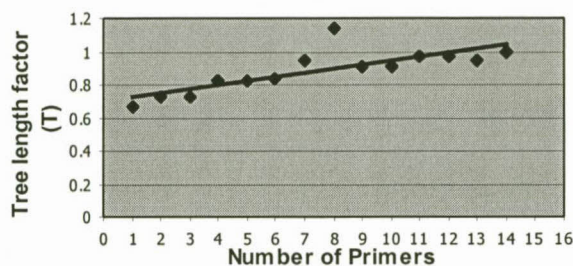


Fig. 5.18. The effect of the number of primers on the tree length factor (T) (Addendum G).

Stability of branches: One of the most difficult things to compare is the stability of a cladogram. Since stability implies that the same OTU's should be grouped together, this phenomenon should form the basis of any formula to calculate the stability of a cladogram. Therefore, the formula used was, $S = C / B$, where S represents the stability factor, C the number of corresponding branches and B the average number of branches in the two trees. Once again an S-value of 1 will represent 100% support for all branches.

A comparison of the S-values for the different numbers of primers used indicated a linear increase in stability, although the increase is very small. This

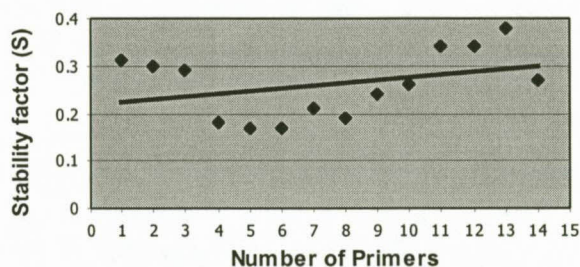


Fig. 5.19. The effect of the number of primers on the stability factor (S)

(Addendum H).

implies that the number of primers does not necessarily play a role in the stability of the cladogram but that the specific primers are also responsible for stability in this analysis.

The effect of different numbers of primers may, consequently, be determined by using the formula,

$$I = (R + L+S) / 3,$$

where I represents the total influence of the resolution (R), tree length (L) and stability (S) on the cladograms.

Although Landry's finding (that the minimum primers to be used for a good RAPD PCR analysis must be not less than twelve), could not be confirmed, there was an increase in stability and reliability with the increase in number of primers in combination. From the graph we can see that each primer combination contributes differently to the combined data set. Therefore the selection of primers is very important in the final evaluation of a RAPD PCR analysis. Thus it is necessary to screen enough primers to do a selection from. Using this equation it is possible to determine the best primer combinations from the initial screening to obtain the best results from the final RAPD PCR analysis.

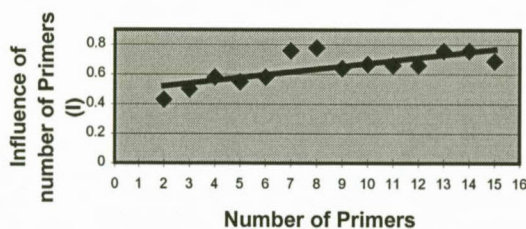


Fig. 5.20. Correlation between number of primers and influence of number of primers on the data set (Addendum I).

Nine equally parsimonious cladograms were obtained from the data matrix (Addendum H) and the consensus cladogram was computed (Fig. 5.21). The tree had a length of 192 and a consistency index of 0.604 (RI= 0.75 and

C= 0.45).

This cladogram clusters species that are closely related and the topology of the cladogram portrays the relationships among the species. The tree topology indicates a separation between the three genera *Karoochloa*,

Merxmuellera and *Schismus*. The positioning of the genera is strong with a bootstrap value of 100 for the *Karoochloa* and *Schismus* separation (Bremer support of 6) and a bootstrap value of 80 for the *Schismus* and *Merxmuellera* separation (Bremer support of 6).

The species of *Merxmuellera* cluster together in a monophyletic clade. In this clade the three *Merxmuellera* species, *M. rufa*, *M. lupulina* and *M. decora*, are clustered very strongly with a bootstrap value of 72 and Bremer support of 3. The RAPD data indicate a strong grouping of *M. drakensbergensis* and *M. guillarmodia* with a bootstrap support of 87.

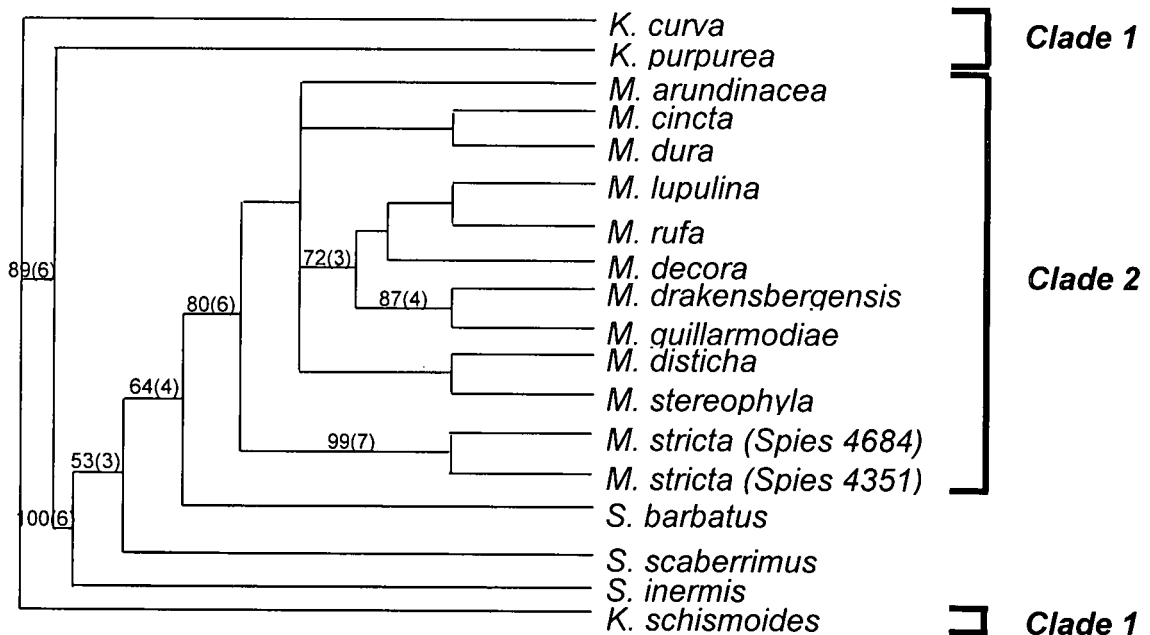


Figure 5.21. Strict consensus cladogram of RAPD data; tree length, 192; consistency index 0.604 (RI= 0.75 and RC = 0.45). Bootstrap values and Bremer support (1) are indicated on the relevant branches in the tree.

The RAPD data indicate that the two species of *Karoochloa* namely, *K. curva* and *K. schismoides*, are basal to the rest of the taxa. The three species of the genus *Schismus* lie intermediate between the two genera *Karoochloa* and *Merxmuellera*.

The mean distance data indicates that internal variations between species in the genera are largest in the genus *Schismus* with the closest relationship being that between species in the genus *Karoochloa*. The mean distances calculated between the genera also indicate that *Schismus* lies intermediate between the genera *Merxmuellera* and *Karoochloa* (Addendum J). The distance data also indicates that *Schismus* has a closer relationship with *Merxmuellera* than with *Karoochloa*.

5.4 Conclusions

With the equation developed in this chapter by using the three parameters resolution (R), tree length (L) and stability (S) of a cladogram, it was not possible to confirm Landry's findings that a minimum of twelve primers must be used in a RAPD PCR study. The equation and its parameters give a clear indication that the choice of primers is very important to get the best results from a RAPD PCR analysis. It is essential that enough primers be screened, from which the selection for the final analysis must be done.

Karoochloa curva and *K. schismoides* are successfully distinguished from the morphologically distinct *K. purpurea* (Clade 1). Therefore, RAPD data are useful for the separation of species in the genus *Karoochloa*. RAPD analysis is more accurate in investigating relationships among populations of a single species, as can be seen with the inclusion of the two *M. stricta* specimens (In Clade 2).

The strong relationship between the *Merxmuellera* species, *M. rufa*, *M. decora* and *M. lupulina* as expected from the morphological data (See chapter 3) was supported by the RAPD analysis (In Clade 2), although the RAPD

analysis shows a slightly closer relationship between *M. rufa* and *M. lupulina*. The distance data (Addendum J) reveal that the difference between *M. decora* and the other two species *M. rufa* and *M. lupulina* is small, therefore it may be that in future that these three species might be handled as one species with small morphological variation or as a polyploid complex.

The two clusterings in the genus *Merxmuellera* support the grouping of the species as in the morphological data set analysis (See Chapter 3). The clustering of *M. drakensbergensis* and *M. guillarmodiae* in the RAPD analysis was supported by a high bootstrap value of 87 (In Clade 2). The distance data shows that the differences between these two species are very small (Addendum J). The overall evaluation of the RAPD data in the case of genus *Merxmuellra* give us an indication of the genetic interrelationships between the species as observed by the Ellis (1980a, b, 1981a, b, 1982a, b, 1983a) in his studies on the leaf anatomy of the *Merxmuellera* species.

The RAPD analysis, (Fig. 5.21) as well as the distance data (Addendum J) calculated from this data set, indicates that three different species in the genus *Schismus* are being dealt with and that these species had the highest mean variation, in comparison with the species in the other two genera, under investigation. To conclude, it appears as if when a large enough primer set is tested, it is possible to obtain valuable information regarding relationships in and between the species and genera.

CHAPTER 6

SEQUENCING

6.1 INTRODUCTION

The first extensive application of molecular data to grass phylogeny was undertaken by Davis and Soreng (1993), using plastid DNA restriction site variation for thirty one taxa representing the six subfamilies of Clayton and Renvoize (1986). This study marked the beginning of molecular studies on the grass family to obtain clearer perspective of the interaction of the different taxa in this family.

Sequencing segments of the genome can reveal phylogenetic important characteristics. However, the evolution of the sequence and not that of the organism, is reflected in the phylogenetic relationships based on sequence data (Doyle *et al.* 1992).

For larger scale phylogenetic studies, DNA sequencing of slowly evolving protein coding genes or ribosomal RNAs is the more conventional approach in plants as it is in other organisms (Chase *et al.* 1993; Doyle 1993). The occurrence of rRNA throughout nature and the development of DNA sequencing for the rapid determination of the primary nucleotide sequence of rRNA molecules, makes rRNA an excellent tool for inferring evolutionary relationships (Hamby & Zimmer 1992). Hamby and Zimmer (1992) reviewed a broad rRNA survey with more than 60 sequences in the flowering plants.

Variation or the lack of variation between members of a rDNA family, is of theoretical and applied interest. Variation is observed in the internal transcribed spacers (*ITS1* and *ITS2*) and the inter-genomic spacer (*IGS*). The internal transcribed spacer (*ITS*) regions are more variable than the functional *18S*, *5.8S* and *28S* genes but they are more conserved than the *IGS* region (Jorgensen & Cluster 1988). Variation between the *18S*, *5.8S* and *28S* genes is homogenised by processes such as unequal crossing over and gene conversion, which are known collectively as molecular drive (Dover 1982).

In many angiosperm families the internal transcribed spacer regions (*ITS*) of the 18-26S nuclear ribosomal DNA (nrDNA) have proved to be a useful source of information for phylogenetic studies, at specie and generic levels (Buckler & Holtsford 1997). The *ITS1* and *ITS2* spacer regions, which occur in this region, can be amplified with the PCR technique and sequenced, using universal primers (White *et al.* 1990). In most plant groups both *ITS1* and *ITS2* give enough variation to differentiate between closely related species. Variation between *ITS* sequences is caused mainly by point mutations and to a lesser extent by insertions/deletions of nucleotide sequences (Grebenstein *et al.* 1998). Earlier alignment attempts across angiosperm families indicated that plant *ITS1* and *ITS2* have diverged further at the level of their nucleotides than the nrDNA subunits (Yokota *et al.* 1989), with the exception of the expansion segment subregions (Hassouna *et al.* 1984) or the large subunit (26S). Restriction site analysis of the nuclear DNA of closely related species has shown consistently that a high proportion of variable sites match the *ITS* region, as well as intergenic spacer (*IGS*) and external transcribed spacer (*ETS*) regions (Appels & Dvorak 1982).

Properties of the *ITS* region which make it a useful source for informative data, are the following:

- a) *ITS* region is one of the most highly repeated sequences in the plant nuclear genome.
- b) It undergoes rapid concerted evolution (Arnheim *et al.* 1980) by means of gene conversion and unequal crossing over.
- c) The small size of the *ITS* region (<700 bp in angiosperms).
- d) The presence of highly conserved sequences, flanking each of the two spacers, makes the region easily amplifiable (Baldwin *et al.* 1995).

Within the family Poaceae, *ITS* sequences have been successfully used to resolve phylogenetic relationships at the subfamilial (Hsiao *et al.* 1994, 1995a) and tribal (Hsiao *et al.* 1995b) levels.

The aim of this study was to use the information provided by the *ITS* region in determining the phylogenetic relationships within and between the three genera *Karroochloa*, *Merxmuellera* and *Schismus*.

6.2 Results

6.2.1 Sequence variation of *ITS* region.

Sequences of 23 species of the genera *Karroochloa*, *Merxmuellera* and *Schismus*, together with three out-group species *Pentaschistis aurea*, *Prionanthium ecklonii* and *Pentameris macrocalycina*, were used for phylogenetic analysis.

The internal transcribed spacer (*ITS*) length of the entire *ITS* region of the genera *Karroochloa*, *Merxmuellera* and *Schismus* varied from 366 to 479 nucleotides. The *ITS 1* region ranged from 204 to 227 base pairs (bp), and the *ITS 2* spanned 143 to 255 bp. The 5.8S sub-unit was 164 bp in length in all species. Most of the sequence variation occurred in the spacer regions (Addendum K).

The aligned sequences yielded 518 characters (Addendum K), of which 96 were variable sites and 204 were informative. The percentage guanine (G) and cytosine (C) in the 26 specimens ranged from 64% (*Schismus scaberrimus* Spies 4661; *Merxmuellera arundinacea* Spies 4684) to 71% (*M. rangei* AF019862). The average GC content was 67%.

Phylogenetic analysis of aligned *ITS* from all accessions produced nine equally parsimonious trees. In the strict consensus tree (Fig. 6.1) the accessions of *Karroochloa* and *Schismus* each forms a monophyletic group (Bootstrap support value of 74 & 100; Clades 2 and 3).

According to this data the genus *Merxmuellera* is polyphyletic, with two clades (Clades 4 & 5) grouped together and Clade 6 which is separated from clades 4 & 5, by the sister grouping of the genera *Karroochloa* and *Schismus*. The CI values indicate that over 74% of the character state changes observed in the data set, are actual synapomorphies. Bootstrap support values of more than 50% support all the clades.

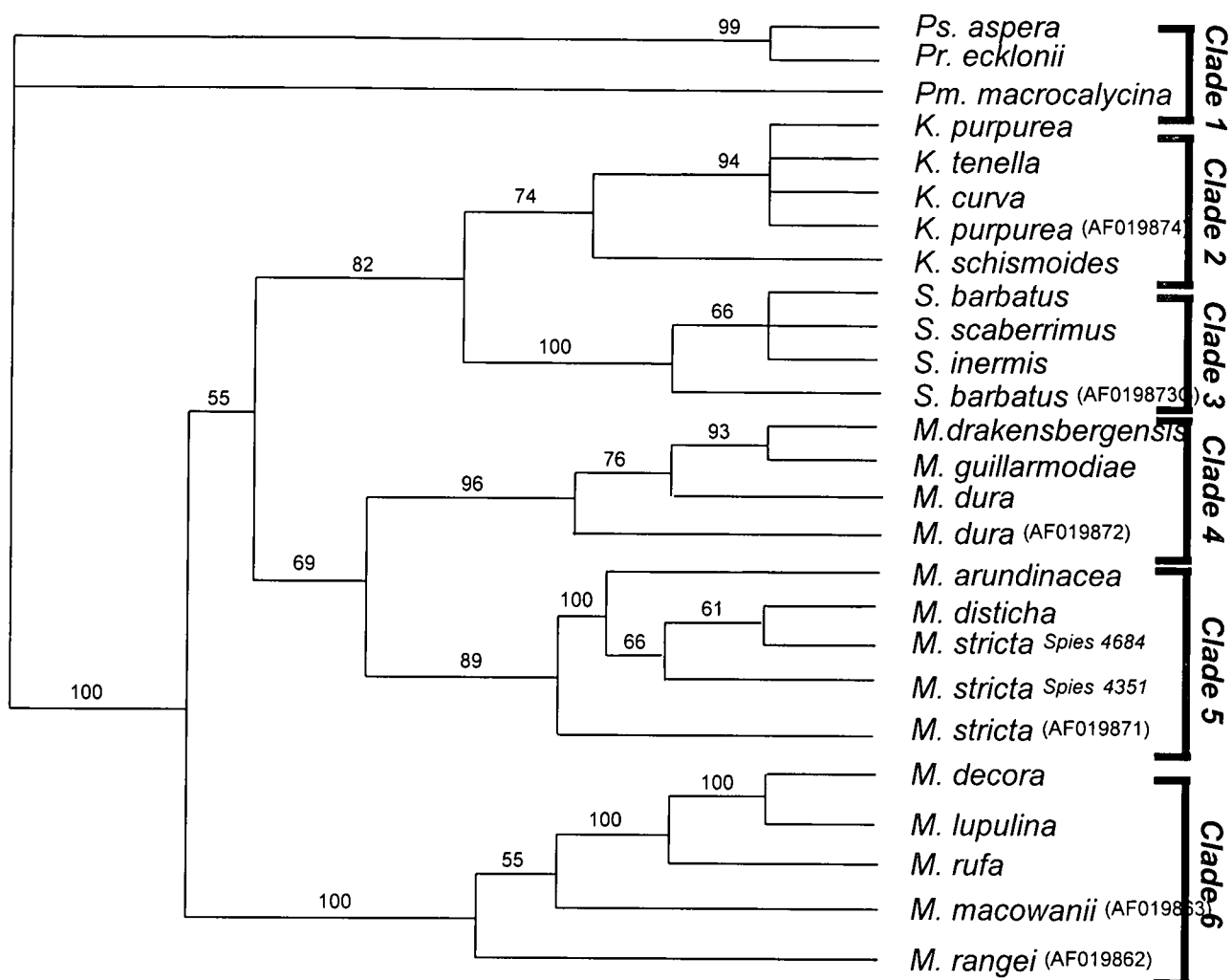


Figure 6.1 Strict consensus cladogram constructed from the nine equally parsimonious cladograms with a length of 572, CI of 0.74 and HI of 0.26. Bootstrap support values are indicated on the relevant branches in the strict consensus tree. Clade numbers are referred to in the text.

Several unique indels were observed for the genera under investigation or between species in a genus; e.g. *Karoochloa* had two unique indels at base pair 243 (10 bp) and at base pair 266 (2 bp) of the total sequence. The indel at base pair 243 also discriminates between *K. schismoides* and the rest of the *Karoochloa* species by that the indel is shorter for *K. schismoides*. *Merxmuellera* indicates three unique indels at base pair 377 (6 bp), base pair

436 (13 bp) and at base pair 449 (39 bp)(Addendum K). For *Schismus* only one unique indel of seven base pairs are recognised at base pair 243.

The genus *Merxmuellera*, *M. decora*, *M. lupulina* and *M. rufa* share four indels at base pair 25 (3 bp), base pair 97 (3 bp), base pair 114 (8 bp), base pair 180 (1 bp) and base pair 197 (4 bp) respectively. *Merxmuellera stricta* and *M. disticha* share five common indels at base pair 190 (13 bp), base pair 222 (5 bp) and base pair 231(5 bp).

6.3 DISCUSSION

Molecular phylogenies are providing new perspectives to our understanding of the origin and evolution of the grass family. Although not all DNA characters are equally informative (e.g. characters which undergo multiple changes, through mutations are less informative) it is still the most advantageous method to collect additional information of phylogenetic value on the species and genera under investigation. The uninformative characters could blur character relations and cause underestimation of branch lengths. Characters such as synonymous changes which are often found on third codon positions in protein coding genes, are expected to be less informative characters over long evolutionary periods, although they may be very useful at low levels of divergence (Doyle 1993). Källersjö *et al.* (1998) indicated that third codon positions to be phylogenetically informative in a study done on 2538 *rbcl* DNA sequences of all the major groups in the flora. Barker and Linder (1995) and Linder *et al.* (1996) also demonstrated that *rbcl* DNA sequences can be successfully applied to produce additional information on the phylogenetic status of grass species with specific reference to the sub family Arundinoideae. When these positions were not used there was a loss of resolution. The influence of the third codon positions on group positioning in a phylogenetic evaluation is much smaller in comparison to the first and second positions.

The first stage of phylogenetic analysis of DNA sequences defines the character homologies on which the whole study is based (Doyle 1993). Character homology is an absolutely critical step. Since the probabilities of

occurrence of both indels and point mutations are unknown, similarities among sequences are often maximised by adding gaps almost randomly. Options as to how the gaps should be treated range from ignoring them completely, to considering them more informative than single nucleotide changes (Lloyd & Calder 1991). In this study the indels and nucleotide substitutions were treated as equally informative.

6.3.1 *ITS* region: variation and GC content

The GC content of *ITS* regions of most grasses is higher than 50%. The average of 67% found in this study, is similar to that of other grass subfamilies (Hsiao *et al.* 1995a, 1998, 1999). The GC content of *M. rangei* (AF019862) growing in the most arid regions of southern Africa is the highest namely 71%. This coincides with the work of Salinas *et al.* (1988) who indicated by reassociation kinetics of single-stranded DNA that grasses growing in arid regions have on average a higher GC content than plants from temperate areas. Although several small gaps (1-5bp) are present in the *ITS* spacers, nucleotide substitution appears to be the main source of variability. Nucleotide substitution was the most common polymorphism observed in this spacer region and this is in agreement with the results obtained in other plant groups investigated (Baldwin *et al.* 1995). The presence of larger indels was suggested by sequence segments, which do not have any homologues in the sequences of the other taxa.

6.3.2 Phylogenetic analyses

Strict consensus tree (Fig. 6.2) computed six distinct clades. Clade 1 comprises all the out-group accessions namely *Pentaschistis aurea*, *Prionanthium ecklonii* and *Pentameris macrocalycina*.

Clade 2 comprises all the accessions of the genus *Karoochloa*. The two annuals, *K. purpurea* and *K. tenella*, as well as the perennial *K. curva* were clustered in a close relationship. The annual species *K. schismoides* that inhabits the dryer area of southern Africa is more distant from the other three

species. This separation may be due to the fact that grasses growing in arid regions have, according to Salinas *et al.* (1988), a higher GC content than grasses growing in temperate regions.

Clade 3, which is also a sister clade of clade 2, comprises all the specimens of the genus *Schismus*. This sister relationship confirms the assumption of Conert and Türpe (1964) that these two species are closely related.

In clade 4 *M. guillarmodiae* and *M. drakensbergensis* indicate a close relationship. The morphological data in Chapter four and the RAPD data in Chapter five also confirmed this close relationship.

In this analysis *M. disticha* and *M. stricta* were clustered together. These two species had the same distribution amplitudes and from both species there is cytogenetic evidence (Chapter 4) of possible hybridisation but these species differ morphologically (Chapter 3).

This data set once more confirms the close relationship between *M. rufa*, *M. lupulina* and *M. decora*, although *M. decora* and *M. lupulina* are satisfactorily separated from *M. rufa* (Bootstrap support of 100%).

6.3.3 Distance data

According to the data set of the *ITS* distances (Addendum L), *Schismus* had the least internal variation (0.02) followed by *Karoochloa* (0.06), with the biggest variation detected in *Merxmuellera* (0.19). This indicates that *Schismus* and *Karoochloa* are genetically most alike.

6.4 CONCLUSIONS

The *ITS* sequence phylogeny leads to the following conclusions:

1. *ITS* sequence data confirm the findings of studies done by Barker (1995a) and Roodt (1999) that *Merxmuellera* is polyphyletic.
2. *ITS* sequence data, as well as the distance calculation, confirm the close relationship between *Karoochloa* and *Schismus* as postulated by de Wet (1956).

3. *ITS* data further increase the possibility that *M. drakensbergensis* and *M. guillarmordiae* must be regarded as one species.

4. *ITS* data also indicates the complex genetic interaction between the three species *M.rufa*, *M.lupulina* and *M. decore* that strengthens the possibility to lump these three species into one species and rather handle them as varieties in a polyploid complex.

5. In an effort to achieve a classification which reflects evolutionary relationships, assessment of other gene regions and broader sampling of the genera under investigation is required to corroborate the *ITS* results. In addition, morphological variation between species in a genus needs to be reassessed in the light of the implications of relationships made by the molecular phylogenetic data.

CHAPTER 7

PHYLOGENY

7.1 Introduction

In order to understand relationships between and within the three genera *Karoochloa*, *Merxmuellera* and *Schismus*, several sets of data have been collected for phylogenetic analysis. These include own observations such as morphological data (Chapter 3), cytogenetic data (Chapter 4), RAPD data (Chapter 5) and molecular data (Chapter 6)

These different data sets are expected to converge onto the true species phylogeny of the group under investigation as was suggested by Miyamoto and Cracraft (1991).

To date, all the evolutionary and systematic research of the genera *Karoochloa*, *Merxmuellelra* and *Schsimus* has consisted of morphological taxonomic work with special interest in spike morphology (Conert & Türpe 1969; Conert 1971; Conert & Türpe 1974). Chromosome counts were carried outdone on a few specimens of *Karoochloa* (*K. purpurea*, De Wet 1954a, Spies & du Plessis 1986b; *K. schismoides*, du Plessis & Spies 1988; *K. tenella*, De Wet 1960), *Merxmuellera* (*M. arundinacea*, De Wet 1960; *M. disticha*, De Wet 1954a) and *Schismus* (*S. barbatus*, du Plessis & Spies 1988; *S. scaberrimus* Spies & du Plessis 1988), but no intensive cytogenetic study was conducted for the three genera under investigation. In 1994 Verboom *et al.* reported on the presence of haustorial synergids present in some specimens of the genus, *Merxmuellera*.

The only published molecular studies of phylogenetic relationships of the genera was conducted in the context of the subfamily Arundinoideae with the inclusion of specimens of a few species of the genera to determine their phylogenetic position in the subfamily Arundinoideae. These data were published by Barker *et al.* (1999) who exploit the rpoC2 sequence region and

Roodt (1999) who used the *ITS* sequence regions to establish the relationships among genera in the Arundinoideae.

7.2 Phylogenetic assessment of the species and genera investigated

7.2.1 *Karoochloa*

Of the four *Karoochloa* species the two annual species namely *K. schismoides* and *K. tenella* inhabit the western temperate region of southern Africa. The two perennial species *K. curva* and *K. purpurea* inhabit the more moderate to moist southern and eastern parts of southern Africa. According to Conert and Türpe (1969), *K. curva* and *K. schismoides* as well as *K. purpurea* and *K. tenella* are morphologically closely related. In the phylogenetic analysis of the morphology data (Fig. 3.25) this phylogenetic lineage was concealed by the annual and perennial status of the species investigated.

In the cytogenetic investigation of the genus *Karoochloa*, only diploid specimens were observed although there are reports of ploidy in the genus (*K. purpurea*, Spies & Du Plessis 1986b, [2n=24]; De Wet 1954a, [2n=24]; *K. schismoides*, Du Plessis & Spies 1988 [2n=24]; *K. tenella*, De Wet 1960 [2n=24]). This high frequency of diploids observed indicates a young polyploid complex. Therefore this is a further confirmation of the possibility that the species must be grouped together because the influence of hybridization and polyploidy had not yet played a immense role in the formation of new species.

The results obtained from the embryo sac study indicate that these species had a very conserved embryo sac structure as well as the embryo sacs all being from the sexual *Polygonum* type. These results also suggest a very close relationship between the species studied.

Although it was not possible to observe in the morphological analysis the expected morphological lineage between *K. curva* and *K. schismoides* as well as *K. purpurea* and *K. tenella*, the RAPD data confirmed the close lineage of these species. Although only three of the four species of *Karoochloa* was included in the RAPD analysis there is a clear indication of the initial finding of a

close lineage between *K. curva* and *K. schismoides* by the basal monophyletic placing of these two species. The RAPD data furthermore indicate a definite distant relationship between the former two species and the species *K. purpurea* confirming with the morphological data.

In the *ITS* sequence data set the three species *K. purpurea*, *K. tenella* and *K. curva* was included in a monophyletic clade with *K. schismoides* as a sister clade. The *ITS* data do not corroborate the RAPD findings of a close lineage between *K. curva* and *K. schismoides* although it gives a clear indication of a close lineage for the species *K. purpurea* and *K. tenella*.

Since the obvious morphological differences between *K. curva* and *K. schismoides* as well as *K. purpurea* and *K. tenella* are minimal, adaptive differences are likely to be physiological. Further experiments, using defined growth conditions, more specimens and the sequencing of more genes, will determine the phenotypic (annual, perennial) and genetic component of the species. Determination of these adaptive genetic differences will permit the future development of a comprehensive model for the evolution of these closely related taxa.

7.2.2 *Merxmuellera*

Merxmuellera are nearly cosmopolitan in distribution throughout South Africa due to the two widespread taxa *M. stricta* and *M. disticha*. *Merxmuellera* inhabits mainly the moderate to moist regions of southern Africa with the exception of *M. rangei* that occurs in the Namib Desert of Namibia.

This genus have discrete centers of taxonomic diversity, namely the Drakensberg Mountain region including the species *M. aureocephala*, *M. davyi*, *M. drakensbergensis*, *M. macowanii* and *M. stereophylla* and the Cape Floral Kingdom including the species *M. arundinacea*, *M. cincta*, *M. decora*, *M. dura*, *M. lupulina*, *M. papposa*, *M. rufa* and *M. setacea*.

Morphologically *Merxmuellera* are divided into two major sister clades (Fig 3.25, Clade 2a, 2b). Clade 2a resembles the close lineage of *M. arundinacea*, *M. cincta*, *M. decora*, *M. disticha*, *M. dura*, *M. lupulina*, *M.*

papposa, *M. rufa* and *M. rangei*. The second lineage (Fig. 3.25, Clade 2b) includes *M. aureocephala*, *M. davyi*, *M. drakensbergensis*, *M. guillarmodiae*, *M. macowanii*, *M. stereophylla* and *M. sticta* in the genus *Merxmuellera*. Only the new accession in the genus namely *M. setacea* was not successfully incorporated into one of the morphology sister clades. Therefore although included in the *Merxmuellera* clade it indicates a morphologically distant relationship to the other species of the genus *Merxmuellera*. *Merxmuellera rufa*, *M. lupulina* and *M. decora* reveal a morphologically close lineage in the Cape Floral Kingdom (Fig. 3.25, Clade 2a) to the genus under investigation (Bootstrap support of 84%). Leaf anatomical data done by Ellis (1983a) support the morphological close lineage observed. *Merxmuellera arundinacea*, *M. cincta*, *M. rangei*, *M. disticha*, and *M. dura* form a second close lineage in the Cape Floral Kingdom group of the genus *Merxmuellera*. This lineage was not support by a bootstrap value of above 50% as well as leaf anatomical data from the studies done by Ellis (1980a, 1982a, 1982b). In the Drakensberg Mountain clade (Fig. 3.25, Clade 2b) another two close morphological relationships were revealed namely the *M. davyi* and *M. macowanii* as well as the *M. drakensbergensis* and *M. guillarmodiae* lineages.

According to the distribution and habitat preferences, such relationships are possible. Ellis also noted the *M. davyi* and *M. macowanii* lineage in his leaf anatomical study (1981b). The second lineage does not corroborate the leaf anatomical findings and postulations of Ellis (1981a).

Cytogenetic studies reveal that *M. rufa*, *M. lupulina* and *M. decora* are all part of an octoploid swarm ($2n=48$). This corroborates the morphological and leaf anatomical (Ellis 1983a) findings of a close relationship between the three species investigated. This strengthens the assumption that these three species must be lumped into one species and rather be treated as varieties of a polyploid complex.

Merxmuellera disticha reveals a tetraploid chromosome number of $2n = 24$. Several chromosome abnormalities were observed that indicate that this species are of hybrid origin. These findings may explain the species successful

adaptation to a variety of habitats over a large distribution area in South Africa and Lesotho as well as the variation in leaf anatomy structure observed by Ellis (1980a).

In this study the species *M. stricta* had two ploidy levels namely $2n=24$ (tetraploid) and $2n=36$ (hexaploid). This species meiotic division was also abnormal in about 20% of the meiotic cells studied. These abnormalities are an indication of a hybrid origin for this species and thus also explain the successful adaptation of this species to different environments in South Africa and Lesotho. The abovementioned observation in the species *M. stricta* could explain the finding in the leaf anatomical study done by Ellis (1980b) where he observed different leaf anatomical types.

Sexual *Polygonum* type embryo sacs were observed for all the specimens studied in the genus *Merxmuellera*. A wide variety of structural differences were observed for the genus *Merxmuellera* in the embryo sac study. This observation correlates the high inter and intraspecific morphological and leaf anatomical variation observed in the genus *Merxmuellera*. This driving force of variation may be the result of hybridization and successive polyploidization of the species in the genus *Merxmuellera* as well as adaptive radiation of the species especially at high altitudes in the Drakensberg Mountain region. The cytogenetic results indicate that the hybrids were successful in their attempt to restore fertility, therefore no asexual apomictic embryo sacs were observed and the observed meiotic abnormalities per specimen were also too low to have an influence on pollen fertility.

The RAPD data (Chapter 7) corroborate the morphological lineage of *M. decora*, *M. rufa* and *M. lupulina*, although the RAPD data reveal a more distant relationship between *M. decora* and the other two species of the lineage. This relationship was not supported by a bootstrap value of above 50%.

The second morphological lineage that the RAPD data supports is that of *M. drakensbergensis* and *M. guillarmodiae*. According to the leaf anatomical study done by Ellis (1981a) this is an unexpected lineage. This lineage in the RAPD analysis is strongly supported by a bootstrap value of 87%.

The following lineages observed in the morphological analysis were not supported by the RAPD data analysis, namely the morphological closely related species *M. disticha*, *M. dura*, *M. arundinacea* and *M. cincta* as well as the second closely associated species *M. davyi* and *M. macowanii*. However the RAPD data reveal two new lineages namely *M. cincta* and *M. dura* as well as *M. disticha* and *M. stereophylla*. These findings also do not corroborate the leaf anatomy observations done by Ellis (1980a, 1981a). The close relationship between *M. drakensbergensis* and *M. guillarmodiae* was also supported by the ITS sequence data. The same clade construction for the RAPD data of *M. rufa*, *M. lupulina* and *M. decora* were observed in the ITS analysis, although the species genetically more distant in this analysis was not *M. decora* but *M. rufa*. In this analysis, the lineage as well as the species distant relationship are well supported by a bootstrap value of 100%.

The ITS data set only reveal one new lineage for the genus *Merxmuellera*, namely *M. disticha* and *M. stricta* (Fig.6.1, in Clade 5). This new lineage was expected if the cytogenetic evidence is taken into consideration (Chapter 4) as well as leaf anatomical evidence done by Ellis (1980a, 1980b). Investigation of Clade 5 in Figure 6.1 indicates close relationship between the two tetraploid specimens studied with a more distant relationship of the hexaploid. The gene bank introduction indicates an even more distant relationship to the previous specimens of *M. stricta* analyzed. This may be explained by the findings of Ellis (1980a, 1980b) where it was proved that more than one leaf anatomical type for these species were present. The cytogenetic data indicate hybridization that implicate the possibility that different closely related ancestors may play a role in the development of the different ploidy levels observed in the cytogenetic study of the species *M. stricta*. This may also be the case in *M. cincta* that also reveal the possibility of hybridization and polyploidy.

The ITS and cytogenetic data indicate that *M. arundinacea* with a chromosome number of $2n=12$ published by De Wet (1960) may be one of the ancestors of the *M. disticha* and *M. stricta* complex.

ITS data indicate that the octoploid ($2n=48$) *M. macowanii* is also related to the *M. rufa*, *M. lupulina* and *M. decora* lineage and therefore it is possible that these species may share an ancestor. This lineage is not supported by morphology data, leaf anatomical data (Ellis 1983a) as well as RAPD data.

The interpretations of the different data sets give a clear indication of the genetic complexity and inter relationships of the species involved in this investigation. More specimens per species as well as all species had to be included to get a better overview of the relationships of the species in the genus *Merxmullera*. There are clear indications of the incorporation of some of the species into one species and are to be handled in future as polyploid complexes rather than different species. To confirm this reduction in number of species it is necessary to sequence more genes to get a clear understanding of the genetic relationship of the species as well as to corroborate the findings of this study.

7.2.3 *Schismus*

Schismus barbatus and *S. inermis* are widely distributed throughout South Africa while *S. scaberrimus* had a more localized distribution in the dryer western parts of South Africa (Chapter 3).

Morphologically the species of *Schismus* investigated resulted in a monophyletic polytomy that indicate the these species had a close relationship.

The genus *Schismus* only reveal diploids in this study although there are reports on ploidy (*S. barbatus*, Du Plessis & Spies 1988 ($2n=24,36$); *S. scaberrimus*, Spies & Du Plessis 1988 ($2n=24,36$)).

Embryo sac structure was variable but all of the *Polygonum* embryo sac type. This was expected due to the fact that ploidy already plays a role in the evolution of the species in the genus *Schismus*.

The RAPD data reveal a strongly supported sister relationship between the three species investigated with strong bootstrap support ranging from 53% to 80% (Fig 5.21).

The *ITS* data of this species was resolved into a terminal polytomy. The distant relationship of the introduction from the gene bank AF 019873G may possibly be due to the fact that it might be of a higher ploidy levels and that hybridization might have played a role in the genetic diversity of the specimen.

The investigation of more specimens and more gene sequences are needed to prove the reliability of the relationships of the species established in this thesis.

7.2.4 Phylogeny of the genera *Karoochloa*, *Merxmuellera* and *Schismus*.

The analyses of the morphology data set reveal a basal sister relationship between the three genera under investigation.

Morphologically and distribution together with cytogenetic data consider the three genera to be closely related with specimens distributed well throughout southern Africa. Some species were more localized than others. This might be the result of introgression of specimens into more specific habitats such as the higher parts of the Drakensberg mountains and the more arid region of the Northern Cape Province and the Namib Desert of Namibia.

Phylogenetic analysis of these data identified three major lineages namely *Karoochloa*, *Merxmuellera* and *Schismus* with indications of intraspecific variation, hybridization and polyploidy in some of the species investigated of the three genera. However, relationships among these groups suggested by DNA data are different from those suggested by morphological data.

The RAPD data indicate a more clear sister relationship between the three genera with the genus *Schismus* the most closely related to both *Karoochloa* and *Merxmuellera* (Fig. 5.21).

In a more intensive study of the genera using data from the nuclear internal transcribed spacer (*ITS*) region, it was found that *Merxmuellera* is polyphyletic, with the smaller genera *Karoochloa* and *Schismus* nested within this genus as sister clades (Fig. 6.1). The polyphyletic nature of *Merxmuellera* corroborates the findings of the molecular analysis done by Barker *et al.* (1995)

and Roodt (1999) as well as the cytogenetic results on haustorial synergids done by Verboom *et al.* (1994) that also indicate a polyphyletic nature for the genus *Merxmuellera*.

The nested placing of the genera *Karoochoa* and *Schismus* in the genus *Merxmuellera* contradict the findings of Conert in 1971 that there is no genus of African grasses to which *Merxmuellera* indicates any relationship.

7.3 Conclusions

Preliminary insight has been provided for relationships among the genera *Karoochoa*, *Merxmuellera* and *Schismus* and among the species of the three genera, although both of the topics require further phylogenetic studies.

Although no formal taxonomic changes were adopted, we propose the following, based on evidence available in this thesis:

- a) In the morphology analysis, Embryo sac and RAPD study carried out on the annual status of *K. tenella* and *K. schismoides* were questioned. This is due to the fact that these two species each are very closely related to a perennial species in the same genus. The only differences between the annual and closely related perennial species are their distribution with the annual species inhabiting the arid parts of the Northern Cape Province and the perennials the more moist southern and eastern parts of Southern Africa. Therefore the annual status of the two species might be due to environmental conditions and thus they are not true annuals. This hypothesis had to be tested under controlled environmental conditions to make a final conclusion on the number of species in the genus *Karoochoa*.
- b) In the genus *Merxmuellera* there are two lineages that were strongly supported by different data sets. The first lineage that needs attention is the *M. rufa*, *M. decora* and *M. lupulina* relationship. These three species revealed a close relationship in all the data sets investigated in this study. The cytogenetic data was of high ploidy levels for all three of the species. Even the leaf anatomical data of Ellis (1983a) indicate a close lineage.

Therefore it is proposed to include these three species into one species and rather treat them as varieties of a polyploid complex than different species.

The second lineage that needs attention is the *M. drakensbergensis* and *M. guillarmodoae* relationship that is strongly supported by the RAPD and *ITS* data sets. This lineage needs more attention to uncover the true phylogeny of these two species. Due to the fact that the DNA data sets support this lineage, the initial morphological classification of these two species may be due to adaptive radiation of the species at high altitudes. Therefore a more intensive study including more specimens and a larger sampling area needs to be initiated to determine the phenotypic and genetic components of the species. Thus it might be possible in future to lump these two species as varieties into the genus *M. drakensbergensis*.

The independent analysis and interpretation of the different data sets indicates inter-relatedness of the species in the genus *Merxmuellera*. Except for the two lineages discussed previously, none of the other species had a specific placement in the genus from one data set to another. The summer rainfall *Merxmuellera* species is undoubtedly interrelated and therefore it is proposed to uphold only two species namely *M. stricta* and *M. disticha* with numerous varieties. In *M. stricta*, three species, *M. drakensbergensis*, *M. guillarmodiae* and *M. stereophylla* should be included as varieties. The same applies to *M. disticha* with which *M. macowanii*, *M. davyi*, and *M. aureocephala* should be combined as varieties.

- c) The survival of the species *S. pleuropogon* must be investigated, since the only known locality where the species is known to occur was visited and due to human disturbance no specimens were found. There was also only one specimen of this species studied and therefore it could also have been a once of hybrid that was not viable. The chance is high that this species should be declared extinct.
- d) The *ITS* sequence results support the polyphyletic nature of *Merxmuellera*, suggested by Barker (1995) and Roodt (1999). The genus *Schismus* appears to be closely related to the genus *Karoochloa* as postulated by de

Wet 1956. The close relationship revealed by ITS data of *Merxmuellera* with *Karroochloa* and *Schismus* does not corroborate the postulation of Conert 1971.

- e) Molecular data, morphological characters, biographical information and RAPD data are complimentary and are all essential for making meaningful phylogenetic inferences.

CHAPTER 8

SUMMARY

In southern Africa, the Poaceae include 194 genera and 967 species and intraspecific taxa, of which 329 are endemic, 847 indigenous and 115 naturalised (Gibbs Russell 1985).

The classification, at present, is based on non-Kranz leaf anatomy and morphology of the spikelet (Clayton & Renvoize 1986). The Arundinoideae are difficult to characterize because they are heterogeneous with numerous isolated or weakly linked genera, whose relationships are highly conjectural.

Most of the species of *Merxmuellera* and *Karoochloa* were previously part of the genus *Danthonia* (Nees & Essenbeck 1841, Steudel 1855, Durand & Schinz 1895). A number of species of *Danthonia* were recently allocated to the new genera *Karoochloa* and *Merxmuellera* (Conert & Türpe 1969, Conert 1971). For a long time, the genus *Schismus* was regarded as very closely related to *Danthonia* and more recently to the genus *Karoochloa*. One *Schismus* species was originally assigned to *Danthonia* (Conert & Türpe 1974).

The genus *Karoochloa* consists of four species, two perennials and two annuals. At present, 20 species are recognised in *Merxmuellera*, 18 are from southern Africa and two species are from the mountains of Madagascar (Barker 1994). The type species of *Schismus*, namely *S. barbatus* grows in southern Africa as well as in northern Africa and Europe. The closely related species, *S. arabicus* also occurs in the Northern Hemisphere and the other three species in this genus are all endemic to South Africa.

The high level of genetic variability allows the grasses to take advantage of new habitats (Ehrendorf 1980). In this investigation, the species *M. stricta* and *M. disticha* revealed wide distribution patterns which overlap and may indicate that these two species are of hybrid origin and, therefore, well adapted to a wide range of climatic conditions, soil types, rainfall and altitude.

In the genus *Merxmuellera*, morphologically distinct groups of species can be identified. The genera *Karoochloa* and *Schismus* form monophyletic groupings and the distance data indicates that morphologically, *Schismus* lies between *Merxmuellera* and *Karoochloa*.

Previous cytogenetic studies have indicated that the genus *Merxmuellera* has basic chromosome numbers of six and seven (Spies *et al.* 1990). In this investigation no indications of a basic chromosome number of seven was found and *Merxmuellera* is thus considered to have a basic chromosome number of six. This genus reveals high ploidy levels that indicate an old polyploid complex was dealt with. In the case of the other two genera *Karoochloa* and *Schismus* young polyploid complexes were dealt with. Only diploids were studied although there are reports on polyploids (Spies & Du Plessis 1986b, 1988).

The chromosome behaviour indicates that *M. stricta* and *M. disticha* could be of hybrid origin.

Embryo sac studies of nine species indicate that all these species are of the *Polygonum*-type and that *Merxmuellera* indicates a variety of embryo sac formations. *Karoochloa*, *Merxmuellera* and *Schismus* can be distinguished clearly on embryo sac structure.

The PCR based RAPD procedure was applied to resolve and investigate the three genera and their species. A strong relationship between the *Merxmuellera* species, *M. rufa*, *M. decora* and *M. lupulina* was clearly illustrated by the RAPD data.

The *ITS* analysis indicated conflicting clustering of some species as well as expected clustering of other species. Ultimately the following argument is applicable: gene trees may not necessarily represent species trees (Doyle 1992).

In this study the percentage lacking data in the combined analysis was too large to be used in the combined analysis. Therefore all the data sets were analysed separately and then compared to each other to make the final conclusions in this thesis.

The close relationships between *M. rufa*, *M. decora* and *M. lupulina* are evident. Therefore, it is suggested that the three species should be combined into one species and be regarded as a polyploid complex, rather than three separate species.

It is clear that all the summer rainfall *Merxmuellera* species are related and the most practical systematic treatment appears to be upholding only two species namely *M. stricta* and *M. disticha*.

The validity of the species *S. pleuropogon* must be investigated because only one specimen, the type specimen, was collected and described by Stapf (1916) and no further specimens were found. Therefore, *S. pleuropogon* may have become extinct or it may have been a once-off hybrid.

Although the genus *Karoochloa* consists of four species, the final analysis indicates that these species are very closely related and that the annual status of *K. schismoides* should be carefully investigated. Such an investigation will indicate whether *Karoochloa* includes four or three species.

Key words: *Karoochloa*, *Merxmuellera*, *Schismus*, Phylogeny.

CHAPTER 9

OPSOMMING

Die Poaceae bestaan uit 194 genera en 967 spesies en intra spesifieke taksa in suiderlike Afrika. Van hierdie spesies is 329 endemies, 847 inheems en 115 genaturaliseer (Gibbs Russell 1985).

Die klassifikasie berus tans op nie-kransige blaaranatomie en morfologie van die blompakkie (Clayton & Renvoize 1986). Die Arundinoideae is 'n moeilike groep om te identifiseer, aangesien daar verskeie geïsoleerde en swak gekoppelde genera bestaan waarvan die verwantskappe onder verdenking is.

Meeste van die spesies in die genera *Merxmuellera* en *Karroochloa* was voorheen in die genus *Danthonia* gegroep (Nees & Essenbeck 1841, Steudel 1855, Durandt & Schinz 1895). Van die spesies is of hergroep, of nuut ingesluit in die genera *Merxmuellera* en *Karroochloa* (Conert & Turpe 1969, Conert 1971). Die genus *Schismus* is deurentyd as naverwant aan die genus *Danthonia* en onlangs aan die genus *Karroochloa* beskou (Conert & Turpe 1974).

Die genus *Karroochloa* bestaan uit vier spesies waarvan twee eenjarig en twee meerjarig is. Op die oomblik is daar 20 spesies bekend in die genus *Merxmuellera* waarvan 18 spesies in suider-Afrika, en twee in die berge van Madagaskar voorkom (Barker 1994). Die tipe spesie van die genus *Schismus*, naamlik *S. barbatus* kom in suider-Afrika en in noord-Afrika sowel as Europa voor. Die naverwante spesie *S. arabicus*, kom net in die noordelike halfmond voor, terwyl daar nog drie spesies van die genus is wat endemies tot suider-Afrika is.

Die hoë vlak van genetiese variasie bevoordeel die grasse in die benutting van nuwe habitate (Ehrendorf 1980). In hierdie studie het twee spesies wat ondersoek is, nl. *M. stricta* en *M. disticha* 'n wye verspreiding getoon. Hierdie verspreiding is 'n aanduiding dat ons moontlik met basters te doen het wat goed aangepas is vir 'n wye verskeidenheid van

klimaatstoestande, grondtipes, reënvalomstandighede en verskeie hoogtes bo seespieël.

Die genus *Merxmuellera* toon verskeie morfologiese groeperings. Die genera *Karroochloa* en *Schismus* het elk 'n monofiletiese groep gevorm en die afstandsdata het aangetoon dat *Schismus* morfologies tussen die genera *Karroochloa* en *Merxmuellera* lê.

Vorige genetiese studies het aangetoon dat die genus *Merxmuellera* 'n basiese chromosoomgetal van ses en sewe het (Spies *et al.* 1990). In hierdie studie is geen aanduidings van 'n basiese chromosoomgetal van sewe gevind nie en daarom word aangeneem dat *Merxmuellera* slegs ses as basiese chromosoomgetal het. In die geval van *Karroochloa* and *Schismus* is slegs diploïedes ondersoek alhoewel daar in die verlede melding gemaak is van poliploïede in die genera (Spies & Du Plessis 1986b, 1988)

Kiemsakke van nege spesies is bestudeer en almal was van die poligonum-tipe. Die genus *Merxmuellera* het 'n verskeidenheid van kiemsakvorms aangetoon. Dit is ook uit die studie duidelik dat ons tussen die kiemsakke van *Karroochloa*, *Merxmuellera* en *Schismus* kan onderskei.

Die RAPD PCR-prosedure is van die metodes wat gebruik is om die drie genera te ondersoek. Die RAPD data het ook die noue verband tussen die spesies *M. rufa*, *M. decora* en *M. lupulina* aangetoon.

Met die *ITS* analise is teenstellende groeperings, sowel as ooreenstemmende groeperings van spesies opgemerk. Die argument van Doyle (1992) geld dus naamlik dat 'n geneboom nie noodwendig die spesieboom ondersteun nie. 'n Geneboom kan dus nie onomstootlik die evolusionêre geskiedenis van spesies bewys nie (Doyle 1993).

In die studie was afwesige data 'n te groot persentasie en dus kon die gesamentlike analise nie in die studie gebruik word nie. Die data is afsonderlik geïnterpreteer en is daarna in vergelyking met mekaar gebruik om die finale gevolgtrekkings te maak.

Vanuit die studie is dit duidelik dat die spesies *M. rufa*, *M. decora* en *M. lupulina*, naverwant is en in een spesie gekombineer behoort te word en

voortaan eerder as 'n poliploïde kompleks behandel behoort te word. Dit is ook duidelik dat die *Merxmuellera* spesies van die somerreëvalgebied almal onderling verwant is en dat hier ook 'n samevoeging behoort plaas te vind met net twee spesies *M stricta* en *M. disticha* met talle variëteite.

Daar moet ook indringend gekyk word na die geldigheid van die spesie, *S. pleuropogon*, aangesien slegs een eksemplaar deur Stapf (1916) versamel is wat die aanvanklike beskrywing gedoen het en nog geen verdere eksemplare versamel is nie. Die kans is dus goed dat die spesie uitgesterf het of 'n eenmalige baster kon gewees het.

Alhoewel die genus *Karoochloa* uit vier spesies bestaan, is die spesies baie na aan mekaar verwant en moet die spesie *K. schismoides* ondersoek word om te bepaal of hulle werklik eenjarig is al dan nie. So 'n ondersoek sal aantoon of *Karoochloa* uit vier of drie spesies bestaan.

Sleutelwoorde: Filogenie, *Karoochloa*, *Merxmuellera*, *Schismus*.

CHAPTER 10

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ADDENDUM A

List of localities and voucher herbarium numbers of specimens investigated in this study. (♣ Indicates chromosome studies, ⊗ indicates embryo sac studies, ° indicates RAPD studies, * indicates DNA sequencing studies and all the specimens listed were morphologically investigated). Grid references are presented using the degree reference system (Edwards & Leistner 1971).

Karoochloa Conert & Türpe

K. curva (Nees) Conert & Türpe

South Africa

Eastern Cape Province.

—3225 (Somerset East): Brintjieshoogte on road from Pearson to Somerset East (-CA), *Cleghorn W.B.* 3156 (STE, PRE).

—3226 (Fort Beaufort): Alice "Woodstock" (-DD), *Griffen M.H.* 172 (PRE).

—3227 (Stutterheim): King Williams Town (-CD), *Story R.* 3689 (Albany).

—3324 (Steytlerville): 25 km from Patensie to Willowmore (-DA), *Spies* 5192 (BFLU).

—3325 (Port Elizabeth): Addo park (-BD), *Liebenberg L.C.C.* 7722 (PRE); 2 km off main road to Addo (-DB), *Smook L.* 3776 (GRA, PRE); Port Elizabeth (-DC), *Dahlstrand K.A.* 1574 (GRA).

—3326 (Grahamstown): Kafferskuil river just outside Riversdale (-AB), *Germishuizen G.* 4226 (PRE); Grahamstown (-BC), *Reed J.E.* 39 (GRA); Grahamstown (-BC), *Schonland S.* 4398 (GRA).

Western Cape Province.

—3019 (Loeriesfontein): 34 km north of Loeriesfontein just after taking turn-off to Lospersplaas (-CD), *Crosby M. 833* (PRE).

—3118 (Vanrhynsdorp): Vanrhynsdorp (-DA), *Maguire B. 945* (PRE).

—3319 (Worcester): Robertson (-DA), *Van Breda 1763* (PRE).

—3320 (Montagu): 5 km from Bonnievale on road to Stormvlei (-CC), *du Toit P.C.V. 2160* (NH, PRE).

—3420 (Bredasdorp): 25 km from Swellendam to Ashton (-AB), *Spies 4518* (BFLU)*[®]*; 10 km from Heidelberg on road to Swellendam (-BB), *du Toit P.C.V. 1997, du Toit P.C.V. 1999* (NH, PRE); 20 km north-west of Witsand on road to Malgas (-BC), *Davidse 35781* (PRE); Bredasdorp (-CA), *du Toit P.C.V. 1945* (PRE, NH).

K. purpurea (L.f.) Conert & Türpe

Lesotho

—2928 (Marakabeis): Road to Mokhotlong west of Sani Pass (-CA), *du Toit P.C.V. 2237* (PRE, NH); Che-che Pass (-CA), *du Toit P.C.V. 2582, du Toit P.C.V. 2583, du Toit P.C.V. 2584* (NH).

—2929 (Underberg): Sani Pass, 3 km from border post on road to Mokhotlong (-CB), *Killick D.J.B. 4627* (PRE, NH).

South Africa

Eastern Cape Province.

—3025 (Colesberg): Plain Venterstad (-DD), *Galpin E.E. 6545* (GRA).

—3026 (Aliwal North): Vlak Plaats (-DB), *Bolus H.H. 14734* (GRA).

—**3027** (Lady Grey): On Jouberts Pass near Lady Grey (-CA), *Wenger M.J.A.* 1802 (PRE); 18 km from Barkly East to Lady Grey (-CD), *Spies* 3370 (BFLU)*; Sterkstroom (-DA), *Theron G.C.* 928 (PRE); Old Lady Grey road above Kraai river (-DC), *Hilliard O.M. & Burt B.L.* 14571 (NU).

—**3028** (Matatiele): Ongeluksnek Pass (-AD), *Hilliard O.M. & Burt B.L.* 18726 (PRE, UNH); Naude's Nek (-CA), *Strever T.* 439 (NH).

—**3126** (Queenstown): Stormberg (-AD), *Rogers F.A.* 4455 (GRA); Molteno (-AD), *Bews* 221 (NU); Buffelsfontein farm (-BC), *Ellis R.P.* 2578 (PRE); Penhoek Pass (-BC), *Spies* 2473, *Spies* 2477 (BFLU); Leeufontein-Nooitgedacht (-DD), *Malthaei* A7596 (GRA).

—**3224** (Graaff-Reinet): Graaff Reinet, Sneeuwberg (-DC), *H B* 520 (GRA).

Northern Cape Province.

—**3119** (Calvinia): 7 km from Nieuwoudtville on road to Loeriesfontein (-AC), *Barker N.P.* 9 (PRE).

—**3123** (Victoria West): Murraysburg (D/CD), *Tyson W.* 510 (NH).

Western Cape Province.

—**3119** (Calvinia): 41 km from Vanrhynsdorp to Nieuwoudtville (-AC), *Spies* 4359 (BFLU)*; 1 km from Calvinia on the road to Williston at picnic spot (-AC), *Spies* 5283 (BFLU); 61 km from Clanwilliam on road through Pakhuis pass (-CA), *Ellis R.P.* 1725 (PRE).

—**3220** (Sutherland): Wolwedans north-west of Sutherland (-AD), *Thompson M.F.* 1810 (STE, PRE); 5 km south of Sutherland on road to Matjiesfontein (-BC), *Ellis R.P.* 2470 (PRE).

—**3222** (Beaufort West): Beaufort West: Karoo National Park (-BC), *Binges B.K.* 363 (PRE).

—**3319** (Worcester): FM-tower at Matroosberg (-BC), *Spies 6244* (BFLU); 69 km from Montagu to Touwsriver (-DB), *Spies 4542* (BFLU)*.

—**3320** (Montagu): 61 km from Montagu to Touwsriver (-CD), *Spies 4536* (BFLU)*[⊗]; 48 km from Montagu to Touwsriver (-CD), *Spies 6241* (BFLU).

—**3420** (Bredasdorp): 10 km from Breede River on road to Malgas (-BC), *Ellis R.P. 1669* (PRE).

Free State.

—**2828** (Bethlehem): Golden Gate National Park (-BC), *Liebenberg L.C.C. 6964*, *Liebenberg L.C.C. 7578* (PRE).

Kwazulu-Natal.

—**2929** (Underberg): Sani Pass (-CB), *Manning J.C.*, *Hilliard O.M. & Burt B.L. 17271* (NU).

***K. schismoides* (Stapf ex Conert) Conert & Türpe**

Namibia

—**2317** (Rehoboth): Great Bushmanland (-DD), *Schlechter M. 114* (GRA).

South Africa

Eastern Cape Province.

—**3325** (Port Elizabeth): Colchester (-DB), *Raal P. 1806* (GRA).

Northern Cape Province.

—**2816** (Oranjemund): Richtersveld (-DD), *Jürgens N. 22744* (PRE): 40 km north of Port Nolloth (-DD), *Pienaar B.J. 1161* (PRE).

—**2817** (Violsdrif): Richtersveld, Eksteensfontein (-CA), *Venter H.J.T. 8047* (BFLU).

—**2917** (Springbok): Kwaganap River 36 km south-east of Port Nolloth on road to Kleinsee (-AC), *Davidse* 33276 (PRE); 14 km from Steinkopf to Port Nolloth (Aninaus Pass) (-CB), Buffels River valley, 67 km west of Springbok on road to Port Nolloth (-CB), *Spies* 2826 (PRE)*; Buffels River valley (-DA), *Davidse* 33291 (PRE); 13 km from Springbok on road to Hondeklipbaai (-DB), *Spies* 2937 (BFLU)*; 10 km east of Springbok on road to Pofadder (-DB), *Spies* 2976 (PRE)*; 2 km from Pofadder to Springbok (-DB), *Spies* 3081 (BFLU)*; 13 km from Springbok to Hondeklipbaai (-DB), *Spies* 3357 (BFLU)*; 13 km from Springbok to Hondeklipbaai (-DB), *Spies* 3371 (BFLU)*; 17 km from Springbok to Hondeklipbaai (-DB), *Spies* 3382 (BFLU)*, *Spies* 4276 (BFLU)*^o; Farm Drogedap 258, 14 km south-east of Springbok (-DD), *Bredenkamp G.J.* 2153 (NU); 28 km south of Springbok on road to Kamieskroon (-DD), *Ellis R.P.* 2203 (PRE).

—**2918** (Gamoep): 13 km south-west of Gamoep on road to Kanidood (-CD), *Davidse* 33306 (PRE).

—**3017** (Hondeklipbaai): Namaqualand, farm Roodeheuvel, 9 km west of Napier (-CD), *Reid C.* 1271 (PRE).

K. tenella (L.f.) Conert & Türp

South Africa

Northern Cape Province.

—**2917** (Springbok): Hester Malan Veld Flower Reserve (-DB), *Rösch & Le Roux* 1471 (NH).

—**2918** (Gamoep): 23 km east of Springbok on road to Pofadder (-CA), *Davidse* 33220 (PRE).

—**3018** (Kamiesberg): Loeriesfontein at junction of Nieuwoudtville Granaatbos (-CD), *Ellis R.P.* 2421 (PRE).

—3119 (Calvinia): 35 km from Vanrhynsdorp to Nieuwoudtville (-AC), *Spies 4350* (BFLU)*[®]; Vanrhyns Pass (-AC), *Spies 6290* (BFLU).

Western Cape Province.

—3118 (Vanrhynsdorp): 4 km north-east of Vanrhynsdorp on road to Nieuwoudtville (-DB), *Davidse 33384* (PRE).

—3218 (Clanwilliam): Clanwilliam district (-BB), *Esterhuizen E.E. 5779* (PRE).

—3219 (Wuppertal): Thornriver in Collibus region (-DA), *Schlechter R.10881* (GRA).

***Merxmuellera* Conert**

***M. arundinacea* (Berg) Coner**

South Africa

Eastern Cape Province.

—3226 (Fort Beaufort): 40 km from Tarkastad on road to Adelaide (-AD), *Ellis R.P. 2573* (PRE).

—3324 (Steytlerville): Kouga mountains, next to track near Graskop (-CB), *Vlok J.H.J. 1200* (PRE); 95 km from Ceres to Sederberg (-AC), *Ellis R.P. 2503* (PRE).

—3325 (Port Elizabeth): Van Stadens Mountain (-DC), *Zehner 4547* (PRE).

—3326 (Grahamstown): Bathurst (-DB), *Britten L. 1889a* (GRA).

Northern Cape Province.

—3017 (Hondekliipbaai): 10 km east from Kamieskroon in Pass (-BB), *de Winter B. 9531* (PRE).

—3018 (Kamiesberg): Kamiesberg, 63 km east of Bitterfontein on road to Kliprand (-DC), *Ellis R.P.* 2429 (PRE).

Western Cape Province.

—3118 (Vanrhynsdorp): Gifberg (-DC), *Spies* 4322 (BFLU)^{o*}; on top of Gifberg at Vanrhynsdorp (-DC), *Spies* 5330 (BFLU); on top of Gifberg at Vanrhynsdorp Between rocks (-DC), *Spies* 5335 (BFLU); road from Elizabethsfontein to Klawer between Kraaifontein and Saferberg (-DD), *Davidse* 33411 (PRE).

—3218 (Clanwilliam): 14 km from Clanwilliam in Pakhuis Pass (-BB), *Ellis R.P.* 709 (PRE); 11 km from Clanwilliam in Pakhuis Pass (-BB), *Ellis R.P.* 1149 (PRE); 10 km from Clanwilliam in Pakhuis Pass (-BB), *Ellis R.P.* 1707 (PRE); Leipoldt's Grave, Pakhuis Pass (-BB), *Taylor H.C.* 11027 (STE, PRE); Pakhuis Pass (-BB), *Spies* 6257 (BFLU); 108 km from Nieuvoordtville to Clanwilliam (-BB), *Spies* 5317 (BFLU); 2 km from Piketberg to Velddrif (-DD), *Spies* 4412 (BFLU).

—3219 (Wuppertal): Swartberg, Ceres (-BC), *Hanekom W.J.* 1396 (PRE).

—3220 (Sutherland): 25 km from Sutherland to Calvinia (-BC).

—3319 (Worcester): Tulbagh (-AC), *Welman A.M.* 726 (GRA, BOL); 26 km north of Villiersdorp on road to Worcester (-CD), *Davidse* 34130 (PRE).

—3320 (Montagu): Montagu (-CC), *Compton R.H.* 18501 (NU).

—3322 (Oudtshoorn): Perdepoort Pass (-CD), *Barker N.P.* 687 (PRE).

—3421 (Riversdale): Riversdale (-AB), *Schlechter R.* 1717 (GRA).

***M. aureocephala* (J.G. Anders) Conert**

South Africa

Kwazulu-Natal.

—**2828** (Bethlehem): Pass between Saddle and Twins (-DB), *Edwards D.* 2132 (NU); road leading to Organ Pipes Pass, Cathedral Peak forest research station (-DB), *Killick D.J.B.* 1727 (NH).

—**2929** (Underberg): Above Carter's Nek (-BC), *Hilliard O.M. & Burt B.L.* 16997 (PRE); Giants Castle Nature reserve (-CB), *Edwards D.* 2284 (PRE); Castle View Farm, 9 km North-west of farm (-CB), *Hilliard O.M. & Burt B.L.* 13544/A (NU); Garden Castle Forest Reserve, Mlambonja Valley (-CB), *Hilliard O.M. & Burt B.L.* 14932 (NU); 9 km north north-west of Castle View farm headwaters, Mlahlangubo River (-CB), *Hilliard O.M. & Burt B.L.* 15212, *Hilliard O.M. & Burt B.L.* 15241 (NU); upper tributaries south of Mkomazi Ridge (-CB), *Hilliard O.M. & Burt B.L.* 15714 (NU); south-east of Giants Castle, headwaters of Elandshoek river (-CB), *Hilliard O.M. & Burt B.L.* 16225 (NU).

M. cincta (Nees) Conert

South Africa

Eastern Cape Province.

—**3325** (Port Elizabeth): Port Elizabeth, near Sea View (DC), *Acocks J.P.H.* 21447 (PRE).

—**3326** (Grahamstown): Grahamstown (-AD), *Britten L.L.* 5875 (GRA); Witteberg (-AD), *Jacot Guillardmond* 9544 (GRA); Howieson's Poort (-AD), *Story R.* 3840 (PRE).

—**3424** (Humansdorp): 16 km from Humansdorp to Cape St. Frances (-BB), *Spies* 3504 (BFLU)*.

—**3318** (Cape Town): Tsitzikama Mountains, path from Hofmans Bosch to Kareedouw (-DD), *Britten L.* 1091 (GRA).

Western Cape Province.

—**3319** (Worcester): Cliff to Ceres town dam (-AD), *Hugo L.* 2328 (PRE).

—3219 (Wuppertal): Pakhuis Pass (-AA), *Loxton A.E.* 183 (PRE).

—3419 (Caledon): Riviersonderend Mountains near Mc Gregor (-BB), *Burgers C.J.* 82 (PRE); Tygerhoek - Riviersonderend. (-BB), *Taylor H.C.* 4492 (PRE).

***M. davyi* (C.E.Hubb) Conert**

South Africa

Mpumalanga.

—2430 (Pilgrim's Rest): Mariepskop (-DB), *Krynauw S.* 780 (PRE);

***M. decora* (Nees) Conert**

South Africa

Western Cape Province.

— 3219 (Wuppertal): 3 km from Algeria to Citrusdal (-AC), *Spies 4407* (BFLU)
o

—3320 (Montagu): 10 km from Barrydale on south side of Tradous Pass (-DC), *Barker N.P.* 34 & 36 (PRE).

—3321 (Ladismith): Garsias pass, north of old toll House (-CC), *du Toit P.V.C.2021*, *du Toit P.C.V 2026* (NH, PRE).

—3322 (Oudtshoorn): 13 km from Riversdale on Garsias Pass (-CC), *Ellis R.P.* 3543 (PRE); Robinson Pass in Outeniqua Mountains between Mosselbaai and Oudtshoorn (-CC), *Davidse 33535*, *Davidse 33536* (PRE); 5 km from George on farm Sandkraal (-CD), *Cook G.W. s.n.* (PRE).

—3419 (Caledon): 21 km from Franschoek to Villiersdorp (-AA), *Spies 4458* (BFLU)*; 5 km from Hermanus on road to Onrusrivier (-AC), *Davidse 33833* (PRE); 10 km from Napier on Road to Stanford (-BD), *Davidse 34094* (PRE);

—3420 (Bredasdorp): 8 km south from Ouplaas to De Hoop nature reserve (-AD), *Spies 4458* (BFLU)*; near gate to Witwater (-BD), *Van Wyk M. 1867* (STE, PRE).

***M. disticha* (Nees) Conert**

Lesotho

—2927 (Maseru): Thaba Putsoa (-BB), *Archibold E.E.A. 578* (GRA); Thaba Putsoa (-BB), *Archibold E.E.A. 584* (GRA); Molimo Nthuse (-DB), *Schmits M. 7310* (PRE).

—2928 (Marakabeis): 33 km north-west of Mokhotlong (-BB), *Wenger M.J.A. 1610* (PRE); Blue Mountain Pass (-BD), *du Toit P.C.V. 2572* (NH); Che-Che Pass, 12 km east of Marakabei. (-CA), *du Toit P.C.V. 2519* (PRE, NH); Pass east of Taung (-CD), *du Toit P.C.V. 2602* (NH); Sehlabathebe National Park (-DD), *Hoener F.K. 1490* (NU).

—2929 (Underberg): Mokhotlong (-AC), *Coetzee J.A. 564*, *Coetzee J.A. 834* (BFLU); Sani Pass on top of escarp (-CA), *du Toit P.C.V. 2207* (NH); sedge moorland along Sani Stream north of the Pass (-CA), *du Toit P.C.V. 2234* (NH); Sani Valley (-CA), *Hilliard O.M. & Burt B.L. 9680* (NH); Sani Valley towards Hodgson's Peak (-CA), *Hilliard O.M. & Burt B.L. 9684* (NU, NH); Sani Pass (-CB), *Killick D.J.B. 4615*, *McWebster M. sn* (NU).

South Africa

Eastern Cape Province.

—3027 (Lady Grey): Aasvoëlkrans (-AC), *Ferreira N.A. F099* (PRE); 43 km from Barkly East to Lady Grey (-CA), *Spies 4751* (BFLU)*[®]; Ben McDhui (-DB), *Hilliard O.M. & Burt B.L. 16473* (NU); Ben Mc Dhui (-DB), *Hilliard O.M. & Burt B.L. 16474* (NU); Doodman's Krans (-DC), *Galpin E.E. 6907* (NH), *Galpin E.E. 6908* (NH), *Galpin E.E. 6909* (NH).

—3028 (Matatiele): 10 km from Rhodes on the road to Naudesnek (-CC), *Spies 4665* (BFLU).

—3125 (Steynsburg): Leeuwfontein (-AC), *Archibold E.E.A. 3000* (GRA); Farm Palmskop, Steynsburg (-BB), *Retief & Germishuizen 312* (PRE); 29 km from Steynsburg to Oviston (-BD), *Spies 6140* (BFLU).

—3126 (Queenstown): Stormberg junction (-AD), *Sim T.R. 13* (GRA).

—3323 (Willowmore): 168 km from Patensie to Willowmore (-DA), *Spies 5219* (BFLU).

—3325 (Port Elizabeth): Redhouse (-DC), *Paterson J.V. 2368* (GRA).

—3326 (Grahamstown): About 9 km on Road to Cradock (-AD), *Britten L. 2979* (GRA); Cradock Road (-AD), *Schonland S. 4386* (GRA).

Western Cape Province.

—3222 (Beaufort West): Beaufort West (-BD), *Gibbs Russell, Robinson & Hermon 494* (GRA, NU); Nieweveld Mountains (-BD), *Gibbs Russel, Robinson & Herman 4201* (GRA).

—3319 (Worcester): Road to Mc Gregor, 15 km from Stormsvlei (-DD), *du Toit P.C.V. 238* (NH).

—3322 (Oudtshoorn): Bassonsrus, upper Cango Valley (-CB), *Moffett R.O. 365* (PRE, STE).

—3421 (Riversdale): 29 km from Dry River to Vermaaklikheid (-AC), *Davidse 33750* (PRE).

Kwazulu-Natal.

—2828 (Bethlehem): Mount Aux Sources (-DB), *Edwards D. 594, Edwards D. 602* (NH, NU); Organ Pipes Pass (-DB), *Edwards D. 1977* (NU); Mount Aux

Sources (-DB), *Hutchinson, Forbes & Verdoorn 112* (NH); Mount Aux Sources (-DB), *Schelpe E.A.C.L.E. 1401, Schelpe E. A.C.L.E. 1446* (NU); Cathedral Peak (-DD), *Schelpe E.A.C.L.E. 176* (NU).

—**2829** (Harrismith): Bushman's Pass, Bushman's Pass (-BC), *West O. 1715* (NH).

—**2929** (Underberg): Giants Castle Game Reserve (-AD), *Mc Allister H.J. 112* (NH); Giants Castle (-AD), *Wright F.B. 947* (NU); summit plateau of Drakensberg in vicinity of Giants Castle Pass (-AD), *Wright F.B. 1081* (NU); south east of Giants Castle (-BC), *Hilliard O.M. & Burt B.L. 16178* (NH); Garden Castle Forest Resort, valley path to Mashai Pass (-CA), *Hilliard O.M. & Burt B.L. 15018* (NU); Sani Pass to the Umkomazana River (-CB), *du Toit P.C.V. 2315* (NH); 9 km north, north-west of Castle View Farm (-CB), *Hilliard O.M. & Burt B.L. 15345* (NU); Sani Pass (-CB), *Hilliard O.M. & Burt B.L. 15513* (NU, PRE); Sani Pass near ruins of old police post (-CB), *Manning J.C., Hilliard O.M. & Burt B.L. 17259* (NH, PRE); Cathedral Peak State Forest (-CC), *Killick D.J.B. 1491* (NH); Underberg (-DA), *Liebenberg L.C.C. 5446* (NH).

M. drakensbergensis (Schweick) Conert

Lesotho

—**2927** (Maseru): Blue Mountain Pass (-BD), *Schimitz M. 6225* (PRE).

—**2928** (Marakabeis): Butha-Buthe (-CA), *Coetzee J.A. 824* (PRE); Che-che Pass. (-CA), *du Toit P.C.V. 2598* (NH, PRE).

—**2929** (Underberg): Sani Pass (-CA), *du Toit P.C.V. 2232* (NH); North of Sani Pass (-CA), *Hilliard O.M. & Burt B.L. 9658* (NU); Qachasnek (-CA), *Liebenberg L.C.C. 5747* (NH); Sehlabathebe, on top of Isenala, just outside the national park (-CC), *Hoener F.K. 2184* (PRE); Impendhle -"Tillietuellem" (-CC), *Huntly K.D. 77* (NU).

—3028 (Matatiele): Second camp above Mphaki ("Mosea") (-AA), *Archibolt E.E.A.* 3365 (GRA).

South Africa

Eastern Cape Province.

—3028 (Matatiele): 12 km from Rhodes to Naudesnek (-CC), *Spies* 4676 (BFLU)*[®]; 16 km from Rhodes to Naudesnek (-CC), *Spies* 4683 (BFLU)*[°]; 22 km from Rhodes on road to Barkley East (-CC), *Spies* 4687 (BFLU)*.

—3127 (Lady Frere): Otto du Plessis Pass, 45 km south of Barkly East (-BB), *Viljoen L.* 194 (PRE).

—3128 (Umtata): Doodmanskrans - Drakensberg (-AB), *Galpin E.E.* 6903 (PRE).

—3226 (Fort Beaufort): Amatola Mountains. Gaika's Kop (-DB), *Hilliard O.M. & Burt B.L.* 18798 (NU, PRE).

Free State.

—2828 (Bethlehem): Near Ox-Bow river campsite (-CC), *Troughton S.C.* b16 (GRA); foot of Brandwag Peek. (-BD/DD), *du Toit P.C.V.* 669 (PRE); Ox-Bow (-DC), *du Toit P.C.V.* 2675 (NU); Clefs Peak Area (-DC), *Killick & Marais* 2183 (PRE); 27 km from Ox-bow Inn on road to Letseng-la-terae (-DD), *Killick D.J.B.* 4511 (PRE).

Kwazulu-Natal.

—2828 (Bethlehem): Mount Aux Sources (-DD), *Edwards D.* 364 (NU, GRA); Giants Castle Game Reserve (-DD), *Edwards D.* 2284 (NU); Cathedral Peak (-DD), *Ellis R.P.* 3189, *Ellis R.P.* 3190, *Ellis R.P.* 1391, *Ellis R.P.* 1398 (PRE);

Cathedral Peak Resort (-DD), *Ellis R.P.* 3304 (PRE); Mount Aux Sources (-DD), *Schelpé E.* 1390 (NU).

—**2929** (Underberg): Sani Pass (-CB), *du Toit P.C.V.* 2313 (NH, PRE); Sentinel path to Mount Aux Sources (-CB), *Edwards D.* 347 (NU); Mount Aux Sources, *Edwards D.* 603 (NU); below Organ Pipes Pass (-CB), *Edwards D.* 1950 (NU); 9 km north north-west of Castle View Farm (-CB), *Hilliard O.M. & Burt B.L.* 13710, *Hilliard O.M. & Burt B.L.* 15239 (NU); Mount Aux Sources (-CB), *Hutchinson, Forbs & Verdoorn* 113 (NH); Bushman's Pass (-CB), *West O.* 1651 (NH); pass between Sani and Sehonghong (-DC), *Ruch M.* 2446 (PRE, GRA).

M. dura (Stapf) Conert

South Africa

Western Cape Province.

—**3119** (Calvinia): 15 km from Nieuwoudtville to Clanwilliam (-AC), *Spies* 5307 (BFLU); 113 km from Clanwilliam to Nieuwoudtville (-AC), *Spies* 6285 (BFLU); Nieuwoudtville Flower Reserve, just east of Nieuwoudtville (-CA), *Davidse* 33393 (PRE); Nieuwoudtville (-CA), *Ellis R.P.* 1719 (PRE); 15 km from Nieuwoudtville to Clanwilliam (-CA), *Ellis R.P.* 2455 (PRE), *Spies* 4361 (BFLU)*, *Spies* 5307 (BFLU); 19 km from Nieuwoudtville to Clanwilliam (-CA), *Spies* 5309 (BFLU).

—**3220** (Sutherland): 2.5 km from Sutherland on road to Calvinia (-BC), *Spies* 4659 (BFLU).

M. guillarmordiae Conert

Lesotho

—**2929** (Underberg): Nyiginye (-BA), *du Toit P.C.V.* 2516 (NH); Highmoor Forest Reserve (-BC), *du Toit P.C.V.* 2500, *du Toit P.C.V.* 2524 (PRE, NH); Mpendhle district. Mulangane ridge, above Carter's Nek (-BC), *Hilliard O.M. & Burt B.L.* 16965 (PRE)*; Sani Pass (-CA), *du Toit P.C.V.* 2206 (NH), *du Toit*

P.C.V. 2242 (NH, PRE); Sani Valley (-CA), *Hilliard O.M. & Burt B.L. 9670* (NH); south of Sani Pass on way to Hodson's (-CB), *du Toit P.C.V. 2268* (NH); Sehlabathebe National Park (-CC), *du Toit P.C.V. 2631*, *du Toit P.C.V. 2642* (NH).

South Africa

Eastern Cape Province.

—**3028** (Matatiele): Rams Gate (-BB), *Strever T. 1336* (NH).

—**3128** (Umtata): Base of Doodmanskranz, Drakensberg (-AB), *Galpin E.E. 6906* (NH).

Free State.

—**2829** (Harrismith): Golden Gate (-DA), *Rossouw L.F. 463* (BFLU).

Kwazulu-Natal

—**2829** (Harrismith): Cathedral Peak Forest Reserve. Organ Pipes Pass (-CC), *Smook L. 1380* (PRE, NH).

—**2929** (Underberg): Kamberg- Highmoor (-BC), *Ruddock 8* (NU).

—**3029** (Kokstad) Giants Castle Game Reserve, Newson's Cottage (-AB), *Wilcox W. 58* (NU); Ngeli, east of Dakotakop (-DA), *Abbot A. 4854* (NH).

***M. lupulina* (Nees) Conert**

South Africa

Western Cape Province.

—**3318** (Cape Town): Jonkershoek. (-DD), *Ellis R. P. 2255*, *Ellis R. P. 2256*

(PRE); Jonkershoek (-DD), *Taylor H.C. 4141* (PRE); Stellenbosh (-DD), *Taylor H.C. 5477* (PRE).

—3319 (Worcester): Du Toitskloof next to tunnel (-AC), *Spies 4601* (BFLU)^{*o*}; 8 km east of Wellington on road to Worcester (-CA), *Davidse 33923* (PRE); Worcester near waterfall (-CB), *Ecklon & Zeyher 136* (PRE); Franchhoek in mountains (-CC), *Schlechter R. 9300* (GRA).

—3419 (Caledon): Lebanon, above stream (-AA), *Kruger F.J. 199* (PRE).

***M. macowanii* (Stapf) Conert**

Lesotho

—2929 (Underberg): Selabathebe National Park (-CC), *Hoener F.K. 1843* (PRE).

—2928 (Marakabeis): Blue Mountain Pass (-BD), *du Toit P.C.V. 2573* (PRE, NH), *du Toit P.C.V. 2574* (NH).

South Africa

Eastern Cape Province.

—3027 (Lady Grey): Witteberg (-DA), *Hilliard O.M. & Burt B.L. 14577* (NU); Ben Mc Dui (-DB), *Hilliard O.M. & Burt B.L. 16559* (NU); Drakensberg summit, Doodmanskrans (-DC), *Galpin E.E. 6903* (GRA); Majuba Nek, Sterkspruit (-CA), *Hepburn J. 357* (GRA); 37 km from Rhodes via Luncheon's Nek (-DD), *Spies 4724* (BFLU)^{*}; 50 km from Rhodes via Luncheon's Nek (-DD), *Spies 4727* (BFLU)^{*o}.

—3028 (Matatiele): Rams Gate (-BB), *Strever T. 1391* (NH); 16 km from Rhodes via Luncheon's Nek (-CC), *Spies 4682* (BFLU)^{*}; 22 km from Rhodes on road to Barkley East (-CC), *Spies 4757* (BFLU)^{*}.

—3128 (Umtata): Maclear (-AB), *Story R. 476* (PRE).

—3226 (Fort Beaufort): Top of Katberg Pass (-BC), *du Toit F. (Dohne RS) A7597* (GRA).

Kwazulu-Natal.

—**2828** (Bethlehem): Mweni Pass, between Cathedral Peak and Natal National Park (-DB), *Edwards D. 843* (NU); Umlambenja river, Cathedral area (-DB), *Schelpe E. 844, Schelpe E. 906* (NU); Estcourt (-DB), *Skead D.M. 180* (NU).

—**2829** (Harrismith): Cathedral Peak, 200 m from the top of Organ Pipes (-CC), *Buthlezi C.N. 403* (NH); Cathkin Peak area (-CC) *Edwards D. 2453* (PRE, NU); 24 km from Nottingham Road on Underberg road (-CB), *Edwards D. 2673* (NU); Cathkin Peak area (-DB), *Edwards D. 2453* (NU).

—**2929** (Underberg): 12 km from Himeville to Sani Pass (-BB), *Arnold T.H. 528* (NU); Highmoor Forest Research Station (-BC), *du Toit P.C.V 2523* (PRE, NH); Kamberg area "Storm Heights" (-BC), *Hilliard O.M. & Burt B.L. 11784* (NU); Garden Castle, Pillor Cave Valley (-CA), *Hilliard O.M. & Burt B.L. 10406* (PRE); Garden castle N.R. Pillar (-CA), *Hilliard O.M. & Burt B.L. 10417* (NU); 9 km north north-west of Castle view farm, head waters of Mlahlangubo River (-CB), *Hilliard O.M. & Burt B.L. 13522* (NU); 9 km north north-west of Castle view farm, head waters of Mlahlangubo River (-CB), *Hilliard O.M. & Burt B.L. 13642* (NU); Upper tributaries south of Mkomazi (-CB), *Hilliard O.M. & Burt B.L. 15873* (PRE,NU); Cobham Forest Reserve (-CB), *Hilliard O.M. & Burt B.L. 15981* (NU); Chameleon Cave area, 8 km north of Castle View farm (-CB), *Hilliard O.M. & Burt B.L. 17869* (NU); Underberg on river bank (-CD), *Tritton R. 32* (NU).

—**2930** (Pietermaritzburg): Banks of Mooi River (-AA), *Wright F.B. 249* (NU); Bushman's River, Daltons Bridge (-CB), *Acocks J.P.H. 10659* (NU); Pietermaritzburg (-CB), *Killick D.J.B. 623* (UNH); Little Noodeberg (-CB), *Killick D.J.B. 660* (NU); Lions River (-CB), *Moll E.J. 993* (NU).

Mpumalanga.

—**2730** (Vryheid): Wakkerstroom (-AD), *Devenish N.J. 1152* (PRE).

***M. papposa* Nees**

South Africa

Eastern Cape Province.

—3324 (Steytlerville): Between Patensie and Willowmore on road to Bainskloof (-DA), *Barker N.P.* 644 (PRE).

—3326 (Grahamstown): Grahamstown (-DC), *Dahlstrand K.A.* 589 (GRA); Howison's Poort (-AD), *Burt-Davyi* 12131 (GRA); Howison's Poort (-AD), *Macwu* 795 (GRA).

Western Cape Province.

—3322 (Oudtshoorn): George (-CD), *Paterson J.V.* 1219 (GRA).

***M. rangei* (Pilg) Conert**

Namibia

—2716 (Witpütz): Aus to Rosh Pinah (-AD), *Ellis R.P.* 5069 (PRE); 100 km south of Aus on Rosh Pinah road (-AD), *Venter H.J.T.* 8932 (PRE).

—2615 (Lüderitz): Lüderitz (-AC), *Giess & Van Vuuren* 844 (PRE).

***M. rufa* (Nees) Conert**

South Africa

Eastern Cape Province.

—3323 (Willowmore): Spitskop (-CC), *Geldenhuys* 145 (PRE).

Western Cape Province.

—3219 (Wuppertal): Cederberg State Forest, Research site 3219 AC/12 (-AC), *Le Maitre D.C.* 292 (STE, PRE); Top of Uitkyk Pass (-AC), *Spies* 4402 (BLFU)*[⊗]*.

—**3318** (Cape Town): Langverwacht above Kuils River, main kloof (-DC), *Oliver E.G.H.* 4683 (STE, PRE).

—**3319** (Worcester): Hawequas State Forest. Du Toits Kloof Pass (-CA), *Forsyth G.G.* 297 (STE, PRE); 23 km south-east of Franschoek on road to Villiersdorp in Franschoek Mountains (-CC), *Davidse* 33855 (PRE); Du Toitskloof (-CC), *Fairall A.R.* 236 (NU); 6 km from Franschoek on road to Villiersdorp (-CC), *Spies* 4451 (BLFU).

—**3418** (Simonstown): Simonstown (-AA), *Schlechter R.* 1671 (GRA); Constantia Peak, on steep North facing slope between rocks (-AB), *du Toit P.C.V.* 1376 (PRE, NH).

—**3419** (Caledon): 8 km north of Grabow, Viljoens Pass, on road to Villiersdorp (-AA), *Davidse* 33507 (PRE); Caledon (-BC), *Acocks J.P.H.* 22800 (PRE).

M. setacea N.P. Barker

South Africa

Western Cape Province.

—**3319** (Worcester): Groot Winterhoek wildernis area, Suurvlakte Plateau north-west of Groenberg (-AA), *Ellis R.P.* 5500 (PRE).

M. stereophylla (J.G. Anders) Conert

Lesotho

—**2928** (Marakabeis): Drakensberge (-CC), *Ellis R.P.* 3186 (PRE).

—**2927** (Maseru): On top of Thaba Ntuso just outside the Sehlabathebe Nature Park (-BD), *Beverly A. & Hoener F.K.* 608 (NU); main road near Bushmans Pass (-BD), *Schmitz M.* 8573 (PRE).

South Africa

Kwazulu-Natal.

—**2929** (Underberg): Kamisberg area, "Storm Heights" (-BC), *Hilliard O.M. & Burttt B.L. 11787* (PRE, NU); Sani Pass (-CA), *du Toit P.C.V. 2208* (NH); Golden Castle Nature Reserve (-CA), *Hilliard O.M. & Burttt B.L. 10508* (NU); south of Sani Pass on way to Hudson's Peak (-CB), *du Toit P.C.V. 2265* (NH); Cabhem Forest Reserve (-CB), *Hilliard O.M. & Burttt B.L. 12508* (NU)*; south of Mkomazi river (-CB), *Hilliard O.M. & Burttt B.L. 15754* (PRE, NU); Cobhem Forest Reserve (-CB), *Manning J.C., Hilliard O.M. & Burttt B.L. 15940* (NU); Sani Pass (-CB), *Manning J.C., Hilliard O.M. & Burttt B.L. 17277* (PRE).

M. stricta (Schrad.) Conert

Lesotho

—**2828** (Bethlehem): Ox-bow (-DC), *du Toit P.C.V. 2673* (NH).

—**2929** (Underberg): Selabathebe National Park (-CC), *du Toit P.C.V. 2630* (NH, PRE); Sehlabathebe National Park (-CC), *Hoener F.K. 1477* (NU); Sehlabathebe National Park (-CC), *Hoener F.K. 2087* (NU); Sehlabathebe (-CC), *Jacot Guillarmond, Getliffe & Mzamane 168* (PRE).

South Africa

Eastern Cape Province.

—**3028** (Matatiele): 22 km from Rhodes to Barkley East (-CC), *Spies 4684* (BFLU)*.*.

—**3224** (Graaff-Reinet): 30 km east of Graaff Reinet (-BB), *Smook L. 3927* (PRE, GRA).

—**3323** (Willowmore): 9 km from Uniondale to Willowmore (-CA), *Spies 3695* (BFLU)*; Willowmore (-CA), *Foucade H.G. 1654* (GRA); 55 km north of Knysna along road to Uniondale (-CC), *Davidse 33939* (PRE)*.

—**3325** (Port Elizabeth): Port Elizabeth (-DC), *Hare T.B. 1940* (NU).

—3326 (Grahamstown): Near Atherstone (-AD), *Britten L.L. 7066* (GRA).

—3424 (Humansdorp): Humansdorp on hills overlooking Gamtoos Valley (-BB), *Cowling R.M. 795* (GRA).

Northern Cape Province.

—3119 (Calvinia): Vanrhyns Pass (-AC), *Spies 6288* (BFLU): 41 km from Vanrhynsdorp on Vanrhyns Pass (-AC), *Spies 4351* (BFLU)*^o*; Beeldhouersfontein, 30 km south east of Murraysburg (-AC), *van den Berg J.A. 2* (PRE); du Toits kloof just outside tunnel (-CA), *Spies 4602* (BFLU).

—3123 (Victoria West): Murraysburg (-AC), *Tyson W. 525* (NH).

Western Cape Province.

—3118 (Vanrhynsdorp): Gifberg (-DC), *Spies 4329* (BFLU); Gifberg Pass (-DC), *Spies 4339* (BFLU)*.

—3218 (Clanwilliam): 11 km out of Clanwilliam on approach to Pakhuis Pass (-BB), *du Toit P.C.V. 1652* (PRE, NH); Skoongesig, Ceres (-AC), *Hanekom W.J. 1008* (PRE); Uitkyk Pass next to water stream (-AC), *Spies 3469* (BFLU)*; hills north-west of Piquetberg (-AD), *Davidse 33333* (PRE)*; Kleinveld north of Rosendal (-CD), *Hugo L. 2259* (PRE).

—3219 (Wuppertal): 44 km from Clanwilliam to Calvinia (-AA), *Davidse 33347* (PRE)*; Top of Uitkyk Pass (-AC), *Spies 4401* (BFLU).

—3220 (Sutherland): 15 km from Sutherland to Matjiesfontein (-BC), *Spies 3147* (BFLU)*; 2,5 km from Sutherland to Calvinia (-BC), *Spies 3839* (BFLU)*.

—3318 (Cape Town): Kirstenbosch (-CB), *Bolus F. 14979* (NH); Wynberg Hill Kirstenbosch (-CD), *Bolus F. 14733* (GRA); Wyngaardt farm near Malmesbury (-DB), *Davidse 34091* (PRE)*; 10 km from Wellington to Worcester in

Bainskloof (-DB), *Spies 4434* (BFLU); Simonsberg above Schoongezicht (-DD), *Taylor H.C. 10258* (PRE, STE); Cape Town (-DD), *van Rensburg W.W. 75* (BFLU).

—**3319** (Worcester): Visgat vicinity (-AA), *Hugo L. 2222* (PRE); Worcester (-AC), *Schlechter R. 9083* (Albany); Robertson (-DC), *Van Breda & Joubert 2000* (PRE); Jonaskop mountain summit (-DC), *Davidse 34110* (PRE)*.

—**3320** (Montagu): 41 km from Montagu to Touwsriver (-CD), *Spies 3618* (BFLU)*; 47 km from Montagu to Touwsriver (-CD), *Spies 3637* (BFLU)*.

—**3418** (Simonstown): Wynberg Hill (-AB), *Davidse 34121* (PRE)*.

—**3419** (Caledon): Caledon (-AB), *Bolus F. 14768* (GRA, NH); 27 km from Villiersdorp on road to Caledon (-AB), *Spies 4651* (BFLU); Shaw's Pass (-AD), *Spies 6227* (BFLU); Caledon near Koude River (-DA), *Davidse 34028* (PRE)*.

—**3420** (Bredasdorp): 13 km from Waenhuiskrans to Bredasdorp (-CA), *Spies 4480*, *Spies 4482* (BFLU).

—**3422** (Mossel Bay): Groot Brakrivier (-AA), *Schlechter R. 5758* (GRA).

Free State.

—**2829** (Harrismith): Golden Gate (-DC), *Potgieter J. 207* (BFLU); Platberg Harrismith (-DC), *Venter H.J.T. 7083* (BFLU).

Kwazulu-Natal.

—**2828** (Bethlehem): Cathedral Peak Forest Research Station (-DB), *Killick D.J.B. 1576* (NU).

—**2829** (Harrismith): Cathedral Peak Station (-CC), *Ellis R.P. 3289* (PRE, NH); Cathedral Peak Forest Resort (-CC), *Killick D.J.B. 1100* (NU, NH); Estcourt (-DD), *West O. 1408* (NH); Estcourt (-DD), *Wright F.B. 231* (NU).

—**2929** (Underberg): Upper Loteni Valley (-AD), *Hilliard O.M. & Burtt B.L. 18161* (NU); Giant's Castle Game Reserve (-AD), *Mc Allister A.J. 84, Smook L. 1387, Smook L. 1391* (NH, PRE); Giants Castle Game Reserve (-AB), *Ward C.J. 6958* (NU, NH). Tabamhlope (-BA), *West O. 1053* (PRE); Garden Castle Forest Reserve (-CA), *Hilliard O.M. & Burtt B.L. 14954* (NU); 9 km north-west of Castle View Farm (-CB), *Hilliard O.M. & Burtt B.L. 13656* (NU); Cobham Forest Reserve (-CB), *Hilliard O.M. & Burtt B.L. 14009* (NU), *Hilliard O.M. & Burtt B.L. 14121* (PRE), *Hilliard O.M. & Burtt B.L. 14124* (NU), *Manning J., Hilliard O.M. & Burtt B.L. 15980* (NU), *Manning J., Hilliard O.M. & Burtt B.L. 16060* (NU), *Manning J., Hilliard O.M. & Burtt B.L. 16084* (NU); Gxalingenwa valley, between Sani Pass and Polela valley (-CB), *Hilliard O.M. & Burtt B.L. 17106* (NU, PRE), *Hilliard O.M. & Burtt B.L. 17108* (NU); Chameleon Cave area (-CB), *Hilliard O.M. & Burtt B.L. 17760* (NU, PRE).

—**2930** (Pietermaritzburg): Inhluzame - Lions River District (-CA), *Edwards D. 3085* (NU); Mpendle District (-CA), *Moll E.J. 669* (NU), *Moll E.J. 1281* (PRE, NU).

Schismus P. Beauv.

S. barbatus (L.) Thell.

Namibia

—**2616** (Aus): Aus on road to Lüderitz Bay (-CB), *Giess W. & van Vuuren D. 640* (PRE).

—**2716** (Witpütz): Farm Spitzkopp (-AB), *Giess W. 13053* (PRE).

South Africa

Eastern Cape Province.

—**3323** (Willowmore): 13 km from Uniondale to Oudtshoorn (Potjiesberg Pass) (-CA), *Spies 6155* (BFLU).

—**3325** (Port Elizabeth): Addo, hill in rhino camp (-DA), *Botha B.P. 6579* (GRA).

Northern Cape Province.

—2816 (Oranjemund): Richtersveld, Ploegberg south of Khubus (-DB), *Oliver, Tölken & Venter 529* (PRE); Holgat River about 40 km north of Port Nolloth on road to Alexander Bay (-DD), *Pienaar B.J. 1124* (PRE).

—2817 (Vioolsdrif): Richtersveld - Vandersterrberg north-east of Khubus (-AC), *Oliver, Tölken & Venter 186* (PRE); large cliff on north side, north of Leliehoek (-AC), *Oliver, Tölken & Venter 367* (PRE); Richtersveld, about 5 km from Eksteensfontein (-CD), *Nicholas A. 2595C* (PRE).

—2824 (Kimberley): Kimberley (-DB), *Terrar E. 52* (NU).

—2916 (Port Nolloth): 11 km west of Port Nolloth (-BD), *de Winter B. 9551* (PRE).

—2917 (Springbok): Kwaganap River 36 km south-east of Port Nolloth on road to Kleinsee (-AC), *Davidse 33276* (PRE); 14 km from Steinkopf to Port Nolloth (Aninaus Pass) (-CB), *Spies 6353* (BFLU); 10 km east of Springbok on road to Pofadder (-DB), *Ellis R.P. 2135* (PRE); 2 km from Pofadder to Springbok (-DB), *Spies 4273* (BFLU)*; 13 km from Springbok to Hondeklipbaai (-DB), *Spies 4277* (BFLU)*^{o*}; 13 km from Springbok to Hondeklipbaai (-DB), *Spies 4278* (BFLU)*; 17 km from Springbok to Hondeklipbaai (-DB), *Spies 4285* (BFLU)*; Springbok (-DB), *van der Westhuizen P.M. 43/78* (PRE); Ydeep River, 35 km south-east of Little Rock Caravan Park (-DD), *Thompson M.F. & Le Roux 24* (PRE, STE).

—2921 (Kenhardt): 10 km west of Kenhardt along stream (-AC), *Smook L. & Harding G.B. 764* (PRE).

—3017 (Hondeklipbaai): 24 km from Soebatsfontein to Kamieskroon (-BA), *Spies 4289* (BFLU)*.

—3018 (Kamiesberg): 14 km from Loeriefontein to Gharies (-AB), *Spies 4215* (BFLU)*.

—3019 (Loeriesfontein): 85 km from Brandvlei om road to Loeriesfontein (-DD), *Ellis R.P. 2425* (PRE).

—3024 (De Aar): Doornkloof Nature Reserve (-BD), *Hahndrick A. 79* (GRA).

—3025 (Colesberg): Tussen-die-riviere Game Reserve, Bethulie (-BD), *Roberts B.R. 5325* (PRE).

Western Cape Province.

—3118 (Vanrhynsdorp): 5 km north of Nuwerus on road to Gharies (-AB), *Rösch & Le Roux 641* (PRE).

—3119 (Calvinia): 55 km from Nieuwoudtville to Clanwilliam (-CC), *Spies 4366* (BFLU)*.

—3217 (Vredenburg): Britannia Bay (-DD), *Acocks J.P.A. 15204* (PRE).

—3218 (Clanwilliam): Wadrif, soutpan west of railway (-AB), *O'Callighan, Van Wyk & Marley 124* (PRE); Pikenierskloof Pass, Piketberg (-DB), *Grant A.L. 4730* (PRE).

—3219 (Wuppertal): 44 km from Clanwilliam to Calvinia (-AA), *Davidse 34039* (PRE)*[⊗].

—3220 (Sutherland): 23 km from Matjiesfontein to Sutherland (-DA), *Spies 4655* (BFLU).

—3320 (Montagu): 41 km from Montagu to Touwsriver (-CD), *Spies 4530, Spies 4531* (BFLU); 24 km from Montagu to Touwsriver (-CC), *Spies 4523* (BFLU)*; 38 km from Montagu to Touwsriver (-CC), *Spies 4524* (BFLU)*; Allexandria (-DD), *Liebenberg L.C.C. 6310* (PRE).

—3221 (Merweville): Layton Garden (-BB), *Shearing D.A.M.B. 1073* (PRE).

—3222 (Beaufordt West): Farm Ardoorns along road to Blouwater (-DD), *Retief & Reid 79* (PRE).

—3321 (Ladismith): 8 km from Ladismith on road to Calitzdorp (-CB), *Barker N.P.705* (PRE).

Free State.

—2925 (Jagersfontein): Petrusberg (-AB), *Spies 6596* (BFLU).

—2926 (Bloemfontein): Bloemfontein on plain (-AA), *Pretorius O. sn* (PRE); Trompsburg, farm Tweefontein (-AA), *Zietsman P.C. 144* (PRE).

***S. inermis* (Stapf) C.E. Hubb**

South Africa

Eastern Cape Province.

—3125 (Steynsburg): Grootfontein Agricultural College (-AC), *Archibold E.E.A.3333* (GRA).

—3324 (Steytlerville): Hankey (-DD), *Paterson F.V. 3212* (GRA).

—3325 (Port Elizabeth): Port Elizabeth (-DB), *Ellis R.P. 596* (PRE); King Neptune Beach (-DB), *Ellis R.P. 2563* (PRE).

Northern Cape Province.

—3017 (Hondekliipbaai): 18 km from Kamieskroon to Leliefontein (-BA), *Spies 4277* (BFLU)*.

—3027 (Lady Grey): Barkley East (-DC), *Galpin E.E. 6906* (GRA).

—3119 (Calvinia): Farm Lokenburg (-CA), *De Winter & Verdoorn 9038* (PRE).

—3123 (Victoria West): Richmond (-BD), *Bolus H.H. 13842* (GRA).

Western Cape Province.

—3318 (Cape Town): Cape Town (-DC), *Spies 4575* (BFLU)*.

—3420 (Bredasdorp): Potberg Nature Reserve (-AD), *Burgers C. 2184* (PRE); De Hoop, Buffelsfontein on road to Ruspunt (-CB), *Van Wyk C.M. 1842* (PRE).

—3421 (Riversdale): Riversdale (-AB), *Schlechter R. 1759* (GRA); 2 km south of Vermaaklikheit on road to Puntjie (-AC), *Davidse 33758* (PRE)*.

—3422 (Mossel Bay): Diaz Beach - Mosselbay (-AA), *Ellis R.P. 2550* (PRE).

***S. scaberrimus* Nees**

South Africa

Eastern Cape Province.

—3226 (Fort Beaufort): Fort Beaufort (-AD), *Ellis R.P. 2573* (PRE).

Northern Cape Province.

—2916 (Port Nolloth): Port Nolloth (-BD), *de Winter 9551* (PRE).

—3017 (Hondekliipbaai): Hondekliipbaai (-BB), *de Winter B. 9531* (PRE).

—3018 (Kamiesberg): Kamiesberg (-DC), *Ellis R.P. 2429* (PRE).

Western Cape Province.

—3220 (Sutherland): 1 km from Clanwilliam on road to Willeston (-BC), *Spies 4660***, *4661* (BFLU)*[⊗]; 1 km from Clanwilliam on road to Willeston (-BC), *Spies 5280* (BFLU); 7 km from Nieuwoudtville to Clanwilliam (-CC), *Spies 5298* (BFLU); 10 km from Nieuwoudtville to Clanwilliam (-CC), *Spies 5304*, *5305* (BFLU); 17 km from Calvinia to Nieuwoudtville (-CC), *Spies 5285*, *5286* (BFLU).

ADDENDUM B

Characters used in cladistical analysis based on morphology.

1. Perennial	(0)	20. Incomplete florets not underdeveloped	(0)
Annual	(1)	Incomplete florets merely underdeveloped	(1)
2. Plants herbaceous	(0)	21. Proximal incomplete florets present	(0)
Plants not herbaceous	(1)	Proximal incomplete florets absent	(1)
3. Leaf blades lanceolate	(0)	22. Lemmas hairy	(0)
Leaf blades linear	(1)	Lemmas hairless	(1)
4. White macro hairs on leaves	(0)	23. Lemma with a germination flap	(0)
No white hairs on leaves	(1)	Lemma without a germination flap	(1)
5. Leaf blades always rolled	(0)	24. Lemma incised	(0)
Leaf blades flat or rolled	(1)	Lemma not incised	(1)
Leaf blades flat or folded	(2)	25. Lemma end in a bristle	(0)
6. Dead leaves split and curl	(0)	Lemma no bristle	(1)
Dead leaves do not split but curl	(1)	26. Lemma awned	(0)
Dead leaves do not split or curl	(2)	Lemma awnless	(1)
7. Ligule membranous	(0)	27. Awns 3	(0)
Ligule a fringe of hairs	(1)	Awns 1 or 3	(1)
8. Inflorescence a single raceme	(0)	28. Median awn different from the laterals	(0)
Inflorescence paniculate	(1)	Median awn the same as the laterals	(1)
9. Rhagis elongated	(0)	29. Awn from the sinus	(0)
Rhagis contracted	(1)	Awn not from the sinus	(1)
10. Inflorescence espatheate	(0)	30. Awns much longer than the body of the lemma	(0)
Inflorescence not espatheate	(1)	Awn about as long as the body of the lemma	(1)
11. Spikelet-bearing axes persistent	(0)	31. Central awn usually geniculate	(0)
Spikelet-bearing axes absent	(1)	Central awn seldom geniculate	(1)
12. Spikelet not compressed laterally	(0)	32. Palea present	(0)
Spikelet compressed laterally	(1)	Palea absent	(1)
13. Spikelet disarticulating above the glume	(0)	33. Palea long	(0)
Spikelet falling with the glume	(1)	Palea short	(1)
14. Hairy callus present	(0)	34. Palea more than 2-nerved	(0)
Hairy callus absent	(1)	Palea 2- nerves	(1)
15. Two glumes present	(0)	35. Two lodicules present	(0)
Glumes absent	(1)	Lodicules absent	(1)
16. Glumes more or less equal	(0)	36. Ligulae fleshy	(0)
Glumes not equal	(1)	Ligulae membranous	(1)
17. Glumes about equalling the spikelet to much exceeding the spikelet	(0)	37. Ligulae ciliate	(0)
Glumes about equal the spikelet	(1)	Ligulae glabrous	(1)
Glumes markedly shorter than the spikelet to about equal	(2)	38. Three stamens present	(0)
18. Glumes awned	(0)	Two stamens present	(1)
Glumes awnless	(1)	39. Ovary hairy	(0)
19. Incomplete florets distal to the female-fertile florets.	(0)	Ovary glabrous	(1)
Incomplete florets not distal to the female-fertile florets	(1)	40. Plant base woolly	(0)
		Plant base not woolly	(1)
		41. Tufted	(0)
		Rhizomatous	(1)
		Stoloniferous	(2)

ADDENDUM C

Distance analysis obtained from the morphological data set of the species *Karroochloa*, *Merxmuellera* and *Schismus*. Values in bold indicate inter- or intragenus averages.

	<i>K.curva</i>	<i>K.pur</i>	<i>K.schis</i>	<i>K.tenela</i>	<i>M.arun</i>	<i>M.aureo</i>	<i>M.cincta</i>	<i>M.davyi</i>	<i>M.decora</i>	<i>M.disticha</i>	<i>M.drakens</i>	<i>M.dura</i>	<i>M.guillar</i>	<i>M.lupu</i>	<i>M.macow</i>	<i>M.papp</i>	<i>M.rangei</i>	<i>M.rufa</i>	<i>M.stereo</i>	<i>M.stricta</i>	<i>M.seta</i>	<i>S.bar</i>	<i>S.iner</i>	<i>S.cabe</i>	
<i>K.curva</i>																									
<i>K.purpurea</i>	0.21																								
<i>K.schismoi</i>	0.21	0.18																							
<i>K.tenella</i>	0.24	0.21	0.03																						
				@0.18																					
<i>M.arun</i>	0.39	0.24	0.24	0.21																					
<i>M.aureo</i>	0.45	0.36	0.30	0.27	0.18																				
<i>M.cincta</i>	0.48	0.33	0.33	0.30	0.09	0.21																			
<i>M.davyi</i>	0.42	0.33	0.27	0.30	0.21	0.03	0.24																		
<i>M.decora</i>	0.42	0.27	0.27	0.30	0.15	0.21	0.18	0.18																	
<i>M.disticha</i>	0.45	0.36	0.30	0.27	0.12	0.18	0.09	0.21	0.21																
<i>M.drakens</i>	0.45	0.42	0.30	0.27	0.30	0.18	0.27	0.21	0.33	0.18															
<i>M.dura</i>	0.45	0.42	0.42	0.39	0.24	0.30	0.21	0.33	0.33	0.12	0.24														
<i>M.guillar</i>	0.42	0.39	0.27	0.30	0.33	0.21	0.30	0.18	0.30	0.21	0.03	0.27													
<i>M.lupuina</i>	0.42	0.27	0.27	0.30	0.15	0.21	0.18	0.18	0.00	0.21	0.33	0.30	0.30												
<i>M.macow</i>	0.42	0.33	0.27	0.30	0.21	0.03	0.24	0.00	0.18	0.21	0.21	0.33	0.18	0.18											
<i>M.papposa</i>	0.52	0.36	0.36	0.39	0.24	0.30	0.21	0.27	0.21	0.18	0.24	0.18	0.21	0.21	0.27										
<i>M.rangei</i>	0.61	0.45	0.45	0.42	0.21	0.33	0.18	0.36	0.30	0.15	0.27	0.15	0.30	0.30	0.36	0.09									
<i>M.rufa</i>	0.42	0.27	0.27	0.30	0.15	0.21	0.18	0.18	0.00	0.21	0.33	0.33	0.30	0.00	0.18	0.21	0.30								
<i>M.stereo</i>	0.61	0.58	0.45	0.42	0.45	0.27	0.42	0.30	0.48	0.33	0.15	0.33	0.18	0.48	0.30	0.27	0.24	0.48							
<i>M.stricta</i>	0.42	0.39	0.27	0.24	0.21	0.15	0.24	0.18	0.30	0.21	0.15	0.33	0.18	0.30	0.18	0.39	0.36	0.30	0.30						
<i>M.setacea</i>	0.30	0.27	0.15	0.18	0.21	0.21	0.24	0.18	0.12	0.21	0.21	0.33	0.18	0.12	0.18	0.27	0.36	0.12	0.36	0.18					
				@ 0.60																	@ 0.22				
<i>S.barbatus</i>	0.70	0.67	0.48	0.45	0.54	0.60	0.45	0.63	0.63	0.42	0.42	0.42	0.45	0.63	0.63	0.42	0.33	0.64	0.33	0.52	0.52				
<i>S.inermis</i>	0.67	0.64	0.51	0.48	0.51	0.57	0.42	0.60	0.60	0.39	0.39	0.39	0.42	0.60	0.60	0.39	0.30	0.61	0.30	0.48	0.48	0.03			
<i>S.scabe</i>	0.61	0.58	0.45	0.42	0.45	0.51	0.42	0.54	0.54	0.33	0.33	0.33	0.36	0.54	0.54	0.33	0.24	0.54	0.24	0.42	0.42	0.09	0.06		
				@ 0.35																		@ 0.46		@ 0.06	

ADDENDUM C-166

ADDENDUM D

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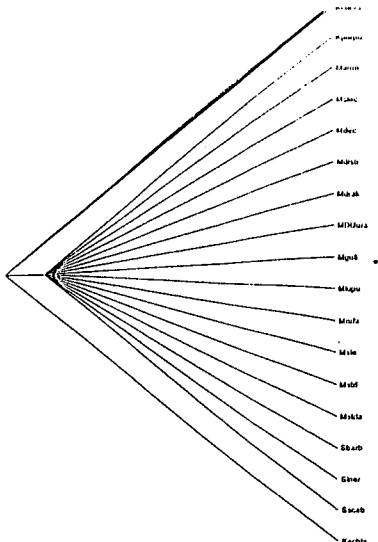
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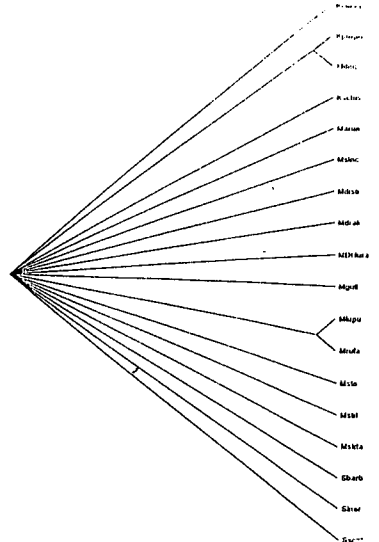
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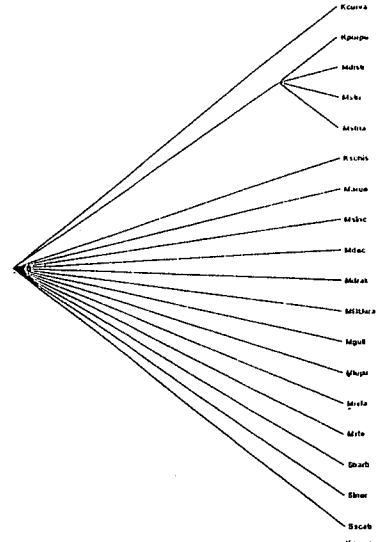
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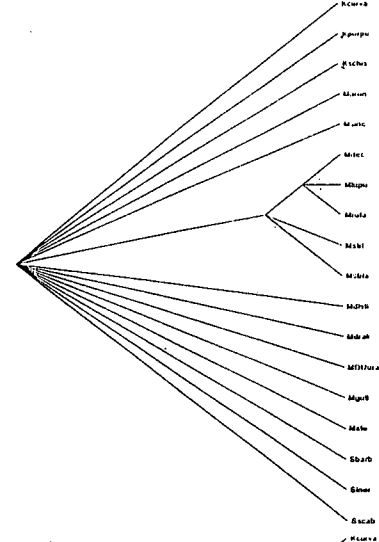
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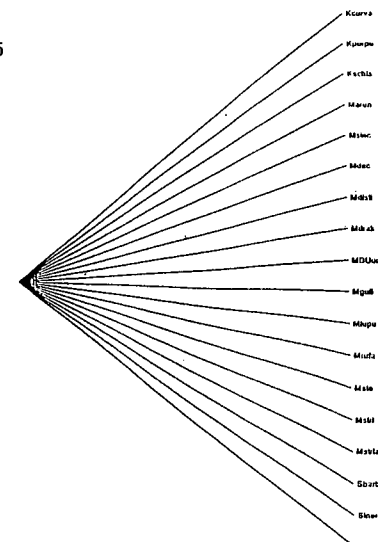
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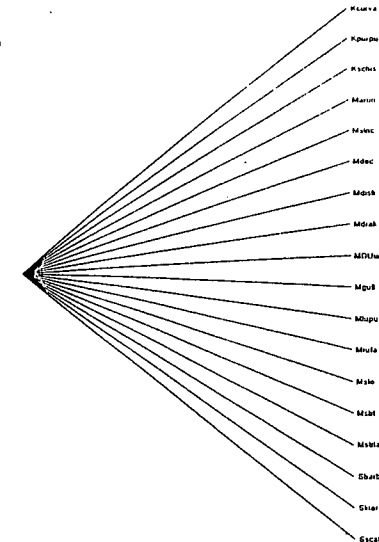
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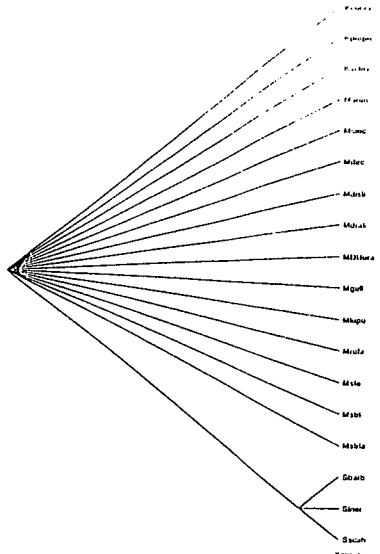
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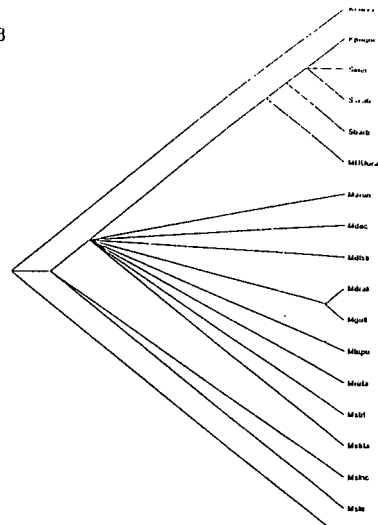
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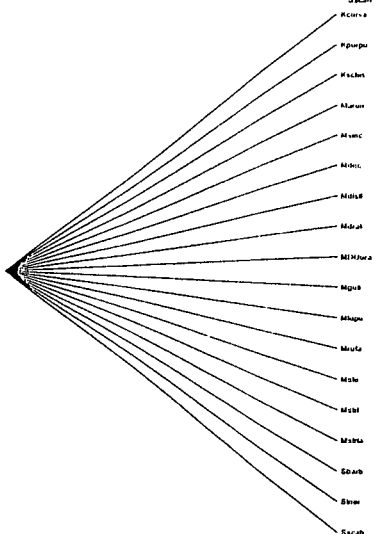
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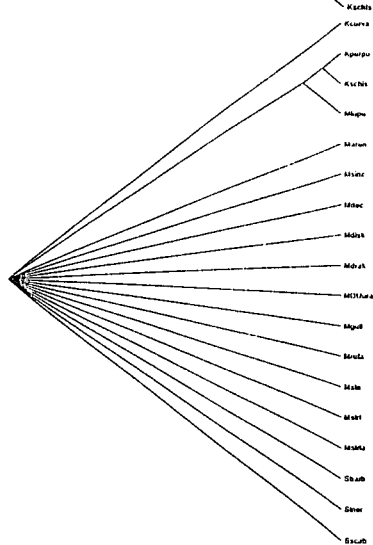
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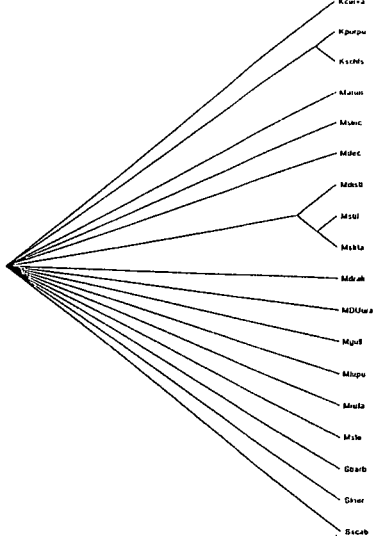
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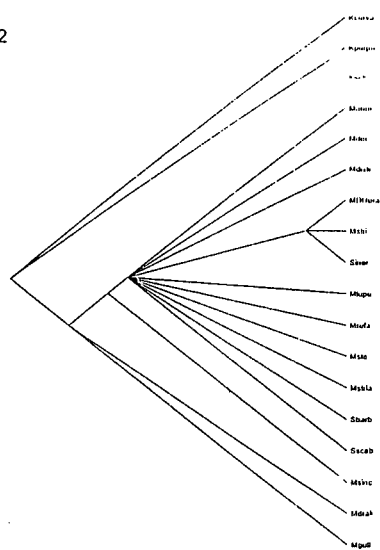
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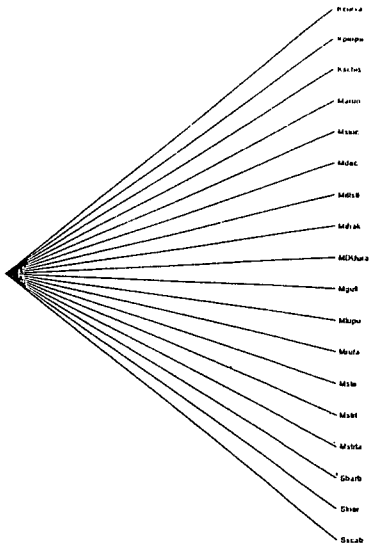
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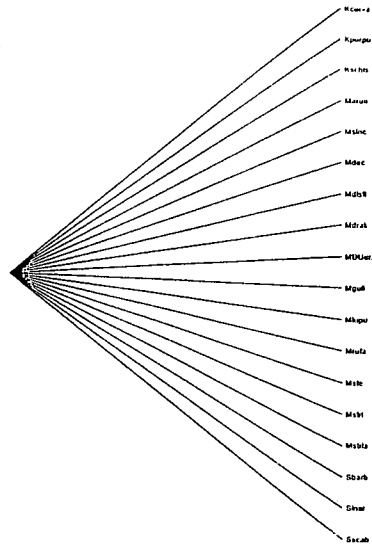
P12



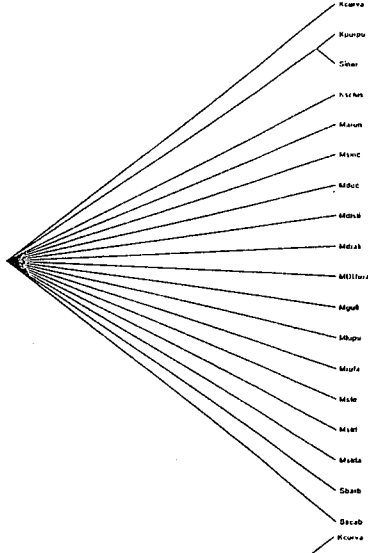
P13



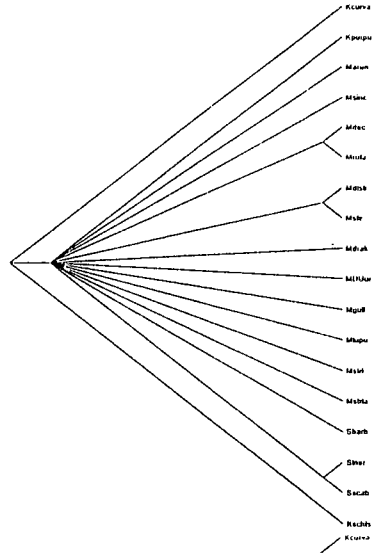
P14



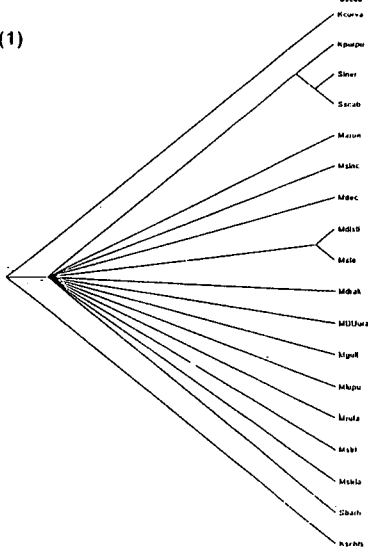
P15



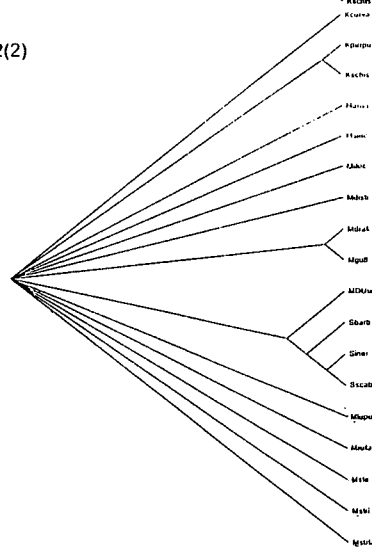
P16



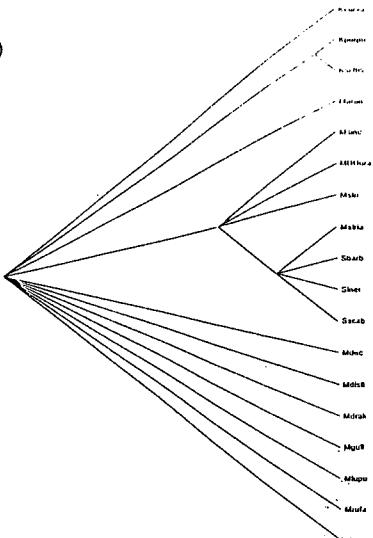
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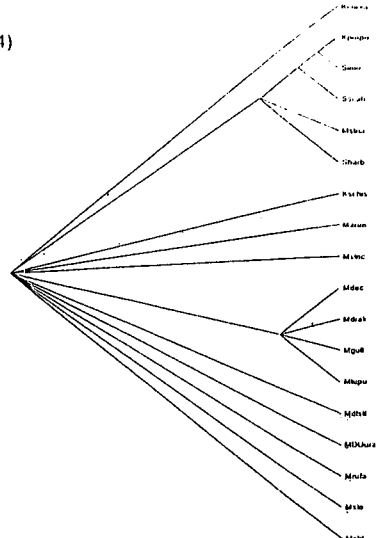
P2(2)



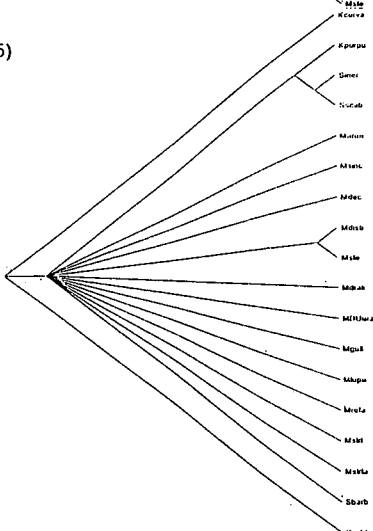
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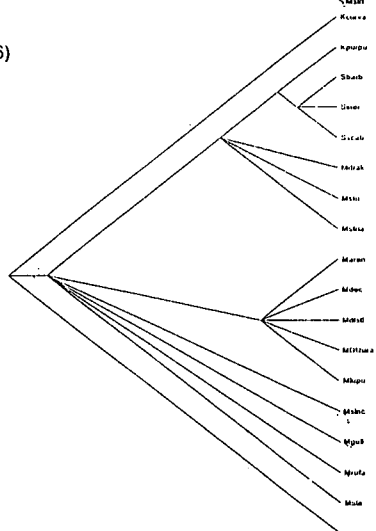
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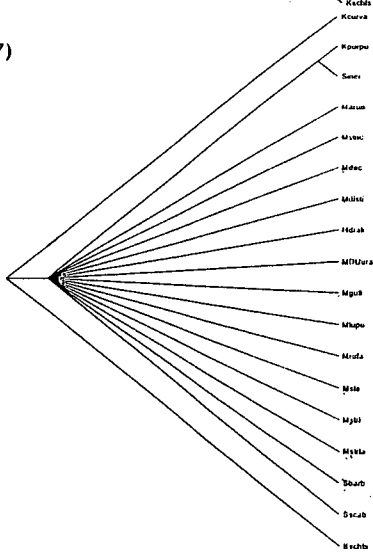
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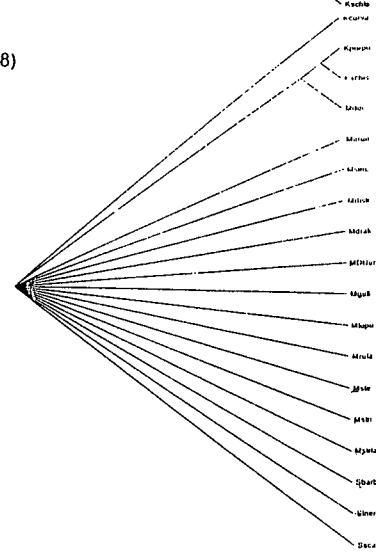
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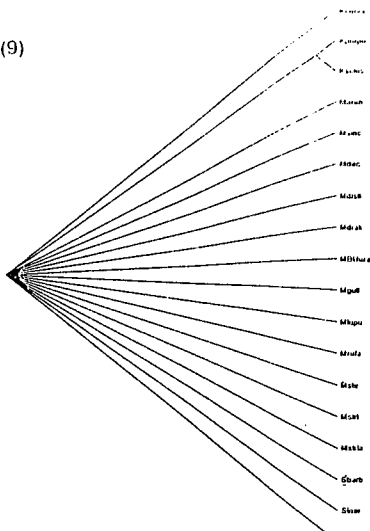
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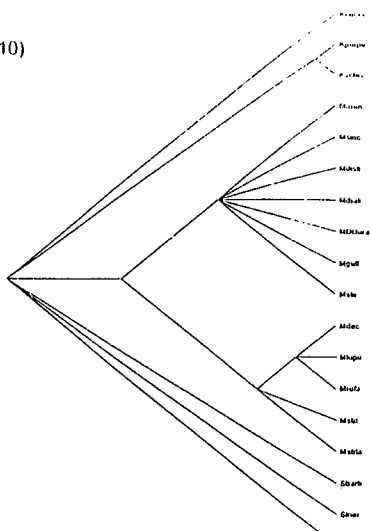
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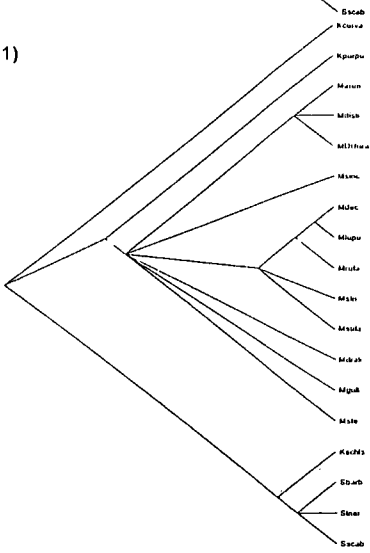
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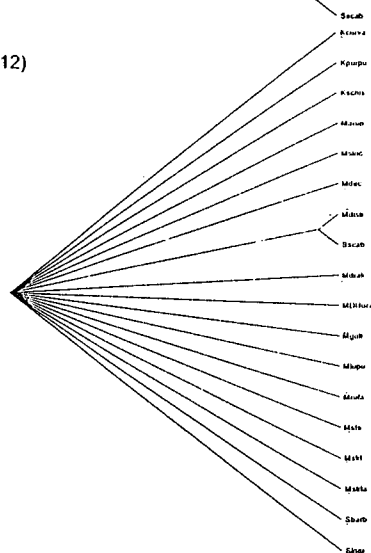
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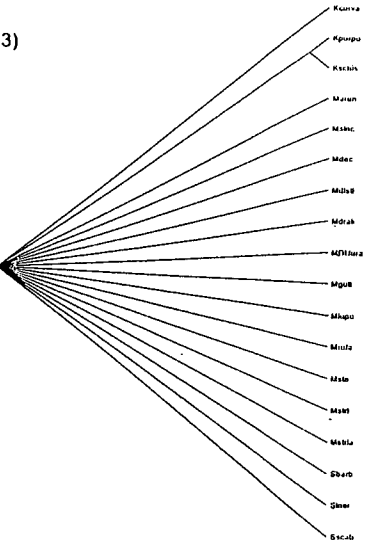
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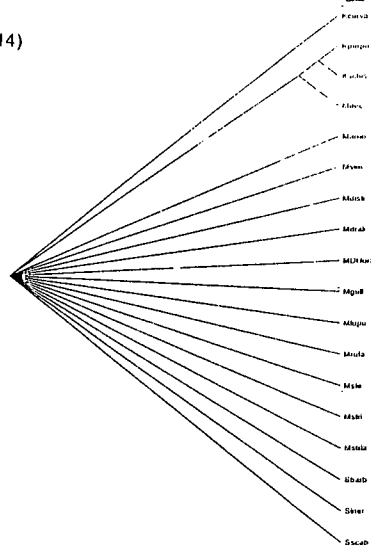
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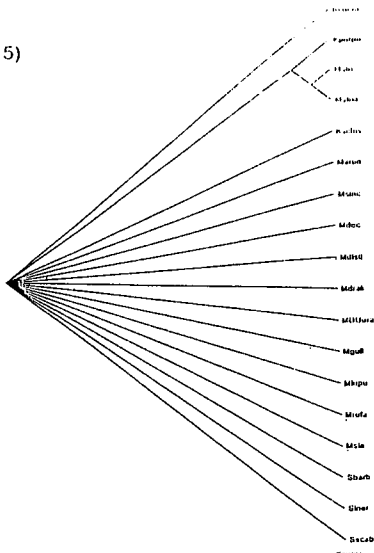
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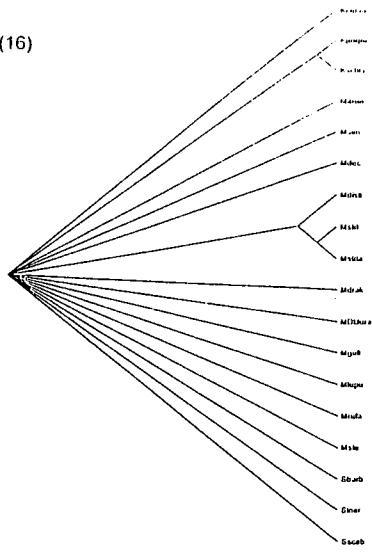
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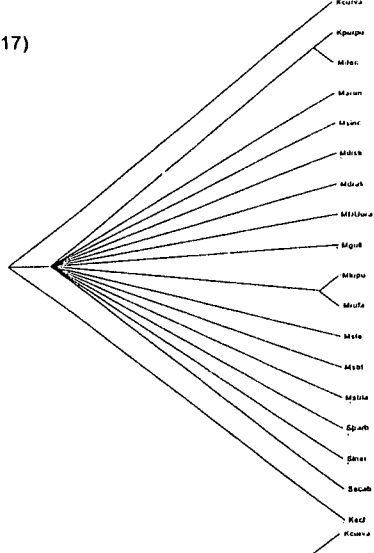
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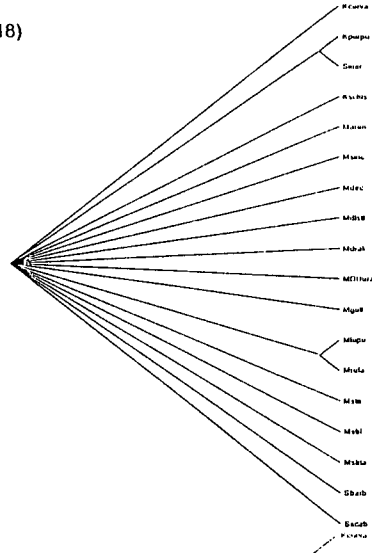
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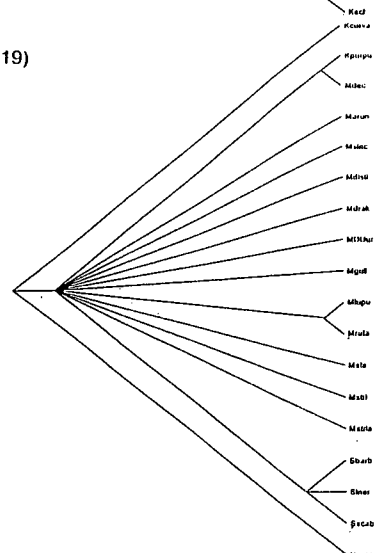
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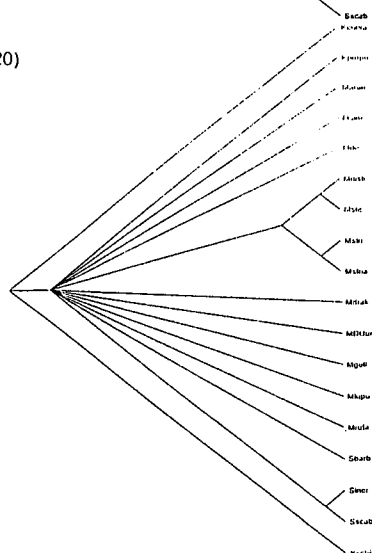
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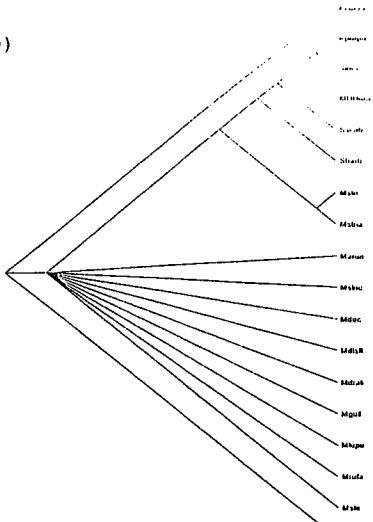
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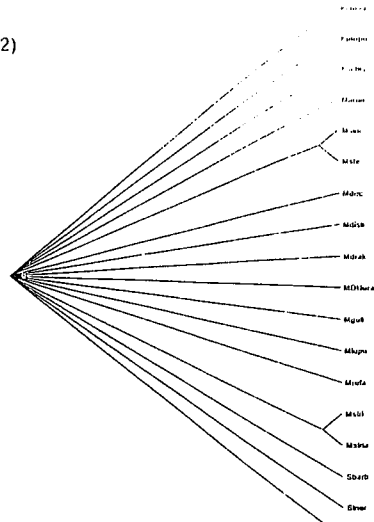
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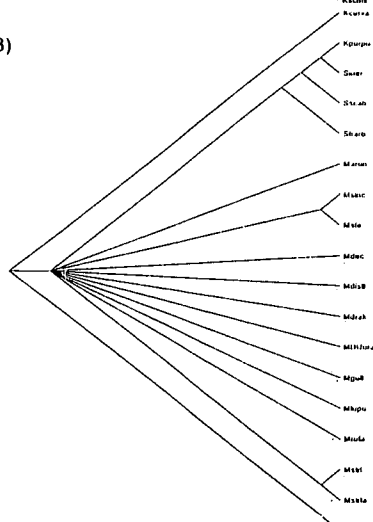
P3(1)



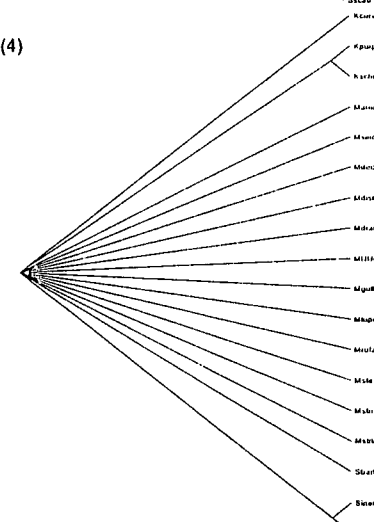
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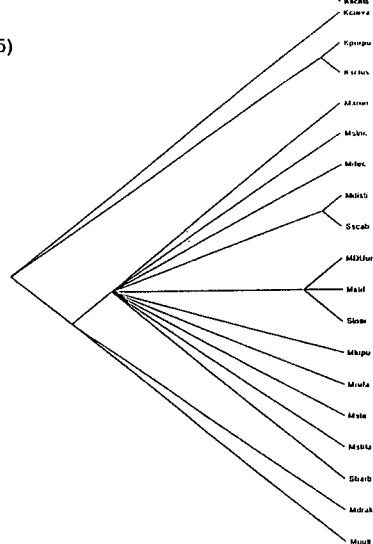
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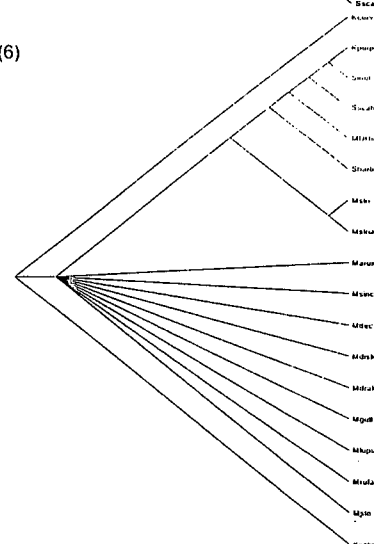
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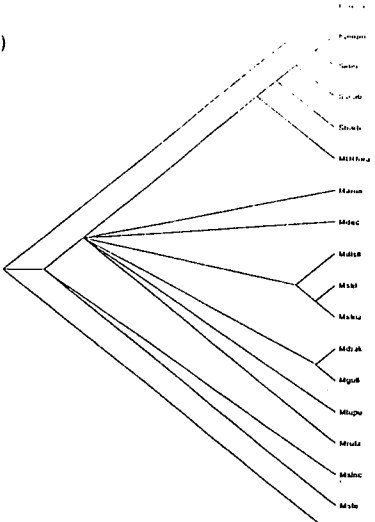
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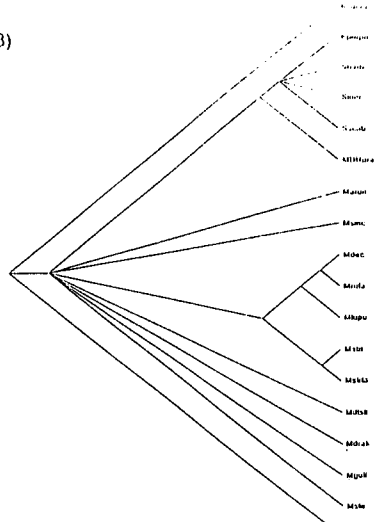
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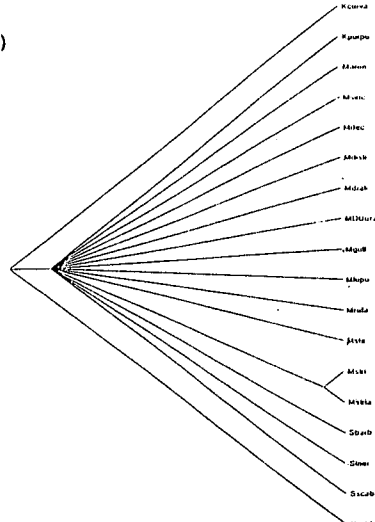
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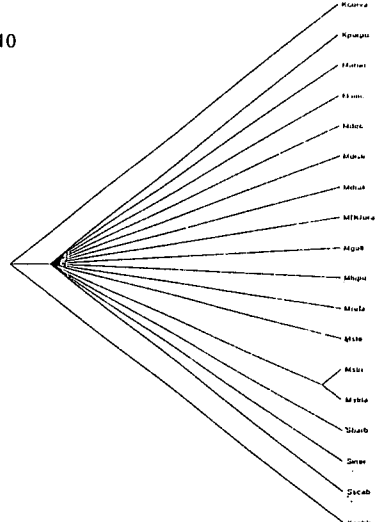
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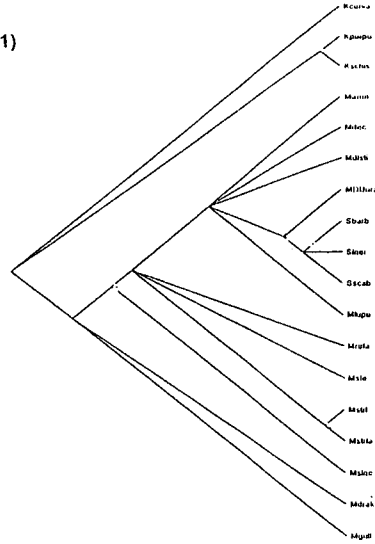
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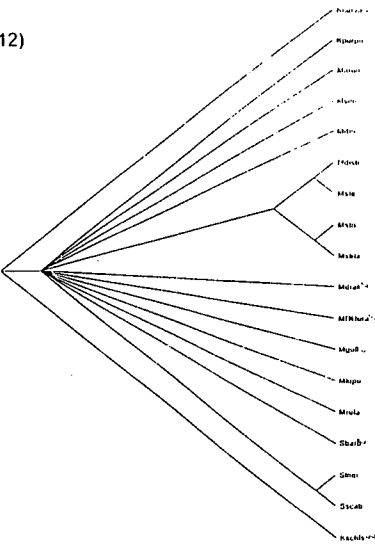
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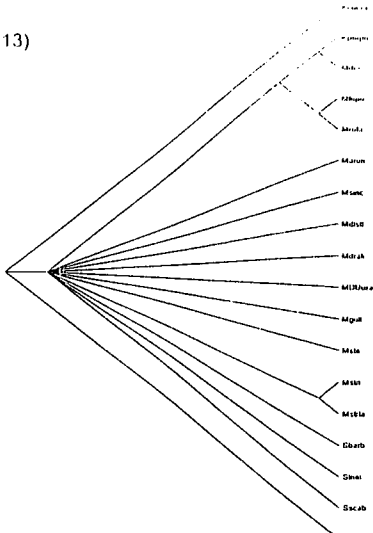
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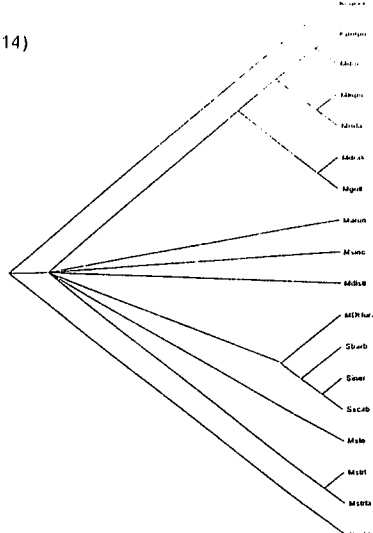
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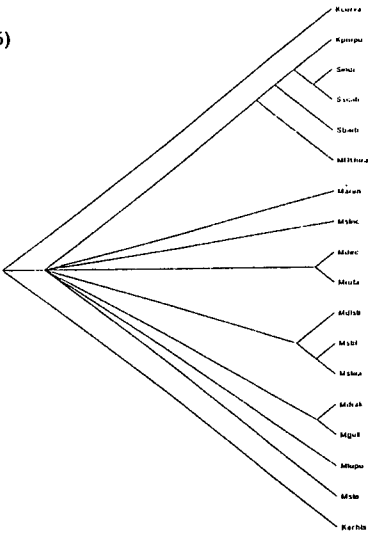
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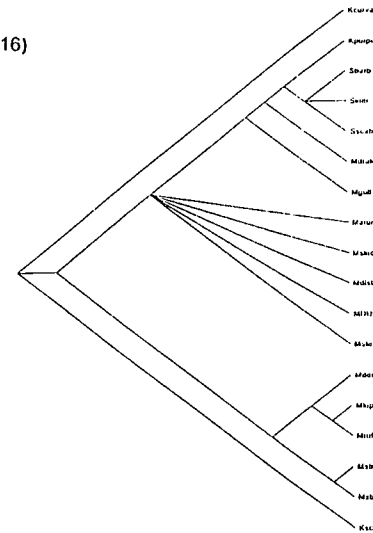
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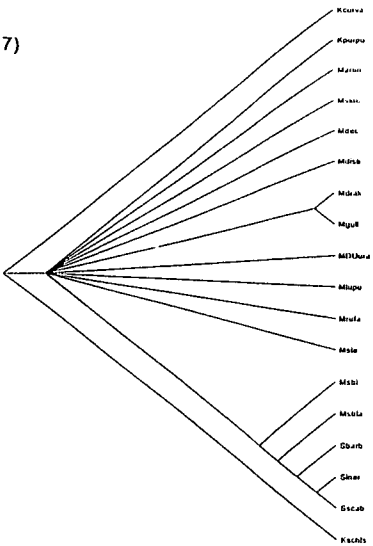
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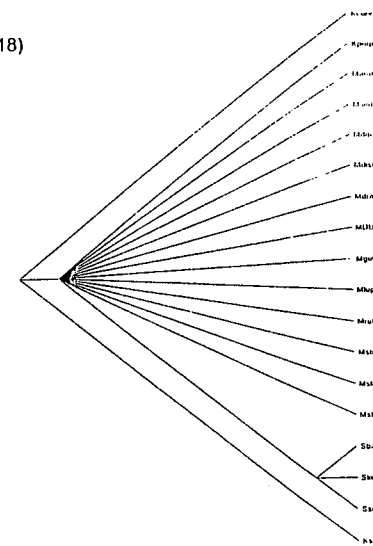
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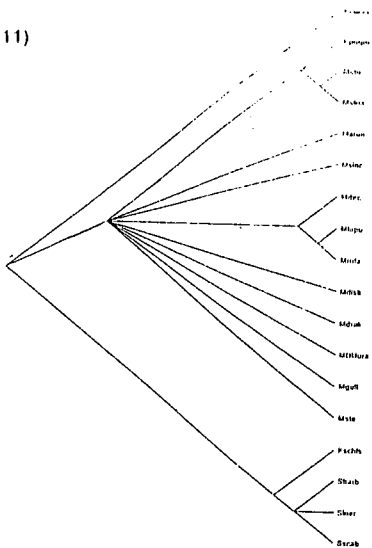
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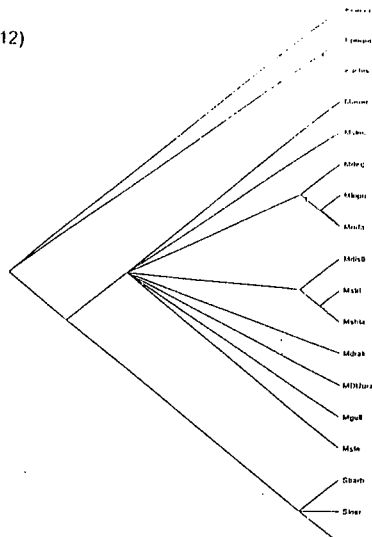
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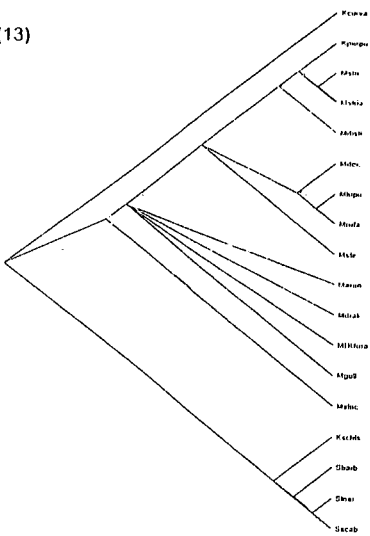
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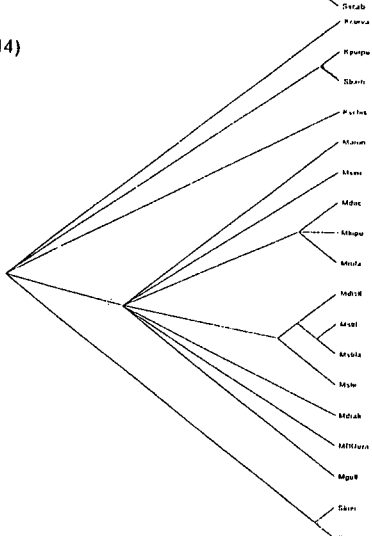
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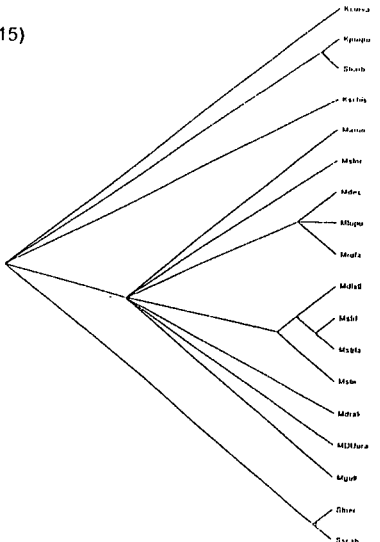
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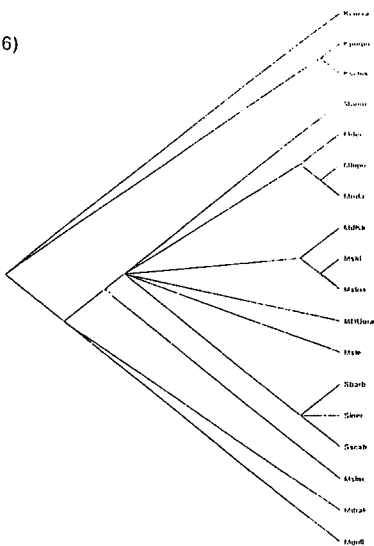
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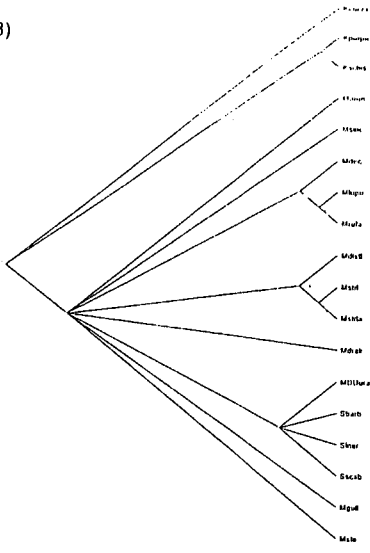
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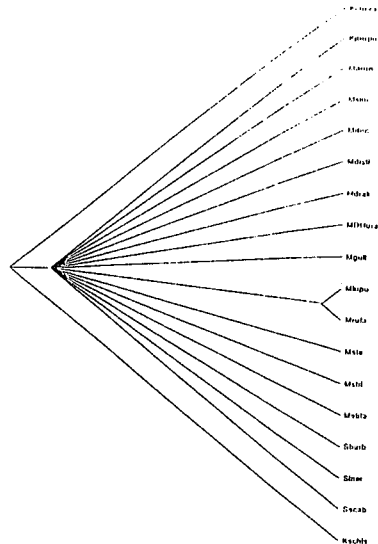
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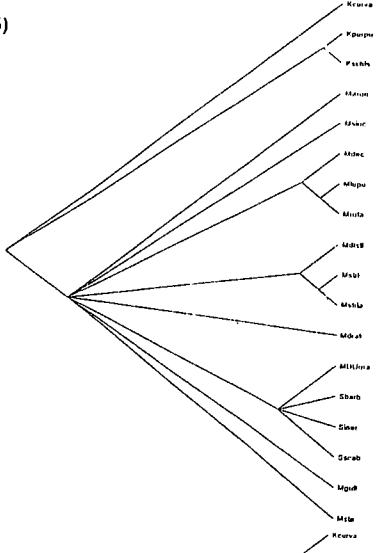
P5(3)



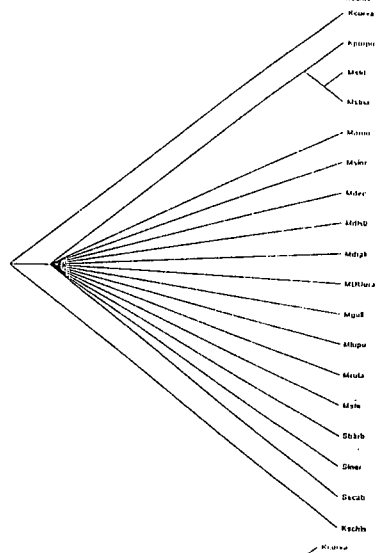
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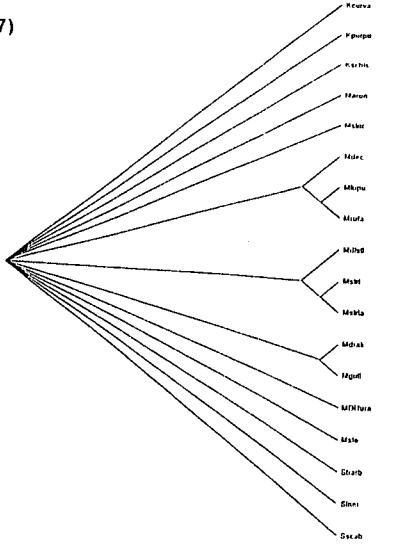
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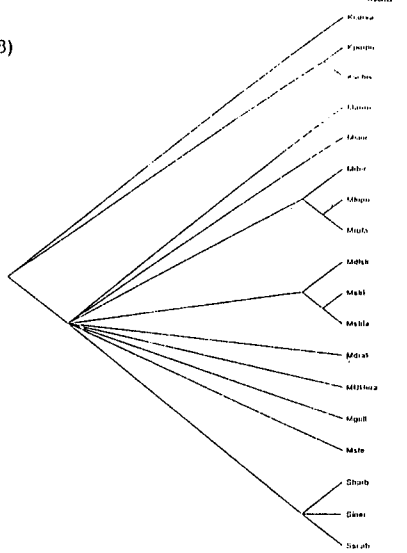
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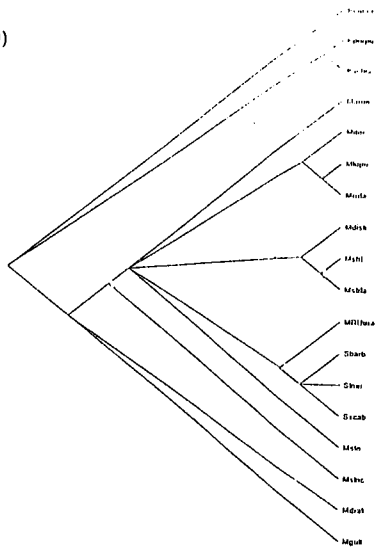
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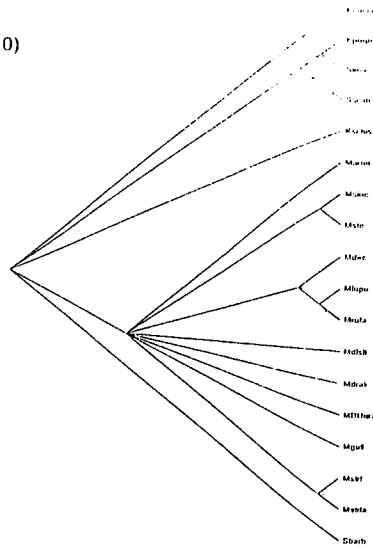
P5(8)



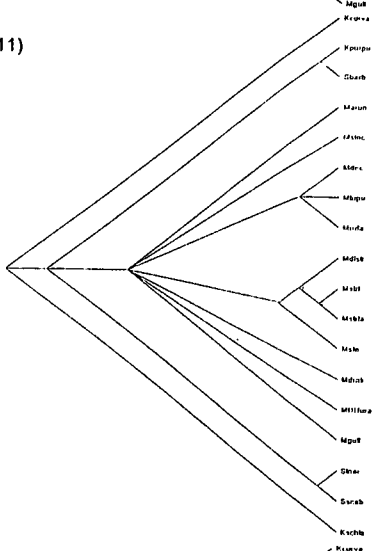
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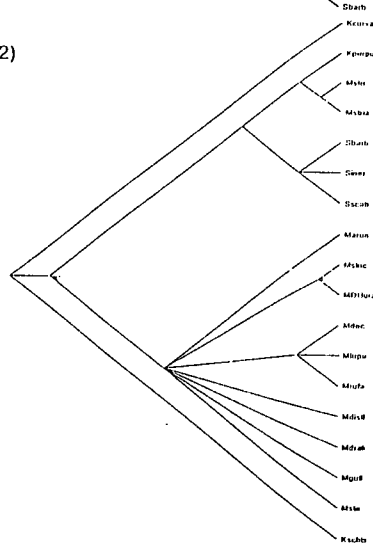
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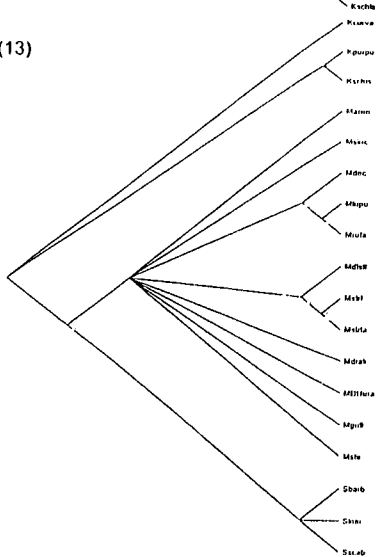
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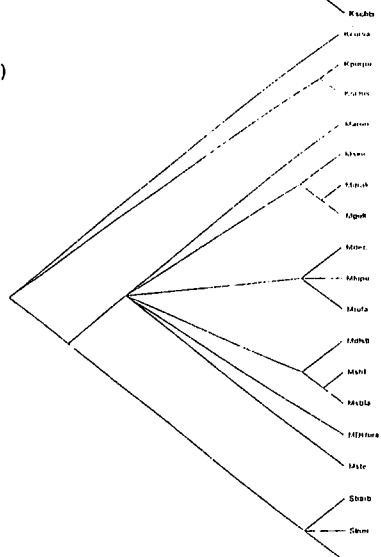
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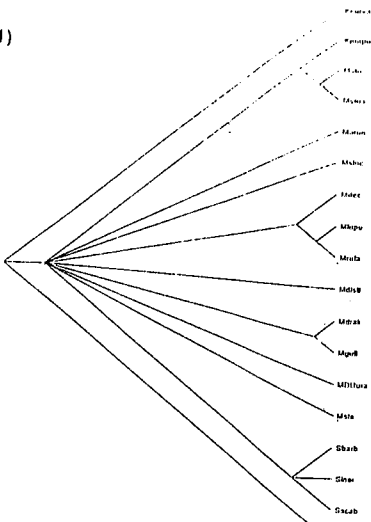
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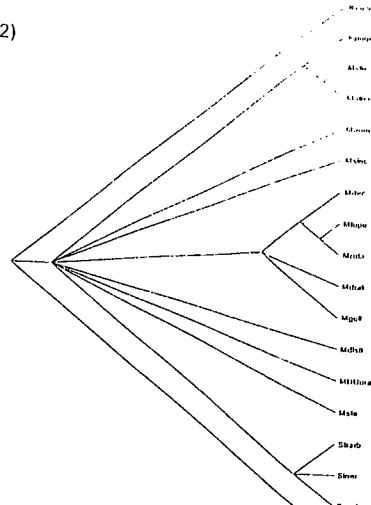
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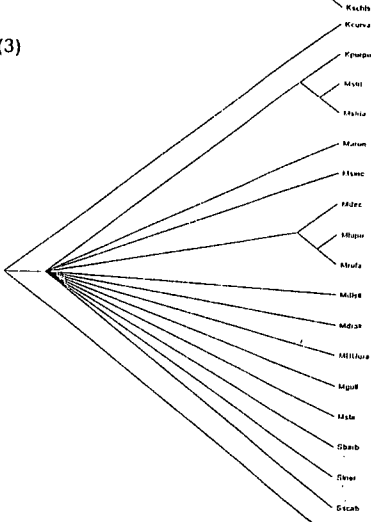
P6(1)



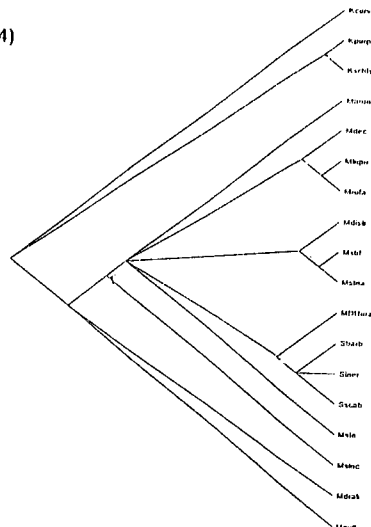
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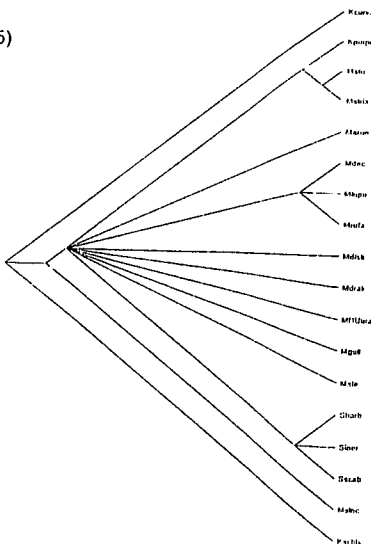
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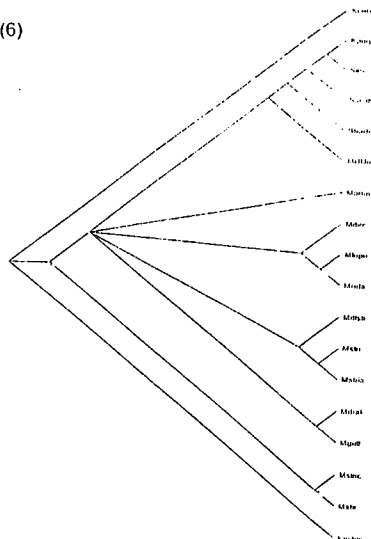
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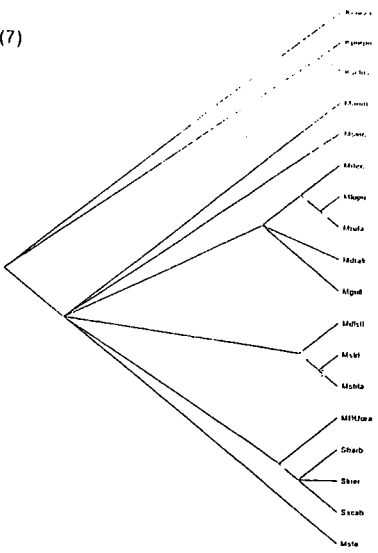
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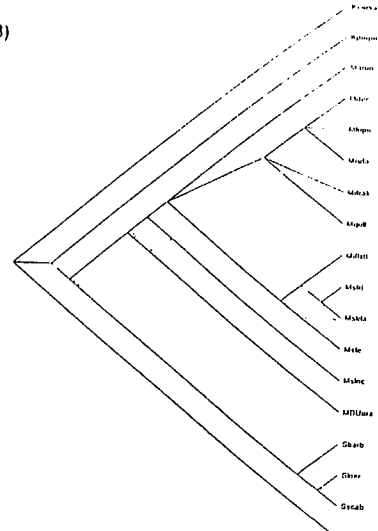
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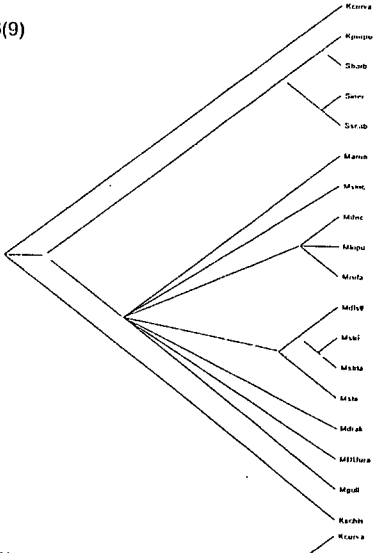
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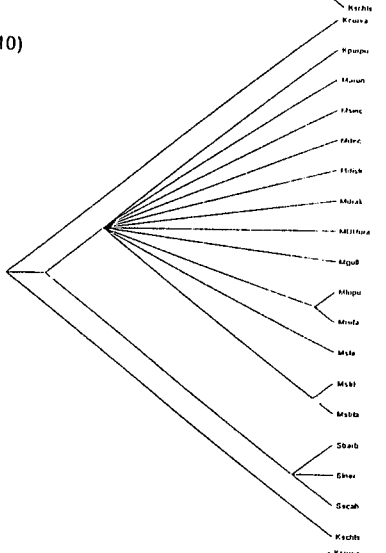
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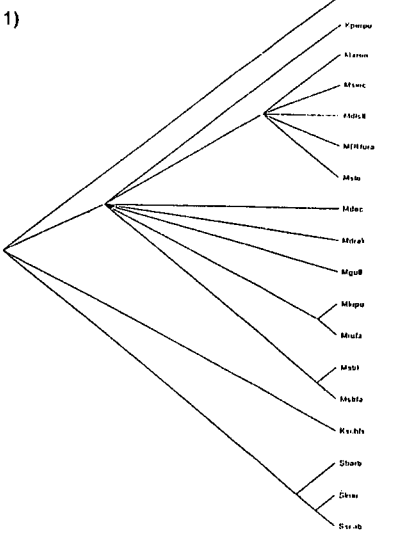
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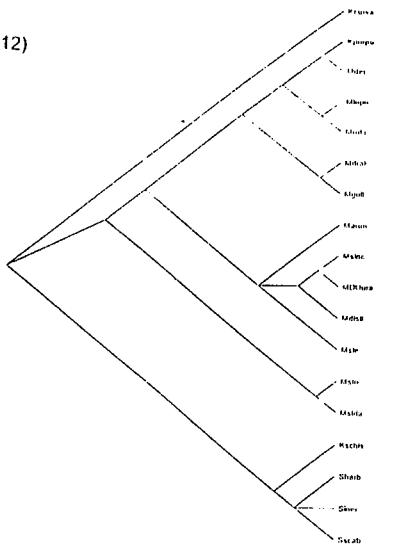
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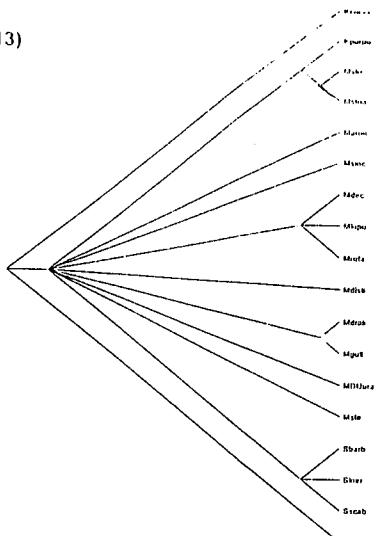
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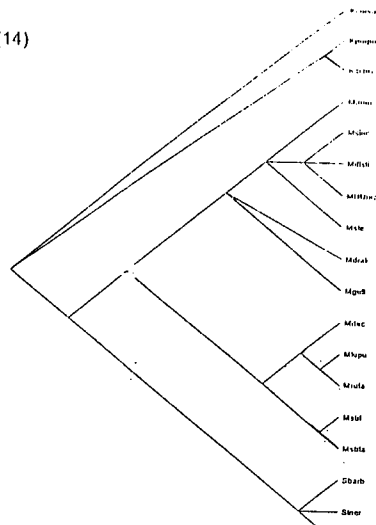
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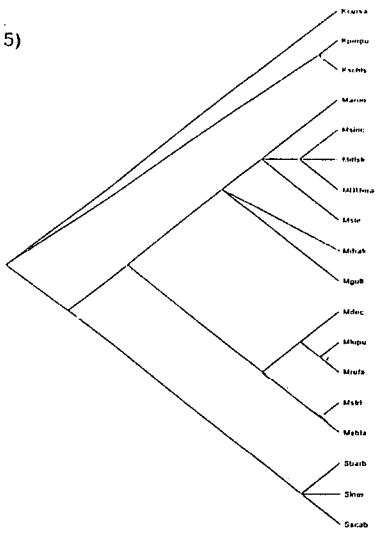
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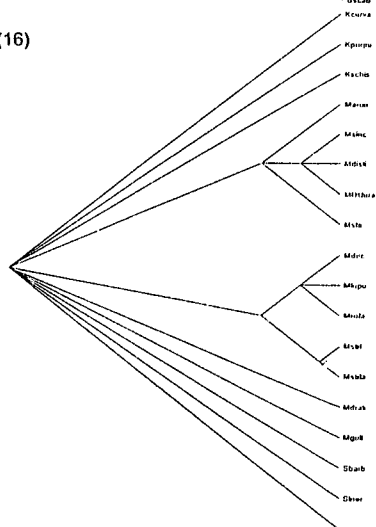
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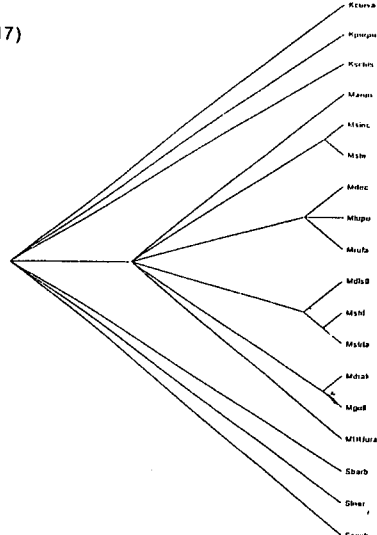
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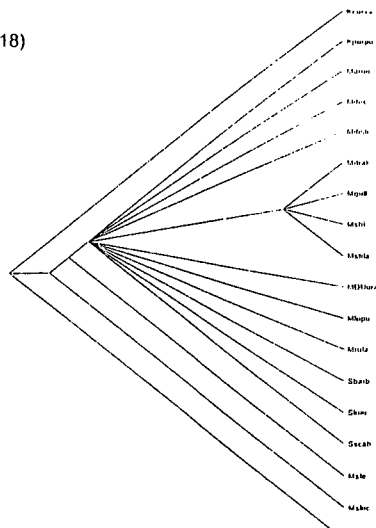
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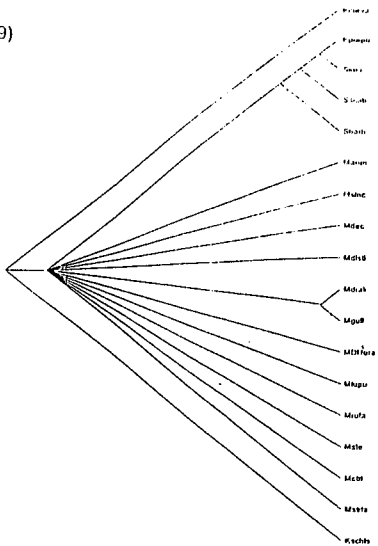
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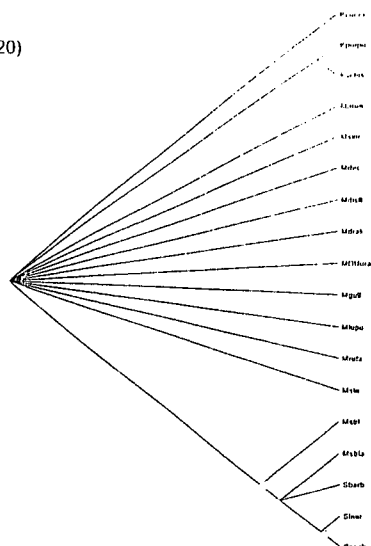
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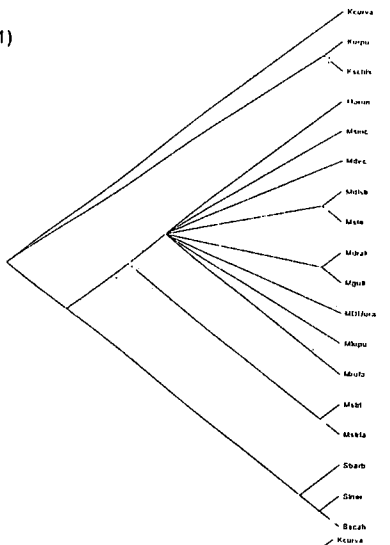
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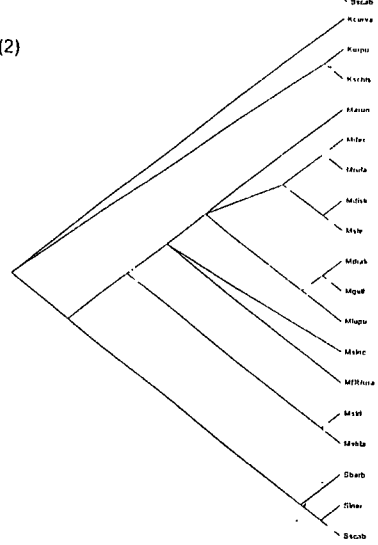
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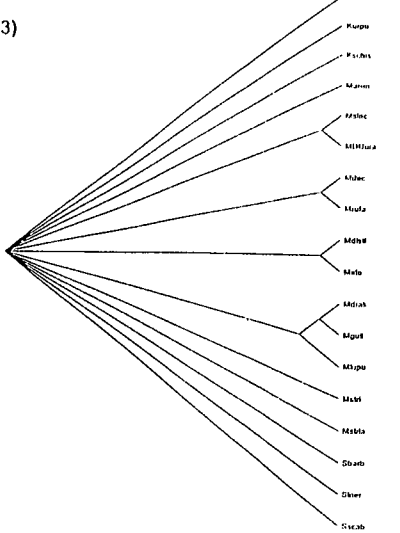
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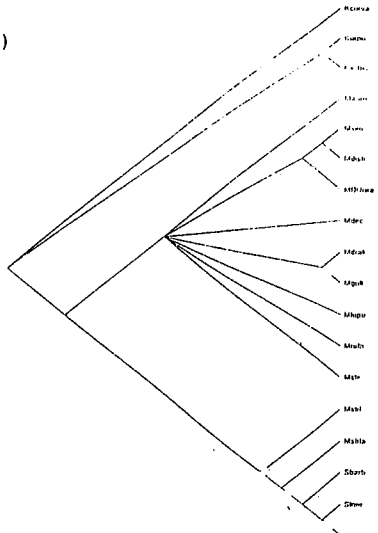
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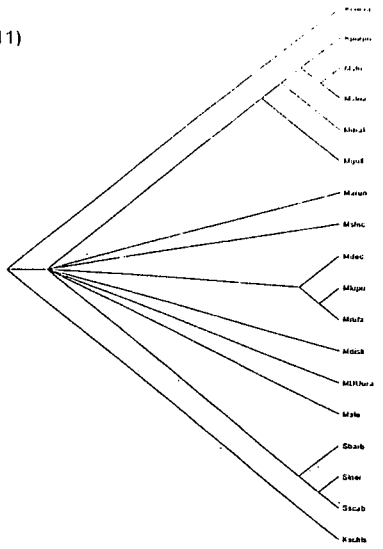
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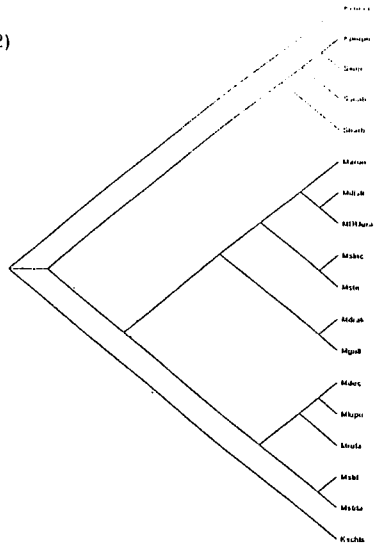
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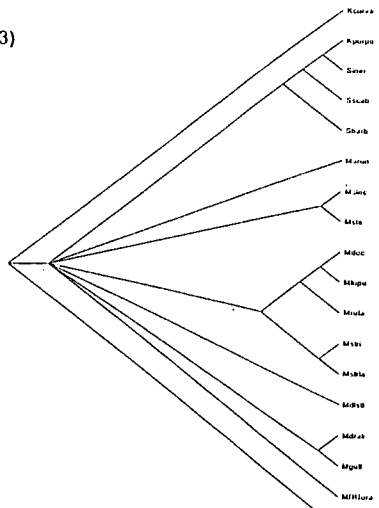
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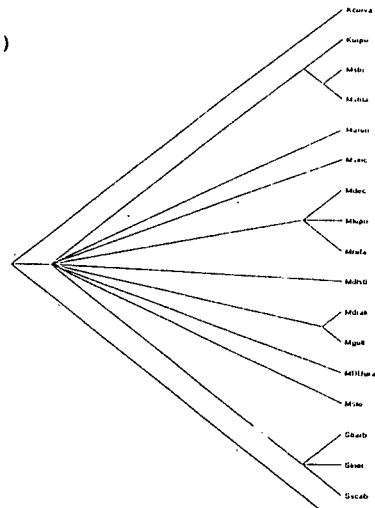
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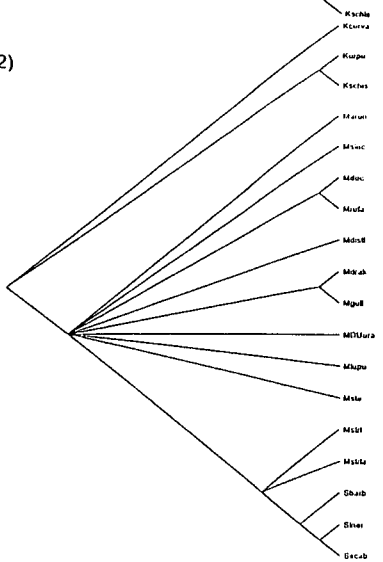
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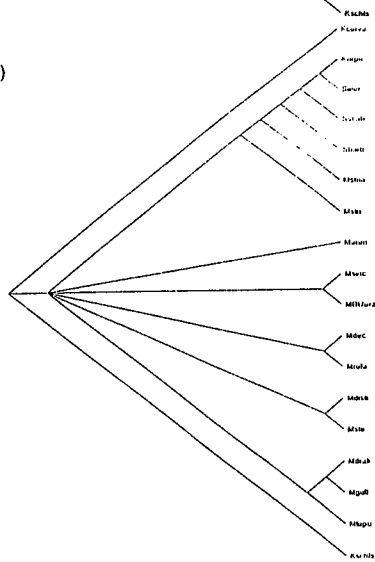
P8(1)



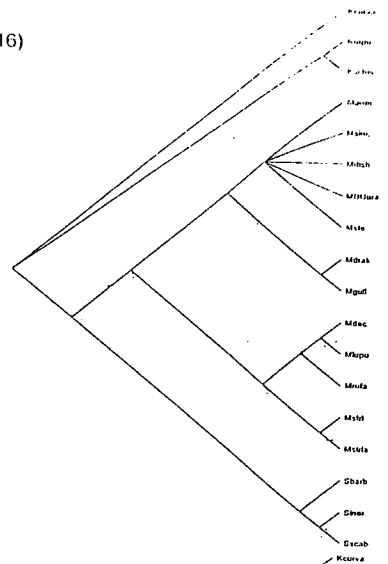
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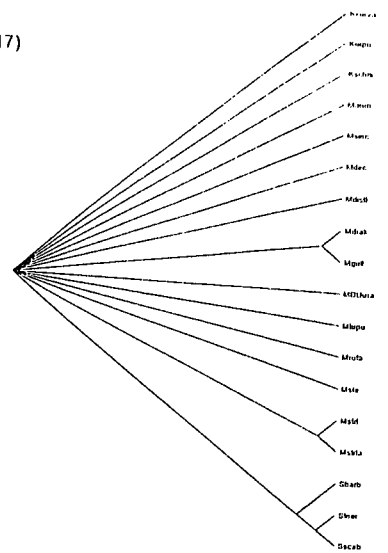
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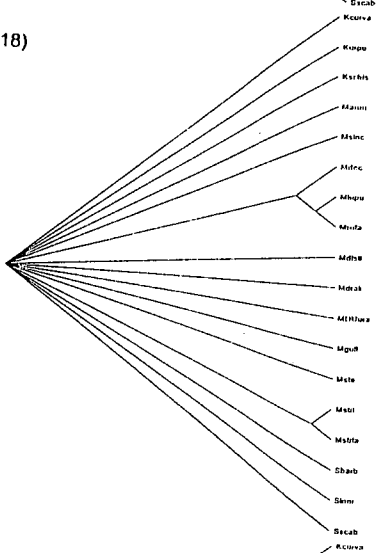
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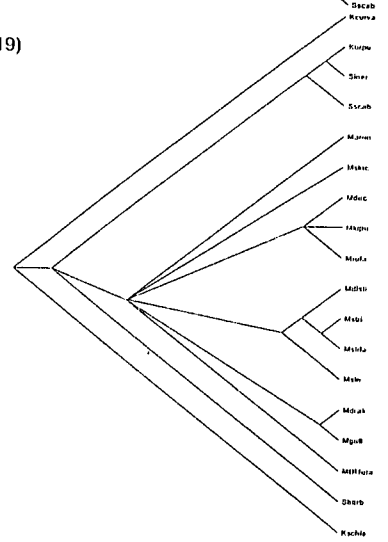
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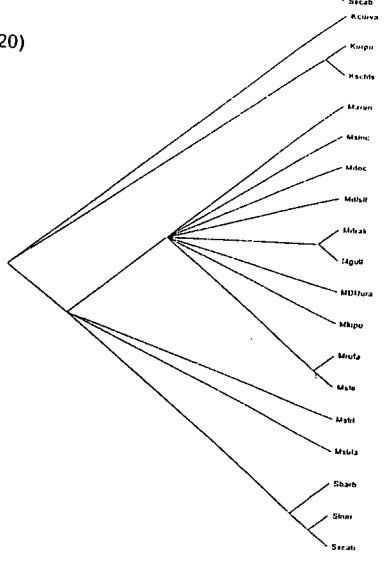
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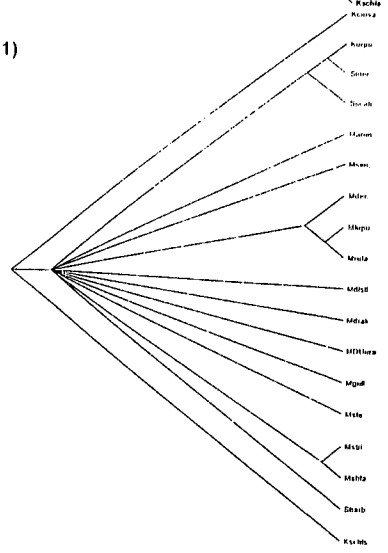
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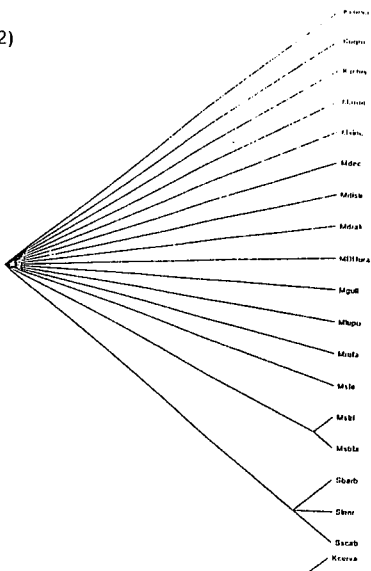
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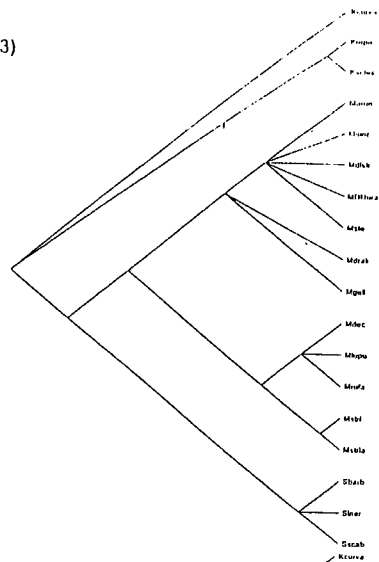
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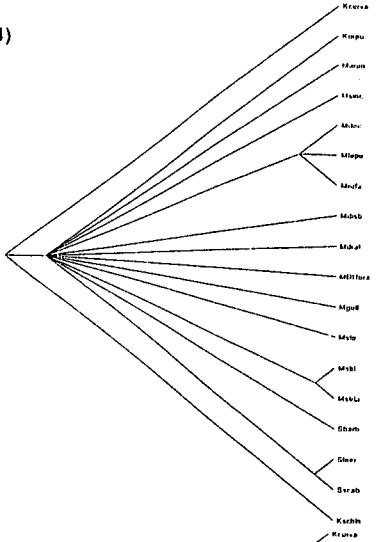
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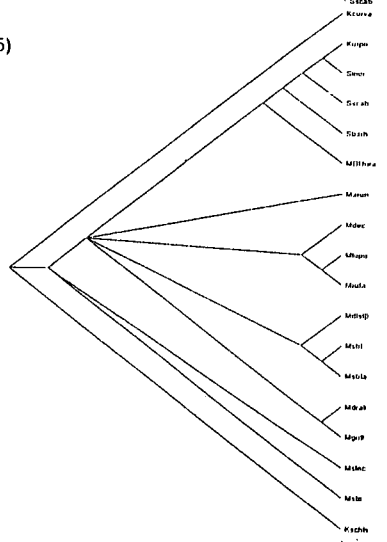
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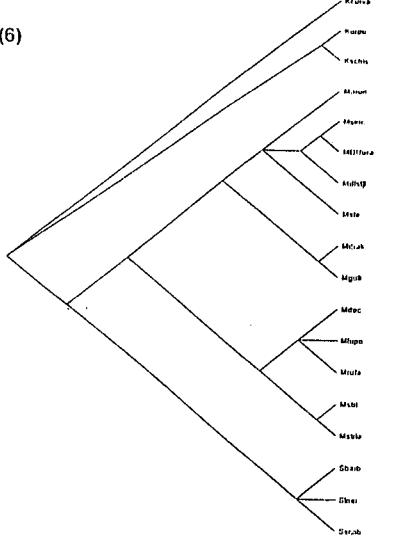
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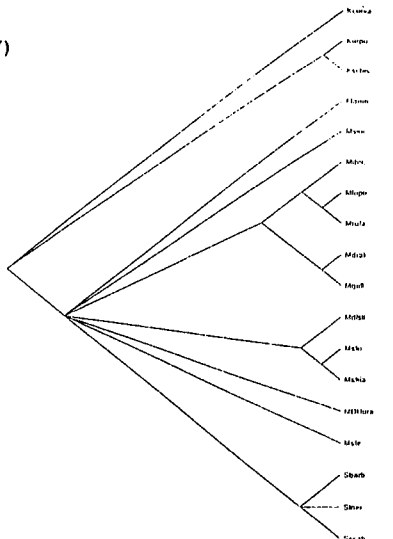
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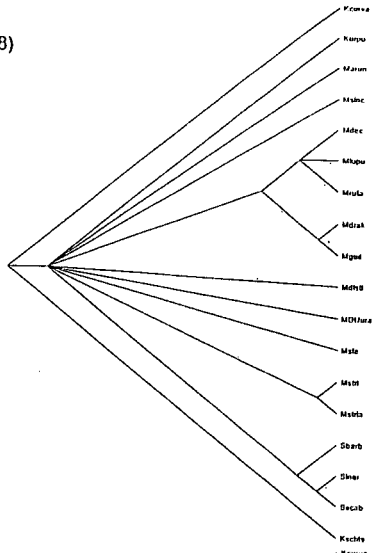
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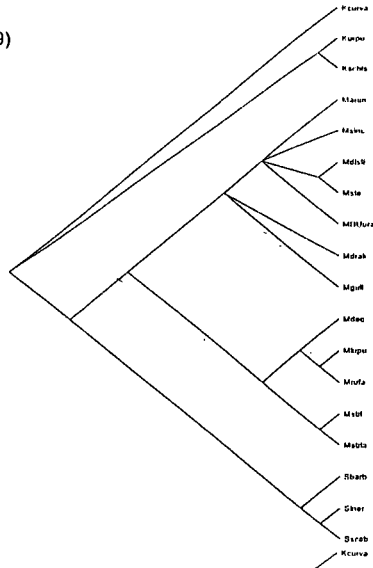
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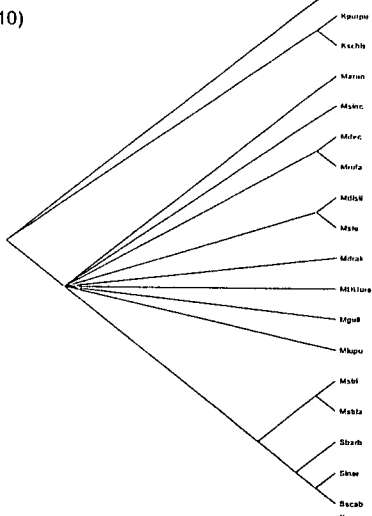
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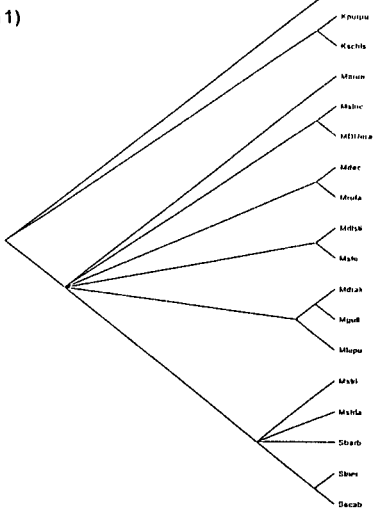
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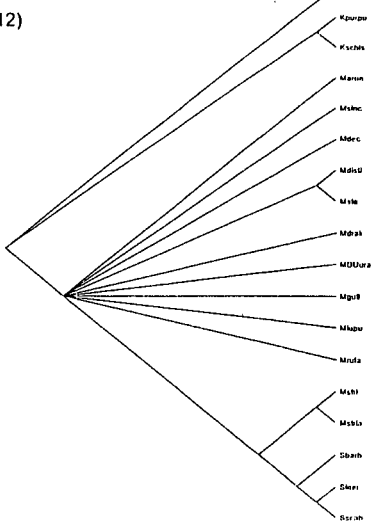
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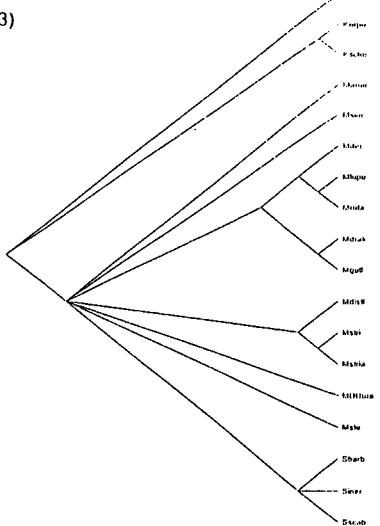
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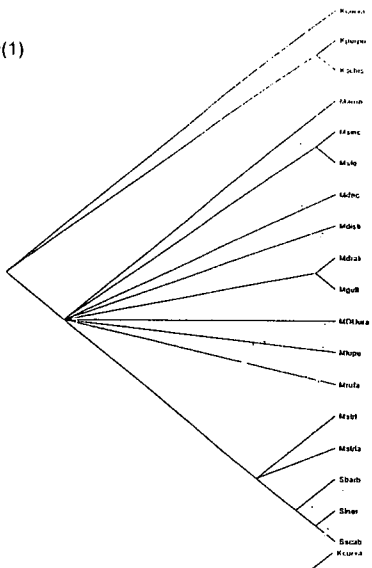
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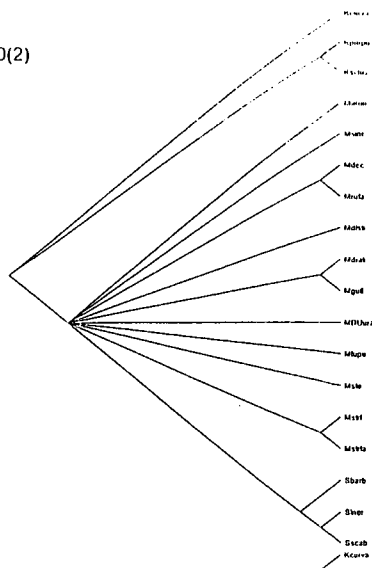
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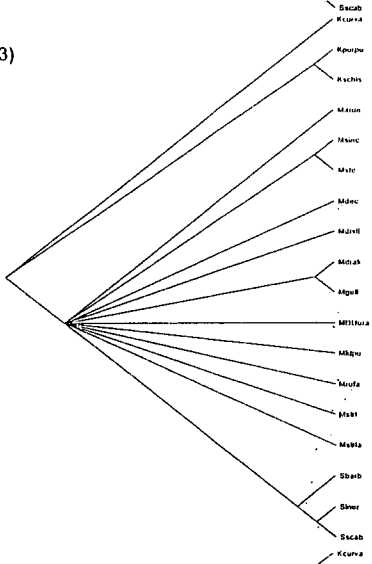
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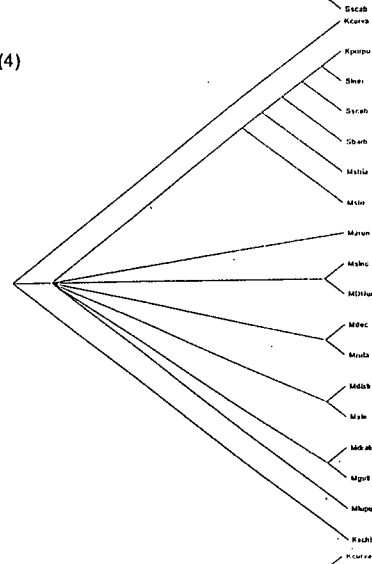
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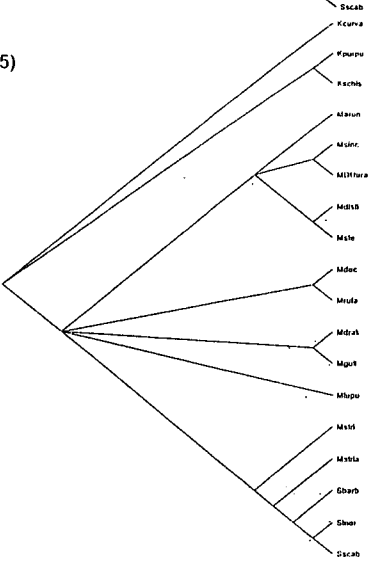
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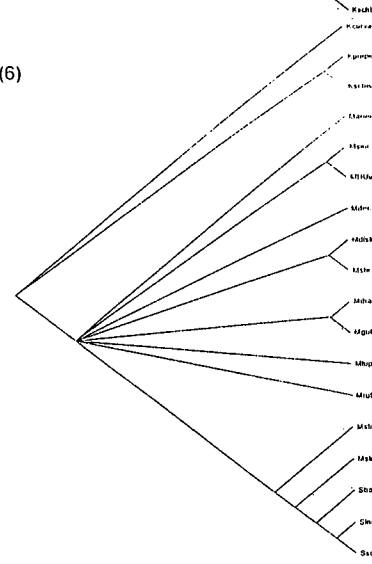
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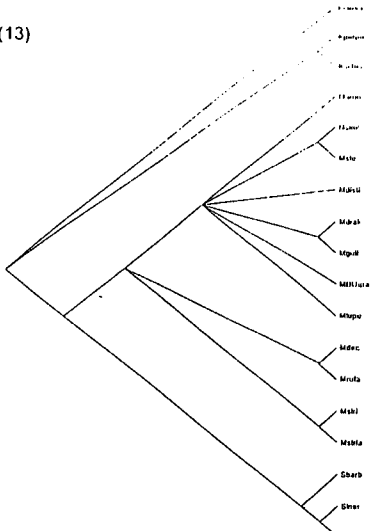
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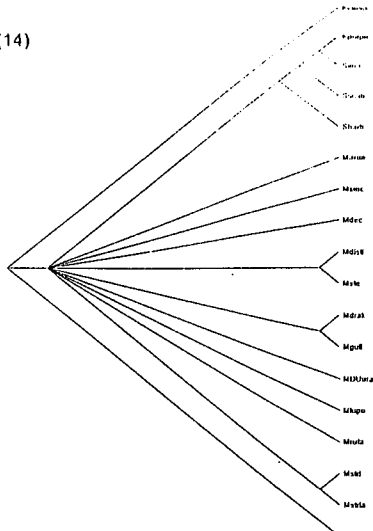
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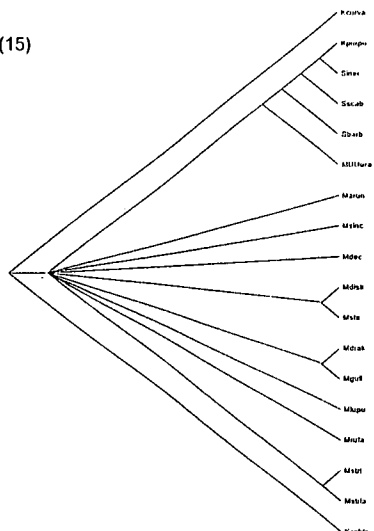
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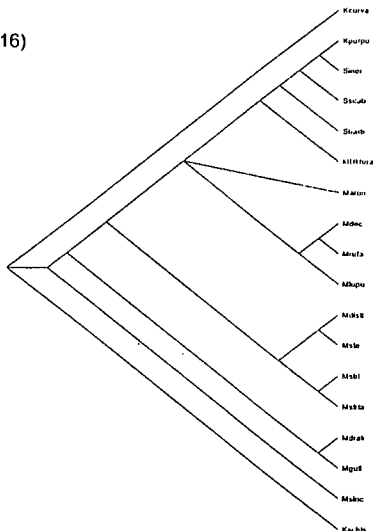
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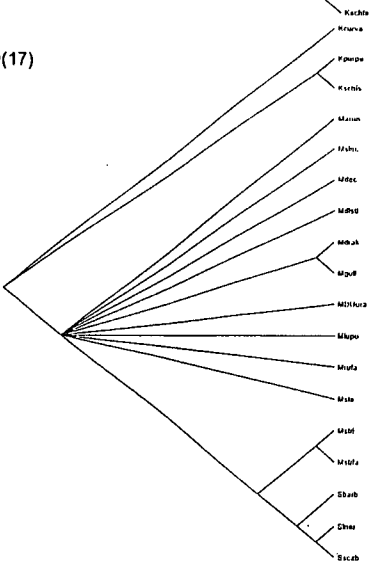
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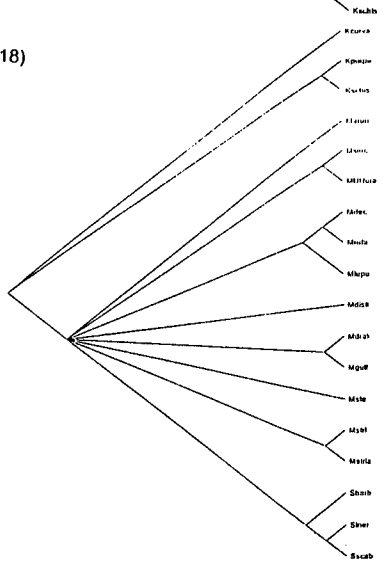
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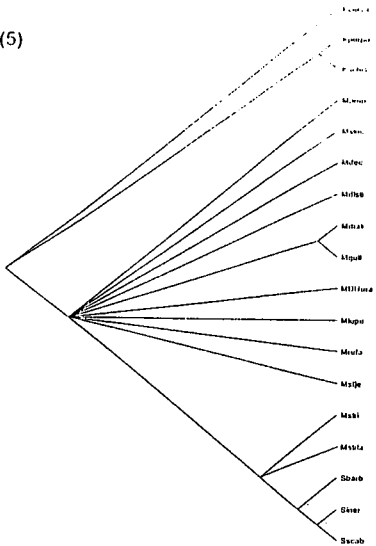
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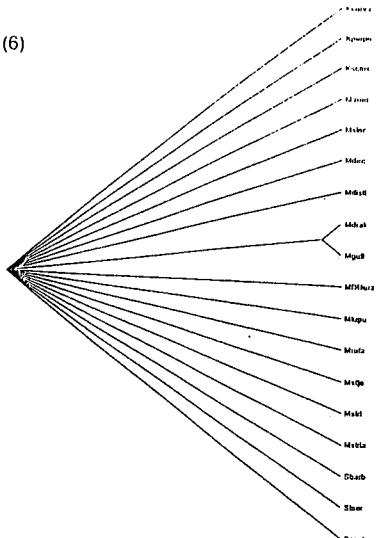
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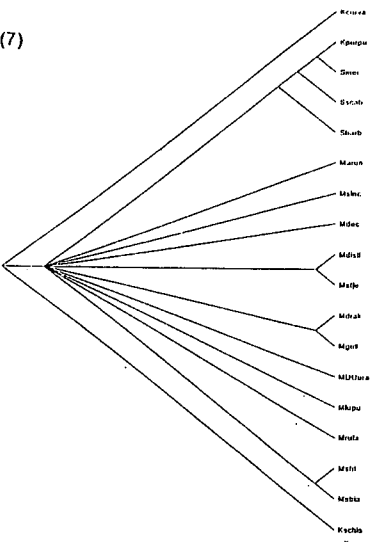
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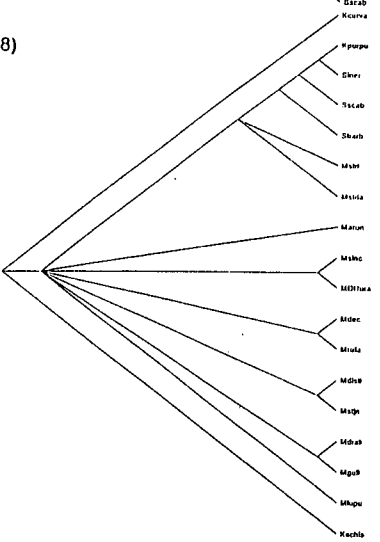
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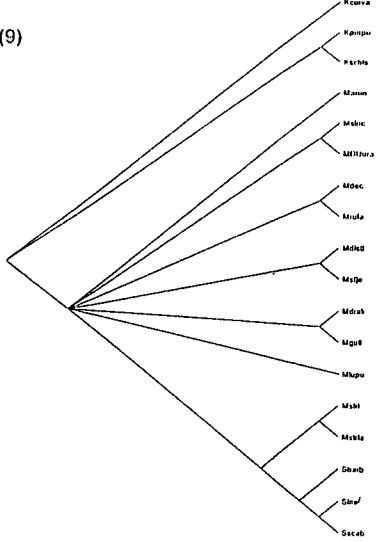
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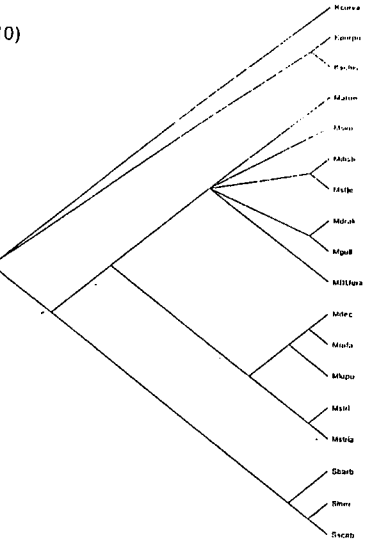
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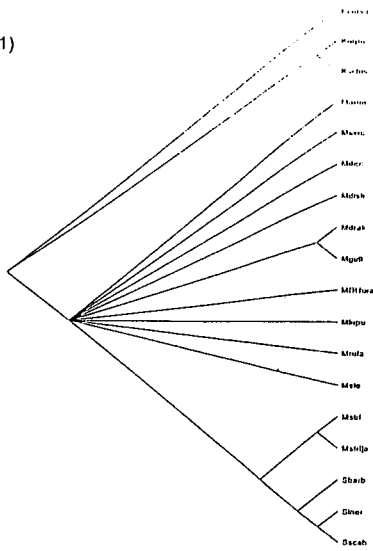
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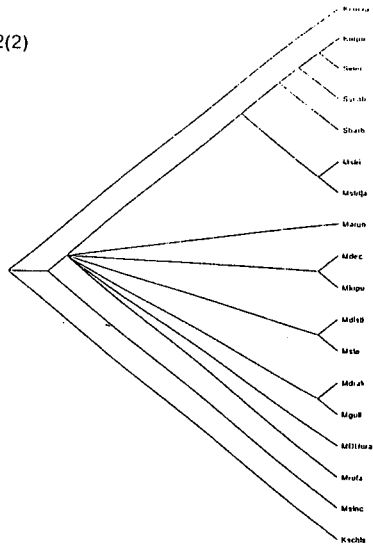
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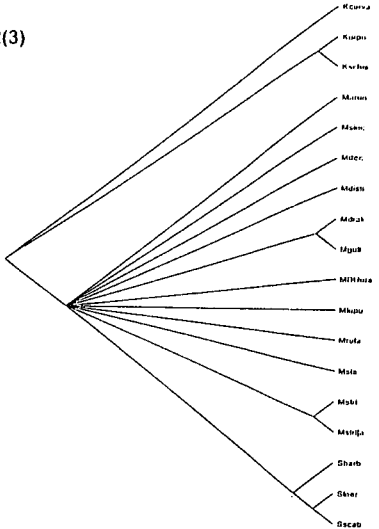
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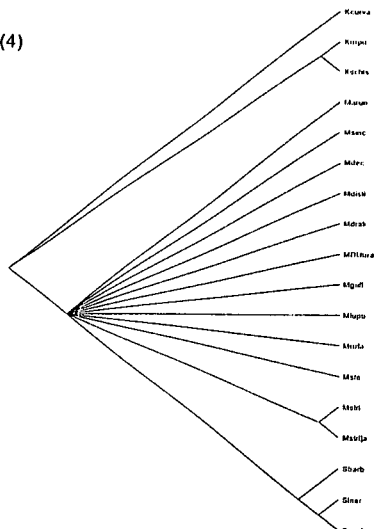
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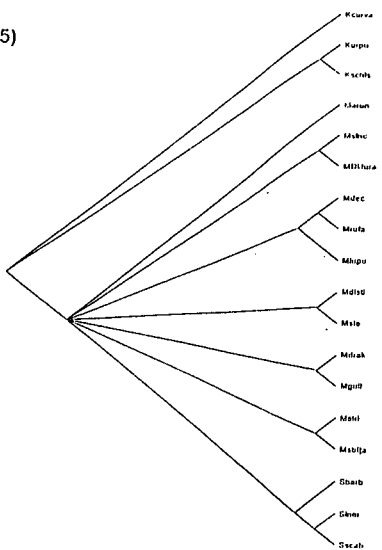
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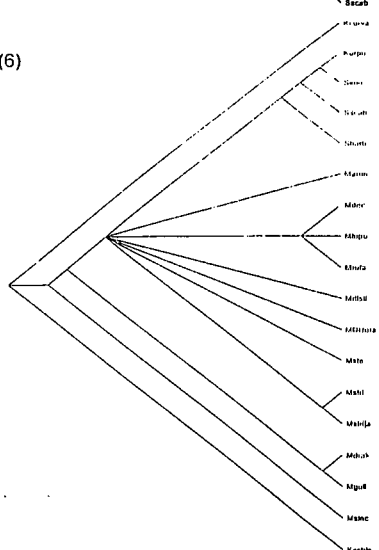
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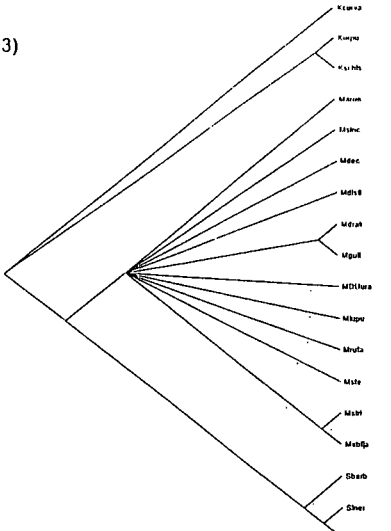
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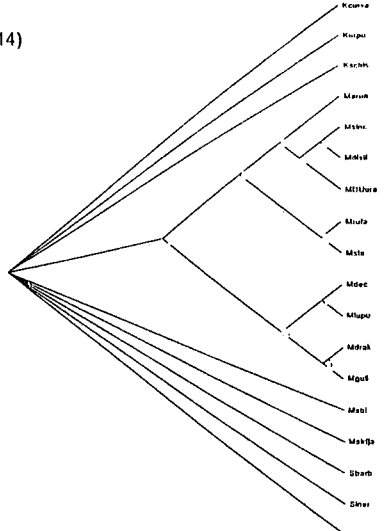
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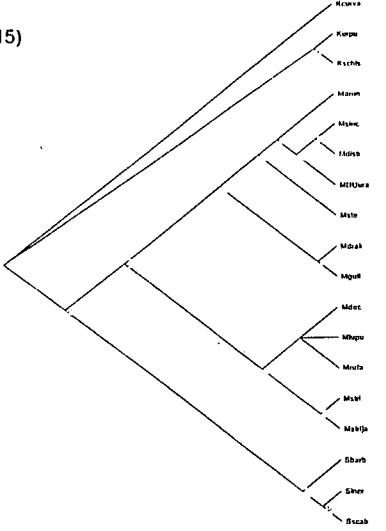
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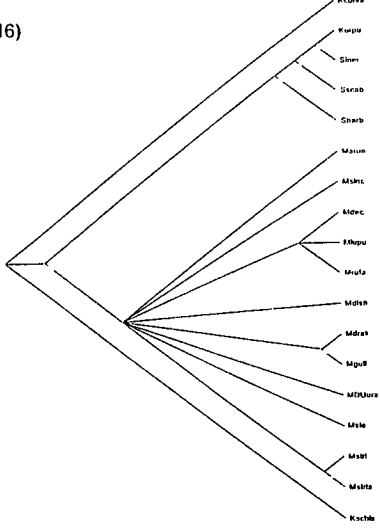
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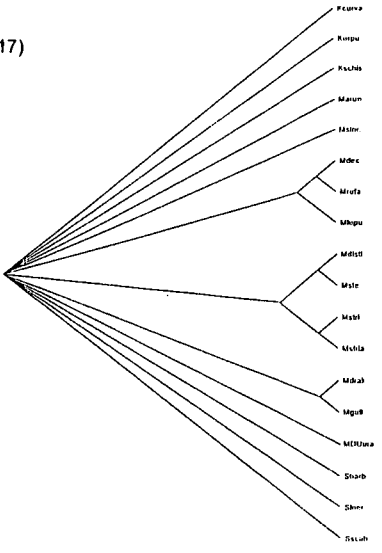
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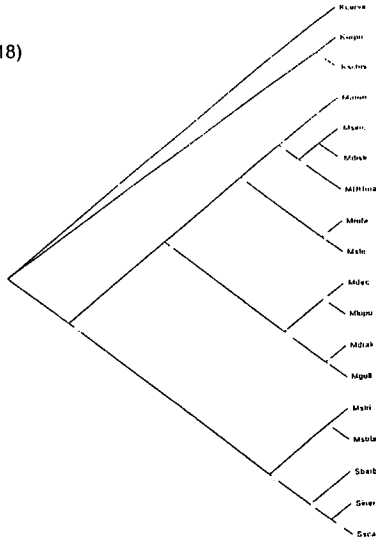
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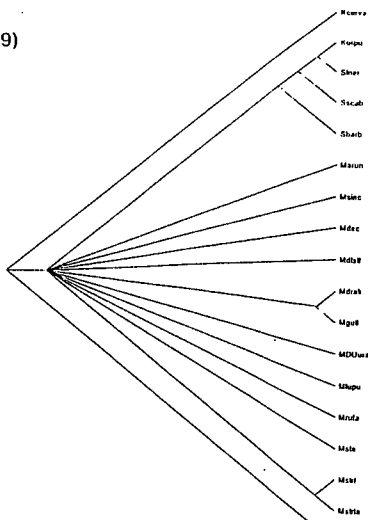
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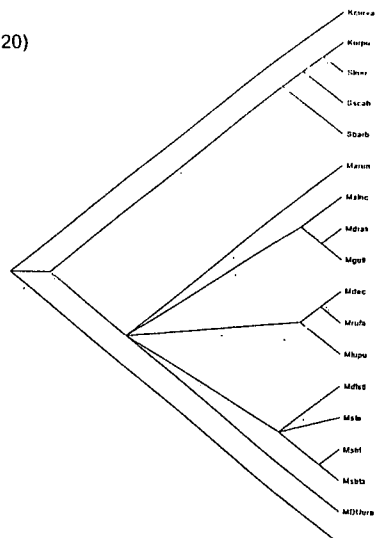
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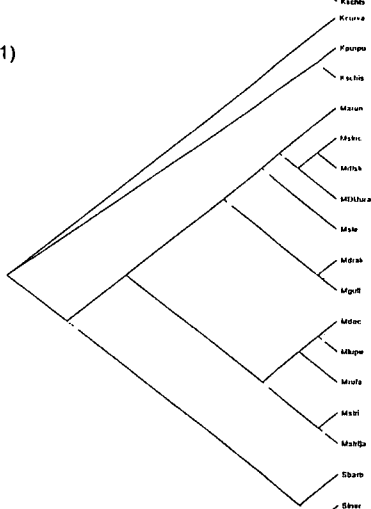
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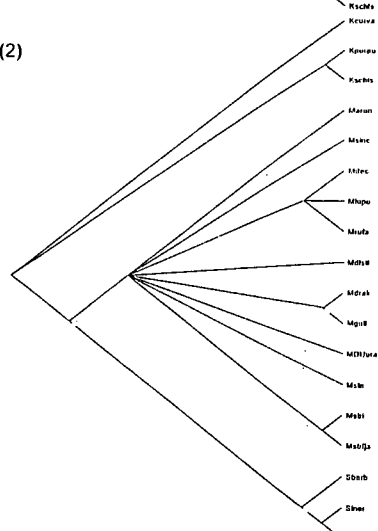
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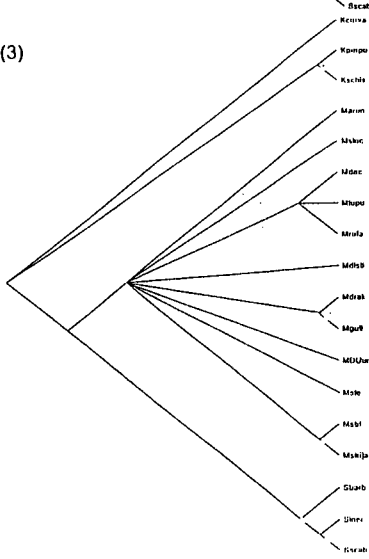
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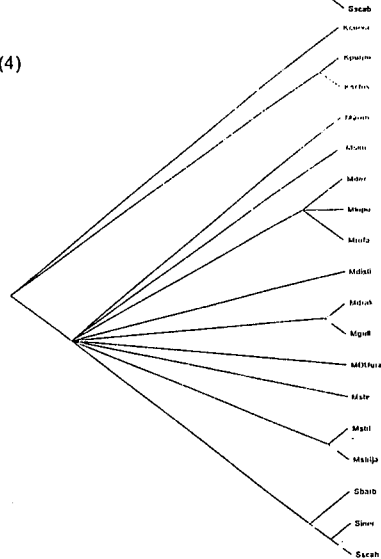
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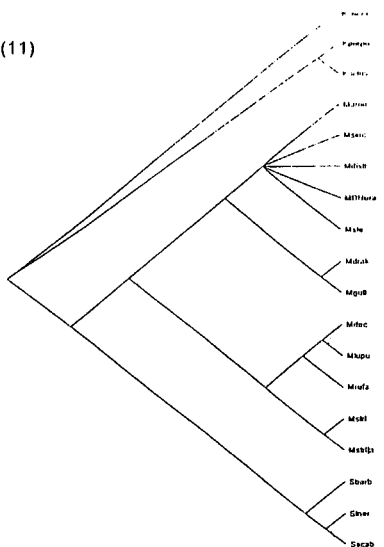
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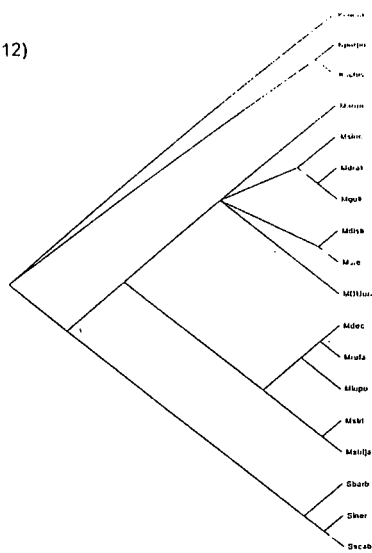
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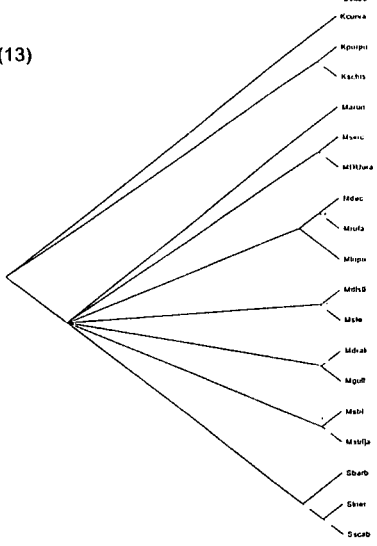
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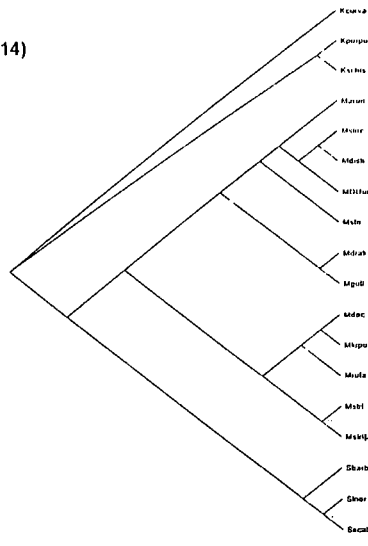
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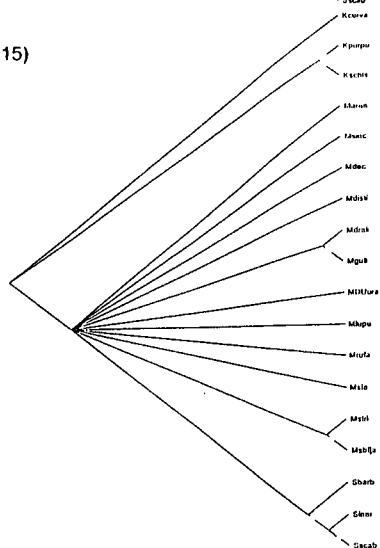
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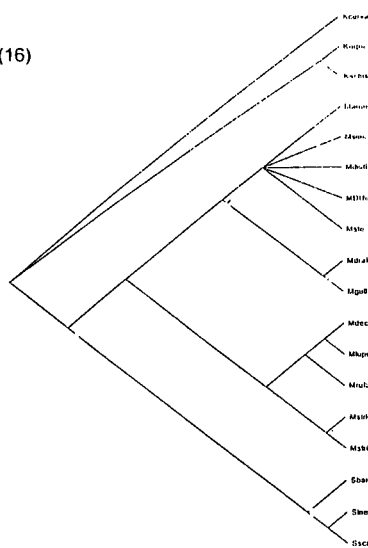
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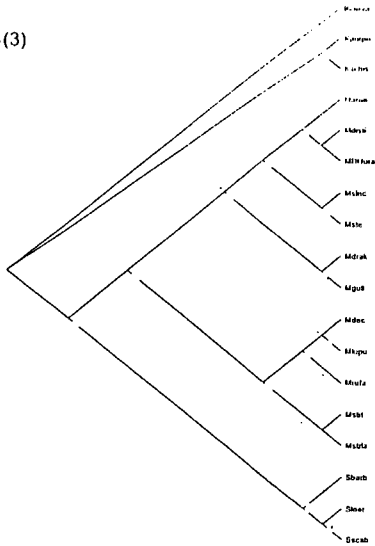
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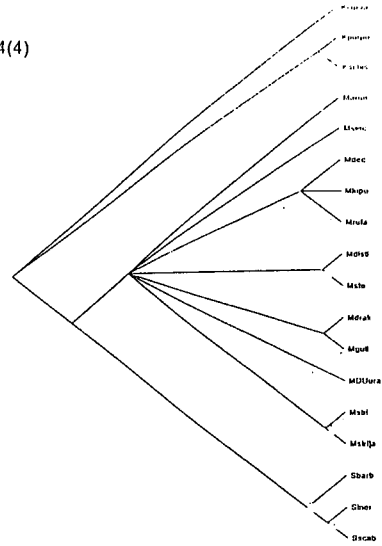
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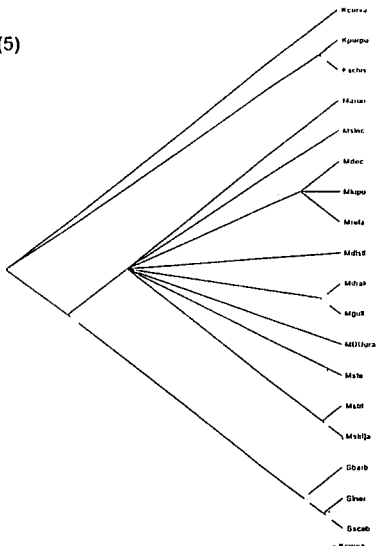
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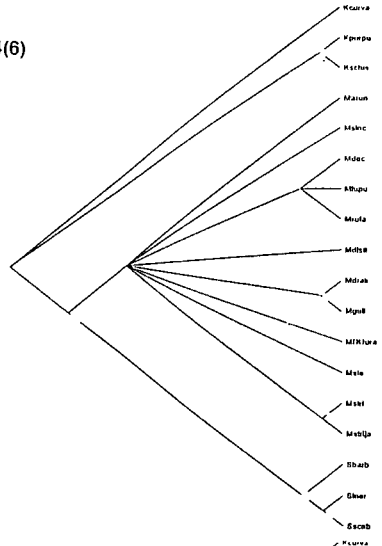
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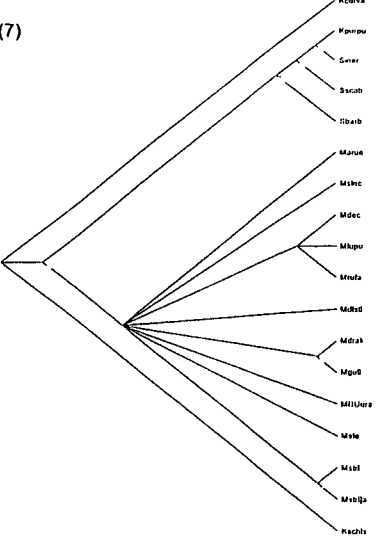
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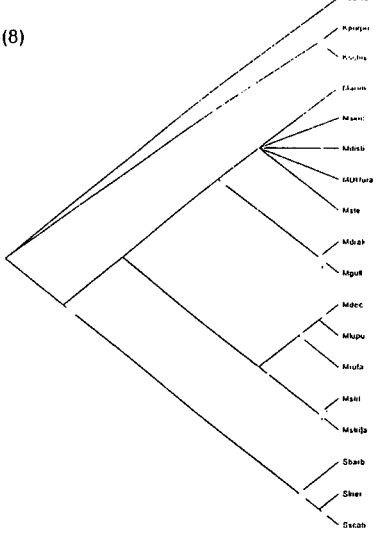
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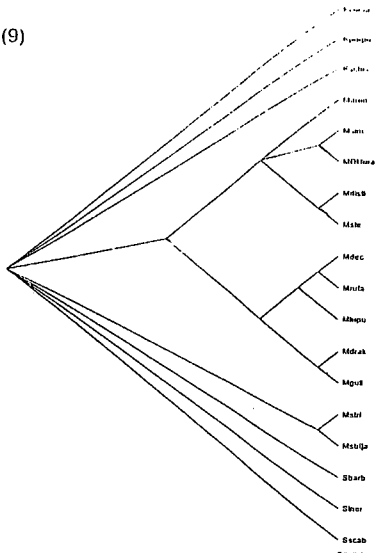
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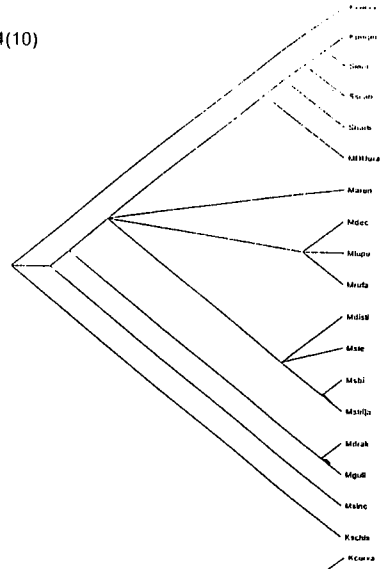
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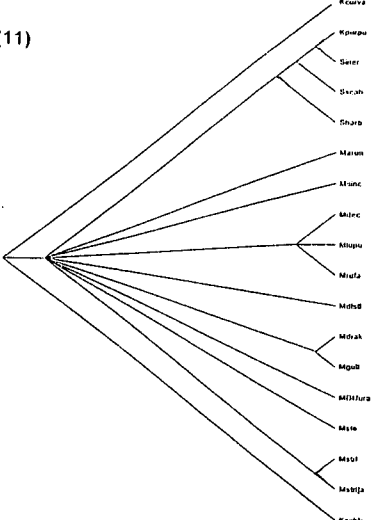
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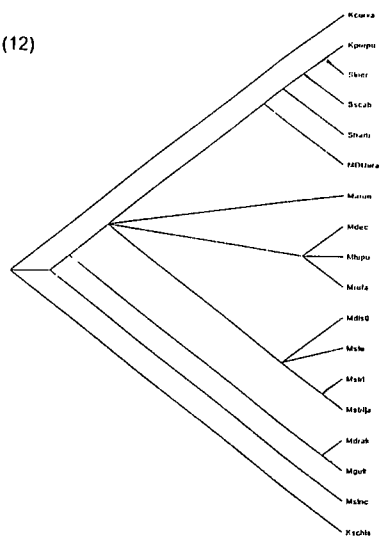
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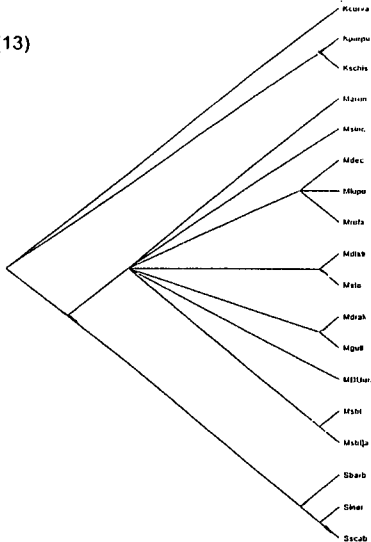
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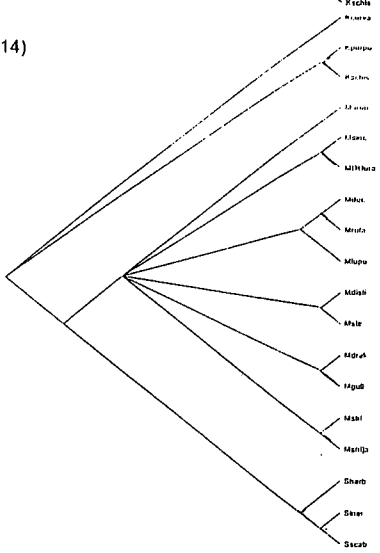
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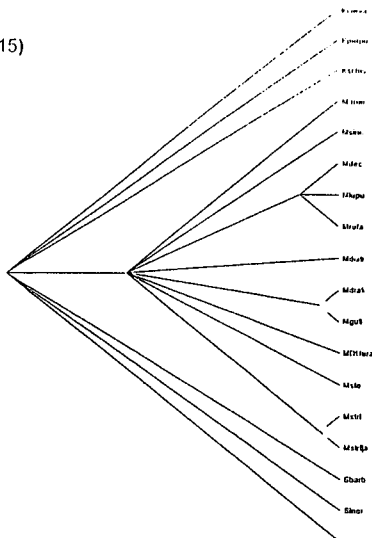
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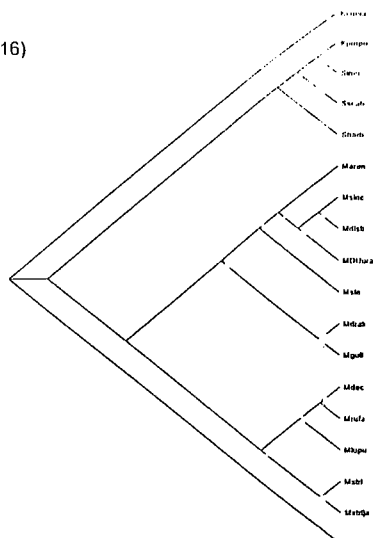
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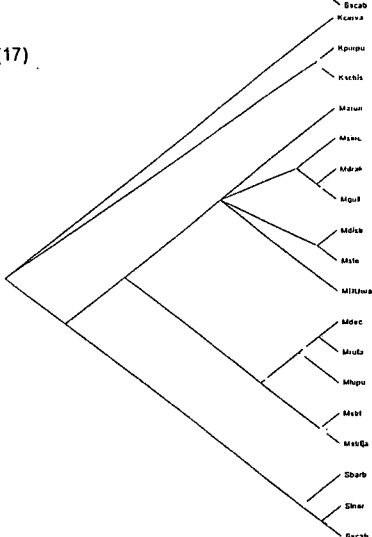
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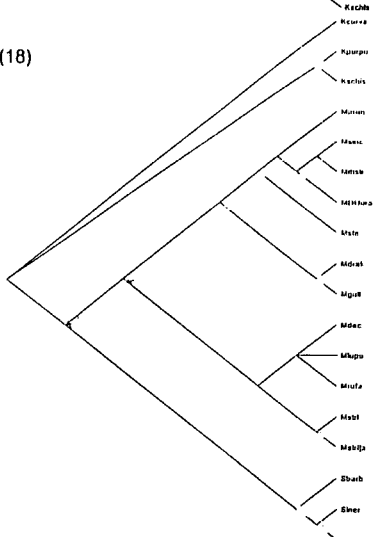
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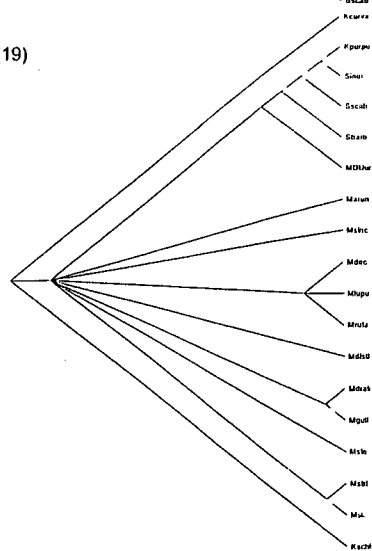
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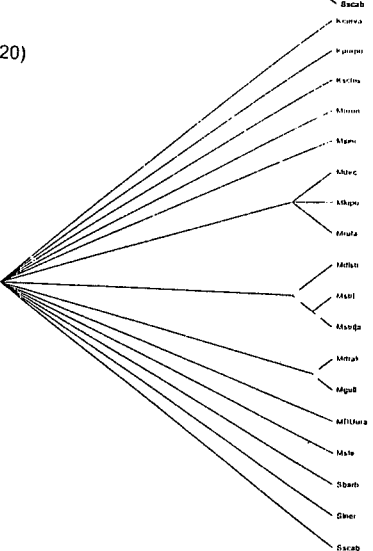
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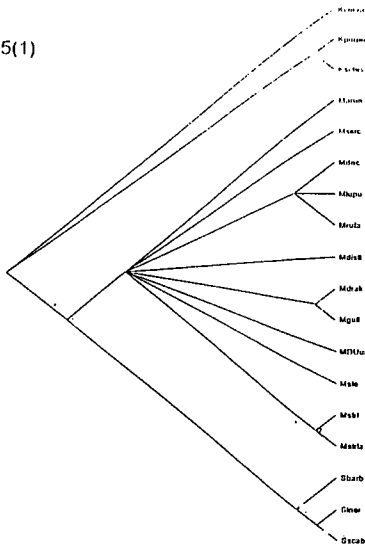
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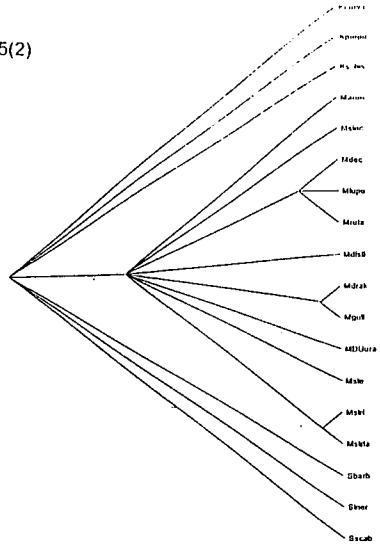
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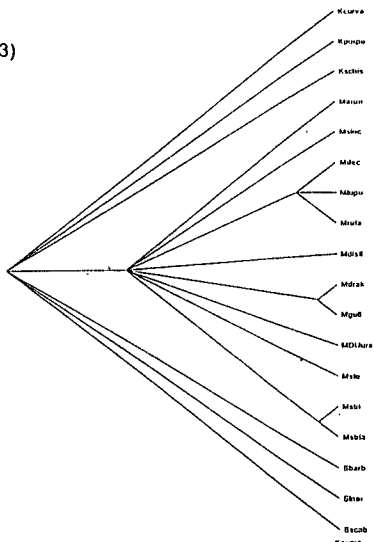
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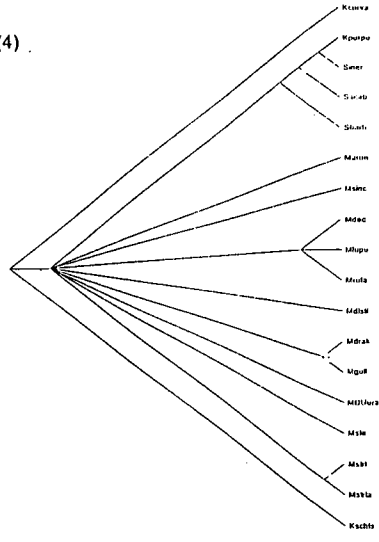
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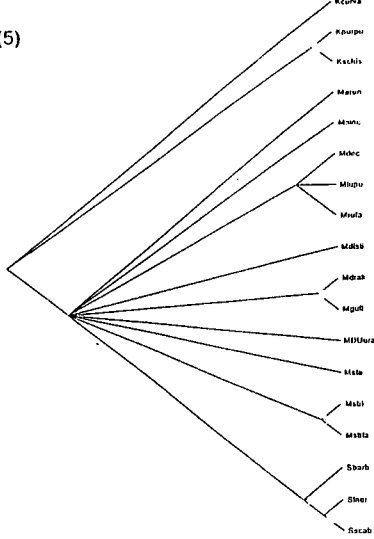
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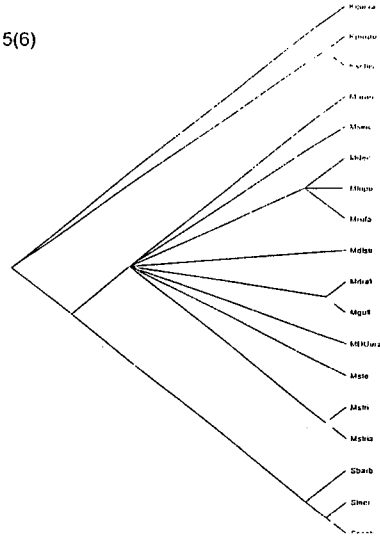
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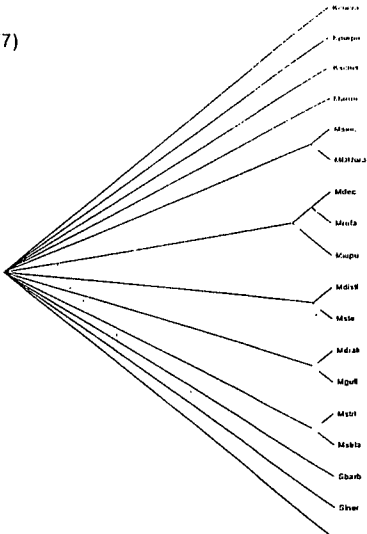
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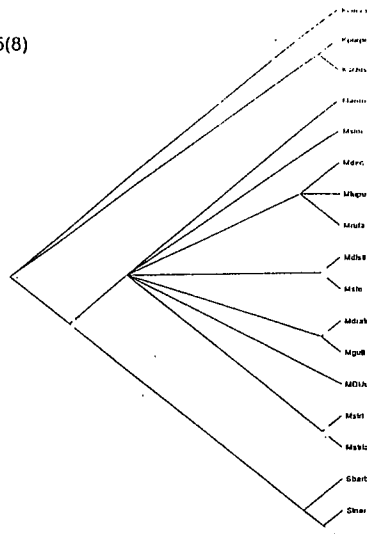
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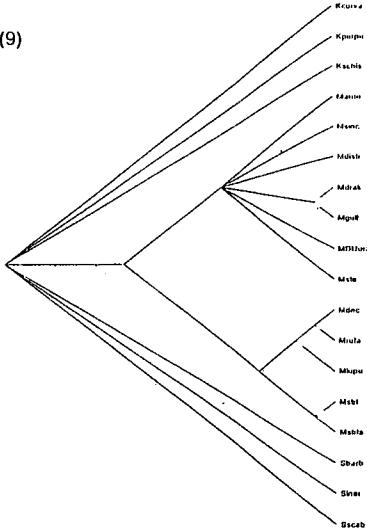
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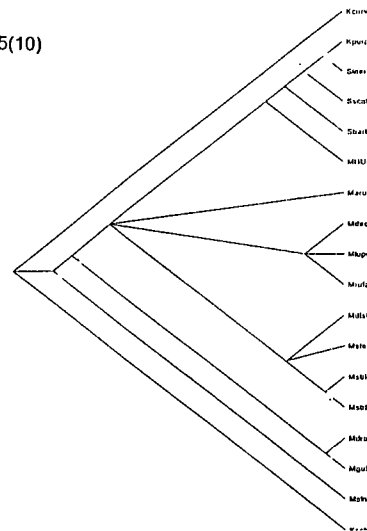
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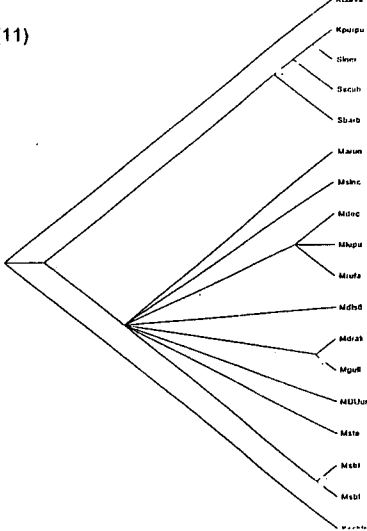
P15(9)



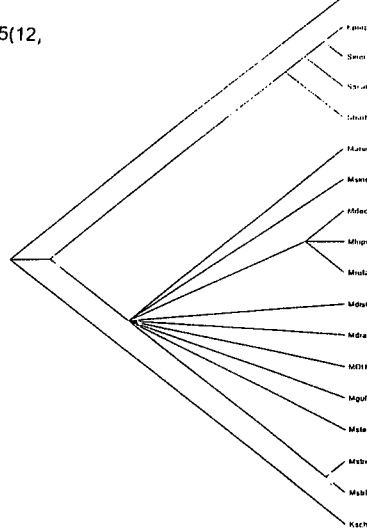
P15(10)



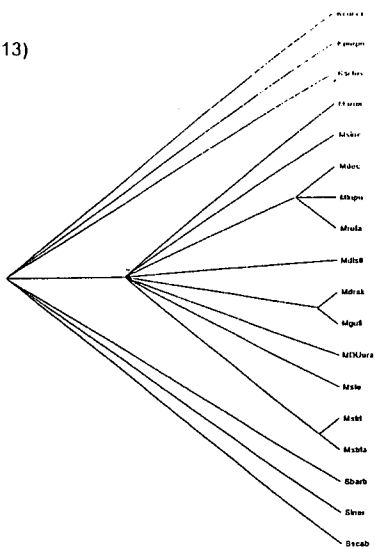
P15(11)



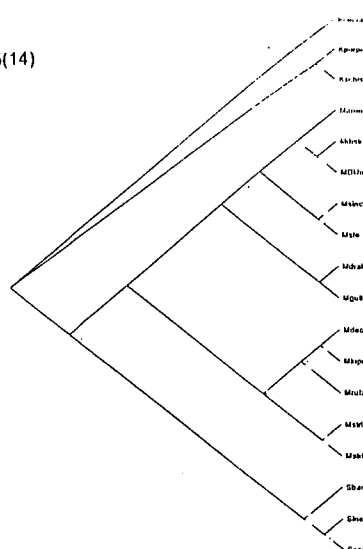
P15(12)



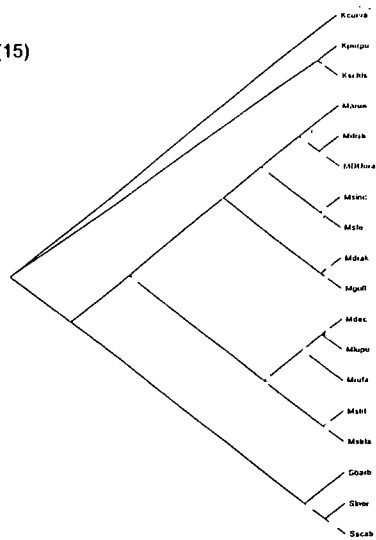
P15(13)



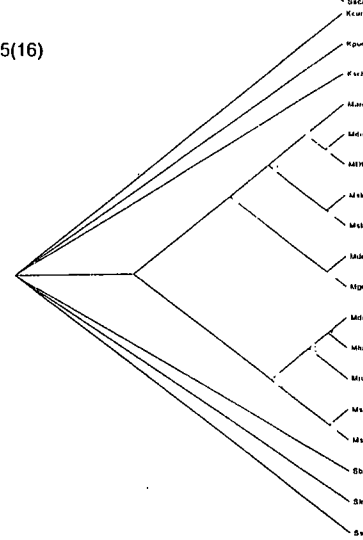
P15(14)



P15(15)



P15(16)



ADDENDUM F

R-VALUES FOR NUMBER OF PRIMERS

1	0.06
2	0.14
3	0.27
4	0.33
5	0.27
6	0.32
7	0.58
8	0.42
9	0.38
10	0.43
11	0.34
12	0.47
13	0.58
14	0.51
15	0.4

ADDENDUM G

T-VALUES FOR NUMBER OF PRIMERS

1	0.67
2	0.73
3	0.73
4	0.82
5	0.82
6	0.84
7	0.94
8	1.14
9	0.91
10	0.91
11	0.97
12	0.97
13	0.94
14	1
15	0.98

ADDENDUM H

S-VALUES FOR NUMBER OF PRIMERS

1	0.31
2	0.3
3	0.29
4	0.18
5	0.17
6	0.17
7	0.21
8	0.19
9	0.24
10	0.26
11	0.34
12	0.34
13	0.38
14	0.27
15	0.21

ADDENDUM I

I-VALUES FOR NUMBER OF PRIMERS

1	0.37
2	0.43
3	0.5
4	0.58
5	0.55
6	0.58
7	0.76
8	0.78
9	0.64
10	0.67
11	0.66
12	0.66
13	0.76
14	0.76
15	0.69

ADDENDUM J

Distance analysis of the RAPD data of the different specimens under investigation. The bold entries indicate the mean variation between and in genera.

	<i>K.curva</i>	<i>K.purpurea</i>	<i>K.schis</i>	<i>M.arun</i>	<i>M.sinc</i>	<i>M.dec</i>	<i>M.distl</i>	<i>M.drak</i>	<i>M.dura</i>	<i>M.gul</i>	<i>M.lupu</i>	<i>M.rufa</i>	<i>M.ste</i>	<i>M.stri</i>	<i>M.stri1</i>	<i>S.barb</i>	<i>S.iner</i>	<i>S.scab</i>
<i>Kcurva</i>																		
<i>Kpurpu</i>	0.08																	
<i>Kschis</i>	0.05	0.11	@ 0.08															
<i>Marun</i>	0.50	0.49	0.52															
<i>Msinc</i>	0.50	0.49	0.53	0.06														
<i>Mdec</i>	0.48	0.47	0.49	0.10	0.12													
<i>Mdisti</i>	0.53	0.52	0.56	0.07	0.08	0.12												
<i>Mdrak</i>	0.48	0.46	0.50	0.08	0.08	0.11	0.11											
<i>Mdura</i>	0.48	0.47	0.50	0.05	0.06	0.11	0.08	0.09										
<i>Mgull</i>	0.46	0.45	0.48	0.06	0.07	0.11	0.11	0.030	0.09									
<i>Mlupu</i>	0.50	0.48	0.51	0.06	0.10	0.06	0.11	0.09	0.09	0.07								
<i>Mrufa</i>	0.49	0.48	0.51	0.07	0.09	0.06	0.10	0.11	0.09	0.09	0.04							
<i>Mste</i>	0.51	0.50	0.53	0.06	0.06	0.12	0.07	0.11	0.09	0.09	0.10	0.08						
<i>Mstri</i>	0.50	0.46	0.50	0.11	0.12	0.12	0.11	0.11	0.11	0.12	0.12	0.11	0.12					
<i>Mstri</i>	0.50	0.47	0.51	0.11	0.12	0.12	0.11	0.11	0.12	0.12	0.12	0.11	0.12	0.01				
			@ 0.49												@ 0.09			
<i>Sbarb</i>	0.48	0.46	0.48	0.13	0.16	0.18	0.16	0.16	0.11	0.16	0.16	0.16	0.16	0.14	0.12			
<i>Siner</i>	0.46	0.40	0.45	0.21	0.22	0.24	0.25	0.23	0.17	0.22	0.22	0.23	0.25	0.20	0.20	0.12		
<i>Sscab</i>	0.48	0.46	0.47	0.20	0.22	0.24	0.22	0.24	0.18	0.23	0.23	0.22	0.23	0.22	0.21	0.11	0.09	
			@ 0.46												@ 0.20			@ 0.11

ADDENDUM K

#NEXUS [ITS1 en ITS2 apart]

BEGIN DATA;

DIMENSIONS NTAX=26 NCHAR=518;

FORMAT DATATYPE=DNA GAP=- MISSING=? SYMBOLS="ACGT" INTERLEAVE;

MATRIX

Aureag	TCGTGACCCGAAA-CCAAAA--C-TGACCGCGAACAAGTCACCTTGTCCGGCCACGCGGCTC
Macrocalycinag	TCGTGACCCGAAA-CCAAAA--C-TGACCGCGAACGCGTCACCTTGTCCGGCCGACGCGCTC
Eckloniig	TCGTGACCCGAAA-CCAAAA--C-TGACCGCGAACAAGTCACCTTGTCCGGTCGCACGGCTC
Purpurea	TCGTGACCCGAAAACCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCTGG
Tenella	TCGTGACCCGAAAACCAAAA--CC-GACCGCGAACGCGTCACGCTGCCCGGTTCGCGCGCTGG
Curva	TCGTGACCCGAAAACCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCTGG
Purpureag	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCTGG
Schismoides	TCGTGACCCGAAAACCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCTGG
Barbatus	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCGGTTCGCGCGCTGG
Scaberrimus	TCGTGACCCGAAA-CCAAAA--CCTGACCGTGAACGTGTACCTTGTCCGGTTCGCGCGCTGG
Inermis	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCGGTTCGCGCGCTGG
Barbatusg	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGT-ACCCTTGTCCGGTTCGCGCGCTGG
Drakens	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCCGG
Guillar	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCCGG
Dura	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCCGGTTCGCGCGCCGG
Durag	TCGTGACCCGAAA-CCAAAA-CC-GACCGCGAATGCGTCACCTTGTCCGG-CGCGCGCCGG
Arundinacea	TCGTGACCCGAAA-CCAATT--CC-GACCGCGATCGCGTCACCTTGTCCGGCCGCGCGCCGG
Disticha	TCGTGACCCGAAA-CCAATA--CC-GACCGCGATCGCGTCACCTTGTCCGGCCGCGCGCCGG
Strictal	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCGGCCGCGCGCCGG
Stricta	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGATCGCGTCACCTTGTCCGGCCGCGCGCCGG
Strictag	TCGTGACCCGAAA-CCAAAA--CC-GACCGCGAACGCGTCACCTTGTCCGGCCGCGCGCTGG
Decora	TCGTGACCCGAAA-CCAAAAAACC---CCGCGAACGCGTCATCA-CTGCCGCCGGGCGTCCGG
Lupulina	TCGTGACCCGAAA-CCAAAAAACC---CCGCGAACGCGTCATCA-CTGCCGCCGGGCGTCCGG
Rufa	TCGTGACCCGAAA-CCAAAAAACC---CCGCGAACGCGTCATCA-CTGCCGCCGGGCGTCCGG
Macowaniig	TCGTGACCCGAAA-CCAAAA-CC-GACCGCGAACGCGTCATCC-CTGCCGCCGGGCGTCCGG
Rangeig	TCGTGACCCGAAA-CCAAAA-CA-GACCGCGAACGCGTCATCC-GTGCCGCCGGGACGCGCCGG

Aureag	ACGCCGCGC-----GGCC-TAGGCCGCCGACCTCCGCGAGGAAGGGGAGCGGGCC
Macrocalycinag	GGCTCGCTCACGC-CGCGTGGCC-TAGGCCGCCGACCTCCGCCAGGATGGGGAGCGGGCC
Eckloniig	ACGCCGCGC-----GGCC-TAGGCCGCCGACCTCCGCAAGGACGGGGAGCGGGCC
Purpurea	GGATCCGTCTCGTTCGCGTGGCCATAGGCCGCCGACCTCCG-TCAGGAGGGGAGCGGGCC
Tenella	GGATCCGTCTCGTTCGCGTGGCCATAGGTAGGCCGACCTCCG-TCAGGAGGGGAGCGGTT
Curva	CCAACCGTCTCGTTCGCGGAGGCCATAGGCCGCCGACCTCCG-TCAGGAGGGGAGCGGGCC
Purpureag	GGATCCGTCTCGTTCGCGTGGC-ATAGGCCGCCGACCTCCG-TCAGGAGGGGAGCGGGCC
Schismoides	GGCTT-GTCCTCGACGTGTGGCCTAAGGCCGCCGACCTCTG-TCAGGAGGAGAGTGGCC
Barbatus	GGCTT-GTCCTCGACGTGTGGCCTAAGGCCGCCGACCTCTG-TCAGGAGGAGAGTGGCC
Scaberrimus	GGCTT-GTCCTCGACGTGTGGCCTAAGGCCGCCGACCTCTG-TCAGGAGGAGAGTGGCC
Inermis	GGCTT-GTCCTCGACGTGTGGCCTAAGGCCGCCGACCTCTG-TCAGGAGGAGAGTGGCC
Barbatusg	GGCTT-GTCCTCGACGTGTGGCCTAAGGCCGCCGACCTCTG-TCAGGAGGAGAGTGGCC
Drakens	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CTAGTTAAACAGCGGGCC
Guillar	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CTAGGCCGCCGAGCGGGCC
Dura	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CTAGGGGGGCGAGCGGGCC
Durag	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CTAGGGGGGCGAGCGGGCC
Arundinacea	GGCTT-GTCCCTGTCGCGTGGCCCATAGGCCGCCGACCTCCG-CTAGGGGGGCGAGCGGGCC
Disticha	GGCTT-GTCCCTGTCGCGTGGCCCAAGGAAGCCGAACTCCG-CTAGGGGGGCGAGCGGGCC
Strictal	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CTAGGGGGGCGAGCGGGCC
Stricta	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CTAGGGGGGCGAGCGGGCC
Strictag	GGCTT-GTCCCTGTCGCGTGGCCCAAGGCCGCCGACCTCCG-CAAGGAGGGGAGCGGGCC
Decora	CCGGGGCTTGTGCGCCGACGGCCCA-CCGGCCC---CGCCGACCTCCGG-----CC
Lupulina	CCGGGGCTTGTGCGCCGACGGCCCA-CCGGCCC---CGCCGACCTCCGG-----CC
Rufa	CCGGGGCTTGTGCGCCGACGGCCCA-GGGCCCC---CGCCGACCTCCGG-----CC
Macowaniig	GGCTTCCGGGGCGCCGACGGCCCA-GGGCCCC---CGACCGGGGGGG-----CC
Rangeig	GGCTTCCGGGGCGCCGACGGCCCA-GGGCCCCGACCTCCGTCGGGGGGGGAGGGGGC

Aureag	GC-AAAAGAACCCACGGCGCCGTACGG-CGT-CAAGGAA-AAC---TGTTATTGCCTTG
Macrocalycinag	GC-AAAAGAACCCACGGCGCCGTACGG-CGT-CAAGGAA-AAC---TGTTATTGCCTTG
Eckloniig	GC-AAAAGAACCCACGGCGCCGTACGG-CGT-CAAGGAA-AAC---TGTTATTGCCTCG
Purpurea	GC-AAAAGAACCCACGGCGCCGGATGG-CGT-CAAGGA---ACACTTGATATTGCCTTG
Tenella	GC-AAAAGAACCCACGGCGGGCGATGG-CGT-CAAGGA---ACACTTGATAAAGCCTAG
Curva	GC-AATTGAACCCACGGCGCCGGATGC-CGT-CATGGA---ACACTTGATATTGCCTTG
Purpureag	GC-AAAAGAACCCACGGCGCCGGATGG-CGT-CAAGGA---ACACTTGATATTGCCTTG
Schismoides	TC-AAAAGAACCAACGGCGCCGAACGG-CGT-CAAGGAGGAACACTTGATATTGCCTTG
Barbatus	TC-AAAAGAACCAACGGCGCCGAACGG-CGT-CAAGGA---ACACTT-ATATTGCCTTG
Scaberrimus	TC-AAAAGAACCAACGGCGCCGTACGG-CGT-CAAGGA---ACACTT-ATATTGCCTTG
Inermis	TC-ATTAGAACCATCGGCGCCGAACGG-CGT-CAAGGA---ACACTT-ATATTGCCTTG
Barbatusg	TC-AAAAGAACCAACGGCGCCGAACGG-CGTCAAGGA---ACACTT-ATATTGCCTTG
Drakens	GC-TTTTGAACGGACGGGGCCGAACGG-CGTCAACGGA---ACACTT-ATATTGCCTTG
Guillar	GC-ATTAGAACCTTTGGCGCCGAACGC-CGTCAACGGA---ACACTT-ATATTGCCTTG
Dura	GC-AAAAGAACCCACGGCGCCGAACGG-CGTCAACGGA---ACACTT-ATATTGCCTTG
Durag	GC-AAAAGAACCCACGGCGCCGAACGG-CGTCAA-GGA---ACACTT-ATATTGCCTTG
Arundinacea	GC-AAAAGTTCCCAGGGCGCCCAACGG-CGTCAACTGC----TAATC-AAAAGAC---
Disticha	CC-AAAAGTTCCCAGGGCGCCCAACGG-CGTCAACTGC----TAATC-AAAAGAC---
Strictal	GC-AAAAGAACCCACGGCGCCGAACGG-CGTCAACTGC----TAATCCACACGACTCTC
Stricta	GC-AAAAGAACCCAGGGCGCCCAACGG-CGTCAACTGC----TAATC-ACACGACTCTC
Strictag	GCCAAAAGAACCCACGGCGCCGTACGGGCGTCAA-GGA---ACACTG-AAATTGCCTTG
Decora	GC-AACAGAACCCACGAAAACGAACGG-CGCTAA-GGA---AACGGGTTATTGCC-TG
Lupulina	GC-AACAGAACCCACGAAAACGAACGG-CGTCAA-GGA---AACGGGTTATTGCC-TG
Rufa	GC-AACAGAACCCACGAAAACGAACGG-CGTCAA-GGA---AACGGGTTATTGCC-TG
Macowaniig	GC-AACAGAACCCACGGCGCCGAACGG-CGTCAA-GGA---ACACTG-TTATTGCCCTG
Rangeig	GC-AACAGAACCCACGGCGCCGACCGG-CGTCAA-GGA---ACACCG-ATATTGCCTTG

Aureag	CGCG----TGGCCGTGG
Macrocalycinag	CGCGCGG-TGGCGG---
Eckloniig	CGCGCGGCCGCGGCTG-
Purpurea	CGCGCGGCTG---CTG-
Tenella	CGCGCGGCTG---CTG-
Curva	CGCGCGGCTG---CTG-
Purpureag	CGCGCGGCTG---CTG-
Schismoides	CGCGCGGCTG---CTG-
Barbatus	CGCGCGGC-GT---TGG
Scaberrimus	CGCGCGGC-GT---TGG
Inermis	CGCGCGGC-GT---TGG
Barbatusg	CGCGCGGC-GT---TGG
Drakens	CGCGCGGC-GG---TGG
Guillar	CGCGCGGC-GG---TGG
Dura	CGCGCGGC-GG---TGG
Durag	CGCGCGGC-GG---TGG
Arundinacea	-GATAC-----CTGG
Disticha	GGCAACG-----
Strictal	GGCAACG-----
Stricta	GGCAACG-----
Strictag	CGCGTGG-TGGC---GG
Decora	CACGGGGCTTGCC----
Lupulina	CACGGGGCTTGCC----
Rufa	CACGGGGCTTGCC----
Macowaniig	CGCGGGGCTG----TGG
Rangeig	CGAGGCGCCG-CGGC--

Aureag	CTGGCCTGCCAGCCGCGC-CGCGCGCAGCGATTCTATAC-TAATC
Macrocalycinag	CTGGCCTGCCGGTTCGCC-CGCGCGCAGCGATTCTATAC-TAATC
Eckloniig	CTGGCCCAGCCGAC-CGCGCGCAGCGATTCTATAC-TAATC
Purpurea	CCGGCCTGCCGGCCGCTC-CGCGCGCAGCGATTCCATACTTAATC
Tenella	CCGGCCTGAAGCCGCTC-CGCGCGCAGCGATTCCATACTTAATC
Curva	CCGGCCTGCCGGCCGCTC-CGCGCGCAGCGATTCCATACTTAATC
Purpureag	CCGGCCTGCCGGCCGCTC-CGCGCGCAGCGATTCCATACTTAATC

Schismoides	CCGGCCTGCCGGCCGCTCACACGACTCTCGG---CAACGGTAATC
Barbatus	CCGGCCTGCCGGACGCTC-CGTGCGCAGCGATTGTATAC-TAATC
Scaberrimus	CCGGCCTGCCGGACGCTC-CGTGCGCAGCGATTGTATAC-TAATC
Inermis	CCGGCCTGCCGGACGCTC-CGTGCGCAGCGATTGTATAC-TAATC
Barbatusg	CCGGCCTGCCGGACGCTC-CGTGCGCAGCGATTGTATAC-TAATC
Drakens	CTGGCCAGCCGGCCGCTG-GGTGCGCAGCGATTGTTTGC-TAATC
Guillar	CTGGCCTGCCGGCCGCTC-GGTGTACAGCGATTGTATGC-TAATC
Dura	CTGGCCTGCCGGCCGCTC-CGTGCGCAGCGATTGTATGC-TAATC
Durag	CTGGCCTGCCGGCCGCTC-CGTGCGCAGCGATTGTATGC-TAATC
Arundinacea	TGTGAATTGCAGAATCCC-GCGAACCATCGAGT---TTT-TGAAC
Disticha	---GATATCTCGGCTCTC-GC-----ATCGA-----TGC-CGTGC
Strictal	---GATATCTCGGCTCTC-GC-----ATCGA-----TGC-CGTGC
Stricta	---GATATCTCGGCTCTC-GC-----ATCGA-----TGA-AGAAC
Strictag	CCGGCTTGCCGGTCTTCC-CACGCGCGGGCATCGTATGC-TAATC
Decora	CCGCGCTGCGGGCCGGAC-GGCGTGCGGGCCGCTTATCT-TAATC
Lupulina	CCGCGCTGCGGGCCGGTTC-GGCGTGCGGGCCGCTTATCT-TAATC
Rufa	CCGCGCTGCGGGCCGCTC-CCCGTGCGGGCCGCTTATCT-TAATC
Macowaniig	CCGGCCTGCCGGCCGCTC-CCCGTGCGGGCATGCTATCT-TAATC
Rangeig	CGGCTC-GCCGGACGCGG-CCCGCGCAGCGATGCTATCT-TAATC

Aureag	CAAAAGACGCTCCCACCCC--ACC
Macrocalycinag	CAAAAGACGCTCCCACCCC--ACC
Eckloniig	CAAAAGACGCTCCCACCCC-AACC
Purpurea	-----CCCCACCG-AACA
Tenella	-----CCCCACCG-AACA
Curva	-----CCCCACCG-AACA
Purpureag	-----CCCCACCG-AACA
Schismoides	-----CACTCCCCACCG-AACA
Barbatus	CCAAAGACGCTCCCACCCT-ACCT
Scaberrimus	-----CGCTCCCACCCT-ACCT
Inermis	-----CGCTCCCACCCT-ACCT
Barbatusg	-----CGCTCCCACCCT-ACCT
Drakens	CAAAAGACGCTGGCACCCC-AACC
Guillar	CAAAAGACGCTCCCACCCC-AACC
Dura	CAAAAGACGCTCCCACCCC-AACC
Durag	-AAAAGACGCTCCCACCCC-AACC
Arundinacea	CAAAAGACACTCCCACCCC--AACC
Disticha	CAAAAGACACTCCCACCCC--AACC
Strictal	CAAAAGACACTCCCACCCC--AACC
Stricta	TCAAAGACACTCCCACCCC--AACC
Strictag	-AAAAGACACTCCCACCCC--AACC
Decora	CGCAAGTTGCGCCGAGGCCTTCT
Lupulina	CGCAAGTTGCGCCGAGGCCTTCT
Rufa	CGCAAGTTGCGCCGAGGCCTTCT
Macowaniig	-CGCTCCCA-CCCCACCG-----
Rangeig	--CCTG----CCCACCCCG-----

Aureag	CCGGGGACGGGACGCGGCGTTTGG-CTCCCCGTGCCGCAAGGCGCGGTGGGCCGAAGTT
Macrocalycinag	CCGGGGCGAGGACGCGGCGTTTGG-CTCCCCGTGCCGAGGGCGCGGTGGGCCGAAGTT
Eckloniig	CCGGGGCGAGGACGCGGCGTTTGG-CTCCCCGTGCCGAGGGCGCGGTGGGCCGAAGTT
Purpurea	--CGGT-GAGGACGTGGTGTTTGG-CTCCCCGCGCCGCGAGGTGCGGTGGGCCGAAGTT
Tenella	--CGGT-GAGGACGTGGTGTTTGG-CTCCCCGCGCCGCGAGGTGCGGTGGGCCGAAGTT
Curva	--CGGT-GAGGACGTGGTGTTTGG-CTCCCCGCGCCGCGAGGTGCGGTGGGCCGAAGTT
Purpureag	--CGGT-GAGGACGTGGTGTTTGGGCTCCCCGCGCCGCGAGGTGCGGTGGGCCGAAGTT
Schismoides	--CGGT-GAGGACGTGGTGTTTGG-CTCCCCGCGCCGCGAGGTGCGGTGGGCCGAAGTT
Barbatus	TTGGGT-GAGGATG-GGTGTATGG-CTCCTCGTGCCGCGAGGCGCGGTGGGCCGAAGTT
Scaberrimus	TTGGGT-GAGGATG-GGTGTATGG-CTCCTCGTGCCGCGAGGCGCGGTGGGCCGAAGTT
Inermis	TTGGGT-GAGGATG-GGTGTATGG-CTCCTCGTGCCGCGAGGCGCGGTGGGCCGAAGTT
Barbatusg	TTGGGT-GAGGATGCGGTGTATGG-CTCCTCGTGCCGCGAGGCGCGGTGGGCCGAAGTT
Drakens	--CGGT-GTCGACGCG-CGTATGG-CTCCCCGTGCCGACGGGCGGTGGGCCGAAGTT

Guillar --CGGT-GAGGACGCG-CGTATGG-CTCCCCGTGCCGCGACGGCGCGGTGGGCGCAAGTT
 Dura --CGGT-GAGGACGCG-CGTATGG-CTCCCCGTGCCGCGACGGCGCGGTGGGCGCAAGTT
 Durag --CGGT-GAGGACGCGCGGTATGG-CTCCCCGTGCCGCGACGGCGCGGTGGGCGCAAGTT
 Arundinacea A--GGT--AGGACGTGGCGTATGG-CTCCCCGTGCCGCGAGGTGCGGTGGACCGAATTT
 Disticha A--GGT--AGGACGTGGCGTATGG-CTCCCCGTGCCGCGAGGTGCGGTGGACCGAATTT
 Strictal A--GGT--AGGACGTGGCGTATGG-CTCCCCGTGCCGCGAGGTGCGGTGGACCGAATTT
 Stricta A--GGT--AGGACGTGGCGTATGG-CTCCCCGTGCCGCGAGGTGCGGTGGACCGAATTT
 Strictag A--GGT-GAGGACGTGGCGTATGG-CTCCCCGTGCCGCGAGGTGCGGTGAGCCGAAGTT
 Decora GGCCGA-GGGGACGCGCGTATTGG-CTCCCCGCGCCGAGGGCGCGGTGGGCGCAAGTT
 Lupulina GGCCGA-GGGGACGCGCGTATTGG-CTCCCCGCGCCGAGGGCGCGGTGGGCGCAAGTT
 Rufa GGCCGA-GGGGACGCGCGTATTGG-CTCCCCGCGCCGAGGGCGCGGTGGGCGCAAGTT
 Macowaniig ----GT-GAGGACGCGCGTATTGG-CTCCCCGCGCCCCAGGGCGCGGTGGGCGCAAGTT
 Rangeig ----GC-GAGGACGCGCGTATTGGCTCCCCGCGCCCCGCGGGGCGCGGTGGGCGCAAGTT

Aureag GGGG-CTGCCGGCGAACCCTGCCGGGCACAGCACATGGTGGGCGAC-----TATGTCTT-
 Macrocalycinag GGGG-CTGCCGGCGAACCCTGCCGGGCACAGCACATGGTGGGCGACCAAAGTT-----GT-
 Eckloniig GGGG-CTGCCGGCGAACCCTGCCGGGCACAGCACATGGTGGGCGACCAA-----GTTGT-
 Purpurea -----CTGCCGGCGTACCGTGCCGGACACAGCACATGGTGGGCGACCAA--GTTGGTTGT-
 Tenella -----CTGCCGGCGTACCGTGCCGGACACAGCACATGGTGGGCGACCAA--GTTGGTTGT-
 Curva -----CTGCCGGCGTACCGTGCCGGACACAGCACATGGTGGGCGACCAA--GTTGGTTGT-
 Purpureag GGGG-CTGCCGGCGAACCCTGCCGGACACAGCACATGGTGGTGCACCTCCAA----GTTGT-
 Schismoides -----CTGCCGGCGTACCGTGCCGGACACAGCACATGGTGGGCGACCAA--GTTGGTTGT-
 Barbatus -----CTGCCGGCGTACCGTGCCGGACTCAGCACATGGTGGGCGACCAA--GTTGG-----
 Scaberrimus -----CTGCCGGCGTACCGTGCCGGACTCAGCACATGGTGGGCGACCAA--GTTGG-----
 Inermis -----CTGCCGGCGTACCGTGCCGGACTCAGCACATGGTGGGCGACCAA--GTTGG-----
 Barbatusg GCGG-CTGCCGGCGTACCGTGCCGGGCACAGCACATGGTGGTGCACCGAAA-----GTTGT-
 Drakens GGGG-CTGCCGGCGTACCGTGCCGGACACAGCACATGGTGGGCGACCAAAG-----TTGA-
 Guillar GGGG-CTGCTGGCGTACCGTGCCGGTCACTCCACATGGTGGGCGACCAAAG-----TTGT-
 Dura GGGG-CTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Durag GGGG-CTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Arundinacea GGGGGCTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Disticha GGGGGCTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Strictal GGGGGCTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Stricta GGGGGCTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Strictag TGG--CTGCCGGCGTACCGTGCCGGTCAACAGCACATGGTGGGCGACCAAAG-----TTGT-
 Decora GGGG-CTGCCGGCGTACCGTGCCGGGCACAGCACATGGTGGGCGACACAAG-----TTGTT
 Lupulina GGGG-CTGCCGGCGTACCGTGCCGGGCACAGCACATGGTGGGCGACACAAG-----TTGTT
 Rufa GGGG-CTGCCGGCGTACCGTGCCGGGCACAGCACATGGTGGGCGACACAAG-----TTGTT
 Macowaniig GGGG-CTGCCGGCGTACCGTGCCGGGCACAGCACATGGTGGGCGACACAAG-----TTGTT
 Rangeig GGGG-CTGCCGGCGTACCGTGCCGGGCACAGCACATGGTGGGCGACACAAG-----TTGTT

Aureag CACGAGTGTGTGCATCGGAACGCGAGCCGGCGCAAGGGCCCTTACGAC-----
 Macrocalycinag CACGAGTGTGTGCCTCGGCACGCGAGCCGGGCACAGCGGCCCTTACGAC-----
 Eckloniig CACGAGTGTGTGCCTCGGCACGCGAGCCGGCGCAGCGGCCCTTAGGAC-----
 Purpurea CACG-GTGTCTGTGTCCTCGGCACGCGAGCCGGCGGAGAAAGGCCCTTAGGACTTGTACGGTGT
 Tenella CACG-GTGTCTGTGTCCTCGGCACGCGAGCCGGGTGAGAAAGGCCCTTAGGACTTGTACGGTGT
 Curva CACG-GTGTCTGTGTCCTCGGCACGCGAGCCGGCGGAGAAAGGCCCTTAGGACTTGTACCGTGT
 Purpureag CACG-GTGTCTGTGTCCTCGGCACGCGAGCCGGCGGAGAAAGGCCCTTAGGAC-----
 Schismoides CACG-GAGTCTGTGTCCTCGGCACGCGTCCGGCGGAGAAAGGCCCTTAGGACTTGTACGGTGT
 Barbatus CA-----
 Scaberrimus CA-----
 Inermis CA-----
 Barbatusg CACG-GTGTGTGTCCTCGGTGCGTAGCCAGCGATACGGCCCTTACGAC-----
 Drakens CACG-GTGTGTGTCATCGGCACGCGAGCGGGCTAGACGGCCCTTAGGAC-----
 Guillar CACG-CTGTGTGTCATCGGCACGCGAGCCGGCTAGACGGCCCTTAGGAC-----
 Dura CACG-GTGTGTGTCATCGGCACGCGAGCCGGCTAGACGGCCCTTAGGAC-----
 Durag CACG-GTGTGTGTCATCGGCACGCGAGCCGGCTAGACGGCCCTTAGGAC-----
 Arundinacea CACG-GTGTGTGTCTCGGCTCGAGCCGCAATACGGCGGTAAGGAC-----
 Disticha CACG-GTGTGTGTCTCGGCTCGAGCGGGCAATACGGCCCTTAGGAC-----

Strictal	CACG-GTGTGTGTCTCGGCTCGCAGCCGGCAATACGGCCCTAAGGAC-----
Stricta	CACG-GTGTGTGTCTCGGCTCGCAGCCGGCAATACGGCCCTAAGGAC-----
Strictag	CACG-GTGTGTGTCTCGGCTCGCAGTCGGCAATACGGCCCTAAGGAC-----
Decora	CTCG-TGTTGTTGGGTCGGCCCGCAGCCGGCGTGACGGCCCTTATGAC-----
Lupulina	CTCG-TGTTGTTGCCCTCGGCCCGCAGCCGGCGAGACGGCCCTTATGAC-----
Rufa	CTCG-GTGCCGTGCCCTCGGCCCGCAGCCGGCGAGACGGCCCTTATGAC-----
Macowaniig	CTCG-GTGCCGTGCCCTCGGCCCGCAGCCGGCGAGACGGCCCTTATGAC-----
Rangeig	CTCG-GTGCTGCGCTCGGCCCGCAGCCGGCGACACGGCCCTTCGGGA-----

Aureag	---CCTCGTACCGTGGC---GCTAGC-----
Macrocalycinag	---CCTCGAACCGTAGC---GCTAGC-----
Eckloniig	---CCTCGAACCGCAGC---GCTAGC-----
Purpurea	GTGCCTCGGTGCGTAGCCAGCGATACGGCCCTTACGACCCCTTTCGACCCGAGCGCACGT
Tenella	GTGGCTCGGTGCGTAGCCAGAGATACGGCCCTTACGACCCCTTTCGACCCGAGCGCACGT
Curva	GTGCCTCGGAGCGTAGCCAGCGATACCGCCCTTACGACCCCTTTCGACCCGAGCGCACGT
Purpureag	-----CCTTTCGACCCGAGCGCACGT
Schismoides	GTGCCTCGGTGCGTAGCCAGCGATACGGCCCTTACGACCCGTTTCGACCCGAGCGCACGT
Barbatus	-----GCGCTAGT
Scaberrimus	-----GCGCTAGT
Inermis	-----GCGCTAGT
Barbatusg	-----CCTTGAGACCCGAGCGCTAGT
Drakens	-----CCTTTCGACCCGAGCGCTTGT
Guillar	-----CCTTTCGACCCGAGCGCTTGT
Dura	-----CCTTTCGACCCGAGCGCTTGT
Durag	-----CCTTTCGACCCGAGCGCTTGT
Arundinacea	-----CCTTTCGACCCGAGCGCATGT
Disticha	-----CCTTTCGACCCGAGCGCATGT
Strictal	-----CCTTTCGACCCGAGCGCATGT
Stricta	-----CCTTTCGACCCGAGCGCATGT
Strictag	-----CCTTTCGACCCGAGCGCATGT
Decora	-----CCA-TCGACCCGAGCGCACGT
Lupulina	-----CCA-TCGACCCGAGCGCACGT
Rufa	-----CCA-TCGACCCGAGCGCACGT
Macowaniig	-----CC--TCGACCCGAGCGCACGT
Rangeig	-----CCC-TCCATCGGAGCGCGAGA

Aureag	CGCTCGGACC
Macrocalycinag	CGCTCGGACC
Eckloniig	CGCTCGGACC
Purpurea	CGCTCGGACC
Tenella	CGCTCGGACC
Curva	CGCTCGGACC
Purpureag	CGCTCGGACC
Schismoides	CGCTCGGACC
Barbatus	CGCTCGGACC
Scaberrimus	CGCTCGGACC
Inermis	CGCTCGGACC
Barbatusg	CGCTCGGACC
Drakens	CGCTCGGACC
Guillar	CGCTCGGACC
Dura	CGCTCGGACC
Durag	CGCTCGGACC
Arundinacea	CGCTCGGACC
Disticha	CGCTCGGACC
Strictal	CGCTCGGACC
Stricta	CGCTCGGACC
Strictag	CGCTCGGACC
Decora	CGCTCGGACC
Lupulina	CGCTCGGACC
Rufa	CGCTCGGACC

Macowaniig
Rangeig

CGCTCGGACC
CGCTCGGACC

;
END
BEGIN PAUP;
END;

ADDENDUM L

Distance analysis obtained from the *ITS* data set of the species *Karoochloa*, *Merxmuellera* and *Schismus*.
 Values in bold indicate inter- or intragenus averages.

	<i>M. arun</i>	<i>M. cinc</i>	<i>M. dura</i>	<i>M. dura(G)</i>	<i>M. draken</i>	<i>M. guillar</i>	<i>M. stricta</i>	<i>M. disti</i>	<i>M. stric1</i>	<i>M. stric(G)</i>	<i>M. decora</i>	<i>M. lupu</i>	<i>M. rufa</i>	<i>M. maco(G)</i>	<i>M. ran(G)</i>	<i>K. purpu</i>	<i>K. curva</i>	<i>K. schis</i>	<i>K. purpu(G)</i>	<i>S. barba</i>	<i>S. scaber</i>	<i>S. inermis</i>	<i>S. barba(G)</i>		
<i>M. arun</i>																									
<i>M. cinc</i>	0.06																								
<i>M. dura</i>	0.05	0.03																							
<i>M. dura(G)</i>	0.07	0.05	0.01																						
<i>M. draken</i>	0.24	0.23	0.20	0.16																					
<i>M. guillar</i>	0.25	0.20	0.21	0.18	0.05																				
<i>M. stricta</i>	0.21	0.20	0.17	0.14	0.11	0.10																			
<i>M. disti</i>	0.20	0.19	0.17	0.17	0.10	0.09	0.06																		
<i>M. stric1</i>	0.17	0.16	0.13	0.13	0.09	0.08	0.04	0.04																	
<i>M. stric(G)</i>	0.15	0.14	0.10	0.10	0.16	0.18	0.13	0.16	0.13																
<i>M. decora</i>	0.33	0.32	0.30	0.26	0.38	0.38	0.35	0.37	0.33	0.27															
<i>M. lupu</i>	0.32	0.31	0.29	0.25	0.37	0.38	0.34	0.36	0.32	0.26	0.02														
<i>M. rufa</i>	0.30	0.28	0.26	0.24	0.35	0.35	0.31	0.34	0.30	0.25	0.04	0.03													
<i>M. maco(G)</i>	0.20	0.19	0.16	0.14	0.26	0.28	0.24	0.27	0.23	0.16	0.17	0.15	0.12												
<i>M. ran(G)</i>	0.26	0.25	0.21	0.19	0.30	0.32	0.28	0.30	0.26	0.21	0.24	0.21	0.19	0.13	@ 0.20										
<i>K. purpu</i>	0.17	0.16	0.13	0.12	0.23	0.24	0.20	0.23	0.19	0.13	0.27	0.26	0.24	0.16	0.21										
<i>K. tenella</i>	0.22	0.21	0.17	0.16	0.25	0.27	0.24	0.26	0.23	0.18	0.30	0.29	0.27	0.21	0.25	0.07									
<i>K. curva</i>	0.18	0.17	0.15	0.14	0.24	0.25	0.22	0.25	0.21	0.15	0.28	0.26	0.25	0.18	0.22	0.02	0.08								
<i>K. schis</i>	0.20	0.19	0.16	0.15	0.24	0.26	0.21	0.24	0.21	0.15	0.28	0.27	0.26	0.18	0.24	0.06	0.11	0.08							
<i>K. purpu(G)</i>	0.19	0.17	0.14	0.13	0.24	0.25	0.21	0.24	0.20	0.15	0.28	0.27	0.25	0.17	0.21	0.02	0.08	0.04	0.09	@ 0.07					
<i>S. barba</i>	0.24	0.24	0.21	0.13	0.10	0.32	0.28	0.28	0.25	0.15	0.34	0.34	0.31	0.20	0.25	0.13	0.19	0.15	0.11		0.14				
<i>S. scaber</i>	0.24	0.18	0.15	0.14	0.27	0.29	0.26	0.28	0.24	0.15	0.31	0.31	0.30	0.20	0.23	0.14	0.20	0.16	0.12		0.14	0.01			
<i>S. inermis</i>	0.24	0.17	0.15	0.14	0.27	0.10	0.26	0.29	0.25	0.16	0.31	0.30	0.29	0.20	0.24	0.15	0.20	0.16	0.13		0.15	0.014	0.02		
<i>S. barba(G)</i>	0.24	0.16	0.13	0.12	0.25	0.27	0.23	0.26	0.22	0.15	0.29	0.28	0.26	0.18	0.22	0.17	0.23	0.19	0.17		0.13	0.03	0.04	0.05	@ 0.03
																@ 0.21								@ 0.23	

ADDENDUM L 223

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